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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) suppresses spheroids attachment on endometrial epithelial cells through the down-regulation of the Wnt-signaling pathway

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ABSTRACT

The environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects embryo development, implantation and fertility in humans. The underlying molecular mechanism by which TCDD suppresses implantation remains largely unknown. We used the trophoblastic spheroids (embryo surrogate)-endometrial cells co-culture assay to study the attachment of trophoblastic spheroids (BeWo and Jeg-3) onto the endometrial epithelial (RL95-2 and Ishikawa) cells. TCDD dose-dependently induced cytochrome P450 1A1 (Cyp1A1) expression in trophoblastic and endometrial epithelial cells. Moreover, TCDD at 1 and 10 nM suppressed β-catenin (a Wnt-signaling molecule) and E-cadherin expression, as well as spheroids attachment onto endometrial cells. Interestingly, activation of the canonical Wnt-signaling pathway via Wnt3a or lithium chloride reverted the suppressive effect of TCDD on β-catenin and E-cadherin expressions in the BeWo and RL95-2 cells, and restored the spheroids attachment rate to be comparable to the untreated controls. Taken together, TCDD induces Cyp1A1 expression, modulates the Wnt-signaling pathway and suppresses spheroids attachment onto endometrial cells.

Key Words: TCDD; Spheroids; Endometrium; canonical Wnt-signaling; Attachment; Implantation
1. INTRODUCTION

Endocrine disruptors (EDs) are naturally occurring compounds or manufactured chemicals that interfere with the production or activity of hormones inside our bodies leading to adverse health effects. EDs can be anti-androgenic, androgenic, estrogenic, aryl hydrocarbon receptor (AhR) agonists, inhibitors of steroidogenesis, anti-thyroid substances, and retinoid agonists [1]. EDs have been linked with developmental, reproductive, immune and other problems in wildlife and laboratory animals. In humans, EDs have been associated with spontaneous abortion, early puberty, alterations of sex ratios, cancers, declining semen quality, male reproductive tract abnormalities and immune system disorders [2]. Moreover, EDs affect embryonic development starting from fertilization. They reduce the developmental rate of farm animal embryos [3], disrupt morphogenesis of rat preimplantation embryos [4] and reduce the numbers of implanted embryos and pups born [5].

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an ED in laboratory species, wildlife and humans [6, 7]. TCDD is produced as an unintentional by-product of many industrial processes that involve chlorine-based chemicals such as waste-burning incineration of various sorts, chemicals and pesticides manufacturing, for example production of polyvinyl chloride plastics (PVPs), as well as pulp and paper mills that use chlorine for bleaching. TCDD is a highly lipophilic, fat-soluble and semi-volatile chemical that is highly persistent in the environment. The tolerable intake of TCDD is about 1 to 4 pg per kilogram of body weight per day [8]. It has been reported that the body TCDD levels can be up to 100,000 times higher than that of the surrounding environment [9]. In China, accumulation of TCDD and estrogen-like pollutants occurs in marine and freshwater fishes cultivated in the Pearl River Delta [10]. Yet, the elimination half-life of TCDD in humans is estimated to be 7 to 9 years [11]. TCDD can damage the immune system and interfere with the
hormonal system. It lowers testosterone levels and cause reproductive and developmental problems in vertebrates [12]. In fact, TCDD elicits a number of toxic and biochemical responses in humans. The most well-known symptom of severe acute intoxication is chloracne, which is a persistent disturbance of epithelial cell differentiation in the skin. Other toxic responses are porphyria, hepatotoxicity [13], and peripheral and central neurotoxicity. Long-term persistence of TCDD in the body can cause teratogenicity, innate and adaptive immune suppression, atherosclerosis, carcinogenesis, hypo- and hyperplasia, diabetes, vascular ocular changes, and neural system damage, including neuropsychological impairment.

The receptor for TCDD, aryl hydrocarbon receptor (AhR) in mammalian tissues, functions as a sensor for a wide range of xenobiotics [14]. The ligand-bound AhR forms a heterodimeric complex with the resident aryl hydrocarbon nuclear translocator (ARNT) that regulates the expression of target genes such as cytochromes P450 (CYP1A1) by interacting with dioxin-responsive elements (DREs) [14, 15]. Yet, AhR activation can inhibit or promote steroid hormone signaling in reproductive tissues, causing estrogenic or anti-estrogenic effects [16]. Environmental AhR ligands have been implicated in promoting endometriosis and endometrial cancer in various species. TCDD was found to inhibit implantation [17] initiated by estrone in animals [18], suggesting that this anti-estrogenic action is a possible interference mechanism of TCDD on blastocyst-uterine interactions.

The Wnt-signaling pathway plays an important role in implantation. A number of microarray studies identified that DKK molecules (Wnt-signaling molecules) were differentially regulated in the human endometrium during implantation [19-22]. These molecules regulate the canonical Wnt-signaling pathway in Xenopus embryos [23]. Wnt-signaling pathway is critical for estrogen-mediated uterine growth [24] and implantation in mice [25, 26]. In fact, suppression of the pathway by GSK-3β
phosphorylation on $\beta$-catenin affects the implantation process in mice [25]. Yet, how TCDD affects implantation and the signaling pathway(s) involved remains largely unknown. In the present study, we investigated the mechanism of TCDD in regulating implantation with the use of the spheroids-endometrial epithelial cells co-culture assay, and delineated the role of canonical Wnt-signaling in regulating the spheroids attachment process.
2. Materials and methods

2.1. Cell culture

Choriocarcinoma Jeg-3 (HTB-36, ATCC, Manassas, VA) and BeWo (CCL-98, ATCC), and endometrial adenocarcinoma Ishikawa (ECACC 99040201, Sigma, St Louis, MO) and RL95-2 (CRL-1671, ATCC) cells were cultured at 37°C in a humid atmosphere with 5% CO₂. Jeg-3 and RL95-2 cells were maintained in DMEM/F12 (Sigma), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, Gibco). Ishikawa was cultured in Minimal Essential Medium (Sigma) supplemented with 10% FBS, L-glutamine and penicillin/streptomycin, while BeWo cells were cultured in RPMI 1640 (Sigma) containing the same supplements. TCDD (Cambridge Isotope Laboratories, USA) with 99% chromatographic purity was dissolved in dimethyl sulfoxide (DMSO). In TCDD exposure experiments including quantitative PCR validation and protein analysis, 1x 10⁵ cells/well were seeded in 12-well tissue culture plates (IWAKI, Japan) in cell culture medium as described above. The cells were then cultured in TCDD containing culture medium for 24 hours. TCDD concentrations ranging from 0.01 to 10 nM in DMSO (0.1% final concentration) were prepared and 0.1% DMSO was used as a negative control. All treatments and controls were repeated 5 times in duplicate.

2.2. RNA extraction and quantitative PCR

Total RNAs from cell lines were extracted using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. RNA samples (300 ng) were reverse-transcribed into cDNA using TaqMan® Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA). A multiplex real-time polymerase chain reaction using 18S as an internal control for the
normalization of RNA loading was performed in a 20 µl reaction mixture with
Assays-on-Demand Gene Expression Assay for human AhR (Hs01054797_g1), Cyp1A1 (Hs00169233_m1) and ribosomal 18S (Hs99999901_s1) TaqMan probes (PE Applied Biosystems). A standard PCR cycling protocol was performed: 1 cycle at 95°C for 10 minutes; and 40 cycles at 95°C for 15 seconds, 60°C for 35 seconds and 72°C for 45 seconds. The house-keeping 18S gene was chosen as the internal control for sample normalization. Relative mRNA expression was quantified using the \( 2^{-\Delta\Delta \text{Ct}} \) method as described elsewhere [27].

### 2.3. Protein extraction and Western blotting

Total protein from the cell lysates was dissolved in RIPA solution (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Antibodies specific for AhR (sc-8088, Santa Cruz, Santa Cruz, CA), CYP1A1 (sc-20772, Santa Cruz), β-catenin (1:2500, BD Bioscience, San Jose, CA), E-cadherin (1:1000, Abcam, Cambridge, MA) and GSK-3β (BD Bioscience) obtained from different sources were used for Western blot analysis. The anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (1:5000, GE Healthcare, Pittsburgh, PA) was used secondary antibody. After thorough washing, proteins on the blotted membrane were visualized by an enhanced chemiluminescence reagent (Santa Cruz). To normalize protein loading, the membranes were stripped and detected for β-actin using anti-β-actin antibody (Sigma).

### 2.4. Cell proliferation assay

Ninety-six well plates were seeded with 3 X 10^3 cells per well in triplicate. The cells were cultured for 3 days with different concentrations of TCDD. CyQUANT® Cell Proliferation Assays were performed to determine the cell number in terms of
DNA content. Briefly, CyQUANT® NF dye reagent was mixed with the 1X Hank’s balanced salt solution (HBSS), and 100 μL of 1X dye binding solution was dispensed into each microplate well for DNA binding. After 1 hour of incubation at 37°C, the fluorescent signal was measured at 535 nm.

2.5. Spheroids-endometrial cell attachment assay

Human choriocarcinoma cells (Jeg-3 and BeWo) and endometrial adenocarcinoma cells (Ishikawa and RL95-2) were cultured at 37°C in a humid atmosphere with 5% CO₂. Adhesion of choriocarcinoma Jeg-3 spheroids to endometrial Ishikawa cells was quantified using an adhesion assay as described [22, 28, 29]. In the co-culture study, Jeg-3 and BeWo cells were treated with 0.1–10 nM TCDD, 50% Wnt3a conditioned medium and 40 mM LiCL for 24 hours. The cell differentiation reagent methotrexate (MTX, 5 μM) was used as positive control [28] and solvent alone (DMSO) was used as negative control in the experiments. Spheroids cells were generated by rotating the trypsinized cells at 4 g for 24 hours. Spheroids ranging in size from 60–200 μm were selected and transferred onto the confluent monolayer of endometrial cells under a dissection microscope. Then, the co-cultures were maintained in the respective medium for 1 hour at 37°C in a humidified atmosphere with 5% CO₂. Non-adherent spheroids were removed by centrifugation at low g-force (10 g) for 10 minutes in media. Attached spheroids were counted under a dissecting microscope and the attachment rate was expressed as a percentage of the total number of spheroids transferred (% adhesion). Images of the cultures were taken by a Nikon Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan).
2.6. Wnt-signaling activation

Wnt3a conditioned medium was obtained from culturing mouse L cells stably secreting Wnt3a protein as described [30]. Briefly, Wnt3a over expressing mouse fibroblast L cells (CRL-2647, ATCC) were cultured in DMEM medium supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. The conditioned medium was collected after 48 hour of confluent culture and the presence of Wnt3a protein was confirmed with specific Wnt3a antibody by Western blotting. The conditioned medium was filter sterilized and stored at -20°C until used.

2.7. Statistical analysis

All the data were analyzed by statistical softwares (SigmaPlot 11.0 and SigmaStat 2.03; Jandel Scientific, San Rafael, CA). The non-parametric analysis of variance on rank test for multiple comparisons followed by the Mann-Whitney U test was used when the data were not normally distributed. A probability value <0.05 was considered to be statistically significant.
3. Results

3.1. Effect of TCDD on AhR and CYP1A1 expression in trophoblastic and endometrial cell lines

The effects of TCDD on AhR and Cyp1A1 expression in trophoblastic (BeWo and Jeg-3) and endometrial epithelial (RL95-2 and Ishikawa) cells were studied. TCDD at 0.01-10 nM did not change AhR expression in the trophoblastic (BeWo and Jeg-3) and endometrial epithelial (RL95-2 and Ishikawa) cells (Figure 1A & B), but strongly induced their transcript and protein expression of CYP1A1 (Figure 1C & D). At 1 and 10 nM of TCDD, there were significant increases (p<0.05) of CYP1A1 transcripts in the four cell lines tested.

3.2. TCDD suppresses spheroids attachment onto endometrial cells

To study how TCDD modulates the attachment process in vitro, we used the spheroids-endometrial cells co-culture assay to study the attachment of trophoblastic spheroids onto endometrial epithelial cells. BeWo and Jeg-3 spheroids of 60–200 μm in size were generated from the trypsinized cells with shaking for 1 day. The spheroids were transferred onto either an RL95-2 or Ishikawa monolayer and co-cultured for an hour and the number of spheroids attached was determined (Figure 2A). TCDD (0.1–10 nM) dose-dependently suppressed BeWo and Jeg-3 spheroids attachment onto the RL95-2 and Ishikawa cells (Figure 2B). At 10 nM TCDD, the attachment rate of BeWo onto RL95-2 and Jeg-3 spheroids onto Ishikawa decreased significantly (p<0.05) from 86% to 50% and 95% to 75%, respectively when compared with the untreated controls. MTX at 5 μM strongly suppressed (p<0.05) spheroids attachment. The average viability of the cell lines in all the groups was >90% as determined by the CyQuant cell proliferation assay (data not shown).
3.3. TCDD suppresses E-cadherin and β-catenin expression in the BeWo and RL95-2 cells

To study whether TCDD regulated canonical Wnt-signaling and extracellular matrix molecule expression in BeWo and RL95-2 cells, the expression levels of β-catenin and E-cadherin were determined. Treatment of the BeWo or RL95-2 cells with TCDD at 1 and 10 nM for 24 hours significantly suppressed (p<0.05) E-cadherin and β-catenin expression (Figure 3A & B) relative to that β-actin. The expression of β-actin protein was used as the loading control.

3.4. Activation of the canonical Wnt-signaling pathway nullifies the suppressive effect of TCDD on spheroids attachment and restores E-cadherin and β-catenin expression

Wnt3a and LiCl treatments were used to activate the Wnt-signaling pathway. Addition of Wnt3a conditioned medium or LiCl (40 μM) restored TCDD-induced reduction of β-catenin and E-cadherin expression, but had no effect on GSK-3β expression in BeWo and RL95-2 cells (Figure 4A). The expression of β-actin protein was used as the loading control. Both Wnt3a and LiCl restored the spheroids attachment rate suppressed by TCDD when compared with the untreated control. Interestingly, Wnt3a but not LiCl itself had a stimulatory effect on spheroids attachment in vitro.
4. Discussion

The present study suggests a possible suppressive mechanism of TCDD on the embryo attachment process by down-regulation of the Wnt-signaling pathway and adhesion molecules. TCDD induced CYP1A1 but not AhR expression in trophoblastic and endometrial epithelial cells. It suppressed spheroids attachment onto endometrial epithelial cells in vitro by reducing β-catenin and E-cadherin protein expression in trophoblastic cells. The effect of TCDD on decreased spheroids attachment could be reversed by Wnt-agonist (Wnt3a and LiCl) treatments, suggesting that the down-regulation of the Wnt-signaling pathway by TCDD could be one of the molecular pathways leading to suppression of spheroids attachment onto the endometrial cells.

TCDD induced CYP1A1 but not AhR expression in all the four cell lines tested. Similarly, TCDD induces CYP1A1 mRNA expression in preimplantation mouse embryos [31] as well as protein expression in explant cultures of human endometrium [32]. Moreover, TCDD causes a significant increase in the secretion of immunoreactive (I) but not bioactive (B) chorionic gonadotropin (CG) from the differentiated but not the undifferentiated human trophoblast cultures [33], suggesting that TCDD-induced pregnancy loss may be associated with a decrease of B/I ratio of CG. Furthermore, TCDD also affects ovarian steroidogenesis and follicle development [34], and has been associated with an increase in spontaneous abortion in animals [35] and implantation loss of fertilized oocytes in humans [17].

To study how TCDD affects the initial attachment of embryos during the implantation process, we used an in vitro spheroids-endometrial epithelial cells cell culture model to simulate this process [22, 28, 29]. It was found that TCDD dose-dependently suppressed trophoblastic spheroids (BeWo and Jeg-3) attachment onto the endometrial epithelial cell lines (RL95-2 and Ishikawa). Although TCDD
at 0.2 nM had been reported to decrease viability of JAr cells [36], we did not observe a significant change in viability of the cell lines studied even after treatment with 10 nM of TCDD in the present study. Similarly, TCDD at 10 nM does not enhance proliferation of Jeg-3 cells as demonstrated by thymidine incorporation [37]. Although TCDD accelerates the differentiation of the blastocyst, it neither alters the development of early mouse morula to blastocysts [38] nor induces apoptosis of preimplantation embryos [31, 39]. A recent study suggested that maternal TCDD exposure disrupts embryo morphogenesis at the compaction stage (8-16 cell), but does not compromise development of the embryo to blastocyst [4]. However, TCDD exposure increases early fetal loss in Cynomolgus macaque [40].

The canonical Wnt-signaling pathway is involved in embryo implantation and early embryonic development [25, 26]. But how the disruption of the Wnt-signaling pathway via reduced β-catenin and E-cadherin expression after exposure to TCDD affects embryo attachment remains largely unknown. In this study, TCDD treatment suppressed E-cadherin and β-catenin expression in the trophoblastic and endometrial cell lines, and addition of Wnt-activator (Wnt3a or LiCl) restored E-cadherin and β-catenin expressions and reversed these suppressive effects on spheroids attachment. In line with this, exposure to TCDD during pregnancy reduced the expression of E-cadherin in the mammary gland [41] and E-cadherin has been identified as an essential molecule for endometrial receptivity [42].

In mice, inactivation of the canonical Wnt-signaling pathway via a reduced level of β-catenin significantly blocked the implantation competency of the embryo [26]. As E-cadherin/β-catenin complexes are important for formation of adherens junctions in endometrial epithelial cells [43], it is likely that the decrease in E-cadherin/β-catenin levels affects spheroid attachment in vitro. To study the role of E-cadherin on spheroid attachment, we used anti-E-cadherin antibody to study the
role of E-cadherin protein in the spheroid attachment assay. Addition of the
anti-E-cadherin antibody (1-4 μg/ml) significantly (p<0.05) suppressed spheroid
attachment in vitro (unpublished data), consistent with the inhibition of implantation
by injection of anti-E-cadherin antibody into the mouse uterine horn [44]. Although
E-cadherin null mouse embryos fail to develop to the blastocyst stage, heterozygous
mutant mice are fertile [45], indicating that a reduced expression of E-cadherin does
not affect implantation. The present study showed that TCDD reduced E-cadherin
expression by ~50% and suppressed spheroid attachment at the same time. These
observations suggest that other genes, apart from E-cadherin, are mediating the
suppressive action of TCDD on spheroid attachment [46].

Taken together, TCDD induces CYP1A1, but not AhR expression in the
trophoblastic and endometrial cell lines. Our in vitro co-culture model suggests that
aberrant expression of Wnt-signaling and adhesion molecules is one of the underlying
molecular mechanisms through which TCDD affects spheroid (blastocyst surrogate)
attachment. These initial findings on TCDD action are being confirmed with the use
of human primary endometrial epithelial cells in our laboratory.

5. Conflict of interest

The author declares that there is no conflict of interest.

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1 References


[34] Heiden TK, Carvan MJ 3rd, Hutz RJ. Inhibition of follicular development, vitellogenesis, and serum 17beta-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin.


[37] Zhang L, Shiverick KT. Benzo(a)pyrene, but not 2,3,7,8-tetrachlorodibenzo-p-dioxin, alters cell proliferation and c-myc and growth factor expression in human placental choriocarcinoma JEG-3 cells. Biochem Biophys Res Commun 1997;231:117-20.


**Figure Legend**

**Figure 1** Effect of TCDD on AhR and Cyp1A1 expression in trophoblastic (BeWo and Jeg-3) and endometrial epithelial (RL95-2 and Ishikawa) cells. (A) Western blotting on the expression of AhR in TCDD (0.01-10nM)-treated RL95-2 cells. (B) The expression of AhR transcript levels in TCDD-treated trophoblastic and endometrial epithelial cells were determined by quantitative PCR. (C) Western blotting on the expression of Cyp1A1 in TCDD (0.01-10nM)-treated RL95-2 cells. (D) The expression of Cyp1A1 transcript levels in TCDD-treated trophoblastic and endometrial epithelial cells was determined by real-time PCR.

**Figure 2** Effect of TCDD on the attachment of trophoblastic spheroids to endometrial epithelial cells. (A) BeWo cells treated with TCDD were trypsinized and shaken for 24 hours to obtain spheroids of 60–200µm in size. The spheroids were put onto an RL95-2 monolayer for an hour and the number of spheroids attached was determined as a percentage of the number of spheroids added. Spheroids derived from JAr cells treated with MTX (5µM) and DMSO (solvent control) were used as positive and negative controls, respectively. (B) The effect of TCDD treatment on spheroids attachment onto endometrial cells was determined at 1 hour after co-culture. TCDD (0.1-10nM) dose-dependently suppresses BeWo and Jeg-3 attachment onto RL95-2 and Ishikawa cells, respectively. The results were pooled from at least 4 independent experiments using more than 2000 spheroids. *, ** denotes significant difference (p<0.05) from the control.

**Figure 3** Effect of TCDD on the expression of E-cadherin and β-catenin in BeWo and RL95-2 cells. (A) BeWo and (B) RL95-2 cells were treated with 0.1-10nM TCDD for 24 hours and the expression levels of E-cadherin and β-catenin proteins
were determined by Western blotting. The protein loading was normalized by β-actin expression. * denotes significant difference (p<0.05) from the control.

Figure 4 Wnt3a and lithium chloride induced the Wnt-signaling pathway, restored β-catenin and E-cadherin expressions and spheroids attachment. (A) Wnt3A conditioned medium and LiCl (40μM) restores β-catenin and E-cadherin, but not GSK-3β expressions in the TCDD treated BeWo and RL95-2 cells. The expression of β-actin protein was used as a loading control. (B) Both Wnt3A and LiCl restored the suppressed spheroids attachment rate by TCDD onto endometrial epithelial cells. Wnt3a alone has a stimulatory effect on spheroids attachment in BeWo and RL95-2 cells. *, ** denotes significant difference (p<0.05 and <0.01, respectively) from the control.
**Figure 1**

**A**

AhR Expression

**B**

BeWo

AhR Expression

**C**

AhR Expression

**D**

Jeg-3

AhR Expression

TCDD (0.001-10nM)

**E**

RL95-2

AhR Expression

**F**

Ishikawa

AhR Expression

TCDD (0.001-10nM)

**AhR Expression**

Control 0.01 0.1 1 10

**Cyp1A1 Expression**

Control 0.01 0.1 1 10

*Significant difference compared to control.
Figure 2

A

Spheroids

Spheroid on RL95-2 cells

B

** BeWo + RL95-2 **

Jeg-3 + Ishikawa

** BeWo only **

RL95-2 only

Attachment rate (%)
Figure 3

A

TCDD (0.1-10nM)
E-cadherin
β-catenin
β-actin

BeWo

Relative protein expression level

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B

TCDD (0.1-10nM)
E-cadherin
β-catenin
β-actin

RL95-2

Relative protein expression level

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Figure 4

A

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<td>LiCl (40μM)</td>
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<td>BeWo + RL95-2</td>
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<td>+</td>
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β-catenin (92 kDa)
E-cadherin (122 kDa)
GSK-3β (73 kDa)
β-actin (42 kDa)

B

BeWo + RL95-2

<table>
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Jeg-3 + Ishikawa

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<th>TCDD (10nM)</th>
<th>Wnt3a-CM</th>
<th>LiCl (40μM)</th>
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