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Early life ethanol exposure causes long-lasting disturbances in rat mesenchymal stem cells via epigenetic modifications

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ABSTRACT

Fetal alcohol syndrome (FAS) is a birth defect due to maternal alcohol consumption during pregnancy. Because mesenchymal stem cells (MSCs) are the main somatic stem cells in adults and may contribute to tissue homeostasis and repair in adulthood, we investigated whether early life ethanol exposure affects MSCs and contributes to the propensity for disease onset in later life. Using a rodent model of FAS, we found that ethanol exposure (5.25 g/kg/day) from postnatal days 4 to 9 in rat pups (mimic of human third trimester) caused long-term anomalies in bone marrow-derived MSCs. MSCs isolated from ethanol-exposed animals were prone to neural induction but resistant to osteogenic and adipogenic inductions compared to their age-matched controls. The altered differentiation may contribute to the severe trabecular bone loss seen in ethanol-exposed animals at 3 months of age as well as overt growth retardation. Expression of *alkaline phosphatase*, *osteocalcin*, *aP2*, and *PPARγ* were substantially inhibited, but *BDNF* was up-regulated in MSCs isolated from ethanol-exposed 3 month-old animals. Several signaling pathways were distorted in ethanol-exposed MSCs via altered trimethylation at histone 3 lysine 27. These results demonstrate that early life ethanol exposure can have long-term impacts in rat MSCs by both genetic and epigenetic mechanisms.

1. Introduction

Fetal alcohol syndrome (FAS) is an ethanol-induced developmental disorder characterized by varying degrees of growth retardation, learning and memory deficits, and motor incoordination [1]. While great efforts have been made to uncover the neurobehavioral anomalies, very little is known about the potential impact of early life ethanol exposure on mesenchymal stem cells (MSCs). Similar to other stem cells, bone marrow-derived MSCs are capable of self-renewal and differentiation. To date, *in vitro* studies indicate that bone marrow-derived MSCs can be induced to become adipocytes, osteocytes, chondrocytes, myocytes, hepatocytes, and neurons [2,3]. Bone marrow-derived MSCs also can function as stromal cells to support hematopoietic stem cells, a principle resource for regeneration of blood and immune system cells [4]. Although these lineage-specific differentiations remain controversial *in vivo*, it has been hypothesized that MSCs are the key somatic stem cell resource to maintain regeneration and homeostasis in adults [5], and aberrant development of MSCs may contribute to impaired tissue homeostasis and increase the risk of disease onset in adulthood. MSCs isolated from adult alcoholic patients are prone to adipogenesis at the expense of osteogenesis [6]. A similar finding in cloned murine bone marrow-derived stem cells treated with ethanol *in vitro* has also been reported [7]. The results indicate that adult human and rodent MSCs are affected by ethanol exposure. However, whether developing MSCs are similarly affected is less clear. High levels of *in utero* ethanol exposure in humans result in shorter statures and delayed mean bone age in children up to 14 years of age [8]. In rodents, prenatal ethanol treatment

Abbreviations: MSCs, mesenchymal stem cells; FAS, fetal alcohol syndrome; BDNF, brain-derived neurotrophic factor; *aP2*, adipocyte protein 2; *PPARγ*, peroxisome proliferator-activated receptor gamma; *Runx2*, runt-related transcription factor 2; *ENSA*, endosulfine alpha.

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decreases the length of individual bones and delays ossification [9]. These findings suggest that FAS-associated growth retardation may be partly attributable to *in utero* alcohol exposure-induced bone malformation. Because bone is constantly remodeling through bone formation by osteoblasts and bone resorption by osteoclasts, and the main resource of osteoblasts are bone marrow MSCs, we speculated that the self-renewal or lineage-dependent differentiation of MSCs may be disturbed by early life ethanol exposure, thus contributing to blunted bone maturation and function and/or increased propensity for various diseases in adulthood.

Epigenetic modifications, including DNA methylation, histone modification, and microRNA, are known to play crucial roles during development and in maintaining homeostasis in adults by regulating tissue-specific gene expression without altering DNA sequences. Aberrant epigenetic modifications have been documented in many environment-related, late-onset diseases such as cancer, autoimmune diseases, neurodegenerative diseases, and drug addiction [10–12]. Recently, it has been shown that epigenetic modifications are also involved in ethanol-induced insults occurring during neurodevelopment [13,14] and in tissue homeostasis and repair [15]. Because accumulating data have demonstrated that stem cells, including MSCs, are tightly regulated by epigenetic modifications [16–18], we hypothesized that ethanol exposure during the perinatal stage may disturb MSCs by interfering with epigenetic modifications and lead to ethanol-induced disorders. To validate our hypothesis, an established *in vivo* binge-like ethanol exposure model system was applied. Rat pups were intubated with ethanol (5.25 g/kg/day) from postnatal days (PD) 4 to 9 (PD 4–9), a developmental stage mimicking the human third trimester and the first year after birth [19]. Bone marrow-derived MSCs were collected at 2 weeks (juveniles), 1 month, and 3 months of age, and the capability of lineage-specific differentiation including neural induction, adipogenesis, and osteogenesis was examined to profile the long-term impact of ethanol exposure. Genome-wide histone modification by histone 3 lysine 27 trimethylation (H3K27me3) modification, the key repressive histone mark to regulate the stem cell pluripotency [17,20], was performed by chromatin immunoprecipitation microarray (ChIP-chip) analysis to identify the early life ethanol-mediated epigenetic modifications in rat MSCs.

2. Materials and methods

2.1. Experimental animals and treatment with ethanol in vivo

Postnatal ethanol intubation procedures were performed as previously described [21]. Briefly, on PD 4, pups within a litter were randomly assigned to either ethanol treatment or control group, each with equal numbers of males and females. Ethanol-containing milk, made fresh daily by diluting 95% (w/v) ethanol in Enfamil® Premium Ready To Use Infant Formula (Mead Johnson), was administered by oral gastric intubation. Ethanol (J.T. Baker) was administered at 5.25 g/kg/day on a 6-day schedule from PD 4–9. Each ethanol dose was divided into two treatments, 2 h apart, during the middle of the light cycle and then followed 2 h later by an additional "milk alone" feeding to offset reduced nursing during intoxication. This regimen of ethanol treatment results in approximately 320 mg/dl of blood alcohol concentration [21]. Control animals were intubated on the same schedule as ethanol-treated animals, except that they were not given milk.

2.2. MSC cultures

MSCs were isolated from bone marrow dissected from control or ethanol-treated rat hind legs at different developmental stages. Isolated MSCs were cultured in maintenance medium, consisting of α-MEM supplemented with 20% (v/v) newborn calf serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin, in a tissue culture incubator with 5% CO_2/95% O_2 atmosphere. Cell cultures were morphologically heterogeneous initially; therefore, all experiments were performed after four passages to obtain a homogeneous phenotype.

2.3. Cell viability assay

In 96-well plates, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) (Sigma–Aldrich) was added to each well containing different number of cells and incubated at 37 °C for 5 h. The reaction was terminated by adding 200 μl of dimethylsulfoxide (DMSO), and the absorbance was measured at 595 nm.

2.4. In vitro lineage-specific differentiation

The induction protocols for neurogenesis, osteogenesis, and adipogenesis and the corresponding lineage-specific immunostaining procedures were done as described previously [22]. Adipogenic differentiation was measured by Oil Red O staining, and osteogenic differentiation was determined by alkaline phosphatase staining.

2.5. Methods for ChIP-chip and ChIP PCR assays

ChIP assay was conducted following the manufacturer's instructions (Upstate). Protocols for the ChIP-chip assay were as described previously [22]. Briefly, anti-H3K27me3 antibody (Upstate)-enriched DNA (2 μg) was labeled, coupled with Cy dye, and hybridized with the customer-made rat CpG island arrays (Agilent Technologies). For ChIP PCR, enrichment was determined by PCR and visualization after electrophoretic separation in agarose gels

Table 1
Gene-specific primer sequences used in the study.

Primers used for RT-PCR	
Gene Name (RefSeq)	Sequences (5′ → 3′)
Osteocalcin (NM_013414)	F: TATGGCACCACCGTTTAGGG
	R: CTGTGCCGTCCATACTTTCG
ALP (NM_013059)	F: TCCCAAAGGCTTCTTCTTGC
	R: ATGGCCTCATCCATCTCCAC
Runx2 (NM_053470)	F: TAACGGTCTTCACAAATCCTC
	R: GGCGGTCAGAGAACAAACTA
Wnt5a (NM_022631)	F: AATAACCCTGTTCAGATGTCA
	R: TACTGCATGTGGTCCTGATA
GAPDH (NM_017008)	F: ACCCAGCCCAGCAAGGATAC
	R: TGGGGTCTGGGATGGAATTG
PPARγ (NM_001145366)	F: CATTTCTGCTCCACACTATGAA
	R: CGGGAAGGACTTTATGTATGAG
aP2 (NM_053365)	F: AGCGTAGAAGGGGACTTGGT
	R: ATGGTGGTCGACTTTCCATC
Trip10 (NM_053920)	F: AGGACACCCCCATCTACACTGA
	R: TCCATCCATCACCCTTGTCTTC
ENSA (NM_001033974)	F: ACCATGTCCCAGAAACAAGAAGAAGA
	R: TCATTCAAATTGGCCACCCGCAAG
JMJD2 (NM_001106663.2)	F: CAGATGTACCAGGTGGAGTTCG
	R: TGAACCGCATGTCTGAAGCT
HIC1 (NM_001107021)	F: CCTCATTTTGCACAAGTGGC
	R: ACCTCGGAAGCAGAGACAGATG
RassF1A (NM_001037555)	F: GCTTCATCAAGGTTCAGCTGA
	R: TCAAAGAGTGCAAACTTGCG
BDNF (NM_012513)	F: CCATTCAGCACAAGGGTCCC
	R: CCCAGGAGCCCAGTCAGGTA
Primers used for ChIP PCR	
*ENSA*_YY1	F: ACTTTGAGGCAGAGGCAGAGA
	R: AAGGATAATGGCTACAGGCACTA
*ENSA*_AML-1α	F: ATTCCCACACCCTACCCAC
	R: GTGAGCAGTCTAAGCCAAGTCT

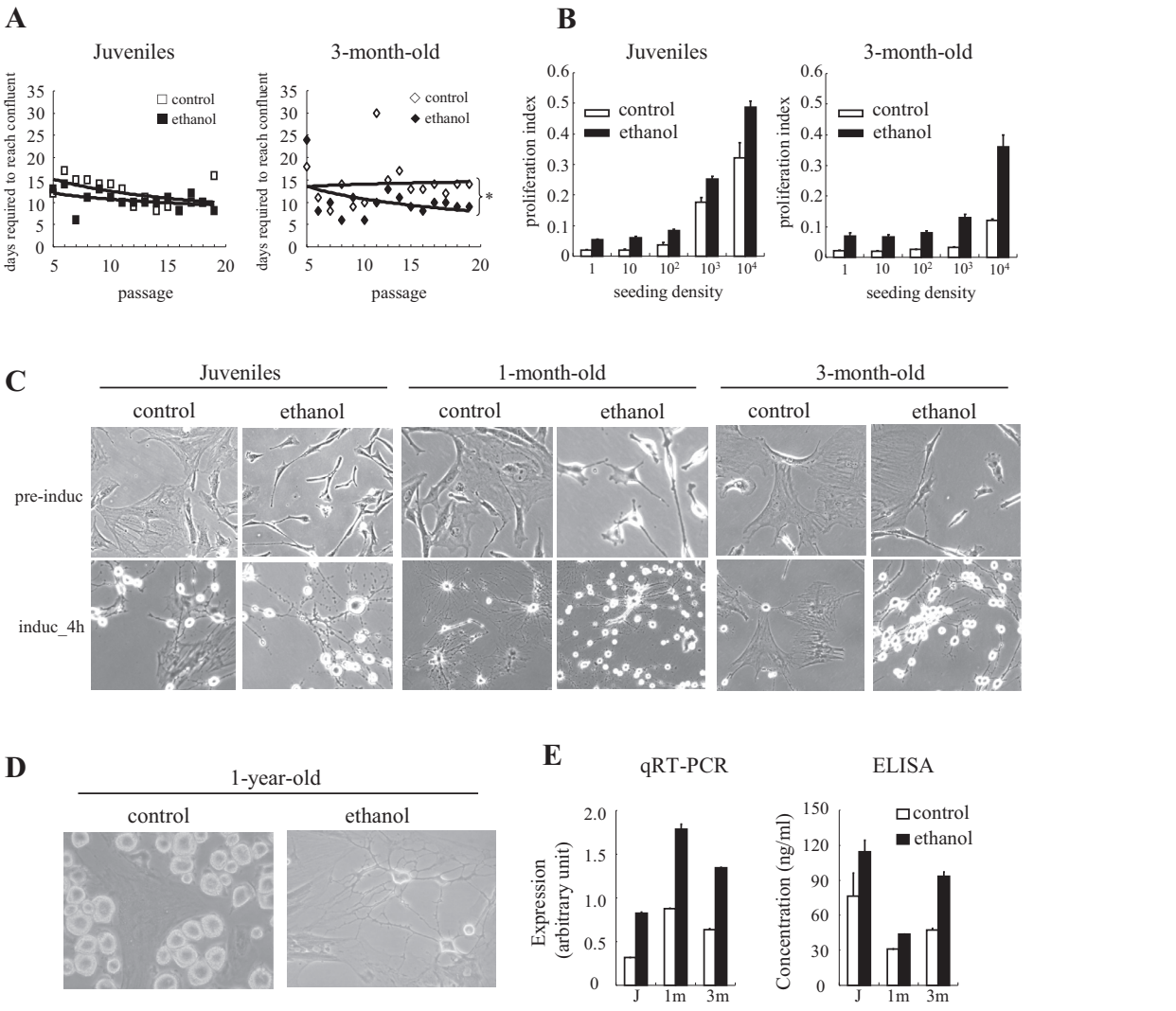

Fig. 1. Early life ethanol exposure has long-term effects on proliferation and induced neural differentiation in rat MSCs. (A) Ethanol exposure accelerates the proliferation rate of MSCs during *in vitro* expansion. $^{*}p < 0.05$. (B) Results of MTT cell viability assays confirm that MSCs obtained from ethanol-exposed animals exhibit higher proliferation rates than their age-matched controls. (C) Morphological changes during pre-induction (pre-induc) phase and 4 h after neural induction (induc_4 h). (D) Representative images showing that MSCs isolated from 12-month-old control animals spontaneously differentiate into adipocytes; whereas, MSCs isolated from age-matched, early ethanol-challenged littermates spontaneously develop the neuron-like morphology. (E) Levels of BDNF mRNA were measured by qRT-PCR, and amounts of secreted protein by ELISA. J: juveniles; 1 m: 1-month-old; 3 m: 3-month-old.

stained with ethidium bromide. Primers used for ChIP PCR are listed in Table 1.

2.6. RT-PCR and quantitative RT-PCR (qRT-PCR)

General RT-PCR and qRT-PCR were used to detect expression levels of target genes as described previously [22]. *GAPDH* was used as loading control. Primer information is listed in Table 1.

2.7. Histology

Trabecular bones collected from both control- and ethanol-exposed animals at different ages were fixed and stained with hematoxylin and eosin for histological analysis. Histology was confirmed by pathologists (PYC and KTY).

2.8. Data analysis

Data are presented as mean ± SEM from at least two independent experiments (different litters) performed in duplicate. Statistical significance was determined by Student *t*-test or analysis of variance and set at $p < 0.05$.

3. Results

3.1. Ethanol exposure alters proliferation and differentiation of MSCs

To determine whether early life ethanol exposure has a long-term impact on the functional maturation of MSCs, bone marrow-derived MSCs were isolated from juvenile, 1-month-old, and 3-month-old rat pups after ethanol challenge from PD 4–9. Prior to induction, the morphology of MSCs obtained from ethanol-exposed and age-matched control animals appeared to be fibroblast-like, regardless of treatment. However, ethanol-exposed MSCs appeared to proliferate more frequently than the age- and passage-matched control MSCs (Fig. 1A and B). MTT assay confirmed that while both treatment groups showed a trend towards an age-dependent decline in proliferation rate, ethanol-exposed MSCs possessed better proliferation capabilities than did controls, even in 3-month-old animals. These data suggest that early life

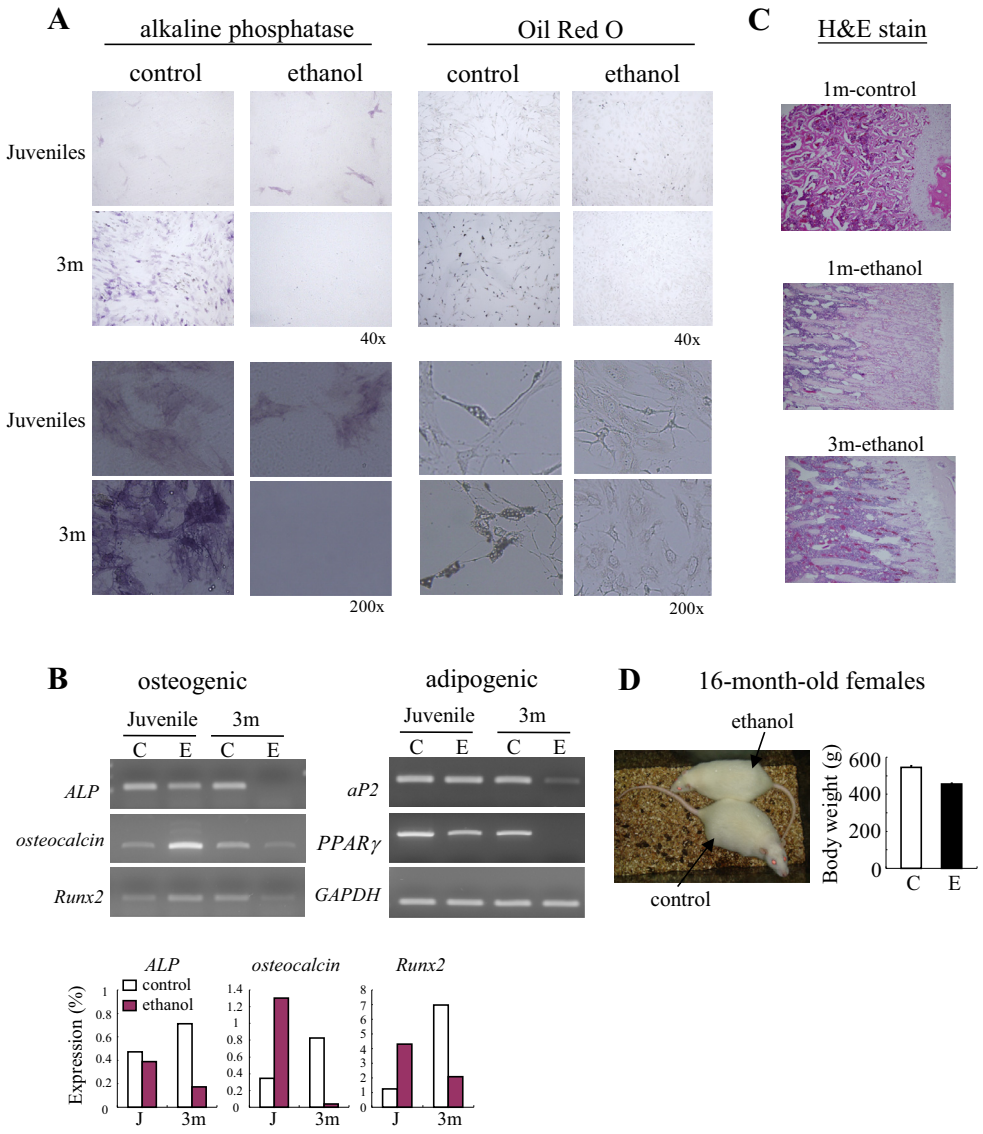

Fig. 2. Both osteogenesis and adipogenesis are permanently suppressed in MSCs isolated from early life ethanol-exposed animals. (A) Alkaline phosphate and Oil Red O staining were used to determine osteogenesis and adipogenesis, respectively. (B) RT-PCR analysis shows that expression of *ALP* (*alkaline phosphatase*), *osteocalcin*, *Runx2*, *aP2*, and *PPARγ* were substantially reduced in 3-month-old ethanol-exposed MSCs. qRT-PCR was performed to provide quantitative data. (C) H&E staining was performed to investigate the bone formation under the influence of early life ethanol exposure. (D) Body size and body weight measured in 16-month-old female littermates. Mean body weight: control (C): 544.3 ± 10.2 g; ethanol (E): 455.6 ± 4.7 g. $p = 0.003$, $n = 3$ litters. Images were taken from one representative litter. J: juveniles; 1 m: 1-month-old; 3 m: 3-month-old; C: control group; E: ethanol-treated group.

ethanol exposure can cause long-lasting disturbances in proliferation of rat MSCs.

We then determined whether the differentiation potency was also altered by early ethanol exposure. First we examined neurogenesis using the neuron-lineage differentiation paradigm. Prior to induction, MSCs were treated with neural pre-induction medium for 1 day. In all three age groups, ethanol-exposed MSCs changed their morphology from a flat, fibroblast-like morphology into a long, thin, spindle-like morphology during the pre-induction condition, whereas most MSCs isolated from control animals remained in the fibroblast-like phenotype (Fig. 1C, pre-induc). Shortly after the medium was switched to the neural induction medium, both control and ethanol-treated juvenile MSCs rapidly developed a neuron-like structure, with a round-up cell body and a few short processes (Fig. 1C, induc_4h). MSCs isolated from more mature control animals appeared less sensitive to neural induction, consistent with an age-dependent decline in multipotency. However, MSCs isolated from 1- or 3-month-old ethanol-exposed animals actively developed into the neuron-like morphology. Moreover, compared to age-matched controls, these ethanol-exposed MSCs survived longer after induced differentiation (data not shown). Furthermore, MSCs isolated from 12-month-old control animals spontaneously and predominantly differentiated into adipocytes in culture, whereas MSCs obtained from the ethanol-exposed, age- and gender-matched animals spontaneously developed a neuron-like structure (Fig. 1D).

These phenotypic differences prompted us to examine whether expression of neurotrophic factors was affected by ethanol exposure. We focused on BDNF, a molecule crucial for neuronal differentiation and survival [23]. We found that expression levels of both BDNF mRNA and protein were up-regulated in ethanol-exposed cells in all three age groups, as demonstrated by qRT-PCR and ELISA, respectively (Fig. 1E). Our data suggest that early life ethanol exposure not only alters MSC proliferation, but also

promotes neuron-like differentiation and survival, in part by increasing BDNF synthesis.

3.2. Early postnatal ethanol exposure induces late-onset suppression of osteogenesis and adipogenesis of MSCs

Next we determined whether other lineage-specific differentiation of MSCs was similarly affected by early life ethanol exposure. Isolated MSCs were treated with osteogenic or adipogenic induction medium for 10 days followed by immunostaining for alkaline phosphatase and Oil Red O, respectively. Fig. 2A shows that MSCs from juvenile animals efficiently differentiated into osteocytes and adipocytes in both control and ethanol-exposed groups. Age-dependent increases in alkaline phosphatase and Oil Red O immunoreactivity were observed in control MSCs. In contrast, MSCs obtained from 3-month-old ethanol-exposed animals were unresponsive to either lineage induction (Fig. 2A).

To address this observation at the molecular level, we assessed expression levels of several genes that control the processes of osteogenesis and adipogenesis. A consistent age-dependent, ethanol-associated drastic decrease in mRNA expression of osteogenesis markers (alkaline phosphatase, osteocalcin, and *Runx2*) and adipogenesis markers (*PPARγ* and *aP2*) was seen in MSCs from 3-month-old ethanol-exposed animals (Fig. 2B). Assay by qRT-PCR confirmed that early life ethanol exposure causes substantial reduction in the expression of osteogenic genes in MSCs from 3-month-old animals.

To evaluate the pathophysiological impact of loss of osteogenic and adipogenic potential of MSCs in ethanol exposure, bone development and body size/weight were measured in both control and ethanol-exposed rats. Histological examination confirmed that the development of trabecular bone was severely distorted in ethanol-exposed animals (Fig. 2C). Ethanol-exposed rats had permanent smaller body sizes and lower weights than their age- and sex-matched control littermates, even at 16 months of age (Fig. 2D). Taken together, our data demonstrate that early life ethanol exposure causes a long-lasting blunting of the osteogenic and adipogenic differentiation of MSCs by interfering with the expression of *alkaline phosphatase*, *osteocalcin*, *Runx2*, *aP2*, and *PPARγ*. This blunted osteogenesis may contribute to the malformation of trabecular bones in adults and FAS-associated growth retardation.

3.3. H3K27me3-associated genes/pathways are affected in ethanol-exposed juvenile MSCs

Epigenetic modification is known to play a crucial role in regulating the self-renewal and differentiation of stem cells [24,25]. We hypothesized that ethanol exposure might disturb epigenetic regulation and contribute to the observed phenotypic anomalies in ethanol-exposed MSCs and animals. We aimed to determine the causal relationship between ethanol and H3K27me3-associated genome, a predominant repressive histone mark that tightly regulates stem cell properties during development and adulthood. We applied ChIP-chip analysis to globally identify genes with changed H3K27me3 association in MSCs isolated from juvenile rat pups. A total of 744 genes were found to have changed H3K27me3 association. KEGG pathway analysis revealed that, in line with its differentiation defect, most of the identified genes were involved in glucose homeostasis and the immune system (Table 2). Components of hedgehog, Notch, Wnt, mTOR, and insulin signal pathways were also sensitive to early life ethanol challenge. Both canonical and non-canonical Wnt signaling cascades contribute to osteoblastogenesis from bone marrow MSCs, thus the dysregulated Wnt signaling pathway may compromise osteogenesis and contribute to the deformed trabecular bone in ethanol-exposed animals.

Table 2
Affected H3K27me3-associated pathways analyzed by KEGG ontology in MSCs from juvenile rats exposed to ethanol early in life.

Rank	Pathway name
1	Maturity onset diabetes of the young
2	Antigen processing and presentation
3	Basal cell carcinoma
4	Type I diabetes mellitus
5	Ribosomes
6	Type II diabetes mellitus
7	Prostate cancer
8	Graft-versus-host disease
9	Allograft rejection
10	Autoimmune thyroid disease
11	Hedgehog signaling pathway
12	Melanogenesis
13	Notch signaling pathway
14	Colorectal cancer
15	Dentatorubropallidoluysian atrophy
16	Endometrial cancer
17	mTOR signaling pathway
18	Insulin signaling pathway
19	Non-small cell lung cancer
20	Glycan structure – degradation

3.4. Expression of target genes is altered in ethanol-exposed MSCs

We next determined expression levels of genes related to the functional maturation of MSCs. Both *ENSA* and Trip10 are involved in glucose homeostasis. Wnt5a can promote osteogenesis and suppress proliferation of MSCs. JMJD2 is a demethylase that controls the methylation status of H3K27. *HIC1* and *RassF1A* are tumor suppressor genes acting via promoting apoptosis. RT-PCR analysis revealed that expression of these genes in juvenile MSCs did not differ apparently between treatment groups. In contrast, levels of mRNAs of the abovementioned genes, except for *JMJD2*, were substantially suppressed in 3-month-old ethanol-exposed MSCs (Fig. 3), further providing a molecular explanation for the suppressed osteogenesis and adipogenesis and enhanced proliferation in these ethanol-exposed cells.

Lastly, anti-H3K27me3 enrichment from within the target gene promoter was examined by ChIP PCR. As shown in Fig. 4, only the putative YY1 binding site, but not that of AML-α, was amplified from the *ENSA* promoter of ethanol-exposed juvenile MSCs by

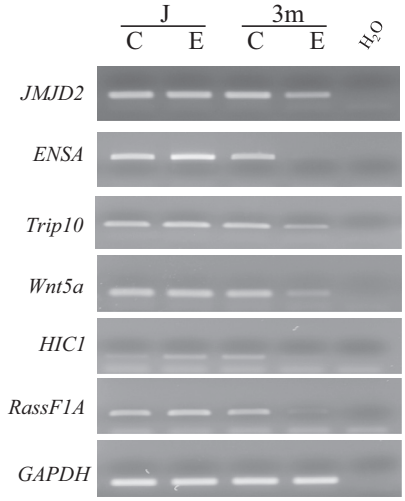

Fig. 3. Early life ethanol exposure is sufficient to cause long-term alterations of target gene expression in MSCs. Target gene expression was determined by RT-PCR. J: juveniles; 3 m: 3-month-old. C: control group; E: ethanol-treated groups.

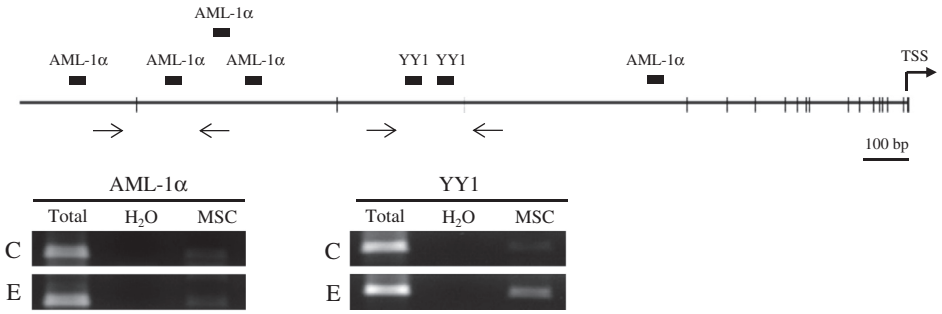

Fig. 4. Ethanol affects H3K27me3 association within the *ENSA* promoter of juvenile MSCs. Top: physical map of the putative transcription factor binding sites on the *ENSA* promoter. Arrow: designated amplification region for ChIP PCR. Vertical short bar: CpG sites. Bottom: ChIP PCR validation of H3K27me3 pull-down on putative AML-1α- and YY1-binding loci. TSS: transcription start site.

anti-H3K27me3 pull-down (Fig. 4). Because YY1 is one of the Polycomb group (PcG) of proteins that are responsible for adding the third methyl group on H3K27, this increased YY1 association further supports that ethanol exposure can act via altered histone modification to affect gene expression in developing MSCs.

4. Discussion

Bone marrow-derived MSCs are an important stem cell resource for adult tissues. Defects in MSCs can contribute to inappropriate maintenance and/or repair of tissues and thus predispose one to diseases. Here we found that early life ethanol exposure caused permanent alterations in proliferation and differentiation of MSCs from rats. Early life ethanol exposure caused long-lasting suppression of osteogenesis and adipogenesis of MSCs, accompanied by distorted bone development and permanently smaller body sizes. However, sensitivity to neural induction appeared to be elevated in ethanol-exposed MSCs. The MSC genome was disturbed by ethanol exposure partly by histone modification as demonstrated by ChIP-chip assay against H3K27me3. Taken together, our data demonstrate that epigenetic modification is one of the mechanisms underlying ethanol-induced, long-lasting MSC dysfunction. In addition, abnormal MSCs are closely associated with osteoporosis and impaired immune and glucose homeostasis in adults, strongly suggesting that FAS children are at high risk to develop these diseases in adulthood.

Alcohol is a risk factor for bone development and homeostasis deficiencies throughout life. Increased osteoporosis and delayed fracture healing have been widely documented in adult chronic alcohol abusers. Offspring from alcoholic mothers often display growth retardation, a characteristic of FAS. While a growing body of evidence demonstrates that ethanol-related bone malformation is due to inhibited osteoblast development and function [7,26,27], the molecular mechanisms are not fully understood. Our study demonstrates that MSCs isolated from animals challenged with early life ethanol exposure are insensitive to either adipogenic or osteogenic induction. Expression of *aP2*, *PPARγ*, and *alkaline phosphatase* was suppressed in ethanol-exposed groups at both developmental stages. While expression of *osteocalcin* and *Runx2* was transiently increased in MSCs from ethanol-exposed juveniles, expression was substantially down-regulated in 3-month-old ethanol-treated MSCs compared to their age-matched controls. Because osteocalcin is produced by osteoblasts, we believe the initial increase in osteocalcin expression in juveniles was due to the fact that the osteogenic transcription factor *Runx2* remained functional in ethanol-challenged MSCs at this age, and thus MSCs could be efficiently induced to become osteoclasts (Figs. 2B and 3). However, by 3 months of age, expression of *Runx2* was drastically suppressed, which, together with the decreased *Wnt5a* expression, rendered the MSCs incapable of differentiating into osteocytes. Thus, no osteocalcin was detected in 3-month-old ethanol-exposed MSCs. Furthermore, histology of bones obtained from 3-month-old ethanol-treated animals showed distorted bone morphology with an impaired maturation process of the epiphyseal growth plate, indicating that early life ethanol exposure can cause long-lasting disturbances in bone formation attributable to suppressed osteogenesis.

There appears to be a vulnerable time window for the deleterious impact of ethanol on bone development and homeostasis. Partial recovery of osteoblasts and bone loss has been seen in human alcoholic abstainers, even after 2 weeks of drink cessation [6]. Binge-like or liquid diet chronic ethanol exposure in adolescent rats leads to altered bone parameters accompanied by altered gene expression, but many abnormalities normalize after a period of abstinence [26]. We found that ethanol exposure from PD 4–9 was sufficient to cause long-lasting alterations in gene expression, even after 3 months of abstinence. These data suggest that PD 4–9 is a vulnerable window for ethanol-induced long-lasting insults in rodent bone development.

Adipocytes play a critical role in systemic metabolism and coordinate lipid and glucose homeostasis [28]. Thus, aberrant adipogenesis has been implicated in human obesity, type II diabetes, and other metabolic disorders. Our study indicates that the ethanol-related blunted adipogenesis was mediated by suppressed *PPARγ* expression and contributed to the permanent smaller body size. In our previous report, human MSC-to-adipocyte differentiation was accompanied by increased expression of *Trip10* and *ENSA* [17]. In support of this finding, MSCs from ethanol-exposed rats had decreased *Trip10* and *ENSA* expression and failed to develop into adipocytes. In addition, our microarray data revealed that genes with ethanol-altered H3K27me3 association are mainly involved in glucose homeostasis and diabetes, further supporting the link between adipogenesis and glucose homeostasis. These data imply that, due to the long-lasted abnormal adipogenesis of MSCs, FAS patients may be prone to develop metabolic or other diseases as they mature. This again reflects the critical developmental window of vulnerability to environmental insults and supports the developmental origins of diseases hypothesis proposed by Ozanne and Constancia [29], which suggests that chronic diseases may originate during early life and the epigenotype may be a contributing mechanism to the pathology in later life.

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