ETHANOL (EtOH)-MEDIATED DIFFERENTIATION OF EMBRYONIC STEM CELLS VIA RETINOIC ACID (RA)-RETINOIC ACID RECEPTOR-GAMMA (RARγ) SIGNALING

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate School

Of Medical Sciences

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2019

ETHANOL (EtOH)-MEDIATED DIFFERENTIATION OF EMBRYONIC STEM CELLS VIA RETINOIC ACID (RA)-RETINOIC ACID RECEPTOR-GAMMA (RARγ) SIGNALING

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Cornell University, 2019

Ethanol (EtOH) is a teratogen, but the mechanisms by which EtOH exerts its teratogenic effects aren't fully understood. Vitamin A (all-*trans* retinol/ROL) can be oxidized to all-*trans*-retinoic acid (RA), which plays a critical role in differentiation and development. Using an embryonic stem cell (ESC) model to analyze effects of EtOH on differentiation, we show that mRNAs associated with differentiation are increased by EtOH and its metabolite acetaldehyde, but not its acid metabolite acetate. EtOH also decreases pluripotency-related mRNA levels. Kinetics assays showed that ALDH2, and not ALDH1A2, is responsible for metabolizing most of the acetaldehyde in ESCs. Using reporter assays, chromatin immunoprecipitation assays, and RARγ-knockout ESC lines generated by CRISPR/Cas9 or homologous recombination, we demonstrate that EtOH signals via RARγ binding to RA response elements (RAREs) in differentiation-associated genes. We also demonstrate that EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcripts, used as examples of direct RA target genes, require expression of the RA-synthesizing enzyme ALDH1A2. This result suggests that EtOH-mediated induction of *Hoxa1* and *Cyp26a1* transcripts requires ROL from serum. The retinol dehydrogenase gene

RDH10 and a functional RARE in the ROL transporter *Stra6* gene are required for EtOH induction of *Hoxa1* and *Cyp26a1* mRNAs, as shown with CRISPR/Cas9 knockout lines. Thus, we identify a mechanism by which EtOH stimulates stem cell differentiation via increased influx and metabolism of ROL for downstream RARγ-dependent transcription. Our data suggest that 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. Furthermore, we suggest that stem cells, which generally cannot produce retinyl esters, may be particularly vulnerable to EtOH teratogenesis.

BIOGRAPHICAL SKETCH

Ryan N Serio was born on November 9, 1978, in New York, New York. He received his Bachelor's of Science in Pharmacy in 2001 and Doctorate of Pharmacy in 2003 from St. John's University College of Pharmacy and Health Sciences. After working in pharmacy for 11 years, Ryan pursued his passion for medical research by first receiving a Master's degree in Biopharmaceutical Technology from St. John's University and then interning for one year in the Muscle and Metabolism Department of Regeneron Pharmaceuticals. Under the mentorship of Dr. Esther Latres, he gained experience culturing and differentiating human myotubes from satellite cells and using proprietary compounds to tests effects on differentiation, and aided in projects related to dyslipidemias, obesity, and diabetes.

Ryan was accepted into the Ph.D. Program for Pharmacology at Weill Cornell Graduate School of Medical Sciences in 2012. He joined the labs of Dr. Lorraine J. Gudas, an expert in retinoid biochemistry and their role in genetics and cell signaling pertaining to health and disease, and co-mentor Dr. Steven S. Gross, an expert on cellular intermediary metabolism and biochemistry. Their combined mentorship enabled Ryan to study the effects of ethanol on stem cell differentiation from both a genetic and biochemical standpoint. The focal point of his research was to delineate the mechanism by which ethanol affects the retinoic acid signaling pathway in stem cells, and how these alterations affect stem cell differentiation.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my mentor, Dr. Lorraine Gudas for accepting me into her lab and for all of her generous guidance as I performed my thesis work. Dr. Gudas helped me immensely in every way in starting my scientific career, from asking the most important scientific questions, designing the proper experiments to test them, analyzing data, writing grants and manuscripts, and homing my presentation skills. I would also like to greatly thank my co-mentor, Dr. Steven Gross, for all of the guidance and assistance he provided with my thesis project. In addition, I thank my thesis Special Committee members Drs. Samie Jaffrey and Michael Kharas, for steering me in the right direction in my project and for their numerous insights, and my Special Committee chair, Dr. Olga Boudker, for helping me in my inchoate stage at Weill Cornell. My research was partially supported by the T32-CA062948 and F31-AA024024 training grants.

I would like to thank the Gudas and Gross labs, past and present, in particular Dr. Kristian Laursen, for all of his guidance and scientific advice throughout my stay, as well as Drs. Alison Urvalek, Changyuan Lu, YuLiang Ma, and everyone I worked with, who made my stay here both fun and intellectually stimulating. Finally, I would like to thank my parents for giving me the best chance to succeed throughout my life, and my family and extended family and friends for all their support throughout my journey.

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LIST OF ABBREVIATIONS

AcH	acetaldehyde
ACN	acetonitrile
DMSO	dimethyl sulfoxide
EtOH	ethanol
ESC	embryonic stem cell
FSC	fetal calf serum
gRNA	guide RNA
H3K27ac	acetylated histone 3 lysine 27
KOSR	knockout serum replacement
LLOD	lower limit of quantitation
LOD	limit of detection
IgG	immunoglobulin G
MRM	multiple reaction monitoring
RA	retinoic acid
RAc	retinyl acetate
RAR	retinoic acid receptor
RAS	reactive aldehyde species
ROL	retinol
ROS	reactive oxygen species
RP-HPLC-MS/MS	reversed phase high performance liquid chromatography-tandem mass spectrometry

RT	retention time
RT-qPCR	quantitative reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
VAM	high vitamin A medium
WT	wild type
4-HNE	4-hydroxynonenal

CHAPTER ONE

INTRODUCTION

Ethanol (EtOH) is a potent teratogen that adversely dysregulates the processes controlling cellular differentiation during embryonic development (1,2). The mechanisms by which EtOH affects the differentiation of stem cells, which form the foundation underlying the teratogenic potential of EtOH, are not well-characterized, despite ongoing efforts to elucidate the factors causing teratogenic phenomena (1,3-9). A brief description of stem cells and their differentiation in response to various morphogenic signals is first warranted before further examining how EtOH interferes with stem cell differentiation.

PLURIPOTENCY AND DIFFERENTIATION OF EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts, and are considered pluripotent in that they contain the potential to differentiate into any cell type in the human body (10,11). Pluripotent cells from the inner cell mass differentiate into primitive ectoderm prior to giving rise to three embryonic germ layers (ectoderm, mesoderm, endoderm) (10). These three layers then establish the body plan of an embryo and eventually a fetus through the coordinated expression of temporally controlled, morphogen-responsive differentiation programs (12). Naïve ESCs express a set of

pluripotency factor genes, including *Oct4*, *Nanog*, and *Sox2* (13) that enable the maintenance of an undifferentiated state characterized by symmetric self-renewal while retaining the capability to form cells comprising the germ layers in addition to more specialized downstream derivatives (14).

Transcriptional control of the stem cell state is maintained by high levels of pluripotency factors that bind throughout the genome at cis-regulatory elements, particularly at promoters and enhancers of target genes (15). Enhancers are genomic regions that are typically comprised of a few hundred base pairs and contain binding motifs for a number of different transcription factors (16). Clusters of large enhancers that are densely occupied by specific transcription factors are called superenhancers (17,18). Superenhancers are instrumental in dictating cell fate, as they contain binding motifs for “master” transcription factors that are expressed in a cell-type and tissue-specific manner and maintain the identity of the particular cell they occupy (17,18). Once one or more of the core transcription factors binds the superenhancer, cooperative binding of other factors occurs within the enhancer region to activate target genes as transcriptional units that program the cell to express the characteristics of a particular fate (17,19).

In ESCs, the five major transcription factors that maintain stem pluripotency through their interactions with superenhancer domains are OCT4, SOX2, NANOG, KLF4, ESRRB, and Mediator (17). While the function of Mediator is mainly to recruit RNA Polymerase to target gene promoters (20), the remaining transcription factors maintain

pluripotency and self-renewal properties intrinsic to stem cells (15). Because of the vital role that the major pluripotency-associated transcription factors play in defining and maintaining the properties of ESCs, altering one or more of these five core pluripotency factors may result in the loss of stem cell identity and precocious differentiation (21-24).

Maintenance of the stem cell state is additionally controlled by epigenetic factors, including DNA methylation patterns and chromatin modifications that provide distinct regulatory signatures that either favor or repress transcription (13,25-28). Histone acetylation is a type of modification that is relevant to the research presented here in that an increase in this modification is often indicative of transcriptionally active chromatin involved in the recruitment of transcription factors (29). Histone acetylation at lysine 9 or 14 on histone 3 (H3K9/14) occurs in euchromatin to activate transcription, and acetylation at lysine 27 (H3K27me3) is often present in active enhancers (30). In contrast, trimethylation of H3 (H3K27me3) is a well-characterized repressive histone modification that maintains many differentiation-associated genes in a transcriptionally silenced state in ESCs (31,32). Many key regulators of ESC differentiation contain a “poised” bivalent chromatin structure, expressing some activating histone modifications (H3K4me3) and some repressive modifications (H3K27me3) that allow for faster, more flexible control of expression (33). As ESCs begin to differentiate, the epigenetic landscape changes so that promoters and enhancers controlled by the core pluripotency factors gradually exhibit more repressive marks (25,31). Those loci containing differentiation-

associated genes then recruit chromatin regulatory proteins that deposit modifications that are associated with transcriptionally active euchromatin that become finely tuned throughout stages of differentiation to express different gene signatures at the appropriate times based on intrinsic factors and external stimuli (25,26,34,35).

Because they represent the most primitive stage of development, ESCs hold great potential as tools for delineating some of the core mechanisms by which EtOH exerts its effects. The potential for widespread phenotypic changes that impact the largest number of downstream processes is greater the earlier that a toxic insult is given in terms of stage of development: the more primordial the stage, the more encompassing the effects. Therefore, it is logical to study the effects of a teratogenic substance like EtOH in highly plastic stem cells representing the most primordial stage of development, like ESCs.

In order to understand how EtOH influences normal ESC differentiation, it is crucial to understand how physiologic differentiation unfolds. Stem cell differentiation is a tightly regulated process (11,15). The loss of pluripotency and onset of differentiation is controlled by the response of ESCs to specific morphogens, such as retinoic acid (RA), that signal in the nucleus to suppress the pluripotency-related programs and to activate transcriptional hubs for the expression of differentiation-associated genes (36). Depending on the type of morphogen, its concentration, and its interactions with additional morphogenic signals, the differentiation of stem cells as they lose pluripotency characteristics is directed toward different lineages at different stages (12).

ETHANOL INTERFERES WITH THE DIFFERENTIATION OF STEM CELLS

Profound changes in embryonic patterning and development can result from small changes in the expression of some of the initial differentiation-associated genes to be expressed when a stem cell begins to differentiate along a specific lineage (37). Several studies have capitalized on the potential of stem cells to form cell types of different terminal fates depending on time- and signal-dependent factors in directed differentiation cell culture models to assess the ability of EtOH to influence differentiation down specified lineages (38-40). Studies using directed differentiation are useful for analyzing the emergence of differentiation defects that occur as a stem cell is in the process of differentiating into a terminal cell of particular interest. However, one must bear in mind that these results cannot always be extrapolated to draw conclusions for general ESC differentiation because of the many differences correlated with each stage of development that are recapitulated in cell culture by using lineage-specific differentiation protocols. Our use of pluripotent ESCs avoids these confounding variables.

The phenotypes observed following EtOH treatment of stem cells have been explored in various studies in disparate contexts. Many studies have probed defects during directed stem cell differentiation into different terminal cell types, including neural, hepatic, and cardiac cells (38-42). In all three cell types, EtOH either delays or diverts differentiation from the terminally differentiated cell of interest.

EtOH was demonstrated to divert differentiation of neural stem cells away from a neuroectoderm fate (39,41,42) and toward a mesoendoderm fate (39). This diversion of differentiation was accompanied by distinct subpopulations of cells that exhibited a high OCT4/SOX2 ratio that gave rise to cells characteristic of mesoendoderm as well as fewer β-tubulin-III-positive cells indicative of neuroectoderm (39). Additional studies showed that EtOH causes defects in directed differentiation of stem cells to hepatic (38) and cardiac progenitor cells (40), respectively. The mechanism for dysregulated differentiation in each case was attributed to suppression of WNT signaling necessary for proper differentiation (38,40,41).

The role of EtOH in embryonic stem cell (ESC) differentiation was probed using naïve ESCs (3), and also with ESCs differentiated using leukemia inhibitory factor (LIF) removal as a trigger for loss of pluripotency (4). VandeVoort et al. used alkaline phosphatase staining and staining for specific human stem cell-related markers (TRA-1-81, TRA-1-60, OCT4, SSEA4) to analyze changes in pluripotency following 0.1% and 1% EtOH exposure to ESCs for two weeks (3), and demonstrated a loss of pluripotency in ESCs treated with EtOH via staining with alkaline phosphatase and TRA-1-81, despite OCT4, SSEA4, and TRA-1-60 staining remaining unaffected by EtOH compared to untreated ESCs (3). The loss of pluripotency in EtOH-treated cells coincided with changes in cell morphology, including a decrease in colony size, differences in densities, increased opaqueness, and changes in shape. In particular, EtOH caused colonies to exhibit irregular borders characterized by dendritic projections (3). This

phenotype is consistent with differentiated cells as opposed to ESCs, which form spherical colonies with smooth borders (3). These results showed that EtOH treatment of pluripotent ESCs may cause differentiation, but the mechanisms by which the cells differentiated remain unknown. A central part of my thesis work was to elucidate the mechanisms causing ESC differentiation by EtOH, given that we could first recapitulate a differentiation phenotype.

There remains some debate over whether EtOH treatment causes ESC differentiation. In a second report studying the effects of EtOH on differentiation, Arzumanyan et al. treated cultured ESCs with 100 mM EtOH for two days followed by differentiation over 6 days by removing Leukemia inhibitory factor (LIF), a signaling protein required for maintaining stemness in cell culture (43), from the medium (4). There was a delay in the decline of specific pluripotency-related transcription factor mRNAs, including OCT3/4, SOX2, NANOG, and SSEA-1, while these transcripts were unaffected in ESCs treated with EtOH for 48 hours (4). These results are inconsistent with the previous findings (3), and argue against the capacity of EtOH to facilitate ESC differentiation. One possibility for these discrepancies is the method of differentiation used by Arzumanyan et al., as depletion of LIF causes a specific differentiation signature characterized by expression of Fgf5, which is a marker of primitive ectoderm, which gives rise to neuroectoderm (44,45). Because EtOH specifically inhibits neuroectoderm differentiation (39,41), it is unsurprising that EtOH would delay differentiation caused by LIF removal, which recapitulates the neuroectodermal phenotype (44,46).

My thesis work does not utilize medium LIF removal as a method of differentiation. Instead, we administer EtOH to ESCs over different time courses and then measure pluripotency- and differentiation-associated transcript levels to initially analyze potential changes related to differentiation. Because I initially do not use a differentiation protocol, I would expect to see a decline in pluripotency factor mRNAs and an increase in transcripts involved in differentiation if EtOH does stimulate ESC differentiation.

ETHANOL IS METABOLIZED TO ACETALDEHYDE, WHICH IS DETOXIFIED BY ALDEHYDE DEHYDROGENASE ENZYMES.

To better comprehend how EtOH affects differentiation, it is imperative to first understand how it is metabolized. EtOH is metabolized through oxidation reactions to additional chemically reactive compounds that also confer biological activity, including acetaldehyde (AcH), acetate, and acetyl coenzyme A (acetyl CoA) (47,48).

EtOH metabolism requires a two-step oxidation process (49,50). First, EtOH is oxidized to the reactive aldehyde species, acetaldehyde (AcH), by either a member of the alcohol dehydrogenase (Adh) family or catalase (49,50). The metabolism of AcH to acetate is carried out primarily by aldehyde dehydrogenase 2 (ALDH2, NCBI #11669) and the metabolism of acetate is performed by acetyl CoA synthetase short chain family member 2 (ACSS2/ACECS1, NCBI #60525) (48). Acetyl CoA then serves as a central metabolite for several biochemical

processes, including lipogenesis, energy production from the tricarboxylic acid cycle, and protein acetylation (51-53). Because distinguishing between the effects of EtOH and its metabolites may provide information on how EtOH affects differentiation, our strategy included the generation of ESC lines in which the enzymes (ALDH2 and ACSS2) that primarily oxidize AcH and acetate are knocked out, respectively, by CRISPR/Cas9-mediated deletion (Figure 1.1). As a result, AcH levels would increase from deletion of ALDH2 and acetate levels would increase from deletion of ACSS2, allowing us to better analyze the effects of each individual metabolite and enzyme on ESC differentiation.

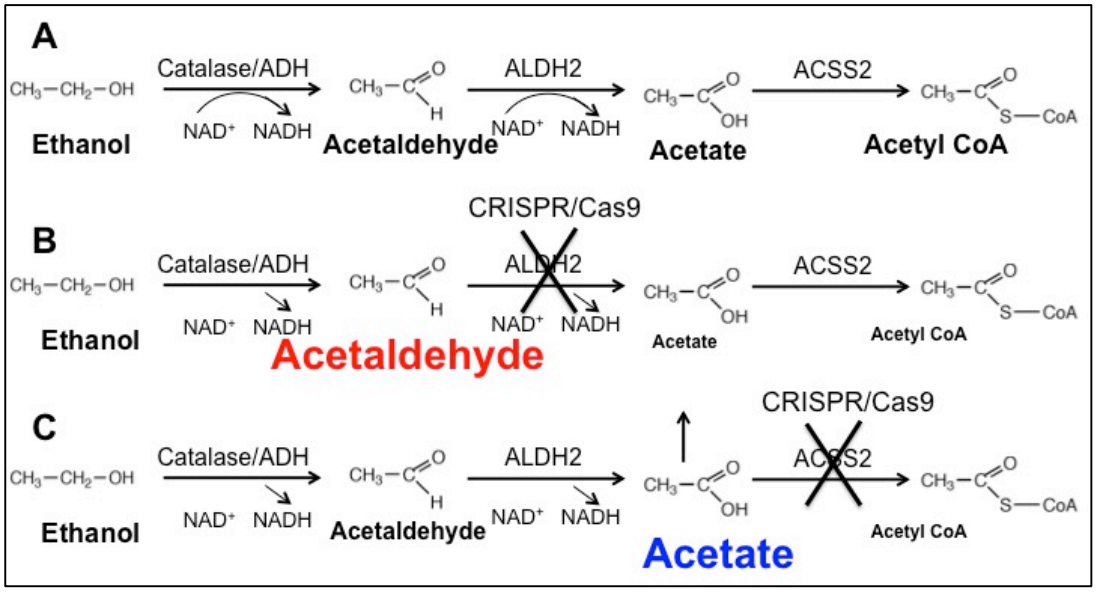

Figure 1.1: Strategy for dissecting the effects of ethanol metabolism on embryonic stem cell differentiation. In WT ESCs, EtOH is first metabolized to AcH by an ADH family member or catalase. AcH is then oxidized to acetate by ALDH2, and acetate is further metabolized to acetyl CoA by ACSS2. Knocking out ALDH2 would prevent AcH metabolism to acetate upon EtOH treatment, leading to increased AcH in cells and decreased acetate compared to WT cells treated with EtOH. Deleting ACSS2 would prevent acetate metabolism to acetyl CoA following EtOH addition, leading to increased acetate levels compared to WT cells treated with EtOH.

AcH can alter the structure and function of many biomacromolecules, including proteins, nucleic acids, and membrane lipids, by forming covalent adducts (54). These adducts can cause a variety of toxic effects such as DNA damage, loss of protein function, or organelle dysfunction (54,55). Although much is known about the mechanisms by which AcH damages cell components, questions in the field of stem cell biology still remain. We attempt to investigate whether AcH and its metabolism, in particular, play roles in ESC differentiation by creating and using an ALDH2-knockout ESC line to

prevent efficient metabolism of AcH to its less toxic downstream product, acetate.

Whereas many types of enzymes are capable of metabolizing EtOH to AcH, members of the aldehyde dehydrogenase family uniquely detoxify acetaldehyde and other reactive aldehydes by converting them to acids (56). In the human adult liver, ALDH2 is expressed at high levels and oxidizes xenobiotic aldehydes such as AcH as well as endogenous aldehydes, including 4-hydroxynonenal (4-HNE) (57-60). ALDH2 executes metabolism of its substrates as a homotetramer through thiol-dependent, nucleophilic attack on the carbonyl carbon of the aldehyde, transferring the hydrogen atom as a proton and electron pair to the reducing equivalent NAD^+ (56). An oxygen atom from a water molecule then forms a covalent bond with the carbonyl group, breaking the thiol bond to generate a terminal acid product (Figure 1.2).

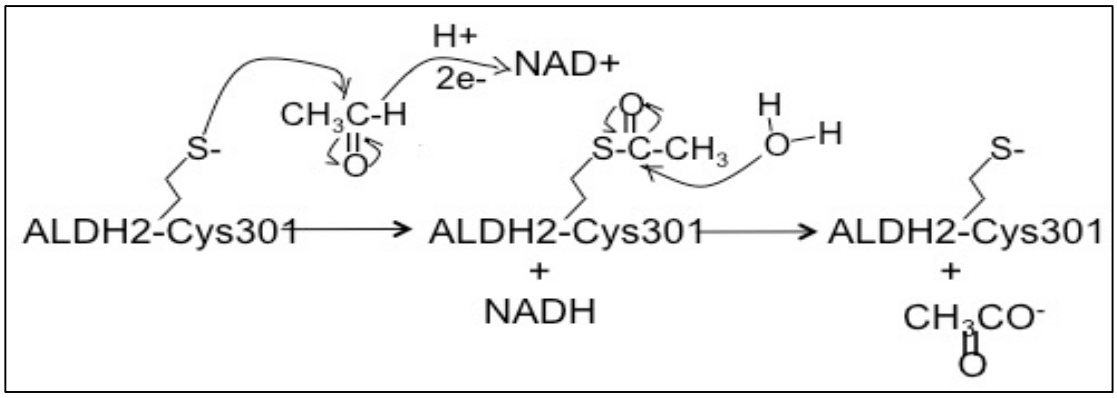

Figure 1.2: Mechanism of ALDH2 activity. A reactive thiol group in the catalytic site of ALDH2 attacks the electrophilic carbonyl group of AcH to form a covalent adduct. A water molecule then bonds with the same carbonyl, dissociating the newly formed acid from the enzyme. The proton and electrons extracted from the aldehyde group are transferred to the NAD+ cofactor in the process.

While other members of the aldehyde dehydrogenase family of enzymes can metabolize AcH, Aldh2 has the greatest affinity for AcH, with a Km of 0.2 μM (61). In humans, the hereditary dissemination of an allele that silences ALDH2 activity is fairly common in certain populations, including in approximately 40-50% of people of Asian descent (62). The most predominant genetic variant associated with ALDH2 inactivation is the ALDH2*2 allele, in which there is a single base pair alteration of glutamate to lysine (E487K) that leads to a dominant negative phenotype (63). This variant is associated with accumulation of AcH upon EtOH ingestion and with more severe complications, including higher risk of cancer development compared to members of the population with functional ALDH2 (64,65). This information is presented here as it shows that ALDH2 is responsible for most of AcH metabolism in humans, and that loss of its function implicates AcH in several EtOH-mediated disease complications with severe phenotypes.

Although most EtOH metabolism occurs in the liver, AcH can produce toxic effects in other tissues (66). Human adult stem cells (67) as well as stem cell progenitors (68) are highly sensitive to the effects of the metabolic byproducts of EtOH. Stem cell compartments that regenerate tissue are vital for maintaining functional integrity of many body compartments (69), and thus must be able to withstand toxic insults from xenobiotics. Adult stem cells are multipotent rather than pluripotent, in that they are limited in the types of cells into which they can differentiate, yet they share several essential core genetic regulatory networks and bioenergetics pathways with ESCs (70),

making ESCs a relevant model system for extrapolating some general functions of stem cell components within the body. In addition, targeting ALDH2 for deletion in ESCs would allow us to distinguish between effects on differentiation that are caused by AcH versus effects potentially caused by EtOH in the absence of oxidative metabolism.

RETINOL METABOLISM IS SIMILAR TO ETHANOL METABOLISM AND CRITICAL FOR DIFFERENTIATION

Central to stem cell differentiation are a group of compounds known as the retinoids (36). Retinoids are signaling molecules that exert paramount effects on development and differentiation, primarily via the vitamin A derivative, retinoic acid (RA).

Retinol (vitamin A/ROL) is a compound that is obtained from the diet and serves as a precursor for RA formation. One well-characterized manner in which ROL enters cells from the bloodstream is via binding to retinol-binding protein-4 (RBP4) followed by binding to STRA6, a retinol transporter protein (71). Upon entry in the cell, ROL releases RBP4 and binds RBP1 (CRBP1), which then translocates to a retinoid oxidation complex consisting of the retinol oxidase, Retinol dehydrogenase-10 (RDH10) and the retinaldehyde reductase, Dehydrogenase/Reductase-3 (DHRS3) (72). RDH10 converts ROL to retinaldehyde, and DHRS3 prevents excessive production of retinaldehyde and its derivative, RA, by serving as a rheostat to perform the reverse catalytic reaction (reduction) when RA

accumulation is sensed (73,74). Retinaldehyde is then converted to RA by the actions of one of three aldehyde dehydrogenase 1 family members, ALDH1A1, ALDH1A2, and ALDH1A3 (75). Importantly, ALDH1A2 is the first ALDH1 family member to be expressed in embryos, and is expressed in high levels on E7.5 in mice (76).

The mechanism of ROL metabolism is similar to that of EtOH, in that the alcohol form becomes oxidized first to an aldehyde and then an acid, with the first step usually catalyzed by an alcohol or retinol dehydrogenase enzyme and the second step catalyzed by an ALDH family member (Figure 1.3). Because of the similarities in metabolism between EtOH and ROL, many studies have focused on elucidating whether EtOH competes with (5-8,77,78) or activates (9,79) the RA synthesis pathway. Before expounding on these studies in more detail, further explanation of the RA signaling pathway is necessary due to the profound effects that RA exerts in activating transcription of a differentiation gene signature.

Once RA is produced, it binds to the CRABP2 protein and translocates to the nucleus (80). Here it serves as a ligand for one of three retinoic acid receptor (RAR) isoforms: RARα, RARβ, and RARγ (81). The RARs form heterodimeric complexes with a member of the retinoid X receptor (RXR) family (RXRα/β/γ) (36). While all three RARs exhibit some degree of functional redundancy in signaling (82,83), RARγ has been shown to be particularly important for ESC differentiation (84,85). When unbound to RA, RARs remain in the nucleus but are devoid of transcriptional properties, as they remain in a repressive state, bound by several co-repressor proteins (25). Upon

binding to RA, coactivators bind the RA-RAR/RXR complex, replacing the corepressors, and transcription occurs rapidly (86,87).

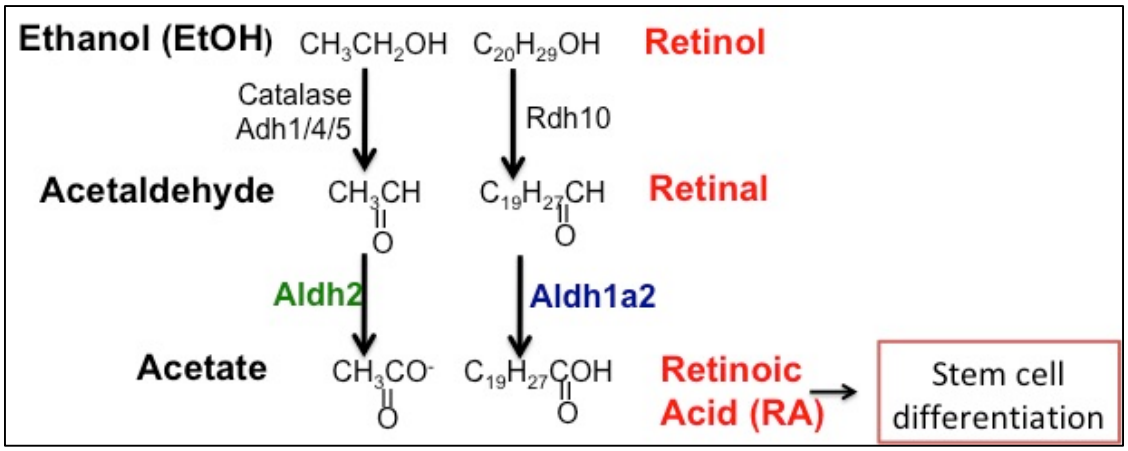

Figure 1.3: Metabolic oxidation of ethanol and retinol in stem cells. Both EtOH and retinol are oxidized to an unstable aldehyde intermediate and then further metabolized to an acid by the actions of an enzyme in the aldehyde dehydrogenase family. Several enzymes are capable of oxidizing EtOH to acetaldehyde in stem cells, including members of the alcohol dehydrogenase (ADH) family and catalase. Aldehyde dehydrogenases primarily metabolize acetaldehyde and retinaldehyde (retinal), with ALDH2 exhibiting the lowest Km for acetaldehyde and ALDH1A2 being the first of the retinoid-oxidizing aldehyde dehydrogenases (ALDH1A1/1A2/1A3) to be expressed in embryos (76).

RA-RAR/RXR complexes bind to cis-elements on DNA referred to as retinoic acid response elements (RAREs) (36,88). RAREs, which are often in enhancers, consist of a consensus sequence of either direct or inverted repeats separated by a short series of nucleotides. Some of the most frequently bound sites are direct repeat (DR)-2 and DR-5, which are annotated in the following manner, for example: 5'-(A/G)G(G/T)TCA-(N_2)-(A/G)G(G/T)TCA-3' (36). Genes that contain RAREs for direct activation by RA are called "primary response", or "immediate early" genes, while targets of RA that become activated as

a result of activation by a primary response gene that is a transcription factor are called “secondary response” genes (86,87). Retinoid metabolism and signaling is further illustrated in Figure 1.4.

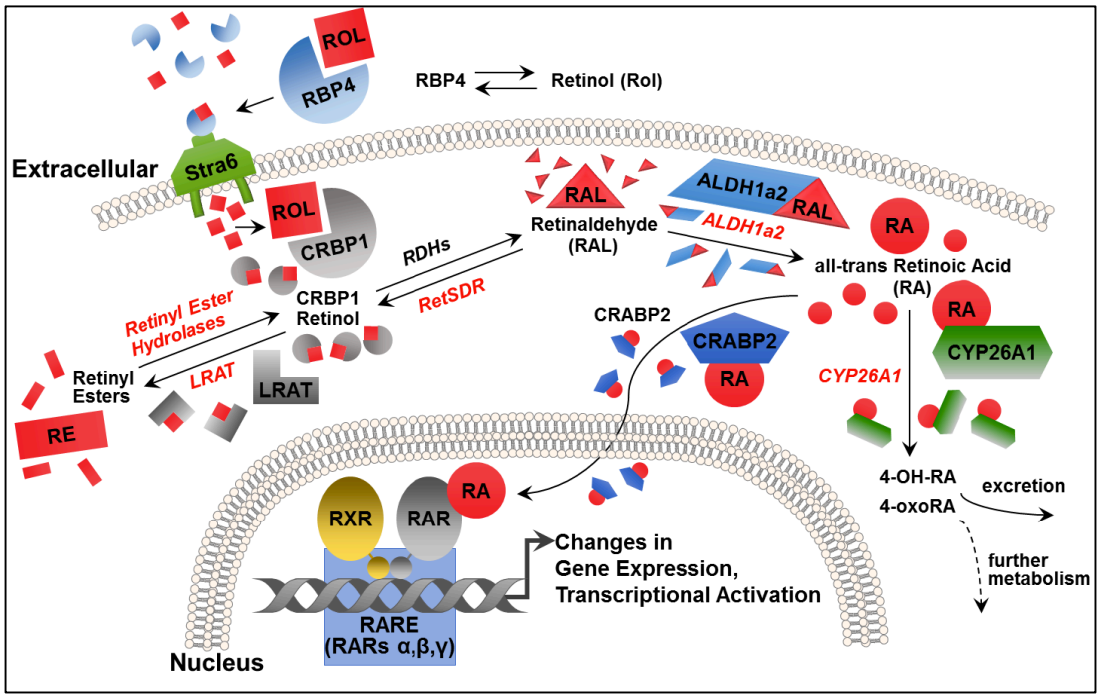

Figure 1.4: Diagram of uptake and intracellular metabolism of retinol. Retinol binds RBP4 extracellularly and is transported into certain cell types by the STRA6 receptor. Intracellular retinol binds CRBP1 and is either metabolized by LRAT to retinyl esters for storage or oxidized to retinaldehyde by RDH10 of the retinoid oxidation complex. Retinaldehyde is converted to RA by ALDH1A2. RA is then either metabolized to polar metabolites by CYP26A1 for excretion, or binds CRABP2 and is translocated to the nucleus. In the nucleus, RA binds one of three RARs, where it activates transcription of primary response genes upon recruitment of coactivators to the RARE-bound complex. Modified from Mongan and Gudas (2007) (88), by Daniel Stummer.

A major family of genes that are important regulators of embryonic development in response to RA signaling is the HOX family (87). *Hox* genes exhibit colinearity in expression, with genes clustered in the 3’ end, including *Hoxa1* and *Hoxb1*, being transcribed first,

followed by derepression and subsequent transcription of genes from 3' to 5' in temporally defined succession (89). The timing of the expression of *Hox* genes from initial activation following the loss of pluripotency through successive stages of differentiation is dependent on both the RA:RAR/RXR binding dynamics and the epigenetic profiles of chromatin state (90,91). Of the *Hox* gene cluster, *Hoxa1* is one of the first primary response genes to be expressed during embryogenesis in response to RA-dependent transcription (87,92), and therefore serves as a salient readout for early RA signaling activity.

ETHANOL AND RETINOIC ACID SIGNALING IN DEVELOPMENT AND DISEASE

Because of the importance of RA in stem cell differentiation and in a wide range of developmental processes (36), RA-dependent signaling pathways have been studied extensively in conjunction with EtOH to investigate potentially toxic effects that may arise from dysregulation of RA signaling by EtOH (1,93). Despite persistent research in this field, there is still controversy regarding the precise mechanisms by which EtOH interacts with RA signaling (5-7,9,78,79). Therefore, it has been difficult to establish the role that RA plays in EtOH-mediated teratogenicity, and my thesis work was performed with the intent of illuminating how EtOH mechanistically interacts with components of the RA synthesis and RA signaling pathways.

Much of the disparate results stem from differences in model systems used, the use of indirect techniques for assessment, or the

differences in the timing of experiments in the differentiation protocols and developmental stages studied (94,95). To further complicate matters, phenotypes are not straightforward, with similar developmental phenotypes presenting in cases of both RA deficiency and RA overexposure (96,97). These include changes in mental status and behavior (93), increased risk for cancers (96,98), craniofacial abnormalities (2,97), defects in brain development (99,100), and inhibition of neurogenesis (101,102).

Phenotypes from cell culture and animal models of EtOH toxicity are complicated to assess, with some studies suggesting that RA signaling is inhibited (6-8,78,94), while other studies suggest that RA signaling is activated by EtOH exposure (9). Prior to the utilization for highly sensitive, mass spectrometry-based approaches for directly detecting RA (103), it was proposed that EtOH competed with ROL for a limiting amount of alcohol dehydrogenase (ADH), due to the similarities in metabolism between EtOH and ROL (94,104,105). In this model, because AcH is a toxic intermediate, the metabolism of EtOH took precedence over RA production to prevent AcH accumulation and the damage that it caused as a result (104). However, under normal physiologic conditions, a member of the retinol dehydrogenase (RDH) family of enzymes, RDH10, was identified to conduct the first step in retinol oxidation rather than members of the ADH family (106). Furthermore, tissue RA levels were later shown to be either unaffected by EtOH or increased by EtOH exposure *in vivo* (9,79).

Since precise measurement of RA levels has become possible with advances in liquid-chromatography-mass spectrometric approaches (103), serum and tissue levels of RA have been quantified following acute and chronic EtOH dosing (9). A 6.5% EtOH diet was given to pregnant dams for 7 days before harvesting embryos at day E19. RA levels were increased by 1.5-20-fold in the hippocampus and 2-50-fold in the cortex, with the degree of fold change directly correlating with the maternal blood alcohol concentration at the time of harvest. Furthermore, adult mice fed a high single dose of EtOH (3.5 g/kg) exhibited high RA levels in the hippocampus, testis, and liver, and mice fed a 6.5% EtOH diet for one month displayed increased RA levels in the hippocampus, cortex, testis, and serum. None of the mice tested in any experiment displayed lower levels of RA in serum or any tissue tested than control dams. These results are consistent with ESC cell culture experiments showing EtOH causing differentiation (3), and they support a model whereby EtOH stimulates differentiation via the activation of RA signaling, a hypothesis that we endeavored to further test in my thesis work in ESCs.

There remain many unanswered questions in understanding how EtOH affects RA signaling. Despite being detected at increased levels in a tissue-specific manner (9), other studies have shown evidence that retinoid administration can partially rescue some of the developmental defects caused by EtOH (5,7,8). These findings have led to a modified version of the competition hypothesis where ALDH1A2 was postulated as the rate-limiting step in both retinaldehyde and AcH metabolism to inhibit RA-mediated differentiation (6,78), despite its

high Km of 650 μM (107). When 0.5% EtOH was administered to Xenopus embryos for 48 hours during the late blastocyst stage, when ALDH1A2 was the predominantly expressed aldehyde dehydrogenase family member, mRNAs of RA-responsive genes were decreased and rescued by 4-methylpyrazole, an ADH inhibitor (78). These results demonstrate that developmental stage must be appreciated when considering any interactions between EtOH exposure and RA signaling.

For my thesis work, we used ESCs to investigate the effects of EtOH on differentiation and to meticulously probe the relationship between EtOH metabolism to RA signaling. Using ESCs allowed us to bypass the many variable intermediate stages associated with EtOH exposure in different systems that are in constant flux during various phases of development. In this manner, our focus has primarily been on identifying the mechanisms of action by which EtOH affects differentiation in cells representing the most primitive stage of development.

Because there is no consensus as to whether EtOH activates or inhibits RA-mediated transcriptional effects associated with differentiation, my thesis work aims to identify which of the phenotypes is present, if either, in ESCs, and delineate the mechanisms by which that phenotype is expressed. We attempt to resolve some of the apparent paradoxes in how EtOH affects stem cell differentiation via a twofold strategy: 1) to identify the role, if any, that EtOH and its metabolism plays in ESC differentiation, and 2) to determine how EtOH (or its metabolites) interact with RA signaling to

exert effects on ESC differentiation. If EtOH or its metabolites are shown to activate differentiation, deleting ALDH1A2 (NCBI #19378), the principal enzyme that metabolizes retinaldehyde to RA (Figure 1.3), could additionally provide us with a unique opportunity to directly test whether RA synthesis is required for EtOH- or AcH-mediated differentiation.

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CHAPTER TWO

ETHANOL PROMOTES DIFFERENTIATION OF EMBRYONIC STEM CELLS THROUGH RETINOIC ACID RECEPTOR-γ

INTRODUCTION

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) (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 physiologic differentiation of stem cells (7,8). Dysregulated RA signaling (from either supra- or subphysiologic 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 owing to 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, while EtOH is metabolized to acetaldehyde (AcH) and then to acetic acid (Figure 2.1A). 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).

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.

METHODS

CELL CULTURE AND REAGENTS

ESCs were cultured as previously described (18). We treated ESCs with 95% EtOH, 1 mM AcH (Calbiochem, San Diego, CA), 1 mM sodium acetate (Sigma, St. Louis, MO) 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% dimethyl sulfoxide (DMSO). AcH was aliqoted from a freshly opened bottle and tubes were stored at -20^{o}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. 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 hours prior to collecting lysates. Reagents were changed twice daily approximately 12 hours apart, with the final reagent change completed 8 hours prior to harvest. EtOH was used at concentrations of 40 mM and 80 mM in various experiments. 103 units/ml of LIF was added to medium for all

experiments, including medium in which Knockout™ SR (Gibco, Dublin, IRE) replaced ESC-grade fetal calf serum.

ALKALINE PHOSPHATASE ASSAY

Cells were plated at a concentration of 250 cells/well in gelatinized 6 well plates and given 72 hours to adhere prior to treatment. Media and reagents were changed every 12 hours. We used 0.1% DMSO-treated and 40 mM EtOH-treated ESCs for quantitation, and we used 1 μM RA-treated cells as a positive control for loss of staining. Alkaline phosphatase (AP) activity was visualized by Fast Red (Sigma) staining, and monitored by bright field microscopy after 96 hours. We randomly selected five colonies from vehicle-treated and EtOH-treated wells for photographic imaging. We then performed intensity quantitation on these images using Image J software.

WESTERN BLOTTING AND ANTIBODIES

ESCs were harvested in 4% SDS lysis buffer, boiled, and resolved on SDS-PAGE gels. Antibodies were applied using the following dilutions: ALDH1A2 (1:500), RARγ (1:1000), RDH10 (1:1000), and actin (1:40,000). H3K27ac (Ab4729; Lot GR183919-2; Abcam, Cambridge, MA) and normal rabbit IgG (sc-2027; Lot A3014; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used for ChIP assays. Aldh1a2 (ab156019; Lot GR117687-8; Abcam), RARγ (ab97569; Lot 102512 ; Abcam), Rdh10 (14644-1-AP; Lot 26;

Proteintech, Rosemont, IL), and β-actin (MAB1501; Lot 2665057; Millipore, Etobicoke, ON) antibodies were used for Western blotting.

RNA ISOLATION AND REAL-TIME PCR

We extracted RNA using TRI Reagent (Sigma) according to the manufacturer's instructions. We then quantified and reverse transcribed 1 μg RNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) and diluted fivefold. We used SYBR Green quantitative PCR Supermix in a 15 μl reaction mix to carry out reactions on a Bio-Rad iCycler using 3 μl of cDNA. Quantification was performed using the Ct method and a standard curve was generated for all runs to assess efficiency. The quantified levels of all mRNA transcripts were normalized using a 36b4 internal control. Table S2.1 displays a list of primers used.

ACTINOMYCIN D ASSAY

Experiments were performed using 2 μg/ml actinomycin D (Calbiochem). Briefly, CCE cells were seeded in 12-well plates and treated for 48 hours with 40 mM EtOH or 1 μM RA. After 48 hours, RNA was isolated from one well containing each of the treatment groups, and actinomycin D was added to the remaining wells for 30, 90, and 240 minutes before lysing cells and extracting RNA for analysis by RT-qPCR. 36b4 was used as an internal control as levels remained stable through all treatment groups.

β-GALACTOSIDASE ASSAY

CCE cells were grown in 6 well plates and transfected the following day with 2-3 ug 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 ug (15:1 ratio sample:control) to normalize β-galactosidase activity to luciferase expression. Cells were treated 48 hours after transfection with DMSO (0.05%), 40 mM EtOH, or 0.5 μM RA for 24 hours with a reagent change 8 hours 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-galactosidase assays.

GENERATION OF CCE-ALDH1A2E5$^{-/-}$ CELL LINE

CRISPR constructs for CCE-ALDH1A2-E5$^{-/-}$ line creation were generated using the pX461-hSpCas9n(BB)-2A-GFP nickase vector. Guide RNAs targeting the sequence TTCACAAGACACGAGCCCAT (A) in the sense strand of exon 5 and the sequence CTCTGGAGTGACCGTGCTTA (B) in the antisense strand of intron 5 of the Aldh1a2 gene were cloned into the BbsI sites of disparate pX461-hSpCas9n(BB)-2A-GFP vectors. Vector A was digested with XbaI overnight, dephosphorylated with shrimp alkaline phosphatase, run on an agarose gel, excised, and purified. Vector B was amplified using primers targeting the hU6 promoter (Fwd: 5'-

TTTGCTAGCGAGGGCCTATTTCCCATGAT -3') and a downstream CRISPR sequence (Rev: 5'-GGTACCGCTAGCGCCATTTGTCTGC-3'). A 400 bp product was excised from an agarose gel, purified, and ligated into vector B. Clones were transformed into DH5α *E. coli*. Clones positive for both gRNAs exhibited an 850 bp band, 400 bp greater than clones which failed to incorporate vector B, following XbaI and PciI double digestion. Following transfection, single cell dilutions were plated and grown for 1 week. Colonies were subsequently grown in 24-well plates and harvested in PBS. and single cell clonal expansion, colonies were harvested in PBS and their DNA was amplified by PCR (Fwd: 5'-TGTGTAGGATGTGCCTCAGTTTC-3'/Rev: 5'-ACCACACATCGCTAAGGACCG-3', 254 bp product), and digested with BanII to genotype CRISPR-edited clones. Clones lacking the restriction site were Sanger sequenced on both alleles. We expanded double positive knockout clones in culture and tested for protein expression by Western blotting.

GENERATION OF CCE-RDH10E2-/- CELL LINE

The CRISPRevolution Synthetic RNA kit was used in generating CCE-Rdh10E2$^{-/-}$ cells (Synthego, San Francisco, CA). A guide (g)RNA targeting the sequence G*C*U*CAUUGAAAGAACCAUGA in the sense strand of exon 2 was purchased (Synthego), with asterisks representing bases modified with 2'-O-methyl groups and 3'-phosphorothionate linkages to stabilize the RNA for transfection. CCE ESCs were transfected for 24 hours with gRNA, Cas9 RNA (TriLink,

San Diego, CA), and mCherry RNA (TriLink), the latter being used to control for transfection efficiency. After the transfection, we grew clones for 8 days following single cell dilutions and transferred to 24 well plates. We then harvested colonies in PBS and extracted DNA for genotyping. DNA was amplified by PCR (Fwd: 5'-CAAGGAGGTTGGCGAGGTCTC-3'/Rev: 5'-GGACAGCTCATTAGACAGGCATCT-3', 254 bp product), and digested with BccI. Clones lacking the BccI cut site were expanded and sequenced on both alleles to confirm a homozygous deletion. We expanded double positive knockout clones in culture and tested for protein expression by Western blotting.

CHROMATIN IMMUNOPRECIPITATION ASSAYS

Experiments were performed as previously described (18) with described antibodies. Briefly, we plated AB1 ESCs overnight in 25 cm^2 plates at a density of $2.5X10^6$ and added 1 μM RA and/or 80 mM EtOH for 24 hours with a media/reagent change 8 hours prior to harvesting for four biological repeats. Cells were cross-linked with 1% formaldehyde for 10 minutes and quenched with 125 mM glycine for 5 minutes. Lysates were prepared in 3 ml PBS, sonicated, and pre-cleared with 50% Protein A Sepharose beads (CL-48; GE Healthcare, Uppsala, Sweden). We used 45-150 μg of DNA for immunoprecipitation experiments conducted with 2-3 μg of antibodies against H3K27ac. An IgG negative control was included with each experiment. qPCR analysis was performed using 3 μl of purified DNA,

which was normalized against 3 μl of input DNA. All values were normalized to the IgG control.

RETINOID EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Lysates were collected in PBS and extracted using 50% acetonitrile (ACN)/butanol and saturated K_2HPO_4 (53). The organic phase was vacuum dried and reconstituted in 100% ACN before loading. Retinoid separation was conducted using HPLC (Agilent 1290 Infinity, Palo Alto, CA) and JetStream electrospray ionization (ESI) in positive ion mode.

AB1 ESCs were grown in 10 cm^2 plates at a density of 330,000 cells/well and treated with 80 mM EtOH or 1 μM RA for 8 hours. Alternately, we treated CCE cells with 40 mM EtOH for 48 hours, switching to high vitamin A medium (VAM, +0.5 μM of exogenous ROL) 6 hours prior to lysis. Retinoid extraction was done in a dark room under red light. Cells were washed twice with PBS after 48 hours and harvested in 3 ml PBS. Lysates were centrifuged and PBS was removed completely. Cells were re-suspended in 500 ul PBS, vortexed and stored at -70^{o}C for up to 2 weeks. An internal standard of 5 μM RAc was added to each sample prior to extraction, and compared to a post-extraction RAc standard to determine extraction efficiency. The post-extraction standard was also compared to a 5 uM RAc standard prepared in 100% ACN to find the recovery rate, which was determined to be 1.5. Cells were extracted using 50% ACN/butanol followed by

saturated K_2HPO_4. The organic phase was collected after centrifugation and transferred to a new tube, which was vacuum dried on organic mode and then re-suspended in 100% ACN, vortexed, centrifuged and transferred to new vials.

LC-MS/MS analysis was performed using an Agilent 6460 Triple Quad LC/MS system (Agilent Technologies, Palo Alto, CA) with an Agilent 1290 Infinity HPLC and a JetStream ESI source. The mass spectrometer was operated in positive ion multiple reaction monitoring (MRM) mode. Retinoids were separated on an Agilent SB-Aq C18 column (2.0 X 150 mm, 1.8 μm) at a flow rate of 400 µl/min. The sheath gas used was N_2 (12 L/min at 400°C). The mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in ACN (mobile phase B). The LC elution gradient was: 0-8 min., 50% B to 95% B; 8-11 min., hold at 95% B; 11-11.1 min, 95% B to 50% B; 11.1-15 min., 50% B. Standards of RA, ROL, and the internal standard RAc (Sigma) were prepared in 100% ACN and pooled to generate calibration curves. The primary mass transition used for RA monitoring was *m/z* 301.2→123.1 with a secondary transition of 301.2→159.1. The MRM transitions used to assay for other retinoids were as follows: RAc and ROL (*m/z* 269.2→93.0), retinaldehyde (*m/z* 285.2→161.0), 4-hydroxy-RA (*m/z* 299.2→95.1), 4-oxo-RA (*m/z* 315.2→91.1, 315.2→165.5), 5,6-epoxy-RA (*m/z* 317.2→159.1), and 5,8-epoxy-RA (*m/z* 317.2→159.1). A peak area of 200 was used to mathematically determine the lower limit of quantitation (LLOQ). Retinoid standards were prepared in 100% ACN. Raw data were

processed using Agilent MassHunter Qualitative and Quantitative Analysis Software 6.0.

The concentration of each standard was first verified by measuring absorption in the UV/Vis range by spectrophotometry (Nanodrop 2000c, Thermo Scientific, Waltham, MA) and applying Beer's law ($A = \varepsilon * l * c$). Absolute values of retinoids were calculated by comparing ion counts in the sample to ion counts from 40 pmoles of its respective standard. The total (in pmoles) was divided by the injection volume (4 μl) to attain the concentration in pmoles/μl in the post-extraction samples. Concentration in the pre-extraction volume was then calculated before obtaining the molar concentrations and amounts of each in moles per 1 million cells.

STATISTICAL TREATMENT OF THE DATA

Statistical analysis was conducted on at least three, independent biological replicates for each experiment using Graph Pad Prism 7.0 software. The means ± SEM were determined. ANOVA was used to determine statistical significance within sets of 3 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.

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 (rt)-PCR on AB1 ESCs 24, 48, and 72 hours after 40 mM EtOH addition (Figure 2.1B). This EtOH dose is representative of human blood concentrations typical of binge drinking (0.184%) (20). Using alkaline phosphatase staining, we demonstrated a reduction of pluripotency in ESCs upon EtOH treatment for 96 hours, as EtOH decreased staining intensity by 7.6% ($p=0.020$) (Figure S2.1A). 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%, $p=0.02$), *Dppa5* (83±4%, $p=0.003$), and *Nanog* (58±7%, $p=0.009$) 24 hours after EtOH addition, while others, including *Oct4* (21) and *Sall4*, were unchanged (Fig. S2.1B).

Transcripts increased by >2-fold by 40 mM EtOH treatment included those of the homeotic (HOX) family (*Hoxa1*, *Hoxb1*, *Hoxa5*) and *Cdx1* (Figure 2.1C, Fig. S2.1C). Because several transcripts increased by EtOH are direct RAR/RXR transcriptional targets (22-24), we then analyzed the primary RA target genes *RARβ2* and *Cyp26a1*. We detected increases after 48 hours of EtOH treatment (Figure 2.1C) compared to 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 40 and 80 mM doses of EtOH elicited similar effects (Fig. S2.1D). EtOH treatment did not increase transcript levels of lineage-specific genes that were also unaffected by RA treatment at 48 hours, such as *Fgf5* (ectoderm) and *Sox17* (endoderm) in ESCs (Fig. S2.1E). Thus, EtOH increases transcript levels of specific RA target genes rather than effecting a broad differentiation phenotype.

To probe for additive effects of EtOH and retinoids we added 40 mM 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 to RA/ROL-treated cells alone at 48 hours (Fig. S2.1F), suggesting that transcript induction of differentiation-associated genes by RA and EtOH converges on the same pathway.

Because EtOH is rapidly oxidized to acetaldehyde (AcH) (Figure 2.1A) (25), we treated CCE cells with either 40 mM EtOH or 1 mM AcH for 72 hours. 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 (Figure 2.1D). In contrast, 1 mM acetate treatment for 48 hours did not increase mRNA levels of *Hoxa1*, *Cyp26a1*, and *RARβ2*, and partially inhibited the EtOH-mediated increases in *Cyp26a1* (Figure 2.1E). Therefore, we conclude

that the EtOH-induced transcript increases result from EtOH metabolism to AcH, and not to acetate.

To determine if EtOH increases *Hoxa1* mRNA levels by enhancing mRNA stability or by increasing transcription, we treated CCE ESCs with 40 mM EtOH or 1 μM RA for 48 hours, isolated RNA immediately from some wells, and added 2 μg/ml of actinomycin D to other wells for 30, 90, or 240 minutes 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* (Figure 2.1F) and -0.043±0.04 (p=0.54) for *Cyp26a1* (Figure 2.1G). 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 from 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 (RARγE8$^{-/-}$) (Fig. S2.2A) (26). We cultured WT and

RARγE8$^{-/-}$ cells with 40 mM EtOH for 48 hours, 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 non-coding RNA *Hotairm1* (8.9±1.3-fold, p=0.003), which regulates transcription of the Hoxa cluster (30), increased in WT ESCs compared to vehicle-treated cells. In contrast, in RARγE8$^{-/-}$ cells deletion of RARγ prevented these mRNA increases (Figure 2.2A, Fig. S2.2B).

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$^{-/-}$ cells (Figure 2.2B). Since *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 RARβ$^{+/-}$γ$^{-/-}$ line (29) treated for 2 hours 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 AB1 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 to vehicle-treated cells (Fig. S2.2C). In contrast, *Hoxa1* and *Hoxb1* transcript levels did not increase in EtOH-treated RARβ$^{+/-}$γ$^{-/-}$ cells±RA (Fig. S2.2D). These data clearly demonstrate that RARγ mediates the effects of EtOH with respect to ESC differentiation.

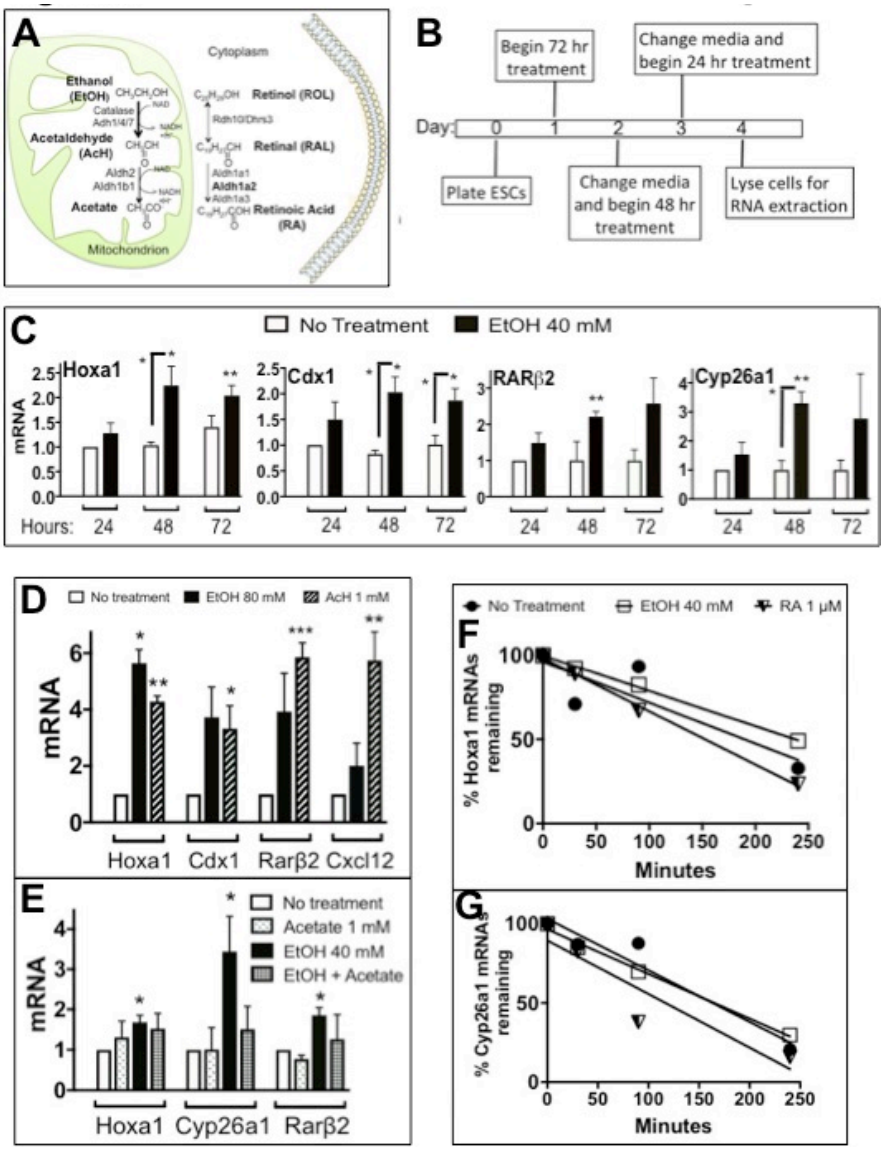


Figure 2.1: Ethanol increases transcript levels of genes necessary for RA-mediated differentiation in ESCs. *A*, Schematic of the metabolic pathways of EtOH and ROL. *B*, Timeline for cell culture experiments. *C*, Fold changes in mRNA levels by 40 mM EtOH; these transcripts are targets of RA. Treatment groups at 48 and 72 hours were compared to untreated ESCs at 24 hours, except where indicated by bar. *D*, Fold changes in mRNA levels by EtOH and 1 mM AcH at 72 hours. *E*, Fold changes in mRNA levels by EtOH or 1 mM acetate±EtOH at 48 hours. *F-G*, RT-qPCR analysis of relative stabilities of *Hoxa1* (*F*) and *Cyp26a1* (*G*) transcripts at 30, 90 or 240 minutes after administering 2 μg/ml of actinomycin D to inhibit transcription. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

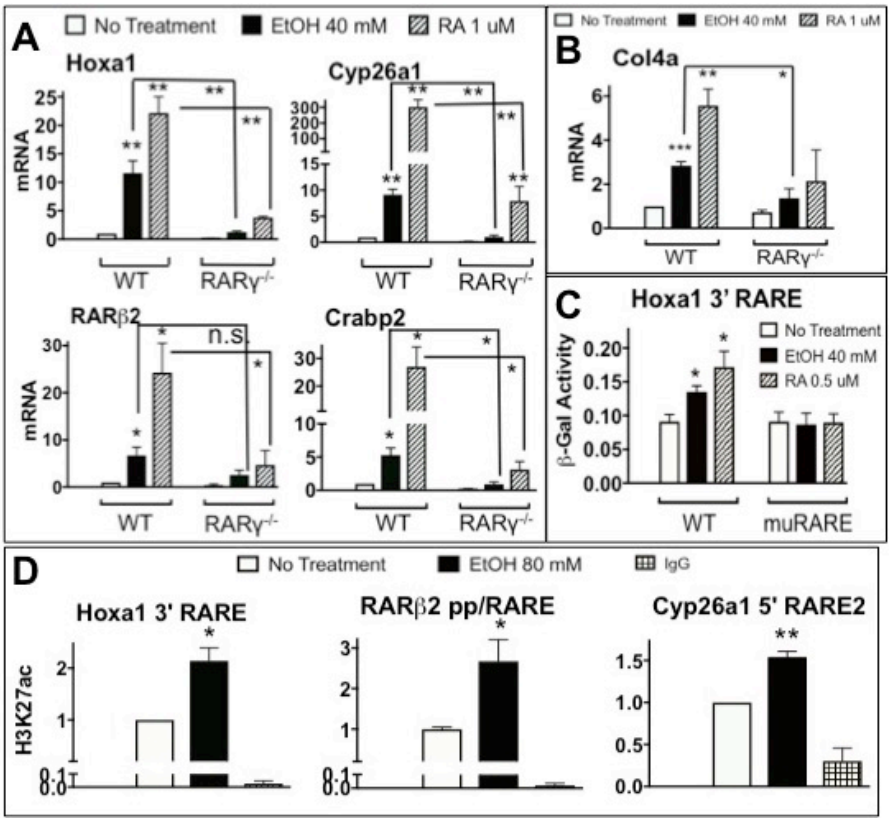


Figure 2.2: RARγ is required for the expression of a subset of RA-target genes induced by ethanol. *A*, Fold changes in transcript levels of RA-inducible genes in WT and RARγE8$^{-/-}$ ESCs at 48 hour treatment with EtOH (40 mM) or RA (1 μM RA). *B*, Fold changes in transcript levels of the late differentiation marker *Col4a* in CCE WT and CCE-RARγE8$^{-/-}$ cells at 48 hour treatment with EtOH (40 mM) or RA (1 μM RA). *C*, β-galactosidase 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 hours ± EtOH (40 mM) or RA (0.5μM), 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 hours, 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-IP input DNA. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

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 CCE 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 hours. We observed a 1.5±0.15-fold (p=0.034) increase in β-galactosidase 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 (Figure 2.2C). We did not observe any increase in β-galactosidase 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, while *RARβ2* contains an RARE near its proximal promoter (18). Genes from the EtOH-treated WT ESCs exhibited >1.5-fold H3K27ac enrichment near RAREs compared to 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*) (Figure 2.2D). 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 (RAL) 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 (Figure 2.3A). 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) (Figure 2.3B-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, since the RARγE8$^{-/-}$ ESC line showed attenuated increases in these transcripts by EtOH compared to those in WT ESCs (Figure 2.3C). 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 (Figure 2.3D). Importantly, Dhrs3 stabilizes the Rdh10-containing retinoid oxidoreductase complex (34). These data show that EtOH increases mRNAs of key genes that metabolize ROL to RAL. In contrast, the *Aldh1a2* mRNA level was not increased by EtOH in WT ESCs (Fig S2.3, Figure 2.1A).

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$^{-/-}$ cell line (Figure 2.4A-B). The absence of ALDH1A2 prevented the EtOH-mediated *Hoxa1* and *Cyp26a1* transcript increases observed in WT ESCs (Figure 2.4C). These data indicate that metabolism of retinaldehyde to RA is required for EtOH to increase *Hoxa1* and *Cyp26a1* transcripts.

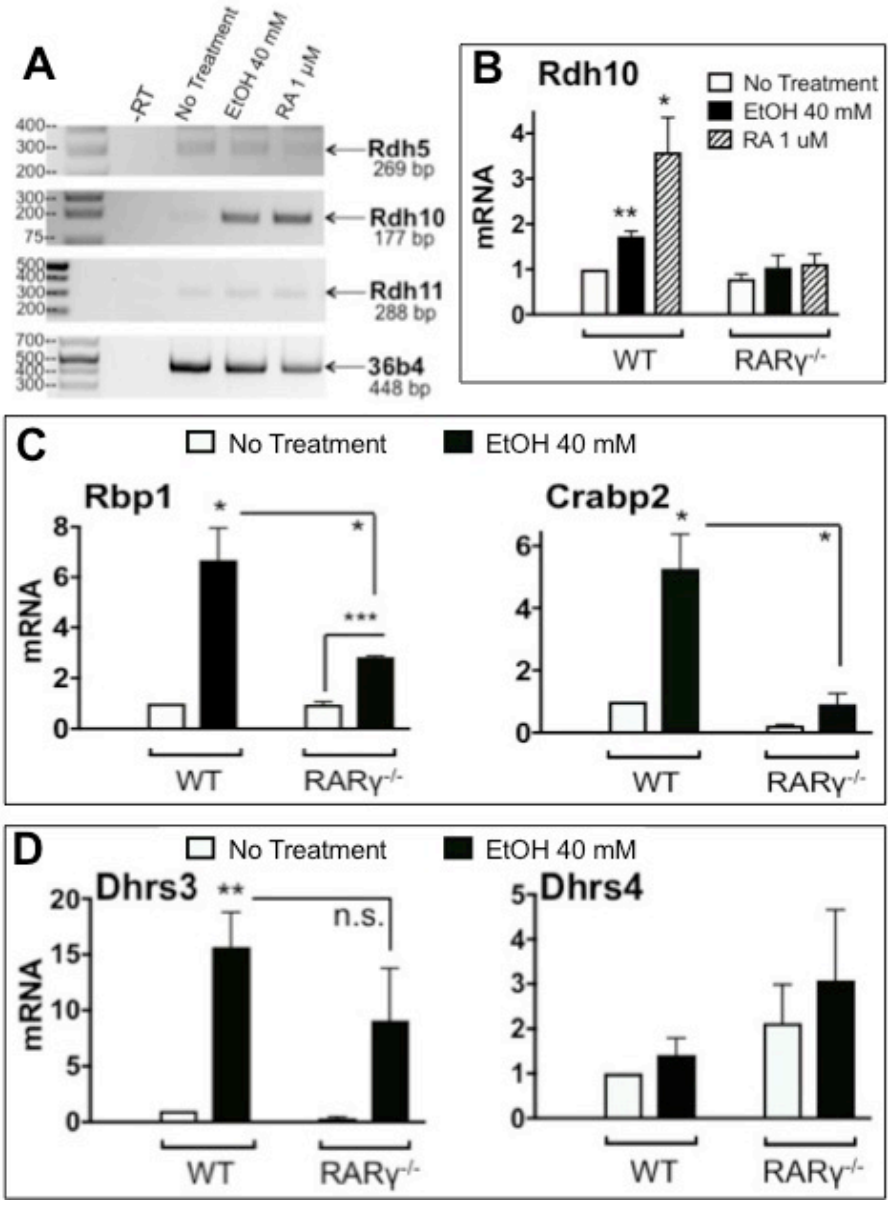


Figure 2.3: Transcripts involved in RA synthesis are upregulated following ethanol addition. *A*, Representative semiquantitative rt-PCR analysis of a panel of retinol dehydrogenase family transcripts expressed in ESCs, ±40 mM EtOH and 1 μM RA (n=2). *B*, Fold changes in Rdh10 transcript levels in WT and RARγE8$^{-/-}$ ESCs at 48 hour treatment with EtOH (40 mM) or RA (1 μM RA). *C-D*, Fold changes in transcript levels of genes associated with retinoid transport (*C*) and retinaldehyde reduction (*D*) in WT and RARγE8$^{-/-}$ ESCs at 48 hour treatment with EtOH (40 mM). Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent biological experiments where n = at least 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

We next cultured WT ESCs in knockout serum replacement medium (KOSR), which, unlike fetal calf serum, does not contain ROL. EtOH did not increase *Hoxa1* and *Cyp26a1* mRNAs in WT ESCs cultured in KOSR medium (Figure 2.4C). 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*) (Figure 2.4C). In contrast, *Hoxa1* transcript levels were similarly increased by 1 μM RA in WT ESCs cultured in either serum-containing or KOSR medium (Fig. S2.4). Collectively, these data clearly demonstrate that ROL, via its two-step oxidation to RA, is required for EtOH-mediated *Hoxa1* and *Cyp26a1* transcript increases.

ETHANOL TREATMENT DOES NOT CAUSE INCREASED RA LEVELS IN EMBRYONIC STEM CELLS.

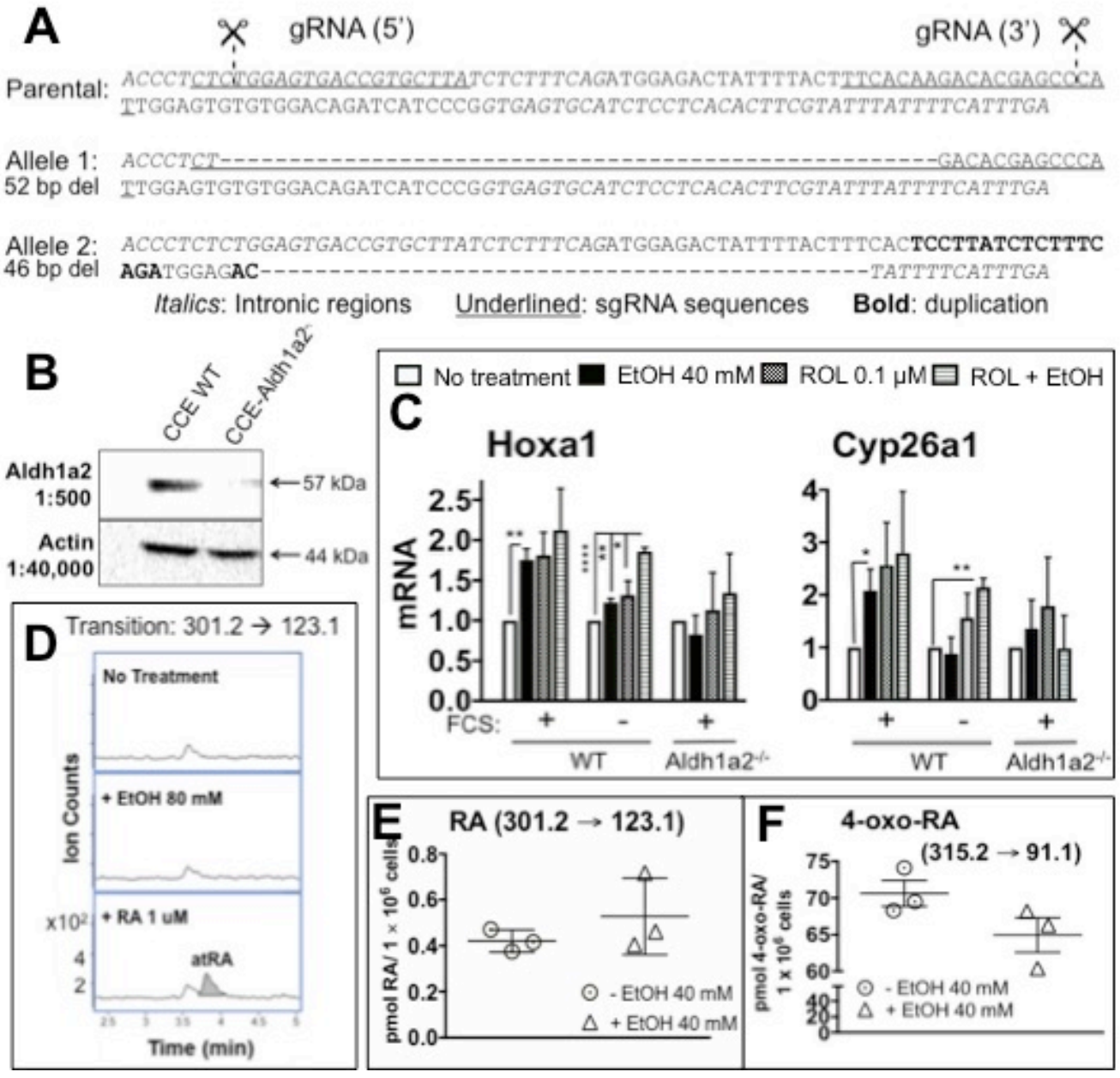
We measured RA levels in EtOH-treated ESCs using reversed phase high performance liquid chromatoraphy-tandem mass spectrometry (RP-HPLC-MS/MS) to determine whether increased intracellular RA levels correlated with the increases in Hoxa1 and *Cyp26a1* transcripts. Using a triple quadrupole mass spectrometer, we detected a peak for 20 pmoles of an RA standard at a retention time of 3.5 minutes (Fig. S2.5A). Calibration curves were generated for RA with a limit of detection (LOD) of 40 fmoles and a lower limit of

quantitation (LLOQ) of 382 fmoles (95 nM for 4 X 10^6 cells, where 1 μl volume=1 X 10^6 cells and 4 μl=injection volume), for the transition m/z 301.2→123.1, and 341 fmoles (85 nM for 4 X 10^6 cells) for a secondary m/z 301.2→159.1 transition (Fig. S2.5B-C). We also generated a calibration curve for 4-oxo-RA, a metabolite of RA (Fig. S2.5D). RA levels in AB1 cells 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 hours (Figure 2.4D, Fig. S2.5E). We observed a second peak at RT 3.25 minutes for transition m/z 301.2→159.1, but this peak did not correspond to any known RA isomer or metabolite (Fig. S2.5F-H).

To increase the sensitivity for RA detection, we next treated WT ESCs with 40 mM EtOH for 48 hours and switched to a high vitamin A containing medium (VAM, +0.5 μM ROL) 6 hours prior to collecting lysates. This medium contained a tenfold higher ROL concentration than that in standard 10% serum-containing medium (0.05-0.1 μM). In WT 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 WT ESCs compared to vehicle-treated cells (Figure 2.4E).

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 (Figure 2.4F). Thus, we did not observe increases in intracellular RA levels by mass spectrometry after EtOH addition.

Figure 2.4: 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 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 to β-actin loading control. *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). Fold changes in transcript levels of genes in ALDH1A2E5$^{-/-}$ ESCs grown in 10% FCS-containing medium are compared to those of ESCs grown in standard medium+10% FCS and in chemically defined KOSR-containing medium. Transcript levels are compared to those of 0.1% DMSO-treated cells set to 1. *D-F,* Reverse-phase liquid-chromatography-tandem MS/MS followed by MRM 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 hours (RT = 3.5 min). Intracellular RA (*E*) and 4-oxo-RA (*F*) concentrations 48 hours after EtOH addition and 6 hours following switch to VAM. Quantitation of RA was calculated in pmoles/1 X 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 standard errors of independent experiments where n = at least 3 biological repeats. *, $p \leq 0.05$, **, $p \leq 0.01$, ****, $p \leq 0.0001$.


A
gRNA (5')
gRNA (3')
Parental:
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Allele 1:
52 bp del
ACCCTCT----------GACACGAGCCCA
TTGGAGTGTGTGGACAGATCATCCCGGTGAGTGCATCTCCTCACACTTCGTATTTATTTTCATTTGA
Allele 2:
46 bp del
ACCCTCTCTGGAGTGACCGTGCTTATCTCTTTCAGATGGAGACTATTTTACTTTCACTCCTTATCTCTTTC
AGATGGAGAC----------TATTTTCATTTGA
Italics: Intronic regions
Underlined: sgRNA sequences
Bold: duplication
B
CCE WT
CCE-Aldh1a2-/-
Aldh1a2 1:500
57 kDa
Actin 1:40,000
44 kDa
C
No treatment
EtOH 40 mM
ROL 0.1 µM
ROL + EtOH
Hoxa1
Cyp26a1
mRNA
FCS:
WT
Aldh1a2-/-
D
Transition: 301.2 → 123.1
No Treatment
+ EtOH 80 mM
+ RA 1 uM
atRA
Ion Counts
Time (min)
E
RA (301.2 → 123.1)
pmol RA/ 1 × 10^6 cells
- EtOH 40 mM
+ EtOH 40 mM
F
4-oxo-RA
(315.2 → 91.1)
pmol 4-oxo-RA/ 1 x 10^6 cells
- EtOH 40 mM
+ EtOH 40 mM

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 measured *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 (Figure 2.5A). 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 upregulated to a greater extent by both EtOH and RA treatment (Fig. S2.6).

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$^{RARE-/-}$) (37). First, we verified that STRA6$^{RARE-/-}$ ESCs display a considerably weaker response to RA stimulation than WT ESCs. Treating STRA6$^{RARE-/-}$ ESCs with 1 μM RA for 48 hours resulted in a 3.13±1.01-fold (p=0.044) increase in STRA6 levels compared to a 13.03±0.95-fold (p=0.0004) increase in WT ESCs, confirming that RA does not robustly increase Stra6 mRNA in STRA6$^{RARE-/-}$ cells (Figure 2.5B). We then detected a 4.68±1.44-fold (p=0.042) increase in *Stra6* mRNA by 40 mM EtOH and

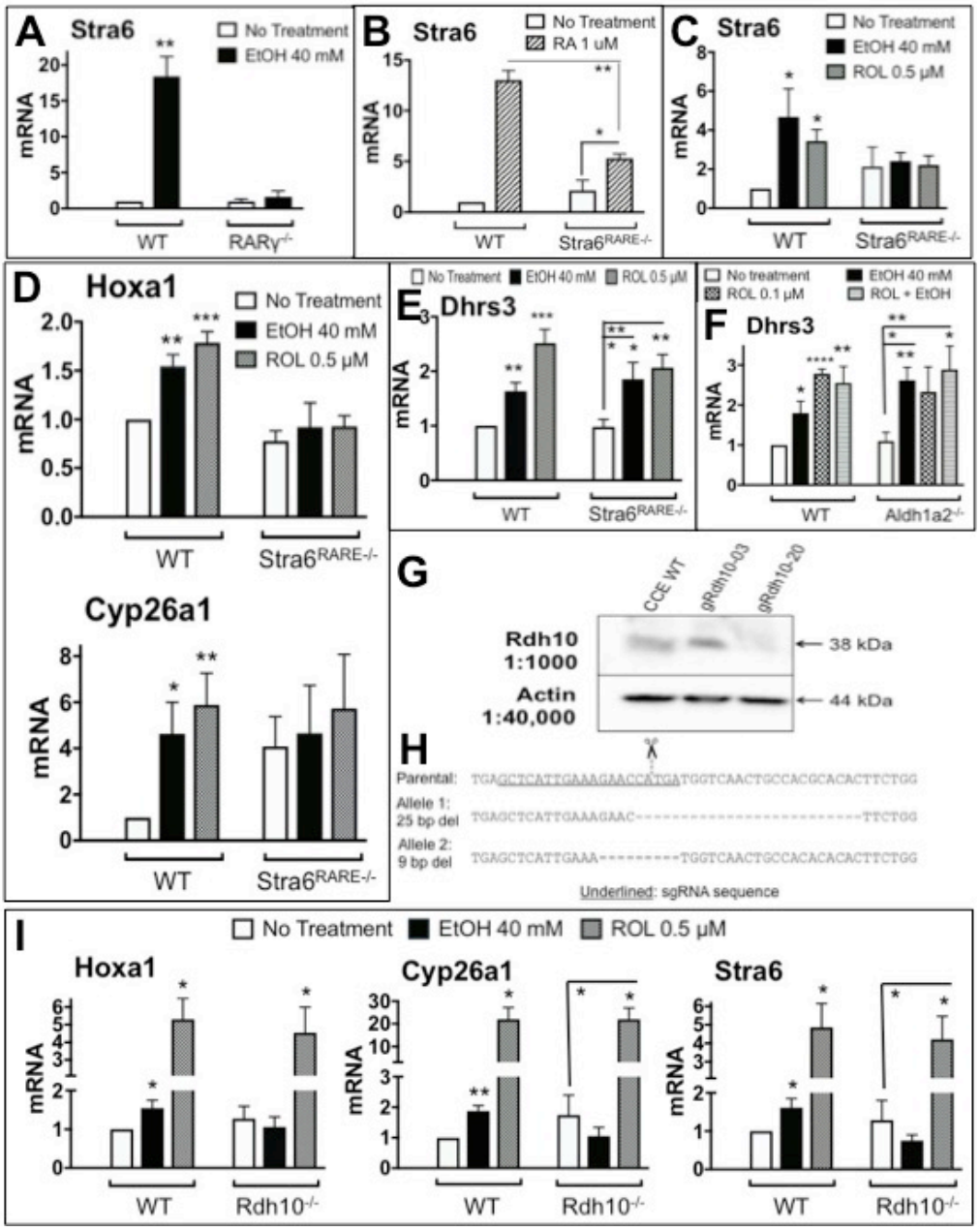
a 3.44±0.59-fold (p=0.015) increase by 0.5 μM ROL in WT ESCs (Figure 2.5C). In contrast, *Stra6* transcripts were not induced by either 40 mM EtOH or 0.5 μM ROL in STRA6$^{RARE-/-}$ cells (Figure 2.5C). While *Hoxa1* and *Cyp26a1* mRNAs were similarly increased by both EtOH (1.55±0.12-fold, p=0.004, *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 (Figure 2.5D). 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=.004) and STRA6$^{RARE-/-}$ (1.86±0.31-fold, p=0.04) ESC lines (Figure 2.5E). 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 to a 1.80±0.3-fold (p=0.037) change in EtOH-treated WT cells (Figure 2.5F). These data indicate that EtOH-mediated increases in *Dhrs3* mRNA levels occur independently of ROL import by STRA6 and RA production from retinaldehyde.

RDH10 IS REQUIRED FOR ETHANOL-DEPENDENT INCREASS IN DIFFERENTIATION-ASSOCIATED GENES.

To demonstrate a 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 (Figures 2.5G-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 to vehicle-treated 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.

Figure 2.5: 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 hour treatment with 40 mM EtOH. *B,* Fold changes in *Stra6* mRNA levels in 1 μM RA-treated WT and STRA6$^{RARE-/-}$ ESCs at 48 hours. *C-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 hour 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 to β-actin loading control. 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 hours (n=4). Transcripts levels in 0.1% DMSO-treated WT cells were set to 1. Treatment groups were compared to 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 standard errors of independent experiments where n = at least 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001, ****, p≤0.0001.


A
Stra6
No Treatment
EtOH 40 mM
mRNA
WT
RARγ-/-
B
Stra6
No Treatment
RA 1 uM
mRNA
WT
Stra6RARE-/-
C
Stra6
No Treatment
EtOH 40 mM
ROL 0.5 µM
mRNA
WT
Stra6RARE-/-
D
Hoxa1
No Treatment
EtOH 40 mM
ROL 0.5 µM
mRNA
WT
Stra6RARE-/-
Cyp26a1
mRNA
WT
Stra6RARE-/-
E
Dhrs3
No Treatment
EtOH 40 mM
ROL 0.5 µM
mRNA
WT
Stra6RARE-/-
F
Dhrs3
No treatment
EtOH 40 mM
ROL 0.1 µM
ROL + EtOH
mRNA
WT
Aldh1a2-/-
G
CCE WT
gRdh10-03
gRdh10-20
Rdh10
1:1000
Actin
1:40,000
38 kDa
44 kDa
H
Parental: TGAGCTCATTGAAAGAACCATGATGGTCAACTGCCACGCACACTTCTGG
Allele 1:
25 bp del TGAGCTCATTGAAAGAAC----------TTCTGG
Allele 2:
9 bp del TGAGCTCATTGAAA---------TGGTCAACTGCCACACACACTTCTGG
Underlined: sgRNA sequence
I
No Treatment
EtOH 40 mM
ROL 0.5 µM
Hoxa1
mRNA
WT
Rdh10-/-
Cyp26a1
mRNA
WT
Rdh10-/-
Stra6
mRNA
WT
Rdh10-/-

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 was 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. 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 (16). 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 one month, Napoli and colleagues 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 (15).

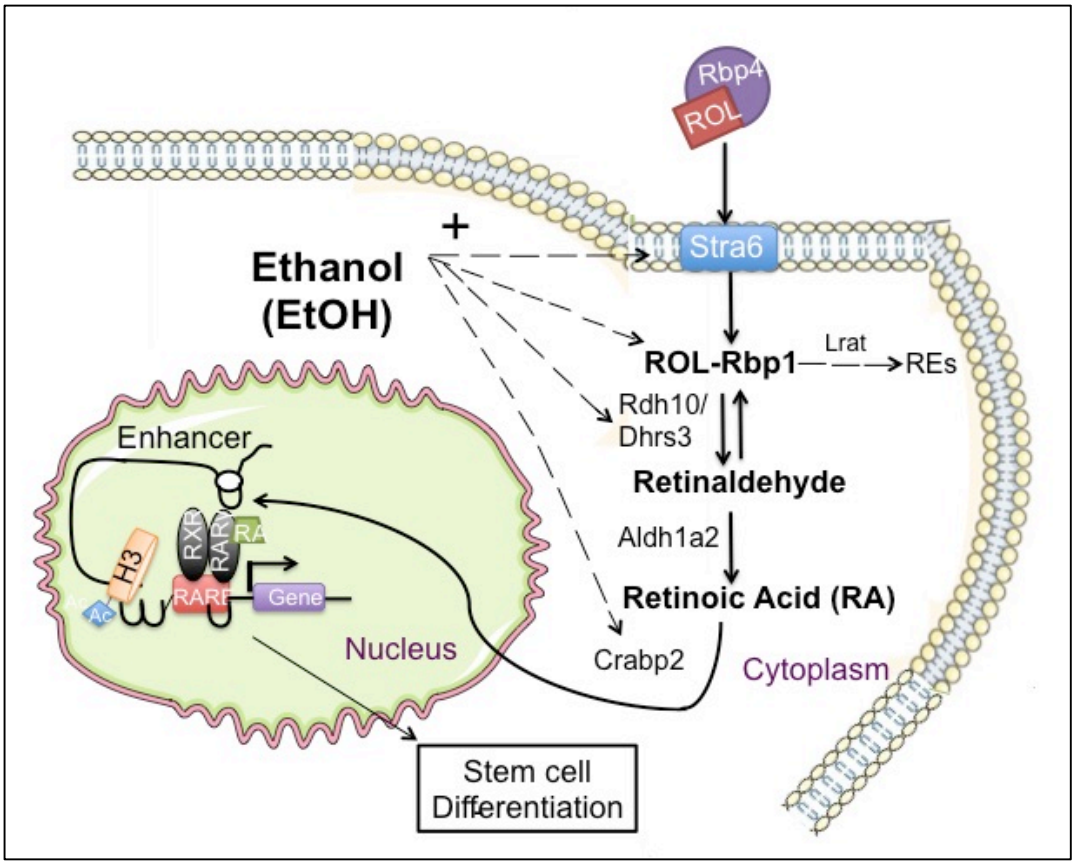


Figure 2.6: Model for ethanol regulation of stem cell differentiation via activation of RA signaling. ROL, in complex with Rbp4, is a substrate for Stra6, which imports ROL into the cell. EtOH increases the mRNAs of *Stra6* and several genes in the RA synthesis pathway, including *Rdh10*, *Dhrs3*, *Rbp1*, and *Crabp2*. Since the LRAT pathway is not functional in ESCs, intracellular ROL is presented to the RA synthesis machinery upon binding to RBP1 rather than stored as retinyl esters. 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.

The contextual relationship between EtOH and RA may also be influenced by developmental stage, contributing to differences in the literature. Shabtai et al. 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 (41). 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 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 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 FACTORS 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 (Figures 2.1C, S2.1B). The loss of pluripotency in EtOH-treated ESCs was confirmed using alkaline phosphatase staining (Figure S2.1A). 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 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 (Figure 2.1D-E).

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 (17,45). RARγ is an essential transcription factor in RA-dependent differentiation of ESCs (27,46,47). Some functional redundancy exists among the three types of RARs in ESCs (26,48,49), but only RARγ was demonstrated to mediate F9 embryonic carcinoma cell differentiation and override activity of other RARs (46). Additionally, the loss of RARγ, but not RARα, was associated with differentiation defects and altered *Hoxa1* expression (28,50), 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 (47). However, whereas RARα is enriched 24-48 hours after RA signaling commences to sustain differentiation, RARγ initiates differentiation via direct activation of primary response genes (22,28,47,50). 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 (Figures 2.2A-B, S2.2).

ETHANOL INDUCTION OF DIFFERENTIATION-ASSOCIATED TRANSCRIPTS IN EMBRYONIC STEM CELLS IS DEPENDENT ON ALDH1A2.

We demonstrate here that EtOH treatment of ESCs likely increases intracellular ROL from the serum to generate RA to activate transcription. This effect of EtOH requires *Aldh1a2* expression, as genetic ablation of *Aldh1a2* prevented EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcripts (Figure 2.4C).

Despite our inability to detect differences in RA levels between EtOH-treated and untreated ESCs (Figure 2.4D-E), depleting medium of ROL caused abrogation of EtOH-mediated transcriptional effects (Figure 2.4C). Precedence for potent RA activity in the absence of detectable RA increases by mass spectrometry is found in the literature. For example, Blaner and colleagues 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 (51). 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 (Figure 2.4C). 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* (Figure 2.3). RDH10 and DHRS3 exist in a bifunctional complex to ensure that RA levels are tightly controlled (34). While 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 *Dhrs3* being required for fine-tuning retinoid oxidoreductase complex activity (34).

In addition, though *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 (Figure 2.4F). 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, Figure 2.6).

STRA6-DEPENDENT RETINOL UPTAKE FROM THE MEDIUM FACILITATES 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 (Figure 2.5A), and loss of *Stra6* RARE function was sufficient to abrogate EtOH-mediated increases in *Hoxa1* and *Cyp26a1* transcripts (Figure 5D). 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 (27,52), 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. Since 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, Figure 2.6). 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 (Figure 2.5I). 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 (52,53).

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 (Figure 2.6). 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 prevention or treatment of EtOH-associated toxicities and diseases.

ABBREVIATIONS

AcH, acetaldehyde; ACN, acetonitrile; DMSO, dimethyl sulfoxide; EtOH, ethanol; ESC, embryonic stem cell; FSC, fetal calf serum; gRNA, guide RNA; H3K27ac, acetylated histone 3 lysine 27; KOSR, knockout serum replacement; LLOD, lower limit of quantitation; LOD, limit of detection; IgG, immunoglobulin G; MRM, multiple reaction monitoring; RA, retinoic acid; RAc, retinyl acetate; RAR, retinoic acid receptor; RARE, reinoic acid response element; ROL, retinol; RP-HPLC-MS/MS, reversed phase high performance liquid chromatography-tandem mass spectrometry; RT, retention time; RXR, retinoid X receptor; VAM, high vitamin A medium; WT, wild type

Table S2.1: Primer sequences used for semiquantitative PCR and qPCR with predicted product size.

Target	Forward (5'→ 3')	Reverse (5'→3')	Product size (bp)
Aldh1a2	GACTTGTAGCAGCTGTCTTCACT	TCACCCATTTCTCTCCCATTTCC	160
Cdx1	GGTAAGACCCGAACCAAGGAC	CAGAAGGCCAGCATTAGTAGG	318
Crabp2	CAGACCGTGGATGGGAGA	ACGGAAGTCGTCTCAGGCA	234
Cxcl12	TTCACTCTCGGTCCACCTCGG	ATCGGTAGCTCAGGCTGACTGG	162
Cyp26a1	GAAACATTGCAGATGGTGCTTCAG	CGGCTGAAGGCCTGCATAATCAC	272
Cyp26a1 RARE2	TTCACTGAGATGTCACGGTCC	TTCCCAATCCTTTAGCCTGA	64
Dhrs3	CCTTCATGGAGAGCCTGACCTTG	GGTCCACGGGAGCAAGAGAAG	209
Dhrs4	TGGTCGTCAGCAGCCGCAAAC	CCTCTGTGACATCCATTAGATTTCC	219
Dppa5	CAGTCGCTGGTGCTGAAATATC	CCAGGTTCTTCAGCTCAAACATG	97
Fgf5	AAAGTCAATGGCTCCCACGAA	CTTCAGTCTGTACTTCACTGG	465
Hnf1β	GCGCCGCAACCGGTTTAAATG	GTGCGTCTGGTTGGAGCTATAG	280
Hotairm1	GGCAGATAACAGGCAACTCCTAA	CCATCCACTCAGTCATCTGTC	184
Hoxa1	TTCCCACTCGAGTTGTGGTCCAAGC	TTCTCCAGCTCTGTGAGCTGCTTGGTGG	220
Hoxa1 RARE	TCTTGCTGTGACTGTGAAGTCG	GAGCTCAGATAAACTGCTGGGACT	268
Hoxa5	CCCCTGGATGCGCAAGCTGCACATT	TTCTCCAGCTCCAGGGTCTGGTAGCGA	105
Hoxb1	AGGAATCGCCTTGCTCGTCAGA	GCGGCGATCTCCACCCTC	223
Klf4	GCACACCTGCGAACTCACAC	CCGTCCCAGTCACAGTGGTAA	53
Nanog	ATGCCTGCAGTTTTTCATCC	GAGCTTTTGTTTGGGACTGG	153
NR2F1	CTGTCCCATCGACCAGCACCACCG	GACAGGTAGCAGTGGCCATTGAGAG	177
Oct4	GAGGAGTCCCAGGACATGAA	AGATGGTGGTCTGGCTGAAC	154
RARβ	GATCCTGGATTTCTACACCG	CACTGACGCCATAGTGGTA	247
RARβ2 pp/RARE	TGGCATTGTTTGCACGCTGA	CCCCCCTTTGGCAAAGAATAGA	284
RARγ	ATGTACGACTGCATGGAATCGT	GATACAGTTTTTGTCACGGTGACAT	366
Rbp1	AATCGCCAACTTGCTGAAGCC	ATCCACTGCGTCCAGCCAC	237
Rdh5	CCACACAACACTACTGGATATCAC	CCAAGACACTGGTGATGTTGAC	269
Rdh10	TCTGGACATCACCTTCTGGAATG	CTCAACTCCAGCAGTGCTGAAC	177
Rdh11	GGCAGGATCCACTTCCATAACC	GCTCTCGAGACCTTCTGTCAG	288
Sall4	CACCACGAAAGGCAACCTGAAG	GGTTCTCTATGGCCAGCTTCCTT	92
Sox2	GAGTGGAAACTTTTGTCCGAGA	CTCCGGGAAGCGTGTACTTA	158
Sox17	AACGCAGAGCTAAGCAAGATGC	TTCTCTGCCAAGGTCAACGCC	193
Stra6	GTTCAGGTCTGGCAGAAAGC	CAGGAATCCAAGACCCAGAA	102
Stra6 (short)	CAGTTTAGGGAGCACACTGTATA	CAGGAATCCAAGACCCAGAA	475
Stra6 (long)	CCTCCGGGGTGACAGATGACTAC	CAGGAATCCAAGACCCAGAA	620
Stra8	CAACTCAGAAAATCCAGAGGAGA	CTCCAGGCACTTCAGCAACAT	145
36b4	AGAACAACCCAGCTCTGGAGAAA	ACACCCTCCAGAAAGCGAGAGT	448

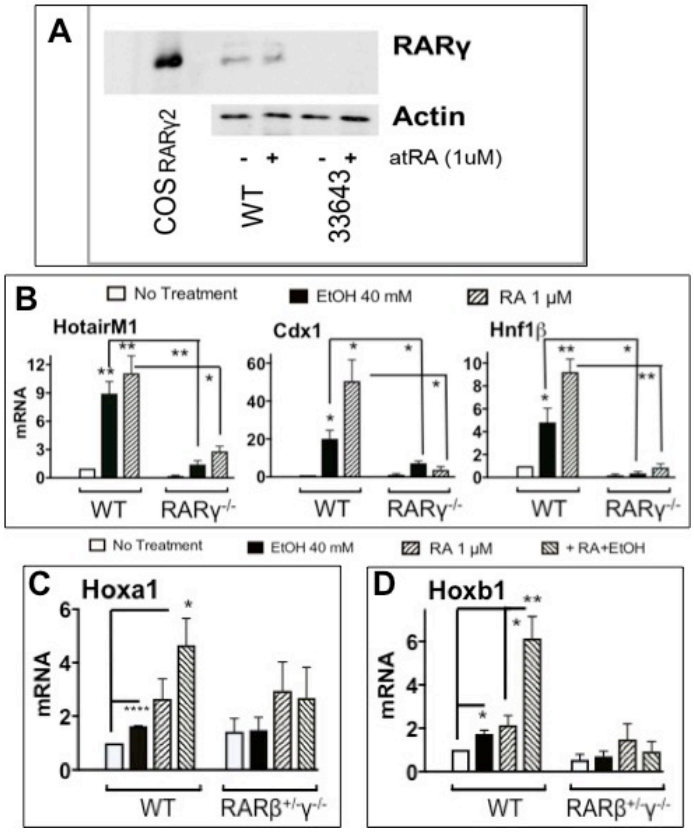


Figure S2.1: Ethanol alters mRNA levels of genes associated with pluripotency and differentiation. *A*, Relative intensities from alkaline phosphatase stained 0.1% DMSO-treated versus 40 mM EtOH-treated WT ESCs. We selected colonies at random and quantified intensities using Image J software. *B*, Relative transcript levels of genes associated with pluripotency and stem cell function in 40 mM EtOH-treated AB1 ESCs. Transcript levels in vehicle-treated cells were set to 100%. *C*, Fold changes in mRNA levels of *Hoxb1* and *Hoxa5* by EtOH. *D*, Fold changes in transcripts of selected EtOH-inducible genes are shown for 40 mM- and 80 mM-EtOH treated samples after 48 hour treatments in CCE ESCs. *E*, Representative semiquantitative rt-PCR analysis of *Fgf5* and *Sox17* transcripts expressed in AB1 ESCs, ±40 mM EtOH and 1 μM RA. cDNA from J1 ESCs treated with 1 μM RA for 72 hours was used as a positive control for *Sox17* transcripts and cDNA from AB1 ESCs cultured in the absence of LIF for 48 hours was used as a positive control for *Fgf5*, respectively (n=2). *F*, Fold changes in transcript levels of *Cdx1*, *Hoxa1*, and *Hnf1β* in WT AB1 ESCs treated with 1 μM RA or 1 μM ROL±40 mM EtOH for 48 hours. Fold changes were expressed as ratios relative to the vehicle-treated groups of the same gene set to 1. Error bars represent standard errors of independent experiments where n = 3 biological repeats except where otherwise indicated. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

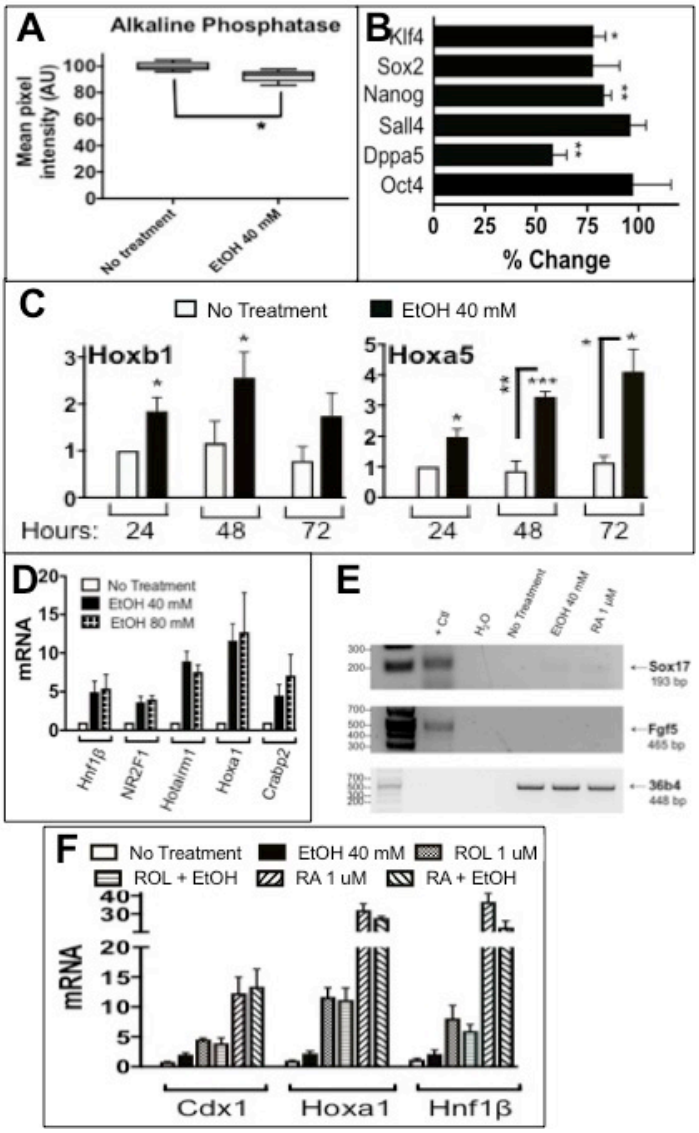


Figure S2.2: mRNA levels of differentiation-associated genes are increased by ethanol in the presence and absence of RA in CCE WT but not RARβ$^{+/-}$γ$^{-/-}$ ESCs. *A*, Western blotting for RARγ in WT and RARγ-edited cell line (Clone #33643) in the presence and absence of RA compared to β-actin loading control. *B*, Fold changes in transcript levels of RA-inducible genes in WT and RARγE8$^{-/-}$ ESCs at 48 hour treatment with EtOH (40 mM) or RA (1 μM). *C-D*, Quantitative analysis of *Hoxa1* (*C*) and *Hoxb1* (*D*) transcripts in AB1 WT and RARβ$^{+/-}$γ$^{-/-}$ ESCs at 2 hours after treatment with EtOH (40 mM), RA (1 μM), or 40 mM EtOH+1 μM RA, compared to 0.1% DMSO-treated cells. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = 3 biological repeats. *, p≤0.05, **, p≤0.01, ****, p≤0.0001.

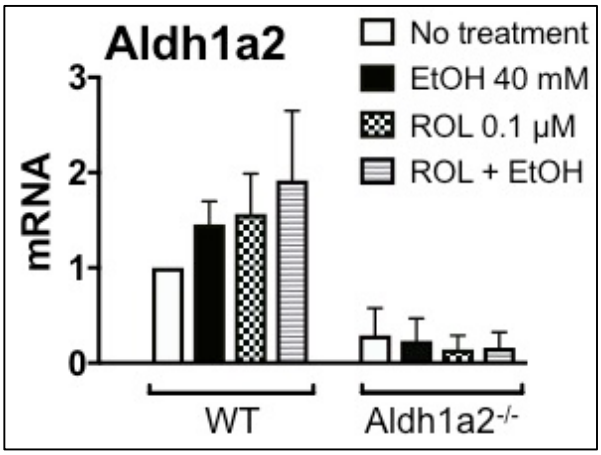


Figure S2.3: *Aldh1a2* transcript levels are not altered by ethanol. Fold changes in transcript levels of *Aldh1a2* in CCE WT and Aldh1a2$^{-/-}$ ESCs treated with 40 mM EtOH, 0.1 μM ROL, and 40 mM EtOH+0.1 μM ROL. Error bars represent standard errors of independent experiments where n = 3 biological repeats.

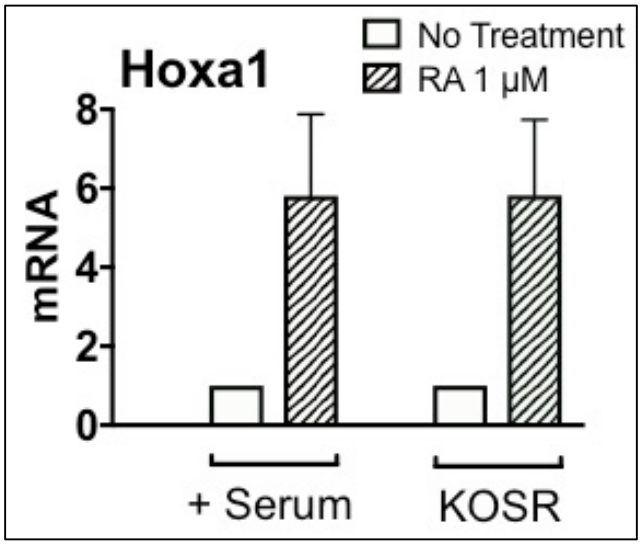


Figure S2.4: *Hoxa1* transcript levels are increased by similar fold changes in WT ESCs cultured in serum-containing and chemically defined medium. mRNA levels of *Hoxa1* following 48 hours of 1 μM RA treatment in standard ESC medium and KOSR. Error bars represent standard errors of independent experiments where n=3 biological repeats.

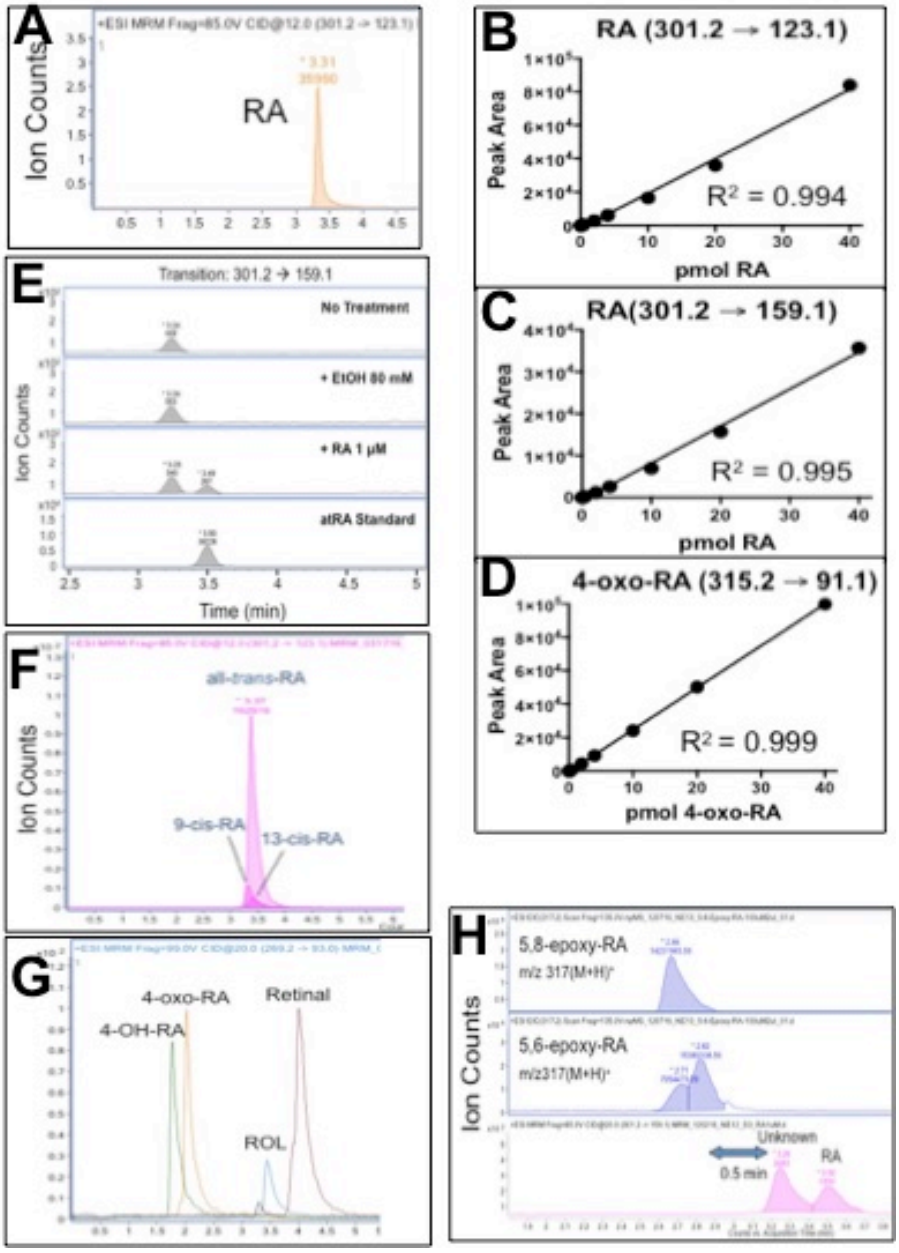


Figure S2.5: Tandem MS/MS characterization of retinoids. *A*, Chromatogram for RA standard (20 pmoles) at primary transition (*m/z* 301.2→123.1) *B-C*, Representative calibration curves for *all-trans* RA at 2 transitions, *m/z* 301.2→123.1 (*B*) and 301.2→159.1 (*C*). *D*, Representative calibration curves for 4-oxo-RA. *E*, RA ion counts at *m/z* 301.2→159.1 transition (RT = 3.5 min.) *F-G*, Chromatograms for RA isomers (*F*) and RA metabolites and other retinoids (*G*). *H*, Ion counts for 5,6- and 5,8-epoxy-RA standards compared to samples treated with 1 µM RA.

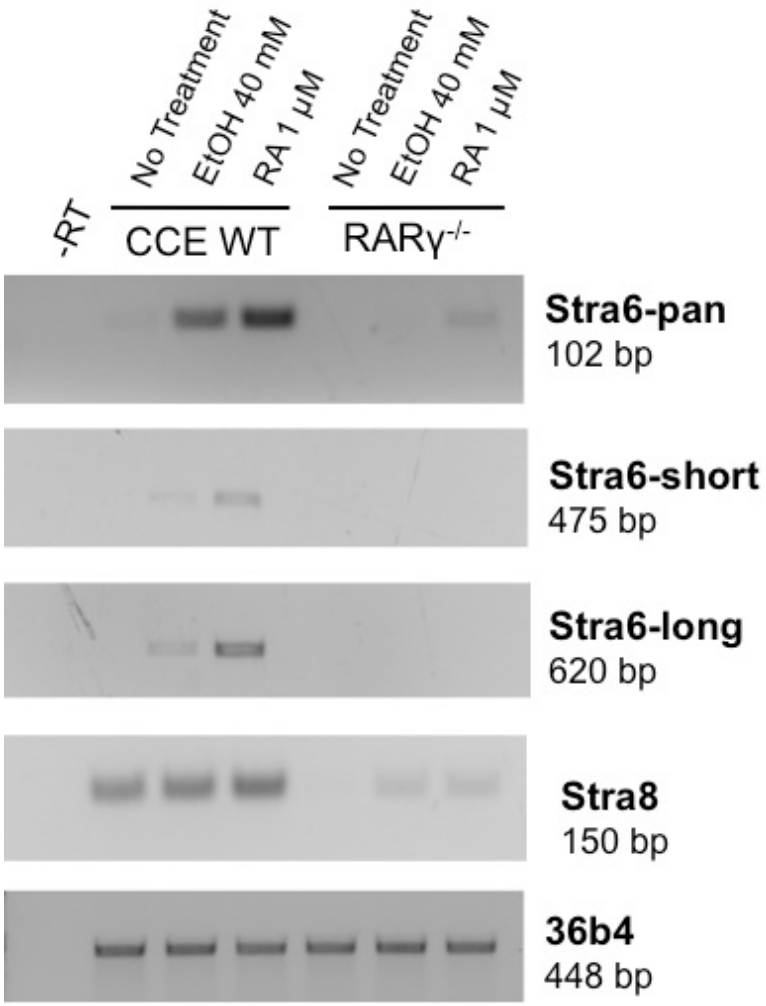


Figure S2.6: Ethanol increases transcript levels of both short and long isoforms of *Stra6*. Representative sample of semiquantitative RT-PCR analysis of transcripts of full length *Stra6* and its short and long isoforms (n=2). *Stra8*, which has similar homology but different function from *Stra6*, was not induced by EtOH. 36b4 was used as an internal control.

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CHAPTER THREE

ETHANOL-INDUCED DIFFERENTIATION OF EMBRYONIC STEM CELLS IS MEDIATED BY ACETALDEHYDE

INTRODUCTION

Alcohol use disorders affect millions of adults in the US every year, and are responsible for approximately 4% of the global disease burden each year (1). Binge drinking is defined as acute intoxication from ethanol (EtOH) ingestion that causes blood concentrations to exceed 0.08% in grams (2). The Centers for Disease Control and Prevention has established that there is no safe level for alcohol intake in pregnancy, yet binge drinking occurs most frequently in adults between the ages of 18-34 (2), when women are in prime child bearing age. EtOH diffuses into the placenta in concentrations similar to those that are present in maternal blood (3,4), and causes a variety of teratogenic effects in embryos, leading to birth defects which fall under the category of fetal alcohol spectrum disorders (FASD) (5). Thus, we used embryonic stem cells (ESCs), which represent the most primordial stage of development, to serve as a model system for investigating whether EtOH or its metabolites can alter differentiation capacity.

Alcohol is metabolized in a two-step oxidation process (6). First, EtOH is oxidized to acetaldehyde (AcH) by members of the alcohol dehydrogenase (ADH) family of enzymes, CYP4E1, or catalase, and

subsequently, AcH is metabolized to acetate by aldehyde dehydrogenase (ALDH) family members (Figure 2.1A) (6-8). Acetate can then either exit the cell through monocarboxylate (MCT1/4, SLC16A1-3) transporters (9) or serve as a substrate for acetyl CoA formation, primarily via the enzymatic actions of cytoplasmic Acyl-CoA synthetase short chain member 2 (ACSS2/ACECS1) (10). Acetyl CoA is a central metabolite in several physiologic processes, including anabolic production of fatty acids and triglycerides (lipogenesis), protein and histone acetylation, and catabolic processes to generate energy, such as its entry into the tricarboxylic acid cycle (Figure 3.1A) (11-13). AcH is a highly reactive aldehyde intermediate (14) that causes damage to nucleic acids, proteins, lipid membranes, and can disrupt the functions of organelles such as mitochondria (15). The major toxic effects of AcH result from formation of covalent modifications with several biomacromolecular species to generate adducts that interfere with their physiologic functions, resulting in protein inactivation and DNA damage and mutations (14).

The primary enzyme that metabolizes AcH, ALDH2 (7), has a Km for AcH of 0.2 μM (16). There is no consensus as to how AcH is metabolized in ESCs, which express ALDH2 as well as ALDH1A2, an enzyme required for retinaldehyde conversion to retinoic acid (RA). Data from some studies have suggested that EtOH can be metabolized by ALDH1A2 at the expense of RA formation at some early stages of embryonic development (17,18).

In this research, we show that ALDH2 primarily metabolizes AcH in ESCs. We also show that EtOH, AcH and 4-hydroxynonenal (4-

HNE), an endogenous aldehyde that requires ALDH2 for metabolism (19), induce transcripts of genes associated with differentiation and RA signaling, such as *Hoxa1*, *Cyp26a1*, and *Stra6*. Furthermore, we show that loss of ALDH2 partially inhibits the EtOH- and aldehyde-mediated induction of these differentiation-associated genes, and also partially inhibits the ability of RA to induce these transcript levels. We conclude that EtOH stimulates differentiation downstream of its metabolism by ALDH2, and thus, that the loss of ALDH2 may result in aberrant differentiation to extraembryonic endoderm by RA.

METHODS

CELL CULTURE AND REAGENTS

CCE ESCs were cultured as previously described (20). Cells were treated with 40 mM 95% EtOH, 1 mM AcH (Calbiochem, San Diego, CA), 1 μM 4-hydroxynonenal (HNE) (Sigma, St. Louis, MO), 1 mM sodium acetate (Sigma) pH=7.4, and 1 μM all-*trans*-RA (Sigma) dissolved in 100% dimethyl sulfoxide (DMSO). AcH was aliqoted from a freshly opened bottle and tubes were stored at -20^{o}C for no more than 2 months. Each aliquot was immediately discarded after a single use. RA was prepared in dim light from a 1 mM stock solution; the final concentration of DMSO was 0.1%. ESCs were seeded in 6 well plates, and reagents were changed twice daily approximately every 12 hours for 48 hours, with the final reagent change completed 8 hours prior to

harvest. 103 units/ml of LIF were added to medium for all experiments.

WESTERN BLOTTING

CCE WT and mutant ESCs were harvested in 4% SDS lysis buffer, boiled, and resolved on SDS-PAGE gels. Antibodies were applied using the following dilutions: ALDH1A2 (1:500, Abcam; ab156019; Lot GR117687-8), ALDH2 (1:2000, Abcam; ab108306; Lot GR97098-10), ACSS2 (1:1000, Cell Signaling; 3658S; Lot 2), RARγ (ab97569; Lot 102512 ; Abcam), and actin (1:40,000 or 1:60,000, Millipore, MAB1501; Lot 2665057).

GENERATION OF ALDH2$^{-/-}$ AND ACSS2$^{-/-}$ EMBRYONIC STEM CELLS

Guide (g)RNAs targeting the sequence GAAGTCGCCGTCGATGGGAA (A) in the sense strand of exon 5 and the sequence TATACCCGCCATGAGCCTGT (B) in the antisense strand of the Aldh2 gene were cloned into the BbsI sites of disparate pX461-hSpCas9n(BB)-2A-GFP vectors. Vector A was digested with XbaI overnight, dephosphorylated with shrimp alkaline phosphatase, run on an agarose gel, excised, and purified. Vector B was amplified using primers targeting the hU6 promoter (Fwd: 5'-TTTGCTAGCGAGGGCCTATTTCCCATGAT -3') and a downstream CRISPR sequence (Rev: 5'-GGTACCGCTAGCGCCATTTGTCTGC-3'). A 400 bp product was excised from an agarose gel, purified, and ligated

into vector B. Clones were transformed into DH5α *E. coli*. Following XbaI and PciI double digestion, clones positive for both gRNAs exhibited an 850 bp band, 400 bp greater than clones that failed to incorporate vector B. Following transfection, we harvested colonies, amplified their DNA by PCR (Fwd: 5'-TGAGCATGGCTGACCCCAAGT-3'/Rev: 5'-AGCCAAATGCCAGGGTTGTTGC-3', 272 bp product), and digested the DNA with BccI to genotype CRISPR-edited clones. Clones lacking the restriction site were sequenced on both alleles and double positive knockout clones were expanded in culture.

The same procedure was followed for the *Acss2* gene, using gRNAs CAGCAATGTTCTCCGTAAAC (A) and GAGTTCACGGTATGTGATCT (B) targeting exon 3, and EcoNI for genotyping clones. The primers used for PCR amplification are as follows: Fwd: 5'-GTTGGAATTTTGTGACTGCTCCTG-3'/Rev: 5'-CCTGTTACCAGATCCATCCATTTC-3', 283 bp product.

GENERATION OF ALDH1A2$^{-/-}$ EMBRYONIC STEM CELLS

gRNAs targeting the sequence TTCACAAGACACGAGCCCAT (A) in the sense strand of exon 5 and the sequence CTCTGGAGTGACCGTGCTTA (B) in the antisense strand of intron 5 of the *Aldh1a2* gene were cloned into the BbsI sites of disparate pX461-hSpCas9n(BB)-2A-GFP vectors. We prepared vectors as described for Aldh2$^{-/-}$ ESC line generation. We harvested clonal colonies in PBS following transfection and amplified their DNA by PCR (Fwd: 5'-TGTGTAGGATGTGCCTCAGTTTC-3'/Rev: 5'-

ACCACACATCGCTAAGGACCG-3', 254 bp product), and digested with BanII to genotype CRISPR-edited clones. We sequenced clones lacking the restriction site on both alleles. We then expanded double positive knockout clones in culture and tested for protein expression by Western blotting.

KINETIC ASSESSMENT OF WILD TYPE, ALDH2-/-, AND ALDH1A2-/- EMBRYONIC STEM CELL LINES

We cultured ESCs in either 6 well plates or 10 cm dishes and harvested in a mild TEN buffer pH=7.2 (50 mM Tris, 150 mM NaCl, 1 mM EDTA) to retain enzymatic activity of cells. We then centrifuged the cells, resuspended them in 250 mM Tris pH=7.8 with vortexing, frozen, and lysed them by performing snap thawing. We then centrifuged the ESCs at high speed, concentrated them using 10K concentrators (Amicon Ultra, Sigma), and then performed two additional concentration steps with 250 mM Tris pH=7.8.We conducted kinetics assays to determine the rate of AcH metabolism in various cell lines using the SpectraMAX 340PC plate reader (Molecular Devices, San Jose, CA) We measured absorbance readings every 60 seconds using a 340 nm primary wavelength for NADH detection and 600 nm secondary wavelength for background determination. The concentrations of reagents used for the assay were as follows: AcH 3.2 mM, NAD 3 mM, Alda1 (Sigma) 15 μM, and diethylaminobenzaldehyde (DEAB) (Sigma) 1 μM. 10 μg/μl of protein lysate was added to each well. AcH was added last each time to prevent early oxidation prior to

measuring readings. The averages of three technical replicates for each sample were used to quantitate rate values in nM•min^{-1}•mg^{-1}protein units, and two biological repeats were performed. Rates were calculated using the slopes of linear regression lines generated for the average each sample in Microsoft Excel.

RNA ISOLATION AND REAL-TIME PCR

We performed RNA extraction using TRI Reagent (Sigma) according to the manufacturer's instructions. We quantified 1 μg RNA and reverse transcribed to make complementary DNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). All cDNA was diluted fivefold. We used SYBR Green quantitative PCR Supermix in a 15 μl reaction mix to conduct reactions on a Bio-Rad iCycler using 3 μl of cDNA. We performed quantification using the Ct method and generated standard curves for all runs to assess efficiency. We normalized the levels of all mRNA transcripts using a 36b4 internal control. Table 3.1 shows a list of primers used.

STATISTICAL TREATMENT OF THE DATA

We conducted statistical analysis on qPCR data on at least three, independent biological replicates for each experiment using Graph Pad Prism 7.0 software, and determined the means ± SEM. We used ANOVA to determine statistical significance within sets of 3 or more groups, and Student's t-test to compare two independent

populations. A two-tailed p value < 0.05 was considered statistically significant.

RESULTS

ALDH2- AND ACSS2-KNOCKOUT EMBRYONIC STEM CELL LINES WERE GENERATED VIA CRISPR/CAS9.

We sought to ablate ALDH2 activity in CCE embryonic stem cells (ESCs) using CRISPR/Cas9 targeted to two sequences in exon 5 of the *Aldh2* gene. Likewise, we targeted two sequences in exon 3 of the *Acss2* gene for CRISPR/Cas9-mediated deletion. We identified 5 clones from guide (g)RNA-Aldh2-treated ESCs that lacked functional ALDH2 protein via Western blotting (Figure 3.1B). We also identified two clones from the gRNA-ACSS2-treated cells that were negative for protein expression (Figure 3.1C). We selected one clone from each knockout line for sequencing on both alleles to ensure loss of both alleles in the targeted exon of the *Aldh2* (Figure 3.1D) and *Acss2* (Figure 3.1E) genes.

ALDH2 PRIMARILY METABOLIZES ACETALDEHYDE IN EMBRYONIC STEM CELLS.

To determine if ALDH2 is responsible for most of the AcH oxidation in wild type (WT) ESCs, we compared the kinetics of NADH production secondary to AcH consumption in ESCs (Figure 3.2A). In some

situations, ALDH1A2, instead of ALDH2, has been reported to metabolize AcH instead of ALDH2 (17,18). Thus, we generated 52 and 46 base pair deletions in each allele of exon 5 of ALDH1A2 to generate an ALDH1A2-knockout ESC line to probe the extent of AcH oxidation by ALDH2 versus ALDH1A2 in ESCs (Figure 3.2B). The cofactor NAD^+ is reduced to NADH upon conversion of AcH to acetate by members of the aldehyde dehydrogenase family of enzymes, making detection of NADH production at a wavelength of 340 nm diagnostic for enzyme activity (21). We determined the rate of AcH (3.2 mM) oxidation by WT ESCs to be 72.4 nmole•min^{-1}•mg^{-1}protein (Table 2). The rate at which *Aldh2*$^{-/-}$ ESCs oxidized AcH was decreased by 94.9% (3.67 nmole•min^{-1}•mg^{-1} protein) compared to that of WT ESCs, whereas we observed only a 20.2% reduction in the rate of AcH oxidation in *Aldh1a2*$^{-/-}$ (57.8 nmole•min^{-1}•mg^{-1}protein) compared to that of WT cells (Figure 3.2C). These results confirm that AcH is primarily metabolized by ALDH2, and not by ALDH1A2, in ESCs.

Table 3.1: List of primer sequences used for qPCR with predicted product size.

Target	Forward (5'→ 3')	Reverse (5'→3')	Product size (bp)
Cyp26a1	GAAACATTGCAGATGGTGCTTCAG	CGGCTGAAGGCCTGCATAATCAC	272
Hoxa1	TTCCCACTCGAGTTGTGGTCCAAGC	TTCTCCAGCTCTGTGAGCTGCTTGGTGG	220
RARβ	GATCCTGGATTTCTACACCG	CACTGACGCCATAGTGGTA	247
Rdh10	TCTGGACATCACCTTCTGGAATG	CTCAACTCCAGCAGTGCTGAAC	177
Stra6	GTTCAGGTCTGGCAGAAAGC	CAGGAATCCAAGACCCAGAA	102
36b4	AGAACAACCCAGCTCTGGAGAAA	ACACCCTCCAGAAAGCGAGAGT	448

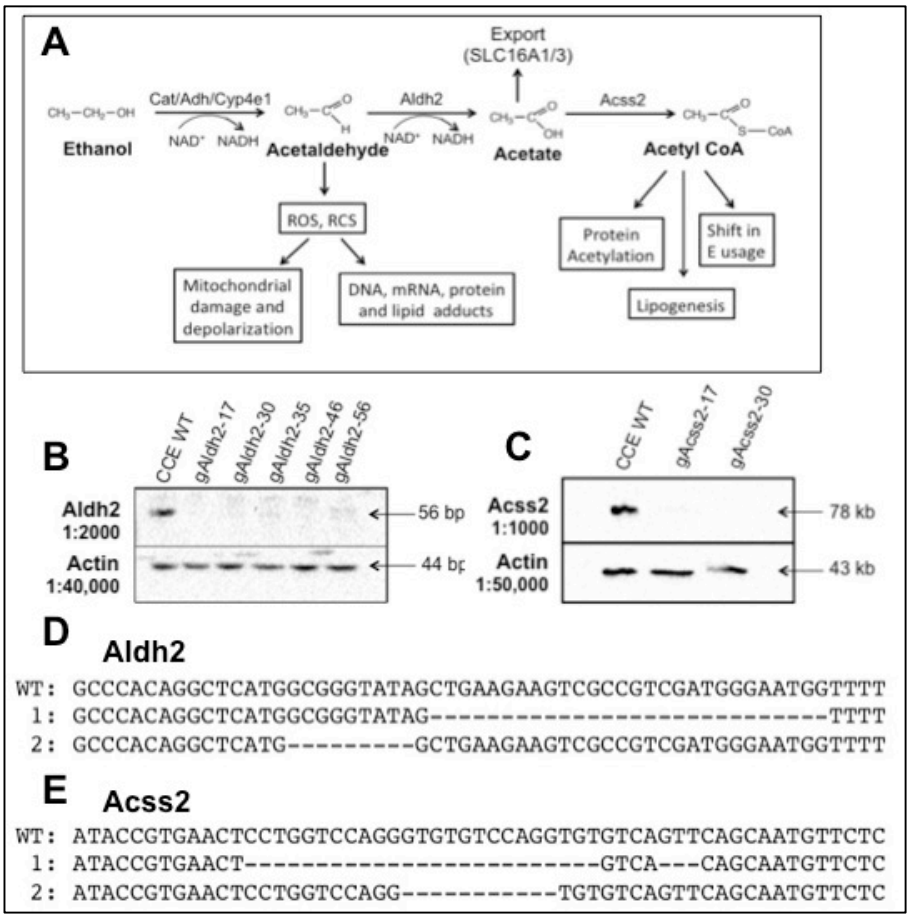

Figure 3.1: ALDH2 and ACSS2 were targeted by CRISPR/Cas9 to generate knockout cell lines. *A*, Schematic of the EtOH oxidation pathway. EtOH is first oxidized to AcH by an alcohol dehydrogenase family member, Catalase, or CYP4E1 using NAD^+ as a cofactor. AcH can then generate reactive carbonyl or oxygen species which damage macromolecules and mitochondria. ALDH2 metabolizes AcH to acetate while reducing another molecule of NAD^+. Acetate is then either exported by a transporter in the SLC16A family or converted to acetyl CoA by ACSS2. Acetyl CoA can then enter into a number of physiologic pathways, including lipogenesis, pathways of energy production, and protein acetylation. *B-C*, Western blotting of ALDH2 (*B*) and ACSS2 (*C*) in WT and CRISPR/Cas9-generated mutant clones. β-actin was used as a loading control in both experiments. *D-E*, Gene sequences for WT and mutated alleles for a selected clone of *Aldh2*$^{-/-}$ (28 and 9 base pair deletions) (*D*) and *Acss2*$^{-/-}$ (28 and 11 base pair deletions) (*E*) ESCs.

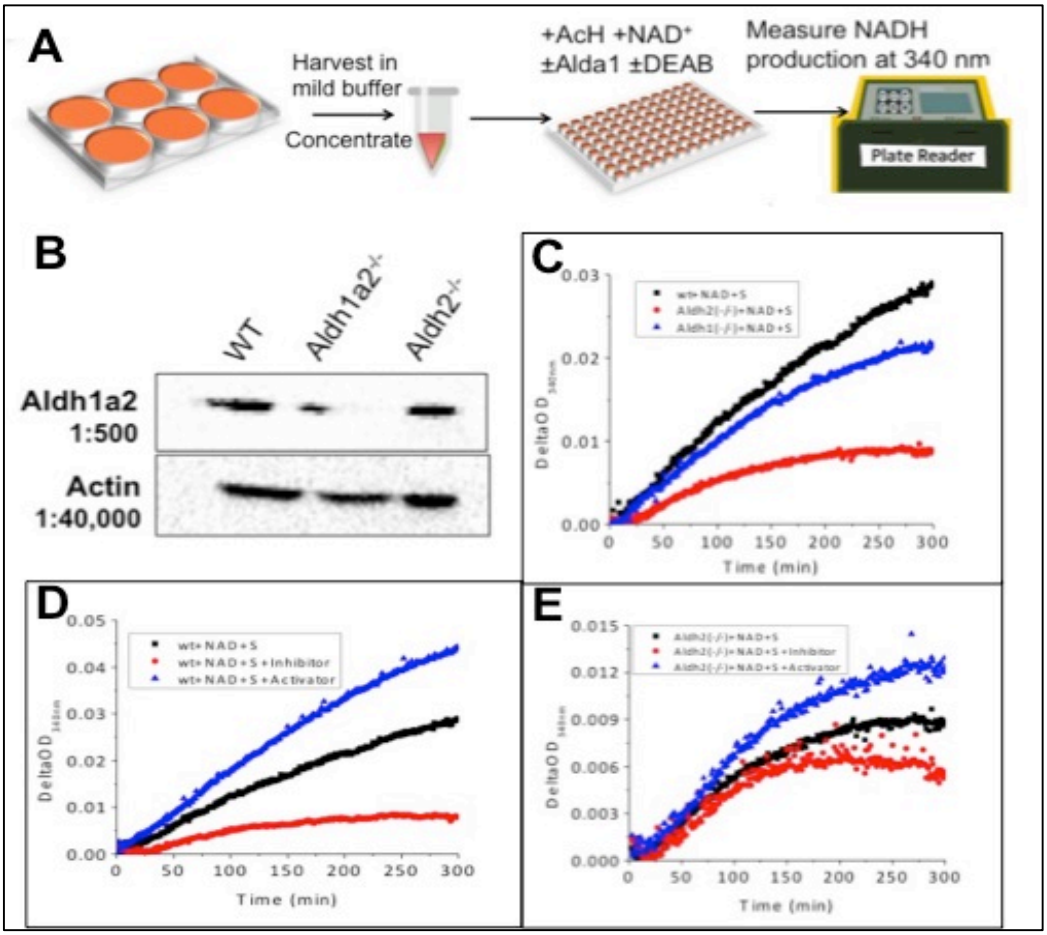

Figure 3.2: ALDH2 primarily metabolizes acetaldehyde in embryonic stem cells. *A*, Diagram of experimental design for kinetics assays. ESCs were lysed, concentrated, and treated with 3.2 mM AcH, 3 mM NAD^+ ±15 μM Alda1 ±1 μM DEAB prior to measuring absorbance at 340 nm (λ for NADH formation) on a plate reader. *B*, Western blotting of Aldh1a2 in WT, *Aldh1a2*$^{-/-}$, and *Aldh2*$^{-/-}$ ESCs, compared to a β-actin loading control. *C*, Kinetic analysis of rate of NADH formation in WT, *Aldh1a2*$^{-/-}$, and *Aldh2*$^{-/-}$ ESCs over 5 hours. *D-E*, Kinetic analysis of rate of NADH formation in WT (*D*) and *Aldh2*$^{-/-}$ (*E*) ESCs treated with only NAD and AcH compared to WT ESCs treated additionally with ALDH2 agonist Alda1 or ALDH2 antagonist DEAB. A representative experiment of n=2 biological replicates is shown.

To ensure that NADH was primarily being produced by AcH conversion to acetate and not other non-specific reactions, we treated the cell lysates with 1 μM diethylaminobenzaldehyde (DEAB), which is a pharmacological inhibitor of ALDH2, or 15 μM Alda1, an ALDH2 agonist, in addition to AcH. Alda1 increased (30.4%, 104 nmole•min^{-1}•mg^{-1}protein) and DEAB decreased (51%, 35.5 nmole•min^{-1}•mg^{-1} protein) the rates of AcH oxidation in WT ESCs compared to that in lysates from WT ESCs treated only with AcH (72.4 nmole•min^{-1}•mg^{-1} protein) (Figure 3.2D). The changes in the rates of AcH oxidation in *Aldh2*$^{-/-}$ ESCs ± Alda1 ±DEAB were small; addition of Alda1 caused an 8.3% increase and addition of DEAB resulted in a 15.3% decrease in AcH oxidation rate. These data suggest that the roles of additional ALDH family enzymes in the oxidation of AcH in ESCs are minimal (Figure 3.2E, Table 3.2).

Table 3.2: Kinetic rates of acetaldehyde oxidation in wild-type and mutant cell lines.

	nmole•min^{-1}•mg^{-1}protein		
	WT	**Aldh2$^{-/-}$**	**Aldh1a2$^{-/-}$**
+ AcH	72.4	3.67	57.8
+ AcH + Alda1	104.0	4.00	-
+ AcH + DEAB	35.5	3.11	-

ETHANOL INCREASES TRANSCRIPTS INVOLVED IN DIFFERENTIATION, BUT ONLY WHEN ALDH2 IS PRESENT.

To probe the phenotypes of ESCs lacking either Aldh2 or Acss2, we first measured cell proliferation of *Aldh2*$^{-/-}$ and *Acss2*$^{-/-}$ compared to WT ESCs. *Aldh2*$^{-/-}$ ESCs displayed a 56.5% lower proliferation rate than WT cells ($p<0.0001$), while *Acss2*$^{-/-}$ ESCs exhibited no change in proliferation rate (Figure 3.3A). To analyze the roles of ALDH2 and ACSS2 in the induction of differentiation-associated transcripts, we measured transcript levels of *Hoxa1*, *Cyp26a1*, and *RARβ2* ±40 mM EtOH for 48 hours. EtOH increased mRNAs of these transcripts in WT (3.7±0.1-fold, $p<0.0001$, *Hoxa1*; 4.4±0.7-fold, $p=0.009$, *Cyp26a1*; 2.0±0.1-fold, $p=0.007$, *RARβ2*) and Acss2$^{-/-}$ (2.5±0.3-fold, $p=0.03$, *Hoxa1*; 3.5±0.8-fold, $p=0.049$, *Cyp26a1*; 1.97±0.3-fold, $p=0.031$, *RARβ2*) ESCs compared to the levels in untreated ESCs (Figure 3.3B). However, levels of these transcripts were not increased by EtOH versus untreated cells in *Aldh2*$^{-/-}$ ESCs compared to WT (2.2±0.3-fold, $p=0.018$, *Hoxa1*; 4.0±0.98-fold, $p=0.027$, *Cyp26a1*; 1.6±0.2-fold, $p=0.044$, *RARβ2*) cells (Figure 3.3C).

To examine whether the reductions in EtOH-mediated induction of differentiation-associated mRNAs in *Aldh2*$^{-/-}$ ESCs were the result of specific increases in oxidative stress secondary to a defect in AcH clearance when ALDH2 was deleted, we treated WT and *Aldh2*$^{-/-}$ ESCs with the free radical scavenger, N-acetylcysteine (NAc). Addition of 2 mM NAc to EtOH-treated cells failed to restore EtOH-mediated mRNA

increases in *Hoxa1* in Aldh2$^{-/-}$ ESCs, ruling out a general oxidative stress signature in preventing *Hoxa1* induction (Figure 3.3D).

We hypothesized that the loss of differentiation-related transcript induction in EtOH-treated *Aldh2*$^{-/-}$ ESCs could have resulted from either a loss of ALDH2 enzyme function or a decrease in acetate, as loss of ALDH2 function leads to a reduced conversion of AcH to acetate following EtOH exposure (Figure 3.3E). Whereas circulating acetate levels in serum are normally 20-50 μM, acetate levels increase to 1-2 mM following exposure to EtOH from the actions of ALDH2 (22). Therefore, we added 1 mM acetate into the cell culture media of WT and *Aldh2*$^{-/-}$ ESCs ± EtOH in an attempt to restore the increases in differentiation-associated mRNA levels. Combining acetate with EtOH did not result in increased transcript levels of *Hoxa1*, *Cyp26a1*, and *RARβ2* genes in either WT or Aldh2$^{-/-}$ ESCs compared to untreated cells (Figure 3.3F), ruling out acetate production from AcH as causative for the increased levels of these differentiation-associated transcripts.

TRANSCRIPTS ASSOCIATED WITH DIFFERENTIATION AND RETINOIC ACIS SYNTHESIS ARE INCREASED BY ETHANOL, ACETALDEHYDE, AND 4-HYDROXYNONENAL.

Both AcH and 4-hydroxynonenal are reactive aldehyde species (RAS), which are substrates for ALDH2 (19). We tested whether treating WT ESCs with 1 mM AcH or 1 μM 4-HNE for 48 hours could recapitulate the increases in differentiation-associated transcripts compared to

untreated ESCs that we observed following EtOH treatment. In WT ESCs, both EtOH and AcH similarly increased *Hoxa1* (EtOH: 1.7±0.2-fold, p=0.003; AcH: 1.8±0.4-fold, p=0.044) and *Cyp26a1* (EtOH: 2.8±0.4-fold, p=0.003; AcH: 2.2±0.5-fold, p=0.048) transcripts compared to untreated cells, and 4-HNE caused *Cyp26a1* mRNA elevations (1.6±0.2-fold, p=0.010) (Figure 3.4A). Neither *Hoxa1* nor *Cyp26a1* transcripts were increased by EtOH, AcH, or 4-HNE in Aldh2$^{-/-}$ ESCs versus untreated ESCs (Figure 3.4A), suggesting that ALDH2 activity is required for increases in differentiation-associated transcripts in the presence of aldehyde substrates.

Because *Hoxa1* and *Cyp26a1* are targets of retinoic acid (RA) (20,23), we measured mRNAs of genes involved in retinol import and oxidation: the retinol transporter *Stra6* and the retinol dehydrogenase *Rdh10*. Addition of either EtOH, AcH, or 4-HNE increased transcripts of both *Stra6* (EtOH: 1.7±0.1-fold, p=0.0003; AcH: 1.4±0.1-fold, p=0.016; 4-HNE: 1.5±0.1-fold, p=0.006) and *Rdh10* (EtOH: 2.5±0.3-fold, p=0.008; AcH: 2.4±0.5-fold, p=0.044; 4-HNE: 1.7±0.1, p=0.002) in WT but not Aldh2$^{-/-}$ ESCs compared to untreated ESCs (Figure 3.4B). We conclude that the aldehyde substrates of Aldh2, AcH and 4-HNE, stimulate mRNA increases of differentiation-associated genes related to retinoid signaling in stem cells, but only when functional ALDH2 is present.

INCREASES IN TRANSCRIPT LEVELS OF RETINOIC ACID-RESPONSIVE GENES ARE PARTIALLY INHIBITED IN ALDH2-NULL EMBRYONIC STEM CELLS.

To determine whether the inhibition of differentiation that we observed in ALDH2-null ESCs are specific to EtOH or extend to more general ESC differentiation, we used 1 μM RA to differentiate WT and *Aldh2*$^{-/-}$ ESCs along an extraembryonic, or epithelial, lineage (24). *Hoxa1, Cyp26a1*, and *RARβ2* are primary RA-responsive genes and were used as readouts for RA signaling activity (20,23,24). We found that *Hoxa1* (-54.6%, p=0.027), *Cyp26a1* (-51.2%, p=0.034), *RARβ2* (-43.2%, p=0.009), and *Stra6* (-41.7, p=0.040) all displayed decreased mRNA levels in *Aldh2*$^{-/-}$ ESCs compared to WT ESCs treated with RA (Figure 3.5). The lesser extent to which differentiation-associated transcripts are increased in response to RA treatment indicate that RA signaling is partially inhibited in Aldh2-null ESCs.

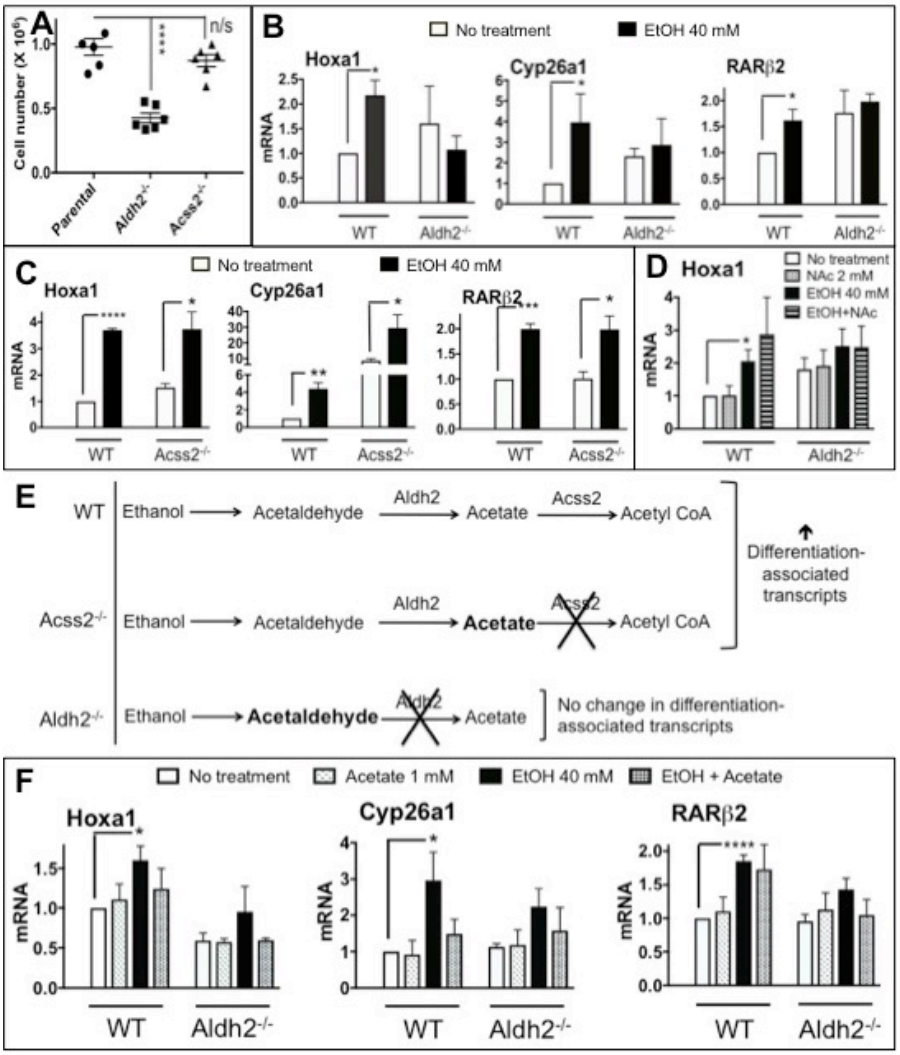

Figure 3.3: Ethanol induces differentiation-associated transcripts, which is inhibited by ALDH2 ablation. *A*, Proliferation rate of WT, *Aldh2*$^{-/-}$, and *Acss2*$^{-/-}$ ESCs at 72 hours. *B*, Fold changes in mRNA levels by 40 mM EtOH in WT and *Aldh2*$^{-/-}$ ESCs. *C*, Fold changes in mRNA levels by 40 mM EtOH in WT and *Acss2*$^{-/-}$ ESCs. *D*, Fold changes in mRNA levels of *Hoxa1* by EtOH, 2 mM NAc, and EtOH±NAc. *E*, Summary of effects of differentiation-associated transcripts by EtOH in WT ESCs vs. ESCs lacking ALDH2 or ACSS2. *F*, Fold changes in mRNA levels by EtOH, 1 mM acetate, and EtOH±acetate in WTand *Aldh2*$^{-/-}$ ESCs. All qPCR experiments were conducted at 48 hours. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001, ****, p≤0.0001.

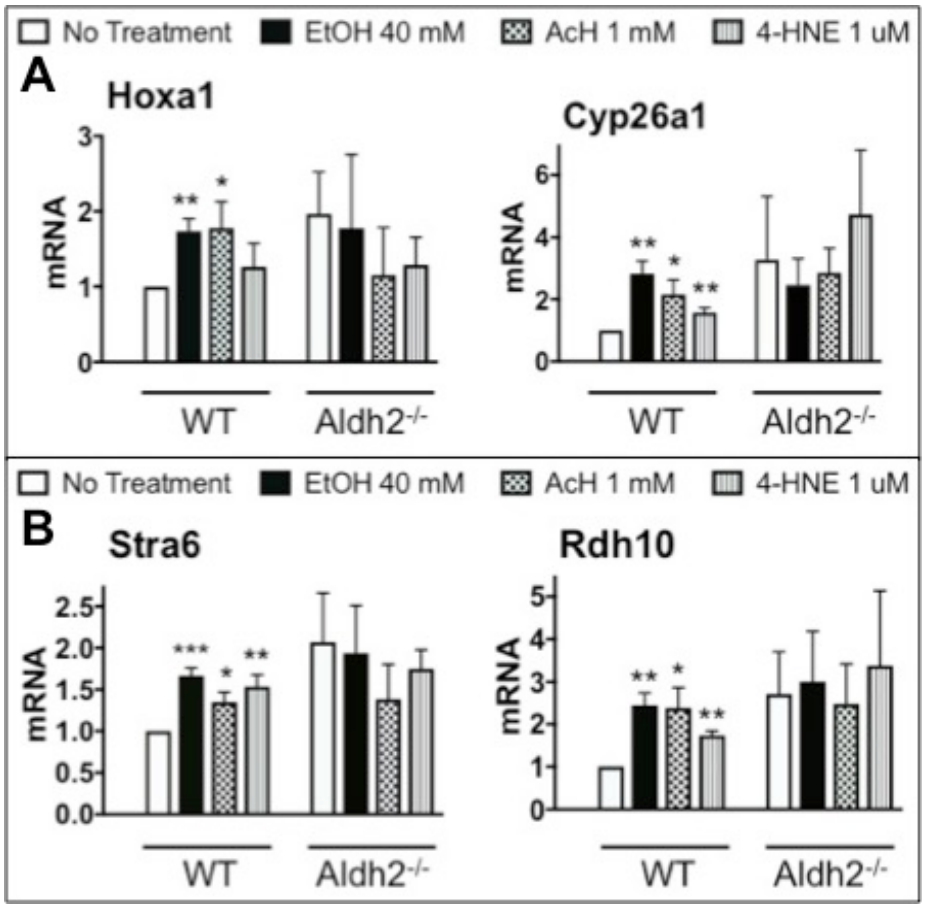

Figure 3.4: Differentiation-associated transcripts are increased by aldehyde substrates of ALDH2. *A*, Fold changes in mRNA levels of genes involved in differentiation by 40 mM EtOH, 1 mM AcH, and 1μM 4-HNE in WT and *Aldh2*$^{-/-}$ ESCs. *B*, Fold changes in mRNA levels of genes involved in RA synthesis by EtOH, AcH, and 4-HNE in WT and *Aldh2*$^{-/-}$ ESCs. All qPCR experiments were conducted at 48 hours. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = at least 3 biological repeats. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

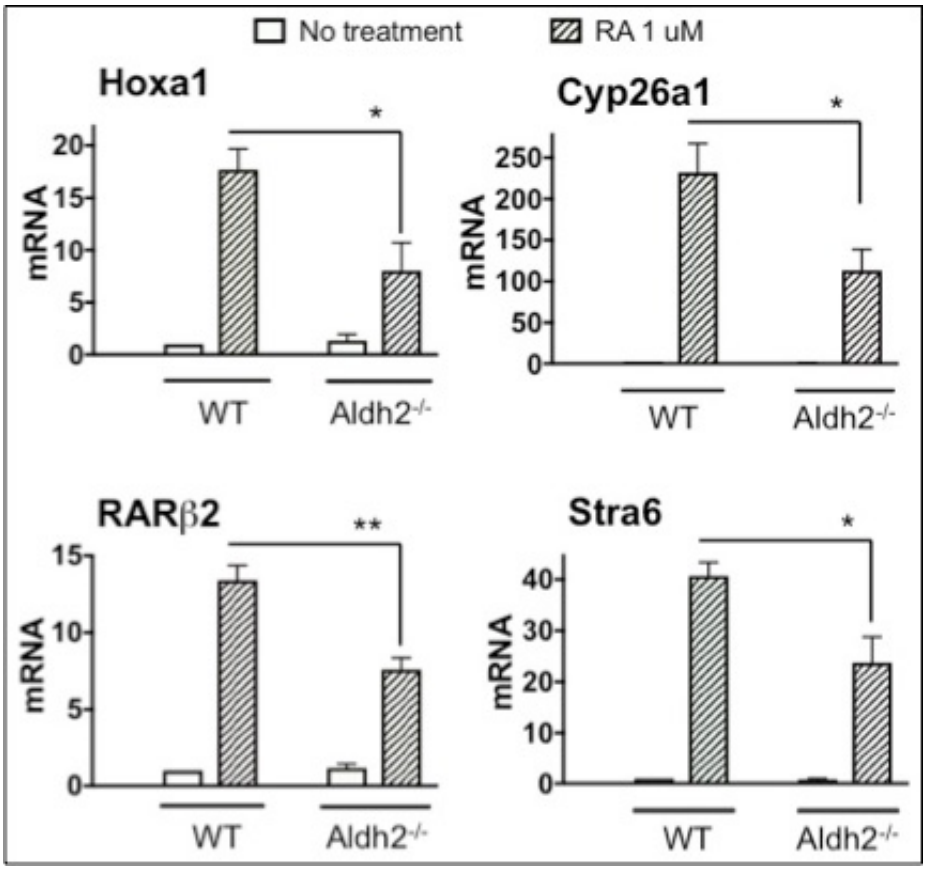

Figure 3.5: Loss of ALDH2 causes partial inhibition of RA-mediated transcript increases. Fold changes in mRNAs by 1 μM RA in WT and *Aldh2*$^{-/-}$ ESCs. B, Fold changes in mRNA levels of genes involved in RA synthesis by RA in WT and *Aldh2*$^{-/-}$ ESCs. qPCR experiments were conducted at 48 hours. Y-axes vary with samples being analyzed, and mRNA levels are shown in arbitrary units. Error bars represent standard errors of independent experiments where n = at least 3 biological repeats. *, p≤0.05, **, p≤0.01.

DISCUSSION

ALDH2 IS THE ALDEHYDE DEHYDROGENASE FAMILY MEMBER THAT PRIMARILY METABOLIZES ACETALDEHYDE IN EMBRYONIC STEM CELLS.

We showed here, using knockout models of Aldh2 and Aldh1a2, that ALDH2 preferentially metabolizes AcH in ESCs (Figure 3.2C). ALDH2, with a Km of 0.2 μM, is the enzyme that primarily metabolizes AcH when it is adequately expressed in cells (16). In comparison, ALDH1A2, which is also expressed in ESCs and is the first retinoid-oxidizing aldehyde dehydrogenase enzyme expressed in the embryo (25), has a very low affinity for AcH (Km = 650 μM) (26).

The importance of ALDH2 in AcH detoxification in humans is best illustrated by analyzing the phenotypes of those lacking its enzymatic activity in at least one allele (*ALDH2*2*) A large population of humans of East Asian descent is genetically predisposed to toxic accumulation of AcH from alcohol consumption from harboring this dominant genetic variant (27), which has been directly linked to increased risk of cancers (28,29) and neurologic sequelae (30). The *ALDH2*2* variant is a glutamate to lysine (E487K) substitution that inactivates ALDH2 by causing conformational changes to its active site and greatly weakening its affinity for the NAD^+ cofactor (31). The severe phenotype caused by the *ALDH2*2* variant upon alcohol exposure earmarks ALDH2 as a critical catalyst for EtOH detoxification in humans.

Despite the important role that ALDH2 plays in EtOH detoxification, some studies have shown that in certain situations, ALDH1A2 can metabolize AcH (17,18). For example, *Xenopus* embryos do not express ALDH2 during gastrulation and EtOH could instead be metabolized by ALDH1A2 during this window of time (18). In ESCs, we showed that loss of ALDH2 was sufficient in halting most AcH oxidation, while deletion of ALDH1A2 caused little change from WT (Table 3.2, Figure 3.2). Therefore, in our ESC model, ALDH2 is the enzyme that primarily metabolizes AcH and thus is a salient protein to target for ablation in our strategy to dissect the functional consequences of EtOH metabolism in ESCs.

While the ALDH2 agonist Alda1 induced activity and the Aldh2 antagonist inhibited activity of AcH metabolism, the same pharmacological agents had no marked effect in *Aldh2*$^{-/-}$ cells. What little effect by the drugs was observed could be explained by either normal variability or by the low activity of a different ALDH family member with redundant properties, such as mitochondrial Aldh1b1, which has a fairly low Km for AcH (30 μM) (32) and is also activated by Alda1 (33) despite its low expression in stem cells and embryos (34).

ALDH2 IS REQUIRED FOR ETHANOL-MEDIATED INCREASES IN MRNAS OF DIFFERENTIATION-ASSOCIATED GENES.

Because EtOH has strong teratogenic potential, understanding its effects in stem cell differentiation is crucial. We found that EtOH increased the mRNA levels of *Hoxa1, Cyp26a1*, and *RARβ2* compared

to untreated WT ESCs, and these EtOH effects were inhibited by deleting the *Aldh2* gene (Figure 3.3). Loss of ALDH2 enzyme additionally led to a robust decrease in proliferation compared to WT cells (Figure 3.3A). In contrast, knocking out ACSS2 had no effect on either growth (Figure 3.3A) or mRNA increases of *Hoxa1*, *Cyp26a1*, and *RARβ2* (Figure 3.3C). Furthermore, substituting acetate into the medium of ALDH2-null ESCs in a concentration that is expected to be present in the serum following EtOH ingestion (22) was insufficient to rescue EtOH-mediated transcript increases (Figure 3.3F). This demonstrates that the increase in differentiation-associated mRNAs by EtOH was not due to an increase in acetate or any downstream metabolite of acetate, but rather the result of either a direct effect by AcH or a secondary effect of ALDH2 activation.

Using EtOH to dissect its effects on stem cell differentiation has resulted in context-dependent changes in pluripotency and differentiation-related factors (35-38). For example, treating ESCs with a wide range of doses, from 0.1%-1% (17.4 mM-174 mM), of EtOH for two weeks caused a loss of pluripotency and spontaneous differentiation, as measured by alkaline phosphatase and TRA-1-81 staining, despite some pluripotency-related genes remaining highly expressed (35). In contrast to these findings, a 48 hour treatment with 100 mM EtOH followed by 6 days of differentiation via Leukemia inhibitory factor (LIF) removal caused a delay in the loss of pluripotency factor-related mRNAs, suggesting that differentiation was also being delayed by EtOH (36). Furthermore, EtOH can interfere with directed differentiation toward specified cell lineages by diverting

differentiation away from neuroectodermal (38,39), hepatic (40), or cardiac (41) fates, indicating a wide scale of defects resulting from exposure during differentiation.

We showed here that differentiation-related transcripts were induced in ESCs upon exposure to EtOH within 48 hours (Figure 3.3). These results are consistent with a model of EtOH stimulating stem cell differentiation (35). The delay in differentiation observed by Arzumnayan et al. (36) may have been unique to the method of differentiation being utilized, via depletion of LIF in cell culture medium. Our lab has shown several transcriptional differences between ESC differentiation by LIF removal compared with differentiation by adding exogenous RA (42,43). Depletion of LIF from culture medium causes ESCs to express the primitive ectoderm marker, Fgf5 (43,44), which increases over time to drive cells along a neuroectodermal fate (45). In contrast, RA treatment of ESCs suppresses Fgf5 mRNAs and differentiates cells along an extraembryonic endodermal, or epithelial, lineage (24,43). EtOH has been demonstrated to robustly inhibit neuroectodermal differentiation (38,39,46), thus it is expected that ESC differentiation secondary to LIF depletion would also be inhibited by EtOH.

ALDH2 OXIDATION OF ACETALDEHYDE AND 4-HYDROXYNONENAL IS REQUIRED FOR DIFFERENTIATION-ASSOCIATED TRANSCRIPT INCREASES.

Reactive aldehyde species (RAS), such as AcH and 4-HNE, are becoming increasingly appreciated for their roles in initiating signaling responses (47,48). Adducts formed from covalent modifications with the electrophilic groups may not only act as toxic stimuli but may also serve as relevant post-translational modifications for proteins to effect downstream signaling pathways (48). What effects these changes have on stem cell biology are still largely unknown on a mechanistic level, thus we explored the potential role that AcH plays in EtOH-mediated differentiation by using an ALDH2-knockout ESC model, in which AcH could not become efficiently metabolized (Figure 3.2).

We showed here that when functional Aldh2 is present, both exogenous AcH and 4-HNE can upregulate the mRNA levels of several differentiation-related genes compared to untreated ESCs, including genes in the RA signaling pathway. Ablating ALDH2, the enzyme primarily responsible for metabolizing both AcH and 4-HNE (19,49) partially inhibited these increases (Figure 3.4), while basal levels of untreated *Aldh2*$^{-/-}$ ESCs trended higher than untreated WT ESCs (Figures 3.3-3.4). This suggests that the inhibition of EtOH-mediated transcript increases is caused by AcH, which is consistent with studies in animal models which demonstrate that AcH induces the same types of developmental defects as EtOH (50-52).

4-HNE, another substrate of ALDH2, is also associated with differentiation in stem cell and cancer cell models (53,54), and exhibited similar effects on differentiation-associated transcripts in WT ESCs, increasing the mRNA levels of *Cyp26a1*, *Stra6*, and *Rdh10* (Figure 3.4). The induction in transcripts compared to untreated cells by 4-HNE was observed in only three of the four genes investigated. This lack of robust induction of differentiation-related genes may be attributed to the high instability and short half-life of 4-HNE in cell culture compared to EtOH (54,55). While we detected AcH metabolism after 4 hours (Figure 3.2C), 4-HNE is undetectable in medium after 45 minutes (55). This may result in less 4-HNE available as a substrate for Aldh2-dependent oxidation and thus a weaker effect on differentiation-associated transcript induction.

The transcript increases versus untreated ESCs that we measured following both AcH and 4-HNE treatment implies that RAS such as AcH and 4-HNE may disrupt stem cell function and predispose ESCs to precocious differentiation. We did not observe these increases in differentiation-associated genes upon treatment with EtOH or aldehyde substrates in Aldh2$^{-/-}$ ESCs. However, basal levels of many of these transcripts were often higher and variable in untreated Aldh2$^{-/-}$ ESCs (Figures 3.3-3.4). Because cells produce and may accumulate 4-HNE and AcH endogenously (14,56), ESCs lacking Aldh2 may be poised for differentiation by endogenous aldehydes and thus do not respond as robustly to external insults as stem cells containing ALDH2.

RAS, including AcH and 4-HNE, cause secondary accumulation of reactive oxygen species (ROS) in cells that effects a wide range of additional non-specific toxic events (15,47). However, we showed that the effects on differentiation-associated mRNAs are unrelated to the ability of AcH to increase ROS in ESCs, as N-acetylcysteine, a radical scavenger, did not prevent the EtOH-mediated increases in *Hoxa1* transcript levels, nor did it affect mRNA levels in Aldh2$^{-/-}$ ESCs (Figure 3.3D).

LOSS OF ALDH2 BLUNTS THE INDUCTION OF DIFFERENTIATION-ASSOCIATED TRANSCRIPTS UPON RETINOIC ACID EXPOSURE.

The RA signaling pathway is critical to stem cell differentiation and embryogenesis (57). Our results show that the differentiation markers that increase at an mRNA level by EtOH and aldehyde treatments are targets of RA, and that transcripts involved in retinol transport, *Stra6* (58), and oxidation, *Rdh10* (59), to produce RA, are also increased (Figure 3.4). In addition, *Aldh2* deletion not only inhibits the increases in EtOH/aldehyde-mediated differentiation-associated transcripts but also blunts the increases in these mRNAs after RA treatment (Figures 3.4-3.5). This suggests that ALDH2 loss is sufficient to impair RA-mediated increases in transcripts associated with differentiation to extraembryonic endoderm (epithelium) (24). This is important as a weakened transcriptional response of key genes expressed early in development by RA has profound consequences on differentiation and embryonic development (57,60). Further research is

necessary to qualify the extent to which stem cell differentiation mediated by EtOH and by retinoids relate to one another, and if RA signaling is directly activated downstream of AcH metabolism.

CONCLUSIONS

Our findings illuminate the role of EtOH on stem cell differentiation through its conversion to AcH and subsequent metabolism by Aldh2. Both EtOH and AcH stimulate increases in differentiation-associated genes, including *Hoxa1*, *Cyp26a1*, *Stra6*, and *Rdh10*. Without ALDH2, we did not measure further increases in these transcript levels upon EtOH or aldehyde treatment. Furthermore, loss of ALDH2 slows proliferation of ESCs and diminishes the responsiveness of several differentiation-related mRNAs to RA. Also pertinent is that AcH and other RAS that are metabolized by ALDH2 may cause premature differentiation. Because the response of differentiation marker transcripts to RA was also partially inhibited, loss of ALDH2 may potentially cause general defects in RA-mediated differentiation along an extraembryonic endoderm lineage, even in the absence of EtOH. These data raise the possibility that the mechanism by which EtOH differentiates ESCs overlaps with RA-activated transcriptional programs, which are crucial to embryonic development and teratogenic when dysregulated (61,62).

ABBREVIATIONS

AcH, acetaldehyde; DMSO, dimethyl sulfoxide; ESC, embryonic stem cell; EtOH, ethanol; gRNA, guide RNA; qPCR, quantitative polymerase chain reaction; RA, retinoic acid; RAS, reactive aldehyde species; ROL, retinol; WT, wild type; 4-HNE, 4-hydroxynonenal

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CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

The research presented here centered on the premise that ethanol (EtOH) affects embryonic stem cell (ESC) differentiation. Determining how EtOH influences differentiation mechanistically is of an urgent nature due to the many complications that arise from EtOH exposure to stem cells during development (1,2). While there is much extant evidence showing that EtOH impacts stem cell differentiation, the literature in the fields of stem cell and developmental biology is comprised of reports that show apparently conflicting data that EtOH may promote (3,4) or suppress (5-8) differentiation. We addressed the relationship between EtOH and differentiation by using embryonic stem cells (ESCs), which allow us to investigate how EtOH affects differentiation in the most primitive and malleable stage of development prior to expression of any differentiation-associated genes.

ETHANOL PROMOTES DIFFERENTIATION OF EMBRYONIC STEM CELLS VIA RETINOIC ACID SIGNALING

RA causes differentiation of stem cells upon binding one of its cognate nuclear receptors, retinoic acid receptor (RAR)-α/β/γ, with RARγ being especially relevant in activating transcriptional signatures driving ESC differentiation (11,12). To test the ability of EtOH to

activate RA signaling, we first treated WT ESCs and ESCs in which RARγ was deleted by CRISPR/Cas9 with EtOH and measured the transcript levels of RA-responsive differentiation-associated genes (13-15). Loss of RARγ completely abrogated the increases in mRNA levels of several differentiation-associated genes by EtOH compared to untreated ESCs, implicating RA:RARγ-dependent signaling in differentiation secondary to EtOH treatment. We showed via a β-galactosidase reporter assay that such increases in mRNAs by EtOH compared to untreated ESCs resulted from transcription, as loss of the *Hoxa1* retinoic acid response element (13) completely blocked these EtOH-mediated increases in *Hoxa1*.

To examine whether RA synthesis is required for EtOH-mediated differentiation, we created CRISPR-Cas9-generated ALDH1A2$^{-/-}$ ESCs to inhibit the final step of RA production from retinol (ROL). We also generated *Rdh10*$^{-/-}$ ESCs, as RDH10 is the major retinol-oxidizing enzyme in most cell types (16). Compared to WT ESCs, increases in differentiation-associated transcripts by EtOH were inhibited in both *Rdh10*$^{-/-}$ and *Aldh1a2*$^{-/-}$ ESCs. This demonstrates that RA synthesis from ROL is required for EtOH-mediated ESC differentiation, and that production of RA in ESCs occurs via sequential oxidation steps conducted by RDH10 and ALDH1A2, respectively.

To assess whether EtOH was causing ESCs to import ROL from the serum to promote differentiation, we first substituted serum-containing medium with medium containing knockout serum replacement devoid of ROL to attempt to differentiate ESCs with EtOH, ROL, and RA. While differentiation-associated mRNAs were increased

by RA compared to untreated ESCs in ESCs grown in both types of medium, these transcripts were not increased by EtOH. Adding ROL back into the medium at a concentration (0.1 μM) expected in serum (17) restored EtOH-mediated transcript induction versus untreated cells to the levels measured in serum-containing medium. These data demonstrate that ROL from serum is required for EtOH-mediated differentiation.

One of the mechanisms by which ROL is imported into some cell types is through the actions of the Stra6 transporter (18,19). To test whether STRA6 was importing ROL from the serum following EtOH treatment, we used an ESC line in which the *Stra6* RARE was deleted (20). Ablation of the *Stra6* RARE prevented EtOH-mediated induction of differentiation-associated mRNAs compared to untreated cells, implicating STRA6 in facilitating ROL import in ESCs following EtOH treatment.

Together, our findings here showed that EtOH, through its metabolism by ALDH2, caused extracellular ROL to enter ESCs from the medium via STRA6 and become metabolized to RA by consecutive oxidation reactions catalyzed by RDH10 and ALDH1A2, respectively. RA then signals in the nucleus through RARγ to activate transcription of genes that cause differentiation.

ETHANOL CAUSES DIFFERENTIATION OF EMBRYONIC STEM CELLS VIA ACETALDEHYDE

We found that several differentiation-associated transcripts were reproducibly increased by 48 hours of treating ESCs with 40 mM EtOH dosed every 12 hours, compared to untreated ESCs. Many of the transcripts studied were targets of retinoic acid (RA), and included mRNAs of primary response genes such as *Hoxa1*, *RARβ2*, and *Cyp26a1*, as well as those of secondary response genes associated with terminal differentiation, such as *Col4a*.

EtOH is oxidized to the biologically active metabolites acetaldehyde (AcH) and acetate (9). Therefore, we attempted to distinguish the differentiation-related effects of the parent compound from its metabolites by designing knockout ESC lines for aldehyde dehydrogenase 2 (ALDH2) and acetyl CoA synthetase short chain family member 2 (ACSS2), which metabolize AcH and acetate, respectively. This gave us a unique opportunity to dissect the roles of individual metabolites and enzymes involved in EtOH metabolism in the context of stem cell differentiation.

We initially expected that if AcH caused ESC differentiation, then loss of Aldh2 would lead to mRNA levels of *Hoxa1*, *RARβ2*, and *Cyp26a1*, used as readouts for differentiation, that were higher than wild type (WT) ESCs upon EtOH treatment, and if acetyl-CoA was responsible for differentiation then the same transcripts would be decreased by EtOH in ACSS2-null ESCs compared to WT ESCs treated with EtOH. What we found was that loss of ACSS2 did not affect

EtOH-mediated mRNA increases of differentiation-associated genes by EtOH, as both WT and *Acss2*$^{-/-}$ ESCs showed similar increases in transcript levels compared to untreated ESCs; however, loss of ALDH2 prevented further EtOH-mediated increases of these mRNA compared to untreated ESCs. Adding additional substrates of ALDH2, including the EtOH metabolite AcH and the endogenous aldehyde 4-hydroxynonenal (4-HNE) (10), also caused increases in differentiation-associated transcripts versus untreated cells in WT but did not further increase mRNA levels compared to untreated *Aldh2*$^{-/-}$ ESCs, in which basal transcript levels generally trended higher than in WT ESCs. To determine whether lack of acetate production rather than an increase in AcH was responsible for the loss of EtOH-mediated differentiation, we added 1 mM acetate into the medium of WT and *Aldh2*$^{-/-}$ ESC to observe if differentiation occurred in *Aldh2*$^{-/-}$ cells by EtOH, but acetate failed to restore the EtOH-mediated induction of differentiation-related transcripts. Furthermore, loss of ALDH2 caused 1 μM RA to induce target genes (*Hoxa1*, *Cyp26a1*, *RARβ2*, *Stra6*) to a lesser extent compared to WT cells, implicating Aldh2 in RA-dependent differentiation. Therefore, we conclude, first, that AcH is required for EtOH-mediated differentiation, and secondly, that because RA signaling was also partially inhibited by ALDH2 deletion, that Aldh2 loss is sufficient for impaired RA signaling along the extraembryonic endoderm lineage. Furthermore, inhibition of both EtOH- and RA-mediated transcripts of differentiation-associated genes by ALDH2 deletion implies a possible common mechanism for ESC differentiation by the two compounds.

FUTURE DIRECTIONS

The research presented here illustrates the mechanisms by which EtOH causes stem cell differentiation via RA-RARγ signaling following ROL import by STRA6 and RA synthesis. Elucidating the processes occurring after AcH formation by EtOH and preceding the activation of RA signaling is worthwhile, and may be amenable to investigation in future studies. Assessing genomewide transcript activity using an RNA-Seq platform is one method by which the differences in EtOH metabolism in the presence and absence of functional ALDH2 activity may be examined. Such an experiment may provide additional details to illuminate the genetic changes that predispose EtOH- and AcH-treated ESCs to RA-mediated differentiation. In addition, the phenotype of *Aldh2*$^{-/-}$ ESCs could be further explored using alkaline phosphatase staining and measurement of pluripotency factor mRNAs compared to WT ESCs. These assays will determine if *Aldh2*$^{-/-}$ ESCs are exhibiting premature differentiation in the absence of exogenous aldehyde treatment.

While loss of *Aldh1a2* and the *Stra6* RARE led to a loss of induction in most differentiation-associated transcripts in EtOH-treated compared to untreated ESCs, *Dhrs3* was a notable exception. Transcripts of *Dhrs3* were increased compared to untreated cells in *Stra6*$^{RARE-/-}$ and *Aldh1a2*$^{-/-}$ ESCs in addition to the WT line. This demonstrates that induction of *Dhrs3* by EtOH occurs independently of RA and therefore could precede the activation of the downstream transcriptional targets of RA. The mechanisms behind its activation

could be further explored by generating a *Dhrs3*-knockout ESC line. Despite serving as a retinaldehyde reductase traditionally associated with shifting reacting equilibrium from RA to ROL production (21), DHRS3 was recently found to be a major component of the retinoid oxidation complex that colocalizes with RDH10 *in vivo* to control ROL metabolism (22,23). Some striking features of DHRS3 include the 13fold increase in ROL oxidation by RDH10 when it colocalizes with DHRS3 in a complex compared to when RDH10 is expressed alone, as well as the ability to increase the half-life of RDH10 (22). Therefore, changes in DHRS3 activity may potentially link EtOH metabolism with the initial events that lead to the inexorable drive toward differentiation once RA-RARγ signaling commences. To date, there are no studies that examine DHRS3 in the context of EtOH treatment or AcH metabolism, so further experimentation exploring this possible connection based on our novel findings may be lucrative, potentially shedding light on some of the processes that lead to the initial activation of RA signaling upon ESC exposure to EtOH.

Because we demonstrated a STRA6 requirement for EtOH-mediated differentiation (Figure 2.5), future experiments related to DHRS3- and RDH10-dependent activity on ROL must take into account the amount of ROL entering the cells. Using a STRA6-knockout ESC line would allow us to demonstrate whether EtOH causes some ROL to enter cells via a different mechanism than STRA6. Intracellular ROL would be measured directly in EtOH-treated WT, STRA6-KO, and STRA6-RARE-KO ESCs, with the latter cell line included to distinguish RA-dependent STRA6 activity from total ROL

intake (Figure 4.1). To ensure that intracellular ROL levels are undetectable before EtOH is administered, ESCs would be grown in knockout serum replacement containing media that lacks ROL, and 0.5 μM would then be added to measure the amount imported into cells under each condition (Figure 4.1). If some ROL enters WT cells in the absence of EtOH or in STRA6-KO cells, then it is possible that this small amount of ROL may be converted to RA via activation of RDH10/DHRS3 complex by EtOH, which could trigger further uptake of ROL through *Stra6* gene activation by RA. We may then overexpress RDH10 and DHRS3 to examine whether expression of either enzyme is sufficient to stimulate differentiation. If differentiation-associated transcripts (*Hoxa1*, *RARβ2*, *Cyp26a1*) are induced by RDH10 or DHRS3 overexpression in the absence of EtOH, then EtOH is potentially acting through this complex to differentiate ESCs.

If we show that ROL import into ESCs is completely dependent on STRA6 (no intracellular ROL detectable in STRA6-KO cells), then we would focus on EtOH effects on the STRA6 protein instead of the RDH10/DHRS3 complex. The crystal structure of STRA6 reveals that the intracellular domain of STRA6 binds to two calmodulin (CaM) proteins (24). CaM is activated by calcium and regulates several transporters and ion channels (25); thus, it is possible that calcium release secondary to oxidative damage by acetaldehyde may be responsible for the initial STRA6 activation by EtOH. The biophysical mechanisms for STRA6 activation are still unknown, and whether or not EtOH is involved in STRA6 activation may lead to lucrative novel

findings that may link acetaldehyde-related cellular stress to EtOH-mediated RA signaling.

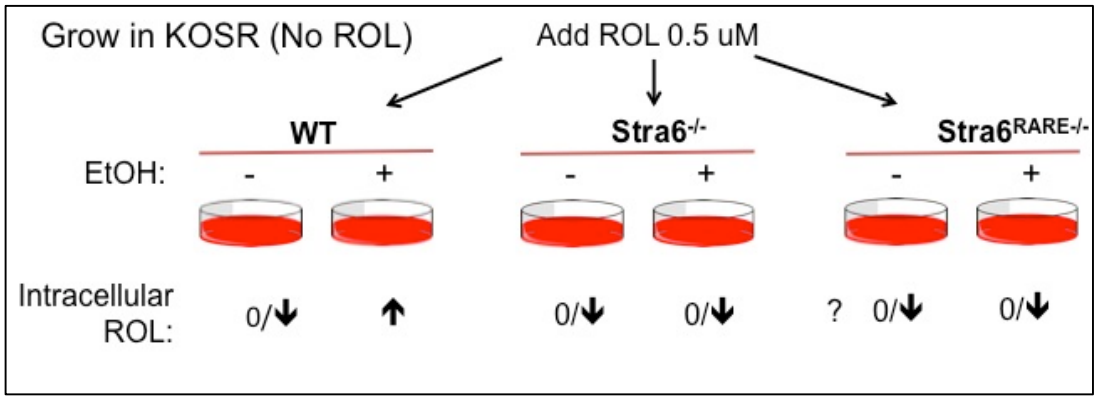

Figure 4.1: Schematic for testing the ability of ethanol to import ROL through STRA6. We would grow WT, *Stra6*$^{-/-}$, and *Stra6*$^{RARE-/-}$ ESCs in medium containing knockout serum replacement rather than ROL-containing serum. We would then add 0.5 μM ROL to each line, except for negative controls, (not pictured) and measure the amount of intracellular ROL that is detectable in each cell line ± 40 mM EtOH. Performing this experiment will demonstrate 1.) whether ROL may be imported in the absence of STRA6, 2.) whether trace amounts of ROL are imported into WT ESCs in the absence of EtOH, and 3.) whether all imported ROL is dependent on RA signaling. The results in this experiment would guide future experiments concerning the effects of EtOH on differentiation via the activities of STRA6 and the RDH10/DHRS3 complex.

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