

ROLE OF RESPONSE GENE TO COMPLEMENT 32 (RGC32) IN THE  
TRANSFORMATION OF CARDIAC FIBROBLASTS TO MYOFIBROBLASTS

by

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(Under the Direction of WAN-I OLIVER LI)

ABSTRACT

Cardiac fibrosis is defined as an abnormal accumulation of excessive extracellular matrix proteins, especially collagens, in the myocardium, which characterizes morphological feature of the structural myocardial remodeling that occurs in several cardiac diseases. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a major contributor to fibrogenic responses, modulating fibroblast activation and promoting extracellular matrix deposition by up-regulating collagen and fibronectin synthesis and by decreasing matrix degradation through inhibition of activities of matrix metalloproteinases (MMPs). The present study was designed to investigate whether response gene to complement 32 (RGC32) is involved in the transformation of fibroblasts to myofibroblasts and whether RGC32 depletion or overexpression alters the expression of smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA), an indicator of myofibroblasts, and ECM proteins. Our results suggest that RGC32 is a TGF- $\beta$  downstream target mediating the myofibroblast transition in the primary mouse cardiac fibroblasts (mCFs). RGC32 depletion attenuates while overexpression enhances the expression of  $\alpha$ -SMA and collagen I.

INDEX WORDS: Cardiac Fibrosis, Cardiac fibroblasts, TGF- $\beta$ , RGC32, Collagen I

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## **CHAPTER 1**

### **Introduction**

Heart injuries from many different causes can end up with a common end-stage of pathologic remodeling and fibrosis, promoting the development of heart failure (Kania et al., 2009). The pathologic remodeling refers to alterations in size, shape, and function of the heart after injury such as myocardial infarction, pressure overload, and volume overload (Sutton et al., 2000). Cardiac fibrosis is a detrimental process that results in a progressive stiffening of ventricle walls, loss of contractility, and abnormalities in cardiac conductance by a disproportionate accumulation of extracellular matrix between muscle fibers or around blood vessels (Goumans et al., 2008). The increased collagen deposition in the myocardium is a hallmark of the cardiac fibrosis.

The muscular compartment of the heart has been almost exclusively focused throughout the history of cardiology since cardiac myocytes are the cells that actually perform the main function of the heart, pumping the blood throughout the body. However, in recent years, the non-myocyte cell populations are increasingly appreciated due to their significant contributions to the performance of the normal and failing heart. In particular, cardiac fibroblasts have been recognized as a critical cell type involved in homeostatic maintenance of extracellular matrix (ECM) and wound healing process (Brown et al., 2005). The homeostasis of cardiac ECM is very important in that its structure serves as a scaffold providing the local environment directly



influencing the behaviors of cardiomyocytes. In keeping with the structural and mechanical role of cardiac ECM, the major constituents are the fibillar collagens I (~80%) and III (~10%), with smaller amounts of collagens IV, V, VI, elastin, laminin, proteoglycans, glycoproteins and other bioactive molecules (Bosman et al., 2003). Myocardial ECM is a highly organized dynamic network whose turnover is tightly regulated by coordinated degradation and synthesis of the various ECM components. Cardiac fibroblasts can regulate the rates of synthesis and degradation by modulating the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in response to the various stimuli. MMPs belong to a family of zinc-dependent proteolytic enzymes and play an important role in degrading the ECM while TIMPs inhibit the action of MMPs (Li et al., 2000). Therefore, MMPs and TIMPs are the key regulators in regulating the homeostasis of ECM. Any factors interrupting this subtle balance between MMPs and TIMPs may initiate the pathologic remodeling by altering ECM turnover.

Myocardial injury leads to the complex sequence of events involving coordinated interactions among multiple cell types in the wound area. Inflammation is an essential step for tissue repair during wound healing, resulting in infiltration of immune and inflammatory cells, degradation and phagocytosis of necrotic myocytes and cellular debris, and recruitment of cardiac fibroblasts within the zone of injury by chemotaxis (Bujak and Frangogiannis et al., 2007). Cardiac fibroblasts proliferate, reconstructing and remodeling the ECM to produce a mature scar. Thus, net ECM degradation through the increased MMP expression dominates the initial phase of the injury response, whereas net ECM synthesis, arising from enhanced collagen synthesis by fibroblasts, dominates the later phase of healing (Brown et al., 2005). During the tissue repair, cardiac fibroblasts can be transformed into myofibroblasts whose capability of

collagen synthesis is markedly increased than that of fibroblasts (Rohr et al., 2011). Myofibroblasts, the specialized cell type that switches from a fibroblast-like state to a contractile smooth muscle-like state migrate to the wound area and secrete ECM proteins such as collagens and fibronectin for wound healing. In normal tissue repair, myofibroblasts can disappear by apoptosis. However, prolonged or persistent inflammation may stimulate profibrotic signaling pathways via overproduction and/or activation of cytokines such as transforming growth factor-beta (TGF- $\beta$ ), causing excessive deposition of ECM in the wound (Krenning et al., 2010).

Cardiac fibrosis plays a dual role in myocardial remodeling after injury. On the one hand, it is a prerequisite for wound healing. On the other hand, it contributes to ventricular remodeling and progression of heart failure. An initially adaptive healing process turns into the potentially maladaptive response in the long term. Therefore, it is critical to find a therapeutic strategy that may prevent the over-proliferation and over-activation of myofibroblasts at the appropriate timing.

Although fibrosis is one of the largest groups of diseases observed in various organs including heart, liver, and kidney, the mechanisms of fibrosis have not been fully understood and no effective therapy has been developed. Previous study from our group reported that response gene to complement 32 (RGC-32) overexpression exacerbated the injury-induced vascular remodeling *in vivo* through a rat carotid artery balloon-injured model (Wang et al., 2011). Vessel wall injuries are also known to cause similar structural changes with those of cardiac injuries, including transition of fibroblasts to myofibroblasts, adventitial fibrosis, and expression of MMPs (Strauss and Rabinovitch et al., 2000). The experiments described in this thesis investigated the role of RGC-32 in cardiac fibroblast-to-myofibroblast transition mediated by TGF- $\beta$  *in vitro* using mouse primary cardiac fibroblast cells.

## **CHAPTER 2**

### **Literature Review**

#### **Cardiac Fibrosis**

Cardiac fibrosis is defined as a progressive accumulation of fibrillar extracellular matrix in the myocardium that is presumed to be derived from fibroblast cells within the heart. Cardiac fibrosis is a consequence of many cardiovascular diseases including hypertension, myocarditis, dilated cardiomyopathy, and myocardial infarction (Chan et al., 2010). Such cardiac injuries trigger inflammatory responses in the myocardium, leading to production of inflammatory cytokines such as TGF- $\beta$ , leukocyte infiltration, fibroblast proliferation, and transformation of fibroblasts to myofibroblasts (Bujak and Frangogiannis et al., 2007). All of these changes contribute to deposition of extracellular matrix proteins, in particular collagens, making up scar tissues. Scar formation itself is an essential step for wound healing to prevent the enlargement of injured area. However, the persistent activation of tissue repair program caused by the elevated level of profibrotic cytokines stiffens ventricles, which impairs myocardial contractile and diastolic functions, ultimately resulting in heart failure (Leask et al., 2010).

Chronic heart failure is a complex clinical syndrome that arises from abnormalities of cardiac structure, function, or both that impair the ability of the heart to fill or to eject blood. It is one of the main public health problems that cause a poor life quality and place an economic burden on the healthcare system worldwide. In the United States alone, heart failure affects 5

million adults and accounts for 400,000 ~ 700,000 deaths per year and \$20 ~ 40 billion in yearly healthcare costs (Brown et al., 2005). Moreover, it is highly lethal with a 5 year mortality in treated patients that is worse than that of many other malignancies despite recent advances in treatment strategies (Hunt et al., 2005). Regardless of its pathogenesis, heart failure progression is mainly caused by left ventricle remodeling. The pathologic changes in heart failure occur in 2 forms: one is cardiomyocyte hypertrophy, necrosis, and apoptosis, and the other is cardiac fibroblast hyperplasia and cardiac fibrosis (Wei et al., 2011). The prominent feature of the remodeling heart is cardiomyocyte hypertrophy. Chronic exposure to myocardial stress causes cardiac muscle to hypertrophy with the myocardium thickening. Since the cardiomyocytes have little or no capacity to proliferate, their only means of growth is by cellular enlargement. Therefore, increase in cardiomyocyte size is a dominant cellular response to virtually all forms of myocardial injury in an effort to protect the integrity of the heart wall (Diwan and Dorn et al., 2007). Cardiac hypertrophy has traditionally been considered as a cellular adaptive response to sustain cardiac output in the face of pathologic stimulus, which offsets increased load, attenuates the progressive dilatation, and stabilizes the contractile function (Sutton et al., 2000). However, prolonged hypertrophy leads to the irreversible outcome of cardiomyocyte degeneration and death to exacerbate the insufficiency of myocardium in the long run (Frey and Olson et al., 2003). In an injured organ, debris is either replaced by cells of the same type or by parenchymal tissue during the wound healing process. In adult mammals, necrotic cardiomyocytes are replaced by fibrous extracellular matrix proteins produced by myofibroblasts, forming an extensive microvascular network that contains cross-linked collagen bundles for wound closure (Bujak and Frangogiannis et al., 2007). However, this scar tissue cannot replace the function of cardiomyocytes, leading to the progression of heart failure.

## **Cardiac Fibroblasts**

The normal heart consists of four major cell types: cardiac fibroblasts, myocytes, endothelial cells, and vascular smooth muscle cells, with fibroblasts making up the largest portion of the cellular mass ranging from 40% to over 60% of the total cell population depending on the species (Souders et al., 2009).

Cardiac fibroblasts are recognized as the cell type primarily responsible for homeostatic maintenance of extracellular matrix (ECM) in the normal heart. They can synthesize and release ECM proteins in response to a variety of hormones and growth factors in the microenvironment and modulate the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Since the ECM provides an important environment for cells to grow, migrate, and differentiate, homeostasis of ECM is tightly regulated to ensure proper cardiac structure and function. The major constituents of cardiac ECM are the collagens I (~80%) and III (~10%), with the ECM being a reservoir of growth factors, proteases, and other molecules, many of which are sequestered in inactive forms (Maquart et al., 2004). The ECM is subject to constant turnover amounting up to 5% per day (Rohr et al., 2011). The dynamic nature of ECM is obtained by the balance between synthesis and degradation of ECM component, which are regulated by enzymes named MMPs and TIMPs. Since MMPs, a family of zinc-dependent proteases, are capable of degrading all the matrix components and their activities are inhibited by TIMPs, the interplay of MMPs, TIMPs, and their regulators determines progression of fibrosis in the heart. During the myocardial matrix remodeling, synthesis increases and/or degradation decreases to yield a net increase in ECM, a process termed fibrosis. This change occurs as a wound healing response to chronic cardiac injury caused by myocardial infarction, pressure overload (hypertension and aortic stenosis), inflammatory response (myocarditis and

dilated cardiomyopathy), or volume overload (valvular regurgitation and congenital heart diseases), which may result in cardiac remodeling and heart failure (Wei et al., 2011).

During the tissue repair, fibroblasts are activated to transform into the specialized form, myofibroblasts, which are not part of the normal cardiac tissue and appear only after the cardiac injury. Transformation to myofibroblast phenotype is strongly promoted by several cytokines and growth factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ). Myofibroblasts have morphological and biochemical features intermediate between those of fibroblasts and smooth muscle cells, expressing the contractile protein  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Smooth muscle  $\alpha$ -actin-containing stress fibers contribute to the force generation and retraction required for wound healing. Myofibroblasts markedly increase the rate of collagen synthesis as compared with the unstimulated fibroblasts in order to consolidate the damaged or infarcted tissues (Petrov, Fagard, and Lijnen et al., 2002). Myofibroblasts disappear by apoptosis in a normal tissue repair, whereas the persistence of myofibroblasts results in the overproduction of ECM proteins, leading to the fibroproliferative disorders (Moulin et al., 2004).

### **Transforming Growth Factor- $\beta$ 1 (TGF- $\beta$ 1)**

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a member of a large family of structurally related cell regulatory proteins including TGF- $\beta$ , bone morphogenetic proteins (BMPs), activin/inhibin, and glial cell line-derived neurotrophic factor (GDNF). Three isoforms of TGF- $\beta$  have been identified in mammalian species, named TGF- $\beta$ 1,  $\beta$ 2, and  $\beta$ 3 (Schiller et al., 2004). Although the three isoforms of TGF- $\beta$  are encoded by three distinct genes located on different chromosomes under control of a unique promoter, they have approximately 80% homology at the level of amino acid sequence and signal through the same cell surface receptors having

similar cellular targets (Inagaki and Okazaki et al., 2007). Since TGF- $\beta$ 1 is the most prevalent and is found almost ubiquitously, it has been most extensively studied for its roles in various pathological conditions. TGF- $\beta$ 1, normally just referred as TGF- $\beta$ , was first identified in the culture medium conditioned by transformed cells and was named for its ability to induce anchorage-independent growth of rodent fibroblasts (Roberts et al., 1980).

TGF- $\beta$  is one of the most pleiotropic and multifunctional peptides eliciting diverse and often contradictory cellular responses from many different cell types (Bujak et al., 2007). TGF- $\beta$  plays an important role in a wide variety of biological processes such as embryonic development, cell growth and differentiation, cell proliferation and apoptosis, migration, adhesion, regulation of the immune response and tissue repair.

TGF- $\beta$  is a homodimeric polypeptide synthesized and secreted as a precursor that contains latency-associated proteins (LAP). This precursor is bound by separately secreted latent TGF- $\beta$  binding proteins (LTBPs), which act as a safeguard against inadvertent activation of TGF- $\beta$ . In addition, TGF- $\beta$  is associated with several ECM components, which serve as a reservoir from which activated TGF- $\beta$  can be readily released without de novo synthesis. TGF- $\beta$  can be activated by proteolytic cleavage, which releases the caged TGF- $\beta$  protein allowing them to reach the receptor.

TGF- $\beta$  exerts its effect by binding to the heteromeric complexes of two different kinds of serine/threonine kinase receptors. After an active ligand binds to a constitutively active type II receptor, type II receptor recruits and phosphorylates type I receptor, which subsequently phosphorylates the receptor-activated Smads (R-Smads), Smad2 and 3. R-smads are direct substrates of the TGF- $\beta$  receptor kinase and interact with the common partner Smad4 (Co-Smad). Smad4-containing Smad complexes then translocate into the nucleus where they act as a

transcription factor and participate in the regulation of the target gene expression (Xiao and Zhang et al., 2008). Smads 2-4 share highly conserved DNA-binding MH1 and transactivation MH2 domains, which facilitate Smad interactions with other proteins (Shi et al., 1998). TGF- $\beta$  signaling has an autoregulatory negative feedback function mediated by anti-smad-like Smad7. In contrast to receptor-mediated Smads, Smad7 stably binds to TGF- $\beta$  receptors and interferes with ligand-induced phosphorylation of Smad2 and 3 (Hayashi et al., 1997). Besides Smad-mediated transcription, TGF- $\beta$  can activate other signaling cascades including extracellular signal-regulated kinase (Erk), c-Jun-N-terminal kinase (JNK), TGF- $\beta$ -activated kinase1 (TAK1), and p38 mitogen-activated protein kinase (MAPK) pathways, allowing versatile and diverse responses of TGF- $\beta$  (Derynck and Zhang et al., 2003). Although the Smad-dependent pathway alone is inherently simple, combinatorial interactions in heteromeric receptors, receptor-related proteins, Smad-interacting proteins, cooperation with sequence-specific transcription factors, and cross-talk with other signaling pathways make TGF- $\beta$  signaling intricate, conferring the amazing diversity of its effects eliciting multiple and often opposing cellular responses.

Many factors have been implicated in the initiation and progression of cardiac fibrosis, including inflammatory cytokines, angiotensin II, endothelin, growth factors and oxidative stress. Among these factors, TGF- $\beta$  has been recognized as an important regulator in cardiac fibroblast activation and fibrous tissue deposition (Sun and Weber et al., 2000). Through numerous studies conducted over the past three decades, TGF- $\beta$  has become a well-established profibrotic cytokine that stimulates the production of extracellular matrix in a number of different organs such as kidney, liver, lung, and heart (Leask et al., 2007).

Cardiac injury such as myocardial infarction triggers an inflammatory response, resulting in free radical formation, initiation of complement cascade, and activation of Nuclear Factor



(NF)- $\kappa$ B and Toll-Like Receptor (TLK)-mediated signaling pathways (Bujak and Frangogiannis et al., 2007). These changes induce cytokine and chemokine synthesis and upregulate the expression of adhesion molecules in the endothelial cells and leukocytes, leading to infiltration in the injured area. Neutrophils and macrophages clear the wound from the dead cells and matrix debris. TGF- $\beta$  plays a role in recruiting monocyte to promote granulation tissue formation and suppressing pro-inflammatory cytokines and chemokines for the resolution of the inflammatory infiltrate. Although the mechanisms responsible for resolution of inflammation are not fully understood yet, the timely repression of synthesis of inflammatory mediators is important for the transition to the fibrous tissue deposition. After the inflammatory mediators are suppressed, fibroblasts predominate in the wound and transform into the myofibroblasts, secreting ECM proteins and forming an extensive microvascular network. TGF- $\beta$  is critically involved in the regulation of fibrous tissue deposition by facilitating acquisition of the myofibroblast phenotype, inducing ECM protein synthesis, and promoting matrix preservation through increased expression of TIMPs and decreased expression of MMPs.

Due to those profibrotic actions of TGF- $\beta$ , treatment regimens aimed at TGF- $\beta$  inhibition such as neutralizing TGF- $\beta$  antibodies and receptor antagonists have been extensively investigated. However, the use of TGF- $\beta$  antagonists to alleviate myocardial fibrosis does not come without risks because of the broad targeting of TGF- $\beta$ . *In vivo* studies have shown that TGF- $\beta$ 1 deficient mice have markedly reduced collagen deposition compared to control mice although those mice suffer from a severe wasting syndrome accompanied by a pronounced inflammatory response and tissue necrosis, resulting in organ failure and death (Kulkarni et al., 1993). Inhibition of TGF- $\beta$  axis is likely to have adverse side-effects since TGF- $\beta$  is able to both stimulate and suppress cell proliferation depending on cell types. Mutational inactivation of the

TGF- $\beta$  and Smad pathway causes uncontrolled cellular growth, particularly in endothelial cell lines (Kim et al., 2000). Additionally, loss of TGF- $\beta$  receptor function has been associated with progression of malignancy and increased tumor growth (Yin et al., 1999). These studies indicate that specific and selective intervention in downstream of TGF- $\beta$  signaling is required for developing antifibrotic therapies. Moreover, appropriate timing of the intervention should be examined because TGF- $\beta$  plays an important role in both inflammatory and fibrotic phases of left ventricle remodeling. Anti-TGF- $\beta$  gene therapy during inflammatory phase enhanced cytokine and chemokine synthesis and increased infiltration, causing exacerbated ventricular dysfunction and increased mortality (Ikeuchi et al., 2004). In contrast, late TGF- $\beta$  inhibition attenuated cardiac hypertrophy and decreased fibrosis.

### **Response Gene to Complement-32 (RGC-32)**

Response gene to complement-32 (RGC-32) was first discovered by differential display during experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS) research (Badea et al., 1998). Myelin and oligodendrocytes were known to be a target of immune-mediated attack in EAE and MS and deposition of C5b-9 was found to be the common characteristic in affected brains of EAE and MS. It was demonstrated that sublytic complement activation on oligodendrocytes downregulates expression of myelin genes and induces cell cycle in culture. During a search for new genes whose expression is altered in response to complement and that may be involved in cell cycle activation, Badea et al. identified 32 new genes, which were designated as RGC-1 to 32 according to the order of identification.

Several studies have been conducted to investigate the genetic and molecular characteristics and the role of RGC-32 in a wide range of cell lines and tissues. The human

RGC-32 gene is found to be located on chromosome 13 and encode a 137 amino acid protein with 92% similarity to both the rat and mouse proteins (Badea et al., 2002). RGC32 mRNA is abundantly expressed in the placenta, skeletal muscle, kidney, pancreas, liver, and aortic endothelial cells while weakly expressed in the heart and brain. RGC-32 expression can be induced by complement activation, particularly C5b-9, steroid hormones, growth factors, and serum (Vlaicu, 2008).

Since RGC-32 was one of those 32 genes initially selected for their capability to induce the cell cycle, its mechanism regulating the cell cycle was first studied. RGC-32 was shown to work as a cell cycle regulator to activate p34<sup>CDC2</sup> kinases and to induce S-phase entry and mitosis (Badea et al., 2002). RGC-32 was translocated to nucleus by complement activation and physically associated with cyclin-dependent kinase p34<sup>CDC2</sup> to increase its kinase activity in aortic smooth muscle cells. Moreover, overexpression of RGC32 can increase DNA synthesis in response to serum growth factor in the OLG-C6 glioma cell lines, suggesting the role of RGC-32 in cell proliferation (Badea et al., 1998).

The involvement of RGC-32 in cell differentiation was found in an attempt to identify the downstream targets of TGF- $\beta$ -induced smooth muscle cell differentiation (Li et al., 2007). Microarray analysis of TGF- $\beta$ -treated neural crest cells showed that RGC-32 expression was increased about 50-fold after 24h treatment with TGF- $\beta$ . RGC-32 knockdown with siRNA inhibited the smooth muscle cell marker genes,  $\alpha$ -SMA, SM22 $\alpha$ , and calponin while overexpression enhanced the promoter activities of  $\alpha$ -SMA, SM22 $\alpha$ , and SM-MHC, indicating that RGC-32 is involved in smooth muscle cell differentiation. In addition, RGC32 mediated the TGF- $\beta$ -induced epithelial-mesenchymal transition (EMT), the conversion from an epithelial to a mesenchymal phenotype, that has been considered as an important source of myofibroblasts in

renal fibrosis, acting downstream of Smad in human proximal tubular cells (Huang et al., 2009).

Alteration in RGC-32 expression has been observed in a wide range of human cancers. RGC-32 expression was upregulated in colon, ovarian, breast, and prostate cancers (Vlaicu, 2008). RGC-32 expression was higher in advanced stages of colon cancer than in precancerous states or initial stages of colon cancer and RGC-32 knockdown induced an increase in acetylation of histones, suggesting RGC32 contributes to the development of colon cancer by regulating the chromatin assembly (Vlaicu et al., 2010). In contrast, RGC-32 mRNA expression was decreased in primary astrocytomas, especially tumors with mutations of p53 (Saigusa, 2007). Overexpression of RGC-32 suppressed the growth of glioma cells, indicating the possible function of RGC-32 as a tumor suppressor for glioma.

### **Type I Collagen**

Collagens are a family of closely related but distinct extracellular matrix proteins, influencing cell behavior and maintaining tissue structure. At least 19 proteins representing more than 30 gene products have been defined as collagens (Lamberg et al., 1996). All collagen molecules consist of three polypeptide chains, called  $\alpha$ -chains, which are coiled around one another to form a triple-helical conformation. In some collagen types, all three alpha chains are identical, while in others the collagen molecule contains two or three different alpha chains.

Type I collagen is the major fibrillar component of ECM produced by fibroblasts in the adult heart (Namba et al., 1997). It is a heterotrimer composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain encoded by COL1A1 and COL1A2 genes respectively. Fibrillar type I collagen is synthesized and secreted as a precursor polypeptide by the fibroblasts and proteolytically processed by removal of N- and C-terminal propeptides through the action of specific proteases.

Collagen monomers are then cross-linked to produce the mature structure, which aggregates to form larger collagen fibrils and helps to form the ECM with other components (Brown et al., 2005). Expression of collagen genes is highly regulated at the transcriptional and post-transcriptional levels.

Excessive synthesis and deposition of collagen in the myocardial interstitium is a hallmark of cardiac fibrosis reducing ventricular compliance due to the inherent stiffness of collagen type I. Because of the central role of type I collagen in the wound-healing process, the molecular regulation of type I collagen gene expression has been of significant importance as a possible way of abrogating irreversible fibrosis and excessive scarring. Numerous efforts have been made to identify factors and signal transduction pathways at the transcriptional level since the excessive accumulation of type I collagen is largely due to an increase in the rate of transcription of the corresponding genes (Verrecchia and Mauviel et al., 2004). Many studies have reported that the expression of type I collagen is increased by abnormal activities of different transcription factors in response to abnormally induced signaling pathways.

TGF- $\beta$  is one of the most potent factors in activating type I collagen gene transcription. The recruitment of Smad, a downstream effector of TGF- $\beta$  signaling, to DNA via MH1 domain is a critical step to activate transcription. Crystal structure of Smad MH1 domain bound to an optimal DNA sequence reveals CAGA nucleotide sequence recognized by Smad (Shi et al, 1998). The COL1A2 promoter contains Smad3/4-binding consensus “CAGA boxes” at TGF- $\beta$  responsive element that are necessary and sufficient to mediate transcriptional responses induced by TGF- $\beta$  (Chen et al., 2000). However, as CAGA boxes are widely distributed in the promoters of mammalian genes such as PAI-1, JunB, and other TGF- $\beta$  inducible genes, and the affinity and specificity of Smad binding to CAGA boxes is relatively low, other transcriptional factors are

required to contribute to the specific and tight Smad-DNA interactions (Hua et al., 1999). It has been shown that interaction of activated Smad2/3 with Sp1, along with p300/CBP and other transcriptional coactivators is required for the maximal stimulation of collagen synthesis (Zhang et al., 2000).

The enhanced activity of Specificity protein 1 (Sp1), a ubiquitous zinc-finger family transcription factor, was observed in numerous diseases characterized by tissue fibrosis. TGF- $\beta$  stimulates human COL1A2 expression by inducing binding of a Sp1-containing complex to TGF- $\beta$  responsive element (TbRE) that contains CAGA box (Zhang et al., 2000). It was shown that there is a cooperative synergy between Smad3/Smad4 and Sp1 at the TbRE site and Smad – and Sp1-binding sites are required for the optimal promoter activation.

p300 and CBP are structurally closely related nuclear proteins originally isolated by their ability to bind to adenoviral E1A oncoprotein and cAMP response element binding protein (CREB), respectively (Eckner et al., 1994; Chrivia et al., 1993). p300 and CBP have largely overlapping functions as shown by the similarities in the phenotypes in p300<sup>-/-</sup> and CBP<sup>-/-</sup> knockout mice (Yao et al., 1998). p300/CBP do not directly bind to specific DNA sequences but function as transcriptional coactivators or adaptor proteins that are recruited to target promoters by sequence-specific DNA-binding proteins. p300/CBP have intrinsic histone acetyltransferase (HAT) activity (Ogryzko et al., 1996). Acetylation of histones alters nucleosomal conformation to decrease the histone affinity for DNA, allowing the transcriptional machinery and factors to access their recognition sites and facilitating the transcription.

It has been reported that ectopic expression of p300 in lung and skin fibroblasts enhanced the Smad-mediated stimulation of collagen gene expression (Ghosh et al., 2000), whereas selective depletion of p300 by p300-specific ribozymes abrogated the TGF- $\beta$ -induced collagen

synthesis, suggesting that abundance of p300 governs the intensity of profibrotic responses elicited by TGF- $\beta$  (Bhattacharyya et al., 2005). Interaction between Smad2/3 with p300 was shown to be essential for Smad-dependent collagen synthesis and acetyltransferase activity of p300 is required for maximum stimulation as highlighted by the fact that fibroblasts transfected with HAT-deleted mutants of p300 revealed the reduced TGF- $\beta$  stimulation of COL1A2 promoter activity (Ghosh et al, 2000).

## References

- Badea, T. C., Niculescu, F. I., Soane, L., Shin, M. L., Rus, H. (1998) Molecular cloning and characterization of RGC-32, a novel gene induced by complement activation in oligodendrocytes. *J. Biol. Chem.* **273**, 26977-26981.
- Badea, T., Niculescu, F., Soane, L., Fosbrink, M., Sorana, H., Rus, V., Shin, M. L., Rus, H. (2002) RGC-32 increases p34<sup>CDC2</sup> kinase activity and entry of aortic smooth muscle cells into S-phase. *J. Biol. Chem.* **277**, 502-508.
- Bhattacharyya, S., Ghosh, A. K., Pannu, J., Mori, Y., Takagawa, S., Chen, G., Trojanowska, M., Gilliam, A. C., Varga, J. (2005) Fibroblast expression of the coactivator p300 governs the intensity of profibrotic response to transforming growth factor- $\beta$ . *Arthritis Rheum.* **52**, 1248-1258.
- Bosman, F. T., Stamenkovic, I. (2003) Functional structure and composition of extracellular matrix. *J. Pathol.* **200**, 423-428.
- Brown, R. D., Ambler, K., Mitchell, M. D., Long, C. S. (2005) The cardiac fibroblasts: Therapeutic target in myocardial remodeling and failure. *Annu. Rev. Pharmacol. Toxicol.* **45**, 657-687.
- Bujak, M., Frangogiannis, N. G. (2007) The role of TGF- $\beta$  signaling in myocardial infarction and cardiac remodeling. *Cardiovas. Res.* **74**, 184-195.
- Chan, E. C., Disting, G. J., Guo, N., Peshavariy, H. M., Taylor, C. J., Dilley, R., Narumiya, S., Jiang, F. (2010) Prostacyclin receptor suppresses cardiac fibrosis: Role of CREB phosphorylation. *J. Mol. Cell. Cardiol.* **49**, 176-185.



- Chen, S. J., Yuan, W., Lo, S., Trojanowska, M., Varga, J. (2000) Interaction of Smad3 with a proximal Smad-binding element of the human  $\alpha 2(1)$  procollagen gene promoter required for transcriptional activation by TGF- $\beta$ . *J. Cell. Physiol.* **183**, 381-392.
- Chrivia, J. C., Kwok, R., P., Lamb, N., Hagiwara, M., Montminy, M. R., Goodman, R. H. (1993) Phosphorylated CREB binds to specifically to the nuclear protein CBP. *Nature* **365**, 855-859.
- Creemers, E. E., Pinto, Y. M. (2011) Molecular mechanisms that control interstitial fibrosis in the pressure-overloaded heart. *Cardiovas. Res.* **89**, 265-272.
- Cutroneo, K. R. (2003) How is type I procollagen synthesis regulated at the gene level during tissue fibrosis. *J. Cell. Biochem.* **90**, 1-5.
- Cutroneo, K. R. (2007) TGF- $\beta$ -induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring. *Wound Rep. Reg.* **15**, S54-S60.
- Derynck, R., Zhang, Y. E. (2003) Smad-dependent and Smad-independent pathways in TGF- $\beta$  family signaling. *Nature* **425**, 577-584.
- Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., Livingston, D. M. (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes & Dev.* **8**, 869-884.
- Ghosh, A. K., Yuan, W., Mori, Y., Varga, J. (2000) Smad-dependent stimulation of type I collagen gene expression in skin fibroblasts by TGF- $\beta$  involves functional cooperation with p300/CBP transcriptional coactivators. *Oncogene* **19**, 3546-3555.

- Ghosh, A. K., Yuan, W., Mori, Y., Chen, S. J., Varga, J. (2001) Antagonistic regulation of type I collagen gene expression by interferon- $\gamma$  and transforming growth factor- $\beta$ : Integration at the level of p300/CBP transcriptional coactivators. *J. Biol. Chem.* **276**, 11041-11048.
- Ghosh, A. K. (2002) Factors involved in the regulation of type I collagen gene expression: Implication in fibrosis. *Exp. Biol. Med.* **227**, 301-314.
- Gouman, M. J., van Zonneveld A. J., ten Dijke P. (2008) Transforming growth factor beta-induced endothelial-to-mesenchymal transition: a switch to cardiac fibrosis? *Trends. Cardiovas. Med.* **18**, 293-298.
- Gusterson, R. J., Jazrawi, E., Adcock, I. M., Latchman, D. S. (2003) The transcriptional co-activators CREB-binding protein (CBP) and p300 plays a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. *J. Biol. Chem.* **278**, 6838-6847.
- Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Wrana, J. L., Falb, D. (1997) The MAD-related protein Smad7 associates with the TGF- $\beta$  receptor and functions as an antagonist of TGF- $\beta$  signaling. *Cell* **89**, 1165-1173.
- Hua, X., Miller, Z. A., Wu, G., Shi, Y., Lodish, H. F. (1999) Specificity in transforming growth factor- $\beta$ -induced transcription of the plasminogen activator inhibitor-1 gene: Interactions of promoter DNA, transcription factor  $\mu$ E3, and Smad proteins. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13130-13135.
- Huang, W., Li, Z., Rus, H., Wang, X., Jose, P. A., Chen, S. (2009) RGC-32 mediates transforming growth factor- $\beta$ -induced epithelial-mesenchymal transition in human renal proximal tubular cells. *J. Biol. Chem.* **284**, 9426-9432.

- Huang, W. Y., Xie, W., Guo, X., Li, F., Jose, P. A., Chen, S. Y. (2011) Smad2 and PEA3 cooperatively regulate transcription of response gene to complement 32 in TGF- $\beta$ -induced smooth muscle cell differentiation of neural crest cells. *Am. J. Physiol. Cell Physiol.* **301**, C499-506.
- Ikeuchi, M., Tsutsui, H., Shiomi, T., Matsusaka, H., Matsushima, S., Wen, J. (2004) Inhibition of TGF-beta signaling exacerbates early cardiac dysfunction but prevents late remodeling after infarction. *Cardiovasc. Res.* **64**, 526-535.
- Inagaki, Y., Okazaki, I. (2007) Emerging insights into transforming growth factor  $\beta$  smad signal in hepatic fibrogenesis. *Gut* **56**, 284-292.
- Kania, G., Blyszczuk, P., Eriksson, U. (2009) Mechanisms of cardiac fibrosis in inflammatory heart disease. *Trends. Cardiovas. Med.* **19**, 247-252.
- Khan, R., Sheppard, R. (2006) Fibrosis in heart disease: understanding the role of transforming growth factor  $\beta_1$  in cardiomyopathy, valvular disease and arrhythmia. *Immunology* **118**, 10-24.
- Kim, S. J., Im, Y. H., Markowitz, S. D., Bang, Y. J. (2000) Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev.* **11**, 159-168.
- Krenning, J., Zeisburg, E. M., Kalluri, R. (2010) The origin of fibroblasts and mechanism of cardiac fibrosis. *J. Cell. Physiol.* **225**, 631-637.
- Kulkarni, A. B., Karlsson, S. (1993) Transforming growth factor beta-1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am. J. Pathol.* **143**, 3-9.

- Lamberg, A., Helaakoski, T., Myllyharju, J., Peltonen, S., Notbohm, H., Pihlajaniemi, T., Kivirikko, K. I. (1996) Characterization of human type III collagen expressed in a Baculovirus system. *J. Biol. Chem.* **271**, 11988-11995.
- Leask, A. (2007) TGF $\beta$ , cardiac fibroblasts, and the fibrotic response. *Cardiovas. Res.* **74**, 207-212.
- Leask, A. (2010) Potential therapeutic targets for cardiac fibrosis: TGF $\beta$ , Angiotensin, Endothelin, CCN2, and PDGF, Partners in fibroblasts activation. *Circ. Res.* **106**, 1675-1680.
- Li, Y. Y., McTiernan, C. F., Feldman, A. M. (2000) Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. *Cardiovasc. Res.* **46**, 214-224.
- Li, F., Luo, Z., Huang, W., Lu, Q., Wilcox, C. S., Jose, P. A., Chen, S. (2007) Response gene to complement 32, a novel regulator for transforming growth factor-beta-induced smooth muscle cell differentiation of neural crest cells. *J. Biol. Chem.* **282**, 10133-10137.
- Lim, H., Zhu, Y. Z. (2006) Role of transforming growth factor- $\beta$  in the progression of heart failure. *Cell. Mol. Life Sci.* **63**, 2584-2596.
- Maquart, F. X., Pasco, S., Ramont, L., Horneback, W., Monboisse, J. C. (2004) An introduction of matrikines: extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. *Crit. Rev. Oncol. Hematol.* **49**, 199-202.
- Namba, T., Tsutsui, H., Tagawa, H., Takahashi, M., Saito, K., Kozai, T., Usui, M., Imanaka-Yoshida, K., Imaizumi, T., Takeshita, A. (1997) Regulation of fibrillar collagen expression and protein accumulation in volume-overloaded cardiac hypertrophy. *Circulation.* **95**, 2448-2454.

- Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953-959.
- Poncelet, A. C., Schnaper, J. W. (2001) Sp1 and Smad proteins cooperate to mediate transforming growth factor- $\beta$ 1-induced  $\alpha$ 2(I) collagen expression in human glomerular mesangial cells. *J. Biol. Chem.* **276**, 6983-6992.
- Roberts, A. B., Lamb, L. C., Newton, D. L. (1980) Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. *Proc. Natl. Acad. Sci. USA.* **77**, 3493-3498.
- Rohr, S. (2011) Cardiac fibroblasts in cell culture system: Myofibroblasts all along? *J. Cardiovasc. Pharmacol.* **57**, 389-399.
- Ruiz-Ortega, M., Rodriguez-Vita, J., Sanchez-Lopez, E., Carvajal, G., Edigo, J. (2007) TGF- $\beta$  signaling in vascular fibrosis. *Cardiovas. Res.* **74**, 196-206.
- Saigusa, K., Imoto, I., Tanikawa, C., Aoyagi, M., Ohno, K., Nakamura, Y., Inazawa, J. (2007) RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest. *Oncogene* **26**, 1110-1121.
- Shi, Y., Wang Y. F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N. P. (1998) Crystal structure of a Smad MH1 domain bound to DNA: insight on DNA-binding in TGF- $\beta$  signaling. *Cell* **94**, 585-594.
- Souders, C. A., Bowers, S. L. K., Baudino, T. A. (2009) Cardiac fibroblast: The renaissance cell. *Circ. Res.* **105**, 1164-1176.

- Spinale, F. G., Coker, M. L., Bond, B. R., Zellner, J. L. (2000) Myocardial matrix degradation and metalloproteinase activation in the failing heart: a potential therapeutic target. *Cardiovas. Res.* **46**, 225-238.
- Schiller, M., Javelaud, D., Mauviel, A. (2004) TGF-beta-induced SMAD signaling and gene regulation: consequences of extracellular matrix remodeling and wound healing. *J. Dermatol. Sci.* **35**, 83-92.
- Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N. P. (1998) Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding on TGF-beta signaling. *Cell* **94**, 585-594.
- Strauss, B. H., Rabinovitch, M. (2000) Adventitial fibroblasts: defining a role in vessel wall remodeling. *Am. J. Respir. Cell Mol. Biol.* **22**, 1-3.
- Sutton, M. J., Sharpe, N. (2000) Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation* **101**, 2981-2988.
- Swaney, J. S., Roth, D. M., Olson, E. R., Naugle, J. E., Meszaros, J. G., Insel, P. A. (2005) Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *PNAS* **102**, 437-442.
- Verrecchia, F., Mauviel, A. (2004) TGF- $\beta$  and TNF- $\alpha$ : antagonistic cytokines controlling type I collagen gene expression. *Cell. Signal.* **16**, 873-880.
- Vlaicu, S. I., Cudrici, C., Ito, T., Fosbrink, M., Tegla, C. A., Rus, V., Mircea, P. A., Rus, H. (2008) Role of response gene to complement 32 in diseases. *Arch. Immunol. Ther. Exp.* **56**, 115-122.

- Vlaicu, S. I., Tegla, C. A., Cudrici, C. D., Fosbrink, M., Nguyen, V., Azimzadeh, P., Rus, V., Chen, H., Mircea, P. A., Shamsuddin, A., Rus, H. (2010) Epigenetic modifications induced by RGC32 in colon cancer. *Exp. Mol. Pathol.* **88**, 67-76.
- Wang, J. N., Shi, N., Xie, W. B., Guo, X., Chen, S. Y. (2011) Response gene to complement 32 promotes vascular lesion formation through stimulation of smooth muscle cell proliferation and migration. *Arterioscler. Thromb. Vasc. Biol.* **31**, e19-e26.
- Wei, L. (2011) Immunological aspect of cardiac remodeling: T lymphocyte subsets in inflammation-mediated cardiac fibrosis. *Exp. Mol. Pathol.* **90**, 74-78.
- Yao, T. P., Oh, S. P., Fuchs, M., Zhou, N. D., Ch'ng, L. E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., Eckner, R. (1998) Gene dose-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell* **93**, 361-372.
- Yin, J. J., Selander, K., Chirgwin, J. M. (1999) TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J. Clin. Invest.* **103**, 197-206.
- Zhang, W., Qu, J., Inagaki, Y., Greenwel, P., Ramirez, F. (2000) Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor  $\beta$ 1 stimulation of  $\alpha$ 2 (I)-collagen (COL1A2) transcription. *J. Biol. Chem.* **275**, 39237-39245.

## **CHAPTER 3**

### **Role of Response Gene to Complement 32 (RGC32) in the Transformation of Cardiac Fibroblasts to Myofibroblasts**

#### **ABSTRACT**

Injury in heart due to various causes can end up with a common end-stage of pathological remodeling and fibrosis, eventually leading to the heart failure. One of the well-known cytokines involved in this process is transforming growth factor- $\beta$  (TGF- $\beta$ ). TGF- $\beta$  is a potent stimulator of the transformation of cardiac fibroblasts into smooth muscle-like myofibroblasts, which migrate to the damaged area and secrete extracellular matrix (ECM) proteins such as collagen I and fibronectin. The aim of the present study was to investigate whether response gene to complement 32 (RGC32) is involved in the transformation of cardiac fibroblast to myofibroblast. We examined the effects of RGC32 depletion or overexpression on myofibroblast formation as assessed by  $\alpha$ -SMA expression, and ECM protein production, using primary cultures of mouse cardiac fibroblasts (mCFs). Data obtained by real-time PCR, western blotting, and immunofluorescent staining suggest that RGC32 is a TGF- $\beta$  downstream mediator in mCFs. RGC32-knockout attenuated the expression of collagen I, fibronectin, and  $\alpha$ -SMA in response to TGF- $\beta$  stimulation, indicating that RGC32 is involved in the TGF- $\beta$ -induced transformation to myofibroblast. On the other hand, RGC32 overexpression enhanced the



expression of  $\alpha$ -SMA, collagen I, and fibronectin, mimicking the effect of TGF- $\beta$ . These findings implicate RGC32 as a novel fibrogenic factor in the cardiac fibrosis.

*Keywords:* Cardiac fibroblasts, Transforming Growth Factor- $\beta$ , Response Gene to Complement 32, Collagen synthesis

## INTRODUCTION

Cardiac fibrosis is defined as a progressive accumulation of fibrillar extracellular matrix (ECM) in the myocardium, leading to an abnormal thickening of heart walls. The regulation of ECM remodeling is primarily mediated by cardiac fibroblasts (CFs), which are the main non-muscle cells in the heart. CFs modulate matrix turnover in non-pathological conditions and their activity is greatly enhanced after an acute cardiac injury or during chronic cardiovascular disease (Olson et al., 2008). During the wound healing response to cardiac injury, fibroblasts are activated to transform into the specialized form, myofibroblasts, which are not part of the normal cardiac tissue and appear only after the cardiac injury (Baum et al, 2011). Smooth muscle  $\alpha$ -actin-expressing myofibroblasts significantly elevate the production of fibrillar collagen and uncontrolled cross-linking of collagen molecules, reducing the left ventricle compliance due to the intrinsic stiffness of collagens. The altered number and function of myofibroblasts have been also implicated in diseases with increased ECM deposition and resultant fibrosis in various organs such as liver, skin, lung, and kidney.

Several cytokines or growth factors seem to function in a coordinated manner to produce a fibrotic microenvironment, leading to the myofibroblast formation (Ong et al., 2009). Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a potent factor that stimulates both fibroblast-to-myofibroblast transformation and collagen production. Therefore, it is of great importance to identify a relay molecule in the TGF- $\beta$  signaling pathway from the cell membrane to the nucleus to seek as a possible way of abrogating irreversible fibrosis and excessive scarring.

Response gene to complement 32 (RGC32) is one of the downstream targets of TGF- $\beta$  (Li et al., 2007). Since the initial report describing RGC32 as a cell cycle regulator involved in

cell proliferation, several studies from our group have reported the functions of RGC32 in relation to smooth muscle differentiation, vascular lesion formation, and renal fibrosis (Badea et al., 2002; Huang et al, 2011; Wang et al., 2011; Li et al., 2011). Evidence suggests that TGF- $\beta$  stimulates RGC32 expression in many different cell types.

We hypothesized that RGC32 promotes the transformation of cardiac fibroblast to myofibroblast, which increases the collagen synthesis in the adult mouse cardiac fibroblasts (mCFs). We used adenoviral RGC32 gene transfer to show that exogenous RGC32 overexpression mimics the effects of TGF- $\beta$  by promoting myofibroblast formation and collagen production in the absence of TGF- $\beta$  treatment. We also confirmed that loss of RGC32 gene markedly inhibited the fibroblast-to-myofibroblast transformation and collagen synthesis in response to TGF- $\beta$  by using the cardiac fibroblasts isolated from RGC32 knockout mice. These results suggest a key profibrotic action of RGC32 serving as a TGF- $\beta$  downstream mediator in adult mCFs.

## **EXPERIMENTAL PROCEDURES**

### **Isolation and culture of mouse cardiac fibroblasts**

Cardiac fibroblasts (CFs) were isolated from hearts of either wild-type or RGC32-knockout C57BL/6 mice. Freshly excised mouse ventricles were washed in PBS to remove red blood cells and blood vessels, transferred to 3 ml serum free Dulbecco's Modified Eagle Medium (DMEM) containing 1 mg/ml of type I collagenase and 2 mg/ml of trypsin, and minced into small pieces. This solution with small pieces of heart tissue was incubated at 37°C in a water bath, and the supernatant was transferred to DMEM containing 20% fetal bovine serum (FBS) and 2 mM L-glutamine every 15 min. CFs collected from 3 digestive cycles were pelleted,

resuspended in fresh DMEM supplemented with 20% FBS, 2 mM L-glutamine, 2000 I.U./ml penicillin and 2000 ug/ml streptomycin, and plated on a T75 tissue culture flask. Cells were allowed to attach for 2 hours at 37°C in 5% CO<sub>2</sub> incubator before changing medium, which removed the weakly adherent cells such as myocytes and endothelial cells. On reaching confluency, cells were detached with trypsin/EDTA and sub-cultured in DMEM supplemented with 20% FBS and 2 mM L-glutamine. Since the phenotype of cardiac fibroblasts can be influenced by growth conditions such as passage and cell density, cells at passage 1-5 were used for experiments.

### **Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. One µg of RNA was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-rad) according to the manufacturer's protocol. 50 ng of cDNA (relative to RNA amount) was amplified in a Stratagene Mx3005P QPCR system (Agilent Technologies) with SYBR Green/Rox PCR master mix (SABiosciences) and primers. mRNA expression of the genes of interest was normalized to the expression of cyclophilin.

### **Western Blotting**

Cardiac fibroblasts isolated from wild type mouse hearts (control) were stimulated with 10 ng/ml of TGF-β up to 48 hr after being starved with serum-free media for 24 hr. At the end of the experiment, proteins were extracted by scrapping in cold PBS and centrifuged to collect pellets. After removing the supernatant, pellets were lysed in RIPA buffer containing proteinase inhibitor and boiled with 5X loading buffer for 5 min. After separation by SDS-PAGE and

transfer to PDGF membrane, the signals were detected with specific corresponding antibodies such as  $\alpha$ -SMA, collagen I, fibronectin, RGC-32, and  $\alpha$ -Tubulin, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The blots were visualized by chemiluminescence (Millipore) and analyzed using Konica SRX-101A film processor.

### **Immunofluorescent Staining**

Cardiac fibroblasts cultured on 12 mm glass coverslips were serum-starved for 24 hr and then stimulated for different time periods up to 48 hr in vehicle (control) or 10 ng/ml of TGF- $\beta$ 1. CFs were washed with cold PBS twice. CFs were then fixed with 1% paraformaldehyde (PFA) for 1 min at room temperature and cold methanol:acetone (1:1) for 10 min on ice. CFs were washed with PBS and then blocked with 5% goat serum in PBS for 1 hr at room temperature. CFs were incubated with 2 primary antibodies from different animal species at 4°C overnight. After washing with PBS, CFs were incubated with secondary antibodies conjugated with FITC or TRITC about 1.5 hr at room temperature. CFs were washed with PBS and then incubated with DAPI for 20 min before microscopic imaging.

### **Statistical Analysis**

Data are expressed as mean  $\pm$  S.E. and all experiments were repeated independently three or more times. Analysis of variance was used to assess the differences among multiple groups. A *t*-test was used to assess the differences between pairwise groups.  $p < 0.05$  was considered a statistically significant difference.

## RESULTS

TGF- $\beta$  is a profibrotic cytokine that induces the transformation of fibroblasts to  $\alpha$ -SMA-expressing myofibroblasts and stimulates the production of extracellular matrix proteins such as collagens and fibronectin. RGC32 has been shown to be a downstream target of TGF- $\beta$  in other mesenchymal cell lines such as neural crest stem cells and C3H10T1/2 fibroblast cells. To determine whether RGC32 plays a role in the TGF- $\beta$ -induced transformation of cardiac fibroblast to myofibroblast, primary cardiac fibroblasts isolated from adult mice were treated with 10 ng/ml of TGF- $\beta$  for the different time points to manipulate the fibrotic microenvironment *in vitro*. The expressions of RGC32,  $\alpha$ -SMA, ECM proteins collagen I and fibronectin were examined by RT-qPCR, western blotting, and immunofluorescent staining to observe its mRNA and protein expressions in response to TGF- $\beta$  stimulation.

Real-time PCR data confirmed that RGC32 is a direct target of TGF- $\beta$  in the primary mouse cardiac fibroblast (mCFs) as it increases in a time-dependent manner in response to TGF- $\beta$  stimulation (Fig. 1). The mRNA expression of RGC32 was increased as early as 2 hr after TGF- $\beta$  treatment and the induction was increased 7-fold after 48 hr of treatment.  $\alpha$ -SMA mRNA was readily increased up to 8-fold by 2 hr and 50-fold after 48 hr of TGF- $\beta$  treatment compared with untreated control (Fig. 1), indicating that TGF- $\beta$  is a potent inducer of fibroblast activation. Commonly used fibrosis markers, Type I collagen and fibronectin also significantly increased in response to TGF- $\beta$  (Fig. 1), suggesting that TGF- $\beta$  induction can manipulate the fibrotic microenvironment for mCFs to be activated and to produce ECM proteins.

Western blotting also showed that protein levels of RGC32,  $\alpha$ -SMA, Collagen I, and fibronectin were increased in response to TGF- $\beta$  stimulation although the fold induction in protein expression was not as great as mRNA expression (Fig. 2). Additional studies by using

immunofluorescent staining visualized that RGC32,  $\alpha$ -SMA, Collagen I, and fibronectin expression in mCFs was increased in a time-dependent manner by TGF- $\beta$  (Fig. 3).

Collagen molecules consist of three polypeptide chains, called  $\alpha$ -chains, which are coiled around one another to form a triple-helical conformation. Type I collagen is composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain encoded by COL1A1 and COL1A2 genes respectively, making up approximately 80% of the cardiac ECM. The next abundant collagen occupying ECM of the heart is type III collagen, accounting for about 10%. To confirm the TGF- $\beta$  induction of the individual propeptides of procollagen type I and III, RT-qPCR was performed to examine the mRNA level of COL1A1, COL1A2, and COL3A1. All three genes were significantly up-regulated by 8 hr treatment with TGF- $\beta$  (Fig. 4).

To determine if RGC32 is functionally important for the transformation of fibroblast to myofibroblast, the cardiac fibroblast cells from RGC32 knockout mice, designated as KO in the figures, was compared with those from wild type (WT) mice. The equal number of cells at the same passages either from WT or RGC32-KO mice were cultured and treated with 10 ng/ml of TGF- $\beta$ . RT-qPCR confirmed that mCFs from RGC32-KO mice did not express RGC32, suggesting a successful deletion of RGC32 gene in the animal model (Fig. 5). RGC32-KO cells have a lower expression of  $\alpha$ -SMA than WT cells in untreated condition. More importantly, the absence of RGC32 gene in mCFs inhibited the expression of  $\alpha$ -SMA even with TGF- $\beta$  stimulation, suggesting that RGC32 may be involved in TGF- $\beta$ -mediated transformation of myofibroblasts.

The comparison of mRNA expression of polypeptide  $\alpha$ -chains between WT and KO mCFs showed that the basal level of COL1A1, COL1A2, and COL3A1 genes was similar in both WT and KO (Fig. 6). When treated with TGF- $\beta$ , RGC32-KO showed a lower mRNA

expression of COL1A1 and COL1A2 genes compared with the untreated KO mCFs while the COL3A1 expression level was kept similar in both untreated and treated groups (Fig. 6). These results suggest that WT mCFs were more responsive to TGF- $\beta$  stimulation than RGC32 KO mCFs in increasing collagen gene expression. Western blotting was performed with the same experimental design to detect the protein level of  $\alpha$ -SMA, collagen I, and fibronectin. As shown in Fig. 7,  $\alpha$ -SMA, collagen I, and fibronectin protein levels were greatly up-regulated in WT mCFs, but not in KO mCFs. These data indicate that TGF- $\beta$  cannot elicit the fibrotic response from RGC32 KO cells as much as WT cells. Therefore, RGC32 as a TGF- $\beta$  downstream mediator may have facilitated the transformation of fibroblasts to myofibroblasts, promoting the ECM protein production.

To test if RGC32 alone can induce myofibroblast transformation promoting the ECM protein production in WT cardiac fibroblasts, adenoviral vectors expressing GFP or RGC32 (named as Ad-GFP or Ad-RGC32 in the figures) was used to transduce WT mCFs. Western blotting was performed to detect protein expression of fibrotic markers including  $\alpha$ -SMA, collagen I, and fibronectin. RGC32 expression was increased by Ad-RGC32 infection confirming the effective transduction of the gene by adenoviral infection (Fig 8). Overexpression of RGC32 in WT mCFs induced a strong expression of  $\alpha$ -SMA, collagen I, and fibronectin, suggesting that RGC32 mimics the effect of TGF- $\beta$  on the myofibroblast formation.

To examine whether introducing RGC32 into RGC32-knockout mCFs can restore the fibrotic function to induce the myofibroblast transition, KO cells were infected with either Ad-GFP or Ad-RGC32 and analyzed with western blotting to detect protein levels of  $\alpha$ -SMA and collagen I. As shown in Fig. 9, a substantial expression of RGC32 in KO cells occurred by adenovirus-mediated gene transfer. Additional stimulation with TGF- $\beta$  enhanced the RGC32



protein expression (upper band of RGC32). TGF- $\beta$  treatment alone partially induced  $\alpha$ -SMA and collagen I protein expression in KO mCFs while Ad-RGC32 transduction mimicked the effect of TGF- $\beta$  as shown by the similar intensity of bands for both proteins (Fig. 9). Interestingly, combination of Ad-RGC32 transduction and TGF- $\beta$  treatment decreased protein level of  $\alpha$ -SMA and collagen I in KO mCFs. These data suggest that TGF- $\beta$ -stimulated transition to myofibroblast occurred in the absence of RGC32 and exogenously overexpressed RGC32 may inhibit TGF- $\beta$  function through a negative feedback mechanism.

## DISCUSSION

Evidences suggest that RGC32 is up-regulated during the development of excess fibrous connective tissue and repair process in various organs including liver and kidney (Gnainsky et al., 2007; Li et al., 2011). The investigation of how halofuginone, a powerful inhibitor of liver fibrosis, alters the expression of TGF- $\beta$ -regulated genes showed that RGC32 expression was up-regulated in rat hepatic stellate cells during liver fibrosis. RGC32 was also identified as a novel fibrogenic factor contributing to the pathogenesis of renal fibrosis through fibroblast activation, acting as a downstream target of TGF- $\beta$ . Many studies have shown that TGF- $\beta$  signaling plays an important role in the progression of cardiac fibrosis. However, the role of RGC32 in the process of TGF- $\beta$ -induced fibroblast activation in the cardiac fibrosis remains unknown.

The experiment described in this thesis investigated the role of RGC32 in the fibroblast activation acquiring the myofibroblast phenotype. Myofibroblast is a specific cell type that has intermediate features between fibroblasts and smooth muscle cells expressing  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and having a higher activity for collagen production than fibroblasts. RGC32 was shown to act as a TGF- $\beta$  downstream target involved in smooth muscle differentiation from

neural crest cells. However, the function of RGC32 in the primary cardiac fibroblast cell type is not known.

The primary mouse cardiac fibroblasts (mCFs) were stimulated with 10 ng/ml of TGF- $\beta$ . RGC32 was increased both in mRNA and protein levels by TGF- $\beta$ . Along with RGC32 induction,  $\alpha$ -SMA, collagen I, and fibronectin were also up-regulated by TGF- $\beta$ . RGC32 seems to be a downstream mediator of TGF- $\beta$  in mCFs. TGF- $\beta$  is capable of inducing transformation of fibroblasts to myofibroblasts confirming the integrity of isolated primary mCFs.

The mCFs isolated from RGC32-KO mice (KO mCFs) were not as responsive as those from wild type mice (WT mCFs) to TGF- $\beta$  stimulation in inducing myofibroblast-related markers and producing less ECM proteins. These data suggest that RGC32 is involved in TGF- $\beta$ -induced transformation of myofibroblasts from mCFs.

Propolypeptides of collagen type I and III, the two major collagen types in the heart, were detected in WT mCFs since the mRNA expression of propeptides reflect the collagen synthesis. In response to 10 ng/ml TGF- $\beta$  treatment, COL1A1, COL1A2, and COL3A1 were increased in WT mCFs in a time-dependent manner. mRNA levels of these three genes in WT mCFs were induced higher than KO mCFs by TGF- $\beta$ , suggesting the capability of KO mCFs to synthesize collagens is reduced compared with WT mCFs.

RGC32 overexpression in WT mCFs enhanced the myofibroblast transition in the absence of TGF- $\beta$  treatment. RGC32 overexpression by adenoviral gene transfer increased the  $\alpha$ -SMA, collagen I, and fibronectin expression, indicating that RGC32 can mimic the effect of TGF- $\beta$  serving as a downstream mediator.

Restoring RGC32 expression or overexpression by adenoviral gene transfer in RGC32 KO mCFs showed that RGC32 restoration can rescue the capability to produce collagen and

mediate fibroblast activation. TGF- $\beta$  stimulation in RGC32-KO cells also showed that TGF- $\beta$  was able to activate myofibroblast markers in the absence of RGC32, suggesting that a compensatory mechanism exists for myofibroblast transition. RGC32 overexpression had the same effect with TGF- $\beta$  treatment in the induction of  $\alpha$ -SMA and collagen I proteins. However, the combination of RGC32 overexpression and TGF- $\beta$  treatment decreased their protein expression compared to the individual manipulation, suggesting that excessive RGC32 may trigger a negative feedback mechanism, which is a common phenomenon in TGF- $\beta$  signaling transduction.

In conclusion, we have identified RGC32 as a novel fibrogenic factor mediating the TGF- $\beta$ -induced cardiac fibroblast activation. Data suggest that the action of TGF- $\beta$  signaling in mediating myofibroblast transformation may be mediated by alternative signaling pathways while RGC32 is knocked out. Exogenously introduced RGC32 appeared to be sufficient to induce fibroblast-to-myofibroblast transformation. However, the molecular mechanism by which RGC32 mediates the TGF- $\beta$ -induced fibrotic responses in mCFs remains to be further investigated.

## REFERENCES

- Badea, T., Niculescu, F., Soane, L., Fosbrink, M., Sorana, H., Rus, V., Shin, M. L., Rus, H. (2002) RGC-32 increases p34<sup>CDC2</sup> kinase activity and entry of aortic smooth muscle cells into S-phase. *J. Biol. Chem.* **277**, 502-508.
- Baum, J., Duffy, H. S. (2011) Fibroblasts and Myofibroblasts: What are we talking about? *J. Cardiovasc. Pharmacol.* **57**, 376-379.
- Bujak, M., Frangogiannis, N. G. (2007) The role of TGF- $\beta$  signaling in myocardial infarction and cardiac remodeling. *Cardiovas. Res.* **74**, 184-195.
- De Jong, S., van Veen, T. A., de Bakker, J. M., Vos, M. A., van Rijen, H. V. (2011) Biomarkers of myocardial fibrosis. *J. Cardiovasc. Pharmacol.* **57**, 522-535.
- Esson, D. W., Popp, M. P., Liu, L., Schultz, G. S., Sherwood, M. B. (2004) Microarray analysis of the failure of filtering blebs in a rat model of glaucoma filtering surgery. *Invest. Ophthalmol. Vis. Sci.* **45**, 4450-4462.
- Gnainsky, Y., Kushnirsky, Z., Bilu, G., Hagai, Y., Genina, O., Volpin, H., Bruck, R., Spira, G., Nagler, A., Kawada, N., Yoshizato, K., Reinhardt D. P., Libermann, T. A., Pines, M. (2007) Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.* **328**, 153-166.
- Guo, X., Jose, P. A., Chen, S. Y. (2011) Response gene to complement 32 interacts with Smad3 to promote epithelial-mesenchymal transition of human renal tubular cells. *Am. J. Physiol. Cell Physiol.* **300**, C1415-C1421.

- Huang, W., Li, Z., Rus, H., Wang, X., Jose, P. A., Chen, S. (2009) RGC-32 mediates transforming growth factor- $\beta$ -induced epithelial-mesenchymal transition in human renal proximal tubular cells. *J. Biol. Chem.* **284**, 9426-9432.
- Huang, W. Y., Xie, W., Guo, X., Li, F., Jose, P. A., Chen, S. Y. (2011) Smad2 and PEA3 cooperatively regulate transcription of response gene to complement 32 in TGF- $\beta$ -induced smooth muscle cell differentiation of neural crest cells. *Am. J. Physiol. Cell Physiol.* **301**, C499-506.
- Inagaki, Y., Truter, S., Ramirez, F. (1994) Transforming growth factor- $\beta$  stimulates  $\alpha 2(I)$  collagen gene expression through a cis-acting element that contains an Sp1-binding site. *J. Biol. Chem.* **269**, 14828-14834.
- Li, F., Luo, Z., Huang, W., Lu, Q., Wilcox, C. S., Jose, P. A., Chen, S. (2007) Response gene to complement 32, a novel regulator for transforming growth factor-beta-induced smooth muscle cell differentiation of neural crest cells. *J. Biol. Chem.* **282**, 10133-10137.
- Li, Z., Xie, W. B., Escano, C. S., Asico, L. D., Xie, Q., Jose, P. A., Chen, S. Y. (2011) Response gene to complement 32 is essential for fibroblast activation in renal fibrosis. *J. Biol. Chem.* **286**, 41323-41330.
- Olson, E. R., Shamhart, P. E., Naugle, J. E., Meszaros, J. G. (2008). Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase Cdelta and intracellular calcium in adult rat cardiac fibroblasts. *Hypertension* **51**, 704–711.
- Ong, V. H., Carulli, M. T., Xu, S., Khan, K., Lindahl, G., Abraham, D. J., Denton, C. P. (2009). Cross-talk between MCP-3 and TGFbeta promotes fibroblast collagen biosynthesis. *Exp. Cell Res.* **315**, 151–161.

- Petrov, V. V., Fagard, R. H., Lijnen, P. J. (2002) Stimulation of collagen production by transforming growth factor- $\beta$ 1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* **39**, 258-263.
- Small, E. M., Thatcher, J. E., Sutherland, L. B., Kinoshita, H., Gerard, R. D., Richardson, J. A., DiMaio, J. M., Sadek, H., Kuwahara, K., Olson, E. N. (2010) Myocardin-related transcription factor-A controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circ. Res.* **107**, 294-304.
- Teekakirikul, P., Eminaga, S., Toka, O., Alcalai, R., Wang, L., Wakimoto, H., Naylor, M., Konno, T., Gorham, J. M., Wolf, C. M., Kim, J. B., Schmitt, J. P., Molkentin, J. D., Norris, R. A., Tager, A. M., Hoffman, S. R., Markwald, R. R., Seidman, C. E., Seidman, J. G. (2010) Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires TGF- $\beta$ . *J. Clin. Invest.* **120**, 3520-3529.
- Vlaicu, S. I., Cudrici, C., Ito, T., Fosbrink, M., Tegla, C. A., Rus, V., Mircea, P. A., Rus, H. (2008) Role of response gene to complement 32 in diseases. *Arch. Immunol. Ther. Exp.* **56**, 115-122.
- Wang, J. N., Shi, N., Xie, W. B., Guo, X., Chen, S. Y. (2011) Response gene to complement 32 promotes vascular lesion formation through stimulation of smooth muscle cell proliferation and migration. *Arterioscler. Thromb. Vasc. Biol.* **31**, e19-e26.

### Legends for figures

**Figure 1: TGF- $\beta$  induced RGC32 and myofibroblast marker mRNA expression.** Primary cardiac fibroblasts isolated from adult mouse (mCFs) were cultured, serum-starved for 24 hr, and treated with 10 ng/ml of TGF- $\beta$  for the times indicated. qPCR results showed that RGC32 and myofibroblast-related markers increased after TGF- $\beta$  treatment. mRNA values were normalized to cyclophilin. Error bars represent SEM. Asterisks (\*) indicate a significant difference from the TGF- $\beta$ -untreated control ( $P < 0.05$ ).

**Figure 2: TGF- $\beta$  induced RGC32 and myofibroblast marker protein expression.** mCFs were cultured, serum-starved for 24 hr, and treated with 10 ng/ml of TGF- $\beta$  for the times indicated. Protein expression was quantified by western blotting.  $\alpha$ -Tubulin was used as an internal control.

**Figure 3: TGF- $\beta$  induction of RGC32 and myofibroblast markers as shown by immunostaining.** mCFs were cultured on 12 mm glass coverslips, serum-starved for 24 hr, and treated with 10 ng/ml of TGF- $\beta$  for the times indicated. Cells were incubated with RGC32 (green),  $\alpha$ -SMA (red), collagen I (green), and fibronectin (green) antibodies followed by the secondary antibodies. DAPI stains nuclei (blue).

**Figure 4: TGF- $\beta$  increased COL1A1, COL1A2, and COL3A1 gene expression in a time-dependent manner.** mCFs were cultured, serum-starved for 24 hr, and treated with 10 ng/ml of TGF- $\beta$  for the indicated times. qPCR data showed that COL1A1, COL1A2, and COL3A1 mRNA expression reached the highest levels after 8 hr of TGF- $\beta$  treatment. mRNA values were

normalized to cyclophilin. Error bars represent SEM. Asterisks (\*) indicate a significant difference from the TGF- $\beta$ -untreated control ( $P < 0.05$ ).

**Figure 5: RGC32-KO attenuated TGF- $\beta$  induction of myofibroblast marker mRNA expression in mCFs.** The equal numbers of mCFs at the same passages either from WT or RGC32-KO mice were cultured, serum-starved for 24 hr, and treated with TGF- $\beta$  for 24 hr. qPCR data showed that WT mCFs were more responsive to TGF- $\beta$  stimulation and produced more  $\alpha$ -SMA mRNA expression than RGC32-KO mCFs. mRNA values were normalized to cyclophilin. Error bars represent SEM. Asterisks (\*) indicate a significant difference from the TGF- $\beta$ -untreated control ( $P < 0.05$ ).

**Figure 6: RGC32-KO attenuated TGF- $\beta$  induction of COL1A1, COL1A2, and COL3A1 mRNA expression in mCFs.** Equal numbers of mCFs at the same passages either from WT or RGC32-KO mice were plated, serum-starved for 24 hr, and treated with TGF- $\beta$  for 8 hr. qPCR data showed that WT mCFs were more responsive to TGF- $\beta$  stimulation than KO mCFs and produced more all three collagen mRNA. mRNA values were normalized to cyclophilin. Error bars represent SEM. Asterisks (\*) indicate a significant difference from the TGF- $\beta$ -untreated control ( $P < 0.05$ ).

**Figure 7: RGC32-KO attenuated TGF- $\beta$  induction of myofibroblast marker protein expression in mCFs.** Equal numbers of mCFs at the same passages either from WT or RGC32-KO mice were cultured, serum-starved for 24 hr, and treated with 10 ng/ml of TGF- $\beta$  for 24h. Protein expression was analyzed by western blotting to detect  $\alpha$ -SMA, collagen I, and



fibronectin. TGF- $\beta$  induced a greater expression of these genes in WT mCFs as compared to KO mCFs.  $\alpha$ -Tubulin was used as an internal control.

Figure 8: **RGC32 overexpression by adenoviral gene transfer markedly increased  $\alpha$ -SMA, collagen I, and fibronectin expression in WT mCFs.** WT mCFs were cultured, infected with either Ad-GFP (control) or Ad-RGC32 for 48 hr, and serum-starved for 24 hr. Protein level was examined by western blotting and  $\alpha$ -Tubulin was used as an internal control.

Figure 9: **RGC32 overexpression by adenoviral gene transfer in KO mCFs mimicked the function of TGF- $\beta$  in promoting the myofibroblast formation and ECM production.** KO mCFs were cultured, infected with either Ad-GFP or Ad-RGC32 for 48 hr, and treated with vehicle or 10 ng/ml of TGF- $\beta$ . Western blotting was performed to detect the expression of myofibroblast-related markers.  $\alpha$ -Tubulin was used as an internal control.

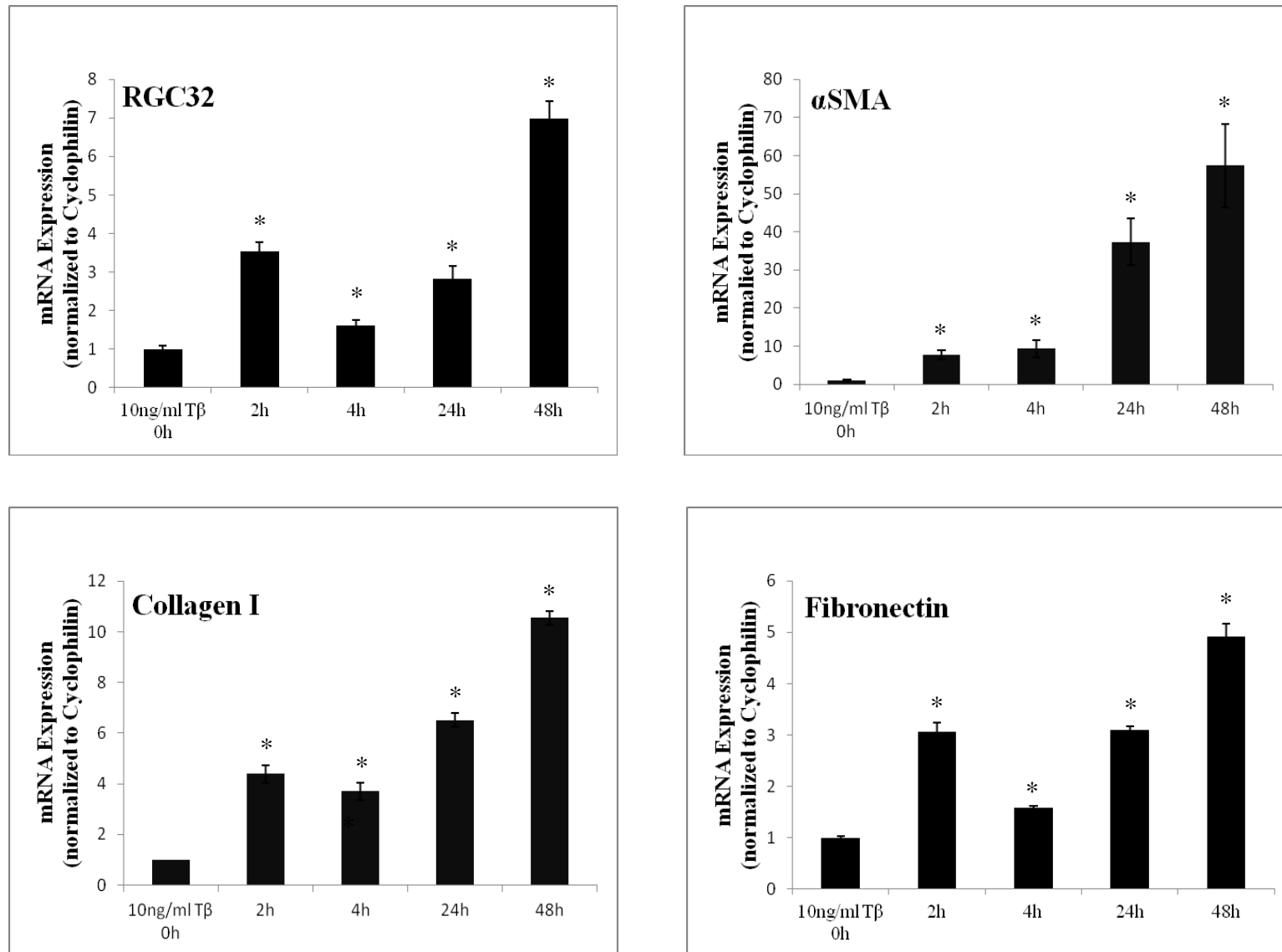


Figure 1

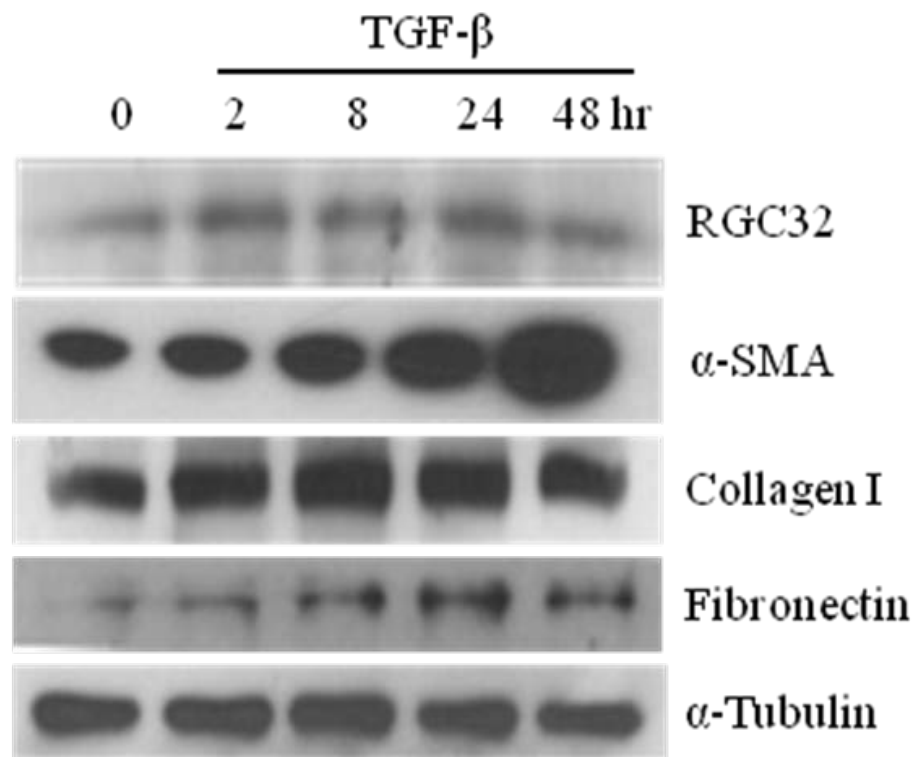


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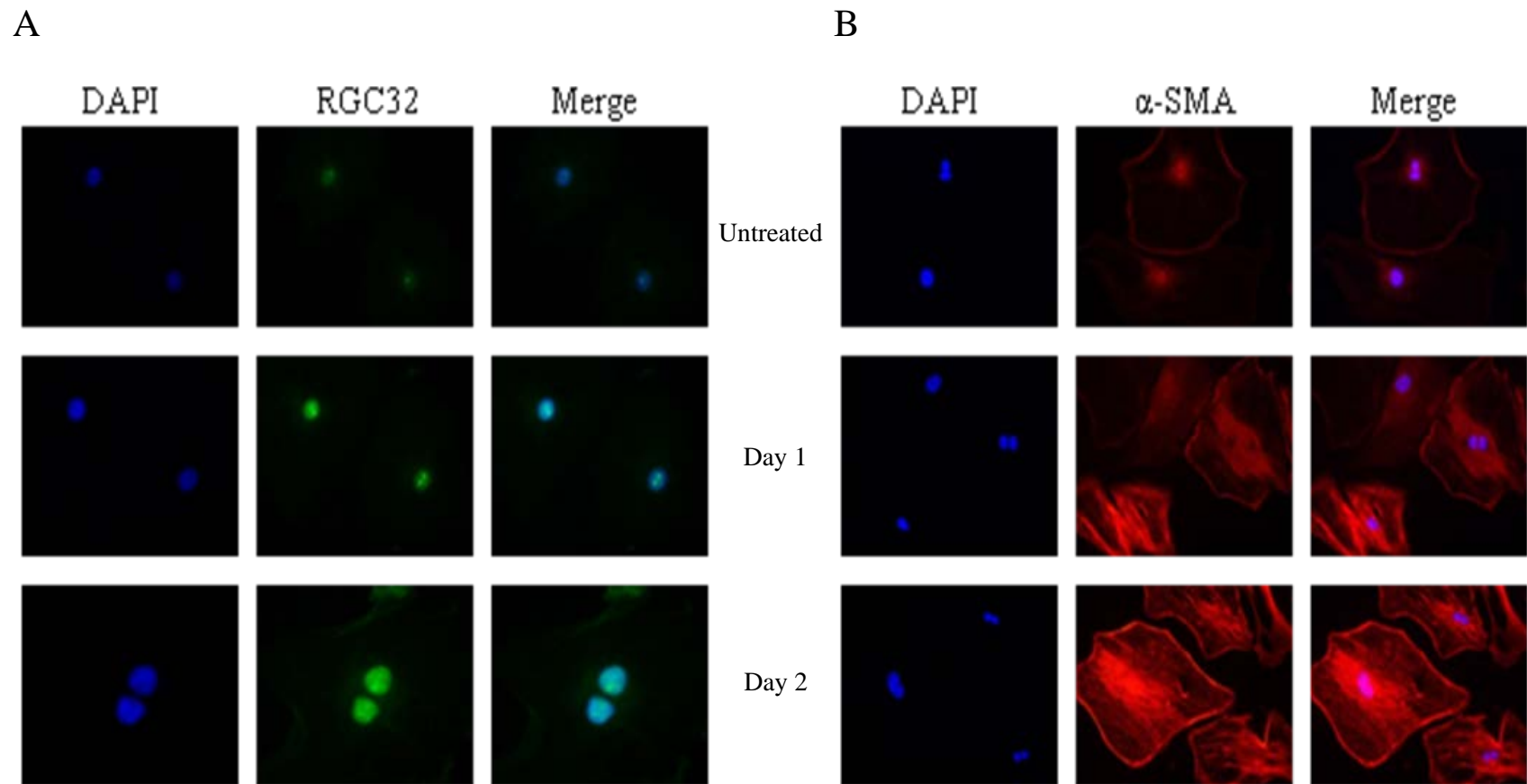
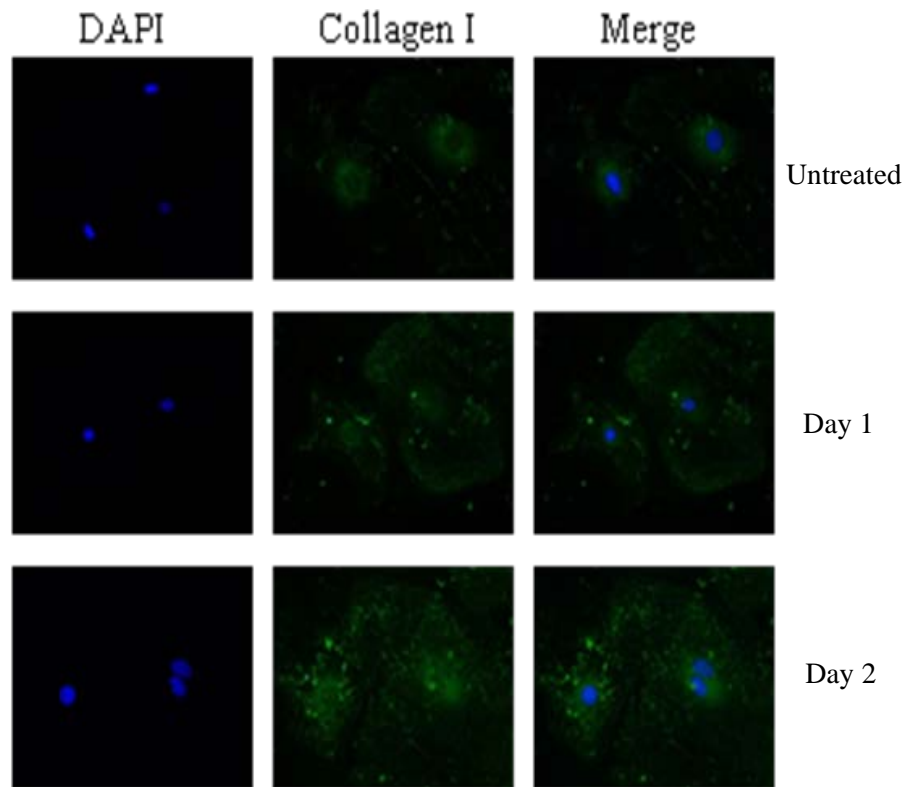


Figure 3

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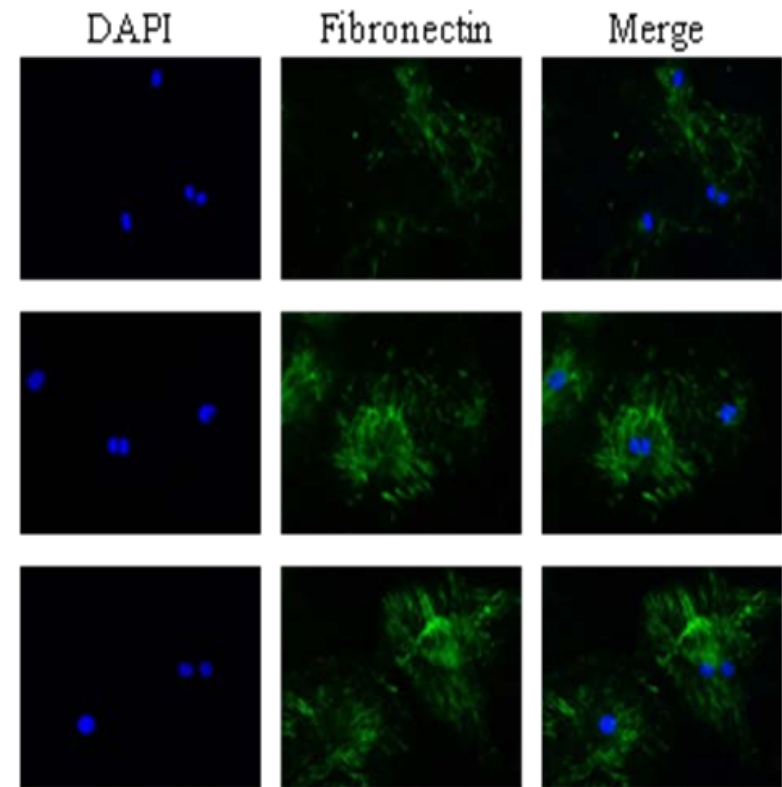


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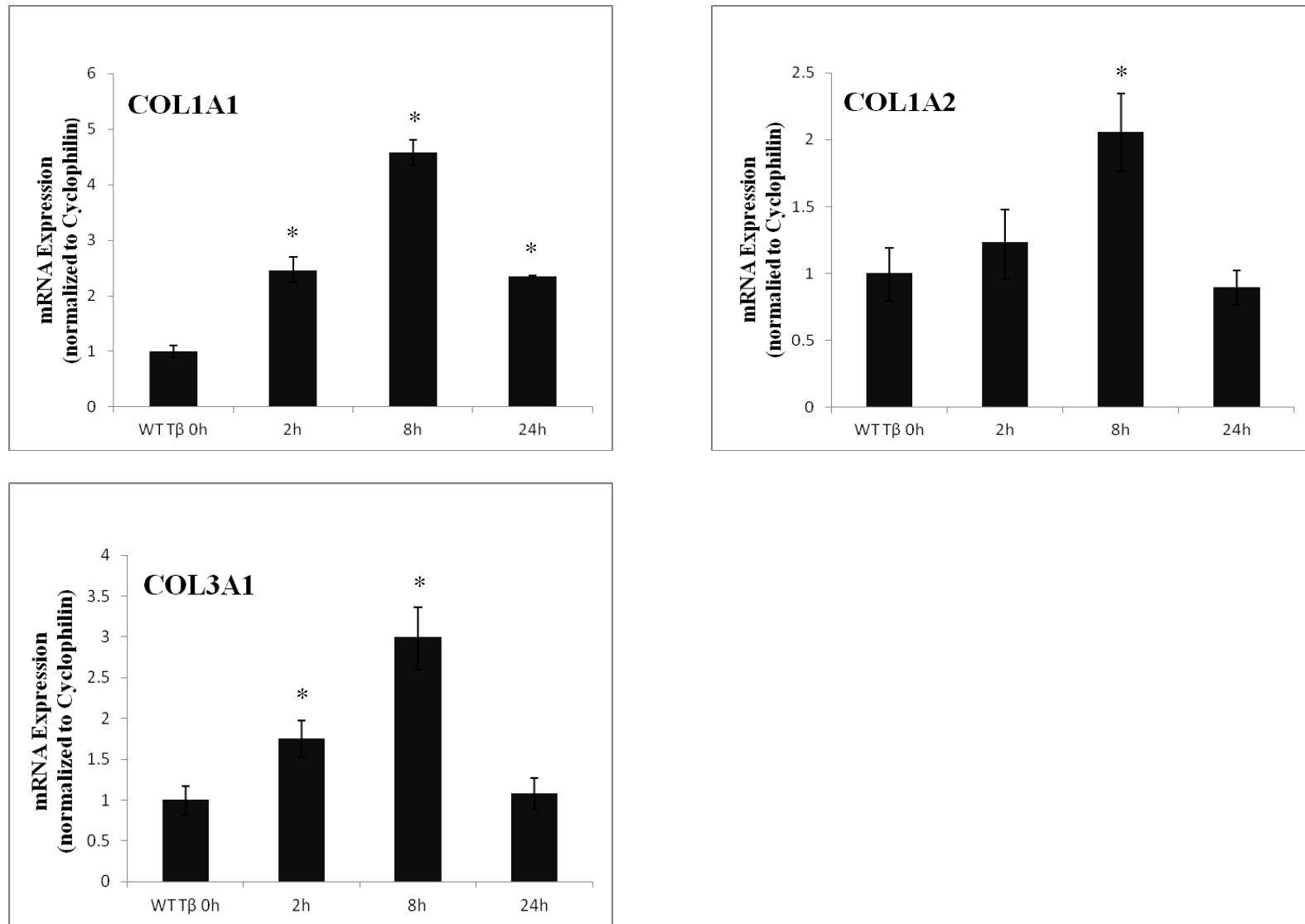


Figure 4

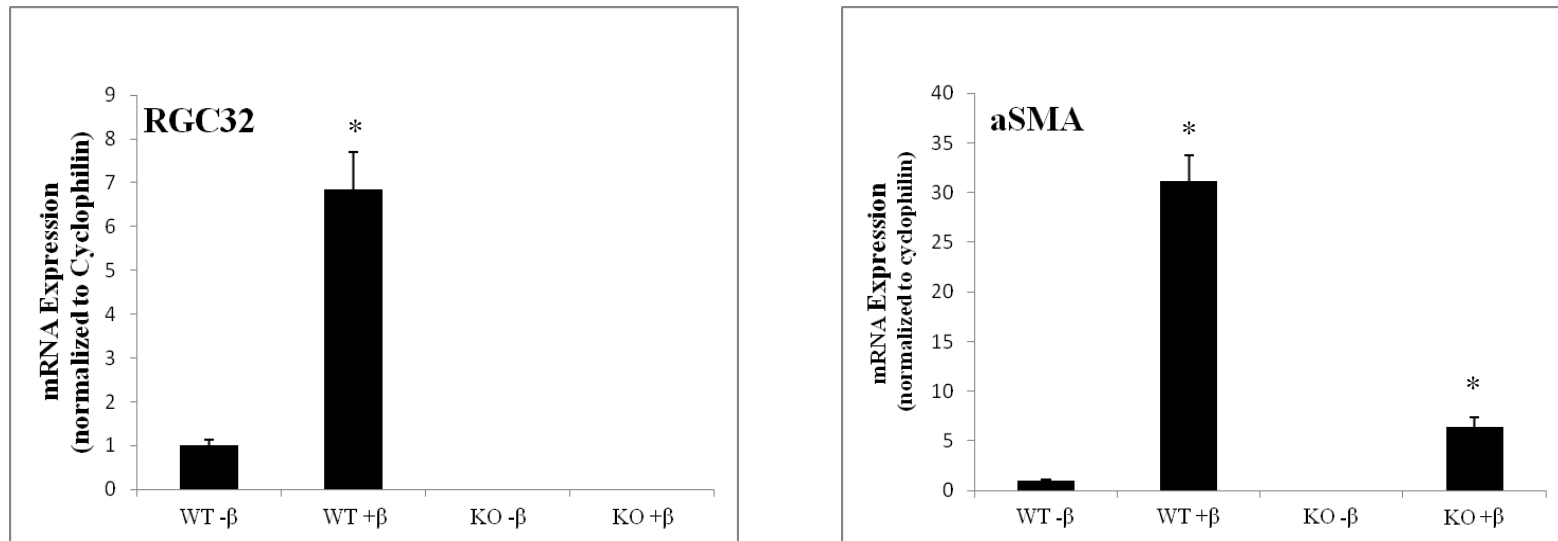


Figure 5

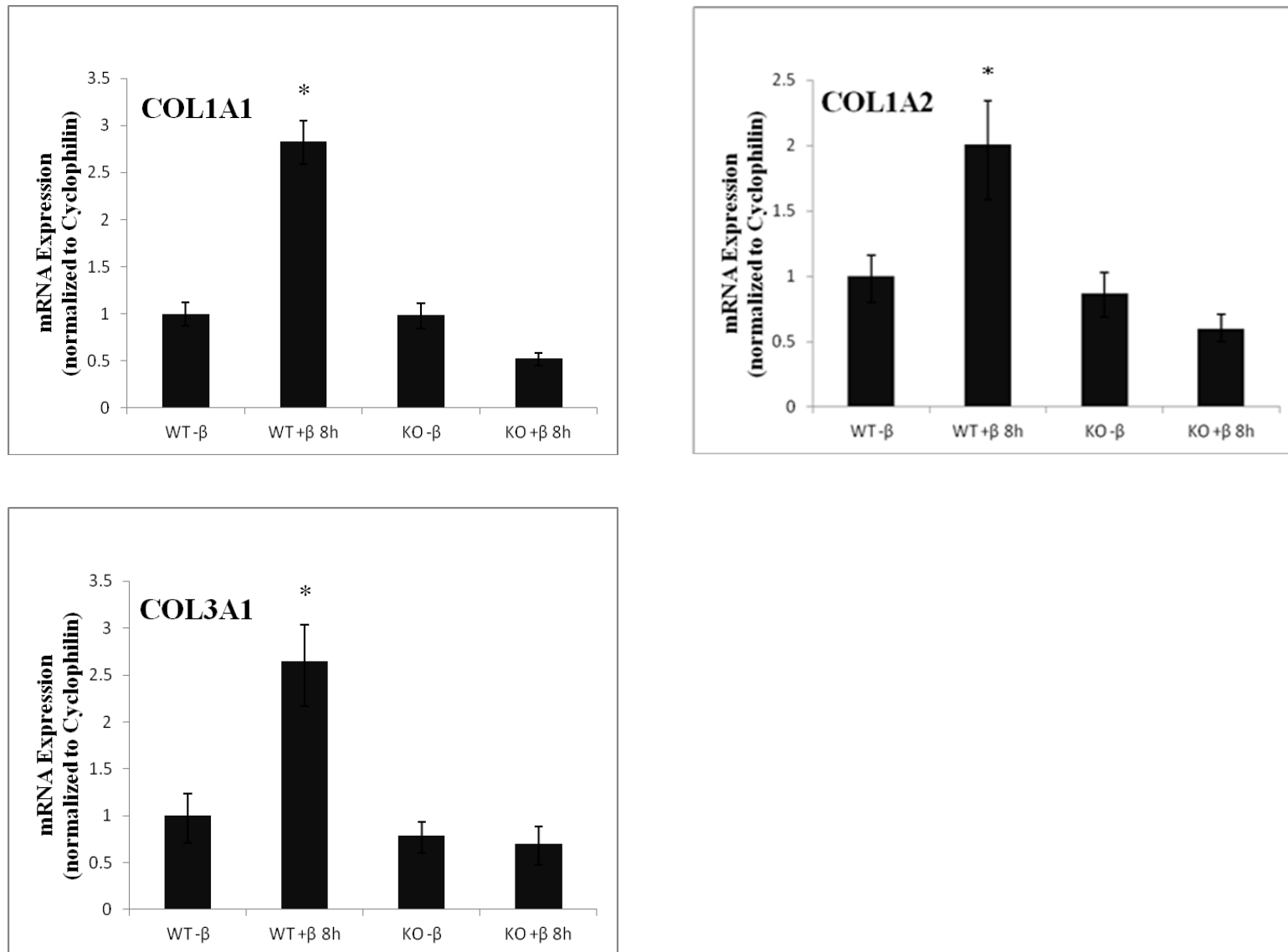


Figure 6



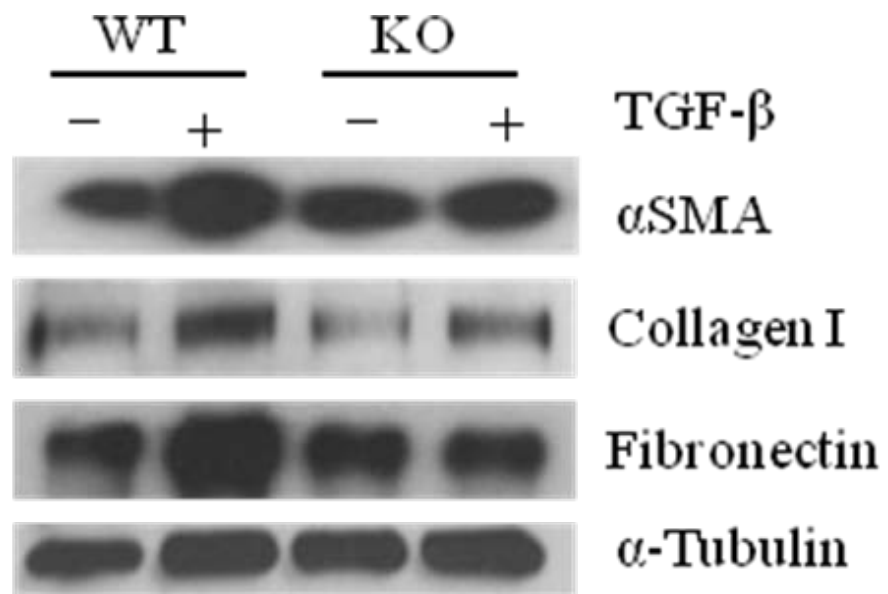


Figure 7

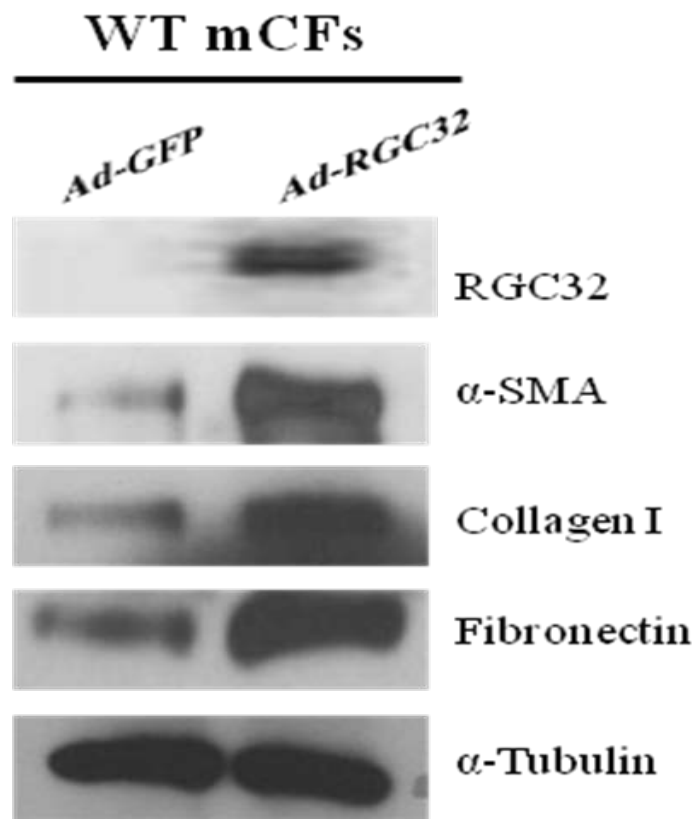


Figure 8

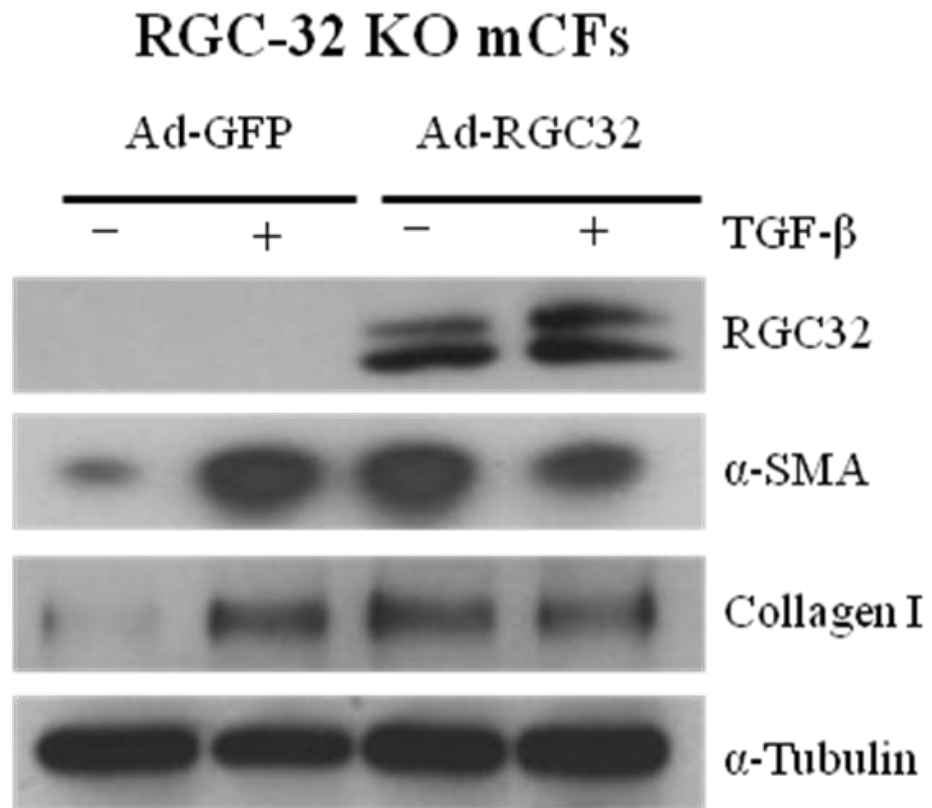


Figure 9