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Microfluidic MeDIP-seq for low-input methylomic analysis of mammary tumorigenesis in mice

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Abstract

Studies of dynamic epigenomic changes during tumorigenesis using mice often require profiling epigenomes using a tiny quantity of tissue samples. Conventional epigenomic tests do not support such analysis due to the large amount of materials required by these assays. In this study, we developed an ultrasensitive microfluidics-based methylated DNA immunoprecipitation followed by next-generation sequencing (MeDIP-seq) technology for profiling methylomes using as little as 0.5 ng DNA (or ~100 cells) with 1.5 h on-chip process for immunoprecipitation. This technology enabled us to examine genome-wide DNA methylation in a C3(1)/SV40 T-antigen transgenic mouse model during different stages of mammary cancer development. Using our data, we identified differentially methylated regions and their associated genes in different periods of cancer development. Our results showed that unique methylomic features were presented in various tumor developmental stages.

Graphical Abstract

An ultralow-input microfluidic MeDIP-seq technology was applied to study genome-wide DNA methylation during mouse tumor development.

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Availability of data

Gene Expression Omnibus: Microfluidic MeDIP-seq data are deposited under accession number GSE109400 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109400>).

Live subject statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Virginia Tech and the animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech.

Conflicts of Interest

There are no conflicts of interest to declare.

Keywords

Microfluidics; DNA Methylation; MeDIP-Seq; Mammary Cancer

Introduction

Epigenetic mechanisms such as DNA methylation, histone modifications, nucleosome positions, and expression of noncoding RNA play critical roles in regulations of cellular processes¹. DNA methylation is the best studied epigenetic modification in mammals. DNA methylation typically refers to the addition of a methyl group at the carbon-5 position of cytosine residues within CpG dinucleotides, forming 5-methylcytosine (5mC). Clusters of CpG sites ("CpG islands" or CGIs) commonly span promoters of housekeeping genes². Promoter CGIs typically remain unmethylated in normal cells and are associated with active gene expression during differentiation, while methylated CGIs are associated with gene repression. DNA methylation is highly involved in biological processes such as embryonic development and tumorigenesis^{3, 4}.

The genome-wide DNA methylation profile (i.e. the DNA methylome) is established early in development for regulation and maintenance of gene expression during differentiation. During the course of disease development such as tumorigenesis, the methylome is altered and disrupted. For example, hypermethylation at various genes in tumors has been recognized as a common epigenetic feature for all types of human cancers. Genes including cell-cycle inhibitor p16-INK4a⁵⁻⁸, DNA-repair genes MLH1 and BRCA1^{9, 10}, and tumor-suppressor genes Rb and p53 have been shown to undergo methylation-associated silencing in tumor cells¹¹⁻¹³. While acquiring hypermethylation at specific promoters, overall human tumors undergo a global genomic hypomethylation. Since DNA methylation protects the integrity of chromosomes, this global DNA hypomethylation potentially contributes to large-scale genetic changes that are hallmarks of tumorigenesis. Therefore, understanding of the dynamics in the methylome during cancer development will improve various aspects of biomedicine, including risk stratification, disease diagnostics, and drug/therapeutic discovery.

Bisulfite sequencing is generally considered the gold standard for DNA methylation analyses^{14, 15}. Bisulfite treatment converts cytosine residues to uracil, but leaves 5mC

residues unaffected. Such treatment introduces changes in the DNA sequence based on the methylation status of individual cytosine residues. Combined with next-generation sequencing (NGS), the approach generates methylomic profile with single-nucleotide resolution. In spite of the ultrahigh resolution, bisulfite sequencing requires a large amount of DNA (1–5 μ g) and the cost associated with deep sequencing is high^{16–18}. It normally requires at least 500 million reads per sample to cover about 95 % of the genome^{19, 20}. This can be prohibitive when various samples with repeats are analyzed²¹. Another alternative to bisulfite sequencing is reduced representation bisulfite sequencing (RRBS)^{22, 23}. Although RRBS provides single-nucleotide resolution, it only covers 5–10 % of genome (mostly CGIs and promoter regions). Enrichment-based technologies have also been applied to study methylomes^{24–28}. These approaches dramatically reduce the required sequencing depth by enriching methylated DNA fragments based on affinity purification. Methylated DNA immunoprecipitation followed by next-generation sequencing (MeDIP-seq) uses a monoclonal antibody specific for 5mC to target single-stranded methylated DNA fragments (sheared by sonication) and identifies these fragments using sequencing^{26–30}. In comparison to bisulfite sequencing, MeDIP requires only 30–60 million reads per sample with a 100–300 bp resolution^{31, 32}. MeDIP-seq provides an unbiased tool for probing DNA methylation events both within CGIs and non-CGI regions throughout the entire genome. There have been a number of microfluidic technologies developed for profiling genome-wide epigenetic changes^{33–41}.

Conventional MeDIP-seq requires 5–20 μ g DNA^{26, 28} due to the low efficiency associated with immunoprecipitation. Given that a diploid mammalian cell typically contains 4–8 pg of DNA, conventional MeDIP-seq requires at least 10^6 – 10^7 cells. The current state-of-the-art MeDIP-seq requires 50 ng DNA, which is roughly equivalent to DNA from 10^4 cells^{31, 42, 43}. In this study, we developed an ultrasensitive microfluidic MeDIP-seq technology for profiling methylomes using as little as 0.5 ng DNA (or ~50–100 cells) with a 1.5 h on-chip process. After demonstrating the principle using a cell line, we then obtained methylomic profiles in a C3(1)/SV40 T-antigen transgenic mouse model during mammary cancer development. Our results indicated that DNA methylomes experienced dynamic changes during the development of mammary tumors.

Results and discussion

The chip device contained 7 reaction chambers that allow to run 7 parallel MeDIP assays at the same time (Fig. 1a). The device and operation were similar to what we demonstrated previously for MOWChIP-seq³⁴. However, the MOWChIP-seq technology focuses on studies of genome-wide histone modifications while the current technology is designed to examine DNA methylation. MeDIP started with flowing a suspension of 5mC-antibody-coated magnetic beads (IP beads) into the microfluidic chamber (~720 nl) and the IP beads were packed against a partially closed microvalve to form a packed bed (Fig. 1b). Sonicated single-stranded DNA (ssDNA) (100–500 bp, in 50 μ l, Supplementary Fig. S1) were then flowed through the bead-packed bed, allowing targeted methylated DNA to adsorb onto the bead surface (Fig. 1c). After MeDIP, we applied alternating pressure pulses at two ends of the microfluidic chamber (at 0.5 p.s.i. and with a duration of 0.5 s for each pulse) for 5 min to create oscillatory washing to effectively remove nonspecific binding (Fig. 1d). Such

washing was conducted in MeDIP buffer. After the oscillatory washing, the beads were retained by a magnet on one side of the chamber while the unbound ssDNA fragments and other debris/waste were flushed out of the microfluidic chamber by MeDIP buffer (Fig. 1e). Finally, the MeDIP beads with adsorbed ssDNA fragments were flushed out of the chamber and collected for off-chip processing.

We first tested the technology using a lymphoblastic cell line GM12878. MeDIP-qPCR was used to quantify the MeDIP efficiency at different antibody coating concentrations (Fig. 2). Fold enrichment represented the ratio of percent input between known methylated DNA loci (GEMIN4 and ZC3H13) and known unmethylated DNA loci (N1 and N2). With 100-ng starting DNA, the fold enrichment increased as antibody coating concentration increased (Fig. 2a). However, with 10-ng starting DNA, the fold enrichment decreased as antibody coating concentration increased (Fig. 2b). This suggests careful optimization of antibody coating conditions (to produce the highest fold enrichment) was necessary for different starting DNA amounts. Too little antibody would be insufficient for DNA adsorption while too much antibody would increase non-specific adsorption.

MeDIP-seq results were obtained using our microfluidic technology with various amounts of starting DNA (100–0.5 ng) from human cell line GM12878. As expected, the quality of methylomic profiles slowly declined when the DNA sample amount decreased from 100 to 0.5 ng (Fig. 3a). The correlation coefficients r between the two replicates were excellent for samples in the range of 100–5 ng (0.93, 0.91, 0.90 for 100-, 10-, 5-ng samples, respectively) (Fig. 3b). Data obtained using 10- and 5-ng samples were also highly correlated with those of 100-ng DNA samples (with average r of 0.91 and 0.90, respectively). For 0.5 ng DNA samples, the correlation between the two replicates was 0.80 and the correlations to data of 100 ng DNA samples were 0.69 and 0.60 for the two replicates. Nevertheless, most of the major MeDIP peaks can be seen in the data of 0.5 ng DNA samples (Fig. 3a, and Supplementary Fig. S2). 0.5 ng DNA samples averagely yielded 72,432 peaks per data set, compared to 105,410 peaks per data set for 100 ng DNA samples. Our microfluidic MeDIP process collected 0.2 ng and 1.2 ng DNA out of the input 10, 100 ng DNA, respectively, with optimized antibody coating conditions. The amount of collected DNA was too low to be measured when the amount of starting DNA was 5 or 0.5 ng. The microfluidic MeDIP process took about 40 min, compared to overnight immunoprecipitation in conventional MeDIP protocols⁴².

To quality-control MeDIP-seq data, saturation analysis was conducted by determining the number of reads needed to generate a genome-wide profile that could be reproduced by another independent set of reads of similar number. We defined saturation sequencing depth as the minimum number of reads needed for each of two subsets of sequencing reads that yielded a >0.95 Pearson correlation coefficient between them. 0.5 ng DNA samples required an average 3.17 million reads to saturate (Supplementary Fig. S3). With the input amount DNA going up, 100 ng samples only required an average of 0.89 million reads to saturate (Supplementary Fig. S3).

MeDIP-seq data were normalized based on the coverage and CpG density in genome-wide windows (of 250 bp each)²⁸. This can be visualized as calibration plots. The 0.5 ng DNA

sample (Supplementary Fig. S4a) and 100 ng DNA sample (Supplementary Fig. S4b) plots showed that most CpG-poor regions were methylated, whereas CpG-rich regions were generally unmethylated, revealing the linear relationships between the MeDIP-seq reads and the density of methylated CpGs.

We obtained methylomic profiles on mammary tumors of different stages that harvested from C3(1)/SV40 T-antigen transgenic mice (Fig. 4a). This transgenic mouse model contains a recombinant gene expressing simian virus 40 early-region transforming sequences under the regulatory control of the rat prostatic steroid binding protein C3(1) gene⁴⁴. The expression of TAg in the mammary epithelium results in progressive lesions and tumor development in all female mice. Atypia of the mammary ductal epithelium develops at about 8 weeks of age, representing low-grade mammary intraepithelial neoplasia (MIN). The atypical lesions progress to high-grade MIN, resembling human ductal carcinoma in situ (DCIS) at about 12 weeks of age. Invasive carcinomas are typically observed at about 16 weeks of age⁴⁵.

Histologic progression of formalin-fixed, paraffin-embedded mammary normal tissue and mammary tumor tissue were stained by Hematoxylin and Eosin then imaged. Mammary tissues were first harvested from 6 week old C3(1)/Tag transgenic mice (Fig. 5a). Early stage of mammary intraepithelial neoplasia progressed into invasive carcinoma beginning at about 16 weeks, with cribriform patterns formed in mammary tumor tissue (Fig. 5b). As tumor progressed, more cribriform patterns were found in 23 weeks mammary tumor tissue (Fig. 5c).

The correlation coefficient r between two replicates was 0.82, 0.99, and 0.81 for 6 week 16 week, 23 week samples, respectively. 23 week samples were more consistent with 16 week samples (average $r = 0.86$) than with 6 week samples (average $r = 0.63$) (Fig. 4b). There were substantial changes in the MeDIP-seq peaks at critical genes with tumor development. For example, when comparing MeDIP-seq profiles of 23 weeks samples (invasive carcinomas tumor tissues) and 6 week samples (healthy mammary tissue), we observed peak decrease (decreased level of methylation or hypomethylation) with development of tumor at oncogene CDK4 (Fig. 5d). In contrast, at tumor suppress gene HIC1, higher peaks (hypermethylation) were observed in mammary tumor tissues of 23 weeks (Fig. 5e).

Using EdgeR test ($p < 0.01$), we were able to identify 29984 differentially methylated regions (DMRs) between 6 and 16 week samples (Supplementary Table S1), 16483 DMRs between 16 and 23 week samples (Supplementary Table S2). 3938 DMRs were included in both sets. This means that 12545 DMRs were newly developed during the period of 16–23 weeks. In addition, we tracked hypermethylation and hypomethylation, which were increase or decrease in the methylation level over the course of mammary tumor development, respectively. When the 16 week samples were compared to 6 week ones, out of the total of 29984 DMRs, 12532 (42%) were hypermethylation and 17452 (58%) were hypomethylation. 9747 (59%) DMRs were hypermethylated and 6736 (41%) DMRs were hypomethylated when comparing 23 week data with 16 week data. Interestingly, 1772 out of the 12532 hypermethylated DMRs found at 16 weeks were also identified as hypermethylated during the period of 16 to 23 weeks (i.e. there were 7975 newly developed

hypermethylated DMRs from 16 to 23 weeks). In contrast, none of the hypomethylated DMRs found during 6–16 weeks were identified as hypomethylated during 16 to 23 weeks (i.e. all the 6736 hypomethylated DMRs developed during 16 to 23 weeks without any prior decrease in the methylation level during 6–16 weeks).

We also divided DMRs to genomic regions including introns, exons, promoters, CpG islands, CpG islands in promoter regions (Fig. 6). There were generally more hypermethylation DMRs than hypomethylation ones in the overall level and all these specific genomic regions for the two analyses (6–16 and 16–23 week). During 6–16 weeks, we found particularly large differences between hyper- and hypo-methylation in CpG islands and CpG islands in promoter regions (Fig. 6a). In comparison, in the 16–23 week analysis, hypermethylation was substantially more prevalent than hypomethylation in all categories of genomic regions analyzed (Fig. 6b). This also led to a much larger margin between hyper- and hypo-methylation in the entire genome in 16–23 weeks than in 6–16 weeks.

Genes associated with DMRs were identified by Genomic Regions Enrichment of Annotations Tool (GREAT)⁴⁶. We were able to identify 14207 genes associated with 29984 DMRs when tumor progressed from 6 weeks to 16 weeks (Supplementary Table S3), and 10147 genes associated with 16483 DMRs when tumor progressed from 16 weeks to 23 weeks (Supplementary Table S4). A large percentage of the DMR-associated genes in 16–23 weeks (84.7%, or 8598 out 10147) were also identified as DMR-associated during 6–16 weeks. GREAT analysis specifically identified enrichment of DMRs near genes involved in biological processes, mouse phenotype, and disease ontology in mammary tumorigenesis (Supplementary Fig. S5). GO terms are defined significant by both the binomial test over genomic regions (top 100 binomial P-value) and the hypergeometric test over genes (top 1500 hypergeometric FDR Q-value).

DNA methylation level is known to have strong association with chondrocyte differentiation activity and cartilage development^{47–51}. Some important biological processes such as regulation of chondrocyte differentiation, regulation of cartilage development, and regulation of stem cell differentiation were found by GREAT in tumor progression in both the periods of 6 to 16 weeks and 16 to 23 weeks (Supplementary Fig. S5a). Mouse phenotype of abnormal endochondral bone ossification was another process related to DNA methylation and breast cancer development^{52–54} that was found in tumor progression during both 6–16 weeks and 16–23 weeks (Supplementary Fig. S5b). Some unique stage-specific biological processes such as negative regulation of G1/S transition of mitotic cell cycle, negative regulation of mitotic cell cycle phase transition were only found when tumor progressed from 6 to 16 weeks. Other biological processes such as cellular lipid metabolic process, cellular response to growth factor stimulus were only found when tumor progressed from 16 to 23 weeks. Mouse phenotypes such as abnormal cervical vertebrae morphology, abnormal palatal shelf fusion at midline were only found when tumor progressed from 6 to 16 weeks while short sternum, chondrodystrophy were only found when tumor progressed from 16 to 23 weeks. Disease ontologies such as nasopharynx carcinoma, adenocarcinoma of prostate were only found when tumor progressed from 6 to 16 weeks and myomatous neoplasm, muscle tissue neoplasm were only found when tumor progressed from 16 to 23 weeks (Supplementary Fig. S5c).

We compared DMR-associated genes involved in our early stage mouse mammary tumors (6 to 16 weeks) to those of early stage mouse intestinal tumor (normal to adenoma) from previous publication²⁶. 15 to 19 weeks isogenic B6-APC^{Min/+} (APC^{Min}) and B6-APC^{+/+} (B6) wild type littermates were used for comparison in their study of mouse intestinal tumors. We identified 37175 DMRs ($p < 0.01$) between normal intestinal tissue and adenoma tissue (Supplementary Table S5), which were associated with 15761 genes (Supplementary Table S6). We found that 11814 of these genes (75.0 %) overlapped with DMR-associated genes involved in 6–16 week mouse mammary tumor development (Supplementary Table S7). This suggests that a largely common set of genes experience methylation variation during development of different types of cancers.

Microfluidics is an emerging platform for conducting low-input epigenomic analyses. In this work, we show that our microfluidic technology dramatically decreases the required DNA amount for MeDIP-seq analysis. Our technology takes advantage of efficient adsorption of methylated DNA fragments in a packed bed of beads and effective removal of nonspecific adsorption using an oscillatory washing process. We envision that our technology will enable a wide range of genome-wide methylomic investigations into animal and patient materials from scarce sources.

Methods

Fabrication of the microfluidic MeDIP device

The microfluidic device was composed of a microfluidic chamber (~720 nl), connecting channels, and a micromechanical valve that could be partially closed to stop magnetic beads while allowing liquid to pass. The chamber was in elliptic shape with a major axis of 6 mm, a minor axis of 3 mm, and a depth of 40 μm . 27 micro-pillars were placed inside the chamber to prevent collapsing of PDMS. Multilayer soft lithography was used to fabricate the microfluidic device^{34, 55}. Briefly, two photomasks (one for fluidic layer, and one for control layer) that had desired microscale patterns were designed with computer aided design software FreeHand MX (Macromedia) and printed on high-resolution (5,080 d.p.i.) transparencies. To make fluidic layer master, 40 μm thick photoresist (SU-8 2025, Microchem) was spun on a 3-inch silicon wafer (978, University Wafer) at 500 rpm for 10s and 2500 rpm for 30s followed by soft bake at 65°C for 1 min and 95°C for 7 min. To make control layer master, 50 μm thick SU-8 was spun at 500 rpm for 10s and 1500 rpm for 30s and followed by the same bake condition as the fluidic master. The coated wafers were exposed to UV light for 17s at 580 mW exposure intensity and a post exposure bake at 65°C for 1 min and 95°C for 7 min. Masters were then developed in SU-8 developer for 2–3 min, rinsed with isopropyl alcohol and blown dry by pressured air. To make fluidic layer stamp, PDMS (General Electric silicone RTV 615, MG chemicals) with a mass ratio of A:B = 5:1 was thoroughly mixed and vacuumed for 1 h. It was then poured onto the fluidic layer master in a Petri dish to a depth of ~5 mm thick. To make control layer stamp, PDMS with a mass ratio of A:B = 20:1 was mixed, vacuumed for 1 h, and spun onto the control layer master at 1100 rpm for 35s to achieve a depth of 108 μm thick. Both PDMS stamps were partially cured at 80 °C for 30 min. The fluidic layer was then peeled off from the fluidic layer master and aligned to the control layer PDMS stamp. Two-layered PDMS was bonded

by baking at 80 °C for 1 h, and then peeled off from the control layer master. Inlets and outlets of the device were punched by a 2 mm hole puncher. Finally, the two-layered PDMS structure and a pre-cleaned glass slide were treated in an oxygen plasma cleaner (PDC-32G, Harrick Plasma) and brought into contact to form closed channels and chamber. The device was then baked at 80 °C for 1 h to strengthen the bonding between PDMS and glass. Glass slides were cleaned in a basic solution (H₂O: 27% NH₄OH: 30% H₂O₂ = 5:1:1, volumetric ratio) at 75 °C for 2 h and then rinsed with ultrapure water and thoroughly air blown to dry.

Setup of the microfluidic device

The microfluidic experiment was monitored by a charge-coupled device (CCD) camera (ORCA-285, Hamamatsu) attached to the port of an inverted microscope (IX 71, Olympus). The reagents were flown into the inlet via a tubing driven by a syringe pump (Fusion 400, Chemyx). The micromechanical valve was actuated by a solenoid valve (18801003–12V, ASCO Scientific), which was connected to a pressure source and controlled by a data acquisition card (NI SCB-68, National Instruments) and a LabVIEW (LabVIEW 2012, National Instruments) program for its switching function. Pressure (30–35 p.s.i.) that was applied to the control channel deformed the PDMS membrane between fluidic channel and control channel and formed a partially closed valve to stop beads while allowing fluid to pass. The oscillatory washing was conducted by connecting the inlet and outlet of the microfluidic chamber to solenoid valves³⁴. Alternating pressure pulses programmed by a LabVIEW program were exerted on the two ends of the chamber to move the beads back and forth.

Cell culture

GM12878 cells were obtained from Coriell Institute for Medical Research. GM12878 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 15% fetal bovine serum, 100 U penicillin, 100mg streptomycin/ml (Invitrogen) at 37°C in a humidified incubator containing 5% CO₂. Cells were sub-cultured every two to three days to maintain exponential growth.

Mouse

Hemizygous transgenic female mice (FVB-Tg(C3–1-TAg)cJeg/JegJ, Jackson Laboratory) were used in this study. Mice were euthanized at age of 6, 16, 23 weeks by exposing mice to 100% carbon dioxide at 1 – 2 l/min for 5 min followed by cervical dislocation. One mouse per stage was used for the MeDIP-seq profiling. Mice were sprayed with 70% ethanol on the ventral side. Skin on the ventral side was cut through, pulled away from mouse body, and pinned down to expose mammary glands. Mammary tumors were identified and cut off. The size and weight of a tumor were recorded.

Preparation of ssDNA

Genomic DNA from GM12878 cells was extracted from 10⁶ cells using Blood & Cell Culture DNA Mini Kit (Qiagen). Mouse genomic DNA was extracted from mouse mammary tissues using DNeasy Blood & Tissue Kit (Qiagen). Extracted genomic DNA was sonicated with a Covaris E220 sonicator for 180 s with 10 % duty cycle, 50 peak incident

power and 200 cycles per burst. The concentration of sonicated DNA was quantified using a Qubit 2.0 fluorometer with dsDNA HS Assay kit (Q32851, Life Technologies). Different sample sizes (0.5~100 ng) were aliquoted and diluted with MeDIP buffer to generate a final volume of 50 μ l for microfluidic MeDIP. 0.5 ng, 5 ng, 10 ng, or 100 ng of the sonicated sample was used as the input. Sonicated DNA samples were freshly denatured into single strand DNA (ssDNA) under 95°C for 15 min and put on ice for 5 min before microfluidic MeDIP.

Preparation of immunoprecipitation (IP) beads

Dynabeads Protein A (10001D, Invitrogen) were used for MeDIP. They are 2.8 μ m superparamagnetic beads with recombinant Protein A (~45 kDa) covalently bound to the surface. For each assay, 5 μ l of 30 mg/ml beads (equivalent to 150 μ g) were washed twice with freshly prepared MeDIP buffer (10 mM monobasic sodium phosphate dihydrate, 10 mM dibasic sodium phosphate, 140 mM NaCl, 0.05%(v/v) Triton X-100) and resuspended in 150 μ l MeDIP buffer containing 5-methylcytosine (5-mC) antibody (pAb) (61255, Active Motif). The antibody concentration for coating was 3.3 μ g/ml for 0.5 ng DNA, 5 μ g/ml for 1 ng DNA, 6.6 μ g/ml for 10 ng DNA, and 40 μ g/ml for 100 ng DNA. Beads were gently mixed with the antibody at 4 °C on a rotator mixer at 24 rpm for 2 h. Antibody-coated beads were washed twice with the MeDIP buffer and then resuspended in 5 μ l MeDIP buffer.

Microfluidic MeDIP

The MeDIP procedure started with rinsing the fluidic chamber with the MeDIP buffer at a flow rate of 20 μ l/min for 30 s. The micromechanical valve was then partially closed. 5 μ l antibody-coated immunomagnetic beads were flown into the microfluidic chamber driven by the syringe pump at 20 μ l/min and aided with a cylindrical permanent magnet (NdFeB, D48-N52, 0.25 inch dia. and 0.5 inch thick, K&J Magnetics). The beads were packed against the partially closed valve to form a packed bed. The MeDIP buffer containing denatured ssDNA fragments with a total volume of 50 μ l was flowed through the packed bed of MeDIP beads at a flow rate of 1.5 μ l/min. After MeDIP, the MeDIP buffer was flowed into the fluidic chamber at a flow rate of 2 μ l/min for 2 min. Two tubings each prefilled with 20 μ l MeDIP buffer were plugged into the inlet and outlet of the chamber for oscillatory washing. Oscillatory washing³⁴ was done by applying pressure pulses (each at 0.5 p.s.i., with a pulse duration of 0.5 s and an interval of 0.5 s between two pulses) alternately at inlet and outlet of the fluidic chamber for 5 min while keeping the micromechanical valve open. The pulsing was controlled by the data acquisition card and a LabVIEW program. After oscillatory washing, beads were retained on one side of the chamber by the magnet. The unbound ssDNA was flushed out of the chamber by flowing MeDIP buffer through the chamber at 2 μ l/min for 2 min. Finally, MeDIP beads were flushed out by MeDIP buffer under a flow rate of 50 μ l/min for about 5 min and collected into a 1.5-ml LoBind Eppendorf tube.

Extraction of MeDIP/input DNA

After microfluidic MeDIP and MeDIP bead collection, supernatant was removed by pipetting while a magnet was used to retain MeDIP beads in the tube. MeDIP ssDNA on beads were mixed with 198 μ l digestion buffer (200 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 1% SDS, 0.1 M NaHCO₃) and 2 μ l of 20 mg/ml proteinase K (26160, Thermo

Scientific). Input ssDNA (50 µl) was mixed with 148 µl digestion buffer and 2 µl 20 mg/ml proteinase K. Samples were then incubated at 50 °C for 4 h. Equal volume (200 µl) of Phenol-chloroform-isoamylalcohol (25:24:1) was added to the sample, mixed by vortexing, and centrifuged at 16,100g for 5 min at room temperature. ssDNA was extracted by collecting ~200 µl aqueous phase to a fresh 1.5 ml Eppendorf tube. 50 µl of 10 M ammonium acetate was then added to the sample. 750 µl of 100% ethanol and 2 µl of 20 µg/µl glycogen (10814010, Invitrogen) were added for ethanol precipitation and ssDNA purification. Samples were placed at -20 °C overnight for ethanol precipitation. Next, samples were centrifuged at 16,100g for 10 min at 4 °C before the supernatant was carefully removed. The ssDNA pellet was washed with 500 µl of 70% ice-cold ethanol without breaking the pellet. The supernatant was then removed after centrifugation at 16,100g for 5 min at 4 °C. The pellet was air dried for 5 min and resuspended in 10 µl DNase-free water. This purified ssDNA was used directly for MeDIP-qPCR or for sequencing library construction. DNA concentrations were measured using a Qubit 2.0 fluorometer with ssDNA Assay kit (Q10212, Invitrogen).

Construction of sequencing libraries

Sequencing libraries were prepared by DNA SMART™ ChIP-Seq kit (634865, Clontech). This kit generates sequencing libraries from low-input ssDNA MeDIP samples (100 pg - 10 ng) without using ligation. Sequencing libraries were amplified by PCR using primers containing Illumina adapters. Amplification cycles used for 0.5, 5, 10, 100 ng GM12878 DNA samples were 17, 13, 13, 12 cycles, respectively. Amplification cycles used for 10 ng mouse DNA samples were 13 cycles. The libraries were purified using Ampure XP beads (A63880, Beckman Coulter). The library fragment size was determined using high sensitivity DNA analysis kit (5067–4626, Agilent) on an Agilent 2200 TapeStation. Sonicated DNA for MeDIP had average fragment size around 200 bp (Supplementary Fig. S1a). After library preparation, the average peak size shifted to 350 bp as library construction added about 150 bp adaptor (75 bp on each end) to the MeDIP DNA (Supplementary Fig. S1b). KAPA library quantification kit (KK4809, Kapa Biosystems) was used to determine effective library concentrations. The final concentrations of libraries submitted for sequencing were ~8 nM (20 µl and 37 ng DNA). The libraries were sequenced on an Illumina HiSeq 4000 with single-end 50 nt read. Typically 15–20 million reads were generated per library.

MeDIP-qPCR data analysis

qPCR was done using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an CFX96 real-time PCR machine (Biorad) with C1000Tm thermal cycler base. All PCR assays were performed using the following thermal cycling condition: 95°C for 10 min followed by 40 cycles of (95°C for 15 s, 56°C for 30 s, 72°C for 30s). GEMIN4 and ZC3H13 are two known positive loci for human while GRICK3 and ZC3H13 are two known positive loci for mouse. N1 and N2 are two known negative loci for human and mouse. Primer concentration was 400 nM. All primers were ordered from Integrated DNA Technologies (Coralville). The MeDIP-qPCR results were represented as relative fold enrichment (Fig. 2), which is the ratio of percent input (Supplementary Fig. S6) between a positive locus and a negative locus. Percent input was calculated using the following

equation: $2^{(C_q^{INPUT} - C_q^{IP})} \times 100\%$, where C_q is amplification cycle number run by qPCR, *INPUT* is the DNA sample without immunoprecipitation, *IP* is DNA sample after MeDIP.

MeDIP-seq data analysis

MeDIP sequencing reads were mapped to the human genome (hg19) or mouse genome (mm10) using Bowtie2 (v2.2.5)⁵⁶ with default parameter settings. Peaks of each MeDIP sample were called against input by MACS (v2.1.0)⁵⁷ with p-value < 10⁻⁵ and other parameters set at default values. Pearson correlations of genome-wide coverage profiles between samples were calculated using Bioconductor R package “MeDIPS” with MeDIPS.correlation function⁵⁸. To identify DMRs between tumor and normal tissues, MeDIPS.meth function from MeDIP package⁵⁸ with parameters set as p.adj = “fdr”, diff.method = “edgeR”, MeDIP = T, CNV = F, minRowSum = 10 and MeDIPS.selectSig function with parameters set as p.value = 0.01, adj = F, ratio = NULL, bg.counts = NULL, CNV = F were used. We first obtained the 250 bp windows that were differentially methylated (only uniquely mapped reads were used), and then we merged neighboring significant windows into a larger continuous region with MeDIPS.mergeFrames function to generate differentially methylated regions. GO analysis was performed by GREAT⁴⁶ with default parameter settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Microfluidic MeDIP-seq device and its operation. **(a)** The microfluidic device (containing 7 independent units). **(b)** Formation of packed bed of IP beads while micro-valve is closed. **(c)** MeDIP by flowing ssDNA fragments through the packed bed. **(d)** Oscillatory washing. **(e)** Retaining IP beads on one side of chamber to remove unbound chromatin fragments and debris.

Figure 2. Fold enrichment (ratio between % input at positive loci and % input at negative loci) under different antibody coating concentrations. **(a)** 10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ with 100 ng DNA from GM12878 cells. **(b)** 6.6 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ with 10 ng DNA from GM12878 cells.

Figure 3.

Microfluidic MeDIP-seq data on GM12878 cell line. **(a)** Genome browser tracks for our microfluidic MeDIP-seq using various amounts of DNA (100–0.5 ng). Two replicates were profiled for each sample. **(b)** Genome-wide correlations among MeDIP-seq data sets of various sample sizes. MeDIP-seq genomic coverage profiles were used for computing correlations. Colors represent Pearson correlation coefficients.

Figure 4.

Microfluidic MeDIP-seq data on transgenic mouse samples. **(a)** Genome browser tracks for microfluidic MeDIP-seq using 10 ng DNA from different stages of mammary tumor harvested from C3(1)/Tag transgenic mice. Two replicates were profiled for each sample. **(b)** Genome-wide correlations among MeDIP-seq data sets of different stages of mammary cancer. MeDIP-seq genomic coverage profiles were used for computing correlations. Colors represent Pearson correlation coefficients.

Figure 5. Histologic progression of mammary tumors in C3(1)/Tag transgenic mice and MeDIP-seq profile comparing 23 weeks tumor to 6 weeks normal tissue. **(a)** Normal mammary tissue in a 6-week-old mouse. **(b)** Invasive carcinoma with cribriform pattern in a 16-week-old mouse. **(c)** Invasive carcinoma with extensive cribriform patterns in a 23-week-old mouse. **(d)** Hypomethylation on oncogene Cdk4 with tumor development. **(e)** Hypermethylation on TSG Hic1 with tumor development.

Figure 6. Fraction of subgenomic regions occupied by DMRs. Fraction is calculated as sum of all the 250 bp windows that were either hypo- or hypermethylated ($p < 0.01$) in given subgenomic region divided by the overall size of that subgenomic region. DMRs were generated by comparing combined data of two replicates on mice of different stages. **(a)** Fraction of subgenomic regions occupied by DMRs for hypo- and hypermethylation (16 weeks mammary tumor compared to 6 weeks mammary normal tissue). **(b)** Fraction of subgenomic regions occupied by DMRs for hypo- and hypermethylation (23 weeks mammary tumor compared to 16 weeks mammary tumor).