

Northumbria Research Link

Citation: Dowson, Christopher and O'Reilly, Steven (2016) DNA methylation in fibrosis. *European Journal of Cell Biology*, 95 (9). pp. 323-330. ISSN 0171-9335

Published by: Elsevier

URL: <http://dx.doi.org/10.1016/j.ejcb.2016.06.003>
<<http://dx.doi.org/10.1016/j.ejcb.2016.06.003>>

This version was downloaded from Northumbria Research Link:
<http://nrl.northumbria.ac.uk/27228/>

Northumbria University has developed Northumbria Research Link (NRL) to enable users to access the University's research output. Copyright © and moral rights for items on NRL are retained by the individual author(s) and/or other copyright owners. Single copies of full items can be reproduced, displayed or performed, and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided the authors, title and full bibliographic details are given, as well as a hyperlink and/or URL to the original metadata page. The content must not be changed in any way. Full items must not be sold commercially in any format or medium without formal permission of the copyright holder. The full policy is available online: <http://nrl.northumbria.ac.uk/policies.html>

This document may differ from the final, published version of the research and has been made available online in accordance with publisher policies. To read and/or cite from the published version of the research, please visit the publisher's website (a subscription may be required.)

www.northumbria.ac.uk/nrl



DNA Methylation in Fibrosis

Christopher Dowson and Steven O'Reilly PhD

Cell Biology group

Faculty of Health and Life Sciences

Northumbria University

Ellison Building

Newcastle Upon Tyne

NE1 3HY

United Kingdom

Email: steven.o'reilly@northumbria.ac.uk

Telephone: 44 1912274780

Abstract

Fibrosis is characterised by an exuberant wound healing response and the major cell type responsible is the myofibroblast. The myofibroblast is typified by excessive ECM production and contractile activity and is demarcated by alpha-smooth muscle actin expression. What has recently come to light is that the activation of the fibroblast to myofibroblast may be under epigenetic control, specifically methylation. Methylation of DNA is a conserved mechanism to precisely regulate gene expression in a specific context. Hypermethylation leads to gene repression and hypomethylation results in gene induction. Methylation abnormalities have recently been uncovered in fibrosis, both organ specific and widespread fibrosis. The fact that these methylation changes are rapid and reversible lends themselves amenable to therapeutic intervention. This review considers the role of methylation in fibrosis and the activation of the myofibroblasts and how this could be targeted for fibrosis. Fibrosis is of course currently intractable to therapeutics and is a leading cause of morbidity and mortality and is an urgent unmet clinical need.

Introduction

Fibrosis is characterised by an accumulation of extracellular matrix (ECM) proteins, particularly type I and type II collagen (Iredale, 2007). This accumulation results in a hardening of the tissue, similar to what you would see as a result of scar tissue formation during the process of wound healing (Wynn, 2008), however, in this case it is pathological and fibrosis of an organ will impair its function (Zeisberg and Kalluri, 2013). In fibrotic tissues, myofibroblasts are the primary contributors to the excessive production of ECM proteins regardless of the aetiology (Zeisberg and Kalluri, 2013). The fibrosis can be organ specific as in liver fibrosis or more widespread as in systemic sclerosis (SSc). Whether fibrosis is organ specific or diffuse it is a major cause of morbidity and mortality. Currently, there is no specific treatment and a deeper understanding of the activation of the myofibroblast is required. It is only recently that the molecular mechanisms of the switch between fibroblast and myofibroblast have come to light.

Myofibroblasts

Myofibroblasts produce ECM proteins such as collagen and ED-A fibronectin. However, the defining characteristic of the myofibroblast is its expression of α -smooth muscle actin (α -SMA); this gives the cell contractility, which allows the cell to aid in the process of wound healing by actively pulling the two edges of a wound towards each other (Hinz *et al.*, 2007).

In a tissue that has been damaged, fibroblasts will differentiate into myofibroblasts and contribute to the repair of the damage (Hinz *et al.*, 2007). Persistent injury leads to the formation of scar tissue and fibrosis. Myofibroblasts can also arise from epithelial cells through a process known as epithelial-mesenchymal transition (EMT) (Wynn, 2008); in the liver, hepatic stellate cells (HSCs) are the major cell type from which myofibroblasts originate (Page *et al.*, 2015). The differentiation of HSCs to myofibroblasts is preceded by a specific set of gene changes and it is alteration in gene methylation that drives this process (Mann *et al.*, 2010). Methylation is an epigenetic modification that can spatially and temporally regulate gene expression. This review aims to examine the role of methylation in myofibroblasts and how this impacts fibrosis.

Pathogenesis of Fibrosis

Fibrosis can be triggered by injury to a tissue; this can be caused by toxicity, autoimmunity, the effect of cytokines, infection or trauma. Regardless of the cause, fibrosis arises when there is a failure of an injury to achieve resolution, thus leading to an accumulation of fibroblasts and overproduction of ECM proteins that can inhibit the function of organs. This loss of organ function can be fatal.

When there is damage to a tissue, resident fibroblasts (and some epithelial cells) adopt a myofibroblast phenotype and, through their contractility and secretion of ECM proteins, aid in wound repair. In fibrosis, the accumulation of myofibroblasts and ECM production becomes excessive and is also accompanied by damage to parenchymal tissues, changes to the microvasculature resulting in reduced perfusion and infiltration of mononuclear cells. Fibrosis can result in the loss of organ function as the resident parenchymal cells are replaced by myofibroblasts and ECM (Zeisberg and Kalluri, 2013).

Methylation

Methylation of deoxyribonucleic acid (DNA) is the process of adding a methyl group to a nucleotide within the DNA. This methyl group is commonly donated by S-adenosyl-L-methionine (AdoMet) and its transfer is facilitated through the action of the DNA methyltransferase (DNMT) family of enzymes (Cheng and Roberts, 2001). Dietary sources of methyl donors (e.g. folate) are important, with deficiency resulting in hypomethylation (Duthie *et al.*, 2000). In humans, four DNMT enzymes have been identified (see Table 1).

The methylation of DNA occurs predominantly at CpG dinucleotides, where a cytosine and guanine are separated by a single phosphate. The methylation occurs specifically on the 5th Carbon atom of the cytosine ring (Meehan *et al.*, 1992), and is then referred to as 5-methylcytosine (5mC) (see figure 1); DNMT3a and DNMT3b both methylate DNA in this manner (Cheng and Roberts, 2001). Reports on the degree of CpG island methylation vary, but numerous sources show it to be somewhere between 60-90% (Meehan *et al.*, 1992; Robertson *et al.*, 1999).

The greatest frequency of CpG sequences is seen in CpG islands, sequences of repeating C and G nucleotides that are typically around 1000 base pairs in length. Approximately 70% of genes have a CpG island in their promotor region (Deaton and Bird, 2011).

DNMT3 and DNMT1 act synergistically, there is evidence to show that methylation by DNMT3 stimulates DNMT1 to further methylate the DNA, in one study this resulted in a fivefold increase in methylation activity over that of DNMT3 alone (Fatemi *et al.*, 2002). Alone, DNMT1 is recruited to sites of DNA where it acts to maintain methylation (Mortusewicz *et al.*, 2005). It also plays a role in DNA replication and knockdown of DNMT1 causes an arrest of intra-S phase DNA replication. It is thought that this mechanism acts to prevent demethylation that would arise from DNA replication in and absence of DNMT1 (Milutinovic *et al.*, 2003). DNMT3L has also been shown to cause a 20-fold increase to the catalytic activity of DNMT3a and DNMT3b (Gowher *et al.*, 2005; Karetta *et al.*, 2006).

The methylation and demethylation of DNA is a form of epigenetic modification; a stable, inherited modification to DNA that does not alter the sequence, but results in a change in gene expression (Ciechomska *et al.*, 2014). DNA methylation is critically involved in X chromosome inactivation and the silencing of retroviral elements of the genome (Moore *et al.*, 2013). Methylation of DNA can result in gene repression (Mohn *et al.*, 2008) and hypomethylation of DNA is associated with elevated gene expression (Karouzakis *et al.*, 2009), however methylation does not always cause a change in expression. Gene repression is achieved through the action of methyl binding proteins such as methyl-Cap binding protein 2 (MeCP2)(Nan *et al.*, 1998), and other members of the methyl-CpG binding domain (MBD) family, which all share this domain, allowing them to bind to methylated-CpG sequences (Hendrich and Tweedie, 2003). Repression of a gene is dependent on the density of the methylation, i.e. how frequently methylated-CpG sequences occur within the gene, and also on the location of the methylation in relation to the promoter region of the gene (Bian *et al.*, 2013; Hsieh, 1994)

Epigenetic changes have been linked to many diseases including cardiovascular disease (Webster *et al.*, 2013); as well as colon, lung, breast and thyroid cancer, where large hypomethylated blocks of the genomes have been found (Hansen *et al.*, 2011) and promoter hypermethylation of classic tumour suppressor genes are found. More recently, methylation changes have been associated with

fibrotic diseases such as hepatic, pulmonary, renal and cardiac fibrosis (Mann *et al.*, 2010; Rabinovich *et al.*, 2012; Tampe *et al.*, 2014; Xu *et al.*, 2015). Numerous genes have been found to be involved in this association and those referred to in this review are listed in Table 2.

Komatsu *et al.* also found hypomethylation in the early stages of liver fibrosis. Global hypomethylation was observed, along with hypomethylation and upregulation of secreted phosphoprotein 1 (Spp1), a gene which induces inflammation. In addition, α Sma, Col1a2, and Timp1 genes were also found to be hypomethylated and upregulated (Komatsu *et al.*, 2012). The upregulation of these genes, which are closely linked to fibrosis, coinciding with upregulation of Spp1 suggest that Spp1 may also play a role in the processes of liver fibrosis. Interestingly it appears TIMP-1, a major fibrotic inducer is regulated by methylation as it can be modified by decabine treatment (Vincent *et al.*, 2015).

In tissue samples from patients with idiopathic pulmonary fibrosis (IPF), it was found that 625 CpG islands were differentially methylated when compared to healthy tissue samples, with 91.2% of them being in intronic, exonic or intergenic regions and 8.8% of them in promoter regions. Many of these differentially methylated CpG islands were found to be in genes involved in apoptosis, cell morphogenesis, regulation of cellular biosynthetic processes, histone acetylation, cellular growth and proliferation, cell morphology, cancer and cell signaling. The IPF samples were then compared to samples from lung cancer patients; the lung cancer samples were found to have 2428 differentially methylated CpG islands when compared to healthy tissue samples. 65% of the differentially methylated CpG islands methylated in IPF samples were also seen in the lung cancer samples (Rabinovich *et al.*, 2012).

Methylation of the promoter region of the Thy-1 gene is associated with the myofibroblast phenotype in the lung, where expression of Thy-1 ceases and α -SMA expression is acquired. Using CCD19Lu lung fibroblasts, Robinson *et al.* found that hypoxia could induce global hypermethylation and that Thy-1 mRNA expression was suppressed. When the methylation was reversed with 5-aza-2'-deoxycytidine, Thy-1 expression was restored. This provides evidence that methylation alterations can influence myofibroblast differentiation (Robinson *et al.*, 2012). It has also been shown in idiopathic lung fibrosis that there is hypermethylation of the p14^{ARF} promoter compared to controls, leading to reduced expression. P14^{ARF} is a pro apoptotic protein, thus, its down regulation promotes resistance to apoptosis in IPF (Cisneros *et al.*, 2012).

In SSc, a prototypical fibrotic disease, in which the skin and lung are fibrotic, promoter hypermethylation of DKK1 and SFRP1 is found in myofibroblasts. This hypermethylation could be reversed with 5-Aza'C and, in a mouse model of fibrosis, pre-treatment with 5-Aza'C ameliorated fibrosis. This occurs because DKK1 and SFRP1 are Wnt antagonists; therefore, a reduction in their levels leads to unabated Wnt signalling, which is known to drive fibrosis (Dees *et al.*, 2014).

Furthermore, there has been described global and gene specific changes in dermal fibroblasts from SSc patients, including hypomethylation in RUNX genes (Altorok *et al.*, 2015). This is particularly interesting as both RUNX1 and RUNX2 have been shown to regulate tissue inhibitor of metalloproteinases 1 (TIMP-1) (Bertrand-Philippe *et al.*, 2004), which has a critical role in systemic sclerosis. A recent study in keloid fibroblasts, which are similar to SSc fibroblasts, with an abundance of ECM molecules, showed massive changes in global methylation with the majority being hypomethylated and 26% being hypermethylated (Jones *et al.*, 2015).

Altorok *et al.* also found numerous differentially methylated sites in SSc fibroblasts when compared to healthy controls. In diffuse cutaneous SSc, 2710 differentially methylated CpG sites were identified, with 61% being hypomethylated, and in limited cutaneous SSc, 1021 differentially methylated CpG sites were identified, with 90% being hypomethylated. Out of the differentially methylated sites in diffuse cutaneous SSc and limited cutaneous SSc, only 203 CpG sites were common between the two forms of the disease. These common sites of differential methylation between the two forms of the disease are likely key in the processes of fibrosis. In addition to the hypomethylation of the RUNX genes, COL23A1 and COL4A2, encoding collagens and MYO1E, encoding myosins, were also hypomethylated in both diffuse cutaneous SSc and limited cutaneous SSc (Altorok *et al.*, 2015).

Hypermethylation of the FLI1 promoter region has also been described in myofibroblasts from SSc patients, thus leading to its repression. FLI1 is a collagen suppressor gene and a negative regulator of fibrosis which is reduced in SSc patient's cells; in normal fibroblasts, reduction of FLI1 via siRNA results in excessive ECM production. SSc fibroblasts were also found to contain higher levels of DNMT1, histone deacetylase (HDAC)-1, HDAC-6, methyl-CpG DNA binding domain (MBD)-1 and MeCP2 than control fibroblasts, which is indicative of the epigenetic modifications occurring within them (Wang *et al.*, 2006).

Demethylation

DNA methylation marks are very stable and can be transmitted through generations, but they are not permanent and can be erased. The ten-eleven translocation (TET) family of proteins are responsible for the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC) (Zhao *et al.*, 2014), and whilst 5mC is typically associated with gene silencing effects, 5hmC is associated with an increase in gene expression (Coppieters *et al.*, 2014). In addition, it has been found that TET can also convert 5mC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito *et al.*, 2011). These alterations made to 5mC by TET are intermediate steps in the process of DNA demethylation; this can occur through the oxidation of 5mC and 5hmC into 5caC, which is then excised by thymine-DNA glycosylase, a base excision enzyme. From the current evidence, it is thought that this results in the initiation of the base-excision repair pathway, replacing what was originally 5mC with a non-methylated cytosine (He *et al.*, 2011; Maiti and Drohat, 2011). There is also evidence that activation-induced deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC) deaminases promote demethylation of 5hmC (Guo *et al.*, 2011). TET can also cause DNA demethylation in a passive manner, DNMT1 shows lower activity at hemi-5hmC sites, meaning that the methylation cannot be maintained throughout the process of cell division (Valinluck and Sowers, 2007). TET3 has recently been shown to regulate activation of hepatic stellate cells (HSCs) into myofibroblasts, silencing of TET3 was found to promote HSC activation and resulted in the downregulation of HIF-1A-AS1, a long non coding RNA; in addition, silencing of HIF-1A-AS1 was found to promote proliferation and reduce apoptosis in LX-2 cells (Zhang *et al.*, 2014).

It was recently shown that HSC transdifferentiation was accompanied by alteration in DNA methylation patterns; and that a decrease in DNMT3b resulted in a pro-fibrogenic phenotype in activated HSCs (Page *et al.*, 2015). In human livers affected by primary sclerosing cholangitis (PSC), where there is fibrosis of the intrahepatic bile ducts, it was shown that there is an increase in TETs

(demethylating enzymes) and DNMTs; however hepatic protein expression of TET2 and TET3 ceased and there was also an induction of DNMT3b expression. Similar findings were also obtained from livers effected by alcoholic liver disease, although there was a reduction in TET2 expression (Page *et al.*, 2015). It is suggested that global and gene specific methylation changes precede the differentiation of the stellate cell. While Page *et al.* found a more methylated genome associated with liver myofibroblast activation, another group, Gotze *et al.*, found *in vitro* that early activation of stellate cells was associated with global hypomethylation (Gotze *et al.*, 2015). These conflicting results could be a result of different genes having their methylation patterns altered; for example, hypomethylation of pro-fibrotic genes and hypermethylation of anti-fibrotic genes could both result in fibrosis. However, it also possible that this could simply be caused by the passage number of the cells that were used in each study, as it has been shown that methylation patterns can change with passage (Shmookler Reis and Goldstein, 1982).

A gene specific hypermethylation occurs in phosphatase and tension homolog deleted on chromosome 10 (PTEN), a negative regulator of HSC activation. Hypermethylation of the PTEN promoter results in lessened expression, as well as enhanced phospho-extracellular-signal-regulated kinase (p-ERK) signalling, which is important in mediating fibrosis. Treatment with 5-aza-2'-deoxycytidine (5-Aza'C), a DNMT inhibitor, was found to decrease aberrant hypermethylation of the PTEN promoter, preventing the loss of expression seen in HSC activation. Furthermore, 5-Aza'C treatment prevented transforming growth factor- β (TGF- β)-mediated upregulation of Col1a1 and α -smooth muscle actin (α -SMA) mRNA as well as α -SMA protein (Bian *et al.*, 2012).

Bone morphogenetic protein endothelial cell precursor-derived regulator (BMPER), a member of the TGF- β superfamily, is an important regulator of fibroblast activation. It was found that BMPER is highly expressed in IPF lung fibroblasts, more so than in healthy lung fibroblasts. Demethylation using 5'-azacytidine resulted in a decrease in BMPER expression in both healthy and IPF fibroblasts; this shows that the expression of BMPER, and therefore fibroblast activation, is regulated through methylation and demethylation. Treatment with 5'-azacytidine to cause demethylation was also found to decrease expression of Collagen 1, hyaluronan and hyaluronan synthase 2 (Huan *et al.*, 2015). Hyaluronan is a polysaccharide component of the ECM (Laurent and Fraser, 1992) whilst hyaluronan synthase 2 is the enzyme which produces it (Jacobson *et al.*, 2000). It can therefore be established that a decrease in BMPER expression appears to result in reduced production of ECM components by lung fibroblasts. This decrease in ECM components also suggests a decrease in fibroblast migration as this is facilitated by the ECM (Huan *et al.*, 2015).

Angiogenic factor with G patch and FHA domains 1 (Aggf1) expression has been found to be suppressed in HSCs and downregulated in liver fibrosis. This suppression of Aggf1 expression was found to be a result of changes in DNA methylation, and treatment with 5'-azacytidine restored expression. Inducing overexpression of Aggf1 was shown to alleviate liver fibrosis both in mice and in HSC cultures. Aggf1 regulates liver fibrosis by binding to the inhibitory SMAD7 protein, this results in reduced binding of SMAD3 to gene promoters and subsequent inhibition of fibrogenesis. When SMAD7 was knocked down, HSC activation was restored (Zhou *et al.*, 2016). This again shows another gene that can induce fibrosis as a result of modification to DNA methylation.

MeCP2

MeCP2 is also of particular interest in fibrosis as there is evidence implicating it in the regulation of genes that play a vital role in the process (Bian *et al.*, 2013). MeCP2 is able to bind to a single methylated CpG dinucleotide and shows weaker binding to methylated non-CpG sequences; MeCP2 binding to methylated CpGs can result in gene repression, however, its suppressive capabilities rely on HDAC1 (Nan *et al.*, 1998). MeCP2 recruits HDAC1 to methylated DNA where it regulates heterochromatic association through interaction with a protein responsible for heterochromatic packaging, Heterochromatin protein-1 (Pandey *et al.*, 2015). Hemi-methylated DNA has been shown to be a poor substrate for MeCP2, meaning there will be less repression (Meehan *et al.*, 1992). It has been found in mouse models that MeCP2 also plays a role in gene activation. MeCP2-null mice had around ~85% of dysregulated genes in their hypothalamus downregulated (2184 out of the 2582 genes). At the site of activated gene promoters, MeCP2 is associated with CREB1, a transcriptional activator (Chahrour *et al.*, 2008).

One way in which MeCP2 has been implicated in the pathogenesis of fibrosis is through its ability to positively regulate expression of the enzyme absent, small, or homeotic disc1-like (ASH1L). ASH1L can methylate H3K4 (histone 3, lysine 4), which functions to upregulate the expression of certain genes, including α -SMA, collagen, TIMP1 and transforming growth factor- β 1 (TGF- β 1) (Bian *et al.*, 2013). These are key proteins associated with the myofibroblast phenotype.

In lung fibroblasts from mice with bleomycin-induced pulmonary fibrosis, Hu *et al.* found that MeCP2 binds to CpG islands in the α -SMA gene, specifically in the promoter region, intron 1 site 1 and intron 1 site 2; binding occurred regardless of whether they were methylated or unmethylated. However, MeCP2 showed a much higher affinity for the methylated promoter region and methylated intron 1 site 2. When over expression of MeCP2 was stimulated using a plasmid, a resulting rise in α -SMA expression was seen. siRNA induced repression of MeCP2 resulted in inhibition of α -SMA, even when treated with TGF- β , which normally stimulates α -SMA expression. This indicates that MeCP2 acts as an activator of α -SMA expression. However, it was also found methylation of the α -SMA promoter region inhibited the stimulatory effect of MeCP2 (Hu *et al.*, 2011).

The same study also found that mice deficient in MeCP2 showed lesser levels of fibrosis when treated with bleomycin than was seen in the wild-type (Hu *et al.*, 2011). In systemic sclerosis we have also seen elevated levels of MeCP2 in fibroblasts (O'Reilly *et al.*, 2016). We also showed that MeCP2 is induced in normal fibroblasts by the addition of TGF- β . Thus, it is clear that MeCP2 plays a role in fibrosis through regulation of gene expression.

TGF- β

TGF- β is also closely involved in the processes of fibrosis and plays a major role in the mediation of fibrosis. Fibroblasts that have been exposed to TGF- β express high levels of connective tissue growth factor (CTGF) and that TGF- β directly activates transcription of CTGF (Grotendorst *et al.*, 1996).

TGF- β has been linked to an increase in the expression of COL1A1 in hepatic stellate cells (HSCs) (Garcia-Trevijano *et al.*, 1999) and also COL1A2 in primary human fibroblasts (Chen *et al.*, 1999). It has also been shown that TGF- β stimulates fibroblast differentiation into the myofibroblast

phenotype, mediated by extracellular-signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK). This change in phenotype coincides with numerous changes to gene expression (Ding *et al.*, 2008). In cells from neonatal Sprague-Dawley rats, TGF- β induced upregulation of COL1A1 was also accompanied by decreased expression of DNMT1 and DNMT3a. This resulted in a decrease in the methylation levels at multiple CpG sites in the COL1A1 promoter (Pan *et al.*, 2013). However, Bechtel *et al.* found DNMT1 expression was induced by TGF- β (Bechtel *et al.*, 2010) but again this may simply be caused by the passage number of the cells used in each study; alternatively it may be caused by differences in the type of cells used, since Pan *et al.* used cardiac cells and Bechtel *et al.* used renal cells and it has been established the methylation patterns vary in different tissues and individuals (Zhang *et al.*, 2013).

TGF- β also induces changes to methylation during EMT. Exposure to TGF- β resulted in an increase in the average methylation values of around 500 CpG islands located in or near gene promoters. Withdrawal of TGF- β stimulation resulted in methylation levels returning to their pre-TGF- β level. This suggests that continuing stimulation by TGF- β is required throughout the process of EMT (Cardenas *et al.*, 2014). Thus, TGF- β can induce both hypermethylation and hypomethylation, illustrating the complexities of the pathways that control and alter methylation patterns. Interestingly, treatment with 5-aza-2'-deoxycytidine abrogates the effects of TGF- β induced myofibroblasts in cardiac cells (Watson *et al.*, 2014).

Micro RNAs

Micro RNAs (miRNAs) are small, non-coding RNAs that are typically around 22-25 nucleotides in length. Their role is to mediate cleavage of messenger RNAs (mRNAs), destabilise mRNAs or repress translation, resulting in the regulation of gene expression. Many of these miRNAs regulate genes that are either pro- or anti-fibrotic; this is achieved through imperfect binding to the 3' untranslated region (UTR) and the 5' UTR of their target mRNAs (O'Reilly, 2016). It is now appreciated that while miRNAs are regulating gene expression, they themselves are regulated by methylation and cross talk occurs between the two.

MiRNA29 is closely linked to fibrogenesis and decreased levels have been associated with fibrosis of the heart, liver, kidney and skin as well as SSc. MiRNA29 has been shown to target the DNMT family, and ultimately results in abnormal methylation. Therefore, a reduction in miRNA 29 could result in hypermethylation of genes that regulate fibrosis. In idiopathic pulmonary fibrosis, there is a reduction in the levels of the miRNA-17-92 cluster. DNMT1 is a target gene for the miRNA-17-92 cluster, so a reduction in miRNAs of this cluster results in increased DNMT1 levels. Transfection of these miRNAs caused a reduction in DNMT1 levels and resulted in globally reduced DNA methylation (O'Reilly, 2016).

Peroxisome proliferator-activated receptor- γ

Peroxisome proliferator-activated receptor- γ (PPAR- γ) has been inversely related to expression of collagen and is a critical regulator of liver fibrosis; it has been suggested as a 'master regulator' (Ghosh *et al.*, 2008).

MeCP2 has been shown to downregulate expression of PPAR- γ (see figure 2) through the promotion of methylation of H3K9 (histone 3, lysine 9) and enhancer of Zeste homolog 2 (EZH2) mediated methylation of H3K27 (histone 3, lysine 27), this allows for the chromatin silencer Heterochromatin Protein 1 α (HP1 α) to interact with the 5' end of the PPAR- γ gene (Mann *et al.*, 2010). This decrease in expression of PPAR- γ ultimately results in reduced inhibition of collagen synthesis and shows that MeCP2 has a critical role in the differentiation of liver myofibroblasts.

In rats, it has been found that ancestral liver damage led to adaptations in their offspring, making them more resistant to liver fibrosis. These adaptations were largely achieved through alterations to DNA methylation and histone deacetylation and resulted in an increase in PPAR- γ , which acts to impede fibrosis, as well a decrease in expression of profibrogenic TGF- β 1 and decreased myofibroblast generation. Crosslinked qChIP analysis on sperm from these rats showed greater levels of H2A.Z and H3K27me3 incorporation into the chromatin at the PPAR- γ promoter (Zeybel *et al.*, 2012). However, how this occurs is not fully known and the mediators of the modulation of the sperm remain to be identified. These acquired and inheritable changes to gene expression reinforce the major role of epigenetic alterations in the processes of fibrosis. Of major interest in this study is the fact that in liver fibrosis patients, methylation at the PPAR- γ promoter could discern those with mild and severe liver fibrosis. It was found that those with severe liver fibrosis had higher levels of PPAR- γ promoter CpG methylation than those with mild fibrosis. This could underpin why some people progress to severe liver fibrosis and others have a more stable, mild disease. It has been known for some time that only a proportion of heavy drinkers develop alcoholic liver disease and this differential methylation may explain why. It has been shown in liver fibrosis that regression can occur if the damaging agent is removed. Therefore factors that promote regression of fibrosis are of interest. This is associated with reduction of TIMPs and induction of MMPs which will have a net effect of breaking down the collagenous scar. It may be in the case of myofibroblast regression that the cells have a reversal of their differentially methylated genes that promotes the reversal. This is yet to be proved experimentally but is an attractive hypothesis. As further research into TETs mediating the demethylation of DNA progresses this will be an interesting area.

Conclusion

Epigenetic changes, primarily methylation, and MeCP2 are intrinsic components in the process of fibrosis. With the evidence presented here showing global alterations to methylation, it seems that this may be one of the primary driving forces behind fibrogenesis and, in combination with MeCP2, HDAC1 and miRNAs, results in changes of expression of critical genes such as collagen, α -SMA, TIMP-1 and TGF- β . The evidence suggests that there is cross talk between methylation and other epigenetic modifications including miRNA, thus adding to the complexity found in myofibroblast activation and fibrogenesis.

Treatment of fibrosis remains to be a challenge; drugs that demethylate DNA, such as 5-Aza'C, may be repurposed for treatment of fibrosis. In cancer cell lines, treatment with the DNMT inhibitors azacytidine and decitabine was found to cause demethylation of genes in a non-random pattern, however, some CpGs appeared to have a resistance to drug-induced demethylation. This resistance was not seen in DNMT1, DNMT3b double knockout cells, indicating that the methylation of resistant CpGs is being maintained by these enzymes. It was also found that whole genome demethylation was higher than gene specific demethylation (Hagemann *et al.*, 2011).

Hydralazine, a vasodilator used to treat hypertension, has also been found to inhibit fibrosis. In C57BL/6 mice with renal fibrosis induced by unilateral ureteral obstruction, treatment with hydralazine produced lessened fibrosis compared to untreated controls and coincided with a reduced accumulation of fibroblasts. These results were mirrored in *CD1* mice which received a dose of folic acid to induce renal fibrosis. It was also found that hydralazine treatment caused a reduction in *Rasa1* methylation, caused by a reduction of DNMT1 expression and an induction of TET3-mediated hydroxymethylation (Tampe et al., 2015). In HL-1 cells derived from mouse atrial cardiomyocytes, hydralazine also caused a decrease in methylation of the SERCA2 promoter by blocking DNMT1 (Kao et al., 2011).

Therefore, one of the greatest challenges in developing a demethylating treatment for fibrosis is ensuring that the desired genes are targeted. Whilst globally reducing methylation levels may potentially alleviate fibrosis, it could also have unintended side effects. To effectively develop and apply a demethylating drug for the treatment of fibrosis is likely going to require a more complete understanding of the processes and interactions between methylation patterns, miRNAs, the MBD family of proteins and HDACs as well as a deeper understanding of the genes involved. In the future, cell-free DNA methylation may possibly be used as a biomarker for fibrotic disease.

Tables

Table 1. - Enzymes of the DNA methyltransferase family that methylate the 5th carbon of cytosine.

<u>Family</u>	<u>Function</u>
DNMT1	Preferentially methylates hemimethylated DNA. Maintains methylation.
DNMT2	Methylates cytosine 38 in the anticodon loop of aspartic acid transfer RNA.
DNMT3a	Add methyl groups to DNA.
DNMT3b	
DNMT3L	Associates with DNMT3a & Dnnt3b and has been shown to increase catalytic activity.
TET1	Oxidise 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine.
TET2	
TET3	
References: (Cheng and Roberts, 2001; Fatemi et al., 2002; Goll et al., 2006; Karetka et al., 2006; Loenarz and Schofield, 2009)	

Table 2. - Differentially methylated genes confirmed in fibrosis.

Gene	Methylation change	References
<i>COL23A1</i> <i>COL4A2</i> <i>MYO1E</i> <i>RUNX</i>	Hypomethylated	Altorok <i>et al.</i> , 2015
α Sma <i>COL1A2</i> <i>Spp1</i> <i>Timp1</i>	Hypomethylated	Komatsu <i>et al.</i> , 2012
<i>COL1A1</i>	Hypomethylated	Pan <i>et al.</i> , 2013
<i>Rasa1</i>	Hypermethylated	Bechtel <i>et al.</i> , 2010
<i>PTEN</i>	Hypermethylated	Bian <i>et al.</i> , 2012
<i>P14^{ARF}</i>	Hypermethylated	Cisneros <i>et al.</i> , 2012
<i>DKK1</i> <i>SFRP1</i>	Hypermethylated	Dees <i>et al.</i> , 2014
<i>BMPER</i>	Hypermethylated	Huan <i>et al.</i> , 2015
<i>Thy1</i>	Hypermethylated	Robinson <i>et al.</i> , 2012
<i>FLI1</i>	Hypermethylated	Wang <i>et al.</i> , 2006
<i>Aggf1</i>	Hypermethylated	Zhou <i>et al.</i> , 2016

Figures

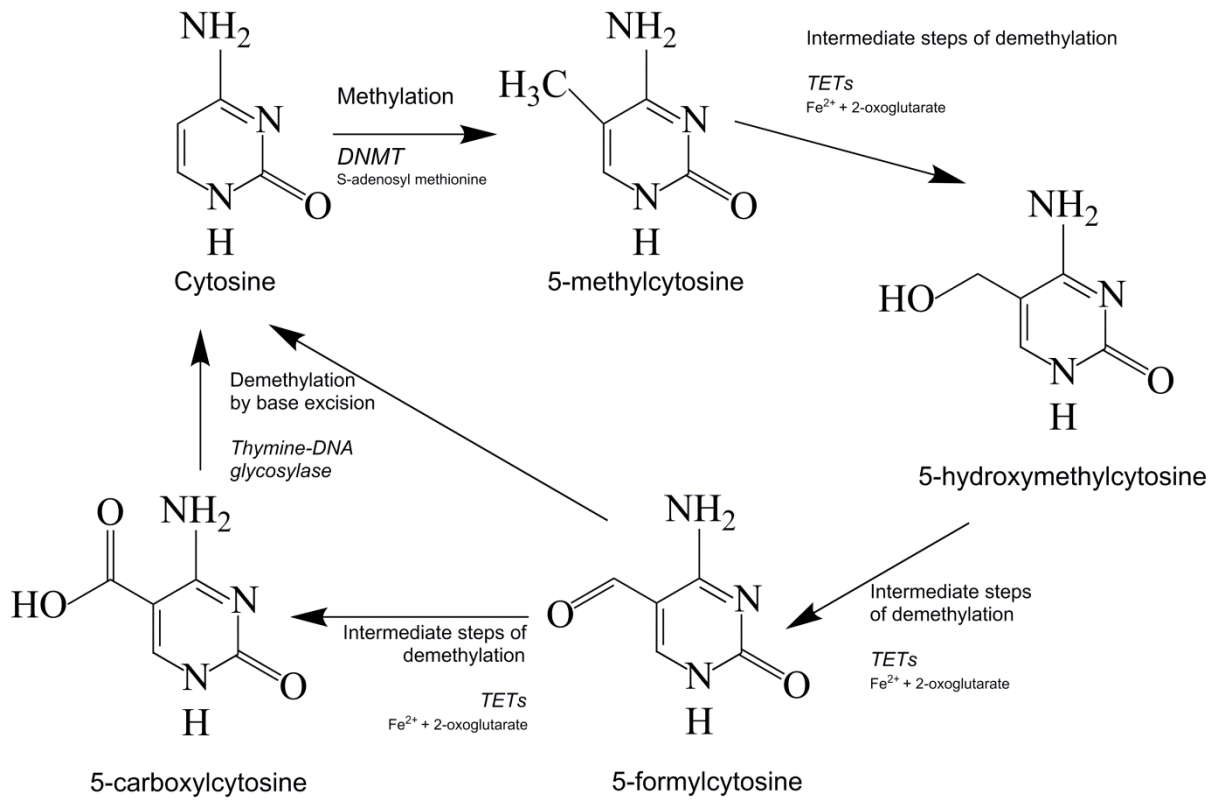


Figure 1 Methylation of the cytosine residue and hydroxymethylation. This is mediated by DNMTs and TETs.

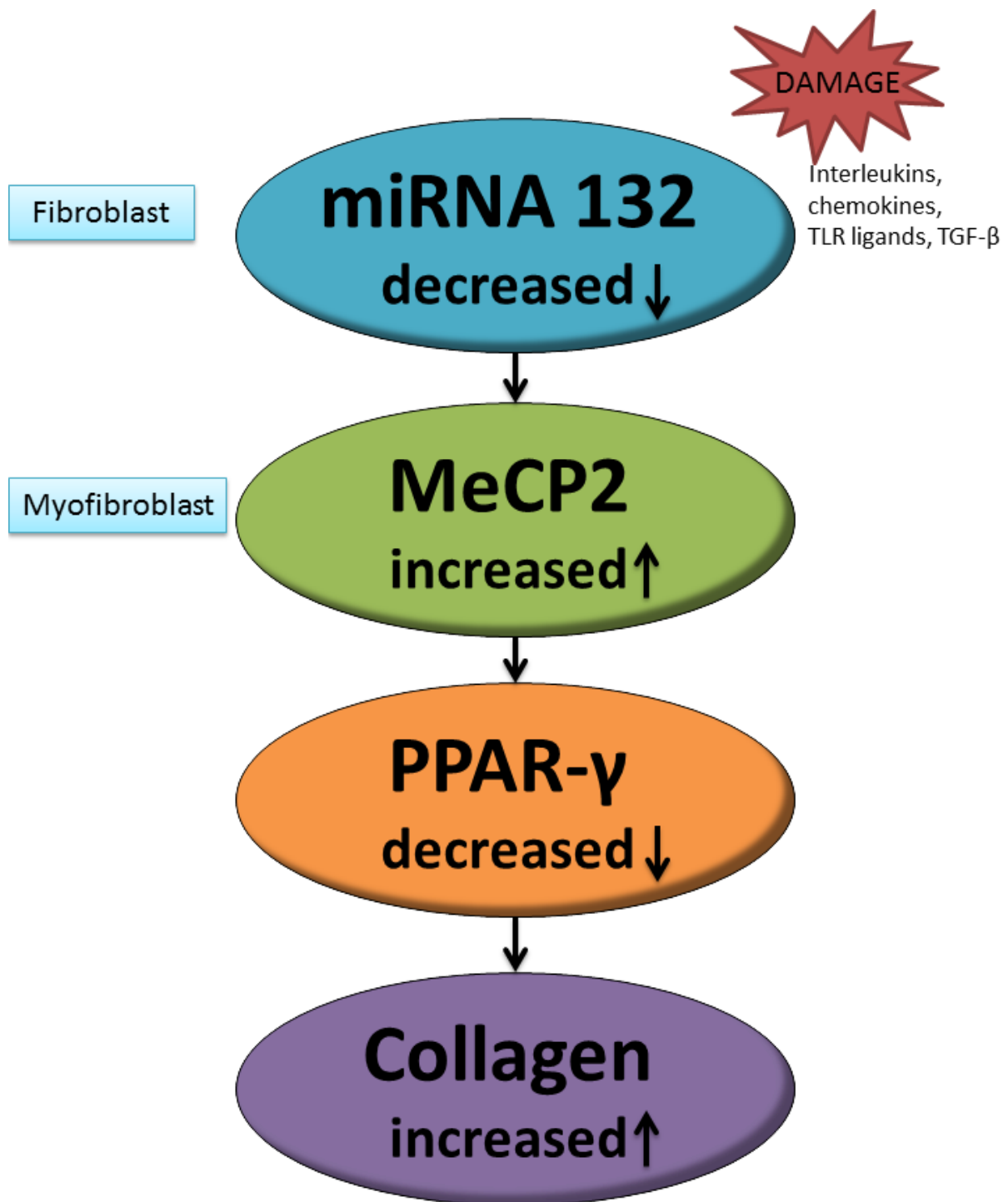


Figure 2 A simple depiction of collagen regulation, decreased levels of miRNA132 results in an increase in MeCP2 expression. MeCP2 then downregulates PPAR- γ expression ultimately causing increased collagen synthesis.

References

- Altorko, N., Tsou, P.S., Coit, P., Khanna, D., Sawalha, A.H., 2015. Genome-wide DNA methylation analysis in dermal fibroblasts from patients with diffuse and limited systemic sclerosis reveals common and subset-specific DNA methylation aberrancies. *Ann Rheum Dis* 74, 1612-1620.
- Bechtel, W., McGoohan, S., Zeisberg, E.M., Muller, G.A., Kalbacher, H., Salant, D.J., Muller, C.A., Kalluri, R., Zeisberg, M., 2010. Methylation determines fibroblast activation and fibrogenesis in the kidney. *Nat Med* 16, 544-550.
- Bertrand-Philippe, M., Ruddell, R.G., Arthur, M.J., Thomas, J., Mungalsingh, N., Mann, D.A., 2004. Regulation of tissue inhibitor of metalloproteinase 1 gene transcription by RUNX1 and RUNX2. *The Journal of biological chemistry* 279, 24530-24539.
- Bian, E.B., Huang, C., Ma, T.T., Tao, H., Zhang, H., Cheng, C., Lv, X.W., Li, J., 2012. DNMT1-mediated PTEN hypermethylation confers hepatic stellate cell activation and liver fibrogenesis in rats. *Toxicol Appl Pharmacol* 264, 13-22.
- Bian, E.B., Huang, C., Wang, H., Chen, X.X., Tao, H., Zhang, L., Lv, X.W., Li, J., 2013. The role of methyl-CpG binding protein 2 in liver fibrosis. *Toxicology* 309, 9-14.
- Cardenas, H., Vieth, E., Lee, J., Segar, M., Liu, Y., Nephew, K.P., Matei, D., 2014. TGF-beta induces global changes in DNA methylation during the epithelial-to-mesenchymal transition in ovarian cancer cells. *Epigenetics* 9, 1461-1472.
- Chahrour, M., Jung, S.Y., Shaw, C., Zhou, X., Wong, S.T., Qin, J., Zoghbi, H.Y., 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* 320, 1224-1229.
- Chen, S.J., Yuan, W., Mori, Y., Levenson, A., Trojanowska, M., Varga, J., 1999. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. *The Journal of investigative dermatology* 112, 49-57.
- Cheng, X., Roberts, R.J., 2001. AdoMet-dependent methylation, DNA methyltransferases and base flipping. *Nucleic acids research* 29, 3784-3795.
- Ciechomska, M., van Laar, J.M., O'Reilly, S., 2014. Emerging role of epigenetics in systemic sclerosis pathogenesis. *Genes Immun* 15, 433-439.
- Cisneros, J., Hagood, J., Checa, M., Ortiz-Quintero, B., Negreros, M., Herrera, I., Ramos, C., Pardo, A., Selman, M., 2012. Hypermethylation-mediated silencing of p14(ARF) in fibroblasts from idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 303, L295-303.
- Coppieters, N., Dieriks, B.V., Lill, C., Faull, R.L., Curtis, M.A., Dragunow, M., 2014. Global changes in DNA methylation and hydroxymethylation in Alzheimer's disease human brain. *Neurobiology of aging* 35, 1334-1344.
- Deaton, A.M., Bird, A., 2011. CpG islands and the regulation of transcription. *Genes & development* 25, 1010-1022.
- Dees, C., Schlottmann, I., Funke, R., Distler, A., Palumbo-Zerr, K., Zerr, P., Lin, N.Y., Beyer, C., Distler, O., Schett, G., Distler, J.H., 2014. The Wnt antagonists DKK1 and SFRP1 are downregulated by promoter hypermethylation in systemic sclerosis. *Ann Rheum Dis* 73, 1232-1239.

Ding, Q., Gladson, C.L., Wu, H., Hayasaka, H., Olman, M.A., 2008. Focal adhesion kinase (FAK)-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. *The Journal of biological chemistry* 283, 26839-26849.

Duthie, S.J., Narayanan, S., Blum, S., Pirie, L., Brand, G.M., 2000. Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. *Nutr Cancer* 37, 245-251.

Fatemi, M., Hermann, A., Gowher, H., Jeltsch, A., 2002. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *European journal of biochemistry / FEBS* 269, 4981-4984.

Garcia-Trevijano, E.R., Iraburu, M.J., Fontana, L., Dominguez-Rosales, J.A., Auster, A., Covarrubias-Pinedo, A., Rojkind, M., 1999. Transforming growth factor beta1 induces the expression of alpha1(I) procollagen mRNA by a hydrogen peroxide-C/EBPbeta-dependent mechanism in rat hepatic stellate cells. *Hepatology* 29, 960-970.

Ghosh, A.K., Wei, J., Wu, M., Varga, J., 2008. Constitutive Smad signaling and Smad-dependent collagen gene expression in mouse embryonic fibroblasts lacking peroxisome proliferator-activated receptor-gamma. *Biochemical and biophysical research communications* 374, 231-236.

Goll, M.G., Kirpekar, F., Maggert, K.A., Yoder, J.A., Hsieh, C.L., Zhang, X., Golic, K.G., Jacobsen, S.E., Bestor, T.H., 2006. Methylation of tRNA^{Asp} by the DNA methyltransferase homolog Dnmt2. *Science* 311, 395-398.

Gotze, S., Schumacher, E.C., Kordes, C., Haussinger, D., 2015. Epigenetic Changes during Hepatic Stellate Cell Activation. *PloS one* 10, e0128745.

Gowher, H., Liebert, K., Hermann, A., Xu, G., Jeltsch, A., 2005. Mechanism of stimulation of catalytic activity of Dnmt3A and Dnmt3B DNA-(cytosine-C5)-methyltransferases by Dnmt3L. *The Journal of biological chemistry* 280, 13341-13348.

Grotendorst, G.R., Okochi, H., Hayashi, N., 1996. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ* 7, 469-480.

Guo, J.U., Su, Y., Zhong, C., Ming, G.L., Song, H., 2011. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* 145, 423-434.

Hagemann, S., Heil, O., Lyko, F., Brueckner, B., 2011. Azacytidine and decitabine induce gene-specific and non-random DNA demethylation in human cancer cell lines. *PloS one* 6, e17388.

Hansen, K.D., Timp, W., Bravo, H.C., Sabunciyan, S., Langmead, B., McDonald, O.G., Wen, B., Wu, H., Liu, Y., Diep, D., Briem, E., Zhang, K., Irizarry, R.A., Feinberg, A.P., 2011. Increased methylation variation in epigenetic domains across cancer types. *Nature genetics* 43, 768-775.

He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., Sun, Y., Li, X., Dai, Q., Song, C.X., Zhang, K., He, C., Xu, G.L., 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333, 1303-1307.

Hendrich, B., Tweedie, S., 2003. The methyl-CpG binding domain and the evolving role of DNA methylation in animals. *Trends in genetics : TIG* 19, 269-277.

- Hinz, B., Phan, S.H., Thannickal, V.J., Galli, A., Bochaton-Piallat, M.L., Gabbiani, G., 2007. The myofibroblast: one function, multiple origins. *Am J Pathol* 170, 1807-1816.
- Hsieh, C.L., 1994. Dependence of transcriptional repression on CpG methylation density. *Molecular and cellular biology* 14, 5487-5494.
- Hu, B., Gharaee-Kermani, M., Wu, Z., Phan, S.H., 2011. Essential role of MeCP2 in the regulation of myofibroblast differentiation during pulmonary fibrosis. *Am J Pathol* 178, 1500-1508.
- Huan, C., Yang, T., Liang, J., Xie, T., Cheng, L., Liu, N., Kurkciyan, A., Monterrosa Mena, J., Wang, C., Dai, H., Noble, P.W., Jiang, D., 2015. Methylation-mediated BMPER expression in fibroblast activation in vitro and lung fibrosis in mice in vivo. *Scientific reports* 5, 14910.
- Iredale, J.P., 2007. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *The Journal of clinical investigation* 117, 539-548.
- Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C., Zhang, Y., 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333, 1300-1303.
- Jacobson, A., Brinck, J., Briskin, M.J., Spicer, A.P., Heldin, P., 2000. Expression of human hyaluronan synthases in response to external stimuli. *The Biochemical journal* 348 Pt 1, 29-35.
- Jones, L.R., Young, W., Divine, G., Datta, I., Chen, K.M., Ozog, D., Worsham, M.J., 2015. Genome-Wide Scan for Methylation Profiles in Keloids. *Dis Markers* 2015, 943176.
- Kao, Y.H., Cheng, C.C., Chen, Y.C., Chung, C.C., Lee, T.I., Chen, S.A., Chen, Y.J., 2011. Hydralazine-induced promoter demethylation enhances sarcoplasmic reticulum Ca²⁺-ATPase and calcium homeostasis in cardiac myocytes. *Laboratory investigation; a journal of technical methods and pathology* 91, 1291-1297.
- Kareta, M.S., Botello, Z.M., Ennis, J.J., Chou, C., Chedin, F., 2006. Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. *The Journal of biological chemistry* 281, 25893-25902.
- Karouzakis, E., Gay, R.E., Michel, B.A., Gay, S., Neidhart, M., 2009. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. *Arthritis and rheumatism* 60, 3613-3622.
- Komatsu, Y., Waku, T., Iwasaki, N., Ono, W., Yamaguchi, C., Yanagisawa, J., 2012. Global analysis of DNA methylation in early-stage liver fibrosis. *BMC Med Genomics* 5, 5.
- Laurent, T.C., Fraser, J.R., 1992. Hyaluronan. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 6, 2397-2404.
- Loenarz, C., Schofield, C.J., 2009. Oxygenase Catalyzed 5-Methylcytosine Hydroxylation. *Chem Biol* 16, 580-583.
- Maiti, A., Drohat, A.C., 2011. Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. *The Journal of biological chemistry* 286, 35334-35338.
- Mann, J., Chu, D.C., Maxwell, A., Oakley, F., Zhu, N.L., Tsukamoto, H., Mann, D.A., 2010. MeCP2 controls an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis. *Gastroenterology* 138, 705-714, 714 e701-704.

- Meehan, R.R., Lewis, J.D., Bird, A.P., 1992. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic acids research* 20, 5085-5092.
- Milutinovic, S., Zhuang, Q., Niveleau, A., Szyf, M., 2003. Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. *The Journal of biological chemistry* 278, 14985-14995.
- Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., Schubeler, D., 2008. Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. *Molecular cell* 30, 755-766.
- Moore, L.D., Le, T., Fan, G., 2013. DNA methylation and its basic function. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 38, 23-38.
- Mortusewicz, O., Schermelleh, L., Walter, J., Cardoso, M.C., Leonhardt, H., 2005. Recruitment of DNA methyltransferase I to DNA repair sites. *Proceedings of the National Academy of Sciences of the United States of America* 102, 8905-8909.
- Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A., 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, 386-389.
- O'Reilly, S., 2016. MicroRNAs in fibrosis: opportunities and challenges. *Arthritis research & therapy* 18, 11.
- O'Reilly, S., Ciechomska, M., Fullard, N., Przyborski, S., van Laar, J.M., 2016. IL-13 mediates collagen deposition via STAT6 and microRNA-135b: a role for epigenetics. *Scientific reports* 6, 25066.
- Page, A., Paoli, P., Salvador, E.M., White, S., French, J., Mann, J., 2015. Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape. *J Hepatol.*
- Pan, X., Chen, Z., Huang, R., Yao, Y., Ma, G., 2013. Transforming growth factor beta1 induces the expression of collagen type I by DNA methylation in cardiac fibroblasts. *PLoS one* 8, e60335.
- Pandey, S., Simmons, G.E., Jr., Malyarchuk, S., Calhoun, T.N., Pruitt, K., 2015. A novel MeCP2 acetylation site regulates interaction with ATRX and HDAC1. *Genes Cancer* 6, 408-421.
- Rabinovich, E.I., Kapetanaki, M.G., Steinfeld, I., Gibson, K.F., Pandit, K.V., Yu, G., Yakhini, Z., Kaminski, N., 2012. Global methylation patterns in idiopathic pulmonary fibrosis. *PLoS one* 7, e33770.
- Robertson, K.D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F.A., Jones, P.A., 1999. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic acids research* 27, 2291-2298.
- Robinson, C.M., Neary, R., Leventale, A., Watson, C.J., Baugh, J.A., 2012. Hypoxia-induced DNA hypermethylation in human pulmonary fibroblasts is associated with Thy-1 promoter methylation and the development of a pro-fibrotic phenotype. *Respiratory research* 13, 74.
- Shmookler Reis, R.J., Goldstein, S., 1982. Variability of DNA methylation patterns during serial passage of human diploid fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* 79, 3949-3953.

Tampe, B., Tampe, D., Muller, C.A., Sugimoto, H., LeBleu, V., Xu, X., Muller, G.A., Zeisberg, E.M., Kalluri, R., Zeisberg, M., 2014. Tet3-mediated hydroxymethylation of epigenetically silenced genes contributes to bone morphogenic protein 7-induced reversal of kidney fibrosis. *J Am Soc Nephrol* 25, 905-912.

Tampe, B., Tampe, D., Zeisberg, E.M., Muller, G.A., Bechtel-Walz, W., Koziol, M., Kalluri, R., Zeisberg, M., 2015. Induction of Tet3-dependent Epigenetic Remodeling by Low-dose Hydralazine Attenuates Progression of Chronic Kidney Disease. *EBioMedicine* 2, 19-36.

Valinluck, V., Sowers, L.C., 2007. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. *Cancer Res* 67, 946-950.

Vincent, Z.L., Mitchell, M.D., Ponnampalam, A.P., 2015. Regulation of TIMP-1 in Human Placenta and Fetal Membranes by lipopolysaccharide and demethylating agent 5-aza-2'-deoxycytidine. *Reprod Biol Endocrinol* 13, 136.

Wang, Y., Fan, P.S., Kahaleh, B., 2006. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis and rheumatism* 54, 2271-2279.

Watson, C.J., Collier, P., Tea, I., Neary, R., Watson, J.A., Robinson, C., Phelan, D., Ledwidge, M.T., McDonald, K.M., McCann, A., Sharaf, O., Baugh, J.A., 2014. Hypoxia-induced epigenetic modifications are associated with cardiac tissue fibrosis and the development of a myofibroblast-like phenotype. *Hum Mol Genet* 23, 2176-2188.

Webster, A.L., Yan, M.S., Marsden, P.A., 2013. Epigenetics and cardiovascular disease. *The Canadian journal of cardiology* 29, 46-57.

Wynn, T.A., 2008. Cellular and molecular mechanisms of fibrosis. *The Journal of pathology* 214, 199-210.

Xu, X., Tan, X., Tampe, B., Nyamsuren, G., Liu, X., Maier, L.S., Sossalla, S., Kalluri, R., Zeisberg, M., Hasenfuss, G., Zeisberg, E.M., 2015. Epigenetic balance of aberrant Rasal1 promoter methylation and hydroxymethylation regulates cardiac fibrosis. *Cardiovasc Res* 105, 279-291.

Zeisberg, M., Kalluri, R., 2013. Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. *American journal of physiology. Cell physiology* 304, C216-225.

Zeybel, M., Hardy, T., Wong, Y.K., Mathers, J.C., Fox, C.R., Gackowska, A., Oakley, F., Burt, A.D., Wilson, C.L., Anstee, Q.M., Barter, M.J., Masson, S., Elsharkawy, A.M., Mann, D.A., Mann, J., 2012. Multigenerational epigenetic adaptation of the hepatic wound-healing response. *Nat Med* 18, 1369-1377.

Zhang, B., Zhou, Y., Lin, N., Lowdon, R.F., Hong, C., Nagarajan, R.P., Cheng, J.B., Li, D., Stevens, M., Lee, H.J., Xing, X., Zhou, J., Sundaram, V., Elliott, G., Gu, J., Shi, T., Gascard, P., Sigaroudinia, M., Tlsty, T.D., Kadlecik, T., Weiss, A., O'Geen, H., Farnham, P.J., Maire, C.L., Ligon, K.L., Madden, P.A., Tam, A., Moore, R., Hirst, M., Marra, M.A., Zhang, B., Costello, J.F., Wang, T., 2013. Functional DNA methylation differences between tissues, cell types, and across individuals discovered using the M&M algorithm. *Genome research* 23, 1522-1540.

Zhang, Q.Q., Xu, M.Y., Qu, Y., Hu, J.J., Li, Z.H., Zhang, Q.D., Lu, L.G., 2014. TET3 mediates the activation of human hepatic stellate cells via modulating the expression of long non-coding RNA HIF1A-AS1. *Int J Clin Exp Pathol* 7, 7744-7751.

Zhao, X., Dai, J., Ma, Y., Mi, Y., Cui, D., Ju, G., Macklin, W.B., Jin, W., 2014. Dynamics of ten-eleven translocation hydroxylase family proteins and 5-hydroxymethylcytosine in oligodendrocyte differentiation. *Glia* 62, 914-926.

Zhou, B., Zeng, S., Li, L., Fan, Z., Tian, W., Li, M., Xu, H., Wu, X., Fang, M., Xu, Y., 2016. Angiogenic factor with G patch and FHA domains 1 (Aggf1) regulates liver fibrosis by modulating TGF-beta signaling. *Biochim Biophys Acta*.