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Alcohol-induced suppression of *KDM6B* dysregulates the mineralization potential in dental pulp stem cells

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Abstract

Epigenetic changes, such as alteration of DNA methylation patterns, have been proposed as a molecular mechanism underlying the effect of alcohol on the maintenance of adult stem cells. We have performed genome-wide gene expression microarray and DNA methylome analysis to identify molecular alterations via DNA methylation changes associated with exposure of human dental pulp stem cells (DPSCs) to ethanol (EtOH). By combined analysis of the gene expression and DNA methylation, we have found a significant number of genes that are potentially regulated by EtOH-induced DNA methylation. As a focused approach, we have also performed a pathway-focused RT-PCR array analysis to examine potential molecular effects of EtOH on genes involved in epigenetic chromatin modification enzymes, fibroblastic markers, and stress and toxicity pathways in DPSCs. We have identified and verified that lysine specific demethylase 6B (*KDM6B*) was significantly dysregulated in DPSCs upon EtOH exposure. EtOH treatment during

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odontogenic/osteogenic differentiation of DPSCs suppressed the induction of *KDM6B* with alterations in the expression of differentiation markers. Knockdown of *KDM6B* resulted in a marked decrease in mineralization from implanted DPSCs *in vivo*. Furthermore, an ectopic expression of *KDM6B* in EtOH-treated DPSCs restored the expression of differentiation-related genes. Our study has demonstrated that EtOH-induced inhibition of *KDM6B* plays a role in the dysregulation of odontogenic/osteogenic differentiation in the DPSC model. This suggests a potential molecular mechanism for cellular insults of heavy alcohol consumption that can lead to decreased mineral deposition potentially associated with abnormalities in dental development and also osteopenia/osteoporosis, hallmark features of fetal alcohol spectrum disorders.

Keywords

alcohol; dental pulp stem cells (DPSCs); epigenetics; DNA methylation; osteogenic differentiation; odontogenic differentiation; mineralization

INTRODUCTION

Heavy alcohol consumption could result in a range of health, social and behavioral problems. Studies have demonstrated the potential toxic effects of alcohol in many organs and have shown deleterious molecular effects on cellular physiology, including the potency and maintenance of stem cells. Based on our previous study demonstrating the epigenetic effect of alcohol on embryonic stem cell pluripotency [1], we tested alcohol's epigenetic effect on the potency and differentiation capability of adult stem cells.

Alcohol consumption has been shown to have detrimental effects on the brain, liver, muscles, skeleton and fetal development [2]. While some studies have indicated that low doses of alcohol consumption may increase bone mass and density [3, 4], chronic heavy alcohol use can dramatically affect bone health and increase the risk of osteoporosis later in life. Different mechanisms have been hypothesized to cause changes related to alcohol abuse, including a direct effect on osteoblasts and osteoclasts, changes in signaling due to oxidative stress, increased fat accumulation in the bone marrow, modulation of regulatory hormones, and/or indirectly through decreased caloric intake [5]. The effects of alcohol consumption on bone health are multifactorial and are related to the duration and dosage of consumption [6]. It has been demonstrated that Fetal Alcohol Syndrome (FAS) patients show high incidences of dentofacial and temporomandibular joint disorders, including midfacial underdevelopment with shortage of bone, delayed dental development, enamel anomalies, and cleft palate or cleft lip [7]. In an animal model, maternal alcohol ingestion before and during gestation caused retardation in cell differentiation within the tooth germ and in calcification of the dentin matrix [8]. The dental anomalies observed may stem from cellular alterations in the basal layer of the epithelium of the tooth germ during odontogenesis [8–11].

Dental pulp stem cells (DPSCs), also known as dental pulp-derived mesenchymal stem cells, are a multipotent adult stem cell population that has a high proliferative potential. DPSCs in this study isolated from adult teeth are easily accessible and cryopreservable for long periods [12, 13]. DPSCs were used as a model of a mineralizing system, *in vitro* and *in vivo*, to

study the effects of EtOH. It has been shown in numerous studies that under various conditions, dental pulp stem cells can be induced towards odontogenic and osteogenic lineages. One study demonstrated that vascular endothelial growth factor (VEGF) gene could promote odontogenic differentiation in DPSCs *in vitro* [14] while another identified that DPSCs undergo osteogenic differentiation through the NF- κ B signaling pathway [15]. DPSCs had the ability to differentiate toward both odontogenic and osteogenic lineages in presence of a carboxymethyl cellulose-hydroxyapatite hybrid hydrogel [16]. Furthermore, medium modification with bone morphogenetic protein 2 was shown to stimulate odontogenic differentiation and formation of an osteo-dentin matrix [17]. Although DPSCs have been long studied for their regenerative capabilities in both dentistry and orthopedics, the molecular mechanisms controlling their stem cell potency have yet to be discovered.

It has been shown that *KDM6B*, a lysine-specific demethylase, plays a key role in osteogenic differentiation by removing H3K27me3 from the promoters of osteogenic genes in human bone marrow stromal cells (BMSCs) [18]. It has also identified *KDM6B* in controlling *HOX* expression through the removal of H3K27me3 in human BMSCs [18]. A recent study has shown *KDM6B* to play a critical role in the epigenetic regulation of odontogenic differentiation in human DPSCs [19]. In DPSCs, *KDM6B* knockdown studies resulted in decreased alkaline phosphatase activity and alizarin red staining, and reduced expression levels of marker genes, including osterix (*OSX*), osteocalcin (*OCN*), and osteopontin (*OPN*) [19]. Decreased levels of these markers and mineralization parallel effects observed in postmenopausal osteoporotic patients, who exhibit significant reductions in the number of osteocytes and osteoblasts, and demineralization of compact and cancellous bone [20]. While DPSCs primarily differentiate to dentin and BMSCs differentiate to bone, both dentin and bone formation share similar mineralization matrix components. Using DPSC model of mineralization will give us insight on the molecular effects of EtOH on mineralization matrix that is similar to bone mineralization model of BMSCs. In this study, we explore the epigenetic effects of alcohol on DPSCs and a possible link between alcohol and mineralization through the dysregulation of *KDM6B*.

MATERIALS AND METHODS

Culture of human DPSCs and EtOH treatment

Early passage DPSCs (P1-P2) were isolated from adult teeth (molars) were cultured in α -MEM supplemented with 10% fetal bovine serum (v/v), 2 mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 50 unit/ml penicillin and 50 μ g/ml streptomycin. Exponentially growing DPSCs were treated with different concentrations of EtOH diluted from absolute EtOH (FW=21.7 M). For acute exposure, cells were fed with media containing given concentrations of EtOH (20 or 50 mM) for 24 or 48 hrs. For chronic exposure, cells were intermittently exposed to EtOH by oneday exposure and one-day withdrawal for up to 2 weeks.

Transcriptomic analysis

Total RNA was isolated with RNeasy kit (Qiagen, Carlsbad, CA). Samples were prepared in biological duplicates. 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 Clinical Microarray Core Facility).

Methyl-DNA Immunoprecipitation and DNA Methylation Array Analysis

Methyl-DNA immunoprecipitation was performed according to a previous standard protocol from our laboratory by using antibody against 5-methylcytidine (Eurogentec, San Diego, CA) [21]. Methylation array analysis was performed according to a previous standard protocol from our laboratory [21]. Genomic profiling was performed by NimbleGen Systems (100718 HG18 CpG Refseq Promoter MeDIP). 3 μ g of sonicated total DNA as input and 4 μ g of DNA sample immunoprecipitated with anti-5-methylcytidine were sent to the UCLA Clinical Microarray core for differential random labeling by priming with Cy3 or Cy5 and hybridization to arrays. From the scaled \log_2 ratio data, a fixed-length window (750 bp) was placed around each consecutive probe, and the one-sided Kolmogorov-Smirnov (KS) test was applied to determine whether the probes were drawn from a significantly more positive distribution of intensity log ratios than those in the rest of the array. Resulting score for each probe was $-\log_{10}$ (p value) from the windowed KS test around the probe and was assigned as "p-value." NimbleScan software (NimbleGen Systems) detects peaks by searching for at least two probes above a p value minimum cutoff ($-\log_{10}$) of 2. Using a custom Unix code, we aligned "ratio peak p -values" to human genome18 (RefSeq.hg18) and created a matrix file.

Bioinformatics analysis

The fastq files generated have been processed using GALAXY and aligned to the human genome, build hg19 (<https://main.g2.bx.psu.edu/>). Further analysis was done using R and bioconductor. After alignment to the genome perl scripts was used to delineate regions of interest on proximal and distal promoters using the reference sequence obtained from the UCSC genome browser (<http://genome.ucsc.edu/>). Subsequently, these regions have been clustered by K-means clustering using Gene Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/>). Clusters identified were visualized using JavaTreeView (<http://jtreeview.sourceforge.net/>). Furthermore, data has been collapsed and correlated to gene expression data within R.

Pathway focused RT-PCR array analysis

Total RNA was purified and the quality of RNA was determined by using Agilent Bioanalyzer. The samples were processed for RT² Profiler PCR array analysis (SABiosciences, Valencia, CA). Three different pathway specific arrays were used- Epigenetic chromatin modification enzyme array, Stress and toxicity array, and Fibroblastic marker array. These arrays also contain genomic DNA contamination control, positive PCR controls, and RT controls. To ensure inter-well and intra-plate consistency, these controls were considered for determining Ct value for each well. Results (Ct values) were normalized against five internal controls (ex: Actin B, GAPDH, 18S rRNA, etc.). Results have been

analyzed by using the company's RT² Profiler PCR Array Data Analysis version 3.5 (SABiosciences, Valencia, CA).

Odontogenic/osteogenic differentiation of human DPSCs and EtOH treatment

DPSCs were grown in mineralization-inducing media containing 100 μ M ascorbic acid, 2 mM β -glycerophosphate and 50 nM dexamethasone. Exponentially growing DPSCs were treated with different concentrations of EtOH diluted from absolute EtOH (FW=21.7 M). For acute exposure, cells were fed with media containing given concentrations of EtOH (20 or 50 mM) for 24 or 48 hrs. For chronic exposure, cells were intermittently exposed to EtOH (one-day exposure and one-day withdrawal) for up to 2 weeks.

Transduction of human DPSCs and EtOH treatment

GP2-293 cells were grown in DMEM containing 10% FBS and transiently transfected with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) to produce retroviral MSCV-KDM6B. DPSCs were cultured to 60% confluency and stably transduced with either control retrovirus or retroviral MSCV-KDM6B to generate KDM6B expressing DPSCs. Exponentially growing DPSCs were grown in mineralization-inducing media as described above and treated with different concentrations of EtOH diluted from absolute EtOH (FW=21.7M). Cells were fed with differentiation media containing given concentrations of EtOH (50 or 100 mM) for indicated period of time.

Quantitative RT-PCR analysis of differentiation markers

For validation of gene expression by quantitative RT-PCR analysis, total RNA was first subjected to DNase digestion with Turbo DNA-free kit (Life Technologies, Grand Island, NY). 2 μ g of total RNA treated with Turbo DNA-free kit (Thermo Fisher Scientific, Waltham, MA) was used to synthesize cDNA by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in 40 μ l reaction mixture. The resulting cDNA was diluted (mixed with H₂O by 1:4) and 1.5 μ l of diluted cDNA was used per well (in 10 μ l reaction volume) in a 384 well plate using LightCycler 480 SYBR Green I master mix (Roche Diagnostics, Indianapolis, IN). PCR was done with specific set of primers (Supplemental Table 1) at annealing temperature of 60°C.

Alkaline phosphatase and Alizarin Red staining

For alkaline phosphatase (ALP) staining, after induction, cells were fixed with 4% paraformaldehyde and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.1 M Tris buffer (pH 9.3). ALP activity assay was performed using an ALP kit according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA) and normalized based on protein concentrations. To detect mineralization potential, cells were induced for 2–3 weeks, fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (Sigma-Aldrich, St Louis, MO, USA). To quantify the calcium mineral deposition, Alizarin Red was destained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 30 min at room temperature.

Implantation of DPSCs into immunocompromised mice

6.0×10^6 DPSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc.) and then implanted into the dorsal surface of 10-week-old immunocompromised mice (Beige nude/nude Xid (III) mice) as previously described (Miura *et al.*, 2004). The implants were harvested at 8 weeks post-implantation, fixed in 4% paraformaldehyde, and then decalcified with 10% EDTA (pH 8.0) for paraffin embedding. Paraffin sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). New mineralized matrix formation was observed under microscope. For quantification, the NIH software Image J was used as previously described [22].

RESULTS

EtOH treatment resulted in significant gene dysregulation and DNA methylomic alterations in human DPSCs

To test our hypothesis that alcohol may have deleterious molecular effects on the maintenance and regulation of adult stem cells, we have used multipotent dental pulp stem cells (DPSCs) and acutely exposed them (24 or 48 hrs) to defined concentration of EtOH (20 or 50 mM). Cell growth analysis showed that 20 or 50 mM EtOH treatment did not induce significant reduction in cell growth (Supplemental Figure 1). To profile molecular signatures that are affected by EtOH treatment in DPSCs, we have performed gene expression microarray analysis on DPSCs after 24 hr EtOH treatment. A genome-wide microarray (Affymetrix Human Genome U133 Plus 2.0 Array platform) was used on biological duplicates to generate gene expression data for DPSCs in order to compare the concentration-dependent effects of EtOH (0, 20 or 50 mM) (GEO Accession: GSE57255). Weighted Gene Correlation Analysis (WGCNA) identified several gene modules that are significantly correlated to EtOH treatment and revealed a certain level of complexity in gene expression level changes in response to EtOH treatment (Fig. 1A). We identified modules with dose-dependent changes in gene expression upon EtOH treatment (Fig. 1B). Also there were modules that showed changes only specific to certain EtOH concentration. We observed differential molecular effects by different concentrations of EtOH, such as 20 mM EtOH showed different pattern of expression changes compared to 50 mM EtOH. This implies that EtOH at different concentration levels may have different physiological impacts through differential molecular responses. Recently, epigenetic effects, such as DNA methylation, of alcohol on gene regulation have been documented [1, 23]. To test our hypothesis that alcohol induces epigenetic alterations and leads to deregulation of gene signatures in DPSCs, we have profiled DNA methylomic changes in DPSCs that are potentially affected by EtOH exposure. We have performed CpG Promoter microarray analysis coupled with methylated DNA immunoprecipitation (MeDIP) on the same set of samples used for transcriptomic profiling. The blue module consisting of genes whose promoters are hypermethylated with EtOH treatment and the turquoise module represents genes whose promoters are hypomethylated upon EtOH exposure (Fig. 1C). Heatmaps show changes in DNA methylation in biological duplicates of DPSCs upon exposure to different concentrations of EtOH (0, 20 and 50 mM) for 24 hrs. We have examined potential effects of EtOH-mediated DNA methylomic changes on actual transcriptomic alterations. We have performed combinatory analysis on genes that were (1) hypermethylated with genes that

were downregulated and (2) hypomethylated with genes that were upregulated (Fig. 1D). We have identified genes that show concordant changes in promoter methylation and gene expression. With 20 mM EtOH treatment, we found that 434 genes were hypermethylated on the promoter whose expressions were downregulated. We also found 469 genes that were hypomethylated and upregulated. The results suggest that the molecular repertoire of gene expression in DPSCs is widely affected by EtOH-mediated DNA methylomic alterations.

Since we were interested in more physiologically relevant effects of EtOH, we focused on 20 mM EtOH's effect that is known to be the DUI level of alcohol in blood (Fig. 2A). We identified two gene modules that were most significantly associated with EtOH treatment—the blue module for genes upregulated with EtOH exposure and the black module for genes downregulated with EtOH treatment (Fig. 2B). To examine potential effects of EtOH on biological process, we have performed DAVID (The Database for Annotation, Visualization and Integrated Discovery) on genes from the blue and black modules. The blue module of upregulated genes upon EtOH treatment was associated with several important signaling pathways, such as MAPK, calcium, WNT and mTOR signaling (Fig. 2C). On the other hand, the black module of downregulated genes showed association with cell cycle, axon guidance, neurotrophin signaling, TGF-beta signaling and p53 signaling (Fig. 2C). To examine if there is any concordance in molecular networks affected by EtOH treatment, we have performed basic expression analysis in Cytoscape. We established the core gene interaction network by combining the genes in the blue or the black module with the network data (Supplemental Figure 2). The blue module contains *CTBP1*, *FAM82A1* (*RMD4*), *CCDC149*, *MYO10*, *FCGR2C*, *SRGAP2*, *DLGAP1*, *PPP2R5C* and *CGLF2*. The black module consists of *STAG2*, *CCT2*, *PKG1*, *MAGIX*, *PDZD3*, *HNRNPD*, *INSIG1*, *FCRL5*, *HLA-E* and *PDLIM5*. We have ranked genes based on fold changes (>2-fold) and *p* values (<0.05) for each EtOH concentration treatment (20 mM or 50 mM) for further selection and validation (Table 1).

EtOH treatment triggered changes in epigenetic modifiers in human DPSCs

To examine EtOH-induced changes in known epigenetic modifiers in DPSCs we have performed a pathway focused RT-PCR analysis and assessed the effect of EtOH on the levels of known epigenetic chromatin modification enzymes, fibroblastic markers, and stress/toxicity genes (Qiagen Inc., Valencia, CA). We have used two different EtOH concentrations (20 and 50 mM) and treated cells acutely for 24 hrs. Heatmaps show the level of genes upon exposure to EtOH relative to non-treatment control (Fig. 3). We observed both EtOH concentration-dependent and concentration-independent changes in the level of genes. We then further attempted to validate the acute changes in gene expression, especially of epigenetic chromatin modification enzymes, by quantitative PCR analysis (Fig. 3D). Among 15 genes we tested (*AURKA*, *DOTIL*, *ESCO2*, *KAT2B*, *KAT7*, *KDM6B*, *MLL*, *NSD1*, *PRMT6*, *SETD1B*, *SETD7*, *SUV39H1*, *FCHO*, *PRMT7*, *DNMT1*, *HDAC4* and *HDAC11*), we found that 8 genes showed the levels of expression concordant between the array result and qRT-PCR result. We found lysine demethylase 6B (*KDM6B*) showed the most noticeable change upon EtOH treatment (2.5-fold downregulated in the RT array analysis and 4.6-fold downregulated in the qRT-PCR analysis) (Fig. 3D). Previously *KDM6B* has been demonstrated to play a role in the control of DPSC and BMSC [18, 19], which suggests

that alcohol-mediated dysregulation of *KDM6B* may have a functional link to the effect of alcohol exposure on osteogenic differentiation of DPSCs.

EtOH treatment induced dysregulation of *KDM6B* and odontogenic/osteogenic differentiation

To examine if EtOH treatment has any functional effect on the mineralization of DPSCs, we have cultured DPSCs under odontogenic/osteogenic differentiation conditions and examined the molecular effect of EtOH on the expression of differentiation-related lineage markers (Fig. 4). It has been previously demonstrated that the induction of *KDM6B* during odontogenic differentiation is immediate. Since *KDM6B* was previously demonstrated as an early responder [19], the fold changes of *KDM6B* expression with and without induction of differentiation treatment at 20 mM EtOH were analyzed at the 2hr time point (Fig. 4A). Upon analysis, EtOH treatment resulted in a significant reduction of *KDM6B* expression level during odontogenic/osteogenic differentiation (2.5-fold downregulated in the qRT-PCR analysis). Furthermore, relative expression levels of the mineralization-associated markers we tested (*ALP*, *BMP2*, *BMP4*, *DLX2*, *OCN*, and *OPN*) at the 24 and 72 hr time periods all exhibited concentration dependent reductions to EtOH, with more significant decreases at the 72 hr time point (Fig. 4B). As *KDM6B* has been demonstrated to play an early role in the control of DPSC fate, we suggest that these differentiation-related markers may be functioning downstream of the epigenetic modifier.

Following the analysis of the acute effects of EtOH in DPSCs, we investigated the chronic effects of EtOH on mineralization. We investigated these long-term effects on differentiation *in vitro* by culturing DPSCs in odontogenic/osteogenic differentiation media for 2 weeks with 20 mM or 50 mM level of EtOH treatment. The effects of EtOH on mineralization were qualitatively visualized through Alizarin Red staining. At the 2-week time point, Alizarin red staining, indicative of calcium accumulation by cells of an odontogenic/osteogenic lineage, was highest in the control group differentiated without EtOH treatment (Fig. 4C). As the concentration of EtOH treatment increased from 20 mM to 50 mM, a progressive level of the decreased staining was observed. Similarly, alkaline phosphatase staining was highest in the control group differentiated without EtOH treatment (data not shown). Much like the results observed with the Alizarin Red staining, the density of the staining progressively decreased with increasing concentrations of EtOH at 20 mM and 50 mM. These results suggest that EtOH has both acute and chronic effects on the mineralization potential of DPSCs.

Suppression of *KDM6B* altered odontogenic/osteogenic potency in DPSCs

Our findings support that EtOH treatment resulted in suppression of *KDM6B* along with inhibition of mineralization in DPSCs. We next investigated whether the expression level of *KDM6B* was directly correlated with odontogenic/osteogenic differentiation potential through associated osteomarkers. It has recently been demonstrated that a knockdown of *KDM6B* in BMSCs and DPSCs resulted in dysregulation of osteogenic and odontogenic differentiation *in vitro* and *in vivo* [18, 19]. As *KDM6B* was significantly related to the odontogenic/osteogenic potential of DPSCs *in vitro*, we performed an *in vivo* study in mice to further investigate *KDM6B* ability to induce mineralization. DPSCs in the control group

were transfected with non-targeting *siRNA* while the experimental group was transfected with *siKDM6B* (Fig. 5A). Subsequently, these cells were implanted into immunocompromised mice and allowed to differentiate. Following an 8-week time period, histological analysis of the H&E staining in the control group indicated the formation of mineralized tissue and minimal connective tissue around tricalcium phosphate/hydroxyapatite (TCP-HA) bone scaffold (Fig. 5B). In contrast, DPSCs transfected with *siKDM6B* showed significant reductions in the formation of mineralized tissue and more prominent formation of connective tissue. When a quantitative analysis of the total *in vivo* mineralized matrix area was performed, the DPSCs transfected with *siKDM6B* showed a 66% reduction in mineral deposition as compared to the control (Fig. 5C). Our results suggest that *KDM6B* is necessary for odontogenic/osteogenic differentiation potential and formation of mineralized tissue.

Ectopic expression of KDM6B restored odontogenic/osteogenic differentiation in EtOH-treated DPSCs

To further assess the direct functional significance of EtOH-induced suppression of *KDM6B* in the effect of alcohol exposure on odontogenic/osteogenic differentiation, we examined the restorative effect of the ectopic expression of *KDM6B* on EtOH-induced dysregulation of mineralization. DPSCs in culture were stably transduced with retroviral *KDM6B* expression construct and control virus. The expression of transduced *KDM6B* was determined by qRT-PCR and Western analysis (Fig. 6A). In addition, the transduced cells were subjected to odontogenic/osteogenic differentiation for 2 weeks. We analyzed the odontogenic/osteogenic differentiation potential of transduced DPSCs through the expression of alkaline phosphatase (ALP), which is frequently used to characterize activity of mineralizing cells [24–28]. Following ALP staining, we found that DPSCs transduced with the control vector showed a significant, progressive reduction in ALP staining at 50 and 100 mM EtOH treatment. In contrast, DPSCs transduced with the MSCV-*KDM6B* construct and treated with 50 and 100 mM EtOH showed enhanced levels of staining as compared to the control cells. Our results suggest that *KDM6B* was able to adequately restore mineralization potential of DPSCs in the presence of varying levels of EtOH.

The resulting transduced cells were subjected to odontogenic/osteogenic differentiation for 24 or 72 hrs and the level of osteogenic marker expression was quantitatively assessed. It was found that the forced expression of *KDM6B* in differentiating DPSCs in the absence of EtOH treatment caused alterations in mineralization-related genes (Fig. 6B). Some of the markers (*DLX2*, *DLX5*, and *IBSP*) were further induced by the expression of exogenous *KDM6B* compared to the control vector. However, the expression of *OCN* and *SPP* were suppressed by the ectopic expression of *KDM6B*. There were slight differences at the level of changes between the 24 and 72 hr differentiation, but the trend of the effect of *KDM6B* expression on marker gene expression was similar between the two time points.

The effect of *KDM6B* expression on the EtOH-induced suppression of odontogenic/osteogenic differentiation genes was determined. As shown in Fig. 6C, the forced expression of *KDM6B* in DPSCs suppressed EtOH-induced inhibition of mineralization *in vitro*. Compared to DPSCs transduced with the control virus, *KDM6B* expressing DPSCs showed

significant induction of differentiation-related genes even in the presence of either 50 or 100 mM EtOH (Fig. 6C). It was noted that the suppressive effect of 50 mM EtOH treatment on gene expression was more readily reversed by the expression of KDM6B than cells with 100 mM EtOH treatment (*ALP*, *BMP2*, *BMP4*, *IBSP*, *DLX5* and *OCN*). The *DLX2* gene was least affected by KDM6B. A higher dose of EtOH (100 mM compared to 50 mM) and a longer exposure (72 hr compared to 24 hr) to EtOH resulted in a reduction of the effect of exogenous KDM6B expression on the recovery of odontogenic/osteogenic gene expression.

We found that the forced expression of KDM6B significantly altered the response of DPSCs to EtOH treatment. As shown in Fig. 6D, we found that EtOH treatment induced the expression of mineralization-related genes in KDM6B expressing cells. The expression of markers was significantly inhibited by 50 mM EtOH treatment when compared within control group (0 mM EtOH) for genes *ALP* ($p=0.04$), *BMP4* ($p=0.003$), *OCN* ($p<0.0001$), and *SPP* ($p<0.0001$) (Fig. 6D). The ectopic expression of KDM6B resulted in significant increases in expression levels of these genes when treated with EtOH (50 mM) compared to non-treated cells (0 mM), particularly at 5.53 fold ($p=0.003$) and 4.94 fold ($p<0.0001$) increases for *OCN* and *SPP*, respectively. It seems that molecular dynamics and timing in signaling between mineralization signals and the regulation of key regulators such as KDM6B is important to ensure proper mineralization of DPSCs. A prolonged expression of KDM6B at a non-physiological level as shown in KDM6B expression cells could cause dysregulation in cellular response and downstream molecular signaling pathways. Overall, our results suggest that the expression of KDM6B in DPSCs can counteract the effects of EtOH-induced inhibition of odontogenic/osteogenic differentiation and promote mineralization in the presence of EtOH.

DISCUSSION

Alcohol abuse appears to lead to periodontal disease, tooth decay and mouth sores that are potentially precancerous [29–32]. Persons who abuse alcohol are at high risk of having seriously deteriorated teeth, gums and compromised oral health in general [29–32]. It is generally believed that alcohol may have toxic effects on various cellular functions, but we are in lack of detailed information about molecular and cellular effects of alcohol on stem cell maintenance and the differentiation process. Our recent publication demonstrated that EtOH exposure induced significant transcriptomic alterations in human embryonic stem cells (hESCs) through DNA methylomic deregulation [1]. Several studies have demonstrated effects of EtOH on DNA methylation, resulting in genetic and phenotypic changes [33–42]. It has been shown that EtOH induced alterations in DNA methylation patterns and inhibited neural stem cell (NSC) differentiation [43]. EtOH induced the hypermethylation of multiple cell cycle genes and increased the expression of DNA methyltransferases in NSCs. These alterations affected growth factor signaling, in conjunction with the down regulation of associated mRNAs and cell cycle proteins [44]. In another study, EtOH exposure prevented the methylation of specific genes related to neural development, including insulin-like growth factor 1 (IGF1), epidermal growth factor-containing fibulin-like extracellular matrix protein (EFEMP1), and SRY-box-containing gene 7 (SOX7) [43]. The hyper- or hypomethylation of specific genes have been shown to significantly affect NSCs differentiation and proliferation. However, it is important to note that many studies related to

EtOH exposure and adult stem cell potency have shown that the intrinsic genetic and epigenetic mechanisms that control cellular fate are potentially of equal significance.

The negative, long term effects of alcoholism on bone mass have been well-established [3]. Studies have shown that heavy chronic alcohol consumption compromises bone quality and increases the risk for osteoporosis. Other studies have shown that alcoholism is associated with a variety of risk factors that may contribute to the pathogenesis of bone disease, including poor nutrition, liver disease, malabsorption, vitamin D deficiency, hypogonadism, parathyroid dysfunction and tobacco use [45]. Recent studies have reported that alcohol may affect bone formation through osteocyte apoptosis, oxidative stress, and Wnt signaling pathway modulation [6]. One study involving alcohol-binged rats suggested that following administration of 20% alcohol/saline solution for 1, 2 or 3 weeks, a stimulation of bone resorption and decrease in bone mineral density was observed. However, it was interesting to note that concurrent administration of risedronate, a bisphosphonate, mitigated the response and maintained trabecular architectural indices [46]. The duration of alcohol treatment resulted in the modulation of expression profiles of RANKL and OPG, genes that regulate osteoclastogenesis. This study sheds light on the deleterious effects of bone metabolism in response to binge drinking. However, the exact mechanisms of how alcohol is related to bone loss remains unknown, but may include both direct and indirect actions, and be related to diet and other lifestyle factors.

While chronic alcohol abuse has been linked to an increase in the risk for osteoporosis, light to moderate consumption has been correlated with a reduction in osteoporotic risk. Some studies have identified that light to moderate concentrations of EtOH exposure has resulted in higher bone mineral density and a reduced risk of osteoporosis [3, 4]. Another study found that light alcohol consumption resulted in increased lumbar spine bone mineral density (BMD) and whole body BMD in postmenopausal women [47]. In animal studies, low alcohol consumption of ethanol (5%, 2h per day) in 4-week old rats showed higher BMD and greater trabecular thickness than compared to the control groups [48]. However, there are few animal models related to the effects of light alcohol consumption in the literature and further investigative studies in animal models should be performed in the future to shed light on these results. In contrast, at longer durations of moderate to high alcoholic exposure, there is a dose dependent effect on bone to increase risk for stress fractures. In a rat study investigating the long-term effects of heavy alcohol consumption on cancellous and cortical bone microarchitecture, ethanol consumption resulted in lower bone mineral density and content, reduced cortical thickness, and a lower femur length as compared to controls [49]. These results suggest that chronic, heavy alcohol consumption results in a decrease in bone size, mass, and density, and negatively alters cancellous bone microarchitecture resulting in decreased skeletal integrity.

In this study, we have used DPSCs as a model to examine the effect of EtOH on mineralization process. Our findings show that EtOH treatment resulted in altered mineralization in DPSCs. The dysregulation of odontogenic/osteogenic differentiation in DPSCs treated with EtOH is reminiscent of previous findings with reductions in bone mineral density and volume. We discovered that EtOH treatment results in the reduction of several known mineralization-related gene expression profiles, including *ALP*, *BMP2*,

BMP4, *DLX2*, *OCN*, and *OPN*. These findings appear to mirror potential biological mechanisms that are significantly dysregulated in the etiology of alcohol-induced osteoporotic events. *In vitro* studies are often difficult to adapt to *in vivo* studies due to their inability to fully simulate *in vivo* conditions. There are no *in vitro* models to study the effects of EtOH on bone formation and resorption [50]. Furthermore, one study identified a significant discrepancy in body weight gain and bone measurements due to the delivery method of EtOH. In this study, intraperitoneal injection resulted in reduced body weight, suppression of periosteal and cancellous bone formation, and decreased mRNA levels for bone matrix proteins as compared to intragastric administration [51]. This study suggests that treatment of DPSCs *in vitro* using EtOH-containing media may provide a dosage that is not replicable physiologically. In addition, the effects of alcohol on epigenetic modifiers and bone loss may not be fully adaptable to human studies, as it fails to take into account comorbidity factors including poor nutrition, vitamin deficiencies, mechanical loading, weight and smoking [50]. The effects of alcohol related bone loss is controversial, with human studies reporting bone loss as well as no bone loss.

Based on our findings on genome-wide, epigenetic effects of EtOH on DPSCs, we analyzed known epigenetic modifiers to elucidate a potential epigenetic link between alcohol and osteoporosis. Consistent with our conclusion, *KDM6B*, a known epigenetic modifier involved in osteogenic and odontogenic differentiation, was found to be significantly dysregulated in response to EtOH. Knockdown of *KDM6B* resulted in similar phenotype as EtOH treatment *in vitro* while concurrently showing similar reductions in the gene expression profiles of known osteomarkers. Thus, it is suggested that dysregulation of *KDM6B* by EtOH reduces odontogenic/osteogenic potency of DPSCs, however the molecular pathways behind the interactions of *KDM6B* and known mineralization-associated markers remains to be discovered.

Recent studies show that *KDM6B* is involved in the control of calcium-induced differentiation [52], regulates osteoblast differentiation [53], contributes to neuronal survival and differentiation [54], odontogenic differentiation of DPSCs [19], and osteogenic differentiation of human BMSCs [18]. It has been reported that calcium-induced differentiation leads to increased binding of *KDM6B* and erasure of repressive marks such as H3K27me3 [52]. It has been demonstrated that BMP4/7-mediated activation of SMAD1/4 may induce the expression of *KDM6B* and trigger osteogenic pathway [18]. Mechanistically, *KDM6B* is recruited to bone morphogenic protein 2 (BMP2) and HOX (homeotic genes) promoters and activate the odontogenic differentiation-related gene expression [18]. *KDM6B* removes epigenetic marks, H3K27me3, from the promoter of osteogenic genes to promote osteogenic commitment [18]. In addition, a recent study has demonstrated the facilitating role of *KDM6B* on odontogenic differentiation [19]. It needs to be experimentally demonstrated if EtOH triggers dysregulation of *KDM6B* via altered regulation of differentiation signal-induced activation of SMAD1/4 pathway and the control of H3K27me3 mark as demonstrated in BMSCs [18].

In conclusion, our findings confirmed that EtOH has a significant impact on the dysregulation of *KDM6B* and provides a potential epigenetic effect of heavy alcohol exposure in DPSCs. These findings will be helpful in identifying molecular mechanisms

associated with alcohol induced osteoporosis in a proper model, as it suggests a potential mechanism of EtOH-induced suppression of mineralization. Further study is needed to evaluate the significance of our findings in fetal development where maternal alcohol consumption results in craniofacial and dental abnormalities, which are hallmark features of fetal alcohol spectrum disorders (FASD).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Molecular effects of alcohol on transcriptome and DNA methylome in human DPSCs
- Identification of KDM6B as a target in alcohol-induced disruption of DPSC potency
- Validation of the role of KDM6B in alcohol-induced disruption in vitro and in vivo

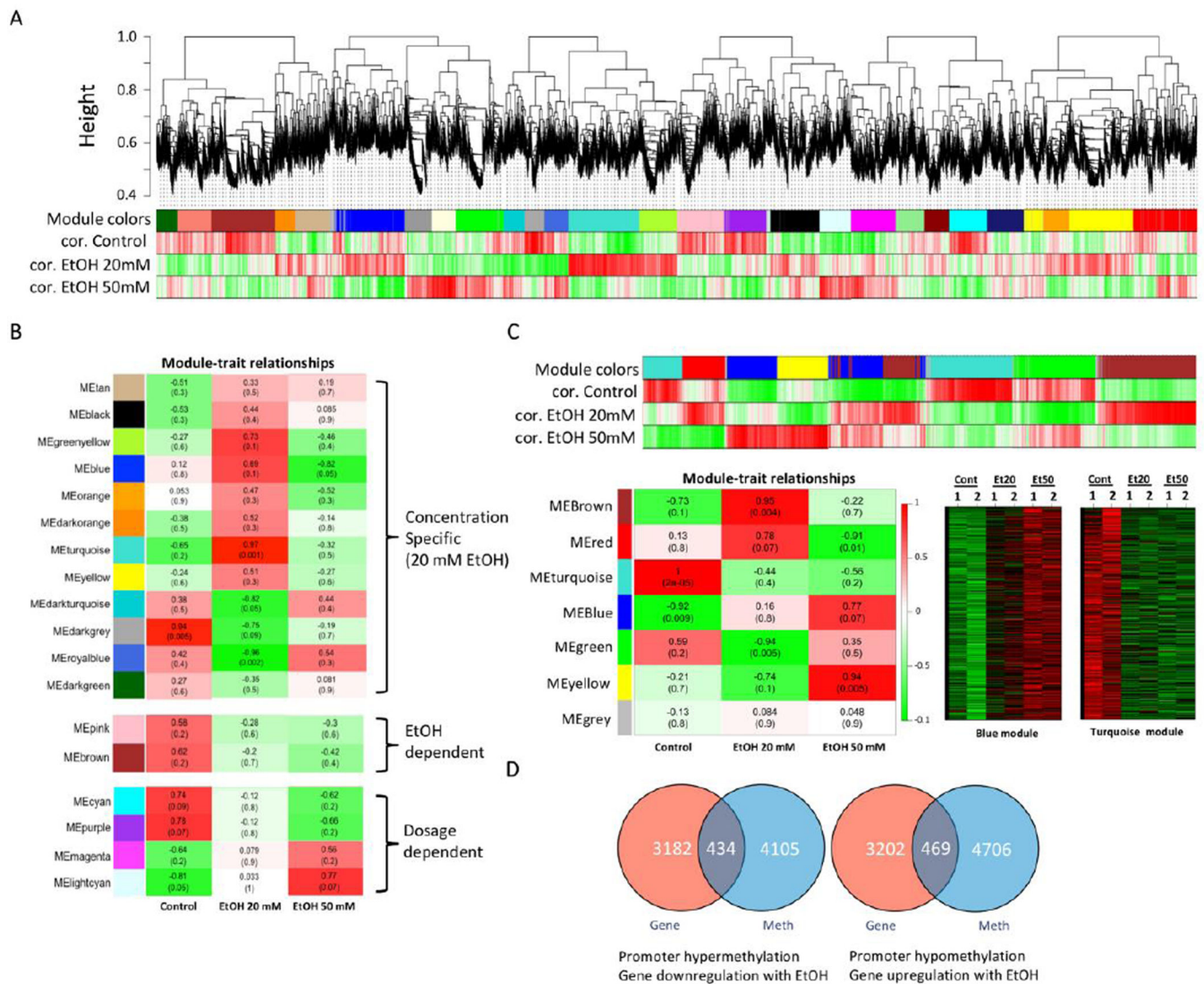


Figure 1. Transcriptome analysis by gene expression microarray

A. Weighted Gene Co-Expression Network Analysis (WGCNA) was performed to identify transcriptomic changes in DPSCs induced by EtOH. Expression microarray data was processed by WGCNA and modules of genes that are correlated together were identified. Modules are clusters of highly interconnected genes. In an unsigned co-expression network, modules correspond to clusters of genes with high absolute correlations. In a signed network, modules correspond to positively correlated genes. A group of genes (module) correlated to Control (cor. Control without EtOH treatment), EtOH 20 mM (cor. EtOH 20 mM) or EtOH 50 mM (cor. EtOH 50 mM) was depicted by different colors (module colors).

B. Intramolecular connectivity measures how connected, or co-expresses, a given gene is with respect to the genes of a particular module. The intramolecular connectivity is interpreted as a measure of module membership. Module-trait relationship map of all modules identified from analysis demonstrating specific EtOH concentration-dependent, EtOH treatment-dependent, or EtOH dosage-dependent gene association modules. **C.** WGCNA analysis on DNA methylomic changes to identify modules associated with EtOH

treatment and epigenetically regulated by EtOH-induced DNA methylation in DPSCs. Most significant modules were selected from the module-trait detection and corresponding heat-maps (blue and turquoise modules) are shown. **D.** Venn diagram analysis of transcriptome vs. DNA methylation to display the number of epigenetically regulated genes. Dosage-dependent hypermethylation and correlated decrease in gene expression due to EtOH. Dosage-dependent hypomethylation and correlated increase in gene expression due to EtOH.

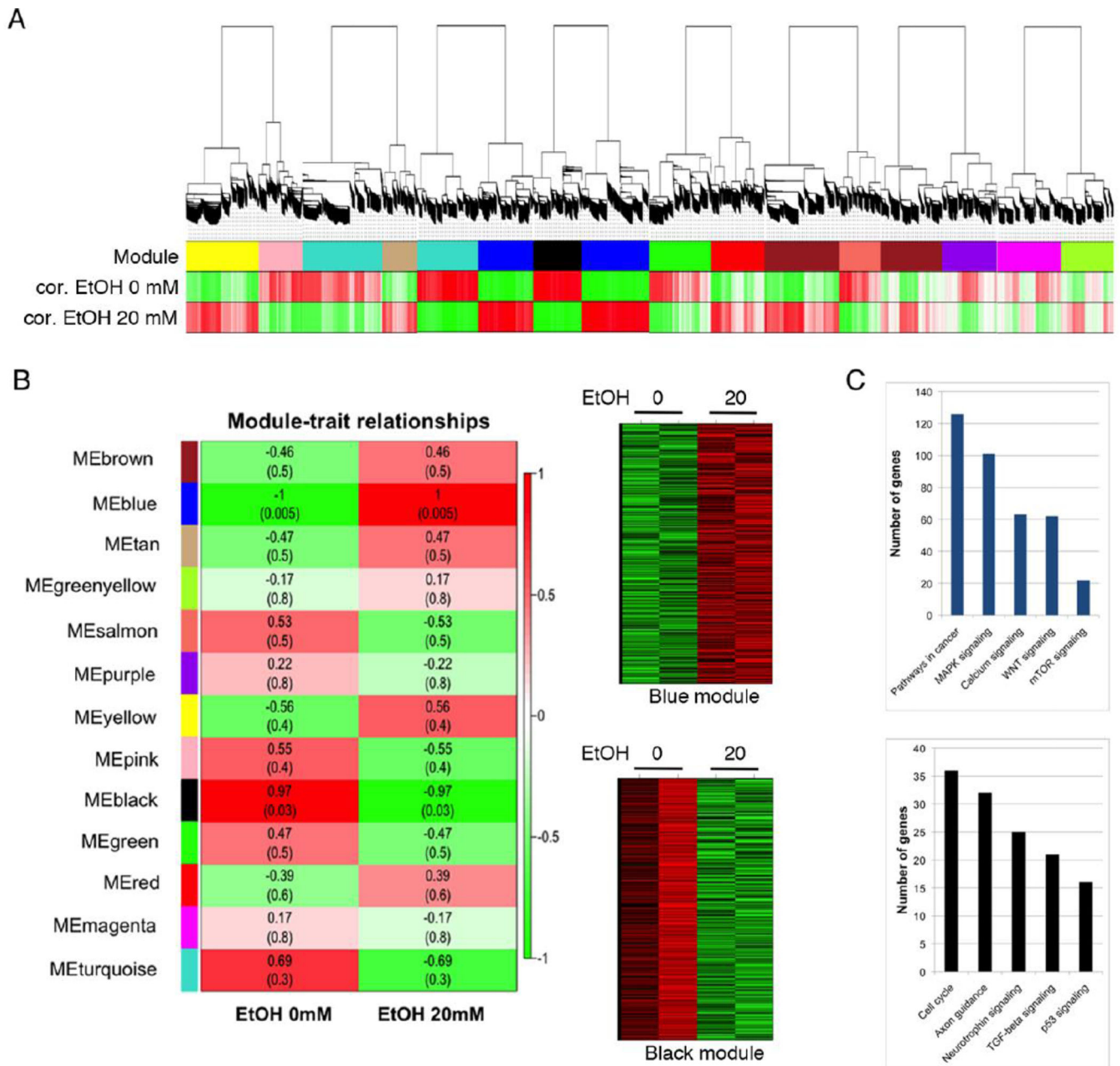


Figure 2. WGCNA on DPSCs treated with 20 mM EtOH

A. WGCNA for transcriptomic changes induced by 20 mM EtOH treatment that is comparable to a 0.08% blood alcohol concentration (BAC) of the DUI level, leads to EtOH-induced gene expression changes in DPSCs. **B.** Module-trait relationship map and heatmap analysis of the black and blue modules, or gene expression profiles, where red indicates up-regulation and green indicates down-regulation. **C.** The Database for Annotation, Visualization and Integrated Discovery (DAVID) gene functional analysis on the blue and the black module.

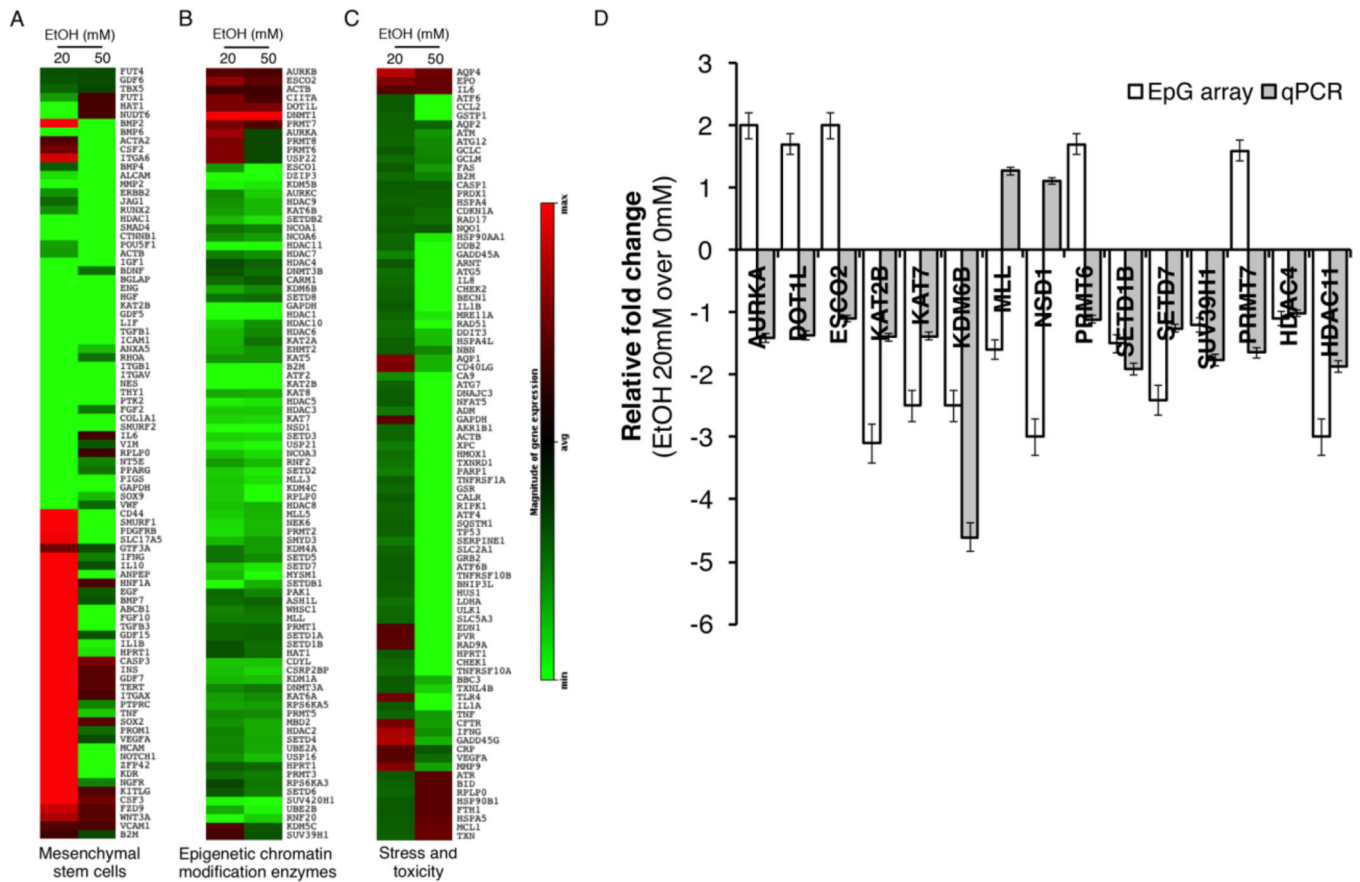


Figure 3. Pathway focused RT-PCR array analysis for genes affected in DPSCs by EtOH treatment

For acute exposure DPSCs were treated for 24hrs with 20 or 50mM EtOH. For chronic exposure cells were treated every other day for 10 days with 20 or 50mM EtOH. Total RNA was prepared and subjected to RT-PCR array analysis. **A.** Fibroblastic marker array, **B.** Epigenetic chromatin modification enzymes array. **C.** Stress and toxicity pathway finder array. Data was analyzed and fold changes against no treatment are presented. **D.** Quantitative RT-PCR analysis was done to validate the result from Epigenetic modifier RT array. Error bar shows the standard error margin (SEM).

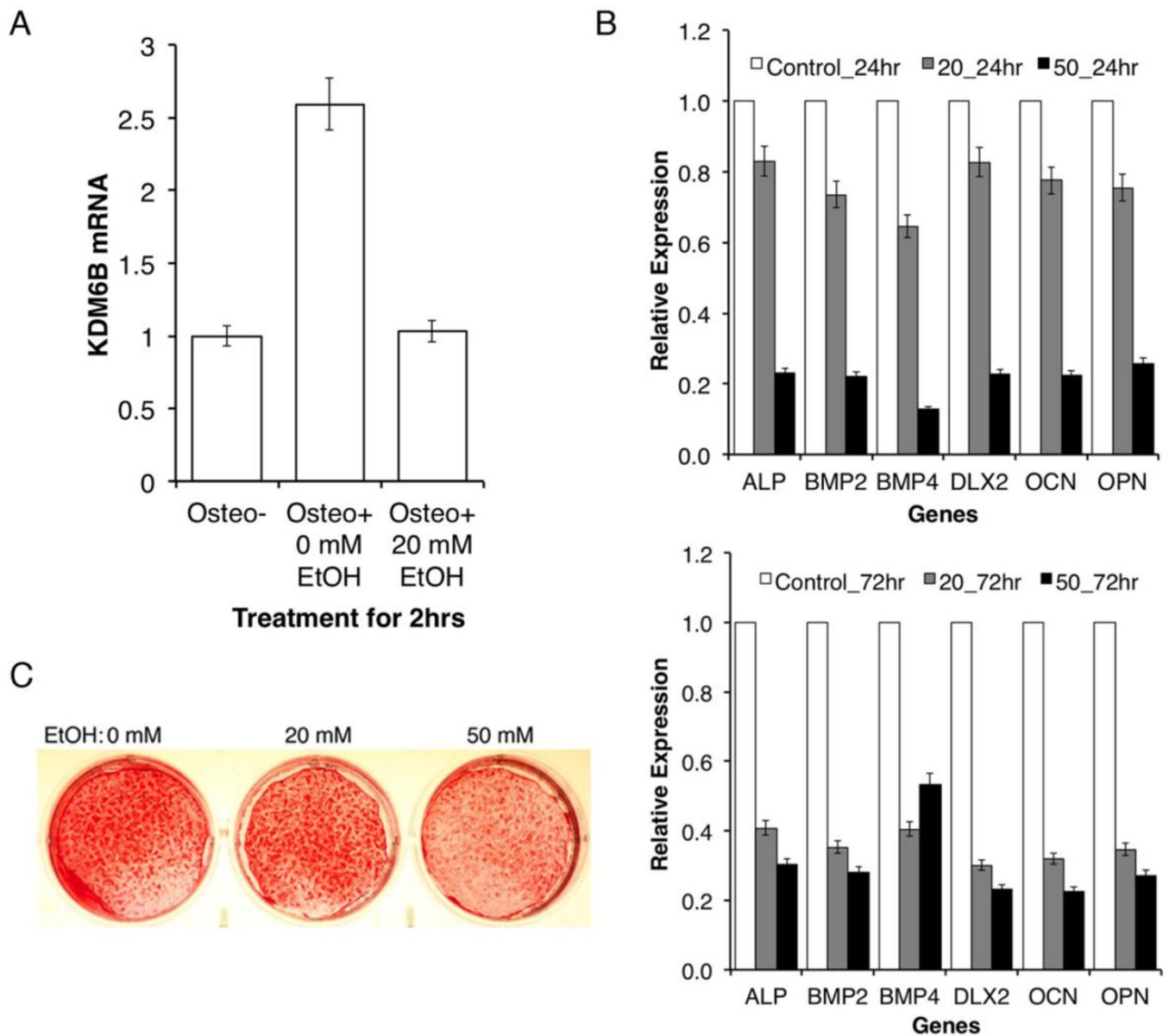


Figure 4. Effect of EtOH on molecular regulation of odontogenic/osteogenic differentiation

A. Comparison of the fold changes of *KDM6B* expression with and without odontogenic/osteogenic differentiation treatment in DPSCs. In response to EtOH, *KDM6B* expression is significantly reduced during differentiation. Error bar shows the standard error margin (SEM). Statistical significance was determined by Student *t*-test ($p < 0.05$). **B.** Comparison of the relative expression levels of osteomarkers in DPSCs cells under differentiation with or without treatment (20 mM or 50 mM EtOH) for 24 hr and 72 hr. At both 24 hr and 72 hr, reduced levels of various osteomarkers were observed. Statistical significance was determined by the one-way ANOVA ($p < 0.05$). Error bar shows the standard error margin (SEM). **C.** Disruptive effects of EtOH on mineralization *in vitro*. Reduced levels of mineralization assessed by Alizarin staining were observed in response to EtOH.

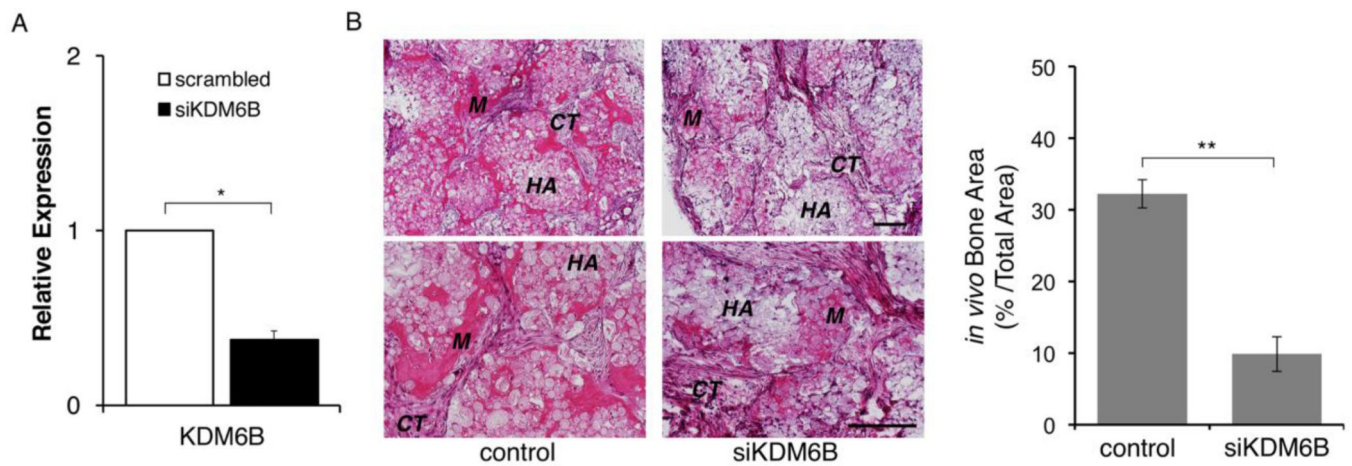


Figure 5. Knockdown of KDM6B resulted in a reduced mineralization potential *in vivo*

A. DPSCs were transiently transfected with a scrambled *siRNA* or *siKDM6B* construct (Thermo Scientific, Waltham, MA). Quantitative RT-PCR analysis showed >2-fold reduction in *KDM6B* in *siKDM6B* cells. Error bar shows the standard error margin (SEM). Statistical significance was determined by Student *t*-test (*: $p < 0.05$). **B.** DPSCs transfected with control (scrambled *siRNA*) or *siKDM6B* cells were implanted into mice as described in Materials and Methods. Formation of mineralized tissue (M) and connective tissue (CT) around HA/TCP (HA) are indicated in H&E staining section. Quantitative measurement showed that *siKDM6B* resulted in about 66% reduction in bone area compared to the control. Statistical significance was determined by Student *t*-test (**: $p < 0.05$). Error bar shows the standard error margin (SEM).

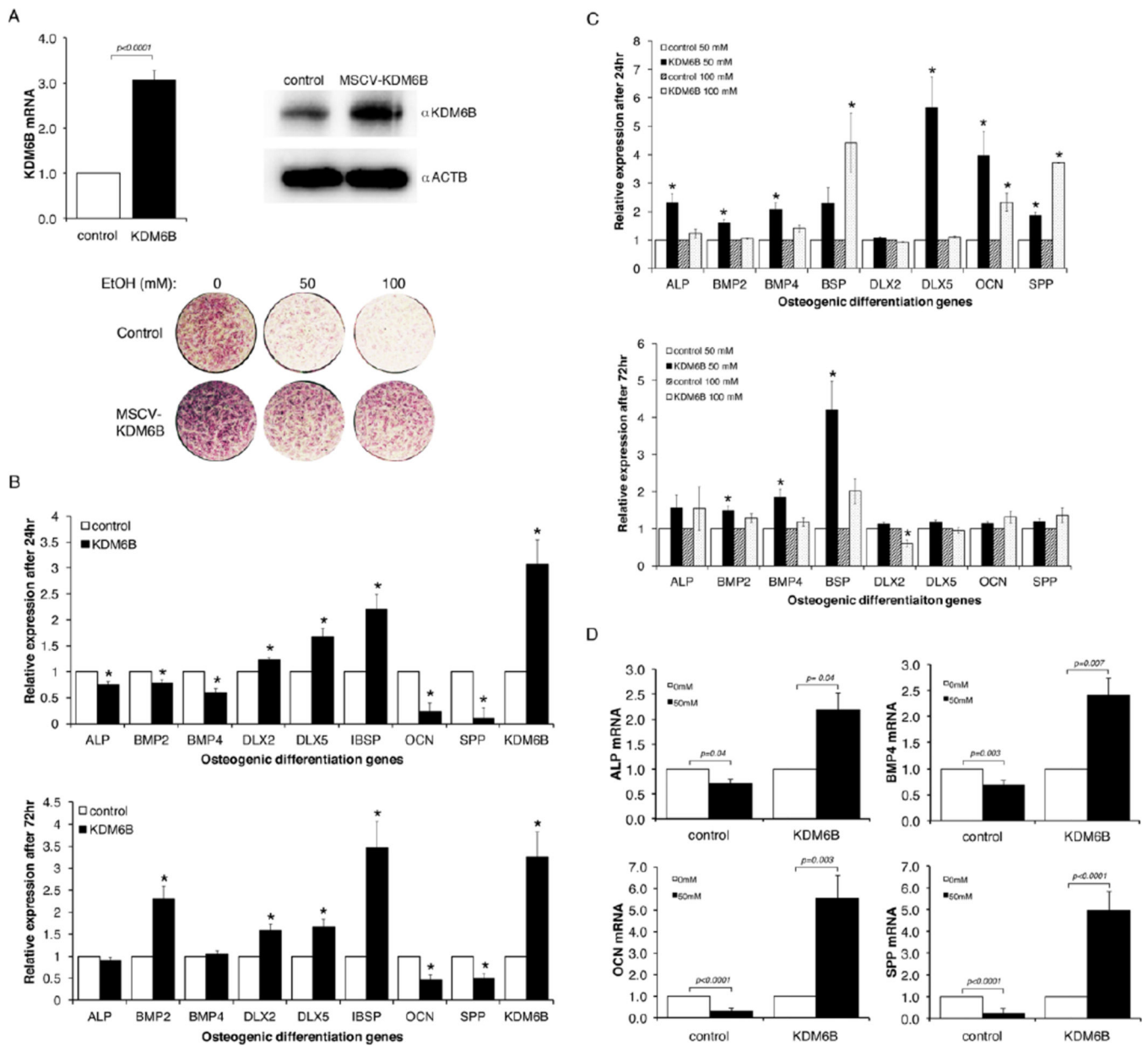


Figure 6. Expression of KDM6B restored differentiation potency in EtOH-treated DPSCs
A. DPSCs were transduced with retroviral KDM6B expression construct (MSCV-KDM6B) or control virus. Three days after transduction cells were lysed and total RNA or protein was purified. The expression of transduced KDM6B was monitored by qRT-PCR or Western analysis using anti-KDM6B (1:1,1000, OriGene Technologies, Inc., Rockville, MD). For protein loading control anti-actin antibody (1:3,000, Sigma, St. Louis, MO) was used. Transduced DPSC cells were plated into 24 well plates and treated with mineralization media containing 0, 50 or 100 mM EtOH for 2 weeks with daily medium change. Differentiation of DPSCs was monitored by alkaline phosphatase staining. **B.** The effect of overexpressing KDM6B in DPSCs on the expression of differentiation-related genes was examined. DPSCs transduced with either control or MSCV-KDM6B were differentiated in

osteogenic media for 24 or 72 hrs and the level of differentiation-related genes was assessed by qRT-PCR in triplicates. Ectopic expression of KDM6B resulted in a differential regulation of lineage marker genes. For both 24 and 72 hr differentiation, *DLX2*, *DLX5* and *IBSP* were consistently induced by KDM6B, but *OCN* and *SPP* were consistently suppressed. Statistical significance was determined by the one-way ANOVA and *p* value <0.05 is considered as significant (with asterisk). Error bar shows the standard error margin (SEM). **C.** Transduced cells were plated into 6 well plates and >90% confluent cells were treated with odontogenic/osteogenic differentiation media containing 50 or 100 mM EtOH for 24 and 72 hrs. Total RNA was isolated and qRT-PCR analysis was performed for various osteogenic marker genes. Comparison was made within samples for control cells and KDM6B expressing cells by determining the relative fold change for 50 or 100mM EtOH treatment. Statistical analysis was done on triplicate samples using the one-way ANOVA and *p* value <0.05 is considered as significant (with asterisk). Error bar shows the standard error margin (SEM). **D.** The effect of EtOH treatment on mineralization-associated gene expression was compared between DPSCs transduced with the control virus and overexpressing KDM6B. DPSCs transduced with either the control virus or MSCV-KDM6B were differentiated for 24 hrs in the presence of 0 or 50 mM EtOH. The levels of mineralization-associated genes were determined by qRT-PCR analysis. Statistical significance was determined by the one-way ANOVA. Error bar shows the standard error margin (SEM).

Table 1List of genes from DPSCs treated with 20 or 50 mM EtOH for 24 hrs ($p < 0.05$).

EtOH 20 mM			
Gene Symbol	Fold change	Gene Symbol	Fold change
ELK4	4.4	DDR2	-3.2
MGC24103	2.9	ITPRIPL2	-2.8
ZCCHC7	2.9	MALAT1	-2.7
SPATS2L	2.8	TMPO	-2.6
SPEN	2.7	ZYG11B	-2.6
SATB2	2.7	KDM6B	-2.5
PPP1R9B	2.5	CALD1	-2.5
NCRNA00182	2.4	ZNF655	-2.5
CLCN5	2.3	C13orf37	-2.4
LRP1	2.3	RPL37A	-2.3
MCTP2	2.3	APOOL	-2.2
RNF167	2.2	LOC100288675	-2.2
RAB3B	2.2	PTPN11	-2.1
KIAA0754	2.2	EIF5B	-2.1
QSER1	2.2	ATG3	-2.1
MAP4K4	2.2	MALAT1	-2.1
NFAT5	2.2	KPNA5	-2.1
LOC286272	2.1	DHFR	-2.0
ANKRD28	2.1	WSB1	-2.0
LOC100289230	2.1	C6orf62	-2.0
RALGPS2	2.1	N4BP2L2	-2.0
RANBP2	2.1	ESCO1	-2.0
ITGA8	2.1	EHBP1L1	-2.0
PIK3C2A	2.0	PTPRO	-2.0
MYCBP2	2.0	ERCC8	-2.0
LOC572558	2.0	GNG12	-2.0
LRCH3	2.0		
PARVA	2.0		
CD44	2.0		

EtOH 50 mM			
Gene Symbol	Fold change	Gene Symbol	Fold change
ELK4	2.9	RPL10L	-4.3
CALD1	2.9	MALAT1	-2.9
SUZ12P	2.3	ATG3	-2.7
MCTP2	2.2	RBM5	-2.3
RASEF	2.1	SNX11	-2.3
GUSBP3	2.0	SSEA2	-2.2

EtOH 50 mM			
Gene Symbol	Fold change	Gene Symbol	Fold change
LRP1	2.0	LAMA3	-2.1
COL12A1	2.0	METTL7A	-2.1
		LRRC27	-2.1
		SLAIN2	-2.1
		MALAT1	-2.1
		SEC62	-2.1
		C11orf31	-2.0
		C13orf31	-2.0
		USP28	-2.0
		DDR2	-2.0
		LARP4	-2.0

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