Beetch, M., Lubecka, K., Kristofzski, H., Suderman, M., & Stefanska, B. (2018). Subtle Alterations in DNA Methylation Patterns in Normal Cells in Response to Dietary Stilbenoids. *Molecular Nutrition and Food Research*, 62(14), [1800193].
<https://doi.org/10.1002/mnfr.201800193>

Peer reviewed version

License (if available):
CC BY-NC

Link to published version (if available):
[10.1002/mnfr.201800193](https://doi.org/10.1002/mnfr.201800193)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the accepted author manuscript (AAM). The final published version (version of record) is available online via Wiley at <https://doi.org/10.1002/mnfr.201800193> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

Subtle alterations in DNA methylation patterns in normal cells in response to dietary stilbenoids

Megan Beetch^{1,a}, Katarzyna Lubecka^{2,a}, Heather Kristofzski³, Matthew Suderman^{4,5}, and Barbara Stefanska^{1*}

¹ Food, Nutrition and Health, Faculty of Land and Food Systems, the University of British Columbia, Vancouver, BC, Canada

² Department of Biomedical Chemistry, Medical University of Lodz, Lodz, Poland

³ Department of Nutrition Science, Purdue University, West Lafayette, IN, USA

⁴ School of Social and Community Medicine, University of Bristol, Bristol, UK

⁵ MRC Integrative Epidemiology Unit, University of Bristol, Bristol, UK

^a These authors contributed equally to this work.

Keywords: diet, DNA methylation, exposure to stilbenoids, healthy cells

The Authors declare no conflict of interest.

*To whom correspondence should be addressed:

Dr. Barbara Stefanska

Assistant Professor

Faculty of Land and Food Systems

University of British Columbia

2205 East Mall, FNH 150

Vancouver, BC V6T 1Z4

Phone: +1 604-822-2524, Fax: +1 604-822-5143

barbara.stefanska@ubc.ca

<http://www.landfood.ubc.ca/person/barbara-stefanska/>

ABSTRACT

Scope: Searching for correlations between dietary polyphenols and risk of chronic diseases has been a challenge due to the lack of quantitative evaluation methods of long-term exposure. We previously observed substantial DNA methylation changes in human cancer cells upon treatment with polyphenols of the stilbenoid class. When induced in normal cells, such molecular changes might persist and reflect chronic exposure.

Methods and Results: Using Illumina-450K microarray, we delineated a genome-wide DNA methylation landscape in MCF10A human immortalized mammary epithelial cells exposed to resveratrol at non-cytotoxic 15 μ M dose for 9 days. We identified subtle alterations suggesting remodeling of DNA methylation patterns rather than switch on/off changes. Using pyrosequencing, we quantitatively measured DNA methylation at 8 CpG sites located within *KCNJ4*, *RNF169*, *BCHE*, *DAOA*, *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP*, upon exposure to resveratrol or pterostilbene and detected similar differences induced by both stilbenoids. Two of the probes, *Runx3* and *Kcnj4*, were successfully verified in whole blood DNA from healthy rats on diets supplemented with stilbenoids.

Conclusions: Our study provides strong support for testing the utility of polyphenol-mediated changes in DNA methylation as quantitative measures of long-term dietary exposures in nutritional epidemiology and clinical trials.

GRAPHICAL ABSTRACT

Subtle alterations in DNA methylation were detected in normal cells exposed to polyphenols of the stilbenoid class, such as resveratrol or pterostilbene, and in whole blood of rats whose diets were supplemented with the compounds. Once confirmed in population-based studies, such molecular markers can be used as an excellent tool to reliably measure long-term polyphenol exposure and establish associations between polyphenol intake and risk of chronic diseases.

INTRODUCTION

Epidemiologic studies have been searching for associations between dietary factors and disease risk for many decades. Such studies compare rates of disease occurrence and dietary consumption in a population to estimate whether there is any association [1]. Thus, the accurate measurements of the intake are key for interpretation of the results. Variations in daily consumption patterns make it challenging to establish individuals' long-term exposures when using usual methods of dietary intake assessment. Self-reporting tools such as food frequency questionnaires, 24-hour recalls, and food diaries, that are commonly employed to estimate dietary consumption, introduce multiple errors, for example via conversion of food intake to nutrient exposure and via ignoring genetic polymorphisms affecting bioavailability. This may account for non-reliable results and lack of correlations between active ingredients and disease [2, 3]. One of the groups of dietary bioactive compounds with promising *in vitro* and *in vivo* effects well-studied in chronic diseases and health maintenance includes polyphenols [4-6]. Despite strong pre-clinical evidence, epidemiologic studies deliver inconsistent results for associations between polyphenol intake and disease risk in humans [5, 7-9].

Quantitative biochemical measurements that would reflect exposure to a certain group of compounds or foods including polyphenols have been under investigation [10]. Urine samples, blood draws, tissue biopsies, and stool specimens can be tested for metabolites, specific protein or gene expression level, or possibly status of epigenetic components, with varying capacities to distinguish acute versus chronic polyphenol intake [11]. Tracking of urinary polyphenols offers advantages relative to blood levels. Due to the short plasma half-life of polyphenol metabolites, blood levels may not consistently represent dietary intake or *in vivo* exposure. Urinary levels, on

the other hand, would be more representative of daily intake as opposed to chronic exposure [12, 13].

Myriad reviews assessing exposure detection indicate a vital need for new or improved methods to accurately measure chronic polyphenol intake in order to draw concrete conclusions about the impact of these dietary compounds in health and disease [11, 14]. Herein, we investigate whether polyphenols mediate molecular changes that could persist and be used as quantitative tools for chronic exposure assessment. We focus on resveratrol (RSV) and pterostilbene (PTS), two major polyphenols of the stilbenoid class, that are abundantly found in grapes and blueberries, respectively [15]. These polyphenols have raised considerable interest because of their possible role in beneficial disease outcomes such as prevention of degenerative diseases and anti-cancer action observed in intervention and preclinical studies [13, 16, 17]. Moreover, mechanistic studies indicate molecular changes, including epigenetics and specifically DNA methylation, that occur in response to the compounds [18-23]. We have recently shown that RSV and PTS modulate gene expression through altering DNA methylation patterns in gene regulatory regions in cancer cells [18]. DNA methylation is one of the components of the epigenome and is characterized by the addition of a methyl group to the 5th position of the cytosine ring within CpG dinucleotides in DNA [24]. This modification leads to changes in gene transcription that do not involve changes to the underlying DNA sequence [24]. Although DNA methylation is the gate-keeper of gene expression providing stable long-term regulation, it is also readily reversible and responsive to environmental factors, demonstrating potential as a useful tool to assess exposure to food bioactives [25]. Furthermore, changes in DNA methylation can easily and accurately be measured

using a straightforward laboratory technique called pyrosequencing which provides a percentage of methylation at a single CpG site resolution.

In the present study, we performed a genome-wide analysis of DNA methylation profiles in MCF10A mammary epithelial cells exposed to RSV and detected numerous subtle changes. Location of these RSV-mediated alterations in gene regulatory regions and enrichment with pathways associated with cardiovascular health, metabolism, cancer, and aging, might indicate a role in stabilizing control mechanisms to maintain a healthy phenotype. Statistically significant alterations of the highest magnitude were identified within regulatory regions of 8 genes, namely *KCNJ4*, *RNF169*, *BCHE*, *DAOA*, *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP*. We further successfully quantitatively validated these changes by pyrosequencing in MCF10A cells exposed to RSV or PTS. Interestingly, the presence of changes within *Runx3* and *Kcnj4* was subsequently confirmed in peripheral blood, an easily accessible source of DNA, of healthy rats exposed to the compounds. Our study provides strong support for testing the utility of polyphenol-mediated changes in DNA methylation as quantitative measures of long-term dietary exposures in nutritional epidemiology and clinical trials.

MATERIALS AND METHODS

Cell culture and incubation with resveratrol (RSV) and pterostilbene (PTS)

Human mammary epithelial MCF10A cell line was purchased from ATCC (CRL-10317, USA). Please see Supporting Information for details on cell culture media and culture conditions. Resveratrol (RSV, Sigma-Aldrich, St. Louis, MO, USA) and pterostilbene (PTS, Cayman Chem., Ann Arbor, MI, USA) were resuspended in ethanol and 10 mM stock solutions were stored at -

20°C. Dilutions of the compounds were freshly prepared prior to adding to the cell medium. Cells were grown in a humidified atmosphere of 5% carbon dioxide at 37°C. 24 h prior to incubation with RSV or PTS, cells were plated at a density of $1-2 \times 10^5$ per a 10-cm tissue culture dish. Cells were exposed to different RSV or PTS concentrations ranging from 0 to 20 μM of RSV or to 15 μM of PTS for 4 days. Cells were then split 1:50, allowed to attach overnight and exposed to the compounds for additional 4 days (9-day exposure).

Illumina Infinium Human Methylation 450K BeadChip microarray

DNA from MCF10A control cells (incubated with ethanol) and cells cultured with RSV for 9 days was isolated using standard phenol:chloroform extraction protocol. Genomic DNA was processed for genome-wide DNA methylation analysis using Infinium HumanMethylation 450K BeadChip as described previously [18, 26]. Please see Supporting Information for details. The methylation score for each CpG was represented as a beta value according to the fluorescent intensity ratio with any values between 0 (unmethylated) and 1 (completely methylated). Raw microarray data and processed data are available from Gene Expression Omnibus (accession number [GSE113299](#)).

Animals and diets

Rats were treated strictly following the animal use protocol #1112000342 approved by the Institutional Animal Care and Use Committee at the involved institutions. A total of 18 male Fischer 344 rats, 4 weeks old, were obtained from Charles River (Indianapolis, IN, USA), housed 2/cage in a temperature-controlled (24°C) room with a 12-h light/dark cycle, and given ad libitum access to water and a chow diet [i.e., choline-sufficient amino acid-defined (CSAA) pelleted diet, Dyets Inc., Bethlehem, PA, USA]. After a week of acclimation period, five-week-old rats were

randomly divided into 3 groups each containing 6 animals (to reach statistical power) and fed the CSAA diet or a CSAA diet supplemented with resveratrol (CSAA+RSV, 1.2 g/kg of diet, 120 mg/kg of body weight per day) or pterostilbene (CSAA+PTS, 1.34 g/kg of diet, 134 mg/kg of body weight per day), pelleted at Dyets Inc. The compounds (BIOTANG Inc., Lexington, MA, USA) were used at equal molar concentrations. Following 20-day feeding period, retro-orbital Plexus sampling was used to collect blood from anesthetized rats. This method involves penetrating the retro-orbital plexus with a capillary tube to promote blood flow from the capillaries behind the eye. Whole blood amounts that did not exceed 10% of circulating blood volume based on body weight were collected from each rat into spray coated K2EDTA tubes (BD & Co., Franklin Lakes, NJ, USA) and stored in -80°C until DNA and RNA extractions were performed.

In order to see a significant difference of 50% between groups at $P < 0.05$ when the variation of the endpoint is 25% of the sample mean, experiments need to have a power of 0.8. This requires a sample size of $n=6$ individual rats per group and was calculated using the biological and analytical variability from previous data [27, 28].

DNA extraction and pyrosequencing

DNA from human mammary epithelial MCF10A cells and rat peripheral blood was isolated using standard phenol:chloroform extraction protocol. DNA bisulfite conversion was performed as previously described [29, 30]. Specific regulatory gene sequences were amplified with HotStar Taq DNA polymerase (Qiagen) using biotinylated primers listed in Supplementary Table S1A (human) and S1B (rat). Pyrosequencing of the biotinylated DNA strands was performed in the PyroMarkTMQ24 instrument followed by data analysis using PyroMarkTMQ24 software (Biotage, Qiagen) [31].

RNA extraction and QPCR

Total RNA isolated using TRIzol (Roche Diagnostics) was processed for cDNA synthesis using 20U of AMV reverse transcriptase (Roche Diagnostics), as recommended by the manufacturer. Light Cycler 480 instrument (Roche) was used for QPCR reaction with the following cycles: denaturation at 95 °C for 10 min, amplification for 60 cycles at 95 °C for 10s, annealing temperature for 10s, 72 °C for 10s, and final extension at 72 °C for 10 min. Reaction mixture consisted of 2 µl of cDNA, 400 nM forward and reverse primers listed in Supplementary Table S1C and 10 µl of Light Cycler 480 SybrGreen I Master (Roche Diagnostics) in a final volume of 20 µl. Quantification of expression levels was performed using a standard curve and analyzed by the Roche LightCycler 480 software.

Statistical analysis

Raw methylation data from Human Methylation 450K microarrays were pre-processed using GenomeStudio and IMA (Illumina Methylation Analyzer for 450K, R/Bioconductor) including quality control, background correction, normalization, probe scaling, and adjustment for batch effect. Differential methylation analysis between sample groups was conducted using linear models (R Bioconductor package limma). Specifically, limma uses an empirical Bayes moderated t-test, computed for each probe, which is similar to a t-test, except that standard errors have been moderated using information from the full set of probes [32]. A methylation difference greater than 0.05 with a moderated t-test $P < 0.05$ was considered statistically significant. Differentially methylated CpG sites were mapped into differentially methylated regions (DMRs) using DMRforPairs algorithm in R/Bioconductor [33, 34] (please see Supporting Information for details).

Statistical analysis of pyrosequencing and viability assays was performed using the unpaired *t*-test with two-tailed distribution. Each value represents the mean $\pm$ S.D. of three independent experiments. Mann-Whitney *U* test was used for statistical analysis of pyrosequencing in *in vivo* rat model with n=6 per group. The results were considered statistically significant when $P < 0.05$.

RESULTS

The landscape of DNA methylation in MCF10A human mammary epithelial cells upon resveratrol (RSV) exposure

The purpose of our study was to define polyphenol-mediated epigenetic response in MCF10A human immortalized mammary epithelial cells, used as an *in vitro* model of normal epithelial cells, and identify molecular changes occurring in response to polyphenols in normal cells. In our previous studies, we observed substantial changes in DNA methylation patterns in breast cancer cells treated with RSV or PTS [18-20]. Based on those pieces of evidence, we hypothesized that mere exposure to dietary polyphenols may leave a stable "footprint" in normal human epithelial cells by remodeling the DNA methylation profile.

As follows, MCF10A mammary epithelial cells were incubated with 15 μ M RSV for 9 days to reflect prolonged exposure to stilbenoids. The dose of RSV was established based on its impact on cell growth (Supplementary Figure S1A). The 15 μ M dose of RSV was not toxic (< 10% of dead cells) and caused a negligible effect on the number of viable cells (<25% decrease in the number of viable cells) compared to control cells treated with ethanol (a vehicle control), as indicated by trypan blue exclusion test and further validated by MTT assay (Supplementary Figure S1A). A similar workflow was applied for PTS where 7 μ M concentration was selected for further

experiments (Supplementary Figure S1B). Simultaneously, these concentrations of RSV and PTS inhibited cancer cell growth by 50% with low cytotoxic effects as demonstrated in our previous study [18]. RSV-exposed and control MCF10A cells were subjected to Illumina 450K Methylation microarray to assess genome-wide DNA methylation effects of RSV exposure. The Illumina 450K microarray provides CpG-specific DNA methylation data for almost 500,000 CpG sequences that corresponds to 99% of all known protein coding genes (Please see Supporting Information for details).

We found 1,324 CpG sites differentially methylated upon 9-day exposure of MCF10A cells to 15 μ M RSV, as compared to control cells ($0.05 \leq \text{differential methylation} \leq -0.05$, $p < 0.05$, limma t test). Chromosomal views of these differences were plotted using the Integrative Genomics Viewer (IGV) visualization tool (Figure 1A). Each vertical bar represents a single differentially methylated CpG site, with red indicating hypermethylation and blue indicating hypomethylation in RSV-exposed versus control cells. Among these changes, 889 CpG sites (67% of total number of changes) corresponding to 602 genes were hypermethylated whereas 435 CpG sites were hypomethylated and located within 305 genes (please see Supplementary Table S2 for differentially methylated CpG sites). The changes in DNA methylation were subtle ranging from 0.05 to 0.20 in differential methylation values. Only 15 hypermethylated CpG sites and 17 hypomethylated CpG sites were identified using a threshold for differential methylation ≥ 0.1 (Figure 1B). A heatmap in Figure 1C for 500 CpG sites with the highest change in DNA methylation between RSV and control further confirms mild alterations. It would suggest remodeling of the methylation patterns of normal cells upon RSV exposure rather than robust on/off switches that mediate a strong biological impact. RSV as a naturally-derived, bioactive

compound at physiologically relevant, non-toxic concentrations could possibly act through indirect mechanisms to induce subtle bidirectional changes in DNA methylation state of normal cells. Nearly half of differentially methylated sites are located in CpG islands (CpGIs), CpGI shores and shelves (Figure 1C). The distribution of hypermethylated and hypomethylated sites relative to TSS is similar with 30% of CpGs located in promoter regions (Figure 1C). Almost 30% of differentially methylated CpG loci are assigned to enhancer regions with equal distribution between hypermethylated and hypomethylated sites (Figure 1C).

Although our prediction was that RSV exposure leaves subtle marks in normal cells by slightly altering DNA methylation but does not have strong biological impact on corresponding genes, the location of changes in regions important for transcriptional regulation (e.g., promoters, enhancers, CpG islands) could indicate long-term regulation of the phenotype. Indeed, we found that differentially methylated genes are associated with cardiovascular and metabolic health (e.g., sodium-calcium exchanger SLC8A1, a family of calcium channel subunits CACNA, mitochondrial acetyl-CoA synthetase ACSS1/ACSS3, aminotransferase ABAT), immune functions (e.g., HLA class of major histocompatibility complex, interferon IFNA14), nervous system functions (e.g., synaptosome associated protein SNAP29, netrin NTN1 and netrin receptors UNC5 promoting neuronal cell survival), and cancer-relevant signaling such as cadherin and WNT pathways (e.g., numerous cadherins and protocadherins, protein kinase PRKCG, AXIN2, HDAC2, SFRP4) (Figure 1D). Alterations in DNA methylation mediated by RSV may therefore serve as a mechanism maintaining active or silenced transcription states crucial for a healthy phenotype.

These results led us to a question whether RSV treatment changes the epigenetic clock age of the normal cells. Maintaining a healthy phenotype could be associated with reversing age-related changes in the DNA methylation patterns. The epigenetic clock age is a type of a molecular age estimation method based on DNA methylation levels. We used Horvath's clock [35, 36] that includes 353 CpG sites, called epigenetic clock CpGs, whose methylation state is correlated with age. Among the 353 clock CpGs, there are 193 positively and 160 negatively correlated CpGs that become hypermethylated and hypomethylated with age, respectively. Using Horvath's R algorithms [35], we calculated DNA methylation age in control and RSV-treated MCF10A cells based on our Illumina 450K data. No significant alterations ($p=0.96199$) in the average DNA methylation age have been found between control (average DNA methylation age = 116.93 ± 0.87) and RSV-exposed (average DNA methylation age = 117.01 ± 2.39) MCF10A cells. Looking through all 353 CpGs, we found 20 sites of the epigenetic clock differentially methylated by RSV exposure. The highest change exceeding 0.05 was detected at cg18984151 located within C3orf75 (TMEM103) which is a subunit of RNA polymerase II (RNAPolII) elongator complex acting as a histone acetyltransferase component of RNAPolII. The locus is positively correlated with age and become hypermethylated with age. RSV treatment led to hypomethylation of this locus, possibly counteracting age-related alterations.

Differentially methylated CpG sites in response to resveratrol (RSV) in MCF10A human mammary epithelial cells are mapped into differentially methylated regions (DMRs)

In further bioinformatics analyses, we screened the genome-wide Illumina 450K data for differentially methylated regions (DMRs). DMRs are defined as regions that contain at least 4 CpG sites (Illumina probes) with a maximum distance of 200 bp between individual sites. Using R

bioconductor DMRforPairs package as described by Rijlaarsdam *et al.* [33], we found 30,417 DMRs among all samples (3 controls, 3 RSV-treated). Since DNA methylation changes in response to RSV are subtle, unsurprisingly we have not reached statistical significance for any of the DMRs. Importantly, there were 124 regions with median methylation levels (M-values) between the samples differed by at least 0.4 that is approximately equal to differential methylation 0.05 (delta beta value). To determine any DMRs between biological replicates, control samples and RSV-treated samples were separately computed in DMRforPairs. Any DMRs found between biological replicates were excluded from the 124 regions resulting in a final list of 80 unique regions differentially methylated in response to RSV in MCF10A cells (Supplementary Table S3).

These 80 regions are referred to as “relevant DMRs”. As observed for differentially methylated CpG sites, 65% of relevant DMRs are located in regions with high regulatory role in gene transcription such as CpG islands and transcription start sites (TSS) (Figure 1E). It is reflected in the chromatin state analysis, where 52% of relevant DMRs are associated with promoters and only 12% with enhancers commonly located in gene body (Figure 1F). As described by Ernst *et al.* [34], we used data generated using 15-state ChromHMM model based on several chromatin marks (e.g., H3K4me3, H3K4me2, H3K4me1, H3K9ac, H3K27ac, H3K36me3, H4K20me1, H3K27me3 and H3K9me3) and selected Broad ChromHMM HMEC mammary epithelial cell track in UCSC Genome Browser (hg19) to determine chromatin states of relevant DMRs in breast cells. DMRs could correspond to the following chromatin states: active, weak or poised promoters, strong or weak enhancers, putative insulators, active or weak transcription, Polycomb-repressed regions or heterochromatin. Active promoters, strong enhancers and active transcription regions are linked to high expression levels, with the latter state determined based on the enrichment of histone marks along transcripts. Nearly 62% of relevant DMRs hypomethylated in response to RSV were

associated with active chromatin states whereas 79% of hypermethylated DMRs were characterized by chromatin states linked to low expression levels including weak and poised promoters, weak enhancers, weak transcription, repressed regions or heterochromatin (Figure 1F).

As for differentially methylated CpG sites upon exposure to RSV, functional pathway analysis for relevant DMRs confirms that RSV-mediated alterations may play a role in maintaining a healthy phenotype. Relevant DMRs are enriched for fatty acid metabolism, (e.g., acyl-CoA dehydrogenase ACADM catalyzing the initial step of fatty acid beta-oxidation, thioesterase PPT2 removing thioester-linked fatty acyl groups, dehydrogenase HSD17B12 catalyzing reactions of the long-chain fatty acids elongation cycle), branch-chain amino acid metabolism (e.g., dehydrogenase ACADM, malonyl-CoA synthetase ACSF3, carboxylase subunit PCCA), immune response to infections (e.g., HLA class of major histocompatibility complex, mRNA splicing regulators SRSF3 and ALYREF), oncogenic pathways (MAP2K3, KRAS, RASA1), and carbon metabolism linked to aging (e.g., ACADM, PCCA, pyruvate kinase PKLR) (Figure 1G).

Differential methylation occurring in response to resveratrol (RSV) or pterostilbene (PTS) in MCF10A mammary epithelial cells may serve as a quantitative measure of the dietary exposure

To investigate whether polyphenol-mediated changes in the DNA methylation patterns of normal cells could be quantitatively detectable, we selected 8 CpG sites for assessment of the methylation levels using pyrosequencing. Pyrosequencing is a quantitative method which measures the percentage of methylation at a single CpG site resolution and requires only PCR of bisulfite converted genomic DNA. In our selection, we took into account the magnitude of the difference in DNA methylation (differential methylation) between RSV-exposed and control cells, statistical

significance and location of change in gene regulatory regions. Hypermethylated CpG sites were located in the promoter or gene body of *KCNJ4* (potassium voltage-gated channel subfamily J member 4), *RNF169* (ring finger protein 169), *BCHE* (butyrylcholinesterase), and *DAOA* (D-amino acid oxidase activator) (Figure 2), whereas hypomethylated loci were located in the promoter of *HOXA9* (homeobox A9), *KRTAP2-1* (keratin associated protein 2-1), *TAGAP* (T-cell activation RhoGTPase activating protein), and CpG island in the body of *RUNX3* (runt-related transcription factor 3) (Figure 3). Functions of the selected genes reflected pathways observed in the integrative analysis of genes containing differentially methylated sites, such as nervous system functions (*KCNJ4*, *BCHE*, and *DAOA*), immune functions (*TAGAP*, and *HOXA9*), cancer and cancer-related signal transduction (*RNF169*, *HOXA9*, and *RUNX3*). Interestingly, the latter group contains a strong oncogene *RNF169* which negatively regulates DNA damage repair, and known tumor suppressor genes *HOXA9* and *RUNX3* which act as transcription factors regulating expression of a gene network linked to inflammatory response and signal transduction. While *RNF169* is hypermethylated (and potentially silenced) in response to RSV, *HOXA9* and *RUNX3* are hypomethylated (potentially activated).

For pyrosequencing, PCR primers were designed so that the tested fragment encompasses the CpG site differentially methylated on the microarray (CpG of interest, marked in square in charts in Figures 2 and 3) and if possible additional neighboring CpG sites (please see gene maps in Figure 2A and 3A). This allows us to obtain a pattern of methylation within a broader DNA region. We call the fragments tested by pyrosequencing “probes” and each probe corresponds to a single gene. Specifically, single CpG sites within *KCNJ4*, *RNF169*, *BCHE* and *DAOA*, all of which identified by Illumina 450K microarray as hypermethylated upon RSV exposure, showed increase in

methylation (Figure 2B). As observed in the array data, pyrosequencing revealed subtle RSV-mediated increases in DNA methylation within CpG sites located in these probes, ranging from 7 to 16% (Figure 2B). More profound changes in pyrosequenced regions were observed within probes hypomethylated in response to RSV, including *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP* (Figure 3B). A robust 9-25% decrease in DNA methylation was detected throughout the entire region of *HOXA9* promoter spanning 13 CpG loci. On the other hand, significant hypomethylation was found mainly at CpG site covered on Illumina within *RUNX3* and *TAGAP*. A probe within *KRTAP2-1* showed the highest >30% decrease in DNA methylation at CpG#1 and CpG#2 (Figure 3B). Pyrosequencing successfully detected all differences in DNA methylation within 8 probes selected based on the microarray data. Thus, these subtle yet significant changes can be assessed by pyrosequencing, a straightforward technique that provides CpG-specific information in a timely manner and can be efficiently used to assess changes in methylation state in relation to exposure to environmental factors including diet [31].

A question arose whether epigenetic alterations are mediated only by RSV or if they are common to other compounds from stilbenoid class of polyphenols. We were especially interested in PTS. RSV is rapidly metabolized to glucuronide and sulfate conjugates that may limit its *in vivo* biological activity, compared to its analog, PTS. Upon oral administration of stilbenoids in *in vivo* models, PTS showed markedly greater total plasma levels of both the parent compound and its metabolites, having almost 80% bioavailability compared to 20% bioavailability of RSV [37, 38].

We therefore exposed MCF10A cells to PTS using the same schedule of treatments as for RSV followed by DNA methylation analysis within 8 selected probes using pyrosequencing. PTS was

used at 7 μ M dose for 9 days. As described in the previous section, PTS at this concentration exerts cancer-specific effects with no toxicity towards normal cells and only a minor effect on the number of viable normal cells (<20% decrease in the number of viable cells) (Supplementary Figure S1B). Probes within *KCNJ4*, *RNF169*, *BCHE* and *DAOA* that were identified and validated in RSV-exposed cells as hypermethylated also showed significantly increased CpG-specific DNA methylation level by 8-34% in response to PTS (Figure 2C). Similarly, pyrosequencing of the tested regions within RSV-hypomethylated genes, *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP*, demonstrated relevant 5-25% decrease in DNA methylation upon PTS exposure (Figure 3C). Collectively, we successfully confirmed using pyrosequencing that all 8 probes are targeted for DNA methylation changes in response to both RSV in PTS in mammary epithelial cells. The subtle changes in DNA methylation after PTS exposure presented similar trends of change as seen after RSV. Changes were slightly stronger for *KCNJ4* and *RNF169* at CpG of interest showing 18% higher increase in DNA methylation levels in response to PTS compared with RSV (Figure 2B and 2C).

The remodeled DNA methylation pattern at *Runx3* and *Kcnj4* promoters detectable in rat peripheral blood in response to exposure to stilbenoids

If the established quantitative molecular changes induced upon long-term exposure to stilbenoids could be detectable using non-invasive methods such as blood test, they would be of potential value in the development of biomarkers of exposure. We therefore tested whether our *in vitro* findings can be replicated in an *in vivo* animal model. In this model, rats were fed a chow CSAA diet or CSAA diet supplemented with RSV (CSAA+RSV, 1.2 g/kg of diet) or PTS (CSAA+PTS, 1.34 g/kg of diet) for 20 days. Compounds were used at equal molar concentrations and the selected

doses have been shown to be effective in attenuating cancer development based on previous studies in animal models [39-42]. At the 20 day-time point, whole blood was collected from 6 rats per group and subjected to DNA isolation, amplification of selected gene fragments, and pyrosequencing.

Four genes out of 8 validated probes in which methylation levels changed in MCF10A cells in response to RSV or PTS, were investigated in rats. The remaining genes were not well annotated in the rat genome and we were not able to obtain DNA sequence for these genes. Because fragments tested in the human genome did not match any sequence in the rat genome, we designed and optimized primers within promoters of *Kcnj4*, *Runx3*, *Bche*, and *Tagap*, in regions where CpG density was the highest (Figure 4A, Supplementary Figure S2). Based on pyrosequencing data, DNA methylation changes within 2 probes were upheld in whole blood of healthy rats upon 20-day RSV or PTS supplementation. Significant hypomethylation within *Runx3* was detected at CpGs #5 and #7 upon exposure to both RSV and PTS (Figure 4B and 4C). Hypermethylation within *Kcnj4* was observed at CpG #2 for RSV and both CpG #1 and #2 for PTS (Figure 4B and 4C). No significant changes in promoter methylation were identified by pyrosequencing in *Bche* or *Tagap* (Supplementary Figure S2).

Our present study establishes for the first time differences in DNA methylation at specific CpG sites after prolonged stilbenoid exposure that are detectable in normal cells and the whole blood of healthy rats. Upon validation in human cohorts, these novel DNA methylation probes may be used as candidates for developing quantitative exposure biomarkers with high application in epidemiologic studies where associations between the intake and disease risk are tested.

Subtle changes in DNA methylation may contribute to corresponding changes in gene expression: additional readout of exposure

In order to test whether the subtle changes in DNA methylation at the candidate genes affect transcriptional readout, we measured gene expression upon RSV treatment. RSV-mediated hypermethylation was reflected in downregulation of 3 out of 4 candidate genes, including *KCNJ4*, *RNF169*, and *DAOA* (trend of reduced expression for the latter) (Figure 5A). This would suggest that maintaining expression of those genes at low levels is beneficial for a healthy phenotype. Indeed, publicly available Oncomine expression data demonstrate up-regulation of *KCNJ4*, *RNF169*, and *DAOA* in breast cancer and other types of cancer compared to normal tissues, confirming potential cancer-promoting role of those genes (Figure 5B). On the other hand, 3 out of 4 genes hypomethylated upon RSV exposure, such as *RUNX3*, *KRTAP2-1*, and *TAGAP*, show increase in expression which suggests their potential health-promoting role (Figure 5C). This is further supported by down-regulation of those genes in breast tumors and other types of cancer compared to normal tissues as indicated by Oncomine expression data in clinical samples (Figure 5D). These results could imply that expression may be used as an additional measure of exposure to stilbenoids. However, not all the candidate genes are affected at the transcription level. Expression of *HOXA9*, a hypomethylated RSV target, stays unchanged (Figure 5C). It is possible that the slight remodeling of DNA methylation may not be enough for certain genes to affect transcription presumably due to co-existing chromatin structure. However, the altered methylation may help stabilize control mechanisms marking the genes to remain silenced (i.e., for cancer-promoting genes) or active (i.e., for guardian of a healthy phenotype). An interesting observation was noted for *BCHE* whose hypermethylation upon RSV treatment corresponded to increase in

expression rather than down-regulation as one would expect (Figure 5A). Additionally, Oncomine expression database clearly shows downregulation of *BCHE* in cancer compared to normal tissue and suggest a health-promoting role for *BCHE* (Figure 5B). An explanation may lie in the regulatory role of the CpG site where RSV-mediated hypermethylation occurs. The CpG site is located within the gene body as indicated in the gene map in Figure 2A. Increased methylation within body of the genes is associated with transcriptional activation and may constitute a signal for gene overexpression [43].

DISCUSSION

Improved quantitative evaluation methods of long-term exposure to dietary bioactive compounds are highly needed. Current measures of dietary exposures introduce inconsistencies in studies evaluating correlations between intake of various bioactive compounds and chronic disease. Polyphenols is a group of compounds with promising results in *in vitro* and *in vivo* studies [4-6], however without clear evidence from epidemiologic investigations [5, 7-9].

If we can provide adequate quantitative biomarkers, we may be able to accurately assess the importance of polyphenol intake in health maintenance and prevention of chronic diseases [11, 14]. An excellent example of how measurement methods influence interpretation of results is a study evaluating polyphenol intake among over 800 older adults living in the Chianti region of Tuscany in Italy [3, 44]. No significant correlations between polyphenol consumption and longevity were found when the intake measured based on dietary questionnaires was taken into account. Interestingly, using total urinary polyphenol concentration as a proxy measure of the intake showed that participants with the highest urinary polyphenol concentration had a lower

mortality rate than individuals with the lowest concentration. The results led to an important conclusion that high dietary intake of polyphenols may be associated with longevity and emphasized the importance of the accurate assessment of the intake.

While urinary metabolites reflect daily consumption, blood levels will be representative of just an hourly intake. Measures of chronic exposure to polyphenols are largely missing. In the present study, we investigated two polyphenols of the stilbenoid class, RSV and PTS, and their potential in inducing molecular changes in normal cells that would be informative of the intake. RSV is highly absorbed by humans but is lowly bioavailable due to its rapid conversion to metabolites [37, 38]. On the other hand, PTS, a naturally occurring dimethylated analog of RSV, is a highly bioavailable [37, 38]. We and others previously observed epigenetic changes, specifically alterations in DNA methylation, at candidate gene loci in human cancer cells treated with RSV or PTS [19-21]. Our recent study further demonstrates remodeling of the DNA methylation patterns in breast cancer cells exposed to polyphenols [18]. These pieces of evidence together with dynamic nature and responsiveness of DNA methylation to environmental factors make it highly probable that normal cells may also respond to long-term exposure to polyphenols by inducing alterations in DNA methylation patterns. Contrary to cancer cells, changes in normal cells would be subtle and without strong biological effects. There is evidence that in normal cells dynamics of DNA methylation allows for long-lasting changes upon a wide range of exogenous and endogenous exposures including toxicants, nutrients, behavioral and social stimuli, leaving so called “methylation memory” [45]. Indeed, we found in the present investigation that exposure of MCF10A mammary epithelial cells to RSV results in alterations at 1,324 CpG sites, of which only 32 sites have a magnitude of the difference in methylation greater than 0.1 (Figure 1). These

changes are induced at non-cytotoxic doses of RSV which supports their value as a “footprint” of exposure rather than a biologically relevant intervention, not necessarily desirable in normal cells. On the other hand, although without the acute biological response, the observed effects on the epigenome might be associated long-term with maintenance of a healthy phenotype. This intriguing hypothesis requires further investigation in future studies.

Although mechanisms underlying stilbenoid-mediated remodeling of DNA methylation patterns remain to be elucidated, the mode of action marking genes by subtle hypermethylation and hypomethylation in the normal cell model seems promising for providing relevant new insights into associations between the epigenome, polyphenol exposure, and health status. We selected 8 probes corresponding to 8 genes, including *KCNJ4*, *RNF169*, *BCHE*, *DAOA*, *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP*, for validation by pyrosequencing as a straightforward laboratory technique to detect changes in DNA methylation with high reproducibility. All probes showed a high concordance between microarray and pyrosequencing (Figure 2 and 3). We also confirmed that exposure to RSV analog, PTS, causes similar changes in DNA methylation at selected probes (Figure 2C and 3C). Hence, these subtle but detectable changes in the DNA methylation pattern of normal epithelial cells exposed to stilbenoids could have potential to be utilized as a valuable substitute or complementary tool in quantification of polyphenol exposure.

Such an assessment approach would be of value only if minimally-invasive specimens serve as a source of DNA. There is evidence of using blood to detect changes in DNA methylation for cancer risk prediction or cancer early detection [46, 47]. For instance, hypomethylation of BLCA-4 repeats detectable in blood cell DNA was associated with higher risk of bladder cancer [48].

Methylation within *CNKSRI*, *IFI44L*, *PENK*, and *WNK2* as well as lack of methylation within *TPO* and *MYTIL* in white blood cell DNA was demonstrated as a potential biomarker for risk prediction of HCC [49-51]. Similar association was found in breast cancer where methylation in *ATM* intragenic loci was linked to high cancer risk [52]. We therefore tested whether the changes in DNA methylation occurring in response to stilbenoids in MCF10A cells are also present in blood of healthy animals on diets supplemented with RSV or PTS. Out of 8 probes established based on our *in vitro* data, 4 probes were found in the rat genome. Of those 4 probes, CpG loci in promoters of *Runx3* and *Kcnj4* were verified and confirmed to have an altered DNA methylation status upon exposure to stilbenoids in rat blood DNA (Figure 4).

Since our studies demonstrate subtle changes in DNA methylation, a question arises whether such small changes can impact transcriptional activity of genes. We found that most but not all of the selected genes were differentially expressed upon stilbenoid-mediated subtle changes in DNA methylation (Figure 5). This clearly indicates that these gentle alterations in DNA methylation can act as a guardian of active or silenced chromatin state, and would explain lack of a strong biological response of normal cells to stilbenoids. This suggestion is further supported by the genome-wide characterization of RSV-mediated changes. We found that differentially methylated CpG sites and corresponding DMRs are located mostly in promoters, CpG islands, and enhancers; regions of transcriptional regulation (Figure 1). Interestingly, genes encompassing differentially methylated sites or regions were enriched with pathways associated with cardiovascular health, metabolism, cancer, and aging, indicating a role in stabilizing control mechanisms to maintain a healthy phenotype.

Our study is the first to establish DNA methylation changes in normal cells and blood DNA of healthy animals which occur upon prolonged exposure to stilbenoid compounds, RSV and PTS. Such quantitative molecular changes possess high potential to be developed into biomarkers of exposure to dietary polyphenols after validation in humans and verification in large cohorts. Recent guidelines for biomarkers in nutritional epidemiology emphasize that combining dietary reporting tools with molecular biomarkers of exposure like those proposed in our study can produce complementary information and increase accuracy of studied associations between diet and health status [10].

AUTHOR CONTRIBUTIONS

M.B., K.L., and B.S. conceived and design the study and performed the experiments. The data were analyzed by all the Authors. M.S. was specifically involved in bioinformatics analysis of the genome-wide data. M.B., K.L., and B.S. wrote the manuscript, and M.S. reviewed it.

ACKNOWLEDGEMENTS

This research was supported by the University of British Columbia VP Academic (10R76632), Purdue University Center for Cancer Research (Biological Evaluation Shared Resource, National Institutes of Health P30CA023168), and USDA National Institute of Food and Agriculture (1005656) Awards, granted to B.S.

The Authors declare no conflict of interest.

REFERENCES

[1] Tarasuk, V. S., Brooker, A. S., Interpreting epidemiologic studies of diet-disease relationships. *The Journal of nutrition* 1997, 127, 1847-1852.

- [2] Tucker, K. L., Assessment of usual dietary intake in population studies of gene-diet interaction. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2007, *17*, 74-81.
- [3] Shim, C. S., Moon, J. H., Cho, Y. D., Lee, M. S., Jeon, H. B., Jin, S. Y., Lee, H. K., Neuroendocrine carcinoma of the ampulla of Vater. *Gastrointestinal endoscopy* 2000, *51*, 593.
- [4] Sinha, D., Sarkar, N., Biswas, J., Bishayee, A., Resveratrol for breast cancer prevention and therapy: Preclinical evidence and molecular mechanisms. *Seminars in cancer biology* 2016, *40-41*, 209-232.
- [5] Grosso, G., Buscemi, S., Galvano, F., Mistretta, A., Marventano, S., La Vela, V., Drago, F., Gangi, S., Basile, F., Biondi, A., Mediterranean diet and cancer: epidemiological evidence and mechanism of selected aspects. *BMC surgery* 2013, *13 Suppl 2*, S14.
- [6] Aires, V., Delmas, D., Common pathways in health benefit properties of RSV in cardiovascular diseases, cancers and degenerative pathologies. *Current pharmaceutical biotechnology* 2015, *16*, 219-244.
- [7] Chao, C., Li, Q., Zhang, F., White, E., Alcohol consumption and risk of lung cancer in the VITamins And Lifestyle Study. *Nutrition and cancer* 2011, *63*, 880-888.
- [8] Chao, C., Haque, R., Caan, B. J., Poon, K. Y., Tseng, H. F., Quinn, V. P., Red wine consumption not associated with reduced risk of colorectal cancer. *Nutrition and cancer* 2010, *62*, 849-855.
- [9] Sutcliffe, S., Giovannucci, E., Leitzmann, M. F., Rimm, E. B., Stampfer, M. J., Willett, W. C., Platz, E. A., A prospective cohort study of red wine consumption and risk of prostate cancer. *International journal of cancer. Journal international du cancer* 2007, *120*, 1529-1535.
- [10] Corella, D., Ordovas, J. M., Biomarkers: background, classification and guidelines for applications in nutritional epidemiology. *Nutricion hospitalaria* 2015, *31 Suppl 3*, 177-188.
- [11] Zamora-Ros, R., Touillaud, M., Rothwell, J. A., Romieu, I., Scalbert, A., Measuring exposure to the polyphenol metabolome in observational epidemiologic studies: current tools and applications and their limits. *The American journal of clinical nutrition* 2014, *100*, 11-26.
- [12] Perez-Jimenez, J., Hubert, J., Hooper, L., Cassidy, A., Manach, C., Williamson, G., Scalbert, A., Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review. *The American journal of clinical nutrition* 2010, *92*, 801-809.
- [13] Arts, I. C., Hollman, P. C., Polyphenols and disease risk in epidemiologic studies. *The American journal of clinical nutrition* 2005, *81*, 317S-325S.
- [14] Spencer, J. P., Abd El Mohsen, M. M., Minihaue, A. M., Mathers, J. C., Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *The British journal of nutrition* 2008, *99*, 12-22.
- [15] Burns, J., Yokota, T., Ashihara, H., Lean, M. E., Crozier, A., Plant foods and herbal sources of resveratrol. *Journal of agricultural and food chemistry* 2002, *50*, 3337-3340.
- [16] Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American journal of clinical nutrition* 2005, *81*, 230S-242S.
- [17] Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *The American journal of clinical nutrition* 2005, *81*, 243S-255S.
- [18] Lubecka, K., Kurzava, L., Flower, K., Buvala, H., Zhang, H., Teegarden, D., Camarillo, I., Suderman, M., Kuang, S., Andrisani, O., Flanagan, J. M., Stefanska, B., Stilbenoids remodel the DNA methylation patterns in breast cancer cells and inhibit oncogenic NOTCH signaling

- through epigenetic regulation of MAML2 transcriptional activity. *Carcinogenesis* 2016, 37, 656-668.
- [19] Stefanska, B., Rudnicka, K., Bednarek, A., Fabianowska-Majewska, K., Hypomethylation and induction of retinoic acid receptor beta 2 by concurrent action of adenosine analogues and natural compounds in breast cancer cells. *European journal of pharmacology* 2010, 638, 47-53.
- [20] Stefanska, B., Salame, P., Bednarek, A., Fabianowska-Majewska, K., Comparative effects of retinoic acid, vitamin D and resveratrol alone and in combination with adenosine analogues on methylation and expression of phosphatase and tensin homologue tumour suppressor gene in breast cancer cells. *The British journal of nutrition* 2012, 107, 781-790.
- [21] Papoutsis, A. J., Borg, J. L., Selmin, O. I., Romagnolo, D. F., BRCA-1 promoter hypermethylation and silencing induced by the aromatic hydrocarbon receptor-ligand TCDD are prevented by resveratrol in MCF-7 cells. *The Journal of nutritional biochemistry* 2012, 23, 1324-1332.
- [22] Lou, X. D., Wang, H. D., Xia, S. J., Skog, S., Sun, J., Effects of resveratrol on the expression and DNA methylation of cytokine genes in diabetic rat aortas. *Archivum immunologiae et therapiae experimentalis* 2014, 62, 329-340.
- [23] Gracia, A., Elcoroaristizabal, X., Fernandez-Quintela, A., Miranda, J., Bediaga, N. G., M., M. d. P., Rimando, A. M., Portillo, M. P., Fatty acid synthase methylation levels in adipose tissue: effects of an obesogenic diet and phenol compounds. *Genes & nutrition* 2014, 9, 411.
- [24] Jones, P. A., Issa, J. P., Baylin, S., Targeting the cancer epigenome for therapy. *Nature reviews. Genetics* 2016, 17, 630-641.
- [25] Lim, U., Song, M. A., Dietary and lifestyle factors of DNA methylation. *Methods in molecular biology* 2012, 863, 359-376.
- [26] Cheishvili, D., Stefanska, B., Yi, C., Li, C. C., Yu, P., Arakelian, A., Tanvir, I., Khan, H. A., Rabbani, S., Szyf, M., A common promoter hypomethylation signature in invasive breast, liver and prostate cancer cell lines reveals novel targets involved in cancer invasiveness. *Oncotarget* 2015, 6, 33253-33268.
- [27] Stefanska, B., Cheishvili, D., Suderman, M., Arakelian, A., Huang, J., Hallett, M., Han, Z. G., Al-Mahtab, M., Akbar, S. M., Khan, W. A., Raqib, R., Tanvir, I., Khan, H. A., Rabbani, S. A., Szyf, M., Genome-wide study of hypomethylated and induced genes in patients with liver cancer unravels novel anticancer targets. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, 20, 3118-3132.
- [28] Siddiqui, R. A., Harvey, K. A., Walker, C., Altenburg, J., Xu, Z., Terry, C., Camarillo, I., Jones-Hall, Y., Mariash, C., Characterization of synergistic anti-cancer effects of docosahexaenoic acid and curcumin on DMBA-induced mammary tumorigenesis in mice. *BMC cancer* 2013, 13, 418.
- [29] Stefanska, B., Huang, J., Bhattacharyya, B., Suderman, M., Hallett, M., Han, Z. G., Szyf, M., Definition of the landscape of promoter DNA hypomethylation in liver cancer. *Cancer research* 2011, 71, 5891-5903.
- [30] Colella, S., Shen, L., Baggerly, K. A., Issa, J. P., Krahe, R., Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. *BioTechniques* 2003, 35, 146-150.
- [31] Tost, J., Gut, I. G., DNA methylation analysis by pyrosequencing. *Nature protocols* 2007, 2, 2265-2275.
- [32] Wilhelm-Benartzi, C. S., Koestler, D. C., Karagas, M. R., Flanagan, J. M., Christensen, B. C., Kelsey, K. T., Marsit, C. J., Houseman, E. A., Brown, R., Review of processing and analysis methods for DNA methylation array data. *British journal of cancer* 2013, 109, 1394-1402.

- [33] Rijlaarsdam, M. A., van der Zwan, Y. G., Dorssers, L. C., Looijenga, L. H., DMRforPairs: identifying differentially methylated regions between unique samples using array based methylation profiles. *BMC bioinformatics* 2014, *15*, 141.
- [34] Ernst, J., Kheradpour, P., Mikkelsen, T. S., Shoresh, N., Ward, L. D., Epstein, C. B., Zhang, X., Wang, L., Issner, R., Coyne, M., Ku, M., Durham, T., Kellis, M., Bernstein, B. E., Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* 2011, *473*, 43-49.
- [35] Horvath, S., DNA methylation age of human tissues and cell types. *Genome biology* 2013, *14*, R115.
- [36] Horvath, S., Erratum to: DNA methylation age of human tissues and cell types. *Genome biology* 2015, *16*, 96.
- [37] Kapetanovic, I. M., Muzzio, M., Huang, Z., Thompson, T. N., McCormick, D. L., Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats. *Cancer chemotherapy and pharmacology* 2011, *68*, 593-601.
- [38] Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., Jr., Walle, U. K., High absorption but very low bioavailability of oral resveratrol in humans. *Drug metabolism and disposition: the biological fate of chemicals* 2004, *32*, 1377-1382.
- [39] Kiskova, T., Ekmekcioglu, C., Garajova, M., Orendas, P., Bojkova, B., Bobrov, N., Jager, W., Kassayova, M., Thalhammer, T., A combination of resveratrol and melatonin exerts chemopreventive effects in N-methyl-N-nitrosourea-induced rat mammary carcinogenesis. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation* 2012, *21*, 163-170.
- [40] Sengottuvelan, M., Viswanathan, P., Nalini, N., Chemopreventive effect of trans-resveratrol--a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis. *Carcinogenesis* 2006, *27*, 1038-1046.
- [41] Paul, S., DeCastro, A. J., Lee, H. J., Smolarek, A. K., So, J. Y., Simi, B., Wang, C. X., Zhou, R., Rimando, A. M., Suh, N., Dietary intake of pterostilbene, a constituent of blueberries, inhibits the beta-catenin/p65 downstream signaling pathway and colon carcinogenesis in rats. *Carcinogenesis* 2010, *31*, 1272-1278.
- [42] Jeyabalan, J., Aqil, F., Munagala, R., Annamalai, L., Vadhanam, M. V., Gupta, R. C., Chemopreventive and therapeutic activity of dietary blueberry against estrogen-mediated breast cancer. *Journal of agricultural and food chemistry* 2014, *62*, 3963-3971.
- [43] Yang, X., Han, H., De Carvalho, D. D., Lay, F. D., Jones, P. A., Liang, G., Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer cell* 2014, *26*, 577-590.
- [44] Warden, B. A., Smith, L. S., Beecher, G. R., Balentine, D. A., Clevidence, B. A., Catechins are bioavailable in men and women drinking black tea throughout the day. *The Journal of nutrition* 2001, *131*, 1731-1737.
- [45] Vineis, P., Chatziioannou, A., Cunliffe, V. T., Flanagan, J. M., Hanson, M., Kirsch-Volders, M., Kyrtopoulos, S., Epigenetic memory in response to environmental stressors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2017, *31*, 2241-2251.
- [46] Friso, S., Udali, S., Guarini, P., Pellegrini, C., Pattini, P., Moruzzi, S., Girelli, D., Pizzolo, F., Martinelli, N., Corrocher, R., Olivieri, O., Choi, S. W., Global DNA hypomethylation in peripheral blood mononuclear cells as a biomarker of cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2013, *22*, 348-355.

- [47] Huang, W. Y., Su, L. J., Hayes, R. B., Moore, L. E., Katki, H. A., Berndt, S. I., Weissfeld, J. L., Yegnasubramanian, S., Purdue, M. P., Prospective study of genomic hypomethylation of leukocyte DNA and colorectal cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2012, 21, 2014-2021.
- [48] Ji, H. X., Zhao, Q., Pan, J. H., Shen, W. H., Chen, Z. W., Zhou, Z. S., Association of BLCA-4 hypomethylation in blood leukocyte DNA and the risk of bladder cancer in a Chinese population. *Pathology oncology research : POR* 2013, 19, 205-210.
- [49] Kao, W. Y., Yang, S. H., Liu, W. J., Yeh, M. Y., Lin, C. L., Liu, C. J., Huang, C. J., Lin, S. M., Lee, S. D., Chen, P. J., Yu, M. W., Genome-wide identification of blood DNA methylation patterns associated with early-onset hepatocellular carcinoma development in hepatitis B carriers. *Molecular carcinogenesis* 2017, 56, 425-435.
- [50] Wu, H. C., Shen, J., Yang, H. I., Tsai, W. Y., Chen, C. J., Santella, R. M., Blood DNA methylation markers in prospectively identified hepatocellular carcinoma cases and controls from Taiwan. *World journal of hepatology* 2016, 8, 301-306.
- [51] Liu, Z., Yan, H., Zhang, J., Blood DNA methylation markers in potentially identified Chinese patients with hepatocellular carcinoma. *Pakistan journal of pharmaceutical sciences* 2016, 29, 1451-1456.
- [52] Brennan, K., Garcia-Closas, M., Orr, N., Fletcher, O., Jones, M., Ashworth, A., Swerdlow, A., Thorne, H., Investigators, K. C., Riboli, E., Vineis, P., Dorronsoro, M., Clavel-Chapelon, F., Panico, S., Onland-Moret, N. C., Trichopoulos, D., Kaaks, R., Khaw, K. T., Brown, R., Flanagan, J. M., Intragenic ATM methylation in peripheral blood DNA as a biomarker of breast cancer risk. *Cancer research* 2012, 72, 2304-2313.

FIGURE LEGENDS

Figure 1. Subtle changes of the landscape of DNA methylation in MCF10A human mammary epithelial cells upon exposure to resveratrol (RSV). The genome-wide DNA methylation patterns were delineated using Illumina 450K microarray platform in MCF10A cells incubated with RSV at 15 μ M for 9 days vs. MCF10A cells treated with ethanol as a vehicle control. (A) Chromosomal view of differentially methylated CpG sites ($P < 0.05$, limma t-test). Each CpG site with significant difference of at least 0.05 between RSV-treated and control untreated cells was plotted into a bar track of a chromosomal view using Integrative Genomics Viewer (IGV) visualization tool. Each bar corresponds to a single, differentially methylated CpG site, with red indicating hypermethylation and blue indicating hypomethylation in RSV vs. control (RSV minus Ctrl). (B) Pie charts of statistically significant differentially methylated CpG sites with the

difference of at least 0.05 or 0.1 between RSV-exposed and control MCF10A cells ($P < 0.05$, limma t-test). Diff. refers to differential methylation (delta beta, RSV minus control). (C) A heatmap showing methylation levels (beta values) for the top 500 differentially methylated CpG loci ($P < 0.05$, limma t-test). The heatmap presents subtle pattern separating data sets obtained from controls (untreated MCF10A cells) and RSV-exposed cells. Columns “a-c” show classification of the CpG sites according to their location relative to transcription start site (TSS) (a), CpG islands (b) and enhancer region (c). (D) Pathways associated with genes containing CpG loci that are differentially methylated upon exposure of MCF10A cells to RSV. Integrative pathway analysis was performed using DAVID knowledgebase. (E) Differentially methylated regions (DMRs) were determined using DMRforPairs algorithm in R/Bioconductor. Regions with median methylation levels (M-values) between the samples differed at least by 0.4 that is approximately equal to differential methylation 0.05 (delta beta value) were considered relevant. Their location relative to transcription start site (TSS), CpG islands and body of the genes is depicted in the pie chart. (F) Chromatin states associated with relevant DMRs were determined using broad ChromHMM HMEC track in UCSC Genome Browser (hg19). DMRs could correspond to the following chromatin states: active, weak or poised promoters, strong or weak enhancers, putative insulators, active or weak transcription, Polycomb-repressed regions or heterochromatin. Active promoters, strong enhancers and active transcription regions are linked to high expression levels, with the latter state determined based on the enrichment of histone marks along transcripts. Weak or poised promoters, weak enhancers, weak transcription, Polycomb-repressed regions or heterochromatin, in turn, are linked to low expression. (G) Genes of overlapping transcripts were determined for the exact DMR region and within a margin of 10000 bp of the region. Pathways associated with genes for all relevant DMRs were determined using DAVID knowledgebase.

Figure 2. Stilbenoid-mediated hypermethylation of CpG loci within *KCNJ4*, *RNF169*, *BCHE*, and *DAOA* in human mammary epithelial cells. Four hypermethylated CpG sites corresponding to 4 genes (probes) in response to resveratrol (RSV) in MCF10A human mammary epithelial cells were identified based on Illumina 450K microarray. The difference in DNA methylation and statistical significance were taken into account in the selection. Each region encompasses a differentially methylated CpG site covered on Illumina 450K microarray (marked in square). The exact position of validated CpG sites relative to transcription start site (TSS) is shown in gene maps (A). The tested region is shaded, and pyrosequenced CpGs are circled and numbered. The putative transcription factor binding sites are indicated as predicted by TransFac. The difference in DNA methylation status at the selected CpG sites was quantitatively validated by pyrosequencing in MCF10A cells exposed for 9 days to 15 μ M RSV (B) and 7 μ M pterostilbene (PTS) (C), a dimethylated analog of RSV. Average methylation status of CpG site covered on Illumina and neighboring CpG sites in the tested fragment of *KCNJ4*, *RNF169*, *BCHE*, and *DAOA* is displayed. All results represent mean $\pm$ S.D. of three independent experiments; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Figure 3. Stilbenoid-mediated hypomethylation of CpG loci within *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP* in human mammary epithelial cells. Four hypomethylated CpG sites corresponding to 4 genes (probes) in response to resveratrol (RSV) in MCF10A human mammary epithelial cells were identified based on Illumina 450K microarray. The difference in DNA methylation and statistical significance were taken into account in the selection. Each region

encompasses a hypomethylated CpG site covered on Illumina 450K microarray (marked in square). The exact position of validated CpG sites relative to transcription start site (TSS) is shown in gene maps (A). The tested region is shaded, and pyrosequenced CpGs are circled and numbered. The putative transcription factor binding sites are indicated as predicted by TransFac. The difference in DNA methylation status at the selected CpG sites was quantitatively validated by pyrosequencing in MCF10A cells exposed for 9 days to 15 μ M RSV (B) and 7 μ M pterostilbene (PTS) (C), a dimethylated analog of RSV. Average methylation status of CpG site covered on Illumina and neighboring CpG sites in the tested fragment of *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP* is displayed. All results represent mean $\pm$ S.D. of three independent experiments; *** P < 0.001, ** P < 0.01, * P < 0.05.

Figure 4. Resveratrol (RSV) and pterostilbene (PTS) change methylation states of CpG sites located in regulatory regions of *Runx3* and *Kcnj4* in rat peripheral blood. Fischer 344 rats were exposed for 20 days to chow diet [choline sufficient amino acid defined (CSAA) diet] supplemented with resveratrol (CSAA+RSV) or pterostilbene (CSAA+PTS). Collected peripheral blood was subjected to DNA isolation followed by DNA methylation analysis by pyrosequencing at loci identified in *in vitro* MCF10A experiment. DNA methylation changes were detected within *Runx3* and *Kcnj4* regulatory regions. The exact position of tested CpG sites is shown in gene maps (A). Average methylation status of CpG sites in the tested fragments of *Runx3* and *Kcnj4* in rats on CSAA diet and CSAA diet supplemented with RSV or PTS is displayed in panels B and C. All results represent mean $\pm$ SEM of n=6 per group; *** P < 0.001, ** P < 0.01, * P < 0.05.

Figure 5. Resveratrol (RSV)-mediated changes in expression of genes which responded to exposure to stilbenoids with subtle alterations in DNA methylation: additional readout of exposure. (A,C) Effect of 9-day exposure of MCF10A mammary epithelium cells to 15 μ M RSV on expression of *KCNJ4*, *RNF169*, *BCHE*, and *DAOA* as well as *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP* that were respectively hypermethylated or hypomethylated by stilbenoids. Gene expression was determined by QPCR. All results represent mean $\pm$ S.D. of three independent experiments; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. (B,D) Cancer gene expression microarray data for *KCNJ4*, *RNF169*, *BCHE*, and *DAOA* as well as *HOXA9*, *RUNX3*, *KRTAP2-1*, and *TAGAP* that were respectively hypermethylated or hypomethylated by stilbenoids. The normal versus tumor gene expression data were obtained from Oncomine and are presented as log₂-transformed median centered per array, and SD-normalized to 1 per array. All the presented changes are statistically significant ($P < 0.05$).

Figure 1

Figure 2

A**KCNJ4**

CpG3: cg23572608

RNF169

CpG2: cg02673002

BCHE

CpG2: cg09406615

DAOA

CpG1: cg01297020

B **C**

A **B** **C**

A

Rat blood_20 days

Runx3

Kcnj4

B **C**

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and exposure to resveratrol (RSV) and pterostilbene (PTS)

Human mammary epithelial MCF10A cell line was purchased from ATCC (CRL-10317, USA) and maintained in DMEM/F12 (1:1) medium (Gibco) supplemented with 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 100 ng/ml cholera toxin (Calbiochem, EMD Millipore, Billerica, MA, USA), 0.01 mg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 500 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 5% horse serum (Gibco), 1U/ml penicillin and 1µg/ml streptomycin (Gibco). MCF10A cell line was routinely verified by morphology, invasion and growth rate. Resveratrol (RSV, Sigma-Aldrich, St. Louis, MO, USA) and pterostilbene (PTS, Cayman Chem., Ann Arbor, MI, USA) were resuspended in ethanol and 10mM solutions were stored at -20°C. Dilutions of the compounds were freshly prepared prior to adding to the cell medium. Cells were grown in a humidified atmosphere of 5% carbon dioxide at 37°C. 24 h prior to incubation with RSV or PTS, cells were plated at a density of $1-2 \times 10^5$ per a 10-cm tissue culture dish. Cells were exposed to different RSV or PTS concentrations ranging from 0 to 20 µM of RSV and 0 to 15 µM of PTS for 4 days. Cells were then split 1:50, allowed to attach overnight and exposed to the compounds for additional 4 days (9-day exposure).

Illumina Infinium Human Methylation 450K BeadChip microarray

DNA from control untreated cells (cultured with ethanol as a vehicle control) and cells cultured with RSV or PTS was isolated using standard phenol:chloroform extraction protocol. Genomic

DNA was quantified using Picogreen protocol (Quant-iT™ PicoGreen® dsDNA Products, Invitrogen, P-7589) and read on SpectraMAX GeminiXS Spectrophotometer. Bisulphite conversion of 500 ng of each DNA sample was performed using the EZ-96 DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research). Then, 4 µL of bisulphite-converted DNA was used for hybridization on the Infinium HumanMethylation 450K BeadChip, following the Illumina Infinium HD Methylation protocol (Illumina). Hybridization and scanning were performed in the University of Chicago Genomics Facility. Raw data processing was performed using the Methylation module (version 1.9.0) of the GenomeStudio software (Illumina; version 2011.1) using HumanMethylation450_15017482_v.1.2.bpm manifest, which normalizes within-sample data using different internal controls that are present on the HumanMethylation 450K BeadChip and internal background probes. The methylation score for each CpG was represented as a beta value according to the fluorescent intensity ratio with any values between 0 (unmethylated) and 1 (completely methylated). Raw microarray data and processed data will be available from Gene Expression Omnibus (accession number pending).

The Illumina 450K microarray platform covers more than 485,000 methylation sites (CpG sequences) per sample at single-nucleotide resolution. This coverage corresponds to 99% of RefSeq genes (all known protein coding genes), with an average of 17 CpG sites per gene region. These CpG sites are distributed across regulatory gene regions including CpG islands, promoters, 5'UTR, first exon, gene body, 3'UTR, and CpG island shelves and open seas. The latter two refer respectively to regions between 2kb-4kb and more than 4kb from CpG islands. CpG islands are regions with high CpG density whose methylation is important in gene regulation.

Viability assays

Cell viability was determined by trypan blue (Sigma-Aldrich, St.Louis, MO, USA) exclusion test. Cells were harvested after incubation with RSV or PTS on day 4 and day 9. Following 3-5 minute incubation with trypan blue, the viable and dead cells were counted under the microscope. The results were confirmed by a viability assay using MTT (Supplementary Figure S1).

Mapping differentially methylated CpG sites into differentially methylated regions (DMRs)

We utilized GenomeStudio (Illumina) to generate final reports that were used as input into the lumi pipeline for pre-processing and normalization of the data in R/Bioconductor. Differentially methylated regions (DMRs) were identified using DMRforPairs algorithm [1]. Default parameters were applied, except min_dM parameter was lowered to 0.4 (lower bound of range). dM value of 0.4 is approximately equal to delta beta-value of 0.05. DMRs were defined as regions containing minimum of 4 probes with maximum of 200bp between them. DMRs were ranked according to dM value (dM indicates the largest median difference (absolute) between any of the sample pairs). No significant DMRs were detected in the analysis of control- versus RSV-treated MCF10A samples, but DMRs considered relevant (dM>0.4) were further assessed.

124 relevant DMRs were detected among all samples (3 controls, 3 RSV-treated). Further, control samples were compared to each other and RSV samples were compared to each other to determine DMRs within biological replicates. No significant DMRs were observed. 9 relevant DMRs were detected among control samples and 35 relevant DMRs were detected among RSV samples. Relevant DMRs detected in biological replicates were removed from further analysis.

We then used broad ChromHMM HMEC track in UCSC Genome Browser (hg19) to determine chromatin states of relevant DMRs in breast cells [2]. DMRs could correspond to the following chromatin states: active, weak or poised promoters, strong or weak enhancers, putative insulators, active or weak transcription, Polycomb-repressed regions or heterochromatin. Active promoters, strong enhancers and active transcription regions are linked to high expression levels, with the latter state determined based on the enrichment of histone marks along transcripts. Weak or poised promoters, weak enhancers, weak transcription, Polycomb-repressed regions or heterochromatin, in turn, are linked to low expression.

REFERENCES

- [1] Rijlaarsdam, M. A., van der Zwan, Y. G., Dorssers, L. C., Looijenga, L. H., DMRforPairs: identifying differentially methylated regions between unique samples using array based methylation profiles. *BMC bioinformatics* 2014, *15*, 141.
- [2] Ernst, J., Kheradpour, P., Mikkelsen, T. S., Shores, N., *et al.*, Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* 2011, *473*, 43-49.

SUPPLEMENTARY FIGURES and TABLES

Supplementary Figure S1. Exposure of MCF10A mammary epithelial cells to resveratrol (RSV) or pterostilbene (PTS) leads to negligible effects on cell viability within a defined range of concentrations. (A,B) Effects of 9 day-exposure of MCF10A mammary epithelial cells to resveratrol (RSV) (A) or pterostilbene (PTS) (B) at a range of concentrations 0-20 μ M for RSV or 0-15 μ M for PTS on cell viability as measured by a trypan blue exclusion test (top panels) and MTT assay (bottom panels). All results represent mean $\pm$ S.D. of three independent experiments; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Supplementary Figure S2. Resveratrol (RSV) and pterostilbene (PTS) effects on methylation states of CpG sites located in regulatory regions of *Tagap* and *Bche* in rat peripheral blood. Fischer 344 rats were exposed for 20 days to chow diet [choline sufficient amino acid defined (CSAA) diet] supplemented with resveratrol (CSAA+RSV) or pterostilbene (CSAA+PTS). Collected peripheral blood was subjected to DNA isolation followed by DNA methylation analysis by pyrosequencing at loci identified in *in vitro* MCF10A experiment. Average methylation status of CpG sites in the tested fragments of *Tagap* and *Bche* in rats on CSAA diet and CSAA diet supplemented with RSV (A) or PTS (B) indicates no significant changes. All results represent mean $\pm$ SEM of n=6 per group.

Supplementary Table S1. Primer sequences used in DNA methylation analysis by pyrosequencing in human mammary epithelial MCF10A cells (A) and rat peripheral blood (B), and in expression analysis by QPCR (C).

Supplementary Table S1A. Primer sequences used in DNA methylation analysis by pyrosequencing in MCF10A mammary epithelial cells.			
Gene	Primer sequences for pyrosequencing	Annealing temperature [°C]	Amplicon length [bp]
BCHE (Assay 3, three CpG sites)	FW 5'-GTTTTTTGAATAATAGGAAGATTTGATG-3' RVBio 5'-AAACTAAAACAACAACCTACATCCTACAT-3' Seq 5'-TGAGTTTTATAGTAATTTGGATT-3'	56	292
DAOA (Assay 1, one CpG site)	FW 5'-TGGTTATGGTAGTTATGTTTTGTGAA-3' RVBio 5'-AAACAAAAAAAAAAAAACCAATATCCACAT-3' Seq 5'-TTTGTGAAAAATTTTATTAATGA-3'	55	129
HOXA9 (Assay 2, thirteen CpG sites)	FW 5'-ATGGGGTTTGTTTTAATTGTG-3' RVBio 5'-CAACCTATATAACTTCTAAAACAATAACTC-3' Seq1 5'-GGGTTTGTTTTAATTGTGG-3' (CpGs 1-4) Seq2 5'-ATAATTAATTAAGTTATTAAGAAGG-3' (CpGs 5-11) Seq3 5'-AATTGAAGTTATAAAAAAGTAGTT-3' (CpGs 12-13)	54	305
KCNJ4 (Assay 2, four CpG sites)	FW 5'-GGTGTTTGGATTTGGGTGGTTTA-3' RVBio 5'-CAACCTAAACATTAACCAAACTCTATCT-3' Seq1 5'-GGTGGTTTATTGAGTAAGG-3' (CpG 1) Seq2 5'-AGGAATTAAATATTTAGGGAAAT-3' (CpG 2-4)	59	292
KRTAP2-1 (Assay 1, three CpG sites)	FW 5'-AGGAATTATATGGGGGAAATAGTTT-3' RVBio 5'-AAATTATACCTAATATCTCCCTCTTCT-3' Seq 5'-AGTTATTAGATAGGGTTTTGA-3'	53	247
RNF169 (Assay 2, three CpG sites)	FW 5'-TGGATTTAGGAGGTTTGGGTTGTA-3' RVBio 5'-ATAACTTTATAACTAAAACCAACCCAC-3' Seq1 5'-AGAAAATGTGGATTAGTGTA-3' (CpGs 1-2) Seq2 5'-AGGTTGTTTTTTTTGGTAG-3' (CpG 3)	59	319
RUNX3 (Assay 1, seven CpG sites)	FW 5'-TAGGGGAAGGTAGTTGATATGGTT-3' RVBio 5'-AACTAAAATAACCTCTTCCCTACT-3' Seq 5'-GGATAATGTATTTTGGGG-3'	54	170
TAGAP (Assay 1, four CpG sites)	FW 5'-TGGATGTGGGATATTGTTATAGAATTAGA-3' RVBio 5'-AAATTCACCATCTCTTTCCTACTT-3' Seq 5'-AGATAGTTTTTTTTTTGAGGAATA-3'	59	285

Supplementary Table S1B. Primer sequences used in DNA methylation analysis by pyrosequencing in rat peripheral blood samples.

Gene	Primer sequences for pyrosequencing	Annealing temperature [°C]	Amplicon length [bp]
Runx3	FW 5'-GGGTAGATGTAAATTGGAATTATTTAAGAA-3' RVBio 5'-CCACCAAATAAAACCCACACCT-3' Seq 5'-AAATAGAATTTATATATTATAGAGG-3'	49	126
Tagap	FW 5'-GGAATTTTTTAAATTTTTTAAGTTGTAGTT-3' RVBio 5'-AAACCAATACTATTAAAAACAACCTCTACT-3' Seq 5'-TTTAAATTTTTTAAGTTGTAGTTTAT-3'	46	116
Bche	FW 5'-GGTTATTTAGAGGAGGTTGTGTATAGG-3' RVBio 5'-TCCTCCTATAAATTCTTCTACTCTACAT-3' Seq 5'-GGTTGTGTATAGGGGAT-3'	55.5	171
Kcnj4	FW 5'-AGGGAAAAGAGAGATAAGAGTGT-3' RVBio 5'-AACCCTAAATTCCATCCCCAACTCTA-3' Seq1 5'-TTTTTATAATTTGATTTTGAATATG -3'	49	219

Supplementary Table S1C. Primer sequences used in expression analysis (QPCR).

Gene	Primer sequences for QPCR	Annealing temperature [°C]	Amplicon length [bp]
BCHE	FW 5'-CCAGAATGGATGGGAGTGATG-3' RV 5'-CCACCGTTTCACTATGGATCTAC-3'	59	123
DAOA	FW 5'-CTGATGGGTGCTGATTCTCTC-3' RV 5'-TGCTCAGAAGAATGCTCCTTT-3'	59	94
HOXA9	FW 5'-CATTAAACCTGAACCGCTGTC-3' RV 5'-CTCTATCAACTGGAGGAGAACC-3'	59	107
KCNJ4	FW 5'-GTGTACTTCGCCAACCTGAG-3' RV 5'-CGGAGAAGATCATGAGCATGTAG-3'	59	103
KRTAP2-1	FW 5'-GAGCACTGGGCTCATCTATT-3' RV 5'-GCCACGAAATCTTAAGAAGAAGG-3'	59	83
RNF169	FW 5'-TCAGAGCACCAATCAAATTAAGC-3' RV 5'-CAGCTTGTGGATTTGATCTTCAG-3'	59	122
RUNX3	FW 5'-GTTTCACCTGACCATCACT-3' RV 5'-GTCCACGGTCACCTTGATG-3'	59	80
TAGAP	FW 5'-GAGAGCAGCCAACGAGAAA-3' RV 5'-CTTAAAGACCACAGCGAGGAG-3'	59	109
GAPDH	FW 5'-TGCACCACCAACTGCTTA-3' RV 5'-AGAGGCAGGGATGATGTTC-3'	59	177

Supplementary Table S2. A list of CpG sites that are differentially methylated upon 9-day exposure of MCF10A mammary epithelial cells to 15 μ M RSV ($0.05 \leq \text{differential methylation} \leq 0.05$, $p < 0.05$, limma t test). Among these changes, 889 CpG sites (67% of total number of changes) corresponding to 602 genes were hypermethylated whereas 435 CpG sites were hypomethylated and located within 305 genes. The changes in DNA methylation were subtle ranging from 0.05 to 0.20 in differential methylation values.

Supplementary Table S3. A list of relevant differentially methylated regions (DMRs) between control MCF10A cells and cells treated with 15 μ M RSV for 9 days. DMRs are defined as regions that contain at least 4 CpG sites (Illumina probes) with a maximum distance of 200 bp between individual sites. Regions in which median methylation levels (M-values) between the samples differed at least by 0.4 which referred as relevant DMRs. Chromosomal positions are indicated in bp, n indicates the number of probes in a region, ID indicates the region ID. Values in the columns per sample indicate median beta values. dM indicates the largest median difference (absolute) between any of the sample pairs and p indicates uncorrected p-value from Mann-Whitney U test ($n=2$) or Kruskal Wallis test ($n>2$), p.adj denotes the multiple testing corrected p-value (method:fdr). Gene symbols of overlapping transcripts are listed for the exact region and within a margin of 10000 bp of the region.

003183058	0.51646375	0.50000787	0.53777358	0.55915204	0.56647887	0.61793984	0.60044027	0.549278116	1.68218037	0.00074478	0.77936147	-2.8973981	18	18742724	18	F
00534179	0.13306564	0.18026619	0.16954464	0.16028456	0.16028456	0.16028456	0.16028456	0.16028456	0.27729116	0.0019947	0.77936147	0.8554261	4	79047000	4	TRUE
00313376	0.20862457	0.17627064	0.23024316	0.20212993	0.22506934	0.27884516	0.26902605	0.2307448	2.95063682	0.0170397	0.77937409	0.95037094	12	71507794	12	R
00629573	0.56875773	0.55676879	0.57684174	0.49418168	0.56911211	0.57593911	0.56910929	0.57684174	2.90030848	0.0170397	0.77937409	0.78502028	13	17678671	13	X
007073483	0.68237096	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	0.70486974	17	71743765	17	TRUE
007811446	0.59085352	0.61497995	0.61497995	0.60910403	0.60910403	0.60910403	0.60910403	0.60910403	0.60910403	0.60910403	0.60910403	0.60910403	9	6149006	9	F
004740004	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	0.47209247	9	6149006	9	F
00688569	0.52325476	0.53191627	0.54488102	0.53129991	0.55323961	0.56412607	0.58388374	0.57555185	4.86549451	0.00179151	0.77936147	1.34447676	9	6149006	9	F
00321775	0.18217351	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	0.17860155	7	75651319	7	F
005140317	0.34770277	0.33977889	0.34588959	0.34041558	0.33947798	0.34111801	0.34079339	0.33940481	2.7643807	0.00179151	0.77936147	0.80413838	22	13880275	22	F
00321775	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	0.19787496	12	13791845	12	F
004201086	0.13604532	0.13180064	0.13604532	0.14079031	0.14079031	0.14079031	0.14079031	0.14079031	0.14079031	0.14079031	0.14079031	0.14079031	9	15886029	9	F
00339292	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	0.48642117	9	15886029	9	F
00517587	0.20168831	0.16247967	0.20077371	0.272147	0.21110338	0.20914389	0.21611749	0.21688764	1.49211448	0.00179151	0.77936147	0.78692049	21	6466081	21	R
00855564	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	0.60420368	21	6466081	21	R
00854432	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	0.15413231	21	6466081	21	R
00894919	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	0.61040883	21	6466081	21	R
00737408	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	0.49098287	21	6466081	21	R
00525027	0.62468083	0.63488121	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	0.64847023	21	6466081	21	R
00295735	0.50518651	0.5782421	0.53827735	0.62051825	0.59966045	0.60709008	0.60420368	0.59966045	0.5782421	0.53827735	0.62051825	0.59966045	21	6466081	21	R
00595913	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	0.23501951	21	6466081	21	R
00264680	0.62735836	0.62588984	0.6786131	0.68149207	0.73907781	0.713174	0.69414038	0.68149207	0.62588984	0.6786131	0.68149207	0.73907781	21	6466081	21	R
00205567	0.5292496	0.50524568	0.54857467	0.57944863	0.61008871	0.64677079	0.64677079	0.61008871	0.5292496	0.50524568	0.54857467	0.57944863	21	6466081	21	R
00788246	0.4472028	0.42009009	0.44970161	0.50473754	0.50481988	0.45714554	0.45714554	0.45714554	0.4472028	0.42009009	0.44970161	0.50473754	22	7618029	22	F
00544255	0.20047834	0.21682313	0.22091214	0.23472835	0.30078052	0.31884058	0.33696055	0.23472835	0.22091214	0.23472835	0.30078052	0.31884058	22	7618029	22	F
00719024	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	0.63811517	22	7618029	22	F
00221401	0.41517795	0.47840789	0.40437793	0.51613141	0.51003819	0.51498803	0.51728787	0.40437793	0.41517795	0.47840789	0.40437793	0.51613141	22	7618029	22	F
00500224	0.65510089	0.62077051	0.64821459	0.64821459	0.64821459	0.64821459	0.64821459	0.64821459	0.65510089	0.62077051	0.64821459	0.64821459	22	7618029	22	F
00479655	0.67113178	0.38931891	0.49039234	0.64454201	0.84226014	0.47840648	0.47840648	0.47840648	0.67113178	0.38931891	0.49039234	0.64454201	22	7618029	22	F
00319929	0.2897088	0.17884309	0.3489626	0.40462187	0.41299173	0.39047274	0.39047274	0.39047274	0.2897088	0.17884309	0.3489626	0.40462187	22	7618029	22	F
00944219	0.45650971	0.70318486	0.6992428	0.42229052	0.3072362	0.51709765	0.51709765	0.51709765	0.45650971	0.70318486	0.6992428	0.42229052	22	7618029	22	F
00898914	0.16475835	0.17077793	0.18477947	0.20659685	0.20758262	0.26888813	0.26888813	0.26888813	0.16475835	0.17077793	0.18477947	0.20659685	22	7618029	22	F
00844841	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	0.61120991	22	7618029	22	F
00104464	0.52811301	0.54402968	0.24725614	0.55153101	0.60999377	0.5422741	0.5422741	0.5422741	0.52811301	0.54402968	0.24725614	0.55153101	22	7618029	22	F
00426319	0.47802974	0.44553117	0.49513786	0.50633965	0.51151614	0.50633965	0.50633965	0.50633965	0.47802974	0.44553117	0.49513786	0.50633965	22	7618029	22	F
003212316	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	0.65004196	22	7618029	22	F
00420044	0.59538494	0.60404005	0.63347446	0.65338403	0.689753	0.67770044	0.689753	0.689753	0.59538494	0.60404005	0.63347446	0.65338403	22	7618029	22	F
00090770	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	0.57008463	22	7618029	22	F
00459951	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	0.9617134	22	7618029	22	F
00210507	0.32014909	0.31746033	0.31746033	0.31746033	0.31746033	0.31746033	0.31746033	0.31746033	0.32014909	0.31746033	0.31746033	0.31746033	22	7618029	22	F
00417970	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	0.6250677	22	7618029	22	F
00515667	0.66279536	0.62588984	0.6786131	0.68149207	0.73907781	0.713174	0.69414038	0.68149207	0.66279536	0.62588984	0.6786131	0.68149207	22	7618029	22	F
00282320	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	0.13352458	22	7618029	22	F
00472962	0.16574393	0.18873304	0.17738272	0.25699311	0.23497902	0.22047465	0.22047465	0.22047465	0.16574393	0.18873304	0.17738272	0.25699311	22	7618029	22	F
00090449	0.46145781	0.42513281	0.42513281	0.42513281	0.42513281	0.42513281	0.42513281	0.42513281	0.46145781	0.42513281	0.42513281	0.42513281	22	7618029	22	F
00463095	0.20077761	0.17458018	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	0.20077761	22	7618029	22	F
00512676	0.24858771	0.29740651	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	0.24858771	22	7618029	22	F
00819637	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	0.40428169	22	7618029	22	F
00317697	0.10079059	0.18250591	0.14735934	0.19182063	0.1870096	0.2138162	0.2138162	0.2138162	0.10079059	0.18250591	0.14735934	0.19182063	22	7618029	22	F
005146330	0.21074664	0.17391237	0.21137376	0.25831174	0.26211577	0.23415899	0.23415899	0.23415899	0.21074664	0.17391237	0.21137376	0.25831174	22	7618029	22	F
00374057	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	0.05640531	22	7618029	22	F
00092904	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	0.52097634	22	7618029	22	F
005146330	0.21074664	0.17391237	0.21137376	0.25831174	0.26211577	0.23415899	0.23415899	0.23415899	0.21074664	0.17391237	0.21137376	0.25831174	22	7618029	22	F
00897012	0.53992488	0.50020247	0.5475471	0.55923805	0.59886199	0.57991252	0.57991252	0.57991252	0.53992488	0.50020247	0.5475471	0.55923805	22	7618029	22	F

02734791	0.20789007	0.34973853	0.2422238	0.2891545	0.3085746	0.21509496	0.27520103	0.37676765	0.20053427	0.77345147	-3.7921483	2	F	0665284	REPOA	NM 12558	Body	chrt:1059442	S	Shore
02800776	0.4801331	0.2332613	0.4744228	0.42995126	0.4048291	0.4473909	0.03518875	0.42512316	0.6052179	0.0595407	0.6185992	4	F	16994610	LEPPLRPL	NM 12558	Body	chrt:1059442	S	Shore
02807767	0.4048003	0.4749488	0.475134	0.5452052	0.4805851	0.5202303	0.04588781	0.34494294	0.0007127	0.7888049	-3.2060966	18	R	2476543	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02807767	0.4048003	0.4749488	0.475134	0.5452052	0.4805851	0.5202303	0.04588781	0.34494294	0.0007127	0.7888049	-3.2060966	18	R	2476543	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.0007123	0.77345147	4	F	10339380	CHSTY	NM 03142	TSS1500	chrt:1047648	S	Shore
02817205	0.2192821	0.2041803	0.2865269	0.2891913	0.3001073	0.2744255	0.03212389	0.2788494	0.47394747	0.00										

[illegible]

q09992309	0.592350103	0.544825598	0.648101266	0.548979512	0.347354138	0.495188454	-0.13261966	0.528782493	-2.59062346	0.036314886	0.817750792	-4.48436982	7	16223374	7	R	ISPD,ISPD	NM	00110143	Body,Body
q15180082	0.681047628	0.54238957	0.709508812	0.4380011119	0.06485914	0.059947335	-0.45671355	0.415958563	-4.4772361	0.003151179	0.773451347	-1.89501961	6	259486542	6	F	TAGAP,TAGAP	NM	1521233N	TSS1500,TSS1500,TSS1500

#	Chr	Start	End	Length	n	ID	Class	990479624	990479624	990479624	990479624	990479624	990479624	Gene.Symbol	dM	p	p.adj	Chromatin state in HMEC cells	Direction	Diff.methylation
1	1	155294935	155295267	333	4	806	gene;tss;islan	0.3337	0.3165	0.3147	0.3198	0.3005	0.3069	FDPS; RUSC1; RU	0.735	0.92	0.999	active promoter	Hypo	-0.012566667
3	19	13365923	13366101	179	4	19914	gene;island	0.8415	0.8263	0.8264	0.8304	0.8448	0.8753	CACNA1A (margin	0.58	0.928	0.999	heterochromatin	Hyper	-0.019866667
4	18	67872746	67872919	174	4	19159	gene	0.0975	0.0799	0.064	0.0678	0.0656	0.0609	RTTN (margin: R1	0.559	0.287	0.999	active promoter	Hypo	-0.0157
6 X	5	152938750	152939907	158	4	41718	tss	0.3389	0.4273	0.4062	0.3732	0.3248	0.3704	RN75L687P; PNC	0.514	0.598	0.999	weak enhancer and weak transcription	Hypo	-0.034666667
9	5	1416793	1417003	211	4	31588	gene;island	0.865	0.883	0.879	0.902	0.8463	0.8868	SLC6A3 (margin:	0.497	0.681	0.999	heterochromatin	Hyper	0.0027
10	3	3885955	3886273	319	4	27685	gene	0.5769	0.5622	0.5504	0.5927	0.5981	0.6194	LRN1; SUMF1 (r	0.496	0.726	0.999	heterochromatin	Hyper	0.040233333
12	13	113299697	113300120	424	5	11947	island	0.8874	0.9093	0.9101	0.9008	0.8769	0.8865	C13orf35 (margin	0.493	0.955	0.999	heterochromatin	Hypo	-0.0142
14	22	36236649	36236760	112	4	26896	gene;tss	0.1561	0.1684	0.1443	0.1485	0.1555	0.2017	RBFOX2 (margin:	0.49	0.758	0.999	strong enhancer	Hyper	0.0123
15	12	25348252	25348358	107	5	9098	gene	0.0617	0.0757	0.071	0.0902	0.0597	0.075	LYRM5; CASC1; K	0.489	0.961	0.999	active promoter	Hyper	0.0055
16	2	183902764	183903098	335	4	22992	gene;island	0.0846	0.0995	0.1031	0.121	0.0995	0.1091	NKCA1P1 (margin:	0.488	0.941	0.999	active promoter	Hyper	0.014133333
17	17	79846205	79846485	281	4	16956	gene;island	0.9395	0.9398	0.9329	0.9267	0.9267	0.9187	RP11-496C9.16; J	0.486	0.825	0.999	transcription elongation	Hypo	-0.031366667
18 X	5	152938698	152939807	210	5	41275	gene;island	0.3471	0.495	0.4909	0.4025	0.3251	0.3986	RN75L687P; PNC	0.481	0.814	0.999	weak enhancer	Hyper	-0.068933333
19	20	3184938	3185276	339	4	25385	gene	0.0706	0.0956	0.0977	0.1055	0.1039	0.097	ITPA; DDRGK1 (r	0.481	0.987	0.999	active promoter	Hyper	0.014166667
20	4	187517794	187518154	361	5	30266	gene	0.9086	0.9186	0.9056	0.9171	0.9107	0.9077	FAT1 (margin: FA	0.479	0.965	0.999	transcription elongation	Hyper	0.0009
21	9	131154899	131155026	128	4	41017	island	0.8899	0.8948	0.8869	0.9001	0.8936	0.919	URML1; RP11-339	0.478	0.304	0.999	weak transcription	Hyper	0.0137
24	1	50513749	50513927	179	4	523	gene	0.9457	0.9455	0.9445	0.9407	0.9412	0.9336	ELAVL4 (margin:	0.473	0.906	0.999	poised promoter	Hyper	-0.006733333
25	7	23571311	23571645	335	5	36976	gene	0.0753	0.0705	0.0721	0.0685	0.0496	0.0642	TRA2A (margin: 1	0.473	0.936	0.999	active promoter	Hypo	-0.011866667
27	6	32138723	32138839	117	5	34121	gene;island	0.8775	0.8982	0.8885	0.8913	0.8791	0.8763	PPT2; PPT2-EGLF	0.467	0.929	0.999	transcription elongation	Hypo	-0.005833333
29	2	112812621	112812775	155	6	23614	tss	0.1282	0.1447	0.1307	0.1251	0.1033	0.1289	TMEM87B (rf	0.464	0.357	0.999	weak promoter	Hypo	-0.015433333
31	1	27986306	27986807	502	5	2929	island	0.0883	0.0983	0.1054	0.0893	0.0895	0.0952	RP11-288L9.4; Rg	0.46	0.835	0.999	weak promoter	Hypo	-0.006
32	1	1248233	1248492	260	4	30	gene;island	0.8317	0.8654	0.8251	0.8496	0.8346	0.8081	PUSL1; ACAP3; C	0.457	0.814	0.999	transcription elongation	Hyper	-0.009966667
33	17	21194718	21194806	89	4	16515	gene	0.8882	0.9079	0.8948	0.9159	0.896	0.9155	MAP2K3 (margin	0.457	0.534	0.999	transcription elongation	Hyper	0.012166667
34	4	118006747	118006832	86	6	36068	tss	0.5244	0.5074	0.5646	0.5254	0.4965	0.5118	TRAM1L1 (margin	0.457	0.334	0.999	weak promoter	Hypo	-0.0209
35	8	80942046	80942378	333	4	39413	gene;island	0.1042	0.086	0.0877	0.0873	0.0846	0.0731	MRP528; TPDS2;	0.456	0.831	0.999	active promoter	Hypo	-0.010966667
36	1	9324688	9325015	328	5	201	gene;island	0.8733	0.8619	0.864	0.8938	0.8602	0.8568	HBP1 (margin: H	0.455	0.922	0.999	weak transcription	Hyper	0.003866667
38	15	101084442	101084565	124	4	13527	gene;island	0.9135	0.9169	0.92	0.9212	0.9069	0.9255	CERS5; RP11-526	0.45	0.948	0.999	heterochromatin	Hyper	0.010166667
42	12	66123127	66123496	370	4	10615	island	0.7677	0.7897	0.8121	0.7954	0.8055	0.8236	(margin: RPSAP5;	0.443	0.912	0.999	weak promoter	Hypo	-0.018333333
44	17	29886672	29888900	219	4	17279	tss	0.967	0.9667	0.9656	0.9601	0.958	0.9557	MR193A; RNUG	0.441	0.786	0.999	weak promoter	Hypo	-0.0085
48	11	77300486	77300650	75	5	7596	tss	0.0927	0.107	0.0923	0.0859	0.0795	0.0904	AQP11; CLNS1A;	0.438	0.994	0.999	weak promoter	Hypo	-0.012066667
49	13	113474153	113474143	261	5	11273	gene;island	0.8901	0.8635	0.8823	0.8762	0.8568	0.8803	ATP11A (margin:	0.437	0.989	0.999	weak transcription	Hypo	-0.007533333
50	5	65222276	65222365	90	4	32148	tss	0.073	0.0961	0.0723	0.0692	0.0512	0.071	ERBB2IP; CTD-20	0.437	0.495	0.999	active promoter	Hypo	-0.007666667
51	5	1218507	1218653	147	4	31577	gene;island	0.8887	0.8777	0.915	0.8958	0.8847	0.8696	SLC19A9; SLC6A1	0.435	0.967	0.999	heterochromatin	Hyper	-0.013766667
52	6	80657412	80657555	144	7	35139	tss;island	0.1027	0.114	0.1042	0.1062	0.1143	0.08	ELOV1; GAPDH	0.434	0.954	0.999	weak promoter	Hypo	-0.0068
54	6	32073324	32073700	377	5	34101	gene	0.8385	0.8416	0.8625	0.8815	0.8666	0.878	TXNB; ATF6B (mi	0.433	0.963	0.999	repressed	Hyper	0.027833333
56	2	108402293	10840362	400	5	22859	gene	0.0679	0.0672	0.0814	0.0782	0.0624	0.0761	RANBP2; CDC11	0.43	0.991	0.999	active promoter	Hyper	6.666667E-05
58	16	19896246	19896644	399	4	15145	tss	0.1745	0.1737	0.1666	0.1694	0.1715	0.136	AC134300.1; GP	0.43	0.977	0.999	weak promoter	Hypo	-0.0193
60	10	27541298	27541418	121	4	5005	tss	0.8902	0.8916	0.9007	0.8841	0.8651	0.8907	RP11-85G18.6; A	0.428	0.901	0.999	weak promoter	Hypo	-0.0142
61	14	101438430	101438663	234	4	12638	tss	0.8365	0.8496	0.8565	0.8689	0.8498	0.8268	ALSD1708.8; SNO	0.428	0.793	0.999	weak enhancer	Hyper	0.000966667
62	13	113832355	113832595	241	4	11296	gene	0.5763	0.5981	0.5896	0.5613	0.5615	0.5532	PROZ; PCID2 (ma	0.427	0.926	0.999	transcription elongation	Hypo	-0.029333333
63	15	81294134	81294324	191	4	13471	gene;island	0.113	0.0991	0.1037	0.1056	0.0959	0.1088	MESDC1; C15orf	0.427	0.619	0.999	active promoter	Hypo	-0.018133333
64	12	85430123	85430336	214	7	9246	gene	0.1151	0.1075	0.1049	0.1087	0.1072	0.1431	LRIO1; TSPAN1	0.426	0.816	0.999	active promoter	Hyper	0.0105
68	16	85670135	85670348	214	4	14901	gene;island	0.8666	0.8708	0.8788	0.8575	0.85	0.852	GSE1 (margin: G5	0.423	0.97	0.999	weak enhancer	Hypo	-0.0189
69	4	81186795	81187198	404	5	30510	tss;island	0.0633	0.0755	0.0702	0.0752	0.0806	0.0801	FGF5 (margin: FG	0.422	0.66	0.999	active promoter	Hyper	0.008966667
70	16	85242446	85242716	271	4	16177	island	0.8999	0.8883	0.906	0.9133	0.9001	0.9137	(margin: CTC-786	0.422	0.723	0.999	weak transcription	Hyper	0.010966667
72	10	13497305	13497396	192	5	5458	tss;island	0.1467	0.1633	0.1723	0.1471	0.1636	0.149	SLC16A1 (margin: 1	0.421	0.841	0.999	poised promoter	Hypo	-0.018333333
73	10	133000130	133000471	342	6	4846	gene	0.5937	0.6187	0.6266	0.6487	0.6572	0.6396	TCERG1L (margin:	0.42	0.977	0.999	heterochromatin	Hyper	0.038833333
74	19	45281006	45281287	282	5	22152	island	0.0487	0.0588	0.0577	0.0606	0.0755	0.077	CBLC (margin: BC	0.419	0.999	0.999	weak enhancer	Hyper	0.015966667
76	14	21152202	21152358	157	4	12298	tss	0.1015	0.0807	0.1029	0.1015	0.1098	0.1033	V_RNA; RNASEA;	0.418	0.99	0.999	active promoter	Hyper	0.009833333
77	11	77300486	77300763	278	7	8696	island	0.0897	0.0886	0.0881	0.0809	0.0659	0.0671	AQP11; CLNS1A;	0.417	0.979	0.999	weak promoter	Hypo	-0.0175
78	17	7893344	7893701	358	4	18011	island	0.0653	0.0572	0.0534	0.0612	0.0679	0.0686	(margin: GUCY2D	0.417	0.985	0.999	weak promoter	Hyper	0.007266667
79	16	89163343	89163800	458	5	14952	gene;island	0.911	0.9163	0.9249	0.9233	0.9267	0.9185	ACSF3 (margin: A	0.417	0.997	0.999	weak transcription	Hyper	0.005433333
81	2	220306470	220306674	205	4	23073	gene;island	0.0449	0.0563	0.0506	0.0503	0.0522	0.0669	SPEG (margin: D	0.415	0.899	0.999	strong enhancer	Hyper	-0.008666667
82	5	86563744	86564119	376	6	32212	tss;island	0.0948	0.1137	0.1182	0.1337	0.1268	0.1389	RASA1 (margin: F	0.415	0.68	0.999	active promoter	Hyper	0.024233333
85	16	2509755	2510115	361	5	15630	island	0.1107	0.1159	0.1232	0.1311	0.1109	0.1162	CCNF; C16orf59;	0.414	0.983	0.999	weak promoter	Hyper	0.0028
86	17	29886672	29887040	378	5	18219	island	0.9664	0.9646	0.9606	0.9576	0.9534	0.9557	MR193A; RNUG	0.414	0.949	0.999	weak promoter	Hypo	-0.0083
88	4	41694736	41694847	112	4	30041	gene	0.2387	0.2549	0.2473	0.2156	0.1895	0.234	UMC1H; RP11-22	0.414	0.95	0.999	heterochromatin	Hypo	-0.0306
90	6	36562212	36562537	326	4	34370	gene	0.0825	0.0698	0.0825	0.0736	0.0685	0.0619	SRSF3 (margin: S	0.414	0.888	0.999	active promoter	Hypo	-0.010266667
91	11	43601969	43602179	211	4	7264	tss;island	0.8309	0.8084	0.8024	0.8192	0.8274	0.8232							