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Hepatic Stellate Cell Transdifferentiation Involves Genome-Wide Remodeling of the DNA Methylation Landscape

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Abstract

Background and aims—DNA methylation is an epigenetic mark that is an established regulator of transcriptional repression with an important role in liver fibrosis. Currently, there is very little knowledge available as to how DNA methylation controls the phenotype of hepatic stellate cell (HSC), the key cell type responsible for onset and progression of liver fibrosis. Moreover, recently discovered DNA hydroxymethylation is involved in transcriptional activation and its patterns are often altered in human diseases. The aim of this study is to investigate the role of DNA methylation/hydroxymethylation in liver fibrosis.

Methods—Levels of 5-mC and 5-hmC were assessed by slot blot in a range of animal liver fibrosis models and human liver diseases. Expression levels of TET and DNMT enzymes were measured by qRT-PCR and western blotting. Reduced representation bisulfite sequencing method was used to examine 5-mC and 5-hmC patterns in quiescent and *in vivo* activated rat HSC.

Results—We demonstrate global alteration in 5-mC and 5-hmC and their regulatory enzymes that accompany liver fibrosis and HSC transdifferentiation. Using RRBS, we show exact genomic positions of changed methylation patterns in quiescent and *in vivo* activated rat HSC. In addition, we demonstrate that reduction in DNMT3a expression leads to attenuation of pro-fibrogenic phenotype in activated HSC.

Conclusions—Our data suggest that DNA methylation/hydroxymethylation is a crucial step in HSC activation and therefore fibrogenesis. Changes in DNA methylation during HSC activation may bring new insights into the molecular events underpinning fibrogenesis and may provide biomarkers for disease progression as well as potential new drug targets.

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Keywords

DNMTs; TETs; hepatic myofibroblasts; liver fibrosis; epigenetics

Introduction

Hepatic stellate cells (HSC) are the predominant cellular origin of fibrogenic α SMA-positive myofibroblasts [1]. In the normal liver the HSC is a quiescent perisinusoidal cell that is found in the Space of Disse where it functions as a store of Vitamin A and is postulated to have immune and stem cell-like properties [2]. Hepatocellular damage, infection or local inflammation triggers HSC to undergo a vast number of changes in gene expression to bring about a phenotypic transdifferentiation, whereby the cell adopts a profibrogenic myofibroblast-like state. The so-called activated HSC (aHSC) becomes highly proliferative, expresses a variety of autocrine and paracrine factors that stimulate the fibrogenic process such as TGF β 1 and PDGF, and secrete fibril forming collagens, collagen cross-linking enzymes and tissue inhibitor of metalloproteinases-1 (TIMP-1) which collectively result in the net deposition and maturation of a fibrotic extracellular matrix [3, 4].

There is gathering evidence that the widespread changes in gene expression that underpin HSC transdifferentiation, and in turn the progression of liver fibrosis, are orchestrated by epigenetic factors including regulators of DNA methylation, histone modifications and non-coding RNAs. DNA is methylated by addition of a methyl group to the 5' position of cytosine (5-methylcytosine) within a cytosine-phosphoguanine dinucleotide (CpG) to form 5-methylcytosine (5-mC). Methylation of CpG dinucleotides is probably one of the most studied epigenetic phenomena, which is known to play a major role in X-chromosome inactivation, imprinting of genes as well as transcriptional silencing of foreign DNA elements. The relationship between methylation and gene expression is complex, with low gene promoter methylation often associated with high levels of gene expression however the causality of this relationship remains unclear.

5-mC can be further modified by enzymatic oxidation to produce 5-hydroxymethylcytosine (5-hmC), which is mainly found in the promoter, enhancer and gene body regions of transcriptionally active genes [5]. 5-mC and 5-hmC are regulated DNA modifications that are under the control of families of enzymes, specifically DNA methyltransferases (DNMT1, DNMT3a, DNMT3b) that regulate annotation of 5-mC and the Ten-eleven translocation methylcytosine dioxygenase (TET) family enzymes (TET1,2 and 3) that oxidise 5-mC to 5-hmC [6, 7]. This regulatory annotation of the CpG dinucleotide provides mechanisms for modifying the cellular epigenome in response to environmental cues and when dysregulated can contribute to altered gene expression in human diseases, of which cancer is currently the best documented [8, 9].

Earlier experiments published by our group demonstrated that pharmacological inhibition of 5-mC, achieved by exposure of freshly isolated HSC to the drug 5-aza-2'-deoxycytidine (5-azadC), blocked transdifferentiation, this being associated with maintenance of expression of the key anti-fibrogenic genes [10]. We concluded that DNA methylation provides an essential blueprint for HSC transdifferentiation and went on to show that the prototypic

methyl-DNA binding protein MeCP2 is critical for the fibrogenic activities of HSC-derived myofibroblasts [11]. Alongside these HSC cell culture-based studies are investigations of DNA methylation signatures in human liver that appear to have the power to stratify patients as either fibrosis progressors or non-progressors [12, 13]. However, as many of the identified sites of differential methylation are found in genes known to regulate the HSC phenotype (e.g. PPAR α , PPAR γ , TGF β 1 etc.), the clinical data raise the possibility that HSC transdifferentiation may be accompanied by remodeling of the DNA methylome.

Here we document global alterations in 5-mC and 5-hmC modifications and their regulatory enzymes (DNMTs and TETs) that accompany liver fibrosis and HSC transdifferentiation. We go on to employ next generation sequencing protocols to identify genome-wide remodeling of 5-mC and 5-hmC marks that are indicative of major epigenetic re-landscaping of the genome during *in vivo* HSC transdifferentiation. In addition, we show that reducing the expression of DNMT3a leads to attenuation of pro-fibrogenic phenotype in aHSC.

Materials and methods

Ethics

Authors hold appropriate licenses for animal experiments, which were issued/approved by local ethical committee and UK Home Office.

Human subjects

Use of human tissue was approved by Newcastle and North Tyneside Local Research Ethics (approval number H10/H0906/41). All samples were collected and used subject to patient's written consent.

Cell isolation

Rat hepatic stellate cells (rHSC) were isolated from normal livers of 350g Sprague-Dawley rats by sequential perfusion with collagenase and pronase, followed by discontinuous density centrifugation in 11.5% Optiprep (Sigma-Aldrich, Gillingham, Dorset, UK). Cells were cultured on plastic at 37°C at an atmosphere of 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine, and 16% fetal calf serum. Cells were harvested on day of the isolation (qHSC) or after 10 days of the culture (aHSC).

Cell isolation- in vivo protocol

For induction of HSC activation *in vivo*, the Sprague-Dawley rats were injected intraperitoneally twice weekly for 4 weeks, with a mixture of CCl₄/olive oil in a 3:1 [vol/vol] ratio at 2 μ l per g body weight. Twenty four hours after the last CCl₄ administration, animals were sacrificed and HSCs isolated using the method as outlined in the previous section.

Experimental Models of Liver Fibrosis

Adult rats were intraperitoneally injected twice-weekly for 4 weeks (chronic) with a mixture of CCl₄/olive oil in a 3:1 [vol/vol] ratio at 2 μ l per g body weight. Bile duct ligation (BDL)

was performed and rats were allowed to develop cholestatic disease and fibrosis. Liver tissues were harvested 14 days after the surgery. Rats were fed methionine-choline-deficient (MCD) or control diet (Research Diets, New Brunswick, NJ) for 8 weeks. The rats were then sacrificed and liver samples collected.

Quantitative PCR

Quantitative real-time PCR assay was established for relative quantification of rat and human TET enzymes and DNA methyltransferases mRNA. Total RNA was purified from isolated cells using the Total RNA purification kit (Qiagen, UK) following manufacturer's instructions. One microgram of total RNA was DNase treated (Promega) and used as template to generate cDNA using a random hexamer primer [p(dN)6] and MMLV reverse transcriptase (Promega). Primers for ECM components, histone lysine methyltransferases and histone lysine demethylases are included in Table 1. SYBR Green quantitative RT-PCR reactions were performed in a total volume of 13 μ l, containing 20ng of cDNA template, 6.5 μ l of SYBR Green JumpStart Taq ReadyMix (Sigma), and 20pmols of forward and reverse primers (Table 1). The PCR reaction was carried out on a 7500 Fast Real-Time PCR System (Applied Biosystem, Warrington, Cheshire, UK) with the following parameters: 1 cycle at 95 °C for 10 sec followed by 40 cycles at 95 °C for 10 sec, 55 °C – 60 °C (primer pair specific annealing temperature, see table 1 and 2) for 30 sec, and finally 72°C for 30 sec. Melt curve analysis was employed to confirm presence of a single PCR product. All reactions were normalized to rat β -actin or human GAPDH internal control, and relative level of transcriptional difference calculated by using the 2^{-C_t} method.

Immunohistochemistry

Activated HSC isolated from CCl₄ treated rats were spun down using the cytocentrifuge at 1000rpm for 5 minutes with medium acceleration. Slides were air-dried for 30 minutes, fixed in 10% formalin for 30 minutes. Slides were then permeabilized with 1% saponin/0.5% BSA/PBS for 10 minutes at room temperature, washed, blocked with 3% BSA for 10min and incubated with the rabbit monoclonal anti actin alpha-smooth FITC antibodies (Sigma, F3777; dilution factor 1:100) for 2h at room temperature. The cells were washed and incubated with DAPI special formulation NucBlue live ready probes reagent (Life Technologies) for 10 minutes at room temperature. The slides were then mounted with ProLong Gold antifade reagent (Life Technologies).

SDS-PAGE and Immunoblotting

Whole-cell extracts were prepared and protein concentration of samples determined using Bio-Rad DC Protein Assay (Bio-Rad, UK). Proteins were separated on 8% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. In order to block nonspecific protein binding blots were incubated with 5% milk for 1h, followed by overnight incubation with primary antibody rabbit anti-TET2 (Abcam, ab135087), TET3 (Abcam, ab139805), DNMT3a (Abcam, ab2850), DNMT3b (Abcam, ab2851), DNMT1 (Epigentek, A-1001-100), TGF β (Cell Signaling, (56E4) Rabbit mAb #3709) at 1:1000, rabbit anti-GAPDH (Abcam, ab9485) or mouse anti- β actin (Abcam, ab8226) both at 1:2000 dilution. Secondary antibody

either against rabbit or mouse was used at 1:2000 dilution. The antibody complexes were detected by using Pierce ECL Western Blotting Substrate (Thermo Scientific).

Reduced representation bisulfite sequencing (RRBS)/ oxidative bisulfite sequencing

Three ug of gDNA per sample (control, bisulfite and oxidative bisulfite conversion) was digested overnight with MspI restriction enzyme (New England Biolabs). The fragmented DNA was then used as input for the NEB DNAultra Library Prep Kit for Illumina, following the manufacture's protocol for methylated adapters, with a size selection for ~200bp fragments. Bisulfite and oxidative bisulfite conversion was performed with TrueMethyl Kit (CEGX, Cambridge); libraries were amplified by PCR of 25 cycles using EpiMark Hotstart Taq DNA polymerase (New England Biolabs). Sequencing was performed on a MiSeq with a V3 cartridge for 100bp single-direction reads yielding ~25million sequences per run. Analysis of RRBS data was performed using bismark (<http://www.bioinformatics.babraham.ac.uk/projects/bismark/>) with bowtie2 aligner against the annotated rat genome (Rnor_5.0) from Ensembl. Analysis was applied to each CpG site individually, yielding the percentage of hydroxymethylation by subtracting the total methylation of the bisulfite and oxidative-bisulfite modified samples at any one individual CpG site. Finally, the identified methylated/hydroxymethylated CpGs from *in vivo* quiescent or activated HSCs were compared by two tailed unpaired Student's t-test and minimal significance set at $p < 0.01$.

Slot blot assay

Genomic DNA (gDNA) was extracted from rat HSC or rat and human liver samples by incubation in lysis buffer (20 mM Tris (pH 8), 20 mM EDTA, 2% SDS and 0.5 mg/ml Proteinase K) overnight at 55°C followed by phenol:chloroform extraction and ethanol precipitation. The extracted DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Loughborough, UK). Double DNA dilutions were then prepared (2ug-250ng) and the slot blot assay was performed using Bio-Dot SF microfiltration apparatus (BIO-RAD) according to the manufacturer's instructions. Following gDNA application and hybridization, membranes were probed with antibodies specific to 5hmC (Active motif, cat #39769, 1:5000) and 5mC (Eurogentec, BI-MECY-0100, 1:2000). To control for loading, membranes were immersed in methylene blue stain and washed with water. In order to quantify 5hmC and 5mC levels GelAnalyzer image processing and analysis software (<http://www.gelanalyzer.com/index.html>) was used.

Small interfering RNA (siRNA) transfection

Rat hepatic stellate cells prepared from 3 separate isolations were cultured for 5 days on plastic and transfected with siRNA designed against DNMT3a (Life Technologies) or negative control siRNA (Ambion) using INTERFERin (Polyplus). The final concentration of siRNA was 100nM. The cells were allowed to grow for 48 hours posttransfection, when they were harvested and RNA and/or whole cell extracts made.

Results

Experimental liver fibrosis is accompanied by loss of 5-hmC and modulation of DNMTs and TETs

To begin to determine if fibrosis is associated with changes in the DNA methylation machinery we examined the hepatic expression of rat DNMTs (DNMT1, DNMT3a and DNMT3b) and TETs (TET2 and TET3 only as TET1 is not expressed in liver or HSC (Suppl Fig 1)) in the methionine-choline-deficiency (MCD) dietary model of steatohepatitis-driven fibrosis [14]. Representative stains for Sirius red and α SMA confirmed fibrosis and myofibroblast differentiation in response to MCD (Suppl Fig 2A). Starting with mRNA isolated from whole liver of MCD, we observed modest yet statistically significant reductions in expression of transcripts for TET2 and DNMT3b, increased expression of DNMT1 and no change for TET3 and DNMT3a, with all changes relative to transcripts quantified in livers from control fed animals (Fig 1A). Protein extracts from the same livers were processed for Western blot detection of DNMTs and TETs. In contrast to the modest changes at the transcript level, TET2, TET3 and DNMT3b proteins were expressed at diminished levels in MCD relative to control livers (Fig 1B). While less dramatic, the opposite effect was found for the DNMT1 and DNMT3a. To determine if these changes in DNMT and TET expression were associated with global shifts in DNA methylation and hydroxymethylation, anti-5-mC and anti-5-hmC specific antibodies were used to probe membranes blotted with increasing dilutions of gDNA extracted from MCD and control livers (Fig 1C). Quantification of slot-blots indicated a significant global reduction in the 5-hmC and 5-mC modifications. The overall loss of 5-hmC is consistent with diminished expression of TETs in MCD livers. The pathogenesis of liver disease following bile duct ligation (BDL) is distinct from that of the MCD model and involves a cholestatic disease process that is associated with fibrosis beginning in the portal tract region prior to involvement of the sinusoids [15] (Suppl Fig 2B). It was therefore of interest to compare effects of BDL-induced liver disease on DNA methylation with those found in the MCD model. Comparing whole liver mRNA between BDL injured and sham operated animals we observed fibrosis-associated reductions in the expression of TET2 and TET3, no change for DNMT1 transcript, upregulation of DNMT3a and reduced expression of DNMT3b (Fig 1D). Loss of expression of TETs in BDL livers was confirmed at the protein level, whereas all three DNMTs were substantively induced relative to sham controls (Fig 1E). Consistent with loss of Tets expression and similar to MCD livers, BDL injury resulted in a reduction of global 5-hmC (Fig 1F). However, in contrast to the loss of 5-mC observed in the MCD model (Fig 1C), 5-mC was globally elevated in BDL livers, which is consistent with the dramatic induction of DNMTs at the protein level (Fig 1D). Repeated administration of carbon tetrachloride (CCl₄) provides a well-established experimental model of chronic liver disease primarily involving the activation of HSC and the deposition of fibrotic matrix in the sinusoidal spaces [16] (Suppl Fig 2C). With this model we observed modest modulation for hepatic TET and DNMT transcripts (Fig 1G). At the protein level CCl₄ injury led to a substantial diminution of TET2 protein and a dramatic induction of DNMT3b (Fig 1H). These alterations in enzyme expression were accompanied by a trend towards loss of 5-hmC but no change in global 5-mC (Fig 1I).

Taken together, the data from these three distinct models of chronic liver disease reveal common underlying modulations of the epigenetic machinery including a consistent loss of expression of TETs and associated reductions in the 5-hmC mark in all three models of liver fibrosis. On the other hand the DNMTs are in general induced at the protein level in fibrotic livers with a particular dramatic induction of the de novo DNA methyltransferase DNMT3b in BDL and CCl₄ models, however this effect was not necessarily accompanied by global changes in 5-mC. We conclude that a general shift towards stabilization of genome DNA methylation and associated suppression of TET-regulated 5-mC-oxidation is a common feature of rat liver fibrosis.

Epigenetic alterations in human chronic liver diseases of distinct aetiologies resemble those associated with rat liver fibrosis

We were next interested to discover if similar epigenetic changes occur in fibrotic human livers. Primary Sclerosing Cholangitis (PSC) is a relatively rare progressive liver disease of poorly defined aetiology that is characterized by concentric obliterative fibrosis of the intrahepatic bile ducts [17]. Transcripts for TETs and DNMTs were modestly upregulated in PSC liver compared to healthy controls (Fig 2A), however in agreement with the animals models hepatic TET2 and TET3 protein expression was lost in PSC, while the DNMT3b enzyme was induced in all 3 patient samples (Fig 2B). Slot blot analyses of 5-mC and 5-hmC revealed a loss of 5-hmC but little change for global 5-mC (Fig 2C). Alcoholic liver disease (ALD) is a much more common cause of hepatic fibrosis than PCS and has a clearly defined aetiology and pathobiology involving steatosis, hepatocyte ballooning, inflammation and both perivenular and pericellular fibrosis [18]. Comparing five ALD with five normal control liver samples, we again observed modest shifts in TET and DNMT transcript expression (Fig 2D) but more profound alterations at the protein level (Fig 2E). TET3 expression was unchanged between diseased and control livers, but by contrast most ALD samples displayed a reduction in TET2. No obvious alterations were seen with DNMT1 or DNMT3a but there was a dramatic induction of DNMT3b as previously observed in the PSC livers and in rat livers following BDL- or CCl₄-induced fibrosis. Levels of 5-hmC displayed a trend towards a reduction, but there was again no clear alteration in global 5-mC (Fig 2F). These data, collected from mechanistically distinct examples of chronic human liver disease, reveal common fibrosis-associated epigenetic changes including reduced TET expression, gain of DNMT3b and reduced 5-hmC that are also observed as characteristic epigenetic features of experimental liver fibrosis in rats.

Induction of DNMT3a and DNMT3b and a global reduction in 5-hmC are features of HSC transdifferentiation

Recent cell-lineage tracing studies in rodents support the concept that transdifferentiation of qHSC to their activated myofibroblastic state represents the major source of fibrogenic myofibroblasts in the diseased liver irrespective of the underlying cause of liver damage [19]. We were therefore interested to determine if HSC transdifferentiation is associated with alterations in the DNA methylation machinery and its 5-mC and 5-hmC marks. With the aim of focusing our investigations on *in vivo* events we elected to initially carry out biochemical measurements in *ex-vivo* HSC either isolated from control (olive oil) or CCl₄-injured rat livers. This *ex-vivo* approach in the absence of culture-activation was also considered

important since cell culture *per se* can result in changes in 5-mC/5-hmC modifications and their regulators [20]. While isolation of aHSC is difficult from mouse liver and requires the use of FACS-sortable cell lineage markers, we consistently find that aHSC are readily isolated in a highly enriched state from diseased rat liver. Here we verify the relative purity of aHSC isolations by cell cytospin staining for α SMA and measurement of the expression of TGF β 1 protein which as expected was absent in HSC isolated from control livers but was highly expressed in HSC isolated from CCl₄-damaged livers (Fig 3A). We also demonstrated minimal expression of F4/80 and albumin transcripts ruling out any significant contamination with either macrophages or hepatocytes respectively (Suppl Fig 3A and B). We next determined the transcript and protein expression for DNMT1, 3a and 3b, TET2 and TET3. Although some statistically significant changes were measured for TET3 (downregulated in aHSC vs qHSC) and DNMT3b (downregulated in aHSC) these effects were at best modest (Fig 3B). Western blot analysis of these enzymes was somewhat variable within treatment groups and we could not identify any consistent expression changes for DNMT1 or TET2 (Fig 3C). TET3 expression was present in four out of five qHSC protein extracts and in only two out of five aHSC extracts indicating that expression of this enzyme may be suppressed during transdifferentiation. By contrast, DNMT3a and 3b proteins were undetectable in qHSC but were expressed in the majority of aHSC samples indicative of a requirement for *de novo* DNA methylation during HSC transdifferentiation (Fig 3C). Slot blot detection for methylation marks detected a significant loss of 5-hmC during HSC transdifferentiation this being consistent with findings in fibrotic human and rodent liver, however there was little alteration in 5-mC (Fig 3D).

As the culture model of HSC transdifferentiation is commonly employed to study the biochemical events that underpin conversion of qHSC to aHSC it was considered relevant to also determine epigenetic changes in this model. DNMT enzymes were expressed at low-to-undetectable protein levels in qHSC and were highly induced in culture-activated HSC (Fig 4A). DNMT1 and DNMT3a transcripts were transiently induced with culture (Fig 4B and C), however DNMT3b transcript was down-regulated compared with levels in freshly isolated cells (Fig 4D), which contrasts with the induction of DNMT3b protein. TET2 and TET3 were down-regulated and unchanged, respectively, at the transcript level. Both TETs were reduced in protein level following 10 days of culture when compared with freshly isolated qHSC (Figs 4A). Hence, the culture model recapitulates the observed increase in DNMT expression found in *ex-vivo* aHSC isolated from CCl₄ damaged livers, but differs in that culture activated HSC exhibit some loss of TETs which may reflect a previous report concerning loss of TET expression as an adaptation to culturing [20]. This culture-associated loss of TETs may explain a gradual daily loss of 5-hmC observed across a time-course of HSC culture (Fig 4G). By contrast, 5-mC levels were relatively unchanged across the different culture time points (Fig 4H).

Genome-wide epigenetic re-landscaping during HSC transdifferentiation

To better define the degree to which HSC transdifferentiation is associated with DNA methylation remodeling of the genome, we sequenced 5-mC and 5-hmC modifications on genomic DNA isolated from *ex-vivo* purified qHSC (olive oil) and aHSC (CCl₄-injured) using the reduced representation bisulfite sequencing (RRBS) protocol which has a bias

towards GC-rich promoter regions [21]. In total we were able to read 14,669,771 and 10,224,679 5-mC sites for qHSC and aHSC respectively, and for 5-hmC 12,133,811 and 8,451,266 sites respectively. When comparing qHSC with aHSC a total of 176,010 5-mC sites were detected in both populations and the sites were distributed throughout the genome indicating a broad distribution of GC-rich regions that are stable through the transdifferentiation process (Fig 5A). When sorted according to genomic location in which they are located, the CpGs with stable modifications are primarily found in intronic regions, with around an 8th in exons and the remainder in 5'UTR (Fig 5B). In qHSC we sequenced 3,484,985 5-mC sites that were unique (or differentially methylated) for the quiescent phenotype and included a high density of reads in chromosomes 5 to 9 and chromosome X (Fig 5C). Over half of methylated CpGs uniquely found in qHSCs were in intronic region, an 8th in exons and almost a quarter in 5'UTR and 3'UTR (Fig 5D). For aHSC, the differentially methylated landscape was distinct from the qHSC profile, with clear evidence of genome-wide remodeling (Fig 5E and F). In particular, new high densities of 5-mC modified sites were found across chromosomes 13 to 19, 20, 21 and also of note at the Y chromosome (Fig 5E and F).

In the case of 5-hmC the data are shown as 5-mC/5-hmC percentages at each CpG site, such that bars rising above the median line are 5-hmC enriched while those below the bar are 5-mC enriched (Fig 6). Again we detected a genome-wide distribution of 5-hmC modifications that are shared between the two phenotypic states (Fig 6A). However, when comparing differentially hydroxymethylated reads for qHSC (Fig 6C) and aHSC (Fig 6E) there is strong evidence for genome-wide remodeling (Fig 6D and F). The differential 5-hmC landscape for qHSC was broadly distributed with no strong peaks identified for any particular chromosomal locations (Fig 6C). By contrast, the differential hydroxymethyl landscape for aHSC was of lower density across the genome, which is consistent with the overall loss of 5-hmC observed in the slot blot 5-hmC assays (Fig 3D). However, we observed a particularly high density of 5-hmC modifications in chromosome 9 that were unique to aHSC (Fig 6E). Tables 2–5 show the top 30 gene loci at which the greatest number of *de novo* differential 5-mC and 5-hmC signatures were recorded in qHSC and aHSC.

Given that RRBS discovered significant changes in DNA methylation/hydroxymethylation associated with HSC activation, we wondered what effect reduction in DNMT3a might have on aHSC phenotype. To this end, we transfected aHSCs with siRNA targeting DNMT3a, which caused a significant reduction in the methyltransferase mRNA and protein (Fig 7A and B). DNMT3a diminution resulted in no change in TGF β 1 (Fig 7C), however it caused a significant reduction in collagen 1A1 mRNA (Fig 7D) and α SMA mRNA and protein (Fig 7B and E) as well as a change in morphology of the aHSC, which adopted a more rounded appearance reminiscent of quiescent phenotype (Suppl Fig 4). We next wondered whether DNMT3a directly regulates these two genes via DNA methylation or via some indirect mode of action. We firstly compared DNA methylation between quiescent and activated HSCs at both genes (Fig 7F and G). Collagen 1A1 promoter was methylated in quiescent HSCs however this methylation was completely absent in activated HSC where this gene is highly expressed (Fig 7F). α SMA gene was methylated at 3' end in quiescent, but not activated HSCs (Fig 7G). As neither gene had DNA methylation present in the activated HSCs, it was unlikely that DNMT3a was exerting its inhibitory effect directly, but rather that an indirect

mechanism regulates diminution of Collagen 1A1 and α SMA in activated HSCs depleted of DNMT3a.

Discussion

Epigenetic mechanisms are now recognised to guide and fine-tune the processes associated with life, beyond that which is specified by the sequence of the genome. The covalent modification of DNA, associated histones as well as non coding RNA coherently operate to establish the epigenetic regulation of gene expression in physiological and pathophysiological conditions.

In this study, we address the role of DNA methylation and hydroxymethylation in human and rat fibrotic liver disease as well as myofibroblasts isolated from normal and fibrotic rat livers. We show that fibrogenesis as found in multiple liver diseases, both in humans and in animal models, is associated with changes in expression of enzymes that regulate DNA methylation (DNMT1, 3a and 3b) as well as hydroxymethylation (TETs). Overall, we show that DNMT1 maintenance methylase and DNMT3a and 3b de novo methylases protein tend to increase their expression in fibrotic liver as compared to normal control (Figures 1B, 1E, 1H, 2B), whereas TET enzymes tend to become downregulated in same instances. Similar trend was found in aHSC/hepatic myofibroblasts that were either isolated from fibrotic rat livers (upregulation of DNMT1, 3a and 3b in Figure 3C) or activated *in vitro* (Figure 4A). We note that reduction in TETs expression led to a reduction in global levels of 5-hmC, however the increase in DNMTs expression did not alter the global amount of 5-mC. The latter may be due to alterations in DNMTs activity and recruitment to the genomic DNA.

It is important to note the extent of similarities in the disease-associated alterations in expression of DNMTs and TETs when comparing the animal models and human disease tissues. Our data suggest that animal models of liver disease are highly representative and therefore a good substitute for studying DNA methylation-related mechanisms in humans. Furthermore, *in vitro* and *in vivo* activated HSCs also follow similar trends, further confirming the validity of use of these cells to study fibrogenesis.

Bearing in mind that expression of DNMT3a and 3b de novo methyltransferases increase with HSC activation, it was of interest to find out whether the process of activation actually depends on activity of these enzymes. Previous studies have shown that distribution of 5-mC and 5-hmC within active transcription start sites in gene promoters are regulated by DNMT1/ DNMT3a, but not DNMT3b. To that end, we carried out a targeted knock down of DNMT3a, which resulted in reduction of Collagen 1A1 and α SMA levels in activated HSCs, as well as changes in their morphology towards a more rounded, quiescent like shape. Therefore, DNMT3a at least in part controls the fibrogenic phenotype of activated HSCs.

In both human and rat models tested (with the exception of TET3 in ALD and TET2 in *in vivo* activated HSCs), there was a uniform decrease in the expression of TET proteins, with a concurrent reduction in global levels of 5-hmC. Using rat HSC isolated from *in vivo* injured or control rats, we show that distribution of DNA methylation and hydroxymethylation across the genome changes dramatically with HSC activation (Figure 5

and 6). While some genomic locations show overlapping methylation and hydroxymethylation patterns that remain unchanged between quiescent and *in vivo* activated HSC activation (Figure 5A and 6A), there are numerous methylated CpG sites that are unique to quiescent HSC (Figure 5C) and other methylated CpG sites that are unique to activated HSC (Figure 5E). The unique 5-mC profiles show major remodeling events between the quiescent and activated HSC; globally chromosomes 1–13 become hypomethylated, and chromosomes 14–19 are hypermethylated in the *in vivo* activated HSCs compared to the control (Figure 5C and E). The X- and Y-chromosomes undergo a reverse methylation between the two groups, whereby the X-chromosome becomes hypomethylated, and the Y-chromosome transitions to a hypermethylated state in activated HSCs. Similarly, there are numerous 5-hmC remodeling events that suggest very specific and significant alterations in hydroxymethylation that are associated with activation of HSCs (Figure 6E). Taken together with previous studies that demonstrated importance of DNA methylation as well as histone modifications in fibrogenesis, these new data highlight the importance of epigenetic modifications that accompany HSC activation, suggesting a major role for epigenetic mechanisms in HSC biology.

It is also worth noting the percentages of genome that are subject to DNA modifications. It is thought that around 10% of mammalian genome is methylated, whereas only around 0.75% is hydroxymethylated. However, such great variances in the overall amount of methylation/hydroxymethylation are not readily detectable using specific antibodies due to the major differences in affinities of 5-mC and 5-hmC antibodies. 5mC antibody has relatively poor affinity for its target, whereas the 5-hmC antibody shows exquisite specificity and very high affinity for its target. As such, direct comparisons of the overall amount of 5-mC and 5-hmC using slot blots are best used as the initial suggestion of the changes that 5-mC and 5-hmC undergo during the process of HSC activation *in vitro* or *in vivo*. These initial findings can then be expanded upon and supported by RRBS and other DNA methylation/hydroxymethylation quantitative methods.

Of note, the RRBS technology used in this study determines the methylation state of each individual CpG measured, and as such provides a single base resolution of DNA sequence.. Because the technology used provides the highest possible degree of resolution, the number of differentially methylated CpGs far exceed the number of genes affected by the change in DNA methylation. Likewise, the same gene can undergo major DNA methylation/hydroxymethylation remodeling, such that it contains many overlapping/unchanged CpG methylation sites in qHSC and aHSC, while it can also harbor numerous 5-mC/5-hmC sites that are only found in the gene in qHSC. Furthermore, there may be many 5-mC/5-hmC sites in that same gene that are uniquely found in aHSC. As an example, LOC310926 gene shows the highest number of overlapping 5-mC and 5-hmC sites in quiescent and activated HSCs. That same gene also has 305 uniquely methylated CpGs only found in the qHSC, whereas in aHSC, LOC310926 carries 251 uniquely methylated CpGs. Although we are able to map 5-mC and 5-hmC sites to highly defined chromosomal locations, due to the relatively poor annotation of the rat genome, a large number of RRBS identified differentially methylated genes are of unknown function. As such, our ability to define specific genomic locations where DNA methylation and hydroxymethylation alterations take place during the process of transdifferentiation, very much exceeds the availability of DNA sequence resources that

are necessary in order to truly appreciate our findings. Despite current limitations, the sequencing data can always be reanalyzed in the years to come, once the rat genome becomes adequately annotated.

A careful search of previously published work shows that DNA methylation in liver has been previously explored by groups other than our own. However, the two published studies either compare normal to fibrotic liver, in absence of purifying *in vivo* activated or control HSCs [22], while other study compares quiescent and day 3 HSC that are undergoing early initiation phase of *in vitro* activation [23]. As such, this is the first study that considers the DNA methylation differences between *in vivo* activated or control HSCs, which are the cells that drive and determine fibrosis progression *in vivo*. This study therefore forms a major incremental step in our understanding of HSC biology.

It is important to note that methylation and hydroxymethylation alterations at specific CpGs that occur with HSC activation may be dynamic and possibly multidirectional. The patterns of 5-mC and 5-hmC that we have uncovered may be a snapshot of a highly active state that might be much more dynamic than previously thought. It is possible that particular CpGs become de novo methylated in order to recruit chromatin remodelling complexes that silence gene expression. Another possibility is that some CpGs become de novo methylated in order to become hydroxymethylated, which would lead to transcriptional activation or an increase in transcriptional elongation, giving directly opposite effect to the aforementioned gene silencing. On the other hand, sites that are already methylated in quiescent HSC may become hydroxymethylated so to induce transcriptional activity or alternatively, further modification by thymine DNA glycosylase leading to generation of unmethylated CpGs. Further studies are required that would take longitudinal snapshots of DNMT/TET activity and associated changes in DNA methylation/hydroxymethylation at targeted CpGs, which would inform about the overall flux in chemical modification of DNA during HSC activation. It is also possible that DNMTs/TETs enter cyclic oscillations at particular CpGs, this in turn allowing for rapid changes towards activation or repression of transcription given appropriate external stimulus.

Taken together, our data suggest that DNA methylation/hydroxymethylation is likely to be an important step in HSC activation and therefore fibrogenesis. This is in agreement with our previous study, which showed that inhibition of 5-mC using 5-azadC prevents HSC activation [11]. Here, we are beginning to unravel the changes that lead to alteration in DNA methylation pattern, including expression of enzymes that regulate the process as well as the genomic sites that are subject to DNA methylation and hydroxymethylation change. DNMTs and TETs therefore may be potential new targets for treatment of fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
aHSC	activated hepatic stellate cells
ALD	alcoholic liver disease
DNMT	DNA methyltransferase
HSC	hepatic stellate cells
MeCP2	methyl-CpG binding protein 2
NHL	normal human liver
PSC	primary sclerosing cholangitis
qHSC	quiescent hepatic stellate cells
rHSC	rat hepatic stellate cells
siRNA	small interfering RNA
αSMA	alpha smooth muscle actin
TET	ten eleven translocation methylcytosine dioxygenase
TGF-β1	transforming growth factor beta1
TIMP-1	tissue inhibitor of metalloproteinase-1

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Figure 1. Levels of 5mC, 5hmC and their regulatory enzymes in animal liver fibrosis models (A) mRNA and (B) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b were assessed in liver samples obtained from controls or rats on MCD diet. (C) Genomic DNA samples were used to evaluate global levels of 5-hmC and 5-mC. Methylene blue staining was used as loading control. The results were analysed using GelAnalyzer software and plotted on the graph. (D) mRNA and (E) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b and genomic DNA (F) were assessed in liver samples obtained from livers of sham operated or bile duct ligated rats. (G) mRNA and (H) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b and genomic DNA (I) were assessed in liver samples obtained from rats treated with olive oil as a control or chronic CCl₄ injured rats. Error bars represent mean values $\pm$ standard error of the mean (SEM) * p <0.05; ** p <0.005; *** p <0.001.

Figure 2. Levels of 5mC, 5hmC and their regulatory enzymes in human liver diseases
 (A) mRNA and (B) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b were analysed in normal human liver samples or from freshly explanted primary sclerosing cholangitis liver (C) Genomic DNA samples were used to evaluate global levels of 5-hmC and 5-mC. Methylene blue staining was used as loading control. The results were analysed using GelAnalyzer software and plotted on the graph. (D-F) analysis of normal human livers or explanted alcoholic liver disease livers as already described in A to C (n=5). Error bars represent mean values $\pm$ standard error of the mean (SEM) * $p < 0.05$; *** $p < 0.001$.

Figure 3. Levels of 5mC, 5hmC and their regulatory enzymes in *in vivo* isolated HSCs from olive oil (control) or chronic CCl₄ treated rats

(A) cytopins were carried out and stained for α SMA, cells were counted and number of α SMA positive cells expressed as percentage of total cell number. SDS-PAGE was carried out on the protein samples and immunoblotted for TGF β 1, GAPDH was used as loading control. (B) mRNA and (C) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b were analysed from *in vivo* activated or control HSCs (D) Genomic DNA samples were used to assess global levels of 5-hmC and 5-mC. Error bars represent mean values $\pm$ standard error of the mean (SEM) * p <0.05; *** p <0.001.

Figure 4. Levels of 5mC, 5hmC and their regulatory enzymes in isolated rat HSCs, which were allowed to spontaneously activate on plastic, *in vitro*
 (A) protein levels of TET2, TET3, DNMT1, DNMT3a, DNMT3b were assessed in quiescent (day 1) and activated HSC (day 10) samples. (B) mRNA was isolated from quiescent HSCs (day 1) or HSCs cultured for 3, 5, 7 and 10 days and level of DNMT1 measured, (C) DNMT3a, (D) DNMT3b, (E) TET2 and (F) TET3 (G-H) gDNA was extracted from different stages of *in vitro* activation (days 1, 3, 5, 7 and 10) and used to measure the amount of 5-hmC (G) or (H) 5-mC by slot-blot. The results were analysed using GelAnalyzer software. Error bars represent mean values $\pm$ standard error of the mean (SEM) * $p < 0.05$.

Figure 5. Genome-aligned 5-mC sites, identified by high-throughput sequencing (RRBS-Seq), were plotted by chromosomal location, displaying their respective methylation percentage (A) Overlapping CpG methylated sites were identified by overlaying the 5-mC sites from the control and CCl₄ groups. (B) The identified overlapping CpG methylated sites were allocated into genomic region with a defined annotated position within the genome. Genomic features corresponding to 5-mC methylation sites identified in (A) were plotted as a percentage of total. (C) Unique 5-mC sites found in the control HSCs only, were plotted onto the genome map (D) Genomic features corresponding to 5-mC methylation sites identified in (D) were plotted as a percentage of total. (E) Unique 5-mC sites found in the *in vivo* activated HSC only, were identified by removing the overlapping sites from the remainder of the 5m-CpG sites from each sample (F) Genomic features corresponding to 5-mC methylation sites identified in (E) were plotted as a percentage of total.

Figure 6. 5-hmC sites, identified by high-throughput sequencing (oxBS-Seq), were plotted by chromosomal location

(A) The amount of hydroxymethylation for each site was obtained by subtracting the methylation obtained from RRBS-Seq from the oxBS-Seq. Overlaying the control and CCl₄ groups identified overlapping sites between the two groups which were plotted onto the genome map. Sites coloured in red show a significant ($p < 0.05$) log2fold-change in hydroxymethylation. (B) Genomic features corresponding to 5-hmC methylation sites identified in (A) were plotted as a percentage of total. (C) Unique 5-hmC sites found in the control HSCs were plotted onto the genome map (D) Genomic features corresponding to 5-

hmC methylation sites identified in (D) were plotted as a percentage of total. (E) Unique 5-hmC sites found in the *in vivo* activated HSC CCl₄ were identified by removing the overlapping sites from the remainder of the 5-hmC sites from each sample. (F) Genomic features corresponding to 5-hmC methylation sites identified in (E) were plotted as a percentage of total.

Figure 7. Rat HSC were transfected with control and DNMT3a siRNA

(A) mRNA levels of DNMT3a were quantified by qRT-PCR in cells treated with control or DNMT3a siRNA. (B) Twenty µg of whole cell protein extract from two preparations of rat HSCs treated with DNMT3a siRNA were separated by SDS-PAGE and immunoblotted for DNMT3a and GAPDH as loading control. qRT-PCR analysis of (C) TGFβ1, (D) Collagen 1A1 and (E) α-SMA expression in cells treated with control or DNMT3a siRNA was performed. Error bars represent mean values ± standard error of the mean (SEM) *p<0.05; **p<0.01; ***p<0.001. (F) DNA methylation pattern at Collagen 1A1 promoter as determined by RRBS was plotted in qHSC and aHSC, as shown by red blocks/bars. DNA methylation becomes erased in aHSCs. (G) DNA methylation pattern at α-SMA as determined by RRBS was plotted in qHSC and aHSC, as shown by red blocks/bars. DNA methylation largely becomes erased in aHSCs.

Table 1

Quantitative PCR primers

Gene	Forward and reverse primer pair sequences	Annealing temperature (°C)
Rat TET2	CCCAGGAAAGCACAGACATAG	58
	AGCACCATTAGGCATTAGCAC	
Rat TET3	TCAGCAACACCTTCATCACAA	58
	TTTTCCTTGGGTGGTTTGTC	
Rat DNMT1	GAGCCCAGCCAGAGTATGC	58
	ATGGCAGAAGGAGGAACAGT	
Rat DNMT3a	CTGGCAAGGCTGTGGAGGTG	58
	GGTGGGGGTGGGGCATAAGC	
Rat DNMT3b	GCTGGTGGCTCTGGGTCTGT	60
	AGGGCGGCTGGGAAGGTCT	
Rat F4/80	GAAAACAAAAGTGCTCCAGTG	57
	ATAGTGCCAGAGTAGATGTC	
Rat Albumin	CCCCAGAACTCCTTTACTATG	57
	AGGCTCTCTCTCCAAATCTCT	
Rat GAPDH	GGGTGTGAACACGAGAAAT	58–60
	ACTGTGGTCATGAGCCCTTC	
Rat β -actin	AGCCATGTACGTAGCCATCC	58–60
	CTCTCAGCTGTGGTGGTGAA	
Human TET2	CTAAGTCCATTCTGATACCA	58
	ATTCTGGCTTCCCTTCATACA	
Human TET3	CCATCCAGCAACCCCGTCTTC	58
	CAAACCTCAGCACCACCGTAGG	
Human DNMT1	AGCCTGAGTGTGGGAAATGT	58
	TCGTCATCTGCCTCCTTCAT	
Human DNMT3a	ACGACAGCGATGAGAGTGAC	58
	TGGTGGAGGTGGTGCGTAGG	
Human DNMT3b	CAAAGACCGAGGGGATGAAG	60
	AAAGAGAGGGTGGAAGGACA	
Human GAPDH	GAAGGTGAAGTCGGAGTC	58–60
	GAAGATGGTGATGGGATTC	
Rat Collagen 1A1	TTCACCTACAGCACGCTTGTG	55
	GATGACTGTCTTGCCCAAGTT	
Rat α SMA	CATGTCGTCCAGTTGGTGAT	55
	CGAAGCGCAGAGCAAGAGA	

Table 2

Genes with 5-mC methylation changes in quiescent HSCs

Symbol	NCBI GenBank entry number	Gene name
Loc310926	NM_001025002	hypothetical protein LOC310926
RT1-CE7	NM_001008845	similar to RT1 class I, CE11; RT1 class I, CE11; RT1 class I, CE7
RT1-CE3	NM_001008841	RT1 class I, CE3
RT1-CE4	NM_001008842	RT1 class I, CE4
Zfp746	NM_001100852	zinc finger protein 746
Chat	NM_001170593	choline acetyltransferase
Rhbdfl	NM_001030034	rhomboid 5 homolog 1 (Drosophila)
Kcnj5	NM_017297	potassium inwardly-rectifying channel, subfamily J, member 5
Chmp4c	NM_001017466	chromatin modifying protein 4C
Ddhd1	NM_001033066	DDHD domain containing 1
Tbc1d5	NM_001134762	TBC1 domain family, member 5; similar to TBC1 domain family, member 5
Akap9	NM_001037093	A Kinase (PRKA) Anchor Protein 9
Corin	NM_182473	corin, serine peptidase
Gkap1	NM_001012160	G kinase anchoring protein 1
Tmem123	NM_001014205	transmembrane protein 123
Prrx2	NM_001105739	paired related homeobox 2
Phlpp1	NM_021657	PH domain and leucine rich repeat protein phosphatase
Phlpp1	NM_021657	PH domain and leucine rich repeat protein phosphatase
Als2	NM_001013413	amyotrophic lateral sclerosis 2 (juvenile) homolog (human)
Fam13b	NM_001106158	family with sequence similarity 13, member B1
Vav2	NM_001106563	vav 2 guanine nucleotide exchange factor
Vash2	NM_001109082	vasohibin 2
Rhoq	NM_053522	ras homolog gene family, member Q
Usp49	NM_001136470	ubiquitin specific peptidase 49
Trim71	NM_001191801	Tripartite Motif Containing 71, E3 Ubiquitin Protein Ligase
Trim3	NM_031786	tripartite motif-containing 3
Nrxn2	NM_053846	neurexin 2
Fam101b	NM_001007611	family with sequence similarity 101, member B
Zfp655	NM_001008362	zinc finger protein 498; zinc finger protein 655; zinc finger with KRAB and SCAN domains 5

Table 3

Genes with 5-hmC hydroxymethylation changes in quiescent HSCs

Symbol	NCBI GenBank entry number	Gene name
Loc310926	NM_001025002	hypothetical protein LOC310926
Chat	NM_001170593	choline acetyltransferase
Ctdspl	NM_001106865	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like
Fam19a5	NM_001191991	Family With Sequence Similarity 19 (Chemokine (C-C Motif)-Like), Member A5
Spry1	NM_001106427	sprouty homolog 1, antagonist of FGF signaling (Drosophila)
Camta1	NM_001195559	Calmodulin Binding Transcription Activator 1
Cul1	NM_001108627	cullin 1
Macf1	NM_001135758	microtubule-actin crosslinking factor 1
Zfp746	NM_001100852	zinc finger protein 746
Zmiz1	NM_001108393	zinc finger, MIZ-type containing 1
Nkx6-1	NM_031737	NK6 homeobox 1
Palm	NM_130829	paralemmmin
Ikzf1	NM_001107237	similar to DNA-binding protein Ikaros form 1 - mouse
Muc19	NM_001309489	Mucin 19, Oligomeric
Phf21b	NM_001130680	PHD finger protein 21B
AABR07030897.1	NM_001134688	coiled-coil domain containing 40
Chrm5	NM_017362	cholinergic receptor, muscarinic 5
Stim2	NM_001105750	stromal interaction molecule 2
Arhgap22	NM_001107297	Rho GTPase activating protein 22
Rreb1	NM_001107348	ras responsive element binding protein 1
Nrxn2	NM_053846	neurexin 2
Kcnj5	NM_017297	potassium inwardly-rectifying channel, subfamily J, member 5
Nfkb1	NM_001276711	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1
Zdhhc14	NM_001039343	zinc finger, DHHC-type containing 14
RGD1560225	NM_001244933	RNA polymerase II regulatory region sequence-specific DNA binding
Il17ra	NM_001107883	interleukin 17 receptor A
Man2a1	NM_012979	mannosidase, alpha, class 2A, member 1
Phlpp1	NM_021657	PH domain and leucine rich repeat protein phosphatase
Phlpp1	NM_021657	PH domain and leucine rich repeat protein phosphatase

Table 4

Genes with 5-mC methylation changes in activated HSCs

Symbol	NCBI GenBank entry number	Gene name
Loc310926	NM_001025002	hypothetical protein LOC310926
Tbc1d5	NM_001134762	TBC1 domain family, member 5; similar to TBC1 domain family, member 5
NA	NM_001107663	similar to MEGF6
Stam2	NM_001012085	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2
Sult1c2	NM_001013177	sulfotransferase family, cytosolic, 1C, member 2; sulfotransferase family, cytosolic, 1C, member 2a
Vav2	NM_001106563	vav 2 guanine nucleotide exchange factor
Csrnp2	NM_001108113	cysteine-serine-rich nuclear protein 2
Sertad4	NM_001108351	SERTA domain containing 4
Dcun1d4	NM_001108359	DCN1, defective in cullin neddylation 1, domain containing 4 (<i>S. cerevisiae</i>)
RGD1559896	NM_001109134	similar to RIKEN cDNA 2310022B05
Mki67	NM_001271366	Marker Of Proliferation Ki-67
Insr	NM_017071	insulin receptor
Rpl5	NM_031099	ribosomal protein L5
Synj1	NM_053476	synaptojanin 1
Nr2f2	NM_080778	nuclear receptor subfamily 2, group F, member 2
Atg3	NM_134394	ATG3 autophagy related 3 homolog (<i>S. cerevisiae</i>)
Arhgap39	NM_173122	preoptic regulatory factor-2

Table 5

Genes with 5-hmC hydroxymethylation changes in activated HSCs

Symbol	NCBI GenBank entry number	Gene name
Loc310926	NM_001025002	hypothetical protein LOC310926
RT1-CE7	NM_001008845	similar to RT1 class I, CE11; RT1 class I, CE11; RT1 class I, CE7
Sult1c2a	NM_001013177	sulfotransferase family, cytosolic, 1C, member 2; sulfotransferase family, cytosolic, 1C, member 2a
Sult1c2a-var1	NM_001013252	signal recognition particle receptor, B subunit; transferrin
Eif4g2	NM_001017374	similar to Eif4g2 protein; eukaryotic translation initiation factor 4, gamma 2; eukaryotic translation initiation factor 4, gamma 2, pseudogene 1
Mib1	NM_001107405	mindbomb homolog 1 (Drosophila)
Mpz1	NM_001007728	myelin protein zero-like 1
Usp3	NM_001025424	ubiquitin specific peptidase 3
Endog	NM_001034938	endonuclease G
Mml3	NM_001107675	mastermind like 3 (Drosophila)
Csrnp2	NM_001108113	cysteine-serine-rich nuclear protein 2
NA	NM_001109175	cyclin O
Ankrd33b	NM_001191565	Ankyrin Repeat Domain 33B
Cnnm3	NM_001106901	cyclin M3
Irf2bp1	NM_001107483	interferon regulatory factor 2 binding protein 1
Dlg1	NM_012788	discs, large homolog 1 (Drosophila)
Shh	NM_017221	sonic hedgehog
Hmga2	NM_032070	high mobility group AT-hook 2
Klf2	NM_001007684	Kruppel-like factor 2 (lung)
RT1-CE10	NM_001008833	RT1 class I, CE10
RT1-CE3	NM_001008841	RT1 class I, CE3
Plekhh3	NM_001037202	pleckstrin homology domain containing, family H (with MyTH4 domain) member 3
Nuak2	NM_001007617	NUAK family, SNF1-like kinase, 2
NA	NM_001107742	solute carrier family 43, member 1
Zbtb12	NM_001134991	Ng35 pseudogene
Brpf1	NM_001191572	Bromodomain And PHD Finger Containing, 1
Kif2a	NM_053376	Kinesin Heavy Chain Member 2A
Rad18	NM_001077673	RAD18 homolog (S. cerevisiae)
Rab4a	NM_013019	RAB4A, member RAS oncogene family