Research Article

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Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape

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Background & Aims: DNA methylation (5-mC) 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 (5-hmC) 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 (RRBS) 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 5-mC/5-hmC 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.

Keywords: DNMTs; TETs; Hepatic myofibroblasts; Liver fibrosis; Epigenetics.
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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 beta 1; TIMP-1, tissue inhibitor of metalloproteinase-1.

Introduction

Hepatic stellate cells (HSC) are the predominant cellular origin of fibrogenic alpha smooth muscle actin (αSMA) 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 pro-fibrogenic 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 transforming growth factor beta 1 (TGFβ1) and platelet derived growth factor, 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 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 metyltransferases (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 HSCs 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. PPARα, PPARγ, TGFβ1 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 transdifferentation. In addition, we show that reducing the expression of DNMT3a leads to attenuation of pro-fibrogenic phenotype in a HSC.

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 350 g 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% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 16% fetal calf serum. Cells were harvested on day of the isolation (quiescent (q)HSC) or after 10 days of the culture (activated (a)HSC).

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 CCl_4/olive oil in a 3:1 [vol/vol] ratio at 2 μl per g body weight. Twenty-four hours after the last CCl_4 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 CCl_4/olive oil in a 3:1 [vol/vol] ratio at 2 μl per gram of 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 (RT-PCR) assay was established for relative quantification of rat and human TET and DNMT enzyme mRNAs. 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 extra cellular matrix 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 μl, containing 20 ng of cDNA template, 6.5 μl of SYBR Green JumpStart Taq ReadyMix (Sigma), and 20 pmols of forward and reverse primers (Table 1). The PCR reaction was carried out on a 7500 Fast RT-PCR System (Applied Biosystem, Warrington, Cheshire, UK) with the following parameters: 1 cycle at 95 °C for 10 s followed by 40 cycles at 95 °C for 10 s, 55–60 °C (primer pair specific annealing temperature, see Table 1 and 2) for 30 s, and finally 72 °C for 30 s. 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 $2^{\Delta\Delta Ct}$ method.

Immunohistochemistry

Activated HSC isolated form CCl_4 treated rats were spun down using the cytocentrifuge at 1000 rpm for 5 min with medium acceleration. Slides were air-dried for 30 min, fixed in 10% formalin for 30 min. Slides were then permeabilized with 1% saponin/0.5% BSA/PBS for 10 min at room temperature, washed, blocked with 3% BSA for 10 min and incubated with the rabbit monoclonal anti-actin alpha smooth FITC antibodies (Sigma, F3777; dilution factor 1:100) for 2 h at room temperature. The cells were washed and incubated with DAPI special formulation NucBlue live ready probes reagent (Life Technologies) for 10 min 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 1 h, 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), TGFβ (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 genomic DNA (gDNA) per sample (control, bisulfite and oxidative bisulfite conversion) was digested overnight with MspI restriction enzyme