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Ethanol exposure affects gene expression in the embryonic organizer and reduces retinoic acid levels

Ronit Yelin[a,1], Racheli Ben-Haroush Schyr[a], Hadas Kot[a], Sharon Zins[a], Ayala Frumkin[b], Graciela Pillemer[a], Abraham Fainsod[a,*]

[a]*Department of Cellular Biochemistry and Human Genetics, Faculty of Medicine, Hebrew University, POB 12272, Jerusalem 91120, Israel*
[b]*Department of Human Genetics, Hebrew University-Hadassah Medical Center, Jerusalem 91120, Israel*

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Abstract

Fetal Alcohol Spectrum Disorder (FASD) is a set of developmental malformations caused by alcohol consumption during pregnancy. Fetal Alcohol Syndrome (FAS), the strongest manifestation of FASD, results in short stature, microcephally and facial dysmorphogenesis including microphthalmia. Using *Xenopus* embryos as a model developmental system, we show that ethanol exposure recapitulates many aspects of FAS, including a shortened rostro–caudal axis, microcephally and microphthalmia. Temporal analysis revealed that *Xenopus* embryos are most sensitive to ethanol exposure between late blastula and early/mid gastrula stages. This window of sensitivity overlaps with the formation and early function of the embryonic organizer, Spemann's organizer. Molecular analysis revealed that ethanol exposure of embryos induces changes in the domains and levels of organizer-specific gene expression, identifying Spemann's organizer as an early target of ethanol. Ethanol also induces a defect in convergent extension movements that delays gastrulation movements and may affect the overall length. We show that mechanistically, ethanol is antagonistic to retinol (Vitamin A) and retinal conversion to retinoic acid, and that the organizer is active in retinoic acid signaling during early gastrulation. The model suggests that FASD is induced in part by an ethanol-dependent reduction in retinoic acid levels that are necessary for the normal function of Spemann's organizer.

Keywords: FAS; FASD; Retinol; Vitamin A; Fetal alcohol syndrome; *gsc*; Cyp26; Embryonic organizer; Gastrula; *Xenopus*

Introduction

Alcohol (ethanol, EtOH) exposure of human embryos during pregnancy increases the probability of spontaneous abortions, causes prenatal mortality and is associated with Fetal Alcohol Spectrum Disorder (FASD; Koren et al., 2003; Sokol et al., 2003). Fetal Alcohol Syndrome (FAS), the most severe form of FASD, is a combination of neurological, anatomical and physiological anomalies of developmental origin, among them, growth retardation, mental retardation, microcephaly, craniofacial dysmorphogenesis and skeletal defects (Chaudhuri, 2000; Jones and Smith, 1973; Lemoine et al., 1968). Some of these malformations have also been observed in animal models such as mouse, chick, frog and zebrafish, as a consequence of alcohol exposure during early embryonic stages, although sometimes, the final outcome does not resemble the human affliction (Blader and Strahle, 1998; Cartwright and Smith, 1995; Rovasio and Battiato, 1995; Sulik, 1985; Sulik et al., 1981). Even though extensive work has been performed to understand the mechanism of EtOH-induced malformations, the molecular pathway(s) leading to FAS are still unknown. Several models have been put forth to explain the EtOH effects including induction of apoptosis, cell adhesion defects, accumulation of free radicals, effects on growth factors and antagonism of retinoic acid (RA) biosynthesis (Deltour et al., 1996; Duester, 1991; Henderson et al., 1989;

* Corresponding author. Fax: +972 2 6415848.
E-mail address: fainsod@cc.huji.ac.il (A. Fainsod).
[1] Present address: Wellman Center for Photomedicine, Massachusetts General Hospital, 55 Fruit Street, BAR 704, Boston, MA 02114, USA.

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Kotch et al., 1995; Olney et al., 2002; Pullarkat, 1991; Singh et al., 1996).

RA is metabolized from Vitamin A (retinol, ROL) and is known to be a key modulator of many developmental processes, and regulates the differentiation of multiple cell types during vertebrate embryogenesis (Durston et al., 1998; Means and Gudas, 1995). The importance of RA in normal embryonic development has been demonstrated by treating embryos with RA or inducing Vitamin A deficiency, both resulting in severe teratogenic effects (Cohlan, 1953; Kessel, 1992; Maden et al., 1996; Wilson et al., 1953). There is extensive similarity between the vitamin A deficient (VAD) phenotype and the phenotype induced by EtOH (Cartwright and Smith, 1995; Dickman et al., 1997; Johnson et al., 1996; Maden et al., 1996). Therefore, EtOH, as a competitive inhibitor of RA metabolism, might divert the enzymes involved in the conversion of ROL to RA from their normal activity to EtOH detoxification, resulting in a transient reduction in RA levels during embryogenesis (Duester, 1991).

In amphibian embryos, gastrulation begins with the appearance of the dorsal blastopore lip where Spemann's organizer is located (Harland and Gerhart, 1997; Spemann and Mangold, 1924). The organizer was defined as the population of cells that, when transplanted into the prospective ventral region of a recipient embryo, was capable of inducing the formation of a secondary body axis. The ectopic organizer, like the endogenous one, provides signals that dorsalize mesoderm, induces convergent-extension movements and specifies the neuroectoderm (Harland and Gerhart, 1997; Keller et al., 1992; Sasai et al., 1995; Smith and Slack, 1983; Spemann and Mangold, 1924). Molecular analysis of the organizer phenomenon has identified numerous genes active in this embryonic structure. Among the organizer-specific genes are transcription factors, growth factors and secreted proteins. It is known that the interactions between these organizer-specific genes and others expressed around the blastopore will determine many aspects of the developing embryo (De Robertis et al., 2000; Lemaire and Kodjabachian, 1996). In *Xenopus*, several RA isomers are present around the blastopore during gastrula stages, but their activity at these early stages has not been determined (Chen et al., 1994; Kraft et al., 1994).

In the present study, we take advantage of molecular approaches in *Xenopus* embryos to elucidate the embryonic structures affected and the mechanism by which EtOH induces its teratogenic effects. We show that the frog embryo recapitulates many aspects of the FAS phenotype and exhibits maximal sensitivity to EtOH exposure during blastula and gastrula stages. EtOH-treated embryos suffer from abnormal convergence-extension movements that delay gastrulation movements and can result in shortening along the anterior–posterior axis. Molecular analysis during gastrula stages identified changes in organizer-specific gene expression, suggesting that Spemann's organizer is targeted by EtOH exposure. Expression analysis of known RA-responsive genes and the use of a RA signaling reporter plasmid further demonstrated opposed effects of EtOH and RA on embryonic development and allowed us to perform rescue experiments. Analysis of transgenic *Xenopus* embryos with the RA reporter plasmid revealed RA signaling activity localized to the dorsal lip region. Our results show that Spemann's organizer is an early target of EtOH exposure, and that this structure exhibits RA signaling during the gastrula stages. These observations suggest that some of the embryonic malformations induced by EtOH can be attributed to the alteration of the normal RA signaling in the organizer.

Materials and methods

Embryo manipulation, microinjection and transgenesis

Xenopus laevis were purchased from Xenopus 1 (USA). 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 MBT (st. 8.5) and either kept in this solution or washed at the specified stages. Treatments of embryos with all *trans*-retinoic acid (1 μM), all *trans*-retinol (70–150 μM), all *trans*-retinaldehyde (5–7.5 μM) and citral (60 μM; 3,4-dimethyl-2,6-octadienal; Schuh et al., 1993) were performed also in 0.1× MBSH soon after MBT.

For the microinjection of reporter plasmids or capped RNAs, 2- to 4-cell embryos were injected radially (4 times). RNA was prepared by in vitro transcription using the RiboMax kit (Promega). Cap analog (Pharmacia) was added to the reaction mixture using a ratio of cap:GTP of 5:1. The template for transcription of *XCYP26* mRNA was prepared from the full length cDNA clone (Hollemann et al., 1998).

Transgenic embryos were generated according to the published procedure (Kroll and Amaya, 1996). For the detection of RA signaling, a reporter plasmid expressing *LacZ* controlled by an RA responsive element (RARE) and the *hsp68* promoter, RAREhsplacZ, was employed (Rossant et al., 1991). Embryos carrying this plasmid were analyzed by staining with Xgal. A green fluorescent protein (GFP) version of the RA reporter plasmid was generated by replacing the *LacZ* coding sequences by the GFP coding sequence. Embryos transgenic with the RAREhspGFP plasmid were analyzed under epifluorescence in a Zeiss Axioplan microscope.

In situ hybridization and probes

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) and the digoxigenin RNA

labeling mixture (Roche). Probes for in situ hybridization were prepared from: clone H7 for *gsc* (Cho et al., 1991), plasmid pXOT30 for *Otx2* (Smith et al., 1993), clone Δ59 for *chordin* (Sasai et al., 1994), clone E13 for *Xnot2* (Gont et al., 1993) and plasmid pSP64TxbraAS for *Xbra* (Smith et al., 1991).

RT-PCR and quantitative PCR

EtOH or RA treated embryos were processed for RNA extraction. RT-PCR reactions were carried out in the exponential phase of the amplification curve as described (Shapira et al., 2000). The primers used were: *histone H4*—CGGGATAACATTCAGGGTATCACT, ATCCATGGCGGTAACTGTCTTCCT for 20 cycles at 55°; *Hoxb4*—GGAGGATGAAGTGGAAGAAAG, GCAGTGTAAACATGACAAATG for 32 cycles at 55°; *Hoxb3*—TTCTCGGACAGCAGGACTATGC, TGGAGTTCTGGCGGCTTTC for 32 cycles at 60.8°; *HoxA2*—TCAATAGTCAGCCGTCGCTTGC, TCTGCTCAAAAGGAGGAGGAATG for 32 cycles at 60.8°; *Hoxb9*—CTCATCAAAACAACCCCTC, TTGTCTCTCGCTCAGGTTC for 32 cycles at 55°.

Quantitative real-time PCR was performed using the ABI Prism 7000 cycler and the ABsolute QPCR SYBR green mixes (ABgene) and analyzed as described (Livak and Schmittgen, 2001). The primers used were: *gsc*—TTCACCGATGAACAACTGGA, TTCCACTTTTGGGCATTTTC; *Otx2*—AAGCCGCAATATAGAAAGGAACA, GGGATTCCTTGTCGCAATTAATA; *chordin*—ACTGCCAGGACTGGATGGT, GGCAGGATTTAGAGTTGCTTC; *GAPDH*—GCTCCTCTCGCAAAGGTCAT, GGGCCATCCACTGTCTTCTG; *Cyp26* CGATTCCTCAAGGTTTGGCTTCA, ATTTAGCGGGTAGGTTGTCCACA.

Analysis of reporter plasmids

RAREhsplacZ radially injected embryos or explants were processed for chemiluminiscent quantitation of reporter activity using the ß-gal Reporter Gene Assay (Roche). Each experiment was repeated a minimum of three times, which involved at least duplicates of 5 embryos or 15 explants each. The protein extracts and the enzymatic reactions were performed according to the manufacturers protocol. The levels of activity were measured on a TD-20/20 Luminometer (Turner Designs).

Results

Increased sensitivity to EtOH during late blastula and early/mid gastrula

In order to take advantage of *Xenopus* embryos to elucidate the early events in EtOH-induced developmental malformations, the conditions for efficient and reproducible EtOH treatment were determined. Embryos were exposed to different concentrations of EtOH from the midblastula transition (MBT, st. 8.5) and allowed to develop until organogenesis (st. 36). EtOH concentrations of up to 1% (vol/vol) resulted in none or very mild external malformations (not shown). *Xenopus* embryos treated with 2%–2.5% EtOH developed with high incidence (>95%) as shortened embryos with small eyes (microphthalmia), reduced cement glands and microcephaly (Figs. 1B,C). Exposure of embryos to 3% EtOH resulted in some instances in embryonic mortality.

For the determination of the developmental stages sensitive to EtOH, embryos were exposed to 2.5% EtOH starting at different developmental stages from MBT up to neurula stages. Embryos exposed during blastula, up to mid-gastrula exhibit severe malformations (st. 8.5, 96.6%, $n = 30$; st. 11, 96%, $n = 26$; Figs. 1B,C). Exposure to EtOH from the mid/late gastrula stages onwards resulted in a gradual reduction in the severity of the developmental malformations induced (st. 12, 50%, $n = 22$; st. 13, 14%, $n = 30$; Figs. 1D,E). Exposure of embryos during neurula stages resulted in no

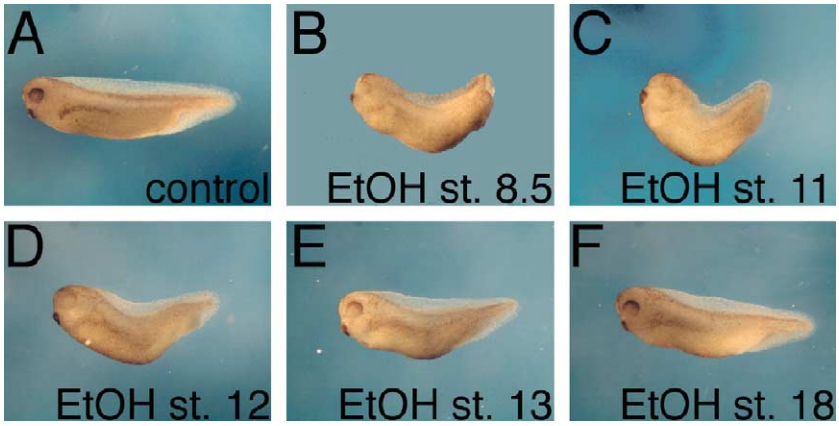

Fig. 1. Maximal sensitivity to EtOH exposure during late blastula/early gastrula. The window of sensitivity for EtOH treatment was determined. Embryos were exposed to 2.5% EtOH from different developmental stages onwards and studied at stage 36. The embryos were incubated in a medium without EtOH (A) or placed in 2.5% EtOH at stages 8.5 (B), 11 (C), 12 (D), 13 (E) and 18 (F).

discernable developmental malformations (st. 18, $n = 30$; Fig. 1F). These observations suggest that during late blastula and early gastrula, *Xenopus* embryos exhibit a high sensitivity to EtOH as determined by the developmental malformations induced.

Exposure to EtOH modifies organizer-specific gene expression

The developmental window of high EtOH sensitivity overlaps with the formation and early function of Spemann's organizer. Therefore, we analyzed several organizer-specific genes for EtOH-induced changes in their expression patterns. Normally, by mid-gastrulation, *chordin*, *gsc* and *Otx2* are expressed in an area restricted to the dorsal blastopore lip, and expressing cells can be detected after invagination (Figs. 2A,C,E). The incubation of embryos in 2.5% EtOH results in increased transcript levels of *chordin* (Fig. 2B; 87%, $n = 49$), *gsc* (Fig. 2D; 88%, $n = 35$) and *Otx2* (Fig. 2F; 83%, $n = 30$), and the cells expressing them are delayed in their invagination through the blastopore. Among the genes with organizer-specific expression, we also studied the homeobox gene *Xnot2* (Gont et al., 1993; von Dassow et al., 1993). Interestingly, the incubation of embryos in 2.5% EtOH eliminated *Xnot2* expression from the dorsal blastopore lip, while the ring of *Xnot2* transcription localized some distance from the blastopore remained unchanged (77%, $n = 31$; Fig. 2H). The specific elimination of *Xnot2* expression from this sub-domain persists during late gastrula stages (st. 12/12.5; not shown). In contrast to the organizer-specific genes, the pan-mesodermal marker, *Xbra* (Smith et al., 1991), was unaffected by EtOH during early gastrula (Fig. 2J; $n = 14$). These results suggested that the organizer is probably a target of the EtOH exposure.

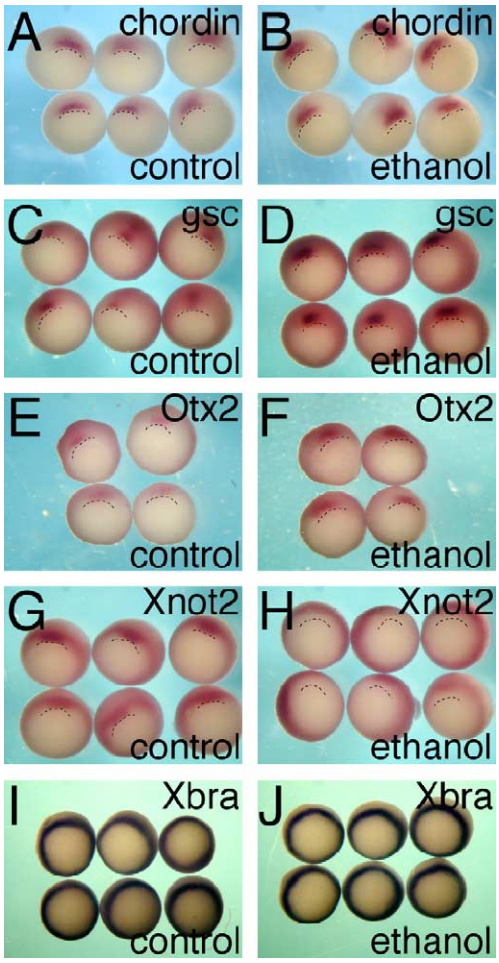

Fig. 2. Spemann's organizer is an early target of EtOH. Organizer-specific gene expression was studied in EtOH-treated embryos. Control (A, C, E, G, I) and EtOH-treated (B, D, F, H, J) embryos were analyzed for organizer specific gene expression at st. 10.5. The expression of *chordin* (A, B), *gsc* (C, D), *Otx2* (E, F), *Xnot2* (G, H) and *Xbra* (I, J) in EtOH-treated embryos is shown.

The changes in *gsc* expression provided a molecular assay for the EtOH effect. Embryos exposed from late blastula to mid gastrula to increasing concentrations of EtOH were scored for changes in *gsc* expression. Up to 0.5% EtOH resulted in normal *gsc* expression in more than 80% of the embryos ($n = 84$). About 50% of the embryos treated with 1% exhibited the EtOH-induced increase in *gsc* expression ($n = 22$), whereas concentrations above 1% resulted in over 90% of the embryos exhibiting *gsc* up-regulation ($n = 63$). These results suggest that gene expression is more sensitive to EtOH than overall embryonic phenotype.

EtOH affects convergent extension during gastrulation

EtOH not only induced the up-regulation of most organizer-specific genes studied, but also affected gastrulation movements. In EtOH-treated embryos, by late gastrula, the *gsc*-positive cells eventually invaginated and migrated rostrally, and by neurula, the *gsc*- and *chordin*-positive cells were localized to their normal position in the rostral region of the embryo as compared to midbrain/hindbrain boundary markers (not shown). These results suggested that the *gsc* and *chordin* positive cells are delayed in reaching their normal rostral domain. To study the possibility that the delay in invagination and rostral migration of the organizer cells is due to the effect of EtOH on convergence-extension movements, exogastrulation was induced in EtOH-treated and control embryos. Exogastrulation in the presence of EtOH resulted in the inhibition of elongation in 94% ($n = 69$) of the cases (Fig. 3B), while in control exogastrulae, almost all (88%, $n = 60$) elongated normally (Fig. 3A). Sibling embryos, not induced to exogasrulate, showed the expected phenotypes of EtOH-treated and control embryos (not shown). These results suggest that EtOH prevents the normal elongation of the embryo during gastrulation. These observations were corroborated by explanting dorsal marginal zones (DMZ). While control DMZs elongated normally (74%, $n = 28$; Fig. 3C), the EtOH-treated dorsal lips remained rounded (60%, $n = 30$; Fig. 3D), again suggesting

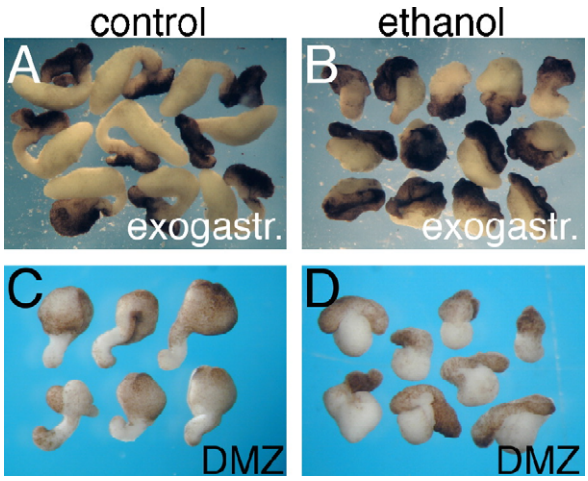

Fig. 3. The effect of EtOH on convergence-extension. Convergence-extension movements and elongation in EtOH-treated embryos were studied in exogastrulae and explanted dorsal marginal zones. Control (A) and EtOH-treated (B) exogastrulae and control (C) and EtOH-treated (D) explanted DMZs.

a defect on convergence-extension affecting the normal elongation of the embryo during gastrulation.

EtOH antagonizes the effects of retinoic acid

We tested the suggestion that increased EtOH levels cause a transient reduction in RA levels. Embryos were treated with EtOH or RA, and the expression of known targets of RA signaling, the *Hox* genes (Krumlauf, 1994), was determined. Embryos were treated with EtOH or RA from late blastula to early neurula, and RNA was prepared for RT-PCR expression analysis (Fig. 4A). EtOH and RA had opposed effects on all *Hox* genes tested. The organizer-specific gene *gsc* was studied by in situ hybridization (Figs. 4B–D) and quantitative real-time PCR (Fig. 5A). Using both approaches, we observed that EtOH induced the up-regulation of *gsc* expression (92%, $n = 39$; Figs. 4C, 5A), while RA gave the opposite effect, repression of *gsc* expression (86%, $n = 37$; Figs. 4D, 5A). The organizer genes *Otx2* and *chordin* were also analyzed by quantitative real-time PCR (Fig. 5A). RA induced their down-regulation while EtOH had the opposite effect and up-regulated them. We also studied the level of expression of *Cyp26* in EtOH treated embryos as a possible mediator of the reduction in RA levels. The analysis of *Cyp26* expression levels was performed by quantitative real-time PCR from whole embryos or from explanted DMZs. In both samples, RA induced the up-regulation of *Cyp26* expression (Fig. 5B). In agreement with the proposed opposite activities of EtOH and RA, alcohol treatment of the embryos resulted in the repression of *Cyp26* expression (Fig. 5B). This observation rules out the possibility of a Cyp26-dependent reduction in RA signaling in EtOH treated embryos.

Quantitative analysis of the opposed effects of RA and EtOH was performed using a RA-reporter plasmid (RAREZ), which contains a RA-responsive element that regulates the expression of the *LacZ* gene (Rossant et al., 1991). *Xenopus* embryos were injected radially with the RAREZ DNA at the 2-cell stage, then at st. 9, they were exposed to RA or EtOH. ß-galactosidase activity was quantified at st. 10.5–11 using a chemiluminiscent assay (Fig. 5C). Embryos treated with RA showed the expected increase in ß-galactosidase activity as compared to control sibling embryos. In contrast, embryos treated with EtOH showed decreased ß-galactosidase activity below control levels, suggesting a reduction in RA signaling. These results further support a developmental effect for EtOH through the inhibition of RA metabolism.

Retinoic acid signaling in Spemann's organizer

Our results suggest that organizer-specific gene expression is under regulation of RA signaling. Therefore, the reduction of RA levels by other means should result in similar effects on organizer-specific gene expression to those observed in EtOH-treated embryos. Partial depletion of retinoic acid was induced by the incubation of embryos in citral (3,4-dimethyl-2,6-octadienal; Schuh et al., 1993). Quantitative PCR analysis of citral-treated embryos revealed an increase in *gsc*, *Otx2* and *chordin* expression, which paralleled the increase observed after EtOH treatment (Fig. 5A). Corroboration of these results was obtained by treatment with citral and analysis of organizer gene expression by

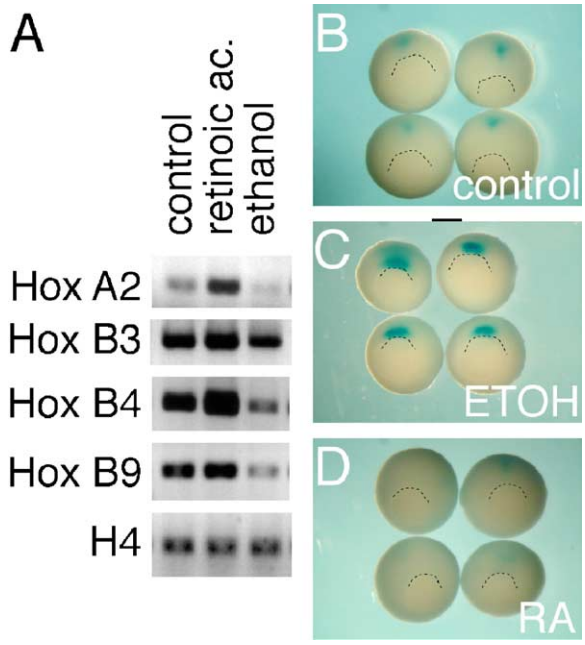

Fig. 4. Opposite effects of EtOH and RA on *Hox* and *gsc* expression. (A) *Hox* gene expression was studied at stage 13 by RT-PCR in embryos treated with EtOH (2.5%) or RA (1 μM). (B–D) In situ hybridization with the *gsc*-specific probe was used to corroborate the opposed effects of EtOH and RA. Changes in the expression pattern of *gsc* (st. 11) were determined in the control (B), EtOH-(C) and RA-treated (D) embryos.

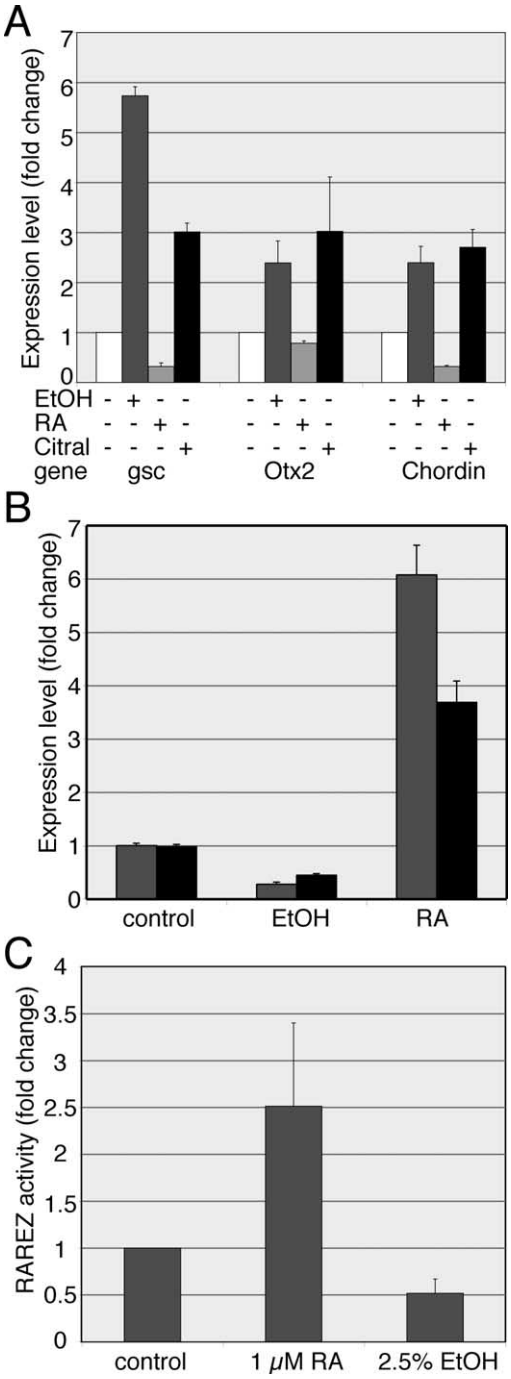

Fig. 5. EtOH and RA have opposed effects on gene expression. (A) Quantitative real-time PCR was employed to study the changes in *gsc*, *Otx2* and *chordin* expression at st. 10.5 as a result of EtOH (2.5%), RA (1 μM) and Citral (60 μM) treatment. $P < 0.05$. (B) Quantitative real-time PCR analysis of *Cyp26* expression as a result of EtOH and RA treatment. Whole embryos (grey) or dorsal marginal zone explants (black) were processed for RNA extraction at st. 10.5. $P < 0.005$. (C) Embryos were injected with the RAREZ, RA reporter plasmid, and subsequently treated with EtOH and RA. Quantitation of the reporter plasmid activity was performed (st. 10.5–11) using a chemiluminiscent substrate for ß-galactosidase. $P < 0.0005$.

in situ hybridization. Citral treatment resulted in the up-regulation and expansion of *gsc* (88%, $n = 16$; Fig. 6B), *Otx2* (37.5%, $n = 17$; Fig. 6D) and *chordin* (100%, $n = 15$; Fig. 6F), and in the elimination of *Xnot2* transcripts from the dorsal blastopore lip (73%, $n = 15$; Fig. 6H), reproducing the results of the EtOH treatment. An alternative way to reduce the level of active RA in the embryo is to overexpress the RA 4-hydroxylase, the cytochrome *P*450, Cyp26 (XCYP26; Hollemann et al., 1998). The overexpression of *Cyp26* results in the up-regulation and expansion of *chordin* (91%, $n = 24$; Fig. 7B) and *Otx2* (82%, $n = 28$; Fig. 7D) expression, in agreement with the results obtained with EtOH and citral. These results further support the suggestion that EtOH is an antagonist of the RA biosynthetic pathway and that Spemann's organizer relies on RA signaling for its normal activity.

The changes in organizer-specific expression and the relationship between EtOH and ROL/RA described above suggest that active RA signaling takes place during gastrula stages in the dorsal lip of the blastopore. In support, injection of the RA reporter plasmid and analysis during neurula stages showed predominant activity of the responsive element along the dorsal midline and in anterior head

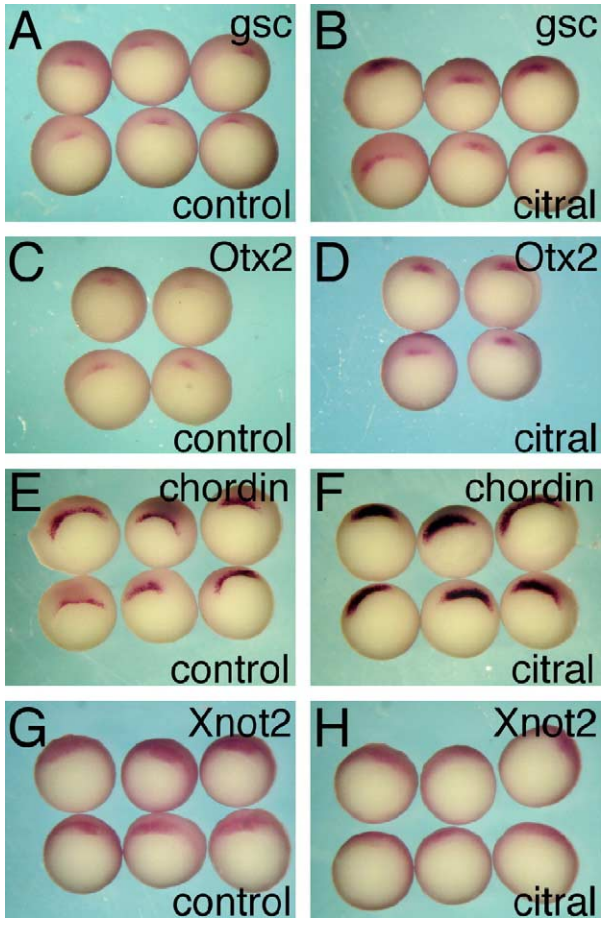

Fig. 6. Citral affects organizer-specific gene expression. In order to reduce the endogenous RA levels, embryos were treated with citral (60 μM). The embryos were processed for in situ hybridization with *gsc* (A, B), *Otx2* (C, D), *chordin* (E, F) and *Xnot2* (G, H) specific probes. Control untreated sibling embryos are marked.

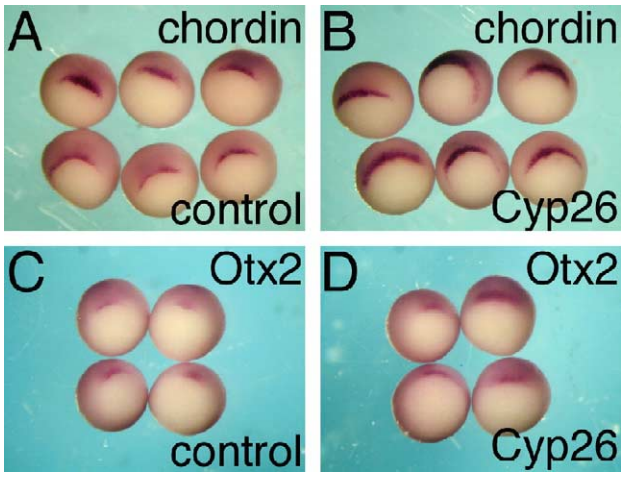

Fig. 7. RA metabolism by Cyp26 overexpression results in changes in organizer-specific gene expression. Active RA levels were reduced by *Cyp26* mRNA injection. At st. 10.5, the embryos were processed for in situ hybridization with *chordin* (A, B) and *Otx2* (C, D) specific probes. Control untreated sibling embryos are marked.

regions (Figs. 8A,B). Analysis of embryos during midgastrula stages revealed ß-galactosidase activity over the DMZ region and in cells involuting from this domain (Fig. 8C). In order to minimize the possible mosaic effects resulting from the injection of expression plasmids, transgenic *Xenopus* embryos were generated with RA-responsive plasmids driving the expression of either *LacZ* or *GFP*. The transgenic embryos also expressed the reporter plasmid in the dorsal blastopore region, Spemann's organizer (61%, $n = 41$; Figs. 8D–F). High levels of RA signaling were detected in *Xenopus* embryos from early gastrulation onwards. Corroboration of these results was obtained by quantifying the reporter activity. RAREZ injected embryos were dissected into dorsal and ventral halves, and the extent of reporter activity was quantified by chemiluminescence (Fig. 8G). The dorsal regions of the embryos exhibited higher reporter activity when compared to whole embryo extracts, while the ventral explants had reduced reporter activity, confirming that the DMZ exhibits higher RA signaling.

EtOH can rescue the effects of exposure to high levels of Vitamin A

To directly test the effect of EtOH on ROL (Vitamin A) conversion to RA, we treated embryos with ROL, EtOH or both. Exposure of *Xenopus* embryos to ROL (70 μM) resulted in severely deformed embryos (Fig. 4B; Schuh et al., 1993), similar to retinoic acid treatment (Durston et al., 1998). Incubation of embryos in EtOH (1.5%) induced the characteristic malformations (Fig. 9C). Combined exposure to EtOH and ROL resulted in extensive rescue of the developmental malformations, lengthening of the embryo and formation of head structures (Fig. 9D). The head malformations were studied in more detail by combined analysis with the *en2*, *Pax6* and *XAG1* markers for midbrain/hindbrain boundary, eye and midbrain and cement gland, respectively. While individual treatment with ROL or EtOH severely altered or inhibited the expression of the marker genes in 96% ($n = 32$; Fig. 9B) and 96% ($n = 31$; Fig. 9C) of the embryos, respectively, combined treatment restored normal expression of these genes in 92% of the

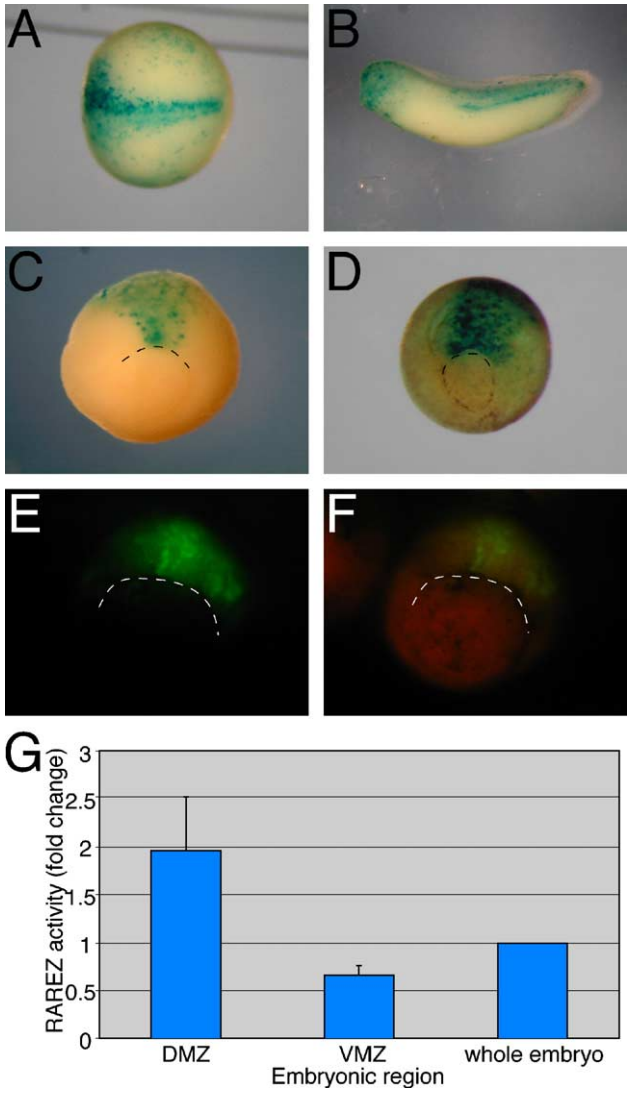

Fig. 8. Retinoic acid signaling in Spemann's organizer. The detection of RA signaling was performed using the RAREZ or RAREGFP reporter plasmids. (A–C) Embryos injected with the RAREZ plasmid and stained for ß-galactosidase activity at stages (A) 13, (B) 30 and (C) 10.5. (D) RAREZ transgenic embryo at st. 11.5 stained for ß-galactosidase activity. (E) Transgenic embryo with the RAREGFP plasmid during early/mid gastrula stages. (F) Same embryo as in (E) illuminated by epifluorescence and normal illumination to demonstrate the location of the GFP signal. The dashed line marks the position of the dorsal lip of the blastopore. (G) Quantitation of the RA signaling using the RAREZ reporter plasmid. At the onset of gastrulation, the DMZ and VMZ regions were explanted from embryos injected with the RAREZ plasmid and were processed for quantification of the ß-galactosidase activity at st. 10.5–11. The control samples were whole embryos extracts. $P < 0.015$.

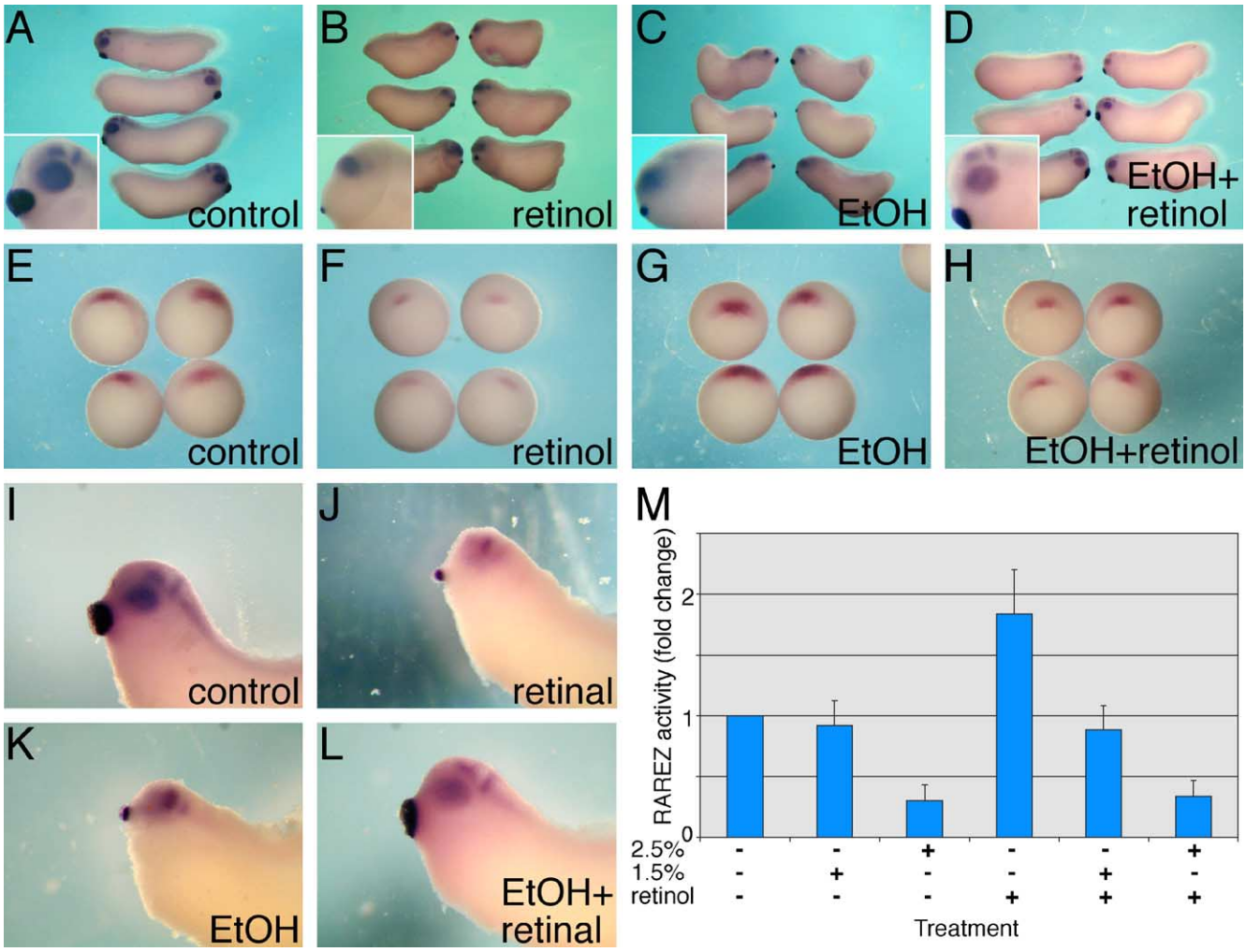

Fig. 9. EtOH can rescue the teratogenic effects of ROL and RAL. The rescue assays included phenotypic, gene expression and reporter plasmid activity assays. (A–D) A combined phenotypic and gene expression assay was performed at st. 33. Embryos were treated with: (A) control, (B) 70 μM ROL, (C) 1.5% EtOH and (D) 70 μM ROL together with 1.5% EtOH. The embryos were processed for in situ hybridization with probes for *en2*, *Pax6* and *XAG1*. The insets are enlargements of the head region. (E–H) Rescue analysis during gastrulation based on the *gsc* expression pattern. The embryos were studied at st. 10.5 after treatment with (E) control, (F) 70 μM ROL (G) 1.5% EtOH and (H) 70 μM ROL and 1.5% EtOH. (I–L) EtOH can rescue the teratogenic effects of RAL. Embryos were studied at st. 33 for head phenotype and the expression of the *en2*, *Pax6* and *XAG1* markers. (I) control, (J) 5 μM RAL, (K) 1.5% EtOH and (L) 5 μM RAL and 1.5% EtOH. (M) Embryos were injected with the RAREZ plasmid, treated with ROL (150 μM), EtOH (1.5% or 2.5%), or a combination of both. The determination of the ß-galactosidase activity was performed at st. 10.5–11. $P < 0.0001$.

embryos ($n = 28$; Fig. 9D). Therefore, EtOH can rescue the teratogenic effects of high ROL levels.

ROL, like RA, repressed the expression of the organizer-specific gene, *gsc*, in 80% of the embryos ($n = 19$; Fig. 9F), while EtOH increased it in 88% ($n = 19$; Fig. 9G). The addition of EtOH to the ROL treatment resulted in a pattern of *gsc* expression similar to control embryos in 84% of the cases ($n = 19$; Fig. 9H). These results support the hypothesis that EtOH suppresses the teratogenic effects of ROL at the molecular level.

Similar results with the head markers were obtained when we performed a combined treatment of EtOH and retinaldehyde (RAL). RAL (5 μM) treatment resulted in the altered or inhibition of the marker gene expression in a large proportion of the embryos (77%, $n = 53$; Fig. 9J), like the EtOH treatment (71%, $n = 53$; Fig. 9K). As observed for ROL, combined EtOH and RAL treatment resulted in restored normal expression of *en2*, *Pax6* and *XAG1* in 65% ($n = 51$) of the embryos (Fig. 9L). These results further support the effect of EtOH as a competitive inhibitor of ROL metabolism. We also used the RAREZ reporter plasmid to determine whether EtOH can modify the ROL effect on RA responsive promoters. While the incubation of *Xenopus* embryos in ROL (150 μM) resulted in increased activity of the RAREZ reporter plasmid (Fig. 9M), EtOH added concomitantly to the embryos, eliminated the positive regulatory effect of ROL in a concentration-dependent manner (Fig. 9M). These results show that EtOH antagonizes the effects of exposure to ROL.

Discussion

EtOH as a teratogen

EtOH causes in *Xenopus* a combination of developmental malformations closely resembling FASD. In this experimental system, we determined that late blastula to early gastrula comprise the developmental window of increased sensitivity to EtOH. During this time period, Spemann's organizer is affected, as judged by embryonic phenotypes and gene expression patterns. We show that the effects of EtOH on gene expression are opposite to those exerted by RA, and that EtOH can reduce the teratogenic effects of ROL (Vitamin A) and RAL, suggesting that EtOH functions in part through the RA pathway. The EtOH, RA, Spemann's organizer connection was further supported by demonstrating that RA signaling is active in the dorsal lip of the blastopore, Spemann's organizer. We provide evidence supporting a "competition model", in which EtOH competes with ROL and RAL for the stores of alcohol- and aldehyde-dehydrogenases (Deltour et al., 1996; Duester, 1991) or additional co-factors common to RA biosynthesis of and EtOH elimination. Based on our results, such competition results in transient reduction in RA levels necessary for normal embryonic development. RA reduction affects several key regulatory genes in the early embryo. The known RA targets, the *Hox* genes (Krumlauf, 1994), showed opposite responses to EtOH and RA, in agreement with the competition model. EtOH and RA also elicited opposed responses on transcriptional activity, even during gastrula stages, of organizer-specific genes and *Cyp26* (RA hydroxylase), and on the transcription of a RA reporter plasmid.

The EtOH embryonic phenotype included shortening along the anterior–posterior axis, microcephally and microphthalmia, phenocopying the common malformations in FAS. The reduction of RA levels by XCYP26 overexpression (Hollemann et al., 1998) or *Raldh2* mutation (Niederreither et al., 1999) results in the compression of the anterior–posterior axis, facial dysmorphogenesis and central nervous system defects overlapping extensively with the Vitamin-A-deficiency (VAD) and FAS defects (Chaudhuri, 2000; Clagett-Dame and DeLuca, 2002). These observations and our results establish a link between reduced RA signaling and EtOH exposure. *Cyp26* expression was down-regulated by EtOH exposure, ruling out a Cyp26-dependent reduction in RA signaling. The exposure of *Xenopus* embryos to RA, RAL and ROL also results in microcephally and microphthalmia (Chen et al., 2001; Durston et al., 1989; Schuh et al., 1993). Recent reports have suggested a plausible explanation for the observation that excess and reduced RA levels might result in similar phenotypes (Weston et al., 2003). Anterior head formation requires the repressor activity of unliganded retinoic acid receptors (RARs) such that loss-of RARα activity or dominant negative co-repressor expression results in abnormal head formation, phenocopying the effect of RA treatment (Koide et al., 2001). RA has also been shown to be required during gastrulation for anterior head structures and craniofacial development, probably through effects on the prechordal plate (Brickell and Thorogood, 1997; Halilagic et al., 2003). The phenotypes obtained by inducing reduced levels of RA by treating *Xenopus* embryos with citral, a RA biosynthesis inhibitor, or overexpressing XCYP26, a retinoic acid 4-hydroxylase, were indistinguishable from exposure to EtOH. These results further support that EtOH, directly or indirectly, causes a transient reduction in RA levels in the developing embryo.

The EtOH/ROL competition hypothesis led to the suggestion that the teratogenic effects of high levels of ROL could be rescued by EtOH exposure. Our results based on a number of assays show that combined exposure to ROL or RAL and EtOH results in normal or almost normal embryos or gene expression patterns in contrast to the effects of each compound independently. In quail embryos, EtOH exposure results in cardiovascular abnormalities, which can also be induced by citral treatment, and they can be partially rescued by RA addition (Twal et al., 1995). Therefore, the embryonic malformations induced by EtOH in both *Xenopus* and quail embryos can be phenocopied by inhibiting the biosynthesis of RA, and the addition of RA, RAL or ROL can perform extensive rescue. The extent of rescue at the level of the antagonism between ROL, RA and EtOH raises the possibility that EtOH might have a pleiotropic effect on embryogenesis by functioning through more that one mechanism.

RA signaling in Spemann's organizer as an early target of EtOH

The developmental window of high EtOH sensitivity overlaps with the formation and early function of the embryonic organizer (Harland and Gerhart, 1997; Levy et al., 2002). Several molecular markers were studied to determine whether the organizer is a target of EtOH. The spatial domain of organizer-specific gene expression expanded, and an increase in their expression levels can be detected. In one instance, *Xnot2* (Gont et al., 1993), the organizer-specific expression of the gene, was repressed. We identified in *Xenopus* embryos changes in the patterns of *gsc* and *Otx2* among others. The same two genes in zebrafish changed during late gastrula/early neurula as a result of EtOH-exposure during late blastula and early gastrula (Blader and Strahle, 1998), further supporting the effect of EtOH on the embryonic organizer. In addition, the organizer is responsible for the formation of dorsal midline structures (Harland and Gerhart, 1997), and analysis of individuals with FAS has led to the suggestion that their midline is malformed (Johnson et al., 1996; Swayze et al., 1997).

In the context of the EtOH/ROL competition model, it was necessary to show that RA signaling is active in the organizer unless the effect is indirect. In addition to changes

in organizer-specific gene expression, we could show: (1) higher RA signaling activity in the dorsal compared to the ventral regions of the embryo during gastrulation. (2) Transgenic embryos generated with the RA reporter plasmid exhibited transcriptional activity predominantly in the organizer based on endogenous retinoids. (3) Additional means for reduction of RA levels gave the same effects on organizer-specific gene expression like EtOH. Furthermore, retinoids have been identified in the organizers of several vertebrate embryos, like Hensen's node in chick (Chen et al., 1992), the node in mouse (Hogan et al., 1992) and the shield in zebrafish (Joore et al., 1997). Also, in agreement with our results, the DMZ of *Xenopus* embryos has been shown to contain higher concentrations of retinoids during late gastrula and neurula stages (Chen et al., 1994; Kraft et al., 1994). The activity of the RA reporter plasmid not only relies on the availability of endogenous retinoids in the organizer region, but it also requires additional components of the retinoic acid signaling pathway necessary to achieve signaling and transcriptional activation. In mouse embryos transgenic for the same reporter plasmid, the anterior limit of expression of the reporter along the primitive streak is the node (Rossant et al., 1991). Reduced RA synthesis due to the mutation of the *Raldh2* gene in mouse and zebrafish embryos has shown a requirement for RA signaling during gastrulation (Grandel et al., 2002; Niederreither et al., 1999). Therefore, not only the embryonic organizer appears to exhibit enhanced RA signaling relative to the rest of the embryo, but some of the embryonic malformations resulting from inhibition of this signal are reminiscent of the FAS phenotype. Together, all these observations suggest that EtOH might impair RA signaling in Spemann's organizer, and this might be one of its earliest effects as a teratogen.

Ethanol impairs gastrulation movements

We observed that cells expressing organizer-specific genes were delayed in their involution and rostral migration, as a result of EtOH-exposure. Analysis of *gsc* expression and the rostral migration of the prechordal plate cells in EtOH-treated embryos showed that during early/mid gastrulation, the *gsc*-positive cells remain at the dorsal blastopore lip, while in untreated embryos, these cells have already invaginated and moved rostrally. The same effect was observed for *chordin* and *Otx2* positive cells. Interestingly, in EtOH-treated embryos, the *gsc*-*chordin*- or *Otx2*-positive cells reach their normal rostral position by neurula stages. The delay does not affect the final position of the cells, as determined by comparison to markers of the midbrain/hindbrain boundary, whose position apparently is unchanged after EtOH treatment (this work; Yelin et al., in preparation). In zebrafish embryos, EtOH affects the movement of the prechordal plate, resulting in the abnormal migration of the *gsc*-positive cells during neurula (Blader and Strahle, 1998). It was suggested that EtOH-treated zebrafish embryos develop cyclopia as a result of an arrest in the movement of the prechordal plate mesoderm. In *Xenopus* embryos, a similar delay in the involution and migration of *gsc*-positive cells can be observed, but apparently, the embryos recover at later stages, preventing the formation of cyclopic embryos (Li et al., 1997).

EtOH exposure also interferes with convergence-extension movements in *Xenopus* embryos, providing a plausible explanation for the delay in the rostral movement of the organizer-derived cells. The abnormal expression of organizer genes could account for the delay or reduced extent of convergence-extension of organizer-derived cells. *Otx2* overexpression interferes with convergence-extension movements (Andreazzoli et al., 1997). Repression of *Xnot2* expression along the dorsal blastopore lip probably also has effects on elongation of the embryo (Yelin et al., in preparation), as this gene has been linked to convergence-extension movements in the notochord (Yamada and Modak, 1998). In conclusion, defective convergence-extension movements can explain in part the reduced length of the embryo along the anterior–posterior axis, while a delay of the prechordal plate in reaching its rostral position could affect the morphogenesis of the head, as observed in individuals with FAS.

Xenopus as a model system for the study of FAS

We routinely exposed *Xenopus* embryos to solutions with 2–2.5% EtOH (vol/vol) to achieve a high incidence of developmental malformations, which allowed us to perform a molecular analysis of the teratogenic effects of alcohol. In the zebrafish study, similar concentrations of EtOH (2.4%) were required to cause the teratogenic effects (Blader and Strahle, 1998). In humans, blood alcohol levels of 0.1–0.3% (gr/100 ml) are observed in clearly drunk individuals, and levels of 0.3–0.5% lead to unconsciousness. In the case of humans, the alcohol level would represent the level in the mother's blood, but no information is available as to the EtOH level in the fetus, which might be higher due to clearance inefficiency. Although the EtOH concentrations used in the *Xenopus* and zebrafish studies are slightly higher than the concentrations that give clear intoxication or unconsciousness in humans, in this case, we refer to the concentrations in the incubation buffer and also here the endogenous levels of EtOH are not known. *Gsc* expression was employed to study the molecular effects of EtOH at concentrations where no phenotype can be observed. Our results show that concentrations as low as 1.5% already result in a high incidence of *gsc* expansion, and 50% of the embryos show effects already in 1% EtOH. These results further support that the concentrations of EtOH employed throughout our experiments are not dramatically different from those affecting human embryos.

It is clear that the origin of the malformations leading to FAS is embryological, but their etiology remains elusive. The analysis of the sensitivity window of *Xenopus* embryos to EtOH-exposure identified a period of high sensitivity

extending from late blastula to mid gastrula. A number of experimental embryological systems have been employed to try and determine this sensitivity window in vertebrates (Blader and Strahle, 1998; Cartwright and Smith, 1995; Chaudhuri, 2000; Sulik, 1984; Webster et al., 1983). In agreement with our results, it is generally believed that maximal EtOH sensitivity in vertebrate embryos is during gastrula and/or early neurula, whereas the *Xenopus* experimental system permitted a more precise determination. Our timed treatments also support the suggestion that although a window for increased sensitivity can be identified, the phenotype of EtOH-treated embryos is cumulative, increasing in severity with longer exposure times. Nevertheless, the most severe phenotypes can only be obtained by exposure of the embryos during late blastula and early gastrula.

The experimental conditions determined for *Xenopus* embryos gave rise to a high incidence of embryos that are microcephalic, microphthalmic and have a shortened rostral–caudal axis. These EtOH-induced malformations are reminiscent of the phenotypes described for individuals affected by FAS. A reduction in head circumference is a common affliction of individuals with FAS (Chaudhuri, 2000; Jones and Smith, 1973). Individuals exposed to EtOH during pregnancy exhibit pre- and post-natal growth retardation that results in reduced stature (Chaudhuri, 2000; Jones and Smith, 1973). In addition, FAS patients exhibit microphthalmia and other craniofacial dysmorphogenesis (Johnson et al., 1996; Stromland and Pinazo-Duran, 2002). This resemblance between phenotypic anomalies induced in *Xenopus* and humans suggests that common embryonic structures and genetic networks are affected by EtOH in vertebrate embryos. Therefore, *Xenopus* embryos can be used as a model system for FAS, taking advantage of the molecular and embryological tools available as demonstrated in the present study.

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