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Molecular Effect of Alcohol on the Osteogenic Potency of Dental Pulp Stem Cells

Author
Hoang, Michael Tuan

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Molecular Effect of Alcohol on the Osteogenic Potency of Dental Pulp Stem Cells

A thesis submitted in partial satisfaction

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in Oral Biology

by

Michael Hoang

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ABSTRACT OF THE THESIS

Molecular Effect of Alcohol on the Osteogenic Potency
of Dental Pulp Stem Cells

by

Michael Hoang

Master of Science in Oral Biology
University of California, Los Angeles, 2018
Professor Yong Kim, Chair

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. Epigenetic changes have been proposed as a molecular mechanism underlying the effect of alcohol on the osteogenic potency of adult stem cells. Through genome-wide gene expression microarray and DNA methylome analysis, we identified that lysine specific demethylase 6B (KDM6B), an epigenetic modifier known to be involved in the osteogenic and odontogenic potency in DPSCs, was significantly downregulated by EtOH. We hypothesize that EtOH downregulates the osteogenic potency of DPSCs through KDM6B and its upstream regulatory factors. In order to investigate the upstream and downstream effects of EtOH on the osteogenic potency of

DPSCs through KDM6B, we performed a pathway-focused RT-PCR array, western blot, immunofluorescence, and ChIP analyses, and *in-vivo* experiments. EtOH treatment during 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. In addition, we investigated upstream regulators and identified a link between Orai1 and the SMAD1 pathway on KDM6B and osteogenic differentiation. We have identified that osteoinduction decreases Ca2+ influx with a concurrent upregulation in Orai1 and Stim1 expression levels, which are further increased in the presence of EtOH during osteogenic differentiation. We believe the changes observed in Orai1 are indicative of cellular compensation in the presence of osteogenic differentiation and EtOH in order to regulate KDM6B expression and ultimately osteogenic potency. Finally, we have observed that EtOH exposure decreases the osteogenic potential in naïve DPSCs. Our study has demonstrated that EtOH-induced inhibition of KDM6B plays a role in the downregulation of osteogenic differentiation in DPSCs. 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.

The thesis of Michael Hoang is approved.

Christine Hong

Shen Hu

Ki-Hyuk Shin

Yong Kim, Committee chair

University of California, Los Angeles

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BACKGROUND

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 (Khalid et al., 2014), 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 (Schuckit, 2009). While some studies have indicated that low doses of alcohol consumption may increase bone mass and density (Feskanich et al., 1999; Jugdaohsingh et al., 2006), 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 (Sampson, 1997). The effects of alcohol consumption on bone health are multi-factorial and are related to the duration and dosage of consumption (Maurel et al., 2012). Recently, ethanol has been shown to have an adverse effect on neural function and be associated with alcohol-related peripheral neuropathy through dysregulation of P2RX3 and neuroactive ligand-receptor interaction.

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 (Church et al., 1997). 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 (Sant'Anna et al., 2005). The dental anomalies observed may stem from cellular alterations in the basal layer of the epithelium of the tooth germ during odontogenesis (Sant'Anna et al., 2005; Kieser, 1992; Jimenez-Farfan et al., 2005, 2005).

In this study, we hypothesize that a genome-wide discovery approach investigating the effects of EtOH on DNA methylation patterns and related gene expression will lead to the identification of key epigenetic modifiers, such as KDM6B. Through the downregulation of KDM6B, we believe EtOH exposure epigenetically reduces the osteogenic potency of DPSCs and influences related upstream regulatory pathways modifying KDM6B expression.

INTRODUCTION

Dental Pulp Stem Cells

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 (Papaccio et al., 2006; Laino et al., 2006). 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 toward odontogenic and osteogenic lineages. One study demonstrated that vascular endothelial growth factor (VEGF) gene could promote odontogenic differentiation in DPSCs in vitro (Zhang et al., 2014) while another identified that DPSCs undergo osteogenic differentiation through the NF-kB signaling pathway (Wang et al., 2013). In

addition, dexamethasone-induced osteogenesis has been demonstrated in rat mesenchymal stem cells through regulation of hedgehog signaling molecules, modulation the phosphorylation of Runx2, and upregulation of transcriptional coactivator with PDZ-binding motif (TAZ) expression (Ma et al., 2013, Phillips et al., 2006, Hong et al., 2009).

DPSCs had the ability to differentiate toward both odontogenic and osteogenic lineages in the presence of a carboxymethyl cellulose- hydroxyapatite hybrid hydrogel (Teti et al., 2015). Furthermore, medium modification with bone morphogenetic protein 2 was shown to stimulate odontogenic differentiation and formation of an osteo-dentin matrix (Atalayin et al., 2016). Although DPSCs have long been studied for their regenerative capabilities in both dentistry and orthopedics, the molecular mechanisms controlling their stem cell potency have yet to be discovered.

KDM6B and its epigenetic effect on osteogenic and odontogenic differentiation

Epigenetics is the study of heritable changes in gene expression without alteration of the underlying nucleotide sequences. Through this manner, genetic activity is regulated through processes such as DNA methylation, post-translational histone tail modification, and modification of chromatin structure (Sakurai et al., 2014). In the regulation of cellular processes and key transcription factors in MSCs, epigenetic processes can alter gene regulatory and coding regions to determine MSC fate. KDM6B, a lysine-specific demethylase, has been shown to be epigenetically involved in a wide variety of cell lineage decisions in MSCs, including neural differentiation genes, macrophage transdifferentiation, and mammalian epidermal differentiation (De Santa et al., 2007, Jespen et al., 2007, Sen et al., 2008)**.** It has been shown that KDM6B plays a key role in osteogenic differentiation by removing H3K27me3 from the promoters of osteogenic genes in human bone marrow stromal cells (BMSCs) (Ye et al., 2012). H3K27me3 has been identified in critical

developmental events such as cell cycle regulation, mammalian X chromosome inactivation, and stem cell identity (Schuettengruber et al., 2007). Another study identified KDM6B in controlling HOX expression through the removal of H3K27me3 in human BMSCs (Ye et al., 2012). A recent study has shown KDM6B to play a critical role in the epigenetic regulation of odontogenic differentiation in human DPSCs (Xu et al., 2013). 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) (Xu et al., 2013). Decreased levels of these markers and mineralization parallel effects observed in postmenopausal osteoporotic patients, as they exhibit significant reductions in the number of osteocytes and osteoblasts, and demineralization of compact and cancellous bone (Pavel et al., 2016).

SMAD Pathway and KDM6B expression

BMP signaling pathway and expression of bone morphogenic proteins, such as BMP-2, -4, -6, -7 and -9, have been known to be potent inducers of osteogenic differentiation in MSCs (Muruganadan et al., 2009). The osteogenic role of BMP signaling has also been shown *in vivo,* with knockout of BMP2-, BMP-4, or SMAD1/5 resulting in varying degrees of skeletal deficiencies and abnormalities (Chen et al., 2012). Similarly, studies in mice have shown BMP signals regulate bone development and remodeling through the stimulation of osteoblasts and osteoclasts (Mishina et al., 2004, Okamoto et al., 2006). BMP signaling pathway involves intracellular mediators known as SMAD proteins, which dimerize and translocate to the nucleus when activated. For example, phosphorylation of SMAD1/5/8 leads to activation of transcription factors such as RUNX2, a master regulator gene for osteogenesis (Ducy and Karsenty, 1995). Recently, BMP4/7 has been shown to strongly induce KDM6B expression in MSCs (Yu et al, 2014). In addition,

knockdown of SMAD1 and SMAD4 has been shown to significantly reduce expression of KDM6B in MSCs (Yu et al., 2014). This study identified SMAD1 as a mediator between BMP signaling and KDM6B expression and provides insight into upstream regulators behind EtOH effects on KDM6B. Interestingly, ORAI1 has been shown to mediate osteogenic differentiation through the BMP signaling pathway in bone marrow stem cells (BMSCs). In this study, BMP2 successfully phosphorylated SMAD1, but not in ORAI1-/- BMSCs, which led to activation of Runx2, Dlx5, and Osx gene expression (Lee et al., 2016).

ORAI1 Pathway and EtOH effects

Calcium (Ca2+) is a highly abundant, intracellular signaling molecule that is intricately involved in the regulation and maintenance of biological processes, including cell differentiation, proliferation, and apoptosis (Apati et al., 2012**,** Tonelli et al. 2012.) Ca2+ signaling and its ability to regulate processes lie in the speed, amplitude, and spatiotemporal patterning of Ca2+ concentration (Berridge et al., 2000). Basic calcium signaling pathways were first reported in mouse embryonic stem cells, where it was demonstrated that ATP and histamine induced a transient increase in intracellular Ca2+, while caffeine and ryanden had no effect (Yanagida et a., 2004). In another study in mESCs, lysophosphatidic acid, a physiological compound involved in serum-dependent cell growth, was shown to induce calcium signaling and intracellular Ca2+ release to increase c-myc expression and increased mESC proliferation (Todorova et al., 2009).

In non-excitable cells, Ca2+ influx is mediated by store-operated Ca2+ entry (SOCE) and store-operated Ca2+ release-activated Ca2+ (CRAC) channels (Prakriya et al., 2006). CRAC channel components, such as ORAI1 and STIM1, regulate the influx of extracellular Ca2+ and release of Ca2+ from the endoplasmic reticulum, respectively.

(Feske et al., 2006). ORAI1 has been known to play an integral role in the nuclear factor of activated T cells (NFAT) signaling pathway necessary for the activation and differentiation of T cells (Srikanth and Gwack, 2013). Recently, the Orai1 pathway has been implicated in oral/oropharyngeal carcinogenesis through the Orai1 and NFAT signaling pathway (Lee et al., 2016). In other studies, mutations in ORAI1 and STIM1 have been shown to be associated with ectodermal dysplasia, defective dentition, and immunodeficiency (McCarl et al. 2009; Picard et al. 2009). In liver cells, ethanol exposure has been shown to increase the expression of STIM1 and ORAI1 at both acute and chronic exposure levels. This ethanol-induced upregulation of SOCs such as ORAI1, and STIM1 may be molecular mechanisms through which EtOH induces liver cell injury (Cui et al., 2013). The concurrent increase in cytoplasmic calcium levels suggests that ethanol increases extracellular Ca2+ influx through the upregulation of calcium channel proteins.

Interestingly, the significance of the ORAI1 pathway has been demonstrated in bone formation (Hwang et al., 2012; Robinson et al., 2012). Orai1 has been suggested to mediate osteogenic differentiation through bone morphogenic protein (BMP) signaling pathway and phosphorylation of SMAD1/5/8 in bone marrow mesenchymal stem cells (Lee et al., 2016). Orai1-/- mice were observed to develop osteopenia through decreased mineral density and trabecular bone volume (Hwang et al. 2012). Osteoblasts, which form bone, and odontoblasts, which form dentin, are similar in nature in their ability to form extraceullular matrices and mineralized hydroxyapatite (Gronthos et al., 2002). Consistent with previous findings in bone-forming osteoblasts, ORAI1 was demonstrated to play a critical role in odontogenic differentiation and mineralization in DPSCs through the regulation of Ca2+ influx (Sohn et al., 2015).

While DPSCs primarily differentiate to dentin and BMSCs differentiate to bone, both dentin and bone formation share similar mineralization matrix components. Using the

DPSC model of mineralization will give us insight on the molecular effects of EtOH on mineralization matrix that is similar to the 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. Furthermore, we hope to elucidate upstream regulatory mechanisms behind EtOH's effects on KDM6B and osteogenesis through Orai1 and the BMP signaling pathway. Through this manner, we suggest that EtOH downregulates the osteogenic potency of DPSCs through its effects on upstream and downstream signaling cascades and cellular processes.

MATERIALS AND METHODS

Culture of human DPSCs and EtOH treatment

Early passage DPSCs (P1–P2) that were isolated from adult teeth (molars) were cultured in α-MEM supplemented with 10% fetal bovine serum (v/v), 2 mM L- glutamine, 100 μM L-ascorbate-2-phosphate, 50 units/ml penicillin and 50 μg/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 (0, 20, 50, or 100mM) for 24, 48, or 72 hours. For chronic exposure, cells were intermittently exposed to EtOH by one-day 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. An equal amount from each sample was subjected to biotinylation using the BioArray High Yield RNA Transcript Labeling System (Enzo Life

Sciences, Farmingdale, NY, USA). An equal amount of cRNA from each sample was labeled, purified and fragmented by using the 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) (Kim et al., 2012). Methylation array analysis was performed according to a previous standard protocol from our laboratory (Kim et al., 2012). Genomic profiling was performed by NimbleGen Systems (100718 HG18 CpG Refseq Promoter MeDIP). 3μg of sonicated total DNA as input and 4μg 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 log2 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. The resulting score for each probe was–log10 (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 cut off (–log10) of 2. Using a custom Unix code, we aligned “ratio peak p-values” to human genome18 (RefSeq.hg18) and created a matrix file.

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 (SA Biosciences, 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 the 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 (SA Biosciences, Valencia, CA).

Odontogenic/osteogenic differentiation of human DPSCs and EtOH treatment

DPSCs were grown in mineralization-inducing media containing 100 μMascorbic acid, 2mMβ-glycerophosphate and 50nM dexamethasone. Exponentially growing DPSCs were grown until confluency and subsequently 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 h. 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 retroviralMSCV-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 = 21.7 M). Cells were fed with differentiation media containing given concentrations of EtOH (50 or 100 mM) for an 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 a Turbo DNAfree kit (Life Technologies, Grand Island, NY). 2μg of total RNA treated with a Turbo DNA-free kit (Thermo Fisher Scientific, Waltham, MA) was used to synthesize cDNA by using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in 40μl of a reaction mixture. The resulting cDNA was diluted (mixed with H2O by 1:4) and 1.5μl of diluted cDNA was used per well (in 10μl reaction volume) in a 384 well plate using a LightCycler 480 SYBR Green I master mix (Roche Diagnostics, Indianapolis, IN). PCR was done with a specific set of primers (Table 1) at an annealing temperature of 60 °C.

Table 1. Primer sequences for qRT-PCR analysis

GENES	FORWARD (5'-3')	REVERSE (5'-3')
ALP	GACCTCCTCGGAAGACACTC	TGAAGGGCTTCTTGTCTGTG
BMP2	GTCAACTCGATGCTGTACCTTGACG	CAACCCTCCACAACCATGTCC
BMP4	CGGCGAAGAAGAATAAGAACTGCCG	CCAGTCATTCCAGCCCACATC
DLX2	CCTGAGAAGGAGGACCTTGA	TTCCGGACTTTCTTTGGCT
DLX5	CGCTAGCTCCTACCACCAGT	GGCTCGGTCACTTCTTTCTC
IBSP	CAGGCCACGATATTATCTTTACA	CTCCTCTTCTTCCTCCTCCTC
KDM6B	GCACTACTGGGAGACCATCA	ACCAGGAACCCGTCAAGTAG
OCN	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGGTCTCTTCACT
ORAI1	GCTCATGATCAGCACCTGCAC	GGGACTCCTTGACCGAGTTG
ORAI2	CTGCATCCTGCCCAATGTG	GAGTTCAGGTTGTGGATGTTGCT
ORAI3	AGCTGTGAGCAACATCCACAAC	CCACGTAGCGGTGCAGTCT
OPN	ATGATGGCCGAGGTGATAGT	ACCATTCAACTCCTCGCTTT
STIM1	TGACAGGGACTGTGCTGAAG	AAGAGAGGAGGCCCAAAGAG
GAPDH	GGTGCTGAGTATGTCGTGGA	CTAAGCAGTTGGTGGTGCAG

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 10mM sodium phosphate for 30 min at room temperature.

Implantation of DPSCs into immunocompromised mice

6.0 × 106 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 a microscope. For quantification, the NIH software Image J was used as previously described (Miura et al., 2004).

Western Blot Analysis

Cells were washed three times with PBS before treatment with ice-cold lysis buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA and 1% Triton X-100). The samples were separated on a 10% SDS polyacrylamide gel and transferred to PVDF membrane by a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked with 5% non-fat milk for 1 hr at room temperature, and then incubated with primary antibodies overnight and proved with the respective secondary antibodies. The signals were obtained using ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Primary antibodies were purchased from the following commercial sources: anti-phospho SMAD1/5/9 (1:1000; Cell Signaling, Danvers, Massachusetts), anti- SMAD1 (1:1000; Cell Signaling, Danvers, MA). The membranes were stripped and then reprobed with rabbit anti-GAPDH antibody (1:3000, Cell Signaling, Danvers, MA).

Immunofluorescence

Cells grown on UV-sterilized glass coverslips were fixed in 4% paraformaldehyde and blocked with 1% BSA for 60min at room temperature. The cells were then incubated at 4C overnight with rabbit monoclonal anti-SMAD1 antibody (catalog 6944, Cell Signaling Technology, USA, dilution 1:300) and rabbit polyclonal antibody anti- pSMAD1/5/9 (catalog 138201, Cell Signaling Technology, USA, dilution 1:300). The next day, the cells were washed three times with PBS-BSA for 10min, then incubated with goat anti-rabbit-FITC (ab6717; Abcam) for 1h at room temperature, and washed as previously described. Finally, the coverslips were mounted onto slides in mounting medium Fluoroshield with DAPI (F6057, Sigma). Images of all samples were acquired under a fluorescence microscope (Olympus, Tokyo, Japan).

Chromatin Immunopercipitation Assay

The assay was performed using a ChIP assay kit (Abcam, San Diego, CA) according to the manufacturer's protocol. Cells underwent formaldehyde treatment for 15min at 37C. All resulting precipitated DNA samples were quantified with Real-time PCR. Data is expressed through the fold enrichment method. Antibodies for ChIP assays were purchased from the following commercial sources: polyclonal anti-SMAD1 (Cell Signaling, Danvers, MA); polyclonal anti-IgG (Abcam, San Diego, CA). The primers for KDM6B are: forward, 5'-TCTGGCTACCTGTGCTGCTA-3'; reverse, 5'-ACAAGCCAGCCTGAGTCCTA-3'.

Ectopic expression of wild-type Orai1

The retroviral pMSCV-CITE-eGFP-Puro vectors encoding ORAI1WT was used to prepare viruses as described previously (Kim et al., 2010). These vectors were transfected into GP2-293 universal packaging cells (Clonetech, Mountain View, CA, USA) along with pVSV-G envelope plasmid using lipofectamine 2000 (Life Technologies). Detailed methods of retrovirus production and infection can be found in previous publications (Shin et al., 2008). Infected DPSCs were selected with 0.5μg/ml puromycin for two weeks and used for experiments.

Single-cell Intracellular Free Ca2+ Imaging

Cells were grown on UV-sterilized glass coverslips prior to imaging. Prior to imaging, cells were loaded with 1mM Fura 2-AM for 45min at 25C, and intracellular Ca2+ measurements were performed. Subsequently, cells were mounted in a RC-20 closed bath flow chamber (Warner Instrument Corp., Hamden, CT) and analyzed on an Olympus 1X51epifluorescence microscope with Slidebook (Intelligent Imaging Innovations, Inc.) imaging software, as previously described (Lee et al., 2016). Cells were perfused with

Ca2+-free Ringer's solution, and Ca2+ stores were passively depleted with 1uM thapsigargin. Fura-2 emission wafs detected at 510nm with excitation at 340 and 380nm, and the Fura-2 emission ratio (340/380) was acquired at every 5s interval after subtraction of background. For each image, 50-100 individual cells were analyzed using OriginPro (Origin lab) analysis software.

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 h) to a defined concentration of EtOH (20 or 50 mM). Cell growth analysis showed that 20 or 50 mM EtOH treatment did not induce a significant reduction in cell growth (Supplemental Fig. 1). To profile molecular signatures that are affected by EtOH treatment in DPSCs, we have performed gene expression microarray analysis on DPSCs after 24 h 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

concentrations. We observed differential molecular effects by different concentrations of EtOH, such as when 20 mM EtOH showed different patterns of expression changes compared to 50mMEtOH. This implies that EtOH at different concentration levels may have different physiological impacts through differential molecular responses. Recently, epigenetic effects, such as DNA methylamine, of alcohol on gene regulation have been documented (Khalid et al., 2014a, 2014b). 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 consists of genes whose promoters are hypermethylated with EtOH treatment and the turquoise module represents genes whose promoters are hypomethylated upon EtOH exposure (Fig. 1C). Heat maps show changes in DNA methylation in biological duplicates of DPSCs upon exposure to different concentrations of EtOH (0, 20 and 50 mM) for 24 h. 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 the potential effects of EtOH on the 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 Fig. 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 (N2-fold) and p values (b0.05) 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 50mM) and treated cells acutely for 24 h. Heat maps 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, DOT1L, 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 that 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 (Ye et al., 2012; Xu et al., 2013), 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 (Xu et al., 2013), the fold changes of KDM6B

expression with and without induction of differentiation treatment at 20 mM EtOH were analyzed at the 2 h 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 h time periods all exhibited concentration dependent reductions to EtOH, with more significant decreases at the 72 h 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. 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 EOH 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 (Ye et al., 2012; Xu et al., 2013). As KDM6Bwas 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 the activity of mineralizing cells (Benoit et al., 2006; Chen et al., 2007; George et al., 2006; Roostaeian et al., 2006; Zhang et al., 2006). 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 theMSCV-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 h 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 h 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 h compared to 24 h) 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 50mMEtOH treatment when compared within the control group (0mMEtOH) for genes ALP ($p=0.04$), BMP4 ($p=0.003$), OCN (p b 0.0001), and SPP (p b 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 (0mM), particularly at 5.53 fold ($p=0.003$) and 4.94 fold (p b 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 nonphysiological 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.

EtOH exposure downregulated KDM6B expression through the SMAD signaling pathway

To investigate whether EtOH exposure had an effect on SMAD1 pathway, an upstream regulator of KDM6B expression, we examined the effects of EtOH on phosphorylation of SMAD1. DPSCs were subjected to osteogenic media and varying levels of EtOH concentration for 24 and 72 h. It was found that in the presence of osteogenic media and no EtOH, pSMAD1 expression in DPSCs was notably increased (Fig. 7A). However, with increasing levels of EtOH in osteogenic media, pSMAD1 expression was markedly decreased with slight differences between 50 and 100mM EtOH concentrations. It was noted that the expression level of SMAD1 itself remained fairly consistent across all samples groups.

For further analysis, the localization and expression level of SMAD1 was investigated through immunofluorescence imaging. DPSCs were subjected to the same conditions as previously stated above and fixed on slides following 24 and 72 h of exposure. We found that without osteo induction, the expression level of pSMAD1 was in DPSCs was insignificant (Fig. 7B). In the presence of osteogenic media without EtOH, the expression level of pSMAD1 was significantly increased and localized in the nucleus, particularly at 24 h. The expression level of pSMAD1 was decreased in the presence of

EtOH at both 24 and 72 h time points. Similarly to our western blot analysis, the expression level and localization of SMAD1 was fairly consistent across all samples.

The effect of EtOH on SMAD signaling pathway and KDM6B expression was confirmed through ChIP analysis. We identified CAGACA and TGTCTG as the major binding sequences for SMAD1 and designed primers for KDM6B accordingly (Fig. 7C). Through fold enrichment analysis method, we found that osteogenic induction promoted recruitment of SMAD1 to the KDM6B promoter region and 6.38 fold ($p=0.001$) increase relative to mock IgG at 24 h (Fig. 7C). Consistently, exposure to EtOH in the presence of osteogenic media resulted in reduced levels of SMAD1 binding to the KDM6B promoter regions at 2.22 fold ($p=0.0001$) and 1.95 fold ($p=0.0004$) for 50 and 100mM EtOH, respectively. Overall, SMAD1 was recruited to the KDM6B promoter region in response to osteo induction and was reduced in the presence of EtOH.

Orai1 signaling pathway as an upstream regulator of KDM6B expression that is affected by EtOH

Based on the current literature and connection to the SMAD pathway through BMP signaling, Orai1 is thought to be upstream of KDM6B (Yu et al., 2014). To validate this, we performed over expression experiments of Orai1 and KDM6B to determine their relative effects on one another. Over expression of Orai1 in DPSCs resulted in a 2.95 fold ($p=0.044$) increase in KDM6B expression (Fig. 8A). However, overexpression of KDM6B no significant effect on the expression level of ORAI1 or Stim1 at 24 or 72 h timepoints.

To investigate whether EtOH has an effect on the Orai1 pathway, we examined the effects of 0, 50, and 100 mM EtOH concentrations on DPSCs in the presence of osteogenic media and expression levels of Orai1, Orai2, Orai3, and Stim1 at 24 h. In response to osteogenic induction at 24 h, Orai1 expression was increased by 1.75 fold ($p=0.006$), with

minimal change in response to EtOH (Fig. 8B). STIM1, a CRAC channel component, expression was increased 2.29 fold (p=0.0001), with increasing expression levels in response to higher levels of EtOH exposure at 2.54 fold (p=0.001) and 3.41 fold (p=0.001), for 50 and 100mM EtOH, respectively. Interestingly, Orai2 and Orai3 exhibited similar increases in response to EtOH in the presence of osteogenic induction. Notably, at 72h, Orai1 expression increased by 3.21 fold (p=0.001) with osteogenic induction in the absence of EtOH. However, with increasing EtOH concentration, Orai1 expression decreased to 2.68 fold (p=0.001) and 2.09 fold (p=0.003) for 50 and 100mM EtOH, respectively. Overall, for Orai2, Orai3, and Stim1, expression levels were reduced at the 72 h.

To investigate the relationship between EtOH and Ca2+ influx through the Orai1 signaling pathway, intracellular measurements on Ca2+ flow were recorded. In response to EtOH in the absence of osteogenic media, Ca2+ influx was markedly reduced from a mean ratio (340/380) of 0.8 to 0.6 and 0.85 to 0.7, at 50 and 100mM EtOH, respectively, at 24 h (Fig. 9A). Images of DPSCs showing SOCE responses, at time point of 630s, indicated that Ca2+ influx levels were higher at 0mM and lower in the presence of EtOH. In the presence of osteogenic media and without EtOH, store operated calcium entry (SOCE) was reduced similarly at both 24 and 72 h (Fig. 9B). In the presence of osteogenic media and EtOH, Ca2+ influx was reduced at 24h from a mean ratio of 0.65 to 0.55 at 24 h. However, at the 72 h timepoint, Ca2+ influx was increased from a mean ratio of 0.65 to 0.75.

Effects of EtOH on osteogenic potential of Naïve DPSC

To further investigate the dysregulatory effects of EtOH, we wanted to determine whether EtOH exposure before osteogenic induction downregulates the osteogenic potential in naïve DPSCs. DPSCs were subjected to 0, 50 and 100 mM EtOH for 24 and 72 h, followed by 24 and 72 h of osteogenic induction, respectively. Osteogenic induction was

confirmed through the increased expression of various osteomarkers, including ALP, BMP2, BMP4, DLX5, OCN, and SPP. We found that with osteo induction following exposure to 50 and 100 mM EtOH at 24 h, osteomarker expression levels were significant reduced (Fig. 10A). In particular, ALP expression level significantly decreased from 2.97 fold (p=0.003) to 2.30 fold (p=0.02) and 2.06 fold (p=0.05), BMP2 decreased from 2.05 fold (p=0.03), to 1.22 fold (p=0.019) and 1.09 fold (p=0.05), and OCN decreased from 1.98 fold (p=0.002)) to 1.62 fold (p=0.05) and 0.97 fold (p=0.01). At 72 h the effects of EtOH on osteogenic potential were much more pronounced. Consistently, we also found that osteomarker expression levels significantly decreased following osteo induction due to 72 h of prior EtOH exposure at both 50 and 100mM concentration levels (Fig. 10B). Most notably, DLX5 decreased from 2.97 fold (p=0.03) to 0.54 fold (p=0.004) and 0.57 (p=0.001), OCN decreased from 2.79 fold (p=0.031) to 0.31 fold (p=0.05) and 0.16 fold (p=0.002), and SPP, which decreased from 2.35 fold (p=0.011) to 1.46 fold (p=0.05) and 0.78 fold (p=0.05). Overall, our results further confirm that EtOH downregulates the osteogenic potency in naïve DPSCs.

SUMMARY AND CONCLUSION

Alcohol abuse appears to lead to periodontal disease, tooth decay and mouth sores that are potentially precancerous (Tezal et al., 2001; Amaral Cda et al., 2008; Yusko et al., 2008; Park et al., 2014). Persons who abuse alcohol are at high risk of having seriously deteriorated teeth, gums and compromised oral health in general (Tezal et al., 2001; Amaral Cda et al., 2008; Yusko et al., 2008; Park et al., 2014). It is generally believed that alcohol may have toxic effects on various cellular functions, but we lack 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 (Khalid et al., 2014a). Several studies have demonstrated effects of EtOH on DNA methylation, resulting in genetic and phenotypic changes (Pandey et al., 2008; Oberlander et al., 2008; Shukla et al., 2008; Ouko et al., 2009; Haycock, 2009; Liu et al., 2009; Moonat et al., 2010; Qiang et al., 2010; Miranda et al., 2010; Kaminen-Ahola et al., 2010). It has been shown that EtOH induced alterations in DNA methylation patterns and inhibited neural stem cell (NSC) differentiation (Zhou et al., 2011). 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 (Hicks et al., 2010). 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) (Zhou et al., 2011). 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 (Feskanich et al., 1999). 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 (Kim et al., 2003). Recent studies have reported that alcohol may affect bone formation through osteocyte apoptosis, oxidative stress, and Wnt signaling pathway modulation (Maurel et al., 2012). 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 (Callaci et al., 2009). 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 (Feskanich et al., 1999; Jugdaohsingh et al., 2006). Another study found that light alcohol consumption resulted in increased lumbar spine bone mineral density (BMD) and whole body BMD in postmenopausal women (Ilich et al., 2002). In animal studies, low alcohol consumption of ethanol (5%, 2 h per day) in 4-week old rats showed higher BMD and greater trabecular thickness than compared to the control groups (Yamamoto et al., 1997). 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 (Johnson et al., 2014). 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 the 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 densityand 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 (Turner, 2000). 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 (Iwaniec and Turner, 2013). 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 co-morbidity factors including poor nutrition, vitamin deficiencies, mechanical loading, weight and smoking (Turner, 2000). 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 a 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 remain to be discovered.

Recent studies show that KDM6B is involved in the control of calcium-induced differentiation (Sen et al., 2008), regulates osteoblast differentiation (Yang et al., 2013), and contributes to neuronal survival and differentiation (Wijayatunge et al., 2014), odontogenic differentiation of DPSCs (Xu et al., 2013), and osteogenic differentiation of human BMSCs (Ye et al., 2012). It has been reported that calcium-induced differentiation leads to increased binding of KDM6B and erasure of repressive marks such as H3K27me3 (Sen et al., 2008). It has been demonstrated that BMP4/7-mediated activation of SMAD1/4 may induce the expression of KDM6B and trigger the osteogenic pathway (Ye et al., 2012). Mechanistically, KDM6B is recruited to bone morphogenic protein 2 (BMP2) and HOX (homeotic genes) promoters and activate the odontogenic differentiation-related gene expression (Ye et al., 2012). KDM6B removes epigenetic marks such as H3K27me3, from

the promoter of osteogenic genes to promote osteogenic commitment (Ye et al., 2012). In addition, a recent study has demonstrated the facilitating role of KDM6B on odontogenic differentiation (Xu et al., 2013). It needs to be experimentally demonstrated if EtOH triggers dysregulation of KDM6B via altered regulation of differentiation signal-induced activation of the SMAD1/4 pathway and the control of the H3K27me3mark as demonstrated in BMSCs (Ye et al., 2012).

BMP4/7 signaling has been shown to induce osteogenesis through the SMAD1 pathway (Yu et al., 2014). In this manner, KDM6B expression is activated via SMAD, which in turn removes gene silencing marks to promote osteoblastogenesis. Our findings have identified an upstream regulatory link between EtOH and KDM6B expression through the downregulation of phosphorylated SMAD1, the active form of SMAD1. BMP signaling has long been associated with the activation of SMAD1 to induce activation of a variety of cell types, including osteoblasts (Miyazono et al., 2005, Massague et al., Sieber et al, 2009, Yu et al, 2014). Recently, Orai1 has been shown to regulate osteogenic differentiation through BMP signaling (Lee et al, 2016). Our results show that EtOH downregulates the osteogenic potential of DPSCs through its impacts on Ca2+ influx, Orai1, and related BMP signaling pathway that involves SMAD1 and ultimately KDM6B. Our results suggest that the osteogenic induction may result in a decrease in Ca2+ influx, which the cell is compensating for by increasing Orai1 and Stim1 expression. As increasing EtOH depresses Ca2+ influx further, the cell responds by increasing expression of Orai1 until Ca2+ influx exceeds basal levels at a chronic timepoint. In this manner, EtOH has been identified as a stressor that negatively affects the osteogenesis and mineral deposition at multiple levels of cellular processes.

In conclusion, our findings confirmed that EtOH has a significant impact on the osteogenic potency of DPSCs through the dysregulation of KDM6B through the Orai1-

BMP signaling pathway 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 spectrumdisorders (FASD).

FIGURES AND DIAGRAMS

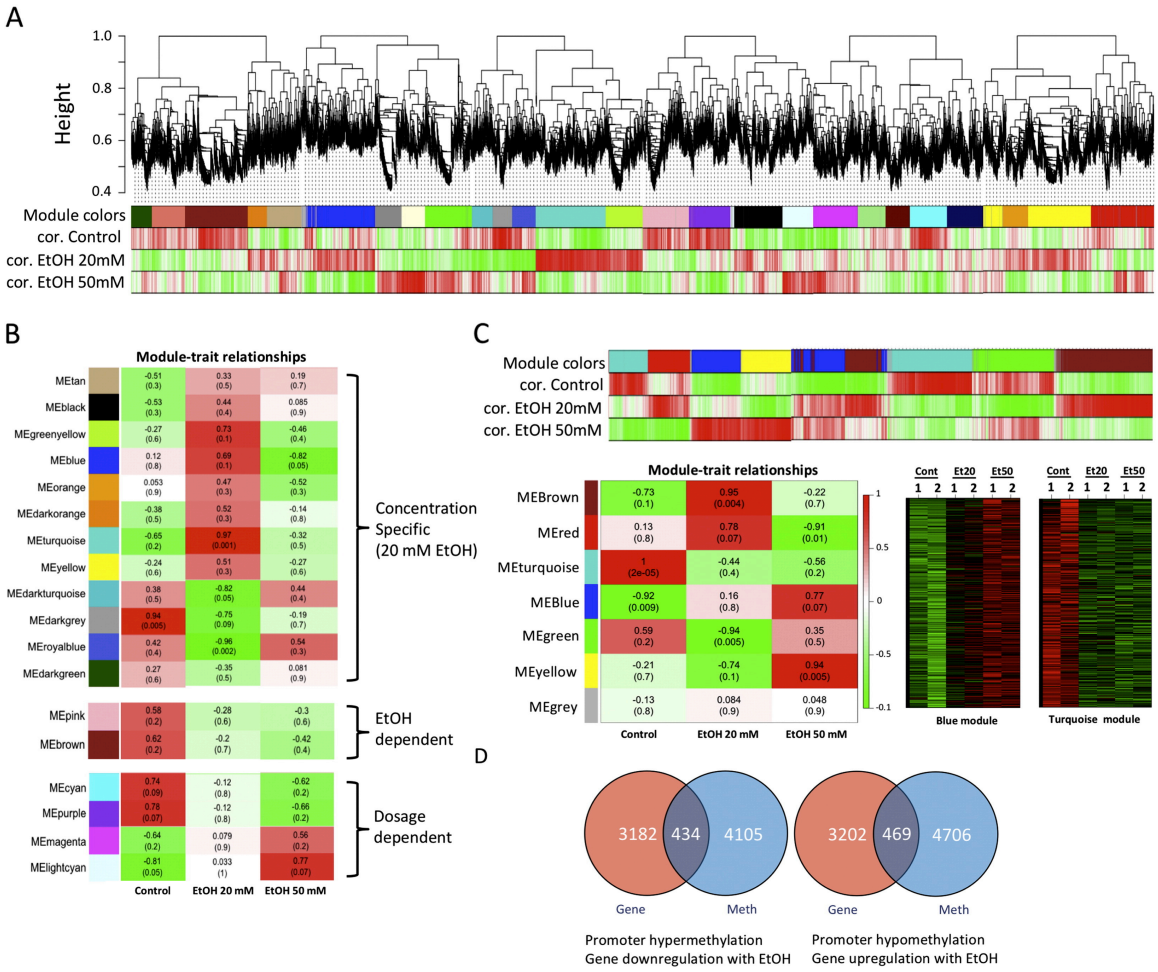

Figure 1: Transcriptome analysis by gene expression microarray. A. Weighted gene co-expression network analysis (WGCNA) was performed to identify transcriptomic changes in DPSCs induced by EtOH. B. Intramolecular connectivity measures how connected, or co-expressed, a given gene is with respect to the genes of a particular module. C. WGCNA analysis on DNA methylomic changes to identify modules associated with EtOH treatment and epigenetically regulated by EtOH-induced DNA methylation in DPSCs. D. Venn diagram analysis of transcriptome vs. DNA methylation displays the number of epigenetically regulated genes.

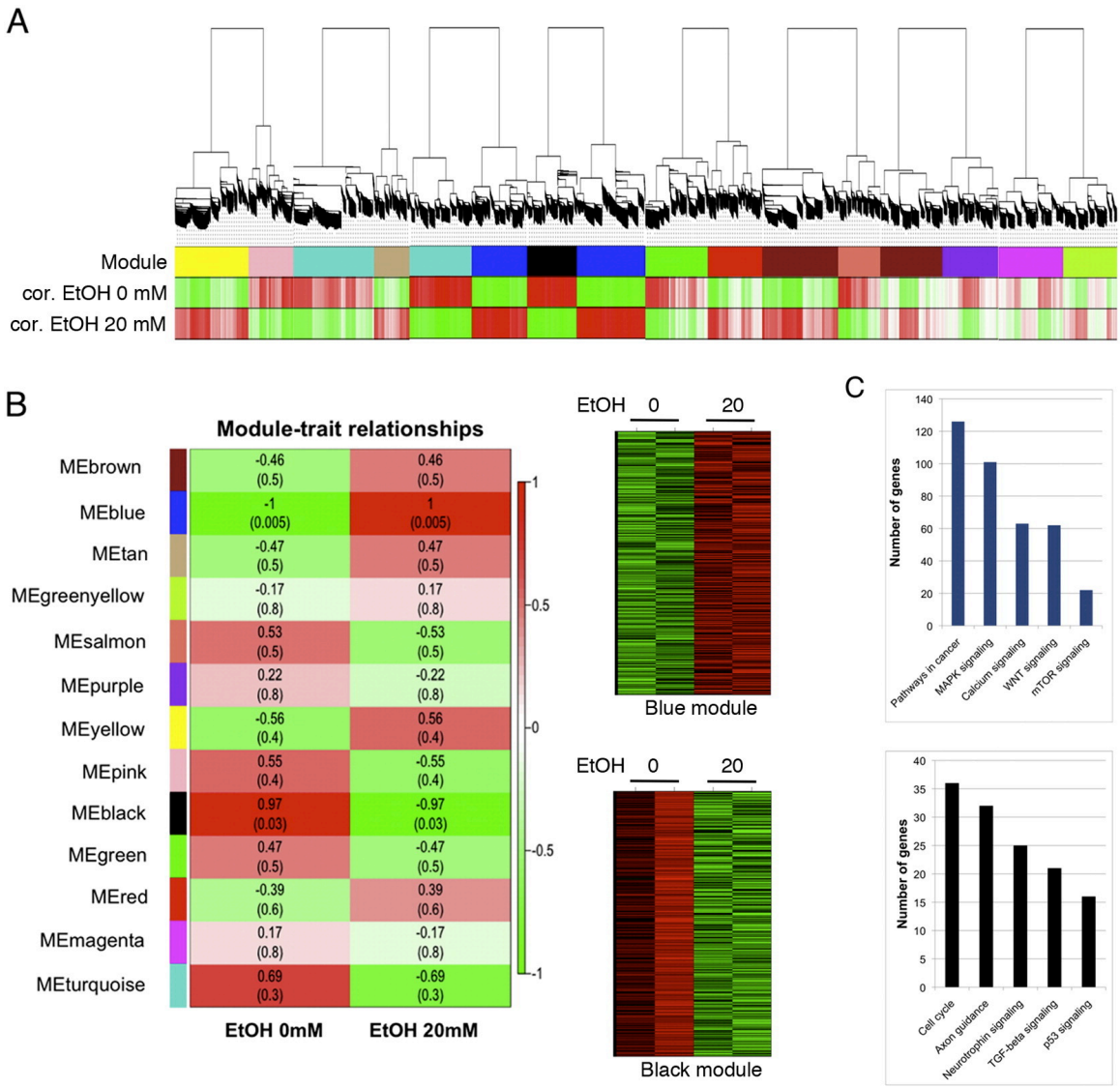

Figure 2: WGCNA on DPSCs treated with 20 mM EtOH. A. WGCNA for transcriptomic changes induced by 20 mM EtOH treatment that is comparable to a 0.08% blood alcohol concentration (BAC) of the DUI level. B. Module–trait relationship map and heat map analysis of the black and blue modules, or gene expression 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.

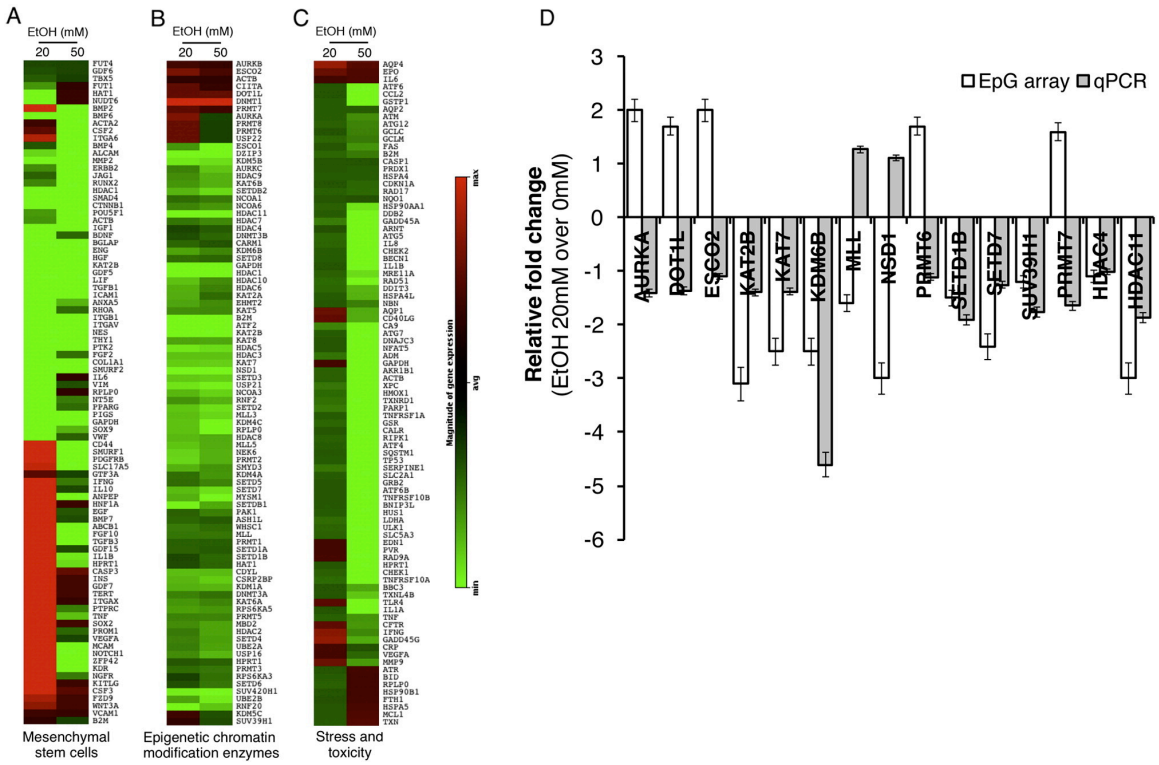

Figure 3: Pathway focused RT-PCR array analysis for genes affected in DPSCs by EtOH treatment. A. Fibroblastic marker array. B. Epigenetic chromatin modification enzymes array. C. Stress and toxicity pathway finder array. Data was analyzed and fold changes against no treatment are presented. D. Quantitative RT-PCR analysis was done to validate the result from the epigenetic modifier RT array. Error bar shows the standard error margin (SEM).

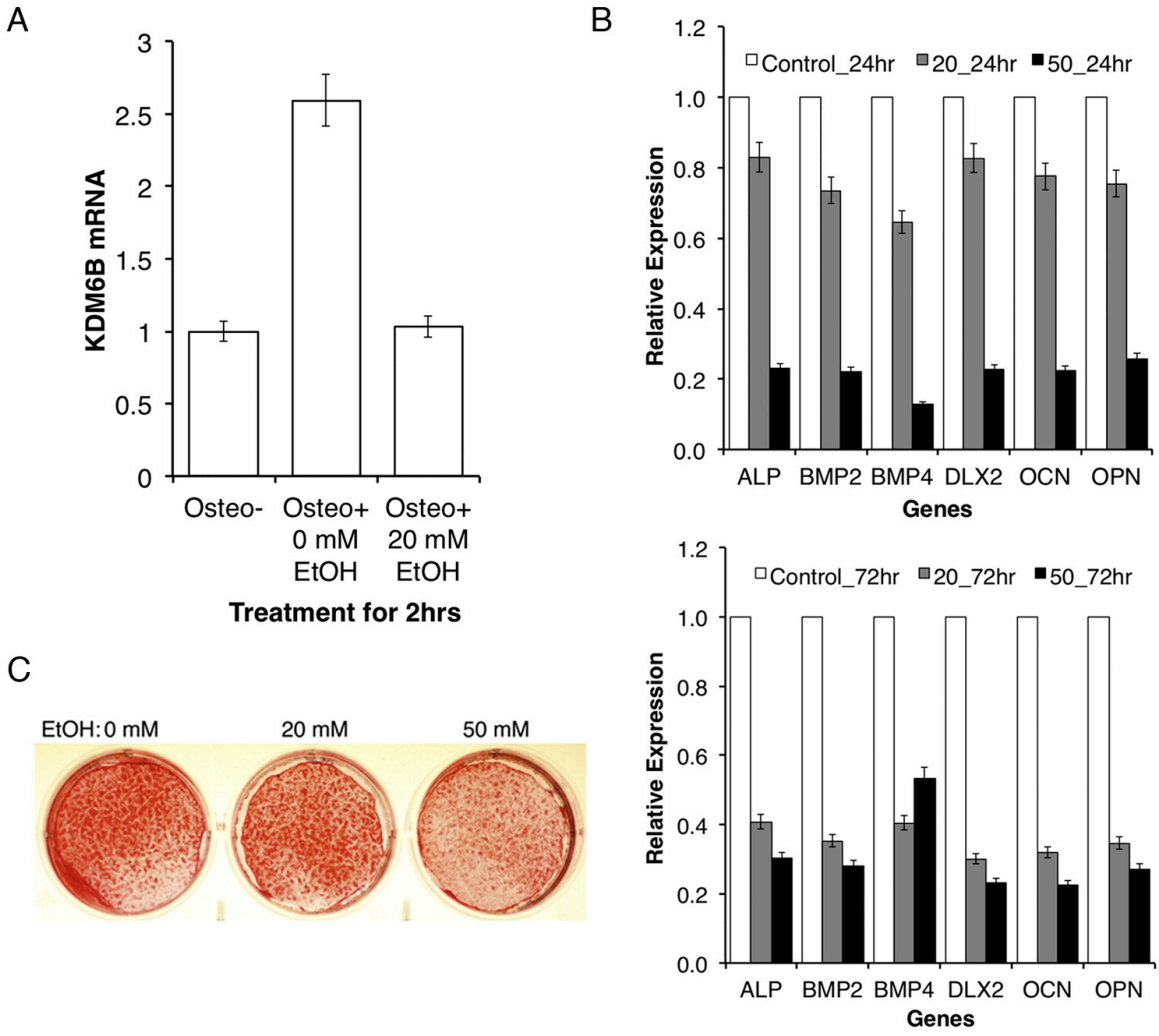

Figure 4: Effect of EtOH on molecular regulation of osteogenic differentiation. A. Comparison of the fold changes of KDM6B expression with and without odontogenic/osteogenic differentiation treatment in DPSCs. Error bar shows the standard error margin (SEM). Statistical significance was determined by Student t-test (p b 0.05). B. Comparison of the relative expression levels of osteomarkers in DPSCs under differentiation with or without treatment (20 mM or 50 mM EtOH) for 24 h and 72 h. Statistical significance was determined by the one-way ANOVA (p b 0.05). Error bar shows the standard error margin (SEM). C. Disruptive effects of EtOH on mineralization in vitro assessed by Alizarin staining.

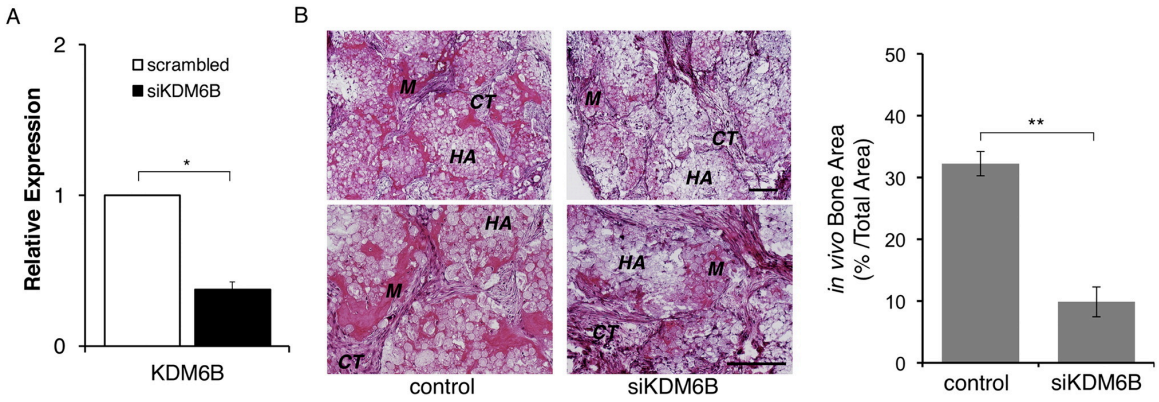

Figure 5: Knockdown of KDM6B resulted in a reduced mineralization potential in vivo. A. Quantitative RT-PCR analysis showed >2 fold reduction in KDM6B in siKDM6B cells. Error bar shows the standard error margin (SEM). Statistical significance was determined by Student t-test (*: $p<0.05$). B. DPSCs transfected 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) is indicated in the 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).

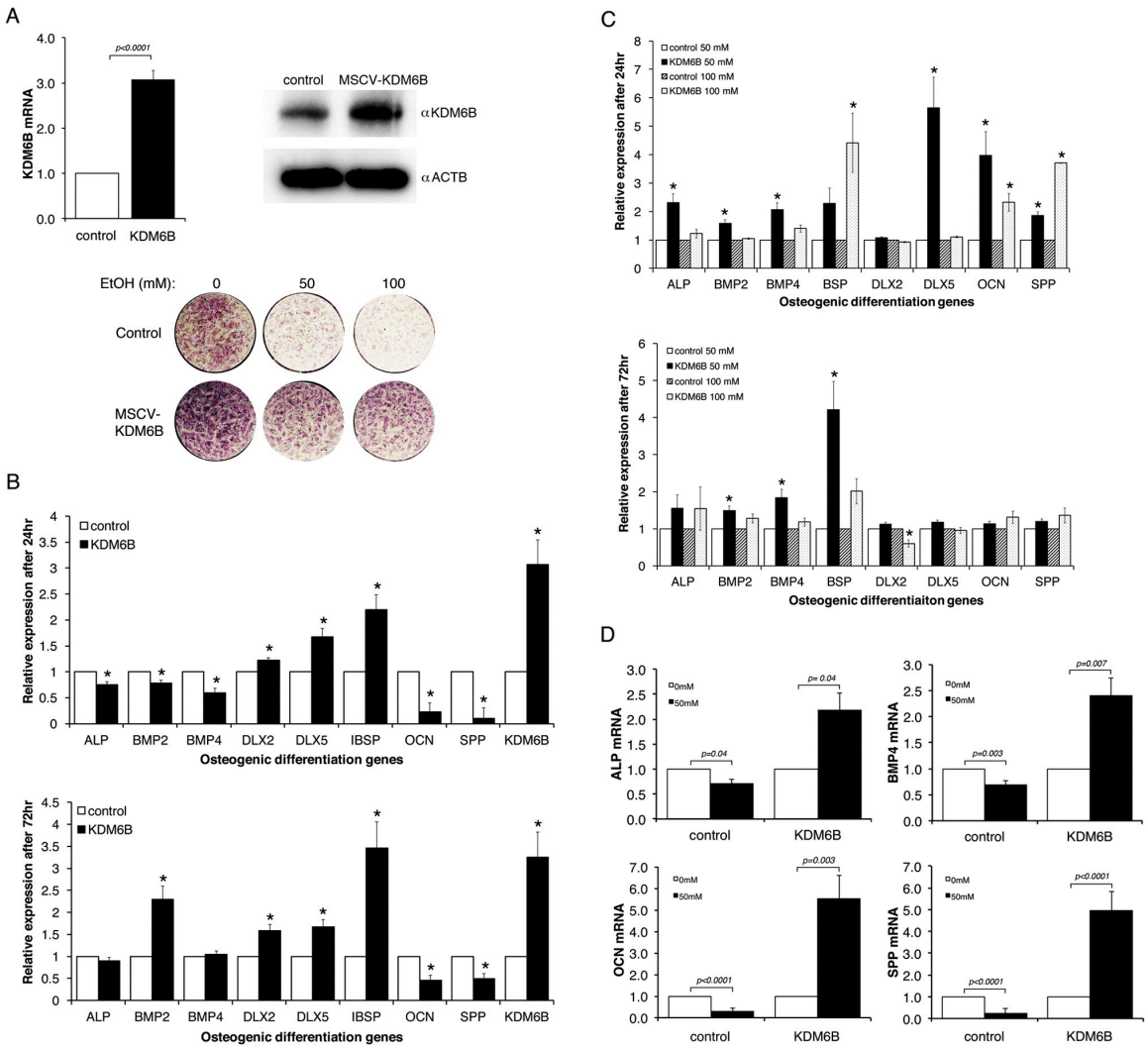

Figure 6: Expression of KDM6B restored differentiation potency in EtOH-treated DPSCs. A. DPSCs were transduced with retroviral KDM6B expression construct (MSCV-KDM6B) or control virus. Three days after, the expression of transduced KDM6B was monitored by qRT-PCR or Western analysis. Transduced DPSC cells were treated with mineralization media containing 0, 50 or 100 mM EtOH for 2 weeks and differentiation of DPSCs was monitored by alkaline phosphatase staining. B. DPSCs transduced with MSCV-KDM6B or control were differentiated in osteogenic media for 24 or 72 h to examine expression of differentiation-related genes. C. Total RNA was isolated from transduced DPSCs treated with odontogenic/osteogenic differentiation media containing 50 or 100 mM EtOH for 24 and 72 h 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 100 mM EtOH treatment. D. The effect of 0 or 50mM 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 h. Statistical significance was determined by one-way ANOVA. Error bar shows the standard error margin (SEM).

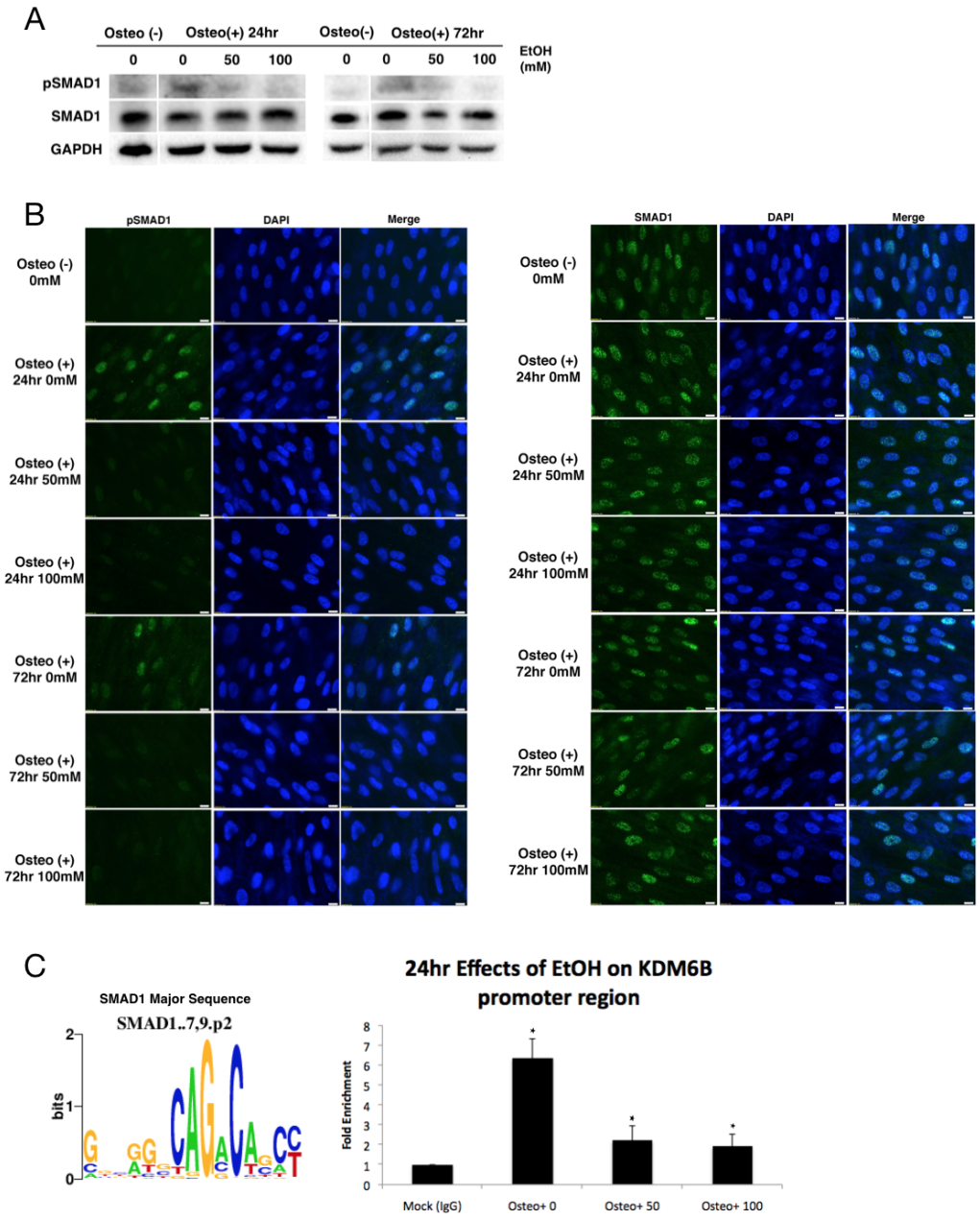

Figure 7: Effect of EtOH on expression level of pSMAD1 and SMAD1 during osteogenic differentiation. DPSCs were treated with mineralization media containing 0, 50, or 100mM EtOH for 24 and 72 h. A. The expression of levels of pSMAD1 and SMAD1 was monitored by Western analysis using anti-pSMAD1 and anti-SMAD1, with anti-GAPDH as the loading control. B. Expression level and localization of pSMAD1 and SMAD1 observed through immunofluorescence images. C. CAGACA and TGTCTG were identified as the major binding sequences for SMAD1 and primers for KDM6B were designed accordingly. ChIP-qPCR analysis was performed using the fold enrichment method for SMAD1 on the KDM6B promoter region at 24 h. Statistical significance was determined by one-way ANOVA and p value < 0.05 is considered as significant (with asterisk). Error bar shows the standard error margin (SEM).

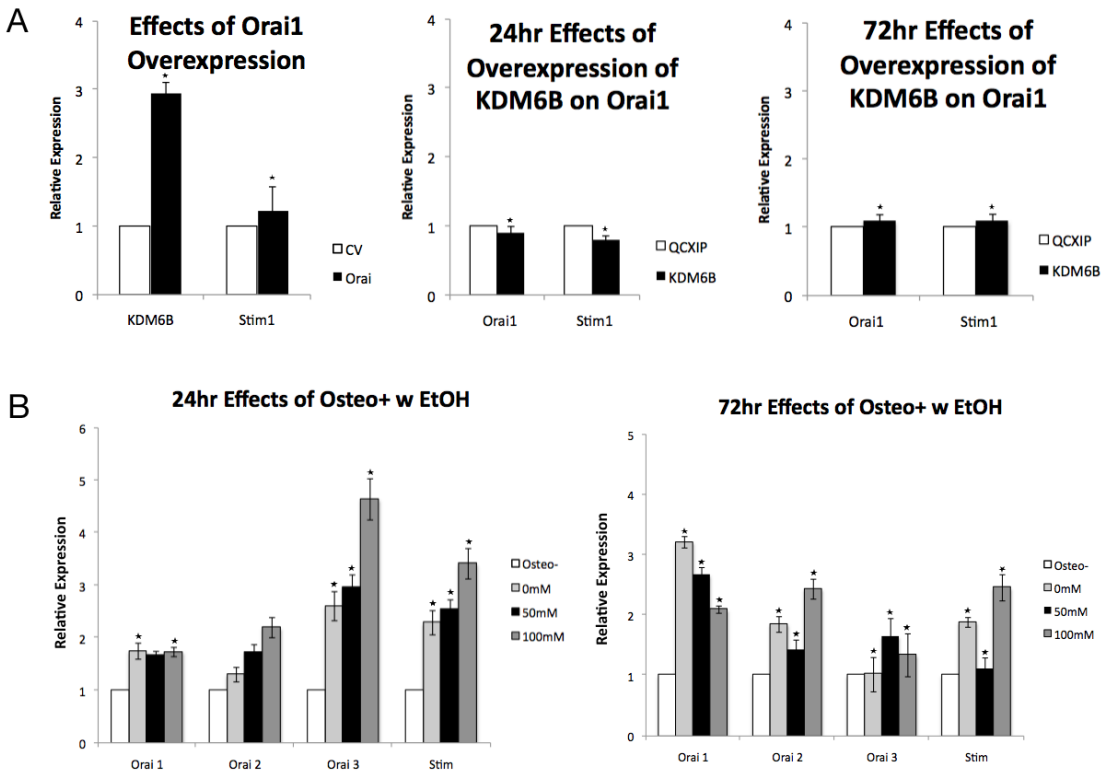

Figure 8: Orai1 signaling pathway as an upstream regulator of KDM6B expression that is affected by EtOH. DPSCs were transduced with retroviral KDM6B expression construct (MSCV-KDM6B) or retroviral ORAI1 expression construct (MO70-ORAI1). A. Total RNA was isolated and qRT-PCR analysis was performed for KDM6B, Orai1, and Stim1 expression levels. Comparison was made within samples for control cells and KDM6B or Orai1 expressing cells by determining the relative fold change. 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). B. DPSCs were treated with mineralization media containing 0, 50, or 100mM EtOH for 24 and 72 h. Total RNA was isolated and qRT-PCR analysis was performed for Orai1, Orai2, Orai3, and Stim1. Comparison was made within samples for control cells and osteo-induced DPSCs by determining the relative fold change for 0, 50, or 100 mM EtOH treatment. Statistical analysis was done on triplicate samples using the one-way ANOVA and p value b 0.05 is considered as significant (with asterisk). Error bar shows the standard error margin (SEM).

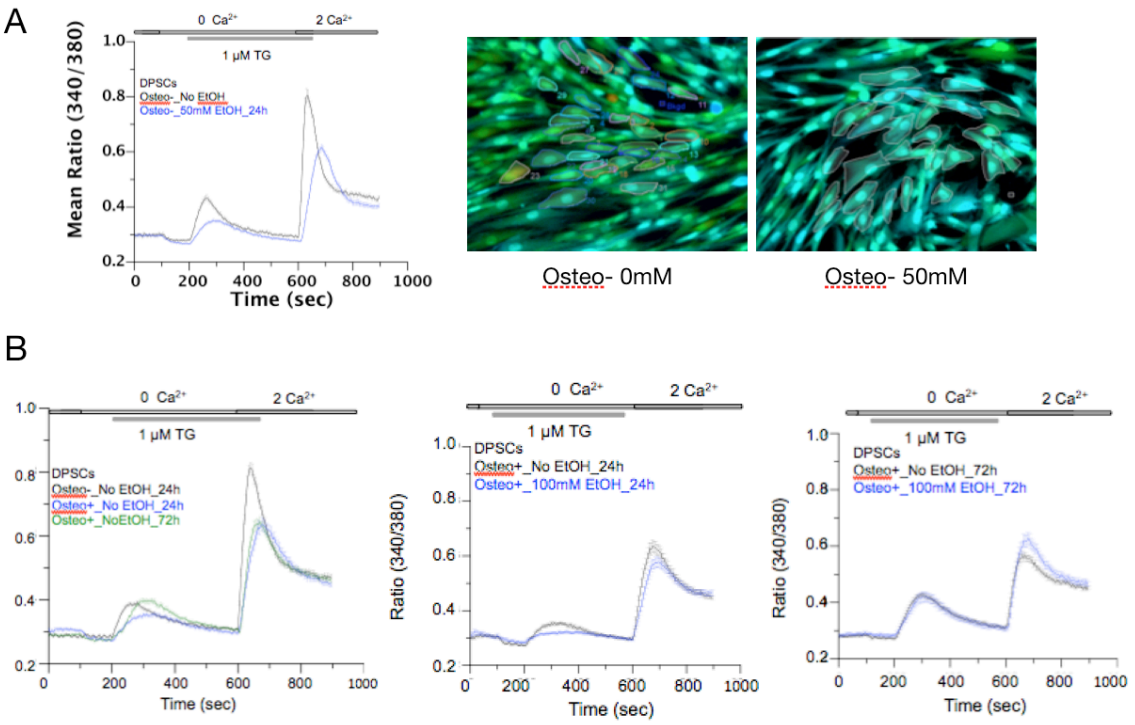

Figure 9: Effect of EtOH on Ca2+ influx in DPSCs with and without osteogenic differentiation. A. DPSCs cultured without differentiation in the absence or presence of 50 mM EtOH for 24 hrs. Imaging started in the presence of 2mM Ca2+ -containing Ringer's solution, which was replaced with Ca2+-free Ringer's solution. 1 μM thapsigargin (TG) was added in Ca2+-free Ringer's solution to deplete the ER Ca2+ stores and SOCE was measured after perfusion with 2mM Ca2+ -containing Ringer's solution. In the presence of 50mM EtOH without differentiation, DPSCs exhibited a significant decrease in Ca2+ influx. Images of DPSCs showing SOCE responses, time point 630sec. Warmer colors show increased Ca2+ levels. B. DPSCs were treated with mineralization media containing 0 or 100mM EtOH for 24 and 72 h. SOCE is reduced after differentiation at 24 and 72 h. In the presence of osteogenic media and EtOH, Ca2+ influx was reduced at 24h and increased at 72 h. All measurements were recorded at a mean ratio of 340/380 and peak values were obtained at the 630sec timepoint.

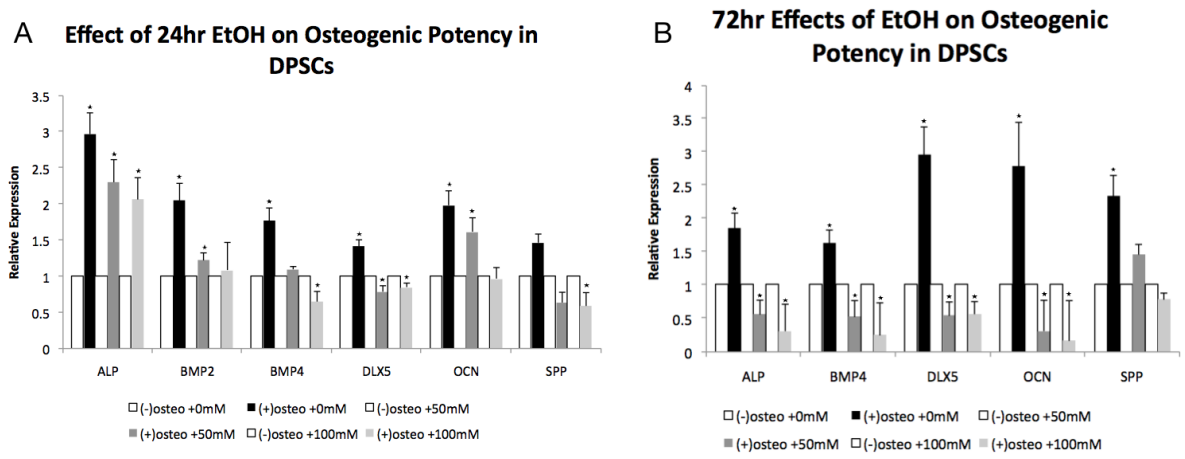

Figure 10: Effects of EtOH on osteogenic potential of Naïve DPSC. DPSCs were treated with 0, 50, or 100mM EtOH for 24 and 72 h prior to exposure to mineralization media for 24 and 72 h. Total RNA was isolated and qRT-PCR analysis was performed for various osteogenic marker genes. Comparison was made between samples for control cells and osteo-induced cells by determining the relative fold change for 0, 50, or 100 mM EtOH treatment. Statistical analysis was done on triplicate samples using the one-way ANOVA and p value b 0.05 is considered as significant (with asterisk). Error bar shows the standard error margin (SEM).

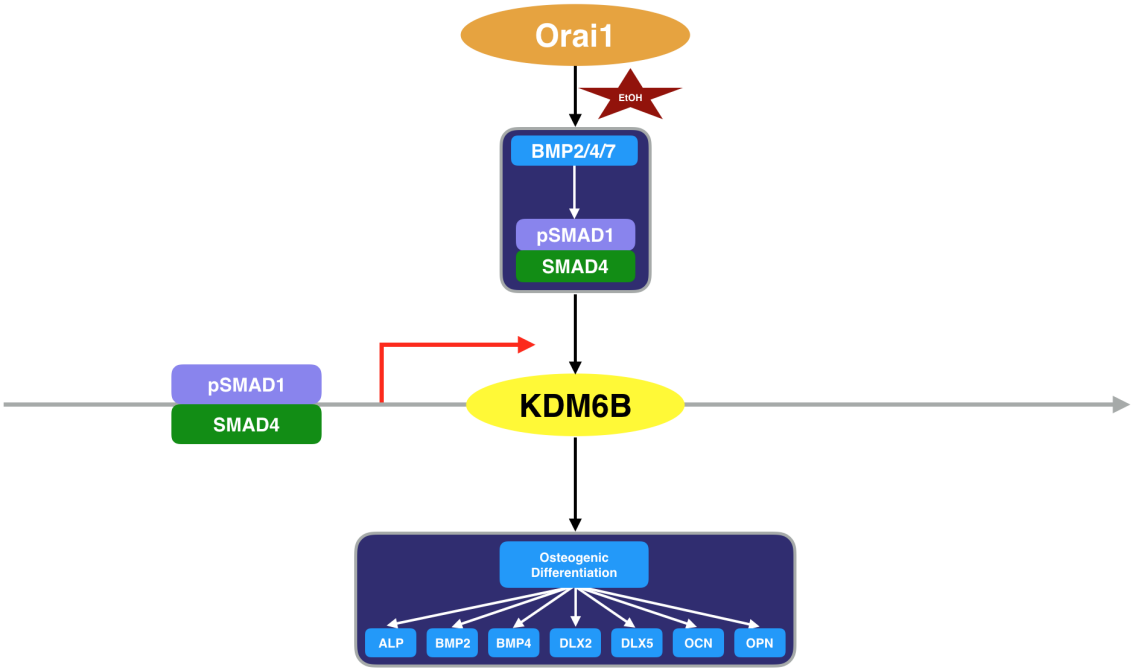

Figure 11: Schematic of EtOH-induced epigenetic regulation of DPSC osteogenic potency through downregulation KDM6B expression. EtOH has been shown to influence upstream and downstream regulators behind the osteogenic potency in DPSCs. Through its effects on Orai1 and Ca2+ influx, EtOH exposure affects the BMP signaling pathway to downregulate KDM6B expression through its effects on the SMAD pathway. Subsequently, the osteogenic potency of DPSCs is downregulated, as observed through the expression of various osteomarkers including ALP, BMP2, BMP4, DLX2, DLX5, OCN and OPN.

REFERENCES

1. Amaral, S., Luiz, R.R., Leao, A.T. The relationship between alcohol dependence and periodontal disease. *J. Periodontol*. 79, 993–998 (2008)
2. Apati et al. Calcium signaling in pluripotent stem cells. *Mol Cell Endocrinol*. 353, 57-67 (2012)
3. Atalayin, C., Tezel, H., Dagci, T., et al. Medium modification with bone morphogenetic protein 2 addition for odontogenic differentiation. *Braz. Oral Res*. 30 (2016)
4. Benoit, D.S., Durney, A.R., Anseth, K.S. Manipulations in hydrogel degradation behavior enhance osteoblast function and mineralized tissue formation. *Tissue Eng*. 12, 1663–1673 (2006)
5. Berridge, M., Lipp, P., Bootman, M. The versatility and universality of calcium signaling. *Nat Rev Mol Cell Biol*. 1, 11-21 (2000)
6. Callaci, J.J., Himes, R., Lauing, K., et al. Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. *Calcif. Tissue Int*. 84, 474–484 (2009)
7. Chen, B., Lin, H., Zhao, Y., et al. Activation of demineralized bone matrix by genetically engineered human bone morphogenetic protein-2 with a collagen binding domain derived fromvonWillebrand factor propolypeptide. *J. Biomed. Mater. Res. A*. 80, 428–434 (2007)
8. Church, M.W., Eldis, F., Blakley, B.W., et al. Hearing, language, speech, vestibular, and dentofacial disorders in fetal alcohol syndrome. *Alcohol. Clin. Exp. Res*. 21, 227–237 (1997)

9. Cui, R., Kan, B., Sun, X., Luo, Z., Guo, R., Guo, X., Yan, M. Role of store-operated Ca2+ channels in primary hepatocytes under conditions of calcium overload and ethanol-induced injury. *Zhonghua Gan Zang Bing Za Zhi*. 11, 860-4 (2013)
10. De Santa, F. et al. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell* 130, 1083-1094 (2007)
11. Ducy, P., Karsenty, G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Molecular and Cellular Biology* 15, 1858-1869 (1995)
12. Feskanich, D., Korrick, S.A., Greenspan, S.L., et al. Moderate alcohol consumption and bone density among postmenopausal women. *J. Women's Health* 8, 65-73 (1999)
13. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG. Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 7090, 179-185 (2006)
14. George, J., Kuboki, Y., Miyata, T. Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds. *Biotechnol. Bioeng*. 95, 404–411 (2006)
15. Haycock, P.C. Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod*. 81, 607–617 (2009)
16. Hicks, S.D., Middleton, F.A., Miller, M.W. Ethanol-induced methylation of cell cycle genes in neural stem cells. *J. Neurochem*. 114, 1767–1780 (2010)
17. Hong, D., Chen, H., Xue, Y., Li, D., Wan, X., Ge, R., Li, J. Osteoblastogenic effects of dexamethasone through upregulation of TAZ expression in rat mesenchymal stem cells. *J. Steroid. Biochem. Mol. Biol*. 116, 86-92 (2009)

18. Hwang SY, Foley J, Numaga-Tomita T, Petranka JG, Bird GS, Putney JW Jr. Deletion of Orai1 alters expression of multiple genes during osteoclast and osteoblast maturation. *Cell Calcium* 52(6), 488-500 (2012)

19. Ilich, J.Z., Brownbill, R.A., Tamborini, L., et al. To drink or not to drink: how are alcohol, caffeine and past smoking related to bone mineral density in elderly women? *J. Am. Coll. Nutr.* 21, 536–544 (2002)

20. Iwaniec, U.T., Turner, R.T. Intraperitoneal injection of ethanol results in drastic changes in bone metabolism not observed when ethanol is administered by oral gavage. *Alcohol. Clin. Exp. Res.* 37, 1271–1277 (2013)

21. Jepsen, K., et al. SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *Nature* 450, 415-419 (2007)

22. Jimenez-Farfan, D., Guevara, J., Zenteno, E., et al. Alteration of the sialylation pattern of the murine tooth germ after ethanol exposure. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 980–988 (2005)

23. Jimenez-Farfan, D., Guevara, J., Zenteno, E., et al. EGF-R and erbB-2 in murine tooth development after ethanol exposure. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 65–71 (2005)

24. Johnson, T.L., Gaddini, G., Branscum, A.J., et al. Effects of chronic heavy alcohol consumption and endurance exercise on cancellous and cortical bone microarchitecture in adult male rats. *Alcohol. Clin. Exp. Res.* 38, 1365–1372 (2014)

25. Jugdaohsingh, R., O'Connell, M.A., Sripanyakorn, S., et al. Moderate alcohol consumption and increased bone mineral density: potential ethanol and non-ethanol mechanisms. *Proc. Nutr. Soc.* 65, 291–310 (2006)

26. Kaminen-Ahola, N., Ahola, A., Maga, M., et al. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet*. 6, e1000811 (2010)

27. Khalid, O., Kim, J.J., Kim, H.S., et al. Gene expression signatures affected by alcohol induced DNA methylomic deregulation in human embryonic stem cells. *Stem Cell Res*. 12, 791–806 (2014)

28. Khalid, O., Kim, J.J., Duan, L., et al. Genome-wide transcriptomic alterations induced by ethanol treatment in human dental pulp stem cells. *Genom. Data* 2, 127–131 (2014)

29. Kieser, J.A. Fluctuating odontometric asymmetry and maternal alcohol consumption. *Ann. Hum. Biol*. 19, 513–520 (1992)

30. Kim, M.J., Shim, M.S., Kim, M.K., et al. Effect of chronic alcohol ingestion on bone mineral density in males without liver cirrhosis. *Korean J. Int. Med*. 18, 174–180 (2003)

31. Kim, J.J., Khalid, O., Vo, S., et al. A novel regulatory factor recruits the nucleosome remodeling complex to wingless integrated (Wnt) signaling gene promoters in mouse embryonic stem cells. *J. Biol. Chem*. 287, 41103–41117 (2012)

32. Kim RH, Lieberman MB, Lee R, Shin KH, Mehrazarin S, Oh JE, Park NH, Kang MK. Bmi-1 extends the life span of normal human oral keratinocytes by inhibiting the TGF-beta signaling. *Exp Cell Res*. 316(16), 2600-8 (2010)

33. Kim, Y., Roubal, I., Lee, Y.S., Kim, J.S., Hoang, M., Mathiyakom, N., Kim, Y. Alcohol-induced molecular dysregulation in human embryonic stem cell-derived neural precursor cells. *PLos ONE* 11(9) e0163812 (2016)

34. Laino, G., Carinci, F., Graziano, A., et al. In vitro bone production using stemcells derived from human dental pulp. *J. Craniofac. Surg*. 17, 511–515 (2006)

35. Lee, S., Park, Y., Song, M., et al. Orai1 mediates osteogenic differentiation via BMP signaling pathway in bone marrow mesenchymal stem cells. *Biochemical and Biophysical Research Communications* 473, 1309-1314 (2016)

36. Lee, S. Rigas, N., Lee, C., Bang, A., Srikanth, S., Gwack, Y., Kang, M., Kim, R., Park, NH, Shin, K. Orai1 promotes tumor progression by enhancing cancer stemness via NFAT signaling in oral/oropharyngeal squamos cell carcinoma. *Oncotarget* 28:43239-43255 (2016)

37. Liu, Y., Balaraman, Y.,Wang, G., et al. Alcohol exposure alters DNAmethylation profiles in mouse embryos at early neurulation. *Epigenetics* 4, 500–511 (2009)

38. Ma, X., Zhang, X., Jia, Y., Zu, S., Han, S., Xiao, D., Sun, H., Wang, Y. Dexamethasone induces osteogenesis via regulation of hedgehog signaling molecules in rat mesenchymal stem cells. *Intl Orthop*. 37(7), 1399-1404 (2013)

39. Massague J., Seoane, J., Wotton, D. Smad transcription factors. *Genes Dev* 19(23), 2783-2810 (2005)

40. Maurel, D.B., Boisseau, N., Benhamou, C.L., et al. Alcohol and bone: review of dose effects and mechanisms. *Osteoporos. Int*. 23, 1–16 (2012)

41. McCarl CA, Picard C, Khalil S, Kawasaki T, Rother J, Papolos A, Kutok J, Hivroz C, Ledeist F, Plogmann K, et al. ORAI1 deficiency and lack of store-operated Ca2+ entry cause immunodeficiency, myopathy, and ectodermal dysplasia. *J Allergy Clin Immunol*. 124(6), 1311–1318 (2009)

42. Miranda, R.C., Pietrzykowski, A.Z., Tang, Y., et al. MicroRNAs: master regulators of ethanol abuse and toxicity? *Alcohol. Clin. Exp. Res*. 34, 575–587 (2010)

43. Mishina, Y., et al. Bone morphogenetic protein type IA receptor signaling regulates postnatal osteoblast function and bone remodeling. *The Journal of Biological Chemistry* 279, 27560-27566 (2004)

44. Miura, M., Chen, X.D., Allen, M.R., et al. A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. *J. Clin. Invest.* 114, 1704–1713 (2004)

45. Miyazono, K., Maeda, S., Imamura, T. BMP receptor signaling: transcription targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev.* 16(3), 251-263 (2005)

46. Muruganandan, S., Roman, A.A. & Sinal, C.J. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cellular and Molecular Life Sciences* 66, 236-253 (2009)

47. Moonat, S., Starkman, B.G., Sakharkar, A., et al. Neuroscience of alcoholism: molecular and cellular mechanisms. *Cell. Mol. Life Sci.* 67, 73–88 (2010)

48. Oberlander, T.F.,Weinberg, J., Papsdorf, M., et al. Prenatal exposure tomaternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics* 3, 97–106 (2008)

49. Okamoto, M., Murai, J., Yoshikawa, H. & Tsumaki, N. Bone morphogenetic proteins in bone stimulate osteoclasts and osteoblasts during bone development. *Journal of Bone and Mineral Research* 21, 1022-1033 (2006)

50. Ouko, L.A., Shantikumar, K., Knezovich, J., et al. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcohol. Clin. Exp. Res.* 33, 1615–1627 (2009)

51. Pandey, S.C., Ugale, R., Zhang, H., et al. Brain chromatin remodeling: a novel mechanism of alcoholism. *J. Neurosci*. 28, 3729–3737 (2008)

52. Papaccio, G., Graziano, A., d'Aquino, R., et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J. Cell. Physiol*. 208, 319–325 (2006)

53. Park, J.B., Han, K., Park, Y.G., et al. Association between alcohol consumption and periodontal disease: the 2008 to 2010 Korea National Health and Nutrition Examination Survey. *J. Periodontol*. 85, 1521–1528 (2014)

54. Pavel, O.R., Popescu, M., Novac, L., et al. Postmenopausal osteoporosis — clinical, biological and histopathological aspects. *Romanian J. Morphol. Embryol*. 57, 121–130 (2016)

55. Phillips, J., Gersbach, C., Wojtowicz, A., Garcia, A. Glucocorticoid-induced osteogenesis is negatively regulated by Runx2/Cbfa1 serine phosphorylation. *Journal of Cell Science* 119, 581-591 (2006)

56. Picard C, McCarl CA, Papolos A, Khalil S, Luthy K, Hivroz C, LeDeist F, Rieux-Laucat F, Rechavi G, Rao A, et al. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. *N Engl J Med*. 360(19), 1971–1980 (2009)

57. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature* 443(7108), 230–233 (2006)

58. Qiang, M., Denny, A., Chen, J., et al. The site specific demethylation in the 5′-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. *PLoS ONE* 5, e8798 (2010)

59. Robinson LJ, Mancarella S, Songsawad D, Tourkova IL, Barnett JB, Gill DL, Soboloff J, Blair HC. Gene disruption of the calcium channel Orai1 results in

inhibition of osteoclast and osteoblast differentiation and impairs skeletal development. Lab Invest. 92(7), 1071–1083 (2012)

60. Roostaeian, J., Carlsen, B., Simhaee, D., et al. Characterization of growth and osteogenic differentiation of rabbit bone marrow stromal cells. *J. Surg. Res.* 133, 76–83 (2006)
61. Sakurai, K., Hoang, M., Kim, Y., Mathiyakom, N., Kim, Y. DNA methylation and chromatin dynamics in embryonic stem cell regulation. *OA Stem Cells* 2(1), 8 (2014)
62. Sampson, H.W. Alcohol, osteoporosis, and bone regulating hormones. *Alcohol. Clin. Exp. Res.* 21, 400–403 (1997)
63. Sant'Anna, L.B., Tosello, D.O., Pasetto, S. Effects of maternal ethanol intake on immunoexpression of epidermal growth factor in developing rat mandibular molar. *Arch. Oral Biol.* 50, 625–634 (2005)
64. Schuckit, M.A. Alcohol-use disorders. *Lancet* 373, 492–501 (2009)
65. Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B. & Cavalli, G. Genome regulation by polycomb and trithorax proteins. *Cell* 128, 735-745 (2007)
66. Sen, G.L., Webster, D.E., Barragan, D.I., et al. Control of differentiation in a selfrenewing mammalian tissue by the histone demethylase JMJD3. *Genes Dev.* 22, 1865–1870 (2008)
67. Shin K.H., Kim R.H., Kim R.H., Kang M.K., Park N.H. hnRNP G elicits tumor-suppressive activity in part by upregulating the expression of Txnip. *Biochem Biophys Res Commun.* 372(4), 880-5 (2008)
68. Shukla, S.D., Velazquez, J., French, S.W., et al. Emerging role of epigenetics in the actions of alcohol. *Alcohol. Clin. Exp. Res.* 32, 1525–1534 (2008)

69. Sieber C., Kopf, J., Hiepen, C., Knaus, P. Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev*. 20, 343-355 (2009)

70. Sohn. S., Park., Y., Srikanth, S. et al. The role of Orai1 in the odontogenic differentiation of human dental pulp stem cells. *Journal of Dental Research* 94(11), 1560-1567 (2015)

71. Srikanth S, Gwack Y. Orai1-NFAT signalling pathway triggered by T cell receptor stimulation. *Mol Cells*. 35(3), 182–194 (2013)

72. Teti, G., Salvatore, V., Focaroli, S., et al. In vitro osteogenic and odontogenic differentiation of human dental pulp stem cells seeded on carboxymethyl cellulose–hydroxyapatite hybrid hydrogel. *Front. Physiol*. 6, 297 (2015)

73. Tezal, M., Grossi, S.G., Ho, A.W., et al. The effect of alcohol consumption on periodontal disease. *J. Periodontol*. 72, 183–189 (2001)

74. Todorova, M.G., Fuentes, E., Soria, B., Nadal, A., Quesada, I. Lysophosphatidic acid induces Ca2+ mobilization and c-Myc expression in mouse embryonic stem cells via the phospholipase C pathway. *Cell. Signal*. 21, 523–528 (2009)

75. Tonelli FM, Santos AK, Gomes DA, da Silva SL, Gomes KN, Ladeira LO, Resende RR. Stem cells and calcium signaling. *Adv Exp Med Biol*. 740, 891–916 (2012)

76. Turner, R.T. Skeletal response to alcohol. *Alcohol. Clin. Exp. Res*. 24, 1693–1701 (2000)

77. Wang, Y., Yan, M., Wang, Z., et al. Dental pulp stem cells from traumatically exposed pulps exhibited an enhanced osteogenic potential and weakened odontogenic capacity. *Arch. Oral Biol*. 58, 1709–1717 (2013)

78. Wijayatunge, R., Chen, L.F., Cha, Y.M., et al. The histone lysine demethylase Kdm6b is required for activity-dependent preconditioning of hippocampal neuronal survival. *Mol. Cell. Neurosci.* 61, 187–200 (2014)

79. Xu, J., Yu, B., Hong, C., et al. KDM6B epigenetically regulates odontogenic differentiation of dental mesenchymal stem cells. *Int. J. Oral Sci.* 5, 200–205 (2013)

80. Yamamoto, A., Sekino, A., Tajima, M., et al. Effect of long-term alcohol administration on bone metabolism in rats. J. *Nutr. Sci. Vitaminol.* 43, 369–375 (1997)

81. Yanagida, E., Shoji, S., Hirayama, Y., Yoshikawa, F., Otsu, K., Uematsu, H., Hiraoka, M., Furuichi, T., Kawano, S. Functional expression of Ca2+ signaling pathways in mouse embryonic stem cells. *Cell Calcium* 36, 135–146 (2004)

82. Yang, D., Okamura, H., Nakashima, Y., et al. Histone demethylase Jmjd3 regulates osteoblast differentiation via transcription factors Runx2 and osterix. *J. Biol. Chem.* 288, 33530–33541 (2013)

83. Ye, L., Fan, Z., Yu, B., et al. Histone demethylases KDM4B and KDM6B promotes osteogenic differentiation of human MSCs. *Cell Stem Cell* 11, 50–61 (2012)

84. Yu, B. The molecular and epigenetic regulation of osteoblast and osteoclast differentiation and the implications in osteoporosis. UCLA Electronic Theses and Dissertations (2014)

85. Yusko, D.A., Buckman, J.F., White, H.R., et al. Risk for excessive alcohol use and drinking-related problems in college student athletes. *Addict. Behav.* 33, 1546–1556 (2008)

86. Zhang, X., Yang,M., Lin, L., et al. Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose-derived stem cells in vitro and in vivo. *Calcif. Tissue Int.* 79, 169–178 (2006)

87. Zhang, W., Liu,W., Ling, J., et al. Odontogenic differentiation of vascular endothelial growth factor-transfected human dental pulp stem cells in vitro. *Mol. Med. Rep.* 10, 1899–1906 (2014)

88. Zhou, F.C., Balaraman, Y., Teng, M., et al. Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcohol. Clin. Exp. Res.* 35, 735–746 (2011)