Tissue-specific DNA demethylation is required for proper B-cell differentiation and function

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There is ample evidence that somatic cell differentiation during development is accompanied by extensive DNA demethylation of specific sites that vary between cell types. Although the mechanism of this process has not yet been elucidated, it is likely to involve the conversion of 5mC to 5hmC by Tet enzymes. We show that a Tet2/Tet3 conditional knockout at early stages of B-cell development largely prevents lineage-specific programmed demethylation events. This lack of demethylation affects the expression of nearby B-cell lineage genes by impairing enhancer activity, thus causing defects in B-cell differentiation and function. Thus, tissue-specific DNA demethylation appears to be necessary for proper somatic cell development in vivo.

Tet2/Tet3 | chromatin | differentially methylated regions | DMRs

DNA methylation takes place at almost all stages of development including the early embryo as well as during lineage commitment and is mediated through a combination of active and passive processes. Recent studies have raised the possibility that demethylation can occur through the involvement of the ten-eleven-translocation family (Tet1, Tet2, and Tet3) that catalyzes the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) as a first step in the pathway (1, 2). Removal of this unusual base may then be accomplished either by further oxidation followed by base excision repair (3) or through replication dilution (4–6). Genetic experiments have demonstrated that Tet enzymes are key players during early development, with Tet3-mediated DNA hydroxylation being involved in epigenetic programming of the zygotic paternal DNA (7, 8), whereas combinations of Tet1 and Tet2 play a role in the demethylation process that takes place during embryonic stem cell differentiation in vitro (2, 9–12).

Tet enzymes also contribute to lineage development. Thus, changes in the pattern of 5hmC have been shown to accompany neurogenesis in vivo (13), and Tet knockdowns indicate that this process may be essential for the normal progression of neuronal differentiation (ref. 14, reviewed in ref. 15). In the hematopoietic system, as well, targeted Tet deletions [Tet1 alone (16), Tet2 alone (17–20), or Tet1 and Tet2 together (21)] appear to alter global 5hmC and 5mC distribution, perturb stem cell self-renewal, cause altered differentiation, and predispose to malignancies (refs. 19, 22, reviewed in ref. 23). None of these studies, however, has addressed the key question of whether demethylation itself is actually required for gene activation and proper lineage differentiation. To this end, we generated a Tet2/Tet3 knockout specific to B-lymphoid development, isolated cells at different stages of differentiation, and analyzed their methylation patterns. Because this approach targets the demethylation machinery in an exclusive manner, it allowed us to evaluate the role of this modification independently of the many transcription factors that drive the process of B-cell differentiation.

Results

It has already been shown that both Tet2 and Tet3 are highly expressed in B lineage cells (24). With this in mind, we generated Tet2/Tet3 mice (18, 23) and crossed them with animals expressing Cre under control of the early B-cell-specific Mb1 promoter (25) to obtain mice with a conditional knockout of these enzymes specifically in the B-cell lineage (Materials and Methods). Reduced representation bisulfite sequencing (RRBS) (26) measures DNA methylation levels at a large number of regulatory regions in the genome with high depth and reproducibility. We wanted to test whether B-cell-specific deficiency of Tet2, Tet3, or a combination thereof would specifically impair DNA methylation during B-cell differentiation.

To this end, we isolated mature naive follicular (Fo) B cells from spleens of 6–to 8-wk-old wild-type and knockout mice by FACS and performed RRBS. Although overall methylation levels were very similar in all samples (Fig. S1A), we identified approximately one thousand four hundred 100-bp tiles that were at least 40% under-methylated in control follicular B cells compared with Tet2/Tet3 double knockouts (DKOs) (P < 10−300, permutation test; Materials and Methods) with lesser effects being observed for each knockout individually. RRBS analysis revealed that these same sites are highly methylated in E7.5 embryos as well as in a variety of adult tissues (Fig. 1). This indicates that they likely become de novo methylated at about the time of implantation and remain modified in most cell types during development, undergoing demethylation exclusively in the B-cell lineage. These results show that Tet2 in combination with Tet3 plays a key role in tissue-specific demethylation.

To further pinpoint the role of these enzymes in this demethylation process, we carried out RRBS analysis on pro-B cells,
bone marrow-derived B-cell precursors. Examination of tiles that are methylated in wild-type pro-B cells and only undergo specific demethylation during differentiation to mature follicular B cells shows that Tet2/Tet3 double deficiency initiated before the pro-B cell stage prevents demethylation at over 95% of these sites. Although our assay (RRBS) is not fully genomic, these results strongly suggest that Tet proteins may be responsible for almost all DNA demethylation that occurs at this stage (Fig. 2A). In contrast, examining sequences that have already undergone demethylation in common lymphoid precursors (CLPs), an early stage of development before B-cell commitment, and before the activation of Cre, reveals that the B-cell–specific Tet2/Tet3 knockout has almost no effect on DNA methylation at these sites (Fig. 2B and Fig. S2A). Thus, even though it has already been demonstrated that demethylation occurring at a given stage of B-cell development is retained further on (27), our experiments support the fundamental idea that once established, the undermethylated state is autonomously maintained without the need for continuous demethylase activity (28), thereby constituting a genuine memory mechanism.

To further validate the requirement of the Tet proteins for DNA demethylation, we examined the Igκ locus using specific primers and Bisulfite sequencing to measure DNA modification at sites that are known to undergo specific demethylation during normal B-cell development (29). The results indicated that Tet2 and Tet3 are indeed required for demethylation of key regulatory sites within the Igκ gene locus (Fig. S2B). Taken together, these findings demonstrate that B-cell lineage demethylation takes place through a Tet-dependent biochemical pathway that likely converts 5mC to 5hmC and further oxidation products, which may then be removed by glycosylation and subsequent base excision repair (3). In keeping with this, genome-wide analysis indicates that these same sites are indeed enriched with DNA methylation levels from wild-type and Tet2/3 low DNA methylation levels. (B) Samples are compared with other wild-type somatic tissues, embryonic day 7.5 (E7.5), liver (Liv), brain (Br), colon (Col), neutrophils (Neut) (n = 2–4) (39). Yellow represents high and blue represents low DNA methylation levels. (B) Hierarchical clustering analysis was performed on RRBS tiles from wild-type and Tet2/3 follicular B cells.

The fact that Tet2/Tet3 deficiency specifically prevents the demethylation that occurs during normal B-cell development presented a unique opportunity to test whether the change in DNA methylation itself plays a role in controlling gene expression in vivo. Because Tet-dependent demethylation seems to take place primarily at putative enhancer elements and not at promoters (Fig. 3), we first restricted our analysis to tiles (n = 814) located within gene domains and compared the expression levels of these genes in the presence or absence of DNA methylation at the enhancer. Strikingly, 23% of these tiles (n = 186), as opposed to a random sample (7%), are located within genes (n = 111) that were found to be inhibited in the Tet2/Tet3 knockout (P < 10−27, z-test of proportions) (Fig. 4A and Fig. S3), with the difference in expression being highly significant (P < 10−39, t test) (Fig. 4B). Furthermore, by then analyzing a published HiC dataset from B-lymphoblastoid cells (38) to detect DMR interactions with distal promoter sequences, we were able to pick up an additional set of 47 genes that are down-regulated in the DKO (Fig. S4). It should be noted that because RRBS only covers a portion of the genome, there are undoubtedly many more, as yet undiscovered, Tet2/3-dependent DMRs that may influence genes in follicular B cells, perhaps explaining the finding that over 1,000 genes are differentially expressed at higher levels in the wild type as shown by RNA-seq (P < 0.05, t test). Furthermore, there are probably other genes that are initially primed by demethylation but still require additional factors to affect expression. Almost all specific genes associated with DMRs have promoters that are completely unaffected by the knockout (Fig. S1C), suggesting that Tet-dependent demethylation represents a regulatory mechanism directed almost exclusively to sequences that most likely have enhancer activity that is sensitive to DNA methylation (39).

We next asked whether these effects of methylation on gene expression are associated with genes actively involved in B-cell development. These genes are differentially marked with histone H3K4me1 in B-lineage as opposed to T-lineage cells, with the highest enrichment seen in mature B cells. Because these same tiles are also specifically enriched for histone H3K27Ac and assume a more open configuration as determined by an assay for transposase-accessible chromatin (ATAC) (33) (Fig. 3B), they likely represent enhancer-like elements (34–37) that normally become activated during B-cell development.

To evaluate the nature of the sites that undergo DNA demethylation during B-cell development, we mapped their locations in the genome, as well as the presence of key chromatin components using published ChIP-Seq data (30–32). Most of the relevant tiles are located within gene coding regions, with only a small percentage being associated with promoters (Fig. 3A). Furthermore, these sites are differentially marked with histone H3K4me1 in B-lineage as opposed to T-lineage cells, with the highest enrichment seen in mature B cells. Because these same tiles are also specifically enriched for histone H3K27Ac and assume a more open configuration as determined by an assay for transposase-accessible chromatin (ATAC) (33) (Fig. 3B), they likely represent enhancer-like elements (34–37) that normally become activated during B-cell development.

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development. Initial gene ontology analysis [Genomic Regions Enrichment of Annotations Tool (GREAT)] (40) indicated that the differentially expressed genes are highly enriched for various aspects of B-cell function, maintenance, and development (Fig. 4C). Many of these genes have also been shown to play a role in hematopoiesis by in vivo genetic analyses (Fig. 5S). Furthermore, the presumed regulatory sites themselves harbor a large variety of binding motifs [Hypergeometric Optimization of Motif EnRichment (HOMER)] (41) for key factors known to be important for B-cell development (e.g., Ebf1, Irf4, E2a, Oct2, Pax5, and Pu.1) (Fig. 5A). ChIP analyses adapted from published data (31, 32) indicate that these factors as well as the chromatin remodeler, Brg1 (33), indeed bind to these sites in mature B cells (Fig. 5B and C).

In light of these findings, we suspected that the lack of demethylation might also affect B-cell development and function. To test this we carried out a series of flow cytometry analyses aimed at assessing lymphoid cell composition in wild type, Tet2, and Tet3 single-knockout as well as double-knockout mice. In the bone marrow we detected a significant (threefold) shift in the ratio between pro-B and pre-B cells in the Tet2/Tet3 knockout indicating a partial block in early B-cell development. This was accompanied by an eightfold decrease in the number of mature recirculating B cells (Fig. 6E and F and Fig. S6D and E). A similar shift was detected in the spleen where B-cell progenitors were present in abnormally large numbers. Two mature B-cell subsets, namely, Marginal Zone (MZ) B cells in the spleen and B1 cells in the peritoneal cavity, were essentially absent (Fig. 6A–C and Fig. S6A and B). Together, these data strongly suggest that demethylation plays a key role in the B-cell maturation process and that its absence leads to an accumulation of more primitive cell types. In addition, RNA-Seq analysis of the Igκ locus in follicular B cells indicated that the DKO causes a change in the light chain repertoire, characterized by a dramatic shift ($P < 10^{-5}$) to more proximal V region rearrangements (Fig. S7).

Despite B-cell developmental defects in Tet2/Tet3 DKO mice, the mice displayed normal numbers of mature splenic follicular B cells (Fig. S6). Thus, we asked whether these cells would be functionally impaired and unable to mount an immune response. Indeed, Tet2/Tet3 knockout mice did not respond to T-dependent immunization with sheep red blood cells (SRBCs), as suggested by the absence of germinal center as well as IgG1+ class switched B cells (Fig. 6D and Fig. S6C). Apart from this, the absence of MZ and B1 cells in the DKO animals implies major defects in the production of natural antibodies and immune defense against blood-borne pathogens. It is noteworthy that in our study, no abnormal phenotypes were observed in single Tet2 or Tet3 knockout animals, suggesting that even partial demethylation of B-cell relevant regulatory regions in vivo may allow normal B-cell development and function (Fig. S6). An unexpected phenotype in the DKO animals was an expansion of myeloid cells in the spleen (Fig. S6B), which progressed over time and is the subject of a separate study.

**Discussion**

As part of the programmed changes in DNA methylation that occur during development, many genes undergo tissue-specific demethylation in association with differentiation, mainly at enhancer-like sequences (42, 43). The physiological role of this demethylation has not yet been elucidated, presumably because the mechanism of this process was not known. In this paper, we demonstrate that almost all demethylation that occurs during B-lineage development requires the specific combination of Tet2 and Tet3 (Fig. 1). Although these enzymes might also exhibit other activities (44–46), the fact that their deletion specifically affects the same tiles that undergo demethylation during B-cell lineage differentiation and are specifically marked with 5hmC (16) (Fig. S1B) provides strong genetic evidence that lineage-specific demethylation is a direct result of Tet activity on its target sequences. These results are in keeping with previous experiments in embryos and tissue culture showing that Tet enzymes indeed bind to enhancer sequences and are required for the generation of 5hmC and subsequent demethylation (47–50) but represent the first demonstration to our knowledge

**Fig. 3.** Characterization of DMRs. (A) Genome distribution of regions demethylated in vitro compared with Tet2/3 FoB cells. (B) Left and Center show ChIP-Seq of demethylated regions $(n = 1,399)$ as a function of distance from their center for H3K4me1 and H3K27ac in hematopoietic stem cells (HSC), common lymphoid progenitors (CLP), B cells, and T cells (GSE60103). Right shows ATAC-seq in CLP, pro-B (GSE66978), and B cells (GSE59992). It should be noted that this accessibility marker is not present at early stages of hematopoiesis and only appears in cells that have undergone demethylation at these sites.

**Fig. 4.** Effect of DNA methylation on expression. (A) Percentage of tiles $(n = 814)$ located in genes with decreased (blue) and increased (red) expression (Tet2/3 < wt FoB cells) associated with DMRs compared with random tiles ($P < 10^{-7}$, z-test of proportions). (B) Heatmap of relative expression levels (RNA-seq) for genes $(n = 111)$ that harbor putative enhancer sequences. Each column shows average data for three biological replicates. (C) Gene ontology of all DMRs $(n = 814)$ by GREAT analysis (40).
that these enzymes also play a role in tissue-specific demethylation in vivo. This, of course, does not rule out the possibility that other enzymatic components (e.g., AID) may also be necessary for demethylation at specific stages of development in vivo (51).

Despite many decades of research, it is still debated whether DNA methylation plays a causative role in development (52). Thus, even though demethylation of regulatory sequences is often correlated with increases in nearby gene transcription in general (53, 54) and in the B-cell lineage in particular (27), it has not been possible so far to determine whether the removal of methyl groups is indeed required for altering transcription patterns. We have used a Tet2/Tet3 knockout to specifically inhibit demethylation in vivo and in this manner have demonstrated that this process is necessary for the proper control of gene expression and for normal developmental progression. Because demethylation is programmed, it must be mediated by transacting factors that recognize the target sequences. Nonetheless, these factors appear to be insufficient by themselves to fully activate gene transcription. This only occurs once demethylation has taken place, probably because this brings about histone acetylation (55, 56) as well as increased chromatin accessibility (Fig. 3B), which may then allow the binding of additional transcription factors (57, 58).

There is evidence that normal hematopoiesis is very sensitive to changes in DNA methylation metabolism, with knockouts of either Dnmt3a, Tet1, or Tet2 causing defects in differentiation while predisposing mice to hematopoietic malignancies (16, 19, 22, 56, 59, 60). In humans, aberrations in these same genes can be detected in hematopoietic cells of leukemic patients (23) as well as in healthy individuals (61), where they may be selected to become preleukemic (62). Our findings help explain how Tet deficiency may contribute to tumorigenesis by preventing enhanced demethylation at genes critically involved in the control of cellular differentiation, thereby promoting precursor cell proliferation.

Materials and Methods

Mice. Tet2α−/−(63), Tet3−/−(23), and Mb1-cre+/- (wt) (25) mice have been described. All mice are C57BL/6 or have been backcrossed to the C57BL/6 background for more than 10 generations. Mice were bred and housed under specific pathogen-free conditions in microisolation cages in a room illuminated from 0700 to 1900 hours (12:12- light-dark cycle), with access to water and chow ad libitum. Genotyping of Tet2, Tet3, and Mb1-cre alleles was performed by PCR amplification of genomic DNA purified from ear snips, using the following primers: Tet2 F GCGCAAGAAAAACAGACAAAG, Tet2 R AAGGGAGGGACTTTCCTCCATCTCAAGAA, Tet3 F CAGTAGGAGGAGTTGACGGTG, Tet3 R TGACAAACCCACACCAACGAA, Mb1-cre F GTGTTAGCTGACCTGTAGAATGC, and Mb1-cre R TGGCTGCACTGACGACCGAAC. It should be noted that the deletion of Tet2 and Tet3 floxed DNA in the Mb1-cre crosses was extremely efficient in both mice and follicular B cells, with no retention of the floxed exons in the Tet3 locus and only 0.5–1% retention in the Tet2 locus as measured by PCR.

Six- to eight-week-old mice were used for all experiments. For T-dependent immunization experiments, mice were injected once i.v. with 109 SRBCs from BioLegend; or mechanic disruption of cell clusters by pipetting (bone marrow, peritoneal washes). Erythrocytes in bone marrow and spleens were lysed with ACK lysis buffer on ice before staining and cell numbers were determined using a hemocytometer (Neubauer) and trypan blue exclusion. Single-cell suspensions for flow cytometry were stained with the following antibodies: αB220-BV785 (RA3-6B2); αCD38-APC (90); αCD1d-PE (1B1) from eBioscience; and goat α-mouse IgM, μ chain specific, from Jackson ImmunoResearch. Single-cell suspensions for cell sorting were obtained using a hemocytometer (Neubauer) and trypan blue exclusion. Single-cell suspensions for flow cytometry were stained with the following antibodies: αB220-BV785 (RA3-6B2); αCD11b-BV711 (M1/70); αCD38-APC (90); αCD117-APC (ACK2); αCD25-PE (PC61) and αCDS-PE (S3.7–3) from BioLegend; αCD1d-PE (1B1) from eBioscience; αCD90-PE/Cy7 (Jo2) and αGl1-PE (A301) from BD; and goat α-mouse IgM, μ chain specific, from Jackson ImmunoResearch. Single-cell suspensions for cell sorting were stained with the following antibodies: αB220-PerCP/Cy5.5 (RA3-6B2); αCD19-BV605 (6D5); αDg-PerCP/Cy5.5 (11-2C6.2a); αCD93-APC and αCD93-Pecy7 (AA4.1); αCD11b-BV711 (M1/70); αCD38-APC (90); αCD117-APC (ACK2); αCD25-PE (PC61) and αCDS-PE (S3.7–3) from BioLegend; αCD1d-PE (1B1) from eBioscience; αCD90-PE/Cy7 (Jo2) and αGl1-PE (A301) from BD; and goat α-mouse IgM, μ chain specific, from Jackson ImmunoResearch. Single-cell suspensions for flow cytometry were stained with the following antibodies: αB220-PerCP/Cy5.5 (RA3-6B2); αCD19-BV605 (6D5); αCD38-APC and αCD93-Pecy7 (AA4.1); αCD25-PE (PC61) and αCD1d-PE (1B1) from eBioscience; αCD90-PE/Cy7 (Jo2) and αGl1-PE (A301) from BD; and goat α-mouse IgM, μ chain specific, from Jackson ImmunoResearch.
wild-type DNA from pro-B or follicular B cells includes small quantities of ShmC as an intermediate in the demethylation process.

Gene Expression. RNA was isolated from snap-frozen FACS-sorted B cells using the Qiagen miRNeasy kit according to the manufacturer’s instructions (Cat. 217084). Fifty nanograms of RNA was taken from different samples and re-versed to cDNA using the cscript kit (Quanta Biosciences 95047). Expression levels of specific genes were tested and normalized to two different housekeeping genes. For RNA-seq analysis, 20-200 ng of RNA was isolated, and the TruSeq RNA Sample Preparation Kit v2 (Illumina) was used for Library preparation. Primers are as follows: housekeeping genes: UBC F TACCCGCA-TATCTTCCCCAGACT, UBC R CTAGAGGATGCGACATTCTA; Gapdh F CCTGGAGG-AAGACCTGCCAAG, Gapdh R CAAACCTGCTCCACGTAGAC.

RNA-seq 50-bp single-end reads were obtained from the HiSeq 2500 and analyzed by TopHat2 v2.0.10 with default parameters (65). Differential expression between wild-type and knockout mice was analyzed by Cuffdiff v2.1.1 with default parameters (Q value ≤ 0.01) (66) and shown to be reproducible between replicates by clustering analysis (data not shown).

Data Analyses. All data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE70538, and a portion has been included as Dataset S1. One-hundred-base pair paired-end sequencing reads from RRBS (GEO) database: Pax5 (GSE38046) (32); Brg1 (GSM1635413) (33); 5hmC (GSE65895) (16) –Motif analysis was carried out by HOMER v4.7.2 (findmotifs.pl) (41). ChIP-seq data were obtained from the publicly available Gene Expression Omnibus database: Pax5 (GSE38046) (32); EBF1, Pu.1, and IRF4 (GSE53595) –Gene ontology analysis was carried out using GREAT (40). H3K4me1 and H3K27Ac; and ENCODE annotation data (GSE31039) (69). One-hundred-base pair tiles and DMRs were calculated with the MethylKit package v0.9.1 (68) using a minimum coverage of 10 per tile, a methylation coverage. Regions with a minimal total coverage of 10 were reported. The DMR set was calculated using a permutation test: the above steps were repeated on random permutations of wild-type and knockout samples, and for each permutation the number of DMRs was calculated. The P value was obtained by measuring the quantile of the original DMR size (in our case the original number of DMRs was the highest among all permutations). Promoter methylation levels were calculated by summing the methylation calls of all single CpGs in the promoter and dividing by the sum of their original number of DMRs was the highest among all permutations). One-hundred-base pair tiles and DMRs were calculated with the MethylKit package v0.9.1 (68) using a minimum coverage of 10 per tile, a methylation difference of 40%, and a Q value ≤0.01. The statistical significance of the DMR set was calculated using a permutation test: the above steps were repeated on random permutations of wild-type and knockout samples, and for each permutation the number of DMRs was calculated. The P value was obtained by measuring the quantile of the original DMR size (in our case the original number of DMRs was the highest among all permutations).
Hi-C datasets (GSE63525) were analyzed using HOMER, filtering out paired reads that map within 1 kb of each other, map to regions with at least 5x higher than background, or are not within 500 bp of a protein site. Interactions with a P value < 0.01 and a modified Z-score > 1.5 were calculated with the analyzeHi-C interactions tool in the HOMER package, using background models of 10,000, 5,000, 2,000, and 1,000 bp bins. Interactions between promoters and DMRs were identified from this list using the HOMER annotateInteractions.pl tool.


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