CHAPTER 7

Targeting TET methylcytosine dioxygenases induces a wide spectrum of anti-fibrotic effects

Rutger A.F. Gjaltema Saskia de Rond, Pytrick G. Jellema, Carlot Kruse, Marianne G. Rots and Ruud A. Bank

Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, The Netherlands

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ABSTRACT

DNA demethylation through oxidation to 5-hydroxymethylcytosine (5hmC) by Ten-Eleven Translocation (TET) methylcytosine dioxygenases has previously been linked to development of fibroproliverative disorders. However, the individual roles of the three known TET enzymes (TET1-3) remain unsolved. Here we characterized the contribution of these enzymes in human fibroblast activation and extracellular matrix expression. We found that expression of each TET enzyme was differentially affected by TGFβ1 signaling, and their depletion resulted in distinctive transcriptional effects of activated fibroblast markers and genes related to collagen homeostasis. Cumulatively, TET2 contained the most prominent pro-fibrotic activity, followed by TET1, while TET3 had repressive effects on selective fibrotic mediators. In addition to 5hmC, TETs can influence transcription independent of their catalytic activity by recruiting enzymes such as the O-GlcNac enzyme OGT. Depletion of OGT did not match the TET-related transcriptional effects. Furthermore, genome-wide distribution of 5hmC detected by 5hMeDIP-seq, revealed a strong presence of gene body linked 5hmC in activated fibroblasts. The overall TET-related transcriptional effects in activated fibroblasts are thus related to its 5hmC activity. Together, these findings shed new light on the roles of TET enzymes and 5hmC in fibroblast activation and ECM synthesis, and reveal targeting TET enzymes as a promising anti-fibrotic target.
INTRODUCTION

Specialized cells secrete growth factors upon tissue injury, triggering activation and initiating the wound healing process, followed by wound contraction and scar tissue formation. In certain cases, wound healing shows an uncontrolled continuation, resulting in fibrotic conditions that can end in organ failure and the need for organ transplantation (1). A typical characteristic of fibrosis is the enhanced deposition of extracellular matrix (ECM), most notably collagen type I, and subsequent stiffening of the affected tissue. This is essentially mediated by increased levels of ECM expression by activated fibroblasts (i.e. myofibroblasts) combined with a reduced proteolytic degradation due to the misbalance of collagenases (MMPs) and their inhibitors (TIMPs), as well as enhanced collagen cross-linking. In the stroma of solid tumors, activated fibroblasts (here known as cancer-associated fibroblasts) can create a pro-metastatic environment, where enhanced ECM secretion and cross-linking increases stroma stiffness, thereby facilitating metastasis of tumor cells (2-4).

TGFβ1 is one of the most prominent inducers of the differentiation of fibroblasts into myofibroblasts. Various pathways (e.g. ALK/SMAD signaling) and molecular mechanisms induced by TGFβ1 signaling influence gene expression and cellular processes leading to myofibroblasts (5). These contractile cells exhibit an upregulated expression of the markers αSMA and SM22α, and show excessive ECM production. Understanding how activation of fibroblasts into myofibroblasts takes place and how collagen synthesis is regulated at the transcriptional level may provide clues to design new treatments against a broad range of pathologies (e.g. fibrosis and cancer).

Epigenetic alterations have been recognized as important contributors to the transcriptional state of a gene. Most notably, the effects of DNA methylation and posttranslational histone modifications and their relation to transcriptional regulation and genome integrity are studied extensively. A simplified explanation for the role of DNA methylation at regulatory elements such as promoters and enhancers is, that it can control access and binding of transcription factors that induce transcriptional activation (6). DNA methylation at selective cytosines (5mC) in a CpG order by DNA methyltransferases is essential for many cellular processes. Alterations in DNA methylation are important contributors to gene deregulation but also to genomic instability, and leads to the establishment of various diseases such as cancer and fibrosis (7, 8).

DNA methylation is a chemical modification that can be actively cleared by three members of the Ten-eleven translocation methylcytosine dioxygenases (TET1-3) that oxidize 5mC into 5hmC (9, 10). Although formation of 5hmC is the primary activity of TETs (11), iterative oxidation of 5hmC by TETs can further generate 5fC and 5caC that can be subjected to removal by thymine DNA glycosylase in conjunction with BER to realize an unmodified cytosine (12-14). However, the majority of the detected 5mC oxidation products is 5hmC; it is about 10- to 100-fold more abundant than 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) (12, 13, 15-17), and is a stable oxidation product that gradually increases after DNA replication (18). Although the intrinsic transcriptional value of 5hmC as a separate modification is largely unclear,
TET enzyme-related transcriptional effects can be classified into two principles. First, oxidized 5mC can prevent the recruitment of specific methylation-recognizing repressive complexes directly or through 5hmC binding proteins (19, 20), which either directly or indirectly affect transcriptional activity. The other is related to recruitment of enzymes or other proteins by TETs that affect chromatin function by e.g. modifying histones, a recruitment that is mostly independent on the oxidative activity of TETs. Examples are the GlcNAc modifying enzyme OGT (21-24), SIN3A (21, 24), and histone deacetylase complexes (24, 25).

DNA methylation changes have been observed in fibrotic conditions, both genome-wide as well as with regard to specific fibrosis-related loci (26-31). Recent evidence supports a role for certain TET family members and 5hmC in fibrosis (32-35). However, these reports did not focus on fibroblast activation and are lacking evidence about the contribution of each of the TET family members in myofibroblast formation. Here we report the contributions of individual TET family members and 5hmC in both fibroblast activation and collagen biosynthesis. We found that TET family members have both overlapping as well as unique transcriptional effects and that that is in line with the genome-wide increase of 5hmC. We also demonstrate that TET2 is crucial for the initiation of these fibrotic processes in both skin and lung fibroblasts, whereas TET3 can be considered as a more anti-fibrotic moderator. Our results add to the mounting evidence that the DNA methylation status is an important contributor to fibroblast activation, and pinpoints TET2 as an attractive target for potent future anti-fibrotic therapies.

MATERIALS AND METHODS

Cell culture and TGFβ1 stimulation
Primary normal human dermal fibroblasts (ScienCell) and primary human pulmonary fibroblasts (ScienCell) were seeded in DMEM (Lonza) supplemented with 10% (heat inactivated) fetal bovine serum (FBS) (Thermo Scientific), L-glutamine and penicillin/streptomycin (Gibco) at 5% CO₂ and 37°C. Prior to experimental conditions, the cells were cultured for 18 hours in starvation medium: DMEM supplemented with 0.5% FBS, L-glutamine, penicillin/streptomycin (Gibco) and 0.17 mM vitamin-C as it is an essential cofactor for collagen synthesis and TET activity (51). Recombinant human TGFβ1 (PeproTech) was reconstituted in 10 mM citric acid pH 3.0 and diluted 20-fold in PBS-BSA (0.1%) to a concentration of 5 μg ml⁻¹. During TGFβ1 stimulation of fibroblasts, reconstituted TGFβ1 was added to starvation medium and was refreshed daily.

TGFβ1 pathway inhibition and mRNA knockdown
SMAD3 specific phosphorylation inhibitor (SIS3) and ALK5 phosphorylation inhibitor (SB431542) (both Sigma-Aldrich) were dissolved in DMSO. Fibroblasts cultured in starvation medium were pretreated for one hour with 20 μM SB431542 or 10 μM SIS3. Afterward, the medium was replaced with starvation medium containing similar concentrations of inhibitors and supplemented with 10 ng/ml TGFβ1 or vehicle control and was incubated for 24 hours. For knockdown experiments, esiRNA (Sigma-Aldrich)
against TET1, TET2, TET3, OGT or a control esiRNA were transfected into fibroblasts with RNAiMax (Thermo Scientific). The day after, the cells were serum-starved for 18 hours and subsequently stimulated with TGFβ1 or treated with vehicle control.

**RNA isolation and qRT-PCR analysis**

Total RNA was isolated from fibroblasts with a RNeasy Plus Mini Kit (Qiagen). Equal concentrations of RNA were reverse transcribed with the RevertAid kit (Thermo Scientific) to obtain cDNA. Primers (Table 1) mixed with SYBR Green (Roche) were used to quantify mRNA expression with a VIA7™ real-time PCR platform (Applied Biosystems). Expression values were calculated compared to YWHAZ housekeeping gene expression using the ΔΔCt method.

**Table 1. Primers used for qRT-PCR detection of mRNA expression**

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<th>Gene ID</th>
<th>Fw 5' - 3'</th>
<th>Rev 5' - 3'</th>
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<tr>
<td>ACTA2</td>
<td>CTGTCCAGCCCATCCTCTCAT</td>
<td>TCATGATGCTGTGTAGGTGTT</td>
</tr>
<tr>
<td>COL1A1</td>
<td>GCCCTCAAGGTATGTGGAGC</td>
<td>ACCTTGTCTGCCAGGTTCAC</td>
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<td>COL1A2</td>
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</tr>
<tr>
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<td>CCCACGTGCGAGGAGGAG</td>
</tr>
<tr>
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</tr>
<tr>
<td>MMP13</td>
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<tr>
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<td>TATGAATGGGAAGCAGATTT</td>
</tr>
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<td>SNAI1</td>
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<td>SOX9</td>
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<td>YWHAZ</td>
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**Low density array detection of collagen synthesis gene expression**

Custom-made microfluidic card-based low density arrays (LDAs) were obtained from Applied Biosystems and contained individual primer probe set to detect mRNA expression of 43 genes related to collagen biosynthesis and homeostasis. Hundred ng of converted RNA from TET1-3 and control KD experiments were diluted in TaqMan PCR master mix (Applied Biosystems), and were run on the VIA7TM real-time PCR
platform according to the manufacturers guidelines. Expression values from biological duplicates were calculated against GAPDH housekeeping gene and Log2 values (Log2(ΔΔCt siTET/ΔΔCt siCtrl)) were plotted in a heatmap using the CIMminer tool (52).

Western blotting and immunofluorescence
Protein was extracted from cells with RIPA buffer (Thermo Scientific) supplemented with proteinase inhibitor cocktail. Equal concentrations of protein was separated on SDS-PAGE and blotted on PVDF membranes. After blocking with 5% skimmed milk (in TBS-T), the membranes were incubated for two hours at room temperature with primary antibodies against TET2, TET3, αSMA, YWHAZ (Abcam), and followed by HRP conjugated secondary antibodies (Dako). Signals were detected with ECL. For immunofluorescence, fibroblasts were seeded in chamber slides (Thermo Fischer Scientific), transfected with esiRNA and stimulated with TGFβ1 or control for 4 days. The cells were fixed with acetone/methanol (1:1 v/v) for 10 minutes at -20°C and left to air dry afterwards. After rehydration in PBS, the cells where blocked in PBS with 10% donkey serum and followed by incubation with primary antibodies against collagen Iα1 (Abcam) or αSMA (Dako) in PBS with 2% BSA. Afterwards, the cells were incubated with isotype-specific Biotin and followed by Streptavidin-Cy3 with DAPI. All imaging was performed with a Leica fluorescence microscope and software.

5hmMeDIP-seq library preparation
Genomic DNA isolated from fibroblasts treated with TGFβ1 or control for 24 hours (5hmC) was sonicated to obtain fragments between 100-350 bps. 5hmMeDIP-seq libraries were generated with a NEXTflexTM Methyl-seq (MeDIP) kit (BIOO scientific) following the manufacturers guidelines. In short, overhangs from fragmented DNA were blunted, filled with poly-A and ligated with barcoded adapters (BIOO scientific). Barcoded single stranded DNA was processed further with a MeDIP-kit (Zymoresearch) with a 5hmC antibody (Active Motif) to obtain 5(h)mC enriched fragments. After PCR amplification libraries were size selected from a 4% agarose gel and confirmed with a Bioanalyzer (Agilent). Next the libraries were multiplexed and clustered using standardized in-house protocols, and deep sequenced to approximately 25 million reads with an Illumina HiSeq 2500 sequencing platform (Genome Analysis Facility UMCG).

Next generation sequencing data analysis
Raw Fastq files or downloads from USCS database (normal human dermal fibroblast ChIP-seq data from H3K4me1; Roadmap-Epigenomics GEO:GSM1003526) were subjected to quality control by FastQC, only when quality was low (for H3K4me1) the sequences were trimmed to obtain high quality reads. The reads were preprocessed by removing sequence artifacts and subsequently mapped to the human reference genome build hg19 with BWA (v0.1). Reads that did not map uniquely to the reference genome were discarded. PCR duplicates of mapped reads were removed with Picard and remaining peaks were called with MACS (v1.0.1). All previous steps were performed with the Galaxy platform (53). Peaks for 5hmC or H3K4me1 showing intersections in both control and TGFβ1 stimulated cells were generated with the
TET enzymes regulate fibrosis

- operate on genomic intervals - tool in Galaxy. USCS Annotations of human (hg19) CpG islands (ExtUnmasked) were imported into Galaxy from the USCS database. CpG shore maps were generated by collecting 2000 bp flanks from both ends of the CGI. To reveal genomic distributions of all genes (including non coding transcripts) or only protein coding genes of called peaks, bed files were lifted to hg38 with USCS lift over tool and analyzed in PAVIS (54) with settings for upstream length 5000 and downstream length 2000 bp. Gene IDs (peak location annotations) were obtained with PAVIS (55), and used as input for Panther to generate ontologies from genomic associations.

Statistics
All data is represented as average ± S.E.M. and were analyzed by two-tailed unpaired Student’s t-test. For each parameter of all data, *P<0.05, **P<0.01, ***P<0.001.

RESULTS

The TGFβ1 pathway differentially regulates TET dioxygenases
TGFβ1, a strong profibrotic cytokine, was used to induce myofibroblast differentiation (Figure 1A). TET1 mRNA expression was reduced by TGFβ1 treatment, peaking at 24

Figure 1. TET dioxygenase expression is affected by TGFβ1 signaling.
(a) mRNA expression of TET1-3 from fibroblasts stimulated with TGFβ1 or control up to 96 h (n=3, *P<0.05, **P<0.01). (b) Western blot detection of TET2, TET3 and αSMA protein level from dermal fibroblasts stimulated with TGFβ1 up to 96 h or control. YWHAZ was used as a loading control. (c) mRNA expression of TET1-3 from dermal fibroblasts co-treated with inhibitors of the TGFβ1 pathway (SB431542 or SIS3), and subsequently TGFβ1 or control for 24 h (n=3, *P<0.05, **P<0.01).
hours, whereas TET3 mRNA expression increased and peaked around 24 hours as well. Both TET1 and TET3 returned to baseline levels at 96 hours. TET2 mRNA levels did not change upon TGFβ1 stimulation. When probed at the protein level, TET2 was reduced at 24 hours and 48 hours when treated with TGFβ1, and returned to levels seen for unstimulated fibroblasts at 96 hours (Figure 1B). TET3 protein followed the same pattern as its mRNA expression. Thus, TET2 is responsive to TGFβ1 only at the protein level, whereas TET3 is responsive at both the mRNA and protein level. Interestingly, changes in TET expression peaked around 24 hours, before αSMA protein expression and myofibroblast differentiation was detected as early as 48 hours (Figure 1B).

Since mRNA expression of the TET family members had different responses in reaction to TGFβ1 stimulation, we wanted to assess whether these differences are related to different TGFβ1 pathway components. To explore this, dermal fibroblasts were treated for 24 hours with TGFβ1 in the presence of the inhibitor SIS3 (to prevent phosphorylation of SMAD3), or in the presence of the inhibitor SB431542 (to inhibit the activation of the TGFβ type I receptor ALK5). TET1 mRNA downregulation was induced by TGFβ1 via ALK5 signaling but was not influence by the TGFβ1 pathway transcriptional regulator SMAD3, which on its turn did affect basal expression of TET1 in unstimulated cells (Figure 1C). As expected, TET2 mRNA expression was not affected by inhibiting either SMAD3 or ALK5. TET3 mRNA expression seemed to follow a typical TGFβ1 pathway signature, as its TGFβ1-induced expression is affected both by ALK5 and SMAD3.

**TET Knockdown show strong but variable anti-fibrotic effects**

DNA methylation has been noted to play a role in the expression of fibrosis-related genes. Therefore we reasoned that TET enzymes may have a role in fibroblast activation and ECM synthesis, possibly by performing 5hmC to activate target genes. To address this, we made use of esiRNA to knock-down (KD) TET1, -2 or -3 mRNA expression (supplemental Figure 1) while cultured with or without TGFβ1 for two days to investigate its effect on fibroblast activation Figure 2A). ACTA2 (also known as αSMA) mRNA was markedly reduced when TET2 was KD; in contrast, it was enhanced after KD of TET1. COL1A1 mRNA expression was reduced in both conditions when either TET1 or TET2 was depleted. This suggest that both TET enzymes prime the chromatin for activation by other factors, such as transcription factors recruited in response to TGFβ1 signaling to drive the expression. Another myofibroblast marker TAGLN (also known as SM22α) is downregulated in activated fibroblasts when either TET2 or TET3 are depleted. Immunocytochemistry detection of αSMA of TET depleted fibroblasts, confirmed the observations seen for mRNA (Figure 2B). Interestingly, Collagen type I protein levels did not decrease after TET1 depletion as compared to control esiRNA, even despite being reduced at mRNA levels. Similar to mRNA expression, TET2 depletion also resulted in a reduced collagen type I deposition (Figure 2C). Finally, as was seen for mRNA, TET3-depleted dermal fibroblasts did not have visible differences of Collagen type I levels compared to fibroblasts transfected with control esiRNA. We conclude from the above that TET2 seems to have the strongest effect on fibroblast activation. To see if this effect on fibroblast activation is a general phenomenon, we repeated TET2 KD experiments in pulmonary fibroblasts. Also here the three markers
Figure 2. Fibroblast activation is affected by TET enzyme depletion.
(A) mRNA expression of ACTA2, COL1A1 and TAGLN from dermal fibroblasts transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05, **P<0.01). Immunocytochemistry detection of αSMA (B) or collagen Iα1 (C) protein levels, counterstained with DAPI, on dermal fibroblasts transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 96 h or control. (D) mRNA expression of ACTA2, COL1A1 and TAGLN from HPFs transfected with esiRNA against TET2 or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05, **P<0.01, ***<0.001).
(ACTA2, COL1A1 and TAGLN) were downregulated in activated fibroblasts as a result of TET2 KD, although not to the same extent as in the dermal fibroblasts (Figure 2D). Furthermore, the effects are more pronounced in unstimulated pulmonary fibroblasts, which are also at a higher baseline than observed for dermal fibroblasts.

**TET knockdown influences expression of ECM biosynthesis-related genes**

Next a custom-made qPCR based low-density array (LDA) was used to screen for individual effects of TET enzymes on an extensive panel of ECM protein genes and modifying enzymes in activated dermal fibroblasts. The resulting heatmap reveals a

![Figure 3. TET-related effects on ECM biosynthesis gene expression.](image)

(A) Heat map of mRNA low-density array obtained expression levels from dermal fibroblasts transfected with esiRNA against TET2 or control, and stimulated with TGFβ1 for 48 h or control. Colors depict Log2 from two replicates normalized to control esiRNA values. (B and C) mRNA expression of COL1A2, COL3A1, COL5A1 and FN1, LOX and MMP13 from dermal fibroblasts transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05, **P<0.01, ***P<0.001).
signature of unique transcriptional effects for the different TETs, whereby KD of either of the TETs affects almost all the genes screened for (Figure 3A). Several of the genes were repeated by qPCR for both TGFβ1-stimulated and control fibroblasts. This showed that COL1A2, COL3A1 and COL5A1 are negatively affected by either TET1 or TET2 depletion in both conditions. Fibronectin (FN1) mRNA expression was also reduced when TET2 was depleted in TGFβ1-stimulated dermal fibroblasts. Expression of the collagen cross-linking enzyme LOX was reduced in activated fibroblasts irrespective which of the TET was depleted, while in control fibroblasts only an effect on expression was seen for TET2 KD. Expression of the collagenase MMP13 in activated fibroblasts was enhanced when TET2 was depleted. Next, we screened for transcriptional regulators with a known role in fibroblast activation or collagen biosynthesis (Figure 4). PPARG (PPARY), a regulator of various fibrosis-related genes, was not affected by depletion of TETs in activated fibroblasts, although slight effects were seen in control fibroblasts. The transcription factor SOX9 expression was affected by TET3 depletion in activated fibroblasts, while SNAI1 (Snail1) expression was enhanced when either TET2 or TET3 was depleted. RUNX was not affected by any of the three TETs. These data reveal that these well-known transcriptional regulators of fibrosis do not correlate to transcriptional effects of fibroblast activation and collagen synthesis as induced by TET KD.

**Figure 4. TET-related effects on fibrosis-related transcription factors.**
mRNA expression of transcription factors PPARG, SOX9, SNAI1 and RUNX1 from dermal fibroblasts transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05, **P<0.01, ***P<0.001).
TET-binding protein OGT has repressive transcriptional effects on specific genes

OGT is an O-Glc-Nac modifying enzyme that can be recruited by all three TET enzymes and promote gene transcription by modifying histones and other proteins (21-23). We wondered if the reduction of fibroblast activation and collagen synthesis was directly related to the TETs, or could also be linked to a block of OGT recruitment. Therefore, we carried out KD of OGT in activated fibroblasts (Supplemental Figure 2A), and assessed the downstream transcriptional effects. Of the classic myofibroblast markers (ACTA2, TAGLN and COL1A1) only ACTA2 mRNA was slightly increased, which was also seen at the protein level (Figure 5B). Several other collagens and FN1 (Supplemental Figure 2B) were also not affected at the mRNA level by OGT depletion. In agreement to the mRNA levels, collagen type 1 protein levels of OGT-depleted activated fibroblasts did not show a convincing difference. (Figure 5A,B). Interestingly, the transcription factors SNAI1 and RUNX1 were upregulated when OGT was depleted (Figure 5C). Similar effects were seen earlier for SNAI1 when TET2 or TET3 was depleted (Figure 4), suggesting that TET-recruited OGT regulates SNAI1. However, as OGT depletion had no general repressive effects on fibroblast activation and collagen synthesis, we reasoned that transcriptional effects of TET depletion mostly rely on 5hmC initiation.

Figure 5: TET-induced transcriptional effects are not due to recruiting OGT

(A) mRNA expression of ACTA2, COL1A1 and TAGLN from dermal fibroblasts transfected with esiRNA against OGT or control, and stimulated with TGFβ1 for 48 h or control (n=3, **P<0.01). (B) Immunocytochemistry detection of αSMA and collagen Iα1 protein levels, counterstained with DAPI, on dermal fibroblasts transfected with esiRNA against OGT or control, and stimulated with TGFβ1 for 96 h or control. (C) mRNA expression of SNAI1 and RUNX1 from dermal fibroblasts transfected with esiRNA against OGT or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05).
TET enzymes regulate fibrosis

TGFβ1 stimulation strongly affects genome-wide distribution of 5hmC in myofibroblasts

5hmC levels are mostly enriched at distal gene-regulatory elements as enhancers, but also at gene bodies not particularly known as CpG-dense regions. To identify genome-wide distribution of 5hmC in the initial activations phase of fibroblasts, we performed 5(h)meDIP followed by genome sequencing of dermal fibroblasts that were treated with TGFβ1 or control for 24 hours. An increase of 5hmC peaks was detected in TGFβ1-stimulated dermal fibroblasts: 65.283 versus 29.859 in control-treated fibroblasts (Figure 6A). Genomic annotations of 5hmC at all genes (protein coding and non-coding regions) or solely at protein coding genes was almost equally split between genic and intergenic regions and with a small increase of 3% of 5hmC at introns in activated fibroblasts at the expense of intergenic regions (Figure 6B). In both conditions only a fraction of 5hmC peaks were annotated in other regions such as promoters and UTRs. A slightly different distribution was seen when we specifically selected for protein coding regions. Here even less promoters were marked by 5hmC and the larger intergenic located 5hmC peaks reflect the exclusion of non-protein coding genes. Annotated 5hmC was found the largest in protein coding regions followed by non-coding regions harboring lincRNA, other RNA and miRNA (Figure 6C).

The majority of 5hmC was detected in areas distant to CpG islands or CpG shores (Figure 6D). Thus corresponding to a low presence in promoter regions and high presence in introns and intergenic regions. No differences in relative peak density were observed between TGFβ1-stimulated and control fibroblasts, although absolute values were more than 2-fold higher for the first. Enhancers are largely present in intergenic regions and gene bodies (introns), which we found to be strongly enriched in 5hmC in both conditions. To find out if these areas display overlap with enhancers, we screened for 5hmC peaks of TGFβ1- or control-treated dermal fibroblast at H3K4me1 positive sites that are largely distributed at enhancers. Interestingly we found only a fraction of the 5hmC peaks from both treatments to have overlap with enhancers, with a fraction more overlap found for TGFβ1 treated fibroblasts (Figure 6E). Peak distributions of 5hmC for various genes related to fibroblast activation and collagen synthesis showed two distinct modes of potential transcriptional regulation (Figure 6F). For instance in TGFβ1-stimulated fibroblasts, COL1A1 and TGFB1 have the strongest 5hmC enrichment in the promoter and downstream regions, whereas FN1 has stronger 5hmC enrichment throughout its intragenic region. For ACTA2 we observed that TET2 binds in the promoter region and overlaps with enhanced 5hmC deposition over time when stimulated with TGFβ1 (Supplemental Figure 3A and 3B). Interestingly, only TET2 depletion had a profound effect on global 5hmC levels in activated fibroblasts (Supplemental figure 3C). Ontology analysis of genes containing 5hmC in activated fibroblasts reveal that the majority falls within metabolic processes, cellular processes and regulation (Figure 6G). Only few 5hmc-containing genes were found to have roles in apoptosis, locomotion and adhesion.
Figure 6. Genome wide 5hmC levels are enhanced in activated fibroblasts.
Next generation sequencing of 5hMeDIP samples from dermal fibroblasts stimulated with TGFβ1 for 24 h of control. (a) Venn diagram of genome wide 5hmC peaks. (b) Genomic annotations of all 5hmC peaks calculated in percentage of distribution for elements in all known genes or only protein coding genes. (c) Proportion of 5hmC peaks detected in various gene types. (d) Relative peak density of 5hmC on CpG islands, CpG shores or on distant regions. Absolute peak numbers are given in the bottom table. (e) Venn diagram of downloaded dermal fibroblasts H3K4me1 ChIP-seq peaks overlapping regions with dermal fibroblasts 5hmC peaks to mark 5hmC present at enhancer regions. (f) Tracks depicting the 5hmC distribution on individual genes. (g) Gene Ontologies, with GO-terms, from 5hmC-annotated genes of TGFβ1-stimulated dermal fibroblasts.
DISCUSSION

It is well established that certain epigenetic processes influence the transcription of genes leading to phenotypic characteristics of fibrotic diseases. Several individual players have previously been identified that are either deregulated or recruited to genes that are decisive for fibrosis development (28, 36-39). Here we characterized the role of TET enzymes in fibroblasts activation and collagen synthesis. As TGFβ1 is one of the most potent inducers of fibroblast activation, we used TGFβ1-stimulated primary dermal and lung fibroblasts to find out if TET enzymes are responsive, and whether depletion of individual TETs affect downstream gene expression. Indeed, TETs reacted at the mRNA and/or protein level to TGFβ1, but not entirely via the same TGFβ1 signaling route. Overall, the TET depletion experiments showed that mainly TET2 has pro-fibrotic properties, whereas both TET1 and TET3 stimulate expression of collagens. Furthermore, activated fibroblast displayed genome-wide more 5hmC compared to unstimulated cells, substantiating a role of TETs in the establishment of a pro-fibrotic phenotype.

TET enzyme expression varies between cell types and developmental stages. We noticed mRNA expression of all three TETs in both normal and activated fibroblasts. Of which at the protein level, TET2 is reduced and TET3 is increased by TGFβ1 stimulation. Other reports found expression of TETs to be either unaffected or reduced in fibrotic tissues or cells, depending on the organ type and model used (32, 35). Our study is novel in that we mainly focused on the TET-induced effects during initiation of fibroblast activation. The LDA data illustrates that the majority of genes related to collagen homeostasis are responsive to depletion of either three TETs. Interestingly, \textit{TET1} KD has the most repressive effects on analyzed genes followed by both \textit{TET2} and \textit{TET3} KD. The overall trend indicates that TET1 and TET2 regulate synthesis of collagens by enhancing expression of collagens and their modifying enzymes, but that mainly TET2 and TET3 affect proteolysis of collagens by repressing expression of several MMPs in activated fibroblasts. Interestingly, it was KD of \textit{TET2} that had the most repressive effects on αSMA and SM22α, suggesting its overall dominance in fibroblast activation in relation to the other two TETs. A previous report designated TET2 as a master regulator for contractility genes in smooth muscle cells (40). As these contractile cells share overlapping features with myofibroblasts, it seems the TET2-induced effects are not cell type specific. As TET3 was highly TGFβ1 responsive both at the mRNA and protein level, we were surprised to find that it did not have a pro-fibrotic role as revealed by αSMA, SM22α and collagen expression analysis. In line with this, none of the ECM genes (collagens and fibronectin) and activated fibroblast markers were repressed when TET3 was depleted. However, \textit{TET3} KD has a positive influence on SOX9 and on \textit{SNAIL} expression independent on \textit{PPARG} expression (which is a known snail repressor (41)), and negatively influences the expression of the MMP inhibitor TIMP1. Therefore we support the view presented by others (32, 34) that TET3 can have protective or balancing roles in fibrosis.

Tissue distribution of absolute 5hmC levels varies greatly (15), while genomic distribution of 5hmC is quite comparable in various cells (stem cells, T-cells and cancer cells) and associates with gene bodies and enhancers, while in lesser extent
to promoters containing CpG islands (42-46). We observed an increase of 5hmC peaks genome-wide for activated fibroblasts. Of these, only a fraction of the 5hmC peaks were found at CpG islands or enhancers, while a large proportion were in gene bodies (introns) or at intergenic regions. As there are no (genome-wide) reports on enhancer usage in activated fibroblasts, the discrepancy in enhancer represented 5hmC peaks in relation to other cells is difficult to explain. The functional role or benefit to gene transcription of 5hmC presence at the gene body is currently not known. As 5mC in gene bodies have been widely found in active genes (6), it would be rational to assume that the decline of 5mC through its oxidation products enhances transcription processes. Although this is not the case for 5fC/5caC in the gene body where they retard elongation of RNA polymerase II (47, 48), 5hmC present at gene bodies of active genes have apparent transcription positive effects (44).

In addition to their role in catalyzing 5hmC, TETs can recruit other enzymes as OGT that may have an effect on gene transcription (21-23). OGT influences transcription by catalyzing the formation of O-GlcNAc on many chromatin-associated factors such as transcription factors, RNA polymerase II CTD, Polycomb and SIN3A/HDAC complexes, histone H2A and H2B, and also on TETs directly (49). For these reason it is difficult to directly correlate our observed OGT KD effects to a reduced TET-related OGT recruitment. However, as most of the O-GlcNAc effects are positive, one would expect that the depletion of OGT to induce repressive effects on transcription. In addition, OGT can also stimulate nuclear translocation, thereby regulating recruitment of TET proteins to chromatin (50). Interestingly, for most genes OGT KD did not negatively influence expression to the same extend as for TET KD. This suggests that the transcriptional effects seen for TET KD are essentially related to reduced 5hmC levels at specific loci, and are not related to OGT recruitment. Interestingly, ACTA2, SNAI1 and RUNX1 are upregulated, which could either be related to reduced activation of repressive Polycomb complexes or SIN3A/HDAC complexes by lowered O-GlcNAc of these complexes or by indirect transcriptional effects. As we found enhanced 5hmC in either the gene body or the promoter in several TET-reactive genes, we conclude that the majority of TET-related transcriptional effects in activated fibroblast are directly related to 5hmC deposition.

This report adds to a growing list of epigenetic processes and regulators that have an evident role in fibroblast activation and ultimately the establishment of fibrosis. However, we are only just starting to understand the interplay of certain DNA and histone modifications on gene transcription, and their collective contribution to regulating decisive complex gene networks. As TET proteins can be part of larger regulatory complexes that affect both DNA and histone modifications, they could have a decisive role in underlying gene expression. An important finding from our study is the strong pro-fibrotic effects related to TET2 presence. As depletion resulted both in reduced ECM expression and in a reduction of activated fibroblast markers, TET2 seems to be a central player in the establishment of fibrotic diseases and could therefore be an interesting therapeutic target for fibrosis and solid tumors alike.
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TET enzymes regulate fibrosis


Perugorria, M.J., Wilson, C.L., Zeybel, M., Walsh, M., Amin, S., Robinson, S., White, S.A., Burt, A.D.,


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SUPPLEMENTAL INFORMATION

Chromatin immunoprecipitation
Genomic binding sites of TET2 and TET3 were obtained by qChIP as explained previously (1). In short fibroblasts were fixed with 1% formaldehyde, lysed and chromatin fragmented by sonication. Antibodies against TET2 and TET3 were coated on Dynabeads and incubated with chromatin overnight. After extensive washing, the enriched fragments were eluted, decross-linked and purified with a QIAquick kit (Qiagen). Real-time PCR was performed on ChIP and input samples with primers and SYBR Green (Roche). qChIP values were calculated as percent relative to input.

5-hydroxymethylated DNA immunoprecipitation
Genomic DNA isolated from fibroblast stimulated with TGFβ1 or control for 24 hours, was isolated with a GeneJet kit (Thermo Scientific) that included RNAse and Proteinase K steps. For 5-hydroxymethylated DNA immunoprecipitation (5hMeDIP), gDNA was diluted in TE buffer and sonicated to obtain fragments between 100 and 650 bps. The fragmented DNA was denatured for 5 minutes at 95°C and diluted in cold IP buffer (10 µM Sodium Phosphphpate, pH 7.0, 140 mM NaCl, 0.05% Triton-X100). Two µg diluted DNA was supplemented with 2 µg anti-5hmC (Active Motif) or rabbit IgG control (Abcam) and incubated overnight at 4°C with agitation. The following day, 20 µl Dynabeads Protein A (Thermo Fisher Scientific) was added to the DNA/antibody mix, and incubated for 2.5 hours at 4°C with agitation. Afterwards, the beads were washed 3 times with IP buffer and eluted with 0.5% SDS in TE buffer. The elute was treated with proteinase K for 1 hour at 50°C, and enriched fragments were isolated with a QIAquick kit (Qiagen). To quantify enriched regions for 5hmC, real-time PCR was performed as described before with primers (ACTA2: FW 5’-agttttgtgctgaggtccctatatg-3’; REV 5’-ttcccaaacaaggaccaaga-3’) on input and 5hMeDIP samples and calculated as percent enrichment relative to input.

DNA isolation and 5hmC dot blot
Genomic DNA was isolated from fibroblasts with a GeneJet kit (Thermo Scientific) that included RNAse and Proteinase K steps. Serial dilutions of DNA were denatured at 95°C and spotted on a nitrocellulose membrane. The DNA was cross-linked to the membrane by exposing to UV light for 5 minutes. Membranes were blocked in skimmed milk (5% in TBS-Tween20) for 1.5 hours, and subsequently incubated with anti-5hmC antibody (Active Motif) for 2 hours. After 1 hour incubation with secondary HRP conjugated antibody (DAKO), chemiluminescent signals were detected with ECL (Thermo Scientific).

REFERENCES

SUPPLEMENTAL FIGURES

Supplemental Figure 1. TET KD efficiency. 
 mRNA expression of TET1, TET2, and TET3 from HDFs transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05; **P<0.01).

Supplemental Figure 2. OGT depletion does not affect transcription of ECM genes. 
 mRNA expression of OGT (A), COL1A2, COL3A1, COL5A1 and FN1 (B) from HDFs transfected with esiRNA against OGT or control, and stimulated with TGFβ1 for 48 h or control (n=3, *P<0.05).
Supplemental Figure 3. TET binding and 5hmC changes by TGFβ1 signaling.
(A) qChIP of TET2 and TET3 at the ACTA2 promoter from HDFs stimulated with TGFβ1 for 6 and 24 h or control (n=2). (B) qPCR of ShMeDIP at the ACTA2 promoter from HDFs stimulated with TGFβ1 for 6 and 24 h or control (n=2). (C) Global 5hmC levels detected by Dotblot of genomic DNA derived from HDFs transfected with esiRNA against either three TETs or control, and stimulated with TGFβ1 for 48 h.
TET enzymes regulate fibrosis
PART III

TRANSCRIPTIONAL MODULATION BY TARGETED REWRITING OF THE EPIGENOME