

# Effects of octylphenol on the expression of cell cycle-related genes and the growth of mesenchymal stem cells derived from human umbilical cord blood

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**Abstract.** Umbilical cord blood (UCB) is defined as blood that exists in the placenta and in the attached umbilical cord following childbirth. Cord blood is now used for research purposes as it contains mesenchymal stem cells (MSCs), multipotent stromal cells which have the ability to differentiate into a variety of cell types. Among endocrine disrupting chemicals (EDCs), octylphenol (OP) is one of the alkylphenols, which are widely used industrial chemicals; these chemicals cause a number of serious side-effects, such as reproductive abnormalities. In this study, we isolated human MSCs from UCB and demonstrate that cultured MSCs express the surface marker, CD34, but not CD105. We further examined the effects of OP on human UCB-derived MSCs following exposure to OP by cell proliferation assay, semi-quantitative RT-PCR and western blot analysis. The results revealed that the transcriptional and translational levels of cyclin D1 were increased, while the levels of p21 were suppressed in the MSCs treated with OP compared with the negative controls. This collapse of the regulation of the cell cycle may directly stimulate the growth of the MSCs under culture conditions. The results from the present study provide further insight into the effects of common EDCs on MSCs derived from human UCB. However, further studies are required to identify the signaling pathways which mediate the effects of EDCs on MSCs.

## Introduction

Umbilical cord blood (UCB) is defined as blood that exists in the placenta and in the attached umbilical cord following childbirth (1). Cord blood is now used for research purposes as it contains stem cells, multipotent stromal cells which have the ability to differentiate into a variety of cell types; thus, cord blood has immense potential for use in the treatment of various diseases (2,3). Cord blood contains all the elements found in whole blood, namely red blood cells, white blood cells, plasma and platelets, which are required for the exchange of gases and nutrients between the mother and baby until birth (4).

Mesenchymal stem cells (MSCs) are multipotent stromal cells and can differentiate into a lineage of cell types, such as osteogenic, adipogenic and chondrogenic cells (5). In a previous study, MSCs were isolated from precursor cells originating from the mesenchyme, and were able to differentiate into different types of blood cells (6). MSCs tend to have a fibroblast-like morphology under normal culture conditions. Previously, cultured MSCs were shown to express the surface molecular markers, CD90 and CD105, but not the CD11b or CD34 surface markers (7). MSCs originate from various adult tissues, such as blood, adipose tissue and UCB (8). Among these stem cells, UCB-derived MSCs in particular, have been investigated for their potential use in clinical studies as their use is considered more acceptable in terms of ethics than other stem cells (9). The use of UCB-derived MSCs in clinical experiments does not pose moral or ethical issues, in contrast to embryonic stem cells (10). In addition, UCB-derived MSCs can escape the immune system and are therefore considered safe for use in transplantation as they are not susceptible to rejection by the host. UCB has been demonstrated to be a good source of MSCs (11).

Endocrine disrupting chemicals (EDCs) are known to interfere with the mammalian endocrine system and cause a number of serious side-effects, such as developmental, reproductive and autoimmune disorders in both humans and wildlife (12). EDCs may be found in personal products, plastic bottles and pesticides. Among the EDCs, octylphenol (OP) is one of the alkylphenols, which are widely used industrial chemicals (13).

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OP has been reported to be persistent in our environment and has been detected in human blood and urine (14). Exposure to OP can increase the risk of productive and developmental diseases, as it has been shown to be estrogenic, which may interfere with the regulation of the endocrine system through hormonal receptors (15). In addition, OP has been reported to deregulate the cell cycle and has been found in all reproductive organs, such as the ovaries and testes, as well as in neurons, and in the hearts of babies (16). Exposure to OP can lead to impaired fertility in mammals. EDCs can interfere with the endocrine system by binding to hormonal receptors, and the accumulation of EDCs in the body through persistent exposure may influence reproductive events over generations. To date, this concern is supported by the investigation of the adverse effects of alkylphenols on genital organ development of fetuses, which is critical for sexual differentiation (17). Alkylphenolic chemicals mimic estrogenic functions in the cells and organs of humans and animals (18). As the use of alkylphenols as industrial chemicals increases, this will increase the exposure to these chemicals in everyday life, which may then cause an increase in the incidence of human health issues, such as cancer (19).

Normal cell proliferation is controlled by the cell cycle, and the cell cycle checkpoints verify whether the processes at each cell cycle phase have been properly completed. This regulation mechanism depends on cell cycle inhibitors and promoters; for example, cyclin D1 is essential for the transition between cell cycle phases, while p21 and p27 inhibit the progression of the cell cycle (12). Genes associated with the cell cycle play a critical role in cell proliferation. However, the functions of cyclin D1 and p21 have been reported to be altered by EDCs, such as bisphenol A or OP, leading to cancer of the reproductive organs in humans; however, the exact mechanisms involved remain unclear (12,20). Thus, EDCs may also exert effects on UCB and the placenta during pregnancy. Studies on the effects of EDCs on UCB are required to elucidate their effects on human health.

The abnormal proliferation of MSCs in human UCB may cause severe and unexpected side-effects to the fetus. In this study, we examined the effects of OP on the expression of cell cycle-related genes, such as cyclin D1 and p21 in MSCs derived from human UCB to elucidate the effects of EDCs on stem cell growth.

## Materials and methods

**Isolation of MSCs.** UCB samples were obtained by Dr Tae-Hee Kim. Using Ficoll-paque PREMIUM (Life Technologies, Rockville Corp., Rockville, MD, USA), we isolated MSCs from UCB following the protocol suggested by GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Following the addition of Ficoll-paque PREMIUM (4 ml/1 tube) to the centrifuge tube, we gently isolated the MSC layers from the blood residue as follows: mixtures of blood with Ficoll-paque PREMIUM were centrifuged at 400 x g for 50 min at 14°C. Following centrifugation, the upper layer was drawn off using a sterile Pasteur pipette. The second layers were transferred to new centrifuge tube and the same process was repeated twice to obtain the pellet containing the MSCs. The protocol for obtaining UCB from human subjects (pregnant women following childbirth)

and the procedure used to isolate the MSCs from UCB were approved by the Institutional Review Board (IRB) of Soonchunhyang University, Bucheon, Korea.

**Culture of MSCs.** The pellet containing the MSCs was suspended in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc.), 100 IU/ml penicillin and 100 mg/ml streptomycin (Corning cellgro/ Mediatech, Inc., Manassas, VA, USA) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The cell culture medium was changed after 7 days following the observation of MSC morphology.

**Cell viability assay.** The effects of OP on MSC viability were determined by MTT assay. Isolated MSCs were plated at a density of 3.0x10<sup>4</sup> cells/well in 96-well plates (Thermo Fisher Scientific Inc., Roskilde, Denmark) with 0.1 ml of cell culture medium. Following incubation for 7 days, the cells were treated with OP (10<sup>-6</sup> M; Sigma-Aldrich Corp., St. Louis, MO, USA) in the medium described above for 6 days. After this treatment, the cells were treated with MTT solution (10 µl of 5 mg/ml; Sigma-Aldrich Corp.) and incubated at 37°C for 3 h. The medium containing MTT was suctioned and the precipitants were solubilized in EtOH. Absorbance was measured at 540 nm using an ELISA reader (VERSA man; Molecular Devices, Sunnyvale, CA, USA). Each experiment was performed in triplicate (n=6).

**Semi-quantitative reverse transcription-polymerase chain reaction (semi-quantitative RT-PCR).** The MSCs were cultured at a density of 3x10<sup>5</sup> cells/well in 6-well plates (Thermo Fisher Scientific Inc.) and were then treated with OP for 6 days. Using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, the concentration of total-RNA was measured using a spectrophotometer (Optizen; Mecasys Co., Ltd., Daejeon, Korea) at 260/280 nm. Total RNA was suspended in diethylpyrocarbonate-deionized water (DEPC-DW) for complementary DNA (cDNA) synthesis. To synthesize the cDNA, a mixture containing murine leukemia virus reverse transcriptase (MMLV-RT; Intron Biotechnology, Sungnam, Korea), nonamer random primers, deoxyribonucleotides (dNTPs), an RNase inhibitor and RT buffer (Intron Biotechnology) was used. cDNA synthesis was performed at 37°C for 1 h and 95°C for 5 min.

The transcripts of CD105, CD34, cyclin D1, p21, and GAPDH genes were amplified as previously described (12,20). The sequences for specific forward and reverse primers are presented in Table I. PCR reactions were performed for 26-30 cycles with denaturation for 30 sec at 95°C, annealing for 30 sec at 58°C, and extension for 30 sec at 72°C using a thermocycler (PTC-100; MJ Research, St. Bruno, QC, Canada). The PCR products (8 µl) were analyzed on a 1.5% agarose gel pre-stained with ethidium bromide (EtBr; Sigma-Aldrich Corp.). The gels were scanned by a Gel Doc 2000 apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and compared to a 100 bp ladder of DNA (Intron Biotechnology). GAPDH was used as the positive control and a PCR reaction without cDNA was used as the negative control (data not shown). Each experiment was performed in triplicate.

Table I. Primer sequences and predicted sizes of the semi-quantitative reverse-transcription PCR products.

Target gene	Sequence (5'→3')	Expected size (bp)
Cyclin D1	F: TCTAAGATGAAGGAGACCATC R: TGACAGGTCCACATGGTCTTCC	354
p21	F: AGGCACCGAGGCACTCAGAG R: TGACAGGTCCACATGGTCTTCC	370
CD34	F: CATCACAGAAACGACAGTCAA R: ACTCCGCACAGCTGGAGG	345
CD105	F: CGGTGGTAGGCTGCAGACCTCACC R: CCTATGGACTTCCTGGTCTTGAGACC	640
GAPDH	F: ATGTTTCGTCATGGGTGTGAACCA R: TGGCAGGTTTTTCTAGACGGCAG	351

F, forward sequence; R, reverse sequence; PCR, polymerase chain reaction.

**Immunoblot analysis.** Following treatment of the MSCs with OP, the total proteins from the cells were harvested using RIPA lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS). Protein concentration was determined using the bicinchoninic acid (BCA) assay (Sigma-Aldrich Corp.). Total protein (50 µg) was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.). The membranes were incubated with cyclin D1 (a mouse monoclonal antibody, 1:2,000 dilution; Abcam, Hanam, Korea), p21 (a mouse monoclonal antibody, 1:4,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (a mouse monoclonal antibody, 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 4 h at room temperature. Primary antibody binding was detected with the secondary antibody of horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibodies (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The expected proteins were visualized using an ECL chemiluminescence system (GenDEPOT; Barker, TX, USA). The band densities were quantified using Gel Doc 2000 (Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate.

**Statistical analysis.** Data are presented as the means ± standard deviation (SD). Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). A statistical analysis was performed using a one-way ANOVA of variance followed by Dunnett's multiple comparison test. P-values <0.05 were considered to indicate a statistically significant difference.

## Results

**Phenotypic characteristics of UCB-derived MSCs.** The MSCs were isolated from UCB and cultured for 7 days. The morphology of the MSCs appeared to be fibroblastic, and the MSCs had thin cell bodies and adherent ability under culture

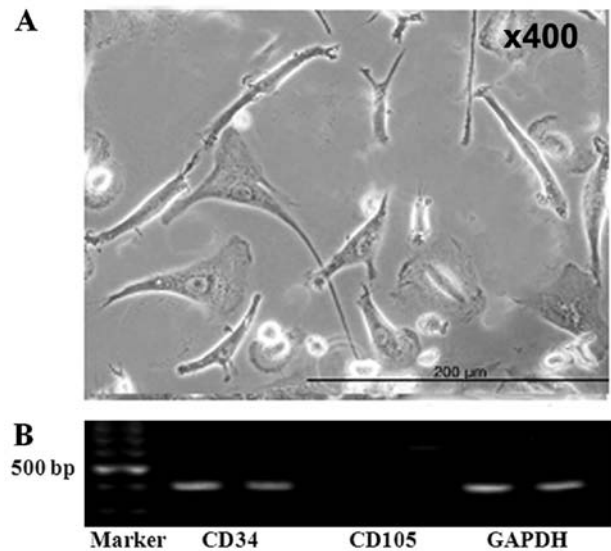


Figure 1. Morphology of mesenchymal stem cells (MSCs) and the expression of cell surface markers in MSCs. (A) Morphology of MSCs derived from human umbilical cord blood 8 days following isolation. (B) Identification of cell surface markers (CD34 and CD105) in MSCs. MSCs were characterized by the positive expression of CD34 and the negative expression of CD105.

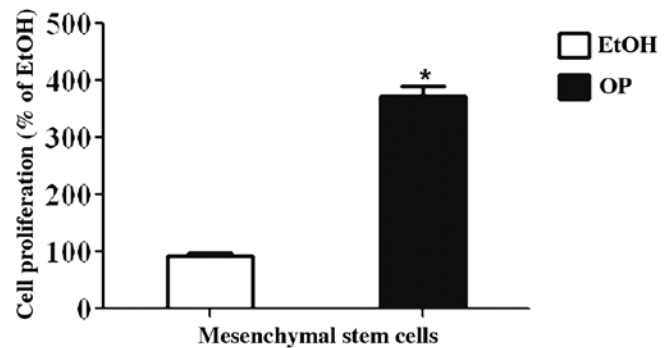


Figure 2. Effect of octylphenol (OP) on the growth of mesenchymal stem cells (MSCs). MSCs were treated with EtOH as the vehicle or OP ( $10^{-6}$  M) for 6 days and cell viability was determined by MTT assay at 540 nm. Data are presented as the means ± SD of triplicate experiments. \*P<0.05 compared to the vehicle treated with EtOH.

conditions, as shown in Fig. 1A. The cultured MSCs were shown to express the surface marker, CD34, but not CD105 (Fig. 1B). We confirmed the morphology of the MSCs and surface markers by semi-quantitative RT-PCR, as shown in Fig. 1B, suggesting that these cells derived from human UCB appeared to be specific MSCs isolated in our culture system.

**Effect of OP on MSC viability.** To determine the ability of OP to promote normal cell proliferation, the MSCs were cultured with OP ( $1 \times 10^{-6}$  M) for 6 days. In this study, OP markedly increased the viability of the MSCs in the culture medium containing OP, as shown in Fig. 2, suggesting that the normal growth potential of MSCs can be altered by EDCs in human UCB. This may cause adverse developmental side-effects to the mother and fetus, although the mechanisms involved warrant further investigation.

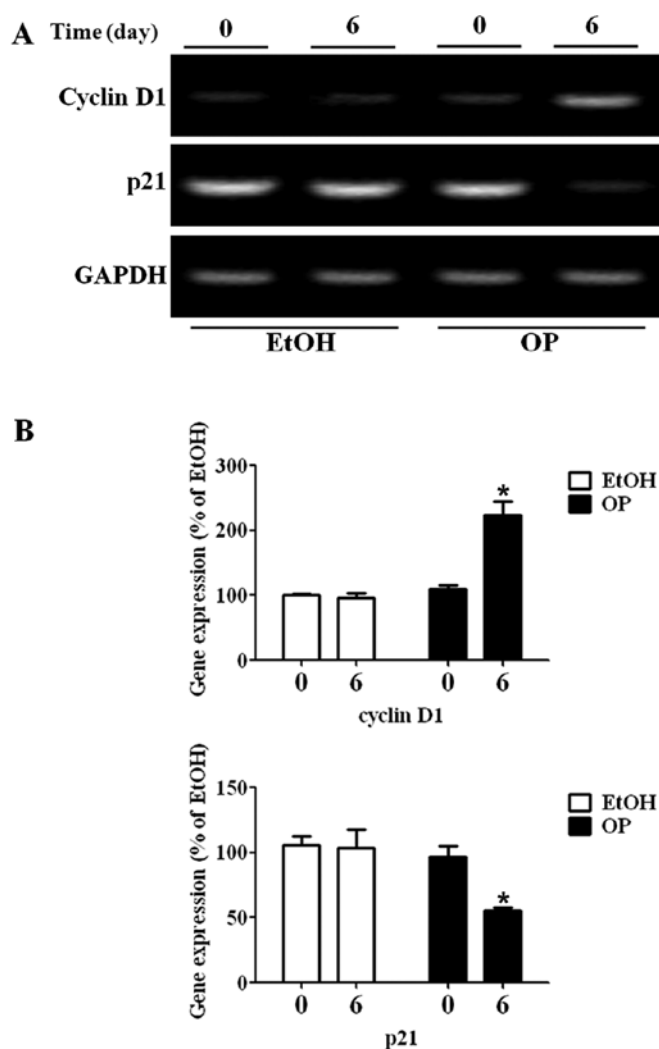


Figure 3. Altered mRNA expression of cyclin D1 and p21 in mesenchymal stem cells (MSCs) following exposure to octylphenol (OP). Transcriptional levels of cyclin D1, p21 and GAPDH were measured by semi-quantitative RT-PCR. (A) PCR products were separated on a 1.5% agarose gel. (B) The quantity of bands in the gel was analyzed using Gel Doc 2000 as described in Materials and methods. \* $P < 0.05$  compared to the vehicle treated with EtOH.

*Altered transcriptional levels of cyclin D1 and p21 by OP.* To evaluate the effects of OP on the mRNA expression of cell cycle-related genes (cyclin D1 and p21), semi-quantitative RT-PCR was performed on the MSCs following treatment with OP. The transcriptional levels of the cyclin D1 and p21 genes were analyzed (Fig. 3A). The mRNA expression of cyclin D1 was enhanced, while the p21 mRNA expression level was decreased following treatment with OP for 6 h (Fig. 3B). These results suggest that OP alters the transcriptional levels of cell cycle-related genes, i.e., cyclin D1 and p21, disrupting cell growth or viability in MSCs derived from human UCB.

*Changes in protein levels of cyclin D1 and p21 induced by OP.* To confirm the transcriptional levels of cyclin D1 and p21, their protein expression was further analyzed in the MSCs by immunoblot analysis following treatment with OP. In parallel with its mRNA expression, the translational level of cyclin D1 protein in the MSCs was significantly increased

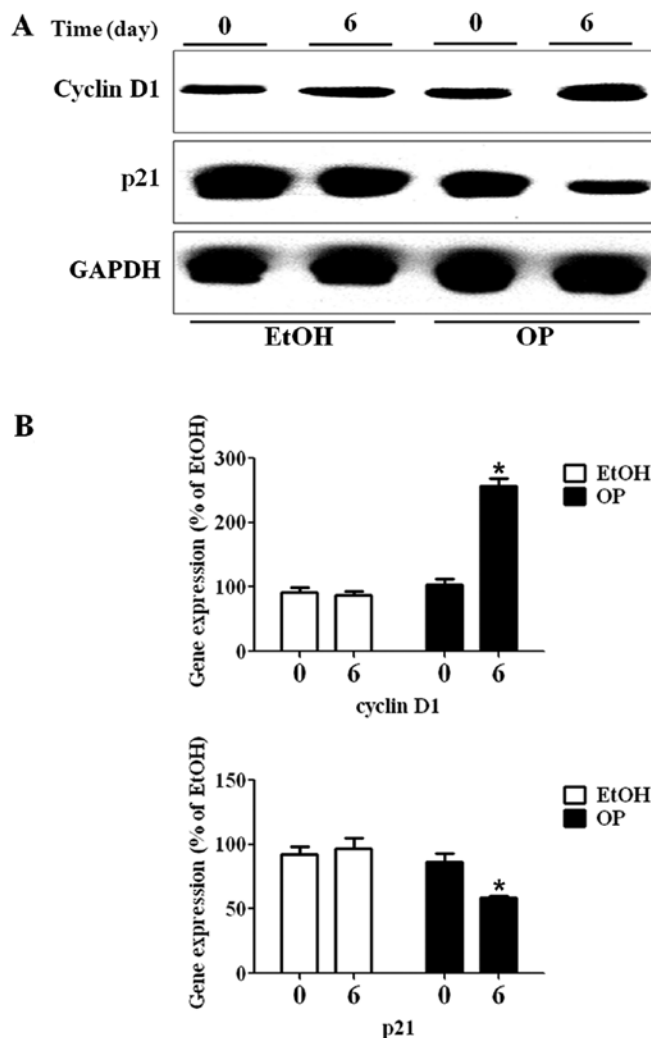


Figure 4. Changes in the protein levels of cyclin D1 and p21 in mesenchymal stem cells (MSCs) following exposure to octylphenol (OP). Protein levels of cyclin D1 and p21 were detected by immunoblot analysis as described in Materials and methods. (A) The antibody binding was visualized using an ECL chemiluminescent reaction. (B) The quantity of the bands on the gel was analyzed by Gel Doc 2000. Data are presented as the means  $\pm$  SD of triplicate experiments. \* $P < 0.05$  compared to the vehicle treated with EtOH.

following exposure to OP for 6 h compared to the negative controls (Fig. 4A). By contrast, the protein level of p21 gradually decreased following treatment with OP for 6 h, as shown in Fig. 4A. The altered protein levels of cyclin D1 and p21 were quantified, as shown in the graphs presented in Fig. 4B, indicating that OP may have a disrupting effect on the cell cycle in MSCs derived from human UCB.

## Discussion

With the development of the scientific industry, alkylphenols have been accumulating in the environment, as well as in the organs and tissues of mammals and humans (21). OP, a commonly used alkylphenol, has been found abundantly in the aquatic environment in waste waters surrounding factories (19), which produce over 50,000 tons per year worldwide (22). These levels in the environment are high enough to be absorbed by the human body; alkylphenols have been detected in human

blood and urine (20). OP has estrogenic activity which can block endogenous hormones and their receptors, particularly in estrogen-related signaling pathways (23). In a previous study, OP has shown affinity to estrogen receptors, inducing strong estrogenic activity, resulting in adverse reactions toward estrogen receptor-expressing organs, such as the breasts and ovaries (24). Therefore, exposure to OP cause serious side-effects, such as reproductive system disorders (25).

In this study, we examined the effects of OP on UCB; cord blood is used for the exchange of gases and nutrients from the mother to the fetus. UCB is known to contain MSCs, which have the ability to differentiate into a lineage of cells for different organs, i.e., adipose tissue, skeletal tissue and cardiac tissue. MSCs can be isolated from bone marrow, as well as cord blood and lungs (26). MSCs are known for their ability to self-renew, and they have thus been the focus of research on treatments for incurable diseases (27). MSCs have been characterized by the presence or absence of cell surface markers, such as CD34 or CD105, respectively (28). Studies have focused on the expansion of MSCs for clinical application (29), although their growth abilities tend to be reduced as time progresses (30). UCB is a promising source of MSCs, as MSCs can be isolated from UCB without posing any ethical issues (31,32).

In this study, we examined the effects of OP on the growth potential of MSCs derived from UCB. The cells were confirmed as MSCs as they expressed surface markers, such as CD34 and did not express CD105. Our results revealed that the treatment of MSCs with OP induced the abnormal cell viability of MSCs, as determined by MTT assay. This result suggests that OP induces the growth of MSCs derived from UCB, although the mechanisms involved have yet to be determined. We hypothesized that OP may cause disruptions in the cell cycle.

We further performed semi-quantitative RT-PCR and western blot analysis to analyze the expression levels of the cell cycle-related genes, cyclin D1 and p21, in MSCs. The cell cycle is regulated by changes in the expression of genes involved in the cell cycle checkpoints in response of stimuli (13). Among the cell cycle-related proteins, cyclins promote the transition of the cell cycle, while cyclin-dependent kinase (CDK) inhibitors, such as p21 and p27, are negative regulators through phosphorylation (12). The specific cyclins for phases of the cell cycle form complexes with CDKs at specific times. The cyclin D1-CDK4 complex accumulates and stimulates cell cycle progression during G1/S transition (12,20). However, p21 is a strong CDK inhibitor which inhibits the activity of cyclin-CDK complexes, and thus functions as a regulator of the G1 cell cycle transition (12).

These results suggest that OP alters the transcriptional levels of cell cycle-related genes, i.e., cyclin D1 and p21, disrupting the cell growth or viability of MSCs derived from human UCB. In parallel with its mRNA expression, the translational level of cyclin D1 protein was significantly increased following the exposure of MSCs to OP. By contrast, the protein level of p21 gradually decreased following treatment with OP. Both the transcriptional and translational levels of cyclin D1 were increased, while the p21 levels were suppressed following treatment of the UCB-derived MSCs with OP. This collapse of the regulation of the cell cycle may directly stimulate the growth of the MSCs under culture conditions. Taken together,

our data demonstrate that the contamination of UCB by EDCs may detrimentally affect the development of the embryo, which may cause birth defects or the birth of a stillborn baby.

In conclusion, in this study, we determined the effects of OP on the growth and viability of MSCs and the expression of cell cycle-related genes in MSCs derived from human UCB. Our results suggest that exposure to OP deregulates the cell cycle by altering the expression of cell cycle-related genes. The data presented in this study provide further insight into the effects of EDCs, which are present abundantly in our environment, and in turn, are also absorbed by UCB and UCB-derived MSCs. Further studies are required to identify the signaling pathways which mediate the effects of EDCs on MSCs derived from human UCB.

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