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2,7-Dibromocarbazole interferes with tube formation in HUVECs by altering *Ang2* promoter DNA methylation status

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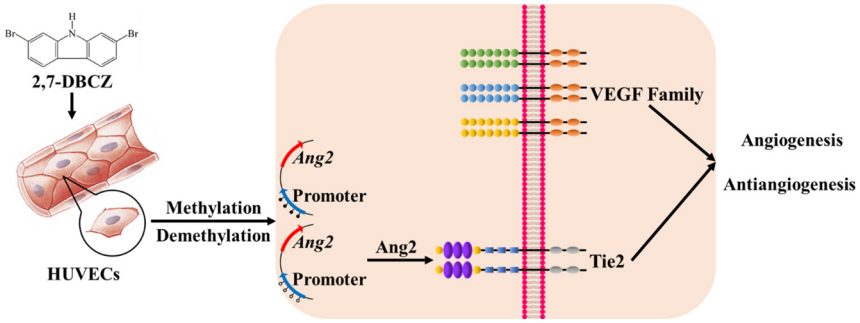
HIGHLIGHTS

- 2,7-DBCZ significantly inhibited the angiogenesis in HUVECs in the non-toxic concentration range.
- *Ang2*-silencing recovered tube formation that inhibited by 2,7-DBCZ.
- 2,7-DBCZ demethylated *Ang2* promoter to change the proportion between Ang2 and VEGFs.

GRAPHICAL ABSTRACT



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ABSTRACT

2,7-Dibromocarbazole (2,7-DBCZ) is one of the most frequently detected polyhalogenated carbazoles (PHCZs) in the environmental media. 2,7-DBCZ has attracted public attention for its potential for dioxin-like toxicity and cardiovascular toxicity. However, researches on the potential mechanism of angiogenesis inhibition by 2,7-DBCZ is still insufficient. Herein, human umbilical vein endothelial cells (HUVECs) were applied to explore the angiogenic effect of 2,7-DBCZ and the potential underlying mechanisms. 2,7-DBCZ significantly inhibited tube formation in HUVECs in the non-toxic concentration range. PCR array showed that 2,7-DBCZ reduced the expression proportion between *VEGFs* and *Ang2*, thereby inhibiting tube formation in HUVECs. Then, small RNA interference and DNA methylation assays were adopted to explore the potential mechanisms. It has been found that angiopoietin2 (*Ang2*)-silencing recovered the tube formation inhibited by 2,7-DBCZ. The DNA methylation status of *Ang2* promoter also showed a demethylation tendency after exposure. In conclusion, 2,7-DBCZ could demethylate the *Ang2* promoter to potentiate *Ang2* expression, thus altering angiogenic phenotype of HUVECs by reducing the proportion between *Ang2* and *VEGFs*. The data presented here can help to guide safety measures on the use of dioxin-like PHCZs for their potential adverse effects and provide a method for identifying the relevant biomarkers to assess their cardiovascular toxicity.

1. Introduction

Polyhalogenated carbazoles (PHCZs) are a group of important organic compounds that have been widely used in photoelectric materials,

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dyes, pharmaceutical products, *etc.* (Dumur, 2015; Parette et al., 2015; Morin et al., 2005; Karon et al., 2014). The sources of PHCZs are also varied and can mainly be divided into natural and artificial sources (Ji et al., 2019). PHCZs have drawn general attention for their structural similarity with dioxin (Guo et al., 2014). They were first found in the sediment of the Buffalo River in the1980s, and >20 PHCZs have been detected at present (Kuehl et al., 1984). The detected concentrations of PHCZs in various environmental media have ranged from 5 to 3500 ng/g (Lisa et al., 2011; Grigoriadou and Schwarzbauer, 2011; Zhu and Hites, 2005; Pena-Abaurrea et al., 2014). Recently, PHCZs have been defined as environmental contaminants for their persistence and bioaccumulation (Wu et al., 2017).

2,7-Dibromocarbazole (2,7-DBCZ) is one of the most frequently detected PHCZs. 2,7-DBCZ is widely used in electronic devices as an intermediate, and has been found in the excretion of cyanobacteria from Ping Chau island, Hong Kong (Karon et al., 2014; Lee et al., 1999). The complex sources of 2,7-DBCZ have made risk assessment of its use very difficult. Fang et al. reported that 2,7-DBCZ could induce significant developmental toxicity in zebrafish, which was similar to dioxin-induced cardiotoxicity, and 2,7-DBCZ could induce malformations such as pericardial edema, elongated and unlooped heart, and swollen yolk sac (Fang et al., 2016). Our previous studies also found that 2,7-DBCZ could promote cardiotoxicity in zebrafish embryos by causing pericardial edema and exhibited dioxin-like toxicity by interfering with aryl hydrocarbon (dioxin) receptor (AhR) related pathways (Ji et al., 2019). However, the potential mechanism by which 2,7-DBCZ induces cardiotoxicity remains unclear.

The development and functional integrity of the vascular system are fundamental requirements for heart function (Touyz and Herrmann, 2018) and angiogenesis is an integral component of the pathogenesis of various disorders including cardiotoxicity (Larrivée and Karsan, 2000). The formation and maintenance of the vasculature are of vital importance to organ development and differentiation during embryonic stages and to processes such as wound healing and reproduction in adult stage (Larrivée and Karsan, 2000). Human umbilical vein endothelial cells (HUVECs) can form vascular structures in response to certain stimuli, thus, they are an effective model to evaluate the angiogenic effect of chemicals (Junji et al., 2004; Zhong et al., 2019). Angiogenesis-related genes are key modulators in the formation of vasculature, and have a variety of effects on the viability, mitogenesis, and differentiation of HUVECs (Larrivée and Karsan, 2000). Vascular endothelial growth factors (VEGFs) compose a family of highly conserved receptor-binding polypeptides that are important regulators of blood vessel formation in health and disease. In vertebrates, VEGFs act through closely related receptor tyrosine kinases (RTKs) to stimulate blood-vessel formation in endothelial cells (Holmes and Zachary, 2005). VEGF-A is the most important growth factor involved in mammalian vascular development and diseases with abnormal growth of blood vessels. Other VEGFs are also involved in the development of lymphatic vessels and disease-related angiogenesis (Li and Eriksson, 2001). The angiopoietin (Ang)-Tie system is also essential in the remodeling and maturation of blood vessels (Wu and Liu, 2010). Abnormal expression of these angiogenesis-related genes would certainly lead to abnormal vascular development. However, the exact genes that play crucial roles in different chemicals induced abnormal vascular development remains uncertain.

Small interfering RNAs (siRNAs) are widely used as an effective biological method to silence genes and are also recognized as an important strategy for exploring gene function (Xia et al., 2002). RNA silencing has been recently applied in cancer therapy by suppressing angiogenesis in the tumor (Zhou et al., 2011; Zhang et al., 2013). Thus, siRNAs provide an effective tool to uncover genes that may play roles in regulating angiogenesis. DNA methylation is an important way to regulate gene expression, and methylation can downregulate the activity of related genes, while demethylation can reactivate them (Meissner et al., 2008; Wolf, 2007). A previous study has shown that aberrant promoter methylation could result in the suppression of gene expression (Kang et al., 2002). Screening the methylation levels of target gene promoters can help to interpret specific epigenetic phenotypes.

In this study, HUVECs were used as an *in vitro* model to evaluate the angiogenic effect of 2,7-BDCZ. Then, qRT-PCR, and RNA silencing were combined to explore the modulators that play key roles in the 2,7-DBCZ-induced antiangiogenesis process. Next, the methylation status of certain promoters was analyzed to interpret the potential underlying mechanism. Overall, our results can provide insight into the mechanism of the effect on angiogenesis induced by 2,7-BDCZ. These data can help guide safety measures on the use of dioxin-like PHCZs for their potential harmful effects and provide a method for identifying the relevant biomarkers for their cardiovascular toxicity.

2. Materials and methods

2.1. Materials and reagents

2,7-Dibromocarbazole (2,7-DBCZ, CAS#: 136630-39-2, 97% purity) was purchased from Beijing Stronger Science Co., Ltd. (Beijing, China). Detailed information regarding 2,7-DBCZ is listed in Table S1. 2,7-DBCZ was dissolved in DMSO (DMSO; Sigma-Aldrich, USA) with a stock concentration of 10^{-1} M and then diluted to working concentrations according to the experiment requirements.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Otwo. Biotech., Inc. (China) and kept in Dulbecco's modified Eagle's medium: F12 (DMEM-F12; HyClone, Logan, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, USA). All cells were kept in a 37 °C incubator with an atmosphere of 5% CO_2 and saturating humidity.

2.3. In vitro viability assays

Cell viability was measured by MTT assays as previously described (Kumar et al., 2018). Briefly, 5000 HUVECs/well were seeded in 96-well plates and allowed to attach. Then HUVECs were treated with different concentrations of 2,7-DBCZ or 0.1% DMSO vehicle control for 24 h. Cell viability was measured by the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) Kit (Promega, USA) according to the manufacturer's protocol.

2.4. Endothelial cell tube formation assays

To evaluate the angiogenic effect of 2,7-DBCZ, endothelial cell tube formation assays were performed as previously described (Singh et al., 2005). Briefly, a 100 μL/well mixture of growth factor-reduced Matrigel (Corning, USA) and DMEM-F12 (1:2) was added into 24-well plates. All the experimental materials and pipettes were precooled. Then the Matrigel-DMEM-F12 matrix was allowed to polymerize for 30 min in a 37 °C incubator. A total of 10^5 HUVECs in complete media containing various concentrations of 2,7-DBCZ or 0.1% DMSO vehicle control were seeded in each Matrigel-coated well. After 24 h, tubular structures were randomly imaged using a phase-contrast microscope at 100× magnification and the tube length and tube size were measured by using ImageJ software.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

The transcriptional effects of 2,7-DBCZ on angiogenesis related genes were measured by qRT-PCR as previously described (Ji et al., 2018). A total of 2×10^5 HUVECs in complete media were seeded in 6-well plates. After the cells were 60–80% confluent, they were exposed to various concentrations of 2,7-DBCZ or 0.1% DMSO vehicle control for

48 h. Then HUVECs were collected and total mRNA was extracted by using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. cDNA reverse transcription reactions were carried out by using the ReverTra Ace® qPCR RT Kit (TOYOBO, Japan). The PCR procedure was carried out on a CFX Connect® Real-Time System (Bio-Rad, USA) by using SYBR® Green Real-Time PCR Master Mix (TOYOBO, Japan). The PCR program was as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer sequences of target genes are shown in Table S2. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *GAPDH* as the reference gene (Livak and Schmittgen, 2001).

2.6. RNA interference of angiogenesis related genes

Genes that mediated the angiogenic effect of 2,7-DBCZ were determined by small RNA interference technology as previously described (Jing et al., 2018). 2×10^5 HUVECs in complete media were seeded in 6-well plates. After the cells were 60–80% confluent, they were transfected with Opti-MEM (Gibco, Rockville, USA) containing Lipofectamine™2000 (Invitrogen, MD, USA) and various concentrations of siRNA (20, 50, 100 nM). The siRNAs were synthesized by Biomics Co., Ltd. (China) and the detailed information is listed in Table S3. The transfection reagent was replaced with complete media after 6 h of treatment. After 48 h, HUVECs were collected to screen the inhibition efficiency by qRT-PCR. The qRT-PCR procedure and prime sequences were the same as those mentioned above. Transfection concentrations with the highest inhibition efficiency were chosen for following experiments.

2.7. In vitro angiogenesis assays after RNA interference

After confirming the optimal siRNA concentrations, the transfected HUVECs were exposed to various concentrations of 2,7-DBCZ or 0.1% DMSO vehicle control to evaluate the angiogenic effect of 2,7-DBCZ. The experimental procedure is the same as the mentioned above.

2.8. Analysis of the methylation status of the Ang2 promoter

To determine the potential mechanism that mediates the angiogenic effect of 2,7-DBCZ, bisulfite sequencing PCR (BSP) was applied to analyze the methylation status of the *Ang2* promoter by Microread Co., Ltd. (China). The experimental procedures are listed in the Supporting Information. Briefly, HUVECs were kept in T25 flasks (Thermo Fisher Scientific). When the cells were 60–80% confluent, they were exposed to 10^{-5} M 2,7-DBCZ or 0.1% DMSO vehicle control. HUVECs were collected for BSP after a 48-h exposure. Then, total DNA was extracted and quantified. After modification, total DNA was amplified with the designed primers by PCR. The purified PCR products were sequenced by BigDye sequencing. The sequencing results were analyzed by BIQ-Analyzer to evaluate the methylation status of the *Ang2* promoter (Bock et al., 2005).

2.9. Statistical analysis

The values are presented as the mean ± standard deviation (SD) from three independent experiments carried out in triplicates. The data were analyzed with Origin 8.0 software (OriginLab Corporation, USA), and the level of significance was set at $p < .05$.

3. Results

3.1. 2,7-DBCZ decreased HUVEC viability

To evaluate the angiogenic effect of 2,7-DBCZ, the cytotoxicity induced by 2,7-DBCZ from 10^{-4} to 10^{-8} M was evaluated to determine the effective exposure concentrations. Results of MTT assay showed that 2,7-DBCZ significantly decreased HUVEC viability at a concentration of 10^{-4} M (Fig. 1). Then, noncytotoxic 2,7-DBCZ at concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M were used in subsequent experiments.

3.2. 2,7-DBCZ inhibited tube formation in HUVECs

Angiogenesis is an integral component of the pathogenesis of vascular disorders, and angiogenic effect of 2,7-DBCZ was measured by *in vitro* tube formation assays. The tube structures formed on Matrigel varied with the exposure concentrations of 2,7-DBCZ (Fig. 2A). 2,7-DBCZ significantly suppressed tube formation in HUVECs. The tube size and tube length formed at the concentration of 10^{-6} M were significantly smaller than those of the 0.1% DMSO vehicle control 1.69-fold and 1.52-fold, respectively (Fig. 2B, C). HUVECs n could not form tube structures even at exposure concentrations of 10^{-5} M.

3.3. 2,7-DBCZ changed the expression levels of angiogenesis-related genes

Angiogenesis-related genes are the modulators in the formation of the vasculature. After exposure to 2,7-DBCZ for 48 h, the expression levels of angiogenesis-related genes changed dramatically (Fig. 3). 2,7-DBCZ promoted the expression of *VEGFA* and *Tie2*, but inhibited *VEGFB* expression at the concentration of 10^{-7} M. At the concentration of 10^{-6} M, 2,7-DBCZ significantly upregulated the expression levels of *VEGFA*, *Ang2*, *Tie2*, and *IL-6* by 1.14-, 1.84-, 1.47, and 1.20-fold, respectively. In addition, the expression level of *VEGFB* down-regulated by 0.79-fold. At 10^{-5} M, 2,7-DBCZ induced the largest disruption to angiogenesis-related genes. The expression levels of *VEGFA*, *Ang2*, *Tie2*, and *IL-6* were higher than those of the 0.1% DMSO vehicle control, with fold increases of 1.45, 2.24, 1.54, and 1.77, respectively. However, 2,7-DBCZ significantly reduced *VEGFB* expression by 0.70-fold.

3.4. Ang2-silencing recovered HUVEC tube formation inhibited by 2,7-DBCZ

The inhibition efficiency of RNA interference was measured to determine the optimal concentration for transfection. According to qRT-PCR results (Fig. S1), siRNA-*VEGFA* version 3 (100 nM), siRNA-*VEGFB* version 2 (50 nM), siRNA-*Ang2* version 2 (50 nM), and siRNA-*IL-6* version 3 (100 nM) induced the highest inhibition efficiency of 71.9%, 96.3%, 52.9%, and 75.2%, respectively. The transfection concentrations maintaining the highest inhibition efficiency were used in the following experiment.

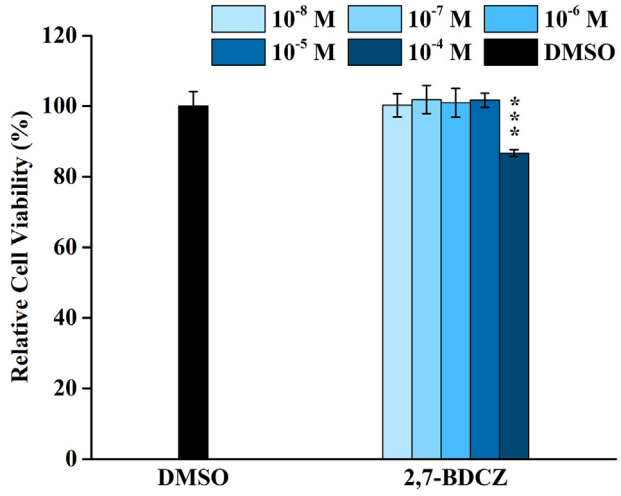

Fig. 1. 2,7-DBCZ decreased HUVEC viability. The results are presented as the percentage of the response with the cell viability of the control group (0.1% DMSO) defined as 100%. Each value represents the mean ± SD of three independent experiments (*$p < .05$; **$0.001 < p < .01$; ***$p < .001$, n = five samples).

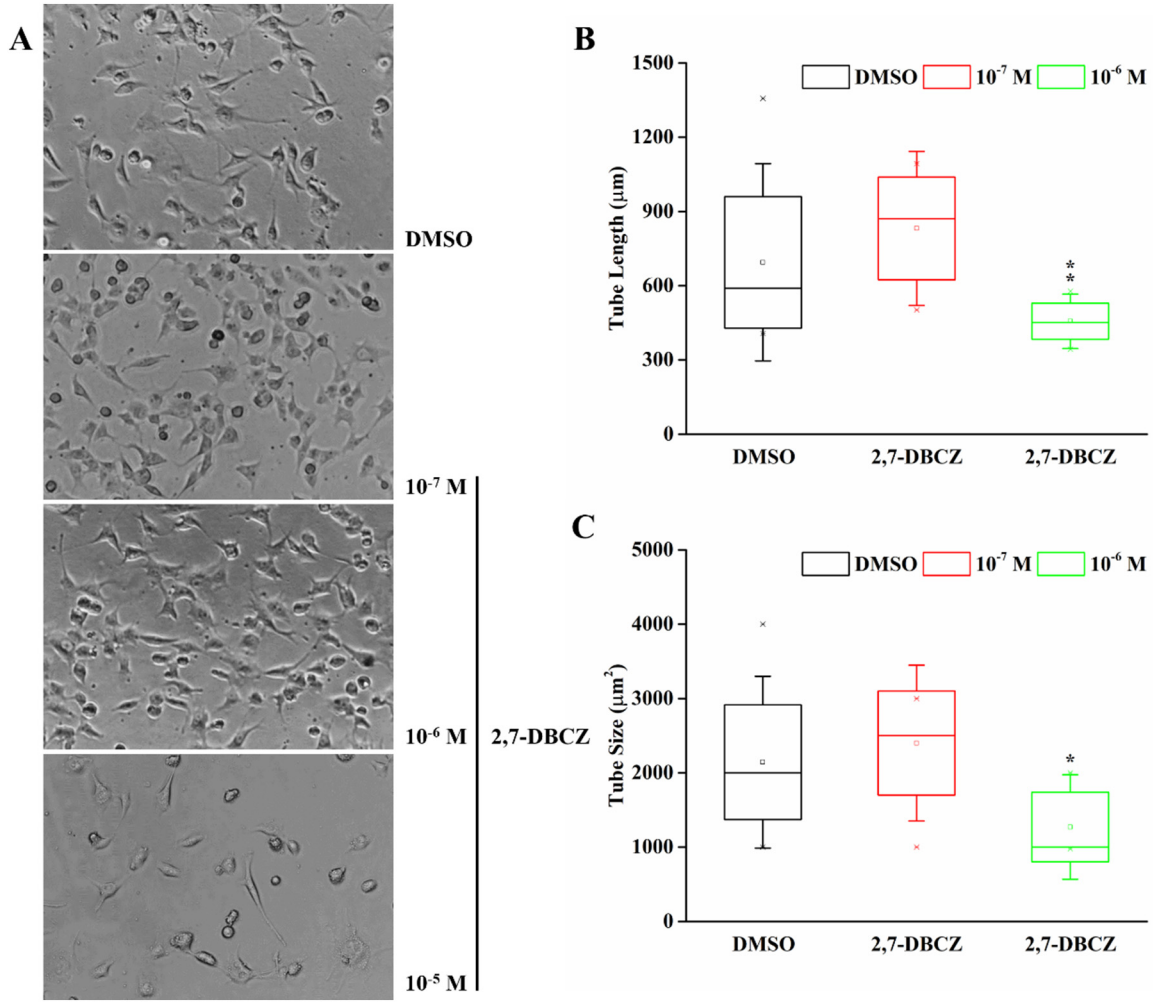

Fig. 2. 2,7-DBCZ inhibited angiogenesis in HUVECs. **A**: 2,7-DBCZ inhibited vascular structure formation in HUVECs increasingly with increasing concentration. Vascular structures were imaged at 100× magnification. The tube size (**B**) and tube length (**C**) were analyzed using ImageJ software. Each value represents the mean ± SD of three independent experiments (*$p < .05$; **$0.001 < p < .01$; ***$p < .001$, n = five samples).

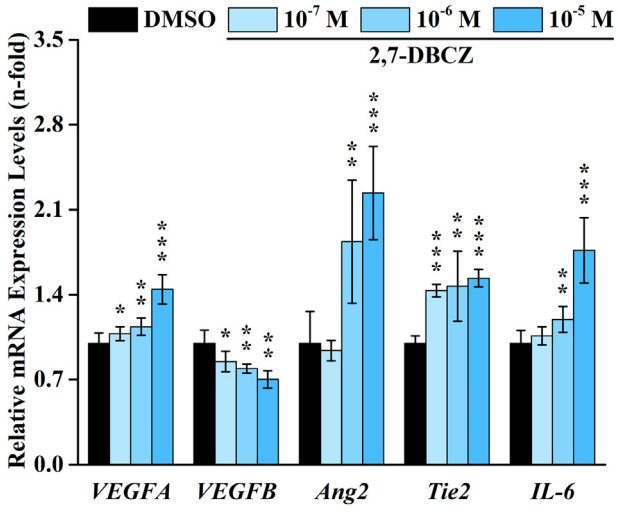

Fig. 3. 2,7-DBCZ changed the expression levels of angiogenesis related genes. The results are expressed as the fold relative to the value of the negative control (0.1% DMSO). The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *GAPDH* as the reference gene. Each value represents the mean ± SD of at least three independent experiments (*$p < .05$; **$p < .01$; ***$p < .001$, n = three samples).

To explore the gene modulators that play a role in the angiogenic effect of 2,7-DBCZ, we exposed various RNA-silenced HUVECs to 10^{-5} M 2,7-DBCZ, and then the treated HUVECs were seeded on Matrigel. As shown in Fig. 4A, *Ang2* silencing plays an important role in recovering 2,7-DBCZ-inhibited tube formation. *VEGFA*, *VEGFB*, and *IL-6* silenced HUVECs could not form tube structures after exposure to 2,7-DBCZ, while *Ang2*-silencing could recover tube formation in HUVECs exposed to 10^{-5} M 2,7-DBCZ. By measuring the tube size and tube length, we found that angiogenesis in HUVECs recovered to a normal level (Fig. 4B).

3.5. 2,7-DBCZ changed the methylation status of the Ang2 promoter

DNA methylation is closely related to epigenetic phenotypes. Thus, the methylation status of the Ang2 promoter was screened to interpret the underlying mechanism of the angiogenic effect of 2,7-DBCZ. The total methylation rates of the Ang2 promoter in the 2,7-DBCZ exposed group and the 0.1% DMSO vehicle control group were approximately the same 91 ± 5% and 94 ± 3%, respectively (Fig. 5). However, the methylation status varied in different promoter regions in the two groups (the regions were divided according to their relative distance to the exon of *Ang2*). After 2,7-DBCZ exposure, the methylation status of region 1 and region 3 were similar to those of the control group. However, the methylation status in region 2 of in the 2,7-DBCZ-exposed group decreased significantly by approximately 11%, which is consistent

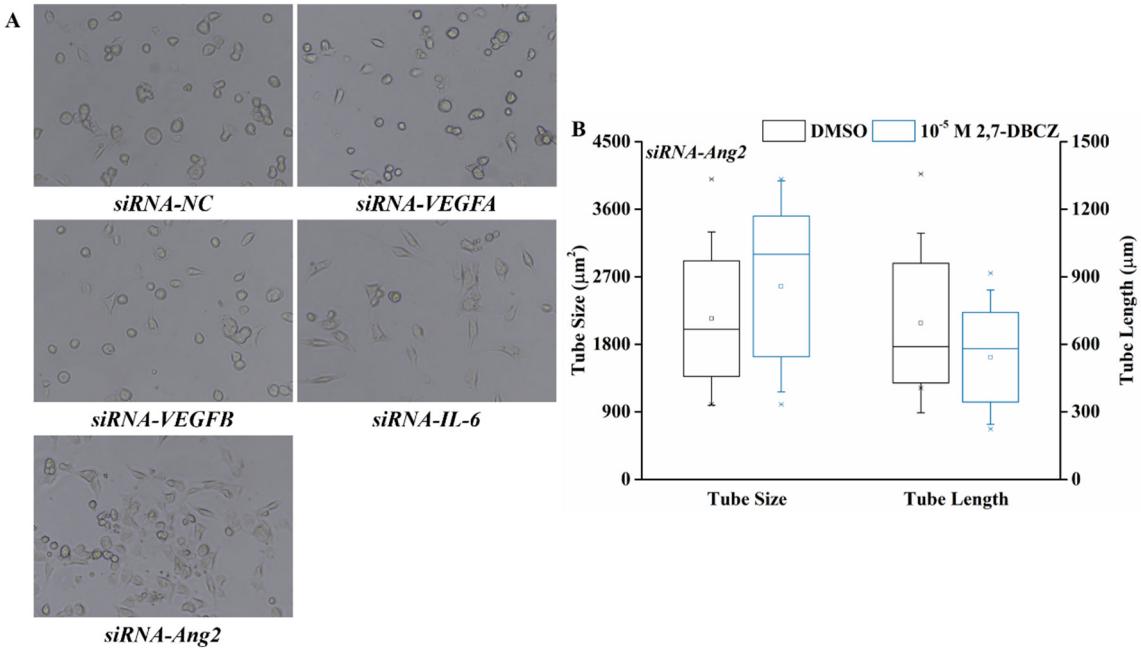

Fig. 4. siRNA-*Ang2* recovered 2,7-DBCZ-inhibited angiogenesis in HUVECs. **A**: Only *Ang2*-silencing recovered angiogenesis in HUVECs after exposure to 2,7-DBCZ. Vascular structures were imaged at 100× magnification. Tube size and tube length (**B**) were analyzed using ImageJ software. Each value represents the mean ± SD of three independent experiments (*$p < .05$, n = five samples).

with the expression level of *Ang2*. These findings suggested that Ang2 promoter demethylation played an important role in the 2,7-DBCZ-induced antiangiogenesis effect.

4. Discussion

Recently, it has become generally accepted to define PHCZs as emerging environmental contaminants for their broad exposure and adverse effects (Wu et al., 2017; Lin et al., 2016). One of the most frequently detected PHCZs, 2,7-DBCZ has been reported to induce developmental toxicity especially cardiotoxicity (Fang et al., 2016). However, few studies have explored the underlying mechanism. Blood vasculature, which comes into direct contract with exogenous chemicals (xenobiotics), is fundamental to the absorption and distribution of xenobiotics. Angiogenesis refers to the formation of capillaries from preexisting vessels in the embryo (Larrivée and Karsan, 2000). The formation of the vascular system and blood supply have key regulatory roles in embryogenesis (Walls et al., 2008; Gore et al., 2012). Disruption of vascular development has been directly correlated with developmental toxicity (Kuehl et al., 1984; Kleinstreuer et al., 2011). 2,7-DBCZ has been reported to exhibit dioxin-like toxicity and can significantly perturb vascular development in zebrafish (Ji et al., 2019; Fang et al., 2016). Dioxin-like 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) was also found to exert developmental effects on angiogenesis and osteogenesis during the early life-stages of salmon and disrupt the normal development of the vascular system (Olufsen and Arukwe, 2011). Previous studies have revealed that AhR can modulate angiogenesis by activating vascular endothelial growth factor (VEGF) transcription in the endothelium and inactivating transforming growth factor-β (TGFβ) transcription (Roman et al., 2009). Given these findings, delineating the molecular mechanisms of the impacts of PHCZs on angiogenesis is of vital importance.

The development and functional integrity of the vascular system are fundamental requirements for heart function (Touyz and Herrmann, 2018) and angiogenesis is an integral component of the pathogenesis of various disorders including cardiotoxicity (Larrivée and Karsan, 2000). Herein, 2,7-DBCZ significantly inhibited tube formation in HUVECs with a dose-effect relationship in a range of selected doses. With increasing concentrations, 2,7-DBCZ gradually suppressed the ability of HUVECs to form tube structures. Yu et al. also reported that TCDD could inhibite the proliferation of HUVECs and human umbilical vein artery cells (HUAECs) in a dose- and time-dependent manner (Li et al., 2015). Disruption of vascular development will inevitably bring about various developmental defects (Kleinstreuer et al., 2011). Angiogenesis-related genes are key modulators in the formation of vasculature. *VEGFA*, *VEGFB*, *Ang2*, *Tie2*, and *IL-6* are crucial genes for angiogenesis and have a variety of effects on the viability, mitogenesis, and differentiation of HUVECs (Larrivée and Karsan, 2000). PCR assays revealed that the expression levels of angiogenesis-related genes in HUVECs were significantly promoted after exposure to 2,7-DBCZ.

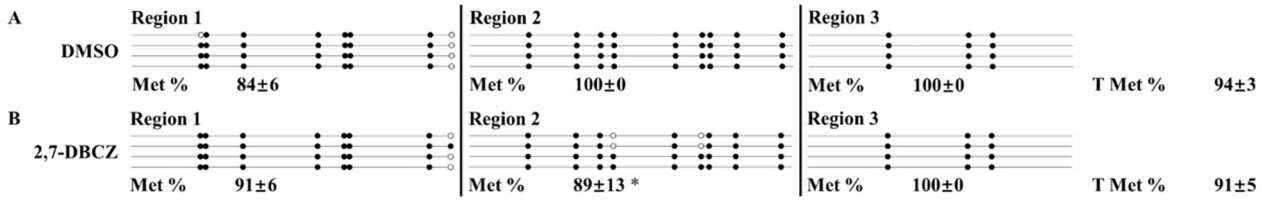

Fig. 5. 2,7-DBCZ enhanced the DNA demethylation rate in the *Ang2* promoter. **A**: The methylation status of the *Ang2* promoter in negative control HUVECs (0.1% DMSO). **B**: The methylation status of the *Ang2* promoter in HUVECs after exposure to 2,7-DBCZ. Four individual HUVECs clones from the different treatment group were sequenced. Each row represents the *Ang2* promoter of each individual clone, and a total of 20 CpG sites (8 on region 1, 9 on region 2 and 3 on region 3) were analyzed. Each circle represents a CpG site within the promoter; black circles represent methylated CpG sites, and white circles represent unmethylated CpG sites. Met % means the average percent of CpG methylation in each region, T Met % means the average percent of total CpG methylation. Each value represents the mean ± SD of four individual clones (*$p < .05$).

VEGFA and *VEGFB* belong to VEGF gene family, which are key regulators of angiogenesis. The Ang-Tie ligand-receptor system is fundamental to the regulation of vascular integrity and quiescence. Ang2 is the dynamic regulator of the Ang-Tie2 axis, which can induce new blood vessel sprout and revascularization by promoting the angiogenic effects of VEGF (Fiedler and Augustin, 2006). As the specific tyrosine kinase receptor in endothelial cells, Tie2 has a pivotal role during the process of the development, maturation, and homeostasis of blood vessels (Yancopoulos et al., 2000). IL-6 is an external factor that can promote the expression of VEGF (Hong et al., 2007). Thus, the change in gene expression is closely related to the disruption of the vascular development. However, the key moderator that regulates the 2,7-DBCZ induced antiangiogenesis is yet uncovered.

RNA interference, a posttranscriptional gene modification initiated by siRNA, has been widely used to cure certain genetic diseases and to determine the functions of target genes (Hwa et al., 2008). siRNA can silence the expression of the target gene in a highly sequence-specific manner. Thus, they can provide an in-depth insight about the specific function of target gene to the corresponding angiogenic phenotype (Bernstein et al., 2001). Herein, various siRNAs were adopted to screen the key genes that play a role in controlling the disruption of vascular development induced by 2,7-DBCZ. The results revealed that after silencing the expression of *VEGFA*, *VEGFB*, and *IL-6*, normal tube fornation in HUVECs was still suppressed by 2,7-DBCZ. However, *Ang2*-silenced HUVECs could be recovered to form tube structures even when exposed to 2,7-DBCZ. This phenomenon pointed to a more direct role of *Ang2* in vascular pathophysiology induced by 2,7-DBCZ. Ang2 is a kind of regulatory factor that controls the rapid vascular adaptive processes and the response of endothelium to exogenous cytokines (Fiedler and Augustin, 2006), thus, it is of great importance in the process of vascular development (Yancopoulos et al., 2000). Aragon et al. had reported that Ang2 could significantly enhance the degree of systolic blood pressure, renal myofibroblast differentiation, and collagen deposition in TCDD exposed mice offspring (Aragon et al., 2008). *Ang2* silencing has been successfully applied in targeted therapy for cancer by suppressing angiogenesis in the tumor (Zhou et al., 2011; Zhang et al., 2013). Liu et al. also reported that *Ang2* inhibition can displayed antiangiogenesis activity in HUVECs (Liu et al., 2012). However, the exact functions of Ang2 are presently poorly understood. Ang2 might work as an agonist or antagonist under specific situations. The role of Ang2 in the regulation of vascular formation depends on the expression related to other angiogenic stimuli (Yuan et al., 2008). Moreover, Ang2 could promote vascular development in combination with VEGF (Fiedler and Augustin, 2006). Ang2 has also been reported to enhance the mRNA expression of *VEGF* and to promote VEGF-mediated angiogenic activity by potentiating the kinase domain region (KDR) in endothelial cells (Chou et al., 2002; Otani et al., 1998). However, in the absence of VEGF, Ang2 conversely leads to vascular regression (Yuan et al., 2008). Herein, *Ang2*-silenced HUVECs recovered the vascular structure suppressed by 2,7-DBCZ. However, the exact regulation network remains unknown.

Gene promoter sequences, as well as the function of their products, are known to correlate with their silencing potential and methylation frequency (Weber et al., 2007), and many genes are known to be silenced by aberrant promoter methylation (Kang et al., 2002). Thus, the methylation status of the CpG islands of *Ang2* was examined by BSP. DNA methylation has a vital impact on the regulation of gene expression and is widely involved in a diverse range of biological processes in vertebrate animals (Jähner and Jaenisch, 1984; Colot and Rossignol, 1999). Previous studies have demonstrated that DNA methylation of the promoter was interpreted by methyl-CpG-binding proteins to strengthen its links with histone modification, and DNA methylation has been targeted to specific regions of the promoter to control gene expression (Klose and Bird, 2006; Yue et al., 2017). Many pieces of evidence implicated DNA methylation as a means of transcriptional silencing (Watt and Molloy, 1988; Suzuki and Adrian, 2008), while demethylation always occurs upon gene activation (Michael et al., 2007). Herein, 2,7-DBCZ enhanced the demethylation of the *Ang2* promoter, and as a result, promoted the expression of *Ang2*. Regarding the angiogenic phenotypes, 2,7-DBCZ suppressed tube formation, while *Ang2*-silencing recovered the inhibited angiogenesis. The exact function of Ang2 dynamically responds to the dose and context of VEGF (Wu and Liu, 2010). Although, 2,7-DBCZ potentiated *Ang2* and *VEGFA* expression in HUVECs, the rising percentage of *VEGFA* expression was much lower than that of *Ang2*. In addition, the expression level of *VEGFB* decreased significantly. In this way, tube formation was inhibited in a relative low proportion of VEGF (Yuan et al., 2008). It has also been reported that Ang-2 upregulation in the absence of other exogenous stimuli might result in vascular destabilization and subsequent vessel regression (Fiedler and Augustin, 2006). In the situation of *Ang2* silencing, the relative proportion of Ang2 and VEGF had increased dramatically. Thus, Ang2 could work as an angiogenic agonist (Fiedler and Augustin, 2006).

TCDD-induced abnormal vascular formation, growth, and function could result in fetotoxicity in many species including mice, rats, monkeys, and perhaps humans (Li et al., 2015; Hernández-Ochoa et al., 2009; Peterson et al., 1993). 2,7-DBCZ has also been reported to cause dioxin-like toxicity and induce cardiovascular anormogenesis in zebrafish (Ji et al., 2019; Fang et al., 2016). Thus, the wide distribution of 2,7-DBCZ in aqueous environments poses great risk to aquatic organisms. AhR is a well-known transcription factor that has an important role in cellular physiology as well as cardiovascular development and homeostasis (Roman et al., 2009; Lahvis et al., 2000). Dioxin-like PCB-77 could also disturb vascular system development in salmon larvae, such as in cardiac edema, by decreasing the levels of VEGF gene transcription (Olufsen and Arukwe, 2011). Pathway-specific cDNA arrays also revealed that TCDD could alter the expression of angiogenesis-related genes (Ahmet et al., 2002). Given these findings, studying the impacts of PHCZs on angiogenesis could lead to good candidates for the identification of biomarkers for their potential effects.

Overall, the results presented here indicated that 2,7-DBCZ could disturb angiogenesis in HUVECs by demethylating the *Ang2* promoter to alter the normal proportion between Ang2 and VEGF. These findings can help to guide safety measures for the use of dioxin-like PHCZs for their potential harmful effects and provide an indicator for the identification of biomarkers for these effects.

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Declaration of competing interest

The author declares no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134156.

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