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Xenopus embryos to study Fetal Alcohol Syndrome, a model for environmental teratogenesis

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Abstract

Vertebrate model systems are central to characterize the outcomes of ethanol exposure and the etiology of Fetal Alcohol Spectrum Disorder (FASD), taking advantage of their genetic and morphological closeness and similarity to humans. We discuss the contribution of amphibian embryos to FASD research, focusing on *Xenopus* embryos. The *Xenopus* experimental system is characterized by external development and accessibility throughout embryogenesis, large clutch sizes, gene and protein activity manipulation, transgenesis and genome editing, convenient chemical treatment, explants and conjugates and many other experimental approaches. Taking advantage of these methods many insights regarding FASD have been obtained. These studies characterized the malformations induced by ethanol including quantitative analysis of craniofacial malformations, induction of fetal growth restriction, delay in gut maturation and defects in the differentiation of the neural crest. Mechanistic, biochemical and molecular studies in *Xenopus* embryos identified early gastrula as the high alcohol sensitivity window, targeting the embryonic organizer and inducing a delay in gastrulation movements. Frog embryos have also served to demonstrate the involvement of reduced retinoic acid production and an increase in reactive oxygen species in FASD. Amphibian embryos have helped pave the way for our mechanistic, molecular and biochemical understanding of the etiology and pathophysiology of FASD.

Keywords

Embryonic development/ *Xenopus*/ Fetal Alcohol Syndrome/ Spemann's organizer
Animal models for the study of FASD

Fetal Alcohol Spectrum Disorder (FASD), as its name implies, is the set of developmental defects that result from exposing human embryos to alcohol during pregnancy (May et al. 2014, Williams et al. 2015, Popova et al. 2016). These developmental defects can take the form of anatomical malformations and may include an extensive neurodevelopmental disorder affecting mental capacity, behavior, social interactions, judgment, concentration ability, hyperactivity, and many other features. Already in the late 19th century and early 20th century, there were reports connecting alcohol exposure to developmental malformations. Oscar Hertwig (1896) studied the teratological effects of chemicals on frog embryos focusing primarily on nervous system malformations. In his conclusions, he suggested that blood borne chemicals like ethanol might cross the placenta to the fetus and give rise to developmental malformations. In a complementary study, Franklin Mall (1908) summarized the analysis of 163 human embryos with developmental defects. He raised the possibility that the generation of human “monsters”, i.e. malformed embryos, could be hereditary but also through external influences and chemicals including alcohol. The outcome of ethanol exposure in experimental model animal embryos was initially described in this time period. Charles Féré (1895) treated chicken embryos with ethanol to study its teratogenic effects. More than a decade afterwards, Charles Stockard (1910) performed a more extensive study of the teratogenic effects of anesthetics including alcohol, also using chicken embryos. He confirmed the malformations arising in the nervous system. Only in the second half of the 20th century, the neurodevelopmental syndrome induced by alcohol was formally described. Initially, Lemoine and co-workwers described the anomalies observed in 127 children of alcoholic parents (Lemoine et al. 1968). This study was subsequently expanded and Fetal Alcohol Syndrome (FAS) was formally suggested including initial guidelines for its diagnosis (Jones et al. 1973,
Jones and Smith 1973). Establishment of the link between alcohol exposure during embryogenesis and the developmental defects observed, brought about the search for mechanisms that could explain this relationship. Since then, multiple models have been suggested to account for the etiology of FASD, and they have been summarized in an accompanying review (Shabtai and Fainsod, this issue). Some of these models focus on cellular mechanisms that can explain elements of the neurodevelopmental disorder, while others concentrate on chemical or biochemical pathways that could explain the molecular etiology of this syndrome. Very early on it became apparent that better understanding of FASD could not rely solely on the study of human patients and it will require extensive use of animal models taking advantage of multiple systems and their particular assets.

The use of animal models to study the effects of alcohol in humans, extends almost throughout the animal kingdom whenever a suitable experimental model is available (Adkins et al. 2017, Park et al. 2017). Although some FASD-related studies have also been performed in invertebrate experimental models (McClure et al. 2011), the majority of studies have taken advantage of experimental vertebrate embryonic models ranging from fish to mammals. Each experimental embryo model has advantages and limitations as an FASD research system (Table 1). In the present review, we will focus on studies performed in amphibian embryos, mainly Xenopus, to further elucidate the effects of ethanol during embryogenesis.

Amphibians are oviparous, egg laying, as opposed to humans that exhibit placental viviparity and give birth to live young. In the context of FASD, this distinction is important, in particular where ethanol etiological studies are concerned. In humans, the ingested alcohol is processed and eliminated to a large extent by the mother. Only the remaining ethanol or its intermediate in the clearance process, acetaldehyde, reach the fetus through the placenta and induce this syndrome (Burd et al. 2007, 2012). In egg-laying animals, the maternal contribution
is restricted to whatever she placed in the egg in preparation for egg laying, i.e. oviposition. Then, in humans, the maternal role is crucial two-fold. In most cases, she is responsible for the fetal exposure to ethanol through ingestion, but not less important, is the maternal role in the clearance of the alcohol ingested. Most of this clearance will take place in the maternal liver.

Ethanol clearance involves two sequential chemical oxidation reactions, first to acetaldehyde and subsequently to acetic acid (acetate; Fig. 1). The first oxidation is performed mainly by middle-chain alcohol dehydrogenases (Crabb and Liangpunsakul 2007). In humans, this reaction is followed by oxidation of acetaldehyde mainly in the liver, primarily by aldehyde dehydrogenase 2 (ALDH2)(Deitrich 2004, Deitrich et al. 2007). The efficiency in alcohol clearance by the mother then becomes a fate changing event. By determining the type and concentration of the ethanol clearance metabolites reaching the embryo, the mother indirectly affects the incidence and severity of the FASD induced. Studies aimed at understanding the genetic contribution to FASD induction have in most instances identified alleles of genes encoding enzymes needed for alcohol clearance, in particular in the maternal genome (Bosron and Li 1986, Höög et al. 1986, Crabb et al. 1989, McCarver et al. 1997, Viljoen et al. 2001, Arfsten et al. 2004, Das et al. 2004, Jacobson et al. 2006, Hurley and Edenberg 2012). Therefore, oviparous FASD model systems mostly avoid the complexities introduced by the maternal genome and her biochemical efficiency and focus almost entirely on the embryo and its response to the alcohol exposure.

**Xenopus embryos as a model of Fetal Alcohol Spectrum Disorder**

For more than a century several species of amphibians, including salamanders and frogs, have served as experimental systems from the description and elucidation of fundamental developmental processes and to study the induction of developmental malformations,
teratogenesis. At the onset of the 20th century, Thomas Hunt Morgan was using frogs and frog embryos to develop experimental, embryological approaches and to study regeneration and the induction of developmental malformations (Morgan 1897, 1901). In parallel, experiments were performed in the laboratories of Hans Spemann and Wilhelm Roux using newt and frog embryos to understand the basic principles of embryonic development. In their experiments on the "organizer," Spemann and co-workers demonstrated the process of embryonic induction which involved cell-cell communication and resulted in neural induction (Fig. 2) (Spemann and Mangold 1924).

In the first half of the 20th century, research was being conducted to develop an animal-based pregnancy test based on the induction of ovulation in *Xenopus laevis* (African clawed frog) females (Hogben 1946). These experiments brought about the introduction and breeding of *X. laevis* in the laboratory. The availability of *Xenopus* eggs led to the development of fertilization protocols, natural and *in vitro*, to obtain embryos. From then on, *Xenopus* embryos have been one of the important experimental systems to study basic embryonic processes, signaling pathways, screening and cloning of numerous developmental genes, nuclear reprogramming, cell cycle regulation and chromatin structure (Fig. 2). In recent years, their use has extended to the establishment of models of human disease (Sater and Moody 2017). The *Xenopus* embryo is accessible throughout its development and all developmental stages can be easily studied. The early *Xenopus laevis* embryo has a diameter of 1.2-1.5 mm. Due to its relatively large size, it can be experimentally manipulated to affect gene and protein expression and function by microinjection of antibodies, proteins, RNA, DNA, and oligonucleotides. Also, the *Xenopus* embryo is very amenable to micro-dissection allowing the transplantation of specific embryonic regions or their growth in culture as explants. Recently, genetic methodologies have been
implemented in *Xenopus*, taking advantage of transgenic and genome editing approaches (Takagi et al. 2013, Tandon et al. 2017).

**Ethanol as a teratogen in Xenopus embryos**

The availability of large numbers of *Xenopus* embryos and the ease in their manipulation and culture led to the development of assay conditions to systematically test the teratogenicity of any chemical compound of interest and to determine and categorize the types of developmental malformations induced. This teratogenesis assay using *Xenopus laevis* embryos is known as the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) (Dumont et al. 1983, Dawson and Bantle 1987). Taking advantage of the FETAX assay, several reports have studied the teratogenic potential of ethanol. One of the central aims of the standardized FETAX assay is to determine the lethal (LC$_{50}$) and teratogenic (EC$_{50}$) concentrations of the compound being studied at several developmental stages. For ethanol, concentrations of 1.44%-1.71% and 0.79%-1.11% (vol/vol) were determined for LC$_{50}$ and EC$_{50}$ in *X. laevis* embryos, respectively (Dawson and Bantle 1987, Dresser et al. 1992, Fort et al. 2003). These results show that overt and efficient induction of developmental malformations in *Xenopus* embryos (>80% of the embryos) requires ethanol concentrations equivalent to 130-190 mM. In humans, blood alcohol levels of 86-100 mM are measured in highly intoxicated individuals and concentrations above are death-inducing. Then, according to these studies, the amounts of ethanol required to observe clear developmental malformations in *Xenopus* embryos are less than double of those observed in intoxicated individuals. These studies support the use of *Xenopus* embryos as a model system to study the etiology of FASD.

**Ethanol-induced craniofacial malformations**
Using *X. laevis* embryos, N. Nakatsuji (1983) investigated the effects of ethanol on normal embryonic development. In particular, he focused on the craniofacial malformations induced by the alcohol exposure. This study took place at a time when animal experiments were starting to focus on establishing experimental model systems that could recapitulate features of the recently described, alcohol-induced syndrome. Nakatsuji studied the malformations induced by ethanol and compared them to malformations characteristic of FAS. Embryos were analyzed from blastula until late tadpole stages. Several parameters were measured in the head region including mouth and brain sizes, the distance between the eyes, and the overall length of the embryo. Nakatsuji (1983) concluded that the head was not affected uniformly by the ethanol exposure. The overall size of the head was reduced, with the anterior head region showing the highest sensitivity to ethanol exposure, in particular, the width of the mouth was the most severely affected. The distance between the eyes was also reduced. Interestingly, according to his measurements, the width of the brain was unaffected. Along the anterior-posterior axis, the length of the head, brain and trunk-tail regions was reduced. These size changes are very similar to those organs and regions affected in children with FAS. In his experiments, Nakatsuji (1983) employed two ethanol concentrations 1% and 2% (vol/vol) and observed concentration-dependent developmental defects. In most of the experiments, over 80% of the treated embryos developed the described anomalies. The high level of reproducibility and severity of the defects observed was dependent on the amount of ethanol used. He concluded that ethanol-treated *Xenopus* embryos recapitulate the craniofacial malformations observed in humans and therefore can be a reliable experimental model to study FAS. These studies, like the FETAX assays, took advantage that *Xenopus* can lay hundreds to thousands of eggs in one day thus providing large samples to study. Also, these embryos are cultured in aqueous conditions allowing the simple addition of ethanol and other compounds to the culture medium.
In the same set of experiments, the effects of ethanol during earlier developmental stages were also analyzed (Nakatsuji 1983). In *X. laevis* embryos, the ethanol treatment delayed the migration of the mesendoderm towards the rostral region during the gastrulation process. The leading edge mesendoderm are the first cells that internalize and differentiate as part of the process of gastrulation. These cells play a central role in the induction of the rostral neuroectoderm which will differentiate as the forebrain, therefore affecting the development of the head (Kiecker and Niehrs 2001, Kaneda and Motoki 2012). The delay in the mesendodermal migration also brought about a retardation in blastopore closure, a landmark of the gastrulation process. In contrast, the expansion of the ectodermal layer that covers externally the embryo (epiboly), continued normally in the presence of ethanol. At neurula stages, it was clear that the neural plate, the future central nervous system, was reduced in size and it exhibited a clear shortening along the anterior-posterior axis and had a delayed deepening of the median groove. Surprisingly, by late neurula, the experimental embryos presented closed neural tubes, and the evidence of the different delays disappeared. These embryos still exhibited a size reduction (Nakatsuji 1983). This study showed that *X. laevis* embryos reliably recapitulate many of the malformations characteristic of children with FAS. Also, effects of ethanol were observed already during gastrula stages. These observations were subsequently corroborated in mouse, chicken, zebrafish and *X. laevis* FASD experimental models (Sulik 1984, Nakatsuji and Johnson 1984, Cartwright and Smith 1995, Blader and Strähle 1998, Yelin et al. 2005, 2007). The detection of these very early effects of ethanol has also helped the study and understanding of the FASD etiology.

*Neural crest defects in alcohol-treated amphibian embryos*
The connection between some of the developmental defects induced by ethanol and the neural crest has been studied for almost four decades (Clarren et al. 1978, Sulik et al. 1981, Colangelo and Jones 1982, Kirby and Bockman 1984). Focusing on a subset of clinical syndromes where multiple tissues were affected, Kirby and Bockman (1984) and Siebert (1983) concluded that many of these tissues had a neural crest origin. They proposed that one of the early events in these syndromes should involve abnormal formation and differentiation of the neural crest cell population. One the syndromes included in their analysis was Fetal Alcohol Syndrome. Soon thereafter, Hassler and Moran published a study on the effects of ethanol on the morphology and differentiation of neural crest cells (Hassler and Moran 1986a, 1986b). Using embryos of the yellow-spotted salamander (*Ambystoma maculatum*), they studied alcohol-induced cellular phenotypes of neural crest cells grown *in vitro*. Neurula-stage salamander embryos were dissected to remove different sections of the developing neural tube. They explanted posterior cranial and trunk neural tube fragments and placed them in culture conditions. Under culture conditions, the neural crest cells migrate away from the neural tube fragment. After six days, they form a monolayer composed of mesenchyme and pigment cells. Meanwhile, the neural crest cells continue to differentiate acquiring a dendritic shape with extensive branching resembling neural cells (Hassler and Moran 1986a, 1986b).

They employed two alcohol exposure protocols to study the effects of ethanol on neural crest differentiation. In the first protocol, the explant cultures were exposed continuously to different concentrations of ethanol (0.05%-0.2%) for six days. This alcohol treatment did not prevent the migration of the neural crest out of the neural tube explants, but it did prevent their differentiation to a branched dendritic morphology. Using antibodies to detect tubulin and actin filaments, they could conclude that the alcohol affected microtubules and microfilaments. The second protocol involved a short ethanol exposure of the cultures, and then they were
allowed to differentiate for six days. This short treatment resulted in the fast retraction of the cell extensions and alteration of cell-cell contacts, resembling the morphology of cells treated according to the first protocol. These results suggested that ethanol functions, in part, by interfering with the structure and function of the cytoskeleton. These early observations on the effect of ethanol on neural crest formation, differentiation and function have been confirmed and expanded in other experimental systems (Smith et al. 2014, Kiecker 2016).

**Elucidating the biochemical basis of Fetal Alcohol Spectrum Disorder**

Multiple models have been proposed to explain the neurodevelopmental anomalies induced by the ethanol exposure. Some models have focused on cellular phenotypes like the reduction in cell adhesion or the induction of apoptosis (Ashwell and Zhang 1996, Chen and Sulik 1996, Ornoy 2007, Smith et al. 2014), while others have pursued more biochemical explanations like the induction of reactive oxygen species or epigenetic changes including abnormal DNA methylation (Diluzio 1964, Reinke et al. 1987, Garro et al. 1991, Cravo et al. 1996, Chen and Sulik 1996, Halsted et al. 2002, Albano 2006). Biochemical analysis of fetuses following ethanol exposure revealed several abnormalities related to vitamin A (Grummer and Zachman 1990). For example, abnormal vitamin A metabolism, changes in the distribution and production of some vitamin A-derived metabolites between tissues was observed. These observations and biochemical considerations led to the proposal that ethanol exposure hampers the biosynthesis of retinoic acid from vitamin A (Duester 1991, Pullarkat 1991). Vitamin A (retinol) is also an alcohol whose conversion to retinoic acid requires two sequential oxidation reactions, first to retinaldehyde and subsequently to the acid form (Duester 2000). The first oxidation reaction is performed by enzymes with an alcohol dehydrogenase activity, while the second reaction requires aldehyde dehydrogenases. The biochemical similarity between retinoic acid
metabolism and ethanol clearance supported the mechanistic proposal that Fetal Alcohol Syndrome is the result of abnormally low retinoic acid signaling levels during embryogenesis (Duester 1991, Pullarkat 1991).

The proposed competition for the alcohol and aldehyde dehydrogenase enzymatic activities would result in low retinoic acid levels during embryogenesis, a condition known to be teratogenic (Hale 1935, Morriss-Kay and Sokolova 1996, Collins and Mao 1999). Low retinoic acid levels, as a result of vitamin A deficiency (VAD), are also known to be teratogenic (Hale 1935, Warkany and Schrafenberger 1946, Wilson and Warkany 1948, 1949, Wilson et al. 1953, Sarma 1959, Underwood 1994, Morriss-Kay and Sokolova 1996). Therefore, the biochemical model for Fetal Alcohol Syndrome suggested that it would be a form of VAD with extensive overlap in the developmental malformations induced. Additional syndromes with phenotypic overlap with FAS like Smith-Magenis, DiGeorge/Velocardiofacial and Matthew-Wood, have also been proposed to arise from reduced retinoic acid signaling (Vermot et al. 2003, Golzio et al. 2007, Elsea and Williams 2011). Most of the evidence comparing FAS to VAD was correlative based on the developmental defects observed, and not on a demonstrated mechanism. Besides the abnormal metabolism of retinoids and their tissue distribution (Grummer and Zachman 1990), cultured mouse embryos were utilized to support a reduction in retinoic acid levels (Deltour et al. 1996). In these experiments, a retinoic acid reporter cell line was employed to show a decrease in retinoic acid detection following exposure of mouse embryos to ethanol.

Almost two decades after *Xenopus* embryos were used to study the craniofacial effects induced by ethanol (Nakatsuji 1983), a series of experiments were performed to further establish this animal model as an experimental model system for FAS research (Yelin et al. 2005). Taking advantage of the large numbers of synchronized embryos obtainable from a
single fertilization in *Xenopus* and the simplicity of initiating or terminating the ethanol treatment, the temporal sensitivity to alcohol exposure was mapped in detail. Large numbers of embryos were placed in ethanol during the midblastula transition (stage 8.5), just prior to the onset of gastrulation (Nieuwkoop and Faber 1967), and taken out of the treatment at different developmental stages, a paradigm termed shift-out. In complementary analysis, groups of embryos were placed in ethanol at different developmental stages, a paradigm called shift-in. The embryos from the shift-out and shift-in groups were incubated until tailbud stages and analyzed for developmental malformations. These studies showed that, *Xenopus* embryos exhibit the highest sensitivity to ethanol in the window between late blastula to early gastrula, a period equivalent to the third week of human embryogenesis. A similar developmental sensitivity window was suspected or exploited in other organisms, including humans, where detailed mapping of the developmental window is more difficult (Sulik 1984, Ernhart et al. 1987, Blader and Strähle 1998). Later alcohol exposures resulted in milder and restricted phenotypes.

The high sensitivity developmental window focused the efforts of subsequent studies on the period surrounding the onset of gastrulation. At this developmental stage, the embryonic organizer, Spemann's organizer in *Xenopus*, is established and begins functioning (Harland and Gerhart 1997). Spemann's organizer represents a small group of cells that secrete multiple instructive and permissive signals. These signals are crucial to establish the basic embryonic axes, anterior-posterior and dorsal-ventral, morphogenetic gradients to pattern these axes, and contribute to the differentiation of the germ layers, ectoderm, mesoderm and endoderm (Harland and Gerhart 1997). *Xenopus* embryos have served as a model system of choice to study the organizer phenomenon (Spemann and Mangold 1924) and the onset of gastrulation. Numerous genes expressed within this region have been investigated by cloning and
manipulation, and several experimental approaches have been developed to research this central embryonic structure (Niehrs 2004). For this reason, extensive information is available about organizer genes that were studied at the onset of gastrulation in alcohol treated embryos. These studies revealed abnormal expression patterns as a result of the ethanol exposure (Yelin et al. 2005, 2007). The effect of ethanol on organizer-specific gene expression was recapitulated by pharmacological inhibition of retinoic acid biosynthesis or enzymatic reduction of retinoic acid levels by CYP26A1 overexpression. Consistently, ethanol exposure resulted in effects opposite to the outcomes from retinoic acid treatment. These observations supported the competition between ethanol and retinoic acid biosynthesis model and identified the organizer as an early target of the ethanol treatment. In support, the organizer in vertebrates is known to contain retinoic acid (Hogan et al. 1992, Kraft et al. 1994, Creech Kraft et al. 1994). Also, using transgenic *Xenopus* embryos carrying a retinoic acid reporter construct (Rossant et al. 1991), it was demonstrated that the full retinoic acid signaling pathway is active in the organizer. This retinoic acid promotes the regulation of organizer-specific genes (Yelin et al. 2005).

For the organizer to have retinoic acid and a functional signaling network, the regulatory ligand has to be produced in the organizer or adjacent tissues. Retinaldehyde dehydrogenase 2 (RALDH2, ALDH1A2), is an ALDH member active in the oxidation of retinaldehyde to retinoic acid (Kumar et al. 2012). In vertebrates, RALDH2 is the enzyme performing the initial production of retinoic acid at the onset of gastrulation (Ang and Duester 1999, Chen et al. 2001, Begemann et al. 2001, Grandel et al. 2002). Vertebrate embryos, mutant in the gene encoding RALDH2 show the earliest embryonic lethality of all mutants in components of the retinoic acid biosynthetic network (Niederreither et al. 1999, Begemann et al. 2001, Grandel et al. 2002). The gene encoding this enzyme begins to be transcribed at the onset of gastrulation in the embryonic organizer or in gastrulating regions surrounding the organizer (Niederreither
et al. 1997, Chen et al. 2001, Begemann et al. 2001, Grandel et al. 2002). These observations suggest that the retinoic acid-producing activity of RALDH2 might be targeted by ethanol. Then, taking advantage of the *Xenopus* FAS model, the ethanol sensitivity was mapped to the embryonic organizer and the effects of ethanol were recapitulated by knocking-down the levels of retinoic acid signaling (Yelin et al. 2005, 2007).

The original ethanol-retinoic acid competition model to explain the etiology of FAS focused on alcohol dehydrogenases (ADHs) of the middle-chain dehydrogenase/reductase family as the main step for the competition by ethanol based on thermodynamic considerations (Deltour et al. 1996). The competition at this enzymatic step would center on the relative affinity of ethanol or retinol (vitamin A) for the same ADH enzyme(s). Several lines of evidence suggest that the competition is actually between retinaldehyde and acetaldehyde for the second oxidation reaction. In recent years, it became clear that during early embryogenesis, different enzymes oxidize retinol and ethanol to their respective aldehyde forms. Oxidation of ethanol to acetaldehyde is performed mainly by ADHs (Deltour et al. 1999). On the other hand, RDH10 has been identified as the main retinol dehydrogenase during early embryogenesis (Strate et al. 2009, Sandell et al. 2012). RDH10 belongs to the short-chain dehydrogenase/reductase (SDR) family (Wu et al. 2002, 2004, Belyaeva et al. 2008) Mutants in the *Rdh10* gene show early embryonic lethality slightly later in development than the lethality observed in *Raldh2* mutants (Rhinn et al. 2011, Sandell et al. 2012). The allocation of the oxidation of retinol and ethanol to different enzyme families greatly reduces the possibility of competition for the same enzyme.

Experiments performed in *Xenopus* embryos further focus the competition between ethanol and vitamin A to the second oxidation reaction carried out by an aldehyde dehydrogenase. In vertebrate embryos, the expression of RALDH2 at the onset of gastrulation correlates with the start of retinoic acid signaling. The embryo is primed to activate this signaling pathway but
requires the last oxidation reaction to take place (Niederreither et al. 1999). This conclusion was demonstrated by early overexpression of an enzyme with retinaldehyde dehydrogenase activity in *Xenopus* embryos which resulted in precocious retinoic acid signaling during blastula stages before the onset of gastrulation (Ang and Duester 1999). Supporting evidence was obtained when *Xenopus* embryos were treated with inhibitors of the ADH or RALDH enzyme families (Kot-Leibovich and Fainsod 2009). Analysis of the response of retinoic acid-regulated genes during early gastrula revealed that ADH inhibition had almost no effect on retinoic acid signaling while inhibition of RALDH induced a reduction retinoic acid target genes. Together, these observations shifted the focus to the RALDH activity as the limiting factor during early gastrula stages and the candidate enzymatic activity hindered by the alcohol exposure.

As RALDH2 is the main enzyme at these developmental stages, this should be the initial activity competed by ethanol, or more precisely, its oxidation metabolite acetaldehyde. *Raldh2* transcription only begins close to the onset of gastrulation. During early gastrula stages, the RALDH2 enzyme is only present in limiting amounts (Chen et al. 2001). According to the modified competition model (Shabtai and Fainsod, this issue), the presence of acetaldehyde would further reduce the availability of RALDH2 for retinoic acid biosynthesis. Taking advantage of the ease of manipulation of *Xenopus* embryos, it was demonstrated that knock-down of the RALDH2 activity rendered the embryo hyper-sensitive to ethanol exposure. Low alcohol concentrations together with RALDH activity knock-down induced phenotypic effects and molecular changes, commonly observed following exposure to higher ethanol concentrations (Kot-Leibovich and Fainsod 2009). In agreement, supplementation of *Xenopus* embryos with RALDH2 activity by mRNA microinjection partially rescued the effects of high ethanol exposure. Manipulation of the RALDH2 activity predictably affected the outcome of
the ethanol exposure, and further demonstrated that the initial competition is at the level of this retinaldehyde dehydrogenase, which appears to be one of the earliest targets of ethanol.

The results that focused the earliest ethanol effects to the onset of gastrulation, in particular to the RALDH2 activity, brought about the need to translate these observations to the human embryo. As research on human embryos is extremely challenging for technical and ethical reasons, the *Xenopus* embryo was used to study the human RALDH2 enzyme (hRALDH2)(Shabtai et al. 2016). Surprisingly, hRALDH2 had not been characterized biochemically. Then, manipulated *Xenopus* embryos were employed to study the activity of the human enzyme in an embryonic setting. In parallel, hRALDH2 was characterized kinetically (Shabtai et al. 2016). Using *Xenopus* embryos overexpressing hRALDH2, it could be shown that this enzyme activates the retinoic acid signaling pathway *in vivo* in a concentration-dependent manner and it requires retinaldehyde to achieve this effect. Importantly, the activity of hRALDH2 in *Xenopus* embryos was hampered by the presence of ethanol. To further support the role of acetaldehyde in the teratogenesis of ethanol, recent studies investigated acetaldehyde itself (Shabtai et al., unpublished). These experiments showed that acetaldehyde induces similar developmental malformations and molecular changes like ethanol or pharmacological inhibition of the RALDH activity. Kinetic analysis revealed that acetaldehyde is a substrate of hRALDH2. Comparison of retinaldehyde and acetaldehyde as substrates of hRALDH2 revealed that the kinetic parameters favor the oxidation of acetaldehyde over retinaldehyde (Shabtai et al., unpublished). These observations further suggest that the competition for the hRALDH2 activity is preferentially diverted towards the oxidation of acetaldehyde.

*Xenopus embryos to study additional developmental defects induced by ethanol*
One of the phenotypes observed in ethanol-treated embryos was a delay in the gastrulation process (Nakatsuji 1983). Analysis of the pattern of expression of organizer-specific genes revealed an increase in transcript levels and more importantly a delay in the invagination and rostral migration of the cells expressing them (Yelin et al. 2005, 2007). The organizer cells that initially invaginate during the process of gastrulation, the leading edge mesendoderm, go on to become the prechordal mesendoderm which plays a central role in the induction and formation of the head and the forebrain (Pera and Kessel 1997, Camus et al. 2000, Kiecker and Niehrs 2001). Further analysis of this effect in *Xenopus* embryos demonstrated that ethanol delays the invagination and migration of the leading edge mesendoderm (Yelin et al. 2007). The prechordal plate cells reach their normal position below the rostral neuroectodermal anlage, but with a temporal delay. To further demonstrate the delay in the migration of the prechordal mesendoderm to their final cranial position, several assays to study morphogenetic movements in *Xenopus* embryos were employed. Incubation of *Xenopus* embryos in high salt conditions affects the internalization of the mesendodermal cells and the gastrulation process proceeds externally thus creating an exogastrula. Ethanol exposure of embryos manipulated to induce exogastrulation prevented the characteristic elongation observed in control embryos (Yelin et al. 2005). Another *Xenopus*-specific assay used to study the effects of ethanol on gastrula morphogenetic movements was to dissect and culture dorsal marginal zone explants. At the onset of gastrulation, the dorsal lip of the blastopore or dorsal marginal zone (DMZ), is where Spemann's organizer resides and it contains the leading edge mesendodermal cells. When explanted and further incubated, the DMZ elongates as part of the normal morphogenetic movements of the cells residing in this region. Also, in this case, DMZ explants exposed to ethanol failed to elongate compared to the control DMZs (Yelin et al. 2005). These results showed that ethanol delays the rostral migration of the prechordal plate by affecting
morphogenetic movements during gastrulation. A similar effect of ethanol on the migration of the prechordal plate has been described in zebrafish (Blader and Strähle 1998). In the zebrafish case, the ethanol treatment induces cyclopia as a result of impaired migration of the prechordal plate. Ethanol has also been shown to affect the migration of other cell types like neural crest cells (Oyedele and Kramer 2013, Smith et al. 2014, Tolosa et al. 2016, Eason et al. 2017). These results suggest that ethanol might have a widespread inhibitory effect on cell migration.

The effects of ethanol on the process of gastrulation and the embryonic organizer were further studied in *Xenopus* embryos. Analysis of the changes induced in organizer-specific gene expression revealed abnormal expression patterns. In some instances, the organizer-restricted expression increased while in others the expression was eliminated. One of the genes consistently up-regulated by the ethanol treatment is *goosecoid* (*gsc*) (Cho et al. 1991, Yelin et al. 2005, 2007). In a series of overexpression experiments, microinjection of *gsc* mRNA up-regulated genes like *chordin* and down-regulated genes like *Xnot2*, recapitulating the effects of ethanol (Yelin et al. 2007). These observations raised the possibility that some of the molecular changes observed as a result of ethanol exposure are secondary to a limited number primary targets like up-regulation of *gsc*. In support, a recent study showed that *gsc* is also a regulator of morphogenetic movements (Ulmer et al. 2017). Similarly, it was determined that the reduction in the *Pax6* expression domain in the eye as a result of ethanol exposure could be recapitulated by *sonic hedgehog* (*shh*) overexpression (Yelin et al. 2007). The pattern of *shh* expression is affected by ethanol exposure (Yelin et al. 2007), and manipulation of *shh* can rescue some of the developmental phenotypes induced by ethanol (Ahlgren et al. 2002). This type of analysis exemplifies the ease to study the primary ethanol effects and their subsequent targets.

*Rescuing the malformations induced by ethanol in Xenopus embryos*
As discussed above, there are multiple models proposed to explain the effects of ethanol exposure during embryogenesis. Some of the models are based on pathophysiological observations of events arising after the alcohol exposure. Alternatively, biochemical mechanisms have been proposed to explain the etiology of FAS. Some studies have been performed in *Xenopus* to try and address the teratogenic mechanism of ethanol. Most of these studies are based on concurrent treatment of embryos with ethanol, and other compounds proposed to have a rescuing effect. As mentioned before, one of the etiological models supported by rescue experiments is the competition between ethanol or its metabolites and vitamin A for the oxidation enzymes. From an early stage, it was shown in *Xenopus* embryos that the effects of ethanol on gene expression can be reproduced by blocking the biosynthesis of retinoic acid, and they are the opposite of the changes induced by treatment with retinoic acid (Yelin et al. 2005). In the same study, it was shown that ethanol causes a number of developmental malformations which can be rescued by retinol or retinaldehyde treatment (Yelin et al. 2005). A similar rescuing effect can be obtained by increasing the level of RALDH2 enzyme in the embryo by mRNA microinjection (Kot-Leibovich and Fainsod 2009). The rescuing effect was determined by focusing on developmental malformations, monitoring the level of the retinoic acid signal, and analyzing the expression pattern of organizer-specific genes. Recent studies have shown that also acetaldehyde treatments can be rescued by supplementation with retinaldehyde or hRALDH2 (Shabtai et al., unpublished). The amenability of *Xenopus* embryos to manipulation and combined treatments provided a convenient experimental system to test this etiological model for FASD.

Another model that has also been studied in more detail using a rescue approach in *Xenopus* embryos is the increase in reactive oxygen species (ROS) as a result of ethanol exposure. Several developmental malformations were the focus of a series of studies centering ROS
induced defects (Peng et al. 2004a, 2004c, 2004b, 2005). This group of researchers focused on ethanol-induced malformations like microencephaly, retarded growth rates, delayed gut maturation, reduced body length and ocular anomalies. In their studies, they could show an increase in ROS and reactive nitrogen species (RNS) (Peng et al. 2004a, 2004b, 2005). The increase in ROS or RNS led to an ethanol concentration-dependent decrease in the expression of ocular, neural and gut marker genes. In this set of studies, four alternative approaches were described that can rescue the ethanol-induced developmental defects.

Focusing on the ethanol-induced microcephaly, they could show that alcohol induced a reduction in *Pax6* and expression of other neural markers (Peng et al. 2004c). The decrease in *Pax6* expression could be rescued by overexpression of catalase which reduced the H$_2$O$_2$ production and reversed the microcephaly. Overexpression of *Pax6* also rescued the microcephalic phenotype and restored normal neural gene expression. This study linked the ethanol exposure to ROS production which in turn affected gene expression, resulting in developmental malformations. In studies done in parallel, it was also shown that ethanol suppressed *Pax6* expression (Yelin et al. 2007). In this study, it was proposed that the reduction in *Pax6* expression involves high *sonic hedgehog* (*shh*) levels and the microcephalic phenotype includes abnormal formation of the first brain ventricle in the forebrain.

Overexpression of catalase and peroxiredoxin 5 was also used to rescue ocular anomalies, delayed gut maturation, and retarded growth (Peng et al. 2004a, 2004b). Overexpression of both enzymes in embryos was shown to reduce ROS *in vivo* efficiently. This inhibition of ROS also efficiently restored normal expression of the molecular markers of the affected tissues. On the other hand, the developmental malformations were only rescued partially (Peng et al. 2004a, 2004b). These rescue results suggest the involvement of additional alcohol induced biochemical changes besides ROS. Also, the antioxidant ascorbic acid (Vitamin C) was used to
rescue the ethanol induced defects (Peng et al. 2005). This study concluded that vitamin C reduces ethanol-induced ROS, rescues the activation activity of NF-κB, and protects the embryo from microcephaly and growth retardation. These studies show again the ease of manipulation of the *Xenopus* embryo to study molecular pathways and mechanisms.

**Limitations of Xenopus embryos as a FAS model**

Several experimental model systems are routinely exploited to characterize and investigate the etiology of ethanol in the induction of FASD. Each one of these experimental models has its unique qualities and drawbacks for these type of studies (Table 1) (Wheeler and Brändli 2009). Nevertheless, besides experimental details of the alcohol exposure protocol, i.e. amount, developmental window, time and mode of exposure, the evolutionary, genetic and molecular similarity to humans are significant (Wheeler and Brändli 2009). The studies described above show that the *Xenopus* embryo recapitulates numerous developmental malformations characteristic of children with FASD. The developmental defects studied until now are mainly morphological malformations characteristic of the severe form, Fetal Alcohol Syndrome. On the other hand, FASD encompasses, besides the anatomical malformations, an extensive neurodevelopmental disorder that includes behavioral, social, functional and mental abnormalities (May et al. 2014, Williams et al. 2015, Popova et al. 2016). In recent years a number of studies focusing on behavioral responses have been described using *Xenopus* embryos (Pronych et al. 1996, Roberts et al. 2000, Blackiston and Levin 2012, Viczian and Zuber 2014). These and many other functional assays can test simple behaviors like light or specific background color avoidance. More advanced assays rely on aversive conditioning training and can test more complex neural functions like associative learning and memory. For now, no
suitable experimental paradigm can examine more behavioral aspects of FASD in *Xenopus* embryos, but new assays are constantly reported.

To a large extent, the studies in *Xenopus laevis* are restricted to embryos and tadpoles due to its long generation time, about 1 year depending on the husbandry conditions. This long generation time limits the use of adult animals that were raised from alcohol exposed or experimentally modified embryos. Also, this long generation time restricts the usefulness of this experimental model for classical genetic studies and screens. Several alternatives have become available in the last decades to allow gene manipulations in *Xenopus laevis*. Overexpression of genes by RNA microinjection is a commonplace approach, allowing analysis of gain-of-function paradigms. For loss-of-function, gene knock-downs and overexpression of dominant negative constructs are routinely employed (Amaya et al. 1991, Heasman 2002). Two additional methodologies are also available in *Xenopus* embryos for gene manipulation. Transgenic manipulation (Kroll and Amaya 1996) and modern genome editing approaches (Tandon et al. 2017, Aslan et al. 2017). All these methods allow extensive genetic manipulation to understand the contribution of targets genes to the process in question, in this case FAS, and determine the hierarchy of genetic networks.

Besides molecular genetic manipulation of embryos and tadpoles, newly metamorphosed froglets are another experimental system of choice. Froglets contain all adult tissues. In *Xenopus laevis*, this post metamorphosis stage can be reached in about three months from fertilization (Edwards-Faret et al. 2017). *Xenopus laevis* are used to study many processes requiring adult tissues and organs like spinal cord regeneration (Edwards-Faret et al. 2017), wound healing (Bertolotti et al. 2013), limb regeneration (Rao et al. 2014), thyroid function (Buchholz 2017) and others.
Another alternative for shorter generation times in Amphibia with many of the advantages of *Xenopus laevis* is its closely related species, *Xenopus tropicalis* (Kashiwagi et al. 2010). The similarity between these two species allows easy implementation of many of the experimental protocols developed for *X. laevis* in *X. tropicalis* embryos. Comparative experimental embryological research has revealed the close similarity between both species (Yanai et al. 2011) such that observations gleaned in one, are almost always applicable to the other. *X. tropicalis* is a diploid species with a generation time of about four months (Kashiwagi et al. 2010). The eggs and adults of *X. tropicalis* are much smaller than those of *X. laevis* making some experimental procedures technically challenging.

**Conclusions**

Amphibian embryos as experimental model systems during embryogenesis have been an important source of insights and the description of developmental processes and signaling pathways. Their use to advance our understanding of the teratogenic etiology of ethanol exposure has focused to a large extent on mechanistic elucidation of FASD. Morphological studies have provided an extensive basis to support the induction of an FASD-like syndrome in ethanol treated amphibian embryos and establishing them as a reliable model system of this disease. These studies have also proceeded to characterize cellular phenotypes like neural crest migration defects or morphogenetic movements during gastrulation, which will translate into anatomical outcomes in older embryos or adults. Amphibian studies, in particular, *Xenopus*, have taken advantage of the ease of manipulation and analysis to extend the phenotypic characterization to molecular effects and abnormal gene expression. With the well characterized embryonic development in *Xenopus*, these studies have identified the onset of gastrulation and in particular the embryonic organizer, Spemann's organizer, as probably the
earliest structure affected by ethanol. Also, taking advantage of the large numbers of embryos and the ease of manipulations *Xenopus* embryos have been employed to study the basic biochemical etiology of the alcohol exposure. These studies have clearly shown an involvement of a reduction in retinoic acid signaling and an increase in reactive oxygen species which could be happening in parallel as a result of the surge in acetaldehyde levels. Reactive oxygen species are a byproduct of the ethanol clearance together with the production of acetaldehyde (Seitz and Mueller 2015, Na and Lee 2017). Then, *Xenopus* embryos have served as an efficient experimental system to advance our understanding of FASD. Some of the conclusions resulting from the *Xenopus* are being validated by establishing the appropriate experimental model in other organisms like mice (see Hicks and Pettreli in this issue).

Gene manipulation studies in frog embryos will begin addressing the genetic component in the induction of FASD. The genomes of both *X. laevis* and *X. tropicalis* have been completely sequenced and are in advanced stages of annotation (Session et al. 2016, Vize and Zorn 2017). Also, gene regulation in these species involves epigenetic modification of chromatin like in humans (Hontelez et al. 2015, Suzuki et al. 2017), providing an excellent system to study the effects of ethanol on chromatin structure in the search for diagnostic biomarkers for FASD. Future studies in *Xenopus* embryos will continue focusing on the elucidation of the biochemistry of the exposure to ethanol and its pathophysiological outcomes. Human enzymes are being studied in an embryonic setting by injection into *Xenopus* embryos, thus increasing the relevance of this experimental system. In the future, studies will take advantage more advanced embryonic stages and froglets to better characterize the developmental malformations induced by ethanol.

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References


Amaya, E., Musci, T., and Kirschner, M. 1991. Expression of a dominant negative mutant of


Blader, P., and Strähle, U. 1998. Ethanol impairs migration of the prechordal plate in the


doi:10.1073/pnas.1103877108.


Sarma, V. 1959. Maternal vitamin A deficiency and fetal microcephaly and anophthalmia;


Figure Legends

**Figure 1. Biochemistry of ethanol clearance.** Schematic representation of the two sequential oxidation reactions required to convert ethanol to acetaldehyde and subsequently acetic acid. The two main enzyme families required for these oxidation reactions are marked.

**Figure 2. *Xenopus* as a model system to study FASD.** Summary *Xenopus* as an experimental system to study FASD. The main embryonic processes elucidated utilizing *Xenopus* embryos are listed. The experimental approaches commonly used in studies involving *Xenopus* embryos are enumerated. The main conclusions obtained from studies of ethanol-treated *Xenopus* embryos are summarized.
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<table>
<thead>
<tr>
<th>Organism</th>
<th>Experiment size(^a)</th>
<th>Ethanol treatment</th>
<th>Time to gastrula</th>
<th>Mutants available</th>
<th>Molecular manipulation</th>
<th>Grafting and explants</th>
<th>Genome editing and transgenesis</th>
<th>Accessibility</th>
<th>Reproduction</th>
<th>Generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenopus laevis</em></td>
<td>Hundreds to thousands</td>
<td>In culture medium</td>
<td>~10 hours</td>
<td>Few</td>
<td>Yes</td>
<td>Yes</td>
<td>Throughout embryogenesis</td>
<td>Egg laying</td>
<td>~1 year</td>
<td>~1 year</td>
</tr>
<tr>
<td><em>tropicalis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>~4 months</td>
<td></td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Hundreds</td>
<td>In culture medium</td>
<td>~5-6 hours</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Throughout embryogenesis</td>
<td>Egg laying</td>
<td>3.5-5 months</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>Tens</td>
<td>In ovo(^b)</td>
<td>~18-19 hours of incubation</td>
<td>Few</td>
<td>Limited</td>
<td>Common</td>
<td>Throughout embryogenesis</td>
<td>Egg laying</td>
<td>5-6 months</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>8-12/litter</td>
<td>Through the mother(^b,c)</td>
<td>~6.5 days</td>
<td>Yes</td>
<td>Difficult</td>
<td>Limited</td>
<td>Surgical</td>
<td>Placental</td>
<td>6-8 weeks</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\); Numbers of embryos in a single experiment.
\(^b\); Could be performed in culture conditions.
\(^c\); In drinking water, gavage, intraperitoneal injection.