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Gene expression signatures affected by alcohol-induced DNA methylomic deregulation in human embryonic stem cells

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Abstract

Stem cells, especially human embryonic stem cells (hESCs), are useful models to study molecular mechanisms of human disorders that originate during gestation. Alcohol (ethanol, EtOH) consumption during pregnancy causes a variety of prenatal and postnatal disorders collectively referred to as fetal alcohol spectrum disorders (FASDs). To better understand the molecular events leading to FASDs, we performed a genome-wide analysis of EtOH's effects on the maintenance and differentiation of hESCs in culture. Gene Co-expression Network Analysis showed significant alterations in gene profiles of EtOH-treated differentiated or undifferentiated hESCs, particularly those associated with molecular pathways for metabolic processes, oxidative stress, and neuronal properties of stem cells. A genome-wide DNA methylome analysis revealed widespread EtOH-induced alterations with significant hypermethylation of many regions of chromosomes.

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Contribution Statement

O.K., J.J.K. and *Y.K.* participated in designing experiments, execution of described works, statistical analysis, interpretation of data and writing the manuscript. *H.-S. K., M. H., O. E., C.L. C.V.* and *T.T.* contributed to the execution of experiments or writing the manuscript.

S.H. provided statistical consultation and bioinformatics support. *I.S.* participated in interpretation of data and writing the manuscript.

Disclosure Declaration

The authors do not have any conflicts of interest.

Undifferentiated hESCs were more vulnerable to EtOH's effect than their differentiated counterparts, with methylation on the promoter regions of chromosomes 2, 16 and 18 in undifferentiated hESCs most affected by EtOH exposure. Combined transcriptomic and DNA methylomic analysis produced a list of differentiation-related genes dysregulated by EtOH-induced DNA methylation changes, which likely play a role in EtOH-induced decreases in hESC pluripotency. DNA sequence motif analysis of genes epigenetically altered by EtOH identified major motifs representing potential binding sites for transcription factors. These findings should help in deciphering the precise mechanisms of alcohol-induced teratogenesis.

Keywords

alcohol; hESCs; epigenetics; WGCNA; EtOH; methylation; potency; differentiation

Introduction

While moderate alcohol consumption by adults has proven health benefits (French and Zavala, 2007; Gunzerath et al., 2004), excessive alcohol consumption has numerous detrimental effects, making it the 3rd leading cause of the global burden of injury and disease (Lim et al., 2012). Moreover, even moderate alcohol consumption during pregnancy is known to cause fetal alcohol spectrum disorders (FASDs), collectively the largest preventable set of birth defects (May et al., 2009; Riley et al., 2011). FASDs are characterized by prenatal and postnatal growth restriction, craniofacial dysmorphology, and structural/functional abnormalities of the central nervous system (CNS) (Hoyme et al., 2005). The severity of the defects depends on pregnant mothers' alcohol (ethanol, EtOH) drinking patterns and doses. For example, many women keep drinking until they realize they are pregnant (4-6 weeks), and some of them quit or decrease their alcohol use only by mid-pregnancy (Day et al., 1989; Floyd et al., 2005; Floyd et al., 2009) therefore many fetuses are exposed to EtOH during early stages of pregnancy. EtOH readily crosses the placenta; consequently, peak fetal blood EtOH levels are similar to the mother (Thomas and Riley, 1998). Although EtOH clearance is increased in pregnancy (Nava-Ocampo et al., 2004; Shankar et al., 2007), EtOH elimination capacity of the fetus is low, particularly in the early stages of pregnancy (Pikkarainen, 1971), and EtOH remains trapped in the amniotic fluid leading to reabsorption by the fetus, thereby prolonging exposure time (Brien et al., 1983; Nava-Ocampo et al., 2004).

Epigenetic modifications, DNA methylation in particular, regulate key developmental processes, including germ cell imprinting, stem cell maintenance/differentiation, and play a crucial role in the early periods of embryogenesis (Bartolomei, 2003; Kiefer, 2007; Kondo, 2006; Surani et al., 2007). DNA methylation is also a fundamental aspect of programmed fetal development, determining cell fate, pattern formation, terminal differentiation and maintenance of cellular memory required for developmental stability (Cavalli, 2006; Kafri et al., 1992). Moreover, aberrant epigenetic changes in response to environmental stimuli have been shown to contribute to developmental disorders (Zhao et al., 2007).

Several hypotheses involving alcohol (ethanol, EtOH)-induced changes in genetic and epigenetic regulation of cells as possible molecular mechanisms of FASDs have been recently advanced (Haycock, 2009; Haycock and Ramsay, 2009; Kleiber et al., 2012; Luo, 2009; Ramsay, 2010; Zeisel, 2011). However, the precise mechanisms by which EtOH alters the transcriptional landscape are still largely unknown. In addition, EtOH dose-dependently influences the molecular, cellular, and physiological regulation of adult stem cells which likely contributes to the deleterious consequences of excessive alcohol consumption in adults (Crews et al., 2003; Crews and Nixon, 2003; Nixon et al., 2010; Roitbak et al., 2011).

Human stem cells may serve as useful models for delineating the molecular effects of EtOH, especially given the ethical issues of administering alcohol to pregnant women. Alcohol researchers have already taken advantage of these models by observing the consequences of EtOH administration on stem cell differentiation (Garic et al., 2011; Miranda, 2012; Nash et al., 2012; Palmer et al., 2012; Vangipuram and Lyman, 2012). However, genome and methylome-wide studies of EtOH's effects on hESCs have not been reported. Here we show that EtOH can induce DNA methylomic changes in hESCs that may have a significant impact on gene regulatory mechanisms potentially involved in stem cell maintenance and differentiation, and by extension, in proper embryo development processes.

Material and Methods

Cell culture

Human embryonic stem cell H1 and H9 were obtained from UCLA Broad Stem Cell Research Center through license agreement with WiCell Research Institute. Cells were initially grown and propagated in hESC growth medium- Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F12 (DMEM/F12) supplemented with 20% Knockout Serum Replacement (KSR), 1 mM glutamate, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and 5 ng/ml basic fibroblast growth factor (bFGF) with mitotically inactivated mouse embryonic fibroblast (MEF) feeder layer. Before treatment, cells were transferred to mTeSR1 medium on matrigel-coated 100 mm plates without feeder cells using standard conditions (Stem Cell Technologies, Vancouver, BC, Canada) (Xu et al., 2001).

Embryoid body (EB) formation

To induce differentiation of hESCs, cells cultured in mTeSR1 medium were dissociated by using Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and washed three times with plain DMEM/F12 medium. Harvested cells were resuspended in hESC growth medium without bFGF supplement and transferred to ultra-low attachment plates to form embryoid bodies (Corning, Inc., Corning, NY). Aggregated embryoid bodies were kept for designated time period up to 10 days with daily media change.

Ethanol treatment

Cultured cells were treated with different concentrations of EtOH (20 or 50 mM) for 24 or 48 hrs. These EtOH concentrations were chosen for their physiological relevance, 20 mM is equivalent to DUI level and 50 mM falls within levels measured in alcoholics (Jones and Sternebring, 1992). The 24 or 48 hrs exposure times were chosen to mimic the likely

prolonged exposure to maternal EtOH, based on the poor EtOH elimination capacity of the fetus during early development (Pikkarainen, 1971). Calculated volume of absolute EtOH (Fisher Scientific, Pittsburg, PA) was diluted in culture media and cells were fed daily with fresh media. Analysis was done in biological duplicates. In some experiments, cells were treated with 1 μM of 5-azacytidine (Sigma, St. Louis, MO) simultaneously with 20 mM EtOH for 24 hrs.

Gene expression microarray

Total RNA was prepared by using RNeasy kit (Invitrogen, Carlsbad, CA). Samples were prepared in biological triplicates. Equal amount from each sample was subjected to biotinylation using BioArray High Yield RNA Transcript Labeling System (Enzo Life Sciences, Farmingdale, NY, USA). Equal amount cRNA from each sample was labeled, purified and fragmented by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). Following the manufacturer's protocols, the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) was applied for gene expression analysis (UCLA Microarray Core Facility).

DNA methylation analysis

Total genomic DNA was isolated and 200 ng of DNA was subjected to methylated DNA quantification assay by using MethylFlash methylated DNA quantification kit (Epigentek Inc., Brooklyn, NY). % 5-methyCytosine was quantified against a positive standard provided. Alternatively, total genomic DNA was isolated and processed for genome-wide DNA methylation sequencing analysis by Zymo Research (Irvine, CA). Briefly, DNA samples were fragmented and end-modified to add an adaptor. After size selection, bisulfite conversion was performed and amplification was done for library construction. Next-generation bisulfite sequencing was done to obtain raw data. After sequence quality check, bioinformatics processing was performed.

Weighted Gene Co-expression Network Analysis (WGCNA) and network construction

WGCNA was performed by following the tutorial written by Langfelder and Horvath (Langfelder and Horvath, 2008). Co-expression networks were built using the R program. Clusters of genes that behaved similarly were grouped into different color modules. Modules were annotated by Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Bioinformatics Microarray Tools (Huang da et al., 2009). Pathway analysis was done by Kyoto Encyclopedia of Genes and Genomes (KEGG). HIVE 0.0.11 was downloaded and ran on Mac OS X 10.7.4. Edges and nodes were normalized to uploaded data. STAMP motif analysis was performed according to the tutorial provided by authors (Mahony et al., 2007; Mahony and Benos, 2007). Top five sequence matches were output in TRANSFAC Transcription Factor Binding Sites database (v.1.1.3).

Quantitative RT-PCR

RNA was purified using the RNeasy Kit. cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). For the PCR reactions, 2X Roche LightCycler 480 SYBR Green I Master Mix was used and analysis was done with the

LightCycler 480 RT-PCR machine. Reactions were done in triplicate using 0.4 μL of cDNA (out of 40 μL of cDNA from 2 μg of total RNA) as a template in a 10 μL reaction volume. Each experiment was repeated at least three times.

Immunofluorescence (IF) analysis

Cells were fixed in 100% methanol for 15 min at room temperature. For staining, samples were permeabilized for 15 min in freshly prepared IF staining solution (PBS containing 0.02% saponin, 1% bovine serum albumin, and 0.05% sodium azide, 0.2% Triton X-100). Samples were then incubated in a 37°C water bath for 1h with a primary antibody diluted in IF solution. Antibodies in ES characterization kit were purchased from EMD-Millipore Chemicals (Billerica, MA) and used in 1:50 dilution as recommended by the manufacturer. The same isotype Ig was used as background control. Samples were transferred to a 1:500 dilution of goat anti-mouse IgG rhodamine (Thermo Scientific, Waltham, MA) in IF solution and incubated for in 37°C water bath for 1h. Processed samples were mounted on a glass slide with mounting medium with DAPI (Vectashield, Burlingame, CA) and visualized with an inverted light microscope (Olympus IX81 and CellSens Dimension software, Center Valley, PA).

Statistical analysis

Group differences were evaluated by unpaired Student's t-test or one-way analysis of variance (ANOVA), where appropriate. Fisher Exact test was used to measure the gene-enrichment in annotation terms. E-value comparing common motif and associated transcriptional factors was calculated based on the Wasserman and Sandelin method (Wasserman and Sandelin, 2004). *P*-values within WGCNA were calculated based on a regression-based *p*-value for assessing the statistical significance between the matrix X and the sample trait T, where matrix X is ‘network nodes times column indices’ of all genes (X = n × m).

Data Access

Gene expression data have been submitted to the NCBI Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45036) under accession number GSE45036.

Results

Transcriptomic profiling of EtOH effects on hESCs

A genome-wide microarray (Affymetrix Human Genome U133 Plus 2.0 Array platform) was used to generate gene expression data for hESCs in order to compare the concentration-dependent effects of EtOH (0, 20 or 50 mM) on two different hESC lines, H1 and H9 (Fig. 1). The H1 and H9 cell lines were derived from male and female fetuses, respectively (Thomson et al., 1998). To find co-expression modules related to EtOH treatment and differentiation status we used the Weighted Gene Co-Expression Network Analysis (WGCNA) (Fig. 1A) (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). We also examined the molecular signatures of differentiation from undifferentiated stem cells to embryonic bodies (EBs) in the absence of EtOH treatment. Comparisons of H1 and H9

heatmaps from representative modules are shown (Fig. 1B). We selected three representative modules that showed the same pattern of expression changes after EtOH treatment in both H1 and H9 hESCs. This is to demonstrate an example of EtOH-induced alterations in gene signatures independent of cell lines. Volcano plots (Fig. 1D) demonstrated similarities in EtOH's effects on expression of genes in the two hESC lines, however, the changes were consistently greater in the H9 compared to the H1 line. Based on these data, we subsequently focused on molecular analysis of differentiation and EtOH application in the H9 hESC line. We also chose to focus on the lower concentration of EtOH (20 mM) because a similar blood level of EtOH, which is just above the driving limit (Jones and Sternebring, 1992), is easily achieved after excessive drinking by women (Bedford et al., 2006). The 24 hr duration of EtOH exposure was chosen because in addition to the low EtOH elimination capacity of the fetus, particularly in the early stages of pregnancy (Pikkarainen, 1971), EtOH remains trapped in the amniotic fluid leading to reabsorption by the fetus and prolonging exposure time (Brien et al., 1983).

Using unsupervised hierarchical clustering with WGCNA consensus analysis we found twenty-four distinct modules (Fig. 1C). Note that two of the representative modules, light-yellow and turquoise, showed consistent gene expression correlations under three different conditions (during differentiation without EtOH treatment, EtOH treatment in undifferentiated hESCs and EtOH treatment in differentiated EBs) (Fig. 1C, indicated with the asterisks). Thus, the light-yellow module showed significant repression in differentiation, whereas a turquoise module exhibited the opposite, suggesting an essential role of genes in these modules during differentiation and also in EtOH-induced molecular alterations. The consensus module trait relationships are represented as correlation values and significance (Fig. S1). Furthermore, to delineate the top genes within these modules, we made a Volcano plot comparing significance and fold change (Fig. 1D). We analyzed natural clusters on the fourteen significant modules using the R package cluster Profiler (Fig. 1E) (Yu et al., 2012). It revealed that the modules formed distinct ontological clusters with little to no overlap. We also identified unique pathways for each module using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (Fig. 1F) (Kanehisa et al., 2012). These pathways include gene regulation, metabolic pathways, neuronal activity and axon guidance as well as pathways involved with epigenetic regulation. Notably, we found that the turquoise module showed most significant association with methyltransferase activity and the light-yellow module showed most significant association with RNA polymerase II activity. This suggests that EtOH can potentially affect transcriptional activity and regulation in hESCs.

Interactions between genes based on co-expression relationships were established by forming a gene network, and these connections are represented by a HIVE plot, as a traditional gene network, and a compressed hub gene network (Fig. 1G-I) (Krzywinski et al., 2012). With highly connected intramodular hub genes centrally located in the module, the connections to the outer rim allow for further elucidating the biological meaning of EtOH related modules. For example, we have identified two top hub genes, *SCUBE3* and *SLC22A5* (presented as red dots in Fig. 1G-I), in a representation of genes downregulated in H9 cells upon EtOH treatment and differentiation. The *SLC22A5* gene functions in the

transport of carnitine into the cell. More specifically, SLC22A5 facilitates the function of protein OCTN2, which is embedded in the cell membrane and acts as a transporter for carnitine (Tamai et al., 1998). Carnitine plays an essential role in transporting fatty acid from cytosol into the mitochondrial matrix to be broken down into usable metabolic energy. The *SLC22A5* gene is therefore an important regulator of fatty acid metabolism, and is necessary for the maintenance of skeletal and cardiac muscle function (Steiber et al., 2004). Mutations in the *SLC22A5* gene can cause systemic primary carnitine deficiency (Sonne et al., 2012). Alcohol has the capacity to upregulate genes involved in fatty acid metabolism similar to effects of glucagon when blood sugar is low. SCUBE3 is a secreted and cell-associated glycoprotein that can form either homo or hetero-oligomers that can attach to the cell surface (Wu et al., 2004). It can act either in an endocrine or autocrine/paracrine fashion in regards to cell signaling. *SCUBE3* can be expressed in human osteoblasts (but not in nonosseus tissues), cardiac tissue, and vascular endothelial cells (Wu et al., 2004). When *SCUBE3* was overexpressed in mice, the animals were diagnosed with cardiac hypertrophy (Yang et al., 2007). Thus, both of the top hub genes have some involvement in cardiovascular function.

EtOH-induced genome-wide DNA methylomic alterations in hESCs

To determine the extent of EtOH's epigenetic effects on the molecular regulation in hESCs, we initially measured the effect of EtOH on global DNA methylation. Recent studies have reported changes in methylation status upon EtOH treatment (Sanchez-Alvarez et al., 2013; Zhou et al., 2011). It has been shown that alcohol exposure alters migration and important basic processes of neural stem cells (Zhou et al., 2011). Their studies also showed that alcohol exposure altered the methylation potential of genes that were initially in a quiescent state (Zhou et al., 2011). To follow up on such studies using hESCs, we performed a biochemical assay for DNA methylation, which revealed a significant increase in global DNA methylation after 48 hrs of EtOH (20 or 50 mM) treatment (Fig. 2A). Global DNA methylation was slightly, but not significantly higher with 50 mM compared to 20 mM EtOH treatment. To further study the molecular effect of EtOH on DNA methylomic signatures in hESCs, we performed a genome-wide DNA methylation analysis in undifferentiated or differentiated hESCs with or without EtOH treatment. For these studies, we again used a 24 hr application of 20 mM EtOH to allow for comparisons with the results of the genome-wide transcriptome studies. Examination of DNA methylation changes that were occurring on the promoters around transcription start site (TSS) or CpG islands (CGIs) revealed that EtOH treatment increased global DNA methylation in undifferentiated hESCs (Fig. 2B and C). Thus, plots of relative DNA methylation levels at the TSS and the CGIs after treatment with 20 mM EtOH (*left panels*) revealed EtOH-induced DNA hypermethylation at the TSS and the CGIs. Similarly, aligned probes plot of methylation showed that treatment of undifferentiated hESCs with 20 mM EtOH resulted in an increase in DNA methylation compared to non-treated control (*middle panels*) and log plots for methylation comparing control cells (*x-axis*) vs. cells treated with 20 mM EtOH (*y-axis*) also showed greater level of DNA methylation after EtOH treatment (*right panels*).

To profile EtOH-induced DNA methylation change on individual chromosomes, we calculated the average level of DNA methylation as shown in Figure 3. We also compared

EtOH effects on DNA methylation in undifferentiated hESCs versus EBs. EtOH treatment of undifferentiated hESCs induced increases in the average levels of DNA methylation in the promoter regions and CpG islands of each chromosome (Fig. 3A and 3B, *top row*). The level of DNA methylation changes specific to EtOH treatment in differentiated hESC EBs was also examined (Fig. 3A and 3B, *middle row*). Interestingly, EtOH had a much smaller effect on DNA methylation in differentiated EBs. The extent of EtOH's effect on DNA methylation on each chromosome was evaluated by calculating a fold-change in DNA methylation level after EtOH treatment against untreated controls (Fig. 3, *bottom row*). Undifferentiated hESCs showed higher fold changes in DNA methylation upon EtOH treatment than differentiated hESC EBs. We also found higher fold changes in EtOH-induced DNA methylation in the promoter regions compared to CGIs. Chromosomes 2, 16 and 18 in undifferentiated hESCs showed the highest increases in DNA methylation in the promoter regions compared to other chromosomes.

Although the average level of DNA methylation per chromosome was increased after EtOH treatment, it does not mean that all the genes in the chromosome are affected the same way. To examine the effect of EtOH on the DNA methylation change in undifferentiated hESCs at the gene level, we calculated the changes in % of genes that are hyper- or hypomethylated upon EtOH treatment, after normalization against untreated cells. Using this type of analysis we observed that EtOH treatment induced overall increases in both hyper- and hypomethylation of the promoter regions in each and all chromosomes (Fig. S2). Interestingly, the % of genes hyper or hypomethylated by EtOH at the promoter regions was relatively similar, whereas a much higher % of genes were hypomethylated at CGIs compared to the % of hypermethylated genes (Fig. S2). Although this first appears counterintuitive to the observed global increases in methylation at promoter regions and CGIs (Figs. 2 & 3), these data demonstrate that while a greater number of genes were hypomethylated, the increases in methylation of a smaller number of genes on individual chromosomes was more than sufficient to produce overall increases in DNA methylation status. These data also underscored the differential effect of EtOH exposure on the methylation of individual hESC genes and, in turn, suggested likely differential influence of EtOH on transcriptional regulation of genes on different chromosomes.

Molecular correlations between EtOH-induced DNA methylomic and transcriptomic alterations in hESCs

In order to relate EtOH-induced methylomic alterations to transcriptional changes we correlated EtOH-induced methylation changes at the promoter regions (Fig. 4 A-C) and the CpG islands (Fig. 4D-F) to the changes in gene expression under three conditions: (a) differentiation (Fig. 4A and D), (b) EtOH treatment of undifferentiated hESCs (Fig. 4B and E), and (c) EtOH treatment of differentiated EBs (Fig. 4C and F). Cutoff was made by β value change >0.5 in DNA methylation and >2-fold in gene expression. From these correlations, we observed that the majority of expression and methylation correlations occurred not only during differentiation alone but also following EtOH treatment of both undifferentiated hESCs and EBs (Fig. 4G).

To determine if EtOH-induced hypermethylation could account for decreases in gene expression we examined the effect of 5-azacytidine (1 μM) on the expression level of genes that were downregulated upon EtOH treatment (Fig. 4H). We chose four genes (*ACADY4*, *FGF17*, *HOXA1* and *PHOSPHO1*) out of top ten genes from combined analysis that were identified as most significantly altered. Co-administration of EtOH with this demethylating agent overcame the gene silencing effect of EtOH (Fig. 4H), suggesting that the changes in level of these genes are mostly due to DNA methylation events.

Next, we developed a heatmap to represent the methylation changes that are occurring at the promoter and intergenic regions as correlated to expression changes (Fig. 4I). We observe that these methylation changes are concentrated around the promoter and transcriptional start site (Fig. 4J) and are inversely correlated to expression changes (Fig. 4K). However, there are several changes in methylation that are not necessarily correlated to changes in gene expression, suggesting interplay between transcript changes and changes in DNA methylation status beyond the simple inverse correlation between epigenetics and transcription. This may be due in part to the fact that genes involved in methyltransferase activity are changing as observed from our ontological analysis in Figure 1F. Thus, EtOH treatment does not only change the transcriptional profile but also the epigenetic profile, which may have more profound implications if these epigenetic changes are permanent and transmissible.

From the combined data set of EtOH-induced transcriptomic and DNA methylomic alterations as shown in Fig. 4, we have further identified a list of genes that are specifically dysregulated by EtOH-induced DNA methylomic changes (Table 1). Genes that are hypomethylated on CpG islands (or promoter regions) and upregulated in gene expression and genes that are hypermethylated on CpG islands (or promoter regions) and downregulated in gene expression are separately listed (β value >0.5 and >2-fold change in expression).

EtOH-induced downregulation of stemness markers

Previous studies have shown that even a single exposure to EtOH during the pre-implantation period enhanced post-implantation fetal death and resorption and retarded normal embryo development (Padmanabhan and Hameed, 1988). Since the pluripotent hESCs are naturally most abundant during the pre-implantation blastocyst stage we hypothesized that EtOH exposure might affect their pluripotency, which in turn would adversely affect their subsequent differentiation. To address this hypothesis, we first examined the effect of EtOH on hESC maintenance by immunofluorescence analysis for stem cell marker proteins. H9 hESCs were treated with EtOH (20 mM) for 24 hrs and subjected to IF analysis. The results showed that the expression of stemness marker proteins (OCT4, TRA-1-60 and SSEA-3) was visibly reduced with EtOH treatment (Fig. S3A). Since fetal alcohol exposure is known to induce programmed cell death in certain embryonic cell populations (Dunty et al., 2001; Ikonomidou et al., 2000; Kilburn et al., 2006), we also examined EtOH's effects on the levels of an early apoptosis marker Annexin V and found noticeable increases in Annexin V immunoreactivity in hESCs after 20 mM EtOH treatment (Fig. S3A). Based on these immunohistochemical data, we next examined EtOH's effects on

the expression of 6 stemness marker genes by quantitative RT-PCR (Fig. S3A). Treatment with EtOH (20 or 50 mM) resulted in significant downregulation of *DNMT3B*, *NANOG*, *OCT4* and *SOX2*, with the remaining *FOXD3* and *TERT* also showing a trend of decreased expression (Fig. S3B). We then reasoned that if pluripotency markers that maintain self-renewing hESCs were downregulated by EtOH, this is likely to have significant effects on the regulation of "downstream" differentiation markers. Analysis of qRT-PCR data for a set of previously described differentiation markers representative of the mesoderm (*BRACHYURY*, *HAND1*, M*EOX1*), definitive and primitive endoderm (*CDX2*, *EOMES*, *GATA4*, *GATA6*, *SOX7*, *SOX17*, *TBX6*) and ectoderm (*NESTIN*, *PAX6*), respectively (Teo et al., 2011) revealed significant up- or down-regulation of 9 out of these 12 markers with EtOH treatment (Fig. S3C). Based on the above data and the profound effects of EtOH exposure during the blastocyst stage on subsequent embryo development and viability (Padmanabhan and Hameed, 1988), we next focused on the effect of EtOH on differentiation-related gene expression in hESCs. To identify specific genes that may be affected by EtOH during differentiation of hESCs, genes that are differentially regulated during EB formation were compared to genes differentially regulated upon EtOH treatment in undifferentiated hESCs (Fig. 5). From this analysis we have identified a subset of genes significantly altered by EtOH treatment that are potentially involved in differentiation of hESCs into EBs (Orange box, Fig. 5). Due to the figure size limitations, only top 10 genes are illustrated in each panel. The complete list of genes for each panel is shown in the Supplemental Table 1.

Validation of genes differentially regulated by EtOH-induced DNA methylation changes

We next validated genes identified from the combined analysis. Based on the observed decreases in stemness markers (Fig. S3), we continued to focus on molecular signatures affected by EtOH-induced DNA methylation changes that may have significant roles in stem cell maintenance and differentiation process. Therefore, we examined EtOH's effect on the subset of genes that are potentially differentiation-related as shown in Fig. 5. DAVID analysis on the identified top 30 genes was performed to examine their functional implication. We found these genes were mostly associated with neuroactive ligand-receptor interaction, vascular smooth muscle contraction, calcium signaling pathways, and energy metabolism. Energy metabolism and its associated pathways are considered to be important cellular processes in maintaining stem cell self-renewal and potency (Cho et al., 2006; Rafalski et al., 2012; Ramm Sander et al., 2013). For validation, the levels of mRNA for selected genes were assessed by qRT-PCR analysis (Fig. 6). Even though we relied on *GAPDH* as a single reference gene (Carnahan et al., 2013), we found that most of the genes in our experimental set (subset of EtOH-induced differentiation related genes with promoter methylation in the middle panel of Fig. 5) were definitively validated. *C2CD2L*, *GUSBP1* and *TMEM217* were significantly upregulated and demonstrated concentration dependency to EtOH treatment (Fig. 6A). By contrast, *P2RX3*, *PIK3C3*, *SLC12A4* and *SLC25A30* were significantly downregulated (Fig. 6B). Thus, most of the genes subjected to qRT-PCR analysis showed the expected trends of gene expression. Of the downregulated genes, *SLC12A4* is involved in K-Cl transport and participates in cell volume homeostasis and cholesterol metabolism (Adragna et al., 2004; Zhou et al., 2004). The gene is localized to chromosome 16, which we identified as one of the hotspots, (see Fig. 3). *P2RX3* codes for a

ligand-gated cation channel, which is involved responses to extracellular ATP, cation transport, Ca-mediated signal transduction, and behavioral responses to pain (Burnstock, 2007). However, the specific functional role of these genes in EtOH-induced hESC self-renewal and potency remain to be investigated.

Common motif analysis and EtOH-related transcription factor discovery

Subsequently, we studied whether there was any sequence specificity to the genes that were being affected by EtOH treatment (Fig. 7). From the genes that are significantly affected by EtOH (shown in yellow Venn-diagram), we wanted to find a subset of genes that is also related to differentiation (shown in purple Venn-diagram). The common genes between genes affected by EtOH treatment and genes altered during differentiation represent genes that may be involved in differentiation profile due to EtOH treatment (middle in Venn-diagram). The common genes were found in all four conditions, CpG and promoter; as well as increased expression (upregulated) and decreased expression (downregulated). To examine if these common genes affected by EtOH share any sequence motifs for transcription factors, we have performed motif analysis using the regulatory sequence analysis tool (RSAT) (Thomas-Chollier et al., 2012). We discovered two major motifs represented within the genes that are commonly associated with differentiation of hESCs and also EtOH exposure. Thus, these motifs may represent potential binding sites of transcription factors that may play a role in differentiation changes that are occurring in hESCs upon exposure to EtOH. Furthermore, these motifs may be used as predictive signatures for genes affected by EtOH treatment. We used Similarity, Tree-building, and Alignment of DNA Motifs and Profiles (STAMP) motif analysis tool (Mahony and Benos, 2007) to determine what transcription factors could be involved in this process (Fig. 7) (Mahony and Benos, 2007). Most significant motifs and associated transcriptional factors that are correlated to EtOH treatment are shown (Fig. 7). Putting together individual module motif and transcription factor analysis, we found transcription factors well known in stem cell maintenance/differentiation such as the FOX, SMAD and POU family to be implicated, as well as transcription factors involved in hepatogenesis, suggesting alteration of differentiation in the hepatic lineage. Furthermore we performed a consensus analysis to examine possible involvement of these transcription factors in EtOH-induced molecular alterations in hESCs. We compared those genes altered by EtOH treatment against genes altered in hESCs treated with short hairpin RNA targeting *OCT4* (*shOCT4*, obtained from publicly available data sets, GEO accession number: GSE34921). We found the significant modules of alterations specifically associated with the stemness transcription factor and EtOH treatment (Fig. S4). This further suggests that EtOH treatment in hESCs can lead to fundamental alterations in the regulatory network of transcription factors involved in stemness and lineage commitment (Fig. S4).

Discussion

The effect of alcohol on development has been extensively studied in many different animal species (Cudd, 2005). In particular, a single exposure to EtOH during the pre-implantation period was shown to enhance post-implantation fetal death and resorption and to retard normal embryo development (Padmanabhan and Hameed, 1988). In humans, fetal alcohol

exposure (FAE) is also correlated with low birth weight, growth, and morphological abnormalities (Day et al., 1989), and in higher rates of spontaneous abortions (Kline et al., 1980; Windham et al., 1997). Numerous other reports have demonstrated genetic, cellular, and biochemical association of alcohol with teratogenesis (Armant and Saunders, 1996; Goodlett and Horn, 2001; Resnicoff et al., 1994; Wozniak et al., 2004). The wide range of physiological and morphological defects associated with FAE suggests that the etiology of FASDs involve a high degree of cellular and molecular heterogeneity. Gastrulation period is considered to be the most sensitive to teratogenic insult, suggesting that differentiating cells might be especially vulnerable to the teratogenic effects of EtOH (Armant and Saunders, 1996).

Here we examined the effect of EtOH on the pluripotency of undifferentiated hESCs as a model of early FAE that includes the pre-implantation period. Specifically, we demonstrated that a 24 hr low dose (20 mM) EtOH treatment significantly reduced the pluripotency (differentiation potential) of hESCs. While to our knowledge no other hESC studies have addressed this issue, somewhat analogous observations were made with rhesus monkey ESCs, albeit at much higher EtOH concentrations and over a course of 4 weeks (VandeVoort et al., 2011). We also, for the first time, demonstrated overall increases in DNA methylation in hESCs, defined the genetic and epigenetic molecular landscapes affected by low dose EtOH exposure, and identified genome-wide hotspots that could potentially be vulnerable to FAE. Furthermore, we have identified landscapes of molecular networks that are potentially deregulated by EtOH exposure through DNA methylomic alterations. This is in contrast to the recently reported lack of methylation changes after exposure of hESCs to 20 mM EtOH (Krishnamoorthy et al., 2013). Interestingly, their reported selective gene subsets are, for the most part, also unchanged in our study, whereas the much larger set of genes which do show methylation changes in our study are not reported by these authors. Furthermore, numerous studies have linked epigenetic mechanisms as potential regulatory events involved in alcohol teratogenesis (Bielawski et al., 2002; Garro et al., 1991; Haycock, 2009; Kaminen-Ahola et al., 2010). Epigenetic imprinting or genome-wide epigenetic reprogramming has been proposed as a mechanism responsible for alcohol-induced teratogenesis in preimplantation embryos (Haycock, 2009; Haycock and Ramsay, 2009). Interestingly, even paternal or maternal alcohol consumption prior to conception has been shown to result in a wide range of birth defects and fetal abnormalities. It is likely that alcohol-induced epigenetic changes in the gametes or within germ line are responsible for pre-conceptional effects of alcohol (Abel, 2004).

We observed numerous instances where EtOH had a differential effect on the transcriptome and methylome of undifferentiated hESCs compared to EBs, which contain differentiating cells encompassing all somatic cell types (Itskovitz-Eldor et al., 2000) including those of neural and hepatic cell lineages. Recent studies have demonstrated inhibitory effects of pharmacologically relevant EtOH concentrations (20-100 mM) on neuronal differentiation of ESCs and specified neural stem cells (NSCs). Specifically, EtOH was shown to inhibit NSC proliferation with dose-dependent increases in apoptosis (Anthony et al., 2008; Fujita et al., 2008; Talens-Visconti et al., 2011) decrease NSC differentiation (Tateno et al., 2004; Zhou et al., 2011) and divert ESC differentiation from neuroectodermal to mesodermal lineage (Ogony et al., 2013; Sanchez-Alvarez et al., 2013). Others utilizing very high EtOH

concentrations (300-2000 mM) also observed increased apoptosis of mouse blastocysts (Huang et al., 2007) and increased endodermal differentiation of EBs (Mayshar et al., 2011). Another recent study that focused on differentiation of human hepatocytes from EBs demonstrated that a 48 hr EtOH (20 mM) treatment perturbed the differentiation of progenitor cells into hepatocytes (Pal et al., 2012). Given our focus on the early onset of hESC differentiation (2-3 days) versus their longer than 21 days of differentiation, direct comparisons are difficult, nevertheless commonalities include EtOH-induced downregulation of the stem cell transcription marker, *OCT4*, and downregulation of several differentiation markers associated with the formation of definitive endoderm and functional hepatocytes (*GATA6 and SOX17*, see Fig. S3). In addition, we used WCGNA to demonstrate many similarities between selective *OCT4* knockdown (Wang et al., 2012) and EtOH effects on undifferentiated hESCs (Fig. S4). Overall, published literature support our findings of a selective impact of EtOH on differentiating cells compared to undifferentiated stem cells.

Our methylome profiling revealed hotspots of DNA methylation on chromosomes 2, 16 and 18. Association of these chromosomes with alcohol dependence has been reported (Dick et al., 2010; Foroud et al., 1998; Treutlein et al., 2009; Wang et al., 2005). Thus, using earlier findings that chromosome 2, especially 2p14-2q14.3, is associated with phenotypes of alcohol dependence, suicide attempts, and conduct disorder, a systematic screen of SNPs with the three comorbid phenotypes yielded evidence of association with 23 genes, likely contributing to the preponderance of reported linkages with alcohol dependence and related phenotypes across chromosome 2 (Dick et al., 2010). Another study utilized a genome-wide association study, discovering 15 SNPs significantly associated with same allele that had shown expression changes in rat brains after long-term alcohol consumption. In the combined analysis, 2 closely linked intergenic SNPs are located on chromosome region 2q35, which has been implicated in linkage studies for alcohol phenotypes, including the CDH13 and ADH1C genes that have been reported to be associated with alcohol dependence (Treutlein et al., 2009). Linkage analysis also detected loci underlying moderate and severe alcoholism implicating chromosome 16 (Foroud et al., 1998). In other studies, patients with carcinoid tumors had frequent history of alcohol consumption (Wang et al., 2005). Allelic loss of chromosome 11q was present in 21% of tumors, chromosome 16q in 13%, and chromosome 18 in 30% (Wang et al., 2005).

In addition to identifying EtOH-induced global changes in genetic and epigenetic molecular landscapes, we were able to narrow down these changes further to four major potential signaling pathways with help of extensive bioinformatics analyses. To our knowledge, this is the first time that energy metabolism, neuroactive ligand-receptor interaction, vascular smooth muscle contraction, and calcium signaling pathways are implicated in EtOH-induced changes in human embryonic stem cells. We provided strong molecular and bioinformatic evidence that even at a low physiologically relevant dose, EtOH may epigenetically alter key genes such as *SLC12A4*, *P2RX3* and others in glycerophospholipid metabolism and regulation of calcium ion transport and/or calcium-mediated signaling, which ultimately may lead to the significant decrease in self-renewal capacity of hESCs.

Scientific findings presented in this report provide us with novel opportunities that have yet to be explored. Elucidating epigenetic molecular signatures and pathways affected by

ethanol will significantly advance our understanding of the mechanisms of ethanol's effect on human stem cells. Moreover, defining the molecular changes that occur in our stem cell model systems can potentially be used to for designing future clinical applications of stem cells to improve human health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Genome wide analysis (genetic and DNA methylomic) of the effects of alcohol on human embryonic stem cells.
- Identification of potential chromosomal hotspots for epigenetic effects of alcohol in hESCs
- Discovery of molecular networks that are affected by alcohol in hESCs

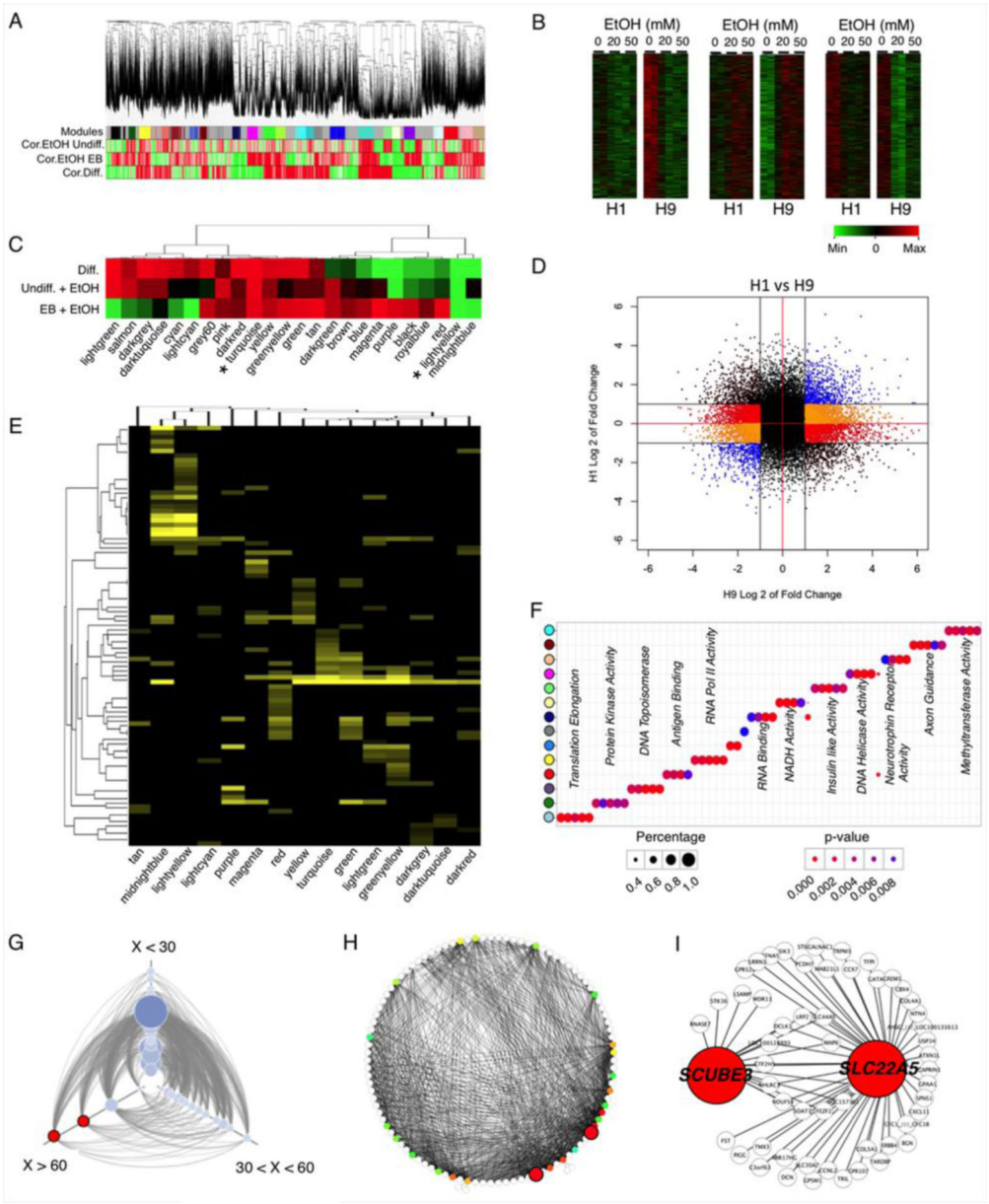


Figure 1. Transcriptomic profiling of gene signatures in EtOH-treated or differentiating hESCs

(A) WGCNA consensus cluster analysis of hESC genes comparing EtOH (20 mM) treatment of hESCs in their undifferentiated or EB states, as well as comparing undifferentiated and differentiated hESCs without EtOH treatment. Correlations of each respective comparison are shown below the module cluster. (B) EtOH dose-response (at 0, 20 and 50 mM) comparison between H1 and H9 cell lines using heatmaps of representative modules. We selected three representative modules that showed the same pattern of expression changes after EtOH treatment in both H1 and H9 hESCs. This is to demonstrate an example of EtOH-induced alterations in gene signatures independent of cell lines. (C) Cluster of the different modules in relation to differentiation and EtOH treatment. Turquoise and light-yellow modules that showed consistent gene expression correlations are indicated by asterisks. (D) A Volcano plot identified the number of the most significantly altered

genes upon EtOH treatment between H1 and H9. (E) The top fourteen modules significantly affected by EtOH were analyzed by R package cluster Profiler and the resulted showed that the modules formed distinct ontological clusters without any overlap. (F) Molecular pathway analysis using KEGG database showed the dot plot of the gene ontology unique to each module of the most affected clusters in (E). Fisher Exact test was adopted to measure the gene-enrichment in annotation terms. When members of two independent groups can fall into one of two mutually exclusive categories, Fisher Exact test was used to determine whether the proportions of those falling into each category differ by group (Huang da et al., 2009). (G) Hive plot of the gene network associated with the turquoise consensus module. Red dots showed two top hub genes- *SCUBE3* and *SLC22A5*. (H) Standard gene network representing the turquoise module. (I) The top hub genes, *SCUBE3* and *SLC22A5*, and implicated genes based on maximal clique centrality.

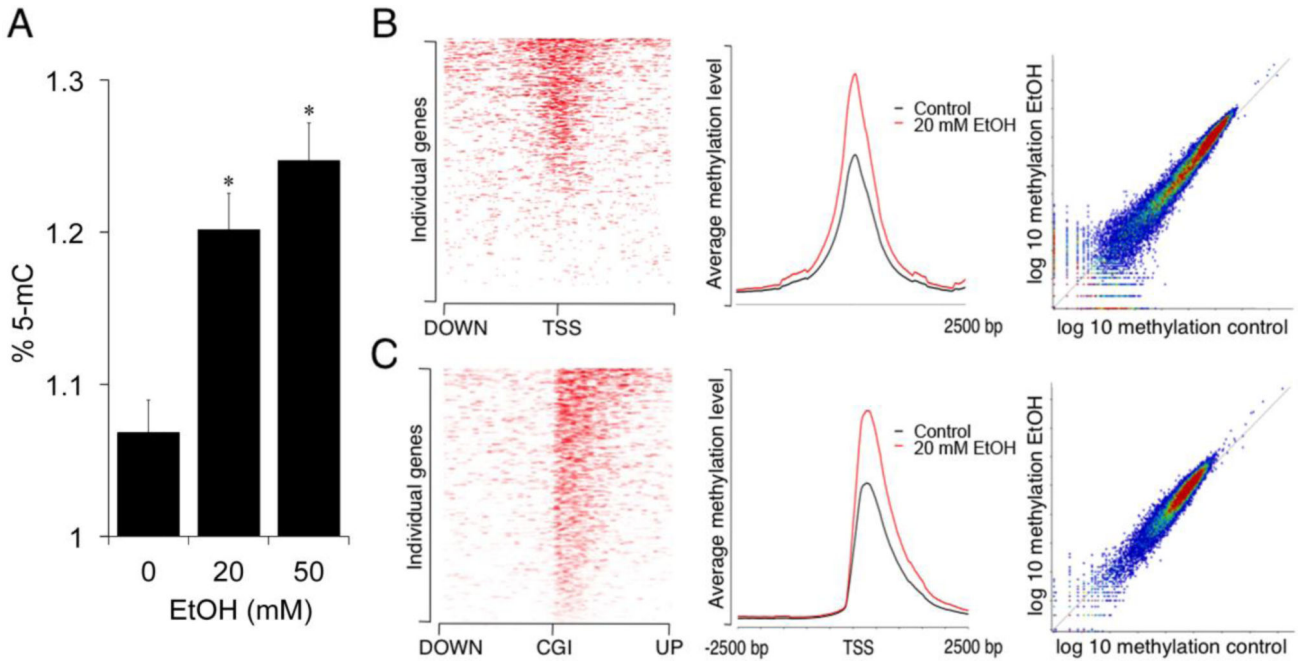


Figure 2. Global DNA methylation changes at the transcription start site (TSS) and CpG islands (CGIs) upon EtOH treatment

(A) EtOH increases DNA methylation in undifferentiated hESCs as measured by changes in % of 5-methylcytosine (5-mC). *, $p < 0.05$ versus 0 mM EtOH (one-way ANOVA). (B) DNA methylation changes after EtOH treatment in undifferentiated hESCs were assessed. Relative DNA methylation levels at the TSS (2.5 kb upstream and downstream) after treatment with 20 mM EtOH were plotted (*left*). It shows the status of DNA hypermethylation at the TSS resulted from EtOH treatment. Aligned probes plot of methylation showed that treatment of undifferentiated hESCs with 20 mM EtOH resulted in an increase in DNA methylation at the TSS compared to non-treated control (*Middle*). The Log plot for methylation at the TSS comparing control cells (*x-axis*) vs. cells treated with 20 mM EtOH (*y-axis*) showed greater level of DNA methylation after EtOH treatment (*right*). (C) DNA methylation changes at CGIs after EtOH treatment were assessed as described in (B). Relative DNA methylation plot at the CGIs after treatment with 20 mM EtOH showed an increase in DNA methylation on the DNA sequences upstream of CGIs (*left*). Aligned probes plot of methylation showed that treatment with 20 mM EtOH resulted in an increase in DNA methylation at the CGIs compared to non-treated control (*Middle*). The Log plot for methylation at the CGIs comparing control cells (*x-axis*) vs. cells treated with 20 mM EtOH (*y-axis*) showed greater level of DNA methylation after EtOH treatment (*right*).

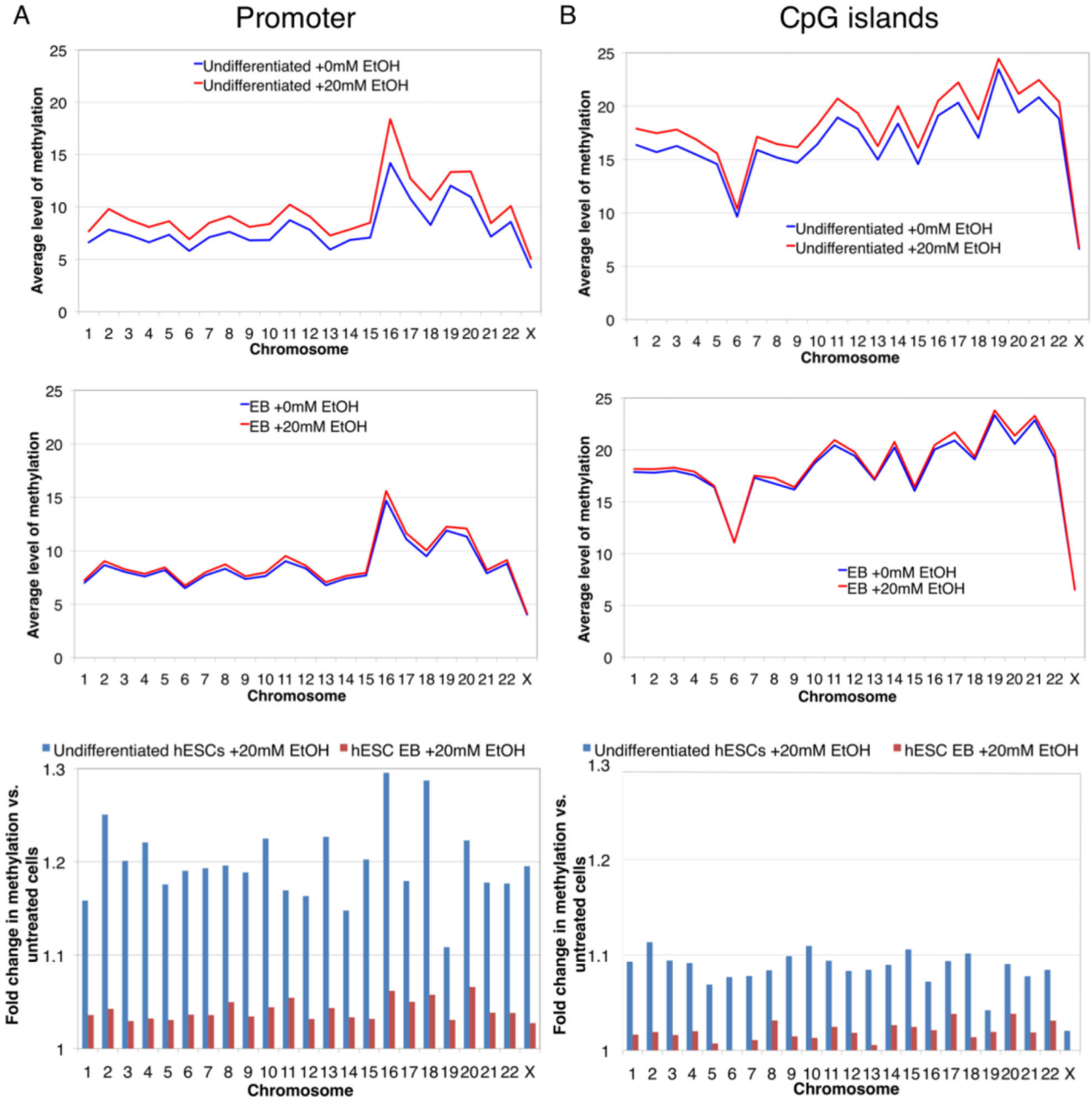


Figure 3. EtOH-induced increases in DNA methylation in undifferentiated hESCs but not in embryoid bodies (EBs)

Comparisons of average relative DNA methylation levels between (A) promoters of genes and (B) CpG islands of genes from undifferentiated hESCs (*top panels*) and hESC EBs (*middle panels*) treated with 0 mM or 20 mM EtOH. Bottom panel shows fold changes in DNA methylation for promoters and CpG islands of genes in EtOH-treated undifferentiated hESCs (*blue bars*) and EBs (*red bars*). Statistical analysis revealed that the EtOH-induced increases in methylation status of Promoter regions (and to a lesser extent CpG islands) were significantly different ($p < 0.05$, unpaired t-test) for undifferentiated hESCs vs. EBs at all 23 chromosomes.

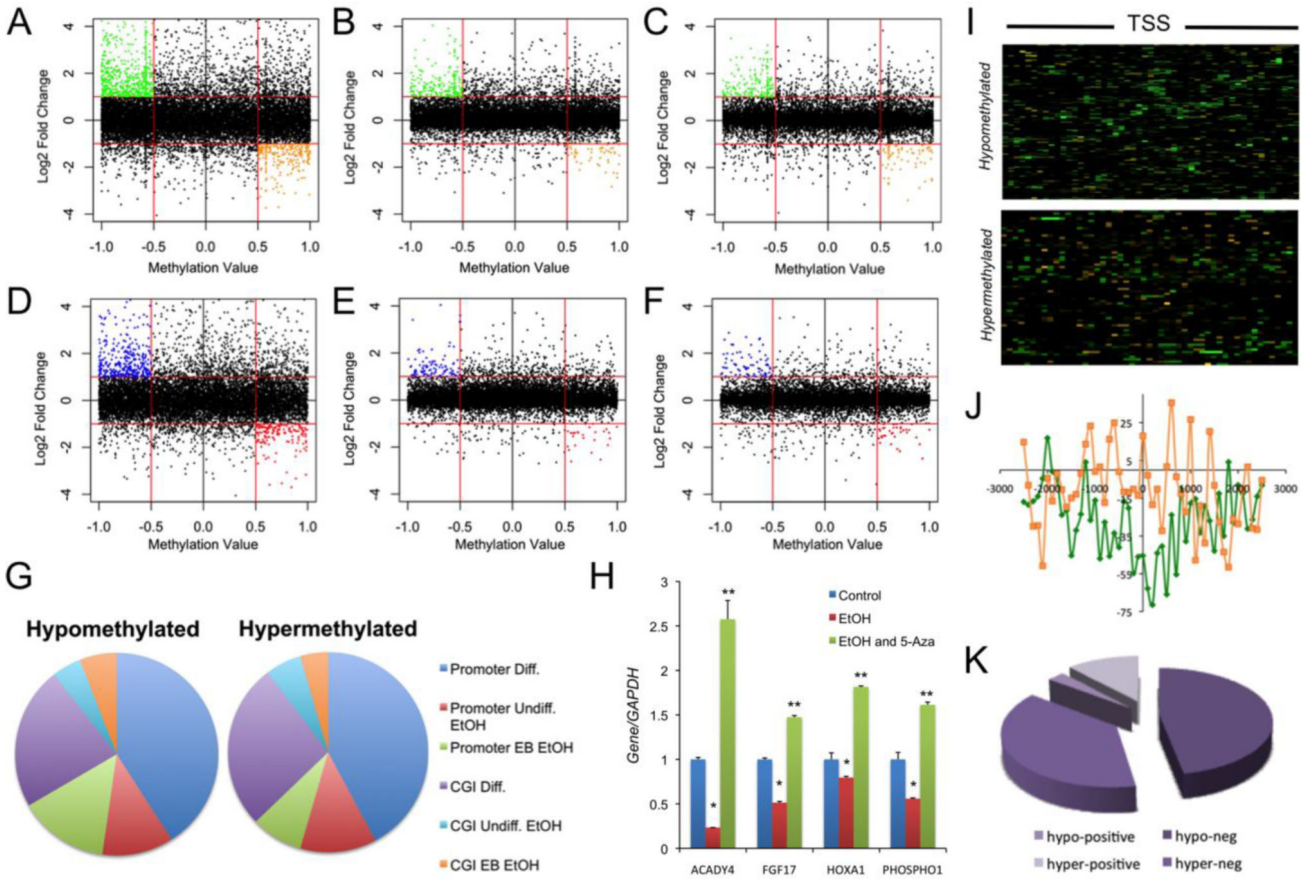


Figure 4. Methylation vs. gene expression in EtOH-treated hESCs

Methylation vs. gene expression of promoter regions during: (A) differentiation of hESCs, (B) EtOH treatment of undifferentiated hESCs and (C) EtOH treatment of EBs. Methylation vs. gene expression of CpG islands during: (D) differentiation of hESCs, (E) EtOH treatment of undifferentiated hESCs, and (F) EtOH treatment of EBs. (G) Summary of hyper and hypo methylated regions and their correlation to expression. (H) Rescue of genes downregulated by EtOH treatment after treatment with 5-azacytidine (5-Aza). Level of *ACADY4*, *FGF17*, *HOXA1* and *PHOSPHO1* gene was quantitatively assessed by qRT-PCR analysis with, without EtOH (20 mM), or after co- treatment with 20 mM EtOH and 1 μM of 5-Aza for 24 hrs. *, $p < 0.05$ vs. control; **, $p < 0.05$ vs. EtOH treatment (one-way ANOVA with Tukey's post hoc). (I) The level of hypo and hypermethylation at 2.5 kb upstream and downstream of the transcription start site (TSS) in EtOH-treated undifferentiated hESCs as shown in (B). The genes represent inverse correlation between methylation and expression-hypermethylated and downregulated (gold) vs. hypomethylated and upregulated (green). (J) A graph representing the average methylation found in I (green represents hypomethylated genes and gold represents hypermethylated genes). (K) Pie chart representing the level of hypo- and hypermethylation in genes both positively and negatively regulated. It shows how much hypo- or hypermethylation refers to either a decreased (negative) or increased (positive) gene expression.

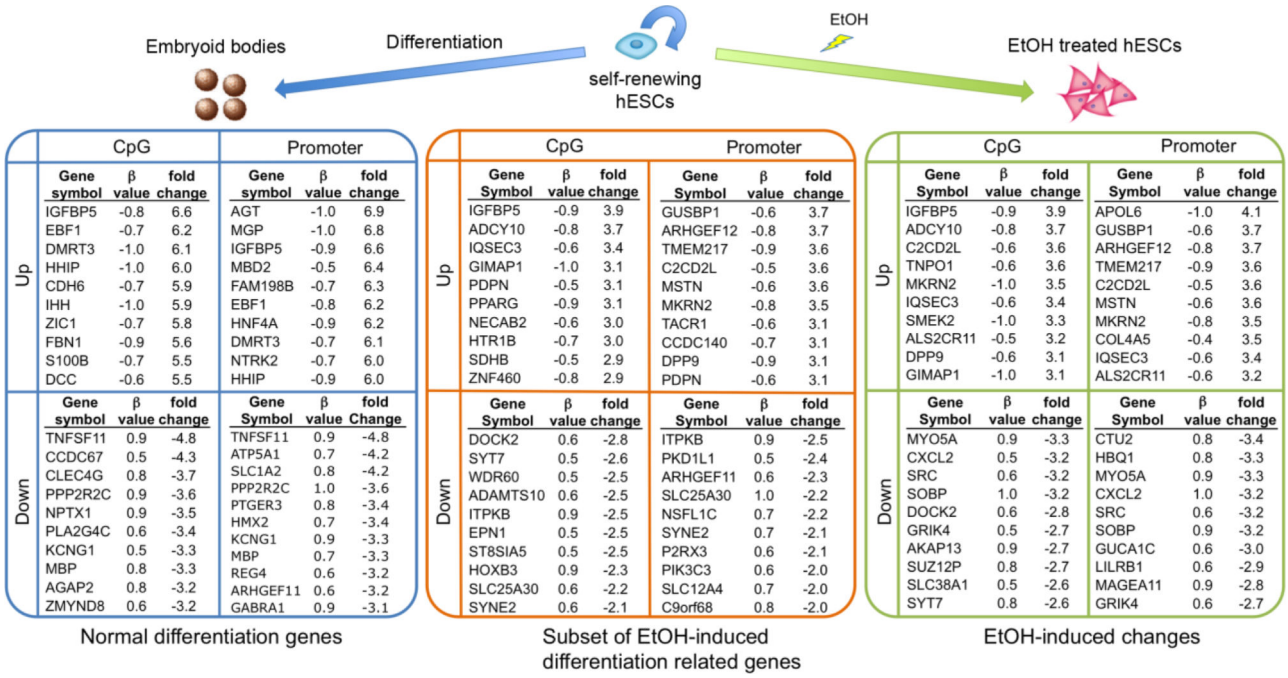


Figure 5. Profiling of molecular signatures specifically affected by EtOH-induced DNA methylation in hESCs

To obtain a list of genes that are epigenetically altered by EtOH-induced DNA methylation changes, genes showing correlative changes in DNA methylation and in gene expression were identified. Genes showing β>0.5 in DNA methylation either on CpG islands or promoter regions with >2-fold change in gene expression were chosen from Fig. 4. Comparison was done to examine the effect of EtOH on the differentiation of hESCs. By combining the list of genes differentially regulated during hESC differentiation and the list of genes dysregulated by EtOH treatment in undifferentiated hESCs, we were able to identify genes that are potentially involved in the differentiation of hESC by EtOH-induced DNA methylation changes (common genes).

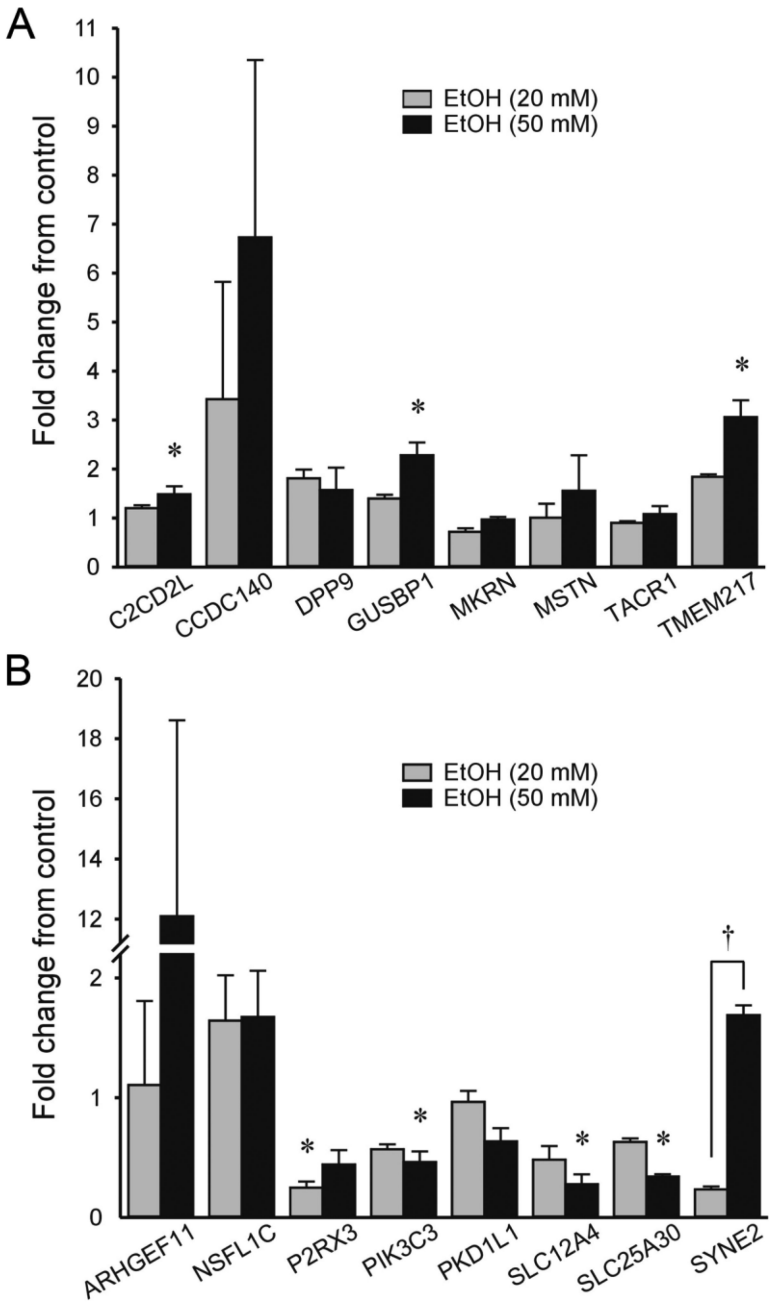
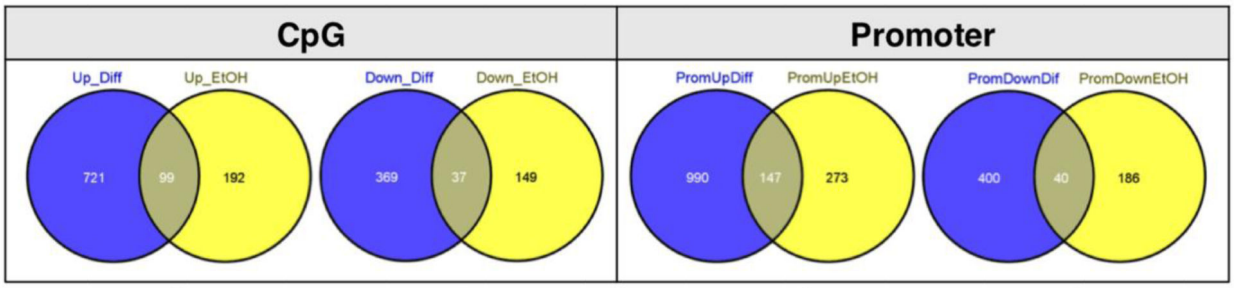


Figure 6. Quantitative RT-PCR validation of gene list

The expression levels of part of genes list obtained from Fig. 5 were validated. hESCs were treated with 0, 20, or 50 mM EtOH for 24 hrs and qRT-PCR performed as described in Materials and Methods. (A) Genes that were upregulated by EtOH-induced DNA hypomethylation. Note significantly increased expression in 3 of 8 genes, with none significantly decreased. (B) Genes that were downregulated by EtOH-induced hypermethylation. Note significantly decreased expression in 4 of 8 genes, with none significantly increased. Bars are mean ± SEM from triplicates; *, $p < 0.05$ vs. control; †, $p < 0.05$ vs. 20 mM EtOH (one-way ANOVA with Tukey post hoc).



	Motif	Associated TFs	E value	Motif	Associated TFs	E value
Up		FOXJ2	2.068e-07		AGL15	4.398e-07
		HSF	7.147e-06		ZEN	2.19e-06
		RIM	4.44e-07		BR-C	1.024e-07
Down		SMAD3	2.976e-04		POU6F	2.586e-05
		MAF	8.674e-04		ZEN	1.449e-07
		ARF	1.152e-03		TAF	2.036e-06

Figure 7. Common motif analysis and EtOH-related transcription factor discovery

Genes that are affected by normal differentiation are shown in purple Venn diagrams (Up_Diff: Hypomethylated and upregulated during differentiation; Down_Diff: Hypermethylated and downregulated during differentiation). Genes that are significantly affected by EtOH are shown in yellow Venn diagrams (Up_EtOH: Hypomethylated and upregulated upon EtOH treatment; Down_EtOH: Hypermethylated and downregulated upon EtOH treatment). Subsets of genes that are affected by both EtOH and differentiation are shown in the middle. These genes are also represented in the middle orange box of Figure 5. Selected genes were further subjected to STAMP analysis to identify potential *cis*-regulatory elements that are present in the differentially regulated genes and transcription factors that could be potentially affected by EtOH treatment. Based on the common gene lists, common motifs and associated transcriptional factors are found for CpG and promoter respectively. E-value is calculated based on the Wasserman and Sandelin method (Wasserman and Sandelin, 2004).

Table 1

Differentially regulated genes in undifferentiated hESCs upon EtOH treatment

CpG Up			CpG Down			Promoter Up			Promoter Down		
Gene Symbol	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**
IGFBP5	−0.9	3.9	MY05A	0.9	−3.3	APOL6	−1.0	4.1	CTU2	0.8	−3.4
ADCY10	−0.8	3.7	CXCL2	0.5	−3.2	GUSBP1	−0.6	3.7	HBQ1	0.8	−3.3
C2CD2L	−0.6	3.6	SRC	0.6	−3.2	ARHGEF12	−0.8	3.7	MY05A	0.9	−3.3
TNPOl	−0.6	3.6	SOBP	1.0	−3.2	TMEM217	−0.9	3.6	CXCL2	1.0	−3.2
MKRN2	−1.0	3.5	DOCK2	0.6	−2.8	C2CD2L	−0.5	3.6	SRC	0.6	−3.2
IQSEC3	−0.6	3.4	GRHC4	0.5	−2.7	MSTN	−0.6	3.6	SOBP	0.9	−3.2
SMEK2	−1.0	3.3	AKAP13	0.9	−2.7	MKRN2	−0.8	3.5	GUCA1C	0.6	−3.0
ALS2CR11	−0.5	3.2	SUZ12P	0.8	−2.7	COL4A5	−0.4	3.5	LILRB1	0.6	−2.9
DPP9	−0.6	3.1	SLC38A1	0.5	−2.6	IQSEC3	−0.6	3.4	MAGEA11	0.9	−2.8
GIMAP1	−1.0	3.1	SYT7	0.8	−2.6	ALS2CR11	−0.6	3.2	GRIK4	0.6	−2.7
PDPN	−0.5	3.1	NCOA3	0.5	−2.6	IL12B	−0.6	3.2	ASB15	0.6	−2.7
PPARG	−0.9	3.1	WDR60	0.5	−2.5	TACR1	−0.6	3.1	SUZ12P	1.0	−2.7
NECAB2	−0.6	3.0	ADAMTS10	0.6	−2.5	SLC25A4	−0.7	3.1	SYT7	0.6	−2.6
C8orf46	−0.7	3.0	ITPKB	0.9	−2.5	CCDC140	−0.7	3.1	UBE2D3	1.0	−2.6
HTR1B	−0.7	3.0	EPN1	0.5	−2.5	DPP9	−0.9	3.1	PTPN22	1.0	−2.5
FOXK1	−0.6	2.9	ST8SIA5	0.5	−2.5	GIMAP1	−0.6	3.1	ITPKB	0.9	−2.5
SDHB	−0.5	2.9	GPR39	0.8	−2.4	PDPN	−0.6	3.1	GRIA3	0.6	−2.4
ZNF460	−0.8	2.9	ZAP70	0.9	−2.4	HAL	−0.9	3.1	ATP8B4	0.6	−2.4
PLVAP	−0.6	2.9	CD6	0.6	−2.4	PPARG	−0.9	3.1	NEU2	0.9	−2.4
PTPN21	−0.9	2.7	TTLL1	0.5	−2.4	CD96	−0.6	3.1	RAB3IP	0.8	−2.4
EML2	−0.6	2.7	SMOC2	1.0	−2.4	NECAB2	−0.7	3.0	PSCA	0.5	−2.3
SERP2	−0.6	2.7	PDK4	0.8	−2.3	HTR1B	−0.7	3.0	ARHGEF11	0.6	−2.3
ZPBP2	−0.8	2.7	HOXB3	0.9	−2.3	IL2	−0.6	3.0	SAR1B	0.7	−2.3
TFB2M	−0.7	2.7	SCAND2	0.6	−2.3	CRB1	−0.6	2.9	RAD21L1	0.9	−2.3
KCNJ10	−0.6	2.7	C10orf90	1.0	−2.3	FOXK1	−0.7	2.9	FNBP4	0.8	−2.3
GREB1	−0.5	2.6	FNBP4	0.6	−2.3	MIA2	−0.6	2.9	DCAF6	0.8	−2.2
GPR75	−1.0	2.6	CARD 14	0.9	−2.2	SDHB	−0.7	2.9	SLC6A4	0.8	−2.2

CpG Up			CpG Down			Promoter Up			Promoter Down		
Gene Symbol	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**	**Gene Symbol**	**Methyl (P value)**	**Expr (fold)**
RAB40AL	−0.6	2.6	CCDC93	0.7	−2.2	PLYAP	−0.6	2.9	CCDC80	1.0	−2.2
ESR1	−0.9	2.6	SLC25A30	0.6	−2.2	REPIN 1	−0.5	2.8	CCDC93	0.9	−2.2
RORA	−0.51	2.6	NTN3	0.7	−2.2	TEX11	−0.7	2.8	OMD	0.6	−2.2

P > 0.5; Fold change >2