Published in final edited form as:

J Mol Cell Cardiol. 2016 March ; 92: 206–213. doi:10.1016/j.yjmcc.2016.02.011.

Epigenetic regulation of cardiac fibrosis

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Abstract

Fibrosis is defined as excess deposition of extracellular matrix (ECM), resulting in tissue scarring and organ dysfunction. In the heart, fibrosis may be reparative, replacing areas of myocyte loss with a structural scar following infarction, or reactive, which is triggered in the absence of cell death and involves interstitial ECM deposition in response to long-lasting stress. Interstitial fibrosis can increase the passive stiffness of the myocardium, resulting in impaired relaxation and diastolic dysfunction. Additionally, fibrosis can lead to disruption of electrical conduction in the heart, causing arrhythmias, and can limit myocyte oxygen availability and thus exacerbate myocardial ischemia. Here, we review recent studies that have illustrated key roles for epigenetic events in the control of pro-fibrotic gene expression, and highlight the potential of small molecules that target epigenetic regulators as a means of treating fibrotic cardiac diseases.

Keywords

Cardiac; Fibrosis; Epigenetics

1. Introduction

Changes in fibroblast gene expression are central to the initiation and maintenance of a fibrotic response. This has been well illustrated in the differentiation of resident cardiac fibroblasts into “activated” myofibroblasts in response to extracellular cues, such as TGF- β 1 [1], IL-18 [2], and platelet-derived growth factor (PDGF)-D [3]. Induction of pro-fibrotic gene expression in cardiac fibroblasts is also triggered by mechanical stress, as seen in pressure overload models of heart failure, and in vitro, with stretching or extended culture duration [4–7]. Stimulation of fibrogenic gene expression in response to stress signals requires the action of sequence-specific DNA binding transcription factors, including SMAD2/3, serum response factor (SRF), myocardin-related transcription factors (MRTFs), and nuclear factor of activated T cells (NFAT) [8]. The molecular mechanisms by which these transcription factors couple to chromatin to drive the changes in gene expression that culminate in excessive production of extracellular matrix (ECM) by cardiac fibroblasts and myofibroblasts remains poorly understood.

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The basic unit of chromatin is the nucleosome, which is a histone octamer wrapped in a 147-bp stretch of DNA. Modifications of nucleosomal DNA or protein, without changes to the underlying nucleotide sequence, can have a profound effect on gene expression, and this mode of gene regulation is referred to as epigenetics. Histone tails are at the nexus of epigenetic control, and are subject to a variety of post-translational modifications (PTMs). These marks are largely mutually exclusive and have dramatic effects on the activation state of gene regulatory sequences (enhancers or promoters), and on expression of genes when these PTMs are found within the gene body. Several lysine residues within the unstructured amino-terminal tails of histones are subject to PTMs, with acetyl marks on the tails of histone H3 (H3) and histone H4 (H4) being the best characterized and most abundant [9]. In mammalian cells, acetylation of eight lysine residues on H3 and five lysines on H4 are readily detected by mass spectrometry [10]. Adding further complexity, histone lysines can be methylated, and sumoylation, ubiquitination, crotonylation, succinylation and malonylation of nucleosomal lysines have also been described [10–13].

The interplay between lysine acetylation and methylation has been studied in detail. Histone acetylation is typically associated with active transcription and, depending on the targeted lysine residue, methylation can lead to either gene activation (e.g. H3K4) or repression (e.g. H3K9 and H3K27) [14]. In general, acetylation or methylation of a given nucleosomal lysine leads to opposing effects on gene transcription (Fig. 1). For example, acetylated H3K9 and H3K27 are associated with gene activation. The mechanisms by which acetyl and methyl groups confer distinct transcriptional responses appear to involve “reader” proteins. For instance, at least two methyl reading chromatin remodelers, which open chromatin, have preferences for methylated H3K4 over other residues. In contrast, repressive methyl binding proteins have preference for methylated H3K9 or H3K27 [15, 16]. Similarly, acetylation of histones that mark active transcription start sites and enhancers can create docking sites for transcriptional coactivator complexes, such as those containing bromodomain and extraterminal domain containing (BET) acetyl-lysine binding proteins (see below). It appears that epigenetic reader, writer, eraser domains have evolved to fulfill functions within feed forward circuits. For example, HATs (e.g. CBP, P300, PCAF), which contain bromodomains that recognize activating acetyl-histone marks, also catalyze the addition of acetyl groups to sites associated with gene induction [17]. Additionally, histone lysine demethylases (KDMs), which contain reader domains for the active H3K4 Me2/3 mark, catalyze removal of methyl groups at H3K9 and H3K27, thereby promoting gene transcription [18].

As small molecules that target histone modifying enzymes and histone readers become increasingly available, a greater understanding of epigenetic pathways in fibroblasts is emerging and suggesting novel strategies for anti-fibrotic therapy. In this article, we focus on the roles of regulators of histone acetylation and methylation in the control of cardiac fibrosis.

2. Histone deacetylases (HDACs) and fibrosis

The 18 mammalian HDACs are grouped into four classes [19]. Coordination of a zinc ion in the catalytic domains of class I, II and IV HDACs is required for catalysis. In contrast, class

III HDACs (sirtuins) utilize nicotinamide adenine dinucleotide (NAD⁺) as a co-factor for catalytic activity.

Myocardial HDAC catalytic activity (class I and class II) is elevated in multiple models of left and right ventricular remodeling, but not during exercise induced cardiac hypertrophy [20, 21]. Importantly, HDAC catalytic activity was also shown to be increased in cultured cardiac fibroblasts stimulated with IL-1 β or fetal bovine serum [20]. The mechanisms governing stress-induced activation of HDACs in cardiomyocytes involves acetylation of class I HDACs by p300 [22], as well as phosphorylation of class I HDACs by casein kinase II [23]. Whether these or other mechanisms regulate HDAC catalytic activity in cardiac fibroblasts awaits further investigation.

Small molecule inhibitors of zinc-dependent HDACs have been shown to block fibrosis in a number of pre-clinical models of cardiac fibrosis [24]. Most of these studies employed pan-HDAC inhibitors such as trichostatin A (TSA). More recently, investigators have taken advantage of newer, isoform-selective HDAC inhibitors. For example, selective class I HDAC (HDAC1, -2, -3) inhibition with Mocetinostat blocked cardiac fibrosis in response to chronic angiotensin II infusion in mice [25], and blunted progression of fibrosis in a chronic rat myocardial infarction (MI) model (with no impact on scar size), resulting in a reduction in LV end diastolic pressure [26]. In this model, Mocetinostat was delivered 3-weeks post-MI and continued for 3 additional weeks, illustrating the potential of class I HDAC inhibition to improve pre-existing cardiac disease. In a 7-day model of mouse MI, administration of the class I HDAC inhibitor, PD-106, post-MI resulted in reduced LV remodeling and improved cardiac function at study endpoint, with concomitant suppression of matrix metalloproteinase-2 and -9 expression [27]. In an ex vivo model of ischemia/reperfusion injury in the rat heart, pretreatment with MS-275, another class I HDAC inhibitor, led to reduced infarct area and significant improvements in LV contractile function [28]. Efficacy of MS-275 was associated with FOXO3a-mediated induction of the antioxidant genes, catalase and mitochondrial superoxide dismutase. In this ex vivo model, it is likely that MS-275 improved LV function, at least in part, by preventing cardiomyocyte death. Mocetinostat, PD-106 and MS-275 are structurally-related HDAC inhibitors that contain a benzamide warhead that confers a high degree of selectivity for inhibition of class I HDACs over other HDAC isoforms [29].

Class I HDAC inhibitors block cardiac fibrosis, in part, by suppressing cardiac fibroblast proliferation. Class I HDAC inhibition in cardiac fibroblasts prevents retinoblastoma protein (Rb) phosphorylation, thereby blocking downstream expression of target genes that promote the G1-to-S transition [25]. The mechanism by which class I HDAC inhibitors block Rb phosphorylation involves induction of the genes encoding p15 and p57, which are cyclin-dependent kinase (CDKs) inhibitors; CDKs phosphorylate Rb. Via this anti-proliferative mechanism, class I HDAC inhibitors prevent stress-induced expansion of ECM-producing myofibroblasts. Class I HDAC inhibitors have also been shown to block differentiation of bone marrow-derived fibrocytes into myofibroblasts [25], and to directly inhibit cardiac myofibroblast activation [26].

Recently, class I HDAC inhibition was shown to stimulate expression of anti-fibrotic microRNA-133 (miR-133), leading to suppression of transverse aortic constriction (TAC)-mediated cardiac fibrosis in mice [21]. By directly associating with the miR-133 promoter, class I HDACs –1 and –2 downregulate expression of this miR in various models of heart failure and, importantly, in cardiac fibroblasts derived from patients with dilated cardiomyopathy. Class I HDAC inhibitors derepress miR-133, leading to suppression of pro-fibrotic miR-133 target transcripts.

Studies in other organ systems, including kidney, lung and liver, have also revealed anti-fibrotic effects of HDAC inhibitors. The findings have been reviewed elsewhere [30, 31].

3. Histone acetyltransferases (HATs) and fibrosis

Histone acetyltransferases (HATs) catalyze the addition of acetyl groups to lysine residues on nucleosomal histone proteins. This PTM leads to altered electrostatic charge of histones, causing chromatin relaxation, and also creates binding sites for proteins with acetyl-lysine binding (reader) domains. HAT activity is paramount for regulation of gene transcription [32, 33]. Twenty-eight human HATs are divided into two types based on cellular location. Type A HATs reside in the nucleus, while Type B HATs localize to the cytoplasm and influence the stability and trafficking of newly synthesized histone proteins. HATs are further grouped into five families based on homology. The most commonly studied families are GNAT (PCAF), GCN5 (p300 and CBP), and MYST (MOZ and TIP60) [34, 35]. In cardiomyocytes, HAT activity is positively regulated by ERK signaling, and in non-cardiac cells, HATs have been shown to be controlled by a growing number of kinases, including AKT, HIPK2 and c-Src [36–39].

The involvement of the p300 HAT in the control of pathological cardiac hypertrophy is well established [40, 41]. In contrast, the function of p300, or other HATs, in cardiac fibrosis remains poorly understood. The natural product HAT inhibitor, curcumin, was shown to reduce perivascular fibrosis in rodent models of cardiac remodeling induced by chronic hypertension or myocardial infarction [42, 43]. However, data obtained with curcumin should be interpreted cautiously, since this compound has many other pharmacological activities unrelated to HAT inhibition, including direct antioxidant activity, inhibition of STAT, MAPK, and NF- κ B signaling pathways, and inhibition of DNA methyl transferases, matrix metalloproteases, and HDACs [44, 45].

Extensive efforts are underway to develop more selective HAT inhibitors [35, 46]. For example, C646 is a pyrazolone-furan that was discovered in a high throughput *in silico* screen based on the structure of the p300 HAT in complex with Lys-CoA [47]. This small molecule is highly selective for p300 over six other HATs, thus illustrating its usefulness as a chemical biological tool [47, 48]. HATs also contain acetyl-lysine reader domains, which facilitate recruitment of these enzymes to specific genomic loci [49]. Selective small molecule inhibitors of the reader domains of the CBP/p300 have been described [50–52]. These compounds, CBP30 and I-CBP112, exhibit a high degree of selectivity for the CBP/p300 acetyl-lysine binding pockets over reader domains in other proteins, and have been

shown to block inflammation and aberrant leukemic self-renewal, respectively. C646, CBP30 and I-CBP112 have yet to be evaluated in the context of cardiac fibrosis.

Given the paucity of information related to HATs and cardiac fibrosis, we focus the remainder of this section on data implicating HATs as drivers of fibrosis in other organ systems. In a model of diabetic renal fibrosis induced by streptozotocin (STZ), C66, a curcumin analog, blocked glomerular and interstitial fibrosis, which was associated with reduced renal mitogen activated protein kinase (MAPK) signaling [53, 54]. Chromatin immunoprecipitation (ChIP) revealed that C66 reduced STZ-mediated recruitment of p300 to the promoters for the genes encoding CTGF, PAI-1 and fibronectin, and this was associated with reduced H3K9/14 acetylation at these sites.

Although HATs have chromatin reader domains, proper recruitment of these enzymes to gene regulatory elements typically requires associations with transcription factors. In cultured rat kidney proximal tubular epithelial cells, high glucose-mediated association of p300 with promoters for ECM genes was shown to require myocardin related transcription factor (MRTF) [55], which is a potent pro-fibrotic co-factor for serum response factor (SRF) [56, 57]. Likewise, in rat kidney mesangial cells, SMAD transcription factors were shown to govern association of p300 with pro-fibrotic genes following TGF- β stimulation, leading to increased acetylation of local H3K9/14 residues [58].

Studies in cultured fibroblasts have also revealed a pro-fibrotic role for p300. Expression of p300 is markedly elevated in skin fibroblasts from systemic sclerosis patients, and knockdown of p300 in cultured fibroblasts using hammerhead ribozymes was shown to significantly blunt TGF- β -mediated pro-fibrotic gene expression [59]. Similarly, anacardic acid, which has been shown to inhibit PCAF, p300 and Tip60 HAT catalytic activity [60], blunted TGF- β -induced collagen1A expression in cultured skin fibroblasts [61].

It is seemingly contradictory that inhibitors of either HATs or HDACs block fibrosis, given their opposing catalytic actions. It is true that HDAC inhibition results in global alterations in histone acetylation, as evidenced by immunoblotting of nuclear lysates, while HAT inhibitors elicit more restricted changes in histone acetylation (Stratton and McKinsey, unpublished). Paradoxically, however, ChIP studies have revealed that HDAC inhibition often results in reduced histone acetylation at gene promoters and enhancers [62]. Thus, at specific genomic loci, HDAC inhibition is able to functionally phenocopy HAT inhibition. This complexity likely reflects crosstalk, where epigenetic modifiers are able to influence the expression, stability, activity, and chromatin localization of one another. For example, class I HDAC activity has been shown to be necessary to allow redistribution of BET proteins to newly activated enhancers and promoters in response cellular stress [63].

Additional evaluation of the role of HATs in fibrosis of the heart is clearly warranted. These investigations should include selective disruption of HATs in cardiac fibroblasts and pharmacological intervention with recently developed, selective HAT inhibitors. Furthermore, since most studies have focused on p300, it will be necessary to extend investigations to the other four families of HATs.

4. Acetyl-lysine readers and fibrosis

Proteins containing bromodomains [64], YEATS domains [65], PHD fingers [66], and PH domains [67] have been shown to bind histones in an acetyl modification-dependent manner, although examples of PHD and PH binding to acetylated histones are infrequent. Analogous reader domains exist for binding to other histone modifications. For example, ten distinct domains have been found to govern binding of proteins to methyl-histones [68]. We will limit our discussion to acetyl-lysine readers.

Forty seven human proteins contain bromodomains. The most well characterized family of proteins that “read” acetyl-lysine marks, without also containing additional catalytic domains for epigenetic modifying activity (e.g., HATs), are the BET proteins (BRD2, BRD3, BRD4 and BRDT). BRD4 and BRDT (testis-specific) uniquely contain carboxy-terminal domains that confer the capacity to activate RNA Polymerase II (Pol II) by recruiting CDK9, a kinase component of the P-TEFb complex; CDK9 phosphorylates serine-2 of the tail of Pol II, leading to transcription elongation [69–71]. However, P-TEFb-independent mechanisms for gene regulation by BET proteins also exist. For example, BRD2 and BRD4 were shown to function as histone chaperones to remove acetyl-histones from the path of elongating Pol II [72, 73]. Furthermore, mass spectrometry studies defining BET partner proteins have suggested that all members of this family can influence chromatin remodeling, methylation, acetylation, and DNA damage response in an acetyl-histone binding-dependent manner [74–76].

An emerging role for BET proteins, specifically BRD4, is in the formation of large, dynamic, cell state-specific enhancers, referred to as super-enhancers (SEs) (Fig. 2). BRD4 disproportionately associates with acetyl-H3K27-containing SEs, which are thought to signal to proximal promoters to stabilize BRD4-containing coactivator complexes near TSSs, and thereby facilitate P-TEFb-mediated Pol II phosphorylation and transcription elongation [77–79].

SEs have also been implicated as signaling hubs for the regulation of enhancer RNAs (eRNAs), which are non-coding RNAs that are transcribed from enhancer DNA by Pol II. Enhancer RNAs are expressed in a signal-dependent manner [80], and regulate gene expression via decondensing chromatin at target promoters, facilitating Pol II recruitment to TSSs of target genes, and promoting direct enhancer/promoter interaction through chromatin looping [81–85]. In keeping with this model, genes that are acted upon by multiple eRNAs have significantly higher expression than eRNA-independent genes [84]. SE-derived eRNAs have been shown to play a critical role in macrophage gene regulation, and expression of these eRNAs is blocked JQ1 (see below), further highlighting a crucial role for BET proteins in SE regulation [86].

Functions for eRNAs in cardiac development and pathological cardiac remodeling are beginning to emerge [87, 88]. For example, in hearts of mice subjected to TAC or MI, expression of eRNAs derived from SEs associated with the genes encoding myocardin, TBX20, and endothelin-1 correlated precisely with production of mRNA transcripts from these genes [89]. In this same report, knockdown of a STAT7-associated eRNA was found to

decrease STAT7 expression in cultured cardiac fibroblasts. Additionally, the long non-coding RNA (lncRNA) MHRT, which has been shown to block pathologic hypertrophy and be downregulated in human heart failure patients, is actually derived from an enhancer within the *Myh7* gene [90]. CARMEN, another SE-derived lncRNA, was shown to be upregulated in a mouse MI model, and in human heart failure [91]. Interestingly, in the mouse genome, CARMEN is located adjacent to another important lncRNA, BVHT (braveheart). Both of these lncRNAs are necessary for cardiac differentiation, and appear to function by binding to SUX12 and EZH2, components of the polycomb repressive complex 2 (PRC2) [91, 92].

SE function has been well-described in endothelial cells, where TNF α treatment leads to NF- κ B-dependent redistribution of BRD4 from basal SEs to *de novo*, stress-activated SEs that promote inflammatory gene activation [77]. However, functions for BRD4-enriched SEs in the control of pathological fibrosis have yet to be described.

Multiple, highly selective small molecule inhibitors of BET bromodomains have been developed [64]. The most well characterized BET inhibitor is JQ1. JQ1 functions by competitively binding BET bromodomains and displacing these acetyl-lysine readers from chromatin, resulting in suppression of downstream signaling events to Pol II [93]. JQ1 was shown to block interstitial cardiac fibrosis and myocyte hypertrophy in mice subjected to TAC [94, 95]. This was accompanied by decreased Pol II activity at pro-fibrotic genes (e.g. α -SMA, PAI-1), as determined by correlating ChIP-Seq evaluation of Pol II elongation with RNA-Seq quantification of mRNA abundance [94].

Small molecule-mediated BET inhibition has also shown promise in models of lung and liver fibrosis. JQ1 was found to blunt PDGF- and TGF- β -stimulated IL-6 secretion, collagen and α -SMA expression, and proliferation and contraction of cultured lung fibroblasts [96]. Inhibition of fibrotic gene expression was also observed upon genetic knockdown of either BRD2 or BRD4, confirming that the effect of JQ1 was due to BET protein inhibition as opposed to an off-target action. Importantly, JQ1 effectively blocked inflammatory cell infiltration and fibrosis in a bleomycin mouse model of idiopathic pulmonary fibrosis [97]. In cultured hepatic stellate cells, which are major drivers of liver fibrosis, JQ1 blocked TGF- β -mediated activation of pro-fibrotic gene expression [98]. Whole-genome analyses implicated the SRF, SMAD3, ETS1 and NF- κ B transcription factors in directing BRD4 to gene regulatory elements for pro-fibrotic genes in stellate cells. *In vivo*, JQ1 was able to reverse pre-existing liver fibrosis in mice exposed to CCl₄; mice were exposed to CCl₄ for three weeks prior to administration of JQ1 for three additional weeks [98].

The mechanistic underpinnings of BET inhibitor-mediated suppression of fibrosis remain unclear. It seems likely that JQ1 and other BET inhibitors will be found to block fibrosis through direct actions on ECM-producing cells (e.g., cardiac fibroblasts and hepatic stellate cells), as well as through indirect actions on other cell types. Regarding indirect actions, inflammation is a key driver of fibrosis [99, 100], and BET inhibitors clearly suppress NF- κ B-dependent activation of pro-inflammatory genes [101]. Studies of isolated cells and tissue-specific knockouts of the genes encoding BET proteins will be required to further delineate the mechanisms by which these reader proteins control pathological fibrosis.

There are currently 14 active or enrolling clinical trials of BET bromodomain inhibitors (www.clinicaltrials.gov), mostly for oncology indications. Bromodomain inhibitors in the clinic include OTX015/MK-8628 (Oncoethix GmbH acquired by Merck), CPI-0610 (Constellation Pharmaceuticals), RVX000222 (Resverlogix Corp), BMS-986,158 (Bristol-Myers Squibb), GSK525762 (GlaxoSmithKline), ABBV-075 (Abbvie), INCB054329 (Incyte Corporation), TEN-010 (Tensha Therapeutic), and BAY 1238097 (Bayer). Only RVX000222 (also known as RVX-208) is being assessed for efficacy in the setting of cardiovascular disease. A Phase III trial is underway with this compound in patients with concomitant type 2 diabetes and coronary artery disease, where the primary endpoint is time to a major adverse cardiovascular event. RVX000222 was previously shown to increase production of APOA1 and HDL, and it was hypothesized that this would lead to decreased atherosclerosis [102]. BET proteins contain tandem bromodomains, and most inhibitors, including JQ1, target both domains. However, RVX000222 selectively targets bromodomain 2 (BD2) of BET proteins [103]. Although both bromodomains of BRD4 can bind acetylated histones, BD1 shows 10-fold higher binding affinity for acetylated histone H4 than BD2 [104]. Similarly, BD2 shows 100-fold higher binding affinity for the acetylated transcription factor NF- κ B over BD1. Thus, it appears that BD2 preferentially governs association of BET proteins with acetylated transcription factors, as opposed to chromatin [105]. Selective inhibition of BD2 results in minimally overlapping and diminished transcriptional effects when compared to inhibition of both BD1 and BD2 with compounds such as JQ1. These results suggest that selective targeting of BD2 may provide a safety profile that is more suitable for chronic therapy, as would be required for the treatment of cardiac fibrosis in heart failure.

5. Histone methylation and fibrosis

Acetylation and methylation of lysine residues in histone tails occurs in a mutually exclusive manner, underscoring the intricacy of the interplay between HATs, HDACs and acetyl-lysine readers with the enzymes and readers that control histone methylation. Addition of up to three methyl groups on specific lysines of nucleosomal histones is governed by histone methyltransferases, which are also referred to as lysine methyltransferases (KMTs). At least thirty three human KMTs are classified into 8 families, KMT1-KMT8. Only KMT4 (DOT1L) lacks the characteristic SET methyltransferase catalytic domain.

DOT1L (KMT4) appears to regulate cardiac hypertrophy and fibrosis. This KMT methylates lysine-79 of histone H3, which is near the H3 and H2B junction, as opposed to the unstructured amino-terminal tail, where most acetylation and methylation sites reside (Fig. 1). Methylation of H3K79 is associated with active gene transcription, and DOT1L mediated H3K79 methylation appears necessary for proper cardiomyocyte development [106]. While global knockout of DOT1L resulted in embryonic lethality, cardiomyocyte-specific deletion of the KMT with an α -MHC-Cre driver led to dramatic and spontaneous cardiac hypertrophy and fibrosis [107]. Consistent with this, DOT1L expression is reduced in failing human heart explants. Since the DOT1L was selectively deleted in cardiomyocytes of mice, it remains unclear whether this KMT directly regulates cardiac fibrosis through activation of resident fibroblasts.

Evidence for direct regulation of fibrosis by KMTs has been provided by studies of other organ systems. Expression of ASH1 (KMT2H), which methylates H3K4, was shown to be elevated in hepatic stellate cells stimulated to undergo myofibroblast differentiation by CCl₄ [108]. Furthermore, this KMT was found to directly bind regulatory elements of pro-fibrotic genes, including those encoding α -SMA, collagen I, tissue inhibitor of metalloproteinase-1 (TIMP1) and TGF- β 1; depletion of ASH1 caused broad suppression of fibrogenic gene expression. In cultured human lung fibroblasts, a small molecule inhibitor of G9a (KMT1C), which methylates H3K9, resulted in restored expression of the anti-fibrotic protein IP-10; derepression of IP-10 occurred through reduction of promoter H3K9 hypermethylation and reduced binding of repressive complexes (HP1, CoREST) to the promoter [109].

SET7/9 (KMT7) has been implicated in TGF- β and high glucose-induced expression of the CTGF, collagen 1A, and PAI-1 genes in a rat mesangial cell model of diabetic nephropathy [110], while inhibition of EZH2 (KMT6), a component of the polycomb repressive complex, resulted in increased collagen release in cultured fibroblasts, and was sufficient to induce fibrosis in mouse dermis [111]. An anti-fibrotic role for EZH2 was further suggested by studies with a small molecule inhibitor of this KMT, termed DZNep [111]. DZNep treatment of cultured skin fibroblasts led to reduced H3K27 methylation at the promoter of the pro-fibrotic transcription factor Fra-2, thus inducing its expression. Furthermore, in mouse models of dermal fibrosis induced by bleomycin or overexpression of TGF- β R1, knockdown of Fra-2 blocked the fibrosis that was induced by DZNep. Cardiac-specific deletion of EZH2 in mice resulted in impaired cardiac development due to decreased H3K27 methylation of regulatory elements for anti-proliferative genes, and consequent inhibition of cardiomyocyte proliferation [112]. In a related study, EZH2 deletion in the heart was shown to derepress Six1 expression in differentiating embryonic cardiac progenitors, leading to abnormal cardiac growth and activation of fibrotic gene expression [113].

There are three clinical trials underway employing KMT inhibitors. The EZH2 inhibitor, EPZ-6438, is being tested in B-cell lymphoma and follicular lymphoma patients, while the DOT1L inhibitor, EPZ-5676, is being evaluated in AML and acute lymphoblastic leukemia patients. Given that EZH2 inhibition and DOT1L disruption are associated with fibrosis in pre-clinical models, it will be interesting to determine if adverse events related to fibrosis are reported in these human studies.

Removal of methyl groups from nucleosomal histones is accomplished by KDMs. More than twenty human KDMs are classed into seven sub-families based on homology. The KDM1 family requires flavin adenine dinucleotide (FAD) as a co-factor for catalytic activity, while JmjC (jumanji) domain-containing demethylase families KDM2/7, KDM3, KDM4, KDM5, and KDM6 are dependent on Fe²⁺/2-oxoglutarate for catalysis [114].

Investigation of KDM activity in fibrosis or fibrosis-related processes has been limited. Expression of JMJD2A/KDM4A was shown to be elevated in human cardiomyopathy, and ectopic expression of this KDM in the heart led to enhanced fibrosis following TAC in mice [115]. In cultured mammary epithelial cells, TGF- β treatment was shown to induce KDM6B expression, and knockdown of this KDM blunted epithelial-to-mesenchymal transition. Conversely, KDM6B overexpression reduced histone methylation at regulatory elements for

pro-fibrotic genes, resulting in enhanced expression of these targets [116]. The H3K9 demethylase JMJD1A was also shown to be important for myofibroblast differentiation of hepatic stellate cells, with knockdown of JMJD1A leading to increased expression of α -SMA and Col1a [117]. Finally, a fatal developmental disease in cattle termed “paunch calf syndrome”, which is characterized by hepatic fibrosis, has been linked to mutations in the histone demethylase, KDM2B [118].

There are only three active clinical trials investigating KDM inhibitors. Two studies are assessing activity of GSK2879552, an inhibitor of LSD1 (KDM1A), in small cell lung carcinoma and acute myeloid lymphoma (AML). The third study is employing tranilcypromine (TCP), an irreversible dual inhibitor of Monoamine-Oxidase (MAO) and LSD1, in patients with AML. LSD1 was recently shown to be a component of a repressive complex containing the NR4A1 orphan nuclear receptor [119]. Formation of this complex on pro-fibrotic gene regulatory elements was found to block TGF- β -mediated gene induction, and limit dermal, lung, liver, and kidney fibrosis in mouse models. As such, it will be important to assess possible pro-fibrotic side effects of LSD1 inhibitors in these human clinical trials.

6. Conclusions

The work highlighted in this review clearly illustrates a crucial function for epigenetic control of gene expression in the pathogenesis of fibrotic disease. However, despite significant advances in our understanding of epigenetic regulation of cardiac fibrosis, major knowledge gaps still exist. The ability to couple state-of-the-art epigenomic technologies (e.g., high resolution, whole genome ChIP-Seq) with pharmacological tool compounds that selectively inhibit specific HATs, HDACs, KMTs, KDMs and reader proteins will undoubtedly reveal novel mechanisms for epigenetic control of cardiac fibrosis and dysfunction. Furthermore, the FDA approval of four HDAC inhibitors for the treatment of cancer (vorinostat, romidepsin, belinostat and panobinostat), and the ongoing clinical evaluation of inhibitors of multiple epigenetic regulators (above), establishes the feasibility of targeting epigenetics to treat human disease.

It is our view that pharmacological manipulation of epigenetic processes with newly described, highly selective inhibitors has the greatest potential to yield fundamental discoveries of fibrotic mechanisms in the heart, and to facilitate clinical translation. Nonetheless, it will be important to complement pharmacology with genetic manipulation of epigenetic regulators in pre-clinical models of pathological cardiac fibrosis, and in cell-based models. Regarding the latter, fibrosis research has long been hindered by the lack of cell-based models that closely recapitulate organ fibrosis *in vivo*. Most *in vitro* models of fibrosis involve culturing fibroblasts from biopsy material or organ explants. However, the high tensile modulus of cell culture plastic results in spontaneous conversion of fibroblasts into myofibroblasts, thus confounding efforts to mimic the agonist-induced fibroblast stimulation that occurs *in vivo* in response to pathological insults. Elastic synthetic polymers and hydrogels that are adjustable with regard to tensile strength have been developed to circumvent this issue [120], and should be applied to studies of epigenetic control of fibroblast activation.

The most straightforward path to clinical evaluation of an epigenetic regulatory compound in humans with cardiac fibrosis would involve “repurposing” a drug that has already proven to be safe in humans in another setting. With a repurposed drug, it would be possible to move directly into a small Phase IIa proof-of-concept (POC) study in patients. However, it will be necessary to determine whether an experimental therapy has “anti-fibrotic activity” in the heart prior to conducting protracted and costly Phase III trials in heart failure patients. We propose that end-stage heart failure patients receiving left ventricular assist devices (LVADs) could be studied in order to determine whether a test therapy is able to blunt fibrosis in the heart (i.e. to establish the pharmacodynamic action of an agent). “Bridge to transplant” LVAD therapy provides a distinctive clinical platform with at least two advantages that would allow for rapid studies of novel anti-fibrotic therapeutics in human heart failure: (1) Bridge to transplant LVAD therapy allows for the collection of human myocardial tissue samples pre- and post-LVAD, thus enabling cellular and molecular analyses of the effects of novel anti-fibrotic interventions; (2) The hemodynamic support platform provided by LVAD therapy, the intense monitoring of LVAD patients, and the anticipated end-result of cardiac transplantation (as early as four months at some centers), allows for some protection against potential un-anticipated negative side effects of new therapeutics, and enables a greater degree of patient safety while conducting early POC studies. Ultimately, the anti-fibrotic therapy would need to be tested in patients with chronic heart failure who are not at end-stage, and who are not scheduled for transplantation. Nonetheless, the LVAD patient studies would provide a valuable gating step, enabling decisions to be made about the long-term potential of a novel anti-fibrotic intervention.

We are in a unique and exciting era of research on epigenetic regulation of fibrosis. Experiments conducted over the next few years have tremendous potential to provide novel insights into the molecular underpinnings of cardiac fibrosis, and to reveal previously unappreciated targets for innovative therapeutic approaches aimed at combating the emerging global health crisis of heart failure.

Acknowledgements

We thank A. V. Ambardekar (UC Denver), S. M. Haldar (Gladstone Institute of Cardiovascular Disease), and Charles Y. Lin (Baylor College of Medicine) for helpful discussions. T.A.M. was supported by NIH (HL116848, HL127240 and AG043822) and the American Heart Association (Grant-in-Aid, 14510001). M.S.S. was funded by a T32 training grant and an F32 fellowship from the NIH (5T32HL007822 and F32HL126354).

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Fig. 1.

Transcriptional consequences of histone H3 tail acetylation and methylation. The amino-terminal tail of histone H3 contains multiple lysine residues that can be acetylated or methylated, including H3K4, H3K9, H3K14, H3K23, and H3K27. Gene activation is indicated with a green circle, while gene repression is indicated with a red hexagon. The impact of methylation on gene expression is based on the generalized conditions of modification with two or three methyl groups (di-, tri-methylated). Methylation of H3K4 is associated with gene activation, while acetylation of this site is linked to both transcriptional activation and repression [121, 122]. The functional consequences of acetylation of H3K79, which is located at the H3-H2B junction of the nucleosome, are not well characterized.

Fig. 2.

A model for control of pro-fibrotic gene expression by BRD4-enriched super-enhancers. Histone acetyltransferases (HATs) and methyltransferases (KMTs) are targeted to gene regulatory elements by interactions with DNA-binding transcription factors (TF), and via intrinsic histone recognition “reader” domains. Acetylation of H3K27, which is governed by HATs such as p300, is a defining feature of super-enhancers. This acetyl-lysine modification creates docking sites for the acetyl-lysine binding protein, BRD4, which facilitates super-enhancer-dependent gene activation through multiple mechanisms (see text). Conversely, methylation of H3K27 by KMTs such as EZH2 leads to assembly of repressive complexes containing methyl-histone reader proteins, including polycomb repressive complex (PRC). Thus, methylation of H3K27 disrupts super-enhancers and promotes gene repression.