Impact of retinoic acid exposure on midfacial shape variation and manifestation of holoprosencephaly in *Twisted gastrulation* mutant mice

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**Running title:** Retinoic acid and holoprosencephaly

**Keywords:** Twisted gastrulation, Bone morphogenetic protein, holoprosencephaly, retinoic acid, apoptosis, oxidative stress.

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ABSTRACT

Holoprosencephaly (HPE) is a developmental anomaly characterized by inadequate or absent midline division of the embryonic forebrain and midline facial defects. It is believed that gene-environment interactions play a role in the widely variable penetrance and expressivity of HPE, although a direct investigation of such effects has been limited. The goal of this study was to examine if mice carrying a mutation in a gene encoding a BMP antagonist *Twisted gastrulation* (*Twsg1*) associated with a low penetrance of HPE are sensitized to retinoic acid (RA) teratogenesis. Pregnant *Twsg1*+/− dams were treated by gavage with a low dose of all-trans RA (3.75 mg/kg). Embryos were analyzed between E9.5 and E11.5 by microscopy and geometric morphometric analysis by microCT. P19 embryonal carcinoma cells were used to examine potential mechanisms mediating combined effects of increased BMP and retinoid signaling. While only 7% of wild type embryos exposed to RA showed overt HPE or neural tube defects (NTD), 100% of *Twsg1* null mutants exposed to RA manifested severe HPE compared to 17% without RA. Remarkably, up to 30% of *Twsg1*+/− mutants also showed HPE (23%) or NTD (7%). The majority of shape variation among *Twsg1*+/− mutants was associated with narrowing of the midface. In P19 cells, RA induced the expression of *Bmp2*, acted in concert with BMP to increase p53 expression, caspase activation, and oxidative stress. This study provides direct evidence for modifying effects of the environment in a genetic mouse model carrying a predisposing mutation for HPE in the *Twsg1* gene. Further study of the mechanisms underlying these gene-environment interactions in vivo will contribute to better understanding of the pathogenesis of birth defects and present an opportunity to explore potential preventive interventions.
INTRODUCTION

Holoprosencephaly (HPE) is a malformation characterized by inadequate or absent midline division of the embryonic forebrain. Incomplete brain septation is accompanied by corresponding midline facial defects in about 80% of the cases (Geng and Oliver, 2009) and, less frequently, jaw defects (Pauli et al., 1983). HPE is the most common defect of the developing forebrain with an incidence of 1 in 250 conceptuses and about 1 in every 10,000 at term (Orioli and Castilla, 2010; Roessler et al., 1996). An important feature of HPE is its incomplete penetrance and expressivity. Even in families with defined mutations some individuals may have no recognizable defects, some have mild forms (referred to as microforms, such as hypotelorism, midfacial hypoplasia, or a single maxillary central incisor), and some are severely affected with cyclopia or proboscis (Roessler et al., 1996). The basis of this phenotypic variability is poorly understood.

HPE can result from widely diverse causes, including both genetic and environmental etiologies. It has been speculated that genetic and environmental factors may have a cumulative effect, accounting for its varied penetrance and expressivity (Ming and Muenke, 2002). The most common genetic cause of HPE in humans are mutations in $SHH$ (Roessler et al., 1996). Some examples of environmental factors that have been associated with development of HPE in humans are ethyl alcohol, poorly controlled maternal diabetes mellitus, retinoic acid (RA) (Cohen and Shiota, 2002), and hypoxia-ischemia (Siebert, 2007). All of these exposures are associated with elevated levels of reactive oxygen species (ROS) (Aoto et al., 2008; Davis et al., 1990; Kay et al., 2000; Ornoy, 2007), suggesting a role for oxidative stress in mediating their teratogenic effects.
Experimental models of HPE to study these interactions are very limited because unlike humans, mice carrying classical HPE gene mutations do not usually show phenotypic variability. For example, disruption of SHH pathway in mice has profound effects on embryonic development with all Shh null embryos manifesting severe HPE (Chiang et al., 1996), while in humans only 37% of carriers of SHH mutations develop HPE (Cohen, 1989). Other, less classical mouse models of HPE, however, do show incomplete penetrance and phenotypic variability, making them potentially more amenable to environmental manipulation with a resultant shift in a phenotypic outcome. For example loss of Bone morphogenetic protein (BMP) antagonists, such as Chordin, Noggin, or Twisted gastrulation (TWSG1) leads to reduction in Shh expression in the ventral neural midline and recapitulates a spectrum of HPE phenotypes in mice (Anderson et al., 2002; Lana-Elola et al., 2011; Petryk et al., 2004). As with BMPs, exogenous RA can also lead to loss of Shh expression and HPE (Helms et al., 1997; Sulik et al., 1995). Although it is currently unknown whether mice with disrupted BMP signaling are more susceptible to RA teratogenic effects, there is evidence that both pathways can cooperate during development, for example during vertebrate limb outgrowth, by inducing interdigital apoptosis (Rodriguez-Leon et al., 1999).

The primary goals of this work were 1) to examine if a mutation in a gene encoding a BMP binding protein TWSG1 confers susceptibility to RA exposure, and 2) whether this effect can be quantified by microCT of the craniofacial region. We chose Twsg1 mouse model because of a relatively low baseline incidence of HPE and an increase in apoptosis as a mechanism of craniofacial defects in these mice (MacKenzie et al., 2009). A secondary goal was to examine potential underlying mechanisms using P19 cells as a validated in vitro model of BMP/RA.
interactions. We hypothesized that \textit{Twsg1}^{-/-} mice would be particularly sensitive to the subteratogenic effects of RA, the midface would be most significantly affected, and the effects of a combined treatment of P19 cells with BMP and RA would be mediated through upregulation of apoptosis.

\section*{RESULTS}

\textit{Twsg1}^{-/-} mice are sensitized to retinoic acid teratogenesis

Our first step in examining the sensitivity of TWSG1-deficient mice to RA was to establish an appropriate treatment dosage that would cause a low but observable incidence defects in WT mice, which could then be used to assess the sensitivity of TWSG1-deficient mice. Since a dose of ATRA of 7.5 mg/kg has been previously shown to cause significant HPE (Kotch et al., 1995), we also tested a lower dose of 3.75 mg/kg. Treatment with 7.5 mg/kg of ATRA was overwhelmingly teratogenic and led to 94\% of WT embryos showing defects with about 2/3 of the embryos showing HPE and 1/3 with a neural tube defect (NTD) (Table 1). However, treatment with 3.75 mg/kg of ATRA led to 7\% of embryos affected with a vast majority of these affected embryos showing HPE (Table 1). Therefore, we selected 3.75 mg/kg of ATRA (referred to as low dose ATRA) as our dose for future experiments with \textit{Twsg1}\textsuperscript{+/+} and \textit{Twsg1}^{-/-} mice.

While only 7\% of WT embryos exposed to low dose ATRA showed overt HPE or NTD, 100\% of \textit{Twsg1} null mutants manifested HPE compared to 17\% without exposure to ATRA (Table 1, Fig. 1, \textit{p}=6x10^{-12}). This rate of defects is far more than what would be expected based solely on adding the prevalence of defects from this dose in WT mice and untreated \textit{Twsg1}^{-/-}
mice. Remarkably, even 30% of heterozygous TwsgI mutants, which are phenotypically normal without ATRA exposure, showed neural defects (predominantly HPE). This represents a statistically significant increase over the incidence seen in wild type embryos with the same dosage (p=0.01) and likewise over the 0% incidence seen in untreated heterozygotes (p=1x10^{-6}). Thus, TWSG1 deficiency increased the teratogenic effect of low dose ATRA in both the homozygous and heterozygous states.

**Geometric morphometric (GM) analysis of facial shape of TwsgI+/- mice demonstrates a continuum of midfacial dysmorphology after exposure to a low dose of ATRA**

While TwsgI-/- embryos exhibited severe HPE phenotypes (cyclopia or proboscis) after in utero exposure to a low dose ATRA, TwsgI+/- showed a range of defects of variable severity. To quantify these defects, GM was employed. The analysis included only TwsgI+/- embryos because severe HPE phenotypes in homozygotes precluded landmark assignment. PC1, which reflects narrowing of the midface, was the only PC that discriminated between treatment groups and accounted for 49% of the total variance. As shown in Fig. 2, while untreated TwsgI+/- embryos clustered with wild type (WT) embryos, those that were affected by low dose ATRA treatment could be clearly discriminated along PC1. Thus, ATRA treatment resulted in a continuum of midfacial narrowing in mice heterozygous for TwsgI mutation.

**ATRA induces the expression of RA-responsive genes in P19 cells**

To test potential mechanisms underlying the acute sensitivity of TwsgI mutant mice to ATRA, we selected P19 mouse embryonal carcinoma cells as an experimental system because they resemble embryonic cells, represent a homogenous cell population that is amenable to
quantitative assays, and have been used by others as a model for BMP/retinoid signaling interactions (Fujita et al., 1999; Glozak and Rogers, 1996; Glozak and Rogers, 1998). P19 cells have been previously reported to be sensitive to retinoids (Xi and Yang, 2008). We were able to confirm this sensitivity by observing the transcriptional induction of several known RA target genes after 1 µM ATRA treatment, including RA receptors alpha and beta (Balmer and Blomhoff, 2002; Sucov et al., 1990), RA hydrolase Cyp26a1 (Loudig et al., 2000), Crbp1 (Xu et al., 2001), and Hox transcription factors HoxA1 and HoxB1 (Balmer and Blomhoff, 2002; Dekker et al., 1993; Dupe et al., 1997) (Fig. 3).

**ATRA upregulates the expression of Bmp2 and its downstream targets in P19 cells**

Since TWSG1’s only known mode of action is through regulation of BMP signaling, it was essential that the P19s be competent to respond to BMPs to mimic what occurs *in vivo*. Although there is some evidence that TWSG1 can promote BMP activity in some species, in mice it appears to act mostly as a BMP antagonist (Larrain et al., 2001; Nosaka et al., 2003; Oelgeschlager et al., 2003; Petryk et al., 2005; Ross et al., 2001; Sotillo Rodriguez et al., 2009; Wills et al., 2006). We examined the expression of several BMP pathway genes and BMP targets after BMP treatment alone, after ATRA treatment alone and after combined treatment (Fig. 4). We found, consistent with previous reports (Heller et al., 1999), that *Bmp2* was upregulated in response to ATRA. The BMP targets *Msx1* and *Msx2* (Davidson, 1995; Liu et al., 2005; Vainio et al., 1993) showed significant induction by BMP2 alone. With ATRA alone, *Msx1* was not induced, while *Msx2* increased 6.2-fold compared to the control group, although not sufficiently to test as statistically significant. However, when BMP and ATRA treatments were combined,
both Msx1 and Msx2 showed dramatically higher induction than with BMP alone (about 2-fold increase for Msx1 and 5-fold increase for Msx2 compared to BMP alone).

The p53 pathway is activated in P19 cells treated with BMP and RA together

The upregulation of the p53 target Trp53inp1 (Tomasini et al., 2003) is indicative of increased p53 transcriptional activity in the cell and activation of the p53 pathway. Following treatment with BMP2, the expression of Trp53inp1 was not significantly changed (Fig. 4). ATRA, however, significantly increased Trp53inp1 expression. In response to a combined treatment with ATRA and BMP2, significantly more expression was observed beyond even that seen with ATRA alone.

RA acts in concert with BMP to increase caspase 3/7 activation in P19 cells

Combined BMP2 and ATRA treatment has been previously shown to induce apoptosis in P19 cells as indicated by assessment of DNA fragmentation using cell sorting or direct electrophoresis; or by microscopic examination of cells for condensed chromatin (Fujita et al., 1999; Glozak and Rogers, 1996; Glozak and Rogers, 1998). We have been able to corroborate this finding by examining caspase 3/7 activation. Treatment of P19 cells with BMP2 and ATRA resulted in a significant increase in the activity of a mediator of apoptosis caspase 3/7 compared to control, BMP2 or ATRA alone (Fig. 5). To examine if oxidative stress can have similar effects, P19 cells were treated with the complex III electron transport inhibitor and inducer of oxidative stress, antimycin A (AMA) (Garcia-Ruiz et al., 1995; Turrens et al., 1985). AMA treatment alone also significantly increased caspase activation, supporting the link between ROS and apoptosis.
**Oxidative stress is increased by retinoic acid treatment in P19 cells**

The antioxidant GSH provides the main cellular defense against oxidative damage and can be depleted and converted to the oxidized form GSSG in conditions of oxidative stress. Hence, GSH/GSSG ratio provides a reliable indicator of the oxidative status of a cell. As expected, treatment of P19 cells with a prooxidant AMA resulted in a significant decrease in GSH/GSSG ratio (Fig. 6). Cells that have been treated with BMP2 alone or low dose ATRA alone did not show any significant changes in GSH/GSSG ratio. On the other hand, a combined BMP2/RA treatment resulted in a markedly lower GSH/GSSG ratio. This result indicates that RA along with BMP2 can induce oxidative stress in this cellular model of early embryonic development.

**DISCUSSION**

The severity of craniofacial abnormalities can vary widely between individuals, despite similar or identical genetic risk factors and environmental exposures. HPE is a prominent example of such phenotypic variability. Several mechanisms have been proposed to explain this phenomenon, such as interaction of two or more HPE genes in generating the phenotype (Nanni et al., 1999), cumulative effects of mutations in non-classical HPE genes that result in concurrent or sequential partial defects in more than one pathway important for forebrain development (Andersson et al., 2006; Ming et al., 2002), presence of genetic modifiers (Nadeau, 2001), stochastic and/or epigenetic contributions (Feinberg and Irizarry, 2010) as well as non-linearities in the properties of signaling pathways (Young et al., 2010). The multifactorial etiology of HPE led to the “multiple hit” hypothesis (Ming et al., 2002), in which genetic predisposition puts individuals at risk for manifesting the disease in the presence of other exposures.
This study provides direct evidence for such modifying effects of the environment in a genetic mouse model carrying a predisposing mutation in Twsg1 gene. Importantly, even haploinsufficiency sensitized the Twsg1 mouse embryos to teratogenic effects of retinoic acid, resulting in HPE, similar to Shh or Gli2 haploinsufficiency predisposing to teratogenic effects of prenatal ethanol exposure (Kietzman et al., 2014). To quantify these effects, we used a 3D geometric morphometric analysis of craniofacial shape by microCT (Chong et al., 2012; Johnson et al., 2006; Nagase et al., 2008). We found that the majority of shape variation in Twsg1+/− mouse embryos with intrauterine exposure to a low dose of ATRA was associated with narrowing of the midface as seen in the human microforms (Roessler et al., 1996), Noggin null mice (Lana-Elola et al., 2011), and in a chick model of HPE (Marcucio et al., 2005; Young et al., 2010). Since the type of dysmorphology, the midfacial narrowing, is similar to that observed in untreated Twsg1−/− embryos (MacKenzie et al., 2009), we propose that RA treatment moves the phenotypes of heterozygotes toward a mutant phenotype.

The exact underlying mechanisms of this increased sensitivity of TWSG1-deficient embryos to a teratogen like RA are unknown. Since the only known action of TWSG1 is through binding BMPs in the extracellular space, and we have previously shown that BMP signaling is increased in the absence of TWSG1 (Ross et al., 2001; Sotillo Rodriguez et al., 2009), we speculate that BMP/RA interactions contribute to the enhanced expressivity of HPE in this mouse model. One potential mechanism of this synergy of BMP and RA lies in the observed induction of the BMP pathway members by RA. RA induces Bmp2 expression as shown both in this study and in previous research (Heller et al., 1999) and may in other settings also induce Bmp4 and Bmp7 (Rodriguez-Leon et al., 1999). The upregulation of Msx genes following a BMP
and ATRA treatment is particularly interesting since the expression of Msx2 has been linked to induction of apoptosis in the craniofacial region, including neural crest cells (Graham et al., 1994) and optic vesicles (Wu et al., 2003).

There is evidence that both BMP and RA pathways cooperate to induce apoptosis in vivo, for example during vertebrate limb outgrowth (Rodriguez-Leon et al., 1999), and in in vitro systems, including P19 cells as shown in this and other studies (Glozak and Rogers, 1996; Xi and Yang, 2008). We have previously shown that in Twsg1−/− embryos increased apoptosis correlates with the degree of severity of craniofacial phenotypes (MacKenzie et al., 2009). Any additional pro-apoptotic factor would be expected to enhance this dysmorphology. In fact, excessive apoptosis is a central common pathway in various craniofacial defects due to either exposure to external noxious agents such as alcohol (Aoto et al., 2008; Sulik, 2005), hypoxia (Smith et al., 2013) or gene mutations (Dixon et al., 2000; Jones et al., 2008; Phelan et al., 1997). This pathological apoptosis appears to be at least partly p53-mediated because genetic or pharmacological inhibition of p53 activation can significantly reduce the frequency of craniofacial defects in Twsg1−/− and other mouse models (Tcof, Pax3) of craniofacial and neural defects (Billington et al., 2011; Jones et al., 2008; Pani et al., 2002). Importantly, teratogens such as RA can also by themselves induce apoptosis in craniofacial primordia (Evrard et al., 2000) and activate p53 (Hosako et al., 2007). In this study, treatment of P19 cells with BMP and ATRA also led to a significant upregulation of the p53 target Trp53inp1. Similarly, in keratinocytes, RA increases the expression of p53 and proapoptotic caspases and sensitizes the cells to apoptosis by lowering their apoptotic threshold (Mrass et al., 2004). Future studies should address whether
BMP and RA pathways can act together to lower the apoptotic threshold during key stages of midline forebrain and facial development in vivo.

Another possible intermediate mechanism underlying hyper-responsiveness of embryos to teratogens is oxidative stress. Studies in animal models and humans have implicated ROS generation in the pathogenesis of craniofacial and other birth defects (Chang et al., 2003; Davis et al., 1990; Dong et al., 2008; Kay et al., 2000; Kotch et al., 1995; Loeken, 2004; Ornoy, 2007). Three known environmental causes of HPE, gestational diabetes, fetal alcohol or RA exposure (Cohen and Shiota, 2002), are all associated with elevated levels of ROS (Aoto et al., 2008; Davis et al., 1990; Kay et al., 2000; Ornoy, 2007). In fact, the ability to remove ROS is thought to be a general mechanism to neutralize environmental toxins. One of the reasons why early embryos may be particularly sensitive to free radical damage is their limited antioxidant capability, partly due to an inherent deficiency of scavengers of ROS, superoxide dismutase and catalase (Davis et al., 1990). Oxidative stress is thought to promote apoptosis, which then disrupts normal development. Supplementation with exogenous antioxidants, including N-acetylcysteine, vitamin C, vitamin E, superoxide dismutase, or catalase in animal models has produced promising results in terms of reducing apoptosis and dysmorphology (Aoto et al., 2008; Kotch et al., 1995; Loeken, 2004; Siman and Eriksson, 1997; Wentzel and Eriksson, 1998). The current study shows that ATRA in combination with BMP can lower GSH to GSSG ratios, indicating induction of oxidative stress in P19 cells. It should also be noted that other cellular processes may also be disrupted by RA and contribute to the phenotypic heterogeneity in Twsg1 mutant embryos, such as premature differentiation induced by increased levels of RA (Laue et al., 2011).
Retinoic acid (an analog of vitamin A) has also been proven to cause birth defects in humans, including central nervous system abnormalities such as HPE (Cohen and Shiota, 2002). The association between vitamin A and birth defects comes from studies in which high doses were used. For example, in a study of 154 human pregnancies, in utero exposure to isotretinoin (prescribed to treat severe cystic acne) was associated with a high risk of congenital malformations (relative risk 25.6) (Lammer et al., 1985). All women took oral isotretinoin at some point during the first 10 weeks after conception. This has led to an increased awareness about teratogenic effects of retinoic acid and reduced exposure, although not complete elimination as it continues to be prescribed for the treatment of acne, sun-damaged skin, psoriasis, prevention of nonmelanoma skin cancer, and for cancer chemotherapy (Mrass et al., 2004). Vitamin A alone however, when used in non-teratogenic doses (as found in over the counter vitamin supplements), has been shown to be safe in the general population.

In summary, TWSG1-deficient mice represent a genetic mouse model of a mutation with low penetrance that sensitizes embryos to environmental influences. The mechanisms underlying these gene-environment interactions are poorly understood. Since similar biological processes appear to be involved in the pathogenesis of a variety of birth defects, and in response to various teratogens, better understanding of these interactions will likely be applicable to other birth defects beyond craniofacial malformation.
MATERIALS AND METHODS

Mice

Mice with a targeted mutation in Twsg1 (Twsg1<sup>ln1.1 Mboc</sup>) were described previously (Petryk et al., 2004). Wild type (WT) mice were purchased from Jackson Lab. All mice were in C57BL/6 background. Presence of a spermatic plug was counted as embryonic day 0.5 (E 0.5). Pregnant females were treated by gavage with all-trans retinoic acid (ATRA, Sigma, St. Louis, MO, USA) in corn oil at doses of 3.75 or 7.5 mg/kg (Kotch et al., 1995) on the morning (10 AM) of E7.5, which is a well defined teratogenic window for the induction of HPE (Higashiyama et al., 2007; Lipinski et al., 2010). Subsequently the pregnant females were euthanized by CO<sub>2</sub> inhalation, embryos were isolated at E9.5 or E10.5, and assessed for external phenotypes under the dissecting microscope, including telencephalic vesicle abnormalities consistent with HPE and neural tube defects. For geometric morphometric shape analysis, embryos were collected at E11.5 and fixed in 4% paraformaldehyde with gluteraldehyde (Schmidt et al., 2010). Mice were housed in SPF conditions. Standard chow and water were provided ad libitum. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Geometric morphometric (GM) shape analysis

GM analysis of craniofacial shape was performed by microCT. Embryos were scanned at 5 micron resolution with a Scanco µCT 35 Scanner (Scanco Medical, Brüttisellen, Switzerland). A detailed description of this technique and computation methods have been previously published (Chong et al., 2012; Young et al., 2010; Young et al., 2007). A set of 45 landmarks were used to define the morphology of the embryonic face and forebrain using established
protocols (Boughner et al., 2008; Parsons et al., 2011; Schmidt et al., 2010). Landmark data are then aligned using a generalized least-squares Procrustes superimposition algorithm to remove size and place all individuals into a common shape space (Mitteroecker and Gunz, 2009). A series of linear combinations of variables is created (principal components or PCs) that explain successively smaller proportions of total variance. PC1 is computed to capture the largest proportion of variation in the original measurements.

**Cell culture and treatments**

P19 mouse embryonal carcinoma cells (ATCC CRL-1825) (Glozak and Rogers, 1996) were cultured in MEM with 10% fetal bovine serum and antibiotics, maintained by splitting ten-fold every 2 days. Cells were treated with recombinant human BMP2 (R&D systems, Minneapolis, MN) and/or All-Trans Retinoic Acid (ATRA). BMP2 was dissolved as a stock at 100 ng/µl in 4 mM HCl, 0.1% bovine serum albumin (BSA). ATRA was dissolved in DMSO and kept as a stock at 10⁻² M. All cells in BMP and ATRA treatment experiments were adjusted with non-solute containing vehicles to final concentrations of 0.0001% (w/v) BSA and 0.01% (v/v) DMSO. Antimycin-A (Sigma, St. Louis, Mo, USA) was prepared in propylene glycol at 6mg/mL.

**Gene expression**

P19 cells were plated into 6 well plates with 20,000 cells/well and allowed to grow overnight. Media were removed and replaced with treatment media containing test compounds. For RA induction experiments, cells were treated either with 1 µM ATRA or with DMSO vehicle in the same v/v dilution. For BMP/RA experiments, cells were treated with vehicle
control, 10 ng/ml BMP2 with DMSO ATRA vehicle control, 1 µM ATRA with BSA BMP2 vehicle control, or BMP2 combined with ATRA. In both sets of experiments, after 24 hours media were removed and 1mL of Trizol (Invitrogen, Carlsbad, CA, USA) was added for RNA isolation according to manufacturer’s instructions. cDNA was prepared from RNA samples by reverse transcription using the Thermoscript reverse transcription system (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using 2x SYBR green mastermix with ROX from SABiosciences on an MX3000p thermocycler (Stratagene/Agilent technologies, La Jolla, CA, USA) and analyzed using the MxPro software (Stratagene). Primers used for the following genes: Bmp2, Crbp1, Cyp26a1, Gapdh, Hoxa1, Hoxb1, Msx1, Msx2, Rara, Rarb and Trp53inp1 are shown in Supplemental Table 1.

**Caspase 3/7 activity assay**

Caspase 3/7 activity was measured using reagents from the Apotox-glo triplex assay kit (Promega, Madison, WI) according to manufacturer’s protocol. Briefly, cells were plated to a white walled clear bottom 96 well plate with 2500 cells per well then test compounds were added, diluted in growth media. Cells were treated with vehicle control, 10 ng/ml BMP2 with DMSO ATRA vehicle control, 1 µM ATRA with BSA BMP2 vehicle control, and BMP2 combined with ATRA. After 24 hours, Caspase activation was measured by addition of luminogenic Caspase substrate and measured in a Centro XS³ LB 960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).
**Glutathione ratio assay**

P19 cells were assayed for ratio of reduced to oxidized glutathione using the GSH/GSSG-glo kit (Promega) according to manufacturer’s instructions. Briefly, cells were plated to a white walled clear bottom 96 well plate with 2500 cells per well then test compounds were added, diluted in growth media. Cells were treated with BMP/RA vehicle control, 10 ng/ml BMP2 with DMSO ATRA vehicle control, 1 µM ATRA with BSA BMP2 vehicle control, BMP2 combined with ATRA, Propylene glycol AMA vehicle control or 70µM AMA. After 16 hours cells were washed once with HBSS then lysed and analyzed for GSH and GSSG content using kit-provided reagents for luminogenic reactions.

**Statistical analysis**

Chi-squared tests, t-tests and ANOVA combined with Tukey’s multiple comparison tests were performed using Prism4 (GraphPad Software, San Diego, CA, USA). Significance was accepted at alpha of 0.05. Fisher’s exact test was used to compare frequencies of embryonic phenotypes between different genotypes and with and without retinoic acid treatments.

**ACKNOWLEDGEMENTS**

P19 mouse embryonal carcinoma cells were a kind gift of Dr. Li-Na Wei. This project was supported by the National Institutes of Health [R01 DE016601 and R56 DE023530 to A.P., R01 DE019638 to R.S.M. and R01 DE021708 to B.H.], Minnesota Medical Foundation [grant #4186-9227-14 to A.P.]. C.J.B. was supported by the Minnesota Craniofacial Research Training Program [R90 DE023058] and the Medical Scientist Training Program [T32 GM008244] from the National Institutes of Health.
COMPETING INTERESTS STATEMENT

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

C.J.B. and A.P. designed the research, analyzed the data, and wrote the paper; C.J.B. and B.S. executed the experiments; R.S.M. and B.H. conducted geometric morphometric analysis by microCT; R.J. and R.S.M. participated in data interpretation and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.
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osteoclastogenesis causes osteopenia in twisted gastrulation-deficient mice through increased


FIGURE LEGENDS

Fig. 1. Phenotypic analysis of *Twsg1* mutants exposed in utero to a low dose (3.75 mg/kg) of ATRA. Lateral views of E9.5 embryos: (A) WT untreated embryo; (B) normal appearance of ATRA treated WT embryo; (C, D) *Twsg1* mutants treated with ATRA showing proboscis (P) and absence of telencephalic vesicles. Frontal views of E10.5 embryos: (E) WT untreated embryo; (F) normal telencephalic vesicles (T; outlined by a dotted line) of ATRA treated WT embryo; (G,H) *HPE* in *Twsg1* embryos treated with ATRA.

Fig. 2. GM analysis of facial shape. (A) Principal component analysis (PCA) based on GM analysis of facial shape in WT and *Twsg1*/*- mouse embryos at E11.5 with or without in utero exposure to a low dose ATRA at E7.5. PC analysis of landmark data shows that the majority of shape variation is associated with narrowing of the midface and that PC1 discriminates between treatment groups. PC1 distinguishes between treated embryos and both WT and heterozygous *Twsg1* mutant embryos. (B) 3D morphing showing variation along PC1; (C) Procrustes distances between groups. P values were obtained by permutation of the Procrustes Distance.

Fig. 3. RA target gene expression in response to ATRA treatment in P19 cells by qPCR. The P19 cell cultures were treated with either DMSO vehicle or 1 µM ATRA for 24 hours, with transcript levels quantified by qPCR. Gene expression was normalized to the expression of *Gapdh*, and shown as a ratio to the average of the DMSO vehicle treated control expression. The induction of RA targets in P19 cells indicates the suitability of the P19 cell model for investigating responses to RA signaling. ***p < 0.001 by Student’s t-test.
Fig. 4. Gene expression levels in response to exogenous BMP2 and ATRA by qPCR. P19 cell cultures were treated for 24 hours with vehicle control (0.00001% BSA, 0.001% DMSO), 10 ng/mL rhBMP2, 1 µM ATRA, or 10 ng/mL rhBMP2 and 1 µM ATRA. Gene expression was normalized to the expression of Gapdh, and shown as a ratio to the mean vehicle treated control expression, with transcript levels quantified by qPCR. ATRA induced both BMP2 and its downstream targets in P19 cells. Combined BMP and ATRA treatment resulted in a significant increase in Trp53inp1 expression. *Significance by Tukey’s test for difference from control (Ctrl); † from BMP treated; ‡ from ATRA treated; 3 marks: p < 0.001; 2 marks p < 0.01.

Fig. 5. Caspase 3/7 activity in P19 cells in response to exogenous BMP2 and ATRA. P19 cells were treated for 24 hours with vehicle control (0.00001% BSA, 0.001% DMSO), 10 ng/mL rhBMP2, 1 µM ATRA, or 10 ng/mL BMP and 1 µM ATRA, no treatment, or 70 µM Antimycin A as a positive control. RA acted in concert with BMP to increase caspase 3/7 activation in P19 cells. * p<0.05, ** p<0.01, *** p<0.001 by Student’s t-test.

Fig. 6. Quantitation of oxidative stress in P19 cells treated with BMP2 and ATRA. Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio was assayed in P19 cells treated for 16 hours with vehicle control (0.0001% BSA, 0.001% DMSO), 10 ng/mL rhBMP2, 1 µM ATRA, or 10 ng/mL BMP combined with 1 µM ATRA. 70µM Antimycin A was tested as a positive control and compared to its vehicle, propylene glycol. As expected, treatment of P19 cells with a prooxidant AMA resulted in a significant decrease in GSH/GSSG ratio. In addition, oxidative stress was increased in P19 cells treated with ATRA in combination with BMP2. *p < 0.05, **p < 0.01.
TRANSLATIONAL IMPACT

Clinical issue
Holoprosencephaly (HPE) is the most common defect of the developing forebrain with an incidence of 1 in 250 conceptuses and about 1 in every 10,000 at term. It is characterized by inadequate or absent midline division of the embryonic forebrain and midline facial defects. A perplexing feature of HPE as well as other craniofacial syndromes in humans is their widely variable penetrance and expressivity even in the case of the same single gene mutation within the same family, with some individuals having severe defects, some mild, and some being unaffected. It is currently unknown what causes manifestation of HPE in genetically at risk individuals, but it has been speculated that environmental factors may play a role. This work addresses the effects of environmental exposure in a mouse model predisposed to HPE.

Results
Twsg1 mutant mice serve as a model of human HPE because of incomplete penetrance and a range of defects among homozygotes. The authors demonstrated that Twsg1 mutants showed increased susceptibility to teratogenic effects of relatively low doses of retinoic acid that in control mice cause few, if any defects. The exposure to retinoic acid occurred at embryonic day 7.5, which is the most sensitive window for teratogen-induced HPE (corresponding to 3rd to 4th week post-fertilization in humans). Remarkably, even Twsg1 haploinsufficiency exacerbated teratogenic effects of prenatal retinoic acid exposure. The majority of shape variation among Twsg1+/− mutants was associated with narrowing of the midface as demonstrated by microCT analysis. Since the only known action of TWSG1 is through binding Bone morphogenetic
proteins (BMPs) in the extracellular space, the authors hypothesized that BMP/RA interactions would contribute to the enhanced expressivity of HPE in this mouse model. P19 cells were used as an in vitro model to begin to understand the mechanisms mediating these gene-environment interactions. In P19 cells, RA induced the expression of \textit{Bmp2} and its downstream targets \textit{Msx1} and \textit{Msx2}, and acted in concert with BMP to increase apoptosis, p53 target gene expression, and oxidative stress, suggesting a role for these pathways in modifying the disease outcome.

**Implications and future directions**

This study provides direct evidence for modifying effects of the environment in a genetic mouse model carrying a predisposing mutation for HPE. Further study of the mechanisms underlying these gene-environment interactions in vivo will contribute to better understanding of the pathogenesis of birth defects and present an opportunity to explore potential preventive interventions.
Table 1. Frequency of HPE and neural tube defects in wild type and Twsg1 mutant embryos with and without *in utero* exposure to ATRA.

<table>
<thead>
<tr>
<th>Type of defect</th>
<th>Wild type</th>
<th></th>
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<tr>
<td></td>
<td>-ATRA</td>
<td>+ATRA 3.75 mg/kg</td>
<td>+ATRA 7.5 mg/kg</td>
<td>-ATRA</td>
<td>+ATRA 3.75 mg/kg</td>
<td>-ATRA</td>
<td>+ATRA 3.75 mg/kg</td>
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<td>HPE or NTD</td>
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<td>30% (n=27)</td>
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<td>60%</td>
<td>0%</td>
<td>23%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td>NTD</td>
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<td>1.4%</td>
<td>34%</td>
<td>0%</td>
<td>7%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Fig. 1

No ATRA

+E9.5

A  +/+  B  +/+  C  +/-  D  -/-

+E10.5

E  +/+  F  +/+  G  +/-  H  -/-

P:

T:

*:
Fig. 2

A. Principal component analysis showing the distribution of (+/+) no RA, (+/-) no RA, and (+/-) RA 3.75 mg/kg.

B. Representation of brain shape distances with three groups:
- WT vs (+/+)
- WT vs (+/-)
- (+/-) vs (+/-)

C. Bar graph indicating shape distance with significance levels:
- WT vs (+/+) (***)
- WT vs (+/-) (***)
- (+/-) vs (+/-) (*)