PATHOGENESIS AND MECHANISMS OF ETHANOL-INDUCED LIMB DEFECTS

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ABSTRACT

Corey S. Johnson: Pathogenesis and mechanisms of ethanol-induced limb defects
(Under the direction of Kathleen K. Sulik, Ph.D.)

Fetal alcohol spectrum disorders encompass a variety of structural and behavioral abnormalities attributable to maternal alcohol consumption. This study was designed to investigate the mechanistic and pathogenic role of retinoic acid (RA) in the genesis of ethanol-induced forelimb defects. The mouse limb bud has proven to be an ideal model with which to study effects of ethanol because its development is more thoroughly understood than other regions or organ systems. The hypothesis that RA-deficient mouse embryos/fetuses manifest similar limb defects and distal limb cell death patterns to those of ethanol treated dams was tested. A RA receptor (RAR) antagonist and an aldehyde dehydrogenase (ALDH) inhibitor produced limb malformations consistent with those following ethanol exposure. Similarly, cell death was observed in the same region of the limb following exposure to each chemical. Secondly, the hypothesis that exogenous RA can prevent ethanol-induced cell death in the limb was tested. A subteratogenic dose of RA was co-administered with ethanol. Limb buds exposed to ethanol and RA exhibited low levels of distal limb cell death, comparable to control limbs, demonstrating that RA acts antagonistically to ethanol in the limb and suggesting that ethanol interferes with RA-mediated development. Also, in support of this premise, the results of *in situ* hybridization analysis reveal that ethanol represses RA-dependant gene expression in the limb at 8 and 18 hours, post treatment. Importantly, however, microarray examination of limb buds within 2-6 hours of ethanol exposure revealed no correlation between the transcriptional changes induced by ethanol and the RAR antagonist. The lack of early RA-mediated gene changes following ethanol exposure indicates that perturbation of RA-dependant developmental pathways is NOT a proximate teratogenic effect of ethanol. Significant changes in cellular functions

and pathways were evident shortly after ethanol exposure, suggesting several possible mechanisms of ethanol teratogenesis that merit future investigation.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AER	Apical ectodermal ridge
ALDH	Aldehyde dehydrogenase
AP	Anterio-posterior
IGF	Insulin-like growth factor
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorders
GD	Gestational day
GO	Gene Ontology
IPA	Ingenuity Pathway Analysis
IPKB	Ingenuity Pathway Knowledge Base
PG	Prostaglandins
LPM	Lateral plate mesoderm
LTR	LysoTracker Red
NBS	Nile Blue Sulfate
qPCR	Quantitative, *real-time* PCR
PD	Proximo-distal
RA	Retinoic acid (all trans)
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROS	Reactive oxygen species
SRM	Subridge mesenchyme
ZPA	Zone of polarizing activity

CHAPTER I
Background

Section 1.1 A brief history of Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders

The first written record cautioning against maternal alcohol consumption comes from the Bible (Judges 13:7), wherein the pregnant mother of Samson was commanded to abstain from "wine and strong drink." Aristotle, perhaps the first embryologist, suggested a connection between maternal alcohol consumption and the "morose and languid" nature of their children in the 4th century BC (reviewed by Warner and Rosett 1975). During the so-called gin epidemic of the mid-1700s in England, when the consumption of gin rose to approximately 1 gallon per capita per year, concerns rose over the possible detriment of alcohol to the developing fetus (Mitchell and Deane, 1962; Abel, 2001). Not until the late 1800s would the anecdotal observations of physicians be subject to scientific inquiry.

In 1848, Samuel Howe presented the first epidemiological evidence that ethanol consumption may be a detriment to embryological development (Warner and Rosett 1975). Later, Sullivan (1899) published a report indicating that alcoholic women incarcerated during pregnancy had lower rates of miscarriage, stillbirths, and children with epilepsy than alcoholic women who were not incarcerated. Although these studies indicated the negative effects of ethanol consumption during pregnancy, the public remained largely ignorant of their importance.

Concurrently, experimental evidence began to contribute to the knowledge of the embryo-toxic effects of ethanol. Fere (1893) exposed chicken embryos to ethanol *in ovo*, noting its embryolethal and dysmorphogenic effects. In 1910, Stockard reported the production of defects in fish embryos exposed to ethanol. He followed this research with studies on guinea pigs, focusing on the effects of both maternal and paternal ethanol exposure to embryonic development (Stockard 1912a; 1912b). Although Stockard's warnings of the potential 'racial degeneration' of man as a

result of alcohol teratogenesis were perhaps overstated, it brought to the public an awareness of the potential health risk of ethanol consumption during pregnancy. Pearl, like Fere before him, found that ethanol had deleterious effects on chick embryos (Pearl 1917). Pearl, and later Stockard considered alcohol a beneficial "selective agent" for the human race.

Not all evidence supported the hypothesis that ethanol was dysmorphogenic. The widely influential epidemiological study of Elderton and Pearson (1910) found no correlation between maternal alcohol consumption and birth defects. This study later gained support from F.B. Hanson, whose meticulous and exhaustive studies on the effects of alcohol on rats found no detrimental effects of ethanol whatsoever (reviewed in Pauly 1996), leaving the question of ethanol's teratogenicity unanswered.

Finally, after a long period of relatively little research on ethanol's effects on reproduction and development, Lemoine et al. (1968), having described over 100 individuals, took notice of the common features of children known to have been exposed to ethanol *in utero*. Although the work of Lemoine et al., was at first met with hesitation, the scientific and medical communities were soon impressed with similar observations by Jones and Smith, leaving unanimity in the affirmation of alcohol's teratogenicity (Jones and Smith 1973; Jones et al., 1973; 1974; reviewed in Armstrong et al., 1998). By 1980, The Research Society on Alcoholism recommended clinical guidelines for diagnosis of Fetal Alcohol Syndrome (FAS; Rosett 1980), and in 1981 the US Surgeon General issued an advisory warning of the dangers of maternal alcohol consumption. The guidelines for FAS identification were recently updated by the Centers for Disease Control and Prevention (CDC, 2005). FAS consists of craniofacial abnormalities, central nervous system deficiencies, and prenatal and/or postnatal growth retardation (Rosett 1980).

Characteristic craniofacial dismorphia in FAS include short palpebral fissures, a relatively long and flat midface, and a retrusive mandible (Clarren et al., 1987). Alcohol exposure also results in growth deficiencies in height and weight (Webster 1989; Streissguth et al., 1991). Children exposed to ethanol *in utero* may also exhibit central nervous system deficits classified as alcohol-

related nerurodevelopmental disorders (ARND). Neural structures commonly affected by ethanol include the basal ganglia, corpus callosum, cerebellum, and hippocampus (reviewed in Mattson et al., 2001). Cognitive deficits involving attention, learning, memory, language, motor, and visuo-spatial abilities are also present. Such cognitive disorders likely have a structural basis, yet definitive evidence is currently unavailable (reviewed in Mattson and Riley 1998). All known ethanol-associated birth defects, structural and behavioral, have been given the umbrella classification, Fetal Alcohol Spectrum Disorders (FASD). Sampson et al. (1997) report that the incidence of FAS in the United States is 2-5 per 1000 births. FASD is suggested to be about 3 times as prevalent as FAS according to the Centers for Disease Control and Prevention.

Embryonic ethanol exposure is known to result in a variety of defects of the heart, ears, eyes, joints, and limbs (Abel 1984). Alcohol-induced limb defects are of particular interest for the current work. Limb defects have often been reported in conjunction with FAS (Spiegel et al., 1979; Jaffer et al., 1981; Cremin and Jaffer 1981; Viljoen et al., 2005). Froster and Baird (1992) indicated that the incidence of limb defects attributable to "severe maternal alcohol problems" is 1.6 per 10,000 live births in a study in British Columbia. These limb defects preferentially affect the right arm and hand. Herrmann and colleagues (1980) have described a number of patients with alcohol-induced limb defects including ectrodactyly, shortened or missing metacarpals and metatarsals, digit hypoplasia, unusually large gaps between digits, camptodactyly, clinocamptodactyly, soft tissue syndactyly, branched digits, and club foot. Others have described amelia (van Rensburg, 1981; Aro, 1983; Pauli and Feldman, 1986). Less dramatic defects of the limb, such as abnormal palmar creases and radioulnar synostosis, are often found in conjunction with FAS (Jones et al., 1973; Speigel 1979).

Section 1.2 Mouse models of FASD: ethanol's dysmorphogenesis

Many model systems have been used to study ethanol's teratogenicity including nonhuman primates, rodents, large mammals, chick, fish, insects, and worms. Of particular use to the study of FASD has been the laboratory mouse. Chernoff (1977) described a mouse model of FAS in which

dams were fed an ethanol-containing diet throughout their pregnancy. Offspring of these mice exhibited craniofacial and heart abnormalities bearing much similarity to ethanol-induced defects seen in humans. Acute ethanol exposure of mouse embryos *in utero* has also been used with particular success to achieve developmental stage-specific defects (Draft et al., 1996; Sulik 1985; Sulik et al., 1981; Kronick 1976). For the study of ethanol's action on the brain, the 'Pup in a Cup' model has been useful. This model allows the study of ethanol exposure to postnatal rodents during a period of time analogous to the third trimester in humans. The use of these various models allows an in depth evaluation of the factors leading to dysmorphogenesis throughout the course of development; variables including dose, length of exposure, diet and nutrition, genetic background, and concomitant drug exposure (West et al., 1994; Abel Hannigan, 1995).

Of the abundant information provided by studies of embryonic ethanol exposure of mice, two pieces of information emerge as especially important to our understanding of the impact of prenatal ethanol exposure in humans. First, genetic background influences the incidence and severity of ethanol-induced malformations. Chernoff (1977) demonstrated that two strains of mice exhibited variable responses to ethanol. One strain had more severe and higher percentages of malformations than another strain. A recent study has shown that genetic variability does, indeed, influence the incidence of FAS in humans (Viljoen et al., 2001), as was predicted by animal studies. Secondly, doses of ethanol administered at different windows in development result in different types of defects, demonstrating specific windows of exposure in which distinct body regions are sensitive to ethanol (West et al., 1994; Coles, 1994; Becker et al., 1996). This observation has allowed investigators to determine the sensitive windows for malformations in humans. For example, exposure to ethanol during gastrulation (day 7 of C57BL/6J mouse development, 3rd week of human development) results in typical FAS craniofacial characteristics (Webster et al., 1980; Sulik et al., 1981; Sulik and Johnston, 1983). Later, on day 8.5 of mouse development (4th week of human development) populations of neural crest are particularly sensitive to ethanol exposure, resulting in facial, great vessel, and pharyngeal arch abnormalities (Sulik et al., 1986). Later, on day 9 of mouse development

(4^{th} – 5^{th} week in humans), limb and urogenital malformations are prevalent following ethanol exposure (Kronick 1976; Webster et al., 1980; 1983; Gage and Sulik, 1991; Kotch et al., 1992). Exposure of mouse embryos to ethanol at later stages of development primarily affects the developing and maturing CNS, causing cell death in the hippocampus and cerebellum (Barnes and Walker, 1981; Goodlet et al., 1990; Bonthius and West, 1990; Maier et al., 1999). The array of malformations that may occur throughout development cautions alcohol use at any stage of pregnancy.

Regarding models for ethanol-induced limb defects, C57BL/6J mice have been particularly useful. Defects caused by prenatal ethanol exposure in these mice include polydactyly (extra digits), syndactyly (fusion of skeletal and/or soft tissues of the digits), and ectrodactyly (missing digits). High incidences of right-sided, forelimb postaxial ectrodactyly are produced by an acute exposure to ethanol on the 9^{th} day of gestation (Kronick 1976; Webster et al., 1983; Kotch et al., 1992). In such malformations, severity ranges from the loss of digit 5, to the loss of digits 2-5 and the ulna.

Studies by Kotch et al. (1992) have provided information regarding the underlying pathogenesis of ethanol-induced limb deficiencies. Examination of gestation day (GD) 12 embryos that were exposed to ethanol on the 9^{th} day of gestation illustrated that some forelimbs were missing a sizable mass of tissue on the posterior aspect of the limb bud, from which the digits are formed. Examination at an earlier time-point demonstrated the presence of excessive amounts of cell death along the apical ectodermal ridge (AER), an embryonic structure involved in limb outgrowth. Kotch et al. (1992) proposed that excessive cell death in the AER accounts for the variety and severity of limb defects seen in the mouse, following embryonic ethanol exposure.

Section 1.3 Cellular effects of embryonic ethanol exposure

The effects of ethanol on the embryo are cell type-dependant, and include changes that may lead to differentiation (Kulyk and Hoffman, 1996), cell death (Sulik et al., 1988), proliferation (Leach et al., 1993), or the inhibition of proliferation (Johnson et al., 2004). It is obvious from the literature that ethanol is capable of eliciting qualitatively different responses, in a cell type-specific manner.

One might surmise that the loss of cells over the course of development, such as occurs following cell death, premature differentiation, or cessation of proliferation, contributes substantially to ethanol-induced dysmorphogenesis. The consequences of cell loss may include alterations in inductive influences, patterning, and the processes of growth and migration.

Bannigan and colleagues (1982, 1984) noted that cell death is commonly observed in specific regions of the mouse embryo following ethanol exposure. Sulik et al. (1988) have since observed that the regions particularly sensitive to ethanol-induced cell death, are those that, as a normal part of development, undergo a limited amount of programmed cell death. Craniofacial (Sulik et al., 1988), urinary tract (Gage and Sulik, 1991), and neural (Kotch and Sulik 1992) defects are characterized by the presence of excessive cell death in their primordia following ethanol exposure. It remains to be determined why cell death occurs in these regions in untreated embryos, and it is equally unclear whether the consequences of excessive cell death are strictly morphological.

Programmed cell death (apoptosis) is a normal part of vertebrate embryogenesis. The occurrence of embryonic cell death may take place for several reasons. First, apoptosis functions in the elimination of transitory structures. For example, the mesonephros is functionally replaced by the metanephros, undergoing the process of cell death to eliminate this structure from the embryo when no longer needed. Secondly, programmed cell death functions in the remodeling of tissues. In the developing hand and foot, a paddle forms which is subsequently reformed into a structure with five digits. Cell death contours the structure, contributing to the formation of a hand from a paddle. Lastly, apoptosis may be a mechanism for restricting proliferation through the abolition of embryonic organizers. In the limb, the anterior neural ridge, isthmus, and facial primordia, it appears that cell death regulates the number of cells responsible for promoting cellular proliferation (Aoto et al., 2002; Chi et al., 2003). Though the exact reason is not known in all instances, programmed cell death occurs in select cell populations in many regions of the developing embryo.

Section 1.3.1 Ethanol's mechanism of action

There are a number of existing hypotheses regarding ethanol's mechanisms of teratogenesis. They can be reduced to two broad categories: maternal effects of ethanol and direct effects of ethanol on the conceptus.

The primary hypotheses concerning the maternal contribution to FASD implicate dysfunction of the placenta or yolk sac. That ethanol has the affect of constricting umbilical vessels in rats, primates, and humans (Jones et al., 1981; Mukberjee and Hodgen 1982; Altura et al., 1983; Savoy-Moore et al., 1989) implicates reduced blood flow as a major contributor. The most obvious consequence of reduced placental blood flow is hypoxia. Though once a popular hypothesis, experimental evidence linking hypoxia to FASD is lacking. However, research indicates that placental transport of amino acids, glucose, vitamins (including folate, thaiamine, and vitamin B_6), and zinc are diminished following ethanol exposure (reviewed in Schenker et al., 1990). Many of these occurances may be a secondary consequence of reduced placental blood flow following ethanol exposure.

There is some discussion in the literature that suggests that ethanol, *per se*, does not mediate FASD. Rather, ethanol's metabolite, acetaldehyde, has been suggested as the proximate teratogen (Veghelyi et al., 1978). The major site of the conversion of ethanol to acetaldehyde occurs in the maternal liver; the embryo has relatively little capability to metabolize ethanol. Investigators have shown that acetaldehyde does, in fact, account for some of the defects associated with ethanol's teratogenesis, although at a much lower incidence than ethanol exposure during the same window of development (Webster et al., 1983). *In vitro* evidence supports the hypothesis that acetaldehyde is, in part, responsible for ethanol's teratogenicicty (Campbell and Fantel, 1983).

Beginning with Stockard (1910), researchers have known that ethanol affects non-mammalian vertebrates, suggesting that the maternal contribution to FASD in mammals may be less significant than some have proposed. Additionally, even mammalian embryos exhibit many ethanol-induced morphological changes when cultured in the presence of ethanol where the maternal

contribution to the effects of ethanol is removed (Brown et al., 1979; Priscott, 1982; Kotch et al., 1996).

Prostaglandins (PG), which are known to be essential in normal development (Persaud 1978; Challis and Patrick 1980), may be elevated following maternal alcohol consumption in several tissue types (Anggard 1983) to the detriment of the embryo. In fact, ethanol-induced growth retardation in mice can be prevented with the application of PG synthesis inhibitors (Pennington et al., 1985), indicating that PG may contribute significantly to the hypoplastic effects of ethanol. In the developing limb, however, PG synthesis inhibition acts synergistically with ethanol to produce malformations in mice (Padmanabhan and Pallot 1995), suggesting that ethanol's effects on PG synthesis may be dependant upon the cellular context or other contributing factors.

Several investigators have implicated altered protein synthesis as a mediator of ethanol's effects. RNA transport and aminoacyl transfer RNA synthases in the embryo are negatively impacted by ethanol (Henderson and Schenker 1977). Additionally, ethanol depresses ribosomal function (Horbach et al., 1989). Some have suggested that ethanol's affect on protein synthesis is primarily a function of the availability of precursors, protein degradation, or hypothermia (reviewed in Schenker et al., 1990; Henderson et al., 1980).

The observation that Ca^{2+} levels rise very quickly after exposure of preimplantation embryos to ethanol (Stachecki and Armant, 1996) has given rise to the hypothesis that ethanol's effects on other developing systems are mediated by intracellular Ca^{2+} signaling (Debelak-Kragtorp et al., 2003). Calcium-mediated stimulation of phospholipase-C (PLC) signaling has been shown to account for ethanol-induced cell death in chick neural crest (Debelak-Kragtorp et al., 2003). Because of the large number of signaling pathways that utilize PLC, the potential targets of ethanol's action are innumerable.

Another cellular mechanism involves plasma membrane fluidity, which is readily altered by ethanol (Chin and Goldstein, 1980; Goldstein et al., 1980). This observation may account for a

number of effects, including ethanol's induction of intracellular Ca^{2+}, altered membrane protein conformation, decreased cell adhesion, and attenuated migration (reviewed in Schenker et al., 1990).

Due to the advancements in the fields of molecular and developmental biology, it has become possible to examine ethanol-induced gene changes in the context of a known developmental system. Importantly, investigators using mouse and chick models have noted that expression of Shh, a major developmental patterning gene is downregulated in response to ethanol exposure (Chrisman et al., 2004; Ahlgren et al., 2002). However, it is not clear whether the decreased expression is the result of ethanol exposure *per se*, or the secondary result of tissue loss that follows ethanol exposure as described by Kotch et al. (1992). That the effect on Shh expression is primary is suggested by the phenotype of the Shh null mutant mouse, which bears limb and craniofacial characteristics of acute ethanol exposure.

Other genes, including those important to gastrulation, are likewise affected by ethanol exposure. In the frog, *Xenopus*, expression of C*hordin*, *gsc,* and *Otx2* is upregulated in response to ethanol, and gastrulation is delayed (Yelin et al., 2005). Murine homologs to these genes are present and important for gastrulation in mouse embryos, although no study has described the response of these genes to ethanol.

A microarray analysis (Dunty 2002) has shown that acute ethanol exposure perturbs a number of significant pathways in the developing brain, including RA, and Wnt signaling pathways. Several other genes involved in the cell cycle-related processes of proliferation, and differentiation and cell death are perturbed following embryonic ethanol exposure (Dunty 2002; Leach et al., 1999; Gu et al., 2001). Though not patterning genes, ethanol-induced perturbation of these cell cycle genes may play an equally important role in dysmorphogenesis, as it is these processes that execute the instructions of developmental patterning. That ethanol affects these essential regulatory pathways and cellular processes is not surprising, given the significant dysmorphogenic effects of ethanol. Gene knock out studies involving components of these pathways have demonstrated their pivotal roles in developmental patterning. Due to the complex relationships among members of these and other

pathways, and the potential for significant changes in cellular constituency, experiments designed to examine the targets of ethanol and other teratogens must consider the biological consequences of exposure as close to the time of exposure as is possible.

Two particularly important genes involved in cell cycle processes are epidermal growth factor (EGF) and insulin-like growth factor (IGF). The perturbation of these highly significant, developmentally regulated growth factors has a considerable effect on growth and development (Thesleff et al., 1995; Rother and Accili 2000). Ethanol targets EGF-dependant cell division by depressing EGF-R phosphorylation (Henderson et al., 1989). Impaired IGF signaling has also been demonstrated in response to ethanol (Resnicoff et al., 1993; 1996; Seiler et al., 2000). These results may be interpreted to support the hypothesis that ethanol-induced changes in cell cycle processes are a result of its perturbation of growth factor signaling.

One of the more compelling hypotheses is that oxidative stress accounts for the teratogenicity of ethanol. Molecules that induce oxidative stress are free radicals (superoxide, hydroxyl ion, and nitrogen dioxide radicals) and reactive oxygen species (ROS; hydrogen peroxide, nitric oxide, and peroxynitrate ion). Ethanol metabolism is suspected of inducing oxidative stress through the accumulation of NADH and mitochondrial generation of hydroxyethyl or superoxide radicals (Goodlet et al., 2005). Ethanol also reduces antioxidant levels, which provide cellular defenses against oxidative stresses such as vitamins C and E, glutathione, superoxide dismutase, and retinol (Reyes et al., 1993; Montoliu et al., 1995; Henderson et al., 1995; Addolorato et al., 1997). The unopposed accumulation of oxidative stressors has two effects. First, it results in the oxidation of macromolecules, which may lead to cellular demise. Secondly, ROS act as signaling molecules in certain cellular contexts, mediating growth factor signaling, Ca^{2+} channel regulation, and gene expression (reviewed in Goldstein et al., 2005; Waring, 2005; Esposito et al., 2004). Thus, the oxidative stress may stimulate a variety of cellular processes by triggering ROS-mediated second messengers, even in the absence of growth factors.

That oxidative stress may mediate ethanol's effects is evidenced by the fact that ROS appear after ethanol exposure in embryonic tissue (Chen and Sulik 1996; Johnson et al., 2004; Goodlet et al., 2005), followed by cell death. Furthermore, the incidence of ethanol induced malformations is diminished by the application of antioxidants (Kotch et al., 1996; Chen et al., 2004; the author's unpublished observations).

Lastly, and of particular relevance to this thesis, is the hypothesis that ethanol-induced malformations result from the inhibition of retinoic acid (RA) synthesis by ethanol. This hypothesis was advanced by Duester (1991) and Pullarkat (1991), who noticed two important pieces of information. First, ethanol and RA utilize the same enzymes for their metabolism. These investigators proposed that ethanol would competitively inhibit the enzyme(s) necessary for the production of RA. Han et al. (1998) have since identified three alcohol dehydrogenase enzymes responsible for synthesizing RA, and have shown that RA production is diminished by ethanol's inhibition of these enzymes. Secondly, Duester and Pullarkat also observed that offspring of laboratory animals exposed to ethanol share morphological similarity with animals whos dams were vitamin A deficient during pregnancy (summarized in Zachman and Grummer 1998).

Deltour and colleagues (1996) have since demonstrated that ethanol lowers RA concentrations in embryos. Because of ethanol's affect on RA concentrations, and RA's integral role in development (described below), reduced RA levels would be predicted to result in developmental abnormalities, a premise that warrants examination as a possible factor in ethanol's teratogenesis.

Section 1.4 Early development of the limb (figure 1.1)

On gestational day (GD) 9, the lateral plate mesoderm (LPM) lies beneath the ectoderm and stretches from the pharyngeal region to the caudal end of the embryo. It is from the LPM that the forelimbs and hind limbs will appear. The forelimb arises from this mesodermal cell population at the level of somites 8-12. The regions of LPM that form anterior (cranial) and posterior (caudal) limbs are known as the limb fields. The earliest known identifier and inducer of forelimb initiation is

Wnt-2b. It is expressed in the LPM and intermediate mesoderm at the anterio-posterior level of the forelimb field (Kawakami et al., 2001). Another gene, Tbx5, is necessary for forelimb initiation (Ahn et al., 2002) and is dependant on RA for its expression (Mic et al., 2004). Together, Tbx5 and Wnt-2b impart identity and induce forelimb bud initiation by stimulating expression of Fgf10 in the LPM of the limb field (Ng et al., 2002). Fgf10 then induces the overlying ectoderm to express Fgf8 (Ohuchi et al., 1997) by way of Wnt-3a in the chick (Ng et al., 2002), and an unidentified signal in the mouse. Together Fgf8 in the ectoderm and Fgf10 in the mesoderm stimulate each other in a positive feedback loop, promoting the proliferation of limb mesenchyme (Xu et al., 1998; figure 1.1). As proliferation begins in the LPM the first signs of the limb bud appear.

While the above process of initiation ensues, the limb bud divides into two transcriptionally distinct regions. The first is characterized by the proximal expression of Meis genes; Meis1 and Meis2 (Ceccini et al., 1997; Capdevila et al., 1999). This region will develop into the stylopod, that region of the forelimb containing the humerus. RA, synthesized in the presumptive forelimb mesoderm, intermediate mesoderm, and somitic mesoderm prior to limb outgrowth (Swindell et al., 1999; Mic et al., 2002), is the signal that regulates where Meis genes will be expressed (Mercader et al., 2000). The second, meis-negative, region permits expression of characteristically distal gene expression and gives rise to more distal limb structures (Mercader et al.,1999; 2000).

A signal from the distal ectoderm prevents Meis expression and is responsible for the distal, Meis-negative, expression domain. This distalizing signal is Fgf8, which arises from a specialized epithelial structure known as the apical ectodermal ridge (AER) and also its primordium, the distal ectoderm. As described above, Fgf8 is first expressed in the ectoderm of the limb field at the initiation stage. As outgrowth ensues, the Fgf8 expression domain becomes restricted, so that on GD 9.5, it is found in the most distal ectoderm in the region of the presumptive AER (Crossley et al., 1995). Fgf8 limits the expression domain of Meis genes through its inhibition of RA signaling. As outgrowth continues into later stages of limb development, Meis genes remain in the proximal limb region as the Meis-negative expression domain, the subridge mesenchyme (SRM), expands distally.

While the proximo-distal (PD) axis is being established, the anterior-posterior (AP) axis begins to emerge. The distal limb is patterned by an organizer found in the posterior mesenchyme (Saunders and Gasseling 1968) known as the zone of polarizing activity (ZPA). The ZPA produces the protein, Shh, which is responsible for the patterning effect of the ZPA (Chiang et al., 2001; Kraus et al., 2001). Shh expression, however, is stimulated by an earlier signal. A proximal source of RA, previously integral to Tbx5 mediated initiation and Meis mediated outgrowth, is next used to stimulate two genes in the posterior half of the limb bud, dHand and Hoxb8 (Lu et al., 1997; Charite et al., 2000 Fernandez-Teran et al., 2000; Mic et al., 2004). Together, dHand and Hoxb8 stimulate Shh expression in the ZPA. The restriction of dHand to the posterior mesenchyme is believed to result from the reciprocal repression between dHand and the Gli3 repressor (Gli3-R). Each of these gene products prevents the expression of the other in their respective posterior and anterior compartments (Welscher et al., 2002). When *Shh* expression increases, it replaces dHand and interacts with Gli3-R in a similar manner. Together, Shh and Gli3-R specify the number of digits produced by the limb as well as the AP identity of those digits.

With the onset of *Shh* expression and the formation of the SRM on GD 9, the regulation of the AP and PD axes becomes coupled as the AER sustains both regions through positive feedback loops. The AER and ZPA are responsible for maintaining their respective axes, and ultimately coordinating the translation of genetic signals into morphological structures. Each of the two organizers maintains one another. Shh, in the ZPA, and Fgf4 and Fgf8, in the posterior AER, form a positive feedback loop (Niswander et al., 1994). So, while RA initiates the establishment of both axes in separate pathways, the two axes become co-dependant. The dorso-ventral axis similarly relates to the AER and the ZPA through signals from the dorsal and ventral ectoderm (reviewed in Capdevila and Izpisúa Belmonte 2001).

As is evident from the above account, RA signaling is necessary for limb initiation, the transition to distal outgrowth, and the establishment of the ZPA. RA is often characterized as a morphogen, emphasizing its influence over the embryo's form and growth. The defining

characteristic of a morphogen is that it acts by producing a concentration gradient. In favor of this hypothesis is the spatial opposition of regions of RA synthesis and degradation. The expression of RALDH2, a major RA synthetic enzyme, is restricted to the trunk and proximal region of the early limb bud (Yashiro et al., 2004; Mic and Duester 2003), forming a "source" of RA. The expression domain of an enzyme responsible for degrading RA, CYP26A1, is opposed to the source at the distal ectoderm of the limb bud forming a "sink" for RA (Fujii et al., 1997). While there is no evidence that the limb responds to a gradient of RA, it is clear that proximal regions depend on RA for normal development, and that distal regions operate without RA or with very low concentrations.

Section 1.5 The components of Retinoic acid synthesis, degradation, and signaling, with particular reference to the limb

All-trans retinoic acid (RA) is synthesized from retinol in a two-step process (figure 1.2). First, retinol is converted to an aldehyde, retinal, in a rate-limiting reaction catalyzed by a class of enzymes known as alcohol dehydrogenases (ADHs). Aldehyde dehydrogenases (ALDHs) then convert retinal to RA (Kim et al., 1992; Blaner et al., 1994). The three alcohol dehydrongenases known to convert retinol to retinal in the mouse embryo are ADH1, ADH3, and ADH4. *ADH 3* is expressed ubiquitously from GD 6.5 day to GD 9.5 (Ang et al, 1996). ADH1 is expressed on the 9th day of gestation in the trunk and mesonephros, while ADH4 is expressed from GD 8.5 through 9.5 in craniofacial regions (Ang et al, 1996; Haselbeck and Duester 1998). Other ADH enzymes are present, however, they are not known to contribute significantly to the production of RA.

There are three ALDHs known to synthesize RA from retinal in the mouse embryo: retinaldehyde dehydrogenase (RALDH) 1, RALDH2, and RALDH3 (Haselbeck et al., 1999; Mic et al., 2000). They are present at different but overlapping windows in development. RALDH1 is present in the cranial regions, including the eye and ear rudiments of the embryo, beginning around GD 9, while RALDH2 appears on the 7th day of gestation. The latter is localized to the LPM and paraxial mesoderm, including the proximal limb mesenchyme during the time of limb bud initiation

and outgrowth (Mic et al., 2002). RALDH3 is exclusively expressed in the ear, eye, and nasal rudiments (Mic et al., 2000; Li et al., 2000). Under normal circumstances, RA is likely produced by the combined activities of RALDH2 and ADH3 enzymes in the limb bud and proximal mesoderm.

Of note is the presence of a third class of enzymes that may participate in the production of RA. These are the short-chain dehydrogenases/reductases (SDR) which include RDH1, RDH5, CRAD1, CRAD2, CRAD3 (RDH9), and retSDR1 (reviewed in Duester et al., 2003). There is very little information regarding these enzymes, though it is clear that some of them have retinol dehydrogenase (ADH) activity while others likely participate in steroid metabolism. RDH5, Crad1, and Crad2 are present in the embryo during limb development (Ulven et al., 2000). Unfortunately, there is no data indicating whether they are localized to the limb.

There are three cytochrome P450 enzymes responsible for the degradation of RA to the inactive forms, 18-OH-RA and 4-OH-RA. CYP26A1 and CYP26B1 are found in the limb bud. However, there expression is slightly different, in that the A1 type is localized to the distal ectoderm, and the B1 type is expressed in the distal mesoderm (Abu-Abed et al., 2002). The third member, CYP26C1, is not found in the developing limb, being localized to the craniofacial and hindbrain regions of the embryo (Tahayato et al., 2003).

Across the developing limb bud, RA receptors (RARs) respond to signals from RA. RARs belong to the nuclear receptor superfamily which includes the peroxisome proliferator activated receptor (PPAR), thyroid hormone receptor (T3R), and vitamin D3 receptor (VD3R). RARs have six distinct domains. A zinc-finger DNA binding domain is present, which is 93-95% conserved among the three types of RAR. The first of the three types was cloned from the human, RARα (Petkovich et al., 1987; Giguere et al., 1987). RARβ had been discovered earlier that year (de The et al., 1987) but was not identified as such until later. Pierre Chambon's laboratory, in the process of cloning the mouse equivalents of the human RARα and RARβ, identified a third RAR, RARγ (Zelent et al., 1989). Later, it was found that each receptor had several splice variants. Some slight temporal and spatial variation has been noted among these receptor isoforms, however, knockout studies have not

revealed significant functional differences among isoforms of the same RAR type (reviewed in Lohnes et al., 1995). Dimerization occurs between receptors as they bind RA and translocate to the nucleus of the cell. There, the RA-RAR dimer complex binds a specific enhancer region of DNA known as a RA response element (RARE; Umesono et al., 1988) that participates in the regulationof the transcription of RA-responsive genes.

A comprehensive study of the expression patterns of RAR was undertaken by Dolle et al. (1989), beginning with GD 10 embryos. These investigators found that RARα and RARγ expression was localized to the mesenchyme of the limb bud. RARβ was found in the most proximal region of the limb and the adjacent flank. By GD 12.5 cartilage has begun to form, and RARγ is localized to the centers of precartilaginous nodules and undifferentiated mesenchyme in the distal limb bud. At this same time, RARα and RARβ maintain their earlier expression domains throughout the limb mesenchyme and in the proximal limb bud, respectively.

Another group of retinoid-binding receptors include the retinoid-X-receptors (RXR; Mangelsdorf et al., 1990) that bind metabolites of RA, primarily 9-cis RA. (Heyman et al., 1992; Levin et al., 1992; Allenby et al., 1993). Although they do not share homology with RAR, they are capable of forming heterodimers with RAR and bind RARE. While heterodimerization between RAR and RXR occurs *in vitro*, it is unknown whether all combinations of RAR and RXR heterodimerize *in vivo*. RXR also form homodimers with themselves and heterodimers with peroxisome proliferator-activated receptors (PPAR), thyroid receptors, and vitamin D receptor (Yu et al., 1991; Berrodin et al., 1992; Bugge et al., 1992; Kliewer et al., 1992a; Kliewer et al., 1992b; Leid et al., 1992; Zhang et al., 1992). The expression of RXRα and β is nearly ubiquitous, while RXRγ is limited to cells of a myogenic lineage during embryogenesis (Mangelsdorf et al., 1992; Dolle et al., 1994). RXRα appears to be necessary for the teratogenic effects of excessive RA *in vivo* (Sucov et al., 1995).

Besides the RARs and RXRs, there are several orphan receptors that are believed to be important in retinoid signaling. Very little is known about them, including their role in limb

development. The retinoid orphan receptors (ROR) are a group of three (RORα, -β, -γ) receptors belonging to the steroid-hormone receptor superfamily (Mangelsdorf DJ, et al., 1995). Like RAR they function by binding a known response element (RORE; Medvedev et al., 1996). RORα and -γ are known to be important to cerebellum and lymph node development, respectively (Dussault et al., 1998, Kurebayashi et al., 2000; reviewed in Jetten et al., 2004), but little else is known of their role in development. Expression of the ROR in the developing limb is unknown.

In addition to those components that synthesize, degrade, and transduce RA, there are binding proteins that regulate the availability and distribution of RA or retinol, intra- and inter-cellularly. Retinol binding proteins (RBP) are typically found in the blood plasma, as they distribute retinol to the cells of the body. Maternal RBP does not cross the placenta, but sufficient retinoids may pass to the embryo as retinyl esters bound to lipoproteins (Quadro et al., 2004).

Intracellularly, types I and II cellular retinol binding proteins (CRBP), and cellular types I and II RA binding proteins (CRABP) control the availability of the retinol and RA, respectively (reviewed in Blomhoff et al., 1990 and Wolf et al., 1991). In the adult, CRBP-I binds retinol and promotes esterification to retinyl esters, which are stored in the liver (Yost et al., 1988). The exact role of CRBPs and CRABPs in embryonic development, however, is the subject of much speculation. The consensus view is that they influence or take part in cytoprotection, cellular uptake of retinol, and modulation of intracellular retinol metabolism (reviewed in Li and Norris 1996, and Siegenthaler 1996). Investigators have shown that overexpression of CRABP-I prevents activation of RA-induced genes, suggesting CRABP I functions to limit the availability of RA (Boylan and Gudas, 1991). Fiorella and Napoli (1991) provide evidence that CRABP-I has a role in catabolism of RA.

CRBP-I and CRABP-II are found in many regions of the developing embryo. Gustafson et al. (1993) have provided an interesting observation in that CRBP-I is localized to the embryonic ectoderm, while CRABP-I is localized the mesenchyme. This is true for the limb bud, as well as for several other regions of the embryo. There appears to be a gradient of CRABP-I in the limb bud, such that its expression (Dolle et al., 1989) and protein (Maden et. al. 1988) is highest in the distal

region and very low in the proximal limb bud. CRABP-II, like CRABP-I, is expressed in limb mesenchyme to the exclusion of the ectoderm (Ruberte, et al., 1992). CRBP-II is present in embryos from GD 6.5-9.5 (Ulven et al., 2000), however there is no information regarding its expression in the limb bud. The reasons for this distribution are unknown and may present a significant obstacle to understanding the functioning of RA.

Section 1.6 Rationale

The teratogenicity of ethanol is well established, however, the means by which it interacts with the developing embryo to produce malformations is unclear. The premise that ethanol causes perturbation of RA-mediated development is supported in many ways. First, RA is necessary for normal development. As has been demonstrated using the hypovitaminosis A model, RAR antagonists, and gene knockouts of RAR and RALDH2, RA is integral to the normal development of a variety of embryonic structures including the limb. Researchers have demonstrated that, particularly in the limb, RA plays a role in signaling to regulators of pattern formation (section 1.4).

Secondly, ethanol exposure lowers the concentrations of RA in adult and developing animals (Sato and Lieber, 1982; Deltour et al., 1996). The means by which ethanol has been proposed to accomplish the reduction of RA concentration, is through the competitive inhibition of the enzyme(s) necessary for RA synthesis (Duester 1991; Pullarkat 1991). As further evidence of the biological relevance of ethanol's impact on RA concentrations, ethanol is known to alter a number of RA signaling components including CRABP-I (Bi et al., 2001), RARα, -β, and -γ (summarized in Zachman and Grummer, 1998). These changes can be seen as an indication of a change in RA concentrations, as many of the components of RA signaling are directly or indirectly regulated by RA.

Lastly, there are many morphological similarities between animals that have undergone development with a retinoid deficiency, and those exposed to ethanol during embryogenesis. Morphological similarity does not provide sufficient evidence to establish a common pathogenesis.

However, it is a requisite piece of evidence. The RAR antagonist AGN 193109 produces craniofacial defects similar to those caused by ethanol (Sulik et al., 1981; Kochhar et al., 1998). In addition to malformations induced by acute ethanol exposure and antagonist treatment, malformations resulting from chronic ethanol exposure and chronic maternal hypovitaminosis A bear similarity to one another (reviewed in Zachman and Grummer, 1998). That malformations resulting from both acute and chronic ethanol exposure recapitulate those of acute and chronic RA signal abrogation, suggests a similarity in their mechanism or pathogenesis.

Section 1.7 Objective and specific aims

The objective of this study was to understand the contribution of RA signal abrogation to the genesis of ethanol-induced limb malformations. The specific aims and hypotheses that this study addressed were as follows:

1: The hypothesis that Retinoic Acid (RA)-deficient mouse embryos/fetuses manifest comparable limb defects and cell death patterns to those embryos/fetuses from ethanol treated dams was tested. To this end, C57BL/6J mouse embryos were exposed to a teratogenic dose of ethanol on gestational day (GD) 9 and 6 hours (9:6), followed by a second dose 4 hours later at GD 9:10. Skeletal abnormalities of the forelimbs were assessed on GD 18 following Alcian Blue/Alizarin Red staining. To ascertain whether the abrogation of the RA signal results in similar morphological abnormalities to those induced by maternal ethanol treatment, disulfiram or BMS-189453 was administered to mice on the 9th day of gestation. GD 18 fetal skeletons were examined and comparisons between each of the treatment and control groups made, based on the type and incidence of defects involving the forelimbs. Because cell death is a strong indicator of ethanol-induced limb dysmorphology (Kotch et al., 1992), Nile Blue Sulfate (NBS) staining of GD 9:14 embryos was used to document and compare the patterns of cell death among the three treatment groups. These *in vivo* studies demonstrate that RA-deficiency-mediated and ethanol-induced limb malformations are pathogenically and morphologically comparable.

2: The hypothesis that maternal RA supplementation can ameliorate ethanol-induced cell death and dysmorphogenesis was tested. Based on the knowledge that ethanol reduces RA concentrations, this study was designed to determine whether exogenous RA could rescue ethanol-induced limb defects and cell death. Pregnant mice were administered 25 mg/kg maternal body weight of RA following ethanol administration. Skeletal examinations were be made on GD 18 to determine whether maternal RA administration reduced the incidence of limb defects. Using NBS as an indicator of cell death, spatial and qualitative assessments were made regarding the effectiveness of RA in diminishing cell death in the presumptive apical ectodermal ridge (AER) of GD 9:14 ethanol-exposed embryos. LysoTracker Red staining and laser scanning confocal microscopy were utilized to confirm these data, and three-dimensional reconstructions were made illustrating the relative distribution and intensity of cell death.

3: The hypothesis that after exposure to ethanol, embryonic forelimbs exhibit transcriptional alterations consistent with impaired RA-signaling was tested. For this work, microarray analyses of limb buds collected at time points ranging from 2-6 hours following maternal administration of ethanol or BMS-189453 were conducted. A dataset of RA-dependant genes was identified through analysis of the limb buds from the BMS-189453 treated embryos. Statistical comparisons were used to match the RA-dependant gene expression profiles from the ethanol-exposed and RAR antagonist-exposed limb buds. To validate microarray results for particular genes, real-time PCR was used. Additionally, *in situ* hybridizations were used to identify the potential changes in expression patterns of selected genes of particular developmental significance. The results of these gene expression studies were useful in determining the contribution of RA signal abrogation in ethanol teratogenesis, as well as providing an overview of the transcriptional changes that follow *in vivo* ethanol exposure.

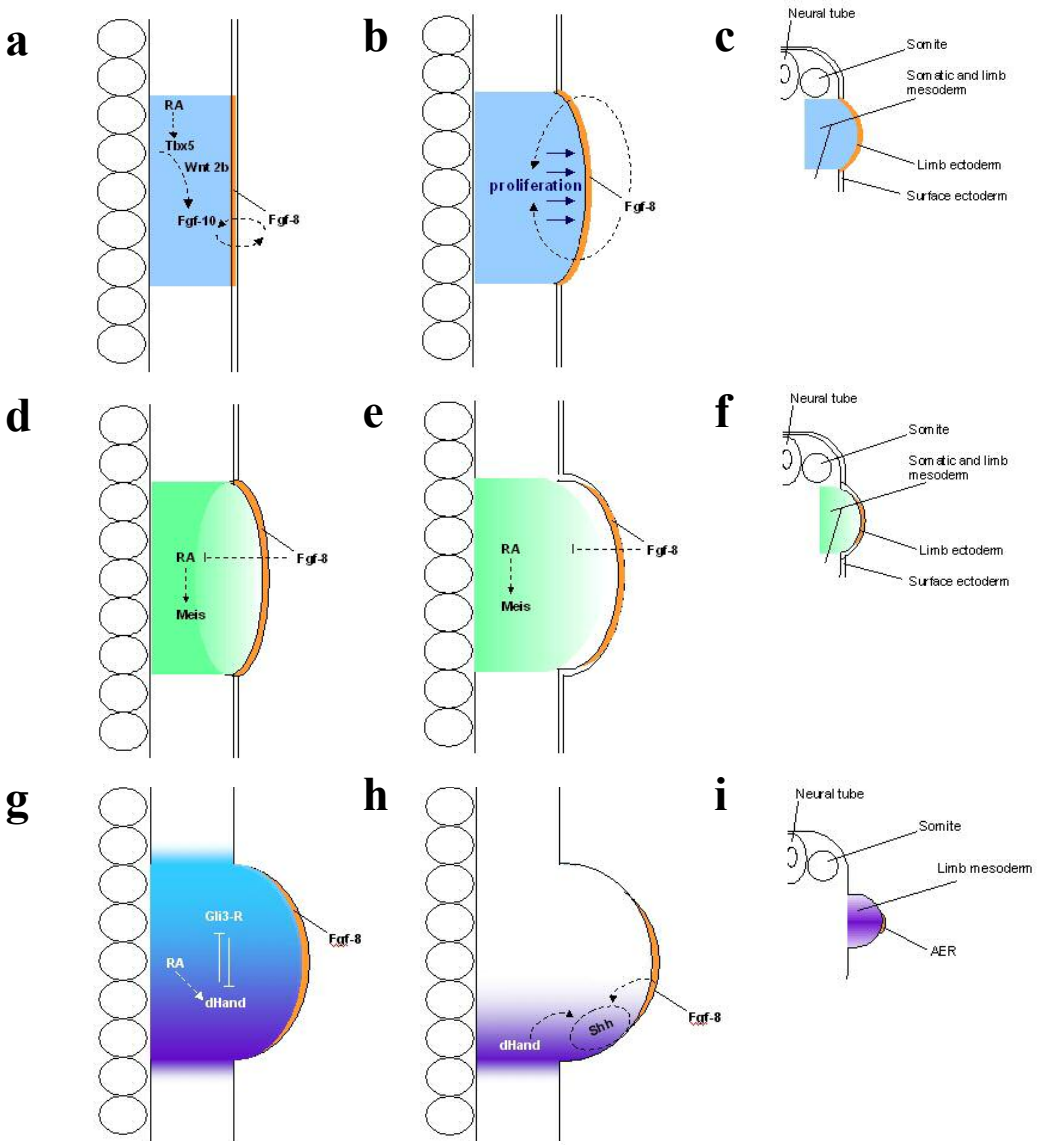


Figure 1.1. **Illustration of the successive, early stages of limb development involving retinoic acid**. (a,b,d,e,g,h): The cranial direction is towards the top of the page and the caudal towards the bottom. The right forelimb is shown developing to the right, and somites and lateral plate mesoderm are to the left of each illustration. (a,b): **Known players in limb bud initiation**: The domain of Fgf10 expression (light blue) is initiated or specified by Wnt-2b and Tbx5. The ectoderm expresses Fgf8 (orange), which maintains Fgf10 in the mesenchyme, causes proliferation, and results in the formation of the limb bud. (d,e): **Initial patterning of the proximodistal axis**: RA-mediated Meis genes expression defines the proximal limb, which is antagonized by Fgf8 in the nascent AER. (g,h): **Patterning of the anterioposterior axis**: RA-mediated dHand expression is limited to the posterior limb mesenchyme by Gli3-R. Shh, and the ZPA that it defines is positioned by dHand and Fgf8 in the posterior border of the developing AER. Shh will later determine the identity and number of posterior digits. (c,f,i): Illustration of a transverse plane through the developing limb corresponding to the developmental stage immediately to it's left.

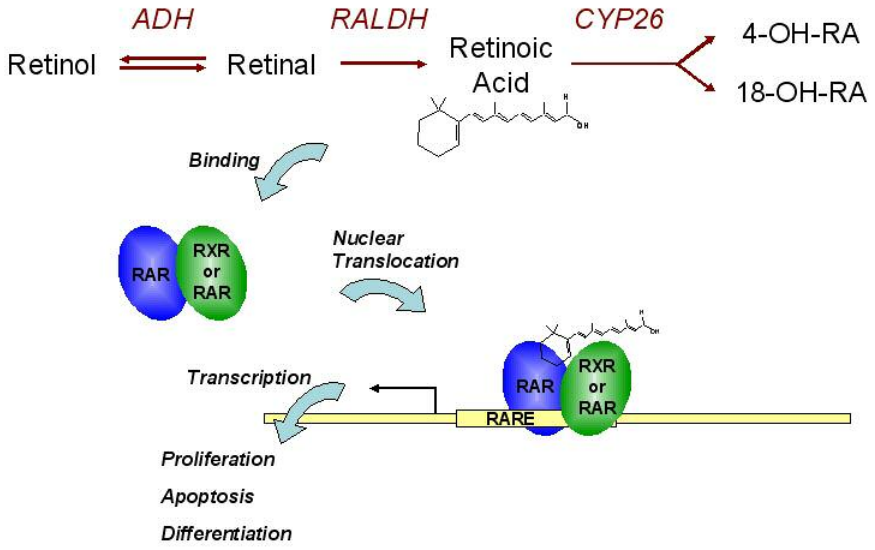


Figure 1.2. The metabolism and biological activity of retinoic acid. Retinoic acid (RA) is produced from retinol by ADH and RALDH enzymes. Catabolism of RA is accomplished by a CYP26A1enzyme. RA binds to cytoplasmic receptors, RAR homodimers or RAR/RXR heterodimers. RA binding allows nuclear translocation. The RA-dimer complex binds RA response elements (RARE) to initiate transcription of RA-responsive genes, resulting in changes in cellular function. Particularly, modifications to the processes of proliferation, apoptosis, or differentiation are among common responses.

CHAPTER II
Perturbation of Retinoic Acid (RA)-mediated limb development suggests a role for diminished RA signaling in ethanol's teratogenesis.

Section 2.1 Abstract

Background: Several lines of evidence link ethanol's teratogenicity to hypovitaminosis A. Many regions affected by ethanol exposure bear similarity to those affected in retinoic acid (RA)-deficient embryos. In the present study, the dysmorphogenic effects of acute embryonic exposure to disulfiram (an aldehyde dehydrogenase inhibitor) or BMS-189453 (a pan-retinoic acid (RA) receptor (RAR) antagonist) on forelimb morphogenesis were examined to determine whether a temporary abrogation of the RA signal would result in limb defects comparable to those caused by acute ethanol exposure. Excessive cell death in the apical ectodermal ridge (AER), a component of ethanol's pathogenesis, was assessed in embryos exposed to BMS-189453 and disulfiram to determine whether they exhibit common effects. Also, the ability of exogenous RA to prevent the effects of ethanol *in vivo* was investigated to further test the hypothesis that ethanol interferes with RA-mediated development. Finally, expression of key developmental regulatory genes for limb development was examined to determine whether those that are RA-dependent were affected by embryonic ethanol exposure.

Methods: Ethanol, disulfiram, or BMS-189453 was administered to C57BL/6J mice on the 9^{th} day of pregnancy. Forelimb morphology was assessed on gestation day (GD) 18 using Alcian blue and Alizarin red staining. Nile blue sulfate (NBS) or LysoTracker Red (LTR) vital staining was utilized to identify cell death in the limbs of GD 9, hour 14 (GD 9:14) embryos. The ability of all-trans RA to prevent ethanol-induced cell death was assessed by co-administration of ethanol and RA on GD 9:6 followed by laser scanning confocal microscopic examination of LTR-staining. This provided a 3-

dimensional representation of cell death in limb buds. *In situ* hybridization and real-time PCR was used to examine gene expression in the ethanol-exposed limb buds.

Results: Treatment with ethanol, disulfiram, or BMS-189453 resulted in postaxial ectrodactyly. Although less frequently observed, intermediate ectrodactyly and other digital defects also occurred in the three treatment groups. Relative to control embryos, excessive NBS staining was evident in the presumptive apical ectodermal ridge (AER) following ethanol, disulfiram, and BMS-189453 exposure. Ethanol-induced NBS staining was prevented by RA supplementation *in vivo*. Within 6 hours of ethanol exposure *dHand* and Shh expression levels were lower than control; within 18 hours Tbx5 was decreased relative to control.

Conclusions: Ethanol-induced forelimb defects and excessive cell death in the AER are recapitulated by abrogation of the RA-signal, strongly implicating a common pathogenesis. The prevention of ethanol-induced cell death by RA indicates that ethanol may compromise the RA signal necessary for AER maintenance. In addition, ethanol-mediated reduction of RA-dependent gene expression is consistent with perturbation of RA-mediated development. Transcriptional repression of the ZPA and AER within only hours of exposure suggests a direct insult to these developmental centers integral to normal limb morphogenesis.

Key words: retinoic acid, ethanol, limb development, fetal alcohol spectrum disorders

Section 2.2 Introduction

Prenatal ethanol exposure results in a constellation of structural and functional abnormalities collectively known as fetal alcohol spectrum disorders (FASD). Although less common than brain and craniofacial defects, prenatal ethanol exposure results in limb defects such as radioulnar synostosis, shortened digits, camptodactyly, clinodactyly, and ectrodactyly (Spiegel et. al 1979; Herrmann et. al 1980; Cremin and Jaffer 1981; Van Rensburg 1981; Froster and Baird 1992).

Hypoplastic nails and abnormal palmar creases are also common defects of the limb (Jones et al., 1973; Tillner and Majewski 1978; Viljoen 2005).

Limb defects have also been reported in an FASD mouse model (Kronick 1976; Kotch et. al 1992). They include postaxial ectrodactyly, intermediate digit ectrodactyly, syndactyly, and abnormally large digital spacing. As reported by Kotch et al. (1992), the pathogenesis of ethanol-induced limb malformations involves localized cell death in the developing apical ectodermal ridge (AER), a region of specialized epithelium that promotes proximo-distal limb outgrowth and participates in the maintenance of the anterio-posterior signaling center, the zone of polarizing activity (ZPA).

Since the dysmorphogenic effects of ethanol upon the embryo were first described (Lemoine 1968; Jones and Smith 1973), many experiments have probed the biochemical, physiological, and developmental processes impacted by ethanol exposure. Investigators have observed that morphological similarities are present between humans with FAS and experimental animals exposed to a vitamin A (retinol) deficient diet (VAD). Pullarkat (1991) and Duester (1991) noted that RA synthesis depends on oxidation of retinol and retinal by enzymes that also metabolize ethanol: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Pullarkat and Duester hypothesized that ethanol's morphological effects result from diminished concentrations of RA, as a result of the competitive inhibition of RA synthetic enzymes by ethanol. Indeed, it has been recognized for some time that ethanol reduces RA concentrations in adult tissue (Van Thiel et al., 1974), a phenomenon that has more recently been demonstrated in the mammalian embryo (Deltour et al., 1996). Another possible explanation for the similarities seen between ethanol-exposed embryos and those of VAD diets, is that ethanol interacts with components of RA signaling. Zachman and Grummer have shown in their investigations (reviewed in Zachman and Grummer 1998) that RA receptors (RARs) and cellular retinol binding protein are disregulated by ethanol in several developmental systems.

It is well established that RA is essential for normal limb development. Of relevance to the present study, is the role of RA in the formation of the ZPA and the AER. RA was initially described as the signal from the ZPA that induced ectopic digits when grafted to the anterior margin of a host limb. The proximate signal was later identified as Shh, a RA-inducible factor. Shh is stimulated by and dependent upon RA (Helms et. al 1994; Stratford et. al 1996). There is some evidence that the RA dependency of Shh may be mediated by dHand, a posteriorly restricted mesenchymal transcription factor. Localization of Shh depends on the RA-dependant dHand localized to the posterior mesenchyme, and Fgf8 secreted from the overlying posterior AER (Lewandoski et al., 2000; Moon and Capecchi 2000). After the ZPA is established, it functions to sustain the posterior AER and a positive feedback loop is established.

RA's function in AER formation is less clearly understood. During the initiation phase of limb bud development, the ectoderm expresses Fgf8. The mesenchyme proliferates in response to Fgf8, and produces Fgf10 which in turn maintains the ectoderm. As limb initiation concludes, the domain of Fgf8 expressing ectoderm narrows as a result of dorsal and ventral ectoderm signals, forming a discrete domain of specialized ectoderm at the ventro-distal margin of the limb bud. With the outgrowth of the limb bud, the influence of Fgf8 is restricted to a distal, mitotically active subpopulation of mesenchyme beneath the developing AER, the subridge mesenchyme (SRM). Fgf8 continues to maintain expression of Fgf10 in the mesoderm (Mahmood et al., 1995; Vogel et al., 1996; Ohuchi et al., 1997; Moon and Capecchi, 2000) necessary for SRM proliferation and AER maintenance. The SRM appears to require the RA-dependent factor, Tbx5 to maintain Fgf10 expression; failure of Tbx5 expression is associated with failed AER maintenance and formation. Like the ZPA, the SRM forms a positive feedback loop with the AER, mediated by Fgf8 and Fgf10. As many investigations have shown, RA is integral to the formation of two important signaling centers, the AER and ZPA.

Both the ZPA and the AER have been investigated regarding their role in ethanol-induced limb malformations. Kotch et al. (1992) demonstrated that excessive cell death in the AER of GD 10

mouse embryos correlated with the occurrence of limb malformations. Subsequent failure of posterior mesenchyme formation was characteristic of many limb buds examined on GD 12. Chrisman et al, (2004) demonstrated that perturbations in the AER and ZPA are accompanied by the loss of their respective molecular markers, Fgf8 and Shh. Other studies suggest that Shh may be important to ethanol-induced malformations. Shh is important in the development of numerous structures, such as the limb, CNS, and craniofacial region (Wilson et al., 2005, Motoyama 2006). Ahlgren et al. (2002) showed that *Shh* is decreased in craniofacial regions following exposure to ethanol and rescue of the ethanol-induced phenotype was possible with application of Shh protein. Given the similarity between the limbs of Shh null mutants and those exposed to ethanol, and the ability of Shh to rescue ethanol's effects in other embryonic structures, a Shh-mediated pathogenesis of ethanol-induced limb malformations seems likely. However, as Chrisman et al. (2004) suggest, perturbation of the AER's ability to sustain Shh expression may be a more direct cause of Shh downregulation.

As an upstream regulator of Shh, perturbation of RA seems a likely mechanism of ethanol-induced limb malformations. However, because of the dissimilarities between limbs exposed to ethanol and those permanently lacking normal RA signaling, ethanol's possible perturbation of RA-mediated limb development hasn't been previously considered. Limb reduction defects related to RA signaling failure were described in *RAR-*α and *RAR-*γ double knockout mice (Lohnes et al., 1994), and included malformations of the scapulae, radius, carpals, and digits. Mice lacking the major RA synthetic enzyme in the limb, RALDH2, that have been given limited dietary supplementation or gavage administration of RA exhibit a similar range of limb defects (Niederreither et al., 2002). We sought to determine whether temporary abrogation of the RA signal results in limb defects comparable to those resulting from ethanol exposure. Studies involving embryonic exposure to disulfiram, an aldehyde dehydrogenase (ALDH) inhibitor (Vallari and Pietruszko 1982) used experimentally to prevent RA synthesis (Stratford et al., 1996; 1997; Xavier-Neto et al., 1999) were also conducted. Additionally, dysmorphogenic effects of exposure to a pan-RAR antagonist, BMS-

189453 (Schulze et. al 2001), were examined. Comparisons of the results of the three treatments sought to establish whether temporary abrogation of the RA signal results in a more limited, "ethanol-like" dysmorphology than other studies have shown. Because excessive cell death in the AER is detectable within hours of ethanol exposure, the presence of abnormal cell death in the limbs of ethanol, disulfiram, or BMS-189453 exposed embryos was compared in order to ascertain whether a common pathogenesis exists. Subsequently, the hypothesis was tested that RA could restore normal limb development to embryos exposed to ethanol. To establish a link between ethanol exposure and abrogation of the RA signal, the RA-dependent genes Tbx5, dHand, and Shh were analyzed using *in situ* hybridization and real-time PCR. Transcripts were examined within hours of ethanol exposure in order to minimize ambiguity regarding the cellular targets of ethanol. These experiments provide evidence that RA-mediated limb development is impacted by ethanol exposure.

Section 2.3 Materials and methods

Animals: C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine and housed in a temperature and humidity-controlled vivarium on a 14 hr light, 10 hr dark cycle. Mice were mated at the beginning of the light cycle at 8 am and inspected for a vaginal plug at 10 am. The presence of a plug was designated as gestational day (GD) 0:0.

In vivo exposure to ethanol: At day 9, hour 6 (9:6) and 9:10, pregnant mice were given intraperitoneal (ip) injections of 25% (v/v) ethanol in phosphate buffered saline (PBS). The ethanol solution was administered at 0.015 ml/g of maternal body weight, resulting in a teratogenic dose of 2.9 g/kg (Sulik et. al 1981). Control animals were treated with two injections of PBS at 0.015 ml/g on GD 9:6 and 9:10. At selected times the embryos or fetuses were removed from the uteri and placed in PBS for subsequent analyses.

In vivo exposure to BMS-189453: BMS-189453, a gift from Bristol-Myers Squibb (Wallingford, CT) has been verified as a pan-RAR antagonist (Chen et al., 1995; Yang

et al., 1999) that is effective in embryonic limb bud cells (Ali-Khan and Hales, 2005). Mice were given a single ip injection of 50 mg/kg BMS-189453 in DMSO; ip injection (0.005 ml/g of maternal body weight) was performed on day 9:10, based on pilot studies of time-dependent effects (data not shown). Control animals were treated with DMSO at 0.005 ml/g at day 9:10. At the time selected for observation, the embryos or fetuses were removed from the uteri and placed in PBS.

In vivo exposure to disulfiram: Mice were given a single 75 mg/kg ip injection of disulfiram in DMSO on day 9:6 of pregnancy. Injection volumes were 0.005 ml/g of maternal body weight. This dose was determined based on pilot studies of dose-dependent effects (data not shown). Control animals were treated with DMSO at 0.005 ml/g at day 9:6. At the time selected for observation, the embryos or fetuses were removed from the uteri and placed in PBS.

Skeletal morphology: GD 18 fetuses dissected from extraembryonic membranes were transferred to, and maintained in 95% (v/v) ethanol for at least 3 days. When ready to stain, the head, skin, and viscera were removed from the trunk. The trunks were transferred to a 0.015% Alcian blue solution in 75% (v/v) ethanol and 20% (v/v) glacial acetic acid for 3-4 days of staining. After clearing in a 1% (w/v) KOH solution for 1-2 days, they were stained for 1 day in 0.025% (w/v) Alizerin Red in 1% KOH. Finally, stained and cleared skeletons were rinsed once in water and transferred to a 1:1 solution of glycerin and 70% ethanol (v/v) for photography and storage. Six litters for each of the PBS and DMSO control groups were examined; ten litters were examined for each experimental group. The numbers of resorptions, live fetuses, and defects were noted for each litter, and the type and severity of limb malformation(s) were noted for each fetus. Percentages of live fetuses at GD 18 and live fetuses with defects were calculated; the percentages of postaxial ectrodactyly, intermediate ectrodactyly, and other defects were determined.

Nile blue sulfate staining: Nile blue sulfate (NBS) is a vital stain that is sequestered into apoptotic bodies and phagolysosomes found in cells neighboring apoptotic cells (Allen et al., 1997). At GD 9:14 or GD 10:0 embryos were dissected from extraembryonic membranes and transferred to a 1:50,000 solution of NBS (Kotch et. al 1992) in lactated Ringers solution. Embryos were then

incubated for 30 minutes at 37°C with agitation every 10 minutes. Following staining, embryos were rinsed in cold Ringers solution and the right forelimbs photographed using a Nikon D70 camera mounted on a Leica DMRB microscope. At least three embryos from each of three litters were stained and photographed for visual comparison.

Whole mount TUNEL staining: Following treatments, embryos at GD 9:14 were dissected from extraembryonic membranes and transferred to 4% paraformaldehyde overnight at 4° C. Embryos were washed 3x in PBS and then washed 3x 30 min in PBX (PBS + 1% Triton X). Embryos were pre-incubated 30 min at 37° C in TdT labeling buffer. The labeling reaction was conducted using the reaction mixture provided by Trevigen (Gaithersburg, MD) for 3 hours at 37 ° C. After a series of PBX washes, a 1:500 dilution of Streptavidin-Fluorescein (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) solution was used to label the TdT enzyme. Embryos were washed and visualized on a TE300 Nikon fluorescence microscope and photographed with a RT Slider Spot Camera. Three embryos from each of three litters were stained and photographed for visual comparison.

LysoTracker Red staining: To examine the ability of RA administration to prevent ethanol-induced cell death, pregnant mice were treated on day 9:6 with ethanol alone (as described above) or with ethanol plus 2.5 mg/ml RA in corn oil. RA was administered by gavage 30 minutes following the first ethanol injection. Control groups included a vehicle control (corn oil and PBS) or a dose of 25 mg/kg RA and 0.015 ml/g PBS. Three embryos from three litters of each control or experimental group were stained with LysoTracker Red (LTR). For visualization of cell death patterns, a procedure previously published by Zucker et al. (1998), was used for confocal microscopic imaging of LTR staining. Stained specimens were cleared in a solution of 1:2 (v/v) benzyl alcohol and benzyl benzoate and then sealed in specially-made aluminum slides. The right forelimbs of embryos were initially visualized using a fluorescent microscope and representative specimens were chosen for subsequent visualization by confocal microscopy. The specimens were imaged using a Leica laser scanning confocal microscope (TCS-SP) with a 10x objective. The LTR dye was excited using the

568 nm laser line; the emission fluorescence was observed between 580-630 nm. Specimens were approximately 1.2 mm thick and were analyzed at 20 μm intervals. Using Leica software, data were prepared for presentation as a maximum projection.

Probe preparation and whole-mount in situ hybridization: At GD 9:12 the embryos were removed from the uteri and placed in cold PBS. Three stage-matched embryos from each of three litters were utilized to provide a reliable sample of embryos. Antisense RNA probes were hybridized to embryos as described by Correia and Conlon (2001). The probes for dHand, Shh, and Tbx5 were kindly provided by E. Olsen, E. Michaud, and V. Papaioannou, respectively. Three embryos from each of three litters were stained and photographed for visual comparison.

RNA extraction, reverse transcription and real-time PCR: Right forelimb buds from a single litter were obtained for quantitative real-time PCR (qPCR). They were put into a microtube, and immediately placed on dry ice before transferring to -80 degree storage. Five samples (1 litter per sample) were used for each treatment or control group. Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen) according to the provided protocol. Traces of genomic DNA were removed using the RNase-free DNase Set (Qiagen). RNA was eluted in 40 ul of RNase-free water. For reverse transcription, 20 ul of reaction mixture from the High Capacity cDNA Archive Kit (Applied Biosystems) was mixed with 400 ng of sample RNA in 20 ul of water. Reverse transcription reactions were run according to the suggested protocol.

TaqMan Universal PCR MasterMix and probes (Applied Biosystems, Foster City, CA, USA) were used for the PCR step: Cyp26a1 - Mm00514486_m1; dHand - Mm00439247_m1; Fgf8 - Mm00438921_m1; Shh - Mm00436527_m1; Tbx5 - Mm00803521_m1. Amplification and detection were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following profile: 1 cycle at 94 degrees for 10 min, and 40 cycles each at 95 degrees for 15 sec and 60 degrees for 1 min.

The threshold cycle (Ct) was determined for each sample and primer combination. The relative expression of each mRNA was calculated by the comparative Ct method, using the value

obtained by subtracting the average Ct value of GAPDH mRNA from the Ct value of each mRNA: the ΔCt. ΔCt calculations were based on mean GAPDH Ct values for each treatment or control group. ΔΔCt values were obtained by subtracting the ΔCt value of a 0 hour group from that of each treatment or control group. Data are represented as fold change: $2^{-(\Delta\Delta Ct)}$. T-test comparisons were conducted on ΔCt values. Because a fraction of embryos develop limb malformations, P values < 0.10 were considered to have biological significance.

Section 2.4 Results

Skeletal morphology

As evident in skeletons stained with Alcian blue and Alizarin red (figure 2.1), each experimental exposure resulted in limb reduction defects. Ethanol administration on GD 9:6 and 9:10 resulted in 67% of fetuses with forelimb defects per litter. Of the affected limbs, 71% exhibited postaxial ectrodactyly, 21% had an intermediate ectrodactyly, and 8% had other limb defects (table 2.1). GD 9:6 disulfiram exposure resulted in 30% of fetuses per litter with a limb defect. Postaxial ectrodactyly was found in 68% of the affected limbs. Intermediate ectrodactyly was observed in 21% of limbs with defects, and the remaining defects were seen in 11% of the affected limbs. BMS-189453 administration on GD 9:6 resulted in no limb defects. However, exposure on GD 9:10 resulted in 72% of fetuses per litter with a limb defect. Of the affected limbs, 41% had postaxial ectrodactyly, 13% had intermediate ectrodactyly, and 47% had other remaining limb defects. These remaining limb defects mainly consisted of large spacing between adjacent digits, a type of defect that was present in all three treatment groups. Control fetuses exposed to PBS or DMSO did not exhibit any limb defects. Ulnar agenesis or distal deficiencies accompanied postaxial loss of three or more digits. In these limbs, there was also a noticeable decrease in the overall size of the limb. Defects of radius, humerus, and scapula were not found in any treatment group. In summary, ethanol, disulfiram, and BMS-189453 treatments produced an array of forelimb malformations common to all treatment groups.

Cell death

Limbs of control embryos exhibited very little NBS staining in the developing AER at GD 9:14. However, excessive NBS staining was obvious in GD 9:14 embryos exposed to ethanol. At 18 hours after the onset of ethanol exposure (GD 10:0) a high level of staining was evident relative to control groups (figure 2.2b). Staining was found at the distal apex of the AER near the center of the preaxial-postaxial axis. A minority of specimens had a slight postaxial shift of this staining pattern.

BMS-189453-treated embryos likewise exhibited a similar localization of NBS to the AER at GD 9:14 and GD 10:0 (figure 2.2c). While ethanol and BMS-189453 both produced similar NBS localization, ethanol-treated embryos were more intensely stained than those treated with BMS-189453. Disulfiram treatment resulted in a cell death pattern similar to BMS-189453 and ethanol treatment by GD 10:0 (figure 2.2d), but were not distinguishable from control limb buds at GD 9:14.

Prevention of ethanol-induced cell death by Retinoic Acid

As with NBS, LTR staining was evident within 8 hours of exposure to ethanol (figure 2.3c). Staining of the AER was intense in ethanol-exposed embryos, contrary to the staining in vehicle and RA-treated embryos (figures 2.3a,b). LTR staining revealed mesenchymal staining within the limb buds of ethanol-treated embryos, a phenomenon not observed using whole mount TUNEL or NBS staining techniques. Control or RA-treated limb buds had very little LTR staining in the AER or mesenchyme. Embryos exposed to both ethanol and RA exhibited a greatly reduced LTR-positive domain, compared to those embryos exposed to ethanol, alone. However, the mesenchymal staining observed in ethanol-exposed embryos was not prevented with RA co-treatment. Whole mount TUNEL and NBS staining techniques provided identical findings with respect to the AER of control and treatment group limbs.

In situ hybridization and real-time PCR

In the forelimb buds of GD 9:12 embryos, dHand was expressed in the postaxial mesenchyme. It was also present in the lateral plate mesoderm proximal and caudal to the limb bud. In most GD 9:12 embryos exposed to ethanol for 6 hours, the dHand expression domain was noticeably smaller and less intense, relative to embryos exposed to PBS (figure 2.4 a,b). Quantitative Real-time PCR (qPCR) indicated that dHand is decreased by ethanol relative to controls at GD 9:12 and GD 10:0 ($P = 0.355$ and $P = 0.084$, respectively; figure 2.5a). BMS-189453 did not have an affect on dHand transcription at either time.

Shh expression was undetectable in the limb buds of GD 9:12 embryos. However, by GD 10:0 a strong signal was present on the postaxial margin of the limb bud in control embryos (figure 2.4g). Embryos exposed to ethanol exhibited little or no observable Shh expression at GD 10:0. In the few instances in which Shh staining was observed in ethanol-exposed embryos a weaker expression domain was present than in the limbs of comparably-staged control embryos. qPCR demonstrated that expression of Shh is extremely low at GD 9:6, the time of initial ethanol exposure (Ct = 32 cycles). In control embryos, six hours later (GD 9:12), expression increased 5.0 fold before climbing an additional 12.8 fold by 14 hours (GD 10). Ethanol decreased *Shh* expression substantially at 6 hours and 18 hours of exposure ($P = 0.006$ and 0.062, respectively; figure 2.5b). BMS-189453 had no affect on *Shh* expression after 2 hours of exposure (GD 9:12), but by GD 10:0, expression was decreased 75% ($P = 0.009$).

Tbx5 expression was present throughout the limb buds of GD 9:12 control embryos and those exposed to ethanol. Ethanol had no discernable effect on the level or distribution of Tbx5 expression in the limb at this time (figure 2.4c,d). However, by GD 10:0 Tbx5 expression was substantially higher in the limb buds of control embryos, compared to the expression of Tbx5 in the limbs of ethanol-exposed embryos (figure 2.4e,f). qPCR confirmed these findings; no substantial change was evident at GD 9:12 following ethanol exposure, but by GD 10:0 ethanol had decreased *Tbx5* transcripts ($P = 0.021$; figure 2.5c). BMS-189453 had no affect on Tbx5 expression at any time examined.

Fgf8 expression was assayed by qPCR. Ethanol exposed limb buds contained 44% of the level of transcripts found in control limb buds at GD 9:12 ($P = 0.070$). By GD 10:0, expression levels were comparable between ethanol and control limb buds (figure 2.5d).

To confirm the RAR antagonist activity of BMS-189453, Cyp26a1expression was examined, as this gene is sensitive to changes in RA (White et al., 1997). In the presence of BMS-189453, Cyp26a1expression was decreased relative to the control group at GD 9:6 ($P = 0.147$) and GD 10:0 ($P = 0.002$; figure 2.5e). BMS-189453 and exogenous RA co-treatment resulted in expression levels intermediate to control and BMS-189453 treatment groups. Ethanol had a minor negative affect on Cyp26a1expression (GD 9:6, $P = 0.2731$; GD 10:0, $P = 0.3871$), but in combination with RA had a robust affect, increasing expression 18 fold. In general, the affect of exogenous RA in combination with ethanol exposure was to enhance the affect of ethanol alone.

Section 2.5 Discussion

This study supports the hypothesis that ethanol perturbs RA-mediated limb development. Unlike previous investigations involving a long-term attenuation of the RA signal in the limb bud, this study demonstrates that a single exposure to a RAR antagonist or ALDH inhibitor results in a narrow range of digital defects. The induction of these defects is associated with dramatically increased cell death in the developing AER and a 44% decrease in Fgf8 expression. The induction of excessive cell death by a RAR antagonist or ALDH inhibitor demonstrates a novel role for RA in AER maintenance. The similarity found in malformations and pathogenesis of acute exposure to BMS, disulfiram, and ethanol is suggestive of a common teratological mechanism. Co-administration of RA prevents ethanol-induced excessive cell death, demonstrating that ethanol's induction of AER cell death is either upstream of RA function or mediated by perturbed RA signaling. In addition, the nascent ZPA, as identified by the RA-inducible genes dHand and Shh, is a target of ethanol exposure. The common changes in RA-regulated gene expression in the developing limb combined with common pathogenic changes and abnormal phenotype produced by ethanol, a RA antagonist, and an

ALDH inhibitor support the hypothesis that one facet of ethanol's mechanism for induction of limb dysmorphogenesis is disruption of RA-mediated development. While it is likely that the pathogenesis of ethanol induced-limb malformations involves perturbation of RA-mediated development, only an examination of the early transcriptional changes following ethanol exposure (prior to 6 hours) will reveal whether RA plays a central role in the mechanism of ethanol's teratogenesis.

The observed distribution of cell death in the developing AER is typical of previous descriptions of teratogenesis following ethanol administration on the 9th day of gestation (Kotch et al., 1992). The mechanism(s) whereby ethanol impacts cellular and developmental targets resulting in changes essential to produce malformations are unknown. The excessive cell death in the AER that occurs following ethanol exposure may result from the direct toxicity of ethanol or from perturbation of supporting cell populations, the ZPA and SRM. In favor of this latter hypothesis, disulfiram and BMS-189453 induce excessive cell death in the AER, presumably as a result of its requirement for RA-dependant supporting cell populations. Because of the close similarity between the pathogenesis of ethanol, BMS, and disulfiram exposures, cell death occurring by a RA-dependent mechanism is favored for ethanol, rather than a direct toxicity. Prevention of ethanol-induced excessive cell death in the AER by exogenous RA further supports this hypothesis.

Though recognized as a component of ethanol's pathogenesis in the limb, there has been no study to date investigating the effects of excessive cell death in the AER on other limb tissues. Since removal of the AER results in apoptosis in the SRM (Sun et al., 2002) and a great number of AER cells die following ethanol administration, it is surprising that cell death is not also prevalent in the SRM. Detailed histological studies over a number of developmental stages are necessary to examine the SRM in regions of overlying AER cell death. Fgf8 expression accompanies cell death in the AER at GD 9:12, but the correlation does not extend to 18 hours as cell death remains abundant in most embryos and Fgf8 levels return to normal by GD 10. This observation is likely the result of the declining proportion of FGF8-expressing AER cells relative to mesenchyme; *in situ* hybridizations,

like that conducted by Chrisman et al. (2004) at later stages following ethanol exposure demonstrates the persistent downregulation of FGF8 in the AER coincident with the loss of cells.

Interestingly, the RA-dependant gene Tbx5, is downregulated following ethanol exposure by GD 10:0. Being responsible for sustaining the AER through Fgf10 expression, it is possible that depressed Tbx5 levels result in the continued cell death observed 24 hours after the onset of ethanol treatment (Kotch et al., 1992). The use of vital stains such as NBS that detect the later stages of apoptosis and the removal of cellular debris resulting form apoptosis (Allen et al., 1997) limits speculation regarding the exact timing of the onset of apoptosis.

Because the AER positions and maintains the ZPA, cell death in the posterior regions of the AER would be expected to reduce Shh expression and cell proliferation in the ZPA. Indeed, as shown in this study, Shh expression is reduced within 6 hours of ethanol administration, and Kotch et al. (1992) demonstrated that proliferation is compromised in those cells that will give rise to the posterior mesenchyme. Despite these observations, excessive cell death is infrequently observed in the region of the AER that overlies the nascent ZPA. This suggests that the ZPA may be a target of ethanol, independent of the effects on the AER. Further precisely timed experiments will be necessary to discern the subpopulation of cells that is the proximate target of ethanol.

William Scott's laboratory has proposed that limb defects arising from GD 9 teratogen exposure disrupts the AER-ZPA epithelial-mesenchymal interaction without interfering with the transcription of Shh. Only when posterior mesenchymal cell loss becomes evident following acetazolamide or cadmium exposure, is Shh transcription lowered (Bell et al., 1999, 2005; Scott et al., 2005). These, and many other teratogens including ethanol induce postaxial ectrodactyly. A previous investigation into the effects of ethanol demonstrated a decrease in Shh expression, at 24-48 hours after initial ethanol exposure (Chrisman et al., 2004) when postaxial tissue loss would be evident. The present study, examining earlier time-points, reveals that ethanol downregulates Shh expression shortly after the onset of exposure. Ethanol-induced reduction of Shh transcripts may either result from the attenuation of Shh expression or from reduced numbers of Shh-expressing cells. Regardless,

the findings of this investigation demonstrate that ethanol decreases the expression of Shh transcripts at earlier stages of limb development than other limb teratogens examined to date.

Cell death observed in the proximal limb mesenchyme was only detectable using LTR staining and clearing procedures. This ethanol-sensitive population has been identified previously (Kotch et al., 1992) by sectioning the limb bud prior to staining. It is notable that the proximal mesenchyme is a location of RA synthesis (Swindell et al., 1999; Mic et al., 2004). As such, these cells are also expected to be a site of ethanol metabolism in the limb bud and may produce toxic concentrations of superoxide leading to cell death. Ethanol induced cell death in this particular subpopulation of cells would be expected to reduce RA availability to the limb bud. However, administration of exogenous RA appears to have little effect on the amount of cell death induced in this subpopulation by ethanol.

Qualitative comparisons of control limb buds with RA-only control limbs buds demonstrates that RA does not, itself, prevent normal programmed cell death in the AER (compare figures 2.3b and 3d). However, RA remarkably decreases the onset of excessive AER cell death caused by ethanol exposure. Given the various responses among different tissue types, it is not surprising that co-administration of ethanol and RA prevented ethanol-induced cell death in the AER, yet did not affect the degree of cell death in other tissues such as limb mesenchyme, or the neighboring somites (figure 2.3). These findings further implicate a pathogenesis for ethanol-induced limb defects, involving the perturbation of RA-mediated development.

Because the co-administration of RA and ethanol leads to embryonic lethality by GD 10:12 (data not shown), assessment of the ability of RA to rescue ethanol-induced limb defects is not possible. It is clear from the literature that co-administration of ethanol and RA do not have a consistent combinatorial affect. Chen et al. (1996) have shown that RA's precursor retinol, in combination with ethanol exhibited an additive affect, reducing viability. The same combination had a synergistic effect, increasing the number of craniofacial malformations over ethanol alone. Twal et al. (1997) found that RA antagonized the effects of ethanol on heart development. We conjecture that

the cardiovascular or another vital embryonic system is adversely affected by an embryolethal combination of treatments.

Disulfiram, known as the prescription drug, Antabuse, causes limb defects in this mouse model and cell death by GD 10:0. The teratogenicity of disulfiram has been noted previously by Webster et al. (1983), although no sensitive region of the embryo was identified. Commonly prescribed to alcoholics to discourage consumption, disulfiram prevents the metabolism of consumed alcohol by inhibiting ALDH, resulting in acetaldehyde accumulation and nausea. A link between disulfiram exposure and human limb defects has been previously suggested (Nora et al., 1977). However, it is difficult to establish a causal link in humans because it is possible that alcohol, which we have shown causes identical malformations, is concurrently present. Results of the present study indicate that disulfiram is, indeed, teratogenic to the limbs, producing intermediate and postaxial ectrodactyly. Preliminary observations also suggest risk of craniofacial abnormalities. Considering the findings of the current study, prescription of disulfiram to women of child-bearing age should be carefully weighed against the evident risk of birth defects.

This investigation has demonstrated the necessity of RA for AER maintenance, while others have demonstrated the role of RA in activating ZPA gene expression (Riddle et al., 1993; Fernandez-Teran et al., 2000). That at least two important regions of the limb bud, the developing AER and dHand and Shh-expressing posterior mesenchyme, are affected by ethanol exposure within hours is suggestive of a common mechanism. As dictated by minor but significant differences in developmental stage, or other variations among embryos, ethanol may preferentially impact one cell population over another, or the same population at different stages giving rise to a variety of defects of the digits. As all of these malformations may occur within a single litter, resolving this question may prove difficult without utilizing whole embryo culture or similar *in vitro* approaches where a narrower range of developmental stages may be isolated. In addition, the relationship between ethanol and Shh deserves much attention. The rapid downregulation of Shh expression by ethanol may be mediated by RA; this and other hypotheses must be scrutinized, as Shh appears to be a

common target of ethanol during organogenesis and perturbation of its signaling may account for a number of ethanol-induced malformations.

Table 2.1. Effects of chemical exposure on forelimbs of GD 18 fetuses. As described in the Materials and Methods, Ethanol was administered at GD 9:6 and 9:10. BMS-189453 was administered on GD 9:10 and disulfiram at GD 9:6.

	Litters Examined	Live Fetuses examined	Mean per litter		
			implantations	live fetuses	live with defects
Control – ethanol vehicle	5	30	7.2 (± 0.837)	6.0 (± 1.225)	0.0 (± 0.000)
Ethanol (2.9 g/kg)	10	70	7.9 (± 2.378)	7.0 (± 2.708)	4.7 (± 3.234)
Control – BMS-189453 vehicle	5	34	8.2 (± 0.447)	6.8 (± 1.304)	0.0 (± 0.000)
BMS-189453 (50mg/kg)	10	56	7.8 (± 1.135)	5.6 (± 2.503)	2.4 (± 2.119)
Control – disulfiram vehicle	5	41	9.2 (± 0.837)	8.2 (± 1.483)	0.0 (± 0.000)
Disulfiram (75 mg/kg)	10	44	6.8 (± 1.229)	4.4 (± 2.716)	1.3 (± 1.829)

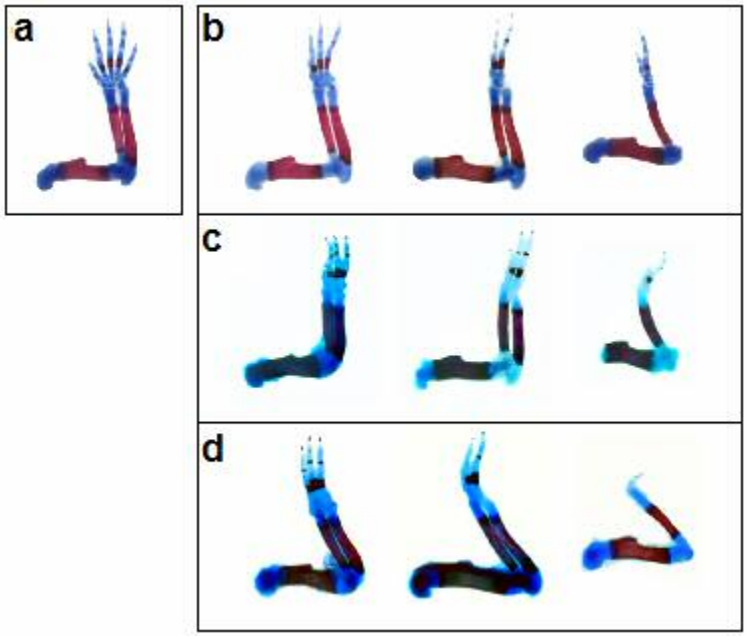


Figure 2.1. Skeletal staining reveals abnormalities in the forelimbs of GD 18 C57BL/6J mice. Postaxial defects are common in mice exposed to ethanol (b), the RAR antagonist, BMS-189452 (c), or the ALDH inhibitor, disulfiram (d) during the 9th day of development. Defects are not found in vehicle control groups (a).

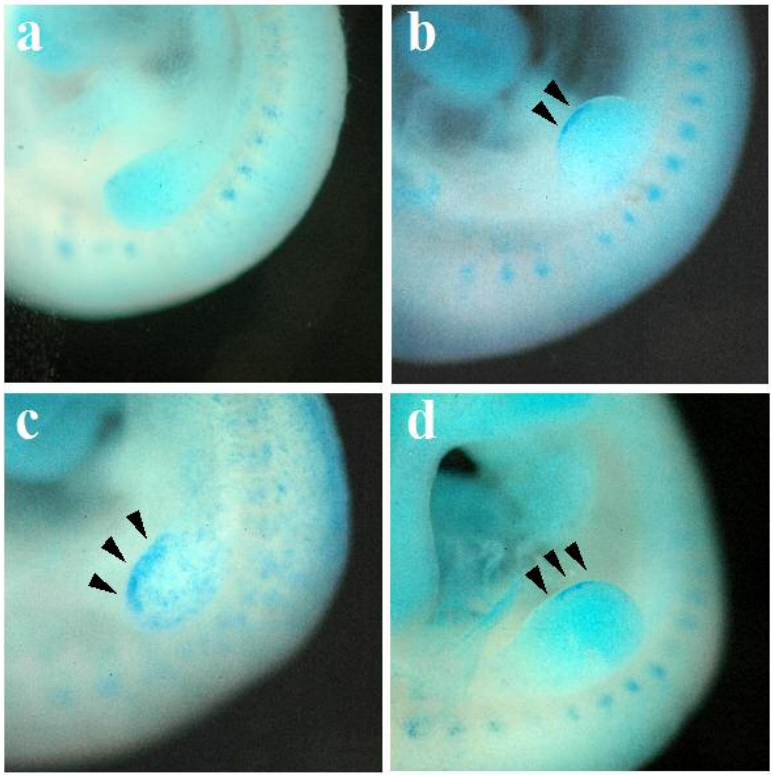


Figure 2.2. NBS staining of GD 10 embryos demonstrates excessive cell death (arrows) in the presumptive AER of the forelimb following exposure to ethanol (b), disulfiram (c), or BMS-189453 (d). A small amount of staining is present in vehicle-exposed embryos. Ethanol was administered on GD 9:6 and 9:10; disulfiram was administered on GD 9:6; BMS-189453 was administered on GD 9:10. The left side of images is the cranial aspect of the embryos; the right forelimb is shown.

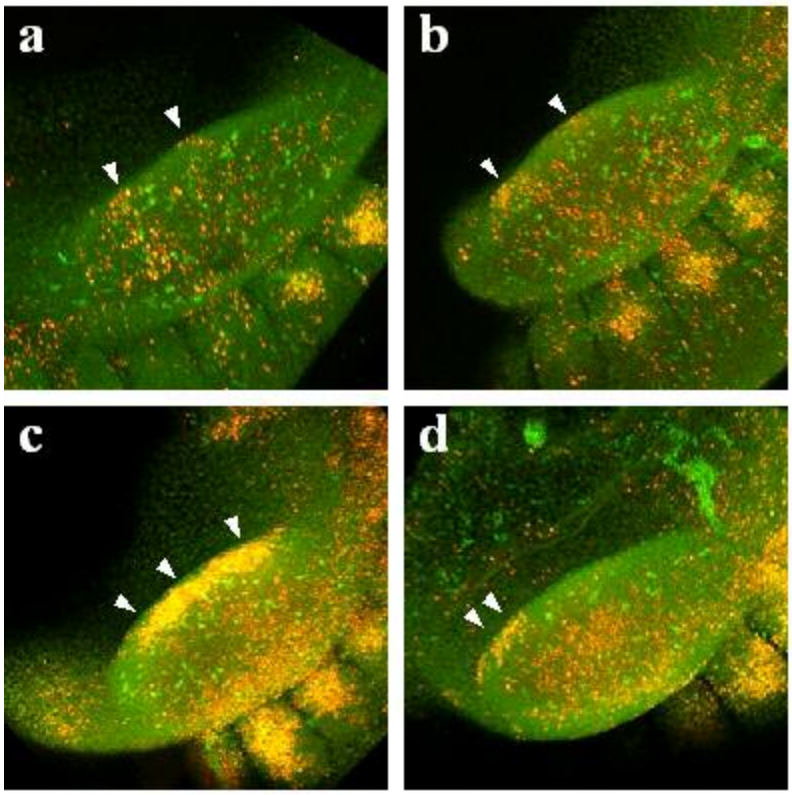


Figure 2.3. Three-dimensional reconstruction of serial optical sections through mouse embryos stained with LystoTracker Red (LTR), obtained by Confocal Laser Scanning Microscopy. LTR staining of GD 9:14 embryos demonstrates the prevention of ethanol's apoptotic effect on the presumptive AER with RA co-administration. Vehicle (a) and RA-treated controls (b) have a small amount of LTR staining (orange). GD 9:6 and 9:10 Ethanol treatment results in intense staining along the presumptive AER (c). Embryos exposed to both ethanol and RA (d) show reduced staining in the same region. Arrows indicate localized LTR staining in the presumptive AER. Green autofluorescence results from gluteraldehyde fixation to allow visualization of the embryo. The right forelimb is shown, with the cranial aspect of embryos towards the top of the page.

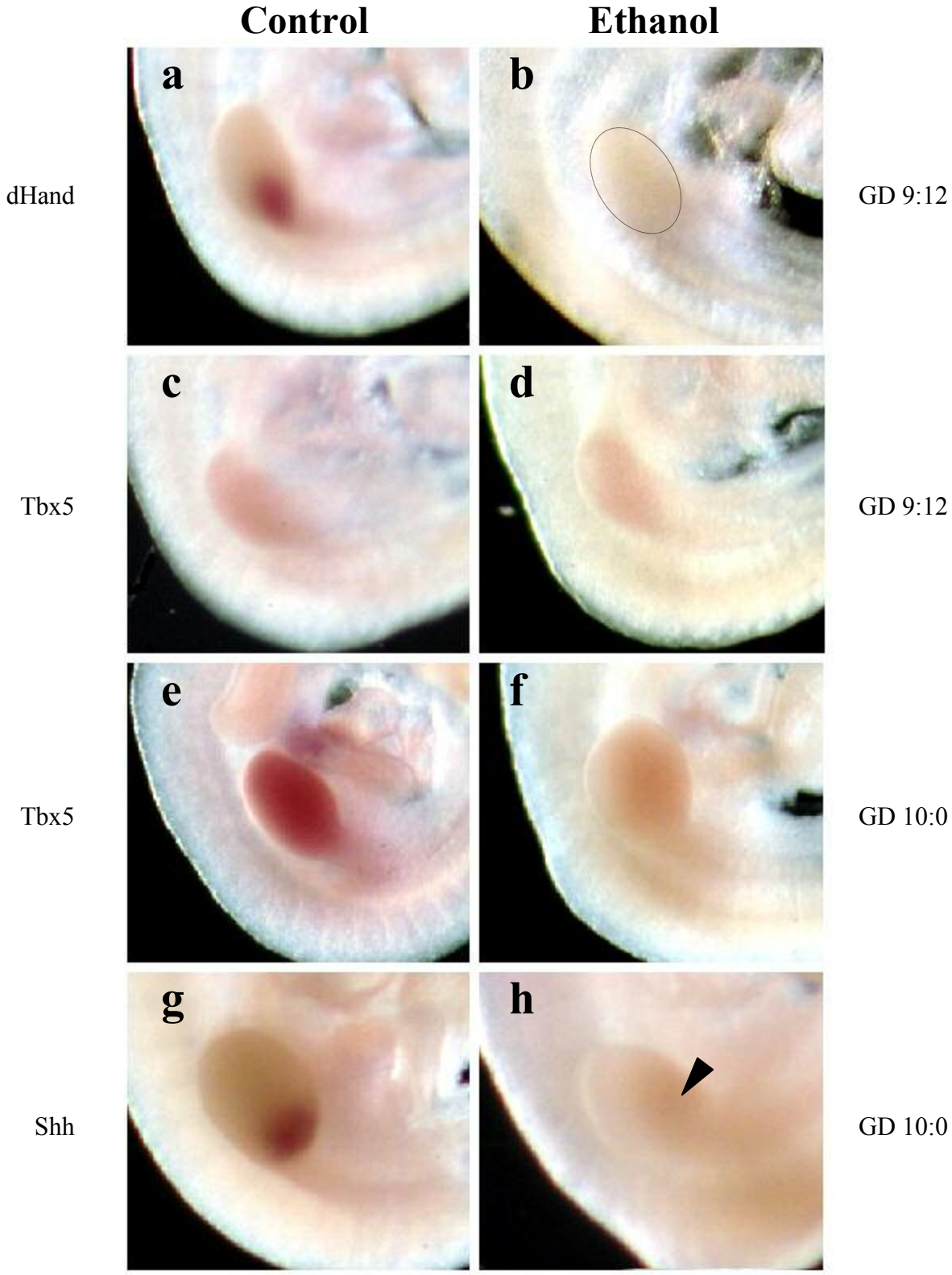


Figure 2.4. *In situ* hybridization of vehicle and ethanol-exposed embryos. dHand expression is shown in embryos exposed to PBS (a) or ethanol (b) for 6 hours. Tbx5 expression is shown in embryos exposed to PBS or ethanol for 6 (c,d) and 18 hours (e,f). Shh expression is shown in embryos exposed to PBS (e) or ethanol (f) for 18 hours. Arrow indicates faint Shh expression in ethanol-exposed embryo.

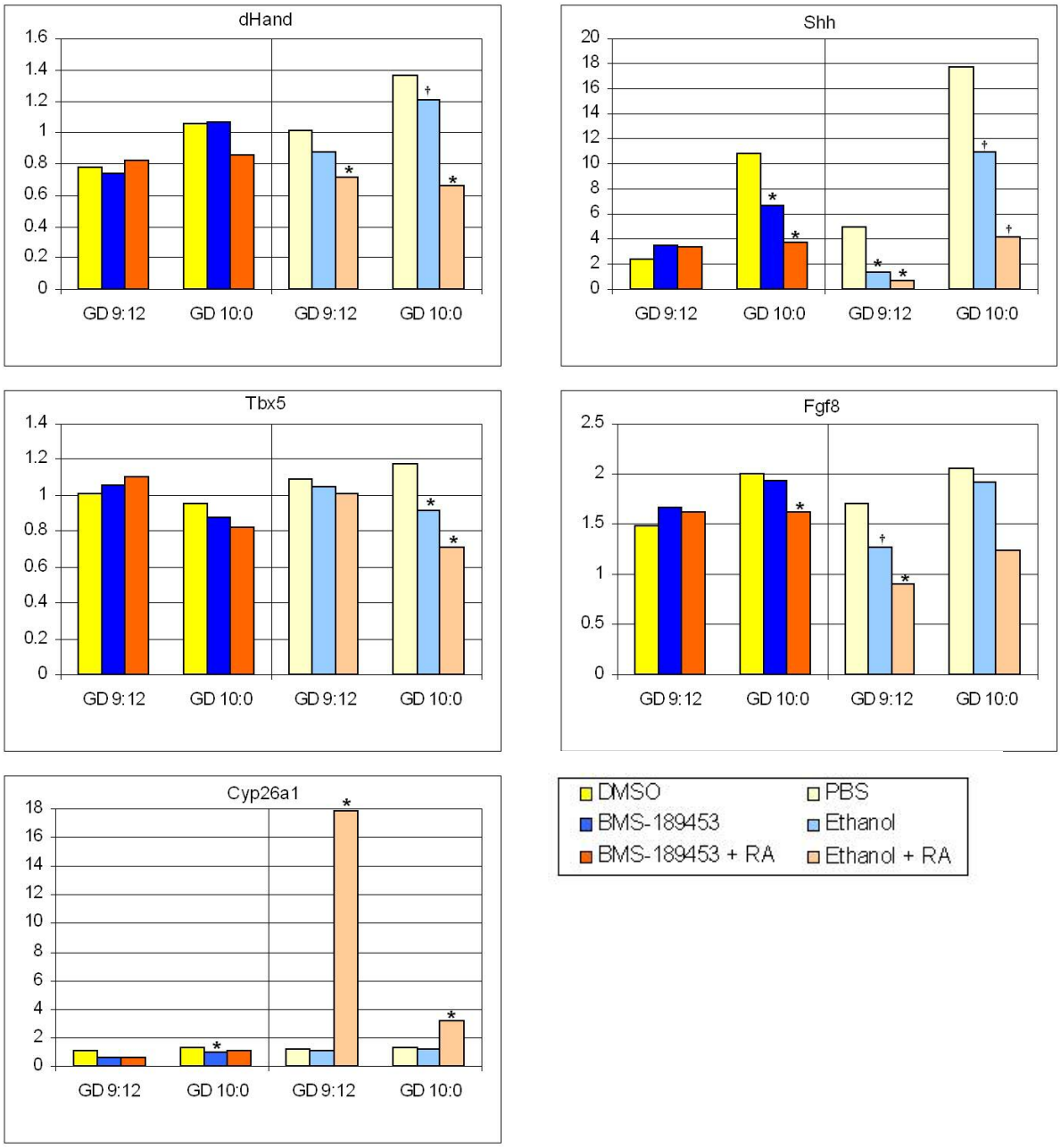


Figure 2.5. Quantitative *real-time* PCR analysis of transcripts found in limb buds exposed to ethanol or BMS-189453 alone, or in conjunction with RA. Data demonstrate the similar effects of ethanol and BMS-189453 on RA-dependent genes, important to limb development. Co-administration with RA has variable effects relative to ethanol or BMS-189453 exposure. Data were converted to fold change and normalized to a no-treatment control group at GD 9:6. * $P < 0.05$; † $0.05 < P < 0.10$.

CHAPTER III
A microarray analysis exploring ethanol's teratogenesis in the mouse forelimb

Section 3.1 Abstract

Background: Fetal alcohol spectrum disorders include a diverse array of behavioral abnormalities and physical malformations. Using a C57BL/6J mouse model, it has been possible to recapitulate many of the effects that maternal ethanol consumption has on human development. In this model, limb defects are produced with high incidence following maternal ethanol exposure on the 9th day of pregnancy. An analysis of ethanol-induced transcriptional changes in the developing embryo was undertaken to provide insights into ethanol's biological responses that occur within hours of exposure.

Methods: Microarray analysis was utilized to examine the transcriptional response of the developing forelimb bud to a dose of ethanol known to result in limb malformations. Transcriptional changes elicited by ethanol shortly after exposure, prior to the overt onset of cell death, were scrutinized. Using Genespring and Ingenuity Pathway Analysis, gene expression was analyzed at 2, 4, and 6 hours following ethanol exposure. Pathway, functional, and network analyses were used to elucidate the biological significance of ethanol-induced transcriptional alterations. Lastly, transcriptional changes resulting from ethanol were compared to those that followed embryonic exposure to a pan-retinoic acid (RA) receptor (RAR) antagonist, BMS-189453, to determine whether a correlation was present between the transcriptional effects of RA signaling abrogation and ethanol exposure.

Results: Following exposure to ethanol, the mouse forelimb bud exhibited transcriptional alterations consistent with changes in IGF-1, JAK/Stat, Wnt/β-catenin, and PPAR signaling pathways. Metabolic pathways were also perturbed, including pyrimidine, purine, and pyruvate metabolism. NF-κB and AP-2α, genes of known importance to limb development, were likewise affected by ethanol exposure. Functional analysis revealed a significant perturbation in cell-cycle control and the

processes of proliferation, cell death, and cell adhesion. Embryonic exposure to a RAR antagonist did not result in transcriptional changes that correlated with those observed at any of the 3 time points following ethanol exposure that were examined.

Conclusions: Ethanol produces transcriptional changes in signaling pathways important to the growth and patterning of the embryonic forelimb bud. Essential processes that mediate and execute developmental programs are impacted by ethanol exposure, indicating that alterations in cell adhesion, proliferation and cell death occur within 2-6 hours after ethanol exposure. A mechanism of ethanol's teratogenesis involving perturbation of RA signaling or synthesis is not consistent with transcriptional changes evident within this time frame. Abrogation of RA signaling at a later time, as has previously been observed, cannot be considered mechanistic given the wide array of ethanol's molecular, cellular, and developmental effects. Importantly, these early transcriptional changes indicate perturbations in processes and signaling pathways that participate in, or contribute to the mechanisms of ethanol-induced forelimb dysmorphology and likely have application to other regions of the embryo that are sensitive to ethanol's teratogenicity.

Section 3.2 Introduction

Fetal alcohol spectrum disorders (FASD) are a diverse array of behavioral and structural abnormalities attributable to gestational ethanol exposure. The structural abnormalities may involve the craniofacies and include the characteristic facial features of fetal alcohol syndrome (FAS) as well as defects of the CNS, cardiovascular, urogenital, and musculoskeletal systems. Contributing to the varied outcome are the timing, amount, frequency, and duration of prenatal ethanol exposure, as well as genetic background and other modifying factors such as maternal nutritional status and concurrent use of other drugs of abuse.

Animal studies, in which such contributing factors can be controlled, have illustrated the developmental stage sensitivity of the various organ systems to ethanol-induced teratogenesis (Sulik and Johnston 1983; Webster et al., 1983; Daft et al., 1986; Gage and Sulik 1991). Of particular

significance for the present investigation are those studies in which ethanol's effect on the developing limbs have been examined. Kotch et al. (1992) showed that acute maternal ethanol treatment of C57Bl/6J mice on the 9th day of gestation (GD 9) yields a high incidence of predominantly right-sided forelimb defects. The most commonly occurring limb defect following ethanol exposure is postaxial ectrodactyly, which may or may not be accompanied by ulnar deficiencies. Intermediate ectrodactyly and wide spacing between the digits are also observed with some frequency.

As with studies of other regions and organ systems, the pathogenesis underlying ethanol-induced limb defects includes excessive cell death in selected cell populations. First shown by Kotch et al. (1992), and confirmed by Chen et al. (2004) and Johnson et al. (Chapter II), within hours of maternal ethanol treatment, excessive cell death occurs in the apical ectodermal ridge (AER), a columnar population of ectoderm cells that is found on the distal margin of the limb bud. Due to its critical role in development, insult to the AER has profound effects on the morphogenesis of the limb. The AER has two known functions. First, it permits distal outgrowth by maintaining the mesenchyme subjacent to the AER, the subridge mesenchyme (SRM), in a proliferative state. Secondly, the AER sustains the zone of polarizing activity (ZPA), the posterior organizing center. Both of these mesenchymal populations, in turn, maintain the AER through a positive feedback mechanism. Whether ethanol's apoptotic affect on the AER is direct or subsequent to insult to adjacent cell populations remains unknown.

Although cell death is a readily notable pathogenic feature, alterations in other morphogenic events including cell proliferation, migration, and signaling are also expected in ethanol-exposed limb buds as these responses are typical of prenatal ethanol exposure as shown by others (Nakatsuji and Johnson, 1984; Jing and Li, 2004; Miller et al., 2002; Lindsley et al., 2006). Chrisman et al. (2004), have demonstrated ethanol-induced transcriptional changes that indicate developmental patterning is disturbed in the limb bud. However, whether these observation result from ethanol exposure or are a consequence of the ethanol-induced cellular deficiencies is not known.

Determination of the proximate cellular and molecular mechanism(s) by which ethanol adversely effects development is an active area of investigation. Among the potential mechanisms under investigation in several laboratories are alterations in placental function (reviewed in Schenker et al., 1990), prostaglandin synthesis (Anggard 1983; Pennington et al., 1985), protein synthesis (Henderson and Schenker 1977; Henderson et al., 1980), membrane fluidity (Chin and Goldstein, 1980; Goldstein et al., 1980), ion influx (Debelak-Kragtorp et al., 2003), cell adhesion (Charness et al., 1994; Ramanathan et al., 1996), retinoic acid (RA) synthesis and generation of oxygen radicals. Of particular note for the current study are the latter two.

The possibility that perturbation of RA-mediated development is a mechanism of ethanol's teratogenesis was advanced by Duester (1991) and Pullarkat (1991). They observed that ethanol may competitively inhibit alcohol dehydrogenases (ADHs), enzymes that both synthesize RA and metabolize ethanol. Owing to RA's integral role in development, a decrease in RA production would be expected to result in dysmorphic limbs. Indeed, Deltour et al. (1996), have documented a reduction in RA levels in embryos following ethanol exposure. Others have noted that transcriptional changes in RA signaling occur following ethanol exposure (reviewed in Zachman and Grummer, 1998), which may indicate a RA-mediated mechanism independent of interference with its production. These data, along with the observation that morphological and pathogenic similarities exist between the effects of ethanol and RAR antagonism in the limb (Chapter II), suggest that embryonic ethanol exposure-induced changes in the synthesis of, or signaling by RA are responsible for ethanol's teratogenesis.

Regarding the production of oxygen radicals, induction of oxidative stress and reactive oxygen species (ROS) in embryonic tissue following ethanol exposure has been documented by several laboratories (Davis et al., 1990; Chen and Sulik, 1996; Johnson et al., 2004). The metabolism of ethanol to acetaldehyde and then to acetate increases the production of superoxide radicals. The presence of excessive superoxide and other ROS results in damage to macromolecules, the perturbation of signaling pathways that utilize ROS as second messengers, and the activation or

deactivation of ROS-responsive transcription factors such as NF-κB and AP-1. These changes may account for many of the malformations seen following embryonic ethanol exposure. This premise is supported by the fact that antioxidants can reduce the incidence of ethanol-induced malformations, including those of the limb (Kotch et al., 1995; Chen et al., 2004; the author's unpublished observations).

To investigate the early biological changes that occur in the limb bud following embryonic ethanol exposure, microarray analysis of transcriptional changes was conducted. To minimize detection of transcriptional adaptations that occur as a consequence of cell death or altered rates of proliferation, the present study focused on limb buds that had been exposed to ethanol for only 2, 4, and 6 hours. Previous descriptions of transcriptional changes following embryonic ethanol exposure have focused on changes in a small number of potential gene targets of ethanol (Yamada et al., 2005; Xu et al., 2005; Chrisman et al., 2004). The strength of a microarray analysis lies in the ability to simultaneously probe several hypothesized mechanisms of ethanol teratogenesis. In an effort to identify or confirm cellular and molecular targets of ethanol, functional and pathway analyses were used to assess transcriptional alterations reflecting ethanol's early effects on metabolism, cell signaling, and cellular activity. Because of the transcriptionally distinct subpopulations of cells within the limb bud, this investigation was expected to identify ethanol-sensitive subpopulations of the limb bud that had not previously been identified as important in the pathogenesis of ethanol-induced limb defects. In addition, transcripts of specific genes known to play a role in redox regulation and response were analyzed to determine if their response would indicate an early transcriptional modulation consistent with ethanol's early production of ROS. Finally, the hypothesis that ethanol perturbs RA-mediated development was tested. Using microarray analysis of ethanol-exposed limb buds to identify ethanol-induced transcriptional changes and a pan-RAR antagonist to identify RA-dependant genes in the limb bud, the transcriptional profiles of ethanol-induced, and RA-dependant genes were compared.

Section 3.3 Materials and methods

Animals: C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine and housed in a temperature and humidity controlled vivarium on a 14 hr light cycle. Mice were mated at the beginning of the light cycle at 8 am and inspected for a vaginal plug at 10 am. The presence of a plug indicated gestation day 0, hour 0 (GD 0:0).

In vivo exposure to ethanol: At day 9, hour 6 (9:6), pregnant mice were given an intraperitoneal (ip) injection of 25% (v/v) ethanol in phosphate buffered saline (PBS), followed by a second injection 4 hours later. The ethanol solution was administered at 0.015 ml/g of maternal body weight. This treatment paradigm results in dysmorphic limbs in a majority of fetuses (Chapter II; Kotch et al., 1992). Pregnant mice were killed at 2, 4, or 6 hours after the initial ethanol injection (GD 9:8, 9:10, or 9:12; henceforth named ethanol +2hr, ethanol +4hr, and ethanol +6hr, respectively). The embryos were then removed from the uteri and placed in cold PBS. Mice killed at 2 and 4 hours after injection did not receive a second dose of ethanol. A control group was treated with a 0.015 ml/g ip injection of PBS at GD 9:6. Control embryos were collected 4 hours after the PBS injection to serve as a control for ethanol treatment groups.

In vivo exposure to BMS-189453: Because the regulation of gene transcription by RA is known to be cell-type specific, and measures of the effects of RA on gene transcription vary widely among microarray experiments (van der Spek et al., 2003), our approach was to directly compare the effects of RA signaling abrogation and ethanol exposure in limb buds. To interrupt RA signaling the pan-RAR antagonist BMS-189453 (Ali-Khan and Hales, 2005; Yang et al., 1999; Chen et al., 1995) was used.

Mice were given a single dose of 10 mg/ml BMS-189453 in DMSO at day 9:10. This 50 mg/kg (0.005 ml/g) dose of BMS-189453 produces limb malformations and a pattern of excessive cell death in the embryonic limb bud that are consistent with the effects of ethanol exposure (Chapter II). A control group was treated with a 0.005 ml/g DMSO at day 9:10. Two hours following injection, the mothers exposed to BMS-189453 or DMSO were killed and the embryos removed from

the uteri in cold PBS.

RNA collection and probe preparation. Previous work in our laboratory has shown that the right forelimb is more susceptible to the dysmorphogenic effects of ethanol than the left (Chapter II; Kotch et al., 1992). The right forelimb was dissected from embryos having 20 to 24 somite pairs and frozen immediately on dry ice and subsequently transferred to -80^{o}C for storage. For each treatment replicate, forelimb buds from 3 litters were pooled and total RNA was extracted using Trizol Reagent (Gibco BRL, Rockville MD) according to the suggested protocol. One microgram of isolated total RNA was amplified using Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX) according to the suggested protocol for a single round of amplification. Prior to fluorescence labeling, samples were examined for purity using the Agilent 2100 Bioanalyzer. Samples with a 28s/18s ratio below 1.7 were rejected. As needed, amino allyl aRNA was labeled with Cy5 from CyDye Post-Labeling Reactive Dye Pack (Amersham Biosciences, Piscataway, NJ) according to the Amino Allyl MessageAmp aRNA Kit protocol. Using the above procedure, a pool of homogenized GD 10:0 embryos was used as the source of reference aRNA. Aminio allyl aRNA was stored in single-use aliquots at -80^{o}C until needed. Reference aRNA was labeled with Cy3.

Hybridization. One microgram each of the fluorescence-labeled aRNA probes were added to a hybridization mix (Hybridizatin Kit Plus; Agilent Technologies, Palo Alto, CA) and denatured at 95^{o}C for 2 minutes. The mixture was hybridized to Agilent Mouse cDNA Microarray G4104A (Agilent Technologies, Palo Alto, CA) slides for 17 hours at 65^{o}C utilizing LifterSlips (Erie Scientific,Portsmouth, NH) to ensure even distribution of the hybridization mixture. Slides were washed according to the protocol provided by Agilent. Slides were dried by centrifugation at 1000rpm for 3 min and scanned at a 10µm resolution using a ScanArray 4000 laser scanner (PerkinElmer Life Sciences, Boston MA). Spot and background intensity values were measured using GenePix Pro 4.1.1.39 (Axon Instruments, Union City CA).

Array analysis. Spots identified on the array that had a mean intensity value greater than the mean local background intensity, plus two standard deviations were considered *present*. In addition,

a gene was only included in the *present* gene list if it's spots had less than 25% saturated pixels in all replicates of at least one treatment group. Data were normalized using GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) using a per-spot and per-chip intensity-dependent (Lowess) normalization. In some instances GeneSpring was further used to normalize the means of treatment group genes to the means of control group genes.

To identify statistically significant pathways, networks, or cellular functions, Ingenuity Pathway Analysis (IPA) was utilized. Information about gene-gene relationships was obtained from the Ingenuity Pathways Knowledge Base (IPKB), unless otherwise cited. For Gene Ontology (GO) analyses, genelists were entered into Onto-Express (Wayne State University, Detroit, MI), which identified statistically over-represented GO terms associated with genes in a genelist.

*RNA extraction, reverse transcription and real-time PCR: The r*ight forelimb buds from a single litter of GD 9:10 mouse embryos exposed to ethanol or PBS for 4 hours or were obtained for quantitative real-time PCR (qPCR). They were put into a microtube, and immediately placed on dry ice before transferring to -80 degree storage. Five samples (1 litter per sample) were used for each treatment or control group. Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen) according to the provided protocol. Traces of genomic DNA were removed using the RNase-free DNase Set (Qiagen). RNA was eluted in 40 ul of RNase-free water. For reverse transcription, 20 ul of reaction mixture from the High Capacity cDNA Archive Kit (Applied Biosystems) was mixed with 400 ng of sample RNA in 20 ul of water. Reverse transcription reactions were run according to the suggested protocol to produce cDNA.

TaqMan Universal PCR MasterMix and probes (Applied Biosystems, Foster City, CA, USA) were used for the PCR step: Hbegf - Mm00439309_g1; Fgfbp1 - Mm00456064_s1; Lrp1 - Mm01160463_g1; AP-2α - Mm00495574_m1; Park7 - Mm01263682_m1. Amplification and detection were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following profile: 1 cycle at 94 degrees for 10 min, and 40 cycles each at 95 degrees for 15 sec and 60 degrees for 1 min.

The threshold cycle (Ct) was determined for each sample and compared to the average Ct value of GAPDH mRNA.

Section 3.4 Results

Transcriptional changes in ethanol-exposed limbs

A gene list of 8571 *present* genes was identified from three experimental groups at 2, 4, and 6 hours post-ethanol exposure and one PBS control group, using the experimental criteria for inclusion described above. An ANOVA identified 367 genes that demonstrated a significantly different ($P < 0.05$) level of expression among the four groups. Hierarchical clustering of all replicate samples demonstrated the reproducibility of samples within each treatment, as samples were most similar to those within the same treatment group (figure 3.1). External array validation was accomplished using qPCR. Of 5 primers, 4 validated the direction of expression changes observed in the microarray results; Hbegf was the exception, shown to be upregulated in response to ethanol using microarray analysis while downregulated using qPCR.

Gene networks were generated to examine the interrelationships of differentially expressed genes, and the cellular functions they may impact. IPA utilized 134 of the 367 statistically significant genes to produce several networks (table 3.1), identifying connections between genes with known interactions. CTNNBI (β-catenin) was identified as a central node in the largest network. Those significantly expressed genes that interact with CTNNBI include: COL18A1, CRABP2, CTNNBIP1, EOMES, FGFBP1, HSPCB, HSPE1, INDO, PDE1C, PKN2, PSEN1, TFAP2A (AP-2α), and WNT4 (figure 3.2). IL-15 was identified as a secondary node in this same network, interacting with ACTG2, COL18A1, CKS1B, HSPE1, PIM1, and STAT3.

A Tukey post hoc analysis of ethanol-responsive genes revealed an 87% similarity between control and ethanol +2hr groups, 39% similarity between control and ethanol +4hr groups, and 95% similarity between control and ethanol +6hr groups (figure 3.3). Of the 367 statistically significant genes identified by ANOVA, IPA utilized 143 genes for functional analysis. This analysis revealed

several significantly affected ($P < 0.05$) *high level function* categories (table 3.2a). Among the overrepresented categories were processes related to cell adhesion, such as 'disassembly of focal adhesions,' 'adhesion of embryonic cells,' and 'adhesion of keratinocytes' (table 3.2b). A large number of processes related to immune system function were identified: formation of lymphocytes, phagocytosis of monocytes, activation of mast cells, chemoattraction of mononuclear leukocytes, cytotoxic reaction of lymphocytes, and the processes of formation, growth, survival, and expansion of T lymphocytes.

Gene ontology (GO) analysis

Because the post-hoc analysis identified only a small number of differentially expressed genes between control and ethanol + 2 or 6 hr groups, further functional analyses focused on the comparison of control and ethanol +4hr groups. Using a gene list of 224 genes found to be statistically different between control and ethanol +4hr groups, a number of overrepresented gene ontology (GO) terms within the categories of *biological process*, *cellular component*, and *molecular function* were identified (table 3.3). The biological processes significantly overrepresented include cell cycle, cytokenesis, protein folding, and apoptosis. ATP binding, receptor activity, and unfolded protein binding comprised the overrepresented molecular functions among genes significantly affected by 4 hours of ethanol exposure. With the transcription of 21 genes affected by ethanol exposure, ATP binding proteins comprised the largest target of ethanol-induced transcriptional modification in the forelimb bud. Seven of these ATP binding proteins play a role in protein phosphorylation, and 11 of them have kinase activity. There is no consensus to the direction of transcriptional alteration among ATP binding proteins resulting from ethanol exposure. Perturbation of gene products involved in receptor activity likewise did not exhibit a clear direction to ethanol-induced disregulation. The significantly overrepresented cellular component terms *membrane* and *integral to membrane* were identified. A comparison of the genes among the molecular function,

biological process, and cellular component categories indicated that 7 of the 10 genes encoding for products with receptor activity are localized to the plasma membrane.

Pathway analyses

To test the hypothesis that known canonical pathways are transcriptionally altered in response to ethanol treatment, pairwise comparisons between PBS control and individual ethanol treatment groups with P-values of 0.05 and 0.01 were conducted. Of the 8571 *present* genes, 280 genes were identified as statistically different from control in the ethanol +2hr treatment group ($P < 0.05$; 32 genes at $P < 0.01$). IGF-1, JAK/Stat, and Parkinson's signaling pathways, as well as those involved in pyrimidine, purine, and pyruvate metabolism are affected in limb buds after 2 hours of ethanol exposure (table 3.4). Members of the significantly affected purine and pyrimidine metabolic pathways were generally downregulated in response to 2 hours of ethanol exposure.

At 4 hours of ethanol exposure, transcriptional changes were significant in 476 of 8571 genes ($P < 0.05$; 91 genes at $P < 0.01$). Canonical pathways affected after 4 hours of ethanol exposure included the following: IL-4, Insulin Receptor, Nitric Oxide, Parkinson's, PI3K/AKT, JAK/Stat, and Wnt/β-catenin signaling pathways.

After 6 hours of ethanol exposure, 232 of 8571 genes were significantly changed relative to control ($P < 0.05$; 46 genes at $P < 0.01$). Canonical pathways that were significantly affected include sterol biosynthesis, estrogen receptor signaling, PPAR signaling, and Parkinson's signaling (table 3.4).

Retinoic acid signaling abrogation in the limb bud

An ANOVA identified 1069 genes that demonstrated a significantly different level of expression among the three ethanol treatment groups and the BMS-189453 treatment group. Hierarchial clustering was conducted on all treatment samples, normalized to the average of their

respective controls groups. Each treatment was reproducible, as samples were most similar to those within the same treatment group (figure 3.4).

Tukey Post Hoc analysis (figure 3.5) demonstrated that transcriptional changes resulting from BMS-189453 exposure were most different from those of the ethanol +4hr treatment group. Of the 1069 genes identified as significantly different among ethanol and BMS-189453 treatment groups, 681 genes (64%) were statistically different between BMS-189453 and ethanol +4hr treatment groups. Ethanol +6hr and BMS-189453 treatment groups were the most similar to one an other, with 245 of 1069 genes (23%) in common. This examination, however, does not distinguish between positive and negative correlation.

To examine the similarity of gene changes following ethanol and BMS-189453 exposures, the Pearson correlation coefficient was determined (table 3.5). All ethanol exposure groups exhibited a negative correlation to the BMS-189453treatment group ($P < 0.0001$) indicating that transcripts were, on average, discoordinately regulated between BMS-189453 and each of the ethanol treatment groups. In contrast, ethanol-to-ethanol treatment comparisons resulted in a positive correlation indicating that significant transcript changes are generally regulated in the same manner across time. Though having a negative correlation, the highest correlation between transcripts was found in the ethanol +4hr and BMS-189453 treatment groups (coefficient = -0.40123).

To examine whether genes were coordinately regulated by BMS-189453 and any of the ethanol treatments, genes found to be significantly affected by both ethanol and BMS-189453 were identified. Six significantly affected gene changes were common to BMS-189453 ($P < 0.05$) and ethanol +2hr groups ($P < 0.05$). Comparisons between BMS-189453 and ethanol +4hr or ethanol +6hr revealed 17 and 9 common genes, respectively. These results are summarized in table 3.1. Combined, these 32 genes represent 8% of the 391 genes significantly affected by BMS-189453 exposure.

Reactive Oxygen Species

The transcription of several genes that impact cellular redox balance were significantly affected by ethanol treatment. LPO, PRODH, SPI1, TNF, and TP53 are identified by IPKB as genes with pro-oxidant activity, and were found to be significantly upregulated among ethanol treatments (table 3.6a). Additionally, several genes with antioxidant activity were downregulated, including FANCC, GLRX, PARK7, PRDX2, and SOD1.

IPKB was used to identify significantly affected genes that respond to ROS. While those genes that influence REDOX balance are modulated by ethanol, those that respond to ROS were not consistently regulated indicating a response to either an oxidative or reducing environment.

Limb-specific gene changes

Examination of the upstream regulators and downstream targets of NF-κB transactivation revealed a coordinated effort towards activation and against repression. Table 3.6b lists those genes that are significantly affected in one or more of the ethanol treatment groups that are regulated by, and modulate the activity of NF-κB. Positive regulators of the activity of NF-κB were upregulated and negative regulators of its activity were downregulated. Additionally, those genes in the dataset that are transcriptionally upregulated by NF-κB transactivation were upregulated in response to ethanol.

Though microarray analysis is not ideal for identifying single gene changes, coordinated regulation of several genes may justify attention to single gene changes. Of particular interest is the transcription factor TFAP2A (AP-2α). The genes significantly affected by ethanol exposure that were identified as downstream targets of AP-2α include STX7, ARBP, TNF, and RPS5. AP-2α represses transcription of each of these genes. Following ethanol exposure +4hrs, AP-2α is downregulated, and each of the genes regulated by AP-2α were upregulated.

Section 3.5 Discussion

Administration of a teratogenic dose of ethanol results in excessive cell death in the AER that is detectable using vital stains at 4 to 24 hours following the onset of maternal ethanol exposure (Kotch et al., 1992; Chen et al., 2004; Chapter II). This study examined the ethanol-exposed forelimb bud at 2, 4, and 6 hours. Microarray examination at these time-points has revealed transcriptional alterations that are potential mediators of ethanol-induced forelimb dysmorphogenesis.

Functional Analysis

Significant findings include the perturbation of genes involved in several cellular functions such as cell death, cellular compromise, and alterations in cellular growth and proliferation in the ethanol-exposed forelimb. These changes might be considered typical effects of ethanol on embryonic tissues subject to dysmorphogenesis (Kotch and Sulik 1992; Armant and Saunders 1996), though effects on proliferation and cell cycle have not been directly observed in the limb bud. A recent report mapping the changing patterns of cell death and proliferation in the mouse limb bud underscore the complex and dynamic nature of these processes (Fernandez-Teran et al., 2006) that play a role in patterning the limb, and draw attention to the variety of potential targets of teratogens. Transcriptional changes related to small molecule biochemistry, such as occurs with genes important to the biochemical modification, transport, and synthesis of amino acids, glucose, and vitamins were expected following ethanol exposure, as previous investigators have shown these processes to be altered by ethanol exposure (Padmanabhan et al., 2002; Shibley and Pennington, 1997; Schenker et al., 1990). Metabolic perturbations are expected to have severe consequences for cell growth and proliferation.

Particularly notable is the affect of ethanol on cell adhesion. Because the arrangement and rearrangement of cells is fundamental to the construction of embryonic structures, the adhesion of cells to one another and to extracellular matrix is essential for normal development. As has been demonstrated by the studies of Charness et al. (1994) and Ramanathan et al. (1996), the function of

certain adhesion molecules are affected by ethanol. Whether the genes products shown to be affected in this study are responsible for a loss of cell adhesion or are responding to changes in cell adhesion cannot be ascertained. Although ethanol-induced changes in the process of patterning of embryonic tissues is observed, effects upon the fundamental processes of cell-to-cell and cell-matrix interactions should not be overlooked as mediators of dysmorphogenesis.

As demonstrated by functional and network analyses, genes known to function in the immune function of monocytes, mast cells, leukocytes, and lymphocytes were found to be affected by ethanol exposure. One might speculate that ethanol's induction of phagocytosis resulting from apoptosis may be responsible for the significant transcriptional modulation of a number of immune system-associated genes. Additionally, other systems likely utilize many of these same genes during development.

To further probe the changes resulting from ethanol exposure, the 4 hour time point was chosen for GO analysis. Ethanol's affect on the cell cycle is supported by a growing literature base showing that embryonic ethanol exposure influences the cellular processes of proliferation, differentiation, and apoptosis (Kotch and Sulik 1992; Armant and Saunders 1996; Johnson et al., 2004). One way in which ethanol interferes with the cell cycle is through interactions with regulators of second messenger release. Ethanol increases intracellular Ca^{2+} concentrations, and its proliferative affect on preimplantation embryos depends on the release of intracellular stores of Ca^{2+} (Stachecki and Armant, 1996; Stachecki et al., 1994). Because of the role of Ca^{2+} as second messengers in growth factor signaling, ethanol can potentiate such signaling cascades in the absence of canonical ligands. On the other hand, others have shown that ethanol interferes with IGF-I growth factor signaling independent of Ca^{2+} perturbation in select cell types (Zhang et al., 1998; Cui et a.l., 1997). Ethanol prevents autophosphorylation of IGF type I receptors, preventing mitogenic and pro-survival signals from being transmitted to the cell, resulting in apoptosis (Resnicoff et al., 1993). A third means by which ethanol may alter growth factor signaling is through the generation of oxidative stress. Oxidative stress not only alters the cell cycle through damage to macromolecules, but may

more directly interfere with signaling events. In select signaling cascades ROS act as second messengers (reviewed in Sauer et al., 2001), and have been confirmed to result from ethanol exposure in limb bud mesenchyme cultures (Johnson et al., 2004). It is, thus, reasonable to conclude that ethanol-induced ROS activate signaling pathways that utilize ROS as second messengers in the limb and modulate transcription of genes, such as those involved in cell cycle control.

Several genes significantly affected by ethanol were identified that either regulate redox changes or respond to such changes. Although no transcriptional response to redox change was evident among significant genes during the examined time points, the seemingly coordinated regulation of genes with anti- and pro-oxidant activities towards an oxidizing environment is intriguing. To investigate this phenomenon more fully, the transcript and protein levels of the most significant modulators of redox status such as glutathione and thioredoxin must be examined. In general, the trend of expression of genes that influence redox suggests a mechanism of ethanol-induced gene regulation that contributes to ethanol's pro-oxidant activity in addition to accepted routes of ROS generation through ethanol metabolism.

The observation that genes whose products bind unfolded proteins, as well as those that are involved in the process of protein folding are altered by ethanol exposure is not surprising, given that several levels of protein synthesis are affected by ethanol. These include RNA transport and aminoacyl transfer RNA synthase activities (Henderson and Schenker 1977), ribosomal function, precursor availability, and protein degradation (reviewed in Schenker et al., 1990). The cell membrane is another of ethanol's cellular targets (Goldstein and Chin, 1981). Since ethanol effects the fluidity and lipid organization of cell membranes, it likely disturbs the conformation of transmembrane proteins such as ion channels and ligand receptors (Sanchez-Amate et al., 1992; Michaelis et al., 1983, Goldstein and Chin, 1981). That expression of gene products found in the cell membrane is significantly altered by ethanol indicates that regulatory mechanisms may be activated in response to effects on membrane proteins that coincide with membrane destabilization.

Since most of the functions described by ethanol's transcriptional profile have been previously described in various systems, our results are internally validated. Ethanol's variety of affect on processes, functions, and subcellular locations supports the hypothesis that it has a broad array of targets within the cell. Importantly, this observation leads to the conclusion that ethanol's mechanisms of action are "universal;" the diversity in response then, is a function of cellular phenotype: the cell type, stage in the cell cycle, and presence or absence of signaling pathways inducible by such mechanisms. Early compromises in cellular integrity, damage to proteins, and effects upon cell adhesion and membrane fluidity are likely contributors to ethanol's effects. Disruption of membrane proteins, particularly those with receptor activity, would lead to perturbation in growth factor signaling pathways, the perturbation of which many believe to have the most profound influences over the course of limb development and patterning.

Pathway Analysis

Examining affected pathways was expected to reveal possible targets of ethanol that may impact development of the forelimb bud. One such pathway, significantly overrepresented at 2 hours of ethanol exposure, is the JAK/Stat signaling pathway. Other have shown that the activity of the JAK/Stat signaling pathway is inhibited following ethanol exposure in adult tissues (Chen et al., 2001; 1999; Nguyen et al., 2000). Of the significantly downregulated genes in this pathway, the activity of PI3 Kinase (PIK3R3) is inhibited by ethanol (Zhang et al., 1998; Resnicoff et al., 1994). Our data suggest that the observed inhibitory affect of ethanol on PI3 Kinase activity and the JAK/Stat pathway is, in part, a result of transcriptional repression. The means by which such repression occurs is unknown, but is likely secondary to changes in growth factor signaling. PI3K signaling is important in mediating the anti-apoptotic activity of FGF8, which is released from the AER. Such ethanol induced-disregulation implicates PI3K and it's regulators in ethanol's apoptotic affect.

The significantly affected genes identified in Parkinson's signaling are PARK7 (DJ-1), SNCA (Park1; α-synuclein), and UBE1. Both PARK7 and SNCA seem to be important to membrane trafficking and have an anti-apoptotic function. PARK7 is hypothesized to have a multifunctional role, perhaps responding to both oxidative stress and protein misfolding (reviewed in Bonifati et al., 2004). After exposure to oxidative stress, PARK7 becomes localized to the mitochondria in cells where it has a neuroprotective affect (Canet-Aviles et al., 2004). Non-neural cells are known to express members of the Parkinson's signaling pathway, but such information for the embryo is lacking. It is unclear how ethanol might downregulate the represented members of this pathway, but a response to stress and the repression of anti-apoptotic mechanisms across ethanol +2hr, +4hr, and +6hr time points is suggested.

Despite changes in a variety of genes involved in metabolism, few metabolic pathways were perturbed by ethanol. Decreased demands of cells for DNA may account for the downregulation of purine and pyrimidine pathways at 2 hours following initial ethanol exposure, as DNA synthesis often decreases following embryonic ethanol exposure (Thompson and Folb, 1982; Dreosti et al., 1981). ADH5, important to pyruvate metabolism, metabolizes ethanol to acetaldehyde, while aldehyde dehydrogenases like ALDH1A1 oxidize acetaldehyde to acetate. These enzymes also participate in the formation of retinoic acid (RA) from retinol. ADH5 participates in both ethanol and RA metabolism, and is ubiquitously expressed throughout the embryo (Molotkov et al., 2002). Unfortunately, little is known about the regulation of ADH5 that would explain ethanol's positive effect.

At 4 hours post ethanol treatment, several signaling pathways containing the serine/threonine kinase AKT3 are upregulated: IL-4, insulin receptor, PI3K/AKT, and JAK/Stat signaling pathways. Generally AKT3 serves as a stimulator of cell growth and proliferation, while repressing apoptosis in these pathways. Additionally, the protein serine/threonine kinase RPS6KB1 found significantly upregulated in insulin receptor and PI3K/AKT signaling pathways stimulates protein synthesis and is generally associated with proliferation. The transcriptional activation of a number of pathways that

result in the repression of pro-death signals and the activation of pro-survival signals is consistent with the observation that most limb bud cells resist ethanol toxicity (Kotch et al., 1992; Chapter II).

Wnt/β-catenin signaling, essential for normal limb development, is significantly affected by ethanol exposure at 4 hours. Because the Wnt/β-catenin signaling pathway shares many of its members with other signaling pathways, it is difficult to predict the overall significance of the changes in gene expression. ILK and integrin signaling pathways, for example, overlap with Wnt signaling making it difficult to identify gene expression changes with a single pathway without others that may overlap (Novak and Dedhar 1999). It is likely that the apparent perturbation of the Wnt/β-catenin pathway involves a combination of, and cross-talk among different pathways. Besides the canonical Wnt/β-catenin signaling pathway, there are several poorly characterized, non-canonical Wnt pathways that exclude a function for β-catenin but utilize other members of the canonical pathway. Both canonical and non-canonical Wnt signaling pathways are important to normal limb development, and both may be perturbed by ethanol. Ultimately, perturbation of the expression levels of the pathway's effectors, the transcription factors LEF1 and TCF3, would mediate Wnt-signaling resulting from ethanol exposure.

IPA's largest network, generated from data across ethanol treatment groups, likewise pointed towards Wnt/β-catenin signaling. As the principle node, β-catenin has known interactions with 13 significantly affected genes including those with diverse functions and belonging to several signaling pathways. β-catenin is a gene with roughly 3 times as many identified outgoing connections as incoming, indicative of a gene that is important in cellular regulation, as opposed to those with mostly incoming connections that often have a metabolic function (Rung et al., 2002). β-catenin is an integral part of the Wnt/β-catenin signal transduction pathway and is essential for normal limb development. Importantly, β-catenin in the mesenchyme subjacent to the AER is responsible for maintenance of the AER and failure of β-catenin-mediated Wnt signaling results in limb truncation (Hill et al., 2006). Two other members of canonical Wnt signaling are represented in this network, LRP1 and WNT4. LRPs (low density lipoprotein-related proteins) were first recognized for their role

in the regulation of cholesterol uptake, transport, and metabolism, but more recently have been identified as playing an important function in embryogenesis, including limb development (Simon-Chazottes et al., 2006; Adamska et al., 2005). Wnt proteins are secreted ligands that initiate signal transduction. Though the role of WNT4 has not been investigated in the limb, other WNT family members are integral to several stages of limb development including initiation, patterning, AER formation, morphogenesis, and later stages of skeletal development (Reviewed in Yang, 2003). Knockout of WNT pathway members results in postaxial forelimb digit loss, the most common ethanol-induced limb malformation (Adamska et al., 2005; Kotch et al., 1992) making the Wnt/β-catenin pathway a likely target of ethanol and a subject of future research efforts.

After 6 hours of ethanol exposure, the number of significantly affected pathways and significant genes has decreased. Though statistically significant, the pathways contain a small number of genes precluding a discussion of biological significance and trends in the expression of pathways. Thus, ethanol's critical transcriptional effects occur primarily within the first 6 hours of acute exposure. It would be expected that later transcriptional changes would be evident following the loss of cells and as proportions of various cell types are altered as a result of selective cell death and perturbation of normal patterns of proliferation. Microarray studies of ethanol exposure that examine tissue after such changes occur are useful in determining the secondary and tertiary effects of ethanol on the embryo (Da Lee et al., 2004), however, the results of this study indicate that transcriptional changes revealing the mechanism(s) of ethanol teratogenesis may be evident prior to 2 hours.

Perturbation of RA-mediated development by ethanol

Because of morphological similarities between ethanol-exposed embryos and those exposed to a vitamin A deficient diet, it has been postulated that ethanol's mechanism of action involves perturbation of the RA signal. The studies of Grummer and Zachman demonstrate that chronic ethanol exposure modulates expression of RARs and binding proteins in embryonic tissues (reviewed

in Zachman and Grummer, 1998). Others have postulated that ethanol-induced malformations arise from the competitive inhibition of RA synthetic enzymes by ethanol (Duester 1991; Pullarkat 1991). Our laboratory has provided evidence for the perturbation of RA-mediated limb development. Administration of a RAR antagonist or ALDH inhibitor to mice on the 9th day of gestation produces the same types of limb defects as those embryos exposed to ethanol. In addition, gene expression of key developmental regulators of limb development is altered in response to ethanol in a manner consistent with RA deficiency. However, the results of the present study indicate that transcriptional changes that occur 2-6 hours following ethanol exposure do not bear any resemblance to those of the RAR antagonist.

The onset of excessive cell death was used as a common endpoint between the two treatments. In previous studies using vital staining, it was determined that ethanol-induced cell death was most intense around 8 hours. A teratogenic does of BMS-189453, however, produced comparable amounts of cell death at 4 hours. Choosing time points prior to the onset of excessive cell death was essential in order to minimize transcriptional changes resulting from compositional changes in cell types that result from altered rates of proliferation and the occurrence of excessive cell death in the limb bud. The observation that there is no similarity in transcriptional profiles between ethanol and the RAR antagonist indicates several things. First, any interaction between ethanol and the RA synthetic or signaling apparatus that results in transcriptional modifications occurs several hours after ethanol exposure. Such long-term effects cannot be considered mechanistic, as there are clearly significant changes in genes affecting metabolism, cell signaling, and cell cycle prior to such an interaction. Previous observation that BMS-189453 induces cell death in the AER, and RA prevents ethanol-induced cell death (Chapter II) indicates that RA is necessary for AER maintenance and can maintain the AER in the presence of ethanol. In light of the present findings, it is likely that ethanol does not impacts RA directly, but perhaps downstream targets of RA in the AER.

Limb-specific gene changes

Because the developing limb has been the subject of much study, a large cadre of genes involved in its development has been identified and their expression domains mapped. Our results indicate that several genes localized to the SRM respond to ethanol. IGF-I and several of its binding proteins are expressed in the developing limb bud and are significantly affected by ethanol exposure. IGF-I signaling is active in the SRM and is responsible for its proliferation in response to FGFs emanating from the AER during normal development (Dealy et al., 1996; Dealy and Kosher 1996). IGFBP-5 is expressed in the AER (van Kleffens et al., 1998) and is upregulated in regions exhibiting cell death (Allan et al., 2000). This observation is consistent with findings that show excessive cell death in the AER and our observation that IGFBP-5 is upregulated in response to ethanol exposure. IGFBP-1 has not previously been detected in the limb but has a mixed function, potentiating or inhibiting the effects of IGF-I (Jones and Clemmons, 1995). The observed downregulation of the IGF-I pathway may indicate a loss of proliferative activity in the SRM. It is not possible to resolve whether the decrease in IGF-I pathway genes is responsible for cell death, but it does indicate a perturbation of the epithelial to mesenchymal signaling that sustains the proliferative activity of the SRM.

NF-κB is also localized to the SRM (Bushdid et al., 1998; Kanegae et al., 1998). Many stimuli activate NF-κB, including moderate levels of oxidative stress. NF-κB is a transcription factor that responds to a number of signals leading to proliferation, cell death, and differentiation, depending on cellular context. In the limb, NF-κB is associated with the proliferative SRM, where active NF-κB is required to maintain the mesenchyme in a proliferative state, essential for outgrowth. AP-2α plays an important role in limb development and is expressed in the SRM (Moser et al., 1997). Like NF-κB, AP-2α is maintained by the AER (Bushdid et al., 1998; Kanegae et al., 1998; Shen et al., 1997). It is not surprising that changes were found in genes expressed in the SRM, since ethanol induces cell death in the population of cells that supports the SRM.

In summary, a number of novel targets of ethanol were identified. Perturbation of Wnt/β-catenin, PI3K, and JAK/Stat signaling may be highly significant in ethanol's teratogenic affect.

Several previously known targets of ethanol were confirmed, such as cell adhesion, cell membrane, protein synthesis, metabolic pathways, and genes controlling ROS. Surprisingly, there was no consensus in the reaction of ROS-sensitive genes to ethanol, but those genes influencing ROS were regulated in a manner that would be expected to yield a pro-oxidant environment. RA-mediated development was not perturbed by ethanol in the earliest hours following exposure; it is more likely that the primary effectors of ethanol secondarily impact RA-mediated development. Lastly, a number of SRM-specific genes are perturbed following ethanol exposure indicating that this region is responding to ethanol's perturbation of growth factor signaling and disruption of epithelial-mesenchymal interactions.

Table 3.1. Network genes identified by IPA using a genelist of 367 statistically significant genes identified by ANOVA. ANOVA identified 367 genes that demonstrated a significantly different ($P < 0.05$) level of expression among ethanol +2hr, +4h, +6hr, and control groups. Networks are comprised of genes with known direct or indirect interactions with at least one network gene. The major 'high level functions' identified among network genes are displayed to the right.

Network	Genes	Functions
1	ACTG2, CKS1B, COL18A1, CRABP2, CTNNB1, CTNNBIP1, EOMES, F9, FGFBP1, HSPCB, HSPE1, IL15, INDO, LRP1, PDE1C, PIM1, PKN2, PSEN1, STAT3, TFAP2A, WNT4	Embryonic Development, Gene Expression, Tissue Development
2	ABCC3, APOA5, CDC37, COL5A2, CRISP2, Cyp2j5, ELF3, FCGR3A, GFER, GNAI2, HSPCB, IL18BP, KLF15, RND2, SCARB1, Wap	Cellular Movement, Cancer, Tumor Morphology
3	ANKRD1, C12orf8, CD3E, COL5A2, COX6A1, CTCF, DSTN, EED, LRRN1, MAPK6, MUT, PCGF2, Plf, PSAT1, ULK2	Cancer, Cell Cycle, Cell Morphology
4	CBX1, CKS1B, DLX1, F8A1, GATA6, HEXA, LMAN1, MCM6, NCOR1, PECI, PIG8, SF3A2, TOMM40, USP33, Wap	Genetic Disorder, Lipid Metabolism, Metabolic Disease
5	ABI2, AK1, CCNL2, CTSH, ELAVL2, HBEGF, HSPCB, LOC340571, MB, NAPSA, NEIL1, PRODH, RPS15, TAF1B	DNA Replication, Recombination, and Repair, Gene Expression, Cancer

Table 3.2. Functional analysis demonstrating processes overrepresented among a genelist of statistically significant genes identified by ANOVA. ANOVA identified 367 genes that demonstrated a significantly different ($P < 0.05$) level of expression among ethanol +2hr, +4h, +6hr, and control groups. a) High level functions identified by IPA grouped according to the number of genes within each function. b) specific processes within high level functions are identified demonstrating particular functions and their associated genes.

a) High level functions	Genes	Percent
Small Molecule Biochemistry	17	31.5
Cell-to-Cell Signaling and Interaction	15	27.8
Cellular Development	13	24.1
Hematological System Development and Function	11	20.4
Molecular Transport	10	18.5
Cancer	10	18.5
Cellular Growth and Proliferation	9	16.7
Tissue Morphology	8	14.8
Organismal Injury and Abnormalities	8	14.8
Nervous System Development and Function	8	14.8
Immune and Lymphatic System Development and Function	8	14.8
Immune Response	7	13.0
Hematological Disease	7	13.0
Cellular Compromise	7	13.0
Cellular Assembly and Organization	7	13.0
Cell Death	7	13.0
Cardiovascular System Development	7	13.0

b) Process	P-value	Genes
Disassembly of focal adhesions	1.50E-03	COL18A1, GNAI2, LRP1, THY1
Formation of lymphocytes	4.66E-03	IL15, LCP2, PIM1
Oxidation of essential amino acids	6.99E-03	INDO, LPO
Phagocytosis of monocytes	6.99E-03	C1QR1, PF4
Modification of essential amino acids	8.42E-03	CLK1, INDO, LPO, SGK
Activation of Ttf1 binding site	1.03E-02	GATA6, STAT3
Squamous-cell carcinoma	1.06E-02	BRCA2, CTNNB1, ODC1, RNF6
Survival of T lymphocytes	1.32E-02	CD3E, IL15, PIM1, STAT3, WNT4
Transduction of calcium	1.42E-02	CD3E, HBEGF
Ovarian tumor	1.84E-02	COL18A1, CTNNB1, GNAI2
Activation of promoter fragment	1.85E-02	CTNNB1, STAT3
Adhesion of embryonic cells	1.85E-02	CTNNB1, EPAS1
Fibrosis of mice	1.85E-02	CDC37, PF4
Growth of T lymphocytes	1.90E-02	CD24, CD3E, IL15, LCP2, PIM1
Activation of mast cells	2.09E-02	FCGR3A, LCP2, THY1
Adhesion of keratinocytes	2.34E-02	PKN2, STAT3
Developmental disorder of epithelial cells	2.34E-02	CDC37, CTNNB1
Formation of T lymphocytes	2.34E-02	LCP2, PIM1
Activation of LEF1 binding site	3.45E-02	CTNNB1, PSEN1
Chemoattraction of mononuclear leukocytes	3.45E-02	IL15, PF4
Patterning of embryonic tissue	3.45E-02	CTNNB1, PSEN1
Bleeding of mice	3.96E-02	F9, FCGR3A, LCP2
Tumorigenesis of mammary gland	3.96E-02	BRCA2, CDC37, CTNNB1
Cytotoxic reaction of lymphocytes	4.07E-02	FCGR3A, IL15
Differentiation of neuronal progenitor cells	4.07E-02	DLX1, PSEN1
Morphogenesis of endothelial cells	4.07E-02	COL18A1, ODC1
Vascularization of organ	4.07E-02	MB, ODC1
Expansion of T lymphocytes	4.37E-02	CD24, IL15, LCP2, PIM1
Bleeding	4.60E-02	EPAS1, F9, FCGR3A, LCP2, PSEN1

Table 3.3. Gene ontology (GO) analysis identifying significantly significant overrepresentation of biological process, cellular component, and molecular function GO terms within a gene list of 224 genes found to be statistically different between PBS control and ethanol +4hr treatment groups by Tukey post hoc analysis. P-value represents the result of the Bonferroni multiple testing correction. Data with corrected P-value < 0.05 and 5 or more genes were included.

Functional profile	P-value	Genes (%)
Cellular component		
integral to membrane	4.79E-02	26 (11.61%)
Molecular function		
ATP binding	2.07E-02	21 (9.38%)
receptor activity	2.46E-02	10 (4.46%)
unfolded protein binding	1.35E-02	5 (2.23%)
Biological Process		
biological process unknown	1.82E-02	14 (6.25%)
cell cycle	1.98E-02	7 (3.13%)
cytokenesis	1.08E-02	5 (2.23%)
protein folding	1.99E-02	5 (2.23%)
apoptosis	4.37E-02	5 (2.23%)

Table 3.4. Significant (P< 0.05) canonical pathways affected in mouse limb buds following ethanol exposure. Pathways and genes in italics were derived from a genelist of significant genes, P <0.05. Pathways and genes in normal typeface were derived from a genelist of significant genes, P <0.01. * indicates that pathways and genes were significant for both analysis.

Canonical Pathway	Significance	Genes
Ethanol + 2 hours		
Parkinson's Signaling*	*2.98E-3* / 3.31E-2	*SNCA* ▼, *PARK7* ▼, *UBE1 ▼
IGF-1 Signaling	*5.58E-3*	*IGFBP5*▲, *IGFBP1*▼, *SRF*▼, *NEDD4*▼, *PIK3R3*▼
Purine Metabolism	*3.02E-2*	*PRIM1*▼, *ABCD3*▲, *PDE1A*▲, *PDE1C*▼, *POLR3A*▼, *ENTPD7*▼, *ENTPD6*▼, *POLR1B*▼, *ADSS*▼
Pyruvate Metabolism	*3.53E-2*	*ADH5*▲, *ALDH1A1*▼, *CACH-1*▲, *AKR1B10*▲
Pyrimidine Metabolism*	*3.92E-2* / 2.85E-2	*PRIM1▼, *POLR3A*▼, *ENTPD7*▼, *ENTPD6*▼, *DUT*▼, *POLR1B▼
JAK/Stat Signaling	*4.91E-2*	*STAT5A*▲, *SOCS2*▲, *PIK3R3*▼
Ethanol + 4 hours		
IL-4 Signaling	1.83E-2	AKT3▲, RPS6KB1▲
Insulin Receptor Signaling	2.02E-2	AKT3▲, SGK▲, RPS6KB1▲
JAK/Stat Signaling	*4.06E-2*	*STAT2*▲, *STAT5A*▲, *AKT3*▲, *SOCS6*▲
Nitric Oxide Signaling in the Cardiovascular System	1.56E-3	FIGF▼, AKT3▲, HSPCB▲
Parkinson's Signaling	4.21E-3	SNCA▼, PARK7▼
PI3K/AKT Signaling	1.35E-2	AKT3▲, HSPCB▲, RPS6KB1▲
Wnt/β-catenin Signaling	*1.64E-2*	*DVL2* ▼, *LRP1*▲, *CDH1* ▼, *LEF1*▲, *WNT4*▲, *AKT3*▲, *TCF3* ▼, *TP53*▲
Ethanol + 6 hours		
Estrogen Receptor Signaling	1.51E-2	MNAT1▲, NCOR1▲
Parkinson's Signaling	*2.21E-2*	*SNCA* ▼, *UBE1*▼
PPAR Signaling	6.15E-3	NCOR1▲, MAP3K7▼
Sterol Biosynthesis	3.69E-2	GGPS1▲

Table 3.5. Pearson correlation coefficients demonstrating the correlations between BMS-189453, ethanol +2hr, +4hr, and +6hr treatment groups. The BMS-189453 treatment group negatively correlates with each ethanol treatment group ($P < 0.0001$).

Pearson Correlation Coefficients, N = 1069

	BMS-189453	*Ethanol +2hr*	*Ethanol +4hr*	*Ethanol +6hr*
BMS-189453	1.0000	-0.2776	-0.4012	-0.1471
Ethanol +2hr	-0.2776	1.0000	0.6481	0.7333
Ethanol +4hr	-0.4012	0.6481	1.0000	0.5135
Ethanol +6hr	-0.1471	0.7333	0.5135	1.0000

Table 3.6. (a) Genes identified by IPKB as involved in affecting intracellular redox balance. Statistically significant (P<0.05) genes compiled from pair wise comparisons of PBS control vs ethanol +2hr, +4hr, or +6hr groups. Genes with a pro-oxidant activity are generally upregulated and genes with antioxidant activity are generally downregulated in response to ethanol. (b) Statistically significant (P<0.05) genes compiled from pair wise comparisons PBS control vs ethanol +2hr, +4hr, or +6hr groups identifying genes regulated by NF-κB, and regulators of NF-κB. Genes that positively regulate NF-κB are upregulated indicating NF-κB is activated in response to ethanol exposure. Genes regulated by NF-κB are likewise coordinately regulated, further indicating elevating NF-κB activity following ethanol exposure. * = indicates the exception to the trend. 2 = indicates significant in ethanol +2hr compared to PBS control. 4 = indicates significant in ethanol +4hr compared to PBS control. 6 = indicates significant in ethanol +6hr compared to PBS control.

(a) Genes affecting redox balance	Log ratio, fold change
Pro-oxidant activity	
LPO	2.167^{2}
PRODH	1.311^{4}
SPI1	1.223^{6}
TNF	1.637^{4}
TP53	1.952^{4}
NCF2*	-1.171^{2}, -1.287^{6}
Antioxidant activity	
FANCC	-1.1311^{2}
GLRX	-1.217^{4}
PARK7	-1.111^{2}, -1.873^{4}
PRDX2	-1.333^{6}
SOD1	-1.449^{2}, -1.475^{6}
BCL2*	1.479^{2}

(b) Gene indicators of NF-κB activity	Log ratio, fold change
Targets of positive regulation by NF-κB	
BCL2	1.479^{4}
IL7R	2.364^{2}, 2.180^{6}
RUNX1	1.669^{2}, 1.708^{4}, 1.325^{6}
STAT5A	1.737^{2}, 3.311^{4}
TNF	1.637^{4}
TNFRSF10B	1.358^{4}
VEGF	1.156^{6}
Positive regulators of NF-κB activity	
CYR61	1.472^{4}
FCER2	1.528^{4}
LCP2	1.975^{2}
PSMB8	1.123^{4}, 1.044^{6}
RPS6KB1	1.450^{4}
TNF	1.637^{4}
TNFRSF10B	1.358^{4}
VEGF	1.156^{6}
Negative regulators of NF-κB activity	
NEDD8	-1.253^{6}
PTEN	-1.340^{4}

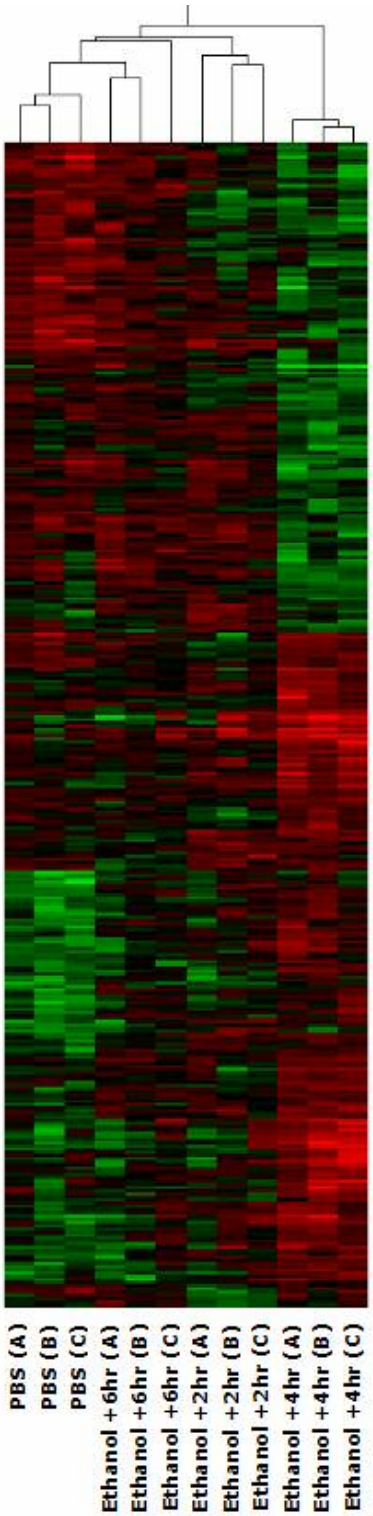
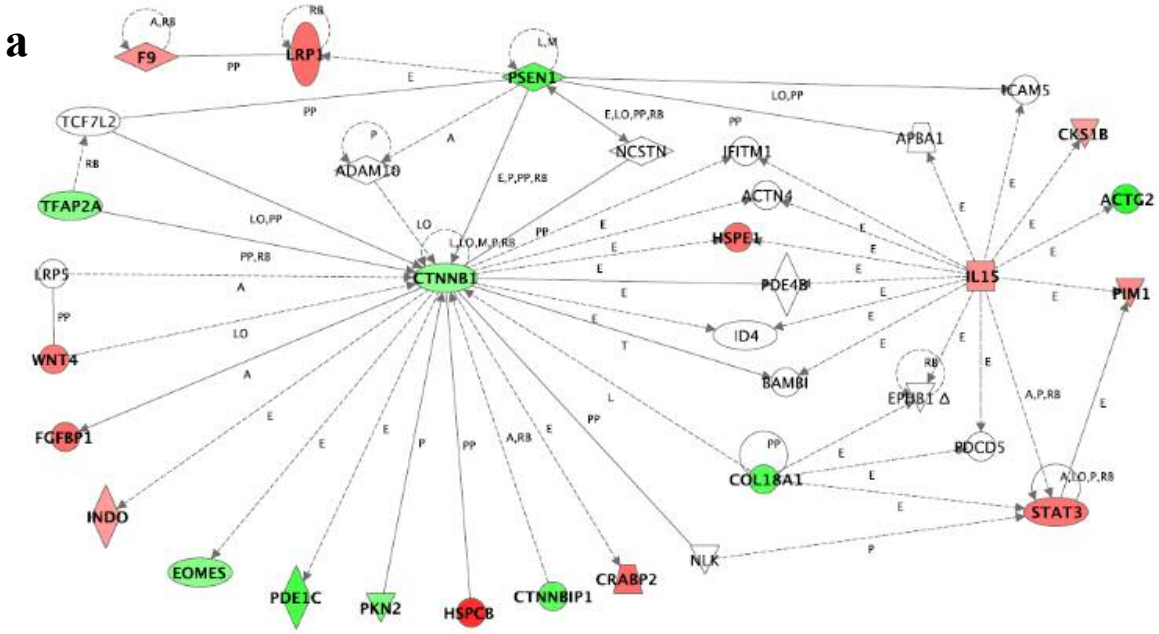


Figure 3.1. GeneSpring hierarchial clustering analysis. A genelist of differentially expressed genes identified by ANOVA ($P < 0.05$) was used to generate data. Ethanol and PBS control groups are listed beneath each column, with the replicate letter A-C in parentheses. Dendrogram at top demonstrates reproducibility of each treatment group.

a



b

Gene	GenBank accession	Log Ratio, Log Fold-change		
		Ethanol +2hr	Ethanol +4hr	Ethanol +6hr
ACTG2	AA756136	-2.079	-1.859	-1.391
CKS1B	AA162721	1.011	1.451	-1.155
COL18A1	AA437677	-1.645	-1.577	-1.093
CRABP2	AA051397	1.527	2.004	1.218
CTNNB1	AA822077	-1.074	1.934	-1.258
CTNNBIP1	AA619808	-1.536	-1.835	-1.074
EOMES	AA606660	-1.166	1.231	-1.227
F9	AA267985	1.148	1.604	-1.081
FGFBP1	AA387137	1.526	1.856	1.530
HSPCB	W08790	2.236	3.738	1.555
HSPE1	AA450526	1.511	2.463	1.002
IL15	AA709787	1.179	-1.522	1.193
INDO	AA871317	1.035	-1.235	-1.148
LRP1	W89938	1.511	1.938	1.610
PDE1C	AA672740	-1.838	-1.560	-1.323
PIM1	AA108991	1.223	1.735	1.143
PKN2	AA413696	-1.295	1.027	1.122
PSEN1	AA087434	-1.718	-1.754	-1.182
STAT3	AA049185	1.443	-1.174	1.173
TFAP2A	AI594276	-1.172	-1.613	-1.112
WNT4	AI894060	1.398	1.862	1.302

c

Edge Lables

Symbol	Relationship
A	Activation/Deactivation
RB	regulation of Binding
PR	Protein-mRNA binding
PP	Protein-Protein binding
PD	Protein-DNA binding
E	Expression
I	Inhibition
L	Proteolysis
M	Biochemical Modification
P	Phosphorylation / Dephosphorylation
T	Translocation
LO	Localization

Figure 3.2. Genes displayed in the largest network of associations identified by IPA. (a) Network displaying associations among genes identified as significantly expressed by ANOVA, among PBS control and ethanol treatment groups. Refer to legend (c) for identification of associations. β-catenin (CTNNB1) and IL-15 are central nodes associated with several genes transcriptionally altered by ethanol exposure. Color represents average of expression values among ethanol treatments. Intensity of color corresponds to intensity of expression; red nodes are upregulated genes and green nodes are downregulated, relative to PBS control. (b) Expression levels of network genes have been normalized to PBS control values.

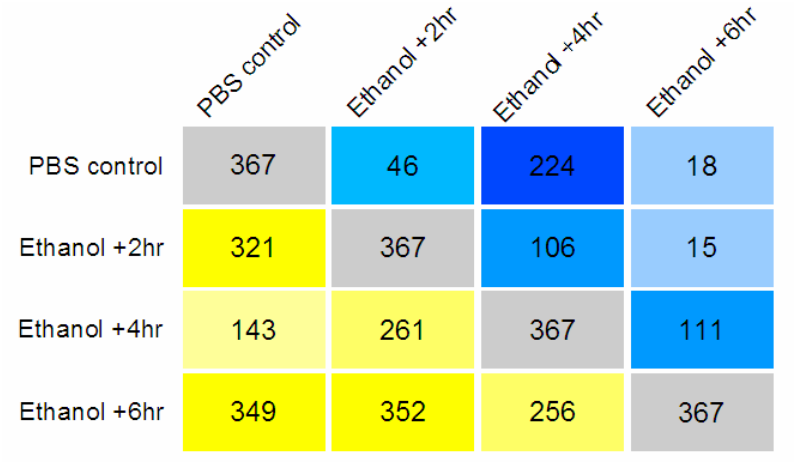


Figure 3.3. Tukey post-hoc analysis. Pair-wise comparisons of PBS control and ethanol treatment groups illustrating the number of genes statistically different (blue boxes) and similar (yellow boxes).

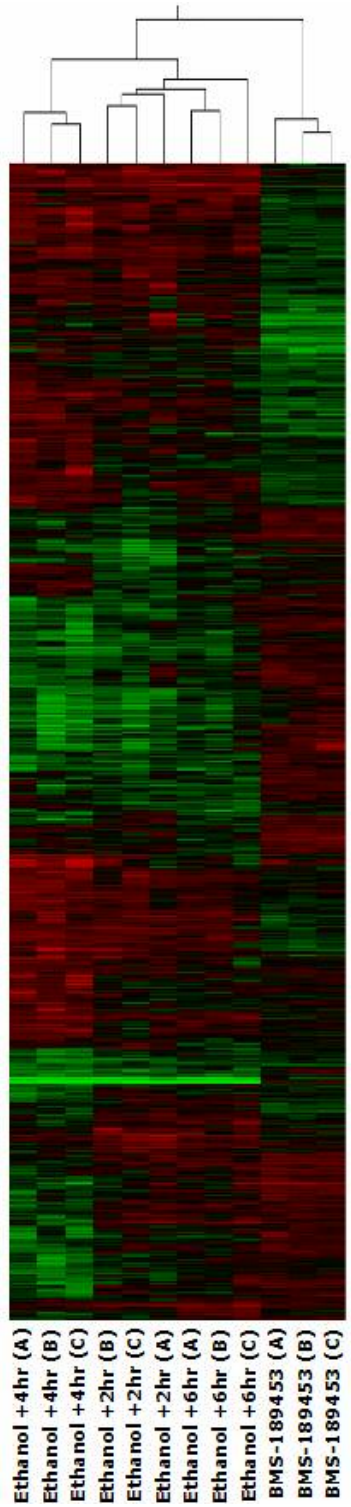


Figure 3.4. GeneSpring hierarchial clustering analysis. Ethanol +2hr, +4hr, and +6hr treatment groups were normalized to the PBS control group. BMS-189453 treatment group was normalized to a DMSO control group. A genelist of differentially expressed genes identified by ANOVA ($P < 0.05$) was used to generate data. Ethanol and BMS-189453 treatment groups are listed beneath each column, with the replicate letter A-C in parentheses. Dendrogram at top demonstrates reproducibility of each treatment group.

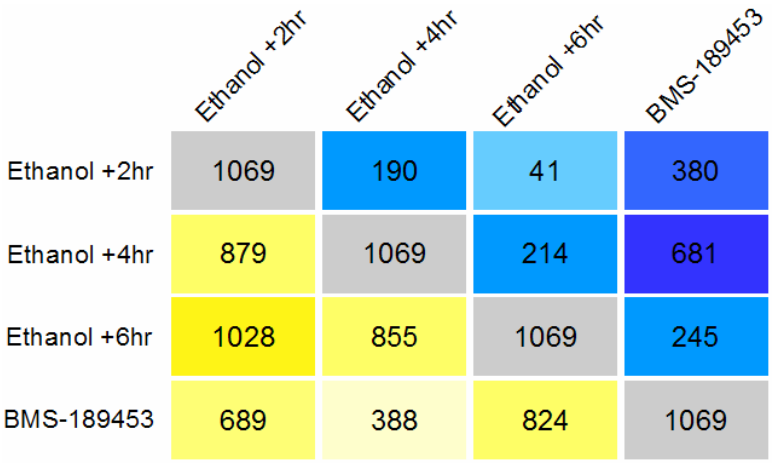


Figure 3.5. Tukey post-hoc analysis. Pair-wise comparisons of BMS-189453 and ethanol treatment groups illustrating the number of genes statistically different (blue boxes) and similar (yellow boxes). Expression values for ethanol and BMS-189453 treatment groups were normalized to their respective controls prior to ANOVA and Tukey post-hoc analysis.

Chapter IV
Conclusions

Based on the proposal by Duester (1991) and the observations that ethanol and RA deficiency share a common dysmorphogenic character, the present investigation has sought to examine whether the dysmorphogenic effect of ethanol on embryos is mediated by RA, using the limb as a model.

Since the effects of acute attenuation of the RA signal has not been examined in the limb bud, this study first established that administration of BMS-189453, a RAR antagonist, and disulfiram, an ALDH inhibitor, produced limb defects. These results show that ethanol-induced limb defects are consistent with perturbation of RA. Further experiments that evaluated the production of excessive cell death in limb buds exposed to ethanol, BMS-189453, and disulfiram demonstrated a similar effect among the three treatments on the AER. Despite assumed differences in uptake, distribution, placental transfer, and clearance rates, the three compounds examined produced a similar response indicating a common pathogenesis.

As further evidence that ethanol perturbs RA function in the limb bud, an additional study demonstrated the ability of exogenous RA to prevent the cytotoxic effect of ethanol in the AER. These results indicate that perturbed RA signaling mediates ethanol-induced cell death within the examined time frame. Interestingly, the somites and proximal limb mesenchyme, subject to ethanol's toxicity, were not prevented from undergoing apoptosis by exogenous RA administration, indicating that the molecular response to ethanol varies according to cell type.

The final series of experiments determined whether ethanol exposure perturbed RA-dependent gene expression. To examine the effects of ethanol on relatively late (up to 18 hours, post treatment) changes in gene expression, whole mount *in situ* hybridization and qPCR were used. Ethanol-induced changes in the expression levels of Shh, dHand, and Tbx5 were consistent with

disregulation of RA signaling. However, when exogenous RA was administered with ethanol, these ethanol-induced gene expression changes were not prevented as expected. These results neither confirm, nor defeat the hypothesis that ethanol interferes with RA-mediated development. The concentration of RA used was, perhaps, toxic to the proximal mesenchyme, while concentrations in the distal limb were reduced by the endogenous enzymes, Cyp26a1 and Cyp26b1. Experiments utilizing lower concentrations of RA are likely to reveal a preventative affect on ethanol-induced gene changes in the proximal mesenchyme.

Additionally, a global transcriptional analysis was conducted after 2, 4, and 6 hours of ethanol exposure. These early transcriptional changes in limb buds were compared to those resulting from BMS-189453 exposure. Surprisingly, there were many differences and few similarities between BMS-189453 and each ethanol treatment group; the majority of genes were discoordinately regulated between groups. Thus, despite transcriptional evidence of ethanol's perturbation of RA-mediated gene expression gained from examination of later time points, the microarray analysis demonstrated that ethanol's induction of early transcriptional changes were not consistent with those resulting from the RAR antagonist.

These results present a paradox. The induction of limb defects and excessive cell death in the AER by BMS-189453 and disulfiram, the prevention of ethanol induced cell death by RA, and the ethanol-induced changes in RA-mediated gene expression, are consistent with a perturbation of RA signaling by ethanol. On the other hand, the global analysis of transcriptional alterations induced by ethanol suggests otherwise. These results may be reconciled; it is possible that the proximate molecular events caused by ethanol exposure in the limb (i.e. its mechanism) are distinct from later effects (its pathogenesis) that lead to perturbation of RA signaling and excessive cell death at 8 hours, and GD 18 dysmorphology.

Another apparent inconsistency observed in this investigation is informative to the examination of ethanol's mechanism. The differential effects of RA and ethanol co-administration in different regions of the limb bud suggest disparate mechanisms of ethanol-induced cell death between

the proximal limb bud and the AER. Since the proximal mesenchyme is a site of RA synthesis and a likely site of ethanol metabolism, there may be competitive inhibition for ADH and/or ALDH, resulting in decreased RA concentrations and producing an intracellular environment where ethanol free radicals or ROS are abundant. ROS may be responsible for mediating the effects of ethanol in the proximal mesenchyme, while decreased RA may secondarily affect the maintenance of the AER and mediate its response to ethanol.

Under this proposed model, the early effects of ethanol would not be mediated by a deficiency of RA, but rather consist of the full spectrum of changes produced by alterations in the redox potential (such as modulation of NF-κB signaling), disruption of growth factor signaling and alterations in cell-cell interactions as observed in our assessment of the transcriptome. To determine if this model is valid there are a number of experiments that would need to be performed. It must be determined, for example, whether there is evidence of free radical damage, and its temporal and spatial limits must be mapped. Additionally, a microarray analysis could be conducted to explore the ability of antioxidants to prevent ethanol's induction of transcriptional changes.

Testing this model is important because the perturbation of RA-mediated development by ethanol is expected to have application to malformations in other regions/organ systems in addition to the limb. RA is important to craniofacial development in the regulation of many of the same genes or gene family members that are present in the limb. Just as dHand, Fgf8, and Shh are integral to limb development, they are also essential to craniofacial patterning and may interact with RA in much the same manner as in the limb bud (Abu-Issa et al., 2002; Ribes et al., 2006; Zhang et al., 2006). Other ethanol-sensitive regions contain a similar cadre of RA-regulated genes, including the developing heart. In heart tissue, however, RA is known to have the opposite affect on dHand expression as it does in the limb (Li and Li, 2006). As a result of RA's many region-specific roles in development, perturbations of RA-mediated development may explain a variety of ethanol's effects in developing embryos.

The microarray analysis presented herein is one of the first descriptions of the transcriptional response to the onset of ethanol exposure. As such, the present study provides future researchers with an abundance of research avenues. Importantly, transcriptionally altered pathways and cellular functions were represented as in a previous expression analysis of the developing mid- and hindbrain exposed to ethanol (Dunty, 2002). In both studies, ethanol elicited changes in IGF-I and Wnt/β-catenin signaling as well as significant alterations to the expression of genes involved in cell cycle control, apoptosis, and, cell adhesion. Despite affecting disparate regions of the embryo, it is becoming clear that ethanol has some common effects on embryonic tissue. Such consistent transcriptional modifications may be reflective of ethanol's mechanism of action. The plethora of gene changes that occur following ethanol exposure are difficult to interpret. On one hand, a portion of the transcriptional changes reflect ethanol's affect on the embryo, and on the other hand, some transcriptional alterations represent the embryo's adaptive response to ethanol. It is the former changes that are of particular interest to discern ethanol's mechanisms. The challenges in determining the mechanisms of ethanol's action with microarray data include eliminating irrelevant changes and discerning downstream gene expression changes from causative changes. Further experiments designed to limit the irrelevant factors could compare anterior and posterior mesenchyme of limb buds, AER and surface ectoderm, forelimb and hind limb, or forelimbs of ethanol-sensitive and insensitive mouse strains.

Despite such limitations, the results of the functional analysis provide interesting candidates for ethanol's proximate, mechanistic effects besides its generation of ROS. One possible mechanism of ethanol's action is loss of cellular adhesion. Several adhesion-related functional categories are affected by ethanol exposure in the limb bud. As Charness et al. (1994) has demonstrated, ethanol interferes with intercellular junctions. Such changes not only have the obvious consequences on the ability of cells to remain attached to on another, to matrix, or to migrate, but also result in changes in cell signaling. One possible mediator is β-catenin. This protein plays in integral role in stabilizing adherins junctions, and it is also a major player in Wnt signaling. Ordinarily, these two roles of β-

catenin are separated to distinct intercellular compartments (Perez-Moreno et al., 2003; Gottardi and Gumbiner 2004), but disruption of the ability of cadherins to bind one another may trigger β-catenin's release and allow its participation in the regulation of Lef/Tcf transcription factors. Perturbation in Wnt/β-catenin signaling components leads to dysmorphology like that produced by ethanol (Simon-Chazottes et al., 2006), perhaps in a RA-dependant manner. Experiments designed to assay cell adhesion and Left/Tcf activity in the presence of ethanol would provide insight into this hypothesis.

The advantage and value of the present study lies in the use of a narrow range of cell types and its early examination of transcriptional responses to ethanol. Using only the right forelimb, this study eliminates transcriptional changes from organ systems that do not respond to ethanol. A major limitation of the present study lies in its use of mixed cell types. Within the mixed cell population in question, AER cells and proximal mesenchymal cells die, while the remaining mesenchyme and ectoderm resist the apoptotic effects of ethanol. Other cells, such as those of the ZPA and SRM, likely undergo perturbation of their proliferative pattern. It is likely that all of these subpopulations respond differently to ethanol, making the attribution of gene changes to a single cell type difficult. Using laser-capture microscopy with current mRNA amplification techniques, a study comparing the responses of AER and non-AER cells to ethanol could be possible. Such a study may provide greater resolution into the specific effects of ethanol on a single cell-type and generate hypotheses that may explain the resistance or sensitivity of a given cell type. Despite its limitations, the use of the whole limb bud has provided invaluable information regarding the signaling pathways and cellular functions affected by ethanol exposure within the limb bud, which may not have been otherwise observed. Few systems in vertebrate morphogenesis develop in isolation, ambivalent to adjacent tissues; a whole-limb bud approach potentially opens a window into ethanol's affect on the inductive interactions of adjacent tissues.

In conclusion, this work has made several important contributions to the study of FASD. RA was shown to play a role in the pathogenesis of ethanol induced limb defects, a finding that is likely to have applicability to other regions of the embryo. Ethanol-induced transcriptional changes,

although consistent with RA-signaling perturbation after several hours following maternal ethanol treatment, did not correlate to those changes occurring shortly after ethanol exposure. Therefore, our data do not support a mechanistic role for perturbation of RA-mediated development. However, the microarray analysis has provided an invaluable description of the early molecular changes following ethanol exposure and provides the impetus for further investigation into the role and interaction among ethanol's proximate molecular effects: induction of ROS, loss of cell adhesion, and perturbation in Wnt/β-catenin signaling.

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