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Original Article

Pueraria mirifica inhibits 17β-estradiol-induced cell proliferation of human endometrial mesenchymal stem cells

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ABSTRACT

Objective: The notion that the human endometrium may contain a population of stem cells has recently been proposed. The mesenchymal stem cells (MSCs) in the endometrium are believed to be responsible for the remarkable regenerative ability of endometrial cells. Estrogens influence the physiological and pathological processes of several hormone-dependent tissues, such as the endometrium. *Pueraria mirifica* (PM) is a herbal plant that contains several phytoestrogens, including isoflavones, lignans, and coumestans, and is known to exert an estrogenic effect on animal models. The present study investigated the effects of PM on the proliferation of human endometrial MSCs (hEN-MSCs).

Materials and methods: The hEN-MSCs were isolated from human endometrial tissue. The surface markers of these hEN-MSCs were identified through reverse transcription-polymerase chain reaction analysis. The proliferation potential of hEN-MSCs was measured through a cell proliferation assay. Multilineage differentiation ability was confirmed through Oil red O and von Kossa staining.

Results: This study demonstrated that 17β-estradiol-responsive MSCs with *Oct-4*, *CD90*, and *CD105* gene expression can be derived from the human endometrium and that PM exerts biological effects on hEN-MSCs, specifically, enhanced cell growth rate, through the estrogen receptor. Furthermore, PM at 1500 and 2000 μg/mL significantly increased cell proliferation compared with the vehicle control, and PM concentration at 1000 μg/mL significantly inhibited the enhanced cell growth rate induced by 17β-estradiol in hEN-MSCs.

Conclusion: This study provides new insights into the possible biological effects of PM on the proliferation of hEN-MSCs.

Introduction

The human endometrium is a dynamic tissue that undergoes more than 400 cycles of regeneration, differentiation, and shedding during a woman's reproductive years. The human endometrium might contain a population of stem cells that are responsible for its remarkable regenerative ability [1–3] and play a role in uterine physiology and pathology [4,5].

Estrogen regulates several endometrial functions including proliferation, differentiation, and secretory functions, through estrogen receptors [6,7]. In clinical settings, estrogen is considered the first-line drug for the prevention and treatment of multiple conditions affecting women's health. The serum estrogen level is highly associated with many diseases. For example, decreased estrogen serum levels may contribute to the development of Alzheimer disease [8], cardiovascular diseases [9], osteoporotic fractures [10], and the signs and symptoms of menopause. By contrast, increased estrogen serum levels may result in obesity, breast cancer [11], and endometriosis [12,13].

Phytoestrogens are plant-derived compounds that chemically resemble the human reproductive hormone estrogen and have

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estrogenic or antiestrogenic activity. *Pueraria mirifica* (PM) is a herbal plant that contains several phytoestrogens, including isoflavones, lignans, and coumestans [14], and has an estrogenic effect on hormone-sensitive tissues and organs, such as antioxidant properties in ovariectomized rats [15], prevention and treatment of postmenopausal osteoporosis [16], prevention of bone loss in ovariectomized rats [17], and cortical bone loss in naturally menopausal monkeys [18], stimulating effects on the luteinizing hormone and the follicle-stimulating hormone in gonadectomized rats [19], increasing the length of the follicular phase and total menstrual cycle, inhibiting ovulation in monkeys [20], and improving signs of vaginal atrophy and restoring vaginal epithelium in postmenopausal women [21].

Mesenchymal stem cells (MSCs) are versatile and can be used for various clinical applications because of their high potential for differentiation into various cell types and easy expansion of cell population *in vitro* [22]. No evidence on the direct effect of PM on the proliferation of endometrial MSCs has yet been reported. The isolation method for MSCs derived from human eutopic and ectopic endometrial tissues has been previously reported [23,24]. This provides an opportunity to analyze the effects of 17β-estradiol and PM on endometrial MSCs. Therefore, the present study investigated the possible effect of PM on the proliferation of endometrial MSCs.

Materials and methods

Isolation and culture of mesenchymal stem cells derived from human endometrial tissue

Human endometrial MSCs (hEN-MSCs) were isolated from human endometrial tissue, as previously described [23]. The study protocol was approved by the Institutional Review Board of the Kuo General Hospital, Tainan, Taiwan. Cell cultures were developed in the basic MSU-1 (phenol red-free) medium [25,26] with 5% fetal bovine serum (FBS), 2 mM N-acetyl-L-cysteine, and 0.2 mM L-ascorbic acid-2-phosphate (Asc-2P). Cell viability was determined using trypan blue dye exclusion staining. When the cells reached 80% confluence, they were trypsinized and subcultured. Early passage cells (passage 2) were used in the following experiments.

In vitro differentiation of hEN-MSCs

These hEN-MSCs were tested for differentiation potential, such as adipogenesis and osteogenesis. The cells were first subcultured and incubated in a modified Eagle's medium (referred to as D-medium) [27] with 10% FBS. The next day, differentiation induction was initiated by changing the medium with different supplementations [23,25,28].

Adipogenesis

These hEN-MSCs were plated at the seeding density of 5×10^4 cells/cm^2 in 35-mm Petri dishes. The next day, cells were incubated in IDII medium for 2 days, then in I medium for 1 day. The cycle was repeated 4 times. 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. I medium is D-medium supplemented with 10% FBS and insulin (10 μg/mL). Lipid vacuoles developed within adipocytes after induction were verified by Oil Red O staining (in red color).

Osteogenesis

These hEN-MSCs were plated at the seeding density of 5×10^4 cells/cm^2 in 35-mm Petri dishes. The next day, cells were induced to differentiate in DAG medium for 2 weeks, with medium change once every 3 days. The DAG medium contains dexamethasone (0.1 mM), Asc-2P (50 μM), and β-glycerophosphate disodium (10 mM) in D-medium with 10% FBS. The formation of calcified extracellular matrix by osteoblasts was visible and can be confirmed by von Kossa staining (in black color).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cells (5×10^5) using 1 mL of TRIzol. RNA (1 μg) was transcribed into cDNA with a Reverse Transcription System kit, and cDNA was amplified with gene-specific primers in a PCR machine. The primers included *CD90* (forward, 5′- TCGCTCTCC TGCTAACAGTCT; reverse, 5′- CTCGTACTGGATGGGTGAACT), *CD105* (forward, 5′- TGTCTCACTTCATGCCTCCAGCT; reverse, 5′- AGGCTGTC CATGTTGAGGCAGT) and *Oct-4* (forward, 5′- GGCTTGGAGACCTC TCAGCCTG; reverse, 5′- TGCAGCAAGGGCCGCAGCTTAC). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a PCR machine. The protocol follows the sequence of 1-min denaturation at 95 °C, reactions cycled 30 times with 30-s denaturation at 95 °C, 30-s annealing at 52 °C, and 1-min extension at 72 °C. The last polymerization step was performed at 72 °C for 10 min. The amplified products were separated on 2% agarose gels and stained with ethidium bromide.

Cell proliferation assay

To examine the effect of 17β-estradiol and PM on cell proliferation, the hEN-MSCs were treated with various concentrations of 17β-estradiol (1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, and 1×10^{-10} M) or PM (50, 100, 500, 1000, 1500, and 2000 μg/mL) for 4 days. PM (Lot no. 112747) was kindly provided by Pro OPS Pharma Inc. Cell numbers were counted using the cell counting chamber under a microscope. Proliferation of hEN-MSCs was calculated by dividing cumulative population doubling level (cpdl) by the number of days elapsed during culture. The cpdl in continual subculture and growth from a known number of cells (1×10^5 cells cultured in a 10-cm dish) was calculated as $\ln(Nf/Ni)/\ln 2$, where *Ni* and *Nf* are initial and final cell numbers, respectively, and ln is the natural logarithm. At least three independent experiments were performed for each study.

Cell growth rate assay

The growth rate of a cell culture was measured by the number of cell population doubling per day. Ratios of the value for treated cells to that for control cells were calculated and shown as relative values. At least three independent experiments were performed for each study.

Statistical analysis

Results shown were obtained from at least three separate experiments. The significance of difference between treatments was assessed using the Mann–Whitney test of nonparametric statistics and was conducted using SPSS for Windows 13.0 statistics program. A p value of <0.05 was considered significant. All data are presented as mean ± SD.

Results

Isolation and identification of hEN-MSCs

The hEN-MSCs were developed and isolated from human endometrial tissue as described in the Materials and Methods. These hEN-MSCs were serpiginous or fibroblast-like in cell shapes and showed contact-insensitive growth (piling up) (Fig. 1A left)

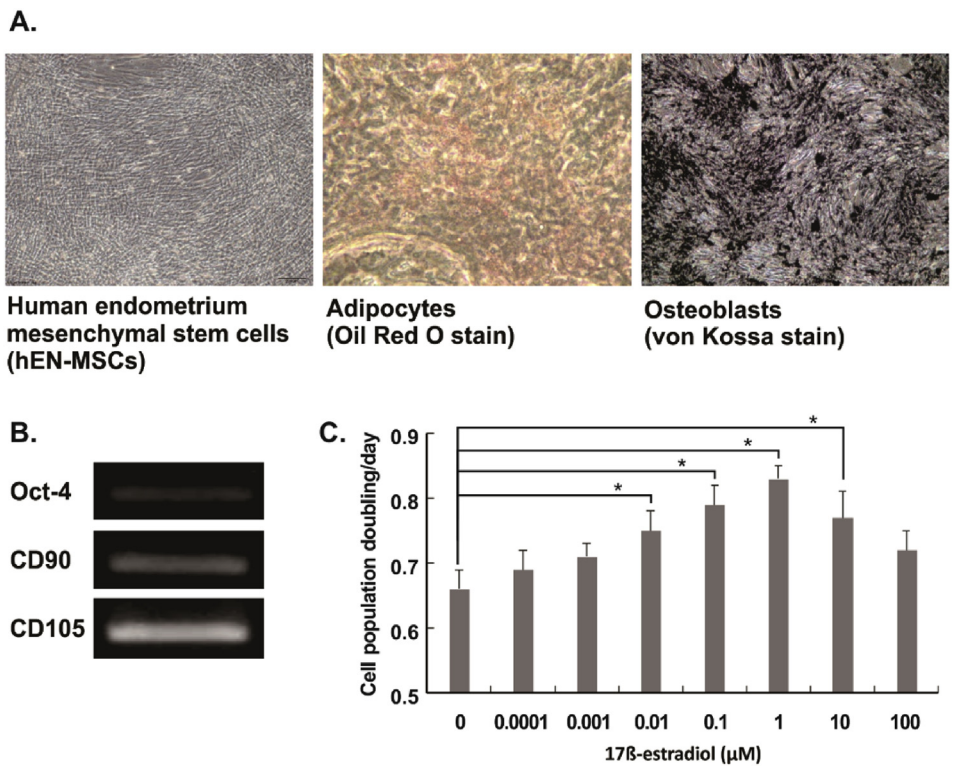

Fig. 1. Derivation of 17β-estradiol responsive human endometrial mesenchymal stem cells (hEN-MSCs) and characteristics of hEN-MSCs. (A) hEN-MSCs were mostly fibroblast-like in morphology and showed contact-insensitive growth (left). They were capable of adipogenesis (shown by Oil Red O staining of lipid droplets, red color) (middle) and osteogenesis (shown by von Kossa staining of calcified extracellular matrix, black staining) (right). (B) By RT-PCR analysis, *Oct-4*, *CD90*, and *CD105* mRNA expression was found in hEN-MSCs. (C) The cells were treated with various concentrations of 17β-estradiol (1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, and 1×10^{-10} M) during 4 days of cell growth for the proliferation assay. Values are presented as the mean ± SD (n = 3, different cultures). *P < 0.05.

similar to some MSCs reported previously [23,25,28]. The ability to express CD90 and CD105 and differentiate into multiple mesenchymal lineages is a qualifying criterion for cells to be considered MSCs [29]. In this study, a stem cell marker (Oct-4) and MSC markers (CD90 and CD105) were found to be expressed in hEN-MSCs by RT-PCR analysis (Fig. 1B). After induction with proper medium supplementations, hEN-MSCs were found to differentiate into adipocytes and osteoblasts on positive staining with Oil Red O (for lipid droplets) and von Kossa (for calcified extracellular matrix) stain, respectively (Fig. 1A middle and right panels).

17β-estradiol enhanced proliferation of hEN-MSCs in a dose-responsive manner

To investigate the effect of estrogen on the proliferation of hEN-MSCs, the cells were treated with various concentrations of 17β-estradiol ranging from 10^{-10} M to 10^{-4} M for 4 days. Results showed that the 17β-estradiol treatment of hEN-MSCs significantly increased cell growth in the dose range from 10^{-8} M to 10^{-5} M in a dose-responsive manner (Fig. 1C). hEN-MSCs are highly 17β-estradiol-responsive and represent a good cell model for studying estrogenic actions on endometrial cell function and proliferation. The effective concentration of 17β-estradiol at 10^{-6} M was used for subsequent experiments.

Effects of PM on proliferation of hEN-MSCs

To investigate the possible effect of PM on the proliferation of hEN-MSCs, the growth response of hEN-MSCs to a range of PM concentrations (50, 100, 500, 1000, 1500, and 2000 μg/mL) was analyzed. Results showed that PM treatment at concentrations 50, 100, 500, and 1000 μg/mL had no significant effect on the proliferation of hEN-MSCs; however, PM was found to significantly increase the proliferation of hEN-MSCs at concentrations 1500 and 2000 μg/mL (Fig. 2A). These results clearly indicate that PM had a biological effect on hEN-MSCs with respect to enhanced cell proliferation. The effective concentration of PM at 2000 μg/mL was used for subsequent experiments.

PM enhanced cell growth rate through estrogen receptors in hEN-MSCs

To investigate whether the effects of PM on cell proliferation are mediated through the estrogen receptor, we treated the cells with PM concentration at 2000 μg/mL or PM concentration at 2000 μg/mL in combination with estrogen antagonist (ICI 182, 780; 1 μM). PM concentration at 2000 μg/mL significantly increased the cell growth rate of hEN-MSCs, and this effect could be blocked by ICI 182, 780. These results clearly indicate that the effect of PM on cell proliferation may be reversed by an estrogen antagonist, ICI 182,780 (Fig. 2B), indicating an estrogen-receptor-dependent mechanism.

PM inhibits 17β-estradiol increased cell growth rate in hEN-MSCs

17β-estradiol significantly induced hEN-MSC proliferation in a dose-dependent manner and reached a plateau at 10^{-6} M (Fig. 1C). Therefore, 17β-estradiol was used at 10^{-6} M without or with PM concentrations at 50, 100, 500, 1000, 1500, and 2000 μg/mL to analyze hEN-MSC growth rate. Results showed that treatment of hEN-MSCs with PM concentrations at 50, 100, 500, 1500, and 2000 μg/mL did not affect the increase in cell proliferation induced

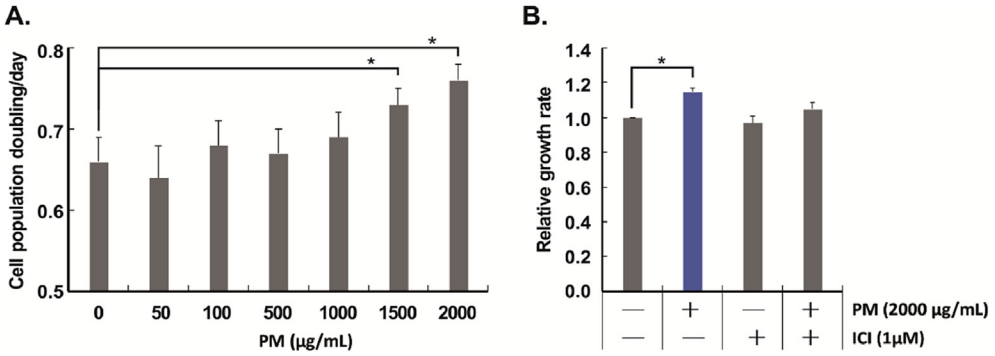

Fig. 2. *Pueraria mirifica* (PM) enhanced cell growth rate through estrogen receptor in hEN-MSCs. (A) These cells were treated with various concentrations of PM (50, 100, 500, 1000, 1500, and 2000 μg/mL) during 4 days of cell growth for the proliferation assay. (B) The growth rate of hEN-MSCs can be further enhanced by PM treatment (2000 μg/mL). The effect can be reversed by an estrogen antagonist (ICI 182, 780; 1 μM) (*P < 0.05; ICI 182, 780 treated cells compared with no treatment control or PM-treated cells).

by 17β-estradiol. However, the PM concentration at 1000 μg/mL significantly inhibited the enhanced cell growth rate induced by 17β-estradiol in hEN-MSCs (Fig. 3). Experiments were repeated three times with consistent results, and a representative result is discussed herein.

Discussion

The primary objective of this study was to test if hEN-MSCs derived from endometrial tissue are responsive to 17β-estradiol. The present study demonstrated that hEN-MSCs with 17β-estradiol

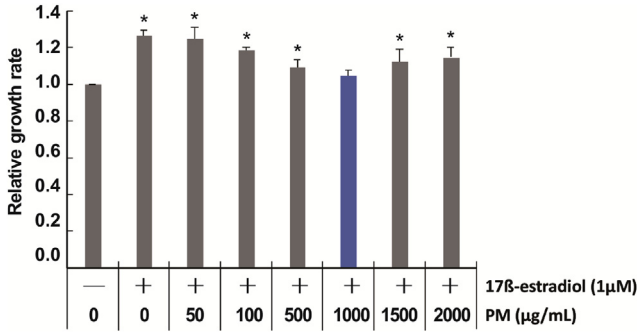

Fig. 3. PM inhibits 17β-estradiol-induced cell growth rate in hEN-MSCs. Treatment of 17â-estradiol at 1 μM without or with PM concentrations at 50, 100, 500, 1000, 1500, and 2000 μg/mL to analyze hEN-MSC growth rate. The PM concentration at 1000 μg/mL significantly inhibited the increase in cell proliferation induced by 17β-estradiol in hEN-MSCs. Values are presented as the mean ± SD (n = 3, different cultures). *P < 0.05.

treatment showed significantly enhanced cell proliferation in the dose range from 10^{-8} M to 10^{-5} M in a dose-responsive manner (Fig. 1C). The growth-promoting effect of 17β-estradiol on hEN-MSCs has not been reported previously. Furthermore, this study found that PM had biological effects on hEN-MSCs with respect to enhanced cell growth rate through estrogen receptors, as summarized in Fig. 4. In this study, treatment with PM at 0, 50, 100, 500, and 1000 μg/mL did not produce significant changes in the growth rate of hEN-MSCs. However, PM concentrations at 1500 and 2000 μg/mL significantly increased the cell proliferation compared with the vehicle control (Fig. 2A). The addition of 1 μM 17β-estradiol plus PM concentrations at 0, 50, 100, 500, 1500, and 2000 μg/mL did not change the growth rate of hEN-MSCs compared with 17β-estradiol alone. In contrast, cell proliferation induced by 1 μM 17β-estradiol was reversed when supplemented with PM concentration at 1000 μg/mL (Fig. 3).

Stem cells are undifferentiated cells with unlimited or prolonged self-renewal capacity and the ability to produce differentiated descendants. In recent years, the notion that human endometrium may contain a population of stem cells has been proposed [1–3,23]. These stem cells may play an important role in endometrium physiology and pathology [4,5]; therefore, a better understanding of stem cell biology may prove helpful in treating these conditions. MSCs have been derived and identified from various tissues including uterine myoma tissue [25], amniotic fluid [28], cord blood [30], adipose tissue [31], lipoma [32], and eutopic and ectopic endometrial tissues [23,24]. MSCs in endometrium were believed to be responsible for the remarkable regenerative ability of endometrial cells [33]. Several fundamental studies have

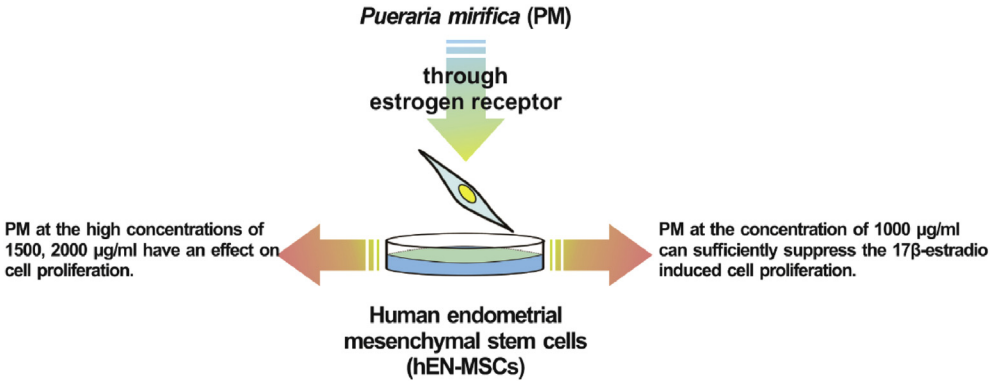

Fig. 4. A diagram showing the biological effects of PM on hEN-MSCs. These effects include the following: (1) PM had a biological effect on hEN-MSCs with respect to enhanced cell growth rate through estrogen receptors. (2) PM concentrations at 1500 and 2000 μg/mL significantly increased cell growth rate. (3) PM concentration at 1000 μg/mL can sufficiently inhibit 17β-estradiol-induced proliferation of hEN-MSCs.

demonstrated that endometriosis is associated with abnormal endometrial proliferation, and endometrial MSCs may play an important role in disease pathology [23,24,33]. In the present study, hEN-MSCs derived from human endometrial tissue were successfully isolated, providing a powerful tool for investigating disease origin. These hEN-MSCs clearly show characteristics of MSCs as defined by the International Society for Cellular Therapy [29], namely 1) plastic-adherent, 2) expression of CD90 and CD105, and 3) multipotent differentiation potential *in vitro* (Fig. 1A, B).

Estrogens are steroid hormones with various functions in many target tissues, influencing the pathological processes of several hormone-dependent tumors, such as testicular, breast, ovarian, and endometrial tumors [34,35]. The effect of estrogen on the proliferation and differentiation of endometrial stem cells through estrogen receptors was observed and found to be concentration-dependent [36]. Phytoestrogens are plant-derived compounds similar to mammalian estrogens, which have become a popular dietary supplement to decrease the symptoms of menopause. PM contains several phytoestrogens, including isoflavones, lignans, and coumestans [14]. In several studies using animal models, when orally administered, PM exhibited an estrogenic effect on hormone-sensitive tissues and organs. However, the effect of PM at the cellular level of estrogen-responsive tissues and organs has not been elucidated. In the present study, we employed a stem cell model, hEN-MSCs, to study the estrogenic action of PM on cell proliferation. As a major finding, the results support that PM at the concentration of 1000 μg/mL can sufficiently inhibit 17â-estradiol-induced proliferation of hEN-MSCs.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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