Ten-Eleven Translocation-2 (TET2) Is a Master Regulator of Smooth Muscle Cell Plasticity

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Background—Smooth muscle cells (SMCs) are remarkably plastic. Their reversible differentiation is required for growth and wound healing but also contributes to pathologies such as atherosclerosis and restenosis. Although key regulators of the SMC phenotype, including myocardin (MYOCD) and KLF4, have been identified, a unifying epigenetic mechanism that confers reversible SMC differentiation has not been reported.

Methods and Results—Using human SMCs, human arterial tissue, and mouse models, we report that SMC plasticity is governed by the DNA-modifying enzyme ten-eleven translocation-2 (TET2). TET2 and its product, 5-hydroxymethylcytosine (5-hmC), are enriched in contractile SMCs but reduced in dedifferentiated SMCs. TET2 knockdown inhibits expression of key procontractile genes, including MYOCD and SRF, with concomitant transcriptional upregulation of KLF4. TET2 knockdown prevents rapamycin-induced SMC differentiation, whereas TET2 overexpression is sufficient to induce a contractile phenotype. TET2 overexpression also induces SMC gene expression in fibroblasts. Chromatin immunoprecipitation demonstrates that TET2 coordinately regulates phenotypic modulation through opposing effects on chromatin accessibility at the promoters of procontractile versus dedifferentiation-associated genes. Notably, we find that TET2 binds and 5-hmC is enriched in CArG-rich regions of active SMC contractile promoters (MYOCD, SRF, and MYH11). Loss of TET2 and 5-hmC positively correlates with the degree of injury in murine models of vascular injury and human atherosclerotic disease. Importantly, localized TET2 knockdown exacerbates injury response, and local TET2 overexpression restores the 5-hmC epigenetic landscape and contractile gene expression and greatly attenuates intimal hyperplasia in vivo.

Conclusions—We identify TET2 as a novel and necessary master epigenetic regulator of SMC differentiation.

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Key Words: cell differentiation | epigenomics | gene expression regulation | hyperplasia | muscle, smooth

Unlike most mature cells, smooth muscle cells (SMCs) are remarkably plastic and can dedifferentiate in response to environmental cues,1,2 adding a layer of complexity to the regulation of gene expression. Although several transcription factors have been identified, a global mechanism that coordinately regulates SMC phenotype has yet to be uncovered. How SMC genes become silenced and then reactivated is unknown and is an area of intense investigation. Recent demonstration that the ten-eleven-translocation (TET) family of proteins is involved in DNA demethylation3–5 prompted us to evaluate the role of the TET proteins in the modulation of SMC phenotype.

The TET proteins (TET1–TET3) are a recently discovered family of DNA demethylases. TET proteins oxidize 5-methylcytosine (5-mC) to generate 5-hydroxymethylcytosine (5-hmC), frequently called the sixth DNA base, in mammalian cells.4,5 Through the base excision repair pathway, 5-hmC is then converted to unmethylated cytosine, leading to DNA demethylation and gene activation.6–8 Therefore, the 5-hmC modification and the TET enzymes have emerged as key activators of gene expression. Studies of TET proteins and 5-hmC function in embryonic stem cells (ESCs) demonstrate that they play a major role in maintaining cellular pluripotency through the regulation of lineage-specific genes.4,9–11 In contrast to this role in ESC pluripotency, the TET proteins (and their 5-hmC products) have an opposing role in adult stem cells and somatic tissues. TET2 mutations have been described in several types of hematopoietic disorders in which the loss of TET2 has been shown to promote hematopoietic stem cell self-renewal.12 TET2 and 5-hmC levels are increased during neurogenesis,13 and more recently, loss of TET2 and 5-hmC was demonstrated to be a key epigenetic event associated with...
aggressive melanoma. Collectively, these studies suggest a cell- or tissue-specific effect of the TET proteins and the 5-hmC mark on gene activation.

Here, we present data using both in vitro and in vivo knock-down and overexpression approaches to functionally characterize the role of TET2 and its 5-hmC product in regulating the SMC phenotype. We find that TET2 expression is necessary for SMC differentiation and that TET2 binds to the SRF and MYOCDS promoters under differentiation conditions. Additionally, we show that TET2 regulates the cellular program by directly modulating chromatin accessibility of target genes. Furthermore, we report that TET2-activated gene promoters are enriched with the 5-hmC mark, resulting in strong gene activation. Overall, we demonstrate a novel epigenetic pathway by which contractile and synthetic genes are regulated simultaneously in SMCs. In addition to demonstrating a critical role for TET2 in adult somatic cells in health and disease, our study suggests that targeting the TET2-5-hmC pathway may be a therapeutic strategy for the treatment of diseases associated with aberrant SMC differentiation.

Methods
Please refer to the online-only Data Supplement for an extended description of the materials and methods. Primer sequences for qualitative polymerase chain reaction (qPCR) analysis of gene expression are provided in Tables I and II in the online-only Data Supplement.

Human Atherosclerotic Tissues
Research protocols were approved by the Institutional Review Boards of the West Haven VA Hospital, Yale University, and the New England Organ Bank. Informed consent was obtained, and human coronary arteries were obtained as previously described. The presence of atherosclerosis was documented at the time of cardiac procurement by an experienced cardiac surgeon. Pliable, translucent coronary arteries were deemed normal and designated healthy controls. Opaque coronary arteries were classified as atherosclerotic to various degrees (mild, moderate, and severe). The macroscopic diagnosis was confirmed by histology.

Femoral Artery Wire Injury
All experiments were approved by the Institutional Animal Care and Use Committees of Yale University and performed in adherence to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Femoral arteries were injured as previously described. For localized virus delivery, viruses (1×10⁷ plaque-forming units) were deliv-
ered to the artery at the time of injury by painting the virus mixture (15 µL concentrated viral supernatant and 35 µL Pheronic-127 gel) circumferentially around the injured femoral artery. The injured and uninjured femoral arteries were collected for cryosectioning 3 weeks after surgery.

Cell Culture
Human coronary artery SMCs (hCASMCs) were purchased from Cascade Biologics (Portland, OR) and cultured as previously described. MRC5 cells were purchased from ATCC and cultured in 10% FBS. Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 basal medium and associated supplements (Lonza Biologicals). Cells from passages 4 through 7 were used for all experiments.

Chromatin Immunoprecipitation–qPCR
Chromatin immunoprecipitation was performed as previously described with slight modifications. Briefly, 5x10⁷ cells were cross-linked and used for each immunoprecipitation. DNA was sheared to 500 to 1000 bp by sonication. Protein G Dynabeads (Invitrogen) were used to pull down the antibody-antigen complex immunoprecipitated with antibodies against TET2 (Abcam), H3K4me3, and H3K27me3 (Cell Signaling). H3 (Cell Signaling) and IgG were also included as positive and negative controls, respectively. Immunoprecipitated DNA was extracted with phenol-chloroform, precipitated with ethanol, and eluted. Recovered DNA was analyzed by qPCR. Primers spanning the promoter regions (within 2500 bp of transcription start site) of the MYH11, MYOCDS, SRF, and KLF4 genes were used to amplify input and immunoprecipitated DNA. Primers were designed to span a CArG [CC(A/T)GG] element; sequences are listed in Table III in the online-only Data Supplement. All samples were performed in at least triplicate from at least 2 independent experiments, and data were normalized to percent input.

Methylated-DNA Capture
Enrichment of methylated DNA was performed with the methylated-DNA capture (MethylCap) kit (Diagenode). Genomic DNA was diluted to 0.1 µg/µL and then sheared to 200 to 500 bp. Methylated DNA was captured by incubation with a His-tag-GST-MBD protein (the methyl-binding domain of the human MeCP2 protein fused with a glutathione-S-transferase and containing an N-terminal His-tag) coupled to magnetic beads. The beads were washed and DNA eluted. The methyl-binding domain–bound (methylated) and input fractions were analyzed by qPCR to confirm enrichment of the methylated genes. Primer sequences can be found in Table III in the online-only Data Supplement.

Hydroxymethylated DNA Immunoprecipitation
Immunoprecipitation of 5-hmC was performed with the hydroxymethylated DNA immunoprecipitation kit (Diagenode) per the manufacturer’s instructions. Samples were sonicated as for methylated-DNA capture, and 1 µg fragmented genomic DNA was immunoprecipitated with a 5-hmC antibody. The DNA-antibody mixture was incubated with magnetic beads and washed, and the DNA was isolated. End-point analysis was performed with locus-specific hydroxymethylated DNA immunoprecipitation–qPCR. Primer sequences are listed in Table III in the online-only Data Supplement.

Glucosylation-Coupled Methylation-Sensitivity qPCR
Sequence-specific detection of 5-hmC and 5-mC as a percentage of total C was performed with the Quest 5-hmC Detection Kit (Zymo Research) based on published methods. Genomic DNA (1 µg) was treated with T4 Phage β-glucosyltransferase, a highly specific 5-hmC glucosyltransferase, that adds a glucose moiety to 5-hmC (generating glucosyl-5-hmC). Glucosylated genomic DNA was then digested with MspI, which recognizes and cleaves mC, 5-mC, and 5-hmC but not glucosyl-5-hmC. Locus-specific detection of 5-hmC was determined by qPCR using primers designed to flank at least 1 MspI site. Primer sequences used are listed in Table IV in the online-only Data Supplement.

Dot Blot
Cellular genomic DNA was isolated with the Qiagen kit; genomic DNA from tissue sections was isolated with the Pinpoint Slide DNA Isolation System (Zymo Research). DNA was blotted onto a nitrocellulose membrane and incubated with anti–5-hmC (1:10000, Active Motif) overnight at 4°C. After incubation with horseradish peroxidase-conjugated IgG secondary antibody, the signal was visualized with enhanced chemiluminescence reagents. Methylene blue staining of the membranes was used to assess equal DNA loading.

Statistical Analysis
Data are expressed as mean±SD. Comparisons between 2 groups were calculated with the Mann-Whitney test. The Kruskal-Wallis test,
followed by the Dunn multiple-comparison test, was performed for data between 2 groups. P values were 2 tailed, and values of P<0.05 were considered to indicate statistical significance. Analyses were performed with Prism 4.0 software (GraphPad). Intraclass correlation coefficients were calculated with an online tool (http://department.obg.cuhk.edu.hk/researchsupport/IntraClass_correlation.asp).

**Results**

TET2 and 5-hmC Are Upregulated During SMC Differentiation

The role of the TET family of enzymes in an adult somatic cell such as SMCs is yet to be determined. We evaluated the expression of TET proteins in hCASMC cultures and found TET2 to be the most highly expressed isoform in these cells (Figure 1A). Remarkably, TET2 transcript and protein levels were significantly higher in hCASMCs compared with human ESCs. To explore a possible role for the TET family members in modulating the SMC phenotype, we induced SMC differentiation by treatment with the mTORC1 inhibitor rapamycin21,22 or dedifferentiation with platelet-derived growth factor-BB (PDGF-BB).23 Both TET2 transcript and protein levels were elevated with differentiation but repressed with PDGF-BB treatment (Figure 1B and Figure I in the online-only Data Supplement). The changes in TET2 were of greater magnitude and of clear statistical significance compared with the modest changes observed with TET1 and TET3 (Figure 1B and Figure IA–ID in the online-only Data Supplement). Notably, TET2 expression closely correlated temporally with SMC differentiation markers such as smooth muscle-myosin heavy chain (MYH11) and smooth muscle actin (ACTA2) but inversely correlated with KLF4 expression (Figure 1B and Figure IE–IG in the online-only Data Supplement). Given its abundant expression and modulation during SMC differentiation, we focused our further studies on TET2. We determined that Tet2 is abundantly expressed in multiple SMC-rich murine tissues (Figure II in the online-only Data Supplement). In the vasculature, Tet2 was highly expressed in SMCs isolated from the medial layer of normal mouse aorta but dramatically decreased with culture in 10% serum, in parallel with Myh11 expression (Figure 1C). These levels were partially rescued with rapamycin (Figure 1C). Because the enzymatic function of the TET proteins is to convert 5-mC to 5-hmC,4,5,9 we measured 5-hmC levels in SMCs. Genomic dot blot analysis revealed a global increase in 5-hmC levels in differentiated SMCs and reduced 5-hmC levels in dedifferentiated cultures (Figure 1D). These results demonstrate that both TET2 and its 5-hmC product correlate with the SMC differentiated phenotype.

TET2 and 5-hmC Levels Are High in Mature SMCs and Are Reduced After Vascular Injury and Disease

To understand the physiological role of TET2 and 5-hmC in vivo, we examined TET2 and 5-hmC expression in murine and human models of vascular injury and disease. Intimal hyperplasia was induced with the mouse femoral artery injury model.19 In the uninjured vessels, Tet2-expressing cells made up >95% of the total number of medial cells (n=4). Tet2-expressing cells were reduced in the injured medial layer, and only 16% of cells in the neointima were Tet2 positive at 3 weeks after injury (n=5; Figure 2A, quantified in Figure 2B).

RNA analysis further confirmed that vessel injury reduced the levels of Tet2 and contractile genes in the media and neointima (Figure 2C). The 5-hmC staining (Figure 2A, quantified in Figure 2D) and genomic levels (Figure 2E) closely paralleled Tet2 expression patterns. These results support our in vitro findings that high Tet2 and 5-hmC levels are associated with the mature, differentiated SMC phenotype, whereas significant loss of Tet2 and 5-hmC is characteristic of dedifferentiated SMC.

We next sought to assay TET2 and 5-hmC levels in human vascular disease. SMC dedifferentiation is a major contributor to atherosclerosis.26,27 We analyzed human coronary artery biopsies from 16 individuals with normal arteries or demonstrating mild, moderate, or severe atherosclerosis. MYH11 staining was used to identify differentiated SMCs within the individual patient samples. MYH11 intensity inversely correlated with the severity of disease, and TET2 expression correlated closely with the MYH11 staining (Figure 3A, quantified in Figure 3B). Consistently, global 5-hmC levels were also significantly reduced in atherosclerotic samples (Figure 3C). Taken together,
these results implicate TET2 and its product, 5-hmC, as novel biomarkers for diseases associated with SMC dedifferentiation.

**Disruption of TET2 Results in Impaired SMC Differentiation**

To assess a causal role for TET2 in SMC phenotypic modulation, we generated shRNA constructs targeting the human TET2 gene (shTET2; Figure IIIA in the online-only Data Supplement). TET2 knockdown was highly efficient and specific (Figure IIIB in the online-only Data Supplement). Significant reductions in SMC differentiation gene expression and 5-hmC levels were observed with TET2 knockdown in hCASMCs (Figure 4A–4C). We found that TET2 is an obligate effector of rapamycin-induced differentiation because TET2 knockdown prevented rapamycin-induced contractile gene expression (Figure 4D) and morphology (Figure IIIC in the online-only Data Supplement). Additionally, we observed enhanced proliferation in shTET2 cultures as determined by propidium iodide staining and flow cytometry (Figure 4E), and mRNA levels for cyclins and cyclin-dependent kinase inhibitors supported these observations (data not shown). TET2 knockdown also significantly increased the expression of markers associated with the synthetic SMC phenotype such as KLF4,28-31 KLF5,32 osteopontin,33 and embryonic type nonmuscle myosin heavy chain-B (MYH10)34 compared with control transfected (shCTRL) cells (Figure 4F and Figure IIID in the online-only Data Supplement). On the other hand, TET2 overexpression promoted many aspects of differentiation in hCASMCs, including increased contractile gene levels, elevated global 5-hmC expression (Figure 4G and 4H and Figure IV A in the online-only Data Supplement), a contractile morphology even in the absence of differentiation stimuli, a decrease in KLF4 and other dedifferentiation gene expression levels, and antiproliferative gene expression (Figure IVB–IVD in the online-only Data Supplement). Together, these results suggest that TET2 is both necessary and sufficient for SMC differentiation.

**Ectopic Expression of TET2 Directs SMC Differentiation**

Because TET2 knockdown and overexpression had such profound effects on the SMC phenotype, we investigated whether ectopic expression of TET2 is sufficient to convert fibroblasts into mature SMCs. The human fetal lung fibroblast line MRC5 expressed moderate levels of TET2 at baseline compared with hCASMCs (Figure 4I). We found that TET2 is an obligate effector of rapamycin-induced differentiation because TET2 knockdown prevented rapamycin-induced contractile gene expression (Figure 4D) and morphology (Figure IIIC in the online-only Data Supplement). Additionally, we observed enhanced proliferation in shTET2 cultures as determined by propidium iodide staining and flow cytometry (Figure 4E), and mRNA levels for cyclins and cyclin-dependent kinase inhibitors supported these observations (data not shown). TET2 knockdown also significantly increased the expression of markers associated with the synthetic SMC phenotype such as KLF4,28-31 KLF5,32 osteopontin,33 and embryonic type nonmuscle myosin heavy chain-B (MYH10)34 compared with control transfected (shCTRL) cells (Figure 4F and Figure IIID in the online-only Data Supplement). On the other hand, TET2 overexpression promoted many aspects of differentiation in hCASMCs, including increased contractile gene levels, elevated global 5-hmC expression (Figure 4G and 4H and Figure IV A in the online-only Data Supplement), a contractile morphology even in the absence of differentiation stimuli, a decrease in KLF4 and other dedifferentiation gene expression levels, and antiproliferative gene expression (Figure IVB–IVD in the online-only Data Supplement). Together, these results suggest that TET2 is both necessary and sufficient for SMC differentiation.

**Figure 2.** Ten-eleven-translocation-2 (TET2) is downregulated during vascular injury. A, TET2 and 5-hydroxymethylcytosine (5-hmC) immunostaining of cross sections from uninjured contralateral control mouse femoral arteries (top row) or injured femoral arteries (bottom row) collected 3 weeks after wire injury. Nuclei are stained with DAPI. B, Quantification of TET2-positive cells divided by total number of DAPI-positive cells in the uninjured (n=4) compared with injured (n=5) mouse femoral arteries. **P<0.01 vs uninjured media. C, Quantitative polymerase chain reaction for Tet2, Myocd, and Myh11 gene expression in uninjured compared with injured femoral arteries 3 weeks after injury. RNA was isolated from media and neointima from sections on slides with the Pinpoint isolation system. The adventitia was removed to ensure that only the media and neointima were used for analysis. *P<0.05 vs uninjured arteries. D, Quantification of 5-hmC–positive cells divided by total number of DAPI-positive cells in the uninjured (n=4) versus injured (n=5) femoral arteries. **P<0.01 vs uninjured media. E, Dot blot for global 5-hmC levels of uninjured and injured samples (n=4). Genomic DNA was isolated from tissue sections with the Pinpoint isolation system as in C. Fold induction levels were calculated from pixel intensity with Image Lab Software ("P<0.01). Scale bar, 50 μm. Error bars represent mean±SD; n represents the number of individual mice. Two or more slides (12–18 sections) per animal were used for analysis. M indicates media; and N, neointima.
imunostaining, and qPCR (Figure 4I–4K and Figure VA in the online-only Data Supplement). Significant increases in Myocd, Srf, and Myh11 gene expression were also observed with TET2 overexpression in mouse NIH3T3 fibroblasts (Figure VB in the online-only Data Supplement). The fibroblast marker S100a4 was also reduced with TET2 overexpression in MRC5 and NIH3T3 cells (Figure VA and VB in the online-only Data Supplement). These results demonstrate that activation of an SMC program in non-SMCs can be achieved with TET2. Interestingly, TET2 overexpression in human umbilical vein endothelial cells upregulated only some SMC-specific genes such as Myh11 and Acta2, whereas Srf and Tagln remained unchanged. Myocd mRNA was not detectable (data not shown), and levels of the endothelium-specific genes Pecam1 and Cdh5 remained relatively unchanged (Figure VC and WD in the online-only Data Supplement).

**Localized Delivery of TET2 Enhances SMC Differentiation and Can Improve Vascular Repair**

To assess the role of TET2 in SMC differentiation in vivo, we performed localized delivery of control, TET2-knockdown, or TET2-overexpressing viruses to the site of femoral arterial wire injury. At 3 weeks after injury, injured femoral arteries treated with a control virus showed moderate intimal hyperplasia, whereas local delivery of the TET2 knockdown virus markedly increased the neointimal area (Figure 5A). In stark contrast, arteries transduced with the TET2-overexpression virus showed significant suppression of neointimal hyperplasia (Figure 5A and 5B and Figure VIA in the online-only Data Supplement). No difference in medial area was observed between the 3 groups (Figure VIB in the online-only Data Supplement). Consistent with the intimal hyperplastic phenotypes, Tet2, Myocd, and Myh11 mRNA levels were reduced with TET2 knockdown but increased with TET2 overexpression relative to control virus (Figure 5C). Myh11, Tet2 and 5-hmC levels correlated inversely with the extent of injury as demonstrated by immunostaining (Figure 5D, top). Expression of these genes was further reduced in the injured arteries transduced with the TET2-knockdown virus (Figure 5D, middle). In the injured femoral arteries transduced with TET2-overexpression viruses, enhanced expression of Myh11 (Figure 5D) and Acta2 (Figure VIVA in the online-only Data Supplement) was noted relative to the control injured samples. In addition, Tet2 and 5-hmC nuclear staining was also more evident throughout these injured samples (Figure 5D, bottom).

To further assess the effect of TET2 knockdown or overexpression on the injured vessels, we examined whether TET2 modulation affected endothelial recovery or changes in mononuclear cell infiltration. Complete endothelial recovery was observed in all treatment groups as detected by CD31 staining, and no differences in leukocyte (CD45) or macrophage (CD68) staining were noted (Figure VIIA in the online-only Data Supplement). We observed significant upregulation of Col1a1, Col4a1, Col8a1, and Col8a2 in the control injured and TET2-knockdown injured samples compared with uninjured samples, whereas significant decreases in Col4a and Col8a2 expression were observed in the injured TET2-overexpression samples compared with injured control samples (Figure VIIB and VIID in the online-only Data Supplement). No significant differences in the cyclins or cyclin-dependent kinase inhibitors were observed between the groups at 3 weeks (Figure VIIIC and VIIID in the online-only Data Supplement). Collectively, these data highlight that loss of TET2 diminishes the 5-hmC epigenetic landscape and plays a causative role in intimal hyperplasia after injury, whereas reintroduction of TET2 potently antagonizes intimal hyperplasia.

**TET2 Binds to Key SMC Promoters and Modifies Histones at These Promoters**

The effects of TET2 on SMC phenotypic modulation suggested the possibility of a direct interaction between TET2 and master SMC genes that control SMC differentiation. To test this notion, we used chromatin immunoprecipitation coupled with qPCR. We used an anti-TET2 antibody to immunoprecipitate protein/DNA complexes from hCASMCs treated with vehicle, rapamycin, or PDGF-BB for 24 hours and...
qPCR amplified CARG-containing regions of the human SRF, MYOCD, and MYH11 promoters. Consistent with increased TET2 expression in SMC differentiation, TET2 binding to these promoters greatly increased during SMC differentiation induced by rapamycin (Figure 6A).

Because TET2 is known to promote DNA demethylation, we further sought to understand TET2-regulated epigenetic changes in SMC genes and evaluated the chromatin state of various SMC loci. We performed chromatin immunoprecipitation–qPCR, immunoprecipitating with antibodies recognizing the H3K4me3 (active) or H3K27me3 (inactive) marks and amplifying critical CARG-containing promoter regions of contractile SMC genes. Chromatin immunoprecipitation analysis of control, untreated hCASMCs cultured in 10% FBS revealed a euchromatic conformation at the MYOCD, SRF, and MYH11 gene promoters (H3K4me3/H3K27me3 ratios of 2.5, 9, and 1.9, respectively; Figure VIIIA–VIIIC in the online-only Data Supplement). No significant difference between the ratio of H3K4me3/H3K27me3 marks was observed at the KLF4 promoter in SMCs at baseline (Figure VIIID in the online-only Data Supplement). As expected, extensive H3K4me3 enrichment at contractile gene loci in SMCs was seen after rapamycin-induced differentiation, whereas PDGF-BB promoted an open chromatin conformation in the KLF4 gene promoter (Figure VIIIIE in the online-only Data Supplement). TET2 knockdown significantly diminished chromatin accessibility and increased the H3K27me3 mark at the MYOCD, SRF, and MYH11 gene loci in hCASMCs, so that H3K4me3/H3K27me3 ratios were decreased by 3- to 8-fold (Figure 6B–6D). In contrast, H3K4me3/H3K27me3 ratios were increased by >4-fold at the KLF4 loci after TET2 knockdown (Figure 6E). These results are consistent with the differentiation defects observed with TET2 knockdown in hCASMCs (Figure 4) and indicate that TET2 differentially regulates the chromatin accessibility of contractile and dedifferentiation genes in an opposing manner.

Promoters of Genes Upregulated by TET2 Are Enriched for 5-hmC

5-hmC levels have been associated with gene activation; therefore, we next used several methods to quantify locus-specific 5-hmC expression at key contractile SMC promoters. We determined the effect of TET2 knockdown on locus-specific 5-hmC levels by glucosylation-coupled methylation-sensitivity qPCR and hydroxymethylated DNA immunoprecipitation-qPCR. The MYOCD, SRF, and MYH11 promoters become hypermethylated in shTET2 cells compared with shCTRL cells (Figure 6F–6H). In contrast, H3K4me3/H3K27me3 ratios were increased by >4-fold at the KLF4 loci after TET2 knockdown (Figure 6E). These results are consistent with the differentiation defects observed with TET2 knockdown in hCASMCs (Figure 4) and indicate that TET2 differentially regulates the chromatin accessibility of contractile and dedifferentiation genes in an opposing manner.
Liu et al. TET2 Regulates SMC Plasticity

Online-only Data Supplement). Taken together, these data indicate that TET2 regulates SMC phenotype through the regulation of 5-hmC, which, in turn, affects chromatin modification of key SMC genes. These data support a primary role for 5-hmC as a distinct epigenetic mark in SMC differentiation.

**Discussion**

In the present work, we identify TET2 as a novel epigenetic master regulator of SMC plasticity that acts upstream of both MYOCDSRF and KLF4. Using both in vitro and in vivo approaches, we demonstrate that TET2 is necessary and sufficient for SMC differentiation and that ectopic expression of TET2 is sufficient to direct SMC differentiation in fibroblasts. Endogenous TET2 is regulated by stimuli that modulate differentiation, including PDGF-BB and mTORC1 inhibition in vitro and after vascular injury in vivo. We report a striking correlation between diminished TET2 and 5-hmC expression and severity of atherosclerosis in human coronary arteries. Most notably, we demonstrate a causal role for TET2 in SMC phenotypic modulation in response to arterial injury because TET2 loss of function exacerbates intimal hyperplasia after injury, whereas TET2 overexpression greatly attenuates intimal hyperplasia.

The unique phenotypic plasticity of SMC is known to be transcriptionally regulated, with MYOCDSRF and KLF4 having been identified as key drivers of this plasticity, promoting the contractile and dedifferentiated phenotypes, respectively. Although these proteins interact with other factors that modulate chromatin, a unifying upstream regulator that promotes global changes in chromatin at both contractile and synthetic gene promoters has never before been identified. It is therefore particularly notable that we now report that TET2 functions as an upstream regulator of these genes. The epigenetic changes regulated by TET2 have broad effects on chromatin, resulting in induction of MYOCDSRF and contractile genes, with concomitant repression of KLF4 and other dedifferentiation-associated genes. TET2 broadly affects SMC phenotype in vitro, including gene expression, morphology, and proliferation, and this translates to corresponding effects on intimal hyperplasia in vivo, including changes in expression of Myocd, Myh11, and collagen gene expression. The TET2 knockout mouse is viable and fertile.
Figure 6. Ten-eleven-translocation-2 (TET2) binds to smooth muscle cell (SMC) promoters to regulate 5-hydroxymethylcytosine (5-hmC) and to modify histones at SMC loci. A, Chromatin immunoprecipitation (ChIP) assay demonstrating TET2 enrichment at the SRF, MYOC, and MYH11 promoters during SMC differentiation. Human coronary artery SMCs (hCASMCs) were treated with rapamycin or platelet-derived growth factor BB (PDGF-BB) for 24 hours. Data are presented as mean relative enrichment over input ±SD of 3 biological repeats. *P<0.05, **P<0.01. B through E, H3K4me3 and H3K27me3 ChIP–quantitative polymerase chain reaction (qPCR) in shCTRL (gray bars) or shRNA constructs targeting the human TET2 gene (shTET2; white bars) hCASMCs at various gene promoters. Primers were designed to encompass the CArG elements (denoted by the small gray box). PCR products are indicated by numbering at the ends of the lower bar. Data are presented as mean relative enrichment over input ±SD of 4 biological replicates. *P<0.05, **P<0.01, ***P<0.001 vs the shCTRL group. F, H, and J, DNA methylation as quantified by methylated-DNA capture. G, I, and K, 5-hmC levels as determined by glucosylation–coupled methylation-sensitivity qPCR or hydroxymethylated DNA immunoprecipitation–qPCR. Data are presented as mean±SD of 4 independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs the shCTRL group.
but susceptible to spontaneous myeloid leukemias.\footnote{22} Although it was dispensable during development, our data suggest that TET2 plays an important role during the response to vascular injury in the adult.

Mechanistically, we demonstrate that TET2 binding directly to the SRF, MYOCD, and MYH11 promoters is strongly enriched in differentiated SMCs and coordinately regulates phenotypic modulation through opposing effects on chromatin accessibility at contractile compared with synthetic gene promoters. We propose that the potent effects of TET2 are attributable to its multiple modes of action, including binding directly to SM-specific promoters, regulating expression of the key transcription factors MYOCD and SRF, and inhibiting the repressor KLF4. TET2 alters DNA methylation, 5hmC modification, and histone methylation at these promoters.

Most studies of TET proteins to date have focused on embryonic and hematopoietic stem cells. Our study is the first to address the function of the TET proteins and the 5-hmC epigenetic mark in myocytes. We provide the first evidence that the 5-hmC modification correlates with the mature SMC phenotype and marks active contractile SMC gene promoters. Recent studies indicate that 5-hmC may represent more than just an intermediate base in the TET-mediated demethylation pathways; rather, it may have a larger role in regulating self-renewal and lineage commitment.\footnote{14,22,47} Genome-wide analyses in mouse ESCs have shown that altering DNA methylation via TET1 and 5-hmC also affects histone methylation,\footnote{44,45} adding to the complexity by which the TET proteins may regulate gene expression. We find that modulation of TET2, and subsequently 5-hmC, results in robust changes in histone methylation. A summary of our epigenetic data and schematic model is shown in Figure 7. The finding that 5-hmC modulates not only local DNA cytosine methylation but also histone methylation suggests that 5-hmC, generated by TET2, does not simply represent an intermediate in the 5-mC demethylation process but functions as an epigenetic mark that modulates the SMC phenotype through recruitment of as-yet unidentified factors to promote chromatin remodeling.

High-throughput screening in mouse ESCs revealed that 5-hmC–expressing regions are enriched for both the H3K4 and H3K27 trimethylation marks,\footnote{45} where TET1 has been shown to maintain ESC identity by promoting the transcription of pluripotency-related factors while simultaneously participating in the silencing of developmental genes.\footnote{44} We now identify a similar pattern of chromatin regulation in SMCs and demonstrate that TET2 and 5-hmC have a similar dual function in adult SMCs: TET2 promotes a euchromatic conformation at promoters of contractile-associated genes while concomitantly inducing a repressive chromatin state at promoters of dedifferentiation-associated genes. Furthermore, using the recently established glucosylation-coupled methylation-sensitivity qPCR technique and a modified hydroxymethylated DNA immunoprecipitation protocol with the newly developed 5-hmC antibody, we were able to specifically and efficiently label 5-hmC at SMC gene promoter regions. This locus-specific analysis found that 5-hmC is abundant in active SMC promoters and low in promoters that are not transcribing, consistent with the notion that promoter hydroxymethylation leads to demethylation and gene activation.\footnote{11,44,45}

The mechanisms by which TET2 mediates opposing effects on differentiation and dedifferentiation genes require further investigation. By activating the promoters of key transcription factors, including MYOCD, SRF, and MYH11, while repressing the KLF4 promoter, TET2 serves as an epigenetic master regulator of SMC differentiation. The concomitant activation of the promoters of MYOCD/SRF target genes (MYH11 and ACTA2) may account for the potent prodifferentiation effects of TET2 (Figure 6). We noted TET2 binding to promoter regions containing CArG elements in contractile promoters, including a putative CArG element in the human MYOCD promoter, but important questions such as whether TET2 also interacts with non–CArG-rich regions of these promoters and what directs TET2 repression of synthetic gene promoters require further investigation.

Our data reveal that TET2 and 5-hmC levels are modulated by environmental signals in SMCs. TET2 mRNA, protein, and 5-hmC levels are repressed by PDGF-BB, the major stimulus of SMC dedifferentiation in vivo and in vitro. Additionally, our data identify TET2 as a critical effector of the mTORC1 pathway in modulating SMC phenotype. We report that TET2 expression is regulated in part by the mTORC1 pathway because rapamycin induces TET2 expression at both the mRNA and protein levels and TET2 knockdown prevents rapamycin-induced differentiation. We have previously shown that mTORC1 inhibition promotes SMC differentiation at the level of transcription,\footnote{21,22} and rapamycin-eluting stents are highly effective against restenosis.\footnote{46} Our data that local TET2 delivery to an injured artery prevents intimal hyperplasia suggest that manipulation of TET2 and 5-hmC expression may
have important therapeutic implications for other diseases involving aberrant SMC plasticity, including cancer, aneurysm, transplant vasculopathy, and hypertension.

Although TET2 mRNA has been reported to be expressed by many tissues, we noted that TET2 is very highly expressed in SMCs relative to other TET family members. Our data suggest that despite persistent expression levels of TET1 and TET3, these enzymes do not compensate for TET2 knockdown or downregulation by PDGF-BB in SMCs. We also observed robust TET2 expression in SMC-rich tissues and in cultured SMCs relative to other cell types (fibroblasts and endothelial cells). TET2 overexpression was sufficient to induce a program of SMC-specific gene expression in human and mouse fibroblasts, similar to what has been reported for MYOC/D overexpression in myoblasts, ESCs, and 10T1/2 and 3T3 cells. We found very low-level TET2 expression in human umbilical vein endothelial cells compared with SMCs. We achieved a modest overexpression of TET2 in human umbilical vein endothelial cells that was still below that seen in SMC at baseline, and this induced only a subset of SMC genes. It is therefore possible that there is a threshold effect such that high levels of TET2 regulate SMC-specific genes. It is also feasible that as-yet unidentified cell type-specific factors or other permissive chromatin modifications cooperate with TET2 in SMCs and in fibroblasts.

Conclusions

This is the first report of TET protein function in myocytes and the first demonstration of a TET protein regulated by the mTORC1 signaling pathway. Our data reveal that modulating TET2 and, in turn, 5-hmC levels results in broad epigenetic changes in methylation and histones indicative of altered chromatin formations at both differentiation- and dedifferentiation-specific SMC genes. Our findings support TET2 as a master epigenetic regulator of SMC phenotypic modulation. Altering key components of the TET2-5-hmC pathway may be a therapeutic target for treating diseases involving aberrant SMC plasticity that is involved in many cardiovascular associated disorders.

Acknowledgments

We thank Drs Michael Simons and William Sessa for comments on the manuscript. We thank Drs Yibing Qyang for hESC extracts and MRC5 cells and Anne Eichmann for HUVECs.

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Disclosures

None.

References

Vascular smooth muscle cells (SMCs) retain the plasticity to dedifferentiate in response to extracellular cues, including inflammation and growth factors. Dedifferentiated SMCs become proliferative and migratory, secrete copious extracellular matrix, and lose contractility. This contributes to many cardiovascular pathologies, including intimal hyperplasia/restenosis, atherosclerosis, aneurysm, transplant arteriosclerosis, and cancer. Genes associated with differentiated SMC, including contractile apparatus proteins, are known to be transcriptionally regulated. Our work now reveals the protein ten-eleven translocation 2 (TET2) as a novel epigenetic master regulator of SMC plasticity. TET family proteins were recently found to regulate plasticity and that targeting TET2/5-hydroxymethylcytosine may have therapeutic potential for smooth muscle pathologies.

Clinical Perspective

Vascular smooth muscle cells (SMCs) retain the plasticity to dedifferentiate in response to extracellular cues, including inflammation and growth factors. Dedifferentiated SMCs become proliferative and migratory, secrete copious extracellular matrix, and lose contractility. This contributes to many cardiovascular pathologies, including intimal hyperplasia/restenosis, atherosclerosis, aneurysm, transplant arteriosclerosis, and cancer. Genes associated with differentiated SMC, including contractile apparatus proteins, are known to be transcriptionally regulated. Our work now reveals the protein ten-eleven translocation 2 (TET2) as a novel epigenetic master regulator of SMC plasticity. TET family proteins were recently found to regulate plasticity and that targeting TET2/5-hydroxymethylcytosine may have therapeutic potential for smooth muscle pathologies.
Ten-Eleven Translocation-2 (TET2) Is a Master Regulator of Smooth Muscle Cell Plasticity
Renjing Liu, Yu Jin, Wai Ho Tang, Lingfeng Qin, Xinbo Zhang, George Tellides, John Hwa,
Jun Yu and Kathleen A. Martin

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Methods

Virus production: TET2 shRNA retroviruses and TET2 over-expressing lentiviruses were generated using standard protocols. Plasmids were transfected into HEK293 cells using Fugene 6, and the viral supernatant collected by ultracentrifugation. Viruses were titered using published methods. The TET2 adenovirus was purchased from Welgen, Inc. (Worcester, MA). TET2 knockdown cells were collected 48 hours post-infection. Cells were infected with the TET2 overexpression virus for 48 hours prior to puromycin selection for 7-10 days. Efficiency of knockdown and overexpression of TET2 was assessed by qPCR and western blots.

Immunocytochemistry and immunofluorescence: Cells and tissues were fixed and stained using standard protocol. Samples were incubated with antibodies against TET2 (1:250, Sigma-Aldrich), 5-hmC (1:5000, Active Motif), MYH11 (SM-MHC) (1:1000, Abcam), ACTA2 (SM-α-actin) (1:500, Sigma), CDH5 (VE Cadherin) (1:500, Santa Cruz), CD31 (1:400, BD Pharmingen), CD68 (1:400, AbD Serotec), CD45 (1:400, BD Pharmingen). Images were taken using a Nikon A1 spectral confocal laser scanning microscope.

Quantitative morphometry: Tissue morphology was visualized by elastic-Van Gieson (EVG) staining. The areas within the lumen and the areas circumscribed by the internal elastic lamina (IEL) and external elastic lamina (EEL) were determined by tracing along the respective vessel regions. The media was defined as the region between the EEL and the IEL, and the neointima was measured as the region between the lumen and the IEL. Each slide consisted of six sections that were cut 50 µm apart, covering 300 µm of the vessel length. Two or more slides (12-18 sections) per subject were analyzed using a Nikon i80 microscope. Multiple measures in the same subject had high concordance (i.e. intra-class correlation coefficient near 1). The intra-class
correlation coefficient of the same rater single measure reliability ranged from 0.9566 to 0.9914 in the animal studies and was 0.9476 in the clinical study.

qPCR: Total RNA was extracted from cells using the RNeasy mini kit (Qiagen). cDNA was prepared using the SsoFast first strand cDNA synthesis kit (Bio-Rad) and PCR was performed with SYBR green PCR master mix (Bio-Rad). All samples were run in triplicate, from at least three independent experiments, and normalized to β-actin. RNA isolation from tissue slides was performed using the Pinpoint™ Slide RNA Isolation System II (Zymo Research) following the manufacturer’s instructions. Six sections per mouse were used for analysis, and samples were run in triplicate and normalized to β-actin. Primer sequences are listed in Supplementary Table 1-2.

Western blotting: Cell lysates and tissues were lysed with RIPA buffer. Primary antibody used included β-tubulin (1:1000, Santa Cruz), TET1 (1:500, Abcam), TET2 (1:500, Abcam), TET3 (1:500, Abcam), SRF (1:500, Abcam), MYH11 (1:1000, Abcam), ACTA2 (1:500, Abcam), KLF4 (1:1000, Abcam), KLF5 (1:1000, Abcam), OPN (1:1000 Abcam), MYH10 (1:1000, Abcam), CNN1 (1:1000, Sigma), CDH5 (1:500, Santa Cruz). Samples were collected from at least three independent experiments.

Flow Cytometry: Cells were resuspended in PBS and fixed overnight at 4°C with 70% ethanol. The cells were stained with propidium iodide containing RNaseA 1 h prior to analysis. Flow cytometry was performed using BD LSR II flow cytometer and analyzed using FlowJo.
Supplementary Figure 1: TET expression during SMC phenotypic modulation

(A) Western blot of TET1 and TET3 levels in hCASMC treated with 50 nM rapamycin or 5 ng/ml PDGF-BB. (B-H) qPCR analyses of TET1/2/3, MYH11, ACTA2 and KLF4 expression in hCASMC treated with 50 nM rapamycin or 5 ng/ml PDGF-BB. All data were normalized to β-
actin. Error bars represent mean ± SD for three independent experiments. *P<0.05, **P<0.01 ***P<0.001.

Supplementary Figure 2: TET2 expression is high in SMC-rich tissues

Protein lysates were prepared from 10 different tissues from two mice and levels of Tet2 expression were examined by Western blot.
Supplementary Figure 3: Disruption of TET2 results in impaired SMC differentiation and promotes a dedifferentiated phenotype

(A) TET2 protein levels following knockdown using three different constructs in hCASMC, *P<0.05 compared to shCTRL. (B) Western blot and qPCR demonstrating that TET2 knockdown does not affect TET1 or TET3 expression. Expression was normalized to hESC, denoted at 1.0 by the dotted line. (C) Contractile morphology (denoted by arrowheads in shCTRL cells treated with rapamycin) is absent in shTET2 cells after 48 h treatment with 50 nM rapamycin. (D) qPCR analysis for the expression of dedifferentiation-associated mRNAs following TET2 knockdown. Data normalized to β-actin and represent mean ± SD from three independent knockdown experiments. *P<0.05 relative to shCTRL.
Supplementary Figure 4: TET2 overexpression promotes SMC differentiation

(A) mRNA levels showing TET2, SRF, MYOCD and smooth muscle contractile gene expression in CTRL and TET2 overexpressing hCASMC. (B) Phase contrast microscopy showing morphology of control and TET2 overexpressing hCASMC. An elongated morphology (indicated by the arrowheads) characteristic of differentiated SMC was observed with TET2 overexpression. (C) mRNA levels of SMC dedifferentiation genes in TET2 overexpressing hCASMC compared to controls. (D) mRNA levels of cyclins and cyclin-dependent kinase inhibitors with TET2 overexpression in hCASMC. All qPCR data were normalized to β-actin and are presented as mean ± SD from three independent overexpression experiments, *P<0.05, **P<0.01, ***P<0.001 relative to CTRL.
Supplementary Figure 5: Ectopic expression of TET2 directs smooth muscle cell differentiation

qPCR of TET2, smooth muscle contractile and fibroblast-specific gene expression in MRC5 human MRC5 (A) and NIH3T3 mouse (B) fibroblasts infected with the TET2 overexpression virus. (C) qPCR of TET2, smooth muscle contractile and endothelial cell specific gene expression in HUVEC cells infected with the TET2 overexpressing virus. (D) Western blot of TET2, SRF, MYH11 and CDH5 of TET2 infected HUVEC. All qPCR data were normalized to β-actin. Data are presented as mean ± SD. Data shown in (A) is representative of four independent experiments. Data shown in (B-D) are representatives of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 relative to CTRL. Cells were infected with the TET2 overexpressing virus for 2 days and then stably selected with puromycin treatment for 7 days.
Supplementary Figure 6: Morphometric analysis of TET2 knockdown and overexpression following femoral artery wire injury.

Quantification of neointimal (A) and medial (B) areas from each group in Figure 5 using computer-assisted image analysis as in Methods. Sample numbers are as in Figure 5B. Six to eight sections from each individual mouse were used for calculations. Data are presented as mean ± SD.
Supplementary Figure 7: TET2 knockdown or overexpression does not affect endothelial recovery or inflammatory cell infiltration

(A) Immunofluorescence staining of CD31 (endothelial), CD45 (leukocytes) and CD68 (macrophages) on injured mouse femoral arteries that received either the control, TET2 knockdown or TET2 overexpression viruses. Scale bar = 50 µm. (B,D) qPCR of collagen genes in CTRL, TET2 knockdown and overexpression samples. mRNA from uninjured mouse femoral arteries was used as a reference sample and set to 1.0 (denoted by the dotted line). (C,E) qPCR of cyclin and cyclin dependent kinase inhibitor genes in CTRL, TET2 knockdown and overexpression samples. mRNA from uninjured mouse femoral arteries was used as a reference sample and set to 1.0 (denoted by the dotted line). mRNA was isolated and pooled together from ten sections from five individual mouse from each group. All qPCR data were normalized to β-actin and shown are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001 relative to CTRL.
Supplementary Figure 8: Histone modification associated with SMC phenotypic plasticity

(A-D) ChIP-qPCR was performed on hCASMC with antibodies to H3K4 or H3K27 trimethylation and key promoter regions were amplified by qPCR. Data are presented as mean relative enrichment over input ± SD of four biological repeats. (E) H3K4 and H3K27 trimethylation ChIP-qPCR was performed on hCASMC treated with 50 nM rapamycin or 5
ng/ml PDGF-BB for 24 hours. Ratio of K4/K27 in control, untreated hCASMC is denoted by the dotted line. Primers (locations indicated by blue numbers, yielding products represented by the blue bars) were designed to encompass the CArG sequence (denoted by the gray box). Data shown are presented as mean ± SD from four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 
Supplementary Figure 9: TET2 regulates 5-hmC at SMC gene promoters

(A) 5-hmC levels in the *MYOCD*, *SRF* and *MYH11* gene promoters in TET2 overexpressing hCASMC as determined by GlucMS-qPCR or hMeDIP. (B) Immunocytochemistry and DAPI staining of control, TET2 knockdown and TET2 overexpressing hCASMC with an anti-5-hmC antibody. Data shown are presented as mean ± SD from four independent experiments. *P<0.01, ***P<0.001. Scale bar, 50 μm.
**Supplementary Table 1: Primers for qPCR (Human)**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>TET1</td>
<td>GCAGCGTACAGGCCACCACT</td>
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MYOCD (QT00072884) and MYH11 (QT00069391) were QuantiTect primers purchased from Qiagen.

* Denotes primers from Origene.
**Supplementary Table 2: Primers for qPCR (Mouse)**

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<td>Myocd</td>
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<td>AGCTCAAGGACTTGACCAG</td>
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Myh11 (QT01060843) was QuantiTect primers purchased from Qiagen

* Denotes primers from Origene.
Supplementary Table 3: Primers for ChIP-qPCR/hMeDIP-qPCR/MethylCap

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CIRCULATIONAHA/2013/002887
Supplementary Table 4: Primers for GlucMS-PCR

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References


