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## Embryoid body test with morphological and molecular endpoints implicates potential developmental toxicity of *trans*-resveratrol

Iris Q. Kim and Yusuke Marikawa

Developmental and Reproductive Biology Graduate Program, Institute for Biogenesis Research, University of Hawaii John A. Burns School of Medicine, Honolulu, HI 96813, USA

### Abstract

Developmental toxicity of compounds, which women of reproductive age are exposed to, should be assessed to minimize the incidence of miscarriage and birth defects. The present study examined the potential developmental toxicity of resveratrol, a dietary supplement widely marketed with various health claims, using the P19C5 embryoid body (EB) morphogenesis assay, which evaluates adverse effects of chemical exposures on tissue growth and axial elongation. Resveratrol (*trans* isoform) impaired morphogenesis at 4  $\mu\text{M}$  and higher, creating smaller and rounder EBs, whereas *cis* isoform, and glucuronated and sulfonated metabolites did not. *Trans*-resveratrol also altered expression levels of developmental regulator genes involved in embryonic patterning, such as *Wnt3a*, *Tbx6*, and *Cyp26a1*. To investigate the mechanisms of *trans*-resveratrol action, the roles of estrogen receptor, sirtuin 1 (SIRT1), and DNA replication in EB morphogenesis were examined. Neither activators of estrogen receptors (diethylstilbestrol [18  $\mu\text{M}$ ] and raloxifene [8  $\mu\text{M}$ ]) nor activator of SIRT1 (SRT1720 [2.4 – 3.2  $\mu\text{M}$ ]) caused morphological and molecular alterations that are comparable to *trans*-resveratrol (10  $\mu\text{M}$ ). By contrast, a reduction in the DNA replication rate with aphidicolin (0.4  $\mu\text{M}$ ) or hydroxyurea (40  $\mu\text{M}$ ) created smaller and rounder EBs and altered the expression levels of *Wnt3a*, *Tbx6*, and *Cyp26a1* in a manner similar to *trans*-resveratrol. Consistently, *trans*-resveratrol significantly reduced the rate of EdU incorporation in P19C5 cells. These results suggest that a reduction in the DNA replication rate is one of the mechanisms by which *trans*-resveratrol impacts EB development. This study provides mechanistic insight for further investigations on the developmental toxicity of *trans*-resveratrol.

### Keywords

Resveratrol; Dietary supplement; Developmental toxicity; Morphogenesis; *In vitro* assay; Stem cell

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Corresponding author: Yusuke Marikawa, 651 Ilalo Street, Biosciences Building, 163A, Honolulu, HI 96813, Tel: (808) 692-1411, Fax: (808) 692-1962. marikawa@hawaii.edu.

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## 1. Introduction

Numerous chemical compounds exhibit developmental toxicity, causing embryonic death or malformation in the reproductive tract of the pregnant mother. Whether a given compound adversely affects embryo development depends on its chemical nature as well as concentration, duration, and timing of exposure *in utero* (Jelinek, 2005; Schardein and Macina, 2006; Daston et al., 2010; Friedman, 2010). Pharmaceutical compounds are usually investigated for their developmental toxicity through experimentations using pregnant model animals and through human case and cohort studies. By contrast, most non-pharmaceutical compounds, such as herbicides, pesticides, cosmetics, industrial byproducts, excipients, and dietary supplements, are much less studied for developmental toxicity. Because the amount and frequency of exposure are not strictly regulated or monitored for non-pharmaceutical compounds, it is difficult to assess their developmental toxicity through human studies. Although experimentations with pregnant animals are routinely conducted in developmental toxicity research, testing of individual non-pharmaceutical compounds would sacrifice enormous numbers of animals, which is not only costly but also unethical from an animal welfare standpoint. To reduce such burden of animal-based research, non-animal alternatives, namely *in vitro* tests, are highly desired to evaluate the developmental toxicity of compounds. While *in vitro* tests alone may not be sufficient to fully predict potential harm to embryos *in utero*, they can yield valuable information on concentration-response relations between specific adverse outcomes and compound exposures. *In vitro* tests can also provide insight into the mechanisms of developmental toxicity, because they are more amenable to molecular interrogations than *in vivo* tests. The information obtained from *in vitro* tests can serve as the foundation for designing animal- and human-based studies in an effective manner.

One of the *in vitro* tests to evaluate the developmental toxicity of compounds utilizes embryoid body morphogenesis of the mouse P19C5 stem cell line (reviewed in Marikawa, 2018). P19C5 cells possess developmental characteristics similar to the epiblast, the pluripotent embryonic precursor of the entire fetal body. P19C5 cells can be induced to differentiate *in vitro* as embryoid bodies (EBs) by aggregation culture in hanging drops. During the first two days of culture, P19C5 EBs grow as spherical cell aggregates. By the fourth day of culture, EBs have transformed into an elongated shape with a distinct morphological polarity (Lau and Marikawa, 2014). Spatial and temporal gene expression profiles suggest that the *in vitro* development of EBs represents gastrulation, the morphogenetic process of body patterning and elongation along the cranial-caudal embryonic axis. Morphological and molecular changes in EBs are controlled by key morphogenetic signals, such as Wnt, Nodal, Fgf, and retinoic acid, in a manner consistent with their regulatory roles in gastrulation (Li and Marikawa, 2015). Importantly, *in vitro* development of P19C5 EBs is impaired by chemical exposures that are known to cause developmental toxicity *in vivo* (Warkus et al., 2016; Warkus and Marikawa, 2017). As a reference list for developmental toxicity validation, Daston et al. (2014) compiled 39 chemical exposures, i.e., *in vivo* concentrations of specific compounds that exhibit adverse effects on embryos or lack thereof. EB growth and morphogenesis, which are quantitatively measured using morphometric parameters of EBs at the end of 4-day culture, are

significantly altered by the adverse exposures of the Daston reference list, but not by the non-adverse exposures, with a total concordance of 71.4 to 82.9% (Warkus and Marikawa, 2017). P19C5 EBs also provide insight into the molecular mechanisms of developmental toxicity through the examination of how gene expression profiles are altered by chemical exposures (Li and Marikawa, 2016; Warkus and Marikawa, 2018). Thus, P19C5 EBs can be effectively used as an *in vitro* model to investigate the developmental toxicity of compounds.

The objective of the present study is to examine the developmental toxicity of a dietary supplement, resveratrol, using the P19C5 EB model. Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a natural compound produced by several plants, including grapes and blueberries (Signorelli and Ghidoni, 2005). Numerous *in vitro* and animal studies have implicated the beneficial effects of resveratrol against various diseases, such as cancers, cardiovascular diseases, inflammatory diseases, and diabetes (Baur and Sinclair, 2006; Park and Pezzuto, 2015). Several molecules have been suggested as targets of resveratrol, including the estrogen receptor (Gehm et al., 1997; Bowers et al., 2000), sirtuin 1 (SIRT1; Howits et al., 2003), phosphodiesterase (PDE; Park et al., 2012), AMP-activated protein kinase (AMPK; Baur et al., 2006), DNA polymerases (Stivala et al., 2001), and ribonucleotide reductase (Fontecave et al., 1998). Nonetheless, the molecular mechanisms underlying the therapeutic properties of resveratrol are still elusive (Kulkarni and Cantó, 2015). While most studies on resveratrol focus on its beneficial effects, the information on its potential developmental toxicity is scarce. Most anti-cancer drugs that are approved by the Food and Drug Administration (FDA) are placed under the Pregnancy Risk Category X, i.e., contraindicated for use during pregnancy due to their developmental toxicity. Because resveratrol suppresses proliferation and survival of various types of cancer cells (Jang et al., 1997; Park and Pezzuto, 2015; Singh et al., 2015), it is possible that developing embryos may also be susceptible to resveratrol exposure. Resveratrol is sold as a dietary supplement with health benefit claims that are not approved by the FDA, and is consumed by people, including women of reproductive age, without a physician's prescription or monitoring. As a result, human studies are essentially impossible to establish an association between resveratrol intake and adverse reproductive outcomes. Intensive investigations are warranted through well-controlled experiments to examine the developmental toxicity of resveratrol. Specifically, the elucidation of the lowest-observed-adverse-effect level (LOAEL) that impairs embryo development and the molecular mechanisms underlying the adverse effects should help assess a potential risk of resveratrol for developing embryos.

Here, we first determined the concentration-response relationship of resveratrol, based on the morphogenetic impact on P19C5 EBs. Next, we examined gene expression profiles of resveratrol-treated EBs to evaluate which developmental regulators were affected by exposure to resveratrol. Lastly, we compared the morphological and molecular impact of resveratrol with other compounds, namely modulators of the estrogen receptor, SIRT1 activity, and DNA replication, to gain insight into the molecular mechanisms underlying the adverse effects of resveratrol. The data provided by the present study serve as a stepping-stone for future studies on the developmental toxicity of resveratrol, which should help determine whether this dietary supplement is to be consumed, and if so how much, by women of reproductive age.

## 2. Materials and methods

### 2.1. Chemicals

All chemical compounds used in the present study were commercially obtained, namely from Selleck Chemicals (Houston, TX), Santa Cruz Biotechnology (Dallas, TX), Sigma-Aldrich (St. Louis, MO), and Cayman Chemical (Ann Arbor, MI), and their details are described in Table 1. Chemical structures of some of the compounds are shown in Fig. 1, A. Note that *trans*-resveratrol was obtained from three different suppliers to confirm the consistency of the morphogenetic and molecular effects. One of them was a part of the Anti-Diabetic Compound Library (Selleck Chemicals), which consists of 33 chemicals that are widely used for diabetes research and treatment, including *trans*-resveratrol. The preparation of *cis*-resveratrol may contain 1–5% of *trans*-resveratrol, according to the product information of the supplier ([www.caymanchem.com/product/10004235](http://www.caymanchem.com/product/10004235)).

### 2.2. Cell culture

P19C5 cells were propagated and used to generate embryoid bodies (EBs), according to the method previously described (Lau and Marikawa, 2014). Briefly, cells were dissociated with Trypsin-EDTA, and suspended in the culture medium at the density of 10 cells/ $\mu\text{L}$  with the specified amount of a test chemical, containing the final concentration of 1% dimethyl sulfoxide (DMSO). Drops (20  $\mu\text{L}$  each) of cell suspension were spotted on the inner surface of Petri dish lids for hanging drop culture. EBs were removed from hanging drops for morphometric analyses at Day 4 of culture and gene expression analyses at Days 0, 1, 2, 3, and 4 (Fig. 1, B). Experiments to assess the morphological impact on EBs by chemical treatments were conducted in three biological replicates using different collections of cell suspensions. For each replicate, 16 hanging drops were generated per treatment in parallel with 16 control (i.e., 1% DMSO only) hanging drops.

### 2.3. Morphometric analyses

EBs were placed together in a dish filled with phosphate-buffered saline for photography, using an AxioCam MRm digital camera connected to an Axiovert 200 microscope with Hoffman modulation-contrast optics (Carl Zeiss, Thornwood, NY). Image files were converted to JPEG format and opened in the ImageJ program (<http://rsb.info.nih.gov/ij>) for morphometric analyses. Morphological parameters, specifically area and circularity ( $= 4 \times \pi \times \text{area} / \text{perimeter}^2$ ), were measured on ImageJ by manually tracing the circumference of individual EBs using the polygon selection tool. Circularity was converted to Elongation Distortion Index ( $\text{EDI} = 1 / \text{circularity} - 1$ ), which is more reflective of the extent of EB elongation: the more an EB elongates, the higher its EDI (Marikawa et al., 2009). Area was used as a proxy for the size of EB, whereas EDI was used to gauge the extent of EB axial elongation. Because the average area and EDI of control EBs were slightly different among experimental replicates, normalization was performed against the average values of control EBs in each replicate to calculate relative area and relative EDI, expressed as a percentage (i.e., control = 100%). Relative area and relative EDI data from all replicates were compiled, and their averages are shown with standard deviation. In the present study, a chemical treatment was defined as having an adverse morphogenetic effect when Day 4 EBs exhibited

average relative area and/or average relative EDI that was lower than that of control by more than 30%.

#### 2.4. Gene expression analyses

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the relative expression levels of developmental regulator genes (Table 2). Total RNA was extracted from EBs using TRI reagent (Sigma-Aldrich) 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 sec, 60°C for 20 sec, and 72°C for 40 sec. 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  $\beta$ -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 the previous studies to evaluate gene expression levels in P19C5 EBs under various experimental conditions (Lau and Marikawa, 2014; Li and Marikawa, 2015, 2016; Yuan and Marikawa, 2017; Warkus and Marikawa, 2018). In addition, microarray analyses of EBs at Days 0 (dissociated cells immediately before aggregation), 1, 2, and 4 showed that the Actb transcript was expressed at a similar level during the course of development (Supplementary material). 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 EBs at Days 1 to 4, and compound-treated EBs 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 and Marikawa, 2018), and the averages of the three replicates are shown with standard deviations.

#### 2.5. Cell viability assay

Impact on cell proliferation and viability was assessed with the CellTiter-Glo Luminescent Cell Viability Assay System (Promega), as described previously (Warkus et al., 2016). Briefly, cells were seeded in 96-well plates at a density of 100 cells/well in 100  $\mu$ L of culture medium containing 1% DMSO with or without a test compound. After 4 days of culture, cells were treated with CellTiter-Glo Reagent. The resulting luminescence was measured as a readout of ATP amount, which serves as a quantitative proxy for the number of metabolically active cells. The intensity of the luminescence was normalized to the control level (1% DMSO without a test compound) in each set of experiments and reported as relative light units. All experiments were repeated independently three times, and the results are shown as mean  $\pm$  standard deviation.

#### 2.6. SIRT1 activity assay

SIRT1 activity was measured using the SIRT-Glo Assay System (Promega), which utilizes an acetylated, cell-permeable, luminogenic peptide as an SIRT substrate, according to the manufacturer's instruction. Briefly, purified SIRT1 enzyme (Cayman Chemical) was incubated with EX527 at various concentrations for 30 minutes at room temperature, and

then mixed with the substrate, followed by luminescence measurement. The intensity of the luminescence was normalized to the control level (no EX527; set as 100%) in each experiment and reported as SIRT1 activity. Experiments were repeated independently three times, and the results are shown as mean  $\pm$  standard deviation.

## 2.7. EdU assay

The efficiency of DNA replication was determined based on incorporation of EdU (5-ethynyl-2'-deoxyuridine) using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Molecular Probes, Eugene, OR), according to the manufacturer's instruction. Cells were plated in 24-well plates at the density of 45,000 cells/well, and cultured in 450  $\mu$ L of medium containing a test compound or vehicle only (1% DMSO). 24 hours later, 50  $\mu$ L of culture medium containing 1 mM EdU was added into each well, and cultured for one more hour. Cells were then fixed in 3.7% formaldehyde, and stained for EdU (with Alexa Fluor 488 Azide) and nucleus (with Hoechst 33342). Images of the EdU and nuclear staining were captured with a fluorescence microscope using the same exposure length for all specimens of the same set of experiment (i.e., control and compound-treated cells), and opened in ImageJ for further analyses. The mean signal intensity of the EdU staining was normalized by the total nuclear area (measured after binary conversion of the Hoechst 33342 signal), and was reported as relative EdU intensity. Experiments were repeated independently three times, and the results are shown as mean  $\pm$  standard deviation.

## 2.8. Statistical analyses

All adverse morphogenetic effects shown in the present study were statistically significant ( $P < 0.01$ ), based on two-sample t-test that was performed between compound-treated group and the control group. For temporal gene expression analyses, two-sample t-test was performed between control and compound-treated groups for each time point to determine significant changes in relative expression levels ( $P < 0.05$ ). For comparisons at Day 2, relative expression levels were normalized against the control in each set of experiment, and the averages of the three replicates were compared between the two treatment groups by two-sample t-test to determine significant differences in relative expression levels ( $P < 0.05$ ). For cell viability assay, SIRT1 activity assay, and EdU assay, two-sample t-test was performed between control and compound-treated groups to determine significant differences ( $P < 0.01$ ).

## 3. Results

### 3.1. Embryoid body morphogenesis is impacted by trans-resveratrol

P19C5 EBs were exposed to individual chemicals of the Anti-Diabetic Compound Library, including *trans*-resveratrol (Fig. 1, A; hereafter simply referred to as "resveratrol" unless otherwise stated) at the concentrations of 0.1, 1, 10, and 100  $\mu$ M, and analyzed for morphometric parameters (area and EDI) at Day 4. For resveratrol, adverse morphogenetic effects (see Materials and methods) were observed when EBs were exposed to 10  $\mu$ M or higher, but not to 1  $\mu$ M or lower concentrations (Fig. 2, A). At exposure to 10  $\mu$ M, EBs were smaller (i.e., lower relative area) and rounder (i.e., lower relative EDI) than the control. Cells did not survive to form EBs when treated at 100  $\mu$ M. To further refine the concentration-

response relationship, EBs were exposed to 2, 4, 6, 8, and 10  $\mu\text{M}$  of resveratrol (Fig. 2, B, C). Here, we used another lot of resveratrol from a different supplier to verify the morphogenetic effects (Table 1). At 10  $\mu\text{M}$ , EBs were smaller and rounder (Fig. 2, C), similar to the effects observed with the compound from the library. Resveratrol also yielded adverse morphogenetic effects at 4  $\mu\text{M}$  and higher, showing concentration-dependent gradual reduction in both area and EDI (Fig. 2, B). The smaller EB size raises the possibility that cell proliferation was reduced by resveratrol during hanging drop culture. Consistently, resveratrol also diminished proliferation of undifferentiated P19C5 cells, as the numbers of viable cells were significantly lower in monolayer culture after 4 days of treatment at 4  $\mu\text{M}$  or higher (Fig. 2D).

To examine specificity of the resveratrol effects, we evaluated the morphogenetic impact of other related chemicals, namely a geometric isomer (*cis*-resveratrol) and major metabolites of *trans*-resveratrol (resveratrol 3-glucuronide, resveratrol 4'-glucuronide, and resveratrol 3-sulfate) (Fig. 1, A). *cis*-Resveratrol is found in various plants along with *trans*-resveratrol, whereas the metabolites are generated in the liver by glucuronidation and sulfation after ingestion of *trans*-resveratrol (Baur and Sinclair, 2006). We examined the morphogenetic effects of these chemicals at 1, 10, and 100  $\mu\text{M}$ , in parallel with another lot of *trans*-resveratrol that was obtained from the same supplier for the *cis*-isomer and the metabolites (Table 1). Consistent with the earlier observations, *trans*-resveratrol caused adverse morphogenetic effects at 10  $\mu\text{M}$  (smaller and rounder EBs) and at 100  $\mu\text{M}$  (cell death), but not at 1  $\mu\text{M}$ . By contrast, *cis*-resveratrol adversely affected EB morphogenesis only at 100  $\mu\text{M}$  (smaller and rounder) (Fig. 2, E). Note that according to the product information from the supplier, the preparation of *cis*-resveratrol may contain 1–5% of *trans*-resveratrol (see Materials and methods). Thus, it is unclear whether the adverse effects caused by 100  $\mu\text{M}$  of *cis*-resveratrol were partly due to the *trans*-resveratrol contaminant, which was possibly present at 1–5  $\mu\text{M}$ . Regardless, the result clearly indicates that *cis*-resveratrol was much less potent than *trans*-resveratrol to cause adverse morphogenetic effects. No adverse effect was observed with any of the three metabolites at all the concentrations tested (Fig. 2, E), suggesting that they are much less potent than unmetabolized *trans*-resveratrol in causing developmental toxicity. The cytostatic effect to reduce proliferation of undifferentiated cells was also specific to *trans*-resveratrol, as shown by the cell viability assay in monolayer culture (Fig. 2, D)

### 3.2. Resveratrol alters gene expression profiles in embryoid bodies

To gain mechanistic insight into how resveratrol exerts the adverse morphogenetic effects, we compared gene expression profiles between control and resveratrol-treated EBs. Resveratrol was evaluated at 10  $\mu\text{M}$  for this study, because this concentration robustly affected both relative area and relative EDI. Twelve developmental regulator genes were examined, whose functions and expression patterns during mouse gastrulation are summarized in Table 2.

In control EBs, a pluripotency maintenance factor (*Pou5f1*) was markedly down-regulated by Day 1, and the genes critical for the initial stage of gastrulation (*Wnt3*, *Brachyury*, *Cdx1*, and *Fgf8*) were up-regulated with the highest peak at Day 1 (Fig. 3, A). The expression

patterns of these genes were essentially unaffected by resveratrol treatment, although the peak level of *Fgf8* was slightly reduced with statistical significance. By contrast, drastic alterations by resveratrol were observed for the genes that control the differentiation of axial stem cells at the posterior end during the later stages of gastrulation (*Wnt3a* and *Tbx6*). The Day 2 peak expression of these genes was markedly diminished by resveratrol. For the HOX genes, which are the key regulators of anterior-posterior axial patterning, the anterior class (*Hoxa1*) was mostly unaffected, whereas the central class (*Hoxc6*) and the posterior class (*Hoxb9*) were diminished on Days 3 and 4 by resveratrol. During embryogenesis, expression of HOX genes and other axial patterning genes are regulated by retinoic acid signaling, whose activity and localization are strictly controlled by synthesis (involving *Aldh1a2*) and degradation (involving *Cyp26a1*) of retinoic acid (Piersma et al., 2017). In EBs, the expression pattern of *Aldh1a2* was mostly unaffected by resveratrol. By contrast, the expression level of *Cyp26a1* was strikingly elevated on Days 1 and 2 (Fig. 3, A). Thus, the expression patterns of developmental regulators were differentially affected by resveratrol. Specifically, genes crucial for the early stage of gastrulation were largely unaffected, whereas those functioning in the posterior end at the later stages were significantly altered.

We then examined whether gene expression profiles are altered by lower concentrations of resveratrol that affected EB morphogenesis mildly (6  $\mu$ M) or did not affect significantly (2  $\mu$ M) (Fig. 2, B). Analyses were focused on the expression levels of *Wnt3a*, *Tbx6*, and *Cyp26a1* on Day 2, because they were most dramatically affected by 10  $\mu$ M resveratrol. Neither of *Wnt3a*, *Tbx6* nor *Cyp26a1* was significantly altered by 2  $\mu$ M resveratrol compared to control (0  $\mu$ M). However, 6  $\mu$ M resveratrol reduced *Wnt3a* and increased *Cyp26a1* significantly, but by a lesser extent than 10  $\mu$ M resveratrol (Fig. 3, B). Thus, the concentration-response relationship of resveratrol to alter gene expression levels was largely in line with the morphogenetic effects.

We also examined the impact of the *cis* isoform and the *trans*-resveratrol metabolites on the expression of *Wnt3a*, *Tbx6*, and *Cyp26a1*. While *trans*-resveratrol consistently altered the levels of *Wnt3a*, *Tbx6*, and *Cyp26a1*, none of the other compounds did (Fig. 3, C). This result further corroborates that the molecular changes caused by resveratrol correlate with its morphogenetic effects.

### 3.3. Estrogen receptor-modulating agents differ from resveratrol in the molecular impact

Resveratrol resembles the structure of an agonist of the estrogen receptor, diethylstilbestrol (DES; Fig. 1, A), and has been shown to activate the estrogen receptor (Gehm et al., 1997; Bowers et al., 2000). As shown in the previous study, DES impairs P19C5 EB morphogenesis (Warkus et al., 2016). EB morphogenesis is also affected by raloxifene (Fig. 1, A; Warkus et al. 2016), which is a selective estrogen receptor modulator (SERM) that potentiates activation of the estrogen receptor. This raises the possibility that the developmental toxicity induced by resveratrol may be mediated through activation of the estrogen receptor. To test this possibility, we evaluated the molecular impacts of DES and raloxifene using the P19C5 EB model.

First, the dose-response relationships were refined for the two agents to determine which concentrations exert adverse morphogenetic effects similar to 10  $\mu$ M resveratrol (i.e., average



relative area = 40–50% and average relative EDI = 20–30%; Fig. 2, B). Exposures to DES at 18  $\mu\text{M}$  and raloxifene at 8  $\mu\text{M}$  yielded smaller and rounder EBs that were comparable to those exposed to resveratrol (Fig. 4, A, B), although raloxifene-treated EBs appeared more opaque compared to resveratrol- or DES-treated EBs.

Next, we examined the gene expression profiles in EBs treated with DES at 18  $\mu\text{M}$  and raloxifene at 8  $\mu\text{M}$ . Expression analyses were focused on the genes that were significantly altered by resveratrol, specifically *Fgf8*, *Wnt3a*, *Tbx6*, *Hoxc6*, *Hoxb9*, and *Cyp26a1* (Fig. 3, A). DES increased the level of *Fgf8* on Day 2, reduced *Wnt3a* at Day 2, reduced *Tbx6* on Day 2, and reduced *Cyp26a1* on Day 1 (Fig. 4, C). No statistically significant alteration was observed for *Hoxc6* and *Hoxb9*. For raloxifene treatment, *Fgf8* was increased on Day 2, whereas *Cyp26a1* was reduced on Day 1 (Fig. 4, D). The other genes, including *Wnt3a* and *Tbx6*, were not significantly affected by raloxifene. When compared with the effects of resveratrol, the molecular impacts of DES and raloxifene were markedly different (Fig. 4, E). Out of 9 types of significant alterations caused by resveratrol, only 2 (i.e., reduction in the *Wnt3a* and *Tbx6* levels at Day 2) were shared by DES and none was shared by raloxifene. Most prominently, *Cyp26a1* was markedly elevated by resveratrol on Day 1, whereas it was reduced by DES and raloxifene. Thus, despite the similarity in morphogenetic effects (i.e., creating smaller and rounder EBs), resveratrol altered gene expression profiles differently from DES and raloxifene, suggesting that the developmental toxicity of resveratrol is not mediated by activation of the estrogen receptor.

#### 3.4. Activation of SIRT1 is not sufficient to cause morphogenetic or molecular effects similar to resveratrol

SIRT1, which is a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, was initially implicated as a direct target of resveratrol (Howitz et al., 2003), although more recent studies suggest that this is not the case (Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). Regardless, activation of SIRT1 appears to be essential for the actions of resveratrol in several experimental settings (Kulkarni and Cantó, 2015). Thus, we investigated whether activation of SIRT1 is involved in the effects of resveratrol in P19C5 EBs.

First, we examined whether SRT1720, a pharmacological activator of SIRT1 (Milne et al., 2007), exhibits morphogenetic and molecular effects comparable to resveratrol. While various concentrations of SRT1720 were evaluated, none of them were able to make a morphological impact similar to resveratrol. At 2.4  $\mu\text{M}$  or lower, SRT1720 did not show adverse morphogenetic impact (Fig. 5, A). By contrast, no cells survived to form EBs when exposed to 3.6  $\mu\text{M}$  of SRT1720. At intermediate concentrations (2.8 and 3.2  $\mu\text{M}$ ), mixed responses were observed: cells in some hanging drops did not survive to form EBs (i.e., dead), whereas those in the other drops formed EBs (i.e., live) (Fig. 5, B). Notably, EBs that survived were relatively in good shape. No adverse morphogenetic effects were observed for EBs exposed to 2.8  $\mu\text{M}$ , whereas those exposed to 3.2  $\mu\text{M}$  exhibited reduced relative EDI, although it was not as extensive as resveratrol (Fig. 5, C). Furthermore, unlike resveratrol, SRT1720 treatments (2.4, and 3.2  $\mu\text{M}$ ) did not significantly alter the expression levels of

*Wnt3a*, *Tbx6*, and *Cyp26a1* (Fig. 5, D). These results suggest that activation of SIRT1 is not sufficient to account for the adverse effects of resveratrol.

Next, we tested whether activation of SIRT1 is required for the effects of resveratrol. EX527 is a pharmacological inhibitor of SIRT1 (Napper et al., 2005), and has been shown to alleviate the impact of resveratrol in several experimental systems (Kim et al., 2011; Price et al., 2012; Desquiret-Dumas et al., 2013; Joe et al., 2015; Safaeinejad et al., 2017). For example, the effects of resveratrol (25  $\mu$ M) to promote mitochondrial function are abrogated by cotreatment with EX527 (10  $\mu$ M) in skeletal muscle cells (Price et al., 2012). Thus, we investigated whether co-treatment with EX527 can rescue resveratrol-treated EBs from the adverse effects. EX527 that we obtained from a chemical supplier was effective in inhibiting the SIRT1 activity in an *in vitro* assay at the concentrations between 2.5  $\mu$ M (by >65%) and 40  $\mu$ M (by >95%) (Fig. 5, E). However, treatment of EBs with EX527 alone at the high concentration (40  $\mu$ M) caused adverse morphogenetic effects (Fig. 5, F). Therefore, for the rescue experiment, we used low (2  $\mu$ M) and medium (10  $\mu$ M) concentrations of EX527, the latter of which was still able to inhibit the SIRT1 activity by about 90% (Fig. 5, E). Nonetheless, both concentrations of EX527 did not significantly alleviate the morphogenetic effects of resveratrol (Fig. 5, G, H). By contrast, the molecular impact of resveratrol was slightly but significantly alleviated by EX527 at 10  $\mu$ M. The repressed expression of *Wnt3a* and *Tbx6* by resveratrol was slightly restored by co-treatment with EX527, whereas the heightened expression of *Cyp26a1* was slightly diminished (Fig. 5, I). These results suggest that the activity of SIRT 1 is required, to a certain extent, for the molecular impact of resveratrol on EBs.

### 3.5. Resveratrol reduces the rate of DNA replication to cause morphogenetic and molecular effects

To further explore possible targets linked to the effects of resveratrol, we evaluated the role of DNA replication. Resveratrol has been shown to directly bind to and inhibit the activity of DNA polymerases (Stivala et al., 2001; Locatelli et al., 2005). Resveratrol also inhibits ribonucleotide reductase, which is an enzyme required to generate deoxyribonucleotides for DNA replication (Fontecave et al., 1998). Direct actions of resveratrol on these enzymes on DNA replication have been suggested to account for the anti-cancer activity of resveratrol (Pirola and Fröjdö, 2008).

To examine how a reduction in the DNA replication rate may affect the EB development, we employed aphidicolin and hydroxyurea, which are pharmacological inhibitors of DNA polymerases and ribonucleotide reductase, respectively. These inhibitors exhibited morphogenetic effects on EBs in a concentration-dependent manner (Fig. 6, A, B). EBs treated with aphidicolin at 0.4  $\mu$ M or hydroxyurea at 40  $\mu$ M were smaller and rounder, similar to resveratrol treatment at 10  $\mu$ M (i.e., relative area = < 60% and relative EDI = < 30%). Thus, we analyzed gene expression profiles of EBs treated with aphidicolin and hydroxyurea at these concentrations, focusing on *Fgf8*, *Wnt3a*, *Tbx6*, *Hoxc6*, *Hoxb9*, and *Cyp26a1*. The expression patterns of these genes were significantly altered by both aphidicolin and hydroxyurea (Fig. 6, C, D). When compared with resveratrol, the impacts of aphidicolin and hydroxyurea were markedly similar. Out of 9 types of significant alterations

caused by resveratrol, 7 were shared by aphidicolin and 6 were shared by hydroxyurea, including the dramatic up-regulation of *Cyp26a1* at Days 1 and 2 (Fig. 6, E). These observations raise the possibility that the major impact of resveratrol is a reduction in the rate of DNA replication, comparable to aphidicolin and hydroxyurea.

To assess whether resveratrol indeed impairs DNA replication in P19C5 cells, we examined its impact on the incorporation of EdU, a deoxyribonucleotide analog. Efficiency of EdU incorporation was significantly lower than control, when cells were treated with resveratrol (10  $\mu$ M), aphidicolin (0.4  $\mu$ M), or hydroxyurea (40  $\mu$ M) (Fig. 7, A, B). By contrast, neither DES (18  $\mu$ M) nor raloxifene (8  $\mu$ M) significantly reduced EdU incorporation, even though both types of exposures created smaller EBs (Fig. 4, A, B) and diminished cell proliferation in monolayer culture (Fig. 7, C). These results suggest that resveratrol reduces the rate of DNA replication, possibly through inhibition of DNA polymerase and/or ribonucleotide reductase, to cause the distinct morphogenetic and molecular effects in EBs.

#### 4. Discussion

In the present study, the potential developmental toxicity of resveratrol was evaluated using the P19C5 EB morphogenesis model. Resveratrol has been marketed as a dietary supplement with numerous health benefit claims, such as protective effects against cancers, cardiovascular diseases, diabetes, and aging (Baur and Sinclair, 2006; Park and Pezzuto, 2015). Animal studies have also suggested that resveratrol provides protective effects for embryos against teratogenic insults, such as exposures to ethanol, dioxin, and maternal diabetes (Jang et al., 2008; Kumar et al., 2011; Singh et al., 2011, 2012; Vega et al., 2016; Bariani et al., 2017). However, the developmental toxicity of resveratrol by itself has not been thoroughly investigated. Whether a given compound exhibits beneficial or adverse developmental effects is dependent on the dosages or concentrations of exposures (Daston et al., 2010). Thus, it is crucial to determine dose-response relationships for adverse impact of resveratrol as well as other dietary supplements that may be consumed by women of reproductive age. *In vitro* tests, such as the EB morphogenesis assay, are generally less costly and more humane than animal experimentations, so they may be used as alternatives at the initial stage of investigations to evaluate the LOAEL and molecular mechanisms of potential developmental toxicity, as in the present study. The information acquired from *in vitro* tests would be valuable in designing adequate and effective *in vivo* studies with animals and humans.

Adverse morphogenetic effects were observed when P19C5 EBs were treated with *trans*-resveratrol at 4  $\mu$ M and higher, raising the possibility that *in utero* exposure to such concentrations would impair embryo development. The key question is whether such concentrations actually occur in people who take resveratrol as a dietary supplement. Pharmacokinetic studies in humans have shown that orally taken resveratrol is efficiently absorbed, but is rapidly metabolized into glucuronated and sulfated forms (Walle et al., 2004; Sergides et al., 2016), which in the present study did not exhibit adverse impact on EBs. Nonetheless, a significant amount of the unmetabolized form, *trans*-resveratrol, is still found in the plasma. Based on the studies conducted with healthy volunteers, a single oral dose of 1 and 5 g *trans*-resveratrol resulted in average peak plasma concentrations ( $C_{\max}$ ) of

unmetabolized resveratrol at 0.4  $\mu\text{M}$  and 2.4  $\mu\text{M}$ , respectively (Boocock et al., 2007). Repeated oral intakes of 1 and 5 g per day for 29 days resulted in higher  $C_{\text{max}}$  of *trans*-resveratrol, namely 0.6  $\mu\text{M}$  and 4.2  $\mu\text{M}$ , respectively (Brown et al., 2010), which are not much lower than those which affected EB development. Similar concentrations may occur in developing embryos, as resveratrol has been shown to cross the placenta in rodents and non-human primates (Bourque et al., 2012; O'Tierney-Ginn et al., 2015). It is of note that the plasma concentrations in the above studies were measured in healthy individuals. Some people may have a condition that diminishes metabolism, e.g., liver diseases or concomitant intake of other drugs that interfere with metabolizing enzymes, which in turn may raise plasma concentrations of unmetabolized resveratrol. Thus, further investigations are warranted to determine the plasma concentrations of resveratrol in people with diverse backgrounds, including women of reproductive age. It is also important to evaluate the stability of resveratrol in the culture medium during 4 days of EB development. The concentrations and effectiveness of compounds may be reduced during culture by various factors, such as degradation, metabolic conversion by the cells, and binding to the culture vessel (plastic) and serum proteins. In that case, actual concentrations of resveratrol that affected EB morphogenesis might be lower than 4  $\mu\text{M}$ , which would further raise a concern regarding the developmental toxicity of this dietary supplement.

Analyses of gene expression profiles in EBs revealed that resveratrol affected several developmental regulators in a differential manner. Gene expression associated with the initial step of gastrulation was largely unaffected, whereas gene expression relevant for the later step was markedly diminished by resveratrol. Interestingly, *Cyp26a1* was dramatically elevated by resveratrol on Day 1 by about 5-fold higher than control. *Cyp26a1* encodes the cytochrome P450 enzyme responsible for oxidative inactivation of retinoic acid (RA), and *Cyp26a1* knockout mouse embryos exhibit morphological and molecular abnormalities similar to those caused by excess RA (Abu-Abed et al., 2001; Sakai et al., 2001). Heightened expression of *Cyp26a1* in resveratrol-treated EBs may reduce the endogenous level of RA and diminish expression of *Hoxc6* and *Hoxb9*. However, a reduction in RA signaling alone cannot fully account for the effects of resveratrol on other genes. For example, the expression of *Cdx1* and *Hoxa1*, both of which are known transcriptional targets of RA receptors (Langston and Gudas, 1992; Houle et al., 2003; Li and Marikawa, 2016), was not significantly altered by resveratrol. Also, the Day 2 peak expression of *Wnt3a* and *Tbx6* is not abolished by BMS493, a pharmacological inhibitor of RA signaling (Li and Marikawa, 2015). Thus, resveratrol is likely to impact other molecular pathways in addition to RA signaling.

The molecular targets of resveratrol that are responsible for its various beneficial effects are still elusive, although several candidate molecules have been proposed (Kulkarni and Cantó, 2015). In the present study, we investigated whether such candidates, specifically the estrogen receptor, SIRT1, and DNA polymerase, are involved in the adverse effects of resveratrol on EBs. Despite having a similar morphological impact, DES and raloxifene did not alter the gene expression profiles in a manner comparable to resveratrol, suggesting that the activation of the estrogen receptor is not responsible for the effects of resveratrol. Activation of SIRT1 by SRT1720 did not yield morphological or molecular effects that are comparable to resveratrol either, although inhibition of SIRT1 by EX527 slightly alleviated

the molecular impact of resveratrol. By contrast, a reduction in the DNA replication rate by aphidicolin or hydroxyurea caused morphogenetic and molecular impacts strikingly similar to resveratrol. Note that DES and raloxifene diminished cell proliferation but did not cause similar gene expression changes. Thus, the distinct alteration of gene expression profiles, such as up-regulation of *Cyp26a1* and down-regulation of *Wnt3a* and *Tbx6*, are not simply due to delayed cell cycle or general cytostatic effects. Rather, the alterations are caused more specifically by a reduced rate of DNA replication, although the molecular mechanisms are presently unclear. Also, note that the molecular impact of aphidicolin and hydroxyurea was not entirely identical to resveratrol: *Fgf8* at Day 1 was increased by the former two but lowered by the latter. This suggests that resveratrol has additional targets linked to the adverse effects on EBs. Among the other potential targets, of particular interest for future studies, are AMPK (Baur et al., 2006; Dasgupta and Milbrandt, 2007), PDE (Park et al., 2012), mechanistic target of rapamycin (mTOR; Liu et al., 2010), and mitogen-activated protein kinase (MAPK; Miloso et al., 1999), as they are key regulators of signaling pathways essential for proper embryo development.

One of the health benefit claims of resveratrol is amelioration of diabetes (Szkudelski and Szkudelska, 2015; Öztürk et al., 2017). Diabetes is characterized by hyperglycemia and impairment in insulin action, and affects about 8.5% of adults aged 18 and older in the entire world (WHO, 2017). Diabetes in pregnant women during the pre-gestational or the first trimester stage significantly increases the chance of congenital malformations in the offspring, collectively known as diabetic embryopathy (Allen and Armson, 2007). To minimize diabetic embryopathy, it is important to control diabetes before and during pregnancy. However, treatment of diabetes must be conducted in a careful manner because medications by themselves could be developmentally toxic. The present study was initiated as a part of the screening of the Anti-Diabetic Compound Library to assess the developmental toxicity of various medications for diabetes. While the outcome of the screening is to be reported elsewhere in the future, the adverse effects of resveratrol on EBs raises a concern for this dietary supplement, which is widely available without prescription. Animal studies suggest that resveratrol ameliorates some of the adverse impact of maternal diabetes on embryos (Singh et al., 2011, 2012, 2013). Nonetheless, in these studies, the plasma concentrations of resveratrol, to which embryos may be exposed, are not shown. Further investigations are warranted to determine the proper dosages of resveratrol that yield beneficial effects without reaching the LOAEL to impair embryo development. Importantly, in spite of many studies of the beneficial impact of resveratrol, its therapeutic application has not been approved by the FDA for any indication, and its pregnancy risk has not been thoroughly investigated. Thus, women of reproductive age should be strongly warned about the use of resveratrol.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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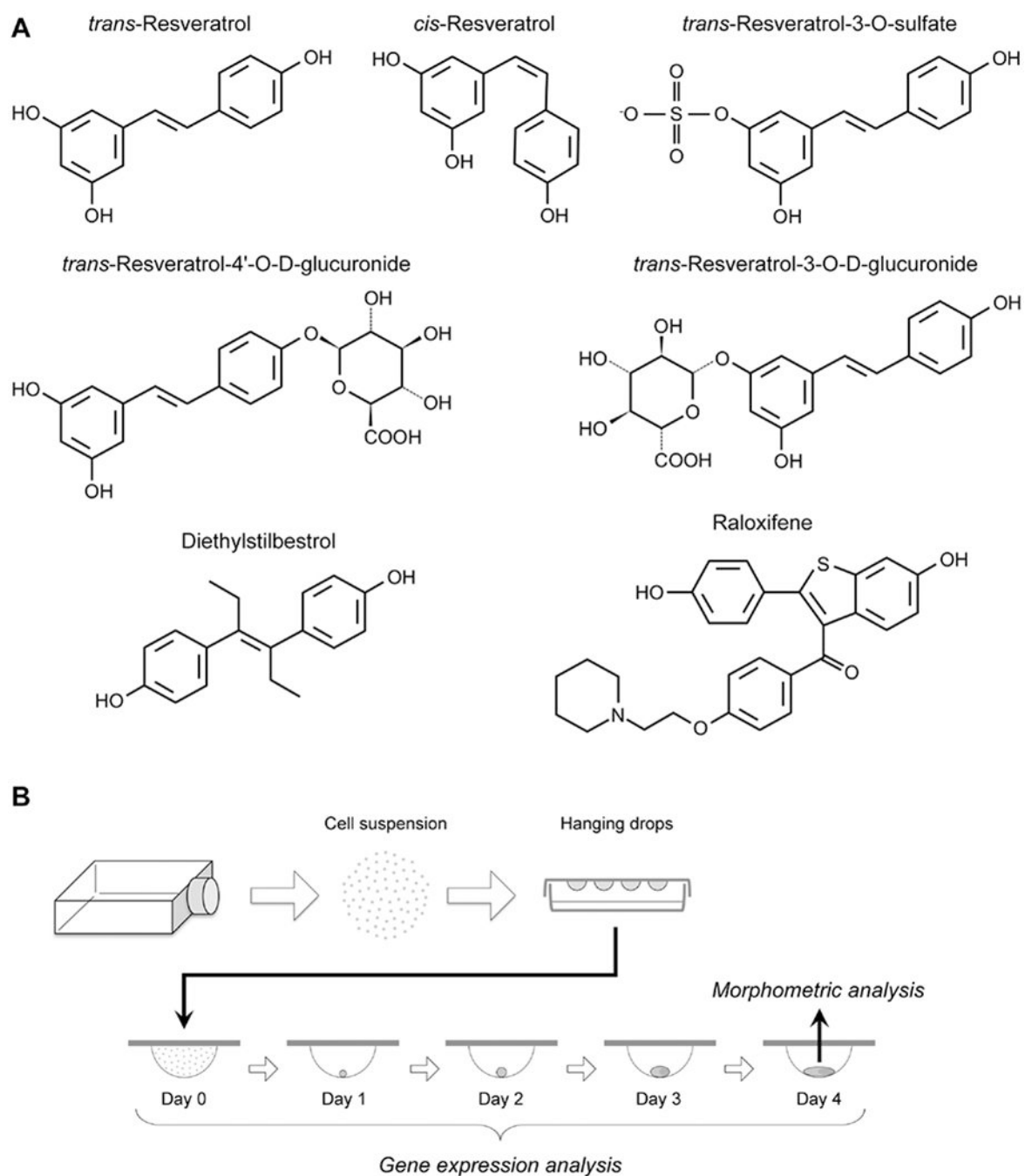
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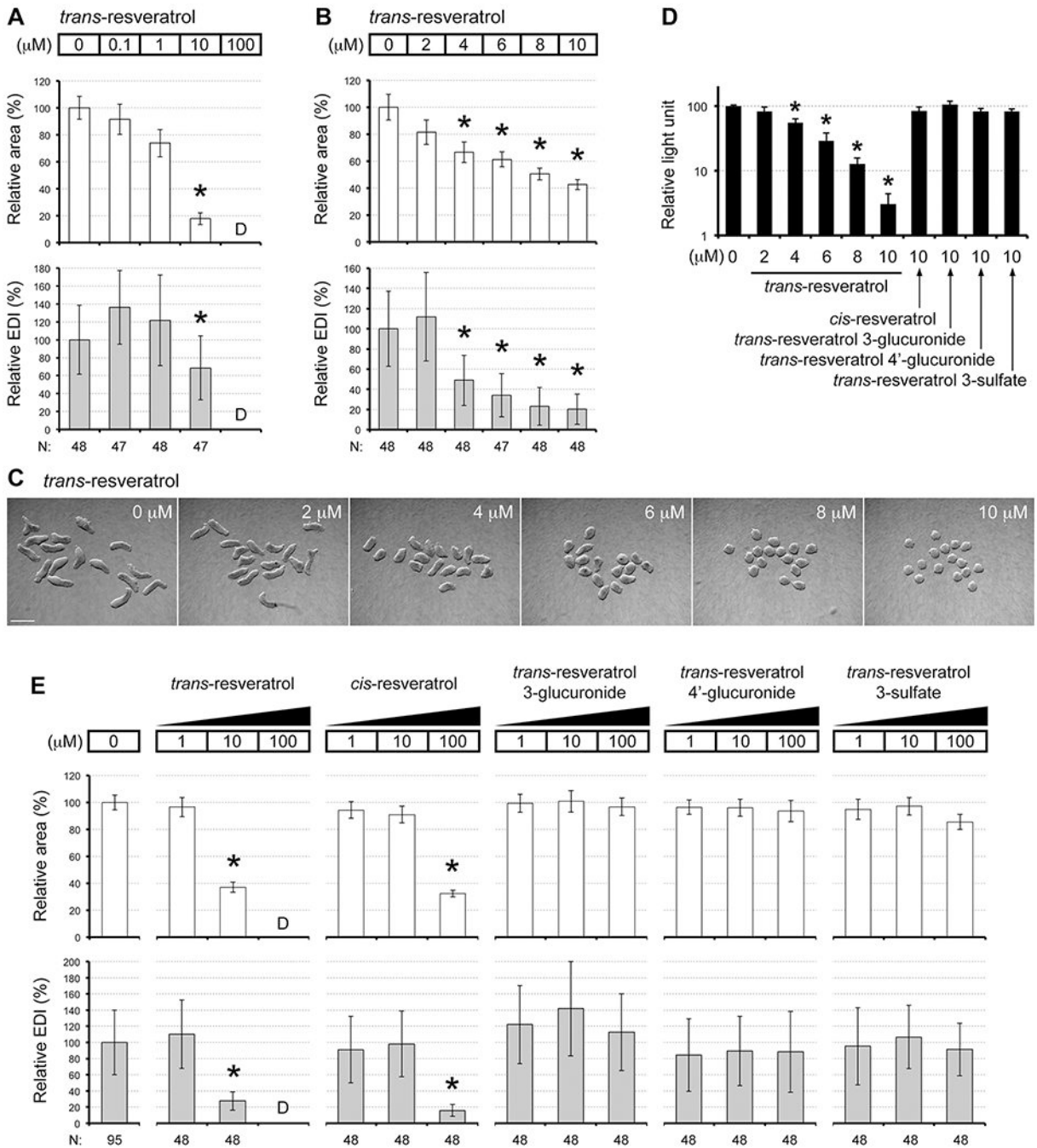
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### Highlights

- Developmental toxicity of resveratrol is examined using morphogenetic embryoid body
- *trans*-resveratrol, but not cis isoform or metabolites, impairs morphogenesis
- Resveratrol alters gene expression patterns of gastrulation regulators
- Activation of the estrogen receptor or SIRT1 is not the major action of resveratrol
- Reduction in DNA replication rate accounts for the resveratrol action



**Fig. 1.** The chemical structures of the compounds evaluated in the present study (A), and the experimental scheme to assess the morphogenetic and molecular impact of compound exposures using P19C5 embryoid bodies (B).



**Fig. 2.** Embryoid body morphogenesis is impacted by *trans*-resveratrol. (A, B) Morphometric parameters of Day 4 embryoid bodies (EBs) treated with resveratrol at different concentrations. Graphs show averages of relative area (white columns) and relative EDI (gray columns) with error bars of standard deviation. Numbers at the bottom (N) are numbers of EBs scored. No area or EDI value is available when EBs were dead (noted as “D”). Asterisks indicate adverse impacts, which are defined as reduction in average area or average EDI by more than 30% relative to controls. All adverse impacts are statistically

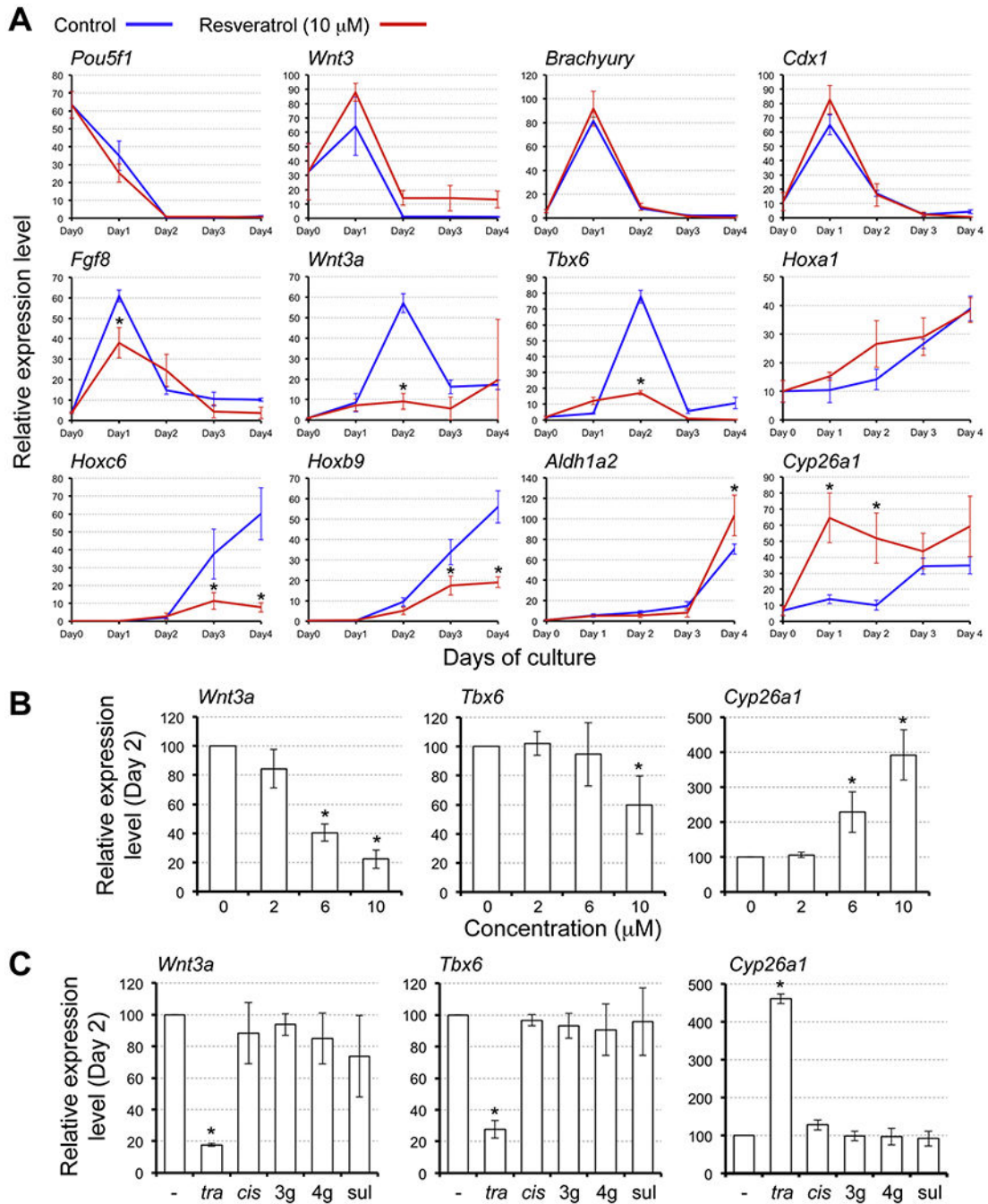
significant ( $P < 0.01$ ; two-sample t-test). (C) Representative images of EBs from one set of experiment, showing control EBs ( $0 \mu\text{M}$ ) and those treated with different concentrations of resveratrol. Scale bar =  $500 \mu\text{m}$ . (D) Impact of resveratrol on cell proliferation, evaluated by the cell viability assay (mean + standard deviation;  $n=3$ ). Cells in monolayer culture were exposed to the compounds for 4 days. Asterisks indicate significant difference ( $P < 0.01$ ; two-sample t-test) in mean relative light unit between control and compound treatment. (E) Morphometric parameters of Day 4 EBs treated with resveratrol-related compounds at different concentrations, presented in the same form as described for (A, B) above.

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**Fig. 3.**

Resveratrol alters gene expression profiles in embryoid bodies. Expression levels of developmental regulator genes were determined by quantitative RT-PCR analyses. (A) Temporal expression profiles in EBs over the 4-day culture period. Horizontal axes represent days of culture whereas vertical axes represent relative expression levels in arbitrary units. Blue and red lines correspond to the relative expression levels (mean  $\pm$  standard deviation;  $n=3$ ) in control EBs and EBs treated with 10  $\mu$ M *trans*-resveratrol, respectively. Asterisks indicate significant reduction or increase ( $P < 0.05$ ; two-sample t-test) in mean relative

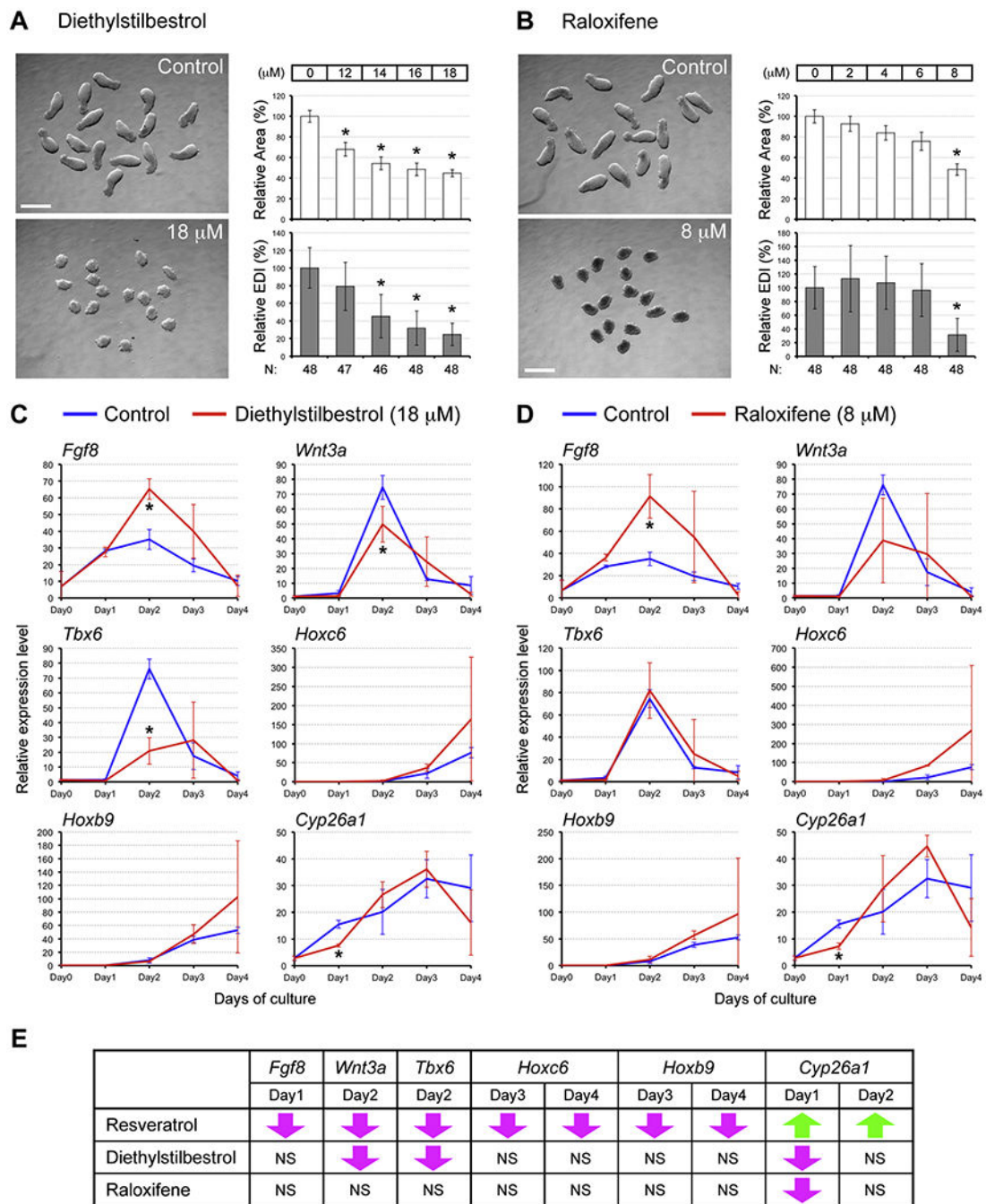
expression levels by resveratrol treatment on a given day of EB culture. (B) Relative expression levels (mean  $\pm$  standard deviation; n=3) in Day 2 EBs treated with *trans*-resveratrol. (C) Relative expression levels (mean  $\pm$  standard deviation; n=3) in Day 2 EBs treated with resveratrol-related compounds at 10  $\mu$ M. -: compound, tra: *trans*-resveratrol, cis: *cis*-resveratrol, 3g: *trans*-resveratrol 3-glucuronide, 4g: *trans*-resveratrol 4'-glucuronide, sul: *trans*-resveratrol 3-sulfate. Asterisks in (B, C) indicate significant reduction or increase ( $P < 0.05$ ; two-sample t-test) in mean relative expression levels as compared to the control (0 in B, – in C).

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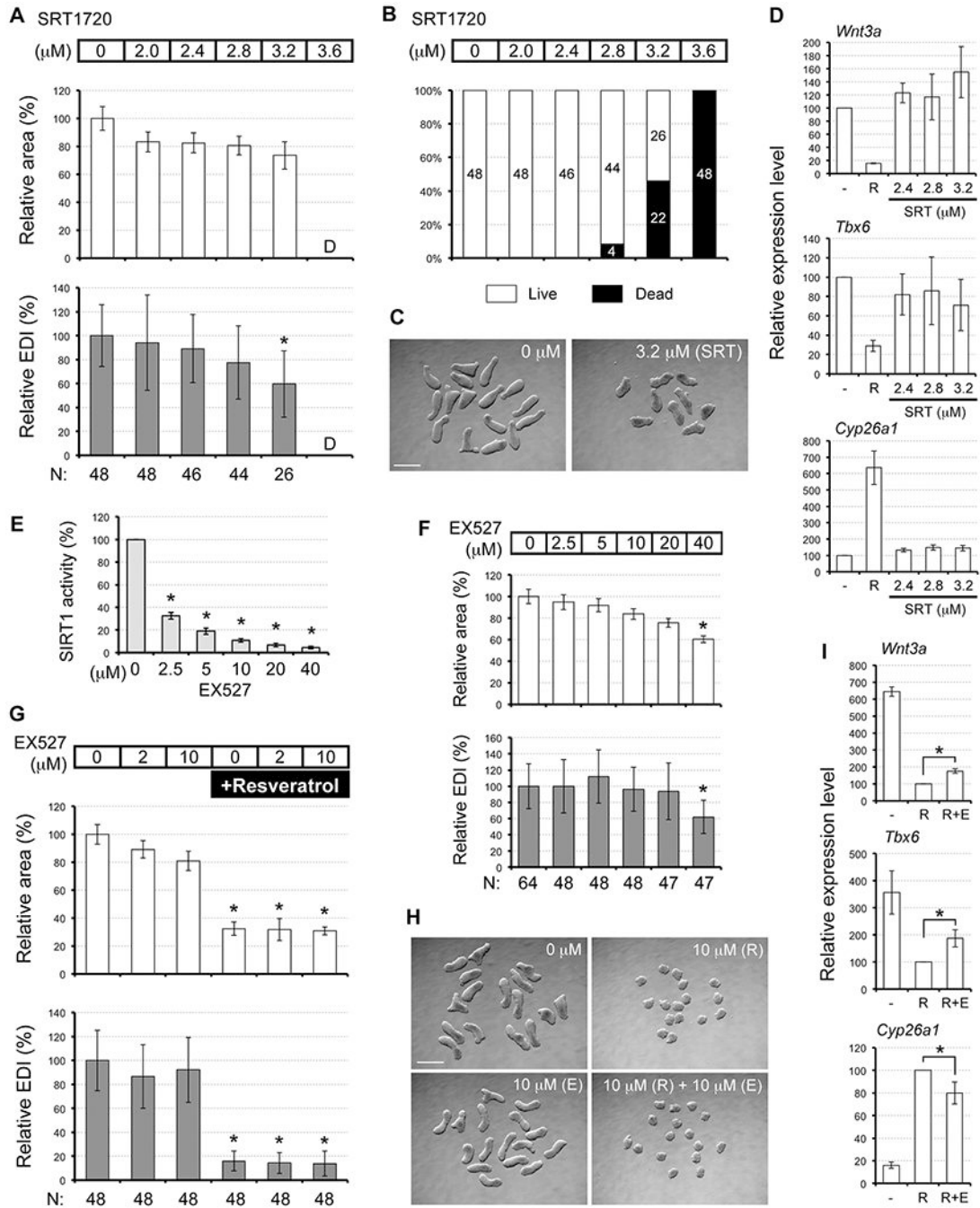
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**Fig. 4.** Estrogen receptor-modulating agents differ from resveratrol in the molecular impact. (A, B) Representative images (left) and morphometric parameters (right) of Day 4 EBs treated with diethylstilbestrol (DES; A) and with raloxifene (B). Graphs in (A, B) show averages of relative area (white columns) and relative EDI (gray columns) ± standard deviation. The numbers of EBs scored are shown at the bottom (N). Asterisks indicate adverse impacts. Scale bars = 500 μm. (C, D) Impact of DES (18 μM; C) and raloxifene (8 μM; D) on relative expression levels (mean ± standard deviation; n=3) of the developmental regulator genes

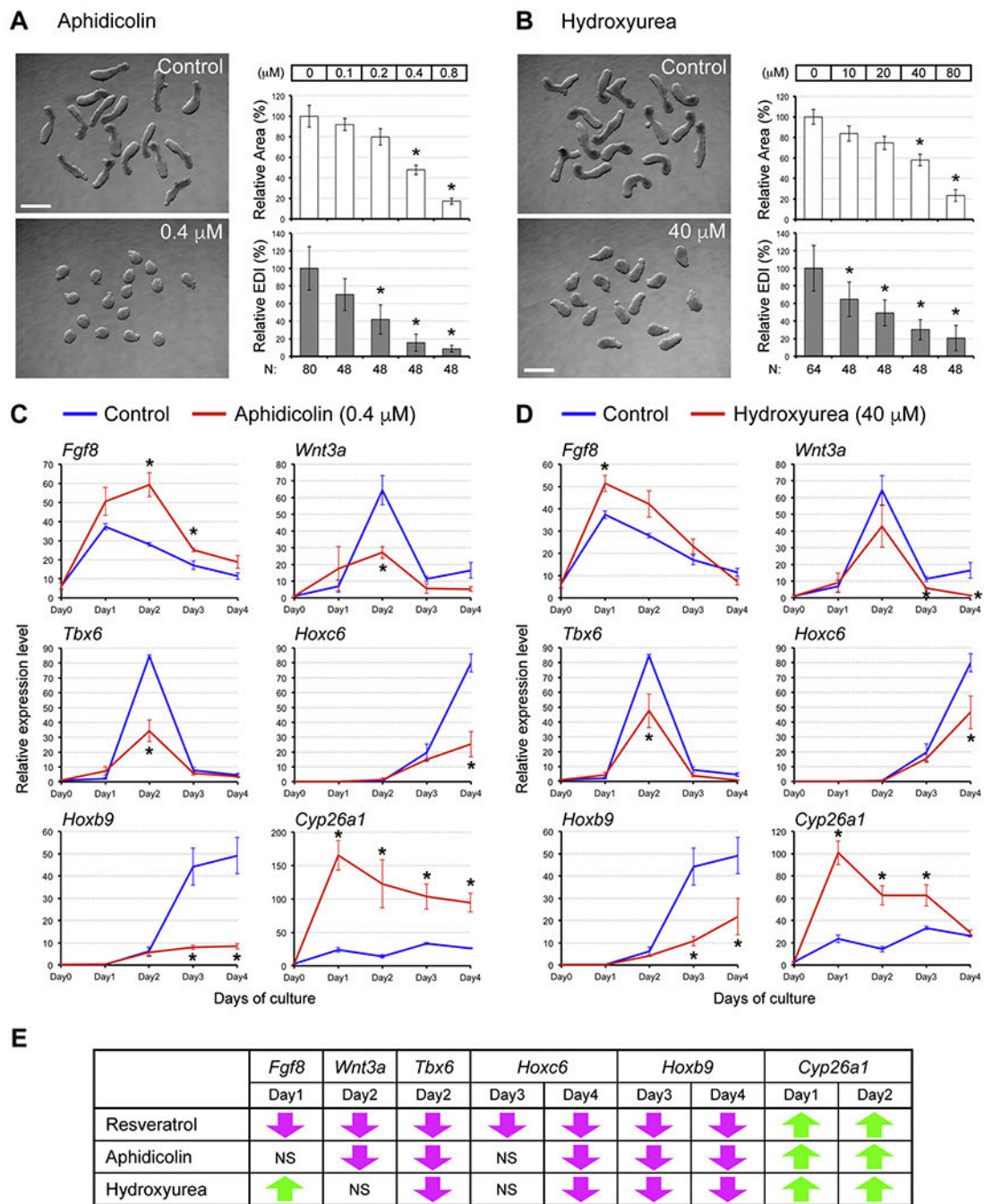


over the 4-day culture. Asterisks indicate significant changes ( $P < 0.05$ ; two-sample t-test) in mean relative expression levels by the estrogen receptor-modulating agent. (E) Comparisons of gene expression changes between resveratrol and the estrogen receptor-modulating agents. Downward magenta arrows indicate significant reduction compared to the control level, whereas upward green arrows indicate significant elevation. NS: No significance.



**Fig. 5.** Activation of SIRT1 is not sufficient to cause morphogenetic or molecular effects similar to resveratrol. (A) Morphometric parameters of Day 4 EBs treated with SRT1720. Graphs show averages of relative area and relative EDI ± standard deviation. The numbers of EBs scored are shown at the bottom (N). Area or EDI value is not available for 3.6 μM treatment, as EBs died or did not form (noted as “D”). (B) Percentage of hanging drops with different concentrations of SRT1720 that yielded live EBs. Numbers of hanging drops are indicated in columns. (C) Representative images of Day 4 EBs. In this set of experiment, nine out of 16

hanging drops with 3.2  $\mu\text{M}$  SRT1720 (SRT) yielded live EBs, whereas all 16 control drops yielded live EBs. Scale bar= 500  $\mu\text{m}$ . (D) Relative expression levels (mean  $\pm$  standard deviation; n=3) of developmental regulator genes in Day 2 EBs treated with SRT1720 (SRT), compared to resveratrol (R; 10  $\mu\text{M}$ ) in the same sets of experiments. (E) Inhibition of SIRT1 by EX527. Purified SIRT1 protein is exposed to EX527 for 30 minutes and incubated with a luminogenic substrate to assess the SIRT1 activity. Luminescence intensity of EX527-treated SIRT1 is normalized by that of untreated SIRT1 (0  $\mu\text{M}$ ; set as 100%) and presented as SIRT1 activity (mean  $\pm$  standard deviation; n=3). Asterisks indicate significant reduction ( $P < 0.01$ ; two-sample t-test). (F) Morphometric parameters of EBs treated with EX527. Asterisks indicate adverse morphogenetic impacts. (G) Morphometric parameters of Day 4 EBs treated with EX527 with or without 10  $\mu\text{M}$  resveratrol. Asterisks indicate adverse morphogenetic impacts. (H) Representative images of Day 4 EBs treated with/without resveratrol (R) and with/without EX527 (E). Scale bar= 500  $\mu\text{m}$ . (I) Relative expression levels (mean  $\pm$  standard deviation; n=3) of developmental regulator genes in Day 2 EBs treated with resveratrol (R; 10  $\mu\text{M}$ ) and with or without EX527 (E; 10  $\mu\text{M}$ ). Expression levels are normalized by the resveratrol treatment, which is shown as 100. Asterisks indicate significant difference ( $P < 0.05$ ; two-sample t-test) in mean relative expression levels between R and R+E.



**Fig. 6.** Pharmacological inhibitors of DNA replication cause morphogenetic and molecular effects similar to resveratrol. (A, B) Representative images (left) and morphometric parameters (right) of Day 4 EBs treated with aphidicolin (A) and hydroxyurea (B). Graphs in (A, B) show averages of relative area (white columns) and relative EDI (gray columns) ± standard deviation. The numbers of EBs scored are shown at the bottom (N). Asterisks indicate adverse impacts. Scale bars = 500 μm. (C, D) Impact of aphidicolin (0.4 μM; C) and hydroxyurea (40 μM; D) on gene expression patterns in EBs. Relative expression levels of

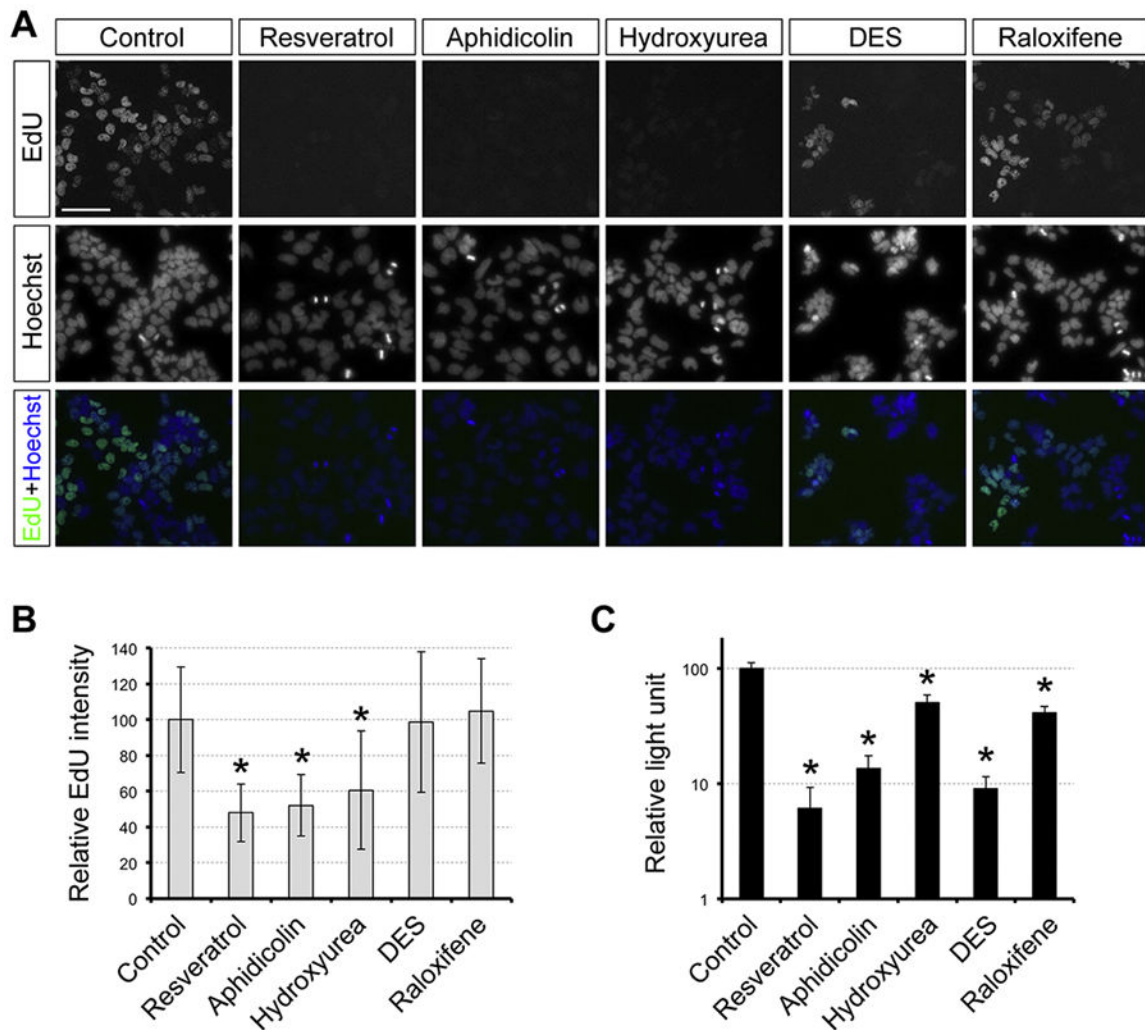
the developmental regulator genes over the 4 days of culture are shown (mean  $\pm$  standard deviation; n=3). Asterisks indicate significant change ( $P < 0.05$ ; two-sample t-test) in mean relative expression levels by the pharmacological inhibitors of DNA replication. (E) Comparisons of gene expression changes between resveratrol, aphidicolin, and hydroxyurea. Downward magenta arrows indicate significant reduction compared to the control level, whereas upward green arrows indicate significant elevation. NS: No significance.

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**Fig. 7.** Resveratrol reduces the rate of DNA replication. (A) Evaluation of DNA replication rate by the EdU assay. P19C5 cells in monolayer culture were treated with vehicle control (1% DMSO), *trans*-resveratrol (10  $\mu$ M), Aphidicolin (0.4  $\mu$ M), hydroxyurea (40  $\mu$ M), diethylstilbestrol (DES; 18  $\mu$ M), and raloxifene (8  $\mu$ M) for 24 hours, followed by incubation with EdU (100  $\mu$ M) for 1 hour. Representative images of EdU labeling and nuclear staining (with Hoechst 33342) are shown. Scale bar = 50  $\mu$ m. (B) Relative EdU intensity (mean  $\pm$  standard deviation; n=3) to evaluate the rate of DNA replication under the compound exposures described in (A). Asterisks indicate significant difference ( $P < 0.01$ ; two-sample t-test) in mean relative intensity between control and compound treatment. (C) Impact of compound exposures on cell proliferation, evaluated by the cell viability assay (mean  $\pm$  standard deviation; n=3). Cells in monolayer culture were exposed to the compounds for 4 days at the same concentrations as described in (A). Asterisks indicate significant difference ( $P < 0.01$ ; two-sample t-test) in mean relative light unit between control and compound treatment.

**Table 1.**

Chemicals used for the present study

Compound Name	CASRN	Vendor (catalog number)	Stock concentration <sup>*1</sup>	Figures <sup>*2</sup>
<i>trans</i> -Resveratrol	501-36-0	Selleck (L2900; Anti-diabetes Compound Library)	10 mM	2A
		Santa Cruz (200808)	50 mM	2BC, 3AB, 5DEFG
		Cayman (70675)	10 mM	2DE, 3C
<i>cis</i> -Resveratrol	61434-67-1	Cayman (10004235)	10 mM	2DE, 3C
Resveratrol-3-O-sulfate	858127-11-4	Cayman (14942)	10 mM	2DE, 3C
Resveratrol-3-O-D-glucuronide	387372-17-0	Cayman (13832)	10 mM	2DE, 3C
Resveratrol-4'-O-D-glucuronide	387372-20-5	Cayman (13833)	10 mM	2DE, 3C
Diethylstilbestrol	56-53-1	Santa Cruz (204720)	50 mM	4AC
Raloxifene	82640-04-8	Santa Cruz (204230)	50 mM	4BD
SRT1720	925434-55-5	Sigma-Aldrich (567860)	10 mM	5ABCD
EX527	49843-98-3	Sigma-Aldrich (E7034)	50 mM	5EFG
Aphidicolin	38966-21-1	Cayman (14007)	1 mM	6AC, 7ABC
Hydroxyurea	127-07-1	Sigma-Aldrich (H8627)	100 mM	6BD, 7ABC

CASRN, Chemical Abstracts Service Registry Number

<sup>\*1</sup> All compounds are dissolved in dimethyl sulfoxide (DMSO), except for hydroxyurea (dissolved in H<sub>2</sub>O)<sup>\*2</sup> Figure numbers that show experimental data using the corresponding chemical stocks

**Table 2.**

Developmental regulator genes examined in the present study

Gene Name	Characteristics*	Primer Sequences (5' → 3')	References
<i>Actb</i>	a. Cytoskeletal actin b. Ubiquitous c. House-keeping	F: GAGAGGGAAATCGTGCGTGACATC R: CAGCTCAGTAACAGTCCGCCTAGA	
<i>Aldh1a2</i>	a. Aldehyde dehydrogenase b. Trunk region c. Retinoicacidsynthesis	F: CTTGCCTCACAACAAGTGAGCTTC R: TCACCCAGGTTAGAGACTGGCTTC	Haselbeck et al., 1999
<i>Brachyury</i>	a. T-box transcription factor b. Early primitive streak c. Mesendoderm specification	F: CCTCGGATTCACATCGTGAGAGTT R: AGTAGGTGGGCGGGCATTATGACT	Herrmann, 1991
<i>Cdx1</i>	a. Homeodomain transcription factor b. Early primitive streak c. Axial patterning	F: TCAGGACTGGACATGAGGTAGAGG R: TGGGAAGGTGGGCATGAGCAGGTA	Meyer and Gruss, 1993
<i>Cyp26a1</i>	a. Cytochrome P450 oxidase b. Posterior end c. Retinoicacid catabolism	F: CGGAGCTGTGTAGGCAAAGAGTTT R: CCTGGAAGTGGGTAAATCTTGCAAG	Sakai et al., 2001
<i>Fgf8</i>	a. Fgf signaling ligand b. Posterior end c. Mesendoderm specification	F: GTTGCACCTTGCTG GTTCTCTGCCT R: AGTCCTTGCCCTTGCCGTTGCTCT	Ohuchi et al., 1994
<i>Hoxa1</i>	a. HOX transcription factor b. Anterior class c. Axial patterning	F: CCCTTTCCTCCACACTGTCTTGT R: AAGACCCGTAAACTCTGCTCTGGA	Wellik, 2009
<i>Hoxb9</i>	a. HOX transcription factor b. Posterior class c. Axial patterning	F: AAGCAGGGAGIGGI I I IA IGAAGG R: GGGATAGGAATGTATGAATGGGGA	Wellik, 2009
<i>Hoxc6</i>	a. HOX transcription factor b. Central class c. Axial patterning	F: TTCGCCACAGGAGAATGTCGTGTT R: CGAGTTAGGTAGCGGTTGAAGTGA	Wellik, 2009
<i>Pou5f1</i>	a. POU domain transcription factor b. Epiblast c. Pluripotencymaintenance	F: AGGCAGGAGCACGAGTGGAAAGCA R: GGAGGGCTTCGGGCACTTCAGAAA	Nichols et al., 1998
<i>Tbx6</i>	a. T-box transcription factor b. Posteriorend c. Axial stem cell differentiation	F: GGCCTCTCTCCACCCTTTAGTTC R: CACTAGTAACAAGCCCCCAGGAG	Chapman et al., 1996
<i>Wnt3</i>	a. Wnt signaling ligand b. Early primitive streak c. Initiation of gastrulation	F: CAGATGCCCGCTCAGCTATGAACA R: AGCAGCACCAAGTGAAGACGCAAT	Liu et al., 1999
<i>Wnt3a</i>	a. Wnt signaling ligand b. Posteriorend c. Axial stem cell differentiation	F: GCCACAAGAGCTTCTGATTGGTA R: CCAGGCAGAAAGACAGTCAGTCACC	Takada et al., 1994

\* Characteristics are described based on: a. Molecularfunction, b. Major expression domains around the gastrulation stage (mouse embryonic stages from E5.5to E8.5), and c. Functional significance in earlyembryo development