Expression and Localization of FAST2; a Transcriptional Co-Activator of Procollagen Gene in Cardiac Fibroblasts

By

Julie Crystal Roth

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the degree:

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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MASTER OF SCIENCE

For my Mom 

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I. ABBREVIATIONS

A_0	angiotensinogen
ACE	angiotensin converting enzyme
Ang II	angiotensin II
AT_1	angiotensin type I receptor
AT_2	angiotensin type II receptor
ARE	activin response element
BMP	bone morphogenic protein
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CHF	congestive heart failure
CMP	cardiomyopathy
CRE	cAMP response element
CBP/CREB	cAMP response element binding protein
Co-Smad	common mediator Smad
ECM	extracellular matrix
EMSA	electro(phoretic) mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAST	forkhead activin signal transducer
GAG	glycosaminoglycan
GTF	general transcription factor
HAT	histone acetyl transferase

HCM	hypertrophic cardiomyopathy
HDAc	histone deacetylase
HTH	helix-turn-helix DNA binding motif
IL-1α	interleukin-1α
INF-γ	interferon-γ
I-Smad	inhibitory Smad
JNK	c-jun N-terminal kinase
LAP	latency associated peptide
LTBP	latent TGF-β-binding protein
MH-1, MH -2	Mad homology region-1, Mad homology region -2
Mad	mothers against decapentaplegic protein: an R-Smad homologue in *Drosophila melanogaster*
MAPK	mitogen activated protein kinase
MI	myocardial infarction
MMP	matrix metalloproteinase
PARCF	primary adult rat cardiac fibroblast
RAAS	renin angiotensin aldosterone system
R-Smad	receptor-mediated Smad
SARA	Smad anchor for receptor activation
Smad	Sma/MAD homologue
TALE	three amino acid loop extension
TβR-I	transforming growth factor-β receptor type I
TβR-II	transforming growth factor-β receptor type II

TGF-$\beta_{1,2,3}$	transforming growth factor-β subtypes
TGIF	tumor growth interacting factor
TIMP	tissue inhibitor of matrix metalloproteinase
TRAP1	TGF-β receptor associated protein 1
VDR	vitamin D receptor
VHD	valvular heart disease
WH	winged helix proteins

II. ABSTRACT

FAST2 *(forkhead activin signal transducer 2)* is a winged-helix (WH) protein known to complex with and co-activate Smad proteins in mammalian cells. Elevated receptor-mediated Smad (R-Smad) protein expression and activation in cardiac fibroblasts is positively correlated to elevated deposition of cardiac fibrillar collagen species in experimental heart failure models. R-Smad signaling is known to influence collagen expression in fibroblasts, however, the role of FAST2 in collagen deposition by cardiac fibroblasts is unknown. We investigated whether FAST2 is expressed in adult rat heart and addressed its role as a putative Smad transcription co-factor. Using primary adult rat cardiac fibroblasts (PARCFs), Western analysis revealed the presence of FAST2 in both cytosolic and nuclear adult cardiac fibroblast protein fractions. Upon stimulation with angiotensin II (Ang II), FAST2 expression significantly increased within the nucleus when compared to Ang II stimulated cytosolic fractions and control nuclear fractions, and a positive correlation between FAST2 protein and procollagen synthesis was observed. To establish the specific effect of FAST2 expression on procollagen expression in fibroblasts, exogenous myc-tagged FAST2 cDNA was transfected into PARCFs. Concomitant FAST2 and procollagen expression were measured by Western blot analysis, and the same sections were subjected to Hoechst nuclear staining. Nuclear accumulation of overdriven FAST2 was associated with enhanced procollagen expression. Stimulation of adult fibroblast cultures with

INF-γ was associated with diminished FAST2 and procollagen expression when compared to untreated quiescent controls and to Ang II + losartan treated cells. To determine whether FAST2 associates with Smad 2 (an R-Smad present in cardiac tissues), we used co-immunoprecipitation techniques, implementing whole cell cardiac fibroblast lysates. We observed that Smad 2 forms a complex with FAST2 protein (reciprocal Western analysis). Thus Smad 2 binds FAST2 in adult rat cardiac fibroblasts. We also demonstrated that overdriven FAST2 protein expression was associated with stimulated procollagen synthesis in these cells. Electrophoretic mobility shift techniques examined whether FAST2 bound to the promoter region of rat procollagen α-2(I). Computer searches of the Genbank, RefSeq and PDB databases as well as analysis of the procollagen type I promoter region revealed the existence of a putative FAST2 binding sequence (AATxxACA) in this gene. A 20-mer ^{32}P end-labeled DNA fragment containing the consensus FAST2 binding sequence was added to nuclear extract preparations obtained from COS-7 cells transfected with the FAST2 mammalian expression vector (pCMV5β-mycFAST2). To determine band specificity, we employed competitive inhibition assays by sequentially adding greater concentrations of unlabeled oligomer to samples. A monoclonal primary antibody raised against the myc tag was exploited for the supershift technique to determine the identity of the protein bound. We found that FAST2 bound directly to the consensus sequence (AATxxACA) present within the procollagen promoter region.

These results support the hypothesis that FAST2 is a transcriptional partner of the Smad complex in cardiac fibroblasts and that its expression is directly linked to procollagen synthesis in these cells. As Smads transduce TGF-β_1 signaling in these cells, it is likely that FAST2 plays a significant role in the regulation of TGF-β_1-mediated cardiac fibroblast collagen synthesis.

III. INTRODUCTION

Congestive heart failure (CHF) is a major health problem. In the United States it represents the most frequent hospital discharge diagnosis among the elderly each year **(1)**. Furthermore, over three hundred thousand people are reported to suffer from this disease in Canada **(2)**. Although there have been numerous important therapeutic advances in recent years for the treatment of heart failure such as the use of angiotensin converting enzyme (ACE) inhibitors, it is likely that the number of cases of CHF will continue to rise. This is due to anticipated shifting population demographics toward increased elderly populations **(72)**. Current therapeutic agents seem to only delay the fatal aspects of the disease **(197)**. Therefore, a critical need for the development of new therapeutic approaches (i.e. biological target molecules) exists.

CHF is a syndrome defined as insufficient cardiac output to meet the metabolic needs of the body **(69)**. "Syndrome" *per se* indicates that the onset of CHF is the end point of many distinct disease types including myocardial infarction (MI), coronary artery disease (CAD), and hypertrophic cardiomyopathy (HCM) **(17)**. Myocyte dropout and thus increased workload on the surviving myocytes may mark the progression of cardiac hypertrophy **(196)**. Increased cardiac loading is associated with concentric ventricular hypertrophy as well as ventricular dilatation and sphericulization **(5)**. Aside from gross geometric ventricular remodeling, compensatory cardiac modifications occur to transiently meet increased myocardial loading. For

example, increased myocyte mass and induction of smooth muscle cells occurs in concert with fibroblast and myofibroblast proliferation **(192)**. Cardiac fibroblasts and myofibroblasts function to secrete extracellular matrix (ECM) proteins and maintain ECM function *in vivo*. In particular, this is accomplished via tightly controlled synthesis and deposition of fibrillar collagens and other ECM components in healthy heart; in the failing heart, these cells are major contributors to cardiac matrix or ECM remodeling **(180)**. A loss of balance between extracellular matrix component deposition (mainly the fibrillar collagens, type I and III) and enzymatic degradation or removal of these proteins via collagenase action (by members of a family of matrix metalloproteinases [MMPs]) has been hypothesized to contribute to the pathogenesis of some forms of heart disease **(110, 232)**. These events are directly linked to increased wall stiffness and contribute to diastolic dysfunction while decreased cardiac myocyte intercommunication results in systolic inadequacy eventuating in decreased cardiac output **(235)**. The renin-angiotensin-aldosterone axis (RAAS) is known to play a major role in mediating cardiac matrix remodeling **(216)**. In heart, Ang II is a pleiotropic agent that modulates the proliferation of cardiac fibroblasts and may be one trigger for phenotypic transformation of these cells into myofibroblasts **(34)**. This hormone also modulates cardiac metabolism and the bioavailability of TGF-β_1 **(87, 132)**. Fibroblasts and myofibroblasts express ACE and Ang II receptors. They serve as the sole source of fibrillar collagens, which are the major components of the cardiac matrix **(216)**. Chronic suppression of Ang II

production or receptor-mediated effects are associated with improved cardiac survival in part by normalizing ECM deposition **(181, 187, 208)**.

TGF-β_1 is the prototypic member of the TGF-β superfamily that includes a number of structurally related polypeptide growth factors including the activins and bone morphogenic protein (BMP) **(249, 250)**. These growth factors possess diverse roles in multiple tissues during embyogenesis and development as well as in adult tissues **(156)**. Although TGF-β_1 governs diverse biologic effects, the immediate post-receptor signaling pathway is relatively simplistic **(250)**. TGF-β_1 signaling involves the dimerization and activation of two serine-threonine kinase membrane-spanning receptors, TGF-β receptor type I and II (TβRI and TβRII), and is initiated by ligand binding to the latter **(178)**. TβRII activates TβRI by phosphorylation, and this protein propagates the signal through a family of downstream intracellular effector proteins known as Smads. Three classes of mammalian Smad proteins have been identified; receptor-regulated Smads (R-Smads, Smad 2 and 3), a common mediator Smad (Co-Smad, Smad 4), and inhibitor Smads or anti-Smads (I-Smads, Smad 6 and 7) **(10, 93, 121)**. R-Smads are directed to the type I TGF-β receptor for activation by the kinase receptor by a hydrophobic FYVE sequence domain protein, SARA (*Smad anchor for receptor activation*) **(221)**. Following phosphorylation of specific serine residues (the SSXS motif) located at the C-terminus, an R-Smad is released from SARA and forms a dimer with Co-Smad 4 which then translocates to the nucleus **(155, 250, 259)**. Within the nucleus the cardiac R-Smad/ Co-Smad dimer recruits

transcriptional cofactors which act to regulate the activation of target genes **(11) (52)**. The multiplicity of Smad-specific signals may be conferred via Smad co-activator and co-repressor protein partners in the nucleus **(103)**. Of these, the winged-helix (WH) superfamily is well characterized and is comprised of a large number of putative transcription factors containing a unique helix-turn-helix motif **(73)**. This conformation allows WH proteins to effectively associate with target gene sequences **(29)**. FAST-1 and FAST2 are WH family member homologues found in various species. FAST-1 is known to mediate the transcriptional activity of activin in *Xenopus* embryos, and FAST2 is a recently discovered mammalian protein that mediates the TGF-β_1/Smad signal in embryonic tissues **(37, 122, 137)**. As it has been previously shown that TGF-β/ Smad signaling is involved in collagen production in PARCFs, we speculated as to the role of FAST2 in the modulation of the promoter region of the constitutively expressed gene collagen α-2 (I). It has also been observed that a FAST2 binding site (AATxxACA) exists in relative proximity to a Smad binding element (SBE) in this gene **(11, 122, 214)**. Therefore we sought to determine whether FAST2 is expressed in PARCFs and to begin investigation into its regulatory effects on the function of these cells *in vitro*.

IV. Literature Review

1. Extracellular Matrix and the Heart

The extracellular matrix (ECM) of all mammalian organ systems is comprised of a large variety of polysaccharides, proteins and proteoglycans, which form an intricate, continuous network between parenchymal cells **(50)**. The majority of these macromolecules are secreted locally by fibroblasts and their derivative cell types that are found throughout the body as well as in abundance within the myocardium **(4)**. The ECM was once thought to serve essentially only as an inert scaffolding, stabilizing tissue structure. It has been shown, however, that the vertebrate ECM plays an active role in the regulation of the behavior of those cells it contacts, influencing development, migration and proliferation **(16)**. Within the myocardium, approximately 75% of total tissue volume is made up of cardiac myocytes. This, however, only makes up about one third of the total cell number while the remaining two thirds are non-myocytes existing within the interstitium. These non-myocytes include fibroblasts, endothelial cells and vascular smooth muscle cells. Cardiac fibroblasts form the largest group among these non-myocyte cells **(1)**.

1.1 Components of the Extracellular Matrix

There are two main classes of extracellular macromolecules that make up the matrix. The first class is composed of polysaccharide chains known as glycosaminoglycans (GAGs) which are frequently linked by covalent bonds to proteins forming proteoglycans **(200)**. GAGs, proteoglycans and hyaluronic acid work to hydrate the ECM and thereby facilitate cardiac contractility and optimize the pumping action in healthy heart tissue **(95, 191)**. The second class which comprise the fibrillar proteins that impart two main functions in the interstitium i.e., adhesion (laminin and fibronectin) and tensile strength (elastin and collagen) **(4)**.

1.1.1 Laminin

Laminin is the first ECM protein to be synthesized during embryonic development. It is a large (~850 kDa) flexible complex consisting of three long polypeptide chains held together by disulfide bonds forming an asymmetric cross **(153)**. Laminin contains four or more different structural domains that allow adherence to various molecules including those for collagen type IV, heparin, entactin, and laminin receptor proteins **(220)**. In adult cardiac tissue, laminin is located along the length of the basement membrane and is concentrated in areas of morphological specialization including the sarcomeric Z bands **(118)**. Laminin is known to mediate cell adhesion, migration, growth and differentiation and plays an intrinsic role in the survival of cardiac myocytes in culture **(142, 143)**.

1.1.2 Fibronectin

Fibronectin is a large glycoprotein with multiple sub-types but is essentially a dimer composed of two subunits joined together by a disulfide bond near their carboxy-termini. Each subunit contains complex folded rod-like domains that contain binding sites for heparin, collagen and cell adhesion. Fibronectin therefore serves as a liaison for cardiac myocytes to the interstitial collagen knit **(100)**. Plasma fibronectin is a soluble form of the protein and participates in blood clotting, wound healing and phagocytosis. The remaining isoforms are all highly insoluble and assemble upon the surface of cells. These are then deposited into the ECM as fibronectin filaments. These various isoforms are, however, encoded by a single large gene containing approximately 50 exons **(4)**. The diverse array of fibronectin isoforms is obtained by the method of alternative splicing in three regions of the primary RNA transcript. The predominant isoform expressed seems to rely upon the specific cell type and developmental stage of the tissue **(198)**.

1.1.3 Elastin

Vertebrate tissues such as skin and blood vessels require strength and structure as well as elasticity in order to maintain proper function. The networks of elastic fibers allow for the resilience required to recoil after a transient stretch

and interwoven inelastic fibers prevent tearing **(43)**. Elastin is the main element of elastic fibers and is a highly hydrophobic protein. It is secreted into the extracellular space and the completed elastic fibers remain near the plasma membrane becoming highly cross-linked and form an extensive web of fibers and sheets **(43)**. The aorta and major arteries contain a large amount of elastic fibers. Not only conferring tensile strength, elastin allows for the storage of potential energy by elastic expansion of the aortic wall during the rapid ejection phase of systole. This energy storage allows for the conversion of pulsatile to constant blood flow through the systemic arterial system via the transfer of potential energy **(20)**.

1.1.4 Collagen

The collagens are a family of highly characteristic fibrous proteins found in all multicellular organisms and they are the most abundant protein found in mammals (25% of total protein mass). The representative feature of all collagens is their long, stiff, triple-stranded helical structure of each molecule. In the myocardium, collagen fibers are laid out in an intricate network consisting of the epimysium, the perimysium and the endomysium, and are continuous with chordae tendineae and valve leaflets **(188)**. Collagen is an important structural component of the heart. Studies have shown that its tensile strength, which is proportional to collagen strut thickness, alignment, configuration and location confer myocardial stiffness and maintenance of myocyte alignment on a beat-to-

beat basis **(25)**. Collagen proteins also have other roles within the myocardium. They contribute to efficient force transduction, prevention of muscle fiber slippage, protection from myocytic over stretching, and storage of potential energy during cardiac systole **(110, 237)**.

1.2 Synthesis of Collagen

Cardiac fibroblasts synthesize, then translate fibrillar collagen mRNAs on membrane bound ribosomes. These individual polypeptide chains contain a short amino-terminal peptide sequence signal and additional amino acids called propeptides at both ends. They are then directed into the lumen of the endoplasmic reticulum (ER) as large precursors called pro-α chains **(26)**. Within the ER, the nascent pro-α chains undergo hydroxylation of selected proline and lysine amino acid residues. Further modifications are made such as glycosylation of certain hydroxylated prolines that enable the subsequent spontaneous assembly of three pro-α chains into mature procollagen proteins **(4)**. The procollagen molecules are then transported to the cellular plasma membrane via a secretory vesicle and, within the extracellular space, the secreted procollagens are converted into mature collagen molecules by the removal of the propeptides by proteolytic enzymes. The characteristic triple helix structure is formed from three collagen molecules, organized individually as α-helices, wound together in a ropelike superhelix **(62)**. All collagens are proline and glycine rich, this feature being a fundamental structural requirement. The amino acid proline contains a ring

structure that acts to stabilize the helical conformation in each α-helix. Glycine, the simplest and smallest amino acid, is located in regular intervals, every third residue, along the central region of each α-chain. The significance of this arrangement of glycines is that it allows the three α-helices to coil tightly together to form the final superhelix **(32)**. This superhelix is composed of either three identical α-chains (homotrimers) or different chains (heterotrimers) with the exception of the collagen isoform IV, which is a homodimer **(179)**.

The propeptides serve two functions. The first role is to guide the procollagen to the ER previously mentioned, while the second function is to prevent these molecules from self-assembling into large intracellular collagen fibrils. This could create unfavorable conditions in the cell when these fibrils are unneeded. Collagen fibrils are composed of many collagen molecules assembled into a 10-300 nm structure. These fibrils form close to the cell surface and aggregate together creating a collagen fiber **(4)**.

1.3 Collagen Sub-types

To date there have been about 25 distinct collagen α chains identified, each encoded by a separate gene. Different combinations of these genes are expressed in different tissues **(32)**. Although the potential number of permutations of collagens reaches approximately ten thousand triple stranded collagen molecules, only 15 types have been isolated and characterized to date. The main isoforms found in heart tissues are types I, III, IV, V and VI although it is the

fibrillar collagens (types I and III) which account for 90% of the collagens found within the myocardium **(21, 179)**. Type I collagen has the tensile strength of steel and accounts for nearly 75% of the total collagen makeup in the myocardium **(33, 232)**. This type of collagen forms aggregates of relatively thick fibers and has the molecular formula $[\alpha 1(I)]_2\alpha 2(I)$ **(4)**. Type III collagen is much more distensible than type I and forms aggregates of finer fibers. Other than heart tissue, type III collagen is found in the skin and blood vessels and has a molecular formula of $[\alpha 1(III)]_3$ **(234)**. The remaining cardiac collagen isoforms (IV, V, and VI) only amount to about 10% of the total collagen complement. Collagen type IV is a basement membrane protein found in myocytes, fibroblasts and most other cardiac cell types where it plays a crucial role in cell adhesion and molecular transport **(21, 179)**. The type V collagen isoform co-exists in the basement membranes in association with type IV collagen as well as with types I and III in the interstitium **(21)**. Collagen type VI intermingles in the interstitium where it appears to coat the surface of the fibrillar collagens **(21)**.

Table 1: Collagen Sub-types and their Properties.

	Type	Molecular Formula	Polymerized Form	Distribution
Fibril-Forming (Fibrillar)	I	$[\alpha1(I)]_2\ \alpha2(I)$	fibril	Bone, skin, tendon, ligaments, cornea, internal organs
	II	$[\alpha1(II)]_3$	fibril	Cartilage, vitreous humor, notochord
	III	$[\alpha1(III)]_3$	fibril	Skin, blood vessels, internal organs
	V	$[\alpha1(V)]_2\ \alpha2(V)$	fibril (with type I)	As for type I
	XI	$\alpha1(XI)\ \alpha2(XI)\ \alpha3(XI)$	fibril (with type II)	As for type II
Fibril Associated	IX	$\alpha1(IX)\ \alpha2(IX)\ \alpha3(IX)$ with type II fibrils	lateral association	Cartilage
	XII	$[\alpha1(XII)]_3$ with some type I fibrils	lateral association	Tendon, ligaments
Network-forming	IV	$[\alpha1(IV)]_2\ \alpha2(IV)$	sheetlike network	Basal laminae
	VII	$[\alpha1(VII)]_3$	anchoring fibrils	Beneath stratified squamous epithelia

Types I, IV, and XI are each composed of more than one type of α-chains while types II, III, VII and XII are homotrimers. The above table lists only nine of the approximate 15 sub-types. (Adapted from Alberts *et al.* 1994)

1.4 Fibrillar Collagen Degradation

At birth, collagen concentrations are equal on both sides of the myocardium. As the heart ages, there is a 30% increase in collagen proteins due to the decreased workload and regression of myocyte size **(62)**. To effect these changes, the cardiac ECM must be able to remodel, and collagen turnover may episodically increase during such incidences. Thus the effective half-life of fibrillar collagens may be well below the dogma of 160 days in the diseased or remodeling heart **(86)**. Degradation of collagen fibers is an important process in normal matrix metabolism and has been shown to occur via three different pathways. Neutrophil serine proteases have been observed to degrade fibronectin, laminin and type IV collagen **(19)**. Fibroblasts contain the phagocytic machinery known to degrade fibrillar collagens and this process is enhanced and inhibited by various cytokines such as transforming growth factor-β (TGF-β) and interleukin 1α (IL-1α), respectively **(65, 227)**. However, fibrillar collagens are highly cross-linked structures typically exceedingly resistant to proteolytic degradation, therefore cleavage of collagen fibers by members of a family of matrix metalloproteinases (MMPs) is an important process in collagen turnover **(56, 129)**.

1.4.1 Matrix Metalloproteinases

The MMPs are a family of zinc dependant enzymes that possess a high affinity for the ECM component thereby playing a pivotal role in tissue remodeling processes **(55)**. All MMP isoforms share similar structural domains, the N-terminal propeptide domain, the catalytic domain, and the C-terminal domain **(210)**. The catalytic domain contains a highly conserved zinc-binding region that is essential for maintaining enzymatic activity **(210)**. The MMP family is subdivided into three groups based on their substrate preference **(35)**. The collagenases are the only sub-type that are capable of digesting the fibrillar collagens of the ECM and include MMP-1, MMP-8, and MMP-13 **(210)**. All of these collagenases, known as interstitial collagenases, have been detected throughout the left ventricular myocardium of humans **(211)**. The two other groups are the gelatinases, which include MMP-2 and MMP-9, and the stromelysins, MMP-3, MMP-10, and MMP-11 **(74, 168)**.

2. Cardiovascular Disease and Matrix Remodeling

Cardiac, or more specifically, left ventricular remodeling is characterized by structural changes involving ventricular chamber size, wall thickness, and cellular composition **(106)**. These changes are regulated by mechanical, neurohormonal, and genetic factors **(180, 190)**. Cardiac remodeling can be either physiological (adaptive) as occurs during normal growth or intensive atheletic

training, or pathological (maladaptive) due to various causes such as MI, cardiomyopathy (CMP), hypertension, and valvular heart disease **(218)**.

2.1 Cardiovascular Disease

Congestive heart failure results from diverse etiologies via multiple pathophysiological mechanisms. Regardless, the common endpoint is chamber dilatation, deficiency in contractility, and stiffening of the myocardium. The more prevalent diseases leading to CHF are valvular heart disease, hypertrophic cardiomyopathy and transmural myocardial infarction **(105)**.

2.1.1 Valvular Heart Disease

Valvular heart disease (VHD) is the common name for seven different major forms of disease affecting the various valves within the heart and the great arteries **(230)**. These classes include both valves that are stenotic and incompetent although most valves that are stenotic are also incompetent **(47)**. VHD can be caused by a multitude of factors such as congenital disease, calcification, rheumatic fever, infective endocarditis, collagen based diseases, and trauma **(27, 148, 199)**. General characteristics of these diseases are lesions, which occur mainly in males, angina, exertional dyspnea, syncope, and arrhythmia and in some cases, sudden death **(131)**. The majority of the types of valvular diseases result in an increase of left ventricular and left atrial volume. This increase in

volume leads to an increase in both left ventricular and atrial end diastolic pressure, a major inducer of hypertrophy via increase in mechanical load **(226)**. Common treatments of these diseases are valve replacement surgery, digitalis and β-blocker administration, and anti-coagulant therapy **(15, 115, 184)**.

2.1.2 Hypertrophic Cardiomyopathy

Cardiomyopathies (CMP) are a family of heart muscle disorders that affect both children and adults and can result in morbidity and premature death **(219)**. The various forms of CMPs are hypertrophic, dilated, and restrictive. Hypertrophic cardiomyopathy (HCM) is a heterogeneous disorder of clinical and genetic origin **(149)**. This disease is characterized by an increase in left ventricular mass in the absence of a secondary cause such as hypertension, as well as exhibiting myofibrillar and myocyte disarray **(150, 151)**. The area affected by hypertrophy involves mainly the ventricular septum rather than the free wall, and in 95% of cases reported hypertrophy is asymmetric (ventricular septal, apical, and mid-ventricular). This disease can also be classified based on hemodynamic assessments, being either obstructive or non-obstructive **(204)**. The clinical symptoms that present vary according to the patient's state at the time. Upon exertion, symptoms include dyspnea, angina, presyncope, syncope, and fatigue. At rest the most common indications of HCM include orthopnea, nocturnal dyspnea, and swelling of the lower extremities. Palpitations, arrhythmias and sudden death can occur at any time, although many cases are asymptomatic **(242)**.

Onset of this disease is mainly age-dependant with the majority of cases being diagnosed in adolescence or early adulthood **(150)**. Treatment of HCM is dependent on the type presented. Obstructive HCM can be treated with negative inotropic agents, myectomy, or implantation of a pacemaker. Patients suffering from non-obstructive HCM are treated with digoxin, to improve systolic function, diuretics to decrease ventricular pressure or transplantation **(149)**.

2.1.3 *Myocardial Infarction*

Myocardial infarction (MI) is defined as the death of a segment of the heart muscle following the interruption of blood flow **(236)**. This occurs within minutes of occlusion and reduces ventricular pump performance in proportion to the amount of ischemic tissue **(6)**. This loss of functioning myocardium provokes the redistribution of work load to the remaining viable heart muscle to maintain physiological cardiac output and blood flow **(175)**. Cessation of blood flow to an area of the myocardium may be due to coronary thrombosis or vasospasm or embolization of one of the coronary arteries. The coronary arteries, particularly the left coronary artery, can also be blocked by atherosclerotic plaque, confining the majority of infarction to the left ventricle. A patient suffering from an acute MI presents such symptoms as sudden severe chest pain radiating to the arm and throat, arrhythmias, dyspnea, syncope, and either high or low blood pressure **(17)**. MI involving coronary stenosis due to the presence of atherosclerotic plaques usually occurs in men aged 40 and above and the risk in women following

menopause is equalized to the risk found in men **(17)**. MI of this nature can also occur at a younger age due to genetic predisposition. Patients suffering from MI with normal coronary arteries are usually younger than those with coronary artery disease and are cigarette smokers or cocaine users **(223).** Standard therapy is administration of thrombolytics, aspirin, nitrates, and β-blockers **(163, 203)**.

2.2 Extracellular Matrix Remodeling

The process of ventricular remodeling is not well understood. It is defined as a repair process after an ischemic, toxic or inflammatory event in the heart or following hemodynamic overload, commonly resulting in fibrosis **(147)**. This change in shape and composition is thought to compensate in acute or chronic alterations from normal demand on the myocardium **(147)**. The term "slippage of myofilaments" has been coined referring to the destruction of connective tissue and may represent the initial remodeling event. Net removal or shuffling of matrix components is known to diminish the functional capacity of the heart **(161)**. Myofibrillar slippage will result in reduced systolic performance leading to increased diastolic stiffness as a consequence of the changes of interstitial cell matrix composition. Cells of the myocardium also endure alterations such as hypertrophy or hyperplasia and may undergo a process known as programmed cell death or apoptosis **(76)**. At a subcellular level, gene expression "switches" to a fetal phenotype in cardiomyocytes evidenced by elaboration of atrial natriuretic peptide and skeletal α-actin. In cardiac fibroblasts fibrillar collagen is synthesized

at abnormal levels **(53)**. Cardiovascular remodeling is affected by various factors including mechanical, neurohormonal and genetic influences.

2.2.1 Mechanical Factors

The heart is constantly experiencing mechanical disturbances including shear and tensile stresses. During embryonic development, cells of the cardiovascular system respond to these mechanical forces in a positive manner and it is used as a stimulus for synthesis of ECM via cardiac fibroblasts. The adult myocardium is also exposed to constant mechanical forces. Cyclical stresses during the normal cardiac cycle do not create pathology in the myocardium, however, changes in either blood pressure or volume will stimulate cardiac remodeling **(228)**. Therefore, it has been suggested that the mechanical environment of the heart is a key modulator of cellular function. ECM remodeling occurs in blood vessels and in the heart in response to increased mechanical load originating from such causes as elevated blood pressure or decreased cardiac output. There is strong evidence supporting the idea that increased mechanical forces have a direct effect on myocyte hypertrophy, however, the regulation of collagen deposition appears to involve additional players **(22)**. Studies have shown that a change in mechanical load in the heart activates the release of pro-fibrotic growth factors such as transforming growth factor-β **(228)** and angiotensin II **(234)**. These factors play a significant role in the increased production of total protein and fibrillar collagens type I and III **(23)**. Volume and

pressure overload will stimulate collagen synthesis, however, in the case of volume overload or the early phases of pressure overload, collagen deposition is matched by myocyte hypertrophy. Remodeling in this manner results in an enlarged ventricle with a normal collagen composition. With chronic pressure overload the myocardium remodels differently as collagen synthesis exceeds the hypertrophic response resulting in fibrosis **(22)**.

2.2.2 Genetic Factors

Members of the mitogen activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK) and c-*jun* NH2-terminal kinase (JNK) play key roles in the regulation of cell growth, apoptosis and gene expression **(44)**. Although the role of MAPKs in cell growth regulation is crucial, its mechanism remains poorly understood. Recent studies of the activities of these kinases in cardiac hypertrophic or hypertensive rats have shown that JNK activity is chronically enhanced. This induction of JNK activity is followed by the upregulation of activator protein-1 (AP-1) activity. AP-1, a heterodimer of c-Fos and c-Jun, will then bind to a specific sequence in target promoters **(114)**. Presently, data have shown that AP-1 transcriptional activation is mediated by TGF-β and involves an interaction with another important family of transcription factors important in cardiac remodeling, the Smad proteins **(134)**. As ERK and JNK activity are elevated in hypertensive rats when compared to normotensive

age-matched controls, these MAPKs may be a useful target for controlling cardiac remodeling **(114)**.

Another genetic factor involved in cardiac remodeling is apoptosis, or programmed cell death **(171)**. The process of apoptosis involves discrete genetic and molecular programs, *de novo* protein synthesis, and expression of a unique cellular phenotype. Certain myocytes within the myocardium undergo apoptosis following such traumas as acute MI, and ischemic and non-ischemic heart failure **(68)**. Programmed cell death is potently stimulated by oxygen radicals, cytokines (FAS/ TNFα receptor signaling), stress conditions (chemical and physical) and hormones (Ang II) **(68)**. Apoptosis of ventricular myocytes will result in progressive pump failure, arrhythmias, and remodeling. It is thought that MAPKs are a potential target of preventative therapies **(67)**.

2.2.3 Neurohormonal Factors

Ventricular remodeling involves the activation of both the adrenergic and RAAS systems following increased wall stress. Studies indicate that myocardial contractility is vastly decreased during CHF, partially due to alterations in β–adrenergic receptor-mediated signal transduction **(197)**. These changes in signaling result in a decreased production of cyclic AMP (cAMP) which, in turn, decreases intracellular calcium concentration. The reduction of intracellular calcium contributes to the attenuation of contractile force **(31)**. In the failing

heart, mRNA message levels for both β-1 and β-2 receptors are decreased as well as protein expression levels. However, α-1 receptor density remains unchanged or is slightly increased during heart failure and has been associated with myocyte growth and cardiac hypertrophy **(141)**.

Considerable effort has been focused on the humoral mechanisms responsible for transducing the increased hemodynamic load in cases of CHF into adaptive cardiac remodeling. Accumulating evidence indicates that the RAAS signaling system and its primary effector peptide, angiotensin II, are powerful contributors of cardiac remodeling **(57)**. Reparative fibrosis, which is secondary to myocyte necrosis and reactive fibrosis contributes to this remodeling and Ang II appears to play a key role in regulating this pathology **(234)**. In the circulating RAAS, the liver derived precursor, angiotensinogen (A_0) is cleaved by the kidney derived protein renin to form angiotensin I. Angiotensin I is converted to the biologically active product, Ang II, by either angiotensin converting enzyme (ACE), cathepsin G or Tonin **(189)**. Ang II is a potent vasoconstrictor and promotes the production of aldosterone, an important player in renal function. In addition to this systematic Ang II production, a local RAAS system exists in the cardiovascular system **(46)**. Although the components of the RAAS are localized in cardiac cells, this system cannot function without the final effector protein, the Ang II receptor. In human and rat myocardium, Ang II receptors have been identified in human and rat atria, ventricles, myocytes and cardiac fibroblasts **(57)**. Biochemical and pharmacological studies have characterized two Ang II receptor subgroups, angiotensin type I (AT_1) and angiotensin type II (AT_2) **(246)**.

The AT_1 receptor is a seven transmembrane domain protein and is further subdivided into AT_{1A} and AT_{1B} classes **(81)**. These Ang II receptor isoforms differ by 18 to 22 amino acids yet maintain similar binding profiles for Ang II ligand and receptor antagonists such as losartan **(81, 83)**. The effect of short-term AT_1 receptor blockade has been previously studied and this therapy has been associated with the normalization of collagen turnover in hearts affected by either MI or cardiomyopathy **(54)**. Thus, antagonism of the AT_1 receptor is another method of reducing or reversing remodeling.

Until recently, it was generally accepted that Ang II signaling was mediated mainly through the AT_1 receptors in the myocardium. Ironically, it had been observed that increased mechanical load causes the upregulation of both AT_1 and AT_2 mRNA and protein expression **(113)**. New evidence however, has demonstrated that the re-expressed AT_2 receptor, located on cardiac fibroblast surfaces, exert an inhibitory effect on Ang II-induced mitogen signals **(222)** thus introducing a novel mode of remodeling control.

Data obtained from several different cell types including cardiac fibroblasts have suggested that specific growth factors, primarily TGF-β_1 and platelet derived growth factor (PDGF), may indirectly mediate the stimulatory effects of Ang II **(116, 228, 234)**. In addition to increasing TGF-β_1 secretion, Ang II may play a role in stimulating the conversion of latent TGF-β_1 propeptide into its active form **(34)**. Also, simultaneous treatment with Ang II and a TGF-β_1 neutralizing peptide in cultured cardiac fibroblasts reduces collagen type I and III mRNA expression. Together these data suggest that Ang II may act upon

fibroblasts indirectly through an autocrine/ paracrine loop via TGF-β_1 to induce fibrotic protein synthesis **(116, 176)**.

2.3 The TGF-β / Smad Signaling Pathway

Two novel concepts or paradigms are emerging to explain the mechanism by which cells process cytokine signaling. First, it is becoming clear that cytokine signaling pathways are not discrete units but pieces of a complex network, acting through multiple cross-talk and feedback interactions. Secondly, although it has long been clear that a specific response to a pleuripotent factor is dependent on cell type, it is now becoming evident that this variable response may rely upon the specific expression of a specific subset of transcription cofactors within a given cell type **(157)**. Thus cofactor specificity confers a characteristic cellular response, wherein that response is variable.

The TGF-β superfamily of cytokines consists of many structurally related peptides that function as either growth or differentiation factors. Members of the TGF-β superfamily are produced by numerous cell types and include the TGF-βs, the bone morphogenic proteins (BMP), the activins, and anti-Müllerian hormones **(154)**. Each member of this family is capable of regulating a vast array of cellular processes including cell proliferation, lineage determination, motility, adhesion and cell death **(156)**. Several of these modes of regulation stem from changes in the expression of key target genes; hence, understanding transcriptional control via the TGF-β family is of great importance **(158)**.

All members of the TGF-β superfamily are synthesized as latent precursors containing a C-terminal domain that is cleaved upon secretion from the cell **(51)**. This propeptide, known as the latency associated peptide (LAP), remains covalently bound to the secreted ligand and keeps it in a latent form by obstructing its binding to receptors **(75)**. A large glycoprotein is the third component of the latent TGF-β complex. Latent TGF-β-binding protein (LTBP) is linked by a disulfide bond to LAP and while not essential for latency, plays a role in secretion and storage in the ECM, and for the eventual activation of the ligand **(164)**.

2.3.1 TGF-β Isoforms and Function

Mature TGF-β proteins are 25 kDa homodimers, with each monomer consisting of 112 amino acids, 9 of which are cysteine residues **(51, 154)**. The three main isoforms are called TGF-β_1, -β_2 and -β_3. However, in rare cases TGF-β heterodimers of β_1/β_2 and β_2/β_3 have been identified **(12)**. All the TGF-β isoforms contain 72% to 80% sequence conservation, with the N-terminal region being the most divergent **(130)**. TGF-β_1, which has been isolated in both cardiac myocytes and fibroblasts, has been implicated in numerous fibrotic disorders such as cirrhosis, glomerulonephritis, fibrosis of the lung and vascular restenosis **(24, 60, 64)**. Increased expression of TGF-β_1 was observed using ELISA and Northern blot techniques within the bordering and scar regions of the myocardium following MI, suggesting a role for this cytokine in post-MI repair **(86)**.

2.3.2 TGF-β Receptors and Ligand Interaction

TGF-β and its related factors signal through the transmembrane serine/threonine receptors referred to as the TGF-β receptor family. The members of this family are divided into two sub-populations, type I (TβR-I) and type II (TβR-II) receptors, based on their structural and functional properties **(156)**. However, the overall structure of all these receptors are the same and include an N-terminal signal sequence, a short cysteine-rich extracellular region, a single hydrophobic membrane spanning domain, and a kinase containing cytoplasmic region **(159)**.

The relatively short (~150 amino acids) extracellular domain of these receptors contains 10 or more cysteine residues that determine the exterior folding pattern. Three of these residues are characteristically clustered near the transmembrane domain while spacing varies for the others **(251)**. Unlike the other domains, the transmembrane domain and the cytoplasmic juxtamembrane region themselves bear no singular structural feature. However this region of TβR-II (Ser 213) is phosphorylated by a kinase in a ligand-independent manner and is required for signal propagation **(144)**. TβR-II phosphorylates the Ser165 in the juxtamembrane region of TβR-I in a ligand-dependent fashion. It is the latter phosphorylation event that appears to selectively modulate the intensity of the different TGF-β responses **(209)**.

A highly conserved 30 amino acid region immediately preceding the protein kinase domain is a feature unique to the TGF-β type I receptors. It is termed the "GS region" as it contains characteristically an "SGSGSG" domain **(109)**. Directly after this sequence lies a leucine-proline motif found on all members of the type I receptor family. This motif serves as a binding site for the immunophilin FKBP12 which may serve as a negative regulator of receptor signal function **(36, 40)**. It has been shown that mutation of the amino acid in the second to last position in the GS domain, which is always a glutamine or threonine, elevates receptor activity *in vitro*, allowing constitutive signal activity **(241)**. Therefore the GS domain is a key regulatory region which controls catalytic activity of the type I receptor kinase or substrate interaction **(156)**.

Both type I and type II TGF-β receptors contain a serine-threonine kinase domain **(159)**. Type I receptors have been observed to phosphorylate their only known substrates, the Smad proteins, on their serine residues while type II receptors phosphorylate themselves on serine and threonine moieties **(146)**. Another difference between the receptor subtypes is that only the TβR-II contains a short C-terminal extension following the kinase domain. This extension may be phosphorylated, however, its deletion does not impair signaling **(240)**. This is in vast contrast to the significant role the C-terminal extension plays in tyrosine kinase signal transduction **(225)**.

TGF-β activates signaling by binding to and causing dimerization of TβR-I and TβR-II receptors. There are two general modes by which ligand binding have been observed: sequential binding and cooperative binding **(156)**.

Sequential binding involves TGF-β binding first to TβR-II and this complex then associating with TβR-I. The type I receptors can recognize the difference between free and bound ligand, complexing only with receptor bound ligand **(8, 58)**. The second binding mode is not typical of TGF-β binding but that of BMP and its receptors. These receptors bind with high affinity to the BMP ligand when expressed together but weakly when expressed separately **(140)**.

2.3.3 Smad Proteins as Mediators of TGF-β Signaling

According to the current dogma, the Smad family of proteins are substrates for the transduction of TGF-β receptor signaling. These proteins play a crucial role in the propagation of TGF-β receptor signals to target genes within the cellular nucleus **(156)**. The first member of the Smad family was identified in *Drosophila* as the gene product of *Mad (mothers against decapentaplegic)* **(202)**. Its discovery led to the identification of related genes in nematodes and vertebrates. Three homologous proteins were identified in *C. elegans* and were named Sma-2, Sma-3 and Sma-4 as their mutation caused a small body size **(194)**. Following this discovery, many related genes were also identified in humans and were aptly named Smads meaning Sma/Mad related **(85)**. Evidence has been accumulating to show that Smad proteins are the downstream effectors of the TGF-β receptors **(133, 138)**.

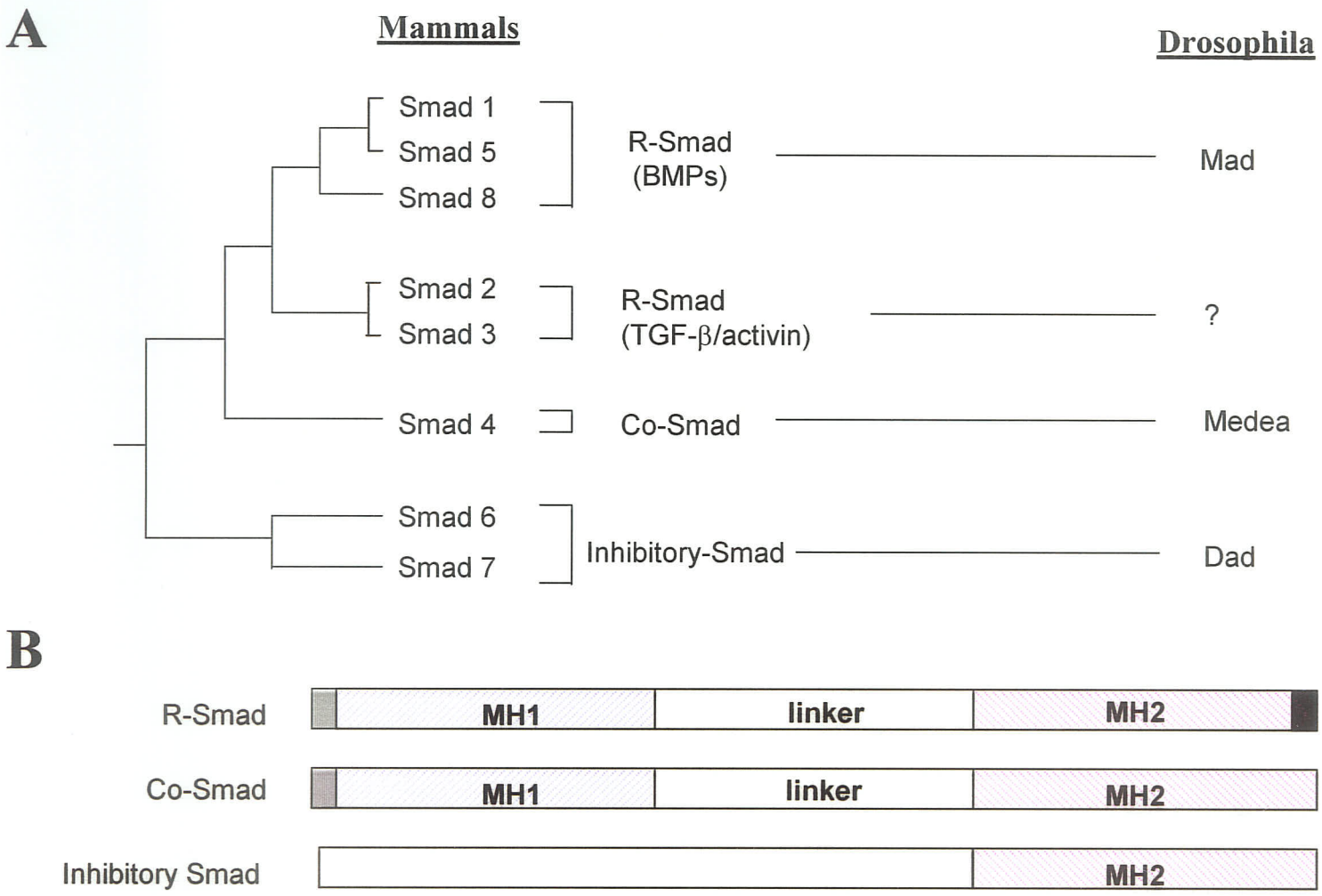

Figure 1. **A**. Evolution of the Smad protein family based on their structure and function **B**. Schematic representation of the three basic Smads, highlighting differences in phosphorylation sites and MH1/MH2 linker placement

2.3.4 Structure of Smads: MH-1 and MH-2 domains

Smad proteins associated with TGF-β signaling are divided into three distinct subclasses (Fig. 1A), the pathway specific or receptor-mediated Smads (R-Smads, Smad 2 and 3), the common mediator Smads (Co-Smads, Smad 4) and the inhibitory Smads (I-Smads, Smad 6 and 7) **(156)**. The Smad family of proteins contain two highly conserved regions at their N- and C-terminal that are referred to as Mad homology region 1 (MH-1) and MH-2, respectively (Fig. 1B). These regions are linked together by a non-conserved "linker" region of variable length **(185)**. The MH-1 domain consists of approximately 130 amino acid residues and is only highly conserved in R- and Co-Smads but not the inhibitory Smads **(156)**. The MH-1 domain of an R-Smad protein contains a β-hairpin loop which is responsible for DNA recognition upon activation **(138, 205)**. The MH-1 domain is also known to inhibit MH-2 binding to TβR-I **(90)**. The MH-2 domain is approximately 200 residues in length and is responsible for Smad/receptor interaction, R-Smad/Co-Smad interaction, DNA binding protein interaction and transcriptional activation (Fig. 2) **(156)**. These functions are conferred by the presence of receptor phosphorylation sites required for R-Smad activation (i.e., Co-Smad binding and subsequent translocation) **(120, 146)**. Overexpression of the MH-1 domain has been demonstrated to inhibit R-Smad/Co-Smad association **(89)**. Specificity of receptor binding is conferred by a sequence of amino acids located within the L3 loop of the MH2 domain **(39)**. The highly variable (i.e. not conserved) MH-1/MH-2 linker region is purposeful mainly in the R-Smad family.

Its domain contains MAPK phosphorylation sites, which when activated by MAPK signaling, inhibit Smad nuclear translocation **(119, 255)**.

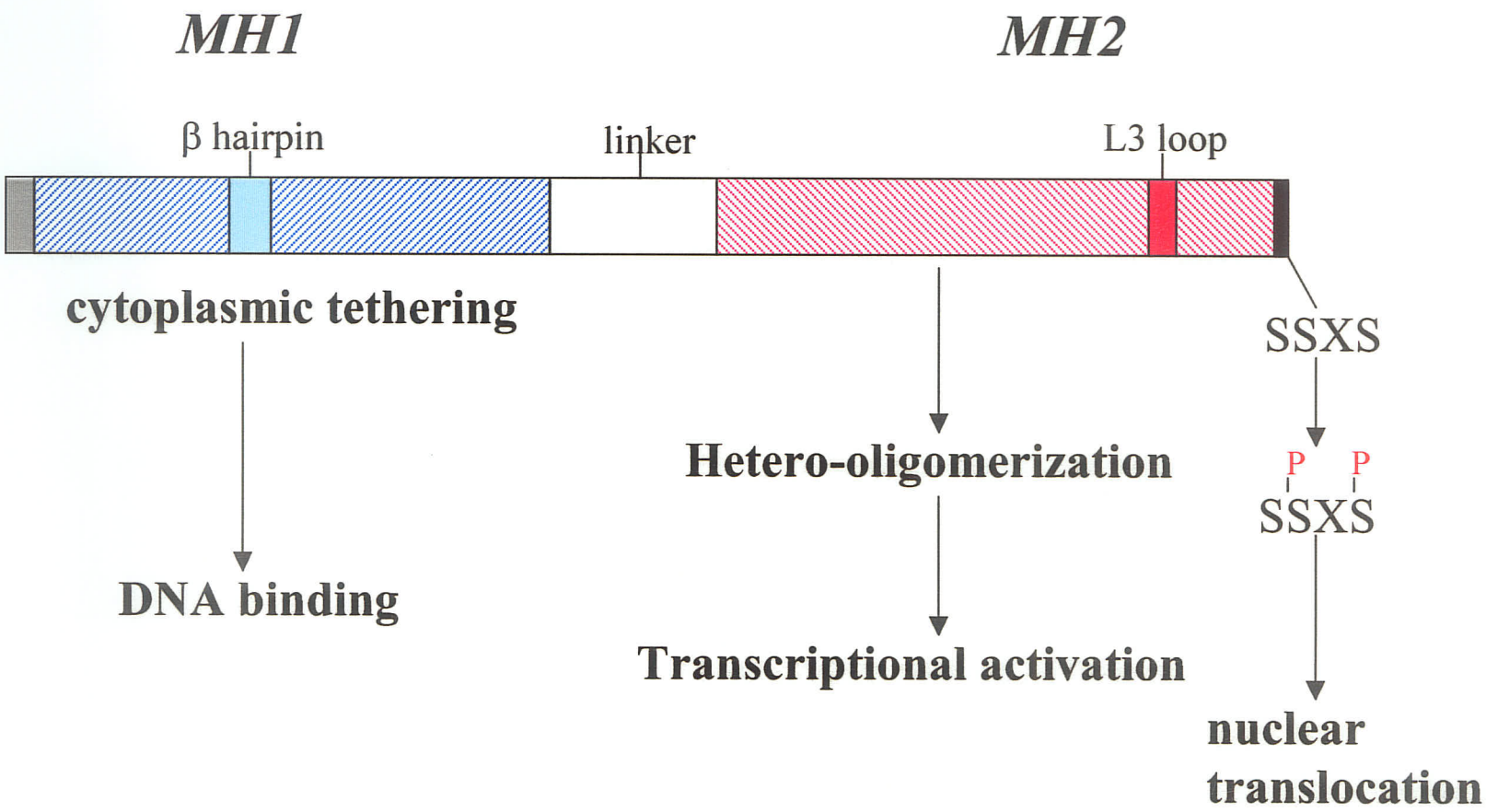

Figure 2. Schematic representation of R-Smad 2 protein structure. Regions within the protein that participate in DNA/protein interaction are labelled.

2.3.5 The Smad Protein Signaling Pathway

R-Smads are cytoplasmic proteins that become activated upon association with the C-terminal kinase domain of the type I receptors **(120)**. These Smads contain a highly conserved "SSXS" motif at their carboxy-termini with the last two serine residues becoming phosphorylated upon activation **(120, 146)**. Blocking R-Smad phosphorylation prevents two subsequent signal-dependent actions, association with Co-Smads and translocation to the nucleus **(244)**. The protein SARA contains a lipid-binding FYVE domain and functions to recruit unphosphorylated R-Smads to the transmembrane spanning TGF-β receptors **(221)**. Association of R-Smads with the type I receptor is transient and upon phosphorylation of the two C-terminal serine residues the R-Smad protein is released from both the TGF-β receptor and SARA protein **(146, 170, 221)**.

Following its release from the type I receptor, R-Smads form a heterodimer with the common mediator Smad, Smad 4 **(244)**. The Smad 4 chaperone protein, TGF-β receptor associated protein 1 (TRAP1), mediates the association of Smad 2 and Smad 4 **(252)**. TRAP1 binds to inactive TβR-II and dissociates upon receptor activation, altering its conformation to form a transient complex with Smad 4. This TRAP1/ Smad 4 complex is observed only in the absence of Smad 2 suggesting that it is a transient complex visualized only in the absence of an acceptor R-Smad **(252)**. Upon signal activation, cytosolic R-Smads form heterodimers with Co-Smads and regulated translocation of this complex to the cellular nucleus is a key event leading to the induction of target gene

transcription **(96, 138, 185)**. The Co-Smads are structurally similar to R-Smads but do not contain the SSXS motif at their C-terminal end as their R-Smad counterparts (Fig. 1). Lacking this SSXS-carboxy region results in the inability to associate with type I receptors, implicating a novel function for this Smad subtype **(146, 170, 259)**. To date, Smad 4 is the only known vertebrate common-mediator protein **(9, 48)**.

Translocation is recognized as a receptor activation dependent phenomenon, as mutations within the sites for type I receptor phosphorylation prevent this event **(244)**. It has also been observed that although phosphorylation triggers both Co-Smad association and translocation, dimerization with a Co-Smad is not required for R-Smad nuclear entry **(244)**. How these Smad proteins propagate the signal to target genes is still relatively unclear. Smad 2, in contrast to Smad 3, contains an extra amino acid sequence at its N-terminal domain that makes it unable to bind to DNA with great affinity. In such cases it is Smad 4 that is responsible for DNA interaction **(122)**. This, along with the recent discovery of various response elements within target genes, suggests that transcriptional co-factors are required for competent gene transcriptional regulation **(37)**.

The third subclass of Smad proteins, the inhibitory Smads, contain the MH2 domain of the other Smad types, but similar to Co-Smads lack the SSXS motif. The I-Smads are unique, however, as they do not share any homology in their N-terminals (Fig.1) **(102, 169)**. Similar to R-Smads, Smad 6 and 7 directly interact with the type I receptor. Since these Smads do not contain the C-terminal phosphorylation sites, the association with the receptor is more stable and thus

impedes signal transduction by competitive inhibition with R-Smads **(91, 102, 169)**. Smad 7 is localized within the nucleus prior to TGF-β stimulation. Translocation to the cytosol is observed following this stimulation **(104)**. Western analysis of the cytosolic fraction of adult rat primary cardiac fibroblasts following TGF-β_1 stimulation indicate an increase in Smad 7 expression after 60 minutes and a return to basal levels at 120 minutes **(231)**. These proteins may therefore act as an auto-regulatory negative feedback system in TGF-β signal transduction. The two vertebrate I-Smads function as inhibitors differently. Smad 7 is a general inhibitor of R-Smad activation whereas Smad 6 selectively inhibits Smad 2 but not Smad 3 **(102, 169)**.

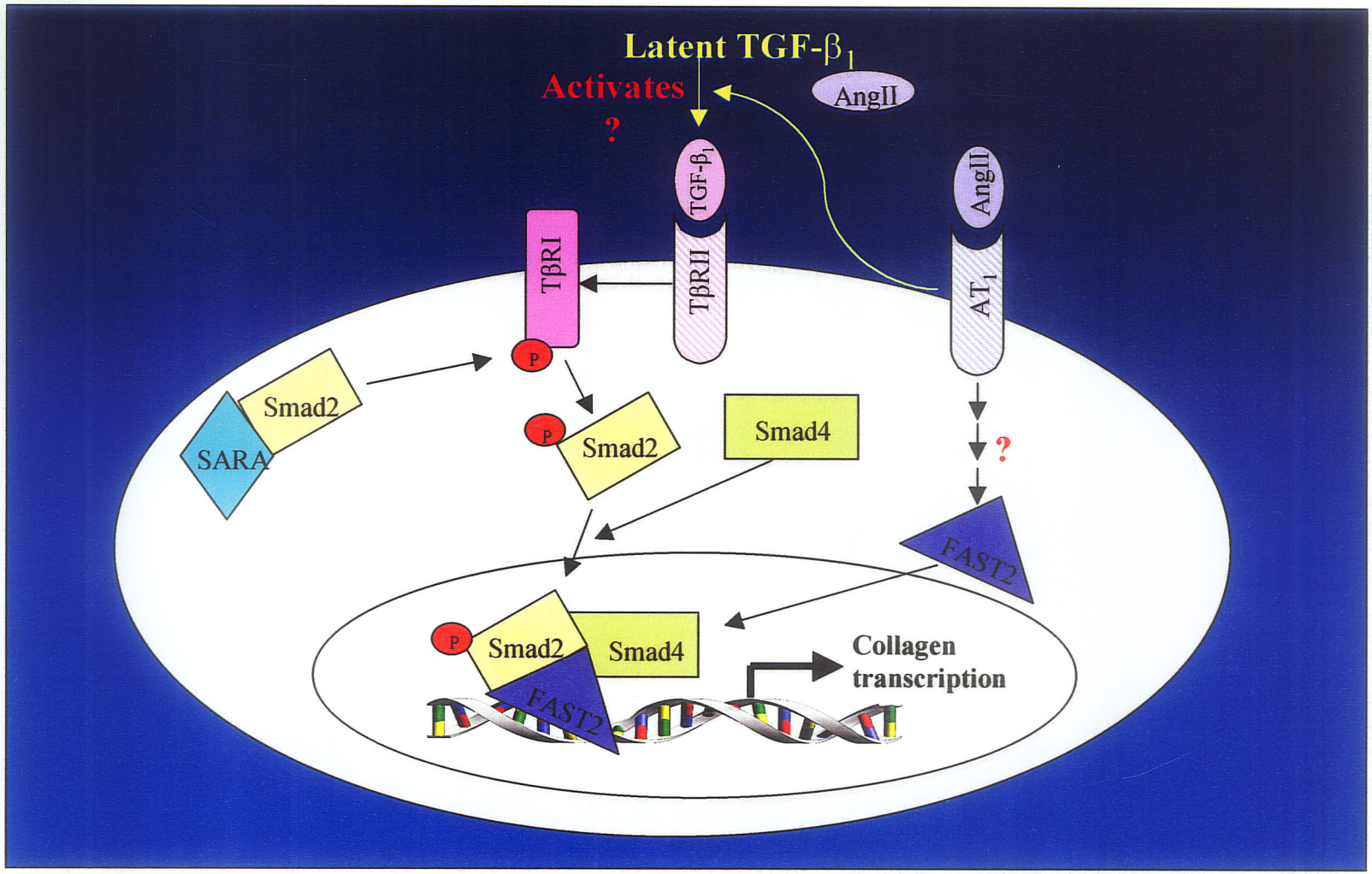

Figure 3: Schematic representation of the putative TGF-β/Smad signaling pathway. FAST2 has been shown be to rapidly translocate into the nucleus after angiotensin II stimulation. The precise pathway regulating this phenomenon is undefined.

2.3.6 Other TGF-β Downstream Factors

In addition to Smad proteins, G proteins and MAPKs have been thought to function as downstream effectors of the TGF-β signaling pathway **(229)**. There are several classes of G proteins including the small G proteins and the heterotrimeric signal transducing proteins **(28, 79)**. These latter G proteins, G_s and G_i modulate the activity of adenylyl cyclase and G protein function may be modified by bacterial exotoxins **(79)**. These toxins can also affect some of the biological activities of TGF-β **(97)**. Experiments have shown that stable transfection of $G_{i\alpha 1}$ into NIH 3T3 fibroblasts, a cell line exhibiting little responsiveness to TGF-β, restores TGF-β responsiveness to control levels **(111)**. Many studies have shown that the small G proteins (Ras, Rac, Rho and Cdc42) play a central role in the downstream signaling pathway of receptors, such as angiotensin type I receptor (AT_1 receptor). The association of the small G protein, Ras, and tyrosine receptors requires protein intermediates such as Grb2 **(160)**. Other proteins are required to activate the membrane bound Ras which leads to the activation of members of the MAPK family (ERKs, p38-MAPK and JNKs) **(59, 160)**.

Small G proteins and the MAPK cascade are also involved in serine/threonine kinase receptor signaling. This was demonstrated using a TGF-β sensitive cell line and showing small G proteins are activated by TGF-β **(165)**. TGF-β has been shown to activate all three of the MAPK cascades **(71)**. Collectively these results indicate that signal transduction by the TGF-β family

can be influenced by the small G proteins and the MAPKs **(71)**. Smad proteins are also known to interact with the MAPK cascade **(7)**. Whether or not the integration of actions between the Smad and MAPK proteins are a general feature in TGF-β signaling remain unknown. However, it has been suggested that the specificity of ligand response is determined not only by activated TβRI/TβRII complexes but also by the combination of downstream effectors present in the cell. This is a concept that is supported by evidence for cross-talk between the AngII/ TGF-β signaling pathways **(87, 229)**.

Another mode of transmodulation between TGF-β and the membrane receptor for INF-γ is thought to occur but its mechanism is unknown **(212)**. INF-γ signals through the INF-γ receptor and the associated protein tyrosine kinase Jak1. Jak1 then mediates the phosphorylation and activation of the transcription factor Stat1 **(101, 195, 212)**. Activation of Stat1 is thought to induce the expression of Smad 7, thus preventing the interaction of R-Smads with the TGF-β type I receptor **(91, 169, 224)**.

3. Transcriptional Co-Factors

Eukaryotic transcription is highly regulated by two classes of transcription factors. General transcription factors (GTFs) bind to an unspecific core promoter sequence in close proximity to the transcription initiation site. Together with an RNA polymerase they form the pre-initiation complex **(82)**. The second class includes sequence-specific transcription factors that bind to regulatory DNA

elements located at various distances upstream from the transcription start site. These DNA regulatory elements contain a specific DNA sequence necessary for transcription factor binding. Factors bound to this region will interact with other downstream factors to either activate or inhibit gene transcription **(166)**. Both of these classes of transcription factors contact RNA polymerases, either directly or via mediators in an initiation complex **(45)**.

3.1 DNA Binding Motifs

DNA-protein interactions are highly specific and among the tightest molecular interactions found in nature **(88)**. X-ray crystallography and NMR spectroscopy techniques of approximately 20 gene regulatory proteins found to complex with DNA were used to examine the structure of these proteins. It was discovered that these proteins contain one of a small subset of structural motifs and interact with the major groove of DNA using either α-helices or β-sheets **(177)**.

3.1.1 The Helix-Turn-Helix Motif

The helix-turn helix (HTH) was the first DNA binding motif to be discovered and has been found to be the most common form **(167)**. This motif is constructed of two α-helices linked together connected by a short sequence of amino acids making up the "turn" of this structure **(89)**. The two helices are

maintained at a fixed angle, via interactions of the helices, to allow for accurate interaction with the DNA binding sites. Interaction with DNA by this type of transcription factor occurs through the carboxy-terminal helix known as the *'recognition'* helix and the varying amino acid side chains of each protein **(128)**. The remaining protein structure varies greatly allowing each protein to present itself to the major groove uniquely. The polypeptide outside of the motif also helps to fine tune the protein/DNA interaction **(193)**.

Homeodomain transcription factors are a separate class of HTHs. It was noted that several sequences of homeotic selector genes contained a conserved 60 amino acid residue sequence known as the homeodomain **(201)**. The structure of a homeodomain protein bound to a specific DNA sequence differs from the previously described HTHs. The HTH motif of homeodomain proteins is always surrounded by the same structure and therefore will present itself to the DNA sequence in the same fashion **(253)**.

3.1.2 The Zinc Finger Motif

The zinc finger motif is structurally more complex than the HTH, which is comprised solely of amino acid residues. This second class of binding motif utilizes one or more atoms of zinc as a structural component, lending to its name **(4)**. Previously, due to a schematic depiction of these proteins, these motifs were known as zinc fingers. Subsequent structural analyses have shown that these proteins are structurally different and can be placed into various classes **(125)**.

One subtype of zinc finger is a relatively simple structure consisting of an α-helix and a β-sheet that is held together by a zinc molecule. These proteins are usually found clustered together in a manner that does not allow the α-helices to contact the major groove. This long repeated series of zinc fingers allows for strong and specific DNA-protein interaction **(186)**.

Another class of zinc finger is found to be a member of a large family of intracellular receptor proteins forming a structure similar to the HTH motif. This structure consists of two α-helices held together by two zinc molecules. This subclass of zinc fingers form dimers that allow one of the two α-helices of each subunit to interact with the DNA major groove. It is good to note that both of these zinc finger subtypes use zinc as a structural element and that both use an α-helix to recognize DNA **(126)**.

3.1.3 The Leucine Zipper Motif

Many of the regulatory proteins function as dimers as it is a simple way to achieve strong and specific interactions with DNA. Often, these regulatory proteins are segmented into two areas, one that is responsible for dimerization and one that makes contact with DNA. A third separate motif known as the leucine zipper combines both of these functions **(127)**. The leucine zipper is composed of two HTH α-helix monomers joined together to form a short coiled-coil. These helices are held together by hydrophobic interactions of amino acid side chains which often contain leucine residues **(173)**. Just past the dimerization interface,

the two α-helices separate forming a Y shaped structure that contacts DNA in a clothespin-like manner.

Leucine zippers can either form homodimers or heterodimers. Heterodimers usually form between two proteins that possess distinct DNA-binding specificities. This vastly increases the number of DNA-binding sequences that these proteins can recognize, an example of *'combinational control'*. Combinational control refers to the process by which combinations of proteins, rather than individual proteins, control cellular processes **(126)**. As there are many types of leucine zipper proteins it is thought that the majority form homodimers, otherwise the amount of crosstalk possible between the gene regulatory circuits could create havoc in the cell **(162)**.

3.1.4 The Winged Helix Motif

As previously described, transcription factors are usually classified according to the structural motif involved in DNA binding or, in some cases, the oligomerization domain. Within the last ten years, a structurally unique motif was identified in the *Drosophila* fork head gene product and in rat hepatocyte nuclear factor 3 (HNF3) **(112)**. The *Drosophila* fork head gene is responsible for proper terminal structure formation during embryonic development. *In situ* studies revealed the early presence of both forkhead mRNA and protein in endodermal tissues, the protein being localized mainly within the nucleus. This nuclear localization suggested that it might function as a transcriptional regulator **(239)**.

Following the discovery of forkhead, a small family of hepatocyte enriched DNA binding transcription factors were identified in rodents, the HNF-3 family **(123, 124)**. Upon inspection of the HNF-3 110 amino acid DNA binding domain it was noted that the sequence did not bear any resemblance to any known transcription factor motifs **(124)**. When compared to the *Drosophila* forkhead protein DNA binding sequence, it was discovered that there was a high degree of sequence homology. Thus, forkhead and HNF-3 are considered to be the founding members of the family of transcription factors, for structural reasons, named winged helix proteins (WH) **(123, 238)**. Members of the WH family are expressed throughout a wide range of tissues during various stages of development and act to control the regulation of cell determination, cell proliferation and differentiation **(243, 254)**.

The topological structure of the winged helix motif is a compact α/β structure consisting of two wings, (W1 and W2), three α-helices (H1, H2 and H3) and three β-sheets (S1, S2, and S3) **(42)**. The N-terminal domain contains a largely helical structure while the C-terminal domain is composed of two of the three β-strands arranged in a twisted anti-parallel β-sheet motif and the two characteristic loops or "wings", W1 and W2 **(152)**. WH proteins are members of the HTH superfamily but they differ in the length of the "turn" connecting helices H2 and H3. This structural variation eliminates stereochemical-binding restrictions found in canonical HTH proteins. The angle between the H2 and H3 helix ranges in typical WH proteins from 100° in the biotin operator repressor protein BirA, to 150° in the transcription factor DP2. In contrast, 120° is the typical angle found in the HTH proteins **(123)**. The majority of WH proteins

present the H3 helix, known as the recognition helix, to the major groove of target DNA sequences. Fourteen protein-DNA contacts occur, five within the length of the recognition helix, four involving the wing W2 and the minor groove of DNA, and five others distributed throughout the length of the polypeptide chain **(73)**. Binding of this transcription factor causes a deformation in the DNA structure, inducing a 13° bend by narrowing the major groove to allow it to embrace the H3 helix **(108)**. Frequently WH proteins exhibit a length of rosette-like hydrophobic residue side-chains. These side-chains are thought to mediate protein-protein interactions in conjunction with DNA binding **(73)**.

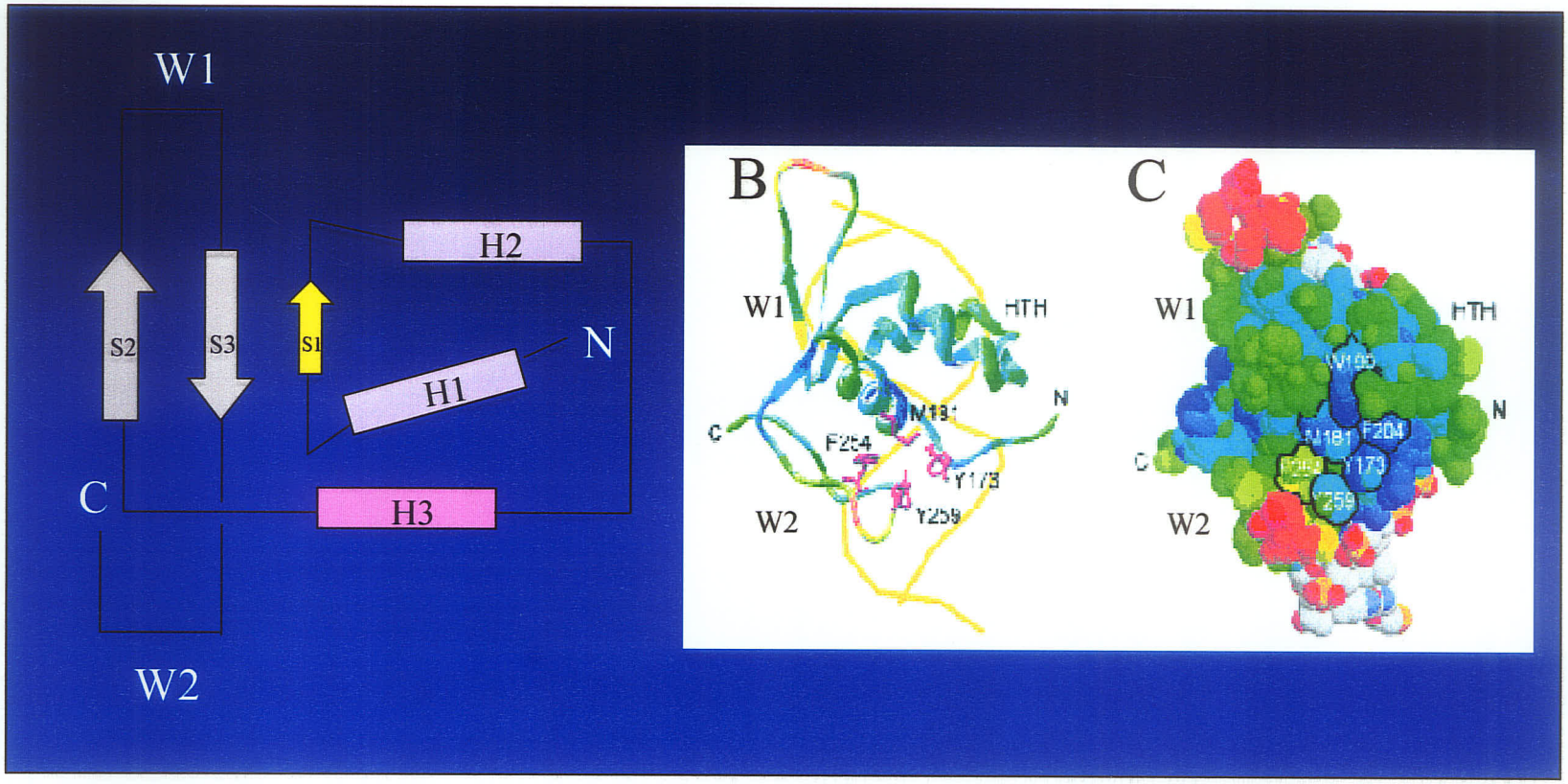

Figure 4. Graphic depiction of a winged-helix protein. **A.** The structure is composed of three α-helices (H1-H3), three β-structures (S1-S3) and two wings (W1-W2). The H3 helix is considered to be the DNA recognition motif. **B.** Backbone model; DNA strands are yellow; side chains are shown in magenta for selected surface hydrophobic residues. **C.** Space-fill model. (Adapted from Stevens *et al.* J. Biol. Chem. 2000.)

3.2 Smad Transcriptional Co-Activators and Co-Repressors

Although both R-Smads and Co-Smads can bind to the promoter regions of various genes at a specific Smad binding element (SBE), regulation of transcription usually occurs via a partnership with additional proteins **(258)**. Many studies have been performed to identify how these Smad binding proteins modulate the TGF-β signal. Positive regulators of the TGF-β pathway signal include both upstream accessory regulators such as SARA, and downstream effectors that function as either general or tissue-specific transcriptional regulators **(262)**. In addition to positive regulators there have been numerous proteins identified that will attenuate TGF-β signaling by interfering with Smad function, for example anti-Smads. These proteins are required for the prevention of inappropriate activation of TGF-β or play a role in a negative feedback loop **(262)**.

3.2.1 Positive Modulators and Effectors

3.2.1.i AP-1 and TFE3

The members of the AP-1 family, c-Jun and c-Fos, have now been shown to physically interact with Smads. These proteins have long been thought to play a role in TGF-β target gene regulation due to the identification of intact AP-1 binding sites within the promoter regions of several TGF-β responsive genes such as PAI-1 **(260)**. c-Jun binds directly to amino acid residues within the variable

linker region of Smad 3 while c-Fos binds to the MH2 domain. This ligand induced complex, which also includes Smad 4, binds to overlapping or adjacent AP-1 sites and SBEs in target gene promoters. Activation of transcription by this complex has been demonstrated by utilizing the classical TGF-β responsive promoter, PAI-1 **(245)**.

Another HTH transcription factor known to induce the transcription of PAI-1 in response to TGF-β signaling is TFE3. TFE3 has been shown to associate with Smads 3 and 4, and the three proteins bind to specific DNA sequences within the PAI-1 promoter and contribute to maximizing TGF-β target gene activation **(98)**. Recently, data has been obtained to suggest that Smad 3 binds directly to TFE3 and that this interaction is enhanced after the C-terminal serines of Smad 3 are phosphorylated **(99)**. The DNA binding sites used by TFE3 and Smads differ from those employed by the AP-1 family of factors, indicating distinct mechanisms of generating specificity at Smad-responsive promoters. It has not been reported if TFE3 interacts with AP-1 family members **(262)**.

3.2.1.ii CREB binding protein/p300

CRE binding protein (CBP/CREB) and p300 are well characterized transcriptional co-activators known to enhance gene activation via a host of unrelated proteins through two different mechanisms **(80)**. First, CREB and p300 help to mediate the interaction of sequence-specific transcription factor complexes to the basal transcription machinery required for transcriptional

activation **(66)**. The second transcription enhancing feature these proteins possess is that they have histone acetyl transferase (HAT) activity **(174)**. Acetylation of the core histone amino-terminal ends alters nucleosomal structure by "unwinding" the chromatin, thereby increasing the accessibility to the transcriptional machinery **(133)**.

Many studies have shown that CBP/p300 will bind directly to the phosphorylated MH2 domain of the R-Smads, Smad 2 and Smad 3 **(107)**. These studies used a variety of TGF-β-responsive reporter gene complexes in cell culture including p3TP-Lux, SBE-Luc and PAI1-Luc **(183)**. Definitively, the specificity and ultimate requirement of CBP/p300 in these assays was confirmed using the adenoviral protein E1A. E1A protein is an inhibitor of CBP/p300 enhancement and it was observed that inhibition of CBP/p300 blocks the increase of Smad-dependant transactivation by this complex **(172)**.

3.2.1.iii Vitamin D Receptor

Another transcriptional co-activator that specifically interacts with R-Smad, Smad 3, is the vitamin D receptor (VDR). VDR is a vitamin D regulated nuclear receptor which functions with co-activators of the steroid receptor co-activator 1/transcriptional intermediary factor 2 family **(262)**. The VDR-Smad 3 interaction is unique, as the VDR appears to bind to the MH1 domain of Smad 3, not the typical protein-protein interacting domain, MH2 **(256)**. Additionally, activation of TGF-β signaling appears to result in Smad 3 mediated enhancement

of VDR dependant transcription. Therefore it is now known that Smad 3 transcriptional effects are not limited to the activation of transcription of TGF-β target genes, but are also involved in co-operative enhancement of other signal related pathways **(257)**.

3.2.1.iv Forkhead Transcription Factors

Currently there are approximately 90 members of the Forkhead superfamily with orthologues expressed in a vast array of species ranging from yeast to man **(117)**. All members exhibit a large degree of sequence homology within their "winged-helix" DNA binding domain. Forkhead activin signal transducer 1 (FAST-1) is a newly discovered WH protein identified by its ability to mediate transcriptional induction of activin, a member of the TGF-β superfamily **(37)**. This transcriptional induction by TGF-β and activin has been shown to involve the TGF-β downstream effector proteins, the Smads. In fact, this discovery not only confirmed a role for Smads inside the nucleus, but was also the first indication that the majority of direct Smad binding proteins are transcriptional regulators **(262)**. FAST-1 interacts directly with R-Smad, Smad 2, to form a transcriptionally active complex on the promoter region of the *Xenopus mix.2* gene at a site known as the activin response element **(38, 139)**. Subsequently, Smad 3 and Smad 4 were shown to be present within the complex. Smad 2 and 3 associate with FAST-1 however this interaction is not known to

occur with Smad 4, as its role resides in DNA binding specificity **(139)**. It has yet to be determined whether or not FAST-1 alone has any transcriptional activity.

Several FAST-1 homologues have been identified in various species including mouse and human. Studies have defined the conserved DNA binding sequence for these transcription factors (TGT G/TT/G ATT), which has also been identified in the activin response element (ARE) of the *Xenopus Mix.2* gene. Additionally, the importance of Smad binding elements adjacent to the FAST-1 binding sequence has been noted **(261)**. Cloning and further analysis of FAST2, a murine FAST-1 homologue, has indicated that its transcriptional regulation is elegant and complex. In a similar fashion to FAST-1 DNA binding, FAST2 complexes with Smad 2 on the *goosecoid (gsc)* promoter to activate target gene transcription. However the FAST2/ Smad 3 complex works to inhibit transcription from the same promoter. This is the first case that suggests that Smad 2 and Smad 3 may have opposing roles within selected systems **(122)**.

3.2.2 Negative Modulators and Effectors

3.2.2.i TGIF

TGIF (tumor growth interacting factor), a ubiquitously expressed homeodomain binding protein belonging to the TALE (three amino acid loop extension) family of proteins, has been shown to repress TGF-β-induced transcriptional activity by associating with histone deacetylases (HDAc) **(247)**. TGIF has also been shown to interact with both Smad 2 and Smad 3 **(18, 247)**.

For these genes, the expression level of TGIF appears to set a maximal response to TGF-β signaling by competing with CBP/p300 for binding to the Smad complex **(158)**. TGIF can bind to the TGF-β target gene promoter element independently of the Smad complex. It is, however, unclear whether this DNA-binding function is involved in the context of Smad-dependant transcription **(248)**.

3.2.2.ii Ski and SnoN

Ski protein was originally discovered as the gene product of a retroviral oncogene *(v-ski)* that is responsible for transformation of chick embryo fibroblasts and muscle hypertrophy in mice **(158)**. Recently, the cellular counterpart, c-Ski and the related protein SnoN, was discovered to function as a co-repressor that recruits HDAcs via an adaptor protein, N-CoR **(145)**. Studies have shown that both c-Ski and SnoN interact with Smad 3 and Smad 4 proteins and act as Smad2/3 co-repressors **(3)**. Unlike TGIF, which is induced by TGF-β stimulation, the interaction of Smads with the Ski and SnoN proteins has been observed under basal conditions and decreases to non-detectable levels after the first hours of TGF-β stimulation **(215)**. This effect is cell type dependant, and may be mediated by TGF-β induced, proteasome-mediated degradation of these two proteins **(158)**.

TGF-β regulation of transcription therefore depends on the ability of Smad proteins to recruit and complex together with proteins possessing different chromatin-remodeling activities. TGIF functions as a negative regulator of TGF-β

signaling while Ski and SnoN serve to protect against agonist-independent gene activation by Smad proteins. It is thought that a wave of SnoN expression may function as a negative feedback mechanism, terminating TGF-β responses **(215)**. This change in co-factor expression is also thought to occur with TGIF levels, affecting TGF-β signaling **(248)**. Therefore, the relative expression levels of these regulator proteins that interact with Smads can be modulated by the same and other signaling pathways, resulting in the negative regulation of TGF-β signaling **(158)**.

V. Materials and Methods

1. Primary adult rat cardiac fibroblast isolation and culture

Primary adult rat cardiac fibroblasts (PARCFs) were isolated from adult male Sprague-Dawley rats weighing between 200-250 g as previously described with minor modifications **(55, 148)**. Briefly, the adult heart was digested by perfusion at a flow rate of 5 ml/min with recirculating Joklik's medium containing 0.1% collagenase for 25-30 minutes at 37°C. The softened heart tissue was then minced and exposed to 0.1% collagenase for another 10 min at 37°C. Cells were collected by centrifugation (2000 rpm for 10 minutes) and resuspended (DMEM/F12 + 10% fetal calf serum + 1 μL/mL gentamicin). The cells were then plated on 100 mm non-coated culture dishes and incubated at 37°C with 5% CO_2 for 2-3 hours to allow for fibroblast adhesion. Cells were then washed 3 times with DMEM/F12 and allowed to grow to 70% confluency before passage. Fibroblasts (purity of ≥ 95%) passaged twice at 50 – 70% concluency were used in all following experiments. Prior to any stimulation, cells were incubated in serum-free media (DMEM/F12 + 1:1000 gentamicin) for 2 hours at 37°C. Fibroblasts were stimulated with angiotensin (10^{-6} M) for 15 or 30 minutes. Equimolar amounts of losartan (10^{-6} M) and/ or interferon-γ were added 1 hour prior to angiotensin stimulation to obtain effective AT_1 blockade and Smad 2 inhibition, respectively **(84, 136, 206)**.

2. Transfection of COS-7 and cultured primary adult rat cardiac fibroblast cells with pCMV5β-mycFAST2

COS-7 cells or primary cardiac fibroblasts were passaged onto cover slips for immunostaining while cells to be used for western analysis were seeded onto 100 mm dishes 24 hours prior to transfection and allowed to proliferate overnight at 37°C to a maximum confluency of 50%. The mammalian myc-tagged expression vector pCMV5β-mycFAST2 was transfected into these cells using the Effectene Transfection reagent Kit (Qiagen, Mississauga, Ontario) according to the manufacturers instructions. A transfection efficiency of ~20% was obtained in the COS-7 preparations and 10% in primary cardiac fibroblasts.

3 Immunofluorescence microscopy for myc, FAST2 and procollagen in control and transfected COS-7 and cultured fibroblasts.

Cells were washed 3 times with 1X PBS, fixed with 1% paraformaldehyde (15 minutes, room temperature) and permeablized with 0.1% Triton X-100 (15 minutes, room temperature) **(85)**. Primary antibodies for myc and FAST2 were diluted 1:100 in 3% BSA in PBS, while the procollagen monoclonal antibody SP1.D8 was diluted 1:20. 100 µL of these respective dilutions were added to each coverslip and incubated overnight in a humidity chamber at 4°C.

Cells were again washed (3 x 5 minutes) with PBS. Biotinylated anti-mouse, anti-rabbit and anti-mouse IgG Texas Red™ linked whole antibody secondary antibody (myc, FAST2 and procollagen, respectively) was added (1:20 in PBS + 3% BSA). The cells were allowed to incubate for 90 minutes at room temperature followed by incubation with FITC-labeled streptavidin also for 90 minutes at room temperature. Nuclear staining was achieved by adding 100μL/ well Hoechst dye number 33342 (10μg/mL) for 20 seconds followed by additional washes (3 x 5 minutes) with PBS. The coverslips were mounted onto slides using 10 μL of Vectashield mounting medium and examined under an epifluorescence microscope, and photographed on Provia Fujichrome 400 color film.

4. Nuclear and cytosolic cellular protein fractionation and total protein extraction from cultured cardiac fibroblasts and COS-7 cells

Adult rat cardiac fibroblasts or COS-7 cells were allowed to reach 90 % confluency at P_2 in 100 mm dishes. Cells to be stimulated were placed into serum-free medium 24 hours prior to use. Medium was aspirated and plates were set on ice. To fractionate the cellular proteins, cells were washed gently with 10 mL of ice-cold 1x PBS and carefully aspirated to completely remove all media. 4 mL of cold Nuclei EZ Lysis Buffer (SIGMA®, St. Louis, MO) was added to each dish.

Using a rubber policeman, cells were scraped in order to lyse and harvest the protein. The lysate was vortexed briefly and incubated on ice for 5 minutes. Nuclei were collected by centrifugation at 500x gravity for 5 minutes at 4°C. The supernatant (cytosolic protein fraction) was carefully removed and the pellet (nuclear protein fraction) was set on ice. The nuclei were lysed using a Tris-EDTA buffer pH 7.4 containing protease inhibitors buffer (0.1M Tris-EDTA pH 7.4, 1mg/ mL aprotinin, 1mg/ mL leupeptin, 1μg/ mL pepstatin). Cells in which total protein was collected were washed in ice cold PBS that was then removed by aspiration. 1-2 mL of PBS was added and the cells were scraped with a rubber policeman. Collected cells were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. The pellet was then resuspended in 100-200 μL of Tris-EDTA/ protease inhibitor cocktail and placed on ice for 30 minutes with intermittent pipetting to lyse the cells. Total, cytosolic and nuclear protein concentrations were calculated using the bicinchoninic acid (BCA) assay described elsewhere **(207)**.

5. Western analysis of FAST2 and procollagen from total cellular protein and cytosolic and nuclear protein fractions obtained from cultured cardiac fibroblasts and transfected COS-7 cells

Prestained broad range (Bio-Rad, Hercules, CA) SDS-PAGE standard and 20 μg of total protein and cytosolic and nuclear fractions, obtained from rat cardiac fibroblasts or COS-7 cells, were separated on a 10% SDS gel.

The separated proteins were transferred onto 0.45 μM polyvinylidene difluoride (PVDF) membrane. The membrane was blocked overnight at 4°C and for 1 hour at room temperature the next day with Tris-buffered saline with 0.2% Tween-20 (TBS-T) containing 5% skim milk powder. Without washing, primary FAST2 or SP1.D8 antibody diluted with 5% skim milk blocking buffer (1:500 and 1:1000 respectively) were added to the membrane for 1 hour at room temperature with shaking. The membrane was washed (4 x 15 minutes) with TBS-T and the secondary HRP-labeled antibody (diluted 1:10 000 in skim milk buffer) was added for 1 hour at room temperature with shaking. The FAST2 and procollagen bands were visualized by enhanced chemiluminescence (ECL+Plus) as per manufacturer's instructions (Amersham Life Sciences Inc. Arlington Heights, IL). Autoradiographs of the membranes were quantified using a CCD camera imaging densitometer (Bio-Rad, model GS 670). Accuracy of protein loading was determined by Coomassie blue staining following autoradiography.

6. Co-Immunoprecipitation of the Smad 2/ FAST2 Complex

Total cell lysate was obtained from adult rat primary cardiac fibroblasts by the method previously described although the cells were scraped in RIPA buffer instead (1 x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) **(85)**. Approximately 1 ml of lysate was pre-cleared by adding 20 μl of protein A/G slurry to eliminate non-specific binding. This was incubated at 4° C for 30 minutes.

The beads were then pelleted by centrifugation at 2500 rpm and the supernatant transferred to a new tube. To 1 ml of the supernatant, various amounts of Smad 2 primary antibody were added (0.2 μg, 0.5 μg, and 1.0 μg) to this mixture and allowed to incubate overnight at 4° C. The pellet was collected by centrifugation at 2500 rpm for 5 minutes at 4° C, and washed 4 times with PBS. The pellet was then resuspended in 40 μl of 1 x electrophoresis buffer (1.0 ml glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml, 10% SDS, 1.25 ml 1.0M Tris-HCl pH 6.7, 2 mg bromophenol blue) and boiled for 3 minutes. Various amounts of sample were loaded onto a 10% SDS-PAGE gel, run for 1 hour then transfered to a PVDF membrane. Membranes were then blocked as previously described **(85)**. The membrane was then probed with the FAST2 primary antibody at a 1:500 dilution and ECL-plus was utilized to visualize the bands.

7. Electrophoretic mobility shift assay using the nuclear extract obtained from pCMV5β-myc-FAST2 transfected COS-7 cells

Nuclear extracts were prepared from COS-7 cells transiently transfected with pCMV5β-mycFAST2 under optimal conditions. Two 20 nucleotide complimentary oligomers were synthesized (Gibco-BRL, Burlington, Ontario) with the FAST2 consensus binding site 5'-CTTTTCCCATATAAATAGGG-3' (*accession number X66209*) found within the rat alpha-2 (I) procollagen promoter region and used to detect DNA-protein interactions.

The two strands were annealed by adding equimolar amounts of DNA together in 1X Annealing buffer (100 nM Tris-HCl pH 7.5, 1 M NaCl, 10 mM ETDA, DEPC- treated water to volume) and heated to 65° C for 10 minutes and allowed to cool to 25° C slowly. ^{32}P end-labeling was achieved using a kit according to the manufactuers instructions (T_4 polynucleotide kinase kit, Gibco-BRL, Burlington, Ontario). Binding reactions were incubated for 15 minutes at room temperature and contained 10, 15 or 20 μg of nuclear extract in the presence of 1.32 μg labeled probe (cpm ≥ 10^6), 2.4 μg poly(dIdC), 2 μL 10% NP-40, 20 mM HEPES, pH 7.9, 5% glycerol, 1 mM EDTA, 5 mM dithiothreitol and 10 μg of bovine serum albumin. FAST2 supershift experiments were carried out with a monoclonal mouse anti-myc tag primary antibody (3, 5 and 10 μg). Primary antibody was allowed to incubate at room temperature in the lysate/ oligo binding reactions for 1 hour prior to gel loading.

The DNA-protein reaction mixtures were subjected to electrophoresis with a 5% native polyacrylamide gel in 1× Tris-Borate EDTA buffer, pH 8.0 **(49)**. Gels were dried for 30 minutes at 80°C in a Beckman lyophilizer. The bands were then visualized using the phospho-luminescent imager Storm-860.

8. Reagents

The primary antibody against FAST2, and the expression vector pCMV5β-mycFAST2 were generated in the laboratory of Dr. Liliana Attisano (U of Toronto, Canada). A second primary FAST2 antibody, Smad 2 antibody and protein A/G agarose beads were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California). Procollagen antibody (SP1.D8) was obtained from the Developmental Studies Hybridoma Bank (U of Iowa, IA). Biotinylated anti-rabbit and anti-mouse secondary antibodies, anti-rabbit and anti-mouse linked Texas Red conjugate, FITC-labeled streptavidin, and HRP-conjugated anti-rabbit secondary antibodies were purchased from Amersham Life Science Inc (Arlington Heights, IL). Losartan was a kind gift from Merck via Dr. Ronald Smith (Rahway, New Jersey). Angiotensin II, Tris, Hepes, BSA, Aprotinin, Leupeptin, Pepstatin, Triton X-100, BCA assay kit, Paraformaldehyde, Tween-20, SDS, NP-40, Sodium deoxycholate, NaCl, EDTA, Dithiothreitol, Glycerol, β-mercaptoethanol and dIdC were purchased from Sigma-Aldrich, (Oakville, ON, Canada). PVDF Western blotting membrane was purchased from Roche Diagnostics (Indianapolis, IN). Interferon-γ was obtained from Boehringer-Mannheim (Laval, QC, Canada). All culture dishes and flasks were purchased from Fisher Scientific (Nepean, Ontario, Canada) while all culture media was obtained from Gibco-BRL (Burlington, Ontario, Canada). The COS-7 cells were purchased from the American Type Culture Collection.

9. Statistical analysis of data

All values are expressed as means ± SEM. One way analysis of variance (ANOVA) followed by Student-Newman-Keuls method was used for comparing the differences among multiple groups (SigmaStat). Significant differences among groups were defined by a probability less than 0.05.

VI. Results

1. Identification of exogenous FAST2 protein in COS-7 cells transfected with pCMV5β-mycFAST2 through the use of two different primary anti-FAST2 antibodies.

To determine the effectiveness of the primary polyclonal anti-rabbit FAST2 antibody we received from the laboratory of Dr. Attisano (University of Toronto, ON), we transiently transfected COS-7 cells for 24 hours by lipofection techniques with the plasmid pCMV5β-mycFAST2 [Fig. 5]. This plasmid contains the DNA sequences for FAST2 (*accession number AF069303*) and an N-terminal myc-tag which lies directly upstream of the FAST2 sequence **(122)**. The presence and localization of both the myc-tag and FAST2 was determined by double immunofluorescent staining using an anti-myc primary antibody and either the commercially offered FAST2 antibody or one created independently. The results indicated that FAST2 protein could be detected equally well with both primary antibodies as both the myc-tag and FAST2 co-localized, ending any argument over the specificity of the antibodies. All future experiments were performed using the independantly manufactured FAST2 antibody. This experiment also demonstrates that FAST2 is present in abundance in transfected cells although lesser quantities are observed in non-transfected cells [Fig. 6]. This suggests FAST2 is found endogenously within COS-7 cells. Both the myc-tag and the majority of FAST2 are localized within the nucleus of these cells suggesting that

FAST2 is principally a nuclear protein or that the antibodies detect mainly a nuclear form of the protein.

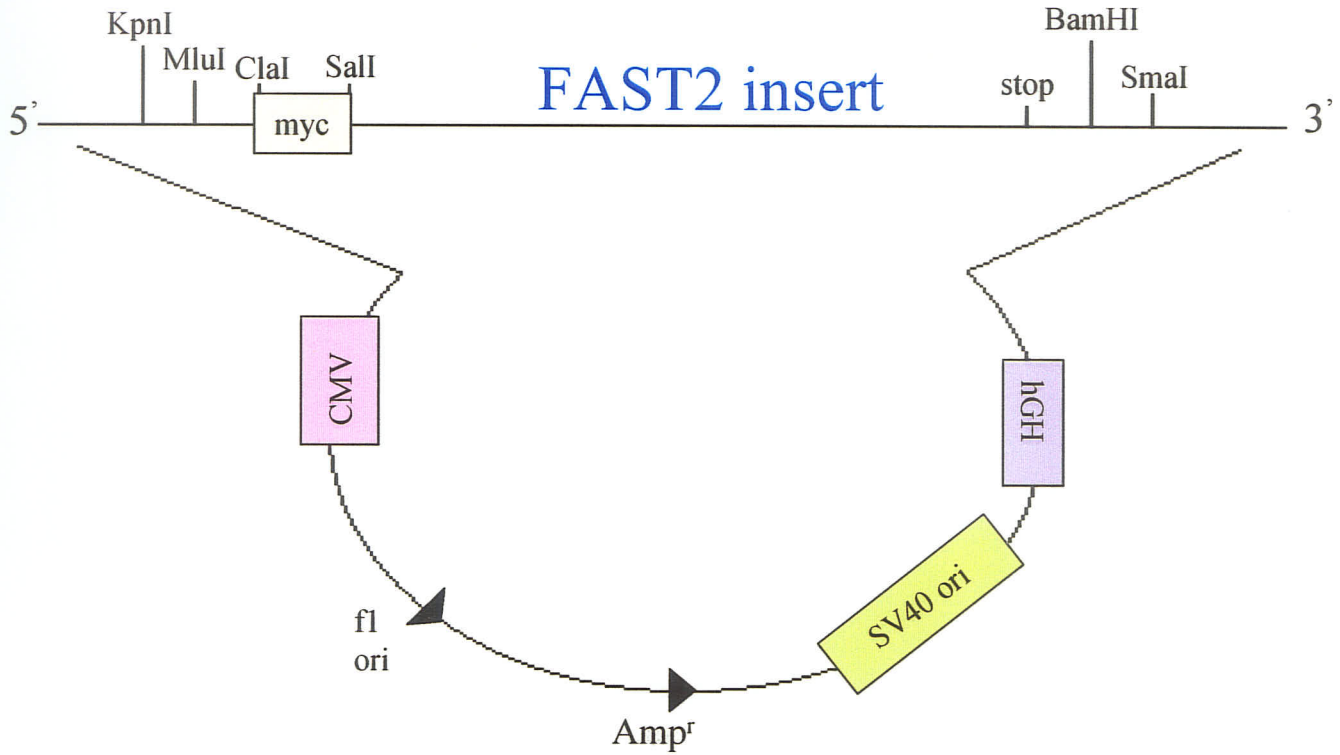

Figure 5. Schematic representation of the mammalian expression vector pCMV5β-mycFAST2, as employed in the current study. Construction of vector is described in Labbé *et al* (1998) and was performed in the Attisano laboratory (University of Toronto).

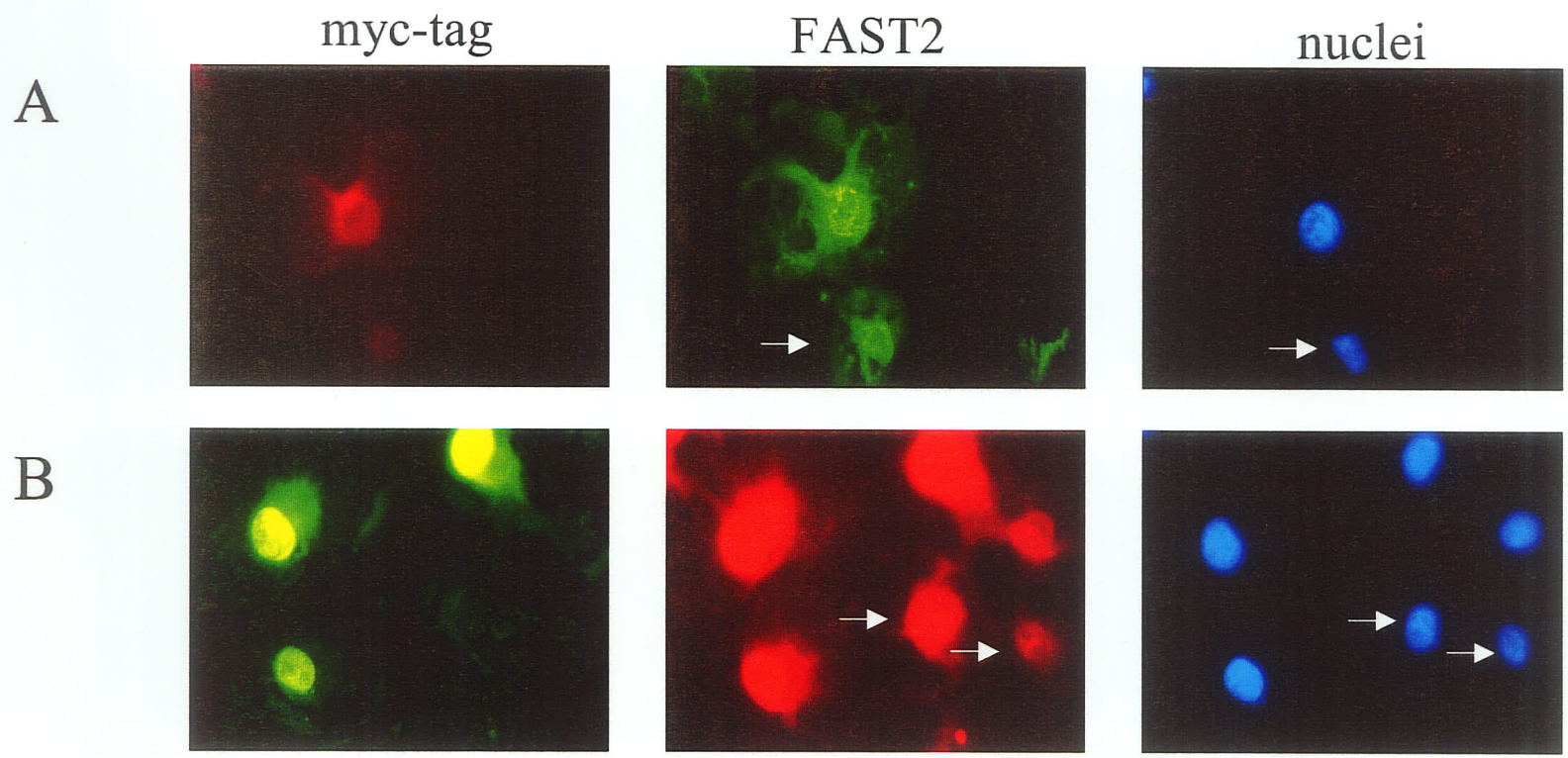

Figure 6: Immunocytochemistry of cultured COS-7 cells transfected with the plasmid pCMV5β-mycFAST2 using Effectene™ (Qiagen- lipid-based delivery reagent). The myc-tag and FAST2 were detected using an anti-myc antibody as well as two different anti-FAST2 primary antibodies. **A.** FAST 1/2 antibody purchased from (Santa Cruz) **B.** FAST2 antibody raised independently (Dr. Liliana Attisano, University of Toronto). Each antibody was used at a 1:50 dilution. Nuclei were stained with Hoechst 33342. Transfection efficiency was ~20% in these cultures. Arrows indicate cells that were not transfected.

2. Detection, quantification and localization of FAST2, a novel protein expressed in cultured adult rat cardiac fibroblasts

We investigated the possibility that FAST2 is a transcriptional cofactor of the Smad signaling pathway leading to the production of procollagen in adult rat heart. Western analysis was used to determine the presence of FAST2 (52 kDa band) in nuclear and cytosolic extracts from control adult rat cardiac fibroblasts [Fig. 7, lanes 1 - 4]. Quantitative densitometric scanning [Fig. 8] indicated that FAST2 expression within the nucleus when compared to the cytosolic fraction is significantly higher. Fibroblasts are the sole cell type responsible for fibrous tissue formation in the heart. Fibroblasts and myofibroblasts (i.e., phenotypically transformed and hyper-synthetic contractile fibroblasts) obtained from adult rat cardiac scar tissue are well known to express genes that encode components requisite for Ang II metabolism **(229)**. Myofibroblasts exhibit higher baseline expression of AT_1 receptors when compared to relatively quiescent cardiac fibroblasts. PARCFs were subjected to Ang II stimulation, which is intended to model the failing heart, prior to protein fractionation revealed that Ang II treatment was associated with a modest decrease in FAST2 expression in the cytosolic fraction [Fig. 7, lanes 5 & 6, and Fig. 8]. A significant increase in FAST2 expression within the nuclear fraction is observed after the 15 minute Ang II stimulation [Fig. 7, lanes 8 & 9 and Fig. 8]. This finding may suggest that Ang II acts to elicit the translocation of cytosolic FAST2 into the nucleus. Immunofluorescent staining patterns of FAST2 are shown in control [Fig. 9A] and Ang II stimulated cultured adult rat cardiac fibroblasts [Fig. 9B]. Double-

staining of cellular nuclei reveal that most of the FAST2 protein is within or proximal to the nucleus in the Ang II stimulated cells although it is fairly ubiquitous throughout control (unstimulated) cells [Fig. 9A, i-iv]. We observed that FAST2 becomes much more centrally located within the nucleus upon Ang II stimulation [Fig 9B, v-viii]. This finding corresponds with the above Western result suggesting translocation of this protein to the nucleus. The function of FAST2 remaining in the cytosol after Ang II treatment is unclear.

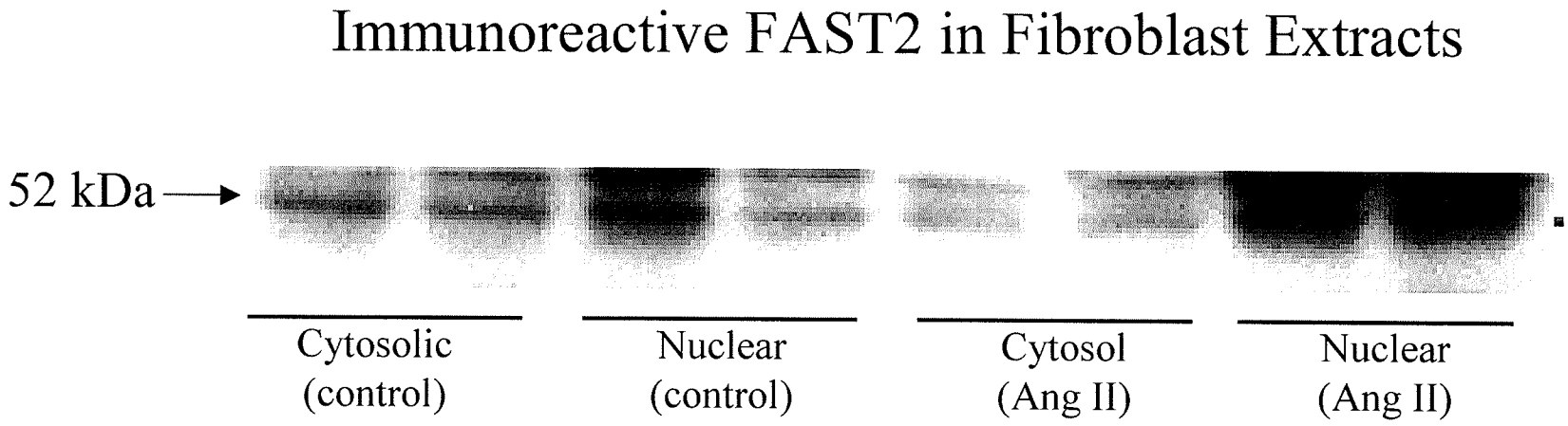

Figure 7. Representative Western blot analysis of immunoreactive FAST2 (Attisano) from nuclear and cytosolic extracts obtained from PARCF. Nuclear and cytosolic fractions were extracted from control (unstimulated and serum-starved) and Ang II stimulated cells (10^{-6}M for 15 min). An averaged sampling of multiple treated cultures revealed increased band intensity (i.e., translocation) of immunoreactive FAST2 to the nuclear fraction in Ang II treated cells when compared to averaged values from control nuclear fractions.

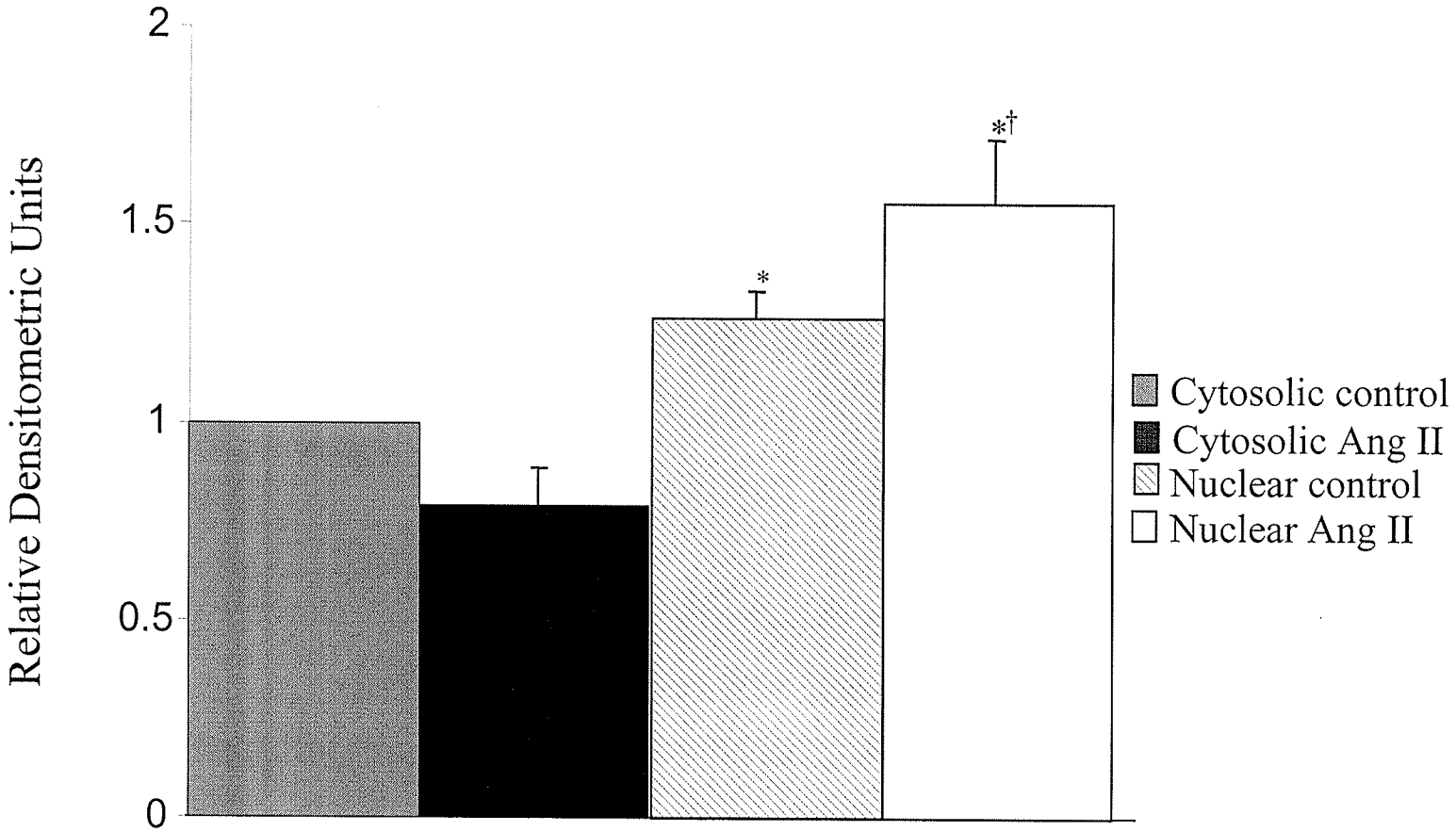

Figure 8. Histographic depiction of quantified immunoreactive FAST2 band intensity from quiescent or Ang II treated PARCFs. Data is described as the mean ± SEM of n = 3 samples per group. *P< 0.05 when compared to values of cytosolic fraction of Ang II stimulated cells. † P< 0.05 as compared to values from nuclei extracted from quiescent cells. A modest decrease in cytosolic immunoreactive FAST2 was noted in the Ang II treated group when compared to values for quiescent cytosolic fraction.

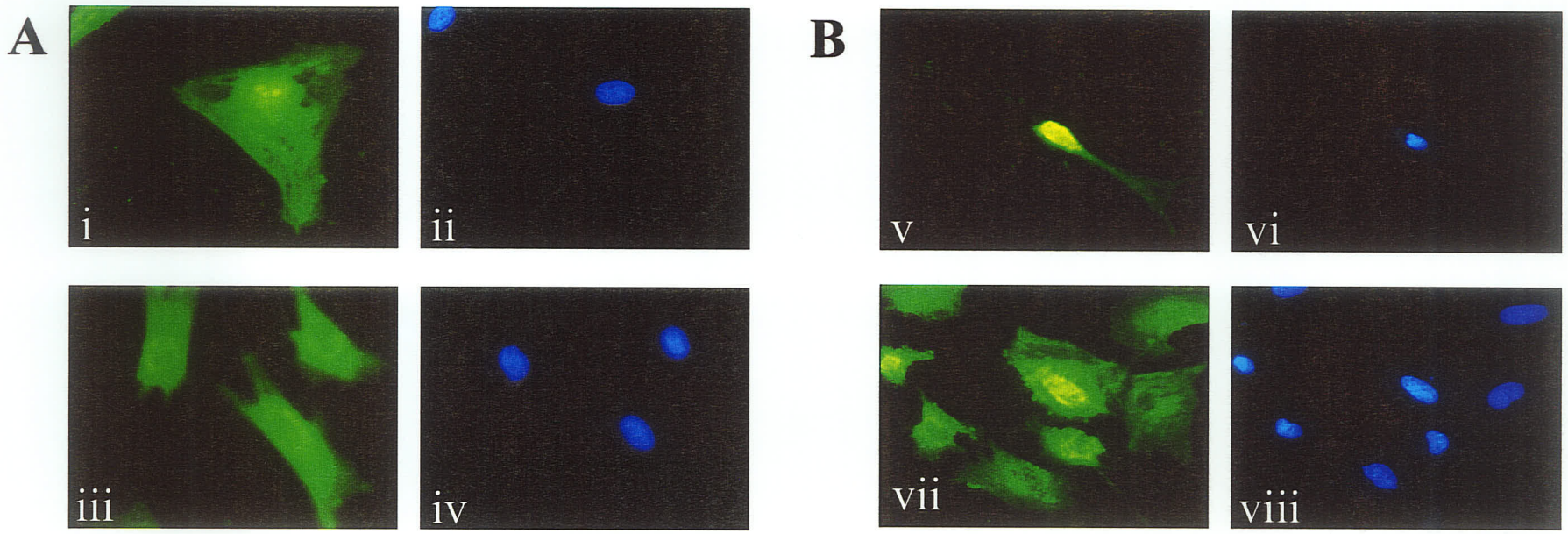

Figure 9: Immunocytochemical staining for FAST2 localization in PARCFs. **A.** Panels i and iii show unstimulated control myofibroblasts stained with anti-FAST2 at a 1:100 dilution; panels ii and iv depict the same PARC myofibroblast cells stained with Hoechst nuclear stain 33342 @ 1μM (ii & iv) (identical fields). **B.** Ang II stimulated PARCF myofibroblasts (10^{-6}M) stained for FAST2 (v and vii) and again with Hoechst 33342 (vi and viii). We noted relatively increased staining of immunoreactive FAST2 in the nuclei of stimulated cells vs unstimulated cells. Marked FAST2 staining was observed in the cytosol of unstimulated cells. Magnification x400 using an Nikon E600 epifluorescence microscope.

3. Effect of angiotensin II and losartan treatment on the expression of endogenous FAST2 and procollagen

Once the presence of immunoreactive FAST2 protein was determined in PARCFs, we tested the effect of Ang II receptor agonists and antagonists on FAST2 and procollagen steady-state protein expression. Western blot analysis of total protein isolated from either unstimulated (control), Ang II treated, or Ang II + losartan treated (10^{-6} M) adult cardiac fibroblasts revealed that total cellular immunoreactive FAST2 (indicated by differential band strength in total extracts subjected to PAGE analysis) is unchanged (despite a modest increase with 15 minutes Ang II treatment) when compared to controls [Fig.10A, lanes 1 & 2]. The modest changes were abrogated with losartan treatment (lane 3). Quantitative densitometric scanning [Fig. 11] highlights these findings. Western examination [Fig. 10B, lanes 4 & 5] and densitometric evaluation of procollagen [Fig. 11] demonstrated that despite the lack of a significant increase in FAST2, that immunoreactive procollagen expression is significantly increased with Ang II treatment. It is well known that PARCFs continuously synthesize procollagen which is normally rapidly degraded in the intracellular space upon lack of hydroxylation by 4-prolyl hydroxylase **(182)**. We hypothesize that rapid induction of the supporting metabolic enzymes for procollagen to achieve increased net expression in a rapid time frame is possible; this is unlikely the case with regard to FAST2. Losartan treatment significantly reduced procollagen expression when compared to the angiotensin-stimulated group [Fig. 10B, lane 6] [Fig. 11] but not when compared to control values.

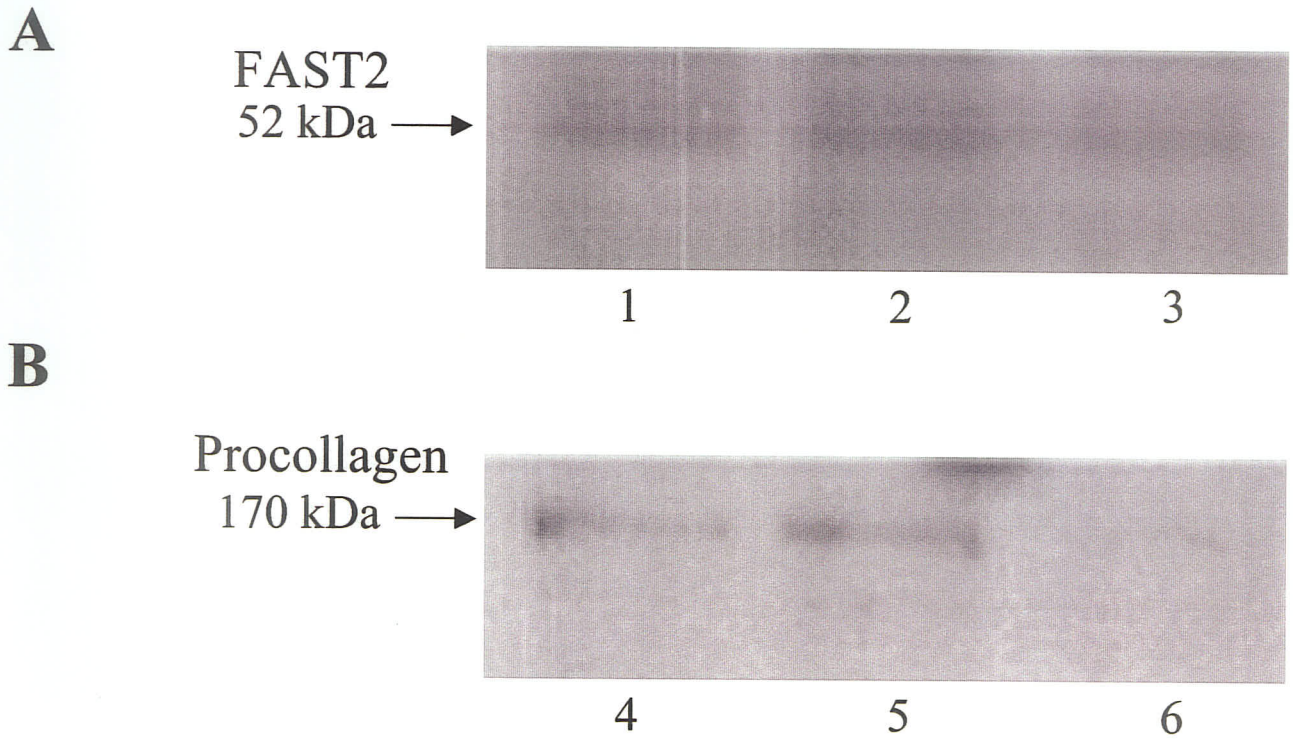

Figure 10. Western blot analysis of FAST2 and procollagen protein from PARCF total protein extracts. **A.** Representative autoradiographs of immunoreactive FAST2 and **B.** immunoreactive procollagen protein from control *(lanes 1 & 4)*, angiotensin II stimulated, 10^{-6} M, 15 min., *(lanes 2 & 5)*, and angiotensin II + losartan treated, each 10^{-6} M, *(lanes 3 & 6)* groups. Coomassie staining of the transferred protein on PVDF membrane revealed even protein loading across the all lanes.

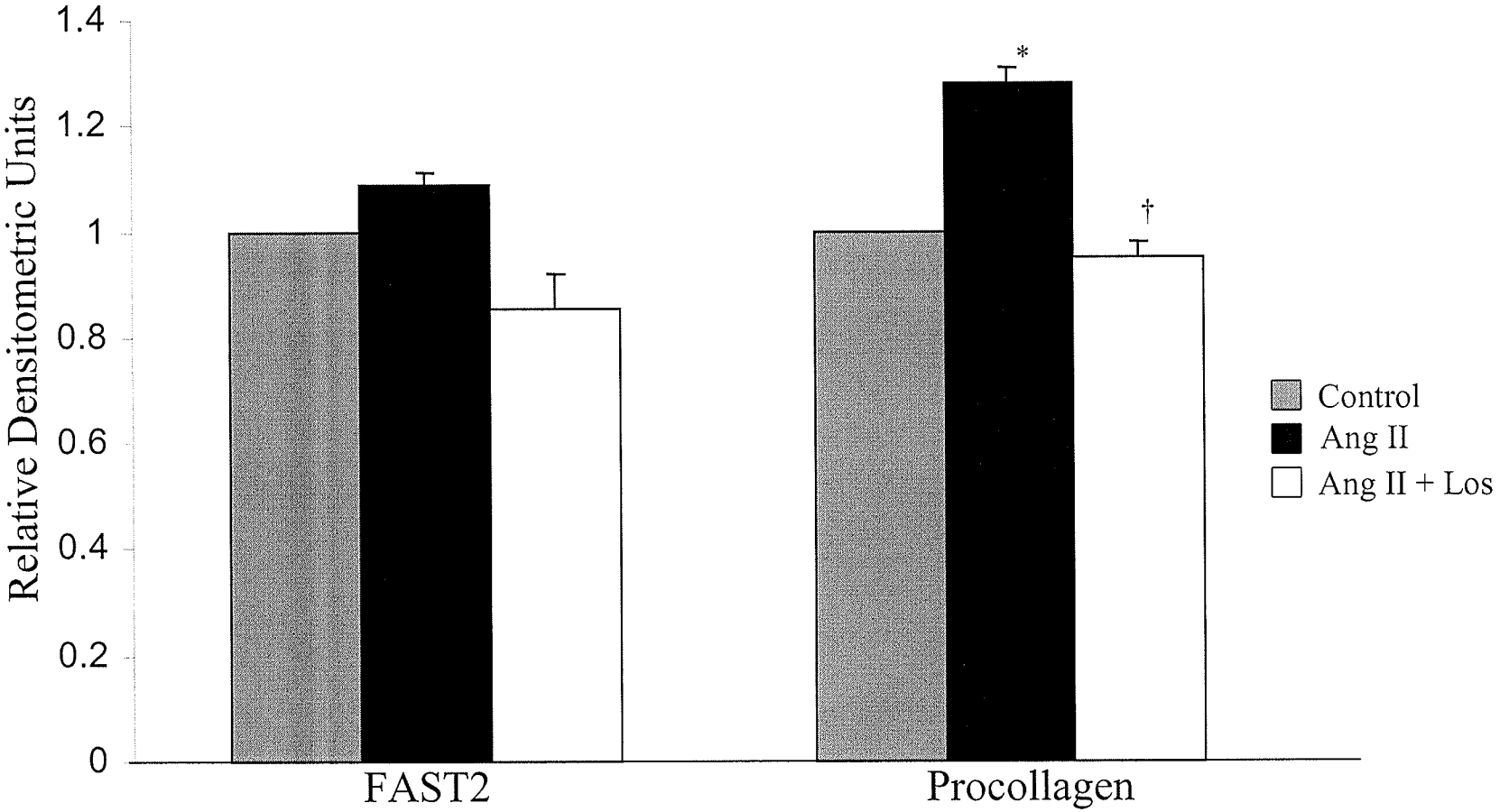

Figure 11: Histograms of the quantified data of immunoreactive FAST2 and procollagen band intensity from Western blots. Data is shown as the mean ± SEM of n = 3 samples. *P< 0.05 compared to control. †P< 0.05 compared to values from Ang II stimulated cells.

4. Effect of exogenous FAST2 expression on the deposition of procollagen

We wished to investigate the relationship between exogenous FAST2 protein over-expression and procollagen metabolism in PARCFs. Immunofluorescence studies of PARCFs transiently transfected with the myc-tagged mammalian expression vector, pCMV5β-mycFAST2, were carried out. Immunostaining of FAST2, procollagen and PARCF nuclei revealed that control cells exhibited a characteristic cytosolic staining pattern for FAST2 as observed in our baseline determinations [Fig. 12A]. Twenty-four hours following transfection with the above plasmid [Fig. 12B], FAST2 becomes concentrated within the nucleus, in agreement with the results shown in Fig. 9. Interestingly, over-expression of only the exogenous FAST2 protein, unlike stimulation that may increase other proteins, induces a significant increase in the deposition of procollagen protein suggesting that FAST2 expression is positively correlated with procollagen accumulation.

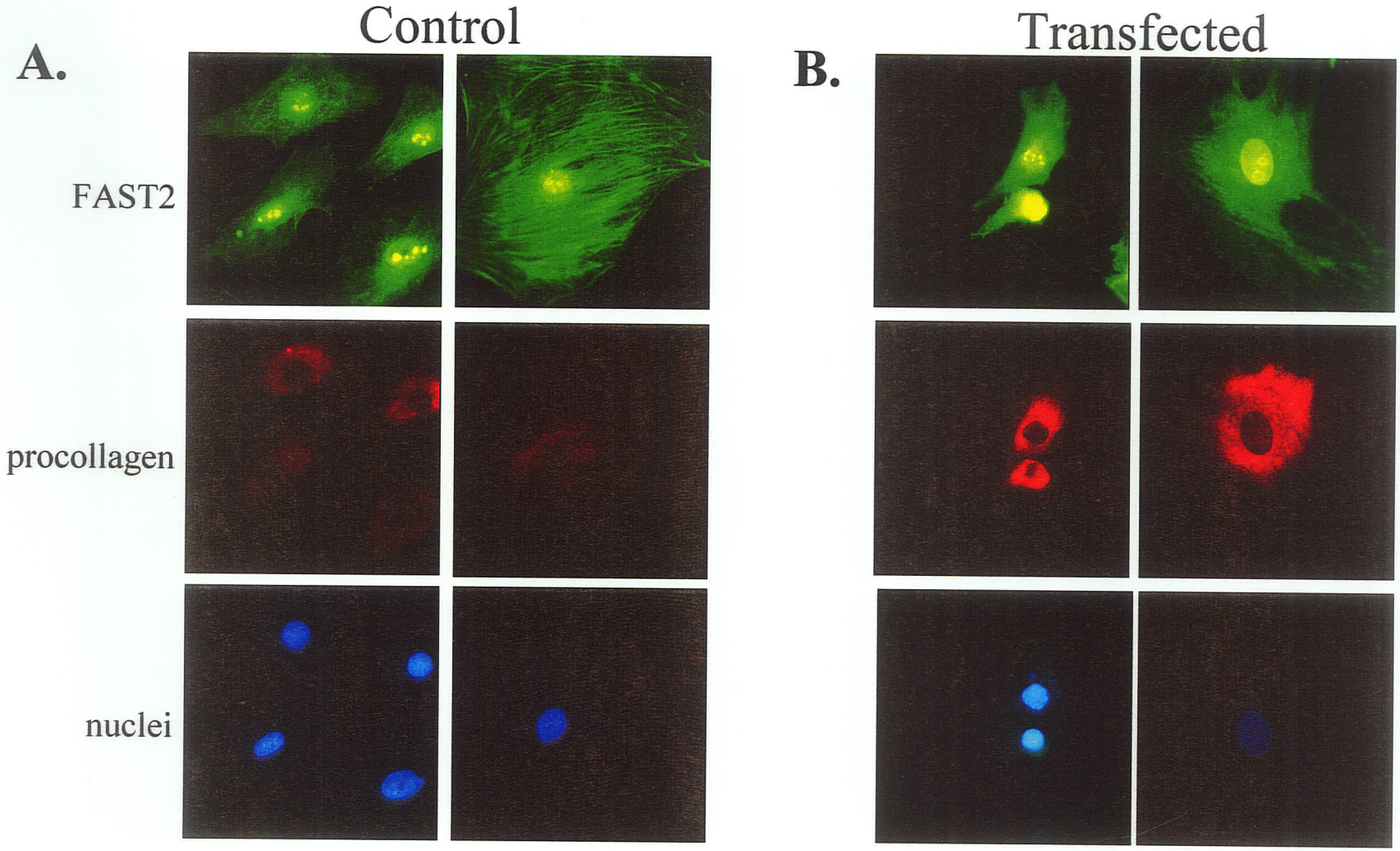

Figure 12. Immunocytochemistry of immunoreactive FAST2 and procollagen in PARCFs. **A.** Control (non transfected) fibroblasts **B.** Fibroblasts transfected with pCMV5β-mycFAST2 using overnight Effectene™ treatment. FAST2 was detected using an anti-rabbit primary antibody at a dilution of 1:50, and procollagen was detected using the monoclonal mouse primary antibody Sp1.D8 at a 1:20 dilution. Nuclei were visualized by staining with Hoechst 33342. Transfection efficiency using this lipid-based method was ~ 20%.

5. The effect of INF-γ on FAST2 and procollagen protein expression when used in concert with losartan

As it is known that TGF-β and INF-γ have opposing effects on a diverse array of cellular functions, we sought to examine the effect of INF-γ on FAST2 and procollagen expression when given together with losartan **(14, 99, 208, 219)**. We also wished to examine immunoreactive FAST2 in nuclear extracts of stimulated PARCFs to examine translocation events. Western analysis of protein obtained from the nuclear fraction of PARCFs [Fig. 13] shows the FAST2 52 kDa band is substantially increased with Ang II treatment while this trend is normalized with 1 hour losartan pre-treatment (250U/mL). Combining losartan with INF-γ treatment completely abrogates the increase in band intensity associated with Ang II stimulation. Densitometric scanning and statistical analysis revealed that immunoreactive FAST2 is unchanged in the presence of Ang II + losartan vs control [Fig. 14]. Furthermore, the combination of losartan and INF-γ revealed a significant reduction of immunoreactive nuclear FAST2 *vs*. control. Double-immunofluorescence staining for FAST2, procollagen and nuclei [Fig. 15] confirmed this trend (Western blot results) of nuclear FAST2 translocation in the various treatment groups as well as revealing a dramatic decrease in procollagen deposition to negligible levels in those cells treated with losartan + INF-γ. These results may indicate that a reduction in activated R-Smad by suppression of R-Smad phosphorylation with INF-γ stimulation can further decrease both FAST2 nuclear accumulation and procollagen protein expression in

these cells. Future experiments should examine the effect of INF-γ on FAST2 and procollagen alone.

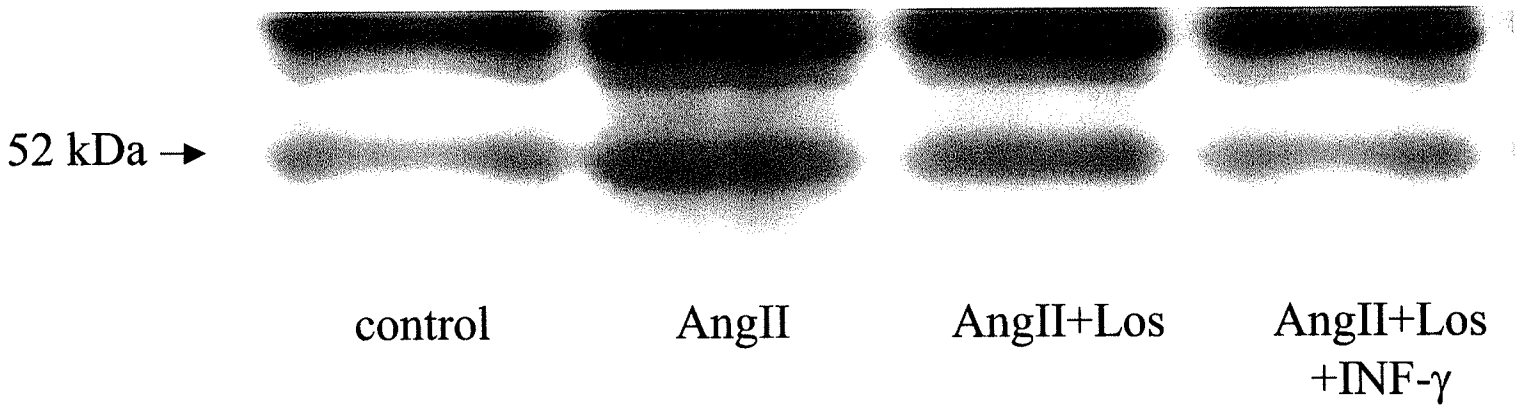

Figure 13. Western blot analysis of FAST2 from nuclear extracts obtained from cultured adult rat cardiac fibroblasts. Representative autoradiograph of FAST2 protein expression under various stimulant conditions. Losartan (10^{-6} M) and INF- γ (250 IU/mL) were administered 1 hour prior to 15 minute AngII (10^{-6} M) stimulation. Coomassie staining revealed even loading protein loading pattern across all lanes (redo in progress).

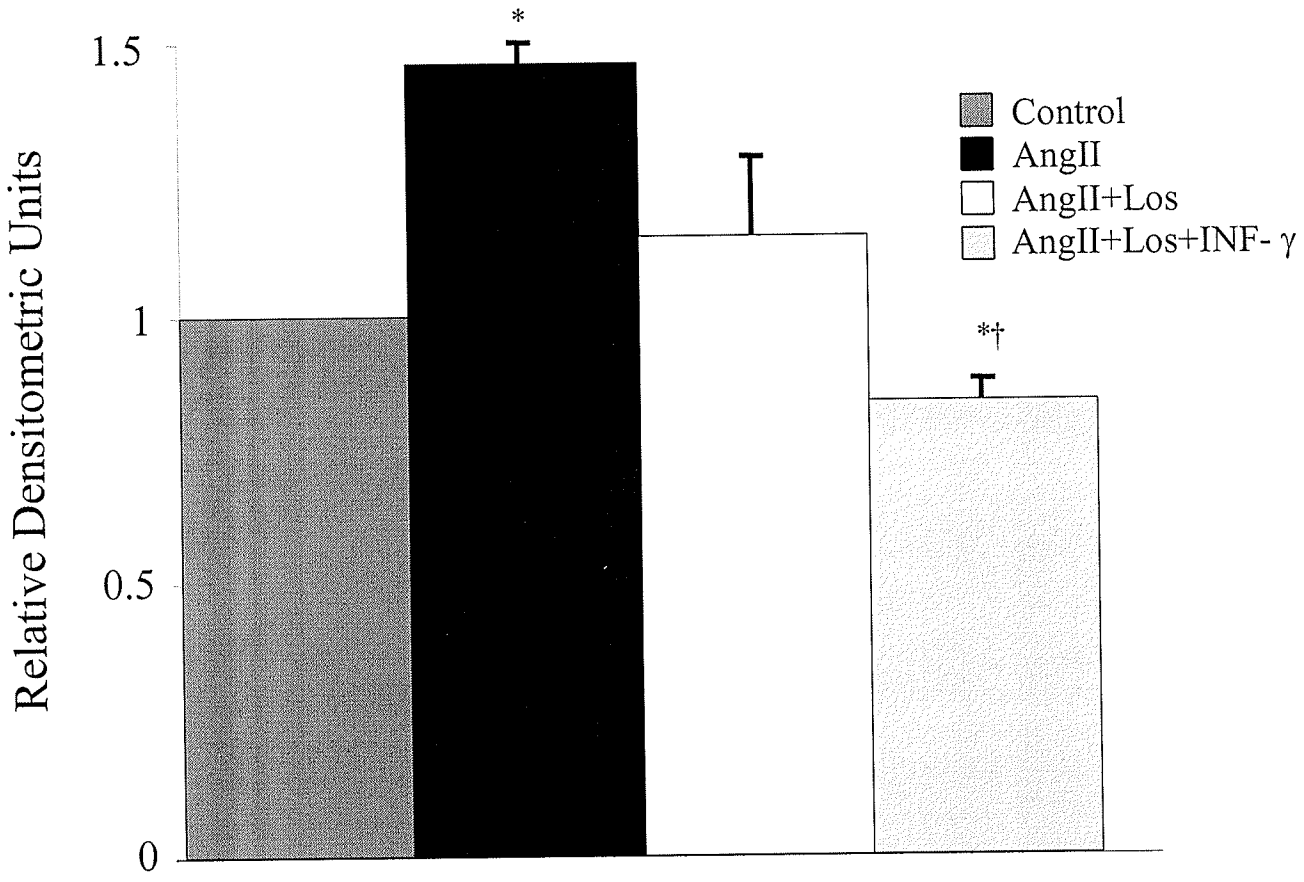

Figure 14: Histograms of quantified data of immunoreactive FAST2 protein expression depicted as the mean ± S.E.M. of n = 4. *P< 0.05 vs control values, †P< 0.05 vs Ang II stimulated.

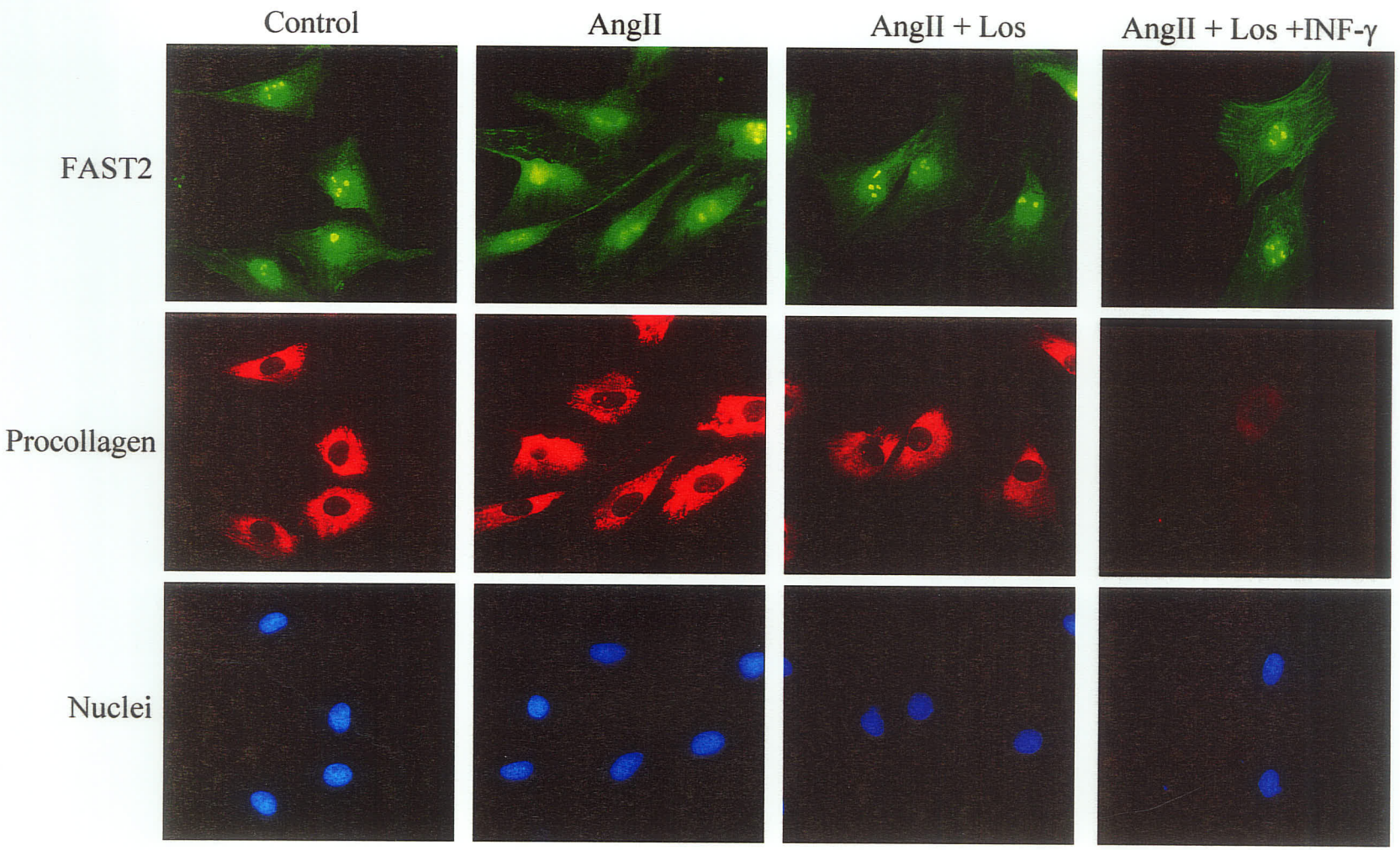

Figure 15: Representative IF staining of FAST2, procollagen, and nuclei in isolated adult rat cardiac fibroblasts from control, Ang II treated, Ang II + losartan (Los)and AngII + Los + interferon-γ (INF-γ) treated groups. Cells were stimulated with 10^{-6} M Ang II for 15 minutes, 10^{-6} M and 250 IU/mL Los and INF-γ respectively for one hour prior to 15 min stimulation with Ang II. Nuclei were stained with Hoescht 33342 nuclear stain.

6. Co-immunoprecipitation techniques indicate that FAST2 protein directly binds to the R-Smad, Smad 2 in adult rat cardiac fibroblasts

In order to determine if the WH protein FAST2 is involved in the TGF-β/ Smad signaling pathway as a transcriptional co-factor, we employed reciprocal co-immunoprecipitation assays. Agarose beads coupled with protein A/G, a protein that binds to the Fc region of an antibody, were incubated with a Smad 2 primary antibody and allowed to complex with proteins found within the nuclear lysate of adult rat cardiac fibroblasts. Western analysis using the FAST2 and Smad 2 directed antibodies of the resulting complexes indicated that the FAST2 protein does indeed form a complex with the TGF-β downstream effector protein Smad 2 [Fig. 16]. Increasing concentrations of Smad 2 antibody were added to the A/G coated beads and it was observed that a correspondingly increased amount of FAST2 protein was precipitated. This may indicate that FAST2 associates with the Smad 2 protein in a 1:1 ratio. Various amounts of precipitate were loaded into the PAGE gel as it was not possible to obtain the protein concentration due to the limited sample obtained.

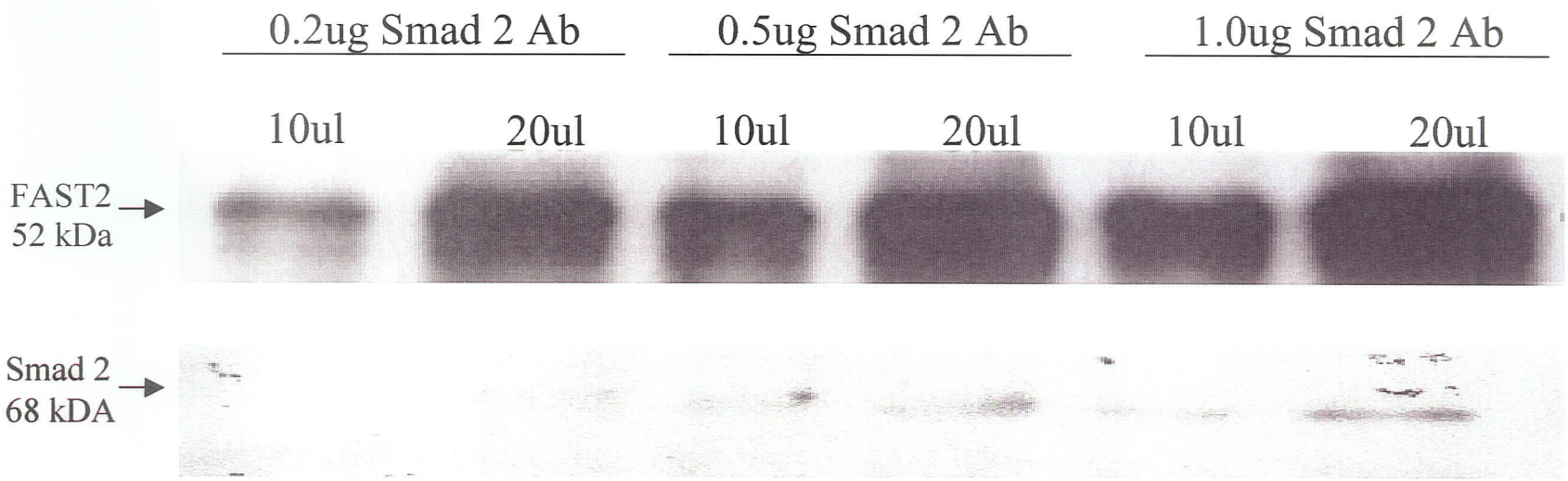

Figure 16: Co-immunoprecipitation of FAST2 using a Smad 2 primary antibody. Total cardiac fibroblast lysate was mixed with Smad 2 primary antibody labeled protein A/G agarose beads. The lysate was centrifuged to precipitate out Smad 2 and any proteins which are bound to it. The precipitate was separated by electrophoresis on a denaturing polyacrylamide gel. A FAST2 primary antibody (1:500 dilution) was used to detect the occurrence of FAST2/ Smad 2 binding. The filter was stripped and re-probed with a Smad 2 antibody (1:250 dilution) as a control. Coomassie staining of the transferred protein on PVDF membrane revealed even protein loading across all lanes.

7. Electrophoretic mobility shift techniques determine that FAST2 is a transcriptional activator of the procollagen gene in adult rat cardiac fibroblast

COS-7 cells were transiently transfected with the pCMV5β-myc-FAST2 expression vector for various time points in order to determine the optimum transfection time to obtain maximum FAST2 expression [Fig. 17]. The optimum transfection time point for FAST2 protein expression was 36 hours, using cell death as our endpoint (data not shown). In order to ensure that any band we detect is specific, we incubated the labeled oligonucleotide with increasing amounts of cold (unlabeled) oligonucleotide with 10 μg of nuclear extract obtained from the transfected COS-7 cells. We observed that the cold oligonucleotide competes with the labeled oligonucleotide [Fig. 18, lanes 2-4] to undetectable levels at a 1:500 ratio. These results indicate that the shifted band is specific. Increasing the concentration of nuclear protein loaded into the lanes resulted in an increase in band intensity [Fig. 18, lanes 5-7].

Supershift assays were used to determine the identity of the protein bound to the procollagen promoter oligonucleotide. To ensure that the FAST2 protein bound to the oligonucleotide is indeed the exogenous mouse FAST2 we overexpressed in the COS-7 cells, the supershift was carried out using the primary antibody raised against the myc-tag. Although a supershifted band was not observed, increasing the concentration of myc antibody used in each reaction led to a reduction of the intensity of the shifted band, and an increase in aggregated proteins in the gel wells [Fig. 18, lanes 8-10]. These results indicate that the

supershifted band does exist, but remains in the wells of the PAGE gel due to the large size. In summary, our data support the hypothesis that FAST2 binds to the promoter region of the collagen gene and is likely linked to the activation of procollagen expression following Ang II/ TGF-β stimulation in PARCFs.

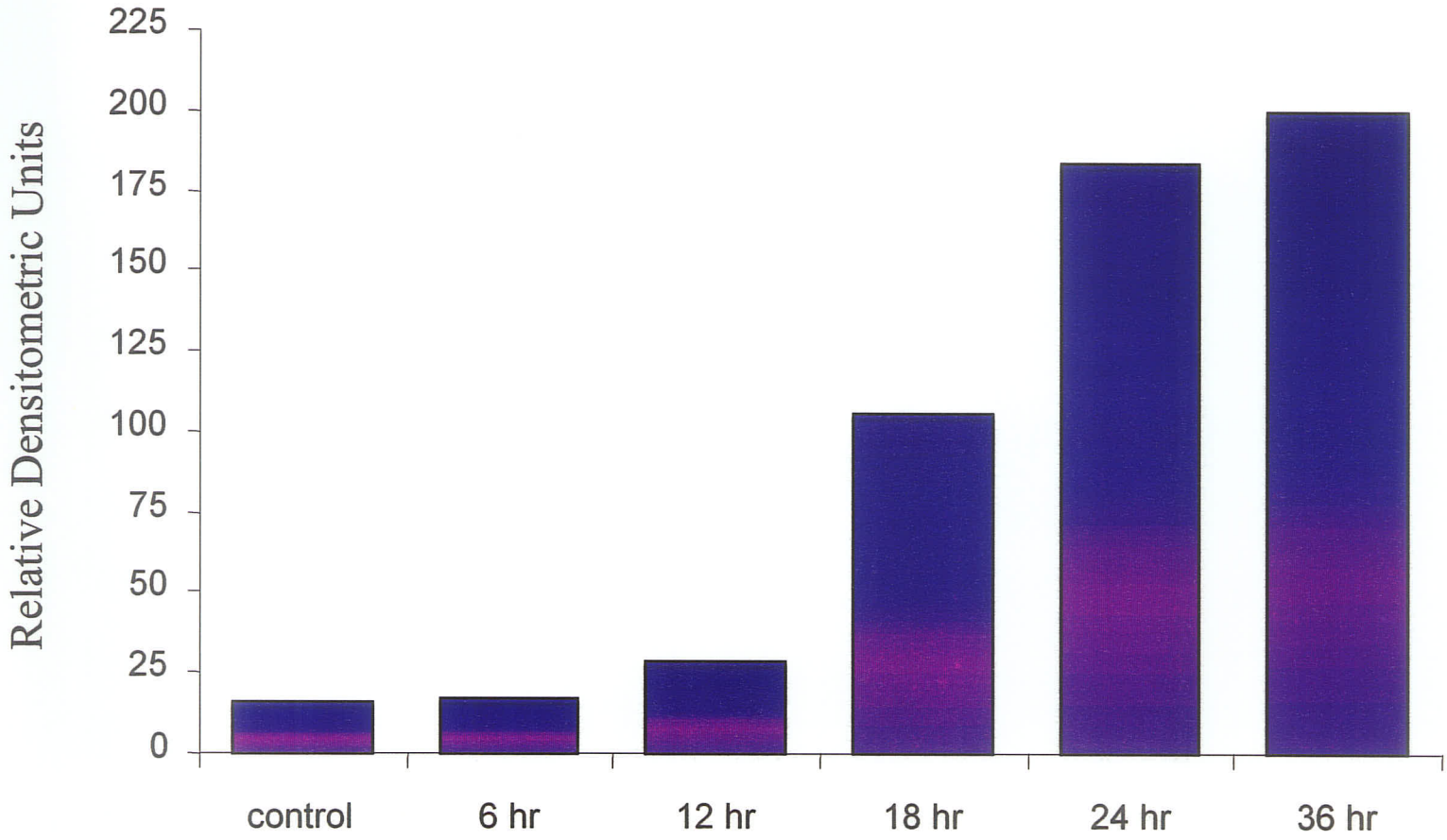

Figure 17: Histogrammatic depiction of Western analysis of time course transfection of pCMV5β-mycFAST2 into COS-7 cells. COS-7 cells were transfected by lipofection techniques, nuclear extract obtained and immunoreactive FAST2 was quantified in extract samples. To differentiate between exogenous (i.e., myc labeled) and endogenous FAST2 protein, membranes were probed with myc primary antibody (1:1000).

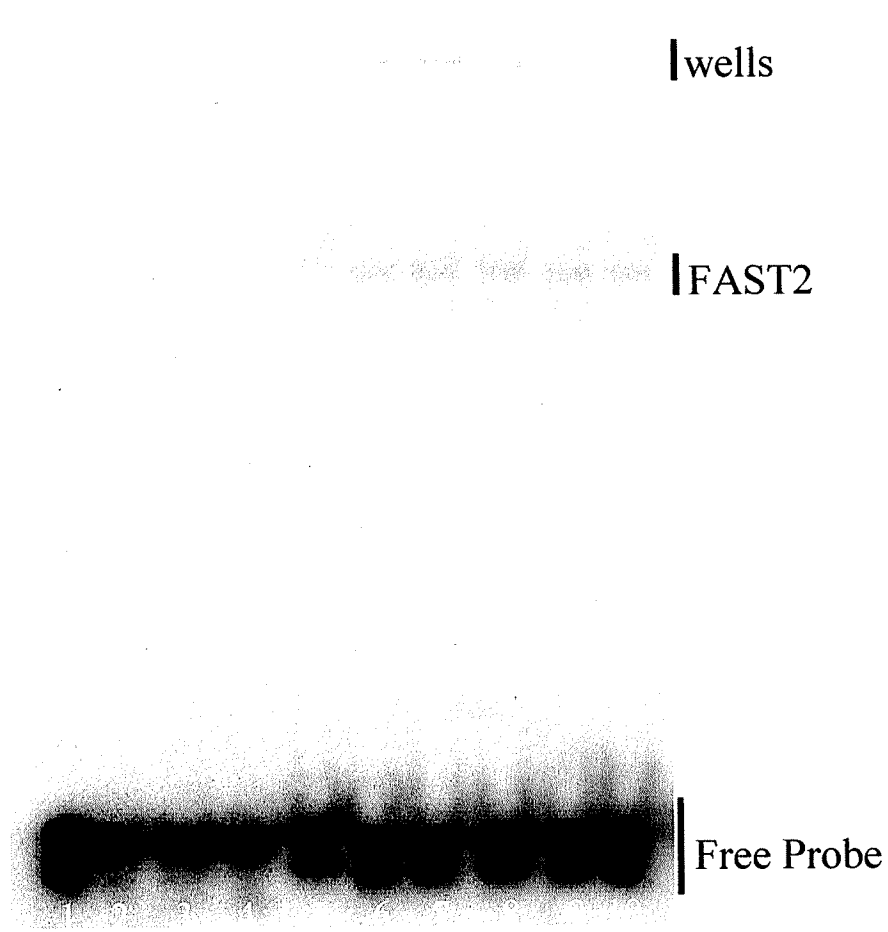

Figure 18: Electromobility shift assay. COS7 cells were transfected with pCMV5β-mycFAST2 and the nuclear fraction was extracted. Protein/DNA complexes were visualized via phosphor-imaging techniques. Free probe was run to orient the gel *(lane 1).* Hot and cold probe competition was used to verify the band of interest at 1:100, 1:200, and 1:500 ratios *(lanes 2-4 respectively).* Varying concentrations of nuclear isolate loaded to assay for favorable conditions *(10ug, lane 5; 15ug, lane 6; 20ug, lane 7).* Primary antibody raised against the myc epitope was used in varying amounts for the supershift in wells loaded with 15ug of nuclear extract *(3ug, lane 8; 5ug, lane 9; 10ug, lane 10)*

VII. DISCUSSION

The major findings of the current work are 1) expression of immunoreactive FAST2 was revealed in cytosolic and nuclear fractions of adult cardiac fibroblasts, 2) that treatment of cardiac fibroblasts with a well-known cardiac trophic agent (Ang II) led to a rapid increase in translocation of immunoreactive FAST2 to the cellular nuclei which is correlated to enhanced production of intracellular procollagen monomers *vs.* basal expression, 3) that overdriven exogenous FAST2 expression in primary fibroblasts is positively correlated to enhanced production of intracellular procollagen monomers *vs.* that in non-transfected cells, 4) that combined losartan and INF-γ treatment of cardiac fibroblasts is associated with both reduced procollagen expression and FAST2 nuclear localization *vs.* untreated cells, 5) that FAST2 forms a complex with the R-Smad Smad 2 and is involved in TGF-β/ Smad signaling, and 6) that FAST2 binds directly to the promoter region of the procollagen gene and may therefore function as a co-activator of procollagen expression.

The pathogenesis of many different types of heart failure is marked by enhanced deposition of cardiac matrix. For example, matrix remodeling of the infarct site as well as in remote surviving myocardium after a relatively large myocardial infarction is a dominant feature of post-MI heart disease, and these events herald the onset of heart failure in the clinical and experimental models **(86, 94)**. In the sequelae of events that succeed cardiac parenchymal tissue damage, monocytes and macrophages are recruited to the site of injury where they

become activated. Within 3-7 days of the initial infarct, fibroblasts arrive at the site of repair where they may undergo phenotypic transformation to myofibroblasts **(70, 233)**. In both healthy and diseased cardiac tissues, fibroblasts and myofibroblasts are the major cell types responsible for the synthesis and metabolism of fibrillar collagens **(61)**. A significant contribution to the pathology of the heart following MI is attributed to excessive fibroblast activity, and we have focussed on these cells in the present study **(217)**. Studies performed in our laboratory have previously observed an increased level of active TGF-β_1 in post-MI hearts **(86)**. TGF-β_1 is a potent stimulant of cardiac matrix deposition via signaling through the Smad family of effector proteins **(86, 135)**. The promoter regions of many constitutively expressed genes, including that of fibrillar collagen type I, contain Smad binding elements [Fig. 19] **(77)**. As R-Smad 2 cannot bind to the DNA promoter region of cardiac target genes with high affinity due to its unique amino acid sequence at the N-termini, recent evidence has led to a general understanding that the inclusion of an additional co-activator (or co-repressor) is required for effective R-Smad DNA binding. This co-factor may also influence target gene expression **(11, 122)**. In this respect, several co-activators have been identified (FAST 1, FAST2, TFE3, AP-1, ATF2) which function by associating with the R-Smad/ Co-Smad dimer to regulate Smad signaling in embryonic tissues **(158)**. Northern blot analysis has revealed that FAST2 is expressed within specific temporal points during mouse embryo development **(122)**.

aaaaagaatg gaaccaattt aagaagccct gtagccacgt ccctcccccc tcggctccct

cccctgcgcc cccgcagtct cctcccagca ccgaggcctg ggccctggct gccctcccat

tggtggagac gtttttggag gcacctccgg ctggtgaaac ttttccatata taaatagggc

aggtctgggc tttattattt tagcaccacg gcagcgggag gtttcgacta agttggaggg

aacggtcacg atcgatgcct cggcccgcca ggtgatacct ccgctggtga cccaggggct

ctgcgacaca aggagtctgc atgtctaagt ggtagacatg ctcagctttg tggatacgcg

aactctgttg ctgcttgcag taacgtcgtg cctagcaaca tgccaa

- GenBank accession number X66209

SBE

a

SBE

FBS

SBE

SBE

Figure 19. DNA sequence of Rat α-2(1) procollagen promoter contains a *FAST2 Binding Site (FBS) and Smad Binding Elements (SBE)*

However the presence of a FAST2 binding domain [Fig. 19] within the promoter region of the constituitive transcriptionally active adult rat collagen α-2 (I) DNA [Genbank accession number X66209] provided a rational basis for the hypothesis that this cofactor may be utilized by adult fibroblasts in the regulation of collagen expression. The current results support this hypothesis.

Using COS-7 cells (a transformed cell line derived from African green monkey kidney fibroblasts known for their ability to readily incorporate exogenous DNA) we demonstrated that in pCMV5β-mycFAST2 transfected cells FAST2 protein localized predominantly in the nucleus. Observing the co-localization of the myc-tag by double-immunostaining assays validated the detection of FAST2. We determined that FAST2 protein can be detected by both commercially available (Santa Cruz) and an independently produced FAST2 primary antibody (laboratory of Liliana Attisano, U of Toronto) [Fig. 6].

To address the question of whether FAST2 protein is expressed in adult cardiac fibroblasts, we chose to use cultured adult primary cardiac fibroblasts obtained from male Sprague-Dawley rats as our model. This model allows us to eliminate confounding influences on collagen synthesis found *in vivo,* such as uncontrolled stimulation of the effector hormones of the RAAS, arterial hypertension, or elevated coronary perfusion pressure which contribute to perivascular fibrosis of intramural coronary arteries **(30).** It may also be argued that the usage of PARCFs *vs.* cultured fetal or neonatal cells is a better approximation of the physiological reality of those fibroblasts contributing to cardiac fibrosis. Cultured adult cardiac fibroblasts have been observed to express

mRNAs for collagens type I and III and actively secrete mature fibrillar collagens **(13, 61)**. With this knowledge and using Western blot analysis, we observed that FAST2 protein is expressed in PARCFs. The staining pattern revealed by our antibody was marked by brightly stained cellular nuclei with lesser staining within the cytosolic space [Fig. 4]. Baseline steady-state FAST2 expression in adult cardiac fibroblasts is undefined, and our data extends the current literature in this respect. A role for FAST2 in development is supported in recent studies, wherein these investigations used murine embryonic cells and embryonic carcinoma cells as models **(122)**. In the current study, we confirmed the rapid induction of procollagen synthesis with Ang II stimulation in PARCFs **(30)**. We also sought the relationship between FAST2 protein activation (i.e., nuclear translocation events) and Ang II stimulation in these cells **(86)**. We have previously shown that angiotensin II upregulates the expression of the key players of the TGF-β/ Smad signaling pathway (active TGF-β_1, Smad 2, -3 and -4) during the chronic phase of myocardial infarct scar healing. We have also demonstrated that chronic AT_1 antagonism via losartan treatment normalizes expression of Smad 2 expression and increased collagen turnover **(84, 255)**. These results indicate that the TGF-β signaling pathway components may assist in ECM remodeling and regulate scar formation via their activation but as previously discussed require the presence of an additional partner. Following 15 minute Ang II stimulation (10^{-6} M) in PARCFs we noticed a slight decrease in immunoreactive FAST2 staining within the cytosol. This observation may indicate a translocation of FAST2 from the cytosol into the nuclear region upon

stimulation. Western analysis of the PARCF nuclear fraction indicated that FAST2 expression is significantly increased when compared to control values. Although our results imply a cytosolic/ nuclear translocation event, we cannot rule out an increase in FAST2 bioavailability from an undefined cellular compartment as the decrease we observed in cytosolic expression was modest. These results are substantiated by immunocytochemical analysis [Fig. 9], which demonstrate FAST2 concentrating within the nucleus following identical stimulation procedures.

TGF-β_1 is a potent inducer of fibrillar collagen production and other principal ECM components in various cell types **(156)**. It has been shown previously that in cultured cardiac fibroblasts, TGF-β_1 stimulates collagen deposition and augments the synthesis of fibronectin and proteoglycans thus mimicking the changes which transpire during the development of cardiac fibrosis **(41, 92)**. It is conceivable that activation of TGF-β_1 by Ang II also effected FAST2 in ensuing stimulatory cellular events. Similar expression trends of FAST2 and procollagen were noted utilizing Western analysis of total protein from cultured cardiac fibroblasts (although changes in band intensity of total cell immunoreactive FAST2 were modest in very short stimulation times). However, the assay used in this experiment is not designed to test movement or activation of FAST2. On the other hand, in another series of studies, simply over-driving FAST2 protein *in vitro* by pCMV5β-mycFAST2 transfection established a causative sequelae of events, linking enhanced nuclear FAST2 and procollagen synthesis.

It is known that TGFβ and INFγ have opposing effects on a diverse array of cellular functions **(14)**. TGF-β signals through serine-threonine kinase receptors while INF-γ activates the transcription factor Stat1 through the INF-γ receptor along with a tyrosine kinase associated protein Jak1 **(101, 213)**. It has been demonstrated that through the Jak1/ Stat1 signaling pathway, INF-γ induces the expression of the inhibitory Smad, Smad 7 **(224)**. We have shown (Wang, *et al,* submitted) that an increase in Smad 7 expression is associated with decreased collagen synthesis in adult rat heart 4 and 8 week post-MI. There is evidence of interplay between the TGF-β/ Smad and Smad/ JNK signaling pathways **(63)** and that this cross-talk between the pathways leads to the inhibition of Smad 2 phosphorylation **(224)**. Recent findings however, refute this ideology and hypothesize the interplay occurs at the gene promoter site level **(78)**. We wished to observe the effect of TGF-β signaling blockade on the expression of FAST2 and procollagen following Ang II stimulation. Western blot anaylsis of adult rat primary cardiac fibroblast nuclear lysate [Fig. 13, lane 4] showed that those cells treated 1 hour prior to Ang II stimulation with INF-γ (250 IU/ml) and losartan (10^{-6} M) exhibited attenuated immureactive FAST2 was significantly below decreased *vs* control. Histograms of the quantified data [Fig. 14] show that this decrease in expression is significant when compared to both Ang II stimulated cells and control levels. Treatment with losartan alone [Fig. 13, lane 3 and Fig.14] did not significantly lower FAST2 expression *vs.* control values. Immunofluorescence studies [Fig. 15] corraborated the results of the Western blot study. Cells treated with Ang II + losartan were found to have markedly reduced

expression of procollagen when compared to both control and Ang II stimulated groups. Decreased procollagen expression was marked in cells treated with INF-γ prior to stimulation; a decrease in FAST2 nuclear localization was also observed in these cells. These data indicate that FAST2 activation (i.e., nuclear translocation) in primary adult rat cardiac fibroblasts is robustly regulated via the R-Smad linked signaling pathway. Further experiments examining the effect of INF-γ alone on these paramerters will be performed in the future.

MASGWDLASTYTPTTPSPQLALAPAQGYLPCMGPRDNSQLRPPEAESLSK

TPKRRKKRYLRHDKPPYTYLAMIALVIQAAPFRRLKLAQIIRQVQAVFPFF

RDDYEGWKDSIRHNLSSNRCFHKVPKDPAKPQAKGNFWAVDVSLIPAEAL

RLQNTALCRRWQNRGTHRAFAKDLSPYVLHGQPYQPPSPPPPPREGFSIKS

LLGDPGKESTWPQHPGLPGQSTAAQAGTLSKGEEGMGTGPSSSSETPLWPL

CSLPGPTIIEGESSQGEVIRPSPVTPDQGSWPLHLLEDSADSRGVPRRGSRAS

LWGQLPTSYLPIYTPNVVMPLATLPTTSCPQCPSSASPAYWSVGTESQGSQD

LLCDLDSLFQGV**PPNK**SIYDVWVSHPRDLAAPAPGWLLSWYSM

SID

- GenBank accession number AF069303

Figure 20. The amino acid sequence of FAST2 contains a *Smad Interacting Domain* (SID) at the N-terminal domain.

Although our previous Western and immunofluorescence data indicate that rapid FAST2 nuclear translocalization is positively correlated to procollagen synthesis, these data were limited in explanation to only associative events. We wished to examine, in a direct manner, the possibility that FAST2 is a modulator of the TGF-β/Smad signaling pathway in cardiac fibroblasts. We determined the presence of a Smad interacting domain within the amino acid sequence of FAST2 protein [Fig. 20] **(35)**. An association of these two proteins would indicate a direct involvement of FAST2 in this pathway. In studies wherein we employed co-immunoprecipitation techniques, this hypothesis was confirmed as we showed that FAST2 bound Smad 2 [Fig. 16]. Increasing the amount of Smad 2 antibody (used to precipitate complexing proteins) and increasing the volumes of preciptated complexed proteins loaded in the Western gel wells was also associated with a linear increase in detectable FAST2. This provides support for the hypothesis that FAST2 associates with the Smad 2 protein with 1:1 stoichiometry. Although the experiments were not performed, it is also hypothesized that other Smad proteins (Co-Smad 4) and transcriptional cofactors (p300/CBP) may be present in the Smad 2 complex. This FAST2/Smad 2 protein association, however, does not prove that FAST2 is directly involved in the process of procollagen transcription.

As FAST2 may function in PARCFs as a transcriptional co-activator we chose to investigate whether FAST2 directly binds the promoter element within the procollagen gene. Despite one group's efforts to show that FAST2 mRNA was not detectable in adult mammalian tissues, we confirmed the presence of a

FAST2 binding site (AATxxACA) in the mammalian procollagen 5' flanking region along with proximal SBE sites [BLAST search; Fig. 19] **(121)**. We synthesized a 20-mer oligonucleotide identical in sequence to a segment of the rat α-2(1) procollagen promoter which contains the putative FAST2 binding site. When combined with nuclear extract isolated from FAST2 transfected COS-7 cells, one specific band was observed. The results of the EMSA and supershift assays indicate that FAST2 protein binds directly to the procollagen promoter [Fig. 18]. Therefore we conclude that FAST2 may be a transcriptional co-activator of procollagen synthesis in mammalian fibroblasts. To date, no other co-activating transcription factors have been identified that are involved in Smad signaling in PARCFs that may regulate procollagen synthesis.

We have identified a novel protein in the adult myocardium and have begun to address its role as a transcriptional co-activator of the procollagen gene. Our findings do not rule out the possibility that other Smad cofactors are present and operating in cardiac fibroblasts. A major avenue for conventional treatment of patients suffering from post-MI heart failure consists of administration of either ACE inhibitors, AT_1 receptor antagonists or their combination. Among other end-points, these treatments are used to ameliorate ongoing ECM remodeling in the interstitium of myocardium remote to the infarct scar as well as the infarct scar itself following infarction. Nevertheless it is becoming evident that crosstalk between various pathways may allow Ang II/TGF-β signaling to bypass these blockades and allow scar formation to occur regardless **(85)**. By delving more deeply into the effector proteins of the Smad signal pathway and designing

methods to abrogate or interfering with their function, blockade of the net endpoint of those signals could be achieved; as a result, blockade bypass is less likely to occur. Therefore the clinical implications for an understanding of transcriptional control of procollagen are relevant as we have shown that modulation of this protein will affect procollagen expression levels. Therefore these findings lend themselves to possible theraputic and preventative modalities for fibrosis in those patients suffering from MI, which is a necessity to ease the socioeconomic burden in industrialized nations. Further elucidation of the components involved in the Smad signaling pathway will also be of great importance and are currently being investigated.

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