

# Acute Alcohol Exposure during Mouse Gastrulation Alters Lipid Metabolism in Placental and Heart Development: Folate Prevention

Kersti K. Linask\* and Mingda Han

**Background:** Embryonic acute exposure to ethanol (EtOH), lithium, and homocysteine (HCy) induces cardiac defects at the time of exposure; folic acid (FA) supplementation protects normal cardiogenesis (Han et al., 2009, 2012; Serrano et al., 2010). Our hypothesis is that EtOH exposure and FA protection relate to lipid and FA metabolism during mouse cardiogenesis and placentation. **Methods:** On the morning of conception, pregnant C57BL/6J mice were placed on either of two FA-containing diets: a 3.3 mg health maintenance diet or a high FA diet of 10.5 mg/kg. Mice were injected a binge level of EtOH, HCy, or saline on embryonic day (E) 6.75, targeting gastrulation. On E15.5, cardiac and umbilical blood flow were examined by ultrasound. Embryonic cardiac tissues were processed for gene expression of lipid and FA metabolism; the placenta and heart tissues for neutral lipid droplets, or for medium chain acyl-dehydrogenase (MCAD) protein. **Results:** EtOH exposure altered lipid-related gene expression on E7.5 in comparison to control or FA-supplemented groups and remained altered on E15.5 similarly to changes with HCy, signifying FA deficiency. In comparison to control

tissues, the lipid-related *acyl CoA dehydrogenase medium length chain* gene and its protein MCAD were altered with EtOH exposure, as were neutral lipid droplet localization in the heart and placenta. **Conclusion:** EtOH altered gene expression associated with lipid and folate metabolism, as well as neutral lipids, in the E15.5 abnormally functioning heart and placenta. In comparison to controls, the high FA diet protected the embryo and placenta from these effects allowing normal development.

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**Key words:** alcohol; folic acid; homocysteine; heart development; placenta; lipid metabolism; MCAD; mouse embryo

## Introduction

Alcohol use during pregnancy is a significant public health problem world-wide. Nearly half a million women in the United States reported drinking alcohol during pregnancy (Bailey and Sokol, 2008) and nearly 20% admitted to binge drinking (Floyd and Sidhu, 2004). Recently, an increase in binge drinking has been reported among young women of child-bearing age. Recent assessment of fetal alcohol syndrome (FAS) within the population suggests that alcohol-related effects are higher than previously indicated: The Centers for Disease Control and Prevention (CDC) report that studies using in-person assessment of school-aged children in several U.S. communities report

estimates of FAS in 6 to 9 of 1000 children (CDC Data and Statistics: <http://www.cdc.gov/ncbddd/fasd/data.html>).

Alcohol use during pregnancy and its effects on neural and craniofacial development have been the primary focus of numerous campaigns to encourage women not to drink alcohol if she is planning on becoming pregnant or during pregnancy. The effects on cardiac development usually are not discussed. Although animal studies indicate that in utero alcohol exposure is a teratogen for cardiogenesis, the results from epidemiologic studies are mixed (Zhu et al., 2015). A large National Birth Defects Prevention Study estimated associations between congenital heart defects (CHDs) and case or control mother reports of periconceptional alcohol consumption (i.e., any consumption) with expected delivery dates during 1997 to 2007. The results of the study suggested that there are no statistically significant increased risks between measures of maternal alcohol consumption and most CHDs examined (Zhu et al., 2015).

However, it was acknowledged that these findings may reflect limitations with retrospective exposure assessment or with unmeasured confounders. It was concluded that additional studies with improvement in measurement of alcohol consumption were recommended. Earlier epidemiological studies indicated that 54% of live-born children with FAS, a subset of fetal alcohol spectrum of disorders (FASD), have some kind of a cardiac anomaly (Abel, 1990). The malformations include valvuloseptal defects, stenosis of the pulmonary artery, tetralogy of Fallot, including d-transposition of the great arteries (Grewal et al., 2008). It

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is noted that children with FAS are not typically exposed to only one dose of alcohol during gastrulation as done in our study, but potentially are exposed at multiple times during early pregnancy, or even throughout pregnancy. We chose to use a single, acute exposure to be more precise in regards to the timing of our exposure and its effects. Our acute early alcohol (ethanol, EtOH) exposure resulted in 86% of embryos displaying cardiac abnormalities in the mouse model (Serrano et al., 2007, 2010).

Based on alcohol exposure studies using the pregnant mouse model or chick embryos, the risk would be high for cardiac birth defects being induced by alcohol even with only one exposure occurring early in pregnancy during gastrulation (Serrano et al., 2007, 2010; Karunamuni et al., 2014). The published animal results extrapolated to human pregnancy indicate that exposures between 16 to 19 days after conception, that is, during gastrulation, is a sensitive period for the induction of heart defects. This timing of exposure during gastrulation is before a woman usually recognizes her pregnancy. Pregnancies are typically confirmed 5 to 6 weeks after conception, or as clinically defined, in the 7<sup>th</sup> or 8<sup>th</sup> week of human pregnancy. Based on the severity of cardiac defects that are induced in the mouse with a binge level of alcohol, the EtOH induced cardiac defects most likely would not be viable and would be lost during human pregnancy.

Thus, they would not be counted in analysis of defects observed in live human births. Of interest, a meta-analysis that assessed the association between maternal reproductive history and CHD risk, the study provided evidence that a history of abortion was associated with a 24% higher risk of CHD. When defined by abortion category, CHD risk increased by 18% and 58% with a history of spontaneous abortion and induced abortion, respectively (Feng et al., 2015).

Although studies have been carried out defining EtOH-induced cardiac defects in the mouse fetus, these analyses in comparison to those focusing on neural and neural crest-related anomalies are limited in number. The analyses usually have focused on exposure beginning on mouse embryonic day (E) 8.0 of gestation or later, that is, after a tubular heart structure already is present, and primarily report results by report phenotypic descriptions of altered pathology. In a review paper of EtOH teratogenicity in mice (Becker et al., 1996), due apparently to a paucity of cardiac data relating to early exposure on E7.0, there is no discussion of heart defects, but only of neural- and neural crest-associated, and craniofacial anomalies. A few studies focused on analysis of protein and DNA content of hearts of different strains of chick embryos at Hamburger and Hamilton stage 15 (Cavieres and Smith, 2000) and in the mouse after E8 after alcohol exposure (Becker et al., 1996; Green et al., 2007).

Alcohol exposure induces similar cardiac defects in vertebrate animal models as in the human depending

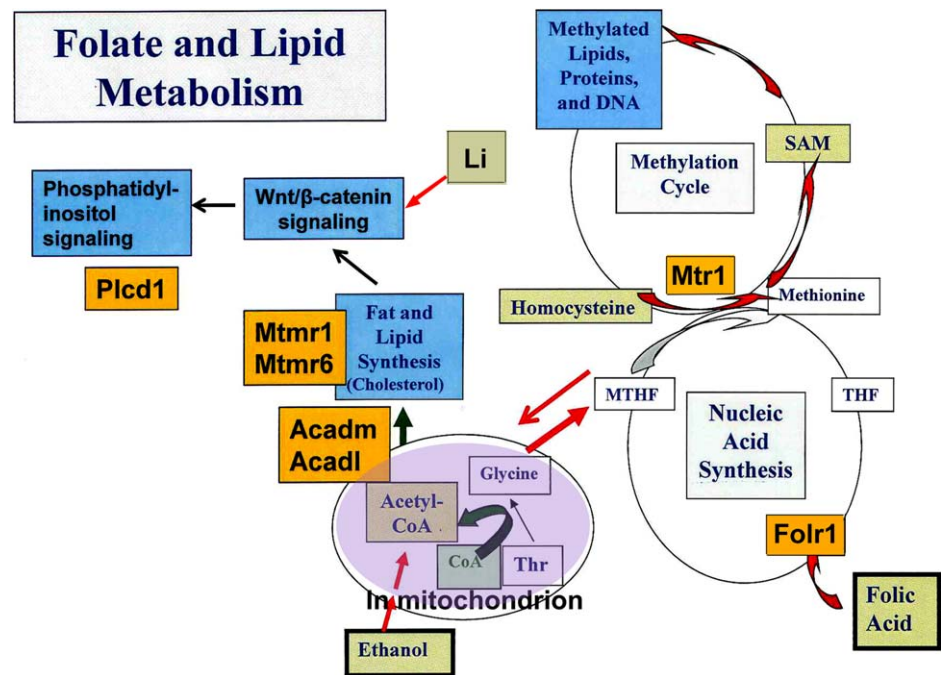
upon developmental timing of exposure and dose (Becker et al., 1996; Cavieres and Smith, 2000). Our published data using the mouse model defined that gastrulation is a critical, sensitive window for alcohol exposure inducing viable cardiac defects that are associated with abnormal development of the second heart field that forms the right ventricle, tricuspid valve, outflow tract, including semilunar valves (Serrano et al., 2010). A recent study also exposed chick embryos to alcohol at gastrulation and reported abnormal embryonic cardiac physiology (Karunamuni et al., 2014). We demonstrated that the early EtOH exposure leads to changes in inhibitory canonical Wnt signaling in the heart fields (Serrano et al., 2010).

Additionally, changes in normal placental development were noted in the mouse model with respect to alcohol exposure, resulting in higher placental resistance and intrauterine growth restriction (IUGR). EtOH exposure differentially misexpressed nonmuscle myosin (NMM)-IIA and-II B, proteins shown to be important for placenta and heart development (Tullio et al., 1997; Wang et al., 2010; Han et al., 2012). Poor placental development has been associated with the IUGR observed with FAS (Gundogan et al., 2008), as well as with the folate metabolism-related gene *Mthfr* in transgenic mouse studies (Pickell et al., 2009). A subsequent study from our lab using the human first-trimester trophoblast cell line (HTR-8/SVneo cells) demonstrated that EtOH adversely affected trophoblast cell migration (Han et al., 2012), but the adverse effects were prevented by folic acid (FA). Decreased trophoblast migration would perturb normal placental development. Thus, as based on our published results, as well as those by others, we focused on EtOH's effects on the heart and placenta in relation to lipid and folic acid metabolism.

Importantly, when we provided to the pregnant mouse the high dose FA dietary supplementation, that is, 10.5 mg/kg FA beginning with morning of conception before alcohol exposure on E6.75 and maintained the supplemented diet, normal heart and placental development was protected and function was normal. This is a higher dose than currently available in prenatal vitamins for women (Serrano et al., 2010; Huhta and Linask, 2015), but a dose used in an epidemiological study that also showed a protective effect on the cardiovascular system in human pregnancy (Czeizel et al., 1999).

There are several hypotheses for how alcohol disrupts development, and many mechanisms have been implicated, including effects on lipid metabolism, zinc, lipid rafts, L1 cell adhesion molecule, alcohol dehydrogenase, and catalase (Lindi et al., 2001; Goodlett et al., 2005). We previously provided evidence that lithium (Li<sup>+</sup>), homocysteine (HCy), and alcohol exposure all induce similar cardiac and placental defects that can be prevented by FA supplementation (Serrano et al., 2007, 2010; Han et al., 2009), and because we recently demonstrated that Li<sup>+</sup> and HCy exposure altered lipid metabolism in abnormally developing

**FIGURE 1.** Schematic diagram of intersection of folate and lipid metabolism. Genes analyzed in our study are shown in orange boxes. Folate uptake by means of *Folr1* facilitates folate-mediated, one-carbon metabolism important in methylation reactions leading to methionine synthesis and S-adenosylated methionine (SAM) and in nucleic acid synthesis. In the embryo, alcohol (EtOH) is slowly metabolized to acetaldehyde and eventually to acetyl CoA formation in mitochondria serving as a metabolite in fat and lipid synthesis.



hearts and placentas (Han et al., 2016), our present hypothesis is that embryonic alcohol exposure alters lipid metabolism and lipid related gene expression, as well as genes associated with the FA cycle (Fig. 1). Genes analyzed included *folate receptor 1 (Folr1)*; *phospholipase C, delta 1*, which is a second messenger within the Wnt pathway; *5-methyltetrahydrofolate-homocysteine methyltransferase (Mtr1)*, which codes for the enzyme methionine synthase enzyme converting Hcy to methionine in the FA cycle; *medium-chain acyl CoA dehydrogenase (Acadm)* and *long-chain acyl CoA dehydrogenase (Acadl)*, which are both involved in mitochondrial  $\beta$ -oxidation of fatty acids to provide energy for cellular reactions.

The protein product of *Acadm* (medium chain acyl dehydrogenase, MCAD) is essential for complete fatty acid oxidation. MCAD deficiency is the most common inherited disorder of mitochondrial fatty acid  $\beta$ -oxidation in humans and is associated with sudden unexpected deaths in infancy (Lovera et al., 2012). Mouse models of disorders of mitochondrial fatty acid  $\beta$ -oxidation show clinical signs that include Reye-like syndrome and cardiomyopathy, and many are intolerant to cold (Schuler and Wood, 2002). Human myotubularin and several other members of the large family of homologous proteins called the myotubularin-related proteins (Mtmrs) are potent lipid phosphatases showing specificity for phosphatidylinositol 3-phosphate (Tronchere et al., 2003). Changes in the specific phosphatase levels can dramatically change the cellular response to extracellular stimulatory factors and alter the spatio-temporal localization of proteins that regulate cellular responses to its environment. We thus also ana-

lyzed *Mtmr-1* and *Mtmr-6* changes in the early embryo and embryonic heart on E15.5. We here demonstrate that alcohol exposure perturbs both lipid and FA metabolism in the heart and placentas that had developed abnormally and the adverse effects were prevented by dietary folate supplementation.

## Materials and Methods

### MOUSE MODEL, HUSBANDRY, AND ALCOHOL EXPOSURE

The C57Bl/6 mouse strain (Jackson Laboratories) was used. All protocols pertaining to handling of mice were approved by the Institutional Animal Care and Use Committee of the USF Morsani College of Medicine.

### HUSBANDRY AND BREEDING

The adult mice were housed at an ambient temperature of 22°C with a 12-hr light/dark cycle and access to food and water ad libitum. For timed matings, mature male and female mice were housed overnight and the presence of a vaginal plug the following morning was taken as evidence of mating and designated as embryonic day 0.5 (E0.5).

### ALCOHOL AND HCY EXPOSURES

As in our previous studies, treated pregnant mice on E6.75 were randomly allocated to receive intraperitoneally (i.p.) an injection of either a single dose of 100  $\mu$ l of 75  $\mu$ M Hcy (Han et al., 2009) or the pregnant mice were administered a binge drinking level of EtOH (Serrano et al., 2010) (i.e., two intraperitoneal, i.p., injections of 306  $\mu$ l of 2.9 g EtOH/kg maternal weight administered at 3 PM and at 6 PM, defined as E6.75). Care was taken that the injections were not made deep into the abdomen. Control

**TABLE 1.** Embryonic Parameters of EtOH Exposure and Folate Protection

(Modified from Serrano and others, 2010)

PARAMETERS		Saline Control	EtOH	EtOH/FA
Litter (Embryos), n		8 (42)	5 (30)	5 (23)
Morphologic	CRL, mm	15.20	12.60	14.43
	BW, g	0.40	0.37	0.44
	PW, g	0.13	0.10	0.12
Valvular Function	SLV	0%	77.00%	0%
	AVVR	0%	7.00%	0%
Cardiac Function	MPI	0.46	0.62	0.53
	*RR, ms	345.00	293.00	303
	*E/A ratio	0.32	0.29	0.31
	*OF, cm/sec	36.07	26.37	23.71
Arterial Doppler	*UAPI	1.32	1.75	1.89
	*DAPI	1.46	1.75	1.84
Venous Doppler	*DVPI	0.85	1.21	1.22
	Umbilical Pulsations	None	None	None

Median of the variables in each of the groups is shown.

FA, folic acid, 10 mg/kg; CRL, crown-rump length; BW, body weight; PW, placental weight; SLV, semilunar valve regurgitation; AVVR, atrioventricular valve regurgitation; MPI, myocardial performance index; RR, cardiac cycle length; OF, ventricular outflow tract; E/A ratio, inflow velocity during early ventricular filling/inflow velocity during atrial contraction; UAPI, umbilical artery performance index; DAPI, descending aorta pulsatility index; DVPI, ductus venosus pulsatility index; Cardiac-placental function parameters show values of ethanol exposure in comparison with untreated control group, as based on Kruskal Wallis test.

Significant difference (\*) is based on probability values of <0.05

pregnant mice received either one i.p. injection for HCy related experiments or two injections of physiological saline with same timing of exposure used for the EtOH experiments. On E15.5, the circulations of the pregnant mice and embryonic hearts were examined in utero using Doppler ultrasonography (Gui et al., 1996; Linask and Huhta, 2000).

The Doppler ultrasound parameters of EtOH exposure or of HCy in comparison to control embryos have been published and discussed in detail (Han et al., 2009; Serrano et al., 2010). In the present study, ultrasonography was used as a tool to define the E15.5 embryos in which cardiac and placental abnormalities were developing. The characteristics of the cardiac and placental ultrasound parameters were as previously published. The two i.p. saline exposure doses administered to control mice on either maintenance or high FA diets on E6.75 did not adversely affect the pregnant mice, and embryonic development was normal. Table 1 shows percentages of normal

and abnormal embryos that are obtained, a summary of the morphometric data, and parameters of heart function from our previously published work.

#### FOLATE MOUSE DIETS

The special animal chow supplemented with 10.5 mg/kg was specially ordered from Harlan Laboratories. This dose was chosen and based on human population trials for protection of craniofacial anomalies (Czeizel et al., 1999). Control mice received the baseline diet 3.3 mg FA/kg, as defined by Harlan. The 3.3 mg FA/kg dose maintains health of the pregnant dam, but did not prevent cardiac defects. The calculations for FA level in the FA-supplemented diets were based on the metabolic body weight (BW) of mice because of the BW difference between humans and mice. (Heusner, 1987). For mice it is calculated as  $BW^{0.75}$  (Harlan Laboratories, Madison, WI). The high FA level represents a supplement of 460 ng/g BW considering metabolic body size (Harlan provided).

Dams were randomly assigned to the experimental group with diet supplemented with the high 10.5 mg/kg or to a control group that received the health maintenance chow of FA content (3.3 mg/kg). On E0.5, pregnant mice assigned to the experimental groups were placed on the FA supplemented diets; on E6.75 experimental pregnant females received EtOH, or where indicated, HCy, or the control dams, physiological saline. Thus, one group of control pregnant mice received the high folate diet, with no alcohol or HCy injection to determine effects of high folate alone. We did not measure actual folate levels reached in the embryos with folate supplementation.

#### DOPPLER ULTRASONOGRAPHY

On E15.5, Doppler ultrasonographic (echo) examinations were performed with a 40 MHz transducer, as previously described (Gui et al., 1996; Serrano et al., 2010) using the Vevo 770 or Vevo 2100 instrumentation (VisualSonics, Toronto, Canada).

#### REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ANALYSIS

Any alterations in gene expression were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using either E7.5 whole embryonic tissue or in micro-dissected heart tissues of embryos at E15.5. Data were normalized in each case to the internal control  $\beta$ -actin. We analyzed individually at least four to five whole embryos on E7.5 or four to five hearts micro-dissected from E15.5 embryos within the specified exposure groups. The analyzed embryos were from different litters. The E15.5 EtOH- or HCy-exposed hearts displayed the same types of cardiac defects observed in our earlier studies, specifically semilunar valve regurgitation and abnormal myocardial function and umbilical artery blood flow.

The gels that are shown for each gene are representative of the patterns in expression that were obtained. We did not carry out relative quantitation using densitometric

readings from scans of the gels to show average densitometric readings and standard deviations, because it is to be noted that not all embryos within a litter in utero are at the same time-point of development at time of exposure and embryos analyzed were from different litters. Thus, intra- and inter-litter variability was noted, but the patterns of gene expression changes, that is, an increase or decrease with the experimental manipulations, were similar.

Changes in gene expression data in the embryonic heart were analyzed for genes associated with the folate pathway (*Folr1* and *Mtr1*); with phosphatidylinositol signaling (*phospholipase C delta 1*), with lipid metabolism as lipid-phosphatases (*Mttr-1* and *Mttr-6*) and with fatty acid synthesis (*Acadm*, *Acadl*).

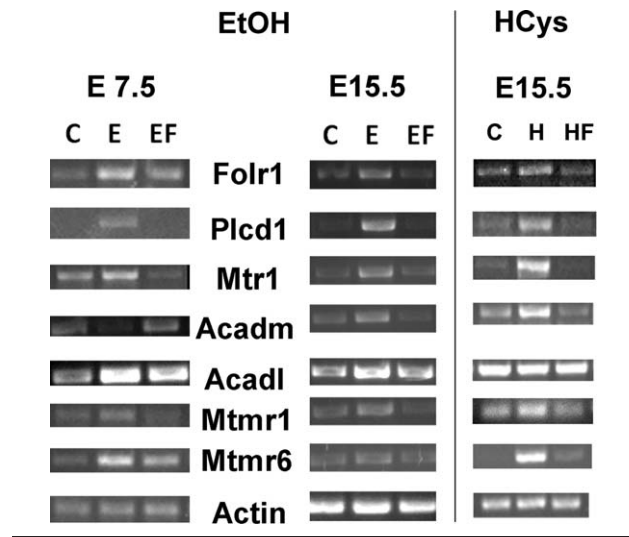
#### IMMUNOHISTOCHEMISTRY

We analyzed the expression of MCAD, which is the protein product of the *Acadm* gene, in the heart as a means of defining changes in lipid metabolism. Three embryonic E15.5 hearts were analyzed for each experimental condition. The *Acadm* gene relates to fatty acid oxidation, and MCAD protein deficiency is the most common inherited disorder of mitochondrial fatty acid  $\beta$ -oxidation in humans and is associated with cardiomyopathy. The immunohistochemistry was carried out on paraffin sections according to standard methodology previously described (Han et al., 2009). MCAD antibodies were obtained from Sigma (St. Louis, MO). Duplicate sections that were to be compared for each condition were placed on the same slide and were processed at the same time.

#### OIL RED O STAINING FOR LIPID DROPLET DISTRIBUTION

Three to five embryonic hearts and placentas of experimental and control embryos were microdissected, frozen, cryosectioned, and prepared for Oil Red O (ORO) staining. ORO is a fat-soluble dye that detects neutral lipids (triglycerides, diacylglycerols, and cholesterol esters) in cryosectioned E15.5 tissues. This method estimates tissue lipid content and displays localization (Mehlem et al., 2013). Lipid droplets stain red. Polar lipids (phospholipids, sphingolipids, and ceramides) are not stained (Fowler and Greenspan, 1985).

Analysis of lipid droplet organization within tissues was done in a blinded manner by coding of the tissue sections of the different treatments and was carried out independently by the investigators and compared. We did not quantify numbers of lipid droplets in the tissues, because not all embryos within a litter in utero are at the same time-point of development at time of exposure and embryos analyzed were from different litters. We show representative sections of the typical pattern of ORO localization of the heart and placenta within the various experimental and control groups. Histochemistry and microscopy was carried out as previously reported (Han et al., 2009).



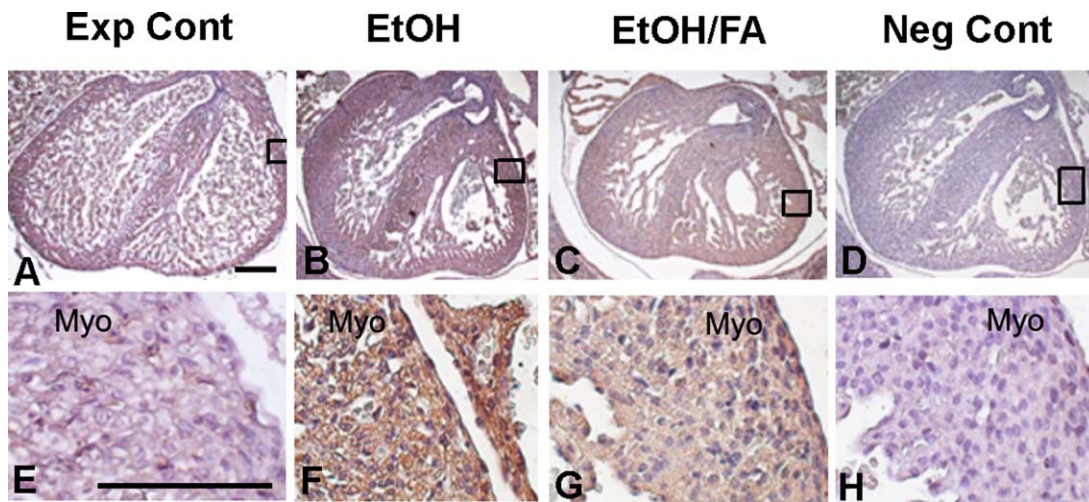
**FIGURE 2.** RT-PCR analysis of gene expression in the embryo at E7.5 after EtOH exposure on E6.75 and in the heart over a week later on E15.5. RT-PCR gel results on E15.5 in the alcohol-exposed heart are compared with HCys exposure. Folate- and lipid-related genes as described in text were analyzed. Note similarity of effects on gene expression with EtOH and folate deficiency induced by elevation of HCys. C, control; E, EtOH exposure; EF, EtOH with FA supplementation.

## Results

### ONE-TIME ALCOHOL EXPOSURE ON E6.75 WITH FOLATE (FA) SUPPLEMENTATION INITIATED ON E0.5

Comparison of alcohol effects and those of FA nutritional deficiency. Using RT-PCR analysis (Fig. 2), the expression changes of previously specified genes in Methods section associated with lipid and FA metabolism were analyzed with respect to alcohol exposure in comparison with those induced by exposure to HCys that is considered a hallmark of FA nutritional deficiency. As seen in Figure 2, in the E7.5 embryo, gene expression of the genes analyzed was altered already a half day after exposure.

A half day after EtOH exposure in E7.5 whole embryos, most of the above-listed genes are upregulated in the embryo or cardiac tissue (Fig. 2). *Acadm* was initially down-regulated, suggesting also a decrease in MCAD protein after exposure. By E15.5, it showed an up-regulation in comparison to control hearts, possibly by a biochemical feedback mechanism to compensate for the initial decrease in *Acadm* gene expression. This over-expression that resulted over a week later after alcohol exposure may be deleterious on its own or was not sufficient to overcome the early adverse effects: More than a week after the acute E6.75 exposure, on E15.5 the EtOH-exposed hearts displayed abnormal function and genes continued to show abnormal expression as compared to expression in the control (C) embryonic hearts. Folate dietary supplementation (F) at 10.5 mg/kg was provided to a cohort of pregnant mice on morning of conception before the EtOH



**FIGURE 3.** Cardiac immunolocalization of medium length chain acyl CoA dehydrogenase (MCAD) protein product of *Acadm*. Top row shows lower magnification of the E15.5 heart with bottom row displaying higher magnification of the myocardium. **A,E:** Low protein expression in the control myocardium. After EtOH exposure during gastrulation (**B**), a high level of MCAD is now present in the myocardium (**F**). Folate supplementation morning of conception (**C,G**) decreases MCAD levels in the myocardium, but not to control levels (compare **E,G**). **D,H:** Negative control tissue is shown. Scale bars = 500  $\mu\text{m}$  in **A**; 100  $\mu\text{m}$  in **E**.

exposure (EF) and the supplementation was observed to maintain more control levels of gene expression. The data, in summary, indicated that EtOH exposure partially manifested its effects by creating folate deficiency in embryonic tissues. This is suggested because, when one compares elevation of HCy, indicative of folate deficiency, it modulated these same genes analyzed in a similar manner in the heart as with EtOH exposure. Folate supplementation protected normal development and gene expression. We note that unlike *Acadm*, *Acadl* expression was not changed except in the E7.5 embryo after alcohol exposure. By E15.5, it was at relatively normal levels.

*Effects of alcohol on medium-chain acyl-CoA dehydrogenase (Acadm gene; MCAD protein) in the heart.* MCAD deficiency is the most common inherited disorder of mitochondrial fatty acid  $\beta$ -oxidation in humans. An MCAD-deficient transgenic mouse model (*Acadm*<sup>-/-</sup>) displays sporadic cardiac lesions, organic aciduria and fatty liver, and profound cold intolerance at 4°C with prior fasting (Tolwani et al., 2005).

In our acute EtOH exposure mouse model, with and without dietary FA supplementation, MCAD protein expression on E15.5 localized to the heart (Fig. 3). Within the control E15.5 fetal heart, MCAD protein is present at low levels (Figs. 3A,E). The acute EtOH exposure up-regulated MCAD protein expression to the relatively high levels observed in the heart on E15.5 (Figs. 3B,F). Early dietary FA supplementation decreased the expression closer to control levels, but not completely (Figs. 3C,G). In summary, one embryonic exposure to EtOH early in gestation within a half day after exposure downregulated *Acadm* gene expression. By mid-gestation, however, possibly to compensate for the apparent nutritional deficient

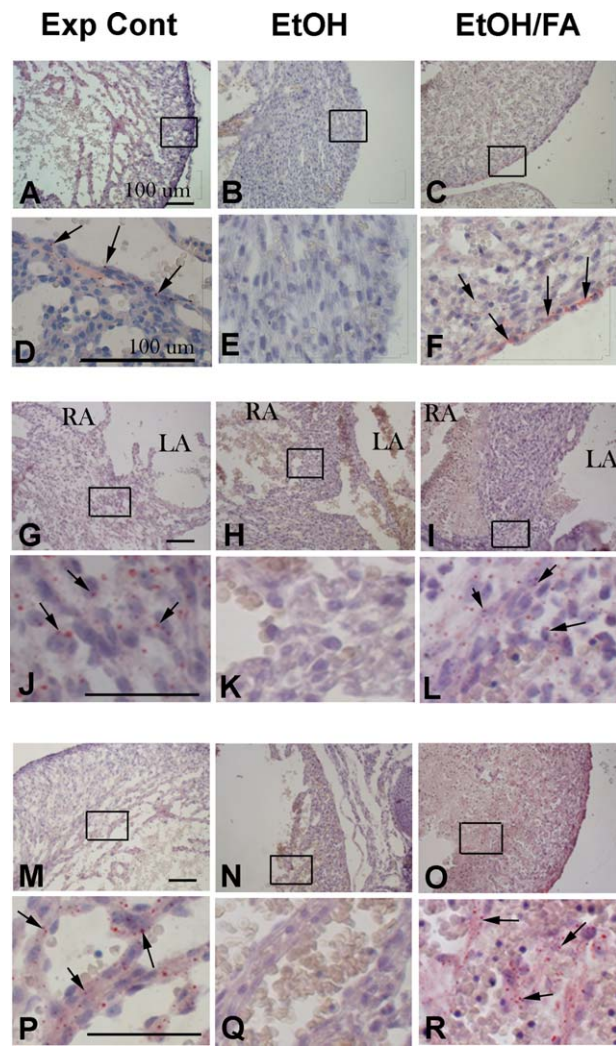
state that was induced earlier, there is significantly higher MCAD protein level expressed in the EtOH-exposed heart in comparison to the control, normal heart. FA supplementation helped to prevent the alcohol's effects, even though it was not able to completely bring the protein expression to control levels. Normal heart function, however, was protected with FA supplementation.

*Effects of alcohol exposure on neutral lipids by Oil Red O (ORO) localization.*

Next we analyzed lipid localization in the heart and placenta after EtOH exposure using a protocol to detect neutral lipids based on ORO staining (Mehlem et al., 2013). Visualization of the morphology and intracellular localization of lipid droplets (LDs) within tissues has become important for understanding lipid biology in disease states.

*ORO localization in the heart. Myocardium* (Figs. 4A–F). In the control and folate-protected hearts, the myocardium displayed a low level of ORO-stained LD localization. A noticeably higher level of ORO-LDs was seen in the sub-epicardial region in the control and high folate-protected hearts with alcohol exposure, especially noticeable in Figure 4F (arrows). Alcohol-exposed hearts were devoid of lipid droplets in all regions (Figs. 4B,E,H,K,N and Q).

*Interventricular septum* (Figs. 4G–L). The superior part of the interventricular septum usually shows a high level of ORO localization (see Fig. 4J). These ORO-localized LDs were absent after the acute, early alcohol exposure (Fig. 4K). The ORO localization in this region was protected with high FA supplementation even with alcohol exposure (Fig. 4L)



**FIGURE 4.** ORO localization of neutral lipid droplets in three regions of the E15.5 mouse heart depicted at two magnifications. Boxed-in regions show area of higher magnification images: **A–F:** The myocardium. **G–L:** Intraventricular septum, superior region. **M–R:** The trabeculae. Arrows point to typical localization of some ORO-stained droplets. Left-hand column shows localization in the experimental control heart (Exp Cont); middle column after acute EtOH exposure during gastrulation; right-hand column depicts the high folate-supplemented hearts with EtOH exposure (EtOH/FA). Scale bars = 100  $\mu\text{m}$  in A, D for top and bottom rows of each region of the heart, respectively. RA, right atrial region; LA, left atrial region.

**Trabeculae** (Figs. 4M–R). Of the regions of the heart, in addition to the superior portion of the interventricular septum, the trabeculae consistently displayed a relatively high deposition of neutral lipids in the control hearts (Fig. 4P). With only maintenance level of FA, EtOH exposure prevented neutral lipid synthesis (Fig. 4Q). Control levels of neutral LDs again were protected by high dose FA supplementation, when provided on morning of conception before the acute EtOH exposure on E6.75 (Fig. 4R).

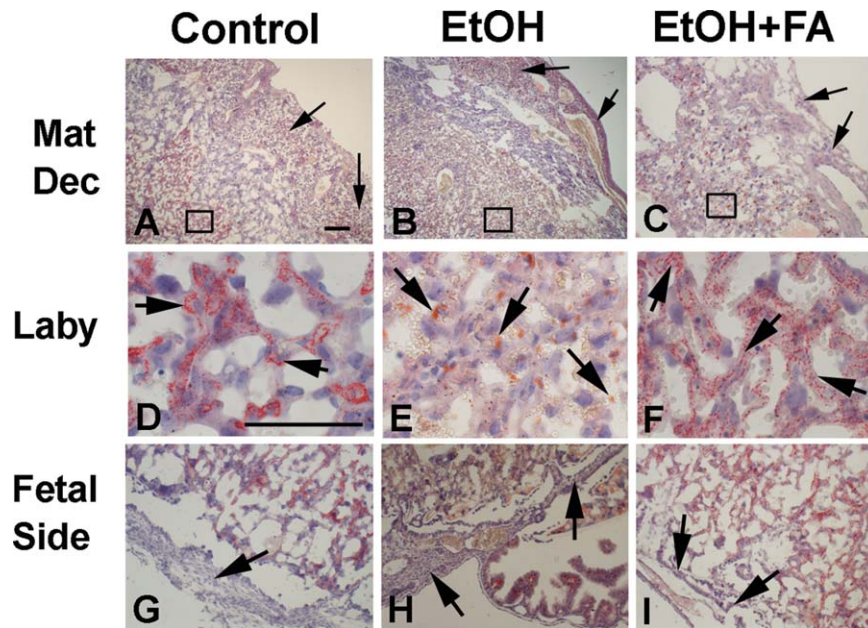
**ORO localization in the E15.5 placenta.** A high number of neutral LDs is present in the control placenta (left column), in the maternal decidua (Mat Dec; Fig. 5A) and predominantly in the labyrinth layer (Laby; Fig. 5D). Neutral lipids were not evident on the fetal side of the placenta (Fig. 5G). In the control labyrinth, the villi display a normal organization and a high level of ORO-LDs uniformly localized within the villi (Fig. 5D). With control maintenance level of FA, after the acute EtOH exposure, the labyrinth shows a disorganization of the villi (Figs. 5B,E), and the ORO localization is present only as sparse, sequestered punctata in the cells (arrows). High-dose folate supplementation (EtOH+FA, third column) protected normal labyrinth development that also displayed now normal villi formation, normal ORO-LD organization, and normal umbilical blood flow.

## Discussion

### ALCOHOL EXPOSURE AND PREGNANCY

FASD is a worldwide problem that unfortunately has lifelong consequences for the child diagnosed with this disorder. FAS, a subset of FASD, includes abnormal neural development, cardiac birth defects, and craniofacial anomalies and results in intrauterine growth retardation. Maternal drinking of alcohol is a direct cause of FAS. Women drink during pregnancy for many reasons, including having little information about the risks of drinking while pregnant and not knowing that early alcohol ingestion, even a one-time exposure as early as gastrulation in the second week of pregnancy (Serrano et al., 2007, 2010), can induce cardiac and neural birth defects. This is much earlier before pregnancy is usually realized at 5 to 6 weeks after conception (Jonsson et al., 2014). The first 3 weeks of pregnancy thus can be considered a high-risk period for induction of both heart and neural birth defects.

An explanation for why more babies do not exhibit cardiac defects postnatally as a result of maternal alcohol consumption may reflect that the cardiac defects are often severe and the affected embryos do not develop to term and if they do, cardiac defects may not be looked for. This should not mean that we can discount alcohol effects on cardiogenesis, as not being relevant to public health. Viable defects can and do occur. The severity of the defect is dependent on dose of alcohol and the timing of exposure during cardiac and placental development during early pregnancy. The earlier the exposure occurs and the higher the level of alcohol, it would be expected to have more serious consequences on the embryo with the possibility that those embryos would not develop to term and be miscarried. Heart and placental function must be relatively normal to have embryonic viability and growth. We also showed that brain development and neural crest cells could be affected by the acute exposure (Manisastry et al.,



**FIGURE 5.** ORO localization of neutral lipid droplets in three regions of the placenta. **A–C:** Maternal decidua (Mat Dec) layer is shown at low magnification in top row under conditions of treatments. Control placenta shows ORO localization throughout the decidua. EtOH exposure enhances ORO localization (B), while folate (FA) supplementation decreases localization, but decidua is thinner and does not return to control tissue organization. **D–F:** The placental labyrinth (Laby) is highly affected by EtOH exposure. Control labyrinth shows a significant amount of lipid droplets within highly organized villi (D); EtOH reduces the ORO to punctata that remain noticeable within a disorganized labyrinth; folate supplementation (EtOH + FA) returns ORO droplet formation to normal levels as well as organization. **G–I:** The fetal side of the placenta does not show ORO-stained droplet localization in the control placenta or with experimental treatments. Bars for low and high magnifications = 100  $\mu$ m.

2006; Chen et al., 2008). Other recent publications with a focus on alcohol and heart development using the avian (Cavieres and Smith, 2000; Karunamuni et al., 2014) and zebrafish (Sarmah and Marrs, 2013) vertebrate models also showed adverse effects of alcohol on early cardiogenesis.

Our previous experimental studies addressing effects of alcohol on heart development using the mouse model demonstrated that acute alcohol exposure during gastrulation affects an important signaling pathway active in cardiomyocyte specification and differentiation and in implantation and placental development, namely the canonical Wnt/ $\beta$ -catenin pathway (Serrano et al., 2010; Han et al., 2012). The results of our present study demonstrating alcohol altering lipids can also impact Wnt signaling. Evidence shows that lipid modifications are necessary for active Wnt signaling (Takada et al., 2006; Steinhauer and Treisman, 2009) by means of two fatty acid modifications (Vrablik and Watts, 2012) implicated in Wnt secretion.

Additionally, it was shown, as for an example, that after lipid modifications had taken place, Drosophila DWnt-1 partitions as a membrane-anchored protein and is sorted into lipid raft detergent-insoluble microdomains of the plasma membrane (Zhai et al., 2004). Of interest, an eleva-

tion of Hcy was reported to significantly increase glomerular endothelial cell permeability by stimulating lipid raft clustering to form redox signaling platforms (Yi et al., 2009). In summary, lipids are critical in fetal development, not only as components of membrane rafts and phospholipids, but also as ligands for receptors and transcription factors in gene regulation and in direct interactions with proteins such as Wnts. When normal lipid synthesis is changed, the potential exists for critical signaling to be altered in the embryo leading to defects.

The heart is a beating, tubular structure by 21 days of human gestation and both the heart and neural tube are undergoing dramatic morphogenesis in the first month. Our animal experimental results extrapolated to human pregnancy demonstrated that acute alcohol exposure at stages equivalent to gestational days 16 to 19 days post-conception induce cardiac defects at a high percentage (Chen et al., 2008; Han et al., 2009). Placing these results in the context that 49% of pregnancies are unplanned (Finer and Zolna, 2011), the first month of pregnancy is a high risk period for induction of cardiac birth defects because not knowing she is pregnant, no precautionary methods may be yet taken by the woman to protect embryonic development. A woman may continue to drink



alcohol or have binge drinking episodes, unknowingly harming embryonic development.

During this same time period, trophoblasts are differentiating to eventually lead to the development of the placenta. Our embryonic exposure during gastrulation thus targets heart development and placental development. The placenta is composed of trophoblast cells originating from the blastocyst trophoblast. Between E4.5 and E7.5, the different trophoblast lineages are differentiating (Rossant and Cross, 2001). The extra-embryonic ectoderm is expanding and forms the chorionic epithelium. Posteriorly, the allantois forms from the embryonic mesoderm and contacts the chorion at ~E8.5. Feto-placental blood vessels grow in from the allantois to form the fetal components of the vascular network of the placental labyrinth. The labyrinth is where nutrient and gas exchange occurs between the maternal and fetal circulations.

Several studies of blastocyst stages indicate that the trophoblast cells overlying the inner cell mass (ICM) and the early extra-embryonic ectoderm act as a pool of stem cells for the trophoblast lineages and respond to ICM signals including to Wnt signaling for their differentiation and proliferation (Sonderregger et al., 2010a, 2010b). The acute exposure experiments targeting E6.75 of gestation thus has the potential to perturb trophoblast differentiation (Monkley et al., 1996; Peng et al., 2008; Fitzgerald et al., 2010). We earlier reported that exposure of incubated human HTR-8/SVneo extravillous trophoblasts to  $\text{Li}^+$ , Hcy, or alcohol (EtOH) altered gene and protein expression and decreased trophoblast cell migration (Han et al., 2012).

Altered migration would affect trophoblast invasion and blood vessel remodeling. The impaired placental development can result in poor gas and nutrient exchange and the observed IUGR. The above studies would suggest the effects of alcohol on the heart-placental axis and changes in umbilical blood flow and placental lipids are associated in the induction of heart birth defects associated with FAS. The importance of the heart-placenta axis and changes in blood flow in cardiovascular development was recently reviewed (Linask, 2013; Linask et al., 2014).

Cardiac anomalies that are observed within FAS have serious consequences for the child and for the family. Often this may involve serious surgeries or a series of surgeries as the child gets older. Neural defects can arise during the entire 9 months of gestation; thus, neural defects are usually the only defects emphasized in the literature provided to the expectant mother. On the basis of our animal studies, development of the heart and brain should be equally treated to educate our population about the consequences of alcohol drinking during pregnancy. Based on data from animal models and epidemiological studies, high dose folate supplementation taken periconceptionally can prevent the adverse effects of alcohol on cardiac development.

This we discussed in a recent review (Huhta and Linask, 2015). The optimal safe dose for human pregnancy

needs to be clinically defined. The association of alcohol during embryonic exposure with elevated Hcy levels and FA prevention (Han et al., 2009; Serrano et al., 2010) has been confirmed since then by multiple studies in the alcohol literature (Shirpoor et al., 2013; Kharbanda et al., 2014; Kruman and Fowler, 2014; Prior et al., 2014). It appears that folate deficiency is associated with the observed dyslipidemia in the fetus and placenta from a binge level of alcohol exposure.

#### RELATIONSHIP OF FOLATE, CHOLINE, AND BETAINE

The present results indicate that EtOH metabolism interacts with FA/Hcy and one-carbon metabolism (Fig. 1). A similar relationship between Hcy-methionine metabolism and effects of EtOH had been reported for steatosis in rat hepatocytes (Kharbanda et al., 2005; Sim et al., 2015). One-carbon metabolism is essential for donating methyl groups for methylation reactions to form S-adenosylmethionine, a primary methyl donor, particularly for DNA methylation that is critical for epigenetic regulation of gene expression.

Another methyl donor in addition to the B vitamins folate and choline is betaine. All three participate in Hcy metabolism and exist in a delicate balance (Ueland et al., 2005). When folate status or intake is low, an elevation of Hcy occurs. In most tissues, remethylation of Hcy to form methionine is catalyzed usually by methionine synthase with cobalamin as a cofactor and 5-methyltetrahydrofolate as the methyl donor. With low folate, Hcy remethylation can be carried out by betaine Hcy methyltransferase. Dimethylglycine is the other product of this reaction.

Choline oxidation leads to the formation of betaine. Choline is also an essential nutrient that functions in cell structure and signaling, lipid transport and neurotransmission. Choline is either supplied by the diet or is synthesized *de novo* from phosphatidylethanolamine. Choline cannot, however, be synthesized at necessary amounts, if both choline and folate levels are low. It is reported that the amount of folate one ingests, dictates how much choline one needs (Jacob et al., 1999). Similarly, it is indicated that folate status affects plasma betaine and dimethylglycine concentrations and the association between betaine and total Hcy during pregnancy (Chiuve et al., 2007; Fernandez-Roig et al., 2013).

The results taken together, indicate optimal folate concentrations will be the overriding factor in determining concentrations of methyl donors available for methylation reactions in the embryo. Any of the methyl donors most likely will be helpful to prevent birth defects, as long as plasma folate concentrations are at a certain level. Alcohol can induce folate deficiency, resulting in elevated Hcy levels, and thus upsets the delicate nutrient balance.

#### ALCOHOL, FOLATE/HCY CYCLE, AND LIPID METABOLISM

Historically a relationship between alcohol exposure and changes in lipid metabolism was accepted (Druse, 1981;

Bearer et al., 1992). Recently, there has been increased interest in meconium lipids with numerous studies showing that meconium fatty acid ethyl esters can be used as biomarkers of gestational EtOH exposure (Zelner et al., 2013). Chronic alcohol consumption is associated with fatty liver disease in adult mammals. Lipid analysis after alcohol feeding in adult mice has established that decreased mitochondrial fatty acid oxidation, coupled with decreased expression of the respective genes, is one of the contributing factors in alcoholic fatty liver (Clugston et al., 2011).

In a study comparing adult C57BL/6J and A/J mice, folate perturbation led to strain-specific differences primarily in the expression profile of the cholesterol biosynthesis pathway and to changes in levels of serum and liver total cholesterol (Kitami et al., 2008). By genetically increasing serum and liver total cholesterol levels in APOE-deficient mice on the C57BL/6J inbred background, the authors significantly improved folate retention during folate depletion, suggesting that homeostasis among the Hcy, folate, and cholesterol and lipid metabolic pathways contributes to the beneficial effects of dietary folate supplementation (Kitami et al., 2008). The dysregulation of genes for fetoplacental lipid metabolism as we observed for alcohol exposure is similarly reported in pregnancy and type 1 diabetes mellitus (Radaelli et al., 2009).

With maternal diabetes or alcohol use, offspring display a higher incidence of CHDs than present in the normal population, as do offspring in pregnancies associated with maternal obesity. In our recent study on acute Li<sup>+</sup> and Hcy exposure resulting in abnormal cardiac and placental physiology, we noted that lipid metabolism was altered in those developing organs and normal physiology and lipid metabolism were protected with FA supplementation (Han et al., 2016). We propose that fetoplacental dyslipidemia may be a common factor in the development of CHDs, including with alcohol exposure that can be prevented with periconceptual folate supplementation.

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