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The Kruppel-like factor KLF15 inhibits connective tissue growth factor (CTGF) expression in cardiac fibroblasts

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Abstract

Cardiac fibrosis is a hallmark feature of pathologic remodeling of the heart in response to hemodynamic or neurohormonal stress. Accumulating evidence implicates connective tissue growth factor (CTGF) as a key mediator of this process. Our group has previously identified Kruppel-Like Factor 15 (KLF15) as an important regulator of cardiac remodeling in response to stress; however, the role of this transcription factor in cardiac fibrosis has not been reported. Here we provide evidence that treatment of neonatal rat ventricular fibroblasts (NRVFs) with the potent pro-fibrotic agent Transforming Growth Factor- β 1 (TGF β 1) strongly reduces KLF15 expression while inducing the pro-fibrotic factor CTGF. Adenoviral overexpression of KLF15 inhibits basal and TGF β 1-induced CTGF expression in NRVFs. Furthermore, hearts from KLF15 $-/-$ mice subjected to aortic banding exhibited increased CTGF levels and fibrosis. From a mechanistic standpoint, KLF15 inhibits basal and TGF β 1-mediated induction of the CTGF promoter. Chromatin Immunoprecipitation (ChIP) and electrophoretic mobility shift assays demonstrate that KLF15 inhibits recruitment of the coactivator P/CAF to the CTGF promoter with no significant effect on Smad3-DNA binding. Consistent with this observation, KLF15 mediated repression of the CTGF promoter is rescued by P/CAF overexpression. Our result implicates KLF15 as a novel negative regulator of CTGF expression and cardiac fibrosis.

Keywords

kruppel; fibroblast; fibrosis; transcription; TGF; CTGF; P/CAF

INTRODUCTION

Chronic pressure overload, volume overload or myocardial injury can cause pathologic cardiac remodeling that frequently results in decompensated heart failure. Cardiac fibrosis is an

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important feature of pathologic remodeling and is characterized by fibroblast proliferation and increased deposition of extracellular matrix (ECM) [1].

Connective tissue growth factor (CTGF) is a multifunctional protein that is expressed in both cardiomyocytes and cardiac fibroblasts [2] and has been implicated in the pathogenesis of diverse pro-fibrotic disease states including atherosclerotic vasculopathy [3] and heart failure [2]. CTGF expression is regulated by diverse stimuli including the potent growth factor, TGF β 1 [2]. The TGF β receptor is a serine/threonine kinase transmembrane heteromeric type I and type II receptor complex. Following receptor activation, signals are transduced to the nucleus through the Smad family transcription factors. Smad proteins can be subclassified into 3 groups: receptor-activated (Smads 1, 2, 3, 5 and 8), co-mediator (Smad 4 and 10) and inhibitory (Smad 6 and 7) [4]. Importantly, TGF β 1-mediated induction of CTGF has previously been shown to be regulated by Smad3 binding to a consensus element in the CTGF promoter [5,6].

Kruppel-like Factors (KLF) are a large family of zinc-finger-containing transcription factors that are crucially involved in mammalian cell differentiation and tissue-development [7]. Accumulating evidence implicates this family in cardiac biology [7]... KLF15 was shown by our group to be highly expressed in the heart, liver, kidney and adipocytes [8]. In addition, we have demonstrated that KLF15 is expressed in cardiomyocytes and acts as an important negative regulator of cardiac hypertrophy [9]. Although KLF15 has been shown to be expressed in cardiac fibroblasts [9], its role in cardiac fibrosis has not been fully understood. In the current study, we provide evidence supporting a role for KLF15 as a transcriptional inhibitor of cardiac fibrosis via its regulation of CTGF expression.

MATERIALS AND METHODS

Plasmid and reagents

Generation of KLF15(−/−) mice and the aortic constriction model have been previously described [9] CTGF-promoter-luciferase was kindly provided by Dr. Andrew Leask (Royal Free and University College Medical School, London, UK) [10]. Human TGF β 1 was from Peprotech (100-21R). Anti-tubulin antibody was from Sigma-Aldrich (St Louis, MO). Other antibodies used in Western blotting and protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ChIP kit was from Upstate biotechnology (Lake Placid, NY). Ad-KLF15-Flag and Ad-GFP were generated by Welgen, Inc. (Worcester, MA). Expression plasmids for KLF15-FLAG, Smad3, Smad4, P/CAF, 3TPlux, and constitutively active T β RI have been described previously [9,11].

Primary fibroblast culture

Primary neonatal rat ventricular fibroblasts (NRVF) were prepared from 2-day old Sprague-Dawley rat pups as described previously [9]. Adenoviral infection was performed on NRVF (Passage 1) at an MOI of 200 for 48 hours (>90% infection efficiency as assessed by GFP signal).

Transient Transfections

Fugene-6 (Roche) was used to transfect primary cardiac fibroblast or 293T cells with CTGF-promoter-luciferase reporter and various combinations of Smad3, Smad4, and KLF15 and subsequent measurement of luciferase activity on a luminometer as previously described [9].

Electrophoretic mobility shift assay (EMSA)

Gel shift was performed as previously described [12]

Northern analysis of gene expression

Total RNA was isolated using Trizol (Gibco-BRL) and subject to Northern analysis as previously described [8,9].

Western blot analysis

Total protein was isolated from NRVF infected with Ad-KLF15 (48 hrs), followed by TGF β 1 treatment for 6 hours. Western blots were performed as previously described [9].

GST-pulldown assay

Recombinant GST-K15 protein was synthesized by the GST Gene Fusion system (Amersham Pharmacia Biotech) and purified by the Bulk GST Purification Module (Amersham Pharmacia Biotech) following manufacturer's protocol. GST-based binding assay was performed as previously described [11].

Co-immunoprecipitation

293T cells were cotransfected with Flag-P/CAF and myc-KLF15 in various combinations using Eugene-6 (Roche) and coimmunoprecipitation performed as previously described [12].

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as previously described [13] using a ChIP assay kit (Upstate Biotechnology) and P/CAF antibody (Santa Cruz). PCR primers for the amplified portion of the rat CTGF promoter were: rCTGFChIP: 5'tcggggcggaggttggtgc3' and rCTGFChIP: 5'tttctaggggccgtgtatctgc3'

Statistical analysis

Data was expressed as mean $\pm$ SEM. Differences between experimental groups were evaluated for statistical significance using Student's t-test for unpaired data. P-values <0.05 were considered statistically significant.

RESULTS

Accelerated cardiac fibrosis in KLF15 null mice subjected to pressure overload

The generation of KLF15 $-/-$ mice and the ascending aortic constriction (AAC) model has been previously described [9]. KLF15 $-/-$ mice hearts subjected to AAC exhibit exaggerated collagen deposition (trichrome staining, Figure 1B) and excess induction of CTGF, a well-described mediator of cardiac fibrosis (Figure 1A). No difference in CTGF expression between $+/+$ and $-/-$ mice was observed in the sham group (data not shown).

KLF15 is downregulated by TGF β 1 and represses CTGF expression in cardiac fibroblasts

To further understand the role of KLF15 in regulation of cardiac fibrosis, we first sought to determine its expression pattern in NRVF under basal and TGF β 1-stimulated conditions. As shown in Figure 1C, TGF β 1 treatment for 6 hours causes significant downregulation of KLF15 expression with concomitant induction of CTGF. Given this expression pattern, we hypothesized that KLF15 might inhibit downstream effects of TGF β 1. As demonstrated in Figure 1D, adenoviral overexpression of KLF15 strongly inhibits both basal and TGF β 1-induced CTGF expression. To determine whether this inhibitory effect occurs at the promoter level, transient transfection studies in primary cardiac fibroblasts were performed using a luciferase reporter containing full length CTGF promoter [10]. Consistent with the effect observed on endogenous gene expression, we found that KLF15 inhibits both basal and TGF β 1-induced activity of the CTGF promoter (Figure 1E). Taken together, these data

demonstrate that KLF15 is expressed in cardiac fibroblasts, is downregulated by TGF β 1, and is inhibits CTGF expression.

KLF15 represses the CTGF promoter via inhibition of P/CAF recruitment

Smad3 has been previously identified as a critical mediator of TGF β -induced CTGF expression [10]. To understand the molecular basis for KLF15's inhibitory effect on CTGF expression we hypothesized that KLF15 may inhibit Smad3 function. As shown in Figure 2A, KLF15 strongly inhibits Smad3/4 mediated induction of a Smad binding element (SBE) - concatomer reporter (3Tplux). We next used EMSA to determine whether KLF15 inhibited the ability of Smad3 to bind target DNA sequences. 293T cells were infected with either control (Ad-GFP/EV) or KLF15 (Ad-GFP/K15), followed by transfection with combinations of flag-Smad3 and/or constitutively active T β RI. Nuclear extracts were harvested, and gel-shift studies performed using a radiolabeled oligonucleotide that included Smad consensus sites. In control virus infected cells (Ad-GFP/EV), active T β RI induced a strong singular DNA-protein complex. The presence of Smad3 in this complex was verified by cold-competition using excess unlabeled oligonucleotide probe and by supershift studies using an anti-flag antibody. In the nuclear extracts from cells overexpressing KLF15, there was no significant change in this Smad3-DNA complex compared to that seen with EV infected cells (Figure 2D). These data suggest that KLF15's ability to inhibit Smad3 activity does not involve inhibition of Smad3-DNA binding.

The fact that KLF15 potently inhibited Smad3 transcriptional activity (Figure 2A) without affecting Smad3-DNA binding (Figure 2D) raised the possibility that KLF15 action may modulate critical coactivators at the CTGF promoter. The protein P/CAF is a potent transcriptional coactivator of Smad3 target genes and has been shown to interact with members of the KLF gene-family [11]. Because coactivators are often present in rate-limiting amounts, we hypothesized that KLF15 may compete for PCAF and thereby reduce Smad3 transcriptional activity. Consistent with this hypothesis, cotransfection of P/CAF abrogated KLF15 mediated repression of Smad3/4 transcriptional activity on the 3Tplux reporter (Figure 2A). P/CAF alone has no significant effect on this reporter (Figure 2A). To assess whether KLF15 interacts with P/CAF, we performed GST-pulldown studies by incubating a GST-KLF15 fusion protein with *in vitro* translated and radiolabeled P/CAF. As shown in Figure 2B, a GST-KLF15 fusion protein can pull-down radiolabeled P/CAF in solution. To assess whether this interaction occurs in intact cells, co-immunoprecipitation was performed in 293T cells cotransfected with myc-KLF15 and flag-P/CAF. Cell lysates were immunoprecipitated with an anti-myc antibody and immunoblotted with anti-flag antibody. As shown in Figure 2C, strong binding was detected between KLF15 and P/CAF. Finally, chromatin immunoprecipitation using a P/CAF-specific antibody (Figure 2E) confirmed that KLF15 can suppress TGF β 1-induced recruitment of P/CAF to the endogenous CTGF promoter in primary cardiac fibroblasts.

DISCUSSION

Fibroproliferative states are a critical feature of cardiovascular pathobiology. As such, a greater understanding of the molecular mechanisms underlying cardiac fibrosis may provide the foundation for novel therapies for heart failure. Our laboratory has previously demonstrated a role for KLF15 in the cardiomyocyte as a novel regulator of hypertrophic remodeling[9]. In this study, we demonstrate that KLF15 functions in the cardiac fibroblast as an important transcriptional inhibitor of cardiac fibrosis and pathologic ventricular remodeling, in part, via its ability to inhibit expression of the potent pro-fibrotic growth factor CTGF. In response to pressure overload, KLF15 $-/-$ mice demonstrate an exaggerated expression of CTGF and significantly increased myocardial collagen accumulation. Mechanistically, we provide evidence that KLF15 can inhibit basal and TGF β 1-induced CTGF expression.

Although the induction of CTGF by TGF β 1 involves multiple molecular effectors [6]. Smad3 is a necessary and critical signaling intermediate as highlighted by the absence of TGF β 1-induced CTGF expression in Smad3(−/−) fibroblasts [14]. We demonstrate KLF15's ability to inhibit Smad3 activity does not involve alteration in Smad3-DNA binding. Rather, we find that a major mechanism by which KLF15 exerts its repressive effects is by regulating the important coactivator P/CAF (p300/CBP associated factor) at the CTGF promoter. P/CAF, a transcriptional co-activator with intrinsic histone acetyltransferase (HAT) activity, has a critical role in cell development, growth and differentiation [15]. With regard to pro-fibrotic pathways, P/CAF has previously been shown to directly interact with Smad3 and potentiate activation of TGF β /Smad signaling [15]. While KLF15 may have multiple mechanisms of action in the cardiac fibroblast, its ability to inhibit Smad3 and P/CAF highlight an important regulatory role at a key nodal point in fibrotic signaling.

In summary, we provide evidence in this study that KLF15 is a novel negative regulator of CTGF expression and cardiac fibrosis. These observations coupled with our previous report in cardiomyocytes [9] identify KLF15 as a novel therapeutic target that may be manipulated to modulate pathologic cardiac remodeling.

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Figure 1. KLF15 is an inhibitor of cardiac fibrosis and CTGF expression

(A) CTGF expression from whole heart RNA extracts of KLF15 $+/+$ and $-/-$ hearts. KLF15 $-/-$ hearts exhibit exaggerated CTGF expression in response to pressure overload. (B) Representative example of Masson's trichrome-stained left ventricular sections from KLF15 $+/+$ and $-/-$ mouse hearts after 1 week AAC. (C) TGF β 1 downregulates KLF15 expression in NRVF. Isolated NRVF were serum starved for 48 hours and stimulated with TGF β 1 (10ng/mL, 6 hours). Total RNA was isolated and Northern analysis performed using indicated cDNA probes. (D) KLF15 inhibits basal and TGF β 1-induced CTGF expression. NRVF were starved for 24 hrs, infected with control adenovirus (Ad-GFP) or Ad-GFP-KLF15 for 24hrs, and treated with TGF β 1 (10ng/mL, 6 hrs). Total RNA and protein were isolated for Northern and Western analysis for CTGF. (E) KLF15 inhibits CTGF promoter activity. NRVF were cotransfected with CTGF-promoter luciferase reporter, KLF15 expression plasmid or empty vector (pCDNA3.1). After transfection, cells were starved for 24hr and treated with TGF β 1 (10ng/mL, 6 hrs). Cell lysates were assayed for luciferase activity, which was normalized to total protein content and expressed as fold-induction over empty-vector control (n=6 per group).

Figure 2. KLF15 inhibits the CTGF promoter by inhibiting P/CAF recruitment

(A) KLF15 inhibits basal and Smad3/4-inducible 3TPlux promoter activity; overexpression of co-activator P/CAF rescues the inhibition. 293T cells were cotransfected with combinations of 3TPlux, KLF15, Smad3, Smad4, and P/CAF expression plasmids. Total transfected DNA was held constant with empty vector (pCDNA3.1). Luciferase activity, normalized to total protein, was expressed as fold induction over empty-vector control (n=6 per group). (B) KLF15 binds to P/CAF in solution. Using *in vitro* transcribed and translated (TNT) P/CAF, direct binding of P/CAF and KLF15 was confirmed in a cell-free system. Input is 2% of the TNT-P/CAF reaction. (C) KLF15 interacts with P/CAF in the intact cell. 293T cells were transfected with the indicated constructs and co-immunoprecipitation was performed as described in Experimental Procedures. (D) KLF15 does not affect Smad3 binding to the CTGF promoter. Nuclear extracts from 293T cells infected with Ad-GFP or Ad-GFP-KLF15 were used in binding with ³²P-labeled wild-type Smad 3 probe (bottom-left gel shift). Cold competition was performed using 100x molar excess of unlabeled oligonucleotide. Supershift studies were performed by pre-incubating reactions with anti-Flag antibody. (E) KLF15 inhibits P/CAF recruitment to the CTGF promoter. NRVF were infected with Ad-GFP or Ad-GFP-KLF15 and stimulated with TGFβ1. ChIP assay was performed for P/CAF recruitment to the endogenous rat CTGF promoter using an anti-P/CAF antibody and indicated PCR primers.