WOUND HEALING IN THE HEART: INVOLVEMENT OF A COMPLEX MOLECULAR CIRCUITRY OF DISCOIDIN DOMAIN RECEPTOR 2, INTEGRIN-β1, ALPHA- SMOOTH MUSCLE ACTIN AND COLLAGEN TYPE I IN ANGIOTENSIN II-STIMULATED CARDIAC FIBROBLASTS

A THESIS PRESENTED BY

HARIKRISHNAN V

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

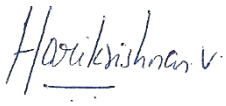
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

2019

DECLARATION BY THE STUDENT

I, Harikrishnan V, hereby certify that I had personally carried out the work depicted in the thesis titled: **"Wound Healing in the Heart: Involvement of a Complex Molecular Circuitry of Discoidin Domain Receptor 2, Integrin-β1, Alpha-Smooth Muscle Actin and Collagen Type I in Angiotensin II-stimulated Cardiac Fibroblasts"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.



Date: 10/10/2019 Harikrishnan.V

CERTIFICATE OF THE GUIDE

Dr K Shivakumar

Division of Cellular and Molecular Cardiology

Sree Chitra Tirunal Institute for Medical Sciences and Technology

Trivandrum 695011, India.

This is to certify that Harikrishnan V of the Division of Cellular and Molecular Cardiology of this Institute has fulfilled the requirements prescribed for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis titled: **"Wound Healing in the Heart: Involvement of a Complex Molecular Circuitry of Discoidin Domain Receptor 2, Integrin-β1, Alpha-Smooth Muscle Actin and Collagen Type I in Angiotensin II-stimulated Cardiac Fibroblasts"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date. Clearance was obtained from the Institutional Animal Ethics Committee for carrying out the study.

Date: 10/10/2019 Dr K Shivakumar

The thesis entitled

WOUND HEALING IN THE HEART: INVOLVEMENT OF A COMPLEX MOLECULAR CIRCUITRY OF DISCOIDIN DOMAIN RECEPTOR 2, INTEGRIN-β1, ALPHA- SMOOTH MUSCLE ACTIN AND COLLAGEN TYPE I IN ANGIOTENSIN II-STIMULATED CARDIAC FIBROBLASTS

Submitted by

HARIKRISHNAN V

For the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

is evaluated and approved by

Dr K Shivakumar (Guide) (Examiner)

ACKNOWLEDGEMENT

I consider myself privileged to have had the opportunity to carry out my doctoral studies in the Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India. I was fortunate to be associated with two Directors Dr Jagan Mohan Tharakan and Dr Asha Kishore and I am grateful to them for their support and the excellent facilities at the Institute. I gratefully acknowledge the financial support received from the Department of Biotechnology, Government of India, for supporting this project and for providing a fellowship.

I am deeply indebted to my esteemed mentor, Dr. K Shivakumar, for his continuous support through all these years and valuable guidance, perceptive encouragement, indispensable suggestions, immense knowledge and motivation, which have helped me greatly in the successful completion of my studies. He has always found time to propose consistently excellent improvements. I am truly thankful for his selfless dedication and critical thinking that have contributed to my academic development. I am indeed fortunate and honored to have been his student.

I express my sincere thanks to Dr. R Renuka Nair for her encouragement and advice. I thank all the members of the Division of Cellular and Molecular Cardiology and the Department of Pathology for their support and help.

I warmly thank Dr Anoop Kumar Thekkuveetil and Dr Srinivas G, the members of my Doctoral Advisory Committee for their help and co-operation. A special thanks to Dr Jackson James of Rajiv Gandhi Centre for Biotechnology, Trivandrum, for their help during the course of my work.

I would like to thank the previous Deputy Registrar Dr S Jayasingh and the Registrar Dr Santhosh, for their support. I wish to take this opportunity to thank all the members of the Division of Academic Affairs for their kind support.

I would also like to thank my seniors, Dr Anupama V and Dr Mereena George for guiding and training me in molecular cardiology. I appreciate the goodwill extended to me by my colleague Allen Sam Titus and my juniors Indraja and Sruthy, for being supportive and creating a joyous environment in the laboratory. A word of thanks would be far too inadequate to express my gratitude to them. I would also like to thank Dr Ajay Godwin, Dr Sherin S and Dr Saifudeen Ismail for their valuable support. Also, I thank my friends in the Department of Biochemistry for their help.

I thank Dr E G Lakatta and Dr Mingyi Wang of the Laboratory of Cardiovascular Science, NIH, USA, for the kind gift of a few reagents used in the study. I thank Dr Randy T Cowling of the Division of Cardiovascular Medicine, University of California-San Diego, La Jolla, California, USA, for providing the cardiac tissue sections of the knockout mice.

I owe a lot to my parents and my sister, who encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true. Above all, I owe it to the Lord Almighty for granting me this opportunity and enabling me to achieve this goal.

CONTENTS

LIST OF FIGURES

List of abbreviations

Ang II	Angiotensin II
ACE	Angiotensin-converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activator protein-1
ARBs	Angiotensin II receptor blockers
AT1 receptor	Angiotensin II type 1 receptor
ATF4	Activating Transcription Factor 4
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
ChIP	Chromatin Immunoprecipitation
DDR1	Discoidin Domain Receptor 1
DDR2	Discoidin Domain Receptor 2
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
ECM	Extracellular Matrix
ECs	Endothelial cells
EMSA	Electrophoretic Mobility Shift Assay
EMT	Epithelial-to-mesenchymal transformation
EPDCs	Epicardial-derived cells
ERK1/2	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin-1
FITC	Fluorescein isothiocyanate
FSP-1	Fibroblast-Specific Protein 1
GPVI	Glycoprotein VI
HRP	Horseradish Peroxidase
IL	Interleukin
ITGA11	Integrin-α11
ITGB1	Integrin-β1

IκB	Inhibitory-kappa B
LAIR1	Leukocyte-associated immunoglobulin-like receptor 1
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
NF-κB	Nuclear Factor-kappa B
NOX	NADPH oxidase
PI3K	Phosphatidylinositide 3-kinases
PKC	Protein kinase C
PLC	Phospholipase C
RAS	Renin Angiotensin System
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
TAC	Transverse aortic constriction
TGF-β	Transforming Growth Factor-β
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TNF-α	Tumour Necrosis Factor-α
TRPC6	Transient Receptor Potential Channel 6
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular Smooth Muscle Cells
YAP	Yes-associated Protein
α-SMA	Alpha-Smooth Muscle Actin

SYNOPSIS

Background of the study:

Cardiac fibroblasts, which are cells of mesenchymal origin, are the only intracardiac source of type I and type III collagens and are importantly involved in the homeostatic maintenance of myocardial extracellular matrix. In response to myocardial injury, normally quiescent cardiac fibroblasts get phenotypically transformed into α-Smooth Muscle Actin (α-SMA)-positive myofibroblasts that aid in wound healing. These cells migrate to the site of injury, proliferate and produce collagen, resulting in the formation of a scar tissue that replaces damaged myocytes to maintain the structural and functional integrity of the heart. However, unlike fibroblasts in non-cardiac tissues, myofibroblasts in the injured myocardium resist apoptosis and continue to synthesize collagen, which, in the long-term, leads to tissue fibrosis, ventricular stiffening and heart failure. Since the phenotypic transition to α-SMA positive myofibroblasts and enhanced collagen type I production are two critical functions of cardiac fibroblasts *post injury*, understanding the molecular mechanisms underlying these two fundamental processes is a clinically important goal.

In this context, collagen receptors in fibroblasts are increasingly implicated in the pathogenesis of fibrosis. Discoidin Domain Receptor 2 (DDR2) and Integrin-β1 are two major collagen receptors in the myocardium. While DDR2 is a fibrillar collagen type I-specific receptor tyrosine kinase whose expression is predominantly in fibroblasts in the cardiac tissue, Integrin-β1 belongs to a family of collagen-binding Integrin receptors that are ubiquitously expressed in fibroblasts and cardiomyocytes. DDR2 and Integrin-β1 have been reported to independently mediate the progression of tissue fibrosis and regulate fundamental cellular processes such as proliferation,

migration and apoptosis resistance. Despite these reports, the existence of a cross-talk between the two collagen receptors and its implications in tissue response to injury and cardiac fibrosis has not been explored.

Objective:

Against this backdrop, the major objective of my study was to test the hypothesis that DDR2/Integrin-β1 crosstalk underlies α-SMA-dependent collagen type I gene expression in Ang II-stimulated cardiac fibroblasts.

Methods:

Primary cultures of cardiac fibroblasts were derived from the ventricular tissue of young adult male Sprague Dawley rats (2-3 months). The cells were cultured in M199 with 10% FBS and characterized using morphological and immunocytochemical criteria. Sub-confluent cultures of cardiac fibroblasts from passage 2 or 3 that were synchronized by serum-deprivation for 24 hours and exposed to Ang II were used as the experimental model. Taqman quantitative Real-time PCR analysis, Western blotting, Immunohistochemistry, Immunocytochemistry, Electrophoretic Mobility Shift Assay (EMSA), Chromatin Immunoprecipitation assay (ChIP), siRNA-mediated gene knockdown and plasmid vector-based overexpression of DDR2, Integrinβ1 and TRPC6 under the regulatory control of the pCMV promoter system were performed following standard protocols. Statistical significance of the data was assessed using one-way ANOVA and Student's *t*-test. $p \leq 0.05$ was considered significant.

Major findings:

Ang II-stimulated Integrin-β1 expression in cardiac fibroblasts is mediated by DDR2

Ang II was found to stimulate integrin-β1 mRNA and protein expression in cardiac fibroblasts. To test the involvement of DDR2 in the regulation of Ang II-stimulated integrin-β1 expression, cardiac fibroblasts transfected with DDR2 siRNA were exposed to Ang II followed by the analysis of integrin-β1 expression. DDR2 silencing significantly attenuated the expression of Ang II-stimulated integrin-β1 mRNA and protein expression. Additionally, knockdown of DDR2 in unstimulated cells reduced basal integrin-β1 expression. Together, the data conclusively demonstrate the role of DDR2 in the regulation of integrin-β1 expression in cardiac fibroblasts under stimulated as well as basal conditions.

DDR2 enhances integrin-β1 expression via ERK1/2-dependent TGF-β1 in cardiac fibroblasts exposed to Ang II:

Next, the mechanisms underlying DDR2-dependent Integrin-β1 expression in Ang II-stimulated cardiac fibroblasts were studied. Knockdown of DDR2 attenuated ERK1/2 activation and TGF-β1 expression in Ang II-treated cardiac fibroblasts. Further, ERK1/2 silencing reduced the expression of TGF-β1, demonstrating a link between DDR2, ERK1/2 and TGF-β1. Further, knockdown of ERK1/2 MAPK and TGF-β1 abrogated the expression of Ang II-stimulated Integrin-β1. Together, the findings demonstrate that DDR2-dependent activation of ERK1/2 MAPK and TGF-β1 mediates integrin-β1 expression in cardiac fibroblasts exposed to Ang II.

The transcriptional regulation of integrin-β1 is mediated by AP-1 downstream of the DDR2-ERK1/2-TGF-β1 axis in Ang II-treated cardiac fibroblasts:

Since ROS was found to mediate Ang II-stimulated integrin-β1 expression, the role of the redox-sensitive transcription factor, AP-1, in the transcriptional regulation of integrin-β1 was probed. Pharmacological inhibition of AP-1 using SR-11302 attenuated Ang II-stimulated Integrin-β1 expression. Ang II induced the nuclear translocation of AP-1, which was attenuated upon silencing of DDR2, ERK1/2 and TGF-β1, demonstrating the role of these factors in acting downstream of DDR2 to activate AP-1. Chromatin immunoprecipitation (ChIP) assay was performed to confirm the role of DDR2-dependent AP-1 in the transcriptional regulation of Integrin-β1. DDR2 knockdown was found to attenuate Ang II-stimulated binding of AP-1 to the Integrin-β1 gene promoter. Considered in tandem, the findings show that, in response to Ang II treatment, DDR2, ERK1/2 and TGF-β1 act in concert to promote AP-1 activation and binding to the Integrin-β1promoter, resulting in the transcriptional upregulation of Integrin-β1.

Regulatory relationship between DDR2 and Integrin-β1 in vivo:

Existence of a regulatory relationship between DDR2 and Integrin-β1 was probed using *in vivo* models. The spontaneously Hypertensive Rat (SHR) is a genetic model of hypertension attributed to excessive activation of the Renin-Angiotensin-Aldosterone System (RAAS) that promotes tissue fibrosis. In cardiac fibroblasts, freshly isolated from 6-month old SHR with established cardiac fibrosis, enhanced DDR2 expression positively correlated with Integrin-β1, associated with an increase in markers of myocardial fibrosis. A direct regulatory relationship between DDR2 and

Integrin-β1 was probed in a DDR2 knockout mouse model. Immunohistochemistry analysis showed that germline deletion of DDR2 significantly decreased Integrin-β1 expression in the myocardium of DDR2 null mice. Since fibroblasts are relatively fewer in the mouse heart, a global reduction in myocardial Integrin-β1 in DDR2 knockout mice suggested a role for DDR2 in the regulation of Integrin-β1 expression in myocytes in addition to fibroblasts. Treatment of H9c2 cardiomyoblasts for 24 h with conditioned media from DDR2-silenced fibroblasts was found to decrease Integrin-β1 expression compared to the control, demonstrating a role for DDR2 in fibroblasts in the regulation of Integrin-β1 expression in myocytes.

DDR2-dependent Integrin-β1 regulates α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts:

Next, the significance of collagen receptor crosstalk in cardiac fibroblast function was explored. Knockdown of DDR2 or Integrin-β1 attenuated Ang II-stimulated increase in α-SMA and collagen type I expression, demonstrating the involvement of these collagen receptors in phenotypic transition and collagen synthesis. Since DDR2 was found to regulate Integrin-β1 expression, the possibility that DDR2-dependent Integrin-β1 mediates α-SMA and collagen type I expression was explored through overexpression studies. Plasmid-based overexpression of Integrin-β1 in DDR2-silenced fibroblasts restored α-SMA and collagen type I expression in the presence of Ang II, suggesting that DDR2/Integrin-β1 crosstalk mediates α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts. Importantly, DDR2/Integrin-β1 crosstalk was also found to mediate the wound healing function of cardiac fibroblasts exposed to Ang II.

α-SMA functions downstream of the DDR2-Integrin-β1 axis to regulate collagen type I expression

Knockdown of α-SMA attenuated Ang II-induced collagen type I expression, demonstrating that cardiac fibroblast phenotypic transition is linked to collagen type I expression. Since α-SMA is involved in myofibroblast mechano-transduction, the role of mechano-sensitive factors in mediating α-SMA-dependent collagen expression was probed. Transient Receptor Potential Channel 6 (TRPC6) is a calcium channel that is reported to regulate fibroblast phenotypic transition and collagen expression. In the present study, overexpression of TRPC6 in α-SMA-silenced cardiac fibroblasts was found to restore collagen type I expression, demonstrating a role for α-SMA-dependent TRPC6 in the regulation of collagen type I expression in Ang II-treated cardiac fibroblasts. Further, the transcriptional regulation of collagen type I downstream of α-SMA and TRPC6 was probed. Yes-associated protein (YAP) is a mechanosensitive transcription factor whose activation is reported to promote tissue fibrosis. Here, inhibition of YAP using Verteporfin abrogated Ang II-induced collagen type I expression, demonstrating a role for YAP in the transcriptional regulation of collagen type I. Knockdown of α-SMA or TRPC6 reduced Ang II-stimulated binding of YAP to collagen type I promoter, demonstrating that α-SMA and TRPC6 coordinately activate YAP to promote its binding to the collagen type I promoter.

Significance of the study:

The findings establish that DDR2-Integrin-β1 crosstalk is a critical determinant of α-SMA-dependent collagen type I expression in Ang II-stimulated cardiac fibroblasts. Notably, the finding that the two collagen receptors act in tandem to regulate phenotypic transition and collagen expression in Ang II-stimulated fibroblasts is novel and of considerable interest. This study uncovers a hitherto unknown role for α-SMA, downstream of the DDR2-Integrin-β1 axis, in the regulation of collagen type I expression in response to Ang II stimulation. This novel observation shows that phenotypic transition is mechanistically coupled to collagen type I expression in cardiac fibroblasts in a setting of myocardial injury. Importantly, the specific localization of DDR2 in cardiac fibroblasts in the myocardium, and its obligate regulatory role in collagen type I expression in these cells, identify it as a potential therapeutic target in the control of cardiac fibrosis.

I. INTRODUCTION

Heart failure is a major health problem and, despite substantial advances in therapeutic strategies, it remains the leading cause of mortality globally (Benjamin Emelia J. *et al.*, 2019). One of the principal characteristics of heart failure is the pathological remodeling of the cardiac tissue due to excessive deposition of collagen type I by cardiac fibroblasts, which leads to myocardial fibrosis, reduced ventricular compliance and cardiac dysfunction. Cardiac fibroblasts are the sole intracardiac source of collagen types I and III, the principal ECM component of the myocardial tissue, and are responsible for extracellular matrix (ECM) homeostasis. These ECM components serve as a scaffold for myocytes and maintain the structural and functional integrity of the heart (Souders *et al.*, 2009). Unlike other organs, the heart has limited regenerative capacity following injury. Hence, reparative processes post-injury involves the removal of damaged myocytes, followed by replacement with a fibrotic scar tissue that preserves myocardial integrity. Cardiac fibroblasts are sentinel cells that respond to pathophysiological stimuli and are primarily involved in the formation of the fibrotic scar tissue. Following myocardial injury, fibroblasts are phenotypically transformed into myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA). These cells migrate to the site of injury where they proliferate and lay down collagen to facilitate wound healing. However, unlike non cardiac fibroblasts, cardiac myofibroblasts resist apoptotic cell death and persist at the site of injury long after termination of the wound healing response, resulting, in the long-term, in adverse myocardial remodeling and pump dysfunction (Eva A Rog-Zielinska *et al.*, 2016).

I.1. *Identification of the problem*

A better understanding of the molecular mechanisms underlying collagen expression in cardiac fibroblasts is essential to devise effective therapeutic strategies to control adverse cardiac fibrosis.

I.1.1. Collagen-binding receptors in ECM remodeling

A large body of evidence points to collagen receptors as important regulators of fibroblast response to tissue injury (Coelho and McCulloch, 2016). In this regard, two major collagen receptors are widely noted for their role in fibrogenesis – Integrin-β1 and Discoidin Domain Receptor 2 (DDR2). While Integrin-β1 is the classical collagen-binding receptor expressed in a variety of cell types, DDR2 is a collagen-binding receptor tyrosine kinase expressed primarily in cells of mesenchymal origin (Jokinen *et al.*, 2004; Ivey and Tallquist, 2016).

DDR2 belongs to a class of Discoidin Domain Receptor tyrosine kinases that constitute a unique class of receptor tyrosine kinases (RTKs) that bind to fibrillar collagens rather than soluble cytokines. Additionally, unlike the quick-on and quick-off pattern of activation of RTKs, the DDRs display slow and sustained activation upon binding to collagen, which influences cell proliferation, migration and differentiation. In contrast to the DDR1 isoform that is expressed primarily in epithelial cells, the DDR2 isoform is expressed primarily in cells of mesenchymal origin and binds to fibrillar collagen types I and III (Leitinger, 2014). In the heart, DDR2 is expressed predominantly in cardiac fibroblasts and is considered a fibroblast-specific marker (Tarbit *et al.*, 2019).

A recent study from our laboratory demonstrated an obligate role for DDR2 in the regulation of collagen type I expression, pointing to its involvement in the pathogenesis of cardiac fibrosis (George *et al.*, 2016). Additionally, Zhao et al (2016) have reported a role for DDR2 in the pathogenesis of idiopathic pulmonary fibrosis.

Integrins constitute a large family of transmembrane glycoproteins that function as receptors for multiple ECM proteins. They are heterodimers consisting of an α subunit and a β subunit. Currently, 18 α subunits and 8 β units have been identified in mammalian cells. These subunits interact to form 24 different subtypes of Integrins. Integrins that bind to fibrillar collagens have β1 as their common subunit. The β1 subunit pairs with cognate α1, α2, α10 and α11 subunits to bind to collagen type I (Jokinen *et al.*, 2004).

Previous studies have reported a role for Integrin-β1 in cell survival, proliferation, migration, differentiation and wound healing (Shibue and Weinberg, 2009; Liu *et al.*, 2010; Aoudjit and Vuori, 2012). In the context of tissue fibrosis, Integrin-β1 is reported to activate profibrotic signaling to mediate the progression of liver fibrosis (Martin *et al.*, 2016).

Despite considerable evidence in support of a role for these collagen receptors in promoting fibrogenesis, the existence of a cross-talk between them and its involvement in the regulation of cardiac fibroblast activation and activity have not hitherto been examined.

I.2. *Broad objective*

Angiotensin II (Ang II), whose intra-cardiac levels are reported to be enhanced following cardiac injury, is a potent pro-fibrotic factor that has been implicated in the phenotypic transition of quiescent fibroblasts to active myofibroblasts. Moreover, Ang II is well known for its stimulatory effects on collagen expression in myofibroblasts (Díez Javier, 2004). Identification of molecular mechanisms underlying phenotypic conversion of cardiac fibroblasts into α-SMA-positive myofibroblasts and enhanced collagen gene expression in response to Ang II can provide novel insights into the molecular basis of cardiac fibrosis.

Against this backdrop, the present study focused on the regulatory relationship between the collagen receptors, DDR2 and Integrin-β1, and investigated the likely role of DDR2-Integrin-β1 crosstalk in mediating α-SMA and collagen type I gene expression in cardiac fibroblasts exposed to Ang II. Further, downstream of the DDR2-Integrin-β1 axis, the study probed the role of α-SMA in the regulation of collagen type I gene expression in Ang II-stimulated cardiac fibroblasts.

I.3. *Specific objectives:*

The following specific questions were addressed in the study:

- The role of DDR2 in mediating Ang II-stimulated expression of Integrin-β1
- Molecular mechanisms underlying DDR2-dependent expression of Integrin-β1 in Ang II-treated cardiac fibroblasts
- Corroboration of the DDR2/Integrin-β1 link in DDR2 knockout mice and Spontaneously Hypertensive Rats (SHR)

- A role for DDR2-Integrin-β1 crosstalk in the regulation of Ang II-stimulated α-SMA and collagen type I expression
- A regulatory role for α-SMA, downstream of the DDR2-Integrin-β1 axis, in mediating Ang II-stimulated collagen type I expression

I.4. *Major findings:*

Using a combination of gene knockdown/overexpression approaches, electrophoretic mobility shift assay and chromatin immunoprecipitation, the present study demonstrated that Discoidin Domain Receptor 2 acts via ERK1/2 MAPK- and TGF-β1-dependent activation of AP-1 transcription factor to enhance the expression of collagen-binding Integrin-β1 in Ang II-stimulated cardiac fibroblasts. Studies on DDR2-knockout mice and Spontaneously Hypertensive Rats corroborated the DDR2-Integrin-β1 link in vivo. The study also demonstrated a role for DDR2-Integrin-β1 crosstalk in the regulation of α-SMA and collagen type I expression and the wound healing ability of cardiac fibroblasts exposed to Ang II. Downstream of the DDR2-Integrin-β1 axis, ILK/Akt signaling was found to regulate α-SMA and collagen type I expression. Importantly, α-SMA was found to activate the Ca^{2+} channel, TRPC6, and the mechanosensitive transcription factor - Yes-associated Protein (YAP), to transcriptionally up-regulate the expression of collagen alpha1(I). These findings demonstrate for the first time that phenotypic transition of cardiac fibroblasts into myofibroblasts is mechanistically linked to collagen type I expression. The observation that DDR2-Integrin-β1 crosstalk underlies α-SMA-dependent collagen expression provides a novel conceptual framework to understand the regulation of collagen expression in cardiac fibroblasts.

II. REVIEW OF LITERATURE

The myocardium is a complex and well-organized system consisting of cellular elements displaying an array of receptors and intracellular signaling molecules that facilitate and regulate cellular response to external stimuli. The myocardial ECM intimately interacts with cellular receptors to play a critical role in physiology and in the pathophysiology of disease (Nepomnyashchikh *et al.*, 2001). Understanding cell-ECM interactions could provide valuable insights into the mechanisms underlying the role of ECM receptors in the progression of myocardial disease.

II.1. *The Heart*

The cardiac tissue is a highly organized structure comprising the parenchyma and stroma. Coordinated and dynamic interaction between the cardiac parenchyma and stromal compartments dictates myocardial function. Parenchyma, made of cardiomyocytes, represents the functional compartment of the cardiac tissue, while the stroma is made up of non-myocyte cells embedded within a connective tissue scaffold. Although cardiomyocytes account for most of the myocardial mass, cells comprising the myocardial stroma account for approximately 70% of the myocardial cell population. These cells include fibroblasts, endothelial cells and vascular smooth muscle cells. Of these, cardiac fibroblasts represent an important cell type that produces and maintains the stromal connective tissue network that contributes to the tensile strength and stiffness of the myocardium (Rohr Stephan, 2012). This connective tissue framework is organized into the endomysium, that surrounds individual cardiomyocytes, the perimysium, that tethers together groups of myocytes, and the epimysium, that encases the cardiac tissue as a whole. Proper functioning of the cardiac connective tissue determines myocardial function while its

disproportionate growth leads to adverse myocardial remodeling and heart failure (Katz, 2010) .

II.2. *Cardiac fibroblasts:*

In understanding cardiac development, physiology, and pathogenesis of disease, the role of cardiac fibroblasts had been overlooked and myocardial dysfunction was viewed mainly in terms of cardiomyocyte loss following injury. However, with the advent of modern lineage mapping technologies, the role of cardiac fibroblasts in myocardial physiology and disease pathogenesis are being increasingly appreciated. These studies have led to the understanding that the role of cardiac fibroblasts is highly contextual and varying, from being helpful under physiological conditions, to being harmful in a pathophysiological setting. Hence, today, it is recognized that cardiac fibroblasts serve not only as a critical player in maintenance of normal cardiac function, but also as a pro-active participant in response to cardiac injury (Lajiness and Conway, 2014).

II.2.1. Morphology and markers:

Fibroblasts are cells of mesenchymal origin and are widely distributed within the connective tissues of vertebrate organisms. Morphologically, fibroblasts are flat, spindle shaped cells consisting of sheet-like extensions originating from the main cell body. A defining feature of the fibroblast that separates it from other cell types is the absence of a basement membrane. When active, fibroblasts display a prominent golgi apparatus and extensive rough endoplasmic reticulum. There is increasing interest in the identification of novel markers that define the quiescent and active states of

fibroblasts. DDR2, a collagen-specific receptor tyrosine kinase was among the first markers used to identify cardiac fibroblasts. DDR2 is robustly expressed in cardiac fibroblasts with a limited expression in cell types such as smooth muscle cells and endothelial cells (Souders *et al.*, 2009). Another major fibroblast-specific marker that has recently been identified is the platelet-derived growth factor receptor α (PDGFRα). Expressed robustly in cardiac fibroblasts during development and in healthy and injured tissue, it is used extensively for the development of inducible Cre-loxP systems, acting as a valuable tool in fibroblast characterization and its role in disease. The transcription factor, TCF-21, has been used successfully as a fibroblast-specific marker to study the embryonic origins of resident cardiac fibroblasts from the epicardium. Together, DDR2, PDGFRα and TCF-21 are the three major markers used for identification of cardiac fibroblasts. In addition to the molecular markers used to identify quiescent cardiac fibroblasts, a different subset of markers is used to identify active fibroblasts, termed as myofibroblasts. These markers include α-SMA and Periostin (Tallquist and Molkentin, 2017).

II.2.2. Origin of cardiac fibroblasts:

Cardiac fibroblasts were considered to be a homogenous cell population. However, recent lineage-tracing studies have revealed that fibroblasts are a complex and heterogenous population of cells having diverse origins that depend upon the developmental stage and physiological conditions. During embryogenesis, fibroblasts are mesenchymal in origin and arise primarily through the differentiation of cells from the proepicardial organ in the developing heart. These cells undergo epithelial to mesenchymal transition to form epicardial-derived cells (EPDCs) that invade the

atrial and ventricular tissue to differentiate progressively into a fibroblast phenotype. Studies have also demonstrated a role for bone marrow-derived multipotent progenitor cells known as mesangioblasts in the developmental origin of cardiac fibroblasts (Lajiness and Conway, 2014).

In the healthy heart, fibroblast numbers are maintained through the proliferation of resident fibroblasts in the myocardium. However, following injury, in addition to resident fibroblasts, recruitment of fibroblasts occurs from epithelial cells through epithelial to mesenchymal transition and from endothelial cells through endothelial to mesenchymal transition. Studies have also shown bone marrow-derived progenitor cells, monocytes, fibrocytes and perivascular cells as being important contributors to the fibroblast population in a setting of cardiac injury (Krenning *et al.*, 2010).

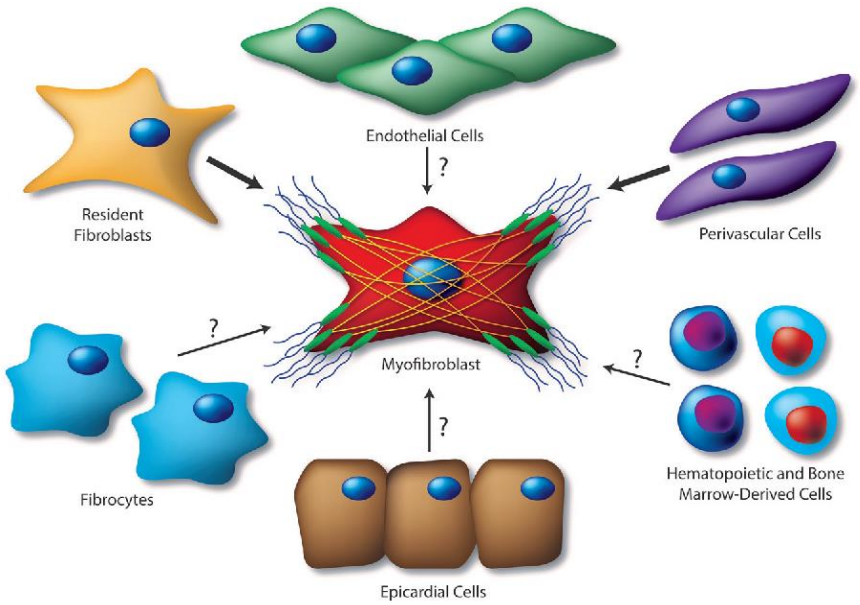


Figure 1: Origin of cardiac fibroblasts (adapted from (Travers Joshua G. *et al.*, 2016)

II.2.3. Organization of fibroblasts in the myocardium:

Although cardiomyocytes constitute the bulk of the myocardial volume, cardiac fibroblasts are the most numerous cell types in the heart. Cardiac fibroblasts are surrounded by a network of ECM proteins comprised primarily of fibrillar collagen, whose three-dimensional network starts to form during fetal development and matures during neonatal development. During this process, a network of collagen fibers produced by fibroblasts surrounds groups of myocytes to form the endomysium. Within the endomysium, cardiac fibroblast forms a network of interconnected cellular processes that surrounds neighboring cardiomyocytes. This arrangement allows fibroblasts to link myocytes with the extracellular collagen network, aiding in the transmission of myocyte contractile force to the collagen network. Cardiac fibroblasts interact with the ECM through Integrins and DDR2. In addition, connexins enable cell-cell contacts. Connexin 43 connects fibroblasts to myocytes while connexin 45 enables fibroblasts to fibroblast connections. These dynamic interactions enable cardiac fibroblasts to maintain proper form and function of the cardiac tissue (Baudino *et al.*, 2006; Goldsmith *et al.*, 2014).

II.3. *Pleiotropic functions of cardiac fibroblasts:*

Cardiac fibroblasts are termed as sentinel cells of the myocardium and performs a multitude of functions in addition to their role of being the primary matrix-producing cells in the cardiac tissue (Souders *et al.*, 2009).

II.3.1. ECM homeostasis:

Cardiac fibroblasts are placed at the center of myocardial ECM remodeling owing to their ability to maintain ECM homeostasis through regulation of ECM secretion and degradation (Krenning *et al.*, 2010). They are the sole source of myocardial collagen types I and III in addition to other collagen types (IV, V, VI), proteoglycans, elastin, glycoproteins and proteases (Baudino *et al.*, 2006; Fan *et al.*, 2012). The primary structural component of the myocardial ECM is collagen type I that accounts for 85% of the myocardial ECM. Having a tensile strength equivalent to that of steel, fibrillar collagen type I maintains the rigidity and tensile strength of the cardiac tissue. Collagen type III accounts for 10-15% of the myocardial ECM and contributes to distensibility of the myocardial tissue (Weber *et al.*, 2013). During injury, myofibroblast-mediated deposition of collagen type I at the site of myocyte loss aids in wound healing and preserves myocardial structural and functional integrity (Weber *et al.*, 2013). Regulation of ECM turnover through balance in ECM synthesis and degradation is important for the maintenance of cardiac function. Increased ECM deposition between layers of myocytes disrupts electrical conduction, leading to impairment of contractility and arrythmias (Gabbiani, 2003; Thompson Susan A. *et al.*, 2011). In this regard, cardiac fibroblasts mediate ECM homeostasis through balancing ECM production with degradation. Fibroblast-mediated degradation of ECM occurs through the activity of matrix metalloproteinases (MMPs) that are regulated in turn by tissue inhibitors of matrix metalloproteinases (TIMPS). Fibroblasts regulate the MMP/TIMP ratio that in turn determines the net proteolytic activity in the myocardium (Spinale, 2007).

II.3.2. Mechanical signaling:

Cardiac fibroblasts are continuously subjected to mechanical stretch in the myocardium. Proper regulation of mechanical signaling is necessary for maintenance of cardiac function. Recent studies have demonstrated a role for mechanical stretch in regulating expression of ECM proteins, ECM receptors and growth factors in cardiac fibroblasts (MacKenna *et al.,* 2000). Mechanical stretch of fibroblasts has also been shown to increase the expression of various matrix metalloproteinases leading to ECM degradation (Tyagi *et al.*, 1998). Mechanical cues are major regulators of cardiac fibroblast activation and act directly on fibroblasts or indirectly through paracrine mediators derived from myocytes exposed to mechanical stretch. For example, mechanical stretch applied on myocyte cultures stimulate Ang II production that in turn stimulates growth factor and cytokine production in cardiac fibroblasts. Further, fibroblast proliferation was reported to increase when exposed to conditioned media derived from myocytes exposed to stretch (Herum *et al.*, 2017). Mechanical forces are translated into changes in fibroblast gene expression through action of ECM receptors such as Integrins and DDR2 (Bayer *et al.*, 2019). Recently, mechanical stretch was also shown to regulate the transcription of pro-fibrotic genes via activation of the transcription factors - Yes-associated protein (YAP) and Myocardin-related Transcription Factor (MRTF) (Finch-Edmondson and Sudol, 2016). In addition to these factors, the cardiac fibroblast AT1 receptor has been shown to be activated by mechanical stretch *in vitro* and *in vivo* in the absence of Ang II, leading to expression of procollagen and MMPs (Li *et al.*, 1998). Together, these receptors participate in mechano-transduction through activation of

intracellular signaling pathways involving various kinases such as FAK, and ILK (Wang *et al.*, 2001; Srivastava and Yu, 2006).

II.3.3. Chemical signaling:

Cardiac fibroblasts are an important source of cytokines, chemokines and other soluble molecules that act in an autocrine or paracrine manner to modulate cardiac function. The pro and anti-fibrotic actions of these soluble factors contribute to the developmental, physiological and pathophysiological activity of fibroblasts. Some of the important chemical factors secreted by cardiac fibroblasts include Ang II, Interleukin-1 (IL-1), Transforming Growth Factor-β (TGF-β), Vascular Endothelial Growth Factor (VEGF) and Insulin like Growth Factor-1 (IGF-1) (Baudino *et al.*, 2006). Ang II is the effector molecule in the Renin-Angiotensin system (RAS) that plays an important role in the regulation of blood pressure and pathogenesis of heart failure. There is a circulatory RAS as well as localized cardiac RAS. In the circulatory RAS cascade, renin cleaves angiotensinogen to Ang I that is processed into Ang II by Angiotensin-converting enzyme. In the cardiac RAS cascade, fibroblasts express renin, angiotensinogen and ACE that allows for local production of Ang II (Dostal and Baker, 1999). In vitro studies have demonstrated that Ang II enhances fibroblast proliferation and expression pro-fibrotic genes. Importantly, Ang II stimulates the expression of TGF-β, endothelin-1 and Interleukin-6 whose concerted actions result in adverse myocardial remodeling (Kawano *et al.*, 2000a). TGF-β is another major factor secreted by cardiac fibroblasts. TGF-β is a potent pro-fibrotic factor that is activated in response to Ang II stimulation and plays an important role in fibroblast activation and expression of collagen and fibronectin (Li

et al., 2013). In addition to these factors, fibroblasts produce fibroblast growth factor and Vascular endothelial growth factor (VEGF) that act on vascular endothelial cells to promote angiogenesis and collateral vessel formation in the injured myocardium (Nehls *et al.*, 1998).

II.3.4. Electrophysiological signaling:

Cell junctions formed by connexins are important for inter-cellular communication and play a similar role in physical communication between fibroblasts and other cells in the cardiac tissue. It has been demonstrated that fibroblast-myocyte coupling can occur through connexin 43 and connexin 45. Functional coupling of fibroblasts to neighboring fibroblasts occurs through connexin 40 and 43. Such functional coupling between fibroblasts and other cells connects different regions of myocytes that would otherwise remain electrically isolated by connective tissue. Cardiac fibroblasts also express ion channels such as K^+ channels and Na^+ channels that influence myocyte function (Souders *et al.*, 2009).

II.4. The cardiac myofibroblast:

Myofibroblasts are defined as fibroblasts that exhibit a prominent endoplasmic reticulum and contractile α-SMA filaments (Souders *et al.*, 2009b). α-SMA expression is used as the standard marker for identification of myofibroblasts (Wang *et al.*, 2012). Expression of α-SMA enables migration and contraction of the scar tissue that preserves the functional integrity of the myocardium post-injury (Eva A Rog-Zielinska *et al.*, 2016). Typically, myofibroblasts are characterized by the expression of a specific set of cytoskeletal markers including α-SMA. These help in

distinguishing myofibroblasts from Vascular Smooth Muscle Cells (VSMCs) expressing α-SMA. In the normal tissue, smooth muscle cells express differentiation markers such as smooth muscle myosin heavy chain, desmin, h-caldesmon and smoothelin that are unique to these cell types and are not expressed in myofibroblasts (Xie *et al.*, 2011). However, during fibrosis, tissue injury or in cell culture, smooth muscle cells lose these specific markers and attain a myofibroblast-like contractile state, expressing α-SMA and producing collagen (Rzucidlo *et al.*, 2007). Hence, there are inherent difficulties in identifying and distinguishing myofibroblasts from smooth muscle cells. To add to this complexity, both smooth muscle cells and endothelial cells express vimentin that is expressed by myofibroblasts. Currently, co-staining with desmin (for identification of smooth muscle cells, myocytes), CD31 (for identification of endothelial cells) and α-SMA (for identification of myofibroblasts) is used to identify and distinguish these major cell types that populate the cardiac tissue (Pinto Alexander R. *et al.*, 2016). Recent studies have suggested periostin to be specifically expressed by myofibroblasts. Transgenic mice expressing β-galactosidase under the control of periostin promoter is used to distinguish myofibroblasts from other cell types in the myocardium (Tallquist and Molkentin, 2017).

II.4.1. Myofibroblasts and their role in wound healing:

Cardiomyocytes are terminally differentiated cells that fail to regenerate post-injury and are eventually lost. In this scenario, cardiac fibroblasts facilitate the process of wound healing through a mechanism involving three overlapping phases - the adaptive phase, inflammatory phase and fibrogenic phase. The early adaptive phase of the wound healing response is characterized by an alteration in the MMP/TIMP ratio, leading to changes in proteolytic activity in the cardiac tissue. An increase in MMP expression and activity facilitates ECM degradation, allowing infiltration of inflammatory cells to the site of injury. Here, the dead myocytes and cellular debris are cleared through phagocytosis by inflammatory cells. The subsequent inflammatory phase is marked by the production of Ang II by inflammatory macrophages at the site of injury. These cells express renin and ACE that aid in the local intra-cardiac generation of Ang II. Excessive Ang II production upregulates TGF-β expression that acts in concert with other inflammatory cytokines to activate fibroblasts. Consequently, phenotypically quiescent cardiac fibroblasts are activated to α-SMA positive myofibroblasts that migrate to the site of injury and proliferate. The fibrogenic phase succeeds these events wherein, myofibroblasts deposit matrix proteins such as collagens type I and III, fibronectin and proteoglycans at the site of myocyte loss. The deposition of these proteins by myofibroblasts lead to the formation of a collagen-rich scar tissue that aids in wound healing of the cardiac tissue. This phase is also known as reparative fibrosis (Travers Joshua G. *et al.*, 2016).

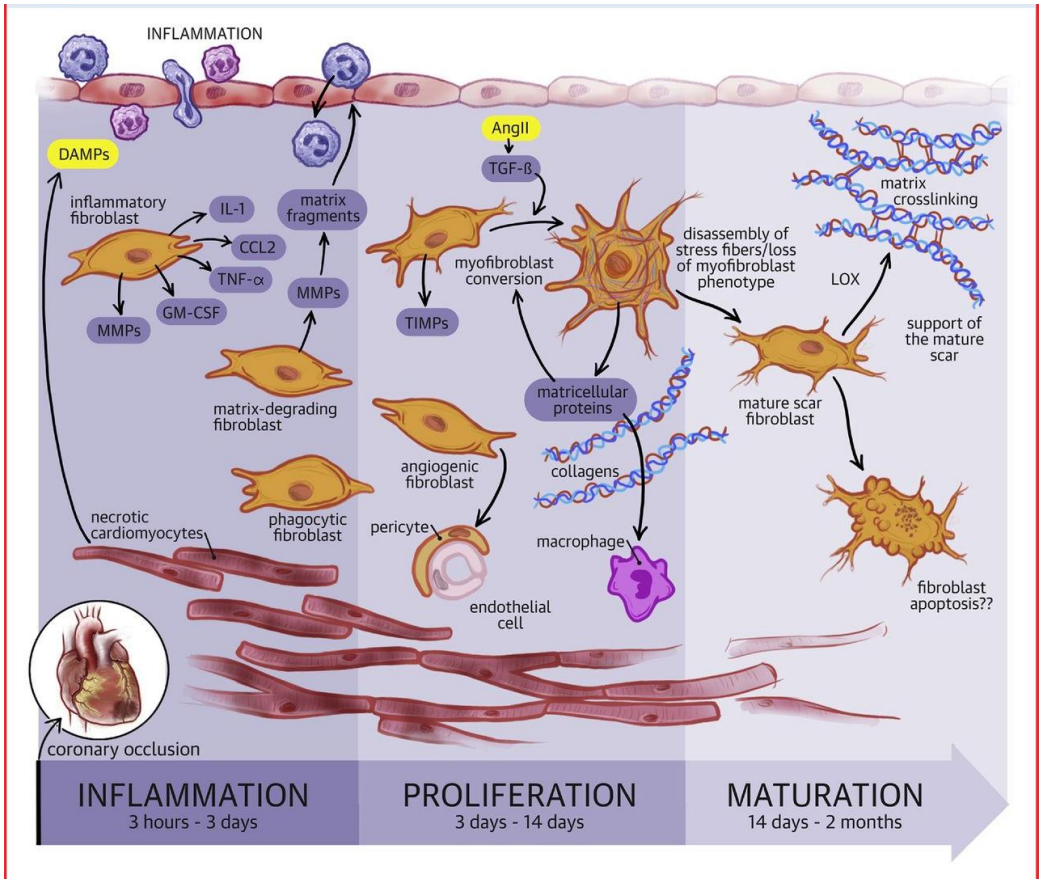


Figure 2: Fibroblast-mediated wound healing response adapted from: (Humeres and Frangogiannis, 2019)

II.4.2. Myofibroblasts and their role in cardiac fibrosis:

Owing to the inherent ability of cardiac fibroblasts to resist apoptotic cell death, these cells continue to proliferate and deposit collagen long after the termination of the wound healing response following injury. Myofibroblasts have been shown to persist for months and even years in an infarct scar (Willems *et al.*, 1994). Soluble signals generated at the site of injury traverse the cardiac interstitium to sites distant from the zone of injury where they activate fibroblasts, resulting in reactive fibrosis. Fibrosis is central to tissue remodeling in the diseased myocardium. In hypertensive

heart disease, diffuse patterns of interstitial fibrosis are seen in association with focal myocyte degeneration (Okoshi *et al.*, 1997). In the ischemic myocardium, diffuse scarring is observed at areas distant from the site of injury and are indicative of reactive fibrosis (Sun and Weber, 2000). Likewise, hypertrophic cardiomyopathy is extensively associated with fibrosis (Creemers and Pinto, 2011). Tissue fibrosis has multiple adverse effects in the myocardium. Firstly, excessive deposition of fibrillar collagen causes the collagen tendrils to ensnare surrounding myocytes and reduce myocyte workload which, in the long-term, leads to myocyte atrophy. Notably, myocyte loss through apoptosis following injury and atrophy of the remaining myocytes contribute to the progressive nature of heart failure (Kamalov *et al.*, 2013).

Secondly, excessive deposition of stiff and heavily cross-linked collagen type I by myofibroblasts increases the passive stiffness of the cardiac tissue that leads to diastolic dysfunction (Yamamoto *et al.*, 2002). Thirdly, fibrous tissue disrupts the propagation of electrical signals in the heart, leading to arrythmias. Connexin channels allow current flow between myocytes and hence play a major role in electrical impulse propagation through the cardiac tissue. Deposition of collagen disrupts these connexin-mediated cell-cell contacts, leading to arrythmias (Morita *et al.*, 2014). Also, expression of α-SMA by myofibroblasts contributes to abnormal impulse conduction (Rosker Christian *et al.*, 2011). Fourthly, the heart is an obligate aerobic organ that needs an uninterrupted supply of oxygen and nutrients. In the normal course, this is met by the coronary vessels that supply oxygen and nutrients to the myocardium. However, perivascular fibrosis mediated by adventitial fibroblasts in the coronary vessel wall may limit oxygen and nutrient diffusion to the

surrounding cardiac tissue, leading to myocyte necrosis (Kai *et al.*, 2006). Collectively, myofibroblast-mediated adverse remodeling of the heart through excessive deposition of fibrillar collagen leads to reparative and reactive forms of fibrosis that adversely impact cardiac pump function. These actions of myofibroblasts have led to the clinical recognition of the concept of 'interstitial heart disease' (Weber *et al.*, 1989).

II.5. The cardiac extracellular matrix:

Myocardial cells are tethered within an extracellular matrix (ECM) consisting of a network of stable structural proteins and macromolecules. Fibrillar collagen is the dominant protein within the cardiac ECM and constitutes ~85% of the collagen in non-human primates and rodents. Collagen types III and V account for ~10-15% and 5%, respectively. These relative levels among different collagen types are kept in balance in the normal myocardium. However, at sites of tissue fibrosis, collagen type I accounts for ~90% of the total collagen content. Cardiac fibroblasts are the sole source of these fibrillar collagen types and are responsible for collagen homeostasis through maintaining a balance between collagen synthesis and degradation (Weber, 1989). The myocardial ECM is organized into three interconnected layers of epimysium, perimysium and endomysium. The endomysium surrounds and interconnects myocytes while the perimysium weaves together groups of cardiomyocytes and organizes them into muscle bundles. The epimysium encases the entire cardiac tissue. The collagenous ECM integrates and preserves cardiac function. The organized collagen scaffold promotes the transmission and co-ordination of forces generated within myocytes to enable cardiac contraction and

relaxation. The collagenous scaffold also prevents myocyte slippage and preserves ventricular geometry, preventing wall thinning, deformation and rupture. Owing to extensive cross-linking of the collagen fibers by lysyl oxidases during the process of collagen fibrillogenesis, the collagen fibers have a high tensile strength that enables them to resist deformation and prevent sarcomeres from stretching beyond optimal lengths. Under normal conditions, collagen fibers are not physically disrupted until ventricular distension exceeds 100 mmHG and the complete rupture of infarcted ventricle occurs at pressures exceeding 600 mmHG. Additionally, collagen forms intercellular struts that links ECM to intracellular cytoskeletal proteins. These linkages enable myocardial stretch to translate into changes in gene expression.

While fibrillar collagens provide strength and distensibility to the myocardium, non-fibrillar collagens types IV and VI populate the basement membrane of cardiomyocytes. Collagen type IV interacts with laminin, perlecan and entactin to form a sheet-like scaffold in the basement membrane. Collagen type VI interacts with collagen type IV and collagen type I, forming an anchor between the basal laminae and interstitium (Weber, 1989). Cellular fibronectin is another major component of the cardiac ECM with roles in cardiac development and disease. Fibronectin contains a number of binding sites for cell receptors and pro-fibrotic factors such as TGF-β and growth factors such as VEGF, bone morphogenetic protein (BMP1) and fibroblast growth factor (FGF). Hence, it acts as a critical regulator of cell signaling in the myocardium. Alternative splicing of fibronectin to the ED-A splice variant occurs during wound healing and fibrosis and is reported to mediate fibroblast activation (Alandi *et al.,* 2016).

II.6. Collagen receptors in matrix biology:

Collagen receptors are important mediators of cardiac fibroblast response to tissue injury. Collagens are triple helical molecules and they interact with their cognate receptors through specific motifs. Their binding to cognate receptors on fibroblasts regulate a wide range of cellular functions such as adhesion, migration, proliferation and differentiation. Four classes of collagen-binding receptors are present. Discoidin Domain Receptors (DDRs), Integrins, glycoprotein VI (GPVI) and leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1). Collagen binds to these classes of receptors in its native triple helical conformation. Integrins and DDRs represent the most ubiquitously expressed collagen-binding receptors. Although they are structurally unrelated, both Integrins and DDRs bind to specific peptide sequence motifs within the native collagen molecule and share common cellular functions. GPVI and LAIR-1 are structurally-related collagen receptors that perform opposite cellular functions. While GPVI serves as a platelet activator, LAIR-1 inhibits their activity (Leitinger, 2011).

II.6.1. Discoidin Domain Receptors:

DDRs are fibrillar collagen-binding receptor tyrosine kinases. Unlike other receptor tyrosine kinases that bind to soluble ligands, DDRs bind to collagen and exhibit a slow and sustained activation post-binding. Since collagen is abundantly present, the slow activation kinetics of DDRs has been postulated to be an evolutionary adaptation to avoid rapid and excessive receptor activation in the presence of excess collagen. Hence, DDRS may need a specific threshold of collagen concentration or an adequate exposure time for ample activation. Structurally, DDRs contain a ligand-

binding extracellular domain (ectodomain), a single transmembrane domain and an intracellular tyrosine kinase domain. The ectodomain of DDRs consists of two domains: the N-terminal DS domain and a globular domain that is unique to the DDR family. The DS domain is homologous to the Discoidin 1 protein found in *Dictyostelium discoideum*. The external domain is linked to the cytosolic domain by a single transmembrane region. The cytosolic domain consists of a C-terminal tyrosine kinase domain and a large juxtamembrane domain. As opposed to most receptor tyrosine kinases that are expressed as monomers in the absence of ligand binding, DDRs are expressed as constitutive dimers on the cell surface. Upon binding to collagen, they undergo autophosphorylation at their cytosolic tyrosine domains and regulate key cellular functions. Despite being unique, the biochemical and cellular mechanisms activated by DDRs remain poorly defined (Carafoli and Hohenester, 2013).

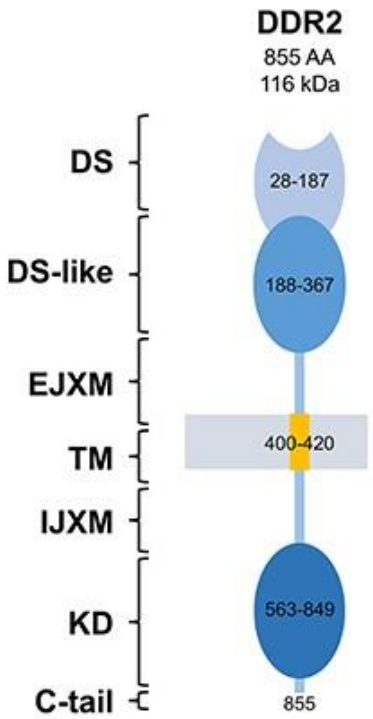


Figure 3: Structure of Discoidin domain receptor 2

II.6.2. Types and features of DDRs:

The DDR family of receptors comprises two isoforms - DDR1 and DDR2. DDR1 is expressed mainly in epithelial cells while the expression of DDR2 is limited to cells of mesenchymal origin such as fibroblasts. The DDRs bind specifically to fibrillar collagens. DDR1 binds to collagen type I, IV and V while DDR2 binds primarily to collagen type I and, to a lesser extent, to collagens II, III and V (Leitinger, 2011). The function of DDRs is similar to that of collagen-binding integrins in that, they recognize specific peptide motifs within collagen. DDR1 and DDR2 bind to the GVMGFO motif present in collagen types I-III. DDR2 is also known to bind to

additional motifs, however, the exact sequences of these peptide motifs have not been determined (Carafoli and Hohenester, 2013).

II.6.3. Discoidin Domain Receptor 2:

DDR2 is a fibrillar collagen-binding receptor tyrosine kinase predominantly expressed in cells of mesenchymal origin. It mediates critical cellular processes, including cell proliferation, migration, adhesion and ECM remodeling. Over-expression of DDR2 is associated with progression of atherosclerosis, osteoarthritis and tumors (Borza and Pozzi, 2014). In humans, the gene encoding DDR2 is located on chromosome 1 at position 1q23.3 and consists of 19 exons that encode a single mRNA transcript. Similar to other RTKs, DDR2 undergoes receptor autophosphorylation within tyrosine residues located in its cytosolic domain. However, in contrast to other RTKs, the activation process in DDRs is slow and sustained (Carafoli and Hohenester, 2013). The downstream signaling cascades activated by DDR2 have been characterized. In vitro studies show Src kinase to associate with the DDR2 cytosolic domain and contribute to DDR2 activation in COS7 cell lines and primary rat hepatic stellate cells. Src phosphorylates DDR2 on three critical tyrosine residues, Y736, Y740 and Y741 located within the activation loop of DDR2 kinase domain. This results in subsequent autophosphorylation activity of DDR2 at other tyrosine residues (Yang *et al.*, 2005). Mutational studies demonstrate that the substitution of Y740 for phenylalanine results in constitutive DDR2 kinase activity, suggesting an inhibitory function for this residue in the regulation of DDR2 activation. DDR2 has been shown to require Src activity to be maximally phosphorylated. Further, Src and its adaptor protein Shc, function as key

signaling intermediates in DDR2 signal transduction (Yang *et al.*, 2005). In this regard, Src is reported to mediate DDR2-dependent activation of the matrix metalloproteinase-2 promoter (Ikeda *et al.*, 2002). Several research groups have characterized the downstream signaling components activated by DDR2, including Src, Shc, ERK ½, JNK and PI3K (Yang *et al.*, 2005; Ruiz and Jarai, 2011). A role for DDR2 in the activation of ERK1/2 MAPK-mediated nuclear translocation of SNAIL transcription factor and its involvement in EMT has been reported (Zhang *et al.*, 2013). Further, studies also show DDR2 to mediate Runx2 transcription factor activation in osteoblasts via activation of p38 MAPK (Zhang *et al.*, 2011a). The regulation of DDR2 by chemical and mechanical factors has also been studied. Mechanical stretch has been shown to enhance DDR2 expression via the action of Ang II in vascular smooth muscle cells (Shyu Kou-Gi *et al.*, 2005).

II.6.4. Role of DDR2 in development and disease:

Targeted germline deletion of DDRs has helped elucidate their role *in vivo*. DDR2 germline deletion led to reduced proliferation of chondrocytes and the shortening of bones. It is also observed that DDR2 knockout mice exhibit dwarfism, mostly due to its involvement in the regulation of skeletal growth (Al-Kindi *et al.*, 2014). A study by Kano et al., 2008 reported spontaneous mutations arising in the DDR2 gene in mice, leading to a loss in DDR2 function. These mice, termed as *Slie* mice, are infertile, pointing to a role for DDR2 in gonadal function (Kano *et al.*, 2008). A role for DDR2 in the regulation of skeletal development was found in chondrodysplasia, a rare autosomal recessive disease, characterized by stunted growth, skeletal abnormalities and premature calcification (Holt *et al.*, 2012). DDR2 has also been

reported to promote skeletal growth through regulating chondrocyte maturation and osteoblast differentiation (Zhang *et al.*, 2011).

The role of DDR2 during development has been characterized through analysis of mutations in the DDR2 gene and mapping them to phenotypic characteristics. A loss of DDR2 function has been linked to progression of osteoarthritis and rheumatoid arthritis (Wang *et al.*, 2002; Zhao *et al.*, 2014). DDR2 has been shown to play a major role in the progression of cancer. It is reported to enhance tumor cell metastasis though the collagen rich ECM by promoting the process of EMT(Ren *et al.*, 2014). Additionally, in the breast cancer microenvironment where mesenchymal stem cells are in contact with breast cancer cells, DDR2 expression in mesenchymal stem cells has been reported to induce activation of DDR2 in breast cancer cells to promote tumor metastasis (Gonzalez *et al.*, 2017).

II.6.5 DDR2 in cardiovascular biology:

Germline deletion of DDR2 led to a decrease in cardiac size, reduced sarcomere lengths and a mild impairment in ventricular function (Cowling *et al.*, 2014). DDR2 is used to characterize cardiac fibroblasts in the developing heart and is a cardiac fibroblast marker (Souders *et al.*, 2009). Characterization studies revealed that DDR2 expression is localized within the epicardium on embryonic day 11, which then progresses to expression within the myocardium and ventricular septal walls by embryonic day 16. This expression pattern of DDR2 is consistent with the migratory movement of cardiac fibroblasts in the developing heart where cells from the epicardium undergo EMT and migrate to the myocardium where they differentiate into cardiac fibroblasts (Morales *et al.*, 2005). Since DDR2 is a receptor for fibrillar

collagen type I, the most abundantly expressed myocardial ECM protein (Leitinger, 2011), exploration of mechanisms underlying DDR2-mediated collagen expression in cardiac fibroblasts is important.

In the vessel wall, DDR2 expression is localized to adventitial fibroblasts and VSMCs (Kuwabara Jill T. and Tallquist Michelle D., 2017). Collagen remodeling plays a major role in arterial remodeling and the progression of hypertension. However, although a role for the DDR1 isoform in the progression of atherosclerosis has been reported (Franco Christopher *et al.*, 2008), a role for DDR2 in adverse vascular remodeling and atherosclerotic disease progression has not been reported. Additionally, the role of DDR2 in vascular cells remains unclear since there are conflicting reports on its involvement in smooth muscle cell proliferation, migration and collagen synthesis (Hou *et al.*, 2012).

II.7. Integrins:

Integrins are transmembrane receptors that act as bridges connecting the cell to the ECM. A primary function of integrins is to connect the ECM with the cellular cytoskeletal network. Structurally, integrins are heterodimers comprising two subunits termed the α subunit and the β subunit. In mammals, there are 18 different α and 8 β subunits that heterodimerize in various combinations, giving rise to 24 different types of Integrins. Each integrin subunits generally consists of a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail domain. The cytoplasmic tails of various β subunits are homologous. Generally, integrins transmit signals and bind to intracellular cytoskeletal proteins via the cytoplasmic tail of the β subunits. Integrins represent an important component of the

cell adhesion complex and bridges two networks across the cell plasma membrane: the ECM network and the intracellular actin network. Integrins do not inherently possess enzymatic activity and therefore rely on signaling adaptors such as Integrin-linked kinase (ILK), Focal adhesion kinase (FAK), talin, vinculin and paxillin to modulate biochemical pathways and cell function (Schwartz, 2001).

II.7.1. Integrin signaling:

Integrins are bidirectional signaling receptors. When bound by a ligand, integrins can activate a host of intracellular biochemical signaling events. Termed as outside-in signaling, this couples ECM signals outside the cells to changes in gene expression, resulting in cell growth, proliferation, differentiation and migration. Another type of signaling involving integrins is termed inside out signaling. In this mode of signaling, regulatory molecules activate signaling events that change the conformation of integrins from an inactive to active state or vice-versa. This enables integrins to bind to ECM ligands (Humphries *et al.*, 2019).

II.7.2. Integrin activation:

The affinity of Integrins for extracellular ligands is regulated through modulating integrin activation via an inside-out signaling mechanism. This mechanism was first appreciated in platelets where integrin activation plays a critical role in platelet adhesion and coagulation (Du *et al.*, 1991). In its inactivated state, integrins appear at a bent conformation near the cell membrane through inhibitory interactions between the α and β subunits (Cooper and Giancotti, 2019). A large body of evidence point to talin as a critical effector of integrin activation. X-ray crystallographic studies have

shown that the binding of a FERM domain of talin to a conserved motif on the β cytoplasmic tail causes conformational changes in the structure of the β subunit, that prevents inhibitory interactions with the α subunit. Thus, binding of talin to β subunit cytoplasmic tail activates integrin (Calderwood, 2004). This is supported by studies in knockout mice that show the importance of talin-integrin interaction in platelet adhesion (Nieswandt *et al.,* 2007). The activation of platelet integrin αIIbβ3 remains the best characterized pathway demonstrating mechanisms in talin-mediated integrin activation. This signaling involves thrombin-triggered activation of PKC that in turn activates small GTPase RAP1. RAP1 GTPase then binds to talin, resulting in tailin activation and binding to integrin β subunit cytoplasmic tail, leading to integrin activation (Petrich *et al.,* 2007). The kindlin family of proteins are also shown to be involved in integrin activation. Following binding of these activating proteins to integrin-β cytoplasmic subunit, conformational changes are propagated across the transmembrane domain, resulting in increased affinity of integrins to ECM ligands (Moser *et al.,* 2008).

II.7.3. Mechanisms in integrin-mediated signaling:

The binding of small clusters of integrins to ECM ligands results in adhesion complexes being formed at the cell membrane surface. These focal adhesions formed by integrins act as a link between the ECM and intracellular actin cytoskeleton. Ligand binding to integrins result in activation of the outside-in signaling cascade and is mediated by a wide variety of signaling molecules (Harburger and Calderwood, 2009).

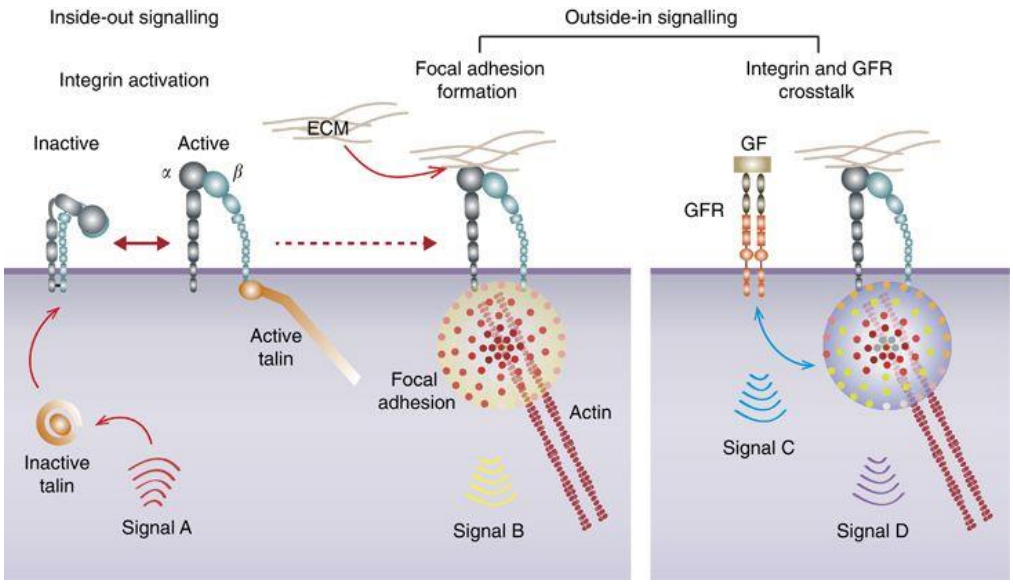


Figure 4: Mechanisms in Integrin activation (adapted from Hamidi *et al.*, 2016)

II.7.4. Integrin signaling through Focal Adhesion Kinase:

Focal Adhesion Kinase (FAK) is a unique non-receptor tyrosine kinase that has been implicated in mediating integrin-dependent signal transduction in a variety of cell types. FAK has been found to colocalize with integrins at focal adhesions and is activated through tyrosine phosphorylation upon the binding of integrins to ECM ligands. FAK is divided into three domains. The N-terminal domain contains sites for binding to the cytoplasmic tail of integrin-β1. The C-terminal domain contains sites that interact with intracellular cytoskeletal network. The kinase domain of FAK is flanked by these N and C-terminal domains (Mitra *et al.*, 2005). Initial evidence of a role for FAK in integrin signaling came from studies that showed FAK phosphorylation and activation upon integrin binding to fibronectin (Schlaepfer *et*

al., 1997). Using peptide mimetics that mimic β1 integrin cytoplasmic tails, FAK has been demonstrated to bind to the cytoplasmic tail of integrin-β1 (Santos *et al.*, 2012). Multiple models positing the mechanism of integrin-dependent activation of FAK have been proposed. One study has demonstrated that membrane distal sequences of integrin-β1 plays a critical role in FAK activation *in vivo* (Hayashi *et al.*, 2002) An alternative model is that integrin-dependent FAK activation is mediated by cytoskeletal proteins like talin . Integrins bind to talin, which in turn binds to FAK. Following ligand binding, integrin acts via talin to activate FAK. However, talin binding to FAK is independent of FAK activation, suggesting that talin is an upstream mediator of FAK. In support of this, microbeads coated with anti-integrin antibodies induce clustering of integrins. Purification and analysis of these beads show that integrins are bound to talin and FAK (Chen *et al.*, 1995). Activation of FAK leads to its association with adaptor molecules such as src and Grb2, leading to activation of downstream signaling cascades involving MAP kinases. These events play a major role in promoting cell adhesion, migration and proliferation (Harburger and Calderwood, 2009).

II.7.5. Integrin signaling through Integrin-linked Kinase:

Integrin-linked kinase (ILK) is another key effector kinase that binds to the cytoplasmic tail of Integrin-β1 and mediates signaling events. ILK functions as an enzymatic and scaffolding protein in association with integrin-linked complexes and aids in integrin-mediated mechano-transduction. As a scaffolding protein, ILK forms a complex with the protein PARVIN and PINCH to form a heterotrimeric complex known as the IPP (ILK, PINCH, PARVIN) complex. Integrins inherently lack

enzymatic functions and depend upon ILK and FAK to activate downstream signaling events. ILK is a serine/threonine kinase that binds to the cytoplasmic tail of Integrin-β1 found at focal adhesions and costameres. ILK activation occurs through binding of phosphoinositide-3,4,5-phosphate (PIP_3) to the pleckstrin homology domain of ILK. Activation of PI3K activates ILK, leading to phosphorylation of Rac1, Akt and GSK-3β (Legate *et al.*, 2006). In vivo studies using ILK knockout mice have demonstrated their role in the progression of concentric hypertrophy, wound healing and fibrosis (Lu *et al.*, 2006; Kavvadas *et al.*, 2010; Serrano *et al.*, 2012).

II.7.6. Integrins in the injured myocardium:

Integrins serve to link the cells to the surrounding ECM. Since myocardial injury triggers ECM remodeling, integrins have emerged as critical regulators of this process. Previous studies have attempted to analyze the expression patterns of various α and β subunits of Integrins post-MI. These studies show that while α1 integrin expression increased for a significant duration in the peri-infarct zone post-MI, the expression of α5 integrin increased during the healing process and decreased during the remodeling process (Nawata *et al.*, 1999), suggesting that different α subunits may play varied roles in the myocardium, post-MI. Integrin-β1has been reported to be a critical effector of the wound healing response in the dermal tissue and the lung, post-injury (Liu *et al.*, 2010; Schnittert *et al.*, 2018). The expression of integrin-β1 was low during the initial phase following MI. However, its expression increased at the infarct zone at 3 days post-MI and peaked at 7 days, after which its expression gradually decreased and returned to baseline (Sun Mei *et al.*, 2003). In

another study on heterozygous Integrin-β1 knockout mice, MI increased Integrin-β1 expression at the peri-infarct and non-infarct zone at one-month post-MI. Following MI, the Integrin-β1 knockout mice exhibited enhanced myocyte apoptosis fibrosis and cardiac function compared to the wild type (Krishnamurthy *et al.*, 2006). Integrin-β1 exhibits cell-type specific expression of isoforms in the myocardium. The β1A isoform is expressed primarily in cardiac fibroblasts and inflammatory cells while the β1D isoform is preferentially expressed in cardiomyocytes. Integrin-β3 is another β integrin whose expression is enhanced in endothelial cells and smooth muscle cells in peri-infarct vessels (Chen *et al.*, 2016). Together, the temporal and cell-type specific expression of α and β integrins point to their involvement in healing and remodeling of the myocardial ECM following injury.

II.7.7. Integrins in fibrosis:

Integrins are critical regulators of ECM remodeling and play a major role in fibrotic disease progression in various tissues. In mouse models of systemic sclerosis, integrin-dependent activation of dermal fibroblasts has been shown to promote collagen production, leading to dermal fibrosis. Pharmacological inhibition of Integrin β1 and β3 in these mouse models have been reported to inhibit fibrotic disease progression in systemic sclerosis (Ray, 2013). A direct role of integrin β1 in fibrogenesis was demonstrated by using integrin-β1 conditional knockout mice wherein, loss of integrin β1 conferred resistance to bleomycin-induced dermal fibrosis (Liu *et al.*, 2010a). Systemic delivery of an integrin αvβ1 inhibitor reduced fibrotic disease progression in mouse models of carbon tetrachloride-induced liver fibrosis and bleomycin-induced lung fibrosis. αV integrins are known to promote

fibrosis via activation of TGF-β. TGF-β isoforms are synthesized as precursor proteins that have a latency-associated peptide (LAP) domain that prevents TGF-β from interacting with its receptors. Binding of αV integrins to the LAP domain causes a conformational change that releases TGF-β into the ECM. Notably, inhibitor compounds that prevent interaction of integrin αv with TGFβ1 LAP has been demonstrated to inhibit tissue fibrosis via decreasing the levels of active TGFβ1 in the ECM (Reed *et al.*, 2015). In vivo studies focusing on the role of αvβ3 and αvβ5 in fibrosis have shown that inhibition of these integrins enhanced collagen synthesis and augmented fibrosis, suggesting that different integrin subunits have varied roles in collagen expression (Bagnato *et al.*, 2018). In vitro silencing of integrin α8 led to an increase in collagen type I mRNA levels whereas knockout of integrin α8 did not alter collagen synthesis in vivo (Chen *et al.*, 2016). Studies have demonstrated a role for integrin α1β1 in promoting α-SMA expression and collagen synthesis. The activation of integrin α1β1 has also been demonstrated to promote differentiation of pericytes into myofibroblasts in vivo, promoting vascular fibrosis (Zeltz and Gullberg, 2016). Studies have explored the role of integrin α11 (ITGA11) subunit in fibrogenesis of the lung, liver and kidneys. Expression of ITGA11 colocalized with α-SMA, demonstrating its localization in myofibroblasts. Further, in vitro knockdown of ITGA11 reduced TGFβ-induced α-SMA and collagen type I expression in hepatic stellate cells (Bansal, Nakagawa, Yazdani, Baarlen, *et al.*, 2017). The in vivo expression of ITGA11 in lung and liver tissues also corelated with the progression of fibrosis in these tissues (Bansal, Nakagawa, Yazdani, Baarlen, *et al.*, 2017). In the heart, transgenic overexpression of ITGA11 was found to promote collagen production and tissue fibrosis (Leask, 2018). Integrin-β1 is a common β

subunit of collagen-binding integrins that is demonstrated to regulate the pro-fibrotic phenotype of myofibroblasts through promoting the expression of α-SMA and collagen type I via YAP and PAK-1 signaling. Further, the expression of ITGB1 corelated with the progression of liver fibrosis, demonstrating its role in fibrotic disease progression (Martin *et al.*, 2016).

II.7.8. Signaling cooperativity between DDRs and Integrins:

Receptor tyrosine kinases are known to function cooperatively with other signaling pathways to modulate cellular function. DDRs and Integrins represent two major collagen- binding receptors in the cell. While previous studies have focused on the regulatory links between the DDR1 isoform and integrin, the regulatory relationship between DDR2 and integrins remain elusive. DDR1 and integrins have been reported to cooperatively regulate critical cellular functions such as cell adhesion and differentiation (Shintani *et al.*, 2008; Xu *et al.*, 2012). Signaling cooperativity between DDR1 and integrin α2β1 has been reported to promote epithelial to mesenchymal transition, promoting metastasis of pancreatic cancer cells. In these cells, both DDR1 and integrin α2β1 coordinately regulate expression of N-cadherin to promote cell scattering (Shintani *et al.*, 2008). In another study, signaling effectors activated by DDR1 and integrin α2β1 converge to result in upregulation of polycomb protein Bmi-1 to promote embryonic stem cell renewal (Suh and Han, 2011a). DDR1 is reported to modulate integrin expression and activation. Overexpression of DDRs led to activation and enhanced binding of integrin integrin α1β1 and α2β1 to collagen type I. However, changes in integrin expression levels were not observed (Xu *et al.*, 2012). In another study, overexpression of DDR1 increased the expression

of integrins α1β1 and α2β1, resulting in enhanced binding to collagen type I (Staudinger *et al.,* 2013). In addition to studies that demonstrate a positive regulatory relationship between DDR1 and integrins, few studies have reported existence of a negative regulatory relationship between DDR1 and integrins. For example, DDR1-mediated suppresion of integrin α2β1 activation was found to attenuate migration in MDCK cells (Wang *et al.,* 2006). DDR1 was also found to inhibit integrin α2β1-induced cell spreading via negative regulation of the Rho-family GTPase Cdc42 (Yeh *et al.*, 2009).

II.8. Role of α-SMA in cell signaling:

The actin cytoskeleton plays a fundamental role in the regulation of cell migration and contractile force generation. Eukaryotic cells contain three actin isoforms - α, β and γ. Of these, the β and γ isoforms are expressed ubiquitously in all cell types while expression of the α actin isoform is restricted to specialized muscle or muscle-like cells. α-actin has three variants – cardiac, skeletal and smooth. The smooth muscle variant of α-actin is predominantly expressed in smooth muscle cells. Expression of α-SMA is known to confer functions of motility and contractility to the cell. In a setting of tissue injury, de-novo expression of α-SMA in fibroblasts aid in fibroblast migration to the site of injury. Additionally, α-SMA-dependent contractile ability helps myofibroblasts in preserving structural integrity of the fibrotic scar tissue (Rockey *et al.*, 2013).

However, the role of α-SMA in the regulation of cellular signaling events remain largely elusive. In general, cellular actin is known to control the expression of smooth-muscle specific genes via regulating the nuclear translocation of the MRTF

transcriptional co-activator. MRTF functions in association with the transcription factor SRF to regulate critical cellular functions. Increased G actin levels sequester MRTF in the cytoplasm, preventing its association with SRF. In response to a stimulus, rapid polymerization of G actins to F actin results in release and nuclear translocation of MRTF, resulting in its association with SRF to drive gene transcription (Olson and Nordheim, 2010a). This phenomenon is reported to regulate the expression of collagen type I and tenacin C mRNA in chondrocytes (Parreno *et al.*, 2014). Actin expression is reported to mediate insulin-induced DNA synthesis in cultures of L6 skeletal myoblast cell lines via activation of ERK MAPK, p-38 MAPK and upregulation of c-fos expression. Additionally, the study reported that latrunculin-mediated depolymerization of cellular actin inhibited cell cycle progression in L6 cell lines via upregulation of p-21 kinase expression (Tsakiridis *et al.*, 1998). In agreement with these studies on the role of cellular actin in regulation of gene expression, another study demonstrated that actin cytoskeleton disassembly attenuated the expression of TGF-β receptor type II, leading to inactivation of SMAD2/3 signaling and collagen expression in dermal fibroblasts (Qin *et al.*, 2018). In contrast to the reported role of α-SMA in cell migration and proliferation, renal myofibroblasts isolated from α-SMA knockout mice showed a decrease in cell proliferation and migration compared to the α-SMA wild type (Takeji *et al.*, 2006a). These results demonstrate cell-type specific roles for α-SMA in the regulation of cellular events.

II.8.1. Role of YAP and TRPC6 as critical effectors in pro-fibrotic signaling:

YAP is an important mediator of the Hippo signaling pathway. Activation of hippo signaling in cells restricts proliferation and thereby check organ growth and morphogenesis. These actions triggered in response to activation of hippo signaling are mediated by two transcriptional coactivators: YAP and transcriptional coactivator with PDZ binding motif (TAZ). Under conditions demanding restrictive proliferation, hippo-induced activation of the large tumor suppressor gene 1 and 2 (LATS1/2) phosphorylates YAP that promotes YAP binding and sequestration to 14-3-3 proteins in the cytoplasm. In response to a mitogenic stimulus, YAP gets de-phosphorylated and migrates to the nucleus where it binds to the TEA domain transcription factor (TEAD) family to promote gene transcription (Boopathy and Hong, 2019).

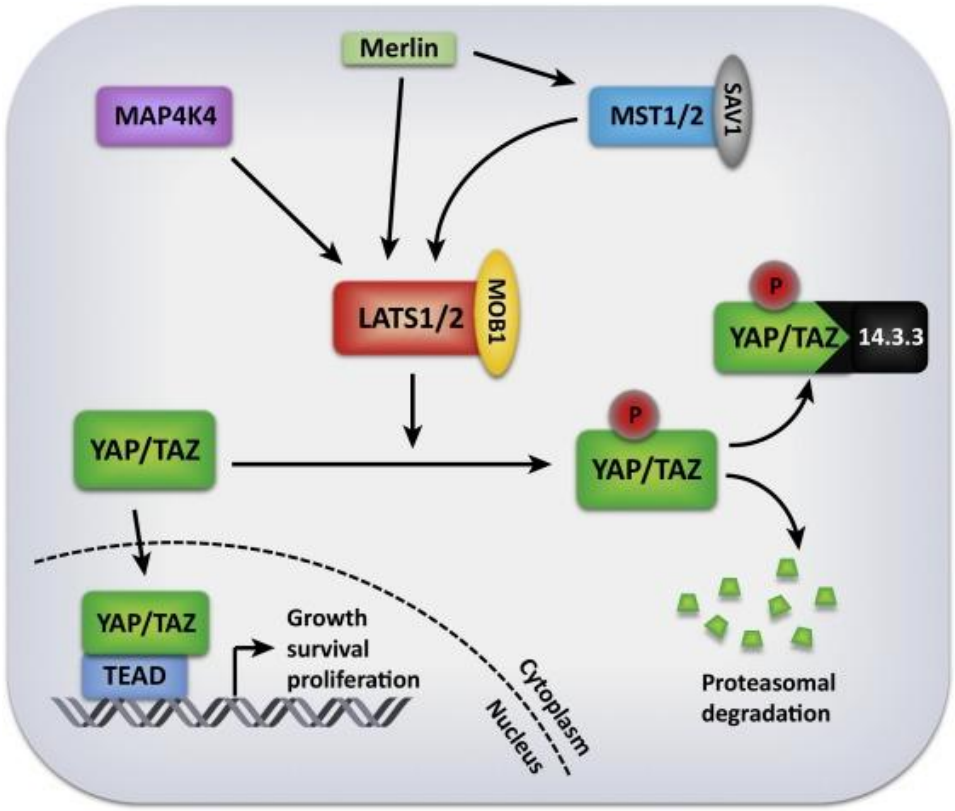


Figure 5: Mechanisms in activation of Yes-associated protein

Recent studies have reported a role for YAP in mediating pro-fibrotic signaling (Noguchi *et al.*, 2018). Blockade of YAP signaling was found to prevent TGF-β-induced fibroblast activation, proliferation and collagen expression (Xu *et al.*, 2016). Moreover, in vivo studies demonstrate YAP activation in myofibroblasts isolated from mice models of unilateral ureter obstructive (UUO) nephropathy. In these UUO mice models, inhibition of YAP using verteporfin decreased YAP expression levels in renal cells and attenuated renal fibrosis (Liang *et al.*, 2017; Gui *et al.*, 2018). The activation of YAP was found to be regulated by tissue stiffness and TGF-β. A higher degree of YAP activation was observed in fibroblasts cultured on stiff matrices, compared to cells cultured on soft matrices, suggesting that the stiff ECM conditions in a setting of fibrosis could promote YAP activation, leading to fibrotic disease

progression (Szeto *et al.*, 2016). The ability of YAP to sense substrate stiffness could be due to its coupling with integrin signaling as integrin-β1 is reported to activate YAP in hepatic stellate cells, leading to liver fibrosis. YAP is also reported to promote pro-fibrotic signaling in the lung. Fibroblast-specific activation of YAP has been showed to promote fibrosis (Liu *et al.*, 2014). However, YAP knockout in cardiomyocytes resulted in reduced cardiac hypertrophy and enhanced myocyte apoptosis and cardiac fibrosis in mouse models transverse aortic constriction (Byun *et al.*, 2019). This finding demonstrates that the role of YAP as a promoter of fibrotic signaling cascade is dependent on the cell-type in which it is activated.

Calcium dependent mechanisms play an important role in mediating pro-fibrotic signaling. In this regard, TRP channels are non-selective calcium entry channels that are expressed in a wide variety of cell types, including fibroblasts. In the cardiac tissue, TRP channels participate in physiological functions such as pacemaking, impulse conduction and propagation. Moreover, TRP channels are involved in cardiac pathophysiology such as arrythmias, fibrosis and inherited cardiomyopathies. In mammals, the TRP receptors consists of six major families: TRPA, TRPC, TRPM, TRPML, TRPP and TRPV (Hof *et al.*, 2019). Within the TRP family, TRPC6 channel expressed in myofibroblasts has been implicated in tissue fibrogenesis. A previous study by Davis et al. demonstrated the role of TRPC6 in the regulation of fibroblast phenotypic transformation, collagen expression and wound healing. In this study, adenoviral-mediated overexpression of TRPC6 facilitated the differentiation of cardiac and dermal fibroblasts to the alpha-SMA-positive myofibroblast phenotype. Subsequently, genetic deletion of TRPC6 in mice prevented Ang II-

stimulated myofibroblast formation and collagen expression and delayed the dermal wound healing response (Davis *et al.,* 2012). Data from in vivo studies show that pressure overload induced by UUO promoted renal fibrosis with a concomitant upregulation of TRPC6 along with other markers of fibrosis. The selective inhibition of TRPC6 using the inhibitor BI749327, attenuated renal fibrosis in this mouse model (Wu *et al.,* 2017). Additionally, TRPC6 knockout mice developed significantly attenuated lung fibrosis following bleomycin treatment compared to wild-type demonstrating its role in the pathogenesis of pulmonary fibrosis (Dietrich *et al.*, 2015).

III. MATERIALS AND METHODS

III.1 *Materials*

III.1.1 Fine chemicals

Medium199 (M199), albumin (from Bovine serum), collagenase type IA (from *Clostridium histolyticum*), trypsin (from porcine pancreas), pancreatin (from porcine pancreas), DNase I (bovine pancreas), HEPES, EDTA, DMSO, amphotericin B solubilised, D-(+)-dextrose, Mannitol, calcium chloride, disodium hydrogen phosphate, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, phenol red, sodium hydroxide, Hoechst 33342, TRI-reagent, DEPC, DNase amplification kit, protease inhibitor cocktail, SDS, trizma base, agarose, glycine, sodium acetate, acrylamide, bis-acrylamide, mercaptoethanol, TEMED, APS, Angiotensin II, Bay 11-7085, PD98059, SB431542 hydrate, VAS2870, Chelerythrine, U73122, Diphenyleneiodoniumb chloride, NAC, Ponceau S, monoclonal anti-vimentin antibody, anti-human von Willebrand antibody, immunostaining kit for desmin, ant-FLAG antibody, anti-β-actin primary antibody, anti-rabbit and anti-mouse secondary antibodies and Gen Elute Blood genomic DNA kit were purchased from Sigma-Aldrich, USA. Random primers, reverse transcriptase, RNAase inhibitor, dNTPs and SB203580 were obtained from Promega (Madison, WI, USA). The PureLink RNA isolation kit and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA). The Low cell# ChIP kit protein A × 48 was from Diagenode (Denville, NJ, USA). NE-PER Nuclear and Cytoplasmic Extraction Reagents, Chemiluminescent nucleic acid detection module, Pierce Biotin 3′-end DNA labeling kit, SYBR Green Master Mix and TaqMan probes for mRNA

expression were from Thermo Fisher Scientific (Waltham, MA, USA). DDR2, transforming growth factor-β (TGF-β1), transient receptor potential cation channel subfamily C member 6 (TRPC6) and control siRNAs were from Ambion (Foster City, CA, USA). Integrin-β1 and α-SMA siRNAs were custom-designed from Eurogentec (Liege, Belgium). The rat DDR2/CD167b Gene ORF cDNA clone expression plasmid was obtained from Sino Biologicals (Beijing, China). The TRPC6 (NM_053559) Rat Tagged ORF Clone was from Origene (Rockville, MD, USA). Pcax Itgb1-FLAG was a gift from Dennis Selkoe and Tracy Young-Pearse (Addgene plasmid # 30153, http://n2t.net/addgene:30153; RRID:Addgene 30153) (Young-Pearse *et al.*, 2007). Opti-MEM and fetal bovine serum (FBS) were from GIBCO (Waltham, MA, USA). All cell culture ware was purchased from BD Falcon (Corning, NY USA). Primary antibodies against DDR2, TGF-β1, extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) and c-Jun were obtained from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies for collagen α1 type I and TRPC6 were from Santa Cruz Biotechnology (Dallas, TX, USA). The primary antibodies for Integrin-β1, α-SMA and c-Fos were from Abcam (Cambridge, UK). β-Tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Elabscience (Houston, TX, USA). All antibodies were used after dilution (1:1000), except c-Fos and c-Jun (1:50) for chromatin immunoprecipitation (ChIP) and α-SMA (1:7000) for western blotting. The H9c2 cell line was obtained from the American Type Culture Collection. XBT X-ray Film was from Carestream (Rochester, NY, USA).

III.1.2 Routine Chemicals

Methanol, isopropanol, chloroform and HCl were from Merck, India. Ethanol absolute was from Commercial Alcohols, Canada. Glycerol was purchased from Sisco Research Laboratories, India. Skim milk was obtained from Hi-media, Mumbai, India. Gentamicin and Benzyl Penicillin were from Cadila pharmaceuticals, India and Alembic Limited, India respectively.

III.1.3 Cell culture ware

35 mm and 100 mm cell culture-treated dishes, 12-well plates and 24-well plates were purchased from Becton Dickinson, USA. 15 ml and 50 ml polypropylene centrifuge tubes were from Tarson. Membrane filter was procured from Millipore, USA. Cell scrapers & T-Flask were from Nunc, USA. 0.6 ml, 1.5 ml/2.0 ml tubes, tips for measuring fine volumes were from Axygen.

III.2 *Equipments used*

Epoch ELISA reader (Bio-Tek instruments, USA), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Thermo Electron corporation, USA), weighing balance (Sartorius, Germany), water bath (Julabo, India), ice-flaker (Blue star), pH meter (Cyberscan, India), CO2 incubator (Sanyo, Japan), phase-contrast microscope (Nikon, Japan), fluorescence microscope (Zeiss Axioskop 2 Plus), laminar air flow chamber (Thoshiba, India), magnetic stirrer (Schott, Germany), Gel Doc XR+ System (Bio-Rad, USA), 7500 Real-Time PCR System and PRISM 377 DNA Sequencer (Applied Biosystems, USA), UV-Transilluminator (Bangalore Genei, India), Bioruptor (Diagenode), Mini-PROTEAN Tetra Cell and

Powerpac universal (Biorad laboratories, USA), Direct-Q 3 water purification system (EMD Millipore, USA), UP50H ultrasonic processor (Hielscher, Germany), mix mate (Eppendorf, USA), thermo aluminum bath (FINEPCR, Korea), tube rocker (Benchmark scientific, India).

III.3 Composition of media, reagents and buffers

III.3.1 Acrylamide 30%

29.2 g of acrylamide and 0.8 g of N, N'-Methylene bisacrylamide were dissolved in 100 ml of deionized water.

III.3.2 Agarose gel (1%) for electrophoresis of DNA or RNA samples

For DNA and RNA – 200 mg agarose was dissolved in 20 ml of 0.5X TBE.

III.3.3 Alkaline lysis solution

6 µl of 10 M NaOH and 15 µl of 20% SDS were mixed and made up to 300 µl with sterile water.

III.3.4 Blocking solution

5% (w/v) skim milk or BSA was dissolved in 1X TBST containing 0.1% Tween-20.

III.3.5 Fibroblast growth medium (pH 7.4)

M199 with Earle's salts containing FBS (10%), benzyl penicillin (50U/ml) and gentamycin (0.04 mg/ml) was used for maintaining cardiac and adventitial fibroblast cultures.

III.3.6 Cell Lysis buffer for protein isolation for western blotting (1X)

Cell lysis buffer (10X) was diluted with deionized water to get a 1X solution.

III.3.7 DAB substrate solution

900 µl of A and 100 µl of B were mixed well and added to the membrane.

III.3.8 DEPC-treated deionized water

1 ml of DEPC was added to one litre of deionized water, stirred until the globules were dissolved, kept at 37°C overnight and autoclaved twice.

III.3.9 Dissociation medium for cardiac fibroblast isolation

Each 100 ml of dissociation medium was made up of sodium chloride (0.68 g), HEPES (0.555 g), sodium dihydrogen phosphate (0.140 g), glucose (0.100 g), potassium chloride (0.04 g), magnesium sulfate (0.02 g), BSA (100 mg) and 10 µl of 1 mM CaCl2. pH was adjusted to 7.4. Deoxyribonuclease I (100 µl) and amphotericin B (200 µl) were added to 100 ml of the medium under sterile conditions at the time of isolation. The dissociation medium had collagenase type IA (40 mg), trypsin (70 mg) and pancreatin (2 mg) per 100 ml.

III.3.11 Electrode buffer/ running buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Tris base (1.5135 g), glycine (7.2 g) and SDS (0.5 g) were dissolved in 500 ml of deionized water.

III.3.12 Ethidium bromide (EtBr) (Stock solution)

1 mg of EtBr was dissolved in 1 ml of de-ionized water. 5 µl of this stock solution was added to 20 ml of 1% agarose for DNA/RNA electrophoresis.

III.3.13 EDTA (0.5 M, pH 8.0)

930 mg EDTA was dissolved in 5 ml DEPC-treated deionized water.

III.3.14 Bis-benzimide H 33324 (Hoechst 33342)

10 mg of Hoechst 33324 powder was dissolved in 5 ml of sterile distilled water to get a final concentration of 10 mM. A working concentration of 10 µM was used for the experiments.

III.3.15 Phosphate-buffered saline (PBS) (pH 7.4)

Sodium chloride (0.8 g), potassium chloride (0.02 g), disodium hydrogen phosphate (0.186 g) and potassium dihydrogen phosphate (0.02 g) were dissolved in 100 ml of deionized water.

III.3.16 LB medium

10 g tryptone, 5 g yeast extract and 10 g NaCl were dissolved in 800 ml distilled water. The pH was adjusted to 7.0 with 1 N NaOH. The volume was made up to 1 litre with distilled water and sterilized by autoclaving.

III.3.17 Non-denaturing gel (6%)

5.93 ml water, 2 ml of 30% acrylamide, 2 ml of 5X TBE, 35 µl of 20% APS and 10 µl of TEMED were mixed.

III.3.18 Paraformaldehyde (4%)

4 g paraformaldehyde was dissolved in 100 ml PBS by keeping at 60ºC.

III.3.19 Potassium acetate

To 29.4 g of potassium acetate in 40 ml of water, 50 ml of glacial acetic acid was added, pH was adjusted to 5.2 using glacial acetic acid and made up to 100 ml.

III.3.20 Resolving Gel for SDS – PAGE (10%)

3.3 ml of 30% acrylamide, 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulfate and 4 µl TEMED were added to 4.0 ml of deionized water.

III.3.21 SDS gel-loading buffer (6X)

SDS (9% w/v), bromophenol blue (0.03%), ß-mercaptoethanol (9%) and glycerol (50% v/v) were dissolved in 18.75 ml of 1 M Tris HCl- pH 6.8 (375 mM).

III.3.22 Serum-free medium

M199 containing antibiotics (50 U/ml penicillin and 0.04 mg/ml gentamycin).

III.3.23 Stacking gel buffer (pH 6.8)

30 ml from resolving buffer was measured, adjusted the pH to 6.8 using HCl and was made up to 45 ml and stored at room temperature.

III.3.24 Stacking gel for SDS – PAGE (5%)

0.670 ml of 30% acrylamide, 0.500 ml of 1 M Tris (pH 6.8), 0.04 ml of 10% SDS, 0.04 ml of 10% ammonium persulfate and 4 µl TEMED were added to 2.7 ml of deionized water.

III.3.25 TEG buffer

1.25 ml of 1 M Tris (pH 8.0), 1 ml of 0.5 M EDTA and 2.5 ml of 1 M glucose were mixed and made up to 50 ml with water.

III.3.26 Towbin's buffer (Transfer buffer)

1.5135 g of Tris base, 7.2 g of glycine and 100 ml of methanol were mixed and made up to 500 ml with deionized water.

III.3.27 Tris borate EDTA buffer (TBE) (5X, pH 8.3)

27.315 g of Tris base, 13.91 g of boric acid and 1.861 g of disodium EDTA (0.5 M, pH 8.3) were mixed and made up to 1L with deionized water.

III.3.28 Tris-buffered saline (10X, pH 7.6)

24.2 g of Tris base and 80 g of sodium chloride were dissolved in water. After adjusting the pH to 7.4 using conc.HCl, the solution was made up to 1 L with deionized water.

III.3.29 Tris-buffered saline with Tween-20 (TBST) [1X]

1X TBS containing 0.1% Tween-20.

III.3.30 Trypsin-EDTA solution

25 mg of trypsin and 3.8 mg of EDTA were dissolved in 10 ml of PBS (pH 7.4).

Methods

III.4 Isolation, culture and characterization of fibroblasts

III.4.1 Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague-Dawley rats (2-3 months old) following the method of Kumaran and Shivakumar, 2002 (Kumaran and Shivakumar, 2002), with some minor modifications. Handling of animals and experimental procedures was in accordance with Institutional Animal Ethics

Committee approval (SCT/IAEC122/AUGUST/2014/85). Animals were euthanized (Thiopentone sodium, 5-10 mg/Kg, intraperitoneal), the heart excised, atria removed and the ventricles were used for isolation of fibroblasts. The ventricular tissue was washed in PBS, minced into bits of approximately 1-3 mm size and subjected to a series of digestions in Dissociation Medium containing Collagenase type IA (0.4 mg/ml), Trypsin (0.6 mg/ml) and Pancreatin (0.020 mg/ml). Digestion was aided by gentle shaking of the flask containing tissue bits in an orbital shaker maintained at 37°C. The supernatants were centrifuged at 1500 rpm for 5 minutes. The cell pellets were pooled, re-suspended in M199 supplemented with 10% FBS, seeded on two 35 mm cell culture dishes and incubated at 37ºC for 2.5 hours in a humidified CO2 incubator with 95% air-5% CO2. At the end of this period, the supernatant containing unattached cells and debris was discarded, the dishes with the adherent fibroblasts were rinsed 3-4 times and incubated with M199 containing 10% FBS. The pre-plating step ensured preferential attachment of cardiac fibroblasts. At 24 hours after isolation, the dishes were washed and incubated with M199 containing 10% FBS and maintained in a CO2 incubator at 37ºC.

III.4.2 Sub-culture of cardiac fibroblasts

At confluence, the culture supernatant was removed, cells were washed with PBS and trypsinised at 37^0C with trypsin-EDTA solution. Trypsinisation was stopped by addition of M199 containing 10% FBS and the detached cells were collected by centrifugation at 1500 rpm for 5 minutes. The cell pellet was suspended in M199 containing 10% FBS and seeded on fresh culture dishes at a split ratio of 1:3.

III.4.3 Characterization of cardiac fibroblasts in culture

Fibroblastic nature of the cells in culture was ascertained by morphology and immunocytochemistry. Sub-confluent and confluent cultures were examined under an inverted phase contrast microscope for morphologic characteristics.

Immunocytochemical analysis of DDR2, vimentin, desmin and von Willebrand factor: Cells from passage 2 or 3, grown to about 50% confluence, were washed with PBS and fixed in 4% ice-cold paraformaldehyde for 20 minutes. The fixed cells were blocked with PBS containing 5% FBS and 0.2% Triton X-100 for 30 minutes. The cells were then incubated overnight in primary antibody diluted in blocking solution. Following PBS wash, cells were incubated in FITC-labelled secondary antibody at room temperature for 90 minutes. After PBS wash to remove unbound stain, nuclei were counterstained with Hoechst 33342 and observed under a fluorescence microscope. Anti-desmin and anti-von Willebrand factor antibodies were used at 1:500 dilution and immunostaining for anti-vimentin was done using a commercially available kit.

III.5 *Experimental model*

Sub-confluent cultures of cardiac fibroblasts in M199 at passage 2 or 3 were used for the experiments. Primary cultures of adult rat cardiac fibroblasts treated with 1 µM Ang II were used.

III.6 *Determination of cell number*

Sub-confluent cardiac fibroblast cultures were synchronized by serum-deprivation for 24 hours. Cell counts were determined before and after 24 hours of treatment using a Neubauer counting chamber.

III.7 *RNA interference*

Cells were seeded on 12-well plates at 8 X 10^4 cells per well. 24 hours after sub-culture, cells were incubated in Opti-MEM containing the pre-designed siRNA (5 pmol DDR2; 5 pmol ERK ½ MAPK siRNA, 5 pmol TGF-β1; 30pmol α-SMA siRNA; 5 pmol TRPC6 siRNA or 5 pmol Integrin-β1 siRNA) and Lipofectamine (2 µl per well) for 19 hours. The control groups were transfected with scrambled siRNA. Following an additional incubation in M199 with 10% FBS for 12 hours, the cells were serum-deprived for 24 hours. Post serum-deprivation, cells were incubated with 1 µM Ang II. Cell lysate was prepared in 2X SDS lysis buffer and denatured in boiling water bath for 5 minutes and stored at -20ºC until use.

III.8 *Over-expression of DDR2, Integrin-β1 and TRPC6*

DDR2 cDNA clone was purchased from Sinobiologicals, China. Integrin-β1 plasmid was purchased from Addgene. TRPC6 cDNA clone was purchased from Origene, USA. Strong constitutive expression of DDR2, Integrin-β1 or TRPC6 was achieved under a pCMV promoter.

III.8.1 Plasmid amplification

- 100 µl nuclease-free water was added to reconstitute the supplied 10 µg lyophilized DNA.
- 1µl of reconstituted plasmid DNA was added to 100 µl DH5-α. Provided a gentle tap and incubated in ice for 30min.
- The vial was kept in water bath for 40 seconds at 42ºC to provide a heat shock, immediately snap-cooled on ice and then mixed with 250 µl LB broth (without ampicillin).
- After incubation at 37ºC for 1 hour at 175 rpm, the broth was plated on kanamycin-containing medium (50 µg/ml) and incubated overnight at 37ºC.
- Post-incubation, one colony was inoculated into kanamycin-containing LB broth (5 ml) and incubated for 6-8 hours at 37ºC in a shaker (175 rpm) to set up a starter culture.
- Further, 100 ml kanamycin-containing LB broth was inoculated with 500 µl of starter culture and incubated for 12-16 hours at 37ºC (175 rpm).
- *Preparation of glycerol stock:* 850 µl culture broth was added to 150 µl sterile glycerol and vortexed vigorously for 5 seconds and kept at -80ºC until use.

III.8.2 Plasmid isolation

Qiagen plasmid Midi kit was used to isolate plasmid DNA.

- Harvested bacterial cells by centrifugation at 6000 x *g* for 15 minutes at 4ºC.
- Added the RNase A solution to re-suspension buffer to a final concentration of 100 µg/ml. Re-suspended the bacterial pellet in 4 ml re-suspension buffer.
- Added 4ml lysis buffer, mixed thoroughly to facilitate homogenous mixing and incubated for 5 minutes at 25ºC.
- Added 4 ml pre-chilled neutralization buffer, mixed thoroughly and incubated on ice for 15 minutes. Precipitation of genomic DNA, proteins, cell debris and potassium dodecyl sulphate was facilitated by addition of neutralization buffer.
- Centrifuged at ≥20000 x *g* for 30 minutes at 4ºC and removed the supernatant containing plasmid DNA.
- Equilibrated a Qiagen-tip100 by applying 4 ml equilibration buffer. The presence of detergent facilitated the flow of buffer through the column. Applied the supernatant from Step 5 to the Qiagen-tip and allowed it to enter the resin by gravity flow.
- Washed the Qiagen-tip with 10 ml wash buffer, which was sufficient to remove all contaminants in plasmid DNA preparation.
- Eluted the DNA with 5 ml elution buffer into 15 ml centrifuge tube and precipitated by adding 3.5 ml isopropanol. Mixed and centrifuged immediately at ≥15000 x *g*. Washed the pellet with 2 ml 70% ethanol and

centrifuged at ≥15000 x *g* for 10 minutes. Carefully decanted the supernatant without disturbing the pellet. Air-dried the pellet and re-dissolved the DNA in 250 µl nuclease-free water. Concentration of plasmid DNA and purity were assessed spectrophotometrically.

III.8.3 Plasmid verification

- Amplification of DDR2 ORF clone was carried out using forward and reverse DNA vector sequencing primers provided in the kit.

  Reaction was set up with the following components:

Water	18.5 µl
10X buffer	2.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
dNTP	0.5 µl
DNA template	1 µl
Sigma Taq polymerase	0.5 µl

The following conditions were employed: 95ºC for 3 minutes followed by 95ºC for 30 seconds, 55ºC for 40 seconds and 72ºC for 2 minutes, repeated over 27 cycles, and final extension at 72ºC for 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel.

- Restriction digestion

  The DDR2 cDNA is located within the KpnI and XbaI sites of the pCMV plasmid. The Integrin-β1 cDNA is located within the EcoRI and NotI sites of the pCMV plasmid. The presence of the cDNA insert in the vector was confirmed by restriction digestion followed by determination of the cDNA insert size on a 1.2% agarose gel. The restriction digestion mixture, consisting of :

10X restriction buffer	2 µl
Plasmid	17.5 µl
Respective restriction enzymes	0.5 µl

was incubated for 2 hours at 37ºC. The post-restriction digestion product was electrophoresed on 1.2% agarose gel.

III.8.4 Transfection of overexpression vectors

1-2µg/µL of purified DDR2, Integrin-β1, TRPC6 or empty control over-expression vectors were mixed with 2µL Lipofectamine2000 in100 µl Opti-MEM, which was added to the cultures for incubation in M199+10% FBS for 8 hours. After a recovery period of 12 hours, cells were treated with 1µM Ang II.

III.9 *Electrophoretic Mobility Shift Assay (EMSA)*

DNA-binding activity of AP-1 in cardiac fibroblasts was assessed by EMSA as described below.

III.9.1 Preparation of nuclear extract

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit following manufacturer's instructions. Sub-confluent cardiac fibroblast cultures in M199 were treated with Ang II, with and without inhibitors, for 30 minutes or 3 hours, harvested by trypsinization, washed in PBS and pelleted. The cell pellet was re-suspended in 75–150 µl (depending on packed cell volume) cytoplasmic extraction reagent I provided with the kit. The mixture was vortexed and incubated on ice for 30 minutes. After addition of cytoplasmic extraction reagent II, the mixture was vortexed, incubated on ice for 5 minutes and centrifuged at 16,000 x g for 5 minutes at 4^0C. The pellet was re-suspended in nuclear extraction reagent, incubated on ice for 2 hours with intermittent vortexing and centrifuged at 16,000 x g for 10 minutes at 4^0C. The supernatant (nuclear fraction) was aliquoted and stored at -80^0C until use. Protein concentration of the nuclear extracts was determined by BCA assay.

III.9.2 Primers

Single-stranded oligos carrying the consensus sequence for the AP-1 binding site 5'-GGGGACTTTCC-3' were used.

III.9.3 Primer labelling

Single-stranded oligos for AP-1 (forward) were biotin-labelled using Biotin 3' end DNA labelling kit. The reaction components were added in the following order:

Component	Volume in µl	Final concentration
Ultrapure water	25	-
5X TdT reaction buffer	10	1X
5 pmol 3'-OH ends of unlabelled DNA	5	0.5 pmol
Biotin-11-UTP (5 µM)	5	0.5 µM
Diluted TdT(2U/µl)	5	0.2 U/µl

Reaction components were incubated for 30 minutes at 37ºC. Following addition of 2.5 µl of 0.2 M EDTA to stop the reaction, 50 µl of chloroform: isoamyl alcohol (24:1) was added and briefly centrifuged at high speed to separate the phases. The top aqueous phase containing the biotin-labelled oligos were collected.

III.9.4 Annealing of AP-1 oligos

Equal volumes of both complementary oligos (biotinylated forward primer and non-biotinylated reverse primer) were mixed at equimolar concentrations, incubated on thermal block at 90ºC for 2 minutes, allowed to cool and stored at -20ºC until use.

III.9.5 DNA binding

15 µg nuclear protein was incubated with 200 femtomoles of double-stranded biotinylated probes for AP-1 and other components of the Light Shift Chemiluminescent kit for 60 minutes at 37ºC. 2 µl loading dye was added and mixed gently. Samples were loaded on 6% non-denaturing acrylamide gel and electrophoresed in TBE buffer at 100 V until the bromophenol blue moved more than 3/4th the gel. Electrophoretic gel transfer onto a nylon membrane was carried out at 380 mA for 80 minutes, followed by UV cross-linking at 254 nm for 10 minutes.

III.9.6 Detection of biotin-labeled oligos

After blocking for 15 minutes, the membrane was incubated in streptavidin-conjugated-HRP in blocking buffer (1:300) for 1 hour with gentle shaking. The bands were visualized by enhanced chemiluminescence by adding Luminol/Enhancer solution in equal ratio.

III.9.7 Competition assay

Specificity of binding was ascertained by competition with 200-fold excess of unlabelled AP-1 oligonucleotide.

III.10 *Western blot analysis*

Western blot analysis was carried out following standard protocol. Cardiac fibroblasts were subjected to the indicated treatments and lysed in cell lysis buffer. The lysates were sonicated, vortexed vigorously, kept on ice for 30 minutes and centrifuged at 16,000 x g for 20 minutes at 4°C to remove cell debris and the supernatant was stored at –80^{0}C until use. The lysates were denatured by incubation

with 6X SDS-gel loading buffer at 100ºC for 5 minutes in a water bath. 25 µg of protein was electrophoretically fractionated at 200 V on 10% SDS-PAGE minigels and electroblotted onto nitrocellulose or PVDF membrane for 60 minutes at 100 V. The membrane was blocked for 1 hour with 5% skim milk or BSA (in case of phosphoproteins) and incubated overnight with mild shaking at 4^0C with the primary antibody prepared in 5% BSA in 1X TBST at 1:1000 dilution. Unbound primary antibody was removed by washing (10 minutes x 4 times) with 1X TBST. Immunoblots were exposed for 1 hour to HRP-conjugated anti-mouse/anti-rabbit secondary antibody at 1:10,000 dilution in 5% skim milk containing TBST, and unbound secondary antibody was removed by washing (10 minutes x 3 times) with TBST. ECL or DAB substrate was used to detect the antigen-antibody complex. The membrane was then stripped overnight by washing with TBST on a rocking platform, re-probed with anti-β-actin antibody (1:1000) and was developed as described above. Protein expression was quantified using Bio-Rad Gel Doc XR+ System and normalized to β- actin.

III.11 *Real-time PCR analysis*

III.11.1 Isolation of total RNA

Sub-confluent cultures of cardiac fibroblasts were subjected to the indicated treatments for 6 or 12 hours and total RNA was isolated using the Purelink RNA mini kit, according to the manufacturer's instructions. The yield and purity of the isolated RNA were determined spectrophotometrically. Intactness of RNA was ascertained by 1% agarose gel electrophoresis.

III.11.2 cDNA synthesis

4 µg of isolated RNA was subjected to DNase I (amplification grade) treatment, as per manufacturer's instructions, to remove any genomic DNA contamination. Following DNase treatment, 2 µg of total RNA was reverse transcribed to cDNA. The cDNA synthesizing mixture consisted of:

5X RT buffer	6.0 µl
dNTPs	2.5 µl
Random primers	2.0 µl
RNase inhibitor	0.5 µl
M-MLV RT	1.0 µl
Total RNA	12.0 µl
DEPC-treated water	6.0 µl
	30 µl

2 µg total RNA was made up to 12 µl with DEPC-treated water and 2.0 µl random primer was initially mixed and incubated for 5 minutes at 70^0C. The mixture was then snap-cooled on ice and mixed with the remaining constituents listed above. The reaction mixture was incubated for 60 minutes at 37^0C, then at 90^0C for 5 minutes and cooled on ice to inactivate M-MLV reverse transcriptase. The cDNA preparations were stored at -20^0C until use.

III.11.3 Real-time PCR analysis

Taqman quantitative Real-time PCR analysis was carried out using 100 ng of cDNA, Taqman master mix, Taqman primers and specific FAM- or VIC-labelled probes. PCR reactions were performed under the following thermal cycling conditions: 95°C for 10 minutes followed by denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute for each of 40 cycles. Gene expression was quantified by ΔΔCt method, with 18S rRNA or β-actin as reference gene.

ΔΔCt method to calculate relative fold-change in target gene expression with reference to 18S rRNA

- Average Ct of each experimental group is recorded.
- ΔCt was calculated for each sample using the following formula

  ΔCt= Ct target-Ct reference gene
- ΔΔCt was calculated as

  ΔΔCt= (Ct target–Ct reference) sample – (Ct target-Ct reference) control
- Relative fold of target mRNA levels of treated with respect to control = $2^{-\Delta\Delta Ct}$

III.12. *Chromatin immunoprecipitation assay*

ChIP assay was performed using LowCell number ChIP kit from Diagenode, according to the manufacturer's protocol.

III.12.1 Binding antibodies to magnetic beads

10 µl of the pre-washed Protein A-beads was mixed with 90 µl of Buffer A and 3 µg of the specific antibody c-FOS, c-JUN, YAP or normal human IgG, centrifuged at 40 rpm for 2 hours at 4°C and the pellet was used for immunoprecipitation.

III.12.2 Cell collection and DNA-protein cross-linking

Cardiac fibroblasts were treated with 1 µM of Ang II for the indicated durations. The cells were trypsinized and the cell pellet, suspended in 500 µl of PBS, was mixed with 13.5 µl of 36.5% formaldehyde and 57 µl of 1.25 M glycine and incubated for 8-10 minutes at room temperature for fixing the protein-DNA complex. The mixture was centrifuged and the pellet was washed twice with 0.5 ml ice-cold PBS.

III.12.3 Cell lysis and chromatin shearing with Bioruptor

The cell pellet was suspended in 130 µl of Buffer B containing protease inhibitor and sodium butyrate (NaBu) and the samples were sonicated using Bioruptor for 5 runs, 10 cycles (30 second ON / 30 seconds OFF) to shear the chromatin into fragments of size between 500 and 600 bp. 130 µl of sheared chromatin was mixed with 870 µl of Buffer A and the shearing efficiency was assessed on 1% agarose gel.

III.12.4 Magnetic Immunoprecipitation

To the antibody-coated beads, 100 µl of diluted sheared chromatin was added and incubated overnight at 4°C on a rotator. 10 µl of sheared chromatin was stored as input sample at 4°C. After overnight incubation, the immunoprecipitated samples were washed using 100 µl each of ice cold Buffer A (X 3 times) and Buffer C and the beads were captured in a magnetic rack. Input samples were centrifuged and both the input and IP samples were processed for the remaining steps.

III.12.5 DNA isolation

The tubes were removed from the magnetic rack and 100 µl of complete DNA isolation buffer (DIB) was added to re-suspend the beads. 99 µl of complete DIB was added to 1 µl of input DNA sample. The tubes were incubated at 55°C for 15 minutes followed by 100°C for 15 minutes and centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was aliquoted and stored at -20°C until use.

III.12.6 Quantitative PCR & Data analysis

The final step involved amplifying and analyzing the immunoprecipitated DNA. Amplification of immunoprecipitated DNA was carried out by PCR using primers for Itgb1 and collagen alpha1(I). 5'-TCAGGACCTCTAGAAGAGCAG-3' and 5'-CTTCCTTCCTTCCTTCCTTCC-3' were used as the forward and reverse primers, respectively, corresponding to a 300 bp region of the Itgb1 promoter that includes the predicted AP-1 binding sites on the Integrin-β1 gene. CTCAGCACTTTCCTCTTTCT-3' and 5'-GCCACCTCATCTTTAGGAAA-3' were used as the forward and reverse primers, respectively, corresponding to a 188 bp

region of the collagen alpha1(I) promoter that includes the predicted YAP binding sites on the collagen alpha1(I) gene. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). ChIP with a non-specific antibody (normal rabbit IgG) served as negative control.

PCR Reaction was set up using the following components

Water	12.5 µl
10X buffer	5.0 µl
MgCl2	1.5 µl
Forward primer (5 pmol)	1.0 µl
Reverse primer (5 pmol)	1.0 µl
dNTP	1.5 µl
DNA template	2.0 µl
Sigma Taq polymerase	0.5 µl
	25 µl

under the following conditions:

For analysis of AP-1 binding to Integrin-β1 gene promoter - 94°C for 3 minutes followed by 94°C for 30 seconds, annealing at 44°C for 30 seconds and extension at 72°C for 1 minute, repeated over 35 cycles, and final extension at 72°C for 7 minutes.

For analysis of YAP binding to the collagen alpha1(I) promoter - 94°C for 3 minutes followed by 94°C for 30 seconds, annealing at 47.1°C for 30 seconds and extension at 72°C for 1 minute, repeated over 40 cycles, and final extension at 72°C for 7 minutes. The amplification product was electrophoresed on 1.2% agarose gel.

III.13. *Scratch wound assay*

Cells were seeded on 35 mm culture dishes and grown to ~90% confluence. DDR2 or Integrin-β1 knockdown by RNA interference was performed as described under Methods. Over expression of Integrin-β1 in DDR2-silenced cells was performed as described under Methods. Following serum deprivation, a single scratch gap was created using a sterile 200 µl pipet tip. After wash with 10% phosphate-buffered saline, the cells were treated with Ang II in serum-free M199 for the indicated durations. About 3-4 fields were examined per dish and images of the wound closure pattern in different groups were acquired using a Nikon inverted phase contrast microscope. Quantification of wound healing was calculated based on the following formula: Length of wound closure = (Length of wound gap at t h - Length of wound gap at t_0) / Length of wound gap at t_0, where th is the time of wound gap measurement at 12 h or 24 h and t_0 is the time of wound gap measurement at the initial time point of 0 h.

III.14. Immunohistochemical Analysis

The ventricular sections of the DDR2 knockout mice were deparaffinized in xylene for 15min (3 times), followed by rehydration in descending grades of alcohol. The hydrated sections were blocked for endogenous peroxidase with 3% hydrogen

peroxide in methanol. The target antigen was retrived using 0.01M freshly prepared sodium citrate buffer (pH-6.0) at 95^0 C for 20 minutes. The sections were blocked with 3% BSA and incubated with primary anti-integrin-β1 antibody at 4^oC overnight. The dilution of the anti-Integrin-β1 primary antibody was 1:300. After washing with PBS, the sections were treated with HRP conjugated secondary antibody (dilution 1:200) for an hour at room temperature. After washing off unbound secondary antibody, the sections were treated with the chromogen, diaminobenzidine (DAB) (Sigma). Integrin-β1 levels in these sections were analyzed by DAB staining and quantified using Fiji-Image J software.

III.15. Sirius red staining

The extent of myocardial fibrosis in 6-month-old SHR was determined by Sirius red staining of cardiac cross sections. Briefly, deparaffinized and rehydrated cardiac sections were stained with 1% Sirius red in saturated aqueous solution of picric acid for 90 minutes followed by wash with two changes of acidified water.

III.16 Statistical analysis

Data were expressed as Mean ± SD. Statistical analysis was performed using Student's *t*-test for comparisons involving two groups. For comparisons involving more than two groups, the data were analyzed by ANOVA. $p \leq 0.05$ was considered statistically significant.

IV. RESULTS

IV.1. Characterization of adult rat cardiac fibroblasts

IV.1.1. Morphological analysis

Cardiac fibroblasts isolated from 2-3 month old adult male rat ventricular tissue were grown in culture as described under Methods. Pre-plating for 150 minutes following isolation ensured selective adhesion of fibroblasts, which constituted >99% of the cultures. At 150 minutes after isolation, the cells had a dense nest-like morphology (Figure 6) and, by 24 hours, the cells attained spindle-like appearance. At confluence, the cultures exhibited a monolayer pattern (Figure 7). Morphological analysis and immunocytochemical staining established the fibroblastic nature of the cells (Figures 8-10). Absence of 'cobblestone' or 'hill and valley' morphology ruled out contamination of cultures by ECs and VSMCs, respectively. Cells from passage 2 or 3 were used for the experiments.

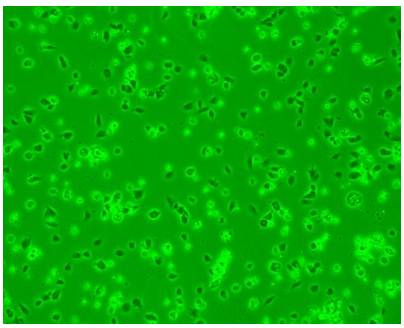


Figure 6: Phase contrast micrograph of adult rat cardiac fibroblasts at 150 minutes after isolation (10X magnification)

Cardiac fibroblasts, isolated as described under Methods, were pre-plated for 150 minutes in M199 containing 10% FBS. At the end of this period, cultures enriched with adherent fibroblasts were rinsed and incubated with M199 containing 10%FBS.

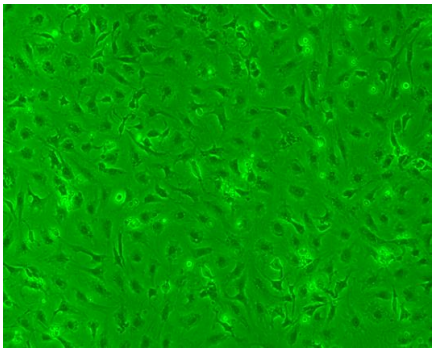


Figure 7: Phase contrast micrograph of adult rat cardiac fibroblasts at confluence (10X magnification)

Cardiac fibroblasts grown to confluence in M199 containing 10% FBS formed a monolayer with spindle-shaped morphology.

IV.1.2. Characterization of cardiac fibroblast cultures using immunocytochemistry

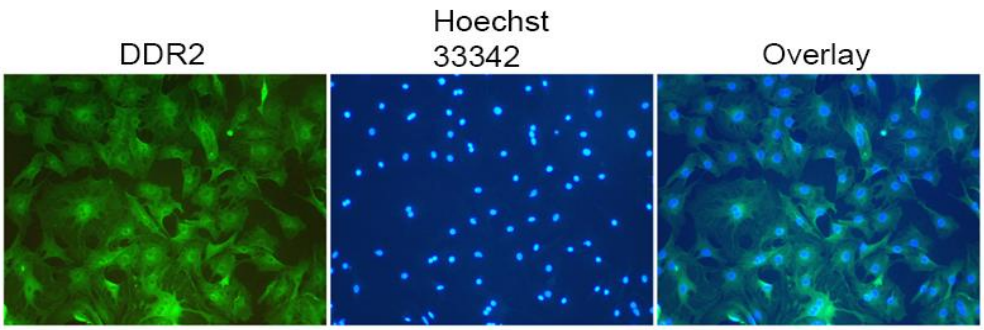


Figure 8: Fluorescent micrographs of DDR2-positive adult rat cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-DDR2 antibody. Nuclei were counter-stained with Hoechst 33342.

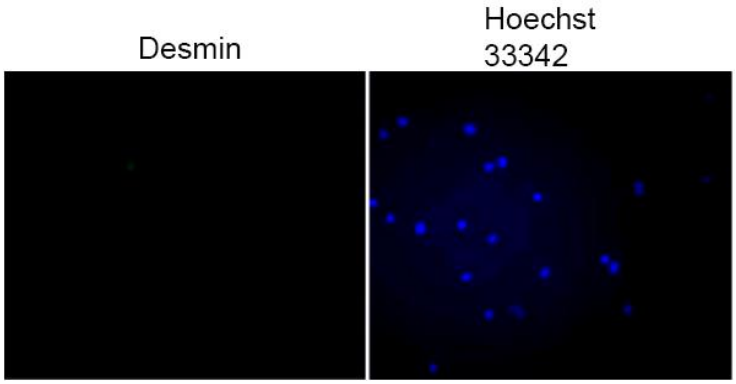


Figure 9: Fluorescent micrographs of desmin-negative adult rat cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-desmin antibody. Nuclei were counter-stained with Hoechst 33342.

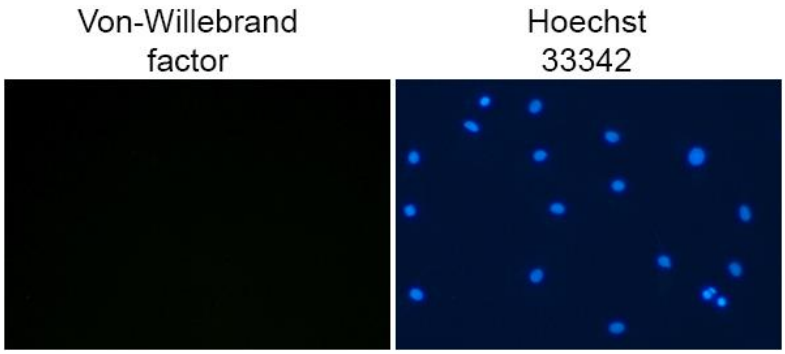


Figure 10: Fluorescent micrographs of von-Willebrand factor-negative adult rat cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-von-Willebrand factor antibody. Nuclei were counter-stained with Hoechst 33342.

IV.2. Experimental model

Cardiac fibroblasts from passage 2 or 3 exposed to Ang II (1µM) served as the experimental model.

IV.3. Effect of Ang II on Integrin-β1 gene expression in cardiac fibroblasts

The effect of Ang II on Integrin-β1 mRNA and protein expression in cardiac fibroblasts was determined by Real-time PCR analysis and western blot, respectively. Sub-confluent cultures of cardiac fibroblasts were exposed to Ang II at a concentration of 1 µM. Total RNA was isolated using Purelink RNA Mini Kit and its purity was ascertained by 1% agarose gel electrophoresis (Figure 11). Intact 18S and 28S rRNA bands confirmed the intactness of the isolated RNA.

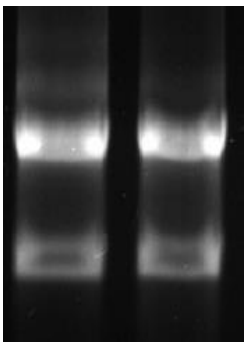


Figure 11: Agarose gel electrophoresis of RNA

RNA, isolated from control and Ang II-treated cardiac fibroblasts, was subjected to 1% agarose gel electrophoresis. Intact 28S and 18S rRNA bands were documented using Bio-Rad Imaging System.

In order to get rid of genomic DNA contamination, total RNA was subjected to DNase I treatment. 2 µg of DNase I-treated RNA was reverse transcribed to cDNA with random hexamer primers and M-MLV reverse transcriptase. mRNA expression was determined by Real-time PCR following co-amplification of the cDNA over 40 cycles with primers specific for rat Integrin-β1 and β-actin. Ang II (1 µM) was found to markedly stimulate Integrin-β1 mRNA expression at 6 h post-treatment (Figure 12).

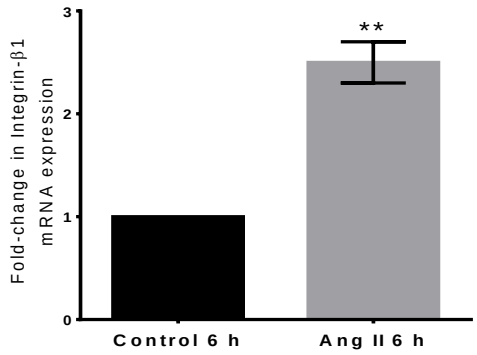


Figure 12: Ang II increased Integrin-β1 mRNA expression in cardiac fibroblasts
*Cardiac fibroblasts were exposed to Ang II (1µM). Integrin-β1mRNA expression was examined by Taqman quantitative Real-time PCR analysis. β-actin was used as endogenous control. ** p< 0.01 vs Control (unpaired, two-tailed Student's t-test), n=3, error bars represent SD.*

To determine whether the increase in Integrin-β1 mRNA expression translates into comparable changes in Integrin-β1 protein levels, total protein was isolated from cardiac fibroblasts exposed to Ang II and Integrin-β1 protein expression was determined by western blot analysis, using β-actin as loading control. A 2-fold

increase in Integrin-β1 protein was observed at 12 h following Ang II treatment (Figure 13).

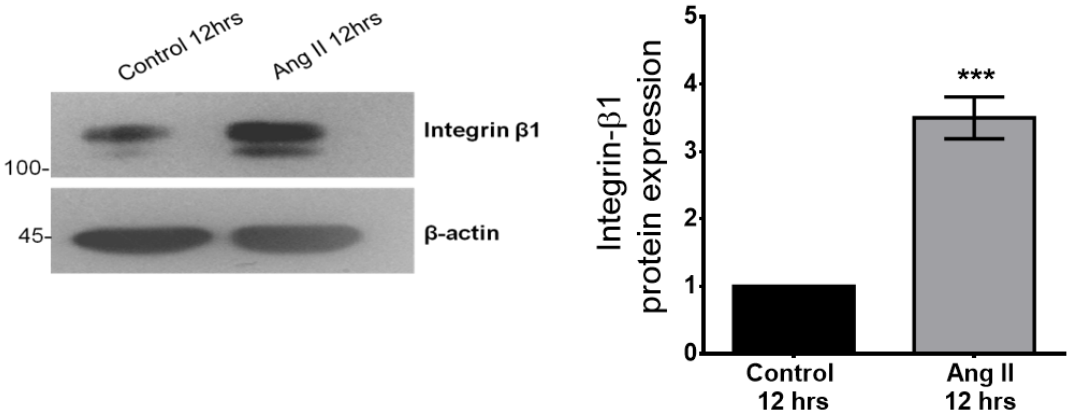


Figure 13: Effect of Ang II on Integrin-β1 protein expression in cardiac fibroblasts

Sub-confluent quiescent cultures of cardiac fibroblasts in M199 were stimulated with Ang II (1μM). Protein was isolated at 12 h post-treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as the loading control. A representative blot from one of three independent experiments is shown. ***p< 0.001 *vs Control (unpaired, two-tailed Student's t-test), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.4. Ang II-dependent DDR2 mediates Integrin-β1 expression in cardiac fibroblasts

Crosstalk between receptor tyrosine kinases and Integrins have been reported to influence the progression of atherosclerosis and cancer (Soung *et al.*, 2010). Discoidin Domain Receptor 2 (DDR2) is a mesenchymal cell-specific collagen-binding receptor tyrosine kinase, expressed predominantly in cardiac fibroblasts in

the myocardium (Souders *et al.*, 2009). Integrin-β1, on the other hand, is a collagen-binding receptor expressed in a wide variety of cell types (Chen *et al.*, 2016). Although DDR2 and Integrin-β1 have been implicated in the progression of fibrosis in the lung, liver and dermal tissue (Liu and Leask, 2013; Martin *et. al*, 2016; Zhao *et al.*, 2016), evidence of a mechanistic link between the collagen receptors that act to promote pro-fibrotic signaling events in cardiac fibroblasts is lacking.

IV.4.1 Ang II-stimulated Integrin-β1 expression is regulated by DDR2

A role for Ang II-induced DDR2 in mediating Integrin-β1 expression in cardiac fibroblasts was checked. siRNA-mediated knockdown of DDR2 was found to significantly attenuate Ang II-induced Integrin-β1 expression (Figure 14).

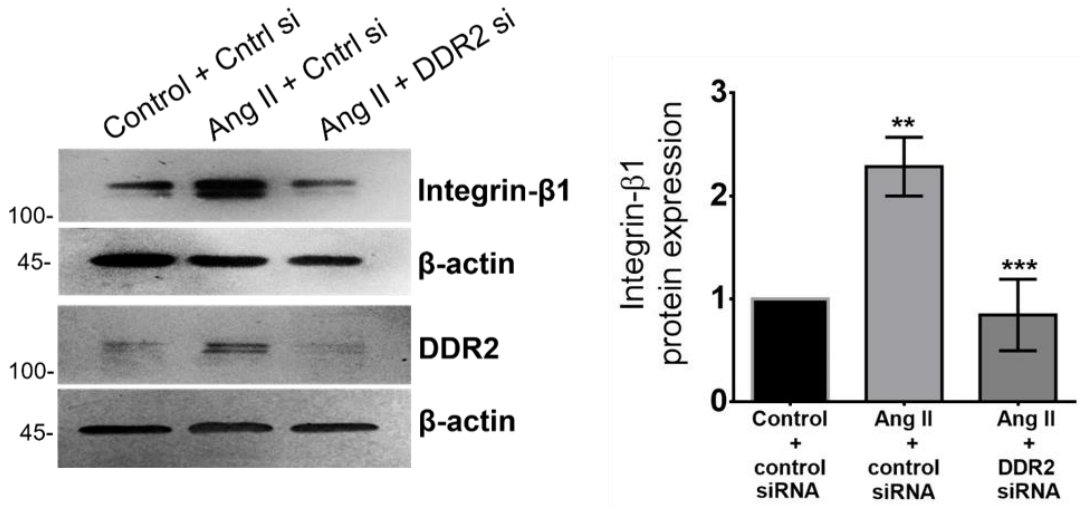


Figure 14: DDR2 was found to mediate Ang II-stimulated Integrin-β1 expression in cardiac fibroblasts

*Cardiac fibroblasts were transiently transfected with DDR2 siRNA or control siRNA. Following treatment of the transfected cells to Ang II for 12 h, Integrin-β1 protein expression was analyzed by western blotting, with β-actin as the loading control. ***

*p< 0.01 vs. control, *** p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.4.2. The basal expression of Integrin-β1 is dependent on DDR2:

A role for DDR2 in regulating the basal expression of Integrin-β1 in addition to its involvement in the regulation of Ang II-stimulated Integrin-β1 expression was checked. A combination of gene silencing and overexpression approaches demonstrated an obligate role for DDR2 in the regulation of Integrin-β1 expression in cardiac fibroblasts under Ang II-stimulated and basal conditions (Figures 15-17).

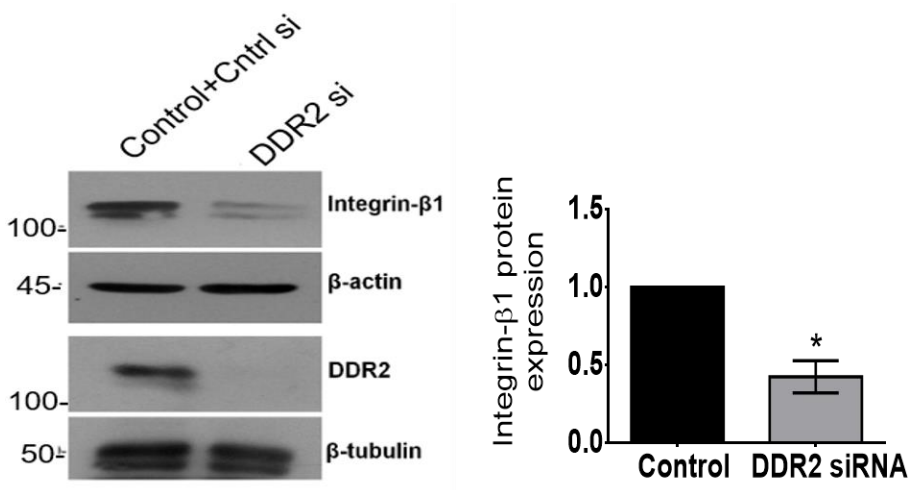


Figure 15: DDR2 knockdown attenuated basal expression of Integrin-β1

*Cardiac fibroblasts were transiently transfected with DDR2 siRNA or scrambled siRNA (control). Following revival and serum deprivation of the transfected cells for 24 h, Integrin-β1 protein expression was examined by western blot analysis and normalized to β-actin. * p< 0.05 vs. control (unpaired, two-tailed Student's t-test), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

.

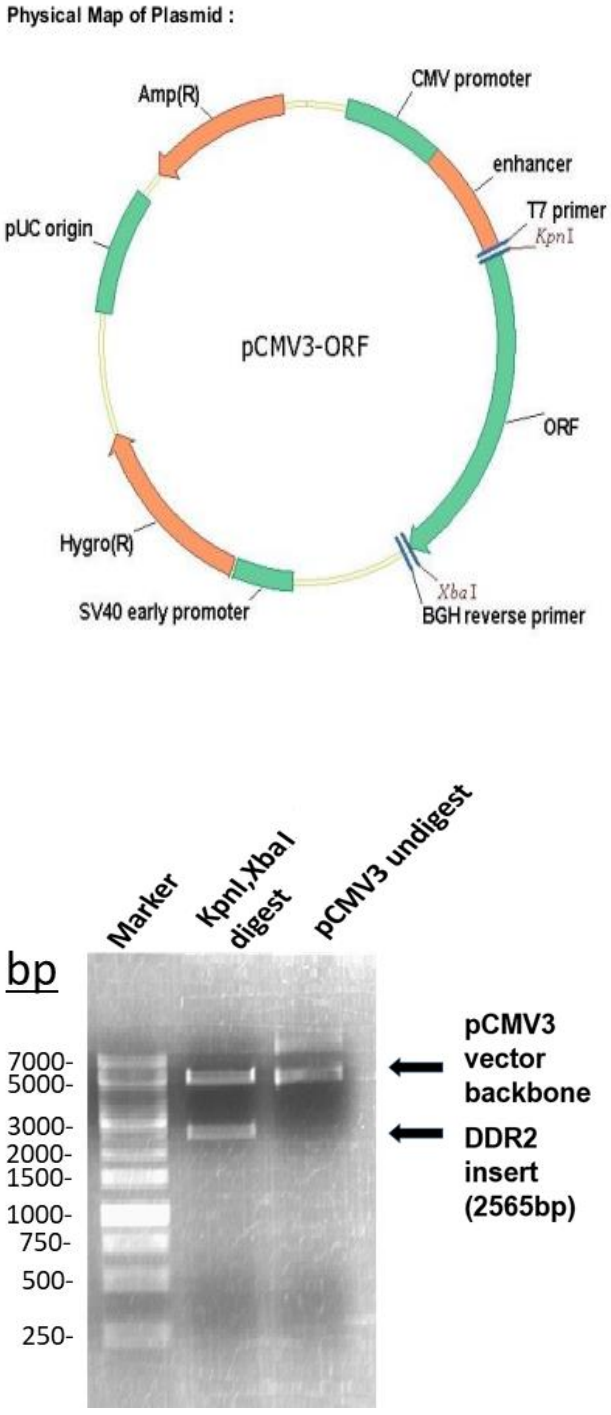


Figure 16: Plasmid map and confirmation of DDR2 insert in the pCMV3 plasmid vector

The DDR2 ORF clone was transformed into competent DH5-α bacterial cells and was amplified. Since the plasmid vector encoded an Ampicillin resistance gene,

transformed colonies in Ampicillin containing LB agar was selected and the plasmid was further amplified by inoculation in LB media. Plasmid isolation was carried out using Qiagen Midi-Prep plasmid isolation kit. The purity and concentration of the plasmid was checked spectrophotometrically. The presence of the DDR2 insert within the pCMV3 vector was checked by double site restriction digestion using XbaI and KpnI restriction enzymes. The digest was then examined using agarose gel electrophoresis.

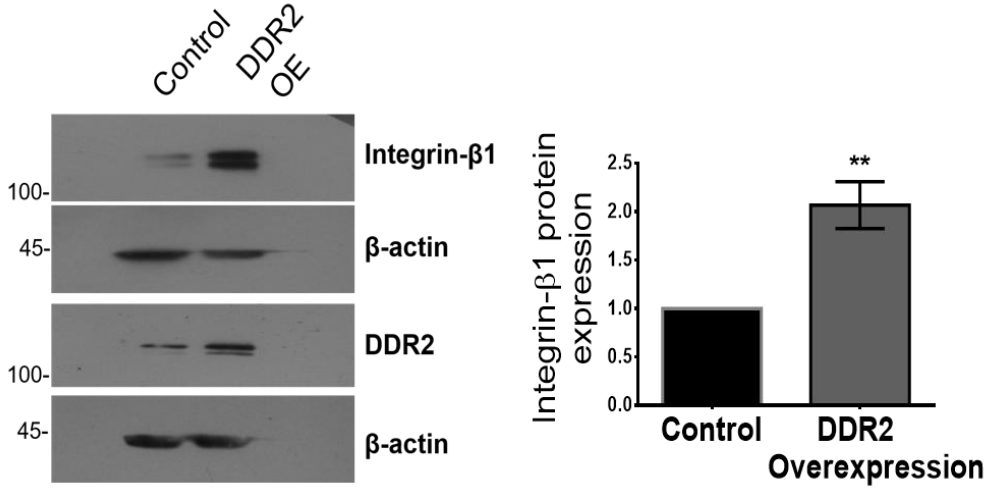


Figure 17: DDR2 overexpression restored basal Integrin-β1 levels

*Subconfluent cultures of cardiac fibroblasts in M199 were transiently transfected with DDR2 overexpression vector or empty plasmid vector (control). Following revival and serum deprivation of the transfected cells for 24 h, Integrin-β1 protein expression was examined by western blot analysis and normalized to β-actin. ** p< 0.01 vs. control (unpaired, two-tailed Student's t-test), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.5. Ang II upregulates Integrin-β1 expression via Nox-dependent ROS, Protein Kinase C, Phospholipase C and p38 MAPK

Our previous studies had demonstrated a role for NADPH oxidase (NOX)-dependent reactive oxygen species (ROS), Phospholipase C, Protein kinase C and p38 MAPK

in the regulation of Ang II-stimulated DDR2 expression in cardiac fibroblasts (George *et al.*, 2016). Since DDR2 was found to regulate Integrin-β1 expression, a role for the NOX-dependent ROS, PKC, PLC and p38 MAPK pathway in the regulation of Ang II-stimulated Integrin expression via DDR2 was hypothesized. Hence, the action of inhibitors of NOX-dependent ROS, PLC, PKC and p38 MAPK on Integrin expression was probed. Inhibitors of NOX-dependent ROS, PLC, PKC and p38 MAPK were found to attenuate the expression of Ang II-stimulated Integrin-β1 (Figures 18-21).

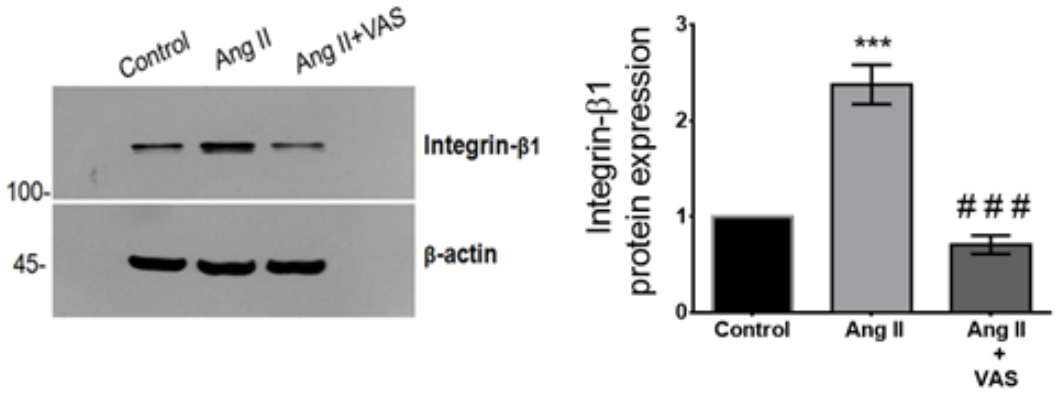


Figure 18: NOX inhibition by VAS2870 attenuated Ang II-induced Integrin-β1 protein expression

*Subconfluent quiescent cultures of cardiac fibroblasts in M199 were pre-treated with chemical inhibitor for NOX - VAS2870 (1μM) for 1 h and, subsequently, with Ang II. Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as loading control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

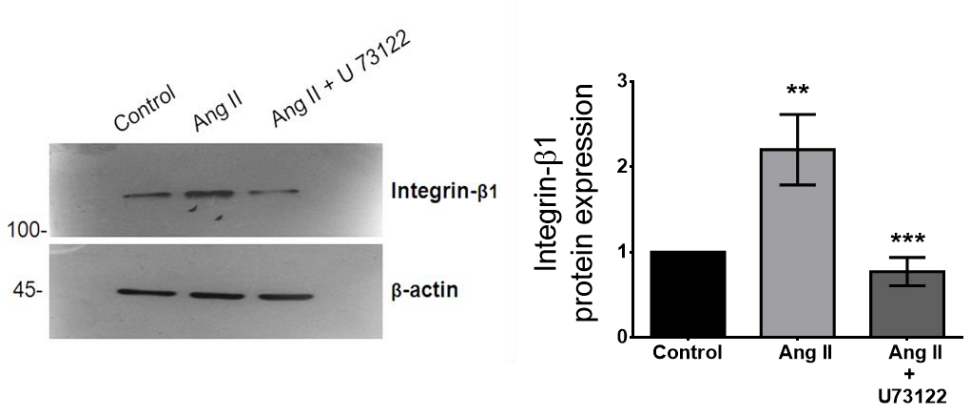


Figure 19: PLC inhibition using U73122 prevented Ang II-induced Integrin-β1 protein expression

*Subconfluent quiescent cultures of cardiac fibroblasts in M199 were pre-treated with chemical inhibitor for PLC – U73122 (1µM) for 1 h and, subsequently, with Ang II. Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as loading control. ** p< 0.01 vs. control, *** p< 0.001 vs. Ang II. (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

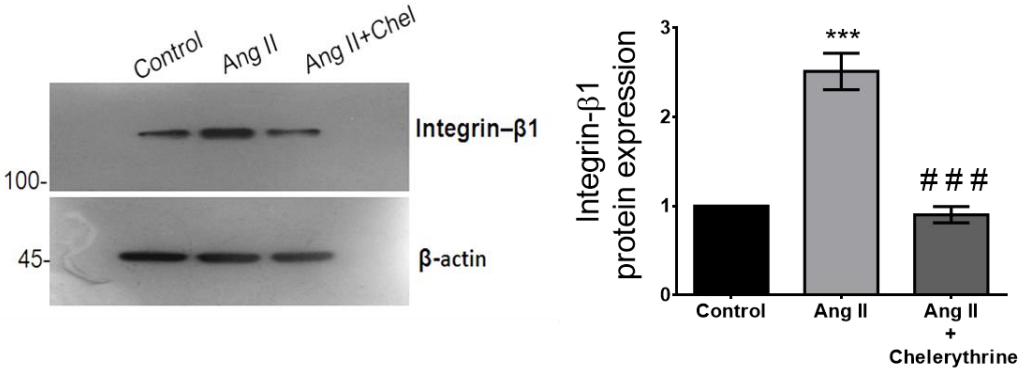


Figure 20: PKC inhibition by chelerythrine prevented Ang II-induced Integrin-β1 protein expression

*Subconfluent quiescent cultures of cardiac fibroblasts in M199 were pre-treated with chemical inhibitor for PKC – Chelerythrine (1µM) for 1 h and, subsequently, with Ang II. Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as loading control. *** p<*

0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.

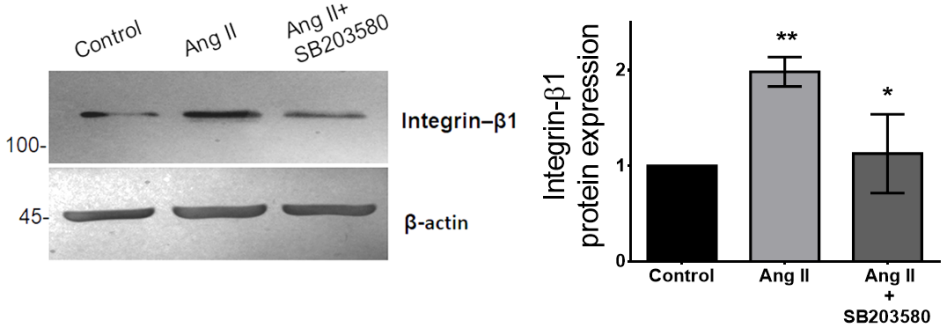


Figure 21: Inhibition of P38 MAPK attenuated Ang II-induced Integrin-β1 protein expression

*Subconfluent quiescent cultures of cardiac fibroblasts in M199 were pre-treated with chemical inhibitor for p38 MAPK – SB203580 (10μM) for 1 h and, subsequently, with Ang II. Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as loading control. ** p< 0.01 vs. control, * p< 0.05 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

Thus, Ang II-stimulated NOX-ROS acts via the PLC, PKC and p38 MAPK pathway to enhance DDR2, which in turn regulates Integrin-β1 expression in cardiac fibroblasts exposed to Ang II.

IV.6. DDR2 regulates the expression of Integrin-α11

Integrin-α11 is the cognate alpha integrin subunit that heterodimerizes with β1. Integrin-α11β1 is reported to be the dominant collagen-binding integrin expressed in fibroblasts (Bansal, Nakagawa, Yazdani, van Baarlen, *et al.*, 2017). In the present study, DDR2 knockdown attenuated the mRNA expression of Integrin-α11 (Figure

22), demonstrating the role of DDR2 in the regulation of α11 as well as the β1 subunit. However, Integrin-β1 is reported to be the major subunit of Integrin involved in signal transduction, with reported roles in fibroblast phenotypic transition, collagen synthesis and dermal wound healing, while the α subunit dictates ligand specificity (Martin et al., 2016). Importantly, although Integrin-β1 is reported to mediate pro-fibrotic signaling in hepatic stellate cells, its role in mediating pro-fibrotic signaling in cardiac fibroblasts remains unclear. Hence, subsequent investigations in this study focused on Integrin-β1.

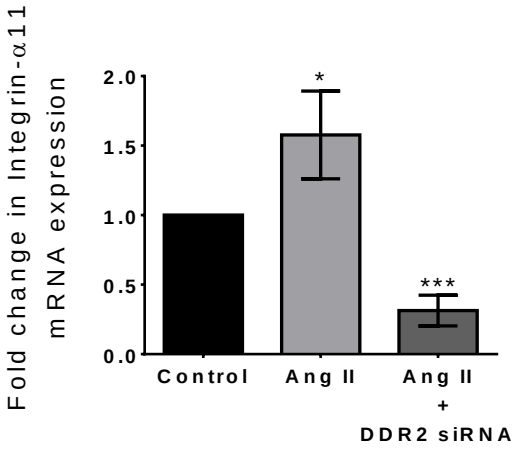


Figure 22: DDR2 knockdown attenuated Ang II-stimulated increase in Integrin-α11 mRNA

*Subconfluent quiescent cultures of cardiac fibroblasts were transiently transfected with DDR2 siRNA or scrambled siRNA.Following exposure of the transfected cells to Ang II for 6 h, Integrin-α11 mRNA expression was analyzed by RT-qPCR analysis. 18S rRNA was used as the endogenous control. * p< 0.05 vs. Control, ***p<0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD.*

IV.7. Mechanisms in DDR2-dependent regulation of Integrin-β1 in Ang II-treated cardiac fibroblasts

IV.7.1 DDR2 acts via the ERK1/2 MAPK-dependent TGF-β1 to enhance Integrin-β1 expression in cardiac fibroblasts exposed to Ang II

Since DDR2 was found to be an obligate regulator of Integrin-β1 expression, the signaling effectors acting downstream of DDR2 to mediate Integrin-β1 expression in cardiac fibroblasts exposed to Ang II were examined. Based on earlier observations from our laboratory demonstrating a role for DDR2 in the activation of ERK1/2 MAPK (George *et al.*, 2016) and the reported synergistic signaling between ERK1/2 MAPK and TGF-β1 (Hough *et al.*, 2012), a role for DDR2-dependent activation of ERK1/2 MAPK and TGF-β1 in the regulation of Integrin-β1 expression in Ang II-treated cells was explored. While siRNA-mediated silencing of DDR2 reduced Ang II-induced phosphorylation of ERK1/2 MAPK and TGF-β1 expression (Figures 23 and 24), pharmacological inhibition of ERK1/2 MAPK using PD98050 or its siRNA-mediated silencing attenuated Ang II-induced increase in TGF-β1 and Integrin-β1 expression (Figures 25-27). Further, inhibition of TGF-β1 using SB431542 or siRNA reduced Ang II-stimulated Integrin-β1 expression (Figures 28 and 29). Together, the data demonstrate the involvement of DDR2-dependent activation of the ERK1/2 MAPK / TGF-β1 signaling pathway in the regulation of Integrin-β1 expression in response to Ang II in cardiac fibroblasts.

IV.7.2. siRNA-mediated knockdown of DDR2 attenuated Ang II-stimulated activation of ERK1/2 MAPK and TGF-β1 expression in cardiac fibroblasts

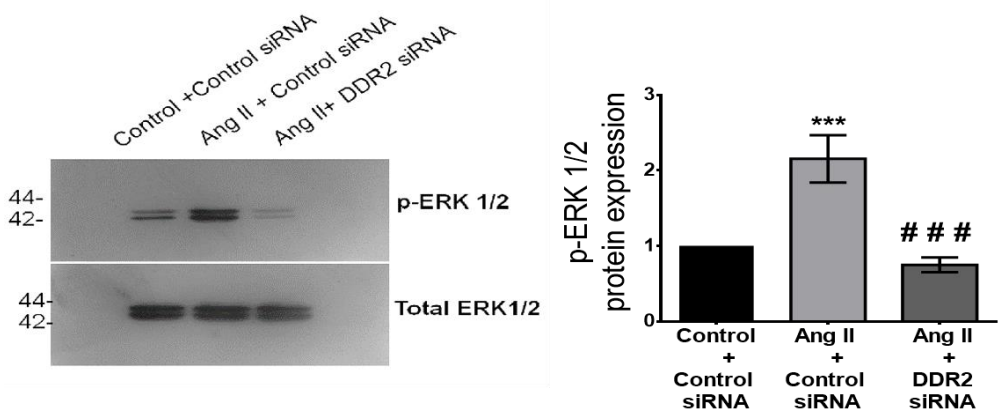


Figure 23: DDR2 knockdown by RNA interference attenuated Ang II-induced activation of ERK1/2 MAPK

*Cardiac fibroblasts were transiently transfected with DDR2 or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h. Phospho-ERK1/2 activation was examined by western blot analysis and normalized to total ERK1/2 levels. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bar SD. The molecular weights are indicated in KDa.*

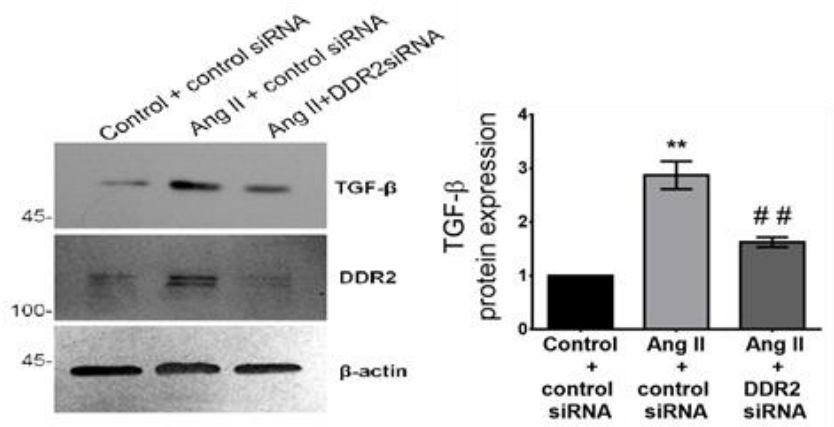


Figure 24: DDR2 knockdown by RNA interference reduced Ang II-induced expression of TGF-β1

*Cardiac fibroblasts were transiently transfected with DDR2 or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, TGF-β1 expression was examined by western blot analysis and normalized to β-actin levels. ** p< 0.01 vs. control, ## p< 0.01 vs. Ang II. (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

These findings show that DDR2-silencing attenuated Ang II-stimulated increase in ERK1/2 MAPK phosphorylation and expression of TGF-β1. Next, using the pharmacological inhibitor PD98059 and ERK1/2 siRNA, the role of ERK1/2 in the regulation of TGF-β1 and Integrin-β1 was probed.

IV.7.3. Inhibition of ERK1/2 MAPK attenuated TGF-β1 and Integrin-β1 expression

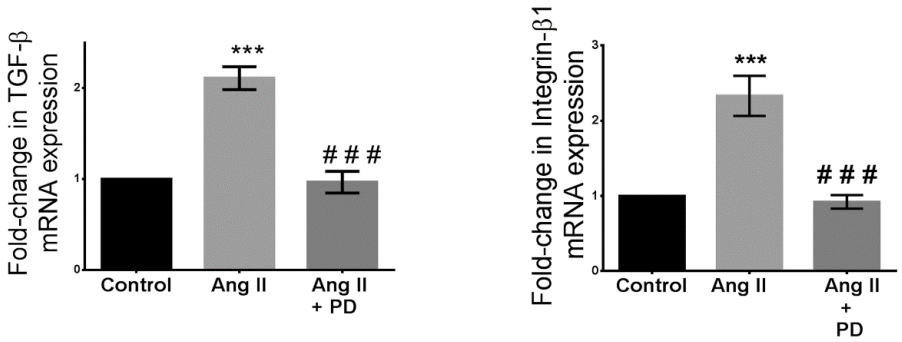


Figure 25: Inhibition of ERK ½ MAPK using PD98050 reduced the expression of TGF-β1 and Integrin-β1 mRNA

*Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated with ERK1/2 MAPK inhibitor (PD98050) for 1 h and, subsequently, with Ang II. TGF-β1 mRNA levels were determined by RT- qPCR analysis at 6 h of Ang II treatment. β-actin served as the endogenous control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II. Integrin-β1 mRNA levels were determined by RT-qPCR analysis at 6 h of Ang II treatment. β-actin served as the endogenous control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD.*

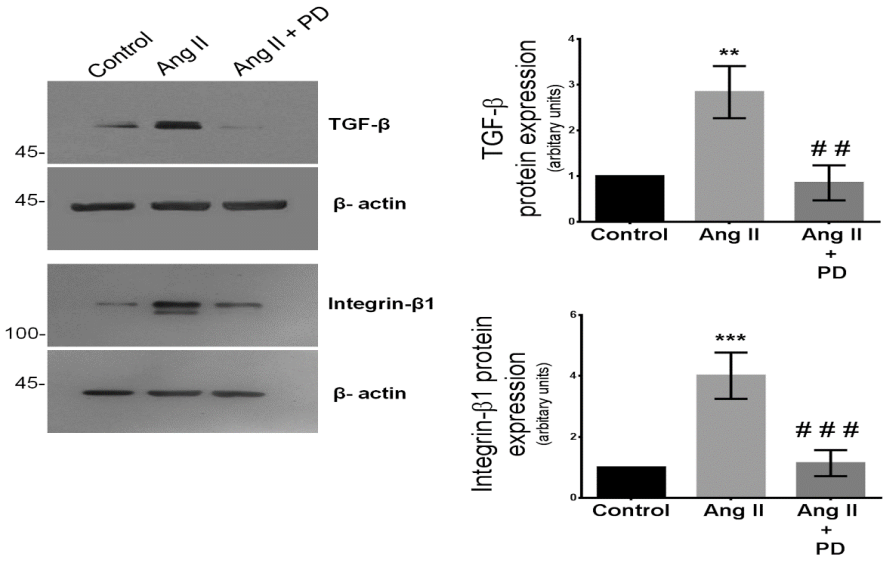


Figure 26: Inhibition of ERK ½ MAPK using PD98050 reduced Ang II-induced expression of TGF-β1 and Integrin-β1 proteins

*Subconfluent quiescent cultures of cardiac fibroblasts in M199 were pre-treated with ERK1/2 MAPK inhibitor (PD98050) for 1 h and, subsequently, with Ang II. Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of TGF-β1, with β-actin as loading control. ** p< 0.01 vs. control, ## p< 0.01 vs. Ang II. Integrin-β1 protein expression was examined at 12 h post-Ang II treatment by western blot analysis, with β-actin as loading control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

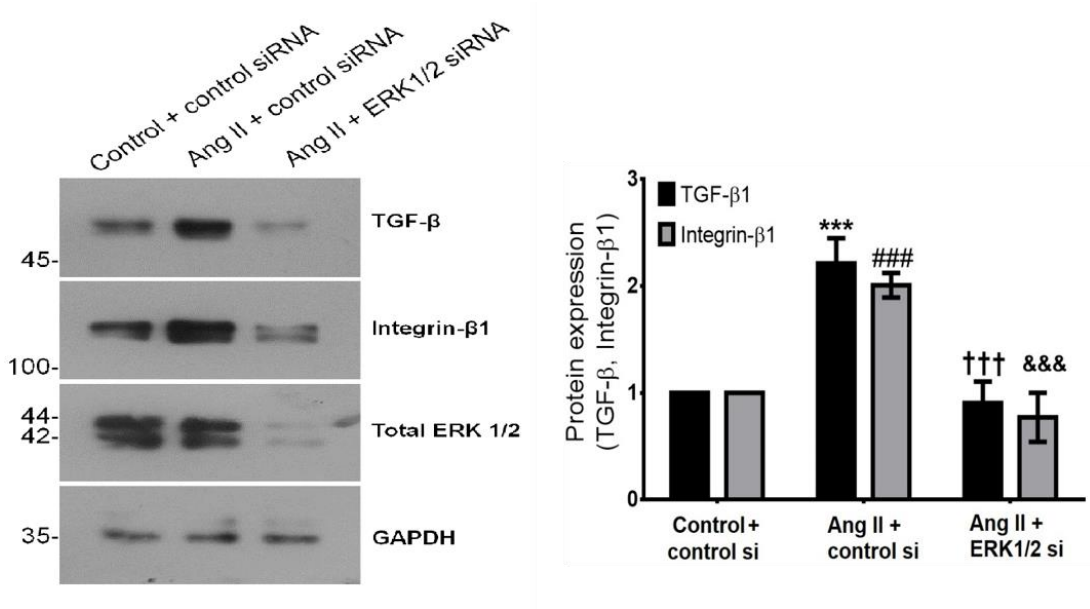


Figure 27: siRNA-mediated inhibition of ERK ½ MAPK attenuated Ang II-induced expression of TGF-β1 and Integrin-β1 proteins

*Cardiac fibroblasts were transiently transfected with ERK1/2 siRNA or scrambled siRNA (control). Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of TGF-β1, with β-actin as loading control. *** p< 0.001 vs. control, ††† p< 0.001 vs. Ang II. Integrin-β1 protein expression was examined at 12 h post-Ang II treatment by western blot analysis, with β-actin as loading control. ### p< 0.001 vs. control, &&& p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

These findings demonstrate a role for DDR2-dependent activation of ERK1/2 MAPK in the regulation of TGF-β1 and Integrin-β1 expression. Next, the role of TGF-β1, acting downstream of DDR2 and ERK1/2 MAPK, in the regulation of Integrin-β1 expression was analyzed.

IV.7.4. TGF-β1 acts downstream of DDR2-dependent ERK1/2 MAPK to regulate Integrin-β1 expression in Ang II-treated cardiac fibroblasts

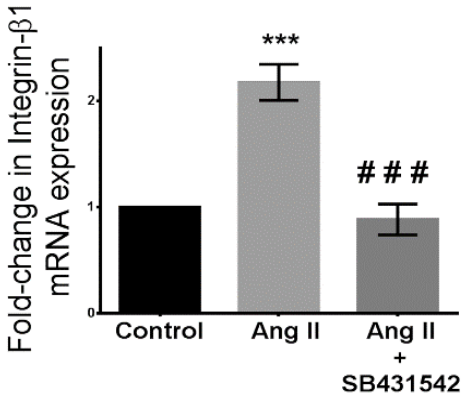


Figure 28: Inhibition of TGF-β using SB431542 attenuated Ang II-induced expression of Integrin-β1 mRNA

*Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated with TGF-β1 inhibitor (SB431542) for 1 h and, subsequently, with Ang II. Integrin-β1 mRNA levels were determined by RT-qPCR analysis at 6 h of Ang II treatment. β-actin served as the endogenous control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD.*

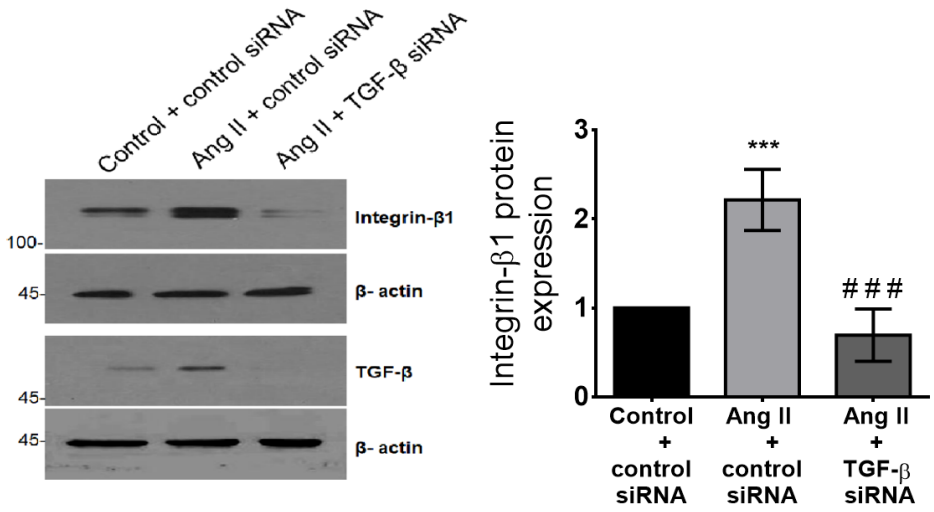


Figure 29: siRNA-mediated inhibition of TGF-β1 attenuated Ang II-induced expression of Integrin-β1

Cardiac fibroblasts were transiently transfected with TGF-β1 siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, Integrin-β1 protein expression was examined by western blot analysis, with β-actin as loading control. ***** *p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

Together, the findings demonstrated that DDR2-dependent activation of ERK1/2 MAPK promotes expression of TGF-β1 that in turn enhances Integrin-β1 expression in cardiac fibroblasts exposed to Ang II.

IV.8. Transcriptional regulation of Integrin-β1 by AP-1 via DDR2-dependent ERK1/2-TGF-β1 signaling in Ang II-stimulated cardiac fibroblasts

Since ROS was demonstrated to mediate Ang II-induced Integrin-β1 expression, the involvement of AP-1, a redox-sensitive transcription factor, in the transcriptional regulation of Integrin-β1 expression was probed. Inhibition of AP-1 with the pharmacological inhibitor, SR11302, significantly reduced Ang II-induced Integrin-β1 mRNA and protein expression (Figure 30). Next, the involvement of DDR2, ERK1/2 and TGF-β1 in the nuclear translocation of AP-1 was analyzed by EMSA. Ang II induced the activation and nuclear translocation of AP-1 that occurred maximally at 30 min (Figure 31) and was reduced by 3 h post-treatment (Figure 32). Further, inhibition of DDR2, ERK1/2 or TGF-β1 significantly attenuated Ang II-induced activation of AP-1 (Figure 31 and 32.).

To confirm the role of DDR2-dependent AP-1 in the transcriptional upregulation of Integrin-β1, ChIP assay was performed. Subconfluent cultures of cardiac fibroblasts were transiently transfected with DDR2 siRNA or control siRNA. Following Ang II treatment for 30 min, the cells were harvested and chromatin was sheared as mentioned under Methods. The shearing efficiency was checked (Figure 33). Cross-linked chromatin extracted from Ang II-treated cells and DDR2 siRNA-transfected cells exposed to Ang II were immunoprecipitated using anti-c-Fos and anti-c-Jun antibodies. The amplification of input chromatin prior to immunoprecipitation served as positive control and chromatin immunoprecipitation using a non-specific antibody (normal rabbit IgG) served as negative control. The results, after normalization to

input DNA, confirmed enhanced binding of c-Fos and c-Jun to the Integrin-β1 gene promoter in response to stimulation with Ang II. Further, AP-1 binding activity was attenuated upon DDR2 silencing, confirming the role of DDR2-dependent AP-1 in the transcriptional regulation of Integrin-β1 (Figure 34).

IV.8.1. AP-1 is involved in the transcriptional regulation of Ang II-stimulated Integrin-β1

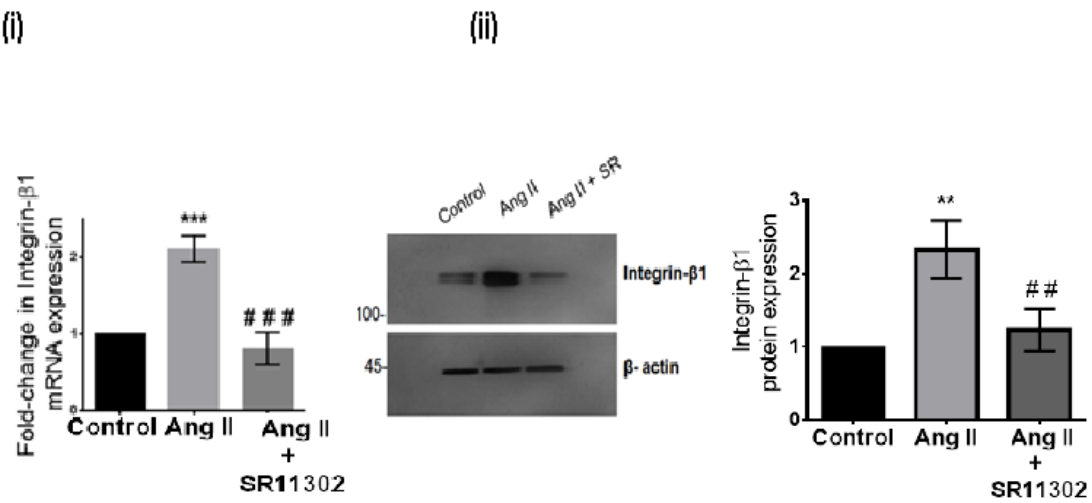


Figure 30: AP-1 inhibition by SR11302 downregulated Ang II-stimulated increase in Integrin-β1 mRNA and protein levels

Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated with AP-1 inhibitor (SR 11302) for 1 h and, subsequently, with Ang II. ***(i)*** *Integrin-β1 mRNA levels were determined by RT-qPCR analysis at 6 h of Ang II treatment. β-actin served as the endogenous control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II.* ***(ii)*** *Protein was isolated at 12 h post-Ang II treatment and subjected to western blot analysis for detection of Integrin-β1, with β-actin as loading control. ** p< 0.01 vs. control, ## p< 0.01 vs. Ang II. (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.8.2. DDR2, ERK1/2 MAPK and TGF-β1 regulate the nuclear transclocation of AP-1 in Ang II-treated cardiac fibroblasts

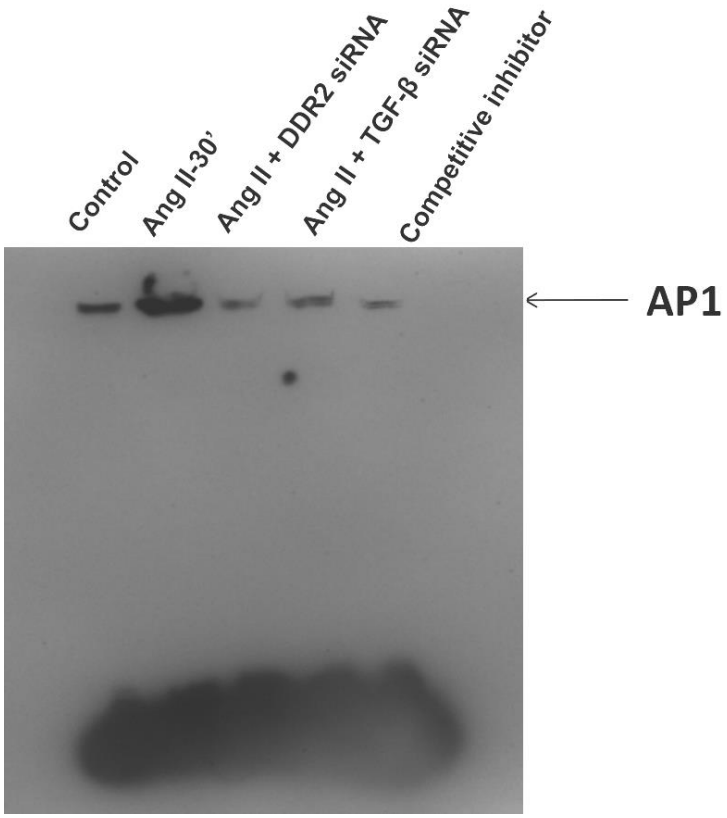


Figure 31: DDR2 and TGF-β1 knockdown reduced Ang II-stimulated nuclear translocation of AP1

EMSA was performed as described under Methods. Ang II enhanced AP-1 nuclear translocation at 30 minutes post-treatment, which was attenuated upon siRNA-mediated silencing of DDR2 and TGF-β1, respectively.

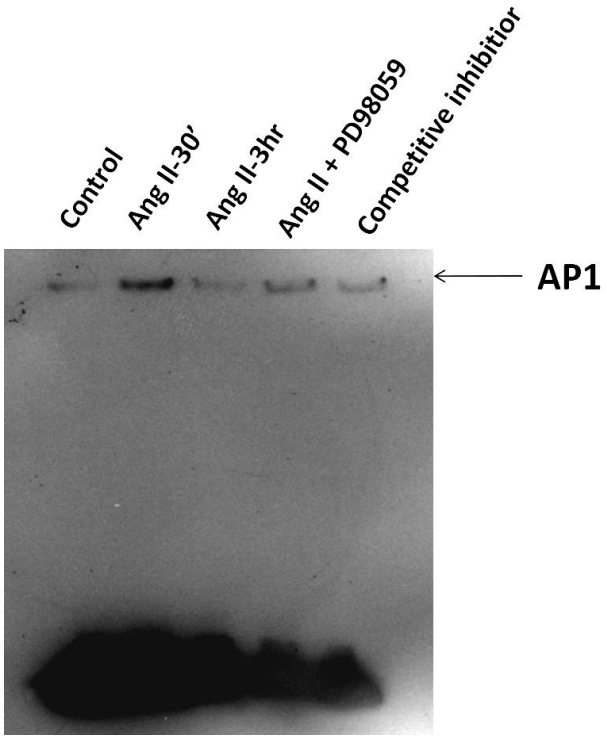


Figure 32: Inhibition of ERK1/2 MAPK using PD98059 reduced Ang II-induced nuclear translocation of AP-1

Ang II enhanced AP-1 nuclear translocation at 30 minutes post-treatment, which was attenuated upon ERK1/2 MAPK inhibition using PD98050.

IV.8.3. Chromatin immunoprecipitation assay (ChIP)

To confirm the binding of AP-1 subunits c-FOS and c-JUN binding to the Integrin-β1 promoter in Ang II-treated cells in vivo, ChIP was performed, which would report the binding of c-FOS and c-JUN subunist of AP-1 to the chromatin region corresponding to the AP-1 promoter-binding site within the Integrin-β1 gene. Cardiac fibroblasts with and without Ang II treatment for 3 hours were lysed and sheared to obtain chromatin fragments of 500 to 600 bp (Figure 33). Ang II enhanced the binding of c-FOS and c-JUN to the AP-1 promoter. Further, DDR2 silencing

reduced the binding of c-FOS and c-JUN to the AP-1-binding region of Integrin-β1 promoter, demonstrating the role of DDR2 in mediating Ang II-stimulated activation and binding of the transcription factor AP-1 to the Integrin-β1 promoter (Figures 33 and 34).

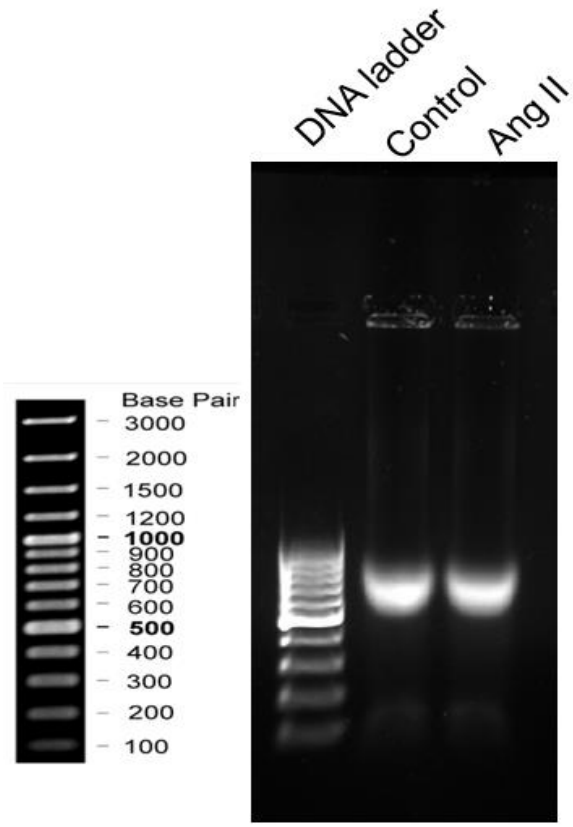


Figure 33: Assessment of chromatin shearing efficiency

Chromatin isolated from cardiac fibroblasts was sheared in a Bioruptor to obtain fragments of 300 bp that were analysed on 1.2% agarose gel.

Figure 34: DDR2 knockdown reduced Ang II-stimulated binding of AP-1 subunits c-FOS and c-JUN to the Integrin-β1 gene promoter

DNA binding of AP-1 subunits, c-FOS and c-JUN, to the ITGB1 gene promoter was confirmed by ChIP using anti-c FOS and anti-c JUN antibodies, respectively. A non-specific anti-rabbit IgG was used as negative control. A representative image showing the PCR amplification product is given.

IV.9. Validation of the DDR2-Integrin-β1 relationship in vivo

The findings from the study pointed to the role of DDR2 in the regulation of Integrin-β1 expression. The link between DDR2 and Integrin-β1 was further analyzed in a Spontaneously Hypertensive Rat (SHR) model and a DDR2 knockout mouse model.

IV.9.1. DDR2 expression is positively correlated with elevated Integrin-β1 in a Spontaneously Hypertensive Rat model

Western blot analysis of cardiac tissue from 6-month-old Wistar and SHR with cardiac fibrosis, as demonstrated by picrosirius red staining, pointed to an association between DDR2 and Integrin-β1 that corelated with the markers of tissue fibrosis – α-SMA and collagen type I (Figures 35 and 36). Further, in cardiac fibroblasts isolated

from 6-month old SHR, a significant increase in the expression of Integrin-β1 protein expression positively correlated with elevated levels of DDR2 (Figure 37). These results pointed to an association between the two collagen receptors and their positive correlation with fibrotic disease progression in the SHR model.

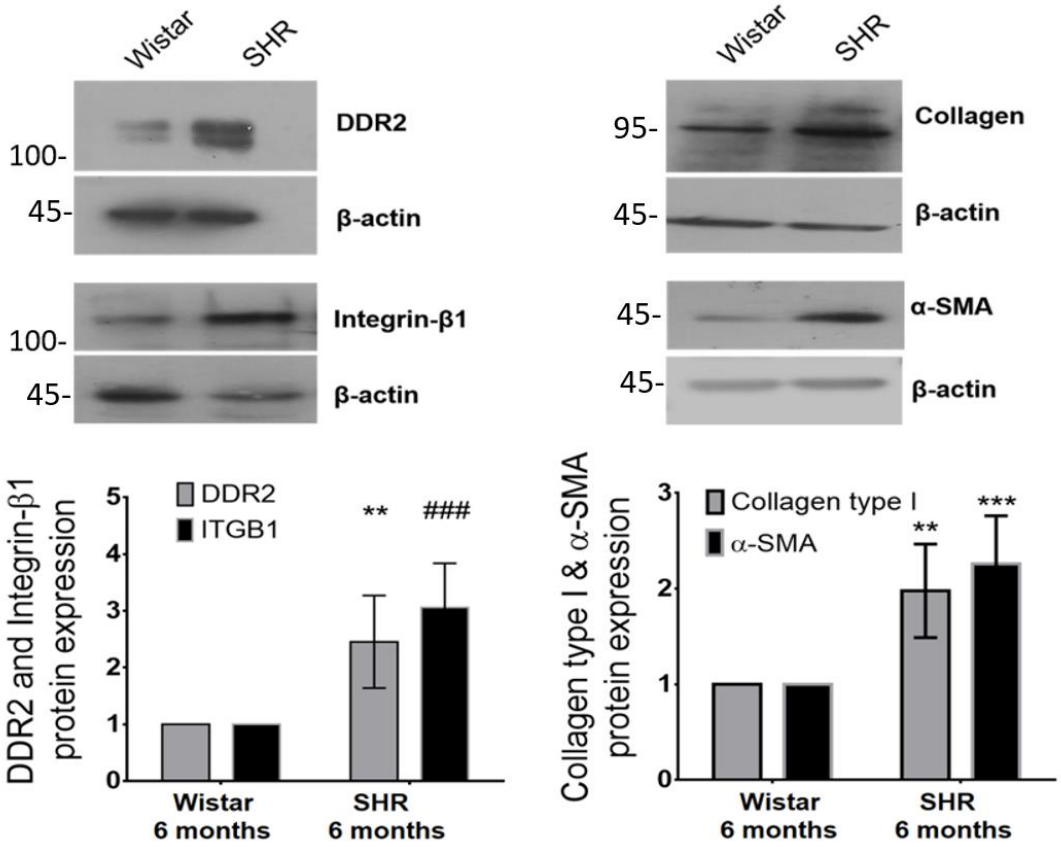


Figure 35: Western blot analysis of DDR2, Integrin-β1, collagen alpha1(I) and α-SMA in cardiac tissue of Wistar and SHR.

*Cardiac tissues isolated from 6-month old Wistar and SHR were analyzed for the expression of DDR2, Integrin-β1, collagen alpha1(I) and α-SMA by western blot analysis. ** p< 0.01 vs. Wistar for DDR2. ### p< 0.001 vs. Wistar for Integrin-β1 (ITGB1). ** p< 0.01 vs. Wistar for collagen alpha1(I). *** p< 0.001 vs. Wistar for α-SMA. (unpaired, two-tailed Student's t-test), n=5, error bars represent SD. The molecular weights are indicated in KDa.*

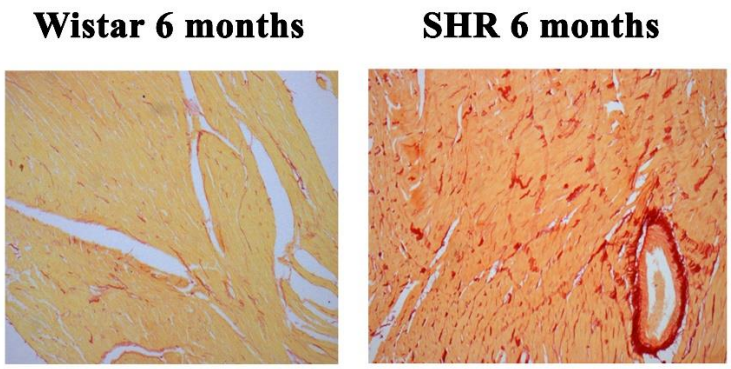


Figure 36: Picrosirius red staining for collagen

Myocardial tissue sections of 6 month-old Wistar and SHR rats were stained for collagen using picrosirius-red (10x magnification).

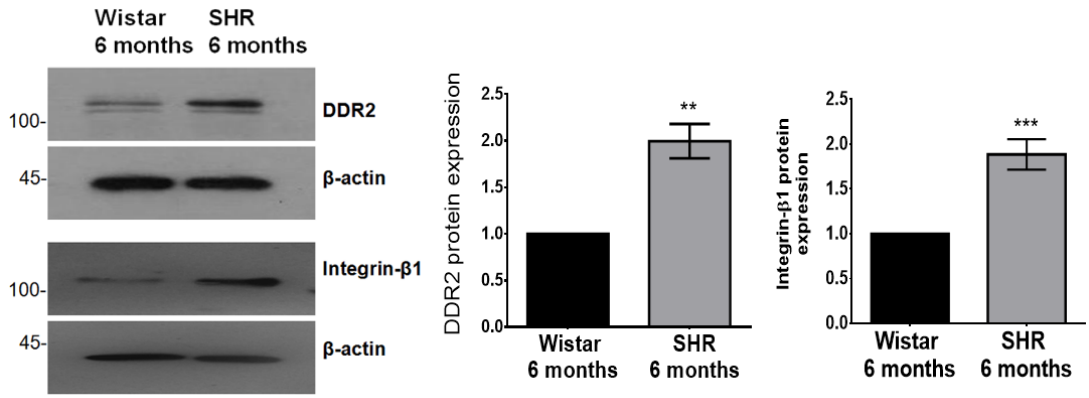


Figure 37: Correlation of DDR2 with Integrin-β1 levels in cardiac fibroblasts isolated from Wistar and SHR

*Cardiac fibroblasts were isolated from 6-month old Wistar and SHR rats. The cells were pre-plated for 2.5 h followed by protein isolation and western blot analysis for the detection of DDR2 and Integrin-β1. β-actin was used as loading control. ** p< 0.01 vs. Wistar, *** p< 0.001 vs. Wistar. (Unpaired two-tailed Student's t-test), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.9.2. DDR2 knockout significantly reduces myocardial Integrin-β1 in mice

Validation of the DDR2-Integrin-β1 link was demonstrated in vivo using knockout mice carrying a germline deletion of DDR2. Previously, knockin of a MerCreMergene casette targeting exon 3 within the DDR2 allele was used for germline deletion of DDR2 in mice. The generation, validation and initial observations on the DDR2-null mice used in this study were reported previously by Cowling et.al (Cowling *et al.*, 2014). In the present study, the effect of DDR2 knockout on Integrin-β1 expression was checked using this mouse model. Immunohistochemistry analysis of myocardial tissue sections using anti-Integrin-β1 antibody demonstrated a significant reduction in myocardial Integrin-β1 expression in the DDR2 null mice (Figure 38). Since fibroblasts are reportedly fewer in number in the mouse cardiac tissue (Pinto *et al.*, 2016), such a striking and global reduction in Integrin-β1 immunostaining intensity suggested a decrease in Integrin-β1 expression in cardiomyocytes as well as fibroblasts.

Hence, preliminary experiments were performed to explore the role of DDR2 on Integrin-β1 expression in myocytes. Rat ventricular H9c2 cells were used as a myocyte model system to study the effect of cardiac fibroblast-specific DDR2 in the regulation of Integrin-β1 expression in myocytes. To this end, the effect of rat ventricular fibroblast-conditioned media on Integrin-β1 expression in cultures of rat ventricular H9c2 cells was probed. Exposure of H9c2 cells for 24 h to fibroblast-conditioned medium enhanced basal Integrin-β1 expression. Importantly, exposure of H9c2 cells to conditioned medium from DDR2-silenced cardiac fibroblasts failed to cause an increase in Integrin-β1 expression (Figure 39), suggesting a role for

DDR2 in cardiac fibroblasts in the regulation of Integrin-β1 expression in cardiomyocytes through a paracrine signaling mechanism.

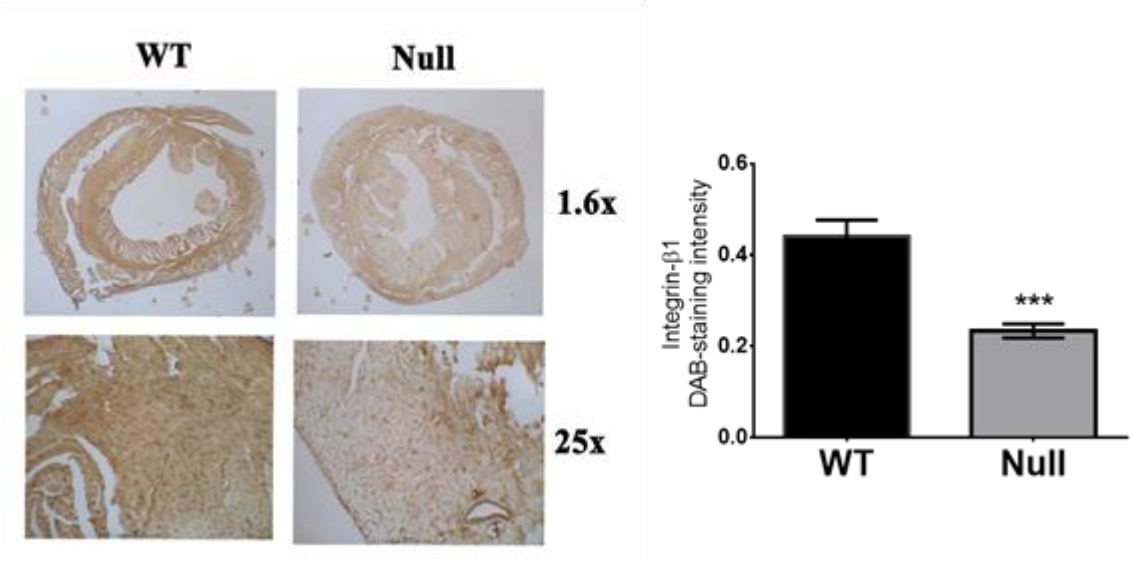


Figure 38: Integrin-β1 Immunostaining in WT and DDR2-null mice

*Immunostaining using DAB showing Integrin-β1 protein in cardiac tissue sections of 10 week-old Wild Type (WT) and DDR2-null mice. *** p< 0.001 vs. WT (unpaired, two-tailed Student's t-test), n=5, error bars represent SD.*

IV.9.3. Conditional media experiment demonstrating a role for rat ventricular fibroblast-derived DDR2 on Integrin-β1 expression in rat ventricular H9c2 cells

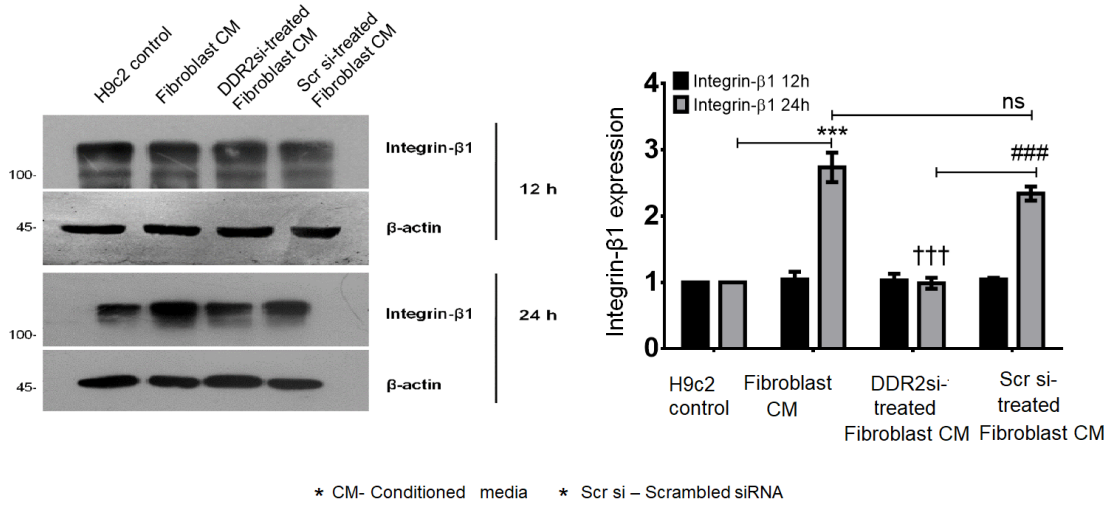


Figure 39: Paracrine regulation of myocyte Integrin-β1 by DDR2

*H9c2 cardiomyoblasts were treated with conditioned media (CM) from control or DDR2-silenced or scrambled siRNA-treated cardiac fibroblasts for 12 h or 24 h. H9c2 in M199 without serum was used as positive control for basal Integrin-β1 protein expression in H9c2 cells. Integrin-β1 protein expression was examined by western blot analysis and normalized to β-actin. *** p< 0.001 vs. H9c2 control at 24 h, ††† p< 0.001 vs. Fibroblast CM at 24 h, ### p< 0.001 vs. DDR2-siRNA-treated fibroblast CM at 24 h, ns-not significant. (Two-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.10. Ang II-stimulated Integrin-β1 regulates α-SMA and collagen type I expression in cardiac fibroblasts

Following myocardial injury, fibroblast activation to the myofibroblast phenotype is marked by enhanced expression of α-SMA along with an increase in the synthesis of collagen type I that accumulates, leading to tissue fibrosis (Eva A. Rog-Zielinska *et al.*, 2016). Owing to the role of collagen receptors in the pathogenesis of tissue fibrosis, the role of DDR2-dependent Integrin-β1 in the regulation of α-SMA and collagen type I in Ang II-stimulated cardiac fibroblasts was analyzed. The validation of Integrin-β1 silencing by siRNA interference is shown in (Figure 40). Knockdown of DDR2 and Integrin-β1 attenuated Ang II-induced α-SMA and collagen alpha1(I) expression (Figures 41-43), demonstrating their role in the regulation of fibroblast activation and activity. Interestingly, knockdown of Integrin-β1 also significantly attenuated Ang II-induced expression of DDR2 protein (Figure 44). Since DDR2 regulates Integrin-β1 expression, this finding demonstrating a mutual regulatory relationship between the collagen receptors.

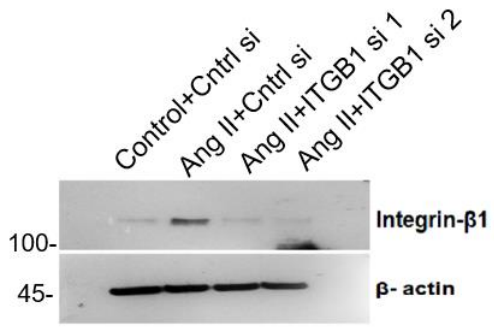


Figure 40: Knockdown efficiency of Integrin-β1

Silencing efficiency of Integrin-β1 siRNA1 or siRNA2 on Integrin-β1 protein expression was checked. siRNA2 was used for silencing Integrin-β1 subsequently.

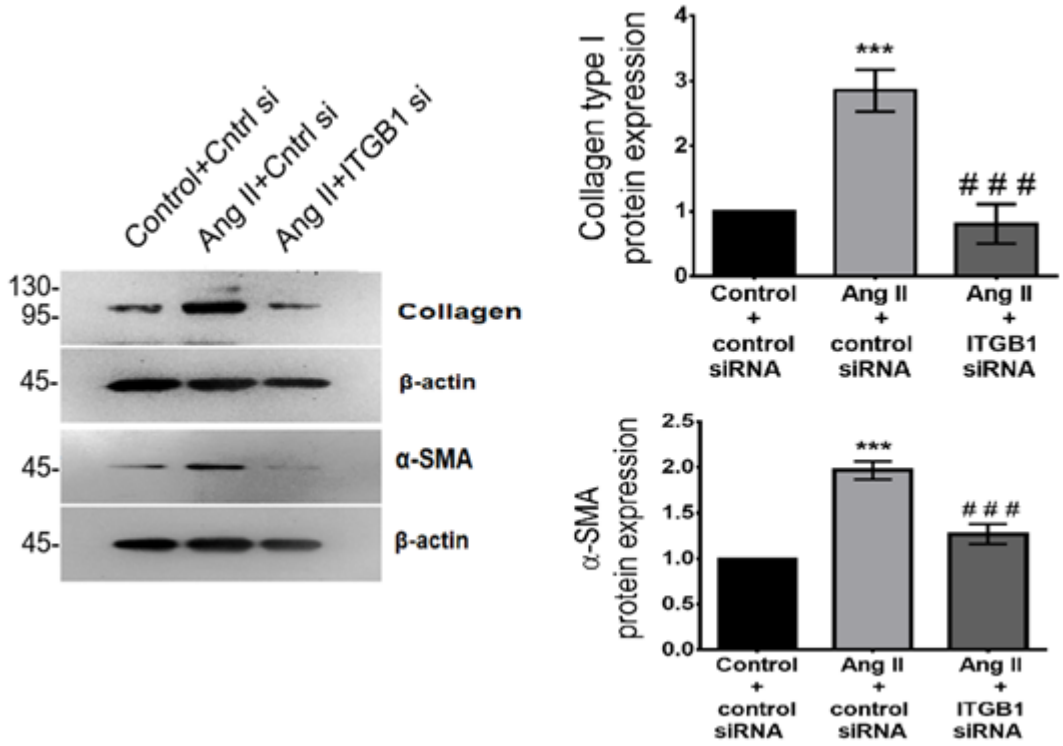


Figure 41: Integrin-β1 was found to regulate Ang II-stimulated expression of collagen alpha1(I) and α-SMA in cardiac fibroblasts

*Cardiac fibroblasts were transiently transfected with Integrin-β1 siRNA or scrambled siRNA. In these cells, following Ang II treatment for 12 h, collagen alpha1(I) and α-SMA protein levels were analyzed, with β-actin as loading control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

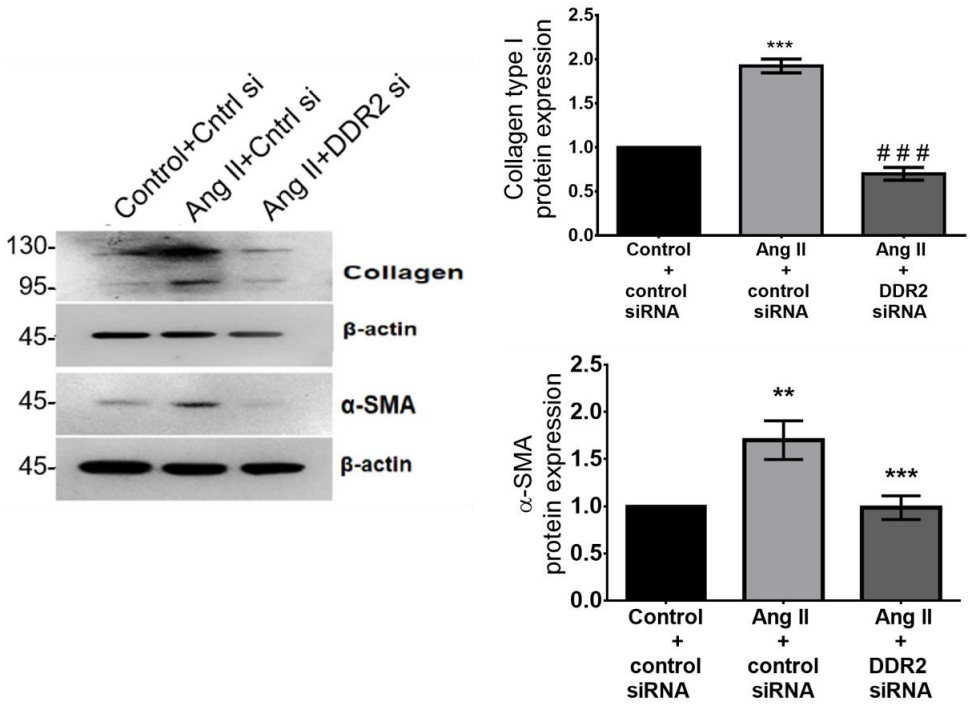


Figure 42: DDR2 was found to regulate Ang II-stimulated expression of collagen alpha1(I) and α-SMA in cardiac fibroblasts

*Cardiac fibroblasts were transiently transfected with DDR2 siRNA or scrambled siRNA. The transfected cells were exposed to Ang II for 12 h. collagen alpha1(I) and α-SMA protein levels were analyzed, with β-actin as the loading control. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (for collagen alpha1(I)). ** p< 0.01 vs. control, *** p< 0.001 vs. Ang II (for α-SMA). (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

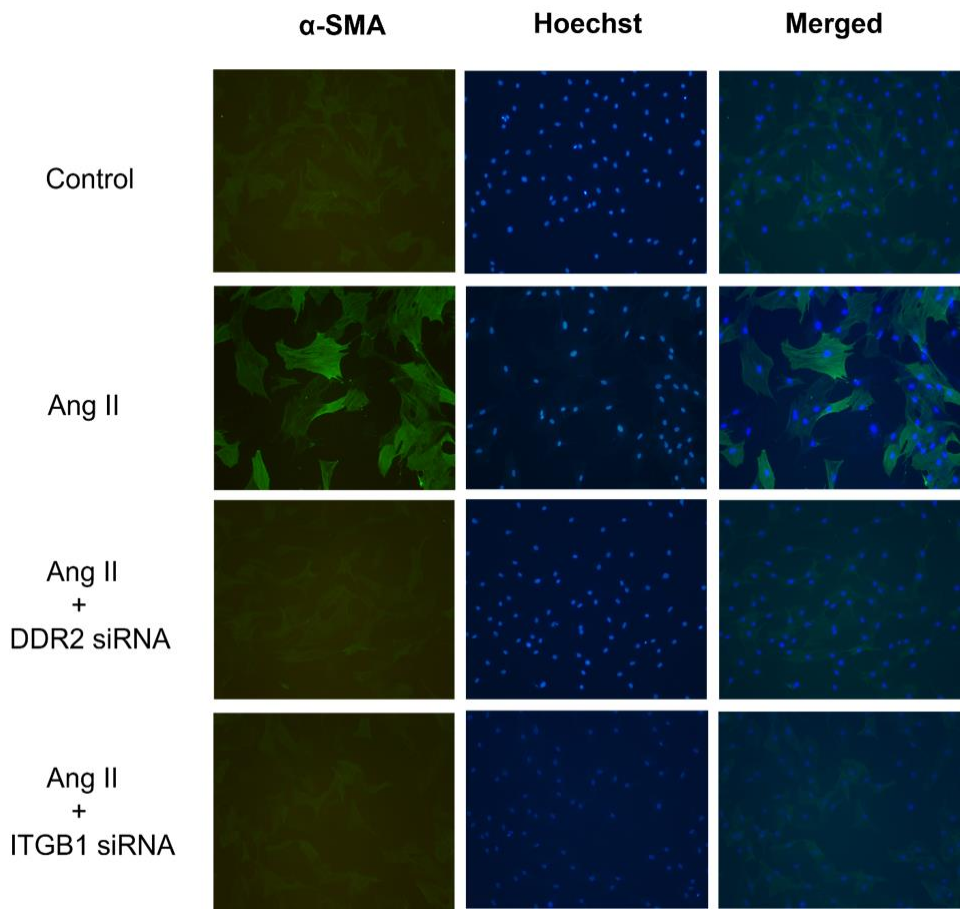


Figure 43: Knockdown of DDR2 or Integrin-β1 attenuated the expression of α-SMA in Ang II-treated cardiac fibroblasts

Cardiac fibroblasts were transiently transfected with DDR2 siRNA or Integrin-β1 siRNA. Following exposure to Ang II for 12 h, α-SMA expression was examined by immunocytochemistry analysis.

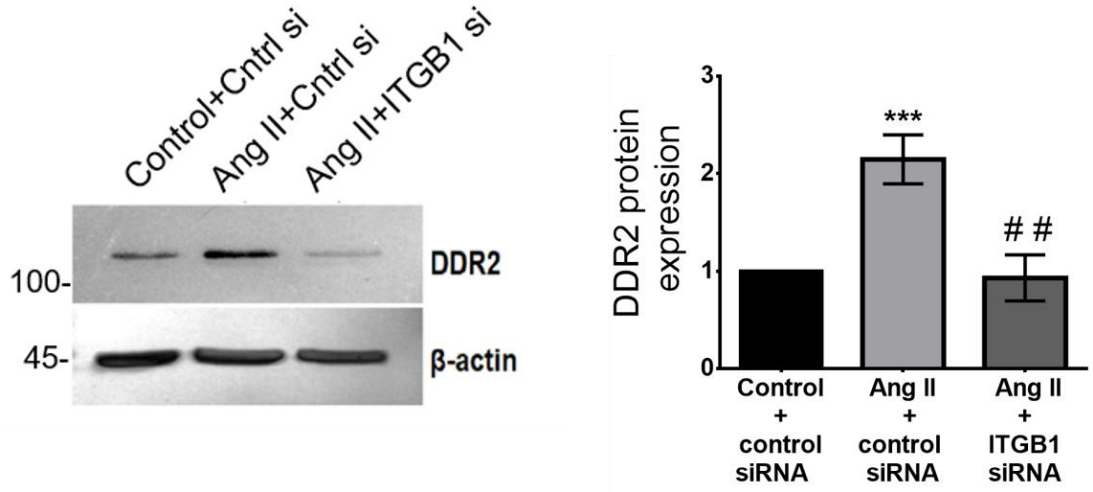


Figure 44: Integrin-β1 knockdown attenuated Ang II-induced expression of DDR2

*Cardiac fibroblasts were transiently transfected with Integrin-β1 siRNA or scrambled siRNA. Following exposure to Ang II for 12 h, DDR2 protein expression was examined by western blot analysis and normalized to β-actin. *** p< 0.001 vs. control, ## p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.11. DDR2-Integrin-β1 axis regulates α-SMA and collagen type I expression in Ang II-stimulated cardiac fibroblasts

Since DDR2 was found to regulate the expression of Integrin-β1 and both these collagen receptors were individually demonstrated to regulate alpha-SMA and collagen, the possibility that DDR2 acts via Integrin-β1 to mediate α-SMA and collagen type I expression was explored using a combination of gene silencing and overexpression approaches. Overexpression of Integrin-β1 in DDR2-silenced cells restored α-SMA and collagen alpha1(I) expression in cardiac fibroblasts exposed to

Ang II (Figure 46). However, DDR2 overexpression in Integrin-β1-silenced cells failed to restore α-SMA and collagen alpha1(I) expression in Ang II-treated cells (Figure 47), showing that DDR2 acts via Integrin-β1 to mediate the expression of Ang II-stimulated α-SMA and collagen type I in cardiac fibroblasts.

IV.11.1. Overexpression of Integrin-β1 in DDR2-silenced cardiac fibroblasts restored the expression of collagen alpha1(I) and α-SMA in Ang II-treated cardiac fibroblasts

Characterization of Integrin-β1 (Itgb1) overexpression plasmid

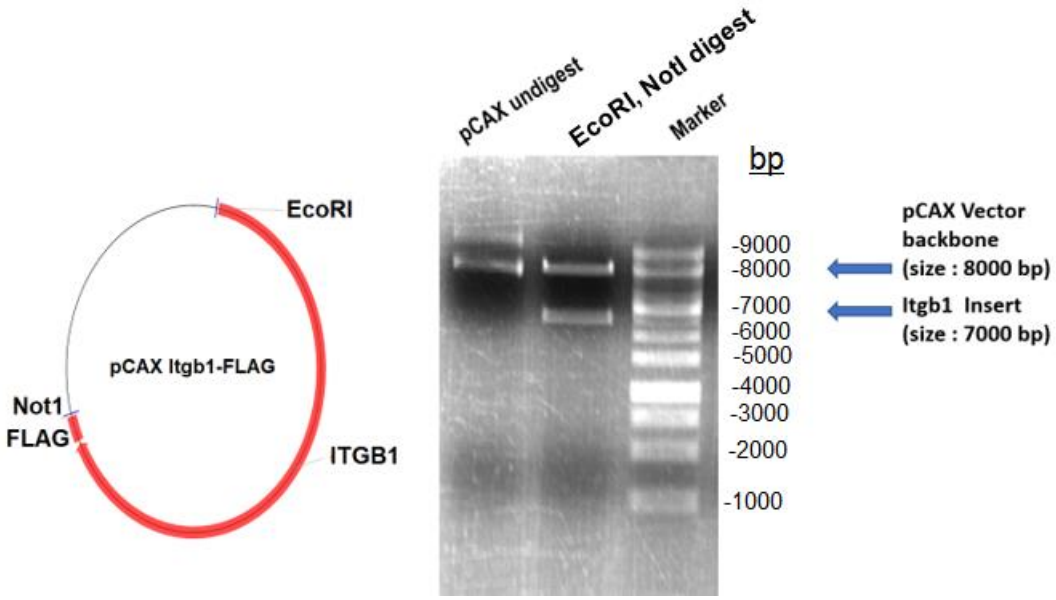


Figure 45: Plasmid map and confirmation of Itgb1 (Integrin-β1) insert in the pCAX plasmid vector

The Itgb1 ORF clone was transformed into competent DH5-α bacterial cells and was amplified. Since the plasmid vector encoded an Ampicillin resistance gene, transformed colonies in Ampicillin containing LB agar was selected and the plasmid

was further amplified by inoculation in LB media. Plasmid isolation was carried out using Qiagen Midi-Prep plasmid isolation kit. The purity and concentration of the plasmid was checked spectrophotometrically. The presence of the Itgb1 insert within the pCAX vector was checked by double site restriction digestion using EcoRI and NotI restriction enzymes. The digest was then examined using agarose gel electrophoresis.

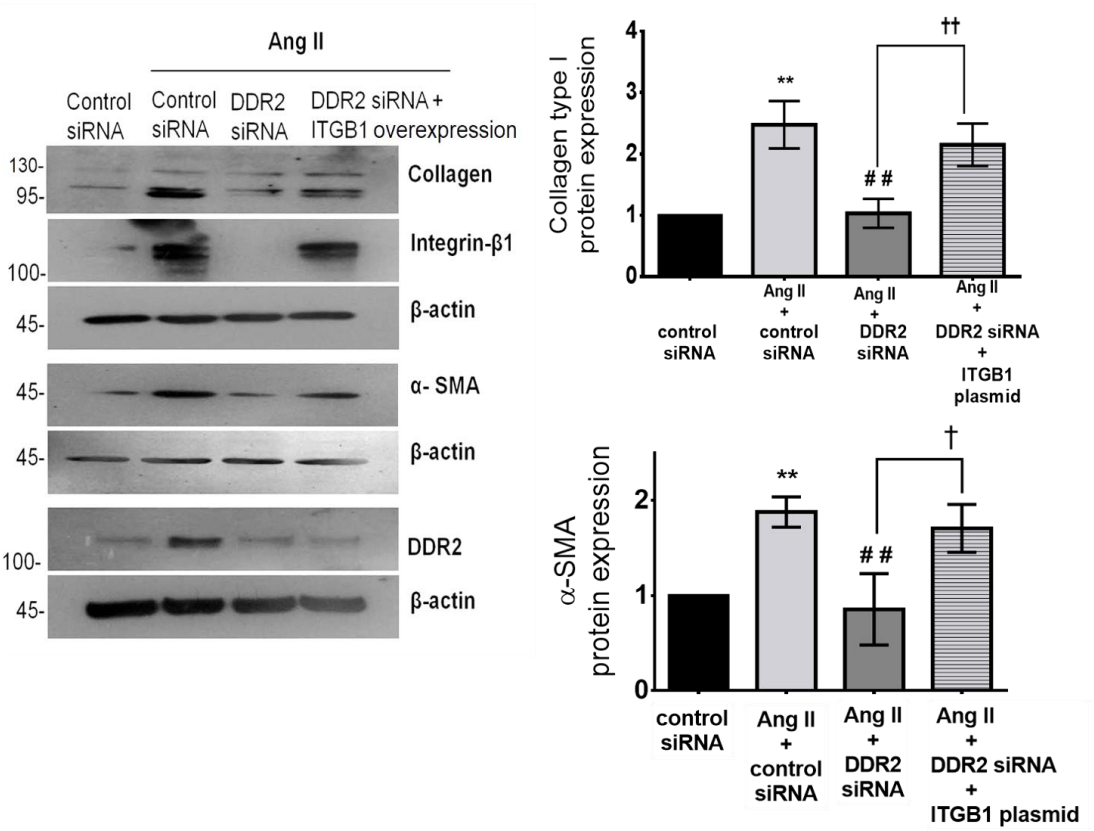


Figure 46: DDR2-dependent Integrin-β1 is a determinant of α-SMA, collagen type I expression in Ang II-treated cardiac fibroblasts

*Cardiac fibroblasts were transiently co-transfected with DDR2 siRNA and Integrin-β1 over-expression vector. Following revival phase and serum-deprivation, the transfected cells were treated with Ang II for 12 h. Collagen alpha1(I) protein expression was analyzed by western blotting and normalized to β-actin. ** p< 0.01*

*vs. control, ## p< 0.01 vs. Ang II, †† p< 0.01 vs. Ang II + DDR2 siRNA, ns-not significant vs. Ang II (One-way ANOVA). α-SMA protein expression was examined and normalized to β-actin. ** p< 0.01 vs. control, ## p< 0.01 vs. Ang II, † p< 0.05 vs. Ang II + DDR2 siRNA, ns-not significant vs. Ang II. (Two-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.11.2. Overexpression of DDR2 in Integrin-β1-silenced cardiac fibroblasts failed to restore the expression of collagen alpha1(I) and α-SMA in Ang II-treated cardiac fibroblasts

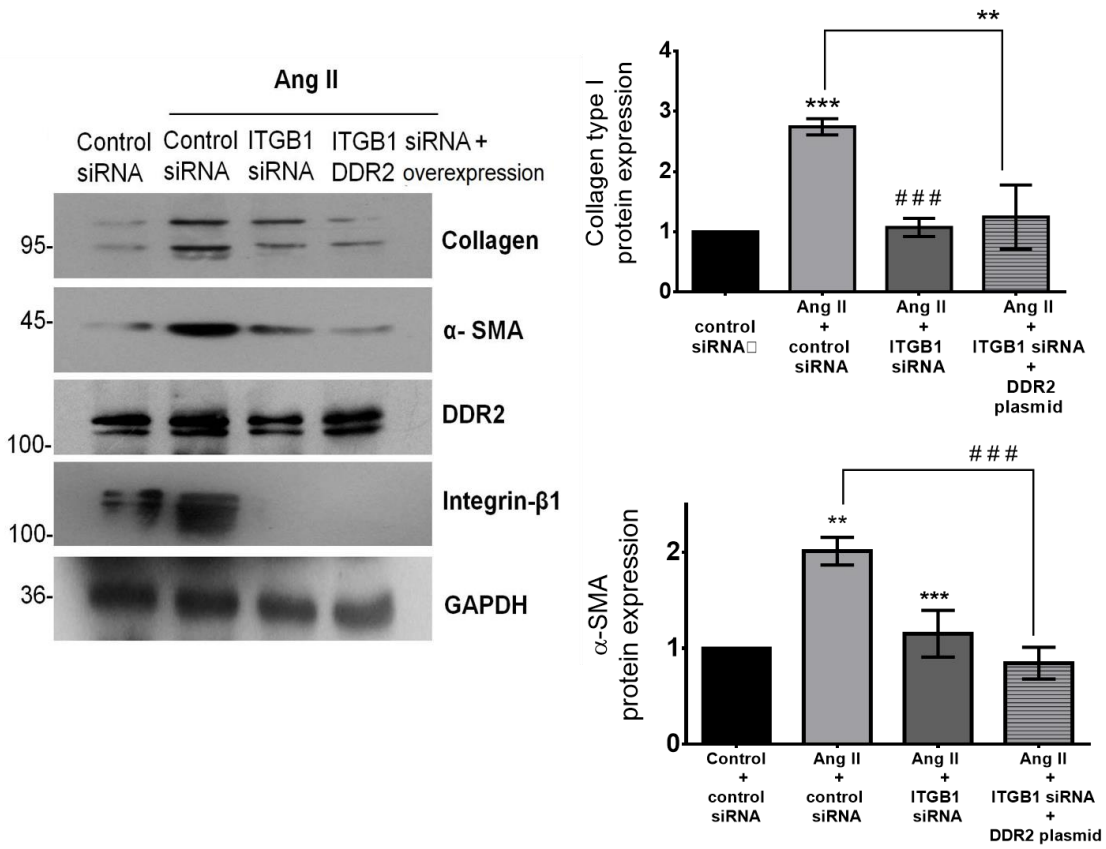


Figure 47: Overexpression of DDR2 in Integrin-β1 silenced cells failed to restore collagen type I and α-SMA expression in Ang II-treated cardiac fibroblasts

*Cardiac fibroblasts were transiently co-transfected with Integrin-β1 siRNA and DDR2 over-expression vector. Following revival phase and serum-deprivation, the transfected cells were treated with Ang II for 12 h. Collagen alpha1(I)protein expression was examined by western blot analysis and normalized to GAPDH. *** p< 0.001 vs. Control, ### p< 0.001 vs. Ang II, ** p< 0.01 vs. Ang II, ns is not significant vs. Ang II + ITGB1 siRNA. α-SMA protein expression was examined by western blot analysis and normalized to GAPDH. ** p< 0.01 vs. Control, *** p< 0.001 vs. Ang II, ### p< 0.001 vs. Ang II, ns-not significant vs. Ang II + ITGB1 siRNA. (Two-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.12. Wound healing assay:

An in vitro wound healing assay was performed to establish a functional role for DDR2-Integrin-β1 crosstalk in cardiac fibroblasts. While Ang II-induced wound closure was impaired upon knockdown of either DDR2 or Integrin-β1, overexpression of Integrin-β1 in DDR2-silenced cells restored the wound healing response of cardiac fibroblasts (Figure 48).

Together, the data underscores the centrality of DDR2 in cardiac fibroblast function, convincingly establishing that DDR2-dependent Integrin-β1 expression mediates α-SMA and collagen alpha1(I) expression and is critically involved in mediating the wound healing response in Ang II-treated cardiac fibroblasts.

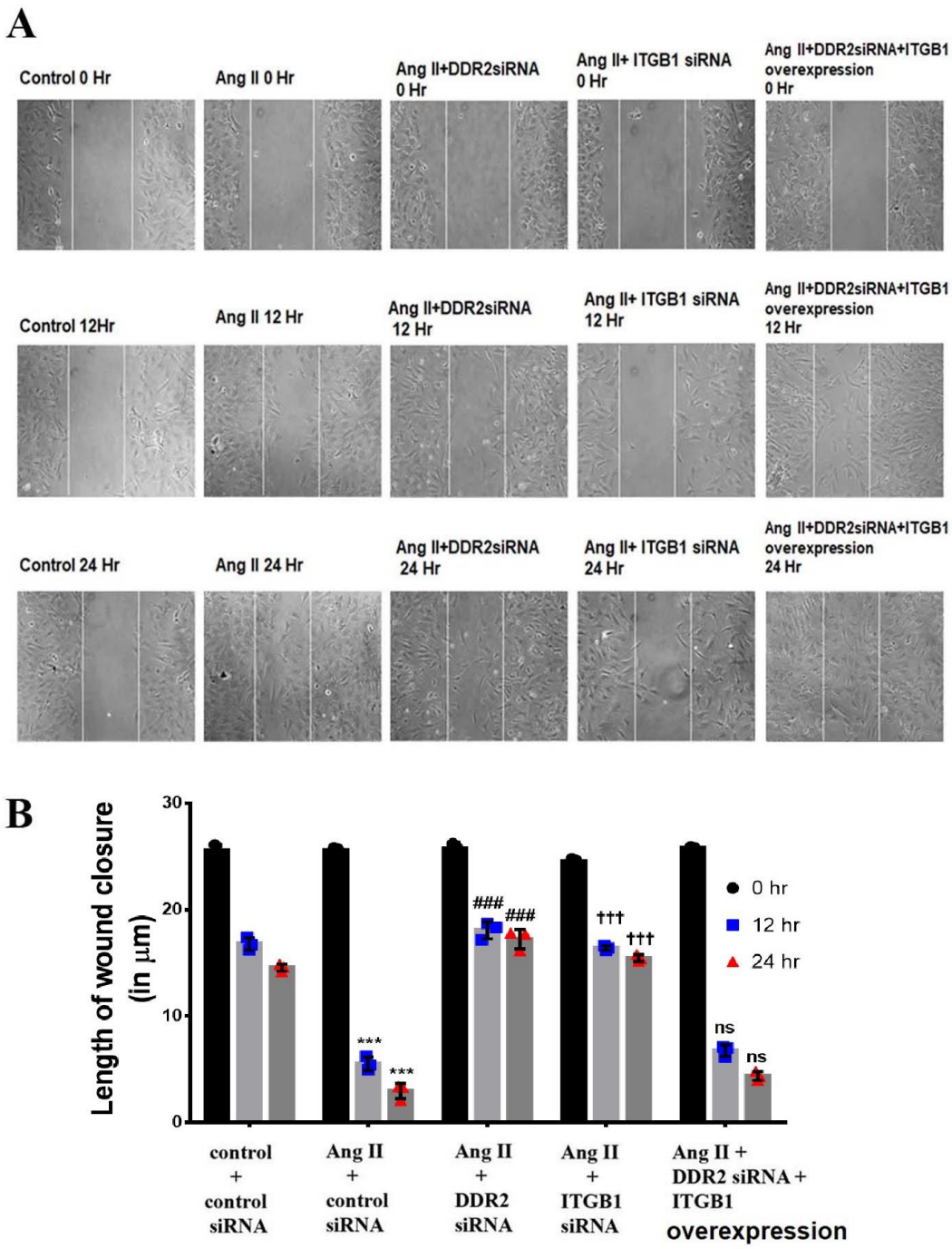


Figure 48: DDR2-dependent Integrin-β1 is a determinant of wound healing in Ang II-treated cardiac fibroblasts

Wound healing ability of cardiac fibroblasts is impaired upon knockdown of DDR2 or Integrin-β1 and is restored upon overxpression of Integrin-β1 over-expression in DDR2-silenced cells. Scratch wound assay was performed as described under Methods. Cardiac fibroblasts were transiently transfected with DDR2 siRNA or

*Integrin-β1 siRNA or co-transfected with DDR2 siRNA and Integrin-β1 over-expression vector. Following revival phase and serum deprivation, the transfected cells were exposed to Ang II and their wound healing ability were examined at 0 h, 12 h and 24 h. The length of wound closure was determined in μm and was quantified as described under Methods. 3-4 fields were examined per dish. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II, ††† p< 0.001 vs. Ang II, ns-not significant vs. Ang II. (Two-way ANOVA), n=3, error bars represent SD.*

IV.13. Schematic diagram based on the findings presented above

Based on the findings presented, the following set of mechanistic events emerge (Figure 49):

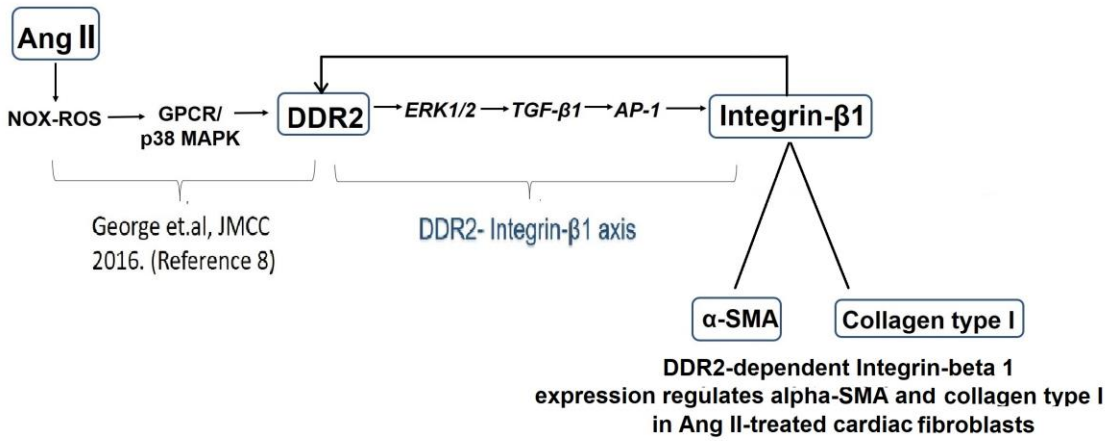


Figure 49: Schematic representation

IV.14. Functional coupling between phenotypic transition and collagen type I expression in cardiac fibroblasts

IV.14.1. α-SMA functions downstream of the DDR2-Integrin-β1 axis to regulate collagen type I expression via the TRPC6-YAP signaling pathway in cardiac fibroblasts exposed to Ang II

The role of α-SMA, downstream of the DDR2-Integrin-β1 axis, in the regulation of collagen type I expression in Ang II-treated cardiac fibroblasts was explored. α-SMA knockdown significantly attenuated collagen alpha1(I) expression in Ang II-treated cardiac fibroblasts (Figure 50) demonstrating that phenotypic conversion of cardiac fibroblasts into α-SMA positive myofibroblasts is functionally coupled to enhanced collagen type I expression. Probing the mechanisms that mediate α-SMA-dependent collagen type I expression, the role of TRPC6 was analyzed. TRPC6 is a Ca^{2+} channel reported to positively influence fibroblast phenotypic conversion to α-SMA-positive myofibroblasts and activate pro-fibrotic signaling in cardiac fibroblasts (Davis *et al.*, 2012). Hence, the role of TRPC6 as a mediator of α-SMA-dependent collagen type I expression in Ang II-treated cardiac fibroblasts was examined. α-SMA knockdown reduced the expression of Ang II-induced TRPC6 (Figure 51) while silencing TRPC6 significantly attenuated the expression of Ang II-stimulated collagen alpha1(I) expression (Figure 52). Together, the findings demonstrate a role for α-SMA and TRPC6 in the regulation of collagen alpha1(I) expression.

IV.14.2. α-SMA regulates Ang II-stimulated collagen type I expression in cardiac fibroblasts

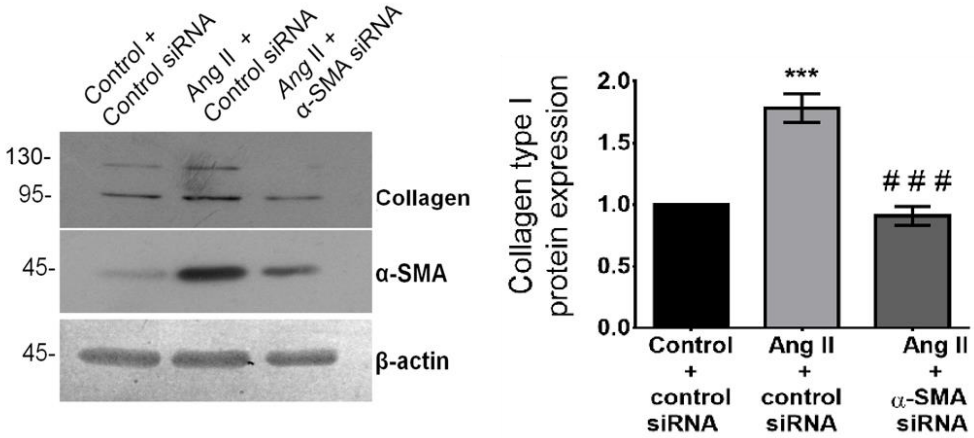


Figure 50: Knockdown of α-SMA attenuated Ang II-induced expression of collagen type I

*Cardiac fibroblasts were transiently transfected with α-SMA siRNA or scrambled siRNA. The transfected cells were exposed to Ang II for 12 h and collagen alpha1(I) protein expression was analyzed by western blotting and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.14.3. α-SMA regulates the expression of Ang II-stimulated TRPC6

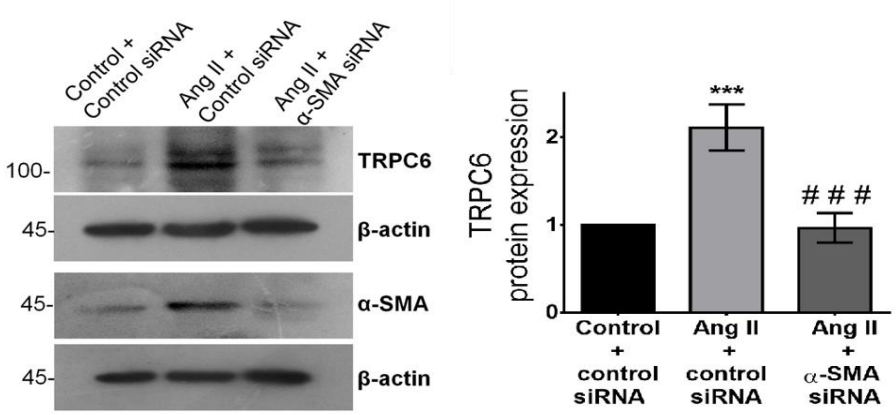


Figure 51: Knockdown of α-SMA attenuated Ang II-induced expression of TRPC6

*Cardiac fibroblasts were transiently transfected with α-SMA siRNA or scrambled siRNA. The transfected cells were exposed to Ang II for 12 h and TRPC6 protein expression was analyzed by western blotting and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.14.4. TRPC6 regulates the expresison of Ang II-stimulated collagen type I

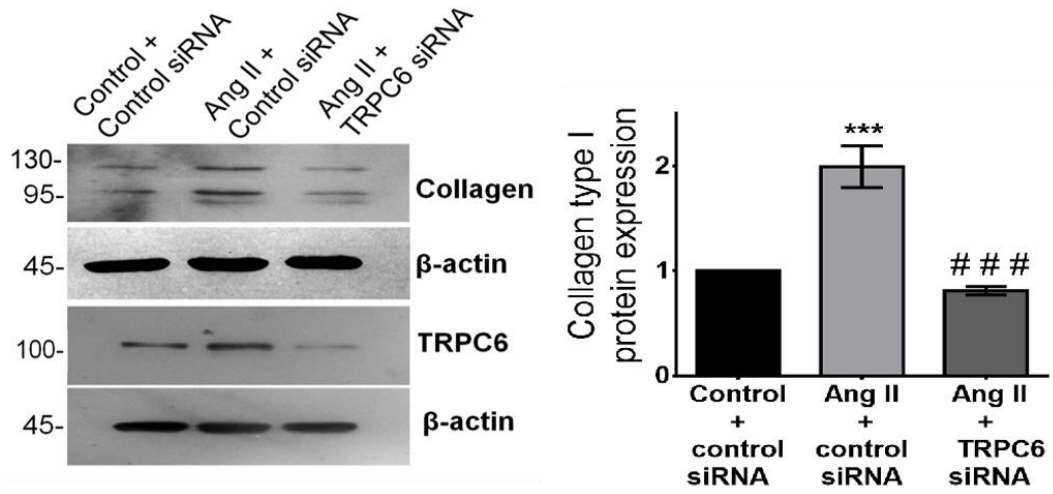


Figure 52: Knockdown of TRPC6 attenuated Ang II-induced expression of collagen type I

*Cardiac fibroblasts were transiently transfected with TRPC6 siRNA or scrambled siRNA. The transfected cells were exposed to Ang II for 12 h and collagen alpha1(I) protein expression was examined by western blot analysis and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.14.5. α-SMA acts via TRCP6 to regulate collagen type I expression in Ang II-treated cardiac fibroblasts

Since α-SMA and TRPC6 were found to mediate Ang II-stimulated collagen type I expression, using an overexpression-based approach, the possibility of α-SMA acting via the calcium channel TRPC6 to regulate collagen alpha1(I) expression in cardiac fibroblasts exposed to Ang II was analyzed. Overexpression of TRPC6 in α-SMA silenced cells restored collagen type I expression, demonstrating a role for α-SMA-

dependent TRPC6 in the regulation of collagen type I expression in Ang II-treated cardiac fibroblasts (Figure 53).

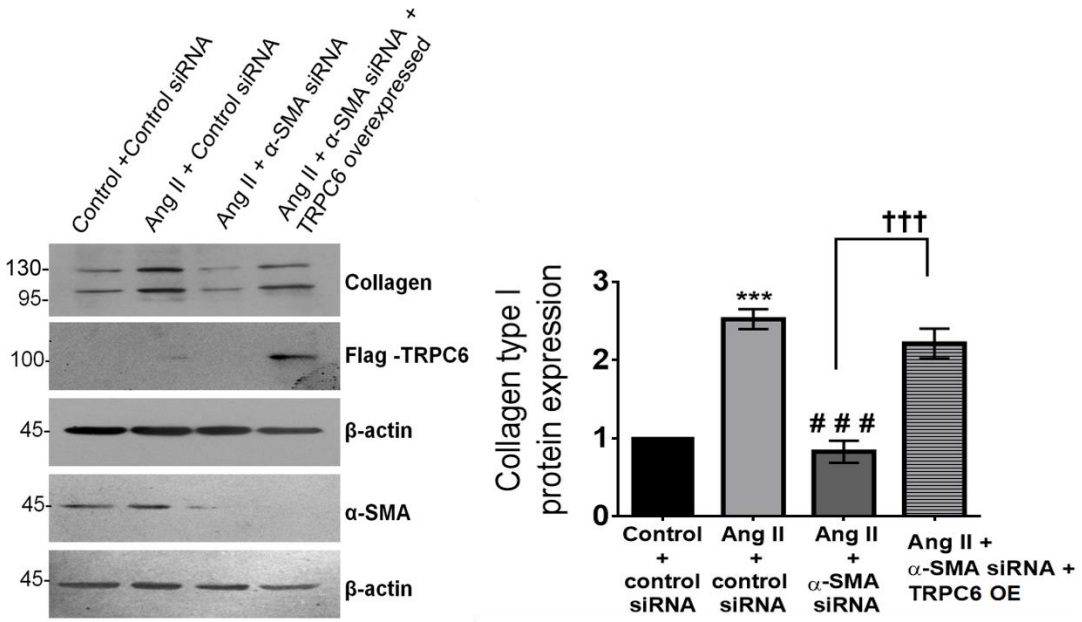


Figure 53: Overexpression of TRPC6 in α-SMA silenced cells restored collagen type I expression in Ang II-treated cardiac fibroblasts

*Cardiac fibroblasts were transiently co-transfected with α-SMA siRNA and TRPC6 over-expression plasmid. Following revival and serum deprivation, the transfected cells were exposed to Ang II for 12 h. Collagen alpha1(I) protein expression was examined by western blot analysis and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II, ††† p< 0.001 vs. Ang II + α-SMA siRNA, ns is not significant vs. Ang II. (Two-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.15. α-SMA-TRPC6-dependent transcriptional regulation of collagen type I is mediated by YAP transcription factor

The transcriptional regulation of collagen type I downstream of the α-SMA-TRPC6 signaling pathway was probed. Yes-associated protein (YAP) is a mechanosensitive transcription factor that responds to changes in actin filament dynamics and mediates pro-fibrotic signaling in the liver (Martin *et al*., 2016). The activation of YAP is dependent on inhibitory phosphorylation at multiple Serine and Threonine residues. Phosphorylation of YAP at Serine 127 is reported to inhibit YAP activation by tethering it to 14-3-3 proteins in the cytoplasm and is used to determine the activation status of YAP (Zhao *et al.*, 2007). In the present study, Ang II enhanced the activation of YAP at 6 h post-treatment, as evidenced by a decrease in the phosphorylation of YAP at S127 (Figure 54). Importantly, α-SMA and TRPC6 knockdown attenuated Ang II-induced YAP activation at 6 h, showing that the activation of YAP is dependent on α-SMA and TRPC6 (Figures 55 and 56). A direct regulatory role for α-SMA and TRPC6 in the regulation of YAP-dependent transcriptional activation of collagen type I was confirmed by ChIP that showed reduced binding of YAP to the collagen alpha1(I) gene promoter upon α-SMA and TRPC6 knockdown in Ang II-stimulated fibroblasts. Considered in tandem with the observation that inhibition of YAP inhibition by YAP-specific inhibitor Verteporfin attenuated Ang II-stimulated expression of collagen alpha1(I), the results demonstrate a novel role for YAP activation downstream of α-SMA and TRPC6 in the transcriptional regulation of collagen type I expression in cardiac fibroblasts (Figures 57 and 58).

IV.15.1. Ang II enhances YAP activation in cardiac fibroblasts

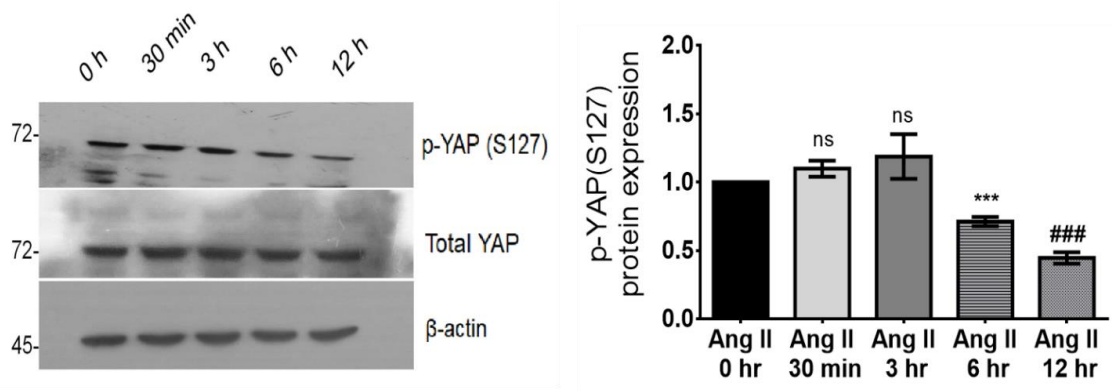


Figure 54: Ang II -treatment enhanced activation of YAP at 6 h

*Cardiac fibroblasts were serum-deprivedfollowed by treatment with Ang II. YAP phosphorylation at S127 was analyzed at 0 h, 30 min, 3 h, 6 h and 12 h post Ang II treatment by western blot analysis and normalized to Total YAP. ns is not significant vs Ang II 0 h, ***p< 0.01 vs Ang II 0 h, ### p<0.001 vs. Ang II 0 h. (unpaired, two-tailed Student's t-test). n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.15.2. α-SMA and TRPC6 mediate Ang II-stimulated YAP activation

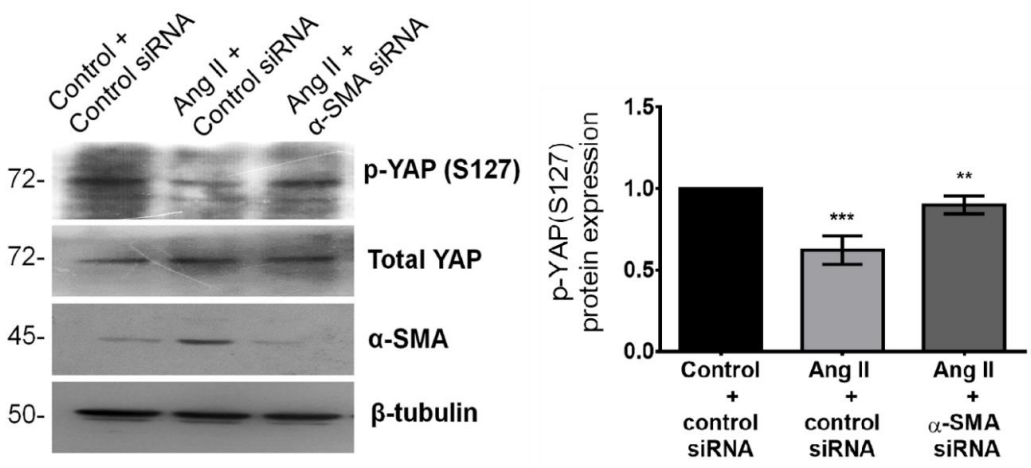


Figure 55: α-SMA silencing enhanced YAP phosphorylation leading to inactivation of YAP

*α-SMA siRNA-transfected cells were exposed to Ang II for 6 h and phosphorylation of YAP at S127 was examined by western blotting and normalized to Total YAP. ***p< 0.001 vs control, **p< 0.01 vs Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

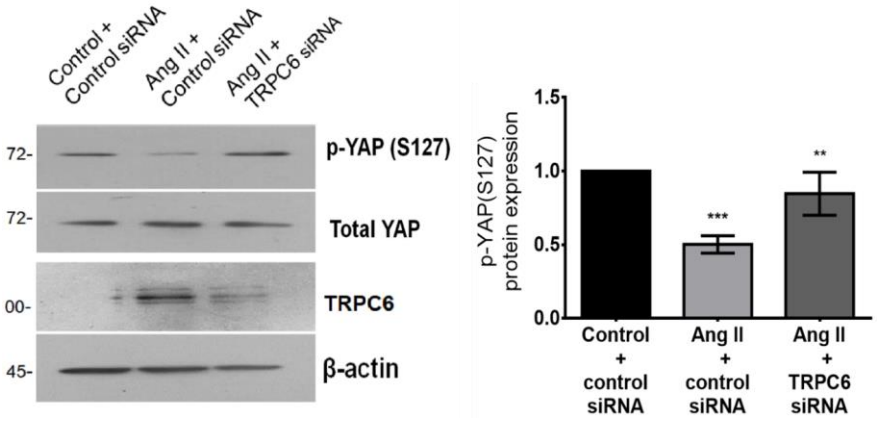


Figure 56: TRPC6 silencing enhanced YAP phosphorylation leading to inactivation of YAP

*TRPC6 siRNA-transfected cells were exposed to Ang II for 6 h and the phosphorylation of YAP at S127 was examined by western blotting and normalized to Total YAP. ***p< 0.001 vs control, **p< 0.01 vs Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.15.3. The YAP transcription factor mediates Ang II-stimulated collagen type I expression

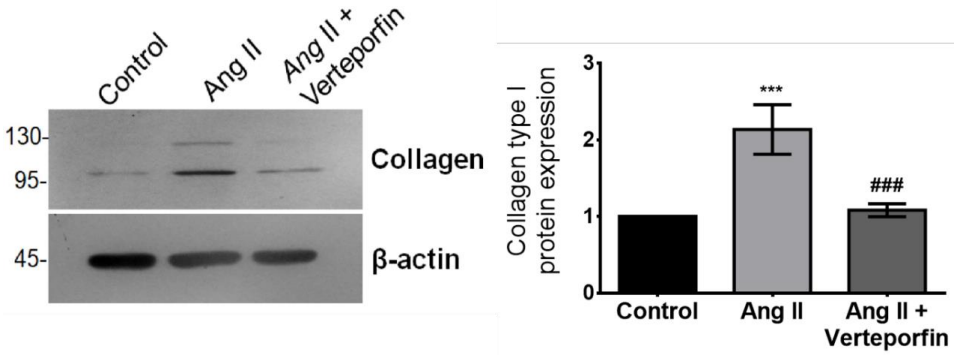


Figure 57: Inhibition of YAP using Verteporfin reduced the expression of collagen type I in Ang II-treated cardiac fibroblasts

*Sub-confluent quiescent cultures of cardiac fibroblasts were pre-treated with YAP inhibitor (Verteporfin) for 1 h and were subsequently treated with Ang II. Protein was isolated at 12 h treatment and subjected to western blot analysis for detection of collagen alpha1(I). β-actin was used as loading control. ***p< 0.001 vs control, ###p< 0.001 vs Ang II (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.15.4. ChIP assay demonstrates a positive regulatory role for YAP in the transcriptional regulation of collagen type I

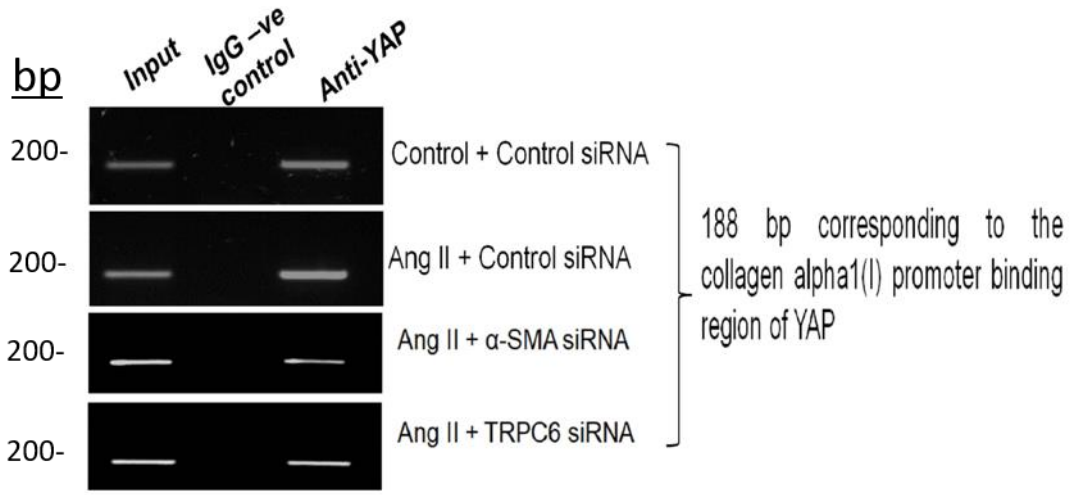


Figure 58: Knockdown of α-SMA and TRPC6 reduced Ang II-stimulated binding of YAP to the collagen alpha1(I) promoter

Cardiac fibroblasts were transiently transfected with α-SMA siRNA, TRPC6 siRNA or scrambled siRNA. Following treatment with Ang II for 6 h, ChIP assay was performed as described under Methods. The binding of YAP transcription factor to the collagen alpha1(I) gene promoter was confirmed by chromatin immunoprecipitation using anti-YAP antibody. A non-specific anti-rabbit IgG was used as the negative control. A representative image depicting the PCR amplification product is shown.

IV.16. Preliminary evidence indicating a role for TRPC6-mediated Ca^{2+} influx in the regulation of YAP activation and collagen type I expression

A previous study by Davis et.al had reported that overexpression of TRPC6 in cardiac fibroblasts enhances intracellular Ca^{2+} levels. Moreover, the study implicated a role for TRPC6-mediated Ca^{2+} influx in mediating cardiac myofibroblast differentiation (Davis *et al.*, 2012). Since the present study focused on the involvement of TRPC6 in mediating collagen type I expression via YAP transcriptional activation, preliminary experiments were performed to investigate the involvement of TRPC6-dependent calcium (Ca^{2+}) in the activation of YAP and regulation of collagen alpha1(I) expression in fibroblasts. Ang II-stimulated YAP activation and collagen alpha1(I) expression was significantly reduced upon chelation of extracellular Ca^{2+} using EGTA (1mM), a specific Ca^{2+}-chelator (Figure 59), demonstrating a role for Ca^{2+} influx in the regulation of YAP activation and collagen type I expression in cardiac fibroblasts. To check whether these Ca^{2+}-mediated events are dependent on the activity of TRPC6, an overexpression study was performed. While overexpression of TRPC6 in fibroblasts enhanced YAP activation and collagen alpha1(I) expression, these effects were significantly attenuated in TRPC6 overexpressing fibroblasts exposed to EGTA (Figure 60), suggesting a role for TRPC6-mediated Ca^{2+}influx in regulating the activation of YAP and collagen type I expression.

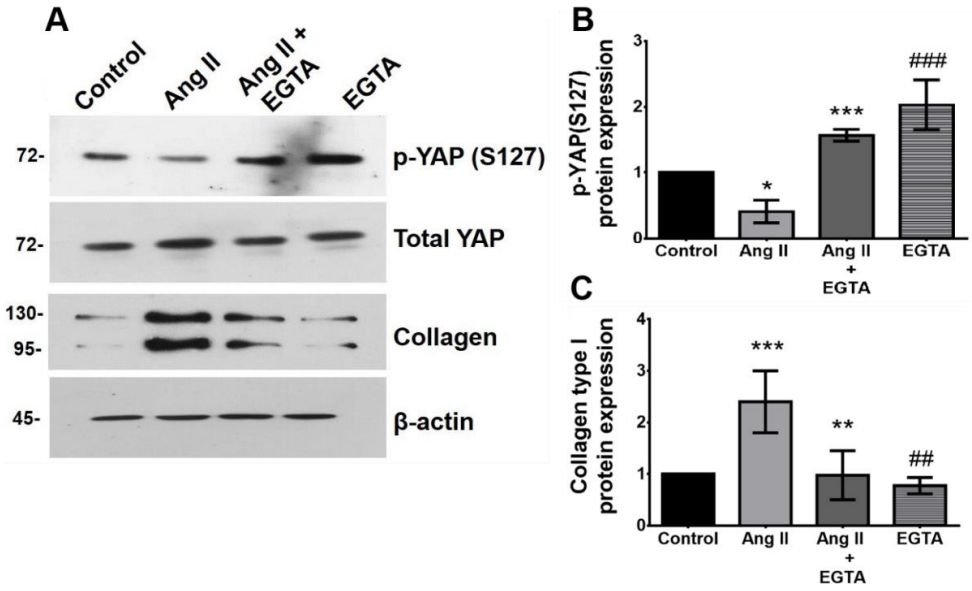


Figure 59: YAP activation and collagen alpha1(I) expression in response to Ang II is dependent on extracellular Ca^{2+} influx

Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated for 1 h with EGTA (1mM) for 1 h followed by Ang II treatment. ***(A, B & C)*** *Phosphorylation of YAP at S127 residue was analyzed by western blotting at 6 h post-Ang II treatment. Total YAP was used as loading control. *p< 0.05 vs. control, ***p< 0.001 vs. Ang II, ### p< 0.001 vs. Ang II. Collagen alpha1(I) expression was examined at 12 h post-Ang II treatment. β-actin was used as loading control. ***p< 0.001 vs. control, **p< 0.01 vs Ang II, ## p< 0.01 vs Ang II. (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

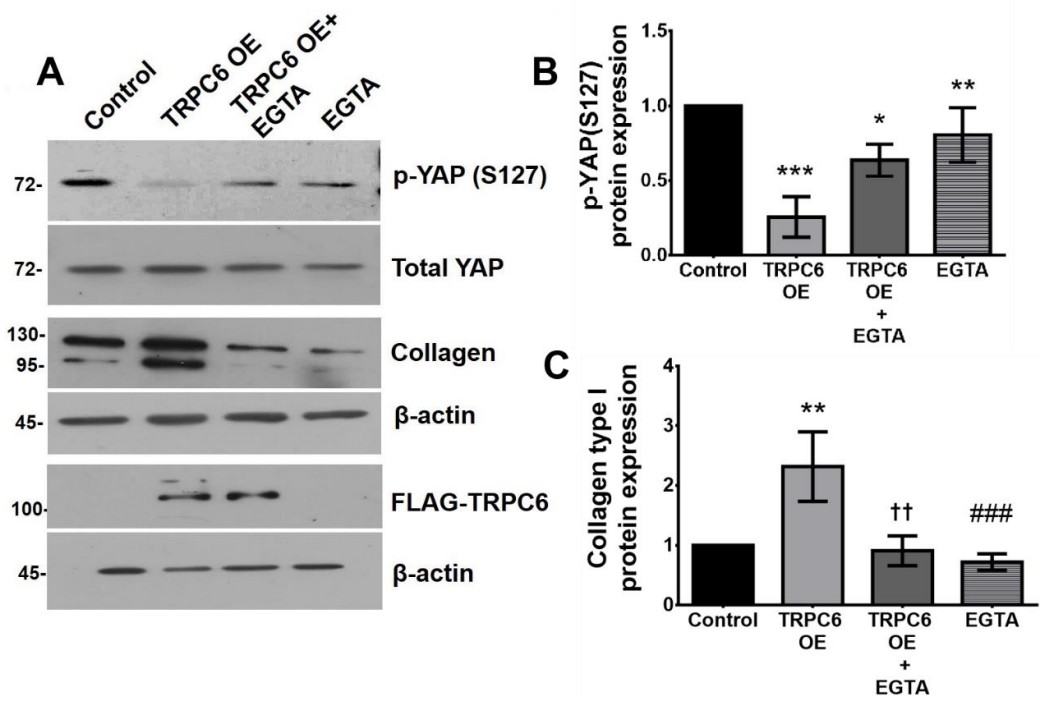


Figure 60: TRPC6-mediated Ca2+ influx was found to regulate YAP activation and collagen alpha1(I) expression

*Cardiac fibroblasts were transiently transfected with TRPC6 overexpression plasmid or empty control vector. Following exposure of the TRPC6 overexpressing cells to EGTA (1mM), YAP phosphorylationat S127 residue was analyzed by western blotting at 6 h post-EGTA treatment with Total YAP as loading control. *** p< 0.001 vs control, * p< 0.05 vs. TRPC6 OE, ** p< 0.01 vs TRPC6 OE. Expression of collagen alpha1(I) protein was analyzed by western blotting at 12 h post-EGTA treatment. β-actin was used as the loading control. ** p< 0.01 vs. control, †† p< 0.01 vs. TRPC6 OE, ### p< 0.001 vs. TRPC6 OE. (One-way ANOVA), n=3, error bars represent SD. The molecular weights are indicated in KDa.*

IV.17. Proposed mechanism for DDR2-Integrin-β1 crosstalk in α-SMA-dependent collagen type I expression in Ang II-treated cardiac fibroblasts

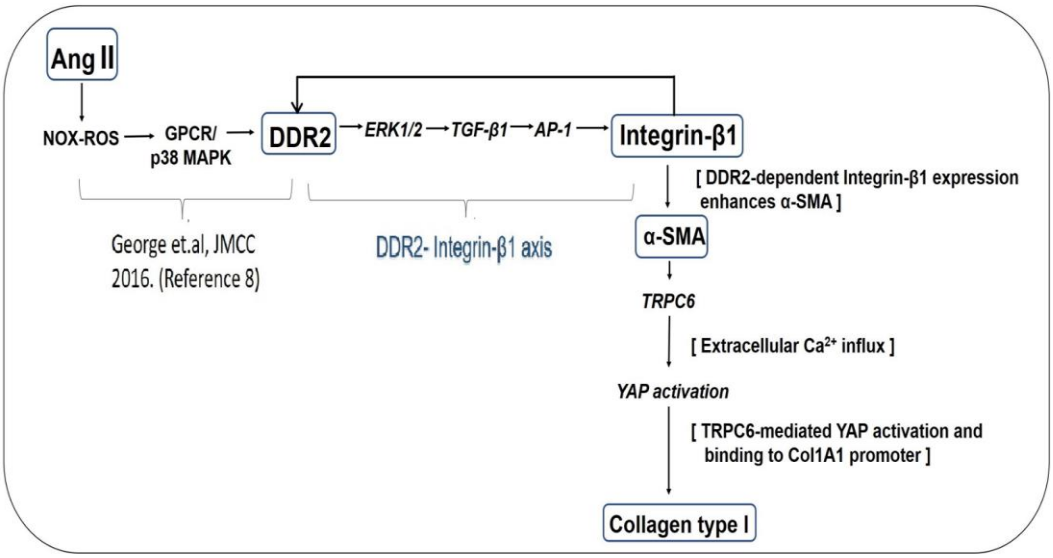


Figure 61: A schematic representation of the probable sequence of signaling events leading to enhanced collagen type I expression in Ang II-treated cardiac fibroblasts.

To summarize the findings, in Ang II-stimulated cardiac fibroblasts, DDR2 acts via ERK1/2 MAPK-dependent TGF-β1 signaling and AP-1 activation to transcriptionally promote Integrin-β1 expression, which in turn enhances α-SMA expression. Notably, α-SMA acts downstream of the DDR-Integrin-β1 axis to regulate collagen alpha1(I) gene expression via the calcium channel TRPC6 and pro-fibrotic transcription factor YAP. The data also indicate the existence of a reciprocal regulatory relationship between DDR2 and Integrin-β1 in which Integrin-β1 functions downstream of DDR2, linking DDR2 to α-SMA, collagen type 1 and wound healing. A schematic representation of these events is presented (Figure 61).

V. DISCUSSION

Cardiovascular disease is a major cause of mortality globally (Benjamin Emelia J. *et al.*, 2019). Myocardial injury triggers a series of events that initiates cellular response to tissue injury in cardiomyocytes and cardiac fibroblasts, culminating in myocardial remodeling (Tallquist and Molkentin, 2017). While the cellular response to stress or injury in myocytes, is marked by hypertrophy or apoptosis, the response evoked in fibroblasts is marked by hyperplasia in these otherwise phenotypically quiescent cells. Importantly, while myocyte hypertrophy can be physiological, elicited in response to growth signals and exercise, or pathological as in response to hypertension, infarction and valvular heart disease, cardiac fibroblast hyperplasia is invariably pathological.

Fibroblasts constitute the predominant cell types within the myocardium and are the sole source for myocardial collagen types I and III that represent the stromal compartment of the cardiac tissue (Eva A Rog-Zielinska *et al.*, 2016). Under normal conditions, fibroblasts maintain a balance between the stromal compartment of the myocardium and the parenchymal compartment comprising myocytes, through homeostatic maintenance of collagen turnover. Following cardiac injury, fibroblast hyperplasia accelerates the synthesis of collagen type I, resulting in the formation of a scar tissue that preserves myocardial function (Baudino *et al.*, 2006). However, owing to the relative ability of cardiac fibroblasts to resist apoptosis, these cells continue to deposit collagen, disrupting the balance between cardiac parenchyma and stroma. Excessive collagen production in the long-term leads to fibrosis, pump dysfunction and heart failure (Baudino *et al.*, 2006).

The action of Ang II, whose intra-cardiac levels are elevated following myocardial injury, is reported to be a critical regulator of fibroblast function. In the context of cardiac fibrogenesis, the stimulatory effect of Ang II on collagen type I production is of significant interest as fibrillar collagen accumulation in the myocardium following myocardial injury leads to tissue fibrosis (Kawano *et al.*, 2000b). Although cardiac myofibroblasts have long been recognized as principal effector cells in fibrogenesis through mediating enhanced synthesis of collagen type I (Souders *et al.*, 2009b), mechanistic insights into signaling pathways that stimulate collagen expression are inadequate, which is underscored by the lack of adequate treatment modalities that target tissue fibrosis (Zhao *et al.*, 2016).

Admittedly, understanding mechanisms underlying collagen expression in fibroblasts in response to injury could aid in uncovering potential targets to control fibrotic disease progression. In this context, collagen receptors, DDR2 and Integrin-β1, are increasingly recognized for their role in fibrogenesis (Borza and Pozzi, 2014). The present study sought to examine the crosstalk between DDR2 and Integrin-β1 and examined the involvement of this crosstalk in the regulation of α-SMA and collagen type I gene expression in cardiac fibroblasts exposed to Ang II.

V.1. A role for collagen receptor crosstalk in collagen gene expression

Since collagen type I is the most abundant ECM protein in the myocardium (Weber, 1989), receptors that bind to collagen type I play an important role in cardiac physiology and pathology. DDR2 and Integrin-β1 are the two major transmembrane receptors binding to collagen type I (Xu *et al.*, 2012). Of these, DDR2 is localized specifically in cardiac fibroblasts and Integrin-β1 is expressed ubiquitously in

fibroblasts as well as in cardiomyocytes in the cardiac tissue (Chen *et al.*, 2016; Travers Joshua G. *et al.*, 2016). The actions of DDR2 and Integrin-β1 have individually been reported to mediate wound healing post-injury as shown by knockout studies wherein, germline deletion of DDR2 or Integrin-β1 in mouse models led to a delayed wound healing response in the dermal tissue (Liu *et al.*, 2010; Olaso, Lin, *et al.*, 2011). Additionally, signaling mechanisms activated by DDR2 and Integrin-β1 have individually been reported to promote pro-fibrotic signaling events, leading to the pathogenesis of liver fibrosis and idiopathic pulmonary fibrosis (Martin *et al*., 2016; Zhao *et al.*, 2016). Although DDRs and Integrins are known to participate in a cooperative manner to regulate collagen type I-mediated tumor cell migration, adhesion and embryonic stem cell renewal (Suh and Han, 2011), these studies stress the role of the DDR1 isoform and its relation to Integrin-dependent signaling pathways. However, to the best of our knowledge, a role for the DDR2 isoform in the regulation of Integrin-β1 expression and their cross-talk in mediating pro-fibrotic signaling in cardiac fibroblasts has not hitherto been studied.

An important outcome of the present study is the demonstration of an obligate role for DDR2 in the regulation of Ang II-stimulated Integrin-β1 expression, as shown by an abrogation of the stimulatory effect of Ang II on Integrin-β1 expression upon DDR2 knockdown. Our previous studies had demonstrated that inhibitors of NADPH oxidase (NOX)-dependent reactive oxygen species (ROS), Phospholipase C, Protein kinase C and p38 MAPK inhibited Ang II-stimulated DDR2 expression in cardiac fibroblasts, implicating the ROS-GPCR-p38-MAPK signaling pathway in Ang II-induced increase in DDR2 (George *et al.*, 2016). In the present study, these

inhibitors were found to significantly reduce Ang II-induced Integrin-β1 expression as well. Since DDR2 regulates Integrin-β1, as shown in the present study, the findings collectively show that Ang II activates the ROS-GPCR signaling cascade to enhance DDR2, which in turn enhances the expression of Integrin-β1 in cardiac fibroblasts.

A combination of DDR2 knockdown and overexpression approaches revealed that basal Integrin-β1 expression is also modulated by DDR2. It is pertinent to note that DDR1 and DDR2 are reported to mediate Integrin α1β1 and α2β1 activation, promoting the adhesion of HEK293 cells to collagen (Xu *et al.*, 2012). However, changes in Integrin expression levels per se were not observed in these earlier studies. This could be because HEK293 are epithelial cells that inherently lack DDR2 expression. To the best of our knowledge, the present study is the first to demonstrate an obligate regulatory role for collagen receptor DDR2 in mediating the expression of collagen-binding Integrin-β1 in cardiac fibroblasts.

Interestingly, the knockdown of Integrin-β1 led to a reduction in Ang II-induced DDR2 expression, revealing a reciprocal regulatory relationship between DDR2 and Integrin-β1. Such a feedback loop between Integrin-β1 and DDR2 could serve to maintain constitutive levels of DDR2 expression, which is required to sustain a state of high proliferative activity, migration and collagen synthesis through RTK signal amplification post stimulation with Ang II.

Integrins function as obligate heterodimers, consisting of an α-subunit and a β subunit. While the α-subunit of Integrin dictates ligand specificity, the β-Integrin

subunit is the predominant signaling arm that mediates intracellular signaling through activation of ILK and FAK (Chen *et al.*, 2016). Integrin-α11β1 is the predominant collagen-binding Integrin in fibroblast (Talior-Volodarsky *et al.*, 2012). The present study demonstrated a role for DDR2 in regulating the expression of Integrin-α11 in addition to β1, highlighting its involvement in the coordinated regulation of Integrin-α11 as well as β1. It is pertinent to note that studies have reported individual roles for both α11 and β1 in mediating pro-fibrotic signaling (Liu and Leask, 2013; Bansal, Nakagawa, Yazdani, van Baarlen, *et al.*, 2017b). However, while Integrin-α11 is reported to promote cardiac fibrosis (Leask, 2018), the role of Integrin-β1, constituting the major signaling arm of Integrin, remains unclear. Hence, subsequent studies focused on the regulatory link between DDR2 and Integrin-β1

V.2. Mechanisms in DDR2-dependent Integrin-β1 expression in Ang II-stimulated cardiac fibroblasts

The signaling effectors acting downstream of DDR2 to regulate Integrin-β1 expression in Ang II-stimulated cardiac fibroblasts were explored. Our previous study reported a role for ERK1/2 acting downstream of Integrin-β1 (George *et al.*, 2016c). Further, DDR2 silencing has been reported to attenuate TGF-β1 expression in primary lung fibroblasts (Zhao *et al.*, 2016). In this study, we demonstrate a role for DDR2-dependent activation of ERK½ MAPK and TGF-β1 in the regulation of Integrin-β1 expression. It is pertinent to note that although previous studies have reported ERK1/2 MAPK activation (George *et al.*, 2016a) and TGF-β1 expression (Zhao *et al.*, 2016) to be under the regulatory control of DDR2, a role for DDR2-

dependent ERK1/2 MAPK-activation and TGF-β1 in the regulation of Integrin-β1 expression has not been reported.

V.2.1. DDR2-dependent transcriptional regulation of Integrin-β1 expression by AP-1

Since NOX-dependent ROS was found regulate Integrin-β1 expression, a role for redox-sensitive transcription factors in the transcriptional regulation of Integrin-β1 was analyzed. NFkB and AP-1 constitute the major redox-sensitive transcription factors (Anupama *et al.*, 2016). While NFkB-dependent transcriptional regulation of Integrin-β1 has been reported (Ahmed *et al.*, 2013), a role for AP-1 in the transcriptional upregulation of Integrin-β1 has not been probed.

AP-1 transcription factor is a dimer comprising c-FOS and c-JUN as subunits (Anupama *et al.*, 2016). c-Jun is reported to enhance fibroblast proliferation in idiopathic pulmonary fibrosis and AP-1 activation has been demonstrated to drive fibrotic disease progression in lung and liver (Wernig *et al.*, 2017).

In the present study, inhibition of AP-1 was found to attenuate Ang II-stimulated Integrin-β1 expression, suggesting a role for AP-1 in the transcriptional regulation of Integrin-β1. Since DDR2-dependent ERK1/2 MAPK and TGF-β1 were found to regulate Integrin-β1 expression, a role for these signaling effectors in promoting AP-1 activation and nuclear translocation was probed. DDR2, ERK1/2 and TGF-β1 were found to activate AP-1, as demonstrated by EMSA. Moreover, a direct role for DDR2 in AP-1-mediated transcriptional regulation of Integrin-β1 was demonstrated by ChIP that demonstrated DDR2 involvement in mediating AP-1 binding to the Integrin-β1 gene promoter. Together, the findings suggest DDR2-dependent

activation of ERK1/2 MAPK and TGF-β1 to coordinately facilitate the nuclear translocation and binding of AP-1 to the Integrin-β1 promoter, resulting in the transcriptional up-regulation of Integrin-β1 in cardiac fibroblasts exposed to Ang II.

V.3. Validation of the DDR2-Integrin-β1 link in vivo

Cardiac stress resulting from pressure overload or myocardial infarction has been reported to enhance the expression of Integrin-β1 (Chen *et al.*, 2016). However, an association between the collagen receptors Integrin-β1 and DDR2 in a setting of myocardial fibrosis has not been demonstrated. The present study used a Spontaneously Hypertensive Rat (SHR) model of myocardial fibrosis for studying an association between DDR2 and Integrin-β1 expression in cardiac fibroblasts. SHR is widely used to study the pathogenesis of myocardial fibrosis (Ren *et al.*, 2017) and a role for the systemic renin-angiotensin system in promoting hypertension-induced reactive fibrosis in SHR is well documented (Brilla *et al.*, 1993). Here, consistent with the demonstration of a regulatory link between DDR2 and Integrin-β1 in vitro in Ang II-treated cardiac fibroblasts, our data pointed to a clear association between DDR2 and Integrin-β1 expression in cardiac tissue as well as in cardiac fibroblasts isolated from 6-month-old SHR. Importantly, the expression levels of DDR2 and Integrin-β1 correlated with markers of myocardial fibrosis - alpha-SMA and collagen type I - pointing to their role in cardiac fibrogenesis.

The link between DDR2 and Integrin-β1 in vivo was probed in DDR2 knockout mice carrying a germ-line deletion of DDR2. A significant reduction in Integrin-β1 immuno-staining intensity was observed in myocardial tissue from DDR2-null mice compared to the wild-type. As cardiac fibroblasts constitute only about 10% of the

myocardial cell population in mice (Pinto *et al.*, 2016), and cardiomyocytes also express Integrin-β1, the striking global reduction in Integrin-β1 immuno-staining intensity suggested a decrease in Integrin-β1 expressi on in myocytes as well as in cardiac fibroblasts. Since cardiac fibroblasts serve as a major source of paracrine factors that regulate gene expression in myocytes (Moore-Morris *et al.*, 2015), this observation raised the possibility that in addition to regulating Integrin-β1 expression in fibroblasts, cardiac fibroblast-specific DDR2 could regulate Integrin-β1 expression in cardiomyocytes as well, via DDR2-dependent paracrine signaling mechanisms.

In support of this, data from conditioned media experiments showed that cardiac fibroblast-derived conditioned media upregulated Integrin-β1 protein expression in H9c2 cardiomyoblasts. On the contrary, conditioned media derived from DDR2-silenced cardiac fibroblasts failed to enhance the expression of Integrin-β1 in H9c2 cardiomyoblasts. In tandem with the in vivo observations on DDR2 null mice, these data confirmed a regulatory role for DDR2-dependent paracrine signaling in the regulation of myocyte Integrin-β1 expression in addition to the regulation of fibroblast Integrin-β1, which could explain the global reduction in Integrin-β1 expression in the DDR2-null mice. This observation is important since it is consistent with the role of Integrin-β1 as a critical determinant of myocardial size and organ growth (Ieda *et al.*, 2009). A previous study utilizing this DDR2 knockout mouse model had demonstrated, by echocardiography, reduced left ventricular chamber dimensions (Cowling *et al.*, 2014). Cardiomyocyte length was atypically shorter in the DDR2-null mice, resulting in decreased heart size and weight. Considering the role of Integrin-β1 in myocyte growth (Ieda *et al.*, 2009) and the role of fibroblast-specific DDR2 in regulating myocyte Integrin-β1 expression, it is tempting to

speculate that DDR2-dependent paracrine signaling mechanisms regulating myocyte Integrin-β1 expression may influence myocyte growth responses. However, this warrants further investigation.

V.4. DDR2-Integrin-β1 crosstalk in the regulation of α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts

Further, the study examined the functional significance of DDR2-Integrin-β1 crosstalk in cardiac fibroblasts exposed to Ang II. Previous studies have implicated a role for DDR2 as well as Integrin-β1 in mediating fibrotic disease progression. While conflicting reports exist on the role of DDR2 in the pathogenesis of liver fibrosis (Olaso, Arteta, *et al.*, 2011; Luo *et al.*, 2013), DDR2-specific silencing conferred resistance to bleomycin-induced pulmonary fibrosis in mouse models (Zhao *et al.*, 2016). With regard to Integrin-β1, it has been shown to promote pro-fibrotic signaling in myofibroblasts in the liver tissue (Martin *et al*., 2016). However, despite several reports on synergism between DDRs and Integrins (Xu *et al.*, 2012) and various studies relating both the collagen receptors to fibrogenesis, the role of signaling cross-talk between DDR2 and Integrin-β1 resulting in the activation of a pro-fibrotic signaling cascade in the myofibroblast in general and cardiac myofibroblast in particular has not been explored.

Our data demonstrated that, while knockdown of DDR2 and Integrin-β1 attenuated Ang II-stimulated α-SMA and collagen type I expression, overexpression of Integrin-β1 in DDR2-silenced cells restored the expression of Ang II-stimulated α-SMA and collagen type I. However, DDR2 overexpression in Integrin-β1-silenced cells failed

to restore the expression of α-SMA and collagen type I. This indicates that DDR2 acts via Integrin-β1 to promote α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts. The data highlight the significance of DDR2-Integrin-β1 crosstalk in the regulation of fibroblast activation and activity.

Consistent with the finding relating the DDR2-Integrin-β1 axis to the regulation of collagen expression, it is pertinent to note that Cowling et al. had previously reported a reduction in basal collagen synthesis and the rate of collagen deposition in vivo in this DDR2 knockout mice model (Cowling *et al.*, 2014). Together with the data presented in this study, these findings correlate reduced expression levels of Integrin-β1 in DDR2-null mice with a reduction in collagen synthesis and deposition, which in turn points to a potential role for DDR2 / Integrin-β1 crosstalk in mediating pro-fibrotic signaling in the myocardium.

V.4.1. A functional role for DDR2-Integrin-β1 crosstalk in wound healing:

While previous findings from this laboratory have reported DDR2 to be necessary for cardiac fibroblast-mediated wound healing response (George *et al.*, 2016), a role for Integrin-β1 in wound healing in cardiac fibroblasts has not been analyzed. We found that while siRNA-mediated silencing of DDR2 or Integrin-β1 reduced the wound healing ability of fibroblasts exposed to Ang II, overexpression of Integrin-β1 in DDR2-silenced fibroblasts restored their wound healing ability, demonstrating a role for collagen receptor crosstalk in cardiac fibroblast function in a context of tissue response to injury.

V.5. A role for α-SMA, downstream of the DDR2-Integrin-β1 axis, in the regulation of collagen type I expression

V.5.1. α-SMA acts downstream of the DDR2-Integrin-β1 axis to regulate collagen alpha1(I) expression in Ang II-treated cardiac fibroblasts

Expression of α-SMA is a hallmark of fibroblast to myofibroblast differentiation and typically coincides with enhanced collagen type I expression in myofibroblasts (Takeji *et al.*, 2006b). Traditionally, the physiological functions of α-SMA in myofibroblasts have been linked to fibroblast motility and contraction (Tallquist and Molkentin, 2017). However, recent reports suggest a role for α-SMA in the regulation of cellular signaling events such as the activation of ERK1/2 MAPK (Rockey *et al.*, 2013). A role for α-SMA in the metastasis of lung adenocarcinoma through upregulation of the expression of FAK and HGFR in adenocarcinoma cells has been reported (Lee *et al.*, 2013). Further, the F/G actin ratio is known to influence the regulation of genes involved in smooth muscle differentiation, although a specific role for α-SMA in this process has not been defined (Olson and Nordheim, 2010). These observations underscore the unexplored existence of a regulatory role for α-SMA in gene expression.

In this study, α-SMA knockdown was found to abrogate Ang II-stimulated collagen type I expression in cardiac fibroblasts, indicating a role for α-SMA in the regulation of collagen type I expression. The finding highlights a novel regulatory event in myofibroblasts that mechanistically links cardiac myofibroblast phenotypic transition to enhanced collagen type I expression.

V.5.2. Mechanisms underlying α-SMA-dependent collagen type I expression in cardiac fibroblasts exposed to Ang II

TRPC6 is a cation-selective Ca2+ channel expressed in cardiac fibroblasts and in myocytes (Hof *et al.*, 2019). Pharmacological targeting of TRPC6 is reported to ameliorate progression of renal fibrosis (Wu *et al.*, 2017). Importantly, TRPC6 is reported to play a critical role in cardiac fibroblast phenotypic transition to α-SMA-positive myofibroblasts, and in cardiac fibrogenesis (Davis *et al.*, 2012). These findings suggest a link between α-SMA and TRPC6 and collagen. Therefore, a role for TRPC6 in mediating α-SMA-dependent collagen type I expression in Ang II-treated cardiac fibroblasts was probed in the present study. We found that, while TRPC6 silencing attenuated collagen type I expression in Ang II-treated cardiac fibroblasts, TRPC6 overexpression in α-SMA-silenced cells restored collagen type I expression in Ang II-treated fibroblasts, demonstrating a role for α-SMA-TRPC6 signaling in the regulation of collagen type I expression. The finding is novel insofar as it links α-SMA – a cytoskeletal actin filament - to the regulation of collagen type I via the calcium channel TRPC6. It is pertinent to note that a previous study by Davis et.al had reported TRPC6 to promote the expression of α-SMA in cardiac fibroblasts (Davis *et al.*, 2012). However, in the present study, α-SMA is shown to function upstream of TRPC6. These findings point to a possible feedback loop functioning in fibroblasts where, α-SMA would promote TRPC6 expression and, TRPC6 in turn would enhance expression of α-SMA in a feedback loop, facilitating myofibroblast function post-injury.

Further, since DDR2, Integrin-β1, α-SMA and TRPC6 are involved in mechano-transduction (Takeji *et al.*, 2006a; Liu *et al.*, 2014; Bayer *et al.*, 2019; Hof *et al.*, 2019), a role for the mechanosensitive transcription factor YAP in the transcriptional regulation of collagen type I, downstream of the α-SMA-TRPC6 signaling cascade, was analyzed. YAP is reported to be a pro-fibrotic transcription factor, regulating pulmonary fibroblast activation to promote idiopathic pulmonary fibrosis (Liu *et al.*, 2014). Notably, YAP has been reported to function downstream of Integrin-β1 to promote pro-fibrotic signaling in myofibroblasts in the liver (Martin *et al*., 2016). In the present study, YAP inhibition with Verteporfin attenuated Ang II-stimulated collagen type I expression in cardiac fibroblasts. Further, ChIP assay demonstrated the role of α-SMA and TRPC6, functioning downstream of the DDR2-Integrin-β1 axis, in Ang II-induced YAP activation and binding to the collagen type I gene promoter, enhancing collagen type I transcription. Together, the data demonstrates a novel role for α-SMA-dependent TRPC6 in mediating YAP activation to promote collagen alpha1(I) expression in Ang II-treated cardiac fibroblasts. Importantly, the findings demonstrate that fibroblast phenotypic activation to α-SMA positive myofibroblasts to be functionally coupled to enhanced collagen type I expression, promoting tissue repair post-injury in the short term, and adverse fibrotic remodeling in the long-term.

V.6. Significance of the study:

This study provides conclusive evidence of an obligate role for collagen receptor crosstalk involving DDR2 and Integrin-β1 in Ang II-stimulated cardiac fibroblast function, which is a major determinant of fibrotic remodeling following cardiac injury.

The findings also shed light on the mechanistic coupling of two distinct cellular events involving phenotypic transition and expression of collagen type I in Ang II-treated cardiac fibroblasts. The regulatory role of α-SMA in collagen gene expression is a novel finding of considerable significance insofar as α-SMA has not hitherto been linked to regulation of collagen gene expression.

Lastly, the demonstration that DDR2-Integrin-β1 crosstalk underlies α-SMA-TRPC6-YAP-dependent collagen type I expression in cardiac fibroblasts offers significant insights into the complex regulatory mechanisms underlying collagen gene expression in the myocardium.

The specific localization of DDR2 in fibroblasts and its obligate role in the activation of quiescent cardiac fibroblasts and in collagen gene expression identify it as a potential drug target in the control of cardiac fibrogenesis.

V.7. Limitations of the study and future directions:

Although the study presents evidence of a crosstalk between DDR2 and Integrin-β1, and demonstrates the link between DDR2 and Integrin-β1 in vivo, a role for DDR2-Integrin-β1 crosstalk in the pathogenesis of cardiac fibrosis in an in vivo setting has not been demonstrated in this study. This would require gain of function and loss of function studies in an in vivo disease model wherein cardiac fibroblast-specific knockin of Integrin-β1 in a DDR2 knockout mouse in a setting of injury would establish the role of collagen receptor crosstalk in collagen expression and cardiac fibrogenesis. Further, while the study has shown DDR2 to mediate the enhanced expression of Integrin-β1 in response to Ang II, further studies utilizing FRET are needed to identify whether a physical association between the two collagen receptors takes place in cardiac fibroblasts exposed to Ang II. Future studies should also probe the role of α-SMA in the regulation of collagen type I expression using α-SMA knockout mice models.

VI. SUMMARY AND CONCLUSIONS

Adverse myocardial remodeling following myocardial injury is associated with dysregulation of collagen synthesis, leading to fibrotic disease progression and ventricular dysfunction (Moore-Morris *et al.*, 2015). Hence, exploration of molecular mechanisms that underlie collagen expression in fibroblasts in a context of injury is a clinically relevant goal. Collagen receptors, DDR2 and Integrin-β1, have increasingly been implicated in tissue fibrosis and remodeling. However, studies attempting to explore the existence of a crosstalk between DDR2 and Integrin-β1 and its implications in fibroblast activation and activity remain obscure. In this context, the aim of the present study was to explore the existence of a crosstalk between DDR2 and Integrin-β1. Further, the study analyzed the involvement of collagen receptor crosstalk in the regulation of α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts.

Major findings of the study:

- Ang II enhances the expression of Integrin-β1
- DDR2 mediates Ang II-stimulated Integrin-β1 expression, demonstrating a crosstalk between the collagen receptors
- DDR2-dependent activation of AP-1 via the ERK1/2 MAPK-TGF-β1 pathway transcriptionally enhances Integrin-β1 expression in Ang II-treated cardiac fibroblasts
- The DDR2/Integrin-β1 link was evident in DDR2 knockout mice and Spontaneously Hypertensive Rats
- DDR2/Integrin-β1 crosstalk mediates α-SMA expression in Ang II-stimulated cardiac fibroblasts

- Importantly, downstream of the DDR2/Integrin-β1 axis, α-SMA mediates enhanced collagen expression via the Ca2+ channel TRPC6, demonstrating that phenotypic transformation of cardiac fibroblasts into myofibroblasts is mechanistically coupled to enhanced collagen type I expression in Ang II-treated cardiac fibroblasts
- Ang II-stimulated α-SMA acts via TRPC6-dependent calcium influx to promote the activation and binding of the mechanosensitive transcription factor, YAP, to the collagen type I gene promoter
- The DDR2/Integrin-β1 crosstalk has a role in wound healing in Ang II-stimulated cardiac fibroblasts

VII. PUBLICATIONS

1) **Harikrishnan V**, Allen Sam Titus, Randy T Cowling, K Shivakumar, Collagen receptor cross-talk determines α-Smooth Muscle Actin-dependent collagen gene expression in Angiotensin II-stimulated cardiac fibroblasts.
J Biol Chem, 2019, *doi: 10.1074/jbc.RA119.009744*

2) Mereena George Ushakumary[#], Mingyi Wang[#], **Harikrishnan V[#]**, Allen Sam Titus, Jing Zhang, Lijuan Liu, Robert Monticone, Yushi Wang, Julie A. Mattison, Rafael de Cabo, Edward G. Lakatta, K. Shivakumar, Discoidin domain receptor 2: A determinant of metabolic syndrome-associated arterial fibrosis in non-human primates. [# - Equal contributors as first author]
PLOS ONE, 2019 *[Accepted, In Press]*

3) Allen Sam Titus, **Harikrishnan V**, K Shivakumar, Identification of a common regulatory pathway that determines cell survival and cell cycle progression in cardiac fibroblasts.
[Manuscript submitted to ***J Biol Chem***]

VIII. REFERENCES

Ahmed KM, Zhang H, and Park CC (2013) NF-κB regulates radioresistance mediated by β1-integrin in three-dimensional culture of breast cancer cells. *Cancer Res* **73**:3737–3748.

Alandi IV, Nieman M, Molkentin JD, and Blaxall BC (2016) Inhibiting Fibronectin Improves Cardiac Function in a Mouse Model of Heart Failure. *The FASEB Journal* **30**:939-970.

Al-Kindi A, Kizhakkedath P, Xu H, John A, Sayegh AA, Ganesh A, Al-Awadi M, Al-Anbouri L, Al-Gazali L, Leitinger B, and Ali BR (2014) A novel mutation in DDR2 causing spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED-SL) results in defective intra-cellular trafficking. *BMC Medical Genetics* **15**:42-58

Anupama V, George M, Dhanesh SB, Chandran A, James J, and Shivakumar K (2016) Molecular mechanisms in H2O2-induced increase in AT1 receptor gene expression in cardiac fibroblasts: A role for endogenously generated Angiotensin II. *J Mol Cell Cardiol* **97**:295–305.

Aoudjit F, and Vuori K (2012) Integrin Signaling in Cancer Cell Survival and Chemoresistance. *Chemother Res Pract* **2012**:289-300

Bagnato GL, Irrera N, Pizzino G, Santoro D, Roberts WN, Bagnato G, Pallio G, Vaccaro M, Squadrito F, Saitta A, Altavilla D, and Bitto A (2018) Dual αvβ3 and αvβ5 blockade attenuates fibrotic and vascular alterations in a murine model of systemic sclerosis. *Clin Sci* **132**:231–242.

Bansal R, Nakagawa S, Yazdani S, Baarlen J van, Venkatesh A, Koh AP, Song W-M, Goossens N, Watanabe H, Beasley MB, Powell CA, Storm G, Kaminski N, Goor H van, Friedman SL, Hoshida Y, and Prakash J (2017) Integrin alpha 11 in the regulation of the myofibroblast phenotype: implications for fibrotic diseases. *Exp Mol Med* **49**:e396–e396.

Baudino TA, Carver W, Giles W, and Borg TK (2006) Cardiac fibroblasts: friend or foe? *American Journal of Physiology-Heart and Circulatory Physiology* **291**:H1015–H1026.

Bayer S, Grither W, Brenot A, Hwang P, Barcus C, Ernst M, Pence P, Walter C, Pathak A, and Longmore G (2019) DDR2 controls breast tumor stiffness and metastasis by regulating Integrin mediated mechanotransduction in CAFs. *eLife* **8**:457-470

Benjamin Emelia J., Muntner Paul, Alonso Alvaro, Bittencourt Marcio S., Callaway Clifton W., Carson April P., Chamberlain Alanna M., Chang Alexander R., Cheng Susan, Das Sandeep R., Delling Francesca N., Djousse Luc, Elkind Mitchell S.V., Ferguson Jane F., Fornage Myriam, Jordan Lori Chaffin, Khan Sadiya S., Kissela Brett M., Knutson Kristen L., Kwan Tak W., Lackland Daniel T., Lewis Tené T., Lichtman Judith H., Longenecker Chris T., Loop Matthew Shane, Lutsey Pamela L., Martin Seth S., Matsushita Kunihiro, Moran Andrew E., Mussolino Michael E., O'Flaherty Martin, Pandey Ambarish, Perak Amanda M., Rosamond Wayne D., Roth Gregory A., Sampson Uchechukwu K.A., Satou Gary M., Schroeder Emily B., Shah Svati H., Spartano Nicole L., Stokes Andrew, Tirschwell David L., Tsao Connie W., Turakhia Mintu P., VanWagner Lisa B., Wilkins John T., Wong Sally S., Virani Salim S., and null null (2019) Heart Disease and Stroke Statistics—2019 Update: A Report From the American Heart Association. *Circulation* **139**:56–e528.

Boopathy GTK, and Hong W (2019) Role of Hippo Pathway-YAP/TAZ Signaling in Angiogenesis. *Front Cell Dev Biol* **7**:342-367

Borza CM, and Pozzi A (2014) Discoidin domain receptors in disease. *Matrix Biology* **34**:185–192.

Brilla CG, Reams GP, Maisch B, and Weber KT (1993) Renin-angiotensin system and myocardial fibrosis in hypertension: regulation of the myocardial collagen matrix. *Eur Heart J* **14**:57–61.

Byun J, Re DPD, Zhai P, Ikeda S, Shirakabe A, Mizushima W, Miyamoto S, Brown JH, and Sadoshima J (2019) Yes-Associated Protein (YAP) mediates adaptive cardiac hypertrophy in response to pressure overload. *J Biol Chem* jbc.RA118.006123.

Calderwood DA (2004) Integrin activation. *Journal of Cell Science* **117**:657–666.

Carafoli F, and Hohenester E (2013) Collagen recognition and transmembrane signalling by discoidin domain receptors. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1834**:2187–2194.

Chen C, Li R, Ross RS, and Manso AM (2016) Integrins and Integrin-related Proteins in Cardiac Fibrosis. *J Mol Cell Cardiol* **93**:162–174.

Chen H-C, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, and Guan J-L (1995) Interaction of Focal Adhesion Kinase with Cytoskeletal Protein Talin. *J Biol Chem* **270**:16995–16999.

Coelho NM, and McCulloch CA (2016) Contribution of collagen adhesion receptors to tissue fibrosis. *Cell Tissue Res* **365**:521–538.

Cooper J, and Giancotti FG (2019) Integrin Signaling in Cancer: Mechanotransduction, Stemness, Epithelial Plasticity, and Therapeutic Resistance. *Cancer Cell* **35**:347–367.

Cowling RT, Yeo SJ, Kim IJ, Park JI, Gu Y, Dalton ND, Peterson KL, and Greenberg BH (2014b) Discoidin domain receptor 2 germline gene deletion leads to altered heart structure and function in the mouse. *Am J Physiol Heart Circ Physiol* **307**:H773-781.

Creemers EE, and Pinto YM (2011) Molecular mechanisms that control interstitial fibrosis in the pressure-overloaded heart. *Cardiovasc Res* **89**:265–272.

Davis J, Burr AR, Davis GF, Birnbaumer L, and Molkentin JD (2012) A TRPC6-dependent pathway for myofibroblast transdifferentiation and wound healing in vivo. *Dev Cell* **23**:705–715.

Dietrich A, Hofmann K, Koenigshoff M, and Gudermann T (2015) Dissecting the Role of TRPC6 Channels in Pulmonary Fibrosis. *The FASEB Journal* **29**:863.24.

Díez Javier (2004) Profibrotic Effects of Angiotensin II in the Heart. *Hypertension* **43**:1164–1165.

Dostal DE, and Baker KM (n.d.) The Cardiac Renin-Angiotensin System. 8.

Du X, Plow EF, Frelinger AL, O'Toole TE, Loftus JC, and Ginsberg MH (1991) Ligands "activate" integrin αIIbβ3 (platelet GPIIb-IIIa). *Cell* **65**:409–416.

Fan D, Takawale A, Lee J, and Kassiri Z (2012) Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair* **5**:15.

Finch-Edmondson M, and Sudol M (2016) Framework to function: mechanosensitive regulators of gene transcription. *Cell Mol Biol Lett* **21**:242-257

Franco Christopher, Hou Guangpei, Ahmad Pamela J., Fu Edwin Y.K., Koh Lena, Vogel Wolfgang F., and Bendeck Michelle P. (2008) Discoidin Domain Receptor 1 (Ddr1) Deletion Decreases Atherosclerosis by Accelerating Matrix Accumulation and Reducing Inflammation in Low-Density Lipoprotein Receptor–Deficient Mice. *Circulation Research* **102**:1202–1211.

Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. *The Journal of Pathology* **200**:500–503.

George M, Vijayakumar A, Dhanesh SB, James J, and Shivakumar K (2016a) Molecular basis and functional significance of Angiotensin II-induced increase in Discoidin Domain Receptor 2 gene expression in cardiac fibroblasts. *J Mol Cell Cardiol* **90**:59–69.

Goldsmith EC, Bradshaw AD, Zile MR, and Spinale FG (2014) Myocardial fibroblast–matrix interactions and potential therapeutic targets. *J Mol Cell Cardiol* **70**:92–99.

Gonzalez ME, Martin EE, Anwar T, Arellano-Garcia C, Medhora N, Lama A, Chen Y-C, Tanager KS, Yoon E, Kidwell KM, Ge C, Franceschi RT, and Kleer CG (2017) Mesenchymal Stem Cell-Induced DDR2 Mediates Stromal-Breast Cancer Interactions and Metastasis Growth. *Cell Reports* **18**:1215–1228.

Gui Y, Li J, Lu Q, Feng Y, Wang M, He W, Yang J, and Dai C (2018) Yap/Taz mediates mTORC2-stimulated fibroblast activation and kidney fibrosis. *J Biol Chem* **293**:16364–16375.

Hamidi H, Pietilä M, and Ivaska J (2016) The complexity of integrins in cancer and new scopes for therapeutic targeting. *Br J Cancer* **115**:1017–1023.

Harburger DS, and Calderwood DA (2009) Integrin signalling at a glance. *Journal of Cell Science* **122**:159–163.

Hayashi I, Vuori K, and Liddington RC (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nat Struct Mol Biol* **9**:101–106.

Herum KM, Choppe J, Kumar A, Engler AJ, and McCulloch AD (2017) Mechanical regulation of cardiac fibroblast profibrotic phenotypes. *MBoC* **28**:1871–1882.

Hof T, Chaigne S, Récalde A, Sallé L, Brette F, and Guinamard R (2019) Transient receptor potential channels in cardiac health and disease. *Nat Rev Cardiol* **16**:344–360.

Holt DW, Henderson ML, Stockdale CE, Farrell JT, Kooyman DL, Bridgewater LC, and Seegmiller RE (2012) Osteoarthritis-like changes in the heterozygous sedc mouse associated with the HtrA1–Ddr2–Mmp-13 degradative pathway: a new model of osteoarthritis. *Osteoarthritis and Cartilage* **20**:430–439.

Hou G, Wang D, and Bendeck MP (2012) Deletion of discoidin domain receptor 2 does not affect smooth muscle cell adhesion, migration, or proliferation in response to type I collagen. *Cardiovascular Pathology* **21**:214–218.

Hough C, Radu M, and Doré JJE (2012) Tgf-beta induced Erk phosphorylation of smad linker region regulates smad signaling. *PLoS ONE* **7**:e42513.

Humeres C, and Frangogiannis NG (2019) Fibroblasts in the Infarcted, Remodeling, and Failing Heart. *J Am Coll Cardiol Basic Trans Science* **4**:449–467.

Humphries JD, Chastney MR, Askari JA, and Humphries MJ (2019) Signal transduction via integrin adhesion complexes. *Current Opinion in Cell Biology* **56**:14–21.

Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong T-T, Shaw RM, and Srivastava D (2009a) Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell* **16**:233–244.

Ikeda K, Wang L-H, Torres R, Zhao H, Olaso E, Eng FJ, Labrador P, Klein R, Lovett D, Yancopoulos GD, Friedman SL, and Lin HC (2002) Discoidin Domain Receptor 2 Interacts with Src and Shc following Its Activation by Type I Collagen. *J Biol Chem* **277**:19206–19212.

Ivey MJ, and Tallquist MD (2016) Defining the Cardiac Fibroblast. *Circ J* **80**:2269–2276.

Jokinen J, Dadu E, Nykvist P, Käpylä J, White DJ, Ivaska J, Vehviläinen P, Reunanen H, Larjava H, Häkkinen L, and Heino J (2004) Integrin-mediated cell adhesion to type I collagen fibrils. *J Biol Chem* **279**:31956–31963.

Kai H, Mori T, Tokuda K, Takayama N, Tahara N, Takemiya K, Kudo H, Sugi Y, Fukui D, Yasukawa H, Kuwahara F, and Imaizumi T (2006) Pressure overload-induced transient oxidative stress mediates perivascular inflammation and cardiac fibrosis through angiotensin II. *Hypertens Res* **29**:711–718.

Kamalov G, Zhao W, Zhao T, Sun Y, Ahokas RA, Marion TN, Darazi FA, Gerling IC, Bhattacharya SK, and Weber KT (2013) Atrophic cardiomyocyte signaling in hypertensive heart disease. *J Cardiovasc Pharmacol* **62**:877-890

Kano K, Marín de Evsikova C, Young J, Wnek C, Maddatu TP, Nishina PM, and Naggert JK (2008) A Novel Dwarfism with Gonadal Dysfunction Due to Loss-of-Function Allele of the Collagen Receptor Gene, Ddr2, in the Mouse. *Mol Endocrinol* **22**:1866–1880.

Katz AM (2010) *Physiology of the Heart*, Lippincott Williams & Wilkins.

Kavvadas P, Kypreou KP, Protopapadakis E, Prodromidi E, Sideras P, and Charonis AS (2010) Integrin-linked kinase (ILK) in pulmonary fibrosis. *Virchows Arch* **457**:563–575.

Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE, and Hsueh WA (2000a) Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. *Circulation* **101**:1130–1137.

Krenning G, Zeisberg EM, and Kalluri R (2010) The origin of fibroblasts and mechanism of cardiac fibrosis. *Journal of Cellular Physiology* **225**:631–637.

Krishnamurthy P, Subramanian V, Singh M, and Singh K (2006) Deficiency of beta1 integrins results in increased myocardial dysfunction after myocardial infarction. *Heart* **92**:1309–1315.

Kumaran C, and Shivakumar K (2002) Calcium- and superoxide anion-mediated mitogenic action of substance P on cardiac fibroblasts. *American Journal of Physiology - Heart and Circulatory Physiology* **282**:H1855–H1862.

Kuwabara Jill T., and Tallquist Michelle D. (2017) Tracking Adventitial Fibroblast Contribution to Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **37**:1598–1607.

Lajiness JD, and Conway SJ (2014) Origin, development, and differentiation of cardiac fibroblasts. *J Mol Cell Cardiol* **0**:2–8.

Leask A (2018) A sticky wicket: Overexpression of integrin alpha 11 is sufficient for cardiac fibrosis. *Acta Physiologica* **222**:e13025.

Lee HW, Park YM, Lee SJ, Cho HJ, Kim D-H, Lee J-I, Kang M-S, Seol HJ, Shim YM, Nam D-H, Kim HH, and Joo KM (2013) Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma. *Clin Cancer Res* **19**:5879–5889.

Legate KR, Montañez E, Kudlacek O, and Fässler R (2006) ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat Rev Mol Cell Biol* **7**:20–31.

Leitinger B (2014) Discoidin domain receptor functions in physiological and pathological conditions. *Int Rev Cell Mol Biol* **310**:39–87.

Leitinger B (2011) Transmembrane Collagen Receptors. *Annual Review of Cell and Developmental Biology* **27**:265–290.

Li Q, Muragaki Y, Hatamura I, Ueno H, and Ooshima A (1998) Stretch-induced collagen synthesis in cultured smooth muscle cells from rabbit aortic media and a possible involvement of angiotensin II and transforming growth factor-beta. *J Vasc Res* **35**:93–103.

Li Y-S, Ni S-Y, Meng Y, Shi X-L, Zhao X-W, Luo H-H, and Li X (2013) Angiotensin II Facilitates Fibrogenic Effect of TGF-β1 through Enhancing the Down-Regulation of BAMBI Caused by LPS: A New Pro-Fibrotic Mechanism of Angiotensin II. *PLOS ONE* **8**:e76289.

Liang M, Yu M, Xia R, Song K, Wang J, Luo J, Chen G, and Cheng J (2017) Yap/Taz Deletion in Gli+ Cell-Derived Myofibroblasts Attenuates Fibrosis. *JASN* **28**:3278–3290.

Liu F, Lagares D, Choi KM, Stopfer L, Marinković A, Vrbanac V, Probst CK, Hiemer SE, Sisson TH, Horowitz JC, Rosas IO, Fredenburgh LE, Feghali-Bostwick C, Varelas X, Tager AM, and Tschumperlin DJ (2014) Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **308**:L344–L357.

Liu S, and Leask A (2013) Integrin β1 Is Required for Dermal Homeostasis. *Journal of Investigative Dermatology* **133**:899–906.

Liu S, Shi-wen X, Blumbach K, Eastwood M, Denton CP, Eckes B, Krieg T, Abraham DJ, and Leask A (2010a) Expression of integrin β1 by fibroblasts is required for tissue repair in vivo. *J Cell Sci* **123**:3674–3682.

Lu H, Fedak PWM, Dai X, Du C, Zhou Y-Q, Henkelman M, Mongroo PS, Lau A, Yamabi H, Hinek A, Husain M, Hannigan G, and Coles JG (2006) Integrin-linked kinase expression is elevated in human cardiac hypertrophy and induces hypertrophy in transgenic mice. *Circulation* **114**:2271–2279.

Luo Z, Liu H, Sun X, Guo R, Cui R, Ma X, and Yan M (2013) RNA Interference against Discoidin Domain Receptor 2 Ameliorates Alcoholic Liver Disease in Rats. *PLOS ONE* **8**:e55860.

MacKenna D, Summerour SR, and Villarreal FJ (2000) Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis. *Cardiovasc Res* **46**:257–263.

Martin K, Pritchett J, Llewellyn J, Mullan AF, Athwal VS, Dobie R, Harvey E, Zeef L, Farrow S, Streuli C, Henderson NC, Friedman SL, Hanley NA, and Hanley KP (2016) PAK proteins and YAP-1 signalling downstream of integrin beta-1 in myofibroblasts promote liver fibrosis. *Nat Commun* **7**:1–11.

Mitra SK, Hanson DA, and Schlaepfer DD (2005) Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* **6**:56–68.

Moore-Morris T, Guimarães-Camboa N, Yutzey KE, Pucéat M, and Evans SM (2015) Cardiac fibroblasts: from development to heart failure. *J Mol Med* **93**:823–830.

Morales MO, Price RL, and Goldsmith EC (2005) Expression of Discoidin Domain Receptor 2 (DDR2) in the Developing Heart. *Microscopy and Microanalysis* **11**:260–267.

Morita N, Mandel WJ, Kobayashi Y, and Karagueuzian HS (2014) Cardiac fibrosis as a determinant of ventricular tachyarrhythmias. *J Arrhythm* **30**:389–394.

Moser M, Nieswandt B, Ussar S, Pozgajova M, and Fässler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med* **14**:325–330.

Nawata J, Ohno I, Isoyama S, Suzuki J, Miura S, Ikeda J, and Shirato K (1999) Differential expression of α1, α3 and α5 integrin subunits in acute and chronic stages of myocardial infarction in rats. *Cardiovasc Res* **43**:371–381.

Nehls V, Herrmann R, Hühnken M, and Palmetshofer A (1998) Contact-dependent inhibition of angiogenesis by cardiac fibroblasts in three-dimensional fibrin gels in vitro: implications for microvascular network remodeling and coronary collateral formation. *Cell Tissue Res* **293**:479–488.

Nepomnyashchikh LM, Lushnikova EL, and Semenov DE (2001) Relationships between Myocardial Parenchyma and Stroma: Regenerative and Plastic Insufficiency of Cardiomyocytes and Development of Diffuse Cardiosclerosis. *Bulletin of Experimental Biology and Medicine* **132**:699–704.

Nieswandt B, Moser M, Pleines I, Varga-Szabo D, Monkley S, Critchley D, and Fässler R (2007) Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo. *Journal of Experimental Medicine* **204**:3113–3118.

Noguchi S, Saito A, and Nagase T (2018) YAP/TAZ Signaling as a Molecular Link between Fibrosis and Cancer. *Int J Mol Sci* **19**:124-138

Okoshi MP, Matsubara LS, Franco M, Cicogna AC, and Matsubara BB (1997) Myocyte necrosis is the basis for fibrosis in renovascular hypertensive rats. *Brazilian Journal of Medical and Biological Research* **30**:1135–1144.

Olaso E, Arteta B, Benedicto A, Crende O, and Friedman SL (2011) Loss of discoidin domain receptor 2 promotes hepatic fibrosis after chronic carbon tetrachloride through altered paracrine interactions between hepatic stellate cells and liver-associated macrophages. *Am J Pathol* **179**:2894–2904.

Olaso E, Lin H-C, Wang L-H, and Friedman SL (2011) Impaired dermal wound healing in discoidin domain receptor 2-deficient mice associated with defective extracellular matrix remodeling. *Fibrogenesis Tissue Repair* **4**:540-558

Olson EN, and Nordheim A (2010a) Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol* **11**:353–365.

Parreno J, Raju S, Niaki MN, Andrejevic K, Jiang A, Delve E, and Kandel R (2014) Expression of type I collagen and tenascin C is regulated by actin

polymerization through MRTF in dedifferentiated chondrocytes. *FEBS Letters* **588**:3677–3684.

Petrich BG, Fogelstrand P, Partridge AW, Yousefi N, Ablooglu AJ, Shattil SJ, and Ginsberg MH (2007) The antithrombotic potential of selective blockade of talin-dependent integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb–IIIa) activation. *J Clin Invest* **117**:2250–2259.

Pinto AR, Ilinykh A, Ivey MJ, Kuwabara JT, D'Antoni ML, Debuque R, Chandran A, Wang L, Arora K, Rosenthal NA, and Tallquist MD (2016) Revisiting Cardiac Cellular Composition. *Circ Res* **118**:400–409.

Qin Z, Fisher GJ, Voorhees JJ, and Quan T (2018) Actin cytoskeleton assembly regulates collagen production via TGF-β type II receptor in human skin fibroblasts. *Journal of Cellular and Molecular Medicine* **22**:4085–4096.

Ray K (2013) Connective tissue diseases: Integrins crucial for the onset of fibrosis in systemic sclerosis—a new therapeutic target? *Nature Reviews Rheumatology* **9**:637–637.

Reed NI, Jo H, Chen C, Tsujino K, Arnold TD, DeGrado WF, and Sheppard D (2015) The αvβ1 integrin plays a critical in vivo role in tissue fibrosis. *Sci Transl Med* **7**:288ra79.

Ren T, Zhang W, Liu X, Zhao H, Zhang Jian, Zhang Jing, Li X, Zhang Y, Bu X, Shi M, Yao L, and Su J (2014) Discoidin domain receptor 2 (DDR2) promotes breast cancer cell metastasis and the mechanism implicates epithelial–mesenchymal transition programme under hypoxia. *The Journal of Pathology* **234**:526–537.

Ren X-S, Ling L, Zhou B, Han Y, Zhou Y-B, Chen Q, Li Y-H, Kang Y-M, and Zhu G-Q (2017) Silencing salusin-β attenuates cardiovascular remodeling and hypertension in spontaneously hypertensive rats. *Sci Rep* **7**:43259.

Rockey DC, Weymouth N, and Shi Z (2013b) Smooth muscle α actin (Acta2) and myofibroblast function during hepatic wound healing. *PLoS ONE* **8**:e77166.

Rog-Zielinska Eva A, Norris RA, Kohl P, and Markwald R (2016) The Living Scar – Cardiac Fibroblasts and the Injured Heart. *Trends Mol Med* **22**:99–114.

Rohr Stephan (2012) Arrhythmogenic Implications of Fibroblast-Myocyte Interactions. *Circulation: Arrhythmia and Electrophysiology* **5**:442–452.

Rosker Christian, Salvarani Nicolò, Schmutz Stephan, Grand Teddy, and Rohr Stephan (2011) Abolishing Myofibroblast Arrhythmogeneicity by Pharmacological Ablation of α-Smooth Muscle Actin Containing Stress Fibers. *Circulation Research* **109**:1120–1131.

Ruiz PA, and Jarai G (2011) Collagen I Induces Discoidin Domain Receptor (DDR) 1 Expression through DDR2 and a JAK2-ERK1/2-mediated Mechanism in Primary Human Lung Fibroblasts. *J Biol Chem* **286**:12912–12923.

Rzucidlo EM, Martin KA, and Powell RJ (2007) Regulation of vascular smooth muscle cell differentiation. *Journal of Vascular Surgery* **45**:A25–A32.

Santos ARC, Corredor RG, Obeso BA, Trakhtenberg EF, Wang Y, Ponmattam J, Dvoriantchikova G, Ivanov D, Shestopalov VI, Goldberg JL, Fini ME, and Bajenaru ML (2012) β1 integrin-focal adhesion kinase (FAK) signaling modulates retinal ganglion cell (RGC) survival. *PLoS ONE* **7**:e48332.

Schlaepfer DD, Broome MA, and Hunter T (1997) Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* **17**:1702–1713.

Schnittert J, Bansal R, Storm G, and Prakash J (2018) Integrins in wound healing, fibrosis and tumor stroma: High potential targets for therapeutics and drug delivery. *Advanced Drug Delivery Reviews* **129**:37–53.

Schwartz MA (2001) Integrin signaling revisited. *Trends in Cell Biology* **11**:466–470.

Serrano I, Díez-Marqués ML, Rodríguez-Puyol M, Herrero-Fresneda I, Raimundo García DM, Dedhar S, Ruiz-Torres MP, and Rodríguez-Puyol D (2012) Integrin-linked kinase (ILK) modulates wound healing through regulation of hepatocyte growth factor (HGF). *Exp Cell Res* **318**:2470–2481.

Shibue T, and Weinberg RA (2009) Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci USA* **106**:10290–10295.

Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ, and Johnson KR (2008) Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. *J Cell Biol* **180**:1277–1289.

Shyu Kou-Gi, Chao Ya-Meng, Wang Bao-Wei, and Kuan Peiliang (2005) Regulation of Discoidin Domain Receptor 2 by Cyclic Mechanical Stretch in Cultured Rat Vascular Smooth Muscle Cells. *Hypertension* **46**:614–621.

Souders CA, Bowers SLK, and Baudino TA (2009a) Cardiac fibroblast: the renaissance cell. *Circ Res* **105**:1164–1176.

Soung YH, Clifford JL, and Chung J (2010) Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *BMB Rep* **43**:311–318.

Spinale FG (2007) Myocardial Matrix Remodeling and the Matrix Metalloproteinases: Influence on Cardiac Form and Function. *Physiological Reviews* **87**:1285–1342.

Srivastava D, and Yu S (2006) Stretching to meet needs: integrin-linked kinase and the cardiac pump. *Genes Dev* **20**:2327–2331.

Staudinger LA, Spano SJ, Lee W, Coelho N, Rajshankar D, Bendeck MP, Moriarty T, and McCulloch CA (2013) Interactions between the discoidin domain receptor 1 and β1 integrin regulate attachment to collagen. *Biol Open* **2**:1148–1159.

Suh HN, and Han HJ (2011a) Collagen I regulates the self-renewal of mouse embryonic stem cells through α2β1 integrin- and DDR1-dependent Bmi-1. *J Cell Physiol* **226**:3422–3432.

Sun Mei, Opavsky M. Anne, Stewart Duncan J., Rabinovitch Marlene, Dawood Fayez, Wen Wen-Hu, and Liu Peter P. (2003) Temporal Response and Localization of Integrins β1 and β3 in the Heart After Myocardial Infarction. *Circulation* **107**:1046–1052.

Sun Y, and Weber KT (2000) Infarct scar: a dynamic tissue. *Cardiovasc Res* **46**:250–256.

Szeto SG, Narimatsu M, Lu M, He X, Sidiqi AM, Tolosa MF, Chan L, Freitas KD, Bialik JF, Majumder S, Boo S, Hinz B, Dan Q, Advani A, John R, Wrana JL, Kapus A, and Yuen DA (2016) YAP/TAZ Are

Mechanoregulators of TGF-β-Smad Signaling and Renal Fibrogenesis. *JASN* **27**:3117–3128.

Takeji M, Moriyama T, Oseto S, Kawada N, Hori M, Imai E, and Miwa T (2006) Smooth muscle alpha-actin deficiency in myofibroblasts leads to enhanced renal tissue fibrosis. *J Biol Chem* **281**:40193–40200.

Talior-Volodarsky I, Connelly KA, Arora PD, Gullberg D, and McCulloch CA (2012) α11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovasc Res* **96**:265–275.

Tallquist MD, and Molkentin JD (2017) Redefining the identity of cardiac fibroblasts. *Nature Reviews Cardiology* **14**:484–491.

Tarbit E, Singh I, Peart JN, and Rose'Meyer RB (2019) Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. *Heart Fail Rev* **24**:1–15.

Thompson Susan A., Copeland Craig R., Reich Daniel H., and Tung Leslie (2011) Mechanical Coupling Between Myofibroblasts and Cardiomyocytes Slows Electric Conduction in Fibrotic Cell Monolayers. *Circulation* **123**:2083–2093.

Travers Joshua G., Kamal Fadia A., Robbins Jeffrey, Yutzey Katherine E., and Blaxall Burns C. (2016) Cardiac Fibrosis. *Circulation Research* **118**:1021–1040.

Tsakiridis T, Bergman A, Somwar R, Taha C, Aktories K, Cruz TF, Klip A, and Downey GP (1998) Actin Filaments Facilitate Insulin Activation of the Src and Collagen Homologous/Mitogen-activated Protein Kinase Pathway Leading to DNA Synthesis and c-fos Expression. *J Biol Chem* **273**:28322–28331.

Tyagi SC, Lewis K, Pikes D, Marcello A, Mujumdar VS, Smiley LM, and Moore CK (1998) Stretch-induced membrane type matrix metalloproteinase and tissue plasminogen activator in cardiac fibroblast cells. *Journal of Cellular Physiology* **176**:374–382.

Wang C-Z, Su H-W, Hsu Y-C, Shen M-R, and Tang M-J (2006) A Discoidin Domain Receptor 1/SHP-2 Signaling Complex Inhibits α2β1-Integrin–mediated Signal Transducers and Activators of Transcription 1/3 Activation and Cell Migration. *Mol Biol Cell* **17**:2839–2852.

Wang H, Haeger SM, Kloxin AM, Leinwand LA, and Anseth KS (2012) Redirecting Valvular Myofibroblasts into Dormant Fibroblasts through Light-mediated Reduction in Substrate Modulus. *PLoS One* **7**.

Wang H-B, Dembo M, Hanks SK, and Wang Y (2001) Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *PNAS* **98**:11295–11300.

Wang J, Lü H, Liu X, Deng Y, Sun T, Li F, Ji S, Nie X, and Yao L (2002) Functional Analysis of Discoidin Domain Receptor 2 in Synovial Fibroblasts in Rheumatoid Arthritis. *Journal of Autoimmunity* **19**:161–168.

Weber KT (1989) Cardiac interstitium in health and disease: The fibrillar collagen network. *J Am Coll Cardiol* **13**:1637–1652.

Weber KT, Jalil JE, Janicki JS, and Pick R (1989) Myocardial Collagen Remodeling in Pressure Overload Hypertrophy-A Case for Interstitial Heart Disease. *Am J Hypertens* **2**:931–940.

Weber KT, Sun Y, Bhattacharya SK, Ahokas RA, and Gerling IC (2013) Myofibroblast-mediated mechanisms of pathological remodelling of the heart. *Nat Rev Cardiol* **10**:15–26.

Wernig G, Chen S-Y, Cui L, Van Neste C, Tsai JM, Kambham N, Vogel H, Natkunam Y, Gilliland DG, Nolan G, and Weissman IL (2017) Unifying mechanism for different fibrotic diseases. *Proc Natl Acad Sci USA* **114**:4757–4762.

Willems IE, Havenith MG, De Mey JG, and Daemen MJ (1994) The alpha-smooth muscle actin-positive cells in healing human myocardial scars. *Am J Pathol* **145**:868–875.

Wu Y-L, Xie J, An S-W, Oliver N, Barrezueta NX, Lin M-H, Birnbaumer L, and Huang C-L (2017) Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho. *Kidney International* **91**:830–841.

Xie C-Q, Ritchie RP, Huang H, Zhang J, and Chen YE (2011) Smooth muscle cell differentiation in vitro: Models and underlying molecular mechanisms. *Arterioscler Thromb Vasc Biol* **31**:1485–1494.

Xu H, Bihan D, Chang F, Huang PH, Farndale RW, and Leitinger B (2012) Discoidin Domain Receptors Promote α1β1- and α2β1-Integrin Mediated

Cell Adhesion to Collagen by Enhancing Integrin Activation. *PLOS ONE* **7**:e52209.

Xu J, Li P-X, Wu J, Gao Y-J, Yin M-X, Lin Y, Yang M, Chen D-P, Sun H-P, Liu Z-B, Gu X-C, Huang H-L, Fu L-L, Hu H-M, He L-L, Wu W-Q, Fei Z-L, Ji H-B, Zhang L, and Mei C-L (2016) Involvement of the Hippo pathway in regeneration and fibrogenesis after ischaemic acute kidney injury: YAP is the key effector. *Clin Sci (Lond)* **130**:349–363.

Yamamoto K, Masuyama T, Sakata Y, Nishikawa N, Mano T, Yoshida J, Miwa T, Sugawara M, Yamaguchi Y, Ookawara T, Suzuki K, and Hori M (2002) Myocardial stiffness is determined by ventricular fibrosis, but not by compensatory or excessive hypertrophy in hypertensive heart. *Cardiovasc Res* **55**:76–82.

Yang K, Kim JH, Kim HJ, Park I-S, Kim IY, and Yang B-S (2005) Tyrosine 740 Phosphorylation of Discoidin Domain Receptor 2 by Src Stimulates Intramolecular Autophosphorylation and Shc Signaling Complex Formation. *J Biol Chem* **280**:39058–39066.

Yeh Y-C, Wang C-Z, and Tang M-J (2009) Discoidin domain receptor 1 activation suppresses alpha2beta1 integrin-dependent cell spreading through inhibition of Cdc42 activity. *J Cell Physiol* **218**:146–156.

Young-Pearse TL, Bai J, Chang R, Zheng JB, LoTurco JJ, and Selkoe DJ (2007) A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J Neurosci* **27**:14459–14469.

Zeltz C, and Gullberg D (2016) The integrin-collagen connection--a glue for tissue repair? *J Cell Sci* **129**:653–664.

Zhang K, Corsa CA, Ponik SM, Prior JL, Piwnica-Worms D, Eliceiri KW, Keely PJ, and Longmore GD (2013) The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis. *Nature Cell Biology* **15**:677–687.

Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, and Yao L (2011a) An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. *Journal of Bone and Mineral Research* **26**:604–617.

Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai Z-C, and Guan K-L (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21**:2747–2761.

Zhao H, Bian H, Bu X, Zhang S, Zhang P, Yu J, Lai X, Li D, Zhu C, Yao L, and Su J (2016) Targeting of Discoidin Domain Receptor 2 (DDR2) Prevents Myofibroblast Activation and Neovessel Formation During Pulmonary Fibrosis. *Mol Ther* **24**:1734–1744.

Zhao W, Zhang C, Shi M, Zhang J, Li M, Xue X, Zhang Z, Shu Z, Zhu J, Mu N, Li W, Hao Q, Wang Z, Gong L, Zhang W, and Zhang Y (2014) The Discoidin Domain Receptor 2/Annexin A2/Matrix Metalloproteinase 13 Loop Promotes Joint Destruction in Arthritis Through Promoting Migration and Invasion of Fibroblast-like Synoviocytes. *Arthritis & Rheumatology* **66**:2355–2367.

IX. ANNEXURES

Urkund Analysis Result

Analysed Document: THESIS FOR PLAGIARISM CHECK.doc (D57166851)
Submitted: 10/17/2019 11:42:00 AM
Submitted By: neethum@sctimst.ac.in
Significance: 0 %

Sources included in the report:

https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0098483&type=manuscript
https://europepmc.org/articles/3916944
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3759374/
71561937-aa0a-4042-93b2-24bdb2b528ca

Instances where selected sources appear:

5

Hit and source - focused comparison, Side by Side:

Left side: As student entered the text in the submitted document.
Right side: As the text appears in the source.

Instances from: https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0098483&type=manuscript

5	62%	5: https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0098483&type=manuscript	62%
cardiac fibroblasts were pre-treated for 1 h with EGTA (1mM) for 1 h followed by Ang II		cardiac fibroblasts were pre-treated with beraprost (10 μM) for 4 h followed by Ang II (100	

Instances from: https://europepmc.org/articles/3916944

3	55%

Effect of Ang II on Integrin-β1 gene expression in cardiac fibroblasts

The effect of Ang II on

3: https://europepmc.org/articles/3916944	55%

Effect of Ang II on miR-21 level in adult rat cardiac fibroblasts

To investigate the effect of Ang II on

Instances from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3759374/

1 100%

into myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA).

1: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3759374/ 100%

into myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA)

4 45%

p> 0.001 vs. control, ### p> 0.001 vs. Ang II.

Figure 42: DDR2 regulates Ang II-stimulated expression of collagen alpha1(I) and α-SMA in cardiac fibroblasts

4: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3759374/ 45%

P>0.01 vs control group, ##P>0.01 vs Ang II group.

Metformin Inhibits Ang II-Induced Expression of Collagen I and Collagen III in Cardiac Fibroblasts

Instances from: 71561937-aa0a-4042-93b2-24bdb2b528ca

2	100%

of a large extracellular domain, a single transmembrane domain and a short cytoplasmic

tail

2: 71561937-aa0a-4042-93b2-24bdb2b528ca	100%

of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail.