Collagen receptor cross-talk determines α-smooth muscle actin-dependent collagen gene expression in angiotensin II-stimulated cardiac fibroblasts

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Running title: DDR2-Integrin-β1 cross-talk in collagen type I expression

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Keywords: cardiac fibrosis; cardiac fibroblast; collagen type I; discoidin domain receptor 2; integrin-β1; α-smooth muscle actin; connective tissue; fibrosis; tissue damage; gene regulation
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Abstract

Excessive collagen deposition by myofibroblasts during adverse cardiac remodeling leads to myocardial fibrosis that can compromise cardiac function. Unraveling the mechanisms underlying collagen gene expression in cardiac myofibroblasts is therefore an important clinical goal. The collagen receptors, discoidin domain receptor 2 (DDR2), a collagen-specific receptor tyrosine kinase, and integrin-β1, are reported to mediate tissue fibrosis. Here, we probed the role of DDR2-integrin-β1 cross-talk in the regulation of collagen alpha1(I) gene expression in angiotensin II (Ang II)-stimulated cardiac fibroblasts. Results from gene silencing/overexpression approaches, electrophoretic mobility shift assays and ChIP revealed that DDR2 acts via extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK)-dependent transforming growth factor-β1 (TGF-β1) signaling to activate activator protein-1 (AP-1) that in turn transcriptionally enhances the expression of collagen-binding integrin-β1 in Ang II-stimulated cardiac fibroblasts. The DDR2-integrin-β1 link was also evident in spontaneously hypertensive rats and DDR2-knockout mice. Further, DDR2 acted via integrin-β1 to regulate α-smooth muscle actin (α-SMA) and collagen type I expression in Ang II-exposed cardiac fibroblasts. Downstream of the DDR2-integrin-β1 axis, α-SMA was found to regulate collagen alpha1(I) gene expression via the Ca2+ channel, transient receptor potential cation channel subfamily C member 6 (TRPC6), and the profibrotic transcription factor, Yes-associated protein (YAP). This finding indicated that fibroblast-to-myofibroblast conversion is mechanistically coupled to collagen expression. The observation that collagen receptor cross-talk underlies α-SMA-dependent collagen type I expression in cardiac fibroblasts expands our understanding of the complex mechanisms involved in collagen gene expression in the heart, and may be relevant to cardiac fibrogenesis.
Introduction

Cardiac fibroblasts, the only intra-cardiac source of collagen types I and III, are a major determinant of wound healing and its unintended consequences in the injured myocardium. In a setting of cardiac muscle damage, normally quiescent cardiac fibroblasts are phenotypically transformed into α-smooth muscle actin (α-SMA)-expressing myofibroblasts that infiltrate the site of injury, proliferate and produce matrix components, facilitating the formation of a collagen-rich scar tissue that prevents ventricular rupture and preserves myocardial integrity (1). However, active cardiac fibroblasts persist in the infarct scar long after termination of the healing response, which leads to excessive collagen deposition and fibrosis that contribute significantly to compromised cardiac function post injury (2). Expression of α-SMA and enhanced collagen deposition, which characterize active myofibroblasts, are critical steps in cardiac tissue repair that profoundly impact cardiac remodeling associated with conditions such as hypertension and myocardial infarction (3). In this regard, Angiotensin II (Ang II), whose intracardiac generation is reported to be enhanced following myocardial injury, is a potent pro-fibrotic factor in the myocardium with marked stimulatory effects on collagen expression in cardiac fibroblasts (4). Identification of mechanisms involved in cardiac fibroblast activation and collagen gene expression, especially in response to Ang II, can therefore provide novel insights into the molecular basis of cardiac fibrogenesis and uncover strategies to optimize cardiac fibroblast function following myocardial injury.

In this context, collagen receptors are increasingly implicated in physiological processes and in the pathophysiology of diseases that primarily affect collagen homeostasis (5). Discoidin Domain Receptor 2 (DDR2) is a mesenchymal cell-specific fibrillar collagen-receptor tyrosine kinase expressed predominantly in fibroblasts and is a fibroblast-selective marker (6, 7) while collagen-binding Integrin-β1 is expressed in a variety of cell types. DDR2 and Integrin-β1 are individually reported to regulate collagen expression and extracellular matrix remodeling (8, 9) and cellular processes...
such as cell survival (10, 11), proliferation (12), migration (13, 14) and differentiation (15, 16). However, despite the reported synergy between receptor tyrosine kinases and integrins in promoting angiogenesis, tumor metastasis and atherosclerosis (17), the possibility that a cross-talk between these two collagen receptors may underlie critical cellular functions in the heart has not been explored. Importantly, the regulatory role of DDR2 in Integrin-β1 gene expression and its implications in the regulation of cardiac fibroblast function have not hitherto been reported.

This study explored the involvement of collagen receptor cross-talk in the regulation of collagen alpha1(I) gene expression in Ang II-stimulated cardiac fibroblasts. Specifically, we probed the regulatory role of DDR2 in Integrin-β1 gene expression and, downstream of the DDR2-Integrin-β1 axis, investigated a hitherto unknown role for α-SMA, a cytoskeletal protein traditionally implicated in cell motility and contraction (18), in the regulation of Ang II-stimulated collagen alpha1(I) expression. To the best of our knowledge, this report is the first to present robust evidence that DDR2-Integrin-β1 cross-talk underlies α-SMA-dependent collagen type I expression in cardiac fibroblasts. The obligate role of α-SMA, downstream of the DDR2-Integrin-β1 axis, in collagen expression implies that phenotypic transformation of cardiac fibroblasts and collagen type I expression are mechanistically coupled, representing an exquisite continuum of cellular events, culminating in enhanced collagen expression in Ang II-stimulated cardiac fibroblasts.

RESULTS

**DDR2 mediates Integrin-β1 expression in cardiac fibroblasts exposed to Ang II**

Ang II was found to significantly enhance Integrin-β1 protein expression at 12 h post-treatment (Fig. 1A, B). To test the possibility that DDR2-mediates Ang II-stimulated Integrin-β1 expression in cardiac fibroblasts, cells transiently transfected with DDR2 siRNA were exposed to Ang II for 12 h, followed by analysis of Integrin-β1 expression. Cell viability post-transfection was affected only minimally (data not shown). DDR2 knockdown significantly attenuated Integrin-β1 expression, indicating a role for DDR2 in the regulation of Ang II-
dependent Integrin-β1 expression in cardiac fibroblasts (Fig. 1A, B). Interestingly, while DDR2 knockdown reduced basal levels of Integrin-β1 expression (Fig. 1C, D), over-expression of DDR2 in un-stimulated cells enhanced Integrin-β1 expression (Fig. 1E, F), which indicates the regulatory role of DDR2 in Integrin-β1 expression even in un-stimulated cardiac fibroblasts maintained under serum-free conditions.

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 (8). In the present study, these inhibitors were found to significantly reduce Ang II-induced Integrin-β1 expression as well (Supplementary Fig. 1A–H). 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.

Integrin α11β1 is reported to be the dominant collagen-binding integrin in cardiac fibroblasts (19). In the present study, DDR2 knockdown attenuated Ang II-induced expression of Integrin-α11 as well, demonstrating a role for DDR2 in the co-ordinated regulation of the α11 and β1 integrin subunits (Fig. 1G).

Subsequent investigations focused on the regulation of Integrin-β1 since the β1 subunit is the major signaling transducer with reported roles in myofibroblast formation and collagen production (20) while the α subunit confers ligand specificity (21). Moreover, the role of Integrin-β1 in mediating pro-fibrotic signaling events in cardiac fibroblasts remains largely unclear.

**DDR2 acts via ERK1/2-dependent TGF-β1 to increase Integrin-β1 expression in Ang II-stimulated cardiac fibroblasts**

Next, the signaling effectors downstream of DDR2 that promote Integrin-β1 expression were explored. Based on our earlier observation that DDR2 induces ERK1/2 MAPK (8) and the reported synergy between ERK1/2 MAPK and TGF-β1 signaling, a role for DDR2-dependent ERK1/2 MAPK activation and TGF-β1 in the regulation of Integrin-β1
expression was probed. siRNA-mediated knockdown of DDR2 reduced Ang II-induced phosphorylation of ERK1/2 MAPK and TGF-β1 expression (Fig. 1A, B). ERK1/2 MAPK inhibition attenuated the Ang II-induced increase in TGF-β1 and Integrin-β1 expression (Fig. 1H, I for protein & supplementary figure 1 I, J for mRNA) while TGF-β1 inhibition reduced Ang II-induced increase in Integrin-β1 expression (Fig. 1J, K for protein and supplementary Fig. 1K for mRNA). Together, the data show the involvement of DDR2-dependent ERK1/2 MAPK activation and TGF-β1 in the regulation of Integrin-β1 expression in response to Ang II in cardiac fibroblasts.

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

Since AP-1 is a redox-sensitive transcription factor (22) that is reported to be regulated by ERK1/2 MAPK (23) and TGF-β1 (24), the transcriptional regulation of Integrin-β1 expression by AP-1 was probed. Inhibition of AP-1 with SR11302 significantly reduced Ang II-induced Integrin-β1 mRNA and protein expression (Fig. 2A-C). EMSA showed Ang II-induced nuclear translocation of AP-1, which occurred maximally at 30 min and was reduced by 3 h (Fig. 2D). Further, inhibition of DDR2, TGF-β1 (using siRNA) and ERK1/2 MAPK (using PD98059) reduced Ang II-induced activation of AP-1 (Fig. 2D, E), demonstrating their involvement in AP-1 activation in response to Ang II.

A ChIP assay was performed to confirm the role of DDR2 in the transcriptional upregulation of Integrin-β1 by AP-1. Subconfluent cultures of cardiac fibroblasts were transiently transfected with DDR2 siRNA. Following Ang II treatment for 30 min, the cells were harvested and chromatin was sheared as mentioned under Methods. Cross-linked chromatin preparations from Ang II-treated cells and DDR2 siRNA-transfected cells exposed to Ang II were immunoprecipitated with 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. Our results, after normalization to input DNA, confirmed enhanced binding of c-Fos and c-Jun to
the Integrin-β1 (ITGB1) gene promoter. Further, AP-1 binding activity was attenuated upon DDR2 knockdown, confirming the role of DDR2-dependent AP-1 in the transcriptional regulation of Integrin-β1 (Fig. 2F).

**Corroborations of the DDR2-Integrin-β1 link in vivo**

*Integrin-β1 expression is positively correlated with elevated DDR2 in Spontaneously Hypertensive Rats*

Western blot analysis of whole heart tissue from 6-month old SHR with myocardial fibrosis, as evidenced by picrosirius red staining, demonstrated an association between DDR2 and Integrin-β1, correlating with markers of fibrosis, α-SMA and collagen type I (Fig 3. A-E). In cardiac fibroblasts isolated from 6-month old SHR, a significant elevation in Integrin-β1 expression positively correlated with elevated DDR2 (Fig. 3F-H). These findings point to a possible link between the two collagen receptors in this model of hypertensive heart disease.

**DDR2 knockout significantly reduces myocardial Integrin-β1 in mice**

The regulatory role of DDR2 in Integrin-β1 expression was evident in vivo in knockout mice carrying a germline deletion of DDR2. Previously, knockin of a MerCreMer gene targeting exon 3 of the DDR2 allele was used for germline deletion of DDR2 in mice. We have previously described the generation, validation and initial observations of the DDR2-null mice (25). Immunohistochemical analysis of cardiac tissue sections using an anti-Integrin-β1 antibody demonstrated a significant reduction in myocardial Integrin-β1 expression in the DDR2-null mice (Fig. 3I, J). This striking global reduction in Integrin-β1 staining intensity pointed to a decrease in Integrin-β1 in myocytes in addition to fibroblasts that are reportedly fewer in number in mouse hearts (26). Preliminary experiments were therefore performed to explore the influence of fibroblast-specific DDR2 on myocyte Integrin-β1 expression. To this end, the effect of rat cardiac fibroblast-conditioned media on Integrin-β1 expression in rat ventricular H9c2 (cardiomyoblast) cell line was analyzed. Exposure of H9c2 cells to fibroblast-conditioned medium for 24 h enhanced Integrin-β1 expression but, notably, conditioned medium from DDR2-silenced cardiac fibroblasts did not enhance Integrin-β1 expression in
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H9c2 cells (Fig. 3K, L), suggesting a role for fibroblast-specific DDR2 in Integrin-β1 expression in these cells.

**DDR2-Integrin-β1 cross-talk regulates α-SMA and collagen alpha1(I) expression in Ang II-treated cardiac fibroblasts**

DDR2 or Integrin-β1 knockdown attenuated Ang II-induced α-SMA and collagen alpha1(I) expression (Fig. 4 A-F). Interestingly, Integrin-β1 knockdown also significantly reduced the expression of Ang II-induced DDR2 (Fig. 4G, H), demonstrating the existence of a reciprocal regulatory relationship between the collagen receptors since DDR2 knockdown abrogated Integrin-β1 expression as well (Fig. 1A). The validation of Integrin-β1 knockdown by RNA interference is shown in Fig. 4 I.

While DDR2 knockdown attenuated the Ang II-induced increase in α-SMA and collagen alpha1(I), plasmid-based overexpression of Integrin-β1 (ITGB1) in DDR2-silenced cells restored the expression of α-SMA and collagen alpha1(I) upon Ang II treatment (Fig. 5 A-C). However, DDR2 overexpression in Integrin-β1-silenced cells failed to restore α-SMA and collagen alpha1(I) expression in Ang II-treated cells (Fig. 5 D-F), showing that DDR2 acts via Integrin-β1 to mediate α-SMA and collagen type I expression.

Scratch wound assay showed that, while knockdown of either DDR2 or Integrin-β1 impaired Ang II-induced wound closure, overexpression of Integrin-β1 in DDR2-silenced cells restored the wound healing response (Fig. 6A, B).

Together, the data show the involvement of the DDR2-Integrin-β1 axis in the regulation of α-SMA, collagen type I and wound healing.

**α-SMA acts downstream of the DDR2-Integrin-β1 axis to regulate collagen alpha1(I) expression via the TRPC6-YAP pathway in Ang II-treated cardiac fibroblasts**

The role of α-SMA as a determinant of collagen type I expression in cardiac fibroblasts exposed to Ang II was examined. α-SMA knockdown significantly reduced Ang II-dependent collagen alpha1(I) expression in cardiac fibroblasts (Fig. 7A, B). We probed the role of TRPC6, a Ca^{2+} channel in fibroblasts with a demonstrated role in myofibroblast transformation (27), as a mediator of α-SMA-dependent collagen type I expression in cardiac fibroblasts.
expression in Ang II-treated cardiac fibroblasts. α-SMA knockdown significantly reduced Ang II-induced TRPC6 expression (Fig. 7C, D). TRPC6 knockdown in turn significantly attenuated Ang II-stimulated collagen alpha 1(I) expression (Fig. 7E, F). Notably, plasmid vector-based overexpression of TRPC6 in α-SMA-silenced cardiac fibroblasts rescued collagen alpha1(I) expression upon Ang II treatment (Fig. 7G, H), confirming the regulatory link between α-SMA, TRPC6 and collagen type I.

α-SMA-dependent transcriptional regulation of collagen type I expression

The transcriptional regulation of collagen type I downstream of the α-SMA-TRPC6 signaling axis was checked. Yes-associated Protein (YAP) is a mechanosensitive transcription factor that responds to changes in actin filament organization and mediates pro-fibrotic signaling (28, 29). The activation of YAP is dependent on inhibitory phosphorylation at multiple residues (30). Phosphorylation at Serine 127 is reported to inhibit YAP activation by tethering it to the 14-3-3 protein in the cytoplasm and, hence, is used to ascertain YAP activation. In the present study, Ang II enhanced YAP activation at 6 h post-treatment, as evidenced by a decrease in YAP phosphorylation at S127 (Supplementary Fig. 4G, H). Importantly, knockdown of α-SMA and TRPC6 attenuated Ang II-stimulated YAP activation, showing that YAP activation is dependent on α-SMA and TRPC6 (Fig. 8A-D). A direct role for α-SMA and TRPC6 in YAP activation was confirmed by ChIP that showed reduced binding of YAP to the collagen type I gene promoter upon α-SMA and TRPC6 knockdown in Ang II-stimulated cells (Fig. 8E). Together with the finding that inhibition of YAP by Verteporfin attenuated Ang II-stimulated expression of collagen alpha1(I) (Fig. 8F, G), the observations indicated a role for YAP activation downstream of α-SMA and TRPC6 in the regulation of collagen type I expression in Ang II-treated cardiac fibroblasts.

A previous study by Davis et al. (2012) had demonstrated a role for TRPC6-mediated Ca$^{2+}$ influx in cardiac myofibroblast differentiation (27). Since the present study focused on TRPC6 and its role in collagen expression via activation of YAP, preliminary experiments were performed to confirm
the involvement of Ca\(^{2+}\) in the activation of YAP and regulation of collagen type I expression. Ang II-stimulated collagen type I expression and YAP activation were significantly attenuated upon chelation of extracellular Ca\(^{2+}\) using EGTA (1mM), a specific Ca\(^{2+}\)-chelator (32), demonstrating the role of Ca\(^{2+}\) in regulating collagen type I expression and activation of YAP in fibroblasts (Fig. 9A-C). Furthermore, while overexpression of TRPC6 in cardiac fibroblasts enhanced collagen expression and YAP activation, these effects were attenuated in EGTA-treated fibroblasts overexpressing TRPC6 (Fig. 9D-F), further supporting a role for TRPC6-mediated Ca\(^{2+}\) in the regulation of YAP activation and collagen type I expression. These findings were corroborated using SKF96365, which blocks TRPC-dependent Ca\(^{2+}\) entry (33) and is reported to prevent Ca\(^{2+}\) influx and fibronectin expression in Ang II-treated cardiac fibroblasts (34). Here, while overexpression of TRPC6 in cardiac fibroblasts enhanced YAP activation and collagen expression, these effects were attenuated in SKF96365-treated cardiac fibroblasts overexpressing TRPC6 (Fig 9G-J). This observation further supports the role of TRPC6-dependent Ca\(^{2+}\) in mediating YAP activation and collagen expression in Ang II-treated cardiac fibroblasts.

A schematic representation of the plausible sequence of events that underlie enhanced collagen type 1 expression in cardiac fibroblasts exposed to Ang II is depicted in Fig. 10G.

**Effect of siRNA on un-stimulated cells:**

In all the experiments, the effect of siRNA on cells maintained under un-stimulated (basal) conditions was also analyzed (Supplementary Fig. 1-4).

**DISCUSSION**

Heart failure remains a major cause of morbidity and mortality worldwide despite significant advances in therapy and prevention (35). Apart from progressive myocyte hypertrophy and apoptosis that compromise cardiac function, sustained activation of cardiac interstitial fibroblasts in response to injury leads inadvertently to interstitial fibrosis that contributes to the onset and progression of heart failure (36). There is increasing appreciation that therapy directed at cardiac fibrosis may retard heart failure and other
In the current clinical setting, blocking the actions of Ang II, a potent inducer of cardiac fibrosis, is a preferred treatment modality to reduce adverse myocardial remodeling following myocardial infarction. However, Ang II blockade remains only partially effective and can potentially entail unfavorable effects (38, 39), which necessitates identification of other appropriate therapeutic targets through exploration of the molecular basis of cardiac fibrogenesis.

It has been reported that a synergy between receptor tyrosine kinases (RTKs) and integrins underlies the regulation of many pathophysiological events (17). For example, cross-talk between Insulin-like Growth Factor-1 (IGF-1) and Integrin-αvβ3 is reported to impact atherosclerotic lesion formation (40). In cancer cells, Epidermal Growth Factor Receptor (EGFR)-mediated signaling cooperates with Integrin-αvβ5 to promote cell adhesion, migration and metastasis (41). Additionally, antagonism of Integrin-αvβ3 prevents basic Fibroblast Growth Factor (bFGF)-induced angiogenesis in vivo(42). Together, these reports highlight the importance of RTK-integrin cross-talk in the modulation of cell type-specific biological responses.

In the present study, we show that DDR2 knockdown abrogates the effect of Ang II on Integrin-β1 expression, which demonstrates the obligate role of DDR2 in Integrin-β1 expression in Ang II-stimulated cardiac fibroblasts. A combination of DDR2 knockdown and overexpression approaches established the regulatory role of DDR2 in Integrin-β1 gene expression even in unstimulated cells. It is pertinent to note that previous studies exploring links between DDRs and Integrin-β1 had focused mainly on the role of the DDR1 isoform in mediating Integrin-β1 activation and its effects on cell migration, protein trafficking and epithelial-mesenchymal transition (43). The focus of the present study was on DDR2 rather than DDR1 since the DDR2 isoform is found in cells of mesenchymal origin, like cardiac fibroblasts (44), while DDR1 is expressed mainly in epithelial tissues (45). It was reported that DDR1 and DDR2 induce α1β1 and α2β1 integrin activation to promote adhesion of HEK293 cells to collagen (46). However, no difference in expression levels of the integrins was observed in their study, possibly due to cell type-specific...
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differences. To the best of our knowledge, ours is the first demonstration of the involvement of DDR2 in Integrin-β1 gene expression in cardiac fibroblasts. Interestingly, Integrin-β1 knockdown attenuated Ang II-induced DDR2 expression, suggesting that DDR2 and Integrin-β1 are locked in a cycle of mutual regulation. This feedback loop between Integrin-β1 and DDR2 could function to maintain the expression levels of DDR2 in cardiac fibroblasts, enabling RTK signal amplification post-Ang II stimulation.

The Spontaneously Hypertensive Rat (SHR) represents a genetic model of hypertensive heart disease with a marked degree of myocardial fibrosis (47). A role for the renin-angiotensin system in mediating hypertension-induced fibrosis in SHR is well appreciated (48). The present study provides evidence of a positive correlation between DDR2 and Integrin-β1 in the myocardium and in cardiac fibroblasts isolated from 6 month-old SHR with myocardial fibrosis, which was consistent with the demonstration of the DDR2-Integrin-β1 link in Ang II-treated cells. Further, in a knockout mouse model carrying a germ-line deletion of DDR2, we observed a significant reduction in Integrin-β1 staining intensity in cardiac tissue from DDR2-null mice. Since fibroblasts constitute only about 10% of the total myocardial cell population in mice (26) and cardiac myocytes also express Integrin-β1 (49), the global reduction in Integrin-β1 staining intensity seems to suggest a decrease in Integrin-β1 in cardiomyocytes as well as fibroblasts. This raises the possibility that fibroblast-specific DDR2 contributes to Integrin-β1 expression in myocytes via DDR2-dependent paracrine signaling. Cardiac fibroblasts are a major source of humoral factors that regulate transcriptional activity within myocytes (50). Therefore, a loss of DDR2 in fibroblasts could repress paracrine factors regulating myocyte Integrin-β1 expression. In support of this, conditioned media derived from cultured rat cardiac fibroblasts upregulated Integrin-β1 expression in rat ventricular H9c2 cells. However, conditioned media from DDR2-silenced cardiac fibroblasts failed to enhance Integrin-β1 in H9c2 cells, suggesting that DDR2-mediated paracrine signaling may regulate Integrin-β1 expression in myocytes. This observation is important since it is consistent with the role of Integrin-β1 as an important
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determinant of cardiac size and organ growth (51). Our previous study on this mouse model had demonstrated, by echocardiography, reduced left ventricular chamber dimensions (25). Cardiomyocyte length was atypically shorter in the DDR2-null mice, resulting in decreased heart size and weight. It is tempting to speculate that the reduction in heart size in DDR2-null mice indicates the importance of DDR2 not only in the regulation of cardiac fibroblast function but also in regulating myocardial size. The possibility that cardiac fibroblasts are involved in myocardial growth via DDR2-dependent paracrine mechanisms warrants investigation.

Having established the regulatory role of DDR2 in Integrin-β1 expression, we probed the mechanisms by which DDR2 regulates Integrin-β1. We found that DDR2-dependent activation of ERK1/2, as previously shown by us (8), enhanced TGF-β1 expression in Ang II-treated cells. Further, inhibition of ERK1/2 MAPK or TGF-β1 attenuated Ang II-induced Integrin-β1 expression, suggesting that DDR2-dependent ERK1/2-TGF-β1 signaling regulates Integrin-β1. The transcriptional regulation of Integrin-β1 in Ang II-treated cardiac fibroblasts was probed. We focused mainly on AP-1, a redox-sensitive transcription factor known to be a target of Ang II (52). c-Jun, a component of the AP-1 complex, is reported to enhance the proliferation of fibroblasts in idiopathic pulmonary fibrosis (53). Moreover, AP-1 is a target of MAPK signaling (22) and TGF-β1 (54). We observed that DDR2 mediates AP-1 activation via ERK1/2 and TGF-β1 signaling, resulting in AP-1 binding to the Integrin-β1 gene promoter and transcriptional upregulation of Integrin-β1. This study provides evidence that Ang II stimulates the expression of DDR2, which in turn enhances Integrin-β1 expression in cardiac fibroblasts via ERK1/2- and TGF-β1-mediated AP-1 activation.

An important outcome of the study was the elucidation of the obligate role of DDR2-Integrin-β1 cross-talk in α-SMA and collagen expression. Since we found DDR2 to influence Integrin-β1 expression, we examined the role of DDR2-dependent Integrin-β1 expression in α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts. Consistent with previous studies (29), we found that Integrin-β1 knockdown attenuated α-SMA and collagen type I expression. However,
we also found that, while DDR2 knockdown inhibited α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts, overexpression of Integrin-β1 in DDR2-silenced cells restored α-SMA and collagen type I expression. Interestingly, DDR2 overexpression in Integrin-β1-silenced cells did not restore α-SMA and collagen type I, clearly indicating the centrality of Integrin-β1 as an indispensable factor that mediates DDR2-dependent phenotypic transformation of cardiac fibroblasts and collagen expression.

Integrin-β1 is a common subunit of collagen-binding integrins, which generally acts in association with integrin alpha subunits (α1β1, α2β1, α10β1, α11β1) to activate downstream signaling events (55). However, different studies have stressed the regulatory role of the α and β subunits in tissue fibrosis individually. For example, the genetic ablation of Integrin-β1 per se has been shown to attenuate liver fibrosis and impede dermal wound healing (29, 56). Further, myocardial Integrin-β1 expression is reported to be upregulated post-MI (57). On the other hand, although germline deletion of integrin α11 has been reported to abrogate diabetes-related cardiac fibrosis (58), the role of Integrin-β1 in mediating pro-fibrotic signaling events in cardiac fibroblasts remains largely unclear, a lacuna that this study addressed.

To further evaluate the functional significance of DDR2-Integrin-β1 cross-talk in cardiac fibroblast function in a context of injury, its involvement in fibroblast response to injury was probed by a scratch-wound assay. We found that knockdown of DDR2 or Integrin-β1 reduced the wound healing ability of fibroblasts exposed to Ang II. Importantly, overexpression of Integrin-β1 in DDR2-silenced cells restored their wound healing function, demonstrating the role of collagen receptor cross-talk in cardiac fibroblast function. It is pertinent to note in this context that, in our previous study, collagen synthesis and the rate of collagen deposition were found to be markedly reduced in DDR2-null mice (25). Together, the findings show that reduced expression of Integrin-β1 in DDR2-null mice reduces collagen synthesis and deposition, and wound healing.

This study uncovers a hitherto unknown role for α-SMA, downstream of Integrin-
β1, in the regulation of collagen type I expression in Ang II-treated cardiac fibroblasts. Increased collagen synthesis is typically correlated with the transition of cardiac fibroblasts to an α-SMA-positive myofibroblast phenotype (1). α-SMA expression marks myofibroblast differentiation and plays a critical role in wound healing post injury. Traditionally, the functional roles ascribed to α-SMA involve cell motility and contraction of the fibrotic scar tissue in a setting of injury. However, recent studies exploring a role for α-SMA in cell signaling report α-SMA-dependent activation of ERK1/2 MAPK (18). In lung adenocarcinoma cells, the expression of Focal Adhesion Kinase (FAK) and Hepatocyte Growth Factor Receptor (HGFR or c-MET) has also been shown to be regulated by α-SMA, which leads to augmented metastasis (59). In general, the cellular F/G actin ratio is reported to influence the transcription of smooth muscle differentiation-related genes in cells (60). However, a specific role for α-SMA in the regulation of gene transcription remains largely unexplored. We found that knockdown of α-SMA attenuates Ang II-induced collagen type I expression, showing that α-SMA, beyond its structural importance in myofibroblasts, could be a critical mediator of signaling processes and gene expression following injury. This finding, for the first time, sheds light on a molecular regulatory event that causally links the phenotypic transition of cardiac fibroblasts to enhanced collagen type I production.

Further, we dissected the molecular mechanisms underlying α-SMA-dependent collagen expression. α-SMA constitutes the mechanosensitive stress fibers in myofibroblasts and couples mechanical stretch to Ca\(^{2+}\) influx via cation channels (61). TRPC6 is a cation channel in fibroblasts that mediates Ca\(^{2+}\) influx and is reported to promote fibroblast differentiation into myofibroblasts (31). Since α-SMA mediates collagen expression, as shown here, we proposed a causal role for TRPC6 in mediating the α-SMA-dependent increase in collagen type I in Ang II-treated cardiac fibroblasts. In support of this, we observed that, while TRPC6 knockdown abolished collagen type I expression in Ang II-treated cardiac fibroblasts, overexpression of TRPC6 in α-SMA-silenced cells restored collagen type I expression in Ang II-treated cells, demonstrating the importance of α-SMA-TRPC6 signaling in the regulation of...
collagen type I expression. Further, we probed the transcriptional events downstream of α-SMA and TRPC6 that regulate collagen type I. Considering the role of DDR2, Integrin-β1, α-SMA and TRPC6 as mediators of mechanotransduction, a role for the mechanosensitive transcription factor Yes-associated protein (YAP) in the transcriptional regulation of collagen type I seemed plausible. Here, we show that YAP is a positive regulator of collagen expression since inhibition of YAP with Verteporfin led to a reduction in collagen type I expression in Ang II-stimulated cardiac fibroblasts. Further, α-SMA and TRPC6, functioning downstream of the DDR2-Integrin-β1 axis, were found to mediate Ang II-induced activation and binding of YAP to the collagen type I promoter, facilitating the transcriptional upregulation of collagen type I. Consistent with its role as a Ca2+ channel mediating Ca2+ influx in cardiac fibroblasts (31), TRPC6 was found to regulate YAP and collagen via Ca2+. This is the first demonstration of a role for mechanosensitive α-SMA and TRPC6 in promoting YAP activation to enhance collagen expression. Together, the findings suggest a role for YAP in cardiac fibrogenesis, consistent with the reported role of YAP in mediating Integrin-β1-dependent liver fibrosis (29). Further, our observations imply that YAP may transcriptionally link α-SMA to changes in gene expression.

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 enhance Integrin-β1 that in turn increases α-SMA expression. Notably, α-SMA regulates collagen alpha1(I) gene expression via the mechanosensitive effectors TRPC6 and YAP. The data also indicate the existence of a reciprocal regulatory relationship between DDR2 and Integrin-β1 in which Integrin-β1 acts downstream of DDR2, linking DDR2 to α-SMA, collagen type 1 and wound healing. The link between DDR2 and Integrin-β1 is also evident in a genetic model of hypertensive heart disease and in DDR2-null mice. Future studies should address the involvement of these regulatory mechanisms in the pathogenesis of myocardial fibrosis in animal models of human disease using knockout strategies.

In conclusion, this communication provides credible evidence of an obligate
role for collagen receptor cross-talk in Ang II-stimulated cardiac fibroblast function, which is a major determinant of tissue remodeling following myocardial injury. The findings also uncover a mechanistic coupling of the two distinct cellular processes of collagen production and phenotypic transformation of interstitial fibroblasts into an active state. The regulatory role of α-SMA in collagen gene expression is a novel and notable finding of considerable significance insofar as α-SMA has not hitherto been linked to regulation of gene expression. Lastly, while it is reasonable to believe that other signaling pathways may also contribute to the regulation of collagen expression or converge upon the regulatory cascade described here, the demonstration that DDR2-Integrin-β1 cross-talk underlies α-SMA-TRPC6-YAP-dependent collagen type I expression in cardiac fibroblasts significantly advances our knowledge of the complex mechanisms underlying collagen gene expression in the heart and provides a new paradigm to understand the molecular basis of myocardial fibrosis. Future strategies to therapeutically target cardiac fibrogenesis may lie within this inherent complexity of regulatory mechanisms.

EXPERIMENTAL PROCEDURES

Materials

Angiotensin II, PD 98059, chelerythrine, U73122, VAS2870, SB431542 hydrate, EGTA and M199 were obtained from Sigma-Aldrich, (St. Louis, MO, 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) (62). 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 alpha1 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-phosphatedehydrogenase (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. SKF96365 was a kind gift from Prof. Gaiti Hasan (NCBS, Bangalore). XBT X-ray Film was from Carestream (Rochester, NY, USA). The study on rats was approved by the Institutional Animal Ethics Committees of SCTIMST and the study on mice was approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

**Isolation of cardiac fibroblasts**

Cardiac fibroblasts were isolated from young adult male Sprague–Dawley rats (2–3 months old) as described earlier (63). Subconfluent cultures of cardiac fibroblasts from passage 2 or 3 were used for the experiments. Cells were serum-deprived for 24 h prior to treatment with 1 μM Ang II.

**Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis**

Subconfluent cultures of cardiac fibroblasts were subjected to the indicated treatments and total RNA was isolated using the PureLink RNA isolation kit (Invitrogen), according to the manufacturer’s instructions. Following DNase I treatment, 2 μg of total RNA was reverse transcribed
to cDNA with random primers and M-MLV reverse transcriptase. TaqMan RT-qPCR analysis was carried out using the ABI prism 7500 Sequence Detection System (Applied Biosystems, CA, USA) with specific FAM-labeled probes for Itgb1 (Assay ID: Rn00566727_m1) and TGF-β1 (Assay ID: Rn00572010_m1), and VIC-labeled probes for β-actin (Rn00667869_m1). RT-qPCR analysis using SYBR Green gene expression assay was used for analyzing fold change in Integrin-α11 expression with ACCGCACGGCATTTGGCAT as the forward primer and TCGTGGGATTCCCCGTCCGT as the reverse primer. 18S rRNA was used as the endogenous control with CCCGCGAGTACAACCTTCT as the forward primer and CGTCATCCATGGCGAACT as the reverse primer. PCR reactions were performed under the following thermal cycling conditions: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method.

**Western blot analysis**

Subconfluent cultures of cardiac fibroblasts in serum-free M199 were treated with Ang II (1 μM) and relative protein abundance was determined by western blot analysis following standard protocols (64) and using β-actin, β-tubulin or GAPDH as loading control. Enhanced chemiluminescence reagent was used to detect the proteins with X-ray Film.

**RNA interference and overexpression**

Cardiac fibroblasts at passage 3 were seeded on 60 mm dishes at equal density. After 24 h, the cells were incubated in Opti-MEM for 5–6 h with Ambion pre-designed Silencer-Select siRNA, custom-designed siRNA from Eurogentech or scrambled siRNA at the given concentrations (10 nM for DDR2, ITGB1, TRPC6 and TGF-β1 and 50 nM for α-SMA) and Lipofectamine 2000 (8 μl).

Constitutive expression of DDR2 and TRPC6 was achieved under the control of a CMV promoter. Constitutive expression of Integrin-β1 was achieved under the control of an SV-40 promoter. The DDR2
and Integrin-β1 plasmids were verified by restriction mapping, while the TRPC6 plasmid was verified by PCR amplification using the primers provided in the kit. For overexpression, the plasmid vector for DDR2 (1 μg/μl) was transfected using Lipofectamine 2000. For cotransfection experiments, a mixture of siRNA (5 nM) and plasmid DNA (1 μg/μl) was used and transfected using Lipofectamine 2000. Following a post-transfection recovery phase in M199 with 10% FBS for 12 h, the cells were serum-deprived for 24 h and then treated with Ang II (1 μM) for the indicated durations. Cell lysates were prepared in Laemmli sample buffer, denatured and used for western blot analysis.

**Electrophoretic mobility shift assay (EMSA)**

The DNA-binding activity of activating protein-1 (AP-1) was assessed by EMSA using the LightShift Chemiluminescent EMSA Kit. Subconfluent cardiac fibroblast cultures were serum-starved for 24 h followed by incubation in M199 with or without Ang II (1 μM), and nuclear extracts were prepared using the NE-PER nuclear extraction kit. The protein concentration of the nuclear extracts was determined using the bicinchoninic acid (BCA) protein assay method. Single-stranded oligos containing the consensus sequence for the AP-1 binding site 5′-CGCTTGATGACTCAGCCGAA-3′ were biotinylated using the Thermo Scientific 3′-end biotin labeling kit and annealed with their complementary strand at 90°C. The nuclear extracts were incubated with the biotinylated probes and components of the Light Shift Chemiluminescent kit at 37°C for 60 min and electrophoresed on a 6% non-denaturing gel. After transfer to nylon membrane, the DNA was UV cross-linked at a wavelength of 254 nm for 10 min. After blocking, streptavidin-conjugated horseradish peroxidase was applied and the bands were visualized by enhanced chemiluminescence.

**ChIP assay**

The ChIP assay was performed with the Low Cell Number ChIP kit, according to the manufacturer’s protocol. Briefly, after treatment of cardiac fibroblasts with 1 μM Ang II for 30 min, the cells were cross-linked with 1% formaldehyde, lysed and sonicated in a DiagenodeBioruptor to generate ~600 bp DNA fragments. The respective lysates were incubated with
anti-c-Fos/anti-c-Jun/YAP antibody overnight at 4°C with rotation. Immune complexes were precipitated with protein A-coated magnetic beads. After digestion with proteinase K to remove the DNA-protein cross-links from the immune complexes, the DNA was isolated and subjected to PCR using primers for Itgb1 and collagen alpha1(I). 5’-TCAGGACCTCTAGAAGAGCAG-3’ and 5’-CTTCCTTCTTCCTCCTCC-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. 5’-CTCAGCACTTTCTGCTTT-3’ and 5’-GCCACCTCATCTTTAGAAA-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. The PCR products were subjected to electrophoresis on a 1% agarose gel.

**In vivo study and histology**

The generation of DDR2 null mice and genotyping were described in our previous study (25). Mouse tissue sections were prepared as described previously. Briefly, hearts were collected from age-matched male WT and DDR2-null mice, fixed in 4%-buffered paraformaldehyde for 2 days, embedded in paraffin, cross-sectioned and mounted onto slides. Integrin-β1 levels in these sections were analyzed by 3, 3'-diaminobenzidine (DAB) staining and quantified using Fiji-Image J software.

**Scratch wound assay**

Cells were seeded on 35 mm culture dishes and grown to 70–80% confluence. Gene knockdown using siRNA and overexpression using plasmid vectors were performed as described above. Following serum deprivation, a single scratch gap was created using a 10 μl pipet tip. After washing with phosphate-buffered saline, the cells were treated with Ang II (1 μM) in serum-free M199 for the indicated durations. Approximately 3–4 fields were examined per dish and images of the wound closure pattern in the treated and control groups were acquired using a Nikon inverted phase contrast microscope.
DDR2-Integrin-β1 cross-talk in collagen type I expression

microscope. Quantification of wound healing was calculated based on the following formula: \( \text{Length of wound closure} = (\text{Length of wound gap at } t_h - \text{Length of wound gap at } t_0) / \text{Length of wound gap at } t_0 \), where \( t_h \) 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.

**Conditioned-media experiments**

Cardiac fibroblasts isolated from male Sprague-Dawley rats were used. Rat ventricular H9c2 cells cultured in M199 + 10% FBS was used as an in vitro model for myocytes. DDR2 knockdown in cardiac fibroblasts was achieved using siRNA. Fibroblasts transfected with scrambled siRNA and fibroblasts maintained in M199 without serum served as controls. Six hours post-transfection, the transfection mix was replaced with M199 without serum and the fibroblast culture was maintained for 24 h. Subsequently, H9c2 cells were exposed to the fibroblast-derived conditioned media from each of the groups. Lysates were collected at 12 h and 24 h and Integrin-β1 protein expression was analyzed and quantified after normalization to β-actin expression.

**Statistical analysis**

Data are expressed as Mean ± SD. Statistical analysis was performed using Student's \( t \) tests for comparisons involving 2 groups. For comparisons involving more than 2 groups, the data were analyzed by one-way ANOVA. \( p<0.05 \) was considered significant. The in vitro data presented are representative of 3 independent experiments (n=3). The data presented on experiments involving Spontaneously Hypertensive Rats (SHRs) are representative of myocardial tissue sections obtained from 3 age-matched male SHRs. The in vivo data on DDR2-null mice are representative of myocardial tissue sections obtained from 5 age-matched male mice.
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ACKNOWLEDGMENTS

This work was supported by a research grant to SK from the Department of Biotechnology, Government of India (BT/PR23486/BRB/10/1589/2017). SK gratefully acknowledges Dr Barry H Greenberg, Division of Cardiovascular Medicine, Department of Medicine, University of California-San Diego, La Jolla, California, USA, for providing the DDR2 knockout mice tissue samples. The authors thank Dr Deepthi AN and Dr Neethu Mohan for assistance in the analysis of IHC sections. The authors thank Prof. Gaiti Hasan (National Centre for Biological Sciences, Bangalore) for providing SKF96365. HV thanks the Department of Biotechnology, Government of India, for a Fellowship. SK, HV and AST acknowledge the facilities provided by SCTIMST.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Figures and Figure legends:

Fig 1: DDR2 mediates Integrin-β1 expression in cardiac fibroblasts exposed to Ang II. (A,B) Cardiac fibroblasts were transiently transfected with DDR2 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< 0.01 vs. control, *** p< 0.001 vs. Ang II. Phospho-ERK1/2 activation was examined by western blot analysis and normalized to total ERK1/2 levels. ## p< 0.01 vs. control, ### p< 0.001 vs. Ang II. TGF-β1 protein expression was examined by western blot analysis, with β-actin as loading control. †† p< 0.01 vs.
DDR2-Integrin-β1 cross-talk in collagen type I expression

control, ††† p< 0.001 vs. Ang II. (C,D) 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 at baseline was examined by western blot analysis and normalized to β-actin. * p< 0.05 vs. control. (E,F) 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. (G) Cardiac fibroblasts were transiently transfected with DDR2 siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 6 h, Integrin-α11 mRNA expression was examined by RT-qPCR analysis. 18SrRNA served as the endogenous control. (H,I) Cardiac fibroblasts were transiently transfected with ERK1/2 siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, TGF-β1 and Integrin-β1 protein expression was examined by western blot analysis with GAPDH as loading control. For TGF-β - *** p< 0.001 vs. control, ††† p< 0.001 vs. Ang II. For Integrin-β1 - ### p< 0.001 vs. control, &&& p< 0.001 vs. Ang II. (J,K) 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. Data are representative of three independent experiments, n=3. Error bars represent SD.
DDR2-Integrin-β1 cross-talk in collagen type I expression

Fig 2:

A

Fig 2: Transcriptional regulation of Integrin-β1 by AP-1 via DDR2-dependent ERK1/2 MAPK/TGF-β1 signaling in Ang II-stimulated cardiac fibroblasts (A)

Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated with AP-1 inhibitor (SR 11302) 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. (B,C) 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<
DDR2-Integrin-β1 cross-talk in collagen type I expression

0.01 vs. Ang II. (D) EMSA was performed as described under Methods. Ang II enhanced AP-1 nuclear translocation at 30 minutes post-treatment, which was attenuated at 3 hr. ERK1/2 MAPK inhibition using PD98059 reduced Ang II-induced AP-1 activation at 30 min. (E) 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. (F) DNA binding of AP-1 subunits, c-FOS and c-JUN, to the Integrin-β1 (ITGB1) gene promoter was confirmed by ChIP using anti-c FOS and anti-c JUN antibody, respectively. A non-specific anti-rabbit IgG was used as negative control. A representative image showing the PCR amplification product is given. Data are representative of three independent experiments, n=3. Error bars represent SD.
DDR2-Integrin-β1 cross-talk in collagen type I expression

Fig 3:
DDR2-Integrin-β1 cross-talk in collagen type I expression

Fig 3: Corroboration of the DDR2-Integrin-β1 link in vivo (A-D) Cardiac tissues of 6-month old Wistar and SHR were probed for DDR2, Integrin-β1, collagen alpha1(I) and α-SMA expression 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, (n=5). (E) Cardiac tissue sections of 6 month-old Wistar and SHR rats were stained for collagen using picrosirius-red (25x magnification), (n=4). (F,G,H) 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 subjected to western blot analysis for detection of DDR2 and Integrin-β1, with β-actin as loading control. ** p< 0.01 vs. Wistar for DDR2, *** p< 0.001 vs. Wistar for Integrin-β1, (n=3). (I,J) DAB staining showing Integrin-β1 protein in myocardial tissue sections of 10 week-old Wild Type (WT) and DDR2-null mice. *** p< 0.001 vs. WT, (n=5). (K,L) Sub-confluent quiescent cultures of rat ventricular H9c2 cells were treated with conditioned media (CM) derived from control rat cardiac fibroblasts or DDR2-silenced rat cardiac fibroblasts or control siRNA-treated rat cardiac fibroblasts for 12 h or 24 h. Quiescent cultures of H9c2 in M199 without serum was used as control for basal Integrin-β1 protein expression in H9c2 cells. Integrin-β1 protein expression in H9c2 cells 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. Three rats (Sprague Dawley) were used for cardiac fibroblast isolation for the conditioned-media experiments (n=3). Error bars represent SD.
Fig 4: Ang II-induced Integrin-β1 regulates DDR2, α-SMA and collagen type I expression in cardiac fibroblasts (A-C) Cardiac fibroblasts were transiently transfected with DDR2 siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II 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 - for collagen alpha1(I)],[** p< 0.01 vs. control, *** p< 0.001 vs. Ang II - for α-SMA]. (D-F) Cardiac fibroblasts were transiently transfected with Integrin-β1 (ITGB1) siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II 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. (G,H) Cardiac fibroblasts were transiently transfected with Integrin-β1 (ITGB1) siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, DDR2 protein expression was examined by western blot analysis and normalized to β-actin.*** p< 0.001 vs.
DDR2-Integrin-β1 cross-talk in collagen type I expression

control, ## p< 0.01 vs. Ang II. (I) A representative image of Integrin-β1 siRNA validation is given. Knockdown efficiency of Integrin-β1 siRNA1 or siRNA2 on Integrin-β1 protein expression was checked. siRNA2 was used for silencing Integrin-β1. Data are representative of three independent experiments, n=3. Error bars represent SD.
Fig 5: DDR2-dependent Integrin-β1 expression is a determinant of α-SMA and collagen type I expression in Ang II-treated cardiac fibroblasts. (A-C) Cardiac fibroblasts were transiently co-transfected with DDR2 siRNA and Integrin-β1 (ITGB1) plasmid over-expression vector. 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< 0.01 vs. control, ## p< 0.01 vs. Ang II, †† p< 0.01 vs. Ang II + DDR2 siRNA. α-SMA protein expression was examined by western blot analysis and normalized to β-actin. ** p< 0.01 vs. control, ## p< 0.01 vs. Ang II, † p< 0.05 vs. Ang II + DDR2 siRNA. (D-F) Cardiac fibroblasts were transiently co-transfected with Integrin-β1 (ITGB1) siRNA and DDR2 over-expression vector. Following revival and serum-deprivation, the transfected cells were exposed to Ang II for 12 h. Collagen alpha1(I) protein expression was examined by
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western blot analysis and normalized to GAPDH. *** p< 0.001 vs. Control, ### p< 0.001 vs. Ang II, ** p< 0.01 vs. Ang II. 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. Data are representative of three independent experiments, n=3. Error bars represent SD.
Fig 6:

A

Control 0 Hr | Ang II 0 Hr | Ang II+DDR2siRNA 0 Hr | Ang II+ITGB1 siRNA 0 Hr | Ang II+DDR2siRNA+ITGB1 overexpression 0 Hr

Control 24 Hr | Ang II 24 Hr | Ang II+DDR2siRNA 24 Hr | Ang II+ITGB1 siRNA 24 Hr | Ang II+DDR2siRNA+ITGB1 overexpression 24 Hr

B

Length of wound closure (in μm)

- control + control siRNA
- Ang II + control siRNA
- Ang II + DDR2 siRNA
- Ang II + ITGB1 siRNA
- Ang II + DDR2 siRNA + ITGB1 overexpression

0 hr | 12 hr | 24 hr

### | ### | +++

††† | ††† | ns

ns | ns | ns
Fig 6: DDR2-dependent Integrin-β1 expression is a determinant of wound healing in Ang II-treated cardiac fibroblasts. (A,B) Wound healing ability of cardiac fibroblasts is impaired upon DDR2 or Integrin-β1 knockdown and is restored upon Integrin-β1 overexpression 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 a mixture of DDR2 siRNA and Integrin-β1 over-expression vector. Following revival and serum deprivation, the transfected cells were exposed to Ang II and the wound healing ability of these cells were examined at 0 h, 12 h and 24 h. The length of wound closure was determined in μm and 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. Scale bar is represented as 25μm. Magnification=10x. Data are representative of three independent experiments, n=3. Error bars represent SD.
Fig 7: α-SMA-dependent TRPC6 regulates collagen type I expression in Ang II-treated cardiac fibroblasts
Cardiac fibroblasts were transiently transfected with α-SMA siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, (A,B) 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. (C,D) TRPC6 protein expression was examined by western blot analysis and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. Ang II. (E,F) Cardiac fibroblasts were transiently transfected with TRPC6 siRNA or scrambled siRNA (control). Following exposure of the transfected cells to Ang II for 12 h, collagen type 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. (G,H) Cardiac fibroblasts were transiently co-transfected with α-SMA siRNA and TRPC6 plasmid over-expression vector. Following revival of the transfected cells and serum deprivation, cardiac fibroblasts were exposed to Ang II for 12h. 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.
Fig 8: α-SMA-dependent TRPC6 regulates collagen type I expression via YAP activation in Ang II-treated cardiac fibroblasts. Cardiac fibroblasts were transiently transfected with α-SMA siRNA, TRPC6 siRNA or scrambled siRNA (control). (A,B) Following exposure of the α-SMA siRNA-transfected cells to Ang II for 6 h, phosphorylation of YAP at S127 was examined by western blot analysis and normalized to Total YAP. ***p< 0.001 vs control, **p< 0.01 vs Ang II. (C,D) Following exposure of the TRPC6 siRNA-transfected cells to Ang II for 6 h, phosphorylation of YAP at S127 was examined by western blot analysis and normalized to Total YAP.***p< 0.001 vs control, **p< 0.01 vs Ang II. (E) Cardiac fibroblasts were transiently transfected with α-SMA siRNA, TRPC6 siRNA or scrambled siRNA (control). Following Ang II treatment for 6 h, ChIP assay was performed as described under methods. DNA binding of YAP to the collagen alpha1(I) gene promoter was confirmed using anti-YAP antibody. A non-specific anti-rabbit IgG was used as negative control. A representative image showing
the PCR amplification product is shown. (F,G) Sub-confluent quiescent cultures of cardiac fibroblasts were pre-treated with the YAP inhibitor (Verteporfin) 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 collagen alpha1(I), with β-actin as loading control. ***p< 0.001 vs control, ###p< 0.001 vs Ang II. Data are representative of 3 independent experiments, n=3. Error bars represent SD.
Fig 9: Subconfluent quiescent cultures of cardiac fibroblasts were pre-treated with EGTA (1mM) for 1 h and, subsequently, with Ang II. (A, B & C) Phosphorylation status of YAP at S127 was examined by western blot analysis at 6 h post-Ang II treatment and normalized to Total YAP. *p< 0.05 vs. control, ***p< 0.001 vs. Ang II, ### p< 0.001 vs. Ang II. Expression of collagen alpha1(I) was analyzed at 12 h post-Ang II treatment with β-actin as loading control. ***p< 0.001 vs. control, **p< 0.01 vs Ang II, ## p< 0.01 vs Ang II. (D, E & F) Cardiac fibroblasts were transiently transfected with TRPC6 overexpression plasmid or control plasmid. Following exposure of the transfected cells
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to EGTA (1mM), phosphorylation status of YAP at S127 was examined by western blot analysis at 6 h post-EGTA treatment and normalized to Total YAP. *** p< 0.001 vs control, * p< 0.05 vs. TRPC6 OE, ** p< 0.01 vs TRPC6 OE. Collagen alpha1(I) protein expression was examined by western blot analysis at 12 h post-EGTA treatment and normalized to β-actin. ** p< 0.01 vs. control, †† p< 0.01 vs. TRPC6 OE, ### p< 0.001 vs. TRPC6 OE. (G, H, I & J) Cardiac fibroblasts were transiently transfected with TRPC6 overexpression plasmid or control plasmid. Following exposure of the transfected cells to SKF96365 (10µM), phosphorylation status of YAP at S127 was examined by western blot analysis at 6 h post-treatment with SKF96356 and normalized to Total YAP. * p< 0.05 vs control, ** p< 0.01 vs. TRPC6 OE, # p< 0.05 vs. TRPC6 OE. The control and SKF96365 blot from a separate run have been juxtaposed. Collagen alpha1(I) protein expression was examined by western blot analysis at 12 h post-treatment with SKF96365 and normalized to β-actin. *** p< 0.001 vs. control, ### p< 0.001 vs. TRPC6 OE, ††† p< 0.001 vs. TRPC6 OE. Data are representative of 3 independent experiments, n=3. Error bars represent SD.
Fig 10: (A) A schematic representation of the plausible sequence of events that underlie enhanced collagen type I expression in cardiac fibroblasts exposed to Ang II.
Collagen receptor cross-talk determines α-smooth muscle actin-dependent collagen gene expression in angiotensin II-stimulated cardiac fibroblasts
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J. Biol. Chem. published online November 7, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009744

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