The effect of myocardin and Smad3 overexpression in ventricular myofibroblasts: cellular contractility and collagen production

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List of Abbreviations

α-SMA	Alpha smooth muscle actin
ACE	Angiotensin converting enzyme
AT_1	Angiotensin 1
BMP	Bone morphogenic protein
Co-Smad	Common Smad
CT-1	Cardiotrophin-1
GDF	Growth and differentiation factor
gp130	Glycoprotein 130
HAT	Histone acetyltransferase
I-Smad	Inhibitory Smad
LIF	Leukemia inhibitory factor
PDGF	Platelet derived growth factor
MI	Myocardial Infarction
MRTF-A	Myocardin related transcription factor A
MRTF-B	Myocardin related transcription factor B
NES	Nuclear export signal
NLS	Nuclear localization signal
PAI-1	Plasminogen activator inhibitor-1
R-Smad	Receptor Smad
SBE	Smad binding element
SCF	Stem cell factor
SRF	Serum response factor

SSXS	Serine serine any amino acid serine
TAD	Transcriptional activation domain
TGF-β	Transforming growth factor beta
TGF-βRI	Transforming growth factor beta receptor I
TGF-βRII	Transforming growth factor beta receptor II
TRAP-1	Transforming growth factor beta receptor I associated protein 1

I Abstract

The incidence of cardiovascular disease has reached epidemic proportions in North America. Specifically, myocardial infarctions (MI) are a major contributor to heart failure which greatly influences morbidity and mortality rates in developed nations. In the post-MI heart, cardiac fibroblasts migrate to the damaged area, convert to myofibroblasts and contribute to infarct scar contraction. As well, cardiac myofibroblasts are hypersynthetic for matrix components eg, collagen, and *de novo* production of fibrillar collagens lessens the chance for acute scar rupture. TGF-β_1 is important in the initiation of cardiac healing and fibrosis. Canonical TGF-β_1 signaling occurs with the activation of receptor-operated Smads (R-Smads) including Smad3. The current study addresses the question of whether Smad3 and/or myocardin influence myofibroblast contractility. We believe that myocardin is a Smad3 binding partner and cofactor and thus contributes to Smad associated healing and fibrotic events in the heart. In mesenchyme-derived cells, myocardin exists as a nuclear protein and is a cardiac and smooth muscle specific transcriptional coactivator of serum response factor (SRF). This transcription factor has been shown to bind to Smad3 in COS-7 cells (a green monkey kidney fibroblast-like cell line) and we suggest that it may contribute to fibroproliferative events. Precisely how Smad3/myocardin facilitates post-MI wound healing and/or contributes to inappropriate post-MI fibrosis is unknown.

Very little work has been done to address myocardin expression in cardiac ventricular myofibroblasts. While a number of previous studies address TGF-β/Smad signaling in cardiac myofibroblasts, none have addressed the effects of overexpressed Smad3 on cellular contractility and collagen secretion. As Smad3 and its endogenous inhibitor Smad eg, I-Smad7, contribute significantly to TGF-β signaling in myofibroblasts, we rationalize that they must be important in

the regulation of many fibroproliferative processes. Our goals were first to measure/determine myocardin expression in primary ventricular myofibroblasts; second, to explore a putative interaction between Smad3 and myocardin; third to examine a possible link between TGF-β_1 stimulation, myocardin and Smad3. Finally, we sought to examine the effect of overexpressed Smad3, Smad7 and myocardin on contractility and collagen production. These experiments were conducted by using RT-PCR, co-immunoprecipitation, adenoviral overexpression of Smad3, Smad7 and myocardin, Western blot analysis, collagen gel deformation assays (contractility studies) and finally, Pro-collagen 1 N-terminal Peptide (P1NP) secretion as a measure of mature collagen production.

We document the novel expression of myocardin in ventricular myofibroblasts and provide evidence that myocardin may serve as a Smad3 cofactor in cardiac myofibroblasts. Further, myocardin overexpression is linked to increased contractility in myofibroblasts compared to LacZ infected controls, and that TGF-β_1 acutely stimulated myocardin expression followed by a dramatic reduction 1 hour thereafter. Overexpressed Smad3 alone led to increased contractility in primary ventricular myofibroblasts. Thus the effect of increasing myocardin expression had a comparable effect to that of increased Smad3 alone with this endpoint. Finally, overexpression of both Smad3 and myocardin in the presence of TGF-β_1 led to an additive stimulation of contractility in cells when compared to the effect of TGF-β_1 stimulation alone. Overexpressed Smad7 alone was associated with decreased secretion of type I collagen when compared to the control; when cells overexpressing Smad7 are stimulated with TGF-β_1, collagen secretion is dramatically reduced when compared to cells treated with TGF-β_1.

In an addition series of experiments we addressed reverse mode NCX1 function as a means of Ca^{2+} entry to the cytosol of myofibroblasts upon their excitation. We have previously shown the

stimulatory effect of TGF-β_1 on myofibroblast contractility, and we now report that overexpression of Smad3 alone led to increased mRNA expression of NCX1. Thus it is possible that TGF-β_1 signaling via Smad3 may influence Ca^{2+} movements and thus contractile performance in ventricular myofibroblasts.

II Introduction

Cardiovascular disease is the number one killer in Canada and is a risk factor with the ability to cause many secondary ailments. This disease is therefore a concern for millions of Canadians (Heart and Stroke Foundation of Canada http://ww2.heartandstroke.ca/Page.asp?PageID=1366&ArticleID=5533&Src=blank&From=SubCategory). Although heart failure can develop secondarily to a variety of ailments, a MI is the most common origin. Post-MI, the heart undergoes significant remodeling of the extracellular matrix and of the myocytes themselves. During this remodeling, a phenotypic switch from fibroblasts to myofibroblasts occurs. Myofibroblasts contribute to the healing of the damaged heart tissue – they are hypersynthetic for most matrix component proteins and their appearance is associated with elevated fibrillar collagens in the atria and ventricles following a MI (1;2). The healing of the infarct scar is influenced by myofibroblasts not only via increased deposition of matrix (which protects from ventricular rupture) but also their contractilily contributes to scar shrinkage in the weeks and months following a MI (3). Further, myofibroblast contraction may contribute to inappropriate wound healing beyond the point of "normal" healing as contraction in lung myofibroblasts has been recently associated with conversion and release of latent TGF-β_1 normally held in the surrounding matrix (4). Although this initial collagen deposition is advantageous, myofibroblasts continue their secretion and deposition of collagen to the point where it causes adverse effects. This excess collagen causes the heart to physically stiffen, leading to global impairment of cardiac pump function – continued cardiac fibrosis exacerbates nonspecific interstitial fibrosis, and this contributes to decompensated heart failure (5).

Myocardin is a transcription factor that is a cardiac restricted nuclear protein and has been shown to have an effect on cytoskeletal regulation, cell proliferation and cardiac

hypertrophy (6;7). Studies have proven that myocardin is a transcriptional co-activator of SRF (Serum Response Factor) in smooth muscle cells (8) but its expression in ventricular myofibroblasts is unknown. In smooth muscle cells, myocardin binds to Smad3 and may be able to function without SRF or a CArG (C and A rich G sequences) box in this manner (9). Recent work has indicated that myocardin may be directly implicated in the fibroblast to myofibroblast conversion in ventricular fibroblasts, as its forced induction is associated with the secondary upregulation of muscle-like genes including SERCA2, L-type Ca^{2+} channels in fibroblasts, and K_{ir} channels (10). While little is known about the mechanisms of intracellular Ca movement associated with migration, secretion, and contraction, ventricular fibroblasts and myofibroblasts are known to express Na^{+}-Ca^{2+} Exchanger type 1 (NCX1) (11). Romero and coworkers have shown that reverse-mode NCX1 function is important for Ca^{2+} entry to human lung myofibroblasts in response to TGF-β_1 activation of cells (12). As this cytokine is among the most powerful stimuli for secretion of fibrillar collagen in heart (13), we suggest that there is a linkage between Ca^{2+} entry and fibroproliferative events in these cells.

The TGF-β/Smad signaling pathway is an important player in the healing process in post-MI heart. Specifically, TGF-β_1 is a pro-fibrotic cytokine that is responsible for the differentiation of fibroblasts to myofibroblasts, ventricular remodeling, a down-regulator of proliferation and is involved in the development of cardiac hypertrophy (14;15). Smad3 is a receptor Smad involved in this pathway that when activated by TGF-β, binds to co-Smad4 and is translocated into the nucleus where it is involved with the transcription of various genes, including those involved with contractility and fibrosis (16;17). Another component of this pathway is Smad7 - which functions as an inhibitory factor that blocks the activation of receptor Smads. This is accomplished by Smad7 interacting with the activated TGF-βRI and forming a stable complex

which inhibits the phosphorylation and therefore activation of the R-Smads (18;19). The role that Smad7 plays in contractility and the development of fibrosis is not fully understood but there is evidence that there are decreased levels of Smad7 in scar tissue (20). Smad7 has also been proven to have the ability to inhibit the contractility of myofibroblasts when stimulated with TGF-β_1 (21).

Currently there is very little data that addresses the mechanisms via which R-Smad eg, Smad3, and myocardin influence myofibroblast contractility and collagen secretion. The current thesis was carried out to address specific questions surrounding direct effects of Smad3 on these endpoints, and to also explore the function of myocardin in ventricular myofibroblasts. We also investigated the effects of adenoviral overexpression of myocardin, Smad3 and Smad7, separately and in combination, on contractility and collagen secretion, with and without TGF-β_1 stimulation.

We have observed that myocardin is expressed in ventricular myofibroblasts and specifically binds to Smad3. We also discovered that Smad3 and myocardin separately stimulate contractility of cardiac myofibroblasts. As well, when Smad3 is co-overexpressed with Smad7 and stimulated with TGF-β_1, Smad3 overrides the inhibitory effects of Smad7 on contractility and is comparable to that of TGF-β_1 stimulated control cells. We also found that overexpressed Smad3 is associated with elevation of NCX1 mRNA expression. In other studies we confirmed that overexpressed Smad7 alone decreased collagen secretion when compared to the controls, and that this treatment markedly reduces collagen secretion normally evident with TGF-β_1 stimulation. Finally, when cells overexpressed with both Smad3 and Smad7 and stimulated with TGF-β_1, the inhibitory effects of Smad7 were no longer obvious.

III Statement of Hypothesis

Previous studies have shown that Smad3 and myocardin bind and function as cofactors in COS-7 cells (9). Other studies indicate that myocardin participates in TGF-β_1 signaling and can synergistically stimulate Smad3-induced Smad Binding Element (SBE) promoter activity that is independent of a CArG box (9) in smooth muscle cells. We hypothesized that myocardin would bind to both phosphorylated and non-phosphorylated Smad3 in primary ventricular myofibroblasts, and that these proteins are cofactors in these cells. A corollary of this working hypothesis is that p-Smad3 and myocardin will show similar expression responses to TGF-β_1. Previous work performed in our lab has shown that cells overexpressing Smad7 inhibit the rate of contraction when stimulated with TGF-β_1 (21). Thus we hypothesisized that both overexpressed Smad3 and myocardin with TGF-β_1 stimulation would stimulate contractility in ventricular myofibroblasts, and that overexpressed Smad3 expression will increase type I collagen secretion from these cells.

IV Literature Review

1.0 Heart disease and its impact on society

Cardiovascular disease accounts for more deaths in Canada every year than any other disease and is responsible for 74,626 deaths up to the year 2002 (the most recent available data year Statistics Canada) (http://ww2.heartandstroke.ca/Page.asp?PageID=33&ArticleID=1077&Src=news&From=SubCategory). Heart disease is a major risk factor for millions of Canadian citizens and there are more than 75,000 heart attacks per annum, with 1 in 3 of those people dying due to complications from heart disease every year (Heart and Stroke Foundation of Canada http://ww2.heartandstroke.ca/Page.asp?PageID=1366&ArticleID=5533&Src=blank&From=SubCategory). Cardiovascular disease also imparts a large economic burden on the health care system, requiring $18 billion a year which includes the price of direct medical costs and supplies as well as indirect medical costs like disability payments. Given the financial burden that is caused by cardiovascular disease, the research and development of new treatments and medicines will lessen that burden as well as treat this disease with more efficient medications and medical procedures.

1.1 Development and causes of heart disease

Heart disease can be caused by a variety of different pre-existing conditions including hypertension, cardiomyopathies and myocardial infarction (MI) but there are many other factors that play into whether a person will develop heart disease. Many lifestyle choices affect a person's chances of developing this disease, for example, poor diet, little exercise and smoking. Uncontrollable factors such as genetics, sex and age may also contribute to a person developing the above conditions. Although many diseases can contribute to heart failure and death, the most

overwhelming contributor is MI (22). The high incidence of MI provides ample rationale to investigate post-MI events that contribute to heart failure, including remodeling of the extracellular matrix (matrix), myofibroblast function, collagen production and the development of fibrosis and healing of the infarct scar.

1.2 Myocardial infarction

MI occurs when the blood supply to the heart is reduced (ischemia) to less than its normal blood flow which causes a lack of oxygen and nutrients required in order to maintain normal function. The blood supply is typically restricted in the left coronary artery (23) and this can occur because of a blood clot or an atherosclerotic plaque that has narrowed the vessel to the point where blood flow is restricted to dangerous levels (24). Abnormalities in the function of the heart are typically seen within 24-48 hours after the MI but can be seen at even earlier time points (25). Once blood flow has been restored eg, the blood clot has been dissolved or a portion of the plaque has broken free allowing an increase in blood flow, oxygen and nutrients are reperfused back into the tissue which causes a sudden production of free oxygen radicals (23). Free oxygen radicals can contribute to the formation of detrimental substances which can promote signaling in apoptosis pathways as well as further cellular damage and cell death in the myocardium (23).

1.3 Initial wound healing in the damaged myocardium

The ischemic conditions produced during a MI causes damage to the tissue in the heart which causes the heart to undergo four main phases of wound healing. The first phase is the death of the cardiomyocytes which can occur by two pathways: apoptosis or necrosis. The peak

of cardiomyocyte apoptosis occurs at 6-8 hours after a MI and the peak for necrosis occurs between 12 hours to 4 days after a MI (26). The death of cardiomyocytes releases many different substances into the bloodstream like creatine kinase, troponin-T and fatty acid binding protein (27) which act as markers for repair and are also responsible for initiating the second phase of wound healing.

The second phase of healing is marked by an immune response where monocytes begin to enter the area only 1 hour after the damage has occurred. When the monocytes actually reach the damaged area, the monocytes convert into macrophages by a phenotypic switch (25). Granulocytes migrate into the area of infarction as early as 6-8 hours post-MI with peak migration at 24-48 hours post-MI. Granulocytes are responsible for removing dead myocytes and at this time other immune system components including macrophages, lymphocytes and plasma cells migrate to the same area and help the granulocytes as well as increase their efficiency (27). The substances listed in the first phase (creatine kinase, troponin-T and fatty acid binding protein) which were released into the blood stream also cause the body to have another type of immune response which begins between 12–16 hours after an MI (27). Specific cytokines of the immune system are released in this immune response like interleukin 6 and 8 (IL-6 and IL-8) as well, another section of the immune system is activated called the complement system which is responsible for helping the body remove dead cells and debris (27-29).

The third phase of healing begins at 48-72 hours after infarction where the deposit of new extracellular matrix proteins can be seen in the border of the infarcted tissue first and then within the infarcted tissue itself (27). This new tissue is granulation tissue which creates new capillaries that grow and enter the wound (30). The matrix proteins help prevent the infarcted zone from rupturing as well as increasing the strength of the granulation tissue. Fibrin is the first protein to

be deposited which is followed by other proteins such as fibronectin (31;32). Just a few days post-MI, myofibroblasts have migrated into the area of infarct and have started to produce and deposit various types of collagens, mainly types III and I (27). Cross-linking of all of the deposited collagens takes a few weeks to complete and increases the tensile strength of the fibers to help the healing of the infarct (33). In order to promote the healing process, collagen turnover in the damaged myocardium occurs very quickly when compared to the normal rate of turnover which is approximately 4 months (34).

The fourth and final phase of healing is characterized by scar maturation. The scar tissue that is formed is directly proportional to the size of the original wound and is also related to how effectively the scar has contracted (35). This contraction is achieved by many different cell types leaving the infarct site, including the granulocytes which migrated to the site in the second phase, which is most likely carried out by apoptosis (36). The exceptions to this process are the myofibroblasts where the majority stay in the infarct zone and continue assisting in the healing process (37). The myofibroblasts help the damaged area regain some lost contractility due to cardiomyocyte death. Myofibroblasts can still be detected in the heart many years, even decades post-MI. It is important to note that since new cardiomyocytes are not created in order to replace the damaged ones, the scar tissue that was formed during the healing process is permanent (33).

1.4 Modifications in the post-MI myocardium

Studies have shown that the extent of the remodeling that occurs in the post-MI myocardium depends on the size of the infarct zone that is created during the event (38). Likewise, the extent of the hypertrophy that occurs is dependent on the size of the infarct (39). In the case of a large MI, severe hypertrophy occurs which causes a significant decrease in

cardiac function and performance. As well, the heart undergoes ventricular remodeling in order to increase the blood volume it is able to hold (40). Muscle fibers are also affected post-MI as they undergo a change in alignment which affects the efficiency at which the heart is able to pump. The composition of tissues also changes post-MI to drastically increase the amount of connective tissue that is present (41).

The infarcted area in the heart no longer contributes to the rhythmic contractions that the heart produces and the undamaged cardiac tissue now must compensate by adapting to these new conditions. The remaining cardiac tissue must now increase its workload in order to maintain the same functions as before the MI. The heart accomplishes this by changing the structure and thereby functions of the cardiomyocytes, fibroblasts and endothelial cells (42). Cardiomyocyte hypertrophy is initially favorable because it decreases the amount of wall stress which increases cardiac performance but in the long run progressive hypertrophy can lead to adverse effects and the development of heart failure. As well, cardiomyocytes undergo many changes including an increase in the synthesis of proteins, a sudden increase in the levels of heat shock proteins and proto-onco genes as well as changes in gene expression (2). Fibroblasts, which are responsible for the production and secretion of the components of the ECM, mainly secrete collagen type III and type I (27). As these components are deposited and new ECM is formed, it influences the mechanical signals that are sent from the ECM to the inside of the cell. The mechanical signals have an effect on the fibroblasts in which they undergo a phenotypic switch into myofibroblasts which produce large amounts of collagen (1;2). Endothelial cells cover the inner vascular vessel walls and multiply after an MI in an effort to compensate for the loss in cardiomyocytes but it results in the myocytes regressing back to a developing state (27). This state produces a

cardiomyocyte phenotype which requires less energy to function but also has a decrease in its overall performance (27).

1.5 Cardiac myofibroblasts

In a normal heart, cardiac myofibroblasts are present in the tissue and they help maintain the structure of the cardiomyocytes. Post-MI, cardiac myofibroblasts are responsible for producing and secreting collagen which initially helps prevent the scar from rupturing. Myofibroblasts were first discovered in 1971 and were defined as a cell that had the characteristics of both fibroblasts and smooth muscle cells (43). Through further research, myofibroblasts are currently defined as phenotypic variants of fibroblasts and although they are similar in many respects, there are many distinct differences between them. The main characteristic that differentiates the two are that only myofibroblasts express the smooth muscle embryonic isoform of myosin heavy chain (SMemb) (44). Myofibroblasts have many structural characteristics including: well-defined rough endoplasmic reticulum, granules that produce collagen that are made in its golgi apparatus and gap junctions (45). Molecular characteristics of myofibroblasts include the expression of vimentin, α-SMA, TGF-β receptors, AT_1 receptors, angiotensin converting enzyme (ACE), CT-1 and its receptor complex gp130, LIF receptors as well as fibrillar collagens (46-49). α-SMA is believed to be an important component of myofibroblasts because it has been suggested that it plays a role in its ability to contract and close the wound that is created during an MI (3).

Cultures of cardiac myofibroblasts can be achieved by plating out isolated cardiac fibroblasts in a media and serum environment *in vitro*. It has been suggested that cardiac fibroblasts will first differentiate into proto-myofibroblasts (partially differentiated fibroblasts)

before finally differentiating into myofibroblasts, when plated at low density *in vitro* (50-53) and when the correct cytokines are present. Proto-myofibroblasts can be seen in both *in vivo* and *in vitro* conditions and do not yet express αSMA (like fibroblasts) but can form stress fibers (like myofibroblasts) which contain β and γ forms of actin (50). Cytokines that are known to cause cardiac fibroblast differentiation are PDGF, stem cell factor (SCF) (54) and TGF-β (51). Another factor known to cause cardiac fibroblast differentiation is mechanical stress that occurs in *in vivo* conditions (50).

1.6 Wound contraction, collagen production and contractility

Wound healing is a complex process that can be defined as the edges of the wound coming together in order to minimize the area of the wound. It involves many different cell types, including fibroblasts and myofibroblasts, as well as various signaling molecules. While many of the components of this system are known, the exact interaction between the fibroblasts/myofibroblasts and the extracellular matrix must still be determined (55). There are two widely accepted but opposing theories on wound healing: the cell contraction theory and the cell traction theory. Many *in vitro* studies have been conducted in which one or the other of the theories is supported and the variability seen in these experiments are probably due to the use of different substrates. Therefore many more studies are required, especially *in vivo*, in order to draw any accurate conclusions.

The cell contraction theory states that myofibroblasts are responsible for pulling the edges of the wound together, contracting like smooth muscle cells do, by pulling the collagen fibers in towards the cell bodies (55). Conversely, the cell traction theory states that fibroblasts, not myofibroblasts, cause the closing of the wound by the tractional forces that are created when

they remodel themselves. It is has been suggested that these forces cause the gathering of collagen fibers which in turn causes the wound to contract (56).

In a study by McGrath and Hundahl, the level of myofibroblasts were studied in the granulation tissue throughout the entire wound healing process and they found the number of myofibroblasts correlated with the rate of contraction and therefore supported the cell contraction theory (57). However, after looking at all of the available data, they found their study actually combined the two theories by concluding that the fibroblasts begin the wound healing process by remodeling and initiating the preliminary closing of the wound but that the myofibroblasts were responsible, at least in part, for the wound contraction. Another independent study conducted by Darby *et al.* also supported these findings and conclusions (58).

2.0 TGF-β/Smad signaling

2.1 Introduction

TGF-β (Transforming Growth Factor Beta) is a multifunctional growth factor which is part of a large superfamily of signaling molecules consisting of smaller subfamilies including: BMPs (Bone Morphogenic Proteins), GDFs (Growth and Differentiation Factors), activins, Vg1 and TGF-β as well as others which are vastly different from the families named above. These signaling molecules are responsible for many cellular processes including proliferation, differentiation, wound repair, extracellular matrix remodeling, endocrine and immune system functions (59;60).

TGF-β is expressed in both cardiac myocytes and myofibroblasts (61) and is a stimulus for differentiation, an inhibitor of proliferation and acts as a pro-fibrotic cytokine causing the production and deposition of collagen (14). Malfunctions in the TGF-β signaling pathway can cause many different diseases including various immunological diseases, cancer, vascular

modifications and tissue fibrosis (62;63). Disturbances in the TGF- β pathway also contribute to ventricular remodeling, cardiac hypertrophy and the development of heart failure which includes transcription of various genes that play a role in cell proliferation, differentiation, motility and collagen production (15;46;61).

2.2 TGF-β isoforms and receptors

There are three different and distinctive isoforms of TGF-β which are TGF-β_1, TGF-β_2 and TGF-β_3 (64). Although it has been shown that all isoforms of TGF-β are very similar to each other in structure and function, the effects TGF-β has on a cell differs with cell type as well as with the effect of additional cytokines (64). TGF-β signaling requires two receptors, TGF-βRII and TGF-βRI, in order to bind TGF-β and transmit a signal. TGF-βRII receptors have three main domains: a short extracellular segment, a single-pass transmembrane region and a serine/threonine rich cytoplasmic region (60). This receptor has a molecular weight of 70 kDA and its serine/threonine rich cytoplasmic region is able to undergo autophosphorylation in order to become active and allow the active recruitment and dimerization with TGF-βRI (59). TGF-βRI is a 55kDA protein that has four main domains: an extracellular region, a serine-glycine rich region, a cytoplasmic segment and a serine/threonine kinase domain (59). The receptors, although similar in certain domains, have differences that allow them to be in two distinct classes. For example, these two receptors, share only 40% amino acid sequence homology in the kinase domains (60). These differences are believed to allow for specific cytokine/receptor interactions (59).

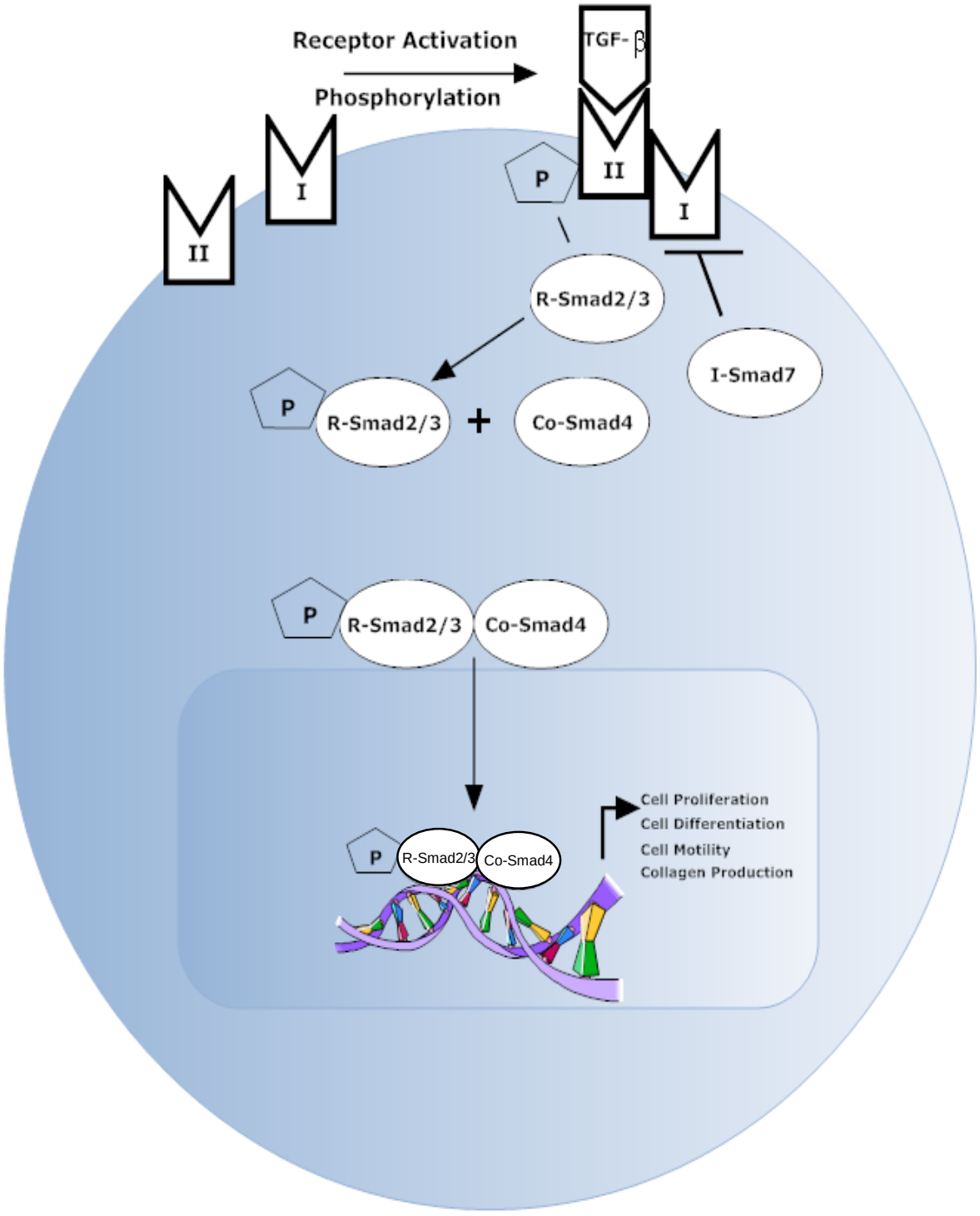

Figure 1. TGF-β1 signaling in cardiac myofibroblasts, with emphasis on the canonical R-Smad/Co-Smad pathway. Excitatory R-Smads bind to Co-Smad4 and translocate into the nucleus where they bind Smad binding elements in promoter regions in target genes. I-Smad7 inhibits R-Smad signaling. I – TGF-β receptor I; II – TGF-β receptor II; P – Phosphate group.

2.3 TGF-β signaling - receptor regulation

TGF-β binds to TGF-βRII which causes TGF-βRI to be recruited. Once they are in close proximity to each other, TGF-βRI become transautophosphorylated which causes the type I receptor to become activated and capable of transmitting the TGF-β signal (65). While in many different cell types the receptors are internalized and returned back to the surface, TGF-β signaling regulates the level of receptors by endocytosis which down-regulates the amount of available receptors and therefore down-regulates the TGF-β signaling and its effects when required (66).

2.4 TGF-β signaling proteins

TGF-β signaling is carried out by Smad proteins. Mammalian Smad proteins have their name derived from the first members of the family discovered, the products of *sma* genes in *C. elegans* and the *mad* genes in *Drosophila* (67). There are three distinct categories of Smad proteins: receptor Smads (R-Smads) which are Smad1-3, 5 and 8 and are activated by phosphorylation by the TGF-βRI, co-smad (Co-Smad4) which is responsible for translocating receptor Smads into the nucleus, and inhibitory Smads (I-Smads) which are Smad6 and 7 and act as a negative regulatory feedback loop. All Smad proteins have two domains: an N-terminal Mad Homology 1 (MH1) domain and a C-terminal Mad Homology 2 (MH2) domain. The MH1 domain is highly conserved between R-Smads and the Co-Smad whereas the I-Smads have only a low sequence similarity (67). I-Smads are similar to R-Smads in many respects but the MH1 domain in the I-Smad is poorly conserved (59).

2.5 The role of R-Smad3 in development of fibrosis and contractility

As described above, Smad3 is a R-Smad that when phosphorylated by TGF-βRI becomes activated. Once translocated into the nucleus, Smad3 has been shown to be involved in the transcription of many genes – including collagen (68) and α smooth muscle actin (69) - which are involved in the development of fibrosis and contraction. It has been shown that Smad3 null mice have a decreased mRNA expression for type I procollagen as well as hydroxyproline in the lungs when compared to the wild-type mice (17). Other studies have linked TGF-β_1 overexpression to tissue fibrosis, and that this link is ablated in Smad3 null mice (70).

Smad3 has also been shown to be involved in contractility. Previous studies in mouse embryo fibroblasts and lung fibroblasts have shown that Smad3 is responsible for TGF-β mediated contraction (16;69). Smad3 is a transcription factor responsible for regulating the transcription of α Smooth Muscle Actin (αSMA) which encodes for an important cytoskeletal contractile protein (69). Smad3 is expressed in myofibroblasts, which are responsible for wound healing and fibrosis (71). Myofibroblasts also possess contractile properties and express high levels of αSMA which is believed to be generally responsible for contraction (72-74). When TGF-β signaling occurs, Smad3 becomes activated and is translocated into the nucleus where it leads to the activation of αSMA transcription which is believed to give myofibroblasts increased contractility (69).

2.6 Smad7 involvement in the development of fibrosis and contractility

As explained above, Smad7 is an I-Smad that inhibits R-Smad phosphorylation and activation. Smad7 accomplishes this by interacting with the activated TGF-βRI and forms a stable complex which inhibits the phosphorylation and therefore activation of the R-Smads

(18;19). Smad7 has been shown to inhibit growth as well as stimulate apoptosis and morphogenesis in embryonic lung in the presence of TGF-β (75;76). Other cytokines that influence the activities of Smad7 are tumor necrosis factor α (TNF-α) and interferon γ (77;78).

The role of Smad7 in the development of fibrosis and contractility is still not completely understood. Smad7 expression in cardiac myofibroblasts is decreased in both the scar tissue as well as the remnant heart tissue which suggests that it may play a role in the development of fibrosis (20). As well, overexpressed Smad7 in cardiac myofibroblasts has been shown to reduce collagen synthesis when stimulated by TGF-β (20). Further studies are required in order to yield a better understanding of the functions of Smad7 in fibrosis and contractility.

2.7 Mechanism of R-Smad signaling

When TGF-βRI is phosphorylated, the receptor is then able to activate R-Smads with the help of two proteins which will then transmit the signal. The first protein that is required for R-Smads to become phosphorylated is SARA (Smad Anchor for Receptor Activation). SARA's function is to recruit R-Smads to the TGF-βRI by interacting with the receptor complex (79). The second protein is Disabled-2 (Dab-2) which facilitates the interaction of the TGF-βI receptor and the R-Smads and is required for the activation of the R-Smads (80). The R-Smads are phosphorylated at a SSXS sequence and are then active and thereby available to bind to co-smad4 (81). I-Smads do not contain this SSXS sequence and therefore cannot be phosphorylated (81) (59).

2.8 Entry of the R-Smad/Co-Smad4 complex into the nucleus

R-Smad phosphorylation is usually attended by heterodimerization with Co-Smad4 in the cytoplasm of the cell. Smad4 itself is not phosphorylated because it lacks the SSXS domain which allows phosphorylation to occur (82). Co-Smad4 requires a cytoplasmic protein called TRAP1 (TGF-βRI Associated Protein-1) which binds to inactive heteromeric TGF-β receptors and is released upon receptor activation (83). TRAP-1 transports Smad4 to R-Smads and then forms a heterotrimeric complex and will then be able to be translocated into the nucleus (84).

All nuclear transport occurs through the nuclear pores, but depending on the size of the substance the transport may be passive or active. Sequence analysis of the Smad proteins has shown that they do not contain any known traditional Nuclear Localization Sequence (NLS) but Smad3 does contain what is believed to be a nuclear localization signal in its N-terminal domain (85). Smad4 has been shown to contain a Nuclear Export Signal (NES) which is leucine-rich and controls the subcellular distribution of Smad4 (86).

Once the R-Smad/Co-Smad complex is inside the nucleus, the complex can interact and bind with DNA to influence the transcription of certain genes. Smads interact with DNA by binding with Smad Binding Elements (SBE) which have the sequence 5'-AGAC-3'. The interaction with the SBE is with the MH1 domain of the R-Smads as well as for the Co-Smads (62). SBEs have been found in many different promoter regions that are involved in the TGF-β pathway including: α2(I) procollagen, type VII collagen and PAI-1 (62). It has also been shown that the R-Smad/Co-Smad complex can bind DNA directly without SBE's with the help of transcriptional activators and other cofactors (62).

2.9 Regulation of R-Smad transcription

R- and Co-Smads are transcription factors that are able to bind DNA through Smad binding elements (SBE) that are located on the promoter regions of target genes (62). Both groups of Smads have been shown to bind using a β-hairpin loop which allows for the direct interaction of the Smads with the DNA (87). Smad3 and Smad4 have been shown to bind to GC-rich sequences which indicates that Smad-SBE binding may not be restricted to just the SBE sequence but further studies are required in order to determine what other sequences can act as SBEs (88). While it is not known if transcription factors are able to compete for binding with Smads, it is known that Smads bind to DNA with low affinity (87) so other transcription factors are required in order to stabilize the interaction as well as activate gene transcription (89). An example of this synergistic activity is the interaction of Smad2 and Forkhead Activin Signal Transducer -1 (FAST-1) being strengthened by the binding of Smad4 (59).

Transcription is also regulated by many activators and repressors which can increase or decrease the amount of a certain gene being transcribed. For example, CBP/p300 is a co-activator that is known to possess histone acetyltransferase (HAT) activity which interact with DNA and unravel the DNA by acetylating the core histones in order to allow it to be more accessible to transcription factors like R-Smads (90;91). Increased accessibility of DNA allows for better binding of transcription factors and therefore an increase in transcription is likely to occur.

3.0 Myocardin and MRTFs

3.1 Introduction

Myocardin, named for its critical function in gene expression in the myocardium as well as its apparently specific expression in cardiac tissues, was first discovered in 2001 by Eric Olson's group (8). This group was seeking to identify novel genes that were responsible for cardiac development and/or transcription factors that control the production of specific cardiac genes (8). There are three components of the myocardin coactivator family: myocardin, MRTF-A and MRTF-B (Myocardin Related Transcription Factor A and B). Myocardin is a nuclear-restricted protein that is a cardiac and smooth muscle-specific transcriptional coactivator of serum response factor (SRF) (8) Myocardin is expressed at the same time as the earliest known marker for cardiac development (Nkx 2-5). It is expressed during the developmental stages in the cardiovascular system as well as in smooth muscle cells and some internal organs but after birth its expression is completely restricted to the heart (8).

MRTF-A and B are found in the cytoplasm and are involved with regulating actin dynamics, cytoskeletal proteins and cell contraction. MRTF-A is found in many developing and adult tissues and is found in its highest concentrations in mesenchymal, epithelial and muscle cells (92). Conversely, MRTF-B is found in its highest concentrations in neural structures and the brachial arch arteries in the cardiovascular system (93) (92). Together, myocardin and both MRTFs all are involved in migration, myogenesis, cytoskeletal regulation, cell proliferation and cardiac hypertrophy (6) (7).

3.2 Structure of myocardin and MRTFs

Myocardin is a 938 amino acid sequence that encodes for a 101.8 KDa protein in the rat (94). MRTF-A and B are 929 and 1039 amino acids which correlates to 98.9KDa and 118.1 KDa

proteins, respectively. There are 5 major structural domains in myocardin and its related transcription factors which differ slightly between the different members (6). The 5 domains are the RPEL domain, the leucine zipper, the SAP domain, the B1 domain and the TAD (Transcriptional Activation Domain) domain (6). There are three RPEL domains found at the N terminal region of the sequence for all of the family members but this region has different functions in the MRTFs than myocardin (95). In the MRTFs, this domain allows for the binding of globular monomeric actin, but myocardin's RPEL domain has diverged from that of the MRTFs and cannot bind actin (7). The function of this domain in myocardin must still be determined. The leucine zipper domain allows myocardin to bind to its related transcription factors (at the same domain) which may allow myocardin to affect actin dynamics because of its inability to bind actin although this is still a theory which remains to be tested. The B1 domain in all family members is a 7 residue sequence that allows for the binding to SRF and thereby allows these transcription factors to exert their effects (8). Myocardin, along with MRTF-A and MRTF-B contain a SAP (SAF-A/B, Acinus, PIAS) domain which is named for the three proteins in which the sequence was originally found (96). The SAP domain is a 35 amino acid sequence that is found in many nuclear proteins and acts as a mediator between myocardin and its interaction with other transcription factors and is involved in cellular processes including apoptosis, chromosomal functions and organization of the nucleus (96). In MRTF-A and MRTF-B this region is responsible for its localization in the cytoplasm as well as its ability to bind to actin (96) (6). The last domain is in the C terminal end of the sequence and is a strong transcriptional activation domain for all of the members of the myocardin family (7).

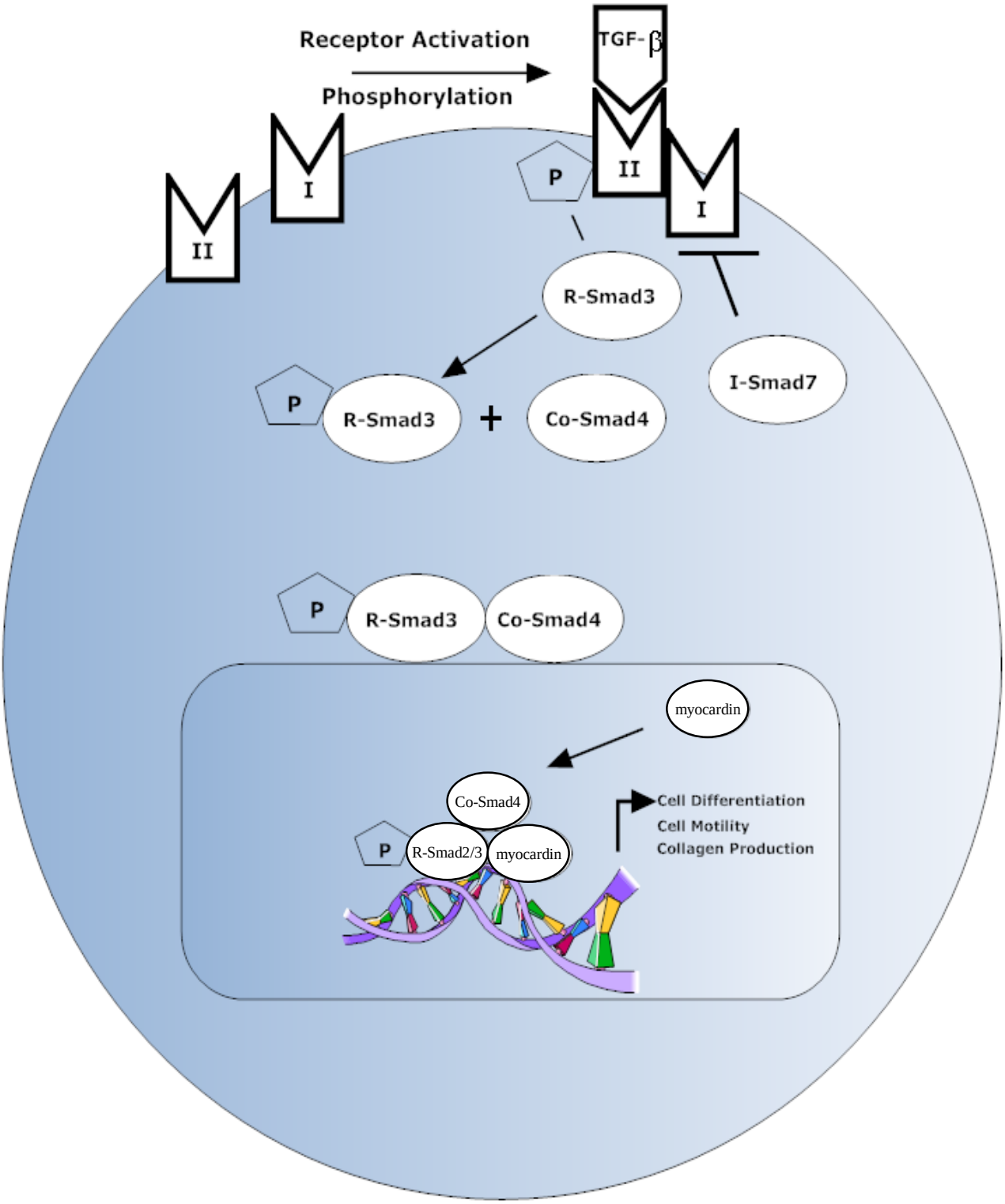

Figure 2. Model of TGF-β signaling with myocardin functioning as a putative R-Smad coactivator in cardiac myofibroblasts. I – TGF-β receptor I; II – TGF-β receptor II; P – phosphate group.

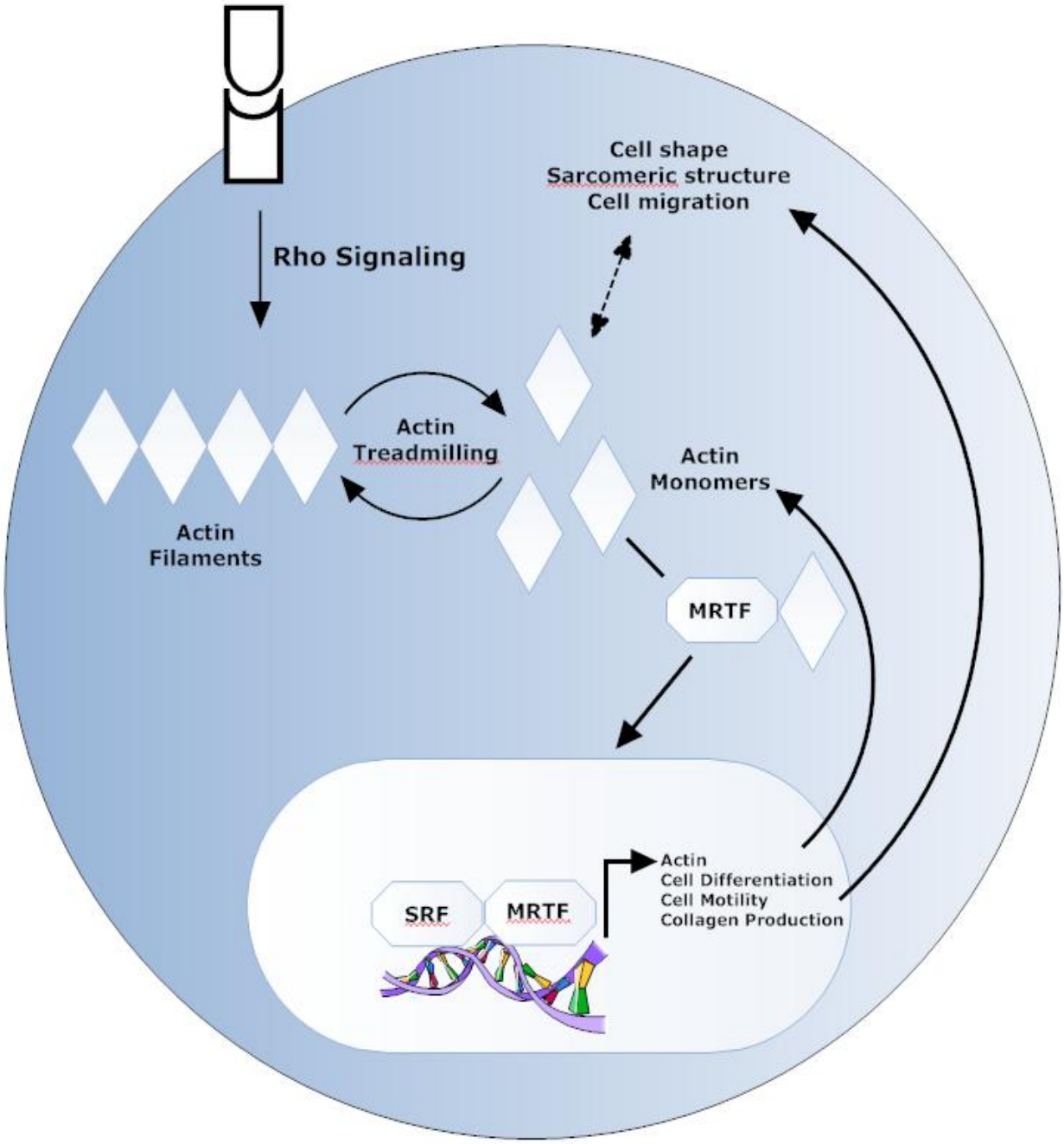

Figure 3. Background relevant to myocardin-related transcription factors (MRTFs) signaling in cardiac myofibroblasts. MRTFs are believed to be important in promoting a more muscular phenotype in cells, such as myofibroblasts. Cytokines binding to the cell surface receptors stimulate an increase in the production of actin filaments by Rho signaling - MRTFs are known to translocate to the nucleus by an unknown mechanism. They then become involved in transcription. This leads to a regulatory feedback loop to regulate cellular activities.

3.3 SRF: Structure, function and interaction with myocardin family

Serum response factor is a transcription factor of 508 amino acids and is also a member of the MADS (MCM1, Agamous, Deficiens, SRF) family (97). This portion of the sequence is responsible for the binding of DNA as well as other cofactors including myocardin and its related transcription factors (98). SRF binds to DNA at a specific sequence called a CArG (C and A Rich G sequences) box and is involved in many different cellular process including cell proliferation, migration, muscle differentiation, myogenesis and regulation of the cytoskeleton (6). In order for the transcription of specific genes by SRF, it requires other cofactors, like myocardin, to generate specific signals (99).

3.4 CArG-dependant and -independent signaling of myocardin

When myocardin was first discovered, it was believed that it would only function in cooperation with SRF and that without SRF, myocardin and its related transcription factors would have no transcriptional activity (92). Through further studies it has been shown that there may be an alternative signaling pathway in which SRF is not required. This pathway proposes that myocardin heterodimerizes with Smad3 and thereby binds to DNA in a CArG-box independent manner and still has transcriptional activities without SRF being involved which was shown in 10T1/2 cells (9). Although this alternative pathway that has been proposed, it should be noted that it has not been completely explored and that Smad3 can also bind to CArG-box sequences on DNA so the pathway may not be completely CArG-box independent (9). This line of research is far from clear, and further work needs to be conducted to either prove or disprove these ideas.

3.5 Myocardin and cytoskeletal signaling

Through the Rho signaling pathway, myocardin has been shown to be involved in many cellular processes including maintaining cellular structure, the cell's ability to migrate as well as the structure of its sarcomeres (6). This control occurs by an extracellular mitogen binding to its receptor and causing actin to polymerize through the Rho pathway. MRTF's, through a mechanism that is still unclear, moves into the nucleus and causes the transcription of various genes. This includes the transcription of actin which affects cell structure, migration and sarcomeric structure. The transcription of these genes also acts as a feedback loop in order to down-regulate SRF's activities (95).

3.6 Myocardin and cardiac hypertrophy

Myocardin has been shown to be involved in the signaling pathway that results in the hypertrophy of cardiac myocytes but no data has been presented to date concerning myocardin's function in myofibroblasts (100). There are three proposed mechanisms for how myocardin is involved in cardiac myocyte hypertrophy. The first mechanism involves p300 which is a coactivator involved in transcription that possesses histone acetyltransferase activity (101;102). p300 has been shown to interact with myocardin in the TAD domain and causes an increase in myocardin's transcriptional activity (100). It is also interesting to note that myocardin deletion mutants, which have a portion of the TAD domain missing, results in the failure of p300 binding which causes a decrease in the expression of hypertrophic genes (100). This finding infers that myocardin is required for hypertrophic gene transcription and expression (8). The second mechanism is that an increase in hypertrophic signaling will cause an increase in the amount of myocardin being transcribed and therefore results in an increase in the amount of myocardin

protein and consequently transcription of hypertrophic genes (103). The third mechanism proposed is that increased hypertrophic signaling causes myocardin to become more active, probably due to modifications to myocardin like acetylation or phosphorylation and thereby playing a role in hypertrophy (103).

3.7 The Interaction of Myocardin and Smad3

Myocardin and Smad3 were first shown to interact directly with each other, in both *in vitro* and *in vivo* in 10T1/2 cells (9). Only a few studies have been conducted since then which examine this interaction. Smad3 binds full length myocardin (6) and they have a synergistic effect on SBE-mediated transcription that is independent of CArG boxes (9). As well, it has been determined that myocardin is likely an essential transcription factor which is required for TGF-β signaling and is a major component that regulates the transcription of specific genes (9). It is unclear whether R-Smad phosphorylation is a required step for binding and/or activation of this complex. Furthermore whether Smad3 and myocardin are cofactors in cells other than smooth muscle is unclear.

V Materials and Methods

1.0 Materials

Media used for cell culture (Dulbecco's Modified Eagle Medium, DMEM/F12, and SMEM), fetal bovine serum (FBS) and antibiotic penicillin were purchased from GIBCO BRL (Grand Island, NY). Collagen solution for the gel contraction assay was obtained from Stem Cell Technologies (Vancouver, BC). Surgical blades were obtained from Becton-Dickinson Acute Care (Franklin Lakes, New Jersey). Whatman pH indicator paper was purchased from Whatman International Ltd. (Kent, UK). Culture plates and dishes were received from Fisher Scientific (Whitby, ON). PCR MasterMix was purchased from Promega (Madison, WI). Protein assay supplies (Bicinchoninic acid and copper II sulphate) were ordered from Sigma-Aldrich (Oakville, ON). Pre-stained molecular weight ladder (SeeBlue2) was purchased from Invitrogen (Burlington, ON). Primary antibodies were received from the following companies: phosphorylated Smad3 from Cell Signaling (Danvers, MA), Smad3 from Upstate (Lake Placid, NY), Smad7 from Santa Cruz (Santa Cruz, CA), myocardin from Abcam (Cambridge, MA), Actin from Santa Cruz (Santa Cruz, CA). Secondary antibodies were obtained from Bio-Rad Laboratories (Hercules, CA). Polyvinylidene difluoride (PVDF) membranes were received from Millipore (Billerica, MA). The chemiluminescence kit (ECL+ Plus) and Immunoprecipitation Starter Pack was purchased from GE Healthcare (Buckinghamshire, England). Recombinant human TGF-β_1 was received from R&D Systems (Minneapolis, MN). The P1NP EIA was purchased from IDS (Boldon, England).

2.0 Methods

2.1 Rat cardiac myofibroblast isolation and culture

Myofibroblasts were isolated from adult male Sprague-Dawley rats with weights between 150 – 200 grams and the methods of Brilla *et al.* (104) were followed with minor modifications. Briefly, the rat heart was hung on a Langendorff apparatus and perfused with recirculated Joklik's medium at a flow rate of 5 mL/minute at 37°C and then perfused with 0.1% collagenase in SMEM media for 20 – 25 minutes. Released cells were then collected by centrifuging at 2000 rpm for 5 minutes and resuspended in 10% FBS DMEM/F12 media containing 100mM ascorbic acid, 100 U/mL penicillin and 100 ug/mL streptomycin. Cells were then plated and allowed 2 hours to adhere after which cells were washed twice with 1x phosphate buffered saline (PBS) in order to remove any non-adhering cells and myocytes and maintained in 10% FBS DMEM/F12. The cells used in these experiments were of passage 0 (P0), P1 and P2. By using this method, it has been determined that >95% of the cells in the culture are fibroblasts by staining for procollagen type I (a major product of fibroblast cells)(105;106). This was shown by phenotyping experiments to show the percentage of cell that were endothelial cells and fibroblasts. It was shown that <1% of the cells stained positive for desmin (a protein seen specifically in smooth muscle cells), factor VIII and α-SMA (a protein only seen in SMCs and myofibroblasts).

2.2 Infection of P1 myofibroblasts using LacZ, Smad3, Smad7 and myocardin adenoviruses

Optimal MOI's (Multiplicity Of Infection) were determined to be 10 viral particles/cell (vp/cell) for the Smad3 adenovirus and 100 vp/cell for the LacZ, Smad7 and myocardin adenoviruses with an infection time of 24 hours for all of the viruses. All the adenoviruses were

titred by the $TCID_{50}$ method. The myocardin adenovirus used for the following experiments encodes the human form of myocardin that has 81% homology to the rat isoform of myocardin and was obtained from Dr. A.A.F. de Vries. Optimal MOI's were determined by Western Blot analysis for all viruses except the myocardin adenovirus which was analyzed by RT-PCR due to poor antibody quality when used for Western Blot analysis. The optimal MOI's for the LacZ and Smad7 adenoviruses were determined previously in our laboratory. P1 myofibroblasts were plated in a variety of dishes and allowed to adhere overnight. Cells were then starved for 24 hours and infected with the optimal MOI listed above in 10 mL of serum-free DMEM F12 media for 24 hours. Cells were then used for a variety of experimental conditions listed below and the results were analyzed.

2.3 Protein extraction and assay

Different treatment groups of cells were washed 3 times with ice cold 1x PBS. Cells were lysed by the addition of 120 µL RIPA buffer (pH=7.6) which contains 150 mM NaCl, 1.0% nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1mM Na_3VO_4 and 1mM EGTA) and protease inhibitors (4µM leupeptin, 1µM pepstatin A and 0.3 µM aprotinin). Cells were then scraped and collected where they lysed for 1 hour and then sonicated three times for five seconds each. The lysate was centrifuged at 14,000 rpm at 4 °C for 15 minutes and the supernatant was collected and stored at -20°c. The concentration of each protein sample was calculated using the bicinchoninic acid (BCA) method which is described previously (107).

2.4 Western blot and analysis of target proteins

Cell lysates were prepared for analysis by mixing with Laemmli loading buffer (125 mM Tris-HCl (pH=6.8), 5% glycerol, 2.5% SDS, 5% 2-mercaptoethanol and 0.125% bromophenol blue) and boiled for 5 minutes. Equal amounts of each protein sample (30 μg) were separated on an 8-10% SDS-polyacrylamide by gel electrophoresis (SDS-PAGE) with 15 μL of prestained low molecular weight marker. Separated proteins were then electophoretically transferred onto a 0.2 μM polyvinylidene difluoride (PVDF) membrane and blocked with 10% skim milk in Tris-buffered saline with 0.2% Tween 20 (TBS-T) overnight at 4°C. The membranes were then probed with primary antibodies for one and a half hours at the concentration of 1:1000 for Smad3 in 5% skim milk. Secondary antibody labeled with horseradish peroxidase (HRP) was incubated for one hour at room temperature at a concentration of 1:10000 in 0.2% TBS-T. The membrane bands were then visualized by ECL Plus Western Blotting Detection System according to the protocol and developed using X-ray film. Equal loading of the protein samples was confirmed by probing for actin at a concentration of 1:1000.

2.5 RT-PCR

RNA was isolated from adult P0, P1 and P2 fibroblasts using the GenElute Mammalian Total RNA Extraction Miniprep Kit and the end product was analyzed by spectrophotometry and agarose gel electrophoresis. Reverse transcription was then conducted by using 1 μg of the isolated RNA and the Superscript III First-Strand Synthesis System to create a cDNA template. PCR was completed using Promega PCR Master Mix, HeLa specific primers (provided in the Superscript III First-Strand Synthesis System kit), rat myocardin specific primers (5’ TTCCTGTGCACACTGCTGTAAAG 3’ and 5’ GCTGAAGCTTGTGCTGCCAAAG 3’)

(sequence obtained from Dr. Eric Olson's laboratory, UT Southwestern, Texas), human myocardin specific primers (5' TTCAGAGGTAACACAGCCTCC 3' and 5' TGATCCTCTCTAGCGTCTGCT 3') (sequence obtain from Vardes *et al.*(108) or NCX1 specific primers (5' CTGGGATCTTCTGCTCCAG 3' and 5' CCTCCACCTCTGGCAGTC 3') (sequence obtained from Dr. Mark Hnatowich) using the following thermocycler conditions for rat and human myocardin: 33 cycles for the rat isoform and 23 cycles for the human isoform of 10 seconds denaturing at 95°C, 30 seconds annealing at 58°C and 45 seconds of elongation at 72°C with an initial denaturing of 2 minutes at 95°C and a final extension of 5 minutes at 72°C. Conditions for the HeLa controls were: 40 cycles of 10 seconds denaturing at 95°C, 30 seconds of annealing at 55°C and 45 seconds of elongation at 72°C with an initial denaturing of 2 minutes at 95°C and a final extension of 5 minutes at 72°C were run as per the Superscript III First-Strand Synthesis System protocol. Thermocycler conditions for NCX1 were: 33 cycles of 45 seconds at 95°C, 45 seconds annealing at 55°c and 1 minute and 30 seconds of elongation at 72°C with an initial denaturing of 1 minute at 95°C and a final extension of 5 mintues at 72°C. The manufacturer's protocol was followed for all of the above kits. 10 µL of Trackit 1Kb Plus DNA Ladder (0.1 µg/uL) was run in addition to 5 µL of HeLa positive and negative controls and 25 µL of the myocardin and NCX product were then analyzed by agarose gel electrophoresis (0.8% gel at 120 V for 60 minutes) and photographs were taken with a Polaroid camera.

2.6 Co-immunoprecipitation assay

Immunoprecipitation was completed by using the Immunoprecipitation Starter Pack kit (GE Healthcare). Briefly, P1 ventricular myofibroblasts were isolated and lysed using the conditions explained under "2.3 Protein extraction and assay" except cells were lysed in 1 mL of

lysis buffer instead of 120 µL. The cell lysates were then aliquoted to 500 µL/tube and 1 µg of the myocardin antibody was added to each tube and rotated for 2 hours at 4°C. The immune complexes were then precipitated using 50 µL of Protein G Sepharose 4 Fast Flow (which is in a 50% slurry) and rotated for 1 hour at 4°C. The samples were then centrifuged for 20 seconds at 12000 g and the pellet was washed 3 times with 1 mL of lysis buffer and once with wash buffer (50 mM Tris, pH 8) (samples were centrifuged between each wash for 20 seconds at 12000 g and the supernatant was removed). The final pellet was resuspended in 30 µL of sample buffer (containing 1% SDS, 100 mM DTT and 50 mM Tris at pH 7.5) and heated to 95°C for 3 minutes to allow for dissociation of the beads and the protein. The sample was centrifuged for the final time for 20 seconds at 12000 g and the supernatant removed. The supernatant was prepared for SDS-PAGE analysis by adding 1 µL of 0.1% bromophenol blue and 18 µL of 40% glycerol. Protein samples were then analyzed according to the conditions as explained elsewhere.

2.7 "Floating" collagen gel contraction assay: myofibroblast contractility

To the collagen gel matrixes to measure contraction, 7 mL of chilled type I collagen (3 mg/mL) was mixed with 2 mL of 5x concentrated DMEM F12. The solution was then adjusted to a pH of 7.4 with 1M NaOH and the volume was brought up to 10 mL. 600 µL of the collagen gel was then placed into each well of a 24 well culture dish and allowed to solidify overnight at 37°C. P1 myofibroblasts were then seeded at a density of $1x10^5$ cells/well and were given until the next day to grow up and attach to the matrix. The cells were then serum-starved for 24 hours and then infected with adenovirus for 24 hours before continuing with the experiment. The gels were then cut away from the well of the culture dish with a surgical scalpel blade before stimulation with cytokines occurred – hence the moniker "floating gel". Photographs were then

taken at 0 and 24 hours after stimulation. All photographs were analyzed with the IDL based MeasureGel software and results were expressed as means ± SE.

2.8 Procollagen type I N-terminal peptide secretion assay using enzyme immunoassay

The enzyme immunoassay eg, P1NP EIA, was completed by using the Rat/Mouse P1NP EIA kit from ImmunoDiagnostic Systems (IDS) and following the kit directions – the secretion of the globular cleaved N-terminal peptide is a reliable marker for collagen type I secretion. As collagen synthesis in myofibroblasts is complex and is subjected to both recycling of monomeric protein components and numerous post-translational modifications, secretion of the mature protein is the most reliable indicator of collagen synthesis in these cells. P1 cardiac myofibroblasts were grown in 150 mm cell culture dishes until they reached 70-80% confluency when they were serum starved for 24 hours. Some groups were then infected with adenoviruses for another 24 hours before stimulation with TGF-β_1 occurred. TGF-β_1 was replenished every 12 hours for a total of 24 hours. The cells and media were scraped from the culture plates and 5 µL of homogenized sample (cells and media) was added to the 96-well antibody coated plate in duplicate and 45 µL of sample diluent was added to each sample. 50 µL of P1NP Biotin was added to all standards, controls and samples and shaken at 500-700 rpm for 1 hour at room temperature. All wells were then washed three times with 250 µL of wash solution and 150 µL of enzyme conjugate was added to all of the wells and allowed to sit at room temperature for 30 minutes. The wells were then washed again three times with wash solution and 150 µL of TMB substrate is added to all of the wells and given another 30 minutes at room temperature. Finally, 50 µL of stop solution is added to all of the wells and the plate is read on a spectrophotometer at

450 nm within 30 minutes of adding the solution. The OD readings are then used to calculate the amount of collagen/sample from the standard curve.

2.9 Statistical analysis

All data is expressed as mean ± standard error. Wherever appropriate, statistical significance among samples was determined using one-way ANOVA and Student-Newman-Keuls tests to compare differences between groups. Statistical significance is defined for each significant group, and are listed in each legend to Figures.

VI Results

1.0 Effect of passage on myocardin mRNA expression in cardiac fibroblasts (P0 cells) and myofibroblasts (P1 and P2 cells)

Myocardin has been shown to be involved in many different processes including cell structure, migration and cardiac hypertrophy (6). The relative amounts of myocardin in P0, P1 and P2 cardiac myofibroblasts was determined by RT-PCR. For RT-PCR, RNA was isolated from P0, P1 and P2 myofibroblasts and then converted into cDNA in order to do primer specific reverse transcriptase PCR. HeLa RNA was used as a control to show the reverse transcription process was occurring. Myocardin cDNAs were amplified, run on a DNA gel and the band intensities were measured and compared by densitometer (Figure 4). Myocardin mRNA expression is progressively increased with increasing number of passages, and thus myofibroblasts (P1 and P2 cells) expressed significantly higher myocardin than fibroblasts (P0 cells). Further, the supermature P2 cells expressed significantly more myocardin mRNA than did the P1 myofibroblasts.

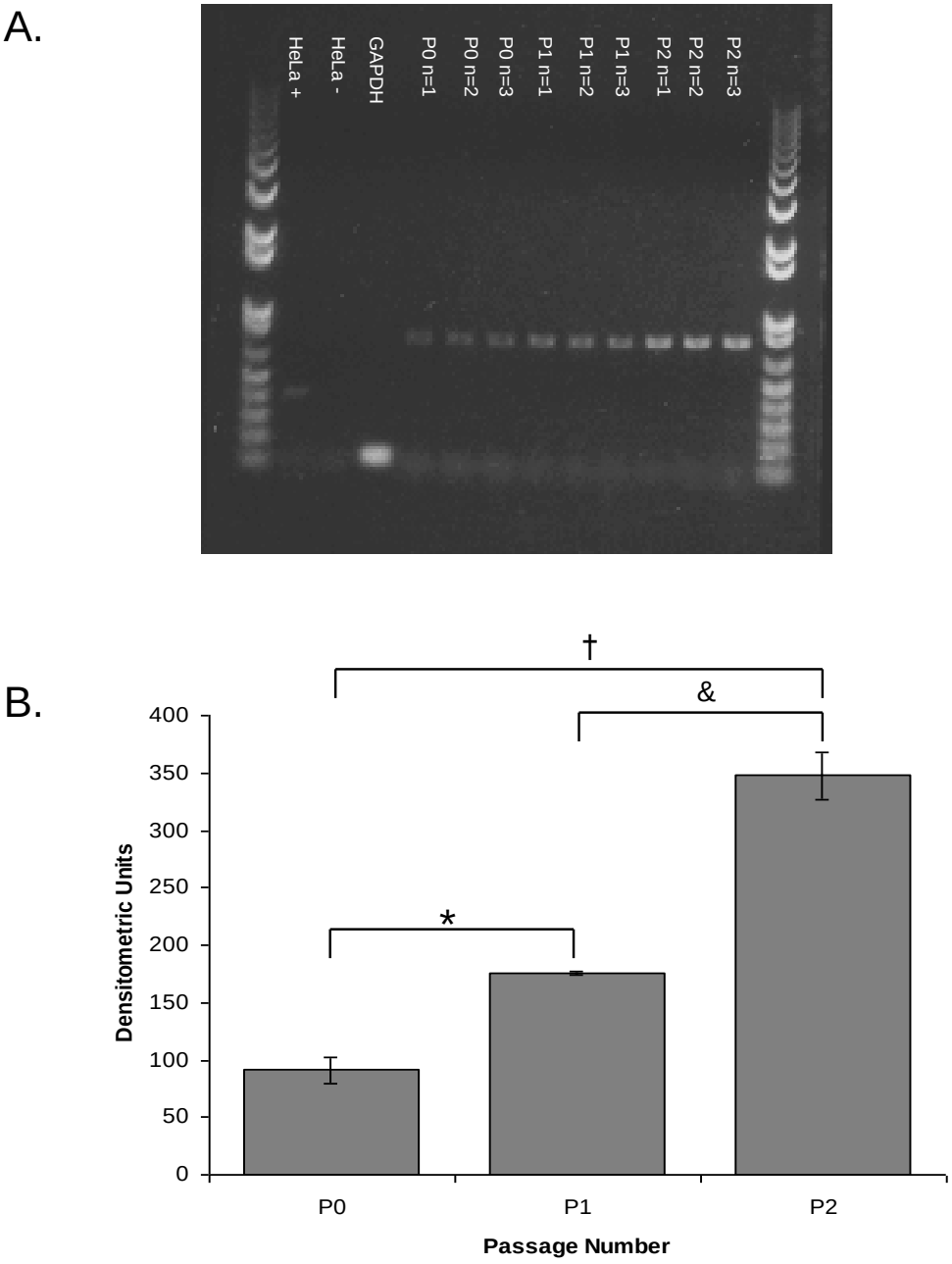

Figure 4. Effect of passage on relative myocardin mRNA expression in ventricular fibroblasts (P0) and myofibroblasts (P1 and P2). RNA was isolated from adult P0, P1 and P2 cells and reverse transcribed. PCR was then conducted with myocardin specific primers and the product was run on a DNA gel and analyzed with densitometry (Quantity One - Biorad) **Panel A**: DNA gel showing relative levels of myocardin in each passage number. **Panel B**: Graph of quantified data from the sample groups shown in panel A. $^{*}P \leq 0.021$ *vs.* P0, $^{\&}P \leq 0.009$ *vs.* P1, $^{\dagger}P \leq 0.001$ *vs.* P0; data are expressed as mean ± SEM (n=3).

2.0 The putative interaction of myocardin and Smad3 in P1 myofibroblasts

Previously it has been determined that myocardin and Smad3 interact with each other in COS-7 cells (9) but whether this relationship exists in primary ventricular myofibroblasts is unclear. Our results (Figure 5) provide data to indicate the Smad3 and myocardin co-immunoprecipitate when pulled down with the appropriate antibody. The precipitating antibody was 1 µg of anti-myocardin and the resulting samples from the immunoprecipitation were run on a Western blot and probed for anti-p-Smad3 and anti-total Smad3. We found that myocardin and Smad3 interact with each other in primary myofibroblasts, and to our knowledge, this is the first result of its kind. With respect to significance of this interaction to cellular function, our results are quite limited. We are unable to resolve whether p-Smad3 or Smad3 is more important in binding myocardin.

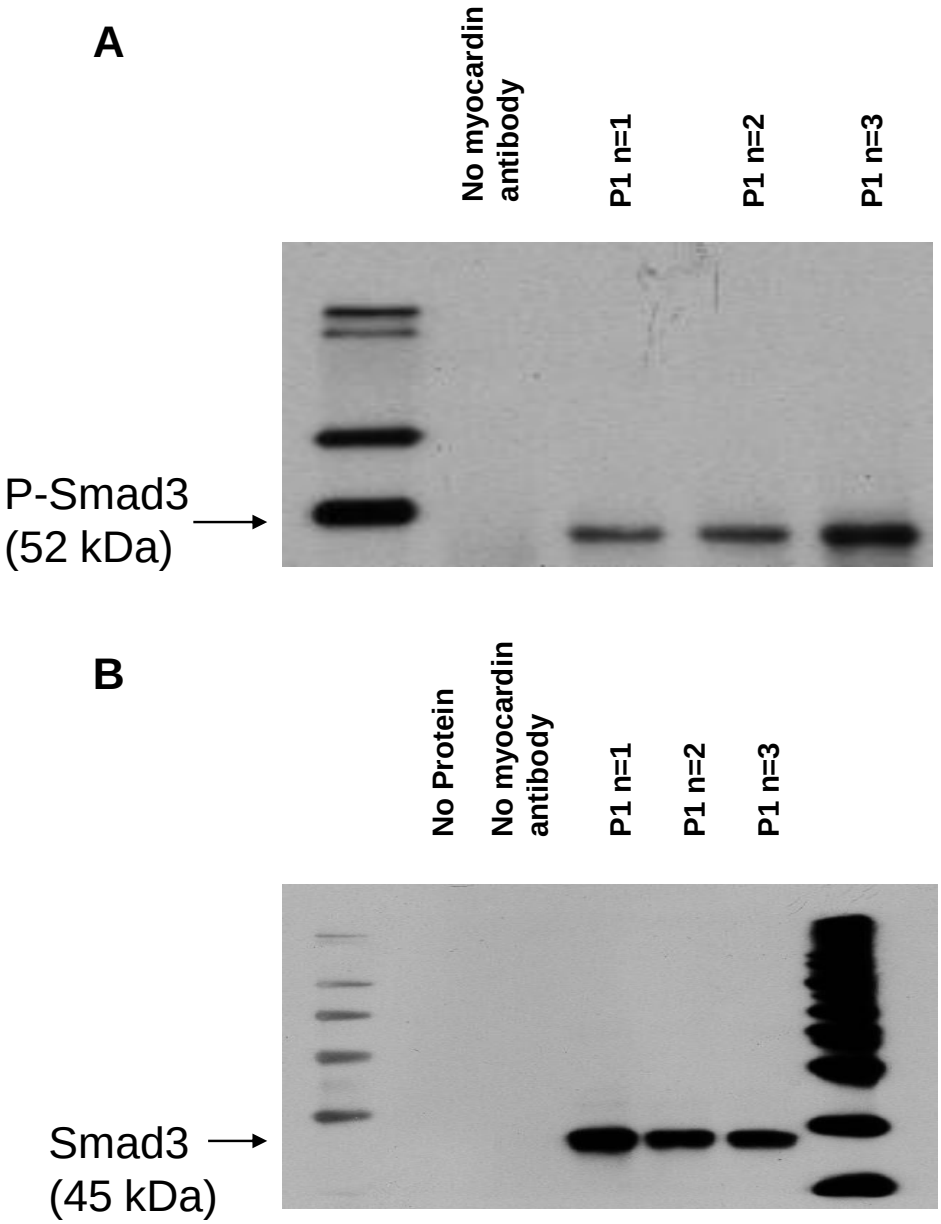

Figure 5. The interaction of myocardin and Smad3 in P1 cardiac myofibroblasts. Cells were grown to approximately 80% confluency and then collected in order to perform the immunoprecipitation by using the Immunoprecipitation Starter Pack (GE Healthcare). The myocardin antibody was used to precipitate out interacting proteins and these proteins were then run on a Western gel and probed with Smad3 or p-Smad3 to reveal the interaction. **Panel A**: Western blot analysis showing the interaction of p-Smad3 and myocardin. **Panel B**: Western blot analysis showing the interaction of total Smad3 and myocardin.

3.0 Optimization of the adenoviral overexpression of Smad3 and myocardin in P1 ventricular myofibroblasts

To test for optimal conditions for adenoviral overexpression, eg, drive overexpression of the target protein without killing or damaging host cells, primary ventricular myofibroblasts were grown to 70-80% confluency and then serum-starved for 24 hours. The cells were then infected with different titers of virus (measured by multiplicity of infection) and allowed to infect the cells for an additional 24 hours. The cells were then collected, proteins harvested and the samples analyzed by Western blot for Smad3 (and RT-PCR for myocardin due to poor quality of available commercial primary antibodies). The results shown in Figure 6 demonstrates that the Smad3 adenovirus is greatly overexpressed at 10 MOI and has approximately 40 times the expression of Smad3 when compared to uninfected P1 cardiac myofibroblasts. Figure 7 shows that at 100 MOI of adenoviral myocardin, mRNAs encoding for human myocardin were elevated when compared to the non-infected and LacZ infected controls.

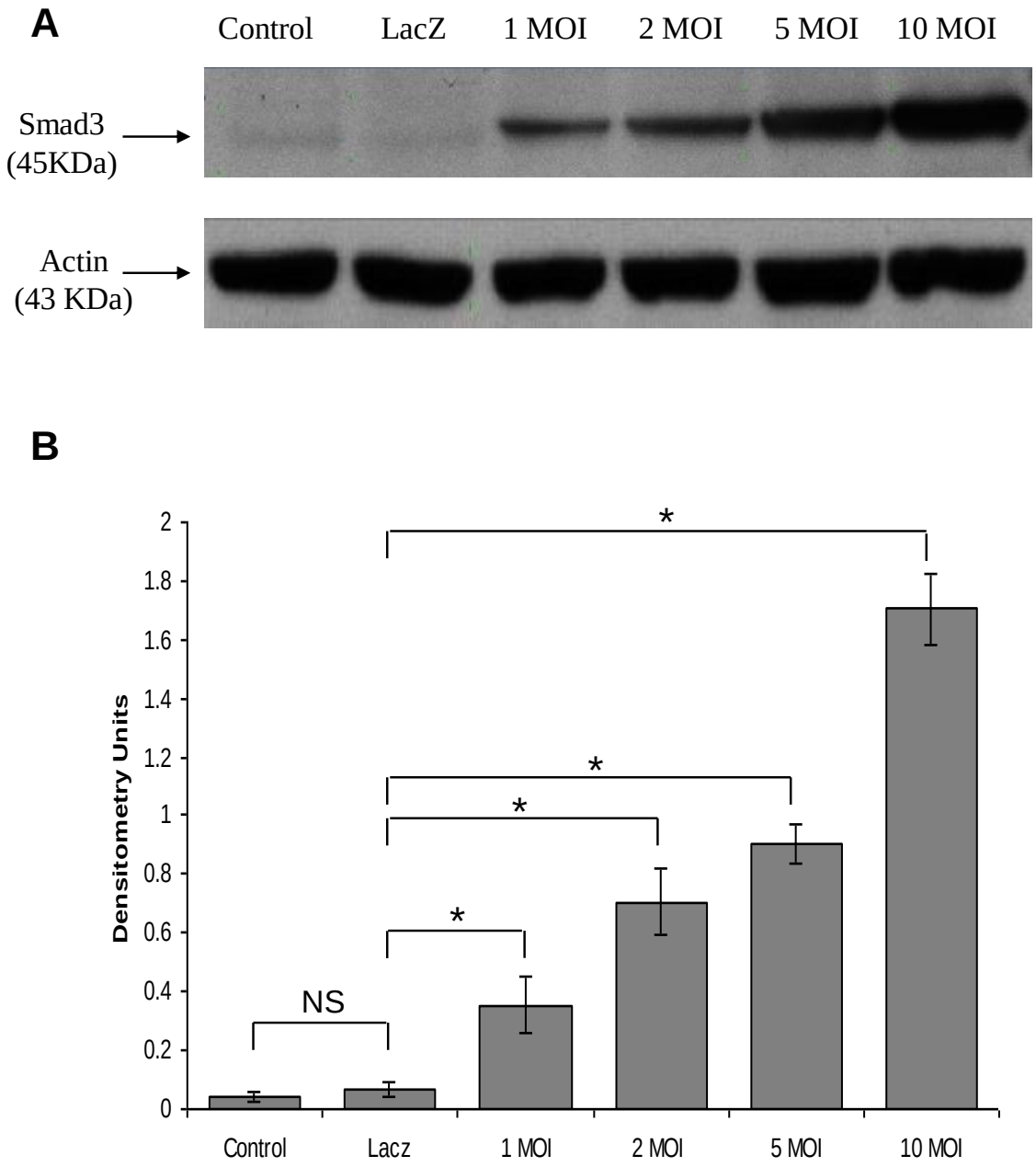

Figure 6. Optimization of the adenoviral overexpression of Smad3 in P1 cardiac myofibroblasts. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 24 hours. Cells were then infected with various MOI's of Smad3 adenovirus and allowed 24 hours of infection. The cells were collected and analyzed by Western blot. **Panel A**: Representative Western blot showing levels of Smad3 (45 kDa) and actin (43 kDa). **Panel B**: Graph of quantified data from the sample groups shown in panel A (data quantified by densitometric scanning). NS – not significant, *P<0.001 *vs.* LacZ; data are expressed as mean ±SEM (n=3). All bands were normalized to actin (loading control).

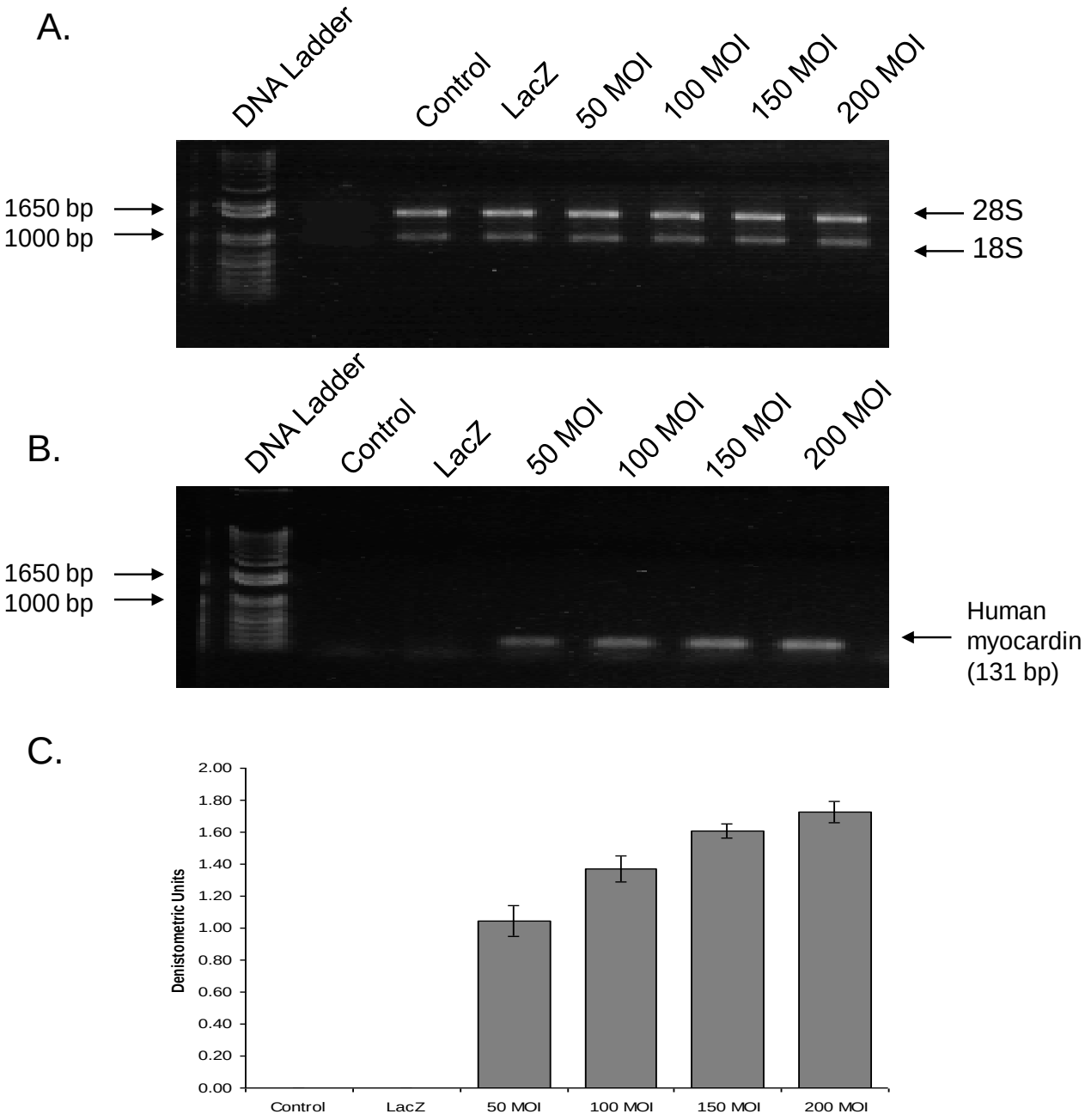

Figure 7. Optimization of adenoviral transduction of human myocardin in P1 cardiac myofibroblasts. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 24 hours. Cells were then infected with various MOI's of human myocardin adenovirus and allowed 24 hours of infection. The cells were collected and analyzed by RT-PCR. **Panel A**: Representative RNA gel showing equal loading and high quality RNA. **Panel B**: Representative DNA gel showing the level of human myocardin at different MOI's. **Panel C**: Graph of quantified data from the sample groups shown in panel B (data quantified by densitometric scanning). Data are expressed as mean ±SEM (n=3). All bands were normalized to the 28S band of RNA (loading control).

4.0 Effects of TGF-β_1 over 48 hours on the expression of p-Smad3 and myocardin

TGF-β_1 has been shown to be involved in many different cellular processes which include differentiation, proliferation, wound repair and extracellular remodeling (59;60). We have examined the effects of TGF-β_1 stimulation on the expression of p-Smad3 and myocardin mRNA expression in order to demonstrate how TGF-β_1 signaling affects certain transcription factors (Figure 8). Ventricular myofibroblasts were seeded in 100 mm dishes and grown to 70-80% confluency and then serum-starved for 48 hours in serum-free media. The cells were then stimulated with TGF-β_1 at different time points before being harvested and analyzed by Western blot analysis or RT-PCR. We found that p-Smad3 levels were rapidly increased after treatment and reached its maximum phosphorylation state between 15 minutes and 1 hour from onset of TGF-β_1 stimulation. After 1 hour, the level of p-Smad3 gradually decreased with each time point until 48 hours where it did not differ from TGF-β_1 stimulation. In contrast, we found a completely different pattern of myocardin mRNA expression with stimulation. The results in Figure 9 indicates that myocardin mRNA expression was comparable to control between time 0 and 1 hour of stimulation and then was significantly reduced at later time points in response to TGF-β_1 stimulation.

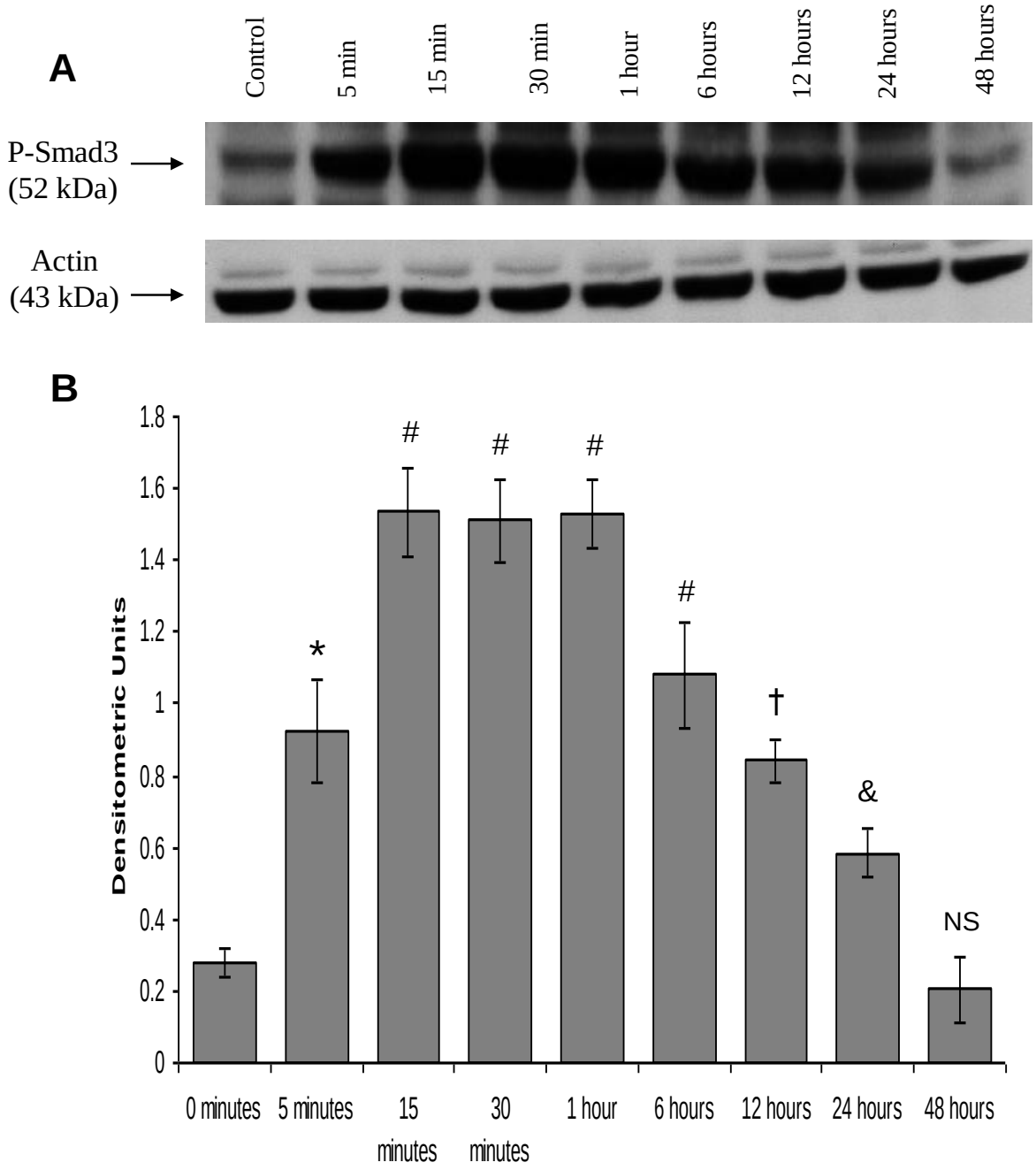

Figure 8. The effect of TGF-β_1 (10 ng/ml) stimulation over 48 hours on the expression of p-Smad3. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 48 hours. Various times of TGF-β_1 stimulation were carried out and the protein was collected and analyzed by Western blot. **Panel A**: Representative Western blot showing levels of p-Smad3 (52 kDa) and actin (43 kDa). **Panel B**: Graph of quantified data from the sample groups shown in panel A (data quantified by densitometric scanning). $^{*}P \leq 0.002$ *vs.* 0 minutes, $^{\#}P < 0.001$ *vs.* 0 minutes, $^{\dagger}P \leq 0.003$ *vs.* 0 minutes. $^{\&}P \leq 0.052$ *vs.* 0 minutes; data are expressed as ± SEM (n=3). All bands were normalized to actin (loading control).

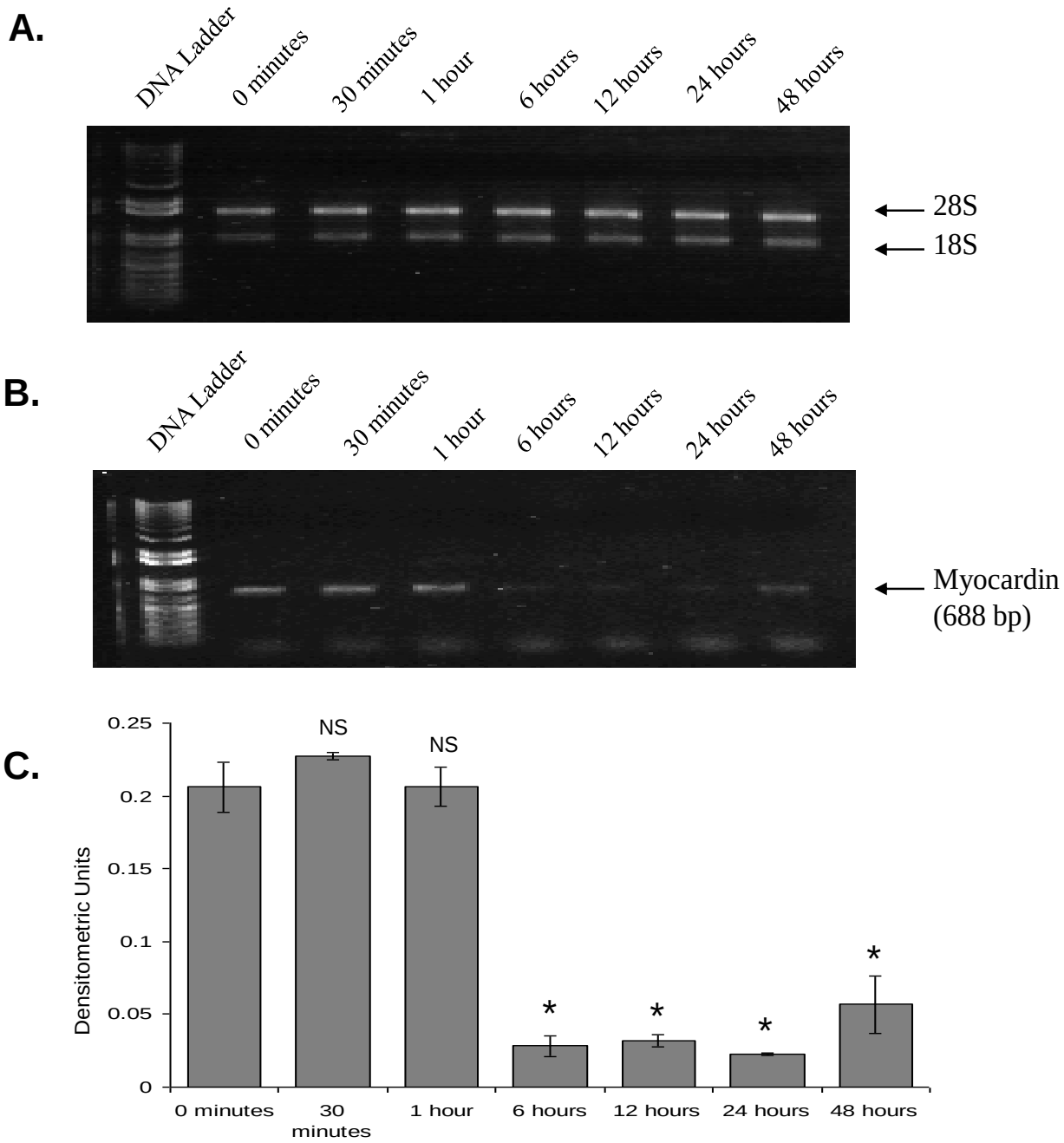

Figure 9: The effect of TGF-β_1 (10 ng/ml) over 48 hours on the expression of myocardin. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 48 hours. Various times of TGF-β1 stimulation were carried out and the RNA was collected and analyzed by RT-PCR. **Panel A**: Representative RNA gel showing equal loading and high quality RNA. **Panel B**: Representative DNA gel from the sample groups shown in panel A (data quantified by densitometric scanning). **Panel C**: Graph of quantified data from the sample groups shown in panel B (data quantified by densitometric scanning). $^{*}P \leq 0.001$ vs. 0 minutes, NS – not significant *vs*. 0 minutes; data are expressed as mean ± SEM (n=3). All bands were normalized to the 28S band of RNA (loading control).

5.0 Effects of overexpressed Smad3, Smad7 and myocardin (with and without TGF-β_1 stimulation) on ventricular myofibroblast contractility

Type I collagen gel deformation assays have been used in previous studies (109) to infer the degree of myofibroblast contractility. The advantage of this assay system is that it provides an averaged effect of a large number of cells and features high reproducibility. We examined the effects of adenoviral overexpression of Smad3 and Smad7 on contractility in ventricular myofibroblasts (Figure 10). We prepared a gel (3 mm) of type I collagen in a 24 well culture dish and seeded $1x10^5$ cells onto this matrix. The cells were then allowed to adhere to the matrix for 24 hours in 10% DMEM/F12 media, and serum-starved for another 24 hours before being infected with various adenoviruses (LacZ, Smad3, Smad7 and/or myocardin) for another 24 hours. The gel matrixes were then cut away from the culture dish and treated with vehicle or TGF-β_1 (10 ng/mL) for 24 hours. Photographs of the wells were taken at 0 and 24 hours after stimulation and measured. We found that overexpressed Smad3 alone was associated with significantly elevated contractility eg, collagen gel contraction vs. control – including uninfected control and LacZ infected cells. No significant differences between the TGF-β_1 stimulated and the Smad3 overexpressed plus TGF-β_1 stimulated group was noted (Figure 10). Figure 11 indicates that overexpressed Smad7 had no effect when compared to control or LacZ infected cells but it did significantly suppress TGF-β_1-mediated contraction. Co-infection of Smad3 and Smad7 showed no significant difference when compared to Smad7 infection alone whereas the Smad3 plus Smad7 plus TGF-β_1 stimulated group show results that are similar to that of the TGF-β_1 stimulated group (Figure 12). In comparison, Figure 13 indicates myocardin overexpression alone was associated with an increase in myofibroblast contractility, and that this increase in contractility is comparable to that induced by Smad3. TGF-β_1 stimulation alone led

to a further relative increase in contractility, while TGF-β_1 contractility was slightly (but significantly) increased further in the presence of myocardin and Smad3 (Figure 13).

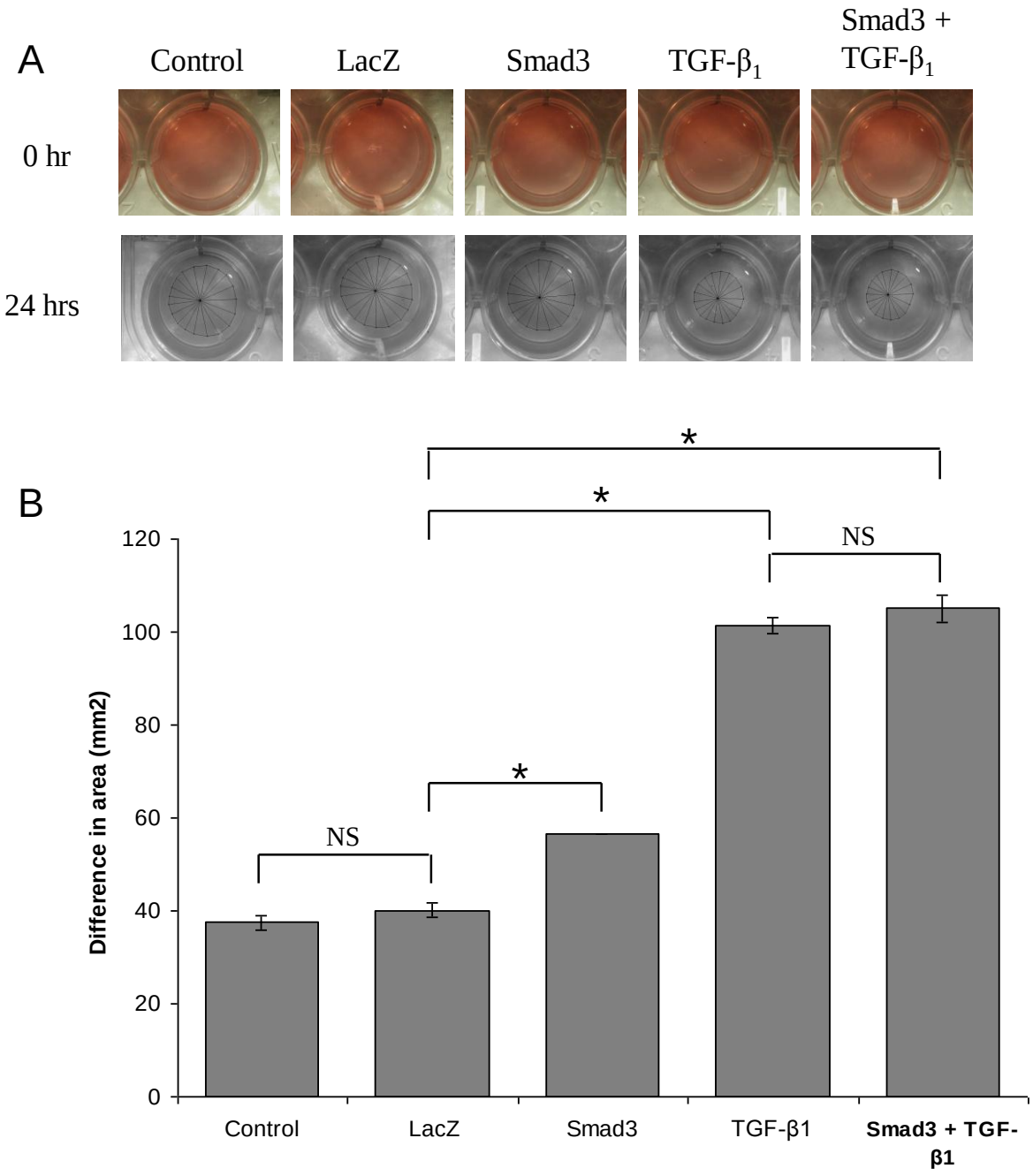

Figure 10. Effect of overexpressed Smad3 on P1 cardiac myofibroblast contractility. **Panel A**: Representative contraction gel assays with and without TGF-β_1 stimulation. **Panel B**: Graphical representation of analyzed contractility data from IDL based computer software. *P<0.001 *vs.* LacZ; data are expressed as mean ± SEM (n=3).

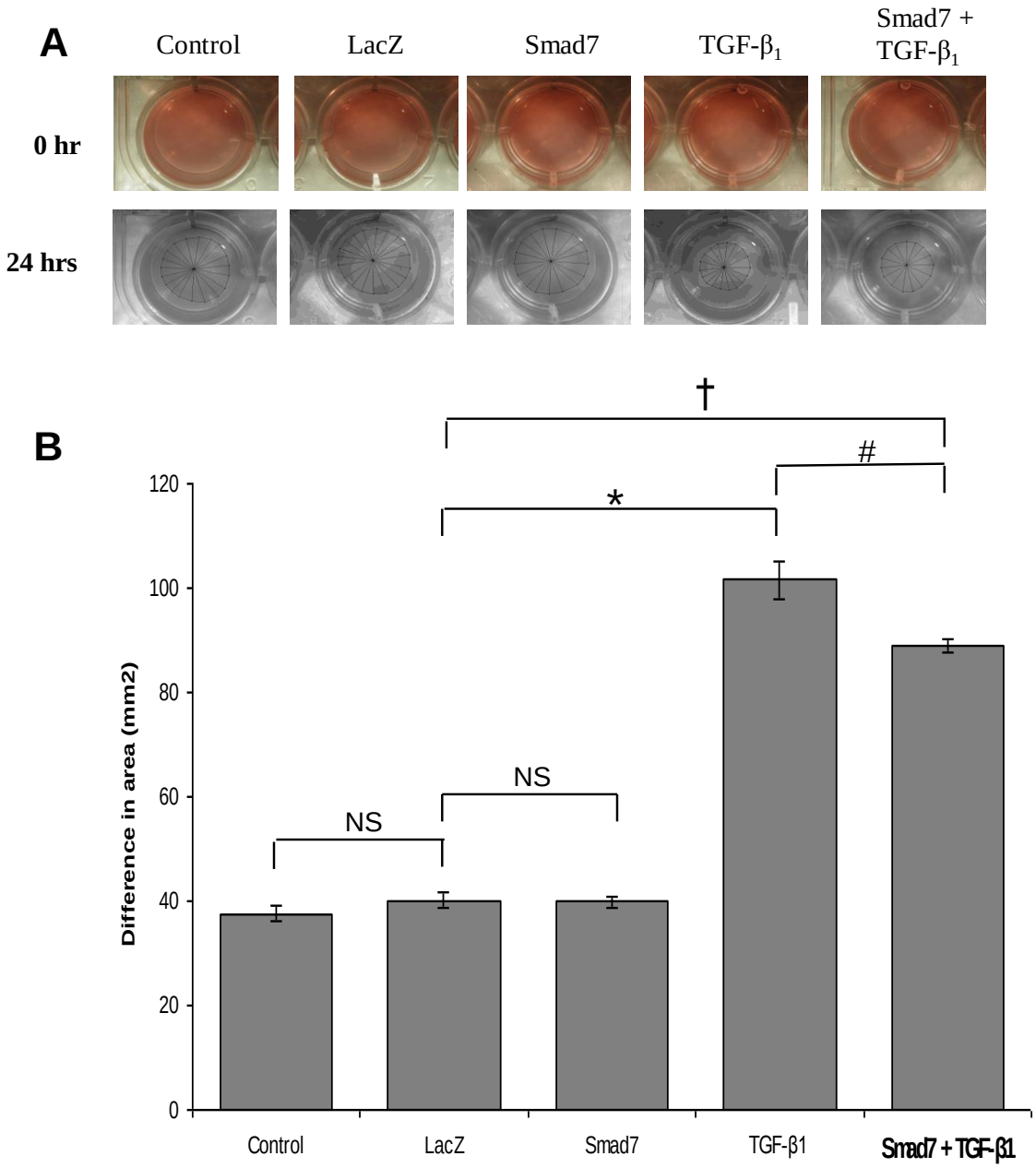

Figure 11. Effect of overexpressed Smad7 on P1 cardiac myofibroblast contractility. **Panel A**: Representative contraction gel assays with and without TGF-β_1 stimulation. **Panel B**: Graphical respresentation of analyzed contractility data from IDL based software. *P<0.001 *vs*. LacZ, $^{\#}$P <0.001 *vs*. LacZ, †P <0.001 *vs*. LacZ; data are expressed as mean ± SEM (n=3).

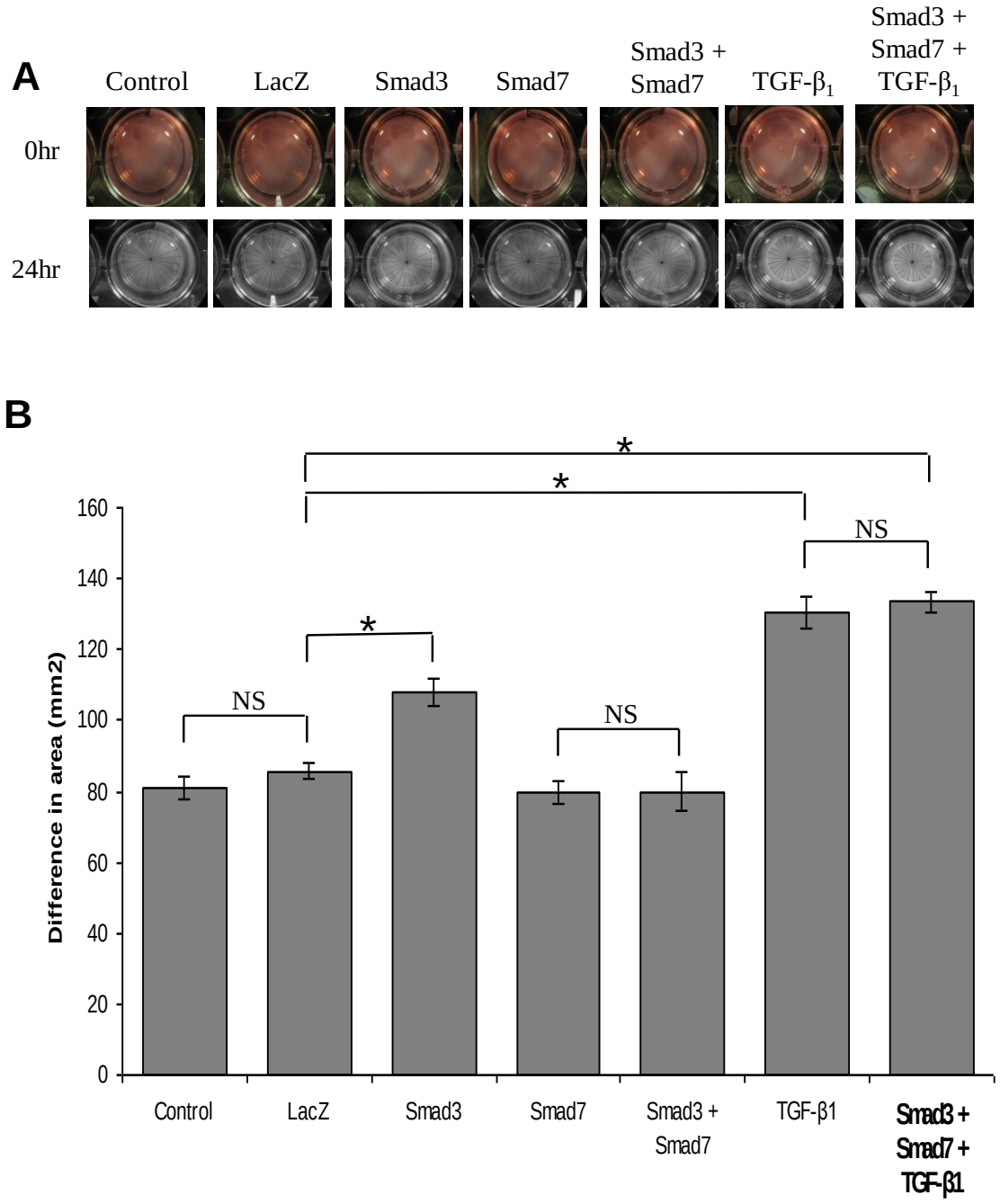

Figure 12. Effect of overexpressed Smad3 and Smad7 on P1 cardiac myofibroblast contractility. **Panel A**: Representative contraction gel assays with and without TGF-β_1 stimulation. **Panel B**: Graphical representation of analyzed data from IDL based computer software. *P<0.001 *vs*. LacZ, ; data are expressed as mean ± SEM (n=3)

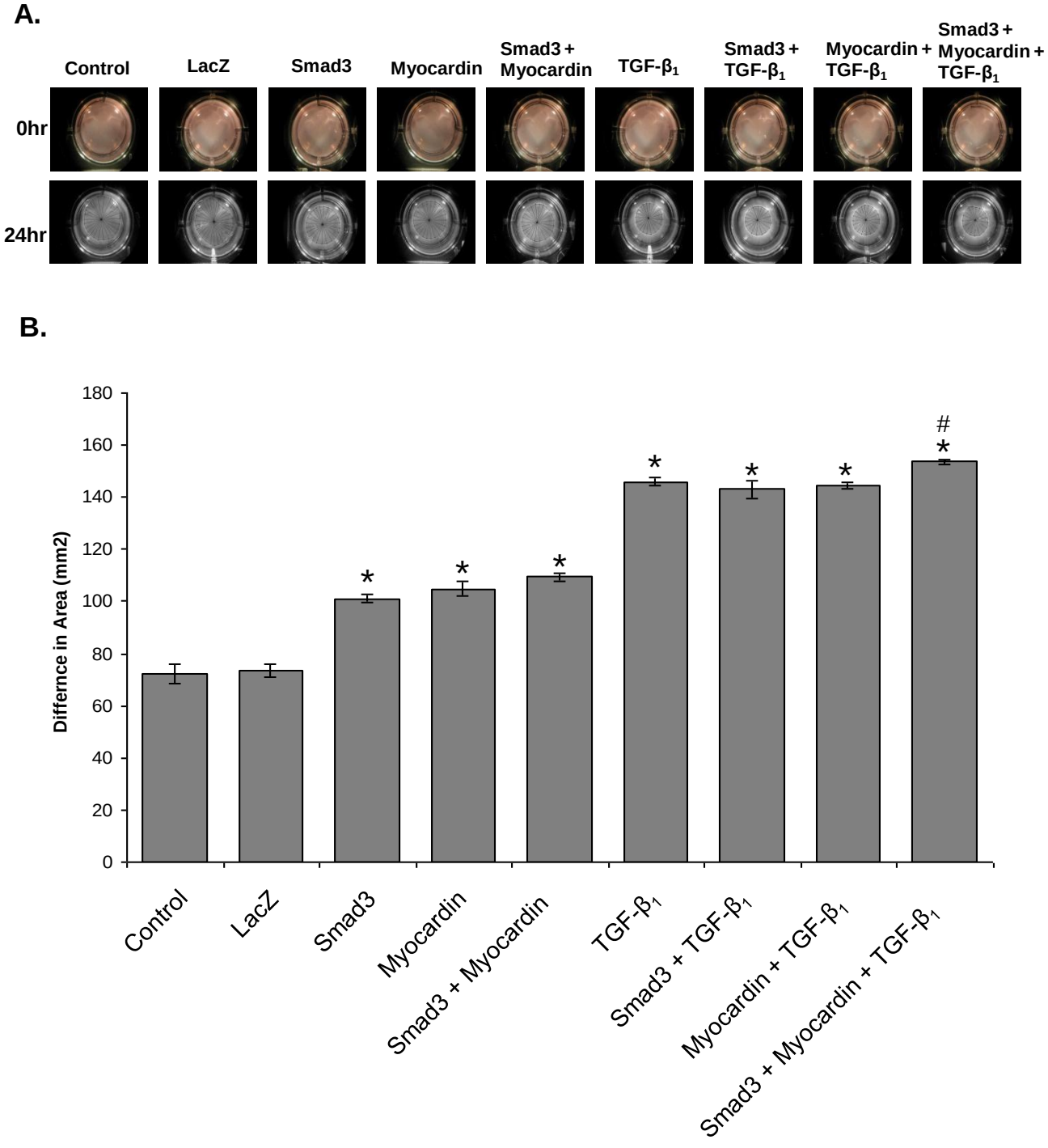

Figure 13: The effects of overexpressed myocardin and Smad3 on P1 cardiac myofibroblast contractility. **Panel A**: Representative gel contraction assays with and without TGF-β_1 stimulation. **Panel B**: Graphical representation of analyzed contractility data from IDL-based software. *P<0.001 *vs.* LacZ, $^{\#}$ P $\leq$0.037 *vs.* TGF-β_1; data are expressed as mean ± SEM (n=3).

6.0 The effect of overexpressed Smad3 and myocardin on the expression of NCX1

We have recently shown that Na^{+}-Ca^{2+} exchanger (NCX1) is expressed in ventricular myofibroblasts (11) and others have demonstrated that reverse-mode NCX1 function is an important transport modality for the entry of Ca^{2+} for fibroblast contraction (110). We examined the effects of overexpressed Smad3 (Figure 14) and myocardin (Figure 15) on NCX1 mRNA expression. P1 cardiac myofibroblasts were seeded in 60 mm cell culture dishes, starved for 24 hours and treated with different titres (MOI) of adenoviral vectors (target genes). RT-PCR was performed using NCX1 specific primers to measure NCX1 mRNA expression. We found that myocardin did not have any effect on NCX1 mRNA expression whereas overexpressed Smad3 was associated with elevated levels of NCX1 mRNAs.

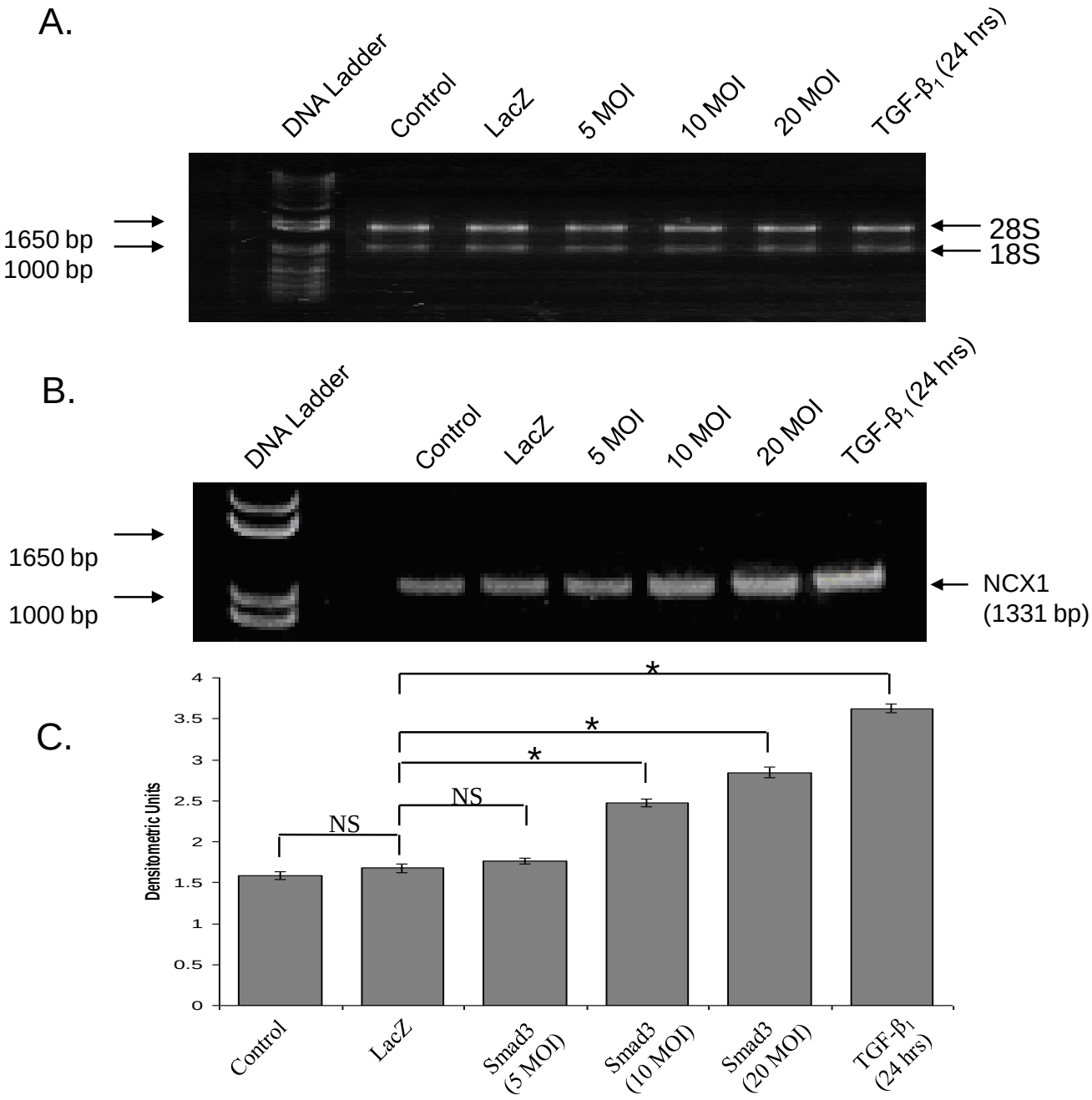

Figure 14. The effect of increasing levels of Smad3 on the expression of NCX1 by RT-PCR. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 24 hours. Cells were then infected with various MOI's of Smad3 adenovirus and allowed 24 hours of infection. The RNA was collected and analyzed by RT-PCR. **Panel A**: Representative RNA gel showing equal loading and high quality RNA. **Panel B**: Representative DNA gel showing levels of NCX1. **Panel C**: Graph of quantified data from the sample groups shown in panel B (data quantified by densitometric scanning). NS – not significant, *P<0.001 *vs.* LacZ; data are expressed as mean ±SEM (n=3). All bands were normalized to 28S band RNA levels (loading control)

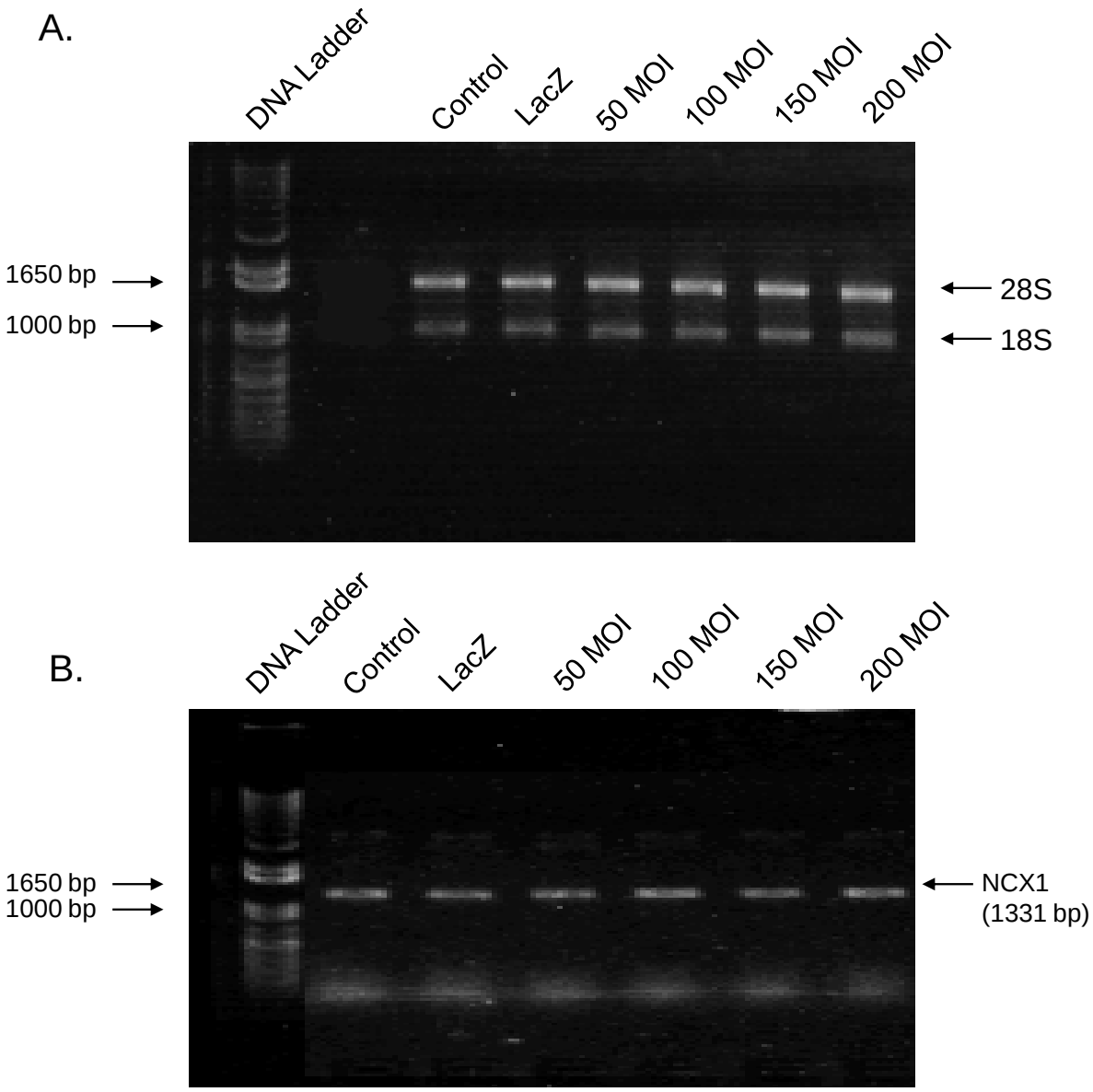

Figure 15. The effect of increasing levels of myocardin on the expression of NCX1 by RT-PCR. Cells were grown to 70-80% confluency and rendered quiescent by serum starving for 24 hours. Cells were then infected with various MOI's of human myocardin adenovirus and allowed 24 hours of infection. The RNA was collected and analyzed by RT-PCR. **Panel A**: Representative RNA gel showing equal loading and high quality RNA. **Panel B**: Representative DNA gel showing levels of NCX1. No significant differences were seen among any groups (n=3). All bands were normalized to 28S band RNA levels (loading control).

7.0 Effects of overexpressed Smad3 and Smad7 on collagen secretion

TGF-β_1 is involved in cellular processes including differentiation, proliferation, wound repair and extracellular remodeling (59;60). We have examined the effects of TGF-β_1 stimulation on collagen secretion in response to the overexpression of transcription factors Smad3 and Smad7 (Figure 16). Some groups (transfected and non-transfected) were then stimulated with TGF-β_1 before being harvested and analyzed for collagen secretion (procollagen type 1–N-terminal peptide using an enzyme-linked immunoassay). This assay has the advantage of high reproducibility as well as excluding the need to incorporate radioactive reagents for detection. As expected, TGF-β_1 treated cells released significantly more collagen when compared to non-stimulated controls. However, Smad3 stimulation alone had no effect on collagen secretion. Notably, Smad3 plus TGF-β_1 treatment led to a significant elevation of collagen secretion when compared to TGF-β1 treated cells alone. Another significant result demonstrated that Smad7 plus TGF-β_1 had collagen levels that were not significantly different from that of the control.

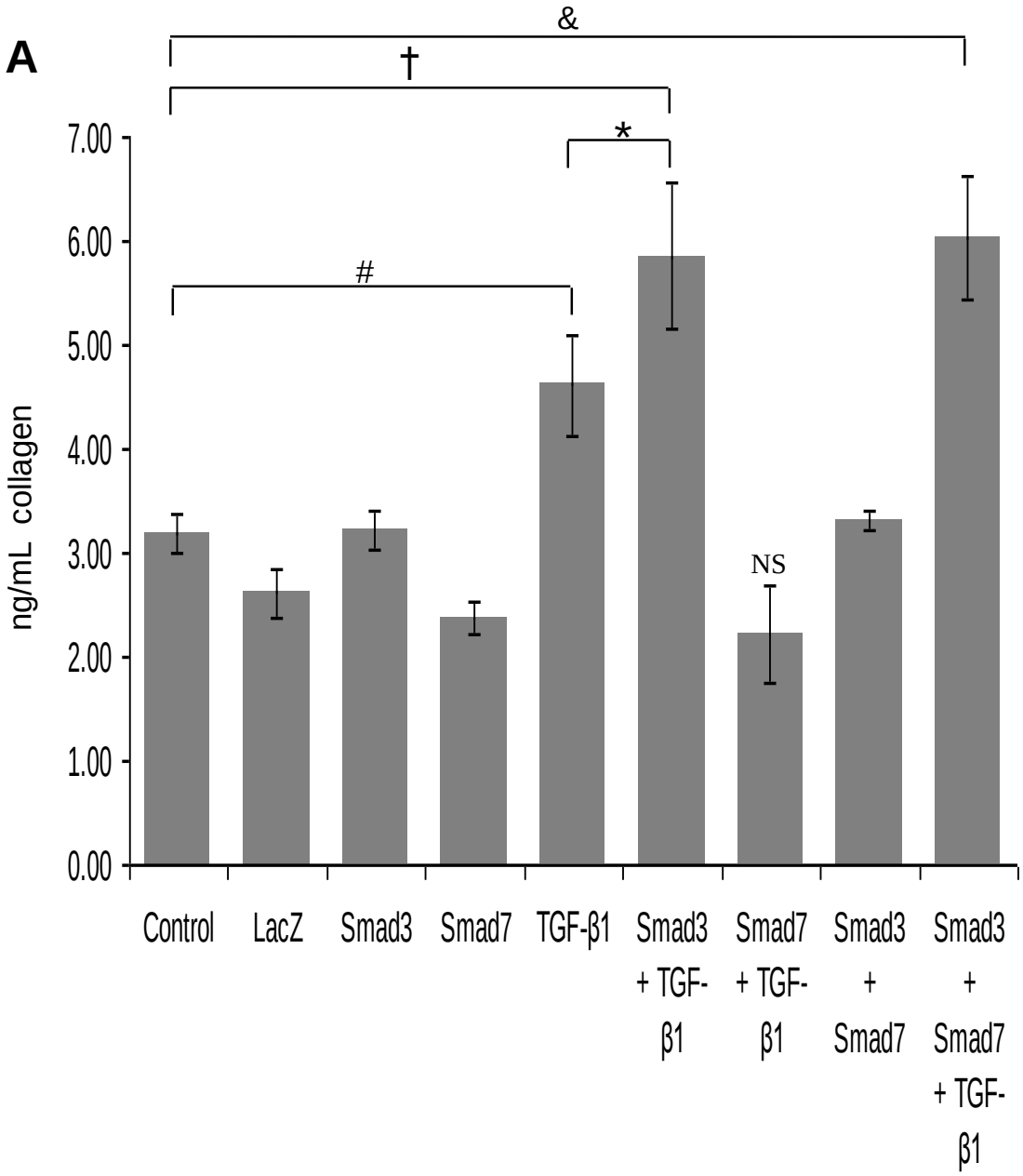

Figure 16. Effect of overexpressed Smad3 and Smad7 with and without TGF-β_1 on collagen secretion. Cells were grown to 70-80% confluency and serum starved for 48 hours and then some groups infected with adenovirus for another 24 hours. Some groups were then stimulated with TGF-β_1 for 24 hours before the cells were harvested and the assay was performed. **Panel A.** Graph of quantified data from spectrophotometer results. NS – Not significant, $^{\#}P<0.046$ *vs.* control, $^{*}P\leq 0.049$ *vs.* TGF-β_1, $^{\dagger}P<0.001$ *vs.* control, $^{\&}P<0.001$ *vs*. control; data are expressed as ±SEM (n=3).

VII Discussion

In this study, we sought to expand knowledge of R-Smad signaling in primary ventricular myofibroblasts by addressing the question of how Smad3 affects contractility and collagen secretion. Further, we investigated the putative interaction of Smad3 and myocardin, and thus laid the groundwork for demonstrating the role of R-Smad cofactor for the latter. As Smad3 is phosphorylatable, this is a known regulatory means by which Smads enter the nucleus, we also treated Smad3 and myocardin transfected cells with TGF-β_1. To ensure our system was functioning, we also investigated parallel effects of the inhibitory Smad7 on myofibroblast contractility. In effect, the Smad7 studies served as another means of controlling our R-Smad experiments by using a transcription factor with opposite effects. Finally we addressed secretion of collagen type I to determine whether simply overdriving Smad3 expression was sufficient to elevate deposition of this important fibrillar collagen species in these cells. Our main findings are centred on R-Smad3 and myocardin-stimulated elevations in myofibroblast contraction. Both nuclear transcription factors stimulate myofibroblast contraction when compared to control samples, and the novelty of these findings lies in their partial explanation of TGF-β_1 –mediated effects. Further, in noting a positive pattern of induction of NCX1 in response to Smad3 overexpression, we have begun to address a putative mechanism for Smad3-linked increased contractility in ventricular myofibroblasts. This hypothesis is supported by the novel finding (12) that implicates reverse-mode NCX1 activity to Ca^{2+} entry and enhanced function in fibroblasts.

Myocardin as a putative R-Smad cofactor

Myocardin is a novel transcription factor discovered in 2001 by Eric Olson and his group (8). To date, there is no published work to address myocardin expression in cardiac myofibroblasts, although there is some data from closely related cell types. Myocardin is expressed in cardiac myocytes (6;9;100) but not in kidney myofibroblasts (111). In contrast, our RT-PCR data shows that myocardin is expressed in ventricular myofibroblasts and that increased passaging of the cells leads to increased expression of myocardin. The increase in expression from P0 cells to P1 and from P1 to P2 is ~ 65%. This finding may be significant for two reasons. The first is that, in the context of the work of C.A. McColloch, fibroblasts plated out on a non-compressible substrate eg, as in an uncoated plastic plate, tend to transition to mature and supermature myofibroblasts with time and passage (112). The maturity of these cells is determined by the appearance and density of actin stress fibers in these cells. Second, myocardin expression has been linked to the induction of muscle-associated proteins in smooth muscle cells, and these include Kir channels, NCX1, and L-type Ca^{2+} channels (113).

Although we did not include this class of proteins in our current study, MRTFs play an important role in cytoskeletal regulation and cellular contractility (95). Using a pathway that involves Rho signaling and Smad proteins, MRTFs may regulate actin filaments in cells and may therefore have an effect on cell shape and migration in cardiac myofibroblasts (95). Myocardin is a known factor to influence the development of cardiac hypertrophy (100). Studies have shown that common hypertrophic signals cause an increase in the expression and activity of myocardin (114). Adenoviral overexpression of myocardin in neonatal cardiac myocytes demonstrates that myocardin stimulates the formation of hypertrophy (115). This data supports

the hypothesis that myocardin may regulate the phenotypic switch of fibroblasts to myofibroblasts as well as ramping up the production and secretion of collagen.

While limited information regarding the interaction of myocardin and Smad3 exists (9), questions regarding their interaction in ventricular myofibroblasts remain unaddressed to the present day. Myocardin has been shown to bind to full-length Smad3 in cardiomyocytes and COS-7 cells (9). Specifically, myocardin binds at the N-terminal and C-terminal ends of Smad3 with an apparent greater association with the C-terminal end in COS-7 cells (9). Our data indicates that both Smad3 and phosphorylated Smad3 (p-Smad3) and myocardin specifically bind, and this provides a novel means of Smad activation apart from canonical Smad signaling. Previous experiments by other laboratories have shown that "unactivated" receptor Smads are capable of forming heterodimers and heterotrimers and are then able to translocate to the nucleus in the absence of Co-Smad participation (116). Therefore, it is likely that "unactivated" R-Smads are found in the nucleus along with their phosphorylated form. Although it may be possible that the dominant form of Smad3 in the nucleus is phosphorylated, myocardin may not discriminate between this form and unphosphorylated Smad3. Results from other laboratories suggest that the C-terminal end of Smad3 is the site of high-affinity binding, and it is suggested that the phosphorylation site of Smad3 may cause that increased binding affinity. Further studies are required in order to determine the precise epitope within both Smad3 and myocardin that is required for their functional interaction. This notwithstanding, the interaction of these two transcription factors may impact on cytoskeletal regulation, cell proliferation, collagen production and cardiac hypertrophy (6) in ventricular myofibroblasts.

Linkages - Smad3, myofibroblast contractility and the pathogenesis of heart failure

There are currently three main methods of collagen gel deformation that are used to infer contractility and extracellular matrix remodeling: the stressed relaxed collagen matrix, the floating collagen matrix and the anchored collagen matrix (117). We have used the floating collagen matrix model in order to study the *in vitro* effects of myofibroblast contractile responses in a 3D environment. It has been previously shown that TGF-β_1 stimulation increases the contractility of fibroblasts when compared to non-stimulated cells (21;118;119) which corresponds to our findings. However, our main finding in the contractility study was that overexpressed Smad3 alone, with no stimulation of any cytokines, increased the contractility of cardiac myofibroblasts by approximately 1.5 fold (66%) when compared to the unstimulated control. Other groups have come to the conclusion that Smad3 is responsible for TGF-β_1 mediated gel contraction in human lung fibroblasts (69) but no studies have shown that Smad3 alone increases contractility, and again, not in ventricular myofibroblasts. Previous findings from our laboratory have shown that Smad3, along with other transcription factors and proteins, is upregulated in cardiac myofibroblasts in 8 week post-MI rat heart, as well as in earlier time points (120). Eight weeks after MI is a critical time point in this experimental model, as it coincides with the onset of moderate heart failure and with overt fibrosis of not only the infarct scar, but the remnant myocardium itself. As interstitial fibrosis is a well-known cardiac disease modifier (5), we feel that the connection between early (and likely causal) elevated expression of R-Smads is significant. The increased levels of Smad3 and subsequent increase in contractility may increase the tensile resting force in the matrix, thereby stretching cells in the parenchyma. This may, in turn exacerbate and potentiate TGF-β_1 release from adjacent cells eg, myofibroblasts and myocytes in order to compensate with the development of fibrosis. Thus,

this may be a positive feedback system that contributes to a rapid decompensation mechanism for the heart as it slips into overt heart failure marked by accelerated hypertrophy and matrix remodeling.

The contrast between effects of Smad3 and myocardin

Our second major finding was that increased expression of myocardin alone led to increased ventricular myofibroblast contractility that was comparable to that mediated by Smad3 overexpression. Although both transcription factors may cause the same effect in regards to contractility, they may do so via different mechanisms. In particular, the difference in the induction of NCX1 between the two factors was notable. Despite myocardin's inability to activate NCX1 mRNA expression, it is possible that myocardin could increase NCX1 function by a mechanism independent of transcription such as phosphorylation. Further, myocardin-linked alterations in mRNA expression may be discoordinate with NCX1 protein expression. Finally, and perhaps most likely, elevated myocardin could force the cells towards a more muscular phenotype by influencing the transcription of other genes such as αSMA.

The effects of Smad7

Overexpression of Smad7 typically opposes that of R-Smads, and our current data upholds earlier findings (20). In general we confirmed that Smad7 decreased contractility when compared to the TGF-β_1 stimulated cells, confirming our previous work (21). As well, when cardiac myofibroblasts were coinfected with both Smad3 plus Smad7 adenoviruses, contractility was not significantly different from that of the control, but when stimulated with 10 ng/ml TGF-β_1, contractility was comparable to TGF-β_1 alone group. Thus, ectopic overexpressed Smad3 is

able to override the effects of Smad7 when stimulated with TGF-β_1. This finding indicates that Smad3 overexpression in heart failure may be a dominant contributor to decompensated heart failure.

Confirmation of the functionality and responsiveness of ventricular myofibroblasts

TGF-β_1 treatment of our cells resulted in Smad3 phosphorylation. Phosphorylated Smad3 probing indicated rapid activation with its maximum phosphorylation level beginning at 15 minutes and lasting to 1 hour of stimulation. The response of myocardin to TGF-β_1 was determined through RT-PCR, as no high-quality myocardin antibodies are commercially available for Western blot analysis. TGF-β_1 stimulation led to marked reduction of myocardin mRNA expression which remained low out to 48 hours. Recent studies have shown that in human embryonic fibroblasts, myocardin expression peaks in response to TGF-β_1 at a concentration of 1 ng/mL at 4 hours of stimulation (121). Other work indicates that in response to hypertrophic stimuli, myocardin expression and activity levels are increased (114). Therefore, and despite our current results, the burden of evidence seems to point to TGF-β_1 mediated increase in phosphorylation of Smad3 in parallel with an increase in the production and activity of myocardin in other cell types. Using general terms, we speculate that both transcription factors at increased levels, at least in acutely treated cells, may allow cells to response rapidly to transcribe target genes that govern cellular function. Markedly decreased myocardin expression may be due to activation of an autoregulatory mechanism whereby it may repress its own expression (121). This may also assist in explaining the lack of effect seen with overexpressed myocardin on mRNA levels of NCX1 (Figure 15).

Previous studies examining Smad3 and its involvement in collagen secretion and fibrosis have shown that TGF-β_1 and the Smad signaling pathway are essential for the transcription of collagen genes and the development of fibrosis (122;123). In the development of cardiac fibrosis, Smad3 has been demonstrated to be a critical player in TGF-β_1 mediated fibrosis (124). Similarly 24 hour stimulation of cells with TGF-β_1 revealed increased type I collagen secretion vs. controls, and thus we conclude that our cell model system was responding and functioning in a predictable manner. Prior work focusing on the effects of Smad3 on fibrosis have shown that Smad3 null mice expressed decreased amounts of mRNA encoding for type I pro-collagen (17). As well, these mice revealed decreased levels of pulmonary hydroxyproline when compared to the wild-type mice (17). It seems likely that elevated Smad3 post-MI contributes to increased fibrosis and unchecked and inappropriate matrix deposition which is commonly associated with overt heart failure.

Previous studies from our laboratory have shown that Smad7 is responsible for inhibiting collagen production (13). Our new data is consistent with previous findings and shows that Smad7 inhibits TGF-β_1 –mediated collagen secretion. To date, no studies have shown the effects of co-overexpression of both Smad3 and Smad7 on collagen secretion. We found that cells that were co-infected with both Smad3 and Smad7 adenoviruses and were stimulated with TGF-β_1 showed no significant difference when compared to cells stimulated with TGF-β_1 and overexpressing Smad3. Thus the current results are in parallel with the contractility data, and emphasize the dominant effects of Smad3 *vs.* those of Smad7.

VIII Conclusions

1. We have demonstrated that myocardin is expressed in cardiac myofibroblasts and binds to p-Smad3 and Smad3.
2. Our investigation is the first to demonstrate that overexpressed Smad3 alone increases the contractility of cardiac myofibroblasts as well as NCX1 mRNA expression. This is an important observation as reverse-mode NCX1 activity is critical for normal fibroblast function.
3. Smad3 overexpression overrides the effects of overexpressed Smad7 which resulted in elevated contractility.
4. Myocardin overexpression alone increases the contractility of cardiac myofibroblasts as well as has a synergistic effect when co-overexpressed with Smad3 and TGF-β_1.
5. Similar to the contractility study, co-overexpression of Smad3 and Smad7 resulted in sustained elevation of type I collagen secretion - thus Smad3 overrode Smad7 effects with respect to this end-point.

IX Future Directions

One of our most exciting observations of our studies is that myocardin is expressed in ventricular myofibroblasts and its expression was elevated with increasing passage number eg, in the shift from fibroblast to myofibroblast. Others have shown that myocardin "pushes' the myocyte phenotype in smooth muscle (125) which is marked by the specific induction of L-type Ca channels, NCX1, and K_{ir} channels. In this regard, experiments to explore the link between the expression of these genes, as well as whether myocardin is responsible for the phenotypic switch from fibroblasts to myofibroblasts would be worthwhile. This linkage might be accomplished by building an adenoviral knock-down of myocardin. By decreasing its expression levels in myofibroblasts, experiments could determine if the myofibroblasts take on the non-contractile fibroblast-like phenotype. As culturing fibroblasts on compressible matrices is also associated with a slowing of this phenotypic switching (126), we would explore the possibility that these plating conditions plays a role in regulating down-regulation of myocardin.

As well, we would like to investigate the specific nature of the interaction between myocardin and p-Smad3. As these two proteins were shown to co-precipitate by binding, evidence from other laboratories indicate that myocardin binds full-length Smad3 in smooth muscle cells (6), the Smad3 phosphorylation site may not be critical for binding. In order to determine what regions of Smad3 are required for binding to myocardin, site-directed mutagenesis of the likely epitopes, including key phosphorylation sites of Smad3 could be conducted to see if this is the regulatory factor. If there is still an interaction, deletion studies could also be conducted in order to find the specific region(s) required for binding. As well, once a high quality myocardin antibody is obtained, the actual protein levels of myocardin in the

TGF-β_1 time trial could be determined in order to see how comparable they are to the levels of p-Smad3.

X Methodological limitations of the current study

Throughout the current investigation, we have relied upon data generated from experiments to gauge mRNA expression of NCX1 and myocardin, particularly in response to 10 ng/ml TGF-β_1 or adenoviral overexpression of Smad3. This trend is governed by purely practical considerations – in both cases, the lack of a properly functioning primary anti-myocardin antibody. While we acknowledge that steady-state mRNA expression only provides an early glimpse at transcriptional activation of these two target genes, we feel that as a first step, this data is clearly much better than speculation alone. We will pursue the issue of changes in protein abundance in future work in order to gain a rounded set of data to address the expression of these two genes by the development of specific Western blot protocols. A superior (but some may argue it as a half-measure) approach lies in the use of real time or so-called qRT-PCR to gain information about the relative mRNA abundance of target genes at the early stages of the PCR reaction. Using this method, one gains the advantages of enhanced sensitivity eg, ability to detect a two-fold increase in copy number *vs*. a detection limit of 5- to 10-fold changes in copy number of the target gene. On the issue of loading controls for our traditional PCR studies and based on our preliminary data, we feel that GAPDH, despite its status as a constitutively expressed gene, is probably markedly influenced by Smad3 and/or TGF-β_1. This could be addressed by the use of β-tubulin as the "housekeeping gene".

Other limitations within the study include i) the reliance on adenoviral overexpression of Smad3, Smad7 and myocardin target genes to observe the effects of forced expression and ii) the assumption that the amount of Smad7 overexpression is sufficient to balance the effects of Smad3 overexpression. The former issue may be effectively dealt with using knock-out or knock-in transgenic mouse models and/or RNAi technology, and we have initiated these studies.

The latter question, and especially that surrounding Smad7, has been addressed by our group in the past (largely based on the work by Dr. Baiqiu Wang *et al*., *Am J Physiol*, 2007). We have found that at 100 MOI of Smad7 adenovirus is an effective titer and is sufficient to suppress the phosphorylation of Smad2 in ventricular myofibroblasts (when stimulated with 10 ng/ml TGF-β_1). As well, it is also effective against the induction of fibrillar collagen expression with which it is normally associated. While this bioassay is not definitive nor directly addresses a supposed balance between overexpression of Smad7 and Smad3 in our studies, it does illustrate the bioactivity of Smad7 associated with suppression of that particular endpoint. In combination with the known effects of overexpressed Smad3 derived from our current work, we felt the current MOI doses of each virus to be logically derived.

XI List of References

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