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Cloning and characterisation of the human gene gremlin promoter

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Cloning and characterisation of the human gene gremlin promoter

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RÉSUMÉ

L'ostéoarthrose (OA) est une maladie articulaire invalidante caractérisée par la perte de l'intégrité du cartilage articulaire. Les recherches tentent de comprendre les mécanismes moléculaires de la maladie afin de trouver des inhibiteurs efficaces pouvant prévenir la dégradation du cartilage articulaire. Les BMPs (bone morphogenic proteins) jouent un rôle dans le processus pathophysiologique de cette maladie. Cette étude cible le rôle d'un antagoniste des BMPs, le gremlin.

Nous avons étudié la régulation de l'expression de gremlin par le clonage et la caractérisation de son promoteur et en déterminant si gremlin pouvait jouer un rôle autre qu'antagoniste des BMP, en affectant l'expression d'autres gènes par l'activation d'une cascade de signalisation dans la cellule.

Les résultats ont identifié une région importante dans le promoteur de gremlin qui affecte son activité basale et induite, et ont montré que le gremlin ne pouvait pas affecter l'expression génique et l'activation de signalisation intracellulaire indépendamment des BMPs. Cette étude démontre que le rôle de gremlin dans l'OA en est un essentiellement d'antagoniste des BMPs.

Mots clés:

Osteoarthrose

Cartilage

Chondrocyte

Gremlin

BMP

BMP antagoniste

SUMMARY

Osteoarthritis (OA) is a disease that affects the integrity of the articular cartilage which leads to serious health issues for many individuals. Research is focused on understanding the molecular mechanisms which lead to this loss in integrity in the hopes of finding a way to turn the tide. The bone morphogenetic proteins (BMPs) have been shown to play a role in the progression of this disease and this study focuses on one of their antagonists, gremlin.

We therefore decided to study what affects the expression of this protein through the cloning and characterization of its promoter region. We also studied the role of this protein in the disease, can it influence gene expression and can it initiate a signalling cascade within the cell on its own.

The results identified a region important for basal and induced activity of its promoter .The results also demonstrated that the main role of this protein in the progression of OA is through BMP antagonism. Gremlin does not initiate a signalling cascade and affect gene expression on its own.

Key words:

Osteoarthritis

Cartilage

Chondrocyte

Gremlin

BMP

BMP antagonist

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LIST OF ABREVIATIONS

BMP:	BONE MORPHOGENETIC PROTEINS
CSA:	CYCLOSPORIN A
ECM:	EXTRACELLULAR MATRIX
FGF:	FIBROBLAST GROWTH FACTOR
GRM:	Gremlin
IGF:	Insulin-like growth factor
IFN-γ:	INTERFERON GAMMA
IL-1β:	INTERLEUKIN-1-BETA
iNOS:	Inducible nitric oxide synthase
MMP:	MATRIX METALLOPROTEASE
NO:	Nitric Oxide
OA:	OSTEOARTHRITIS
PDGF:	Platelet derived growth factor
TNF-α:	Tumor necrosis factor-α
TIMP:	Tissue inhibitor metalloproteases

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INTRODUCTION

I. OSTEOARTHRITIS (OA)

Osteoarthritis (OA) is a disease affecting over 500 000 individuals every year in the United States alone and current estimates show that over 20 million people in the United States are dealing with this affliction (1). This disease affects approximately 15% to 20 % of the population and of this number 65 % are 60 years of age or older. Moreover, one of the risk factors is obesity and thus with the advent of an aging baby boomer generation, the large amount of overweight children and adults, the number of cases of OA will only increase in the future, placing an even greater strain on the already stressed medical infrastructure. The cost of treating these patients will be a huge burden to governments and insurance companies around the world.

The causes of OA differ between individuals and are related to various influences such as genetics, obesity, injury, muscle weakness, use over time of the joint which in addition to the previous influences can lead to early onset of OA (2). These changes can lead to disability which greatly reduces the quality of life of those affected. The changes in the weight bearing joints are manifested through pain, stiffness and an overall loss of motion. OA is a condition resulting from the gradual degradation of the articular cartilage but also involving the other tissues of the joint such as the synovial membrane and the subchondral bone (3). In cartilage, there is a breakdown in the matrix resulting in fibrillation, fissure appearance, gross ulceration and full thickness loss of the joint surface (3). These changes in the cartilage are usually coupled with alterations within the subchondral bone in the form

of bone remodeling and the formation of osteophytes (4). The changes also affect the synovial membrane leading to an inflammation of this tissue causing the release of inflammatory mediators into the synovial fluid which diffuses to the cartilage (4).

The progression of the condition hinges upon the homeostasis of the two general processes involved in the joint makeup, the anabolic and the catabolic processes. The anabolic process involves the proteins and molecules which are necessary for the maintainence and management of the cartilage integrity. Examples are the collagens, the proteoglycans and other matrix proteins. The catabolic process involves proteases, cytokines and molecules involved in the breakdown and degradation of the articular cartilage matrix which undermines the structural integrity of the joint. At an early stage of the disease, there is an upregulation in the anabolic process followed by an increasing production of the catabolic factors (5). Eventually the catabolic process becomes the dominant one leading to matrix breakdown, then inflammation and pain (5).

II. NORMAL JOINT CARTILAGE

A-CHONDROCYTES

The structure of normal human adult cartilage is formed of layers that can be divided into different zones; the superficial or tangential zone, the transitional or middle zone, the radial or deep zone and the calcified zone (Figure 1). The transitional areas between these zones is blurry while the one between uncalcified and calcified is sharp and clear and is named the "tidemark"(Figure 1). There is only one cell type in the articular cartilage and it is named chondrocyte (6). Depending on the zone in the cartilage the density of the chondrocytes varies: the highest densities are found in the superficial zone and the least dense in the deep zone (6). The shape of the chondrocytes also changes from zone to zone in relation to the

density, they are most dense in the superficial zone (Figure 1) where due to higher density of cells they exhibit flattened disc like shapes. The chondrocytes begin to take a more circular shape in the radial zone (figure 1) and seem to be randomly dispersed, while as we approach the end plate (Figure 1) they are arranged perpendicularly to the articular surface and grouped in 2-6 cells (6).

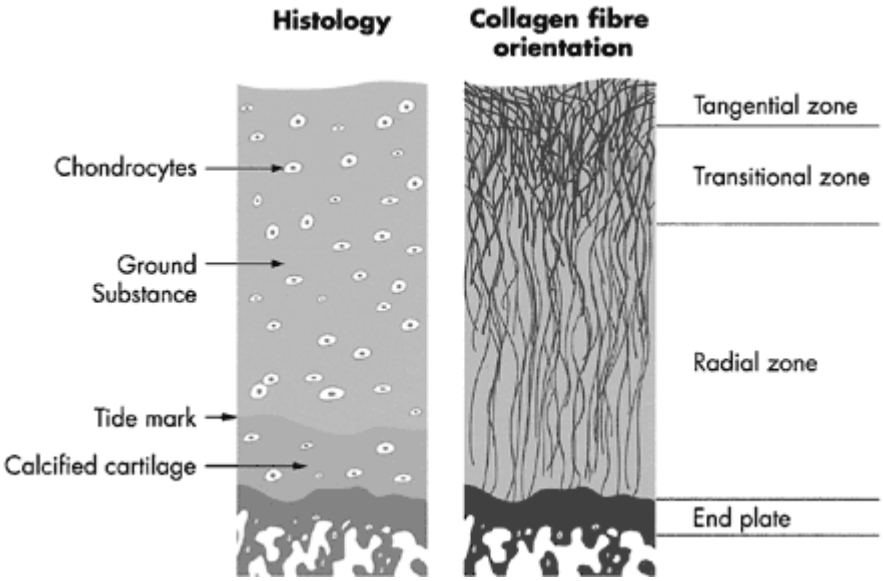


Figure 1: Zones of the articular cartilage

(www.kneejointsurgery.com/.../anatomy.html)

B- EXTRACELLULAR MATRIX (ECM)

The human articular cartilage is formed of an ECM which is heavily hydrated and composed of 2-3% cells (7). These chondrocyte cells lack cell to cell contact (7). Communication occurs through the ECM. The lack of blood vessels and nerve signals means that the delivery of nutrients occurs through diffusion (7). The chondrocytes embedded within the matrix assures the maintanance of the area surrounding them; they are

the keepers of the ECM and are responsible for the metabolic environment (7). The ECM is made up of different compartments and the area next to the chondrocytes is called the pericellular or lacunar matrix (8). This area is characterized by proteoglycan aggregates which bind the cell through the interaction of hyaluronic acid with the chondrocyte cells by CD-44-like receptors (8). The pericellular environment is also characterized by a relative lack of fibrillar collagens (8). Next to the pericellular area is the territorial or capsular matrix which is characterized by a large amount of fibrillar collagen that encases the chondrocytes forming a supporting framework for these cells (6). As we move further away from the chondrocyte, the final compartment is called the interterritorial area and it is at this location that the largest amounts of fibrillar collagen and communication between the chondrocytes are found (6). This area occurs through microfilaments and specific matrix molecules like anchorin and CD-44 like receptors on the cellular surface (9). The ECM environment is held together by chondrocytes which rarely divide after adolescence; therefore each loss of a chondrocyte causes a loss in the integrity of the underlying structure of the ECM. The different compartments of the ECM can be seen in Figure 2. It is important to remember that the further one goes from the chondrocyte, the more collagen is present.

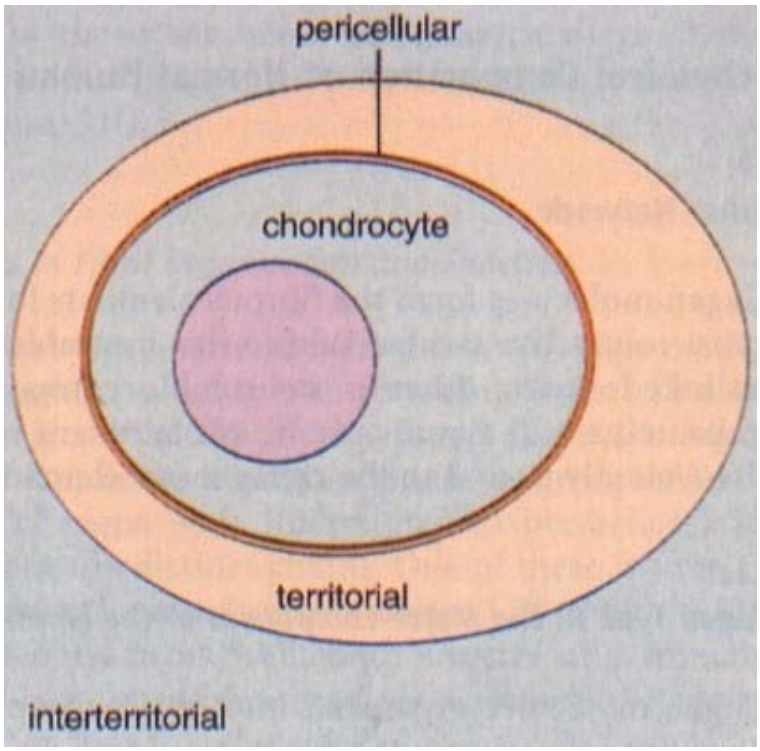


Figure 2: Compartments of the ECM (1)

1-COLLAGEN

The collagen framework found in the articular cartilage varies between the different zones and it accounts for most of the dry weight of this region (10). Once this framework is established during development, it cannot be replaced or created anew, it is up to the chondrocytes to maintain this fibrillar network which gives strength and adaptability to the cartilage (11). The concentration of the collagen in the articular cartilage is directly dependent upon the density of the chondrocytes found within. In the superficial zone, analysis under transmission electron microscopy shows that the collagen framework resembles the shape and direction of the chondrocytes in that they are parallel to the articular cartilage and thin in diameter (11). As we move deeper into the cartilage, the diameter of the fibrils increases in size as do the size and shape of the chondrocyte cells,

reflecting an inherent larger space dimension in these areas (11). The fibrous elements found in this region are made up predominantly of collagen II but also of collagen type IX, XI and small amounts of type V, other small non collagenous proteins including proteoglycan and glycoproteins (12). During maturation the content of the fibrils change from a predominantly collagen type IX and makeup to a 90% of the collagen type II constitution reflecting an increase in strength of the fibrils needed with age (14). The type of collagen found closest to the calcified zone in the articular cartilage is the collagen type X (14). As we move further away from the chondrocyte into the interterritorial matrix the collagen fibrils become coarser in their constitution when observed under transmission electron microscopy and more type II collagen in this region is found (15). The fibrils are made up of a mixture of the three types of collagen, type IX, II, XI with this template being the basic one during early development (15). The collagen framework provides the structural support the chondrocytes require and enables the articular joint a manner of flexibility in response to joint load bearing (16). The ability of the chondrocytes to reproduce the dense fibril network decreases with age and it has been observed that only some newly synthesized collagen type II is produced after maturity in response to injuries (12). Besides this fibrillar collagen network there are a number of other proteins that play an important role in the integrity of the articular cartilage, these include proteoglycans, glycoproteins and non-glycosylated proteins (17). The most abundant proteoglycan found in the articular cartilage joint is called aggrecan (18).

2-AGGRECAN

Proteoglycans are proteins with glycosaminoglycan domains; they are found predominantly in the ECM and consist of most of the space in the ECM. These proteins along with water

allow the articular cartilage to compress under load stress thereby giving the joint flexibility (19). Aggrecan makes up 90% of the proteoglycan found in the articular cartilage and has a mass of about 230 kilodaltons (19). Most of aggrecan weight comes from the glycosaminoglycan domains that are made up mostly of keratan sulfate and chondroitin sulfate (20). Each glycosaminoglycan domain can be made up of over 100 different chondroitin sulfate and keratan sulfate chains (20). It is this high content of chondroitin sulfate and keratan sulfate which gives the aggrecan the ability to interact with hyaluronic acid in the ECM (20). Once the aggrecan molecule is synthesized by the chondrocytes and is secreted into the ECM, it aggregates with other aggrecan molecules as it name implies. This aggrecation of over 200 aggrecan molecules and link proteins is essential in forming the link between the aggrecan and hyaluronic acid (8). This proteoglycan aggregation maintains cellular contact through the hyaluronan interaction with CD-44 like receptors on the cell surface. The formation of these large aggregate complexes allows the articular cartilage to resist compression when under stress (8). There are two types of aggregate populations, one that is lighter, weighing approximately 60 kilodaltons, while another termed “superaggregates”, weighing 120 kilodaltons, is found in the middle region of the articular cartilage (21). Interestingly, during the onset of OA there is a distinct loss in superaggregates found in the articular cartilage which demonstrates how the integrity of the ECM is compromised (21).

3-SMALL NON-AGGREGATING PROTEOGLYCANS

The aggrecans are not the only proteoglycans to populate the ECM, there are other small non-aggregating proteoglycans like biglycan and decorin (22). These two proteins are part of a group of proteins called Small Leucine-Rich Repeats Proteoglycans (SLRP) that can be

divided into two subfamilies depending upon which type of side chain is attached to it, either keratan sulfate or dermatan sulfate side chain (23). Both decorin and biglycan are populated with dermatan sulfate side chains and have been found in articular cartilage (24). Examples of proteoglycans with keratan sulfate side chains found in articular cartilage are fibromodulin and lumican (25). Depending upon the stage of development these two proteins are found in the articular cartilage with or without their keratan side chain, fibromodulin has a keratan side chain only in the fetus or in a juvenile but loses it later on in the life cycle (25).

The importance of these small leucine rich repeat proteoglycans in the ECM is demonstrated in SLRP-null mice which develop abnormalities in the articular cartilage (17). The SLRP's interact with many components of the ECM such as collagens, fibronectin and heparin to help form the support structure network for the chondrocytes (17).

4-OTHER STRUCTURAL PROTEINS OF THE ECM

There are other types of proteins that play an important role in the structural support of the ECM that are neither collagenous nor proteoglycan in type (17). These proteins are also necessary to help maintain the integrity of the ECM by their interactions with the other proteins present within the matrix like collagens and proteoglycans (17). An example of this type of protein is COMP, cartilage oligomeric matrix protein, which is a member of the thrombospondin superfamily and has a mass of 85 kilodaltons (26). This pentameric protein has been shown to interact with collagen through its carboxy-terminal regions (27). When a mutation occurs within this gene, two conditions have been known to occur, pseudoachondroplasia or epiphyseal dysplasia (27). During the early stages of OA, it has been shown that COMP is degraded and fragments of this protein have been found in

synovial fluid (28). These fragments are used as biomarkers for early signs of OA in patients (28).

Anchorin is also a structural protein found in the ECM, it has been shown to mediate chondrocyte interactions with the matrix from its position on the cell membrane surface (29). Finally, fibronectin is another of these structural proteins with an essential role in matrix support (30). It seems to mediate interactions between the cellular membrane and other matrix proteins like collagen type II and thrombospondin (30). During the late stages of OA, fragments of degraded fibronectin are found in the joint fluid and have been shown to increase the catabolic breakdown of the matrix and decrease the synthesis of aggrecans (31). Figure 3 depicts the different matrix proteins and how they interact with each other and with the chondrocyte (1).

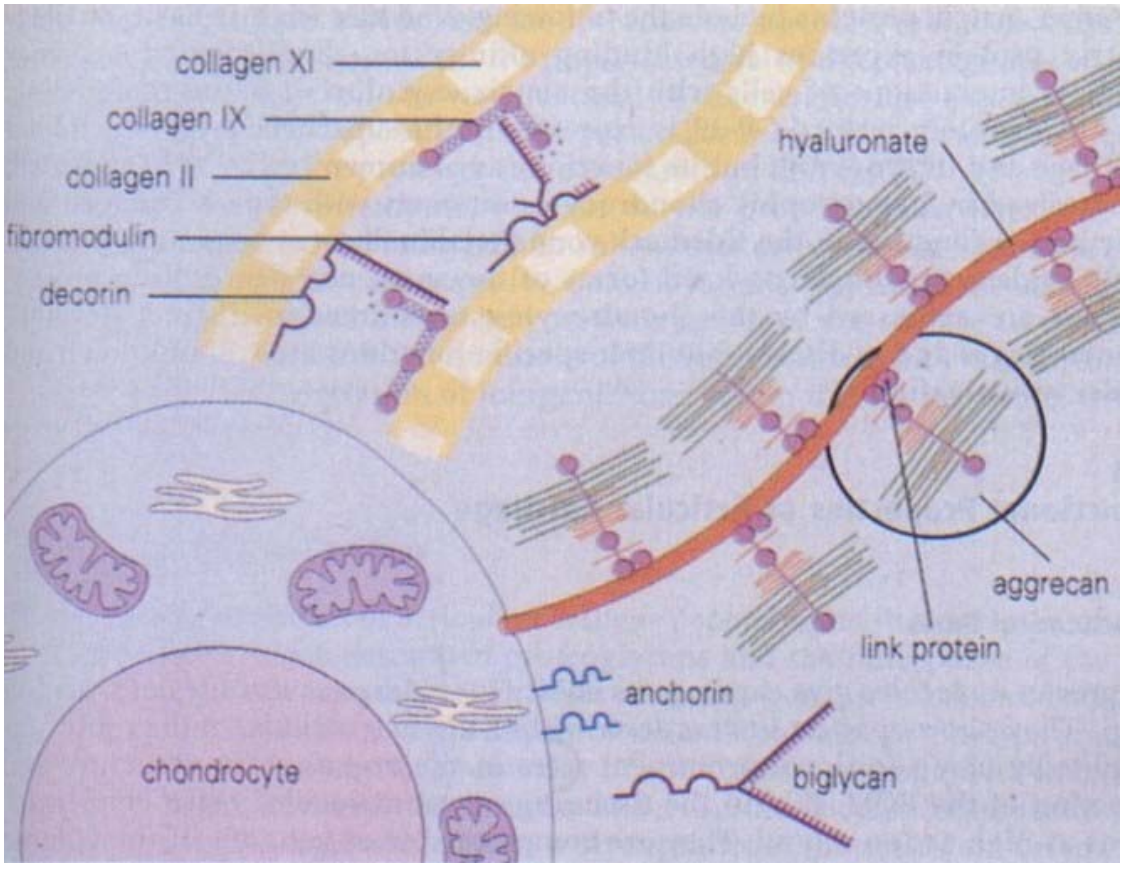


FIGURE 3: PROTEIN INTERACTION IN THE ECM (1)

III. OA CARTILAGE

OA is the result of the action of a number of different factors, which lead to cartilage degeneration over time. The catabolic factors involved in the pathophysiology of OA include the proteases, the pro-inflammatory cytokines and some growth factors. The major family of proteases that have been shown to be involved in OA are the matrix metalloproteases (32). Proteases from this family are able to degrade the major cartilage matrix macromolecules (32). From this family of proteases three groups have been found to be elevated in OA, the collagenases, the stromelysins and the gelatinases (32). Collagenases are responsible for the degradation of collagen, the stromelysins have been linked to proteoglycan degradation and the gelatinases are involved in the degradation of denatured collagen (32).

A. CATABOLIC FACTORS

1-MATRIX METALLOPROTEASES (MMPs)

The MMP family consists of the classical MMPs, the membrane bound MMPs called MT-MMP, the ADAMs (a disintegrin and metalloprotease) also called the adamlysins and the ADAMTS which are a disintegrin and metalloprotease with a thrombospondin motif (33). In cartilage, studies have shown that MMP, MT-MMP and ADAMTS are implicated during the disease process (33). There are over 20 members in the MMP family that include the collagenases, the stromelysins, the gelatinases, some elastases and the aggrecanases ADAMTS-4 and ADAMTS-5 (33). MMPs depend upon the presence of a zinc ion in their active site for their catalytic activity (33). Usually, these proteases are active at neutral pH and are either secreted into the extracellular matrix or as for the MT-MMP found on the

cellular membrane surface (34). Their ability to maintain activity at a neutral pH enables them to play an important role in the overall protein turnover taking place in the ECM (33). Unlike most proteases, MMP are examples of multidomain proteases with additional protein modules that coordinate the localization and character of the protease itself (35). For example the C-terminal domain of collagenases called hemopexin is responsible for its ability to bind to triple-helical collagen (35). The cooperation between this domain and its catalytic domain is still unclear but it is evidently necessary to allow for the triple helical collagen strands to be separated and cleaved (35). Another example of multidomain functionality is found in the gelatinases which contain additional fibronectin type II repeats that are important in the binding of this proteases with its substrate denature collagen (35). These unique characteristics allow the MMPs the ability to be specific and diverse in their role as mediators of macromolecule turnover in the ECM.

i. Collagenases

Three main collagenases have been shown to be elevated in OA, collagenase-1 (MMP-1), collagenase-2 (MMP-2) and collagenase-3 (MMP-13) (32). This family of MMP can be separated into groups based on substrate specificity and their location in the cell (36). The three types of collagenases all cleave type II collagen to varying degrees, with collagenase-3 being five times more likely to cleave collagen II than collagenase-1 (37). Collagenase-1 has higher substrate specificity for collagen type III while collagenase-2 has a higher specificity for collagen type I (37). The presence of these three collagenases varies during the progression of OA. Collagenase-1 has been shown to be involved mainly in the catabolic or inflammatory phase of OA while collagenase-3 has been shown to be involved in the remodelling phase (37). The role of collagenase-2 in OA is still being investigated and like many proteases, this role cannot be narrowed down to one specific activity but to a

general activity important in maintaining the balance between the anabolic/catabolic mechanisms in the ECM. It has been shown in immunohistochemical studies in animal models that with the increase of lesions in OA, the levels of collagenase-1 increased in the superficial zones of the cartilage while the presence of collagenase-3 increased in the deeper zones (38). Interestingly, it has been shown that in OA, collagenase-1 is found also in the synovial fluid further indicating its role in the inflammation process (38).

ii. Stromelysins

Like the collagenases three stomelysins have been described in human articular tissue, stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and stromelysin-3 (MMP-11) (37).

Only stromelysin-1 has been found to be elevated in OA tissues; this is shown in histological studies which correlated an increase in OA lesions with an increase in MMP-3 (37). Stromelysin-2 could not be found in OA synovium but studies have shown it to be present at minimal levels in synovial fibroblasts of rheumatoid arthritis patients, while stromelysin-3 has also been shown to be found in synovial fibroblasts of rheumatoid arthritis patients (39). Stromelysin-1 has also been found in synovial fluid and further studies have linked elevated levels of this stromelysin and proteoglycan degredation (39). Stomelysin-1 has also been shown to be involved in the degradation of collagen type IX which plays an important role in the stability of the ECM (40). Collagen type IX is necessary for the linking of proteoglycans and type II collagen in the ECM (40).

iii. MT-MMPs, Gelatinases, ADAMTS and ADAMs

The membrane bound MMPs are bound to the cellular membrane through their C-terminal domains and this allows for restricted protease activity (41). The MT-MMPs were found to be expressed in articular cartilage and their specific role in OA is yet to be determined (41).

So far there have been four MT-MMP expressed in the cartilage and these have been called MT1-MMP through MT4-MMP and all possess collagenase activity (41). One of the roles attributed to these membrane bound MMPs is the activation of other metalloproteases such as 72 kD gelatinase (MMP-2) and collagenase 3 (MMP-13) (42). Other members of the MMP family that are membrane located are the ADAMs which are also multidomain proteases (43). Theses proteases contain a disintegrin unit and a catalytic unit (43). The disintegrin unit allows it to bind to integrins on the cellular membrane surface and in doing so enables it function as a disrupter of the interactions between the matrix and the cell (43). Among the ADAMs studied, the most notable is the ADAM-17, which is also called TACE for Tumor Necrosis Factor alpha Converting enzyme and has been shown to be involved in OA pathology (43).

The ADAMTS are closely related to the ADAMs family but differ in that they do not bind to the cell surface and contain a thrombospondin motif (35). This motif allows for the protease to bind to glycosaminoglycans (35). The most notable of this family of proteases are ADAMTS-4 and ADAMTS-5 also known as aggrecanases. They are responsible for the degradation of the aggrecan structure in the ECM and cleave the aggrecan molecules at glutamic residues, specifically at the residues GLu373 –Ala374 (44). In OA there is evidence of an increasing number of aggrecan fragments found in the synovial fluid which plays a role in the inflammation of the synovium (44). The increasing amount of aggrecan fragments has been linked to the activity of the aggrecanase proteases and the levels of aggrecanase have been shown to be increased by the inflammatory cytokine interleukin-1β (45).

The last group, the gelatinases, has been found in human articular tissue, specifically gelatinase 72kD and gelatinase 92kD; only the gelatinase 92kd has been shown to be increased in OA (37). Although the MMP family is the major player in the progression of OA in terms of cartilage degradation, there are other proteases that have been found to play a role in cartilage turnover which will be discussed later. The regulation and activation of MMPs is essential in OA. It is often a problem in their regulation that plays an important part in the progression of OA and an overexpression in MMP would lead to a complete destruction of the articular cartilage (37).

2-MMP regulation

The regulation of MMPs occurs at both the transcriptional and posttranslational levels (46). The expression of MMPs in normal articular joint tissue is low (46). The expression of these proteases can be induced by proinflammatory cytokines such as interleukin-1(IL-1), tumor neucrosis factor alpha (TNF-α) and certain growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) (37). At the transcriptional level, these cytokines and growth factors affect MMP levels of expression by interacting with the Activator protein site (AP-1) in the promoter region of the MMP genes (47). This activator protein site is present in all of the MMP genes except for MMP-2 (47). Transcriptional factors that bind to this site are members of the Fos and Jun family which bind as heterodimers (Jun/Jun or Fos/Jun) to activate transcription of the MMP genes (47). The cytokine TNF-α has been shown to increase MMP expression by inducing a prolonged increase in Jun and Fos while TGF-β and glucocorticoids suppress MMP expression through the AP-1 site (47). Another transcription site that has been shown to play an important role in the induction of MMP

expression is the polyomavirus enhancer A (PEA-3) site and this site has been shown to act in concert with the AP-1 site in some MMP transcription (37).

At the posttranslational level most of the MMPs are synthesized as proenzymes which are inactive until they are activated usually through cleavage by a protease (46). The activation process of MMPs occurs in two different stages. An intermediate is first generated by an activator and then the partially activated MMP is then fully activated by other intermediates or partially activated MMPs (46). Besides being activated by MMPs themselves, they can also be activated by other factors (46). An example of this is the prohormone convertase, furin, which is a serine protease found in the Golgi apparatus that cleaves MMP-11, MMP-14 and aggrecanases to activate them (47). In the case of the other MMPs, they are activated either through the action of serine proteases or other MMPs (47). These posttranslational modifications are necessary as a regulatory mechanism to control the levels of active MMPs found the ECM (47). The activity of MMPs is also regulated by inhibitors called tissue inhibitors of MMPs or TIMPs (48). These proteins contain two domains, one which binds to the active site of the MMPs and in doing so inhibit their catalytic ability (48). There are currently four TIMPs identified and they are called TIMP 1-4 (48). Only three of these molecules have been found in human articular tissue (48). It has been shown that chondrocytes produce TIMPs -1, -2, -3 and TIMP-3 has been found exclusively in the ECM (48). Most of the TIMPs show little binding selectivity as to which MMP they bind but TIMP-3 has been shown to inhibit specifically ADAM-17 (TACE) and the aggrecanases ADAMTS-4 and ADAMTS-5 (48). Studies have demonstrated that in OA there are elevated levels of TIMP-1 and TIMP-3 and that there is a distinct imbalance between the levels of the TIMPs and MMPs (37). The inhibitory function of TIMPs is not the only role that these molecules may play in OA (49). The activation of MMP-2 requires the presence of TIMP-2

to interact with MT-MMP-1 to cleave the pro-MMP-2 (50). Once again, one can see the importance of maintaining a balance between the factors present in the human articular joint; any disruption in this balance leads to a breakdown in the support matrix structure.

3-SERINE PROTEASES

The serine proteases are also active at a neutral pH as the metalloproteases and contain a catalytic group which is a hydroxyl moiety (51). These proteases are also activators of the MMPs (8). An example of this family of proteases is urokinase-type (uPA) and tissue-type (tPA) plasminogen activators (52). These enzymes are responsible for activating the plasmin protease which has been shown to play a role in the activation of MMPs (52). Elevated levels of plasmin have been seen in OA (53). The PA/plasmin system can be stimulated by the proinflammatory cytokine IL-1β (53). Plasmin has the ability to digest glycoproteins which form part of the supporting structure of the ECM (53). As in the case for MMPs, the serine proteases also have inhibitors which help in maintaining a balance between the catabolic and anabolic mechanisms of the articular joint tissue. The serine protease inhibitor is called the PA inhibitor, and PAI-1 has been shown to decrease in OA cartilage (54). As in the case of the TIMPs, the serine protease inhibitor experiences a decrease in level during the progression of OA, thereby demonstrating again the importance of a balance within the articular joint tissue environment.

4-THIOL PROTEASES

The thiol proteases are actively involved in the degradation of the ECM molecules and studies indicate that they are capable of doing so due to their ability to maintain activity at acidic pH levels (55). An example of this is cathepsin B which is active both in the degradation of collagen and proteoglycan but also has been shown in vivo to be an activator of MMPs (55). As in the case of the other proteases, cathepsin B has inhibitors which are important in its regulation. In OA their levels are decreased, while the levels of cathepsin B are increased (56). Therefore an imbalance is created again which favors the catabolic over the anabolic process.

B. PRO-INFLAMMATORY CYTOKINES

The role of the various proteases in the progression of OA is essential but their expression and subsequent involvement in OA is driven by the effect of cytokines released from an inflamed synovial membrane and the chondrocyte of the articular cartilage (57). Cytokines are small proteins that act as a messenger for cell to cell sharing of information. The term cytokine includes some growth factors, interleukins and interferons (58). In the progression of OA these molecules transmit information from cell to cell depending upon the environment of the articular cartilage matrix at the time. In the catabolic state in which the degradation of the cartilage is taking place, the presence of pro-inflammatory cytokines is elevated which causes the release of degradative enzymes in the tissue matrix (59). In patients with OA, it has been shown that the chondrocyte cells produce IL-1β, IL-6, IL-8 , IL-17, IL-18, TNF-α, the inflammatory mediators prostaglandins and nitric oxide (NO) (59). These factors help increase the levels of the degradative proteases like MMPs thereby destabilizing the balance between the anabolic and catabolic states. Studies have also shown that the pro-inflammatory cytokines IL-1β and TNF-α are the major cytokines involved in

the progression of OA; they increase their own expression as well as the expression of other inflammatory cytokines such as IL-6, IL-8 and the leukocyte inhibitory factor from chondrocyte and synovial cells (60). On the other side of the spectrum there are anti-inflammatory cytokines which are essential in maintaining the balance in the articular tissue. The anti-cytokines help restrict the expression of the degradative enzymes into the ECM. Examples of anti-inflammatory cytokines are IL-4, IL-10 and IL-13 which are essential in moderating inflammatory cytokine and protease synthesis (61, 62). They also play a role in regulating and increasing the expression of proteins which in turn help regulate MMP activity such as TIMPs and IL1-Ra which is an antagonist for the IL-1 receptor (62).

1-Interleukin-1β (IL-1β)

The presence of the pro-inflammatory cytokine IL-1β in OA chondrocytes has been shown and is found predominantly in the superficial layer of the articular cartilage joint (63). The cytokine IL-1β has been linked to causing many different changes in the environment of the articular cartilage leading to the overall destruction of the cartilage in OA (64). IL-1β is synthesized as an inactive precursor and activated by the IL-1β converting enzyme ICE (caspase 1), which is responsible for the mature active form of the cytokine (37). Two IL-1β receptors exist, type I and II, and they have different affinities for the cytokine (63). Type I IL-1R receptor has a higher affinity for IL-1β than the type II IL-1R receptor and is responsible for signal transduction (63). An increased number of this receptor type has been seen in chondrocytes during OA (63). Both of these receptors can exist in a soluble form which acts as an antagonist for the membrane bound form in that it binds IL-1β without signal transduction occurring (65).

This cytokine causes an imbalance in the type of collagen produced by the chondrocytes (37). It increases the synthesis of type I and type III collagen without increasing the amount of type II collagen (37). This affects the integrity of the structure of the ECM in that the concentrations of the different types of collagen are not at the right proportions (37). This has been shown to cause damage to articular cartilage repair (37). Other roles for the cytokine in the progression of OA involved decreasing the synthesis of TIMP-1 and increasing the synthesis of MMPs (66). Similarly, IL-1β has been shown to create an imbalance within the plasminogen system by increasing plasmin synthesis while decreasing the synthesis of the plasmin inhibitor PAI-1 (66). These roles share a common thread in that they all create an imbalance within the articular joint by increasing the catabolic process of the joint over the anabolic system of maintenance. IL-1β causes these changes by activating various signal transduction cascade to influence gene expression. Some of the signal transduction cascades affected by the cytokine are PKA, ERK 1 / 2, p38, SAPK/JNK, PKC and other protein kinases (37).

2-Tumor Necrosis Factor-α (TNF-α)

The pro-inflammatory cytokine TNF-α has been shown to play an important role in the progression of OA and also in the inflammation of the synovial membrane (67). The cytokine is produced in an inactive pro-form and cleaved by a TNF-α converting enzyme called TACE which is part of the adamalysin protease family (67). The TACE levels are increased in OA which is indicative of the overall catabolic state of the joint during the disease (67). Once the cytokine is active, it forms a trimer with other TNF-α molecules to bind to one of two types of specific receptors (68). TNFR55 is the dominant receptor in OA and TNF75 can be found on the cell membrane (69). Both of these receptors can exist in a soluble form (69). The soluble forms of the receptors help the binding of TNF-α to the TNF

receptor on the cell membrane (70). However at high concentrations the soluble forms of the receptor can act as an antagonist by competing with free TNF-α and preventing it from binding to its receptor and initiating a signal transduction (71). The signal transduction occurs by the activation of different signaling pathways such as NFκB, the SAPK/JNK, ERK 1/2 kinase and the PKC (72). The concomitant signaling cascade causes an increase in AP-1 synthesis and proteins involved in AP-1 site binding such as Jun/Fos which have all been demonstrated to be involved in MMP expression (72).

3-Other Pro-Inflammatory Cytokines in OA

The cytokines IL-6, IL-17 and leukemia inhibitory factor (LIF) have all been shown to be elevated in OA although exact roles for each of these cytokines still remain to be determined (37). The role of IL-6 has been linked to progression of OA in that it is induced by the pro-inflammatory cytokines IL-1β and TNF-α (73). LIF has also been shown to be induced by IL-1β and TNF-α and plays a role in the inflammation process by causing the degreadation of proteoglycans and MMP and induction of nitric oxide (NO) (74). IL-17 has also been shown to be elevated in OA and has been linked to the induction of NO (75).

4- NITRIC OXIDE (NO)

The inorganic molecule NO has been implicated in the catabolic process of OA and studies have shown that during the progression of the disease elevated levels of NO can be found in the synovial fluid and articular cartilage (76). The elevated level of NO has been tied to both cytokine induction and constitutive induction by the chondrocytes themselves (77). The role of NO plays in the catabolic mechanism occurs through the inhibition of aggrecan and proteoglycan production (79). Studies have also shown that NO increases MMP production while inhibiting the synthesis of IL-1Ra which contributes to catabolism (76,

79). During OA, an increased level of NO has been observed in the articular cartilage when compared to normal cartilage and an increase in the amount of nitrate/nitrite has been found in the synovial fluid (80). The increase in nitrogen products in the synovial fluid is a result of an increase in the amount of inducible NO synthase (iNOS) in the cartilage which is responsible for NO production (81). Finally, elevated levels of NO have been linked to cell death; the presence of this factor in the articular cartilage was also reported to lead to apoptosis (82). Apoptosis is an important part of OA in that one of the characteristics of OA is a lack of chondrocyte cells in the articular cartilage and this phenomenon has been observed during the progression of the disease (83).

C. ANABOLIC FACTORS IN OA

1-ANTI-INFLAMMATORY CYTOKINES

Three main anti-inflammatory cytokines have been studied in OA: IL-4, IL-13 and IL-10 (10). Each of these has been shown to counter the inflammatory process of the pro-inflammatory cytokines by decreasing the production of inflammatory cytokines and increasing the production of TIMPs (58). The effect that these anti-inflammatory cytokines have on the cell seems to be much more specific in that they require specific conformations of certain types of receptors on the cell membrane to initiate a signal transduction. IL-13 and IL-10 receptors have different conformations depending upon the cell type and this affects the ability of the cytokines to initiate signaling (85). This level of complexity in regards to the receptors points to another difficulty in maintaining its potential.

Another inhibitor of the pro-inflammatory cytokines is IL-1Ra which competitively binds to the IL-1 receptor and inhibits the actions of IL-1β (86). IL-1Ra needs to be at a high

level to inhibit IL-1β activities in the articular tissues, once again pointing to the cartilage's inability to respond or stop the progression of OA (86).

2- GROWTH FACTORS INVOLVED IN CARTILAGE HOMEOSTASIS

The literature suggests that anabolic effects occur through growth factors that synthesize matrix molecules and that are necessary for continued balance in the joint, thus homeostasis.

i. Insulin-like Growth Factor (IGF)

IGF-1 and IGF-2 are important for proteoglycan synthesis in the articular joint and are essential players in cartilage homeostasis in all stages of life (87). IGF-2 has been shown to be predominantly active in early stages of life while IGF-1 has been shown to be active in the adult life stages explaining its studies during OA (87). The presence of these growth factors in the articular joint occurs through their synthesis by the articular tissue cells (37). As it is the case for all factors involved in OA, these growth factors also have specific inhibitory molecules that limit their actions and they are called IGF-binding proteins (IGF-BPs) (88). Studies have shown that the presence of IGF-BPs is elevated in OA through treatment with TNF-α and IL-1β and explains, in part, the reason why the anabolic repair mechanisms fail (89). Another interesting observation is that the levels of IGF-1 are elevated in OA due to to an increase in cytokine presence in chondrocytes during progression of the disease (89). The increase in IGF-1 synthesis does not lead to an increase in cartilage anabolic factor synthesis not because of the limited amount of IGF-1 receptors on the cell surface but because of a high level of IGF-BPs released by the cells (88).

ii. Platelet derived growth factor (PDGF) and fibroblast growth factor (FGF)

PDGF has been shown to also play a role in the balance between the anabolic and catabolic mechanisms of the articular cartilage joint (90). Studies have demonstrated its role in proteoglycan degradation and synthesis (90). The presence of both PDGF/FGF has been found in both synoviocytes and chondrocytes during the progression of OA (90). Although FGF has been shown to increase mitotic activity in chondrocytes by increasing cellular division and proliferation, its precise role in OA is still under investigation (91). One should note that FGF constitutes a family in which different effects could be mediated by different FGF. Studies demonstrate that it is not one growth factor or cytokine alone that helps promote the anabolic mechanism of the articular joint but the presence of all of these factors working in synergy creating an overall anabolic environment (91).

iii. Transforming growth factor-β (TGF-β)

The TGF-β family of proteins consists of 30 members, three of which are TGF-βs, five activins and over 20 bone morphogenetic proteins (92). TGF-β plays an important role in inhibiting cell proliferation in many types of cells such as epithelial and hematopoietic cells and has been shown to limit tumor cell proliferation (92). In OA it plays a role in inhibiting enzyme release from different cells types and promoting cell synthesis of TIMPs (93). TGF-β has been found in both chondrocytes and synoviocytes and is activated during inflammation periods of the joint (94). Another role associated with this growth factor in OA, is the increased synthesis of proteoglycan and specifically the smaller proteoglycans found in the ECM (95).

The two 12.5 polypeptides that make up TGF-β are joined by a disulfide bond (96). The synthesized TGF-β is present in an inactive form called LTBP with a prosegment which designates the protein for the ECM (96). The effects of the TGF-β are often affected by other factors in the ECM which can bind the growth factor, thereby inhibiting it from binding to its receptor to mediate a cellular response (96). The two main receptors used by TGF-β are receptors type I and type II, which are structurally similar to serine/threonine kinases (97). There are far more ligands which are specific for these receptors than there are TGF-β on the cellular surface thereby creating a competition for the ligands to bind the appropriate receptor (97). These receptors are able to cooperate together in complexes with the effector Smad molecules within the cell to affect a greater versatility of signaling (92). Smad molecules also interact with various transcription factors to initiate or inhibit gene transcription (92). Indeed, from a relatively simple pathway of signaling, TGF-β ligand to receptor type I or II to the Smad signaling cascade, the TGF-β superfamily is able to mediate many different cellular functions (98).

The Smads are the principal signaling cascade that is used by TGF-β in the cell and they can be divided into three subgroups 1) R-Smads which are receptor activated Smads and are the first in the cascade, 2) Common Smads (Smad 4) which are the subsequent Smads activated in the cascade, and 3) Inhibitory Smads which regulate and control the signaling cascade from within the cell (92). The R-smads are characterized by a C-terminal SXS motif in which both serines are phosphorylated by the receptor type I (92). TGF-β binding to the receptor type I can lead to the receptor recruitment of the R-smads, such as Smad 2 and Smad 3, and Smad 1, 5 and 8 with BMP signaling (92). So depending upon the type of ligand, a different type of signaling Smad is activated and thus a different type of cellular response is initiated (97). Other than the interaction of the R-Smads with the receptor type

1, R-Smads can also be activated by the presence of various cytoplasmic kinases (99). Many kinases are able to interact with the different Smads, the Protein Kinase C, Calcium/ Calmodulin dependent protein kinase and Akt (99).

The inhibitory Smads, Smad 6 and Smad 7 are able to inhibit the Smad signaling cascade through their ability to interact with the R-Smads (100). They do so by binding to the active site on the receptor type I complex thereby blocking the binding of the R-Smads and the continuous Smad signaling cascade (100). The level of complexity of the signaling in terms of different effects it can have on the cell is increased by the fact as mentioned above that the Smads are able to intereact with many different transcription activators (100). They activate transcription of genes through their cooperation with these other transcription factors (97). Indeed, R-Smads alone, excluding Smad 2, have a relatively low affinity for DNA but this is increased through interactions with a number of different transcription factors (101). They interact with these transcription factors through their MHI or MH2 domains depending upon the type of cofactor it binds to (92). The first cofactor found was the FAST/FoxH1 which is a forkhead transcription factor and was shown to interact with with Smad 2/4 complexes in response to activin binding to the TGF-β receptor (102). An example of this cooperation is seen in endothelial cells in which TGF-β activates the expression of p21, the FoxO cofactor binds to the Smad 3 /4 complexe to help induce transcription of p21 gene (103). In the p21 promoter, Smads are also able to bind to the Sp1 transcription factor site to regulate the expression of the gene indicating a possibility of many Smad complexes binding to one particular promoter in an effort to influence its expression (104).

Finally TGF-β is able to activate signaling cascades other than the Smad; it can activate the MAPK kinases, ERK 1/2, JNK and p38 which can in turn and as discussed above, influence

the Smad signaling cascade (105). This once again demonstrates the level of complexity of the reactions that these ligands can have on the cell and how it is very difficult to anticipate the response of the cell to these ligands (105).

D. Bone Morphogenetic proteins (BMPs)

BMPs are part of the TGF-β superfamily of proteins and have been shown to play a role in embryonic development and other various cellular functions in both adults and infants (99). Over 20 different members of this subgroup have been identified based on their sequence homology (106). BMPs are secreted from the cell with a hydrophobic stretch of 50-100 amino acids (106). This precursor is cleaved upon activation and is characterized by seven cysteines that are used to dimerize with another monomer (106). The BMP precursor is cleaved by convertases such as the serine endoprotease furin (107). Most BMPs have a molecular mass in the range of 20 000 to 30 000 daltons (107). Interestingly, BMP-1 is unrelated to other BMPs in that it is not involved in cell differentiation and growth (107). This BMP acts as a protease and can cleave procollagen fibers, BMP-2, BMP-7 and the antagonist chordin (107).

BMPs have been linked to many different processes within the cell including cell proliferation, differentiation, migration, apoptosis and in angiogenesis (108). Although their name is indicative of their primary role in bone morphogenesis, some of these proteins, in particular BMP-2, -7 and have been shown to stimulate proteoglycan synthesis in chondrocytes (108). This anabolic effect on the articular cartilage is followed by another possible effect in that stimulation by BMPs can lead to dedifferentiation of chondrocytes, calcification and bone formation (108). BMPs have also been shown to stimulate chondrocyte maturation and chondrocyte function which stimulates the production of

collagens type II and type X (107). When BMP-2 and -4 are overexpressed in developing limbs an increase in the number of chondrocytes has been observed and subsequent increase in matrix cartilage (107). BMPs play an important role in chondrogenesis which is the differentiation and maturation of chondrocyte cells by stimulating the expression of the Sox-9 gene which is involved in the evolution of the chondrocyte (107). Studies have shown that BMP-7 is a more potent stimulator of synthesis of cartilage molecules than TGF-β (109). Indeed, in vivo studies have shown that stimulation of the knee joint with BMP-2 and BMP-9 causes an increase in proteoglycan synthesis that is higher than the effect of TGF-β stimulation (110).

Interestingly while the stimulation by BMP is higher, it is shorter in duration than the effect that TGF-β has on the proteoglycan synthesis in the knee joint (110). Another interesting finding is that both TGF-β and BMP signaling converge upon the same effector which is Smad-4 leading to a possible competition for the Smad-4 to initiate changes within the cell (107). This convergence of pathways can either inhibit or increase the effects of the TGF-β and BMP ligands in the cell depending upon the cellular environment at the time.

1-BMP receptor and signaling

The receptors responsible for BMP signaling are two distinct serine/threonine kinase receptors that can pass along the signal to effector Smad proteins, the same type of receptors used by TGF-β ligands; receptor type I and receptor type II (107). The type 1 receptor can be subdivided into four different type; type IA or activin receptor-like kinase ALK-3, type IB or ALK-6, ALK-2 and ALK-1 (108). When the BMP ligand binds to the type I receptor, the receptor type II forms a heterodimer with it and the kinase activity of the type II receptor activates the type I receptor leading to the initiation of the signaling cascade (111).

The different types of receptor type I allows for diversity in signaling which is essential in determining the impact of the ligand binding on the cell. The type of signaling cascade depends upon wether the receptor heterodimer formation occurred prior to or after BMP ligand binding to the type I receptor (107). If the heterodimer formation was already in place before BMP ligand binding, then the Smad signaling cascade is activated (107). When the BMP ligand itself induces the formation of a receptor heterodimer, it is the MAPK pathway that is activated (107). When the BMP ligand binds first to the receptor type II, there is activation of the receptor type I which intitiates the signaling cascade (108). As for the type 1 receptor, the type II can also be subdivided into three different types; BMPR2, ActR-II and ActR-IIB (108). The main reason why BMPs bind to the receptor type II is because the ligands have a low affinity for the type I receptor and that the type II receptor is specific for the BMP ligand (107).

A number of signaling pathways other than Smads have been associated with the BMP ligand, these include the JAK/STAT, the calcium/calmodulin and p38 (106). The latter leads to cell apoptosis but the main signaling cascade used by the BMP ligand is the same as the TGF-β ligand; the SMAD signaling cascade (108). The Smads activated by the receptor type I from BMPs are Smad -1, -5 and -8 which then bind to Smad 4 which aids in the translocation to the nucleus for cellular gene transcription initiation or inhibition (108). As mentioned above the difference between the BMP mediated signaling and the TGF-β mediated Smad signaling is that TGF-β uses Smads 2 and 3 while BMPs use Smad-1 and -5 (107). Both pathways converge upon Smad-4 which is necessary for translocation to the nucleus and DNA binding (107). BMPs activated signaling can be potentiated or inhibited by a number of cofactors within the cell which increases the diversity of the effect BMPs can have on the cell.

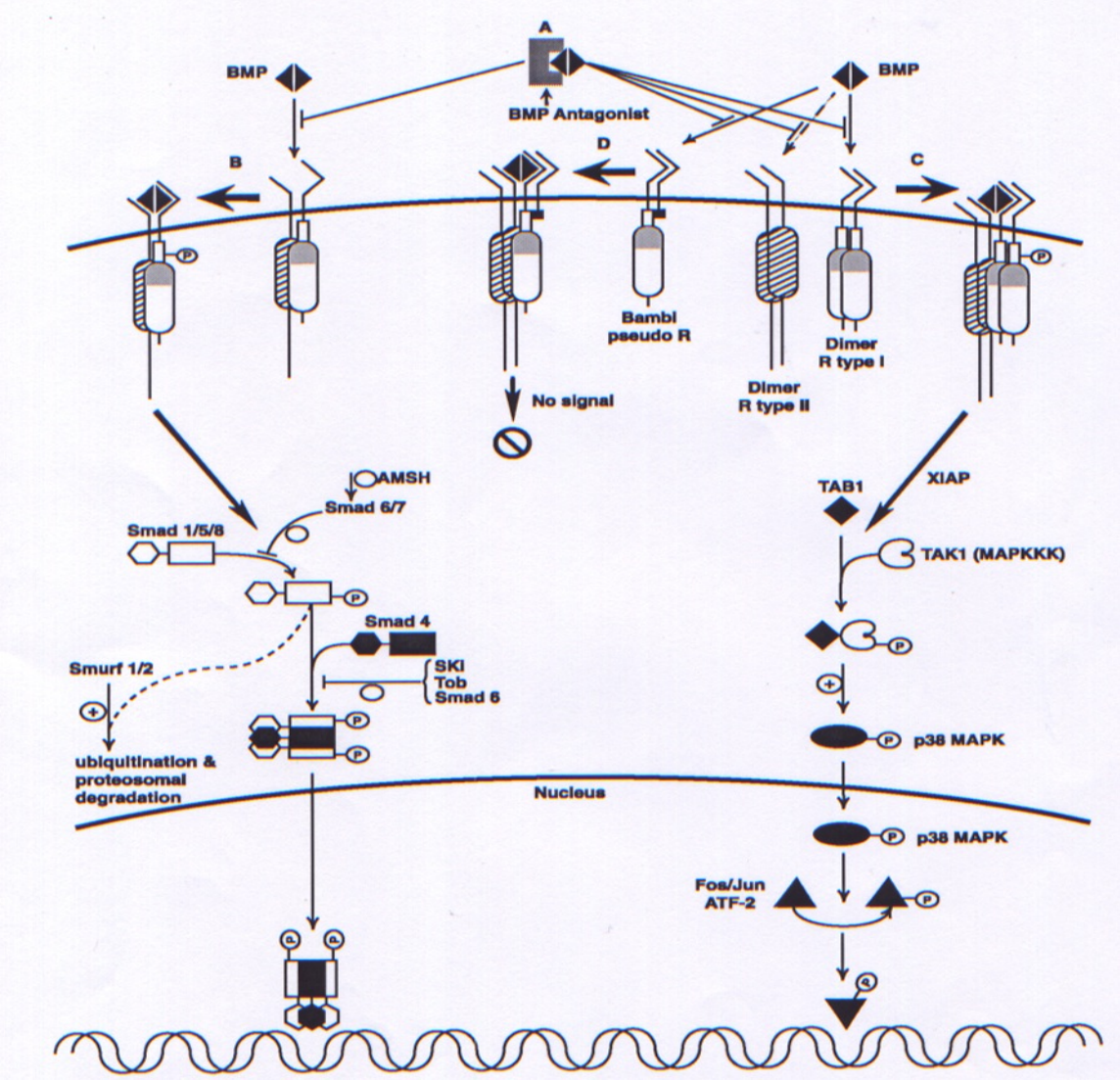


Figure 4. Mechanisms of BMP signaling (107)

Studies have shown that the transcription factor Runx-2/Cbfa-1 interact with Smad-1 and Smad-5, which play an important role in translocation to the nucleus of the BMP signal (112). It has also been show that in chondrocytes, BMP-2 induces Runx-2/Cbfa-1 expression as this cofactor is necessary in BMP-2 signaling (113) Figure 4 illustrates BMP initiated signaling within the cell, the different inhibitors and factors involved and the two

major pathways activated by the BMP ligand; Smad and MAPK kinases (107). As is the case in many pathways in the cell, there are often inhibitors both within the cell and outside that play an important role in the regulation of signaling.

Within the cell there are two main mechanisms of BMP signaling repression: inhibitory proteins that block the activities of the Smad molecules and pseudoreceptors incapable of initiating a cellular response (114). The pseudoreceptor that binds both BMP and TGF-β ligands is called BAMBI and it contains extracellular domains similar to the type I receptor for both ligands (114). The BAMBI receptor is able to associate with the BMP type I receptors thereby inhibiting their actions without interfering with the BMP ligands themselves (114). Studies have shown that BAMBI is co-expressed with BMP-4 indicating a possible negative feedback loop in which the BMP pathway is regulating itself (107). The second pathway is through proteins within the cell blocking the signaling cascade, the major proteins are the inhibitory Smads; Smad 6 and Smad 7 (115). They both contain MH2 domains which are necessary for protein interactions, but lack the MH1 domain which enables them to bind to DNA and affect gene transcription (107). These inhibitory Smads replace Smad 1 and 5, are phosphorylated by the BMP type I receptor, and effectively blocking further signaling (115). They can also bind transcription factors preventing association with other Smads (107). For example Smad 1 binds the repression transcription factor Hoxc-8 to remove it from DNA (116). The inhibitory Smad 6 will bind Hoxc-8 to block the action of Smad 1 allowing the Hoxc-8 transcription factor to continue inhibiting gene expression (116).

Besides the inhibitory Smads there are other proteins that inhibit Smad signaling such as the Ski and Tob (117). The Tob protein is a member of a family of anti-proliferative proteins and inhibits BMP activity in osteoblasts by blocking the activity of Smad-1 and -5 (117).

The Tob gene is expressed in osteoblasts when BMP-2 is increased indicating a negative feedback loop in osteoblasts (117). The other is the Ski protein which is an oncogene and blocks BMP signaling by binding to the MH2 protein binding domain of Smad -1, -4 and -5 before the formation of heterodimeric protein complex forms (118). Studies have shown that the lack of this protein in embryonic development leads to death of mice indicating the importance of this protein in regulating the actions of BMPs (119).

The main intracellular mechanism of BMP inhibition occurs through the blocking of the Smad pathway directly through the inhibition of Smad-protein interactions or the inhibition of Smad-DNA interactions. The following section will discuss the extracellular regulation of BMP actions; the BMP antagonists and their role in OA.

IV. THE BMP ANTAGONISTS

BMP antagonists are molecules that inhibit the activities of BMPs by binding to them and blocking them from binding to the BMP receptors. There are many different types of BMP antagonists; noggin, chordin, follistatin, follistatin-related gene (FLRP), ventroptin, twisted gastrulation (Tsg) and the Dan/Cerberus family of genes which includes the head inducer Cerberus, the tumor suppressor Dan, the protein related to Dan/cerberus (PRDC), caronte, Dante (Dte) and gremlin (107). The number of antagonists that exist to inhibit the functions of the BMPs indicates the level of importance these factors play in development and overall cellular functions. Differing roles have been found for the BMP antagonists depending upon the cell type and the stage in the life cycle at which the BMPs are expressed.The BMP antagonist noggin is a polypeptide of about 22 kDa but is secreted as a homodimer of 64 kDa and has been shown to block with varying degrees of specificity BMP-2, -4, -5, -6 and -7 (107). In osteoblasts the levels of noggin expression are increased in response to elevated

BMPs and in chondrocytes elevated noggin expression has been linked to induction by Ihh (Indian hedgehog) (120). Noggin is able to block the effect of BMPs on collagen and non-collagenous protein synthesis in cells of osteoblastic lineage and also has been show to inhibit chondrogenesis and limb development (107).

The BMP antagonist chordin has a molecular weight of around 105 kDa but is secreted as a 120 kDa molecule (121). This is probably due to posttranslational modifications and it is characterized by four cysteine rich domains (107). In osteoblasts chordin expression is low and has not been shown to play a significant role in these cells differentiation (122). However, in chondrocytes it has been shown to play a role in the maturation of chondrocytes (107). Follistatin was initially identified as an activin inhibitor but it has also been shown to inhibit BMP-4 and is involved skeletal development (123). Recently it has shown that the follistatin gene expression was upregulated in human OA chondrocytes and synoviocytes indicating a possible role for the antagonist in the progression of the disease (123). This study also found that some factors were responsible for its up-regulation (123). The ones with the strongest effects were IFN-γ and TNF-α (123). The study also suggested that follistatin appears at a later stage of the OA process as it is induced by inflammatory cytokines (123). Another indication of a linkage between TNF-α and follistatin is that both have been found in higher levels in the superficial zones of OA cartilage, thus nearest to the inflamed synovial membrane (123). Other factors found that affect the expression of the follistatin are TGF-β which induces expression while BMP-2 down regulates its expression (123). Another antagonist is the Twisted Gastrulation (Tsg) which is able to bind chordin/Sog and BMP-4, creating a tertiary complex that has a higher specificity for BMP-4 than either antagonist alone (107).

The Dan family is a group of related glycoproteins that all share a common function of blocking the action of BMPs (107). This family includes seven members: Dan, Cerberus, PRDC, dante, caronte, gremlin and sclerostin (107). Specifically these proteins share a region of homology that unites them: the presence of a cysteine knot in the carboxyl terminal domain which is important in their functionality and can be seen in the tertiary structure (107). Dan has tumor suppressor activity and the protein is about 19 kDa; when it is secreted it usually dimerizes into a homodimer (124). This protein is expressed in embryonic tissues and is not induced by BMP (107). Cerberus as it names indicates is involved in head formation acting as a head organizer and plays a critical role in neural tissue formation (107). The protein is about 31 kDa in size and like the other members of this family undergoes post-translational modifications which enable it to bind primarily BMP-4 (125). Another role for this antagonist is its ability to bind and prevent the actions of the Wnt 8 protein thereby affecting the Wnt signaling pathway (125). Of note, other members of the Dan family have been shown to be involved in this pathway and also play important roles in development as well as in the regulation of the bone morphogenetic proteins (107). As my project focuses on one member in particular, gremlin, it will be discussed in greater detail.

A. GREMLIN

The BMP antagonist gremlin is a protein that has been studied primarily for its involvement in development during the early stages of the life cycle. New evidence suggests that it may also play a role during the progression of OA and respond to the elevated BMP levels found in the articular cartilage during the disease.

The gremlin gene was first cloned from a Xenopus ovarian library (107). It has a role in limb development and axial patterning (126). The rat homolog is Drm (down-regulated by v-mos), v-mos is a viral oncogene, interestingly other viral oncogenes including v-raf and v-ras were found to effect the expression of gremlin/Drm in rat fibroblasts (127). The Drm gene encodes a 20.7 kDa protein that is glycosylated, increasing it weight to 28 kDa at secretion from the cell (127). Gremlin and its rat homolog Drm share 80% amino acid homology, and all forms of this gene across species share this high level of homology (107). It is interesting to note that Drm can be secreted in a glycosylated form but also in a non-glycosylated form, and that both forms undergo phosphorylation (127). The protein can also be found in a soluble and cell associated form, and both have BMP inhibiting capabilities (107). Gremlin expression has been seen in various types of tissue; such as in the brain, the kidneys, the testis and recently in the articular cartilage and synovial membrane (107). The main activity of this protein is to inhibit BMP activities in these different tissue types (107).

1-Roles of Gremlin in different tissues and stages of development

Gremlin plays a role in fibrosis which is characterized by an increase in the production of the ECM components collagens I and II, proteoglycans, fibronectins and hyaluronic acid (128). This cellular event occurs in all types of tissue: the liver, pancreas, kidney, lung, skin as well as in the synovial membrane (128). Boers et al. used hepatic stellate cells (HSC) which are the liver cells in which fibrosis occurs and determined which genes were upregulated when the cells were undergoing fibrosis (128). Their study demonstrated an increase in the expression of the two BMP antagonists follistatin and gremlin. They suggest that a possible role for gremlin, as a pro-fibrotic factor, is its ability to block BMPs (128). This was based upon results indicating that stimulation with BMP-7 reduces the fibrotic

change of the cells (128). This is interesting considering that gremlin binds preferentially to BMP-2,-4 and -7 (128).

Gremlin overexpression in transgenic mice has been shown to cause osteopenia and to reduce bone density by 20-30% resulting in impaired bone formation (129). Gazerro et al. showed that transgenic mice overexpressing the gremlin gene had disorganized collagen bundles and a 70% decrease in the number of osteoblasts (129). These changes in phenotype were related to the gremlin's inhibition of BMPs (129). However, they postulated that gremlin might have the ability to bind and inhibit other signaling pathways since their results demonstrated a reduction in Wnt signaling (129). Wnt proteins and signaling are involved in many processes that occur in development including cell proliferation and osteoblast differentiation (129). They tested this hypothesis by using another BMP antagonist, noggin, which binds BMP-2,-4 and -7 but with a higher specificity than gremlin in the same type of cells (129). Interestingly, they found that noggin had the same ability to reduce Wnt signaling thereby indicating that gremlin principal role was the antagonism of BMPs (129).

On the other hand, Chen et al. showed a new role for the gremlin protein by demonstrating that overexpression of gremlin in tumor derived cell lines increased the expression of the cell cycle inhibitor p21 (130). They demonstrated that most tumor derived cell lines fail to express the gremlin gene, they induced its expression to observe the effects that this change would have on the cells (130). Data indicated that gremlin has tumor suppressor ability in that its overexpression was able to inhibit the neoplastic phenotype of the tumor cell lines (130). This ability coincided with an increase in the expression of the transcription factor p21 indicating a possible role of gremlin (130). They tested various pathways to determine

how gremlin exerts its effects (130). Data showed that it was not through the signaling cascades p53 or through the MAPK kinases including ERK 1/ 2 and p38 (130). Although, it is well know that gremlin's principal action is to antagonize the activities of BMPs, they stimulated the cells with BMP -2 and -4 and found that they did not increase tumor cell proliferation or growth (130). Another interesting finding was that they failed to detect the presence of these BMPs in the tumor cell lines (130). They concluded that gremlin was exerting its effects through a novel pathway which is able to increase the expression of the p21 cell cycle inhibitor (130). However questions that remain to be answered are: is gremlin causing this change, if so, is it through its own receptor, is it gremlin itself within the cell causing these changes and can gremlin act as a transcription factor itself?

Gremlin's role other than only being an antagonist of BMP was suggested by Stabile et al by showing that gremlin could act as a pro-angiogenic factor (131). Gremlin is a well known BMP antagonist and BMPs have been shown to be involved in angiogenesis, specifically BMP-4 (131). However, the authors demonstrated that the presence of BMP-4 has no influence on gremlin's ability to interact with the endothelial cell receptors, and their results indicate that gremlin exerts its effects through a BMP-independent pathway (131). The authors also suggest that gremlin may be acting through a specific receptor and initiating changes within the cell (131). Lastly gremlin has been shown to be involved in the pathogenesis of OA (123). In this disease, gremlin was found to be upregulated in chondrocytes (123). This upregulation may be linked to the elevated levels of BMPs in the OA pathogenesis.

V. PURPOSE OF THE STUDY

The purpose of this study was to define the role and regulation of gremlin in OA cartilage and to determine if it played a different role other than being a BMP antagonist. To understand gremlin's role in OA, the approach used for this study was threefold. First we wanted to look at what factors control the expression of gremlin through the cloning and characterization of its promoter region. The cloned promoter was used in studies to determine its activity in OA chondrocytes when stimulated with different factors and the promoter was further characterized through the creation of deletion constructs. This enabled us to understand which regions of the promoter were important in controlling the expression of the gremlin gene in chrondrocytes. Secondly, we wanted to determine if increased levels of gremlin could affect directly the expression of other genes thereby influencing the cellular environment. This was accomplished through the use of microarray technology which enabled us to determine which genes were affected in chondrocytes when these cells were stimulated with gremlin. Lastly we wanted to understand if gremlin can mediated signaling cascades on its own and exert an effect on the cell other than through the BMP triggered cascade. This was accomplished through the use of a BMP neutralizing antibody and western blotting.

This three pronged approach enabled us to understand in more depth how gremlin exerts its effects, what can control the expression of this gene and finally to understand if it has a role other than being a BMP antagonist during the progression of OA.

MATERIALS AND METHODS

CELL CULTURES

Human OA cartilage was taken from patients undergoing total knee arthroplasty (123). These patients were evaluated by a certified rheumatologist and diagnosed with OA (123). The diagnosis was confirmed by microscopic analysis and all the specimens represented moderate to severe OA (18). The use of human specimens was approved by the institutional Ethics Committee Board of the Hopital Notre-Dame.

Chondrocytes were taken from full-thickness strips of articular cartilage by sequential enzymatic digestion at 37°C using 1mg/ml of pronase (Boehringer Mannheim Biochemicals, Indianapois, IN). The cells were further incubated in the presence of 2 mg/ml collagenase for 6 h (collagenase type 1A; Sigma Chemical Co., St.Louis, MO) at 37°C with DMEM (Gibco-BRL Canadian Life Technologies, Burlington, Canada) including 10% heat-inactivated Fetal Calf Serum (FCS) (Hyclone, Logan, UT), 100 U/ml penicillin (Gibco-Brl), and 100 μg/ml streptomycin (Gibco-BRL) (29). The cells were then plated in tissue culture flasks containing DMEM with 10% FCS and antibiotics at 37°C in an atmosphere humidified at 5% CO2/95% air, until they became confluent.

For the microarray experiments primary chondrocyte cultures were used while first passage chondrocyte cultures were used in the transfection, western blot and PCR experiments. To assay the effect of gremlin on gene expression, confluent cells were grown in DMEM 0.5 % FCS for 24 hours and subsequently stimulated by gremlin (R&D, Minneapolis, MN) at 10 ng/ml and 100 ng/ml. The cells were incubated for a period of 24 h for RT-PCR assays and 48 h for protein production (123).

RNA EXTRACTION, REVERSE-TRANSCRIPTION (RT) AND REAL-TIME QUANTI-TATIVE PCR (qPCR)

Total cellular RNA was extracted from OA chondrocytes with Trizol Reagent according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). The extracted RNA was treated with the DNA-free DNase Treatment and Removal kit (Ambion, Austin, TX) thereby ensuring the removal of chromosomal DNA (123). The RNA was quantified using the Ribogreen RNA Quantification Kit (Molecular Probes, Eugene, OR). The RT reactions were primed with Random Hexamers with 2 μg of total RNA in a 100 μl final volume. Real-time qPCR analysis was performed with the Rotor-Gene RG-3000A from (Corbett Research, San Fransico, CA) with 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and used according to manufacturer's specifications (123). 25 ng of the cDNA produced from the RT reactions were amplified it in a total volume of 50 μl consisting of 1X Master Mix, uracil N-glycosylae (UNG; 0.5 units; Epicentre Technologies, Madison, WI) and the gene-specific primers (table 1), which were added at a final concentration of 200 nM (123). The tubes were first incubated for 2 minutes at 50°C (UNG reaction), then at 95°C for 15 minutes (UNG inactivation and polymerase activation), then 40 cycles consisting of a denaturation period for 30 seconds, an annealing period for 30 seconds at 60°C, an extension period at 72°C for 30 seconds followed by a data acquisition phase at 77°C for 15 seconds. The Corbett Research software collected the data, processed it and gave it a threshold cycle (Ct) corresponding to the PCR cycle at which an increase in reporter fluorescence above a baseline signal can be detected (123). The change in

Table 1. PRIMER LIST

GENE	GENE SYMBOL	SIZE (bp)	SEQUENCE	SENSE OR ANTISENSE
Apolipoprotein A-II	APOA2	232	TCGCAGCAACTGTGCTACTC	S
			GTTCCAGCCTTCTTGATCAG	AS
Autoimune Regulator	AIRE	252	GTGTACTCACTGCGCCGC	S
			AGGATGCCATCGAAGGTGTG	AS
Cell division cycle associated 7,variant 1	CDCA7	252	TCATCCTCTGATGACAGTTG	S
			TGATTCATCGTCAGAGTCG	AS
Chemokine Receptor 4	CCR4	221	TGTCATGCTCATGAGCATTG	S
			GAACCTTCCACGTCGTGGAG	AS
Dopamine Receptor 4	DDR4	200	ACCAACTCCTTCATCGTGAG	S
			AACCTGTCCACGCTGATG	AS
Fibrinogen Gamma Chain	FGG	249	CATCAGAAGTCAAACAGCTG	S
			CCTGGCACTGTGCTTCAAG	AS
Glial Cells Missing Homolog 1	GCM1	232	AAGATCTACCTGAGACCTGC	S
			TCATGGCTCTTCTTGCCTC	AS
Inhibitor differentiation 1	Id1	243	GCTGTCTGTCTGAGCAGAG	S
			CGGATTCCGAGTTCAGCTC	AS
Inhibitor differentiation 3	Id3	256	CTGTCGGAACGCAGTCTGG	S
			CTGTCTGGATGGGAAGGTG	AS
killer Cell Lectin-Like Receptor Subfamily F Member 1	KLRF1	229	GAGTTCTGCCCAAACATCTC	S
			GATCTCCAGTGTCCTCATAC	AS
Natriuretic Peptide Precursor C	NPPC	299	CATCTCTCCCAGCTGCTG	S
			GCTCCTTTGTATTTGCGC	AS
RAS Protein Activator Like 1	RASAL1	256	GCCTTCTACGTGCTGGATG	S
			ATGTGCCAGAGATGTCTCTG	AS
Retina and Anterior Neural Fold Homeobox 2	RAX2	166	CAAGAAGAAGCACCGGAG	S
			GCGGTTCTGGAACCACAC	AS
Zinc Finger and SCAN Domain Containing 4	ZSCAN4	246	GGAGAGATTCATAGAAGACC	S
			TTCCAGCCATCTTGTTCATC	AS

gene expression following stimulation with the various factors was calculated using the following formula: fold change= 2-Δ(ΔCt), where ΔCt= Ctstimulated - ΔCtGAPDH and Δ(ΔCt)= ΔCtstimulated- ΔCtcontrol (123). The primer efficiencies for the test genes were the same as that for the housekeeping gene GAPDH.

TRANSIENT TRANSFECTION ASSAYS

Calcium Phosphate Protocol

Transient transfection assays for promoter A were performed using the calcium phosphate coprecipitation method developed in our laboratory (132). The cells were grown in 10% DMEM/ 5% streptomycin and penicillin antibiotics. Once confluent they were transfected with a DNA-calcium preparation consisting of 125 mM CACl2 and 3 μg of the plasmid (132). Ater 5 hrs, the cells underwent a glycerol shock for 2 min at room temperature and cells were rinsed with PBS. The cells were then placed in 10% DMEM/5% streptomycin and penicillin for 24 hrs. The following day the cells were stimulated with BMP-2 (10 ng/ml), IFN-gamma (10 ng/ml) and CyclosporineA (CSA) (10 U/ml) for 24 h in 10% DMEM/ 5% antibiotics. Cellular proteins were extracted using the Reporter Lysis Buffer (Promega).

Transfectin Protocol

Transient transfection assays for promoter B were performed with the Transfectin lipid reagent (Bio-Rad, Hercules, CA), chondrocytes from first passage cultures were plated in a 12 well plate at 300 000 cells/well in 10% DMEM/5% streptomycin and penicillin antibiotics and immediately transfected with a mixture of the Transfectin lipid reagent (2 μl)

for 2 μg of plasmid. After 4-5 hrs incubation time, the medium was replaced for fresh DMEM containing 10% FCS and incubated overnight. The following day, BMP- 2 (10 ng/ml), IFN-γ (10 ng/ml), CSA (10 U/ml) were added and the cells were incubated for 48 h at 37 °C. Cellular proteins were extracted using the Reporter Lysis Buffer following the manufacturer specifications (Promega, Madison, WI).

Luciferase Activity

Luciferase activity was measured with the Lumat LB 9507 luminometer (EG&G, Berthold, Bad Wildbad, Germany) (123). The total protein concentration was measured using the bicinchoninic acid method (Pierce, Rockford, IL) (123). Promoter activity was calculated as relative luciferase units per microgram of protein and expressed as a percentage of the control.

Data Analysis

The values are expressed as mean ± SEM and statistical significance was measured using the Mann-Whitney U test, a p value< 0.05 was considered significant.

PROTEOME PROFILER ARRAY

To identify the signaling cascades affected by gremlin stimulation of the OA cells we used the proteome profiler array (R&D systems, Minneapolis, MN). The system uses specific signaling antibodies directed against the different phosphorylated kinases. It allows for the determination of phosphorylation of 9 MAP kinases and 9 serine/threonine kinases which represent the major MAP kinases in the cell (ERK 1/2, JNK 1-3, p38 isoforms, AKT, GSK-3, p-70 and S6). The chondrocyte cells were treated at different times (0, 30 min, 2 h and 6

h) with gremlin at a concentration of 100 ng/ml in the presence of a neutralizing anti-BMP 2/4 antibody (R&D systems, Minneapolis, MN) at a concentration of 25 μg/ml. The results were subsequently analyzed by densitometry analysis using the Total Lab Software (nonlinear dynamics, Durham, NC).

WESTERN IMMUNOBLOT

First passaged OA chondrocytes were seeded in 12 well plates at 300 000 cells/well with 10% DMEM/antibiotics as described above and grown for 72 h to confluence. The cells were then placed in low serum concentration 0.5% FCS /DMEM/antibiotics for 24 h, after this period the medium was replaced for fresh 0.5% FCS/DMEM/antibiotics and the cells stimulated with gremlin 100 ng/ml (R&D systems, Minneapolis, MN) for a period ranging from 10 min to 6 h. Cellular proteins were extracted using 0.5% SDS with inhibitors aprotinin (1 mg/ml), leupeptin (1 mg/ml), PMSF (1mM), sodium-orthocanadate (1 mM) and Pepstatin (1 mg/ml). The protein concentration was determined using the Bicinchoninic acid method (Pierce, Rockford, IL) and 20 μg of the protein were electrophoresed on a discontinuous 4-12% SDS gel polyacrylamide. The proteins were transferred onto a nitrocellulose membrane (Hybond C extra, Amersham). The membrane was placed in a blocking solution consisting of SuperBlock Blocking buffer (Pierce, Rockford, IL) and TBS (Tris 20 mM pH 7.4, NaCl 137 mM) with 5% skim milk for a 24 h period. Following this the membrane was washed in TTBS (Tris 20 mM pH 7.4, NaCl 137 mM and 0.1% Tween 20) twice for 15 min. The first antibody was added and incubated overnight with the membrane. The primary antibodies used were a mouse anti-phosphorylated FAK (Calbiochem, San Diego, CA) diluted 1/10 000, a rabbit phospho-p90 Rsk (Cell signaling, Boston, MA) diluted 1/ 10 000, a mouse anti-phospho-MSK2/RSKB (R&D systems, Minneapolis, MN) diluted 1/5000, a rabbit phospho-GSK 3 α/β (Cell Signalling) diluted

1/5000, a mouse phospho-AKT1 (SantaCruz Biotechnology, SantaCruz, CA) diluted 1/5000 and a mouse phospho p42/44 (Cell Signalling, Boston, Ma) diluted 1/5000. Following the incubation, the membrane was washed 6 times for a period of 5-10 minutes each in TTBS 1X. The secondary antibody, either a anti-mouse or anti-rabbit diluted 1/25 000 to 1/50 000 was added for a period of 1 h and detection was accomplished by chemiluminescence with the SuperSignal ULTRA Chemiluminescent substrate (Pierce, Rockford, IL).

CLONING OF THE 5'FLANKING REGION OF GREMLIN

In silico analysis of the upstream region of the gremlin gene sequence demonstrated two possible promoter regions separated by an intron of 12kb.

Promoter A

The promoter A region of the gremlin gene (GRM A) was obtained using the PromoterFinder DNA walking kit (Clontech, Mountain View, CA) which involves a primary and a secondary nested PCR reaction (133). There are four human genomic librairies in the kit with each one digested by a different restriction enzyme: EcoRI, DraI, PvuII and SspI. These librairies are a pool of specially prepared DNA fragments that enable the isolation and cloning of specific pieces of DNA. The DNA fragments of the librairies are ligated at the 5'-end to a linker to which specific primers can hybridize, a primary primer or outer primer called AP1 which is provided by the kit and a gremlin gene specific primer 5'-CCCAAGAGGAGAAGCAGG-(AS). This primary PCR mixture is used as a template after dilution for the secondary or nested PCR reaction, which uses the nested adaptor primer AP2 provided in the kit and a nested gremlin specific primer 5'-GTGTAGGCTGTGCGGCTC-(AS), which produced a single 2.5 kb PCR product of the region of interest, the gremlin promoter A. The PCR parameters were: 40 cycles at 94°C for

30 seconds, 65°C for 30 seconds and 72°C for 60 seconds. The PCR fragment produced was cloned into pCR II vector (Invitrogen, Carlsbad, CA) using a DNA ligase (Invitrogen, Carlsbad, CA) to ligate the vector and the PCR fragment. The fragment was re-amplified with primers that contained a Sac1 or Bgl II restriction site, 5'-GTCAGATCTAAGAAAGGAAGCACAGAG-(AS) and 5'-CTAGAGCTCAATG GTGTTTACTTACC-(S). These re-amplified fragments were digested with Sac1/Bgl II and subcloned into the SacI/BglII sites of the luciferase reporting pGL3 vector (Promega, Madison, WI) The PCR reaction for the subcloning was as follows: at 95˚C for 1 min followed by 25 seconds at 94˚C and 72˚C for 4 min (7 cycles). The next 32 cycles were heated at 94˚C for 25 seconds, then at 67˚C for 4 minutes and finally the PCR tubes were cooled at 67˚C for 4 minutes.

Promoter B

The second potential promoter, the one furthest upstream of the gremlin gene was cloned using the BD advantage-GC genomic PCR kit (Clontech, Mountain View, CA) to enable the highly GC rich region to be amplified and studied. The primers used to amplify promoter B are gremlin specific primers 5'-CCTTATGCCTTCCTCCTGATTGGACAA TGG(S) and 5'-TCCTGGGTTGGTTTGCGGCCCTCTTC (AS) and the reagents found in the BD advantage kit which uses a Tth DNA polymerase and GC melt to amplify GC rich elements. The PCR parameters were 95˚C for 1 min followed by 7 cycles for 25 seconds at 94˚C and 72˚C for 4 min. The next 32 cycles at 94˚C for 25 seconds, then 67˚C for 4 min and finally the PCR tubes were cooled at 67˚C for 4 min. The amplified PCR fragment (2.1 kb) was isolated and purified using the GFX PCR DNA Gel Band Purification Kit (GE, Mississauga, ON) and cloned into the pCR II vector, the fragment was subsequently cloned

into the luciferase reporter vector pGL3, using the restriction enzymes SacI and SmaI, to give the gremlin promoter B (GRM B).

Promoter B Deletions

The deletions of the GRM B were accomplished through digestion of the pCR II-gremlin promoter with restriction enzymes and subsequent ligation into similarly digested pGL3 vector. The deletion gremlin 1 fragment (GRM1=0.6 kb) was obtained using the restriction enzymes KpnI and SacI, the deletion gremlin 2 fragment (GRM2 =1.6 kb) was obtained using NsiI and SacI, the deletion gremlin 3 fragment (GRM3=1.1 kb) was obtained using NsiI and PstI and the deletion gremlin 4 fragment (GRM4= 0.2 kb) was obtained using KpnI and PstI. These fragments were consequently subcloned into the pGL3 luciferase reporting vector (Promega, Madison, WI).

MICROARRAYS

The first analysis consisted of microarrays performed on 3 different specimens of OA chondrocyte cells. Triplicate arrays were done to account for changes between each sample; if the same gene was affected in all 3 microarrays, it would be considered as having been influenced by gremlin stimulation. Each sample was split into three wells and grown without and with gremlin at 10 ng/ml and 100 ng/ml gremlin (R&D systems, Minneapolis, MN). The cells were grown to confluence, and then put in DMEM/0.5% FCS 24 h before incubation in the presence or absence of gremlin. Gremlin was added with fresh DMEM/0.5% FCS and the RNA was extracted after 24 h using the reagent Trizol (Invitrogen) according to the manufacturers specifications (123). RNA was extracted, purified, dosed, 10 μg of the extracted RNA was sent to Genome Quebec (Montreal, Quebec) for processing and analysis. The array used was the HuU133plus2.0 GeneChip

Affimetrix which contains oligonucleotides that covers all the human genes. The results obtained were expressed as fold change between the control and the gremlin stimulated cells.

The second microarray was prepared in the same manner as the first but this time the BMP2/4 neutralizing antibody (R&D systems, Minneapolis, MN) was added at 25 μg/ml to all the specimens. The array used was the Agilent « 4x44K » which also covers each human gene. The antibody was added 1hr before the addition of gremlin. The RNA was extracted in the same manner as the first microarray above. The second set of microarrays was sent to Genoscience (Marseille, France) and the results were calculated using fold change as an indicator of a change in genetic expression. The presence of the antibody prevents binding of gremlin to BMP-2 thereby allowing it to remain free and illicit its own cellular response as opposed to blocking the BMPs.

RESULTS

A) CHARACTERIZATION AND CLONING OF THE GREMLIN PROMOTERS

Analysis of the 5'UTR

The gremlin gene is located on chromosome 15q13.3 (Genbank) and in silico analysis of the 5'UTR immediately upstream of the ATG start codon suggests the presence of a promoter region (fig.1A). It contains a TATA box, multiple transcription factor binding sites such as AP1, PEA3 and YY1. A survey of the deposited gremlin mRNA sequences in GenBank shows a gap of approximately 12.5 kb when aligning these sequences with the genomic sequence. This suggests the presence of an intron (figure.1A). This information points to another possible promoter region directly upstream of the mRNA start site (figure1A). This figure is a representation of the 5'UTR of gremlin gene with both potential promoters along with the primers used to characterize it.

The activity of the two possible promoter regions would theoretically yield two different transcripts. RT-PCR experiments were done to validate this hypothesis and the results are shown in figure1B. The results indicate the presence of two transcripts which coupled with the in silico analysis lead to the decision to study both possible promoter regions. The first region, gremlin promoter B (GRM B) is situated 12.5 kb upstream from the ATG start site and the second, gremlin promoter A (GRM A), is immediately adjacent to the ATG start site (figure1A).

The RT-PCR confirmed the presence of transcripts from these two promoter regions (Figure1B). These results point to the possibility that there are two different and active promoter regions found in the 5' untranslated region of the gremlin gene. Alternatively, the transcript from promoter A may be the result from an unspliced transcript from promoter B.

FIGURE 1

DETERMINATION OF POTENTIAL PROMOTER ACTIVITY (A and B) OF THE GREMLIN GENE BY RT-PCR TO DETECT THE PRODUCTION OF mRNA

(A) Schematic representation of the 5'untranslated region of the gremlin gene

/ ----- (12 kb INTRON) --------- /

{----- PROMOTER REGION B -----} {-----PROMOTER REGION A -----} **ATG**

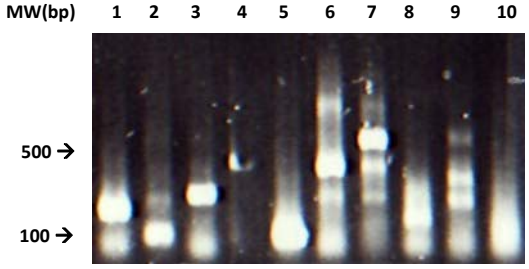
/----------I----------/

→ → → → → → → → ← ← ← ←

PRIMERS 18 17 9 10 11 16 3 15 1 2

(B) Representative gel of RT-PCR results

PRIMERS	FRAGMENT SIZE (bp)
1- 1/ 2	297
2- 16/15	160
3- 11/ 3	374
4- 10/ 3	580
5- 17/ 5	160
6- 17/ 2	560
7- 16/ 2	560
8- 18/15	260
9- 18/ 2	660
10- 18/ 3	170



(A) Schematic representation of the 5' untranslated region of the gremlin gene showing the two different promoter region A and B and the 12.5 kb intron. Also shown are the primers used to determine the activity of the two possible promoters. Primers 1 and 2 were used as control primers for the RT-PCR reaction. **(B)** The total RNA was extracted from OA chondrocytes as explained in the materials and methods and used for RT-PCR to determine transcript expression from both promoters.

The need to confirm these results led us to clone both possible promoter regions to further characterize their activity.

Cloning of the two promoter regions and determination of their basal activity

The details for the cloning of the two promoters are described in the materials and methods section. Cloning of the gremlin promoter A was accomplished using the Promoter Finder DNA Walking Kit (Clontech), which involves a primary, a secondary and or a nested PCR reaction and cloning the fragments into the cloning vector pCRII (Invitrogen). The second promoter region, gremlin promoter B, was isolated through genome amplification, cloning into pCRII (Invitrogen) vector and as with gremlin promoter A, all constructs were then cloned into the luciferase reporter vector pGL3 (Promega) to analyze promoter activity. The pGL3 vector alone without the promoter inserts was used as a control for the experiment. Transfection experiments demonstated that only gremlin promoter B (n=8) was active with an average luciferase activity of 111% when compared to the luciferase activity of the control while gremlin promoter A (n=6) showed no luciferase activity. This result indicates that the transcript from promoter A is likely an unspliced transcript from promoter B.

In conclusion, gremlin promoter region B is the promoter region which controls the expression of gremlin in OA chondrocytes under the conditions used. Promoter A may be active in other cell types under different culture/tissue conditions.

B) CHARACTERIZATION OF THE GREMLIN PROMOTER B

As gremlin promoter B was demonstrated to be the active promoter in OA chondrocytes, further characterization was necessary to elucidate what may regulate its expression. To accomplish this, two methods were used. First, chondrocytes were transfected with the plasmid containing promoter B and incubated in the presence of various factors which could

possibly influence its activity. The three factors chosen were; BMP-2, IFN-γ and cyclosporine A(CsA). The second method was accomplished through the creation of deleted derivatives of the promoter, and measurement of their basal and induced activity

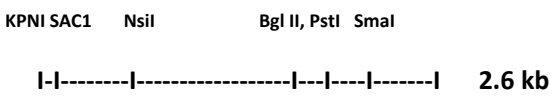
BMP-2 was chosen because it is known to increase gremlin expression in chondrocytes and due to the fact that the main role of gremlin in the cell is to antagonize BMP-2 by binding to it and blocking its actions (123). Cyclosporin A (CSA) is an immunosuppressive drug that inhibits the activity of various cytokines and other genes involved in the immune response through the mediation of the nuclear factor of activated T cells (134). CSA was chosen because it has been shown to be involved in the same cellular processes as gremlin such as angiogenesis and in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis (134). The inflammatory factor IFN-γ was also used because it affects cartilage physiology and has been shown to decrease gremlin expression in OA chondrocytes (123).

The gremlin promoter B (GRM B) was digested with restriction enzymes to create different sized fragments in order to determine which region influences the basal and induced expression of gremlin. The four different fragments produced were called GRM 1, 2, 3 and 4 and are shown in Figure 2. In figure 2A the different restriction enzymes used to create the deleted derivatives can be seen and figure 2B lists the relative basal promoter activity of each deleted derivative in relation to the intact GRM B promoter.

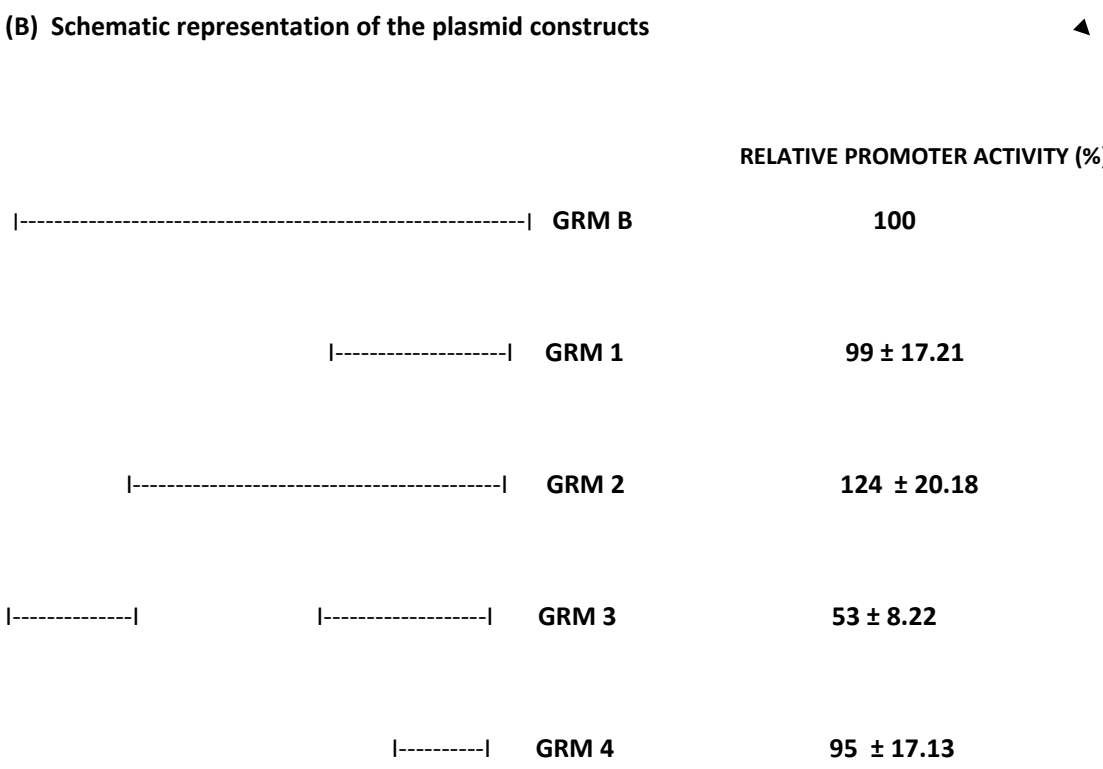
FIGURE 2

PROMOTER ACTIVITY OF THE GREMLIN B PROMOTER AND ITS DELETED DERIVATIVES

(A) RESTRICTION MAP OF THE 2.6 KB GREMLIN B PROMOTER FRAGMENT



(B) Schematic representation of the plasmid constructs



(A) Restriction map of the 2.6 kb gremlin B (GRM B) promoter fragment. The positions of the restriction enzymes on the map are shown. **(B)** The schematic representation of the plasmid constructs and the intact gremln GRM B promoter is shown and the deleted derivatives were constructed as described in the materials and methods section. The table at the right panel illustrates the relative promoter luciferase activity of the constructs in OA human chondrocytes (n=5-8). Promoter activity was measured as unit luciferase/μg of total protein, and data are expressed as relative units of activity with respect to the GRM B promoter, which has been given a value of 100%.

Figure 3 represents the basal and induced activity of the wild type promoter B, while figures 4B, 5B, 6B and 7B show the induced activity of the gremlin promoter deleted derivatives in the presence of BMP2, IFN-γ and CSA. The experiments were designed to analyze the effect of each of these factors by placing the various promoters constructs in the presence of these factors for a period of 24 h and then analyzing the expression levels of the gremlin promoter through luciferase activity. OA chondrocytes (n=5-8) were transfected with the different plasmid constructs and stimulated for 24 h. Promoter activity in the treated cells was compared to that of the intact control promoter GRM B with none of the different factors present and was measured in percentage luciferase activity/ protein concentration with the wild type GRM B promoter given a 100% value.

GRM B INDUCED ACTIVITY

The effects of the different factors on the GRM B promoter can be seen in figure 3B. When induced with BMP-2 the GRM B promoter activity increased by 10%, when induced with IFN-γ the activity of GRM B increased by 6% and induction with CSA 10 U/ml the activity decreased by 5%. Statistical analysis of these results indicates no significant change in expression levels .

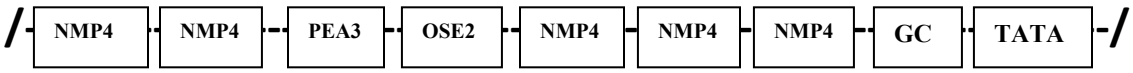
GRM 1 BASAL AND INDUCED ACTIVITY

Deleted derivative GRM 1 was created using the restriction enzymes Kpn1 and Bgl II (fig. 2A). This fragment has a overall basal luciferase activity of 99% (fig. 2B) in relation to the

FIGURE 3

INDUCED GREMLIN PROMOTER B ACTIVITY

(A) Representation of transcription factor sites present on the gremlin promoter B

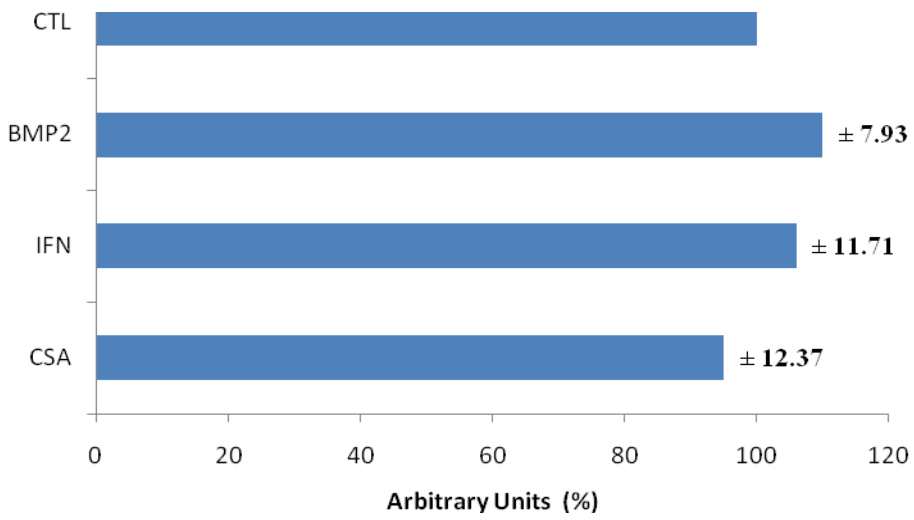


PEA3, OSE2, NMP4 = transcription factors

GC = GC rich region of promoter

TATA = TATA box

(B) Induced gremlin promoter B activity



(A) Representation of various important transcription factors sites found within the gremlin promoter B (GRM B). **(B)** Effect of BMP2, IFN-γ and CSA on promoter activity. Concentrations used for factors are BMP2 (10 ng/ml), IFN-γ (10 ng/ml) and CSA (10 U/ml), OA chondrocytes (n-5-8) were prepared as described in the material and methods section. Promoter activity was measured as unit luciferase/µg of total protein, and data are expressed as relative units of activity with respect to the gremlin promoter B wild type (WT) with no factors added, which has been given a value of 100%.

original promoter B (n=5-8). These results indicate that deletion of over half the promoter segment which includes the transcription factor sites NMP4, SMAD 3/4, OSE2 and PEA 3 has little or no impact on the activity in OA chondrocytes. Figure 4A illustrates the remaining transcription sites on GRM 1: NMP4, SMAD 3/4, OSE2 and PEA 3. NMP4 is the binding site for the NMP4 protein which has been shown to play a role in MMP-13 expression in chondrocytes (31). SMAD site is the downhill effector of the SMAD pathway that can be initiated in response to BMP-2. Figure 4B represents the promoter activity in the presence of the different stimulating factors. BMP-2 increases promoter activity by 11%, IFN-γ decreases it by 11% and CSA decreases it by 31%. These results clearly show that both BMP-2 and IFN-γ do little to change the activity of this promoter construct while CSA appears to have an inhibitory effect on the promoter's activity. Statistical analysis of these results indicates no significant change in expression levels when induced with the factors.

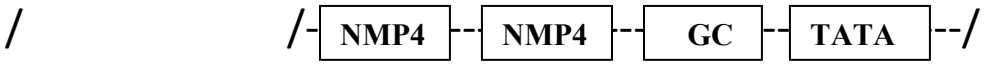
GRM 2 BASAL AND INDUCED ACTIVITY

The second deleted derivative GRM 2 was created using the restriction enzymes Kpn1 and Nsi1 (fig.2A) and its overall activity in human OA chondrocytes (n=5-8) was 124% in relation to the original promoter GRM B (fig.2B). Statistical analysis of this result shows no significant change in expression. The slight increase in expression levels of gremlin could be attributed to the deletion of the region in GRM 2 and the transcription factor sites removed such as NMP4. Figure 5A shows the transcription factor sites remaining on GRM 2: PEA-3, OSE, NMP4, GC rich region and the TATA box. The promoter's induced activity is shown in figure 5B. In the presence of BMP2, its activity decreased by 14%, in the presence of IFN-γ it decreased by 25% and in the presence of CSA it decreased by 35%. These results showed that BMP2 has little effect on its activity while IFN-γ and CSA inhibit

FIGURE 4

INDUCED GREMLIN PROMOTER DELETED DERIVATIVE 1 ACTIVITY

(A) Representation of transcription factor sites present on deleted derivative promoter

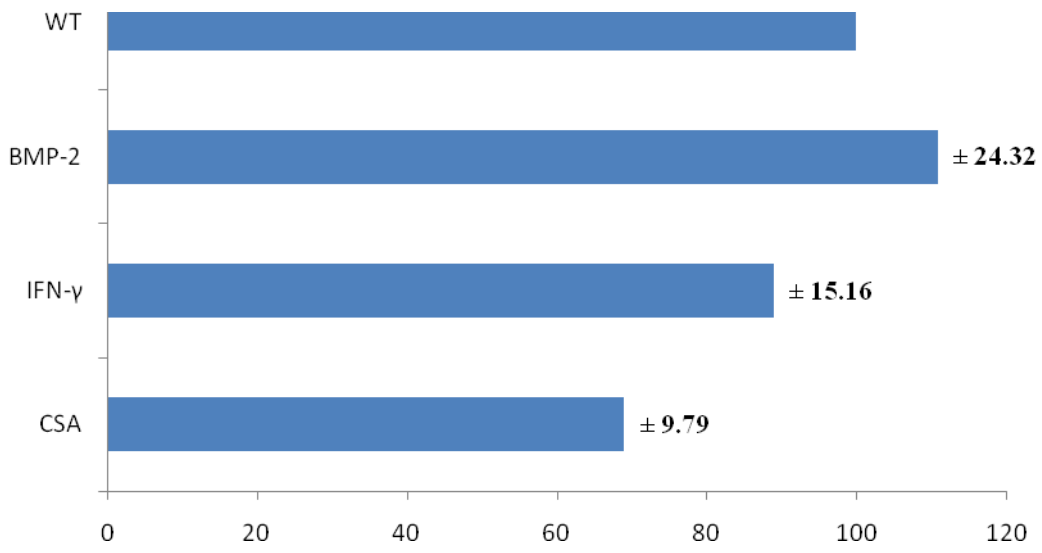


NMP4 = transcription factor

GC = GC RICH REGION IN PROMOTER

TATA = TATA BOX

(B) Induced GRM1 promoter activity

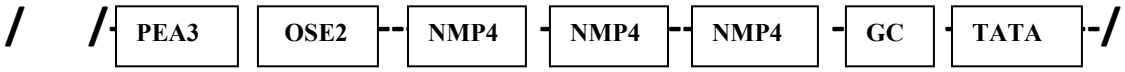


(A) Representation of various important transcription factors sites found within the deleted derivative promoter 1 (GRM 1). **(B)** Effect of different factors BMP-2, IFN-γ and CSA on deleted derivative promoter expression. Concentrations used for factors are BMP-2 (10 ng/ml), IFN-γ (10 ng/ml) and CSA (10 U/ml), OA chondrocytes (n-5-8) were prepared as described in the material and methods section. Promoter activity was measured as unit luciferase activity/µg of total protein, and data are expressed as relative units of activity with respect to the deleted derivative gremlin 1 promoter wild type (WT) with no factors added, which has been given a value of 100%.

FIGURE 5

INDUCED GREMLIN PROMOTER DELETED DERIVATIVE 2 ACTIVITY

(A) Representation of transcription factor sites present on deleted derivative promoter

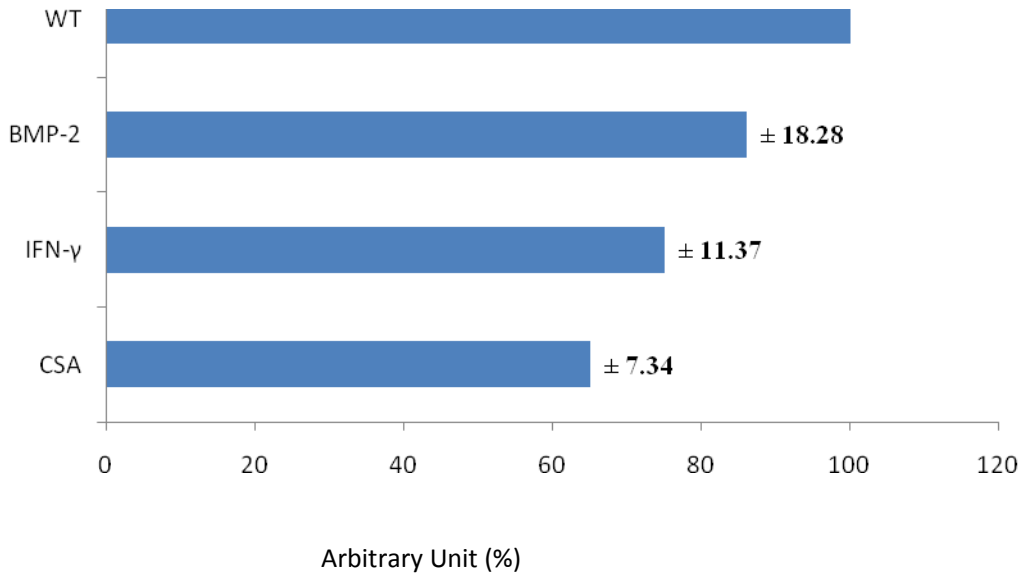


PEA3, OSE2, NMP4 = transcription factors

GC = GC rich region of promoter

TATA = TATA box

(B) Induced GRM2 promoter activity



(A) Representation of various important transcription factors sites found within the deleted derivative promoter 2 (GRM 2). **(B)** Effect of different factors BMP-2, IFN-γ and CSA on deleted derivative promoter expression. Concentrations used for factors are BMP-2 (10 ng/ml), IFN-γ (10 ng/ml) and CSA (10 U/ml), OA chondrocytes (n-5-8) were prepared as described in the material and methods section. Promoter activity was measured as units luciferase activity/µg of total protein, and data are expressed as relative units of activity with respect to the deleted derivative gremlin 2 promoter wild type (WT) with no factors added, which has been given a value of 100%.

the activity of the promoter. Statistical analysis shows no significant change in promoter activity.

GRM 3 BASAL AND INDUCED ACTIVITY

The fragment GRM 3 was created using the restriction enzymes Nsi1 and Pst1 and it has an overall activity of 53% in human OA chondrocytes (n=5-8) in relation to the original promoter GRM B. This is a significant difference in expression levels of gremlin and shows a dramatic decrease in the expression of gremlin when the part of the promoter B between the restriction enzymes NsiI and PstI is deleted. This indicates the importance of this region in maintaining the expression levels of gremlin. The transcription factor sites deleted in this construct are PEA3, OSE2 and NMP4. Figure 6A shows the construct GRM 3 and the transcriptions factors that remained after the deletion reactions such as NMP4, GC rich region and the TATA box region. The promoter's induced activity is shown in figure 6B. Data showed that in the presence of BMP-2, the promoter's activity increases by 13%. When IFN-γ is added the activity of the promoter decreased by 15% and when CSA is added the activity is inhibited by 23%. Statistical analysis of the results shows a significant change in expression levels. The loss of the three transcription factor sites PEA-3, OSE2 and NMP4 causes a dramatic inhibition of the expression of the gremlin B promoter.

FIGURE 6

INDUCED GREMLIN PROMOTER DELETED DERIVATIVE 3 ACTIVITY

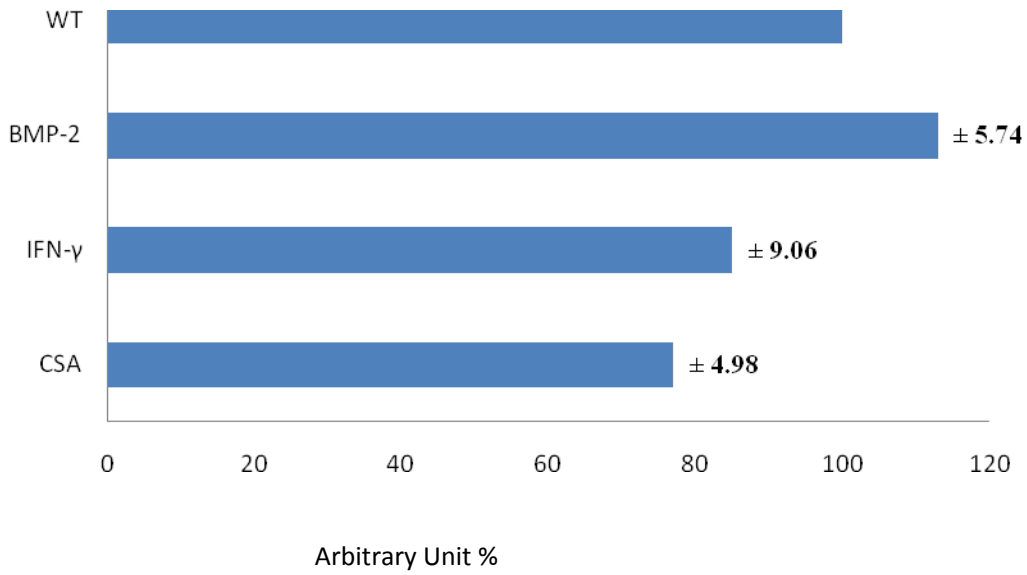
(A) Representation of transcription factor sites present on deleted derivative promoter

NMP4 = transcription factor

GC = GC RICH REGION IN PROMOTER

TATA = TATA BOX

(B) Induced GRM3 promoter activity



(A) Representation of various important transcription factors sites found within the deleted derivative promoter 3 (GRM 3). **(B)** Effect of different factors BMP-2, IFN-γ and CSA on deleted derivative promoter expression. Concentrations used for factors are BMP-2 (10 ng/ml), IFN-γ (10 ng/ml) and CSA (10 U/ml), OA chondrocytes (n-5-8) were prepared as described in the material and methods section. Promoter activity was measured as units luciferase activity/µg of total protein, and data are expressed as relative units of activity with respect to the deleted derivative gremlin 3 promoter wild type (WT) with no factors added, which has been given a value of 100%.

GRM 4 BASAL AND INDUCED ACTIVITY

The fragment GRM 4 was created using the restriction enzymes Kpn1 and Sma1 and its overall activity in human OA chondrocytes (n=5-8) was 99% in relation to the original promoter GRM B (fig. 2B). The deletion of most of the promoter and loss of almost all the transcription factor sites in this case does not seem to cause a significant change in gremlin expression levels. Figure 7A shows the promoter construct GRM 4 and the transcription factors that remained after deletions such as the GC rich region and the TATA box region. Figure 7B gives the activity of the promoter construct stimulated by the different factors. In the presence of BMP-2, the activity of the promoter increased by 15%, in the presence of IFN-γ the activity of the promoter decreased by 17% and in the presence of CSA the activity decreased by 30%. Statistical analysis of the results shows no significant change in expression.

In conclusion there seems to be little difference in basal expression levels of the different deleted derivatives except in the case of fragment GRM 3 which showed a significant decrease in promoter activity. Statistical analysis using the Man Whitney test indicates no significant difference in expression due to the deletions except for the GRM3 deleted derivative**.** Although not statistical significant, trends could be detected. The presence of CSA always triggered a decrease in activity, especially in the deleted derivatives. IFN-γ also induced a decreased activity, which was not as pronounced as that caused by CSA.

FIGURE 7

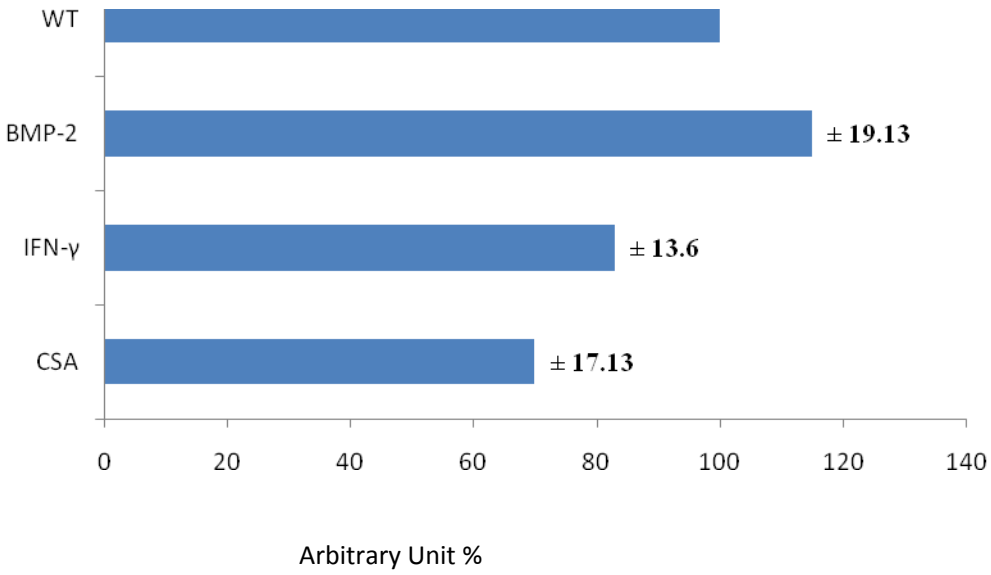
INDUCED GREMLIN PROMOTER DELETED DERIVATIVE 4 ACTIVITY

(A) Representation of transcription factor sites present on deleted derivative promoter

GC = GC RICH REGION IN PROMOTER

TATA = TATA BOX

(B) Induced GRM4 promoter activity



(A) Representation of various important transcription factors sites found within the deleted derivative promoter 4 (GRM 4). **(B)** Effect of different factors BMP-2, IFN-γ and CSA on deleted derivative promoter expression. Concentrations used for factors are BMP-2 (10 ng/ml), IFN-γ (10 ng/ml) and CSA (10 U/ml), OA chondrocytes (n-5-8) were prepared as described in the material and methods section. Promoter activity was measured as units luciferase activity/µg of total protein, and data are expressed as relative units of activity with respect to the deleted derivative gremlin 4 promoter wild type (WT) with no factors added, which has been given a value of 100%.

C) SIGNALIZATION PATHWAYS ACTIVATED BY EXTRACELLULAR GREMLIN

Gremlin is a known BMP-2/4 antagonist and in the literature data have indicated that it might activate transcription independently of its binding to BMP-2/4 (130). If gremlin could activate transcription, most likely it would be through signalling pathway leading to gene expression. The second focus of my research centered upon possible signal transduction pathways activated by gremlin. Although it is well known that it is a BMP-2/4 antagonist, the goal was to discover possible signal transduction pathways that may be elicited by the gremlin protein itself rather than through an intermediate molecule such as BMP. It has been suggested by other researchers that the gremlin protein could bind directly to a receptor and the goal of our research was to find evidence of another signalling cascade other than the Smad pathway which is usually activated by BMP- 2/4 (131).

PROTEOME PROFILER RESULTS

This experiment was done to determine if intracellular signalling pathways were activated in OA chondrocytes upon stimulation with gremlin (100 ng/ml). To this end we used the Proteome Profiler Array (R&D). This array enabled the analysis of many different pathways at once; allowing us to analyze gremlin's effect on the phosphorylation of all three major families of mitogen activated protein kinase (MAPK's, ERK's, JNK's), different p38 isoforms and other intracellular kinases (AKT, GSK 3, p70). The cells were incubated in the presence of gremlin (100 ng/ml) for a period of 10 min, 45 min and 6 h and proteins extracted according to the procedure detailed in the proteome profiler array kit. To negate gremlin primary role of BMP -2/4 antagonist, a BMP-2/4 neutralizing antibody (R&D) (25 U/ml) was added 1hr before the

FIGURE 8

RESULTS OF PROTEOME PROFILER ARRAY : GREMLIN SIGNALLING IN THE CELL

(A) Table of signalling pathways analyzed in proteome profile array kit

-**ERK1, JNK1, JNKpan, p38γ, p38σ, RSK1, GSK 3α/β,**

-AKT 1, ATK 2, ERK 2, JNK 2, p38α, p38β, RSK 2, JNK 3, AKT pan, MSK 2, HSP27, p70 S6 Kinase

(B) Fold changes seen in pathways affected by gremlin stimulation

	CTL Rabbit IgG	p38δ	RSK1	GSK 3α/β	AKT 1
0 min	1	1.0	1.0	1.0	1.0
10 min	0.8	2.1	23.5	2.3	1.9
45 min	6.3	3.4	24.5	2.4	1.6
6 hrs	1.7	2.4	21.5	2.0	1.4

(A) List of signalling pathways analyzed by the proteome profiler array. **(B)** Pathways affected in the chondrocyte OA cells (n=1) by stimulation with 100 ng/ml of Gremlin and pre-incubation with the neutralizing antibody BMP-2/4 (25 U/ml). Results expressed as fold change with respect to the control (0 min) with no factors present, which has been given a vallue of 1. The change in pathway signalling was analysed at 3 different time intervals; 10 min, 45 min and 6 hrs. The cells were prepared as explained in the materials and methods section according to the proteome profiler array kit (R&D systems).

addition of gremlin to cells to block the BMP-2/4 signaling , which would leave the added gremlin free to interact with the cellular surface.

The figure 8A lists the different pathways that were analysed in the proteome profiler array and figure 8B shows those that were activated in response to gremlin stimulation: p38γ, RSK, GSK3α/β and AKT. The p38γ phosphorylation increased in the presence of gremlin, doubling in the first 10 min and rising to 3.4 times at 45 min. The same effect can be seen with GSK3α/β in that its phosphorylation doubled in the presence of gremlin. The most significant increase in activity can be seen with RSK1 which was increased by as much as 24 times in the presence of gremlin, while a mild increase in Akt phosphorylation in the presence of gremlin can be seen. This experiment allowed us to target specifically these different pathways through western blot analysis to confirm whether gremlin does indeed affect these different pathways.

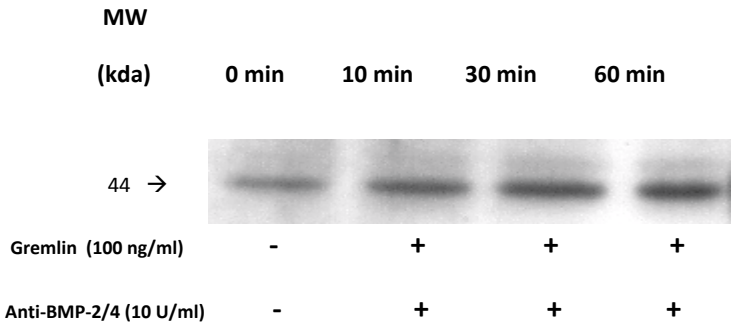
WESTERN BLOT RESULTS

Western blots were used to confirm the results from the proteome profiler array. We also investigated gremlin's effect on the ERK 1/2 (p42/44) phosphorylation due to the importance of this cascade in many of the cell's basic functions and the fact that this pathway is important in OA pathophysiology. Figure 9A is a representative western blot using antibodies against the phosphorylated p42/44 with protein lysates from 3 differerent human OA chondrocyte specimens. These specimens were treated as for the proteome profiler experiments, they were placed 1hr before stimulation with gremlin (100 ng/ml) in the presence of an anti-BMP-2/4 blocking antibody (25 U/ml) which would allow excess gremlin to bind to other targets besides

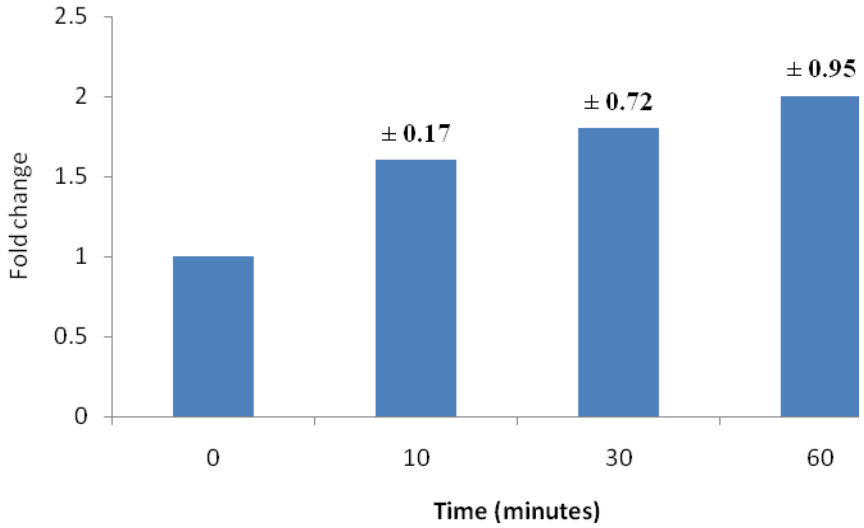
FIGURE 9

WESTERN BLOT ANALYSIS OF p42/44 PHOSPHORYLATION FOLLOWING GREMLIN STIMULATION

(A) Representative western blot of p42/44 phosphorylated



(B) Graph of densitometry calculations of western blot results



(A) Representative western blot of chondrocyte OA (n=3) in the presence of gremlin (100 ng/ml) and the neutralizing antibody BMP-2/4 (25 U/ml). Cells were treated with factors, extracted and western blot analysis was performed using the p42/44 antibody (1/50 000 dilution) according to the procedure in the material and methods section. **(B)** Graph representing densitometry calculations of western blot, fold change calculated with respect to time =0, which has been given a value of 1.

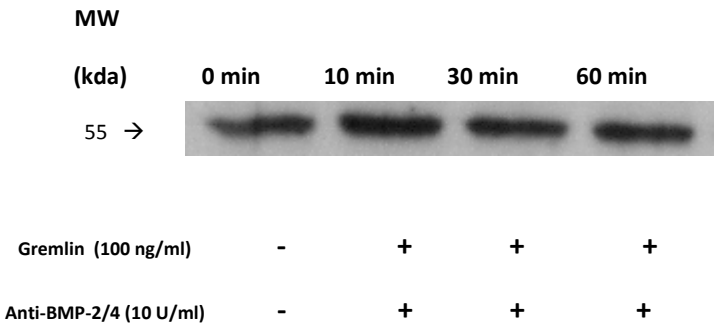
BMP-2/4. The time periods for stimulation with gremlin (100 ng/ml) were 10, 30 and 60 minutes, after which cellular lysates were prepared for western blot.

Figure 9B is a representation of the densitometry results of the western blots. From the graphical representation we can see a slight increase of the p42/44 over time. However, statistical analysis of the results indicated no significant difference between the time intervals. Therefore phosphorylation of the p42/44 pathway is not a likely route for gremlin signalling. Figure 10A shows a representative western blot performed using the antibody Akt and the same three cellular lysates and conditions for the p42/44 above. The results of densitometry analysis of can be seen figure 10B and they clearly show that there is no significant difference in Akt signalling when stimulated with the gremlin protein. The next signalling pathway examined is the GSK 3α/β pathway (figure 11). The cells were prepared in the same manner as above. Figure 11A shows a representative western blot with an anti-GSK 3α/β antibody and figure 11B is a graph of the densitometry measurements of the western blots (n=3). From this graph it is clear that gremlin has no effect on GSK 3α/β signalling. The next signalling pathway tested is the FAK pathway which had been suggested as a possible signalling pathway of the gremlin protein by Stabile et al. during angiogenesis (131). The results of the westerns experiment are seen in figure 12. Figure 12A is a representative western blot analysis performed with an anti-FAK antibody. The same conditions were used as in the previous western blot experiments. Figure 12B is a graph of the densitometry measurements done on the western blots (n=4), it is clear that no significant change occurs in the FAK pathway due to the presence of gremlin. Figure 13A is a representative western blot analysis performed with the anti-RSK antibody. Once again the conditions of the experiment remained the same and densitometry measurements were done on

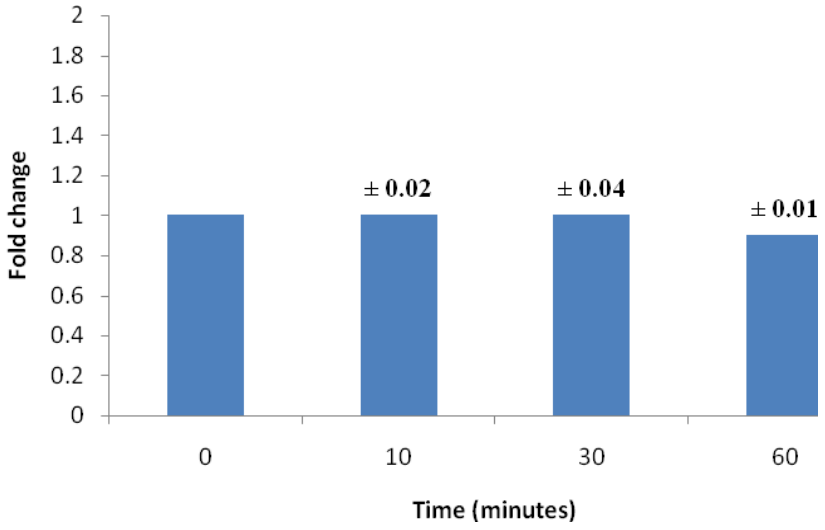
FIGURE 10

WESTERN BLOT ANALYSIS OF AKT 1 PHOSPHORYLATION FOLLOWING GREMLIN STIMULATION

(A) A representative western blot analysis of AKT 1 phosphorylated



(B) Graph of densitometry calculations of western blot results

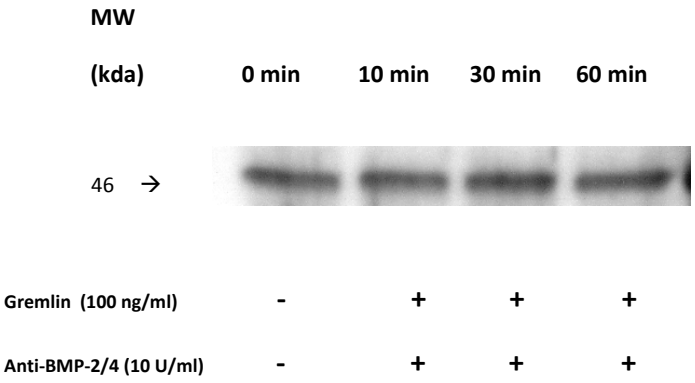


(A) Representative gel of western blot analysis of chondorcyte OA (n=3) in the presence of gremlin (100 ng/ml) and the neutralizing antibody BMP-2/4 (25 U/ml). Cells were treated with factors, extracted and western blot analysis was performed using the Akt 1 antibody (1/ 25 000 dilution) according to the procedure in the material and methods section. **(B)** Graph representing densitometry calculations of western blot gel above, fold change calculated with respect to time =0, which has been given a value of 1.

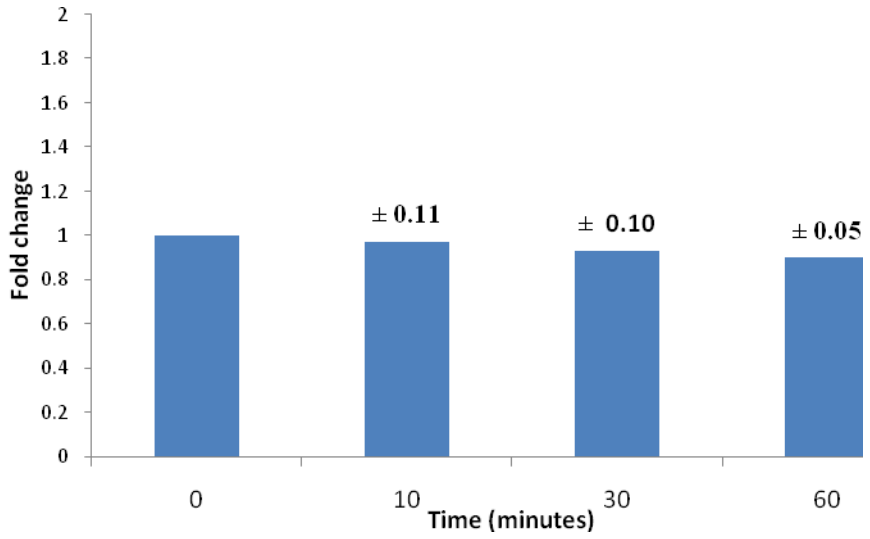
FIGURE 11

WESTERN BLOT ANALYSIS OF GSK 3α/β PHOSPHORYLATION FOLLOWING GREMLIN STIMULATION

(A) A representative western blot analysis of phosphorylated GSK 3α/β



(B) Graph of densitometry calculations of western blot results

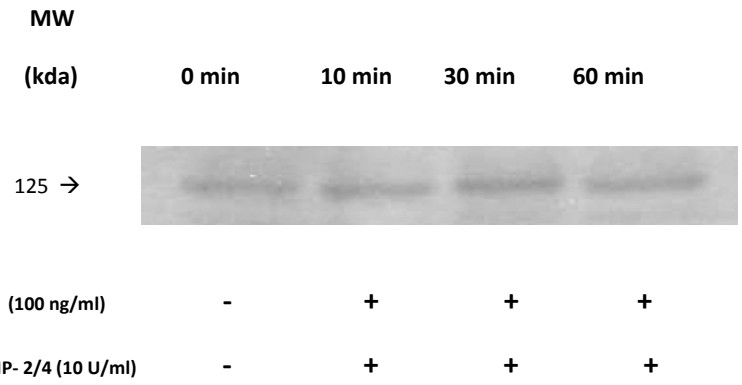


(A) Representative gel of western blot analysis of chondorcyte OA (n=4) in the presence of gremlin (100 ng/ml) and the neutralizing antibody BMP-2/4 (25 U/ml). Cells were treated with factors, extracted and western blot analysis was performed using the GSK 3α/β antibody (1/ 25 000 dilution) according to the procedure in the material and methods section. **(B)** Graph representing densitometry calculations of western blot gel above, fold change calculated with respect to time =0, which has been given a value of 1.

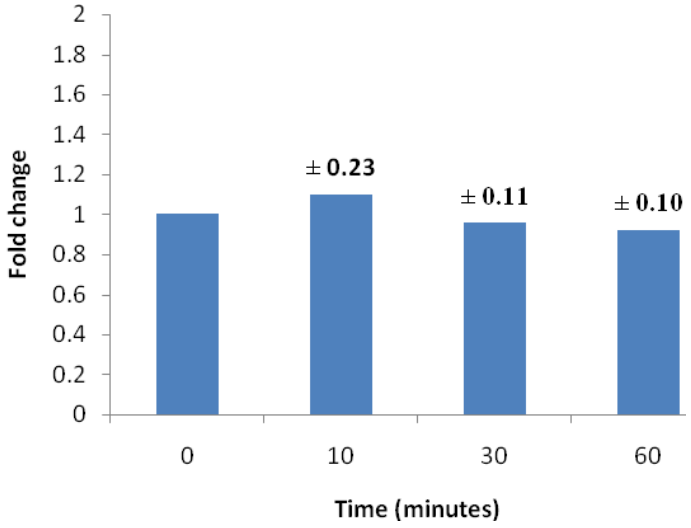
FIGURE 12

WESTERN BLOT ANALYSIS OF FAK PHOSPHORYLATION FOLLOWING GREMLIN STIMULATION

(A) A representative western blot analysis of FAK phosphorylation



(B) Graph of densitometry calculations of western blot results

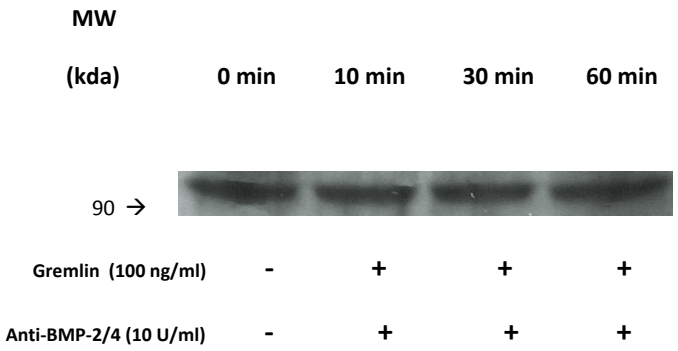


(A) Representative gel of western blot analysis of chondorcyte OA (n=4) in the presence of gremlin (100 ng/ml) and the neutralizing antibody BMP-2/4 (25 U/ml). Cells were treated with factors, extracted and western blot analysis was performed using the FAK antibody (1/ 25 000 dilution) according to the procedure in the material and methods section. **(B)** Graph representing densitometry calculations of western blot gel above, fold change calculated with respect to time =0, which has been given a value of 1

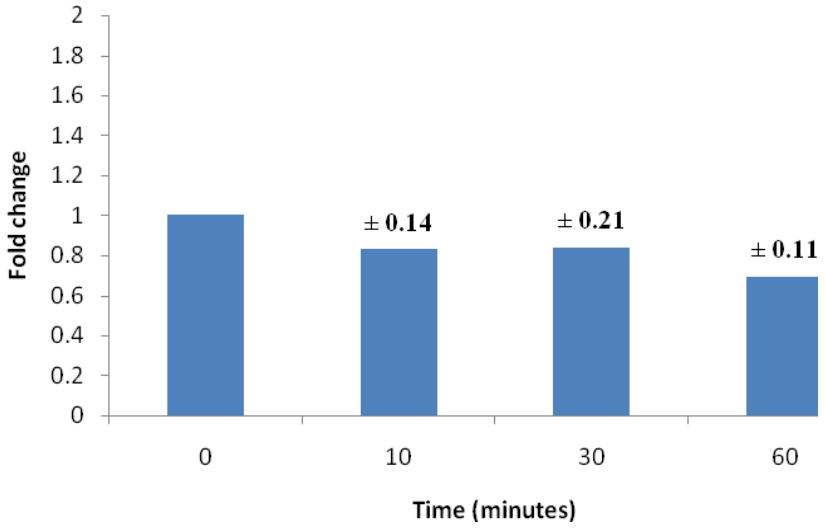
FIGURE 13

WESTERN BLOT ANALYSIS OF RSK PHOSPHORYLATION FOLLOWING GREMLIN STIMULATION

(A) A representative western blot analysis of RSK phosphorylation



(B) Graph of densitometry calculations of western blot results



(A) Representative gel of western blot analysis of chondorcyte OA (n=2) in the presence of gremlin (100 ng/ml) and the neutralizing antibody BMP-2/4 (25 U/ml). Cells were treated with factors, extracted and western blot analysis was performed using the RSK antibody (1/ 25 000 dilution) according to the procedure in the material and methods section. **(B)** Graph representing densitometry calculations of western blot gel above, fold change calculated with respect to time =0, which has been given a value of 1

the werstern blot results (n=2). Results showed in figure 13B clearly reveal no induction of the RSK pathway in the presence of gremlin. In summary these results indicate that gremlin does not activate any of the signalling cascades tested in OA chondrocytes

D) EFFECT OF EXTRACELLULAR GREMLIN ON GENE EXPRESSION

ANALYSIS BY MICROARRAY

The last aim of this project was to determine if gremlin was able to directly affect gene expression and this was tested by microarray. OA chondrocyte (n=3) cells were stimulated with gremlin at concentrations of 10 ng/ml and 100 ng/ml for 24hrs and the RNA was extracted. Non-stimulated cells served as control for the experiment. The extracted RNA was sent to Genome Quebec to be processed and analyzed. Figure 14 lists the different genes whose expression levels changed in the presence of gremlin, at 10 ng/ml gremlin (fig.14A) and at 100 ng/ml (fig.14C), while figure 14B lists the genes affected by the different concentrations (10 ng/ml and 100 ng/ml). From these results we can see that there were many genes affected including some that have been linked to cartilage metabolism, such as annexin, calreticulin and collagen type X. The inhibitors of differentiation Id1 and Id3 were affected in all 3 OA chondrocytes with a significant decrease in fold change, as much as 50%. These lasts results proved interesting because of the inhibitory effect that BMP2 has on the expression of Id1 and Id3. These results needed to be validated by pPCR to determine if this response is attributed to BMP-2 or gremlin.

FIGURE 14

EFFECT OF GREMLIN ON GENE EXPRESSION BY MICROARRAY TESTING

(A) List of genes with expression level changes, measured in fold change, observed between the control and stimulation with gremlin 10 ng/ml.

Gene Title	Gene Symbol	Sample 1	Sample 2	Sample 3
vacuolar protein sorting 8 homolog	VPS8	1.79	0.64	1.25
LIM domain kinase 1	LIMK1	0.59	0.94	0.57
elastin	ELN	0.61	0.87	0.64
collagen, type X	COL10A1	0.63	0.99	1.51

(B) List of genes with expression level changes measured in fold change, observed between stimulation with gremlin 10 ng/ml and gremlin 100 ng/ml.

Gene Title	Gene Symbol	Sample 1	Sample 2	Sample 3
inhibitor of DNA binding 3	ID3	0.47	0.62	0.64
inhibitor of DNA binding 1	ID1	0.34	0.58	0.50
Calreticulin	CALR	1.70	0.65	0.94
Ariadne homolog	ARIH1	0.65	0.91	1.57

(C) List of genes with expression changes, measured in fold change, between the control and stimulation with gremlin 100 ng/ml.

Gene Title	Gene Symbol	Sample 1	Sample 2	Sample 3
hyaluronan synthase 2	HAS2	1.00	1.58	1.63
retinoic acid receptor, alpha	RARA	1.52	1.00	0.64
sorbin and SH3 domain containing 1	SORBS1	0.56	0.80	0.64
atonal homolog 8 (Drosophila)	ATOH8	0.51	0.68	0.56
RAB11B, member RAS oncogene family	RAB11B	1.63	0.82	0.54
inhibitor of DNA binding 3	ID3	0.48	0.47	0.55
inhibitor of DNA binding 1	ID1	0.32	0.37	0.37
Rho GTPase activating protein 29	ARHGAP29	2.03	0.62	0.98
Rho GTPase activating protein 29	ARHGAP29	0.42	1.80	1.05
zinc finger protein 652	ZNF652	1.76	0.44	0.98
BCL2-associated X protein	BAX	1.58	0.67	1.15
CD24 molecule	CD24	0.66	1.63	1.15
FK506 binding protein 10, 65 kDa	FKBP10	1.87	0.63	0.81
chromatin modifying protein 4B	CHMP4B	1.73	0.64	0.98
pyruvate dehydrogenase phosphatase	PDP2	2.75	0.42	1.05
Annexin A1	ANXA1	0.27	3.15	0.77
HBV preS1-transactivated protein 4	PS1TP4	2.44	0.54	1.01
Chromosome X open reading frame 33	CXorf33	1.66	0.54	1.03
guanine nucleotide binding protein-like 3	GNL3L	1.63	0.57	0.80

(A), (B), (C) List of genes affected following stimulation of OA chondrocytes (n=3) by gremlin (10 ng/ml and 100 ng/ml). Non-stimulated cells served as control.The RNA for the microarray was prepared as explained in the materials and methods section and the results presented in fold change, relation to the control which was given an arbitrary value of 1.

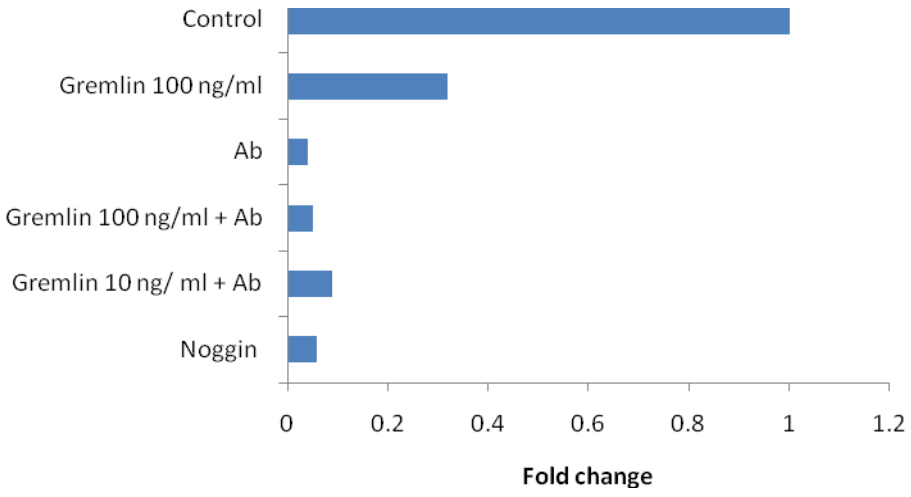
VALIDATION BY qPCR

Quantitative RT-PCR on Id1 and Id3 was done to validate the results of the microarray and the results can be seen in figure 15. The OA chondrocytes were stimulated with gremlin (10 ng/ml and 100 ng/ml) for 24 hrs and the RNA was extracted. To account for the effect of BMP-2/4 antagonism by gremlin, the cells were pretreated 1h before gremlin stimulation with the BMP- 2/4 neutralizing antibody (R&D) at 25 U/ml. Figure 15A shows the results of the qPCR for Id1, confirming the inhibition of Id1 by gremlin. However the inhibition is also enhanced in those cells pretreated with the neutralizing antibody which is indicative of the inhibition passing through the BMP pathway as opposed to being caused by the gremlin protein alone. This hypothesis is further confirmed when noggin which is a more potent inhibitor of BMP-2/4 is added alone and an even more pronounced inhibition of Id1 is seen in the qPCR. In conclusion the inhibition of Id1 is accomplished through gremlin's role as an inhibitor of BMP-2/4 as opposed to gremlin acting through another pathway or through a possible gremlin promoter alone. Figure 15B illustrates the effect of gremlin on Id3, the results show that once again the inhibition of the Id3 gene expression level is accomplished through gremlin's interaction with BMP 2/4 and not by the effect of gremlin alone.

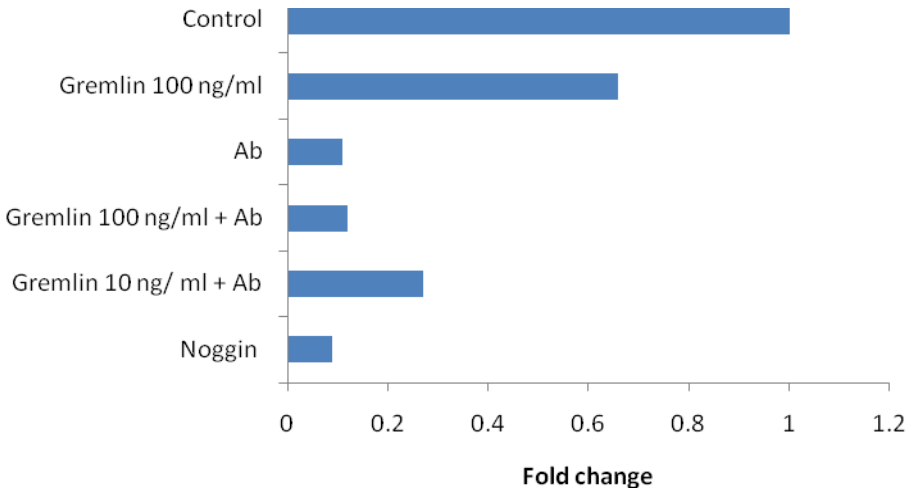
FIGURE 15

VALIDATION OF THE MICROARRAY RESULTS BY RT-PCR

(A) Validation of inhibition of Id1 by gremlin through qPCR



(B) Validation of inhibition of Id3 by gremlin through qPCR



(A), (B) Effect of gremlin on Id1 and Id3 expression in OA chondrocytes (n=1), expressed in fold change. Cells were treated with gremlin 10 ng/ml and 100 ng/ml, the (Ab) neutralizing antibody BMP2/4 (25 U/ml) and noggin 100 ng/ml, according to the procedure in the materials and methods section. Fold change calculated in respect to the unstimulated cells which has been given a value = 1.

SECOND MICROARRAY ANALYSIS

The second series of microarray were done to complement the results of the first microarray. In this experiment, cells were pre-treated with the neutralizing antibody BMP2/4 and then with the gremlin protein at 100 ng/ml. The RNA extracted from the control and treated cells was sent to Genoscience for the processing and analysis of the samples. This would enable us to determine which genes were affected by gremlin stimulation without going through the BMP pathway. The results of this microarray can be seen in figure 16 which shows which genes are upregulated (16A) and those genes down regulated (16B) by stimulation with gremlin and are presented as fold change compared to non-stimulated cells. The genes that were tested by qPCR analysis are highlighted in figures 16A and 16B. These genes were chosen for qPCR because they exhibited the largest variation in fold change during the microarray. The results showed that expression of the tested genes were not affected by gremlin stimulation.

In conclusion, the results from the microarrays could not be validated by the more quantitative method of pPCR. These results indicate that gremlin does not affect genetic expression other than through the mediation of BMP activity.

FIGURE 16

RESULTS OF MICROARRAY ANALYSIS IN THE PRESENCE OF GREMLIN AND THE NEUTRALIZING BMP 2/4 ANTIBODY

(A) List of genes up-regulated in the presence of gremlin and the neutralizing BMP 2/4 antibody

Gene Title	Gene symbol	Sample 1	Sample 2	Sample 3
cytoskeleton associated protein 2	CKAP2	**1.67**	**3.10**	**2.46**
interleukin 1 receptor antagonist, transcript variant 1	IL1RN	**1.62**	**1.93**	**1.55**
natriuretic peptide precursor C ***	NPPC	**2.59**	**2.75**	**1.91**
HRAS-like suppressor 2	HRASLS2	**1.73**	**1.73**	**1.96**
tigger transposable element derived 4	TIGD4	**2.60**	**1.65**	**2.18**
Sp5 transcription factor	SP5	**1.72**	**3.22**	**1.79**
autoimmune regulator ****	AIRE-2	**2.82**	**4.10**	**2.09**
retina and anterior neural fold homeobox like 1***	RAXL1	**2.53**	**4.09**	**1.63**
glycine receptor, alpha 3, transcript variant 1	GLRA3	**2.37**	**2.20**	**3.38**
radical S-adenosyl methionine domain containing 2	RSAD2	**3.24**	**2.57**	**2.24**
radical S-adenosyl methionine domain containing 2	RSAD2	**1.80**	**2.19**	**2.09**
leptin (obesity homolog, mouse)	LEP	**1.66**	**1.53**	**1.50**
myxovirus (influenza virus) resistance 2 (mouse)	MX2	**2.02**	**2.25**	**1.57**
Purkinje cell protein 4	PCP4	**5.41**	**2.10**	**1.72**
RAS protein activator like 1 (GAP1 like) ***	RASAL1	**2.06**	**1.98**	**1.92**
sine oculis homeobox homolog 3 (Drosophila)	SIX3	**2.56**	**2.47**	**3.61**
poly (ADP-ribose) polymerase family, member 4	PARP4	**7.45**	**3.02**	**1.86**
neurogenin 1	NEUROG1	**1.63**	**2.00**	**2.89**
claudin 12	CLDN12	**1.58**	**1.62**	**1.54**
glial cells missing homolog 1 (Drosophila) ****	GCM1	**1.52**	**1.63**	**1.62**
myeloid/lymphoid or mixed-lineage leukemia 2	MLL2	**1.52**	**1.60**	**1.98**
NIMA (never in mitosis gene a)-related kinase 7	NEK7	**1.71**	**1.80**	**1.94**
gonadotropin-releasing hormone 2, transcript variant 2	GNRH2	**2.21**	**1.95**	**1.88**
Ras association (RalGDS/AF-6) domain family 8	RASSF8	**1.76**	**1.57**	**1.81**

(B) List of genes down regulated in the presence of gremlin and the neutralizing BMP 2/4 antibody

Gene Title	Gene symbol	Sample 1	Sample 2	Sample 3
apolipoprotein A-II ***	APOA2	**0.28**	**0.46**	**0.22**
sorbin and SH3 domain containing 1, transcript variant 1	SORBS1	**0.27**	**0.25**	**0.63**
fibrinogen gamma chain, transcript variant gamma-A ***	FGG	**0.69**	**0.42**	**0.38**
cell division cycle associated 7, transcript variant 1 ***	CDCA7	**0.43**	**0.11**	**0.32**
phosphodiesterase 7A, transcript variant 2	PDE7A	**0.65**	**0.44**	**0.37**
killer cell lectin-like receptor subfamily F, member 1 ***	KLRF1	**0.15**	**0.18**	**0.49**
peptidyl arginine deiminase, type II	PADI2	**0.55**	**0.66**	**0.68**
myozenin 2	MYOZ2	**0.09**	**0.58**	**0.58**
growth differentiation factor 9	GDF9	**0.63**	**0.61**	**0.43**
Ras-associated protein Rap1	RBJ	**0.47**	**0.46**	**0.64**
acyl-Coenzyme A oxidase 1, palmitoyl, transcript variant 1	ACOX1	**0.44**	**0.49**	**0.54**
dopamine receptor D4 ***	DRD4	**0.39**	**0.54**	**0.70**
chemokine (C-C motif) receptor 4 ***	CCR4	**0.52**	**0.66**	**0.24**
mitochondrial coiled-coil domain 1	MCCD1	**0.67**	**0.63**	**0.39**
zinc finger and SCAN domain containing 4 ***	ZSCAN4	**0.50**	**0.40**	**0.67**
carboxypeptidase O	CPO	**0.49**	**0.54**	**0.60**
sterile alpha motif domain containing 13	SAMD13	**0.65**	**0.58**	**0.52**
adenosine A3 receptor, transcript variant 1 ***	ADORA3	**0.45**	**0.68**	**0.40**
phosphodiesterase 4B, cAMP-specific.	PDE4B	**0.65**	**0.59**	**0.62**
perilipin	PLIN	**0.69**	**0.46**	**0.62**

(A), (B) Genes up-regulated and down-regulated in chondrocyte OA (n=3) in the presence of gremlin 100 ng/ml and the neutralizing BMP 2/4 antibody (25 U/ml). Cells were prepared for the microarray as described in the materials and method section and results were presented as fold change. *****Validation of the results by RT-PCR indicates no significant change between the control and treated chondrocytes.**

DISCUSSION

The purpose of this project was to characterize gremlin's regulation in OA chondrocytes and to determine whether gremlin has another role in OA. Indeed although gremlin is known to be a BMP antagonist, we hypothesize that it could also regulate other factor expression/production on its own. The first topic was accomplished through the cloning of the promoter, and determining the regions important for basal and induced activity. The second topic was to determine if gremlin by itself might illicit changes in gene expression, i.e. does the presence of gremlin causes an increase or decrease in expression of other genes. This was accomplished through the use of microarrays, which enabled us to determine which genes were directly affected by the presence of gremlin. Moreover we also wanted to determine whether gremlin by itself could activate intracellular signalling, without passing through the BMP-SMAD mediated pathway. Data from this study definitely rule out a role for gremlin other than a BMP antagonist in OA chondrocytes.

The first step in the analysis of gremlin regulation was the characterization of its 5'UTR. This analysis revealed the presence of two mRNA transcripts which suggested the presence of two gremlin promoter regions. The presence of two promoters in the 5'UTR of a gene is not unheard of. Indeed, some well known genes showed multiple promoters, including the retinoic acid receptor α, β, γ which allows for multiple isoforms of the protein; another example is IGF-2 which has developmental and tissue specific regulated promoters (135). It is believed that an organism will gain an added flexibility in the control of a genes expression by transcribing a single gene from multiple promoters (135). It allows for the organism to create diversity in the upstream region of certain genes, this becomes important when the gene is expressed in different tissue types such as gremlin allowing for an added

regulation of the gene (135). The use of multiple promoters by certain genes differs; some genes use only one promoter in one tissue type while using the alternate promoter in another tissue type. For example the α-amylase gene uses a strong upstream promoter in the parotid gland and a weak downstream promoter in the liver (135). This idea of alternate promoters in these genes led us to consider that this could also apply for gremlin as this protein plays a role in different tissues such as cartilage and bone morphogenesis and glucogenesis in the liver; two promoters may be be differentially active in these different tissues or show different activities in the same tissue environment. .

The analysis of the 5'UTR of gremlin however has shown that the mRNA transcripts in OA chondrocytes originated not from two different promoters, but from one promoter. The active promoter was GRM B situated 12.5 kb upstream from the ATG start codon. It is possible that that the promoter found in the upstream region of gremlin is tissue specific, or that the promoters are specific to different stages in the human life cycle. For example, IGF also has multiple promoter regions but only one is active during fetal development while the others become active after birth predominantly in the liver (135). As for gremlin, this molecule plays a role in axial patterning during development and is most active at this stage of the life cycle. Afterwards it becomes almost dormant in expression levels, usually maintaining a minimal expression pattern in the cell. It is possible that the GRM A promoter is not active in OA chondrocytes, but only active during early development or other tissues. It is likely that the presence of transcripts from GRM A comes from an unspliced variant of the transcript from GRM B.

The next step of the study was to clone and characterize the active promoter GRM B, in order to understand which factors affect its expression and which transcription factor sites play an important role in its regulation in OA chondrocytes. The construction of the deleted

derivatives of the gremlin promoter would enable us to learn which regions of the promoter influence the gene expression. Experiments in which we induced gremlin through stimulation with BMP-2, IFN-γ and CSA were performed to discriminate which factors regulate its expression. Previous studies have demonstrated that BMP-2 upregulates gremlin's expression while IFN-γ downregulates its expression (123). CSA was chosen to analyze if it exerts an effect on the gremlin promoter region, given that it plays a role in some similar pathways that gremlin is involved in, for example in the angiogenesis (134).

Analysis of the promoter region through deleted derivative demonstrated that only the deletion seen in GRM 3 showed significant change in basal promoter activity. This was an unexpected finding as the GRM 4 deletion also lacks the deleted region of GRM 3 but shows an activity similar to that of the wild type promoter. GRM 4, in fact, lacks most of the promoter region but retains the GC-rich region. It is possible that the extra region deleted in GRM 4 contains some sites that are recognized by inhibitory transcription factors. There is very little promoter region remaining in GRM 4, but it is enough and essential to maintain expression levels comparable to the wild type GRM B. GC-rich regions as found in GRM 4 are known to bind transcription factor such as Sp1 and the Sp-family of transcription factors (136). Another transcription factor family know to bind GC-rich regions and regulate gene expression is the Kruppel-like factors (KLF) (137). The interaction of these transcription factors may account for the expression of GRM 4 when most of the gremlin promoter has been deleted.

The expression of gremlin following treatment with BMP-2, IFN-γ and CSA yielded similar results in that no significant difference occured when the cells were stimulated with these factors, although some patterns were detected. A surprising result was that the wild type promoter GRM B was only slightly but not significantly activated following BMP-2

treatment. BMP-2 is a known activator of gremlin expression in whole cells. However, the present study was done in vitro with a defined sequence length which may not comprise all the regulatory sites that would normally bind transcription factors at play in the in vivo situation.

Whole cell studies have also shown an inhibitory effect of IFN-γ on gremlin expression. Although an inhibitory pattern was observed with the deleted derivatives, the effect was not statistically significant. As each promoter construct contained the GC-rich region, it is possible the effect of IFN-γ may be operating in this region.

Similarly, an inhibitory pattern occurred with CSA treatment. The inhibitory effect was stronger than that observed with IFN-γ, although it did not reach statistical significance. As was the case with IFN-γ, each deleted promoter construct retained the GC-rich region which may also be essential for the effect of CSA.

The results from these experiments point to a complex regulation of the gremlin promoter. There is a short sequence containing a GC-rich region which seems essential for basal and induced activity of the promoter. Further analysis through mutagenesis experiments of the GC-rich region would be necessary to identify the transcription factor sites directly implicated in promoter regulation. Moreover, a much larger promoter construct could be used in order to reproduce as much as possible the situation seen with the whole cells.

Further, we determined if extracellular gremlin could initiate its own cellular signaling cascade and cellular response. The first indication that this might be possible was seen in the study by Stabile et al. who reported that the FAK signaling pathway was triggered by exposure to gremlin and the possible presence of a gremlin receptor on the cellular surface (131). This, in addition to the data from Chen et al. (130) showing gremlin as a possible tumor suppressor, led us to hypothesize that gremlin could stimulate an intracellular

signaling cascade apart from its role as a BMP antagonist. Such a hypothesis is not unrealistic as many proteins within the cell have multiple roles. It was therefore very possible that gremlin could initiate its own cellular response as well as being a BMP antagonist. For this experiment we used the Proteome Profiler Array which allowed us to identify which signaling cascades might be triggered by gremlin. To account for the interaction between gremlin and BMPs, a neutralizing BMP-2/4 antibody was added to block the BMP 2/4-gremlin interaction allowing gremlin to remain free to illicit a response within the cell. The results of the array identified 3 different pathways which were affected by gremlin, RSK, AKT, GSK 3α/β, but these results were not validated by Western blotting. The FAK pathway was also studied because of the report by Stabile et al. (131) who reported that gremlin triggered this pathway. The p42/44 pathway was also chosen since it is one of the major signaling cascades involved in OA chondrocytes. Data have also shown that these pathways were not activated.

These results have illustrated the importance of using more than one procedure. The lack of correlation between the two procedures (Proteome Profiler and Western blots) may be linked to a lack of sensitivity in the Proteome Profiler Array, where a very small difference could not be reproduced with the Western blots. The proteome profiler is a good initial tool, but the results must always be supported by further experimentation. The conclusion of the signaling experiments is that gremlin does not directly initiate a signal in OA chondrocytes.

The last phase of experiments were done concurrently with the signalling experiments to determine if gremlin could directly affect gene expression and if so which genes would be affected by the presence of this protein. The results from the first microarray indicated a change in the expression of genes Id1 and Id3. Further analysis of the expression of the two

genes through quantitative RT-PCR validated the microarray results. Both experimental procedures demonstrated that gremlin inhibits the expression of these two genes. The next step was to determine whether gremlin was exhibiting this affect through a BMP mediated cascade or through an alternate route. A second microarray analysis was done, but this time, included a neutralizing BMP antibody. This would allow us to pinpoint specifically which genes might be affected by gremlin acting through a signaling cascade other than the SMAD pathway. The results of the second microarray were promising in the fact that a number of genes were affected but the results could not however be validated by quantitative RT-PCR analysis, also done with RNA extracted from cells treated with the neutralizing BMP antibody. This lack of correlation between two experimental procedures again demonstrates a need for different experimental procedures. Microarrays are useful to screen thousands of genes in one session but are not as quantitative as RT-PCR. These results coupled with the results from the signaling experiments led us to conclude that, within our experimental procedures, gremlin mediates its role during the progression of OA essentially as a BMP antagonist and not as an independent regulator of gene expression.

The conclusions of this thesis are that the gremlin promoter region responsible for its activity in OA chondrocytes is situated 12.5 kb upstream from the ATG start codon; that gremlin mediates its effect on the cell through BMP inhibition and does not directly affect gene expression. Gremlin expression has been shown to increase during OA. An increased level of gremlin could antagonize the BMP activity and thus reduce the overall anabolic process which in turn favors the catabolism of the cartilage.

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