A Novel Method to Differentiate Tonsil-Derived Mesenchymal Stem Cells *In Vitro* into Estrogen-Secreting Cells

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Abstract

BACKGROUND: The advantages of tonsil-derived mesenchymal stem cells (TMSCs) over other mesenchymal stem cells (MSCs) include higher proliferation rates, various differentiation potentials, efficient immune-modulating capacity, and ease of obtainment. Specifically, TMSCs have been shown to differentiate into the endodermal lineage. Estrogen deficiency is a major cause of postmenopausal osteoporosis and is associated with higher incidences of ischemic heart disease and cerebrovascular attacks during the postmenopausal period. Therefore, stem cell-derived, estrogen-secreting cells might be used for estrogen deficiency.

METHODS: Here, we developed a novel method that utilizes retinoic acid, insulin-like growth factor-1, basic fibroblast growth factor, and dexamethasone to evaluate the differentiating potential of TMSCs into estrogen-secreting cells. The efficacy of the novel differentiating method for generation of estrogen-secreting cells was also evaluated with bone marrow- and adipose tissue-derived MSCs.

RESULTS: Incubating TMSCs in differentiating media induced the gene expression of cytochrome P450 19A1 (CYP19A1), which plays a key role in estrogen biosynthesis, and increased 17 β -estradiol secretion upon testosterone addition. Furthermore, CYP11A1, CYP17A1, and 3 β -hydroxysteroid dehydrogenase type-1 gene expression levels were significantly increased in TMSCs. In bone marrow-derived and adipose tissue-derived MSCs, this differentiation method also induced the gene expression of CYP19A1, but not CYP17A1, suggesting TMSCs are a superior source for estrogen secretion.

CONCLUSION: These results imply that TMSCs can differentiate into functional estrogen-secreting cells, thus providing a novel, alternative cell therapy for estrogen deficiency.

Keywords Estrogen · Tonsil · Mesenchymal stem cell · Secretion · Cytochrome P450 family 19 subfamily A member 1

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1 Introduction

Estrogen is an important enzyme not only for fertility, but also for glucose metabolism, immune response, bone homeostasis, cardiovascular health, and neural functions [1]. Estrogen deficiency is linked to numerous human diseases. For example, estrogen deficiency-induced postmenopausal syndrome is relatively well established, and estrogen deficiency can cause severe osteoporosis not only in females, but also in males, due to the critical role of estrogen in bone metabolism [2]. In addition, Alzheimer's dementia and hippocampus degeneration have been associated with decreased estrogen levels [3]. Furthermore, estrogen deficiency enhances metabolic dysfunction, thereby increasing the predisposition to type 2 diabetes mellitus, metabolic syndrome, and cardiovascular diseases [4, 5]. The pathways of estrogen biosynthesis are described in Fig. 1. Estrogens are derived from cholesterol, with pregnenolone formed from cholesterol via cytochrome P-450 11A1 (CYP11A1) enzyme activity [6]. The next steps in estrogen biosynthesis are the conversion of pregnenolone to 17α -hydroxypregnenolone and dehydroepiandrosterone and the conversion of progesterone to 17α -hydroxyprogesterone and androstenedione [6]. The conversions of pregnenolone and progesterone are carried out by 17α -hydroxylase, and the conversions of 17α -hydroxypregnenolone and 17α -hydroxyprogesterone are mediated by C17,20 lyase; both enzymes are products of cytochrome P-450 17A1 (CYP17A1) [6]. Furthermore, 3β -hydroxysteroid dehydrogenases (HSD3B) catalyze several reactions in the androgen pathway, leading to androstenedione and testosterone production. They also catalyze the production of progesterone from pregnenolone [6]. The rate-limiting step in estrogen production is mediated by cytochrome P-450 19A1 (CYP19A1), which codes for aromatase [6]. In addition to converting testosterone to estradiol in ovarian granulosa cells, aromatase converts androstenedione to estrone in adipose tissue [6].

Stem cell-based hormone therapy holds great potential for the treatment of various endocrine diseases. Mesenchymal stem cells (MSCs), which are designated as non-haematopoietic, fibroblast like-cells with the capacity to differentiate into mesodermal lineages (i.e., osteocytes, adipocytes, and chondrocytes), reside in various tissues [7]. In addition to the bone marrow and adipose tissues, human palatine tonsil tissues have emerged as a good source of MSCs [8]. Tonsil-derived MSCs (TMSCs) can be easily isolated from abandoned tissues during tonsillectomy and have a relatively high proliferation rate with low immunogenicity [8–10]. The alleviating effect of TMSCs in various diseases has also been demonstrated with

animal-based disease models, such as concanavalin A-induced acute liver injury [11], graft-versus-host disease [12], glucose intolerance [13], and osteoporosis [14]. In addition, TMSCs can be successfully differentiated not only into mesodermal lineage, but also into endodermal and ectodermal lineages [8]. For example, TMSCs can be efficiently differentiated into insulin-secreting cells using the insulin-transferrin-selenium method, and implantation of these insulin-secreting cells can alleviate streptozotocin-induced diabetes mellitus *in vivo* [10]. Furthermore, TMSCs can be successfully differentiated into parathyroid hormone-secreting cells, which are responsive to extracellular calcium levels, and the application of these parathyroid-hormone secreting cells in parathyroidectomised rats on a calcium-free diet results in the near-complete restoration of survival rates and serum levels of parathyroid hormone and ionized calcium [15]. These reports suggested a high differentiating efficiency of TMSCs into endocrine cells.

Recently, bone marrow-derived mesenchymal stem cells (BMSCs) have shown potential for differentiation into estrogen-secreting cells [16, 17]. Considering the excellent potency of TMSCs for differentiation into the endodermal lineage, we investigated the differentiating efficacy of TMSCs into estrogen-secreting cells in the present study. To induce the differentiation of TMSCs into estrogen-secreting cells, we developed a novel method that utilizes retinoic acid, insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and dexamethasone, and demonstrated the ability of TMSCs to be differentiated into estrogen-secreting cells.

2 Methods

2.1 Cell culture

Human TMSCs were isolated from palatine tonsils, as described previously [10, 11], and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Corning, Corning, NY, USA) and 1% penicillin/streptomycin (P/S). The experimental procedures were approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (Seoul, South Korea, ECT-11–53-02). Human adipose tissue-derived mesenchymal stem cells (AMSCs) were purchased from Invitrogen (Carlsbad, CA, USA) and cultured as described previously [10]. Human BMSCs were purchased from PromoCell (Heidelberg, Germany). Cells of passages 5–8 were utilized in the present study.

Fig. 1 Schematic overview of the estrogen biosynthetic pathway. The rate-limiting step in estrogen production is mediated by cytochrome P-450 19A1 (CYP19A1), which codes for aromatase, and is colored

in red in the grey box. CYP11A1, cytochrome P-450 11A1; CYP17A1, cytochrome P-450 17A1; CYP19A1, cytochrome P-450 19A1; HSD3B, 3β-hydroxysteroid dehydrogenases

MCF-7 (a breast cancer cell line) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in DMEM supplemented with 10% FBS and 1% P/S. To examine the degradation of estrogen receptor α , MCF-7 cells were cultured in DMEM with or without β -Estradiol (Sigma Aldrich, St. Louis, MO, USA, 10 nM) for 6 h. To examine the biological effect of estrogen generated by differentiated estrogen-secreting cells, MCF-7 cells were cultured in conditioned medium harvested from differentiated estrogen-secreting TMSCs for 6 h.

2.2 Induction of MSC differentiation for estrogen secretion in vitro

To differentiate MSCs into estrogen-secreting cells, MSCs were cultured in DMEM supplemented with 10% FBS (Corning), 1% P/S, 10 μ M retinoic acid (Sigma Aldrich), 20 ng/ml IGF-1 (Peprotech, Rocky Hill, NJ, USA), 200 ng/ml bFGF (Peprotech, Cranbury, NJ, USA), and 250 nM dexamethasone (Sigma Aldrich). If needed, 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) (Sigma Aldrich, 20 nM) was added to the differentiating media.

2.3 Real-time polymerase chain reaction (PCR)

Total mRNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and complementary DNA was then synthesized using ReverTraAce qPCR RT

master mix with gDNA remover (Toyobo, Japan), as indicated in the manufacturer's protocol. Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 7500 sequence detection system (Applied Biosystems). Relative gene expression was calculated according to a previous report [18]. Primers are listed in Table 1.

2.4 Western blot analyses

Cells were lysed in RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 50 mM NaF, 2 mM Na_3VO_4 , and protease inhibitors (Sigma-Aldrich). Proteins from cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were probed with antibodies against CYP19A1 (Santa Cruz Biotechnology, Dallas, TX, USA), estrogen receptor α (Abcam, Cambridge, UK), and β -actin (Sigma aldrich), followed by corresponding peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The blots were developed by a chemiluminescence reaction (SuperSignal West Pico chemiluminescent substrate, Thermo Fisher Scientific, Waltham, MA, USA), and the bands were detected with a Bio-Imaging Analyzer LAS-4000 (Fuji, Tokyo, Japan).

Table 1 Primers used in real-time PCR

Gene	Primer sequence (5'–3')	References
GAPDH	F: ACACCCACTCTCCACCTTT R: TGCTGTAGCCAAATTCGTTG	[11]
CYP11A1	F: GCAGTGTCTCGGGACTTCG R: GGCAAAGCGGAACAGGTCA	[37]
CYP17A1	F: GTGGAGACCACCACCTCTGT R: GCTGAAACCCACATTCTGGT	[38]
CYP19A1	F: AGGAGGTGACCAATGAATCG R: CACGATAGCACTTTCGTCCA	[39]
HSD3B1	F: GGAATCTGAAAAACGGCGGC R: CTGAGATAAGTAGAACTGTCCTCGGATG	[37]
HSD3B2	F: GGGTGAATCTAAAAAATGGTGAT R: AGATGTAATAGAAATTGACCTCGGACA	[40]
HSD17B1	F: GATCCATCCCAGAGCTTCAA R: CGGATTTTGAGTCCCTTACG	
HSD17B2	F: AGTCTGCCTGCTCATCCTGT R: CAATCACCACTGTACCAG	
HSD17B7	F: GCTGACCCAGGGTGATAAGA R: AGAGGAGAGGCTCCAGTTCC	
HSD17B8	F: GCATCACCCAGGATGAGTTT R: ATGGAACCACGACAACCATT	
STS	F: ACCTTGGGATGAGCTGTCAC R: GTGAAGACACTGCCCTCTCC	

2.5 Enzyme-linked immunosorbent assay

TMSCs (2×10^5) were seeded in 12-well plates and then differentiated into estrogen-secreting cells. After 7 days of differentiation, the media were replaced with testosterone (50 ng/ml, Sigma-Aldrich)-added fresh differentiating media, which did not include FBS, and then the cells were incubated for another 3 days. Testosterone was added as the substrate of CYP19A1 enzyme activity, as described previously [19]. The culture media were harvested, and 17 β -estradiol levels in the culture media were measured with a 17 β -estradiol ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA, ADI-900–008), according to the manufacturer's instructions.

2.6 Statistical analysis

Data were presented as mean $\pm$ standard error of the mean (S.E.M.). Statistical significance was analysed using the Student's *t* test or one-way analysis of variance. Analyses were performed using the GraphPad PRISM 6 statistical software (GraphPad Software). A *p* value < 0.05 was considered significant.

3 Results

3.1 Neither retinoic acid nor high glucose was sufficient to induce CYP19A1 gene expression in TMSCs

A previous study that used BMSCs for differentiation into estrogen-secreting cells reported the successful release of 17 β -estradiol following incubation in a high-glucose (4.5 g/L) DMEM or in combination with retinoic acid (10 μ M) for 1–4 days [16]. Therefore, we first tried to differentiate TMSCs into estrogen-secreting cells using retinoic acid and high glucose, as indicated in the previous study [16]. Because CYP19A1 is the most important and rate-limiting enzyme for estrogen biosynthesis, CYP19A1 gene expression was analysed. Culturing TMSCs or BMSCs in high-glucose DMEM did not significantly increase CYP19A1 gene expression compared to low (1.0 g/L)-glucose DMEM (Fig. 2). Addition of retinoic acid to low-glucose DMEM decreased and increased CYP19A1 mRNA levels in TMSCs and BMSCs, respectively (Fig. 2). Addition of retinoic acid to high-glucose DMEM enhanced CYP19A1 mRNA levels in TMSCs but decreased CYP19A1 mRNA levels in BMSCs (Fig. 2). Basal CYP19A1 gene expression was significantly higher in TMSCs compared with BMSCs (Fig. 2). These data

comprehensively implicate the lack of consistency in the alteration of CYP19A1 gene expression upon treatment with retinoic acid or high glucose.

3.2 A novel differentiation method was developed to induce estrogen-secreting cells in TMSCs

Next, we developed a new method utilizing retinoic acid, IGF-I, bFGF, and dexamethasone, which can increase CYP19A1 transcription for estrogen secretion, based on previous reports [20–23]. We added these four differentiating factors in DMEM with 10% FBS and 1% P/S to induce the differentiation of TMSCs into estrogen-secreting cells. Since the combined treatment of all the factors was more effective in increasing CYP19A1 mRNA levels than any other combination among these factors (data not shown), we established a novel method for estrogen-secreting cells as the combined treatment of retinoic acid, IGF-I, bFGF, and dexamethasone. The prominent morphological changes of TMSCs were not observed during differentiation (data not shown). There were 4.44 ± 0.32 -fold and 32.94 ± 2.68 -fold increases in CYP19A1 transcription in TMSCs and BMSCs, respectively, after 4 days of differentiation using the novel method under low-glucose conditions (Fig. 3A). Because no significant difference was observed in CYP19A1 transcription under high-glucose conditions compared with low-glucose conditions (Fig. 3A), low-glucose DMEM was used hereafter. Basal CYP19A1 gene expression was 16.87 ± 0.15 -fold higher in TMSCs compared with BMSCs, and relative CYP19A1 gene expression was still higher in TMSCs than in BMSCs after induction into estrogen-secreting cells (Fig. 3A). The final differentiation

method to induce estrogen-secreting cells using MSCs is described in Fig. 3B.

3.3 Addition of 12-*O*-tetradecanoylphorbol-13-acetate in the novel differentiating method for estrogen secretion increased CYP19A1 transcription levels, but decreased CYP11A1 mRNA levels in TMSCs

Because forskolin and TPA have also been reported to increase CYP19A1 mRNA and aromatase activity in trophoblast cells [24], we investigated the effect of these factors on CYP11A1, CYP17A1, and CYP19A1 levels in TMSCs. No significant alteration in these three gene transcripts was observed upon the addition of forskolin into the novel differentiating media (data not shown). Culturing TMSCs in the novel differentiating media significantly induced gene expression of CYP11A1 (Fig. 4A), CYP17A1 (Fig. 4B), and CYP19A1 (Fig. 4C). Although TPA addition significantly enhanced CYP19A1 mRNA levels in TMSCs (Fig. 4C), it decreased CYP11A1 mRNA levels (Fig. 4A). Because CYP11A1 plays an important role in the conversion of cholesterol into pregnenolone, which is a precursor of estrogen, decreased CYP11A1 gene levels can lead to decreased estrogen secretion. Therefore, we did not include TPA in the finalized version of the novel differentiating method. We further analysed the efficacy of this method in inducing CYP11A1, CYP17A1, and CYP19A1 gene expression in BMSCs and AMSCs. Consistent with TMSCs, this method was effective in inducing CYP19A1 gene expression in both BMSCs and AMSCs (Fig. 4C). However, the effect of this method on CYP11A1

Fig. 2 No consistent alteration of CYP19A1 gene expression upon retinoic acid or high glucose treatment. Tonsil-derived mesenchymal stem cells (TMSCs) and bone marrow-derived mesenchymal stem cells (BMSCs) were stimulated with a combination of retinoic acid (10 μ M) and high glucose (4.5 g/L) for 4 days, and CYP19A1 mRNA levels were investigated using real-time PCR. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Fig. 3 Development of a novel method to differentiate TMSCs into estrogen-secreting cells. **A** To differentiate TMSCs into estrogen-secreting cells, TMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 10 μ M retinoic acid, 20 ng/ml insulin-like growth factor-1 (IGF-1), 200 ng/ml basic fibroblast growth factor (bFGF), and 250 nM dexamethasone for

4–7 days. High (4.5 g/L) or low (1.0 g/L)-glucose DMEM was used. After 4 days of incubation in the differentiating media containing 10 μ M retinoic acid, 20 ng/ml IGF-1, 200 ng/ml bFGF, and 250 nM dexamethasone, CYP19A1 gene transcription levels in both TMSCs and BMSCs were analysed with real-time PCR. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). *** $p < 0.001$. **B** Schematic diagram of a novel method for differentiation of MSCs into estrogen-secreting cells

and CYP17A1 gene expression was not consistent in BMSCs and AMSCs, as compared with that in TMSCs (Fig. 4A, B). These data comprehensively indicate the effectiveness of the novel method to induce CYP19A1 gene transcription in MSCs. However, this method did not consistently induce CYP11A1 and CYP17A1 gene expression in MSCs.

3.4 Estrogen-secreting TMSCs induced by the novel method were characterised

We further examined gene transcription in relation to estrogen biosynthesis. In addition to elevating CYP11A1 (Fig. 5A), CYP17A1 (Fig. 5B), and CYP19A1 (Fig. 5C) gene expression, 3 β -hydroxysteroid dehydrogenase, type 1 (HSD3B1) gene levels were increased by the novel method (Fig. 5D). However, 3 β -hydroxysteroid dehydrogenase, type 2 (HSD3B2) gene levels were not significantly altered (Fig. 5E). HSD3B catalyses several reactions in the androgen pathway (Fig. 1), leading to the production of androstenedione and testosterone, and also catalyses the conversion of pregnenolone to progesterone [6]. Therefore, elevated HSD3B1 levels favour estrogen biosynthesis. However, mRNA levels of steroid sulfatase (STS), which

hydrolyses estrone 3-sulfate and dehydroepiandrosterone-sulfate to their active un-sulfoconjugated forms [25], were not altered by the novel method (Fig. 5D). Because 17 β -hydroxysteroid dehydrogenase (HSD17B) isoforms reversibly catalyse the final step in the formation of estradiol from estrone (Fig. 6A) [26], gene levels of HSD17B isoform types 1, 2, 7, and 8 were also examined (Fig. 6B–E). HSD17B, type 1 (HSD17B1) gene levels were not altered by the novel differentiating method (Fig. 6B). Gene levels of HSD17B, type 2 (HSD17B2) and HSD17B, type 7 (HSD17B7) were elevated (Fig. 6C, D), whereas HSD17B, type 8 (HSD17B8) gene levels were decreased upon differentiation (Fig. 6E). Therefore, the one direction of conversion between estradiol and estrone may not be dominant.

Then, we investigated CYP19A1 protein levels after differentiation. Despite elevated CYP19A1 mRNA levels (Fig. 5C), CYP19A1 protein expression was not detected by western blotting after 4 days of differentiation (data not shown). Thus, we extended the time for differentiation to 21 days. Elevated CYP11A1 and CYP17A1 mRNA levels by the novel method were maintained during all 21 days (Fig. 7A, B). The mRNA levels of CYP19A1 were maximum after 7 days of differentiation, with a 7.45 ± 0.09 -

Fig. 4 Addition of 12-*O*-tetradecanoylphorbol-13-acetate to the differentiating media significantly elevates CYP19A1 transcription. TMSCs, BMSCs, and AMSCs were cultured in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-added differentiated media for 4 days. The effects of TPA on **A** CYP11A1, **B** CYP17A1, and **C** CYP19A1 gene transcripts were analysed with real-time PCR. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$

fold increase compared with basal levels (Fig. 7C). The protein levels of CYP19A1 were also detected after 7 days of differentiation, and the CYP19A1 band intensity was decreased at 21 days compared with at 7 or 14 days of differentiation (Fig. 7D). Therefore, we concluded that 7 days of differentiation is optimal for differentiating TMSCs into estrogen-secreting cells, considering the rate-limiting step of CYP19A1. Finally, we measured 17 β -estradiol in media secreted from differentiated cells in TMSCs and BMSCs (Fig. 7E). In both MSCs, 17 β -estradiol secretion was significantly increased in the differentiated group compared with the non-differentiated group upon testosterone addition (Fig. 7E). Secretion of 17 β -estradiol was significantly higher in differentiated TMSCs than in differentiated BMSCs (Fig. 7E). The estrogen receptor α is a ligand-activated transcription factor which mediates the pleiotropic effects of 17 β -estradiol, and 17 β -estradiol treatment degrades estrogen receptor by inducing its polyubiquitination [27]. Thus, we incubated MCF-7 cells with conditioned medium harvested from differentiated TMSCs to examine the biological function of secreted estrogen from differentiated cells. Conditioned medium harvested from differentiated TMSCs can induce the degradation of estrogen receptor α similar with β -estradiol (Fig. 7F), indicating its physiological function.

3.5 CYP17A1 and CYP19A1 gene expression returned to basal levels after withdrawal of differentiating factors in the media

We then investigated whether estrogen-secreting TMSCs would remain as differentiated upon the removal of differentiating factors. The differentiating media was changed to DMEM supplemented with 10% FBS and 1% P/S. Both CYP17A1 and CYP19A1 gene levels were dramatically reduced at 3 days after withdrawal from differentiating factors and returned to basal levels at 3 and 7 days, respectively (Fig. 8), which implicates the de-differentiation process. Therefore, we concluded that the differentiation of TMSCs into estrogen-secreting cells was reversible, and that the differentiated cells should be exposed to differentiating media continuously to sustain their estrogen-secreting status.

4 Discussion

Estrogen is a hormone that functions as a critical regulator of mammalian physiology and development [28]. In particular, it plays a crucial role in skeletal growth and bone homeostasis in both men and women. Estrogen deficiency

Fig.5 Alteration of genes related to estrogen biosynthesis upon differentiation. After 4 days of incubation in the differentiating media containing 10 μ M retinoic acid, 20 ng/ml IGF-1, 200 ng/ml bFGF, and 250 nM dexamethasone, **A** CYP11A1, **B** CYP17A1, **C** CYP19A1, **D** 3 β -hydroxysteroid dehydrogenase, type 1 (HSD3B1),

E 3 β -hydroxysteroid dehydrogenase, type 2 (HSD3B2), and **F** steroid sulfatase (STS) gene transcript levels in TMSCs were analysed with real-time PCR. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

leads to bone loss, due to a complex interplay of hormones and cytokines that converge to disrupt the process of bone remodelling [29]. In addition, estrogen regulates glucose and lipid metabolism, and estrogen deficiency can lead to the development of metabolic syndrome and a higher cardiovascular risk in women; this is characterized by lipid profile variations and predominant abdominal fat accumulation [5]. Furthermore, estrogen has been reported to act as a neuroprotective mediator, and estrogen depletion in the brain is now regarded as a significant risk factor for developing Alzheimer's disease neuropathology [3, 30]. Therefore, maintaining proper estrogen levels is important for physiologic and metabolic homeostasis. Although hormone replacement therapy, such as estrogen therapy and estrogen-progesterone combination therapy, is currently used to mitigate perimenopausal and postmenopausal symptoms, the use of oral hormone replacement therapy increases breast cancer, endometrial or ovarian cancer, stroke, and gallbladder disease [31]. Hormone replacement therapy also requires taking a daily pill, which reduces patient compliance. Stem cell therapy can be an alternative option for hormone replacement therapy, if physiological estrogen secretion from stem cells can be achieved. The

present study demonstrated the capacity of TMSCs to secrete estrogen and developed an efficient differentiating method for estrogen-secreting cells.

The ability of TMSCs to secrete estrogen was superior to that of BMSCs. Both CYP19A1 gene expression and estrogen secretion were much higher in TMSCs compared with BMSCs after differentiation. Interestingly, CYP19A1 gene expression was significantly higher in TMSCs than BMSCs and AMSCs at the basal state. CYP19A1 is known to be expressed not only in ovarian follicles in premenopausal women, but also in many extragonadal sites, including the bone, breast, adipose tissue and brain [32]. This tissue-specific expression of CYP19A1 maintains tight local control over the synthesis and action of estrogens [32]. Although CYP19A1 expression and estrogen synthesis in palatine tonsil tissues have not yet been investigated, estrogen synthesis in lymphoid tissues has been reported previously [33]. Mesenteric lymph node and Peyer's patch tissues cultured *in vitro* produced estradiol [33], and the expression and activity of steroidogenic enzymes were detected in the spleen [34]. As a lymphoid tissue, tonsils might be a site of estrogen synthesis *in vivo* considering high CYP19A1 expression. Overall, our data

Fig. 6 Alteration of genes related to estrone and estradiol conversion upon differentiation. **A** Schematic diagram of estrone and estradiol conversion. 17 β -hydroxysteroid dehydrogenase (HSD17B) isoforms catalyse the conversion between estrone and estradiol. After 4 days of incubation in the differentiating media containing 10 μ M retinoic

acid, 20 ng/ml IGF-1, 200 ng/ml bFGF, and 250 nM dexamethasone, **B** HSD17B1, **C** HSD17B2, **D** HSD17B7, and **E** HSD17B8 gene transcript levels in TMSCs were analysed with real-time PCR. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$

suggest that TMSCs are an optimal source for producing estrogen-secreting cells.

In the present study, we newly developed a method for differentiating MSCs into estrogen-secreting cells. Although high-glucose DMEM in combination with retinoic acid has been shown to induce estrogen secretion in BMSCs [16], it did not successfully induce CYP19A1 expression in both TMSCs and BMSCs. The novel method developed in the present study uses a combination of differentiating factors, including retinoic acid, IGF-1, bFGF, and dexamethasone, which are known to elevate CYP19A1 transcription [20–23]. IGF-I has been reported to stimulate CYP19A1 transcription via IGF-1 receptor/phosphatidylinositol 3-kinases/AKT [20]. Treatment of bFGF with various concentrations in cultured buffalo granulosa cells revealed that the highest mRNA expression and immunoreactivity of CYP19A1 were observed with 200 ng/ml at 72 h of incubation [21]. CYP19A1 gene transcription was upregulated with dexamethasone in primary mouse adipose fibroblasts [23] and THP-1 cells [22]. The combination of these factors was able to enhance CYP19A1 transcription in all the MSCs tested, implicating it as a general differentiation method of MSCs into estrogen-secreting cells. Thus, the conversion of testosterone to estradiol, as well as the conversion of androstenedione to estrone, could be achieved with this method in MSCs. However, the altered expression of steroidogenic enzymes other than CYP19A1 was not consistent among different

MSCs. Therefore, the production of estrogen from other precursors, such as cholesterol and progesterone, may not be induced in BMSCs or AMSCs. Another limitation of this method is the reversibility of differentiated estrogen-secreting cells upon the withdrawal of differentiating factors. A similar regression of differentiated cells was also observed in insulin-secreting cells that were differentiated from TMSCs [10]. Thus, devices or hydrogels that allow for a continuous supply of differentiating factors would be necessary for the implantation of estrogen-secreting cells *in vivo*. In addition, decreased CYP19A1 protein levels at 21 days compared with at 7 or 14 days of differentiation can also diminish the estrogen-secreting efficacy after *in vivo* implantation of estrogen-secreting cells. Therefore, for clinical application, cell-based therapy based on this novel differentiating method for estrogen-secreting cells may require sustained supplementation of differentiating factors, and a further modification of the protocol for irreversible differentiation would be needed.

A previous study reported a protocol for generating functional ovarian granulosa-like cells from human embryonic cells and used a step-wise differentiation process with a combination of bone morphogenetic protein 4, Wnt family member 3A, activin-A, and bFGF [19]. However, this combination did not induce CYP19A1 expression or estrogen secretion in TMSCs (data not shown), implicating the requirement of different methods to generate estrogen-secreting cells from TMSCs. Since only a few

Fig. 7 Functional estrogen-secreting cells can be generated from TMSCs after 7 days of incubation in differentiating media. TMSCs were cultured in the differentiating media containing 10 μ M retinoic acid, 20 ng/ml IGF-1, 200 ng/ml bFGF, and 250 nM dexamethasone for 7–21 days. **A** CYP11A1, **B** CYP17A1, and **C** CYP19A1 mRNA levels were analysed using real-time PCR. **D** CYP19A1 and β -actin protein levels were analysed with western blotting. **E** Levels of 17 β -estradiol in media secreted from differentiated cells were measured using an enzyme-linked immunoassay. Testosterone (50 ng/ml) was

added as a substrate for aromatase activity. **F** To investigate the biological function of estrogen produced by differentiated estrogen-secreting TMSCs, MCF-7 cells were cultured in conditioned medium harvested from differentiated estrogen-secreting TMSCs for 6 h. For control, MCF-7 cells were cultured in DMEM with or without β -Estradiol (10 nM) for 6 h. Estrogen receptor α (ER α) and β -actin protein levels were analysed with western blotting. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. C control, D differentiated cells

studies have reported on the generation of estrogen-secreting cells from stem cells [16, 19], the novel method presented in the current study provides a robust foundation for the investigation of MSC differentiation into the estrogen-secreting cells. Because MSCs have excellent immune-modulating capacity and do not need to be co-cultured with feeder cells, they have advantages over embryonic stem cells for clinical implantation. Therefore, the novel method developed in the present study is important and might be applied as a therapy for estrogen deficiency.

TMSCs can be easily isolated from waste tonsil tissues obtained during tonsillectomy [8]. Tonsillectomy is a common surgical procedure worldwide and is usually performed in children under 15 years of age [35]. Therefore, the donor age of TMSCs is typically young. TMSCs also have several advantages over other MSCs; these include the completely noninvasive process of tissue collection, relatively high proliferation rate, excellent differentiation potential, maintenance of MSC-specific characteristics during expansion, and low immunogenicity [8, 10, 36]. Previously, a double intratibial injection of non-

differentiated TMSCs was shown to recover postmenopausal osteoporotic bone mass, but estrogen secretion from undifferentiated TMSCs or serum estrogen levels upon TMSC implantation have not been measured [14]. Although estrogen secretion from murine undifferentiated BMSCs has been reported [17], 17 β -estradiol secretion was not detected in both undifferentiated human BMSCs and TMSCs in the present study. In addition, the addition of testosterone as a substrate for aromatase activity was needed to detect 17 β -estradiol in culture media for differentiated estrogen-secreting TMSCs and BMSCs. This is in accordance with differentiation data of human embryonic stem cells into functional ovarian granulosa-like cells [19]. The discrepancy may be derived from differences in MSC sources or donor variation. The concise properties of estrogen secretion in various MSCs requires further testing with more samples.

In summary, results from the present study suggests that TMSCs are a good source for producing estrogen-secreting cells. A novel protocol for the effective differentiation of MSCs into estrogen-secreting cells was also developed. This may aid in providing the foundation for the clinical

Fig. 8 The differentiation of TMSCs into estrogen-secreting cells is reversible. **A** Schematic illustration of the experiment to examine the reversibility of differentiation. TMSCs were cultured in the differentiating media containing 10 μ M retinoic acid, 20 ng/ml IGF-1, 200 ng/ml bFGF, and 250 nM dexamethasone for 7 days, and then the differentiating media was changed to DMEM supplemented with

10% FBS and 1% P/S. **B** CYP17A1 and **C** CYP19A1 gene levels were analysed with real-time PCR at 3 and 7 days after withdrawal from differentiating factors. Values are expressed as mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. C control, D Differentiated cells

application of MSCs in the estrogen-secreting cell transplantation process for estrogen deficiency.

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Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

Ethical statement The experimental procedures were approved by the Institutional Review Board of Ewha Womans University Mok-dong Hospital (Seoul, South Korea, ECT-11-53-02).

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