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Ethanol promotes differentiation of embryonic stem cells through retinoic acid receptor-γ

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Ethanol (EtOH) is a teratogen, but its teratogenic mechanisms are not fully understood. The alcohol form of vitamin A (retinol/ROL) can be oxidized to all-*trans*-retinoic acid (RA), which plays a critical role in stem cell differentiation and development. Using an embryonic stem cell (ESC) model to analyze EtOH's effects on differentiation, we show here that EtOH and acetaldehyde, but not acetate, increase differentiation-associated mRNA levels, and that EtOH decreases pluripotency-related mRNAs. Using reporter assays, ChIP assays, and retinoic acid receptor-γ (RARγ) knockout ESC lines generated by CRISPR/Cas9 and homologous recombination, we demonstrate that EtOH signals via RARγ binding to RA response elements (RAREs) in differentiation-associated gene promoters or enhancers. We also report that EtOH-mediated increases in homeobox A1 (*Hoxa1*) and cytochrome P450 family 26 subfamily A member 1 (*Cyp26a1*) transcripts, direct RA target genes, require the expression of the RA-synthesizing enzyme, aldehyde dehydrogenase 1 family member A2 (Aldh1a2), suggesting that EtOH-mediated induction of *Hoxa1* and *Cyp26a1* requires ROL from the serum. As shown with CRISPR/Cas9 knockout lines, the retinol dehydrogenase gene *Rdh10* and a functional RARE in the ROL transporter stimulated by retinoic acid 6 (*Stra6*) gene are required for EtOH induction of *Hoxa1* and *Cyp26a1*. We conclude that EtOH stimulates stem cell differentiation by increasing the influx and metabolism of ROL for downstream RARγ-dependent transcription. In stem cells, EtOH may shift cell fate decisions to alter developmental outcomes by increasing endogenous ROL/RA signaling via increased Stra6 expression and ROL oxidation.

Complex regulatory circuitry is required for maintaining the properties of stem cells so that symmetric self-renewal is not diverted prematurely to drive differentiation toward terminal cell fates (1). High levels of blood ethanol (EtOH) lead to aberrant regulation of normal differentiation in developing embryos and fetuses, making EtOH a teratogen (2–4). Unraveling the mechanisms by which EtOH interferes with endogenous cell signaling pathways that control differentiation is critical for understanding EtOH-mediated toxic effects which lead to disease states that arise during development, *i.e.* fetal alcohol spectrum disorders (FASD)[2] (5) and diseases such as cancers, which are caused in part by changes in cell plasticity (6).

Of particular importance in stem cell differentiation is the interaction between EtOH and the vitamin A (retinol, ROL) metabolite all-*trans*-retinoic acid (RA), which lies at the nexus of physiological differentiation of stem cells (7, 8). Dysregulated RA signaling (from either supraphysiological or subphysiological levels) leads to several teratogenic phenotypes in common with EtOH (9–11). Several studies have shown interactions between EtOH and RA signaling (4, 12–16), possibly because of the similarities in metabolism between ROL and EtOH. Both EtOH and ROL metabolism rely on parallel two-step oxidation processes; ROL is metabolized to retinaldehyde and then to RA, whereas EtOH is metabolized to acetaldehyde (AcH) and then to acetic acid (Fig. 1*A*). Some studies have shown that EtOH decreases retinoid production and RAR signaling (13, 14, 16). In contrast, embryos of EtOH-treated mice show higher RA levels in specific sites (15), prompting us to explore the mechanisms underlying the effects of EtOH on RAR signaling in embryonic stem cells (ESCs) in greater depth.

In ESCs, RA is an endogenous agonist for the three retinoic acid receptor (RAR) members (RARα/β/γ) of the nuclear receptor family of transcription factors (8, 17). RA-bound RARs and members of the retinoid X receptor (RXRα/β/γ) family form heterodimers (17), and these RA:RAR/RXR complexes cause displacement of corepressors that maintain chromatin in a transcriptionally inactive state (17, 18). Posttranslational acetylation of histones by histone lysine acetyltransferases, which are components of the multi-protein coactivator complex, increases accessibility for binding of RA:RAR/RXR complexes at cis-acting RA-response elements (RAREs) (19). RAREs are frequently located within promoter and/or enhancer regions that control transcription of lineage-specific genes necessary for stem cell differentiation by bound RA:RAR/RXR complexes (19).

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This article contains Figs. S1–S6, Table S1, and supporting "Materials and methods".

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[2] The abbreviations used are: FASD, fetal alcohol spectrum disorders; AcH, acetaldehyde; ESC, embryonic stem cell; KOSR, knockout serum replacement (medium); n.s., not significant; RA, all-trans retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RE, retinyl esters; ROL, retinol; RT-qPCR, quantitative reverse transcriptase PCR; RXR, retinoid X receptor.

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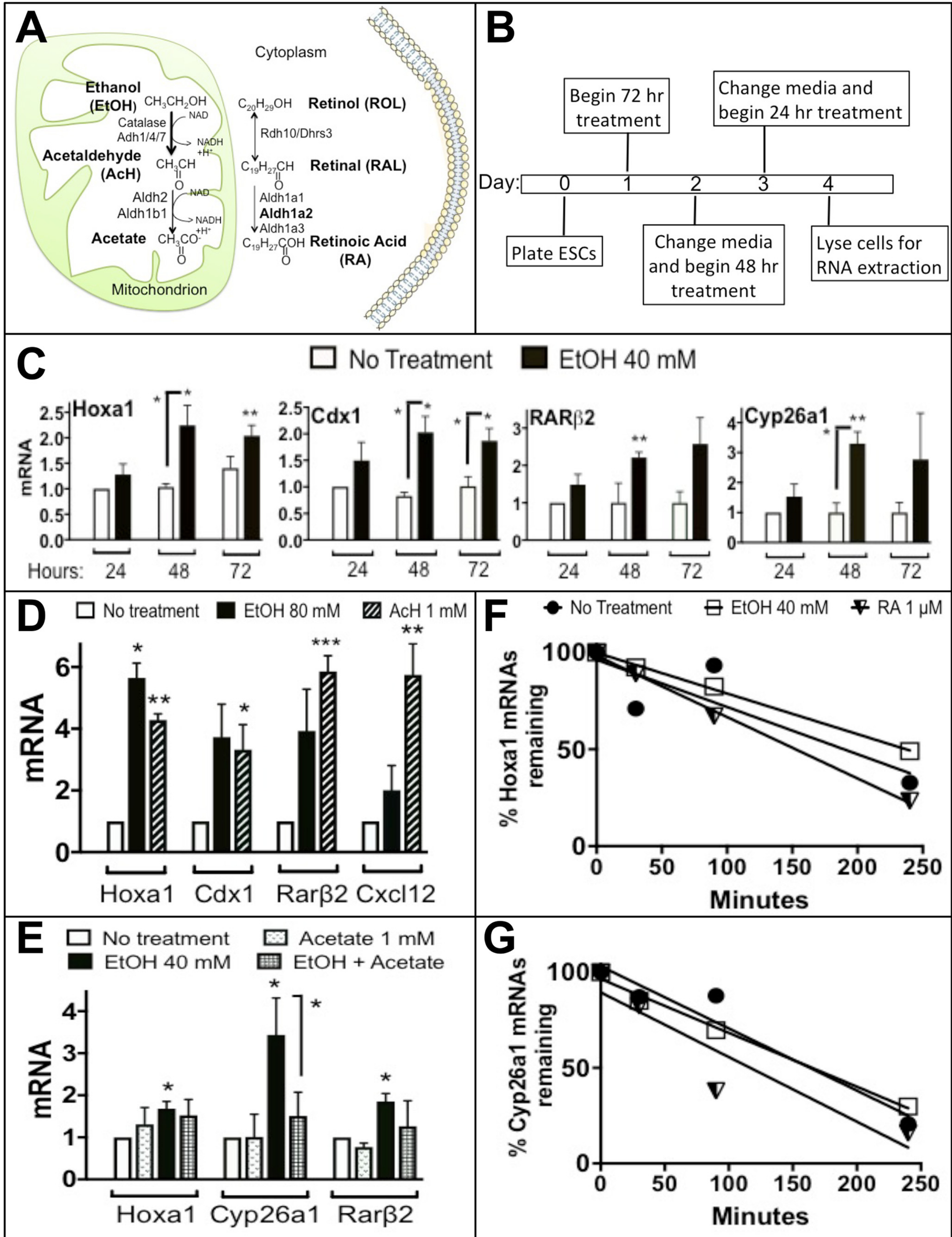
A
Cytoplasm
Ethanol (EtOH)
Acetaldehyde (AcH)
Acetate
Catalase
Adh1/4/7
Aldh2
Aldh1b1
Mitochondrion
Retinol (ROL)
Rdh10/Dhrs3
Retinal (RAL)
Aldh1a1
Aldh1a2
Aldh1a3
Retinoic Acid (RA)
B
Begin 72 hr treatment
Change media and begin 24 hr treatment
Day: 0 1 2 3 4
Plate ESCs
Change media and begin 48 hr treatment
Lyse cells for RNA extraction
C
No Treatment
EtOH 40 mM
Hoxa1
Cdx1
RARβ2
Cyp26a1
mRNA
Hours: 24 48 72
D
No treatment
EtOH 80 mM
AcH 1 mM
mRNA
Hoxa1 Cdx1 Rarβ2 Cxcl12
E
No treatment
Acetate 1 mM
EtOH 40 mM
EtOH + Acetate
mRNA
Hoxa1 Cyp26a1 Rarβ2
F
No Treatment
EtOH 40 mM
RA 1 μM
% Hoxa1 mRNAs remaining
Minutes
G
% Cyp26a1 mRNAs remaining
Minutes

Here we provide evidence that EtOH causes ESC differentiation by increasing RA synthesis by Aldh1a2 following uptake of ROL from the medium by the Stra6 transporter and ROL oxidation by Rdh10. Downstream RA signaling is then dependent on RARγ-mediated transcription via direct RARE activation in primary RA-responsive genes. Elucidating the precise mechanisms underpinning the interactions between EtOH and RA-mediated transcription during ESC differentiation enhances our fundamental understanding of several disease phenotypes during development.

Results

Ethanol decreases pluripotency transcripts and increases transcripts of differentiation-associated genes in embryonic stem cells

To establish the phenotype of alcohol-exposed ESCs we performed quantitative reverse transcriptase PCR (RT-qPCR) on AB1 ESCs 24, 48, and 72 h after 40 mM EtOH addition (Fig. 1*B*). This EtOH dose is representative of human blood concentrations typical of binge drinking (0.184%) (20). Using alkaline phosphatase staining, we demonstrated a reduction in pluripotency in ESCs upon EtOH treatment for 96 h, as EtOH decreased staining intensity compared with 0.1% DMSO-treated cells by 7.6% ($p = 0.020$) (Fig. S1*A*). We additionally compared the mRNA levels of genes associated with pluripotency in EtOH-treated *versus* untreated cells. We measured lower mRNA levels of *Klf4* (78 ± 6%, $p = 0.02$), *Nanog* (83 ± 4%, $p = 0.003$), and *Dppa5* (58 ± 7%, $p = 0.009$) 24 h after EtOH addition, whereas others, including *Oct4* (21) and *Sall4*, were unchanged (Fig. S1*B*).

Transcripts increased by >2-fold by 40 mM EtOH treatment included those of the homeotic (Hox) family (*Hoxa1*, *Hoxb1*, *Hoxa5*) and *Cdx1* (Fig. 1*C*; Fig. S1*C*). Because several transcripts increased by EtOH are direct RAR/RXR transcriptional targets (22–24), we then analyzed additional primary RA target genes, *RARβ2* and *Cyp26a1.* We detected increases after 48 h of EtOH treatment (Fig. 1*C*) compared with vehicle-treated ESCs. We additionally measured transcripts of RA-responsive genes in another ESC WT line, CCE, to rule out any AB1 ESC line-specific effects of EtOH. We found that EtOH also increased transcript levels of RA-responsive genes in CCE cells, and that doses of 40 and 80 mM EtOH elicited similar effects (Fig. S1*D*). We used either 40 or 80 mM EtOH in subsequent experiments. EtOH treatment did not increase transcript levels of lineage-specific genes that were also unaffected by RA treatment at 48 h, such as *Fgf5* (ectoderm) and *Sox17* (endoderm) in ESCs (Fig. S1*E*). Thus, EtOH increases transcript levels of specific RA target genes rather than affecting a broad differentiation phenotype.

To probe for additive effects of EtOH and retinoids we added EtOH to ESCs that were also treated with 1 μM RA or ROL. We used *Hoxa1*, *Cdx1*, and *Hnf1β* as readouts for both RA responsiveness and ESC differentiation and did not detect additional increases in transcript levels compared with RA/ROL-treated cells alone at 48 h (Fig. S1*F*), suggesting that transcript induction of differentiation-associated genes by RA and EtOH converges on the same pathway.

Because EtOH is rapidly oxidized to AcH (Fig. 1*A*) (25), we treated CCE cells with either EtOH or 1 mM AcH for 72 h. AcH caused transcript increases in *Hoxa1* (4.29 ± 0.2, $p = 0.004$), *Cdx1* (3.32 ± 0.82, $p = 0.046$), *RARβ2* (5.86 ± 0.5, $p = 0.0006$), and *Cxcl12* (5.75 ± 1.01, $p = 0.009$), a developmental gene that was not significantly increased by EtOH treatment (Fig. 1*D*). In contrast, 1 mM acetate treatment for 48 h did not increase mRNA levels of *Hoxa1*, *Cyp26a1*, and *RARβ2*, and inhibited the EtOH-mediated increases in Cyp26a1 transcripts (Fig. 1*E*). Therefore, we conclude that the EtOH-induced transcript increases result from EtOH metabolism to AcH, and not to acetate.

To determine whether EtOH increases *Hoxa1* mRNA levels by enhancing mRNA stability or by increasing transcription, we treated CCE ESCs with EtOH or 1 μM RA for 48 h, isolated RNA immediately from some wells, and added 2 μg/ml of actinomycin D to other wells for 30, 90, or 240 min to block transcription. The differences in the derivatives of the linear regression lines between untreated and EtOH-treated WT ESCs were −0.034 ± 0.09 ($p = 0.76$) for *Hoxa1* (Fig. 1*F*) and −0.043 ± 0.04 ($p = 0.54$) for *Cyp26a1* (Fig. 1*G*). The absence of major changes in half-lives of both *Hoxa1* and *Cyp26a1* mRNAs between vehicle-treated and EtOH-treated ESCs suggests that the increases in transcript levels upon EtOH treatment do not primarily result from increased mRNA stability in the presence of EtOH.

RARγ is required for ethanol regulation of genes involved in stem cell differentiation

RARγ controls the expression of several genes that exhibited increased mRNA levels in response to EtOH, including *Hoxa1*, *Cyp26a1*, *RARβ2*, *Crabp2*, and *Hnf1β* (23, 26–28). In addition, our lab has established that RARγ is essential for RA-induced *Hoxa1* transcription through its 3′ RARE (29). To define the role of RARγ in EtOH-mediated transcription in more depth, we used an ESC line in which both alleles of a target sequence in exon 8 of RARγ were deleted by CRISPR knockout ($RAR\gamma E8^{-/-}$) (Fig. S2*A*) (26). We cultured WT and $RAR\gamma E8^{-/-}$ ESCs with 40 mM EtOH for 48 h and found that transcript levels of *Hoxa1* (11.6 ± 2.2-fold, $p = 0.008$), *Cyp26a1* (9.1 ± 1.1-fold, $p = 0.002$), *RARβ2* (6.7 ± 1.8-fold, $p = 0.034$), *Crabp2* (5.3 ± 1.1-fold, $p = 0.018$), *Cdx1* (20.2 ± 4.4-fold, $p = 0.012$), *Hnf1β*, (4.8 ± 1.3-fold, $p = 0.044$), and the long noncoding RNA *Hotairm1* (8.9 ± 1.3-fold, $p = 0.003$) (30), increased in WT ESCs compared with vehicle-treated cells. In contrast, in

Figure 1. Ethanol increases transcript levels of genes necessary for RA-mediated differentiation in ESCs. *A*, schematic of the metabolic pathways of EtOH and ROL. *B*, timeline for cell culture experiments. *C*, -fold changes in mRNA levels by 40 mM EtOH; these transcripts are targets of RA. Treatment groups at 48 and 72 h were compared with untreated ESCs at 24 h, except where indicated by *bar*. *D*, -fold changes in mRNA levels by EtOH and 1 mM AcH at 72 h. *E*, -fold changes in mRNA levels by EtOH or 1 mM acetate ± EtOH at 48 h. *y*-Axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. *Error bars* represent S.E. of independent experiments where $n = 3$ biological repeats. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. *F* and *G*, RT-qPCR analysis of relative stabilities of *Hoxa1* (*F*) and *Cyp26a1* (*G*) transcripts after administering 2 μg/ml of actinomycin D to inhibit transcription.

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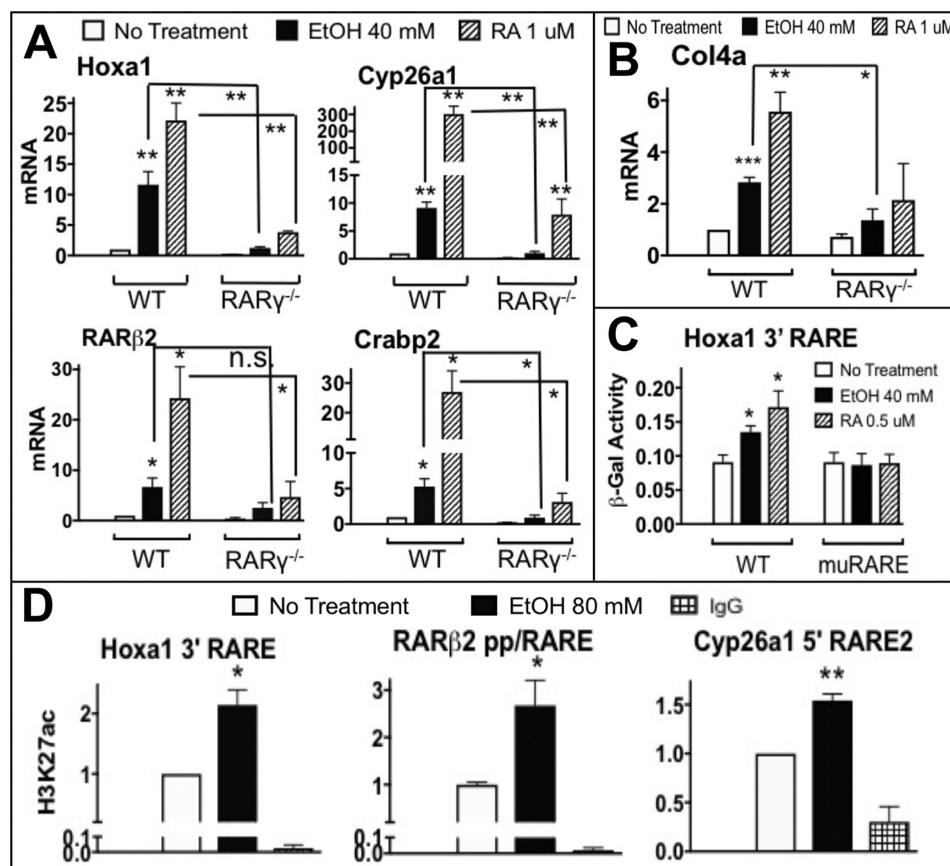

Figure 2. RARγ is required for the expression of a subset of RA-target genes induced by ethanol. *A*, -fold changes in transcript levels of RA-inducible genes in WT and RARγE8$^{-/-}$ ESCs at 48 h treatment with EtOH (40 mM) or RA (1 μM RA). *B*, -fold changes in transcript levels of the late differentiation marker *Col4a* in WT and RARγE8$^{-/-}$ ESCs at 48 h treatment with EtOH (40 mM) or RA (1 μM RA). Treatment groups were compared with untreated ESCs at 48 h, except where indicated by *bar*. *C*, β-gal activity of CCE cells transfected with *Hoxa1* minigene (13.5 kb of Hoxa1 DNA + 6.5 kb of 5′ + 3 kb of 3′ flanking sequences with in-frame fusion of lacZ) with either WT DR5 RARE (CAGGTTCACCGAAAGTTCAAG) or *Hoxa1*-lacZ muRARE (C-cTagcCCGAAAaTTacAG), where *underlined* bases represent consensus RAREs and *lowercase* bases represent mutations; at 24 h ± EtOH (40 mM) or RA (0.5 μM), normalized to luciferase activity of each sample (15:1 test:control). *D*, acetylation state of H3K27 near *Hoxa1, RARβ2*, and *Cyp26a1* RAREs after treating ESCs with 80 mM EtOH for 24 h, relative to DMSO-treated controls set to 1. The RAREs analyzed are located in a 3′ enhancer 4.6 kb downstream of the *Hoxa1* proximal promoter (*pp*), in the *RARβ2* pp, and in a 5′ enhancer 2 kb upstream of the *Cyp26a1* pp. ChIP assays were normalized to pre-immunoprecipitation input DNA. *y*-Axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. *Error bars* represent S.E. of independent experiments where $n = 3$ biological repeats. *n.s.*, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

RARγE8$^{-/-}$ cells, deletion of RARγ prevented these mRNA increases (Fig. 2*A*; Fig. S2*B*).

Moreover, transcripts of the late differentiation marker, Col4a, increased in EtOH-treated WT (2.8 ± 0.19-fold, $p = 0.0006$), but not in RARγE8$^{-/-}$ ESCs (Fig. 2*B*). Because *Col4a* transcripts are only induced in RA-treated ESCs at late times (2–3 days) when the cells are fully differentiated (31), these data demonstrate that EtOH causes ESCs to differentiate along an epithelial lineage.

We confirmed the RARγ requirement for EtOH-mediated ESC differentiation using another RAR$\beta^{+/-}\gamma^{-/-}$ line (29) treated for 2 h with EtOH ± RA. We found that *Hoxa1* and *Hoxb1* transcripts increased by 1.6 ± 0.01-fold ($p < 0.0001$) and 1.7 ± 0.18-fold ($p = 0.014$), respectively, in 40 mM EtOH-treated WT samples, and that RA + EtOH samples displayed a 4.7 ± 0.99-fold ($p = 0.021$) increase in *Hoxa1* and a 6.1 ± 1.0-fold ($p = 0.007$) increase in *Hoxb1* compared with vehicle-treated cells (Fig. S2*C*). In contrast, *Hoxa1* and *Hoxb1* transcript levels did not increase in EtOH-treated RAR$\beta^{+/-}\gamma^{-/-}$ cells ± RA (Fig. S2*D*). These data demonstrate that RARγ mediates the effects of EtOH with respect to ESC differentiation.

RARE activation is necessary for ethanol-mediated Hoxa1 transcription in embryonic stem cells

To determine whether a functional RARE is required for signaling by EtOH we next performed a transient transfection in ESCs using *Hoxa1*-lacZ minigene reporter constructs in which lacZ was cloned into the *Hoxa1* coding sequence (22). We used two different constructs; one contained an enhancer with an intact RARE (WT, AGTTCA) and the other contained an RARE that was inactivated by mutation (*Hoxa1*-lacZ muRARE, AaTTac). We treated these transfected ESCs with vehicle (0.1% DMSO), EtOH (40 mM), or RA (0.5 μM) for 24 h. We observed a 1.5 ± 0.15-fold ($p = 0.034$) increase in β-gal activity in the EtOH-treated and a 1.9 ± 0.28-fold ($p = 0.036$) increase in RA-treated WT ESCs transfected with the construct harboring an intact WT RARE (Fig. 2*C*). We did not observe any increase in β-gal activity in either EtOH- or RA-treated lysates from WT cells transfected with the *Hoxa1*-lacZ muRARE construct. These results show first, that the effects of EtOH occur at the transcriptional level and second, that there is a requirement for a functional RARE to mediate EtOH-induced transcriptional effects on *Hoxa1*.

Enrichment of histone 3 lysine acetylation (acetyl-H3) allows RAREs to become more accessible for the RAR/RXR complex to bind and induce transcription. We performed chromatin immunoprecipitation (ChIP) assays using an antibody against the H3K27ac modification, which identifies transcriptionally active enhancers (32), to examine histone acetylation patterns in chromatin near RAREs of genes which exhibited mRNA increases by EtOH. Both *Hoxa1* and *Cyp26a1* contain at least one RARE at enhancers, whereas *RARβ2* contains a RARE near its proximal promoter (18). Genes from the EtOH-treated WT ESCs exhibited >1.5-fold H3K27ac enrichment near RAREs compared with vehicle-treated ESCs (2.1 ± 0.25-fold, $p = 0.01$, *Hoxa1*; 2.7 ± 0.54-fold, $p = 0.036$, *RARβ2*; 1.6 ± 0.07-fold, $p = 0.001$, *Cyp26a1*) (Fig. 2*D*). These increases in H3K27ac chromatin marks upon EtOH treatment suggest that the chromatin near the RAREs is in a configuration in which transcription is activated.

Ethanol increases transcripts associated with retinol metabolism

To determine whether RA is a required intermediate for the EtOH-mediated increases in differentiation-associated genes, such as *Hoxa1* and *Cyp26a1*, we first measured transcript levels of several genes required for RA synthesis from ROL. ROL is primarily metabolized to retinaldehyde by retinol dehydrogenase-10 (Rdh10) (7). Using semiquantitative RT-PCR, we showed that transcripts of Rdh10, but not Rdh5 or Rdh11, were increased by EtOH in WT ESCs (Fig. 3*A*). By RT-qPCR analysis we also showed EtOH-associated increases in transcript levels of the RARγ target gene *Rdh10* (1.7 ± 0.12-fold, $p = 0.004$) and the intracellular ROL transporter *Rbp1* (*Crbp1*) (6.7 ± 1.3-fold, $p = 0.011$) (Fig. 3, *B* and *C*) in WT ESCs. Crabp2, which transports RA to the nucleus (33), displayed increased transcript levels (5.28 ± 1.1-fold, $p = 0.018$) in WT ESCs upon EtOH addition. *Rbp1* and *Crabp2* exhibited regulation by RARγ, because the RARγE8$^{-/-}$ ESC line showed attenuated increases in these transcripts by EtOH compared with those in WT ESCs (Fig. 3*C*). Transcripts for the retinaldehyde reductase, *Dhrs3*, but not *Dhrs4*, were increased by EtOH treatment (15.7 ± 3.1-fold, $p = 0.009$) in WT ESCs (Fig. 3*D*). Importantly, Dhrs3 stabilizes the Rdh10-containing retinoid oxidoreductase complex (34). These data show that EtOH increases mRNAs of key genes that metabolize ROL to retinaldehyde. In contrast, the *Aldh1a2* mRNA level was not increased by EtOH in WT ESCs (Fig. S3; Fig. 1*A*).

Aldh1a2 is required for ethanol-mediated transcriptional changes

Because EtOH increased transcripts of genes involved in RA synthesis and nuclear transport, we ablated Aldh1a2 activity using CRISPR/Cas9 targeted to two sequences in intron and exon 5 to generate an Aldh1a2E5$^{-/-}$ ESC line (Fig. 4, *A* and *B*). The absence of Aldh1a2 prevented the EtOH-mediated *Hoxa1* and *Cyp26a1* transcript increases observed in WT cells (Fig. 4*C*). These data indicate that metabolism of retinaldehyde to RA is required for EtOH to increase *Hoxa1* and *Cyp26a1* transcripts.

To address the role of serum ROL, we next cultured WT ESCs in knockout serum replacement (KOSR) medium , which, unlike fetal calf serum, does not contain ROL. EtOH did not increase *Hoxa1* and *Cyp26a1* mRNAs in WT ESCs cultured in KOSR medium (Fig. 4*C*). Adding ROL to the KOSR medium at 0.1 μM, typically found in 10% serum-containing medium (35), restored the EtOH-mediated increases in *Hoxa1* (1.7 ± 0.14-fold, $p = 0.01$) and *Cyp26a1* (2.1 ± 0.4-fold, $p = 0.035$) transcripts to levels similar to those measured in serum-containing medium (1.8 ± 0.05-fold, $p < 0.0001$, *Hoxa1*; 2.1 ± 0.17-fold, $p = 0.0005$, *Cyp26a1*) (Fig. 4*C*). In contrast, *Hoxa1* transcript levels were similarly increased by 1 μM RA in WT ESCs cultured in either serum-containing or KOSR medium (Fig. S4). Collectively, these data demonstrate that ROL, via its two-step oxidation to RA, is required for EtOH-mediated *Hoxa1* and *Cyp26a1* transcript increases.

Ethanol treatment did not cause detectable increases in RA levels in embryonic stem cells

We measured RA levels in EtOH-treated ESCs using reversed phase HPLC–tandem MS to determine whether increased intracellular RA levels correlated with the increases in *Hoxa1* and *Cyp26a1* transcripts. Using a triple quadrupole mass spectrometer, we detected a peak for 20 pmol of an RA standard at a retention time of 3.5 min (Fig. S5*A*). Calibration curves were generated for RA with a limit of detection of 40 fmol and a lower limit of quantitation of 382 fmol (95 nM for 4×10^6 cells, where 1 μl volume = 1×10^6 cells and 4 μl = injection volume) for the transition m/z 301.2 → 123.1, and 341 fmol (85 nM for 4×10^6 cells) for a secondary m/z 301.2 → 159.1 transition (Fig. S5, *B* and *C*). We also generated a calibration curve for 4-oxo-RA, a metabolite of RA (Fig. S5*D*). RA levels in ESCs treated with either vehicle or EtOH were too low to detect, but we detected an RA peak in cells treated with exogenous RA for 8 h (Fig. 4*D*; Fig. S5*E*). We observed a second peak at retention time 3.25 min for transition m/z 301.2 → 159.1, but this peak did not correspond to any known RA isomer or metabolite (Fig. S5, *F–H*).

To increase the sensitivity for RA detection, we next treated WT ESCs with EtOH for 48 h and switched to a medium containing high vitamin A (+0.5 μM ROL) 6 h prior to collecting lysates. This medium contained a 5–10-fold higher ROL concentration than that in standard 10% serum-containing medium (0.05–0.1 μM). In ESCs cultured in 0.5 μM ROL we could measure intracellular RA above the sensitivity threshold of the mass spectrometer, but we still detected no changes in RA levels in EtOH-treated ESCs compared with vehicle-treated cells (Fig. 4*E*).

RA is oxidized to 4-oxo-RA (36), so we measured 4-oxo-RA as a surrogate for RA and observed a downward trend in 4-oxo-RA levels after EtOH addition that was not statistically significant (Fig. 4*F*). Thus, we did not observe increases in intracellular RA levels by MS after EtOH addition.

Stra6 is necessary for ethanol-dependent increases in Hoxa1 and Cyp26a1, but not Dhrs3 transcripts

Stra6 is a ROL transporter that is expressed in some, but not all, tissues and cell types (37, 38), and its high expression is associated with a ROL requirement for differentiation (39). If EtOH induces RA-mediated transcription by enabling increased entry of ROL into ESCs for oxidation to RA, then the loss of Stra6 function should abrogate this effect. We first mea-

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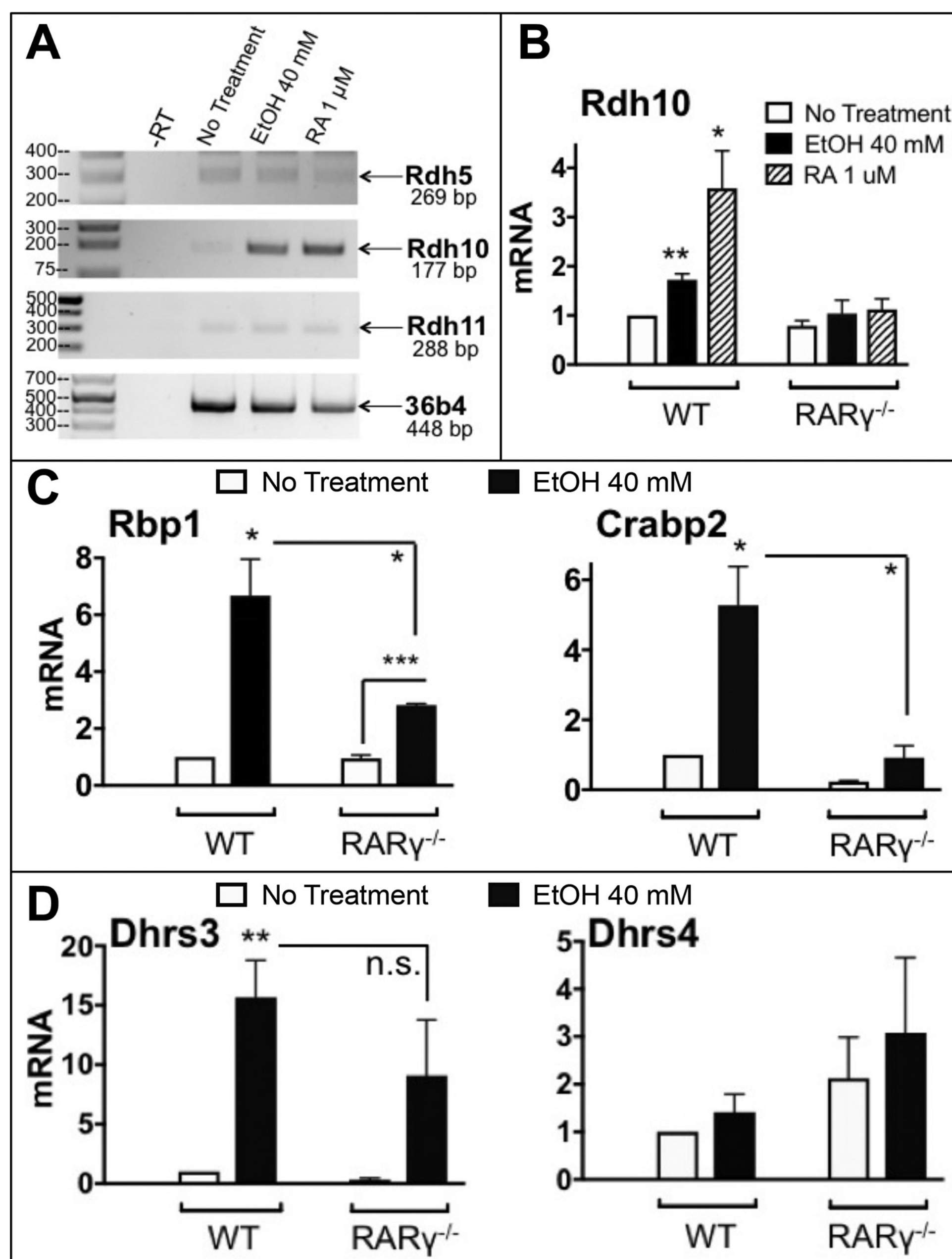

Figure 3. Transcripts involved in RA synthesis are up-regulated following ethanol addition. *A*, representative semiquantitative RT-PCR analysis of a panel of retinol dehydrogenase family transcripts expressed in ESCs, ±40 mM EtOH and 1 μM RA ($n = 2$). *B*, -fold changes in *Rdh10* transcript levels in WT and RARγE8$^{-/-}$ ESCs at 48 h treatment with EtOH (40 mM) or RA (1 μM RA). *C* and *D*, -fold changes in transcript levels of genes associated with retinoid transport (*C*) and retinaldehyde reduction (*D*) in WT and RARγE8$^{-/-}$ ESCs at 48 h treatment with EtOH (40 mM). *y*-Axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Treatment groups were compared with untreated ESCs at 48 h, except where indicated by *bar*. *Error bars* represent S.E. of independent biological experiments where $n =$ at least 3 biological repeats. *n.s.*, not significant; *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$.

sured *Stra6* mRNAs in WT and RARγ$^{-/-}$ ESCs. *Stra6* transcripts were elevated by 18.4 ± 2.8-fold ($p = 0.003$) in WT ESCs treated with EtOH, but were not elevated in the absence of RARγ (Fig. 5*A*). We saw effects of EtOH on the long and short *Stra6* isoforms similar to those we observed with 1 μM RA, with the long isoform increased to a greater extent by both EtOH and RA treatment (Fig. S6).

We compared the effects of EtOH in WT ESCs *versus* ESCs containing biallelic deletions of the *Stra6* RARE, which prevents binding of the RA:RAR/RXR complex (Stra6$^{\text{RARE}-/-}$) (37). First, we verified that Stra6$^{\text{RARE}-/-}$ ESCs display a considerably weaker response to RA stimulation than WT ESCs. Treating Stra6$^{\text{RARE}-/-}$ ESCs with 1 μM RA for 48 h resulted in a 3.13 ± 1.01-fold ($p = 0.044$) increase in *Stra6* levels compared with a 13.03 ± 0.95-fold ($p = 0.0004$) increase in WT cells, confirming that RA does not robustly increase *Stra6* mRNA in Stra6$^{\text{RARE}-/-}$ cells (Fig. 5*B*). We then detected a 4.68 ± 1.44-fold ($p = 0.042$) increase in *Stra6* mRNA by 40 mM EtOH and a 3.44 ± 0.59-fold ($p = 0.015$) increase by 0.5 μM ROL in WT ESCs (Fig. 5*C*). In contrast, *Stra6* transcripts were not induced by either 40 mM EtOH or 0.5 μM ROL in Stra6$^{\text{RARE}-/-}$ cells (Fig. 5*C*). Although *Hoxa1* and *Cyp26a1* mRNAs were similarly

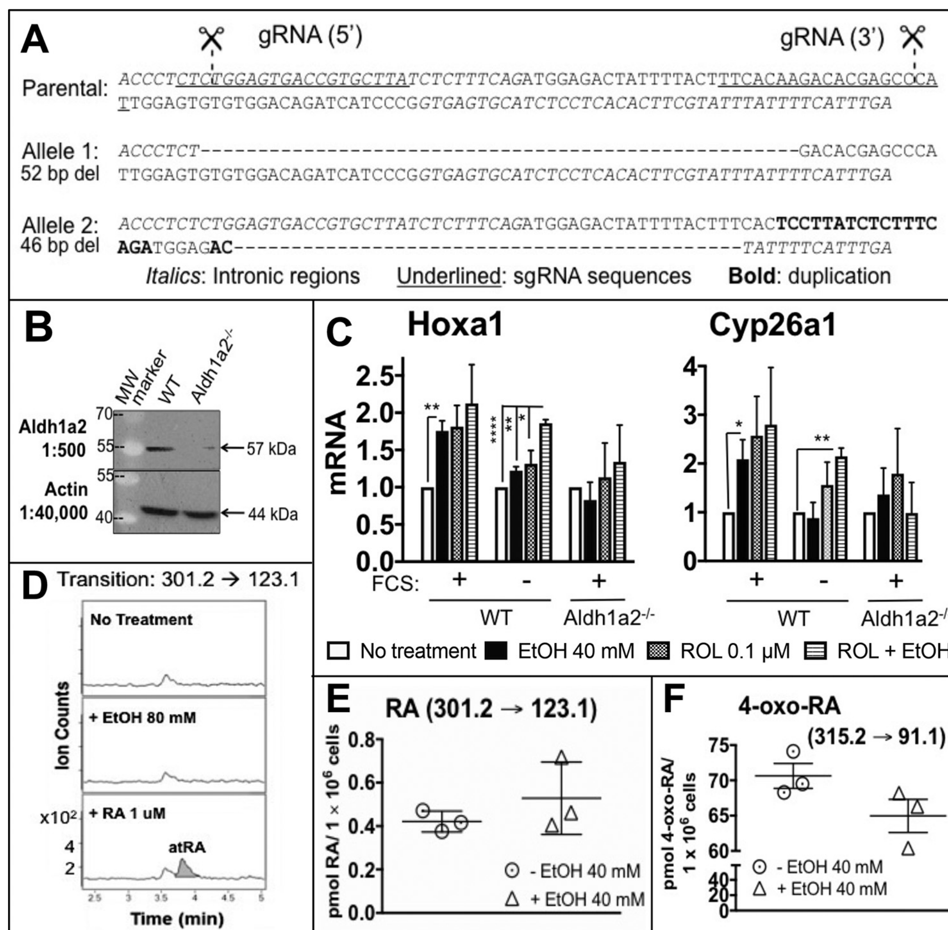

Figure 4. RA synthesis by Aldh1a2 is necessary for ethanol-mediated increases in *Hoxa1* and *Cyp26a1*. *A*, CRISPR/Cas9 deletion strategy using an nCas9 nickase vector. The parental sequence of exon 5 of the *Aldh1a2* gene is shown above with single guide RNA (*sgRNA*) target sequences *underlined*. Sequences of both edited alleles of the Aldh1a2E5$^{-/-}$ ESC line are shown below with deleted nucleotides represented by *dotted lines* and mutated sequences in *bold*. *B*, Western blotting of Aldh1a2 in WT and Aldh1a2E5$^{-/-}$ ESCs compared with β-actin loading control and measured against a molecular weight (*MW*) marker. *C*, quantitative analysis of transcript levels of *Hoxa1* (*left panel*) and *Cyp26a1* (*right panel*) by 40 mM EtOH, 0.1 µM ROL, or EtOH (40 mM) + ROL (0.1 µM). The -fold changes in transcript levels of genes in Aldh1a2E5$^{-/-}$ ESCs grown in 10% fetal calf serum–containing medium are compared with those of WT ESCs grown in standard medium + 10% fetal calf serum (*FCS*) and in chemically defined KOSR-containing medium. Transcript levels are compared with those of 0.1% DMSO-treated cells set to 1. *D–F,* reversed phase LC–tandem MS/MS followed by multiple reaction monitoring analysis of selected retinoids was performed on ESCs ± EtOH. *D*, all-*trans*-RA ion counts after treating ESCs with 80 mM EtOH or 1 µM RA for 8 h (retention time = 3.5 min). Intracellular RA (*E*) and 4-oxo-RA (*F*) concentrations 48 h after 40 mM EtOH addition and 6 h following a switch to medium containing 0.5 µM of additional ROL. Quantitation of RA was calculated in pmol/1 × 10^6 cells after normalizing to cell number, protein count, and recovery rate. An internal standard (5 µM retinyl acetate) not present in biological samples was added to all samples prior to extraction to calculate extraction efficiency. *y*-Axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. *Error bars* represent S.E. of independent experiments where n = at least 3 biological repeats. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

increased by both EtOH (1.55 ± 0.12-fold, $p = 0.004$, *Hoxa1*; 4.64 ± 1.36-fold, $p = 0.024$, *Cyp26a1*) and ROL (1.79 ± 0.11-fold, $p = 0.0004$, *Hoxa1*; 5.89 ± 1.4-fold, $p = 0.008$, *Cyp26a1*), respectively, in WT ESCs we did not detect increases in *Hoxa1* or *Cyp26a1* transcripts upon EtOH or ROL treatment of Stra6-RARE$^{-/-}$ cells (Fig. 5*D*). These data demonstrate that EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcript levels depend on greater ROL uptake via Stra6.

We then measured *Dhrs3* transcript levels and found no differences between EtOH-dependent increases in WT (1.64 ± 0.15-fold, $p = 0.04$) and Stra6$^{RARE-/-}$ (1.86 ± 0.31-fold, $p = 0.04$) ESC lines (Fig. 5*E*). In addition, Aldh1a2 deletion did not prevent EtOH-mediated *Dhrs3* mRNA increases as a 2.39 ± 0.39-fold increase ($p = 0.018$) was observed in EtOH-treated Aldh1a2$^{-/-}$ ESCs compared with a 1.80 ± 0.3-fold ($p = 0.037$) change in EtOH-treated WT cells (Fig. 5*F*). These data indicate that EtOH-mediated increases in *Dhrs3* mRNA levels do not require increased ROL import by Stra6 and RA production from retinaldehyde.

Rdh10 is required for ethanol-dependent increases in differentiation-associated genes

To address the potential requirement for Rdh10-dependent oxidation of ROL for EtOH-mediated increases in differentiation-associated transcripts we used CRISPR/Cas9 to generate deletions in both alleles in exon 2 of the *Rdh10* gene (Fig. 5, *G* and *H*). Adding 0.5 µM ROL to the culture medium caused increases in mRNA levels of *Hoxa1*, *Cyp26a1*, and *Stra6* in WT (5.31 ± 1.19-fold, $p = 0.011$, *Hoxa1*; 22.05 ± 5.17-fold, $p = 0.015$, *Cyp26a1*; 4.86 ± 1.29-fold, $p = 0.016$, *Stra6*) and Rdh10E2$^{-/-}$ (4.55 ± 1.45-fold, $p = 0.05$, *Hoxa1*; 22.02 ± 5.00-fold, $p = 0.014$, *Cyp26a1*; 5.01 ± 1.36-fold, $p = 0.017$, *Stra6*) ESCs. Transcripts of the same genes were unchanged by 40 mM EtOH in the Rdh10E2$^{-/-}$ ESCs compared with vehicle-treated

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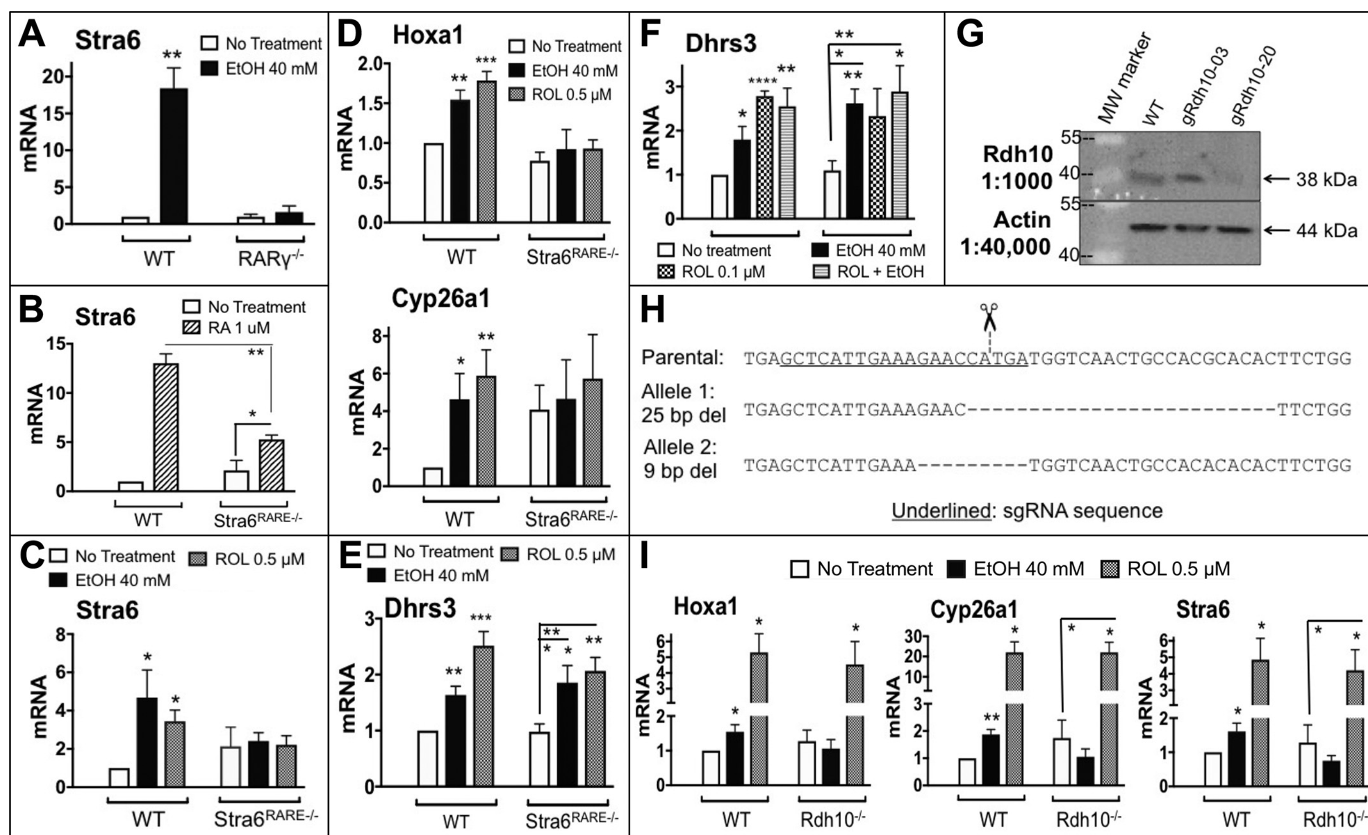

Figure 5. Stra6 and Rdh10 are required for ethanol-mediated increases in *Hoxa1* and *Cyp26a1* transcripts. *A*, -fold changes in transcript levels of the ROL transporter *Stra6* in WT and RARγE8$^{-/-}$ ESCs at 48 h treatment with 40 mM EtOH. *B*, -fold changes in *Stra6* mRNA levels in 1 μM RA-treated WT and Stra6$^{RARE-/-}$ ESCs at 48 h. *C* and *D*, -fold changes in transcript levels of *Stra6* (*C*), and *Hoxa1* (*D*, *top panel*), and *Cyp26a1* (*D*, *bottom panel*) in WT and Stra6$^{RARE-/-}$ ESCs at 48 h treatment with 40 mM EtOH or 0.5 μM ROL. *E*, -fold changes in *Dhrs3* mRNA levels in WT *versus* Stra6$^{RARE-/-}$ ESCs treated with EtOH or 0.5 μM ROL. *F*, -fold changes in *Dhrs3* mRNA levels in WT *versus* Aldh1a2E5$^{-/-}$ ESCs treated with EtOH, 0.1 μM ROL, or ROL + EtOH. *G*, Western blotting of Rdh10 in WT ESC and clones containing gRdh10E2 edits compared with β-actin loading control and measured against a MW marker. Clone 20 was selected for sequencing based on loss of protein expression. *H*, sequences of both edited alleles of the Rdh10E2$^{-/-}$ ESC line are shown below the WT sequence with deleted nucleotides represented by *dotted lines*. *I*, -fold changes of mRNA levels of *Hoxa1*, *Cyp26a1*, and *Stra6* by EtOH and ROL at 48 h ($n = 4$). Transcripts levels in 0.1% DMSO-treated WT cells were set to 1. Treatment groups were compared with untreated WT ESCs, except where indicated by *bar*. *y*-Axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. *Error bars* represent S.E. of independent experiments where n = at least 3 biological repeats. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

cells despite induction in WT cells (1.55 ± 0.21-fold, $p = 0.038$, *Hoxa1*; 1.87 ± 0.18-fold, $p = 0.009$, *Cyp26a1*; 1.61 ± 0.24-fold, $p = 0.045$, *Stra6*). These data show that oxidation of intracellular ROL by Rdh10 is required for EtOH-dependent increases in *Hoxa1*, *Cyp26a1*, and *Stra6* transcripts.

Discussion

The effects of EtOH on RA levels and signaling are highly debated; either potentiation (15) or inhibition (12–14, 16) of RA signaling in cell culture and animal models has been reported. Early studies relied on indirect assessment of RA activity or addition of exogenous ROL (13, 40), as a sensitive method of detecting RA levels was lacking until more recently. Recent studies have found that fluctuations in retinoid levels following EtOH administration often vary in a sex- or tissue-specific manner (15, 16). For example, Kim *et al.* (16) showed that retinyl esters (REs) were depleted in lungs of adult rats from dams fed 6.7% alcohol between embryonic day 7 and 21, with decreased levels in the ventral prostates and livers of males only. RA levels were not measured in this study, however, and depletion of retinyl esters could imply increased transport and utilization of retinoid stores for RA production in other tissues. Another study used both RE and RA levels as readouts for retinoid activity, revealing a complex physiological response to EtOH (15). Using a 6.5% EtOH-containing diet in mice for 1 month, Napoli and colleagues (15) showed that RE levels were unchanged in the brain and increased in kidneys and testis, yet hippocampal and cortex RA levels were increased by 20-fold and 2-fold, respectively, kidney RA levels were unchanged, and serum and testis RA levels were also increased.

The contextual relationship between EtOH and RA may also be influenced by developmental stage, contributing to differences in the literature. Shabtai *et al.* (41) demonstrated in *Xenopus* embryos that a deficiency in Aldh2 expression during gastrulation may create a competition for limiting amounts of Aldh1a2 enzyme and diminish RA production. Using a zebrafish model of high-dose (100 mM) EtOH exposure during gastrulation, addition of RA partially rescued some toxic effects on anteroposterior axis formation, ear development, and craniofacial cartilage defects but exposure to low-dose (1 nM) RA alone or with EtOH recapitulated other FASD-like developmental defects (12). Exposure to pharmacological doses of retinoids, such as through use of the prescription acne medication isotretinoin, also causes severe birth defects resembling an

FASD-like phenotype (42). Hence, retinoid teratogenicity is complex, as RA is central to cell differentiation and organismal development (7), and phenotypes present similarly whether low or high levels of RA are present (9–11, 42). The effects of EtOH are equally complex and are associated with both increases and decreases in RA levels in accordance with tissue physiology as well as gene expression patterns at different developmental stages.

Our use of ESCs allowed us to determine mechanistically how retinoid signaling in pluripotent stem cells, representing the most primitive stage of development, is affected by EtOH exposure. Additionally, we generally used a dose of EtOH (40 mM) that is representative of a concentration that will be present in the bloodstream of a binge drinking adult (20) to analyze the effects in stem cells without subjecting the cells to concentrations that may be potentially lethal in humans and induce a variety of secondary toxic events.

mRNAs of differentiation genes are increased and pluripotency factor mRNAs are decreased in embryonic stem cells treated with ethanol

Prior studies have shown that EtOH delays or interferes with proper differentiation along specific lineages in cell culture models of directed differentiation (3, 43, 44). Thus, we probed the acute effects of EtOH on selected self-renewal and differentiation-associated genes in undifferentiated ESCs. We detected decreases in some pluripotency marker transcripts, and increases in several differentiation-related transcripts (Fig. 1*C*; Fig. S1*B*). The loss of pluripotency in EtOH-treated cells was confirmed using alkaline phosphatase staining (Fig. S1*A*). Addition of 1 μM RA to cultured ESCs directly increases mRNAs of many lineage factors to cause differentiation along a parietal endoderm (epithelial) lineage (24). We have previously shown that Hoxa1 and Cyp26a1 protein levels are correlated with their mRNA levels (45, 46). We showed here that EtOH addition to cultured ESCs induced transcripts of several differentiation-associated genes, which was recapitulated by administering the EtOH metabolite AcH but not by acetate, suggesting that either EtOH or AcH is responsible for these increases in differentiation-associated mRNAs (Fig. 1, *D* and *E*). We speculate that the inhibitory effect of acetate on EtOH-mediated transcript induction of some differentiation-associated genes (Fig. 1*E*) may result from contributions by acetate-derived acetyl groups to histone acetylation modifications. Moussaieff *et al.* (47) have demonstrated that 1 mM acetate treatment of ESCs delays endodermal differentiation via its conversion to acetyl-CoA and subsequent transfer of these acetyl groups to histone lysines to maintain transcriptionally active chromatin for pluripotency-related genes.

RARγ binding to RAREs is necessary for ethanol-induced increases in mRNA levels of differentiation-associated genes

The activation of genes associated with differentiation by RA via RARs is well-characterized (7, 19). Activation of RAR-controlled transcriptional hubs in stem cells produces localized effects within RA-controlled chromosomal regions in factories of related differentiation genes containing RAREs that configure to their proper spatial position for transcriptional effects (17, 48). RARγ is an essential transcription factor in RA-dependent differentiation of ESCs (27, 49, 50). Some functional redundancy exists among the three types of RARs in ESCs (26, 51, 52), but only RARγ was demonstrated to mediate F9 embryonic carcinoma cell differentiation and override activity of other RARs (49). Additionally, the loss of RARγ, but not RARα, was associated with differentiation defects and altered *Hoxa1* expression (28, 53), which are likely caused by the dynamics of RAR subtype–binding patterns following ligand activation. Both RARα and RARγ occupy a large number of sites genome-wide during ESC differentiation (50). However, whereas RARα is enriched 24–48 h after RA signaling commences to sustain differentiation, RARγ initiates differentiation via direct activation of primary response genes (22, 28, 50, 53). We showed here that the increases in mRNAs induced by EtOH were prevented by ablation of *RARγ*, implicating direct RARγ/RXR-mediated signaling in promoting transcriptional effects of EtOH on differentiation genes (Fig. 2, *A* and *B*; Fig. S2).

Ethanol induction of differentiation-associated transcripts in embryonic stem cells depends on Aldh1a2

We demonstrate here that EtOH treatment of ESCs likely increases intracellular ROL from the serum to generate RA to activate transcription. This transcriptional effect of EtOH requires *Aldh1a2* expression, as genetic ablation of *Aldh1a2* prevented EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcripts (Fig. 4*C*).

Despite our inability to detect differences in RA levels between EtOH-treated and untreated ESCs (Fig. 4, *D* and *E*), depleting medium of ROL caused abrogation of EtOH-mediated transcriptional effects (Fig. 4*C*). Precedence for potent RA activity in the absence of detectable RA increases by MS is found in the literature. For example, Blaner and colleagues (54) demonstrated that *Lrat* (lecithin-retinol acyltransferase) ablation in the livers of mice was associated with increases in several RA response genes despite no detectable changes in RA levels measured by a highly sensitive LC-MS protocol. This is in line with our own findings, as an *Lrat*-deficient state in the liver mimics the natural state of ESCs, which are not equipped for ROL storage as esters (27). Excess retinaldehyde that is not oxidized to RA for downstream transcription would instead be converted back to ROL by Dhrs3 to maintain homeostasis (34).

Restoration of *Hoxa1* and *Cyp26a1* transcript induction by EtOH occurred upon adding ROL back into ROL-depleted medium, showing a retinoid requirement and implying enhanced sensitivity to available ROL in the presence of EtOH (Fig. 4*C*). To determine the mechanism underlying increased sensitivity to available ROL by EtOH, we measured mRNAs of genes in the ROL metabolism pathway and found increases in several, including *Rbp1*, *Crabp2*, *Rdh10*, and *Dhrs3* (Fig. 3). Rdh10 and Dhrs3 exist in a bifunctional complex to ensure that RA levels are tightly controlled (34). Although increasing the Rdh10 level in the presence of ROL proportionally increases detectable RA levels, an increase in both protein components, Rdh10 and Dhrs3, of the oligomeric complex prevents overall levels of RA from rising intracellularly (34). In our study, both Rdh10 and Dhrs3 mRNAs increase following EtOH treatment, with larger increases in Dhrs3, consistent with higher levels of

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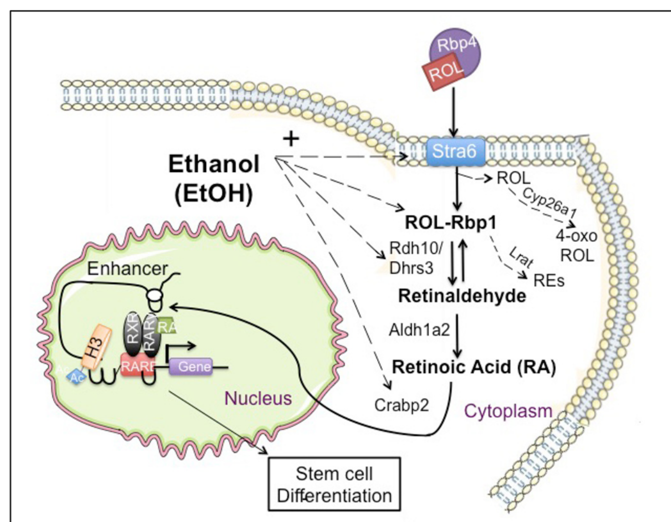

Figure 6. Model for ethanol regulation of stem cell differentiation via activation of RA signaling. ROL, in complex with Rbp4, is a substrate for Stra6, which imports ROL into the cell. EtOH increases the mRNAs of *Stra6* and several genes in the RA synthesis pathway, including *Rdh10*, *Dhrs3*, *Rbp1*, and *Crabp2*. Intracellular ROL is either converted to 4-oxoretinol via Cyp26a1 or presented to the RA synthesis machinery upon binding to Rbp1. ROL is not stored as retinyl esters in ESCs, as Lrat is not expressed in these cells. The Rdh10/Dhrs3 complex oxidizes ROL to retinaldehyde, which serves as a substrate for Aldh1a2-catalyzed oxidation to all-*trans*-RA. Newly formed RA is then transported to the nucleus by Crabp2, where it activates the RARγ/RXR transcriptional complex to stimulate expression of RA-responsive genes necessary for ESC differentiation.

Dhrs3 being required for fine-tuning retinoid oxidoreductase complex activity (34).

In addition, although *Cyp26a1* transcript levels were elevated by EtOH, we do not think that Cyp26a1 is a major contributor to the lack of detectable changes in RA levels after EtOH addition, as levels of 4-oxo-RA, a common polar metabolite formed from Cyp26a1 oxidation of RA, were not increased but rather trended downward (Fig. 4*F*). This finding is consistent with a model of EtOH causing enhanced sensitivity of ESCs to low amounts of RA generated from ROL metabolized by the retinoid oxidoreductase complex (model, Fig. 6).

Stra6-dependent retinol uptake from the medium facilitates efficient conversion of retinol to retinoic acid by Rdh10 for signaling in ethanol-treated embryonic stem cells

The Stra6 transporter, which facilitates ROL intracellular uptake, exhibited increased mRNA levels following EtOH treatment (Fig. 5*A*), and loss of *Stra6* RARE function was sufficient to abrogate EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcripts (Fig. 5*D*). Stra6 has "gatekeeper" functions in ESCs; in the absence of EtOH we speculate that the "gate" remains closed and ROL cannot enter the cells in high enough quantities to facilitate signaling. Given that ESCs express only a very low level of Lrat for ROL storage as retinyl esters (27), ROL entering the cells via the Stra6 transporter should be preferentially oxidized to RA. This suggests that EtOH may exert more toxicity via greater signaling through the RA pathway in cell types that do not express much Lrat. Because RA levels were not increased despite functional effects on expression of differentiation-related genes, it is likely that a steady influx of ROL through Stra6 followed by ROL conversion to RA via Rdh10 occurs, with efficient usage of newly synthesized RA to trigger nuclear signaling and subsequent differentiation through RARγ-mediated transcription (model, Fig. 6). Our findings in Rdh10-null ESCs further support this model. The activation of differentiation-associated genes by EtOH was completely abrogated in Rdh10-null ESCs (Fig. 5*I*). These results suggest that ROL is preferentially oxidized by Rdh10 upon EtOH treatment. Despite the failure of ROL to induce differentiation-associated mRNAs in the absence of a functional Stra6 RARE, induction of these mRNAs in Rdh10-null ESCs by EtOH was similar to that in WT. This suggests that once ROL is imported into ESCs it can still signal in the absence of oxidation by Rdh10, possibly via its efficient intracellular conversion to 4-oxoretinol, which serves as a direct ligand for RARs (55, 56).

Conclusions

Our findings collectively improve our understanding of the mechanisms by which EtOH metabolism affects RARγ signaling and differentiation in stem cells. We have demonstrated that EtOH causes stem cell differentiation via the activation of RA:RARγ-mediated transcription in pluripotent stem cells. We propose a model of enhanced ROL uptake in EtOH-treated ESCs, whereby EtOH causes Stra6-dependent ROL uptake into ESCs, followed by its conversion to RA by Rdh10 and Aldh1a2. RA is then transported to the nucleus to bind RARγ for RA:RAR/RXR-mediated transcription (Fig. 6). Because ESCs represent an early stage in a dynamic cascade of events in early embryogenesis, they serve as a good model for studying EtOH stem cell toxicity. Our lab has previously shown that exogenous RA stimulates target gene transcription in doses as low as 100 pM (24), and thus EtOH effects, via changes in RA signaling, can potentially greatly shift the trajectory of cell fate decisions to alter developmental outcomes. Our data raise the exciting possibility that stem cell–related complications of EtOH exposure may be amenable to manipulation of RAR target genes for the prevention of EtOH-associated toxicities and diseases.

Experimental procedures

Cell culture and reagents

AB1, CCE, RAR$\beta^{+/-}\gamma^{-/-}$, RARγE8$^{-/-}$, and Stra6$^{RARE-/-}$ ESCs were cultured as described previously (24, 26, 29, 37, 45). Cells were treated with 95% EtOH; 1 mM AcH (Calbiochem); 1 mM sodium acetate (Sigma), pH = 7.4; 0.1, 0.5, or 1 μM ROL (Sigma); and all-*trans*-RA (Sigma) at concentrations of 0.1 or 1 μM dissolved in 100% DMSO. AcH was aliquoted from a freshly opened bottle and tubes were stored at −20 °C for no more than 2 months. Each aliquot was immediately discarded after being added to the medium. Retinoids were prepared in dim light from a 1 mM stock solution. We used 1 μM RA for experiments to differentiate ESCs along an extraembryonic endoderm lineage, and 0.5 μM as a positive control for stimulating RARE activation in the β-gal assay. 0.1% DMSO was added to each treatment group not containing RA. ESCs were seeded in 6-well plates and harvested simultaneously for treatments conducted 72, 48, or 24 h prior to collecting lysates. Reagents were changed twice daily ∼12 h apart, with the final reagent change completed 8 h prior to harvest. EtOH was used at concentrations of 40 mM and 80 mM in various experiments. 103 units/ml of leu-

kemia inhibitory factor was added to medium for all experiments, including medium in which KnockOut™ SR (Gibco) replaced ESC-grade fetal calf serum.

β-gal assay

β-gal assays were performed as described previously (22). CCE cells were grown in 6-well plates and transfected the following day with 2–3 μg of either WT *Hoxa1* minigene-lacZ or *Hoxa1*-lacZ muRARE constructs. A pGL3-luciferase construct with an upstream SV40 promoter was simultaneously transfected at 0.1–0.2 μg (15:1 ratio sample:control) to normalize β-gal activity to luciferase expression. Cells were treated 48 h after transfection with DMSO (0.05%), 40 mM EtOH, or 0.5 μM RA for 24 h with a reagent change 8 h prior to harvest. RA doses above 0.5 μM did not cause additional stimulation of reporter assays. Cells were collected in TEN buffer and sonicated to prepare lysates for the β-gal assays.

Generation of Aldh1a2E5$^{-/-}$ and Rdh10E2$^{-/-}$ lines

CRISPR constructs for Aldh1a2E5$^{-/-}$ line creation were generated using the pX461, pSpCas9n(BB)-2A-GFP nickase vector (Addgene #48140). The CRISPRevolution Synthetic RNA kit was used in generating Rdh10E2$^{-/-}$ cells (Synthego, San Francisco, CA). Single cell dilutions were plated and grown for 1 week. Colonies were subsequently grown in 24-well plates and harvested in PBS. Clones were genotyped for genome editing by PCR amplification followed by restriction digestion. Clones lacking the restriction site were Sanger sequenced on both alleles and double-positive knockout clones were expanded in culture (see supporting "Materials and methods").

ChIP assays

Experiments were performed as described previously (18) with described antibodies (see supporting "Materials and methods"). Primers used for PCR analyses are detailed in Table S1.

Retinoid extraction and HPLC–tandem MS analysis

Lysates were collected in PBS and extracted using 50% acetonitrile/butanol and saturated K_2HPO_4 (57). The organic phase was vacuum dried and reconstituted in 100% acetonitrile before loading. Retinoid separation was conducted using HPLC (Agilent, Palo Alto, CA) and Jet Stream electrospray ionization in positive ion mode. Reversed phase HPLC–tandem MS analysis was performed as described previously (58) (see supporting "Materials and methods").

Statistical treatment of the data

Statistical analysis was conducted on at least three independent biological replicates for each experiment using GraphPad Prism 7.0 software. The mean ± S.E. was determined. Analysis of variance (ANOVA) was used to determine statistical significance within sets of three or more groups, and Student's *t* test was used to compare two independent populations. A two-tailed p value <0.05 was considered statistically significant.

Accession numbers

NCBI Gene identifiers for murine genes featured in this study include aldehyde dehydrogenase 1 family member A2 (*Aldh1a2*, ID: 19378), retinol dehydrogenase 10 (*Rdh10*, ID: 98711), retinoic acid receptor γ (*RAR*γ, ID: 5916), and stimulated by retinoic acid 6 (*Stra6*, ID: 20897).

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