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# Methoxyacetic acid inhibits histone deacetylase and impairs axial elongation morphogenesis of mouse gastruloids in a retinoic acid signaling-dependent manner

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## Abstract

**Background:** Teratogenic potential has been linked to various industrial compounds. Methoxyacetic acid (MAA) is a primary metabolite of the widely used organic solvent and plasticizer, methoxyethanol and dimethoxyethyl phthalate, respectively. Studies using model animals have shown that MAA acts as the proximate teratogen that causes various malformations in developing embryos. Nonetheless, the molecular mechanisms by which MAA exerts its teratogenic effects are not fully understood.

**Methods:** Gastruloids of mouse P19C5 pluripotent stem cells, which recapitulate axial elongation morphogenesis of gastrulation-stage embryos, were explored as an in vitro model to investigate the teratogenic action of MAA. Morphometric parameters of gastruloids were measured to evaluate the morphogenetic effect, and transcript levels of various developmental regulator genes were examined to assess the impact on gene expression patterns. The effects of MAA on the level of retinoic acid (RA) signaling and histone deacetylase activity were also measured.

**Results:** MAA reduced axial elongation of gastruloids at concentrations comparable to the teratogenic plasma level (5 mM) in vivo. MAA at 4 mM significantly altered the expression profiles of developmental regulator genes. In particular, it upregulated the RA signaling target genes. The concomitant suppression of RA signaling using a pharmacological agent alleviated the morphogenetic effect of MAA. MAA at 4 mM also significantly reduced the activity of purified histone deacetylase protein.

**Conclusions:** MAA impaired axial elongation morphogenesis in a RA signaling-dependent manner in mouse gastruloids, possibly through the inhibition of histone deacetylase.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**Keywords**

gastruloids; histone deacetylase; methoxyacetic acid; morphogenesis; retinoic acid

## 1 | INTRODUCTION

Teratogenic potential has been linked to various industrial and pharmaceutical compounds, which women of childbearing age may be exposed to (Schardein & Macina, 2006). Understanding how these chemicals affect embryo development at the molecular level may help identify other potentially teratogenic compounds and lead to strategies to alleviate their adverse effects. However, in vivo studies using human and model animals are often too complicated to elucidate the molecular mechanisms of teratogenic compounds. This is largely because the properties and concentrations of compounds are dynamically altered in the mother through absorption, distribution, metabolism, and excretion. By contrast, in vitro models of embryogenesis, such as those created from pluripotent stem cells, are devoid of such maternal influences, and are more amenable to various experimental interrogations for mechanistic analyses. Thus, in vitro approaches can augment in vivo studies by providing valuable insights into the molecular actions of teratogenic compounds.

In vitro gastrulation models called, gastruloids, have been generated from pluripotent stem cells, which recapitulate axial elongation morphogenesis of gastrulation-stage embryos (Beccari et al., 2018; Marikawa, Tamashiro, Fujita, & Alarcón, 2009; van den Brink et al., 2014). In the case of gastruloids made from mouse P19C5 stem cells, three-dimensional cell aggregates grow in size and transform from a spherical to elongated shape with a distinct anterior–posterior axis within 4 days of hanging drop culture (Lau & Marikawa, 2014). Growth and axial morphogenesis of P19C5 gastruloids are significantly impaired by various teratogenic chemicals at the physiologically relevant concentrations (Li & Marikawa, 2016; Warkus & Marikawa, 2017; Warkus, Yuen, Lau, & Marikawa, 2016). Gene expression patterns in gastruloids are also altered by the inhibition of the morphogenetic signals, such as WNT, NODAL, NOTCH, and retinoic acid (RA), in a manner consistent with their functions in normal embryonic patterning (Li & Marikawa, 2015). Accordingly, P19C5 gastruloids have been explored as an in vitro tool to identify molecular pathways linked to the teratogenic effects of various chemicals, such as valproic acid (Li & Marikawa, 2016), fluoxetine (Warkus & Marikawa, 2018), and resveratrol (Kim & Marikawa, 2018).

The purpose of the present study is to investigate teratogenic mechanisms of methoxyacetic acid (MAA), using the mouse gastruloid. MAA (linear formula: $CH_3OCH_2COOH$) is a primary metabolite of organic solvent methoxyethanol (ME) and of plasticizer dimethoxyethyl phthalate (DMEP). Studies using model animals, ranging from rodents to nonhuman primates, have unequivocally demonstrated that MAA, but not ME or DMEP, is the proximate teratogen, which causes various malformations, such as defects in the neural tube, heart, kidneys, limbs, and tail (Brown, Holt, & Webb, 1984; Clarke, Duignan, & Welsch, 1992; Ritter, Scott Jr., Randall, & Ritter, 1985; Scott, Fradkin, Wittfoht, & Nau, 1989; Sleet, Greene, & Welsch, 1988; Terry, Elswick, Stedman, & Welsch, 1994; Yonemoto, Brown, & Webb, 1984). MAA is one of the short-chain fatty acids that activate mitogen-

activated protein kinases and inhibit histone deacetylases (HDAC) to enhance estrogen receptor activity (Jansen et al., 2004). Inhibition of HDAC, in particular, is considered to be involved in the teratogenic effects of MAA (Dayan & Hales, 2014). Nonetheless, how such molecular effects of MAA are linked to the pathogenesis of specific birth defects is not fully understood. A better understanding of the molecular pathways that are affected by MAA is crucial to evaluate the reproductive risk in human, which may result from exposures to the widely distributed ME and DMEP.

Here, we showed that MAA significantly altered morphogenesis and gene expression profile in mouse gastruloids at the teratogenic concentrations. Our studies suggest that enhanced transcription of RA-signaling target genes through inhibition of HDAC is responsible for the adverse effect of MAA to impair axial elongation morphogenesis.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

All chemical compounds used in the present study were commercially obtained, namely from Sigma-Aldrich (St. Louis, MO), Santa Cruz Biotechnology (Dallas, TX), and Calbiochem (San Diego, CA), and their details are described in Table 1.

### 2.2 | Cell culture

P19C5 mouse embryonal carcinoma cells (Lau & Marikawa, 2014) were propagated in culture medium, consisting of 90% minimum essential medium alpha with nucleosides and GlutaMAX Supplement (LifeTechnologies, Carlsbad, CA), 2.5% fetal bovine serum, 7.5% newborn calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Gastruloids were generated as three-dimensional cell aggregates in hanging drops (Figure 1) of culture medium, containing 1% dimethyl sulfoxide (DMSO) with or without a test chemical, according to the method described previously (Lau & Marikawa, 2014). Monolayered cell culture was prepared by seeding 10,000 cells per well in 24-well plates with 500 μl of culture medium the day before chemical treatment.

### 2.3 | Morphometric analyses and criteria for adverse effects

Gastruloids were removed from hanging drops at Day 4 of culture (Figure 1) and placed together in a dish filled with phosphate-buffered saline for photography. Image files in JPEG format were opened in the ImageJ program (http://rsb.infonihgov/ij), and morphological parameters (area and aspect ratio) of each gastruloid were measured by manually tracing the circumference using the polygon selection tool. Aspect ratio is the ratio of the major-to-minor axis of an ellipse that most tightly fits the circumference of a gastruloid, and it is reflective of the extent of axial elongation (Li & Marikawa, 2015). Morphological assessment of chemical-treated gastruloids was conducted in three biological replicates using different collections of cell suspensions. For each replicate, 16 gastruloids were generated per chemical treatment in parallel with 16 control gastruloids (i.e., 1% DMSO only). Because the size and extent of elongation (reflected by area and aspect ratio, respectively) of control gastruloids were different among experimental replicates, normalization was performed relative to the average values of control gastruloids to yield

relative area and relative aspect ratio, expressed as a percentage (i.e., control = 100%). Data of relative area and relative aspect ratio from all replicates were compiled, and their averages are shown with error bars of 95% confidence interval. In the present study, a chemical treatment was defined as having an adverse morphogenetic effect when it reduced the average relative area by >20% or the average aspect ratio by >40% compared with the control, according to the rationale described previously (Warkus & Marikawa, 2017).

### 2.4 | Quantitative reverse transcription-polymerase chain reaction

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to determine the relative expression levels of developmental regulator genes (Table 2). Total RNA was extracted from cell suspension before aggregation (Day 0) and gastruloids (control and MAA-treated) at Days 1, 2, 3, and 4 (Figure 1) using TRI reagent (LifeTechnologies) and Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA), and processed for cDNA synthesis using M-MLV Reverse Transcriptase (Promega, Madison, WI) and oligo-dT (18) primer. Quantitative PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) as follows: initial denaturation at 94 °C for 5 min, followed by up to 45 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 40 s. Data files were opened in CFX Manager software (Bio-Rad) and Ct values were transferred to the Excel program for further analyses. *Actb*, which encodes β-Actin, was used as a housekeeping gene to normalize the expression levels of other genes. *Actb* has been effectively used as a housekeeping gene in previous studies to evaluate gene expression levels in P19C5 gastruloids under various experimental conditions (Kim & Marikawa, 2018; Lau & Marikawa, 2014; Li & Marikawa, 2015, 2016; Warkus & Marikawa, 2018; Yuan & Marikawa, 2017). Additionally, based on the previous microarray analysis data (Kim & Marikawa, 2018), the transcript level of *Actb* is mostly stable from Days 0 to 4 of gastruloid culture, which is comparable or superior to other commonly used housekeeping genes, such as *Gapdh*, *Hmbs*, *Hprt*, *Tbp*, and *Ywhaz* (Figure S1). Gene expression analyses were conducted using three independent sets of samples as biological replicates using different collections of cell suspensions. Each set consisted of 9 samples: Day 0, control gastruloids at Days 1 to 4, and MAA-treated gastruloids at Days 1 to 4, all of which were originated from the same cell suspension. Relative expression levels were calculated for each set of experiment, as previously described (Warkus & Marikawa, 2018), and the averages of the three replicates are shown with error bars of standard deviations.

### 2.5 | Histone deacetylase activity assay

Histone deacetylase (HDAC) activity was measured using the HDAC-Glo I/II Assay System (Promega), which utilizes an acetylated, cell-permeable, luminogenic peptide as an HDAC substrate. Human recombinant HDAC1 protein that carries C-terminal His-FLAG tag was obtained commercially (#10009231; Cayman Chemical, Ann Arbor, MI). The assay was conducted according to the manufacturer's instruction. Briefly, HDAC1 was diluted (1/10,000) in the HDAC-Glo buffer and was mixed with an equal volume of the buffer containing a test chemical at desired concentrations. After 30 min of incubation at 37 °C, luminescence was measured using the Gene Light 55 Luminometer (Microtech, Chiba, Japan).

### 2.6 | RA signaling reporter assay

We previously constructed the RA signaling reporter plasmid RARE-Luc, which contains the firefly luciferase located downstream of the basal promoter with RAR and RXR binding sites (Li & Marikawa, 2016). P19C5 cells were transfected using Lipofectamine 2000 (LifeTechnologies) with RARE-Luc and pRL-TK (Promega), the latter of which encodes *Renilla* luciferase, to normalize transfection efficiency. The luciferase assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) with Gene Light 55 Luminometer, according to the manufacturer's instruction.

### 2.7 | Statistical analyses

All adverse morphogenetic effects shown in the present study were statistically significant ($p < .01$), based on two-sample *t* test that was performed between control and chemical-treated groups. For gene expression analyses, two-sample *t* test was performed between control and chemical-treated groups to determine significant changes in relative expression levels ($p < .05$).

## 3 | RESULTS

### 3.1 | Methoxyacetic acid impairs morphogenesis of mouse gastruloids at teratogenic concentrations

We examined morphological parameters, namely relative area and relative aspect ratio, of mouse P19C5 gastruloids after 4days of culture with various concentrations of MAA. While both parameters were decreased by MAA exposures in a concentration-dependent manner (Figure 2a,b), relative aspect ratio, which represents the extent of axial elongation, was more sensitively affected. For example, at 2 and 4mM, the relative aspect ratio was reduced by 49 and 63%, respectively, whereas the relative area was reduced only by 5 and 14%, respectively (Figure 2b). Note that these concentrations are close to the maternal plasma level of MAA ($C_{max}$=5mM) that causes embryo malformations (Daston et al., 2014; Sleet, Welsch, Myers & Marr, 1996). By contrast, morphogenesis was not impaired by methoxyethanol, a nonteratogenic precursor of MAA, even at much higher concentrations (50 to 200mM) than MAA (Figure 2c). Thus, P19C5 gastruloid morphogenesis was sensitively and selectively affected by MAA in a manner consistent with in vivo situations.

### 3.2 | Methoxyacetic acid alters expression profiles of developmental regulator genes

To gain insights into the molecular mechanisms of MAA teratogenicity, we examined gene expression profiles in gastruloids that were treated with MAA at 4 mM. This concentration was chosen because it is close to the in vivo teratogenic concentration (Daston et al., 2014; Sleet, Welsch, Myers & Marr, 1996), and also it robustly inhibited gastruloid elongation (Figure 2a,b). Transcript levels of specific developmental regulator genes (their molecular characteristics, functions, and expression patterns in the normal mouse embryo are summarized in Table 2) were analyzed by quantitative RT-PCR over the course of 4 days of culture. The expressions of pluripotency maintenance factors (*Pou5f1* and *Nanog*) in MAA-treated gastruloids were downregulated to the basal level by Day 2 of culture in a manner similar to control gastruloids (Figure 3). By contrast, the expression profiles of the other

genes were significantly altered by MAA. Namely, the Day 1 peak expressions of genes that are involved in the initiation of gastrulation (*Wnt3*, *Brachyury*, and *Mixl1*), the Day 2 peak expressions of genes associated with continued gastrulation and caudal patterning (*Snai1*, *Msgn1*, *Tbx6*, *Notch1*, and *Lfng*), and the Day 3 peak expression of the gene essential for segmentation along the body axis (*Meox1*), were all significantly repressed by MAA (Figure 3). It is likely that the diminished expressions of these developmental regulators contributed to the impairment of axial elongation in MAA-treated gastruloids.

However, we found that the expressions of *Cdx1* and *Hoxa1*, both of which are regulators of anterior–posterior axial patterning, were markedly elevated by MAA at Day 1 (Figure 3). Because these two genes are well-known transcriptional targets of RA signaling (Houle, Sylvestre, & Lohnes, 2003; Langston & Gudas, 1992), we further examined the expressions of *Aldh1a2* and *Cyp26a1*, which encode the key regulators of RA metabolism through biosynthesis and degradation, respectively (Table 2). Their expression patterns were significantly changed by MAA: the *Aldh1a2* level remained low at Days 2 and 4, whereas the *Cyp26a1* level was low at Day 1 but significantly increased by Day 4 (Figure 3). These results raise the possibility that MAA alters RA signaling in gastruloids.

### 3.3 | Methoxyacetic acid affects gene expressions and axial morphogenesis in a retinoic acid signaling-dependent manner

As shown above, MAA markedly elevated the expression of *Cdx1* and *Hoxa1* by Day 1 of gastruloid development, which took place under the differentiating condition (i.e., three-dimensional cell aggregation in the presence of 1% DMSO). To test whether MAA can also elevate these RA signaling target genes under a nondifferentiating condition, monolayered cells (without DMSO) were treated with MAA for 1 day and analyzed by quantitative RT-PCR. Cells that were treated with RA at physiological concentrations (5 to 20 nM) exhibited robust up-regulations of *Cdx1* and *Hoxa1*, confirming that these genes are indeed transcriptional targets of RA signaling in P19C5 cells (Figure 4a). MAA also upregulated *Cdx1* and *Hoxa1* in a dose-dependent manner. Notably, upregulations of *Cdx1* and *Hoxa1* by MAA (4 mM) were significantly blunted by co-treatment with BMS493, a pharmacological inhibitor of RA receptors (Figure 4b). These results suggest that MAA activates transcriptions of *Cdx1* and *Hoxa1* in a RA signaling-dependent manner.

To test whether the morphogenetic effect of MAA is also dependent on RA signaling, we co-treated gastruloids with MAA and BMS493. In this experiment, we limited compound treatment only to the first 2 days of gastruloid culture (Figure 5a), because our previous study has shown that BMS493 is detrimental to axial elongation when applied during the last 2 days of culture (Li & Marikawa, 2016). With this treatment regimen, MAA alone robustly impaired axial elongation of gastruloids (Figure 5b). However, when MAA and BMS493 were administered together, gastruloids exhibited significant elongation (Figure 5b). Relative aspect ratio of gastruloids that were co-treated with MAA plus BMS493 was significantly higher than those treated with MAA alone (Figure 5c). Treatment with BMS493 alone had no apparent impact on gastruloid morphology (Figure 5b,c), consistent with our previous study (Li & Marikawa, 2016). These results suggest that MAA diminishes axial elongation of gastruloids in a RA signaling-dependent manner.

### 3.4 | Methoxyacetic acid acts as an inhibitor of histone deacetylase

The morphogenetic and molecular effects of MAA on gastruloids appear to be similar to those of valproic acid (VPA), because VPA diminishes axial elongation and upregulates *Cdx1* and *Hoxa1* in a RA signaling-dependent manner (Li & Marikawa, 2016). VPA is an anti-epileptic drug, and is also a well-known teratogen in human and rodents (Jentink et al., 2010; Tomson et al., 2011). The teratogenic action of VPA is thought to be mediated through the inhibition of histone deacetylase (HDAC) (Phiel et al., 2001). This raises the possibility that MAA also inhibits HDAC to cause teratogenic effects.

To directly test whether MAA acts as a HDAC inhibitor, we performed a cell-free enzyme assay, in which the enzymatic activity of purified HDAC1 protein was measured in the presence of MAA or other compounds. HDAC1 activity was diminished by MAA in a dose-dependent manner, and the activity at 4 mM of MAA was about half of the control level (Figure 6a). By contrast, ME, a nonteratogenic precursor of MAA, did not reduce the HDAC1 activity even at 10 mM (Figure 6a). In this assay, HDAC1 activity was also reduced by trichostatin A (TSA), a potent pharmacological inhibitor of HDAC, as well as VPA, in a dose-dependent manner (Figure 6a). Thus, MAA directly inhibited HDAC1 at the teratogenic concentrations.

The previous study in mouse embryonic stem cells has shown that the inhibition or knockdown of HDAC increases the levels of histone acetylation at the enhancer regions of RA target genes and causes an elevation in their transcriptions (Urvalek & Gudas, 2014). Similarly, treatment of monolayered P19C5 cells with HDAC inhibitors, such as TSA and VPA, upregulated *Cdx1* and *Hoxa1* in a RA signaling-dependent manner (Figure 4a,b). It is possible that HDAC inhibitors upregulate RA signaling targets by sensitizing the enhancers to the basal level of RA, rather than by increasing the amount of RA itself. To test this possibility, the level of RA signaling was measured using the reporter plasmid RARE-Luc (Figure 6b) in monolayered cells that were treated with HDAC inhibitors, including MAA. The reporter signal was robustly increased by the addition of RA at physiological concentrations, validating the effectiveness of the reporter assay (Figure 6c). However, the reporter signals were not elevated by any of the HDAC inhibitors even at the concentrations that up-regulated *Cdx1* and *Hoxa1* (Figure 6c). This suggests that HDAC inhibitors, including MAA, do not increase the amount of RA while enhancing the transcription of RA signaling target genes.

## 4 | DISCUSSION

In the present study, teratogenic actions of MAA were investigated using mouse gastruloids. Gastruloids are three-dimensional aggregates of pluripotent stem cells that recapitulate key features of gastrulating embryos, and have been employed as in vitro models to investigate molecular mechanisms of embryo patterning (Beccari et al., 2018; Li & Marikawa, 2015; Turner et al., 2017; van den Brink et al., 2014; van den Brink et al., 2020) and to analyze embryotoxic chemical exposures (Kim & Marikawa, 2018; Lau & Marikawa, 2014; Li & Marikawa, 2016; Warkus et al., 2016; Warkus & Marikawa, 2017, 2018; Yuan & Marikawa, 2017). Here, we demonstrated that MAA inhibited gastruloid elongation morphogenesis at the in vivo teratogenic concentrations and that the morphogenetic impact of MAA was partly

due to excessive expression of RA signaling target genes. During embryogenesis, the level of RA signaling is spatially and temporally controlled by the coordinated actions of regulatory enzymes, such as ALDH1A2 and CYP26A1, which is pivotal for axial patterning of neural and mesodermal tissues (Piersma, Hessel, & Staal, 2017). As a result, embryo malformations can be induced by disturbance in RA signaling, such as insufficient activation due to vitamin A deficiency or excessive activation due to intake of certain dermatologic medications, including tretinoin (all-*trans* retinoic acid), isotretinoin (13-*cis* retinoic acid), and acitretin (Zasada & Budzisz, 2019). Disturbance in RA signaling may be one of the common mechanisms that are linked to the teratogenic actions of various chemical exposures (Piersma et al., 2017). Accordingly, an adverse outcome pathway framework has been proposed for neural tube and axial defects based on RA signaling (Tonk, Pennings, & Piersma, 2015). Morphogenesis of P19C5 gastruloids is sensitively affected by disturbance in RA signaling caused by exposure to all-*trans* retinoic acid, acitretin, or BMS493 (Li & Marikawa, 2015; Warkus et al., 2016; Warkus & Marikawa, 2017). Thus, the gastruloid may serve as an effective in vitro assay tool to detect chemical exposures that alter RA signaling to cause teratogenesis.

ALDH1A2 and CYP26A1 regulate the amount of RA in an opposing manner: the former mediates biosynthesis and the latter promotes degradation of RA (Piersma et al., 2017). During gastruloid development, the transcript levels of *Aldh1a2* and *Cyp26a1* are dynamically changed in a complementary fashion (Li & Marikawa, 2015). Specifically, *Aldh1a2* is elevated at Days 2 and 4, whereas *Cyp26a1* is elevated at Days 1 and 3. This suggests that the level of RA is dynamically changed during gastruloid development. As shown in the present study, an exposure to MAA significantly altered the temporal expression patterns of *Aldh1a2* and *Cyp26a1*. Most notably, the Day 1 elevation of *Cyp26a1* was markedly suppressed by MAA (Figure 3), which may in turn lead to an increase in the RA level. However, such a possible increase in the RA level is unlikely to have contributed substantially to the up-regulation of the RA signaling target genes (*Cdx1* and *Hoxa1*) by MAA treatment. This is because the reporter assay (Figure 6c) suggests that the RA level was not significantly elevated by MAA treatment in spite of the marked upregulation of *Cdx1* and *Hoxa1*. Nonetheless, this does not exclude the possibility that alterations in the RA level may be involved in other adverse effects induced by MAA exposure.

The morphogenetic and molecular effects of MAA on P19C5 gastruloids are similar to those of VPA (Li & Marikawa, 2016), such that they both diminished axial elongation and upregulated the RA signaling target genes. These similarities are likely to be due to their common molecular actions as HDAC inhibitors. Acetylation and deacetylation of histones are mediated by histone acetyltransferases and HDAC, respectively, and play crucial roles in transcriptional regulation (Lehrmann, Pritchard, & Harel-Bellan, 2002; Mai et al., 2005). Generally, inhibition of HDAC increases histone acetylation, which leads to transcriptional up-regulation. In P19C5 gastruloids, transcriptional up-regulation of the RA signaling targets appear to be responsible for the morphogenetic effects of VPA and MAA, because axial elongation was partly restored by co-treatment with BMS493 (Li & Marikawa, 2016; the present study). However, axial elongation was not completely restored (Figure 5), raising the possibility that other mechanisms are also involved. Inhibition of HDAC increases histone acetylation at the genome-wide level (Wang, Yan-Neale, Zeremski, & Cohen, 2004),

which is likely to up-regulate various other genes. HDAC inhibition can also increase acetylation of nonhistone proteins, such as p53 (Paradis & Hales, 2015), which plays diverse regulatory roles in cell cycle progression and stem cell maintenance (Solozobova & Blattner, 2011). Furthermore, MAA may exert biochemical effects that are unrelated to HDAC inhibition, such as interference with folate-dependent one-carbon transfer reactions (Welsch, Sleet, & Greene, 1987) and activation of PI3K pathway (Bagchi, Hurst, & Waxman, 2009). The roles of these mechanisms in MAA-induced teratogenesis may be further investigated using gastruloids, because such in vitro systems are more amenable for molecular interrogations.

While the teratogenic potential of MAA is evident from model animal studies, it is not clear whether human embryos are also impaired by MAA exposure in a similar manner. Metabolic precursors of MAA, such as ME and DMEP, are widely distributed as an organic solvent and plasticizer, respectively (Bao et al., 2015; Johanson, 2000). As a result, many people, including women of childbearing age, may be exposed to a high level of MAA. In a workplace with daily exposure of 4.5 ppm ME, which is within the permissible exposure limit, MAA concentration in the urine reaches up to 0.6 mM (Shih, Liou, Chen, & Smith, 2001). An epidemiologic study suggests that exposure to ME is associated with increased risks of spontaneous abortion and subfertility in women (Correa et al., 1996). Nonetheless, it is difficult to ascertain from epidemiologic studies that such reproductive outcomes are due to direct actions of MAA on developing embryos, instead of indirect consequences from maternal toxicity. Because experimentations with actual human embryos are nearly impossible for ethical reasons, alternative models are desired that reflect the genomic constitution and biochemical property of human embryonic cells. Accordingly, human pluripotent stem cell lines, which are either derived from preimplantation embryos or created by nuclear reprogramming of somatic cells, have been explored as in vitro models to investigate teratogenic effects of chemical exposures (Flamier, Singh, & Rasmussen, 2017; Kameoka, Babiarz, Kolaja, & Chiao, 2014; Mayshar, Yanuka, & Benvenisty, 2011; Mehta, Konala, Khanna, & Majumdar, 2008; Palmer et al., 2013; West, Weir, Smith, Donley, & Cezar, 2010; Xing et al., 2017). Recent studies reported elongation morphogenesis in three-dimensional aggregates of human pluripotent stem cells (Libby et al., 2020; Marikawa, Chen, Menor, Deng, & Alarcon, 2020; Ninomiya et al., 2020). Such human cell-based morphogenesis models may be exploited to investigate the teratogenic potential of MAA on human embryos, particularly with respect to the mechanism of action (e.g., whether it upregulates the RA signaling targets) and adverse effect concentrations (e.g., whether it impairs morphogenesis at around 5 mM).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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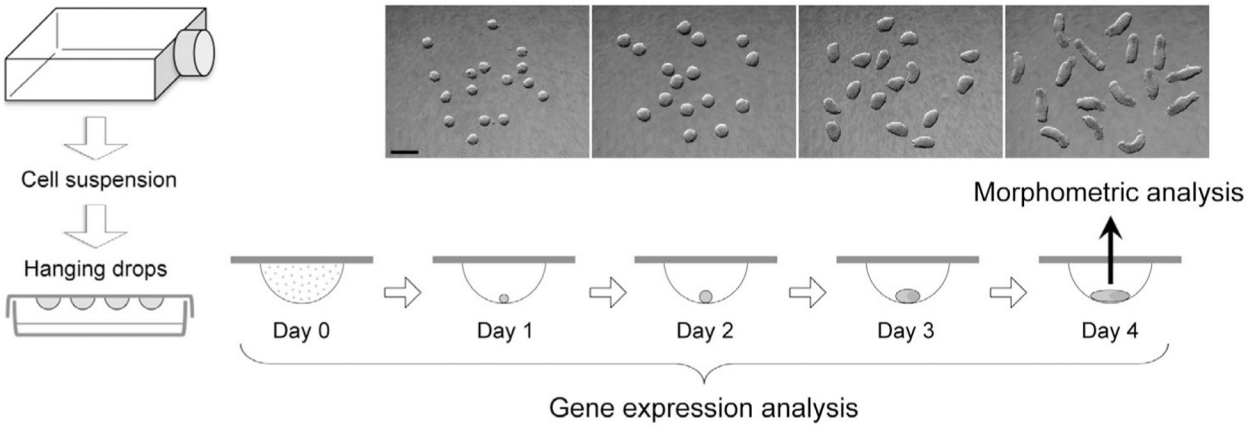


**FIGURE 1.**

The experimental scheme to evaluate the morphogenetic and molecular effects of chemical exposures using gastruloids made from mouse P19C5 pluripotent stem cells. Representative images of gastruloids for each day of culture are shown. Scale bar = 500 μm

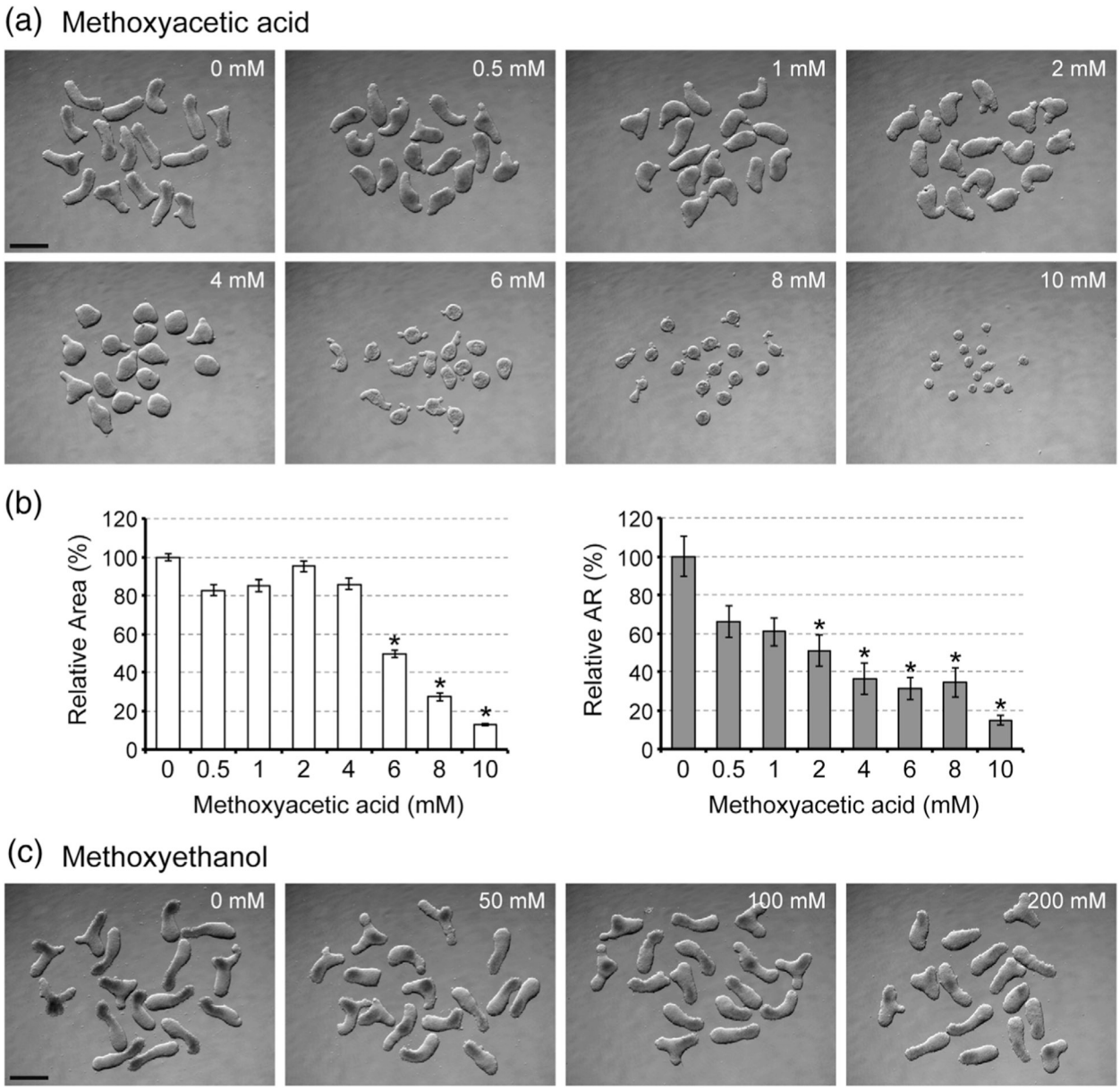


**FIGURE 2.**

Axial elongation morphogenesis of mouse gastruloids is diminished by methoxyacetic acid (MAA). (a) Images of Day 4 gastruloids that were treated with MAA at different concentrations. (b) Morphometric parameters of MAA-treated gastruloids. Graphs show the averages of relative area (left) and relative aspect ratio (AR; right) with error bars of 95% confidence interval ($n = 48$). Asterisks indicate adverse impacts, which are defined as reduction in relative area by >20% or relative AR by >40% compared to the control, which is set as 100%. All adverse impacts were statistically significant ($p < .01$). (c) Images of Day 4 gastruloids that were treated with methoxyethanol at different concentrations. Scale bars = 500 μm

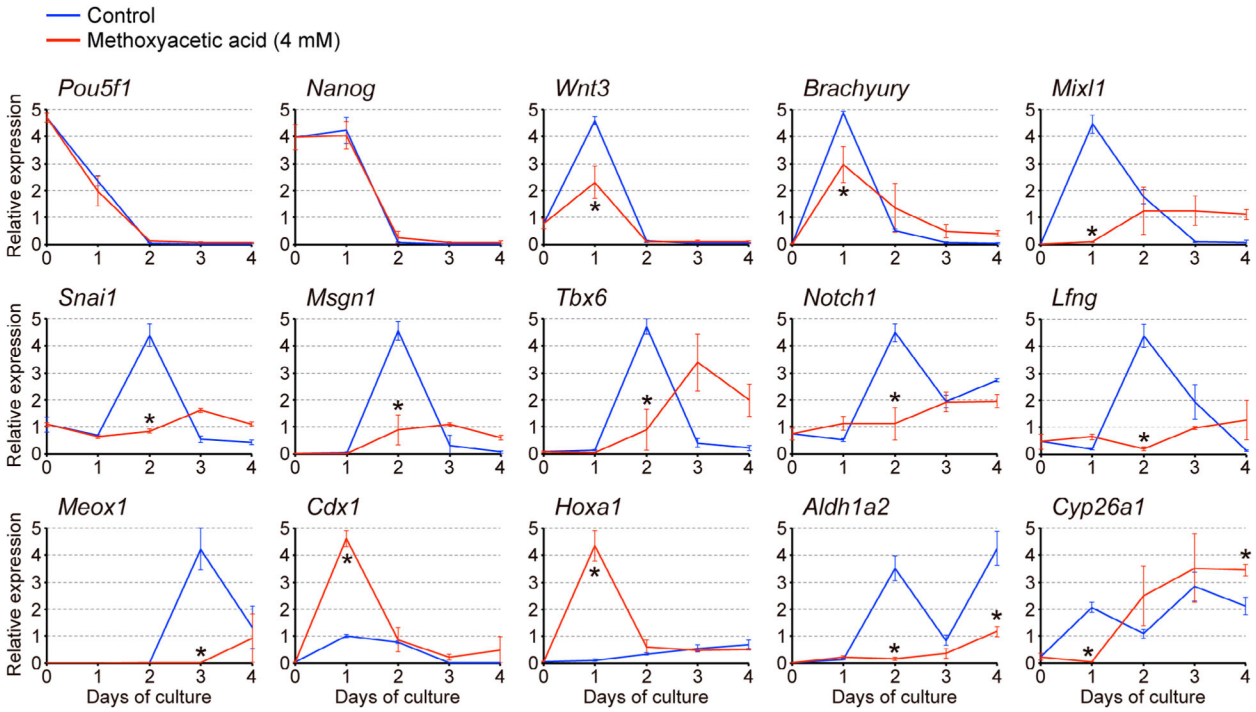


**FIGURE 3.**

Methoxyacetic acid (MAA) alters gene expression profiles in gastruloids. Temporal expression profiles of developmental regulator genes in gastruloids over a 4-day culture period, as determined by quantitative RT-PCR analyses. Horizontal axes represent days of culture, whereas vertical axes represent relative expression levels in arbitrary units, which were normalized against *Actb* as a representative housekeeping gene. Blue and red lines correspond to the relative expression levels (mean ± *SD*; $n = 3$) in control gastruloids and those treated with 4 mM MAA, respectively. Each biological replicate was a pool of nine samples: Day 0, control gastruloids at Days 1 to 4, and MAA-treated gastruloids at Days 1 to 4, all of which were prepared from the same cell suspension. Asterisks indicate a significant reduction or increase ($p < .05$) in mean relative expression levels due to MAA on a given day of culture

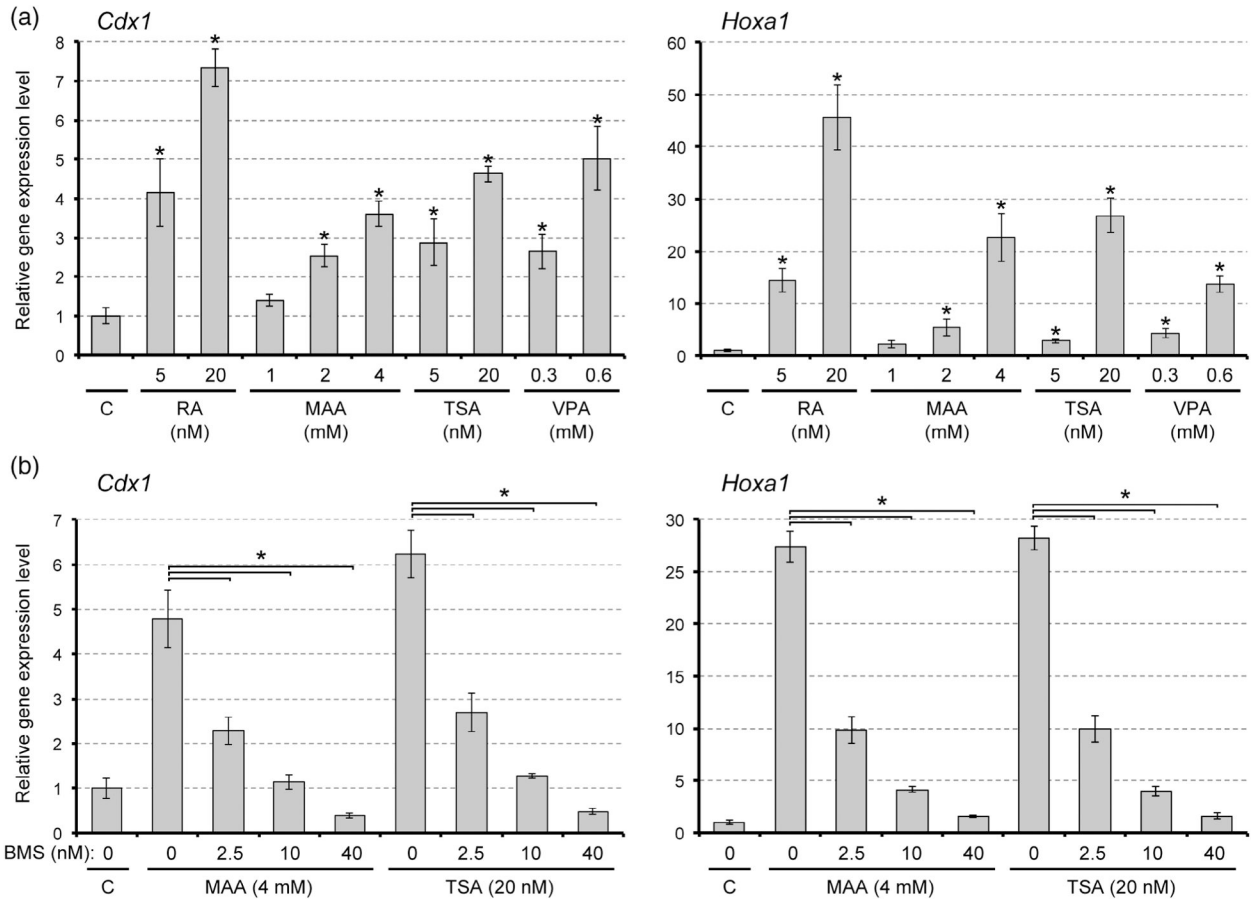


**FIGURE 4.**

Methoxyacetic acid (MAA) and inhibitors of histone deacetylase up-regulate *Cdx1* and *Hoxa1* expressions in a retinoic acid (RA) signaling-dependent manner. Vertical axis represents relative gene expression levels in monolayer culture, as measured by quantitative RT-PCR. Graphs show the relative expression levels (mean ± *SD*; $n = 3$). The control level (C) is set as one. (a) Expression levels after treatment with RA, MAA, trichostatin A (TSA), or valproic acid (VPA) for 1 day. Asterisks indicate a significant increase by a given treatment compared with the control ($p < .01$). (b) Expression levels after co-treatment with BMS493 (BMS) plus MAA or TSA for 1 day. Asterisks indicate a significant difference between the two groups specified by each horizontal line ($p < .01$)

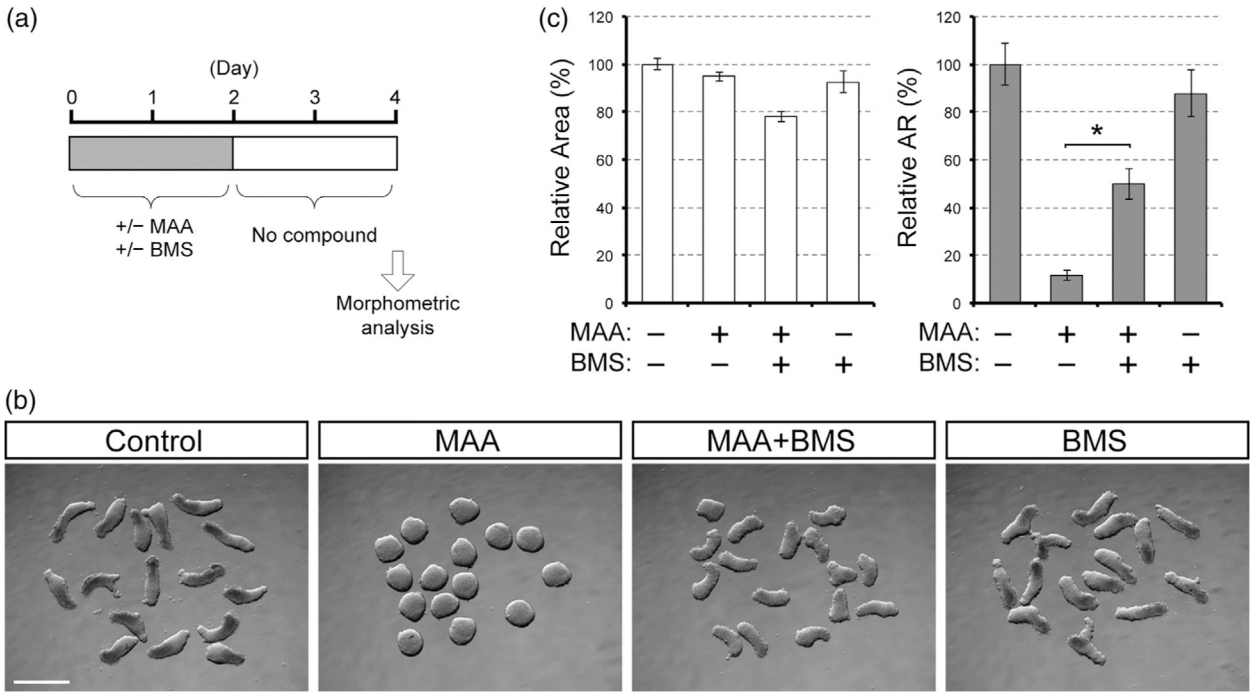


**FIGURE 5.**

The morphogenetic effect of methoxyacetic acid (MAA) is partly alleviated by the suppression of retinoic acid signaling. (a) A regimen for gastruloid treatment with MAA (4 mM) and BMS493 (BMS, 10 nM). (b) Images of Day 4 gastruloids. Scale bar = 500 μm. (c) Morphometric parameters of treated gastruloids. Graphs show the averages of relative area (left) and relative aspect ratio (AR; right) with error bars of 95% confidence interval ($n$ = 48). Asterisks indicate a significant difference ($p < .01$) between the MAA and MAA + BMS groups

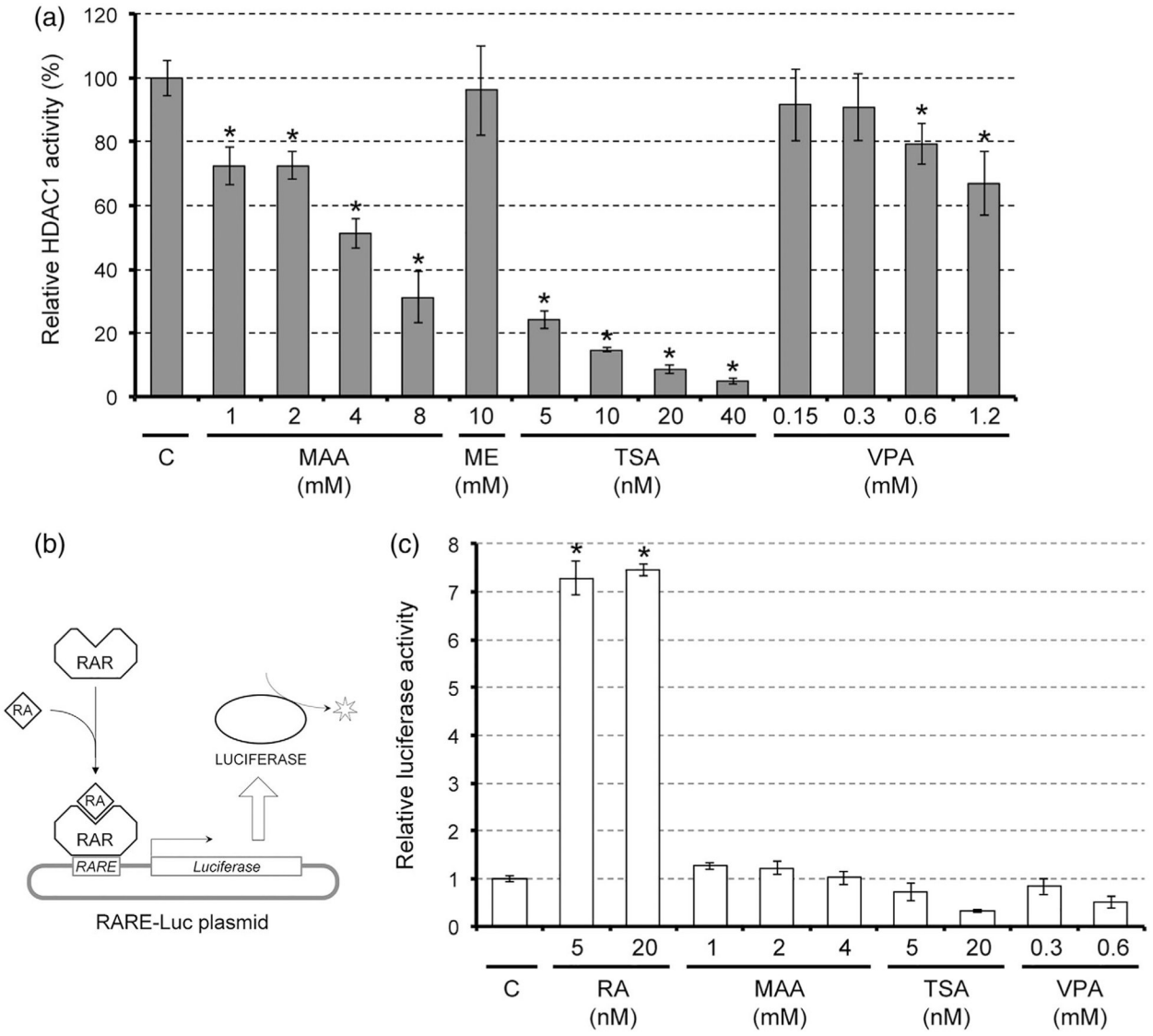


**FIGURE 6.**

Impact of methoxyacetic acid (MAA) on histone deacetylase (HDAC) activity and retinoic acid (RA) signaling. (a) HDAC activity of purified human HDAC1 protein that was incubated with MAA, methoxyethanol (ME), trichostatin A (TSA), or valproic acid (VPA). Asterisks indicate a significant decrease ($p < .05$; $n = 3$) compared to the control (c), which is set as 100%. (b) A schematic diagram, depicting how the RA signaling reporter assay using the RARE-Luc plasmid works. Upon binding of RA, the retinoic acid receptor (RAR) on the RA response element (RARE) in the plasmid activates expression of luciferase, which is then detected using a luminescent substrate. (c) Measurement of RA signaling using the RARE-Luc reporter plasmid in monolayer cells that were treated with chemicals. Graph shows the relative luciferase activity from RARE-Luc (mean ± *SD*; $n = 3$). Asterisks indicate a significant increase ($p < .01$; $n = 3$) compared with the control (C), which is set as one

**TABLE 1**

Chemicals used in the present study

| Chemical name | CASRN | Vendor (catalog number) | Stock concentration (solvent) |
|---|---|---|---|
| Methoxyacetic acid | 625-45-6 | Sigma-Aldrich (194557) | 1 M (water) |
| Methoxyethanol | 109-86-4 | Sigma-Aldrich (284467) | 12.7 M (undiluted) |
| Retinoic acid | 302-79-4 | Sigma-Aldrich (R2625) | 100 μM (DMSO) |
| Trichostatin A | 58880-19-6 | Calbiochem (647925) | 100 μM (DMSO) |
| Valproic acid | 1069-66-5 | Sigma-Aldrich (P4543) | 100 mM (water) |
| BMS493 | 215030-90-3 | Santa Cruz (sc-361124) | 10 mM (DMSO) |

Abbreviations: CASRN, chemical abstracts service registry number; DMSO, dimethyl sulfoxide.

**TABLE 2**

Developmental regulator genes examined in the present study

| Gene name | Characteristics[a] | | Primer sequences (5′ → 3′) | References |
|---|---|---|---|---|
| *Actb* | **a.** | Cytoskeletal actin | F: GAGAGGGAAATCGTGCGTGACATC<br>R: CAGCTCAGTAACAGTCCGCCTAGA | |
| | **b.** | Ubiquitous | | |
| | **c.** | House keeping | | |
| *Aldh1a2* | **a.** | Aldehyde dehydrogenase | F: CTTGCCTCACAACAAGTGAGCTTC<br>R: TCACCCAGGTTAGAGACTGGCTTC | Haselbeck et al., 1999 |
| | **b.** | Trunk region | | |
| | **c.** | Retinoic acid synthesis | | |
| *Brachyury* | **a.** | T-box transcription factor | F: CCTCGGATTCACATCGTGAGAGTT<br>R: AGTAGGTGGGCGGGCGTTATGACT | Herrmann, 1991 |
| | **b.** | Primitive streak | | |
| | **c.** | Mesendoderm specification | | |
| *Cdx1* | **a.** | Homeodomain transcription factor | F: TCAGGACTGGACATGAGGTAGAGG<br>R: TGGGAAGGTGGGCATGAGCAGGTA | Meyer & Gruss, 1993 |
| | **b.** | Primitive streak | | |
| | **c.** | Axial patterning | | |
| *Cyp26a1* | **a.** | Cytochrome P450 oxidase | F: CGGAGCTGTGTAGGCAAAGAGTTT<br>R: CCTGGAAGTGGGTAAATCTTGCAG | Sakai et al., 2001 |
| | **b.** | Posterior end | | |
| | **c.** | Retinoic acid catabolism | | |
| *Hoxa1* | **a.** | HOX transcription factor | F: CCCTTTCCTTCCACACTGTCTTGT<br>R: AAGACCCGTAAACTCTGCTCTGGA | Wellik, 2009 |
| | **b.** | Anterior class | | |
| | **c.** | Axial patterning | | |
| *Lfng* | **a.** | Notch signaling regulator | F: AAGCAGGGAGTGGTTTTATGAAGG<br>R: GGGATAGGAATGTATGAATGGGGA | Shifley et al., 2008 |
| | **b.** | Posterior end | | |
| | **c.** | Somite segmentation | | |
| *Meox1* | **a.** | Homeodomain transcription factor | F: AAAAATCAGACTTCCCAGCGACAG<br>R: TTCACACGTTTCCACTTCATCCTC | Mankoo et al., 2003 |
| | **b.** | Somitic mesoderm | | |
| | **c.** | Somite differentiation | | |

| Gene name | Characteristics[a] | | Primer sequences (5′ → 3′) | References |
|---|---|---|---|---|
| *Mixl1* | **a.** | Homeodomain transcription factor | F: CGACAGACCATGTACCCAGACATC<br>R: TGAGGCTTCAAACACCTAGCTTCA | Hart et al., 2002 |
| | **b.** | Primitive streak | | |
| | **c.** | Mesendoderm specification | | |
| *Msgn1* | **a.** | bHLH domain transcription factor | F: CCAGAAAGGCAGCAAAGTCAAGAT<br>R: TCTGTGAGTTCCCCGATGTACTTG | Yoon & Wold, 2000 |
| | **b.** | Posterior end | | |
| | **c.** | Somite differentiation | | |
| *Nanog* | **a.** | Homeodomain transcription factor | F: GCTTTGGAGACAGTGAGGTGCATA<br>R: GCTACCCTCAAACTCCTGGTCCTT | Mitsui et al., 2003 |
| | **b.** | Epiblast | | |
| | **c.** | Pluripotency maintenance | | |
| *Notch1* | **a.** | Notch signaling receptor | F: GTCTGCAGGCTCCAGTGTTCTGTA<br>R: TCAGTTGGATTTGGATGATGCTGT | Huppert et al., 2000 |
| | **b.** | Posterior end | | |
| | **c.** | Somite segmentation | | |
| *Snai1* | **a.** | Zinc finger transcription factor | F: CCGTCCAGCTGTAACCATGCCTCA<br>R: TGGGAGACACATTGGCCAGGCTGA | Carver et al., 2001 |
| | **b.** | Primitive streak | | |
| | **c.** | Mesoderm development | | |
| *Pou5f1* | **a.** | POU domain transcription factor | F: AGGCAGGAGCACGAGTGGAAAGCA<br>R: GGAGGGCTTCGGGCACTTCAGAAA | Nichols et al., 1998 |
| | **b.** | Epiblast | | |
| | **c.** | Pluripotency maintenance | | |
| *Tbx6* | **a.** | T-box transcription factor | F: GGCCTCTCTTCCACCCTTTAGTTC<br>R: CACTAGTAACAAGGCCCCCAGGAG | Chapman et al., 1996 |
| | **b.** | Posterior end | | |
| | **c.** | Axial stem cell differentiation | | |
| *Wnt3* | **a.** | Wnt signaling ligand | F: CAGATGCCCGCTCAGCTATGAACA<br>R: AGCAGCACCAGTGGAAGACGCAAT | Liu et al., 1999 |
| | **b.** | Primitive streak | | |
| | **c.** | Initiation of gastrulation | | |

[a]Characteristics are described based on: a. Molecular function, b. Major expression domains around the gastrulation stage (mouse embryonic stages from E5.5 to E8.5), and c. Functional significance in early embryo development.