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ORIGINAL ARTICLE

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Early molecular effects of ethanol during vertebrate embryogenesis

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Abstract Fetal alcohol spectrum disorder (FASD) is the combination of developmental, morphological, and neurological defects that result from exposing human embryos to ethanol (EtOH). Numerous embryonic structures are affected, leading to a complex viable phenotype affecting among others, the anterior/posterior axis, head, and eye formation. Recent studies have provided evidence suggesting that EtOH teratogenesis is mediated in part through a reduction in retinoic acid (RA) levels, targeting mainly the embryonic organizer (Spemann's organizer) and its subsequent functions. EtOH-treated *Xenopus* embryos were subjected to an analysis of gene expression patterns. Analysis of organizer-specific genes revealed a transient delay in the invagination of *gsc*- and *chordin*-positive cells that eventually reach their normal rostro-caudal position. Dorsal midline genes show defects along the rostro-caudal axis, lacking either their rostral (*Xbra* and *Xnot2*) or caudal (*FoxA4b* and *Shh*) expression domains. Head-specific markers like *Otx2*, *en2*, and *Shh* show abnormal expression patterns. *Otx2* exhibits a reduction in expression levels, while *en2* becomes restricted along the dorsal/ventral axis. During neurula stages, *Shh* becomes up-regulated in the rostral region and it is expressed in an abnormal pattern. These results and histological analysis suggest the existence of malformations in the brain region including a lack of the normal fore brain ventricle. An increase in the size of both the prechordal plate and the notochord was observed, while the spinal cord is narrower. The reduction in head and eye size was accompanied by changes in the eye markers, *Pax6* and *Tbx3*. Our results provide evidence for the early molecular changes induced by EtOH exposure during embryogenesis, and may explain some of the structural changes that are part of the EtOH teratogenic phenotype also in FASD individuals.

Key words Fetal alcohol syndrome · Spemann's organizer · *XCG1* · teratogen · microcephaly · microphthalmia · head development · eye formation · notochord · prechordal plate · neural tube · craniofacial malformations

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Introduction

Exposure of human embryos during pregnancy to ethanol (EtOH) has been linked to an increase in first semester spontaneous abortions, stillbirth, and the birth of individuals with fetal alcohol spectrum disorders (FASD; Jones and Smith, 1973; Johnson et al., 1996; Chaudhuri, 2000; Kesmodel et al., 2002a, 2002b; Koren et al., 2003; Sokol et al., 2003). Individuals affected with FASD exhibit phenotypes involving changes in the central nervous system, craniofacial deformities, malformed organs including the heart and the eyes, as well as growth and developmental retardation and cognitive and behavioral abnormalities. For these reasons, EtOH is regarded as one of the most widespread and important human chemical teratogens. Although it is well known that EtOH exposure during pregnancy can lead to fetal alcohol syndrome (FAS), fetal alcohol effects (FAE), or milder syndromes, little is known about the developmental and molecular mechanism(s) of EtOH teratogenesis. Questions about the developmental window of sensitivity, the genetic or signaling path-

way(s) affected by EtOH and the region(s) in the embryo (tissues, organs, or structures) targeted by EtOH, were examined taking advantage of different experimental vertebrate developmental systems (Nakatsuji, 1983; Nakatsuji and Johnson, 1984; Cook and Sulik, 1988; Dresser et al., 1992; Grummer and Zachman, 1995; Twal, 1997; Blader and Strahle, 1998; Yelin et al., 2005). Recapitulation of the EtOH-induced teratogenic phenotypes has been observed in multiple instances (Sulik et al., 1981; Runner, 1986; Cook and Sulik, 1988; Dresser et al., 1992; Weston et al., 1994; Becker et al., 1996; Boehm et al., 1997; Gilliam et al., 1997; Peng et al., 2004a, 2004b; Yelin et al., 2005). The complex phenotype resulting from EtOH exposure suggested that this teratogen probably affects several central embryonic processes.

Several models have been put forth to try and explain the teratogenic effects of EtOH including induction of apoptosis, cell adhesion defects, accumulation of free radicals, effects on growth factors, and antagonism of retinoic acid (RA) biosynthesis (Henderson et al., 1989; Duester, 1991; Pullarkat, 1991; Kotch et al., 1995; Deltour et al., 1996; Singh et al., 1996; Olney et al., 2002). One of the models suggested that EtOH might function as a competitive inhibitor of the pathway metabolizing vitamin A (retinol; ROL) to RA (Duester, 1991; Deltour et al., 1996). EtOH conversion to acetic acid (detoxification) and RA biosynthesis from ROL, share the same two oxidation steps and, therefore, the competition between the two pathways for the alcohol and aldehyde dehydrogenase enzymes would lead to continued embryonic development with reduced RA levels, resulting in numerous embryonic malformations (Clagett-Dame and DeLuca, 2002; Marill et al., 2003). Recent evidence supports that the teratogenic effects of EtOH are mediated, partly, through its effects on the RA signaling pathway (Yelin et al., 2005). EtOH and RA induce opposed transcriptional responses on the *Hox* genes, known RA signaling targets, and on organizer-specific genes. Combined EtOH and ROL exposure rescued the teratogenic effects of the independent treatments on the phenotypic outcome (Yelin et al., 2005).

In order to shed light on the EtOH teratogenic phenotype at the molecular level, we studied the changes in gene expression patterns in EtOH-treated embryos. We focused on expression of markers specific for the organizer, the midline, the head, including the brain and the eyes, and the interaction between some of these markers. We observed a delay in invagination and rostral migration of organizer genes, which could result in head and midline defects. This assumption was confirmed by showing abnormal expression patterns of all midline genes tested and an EtOH concentration-dependent restriction in the expression domain of the early head-marker, *Otx2*. In addition to microcephaly, the head exhibits malformations involving the central nervous sysytem and the eyes. The temporal and spatial changes in gene expression patterns suggest that EtOH affects normal organizer functions, followed by midline defects and changes in head-specific features all in agreement with the developmental phenotype of FASD individuals.

Materials and methods

Embryo production, staging, treatment and sectioning

Xenopus laevis frogs were purchased from Xenopus 1 (Dexter, MI). Embryos were obtained by *in vitro* fertilization, incubated in 0.1 × modified Barth's solution (MBSH) and staged according to Nieuwkoop and Faber (1967). EtOH treatments were performed in 0.1 × MBSH (vol/vol). Unless otherwise stated, the embryos were placed in the EtOH solution soon after the midblastula transition (MBT; st. 8.5) and were either kept in this solution or washed at the specified stages.

Optical sectioning was performed by third harmonic generation microscopy (Yelin and Silberberg, 1999; Oron et al., 2004). Embryos were cleared in Murray's solution and were subsequently optically sectioned to 40 μm thick sections. Otherwise, embryos were embedded in paraffin and sectioned for analysis by conventional means.

RNA transcription and *in situ* hybridization

Whole-mount *in situ* hybridization analysis of gene expression was performed as previously described (Epstein et al., 1997). Digoxigenin-labeled RNA probes were prepared from linearized plasmids transcribed *in vitro* using the RiboMax kit (Promega, Madison, WI) and the digoxigenin RNA labeling mixture (Roche, Indianapolis, IN). For double *in situ* hybridization one probe was labeled with digoxigenin and the second one with fluorescein. Double *in situ* hybridization was performed and stained as described (Epstein et al., 1997). Probes for *in situ* hybridization were prepared for: *gsc* (Cho et al., 1991), *Otx2* (Smith et al., 1993), *chordin* (Sasai et al., 1994), *Xnot2* (Gont et al., 1993), *en2* (Hemmati-Brivanlou et al., 1991), *FoxA4b* (FKH1; Dirksen and Jamrich, 1992), *Tbx3* (Li et al., 1997), *Krox20* (Bradley et al., 1992), *XCG1* (Hemmati-Brivanlou et al., 1990a), *Pax6* (Li et al., 1997), *Shh* (Ekker et al., 1995a), *Gbx2* (Tour et al., 2002), and *Xbra* (Smith et al., 1991).

For microinjection of capped RNAs, two to four-cell embryos were injected radially (four times). RNA was prepared by *in vitro* transcription using the RiboMax kit (Promega, Uppsala, Sweden). Cap analog (Pharmacia) was added to the reaction mixture using a ratio of cap:GTP of 5:1. The template for transcription of *gsc* mRNA was prepared from the full length cDNA clone (Cho et al., 1991). *Shh* mRNA was prepared from a cDNA fragment (Ekker et al., 1995a).

Results

The FAS phenotype in Xenopus embryos

The conditions for efficient and reproducible treatment of *Xenopus* embryos with EtOH as a model system to study the etiology of FAS have been established (Nakatsuji, 1983; Dresser et al., 1992; Yelin et al., 2005). Embryos were treated with teratogenic concentrations of EtOH (2%–2.5% vol/vol) from late blastula stages in order to achieve a high efficiency of strongly affected embryos. At stage 41, EtOH-treated embryos were

compared with untreated siblings. Under these conditions, over 95% of the embryos exhibit the same highly penetrant phenotype. Three external malformations are easily discernible in these embryos, which are also part of the common FASD phenotype in humans. These embryos are shorter along the rostro-caudal axis throughout its length, including tail, trunk, and head (Fig. 1B). Head development is impaired resulting in microcephalic embryos (Figs. 1D,1F). Along the mediolateral axis, the malformed head is narrower (Fig. 1F) and, along the anteroposterior axis, regions rostral to the eye, like the cement gland, are reduced in size (Fig. 1D) or are almost completely missing (Figs. 1D,1F), suggesting a reduced forebrain. Additional prominent malformations include abnormal eye development, which in EtOH-treated embryos leads to a microphthalmic phenotype (Figs. 1D,1F). These developmental malformations include the same anatomical regions and very similar defects induced by EtOH in individuals with FAS.

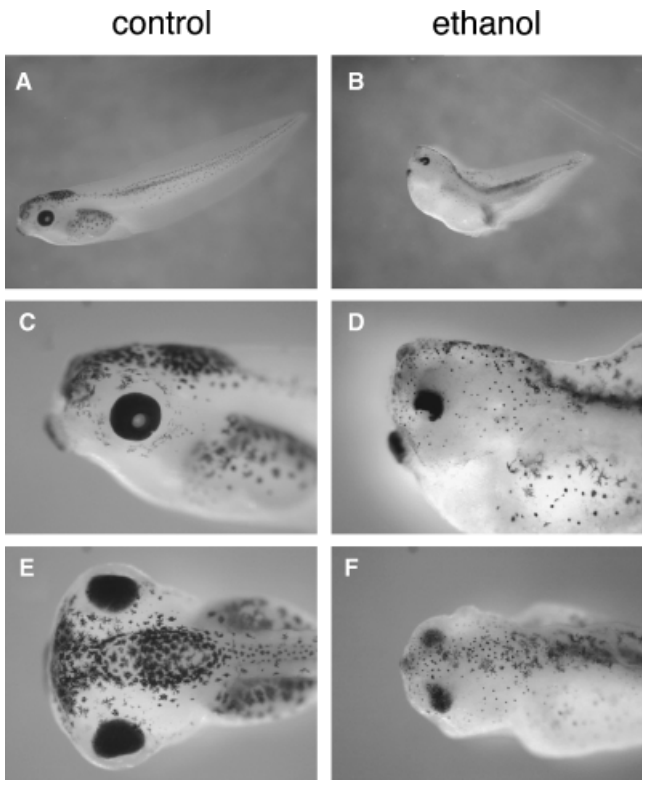

Fig. 1 The teratogenic effects of ethanol (EtOH). Control (**A**, **C**, **E**) and EtOH-treated (2.5% vol/vol) embryos (**B**, **D**, **F**) were analyzed at st. 41. (A, B) Lateral view of the whole embryos to ascertain the shortening induced by EtOH. (C, D) Lateral view of embryos at higher magnification to demonstrate the craniofacial malformations and the microphthalmia induced by EtOH. (E, F) Comparison of heads (dorsal view) to show the EtOH-induced microcephaly.

EtOH affects the embryonic organizer

Based on our previous results which identified Spemann's organizer as one of the earliest embryonic structures affected by EtOH (Yelin et al., 2005), we performed an *in situ* hybridization study with markers specific for this embryonic region. The analysis was undertaken in an effort to understand the chain of events leading from the initial molecular effects of EtOH to the developmental phenotype. Initially, we studied the expression pattern of the organizer-specific gene, *gsc* during gastrula and early neurula. This analysis revealed that the *gsc*-expressing cells are delayed in their involution during gastrulation (90%, $n = 50$; Fig. 2B) and in reaching their rostral position during late gastrula (90%, $n = 50$; Fig. 2D). Eventually, at neurula stages, the *gsc*-expressing cells appear to reach their normal position (92%, $n = 30$; Fig. 2F arrowheads) like in control untreated embryos (Fig. 2E). In order to verify the final position of the *gsc*-positive cells, embryos were hybridized with *Gbx2* (Tour et al., 2002), a marker for the midbrain/hindbrain boundary (MHB) in addition to *gsc,* in order to determine their relative positions. In EtOH-treated embryos, the position of the *gsc*-positive cells (arrowheads) relative to *Gbx2* (arrows), is indistinguishable from their control sibling embryos (Figs. 2E,2F). *Chordin,* another organizer-specific gene also exhibited a delay in the involution and rostral spreading (not shown). When *chordin* (arrowheads) and *en2* (arrows), another marker of the MHB were hybridized simultaneously, it revealed a comparable position of the anterior *chordin* expression domain between control (Fig. 2G) and EtOH-treated embryos already by st. 14 (92%, $n = 30$; Fig. 2H). In this case there is clear up-regulation of *chordin* in its anterior expression domain (Fig. 2H). These results show that EtOH induces a temporal delay in the involution and rostral spreading of anterior organizer-derived cells, namely the prospective prechordal plate.

Xnot2, another organizer-specific gene tested, was an exception such that EtOH exposure eliminates its expression in the dorsal lip of the blastopore during early gastrula stages (Fig. 3D; Yelin et al., 2005). Midline expression of *Xnot2* is restored during late gastrula stages but only caudally to its ring of expression (75%, $n = 32$; Fig. 3B). Expression of this gene is missing in more rostrally localized cells (Fig. 3B), always anterior to the ring of *Xnot2* expression (Gont et al., 1993; von Dassow et al., 1993). These observations suggest that the group of cells that normally express *Xnot2* early in the organizer region lose this ability even as they migrate and spread rostrally.

The elimination of *Xnot2* expression from the organizer was further demonstrated by double *in situ* hybridization with *gsc* and *Xnot2* probes during early/mid gastrula. At these stages, both *gsc* and *Xnot2* expressing cells co-localize to the dorsal blastopore lip and overlap with each other (Fig. 3C). In EtOH-treated embryos,

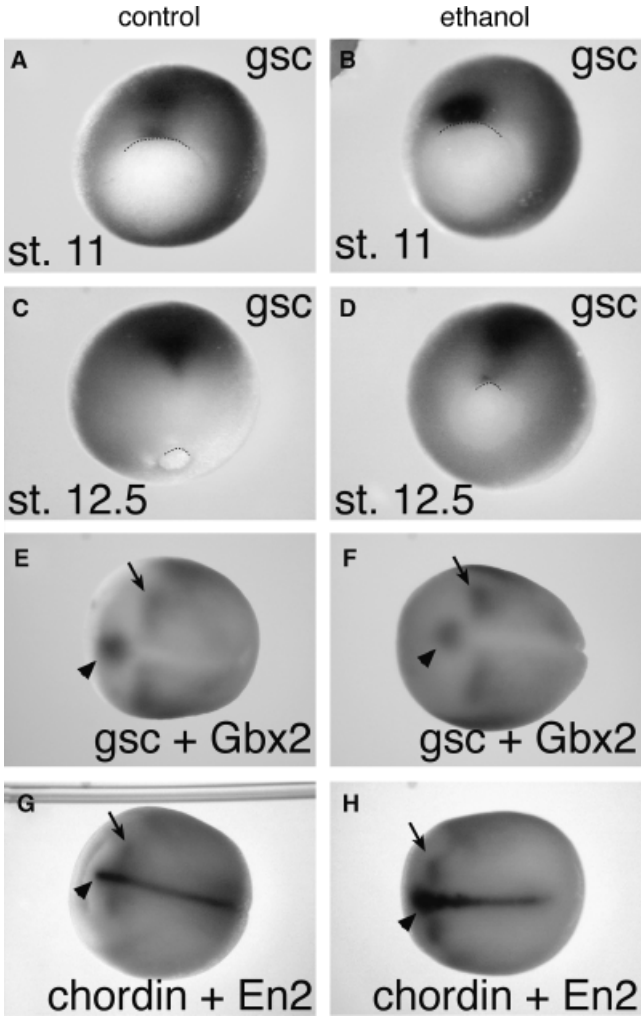

Fig. 2 Delayed morphogenetic movements of organizer-derived cells as a result of ethanol (EtOH) exposure. EtOH-treated embryos were analyzed by *in situ* hybridization during mid-gastrula, late gastrula and early neurula stages in order to determine the fate of organizer-derived cells. (**A–D**) Control and EtOH-treated embryos hybridized with the *gsc* probe to study the migration of the prospective prechordal plate cells. (A, B) Embryos studied during mid-gastrula (st. 11) exhibiting the delay in rostral migration induced by EtOH. (C, D) By late gastrula stages (st. 12.5) the *gsc* signal continues to show a delay in migration in EtOH embryos compared with control embryos. The dotted lines mark the position of the dorsal blastoporal lip. (**E–H**) Localization of the final position of the *gsc*- and *chordin*-positive cells in EtOH-treated embryos (st. 14). (E) Control and (F) EtOH-exposed embryos studied with the *gsc* probe and a *Gbx2* probe to mark the midbrain/hindbrain boundary (MHB; arrows). (G) Control and (H) EtOH-treated embryos hybridized with *chordin* and *en2* probes to localize the position of the former relative to the midbrain/hindbrain boundary (arrows).

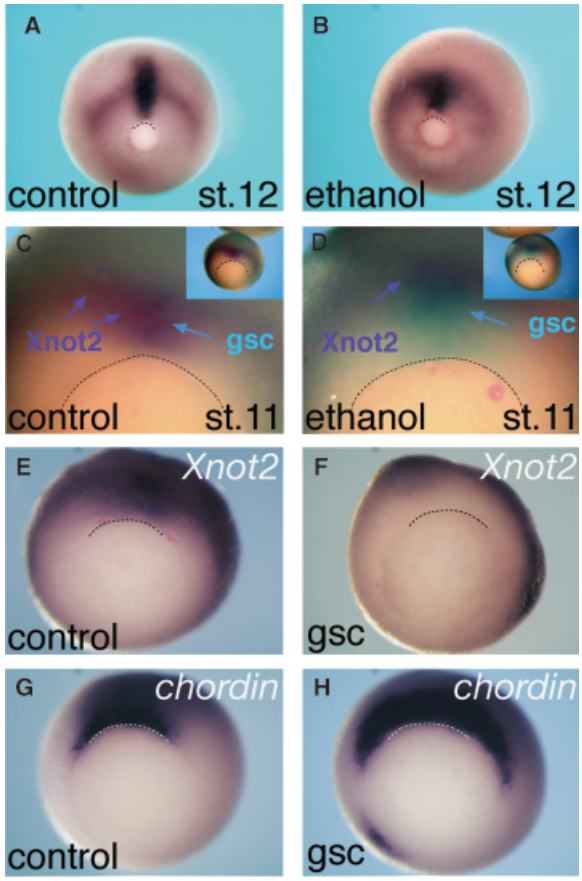

Fig. 3 Abnormal Xnot2 expression following ethanol (EtOH) exposure. (**A**, **B**) *Xnot2* expression at st. 12 following EtOH treatment. (A) Control and (B) EtOH-treated embryos analyzed by *in situ* hybridization with the *Xnot2* specific probe. (**C**, **D**) *Xnot2* and *gsc* expression following EtOH exposure. (C) Control and (D) EtOH-treated embryos were studied by double *in situ* hybridization with *Xnot2* (magenta) and *gsc* (turquoise) probes. Elimination of the *Xnot2* dorsal blastopore signal (magenta) allows the visualization of the *gsc* signal (turquoise). The insets are the same embryos at lower magnification. (**E**–**H**) Xnot2 downregulation by *gsc* overexpression (E, G) Control and (F, H) *gsc* overexpressing embryos were subjected to *in situ* hybridization analysis with the *Xnot2* (E, F) and *chordin* (G, H) probes.

only the *gsc* signal remains in place (82%, $n = 30$; Fig. 3D, turquoise), while *Xnot2* expression (magenta) can only be observed in the ring pattern, in adjacent but non-overlapping domains of expression. These observations show that, as a result of EtOH treatment, while *gsc* is up-regulated, *Xnot2* is down-regulated in the organizer region. Our results suggest a model where *gsc* up-regulation may lead to *Xnot2* repression in agreement with the subsequent localization of both genes, in adjacent but non-overlapping expression domains. In order to test this possibility, *gsc* was overexpressed in *Xenopus* embryos and the pattern of *Xnot2* was determined. As in the EtOH treatment, *gsc* overexpression resulted in *Xnot2* repression in the organizer region (49%, $n = 48$), while the expression domain further away from the blastopore remained unchanged (Fig. 3F). As a control, in sibling embryos we could detect expansion of *chordin* expression (60%, $n = 44$; Fig. 3H) due to *gsc* overexpression, as previously described (Sasai et al., 1994).

The effect of EtOH on organizer-specific gene expression raised the possibility that additional dorsal

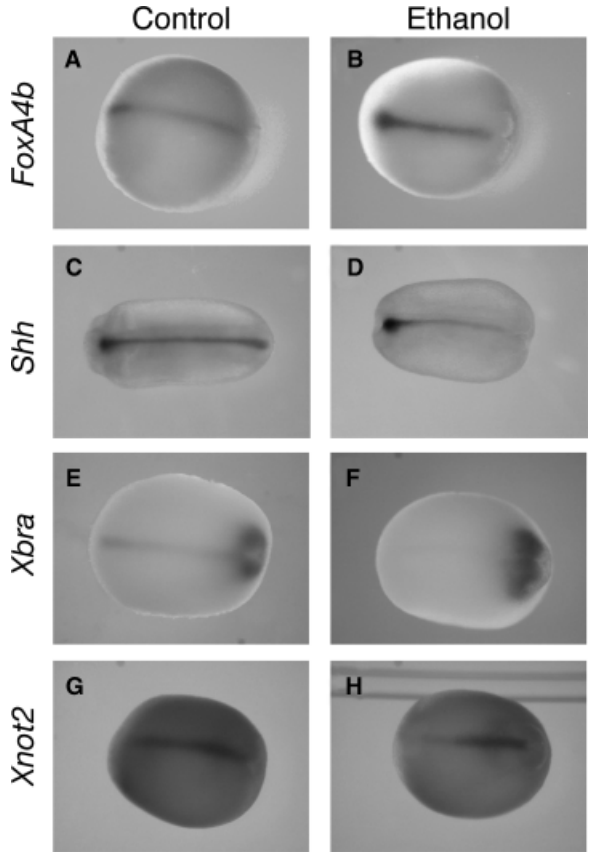

Fig. 4 Ethanol (EtOH) affects midline genes along the rostro-caudal axis. (**A**, **C**, **E**, **G**) Control and (**B**, **D**, **F**, **H**) EtOH-treated embryos were subjected to *in situ* hybridization analysis of changes in midline gene expression during neurula stages (A, B) Embryos analyzed for changes in FoxA4b (FKH1) expression. (C, D) Embryos hybridized with the Shh-specific probe. (E, F) Embryos studied for changes in Xbra expression. (G, H) Analysis of Xnot2 expression during neurula stages.

midline-expressed genes might be affected by this treatment. Analysis by *in situ* hybridization of EtOH-treated embryos during neurula stages revealed that all the dorsal midline genes studied had abnormal expression patterns along the anteroposterior axis. *Chordin* (Fig. 2H) and *FoxA4b* (Fig. 4B; Dirksen and Jamrich, 1992), which are normally expressed along the notochord and the prechordal plate, exhibited rostral up-regulation and caudal down-regulation (92%, $n = 30$; 69%, $n = 13$, respectively). An extreme example of rostral up-regulation, with strong expression along the prechordal plate region, and posterior repression, is exhibited by *Shh* (Figs. 4D, 6H, 6I; Ekker et al., 1995a). *Xbra,* whose normal expression domain at these stages includes the dorsal midline (notochord) and the tailbud (Smith et al., 1991), as a result of EtOH exposure, was down-regulated throughout the length of the embryo and only the tailbud expression remained unchanged (69%, $n = 13$; Fig. 4F). In agreement with its earlier changes in expression (Fig. 3B), *Xnot2* was repressed in rostral regions, with no apparent change in more caudal domains (58%, $n = 12$; Fig. 4H). These results show changes in gene expression patterns along the rostro-caudal axis of the developing embryo and strongly agree with the effects of EtOH along this axis.

The effect of EtOH on head development

EtOH-treated *Xenopus* embryos exhibit microcephaly (Fig. 1F) as do individuals with FAS. In order to begin elucidating the source of this developmental defect, we studied a number of molecular markers important for normal head formation. One of the first genes studied was the head marker *Otx2* (Blitz and Cho, 1995; Pannese et al., 1995) whose expression was analyzed in embryos treated with increasing EtOH concentrations. As expected from the microcephalic phenotype, during late organogenesis/early tailbud stages *Otx2* exhibits a concentration-dependent spatial restriction and reduction in expression levels as a result of the EtOH treatment (77%, $n = 22$; Figs. 5B,5C).

Analysis of the MHB, *en2*, revealed that while expression of this gene appears to remain in its normal position along the anteroposterior axis (Figs. 6E,6F), its dorso-ventral distribution becomes restricted to more dorsal regions of the central nervous system (75%, $n = 24$; Figs. 6B,6C). Analysis of *Shh* revealed a series of changes in its pattern of expression probably related to anatomical changes induced by EtOH. Analysis of st. 22 embryos revealed a thickening in the anterior domain of *Shh*-expression in the region corresponding to the prechordal plate (95%, $n = 40$; Figs. 6H,6I). At later stages, the *Shh* expressing region becomes modified by

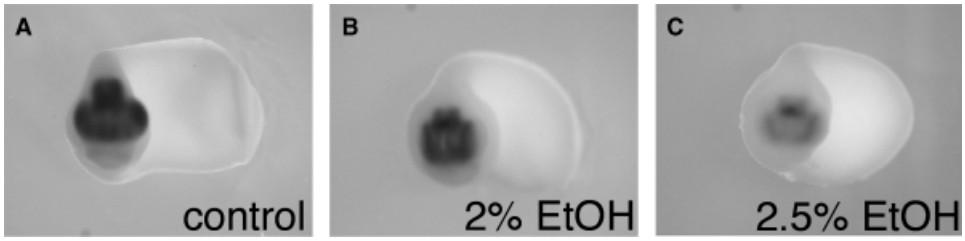

Fig. 5 The head domain is repressed by ethanol (EtOH) exposure. Embryos were treated with increasing concentrations of EtOH and allowed to develop to st. 26. Analysis of the head domain was performed by *in situ* hybridization with the head-specific gene, *Otx2*. (**A**) Control untreated embryo. (**B**) Embryo treated with 2% EtOH. (**C**) Embryo treated with 2.5% EtOH. Frontal views of the embryos.

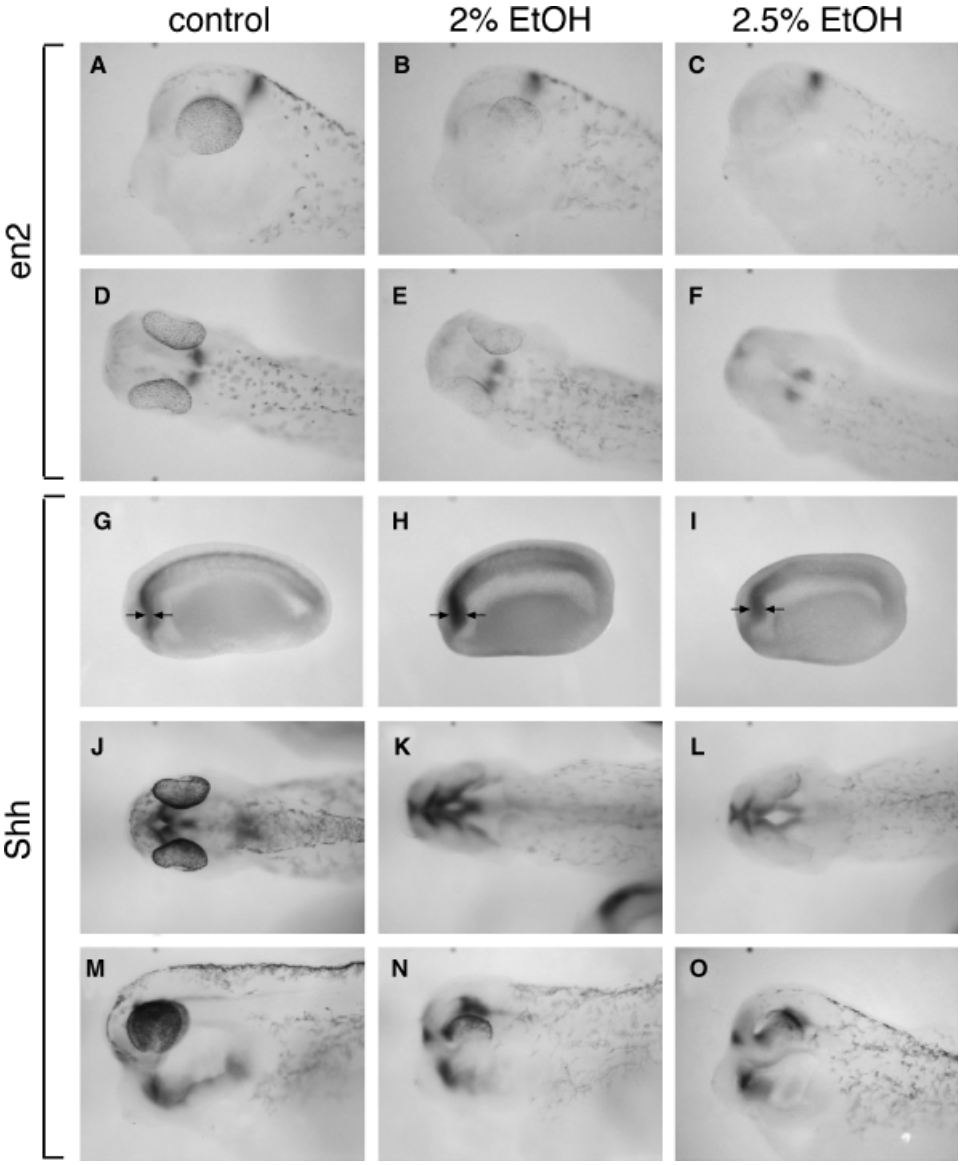

Fig. 6 The effect of ethanol (EtOH) on head structure formation. Embryos were treated with different concentrations of EtOH and allowed to develop to st. 36 (**A–F**, **J–O**) or st. 22 (**G–I**). The embryos were processed for *in situ* hybridization with probes specific for the *en2* (A–F; Hemmati-Brivanlou et al., 1990b) and shh (G–O; Ekker et al., 1995a) genes. (A, D, G, J, M) Control untreated embryos. (B, E, H, K, N) Embryos treated with 2% EtOH. (C, F, I, L, O) Embryos treated with 2.5% EtOH. (A–C, G–I, M–O) Lateral views. (D–F, J–L) Dorsal view. Anterior is to the left. The arrows demarcate the prechordal mesoderm.

EtOH in a concentration-dependent manner. The changes are probably related to the absence of the forebrain ventricle (Fig. 7B, 7F) which results in the abnormal organization of the fore- and midbrain domains, such that new *shh* expression domains apparently form while others are abolished (92%, $n = 24$; Figs. 6K,6L,6N,6O). Most of these changes localized to the diencephalic region.

Further characterization of the head malformations was performed by optical sectioning (Yelin and Silberberg, 1999; Oron et al., 2004) of EtOH-treated embryos ($n = 5$; Fig. 7B) and their control untreated siblings (Fig. 7A). In EtOH-treated embryos there was no detectable forebrain ventricle. The lack of ventricle contributes to the microcephalic phenotype by making the head smaller. In addition, the neural tube became narrower along the dorso-ventral axis, while the notochord is wider than in control sibling embryos (Figs. 7A,7B). The narrowing of the neural tube is in agreement with the observed restriction in the *en2* expression domain (Figs. 6A–6C), while the thickening of the prechordal plate (Fig. 7B) might reflect the up-regulation of *gsc* and *chordin*. Similar conclusions were reached from the analysis of histological sections prepared from paraffin-embedded embryos (Fig. 7F). The reduction in forebrain size and other changes in the head region were studied by *in situ* hybridization with a mixed probe. The hybridization probe included markers specific for the cement gland (XCG1; Hemmati-Brivanlou et al., 1990a), the eye (Pax6; Li et al., 1997), the MHB (en2; Hemmati-Brivanlou et al., 1991) and rhombomeres 3 and 5 (Krox20; Bradley et al., 1992). The hybridization was performed in mildly affected embryos to retain marker expression ($n = 15$). The eye, as marked by *Pax6,* appears to have moved from its normal lateral position to the forefront of the embryo's head (Fig. 7D).

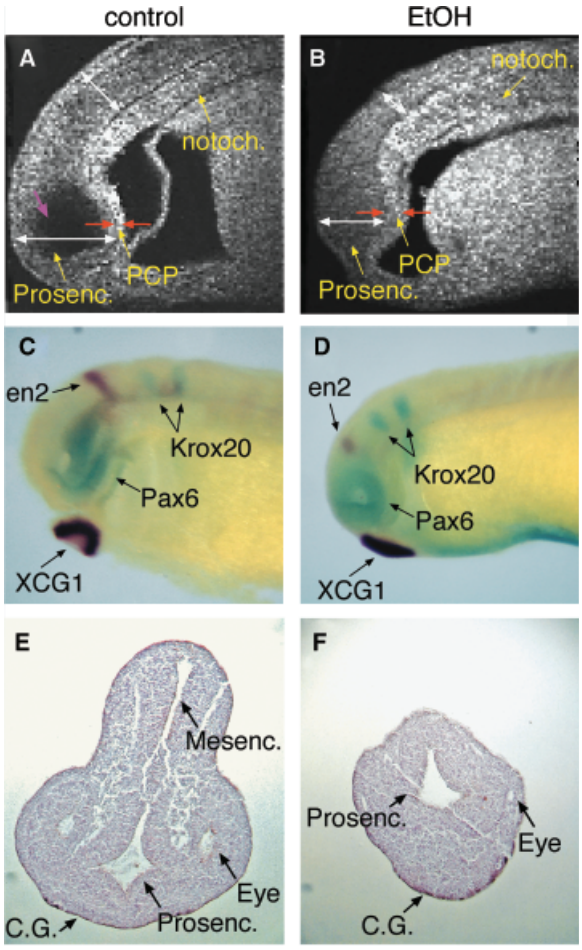

Fig. 7 Ethanol (EtOH) affects forebrain and eye development. Embryos treated with EtOH were subjected to morphological (**A**, **B**), marker gene expression (**C**, **D**), and histological analysis (**E**, **F**). (A) Control and (B) EtOH-treated embryos (st. 26/27) were subjected to optical sagittal sectioning. Prosenc., prosencephalon; PCP, prechordal plate; notoch., notochord; purple arrow, forebrain ventricle; red arrows, prechordal plate width; white arrow, forebrain and spinal cord size. (C) Control and (D) EtOH-exposed embryos (st. 26/27) were subjected to double *in situ* hybridization with markers for the cement gland (XCG1, magenta), eye (Pax6, turquoise), midbrain/hindbrain boundary (MHB; en2, megenta), and rhombomeres 3 and 5 (Krox20, turquoise). (E) Control and (F) EtOH-treated embryos (st.24) were cross-sectioned to perform a histological analysis of the effects of EtOH on head development. The cement gland, prosencephalic, mesencephalic, and eye regions are marked.

This change in position agrees with the suggested reduction in size of the telencephalon and the absence of the forebrain ventricle. The distance from the cement gland to the stripe of *en2* expression following the arch along the front of the embryo is shorter in EtOH-treated embryos (Fig. 7D). This shorter distance also supports a decrease in size of the forebrain. From a lateral view, the stripes of *en2* and *Krox20* expression are shorter along the dorso-ventral axis (Fig. 7D) in agreement with the smaller size of the central nervous system along this axis.

EtOH treatment induces microphthalmia

The effect of EtOH on eye formation was initially studied by histological analysis at st. 24. Cross-sections of EtOH-treated embryos ($n = 6$) were compared with control embryos ($n = 6$). The remaining cement gland was used as a positional marker. The overall size of the histological section across the eyes and the cement gland in EtOH-treated embryos is much smaller than in control embryos (Fig. 7F). The smaller size of the sections can be attributed to the EtOH-induced microcephaly. The eyes in EtOH-treated embryos are rudimentary and very small in agreement with the microphthalmia induced by this treatment.

The effect of EtOH on eye development was further studied using two early markers in the formation of this structure, *Tbx3* and *Pax6* (Li et al., 1997). The forming eyes, as detected by *Tbx3* expression, become reduced in a concentration-dependent manner (Figs. 8B–8D). In embryos exposed to 1% EtOH about 30% ($n = 70$) exhibited the depicted defect. Increasing the EtOH to 2% results in 90% ($n = 70$) of the embryos with microphthalmia, while with 2.5% EtOH, 100% ($n = 70$) of the embryos had eye defects, many of them with undetectable *Tbx3* expression (Fig. 8D).

Analysis of *Pax6* expression was performed using different EtOH concentrations and the embryos were studied at several developmental stages. The expression of *Pax6* clearly supports the reduction in eye structures as a result of EtOH exposure (Fig. 9B). In the instances where the remnant of the eye is still present, two such remnants are clearly distinguishable (Fig. 9). Like *Tbx3*, *Pax6* also exhibits a concentration-dependent response in its elimination by EtOH (data not shown). At later developmental stages, the EtOH-treated embryos exhibit phenotypic microphthalmia as evidenced from the

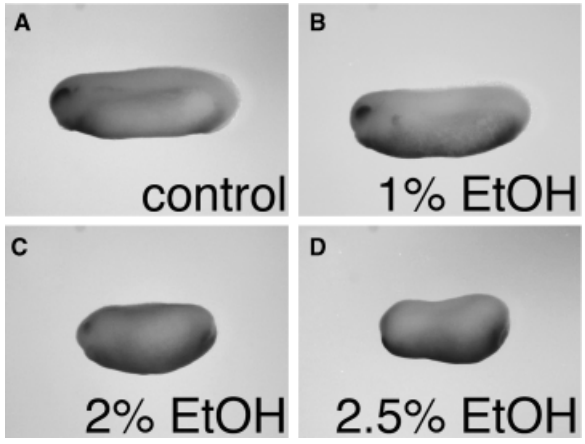

Fig. 8 The effect of ethanol (EtOH) on eye field development. Embryos were treated with increasing concentrations of EtOH. At st. 26 the embryos were fixed and processed for *in situ* hybridization with a probe specific for the *Tbx3* gene. (**A**) Control embryo. (**B**) 1% EtOH (vol/vol). (**C**) 2% EtOH. (**D**) 2.5% EtOH. All embryos in lateral view, anterior to the left.

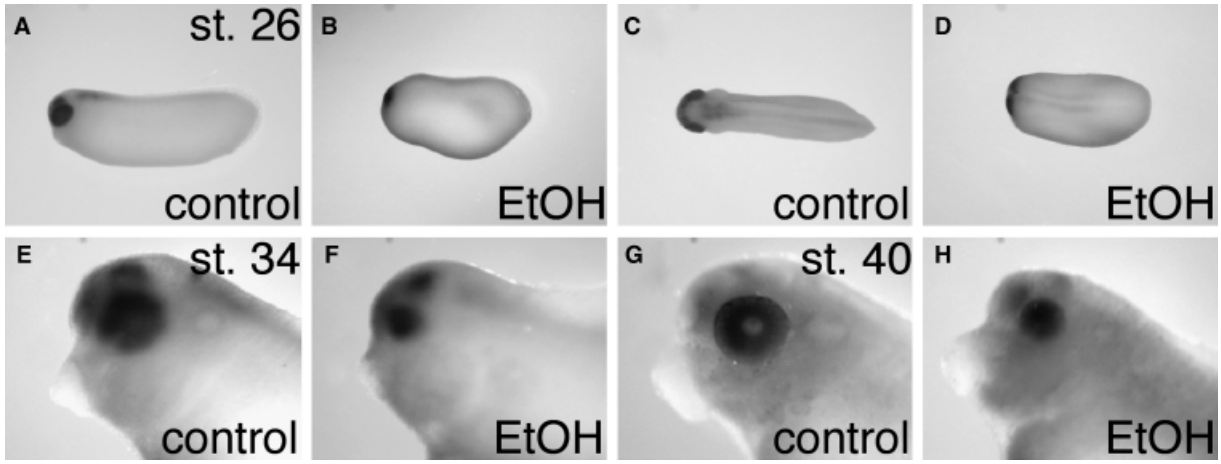

Fig. 9 Microphthalmia and Pax6 down-regulation as a result of EtOH treatment. Control (**A**, **C**, **E**, **G**) and 2.5% ethanol (EtOH;vol/vol)-treated embryos (**B**, **D**, **F**, **H**) were allowed to develop to stages 26 (A–D), 34 (E, F) and 40 (G, H) and, subsequently, processed for *in situ* hybridization with a probe specific for the eye gene, *Pax6*. Panels (A, B, E–H) are lateral views, panels (C, D) are dorsal views. Anterior is to the left.

pattern of *Pax6* expression (87%, $n = 80$; Figs. 9F,9H). Microphthalmia and inhibition of *Pax6* expression have been described in zebrafish (Macdonald et al., 1995; Ekker et al., 1995b) and chicken (Zhang and Yang, 2001) embryos as a result of *Shh* overexpression. Overexpression of *Shh* in *Xenopus* embryos also resulted in *Pax6* down-regulation (83%, $n = 55$; Fig. 10B) establishing a causal relationship between *Shh* and the microphthalmia induced by EtOH.

Discussion

The present study was aimed at performing a molecular characterization during early embryogenesis of the developmental malformations induced by EtOH. This analysis together with the identification of the developmental processes affected by EtOH can provide the basis for our understanding of the etiology of FASD. For experimental purposes, *Xenopus* embryos were treated with 1%–2.5% EtOH (vol/vol), which is equivalent to 0.8–2.0 g/100 ml to achieve a high incidence of similarly and strongly malformed embryos. The lower EtOH concentration, 1% (vol/vol), results in 50% of the embryos with characteristic molecular changes (Yelin et al., 2005). Highly intoxicated humans reach blood EtOH concentrations around 0.2 g/100 ml but the correlation between EtOH ingestion levels and the damage to the embryos has been harder to establish (Koren et al., 2003; Sokol et al., 2003). The conditions used were independently suggested by a number of groups as resulting in highly penetrant and reproducible phenotypes (Nakatsuji, 1983; Dresser et al., 1992; Yelin et al., 2005), and they were also used for zebrafish embryos (Blader and Strahle, 1998). Under these experimental conditions, maximal sensitivity to EtOH exposure is evident during early developmental stages—late blastula and early gastrula, although slighter malformations can still be observed by treatment at later stages (Yelin et al., 2005). Our observations suggest that in *Xenopus,* like in humans, exposure to teratogenic doses of EtOH results in embryos with a compressed rostro-caudal axis and head malformations, including microcephaly and microphthalmia. Both the similarity of the teratogenic phenotype and the critical timing of exposure (early embryogenesis) strongly support the use of frog embryos as a model system for the study of the early events in the induction of FASD (Jones and Smith, 1973; Johnson et al., 1996; Chaudhuri, 2000; Koren et al., 2003; Sokol et al., 2003; Yelin et al., 2005).

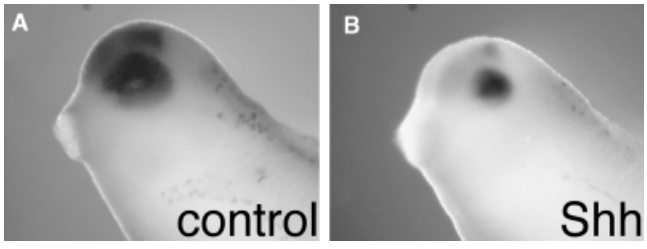

Fig. 10 Pax6 is down-regulated by Shh overexpression. Embryos were injected with *Shh* mRNA and subjected to analysis of *Pax6* expression by *in situ* hybridization at st. 32. (**A**) Control untreated embryo. (**B**) Embryo injected with *Shh* capped RNA.

Analysis of organizer-specific gene expression revealed a delayed invagination of the cells localized in the dorsal blastopore lip during gastrulation. This delayed invagination could be the result of abnormal expression levels of the organizer-specific genes (this work; Yelin et al., 2005). This suggestion is supported by the observation that abnormal *gsc* expression levels results in abnormal gastrulation movements (Niehrs et al., 1993). In the case of EtOH treatment, all organizer-specific genes studied exhibit abnormal expression patterns that may result in delayed gastrulation move-

ments. Defects in morphogenetic movements (convergent-extension) have been described for EtOH-treated embryos (Yelin et al., 2005). This delay can be relevant for the FAS phenotype since the prechordal plate cells have been shown to provide numerous signals important for normal development, including head formation (Bouwmeester et al., 1996; de Souza et al., 1999; Kazanskaya et al., 2000). During early neurula, the dorsal blastoporal cells (*gsc* and *chordin* positive) of EtOH-treated embryos reach later their normal position. Interestingly, the quantitative differences, i.e., up-regulation or down-regulation, observed during gastrulation can still be observed at later stages.

Some of the changes in organizer-specific gene expression might be more direct than others as suggested from the *gsc*/*Xnot2*/*chordin* regulatory interaction. Both *gsc* and *chordin* exhibit up-regulation and expansion while *Xnot2* is eliminated from the early dorsal blastopore lip. Assuming that *gsc* up-regulation is a primary effect of EtOH exposure, then up-regulation of *chordin* could be attributed to the previously described positive regulatory interaction (Sasai et al., 1994). In addition, we show that *Xnot2* is under negative regulation by high levels of *gsc* expression. Therefore, *gsc* up-regulation by EtOH could explain the elimination of *Xnot2* from the early blastopore lip. Once the *gsc*-positive cells invaginate, then the dorsal blastopore lip can resume the expression of *Xnot2*. A similar cross-regulatory interaction probably takes place in the early embryo such that initially *gsc* and *Xnot2* have overlapping patterns of expression in Spemann's organizer and, subsequently, their expression domains become refined and separate in the prechordal plate and notochord respectively. Midline markers also exhibited abnormal expression patterns in EtOH-treated embryos suggesting that, as in the case of *Xnot2*, they might be under regulation of organizer genes affected earlier by the EtOH exposure.

EtOH-treated *Xenopus* embryos like individuals with FAS develop a microcephalic phenotype. This phenotype was initially analyzed by studying the expression pattern of the early head-domain marker, *Otx2* (Blitz and Cho, 1995; Pannese et al., 1995). In agreement with the observed phenotype, the *Otx2* expression domain becomes restricted in a EtOH concentration-dependent manner. The head formed is anatomically abnormal, which was observed by different means. Histological analysis and optical sectioning revealed a lack of the forebrain ventricle in EtOH-treated embryos. The loss of this cavity in the anterior brain could be the result of the prechordal plate reaching its normal position with a delay (Bouwmeester et al., 1996; de Souza et al., 1999; Kazanskaya et al., 2000). In turn, the lack of the ventricle can also explain the abnormal position of the eyes and perhaps even the abnormal pattern of *shh* expression. *Shh* is up-regulated in the anterior region as a result of EtOH exposure and its pattern of expression in the midbrain appears abnormal. The abnormal pattern of expression could also be the result of increased expression in the normal domains but abnormal organization of the domains due to the lack of the forebrain ventricle.

Eye development following EtOH exposure suffers from two main malformations: the eyes are placed abnormally in the head and they are abnormally small, microphthalmic. The abnormal placement of the eyes can again be attributed to the reduction in the forebrain ventricle, which results in a smaller forebrain and the eyes shifting from a lateral position to an extremely rostral one. The size and extent of eye development can be attributed to abnormal expression of the eye morphogenetic genes, *Pax6* and *Tbx3*, the two genes important for eye formation that exhibit EtOH concentration-dependent restriction (Li et al., 1997; Kozmik, 2005). The reduction in both eye-specific marker expression, *Pax6* and *Tbx3,* can be attributed to the increase in *shh* expression levels. We could show that *shh* overexpression down-regulates *Pax6* expression, as previously shown in chicken and zebrafish embryos (Macdonald et al., 1995; Zhang and Yang, 2001). A similar negative regulatory effect of *shh* on the expression of *Tbx3* has been shown in *Xenopus* embryos (Takabatake et al., 2002). In frog embryos, we could always observe two eye fields demonstrating that no cyclopic embryos develop as a result of EtOH exposure as is the case in zebrafish (Fig. 9D; Blader and Strahle, 1998). The difference between *Xenopus* and zebrafish embryos might be that in frogs the prechordal plate cells eventually migrate and are able to split the eye field, while in fish this apparently doesnot happen.

The results described here provide molecular evidence for the teratogenesis of EtOH from its initial effects on the embryonic organizer to the typical molecular, anatomical, and structural changes most strongly affected in individuals with FAS and further support the use of *Xenopus* embryos as a model system to study the etiology of EtOH-induced developmental malformations.

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