Effects of Inhibitory Smad7 on P1 Primary Cardiac Myofibroblast Proliferation

A Thesis Presented to the

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In partial fulfillment of the requirements

for the degree of

Master of Science

By

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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

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LIST OF ABBREVIATIONS

α-SMA	α-smooth muscle actin
BSA	bovine serum albumin
Co-Smad	common-mediator Smad
CKI	cyclin dependant kinase inhibitor
CDK	cyclin dependant kinase
CHF	congestive heart failure
DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix or the matrix
FBS	fetal bovine serum
I-Smad	inhibitory Smad
MI	myocardial infarction
MMP	matrix metalloproteinase
MOI	multiplicity of infection
PAGE	polyacrylimide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PBS	phosphate buffered saline
pRb	phosphorylated retinoblastoma protein
P-Smad	phosphorylated Smad
Rb	retinoblastoma protein
P0	Cells derived directly from the heart and plated without passage
R-Smad	receptor-mediated Smad
SARA	Smad anchor for receptor activation

SMemb	Embryonic isoform of smooth muscle myosin / non-muscle myosin heavy chain
TGF-β	transforming growth factor-β
TβRI	transforming growth factor-β type I receptor
TβRII	transforming growth factor-β type II receptor

I. ABSTRACT

Heart disease of various major etiologies has reached epidemic levels in Canada. Demographic information from Health Canada reveals that in 2001, 180 deaths per 100,000 persons were due to cancer while 230 deaths/100,000 were linked to cardiovascular diseases (OECD - Organization for Economic Co-operation and Development health data, 2002). Myocardial infarction (MI), or heart attack, is the most common etiology of heart failure in North America. In this and other types of heart diseases, cardiac myofibroblasts play a major role in the process of remodeling of the cardiac extracellular matrix (ECM) after myocardial infarction. Activation of transforming growth factor β1 (TGF-β1) receptors leads to activation of various members of the Smad family. These subcellular TGF-β1 effector proteins are now well-recognized to play an important role in the progression of matrix remodeling and tissue fibrosis with inherent modulation of cardiac fibroblast function in heart failure. Inhibitory Smad7 function is an inducible regulatory Smad that is known to inhibit some aspects of the TGF-β1 signal i.e. those mediated via receptor-activated (R-) Smad phosphorylation. We have shown that inactive I-Smad7 resides in the nucleus of quiescent cells and that TGF-β1 serves to release and translocate nuclear I-Smad7 into the cytosol. Activation of I-Smad7 is known to compete for binding of R-Smads to TβRI (TGF-β1 receptor type I as seen on page 13), thereby blocking R-Smads from being phosphorylated during interaction with the receptor. The inhibitory action of I-Smad7 may also increase ubiquitin-mediated degradation of TGF-β1 receptor.

The effects of overexpression of I-Smad7 proteins on cardiac myofibroblast proliferation and fibroblastic phenotype are unclear at present. We undertook investigation of these issues using common assays to provide information about cell proliferation, including ^{3}H-thymidine

incorporation, as well as PCNA expression. Our initial observation characterized the TGF-β1 response in P1 myofibroblasts insofar as we noted significant inhibition of proliferation *vs.* nontreated control values from cells grown in 2% serum alone. In parallel studies, I-Smad7 gene (Ad I-Smad7) was delivered to primary P1 cardiac myofibroblasts by adenoviral transfer at a multiplicity of infection (MOI) of 100 (24 hours). Under these conditions we noted significant overexpression of I-Smad7 by Western analysis which was also incremental with MOI ranging from 25 to 200 at either 24 or 48 hour incubation. The appearance of Ad-LacZ was determined to provide an inference of adenoviral infection efficiency by X-Gal staining. We noted that greatest infection efficiency of Ad-LacZ at 100 MOI at 24 hours, and that the majority of infected cells maintained viability. Overexpression of I-Smad7 adenovirus was associated with a significant decrease of myofibroblast proliferation vs. control values (controls were LacZ [Ad-LacZ] infected cells and non-infected cells). In I-Smad7 infected myofibroblasts we observed reduced ^{3}H-thymidine incorporation *vs.* Ad-LacZ infected controls. Furthermore, our data revealed that I-Smad7 overexpression was associated with a significant increase in expression of 27 kDa p27 protein, while the expression of 110 kDa and 36 kDa pRb and proliferating cell nuclear antigen (PCNA), respectively, was significantly decreased in cells vs. control values associated with LacZ-infected cells. Expression of either p21 or p15 was not altered by exogenous I-Smad7 infection/overexpression. Also, expression of cdk2 and cyclin E was significantly decreased under the same conditions in cultured cells compared to values associated with LacZ-infected cells, while cdk4 and cyclin D expression were not altered by ectopic I-Smad7 overexpression. Results from the immunofluorescence studies of immunoreactive α-smooth muscle actin, SMemb and rhodamine phalloidin- labeled cells indicates that the myofibroblastic phenotype was not altered with ectopic I-Smad7 or Ad-LacZ infection.

Thus our data provides support for the hypothesis that proliferation inhibition caused by TGF-β1 in cardiac myofibroblasts is mediated by secondary I-Smad7 release in these cells. When compared with previous findings from our lab these results support the general hypothesis that I-Smad7 exerts net anti-fibrotic effects in diseased hearts via their effects on primary cardiac myofibroblasts. Therefore, treatments targeting myofibroblast cell proliferation with exogenous I-Smad7 may provide a new therapeutic strategy for the treatment of cardiac fibrosis.

II. INTRODUCTION

The infarcted myocardium undergoes extensive remodeling after MI. An important part of this process is known as cardiac fibrosis [1]. In the early stage of MI, cardiac fibrosis occurs to accelerate wound healing in the infarcted region. However, with the passage of time, interstitial fibrosis of the remnant post-MI heart occurs, and is associated with increased myocardial stiffness. Excessive wound repair is characterized by marked interstitial cardiac fibrosis. Myofibroblast proliferation contributes to the development of cardiac hypertrophy and heart failure [2]. Transforming growth factor β1 (TGF-β1) also plays a significant role in the progression of cardiac fibrosis during the development of cardiac hypertrophy. The precise role of TGF-β1 in regulation of the functional proliferation is highly specific to cell type, as this ligand is pleiotropic. [3].

TGF-β1 signalling is initiated through ligand binding to a transmembrane heteromeric type I, and type II receptor complex which normally displays serine/threonine kinase activity [4]. Following activation of the receptor, the signal is transferred to the nucleus through Smad proteins, a family of transcription factors identified in vertebrates, insects and nematodes [5]. The Smad family consists of receptor-regulated Smads (R-Smad, Smad2 and Smad3), common mediator Smad (Smad4) and inhibitory Smads (Smad6 and Smad7). Upon the ligand binding of TGF-β1, R-Smads are phosphorylated by TGF-β1 type I receptor and oligomerized with Co-Smad4 to form a heterodimeric or heterotrimeric Smad complex. This complex subsequently translocates to the nucleus where it modulates transcription by interacting with DNA binding partners as well as recruiting transcriptional co-activators and co-repressors. TGF-β1 signaling is tightly controlled through multiple mechanisms in the extracellular space, at the membrane, and the cytoplasmic and nuclear space. Net cellular response to TGF-β1 stimulation depends upon the outcome of this well-tuned balance of Smad partners and regulators present in a given context.

I-Smad7 has recently been reported to form a stable interaction with activated TßRI, thereby preventing the binding to and activation of R-Smad through its phosphorylation [6, 7, 8], I-Smad7 stimulates growth, apoptosis and embryonic lung morphogenesis induced by TGF-β1 [9, 10, 11, 12]. Lung fibrosis in rats, which is mediated by consistent activation of TGF-β1, is prevented by the administration of adenovirus I-Smad7 [13]. Overexpression of I-Smad7 is associated with blockade of phosphorylation of Smad2 in renal tubular epithelial cells [14]. Further, data from our lab indicates a relatively early and dramatic decrease in I-Smad7 expression in infarct scar and remnant left ventricular (LV) tissues from post-MI hearts [15]. These findings indicate a potential role of Smad7 in the development of cardiac fibrosis in post-MI heart [15], in which it contributes to de-regulation of cardiac myofibroblast function, in other words, loss of I-Smad7 may de-repress myofibroblasts in the infarcted scar. There is very little information available in the literature to address the effect of I-Smad7 or TGF-β1 on primary cardiac myofibroblast and proliferation. Further, no data is available to describe cardiac myofibroblast phenotype after infection with ectopic I-Smad7 expressing adenovirus. The main goal of the present study is to address the effects of I-Smad7 overexpression on cardiac myofibroblast proliferation.

We hypothesize that normally, in response to TGF-β1, the I-Smad7 response may supercede R-Smad signaling in primary cultured myofibroblasts and is associated with suppression of proliferation. Thus, overexpression of adenoviral I-Smad7 causes decreased proliferation of myofibroblasts due to altered expression of specific cell cycle proteins (p27, p21, p15, phospho-Rb, cyclins and cyclin dependent kinases). We characterized the effect of overdriven I-Smad7 in our *in vitro* cardiac myofibroblast system on expression of cell cycle inhibitors i.e. p21, p27, phospho-Rb and p15. In addition we examined the proliferative effect of the ectopic overexpression of I-Smad7 in cardiac myofibroblast using the ^{3}H-thymidine incorporation technique. We also investigated the

effect of the ectopic overexpression of I-Smad7 on the phenotypic changes of the cardiac myofibroblasts. Furthermore, we examined the effect of overexpression of I-Smad7 on the proliferating cell nuclear antigen (PCNA). We then studied the effects of ectopic overexpression of I-Smad7 on myofibroblast cyclin and cyclin dependant kinase expression patterns, which play a role in the control of cell cycle progression through various phases. Finally, we studied the effect of TGF-β1 on myofibroblast proliferation *in vitro*, using ^{3}H thymidine incorporation.

The goal of our examination of the effect(s) of overexpression of I-Smad7 on proliferation of cardiac myofibroblasts is to elucidate the role of I-Smad7 protein as a player in the specific effect of TGF-β1 i.e. reduced proliferation, in cultured myofibroblasts, and also to provide some insight into I-Smad7's role in cardiac matrix remodeling in fibrosis.

III. STATEMENT OF HYPOTHESIS

I-Smad7 protein functions as an intracellular "brake" for TGF-β1 signaling primarily by the inhibited phosphorylation of R-Smads via the kinase region of activated type II TGF-β receptor. We have previously shown that I-Smad7 is rapidly released from the myofibroblast nucleus to the cytosol in response to extracellular TGF-β1. We predict that the magnitude of the I-Smad7 response for any given cell is variable eg, the release characteristic of fibroblastic cells may not be the same as in myocytes or other cell types. Thus our general working hypothesis is that i) that TGF-β1 leads to inhibition of primary myofibroblast proliferation and that ii) this response depends upon I-Smad7 release. The specific component of this hypothesis to be tested within the context of the current thesis is that enhancement of I-Smad7 expression alone in myofibroblasts is sufficient to override R-Smad signaling in primary cultured myofibroblasts and is associated with suppression of cellular proliferation. Finally, overexpression of I-Smad7 causes decreased proliferation of myofibroblasts in association with altered cell cycle protein expression (p27, p21, p15, phospho-Rb, cyclins and cyclin dependent kinases).

IV. LITERATURE REVIEW

1.0 Etiology of heart failure

Cardiovascular and circulatory diseases are the leading cause of death in Canada [16]. The mechanisms that lead to irreversible pathological remodeling of heart tissue structure contribute to an enhanced risk of adverse cardiovascular events, including myocardial infarction, mechanical dysfunction, symptomatic heart failure and arrhythmias [17]. As a result of the remodeling process, the heart becomes unable to pump enough blood to meet the needs of the body's vital systems, which subsequently break down, leading to further complications, which may lead to death.

1.1 Pathogenesis of myocardial infarction and heart failure

A sudden life-threatening loss of cardiac performance may be induced by various factors. Aside from the aging process which is associated with loss of cardiac function, cardiac dysfunction can occur directly from the development of pathological conditions caused by certain diseases, resulting from the activation of compensatory mechanisms that may become maladaptive, and thereby cause adverse effects on cardiac function.

Acute myocardial ischemia plays a major role in cardiac failure. It is well known that deprivation of (Oxygen) O_2 supply through embedded blood vessels damages the heart muscle and therefore reduces cardiac pump function [18, 19, 20]. Coronary artery occlusion reduces the blood flow that supplies the heart with oxygen and nutrients [20]. Occlusion of coronary arteries may develop via atherosclerotic mechanisms involving pathological stimuli to the arteries [18, 21]. Over time, the injuries that develop from this oxygen deficit cause physical damage to the heart, and may also result in sudden coronary occlusion and MI [19]. When an infarction occurs, the heart

undergoes structural changes to compensate for the loss of tissue. Remodeling of cardiac structure constitutes an important long-term adaptive mechanism, and involves development of hypertrophy, growth of the capillary network, and an increase in interstitial collagen. Structural remodeling is not limited to the infarcted myocardium but, occurs also in the non-infarcted area over time. Cardiac function eventually declines due to transition from adaptive compensatory changes to irreversible maladaptive mechanisms [22]. The nature of the load imposed on the heart contributes to gross ventricular remodeling [23,24]. Aside from MI, the most prominent risk factors for eventual heart failure are hypertension and volume overload, both of which are linked to the development of pathological hypertrophy and fibrosis [18, 23].

Diabetes may also contribute to the development of heart failure even in the absence of coronary artery disease, supporting the concept of primary diabetic cardiomyopathy [23]. The mechanisms that underlie the process of diabetes may also damage the heart muscle. These mechanisms primarily involve the activation of the cardiac renin-angiotensin system, which is linked to hypertension that eventually leads to heart damage [18,24]. Valvular heart disease, another cause of heart failure, develops from stenosis (i.e. narrowing) or improper closing of cardiac valve that controls the blood flow leaving the heart, thereby, disrupting the normal route for blood flow. There are two types of valvular stenosis, mitral or aortic [25]. They both lead to the backup of blood in left atrium during diastole (the filling phase of the heart), and in left ventricle during systole (the emptying phase of the heart), respectively, which eventually leads to hypertrophy and eventual heart failure [25]. Another cause of heart failure is cardiomyopathy. These are mainly genetically defined diseases; the most common cardiomyopathies being hypertrophic cardiomyopathy and dilated cardiomyopathy. Hypertrophic cardiomyopathy is an idiopathic heart disease, affecting the interventricular septum and the left ventricle [26, 27].

Other causes of heart failure include alcoholism as it is associated with chronic hypertension that leads to progressive cardiac damage [28], as well as excessive salt consumption, hyperthyroidism, thiamine deficiency, pneumonia, severe emphysema, high fever, and failure of liver or kidneys. Acute myocarditis is a viral infection of the heart muscle, and can also lead to heart failure [reviewed in 29].

1.2 Post myocardial infarct heart failure

Increasing muscle mass (cardiac hypertrophy), is the adaptive process that occurs after a massive heart attack, usually preceding the development of congestive heart failure (CHF), a major cause of death [30, 31]. and its magnitude is variable depending on the size of infarction [31, 33]. When a large MI occurs, the ventricular chamber may remodel by increasing in volume [33] and undergoing severe hypertrophy, which is associated with increased myocyte size and decreased intrinsic cardiac performance [30]. It is becoming clear that chronic cardiac wound healing and fibrosis development in congestive heart failure is a complex process [15, 35, 36]. Myofibroblast behavior may also potentiate wound healing and eventual cardiac fibrosis. TGF-β_1 is widely studied as a stimulus for fibroblast and myofibroblast function in the setting of myocardial infarction [37]. It is also known to stimulate focal adhesion (FA) supermaturation in myofibroblasts [38]. The myocardial ECM (comprised mainly of fibrillar collagens) is an organized network intimately associated with cardiac function, serving to direct, transmit, and distribute myocyte-generated contractile force [39]. It participates in active restoration of sarcomeric length, via release of stored potential energy in matrix proteins [40, 41, 42, 43] Collagen types I and III (fibrillar collagens) form struts between myocytes and among muscle fibers [40, 41, 44, 45]. Matrix also plays an important role in the regulation of cell death, gene expression and parenchymal cell differentiation [42,43].

Nonetheless, elevated fibrillar collagen expression may be responsible for changing heart function in heart disease based on its adverse influence on myocardial stiffness [46, 47].

The majority of DNA synthesizing cells in the surviving myocardium and infarct scar of experimental animals are fibroblasts and myofibroblasts [48, 49]. Reparative fibrosis or scarring is an adaptative process due to the loss of parenchyma, which are terminally differentiated cells that can not proliferate or regenerate [50]. Continuous parenchymal loss and abnormal collagen accumulation result in decreased passive compliance and impaired cardiac contractility, which potentiates the progression of overt heart failure [50,51,52].

1.3 Healing of infarcted myocardium

Infarct healing is an active process, which can be divided into four phases; the first phase involves cardiomyocyte cell death or myocyte dropout, which occurs by either apoptosis or overt necrosis (6-8 h after infarction) [50]. Myocyte necrosis evokes the second phase of the infarct healing that is characterized by the activation of inflammatory cells, cytokines, and neutrophylic granulocytes, which helps to remove the dead myocytes (12 hours-4 days post-MI). The third phase is marked by onset of structural remodeling of the infarct site, and formation of granulation tissue. Granulation tissue itself is characterized by the appearance of myofibroblasts, enhanced activation of proteolytic matrix metalloproteinases, and neovascularization (2-3 days after infarction). Myofibroblasts are phenotypic variants of quiescent fibroblasts, and are involved in the synthesis and deposition of the ECM proteins. The importance of the matrix protein synthesis includes alterations in the tensile strength of the infarct, and may abrogate cardiac rupture. Enhanced synthesis of fibrillar collagens *de novo* promotes development of cardiac fibrosis, whereas enhanced activities of interstitial collagenase (i.e. MMP-1) and gelatinases (MMP-2 and MMP-9) lead to

degradation of the matrix in acute MI. The net action of these proteases contributes to infarct expansion and myocardial rupture. The process of neovascularization involves the formation of new blood vessels; its role is to increase the blood supply into the infarct area. In the fourth phase of cardiac wound healing, granulation tissue becomes replaced by the scar formation. This phase is characterized by the complete cross-linkage of matrix proteins, and the relative abundance of myofibroblasts, which do not undergo apoptosis at the completion of the wound healing phase (2-3 weeks after infarction) [18, 49, 53, 54].

Therefore, myofibroblasts play very important roles in the scar, where they govern both acute and chronic matrix remodeling in the post MI-period [50].

1.4 Changes in non-infarcted myocardium

Non-infarcted area affects the changes taking place in the infarcted area after an MI, as the infarct scar is unequipped and unable to generate rhythmic contractions as does the remnant heart. The non-ischemic myocardium adapts to the increased workload by changing the structural morphology of the heart muscle [54]. Changes in mechanical loading directly affect the function of cardiomyocytes, endothelial cells and fibroblasts. Cardiomyocyte hypertrophy and apoptosis are prominent features of remnant post-MI heart and these changes are accompanied by proliferation of the endothelial cells lining the capillaries. As the proliferation of endothelial cells cannot compensate for the amount of cardiomyocyte hypertrophy and so the myocytic phenotype shifts into the fetal state [50, 55]. Fetal cardiomyocytes subsequently function with lower energy consumption associated with decreased myocardial function. Concomitantly with the process of cardiomyocyte hypertrophy, mechanical feedback input affects fibroblast function in the remodeling heart. These cells transduce the mechanical stimuli

from matrix to specific intracellular signaling responses that induce an altered pattern of protein expression. This sequence of events then leads to remodeling of the ECM as fibroblasts increase their synthetic output [56, 57]. A big misconception is that for a cell to be classified as a myofibroblast it must express α-SM actin [139]. However, in several locations *in vivo* cells show the morphological characteristics of myofibroblasts, such as stress fibers, but do not express α-SM actin, for example, in the lung alveolar septa and the early phases of granulation tissue formation. Therefore, this shows that there are two types of myofibroblasts, those that do not express α-SM actin, which we propose should be termed 'proto-myofibroblasts'; and those that do express α-SM actin, for which we propose the term 'differentiated myofibroblast'. The distinction between fibroblast and proto-myofibroblast is particularly clear *in vivo*, whereas, when cultured on a plastic substrate in the presence of fetal calf serum, practically all fibroblasts acquire proto-myofibroblastic features [139]. The presence of proto-myofibroblasts in certain adult tissues shows that they can persist and function as an independent cell type; however, proto-myofibroblasts can be induced to express α-SM actin and form differentiated myofibroblasts in response to specific factors, as occurs in certain adult tissues, granulation tissue and fibrocotractive diseases. Mechanical stress with the effect of other factors such as TGF-β1 in a tissue induces fibroblasts to differentiate into proto-myofibroblasts, which also progress to differentiate into fully differentiated myofibroblasts [139] (Figure 1). Over time, cardiac fibrosis develops as a result of the increased production of the ECM proteins, which in most cases seems to overwhelm the altered bioactivity of collagenase matrix metalloproteinases [55].

1.5 Myocytes vs. non-myocytes in the heart

Although cardiomyocytes occupy ~75% of the structural space of the heart, they constitute only one-third of the total cell population. The remaining non-myocytes consist of endothelial,

vascular smooth muscle cells and fibroblasts in the interstitium. Fibroblasts and myofibroblasts represent 90% of the non-myocytes cells [59]. Cardiac myocytes are highly specialized muscle cells composed of bundles of myofibrils that contain myofilaments. Myofibrils have distinct, repeating microanatomical units or sarcomeres which represent the basic contractile units of the myocyte. The sarcomere is defined as the region of myofilament structures between two Z-lines. The distance between Z-lines (i.e., sarcomere length) ranges from about 1.6 to 2.2 μm in human hearts. The sarcomere is composed of thick and thin filaments – myosin and actin, respectively. Chemical and physical interactions between the actin and myosin cause the sarcomere length to shorten, and therefore the myocyte to contract during the process of excitation-contraction coupling. The interactions between actin and myosin serve as the basis for the sliding filament theory of muscle contraction [58]. Cardiac myocytes which carry out the forceful contractions that allow the heart to serve as a pump are thought to be terminally differentiated cells. During cardiac hypertrophy, cardiac myocytes enlarge without proliferating, while cardiac fibroblasts divide and differentiate.

1.6 Fibroblasts vs. myofibroblasts-phenotypic variants of the same cell type

Myofibroblasts play an important role in matrix remodeling by synthesizing and depositing collagen proteins, and they resemble fibroblast cells in some ways. For example, they are characterized with an extensive rough endoplasmic reticulum and Golgi apparatus, but in contrast to fibroblasts, myofibroblasts show elevated expression of α-smooth muscle actin (α-SMA), more extensively developed stress fibers, supermature focal adhesions, and in several cases, desmin and embryonal isoform of myosin heavy chain (SMemb) [53, 61, 62]. Both cell types are known to express vimentin [54]. The expression of α-SMA by myofibroblasts is similar to the contractile myofibrils found in smooth muscle cells, and is thus considered as smooth muscle differentiation

marker along with desmin and smooth muscle myosin heavy chain [54]. More recent findings point to a graded expression of actin and the appearance of stress fibers in fibroblasts, protomyofibroblasts and myofibroblasts. Although fibroblasts might contain actin, these cells do not generate stress fibers, and they do not form adhesion complexes with the matrix [54]. The potential precursor candidates of myofibroblasts are fibroblasts, which under normal physiological conditions represent a heterogeneous population of cells [63, 64]. In fibrosis, a specific fibroblast subpopulation of cells may become predominant, or alternatively fibroblasts may undergo phenotypic modulation [66]. One of the most important factors involved in the phenotypic modulation of fibroblasts to myofibroblasts is TGF-β, a cytokine which is also responsible for the induction of mechanisms implicated in the enhanced collagen synthesis in the post-MI myocardium, which prevents the rupture of the infarct tissue and therefore, contributes to scar formation [18, 51, 62, 65]. These cells can persist in myocardial scars for many years, and it has been suggested that α-SMA contributes to the infarct scar tissue contraction as well as maintenance of scar tonus, serving as an important adaptation to the loss of the myocytes [50]. Tissue contraction during cardiac healing may be also mediated via SMemb expressed by myofibroblasts [61].

2.0 Control of myofibroblast proliferation

Fibroblast proliferation occurs after the first appearance in the infarct zone after an MI. The cell cycle is divided into four distinct phases: G_1, S, G_2 and M. The two G (gap) phases are referred to as growth phases, DNA synthesis occurs during S phase while cell division (mitosis) occurs during M phase [66, 67, 68]. The Mitosis (M) phase is composed of 7 different stages; they are interphase, prophase, prometaphase, metaphase, anaphase, telophase and finally cytokinesis.

Many times a cell will leave the cycle, temporarily or permanently, exiting at the G_1 stage and entering a stage designated G_0 (G naught).

Often G_0 cells are terminally differentiated: they will never re-enter the cell cycle but instead will carry out their function in the organism until they die. Proper progression through the cycle is assured by "checkpoints." These are points in the cell cycle where certain conditions have to be met before the cell can proceed into the next phase [66, 69]. These transitions are governed by the cyclin dependent kinases (CDKs) and by inhibitory and activating phosphorylation events [66, 69]. The activity of these enzymes is dependent on their molecular partners, cyclins and CDK inhibitors (CKI), the levels of which fluctuate depending on the specific phase of the cell cycle, whereas the CDKs themselves are more stably expressed. So far, over 12 cyclins and 9 CDK subunits have been described with the cyclins categorized into 5 groups, A through E [69].

2.1 Major protein classes in cell cycle regulation

Progression through the cell cycle is controlled by check points and different proteins that promote correct timing and ordering of events required for cell growth and cell division. The main proteins that are responsible for controlling the cell cycle are called cyclins, cyclin dependant kinases (Cdks) and cyclin dependant kinases inhibitors. G_1 stage progression is mediated by sequential activation of D type CDKs (cyclin D with CDK4 or CDK6), and cyclinE paired with CDK2 to form a complex [70]. CyclinD is upregulated by growth factors, and is expressed in any cell that is in the cell cycle. A target of the G_1 CDKs is Rb, which is increasingly phosphorylated during mid-late G_1 phase, first by cyclinD-CDK4 and CDK6, then later by cyclin E-CDK2 as cells enter S phase [66, 70]. Phosphorylation and activation of transcription factors, will increase the expression of cyclin D and phosphorylation of Rb, leading to transcription of target genes and cell

cycle progression [67, 71]. Cyclin A and cyclin B are expressed in a phasic manner, with low expression throughout the cell cycle except during the transition from G_2 to M phase [69]. After entry into S phase, Rb is held in its hyperphosphorylated inactive state by cyclinA-CDK2 and by cyclinB-CDK1/CDC2 during M phase. The early events in the cell cycle are dependent on the presence of growth factors, whereas once the cell has entered S phase, the remainder of the cell cycle can be completed even if growth factors are removed. This time point has been designated the restriction (R) point [72]. Beyond this point the cells can not only complete S phase, but also traverse G_2 and M phases in the absence of growth factor stimuli [66, 68, 70, 73] (Figure 3).

Cell cycle progression is not only dependent on CDKs, cyclins and CDK inhibitors. Normal, non-transformed cells must be adherent to the substratum in order for effective proliferation to occur [74]. Additionally, non-adherent cells fail to phosphorylate Rb and fail to activate cyclin E-CDK2 [75]. Therefore, ECM receptors play a critical role in regulating cell cycle progression by facilitating optimal activation of intracellular signalling pathways and induction of cyclins, in particular, cyclinD [74]. The last group of cell cycle regulators is the cyclin dependant kinase inhibitors (CKIs) e.g. p21, p27, p15 and phospho-Rb. These inhibitors antagonise the effects of cyclin dependant kinases (cdks) and lead to inhibition of cell cycle progression (Figure 3). In addition, adhesion may play a role by suppressing total levels of CDK inhibitors p21 and p27 [76]. Cell adhesion exerts its regulatory role primarily at the G_1 restriction point, since the events that are dependent on adhesion also regulate the G_1-S transition. A cell must be able to detach momentarily from the substratum to complete M phase and cytokinesis [76].

2.2 CDK Inhibitor proteins

There are at least 7 different CDKIs in mammalian cells which belonging to two different classes. The first class comprises p21, p27 and p57 that preferentially bind to the G_1/S class of CDKs. The second class of CDKs, referred to as the INK4 (Inhibitor of CDK4) family, is comprised of ankyrin repeat proteins and includes p15, p16, p18 and p19 [77]. These inhibitors act on cyclin D complexed either to CDK4 or CDK6. Recent studies have shown that the cyclin-dependent kinase inhibitors (CKIs) play important roles in cell cycle progression in normal cells. Alterations in the cdk inhibitors also appear to be important in myofibroblast proliferation and therefore affect the process of fibrosis afterwards (Figure 3) [77, 78, 79, 80].

2.2.1 p15

p15 belongs to the CKI family of cell cycle regulators that bind to "cyclin-CDK" complexes and cause cell cycle arrest in G_1, therefore, arresting progression to S phase, where DNA synthesis occurs. P15 regulates the cell cycle pathway by inhibiting cyclin D-CDK4/6 mediated phosphorylation of Rb (Figure 3) [78, 79].

2.2.2 p21

p21 protein is a dual inhibitor of cyclin-dependent kinases and proliferating-cell nuclear antigen (PCNA) both of which are required for passage from G_1 to S phase, p21 is a downstream regulator of the tumor suppressor gene p53 and inhibits CDK 2 and 4. Downregulation of p21 may occur because of loss of function mutations of p53 itself, which may lead to cell cycle arrest or apoptosis, p21 has also been implicated in cell senescence and in cell-cycle withdrawal upon terminal differentiation (Figure 3) [79, 80, 81, 82].

2.2.3 p27

p27 is one of the most notable and well-established members of CKI family, it binds and interacts with all subtypes of "cyclin-CDK" complexes causing cell cycle arrest in G_1 phase, which inhibits cell proliferation. In addition, p27 has been postulated to promote apoptosis, play a role in terminal differentiation of some tissues, and mediate chemosensitivity in solid tumors (Figure 3) [79, 80, 81, 82].

2.2.4 Phosphorylated Retinoblastoma protein (pRb)

The retinoblastoma tumor suppressor protein (phospho-Rb) is a potent inhibitor of mammalian cell growth and the functional inactivation of phospho-Rb is widely presumed to be essential for progression of the cell cycle past the G_1 phase. Phospho-Rb is thought to play a crucial role in the decision-making at the restriction point [83]. The action of phospho-Rb is currently explained by its physical interaction with cellular targets, notably E2F transcription factors. Hyperphosphorylation of phospho-Rb in late G_1 phase of the cell cycle by CDKs abolishes the ability of phospho-Rb to form complexes with E2Fs. Once released from phospho-Rb, the E2Fs are thought to provoke a wave of transcription that is essential for G_1 to S phase progression and subsequent cell division (Figure 3) [84, 88].

2.3 Cyclin proteins

Cyclins were first identified by scientists working on sea urchin eggs, who noted that the concentration of cyclins went up rapidly through most of the cell cycle and then suddenly dropped to zero halfway through the M phase [83]. So far at least 25 mammalian cyclins have now been identified, each one of which is required at a different stage of the cell cycle. It has been suggested

that cyclins act as a switch, turning on mitosis whenever their concentration reaches a certain level [83, 84]. They are broadly classified into G_1 and mitotic cyclins, according to the stage of the cycle where they are produced, G_1 cyclins are relatively short lived proteins, while the mitotic cyclins are longer lived. Cyclins appears to bind and activate CDKs by forming a complex that results in phosphorylation and activation of the CDKs. Activated CDKs then phosphorylate the retinoblastoma (Rb) protein. The Rb protein has a critical role in regulating progression through the G_1 phase and in the event of genomic damage, the cycle may be delayed or abandoned. This activation is required for cells to move from one stage of the cell cycle to the next. Some examples of these cyclins are cyclin D, cyclin E and cyclin A (Figure 3) [83, 84, 85].

2.3.1 Cyclin D

This cyclin is synthesized in late G_1 and appears to be required for progression into S-phase. Cyclin D is upregulated by growth factor, and is expressed in any cell that is in the cell cycle. Overexpression of cyclin D leads to the reduction of length of G_1 and therefore, rapid movement into the S phase. Cyclin D binds to and activates both CDK4 and CDK6 and the formation of all possible complexes between the D-type cyclin and CDK4/6 is promoted by the proteins, p21 (CIP1/WAF1) and p27 (KIP1). The cyclin-dependent kinases phosphorylate the Rb protein leading to release of the E2F transcription factors, resulting in proper G_1/S transition (Figure 3) [86, 87].

2.3.2 Cyclin E

Cyclin E is one of the main members of the cyclin family and is a key regulator of the G_1/S transition in the cell cycle. It activates Cdk2 near the start of S-phase forming a complex that is necessary for the initiation of DNA replication in S-phase. Cyclin E is found associated with the

transcription factor E2F in a temporally regulated fashion. E2F is known to be a critical transcription factor for the expression of some S phase-specific proteins and is thought to be important for a series of others (Figure 3) [86, 87, 88].

2.4 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are a group of Ser/Thr protein kinases that function as central regulators of cell cycle progression from G_1 phase to S phase. They also play an important role in regulation of transcription, mainly in proliferating cell types. Currently 13 CDKs have been identified. CDK activation depends on phosphorylation by a CDK-activating kinase (CAK), (all known CAKs belong to the extended CDK family) [86, 87]. CDKs are activated by cyclin binding, forming a complex. This complex remodels the kinase architecture from an inactive to an active conformation, with respect to substrate binding, positioning of ATP and configuration of the active site. When cells are activated in G_1 phase, they target and phosphorylate Rb, which is increasingly phosphorylated during mid-late G_1 phase (Figure 3) [89, 90].

2.4.1 Cdk 4

CDK4 is a member of the cyclin dependent kinase; and interacts with cyclin D to form a protein complex that promotes the passage of cells through one of the checkpoints (G_1) in the cell growth cycle. This process is achieved by initiating phosphorylation of retinoblastoma protein (Rb) leading to inactivation of the S-phase-inhibitory action of Rb. p21 inhibits the formation of cdk4-cyclinD complex, which inhibits phosphorylation of Rb leading to inhibition of cell cycle transfer from G_1 to S phase (Figure 3)[90, 91, 92].

2.4.2 Cdk 2

Another member of the cyclin dependent kinase family, cyclin-dependent kinase 2 is regulated by phosphorylation and associate with A, E, D1 and D3 cyclins; complex Cdk2 and A-cyclin is present in S and G_2 phases, and is associated with the DNA replication initiation complex. Cdk2 and E-cyclin is active in the G_1 and S-phases and is important for the progression from G_1 to S. p27 plays a major role in antagonizing the effect of cdk2. It does this by abrogating protein complex formation with the cyclins which leads to the inhibition of phosphorylation of Rb, a protein important in the progress of the cell cycle from G_1 to S phase (Figure 3) [90, 91, 92].

3.0 Effect of transforming growth factor β1 (TGF-β1) on fibroblast proliferation

TGF-β1 is a multifunctional peptide that controls proliferation, differentiation, extracellular matrix production (ECM) and other functions in many cell types. Many cells synthesize TGF-β1, and all of them have specific receptors for this peptide. TGF-β1 regulates the actions of many other peptide growth factors and determines a positive or negative direction of their effects, which plays an important role in cardiac remodeling [92, 93]. The TGF-β1 superfamily consists of a variety of structurally related multifunctional cytokines that include TGF-βs, activins/inhibins, glial cell-derived neurotrophic factor (GDNF), growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs) and anti-Mullerian Hormone/Mullerian inhibiting substance (AMH/MIS) [94]. Alteration of the TGF-β1 pathway result in a loss of cell growth regulation, affecting the ability of the cell to proceed through the cell cycle and to differentiate, and hence triggers the progression of various clinical diseases, including auto-immune diseases, vascular disorders, tissue fibrosis and cancer [92, 95]. In the heart, perturbations of TGF-β1 signaling contribute toward initiation and

progression of cardiac hypertrophy, ventricular remodeling, the early response to MI, and heart failure [50,96].

3.1 Receptors

TGF-β1 superfamily exerts their effects through the ligand-induced heterodimerization of specific type I and type II serine/threonine kinase receptors. Most mammalian cells express different members of this receptor family. Five type II receptors and seven type I receptors, which are also referred to as activin receptor-like kinases (ALKs), have been identified [92]. The type II receptors compromise the activin type II and type IIB receptors (ActR-II and ActR-IIB), TGF-β type II receptor (TβR-II), BMP type II receptor (BMPR-II), and AMH type II receptor (AMHR-II) [98]. Type I receptors include ALK-4 and ALK-5 that represent activin and TGF-β1 type I receptors (TβR-I), respectively, ALK-2, ALK-3 and ALK-6 that are recognized as type I receptors for BMP signal transduction. ALK-1 is known as an endothelial specific TGF-β1 type I receptor and ALK-7 still remains to be identified [92]. Both type II and type I receptors contain intracellular domains of serine and threonine residues, whereas their extracellular portions exist in various oligomeric forms such as type II homodimers, type I homodimers, and type II-type I heterodimers. The type II receptor, which is a constitutively active kinase, forms a complex with the type I receptor in a ligand dependent manner. Theoretically, more than thirty different combinations of type II and type I receptor complexes are possible. However, under physiological conditions the outcome in the signaling transduction becomes limited as certain type II receptors tend to interact with certain type I receptors, and because some of the receptors are shared by different TGF-β1 ligands [94].

In TGF-β1 signaling, the formation of TβR-II/ TβR-I receptor complexes triggers phosphorylation of particular serine and threonine residues in the TβR-I. This sequence is termed the

juxtamembrane domain or GS region. The phosphorylation of GS by TβR-II activates TβR-I (Figure 2) [35, 94].

3.2 Mode of action

The "Smad" moniker originates from the fusion of previous nomenclature: *Drosophila mothers against decapentaplegic* (Mad) and *Caenorhabditis elegans* Small body size (Sma) [94, 97]. In vertebrates, Smads were identified as homologs to *Mad* and *Sma* genes [98]. Depending on the mechanism of their action, Smad proteins generally divide into three major groups: the receptor-mediated proteins or R-Smads, the common-mediator proteins or Co-Smads, and the inhibitory proteins or I-Smads. The R-Smad, include Smad2, Smad3 for TGF-β1 signalling. These Smads are directly phosphorylated by TGF-β1 receptors. Co-Smads include Smad4 in mammals, and Smad4α and Smad4β (i.e.Smad-10) in *Xenopus*. Co-Smads promote TGF-β1 signaling by forming heteromeric complexes with R-Smads in the cytoplasm. I-Smads include Smad6 and Smad7, which interfere with TGF-β1 downstream signaling inhibit the signaling functions of the other two Smad groups [33, 92].

TGF-β1 signals through the heteromeric complex of type I and type II transmembrane Ser/Ther kinase receptors. The activation of the receptor complex occurs when type II receptor kinase phosphorylates the GS domain of the type I kinase. The activated type I kinase associates transiently with, and also phosphorylates, such receptor-regulated Smads as Smad2 and Smad3. Once phosphorylated, receptor-regulated Smads dissociate from the receptor, bind Co-Smad and translocate to the nucleus, where they can bind to target promoters in association with DNA-binding cofactors and recruit coactivators to initiate transcription. Alternatively, they can also recruit corepressors, which in turn bind histone deacetylases. As a result, Smads can

both positively or negatively regulate the transcription of specific genes in response to TGF-β1 signaling [99, 100]. Antagonistic I-Smad7 acts in opposition to signal-transducing receptor-regulated Smads and Smad 4, forming a stable association with the activated type I receptors, and preventing the phosphorylation R-Smads [6, 8]. The transcription of the I-Smad7 gene is regulated by TGF-β1 signaling through direct binding of Smad3 and Smad4 to the I-Smad7 promoter. Therefore, I-Smad7 functions in a negative feedback loop to terminate or reduce the strength of the signal (Figure 2) [101, 102].

3.3 Role in myofibroblast proliferation

TGF-β is a secreted signaling molecule with pro-fibrotic properties. It regulates a diverse range of cellular responses, including proliferation, differentiation, migration and apoptosis [103]. Myofibroblasts are the main component of the fibrotic tissue and the main producers of the ECM. TGF-β1 functions responds differently in different organs. In kidneys there is increasing evidence that TGF-β1 is a key mediator of fibrosis in both experimental and human kidney diseases [103]. This is clearly illustrated by the finding that renal fibrosis can be induced by the deliberate expression of TGF-β1 within the normal kidney [104]. TGF-β1 stimulates ECM deposition by both increasing the synthesis of ECM proteins and inducing the production of protein inhibitors, which blocks ECM degradation, TGF-β1 can also stimulate the proliferation and differentiation of fibroblasts, as well as cause phenotypic transformation from fibroblasts to myofibroblasts [103].

In intestinal mucosa, there is increasing evidence that myofibroblasts play a role in the maintenance of normal homeostasis and contribute to wound repair [105]. According to previous findings, myofibroblast differentiation is induced by TGF-β1 secretion *in vivo* [105]. Hepatic

fibrosis is a major cause of hepatic failure and death. This disease is influenced by the effects of TGF-β1 on myofibroblasts and collagen production. Studies have shown that TGF-β1 can induce a prolonged and strong induction of DNA synthesis in hepatic myofibroblasts and therefore, increase its proliferation [105]. In bile duct fibrosis, TGF-β1 was found to be important to activate and induce proliferation of myofibroblasts and increase collagen [106]. TGF-β1 also plays an important role in process of lung fibrosis, in which it initiates myofibroblast proliferation and also increases ECM deposition in interstitium leading to the stiffness of lung tissue [107]. In skin were inhibited under the influence of the effect of TGF-β1 [105]. When compared to myofibroblasts derived from corneal tissue. TGF-β1 induced myofibroblast proliferation. In the heart, transforming growth factor β1 (TGF-β_1) seems to play a significant role in the progression of cardiac fibrosis during the development of cardiac hypertrophy [3]. TGF–β_1 has been shown to profoundly enhance matrix deposition by increasing the synthesis of specific ECM components such as collagen and fibronection. Moreover, TGF–β_1 was found to promote the expression of ECM protease inhibitors [108, 109].

4.0 Effect of I-Smad7 on proliferation and heart function

Inhibitory I-Smad7 is an inducible regulatory Smad protein that antagonizes transforming growth factor β_1 (TGF-β_1) signalling. I-Smad7 has recently been reported to form a stable interaction with activated TβR-I, thereby preventing the binding to and activation of R-Smad [6, 7, 8]. I-Smad7 has been shown to inhibit signal transduction by the TGF-β_1 and activin receptors [6, 8]. I-Smad7 is structurally different from other Smad family members in that it lacks the SSXS phosphorylation motif on the MH2 region and it possesses shorter MH1 domains [8]. I-Smad7 expression is induced by TGF-β_1 in several cell types

[104, 105], Suggesting that I-Smad7 may act via an autoregulatory negative feedback loop. Further, in the mouse model of bleomycin-induced pulmonary fibrosis, overexpressed exogenous I-Smad7 adenovirus treatment of lung tissue was associated with an antifibrotic effect [110]. Nevertheless, the relationship between I-Smad7 and the profibrotic effects of TGF-β_1 remains unknown. I-Smad7 was found to be associated with a significant decrease of collagen synthesis in the presence and absence of TGF-β_1 in primary rat cardiac myofibroblasts [105]. I-Smad7 also abolished TGF–β_1 induced R-Smad2 phosphorylation, and significantly increased expression of MMP-2 protein (the active form of this MMP) [105]. Therefore, modulation of R-Smad phosphorylation by overdriven exogenous I-Smad7 may contribute to the down-regulation of collagen in cardiac myofibroblasts. Thus, treatments targeting collagen deposition through overexpression of I-Smad7 may provide a new therapeutic strategy for cardiac fibrosis [105].

4.1 Mode of action

I-Smad7 interacts efficiently with the activated TGF-β1 type I receptor, and acts as a general inhibitor for TGF-β1 family signalling by preventing R-Smad access to the activated type I receptor [6, 7, 8, 111]. The WD-containing protein, serine/threonine kinase receptor-associated protein (STRAP) interacts with Smad2. STRAP potentiates the inhibitory effects of Smad7, by recruiting I-Smad7 to the TGF-β1 receptor [113, 114]. In the absence of ligand stimulation, I-Smad7 is found predominantly in the nucleus [9, 113, 114]. TGF-β1 stimulation was shown to result in an export of I-Smad7 from the nucleus into the cytoplasm. The MH2 domain of I-Smad7 is sufficient for ligand-induced nuclear export [9].The molecular mechanism through which I-Smads are exported from the nucleus is unknown. I-Smad7 mRNA is potently induced by TGF-β1 and thus may act in an autocrine negative feedback loop to control intensity

and duration of TGF-β1 signaling response. I-Smad7 is a direct target gene; the promoter for the I-Smad7 gene has been characterized, and found to contain critical sequence elements that bind Smads. Thus I-Smad7 alone or together with the other molecules can regulate signaling of TGF-β1 in negative, as well as positive manners (Figure 2)[101, 116, 117, 118].

5.0 Synopsis of the Literature review: putative role of I-Smad7 in myofibroblast proliferation

Decreased I –Smad7 expression contributes to cardiac fibrosis in the post MI heart. I-Smad7 is a pivotal intracellular mediator of TGF-β1 signaling pathway; therefore it affects cardiac myofibroblasts proliferation [16]. In the present study, we characterized the effect of inhibitory I-Smad7 overexpression on primary cardiac myofibroblast proliferation and its influence on phenotypic change.

V. MATERIALS AND METHODS

1. Materials

Culture media (Dulbecco's Modified Eagle Medium, DMEM/F-12, and Minimum Essential Medium, SMEM), fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin) were purchased from Invitrogen (Grand Island, NY). Culture plates and multiwell culture dishes were obtained from Fisher Scientific (Whitby, CA). Prestained low-molecular-weight marker, anti-mouse HRP and anti-rabbit horseradish peroxidase (HRP) conjugated IgGs were supplied by Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) blotting membranes were obtained from Roche Diagnostics (Laval, CA). The enhanced chemiluminescence (ECL Plus) detection system, anti-mouse Ig Texas Red antibody, Streptavidin fluorescein, and anti-sheep/goat Ig, anti-rabbit Ig, and anti-mouse IgG biotinylated antibodies were from Amersham (Arlington Heights, IL). Anti-Flag primary antibody was purchased from Sigma-Aldrich Co. (Oakville, CA). Primary antibodies against PCNA-clone PC10, anti-INK4b (p15) and anti-Cip1/WAF-1/p21 was all obtained from Upstate USA, Inc. (Charlottesville, VA). Monoclonal anti-α-Smooth Muscle-Actin-FITC conjugate was acquired from Sigma Chemical Co. (St.Louis, MO). SMemb primary antibody was purchased from Abcam (Cambridge, MA). Rhodamine phalloidin –F actin – stain was acquired from Molecular Probes (Laiden, Netherlands). Phospho-Rb (Ser780) and p27 Kip1 primary antibodies were both obtained from Cell Signaling (Beverly, MA). Anti-cdk2 unconjugated, anti-cdk4 unconjugated and anti-human TGF-β_1 neutralizing antibody (AF-101-NA) was obtained from R&D (Minneapolis, MN). Anti-cyclin D1 unconjugated and anti-cyclin E unconjugated antibody were acquired from Biosource International, Inc. USA (Camarillo, CA). ^{3}H thymidine (Amersham Pharmacia

Biotech), washed replication-deficient human adenovirus expression vector encoding mouse I-Smad7 under the control of CMV promoter (AdvCMVSmad7) is a gift from Dr. Anita B.Roberts (Laboratory of cell regulation and Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, Maryland, USA). All other materials used were commercially available.

2. Preparation and Culture of Adult Rat Cardiac Myofibroblasts

Adult cardiac myofibroblast cultures were prepared from male 200-250 g Sprague-Dawley rats according to the methods of Brilla *et al.* with minor modifications [Brilla CG,1994, Scheuren N, 2002]. Rat hearts were subjected to Langendorff perfusion i.e. retrograde perfusion at a flow of 5 ml/min at 37°C with recirculatory Joklik's medium containing 0.1% collagenase and 2% bovine serum albumin (BSA) for 20 min. Liberated cells were collected by centrifugation at 2,000 rpm for 5 min, and resuspneded in DMEM-F12. Cells were seeded on 100-mm non-coated culture dishes at 37°C with 5% CO_2, and grown in DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1μl/ml ascorbate. The cardiac fibroblasts attached to the bottom of the culture dishes during the two-hour primary incubation period, and were further maintained in enriched DMEM-F12. Non-adherent myocytes and cells were removed by changing the culture medium. In this study we used myofibroblast cells from the first passage (P1). These cells were passaged by adding 3ml Trypsin to the cultures P0 cardiac myofibroblasts for 3 minutes and left in the hood at 37°C to detach from dishes. 7ml of 10 FBS-DMEM-F12 were added on top of that to neutralize the effect of Trypsin and then the whole 10ml was collected and centrifuged at 2,000rpm for 5 min then resuspneded in 10% FBS-DMEM-F12 as described above.

3. Recombinant adenovirus

Expression of ecotopic I-Smad7 in cardiac myofibroblasts was confirmed by Western blot detection with anti-flag antibody (Sigma, Oakville, Ontario, Canada). AdvCMVLacZ encoding β-galactosidase was used as a control in the experiments.

4. ^{3}H Thymidine Incorporation

Proliferation assays were done on cells cultured on 24-well plates at a density of 0.2×10^{6} cells/well (counted with a hemacytometer) in DMEM/F12 with 10%FBS. After the cells reached ~ 60% confluency, media was replaced with DMEM/F12 + 0% FBS, 10% FBS or 2% FBS. Cells were infected with 100 MOI I-Smad7 adenovirus vector for 24 hours. Other groups of cell were starved for 24 hours then 10 ng/ml of TGF-β1 was added to the media for 24 hours. Cells were then pulsed for 4 h at room temperature with 2.0 μCi/ml [methyl-^{3}H] thymidine (Amersham Pharmacia Biotech). DNA in cell lysate was precipitated with cold 20% trichloroacetic acid (TCA) and filtered through 24mm GF/A filters (Fisher). Beta emission was measured with Cytoscint scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA) and a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA).

5. Protein Extraction and Assay

Infection of cells was terminated by rinsing twice with ice-cold phosphate buffered saline (PBS) prior the addition of 120 μl RIPA lysis buffer (pH= 7.6), containing 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, 1 mM Na_3VO_4, and 1 mM EGTA) and protease inhibitors (4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin). Collected samples were allowed to lyse in RIPA buffer for 1h on ice, and were subsequently sonicated 3 times, 5 seconds each. The insoluble

pellet (membrane fraction) was removed by centrifugation for 15 min at 14,000 rpm at 4°C. Supernatant was collected, and stored at -20°C. The total protein concentration of all samples was measured using the bicinchoninic acid (BCA) method as previously described [Smith PK, 1985].

6. Nuclear protein extraction

Infection of cells was terminated by rinsing them twice in ice-cold phosphate buffered saline (1X PBS) prior to the addition of 1ml ice cold 1x PBS containing 1mM Na orthovanadate. Collected samples were allowed to set on ice then were subsequently Centrifuged for 30 seconds at 9,000 rpm at 4°C. Precipitant was collected, then suspended in 400 μL of hypotonic buffer, containing 20mM HEPES, 20mM NaF, 1mM Na pyrophosphate, 1mM Na orthovanadate, 0.25mM Na molybdate, 40mM β-glycerophosphate, 1mM EDTA 1mM EGTA and 0.5% NP-40. Samples were centrifuged for 30 seconds at 13,000rpm at 4°C. Pellets were re-suspended in 80 μL of high salt and were shaken for 1hour at 4°C. After Centrifugation for 30 seconds at 13,000 rpm at 4°C and supernatant was collected, and stored at –80°C.

7. Western Blot Analysis of Target Proteins

Aliquots from cell lysates were mixed 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 min. Equal amounts of protein samples (15 μg) were resolved by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with prestained low-molecular-weight markers (10 μl). Separated proteins were electrophoretically transferred onto 0.45 μM polyvinylidene difluoride (PVDF) membranes. Blots were blocked overnight at 4°C in Tris-buffered saline with 0.2 % Tween 20 (TBS-T) containing 5 % skim milk, and probed with primary antibodies for 1h at room

temperature. For p21, p27, p15 primary antibodies were diluted 1:1000 in 0.2% TBS-T with 5 % skim milk, the rest of the primary antibodies were diluted 1:500 in 0.2 % TBS-T with 5% skim milk, and included the antibodies against Flag, PCNA, phospho-Rb, cdk2, cdk4, cyclin E, cyclin D and β-tubulin. After that blots were washed three times with 0.2% TBS-T, each time for 5 minutes. The incubation period of secondary antibodies was 1 h at room temperature with the dilution 1:2000 in 0.2 % TBS-T containing 1% skim milk. Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-mouse IgG for detection of flag, PCNA, p15, p21, cdk2, cdk4, cyclin E, cyclin D and β-tubulin, and anti-rabbit IgG for detection of p27 and phospho-Rb. Protein bands on Western blots were visualized by ECL Plus according to the manufacturer's instructions, and developed on X-OMAT film (Kodak, Rochester, N.Y). Even protein loading was confirmed by immunoblotting analysis against β-Tubuline, by incubating PVDF membranes into Ponceau S solution (0.1 % Ponceau S in 5 % acetic acid).

8. Immunofluorescence Assay

Adult primary cardiac myofibroblasts were plated on coverslips (Fisher Scientific, size22*22), and allowed to grow for 24 h in DMEM-F12 containing 10 % FBS until ~60% confluent. Media was replaced with DMEM-F12 (0% FBS, 2% FBS or 10% FBS) and cells were infected with 100 MOI I-Smad7 adenovirus vector for 24 hours. Immunofluorescent staining was performed as previously described [6]. This technique was applied to detect any changes in phenotypes of myofibroblasts after being exposed to I-Smad7 adenovirus transfection. Briefly, cells were washed twice with cold PBS, fixed in 4 % paraformaldehyde, rendered permeable with 0.1 % Triton X-100 in PBS, and incubated with SMemb, α-smooth muscle actin (α-SMA), PCNA and rhodamine phalloidin primary antibodies over night at 4°C. After being washed with cold PBS, cells

were incubated with the biotinylated anti-mouse IgG secondary antibody. The incubation period of the secondary and tertiary antibodies was 90 min. All antibodies were diluted at 1:20 in PBS containing 1 % bovine serum albumin (BSA). After being washed with cold PBS, cells were immersed in the nuclear dye Hoescht No. 33342 (10 μg/ml) for visualization of cellular nuclei and subjected to additional wash with PBS. The slides were examined under a microscope equipped with epifluorescence optics, and photographed on Provia Fujichrome 400 color film or with a Nikon Coolpix 990 digital camera.

9. Statistical Analysis

All values are expressed as means ± SE. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* analysis was used to compare the differences among multiple groups (SigmaStat, Point Richmond, CA). Significant differences among groups were defined by $P \leq 0.05$.

VI. RESULTS

1.0 Characterization of optimal I-Smad7 adenovirus infectivity

We examined the effect of exogenous I-Smad7 in the proliferation of primary cells, in the presence of low and normal serum levels, and thus we needed to ensure that both viral and test gene proteins were optimally amplified in primary myofibroblast cultures. To test the efficiency of the viral transfection, cultures were infected with ectopic replication-deficient human adenovirus, expressing mouse I-Smad7 with CMV promoter (AdvCMVSmad7). Two different time points were examined eg, 24 and 48 hours [Figures 4 and 5]. Expression of ectopic I-Smad7 in cardiac myofibroblasts was confirmed by Western blot detection with anti-flag antibody [Figure 4]. AdvCMVLacZ encoding galactosidase was chosen as a control for viral infection in these experiments and X-Gal staining was used to confirm infection efficiency [Figure 5]. While I-Smad7 adenoviral infection was expressed using various MOI at different time points, we noted that it was optimally expressed at 100 MOI for 24 hrs [Figure 4]. We found that the appearance of X-gal as a marker of viral expression in primary P1 myofibroblasts was optimal at 24 hours using 100 MOI of virus [Figure 5].

2.0 Effect of TGF-β1 on cells incubated in low (2%) serum conditions

P1 myofibroblasts were cultured under a number of conditions – i) serum-free medium with no treatment, and ii) in the presence of 2 % FBS-DMEMF-12 in the presence or absence of 10 ng/ml TGF-β for 24 hours. All cultures were pulsed for 4 h at room temperature with 2.0 μCi/ml [methyl-^{3}H] thymidine prior to stimulation. We confirmed that myofibroblasts underwent significant proliferation in the presence of 2% serum vs. cells exposed to serum-free media ($P < 0.05$) and that 10 ng/ml TGF-β1 led to a significant inhibition of cardiac myofibroblast proliferation when

compared to values in cells treated with 2% serum alone (2% FBS-DMEMF12), as shown in Figure 6. We also noted that thymidine incorporation was not significantly different among serum-starved cells and TGF-β1 treated cells in low-serum conditions. These data are consistent with previous data shown in our lab which indicated that TGF-β1 inhibits myofibroblast proliferation (16).

2.1 Overexpressed I-Smad7 is associated with decreased cardiac myofibroblast proliferation

The effect of I-Smad7 overexpression on primary myofibroblast ^{3}H-thymidine incorporation in low-serum conditions FBS-DMEM-F12 was tested. Thymidine incorporation was significantly inhibited by I-Smad7 *vs.* Lac-Z infected controls in 2% FBS-DMEMD-F12 as well as non-infected 2% serum-treated and 10% serum-treated cells [Figure 7].

3.0 Effect of overexpressed I-Smad7 on cell cycle inhibitor protein expression

To examine the association between I-Smad7 and myofibroblast proliferation, we addressed cell cycle inhibitor protein expression using myofibroblasts overdriven with I-Smad7 as well as viral-infected and non-infected controls. Measurement of members of this class of proteins allowed us to provide an association to mechanisms that may determine cell cycle turnover. Specifically, we examined the expression of p27, p21, p15 and phospho-Rb in passage 1(P1) cultured cells.

3.1 Expression of p27

The p27 protein inhibits cell progression from G_1 phase to S phase and thus provides input to the control of cell proliferation [83, 84, 85]. We measured the expression of p27 in cells after infection with 100 MOI AdvCMVSmad7 for 24 hours using Western blot analysis. Overexpressed I-

Smad7 was associated with a significantly increase of the p27/β-tubulin ratio in myofibroblasts in 2% FBS-DMEM *vs.* values derived from Lac-Z infected controls, as well as non-infected 10% serum treated cells and 2 % non-infected serum-treated cells [Figure 8].

3.2 Expression of phosphorylated Rb

In a manner somewhat similar to p27, phosphorylated Rb influences progression of the cell cycle from G_1 phase to S phase. Thus, we tested the expression of phospho-Rb in 2% serum treated primary (P1) myofibroblasts after infection with I-Smad7 adenovirus at 100 MOI for 24 hours and in viral infected and non-infected controls. Overexpressed I-Smad7 was associated with significantly decreased expression of the phospho-Rb/ β-tubulin ratio in myofibroblasts (in 2% FBS-DMEM media) *vs.* values of Lac-Z infected cells in 2% serum as well as non-infected 2% and 10% serum cultured cells [Figure 9]. Thus I-Smad7 overexpression was associated with a decreased level of phospho-Rb protein under these current culture conditions.

3.3 Expression of p21 and p15

p21 and p15 are cyclin kinase inhibitors that interfere with the production of PCNA protein, leading to the inhibition of cell cycle progression and thus proliferation. To test this, we overexpressed I-Smad7 in primary cultured myofibroblasts with 2% serum and assessed the their expression. We found that overexpressed I-Smad7 did not affect p21 and p15 expression when compared to values of either Lac-Z infected controls, non-infected cultures cultured in 2% FBS or 10% cultured cells [Figure 10, Figure 11]. Thus, exogenous I-Smad7 expression does not affect p15 and p21 expression in these cultured cells under the conditions used in the current study.

4.0 Effect of TGF-β1 on PCNA expression.

We tested for PCNA expression in myofibroblasts in serum-free conditions as well as in the presence of low serum (2%) and low-serum plus 10 ng/ml TGF-β1 treatment for 24 hours. After densitometric scanning, the value for PCNA/β-tubulin ratio in the presence of TGF-β was significantly decreased when compared to the control values for 2% FBS-DMEM-F12 [Figure 12]. The ratio value of the TGF-β stimulated cells was not significantly different from that of the serum-free control value.

4.1 Effect of overexpressed I-Smad7 on PCNA expression

PCNA is a reliable indication of cell proliferation. We tested for its expression in adenoviral I-Smad7 infected cells incubated in 2% FBS-DMEM-F12. Relative expression of PCNA, noted as PCNA/β-tubulin ratio of densitometric absorbance, was found to be significantly decreased with I-Smad7 overexpression in myofibroblasts when compared to the Lac-Z infected (and non-infected) controls in 2% FBS-DMEM-F12 [Figure 13]. We also carried out Immunofluorescence staining of cultured cells to detect the immunoreactive intensity of PCNA expression in cells under identical conditions. We found that PCNA staining intensity is diminished in I-Smad7 infected cells control values *vs.* Lac-Z infected controls as well as in 2% and 10% FBS-DMEM-F12 controls [Figure 14]. These results paralleled the findings present in our Western analyses.

5.0 Effect of overexpressed I-Smad7 on cyclin D expression

We investigated the expression of cyclin D after infecting the cells with I-Smad7 for 24 hours. No significant change was observed in the expression of cyclin D compared to the control

values of Lac-Z infected cells, or when compared to 2% or 10% FBS-DMEM-F12 controls [Figure 15]. These data indicate that cyclin D is not affected by the overexpression of I-Smad7 in cardiac myofibroblasts.

5.1 Effect of overexpressed I-Smad7 on cyclin E expression

To further investigate the putative association of cyclin protein expression in myofibroblast proliferation we investigated the expression of cyclin E in cardiac myofibroblasts after infection with viral I-Smad7. Cyclin E expression was significantly decreased when compared to the control values of Lac-Z infected cells as well as values of 2% FBS and 10% FBS-DMEM-F12 cultured cells controls [Figure 16]. Thus cyclin E expression is inhibited by over expression of viral I-Smad7 under the current conditions.

6.0 Effect of overexpressed I-Smad7 on cyclin dependent kinases (Cdks)

Cyclin dependent kinases are proteins that regulate the progression of the cell cycle from one stage to another by forming a complex with the cell cyclins and are thus implicated in cell proliferation. We examined the effect of overexpression of I-Smad7 on Cdk2 and Cdk4 in myofibroblasts.

6.1 Cdk2 expression

Cdk2 forms a complex with cyclin E and leads to the progression of cell cycle from G_1 phase to S phase. We infected primary cultured cardiac myofibroblasts (in 2% serum) with 100 MOI I-Smad7 adenovirus and carried out Western analysis. We found that expression of Cdk2 (manifest as the Cdk2/β-tubulin ratio) was significantly decreased when compared to virally infected and non-infected control values [Figure 17].

6.2 Cdk4 expression

Cdk4 is able to form a complex with cyclin D and plays a role in the progression of the cell cycle. We infected primary cultured cardiac myofibroblasts (as mentioned above) with I-Smad7 adenovirus and analyzed expression of Cdk4/β-tubulin ratios. As opposed to the results when testing for Cdk2 expression, we noted that Cdk4 was not significantly different vs. control values from any group [Figure 18].

7.0 Effect of overdriven I-Smad7 on cardiac myofibroblast phenotype (P1 cells)

We investigated the effect of infection of cells with I-Smad7 to determine whether the infection *per se* leads to any obvious phenotypic changes in the cardiac myofibroblastic phenotype. We tested three different cell markers (α-SMA, SMemb and rhodamine phalloidin stain for F-actin) to examine cell phenotype and cytoskeletal characteristics.

7.1 Light microscopy – cellular viability

To test cell viability, we cultured primary myofibroblasts in 0% FBS, 2%FBS and 10% FBS-DMEM-F12, then we infected cells in 2% FBS-DMEM-F12 with 100 MOI I-Smad7 adenovirus for 24 hours. In addition, we infected the cells with 100 MOI Lac-Z adenovirus for 24 hours to serve as our control. We used a light microscope to examine cell confluency and proliferation. The number of cells was greater (i.e., the level of confluency) in 10% FBS-DMEM-F12 compared to 2% and 0% FBS-DMEM-F12 and that the cells were viable in all virally infected or non-infected cell cultures [Figure 19].

7.2 Smooth muscle actin (α-SMA) localization

We investigated the expression of this marker by using Immunofluorescence staining. We infected primary cultured myofibroblasts in 2% FBS-DMEM-F12, with 100 MOI I-Smad7 adenovirus for 24 hours. The cells were then stained with primary α-SMA antibody. Expression of α-SMA was strong in all first passage (P1) myofibroblasts with or without overdriven I-Smad7, and this is unchanged from controls [Figure 20]. These data provide an indication that overexpression of I-Smad7 does not changes the phenotype of myofibroblasts, despite the relative reduction in cell number in the representative fields.

7.3 Myosin non-muscle heavy chain (SMemb) localization

In I-Smad7 infected cultures in low serum (2% FBS-DMEM-F12) and exposed to SMemb antibody, we found that SMemb is expressed in all groups [Figure 20] and that I-Smad7 infection did not influence the expression of this myosin isoform under any of the current conditions.

7.4 Rhodamine Phalloidin (F-actin) localization

We investigated expression/localization of F-actin in P1 myofibroblasts. We overexpressed I-Smad7 using 100 MOI for 24 hour in 2% FBS-DMEM-F12. Then we stained the cells using a rhodamine phalloidin - the data revealed that F-actin was evenly distributed in cells after infection in a manner comparable to Lac-Z infected controls and non-infected controls [Figure 20].

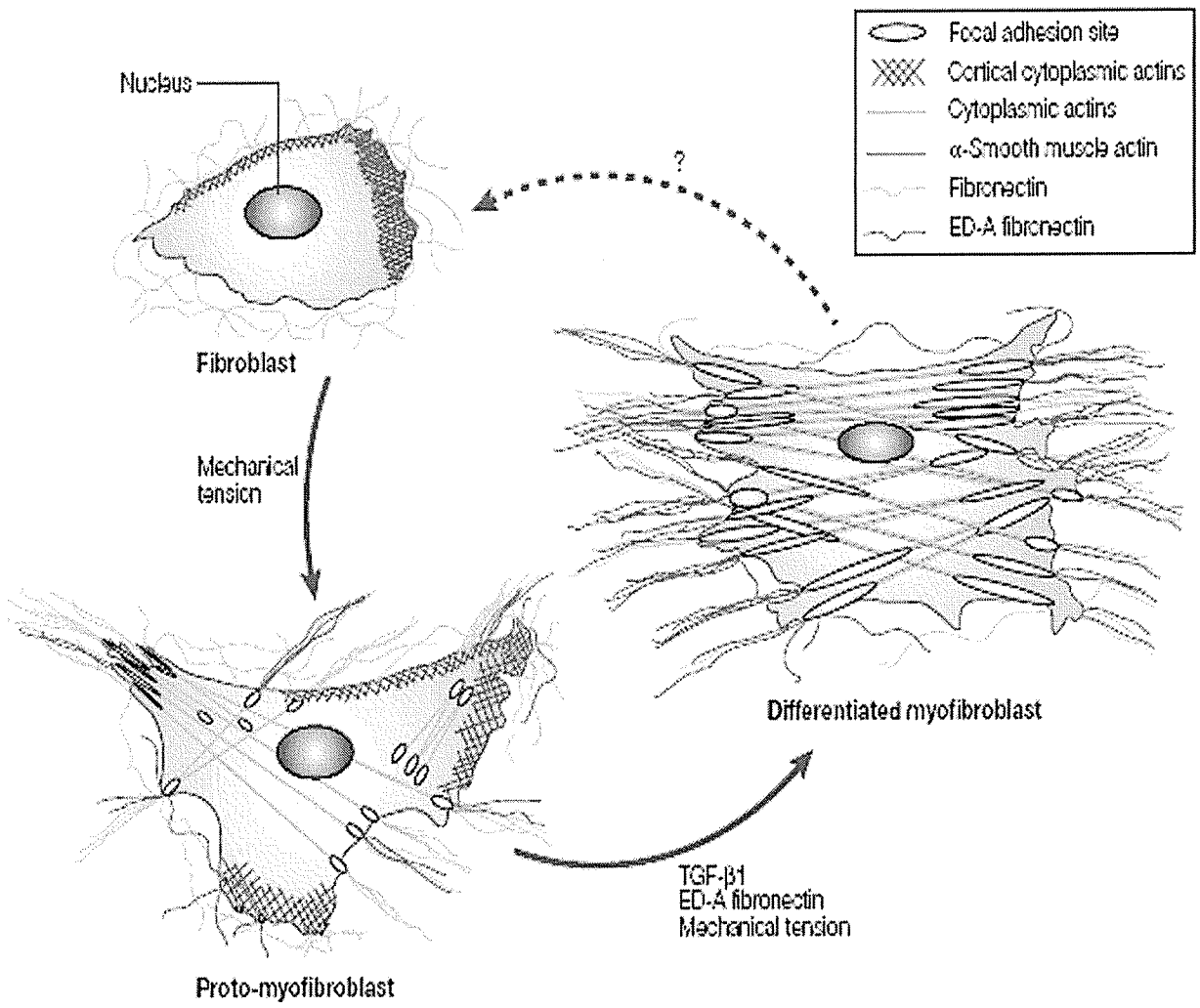


Figure 1. Although they might contain actin in their cortex, relatively quiescent *fibroblasts* do not exhibit stress fibers and do not form adhesion complexes with the extracellular matrix *in vivo*. When the cell faces mechanical stress, fibroblasts differentiate to proto-myofibroblasts: this form expresses actin and exhibit stress fiber formation in their cytoplasm, which terminate in adhesion complexes. *Proto-myofibroblasts* also express and organize cellular fibronectin - including ED-A fibronectin- at the cell surface. *Proto-myofibroblasts* can contract and generate force. This allows the cells to exhibit torsion on the matrix. TGF-β1 increases the expression of ED-A fibronectin, and with mechanical stress modulates differentiation of proto-myofibroblasts into myofibroblasts. *True myofibroblasts* are characterized by extensive expression of α-smooth muscle actin in more developed stress fibers and by large fibronexus adhesion complexes *in vivo*, by supermature focal adhesion *or by SMemb expression in vitro*. *Myofibroblasts* generate contractile force and torsion on the matrix, which is reflected by higher extracellular organization of fibronectin into fibrils [adapted from 139].

Figure 2. TGF-β1 signaling pathway: I-Smad7 antagonizes TGF- β1 signals by blocking R-Smad 2 phosphorylation by the kinase protein of TβR1, and thus, therefore, blocking the formation of Smad2 and/or Smad3 dimer formation and complex with Smad4. Thus R-Smad/Co-Smad trimer (s) confer TGF-β1 signal to the nucleus where transcriptional modification of target genes occurs.

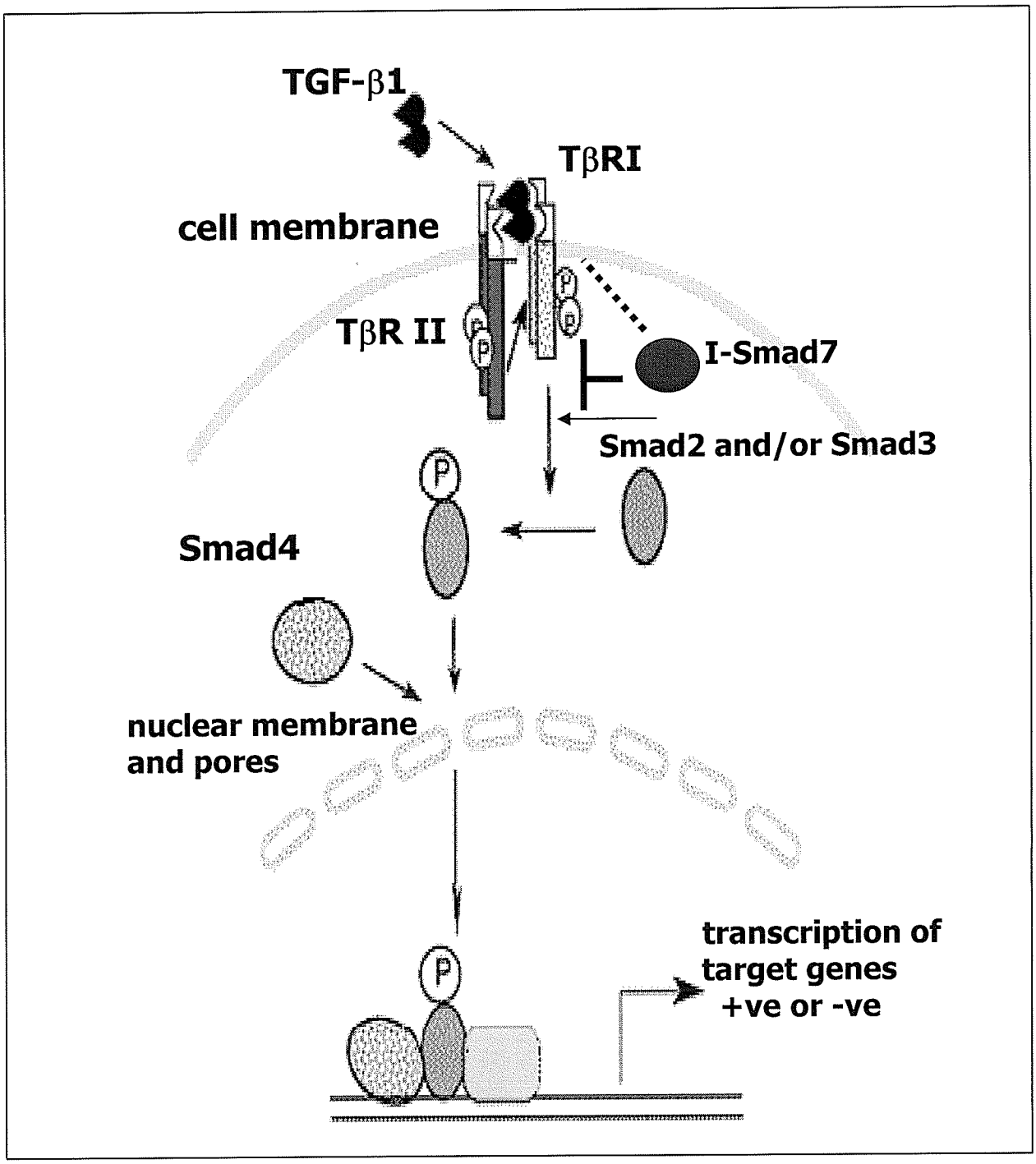
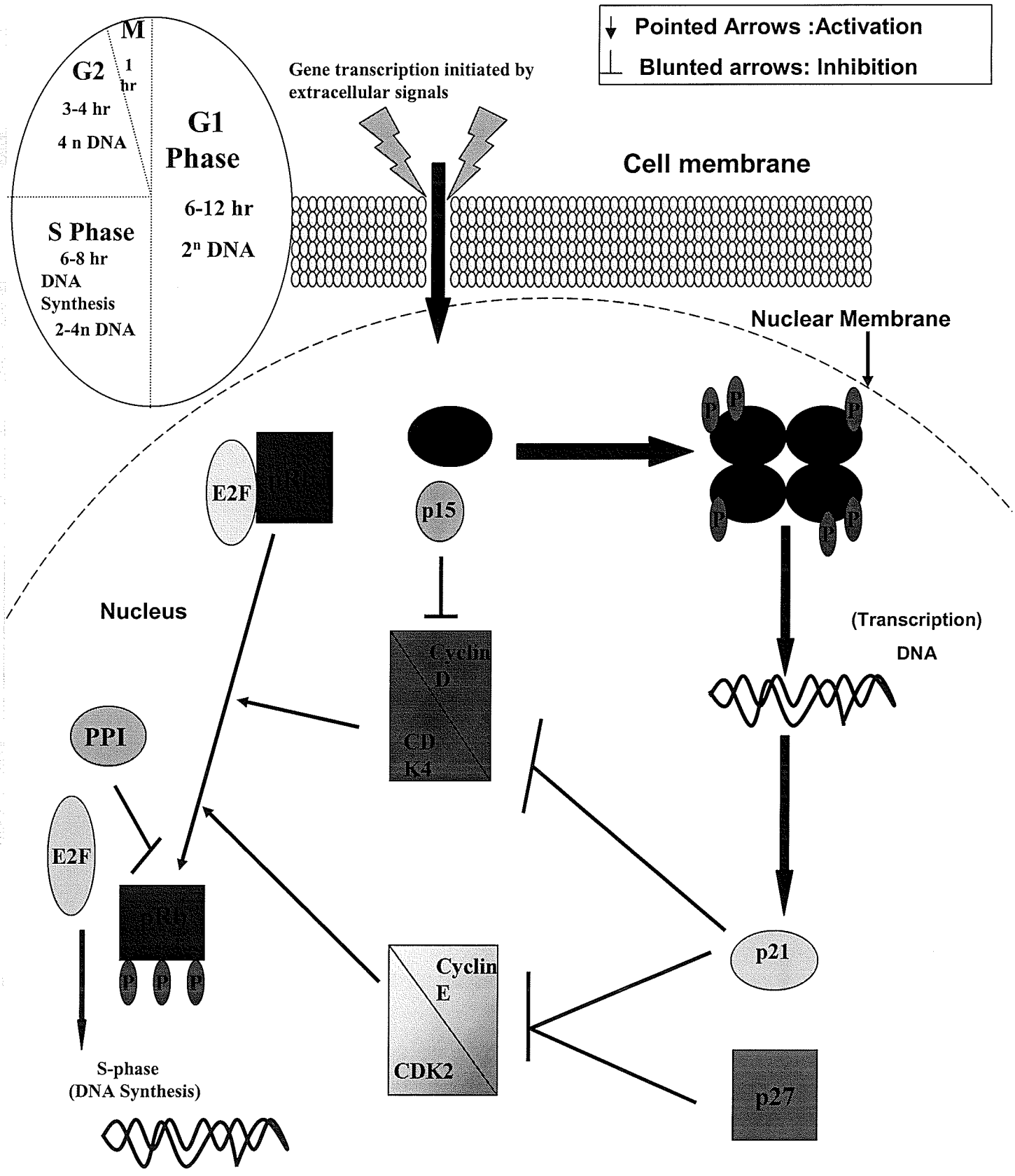


Figure 3. G1 and S phase of the cell cycle, CKIs, Cdks and Cyclins involved in influencing the cell cycle progression, from G1 phase to S phase.



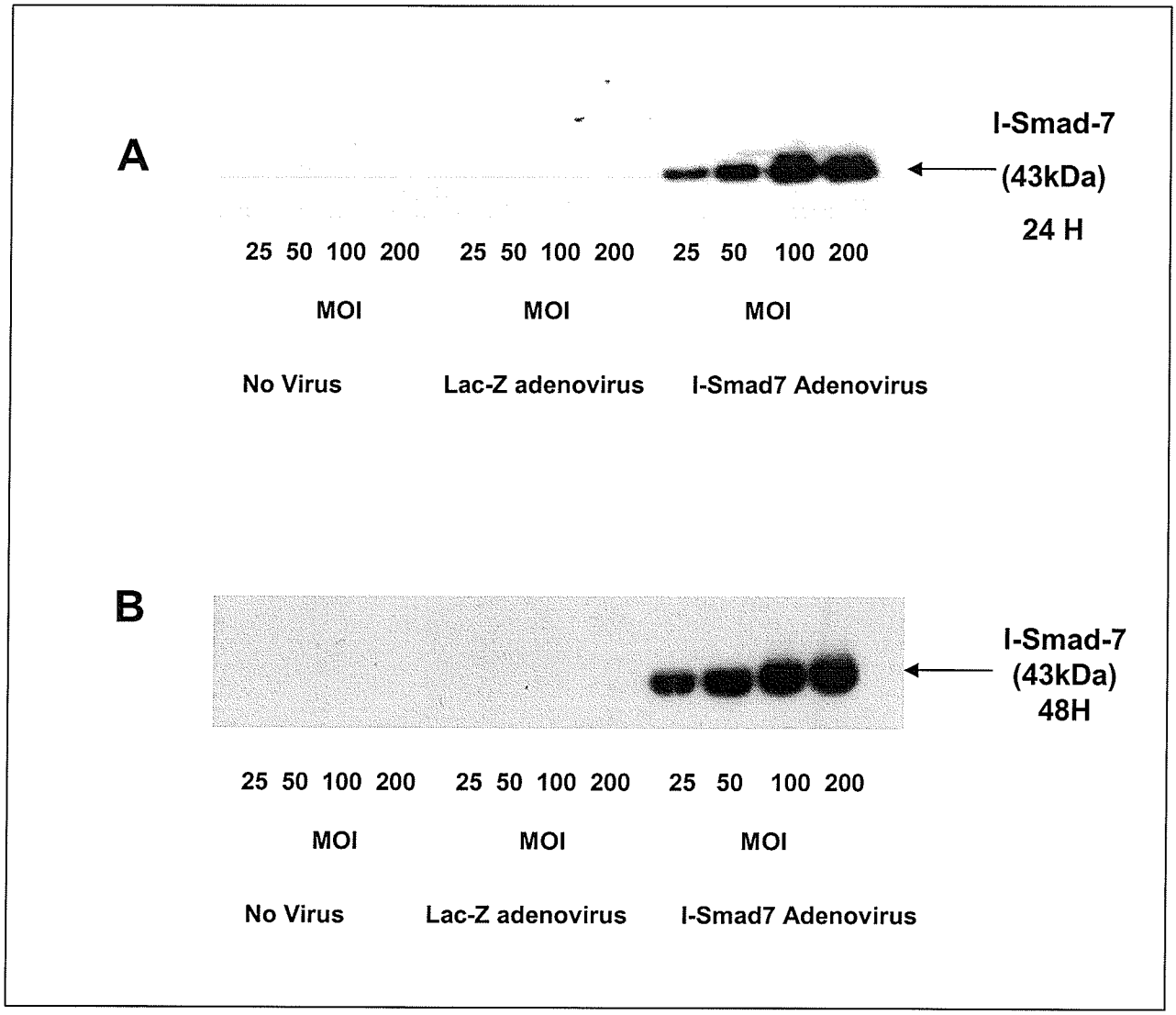


Figure 4. Representative Western blot analysis of I-Smad7 expression in adult primary cardiac myofibroblasts (P1) cultured in 2% fetal bovine serum-DMEM-F12 with or without infection with I-Smad7 adenovirus. ***Panel A***: Dose-dependent effect of 24hr I-Smad7 (43kDa) adenovirus transfection on primary cardiac myofibroblasts. ***Panel B:*** Dose-dependent effect of 48h I-Smad7 (43kDa) adenoviral infection of primary cardiac myofibroblasts. This blot is representative of 4 experiments using identical conditions.
MOI= Multiplicity of infection.

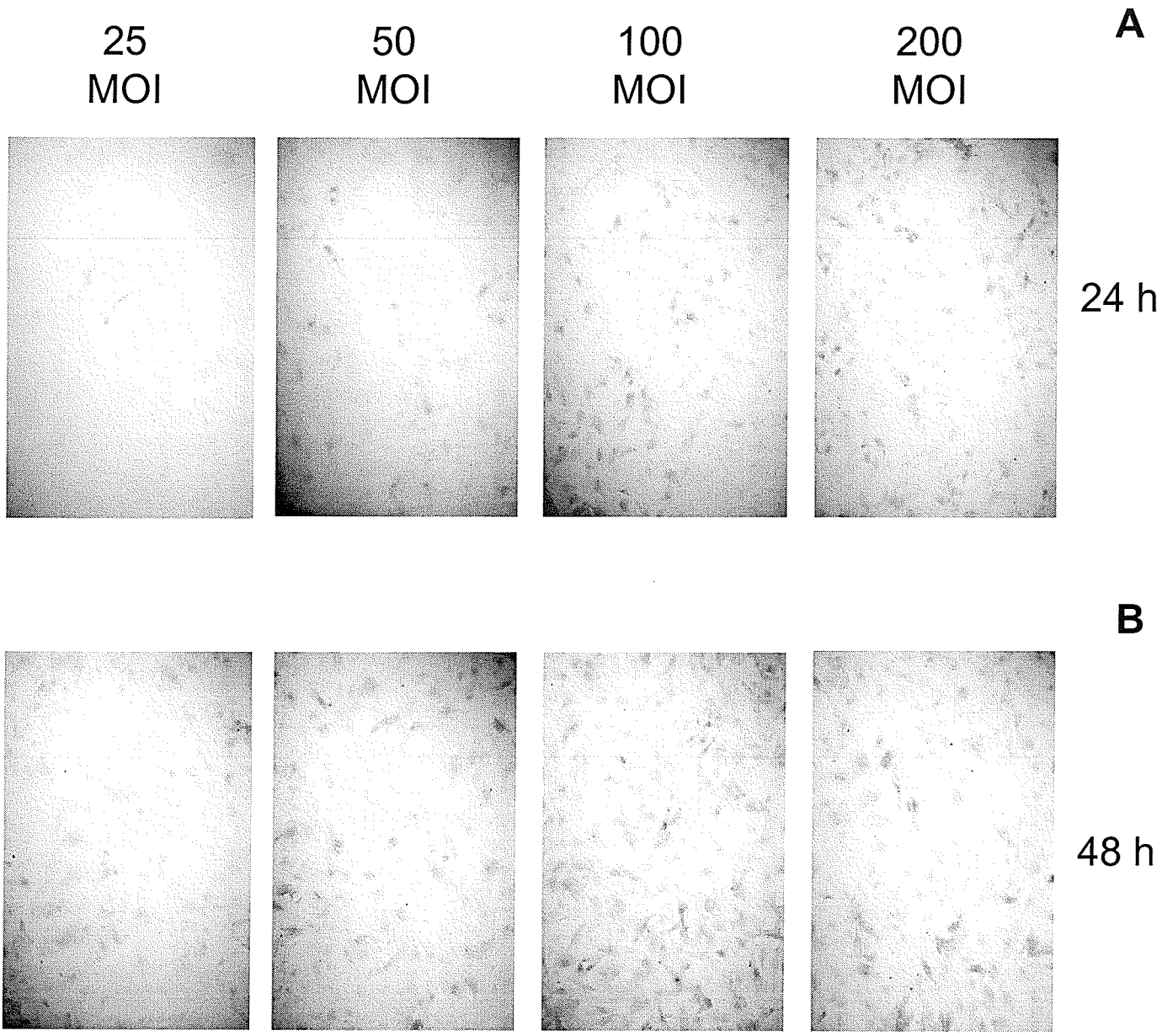


Figure 5. Representative of X-gal staining of cultured adult primary cardiac myofibroblasts in the presence and absence of I-Smad7 adenovirus. ***Panel A*:** Dose-dependent effect of 24 hr Lac-Z adenovirus (43kDa) infection on primary cardiac myofibroblasts. At 100 MOI Lac-Z adenovirus expression was significant. ***Panel B*:** Dose-dependent effect of 48 hr Lac-Z adenovirus infection on primary cardiac myofibroblasts. Data is representative of 4 separate experiments.

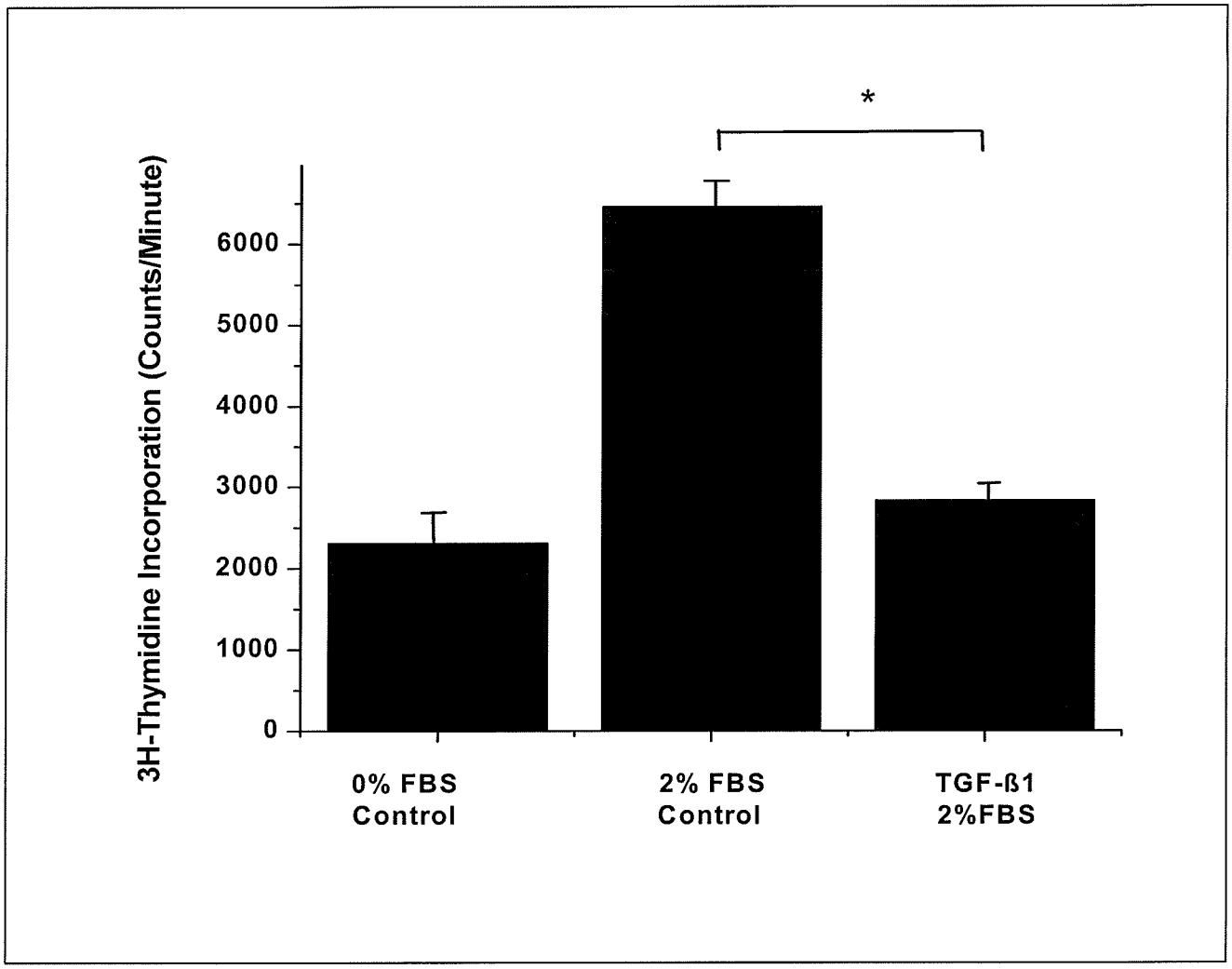


Figure 6. Histographic representation of ^{3}H-thymidine incorporation in (P1) adult primary cultured cardiac myofibroblasts treated with 10ng/ml TGF-β1 for 24h, in the presence or absence of 2% FBS-DMEM-F12. * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; data are expressed as mean ± SEM, for 3 experiments under identical conditions. Data are quantified by densitometric scanning.

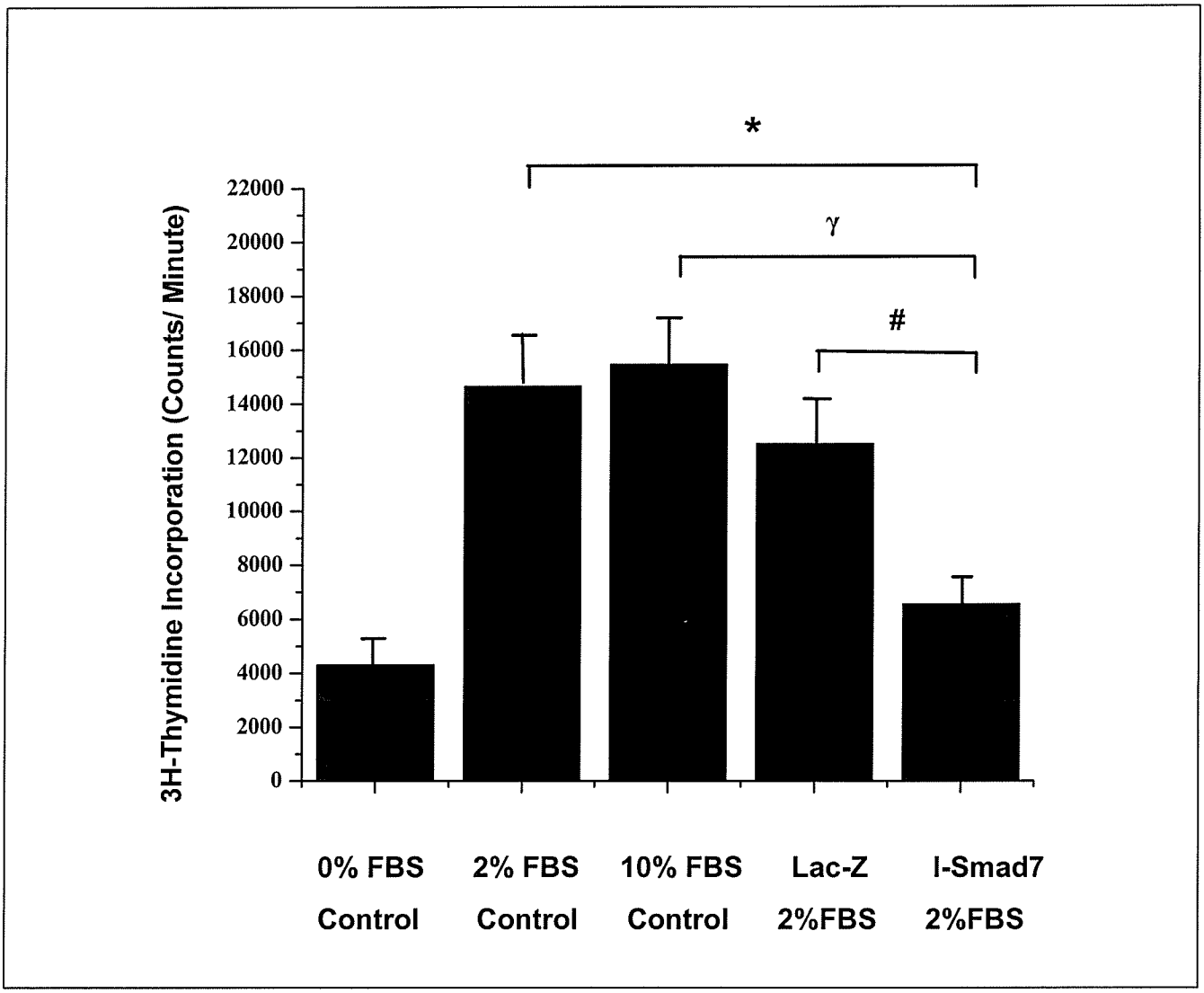


Figure 7. Histographic representation of quantified data from ^{3}H-thymidine incorporation in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24 h (in the presence or absence of 2%FBS-DMEM-F12) and LacZ Control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ; γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 4 experiments. Data are quantified by densitometric scanning.

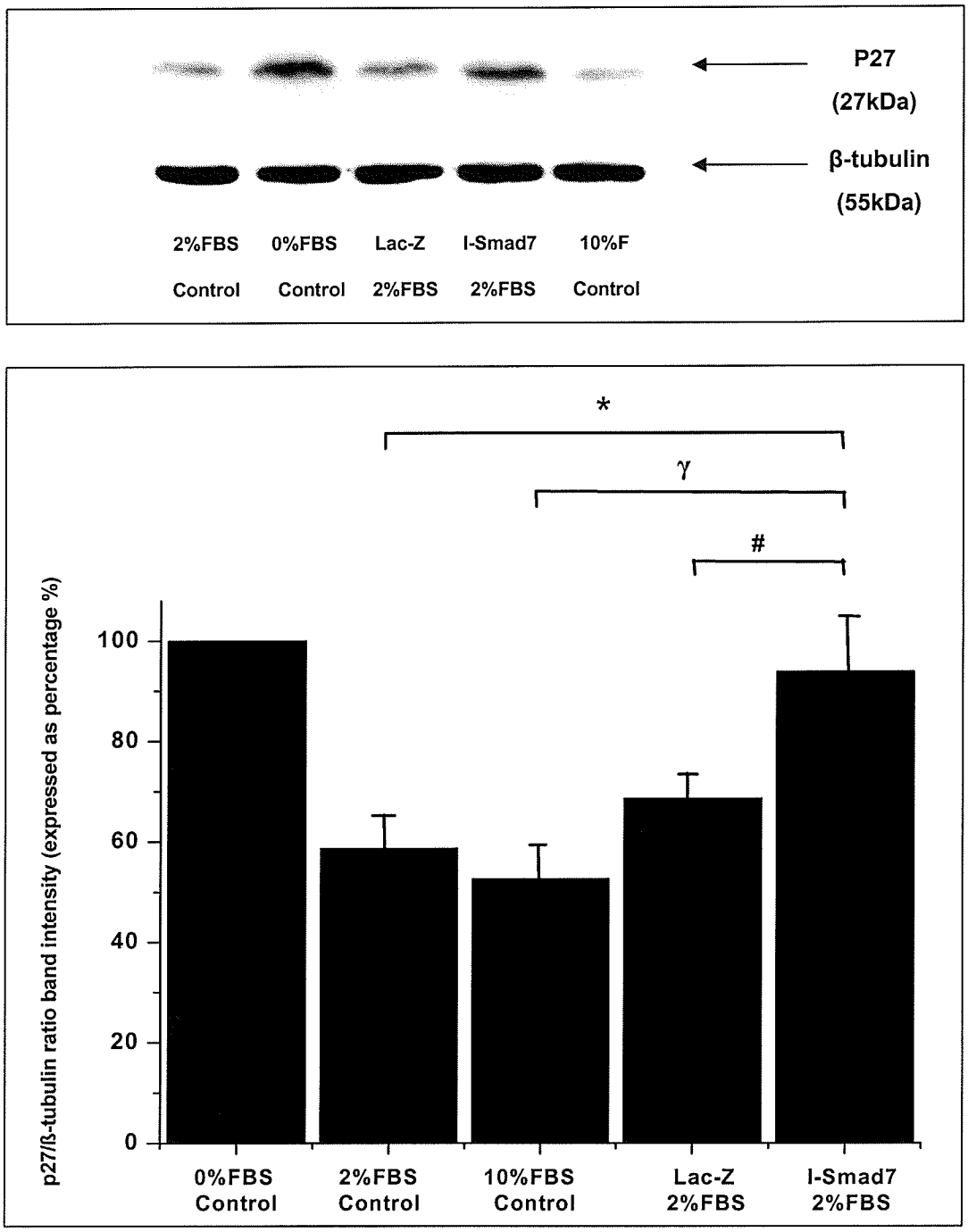
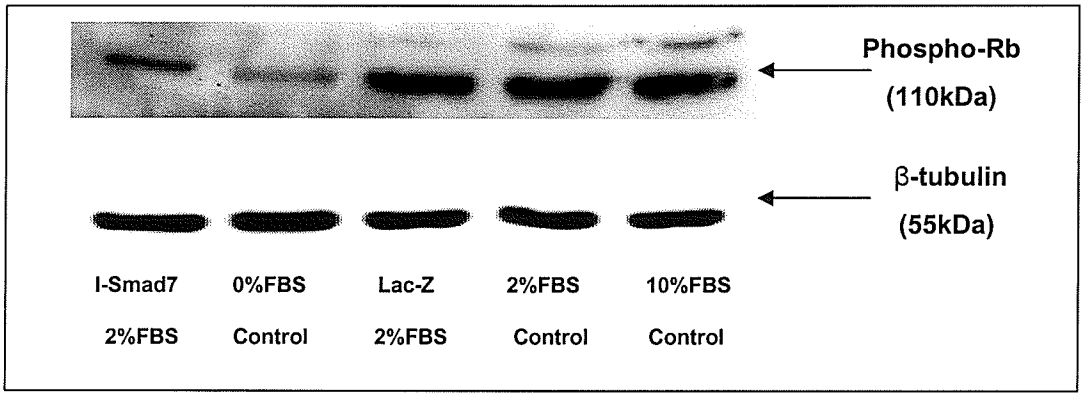


Figure 8: Panel* A:** Representative Western blot analysis of cyclin dependent kinase inhibitor p27 and β-tubulin expression ***Panel B: Histographic representation of p27/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ; γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 5 experiments. Data are quantified by densitometric scanning.



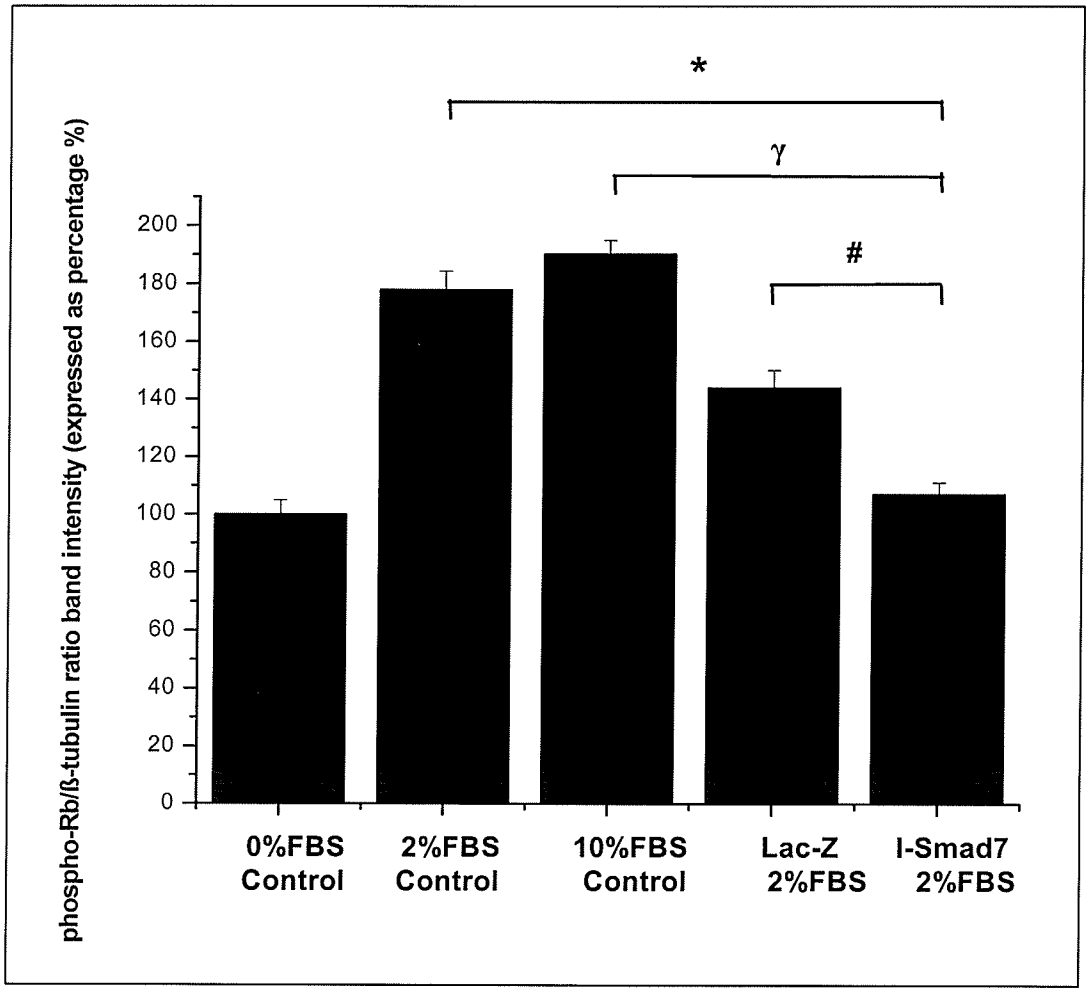


Figure 9: Panel* A:** Representative Western blot analysis of cyclin dependent kinase inhibitor phospho-Rb and β-tubulin expression ***Panel B: Histographic representation of phospho-Rb/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ, γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 5 experiments. Data are quantified by densitometric scanning.

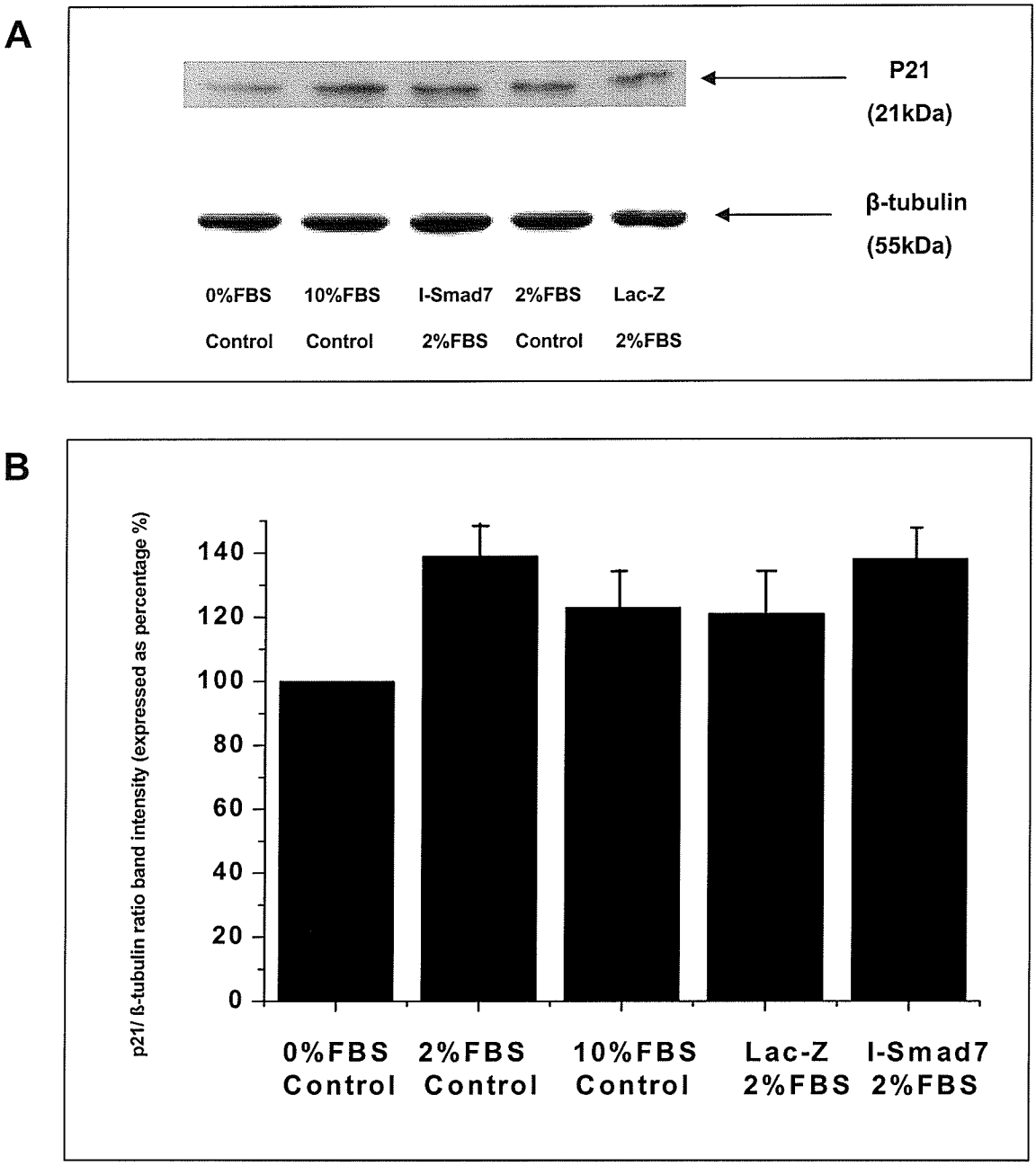
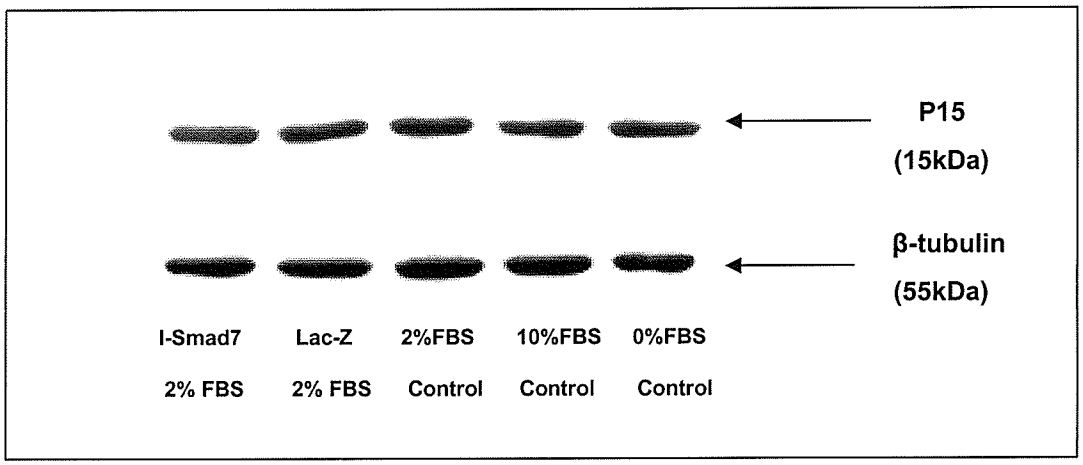


Figure 10: Panel* A:** Representative Western blot analysis of cyclin dependent kinase inhibitor p21 and β-tubulin expression. ***Panel B: Histographic representation of p21/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). Data are expressed as mean ± SEM for a pool of 5 experiments. Data are quantified by densitometric scanning.

A



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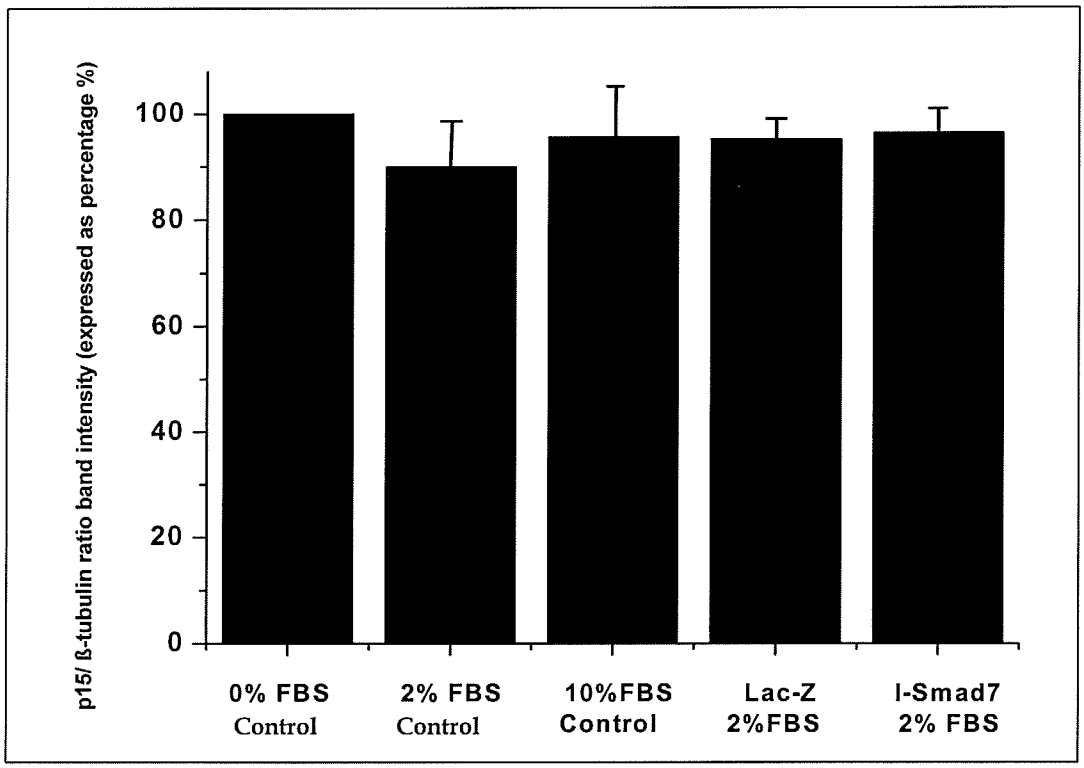


Figure 11: Panel* A:** Representative Western blot analysis of cyclin dependent kinase inhibitor p15 and β-tubulin expression. ***Panel B: Histographic representation of p15/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). Data are expressed as mean ± SEM for a pool of 5 experiments. Data are quantified by densitometric scanning.

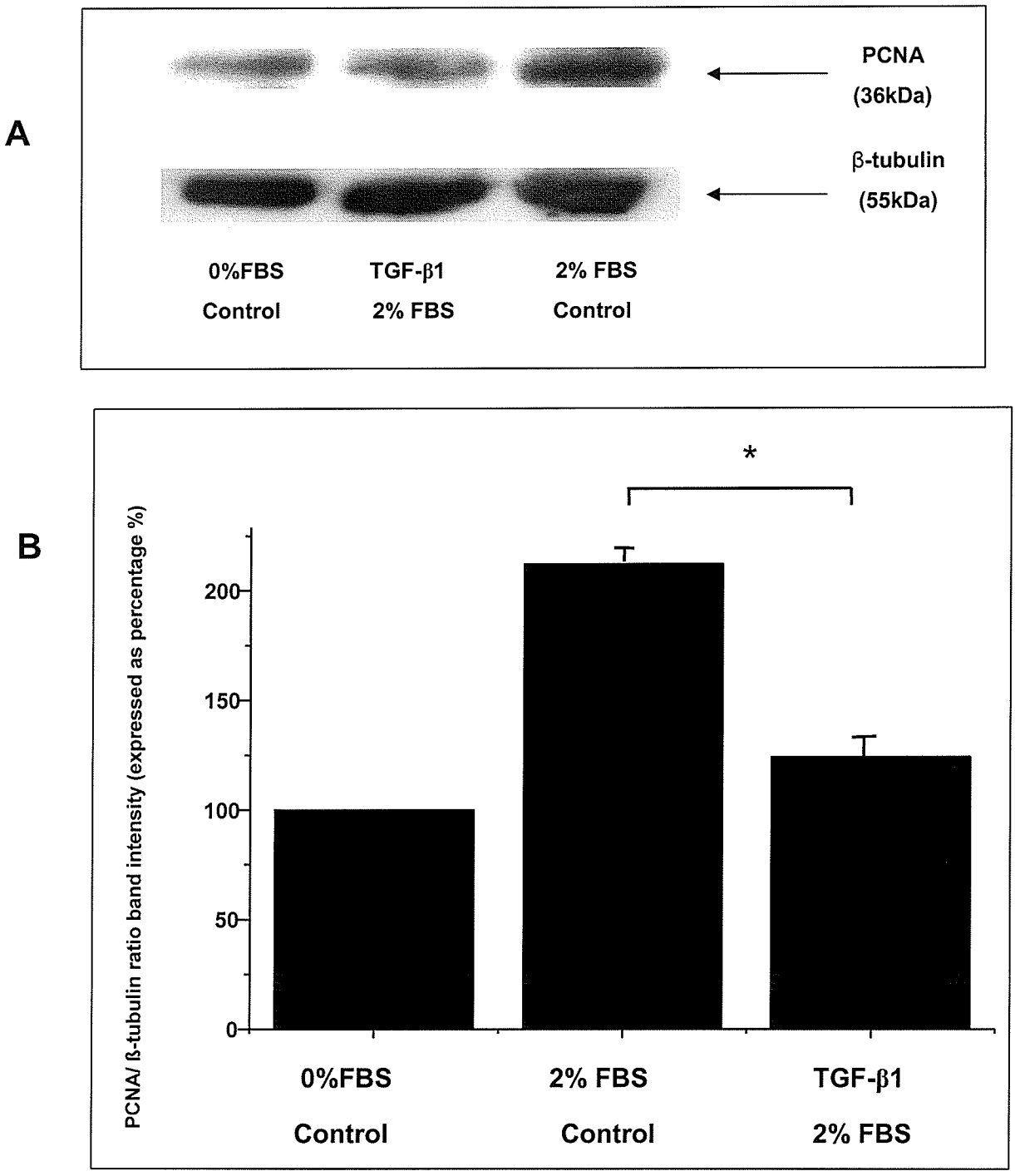


Figure 12. ***Panel A***: Representative Western blot analysis of PCNA and β-tubulin expression. ***Panel B***: Histographic representation of PCNA/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) treated with 10 ng/ml TGF-β1 for 24h (in the presence or absence of 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control. Data are expressed as mean ± SEM, for 3 experiments using identical conditions. Data are quantified by densitometric scanning.

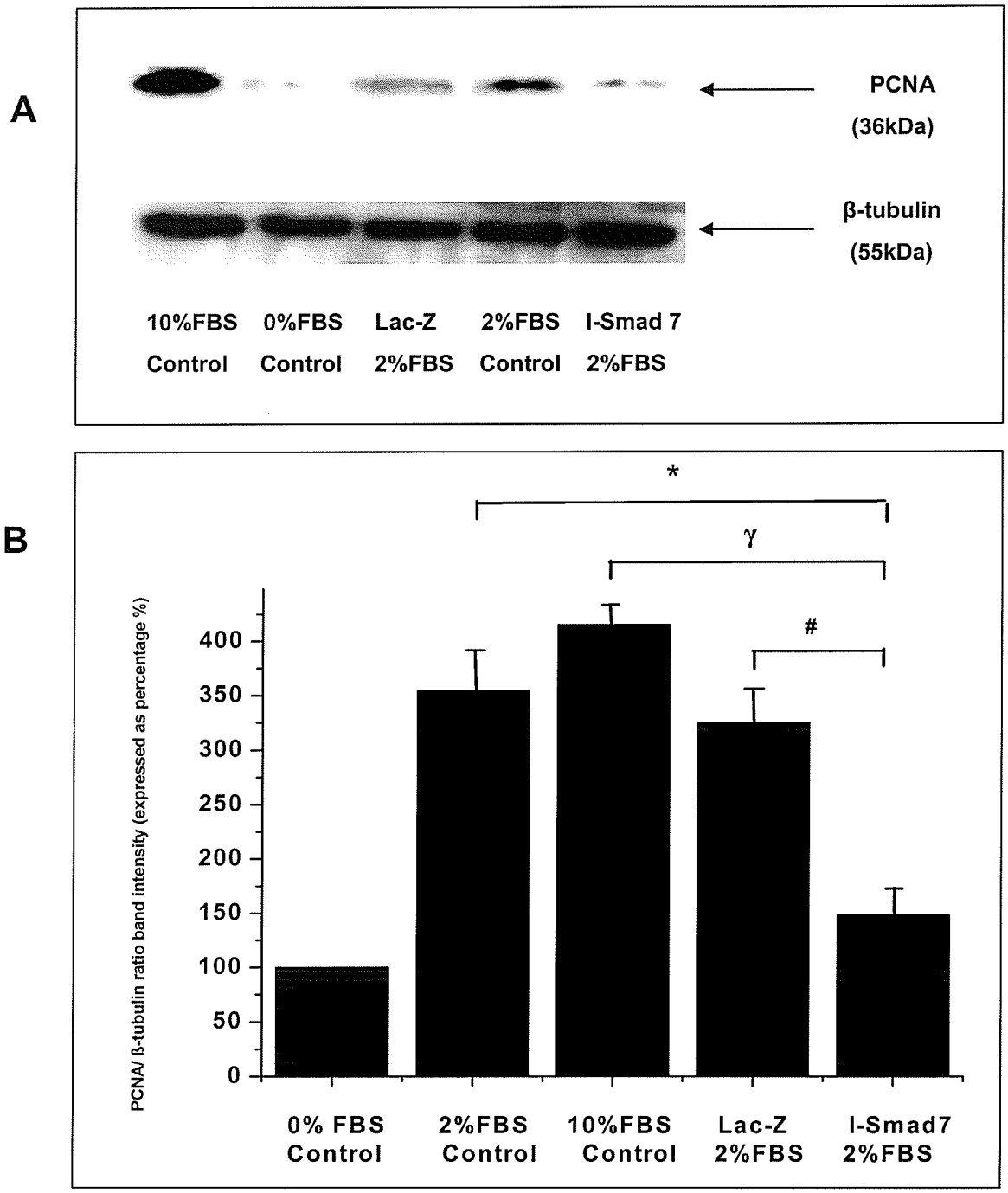


Figure 13. ***Panel A:*** Representative Western blot analysis of PCNA/β-tubulin expression. ***Panel B:*** Histographic representation of PCNA/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ; γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 5 experiments. Data are quantified by densitometric scanning.

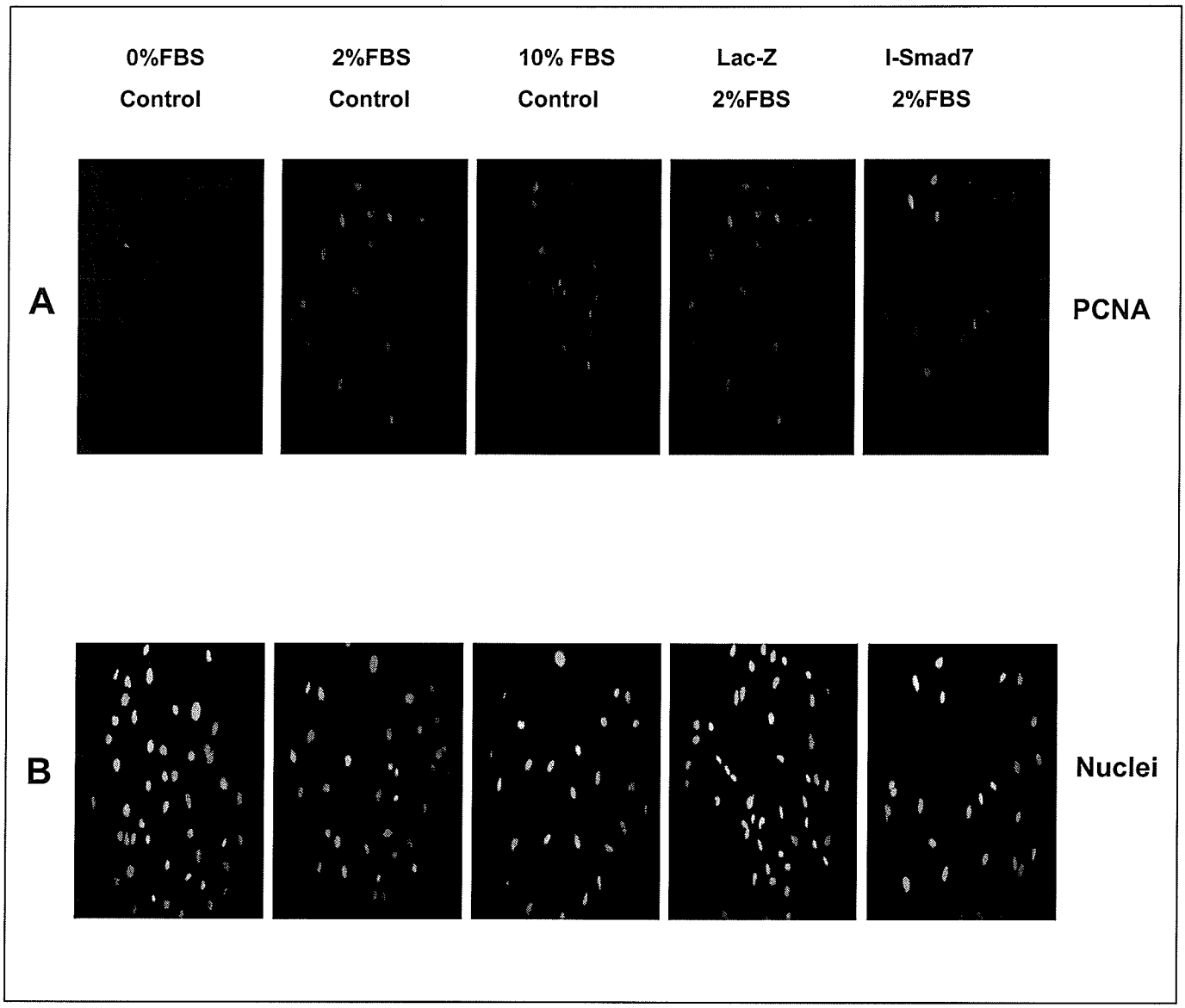


Figure 14: Immunofluorescent PCNA intensity in nuclei (P1) primary cultured cardiac myofibroblasts. ***Panel A***: Immunofluorescent staining of PCNA in cells infected with 100 MOI I-Smad7 adenovirus for 24 hr. ***Panel B:*** Nuclei of identical fields were detected with Hoechst 33342 staining. Original magnification, × 40. (n = 3).

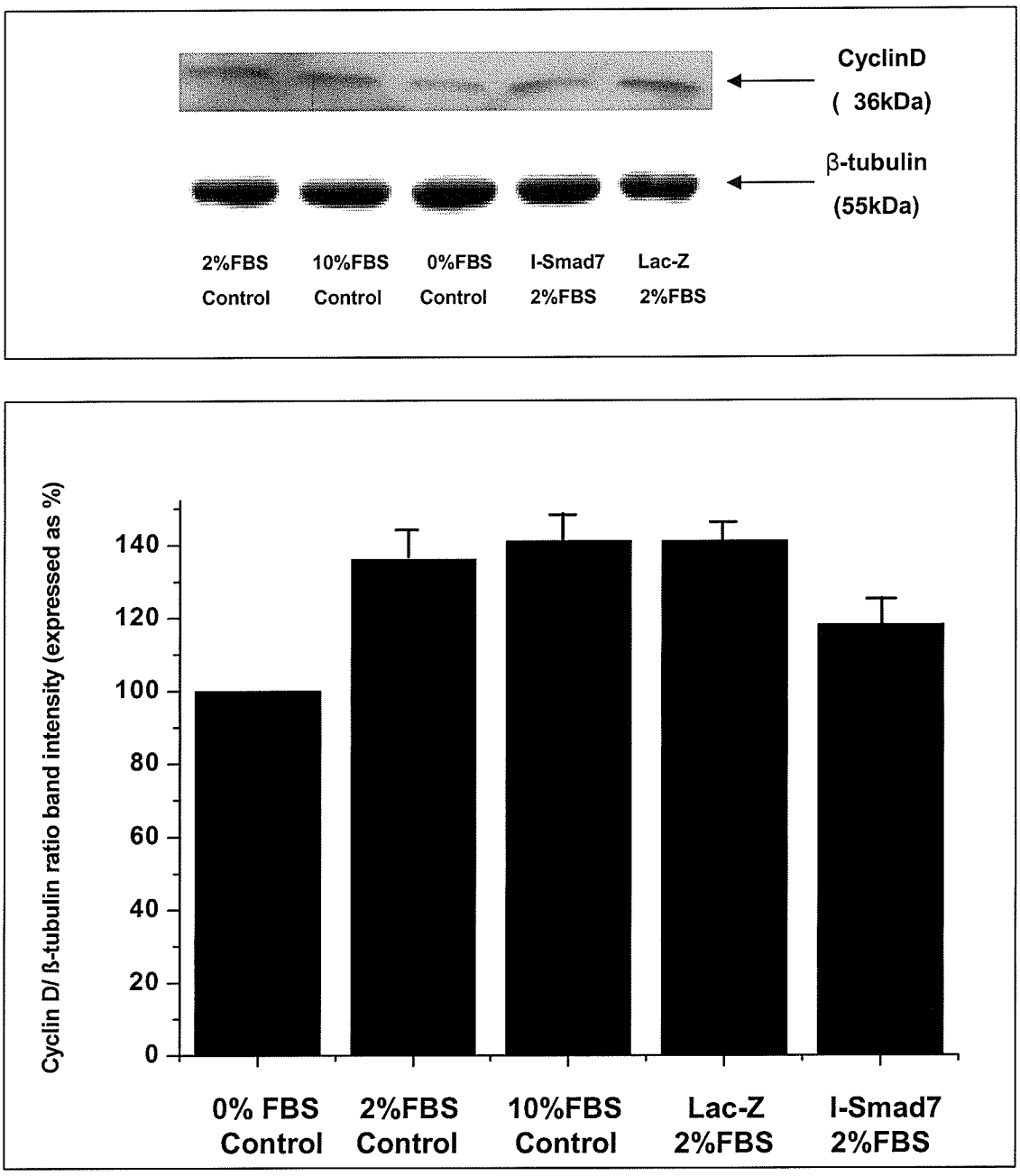


Figure 15: ***Panel*** **A:** Representative Western blot analysis of cyclin D and β-tubulin expression. ***Panel B:*** Histographic representation of cyclin D/ β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). Data are expressed as mean ± SEM for a pool of 5 experiments. Data are quantified by densitometric scanning.

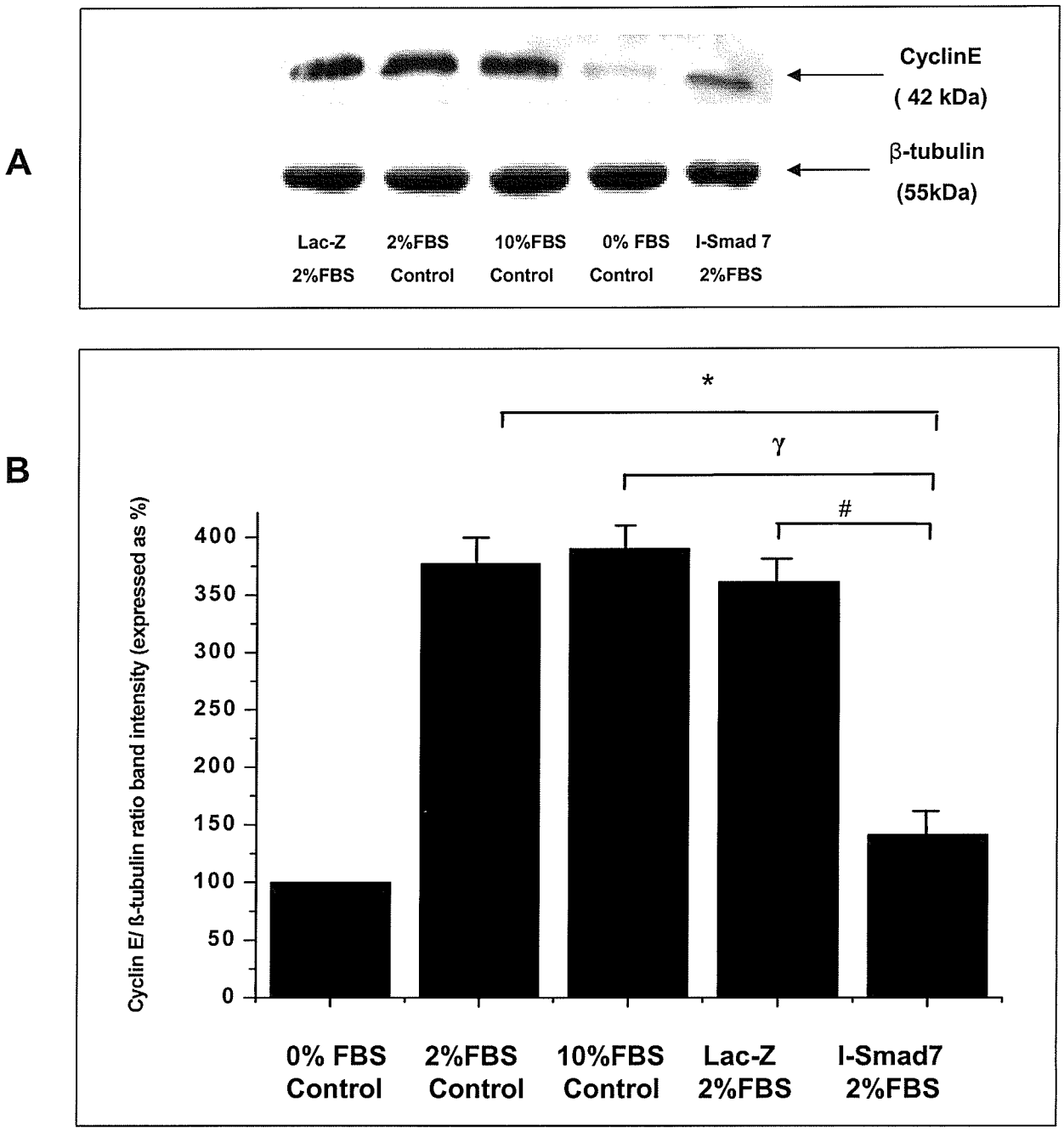


Figure 16: ***Panel*** **A:** Representative Western blot analysis of cyclin E and β-tubulin expression. ***Panel B:*** Histographic representation of cyclin E/ β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ; γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 5 experiments. Data are quantified by densitometric scanning.

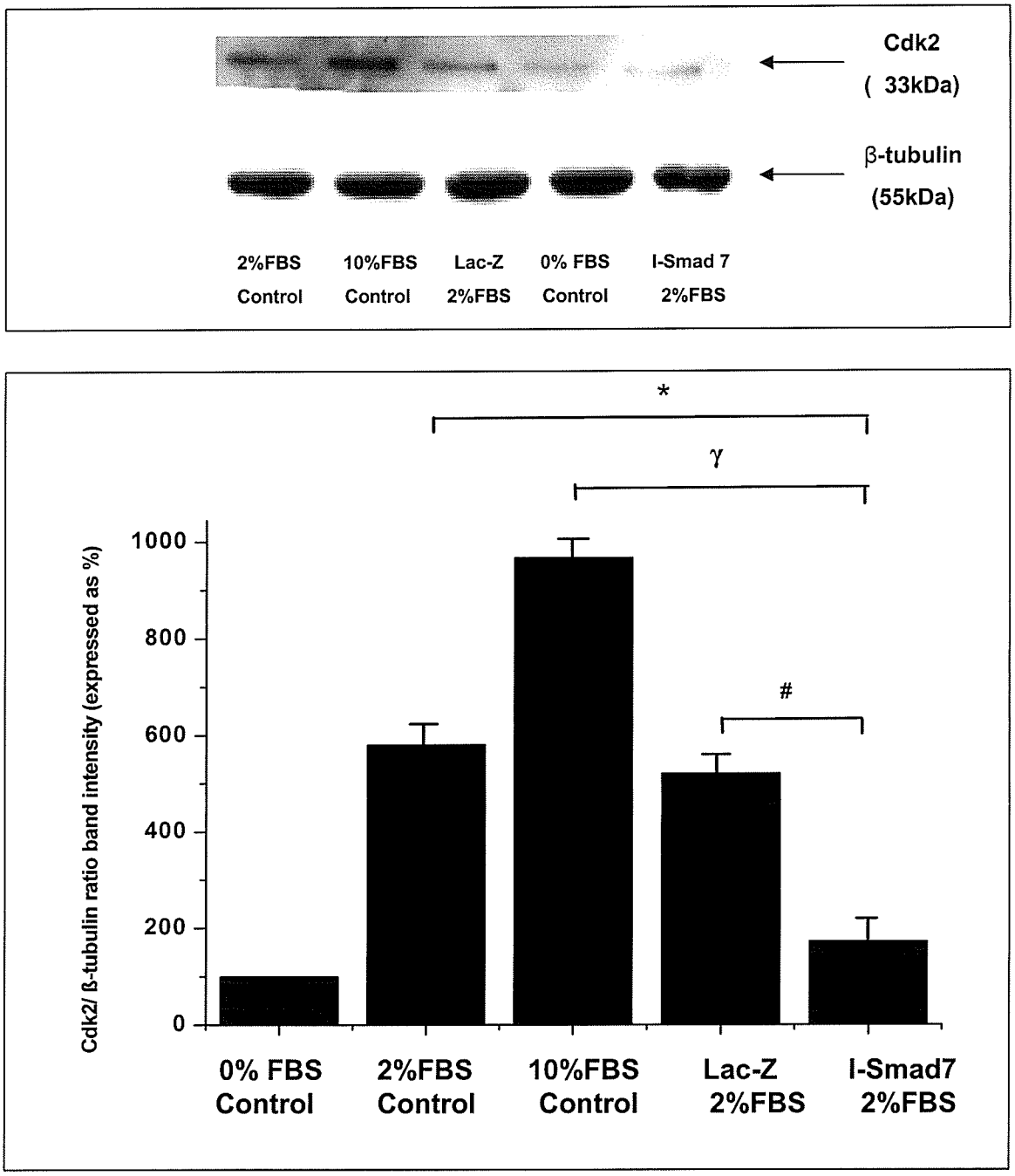


Figure17: ***Panel*** **A:** Representative Western blot analysis of cyclin dependent kinase2 (Cdk2) and β-tubulin expression. ***Panel B:*** Histographic representation of cdk2/β-tubulin band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). * $P \leq 0.05$ vs. 2% FBS-DMEM-F12 control; # $P \leq 0.05$ *vs.* LacZ; γ $P \leq 0.05$ *vs.* 10% FBS-DMEM-F12. Data are expressed as mean ± SEM of 5 experiments. Data are quantified by densitometric scanning.

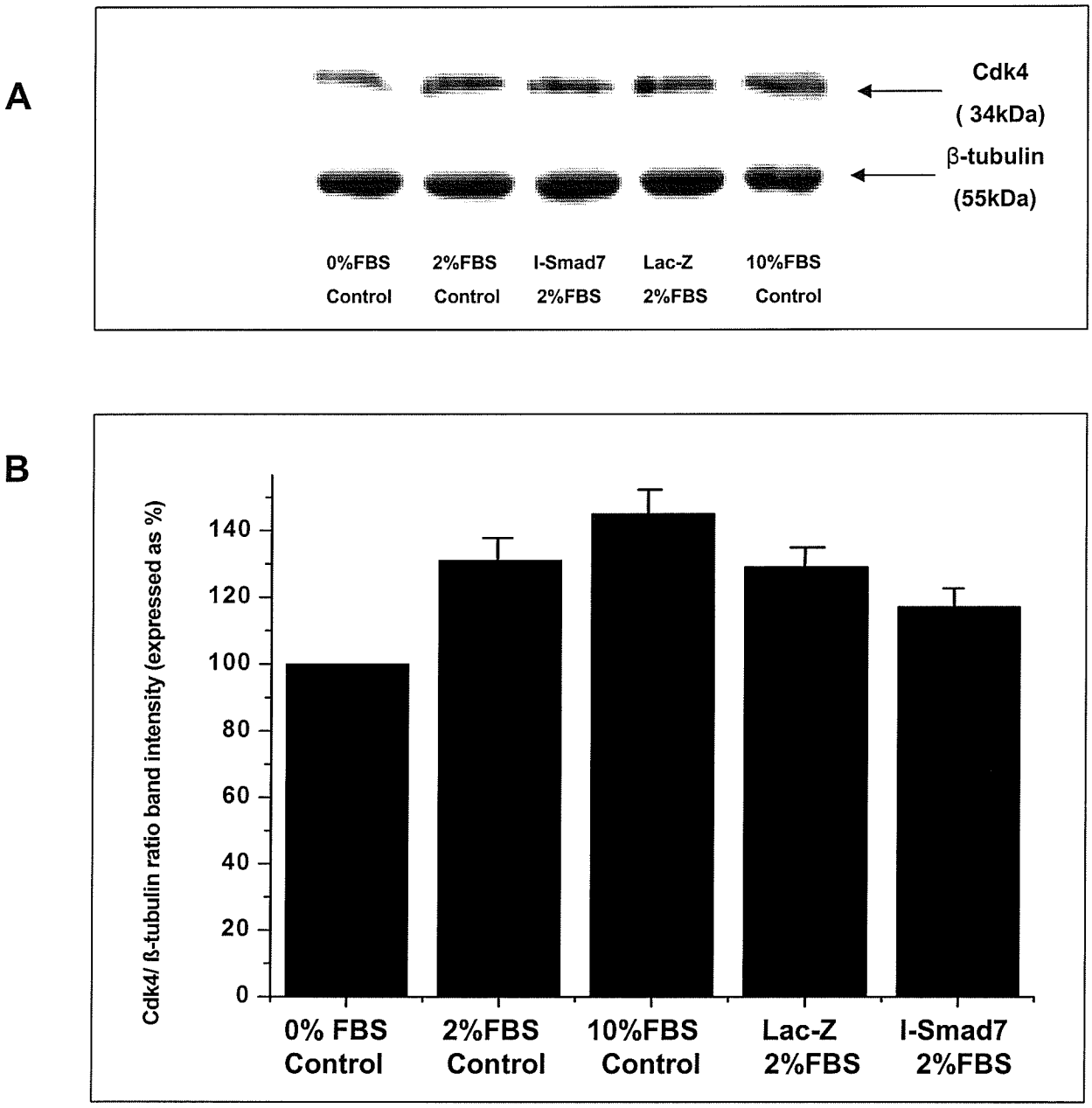


Figure 18. ***Panel*** **A:** Representative Western blot analysis of cyclin dependent kinase4 (Cdk4) and β-tubulin expression. ***Panel B:*** Histographic representation of cdk4/ β-tubulin protein band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24h in the presence or absence of 2% FBS-DMEM-F12 and LacZ control virus (in 2% FBS-DMEM-F12). Data are expressed as mean ± SEM for a pool of 5 experiments. Data are quantified by densitometric scanning.

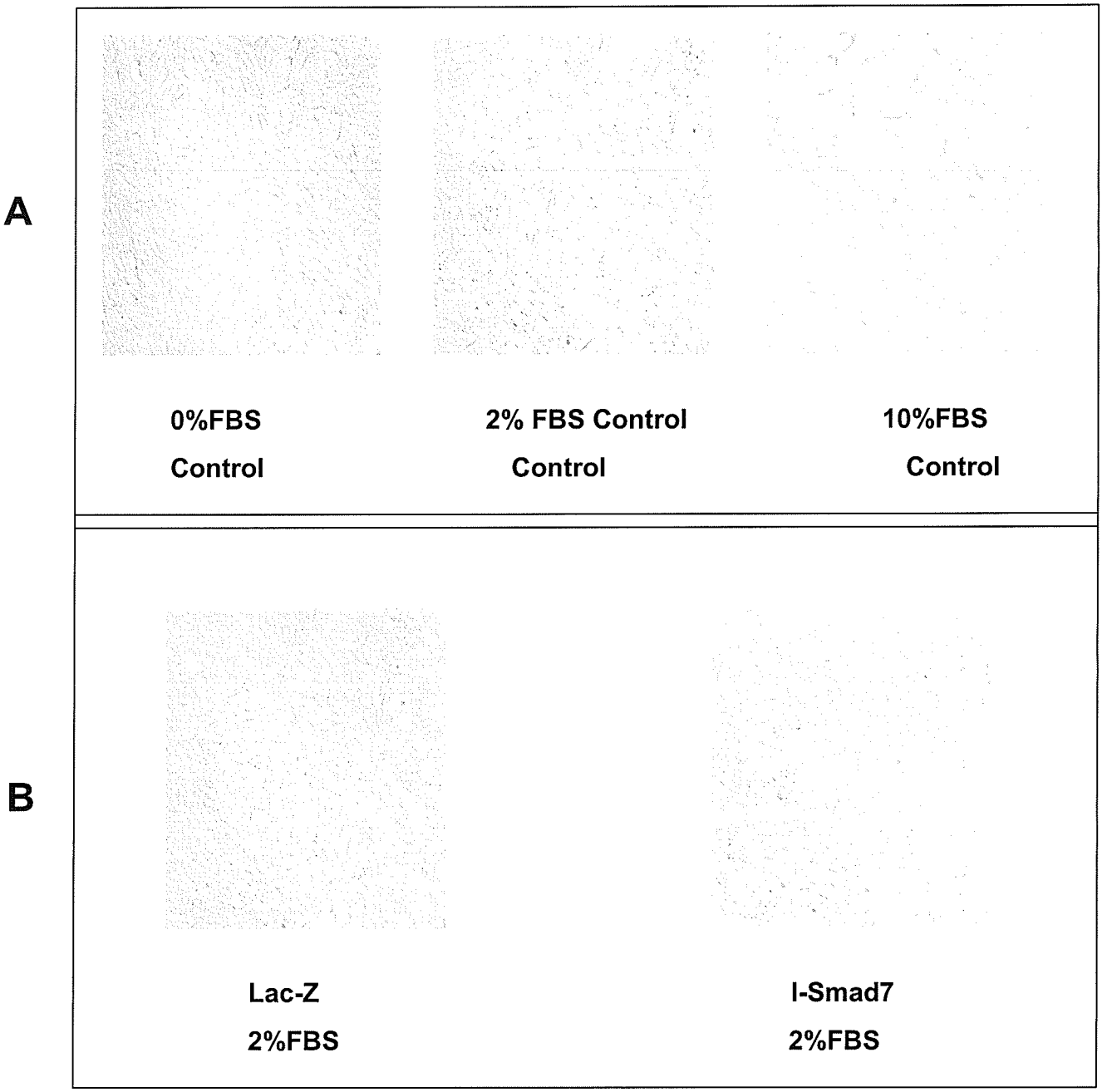


Figure 19. ***Panel A***: Light microscopy of cultured primary cardiac myofibroblasts in 0% FBS, 2% FBS and 10% FBS-DMEM-F12 controls. ***Panel B:*** Light microscopy of 2% FBS-DMEM-F12 cultured primary cardiac myofibroblasts, infected with 100 MOI I-Smad7 adenovirus or Lac-Z adenovirus for 24 hours.

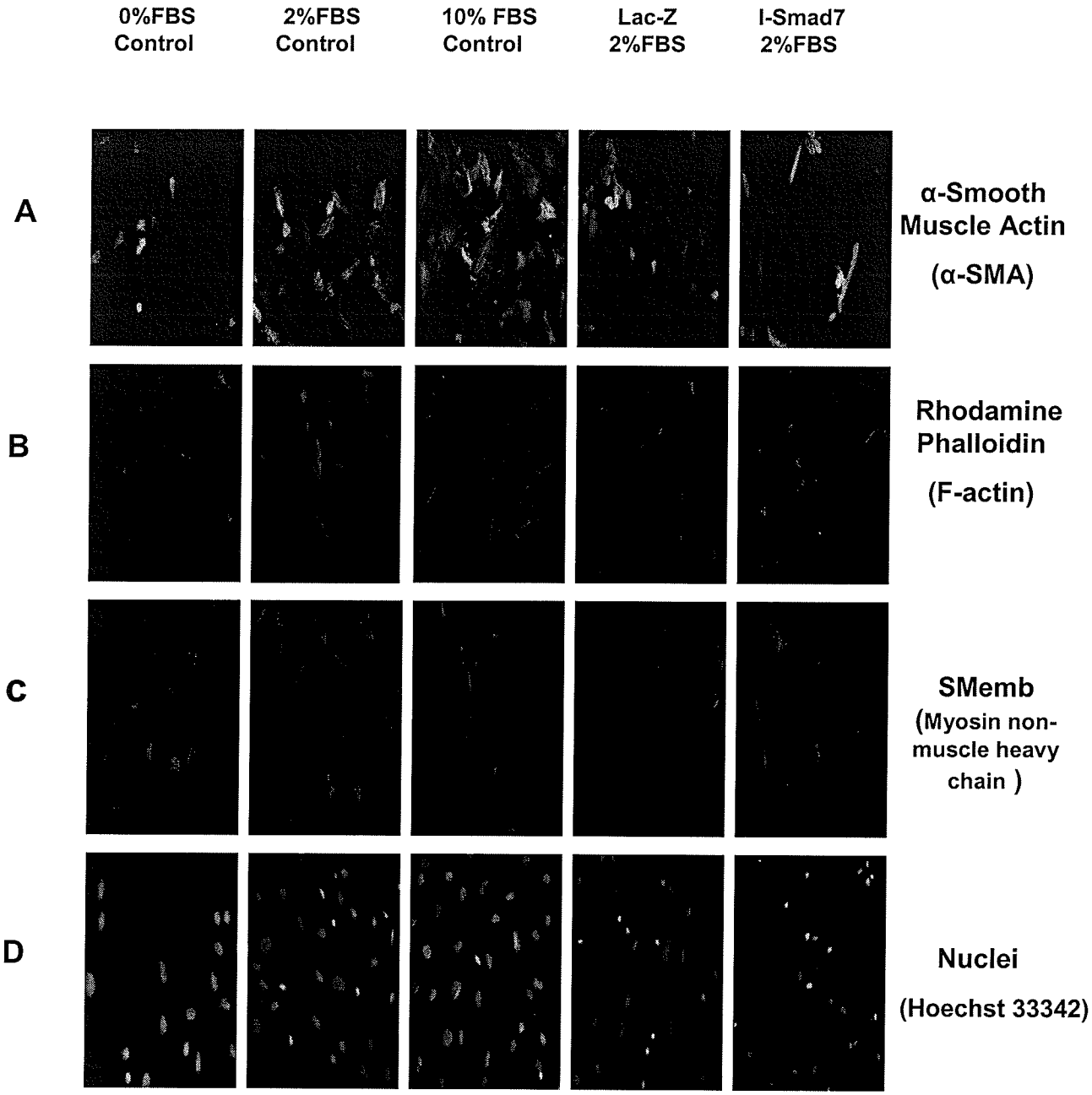
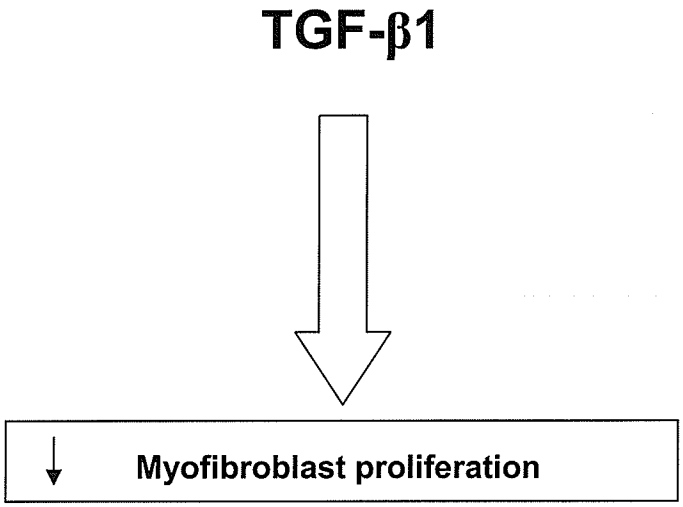


Figure 20: Phenotypic characterization of P1 cultured cells. ***Panel A***: Immunofluorescent detection of immunoreactive α-smooth muscle actin (α-SMA) in cells infected with 100 MOI I-Smad7 adenovirus for 24 hr. ***Panel B:*** Fluorescent staining of rhodamine phalloidin in infected cells. ***Panel C:*** Fluorescent staining of SMemb in infected cells. ***Panel D***: Stained nuclei of identical fields. Original magnification, × 40. (n = 3).

A.



B. TGF-β1 mediated inhibition of myofibroblast proliferation: putative mechanism (s).

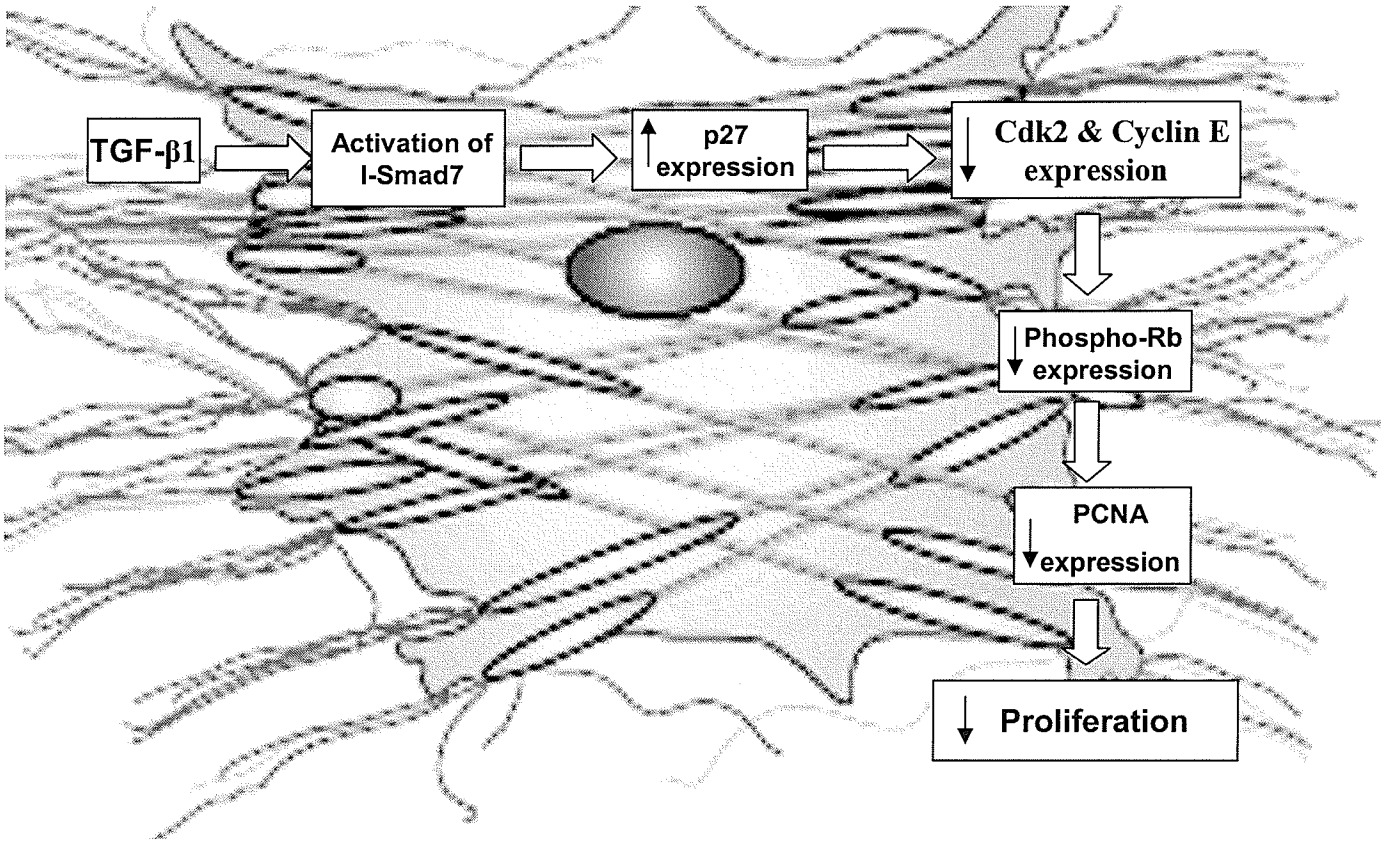


Figure 21: ***Panel A*****:** TGF-β1 causes inhibition of myofibroblast proliferation by an unknown mechanism. ***Panel B:*** Putative mechanism for TGF-β1-mediated inhibition of cardiac myofibroblast proliferation.

VII. DISCUSSION

1. Effect of overexpression of I-Smad7 on cardiac myofibroblast proliferation

To carry out effective wound healing in post-MI heart, fibroblastic cells are known to proliferate within and near to the site of infarction. Granulation tissue is present in acute MI followed by myocardial infarct scar thinning and ultimately in the case of large MI, significant changes to gross ventricular geometry eg, cardiac remodeling. This remodeling process is not limited to cardiac myocytes, but also to the matrix surrounding those cells. Net matrix deposition in diseased heart is usually associated with differentiation of quiescent fibroblasts to highly synthetic myofibroblasts. In this regard, cardiac myofibroblast proliferation is an important contributor to both appropriate infarct wound healing and inappropriate wound healing. For example, in the event of large MI, cardiac fibrosis may ensue in regions including and remote to the site of the infarct scar. In the latter, studies of mechanisms that control the pathological progression of cardiac fibrosis and left ventricular (LV) dysfunction are of vital importance. Thus abnormal proliferation of myofibroblasts in the heart following myocardial infarction (MI) may exert dual effects [125, 126]. It may prevent the injured heart from acute dilatation and rupture of the infarcted region by contributing to formation of new connective tissue to compensate for myocyte death and loss of cardiac wall integrity. As stated above, myofibroblast proliferation may also lead to cardiac fibrosis which is well known to impair cardiac function [125, 126, 127]. The application of antifibrotic agents that target key points in myofibroblast cell cycle progression and proliferation represent promising strategies for this disease. TGF-β1 has been considered to be a major player for fibrotic diseases, including cardiac fibrosis. Previous evidence of blockade of TGF-β1 signal with receptor antisense antibodies, neutralizing antibody, or decorin has highlighted the therapeutic potential by targeting this

molecule in pathological fibrosis [125-126]. It has been well-established that following the binding of the TGF-β1 ligand to TGF-β1 type II receptor, TGF-β1 type I receptor is activated, which leads to the phosphorylation of R-Smads, including Smad2 and Smad3, and subsequently activates a downstream signal transduction pathway. On the other hand, activated TGF-β1 type I receptor also functions to induce the expression of inhibitory Smad7 which antagonizes TGF-β1 signal either by competing for binding of R-Smads to TβRI, thereby blocking R-Smads from interacting with the receptor [6], or by increasing ubiquitin-mediated degradation of TGF-β1 receptor [128]. The discovery of the Smad super-family allows for a new approach for the blockade of TGF-β1 signaling via the use of inhibitory Smad7.

Existing evidence has highlighted the negative regulatory role of I-Smad7 in the activation of R-Smads in a variety of tissues. For example, overexpression of I-Smad7 was shown to attenuate Smad 2/3-mediated inhibition of embryonic morphogenesis [11]. In bleomycin-induced lung fibrosis, overexpressed I-Smad7 was also associated with inhibition of Smad2 phosphorylation, decreased type I procollagen mRNA and net collagen production. Moreover, blockade of activation of Smad2 as well as downregulation of collagen type I, III and IV was observed in renal tubular epithelial cells [14] as well as smooth muscle cells [129]. In hepatic stellate cells, ectopic expression of I-Smad7 also leads to abrogation of Smad2 activation [130]. Data from our lab has shown that I-Smad7 expression was decreased in both scar tissue and remnant tissue in *chronic* post-MI rat hearts, accompanied by decreased cytosolic expression of phosphorylated Smad2 [16]. It also showed that ectopic expression of inhibitory Smad7 appears to inhibit TGF-β1 induced collagen production in primary cardiac myofibroblasts. This anti-fibrotic effect of I-Smad7 involved both TGF-β1 dependent activation of Smad2 and TGF-β1 independent activation of MMP-2. The current investigation has focused on the *acute* effects

of I-Smad7 overexpression *in vitro* – our key finding is that are that overdriven I-Smad7 is associated with significant inhibition of cardiac myofibroblast proliferation, in a manner that parallels the effect of TGF-β1 treatment of these cells (see below). It would follow that an intact TGF-b/I-Smad7 signaling cascade may allow for rapid control of myofibroblast proliferation. Conversely, abnormally low expression of I-Smad7 may play a role in the pathogenesis of cardiac fibrosis by diminishing its anti-proliferative effect(s). While the underlying mechanisms for this phenomenon remain unclear, our current work provides an association between overdriven I-Smad7 and altered expression of different cell cycle regulating proteins i.e. cyclin kinase inhibitors (p27, phosphor-Rb, p21 and p15), cyclin-dependent kinases (Cdk2 and Cdk4) and cyclins D and E.

2. The effect of TGF-β1 on primary cardiac myofibroblasts

TGF-β1 is a cytokine generated within cardiac tissue (and others) that has been implicated as a major stimulator of fibroinflammatory changes in various organs. TGF-β1 has a significant suppressive influence on fibroblast proliferation and is also a powerful stimulus for increased extracellular matrix production in fibroblastic cells [64]; it also modulates the phenotypic conversion of fibroblasts to the muscular myofibroblastic phenotype [64, 103]. We investigated the effects of TGF-β1 on primary cultured cardiac myofibroblasts, and specifically, we confirmed that TGF-β1 significantly inhibits proliferation of these cells, at the dose of 10 ng/ml. It has been shown that TGF-β1 may exert its effects in a highly cell-specific manner and that it increases cell proliferation in higher doses in prostatic stromal cells [135]. This finding, coupled with the current results reinforces the pleiotropic nature of this cytokine, and hints at the complexity and plasticity of its associated intracellular signaling. With regard to myofibroblast

function, we suggest that reduced proliferative capacity is a mechanism facilitating enhanced collagen secretion rather than cell division.

3. Cyclin dependent kinase proteins inhibitors (CKI's) and overexpression of I-Smad7

TGF-β1 plays an important role in tissue fibrosis by enhancing the synthesis of collagen and other components of extracellular matrix and regulating fibroblast differentiation, proliferation and apoptosis [36, 131]. The understanding of TGF-β1 signaling pathways that influence collagen synthesis and cell proliferation has advanced rapidly since identification of the Smad family of proteins [98]. Myofibroblast cell cycle is regulated through the interaction of different proteins [78, 79]. In this study we looked into four of the main cell cycle dependant kinase inhibitor proteins (CKI's). These are p21, p15, p27 and phospho-Rb, which preferentially bind to the G_1/S class of CDKs. Recent studies have shown that the cyclin-dependent kinase inhibitors (CKIs) have important roles in cell cycle progression in normal cells [79, 98]. Alterations in the cdk inhibitors also appear to be important in the regulation of myofibroblast proliferation [76, 78, 79]. In the current study, overexpression of I-Smad7 was associated with a significant increase in p27 expression. This is a salient marker for cell cycle progression inhibition, since p27 inhibits the formation of the cdk2/cyclin E complex. This event facilitates the formation of PCNA and the progression of the cell cycle from G_1 phase to S phase [82, 83].

The importance of phosphorylation of Rb protein is unique and some have argued the ultimate control point for cell cycle regulation [88, 89]. We found that the phospho-Rb expression was significantly decreased by I-Smad7 overexpression in P1 myofibroblasts, thus potentially limiting cell cycle progression at the G_1 phase, as is put forward previously by others [88, 89]. While p21 and p15 proteins also play a regulatory role in the progression of the cell

cycle and both may prevent the formation of the cdk4-cyclin E complex, necessary for the formation of PCNA and phosphorylation of Rb protein [135], the expression of these proteins was unaffected by overexpression of I-Smad7. It is noted that p21 and p15 functions i.e., the blocking the formation of cdk-cyclin complexes, may be substituted by p27 itself [135]. Thus this apparent redundancy might provide a reason as to why both p21 and p15 expression are unchanged in the face of I-Smad7 overexpression. Our current data indicates that p27 and phospho-Rb may be rate limiting factors in regulating cell cycle in primary cardiac myofibroblasts.

4. Cyclins and cyclin dependent kinases (Cdks)

Cdk2-cyclin E activity has long been thought to be essential for the progression from G_1 phase to S phase of the cell cycle [132, 133]. In the cell cycle cyclin E and cdk2 both facilitate phosphorylation of Rb and provide a significant point for the progression of cell cycle [138]. We found that the expression of cyclin E and cdk2 was decreased significantly with overexpression of Smad7 in myofibroblasts. We hypothesis that the cdk2- cyclin E complex may plays a role in regulating the myofibroblast cell cycle, through the ablation of the phosphorylation of Rb coupled with results that points to decreased phospho-Rb in these cells or p27. We suggest that these events represent a series of I-Smad7 dependent events that ablate myofibroblast proliferation. Further, increased p27 expression may cause increased cyclin E-cdk2 complex inhibition and thus decrease phosphorylation of Rb protein; we suggest that these events provide a positive feedback loop with the inhibition of TGF-β1/I-Smad7 mediated myofibroblast proliferation. Cyclin D and cdk4 proteins form a complex that also regulates the phosphorylation of Rb, leading to the progression of the cell cycle from G_1 phase to S phase

[138]. It was of interest to note the lack of significant change in expression of either cdk4 or cyclin D. As this complex is inhibited by the actions of p15 and p21 and as neither of these CKI proteins showed any changes in the expression with I-Smad7 overexpression, it is possible that feedback loop is unaffected by Smad7. The current findings indicate that cdk4-cyclin D complex, p21 and p15 do not play a role in the cell progression from G_1 to S phase in myofibroblasts subjected to I-Smad7 overexpression. As to the question of why the p21/p15 and cdk4/cyclin D axis is unaffected with Smad7 overexpression in myofibroblasts, the mechanism is unknown and will require further study.

5. I-Smad7 effect on PCNA expression and cell protein expression

PCNA is an auxiliary protein of the DNA polymerase and is a proliferation-associated marker [137,138]. Its maximal expression peaks in late G_1 and S phase of the cell cycle. PCNA has been used as a marker of cellular proliferation in different cell types [137, 138]. The current finding that PCNA expression is decreased with I-Smad7 overexpression lends strength to the parallel findings of reduced DNA synthesis (thymidine), and correlates well with enhanced p27 and decreased pRb expression in these cells [Figure 21].

6. Myofibroblast phenotype and I-Smad7

Myofibroblasts are essential for wound healing and wound contraction in the infarcted tissue in the heart. They differentiate from fibroblasts and are characterized by the presence of stress fibers containing α-actin isoforms that is observed in smooth muscle cells [64, 133, 134, 135]. More specifically, fibroblasts are readily identified by their synthesis of specific isoforms of fibronectin but unlike proto-myofibroblasts and fully differentiated myofibroblasts, they do

not exhibit expression of mature and supermature focal adhesion complexes, ED-A fibronectin nor an extensive network of cytoplasmic actins eg, stress fibers [139]. In the current study, key protein markers of the myofibroblastic phenotype were stably expressed in the presence of viral vectors and all cells infected with I-Smad7 adenovirus as well as non-infected groups expressed comparable amounts of α-SMA, SMemb and F-actin. Evidence of stress fiber networks comprised of cytoplasmic actins and associated myosin (SMemb) were present in all groups as noted in Figure 20, despite the relatively low number of cells in the fields shown for serum-free, 2 % serum, and 2% serum plus I-Smad7 treated cells. While many cell types express variable amounts of cortical cytoplasmic actins, notably α-SMA, the stable expression of SMemb myosin in our P1 cultures is significant as it is a reliable additional indicator of the myofibroblastic phenotype [140]. This finding indicates that the stage of differentiation of these cells is unaltered by exposure to adenoviral vectors and more importantly to relatively high levels of I-Smad7 expression. As our data indicate that neither I-Smad7 nor Lac-Z overexpression altered the myofibroblastic phenotype of these cells under our experimental conditions, we were able to safely compare one treated population of cells with either negative or positive controls.

VIII. FUTURE DIRECTIONS

A number of experiments are foreseen that may extend the current data set. As a first step, we would focus only on those proteins that showed remarkable changes in response to I-Smad7 expression. We will likely address the molecular "level" of synthesis that may lead to change in protein expression of p27, cyclin E, cdk2 and phospho-Rb and PCNA and/or studies addressing target protein stability or rate of protein degradation. Furthermore the investigation of mRNA levels of relevant genes or for rates of protein degradation of these genes may lend

insights into transcriptional control mediated by I-Smad7 overexpression in these cells. We may also extend the current study to an *in vivo* level by examining the effect of I-Smad7 adenovirus microinjection to the infarcted scar itself. Presumably, I-Smad7 would then exert its effect on the cardiac myofibroblasts and on the scar healing itself. To this end, the relationship between cyclins, cdks and cyclins kinase inhibitors after treatment with I-Smad7 may be observed *in vivo.*

IX. CONCLUSIONS

We have shown that I-Smad7 overexpression significantly inhibits low serum-induced proliferation in primary cultured cardiac myofibroblasts. Infected cells show high expression of p27 and low expression of cyclin E and cdk2. Both form a complex that is inhibited by p27 and we suggest that this mechanism may lead to inhibition of phosphorylation of Rb which is the final step in progression of the cell from G_1 phase to S phase. Our findings are also supported by significant decreased PCNA expression in transfected cells. I-Smad7 did not alter the phenotype of differentiated P1 myofibroblasts. As TGF-β1 normally inhibits serum-stimulated proliferation of cardiac myofibroblasts cells, we suggest that this may occur via a I-Smad7 directed pathway. As TGF-β1 is one the most potent stimuli for cardiac fibrosis and is known to participate in the differentiation of fibroblastic cells to relatively immobile but synthetic myofibroblasts, we speculate that reduction of proliferative capacity in TGF-β1 treated cells may serve to redirect cellular resources toward enhanced matrix secretion. Finally, while the precise role of I-Smad7 control over myofibroblast matrix deposition is unclear, we speculate that net reduction of matrix production with forced expression of this protein occurs via two mechanisms i) direct reduction of matrix synthesis and ii) reduced number of matrix-producing cells.

Finally, we speculate that the cascade of events that lead to alteration of proliferation of myofibroblasts by I-Smad7 may be as follows: I-Smad7 release leads to increase in CKI p27 formation. This protein blocks cdk2-cyclin E complex formation – the latter is essential for the phosphorylation of Rb to phospho-Rb. A decrease in phosphorylated Rb leads to the inactivation of PCNA function in coupling of DNA strands, leading to inhibition of myofibroblast proliferation (Figure 21).

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