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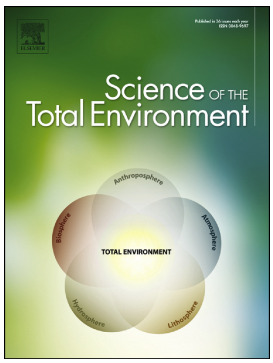

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Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on spontaneous movement of human neuroblastoma cells

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Highlights

1. TCDD has no effect on the proliferation of SK-N-SH cells.

2. TCDD inhibits the spontaneous movement of SK-N-SH cells.

3. TCDD enhances the expression of pro-adhesive genes.

4. AhR mediates the TCDD-induced cellular effect and gene induction.

Abstract

Aryl hydrocarbon receptor (AhR) plays important roles in the interferences of dioxin exposure with the occurrence and development of tumors. Neuroblastoma is a kind of malignant tumor with high mortality and its occurrence is getting higher in dioxin exposed populations. However, there is still a lack of direct evidence of influences of dioxin on neuroblastoma cell migration. SK-N-SH is a human neuroblastoma cell line which has been used to reveal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced dysregulation of certain promigratory gene. Thus, in this study, we employed SK-N-SH cells to investigate the effects of TCDD on the spontaneous movement of neuroblastoma cells, which is a short-range cell migratory behavior related to clone formation and tumor metastasis *in vitro*. Using unlabeled live cell imaging and high content analysis, we characterized the spontaneous movement under a full-nutrient condition in SK-N-SH cells. We found that the spontaneous movement of SK-N-SH cells was inhibited after 36- or 48-h treatment with TCDD at relative low concentrations (10^{-10} or 2×10^{-10} M). The TCDD-treated cells were unable to move as freely as that of control cells, resulting in less diffusive trajectories and a decreased displacement of the movement. In line with this cellular effect, the expression of pro-adhesive genes was significantly induced in time- and concentration-dependent manners after TCDD treatment. In addition, with the presence of AhR antagonist, CH223191, the effects of TCDD on the gene expression and the spontaneous cell movement were effectively reversed. Thus, we proposed that AhR-mediated up-regulation of pro-adhesive genes might be involved in the inhibitory effects of dioxin on the spontaneous movement of neuroblastoma cells. To our knowledge, this is the first piece of direct evidence about the influence of dioxin on neuroblastoma cell motility.

Keywords: Dioxin; Neuroblastoma; Spontaneous movement; Aryl hydrocarbon receptor; Cell

adhesion

1. Introduction

Dioxins are a class of persistent organic pollutants with carcinogenic, mutagenic and genotoxic properties. Aryl hydrocarbon receptor (AhR) is a highly conserved transcription factor expressed in many cell types, which can mediate toxicities of dioxins and structurally similar compounds (Shimizu et al. 2000). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener among dioxins and has been extensively studied on its toxicities and action mechanisms (Van den Berg et al. 2006). It has been evidenced that TCDD exposure has impacts on not only the incidence but also prognosis of cancers. Epidemiological study on the long-term health impacts of TCDD exposure showed that the incidence of all cancers was increased in the population exposed to TCDD in Seveso, Italy (Bertazzi et al. 1989). Moreover, after a two-year chronic exposure to TCDD at a relative low concentration (0.01 μg/kg/day, equivalent to 210 ppt), the rats encountered an increased risk of various squamous cell carcinomas and hepatocellular carcinoma (Kociba et al. 1978). In addition to the increased risk of cancer occurrence, a retrospective cohort study in the United States indicated that workers exposed to TCDD had an increased mortality from various cancers, especially those with the longest occupational exposure to TCDD (Fingerhut et al. 1991). Tumor metastasis involves the invasion and diffusion of tumor cells, which is an important factor determining the prognosis of the tumor, and is the main cause of death in cancer patients (Yamaguchi et al. 2005). However, data obtained in different cancer cells showed distinct influences of TCDD on tumor metastasis (Hsu et al. 2007; Villano et al. 2006).

At the cellular and molecular levels, tumor metastasis relies on the ability of the tumor cells to migrate under different conditions. There are two major types of movement including long-distance cell passive migration and short-range cell spontaneous movement (Aubert et al. 2008). Long-distance migration of tumor cells is induced by nutritional factors, such as oxygen and serum, which mainly

occurs from the primary site to other tissues (Aubert et al. 2008; Victoria et al. 2008). Under the nutrient-rich condition without inducible factors, tumor cells can undergo spontaneous movement with the presence of intrinsic mechanisms driving the movement of cancer cells (Irimia and Toner 2009). Such spontaneous movement is helpful to the mixing of tumor cell clones and the growth of tumors (Waclaw et al. 2015), and it has been considered as an initial stage of cancer cell metastasis and builds basis of long-distance migration of tumor cells (Aubert et al. 2006; Aubert et al. 2008). Such movement of tumor cells is largely influenced by interactions between cells and extracellular matrix (ECM) (Aubert et al. 2008). In addition, the loss of intercellular adhesion and communication can promote the spontaneous movement of cancer cells (Dietrich and Kaina 2010; Wärngard et al. 1996). Moreover, autocrine motility factor/phosphohexose isomerase/neuroleukin secreted by adjacent tumor cells was found to enhance the spontaneous movement by stimulating the expression of the proteases, which ultimately led to increased metastatic potential of the tumor cells (Liotta et al. 1986; Niizeki et al. 2002; Watanabe et al. 1991). It has been found that TCDD treatment can down-regulate the expression of E-cadherin in human breast cancer epithelial cell line MCF-7, suggesting potential impacts of TCDD on the spontaneous movement of tumor cells via regulating cell adhesion-related genes (Diry et al. 2006). However, the effects of TCDD on spontaneous movement of tumor cells are still unclear.

Neuroblatoma is one of the most common malignant tumors during infancy (Wu et al. 2014). Tumor invasion and metastasis are the main causes of death in most patients with neuroblastoma (Boubaker and Delaloye 2003). It has been reported that children (0-14 years old) whose parents were exposed to dioxins may have an increased risk of neuroblastoma (Kerr et al. 2000). Furthermore, it has been reported that maternal exposure to TCDD could lead to an inhibition of AhR mRNA level in the rat fetus (Sommer et al. 1999), and AhR suppression caused the overexpression of the proto-oncogene

MYCN *in vitro* and *in vivo*, which might also affect the occurrence of neuroblastoma (Wu et al. 2014). These pieces of evidence disclose a potential correlation of dioxin exposure or AhR signaling pathway with the occurrence of neuroblastoma. SK-N-SH is a human-derived neuroblastoma cell line. Recently, we have demonstrated that upon TCDD treatment at low concentrations without alteration in cell proliferation and viability, AhR was able to mediate dysregulation of certain functional genes induced by TCDD, including a promigratory gene cell division cycle 42 (CDC42) in SK-N-SH cells (Xie et al. 2013; Xu et al. 2017). However, whether TCDD can affect the migration ability of the neuroblastoma cells is still unknown. As an initial stage of cancer cell metastasis, the spontaneous movement of neuroblastoma cells merits in-depth investigations upon dioxin exposure, which might provide important fundamental evidence for understanding the influences of dioxin on neuroblastoma occurrence and prognosis.

In order to precisely and accurately evaluate the spontaneous movement of neuroblastoma cells, we employed the cultured SK-N-SH cells as an *in vitro* model and tracked their movement alive under an unlabeled and full-nutrient condition. High content analysis has been utilized to monitor the movement of unlabeled living cells in a real-time mode in order to get a movement overview of a group of cells and motility parameters of each single cell (Patsch et al. 2015). Therefore, the high content analysis was used in this study to characterize the spontaneous movement of SK-N-SH cells in a no bias way. In addition, relative low concentrations of TCDD (5×10^{-11}, 10^{-10} and 2×10^{-10} M) were employed in the study, which were close to the average serum concentration in dioxin-exposed populations (10^{-10}-10^{-11} M) in Seveso, Italy, Taiwan and Vietnam (Guo et al. 2004; Needham et al. 1997; Tai et al. 2011; Xie et al. 2013). Furthermore, TCDD-induced dysregulations of spontaneous movement related genes were revealed, and involvements of AhR pathway in the gene expression and the cellular

effects were also explored.

2. Materials and methods

2.1. Cell culture

SK-N-SH cells were purchased from the cell resource center of the Chinese Academy of Medical Sciences (Beijing, China) and were cultured and maintained in Dulbecco′s modified Eagle′s medium (Gibco, Grand Island, New York) supplemented with 10% fetal bovine serum (Corning, New York) and 1% penicillin-streptomycin (Gibco). Cells were cultured at 37 °C in a normal humidified 5% CO_2 incubator.

2.2. Chemical treatment

SK-N-SH cells were seeded onto 6-well plates at a density of 5×10^5 per well for 24 h. TCDD was purchased from the Wellington Laboratory (Ontario, Canada; CAS: 1746-01-6, purity > 98%) and then dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), and the final concentrations of TCDD used were 5×10^{-11}, 10^{-10} and 2×10^{-10} M. Cells were treated with TCDD for 24, 36, or 48 h. CH223191 (Sigma-Aldrich) was an antagonist of AhR (Zhao et al. 2010), whose working concentration was 10^{-6} M in this study. To block the effects of AhR, cells were pretreated with CH223191 for 3 h prior to TCDD treatment as described above. The concentration of DMSO in all treatment groups and control groups was 0.02% in AhR antagonizing experiment and 0.01% in others.

2.3. Cell viability and proliferation assay

Viable cell numbers were determined by a Cell Counting Kit-8 (CCK-8; Sangon Biotech, Shanghai, China) according to the manufacturer′s instructions. In brief, SK-N-SH cells were seeded onto 96-well plates at a density of 2×10^4 cells per well. After 24 h, the cells were treated with TCDD at indicated concentrations for 24 h or 48 h. Subsequently, CCK-8 reagent (10 μL/well) was added into

each well followed by 30-min incubation at 37 °C. The optical density (OD) was measured at 450 nm with the reference wavelength at 650 nm using the Varioskan Flash spectral scan multimode microplate reader (Thermo Scientific, Waltham, MA). The calculation formula is as follows:

Cell viability= 100%×[OD (experimental groups - blank groups) / OD (control groups - blank control groups)]

Experimental groups: containing cells, CCK-8, TCDD or DMSO;

Control groups: containing cells and CCK-8, without TCDD or DMSO;

Blank control groups: containing CCK-8, without cells, TCDD or DMSO.

2.4. Characterization of spontaneous movement using high content analysis

A modified method of spontaneous cell motility assay was employed to characterize the spontaneous movement according to the literature (Patsch et al. 2015). Cells were seeded onto 96-well CellCarrier plates (PerkinElmer, Waltham, MA) at 4×10^3 cells per well and allowed to adhere for 24 h before treatments with 0.01% DMSO or TCDD (5×10^{-11}, 10^{-10} or 2×10^{-10} M). In addition, 10^{-7} M dexamethasone (DEX, Sigma-Aldrich) was employed as a positive control to promote cell adhesion and inhibit cell movement in the experiments, and 0.01% ethanol was used as solvent control (Casulari et al. 2006). Images were acquired by a high content analysis instrument (Operetta, PerkinElmer) using an Olympus LCPLFLN 20 × 0.45 objective in the brightfield and digital phase imaging modalities. Cells were maintained at 37 °C in a live cell chamber with 5% CO_2. Live cell images were obtained from 4 visual fields in each well of 96 plates every 30 min for 48 h, and each group had 12 paralleled wells. These images were analyzed to obtain the trajectories and cell motility parameters of tracked SK-N-SH cells by using the Harmony software (version 4.8, PerkinElmer). The trajectories of tracked cells were derived from 4 fields randomly selected in a well, and for each treatment group, totally 48

images were obtained for further analysis and quantification as shown in Fig. S1. During the whole 48-hour-recording period after TCDD treatment, three cell motility parameters were quantified at 24, 36 or 48 h separately, including the accumulated distance, displacement and speed. The accumulated distance represents the total distance from the original to the end points of cell movement, the displacement represents the straight line distance from the original to the end points of cell movement, and the speed is the total distance of cell movement divided by cell movement time.

2.5. Real-time quantitative reverse transcription-PCR (RT-qPCR)

The total RNA was extracted by GeneJET RNA Purification kit (Thermo Scientific). The concentration of RNA was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific), and then cDNA was synthesized using 2 μg total RNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Then the cDNA was used to perform the RT-qPCR in a QuantStudionTM 6 Flex Real-Time PCR System with the GoTaq® qPCR Master Mix kit (Promega, Madison, WI) according to the manufacturer′s instructions. The amplification conditions were as follows: 95 °C 3 min; 95 °C 10 sec, 60 °C 20 sec, 72 °C 30 sec, 45 cycles. The dissociation curve conditions were as follows: 95 °C 5 sec, 65 °C 1 min, 95 °C 15 sec. The primer sequences of selected cell adhesion-related genes, including PCDHB (protocadherin beta subfamiliy B), PCDHG (protocadherin gamma subfamily G), FZD2 (frizzled-2), FLOT2 (flotillin-2) and DDR2 (discoidin domain receptor tyrosine kinase 2) were shown in Table 1, and the primer sequences of AhR pathway related genes, including CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), AhRR (AhR repressor) and $AChE_T$ (acetylcholinesterase T subunit) were shown in Table S1. These primers were synthesized by Sangon Biotech (Shanghai, China). The primers of PCDHB and PCDHG were designed from the common sequences of the coding regions of PCDHB and

PCDHG gene families, respectively (Table S2). Then the relative expression of these genes was analyzed using the ΔΔCT method (Livak and Schmittgen 2001) with 18S ribosomal RNA as an internal control for normalization.

2.6. Statistical analysis

Graphpad prism software (version 5.0, La Jolla, CA) was used to perform one- or two way analysis of variance (ANOVA) analysis with the Bonferroni correction and one tail unpaired t-test for statistical analyses. All data were expressed by mean ± SEM. Each experiment was performed in triplicate and had three biological repeats. The *p*-values < 0.05 were defined statistically significant.

3. Results

3.1. TCDD treatment at relative low concentrations inhibits spontaneous movement of SK-N-SH cells.

Cell viability and proliferation was first determined 24 h or 48 h after TCDD treatment (5×10^{-11}, 10^{-10} or 2×10^{-10} M) by CCK-8. No significant changes were observed after the TCDD treatment compared to the control, which indicated that such TCDD treatments have no effect on SK-N-SH cell proliferation or viability (Fig. 1).

DEX has been reported to suppress cell migration in SK-N-SH cells, which served as a control to show the reliability of the high content analysis-based cell motility assay. Three cell motility parameters, including the accumulated distance, displacement and speed, were quantified using the living cell unlabeled high content analysis to characterize the cell movement. We found that compared to solvent control, 10^{-7} M DEX could significantly inhibit the accumulated distance and displacement of tracked cells, and there was a decrease trend about the speed of cell movement (Fig. S2 A). Similarly,

after 48-h treatment with 2×10^{-10} M TCDD, the displacement of the tracked SK-N-SH cells was also significantly decreased, and the accumulated distance and displacement only had a downward trend (Fig. S2 B). The data indicated that 2×10^{-10} M TCDD has similar effects on the spontaneous movement of SK-N-SH cells with that of 10^{-7} M DEX.

Then, we further examined the concentration-dependent effect of TCDD on the spontaneous movement. We found that TCDD significantly decreased the accumulated distance of the movement after being treated with 2×10^{-10} M TCDD; whereas we only observed a decreasing trend in 10^{-10} M TCDD group (Fig. 2A). The displacement of the tracked cells was significantly decreased in all TCDD (5×10^{-11}, 10^{-10} and 2×10^{-10} M) treatment groups and the alteration varied in a concentration-dependent manner (Fig. 2B). However, the movement speed of tracked cells was significantly decreased merely in 2×10^{-10} M TCDD group (Fig. 2C). In the time course studies, we found that compared to solvent control, 48-h TCDD treatment at 10^{-10} M led to a mild but significant inhibition of the accumulated distance; however, there was no effect in 24-h and 36-h groups (Fig. 3A). When the cells were treated with 10^{-10} M TCDD for 36 h or 48 h, the displacement of the tracked cells was significantly decreased, and the inhibitory effect was more obvious after 48-h TCDD-treatment (Fig. 3B). However, the movement speed did not significantly change after treatment with 10^{-10} M TCDD (Fig. 3C).

Forty-eight-hour trajectories of the spontaneous movement of 20 cells in TCDD (2×10^{-10} M) or DMSO treatment group were obtained (Fig. 4). In line with the aforementioned quantification data, most of the cells in TCDD group moved within a smaller area than that of DMSO group (Fig. 4). Moreover, the TCDD-treated cells mostly moved around the initial position in a less diffusive way than that of control cells (Fig. 4). Both of the qualitative and quantitative data suggested that the TCDD treatment inhibits the spontaneous movement of SK-N-SH cells.

3.2. TCDD promotes the expression of cell adhesion-related genes via AhR signaling pathway.

Cell adhesion is reversely correlated to the spontaneous movement ability of the cells. Thus, pro-adhesive genes could be candidate responsive genes involved in the TCDD-induced inhibition of the spontaneous movement. Therefore, we further investigated the effects of TCDD on the expression of pro-adhesive genes related to cell-cell adhesion (PCDHB, PCDHG and FLOT2) and cell-ECM adhesion (FZD2 and DDR2). We found that the mRNA levels of all selected genes were significantly up-regulated by ~1.3 folds compared with the control group after 24-h TCDD treatment at 2×10^{-10} M (Fig. 5), while the lower concentrations of TCDD (5×10^{-11} and 10^{-10} M) had no effects after 24-h treatment. In 48-h treatment groups, TCDD at 5×10^{-11} M could significantly up-regulate the expression of PCDHB (Fig. 5A) and PCDHG (Fig. 5B); while TCDD at 10^{-10} M significantly up-regulated the expression of PCDHB (Fig. 5A) and DDR2 (Fig. 5E). However, the mRNA levels of FLOT2 (Fig. 5C) and FZD2 (Fig. 5D) only had an upward trend after 5×10^{-11} and 10^{-10} M TCDD treatment.

Since AhR-dependent pathway is a well-known mechanism for TCDD-induced gene dysregulation. We further examined the AhR activity upon the TCDD treatment in SK-N-SH cells. Here classical responsive genes downstream AhR pathway, including CYP1A1, CYP1B1 and AhRR were examined. In addition, mRNA level of $AChE_T$ was also tested as a control which has been documented to be suppressed upon TCDD treatment at low concentrations in SK-N-SH cells (Xie et al. 2013). The results showed that 2×10^{-10} M TCDD could significantly up-regulate the gene expression of CYP1A1, CYP1B1 and AhRR and down-regulate $AChE_T$ expression, indicating the TCDD could activate AhR pathway in SK-N-SH cells (Fig. S3). We then investigated the role of AhR in the induction of the pro-adhesive genes by TCDD. As the gene up-regulations and AhR activation were documented in 2×10^{-10} M TCDD treatment groups, this condition was used in the following AhR antagonization study.

Consistent with the previous results, 2×10^{-10} M TCDD treatment for 24 h significantly promoted the up-regulation of PCDHB, PCDHG, FLOT2 and DDR2 genes; With the presence of CH223191, the inhibitor of AhR pathway, the effects of TCDD on the cell adhesion-related gene expression were effectively eliminated (Fig. 6). The data indicated that TCDD promoted the transcriptional expression of these pro-adhesive genes via AhR signaling pathway.

3.3. TCDD inhibits the spontaneous movement of SK-N-SH cells via AhR signaling pathway.

In order to examine the involvement of AhR signaling pathway in the suppressive effects of TCDD on the spontaneous movement of the cells, we inhibited AhR pathway using CH223191 prior to the TCDD treatment to see the change in the cell motility parameters. We found that the application of CH223191 could eliminate the inhibitory effect of TCDD on the cell movement (Fig. 7). Interestingly, CH223191 itself could also significantly promote the spontaneous movement of the cells (Fig. 7). These data suggested that AhR might not only mediate TCDD-induced alterations in the spontaneous cell movement, but also participate in maintaining the basal level of the movement.

4. Discussion

In the present study, we found that TCDD could inhibit the spontaneous movement of SK-N-SH cells in time- and concentration-dependent manners, which was evidenced by the restriction of cell movement pattern and inhibition of the cell motility parameters according to the high content analysis-based unlabeled cell motility assay. Among the three test parameters, displacement of the spontaneous movement was a relative sensitive parameter to evaluate this specific cell behavior of human neuroblastoma cells. Regarding the signaling mechanism, AhR was documented to play roles in the TCDD-induced cellular behavior of SK-N-SH cells. We found that TCDD-induced upregulation of pro-adhesive genes, particularly those associated with cell-cell adhesion, such as PCDHG gene family,

was mediated by AhR pathway as well. Such parallel roles of AhR in TCDD-induced cellular effects and dysregulation of related genes suggested potential involvements of AhR-mediated upregulation of the pro-adhesive genes in AhR-mediated inhibition of spontaneous movement in SK-N-SH neuroblastoma cells. However, functional consequences of the newly found dysregulation of the pro-adhesive genes caused by TCDD still need further investigation.

It has been suggested that dioxin exposure increases the occurrence of neuroblastoma in certain populations (Kerr et al. 2000). However, the link between dioxin exposure and neuroblastoma has not been fully evidenced in *in vitro* cellular models, e.g. human neuroblastoma cell lines. In our previous and present studies, the human neuroblastoma cell line, SK-N-SH was employed, and its responsiveness to TCDD treatment was evidenced in terms of inducing gene dysregulations (Xie et al. 2013; Xu et al. 2014; Xu et al. 2017). We did not observe significant effects of TCDD treatment at relative low concentrations on cell viability and proliferation of SK-N-SH (Xie et al. 2013), which is different from other studies using high concentrations of TCDD (Jin et al. 2004; Lee et al. 2002). Apart from proliferation, metastasis ability is another important issue closely related to prognosis of cancers, which has been reported to cause 90% of cancer-related deaths (Sporn 1996). However, the cellular effects of TCDD on metastasis of neuroblastoma cells are still unclear. As a type of short-range cell movement that initiates cancer metastasis, the spontaneous movement of neuroblastoma cells was investigated after TCDD treatment at relative low concentrations. We employed the unlabeled living cell tracking method based on high content analysis to obtain qualitative and quantitative data for characterizing the spontaneous movement, which is different from widely used cellular assays for migration, such as wound healing assay in which extracellular inducible factors are involved. We demonstrated that TCDD treatment has inhibitory effect on the spontaneous movement of SK-N-SH

cells under quiescent condition with full nutrients. We found that in this particular human derived neuroblastoma cell line, short-term TCDD treatment at relative low concentrations was unable to promote the proliferation, nor metastasis initiation. The reason for the increased morbidity of neuroblastoma in dioxin-exposed population still needs further investigation. In our previous report, TCDD could promote the mRNA level of a promigratory gene, CDC42, via AhR in SK-N-SH cells (Xu et al. 2017). Thus, we speculated that TCDD might disrupt the balance between the cell movement and cell adhesion and consequently inhibit the spontaneous movement of SK-N-SH cells under this quiescent condition. While the cellular effect of TCDD on the long-distance migratory ability of neuroblastoma cell line is still under investigation in which traditional wound healing and transwell migratory assays could be used.

The short-range spontaneous movement of tumor cells is reversely correlated to cell-cell adhesion and cell-ECM adhesion mediated by cell adhesion molecules (Crossin 1991). Cadherins, a kind of cell-cell adhesion molecules, mainly involved in the calcium-dependent homophilic cell-cell adhesion mechanisms (Fukata and Kaibuchi 2001). PCDHs are the largest subset of the cadherin superfamily, which are confirmed to mediate both homophilic and heterophilic cell-cell adhesion, change the adhesion and shape of cells by interacting with β1 integrin and actin at the plasma membrane and reduce the spontaneous movement of tumor cells (Banelli et al. 2012). PCDHB are extensively expressed in neuroblastoma. There is also a strong correlation between PCDHB methylation and survival of neuroblastoma patients (Banelli et al. 2012). Previous studies have revealed that the knockdown of PCDHB9, encoding the transmembrane protein protocadherin B9, could significantly reduce cell adhesion to fibronectin in a gastric cancer cell line (Mukai et al. 2017). PCDHG are the most notably expressed in the nervous system, and the homophilic interaction between PCDHG

multimers plays a very critical role in cell-cell adhesion (Reiss et al. 2006; Schreiner and Weiner 2010). That is why we mainly selected PCDHB and PCDHG subfamilies in our study. Recently, flotillins, including those encoded by FLOT1 and FLOT2 genes, have been known as a part of cadherin complexes which play an important role in cell-cell adhesion (Bodin et al. 2014). In mammals, flotillins are more abundant in the nervous system (Bickel et al. 1997). Physiological studies have suggested that flotillins are involved in signal transduction, cytoskeleton remodeling and cell adhesion by accumulating at cell-cell contacts (Bodin et al. 2014). FLOT2 knockdown leads to nonexpression of FLOT1, whereas FLOT1 knockdown had no effects on the expression of FLOT2 (Kurrle et al. 2013). In addition, overexpression of FLOT2 could disturb the distribution of cell–cell adhesion molecules at cell boundaries (Hoehne et al. 2005). These experiments indicate that FLOT2 is more important for the cell-cell adhesion (Kurrle et al. 2013). In addition to cell-cell adhesion, cell-ECM adhesion is also important for the spontaneous movement of tumor cells. Collagen is the most abundant component of ECM (Shoulders and Raines 2009). It was known that overexpression of DDRs could lead to cell adhesion enhancement mediated by collagen-binding integrins (Xu et al. 2012). Additionally, FZD2/Wnt5a is also involved in regulation of cell-ECM adhesion (Matsumoto et al. 2010). Therefore, we focused on the regulation of PCDHB, PCDHG, FLOT2, DDR2 and FZD2 genes in terms of both cell-cell and cell-ECM adhesions in this study. We found that TCDD treatment enhanced the expression of all these genes via AhR, which might result in enhancement in cell-cell and cell-ECM adhesions and probably subsequently cause the aforementioned imbalance between cell adhesion and cell migration; however, the protein levels of these genes and the cell-cell adhesion related functional consequence need further verification.

Our results showed that TCDD at 2×10^{-10} M could not only effectively inhibit the spontaneous

movement of SK-N-SH cells and up-regulate the pro-adhesive genes, but also up-regulate the mRNA levels of AhR-pathway-related genes including CYP1A1, CYP1B1 and AhRR (Fig. S3). Furthermore, the up-regulation of the pro-adhesive genes could be reversed with the presence of AhR antagonist, CH223191. These data supported that the TCDD treatment could activate AhR signaling pathway in SK-N-SH cells, in which AhR protein has been detected previously (Tian et al. 2016). Since the presence of dioxin responsive element (DRE) consensus sequences in the promoter region is one of the features of AhR target genes, we checked the putative promoter of certain genes belonging to PCDHG subfamily, which was the most sensitive subfamily of PCDHs to TCDD treatment in this study. Putative DRE core sequences (5′- GCG TG -3′ or 5′- CAC GC -3′) (Nukaya et al. 2009) could be found in the promoter regions of PCDHGC3, PCDHGA8 and PCDHGA12, which further supported the mediating role of AhR in TCDD-induced dysregulation of PCDHG genes.

5. Conclusion

Our results demonstrated that TCDD has no effect on the proliferation of SK-N-SH cells at the low concentrations used in these experiments. However, TCDD inhibits the spontaneous movement of SK-N-SH cells, which is mediated by AhR. In parallel with this cellular effect, the expression of genes promoting cell-cell and cell-ECM adhesion can be induced by TCDD treatment in concentration- and time-dependent manners via AhR signaling pathway. Taken together, we speculated that excessive expression of pro-adhesive genes may be involved in TCDD-induced inhibition of spontaneous movement of neuroblastoma cells, in which AhR plays important roles. To our knowledge, this is the first study directly revealing the influence of dioxin on the motility of neuroblastoma cells.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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References

Aubert M, Badoual M, Féreol S, Christov C, et al. (2006) A cellular automaton model for the migration of glioma cells. Physical Biology 3(2):93-100, doi: 10.1088/1478-3975/3/2/001.

Aubert M, Badoual M, Grammaticos B. (2008) A model for short- and long-range interactions of migrating tumour cell. Acta Biotheoretica 56(4):297, doi:10.1007/s10441-008-9061-x.

Banelli B, Brigati C, Di Vinci A, et al. (2012) A pyrosequencing assay for the quantitative methylation analysis of the PCDHB gene cluster, the major factor in neuroblastoma methylator phenotype. Laboratory Investigation 92(3):458-465, doi:10.1038/labinvest.2011.169.

Bertazzi PA, Zocchetti C, Pesatori AC, et al. (1989) Ten-year mortality study of the population involved in the Seveso incident in 1976. American Journal of Epidemiology 129(6):1187-1200, doi:10.1093/oxfordjournals.aje.a115240.

Bickel PE, Scherer PE, Schnitzer JE, et al. (1997) Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. Journal of Biological Chemistry 272(21):13793-13802, doi: 10.1074/jbc.272.21.13793.

Bodin S, Planchon D, Rios Morris E, et al. (2014) Flotillins in intercellular adhesion – from cellular physiology to human diseases. Journal of Cell Science 127(24):5139, doi:10.1242/jcs.159764.

Boubaker A, Delaloye AB. (2003) Nuclear medicine procedures and neuroblastoma in childhood: Their value in the diagnosis, staging and assessment of response to therapy. The Quarterly Journal of Nuclear Medicine 47(1):31-40, doi: 10.1007/s00247-002-0851-z.

Casulari LA, Dondi D, Maggi R, et al (2006) Dexamethasone blocks the migration of the human neuroblastoma cell line SK-N-SH. Brazilian Journal of Medical and Biological Research 39:1233-1240, doi: 10.1590/s0100-879x2006000900011.

Crossin KL. (1991) Cell adhesion molecules in embryogenesis and disease. Annals of the New York Academy of Sciences 615(1):172-186, doi:10.1111/j.1749-6632.1991.tb37759.x.

Dietrich C, Kaina B. (2010) The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact

and tumor growth. Carcinogenesis 31(8):1319-1328, doi:10.1093/carcin/bgq028.

Diry M, Tomkiewicz C, Koehle C, et al. (2006) Activation of the dioxin/aryl hydrocarbon receptor (AhR) modulates cell plasticity through a JNK-dependent mechanism. Oncogene 25:5570, doi:10.1038/sj.onc.1209553.

Evan GI, Vousden KH. (2001) Proliferation, cell cycle and apoptosis in cancer. Nature 411(6835):342-348, doi:10.1038/35077213.

Fingerhut MA, Halperin WE, Marlow DA, et al. (1991) Cancer mortality in workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. New England Journal of Medicine 324(4):212, doi: 10.1056/NEJM199101243240402.

Fukata M, Kaibuchi K. (2001) Rho-family GTPases in cadherin-mediated cell-cell adhesion. Nature Reviews Molecular Cell Biology 2(12):887-897, doi:10.1038/35103068.

Guo YL, Lambert GH, Hsu CC, et al. (2004) Yucheng: health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. International Archives of Occupational and Environmental Health 77(3):153-158, doi:10.1007/s00420-003-0487-9.

Hoehne M, Gert de Couet H, Stuermer CAO, et al. (2005) Loss- and gain-of-function analysis of the lipid raft proteins Reggie/Flotillin in Drosophila: They are posttranslationally regulated, and misexpression interferes with wing and eye development. Molecular and Cellular Neuroscience 30(3):326-338, doi: 10.1016/j.mcn.2005.07.007.

Hsu EL, Yoon D, Choi HH, et al. (2007) A proposed mechanism for the protective effect of dioxin against breast cancer. Toxicological Sciences 98(2):436-444, doi:10.1093/toxsci/kfm125.

Irimia D, Toner M. (2009) Spontaneous migration of cancer cells under conditions of mechanical confinement. Integrative Biology 1(8-9):506-512, doi:10.1039/b908595e.

Jin DQ, Jung JW, Lee YS, et al. (2004) 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits cell proliferation through arylhydrocarbon receptor-mediated G1 arrest in SK-N-SH human neuronal cells. Neuroscience Letters 363(1):69-72, doi: 10.1016/S0304-3940(04)00359-3.

Kerr MA, Nasca PC, Mundt KA, et al. (2000) Parental occupational exposures and risk of neuroblastoma: a case–control study (United States). Cancer Cause Control 11(7):635-643, doi:10.1023/A:1008951632482.

Kociba RJ, Keyes DG, Beyer JE, et al. (1978) Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Toxicology and Applied Pharmacology, 46(2):279-303 doi: 10.1016/0041-008X(78)90075-3.

Kurrle N, Völlner F, Eming R, et al. (2013) Flotillins directly interact with γ-catenin and regulate epithelial cell-cell adhesion. PloS One 8(12):e84393-e84393, doi:10.1371/journal.pone.0084393.

Lee YS, Jin DQ, Park SH, et al. (2002) 2,3,7,8-Tetrachlorobenzo-*p*-dioxin inhibits proliferation of SK-N-SH human neuronal cells through decreased production of reactive oxygen species. Free Radical Research 36(12):1283-1289, doi:10.1080/1071576021000016517.

Liotta LA, Mandler R, Murano G, et al. (1986) Tumor cell autocrine motility factor. Proceedings of the National Academy of Sciences 83(10):3302, doi:10.1073/pnas.83.10.3302.

Livak KJ, Schmittgen TD. (2/001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25(4):402-408, doi: 10.1006/meth.2001.

Matsumoto S, Fumoto K, Okamoto T, et al. (2010) Binding of APC and dishevelled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells. The EMBO Journal, 29(7):1192-1204 doi:10.1038/emboj.2010.26.

Mukai S, Oue N, Oshima T, et al. (2017) Overexpression of PCDHB9 promotes peritoneal metastasis and correlates with poor prognosis in patients with gastric cancer. The Journal of Pathology 243(1):100-110, doi:10.1002/path.4931.

Murray IA, Patterson AD, Perdew GH. (2014) Aryl hydrocarbon receptor ligands in cancer: friend and foe. Nature Reviews Cancer 14:801, doi:10.1038/nrc3846.

Needham LL, Gerthoux PM, Patterson Jr DG, et al. (1997) Serum dioxin levels in Seveso, Italy, population in 1976. Teratogenesis, Carcinogenesis, and Mutagenesis 17(4-5):225-240, doi:10.1002/(SICI)1520-6866(1997)17:4/5<225::AID-TCM5>3.0.CO;2-K.

Niizeki H, Kobayashi M, Horiuchi I, et al. (2002) Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells. British Journal of Cancer 86(12):1914-1919, doi:10.1038/sj.bjc.6600331.

Nukaya M, Moran S, Bradfield CA. (2009) The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology. Proceedings of the National Academy of Sciences of the United States of America 106(12):4923-4928, doi:10.1073/pnas.0809613106.

Patsch K, Chiu CL, Engeln M, et al. (2016) Single cell dynamic phenotyping. Scientific reports 6(1):34785, doi:10.1038/srep34785.

Reiss K, Maretzky T, Haas IG, et al. (2006) Regulated ADAM10-dependent ectodomain shedding of γ-protocadherin C3 modulates cell-cell adhesion. Journal of Biological Chemistry 281(31):21735-21744, doi:10.1074/jbc.M602663200.

Schreiner D, Weiner JA. (2010) Combinatorial homophilic interaction between γ-protocadherin multimers greatly expands the molecular diversity of cell adhesion. Proceedings of the National Academy of Sciences 107(33):14893, doi:10.1073/pnas.1004526107.

Shimizu Y, Nakatsuru Y, Ichinose M, et al. (2000) Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proceedings of the National Academy of Sciences, 97(2):779 doi:10.1073/pnas.97.2.779.

Shoulders MD, Raines RT. (2009) Collagen structure and stability. Annual Review of Biochemistry 78:929-958, doi:10.1146/annurev.biochem.77.032207.120833.

Sommer RJ, Marks Sojka K, Pollenz RS, t al. (1999) Ah receptor and ARNT protein and mRNA concentrations in rat prostate: effects of stage of development and 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment. Toxicology and Applied Pharmacology 155(2):177-189, doi:10.1006/taap.1998.8597

Sporn MB. (1996) The war on cancer. The Lancet 347(9012):1377-1381, doi: 10.1016/s0140-6736(96)91015-6.

Tai PT, Nishijo M, Kido T, et al. (2011) Dioxin concentrations in breast milk of vietnamese nursing mothers: a survey four decades after the herbicide spraying. Environmental Science & Technology 45(15):6625-6632, doi:10.1021/es201666d.

Tian WJ, Pei XH, Xie HQ, et al. (2016) Development and characterization of monoclonal antibodies against human aryl hydrocarbon receptor. Journal of environmental sciences 46:259-260, doi:10.1016/j.jes.2016.08.001.

Van den Berg M, Birnbaum LS, Denison M, et al. (2006) The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicological Sciences 93(2):223-241, doi:10.1093/toxsci/kfl055.

Victoria SM, Gilles G, Jessica A, et al. (2008) Rac activation and inactivation control plasticity of

tumor cell movement. Cell 135(3):510-23, doi: 10.1016/j.cell.2008.09.043.

Villano CM, Murphy KA, Akintobi A, et al. (2006) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces matrix metalloproteinase (MMP) expression and invasion in A2058 melanoma cells. Toxicology and Applied Pharmacology 210(3):212-224, doi: 10.1016/j.taap.2005.05.001.

Wärngard L, Bager Y, Kato Y, et al. (1996) Mechanistical studies of the inhibition of intercellular communication by organochlorine compounds. Toxicology - From Cells to Man 18: 149-159, doi: 10.1007/978-3-642-61105-6_16.

Waclaw B, Bozic I, Pittman ME, et al. (2015) A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. Nature 525:261, doi:10.1038/nature14971.

Wang T, Wyrick KL, Meadows GG, et al. (2011) Activation of the aryl hydrocarbon receptor by TCDD inhibits mammary tumor metastasis in a syngeneic mouse model of breast cancer. Toxicological Sciences 124(2):291-298, doi:10.1093/toxsci/kfr247.

Watanabe H, Nabi IR, Raz A. (1991) The relationship between motility factor receptor internalization and the lung colonization capacity of murine melanoma cells. Cancer Research 51(10):2699, doi: 10.1002/1097-0142(19910515)67:10+<2727::AID-CNCR2820671710>3.0.C.

Wu PY, Liao YF, Juan HF, et al. (2014) Aryl hydrocarbon receptor downregulates MYCN expression and promotes cell differentiation of neuroblastoma. PloS One 9(2):e88795-e88795, doi:10.1371/journal.pone.0088795.

Xie HQ, Xu HM, Fu HL, et al. (2013) AhR-mediated effects of dioxin on neuronal acetylcholinesterase expression in vitro. Environmental Health Perspectives 121(5):613-618, doi:10.1289/ehp.1206066.

Xu HM, Xie HQ, Tao WQ, et al. (2014) Dioxin and dioxin-like compounds suppress acetylcholinesterase activity via transcriptional downregulations in vitro. Journal of Molecular Neuroscience 53(3):417-423, doi:10.1007/s12031-013-0167-5.

Xu H, Bihan D, Chang F, et al. (2012) Discoidin domain receptors promote α1β1- and α2β1-integrin mediated cell adhesion to collagen by enhancing integrin activation. PloS One 7(12):e52209-e52209, doi:10.1371/journal.pone.0052209.

Xu T, Xie HQ, Li Y, et al. (2017) CDC42 expression is altered by dioxin exposure and mediated by multilevel regulations via AhR in human neuroblastoma cells. Scientific Reports 7(1):10103, doi:10.1038/s41598-017-10311-3.

Yamaguchi H, Wyckoff J, Condeelis J. (2005) Cell migration in tumors. Current Opinion in Cell Biology 17(5):559-564, doi: 10.1016/j.ceb.2005.08.002.

Zhao B, DeGroot DE, Hayashi A, et al. (2010) CH223191 is a ligand-selective antagonist of the Ah (dioxin) receptor. Toxicological Sciences 117(2):393-403, doi:10.1093/toxsci/kfq217.

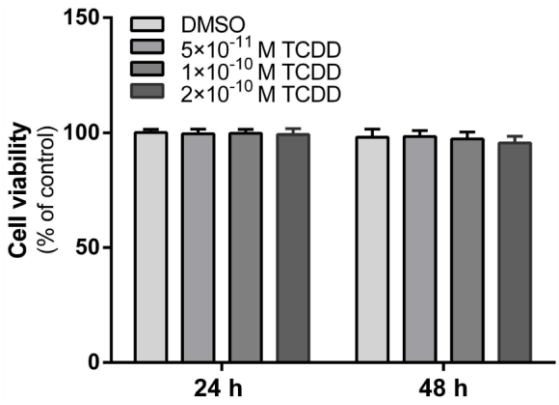

Figure 1 TCDD has no effect on the viability of SK-N-SH cells. SK-N-SH cells were incubated with different concentrations of TCDD (5×10^{-11}, 10^{-10} or 2×10^{-10} M) or 0.01% DMSO (solvent control) for 24 h or 48 h. The cell viability was determined by CCK-8 assay as mentioned in M&M section. The experiments were performed in three independent experiments, and each had six biological repeats. Values are expressed as mean ± SEM. Statistical analysis was done by two-way ANOVA with Bonferroni test.

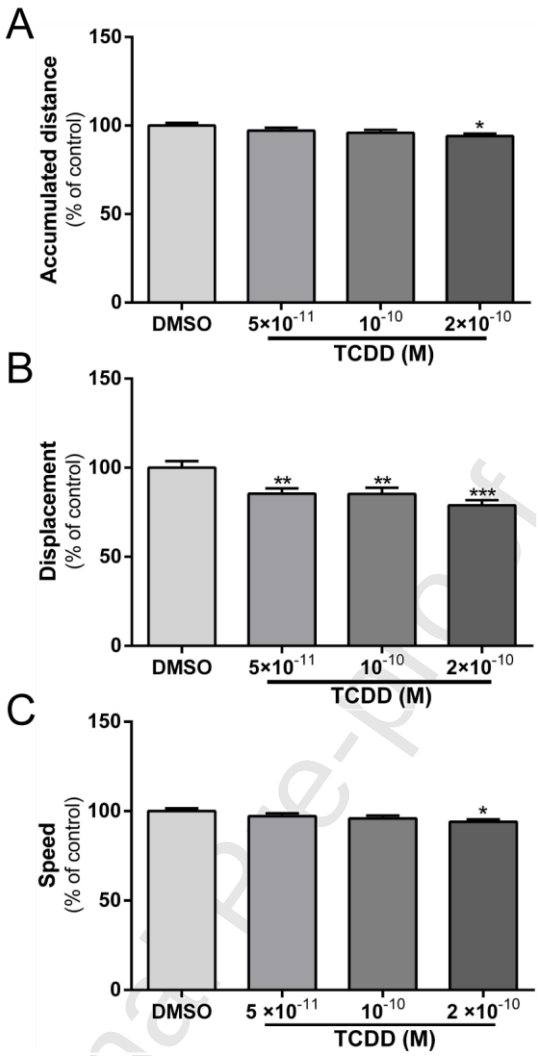

Figure 2 Concentration-dependent effects of TCDD on spontaneous movement of SK-N-SH cells using high content analysis. Cells were incubated with different concentrations of TCDD (5×10^{-11}, 10^{-10} or 2×10^{-10} M) or 0.01% DMSO for 48 h. Quantification of three spontaneous movement parameters obtained using high content analysis as aforementioned in M&M section are shown, including accumulated distance (A), displacement (B) and speed (C). Values are expressed as mean ± SEM from all 48-hour-tracked cells in 12 paralleled wells of 96-well plates in each group (n=600-800). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the DMSO groups (one-way ANOVA with Bonferroni test).

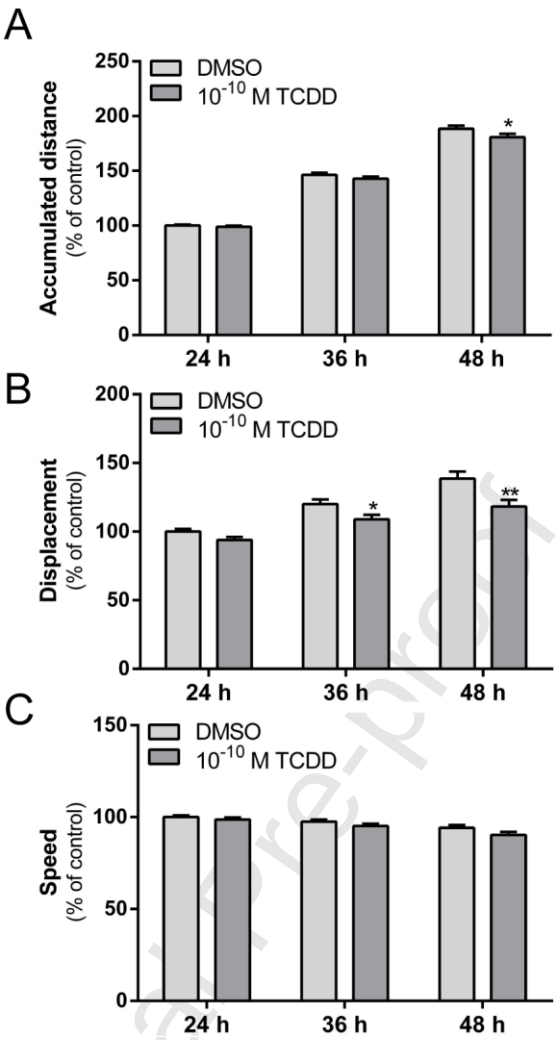

Figure 3 Time course of TCDD effect on spontaneous movement of SK-N-SH cells using high content analysis. Cells were exposed to 10^{-10} M TCDD or 0.01% DMSO for 24 h, 36 h, or 48 h. Quantification of three spontaneous movement parameters were obtained using high content analysis as aforementioned in M&M section, including accumulated distance (A), displacement (B) and speed (C). Values are expressed as mean ± SEM from all tracked cells in 12 paralleled wells of 96-well plates in each group (TCDD group: n=1843 (24 h), 1073 (36 h), and 594 (48 h); DMSO group: n=1855 (24 h), 1111 (36 h) and 630 (48 h)). *$p < 0.05$, **$p < 0.01$ compared with the DMSO control groups (two-way ANOVA with Bonferroni test).

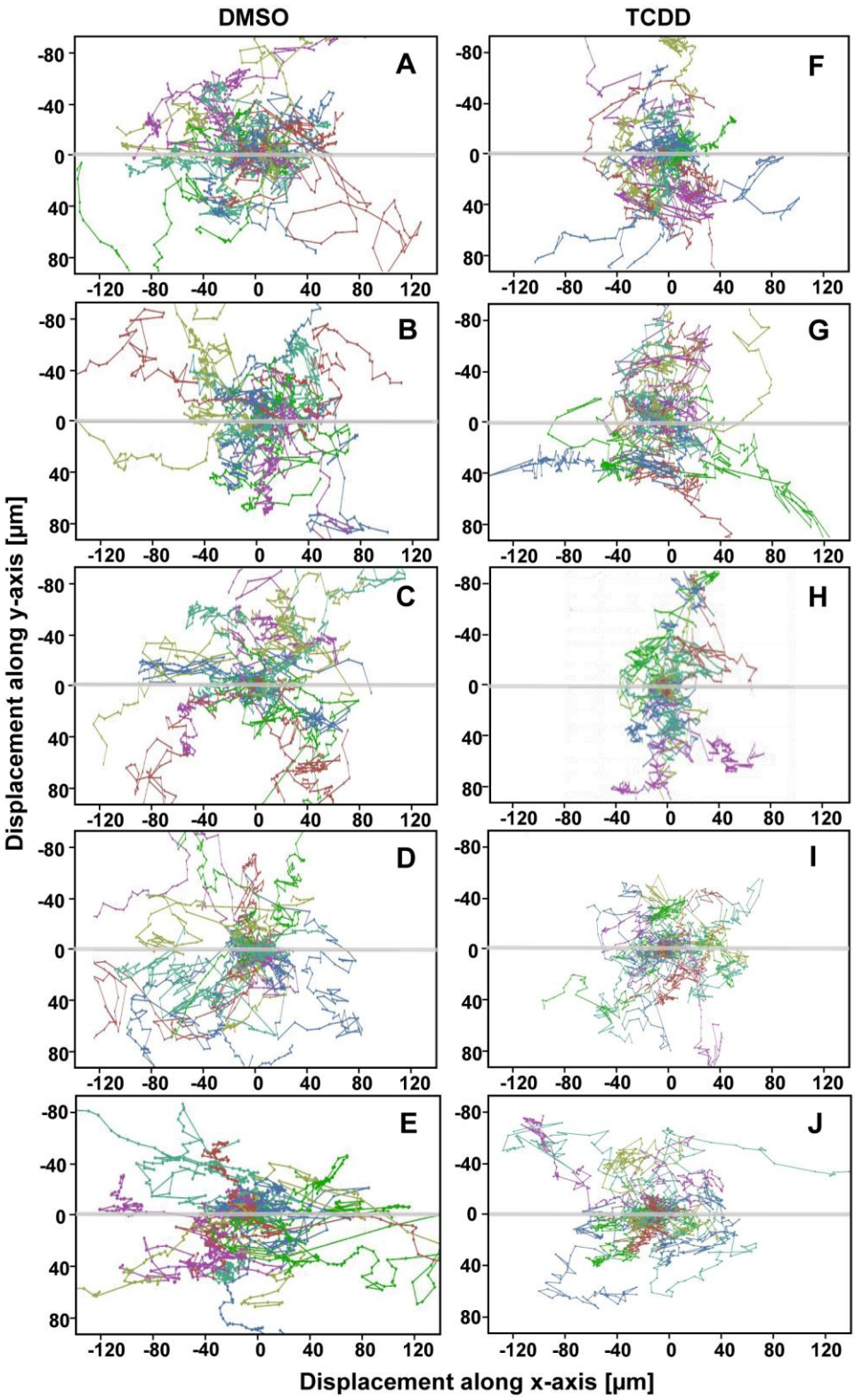

Figure 4 Trajectories of tracked SK-N-SH cells using high content analysis. Cells were incubated with 0.01% DMSO (A, B, C, D and E) or 2×10^{-10} M (F, G, H, I and J) TCDD for 48 h. Each line represents the movement route of single cell within 48 h time period. The starting positions of all tracked cells

were adjusted to the same origin of the coordinate axis according to the actual movement distances as aforementioned in M&M section. Each figure contains twenty tracked cells from four randomly selected fields in a well of 96-well plate (n=20). Trajectories from five representative wells are shown.

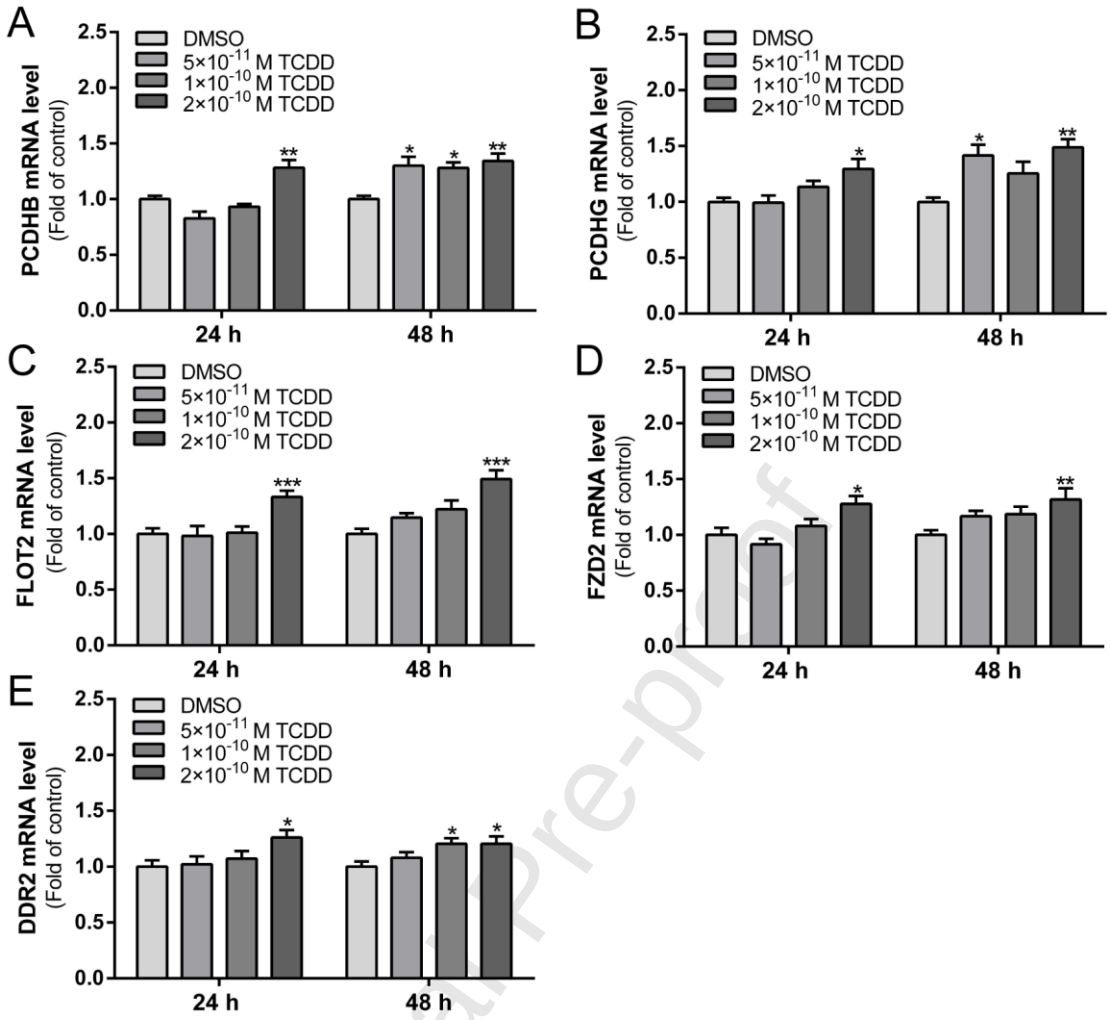

Figure 5 Effects of TCDD on expression of the pro-adhesive genes. SK-N-SH cells were treated with different concentrations of TCDD (5×10^{-11}, 10^{-10}, 2×10^{-10} M) or 0.01% DMSO for 24 h or 48 h. The mRNA expression levels of PCDHB (A), PCDHG (B), FLOT2 (C), FZD2 (D) and DDR2 (E) were determined by qRT-PCR as mentioned in M&M section. Values are expressed as mean ± SEM from triplicated wells of three independent experiments (n=3). Statistical analysis was done by two-way ANOVA with Bonferroni test. *p <0.05, **p < 0.01, ***p < 0.001 compared with DMSO control groups.

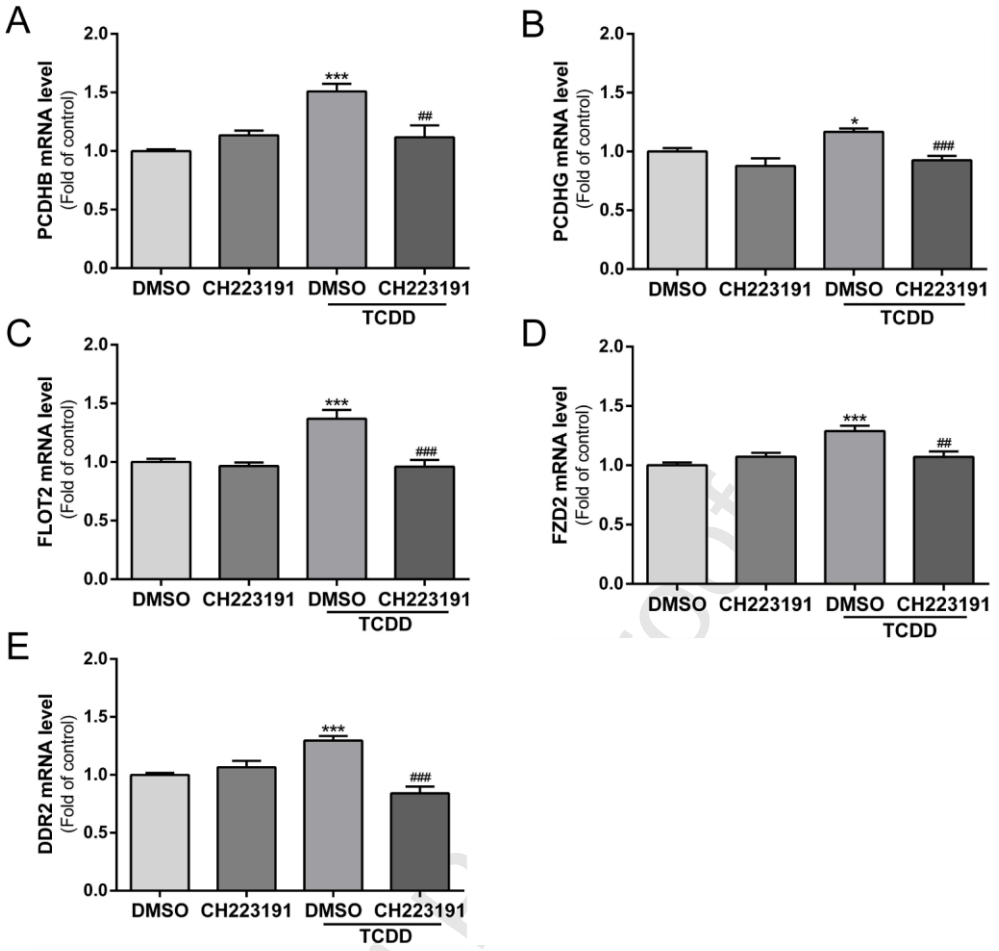

Figure 6 Inhibition of AhR pathway reverses the effect of TCDD on expression of the pro-adhesive genes. SK-N-SH cells were pretreated with 10^{-6} M CH223191 or 0.01% DMSO for 3 h and then treated with 2×10^{-10} M TCDD or 0.01% DMSO for 24 h. The mRNA expression levels of PCDHB (A), PCDHG (B), FLOT2 (C), FZD2 (D) and DDR2 (E) were determined by RT-qPCR as mentioned in M&M section. Values are expressed as mean ± SEM from triplicated wells of three independent experiments (n=3). Statistical analysis was done by one-way ANOVA with Bonferroni test. *p <0.05, ***p < 0.001 compared with DMSO control groups; ##p <0.01, ###p <0.001, compared with TCDD groups.

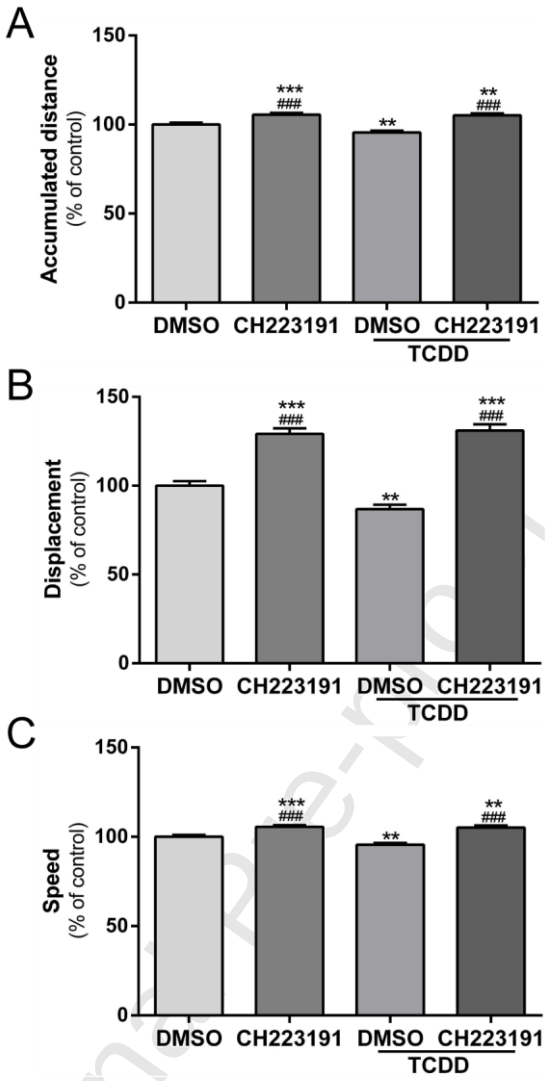

Figure 7 Inhibition of AhR pathway reverses the effect of TCDD on spontaneous movement of SK-N-SH cells using high content analysis. Cells were pretreated with 10^{-6} M CH223191 or 0.01% DMSO for 3 h and then treated with 10^{-10} M TCDD or 0.01% DMSO for 48 h. Quantification of three spontaneous movement parameters obtained using high content analysis as aforementioned in M&M section are shown, including accumulated distance (A), displacement (B) and speed (C). Values are expressed as mean ± SEM from all 48-hour-tracked cells in 12 paralleled wells of 96 well plates in each group (n=1100-1500). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the DMSO control groups; ###p <0.001, compared with TCDD groups (one-way ANOVA with Bonferroni test).

Table 1 Primer sequences of cell adhesion-related genes used in this study

Gene		Sequence	Accession No. In GenBank
18S	Forward	5′-CGCCGCTAGAGGTGAAATTC-3′	NR_003286
	Reverse	5′-TTGGCAAATGCTTTCGCTC-3′	
PCDHB	Forward	5′-TGAGCAGCGAGGCGCTGGTG-3′	Listed in Table S1
	Reverse	5′-GCTCGGTGCAGGGCGCGGAGC-3′	
PCDHG	Forward	5′-CGACTACCGCCAGAATGTCT-3′	Listed in Table S1
	Reverse	5′-TTCTCCTTCTTGCCCGACTT-3′	
FZD2	Forward	5′-GCCCTCATGAACAAGTTCGGT-3′	NM_001466
	Reverse	5′-GTCCTCGGAGTGGTTCTGGC-3′	
FLOT2	Forward	5′-GGCTGTTGTGGTTCCGACTA-3′	NM_001330170
	Reverse	5′-TCTCACAGCGACACAGGATG-3′	
DDR2	Forward	5′-GGCAGCAGTTTGTACTCCCT-3′	NM_001014796
	Reverse	5′-GGTCAATTGGCCTAGCCCTT-3′	

Declaration of interests

√ The authors declare that they have no known competing financial interests or personal relationships
that could have appeared to influence the work reported in this paper.
☐The authors declare the following financial interests/personal relationships which may be considered

as potential competing interests:

Graphical abstract