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Original Article

Bisphenol A at environmentally relevant doses induces cyclooxygenase-2 expression and promotes invasion of human mesenchymal stem cells derived from uterine myoma tissue

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Abstract

Objective: Uterine myoma is the most common benign reproductive tract tumor in women. Despite its high prevalence, the exact pathogenesis of these benign tumors remains unknown. Toward understanding the pathogenic mechanism of these tumors, we attempted to isolate human uterine myoma mesenchymal stem cells (hUM-MSCs), which may be the target cells for tumorigenesis. Furthermore, we tested the response of these hUM-MSCs to the environmental endocrine disruptor, bisphenol A (BPA), which may mimic the action of estrogen in hormone-sensitive organs such as the uterus.

Materials and Methods: The hUM-MSC lines were clonally derived from uterine myoma tissue using the MSU-1 medium supplemented with *N*-acetyl-L-cysteine and L-ascorbic acid-2-phosphate. These hUM-MSCs were characterized by reverse transcription polymerase chain reaction (RT-PCR) analysis for the expression of mesenchymal stem cell (MSC) surface markers (e.g., CD90 and CD105) and the transcription factor Oct-4. The proliferation potential was measured by the cumulative population doubling level and the colony-forming efficiency.

Results: Putative hUM-MSC lines expressed *CD90*, *CD105*, and the stem cell marker gene, *Oct-4*. The cells were capable of differentiating into adipocytes, osteoblasts, and chondrocytes. Bisphenol A treatment of these hUM-MSCs enhanced cell proliferation and colony-forming efficiency in a dose-responsive manner. At an environmentally relevant concentration (10^{-8} M), BPA moreover induced *cyclooxygenase-2 (COX-2)* gene expression and promoted cell migration and invasiveness.

Conclusion: The hUM-MSC cell lines can be isolated from uterine myoma tissues. Bisphenol A could enhance cell proliferation and colony-forming efficiency, induce *COX-2* gene expression, and promote migration and invasion of hUM-MSCs. The results imply that BPA has a detrimental effect on female health by promoting uterine tumorigenesis.

Keywords: bisphenol A; cell invasion; cyclooxygenase-2; mesenchymal stem cells; uterine myoma

Introduction

Stem cells are undifferentiated cells with the capacity for unlimited or prolonged self-renewal. They are able to give rise to differentiated cells. In recent years, tumor stem cells have been reported for many different tumors [1–8]. Many tumor stem cell markers are also markers of normal stem cells in different tissues [9–14], a major evidence supporting the hypothesis that stem cells are the tumor-initiating target cells [15,16].

A uterine myoma (i.e., a uterine fibroid) is a benign tumor of the female reproductive tract. Despite the high prevalence

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of uterine myoma, its underlying pathogenesis is largely unknown [17]. One possible mechanism for uterine myoma formation is the dysregulation of stem cell activity. Evidence for the presence of uterine stem cells has been previously reviewed [18] and reported, and include eutopic and ectopic endometrial tissue [19], endometrial carcinoma [20], myometrium tissue [21], and myoma [22].

Mesenchymal stem cells (MSCs) have been defined by the International Society for Cellular Therapy [23] as possessing three characteristics, namely: (1) they are plastic-adherent; (2) they express CD90 and CD105, but not CD34 and CD45; and (3) they are able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. The most common source of MSCs is the bone marrow [24]. Mesenchymal stem cells have been derived from various tissues such as adipose tissue [25], lipoma [26], cord blood [27], skeletal muscle, dermis [28], synovial membrane [29], placenta [30], amniotic fluid [31], eutopic and ectopic endometrial tissues [19], and peripheral blood [32].

Environmental endocrine disruptors (EEDs) include many environmental estrogens or xenoestrogens that mimic the action of natural estrogen hormone in the body. Bisphenol A (BPA), one of the most important EEDs, is widely used as a material for the production of epoxy resins and polycarbonate plastics. It has been detected in many human tissue compartments such as serum, follicular fluid, amniotic fluid [33,34], urine [35], breast milk [36], saliva [37], and adipose tissue [38] at concentrations ranging from 0.5 nM to 40 nM [34,39–43]. The consequence of BPA exposure in human reproduction remains controversial, although BPA exposure reportedly may be associated with recurrent miscarriages, endometrial hyperplasia, obesity, and polycystic ovarian syndrome (PCOS) [33,44–46].

In this study, we have developed a technique to isolate human uterine myoma tissue-derived mesenchymal stem cells (hUM-MSCs). This provides an opportunity to analyze the effects of BPA on hUM-MSCs.

Materials and methods

Primary culture cells derived from human uterine myoma tissue

Human uterine myoma tissue primary cells were isolated from five patients receiving surgery to treat uterine myoma in the Department of Obstetrics and Gynecology of Kuo General Hospital, Tainan, Taiwan. The use of these tissues has been approved by the Institutional Review Board (IRB). A small amount of uterine myoma tissue (approximately 5 grams) was minced by sterile scissors, and then subjected to overnight digestion at 37°C with collagenase Type I (1 mg/mL) in phenol red-free Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F-12 medium) (5 mL medium per 1 g of tissue). The digested tissue was filtered through a sterile 100-μm polyethylene mesh filter to remove undigested tissues, and again filtered through a 40-μm cell strainer to remove epithelial cells. The primary cells in the filtrate were collected by centrifugation and re-suspended in phenol red-free DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS). On the next day, unattached cells were removed by washing them with phosphate-buffered saline (PBS). The medium was changed every other day, and the cells were allowed to grow until near confluence. After 1 week, the primary cell cultures were trypsinized and stored in liquid nitrogen or subcultured for further experiments.

Isolation and culture of hUM-MSCs

To isolate hUM-MSCs, the primary cell cultures were diluted and seeded in a 96-well plate at a density of one cell per well. All wells were examined under the microscope for the number of attached cells. Wells without cells or with more than one cell were marked and disregarded. The medium was changed to the basic MSU-1 (i.e., phenol red-free) medium [47,48] with 5% FBS, 2 mM *N*-acetyl-L-cysteine (NAC) and 0.2 mM L-ascorbic acid-2-phosphate (Asc-2P). After incubation for 14 days, proliferating cells clonally derived from one single cell were trypsinized and cultured in a 60-mm Petri dish. The medium was renewed every other day and the cells were allowed to grow until near confluence. Three hUM-MSC cultures—hUM-MSC1, hUM-MSC2 and hUM-MSC3—were eventually obtained by this procedure. Early passage cells (i.e., passage 2) were used in subsequent experiments.

Cumulative population doubling level

The cumulative population doubling level (cpdl) in continual subcultures and growth from a known number of cells (1×10^5) were calculated to determine the proliferation potential of hUM-MSCs. The cpdl of each subculture was calculated from the cell count by using the following equation:

$$\ln (Nf/Ni)/ \ln 2$$

in which *Ni* and *Nf* are the initial and final cell numbers, respectively, and ln is the natural logarithm [19,49].

Cell proliferation assay

To examine the self-renewal capacity, the hUM-MSCs were treated for 7 days with various concentrations of 17-β-estradiol (E2), BPA (1×10^{-4} M, 1×10^{-5} M, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, and 1×10^{-10} M dissolved in dimethyl sulfoxide [DMSO]) or treated with the solvent vehicle DMSO (0.1%) as the negative control. The growth rates of the cell cultures were calculated by dividing the cpdl by the number of days that elapsed during the culture [19,47]. At least three independent experiments were performed for each study.

Colony formation assay

To test the effect of BPA on colony-forming ability, the hUM-MSCs cells were plated at a density of 100 viable cells per 10-cm dish in a medium without or with BPA (1×10^{-4} M, 1×10^{-5} M, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, and

1×10^{-10} M). After 21 days, the colonies were stained with 0.5% crystal violet stain. The colonies on the plates were photographed and counted to compare the colony-forming efficiency [19,47]. At least three independent experiments were performed for each study.

Multilineage differentiation potential of hUM-MSCs

The hUM-MSCs cells were tested for their mesodermal multilineage differentiation potential (i.e., adipogenesis, osteogenesis, and chondrogenesis). Cells were first subcultured and incubated in modified Eagle's medium (i.e., the D-medium) [50] with 10 % FBS. The next day, differentiation induction was initiated by changing the medium with different supplementations [19,25,49].

Adipogenesis

The hUM-MSCs were plated at a seeding density of 5×10^4 cells/cm^2 in 35-mm Petri dishes. The next day, the cells were incubated in IDII medium for 2 days, and then in I medium for 1 day. The cycle of treatment (i.e., 3 days) was repeated four times. The IDII medium contains 3-isobutyl-1-methylxanthine (500 μM), dexamethasone (1 μM), indomethacin (1 μM), and insulin (10 μg/mL) in D-medium with 10% FBS. The I medium is the D-medium supplemented with 10% FBS and insulin (10 μg/mL). Oil Red O staining (indicated by red stain) verified the development of lipid vacuoles within adipocytes after induction.

Osteogenesis

The hUM-MSCs were plated at a seeding density of 5×10^4 cells/cm^2 in 35-mm Petri dishes. On the following day, the cells were induced to differentiate in DAG medium for 2 weeks. The medium was changed once every 3 days. The DAG medium contains 0.1 mM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate disodium in D-medium with 10% FBS. The formation of calcified extracellular matrix (ECM) by osteoblasts was visible and can be confirmed by von Kossa staining (indicated by black stain).

Chondrogenesis

Micromass cultures of cells (1×10^5 cells/10 μL) were incubated and formed in 24-well plates for 2.5 hours, and then induced to differentiate in TAI medium for 2 weeks. The medium was changed once every 3 days. The TAI medium contained 10 ng/mL TGF-β1, 50 μM L-ascorbic acid-2-phosphate, and 6.25 μg/mL insulin in D-medium with 10% FBS. The micromass was stained with Alcian blue for the presence of sulfated proteoglycan-rich matrix (indicated by blue stain).

Migration and invasion assay

Cells were inoculated into 24-well Falcon Migration inserts (1×10^4 cells/well) and Matrigel Invasion inserts (1×10^5 cells/well) (8-μm pore size in both inserts). Inserts were placed into Falcon companion plates and incubated for 18 hours for migration and 24 hours for invasion. After incubation, the medium and cells were removed from the top chamber by using cotton swabs and PBS. The number of cells that had invaded or migrated to the underside of the membrane were counted and recorded as the average number of migrating or invading cells per plate in triplicate plates [19,47,48,51].

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cells by using 1 mL TRIzol. The RNA (1 μg) was transcribed into cDNA with a reverse transcription system kit, and cDNA was amplified with gene-specific primers in a polymerase chain reaction (PCR) machine. The primers included *Oct-4* (forward, 5′-GGCTTGGAGACCTCT CAGCCTG; reverse, 5′-TGCAGCAAGGGCCGCAGCTTAC), *CD90* (forward, 5′-TCGCTCTCCTGCTAACAGTCT; reverse, 5′-CTCGTACTGGATGGGTGAACT), *CD105* (forward, 5′-TG TCTCACTTCATGCCTCCAGCT; reverse, 5′-AGGCTGTCCA TGTTGAGGCAGT), *COX-2* (forward, 5′-AGGGCCAGCTTTC ACCAAC; reverse, 5′-AAGGCGCAGTTTACGCTGTC), and *β-actin* (loading control) (forward, 5′-ATGATATCGCCGCGC TCGTCGTC; reverse, 5′-CGCTCGGCCGTGGTGGTGAA). The amplified products were separated on 2% agarose gels and stained with ethidium bromide.

Statistical analysis

The results show the representatives of at least three separate experiments. The significance of difference between treatments was assessed by the Mann-Whitney test of nonparametric statistics and was performed by using the SPSS for Windows 13.0 statistics program (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered significant. All statistical data are presented as the mean ± the standard deviation.

Results

Morphology of the primary cells and hUM-MSCs

Primary cells and hUM-MSCs were isolated from human uterine myoma tissue, as described in the Materials and methods section. Five primary cell cultures and three clonally derived hUM-MSC cell lines—hUM-MSC1, hUM-MSC2 and hUM-MSC3—were obtained by this procedure. In the following experiments, either all three hUM-MSC cell lines or just the hUM-MSC2 cell line was used as the representative cells for some studies. The primary cells were morphologically flat and large (Fig. 1A), whereas the hUM-MSCs were morphologically mostly fibroblast-like, but with a few serpiginous cells (i.e., cells with two long tails), and tended to show contact-insensitive growth (i.e., piling up) (Fig. 1A). As observed previously [25], some MSCs may appear as serpiginous cells. These serpiginous cells may divide symmetrically to give rise to two serpiginous cells or may divide asymmetrically to give rise to one serpiginous cell and one cuboidal cell [19,25,49].

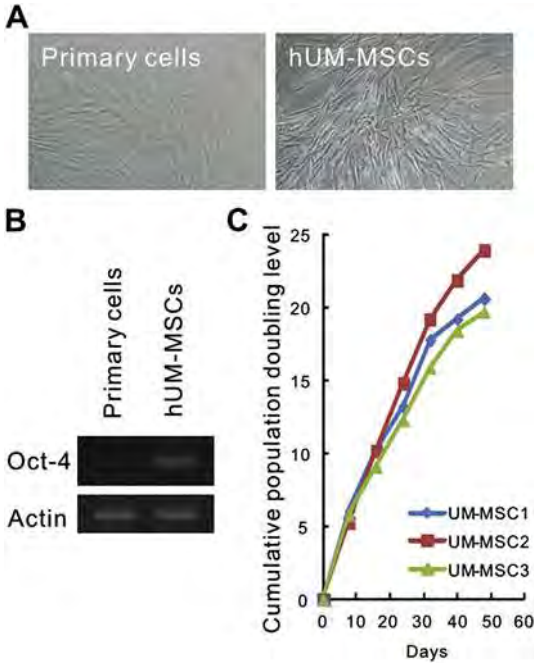

Fig. 1. Morphology and stem cell characteristics of hUM-MSCs. (A) The morphology of primary cells is flat and large, whereas the morphology of hUM-MSCs is fibroblastlike or serpiginous. (B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis shows that, in contrast to primary cells, hUM-MSC2 cells express *Oct-4* mRNA. The hUM-MSC1 and hUM-MSC3 cells have similar results (data are not shown). (C) The cumulative population doubling level of hUM-MSC1, hUM-MSC2, and hUM-MSC3 cells reached 20.6, 23.9, and 19.7, respectively, in 48 days (6 passages). hUM-MSC = human uterine myoma mesenchymal stem cell.

Expression of the Oct-4 gene in hUM-MSCs

In the literature, the transcription factor Oct-4 is a stem cell marker that is expressed in embryonic stem cells, in germ cells [52], in many adult human stem cells [53], and in cancer stem cells [47,48]. Oct-4 expression appears essential for maintaining an undifferentiated state and preventing the differentiation of stem cells [54]. Based on RT-PCR analysis in this study, the *Oct-4* gene is expressed in hUM-MSCs, but not in primary cells (Fig. 1B).

Proliferation potential of hUM-MSCs

Stem cells are characterized by an extensive ability for self-renewal. To determine the proliferation potential of hUM-MSCs, the cumulative population doubling level (cpdl) of three hUM-MSC lines were measured in six passages during 48 days of cell growth. The cpdl for hUM-MSC1, hUM-MSC2, hUM-MSC3 were 20.6, 23.9, and 19.7, respectively (Fig. 1C).

In vitro differentiation and expression of MSC genes of hUM-MSCs

The qualifying criteria for cells to be considered as MSCs are (1) the ability to differentiate into cells of multiple tissue lineages and (2) the expression of CD90 and CD105 [23]. To demonstrate that the hUM-MSCs are capable of multilineage differentiation, they were grown in D-medium with different supplementations to induce differentiation. After induction with specific medium supplementations, as described in the Materials and methods section, the hUM-MSCs differentiated into adipocytes, osteoblasts, and chondroblasts, as indicated by positive staining with Oil Red O (for lipid droplets), with von Kossa (for calcified ECM), and with Alcian blue (for sulfated proteoglycan), respectively (Fig. 2A). By RT-PCR analysis, specific MSC markers (i.e. *CD90* and *CD105*), were expressed in the hUM-MSC2 (Fig. 2B). CD90 and CD105 were also expressed in hUM-MSC1 and hUM-MSC3 (data are not shown).

BPA enhances the growth rate and clonogenic ability of hUM-MSCs in a dose-responsive manner

The growth response of hUM-MSC2 to E2 and BPA concentrations ranging from 10^{-10} M to 10^{-4} M was analyzed. The results showed that E2 concentrations from 10^{-10} M to 10^{-5} M significantly increased cell growth, compared to the vehicle control. The optimum dose was from 10^{-8} M to 10^{-6} M. The BPA treatment (at a concentration of 10^{-8} M to 10^{-5} M) of hUM-MSC2 cells also significantly increased cell growth. Both chemicals inhibited cell growth at 10^{-4} M (Fig. 3A). In the clonogenic assay, E2 and BPA significantly increased the colony formation of hUM-MSC2 cells in a dose-responsive manner at a concentration range of 10^{-10} M to 10^{-6} M (Fig. 3B). In the tested dose range, the effects of E2 were more significant than the effects of BPA. A BPA concentration of 10^{-4} M inhibited clonogenic ability. The effective concentration of BPA at 10^{-8} M is considered an environmentally relevant dose and was used for subsequent experiments.

BPA at an environmentally relevant dose induced cyclooxygenase-2 (COX-2) gene expression in hUM-MSCs cell

In the medical literature, prolonged exposure of breast stem cells to E2 could enhance COX-2 expression [47]. Many

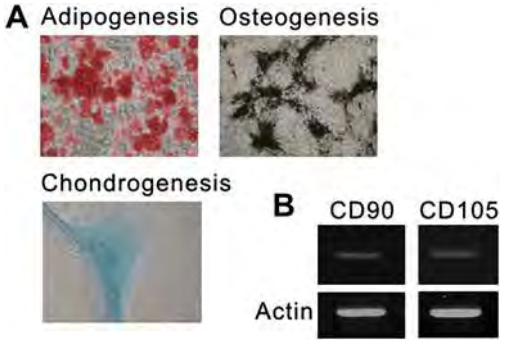

Fig. 2. *In vitro* differentiation and expression of MSC genes of hUM-MSCs. (A) The hUM-MSCs were induced to differentiate into the following mesenchymal lineages: adipocytes (stained red by using Oil Red O staining of lipid droplets); osteoblasts (stained black by using von Kossa staining of calcified extracellular matrix [ECM]); and chondroblasts (stained blue by using Alcian blue staining of sulfated proteoglycans). (B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated *CD90* and *CD105* mRNA expression in the hUM-MSC2 cell lines. BPA = bisphenol A; hUM-MSC = human uterine myoma mesenchymal stem cell.

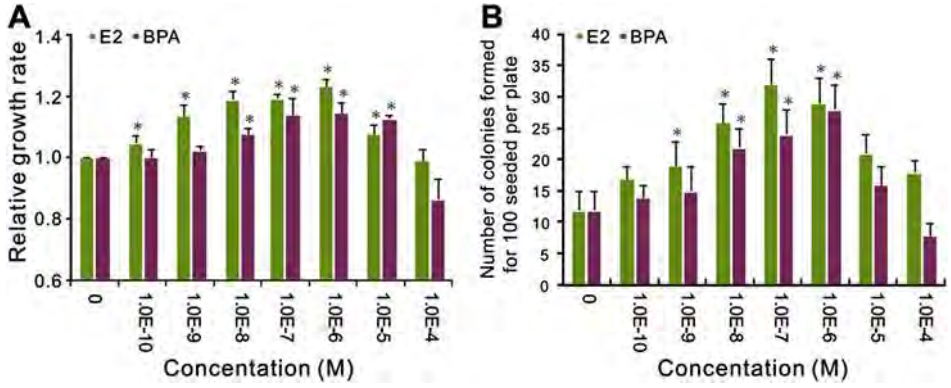

Fig. 3. Bisphenol A (BPA) enhances the growth rate and clonogenic ability of hUM-MSCs. (A) The effect of 17-β-estradiol (E2) or BPA on cell proliferation in hUM-MSC2 cells. The cells treated with various concentrations of E2 or BPA for 7 days were subjected to the proliferation assay. The ratio of the value for the E2-treated or BPA-treated cells to the value for the control cells (i.e., DMSO-treated cells) was calculated. These are shown on the ordinate. Values are presented as the mean ± standard deviation (SD) ($n = 3$, different cultures). (B) The hUM-MSC2 cells treated with various concentrations of E2, BPA, or DMSO (0.1%). After 21 days, colonies of cells were stained with 0.5% crystal violet. The values are presented as the mean ± SD ($n = 3$, different cultures). BPA = bisphenol A; DMSO = dimethyl sulfoxide; E2 = 17-β-estradiol; hUM-MSC = human uterine myoma mesenchymal stem cell. *$p < 0.05$.

environmental endocrine disruptors may mimic the action of natural hormone estrogens such as E2 in the body. Therefore, we investigated the effect of BPA treatment on COX-2 expression in hUM-MSCs. After treatment with BPA (10^{-8} M) for two weeks, the *COX*-2 mRNA level in hUM-MSC2 cells was significantly increased, based on RT-PCR analysis (Fig. 4A). Similar results were obtained for hUM-MSC1 and hUM-MSC3 (data are not shown).

BPA at an environmentally relevant dose enhances the migration and invasion ability of hUM-MSCs cells

The effects of BPA to promote cell migration and invasion ability of hUM-MSCs were examined by using migration and invasion chamber assays. The results reveal that BPA (10^{-8} M) treatment of hUM-MSC2 for two weeks significantly increased cell migration ability, compared to hUM-MSC2 without treatment (2.5% vs. 1.6%, respectively) (Fig. 4B). The same concentration of BPA similarly significantly enhanced the invasion ability of hUM-MSC2 (0.238% vs. 0.086% for the control) (Fig. 4B).

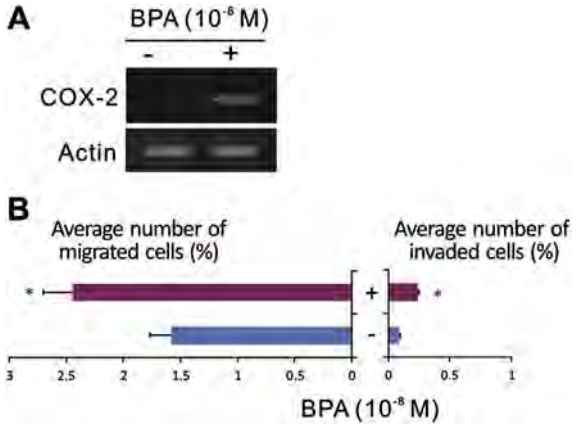

Fig. 4. BPA induces *COX-2* gene expression and promotes the migration and invasion of hUM-MSCs. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis shows that the *COX*-2 mRNA expression is significantly elevated in hUM-MSC2 cells treated with BPA (10^{-8} M) for 2 weeks, compared to the controls (i.e., without BPA treatment). (B) Based on migration and invasion chamber assay, hUM-MSC2 cells treated with BPA (10^{-8} M) for two weeks have significantly enhanced cell migration and invasion. The values are presented as the mean ± standard deviation (SD) ($n = 3$, different cultures). BPA = bisphenol A; hUM-MSC = human uterine myoma mesenchymal stem cell. *$p < 0.05$.

Discussion

This study demonstrated that MSCs can be isolated from human uterine myoma tissue by using the basic MSU-1 medium [47,48,55] supplemented with 5% FBS, 2 mM NAC, and 0.2 mM Asc-2P. The MSU-1 medium without NAC and Asc-2P was first used in developing human breast epithelial stem cells [55]. We previously used a keratinocyte medium supplemented with NAC and Asc-2P to develop MSCs from human and other mammalian adipose tissues [25,26,49,56,57]. The success of developing these MSCs using these media is ascribed to using NAC [58,59] and Asc-2p [60–62] antioxidants which maintain a cellular redox state that is essential for stem cell self-renewal [58].

The hUM-MSCs in this study possessed many MSC phenotypes. First, the cells showed a high proliferation potential (more than 20–24 cpdl for the three clonally derived hUM-MSC cell lines that were examined). Second, the cells were fibroblast-like or serpiginous with a morphology similar to adipose tissue-derived human MSCs [25], lipoma [26], and eutopic or ectopic endometrial tissue [19]. They showed contact-insensitive growth (i.e., piling up). Third, the hUM-MSCs expressed MSC markers (i.e., CD90 and CD105) and the stem cell marker Oct-4, which is expressed in many adult human and canine stem cells [53,56]. Fourth, and most importantly, the hUM-MSCs were capable of differentiating into three major mesodermal cell lineages: adipocytes, osteoblasts, and chondroblasts.

Using the hUM-MSCs, we tested the effect of BPA on gene expression and on phenotypic changes. The results show that BPA increased hUM-MSC proliferation and colony-forming

efficiency in a dose-dependent manner. Furthermore, long-term BPA exposure at environmentally relevant doses induced *COX-2* gene expression and promoted the migration and invasion ability of hUM-MSCs. It has been reported previously that BPA could promote proliferation of MCF-7 cells [63], HeLa cells [64], OVCAR-3 cell line [65], neuroblastoma [66], and JKT-1 cells [67]. The growth-promoting effect of BPA on hUM-MSCs has not been reported previously. In the future, the results derived from experiments using hUM-MSC2 may be confirmed by using the other 2 hUM-MSC lines.

The elevated expression of COX-2 could elicit many effects such as the promotion of tumor progression by inducing invasion and angiogenesis, the suppression of host immunity, the resistance to apoptosis, and the epithelial to mesenchymal transition (see references cited in Wang et al [47]). We previously reported that interleukin-1 beta could induce COX-2 overexpression and promote tumor invasion [51]. The results of this study indicate that long-term exposure of hUM-MSCs to BPA at environmentally relevant doses may induce *COX-2* gene expression and may enhance invasion ability, which is consistent with previous reports [51,68].

In conclusion, we have developed a technique to isolate clonally derived hUM-MSC lines. We further demonstrated that BPA could enhance cell proliferation and colony-forming efficiency, induce *COX-2* gene expression, and promote migration and invasion ability of hUM-MSCs. The results provide evidence that BPA could promote uterine tumor growth. These hUM-MSC lines could be useful for detecting environmental endocrine disruptors or toxicants that promote tumorigenesis.

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