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Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation

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SUMMARY

Human embryos exposed to alcohol (ethanol) develop a complex developmental phenotype known as fetal alcohol spectrum disorder (FASD). In Xenopus embryos, ethanol reduces the levels of retinoic acid (RA) signaling during gastrulation. RA, a metabolite of vitamin A (retinol), is required for vertebrate embryogenesis, and deviation from its normal levels results in developmental malformations. Retinaldehyde dehydrogenase 2 (RALDH2) is required to activate RA signaling at the onset of gastrulation. We studied the effect of alcohol on embryogenesis by manipulating retinaldehyde dehydrogenase activity in ethanol-treated embryos. In alcohol-treated embryos, we analyzed RA signaling levels, phenotypes induced and changes in gene expression. Developmental defects that were characteristic of high ethanol concentrations were phenocopied by a low ethanol concentration combined with partial RALDH inhibition, whereas *Raldh2* overexpression rescued the developmental malformations induced by high ethanol. RALDH2 knockdown resulted in similar RA signaling levels when carried out alone or in combination with ethanol treatment, suggesting that RALDH2 is the main target of ethanol. The biochemical evidence that we present shows that, at the onset of RA signaling during early gastrulation, the ethanol effect centers on the competition for the available retinaldehyde dehydrogenase activity. In light of the multiple regulatory roles of RA, continued embryogenesis in the presence of abnormally low RA levels provides an etiological explanation for the malformations observed in individuals with FASD.

INTRODUCTION

Fetal alcohol spectrum disorder (FASD) encompasses multiple teratogenic effects that result from the exposure of human embryos to alcohol [ethanol (EtOH)] (Koren et al., 2003; Sokol et al., 2003). EtOH exposure during human pregnancy can result in increased rates of abortion and stillbirth rates (Kesmodel et al., 2002a; Kesmodel et al., 2002b), or in individuals with a wide range of developmental, morphological and neurological defects. The strongest manifestation of FASD, also known as fetal alcohol syndrome (FAS), can include: craniofacial malformations, microcephaly, short stature, microphthalmia, heart defects and behavioral and psychological problems (Chaudhuri, 2000). EtOH is widely recognized as one of the most detrimental chemical teratogens afflicting the human population and hence is the focus of intensive study. Several models have been proposed to explain the etiology of the developmental malformations induced by EtOH. Among the mechanisms proposed, it has been suggested that the observed developmental defects might be explained by: the induction of apoptosis, cell adhesion defects, the accumulation of free radicals, effects on growth factors or 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; Yelin et al., 2005).

The enzymatic steps that are required to metabolize EtOH in the body and the enzymatic reactions needed for metabolism of vitamin A [retinol (ROL)] to its active metabolites involve the same enzyme families (Duester, 2000). This observation provides the basis for the model suggesting that EtOH is a competitive inhibitor of ROL metabolism during embryogenesis (Duester, 1991; Deltour et al., 1996; Yelin et al., 2005). During embryogenesis, the most important vitamin A metabolite is RA. In turn, RA is known to be essential for the normal development of many of the structures affected in FAS (Clagett-Dame and DeLuca, 2002; Marill et al., 2003). Taking advantage of Xenopus embryos, we recently obtained evidence supporting the EtOH-RA connection (Yelin et al., 2005). EtOH treatment of frog embryos recapitulated the common phenotypic malformations observed in FAS. Using gene expression assays and an RA reporter plasmid (Rossant et al., 1991), we showed inverse transcriptional responses to RA and EtOH treatments. In addition, we showed that simultaneous exposure to ROL or retinaldehyde (RAL) and EtOH rescues the developmental defects that are normally induced by the individual treatments (Yelin et al., 2005).

The biosynthesis of RA from ROL proceeds through two successive oxidation steps, the first is performed by alcohol dehydrogenases or short-chain dehydrogenases/reductases that generate RAL, and a second step is performed by retinaldehyde dehydrogenases (Duester, 1996; Crabb et al., 2004). EtOH detoxification requires the same two oxidation steps to convert it to acetic acid. Extensive experimental evidence is available supporting the requirement for RA at specified concentrations and stages in order for normal embryogenesis to take place. Excess RA from either ectopic application or mutation of RA-metabolizing enzymes (Cohlan, 1953; Durston et al., 1989; Kessel, 1992; Sakai et al., 2001) and reduced RA from either vitamin A-deficient diets or mutations in RA-synthesizing enzymes (Wilson et al., 1953; Maden et al., 1996; Niederreither et al., 1999) can both result in complex developmental phenotypes that are reminiscent of FAS.

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Therefore, competition between EtOH detoxification and ROL metabolism means that continued embryonic development with abnormally low RA levels results in numerous embryonic malformations.

Among the different alcohol and aldehyde dehydrogenases (ADHs and ALDHs, respectively), retinaldehyde dehydrogenase 2 (RALDH2, also known as Aldh1a2) is of particular interest during early embryogenesis. Mutation analysis has shown that RALDH2 is necessary during vertebrate gastrulation (Niederreither et al., 1999; Grandel et al., 2002). Based on the efficiency of RALDH2 in RAL oxidation, its temporal and spatial pattern of expression and its loss-of-function phenotype in frog, mouse and zebrafish embryos (Haselbeck et al., 1999; Niederreither et al., 1999; Chen et al., 2001; Grandel et al., 2002), it is believed that this enzyme plays a central role during early embryogenesis and that its expression is probably the limiting step in RA biosynthesis at the beginning of RA signaling during gastrulation. Retinoids or RA signaling have been detected in the organizer of vertebrate embryos during gastrulation (Chen et al., 1992; Hogan et al., 1992; Chen et al., 1994; Creech Kraft et al., 1994; Yelin et al., 2005), which is the site of *Raldh2* expression (Chen et al., 2001).

Here, we provide in vivo evidence linking the teratogenic effects of EtOH exposure to the competitive inhibition of normal retinaldehyde dehydrogenase (RALDH) activity, namely of RALDH2, in the early gastrula embryo. Using *Adh4* and *Aldh1* overexpression and known pharmacological inhibitors of ADH and ALDH activities, we were able to show that manipulation of ALDH, but not ADH, activity during early gastrula stages strongly affects the expression of RA-responsive genes. Combined exposure to mildly teratogenic EtOH treatments and ALDH inhibition showed that a reduction in the normal level of ALDH activity predisposes the embryo to enhanced sensitivity to EtOH exposure. However, overexpression of *Raldh2* minimizes the effects of EtOH exposure, thus protecting the embryos from this teratogen. A reduction of RA signaling as a result of oligonucleotide-mediated knockdown of RALDH2 activity was not exacerbated further by concomitant EtOH exposure, identifying RALDH2 activity as the main target of EtOH during early gastrulation. Our results show that RALDH2 activity is present in limiting levels in the early gastrula embryo; EtOH targets RALDH2 activity during early embryogenesis, thus resulting in reduced RA biosynthesis during these crucial developmental stages.

RESULTS

RALDH activity is limiting during early gastrula

The biosynthesis of RA involves two enzymatic oxidation steps, which convert ROL to RAL and then to RA. To elucidate further the etiology of EtOH-induced malformations, it is important to determine, by manipulation of enzymatic activity, which enzyme has the strongest effect on RA signaling during early gastrulation and whether a particular enzyme might be more sensitive to inhibition. We monitored the expression level of the RA-responsive genes *Hoxb1* and *Hoxb4* in embryos where the ADH and ALDH activity was increased or partially inhibited. The effect of *Adh4* or *Aldh1* overexpression (Hoffmann et al., 1998; Ang and Duester, 1999a) was determined by quantitative real-time PCR (qPCR) during early/mid gastrula stages [stage (st.) 10.5-11]. Increasing the ADH activity in the embryo did not significantly affect the expression level of either *Hoxb1* or *Hoxb4* (Fig. 1A). In contrast, increasing the level of ALDH activity results in increased *Hoxb1* and *Hoxb4* transcript levels (Fig. 1A), suggesting that the enzymatic activity of ALDH is present in limiting amounts during these developmental stages.

To further corroborate the enzyme activity that is more limiting at the onset of gastrulation, we turned to a loss-of-function approach. The requirement for ADH activity for *Hox* gene expression was studied by inhibiting these enzymes with 4-methylpyrazole (4-MP, fomepizole) (Collins et al., 1992). Late treatment with 10 mM 4-MP at the blastula stage, which does not induce an overt developmental phenotype (not shown), was followed by analysis of *Hox* expression during early/mid gastrula stages (Fig. 1A). The expression of both *Hoxb1* and *Hoxb4* was only slightly affected by the inhibition of ADH activity, raising the possibility that either these enzymes are present in excess during early gastrula or that their product, RAL, is present in high concentrations. Two additional controls were performed in the context of this experiment. First, sibling embryos were treated with 1 μM RA to demonstrate the expected upregulation of *Hoxb1* and *Hoxb4* (Fig. 1A). Second, to ensure that the 4-MP treatment is effective, we showed that it blocks the upregulation of RA signaling resulting from ROL treatment (supplementary material Fig. S1). Partial inhibition of ALDH activity was induced with 4-diethylaminobenzaldehyde (DEAB) (Russo et al., 1988; Begemann et al., 2004). Embryos were treated with a low concentration of DEAB (30 μM), which is very slightly teratogenic, and analyzed during early/mid gastrula. Analysis of *Hoxb1* and *Hoxb4* expression revealed that this treatment eliminates about 30-50% of the normal transcript levels of these genes (Fig. 1A). These results suggest that, at the onset of gastrulation, ALDH activity is present in limiting amounts and any disruption in its activity level translates, first, into changes in RA signaling levels and ultimately into altered gene expression patterns.

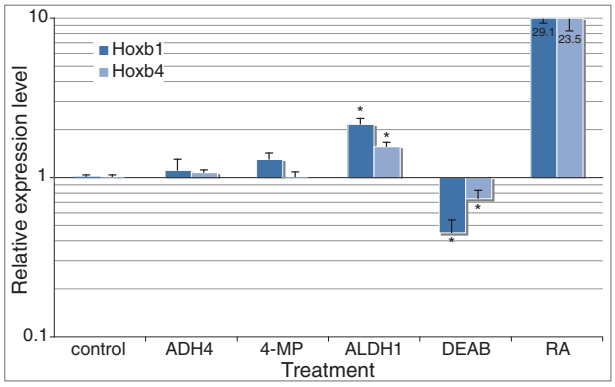

Fig. 1. ALDH is the limiting step in the activation of RA signaling during gastrulation. ADH activity was manipulated in gastrula-stage embryos by either overexpression of *Adh4* or inhibition with 4-MP. In sibling embryos, overexpression of *Aldh1* was used to increase ALDH activity, whereas DEAB treatment was used to inhibit this activity. The level of *Hoxb1* and *Hoxb4* expression as a result of the different treatments was determined by qPCR. Embryos treated with RA were used as a positive control for *Hox* upregulation. *P<0.01 when compared with control expression levels.

Manipulation of RALDH activity recapitulates the FAS phenotype

Next, we focused on providing in vivo biochemical evidence that EtOH or its metabolite, acetaldehyde, is a competitor of RAL for the available RALDH activity. According to our results, RALDH activity is limiting during early embryogenesis and its partial inhibition should result in enhanced competition for the remaining enzymatic activity, thereby sensitizing the embryo to EtOH exposure. Another prediction from the model is that overexpression of *Raldh2*, the main RALDH expressed during early gastrulation, might be capable of rescuing the developmental malformations induced by EtOH exposure by ameliorating the competition. Previously, we described that Xenopus embryos exposed to 1% EtOH (vol/vol; 0.8 grams/100 ml) exhibited no or very mild morphological phenotypes, although molecular effects could be observed readily. By contrast, high EtOH concentrations (2%) recapitulate many of the developmental defects that are characteristic of FAS (Yelin et al., 2005). We tested whether embryos treated with DEAB, which partially inhibits RALDH activity, and exposed subsequently to suboptimal EtOH concentrations can phenocopy the developmental malformations that are characteristic of higher EtOH concentrations. Embryos were treated with suboptimal concentrations of DEAB (20-30 μM) and EtOH (1-1.3%) from late blastula (st. 8.5-9). At early tailbud stages (st. 29), embryos were analyzed both morphologically for developmental malformations and with the *Pax6* and *XAG1* probes for detailed analysis of eye, midbrain and cement gland formation (Fig. 2A-D). Treatment with DEAB (20 μM) alone resulted in a very mild phenotype (Fig. 2B) in the majority of the embryos (74%, *n*=31); EtOH (1.3%) also caused a very weak phenotype (Fig. 2C) (97%, *n*=32). In both instances, the *Pax6* and *XAG1* signal is weaker and the embryos show a slight microcephalic phenotype. Combined EtOH and DEAB treatment resulted in a severe phenotype (Fig. 2D) where the embryos lack all anterior head structures including those expressing *XAG1* and *Pax6* (88%, *n*=33). These embryos are also markedly shorter.

The partial RALDH inhibition results suggest that, by limiting the level of activity of this enzyme, the competition between EtOH and ROL, more specifically acetaldehyde and RAL, becomes more severe. By providing an exogenous source of RALDH activity we should be able to diminish the teratogenic effects of EtOH by relieving the competition for this enzyme. At the developmental stages of interest (i.e. early gastrula) the primary biosynthetic activity that metabolises RAL to RA is provided by RALDH2 (Niederreither et al., 1999; Grandel et al., 2002) and at these stages is restricted (Fig. 1). We performed rescue experiments, where *Raldh2* was overexpressed in EtOH-treated embryos, with the aim of reducing the teratogenic effects of EtOH. *Raldh2* RNA-injected embryos were treated with EtOH and incubated to st. 30 for phenotypic and in situ hybridization analysis, as described. Embryos treated with 2% EtOH exhibited the characteristic developmental malformations, including severe microcephaly, microphthalmia and reduced cement gland formation (96%, *n*=50) (Fig. 2F) (Yelin et al., 2005; Yelin et al., 2007). Injection of *Raldh2* mRNA, which on its own results in the characteristic phenotypes (Fig. 2G) (88%, *n*=25), into EtOH-treated embryos partially rescues the alcohol-induced malformations (Fig. 2H) (80.6%, *n*=36). These results support the hypothesis that EtOH metabolites compete with ROL metabolites for RALDH2 activity, reducing its availability for RA biosynthesis, and therefore supplementation of the embryo with additional RALDH2 has a rescuing effect.

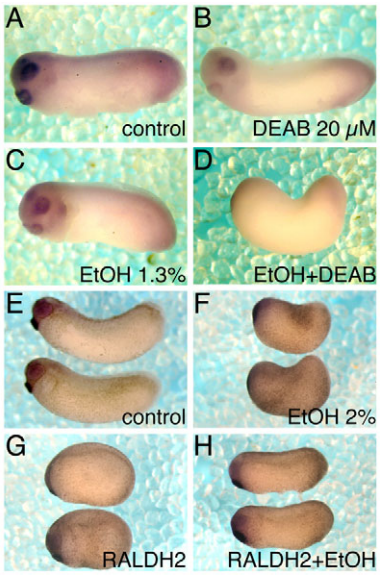

Fig. 2. Manipulation of ALDH activity modulates the EtOH-induced phenotype. Embryos were subjected to different manipulations of ALDH activity in combination with EtOH treatment. Embryos were allowed to develop to early tailbud stages (st. 29) and were analyzed further by in situ hybridization with the *XAG1* (cement gland) and *Pax6* (eyes and midbrain) probes. (A) Control embryo; at the early tailbud stage, the developing cement gland, eyes and midbrain are clearly discernible as the stained areas to the left of the image. (B) Partial ALDH inhibition with 20 μM DEAB. (C) Low EtOH (1.3%) treatment. (D) Combined EtOH and DEAB treatment. (E) Control embryos. (F) Embryos treated with 2% EtOH. (G) Embryos overexpressing *Raldh2*. (H) Rescue of the EtOH effect by overexpression of *Raldh2*.

RALDH2 rescues the EtOH-induced reduction in RA signaling

To determine more directly the changes in RA levels in the embryo that result from partial RALDH inhibition and exposure to weak EtOH exposure, we turned to the RA reporter plasmid system RAREhsplacZ (RAREZ) (Rossant et al., 1991). We have shown previously that this RA reporter is functional in Xenopus embryos and serves to monitor efficiently and quantify relative RA signaling levels using chemiluminiscent β-galactosidase substrates (Yelin et al., 2005). Embryos injected with RAREZ were subjected to DEAB, EtOH or combined treatment (Fig. 3A). Whereas the individual DEAB (20 μM) and EtOH (1.3%) treatments reduced the activity of the reporter plasmid by about 30-40%, their combined exposure blocked the activity of the reporter plasmid by over 60%, which is comparable to treatment with higher EtOH concentrations (2%), corroborating the phenotypic results (Fig. 3A). Further confirmation that EtOH competes for RALDH activity, and not for ADH, in Xenopus embryos was obtained by repeating the sensitization experiment with the ADH inhibitor 4-MP (Fig. 3C). In these embryos, we showed again that partial inhibition of RALDH activity predisposes the embryo to EtOH exposure and strongly reduces the level of RA signaling. In contrast, inhibition of ADH activity (4-MP) has no significant enhancing effect when compared with the EtOH-only treatment (Fig. 3C). These results

again show that, in Xenopus gastrula-stage embryos, RALDH activity is in limiting amounts and, as the embryo has high levels of ADH or RAL (which are not affected by the EtOH exposure), EtOH efficiently competes for RALDH.

To demonstrate further that the observed phenotypic rescue is mediated through changes in RA signaling levels, we performed the *Raldh2* rescue experiment but in this instance the effect on RA signaling was determined during the gastrula stages by using the RA reporter plasmid. Overexpression of *Raldh2* promoted higher RA signaling levels as the extra enzymatic activity metabolized the available RAL stores in the embryo (Fig. 3A), whereas treatment with 2% EtOH strongly reduced RA signaling (~60% inhibition) in the embryo (Fig. 3A). In agreement with the phenotypic analysis, *Raldh2* overexpression reversed the EtOH-induced negative effect on RA signaling (Fig. 3A). These results clearly show that the EtOH-dependent reduction in RA signaling can be rescued by *Raldh2* overexpression by providing a high enough enzyme concentration for both EtOH detoxification and RA biosynthesis. Overexpression of *Aldh1* has been shown to promote premature activation of RA signaling in the embryo by oxidizing the available RAL (Ang and Duester, 1999b). This observation suggested that ALDH1 could fulfill the enzymatic function of RALDH2 in the early embryo. For this reason, the rescue experiment was repeated with overexpression of *Aldh1* instead of *Raldh2*. As expected, *Aldh1* overexpression was also able to increase the level of RA signaling from that reached as a result of the EtOH treatment (supplementary material Fig. S2).

Further corroborating evidence of the increased sensitivity to EtOH induced by partial block of RALDH activity was achieved by studying the expression level of the RA-responsive genes *Hoxb1* and *Hoxb4* during the gastrula stages. Here, the results show that both DEAB (30 μM) and EtOH (1.5%) treatments induce a reduction in both *Hoxb1* and *Hoxb4* expression levels concurrently, whereas the combined treatment affected transcription more severely (Fig. 3B), suggesting an additive effect of both treatments on the RA signaling level. We also studied *Hoxb1* and *Hoxb4* expression during gastrulation in a reversed situation, where we attempted to rescue the effects of EtOH treatment through *Raldh2* overexpression. Treatment with EtOH (1.7%) strongly downregulated the expression of both *Hox* genes (Fig. 3B), whereas *Raldh2* overexpression upregulated both genes (Fig. 3B) and reversed the effects of EtOH, in agreement with the expected changes in RA levels. These observations further confirm the premise that EtOH promotes low RA levels, a condition further worsened by the addition of DEAB, which in turn can be rescued by higher RALDH2 levels.

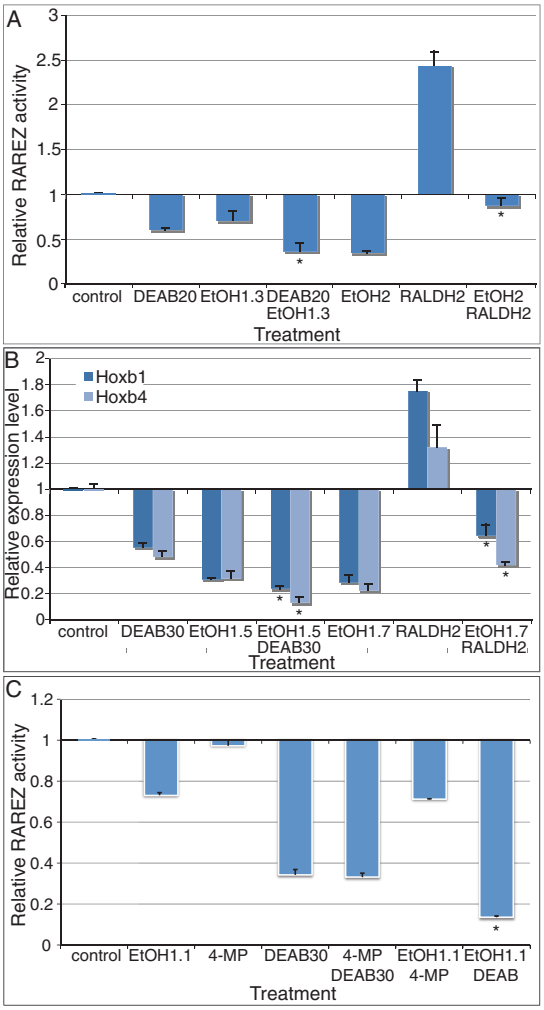

Fig. 3. ALDH manipulation modulates the EtOH effect on RA signaling and *Hox* expression. To demonstrate that ALDH inhibition sensitizes the embryo to EtOH exposure, embryos were treated with DEAB to inhibit ALDH activity or low EtOH concentrations, or a combination of both. In parallel, to demonstrate that the addition of RALDH rescues the EtOH effect, embryos were treated with high EtOH concentrations or injected with *Raldh2* mRNA, or both. (A) Treated embryos were analyzed for the effects on the level of RA signaling by taking advantage of the RA reporter plasmid RAREZ. (B) The effects of the different forms of manipulation on the expression of the RA-responsive *Hox* genes were determined by qPCR. (C) In addition, embryos were treated with 4-MP and low EtOH concentrations to further show that partial inhibition of ADH activity is unable to sensitize embryos to EtOH. **P*<0.01 when compared with the relevant EtOH-only sample.

RA signaling is important for normal gene expression in the organizer and during gastrula

The results show that EtOH has detrimental effects on RA signaling levels and *Hox* expression during the gastrula stages. In agreement, the effect of EtOH on Spemann's organizer, which was identified previously as an early target of EtOH, resulted in upregulation/expansion of organizer-specific gene expression and downregulation of the cytochrome P450 gene *Cyp26A1* during gastrulation (Yelin et al., 2005; Yelin et al., 2007). Therefore, we studied the changes in organizer-specific gene expression following suboptimal EtOH (1.5%) and DEAB (30 μM) treatments. In the case of the organizer-specific genes *Otx2* and *gsc*, EtOH treatment in the presence of partial inhibition of RALDH activity resulted in higher transcript levels than from the individual treatments (Fig. 4A). Analysis of the RA-sensitive gene *Cyp26A1* in embryos treated with EtOH and DEAB showed a stronger downregulation of the gene transcripts compared with that observed with the individual treatments (Fig. 4A). A similar analysis was performed by inducing a reduction in RA levels through the overexpression of the RA hydroxylase *Cyp26A1* (Hollemann et al., 1998; Dobbs-McAuliffe et al., 2004). The combination of EtOH treatment with *Cyp26A1*

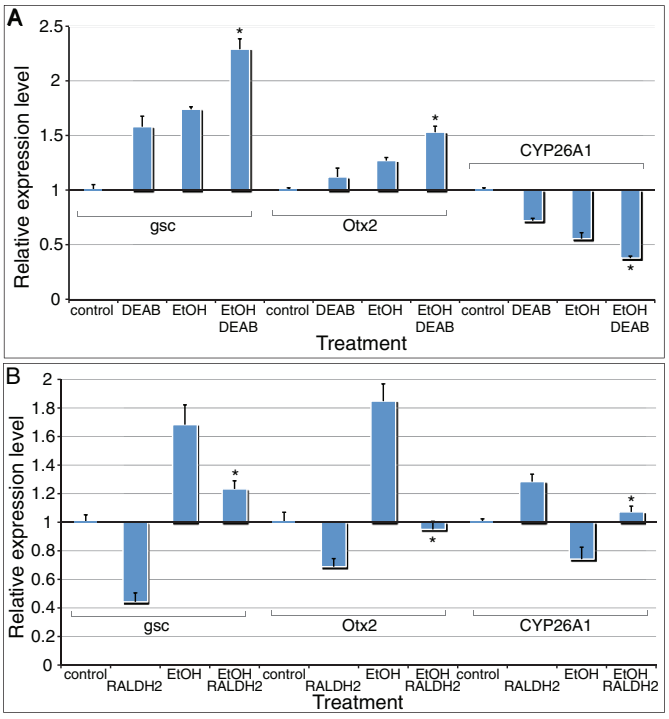

Fig. 4. Organizer-specific gene expression is affected by EtOH treatment and is modulated by RALDH manipulation. The effects of RALDH loss-of-function induced by DEAB-mediated inhibition (A) or RALDH gain-of-function achieved by *Raldh2* mRNA injection (B) were tested in the presence of EtOH (1.5%). To demonstrate the sensitization to EtOH treatment (A) and the rescue of the EtOH effects (B), the expression levels of the early RA-responsive genes *gsc*, *Otx2* and *Cyp26A1* were determined by qPCR. **P*<0.01 when compared with the relevant EtOH-only sample.

RNA injection resulted in very similar effects on *gsc*, *Otx2* and *chordin* expression in the embryo (supplementary material Fig. S3). As expected from the experiments involving DEAB and EtOH treatment, the combination of *Cyp26A1* overexpression and EtOH resulted in stronger upregulation of the organizer-specific genes than from the individual treatments. These results further support the additive effect of EtOH treatment and RALDH inhibition in the reduction of RA levels. The results also suggest the presence of RA signaling during early gastrula, affecting the organizer.

The RALDH2 rescue experiment serves to demonstrate further that additional RALDH activity can counteract the teratogenic effects of EtOH. qPCR analysis of the organizer genes *gsc* and *Otx2* revealed that EtOH (1.5%) induces their previously observed upregulation, whereas *Raldh2* overexpression induces their downregulation – an expected outcome of high RA levels (Fig. 4B). In agreement with EtOH inducing a reduction in RA levels, RALDH2 can rescue the EtOH effect, probably by increasing the amount of RA to almost normal levels (Fig. 4B). In contrast to *gsc* and *Otx2*, which are negatively regulated by RA, *Cyp26A1* is positively regulated by this signaling pathway (Fig. 4B). *Raldh2* overexpression on its own led to an increase in *Cyp26A1* transcript levels, whereas in combination with EtOH exposure, *Raldh2* overexpression rescued the EtOH-induced downregulation of *Cyp26A1* expression (Fig. 4B).

The quantitative study of organizer-specific gene expression was expanded by using in situ hybridization to determine the spatial effects of manipulating the levels of RALDH activity. Embryos treated with EtOH (1.5%) and DEAB (20 µM) were used to study the changes in the *chordin*, *gsc* and *Cyp26A1* expression patterns. Partial inhibition of RALDH activity (with DEAB) resulted in weak expansion/upregulation of *chordin* expression (Fig. 5B) (75%, *n*=24), similar to the effect of EtOH (Fig. 5C) (90%, *n*=21). The combination of EtOH treatment with inhibition of RALDH activity resulted in stronger *chordin* upregulation/expansion than from the individual treatments (Fig. 5D) (82%, *n*=17). Similarly, analysis of the changes in *gsc* expression showed that the individual DEAB or EtOH treatments result in weak upregulation/expansion (Fig. 5F,G) (89%, *n*=28 and 83%, *n*=37, respectively), but EtOH treatment in the presence of RALDH inhibition resulted in stronger expansion/upregulation (Fig. 5H) (84%, *n*=31). We also determined the expression pattern of *Cyp26A1*, which is expressed in the marginal zone, and found that it is positively regulated by RA

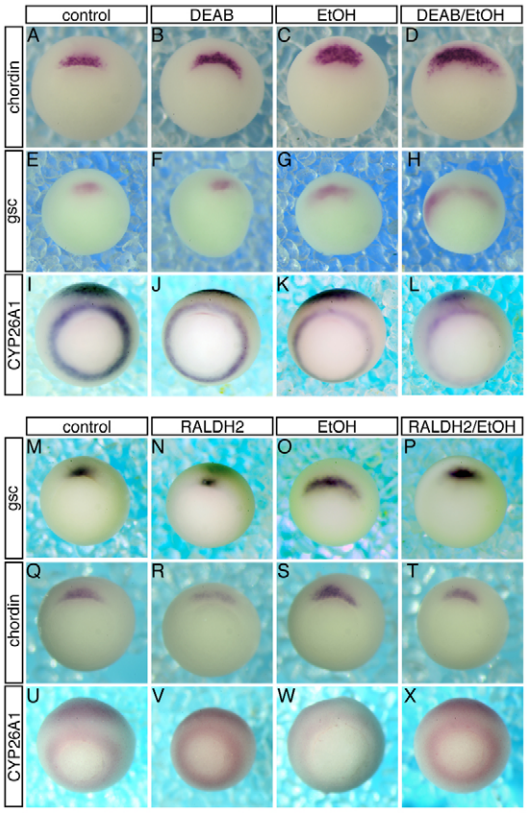

Fig. 5. Manipulation of RALDH modulates the effect of EtOH on gene expression domains during gastrulation. Sensitization to EtOH exposure by RALDH knockdown was shown by using DEAB to enhance the sensitivity to EtOH. Embryos were analyzed by in situ hybridization during early/mid gastrula stages (st. 10.5). Embryos were hybridized with the *chordin* (A-D,Q-T), *gsc* (E-H,M-P) and *Cyp26A1* (I-L,U-X) probes. (A,E,I) Control embryos. (B,F,J) DEAB-treated (20 µM) embryos. (C,G,K) Embryos exposed to EtOH (1.5%). (D,H,L) Combined treatment with DEAB and EtOH shows enhanced sensitivity to the EtOH exposure. Rescue of the EtOH effect was achieved by *Raldh2* overexpression. (M,Q,U) Control embryos. (N,R,V) Embryos injected with *Raldh2* mRNA. (O,S,W) EtOH-treated (1.5%) embryos. (P,T,X) *Raldh2* overexpression rescues the EtOH-induced effects.

(Hollemann et al., 1998). In agreement with this, DEAB or EtOH treatment of embryos resulted in reduced *Cyp26A1* expression (Fig. 5J,K) (96%, *n*=26 and 97%, *n*=41, respectively) whereas the combination of DEAB- and EtOH-induced RA reduction resulted in stronger downregulation of *Cyp26A1* expression (Fig. 5L) (96%, *n*=28). These results further support the suggestion that, by the early gastrula stages, partial inhibition of the RA biosynthetic activity predisposes the embryo to a stronger response to EtOH treatment. In turn, these conclusions strengthen the model in which EtOH competes for the RA biosynthetic enzymes, in particular RALDH2 activity.

The rescuing effect of RALDH2 on EtOH-treated embryos was also studied spatially by in situ hybridization. The negative regulatory effect of *Raldh2* overexpression on *gsc* and *chordin* expression could be observed as a downregulation/restriction of their expression domains (Fig. 5N,R) (83%, *n*=43 and 84%, *n*=32, respectively). By contrast, EtOH (1.5%) had a positive regulatory effect resulting in expansion/upregulation of their expression domain (Fig. 5O,S) (78% for *gsc*, *n*=42; 96% for *chordin*, *n*=28). In addition, *Raldh2* overexpression rescued the effect of EtOH on *gsc* and *chordin* expression, which were restored to an almost normal pattern (Fig. 5P,T) (91%, *n*=35 and 80%, *n*=20, respectively). Further analysis showed that *Raldh2* overexpression increased the expression of the positively regulated RA target *Cyp26A1* (Fig. 5V) (93%, *n*=78), whereas EtOH treatment reduced it (Fig. 5W) (79% *n*=44). As shown for the organizer-specific genes, RALDH2 was also able to rescue the effect of EtOH on *Cyp26A1* expression (Fig. 5X) (86%, *n*=56). Altogether, these results support the hypothesis where, as a result of EtOH treatment and its detoxification, the availability of RALDH2 for RA biosynthesis is restricted and experimentally increasing the level of available enzyme is enough to rescue the EtOH-induced early developmental defects.

EtOH targets RALDH2 during early gastrula stages

Embryonic sensitization to EtOH and the rescue of its effects by manipulation of the available RALDH activity levels demonstrated further that this enzymatic activity is involved in EtOH-dependent teratogenesis. Our analysis suggests that this activity is present in limiting amounts during the onset of gastrulation and that the EtOH metabolite acetaldehyde probably competes for it. During the stages when EtOH has the strongest effect on embryogenesis, that is late blastula/early gastrula (Yelin et al., 2005), RALDH2 has been shown to be central for the biosynthesis of RA and embryonic viability (Niederreither et al., 1999; Chen et al., 2001). These observations suggest that RALDH2 itself might be the early target of EtOH exposure. To conclusively identify whether the endogenous activity that EtOH competes for is that of RALDH2, we designed an antisense morpholino oligonucleotide directed against *Raldh2* (R2MO) (Heasman, 2002). To test the efficiency and specificity of the R2MO, we designed a myc-tagged version of RALDH2. RNA encoding the RALDH2-myc protein was injected together with increasing amounts of the R2MO. Western blot analysis of the injected embryos revealed that the R2MO efficiently inhibits the translation of the injected RNA (by 50-65%) (Fig. 6A). The inhibition of endogenous *Raldh2* translation can be inferred from the strong reduction in RA signaling that results from the co-injection of the RA-reporter plasmid RAREZ (Fig. 6B). The reduction in RA signaling can be rescued by co-injection with RNA that encodes mouse RALDH2 (mRALDH2) (Haselbeck et al., 1999). The *mRaldh2* RNA is not affected by the R2MO designed against the Xenopus sequence and therefore can restore RA signaling in the embryo (Fig. 6B).

To demonstrate that EtOH specifically targets RALDH2, we took advantage of the R2MO. If EtOH affects numerous enzymatic activities, among them RALDH2, then the R2MO-mediated effects should represent only a subset of the EtOH-induced defects. Alternatively, if RALDH2 is the main target of EtOH, then the R2MO should perform most of the RA-inhibitory function and the addition of EtOH should have almost no additive effect, as most RALDH2 activity should have been inhibited by the morpholino oligonucleotide. This analysis was performed by quantitative determination of RA signaling levels taking advantage of the RA reporter plasmid. EtOH treatment (1.3% or 1.7%) of RAREZ-injected embryos resulted in about 20-50% inhibition of

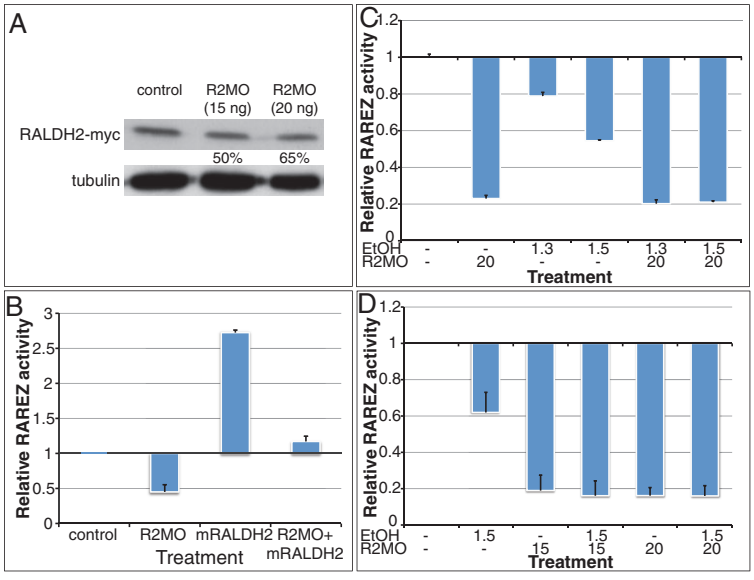

Fig. 6. EtOH targets the RALDH2 activity early during gastrulation. The RALDH2 activity was specifically knocked-down with an antisense morpholino oligonucleotide (R2MO). (A) To show the inhibitory effect of the R2MO on RALDH2 translation a myc-tagged version of this protein was constructed, RALDH2-myc. Capped RNA encoding the RALDH2-myc protein was injected with increasing amounts of R2MO. During mid-gastrula stages, the expression of the protein was determined by immunodetection with the 9E10 monoclonal antibody against myc. (B) The effect of R2MO on RA signaling was determined by co-injection of the RA reporter plasmid, RAREZ. The effect of R2MO on the RALDH2 activity was rescued by co-injection of mRNA encoding the mouse *Raldh2* sequences. (C) Embryos were co-injected with R2MO to knock-down the RALDH2 activity, and RAREZ to monitor the level of RA signaling. Treatment with increasing EtOH amounts did not further reduce the level of RA signaling. (D) Embryos injected with RAREZ and treated with EtOH (1.5%) were co-injected with increasing amounts of R2MO to show that at lower levels of RALDH2 activity inhibition, the inhibitory effect of EtOH can be observed.

RA signaling (Fig. 6C). Embryos injected with the R2MO exhibited about 80% inhibition of RAREZ activity (Fig. 6C). Alcohol treatment of R2MO-injected embryos also resulted in about 80% inhibition of RA signaling, irrespective of the amount of EtOH employed, which was in agreement with RALDH2 being the main enzyme inhibited by EtOH (Fig. 6C). Reducing the amount of R2MO from 20 ng/embryo to 15 ng/embryo caused a slight EtOH-induced reduction (~15%) in RA signaling (Fig. 6D). The weak effect of EtOH at lower R2MO concentrations suggests that, as the efficiency of RALDH2 knockdown decreases, the EtOH target becomes available. These results suggest that the R2MO blocks the appearance of the EtOH target, thus preventing further inhibition of RA signaling by alcohol when the treatments are combined.

DISCUSSION

EtOH as a competitor for vitamin A (ROL) metabolism

One of the models proposed to explain the teratogenic effects of EtOH in FASD is the competition between EtOH and ROL for the ADH and RALDH activities that are necessary for RA biosynthesis in the embryo (Duester, 1991; Deltour et al., 1996; Yelin et al., 2005). According to this model, as a result of the competition between EtOH and ROL, embryogenesis continues in the presence of abnormally low levels of RA signaling. Various vertebrate experimental systems have been developed to demonstrate that abnormally low RA signaling levels during embryogenesis result in developmental malformations (Clagett-Dame and DeLuca, 2002). Some experimental systems have tested restrictions in the amount of vitamin A in the diet, resulting in the vitamin A deficiency syndrome (VAD) (Zile, 2001; Clagett-Dame and DeLuca, 2002; Cui et al., 2003). VAD embryos develop a complicated developmental phenotype, including multiple malformations, some of which are also seen in individuals with FASD. Activation of the RA signaling pathway during vertebrate gastrulation has been shown to rely on the early expression of *Raldh2*, which provides the enzymatic activity that is necessary for RA biosynthesis (Ang and Duester, 1999b; Haselbeck et al., 1999). In agreement with the competition model centering on RALDH activity, mutation of the *Raldh2* gene results in embryonic lethality and developmental defects (Niederreither et al., 1999; Begemann et al., 2001) that are reminiscent of the phenotypes observed in individuals with FASD (Chaudhuri, 2000). The velocardiofacial/DiGeorge syndrome (VCF/DGS), which exhibits developmental malformations observed in FASD, has been suggested to be the result of reduced RA signaling (Vermot et al., 2003). The phenotypic similarity between FASD, VAD, VCF/DGS and the *Raldh2* mutation, and the known or suspected origin of these syndromes, supports the suggestion that reduced RA signaling might be the source of the developmental malformations observed in FASD.

Better support for the EtOH-RA competition model has emerged from studies in Xenopus embryos (Yelin et al., 2005). Taking advantage of either an RA reporter plasmid assay to monitor RA levels or expression analysis of known RA-regulated genes, we were able to detect active RA signaling as early as the onset of gastrulation. Furthermore, in rescue experiments, we showed that combined EtOH and ROL treatment, which on their own are strongly teratogenic, results in embryos that are almost normal (Yelin et al., 2005). These observations demonstrate that high ROL levels can rescue the developmental malformations induced by EtOH, suggesting further that they might be competing for the same enzymatic activities.

RA biosynthesis and EtOH detoxification rely on the same enzymatic activities. Therefore, it was important to determine whether the competition focuses on either the ADHs or RALDHs, or both. Taking advantage of the known inhibitors of both enzymatic activities, we demonstrated that the ADH inhibitor 4-MP had a minimal effect on the expression of known RA target genes, whereas the RALDH inhibitor DEAB had the expected negative effect on both *Hoxb1* and *Hoxb4* expression. An experimental increase of ALDH activity by *Aldh1* overexpression resulted in the predicted upregulation of the same *Hox* genes. Therefore, changes in the level of ALDH activity present in early gastrula embryos result in abnormal RA-dependent gene expression, suggesting that this enzymatic activity acts as a limiting factor at this stage. Alternatively, ADH activity and its oxidation product (RAL) are apparently present in excess in Xenopus embryos and manipulating them has a limited effect. Previous analysis of the RA biosynthetic pathway components in Xenopus embryos revealed that the RA precursors ROL and RAL could be detected readily before gastrulation, probably originating from maternal contributions (Creech Kraft et al., 1994; Costaridis et al., 1996). As described here, the maternal contribution of RAL is supported further by the possibility of inducing premature activation of RA signaling by *Aldh1* and *Raldh2* overexpression (Ang and Duester, 1999b). Apparently, as the Xenopus embryo reaches gastrulation, it has stores of the RA precursors and requires one last enzyme, namely RALDH2, to bring about the synthesis of RA and the activation of this signaling pathway. The appearance of the RALDH biosynthetic activity coincides with the onset of RA signaling, as determined from the analysis of *Raldh2* mutants (Niederreither et al., 1999; Begemann et al., 2001; Chen et al., 2001).

EtOH targets RALDH activity in the gastrula embryo

RA biosynthesis from ROL requires the activity of an ADH or a short-chain dehydrogenase/reductase to produce RAL and, finally, the activity of a RALDH to produce RA. EtOH and its metabolites compete with ROL in both enzymatic steps. The gain- and loss-of-function experiments described above clearly suggest that the RALDH activity might be targeted by the presence of EtOH because the availability of these enzymes is limited in the embryo at the onset of gastrulation (Ang and Duester, 1999b; Niederreither et al., 1999). In a series of experiments using the RALDH inhibitor DEAB, we showed an enhanced detrimental effect of EtOH on RA signaling. The outcome from partial inhibition of RALDH activity was a general enhancement of all EtOH-mediated defects, resulting in more severe developmental phenotypes. The link to RA signaling was strengthened further by using the RA reporter plasmid and expression analysis of RA-responsive genes to determine the effect on RA signaling levels. Exposure to low EtOH levels coupled with partial inhibition of RALDH activity had a strong inhibitory effect on the expression of the RA-responsive *Hox* genes. Furthermore, the expression of the RA reporter plasmid was reduced further by the combined suboptimal treatments (EtOH and DEAB) when compared with the individual treatments. Therefore, partial inhibition of RALDH2 sensitizes the embryo to exposure to low

EtOH concentrations, phenocopying the effects of exposure to higher concentrations of EtOH. The similarity between the malformations induced by a high EtOH concentration and those induced by combined treatment with a low EtOH concentration and DEAB suggests that both treatments induce similar inhibition of RA signaling. The conclusion that pharmacological inhibition of RALDH activity can compensate, in part, for the effect of EtOH further supports the suggestion that acetaldehyde competes with RAL for the available RALDH. Our observations – together with the phenotypic similarity between EtOH-induced malformations, mutations in RA biosynthetic enzymes (Niederreither et al., 1999; Begemann et al., 2001; Grandel et al., 2002) and certain developmental syndromes connected to abnormal RA levels (Clagett-Dame and DeLuca, 2002; Cui et al., 2003) – clearly demonstrate the link between EtOH and the RA signaling pathway during embryogenesis.

The partial inhibition experiments support the role of EtOH as a competitive inhibitor of RA biosynthesis in vivo during embryogenesis. These results confirm that the developmental malformations observed in FASD could stem from embryonic development in the presence of abnormally low RA concentrations, particularly during gastrulation. The competitive inhibition model predicts that the supplementation of additional, exogenous RALDH should be able to reduce the effect of EtOH on RA biosynthesis. The effects of *Raldh2* overexpression, in conjunction with EtOH treatment, were analyzed by different assays to demonstrate that it does indeed have a rescuing effect in the context of EtOH exposure. The expression of the RA-responsive *Hox* genes was partially rescued by the addition of the extra RALDH2 activity. In a similar fashion, RALDH2 restored the RA reporter plasmid activity to almost normal levels. The choice of RALDH2 for the overexpression experiments was based on the observations that the appearance of this enzyme marks the onset of RA signaling during gastrulation and it is the enzyme that is normally affected by EtOH at these stages (Niederreither et al., 1999; Begemann et al., 2001; Chen et al., 2001; Grandel et al., 2002). *Aldh1* overexpression has also been shown to induce premature RA signaling in Xenopus embryos (Ang and Duester, 1999b). However, unlike the *Raldh2* gene which is expressed from early gastrula stages, *Aldh1* expression is only detected during late embryonic stages (Ang and Duester, 1999a; Chen et al., 2001). Recently, the retinol dehydrogenase 10 (*Rdh10*) gene that encodes a member of the short-chain dehydrogenase/reductase family has been studied in detail. *Rdh10* expression begins during the headfold stage [embryonic day (E) 7.5] but it is not expressed in the node and primitive streak of the mouse embryo (Cammas et al., 2007). *Rdh10* was found to be mutated in *trex* mice (Sandell et al., 2007). In contrast to *Raldh2* mutant mice that die before E10.5, *trex* mice die at E13 (Niederreither et al., 1999). Although RDH10 activity is necessary for normal embryogenesis, the stronger phenotype of *Raldh2* mutant mice and the earlier lethality of this mutation suggest that RALDH2 activity might be required earlier during mouse embryogenesis. An alternative explanation for the different phenotypes raises the possibility that *Raldh2* might have a more widespread function in RA biosynthesis in post-implantation mouse embryos (Sandell et al., 2007). These observations suggest that RALDH activity might also be a target of EtOH during mouse embryogenesis.

EtOH affects RA biosynthesis primarily during early developmental stages

We have previously shown that the strongest EtOH-induced malformations were observed when embryos were exposed from the late-blastula to mid-gastrula stages of development (Yelin et al., 2005). In the same study, we showed that organizer-specific, RA-regulated genes were also affected by the EtOH treatment. By using the RA reporter plasmid in transgenic embryos, we were able to detect that the RA signaling pathway was strongly active in the embryonic organizer. Many of the assays employed to demonstrate EtOH-RA competition, RAREZ activity, organizer-specific gene expression and *Hox* transcription, were all performed during the early gastrula stages. The RA reporter plasmid changed its transcriptional activity in accordance with the expected changes in RA signaling levels, such that, in embryos treated with a combination of DEAB and a low EtOH concentration, reporter expression decreased to a level similar to that in embryos subjected to high EtOH concentrations; furthermore, *Raldh2* overexpression reversed the effects of EtOH. The assays were performed in early-gastrula stage embryos indicating that the EtOH-dependent inhibitory competition of RA biosynthesis is already present at this stage of development.

The molecular effects of EtOH exposure on organizer-specific gene expression have been described previously (Yelin et al., 2005; Yelin et al., 2007). As a result of EtOH exposure, we observed an upregulation/expansion in the expression pattern of all the organizer-specific, negatively regulated RA target genes studied. In the present study, we also show that the organizer-specific genes exhibit the expected expansion/upregulation when partial inhibition of RALDH activity (by DEAB) is used to compensate for the use of low EtOH concentrations. Determining the expression levels of organizer-specific genes by qPCR further corroborated these observations. The normal expression of the organizer genes was also rescued by *Raldh2* overexpression in EtOH-treated embryos. The changes in organizer-specific gene expression that result from the combination of EtOH treatment and RA manipulation are in complete agreement with the end result being an overall manipulation of RA signaling. This observation suggests that, in addition to the direct affects of DEAB treatment or *Raldh2* overexpression on RA biosynthesis, EtOH also promotes changes in RA signaling levels.

According to the competitive inhibition model, RA signaling and the processes under its regulation should be more susceptible to manipulation or changes when the concentration of RA is low; further, the maintenance or increase of RA signaling levels are strongly dependent on RA biosynthesis. When these conditions are fulfilled, slight changes in RA signaling levels will result in strong developmental defects. The RA signaling molecule has been detected in the organizer of vertebrate embryos during gastrulation (Chen et al., 1992; Chen et al., 1994). Furthermore, with the aid of a reporter plasmid, an active RA signaling pathway was observed in Spemann's organizer in Xenopus (Yelin et al., 2005). A number of experimental systems have shown that the strongest EtOH-induced developmental malformations are obtained when embryos are exposed to EtOH during the late blastula and early gastrula stages, which is concomitant with the onset of RA signaling (Nakatsuji, 1983; Blader and Strahle, 1998; Yelin et al., 2005) (M. Gur and A.F., unpublished). The observation that late blastula/early

gastrula represents the stage with the highest EtOH sensitivity, and overlaps with the onset of RA signaling, suggests that EtOH has a greater effect when RA concentrations in the embryo are low.

The onset of gastrulation marks the initiation of axis formation and patterning (Harland and Gerhart, 1997). During this stage, the embryonic organizer provides regulatory signals that are important for the establishment and patterning of the early embryonic axes. The localization of RA signaling to the organizer (Chen et al., 1992; Chen et al., 1994; Yelin et al., 2005) leads us to suggest that, even at this early developmental stage, RA might already be involved in the process of axis formation and patterning. The results described here linking RA signaling and EtOH teratogenicity also suggest that it is the early embryonic organizer that is sensitive to high EtOH concentrations. Therefore, EtOH would be affecting one of the signals originating from the embryonic structure that is involved in early axis formation in the embryo. By disturbing the organizer, EtOH affects the main center regulating normal embryogenesis and, as such, will have widespread effects that are reflected in the complex developmental phenotypes observed in individuals with FASD.

TRANSLATIONAL IMPACT

Clinical issue

In fetal alcohol spectrum disorder (FASD) a complex phenotype results from exposure of human embryos to alcohol (ethanol) during pregnancy. When extreme, FASD is also known as fetal alcohol syndrome (FAS) and is characterized by facial defects, including mid-facial malformations, reduced head circumference, short stature, central nervous system defects, and behavioral and psychological problems. It is thought that ethanol may compete with the biosynthesis of retinoic acid (RA), which is necessary for correct anterior/posterior patterning during development. RA is normally produced from vitamin A (retinol) by two oxidation steps. The first is mediated by alcohol dehydrogenase (ADH) and produces retinaldehyde, and the second is mediated by aldehyde dehydrogenase (ALDH) and produces RA. The same two enzymatic steps are required for the clearance of alcohol from the body through a process that produces acetaldehyde followed by acetic acid.

Results

This study uses Xenopus embryos as a model system to understand the influence of ethanol on the biosynthesis of RA during the gastrula stage of embryogenesis. The authors identify RA-synthesizing enzymes that are affected by competitive inhibition with alcohol. As seen with ALDH inhibition, ethanol exposure limited retinaldehyde dehydrogenase (RALDH) activity in frog embryos. Partial inhibition of RALDH activity caused an exaggerated phenotype in embryos exposed to low ethanol concentrations. RALDH2 appears in vertebrate embryos at the onset of gastrulation, when the embryo is most sensitive to ethanol exposure. Supplementation of the embryo with RALDH2 ameliorated the effects of high ethanol exposure during early gastrulation. This provides biochemical evidence that ethanol competes with the enzymes necessary for the normal synthesis of RA during gastrulation, which may result in the developmental abnormalities associated with FASD.

Implications and future directions

RA is important for the direction, differentiation and survival of cells during early embryogenesis. Abnormal changes in RA levels result in developmental malformations, and reduced RA signaling causes a phenotype similar to FAS. This work shows that alcohol competes for enzymes in the RA biosynthesis pathway, suggesting that many alcohol-induced developmental malformations are caused by continued embryonic development in the presence of abnormally low RA levels.

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METHODS

Embryo culture and treatments

Xenopus laevis frogs were purchased from Xenopus I or NASCO (Dexter, MI and Fort Atkinson, WI). Embryos were obtained by in vitro fertilization, incubated in 0.1% MBSH and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Ethanol (EtOH; 1-2% vol/vol), all-trans retinoic acid (RA; 1 μM; Sigma)/4-diethylaminobenzaldehyde (DEAB; 20-30 μM; Sigma-Aldrich) and 4-methylpyrazole (4-MP; 10 mM; Sigma) treatments were performed in 0.1% MBSH. Soon after mid-blastula transition (MBT, st. 8.5), the embryos were placed in the treatment solution and incubated at room temperature until the specified stages.

Constructs, RNA preparation and in situ hybridization

For microinjection of reporter plasmids or capped RNAs, 2- to 4-cell embryos were injected radially. Capped RNA was prepared by in vitro transcription using the Bio-RT kit (Bio-Lab, Jerusalem, Israel). A cap analog (Pharmacia) was added to the reaction mixture using a cap:GTP ratio of 5:1. The template for transcription of *Cyp26A1* mRNA was prepared from the full-length cDNA clone (Hollemann et al., 1998), the template for transcription of Xenopus *Raldh2* mRNA was prepared from pSC2-XRALDH2 (Chen et al., 2001), and the template for transcription of mouse *Raldh2* mRNA was prepared from pGEM-mRALDH2 (Haselbeck et al., 1999).

Xenopus RALDH2 knockdown was induced by injection of a specific antisense morpholino oligonucleotide (R2MO), 5′-CTATTTTACTGGAAGTCATGTCTGG-3′. To test the R2MO, a myc-tagged version of the RALDH2 protein was constructed by PCR amplification of the open reading frame using the primers 5′-TTGGATCCATGACTTCCAGTAAAATA-3′ and 5′-TGATCGATCGGAATTCTTCTGGGGAAT-3′ and subcloned into the *Cla*I and *Bam*HI sites of pCS2-MT.

Whole-mount in situ hybridization analysis of gene expression was performed as described previously (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), plasmid pXAG-1 for *XAG1* (Hemmati-Brivanlou et al., 1990) and pXpax-6-BSSK for *Pax6* (Li et al., 1997).

Quantitative real-time PCR (qPCR)

qPCR was performed using the ABI Prism 7000 cycler and the ABsolute QPCR SYBR green mixes (ABgene). All samples were processed in triplicate and analyzed as described previously (Livak and Schmittgen, 2001). All experiments were repeated at least three times. The primers used were: *gsc*, 5′-TTCACCGATGAACAACTGGA-3′, 5′-TTCCACTTTTGGGCATTTTC-3′; *Otx2*, 5′-AAGCCGCAATATAGAAAGGAACA-3′, 5′-GGGATTCCTTGTCGCAATTAATA-3′; *chordin*, 5′-ACTGCCAGGACTGGATGGT-3′, 5′-GGCAGGATTTAGAGTTGCTTC-3′; *GAPDH*, 5′-GCTCCTCTCGCAAAGGTCAT-3′, 5′-GGGCCATCCACTGTCTTCTG-3′; *Cyp26*, 5′-CGATTCCTCAAGGTTTGGCTTCA-3′, 5′-ATTTAGCGGGTAGGTTGTCCACA-3′; *Hoxb4*, 5′-ATCCAGGAGAAGCCTGTAGATAGA-3′, 5′-TGGCGTGAT-

TAAAGATGAAACATA-3′ and *Hoxb1*, 5′-TTGCCCCAGTGC-CAATGAC-3′, 5′-TCCCCCTCCAACAACAAACC-3′.

Determination of RA signaling levels

Embryos injected radially with the RAREhsplacZ plasmid (RAREZ) (Rossant et al., 1991) were processed for chemiluminiscent quantitation of reporter activity using the β-gal reporter gene assay (Roche). Each experiment was repeated a minimum of three times, which involved at least duplicate experiments in 10 embryos. Protein extracts and enzymatic reactions were performed according to the manufacturer's protocol. The levels of activity were measured on a TD-20/20 luminometer (Turner Designs).

Western blot analysis

Proteins (10-25 μg protein) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto an Immobilon-P Transfer membrane (Millipore, Bradford, PA). Western blot analysis was performed using anti-myc (9E10), goat anti-mouse POD (Jackson Laboratories) and anti-α-tubulin antibodies (Serotec, Oxford, UK) at concentrations of 1:200, 1:10,000 and 1:20,000, respectively.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

The project in its entirety was conceived, designed and performed by H.K.-L. and A.F. Both authors prepared the manuscript.

SUPPLEMENTARY MATERIAL

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