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Transglutaminase Mediation of Pathologic Chondrogenic Differentiation in Cartilage and Aortic Smooth Muscle

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requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

Kristen A. Johnson

Committee in Charge:

Professor Robert Terkeltaub, Chair
Professor Maripat Corr
Professor David W. Rose
Professor Robert Sah
Professor Bruce Torbett

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Chair

University of California, San Diego

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DEDICATION

To Kyle, Kathryn and most of all

their wonderful father Evan Johnson

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LIST OF ABBREVIATIONS

APOE	apolipoprotein E
ATRA	All-*trans* Retinoic acid
α1-AR	α1-adrenergic receptors
BMP	Bone Morphogenetic Protein
CNP	C-type natriuretic peptide
Ca^{2+}	Calcium
cGK	cyclic GMP dependent protein kinase
ECM	extracellular matrix
FCS	Fetal calf serum
FXIIIA	Factor XIIIA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAMSC	human aortic smooth muscle cell
GPCR	G protein coupled-receptor signaling
IGFBP-3	Insulin-like growth factor binding protein-3
Ihh	Indian Hedgehog
IL-1	Interlukin-1
LDLR	low density lipoprotein receptor
MAPK	Mitogen activated protein kinase
MGP	Matrix γ- carboxyglutamic acid protein
MMP-13	Matrix-metalloproteinase -13
MSX2	msh homeo box homolog 2

NPP	Nucleotide pyrophosphatase pyrophosphohydrolase
OA	Osteoarthritis
OPG	Osteoprotegerin
OPN	Osteopontin
PDGF	Platelet derived growth factor
PLA2	Phospholipase A2
PP_i	Pyrophosphate
RUNX2	runt related gene 2
sFXIIIA	soluble Factor XIIIA
SMC	Smooth muscle cell(s)
sTG2	soluble recombinant transglutaminase
TG	Transglutaminase
TG2	Transglutaminase 2
TGFβ	Transforming growth factor β
WT	wild type

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VITA

Education:

2007	Ph.D. Molecular Pathology. University of California, San Diego.
2003-present	Doctoral Student in Molecular Pathology. University of California, San Diego. Advisor: Dr. Robert Terkeltaub
1996	B.A. Biology (*magne cum laude*). Point Loma Nazarene University, San Diego, CA

Research Experience:

2003-present	Doctoral Student in Molecular Pathology. University of California, San Diego. Advisor: Dr. Robert Terkeltaub
1997-2003	Research Associate / Lab Manager, VAMC / UCSD, San Diego, CA Advisor: Dr. Robert Terkeltaub
1996	Lab Instructor / Research Assistant, Point Loma Narazene University Advisor: Dr. Michael McConnell

Honors:

2005	ACR Research and Education Foundation (REF) Medical/Graduate Student Achievement Award

Peer-Reviewed Publications:

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ABSTRACT OF THE DISSERTATION

Transglutaminase Mediation of Pathologic Chondrogenic Differentiation in Cartilage and Aortic Smooth Muscle

by

Kristen A. Johnson

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Professor Robert Terkeltaub, Chair

Transglutaminases (TG) are a group of enzymes which catalyze the transamidation or "crosslinking" of proteins. This generates a covalent, protease resistant bond between two proteins that relies upon the presence and binding of Ca^{2+} to the TG. Of the nine known human isozymes, tissue transglutaminase (TG2) is ubiquitously expressed and most well characterized. A second TG extensively examined is Factor XIIIA (FXIIIA), the tissue form of the blood clotting zymogen FXIII. Both enzymes have other defined functions in addition to the ability to transamidate proteins. In cartilage, FXIIIA and TG2 have an increased expression and activity during aging and osteoarthritis (OA). TG2 is also highly expressed in atherosclerotic lesions and important in artery remodeling.

To understand the consequences of excess TG2 in OA, a detailed molecular structure function study was performed to examine its role in mediating the

progression of chondrocyte hypertrophic differentiation. TG2 is active as a TG in the presence of Ca^{+2} and a functional GTPase when bound by GTP. Using site-directed mutagenesis, external GTP-bound TG2 (Lys^{174}) was required for an effective induction of chondrocyte differentiation, primarily evidenced by increased expression of type X collagen and calcification.

The second TG in cartilage, FXIIIA, was found to also induce chondrocyte hypertrophy, but required TG2 to do so. Site-directed mutagenesis studies revealed the requirement of FXIIIA to bind Ca^{+2} to induce type X collagen, with no reliance upon its catalytic activity. Extracellular FXIIIA bound to $\alpha 1$ integrin, an integrin subunit localized to hypertrophic chondrocytes in OA. This binding was critical for FXIIIA to mobilize TG2, phosphorylate p38 MAP kinase and induce chondrocyte differentiation.

In response to injury, aortic smooth muscle cells (SMC) can differentiate through an osteoblastic / chondrocytic mechanism which leads to matrix calcification. To understand the role of TG2 during this process, $TG2^{-/-}$ SMC were examined. These experiments determined that TG2 promoted calcification of SMC, requiring its catalytic activity. Additionally TG2 can directly regulate the expression of osteopontin, a calcification inhibitor. Collectively the research in this dissertation describes that both TG2 and FXIIA have critical roles in mediating the progression of events that occurs during pathologic cell differentiation.

CHAPTER 1:

INTRODUCTION

TRANGLUTAMINASES AND MATRIX CALCIFICATION

TRANSGLUTAMINASE 2

Transglutaminase 2 (TG2) was first characterized in 1957 (1). It is also known as tissue transglutaminase, transglutaminase C and Gh. The name originated from the first identified function, the transamidation of glutamine side chains (EC 2.3.2.13). This Ca^{2+}-dependent, primary enzymatic action of transglutaminases involves the formation of a covalent bond between two proteins through an available amine group on a lysine side chain (2). Currently there are nine human members of this family. Eight of these members have the ability to not only transamidate proteins, but can incorporate free amines along with its deamidation and isopeptidase activities. Each transglutaminase enzyme has specific substrates and localization (3).

TG2 is a multifunctional enzyme containing various elements which potentially regulate many physiological and pathological events but its exact role is still unclear. Therefore, this is an area of intense study which has led to the elucidation of numerous functions and the proposal of many others. Although TG2 is ubiquitously expressed, the highest expression is found in cells naturally subjected to injury, such as epithelial cells (4). TG2 can be found in the cytosol, nucleus and at the cell membrane or can be externalized into the matrix by a process not understood but may involve biosynthesis of integrins (5).

Crystallization of TG2 revealed the structure of TG2 contains four distinct domains: an N-terminal β-sandwich (containing the fibronectin and integrin

binding site), a catalytic core (containing the catalytic triad) and two C-terminal β-barrel domains (6). Figure 1-1 depicts a drawing of the primary structure of the TG2 protein, indicating the key amino acids known to regulate some of its functions.

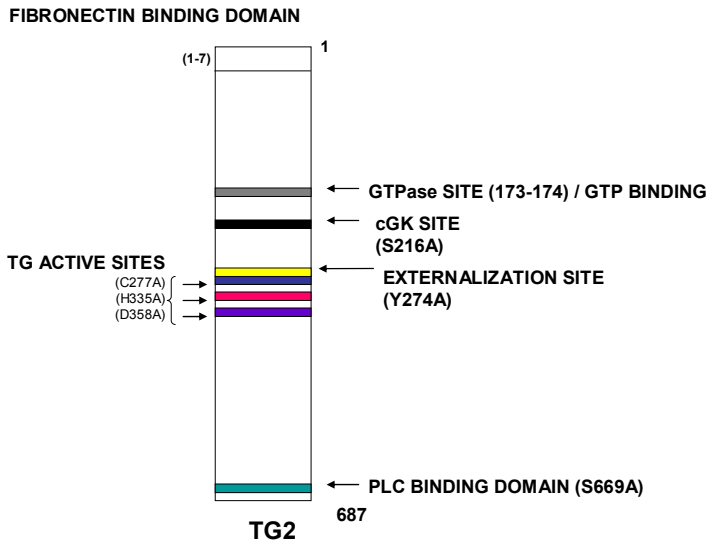


Figure 1-1. The primary structure of TG2. The 687 amino acid protein of TG2 is shown indicating residues critical to the fibronectin binding, the GTPase / GTP binding, the cGK binding, the catalytic core, the externalization site and the PLC binding domain, some of the functions elucidated pertaining to TG2.

Second only to the transamidation activities of TG2, the best characterized function of TG2 is the ability to bind and hydrolyze both GTP and ATP. Characterization of the atypical G protein termed Gh determined the α subunit to be identical to TG2, which was already know to transduce and activate downstream effectors in adrenergic signaling (7). Recently both TG3 and TG5 have been found to also bind and are regulated by guanine nucleotides (8, 9). In TG2 the GTP

binding pocket is distinct from most GTP binding proteins. It is located between the catalytic domain and the first β barrel and involves residues from both domains (6). In the GDP / GTP bound state, the key catalytic amino acid, Cys^{277} is blocked (10). In the Ca^{2+} bound state, the catalytic region is exposed, possibly allowing substrate binding to occur. Therefore the Ca^{2+} binding (critical for transamidation) inhibits the GTP binding and the binding of guanine nucleotides inhibits the catalytic activity (10). The actual amino acids involved in the two activities are distinct (Fig. 1-1) but are regulated by the binding of the either Ca^{2+} or GTP. In addition, the binding of adenine nucleotides inhibits the GTPase activity without altering the TG activity (11). This reciprocal regulation of TG2 is a feature which allows precise tuning of the molecule in specific microenvironments and most likely dictates the many functions of TG2. Inside the cell during physiologic conditions, TG2 is normally in a catalytically latent state and it is not until the local environment has an influx of Ca^{2+} or alteration of nucleotide levels which activates TG2 (12).

Although the primary physiologic role of TG2 is not known, many functions have been elucidated. The homozygous null TG2 mice are viable and have normal size and weight with no severe phenotype (13). This led to the assumption that other mammalian transglutaminases must compensate to some extent for the lack of TG2. $TG2^{-/-}$ mice do have an impaired wound healing, decreased adherence of primary fibroblasts (13), a deficit in the activity of the mitochondrial complex I (14), a decreased ability in the phagocytic clearance of

apoptotic cells (15, 16) and glucose intolerance and hyperglycemia because of reduced insulin secretion (17). It is plausible that under stress or disease, TG2 is critical to the response needed for repair or healing and that further testing of these mice will reveal how TG2 is critical in other physiologic states.

In humans no TG2 deficiency has been reported. Pathologically the dysregultion of TG2 expression or alteration of function has been proposed to be involved in celiac disease, atherosclerosis, cancer, diabetes mellitus, Alzheimer's and Parkison's. At this time, only the involvement of TG2 in celiac disease has been well characterized (18).

It is impossible to discuss all of the accepted functions of TG2, as the molecule mediates many events. Its functions can be divided into two broad categories: those which are mediated through crosslinking / transamidation, and those which are independent of the crosslinking activity. Most of the understanding of TG2 has come from in vitro studies. Early work on TG2 focused on its ability to transamidate proteins. This led to the discovery of many modifications TG2 made on many proteins that affected a wide variety of events. The best example of this is the control of cell life and death (primarily through apoptosis) by transamidation and subsequent activation of RhoA (19). Additionally, polymerization of the cytoskeleton during apoptosis by TG2 crosslinking can stabilize the structure of the dying cells and thus limit the inflammation that may occur with leakage of the cellular components (20).

The Gh / TG2 experimentation has focused on the activation of Gh by G protein coupled receptors (GCPR) to mediate signaling events. For example, the interaction of TG2 with α1-adrenergic receptors (α1-AR) switches off transglutaminase activity and dissociates GTP-bound Ghα / TG2 from Ghβ. Ultimately this simulates PLCδ1, thereby resulting in phosphoinositide hydrolysis and increase in intracellular concentrations of Ca^{2+} (2). Many other pathways are currently being explored which are mediated by Ghα / TG2.

Alternately, TG2 may use both types of functions together in certain circumstances. For successful wound repair, cells must interact with the surrounding ECM to ensure survival and proliferation. Following a puncture wound of a fibroblast monolayer, TG2 was dramatically increased. This suggests that TG2-mediated protein crosslinking is critical for the reconstruction of the extracellular matrix (ECM) (21). As TG2 can also interact with integrins and fibronectin and mediate cell adhesion and cell spreading, without the requirement for its catalytic activity, it again confirms the multi-functionality of this molecule. For example in inflammation, TG2 is involved in the recruitment of inflammatory cells to the wound area. Conversely, in later stages TG2 regulates phagocytosis, activation of TGFβ and therefore limits the inflammation (15, 16, 20).

TG2 can also act as a signal transduction molecule of many growth / differentiation pathways through its intrinsic kinase activity, through the phosphorylation of insulin-like growth factor binding protein-3 (22) but may alter the signaling of other molecules through crosslinking events internally (18, 23).

TG2 may mediate these signaling events across the membrane in the G protein state and then lock the cell in a specific cycle through transamidation of various receptors (23). The known list of TG2 substrates continuously grows each day. Recently a characterization of the substrate specificity of TG2 and FXIIIA (a second extracellular transglutaminase to be discussed shortly) was done (24). Each transglutaminase had sequence specific recognition sites it preferred. As of May of 2005, 115 entries have been made into the TRANSIT database of transglutaminase substrates. Many of these are drugs, drug targets and key proteins known to mediate various disease states. Clearly, understanding the role of TG2 in physiology will help to understand the implication of dysregulation of TG2 in disease. This can only be accomplished through more experimentation.

FXIIIIA

The second transglutaminase, similar to TG2, which can be externalized, is Factor XIIIA (FXIIIA). It is the tissue form of FXIII containing only the A subunit of the clotting factor found circulating in blood. In blood, the primary action of FXIII is to crosslink fibrin molecules and is highly regulated by thrombin. In tissues, FXIIIA is regulated by thrombin or in the presence of high Ca^{+2}. It becomes cleaved at Arg^{37} which allows the binding of Ca^{+2}, thereby promoting catalytic activation. Similar to TG2, FXIIIA must bind Ca^{+2} to stabilize the molecule in the proper

conformation needed to initiate transamidation events. Additionally FXIIIA possesses the conserved catalytic triad found in all transglutaminases (25).

Unlike TG2, there are no nucleotide binding sites which regulate the activity of FXIIIA (25). Structure function studies have been performed examining the regulation of FXIIIA. FXIIIA can be inactivated by the cleavage of thrombin at the C terminus of the molecule or the loss of Ca^{+2} binding due to a decrease in its local concentration. In the absence of these last 200 amino acids, FXIIIA can no longer fold properly to stabilize Ca^{+2} and therefore is catalytically inactive (25). Additionally FXIIIA has been thought to form isomers, specifically through single amino acids. These isomers may increase the stability of FXIIIA. Finally, the binding of metal ions have been reported. Mutations to this site regulate the enzymatic activity of FXIIIA (26).

Primarily the role of FXIIIA has been examined in regards to the clotting factor functions. Recently studies have elucidated the role of FXIIIA in wound healing in angiogenesis. Initial studies found FXIIIA to have a proangiogenic role through promotion of endothelial cell migration and proliferation while inhibiting apoptosis. This action of FXIIIA depended upon the catalytic crosslinking of FXIIIA and the downregulation of TSP-1, a well characterized anti-angiogenic factor (27). In another study the authors continued research to determine the mechanism of action for FXIIIA was through direct binding of the endothelial cell integrin $\alpha V\beta 3$. This binding with FXIIIA promotes the interaction of $\alpha V\beta 3$ with vascular endothelial cell growth factor receptor-2 (VEGFR-2), which leads to its

activation and promotion of epithelial cell proliferation and migration (28). Finally these authors have shown the mediation of FXIIIA in monocytes and fibroblast activity and proliferation in wound healing (29).

Now taken together with the early known roles of FXIIIA, this enzyme, like TG2 is critical to wound healing, particularly in the blood vessels. During the initial stages of wound healing, thrombin activated FXIIIA stabilizes the fibrin clot to limit the bleeding. Secondly this clot is anchored to the wall through further crosslinking mediated by FXIIIA which incorporates fibronectin or other matrix proteins into the wound. Finally, FXIIIA can then promote the proliferation and migration of the endothelial cells to build new vessel walls (29).

In humans, FXIII deficiency is an inherited rare autosomal recessive bleeding disease, primarily associated with mutation in the A subunit. Patients have excessive bleeding at birth from the umbilical cord, impaired wound healing and females have subsequent miscarriages (30, 31). The FXIIIA homozygous null mice show similar characteristics to that of the human disorder. The mice are fertile, but have spontaneous miscarriages. Their survival is also limited due to bleeding episodes, hematothorax, hematoperitoneum and subcutaneous hemorrhage (32). Additionally it has been shown these mice have reduced cardiac remodeling, leading to cardiac rupture after a myocardial infarction (33).

Taken together, both FXIIIA and TG2 have dual roles as transglutaminases and mediators of physiologic processes. Further defining these other events each

are involved in will be critical to understand the pathologies they are involved in as well.

OSTEOARTHRITIS

Osteoarthritis (OA) is the most common form of arthritis and affects roughly 1 in 10 people, primarily those over the age of 45 years of age. Typically this disease has an increasing prevalence with each decade of life but can develop due to other factors such as heredity or tissue injury. OA is not simply a general feature of aging, suggesting that there are particular mediators that lead to the development of this disease (34). Traditionally it was thought of as a "non-inflammatory" arthritis, but recent work has demonstrated there are important inflammatory mediators (35) in the disease process. Additionally non-erosive synovial inflammation can occur even during early stages of the disease (36).

OA can affect any joint, but is most commonly found in the knee, hip, foot and hand. Progression of the disease can lead to a loss of mobility due to joint symptoms including pain and stiffness. Pathologically, there is joint space narrowing, fibrillation of the cartilage, damage to the meniscus, bony outgrowths (osteophytes) as well as weakened or frayed tendons and ligaments. All of these events can lead to activation of sensory neurons that innervate the joint. Currently there are no disease / structure-modifying drugs available and the frequently prescribed anti-inflammatory agents are only partially effective for pain relief (34).

In an osteoarthritic joint although damage can occur in the muscles, tendons and ligaments, the primary tissues involved are the articular cartilage, bone and synovium. In contrast to rheumatoid arthritis, synovial inflammation is secondary to the pathological changes found in bone and cartilage in OA. There is some debate as to whether early cartilage damage or bony changes lead to the development of OA first, but in OA animal model studies, cartilage destruction appears to precede alterations of the bone (37). This dissertation will focus specifically two potential mediators of the alterations that take place in the articular cartilage during OA.

Articular cartilage primarily consists of collagens, proteoglycans and articular chondrocytes. In a normal joint, cartilage is a firm, elastic tissue that acts as a cushion to prevent biomechanical damage that could be caused by severe loading. Maturationally arrested chondrocytes maintain a homeostatic environment to allow proper functioning of the joint. In OA as the cartilage breaks down, the homeostasis is disrupted, leading to an endogenous attempt at repairs which promotes the activation of a cascade of events. Many growth factors, cytokines and enzymes are produced in high amounts leading to a net increase in catabolism, and therefore causing further cartilage breakdown and chondrocyte differentiation (34).

Articular chondrocytes must remain maturationally arrested to maintain the cartilage matrix free of damage and consist specifically of collagens II, VI, IX, XI and aggrecan. While the chondrocytes are metabolically active, there is actually little proliferation. Growth plate or endochondral chondrocytes, which mineralize

under physiologic conditions, have matured past the stage of articular chondrocytes. These cells demonstrate an increase in proliferation, an enlargement or become hypertrophic and finally mineralize. Distinct from normal articular chondrocytes, hypertrophic chondrocytes express high levels of collagen type X, alkaline phosphatase (AP), matrix metalloproteinases 1, 3, 9 and 13, parathyroid hormone related peptide (PTHrP) and Indian Hedgehog (Ihh). In OA, articular chondrocytes leave their arrested state and enter into the differentiation process similar to what is seen in the growth plate, evidenced primarily by the hypertrophic phenotype (Fig 1-2). The molecular mediators which constrain articular chondrocytes in physiologic conditions and those which initiate the chondrocyte differentiation under pathologic conditions (such as OA) are unknown. Understanding these mechanisms is critical for the development of structure modifying drugs for OA (38).

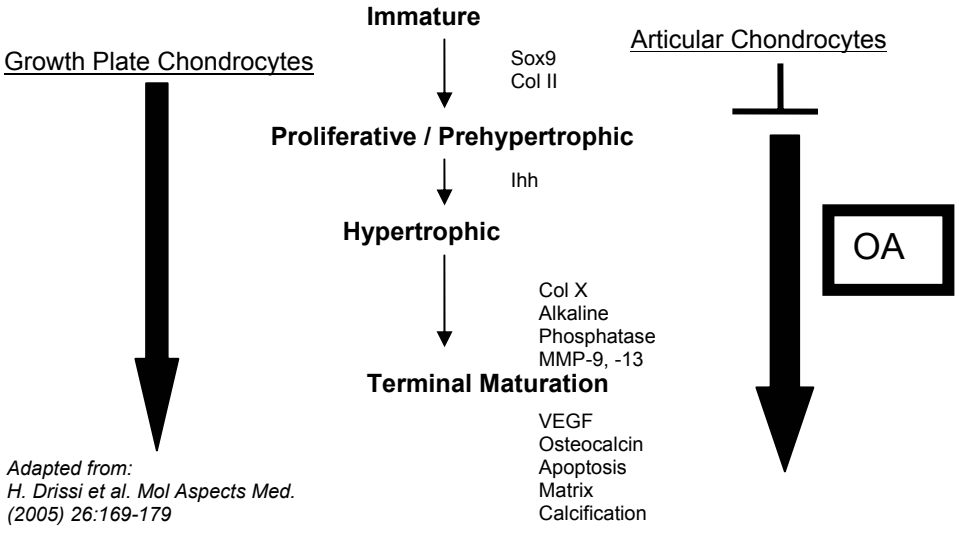


Figure 1-2. Chondrocyte differentiation. A schematic comparing the maturation of chondrocytes physiologically in the growth plate and pathologically in articular cartilage.

Two of the enzymes found to increase during OA and aging in cartilage are TG2 and FXIIIA (39, 40). TG2 and FXIIIA exist in articular cartilage as well as in the growth plate cartilage. Each is highly expressed and externalized in the hypertrophic zone of the growth plate (41, 42), upregulated in OA and co-localize with hypertrophic chondrocytes (39, 40). In both normal and OA cartilage, TG2 appears to be the predominant TG and is catalytically active (43).

After cartilage injury, the crosslinking action of the increased TG2 has been proposed to help stabilize the matrix proteins and prevent further proteolytic or mechanical damage (21, 44). Transglutaminases were initially believed to promote the growth and nucleation of hydroxyapaptite crystals through the formation of crosslinks between matrix proteins (21). In light of the other roles of TG2, specifically its involvement during tissue repair, it is plausible that other actions of excess TG2 may be responsible for the changes found during OA.

In 2003, the Terkeltaub laboratory examined the necessity of TG2 for chondrocyte differentiation. This work described both TG2-dependent and independent pathways which mediate the hypertrophic differentiation of chondrocytes. Stimulation of $TG2^{-/-}$ chondrocytes with IL-1, ATRA or KC/ Groα, led to little to no differentiation or calcification of the matrix (45, 46). Clearly, these two works point to a role of TG2 in cartilage differentiation, but the

mechanism has not been examined sufficiently. Furthermore, the role of the second transglutaminase in cartilage, FXIIIA, has not been elucidated.

SMOOTH MUSCLE CELL (SMC) CALCIFICATION

Calcification of a tissue can occur under physiological and pathological conditions. In bone and growth plate cartilage, the physiologic calcification is initiated by the release of matrix vesicles or apoptotic bodies (47). These contain enzymes and membrane components which shelter and promote the nidus of crystal formation. Eventually the calcium phosphate (hydroxyapatite) crystals rupture the vesicle and mineral is deposited in the surrounding matrix. The formation of the calcification that occurs is dictated by the local environment and the surrounding tissue and cells (47).

During atherosclerosis, aging, hypertension, diabetes and heart failure, calcification of the arteries has been demonstrated to occur (48). Intimal calcification resembles osteogenesis with the appearance of an osteoid and hydroxyapatite matrix (49). Medial calcification is characterized by linear deposits along the elastic lamella or a dense sheet of amorphous crystals (50). The role of this calcification is not clear. In atherosclerosis, it may have a local stabilizing effect on an individual plaque, thereby reducing the inflammation, but causing a destabilization on an adjacent one. Furthermore it can reduce the vascular

distendability in the aorta which can contribute to hypertension, hypertrophy and heart failure (50).

The artery wall is known to be a source and a destination for mesenchymal progenitor cells. Interestingly, in an atherosclerotic plaque, type II collagen expressing cells have been located, suggesting a differentiation of the SMC may have taken place proceeding down the osteogenic or chondrogenic pathway (51). A subpopulation of SMC, originally from bovine aortic medial cells, have been isolated and termed calcifying vascular cells (CVCs) (52). In response to phosphate these CVCs calcify their matrix and while transitioning to an osteoblastic phenotype (52).

Multiple studies have been done examining the mediators of the SMC calcification. Bone morphogenetic proteins (BMPs), TGFβ, osteopontin (OPN), osteoprotegerin (OPG), the sodium dependent phosphate transporter Pit-1, and modulation / regulation of pyrophosphate (PP_i) have all been found to mediate the chondrocytic / osteoblastic transition of SMC and leading to promotion or inhibition of calcification (48). The role of transglutaminases had not been explored in direct regulation of SMC calcification. In light of the role of TG2 in mediating chondrocyte differentiation, it is plausible this enzyme may have an important role. It is known that TG2 is critical for artery remodeling (53). During atherosclerosis, TG2 is increased in the SMC and macrophages (54). Furthermore, leukocyte TG2 has been found to limit the lesion size and constrain the necrotic core expansion in murine induced atherosclerosis (55).

HYPOTHESES

The objective of the second chapter was to determine the consequences of excess TG2 in mediating the progression of chondrocyte hypertrophic differentiation and identify the required amino acid(s). Both functions of TG2 have been found to mediate various cell events. Therefore the hypothesis tested was that externalized TG2 could directly promote articular chondrocyte hypertrophic differentiation dependent upon its binding to GTP.

The objective of the third chapter was to explore the molecular structure of the second transglutaminase FXIIIA and its role in chondrocyte hypertrophic differentiation compared and related to TG2. The hypothesis tested was that increased extracellular FXIIIA expression directly promotes chondrocyte differentiation but is dependent upon Ca^{+2} binding and the presence of TG2.

The objective of the fourth chapter was to examine smooth muscle cells (SMC) from TG2 deficient mice with a particular focus on the role of extracellular TG2 during the mediation of calcification. The hypothesis tested was that catalytically active TG2 is needed to promote the calcification of the SMC.

CHAPTER 2:

EXTERNAL GTP-BOUND TRANSGLUTAMINASE 2 IS A MOLECULAR SWITCH FOR CHONDROCYTE HYPERTROPHIC DIFFERENTIATION AND CALCIFICATION

SUMMARY

Chondrocyte maturation to hypertrophy, associated with up-regulated transglutaminase 2 (TG2) expression, mediates not only physiologic growth plate mineralization but also pathologic matrix calcification and dysregulated matrix repair in osteoarthritic articular cartilage. $TG2^{-/-}$ mouse chondrocytes demonstrate markedly inhibited progression to hypertrophic differentiation in response to both retinoic acid and the chemokine CXCL1. Here, our objectives were to test if up-regulated TG2 alone is sufficient to promote chondrocyte hypertrophic differentiation and to identify TG2 molecular determinants and potential downstream signals involved. TG2 activities, regulated by nucleotides and calcium, include cross-linking of cartilage matrix proteins, binding of fibronectin and hydrolysis of GTP and ATP. Following transfection of TG2 site-directed mutants into chondrocytic cells, we observed that wild type TG2, and TG catalytic site and fibronectin binding mutants promoted type X collagen expression and matrix calcification consistent with chondrocyte hypertrophic differentiation. In contrast, transfected mutants of TG2 GTP binding (K173L) and externalization (Y274A) sites did not stimulate chondrocyte hypertrophy. Recombinant TG2 treatment of bovine cartilage explants demonstrated that extracellular TG2 induced hypertrophy more robustly in the GTP-bound state, confirming an essential role of TG2 GTP binding. Finally, TG2 treatment induced type X collagen in a β1 integrin-mediated manner, associated with rapid phosphorylation of both Rac1 and p38 kinases that

were inhibited by mutation of the TG2 GTP binding site. In conclusion, externalized GTP-bound TG2 serves as a molecular switch for differentiation of chondrocytes to a hypertrophic, calcifying phenotype in a manner that does not require either TG2 transamidation activity or fibronectin binding.

INTRODUCTION

In physiologic endochondral mineralization, the maturation of chondrocytes from resting cells to hypertrophic cells is a critical event in the re-organization of the matrix and enhanced shedding of matrix vesicles that promote calcification (56, 57). The pathologic maturation of chondrocytes to hypertrophy in osteoarthritic (OA) articular cartilage (58, 59) also promotes matrix calcification (60). Chondrocyte hypertrophy is associated not only with expression of the stereotypic marker type X collagen but also with broad alterations in extracellular matrix synthesis, matrix catabolic and calcification-regulatory and angiogenesis-regulating gene expression programs mediating on cartilage beyond calcification (56, 60-63). Genes up-regulated in both hypertrophic growth plate and OA articular chondrocytes include the transglutaminase (TG) isoenzymes TG2 and FXIIIA (40, 42, 43, 64).

Transglutaminases (TGs) catalyze a calcium-dependent transamidation reaction that catalyzes covalent cross-linking of substrates with available glutamine residues to primary amines (EC 2.3.2.13). Both TG2 and FXIIIA, though lacking a

signal peptide, are externalized to the pericellular matrix in both growth plate and articular cartilage (41, 43). In both normal and OA cartilage, TG2 appears to be the predominant catalytically active TG in the extracellular matrix (43). Recently, we observed that young $TG2^{-/-}$ mice have a normal skeletal phenotype and that $TG2^{-/-}$ knee articular chondrocytes become hypertrophic in response to the essential physiologic growth plate chondrocyte differentiation regulator CNP (45). However, $TG2^{-/-}$ chondrocytes exhibited markedly inhibited induction of chondrocyte hypertrophy and calcification in response to CXCL1 (46), a pro-inflammatory chemokine up-regulated in OA cartilages (65). $TG2^{-/-}$ chondrocytes also demonstrated suppressed induction of chondrocyte hypertrophy and calcification in response to retinoic acid (45), an inducer of pathologic calcification in vivo (66). Hence, TG2 is not only a marker of chondrocyte hypertrophy but also a mediator of chondrocyte maturation (45).

TG2 is localized primarily in the cytosol in a catalytically latent form (67). Additionally, TG2 has the potential to translocate to the nucleus, to be externalized and to ultimately co-localize with proteins in the extracellular matrix or on the extracellular side of the plasma membrane (68). TG2 is one of a minority of TG isoenzymes that binds GTP (69) and TG2 dually functions as a TG and GTPase/ATPase (70). These distinct enzyme activities of TG2 are reciprocally regulated, partly at the level of conformation via the binding of Ca^{2+} essential for transamidation catalytic "TG activity" (70). Furthermore, binding of guanine nucleotides to TG2 suppresses catalytic TG activity, whereas binding of adenine

nucleotides inhibits TG2 GTPase activity without altering TG activity (71). Constitutive cytosolic latency of TG2 is maintained through binding to GDP and ADP, but this state is subject to alterations that can promote TG activity, such as cellular influx of Ca^{2+} or externalization of TG2, taking TG2 from a [high GTP, low calcium] to [low GTP, high calcium] microenvironment (71).

Direct up-regulation of TG2 in chondrocytes by transfection markedly increased matrix calcification (40). TG catalytic activity of TG2 has the potential to modulate chondrocyte differentiation and calcification by stimulating increased activity of PLA2 (68) and by promoting conversion of TGFβ from latency to an activate state (64). TG2 can covalently cross-link several collagen subtypes, as well as fibronectin and a variety of calcium-binding and mineralization-regulatory proteins in the pericellular matrix of chondrocytes and osteoblasts (72). Hence, TG2-induced crosslinking of matrix proteins could regulate matrix-cell communication, and TG2 matrix stabilization and cross-linking of calcium-binding matrix proteins such as osteonectin and osteopontin (72, 73) potentially modulates growth of hydroxyapaptite crystals (74, 75). However, in seminal work, Nurminskaya *et al,* observed that hypertrophic chondrocyte-derived or exogenous hepatic TG2 stimulated maturation of preosteoblasts and accelerated mineralization, an effect not dependent on TGFβ activation and not prevented by the competitive transamidation inhibitor putrescine (76).

TG2, acting at the internal and external faces of the plasma membrane, clearly regulates cell migration and differentiation and wound repair (67). In this

context, TG2 can physically interact with the cytosolic tails of certain α integrin subunits (67), and TG2 binds to the gelatin-binding domain of fibronectin in a transamidation-independent manner and acts as an integrin co-receptor for fibronectin (68), thereby modulating cell adhesion (5). TG2 may also function as a protein kinase (77). Significant biologic functions of guanine nucleotide-bound TG2 (68) include TG2 GTPase-related effects exerted on cell signaling externally at the plasma membrane and also intracellularly, where TG2 promotes activation of phospholipase Cδ1 by the α1-adrenergic receptor (78, 79).

In view of the distinct, but potentially complementary means by which TG2 regulates cell differentiation, the objectives of this study were to test the hypothesis that up-regulated TG2 expression is sufficient to promote chondrocyte hypertrophic differentiation and calcification, and if so, to define TG2 molecular determinants and the primary locus (i.e., intracellular or extracellular) for TG2 to directly promote these changes.

RESULTS

Characterization of TG2 site mutants -To investigate TG2 structural determinants for regulation of chondrocyte hypertrophy and calcification, we engineered the initial group of TG2 site directed mutants for study based on the following strategic considerations. First, TG2 Cys^{277} is an essential amino acid for transamidation activity (80) and it participates in a catalytic triad conserved in all

members of the transglutaminase family (70). Therefore we mutated each of the triad amino acids in TG2 (Cys^{277}, His^{335} and Asp^{358}). Second, the hydrophobic pocket involved in the capacity of TG2 to bind and hydrolyze guanine nucleotides, includes Phe-174, Val-479, Met-483, Leu-582, and Tyr-583, while hydrolysis of GTP involves either Lys-173 or Arg-476 (70). Mutation of Lys-173 to Leu has been demonstrated to decrease GTP binding and hydrolysis as well as signal transduction regulatory activity of TG2 (12). Therefore, we generated TG2 K173L. Third, Tyr^{274} and Cys^{277} appear to be critical in mediating the release of TG2 into the extracellular matrix, as recently illustrated by mutagenesis-based studies (81). Hence, we also generated TG2 Y274A to probe for specific effects mediated by externalization of TG2 in chondrocytes but independent of effects on transamidation activity.

For evaluation of effects of direct expression of TG2 site mutant cDNAs we chose to employ readily transfectable SV40-immortalized normal articular chondrocytic CH-8 cells. In transient transfection studies, we confirmed that each point mutation in the TG2 catalytic domain triad (C277G, H335A and D358A) caused a significant decrease in the amount of TG activity when compared to overexpression of the wild type (WT) TG2. There was no significant change in transamidation activity of the GTP binding or externalization site mutants (Figure 1A). Each TG2 mutant was found to express at comparable levels that were above the observed low background expression level detected in CH-8 cells, as assessed by SDS-PAGE/Western blotting (Figure 1B).

TG2 induction of matrix calcification and type X collagen expression mediated by externalization and functional GTP binding- Transfection of WT TG2 into CH-8 cells, under pro-mineralizing conditions imparted by addition to the medium of 1 mM sodium phosphate, confirmed (40) the capacity of TG2 to directly promote calcification by chondrocytic cells (Figure 2A). Mutation of amino acids within the TG catalytic triad did not significantly affect the ability of TG2 to stimulate matrix calcification (Figure 2A). Conversely, both the externalization site mutant and GTP binding site mutant of TG2 were associated with a lower capacity to induce calcification than WT TG2 (Figure 2A).

Next, we tested for potential direct TG2 induction of type X collagen expression, the stereotypic marker for chondrocyte hypertrophy (82). Since P_i promotes chondrocyte hypertrophy (83, 84), we employed conditions where medium was not supplemented with sodium P_i. Transfection of WT TG2 was discovered to be sufficient to induce type X collagen expression (Figure 2B). The capacity of TG2 to induce type X collagen was retained by the catalytic site triad mutants but was depressed for the externalization site mutant and absent in the GTP binding site mutant (Figure 2B).

Qualitative and quantitative assessment of extracellular TG2 levels in the conditioned media were carried out for CH-8 cells transfected with WT TG2 and the panel of TG2 site mutants (Figure 3A-B). The most striking observation was the marked diminution of conditioned medium TG2 levels associated with direct

expression of the externalization site mutant (Y274A) and the GTP binding site mutant (K173L), in comparison to WT TG2 (Figure 3A-B).

In epithelial cells, TG2 externalization has been mechanistically linked with the capacity to bind fibronectin (85). Hence, we concurrently tested the potential role of fibronectin binding in TG2 externalization and induction of type X collagen expression in CH-8 cells. Deletion of the N-terminal seven amino acids of TG2 inhibits binding to fibronectin (85), but poses the risk of marked conformational change. Therefore, we designed point mutants and deletions of limited amino acids in the N-terminal domain of TG2. The first cDNA generated contained a deletion of Leu-5, the second had mutations at Ala-2 and Glu-3, and the third mutations at Leu-7, Glu-8 and Arg-9, since TG2 Glu-8 and Arg-9 have been directly implicated in fibronectin binding (86) (Figure 4A). Each of these mutants was initially tested for the capacity to bind fibronectin (Figure 4B). We observed that all three fibronectin domain mutants were significantly impaired in their ability to bind to fibronectin, with the Del 5 construct retaining less than 5% of this function relative to WT TG2. Significantly, all the other TG2 point mutants tested above also had a significant decrease in fibronectin binding, though to a lesser degree than mutants at the N-terminal fibronectin binding domain (Figure 4B). Next we tested for externalization of the three fibronectin binding domain TG2 mutants after transient transfection into CH-8 cells, as above. Through SDS-PAGE/Western blot analysis, we saw comparable expression levels to the WT TG2 of the fibronectin binding domain mutants, both intracellularly and in the conditioned media (Figure 4C).

Treatment with each of the TG2 fibronectin binding domain mutants also induced type X collagen expression in CH-8 cells (Figure 4D).

Extracellular Mg-GTP- bound forms of TG2 markedly induce type X collagen expression – To complement the preceding experiments based on transfection, and to assess the effects of extracellular TG2 in isolation, we next generated His-tagged WT and mutant TG2 soluble forms (sTG2) to add to chondrocytic cells. First, we observed that WT sTG2 treatment using 0.1 μg/ml protein, induced a less robust increase in type X collagen expression in CH-8 cells relative to controls (Figure 5B, lanes 1 and 5) than was observed in the preceding transfection studies. Additionally, the TG transamidation activity in the cell lysate after transfection with WT sTG2 was five-fold less than that applied with the 0.1μg/ml dose of soluble, extracellular TG2.

As cited above, GTP binding to TG2 induces decreased TG transamidation activity in association with conformational change in TG2 (71). Based on a previous study demonstrating that Mg-nucleotide complexes are primary substrates for the TG2 GTP binding site (71), we added Mg-GTP or non-hydrolyzable Mg-GTPγS to sTG2. We verified that even in presence of calcium, both forms of Mg-GTP complexes induced a five fold reduction in TG transamidation activity (Figure 5A). The sTG2 treated with either Mg-GTP or Mg-GTPγS more robustly induced type X collagen than did WT sTG2 in CH-8 cells (Figure 5B), indicating that hydrolysis of GTP was not necessary for this sTG2 “gain of function”. In parallel

studies, simultaneous addition of Mg-ATP or Mg-ATP and Mg-GTP to WT sTG2 in order to inhibit GTPase activity (71) allowed sTG2 to more robustly induce type X collagen than sTG2 alone (not shown).

Next, we complexed Mg-GTP to additional, mutant forms of sTG2. Under these conditions, addition to cells of Mg-GTP treated soluble mutants of each member of the TG2 catalytic triad induced type X collagen (Figure 6A) and matrix calcification (Figure 6B), essentially comparable to the results for transfected CH-8 cells above. The Mg-GTP-treated TG2 externalization site mutant Y274A, when applied extracellularly, also exerted comparable effects on type X collagen expression and calcification relative to WT sTG2 when complexed to Mg-GTP (Figure 6)**.** In contrast, the exogenous extracellular GTP binding site mutant of sTG2 treated with Mg-GTP, as above, failed to induce type X collagen or matrix calcification by CH-8 cells.

Next, we tested the effects of sTG2 in cartilage organ culture, in order to assess modulation of chondrocyte differentiation by TG2 in a more physiologic milieu. To do so, bovine articular cartilage explants were incubated for five days in the presence or absence of sTG2 (1 μg/ml) with and without Mg-GTP treatment. Frozen sections of cartilage were immunohistochemically stained for type X collagen. Weak type X collagen induction was observed in response to sTG2, with a markedly more robust response demonstrated with sTG2 that was Mg-GTP complexed (Figure 7). Taken together, these results were consistent with

externalized TG2 functioning in the GTP-bound state as a molecular switch for the induction of chondrocyte type X collagen expression.

β1 integrin antibody treatment impairs TG2 induction of type X collagen expression -TG2 interacts with the extracellular domains of β1 and β3 integrin subunits and forms 1:1 complexes on the surface of cells (5). Furthermore, collagen deposition and the enlargement of growth plate chondrocytes during hypertrophy are mediated in part by interactions of the extracellular matrix with β1 integrins (84). Hence, we next tested the role of β1 and β3 integrins during TG2-induced hypertrophy. To do so, chondrocytic cells were pretreated for one hour with 1 μg/ml of antibodies to each integrin prior to treatment with 0.1μg/ml of recombinant WT TG2 or the TG2 K173L mutant for three days. SDS-PAGE/Western blotting of the cell lysates revealed inhibition of WT TG2-induced type X collagen expression when the cells were treated with the β1 integrin antibody, but not after treatment with the β3 integrin (Figure 8).

TG2 mediates cell adhesion and migration partly by acting as an integrin co-receptor for fibronectin (2), and both integrin signaling and certain effects of TG2 on cells are mediated by FAK signaling (77). During modulation of cell-matrix interactions, FAK has been demonstrated to act through small GTPases, such as Rac1 (87), which is also known to be critical in regulation of cardiac hypertrophy (88). Significantly, p38 MAPK, one of the downstream mediators of

integrin signaling (87) is an essential transducer of chondrocyte hypertrophy in vitro (89). We observed that Rac1 phosphorylation was induced within 5 minutes of exposure to 0.1 μg/ml of WT sTG2 (Figure 9) and p38 phosphorylation was induced within 10 minutes of treatment with WT sTG2 (Figure 9). But stimulation of chondrocytic cells with 0.1 μg/ml of the K173L mutant sTG2 revealed that the soluble TG2 GTP binding site mutant had a diminished capacity to induce Rac1 and p38 phosphorylation relative to WT sTG2 (Figure 9). Therefore, soluble extracellular TG2 induced rapid signal transduction in chondrocytes, as well as type X collagen expression and calcification, in a manner dependent on the integrity of the guanine nucleotide binding site.

DISCUSSION

TG2 expression has been implicated as a major, direct mediator of not only osteoblast maturation (76) but also chondrocyte maturation to hypertrophy associated with matrix calcification in response to retinoic acid and CXCR2-binding chemokines (45, 46). Here, we discovered that the ability of extracellular TG2 to bind GTP was central to inductive effects of a "gain-of-function" of TG2 on chondrocyte type X collagen expression and calcification. Significantly, these alterations of chondrocyte differentiation were not dependent upon TG2 transamidation activity or on binding of fibronectin.

Though externalization of TG2 was required to induce type X collagen and calcification it was not sufficient to do so, as illustrated by the failure of exogenous treatment with the TG2 GTP binding site mutant K173 to induce these effects in chondrocytic cells. These results were buttressed by the markedly augmented induction of type X collagen by WT TG2 treated with Mg-GTP. Furthermore, treatment of chondrocytic cells with a soluble form of the poorly externalized Tyr-274 mutant of TG2 also robustly induced type X collagen expression, which was not the case in cells transfected with the Tyr-274 mutant. TG2 can hydrolyze GTP to GDP at a rate comparable to that of other G proteins (71), but based on TG2 crystal structure, it has been proposed that TG2 binding of GDP, or exchange with GTP, are particularly avid (70). Hence, the results of this study suggest that the guanine nucleotide-bound form of TG2 may stably assume an ideal conformation for externalization by chondrocytic cells and also trigger type X collagen expression and calcification selectively in response to specific agonists in the chondrocyte. It will be of interest to determine if such a mechanism mediates our observations that IL-8/CXCL8, which is up-regulated in OA articular cartilages (65, 90) and stimulates chondrocyte hypertrophy, increases TG2 translocation from the cytosol to the conditioned medium in CH-8 cells, an activity not shared by CNP, which induces cultured chondrocyte hypertrophy in a TG2-independent manner (45).

In the growth plate, TG2 has been observed to remain intracellular <u>in situ</u> during growth plate chondrocyte proliferation and maturation, and to become

externalized in the hypertrophic zone (42). Moreover, externalized TG2 has been detected in OA articular cartilages (43). Therefore, our current observations underscore the functional importance of TG2 release by the chondrocyte. TG2 lacks a signal peptide and has not been observed to transition into a cellular secretory pathway through the Golgi. Externalization is mediated by TG2 conformation, regulated in part by the Tyr^{274} and Cys^{277} residues (81), and possibly proceeds by a process similar to that proposed for translocation of TG2 into the nucleus (68). It will be of interest to further understand how chondrocytes externalize TG2, and whether chondrocytes express a tissue-selective chaperone for TG2 release.

In this study, TG2 GTPase activity was not required to switch on chondrocyte type X collagen expression, as demonstrated by results of cell treatment with non-hydrolyzeable GTPγS complexed to sTG2. Moreover, inhibition of GTPase activity of WT TG2 (through Mg-ATP and Mg-GTP addition) was associated with retention of the capacity to promote the induction of type X collagen. We determined that although the fibronectin binding domain of TG2 was not critical for induction of chondrocyte hypertrophy, the interaction between TG2 and β1 integrins may at least partially mediate the induction of hypertrophy in chondrocytic cells. Our exploratory results of signal transduction in response to WT sTG2 as opposed to K173L sTG2 lead us to speculate that conversion of TG2 to a GTP-bound conformational state allows TG2 to be directly recognized at the level of the chondrocyte plasma membrane. Alternatively, conversion of TG2 to a

GTP-bound conformational state may allow TG2 to complex with one or more extracellular matrix proteins and external domains in integrins to induce rapid signaling events including phosphorylation of Rac1 or p38 MAPK. Another possibility is that intercalation of secreted GTP-bound TG2 into the extracellular matrix may indirectly regulate how matrix constituents such as collagens interact with the chondrocyte to stimulate signal transduction and affect cell differentiation.

TG2 interconversion between guanine nucleotide bound transamidation-latent and calcium-bound transamidation-active forms has the corresponding capacity to switch off and switch on a variety of transamidation-related TG2 functions (71). In the maintenance of vascular tone and remodeling of arteries, early steps appear to be mediated in part by smooth muscle cell activation through TG2 effects on protein crosslinking, whereas downstream events may require TG2 G-protein functions (91). As such, our results with clonal chondrocytic cells in fixed culture conditions should not be interpreted to exclude a role for TG2 transamidation activity in modulating chondrocyte differentiation or calcification in the growth plate or in OA cartilage in situ (42, 92).

Significantly, TG2 binding to GTP or GDP renders TG2 highly resistant to proteolytic degradation (71). As such, steady release of bound guanine nucleotides from TG2 outside the cell could help preserve and fine-tune TG2 functional potential in the protease-rich chondrocyte extracellular environment for the prolonged time intervals needed for chondrocytes to mature as well as to modify and calcify their matrix. The known increase in ecto-nucleotide pyrophosphatase

pyrophosp-hohydrolase (NPP) activity (EC 3.6.1.8) in articular cartilages with chondrocalcinosis and / or OA (95) also provides the theoretic potential to promote increased transamidation activity of extracellular TG2 via enhanced extracellular GTP hydrolysis to GMP and PP_i in articular cartilages. Indeed, we observed that total TG transamidation activity was increased on average greater than 40-fold in human knee meniscal fibrocartilages with late stage OA relative to normal menisci (40). In this context, it also is noteworthy that IL-1, which markedly suppresses chondrocyte ecto-NPP expression (93), induces TG2-dependent calcification in association with a TG2-dependent rise in TG activity in chondrocytes, yet IL-1 does not induce chondrocyte hypertrophy in vitro (45). It will be of interest to discern the mechanisms that dissociate TG2-mediated hypertrophy from matrix calcification.

An important component of this study was the confirmation of the induction of type X collagen expression by Mg-GTP bound sTG2 using cartilage organ culture. However, limitations of this study included the primary utilization of SV40-immortalized chondrocytic CH-8 cells. The CH-8 cells were chosen for study because of their human origin and because they maintained a chondrocytic phenotype in culture. Additionally they allowed efficient transfection and useful comparisons of transfected cells and cells treated with exogenous TG2. But CH-8 cells in monolayer culture cannot be held to be a precise surrogate for primary chondrocytes in 3-dimensional non-adherent culture, nor for cartilage in organ culture or in situ. Additional limitations of this study included administration to

chondrocytic cells of recombinant exogenous TG2 prepared from fibroblastic cells, thereby possibly imposing a lack of chondrocyte-specific post-translational processing, secretion, and proteolysis of TG2. CH-8 cells express both FXIIIA and TG2 constitutively, effects that have could modulated observed results including matrix calcification (40) in CH-8 cells. It remains possible that TG2 may have a redundant or directly interactive role in growth plate physiology with other GTP-binding TGs (94) or with FXIIIA (11, 14) which, like TG2, is externalized by mineralizing hypertrophic chondrocytes (41).

In conclusion, we have demonstrated that guanine-nucleotide bound TG2 externalized by chondrocytes acts as a molecular switch to turn on both chondrocyte type X collagen expression and calcification. It will be of interest to define the respective roles of GTP-bound TG2 in not only promoting articular chondrocyte hypertrophy and pathologic calcification driven by OA-associated articular inflammation but also in mediating physiologic growth plate chondrocyte differentiation. Our results add to growing evidence for significant effects of TG2 on differentiation and function of connective tissue cells that are mediated by TG2 binding of GTP and not dependent on TG2 transamidation activity.

The text of chapter 2 is a reprint of the material as it appears in the Journal of Biological Chemistry 280(15):15004-12, 2005. I was the primary researcher and

author and the co-author listed in this publication directed and supervised the research which forms the basis of this chapter.

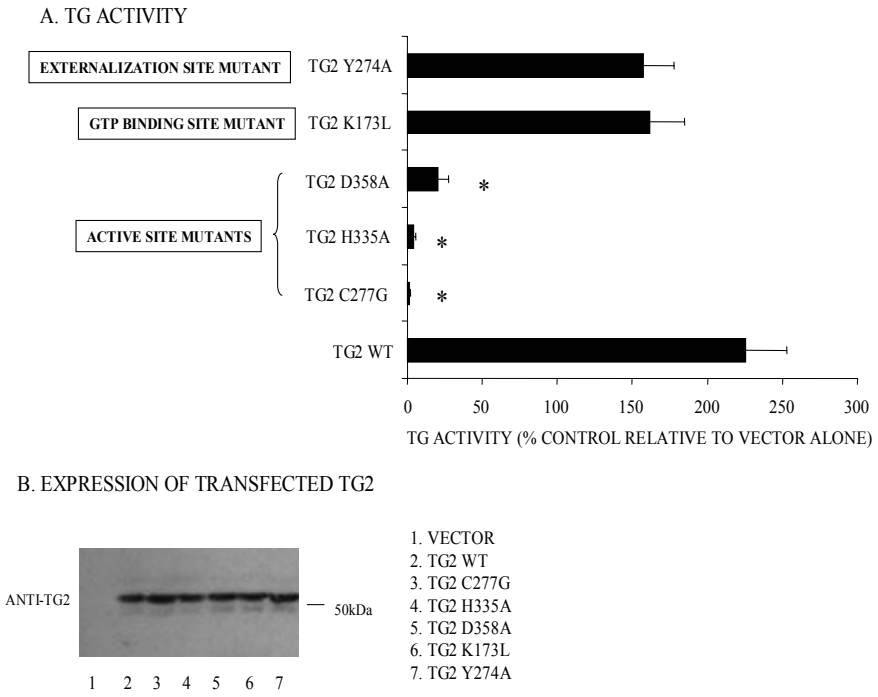


Figure 2-1**.** Changes in TG transamidation activity in association with transfection of specific TG2 mutants in human chondrocytic CH-8 cells. Specific TG2 mutant cDNAs as indicated were transfected using Lipofectamine Plus into aliquots of 2 x 10^5 CH-8 cells plated into a 35 mm culture dish, as described in the Methods. The cells were grown for 72 hours in Medium A, at which time the cell lysates were collected for analysis of TG activity by incorporation of biotinamindopentylamine into *N,N'*- dimethylcasein (Panel A) and TG2 protein levels by SDS-PAGE/Western blotting (Panel B), as described in the Experimental Procedures. Representative of 5 experiments, *$p<0.05$ indicates a significant decrease in TG activity relative to transfection of WT TG2.

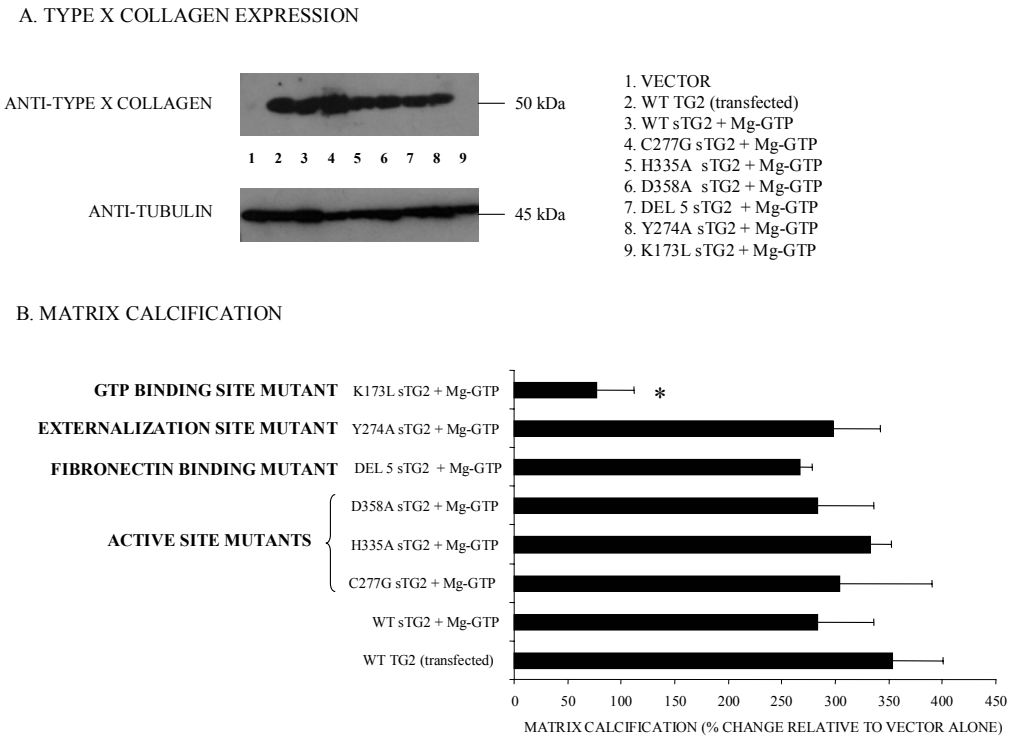


Figure 2-2. TG2 induces matrix calcification and type X collagen without the requirement for a functional TG2 transamidation catalytic site triad but dependent on the GTP binding site in CH-8 cells. Aliquots of 2 x 10^5 CH-8 cells in 35 mm dishes were transiently transfected with WT TG2 or specific TG2 mutants as indicated and carried for 5 days in Medium B, which contained 1 mM supplemental sodium phosphate. The dishes were stained with Alizarin Red and matrix calcification quantified as described in the Methods. Data pooled from 5 experiments performed in triplicate, *$p<0.05$ indicates a significant decrease relative to transfection of WT TG2 (Panel A). Aliquots of 2 x 10^5 CH-8 cells in 35mm dishes were transfected with WT TG2 or specific TG2 mutants and grown for 72 hours in Medium A. Cell lysates were separated by SDS-PAGE and probed for type X collagen and tubulin by Western blotting as described in the Methods. Representative of 5 independent experiments (Panel B).

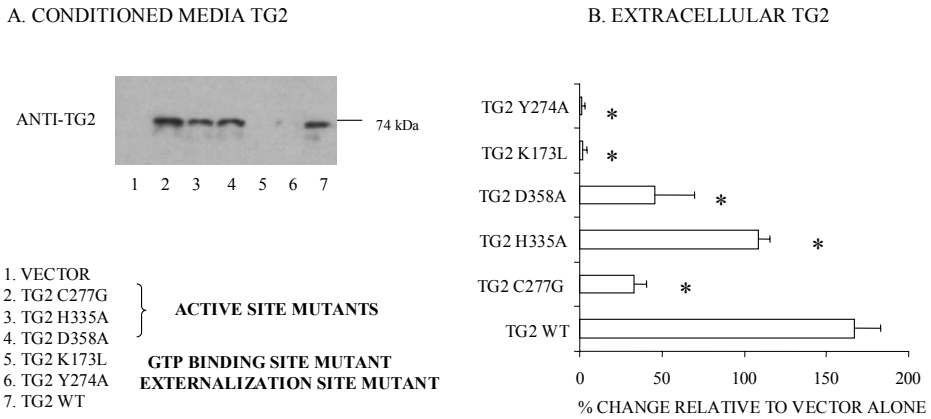


Figure 2-3. Decreased externalization of the TG2 K173L GTP binding site mutant and the TG2 Y274A externalization site mutant. Following transfection of CH-8 cells (2 x 10^5 cells / 35mm dish) the cells were carried for 72 hours. During the last 8 hours, Medium A was removed and replaced with serum free DMEM. Then, the conditioned media were collected, precipitated and separated by SDS-PAGE for Western blotting (Panel A) or quantified for TG2 (Panel B) by ELISA as described in the Methods. (Representative of 5 experiments, *$p<0.05$ indicates a significant change from WT TG2).

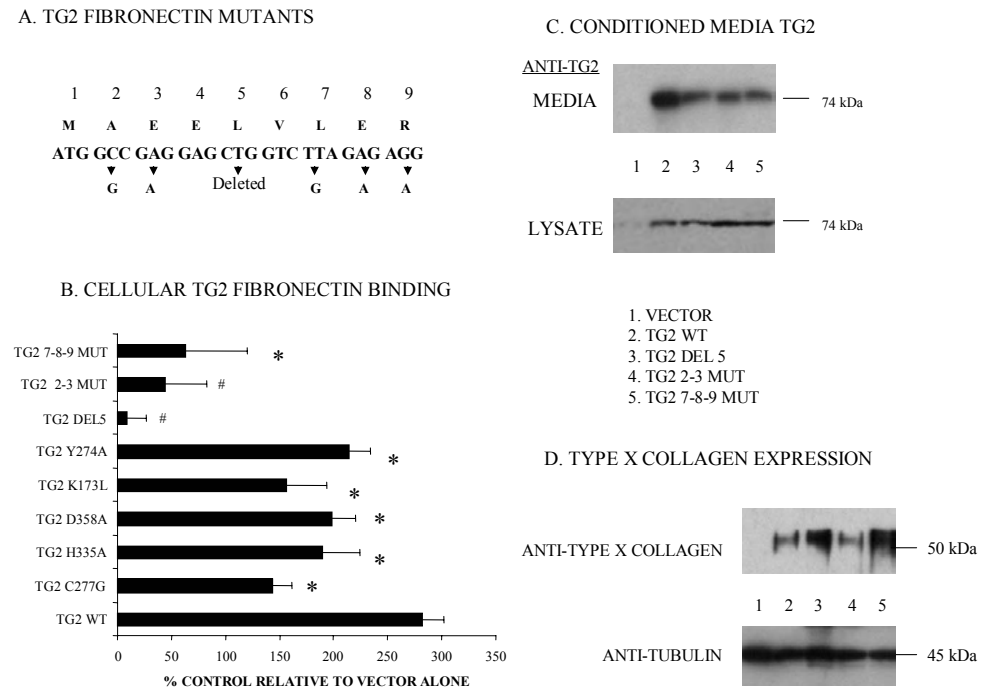


Figure 2-4. Preserved function of the fibronectin binding domain is not required for externalization of TG2 or induction of type X collagen in CH-8 cells. Panel A is a schematic of the first eight amino acids of TG2, with arrows indicating the sites of point or deletion mutants in this fibronectin-binding domain. In the fibronectin-binding studies depicted in Panel B, aliquots of 2 x 10^5 CH-8 cells in 35mm dishes were transfected with the mutants depicted in Panel A or with WT TG2 and carried for 72 hours in Medium A. Cell lysates were harvested, lysed and assessed for the ability to bind to fibronectin coated 96 well plates, with the amount of fibronectin binding by cellular TG2 determined using anti-TG2 antibody CUB7402, as described in the Methods. To evaluate extracellular TG2 in these studies (Panel C), the conditioned media from samples in Panel B were removed, precipitated and analyzed by SDS-PAGE/Western blotting. To assess type X collagen expression (Panel D), CH-8 cells grown as described for Panel B were harvested after 72 hours, and analyzed by SDS-PAGE/Western blotting as described above. Representative of 4 independent experiments. *$p<0.05$, #$p<0.001$ indicate a significant change relative to WT TG2.

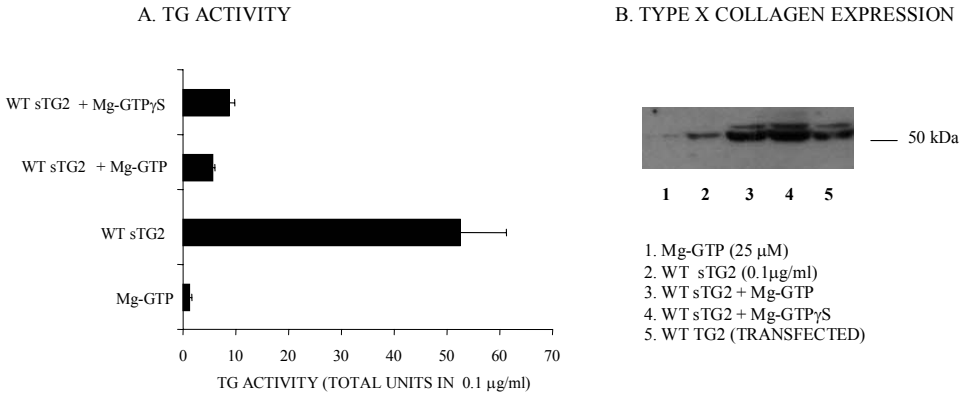


Figure 2-5. Treatment of soluble recombinant TG2 with Mg-GTP inhibits TG transamidation activity but augments the capacity to induce type X collagen expression. Mg-nucleotide complexes as indicated were incubated with 0.1 µg soluble recombinant human TG2 for 30 minutes at 4°C, as described in the Methods. The TG catalytic activity of these preparations was determined as described above (Panel A). Aliquots of CH-8 cells (2 x 10^5 cells in a 35mm dish) were incubated with the Mg-nucleotide complex treated WT TG2 as indicated for 72 hours in Medium A. For comparison purposes, we transfected aliquots of CH-8 cells with WT TG2, as described above for Figure 3. Cell lysates were analyzed for type X collagen expression by SDS-PAGE/Western blotting as described above (Panel B). Representative of 4 independent experiments.

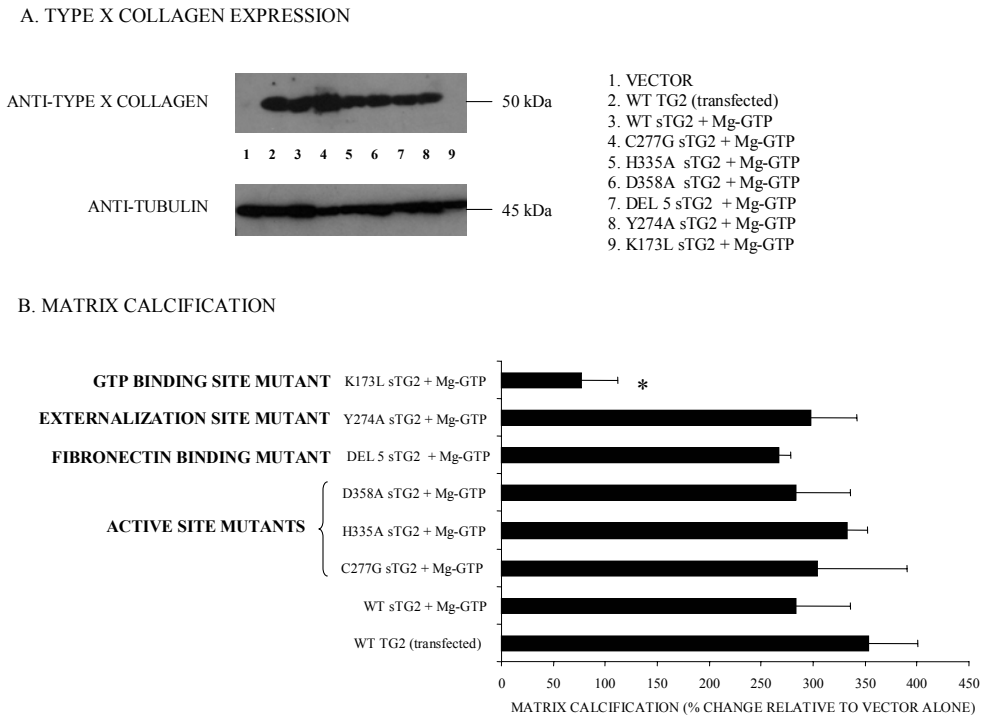


Figure 2-6. Extracellular soluble TG2 K173L GTP binding site mutant treated with Mg-GTP fails to induce type X collagen expression or matrix calcification in CH-8 cells. WT TG2 and soluble recombinant TG2 mutants as indicated were treated with Mg-nucleotide complexes as described above. After 72 hours of incubation in Medium A with each form of soluble TG2, CH-8 cell lysates were collected for analysis of type X collagen expression by SDS-PAGE/Western blotting (Panel A). For studies of matrix calcification (Panel B), the specific TG2 forms previously treated with Mg-GTP were incubated with aliquots of CH-8 cells (2 x 10^5 cells in a 35mm dish) for 5 days in Medium B and quantification of Alizarin Red binding was performed as above. Representative of 3 independent experiments, *$p<0.05$ indicates a significant change relative to soluble WT TG2 treated with Mg-GTP.

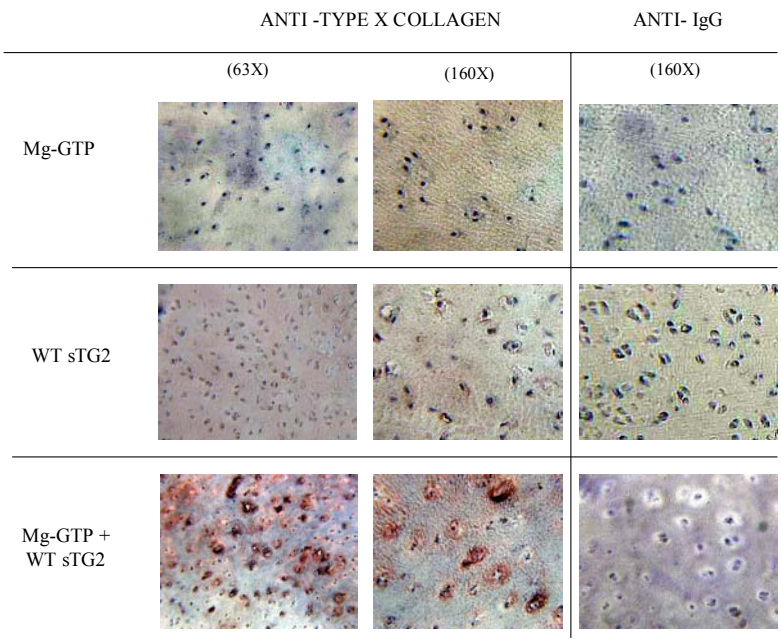


Figure 2-7. Exogenous Mg-GTP bound TG2 markedly stimulates type X collagen expression in normal bovine cartilage organ culture. Normal bovine adult articular knee cartilage was isolated and cut into 2 mm x 2 mm slices. After a 24 hour recovery period in Medium A, Mg-GTP, WT TG2 or Mg-GTP complexed to WT TG2 (1µg/ml) was incubated with the slices for 5 days in medium A. Frozen sections were immunohistochemically stained after fixation in cold acetone, using primary antibody to type X collagen (1:500 dilution) or nonimmune rabbit IgG as a control, as described in the Methods. Results representative of studies of three different bovine knee donors.

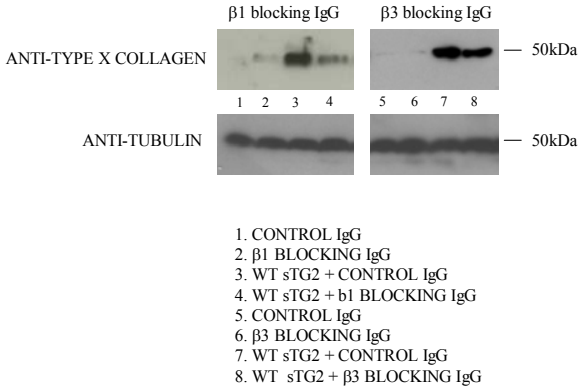


Figure 2-8. Antibody to β1 integrin (but not β3 integrin) inhibits TG2-induced type X collagen expression in chondrocytic cells. CH-8 cells were pretreated with 0.1 μg/ml of antibodies to β1 or β3 integrin (or an isotype control) for 1 hour prior to treatment with sTG2 (pretreated with Mg-nucleotide complexes as described above). After 72 hours of incubation in Medium A with each form of soluble TG2, CH-8 cell lysates were collected for analysis of type X collagen expression by SDS-PAGE/Western blotting, as described in the Methods. Representative of 3 independent experiments.

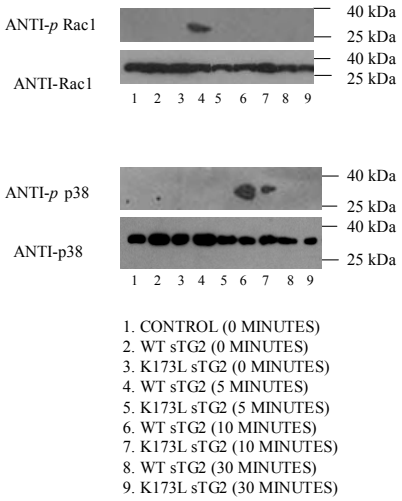


Figure 2-9. Addition of WT sTG2 rapidly induces Rac1 and p38 MAPK phosphorylation in chondrocytic cells in a manner diminished by the K1733L TG2 GTP binding site mutation. CH-8 cells (2 x 10^5 cells / 35mm dish) were starved for 4 hours in serum free medium. We then added fresh Medium A containing 0.1 µg/ml of WT sTG2 or K173L sTG2. At the times indicated, CH-8 cell lysates were collected for analysis of Rac1 and p38 expression and phosphorylation by SDS-PAGE/Western blotting, as described in the Methods. Representative of 3 independent experiments

CHAPTER 3:

FACTOR XIIIA ENGAGES α1β1 INTEGRIN AND MOBILIZES TRANSGLUTAMINASE 2 TO INDUCE CHONDROCTYE HYPERTROPHY

SUMMARY

Two transglutaminases (TGs), Factor XIIIIA (FXIIIA) and TG2, undergo physiologic upregulation in growth plate hypertrophic chondrocytes and pathologic upregulation in osteoarthritic cartilage. Guanine nucleotide-bound TG2 drives chondrocyte maturation to hypertrophy, a state linked to matrix remodeling and calcification. TGs have been demonstrated to interact with certain integrin subunits. During osteoarthritis (OA) the $\alpha 1$ subunit of $\alpha 1\beta 1$ integrin is up-regulated and associated with hypertrophic chondrocytes. Here we examined molecular structure-function of the second FXIIIA in chondrocyte differentiation to determine if there was a distinct mechanism by which FXIIIA and TG2 function. Using both knockout mouse chondrocytes and shRNA transfected into human articular chondrocytes, we determined that FXIIIA induced chondrocyte hypertrophy, but required TG2 expression to do so, whereas extracellular TG2 was sufficient to induce hypertrophy itself. Site directed mutants revealed the FXIIIA endoproteolytic Arg^{37} site and integrity of the Ca^{2+}-binding domain including Ala^{457}, but not intrinsic TG catalytic activity were necessary for FXIIIA to induce chondrocyte hypertrophy. Both transfection of and the addition of exogenous FXIIIA stimulated a rapid movement of TG2 to the cell membrane, a critical feature of TG2-mediated hypertrophy. Here, we demonstrated rapid binding of exogenous FXIIIA to the plasma membrane and physical association with the $\alpha 1$ integrin subunit critical for both TG2 mobilization and phosphorylation within

minutes of the chondrocyte differentiation mediator p38 MAP kinase. Our results identify a unique functional network between two cartilage TG isoenzymes that accelerates chondrocyte maturation without requirement for TG-catalyzed transamidation by either TG.

INTRODUCTION

Transglutaminases (TGs) catalyze a calcium-dependent transamidation reaction that generates covalent crosslinks of available substrate glutamine residues with primary amino groups (EC 2.3.2.13) that modify proteins and protein-protein interactions (95). Expression and cellular release of the most widely expressed TG isoenzyme (TG2), and of the TG isoenzyme FXIIIA, the homodimeric tissue form of the heterotetrameric plasma coagulation protein Factor XIII have been identified in bone and cartilage (27, 41, 95). Moreover, FXIIIA and TG2 have both been implicated in extracellular matrix modification that modulates the capacity of osteoblasts to mature and form bone mineral (96). Changes in FXIIIA and TG2 expression and release also have been identified in the physiologic maturation of growth plate chondrocytes, a process that occurs in a temporal and spatially organized manner that progresses through resting, proliferative and pre-hypertrophic differentiation, to terminal hypertrophic differentiation and cell death (42, 97).

The growth plate chondrocyte hypertrophy gene expression program promotes remodeling of the extracellular matrix, partly through a shift in cartilage-specific collagen expression from type II to type X collagen and by enhanced MMP-13 expression (38). Functional consequences of chondrocyte hypertrophy in the growth plate include calcification mediated partly by matrix vesicle shedding and angiogenesis mediated in part by VEGF expression (56). Though articular cartilage chondrocytes are in a physiologic, maturationally arrested state, pathologic hypertrophic differentiation is observed to develop among osteoarthritic (OA) chondrocytes in situ and has the potential to promote OA progression through dysregulated matrix repair (98) and pathologic calcification (99). Significantly, both FXIIIA and TG2 are molecular markers of chondrocyte hypertrophic differentiation in the growth plate (27, 42). Furthermore, TG2 and FXIIIA expression, as well as total TG catalytic activity, are up-regulated in human knee OA cartilage chondrocytes (40).

Despite lacking signal peptide, TG2 and FXIIIA both are released by chondrocytes and osteoblasts (96). Transamidation of proteins in the extracellular matrix by secreted TGs has the potential to alter cell differentiation and function, specifically exemplified by TG2-catalyzed transamidation of extracellular matrix collagen to promote calcification (100). TG2 is essential to accelerate chondrocyte maturation to hypertrophy in response to signals provided by retinoic acid and CXCL1 (45, 46). Moreover, exogenous nanomolar TG2 is sufficient to directly induce hypertrophic differentiation in chondrocytes in articular cartilage organ

culture (101). In addition, paracrine and juxtacrine effects of TG2 released from chondrocytes modulate osteoblast differentiation through extracellular TG2-induced PKA signaling (76, 96).

Cardinal aspects of the multifunctionality of TG2 include the potential for TG2 to function as an unconventional GTPase and as a cell adhesion protein. TG2 interconverts in a reciprocally regulated manner between a TG catalytically latent guanine nucleotide-bound state and TG catalytically active calcium-bound state (68). Cell surface TG2 serves as an integrin co-receptor for fibronectin (102) and extracellular TG2 promotes integrin clustering that induces RhoA activation (103). In this context, exogenous TG2 must be in a guanine nucleotide-bound state and employs β1 integrin-mediated signaling and rapid activation of p38 MAPK pathway signaling to induce chondrocyte hypertrophic differentiation in vitro (101). TG2 does not require transamidation activity, GTPase activity, or fibronectin binding to promote chondrocyte maturation to hypertrophy (101).

FXIIIA, like TG2, exerts transamidation-dependent and transamidation-independent activities, binds integrins, and functions to modulate both cell adhesion and differentiation. For example, FXIIIA binds αvβ1 and αvβ3 integrin molecules that also are potential substrates for transamidation (104). Moreover, FXIIIA, coordinately with vascular endothelial growth factor receptor 2 (VEGFR-2), modulates cell signaling that drives angiogenesis (28). Significantly, increases in FXIIIA expression and FXIIIA extrusion coincide with and directly stimulate matrix calcification in chondrocytes in situ and in vitro (40, 41, 106). Therefore,

this study examined molecular structure-function of FXIIIA in chondrocyte differentiation. We demonstrate that extracellular FXIIIA promotes chondrocyte hypertrophic differentiation, an activity independent of FXIIIA TG catalytic activity, but dependent on rapid mobilization of TG2 and mediated by FXIIIA-α1 integrin interaction. Our results identify a unique functional network between two TGs that accelerates chondrocyte maturation without the requirement for either TG isoenzyme to catalyze transamidation.

RESULTS

Molecular FXIIIA structure-function in the induction of type X collagen

collagen –To elucidate FXIIIA structure-function in chondrocyte hypertrophy induction, we designed a site mutagenesis strategy that factored in the constitutive latency of FXIIIA as a TG. Specifically, binding of Ca^{2+} to FXIIIA is required for TG catalytic activity, triggered by endoproteolysis of FXIIIA at Arg^{37} by thrombin or alternatively by excess Ca^{2+} alone (95)**.** This process removes a 4 kDa peptide to expose the conserved TG family catalytic triad that includes Cys^{314}. FXIIIa TG activity is subsequently inactivated by thrombin-catalyzed proteolysis at Met^{513}, or alternatively by a decrease in ambient Ca^{2+} (25). We observed catalytic activity of recombinant FXIIIA proteins, most likely due to the presence of Ca^{2+} during amplification[2]. In cartilage, FXIIIA is expressed intracellularly and then released into the pericellular matrix. We first transfected the FXIIIA site mutants previously

characterized to functionally compromise the "activation site" (R37A), the catalytic site (C314A), and the "inactivation site" (M513G) (25) as schematized in Fig. 1. Initial comparison of the human FXIIIA mutants for TG catalytic activity, in the presence and absence of thrombin, verified that individual mutants possessed the predicted, relative changes in TG activity.

Examination of extracellular TG release following transfection of the WT and mutant FXIIIA cDNAs in human chondrocytes revealed an increase in the TG2 release except by the FXIIIA inactivation mutant (Fig. 2A). Extracellular TG2 is sufficient to promote chondrocyte hypertrophy (101). Here, we observed that transfection of FXIIIA WT promoted the expression of type X collagen, but the FXIIIA inactivation mutant did not share this function (Fig. 2B).

Next, we treated cultured human articular chondrocytes with soluble recombinant FXIIIA (sFXIIIA) and TG2 (sTG2). Treatment with either of the TG isoenzymes was sufficient to increase expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-13 (MMP-13), to markedly augment the ratio of type X collagen (the stereotypic marker of chondrocyte hypertrophy) to type II collagen mRNA within 8 hours (Fig. 3A), and to stimulate expression of syndecan-3 within 72 hours (Fig. 3B). In bovine knee articular cartilage in organ culture, sTG2 and sFXIIIA both induced type X collagen (Fig. 3C). Under these conditions, sTG2 but not sFXIIIA stimulated enlargement of the chondrocyte-containing lacunae in articular cartilage explants (Fig. 3C), which suggested differential effects on chondrocyte hypertrophy.

Incubation of chondrocytes with recombinant forms of FXIIIA mutants revealed dependence on the "activation site" R37 residue for extracellular sFXIIIA to induce both TG2 release and type X collagen expression (Fig. 4A, B). Next, we assessed if sFXIIIA must bind Ca^{2+} in order to modulate chondrocyte differentiation, as opposed to alternative effects of the endoproteolytically released N-terminal 4 kDa peptide fragment of FXIII or effects of the activated form of FXIIIA (FXIIIa). To do so, we generated and characterized a FXIIIA 502 truncation mutant that retains the entire Ca^{2+} binding domain and a FXIIIA 481 truncation mutant designed to functionally interrupt the Ca^{2+} binding domain. Additionally, we generated mutants of Ala^{457} and Glu^{490}, both which are essential residues for FXIIIA to bind Ca^{2+} (25). We ascertained that the amino acid 481 truncation and the Ca^{2+} binding domain point mutation at Ala^{457} significantly suppressed the catalytic activity of $FXIIIA^{2}$. Treating chondrocytes with sFXIIIA, we observed that the amino acid 481 truncation mutant and the Ala^{457} mutant failed to induce type X collagen (Fig. 4A) and also failed to stimulate TG2 release from chondrocytes (Fig. 4B). By contrast, the capacity of FXIIIA to induce type X collagen was preserved after mutation of Asp^{270} involved in the binding of cations that inhibit the catalytic activity of FXIIIA (26), or mutation of Arg^{310} involved in the formation of FXIIIA isomers that appear to stabilize FXIIIA (Data not shown, 108).

FXIIIA induction of chondrocyte hypertrophic differentiation requires TG2 - To test if TG2 stimulated chondrocyte hypertrophy in a manner mediated by FXIIIA or vice versa, we studied cultured knee articular chondrocytes from TG2 and FXIIIA knockout mice and congenic controls. We observed that sFXIIIA failed to induce expression of type X collagen in $TG2^{-/-}$ chondrocytes, whereas sTG2 did not require FXIIIA to induce type X collagen (Fig. 5A). In parallel studies, al-trans retinoic acid (ATRA) required both TG2 and FXIIIA to induce type X collagen (Fig. 5A). In contrast, chondrocytes did not require FXIIIA expression to develop type X collagen expression in response to CXCL8, under conditions where TG2 was necessary (Fig. 5A).

In view of the potential for germline *TG2* and *FXIIIA* deletions to result in compensatory mechanisms, parallel studies employed human articular chondrocytes transfected with shRNA cDNAs designed and optimized to specifically decrease expression of TG2 or FXIIIA (Fig. 5B). The TG2 and FXIIIA knockdown experiments confirmed the aforementioned results in mouse TG2 and FXIIIA knockout articular chondrocytes (Fig. 5C, and data not shown). Under these conditions of TG2 and FXIIIA knockdown, each of these TGs accounted for ~50% of total TG activity found in articular chondrocytes (Fig. 5D), consistent with prior results in cultured WT (TG2) and $TG2^{-/-}$ mouse knee articular chondrocytes (45).

FXIIIA induces p38 MAPK pathway signaling dependent on rapid externalization of TG2 -Signaling through the p38 MAP kinase pathway plays a central role in transducing maturation to hypertrophy under a variety of conditions in cultured chondrocytes (46, 109, 110). Moreover, p38 MAP kinase phosphorylation is observed within minutes of the addition to chondrocytes of nanomolar TG2 (101). Hence, we tested if FXIIIA stimulates p38 MAP kinase pathway signaling through rapid externalization of TG2. First, nearly a 100-fold change in the amount of TG2 localized on the cell surface (111) was detected within 1 minute of treatment of chondrocytes with wild type sFXIIIA (Fig. 6A). Under these conditions, sFXIIIA stimulated phosphorylation of p38 MAP kinase within minutes, an activity not shared by the activation site mutant or the sFXIIIA Ala^{457} Ca^{2+} binding mutant of FXIIIA (Fig. 6B). Furthermore, neither of these two FXIIIA site mutants increased TG2 localization at the cell surface (data not shown).

Transfection of shRNA specific for TG2 in chondrocytes, optimized as in Figure 5B-C above, blunted the capacity of FXIIIA to induce phosphorylation of p38 (Fig. 6C). Because TG2 must be in a guanine nucleotide bound state to stimulate chondrocyte hypertrophy (101), we next treated cells with sTG2 in which the GTP binding capacity was abrogated by K173L mutation. Combined treatment with the sTG2 K173L GTP binding mutant and with sFXIIIA failed to induce type X collagen in chondrocytes in which TG2 was knocked down by shRNA transfection (Fig. 6C).

FXIIIA engagement of the α1 integrin subunit is critical for induction of p38 MAPK pathway signaling in chondrocytes –After application of both recombinant TGs to the chondrocytes, we observed binding (within minutes) of the TGs to the chondrocytes, where they remained cell-associated (Fig 7A). In vitro binding assays did not reveal a direct association between TG2 and FXIIIA. Moreover, in the transfection experiments, there was no clear correlation between the extracellular levels of the two TG isoenzymes (Fig. 2A). As such, there was no evidence that TG2 mobilization by FXIIIA was caused by direct interaction with each other.

In fibroblasts, TG2 can bind to the extracellular domain of β1 and β3 integrins (77). Activated FXIIIA can bind to αvβ3 in HUVECs (104). Chondrocytes themselves express multiple integrins. These include α5β1, α1β1, α2β1, α10β1, α6β1, αVβ3 (107). Previously β1 integrin appeared critical for TG2-induced hypertrophy while inhibition of β3 integrin had no effect (101). Immunoprecipitation of chondrocyte cell lysates with an antibody to β1 demonstrated constitutive binding to TG2 but not to FXIIIA. Therefore, we treated chondrocytes with recombinant TG2 and FXIIIA and assessed for interaction between TGs and four α integrins. sTG2 did not detectably bind to α1, α2, α5 or α6 integrin, but sFXIIIA interacted robustly with α1 integrin (Fig 7B).

To assess if the binding of α1 integrin has a critical role in FXIIIA-induced hypertrophy, chondrocytes were pretreated with the α1 integrin blocking antibody FB12, which inhibited the capacity of sFXIIIA but not sTG2 to induce type X

collagen (Fig. 8A). In contrast, the blocking antibody to the promiscuous β1 integrin subunit, which is the only β integrin subunit that heterodimerizes with αl, suppressed type X collagen induction in response to both TG isoenzymes under these conditions (Fig. 8A). Additionally, sFXIIIA-induced p38 MAP kinase activation, along with FAK phosphorylation assessed as a readout for integrin signaling, were inhibited by pretreatment of chondrocytes with α1 integrin blocking antibody FB12 (Fig. 8B).

In OA, α1 integrin expression is augmented, particularly in the hypertrophic chondrocytes (112). Within 5 minutes of treatment of sFXIIIA, we demonstrated an increase in cell surface α1 integrin staining via immunofluorescence (Fig. 9A). Finally, crosslinking of the α1 integrin antibody (TS2/7) with a mouse IgG mimicked the enhanced movement of TG2 to the plasma membrane (Fig. 9B). Furthermore, pretreating the chondrocytes with the α1 integrin blocking antibody FB12 inhibited the ability of FXIIIA to increase TG2 membrane expression (Fig 9B). These data suggested the interaction of FXIIIA and α1 integrin to be critical to FXIIIA-induced hypertrophy.

DISCUSSION

In this study, we identified novel functional implications of up-regulation of FXIIIA expression in the growth plate chondrocyte hypertrophic differentiation program and in osteoarthritic cartilage chondrocytes in situ (27, 113).

Chondrocytes are recognized to have the capacity to release FXIIIA (41, 114, 115). Our data reveal an interaction between FXIIIA and the α1 subunit of α1β1 integrin critical for chondrocyte hypertrophy via TG2 mobilization. Moreover, FXIIIA induced an increase in chondrocyte cell surface α1 integrin. These findings are pertinent in part because up-regulation of α1 integrin develops in the superficial and upper mid-zone in murine osteoarthritic cartilage, a condition in which α1 integrin appears to mediate cartilage matrix remodeling (112).

During examination of the chondrocyte differentiation process, we found that transfection and stimulation of chondrocytes with recombinant FXIIIA increased the movement and release of TG2. We previously demonstrated TG2 effects on chondrocyte maturation to be dependent on TG2 externalization (101). In the absence of TG2 or lack of TG2 externalization, FXIIIA was unable to induce hypertrophy. Both mineralizing hypertrophic growth plate chondrocytes and mineralizing osteoblasts robustly release FXIIIA (41, 96). Our results suggest that one of the functions of FXIIIA release by bone-forming cells is to fine tune TG2 release and thereby regulate TG2-dependent effects on chondrocyte and osteoblast differentiation (95, 115, 117) and transamidation-catalyzed extracellular matrix remodeling in the skeleton (42, 96).

To induce chondrocyte hypertrophy, FXIIIA required intact Ala^{457} for effective binding of Ca^{2+} (25). Transfection studies revealed the "inactivation site" residue M513 to be critical for induction of chondrocyte hypertrophy by FXIIIA. However, we observed that recombinant FXIIIA protein bearing the inactivation

site mutation M513G retained the capacity to induce chondrocyte hypertrophy. The basis for these seemingly paradoxical observations may be that the site mutations of FXIIIA studied alter the conformation of the protein, thereby in all likelihood causing differential modulation of FXIIIA activity inside versus outside of the cell. Alternatively, this finding suggests substantial regulation of FXIIIA function based upon the availability of free Ca^{2+} and effects on FXIIIA conformation, given that FXIIIA catalytic activity was itself not vital to induce chondrocyte hypertrophy.

Chondrocyte maturation to hypertrophy is associated with development of the capacity to endoproteolytically degrade and activate FXIIIA, and to up-regulate release of FXIIIA (76). The externalization of FXIIIA by hypertrophic chondrocytes is augmented by lytic cell death mediated by endoproteolytic activation of FXIIIA (41). Endoproteolysis of FXIIIa at Met^{513} inactivates TG activity through the promotion of Ca^{2+} release from the protein (25, 118). Hence, microenvironmental free Ca^{2+}, and changes in the balance between endoproteolytic activation and inactivation of FXIIIA inside and outside chondrocytes, regulated by thrombin, certain other proteases, could modulate the temporal and spatial organization of chondrocyte maturation to hypertrophy.

In this study, recombinant sTG2 was able to induce chondrocyte hypertrophy in the absence of FXIIIA expression, but not vice versa. These findings indicated TG2 to be the driving force of the functional network of two TG isoenzymes that stimulated chondrocyte maturation. Once outside the cell, FXIIIA rapidly mobilized TG2, evidenced by ~100-fold enrichment of TG2 on the surface

of chondrocytes within minutes of sFXIIIA addition. Changes in subcellular localization of TG2 modulate wound healing, partly through interactions of plasma membrane-bound TG2 with fibronectin and certain integrins that regulate cell adhesion and migration (119). Additionally the plasma membrane-associated TG2 and FXIIIA, in conjunction with increased FXIIIA expression (40), may contribute to the up-regulated activity of the p38 MAP kinase signaling pathway in osteoarthritic cartilage chondrocytes in situ (119).

In cultured chondrocytes, activation of the p38 signaling pathway in response to signals including sTG2, and certain calgranulins and chemokines, plays a central role in promoting maturation to hypertrophy (46, 101, 110, 130). However, it must be noted that p38 signaling does increase the transcriptional activity of the chondrogenic master transcription factor Sox9, an inhibitor of chondrocyte maturation to hypertrophy in vivo and in vitro. Moreover, constitutively activated p38 signaling in MKK6 transgenic mice is associated with reduced chondrocyte proliferation and inhibition of hypertrophic chondrocyte differentiation in growth plates in situ (109).

FXIIIA induced TG2-dependent chondrocyte hypertrophy through its interaction with $\alpha 1\beta 1$ integrin. TG2 has been shown to cluster cell surface integrins and lead to integrin-dependent signaling and activation (103). Thus, it is conceivable that FXIIIA may act to cluster $\alpha 1\beta 1$ integrin and thereby prime chondrocytes for TG2-dependent induction of hypertrophy. Our observation that crosslinking $\alpha 1\beta 1$ integrin through an antibody complex induced TG2 mobilization

supports this notion. Alternatively, it remains possible that FXIIIA might function indirectly by binding to an integrin-associated protein that binds or clusters α1β1 integrin. The latter scenario merits further study in chondrocytes, as ternary complex formation of FXIIIA with αvβ3 integrin and VEGFR-2 on the endothelial cell surface mediates angiogenesis through VEGF-independent VEGFR-2 activation (106).

We observed that exogenous FXIIIA rapidly induced cell surface α1 integrin expression concurrent with TG2 mobilization. TG2 does not possess a signal sequence and it is not known how TG2 moves from the cytoplasm to the plasma membrane. However, TG2 has been shown to associate with β1 integrins (5). Further work will be needed to assess if the translocation of α1β1 integrin to the cell surface may "chaperone" TG2 to the surface of chondrocytes. Significantly, complex formation of TG2 with β1 integrins during biosynthesis is followed by TG2 accumulation on the cell surface in fibroblasts (5).

Further studies will be needed to elucidate which downstream signaling pathways, beyond FAK and p38, transduce chondrocyte maturation in response to FXIIIA and TG2. The functional role of FXIIIA-induced chondrocyte p38 pathway activation within growth plate and articular cartilages may critically depend on the timing and spatial organization of up-regulated FXIIIA release by chondrocytes, as well as the concurrent expression of TG2. In addition, internalization of secreted TG2 followed by nuclear localization of TG2 and its receptor could mediate signaling by TG2 in chondrocytes, as demonstrated with VEGFR-2 in endothelial

cells (121).

Significantly, both sFXIIIA and sTG2 were observed to directly induce type X collagen in non-proliferating single chondrocytes within lacunae in articular explants in this study. Thus, robust release of TG2 and FXIIIA in osteoarthritic cartilage potentially bypasses the conventional growth plate chondrocyte maturation sequence. We speculate that the capacity of TG2 but not FXIIIA to enhance enlargement of the chondrocytes in the lacunae could be attributed to the fact that TG2 and FXIIIA each preferentially mediate the transamidation of unique protein sequences (122).

Limitations in this study included use of monolayer culture conditions for these experiments, necessary not only because of the low yields of primary mouse articular chondrocytes but also to allow subsequent comparative studies with human chondrocytes. Monolayer culture conditions may have imposed significant cell-cell and cell-matrix interactions that would not take place in growth plate and articular cartilages in vivo. Another limitation within much of this study was treatment of cultured chondrocytes with nanomolar amounts of exogenous recombinant FXIIIA and TG2 to assess mechanisms for TG effects on differentiation. Endogenous chondrocyte TG2 and FXIIIA typically reach only high picomolar extracellular concentrations in chondrocytes. However, the movement of secreted endogenous TG isoenzymes to the cell surface is likely to be more efficient than for exogenous TGs. This study did not attempt to define specific effects on chondrocyte differentiation of endogenous intracellular TG2 or

FXIIIA. In this light, guanine-nucleotide bound TG2 does functionally engage several α integrin cytosolic tails (123). We also did not specifically test potential roles, in mediating FXIIIA and TG2 effects on chondrocyte differentiation, of binding or transamidation of TGFβ (124), soluble integrin ligands, or other extracellular matrix proteins. Last, we did not ascertain why recombinant FXIIIA failed to induce type X collagen expression $FXIIIA^{-/-}$ chondrocytes. This finding suggests that FXIIIA exerts significant signaling effects intracellularly in chondrocytes. FXIIIA dimerization of the type 1 angiotensin II receptor via crosslinking of receptor cytosolic tails is a notable example of such FXIIIA intracellular activity (125). Last, we did not assess for potential internalization of exogenous TG isoenzymes by chondrocytes, or signaling effects by intracellular TG2 (121).

In summary, we have established that two TG isoenzymes network to promote and accelerate chondrocyte maturation, but do so in a transamidation-independent manner. Our results add to growing evidence that direct interactions between certain TG isoenzymes and integrins alter cell signaling and differentiation. Our results point to the biologic significance of the robust expression of two TG isoenzymes in association with chondrocyte hypertrophy in vivo. Our results identify relative timing of FXIIIA and TG2 up-regulation in cartilages as a novel mechanism for modulation of chondrocyte differentiation under both physiologic and pathologic conditions.

The text of chapter 3 is a reprint of the material has been submitted to the Journal of Biological Chemistry in October 2006 (revised manuscript). I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis of this chapter.

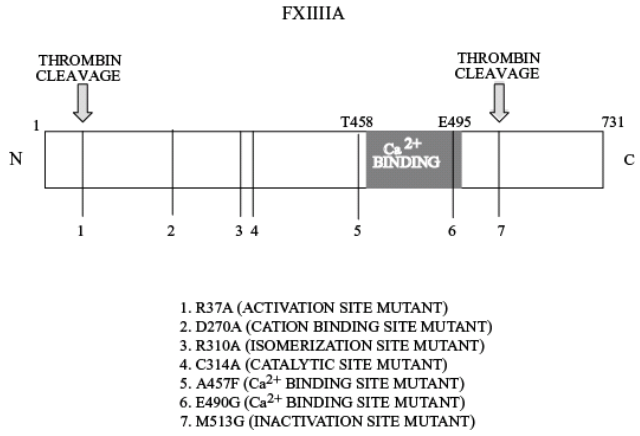


Figure 3-1. FXIIIA site directed mutants generated for structural analysis examination. The schematic depicts features of the primary structure of wild type (WT) FXIIIA and the panel of FXIIIA site directed mutants generated and studied.

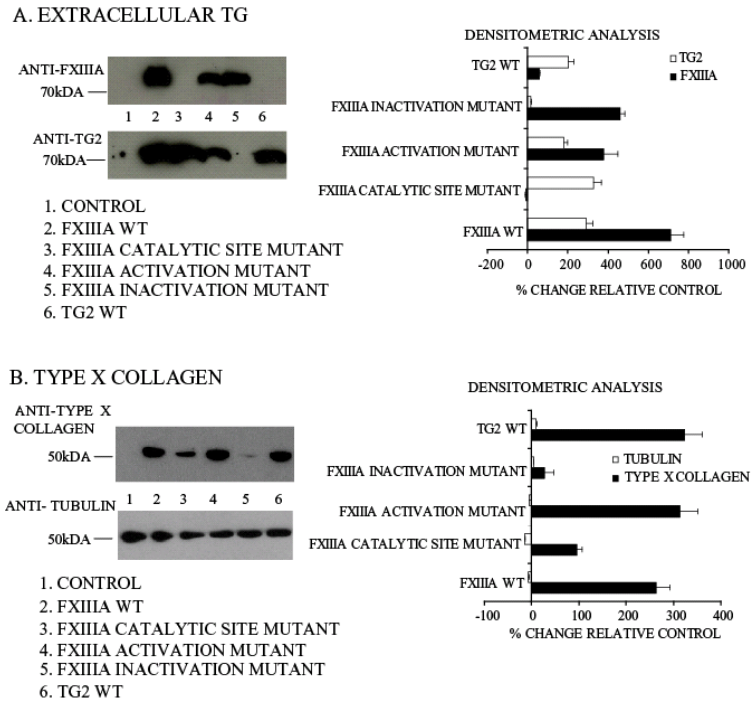
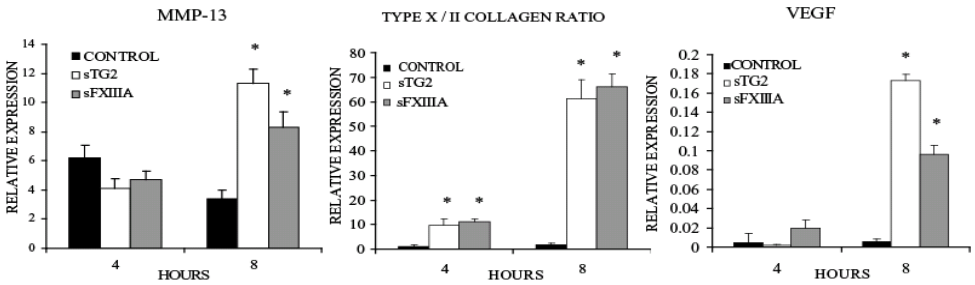


Figure 3-2. Transfection of FXIIIA increases both extracellular TG2 and type X collagen protein expression. To examine the release of FXIIIA and TG2 following overexpression of WT and each mutant FXIIIA, aliquots of human articular chondrocytes (4 x 10^5 cells) were transfected using the AMAXA Nucleofector, as described in the Methods. Following a 72 hour incubation in medium A, conditioned media were collected and separated by SDS-PAGE and analyzed for TG2 and FXIIIA release (A). B, The cell lysates were harvested and separated by SDS-PAGE for comparison of type X collagen and tubulin protein expression. Representative of three donors in three separate experiments (n=9). Densitometric analysis results depict the percent change of expression relative to control of each protein examined by Western Blotting.

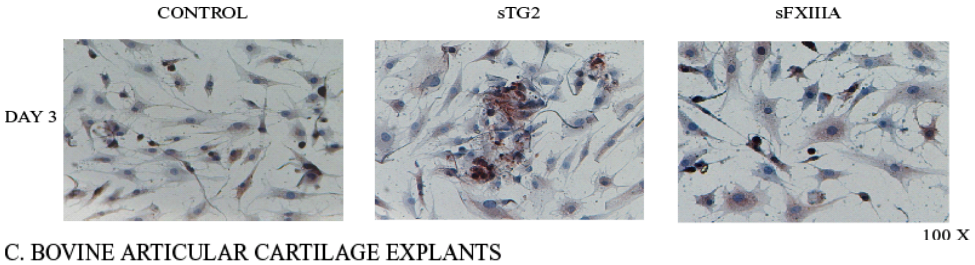
Figure 3-3. Both Exogenous FXIIIA and TG2 induce chondrocyte hypertrophic differentiation. We studied induction by TG2 and FXIIIA of MMP-13, VEGF, type X collagen and Syndecan-3 in cultured chondrocytes. Normal human knee articular chondrocytes plated in 12 well dishes (at 1 x 10^5 cells/well) were incubated 4 or 8 hours with 100 ng/ml of recombinant wild type TG2 or FXIIIA in the ascorbate-containing medium A described in the Methods. A, Using quantitative RT-PCR, we determined chondrocyte mRNA expression levels relative to GAPDH for MMP-13 and VEGF, and the mRNA expression ratio of type X : type II collagen, studying data collected from three separate human donors (n=9, *$p<0.05$). B, Aliquots of 1 x 10^5 human articular chondrocytes plated on glass coverslips were incubated for 24 hours with 100 ng/ml of recombinant wild type TG2 or FXIIIA. The cells were fixed with 4% paraformaldehyde and stained for Syndecan-3 expression). C, Assessment of the induction by TG2 and FXIIIA of Type X collagen in cartilage organ culture. Normal bovine articular cartilage explants in organ culture were incubated with 100 ng/ml recombinant wild type TG2 or FXIIIA for 5 days in medium A, and 10 µm frozen sections were stained for type X collagen expression (representative of 5 donors).

A. QUANTITATIVE PCR



B. HUMAN ARTICULAR CHONDROCYTES

ANTI-SYNDECAN-3



C. BOVINE ARTICULAR CARTILAGE EXPLANTS

ANTI-TYPE X COLLAGEN

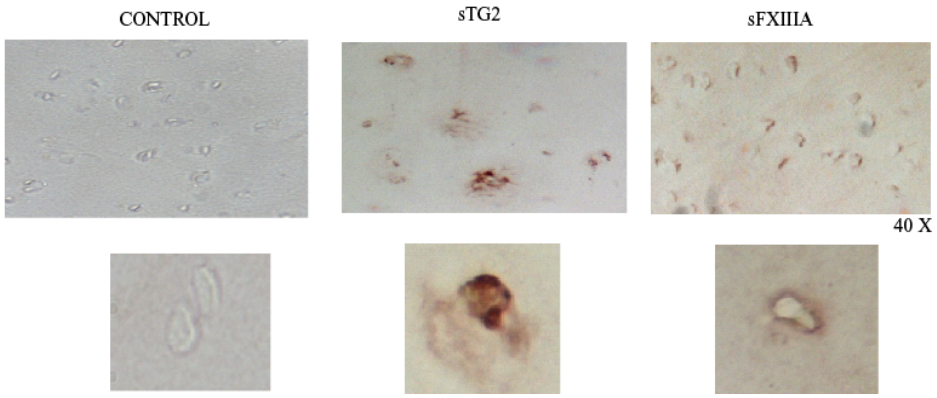
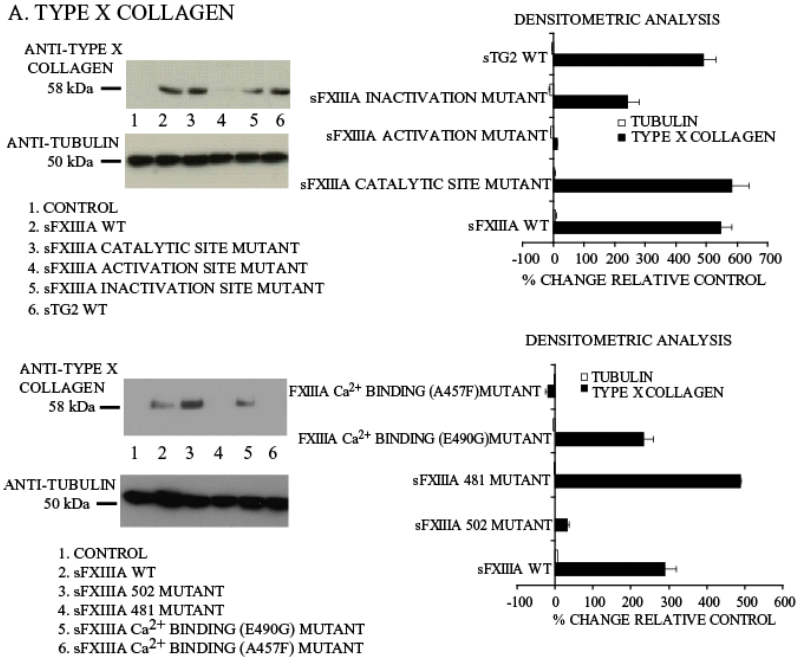


Figure. 3-4. FXIIIA must bind Ca^{2+} to stimulate chondrocyte TG2 release required to induce type X collagen. Aliquots of human articular chondrocytes (1 x 10^5 cells) were incubated with 100 ng/ml of each recombinant protein in medium A for 72 hours, and type X collagen assessed in cell lysates by SDS-PAGE Western blotting (A), and TG2 release into conditioned media quantified by ELISA (B). Representative of three donors in three separate experiments (n=9), with *$p<0.05$ for decrease in TG2 release by sFXIIIA mutant compared to WT sFXIIIA. Densitometric analysis results depict the percent change of expression relative to control of each protein examined by Western Blotting.

A. TYPE X COLLAGEN



B. EXTRACELLULAR TG2

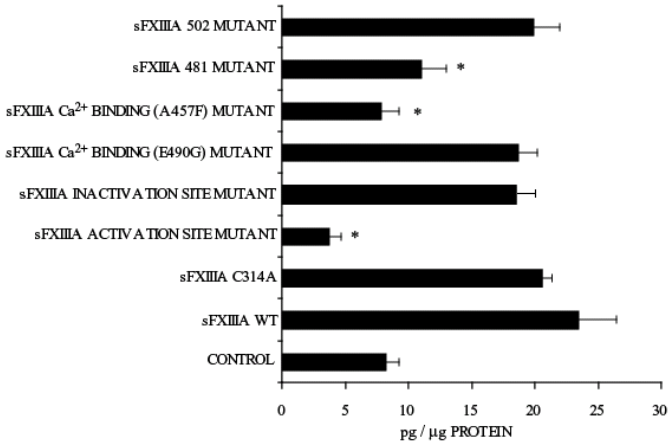
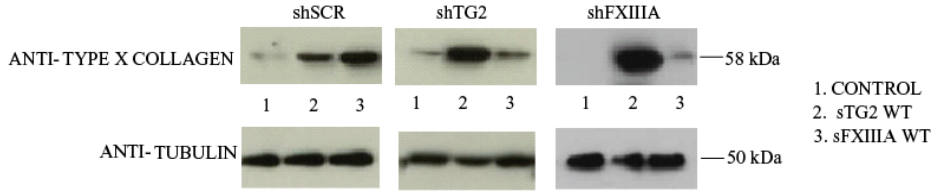
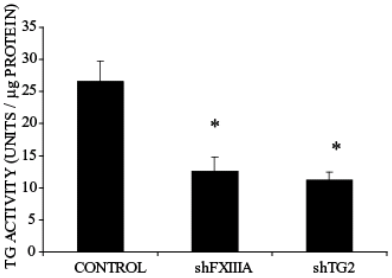


Figure 3-5. FXIIIA-stimulated type X collagen expression is dependent upon TG2. A, Assessment of TG2 and FXIIIA knockout mouse cells. Primary knee chondrocytes were removed from $FXIIIA^{+/+}$, $FXIIIA^{-/-}$, $TG2^{+/+}$, and $TG2^{-/-}$mice. After two weeks in culture, aliquots of $5x10^3$ cells in Medium A were stimulated for 5 days with 10 nM ATRA, 10 ng/ml CXCL8, or 100 ng/ml of sTG2 or sFXIIIA, and then type X collagen was examined by SDS-PAGE/Western blotting, as described in the Methods Representative of three experiments. B-D, Assessment of the effects of TG2 and FXIIIA knockdown in human chondrocytes. Plasmids containing specific shRNA sequences for TG2 and FXIIIA (shTG2 and shFXIIIA) or scrambled sequences (shSCRT, shSCRF or shSCR) were transfected into human articular chondrocytes using the AMAXA Nucleofector, as described in the Methods. Following transfection, aliquots of 2×10^5 cells were transferred to 12 well plates in medium A, followed 24 hours later by addition of 100 ng/ml sTG2 or sFXIIIA where indicated. B, Western blot analyses for TG2 and FXIIIA verified selective TG isoenzyme knockdown by specific shRNAs at 72 hours. C, Type X collagen and tubulin expression after 72 hours of treatment with sTG2 or sFXIIIA. D, Total cell lysates were collected 72 hours after transfection for determination of TG catalytic specific activity, representative of results from 4 human articular chondrocyte donors.
donors.

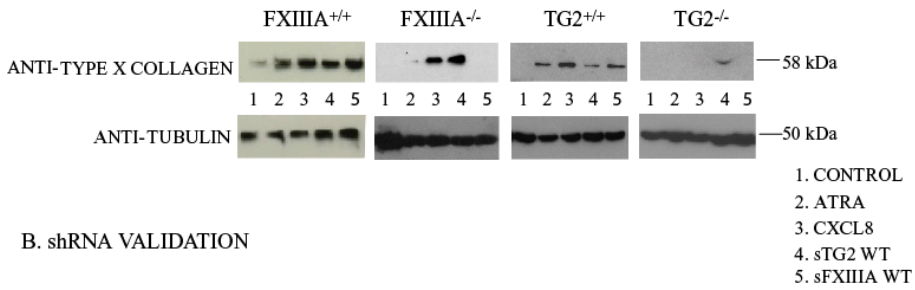
C. TYPE X COLLAGEN



D. TG CATALYTIC ACTIVITY



A. TYPE X COLLAGEN IN MOUSE CHONDROCYTES



B. shRNA VALIDATION

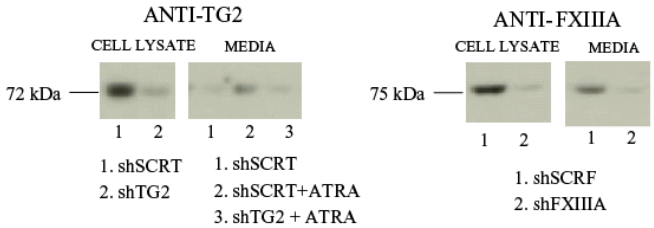
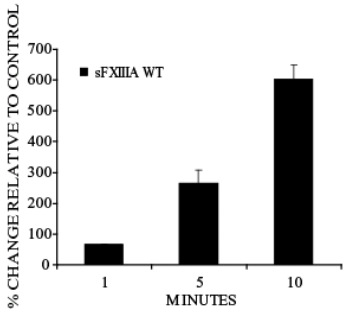
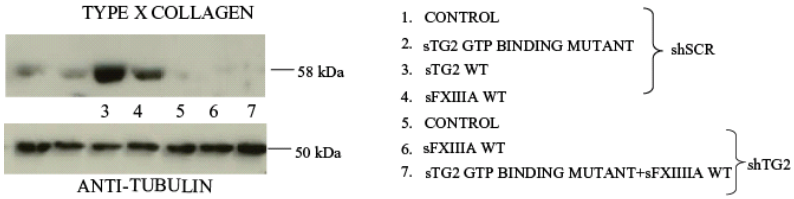


Figure 3-6. Rapid mobilization of TG2 by Ca^{2+}- bound FXIIIA is essential for stimulation of p38 phosphorylation. A, Aliquots of 5 x 10^3 human articular chondrocytes were starved in serum-free DMEM high glucose medium for 2 hours and then stimulated with sTG2 or sFXIIIA. TG2-specific antibodies were used to detect membrane bound TG2, quantified through successive incubations with biotin anti-rabbit and strepavidin-AP antibodies as described in the Methods. B, C, Aliquots of 3 x 10^5 human articular chondrocytes were transfected with scrTG2 or shTG2, as described above. After 24 hours, the cells were starved in serum free DMEM high glucose medium for 2 hours and then stimulated with WT sFXIIIA (or WT sFXIIIA in combination with the sTG2 K173L GTP binding site mutant) for the time indicated. Cell lysates were analyzed by Western blotting for *p*-p38 and total p38 (B) or type X collagen and tubulin (C). Representative of 3 separate experiments using different donors.

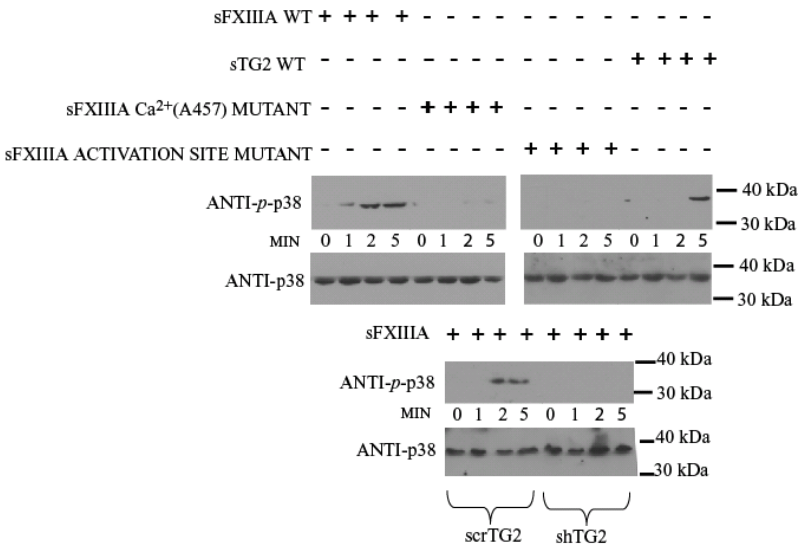
A. PLASMA MEMBRANE BOUND TG2



C. TYPE X COLLAGEN



B. p38 MAP KINASE PHOSPHORYLATION



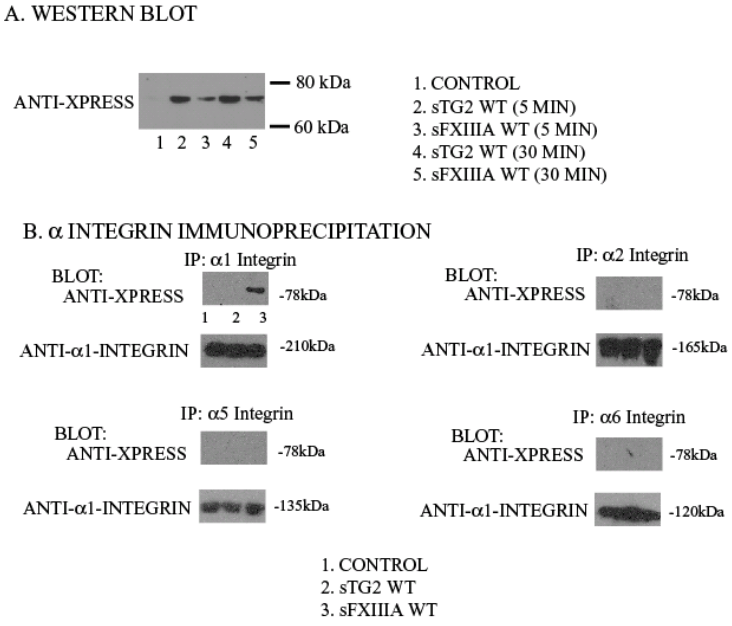


Figure 3-7. Interaction of recombinant FXIIIA with human chondrocytes through α1 integrin. A, Aliquots of 1 x 10^5 human articular chondrocytes were starved for 2 hours in serum free DMEM and then incubated for the indicated times with 100 ng/ml of sTG2 or sFXIIIA. Washed cell lysates were examined for the presence of the Xpress epitope on the recombinant proteins by SDS-PAGE/Western blotting. B, Aliquots of 1 x 10^6 human articular chondrocytes were incubated for three days in medium A containing 100 ng/ml of sTG2 or sFXIIIA where indicated. Cell lysates (200 µg protein) were immunoprecipitated using 1 µg/ml of α1 (clone TS2/7), α2, α5, or α6 integrin-specific antibody, and precipitated proteins were analyzed for the Xpress tag or each respective α integrin by Western blotting. Representative of 3 separate experiments using 3 separate donors.

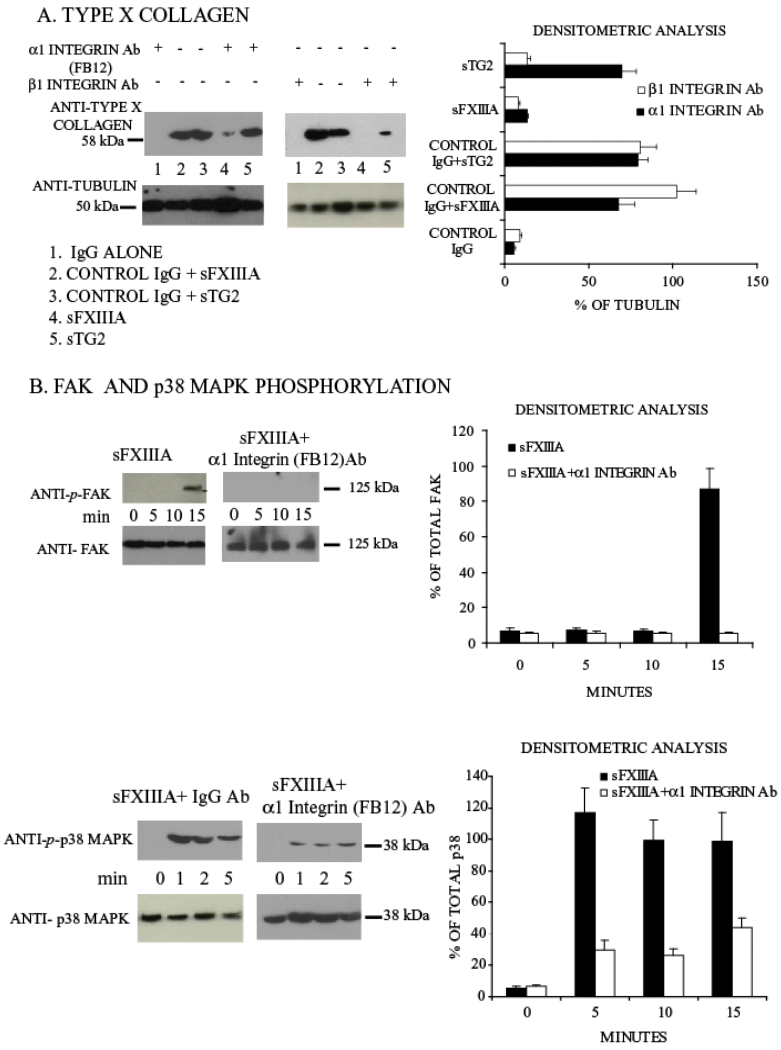


Figure 3-8. FXIIIA induction of type X collagen is mediated through FAK and p38 MAP kinase phosphorylation and dependent upon α1 integrin interactions. A, Aliquots of 1 x 10^5 human articular chondrocytes were starved and pretreated for 2 hours with 1 μg/ml of IgG control, β1 integrin or α1 integrin blocking (FB12) antibodies in serum free DMEM and then incubated for the indicated times with 100 ng/ml of sTG2 or sFXIIIA, with type X collagen assessed by Western blotting of cell lysates as above. B, Aliquots of 1 x 10^5 cells were starved for 2 hours in serum-free DMEM and then incubated for the indicated times with 100 ng/ml of sTG2 or sFXIIIA and 1 μg/ml of αl integrin-blocking antibody (FB12) where indicated, with cell lysates examined for FAK and p38 phosphorylation by Western blotting. Representative of results from 3 experiments employing 3 separate donors. *$p<0.05$. Densitometric analysis results depict the percent change of phosphorylated protein expression relative to total protein of both p38 and FAK as assessed by Western

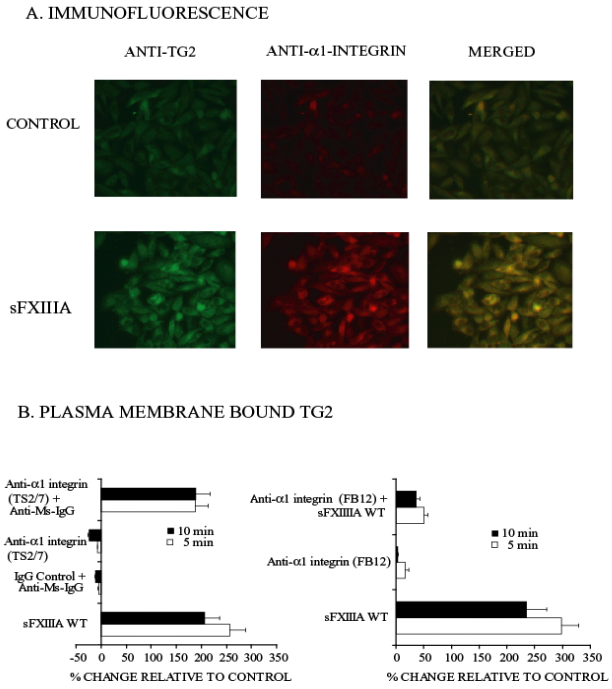


Figure 3-9. Rapid mobilization of TG2 by sFXIIIA and antibody crosslinking of α1 integrin. A, Aliquots of 1 x 10^5 human articular chondrocytes (plated on poly-lysine coated coverslips) were starved in serum-free DMEM high glucose medium for 2 hours and then stimulated with sFXIIIA for 5 minutes. To detect cell surface protein expression, the cells were fixed in 4% PFA prior to staining for TG2 (FITC) and α1 integrin (rhodamine). Immunofluorescent images were visualized as described in the methods. B, To determine if FXIIIA movement of TG2 is integrin dependent, aliquots of 5 x 10^3 human articular chondrocytes were starved in serum-free DMEM high glucose medium for 2 hours and then stimulated with sFXIIIA, the α1 integrin antibody, TS2/7 (with and without crosslinking by anti-mouse IgG) versus an IgG control antibody. Additionally after starvation, the chondrocytes were pretreated with the blocking α1 integrin antibody, FB12 and then stimulated for 5 and 10 minutes with sFXIIIA WT. After fixation of the cells, TG2-specific antibodies were used to detect membrane bound TG2, quantified through successive incubations with biotin anti-rabbit and strepavidin-AP antibodies as described in the Methods.

CHAPTER 4:

THE CATALYTIC ACTIVATION OF EXTRACELLULAR TRANSGLUTAMINASE 2 IS CRITICAL FOR THE INDUCTION OF SMOOTH MUSCLE CELL CALCIFICATION

SUMMARY

Vascular calcification has been documented in association with aging, injury, inflammation, diabetes and atherosclerotic plaque formation. The plasticity of smooth muscle cells (SMC), essential for repair of injuries, can become dysregulated and ultimately promote calcification through an altered phenotype. Transglutaminase 2 (TG2) expression is increased in SMC during atherosclerotic lesion progression and its transamidation activity is important for small artery remodeling. Previous studies also suggest circulating TG2 may stabilize the atherosclerotic plaque while limiting the area of inflammation. To test the hypothesis that the catalytic activity of TG2 is critical for regulation of calcification of SMC, we isolated primary SMC and established aortic cultures from $TG2^{+/+}$ and $TG2^{-/-}$ mice. We demonstrated severely diminished calcification in the SMC and aortic cultures from the $TG2^{-/-}$ mice evidenced by von Kossa staining and decreased calcium content of the cells. Phosphate-induction of calcification caused an increase in the both osteoblastic and chondrocytic gene expression in $TG2^{+/+}$ cells, which was lacking in the $TG2^{-/-}$ SMC. The addition of catalytically active recombinant TG2 was able to partially restore the calcium deposition in the $TG2^{-/-}$ SMC. Furthermore, our data revealed that TG2 can mediate the differentiation of the SMC potentially through its regulation of OPN and stabilizing the contractile state of the cells. Taken together, extracellular TG2 can regulate SMC calcification and differentiation dependent upon its catalytic activity.

INTRODUCTION

Vascular calcification has been documented to be an active process that follows a course similar to osteogenesis (48). It frequently occurs in association with atherosclerosis but can occur in other situations such as aging, inflammation, diabetes or injury to the vessel (50). Evidence suggests there are multiple cell types involved which mediate this active process. These include the smooth muscle cells (SMC), pericytes and mesenchymal stem cells. The SMC do not terminally differentiate which allows for phenotypic changes associated with the need for repair. For example, in human arteries chondrogenic, osteoblastic and adipogenic differentiation of SMC have been recognized (48).

Under physiologic conditions, medial SMC maintain the elasticity of the artery and typically remain in the contractile state. Intimal SMC respond to an injury through transition from the contractile to the synthetic differentiation state which promotes the repair (49). Altered signals or prolonged inflammation may lead to vascular calcification through initiation of an osteoblastic de-differentiation pathway or loss of inhibitory factors which normally maintain the artery wall (50).

Two main types of calcification occur, possibly through distinct mechanisms. Each process appears to involve a differentiation along osteogenic and chondrogenic lineages to promote calcification (126). Intimal calcification is found within large atherosclerotic lesions and may help to limit the localized inflammation (49). In these lesions, type II collagen-expressing chondrocyte-like

cells have been found to deposit hydroxyapatite crystals, the major crystal type in arteries (126), through a matrix vesicle mediated event. The process appears to mimic endochondral ossification where cartilage is gradually replaced by bone. Medial calcification along the elastic laminae is known to occur in aging, diabetes and uremia and leads to stiffness of artery walls. This process is more closely related to intramembranous ossification, where no cartilage precursor is deposited (50).

Although the mechanism of vascular calcification has not been entirely defined, various mediators have been elucidated. Abnormal levels of calcium or phosphate can promote widespread soft tissue calcification (127). High levels of phosphate can be taken up through the sodium dependent transporter Pit-1. This promotes increased expression of RUNX2, a transcription factor which enhances osteogenesis and leads to vascular calcification (128). Matrix γ- carboxyglutamic acid protein (MGP), osteopontin (OPN), pyrophosphate (PP_i) and osteoprotegerin (OPG) have all been elucidated as inhibitors of SMC calcification (48).

Transglutaminase 2 (TG2) is ubiquitously expressed enzyme with multiple functions. Transglutaminases (TGs) catalyze a calcium-dependent transamidation reaction that generates covalent crosslinks of available substrate glutamine residues with primary amino groups (EC 2.3.2.13) that modify proteins and protein-protein interactions. In a reciprocally regulated state, TG2 can also bind and hydrolyze GTP. When bound to GDP / GTP, TG2 can regulate many activities such as the promotion of α1B-adrenoceptor signaling and cell motility (67). Increased

expression in both SMC and macrophages of TG2 is found during the development of atherosclerotic lesions (54) and its crosslinking activity is important in artery remodeling (53). Furthermore we recently demonstrated that TG2 limits lesion size and helps to constrain necrotic core expansion (55).

In the absence of TG2, chondrocytes lose the ability to differentiate and calcify (45). Independently, TG2 can modulate hypertrophic differentiation of cartilage in the GTP-bound state. Therefore we tested the hypothesis that TG2 is needed to promote the osteoblastic / chondrocytic differentiation of SMC which leads to calcification. Our results identify the crosslinking activity of TG2 as a mediator in the promotion of SMC calcification and that TG2 promotes the maintenance of the SMC contractile phenotype.

RESULTS

TG2 is required for phosphate-induced SMC calcification in primary mouse SMC and aortic cultures-TG2 has been demonstrated to be critical for retinoic acid, IL-1 and chemokine induced-chondrocyte calcification (45, 46). Differentiation of SMC from the contractile state can lead to an osteoblastic / chondrocytic phenotype displaying calcification of the matrix (129). To mimic the typical physiologic state of the cells, all primary cultures of SMC were grown on

plates coated with 1μg/ml of laminin which promoted the maintenance of the contractile phenotype.

First we examined the ability of primary SMC to calcify in cells isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice. The addition of phosphate promotes SMC de-differentiation, the appearance of an osteoblastic phenotype and calcification of the matrix (130). We therefore induced calcification with 2.5mM β-glycerophosphate and 50μg/ml of ASC. After 7 days of culture, nodules were found to stain positive with both von Kossa and Alizarin red S (Fig. 4-1A, B) in $TG2^{+/+}$ cultures. The calcium and phosphate deposition were increased over time in these cultures, detected by the Alizarin red S and von Kossa techniques respectively, suggesting an increase in the hydroxyapatite deposition in the cell matrix.

Conversely, the $TG2^{-/-}$ cultures had little to no staining by either technique, indicating the absence of phosphate induced-calcification (Fig. 4-1A, B). By day 14 of culture, the von Kossa positive nodules increased in number in the $TG2^{+/+}$ SMC with a continued absence in the $TG2^{-/-}$ SMC (Fig. 4-1A). Quantification of the free calcium concentration using the phenolsulphonephthalein reagent demonstrated a four-fold increase in the $TG2^{+/+}$ SMC and no detectable increase in the $TG2^{-/-}$ SMC (Fig. 4-1C). During osteoblastic mineralization, a condensation or nodule formation takes place (48). Therefore, the SMC cultures from the mice were examined for nodule formation. The number of von Kossa positively stained nodules / well were significantly decreased on days 7 and 10 in the $TG2^{-/-}$ SMC (Fig 4-1D). In the $TG2^{-/-}$ SMC we did observe a condensation or nodule formation in these cultures

comparable to the $TG2^{+/+}$ SMC, suggesting the lack of von Kossa staining was not due to a defect in the clustering events in the cells (data not shown).

In vitro culture of primary SMC can lead to artificial differentiation of the cells seen typically in vivo only during stress or response to injury (131). To verify the lack of calcification in the $TG2^{-/-}$ was not due to culture conditions alone, we adapted the rat aortic ring culture model to study calcification (132). After culture of 2-3mm sections of the aortas from the $TG2^{+/+}$ and $TG2^{-/-}$ mice for 7 days in media containing 7 units/ml of AP and 2.5mM sodium phosphate, von Kossa staining revealed more intense medial calcification in the $TG2^{+/+}$ sections (Fig 4-2A). Immunohistochemistry for OPN, a calcification inhibitor, revealed a higher protein expression in the $TG2^{-/-}$ aortic sections than in the $TG2^{+/+}$ (Fig 4-2B). Furthermore, staining for type IX / XI collagen, proteins found during calcification and / or cartilaginous differentiation (133, 134), was increased in the $TG2^{+/+}$ and absent in the $TG2^{-/-}$ cultures (Fig 4-2B).

By day 7, ^{45}Ca incorporation was increased by approximately three-fold in the $TG2^{+/+}$ aortic cultures (Fig 4-2C). After decalcification, the $TG2^{-/-}$ aortas had significantly less free calcium at days 5 and 7 (Fig 4-2D). Isolation of mRNA after 9 days of culture of the aortas, revealed a ten-fold increase in the calcification inhibitor OPN, in the $TG2^{-/-}$ cultures compared to the $TG2^{+/+}$ aortas (Fig. 4-2E). Clearly these data indicate TG2 must be present to promote phosphate-induced calcification.

During calcification of SMC, the gene expression patterns often indicate the cells are entering a differentiation pathway similar to that of osteoblast / chondrocyte mechanisms. We therefore examined the mRNA from the $TG2^{+/+}$ and $TG2^{-/-}$ SMC during the course of calcification by quantitative PCR for known regulators of this process. As expected, at days 3 and 7, $TG2^{+/+}$ SMC had an increased tissue non-specific alkaline phosphatase (TNAP) expression which is critical to generate free phosphate needed for calcification. The $TG2^{-/-}$ cells did not share this feature (Fig. 4-3A). Two calcification inhibitors, OPN and MGP had significantly increased mRNA levels in the $TG2^{-/-}$ SMC (Fig. 4-3B, C).

Additionally the $TG2^{+/+}$ SMC displayed an increased mRNA expression of two chondrocytic and osteoblastic transcription factors, RUNX2 and MSX2, compared to the $TG2^{-/-}$ cells (Fig 4-3 D, E). Aggrecan mRNA levels, a chondrocyte matrix protein, was also increased in the $TG2^{+/+}$ SMC (Fig 4-3F). Taken together, these quantitative PCR results indicate an osteoblastic / chondrocytic differentiation has taken place in the $TG2^{+/+}$ cells but was lacking in the $TG2^{-/-}$ cells when both are stimulated with phosphate.

Extracellular, catalytically active TG2 promotes SMC mineralization- TG2 is released from cells by an unexplained, non-conventional mechanism. This allows TG2 to interact with matrix proteins and potentially alter or prepare the matrix for calcification (2). To verify the absence of TG2 protein in the $TG2^{-/-}$ SMC, TG2 levels were assessed by ELISA in the cell lysate (data not shown) and

the conditioned media (Fig.4-4A). Our primary mouse SMC were found to have mRNA expression of TG1, TG2, TG4, TG5 and TG6 (data not shown). Examination of the TG activity revealed a greater than 50% decrease in the $TG2^{-/-}$ SMC that was not induced by PDGF or ATRA (Fig. 4B), suggesting TG2 accounts for the majority of the TG2 activity in SMC.

Previously we examined the role of pyrophosphate (PP_i) and nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) in aortic calcification. In the absence of NPP1 or with a defective PP_i transporter ANK, there was an acceleration of SMC calcification (134). In the $TG2^{-/-}$ SMC, there was no significant difference in the PP_i levels or the NPP activity compared to the $TG2^{+/+}$ (Fig 4C, D), indicating an alternative mechanism for the diminished calcification of these cells.

During calcification an increase in the alkaline phosphatase (AP) activity is commonly found (48). We demonstrated an increase in the mRNA of TNAP, the major AP in SMC (Fig. 4-3A). As the regulation of phosphate is critical to calcification of the matrix, we examined the activity of AP as well as the expression of the type III sodium dependent phosphate transporter Pit-1. There was an increase in the AP activity in the $TG2^{+/+}$ SMC but it was lacking in the $TG2^{-/-}$ cells (Fig. 4-5A). Conversely the $TG2^{-/-}$ had increased levels of Pit-1 protein expression detected by western blotting in comparison to similar levels of the Elastin-Laminin receptor (ELR) in the $TG2^{+/+}$ (Fig 4-5B). These data suggested that even though there is no increase in the AP activity in the $TG2^{-/-}$ cells they still have adequate levels of the phosphate transporter.

To asses the potential of extracellular TG2 in mediation of SMC calcification, recombinant TG2 proteins were given to the TG2 deficient SMC. Using site-directed mutants we compared the two of the major states of TG2. In the presence of calcium, TG2 can transamidate or crosslink proteins. This requires an intact catalytic triad of Cys^{277}, His^{335}, and Asp^{358} (79). Secondly, TG2 can bind and hydrolyze GTP. This relies upon an intact Lys^{173} (79). Therefore the roles of the GTP binding and crosslinking activity of TG2 were examined after reconstitution with mutations at Lys^{173} and Cys^{277}. FXIIIA, a TG which also has catalytic activity similar to that of TG2 but no ability to bind GTP, was used as a control TG. These experiments revealed an increase in the von Kossa staining in the $TG2^{-/-}$ cultures after reconstitution with the sTG2 WT, but still not to the capacity seen in the $TG2^{+/+}$ SMC (Fig 4-6A). Additionally, TG2 appears to rely upon its catalytic activity to mediate these events, as the sTG2 C277G mutant could not restore the calcification and FXIIIA could also partially restore the calcification (Fig 4-6A). Quantification of the free calcium confirmed the von Kossa staining, again revealing upon the reliance of an intact catalytic site of TG2 (Fig 4-6B).

TG2 directly modulates extracellular protein levels of OPN- The $TG2^{-/-}$ SMC appeared to have a dysregulation of OPN mRNA, a calcification inhibitor (Fig 4-3B) and the aortic cultures displayed an increase in both protein and mRNA expression (Fig 4-2B, E). To examine the protein expression of OPN, conditioned media were collected between days 1-17 during the calcification process. The

$TG2^{+/+}$ SMC had a gradual increase in OPN levels during the course of calcification, typical to what is found during osteoblastic differentiation (134). Conversely, $TG2^{-/-}$SMC showed a gradual decrease in OPN levels. The data revealed a significantly higher level in OPN on days 1, 3 and 7 but had a significantly lower level on days 14 and 17 in the $TG2^{-/-}$ SMC when compared to the $TG2^{+/+}$ cells (Fig 4-7A).

Extracellular TG2 can modify the matrix through the transamidation of proteins. This can include TG2 itself as the substrate that becomes crosslinked into various matrix proteins (2), potentially mediating the function of the cells it comes into contact with. To test the effectiveness of stabilized TG2 in the laminin matrix, coverslips coated with laminin were incubated with TG2 for 10 minutes and the remaining soluble TG2 was washed off with PBS. The $TG2^{-/-}$ SMC were then plated on these matrices. The top panels in Figure 4-7B indicate the lack of TG2 in the SMCs, but large amount of sTG2 which attached to the matrix after its addition. Under these conditions, OPN expression clearly was also decreased, evidenced primarily by the lack of membrane-bound and matrix expression, again suggesting the extracellular TG2 itself or through a modification of the matrix can mediate OPN expression (Fig 4-7B).

When human aortic smooth muscle cells (HASMC) were stimulated with sTG2 WT, a 50% decrease was found in the OPN levels in the conditioned media (Fig 4-7C). Interestingly, the catalytic mutant C277G retained this ability to inhibit OPN, while the GTP binding mutant K173L, did not have this effect on OPN (Fig

4-7B). Taken together, these data argue TG2 has a role in modulating OPN and it is specific to TG2 as FXIIIA could not modulate this function.

TG2 regulates differentiation events in mesenchymal cells- The differentiation of SMC from the contractile to the synthetic state is evidenced by an altered protein expression of the cells, including an increased proliferation, migration and expression of OPN and type I collagen (136). This is normally in association with a decreased expression in SM actin and Notch-3. During injury to the vessel well, SMC differentiate to the synthetic phenotype to repair the damage, thus not transitioning through an osteoblastic or chondrocytic pathway (136). As the $TG2^{-/-}$ SMC had high a high basal expression level of OPN, we examined the differentiation state of the SMC.

To do so, passage 2 cells were plated on poly L lysine coated coverslips and stimulated with 10ng/ml of PDGF, an inducer of the synthetic SMC phenotype (137), for 72 hours. The $TG2^{+/+}$ SMC displayed a typical pattern of type I collagen expression, having little to no expression before stimulation and a significant increase after the addition of PDGF (Fig 4-8A). Conversely, the $TG2^{-/-}$ SMC had a higher basal level of type I collagen that could not be stimulated further with the addition of PDGF (Fig 4-8A). Quantitative PCR results revealed a significant decrease in SM-actin and Notch-3 (contractile proteins) and a significant increase in type I collagen and OPN (data not shown).

Previous work has shown the modulation of SMC differentiation state can be mediated by the substrate used for cell growth (131). To examine if this modulation was mediated by TG2, we plated the $TG2^{+/+}$ and $TG2^{-/-}$ SMC on laminin (to promote maintenance of the contractile state), fibronectin (to promote synthetic differentiation) and poly L lysine (similar to the culture conditions on tissue culture plastic which leads to synthetic differentiation) (131). After fixing and staining the cells for type I collagen, we demonstrated a large increase in type I collagen expression, even when the cells are on the "contractile promoting" substrate laminin (Fig 4-8B). These data indicate an altered differentiation state of the $TG2^{-/-}$ SMC.

We previously demonstrated the ability of TG2 to modulate the differentiation of chondrocytes (45, 101). Osteogenic and chondrogenic differentiation are critical mechanisms for SMC calcification (48), and are evident in the $TG2^{+/+}$ SMC. To validate and further explore the role of TG2 mediation of differentiation state mesenchymal cells, we isolated bone marrow stromal cells (BMSC) and primary calvarial osteoblasts from the $TG2^{+/+}$ and $TG2^{-/-}$ mice. The BMSC were cultured under conditions to favor chondrogenesis and we found that the $TG2^{-/-}$ BMSC had a decreased ability to become chondrogenic, evidenced by a decrease in the proteoglycan incorporation and the aggrecan mRNA expression (Fig. 4-9A). Secondly, immature calvarial osteoblasts, when grown under conditions to stimulate osteoblast maturation and mineralization, had a weakened ability to calcify in the absence of the TG2 (Fig 4-9B). Taken together, the lack of

TG2 altered a mesenchymal cell's ability to enter into chondrogenic and osteoblastic differentiation.

DISSCUSSION

In this study we examined the ability of $TG2^{+/+}$ and $TG2^{-/-}$ SMC to calcify when induced by phosphate. We found that TG2 was needed for promotion of SMC calcification. Furthermore by addition of extracellular recombinant WT and mutant TG2 proteins, we found the requirement of catalytically active TG2 to enhance matrix calcification. From our data, TG2 appeared to down regulate the extracellular levels of OPN, a calcification inhibitor, but this ability relied primarily upon the GTP binding conformation. Finally, $TG2^{-/-}$ SMC may have altered differentiation state in vitro, expressing higher levels of the synthetic proteins OPN and type I collagen. Taken together these data argue that TG2 mediates the differentiation of SMC.

After isolation of aortic or primary SMC cultures from the $TG2^{-/-}$ mice and growth under high phosphate conditions, little to no calcification was found when analyzed by von Kossa or alizarin red staining, or after analysis of the free calcium content or the ^{45}Ca uptake. Upon initial examination of $TG2^{-/-}$ mice, no gross phenotype was originally found (13). Recently closer experimentation revealed the development of an autoimmune phenotype due to the impaired ability to phagocytose apoptotic cells (15, 16). Under stress or pathogenic challenges, $TG2^{-/-}$

have an increased inflammatory response (15). Although inflammation of the $TG2^{-/-}$ aortas has not been examined in vivo, it is probable the inflammatory phenotype is evident.

During the phosphate induction of the $TG2^{+/+}$ SMC, an increase in both osteogenic and chondrocytic genes were increased, but these were lacking in the $TG2^{-/-}$ SMC. One possible explanation is a lack of the ability to respond to phosphate, a potent inducer of the osteogenic differentiation pathway (48). The $TG2^{-/-}$ SMC generated or hydrolyzed PP_i similar to the $TG2^{+/+}$ cells as basal levels of NPP and AP activity were comparable. There was also an increased protein expression of the type III sodium dependent phosphate transporter Pit-1. Although we did not specifically measure the phosphate uptake in the $TG2^{-/-}$ SMC it appears that other mechanisms beyond phosphate regulation must primarily be mediated by TG2 to control calcification.

Here we report the addition of extracellular recombinant TG2 led to an increase in the calcification in the $TG2^{-/-}$ SMC. Furthermore, structure-function analysis of TG2 revealed a reliance on the catalytic or crosslinking activity of TG2 to do so. This suggests externalized TG2 may mediate matrix calcification through crossliking matrix proteins. In vivo, the TG2 externalized by the endothelium or the circulating leukocytes during inflammation, could therefore mediate atherosclerotic plaque calcification and limit the inflammatory reactions. Treatment with the catalytically active recombinant TG2 did not completely “rescue” the calcification of the SMC lacking TG2. This suggests either the need for TG2 to be inside the

cells or to be controlling the differentiation state of the cells to promote this phenotype.

Recombinant TG2 was found to decrease the extracellular level of OPN in SMC. This was dependent upon an intact GTP binding site, Lys^{174}. During calcification of the SMC, the $TG2^{-/-}$ cultures had increased protein levels of OPN initially which decreased during differentiation. This was unlike the levels in the $TG2^{+/+}$ which slightly increased during calcification, as expected with an osteoblastic transition (135). Thus our data reveal a regulation of OPN by TG2, independent of its catalytic activity. OPN is an important negative regulator of calcification. It resides primarily in the matrix and signals to the surrounding cells through the $\alpha V\beta 3$ pathway (138). OPN can regulate Ca^{2+} levels, increase carbonic anhydrase II which absorbs bone and inhibit mineralization by binding to the hydroxyapatite crystals (139, 140). OPN is normally located surrounding the sites of calcification (140). TG2 may directly signal to decrease the release or expression of OPN. Alternatively, as OPN is a substrate for TGs (141), crosslinking of OPN by TG2 may regulate its activities or inactivate its functions.

In addition to high osteoblastic / calcifying-cell expression of OPN, the synthetically differentiated SMC express increased OPN (136). We therefore examined the type I collagen expression of the $TG2^{-/-}$ SMC and found a correlated increased level of expression. Additionally, modulation of the synthetic phenotype of the cultured SMC was not possible in the $TG2^{-/-}$ cultures. These data suggest a highly synthetic differentiated state of the $TG2^{-/-}$ SMC. This may in part explain

why there is a lack of calcification in these cells. For if these cells are "committed" toward the synthetic pathway, osteoblastic / chondrocytic differentiation may not occur frequently if at all.

Further examination of the synthetic phenotype is needed, primarily in vivo. Removal of the SMC from the artery wall creates many artifacts, primarily by promoting proliferation and the synthetic phenotype of the SMC (131). Here we cultured the cells on laminin coated dishes until the initiation of calcification to attempt to control this artifact. Even under these conditions, high levels of type I collagen were found in the $TG2^{-/-}$ cells, but that does not rule out another protein that TG2 may affect or that is deficient in the $TG2^{-/-}$ SMC, which alters the differentiation state.

TGFβ has been demonstrated to promote the SMC contractile phenotype through activation of RhoA and then this in turn phophorylates smads (142). TG2 can crosslink RhoA, causing it to become constitutively active. In the SMC, RhoA activates Rho kinase and PKN which both have been demonstrated to promote the transcriptional activation of SMC genes (143). Activated TGFβ levels of the $TG2^{-/-}$ SMC were not significantly different than those in the $TG2^{+/+}$ SMC (data not shown). Additionally, no significant change was found in the RhoA activity between the two cell populations (data not shown). Modulation of the synthetic phenotype by TG2 must therefore be through an alternative mechanism which needs further exploration.

The artery wall is both a destination and a source for progenitor cells (51). In this study we examined the chondrogenic potential of stromal cells and the calcification of calvarial osteoblasts from the $TG2^{-/-}$ mice. In the absence of TG2, both chondrogenesis and the ability of immature osteoblasts to proceed toward terminal differentiation and mineralization were inhibited. Therefore these pathways were inhibited in multiple cell populations, suggesting TG2 regulates the terminal differentiation of mesenchymal progenitors. The lack of chondrocytic or osteogenic differentiation by the $TG2^{-/-}$ SMC, may also be due to a lack or a decreased number of calcifying vascular cells (CVC). These are clonally isolated medial SMC that are selected based upon their ability to calcify (52). In this study we used low passage (2-4) primary SMC and did not attempt to isolate a CVC subpopulation or determine the number of progenitor cells, but these data may enhance the understanding of the mechanism occurring.

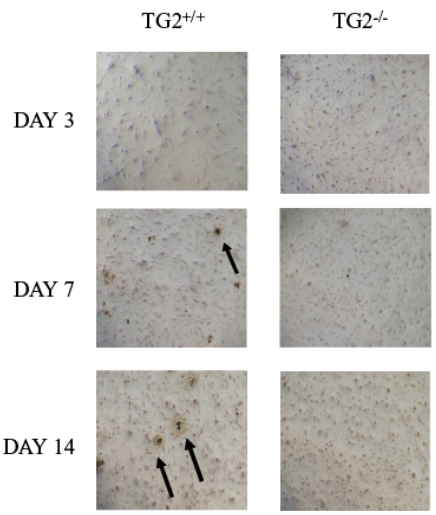
From our data, TG2 is essential for the promotion of calcification and may work on two fronts. First TG2 acts through its matrix crosslinking to stabilize and allow for hydroxyapatite deposition. Preparation of the matrix may be critical as the addition of catalytically active TG2 and FXIIIA, another TG with similar crosslinking activities partially restored the calcification in the $TG2^{-/-}$ cultures. On the other hand, crosslinking may connect TG2 to the matrix to then interact with the cell. TG2 could therefore be able to modify the differentiation state of the cell. This may be through its GTP-bound conformation as this was needed to modulate the OPN levels. Alternatively, TG2 may mediate the signaling inside the cell to

complete these events. Further experimentation is needed to determine the actual mechanism occurring.

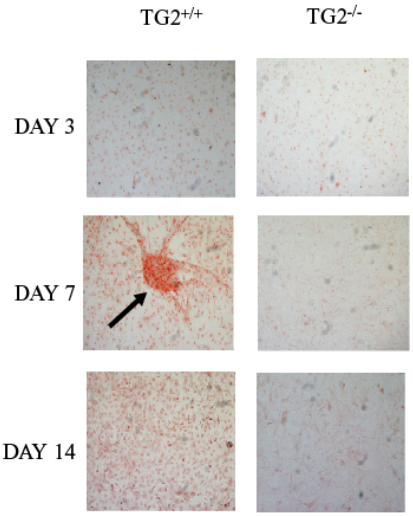
In this work we found that the presence of TG2 in the SMC, as well as in aortic cultures, was necessary to induce phosphate-driven matrix calcification. Catalytically active extracellular TG2 could partially restore the ability of the $TG2^{-/-}$ SMC to calcify. Additionally we found that TG2 modulated the levels of the calcification inhibitor OPN, but this was dependent upon an intact GTP binding site. Finally TG2 appeared to promote osteoblastic and chondrocyte mesenchymal cell differentiation. Elucidation of these actions of TG2 adds to its probable role in mediating pathologic events in the artery wall.

Figure 4-1. $TG2^{-/-}$ mouse primary SMC have significantly decreased matrix calcification. To examine the role of TG2 in SMC mineralization, primary SMC were isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice as described in the methods. Passage 2 SMC from each genotype (5×10^4 cells / well in a 24 well plate (panels A, B, D) or $1x10^5$ cells / well in a 12 well plate (panel C)) were cultured in growth media with the addition 2.5mM β-glycerophosphate and 50μg/ml ASC for 14 days to promote the deposition of calcium phosphate crystals. (A, B) The cells were fixed with 4% paraformaldedye for 10 min on the days indicated and then washed with H_20 (A) or PBS (B). (A) To detect the phosphate deposited in the matrix the fixed cultures were stained using the standard Von Kossa technique and counterstained with nuclear fast red. (B) To visualize the Ca^{2+} deposition in the matrix, Alizarin Red S staining was performed. (C) The free Ca^{2+} concentration of the cultures were determined by incubation of the cell lysate from the SMC on the days indicated in 0.6N HCl for 16 hours. Following this decalcification the Ca^{2+} content was determined using the phenolsulphonephthalein reagent to bind free Ca^{2+} as described. (D) Each 24 well dish considered for panel A ($9mm^2$ wells) was examined microscopically to determine the number of von kossa positive nodules. The numbers represent the total found in each 9 mm^2 well. Panels A, B, representative of 4 experiments, cells pooled from 12 animals each genotype, each experiment. Panels C, D data pooled from 4 experiments,

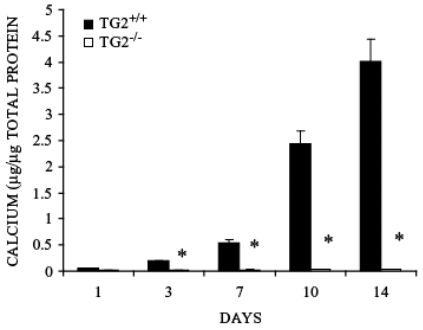
A. VON KOSSA STAINING



B. ALIZARIN RED S STAINING



C. Ca^{2+} CONTENT



D. VON KOSSA POSITIVE NODULES

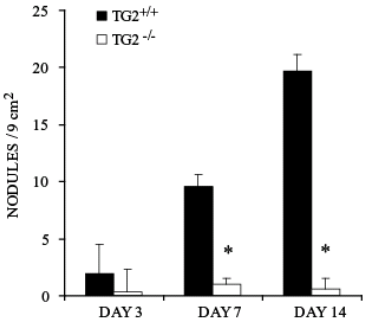
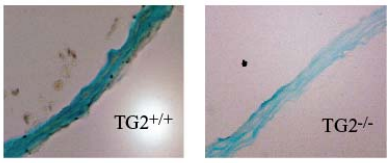
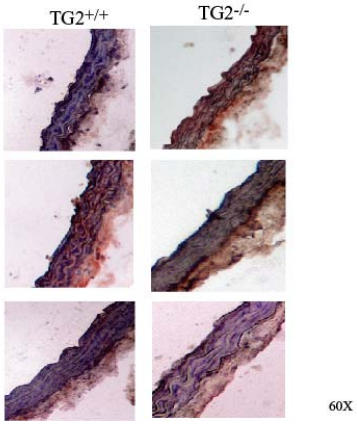


Figure 4-2. Aortic cultures of $TG2^{-/-}$ mice have decreased media calcification. To validate the in vitro findings with SMC, aortas were isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice, cut into 2-3mm slices and cultured in growth media containing 2.5mM NaP_i and 7U/ml alkaline phosphatase. (A) Following nine days of culture, the aortas were washed with PBS and fixed overnight with 4% paraformaldehyde. The tissue was snap frozen, embedded in OCT and 10um sections were sliced. The brown / black positive Von Kossa stained medial regions are evidenced in the $TG2^{+/+}$ sections. Both are counterstained with Fast Green. Representative of 15 animals. (B) To assess the altered differentiation in response to phosphate, the frozen sections were stained with antibodies to the calcification inhibitor OPN, osteoblastic / chondrocytes proteins Type IX / XI and an IgG control. (C) Aortic cultures were incubated as above for 4 and 6 days prior labeling for 24 hours with 0.3µCi/ml ^{45}Ca. The aortas were collected on days 5 and 7, washed three times with PBS, dried, weighed and the ^{45}Ca CPM were quantified in a liquid scintillation counter. Data pooled from 10 animals, replicates of three. (D) The free Ca^{2+} concentration of the aortas were determined after incubation in 0.6N HCl for 16 hours. Following this decalcification the Ca^{2+} content was determined using the phenolsulphonephthalein reagent to bind free Ca^{2+} as described. The aortas were then dried and weighed to determine the mg of Ca^{2+} / mg dry weight. Data pooled from 12 animals, 2 aortic cultures / time point. (E) Total RNA was isolated from the aortic cultures after 9 days of incubation. Relative quantification of OPN expression (to GAPDH) was determined as described above. Data pooled from 5 animals, replicates of three. *$p<0.05$

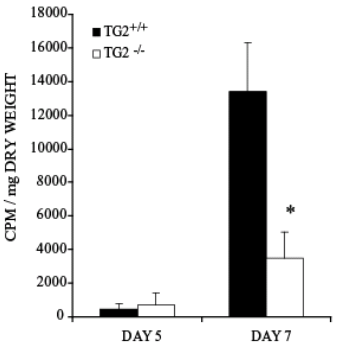
A. VON KOSSA



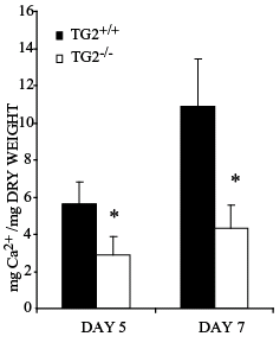
B. IMMUNOHISTOCHEMISTRY



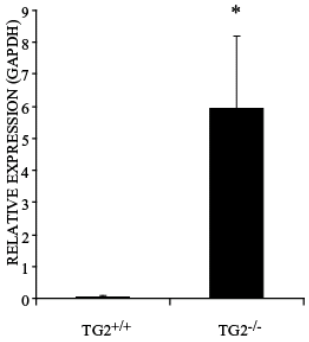
C. ^{45}Ca INCORPORATION



D CALCIUM CONCENTRATION



E. OPN mRNA EXPRESSION



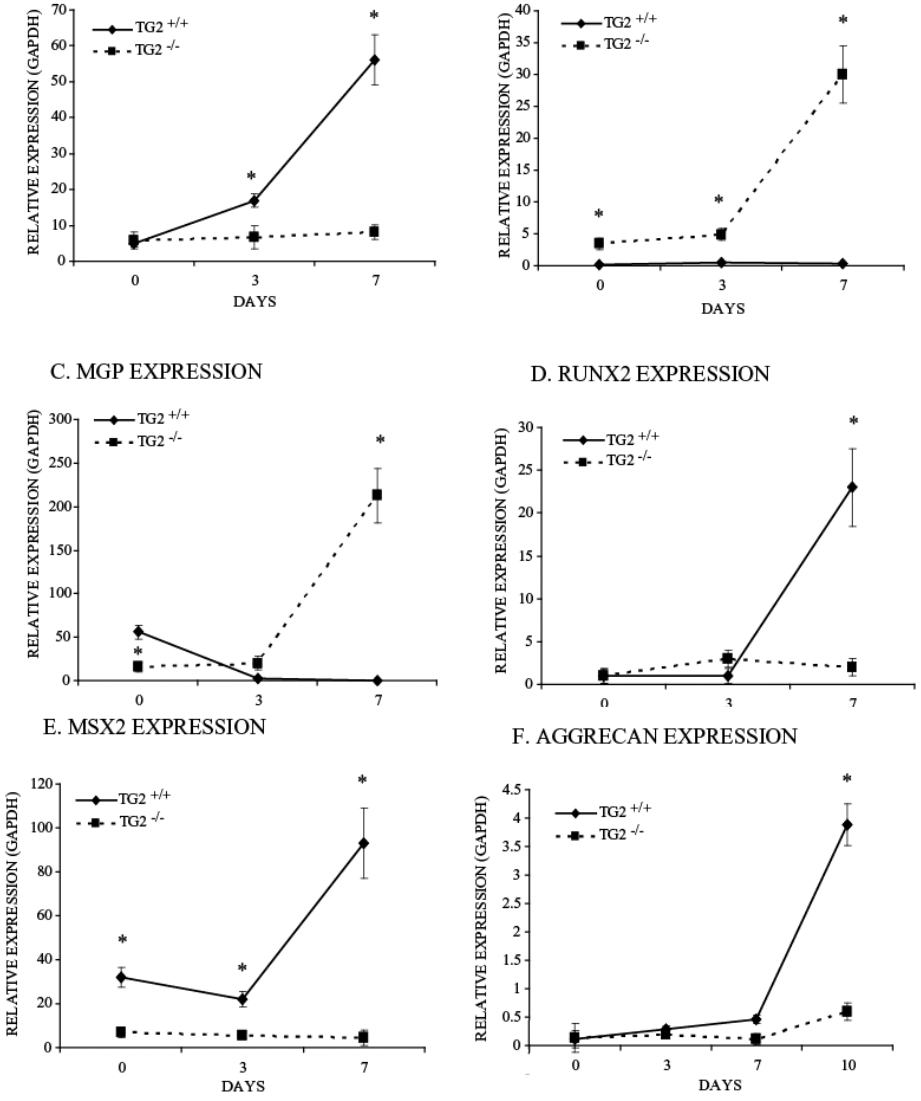


Figure 4-3. During matrix calcification, TG2$^{-/-}$ SMC express increased levels of calcification inhibitors MGP and OPN with a decreased expression of chondrocytic / osteoblastic genes. (A-F) Quantitative PCR was performed to determine the pattern of gene expression during phosphate induced calcification of the TG2$^{+/+}$ and TG2$^{-/-}$ SMC. Total RNA was collected from 5 x 10^4 passage 2 cells plated in a 24 well plate cultured in growth media containing 2.5mM β-glycerophosphate and 50μg/ml ASC for the days indicated. Day 0 represents cells collected prior to the addition of β-glycerophosphate and ASC. Following reverse transcription, each gene depicted was quantified using the LightCycler 2.0 and the data expressed are a relative quantification compared to GAPDH. *$p<0.05$, data pooled from three experiments, replicates of 2.

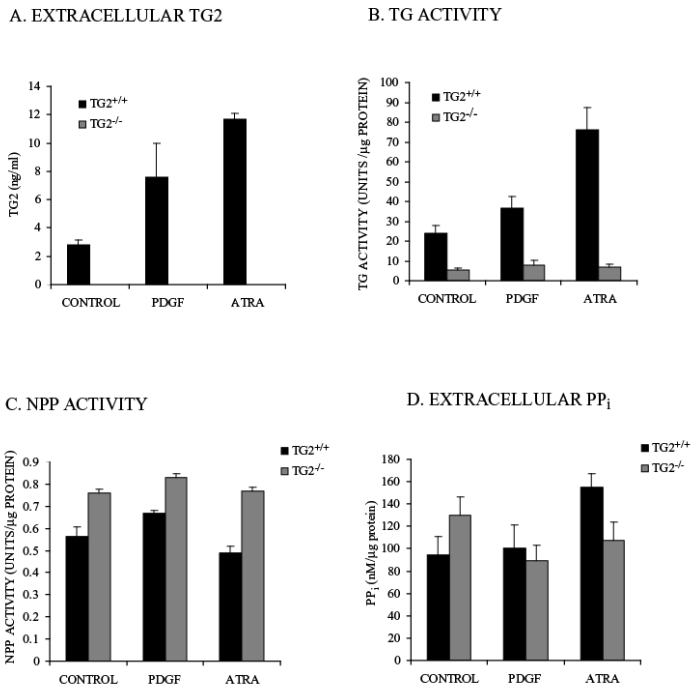


Figure 4-4. Removal of TG2 does not alter the NPP activity of PP_i concentration of SMC. To verify the lack of TG2 protein expression and the level of TG activity in the $TG2^{-/-}$ SMC, 5 x 10^4 passage 3 cells / well (24 well plate) were cultured for 72 hours. Additionally, to determine if TG2 modulates NPP activity and PP_i, known enhancers of SMC calcification, 1x 10^5 passage 3 cells / well (12 well plate) were cultured for 72 hours. Where indicated the cells were stimulated with 10ng/ml of PDGF or 10nM ATRA, mediators of SMC differentiation. (A) The conditioned media was collected, concentrated and bound to a nunc-immuno module plates for detection of the released TG2 protein by ELISA. The cell lysates were collected and extracted for measurement of TG activity (B) and NPP (C). (D) Heat inactivated conditioned media was harvested for measurement of extracellular PP_i.

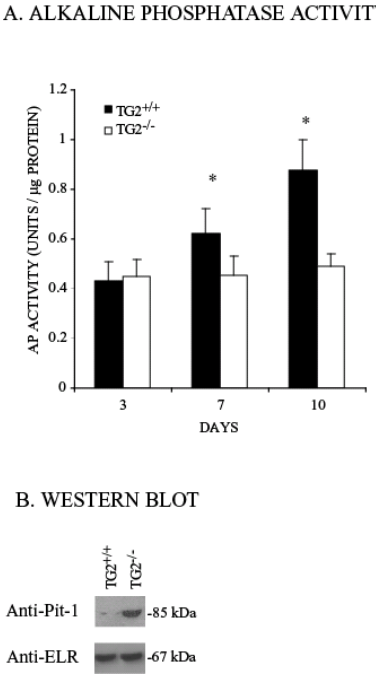


Figure 4-5. $TG2^{-/-}$ mouse primary SMC have significantly decreased AP activity during mineralization but higher Pit-1 levels. Primary SMC were isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice as described in the methods. Passage 2 SMC from each $1x10^5$ cells / well in a 12 well plate (panel A) or 3 x 105 cells / well in a 6 well plate (panel B). The cells were cultured in growth media with the addition 2.5mM β-glycerophosphate and 50µg/ml ASC for up to 10 days to promote the deposition of calcium phosphate crystals. (A) The cell lysates were harvested on the days indicated and the AP activity was determined as described. Data pooled from 3 experiments, replicates of 2. *$p<0.05$. (B) The cell lysates were collected on day 7, the membrane was extracted and then separated by SDS-PAGE. The proteins were transferred and blotted for Pit-1 and ELR. Representative of three experiments.

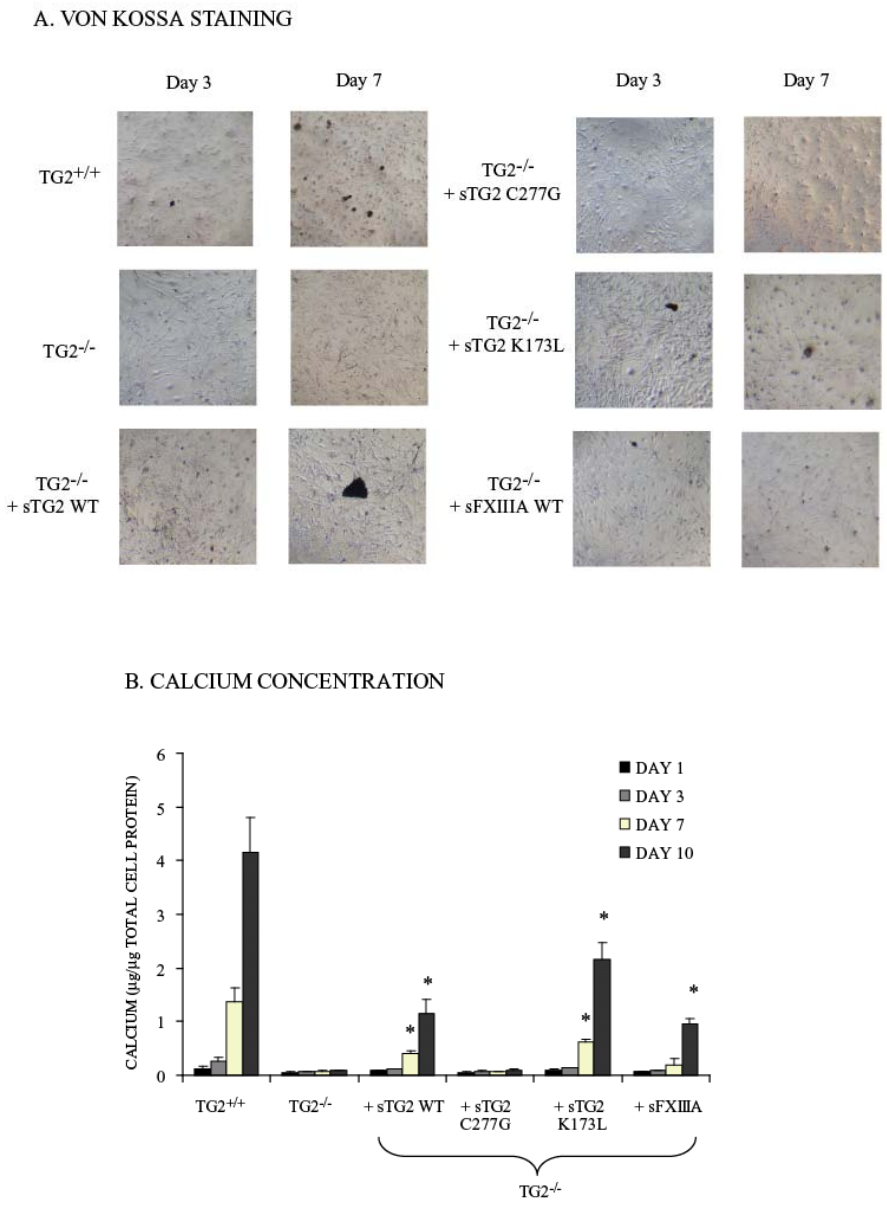


Figure 4-6. Calcification of the SMC matrix is dependent upon TG catalytic activity. To determine the function of TG2 critical to matrix calcification of SMC, cells were cultured in the presence of 100ng/ml WT, mutant TG2 or FXIIIA recombinant proteins. (A) 5x 10^4 passage 2 cells / well (24 well plate) were cultured for 3 and 7 days in the presence of 2.5mM β-glycerophosphate and 50µg/ml ASC. On the days indicated the cells were fixed, washed and stained with the Von Kossa technique. Data representative of 3 experiments, replicates of 3. (B) 1x10^5 SMC / well in a 12 well plate were cultured for 1, 3, 7 or 10 days with and without the addition of 100ng/ml recombinant proteins. The total free Ca^{2+} concentration of the cells was determined as above. Data pooled from three experiments, replicates of 3. *$p<0.05$

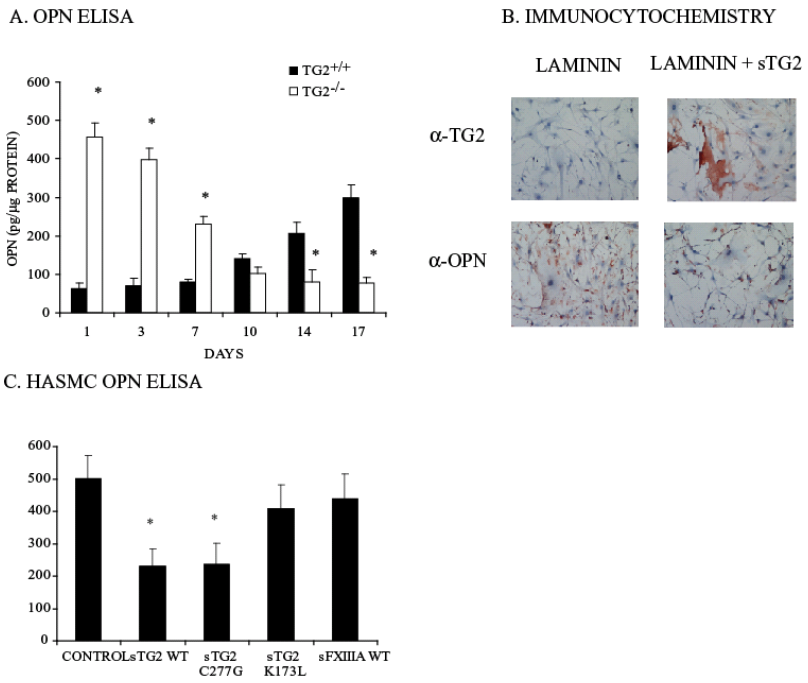


Figure 4-7. TG2 modulates extracellular OPN expression. (A) To determine the regulation of OPN in the $TG2^{-/-}$ SMC during calcification, $5x10^4$ passage 2 cells / well (24 well plate) were cultured up to17 days in the presence of 2.5mM β-glycerophosphate and 50μg/ml ASC. Conditioned media was collected for determination of OPN expression by ELISA as described in the methods. (B) $1x10^5$ cells were grown on coverslips coated with laminin. To crosslink the laminin matrix, recombinant sTG2 WT was added to the laminin coated coverslips for 10 minutes at 37°C and washed off 4 times where indicated. The cells were then plated and cultured for 24 hours prior to fixation with 4% paraformaldehyde. Immunocytochemistry was used to detect the amount of TG2 which was bound to the matrix and the expression of OPN and Type I collagen. Data representative of 3 experiments.(C) HASMC ($5x10^4$) were cultured for 72 hours with and without 100ng/ml of recombinant TG2 (sTG2). The conditioned media was assayed for OPN expression. Data pooled from three experiments, replicates of 3. *$p<0.05$

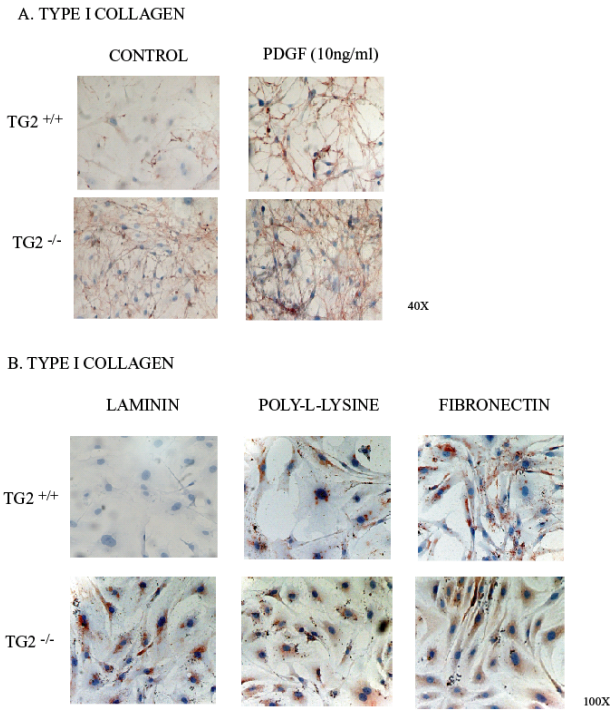


Figure 4-8. $TG2^{-/-}$ SMC have a preference for synthetic differentiation. (A) To examine the differentiation state of the primary SMC, passage 2 SMC ($1x10^5$) were cultured for 72 hours with and without 10ng/ml of PDGF, a potent inducer of synthetic gene expression. After fixation, immunocytochemistry was used to determine the type I collagen expression. (B) To induce the transition from contractile to the synthetic state, $1x10^5$ SMC were cultured on Laminin, Poly Lysine and Fibronectin. After 24 hours, the cells were fixed and stained as above for type I collagen. Data representative of 5 experiments.

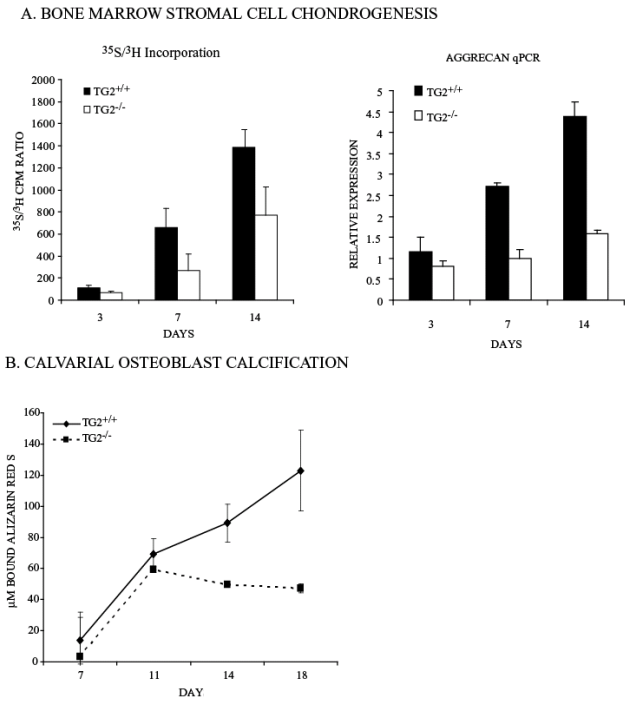


Figure 4-9. TG2 is required for chondrogenic and osteoblastic differentiation. (A) BMSC isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice and cultured for 3, 7 or 14 days with 3 x 10^5 cells in high density conditions in Complete Serum Free media containing 10ng/ml of BMP2. The cells were collected for RNA extraction and qPCR analysis using the LightCycler 2.0. The data depicts the relative expression of each gene compared to GAPDH. Alternatively, the cells were labeled for 24 hours prior to collection with 2mCi/ml of 35S and 1µCi/ml of ^{3}H proline to determine the production of sulfated proteoglycans. Data pooled from 3 experiments, with each experiment consisting of cells pooled from 20 mice of each genotype. (B) Calvarial osteoblasts from $TG2^{+/+}$ and $TG2^{-/-}$ mice were isolated from animals 2-3 days of age. The cells were grown to confluency and then 1 x 10^5 cells were plated in 12 well dishes. The following day, the cells were incubated in αMEM containing 2.5 mM β-glycerophosphate and 50µg/ml of ASC. On the days indicated, the cells were washed with PBS and fixed for 15 minutes at room temperature with 4% PFA. After the last time point, the wells were stained with 0.1% Alizarin Red S, pH 4.0 for 20 minutes. The plates were washed 4X with PBS followed by the addition of 10% cetylpyridium chloride. The released Alizarin Red was quantified at 562nm. Data were pooled from 3 experiments, consisting of 5-6 animals per experiment.

CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

This present dissertation examined the role of TG2 in the differentiation and calcification of chondrocytes and SMC. When these cell types were stimulated with phosphate or grown under hypertrophic conditions, chondrocyte hypertrophic differentiation and calcification as well as SMC calcification were dependent upon the presence of TG2. Furthermore TG2 appeared to restrain the SMC in the contractile state. For in its absence, the cells quickly became more synthetic in vitro. $TG2^{-/-}$ cells have decreased chondrogenesis and delayed osteoblast mineralization. This suggests TG2 can modulate the differentiation of cells of mesenchymal origin. The data clearly represent a critical role involving TG2 in the mediation of these events in vitro, but many details still remain unresolved in an understanding of the mechanisms involved in pathologic changes in vivo.

The actions of extracellular TG2-This current work primarily focused upon the role of extracellular TG2. TG2 exists also in the nucleus, the cytosol and the membrane in addition to the ECM (2). TG2 is regulated by Ca^{2+}, GTP and ATP. Additionally TG2 can bind with multiple integrins such as β1 and β3 integrins and thereby interact with fibronectin and other matrix proteins (2). The movement of TG2 around the cell, to and from each microenvironment must regulate its functions. Under normal, physiological conditions, TG2 is latent, displaying neither TG catalytic nor GTPase activity, due to the presence of adenine nucleotides (71). Throughout this study, extracellular TG2 was important in regulating the

differentiation / calcification of cartilage and SMC, but the precise function or conformation of TG2 mediating these events was different.

To promote chondrocyte hypertrophy and differentiation, TG2 had to be bound to GTP. Furthermore, FXIIIA mediated similar events through the movement of TG2 to the membrane and again relied upon GTP-bound TG2. Originally TGs were thought only to be enzymes which mediated the crosslinking between two proteins, primarily in stabilization of matrix proteins or involved in apoptosis. Now the role of TG2 as a G protein, mediating G protein coupled-receptor signaling (GPCR), is emerging. Recently TG2 has been demonstrated to be a ligand for the orphan GPCR, GPR56 (144). This receptor is a member of the class B or secretin-like family of GPCRs, which also includes receptors such as calcitonin, parathyroid hormone and vasoactive intestinal peptide. The binding of TG2 to GPR56 inhibited the metastasis and tumor growth in melanoma cells (144).

The extracellular environment has a higher level of Ca^{2+} than inside the cell. The exposure of TG2 to Ca^{2+} favors the TG catalytic conformation of TG2 (71). The finding that the GTP binding of TG2 was necessary to induce chondrocyte hypertrophy was therefore quite surprising. This conformation could be required for movement to the cell membrane and therefore could also preferentially bind to integrins or other receptors. No report of the required conformation for TG2 to interact with GPR56 has been made. Additionally recombinant TG2 (or the C-terminal alone) has not been shown to activate signaling of GPR56, leaving the

possibility TG2 may bridge other molecules involved in this signal transduction process (23).

Both the physiologic and pathologic roles of TG2 are not entirely defined. Both FXIIIA and TG2 mediate wound healing, well documented in fibroblasts (2, 13). During normal physiological conditions, basal levels of TGs may be involved in maintaining the articular cartilage matrix which is critical for the prevention of OA. A major anabolic factor in cartilage is TGFβ. TGFβ promotes chondrogenesis and sustains the differentiated cartilage phenotype and can be activated by TG2 (145). Increased levels of activated TGFβ can be found during the disease process and is thought to be indicative of the healing process that is taking place. Additionally, the actions of TGFβ can be inhibited by various matrix proteins, such as the small luecine rich repeat proteoglycan family member Asporin, which can lead to an enhanced development of OA (146).

Physiologically these small alterations in differentiation (as seen by the addition of excess TG2) of partially committed cells do not lead to gross changes in the developing skeleton, for the $TG2^{-/-}$ mice are phenotypically normal at birth (13). Conversely in aged and damaged cartilage, high levels of TGs may lead to altered signaling events, ultimately promoting the further destruction of cartilage. Stress or exposure to aging or injury could reveal more phenotypic changes in the $TG2^{-/-}$ mice.

Externalization of GTP-bound TG2 led to an increase in chondrocyte hypertrophy in differentiated chondrocytes. Previous work tracing TG2 has

localized it to the precartilagenous mesenchyme, the hypertrophic chondrocytes and the surrounding perichondrium in the developing chick limb. More specifically, in the mesenchyme, intracellular TG2 is expressed with a shift to extracellular expression in the hypertrophic chondrocytes (147). The authors found a widespread expression of TG2 during organogenesis with a down regulation as development proceeds. Its reappearance or increased externalization during wound healing (148) may preclude to its role in tissue remodeling or advancement in disease pathology. Although a weak expression of TG2 was found in many tissues of the chick embryo, significant expression was only seen in the limb bud precursors, specifically during hypertrophy. This suggests again that the role of TG2 may primarily be in tissue remodeling during disease and trauma, when it becomes upregulated but may potentially mediate aspects of chondrocyte development.

Elucidation of the role of TG2 during chondrogenesis may lead to an understanding of one of the mediators of terminal differentiation of chondrocytes during chondrogenesis. It may demonstrate a similarity or distinguishing characteristics between chondrogenesis during development and chondrocyte differentiation that takes place during OA. Initial studies using BMSC from the $TG2^{+/+}$ and $TG2^{-/-}$ mice revealed no dependence upon extracellular TG2 in the depression of chondrogenesis (data not shown). These studies pointed to a primary role of intracellular TG2. BMSC express multiple TGs, and therefore a more complex for TG2 may exist that involves a mechanism which is quite distinct from those elucidated here.

The proposed model of action in OA- As OA progresses and damage is done to the cartilage TG2 and FXIIIA are released into the cartilage matrix (40, data not shown). An upregulation of these enzymes initially could be due to changes in cytokine production, such as CXCL8 which can increase the release of TG2 (data not shown). After such release, TGs may have two roles through its two major functions. These are the promotion of chondrocyte differentiation and calcification of the matrix. In the matrix both enzymes are catalytically active, where they may attempt to repair the damaged cartilage through the formation of stable crosslinks of the matrix proteins, but may in actuality only prepare the foundation for optimal crystal deposition.

Ca^{+2} - bound FXIIIA and integrin activation propagated a further movement of TG2 to the membrane and therefore its release. As TG2 reaches the membrane it could interact and bind with integrins (such as β1 or β3 integrin) or other receptors to mediate the signals needed to initiate chondrocyte differentiation. The rapid induction of both FAK and p38 MAP kinase phosphorylation demonstrated in chondrocytes could mediate both integrin mediated and hypertrophic events respectively. These signals may ultimately lead to the differentiation of chondrocytes.

At the same time, TG2 which binds to integrins may act as a bridge to the ECM, similar to its role in fibroblasts (5). This bridge itself may enhance the differentiation of the chondrocytes. Additionally this may stabilize the cell, setting

up a close and concentrated interaction with the now crosslinked matrix proteins, allowing for a nidus of crystal formation, leading ultimately to the calcified matrix seen in OA (Fig. 5-1).

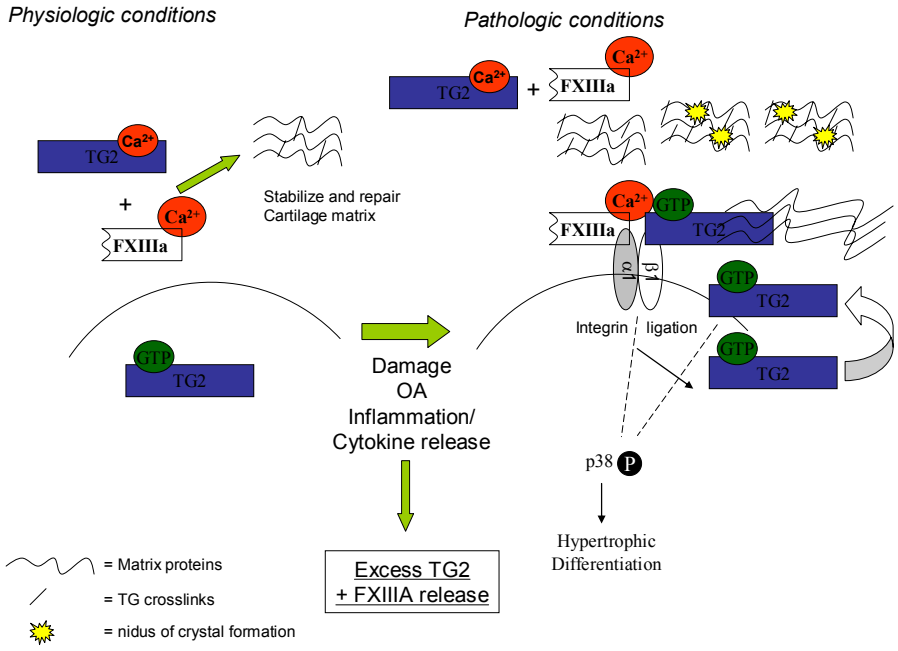


Fig. 5-1. Proposed model of TG functions in physiologic and pathologic conditions in cartilage. See text for discussion

The data presented here during the examination of chondrocytes, revealed a reliance on the GTP bound conformation of TG2 while the catalytic mutant did not propagate the differentiation and calcification. This model is proposing that both catalytically active and GTP-bound TG2 conformations may be critical. The hypothesis is that TG2 initiates the events in its GTP bound form, but also relies upon its transamidation properties to ultimately prepare the matrix to effectively calcify. It is clear that TG2 mediates these events in the absence of its crosslinking

activity, but none of these studies performed were done in the absence of any TG activity. Although low concentrations of serum were used, some TG activity was present. Secondly, FXIIIA was available in all matrices, thus potentially preparing the environment as needed. It may seem unlikely that the extracellular GTP-bound TG2 could be mediating or initiating the differentiation process. In vivo, these events may be primarily initiated inside the cells, where the GTP bound form is prevalent. In the studies presented here, it was clear that extracellular TG2 and FXIIIA could bind to the cell membrane, suggesting the critical events could take place there. The GTP-bound form may also bind to the membrane of its receptors more readily than the Ca^{2+} form, as it is this conformation which enhances the stability and prevents the degradation of TG2 (71).

This model opens many areas of exploration. It would be of interest to determine the key interactions that TG2 and FXIIIA had at the chondrocyte membrane with promotes hypertrophy. Searching for a receptor or protein involved in these events is a large and risky task. It is probable that the mechanisms are similar to those already known. The binding of TG2 could act merely as a scaffolding protein or as a co-receptor for integrins, similar to what has been described for β1 and β3 integrins, stabilizing their interactions with fibronectin (5) and initiate the signaling events. Alternatively, TG2 could bind GPR56 or another member of its family more prevalent in cartilage. Determination of its interaction would help to define the downstream events. Examination of the role of TG2 and FXIIIA in vivo during the disease progression could further elucidate their

responsibilities. Using a mouse OA model, an anterior cruciate ligament tear in the knee of a TG2 / FXIIIA deficient mouse may lead to enhanced progression of OA due to the lack of crosslinking in the matrix, which leads to further instability. Additionally, healing of the initial wound may be slower, thus enhancing the progression of the OA. But at the same time a lack of differentiation and calcification may also be found.

TG2 in the SMC- The degree of dependence upon extracellular TG2 varied among cells types. In cartilage it was mandatory for TG2 to be released (or applied externally) in order to induce chondrocyte hypertrophy. Reconstitution with recombinant soluble extracellular TG2 induced the hypertrophic phenotype, even in the absence of cellular TG2 or FXIIIA. Conversely in SMC, the similar experiments only had a partial restoration of the calcification phenotype. This suggests cellular TG2 is also mediating these events. A mechanism may be involved similar to that which was proposed above for cartilage. Different forms of TG2 may work inside the cell or at the membrane than that which functioned extracellularly.

In SMC, reliance upon the catalytic activity was found to be critical for the induction of matrix calcification. Unlike cartilage, no expression of FXIIIA was found. HASMC express at least 5 different transglutaminases (data not shown), although TG2 accounted for at least 50% of the TG activity. FXIIIA is the only other TG that is externalized (2). The crosslinking of matrix proteins by TG2 may

be more critical in vitro to prepare the matrix for calcification in the SMC than cartilage as chondrocytes do express FXIIIA. Alternatively, if extracellular TG2 binds to a specific receptor in cartilage to mediate the differentiation events, this receptor may be lacking in the SMC. Again this points to the need for cellular TG2 for the cell to fully calcify.

TG2 also mediated a down regulation of the extracellular OPN and relied not upon its catalytic activity to do so, but on the GTP-bound conformation. These data again suggest a model, like proposed above in cartilage (Fig. 5-1), where both conformations of TG2 are critical. Exploration of the TG2 / OPN relationship could be prosperous. OPN is an acidic phosphoprotein normally found in mineralized tissues. It is primarily an inhibitor of apatite crystal growth but can modulate osteoclast function through $\alpha V\beta 3$ integrin (138). OPN has also been found to regulate angiogenesis and modulate migration particularly in metastatic cancers (149). Therefore, TG2's ability to modulate OPN expression could itself be leading to other events which promote the differentiation of SMC. Examination of the actions of TG2 in the SMC from the $OPN^{-/-}$ mice could show an inability of TG2 to mediate synthetic differentiation or calcification.

An interesting phenotype of the $TG2^{-/-}$ SMC was revealed: the cells appeared to be more synthetic in culture. This favored differentiation state of the $TG2^{-/-}$ SMC could dominate the cells and thus inhibit or not allow calcification to occur, particularly as the calcification demonstrated involved the differentiation of the SMC down an osteoblastic / chondrocytic line. SMC are plastic cells which

never fully differentiate in vivo. Their role changes based upon the environmental cues and they adapt or differentiate, perform the needed function and then return to the contractile state to wait for the next signal (48). The finding reported here needs further exploration to determine why there appears to be more synthetic differentiation.

As TG2 interacts with integrins, fibronectin and other matrix proteins, bridging them to the cell in a specific conformation most likely dictates what state the SMC are in. Removal of TG2 obviously alters this. It may be more pronounced in vitro, as growth of any SMC in vitro enhances the synthetic phenotype. A detailed mapping of the mechanisms into synthetic versus osteoblastic differentiation has not been examined. Initially examining which integrin / TG2 interaction is critical may reveal the mechanism at work. Extracellular TG2 did appear to decrease the expression of type I collagen, a major protein found in the synthetic differentiated state of SMC. Other markers or phenotypes of this state need to be examined such as migration, adhesion and proliferation to see if these are enhanced in the $TG2^{-/-}$ SMC and can be restored by extracellular TG2.

TGFβ can promote the development of the SMC contractile phenotype in neural stem cells. Additionally, TGFβ can increase the production of SM genes such as SM-actin and elastin (143). TG2 has a TGFβ response element in its promoter (145). If TG2 is critical for modulating or maintaining the contractile phenotype, the $TG2^{-/-}$ SMC may not respond in the same manner as wild type cells

to TGFβ. Levels of extracellular activated TGFβ and BMP2 were not altered in the SMCs from the TG2 mice (data not shown).

TGFβ has been demonstrated to promote the SMC contractile phenotype through activation of RhoA and then this in turn phophorylates smads (134). Although TG2 can crosslink RhoA and constitutively activate it (19) no alteration of RhoA activity was found in the $TG2^{-/-}$ SMC. Other elements of the potential role of TG2 in modulating the transcriptional pathways of SM specific genes that may be modulated in a similar way need to be explored, particularly as TG2 can be located in the nucleus.

As the mechanisms of SMC calcification are thought to be distinct in medial versus intimal calcification (50), examination of $TG2^{-/-}$ mice (crossed to an $LDLR^{-/-}$ or $APOE^{-/-}$ background), may lead to an understanding of TG2 in atherosclerosis, but would not necessarily support the role of phosphate-induced calcification presented here. BMP2, decorin and RANKL (48) are also mediators of calcification in SMC. Preliminary studies with HAMSC using shRNA to knockdown TG2, point to a deficiency even under BMP2-driven calcification (data not shown). This again argues that cellular or membrane bound TG2 is mediating early events that alter the differentiation of SMC. It will be of interest to continue exploration in these pathways.

All of the data taken together point additional functions of TG2 that were previously uncharacterized. The ubiquitously expressed molecule, although not leading to an overt phenotype in its absence, under the right stimuli can lead to

pathologic conditions. Future studies in vivo may ultimately lead to better treatment studies for multiple diseases.

APPENDIX:

METHODS

Generation of TG2 cDNA Mutants andSoluble Recombinant Forms of TG2-Human TG2 and FXIIIA cDNA in pcDNA4/HisMax was the template for the generation of the following TG2 mutants using the Quikchange II site directed mutagenesis kit (Stratagene, San Diego, CA): Fibronectin binding domain mutants TG2 2-3 MUT (A2G and E3A), TG2 7-8-9 MUT (L7G, E8A and R9A), TG2 DEL5 (L5 deleted), the TG2 GTP binding site mutant K173L (12), the TG2 mutant of an amino acid required for externalization (Y274A) (81), and three TG2 transamidation active site (80) mutants C277G, H335A, D358A, the FXIIIA R37A thrombin activation site mutant, the FXIIIA C314A Catalytic site mutant, the FXIIIA M513G thrombin inactivation site mutant, the FXIIIA D270A cation binding mutant, the FXIIIA R310 isomerization mutant, and two FXIIIA calcium binding mutants, A457F and E490G.

Recombinant forms of His-tagged TG2 and FXIIIA were purified from transfected human fibroblastic HEK-293 cells using the Probond Purification Kit (Invitrogen, San Diego, CA). After purification through binding to nickel columns, dialysis and concentration, the preparation was tested with Limulus Amebocyte Lysate QCL 1000 assay (Cambrex, Baltimore, MD) and determined to have <0.1EU/ml (or below the detection limits) of endotoxin. Where indicated, purified, soluble, recombinant TG2 proteins were used with and without treatment to generate Mg nucleotide complexes (GTP or GTPγS with TG2) as described previously (71). In brief, 1 mM Mg excess was created to maximize the nucleotide

complex formation. To do so, $MgCl_2$ was added to 25 μM GTP or GTPγS and incubated on ice for 30 min with 0.1μg of the soluble TG2 indicated.

$TG2^{-/-}$ and $FXIIIA^{-/-}$ mice-$TG2^{+/+}$ and $TG2^{-/-}$ mice were originally provided by Dr. R. Graham (13). $FXIIIA^{-/-}$ mice and wild type congenic littermate controls were bred from $FXIIIA^{+/-}$ mice previously generated, characterized, and generously provided by Dr. G. Dickneite and colleagues (Aventis Behring GMBH, Germany) (150). All animal housing, experimentation and protocols were approved by the IACUC (Institutional Animal Care and Use Committee) of the San Diego Veteran's Administration Medical Center.

shRNA design and characterization- Ambion's web-based shRNA design program was used to identify 21-mer regions within TG2 and FXIIIA effective for shRNA targeting. Five sequences were originally tested to find an optimal sequence. The 21-mers were then used to generate the 55bp oligos which included two 19bp regions specific to human TG2 or FXIIIA complementary to each other to form the hairpin, a loop sequence separating the complementary domains and a dinucleotide overhang that can hybridize with the RNA target (part of the original 21-mer). The two 55bp complementary oligos were annealed and then ligated into the pSilencer 4.1-CMV neo vector (Ambion, Austin, TX). The scrambled TG2 and FXIIIA shRNAs were randomly generated with the same basepairs as the siTG2. After sequence confirmation, the vectors were transfected into human articular

chondrocytes, using the AMAXA as described. The optimal 19 bp sequences for human TG2, 5'-GAGCGAGAT GATCTGGAAC-3'(1116-1132) and for human FXIIIA, 5'- GAGTTTCTTAATGTCACGA-3' (214-232).

Cell culture conditions, transfections and isolation of bovine cartilage explants- CH-8 cells, an SV40 immortalized clone of normal human knee articular chondrocytes (151)**,** were a generous gift of Dr. M. Hiramoto (Nihon University School of Medicine, Tokyo, Japan). CH-8 cells were cultured in DMEM high glucose supplemented with 10% FCS, 1% glutamine, 100 U/ml Penicillin, 50 μg /ml Streptomycin (Mediatech, Herndon, VA) and maintained at 37°C. Maintenance of chondrocytic differentiation of CH-8 cells was verified by RT-PCR analysis of type II collagen and aggregcan expression. Transient transfection of CH-8 cells was performed using Lipofectamine plus (Invitrogen, San Diego, CA) according to manufacturer instructions, with ~60% transfection efficiency.

Primary articular chondrocytes were isolated by dissection of the tibial plateaus and femoral condyles of the $TG2^{+/+}$, $TG2^{-/-}$, $FXIIIA^{+/+}$ and $FXIIIA^{-/-}$ mice at two months of age, as described (45). Human articular chondrocytes from normal donor knees were isolated as described (46). Passage 1 human articular chondrocytes and mouse chondrocytes were cultured in DMEM high glucose supplemented with 10% FCS, 1% glutamine, 100 U/ml Penicillin, 50 μg /ml Streptomycin (Mediatech, Herndon, VA) and maintained at 37°C.

Transient transfection of human articular chondrocytes was performed using a Nucleofector apparatus (AMAXA, Gaithersburg, MD) and modified by substitution of the transfection reagent with Fugene 6 (Roche, Indianapolis, IN), with achievement of ~70% transfection efficiency in human chondrocytes.

Studies on differentiation and function were performed in Medium A (DMEM high glucose supplemented with 1% FCS, 1% glutamine, 100 U/ml Penicillin, 50 μg/ ml Streptomycin, and 50 μg/ml of ascorbic acid) or Medium B (DMEM high glucose supplemented with 1% FCS, 1% glutamine, 100 U/ml Penicillin, 50 μg/ ml Streptomycin, 1mM Sodium phosphate and 50 μg/ml of ascorbic acid) with 100 μg/ml of sFXIIIA and sTG2 added where indicated.

For organ culture studies, two millimeter by two millimeter slices of cartilage were removed from the patellar groove and femoral condyles of normal bovine knees (Animal Technologies, Tyler, TX). The explants were placed in individual wells of a 96 well tissue culture plate in 200μl of Medium A for 24 hours, after which the explants were incubated for an additional 5 days with treatment as indicated.

Primary mouse smooth muscle cells were isolated from two month old animals. The aortas from the euthanized mice were examined microscopically to remove the adventitia and the aorta was cut open to expose the endothelial layer. The tissue from three animals was pooled for digestion with 1mg/ml collagenase I (Worthington Biochemical, Lakewood, NJ) in 1% FCS media for 10 min to remove

the remaining adventitia and endothelium. The tissue was then removed to fresh media (20% FCS) containing 2mg/ml collagenase, 25% elastase for 1-1.5hr.

The cells were washed three times with 1% media and plated in M231 (Cascade Biologic, Portland, OR) containing the Smooth muscle cell growth supplement (consisting of 5% FCS bFGF, EGF and insulin). This media was used for maintenance and stimulation of the cells (unless indicated otherwise). Each group of cells is stained for SM-actin (>95% positive) and von-willbrand's factor (< 1% positive) to verify the specificity of the cell population. The SMC were plated on tissue culture plates coated with 1μg/cm^2 murine Laminin to promote the stabilization of the contractile differentiation state. The cells were expanded for two passages and then used for experimentation.

Aortic ring cultures were established adapting the rat model (132). Briefly the aortas were removed from the TG2$^{+/+}$ and TG2$^{-/-}$ mice and washed 3 times with 1% FCS containing DMEM high glucose media. 2-3mm pieces were cut and placed in a 96 well plate. These were cultured in SMC growth media containing 2.5mM NaP_i and 7U/ml alkaline phosphatase for 7-9 days as indicated in the figures.

Immunohistochemistry and immunocytochemistry- Frozen sections (5 μm) of the bovine cartilage were fixed in cold acetone for 10 minutes, and sections were immunohistochemically stained using a 1:500 dilution of the type X collagen antibody. Bound antibodies were detected with the avidin-biotin-peroxidase method using the reagents in the Histostain Plus kit (Zymed, San Francisco, CA).

Frozen 10um sections of aortic ring cultures from the $TG2^{+/+}$ and $TG2^{-/-}$ mice were fixed in cold methanol for 10 minutes and then dried for 1 hour. Von Kossa staining was performed and the sections were counterstained for 1 minute in 0.1% Fast Green. For detection of Type IX / XI collagen and OPN, the sections were fixed for 5 minutes in 4% paraformaldehyde. The sections were then permeabilized in 0.1% triton for 5 minutes and blocked with Peroxoblock (Invitrogen, San Diego, CA) for 30sec and then 5% goat serum, 0.5% casein and 0.5% BSA for 30 minutes.

For immunocytochemical analysis of human articular chondrocytes and primary mouse SMC, aliquots of 1×10^5 cells were plated on 18 mm circular glass coverslips and in medium A (chondrocytes) or M231 (SMC). The cells were then fixed for 20 minutes at room temperature with 4 % parformaldehyde and washed with PBS. All primary antibodies were used at a 1:100 dilution. For light microscopy, bound antibodies were detected by the ABC method.

All light microscopy images were visualized on a Nikon microscope using the 4X and 10X objective lenses and with 10X binoculars, and Nikon digital camera images were captured using ACT-2U software. Immunofluorescent staining was detected using 1:100 dilution of anti-mouse IgG-FITC or anti-rabbit-PE (Biosource, Camarillo,CA). Coverslips were mounted with Antifade (Invitrogen, San Diego, CA) and stored at 4°C for < 7 days. Cells were visualized using the Nikon Eclipse TE300 fluorescent microscope using 10X objective and with 10X binoculars. Nikon digital camera images (controlled by the Hamamatsu system)

were captured as JPEG images, and cropped and arranged using Adobe Photoshop and Illustrator software. All imaging was performed at room temperature.

SDS PAGE /Western Blotting, and RT-PCR- For SDS-PAGE / Western blotting analyses, conditioned media were collected and concentrated with tricholoracetic acid (15% vol/vol) for 10 minutes at 4°C. Protein pellets from the concentrated media were washed in 1:1 acetone/ethanol mixture and resuspended in 2% SDS, 0.2M Tris, pH 6.8, and 40% glycerol and protein concentrations determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates were harvested in 10mM Tris (pH 7.6), 150mM NaCl, 0.5mM EDTA, 1mM EGTA, 1% SDS containing a freshly added Complete Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN). Aliquots of 0.01-0.05 mg protein from each sample were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose, as described (45). Anti-type X collagen (Calbiochem, San Diego, CA), anti anti-TG2 and anti-FXIIIA (Neomarkers, Freemont, CA), anti-*p*-FAK ($Try^{567,577}$), anti-FAK, anti-*p*-Rac1, anti-Rac1, anti-*p*-p38, anti-p38 (Cell Signaling, Beverly,MA) and anti-tubulin primary antibodies were used at 1:1000 dilution in Western blotting studies, with luminescent detection employing Supersignal (Pierce, Rockford, IL) according to manufacturer instructions. The monoclonal $\alpha 1$ integrin antibody (TS2/7) (Genetex, San Antonio, TX) was used for immunoprecipitation in addition to immunofluorescent staining. The FB12 $\alpha 1$ integrin antibody (Chemicon / Millipore, Billerica, MA) a validated blocking

antibody was used to pretreat the chondrocytes for 1 hour prior to stimulations where indicated.

Quantification of Matrix Calcification-To quantify matrix calcification by the transfected CH-8 cells or SMC, a previously described Alizarin Red S binding assay was performed, with results further validated in each experiment by direct visual observation of Alizarin Red S staining in each plate (152).

For SMC and aortic culture calcification, samples were decalcified in 0.6N HCl for 24 hours. The calcium level of each sample was determined by a stable interaction with the phenolsulphanethalein dye (Bioassay Systems, Hayward CA). Each sample was corrected for total protein concentration (SMC) or total dried weight (aortic cultures).

Isolation of plastic-adherent bone marrow stromal cells (BMSC)-Mice of the indicated genotypes were euthanized at the ages indicated and the femurs were flushed with 1% FCS- containing DMEM low glucose medium, with the washed cells removed from the femurs subsequently underlayed with a 1.44 g/L density gradient of Ficoll and centrifuged for 20 minutes at 800 x g to deplete hematopoietic cells. The remaining cells were cultured for 14 days in Basal Mesenchymal Stem Cell (MSC) medium (Cambrex, Walkersville, MD) supplemented with 1% glutamine (wt/vol), 100 U/ml Penicillin, 50 μg /ml Streptomycin, and 10% FCS.

For chondrogenesis studies in high density culture, aliquots of 3 x 10^5 cells in a 10 µl volume were placed in a 9 mm dish and allowed to adhere at 37°C for 1 hour followed by the addition of 0.5 ml of MSC medium for 24 hours, after which the medium was replaced with 0.5 ml of Complete Serum Free Medium (CSFM) (Mediatech, Herndon, VA), plus BMP-2 and TGFβ1 where indicated. The medium was replaced every three days.

Isolation of primary calvarial osteoblasts - $TG2^{+/+}$ and $TG2^{-/-}$ mice were taken at 3 days of age for isolation of the calvaria. The sequential collagenase digestion released primary osteoblasts (1 x 10^6 cells / calvaria) as described previously (152). Once confluent the cells were grown in αMEM containing 10% FCS, 1% glutamine, 1% pen / strep, 50µg/ml ascorbic acid and 2.5mM β glycerophosphate to induce mineralization for up to 21 days. The mineral content was quantified using the alizarin red S binding assay.

Assays of TG2 Transamidation Activity, TG2 externalization, and TG2-mediated fibronectin binding activity- TG transamidation activity was determined through 2mM biopentylamine incorporation into 20 mg/ml casein, as previously described (45). For qualitative evaluation of TG2 expression in conditioned media, transfected CH-8 cells were placed in serum free medium for the last eight hours of incubation, and then the conditioned medium was precipitated as described above and separated by SDS-PAGE. TG2 was quantified

in the conditioned medium after binding to a Nunc-Immuno Module plate (Nunc, Rochester, NY) for detection by direct ELISA, using biotin-labeled TG2-specific antibody CUB7402 (153). To assess the ability of CH-8 cells overexpressing specific TG2 mutants to bind to fibronectin, Nunc-Immuno Module plates were coated with 0.5 μg/ml of fibronectin, blocked with 3% BSA and incubated with aliquots of 25 μg in protein of cell lysates and detected as described (153).

Quantitative RT-PCR-Total RNA was isolated as described (45). For quantitative RT-PCR, 1 μl of a 5-fold dilution of the cDNA from reverse transcription reactions was amplified using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics, Indianapolis, IN) with addition of 0.5 μM of each primer in the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Following amplification, a monocolor relative quantification of the target gene and reference (GAPDH) analysis determined the normalized target gene: GAPDH mRNA copy ratios by the manufacturer's LightCycler Software (Version 4.0). The primers employed where designed using LightCycler Probe software, version 2.0 (Roche, Diagnostics, Indianapolis, IN) (Table 1).

OPN ELISA – The OPN ELISA was performed on conditioned media collected from SMC or aortic cultures as previously described (152).

Statistical Analyses- Statistical analyses were performed using the Student's t test (paired 2-sample testing for means) and error bars, where indicated, represented SD.

Table A-1: Primer Sequences for Quantitative mRNA Amplification

Primers employed were designed by the LightCycler probe design software, version 2.0.

NAME	SEQUENCE	ACESSION NUMBER
hMMP-13 F	5’-TGTTCACTTTGAGGATACAGGC-3’	NM_002427
hMMP-13 R	5’-CATAGACAGCATCTACTTTATCACC-3’	NM_002427
hVEGF F	5’-CCTCCCTCAGGGTTTCG-3’	NM_001025366
hVEGF R	5’-CAGAGTCTCCTCTTCCTTCATT-3’	NM_001025366
hType II collagen F	5’-GGTTCCCAGGTGAACG-3’	NM_001844
hType II collagen R	5’-GCCAGGCATTCCCTGAAGA-3’	NM_001844
hType X collagen F	5’-CCAAAGCTTACCCAGCAAT-3’	NM_000493
hType X collagen R	5’-CCAAACATGAGTCCCTTTCAC-3’	NM_000493
hGAPDH F	5’-CTGCCACCCAGAAGACT-3’	BC08351
hGAPDH R	5’-TCCACCACTGACACGTTG-3’	BC08351
mOPN F	5’-CTTCCAAGCAATTCCAATGAAAG-3’	AF515708
mOPN R	5’-TGTGTACTAGCAGTGACGG-3’	AF515708
mAggrecan F	5’-AGGAGATAGGTACAGAAACCT-3’	NM_007424
mAggrecan R	5’-ATCTACTCCTGAAGCAGATGTC-3’	NM_007424
mTNAP F	5’- CCTCAAAGGCTTCTTCTTGC-3’	NM_007431
mTNAP R	5’- GTCCATCTCCACTGCTTCA-3’	NM_007431
mGAPDH F	5'-CATCCCAGAGCTGAACG- 3	DQ403054
mGAPDH F	5'-CTGGTCCTCAGTGTAGCC-3'	DQ403054
mMGP F	5’- GTGGCAACCCTGTGCTAC-3’	NM_008597
mMGP R	5’- CAGGCTTGTTGCGTTCC-3’	NM_008597
mMSX2 F	5’- GAGCCCGGCAGATACTC-3’	NM_013601
mMSX2 R	5’- CCCGCTCTGCTATGGAC-3’	NM_013601
mRUNX2 F	5’-TTTGGACATTTGGAACATTTCTTAGTGTA-3’	NM_009820
mRUNX2 R	5’- TCACATTATGCCTGAAGGAATTGAG-3’	NM_009820

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