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Original Article

Reprogramming human endometrial fibroblast into induced pluripotent stem cells

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Abstract

Objective: A recent breakthrough demonstrated that ectopic expression of four genes is sufficient to reprogram human fibroblasts into inducible pluripotent stem cells (iPSCs). However, it remains unknown whether human endometrial fibroblasts (EMFs) are capable of being reprogrammed into EMF-derived iPSCs (EMF-iPSCs).

Methods: EMFs were obtained from donors in their third and fourth decade of life and were reprogrammed into iPSCs using retroviral transduction with *Oct-4, Sox2, Klf4,* and *c-Myc*.

Results: The EMF-iPSCs displayed the accelerated expression of endogenous Nanog and OCT-4 during reprogramming compared with EMFs. As a result, EMF-iPSC colonies that could be subcultured and propagated were established as early as 12 days after transduction. After 2 weeks of reprogramming, the human endometrial cells yielded significantly higher numbers of iPSC colonies and formed more 3D spheroid bodies than the EMFs. We have shown that human EMF-iPSCs are able to differentiate into neuronal-like cells, adipocytes, and osteocyte-like cells that express specific osteogenic genes.

Conclusion: Human EMFs can undergo reprogramming to establish pluripotent stem cell lines in female donors by the retroviral transduction of *Oct-4, Sox2, Klf4,* and *c-Myc.*

Keywords: differentiation; endometrium; endometrial fibroblasts; gene; inducible pluripotent stem cells

Introduction

Stem cells are considered a promising resource for restorative cell therapy for the treatment of various diseases in a wide range of clinical areas. Recent technical advances have demonstrated that induced pluripotent stem cells (iPSCs) can be generated from mouse embryonic fibroblasts (MEFs) and from human fibroblasts using retroviral transfection of the following four transcription factors: *Oct-4, Sox2, c-Myc,* and

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Klf4 [1–4]. Generally, fully reprogrammed iPSCs display numerous properties similar to those of embryonic stem cells (ESCs). iPSCs share the same morphological features as ESCs as well as the abilities of self-renewal and differentiation into three germ layers [1]. They are indistinguishable from ESCs in morphology, proliferative abilities, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity [1]. Several molecular and functional assays are able to evaluate the similarities between iPSCs and ESCs. These similarities include the reactivation of genes associated with self-renewal and pluripotency; telomerase activity; X chromosome and stage-specific embryonic surface antigens; suppression of somatic genes associated with the cell of origin; silencing of exogenous factors; capabilities of *in vitro* differentiation; demethylation of promoters of pluripotency genes; and *in vivo* teratoma formation, chimera contribution, germline transmission, and tetraploid complementation [5–7]. Other advantages of iPSCs over ESCs include the ability of iPSCs to be derived from the patient's somatic cells, which avoids the potential for immune rejection and the absence of the ethical concerns associated with ESCs. For example, a recent study demonstrated that patient-specific iPSCs derived from dermal fibroblasts of patients with long-QT syndrome can differentiate into functional cardiac myocytes but recapitulated the electrophysiological features of the disorder [8]. Safe induced pluripotent stem clone-derived neural stem cell/neural progenitor cells (NPCs) could be an important source for transplantation therapy in stroke and neurodegenerative diseases [9]. The use of *L-Myc* to replace *c-Myc*, combined with *Oct-4/Sox2/Klf4*, promotes iPSC generation without tumor formation [10]. We previously showed subdural transplantation of iPSCs mixed with fibrin glue did not result in tumor formation after 6 weeks [11]. Therefore, the major advantages of iPSCs over ESCs are that iPSCs can be derived from a patient's own somatic cells, can be used for autologous transplantation without the risk of rejection, avoid the ethical concerns raised by using ESCs, and are considered candidates for cell therapy [1–4].

The human endometrium is responsive to sex steroid hormones and undergoes extraordinary growth in a cyclic manner. The endometrium is shed and regenerated throughout a woman's lifetime. It has been proposed that the human endometrium might contain a population of stem cells that is responsible for its remarkable ability to regenerate [12,13]. Recently, endometrial-derived stem cells from human endometrium were isolated and demonstrated the potential of multipotent capacity to differentiate into different-lineage cells [14,15]. However, the multipotent capacity of the endometrial-derived stem cells of iPSCs should be investigated and further compared with pluripotent stem cells and/or ESCs. More recently, Park et al. [16] showed that human endometrium cells express elevated levels of pluripotent factors, which, with additionally defined factors, result in significantly more efficient and accelerated generation of iPSCs compared with conventional somatic cells [16]. However, whether endometrial fibroblasts (EMFs) can be efficiently reprogrammed into iPSC- or ESC-like cells and possess the pluripotent function of EMF-derived iPSCs (EMF-iPSCs) to differentiate into multiple-lineage cells remains unknown. In this study, we exploited the remarkable capacity of human EMFs to undergo reprogramming to establish pluripotent stem cell lines in female donors by the retroviral transduction of *Oct-4, Sox2, Klf4,* and *c-Myc* (Table 1).

Materials and methods

Tissue collection

Endometrial tissue samples were obtained from women with uterine fibroids who had not undergone hormone therapy (n = 3, aged 37–41 years; menstrual cycle phase: two proliferative phase and one mid-secretory phase). Samples were scraped from the hysterectomy specimens with a curette. The phase of the menstrual cycle was assessed by endometrial histology. This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Taipei Veterans General Hospital, Taiwan. In addition, preoperative written informed consent was obtained from each patient (Table 1).

Isolation of EMFs

EMFs were derived directly from the patients' endometria. The endometrial biopsy was rapidly washed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) in a Petri dish, cut into small fragments, and transferred to a flask [17]. The endometrial tissue was then digested by collagenase P (Roche, Mannheim, Germany) in HEPES-buffered saline for 7 h at 37°C. These cells were plated in a 6-cm tissue culture dish. The expansion medium consisted of Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (LG) (Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS; Gibco) supplemented with 10 ng/mL basic fibroblast growth factor, 10 ng/ml epidermal growth factor, 10 ng/mL platelet-derived growth factor-BB (R&D Systems, Minneapolis, Minnesota, USA), 100 units/mL penicillin, 1000 μg/mL streptomycin, and 2 mM L-glutamine (Gibco).

Induction of iPSCs

iPSCs generated from the EMFs were reprogrammed by the transduction of retroviral vectors encoding *Oct-4, Sox2, Klf4*,

Table 1
Clinical characteristics of patients.

Normal endometrial tissue	Age	Phase	Isolation of EMFs	Reprogramming EMF-iPSCs
Case 1	37	Proliferative	Successful	Successful
Case 2	40	Secretory	Successful	Successful
Case 3	41	Proliferative	Successful	Successful

EMF = Endometrial fibroblasts; EMF-iPSCs = Endometrial inducible pluripotent stem cells.

and *c-Myc* (*OSKM*). Detailed information regarding the procedures of iPSC reprogramming and culture methods are described below.

iPSCs culture, in vitro passage, expansion, storage, and differentiation

Human iPSCs were generated from human endometrial fibroblast derived from healthy donors. The iPSCs were reprogrammed by the transduction of retroviral vectors encoding four transcription factors (*Oct-4, Sox2, Klf4,* and *c-Myc*), as described previously, with some modifications [1,16,18,19]. Briefly, undifferentiated iPSCs were routinely cultured and expanded on mitotically inactivated EMFs (50,000 cells/cm^2) in six-well culture plates (BD technology, Franklin Lakes, NJ, USA) in the presence of 0.3% leukemia inhibitory factor in an iPSC medium consisting of DMEM (Sigma, St Louis, MO, USA) supplemented with 15% FBS (Invitrogen, Carlsbad, CA), 100 mM minimal essential medium nonessential amino acids (Sigma), 0.55 mM 2-mercaptoethanol (Gibco), and antibiotics (Invitrogen). These iPSCs were transfected with pCX-EGFP to constitutively express green fluorescence and were maintained and differentiated *in vitro,* as described previously [20]. Every 3–4 days, colonies were detached with 0.2% collagenase IV (Invitrogen), dissociated into single cells with 0.025% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% chicken serum (Invitrogen) in PBS and replated onto MEFs.

Induced differentiation of EMF-iPSCs into adipogenic, chondrogenic, and neuronal cells

Adipogenic differentiation

First, 1×10^5 EMF-iPSCs at the fifth to eighth passage were treated with adipogenic medium for 14 days. Adipogenic medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 μmol/L hydrocortisone (Sigma-Aldrich), 0.1 mmol/L indomethacin (Sigma-Aldrich), and 10% rabbit serum (Sigma-Aldrich). For the evaluation of adipocytes, cells were fixed with 4% formaldehyde and stained with Oil-red O (Sigma) [13].

Osteogenic differentiation

The 1×10^5 EMF-iPSCs at the fifth to eighth passage were treated with osteogenic medium for 3 weeks. Osteogenic medium consisted of DMEM-LG (Gibco) supplemented with 50 lg/mL ascorbate-2 phosphate, 10^{-8} M dexamethasone, and 10 mM b-glycerophosphate (all from Sigma). Osteogenesis was assessed by alizarin red staining. For evaluation of the mineralized matrix, induced EMF-iPSCs were treated with 2% silver nitrate (Sigma) under UV radiation for 1 h. Cells were fixed with 2.5% sodium thiosulfate (Sigma) for 5 min and washed in dH_2O. Cells were then counterstained with 1% neutral red (Sigma) for 1 min [14].

Neurogenic differentiation

First, 1×10^5 EMF-iPSCs at the 5th to 8th -passage were treated with serum-free modified neurogenic selection medium for two weeks. The serum-free medium for the selection of neural precursor cells contained DMEM/F12 1:1 (Gibco/BRL) and was supplemented with 0.6% glucose, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, 2 μg/ml heparin, 20 ng/ml EGF, and 20 ng/ml bFGF (all from Sigma, St. Louis, MO). For further neural differentiation, EGF was removed from the medium, and the medium was supplemented with 20 ng/ml SHH (R&D), 10 ng/ml BDNF (R&D), and all-trans retinoic acid (100 nM) for another seven days [21].

Karyotypic analysis

To assess whether EMFs (n = 3) and EMF-iPSCs (n = 3) maintain a normal karyotype, passage ten cells were harvested at 70–80% confluency and resuspended in 10 μL of colcemid per mL of media. Cells were incubated at 37°C for 3–6 h, after which cells were resuspended in 0.5 mL of medium and mixed with 0.075 M KCl to a volume of 10 mL. After incubation for 10–15 min at 37°C in a waterbath, cells were resuspended in a total of 10 mL of fixative (methanol:acetic acid ratio: 3:1). Staining with DAPI for G-banding was performed by equilibrating the slides in 0.3 M sodium citrate, which contained 3 M NaCl, for 5 min and subsequently adding two drops of Antifade with DAPI per slide before visualization [15].

Results

Among the adult somatic cells, the human endometrium displays unique features with regard to regenerative potential and plasticity, rendering it an excellent candidate for reprogramming into the pluripotent state. Therefore, human EMFs are thought to be amenable to reprogramming into iPSCs. To gain further insight into the possibility that human EMF are a candidate for iPSCs generation, we followed the approach of Yamanaka's group by introducing four factors in the isolated human EMFs [1,19]. Culture-expanded fibroblasts that are derived from EMFs differentially adopt mesodermal lineages [22]. To improve the pluripotent and multi-lineage differentiation abilities, EMF (Fig. 1A upper left) introduced with *OSKM* were reprogrammed into EMF-iPSCs (Fig. 1A upper right). Undifferentiated iPSCs were cultured on inactivated MEFs and formed colonies highly similar to human ESCs (H9) (Fig. 1A, upper right). EMF-iPSCs formed 3D spheroid bodies at day 14 (Fig. 1A, lower left). These iPSC clones were positively stained for alkaline phosphatase (Fig. 1A, lower right), Oct-4, and SSEA-1 (data not shown). We used a quantitative PCR analysis to examine whether stem cell markers were expressed in EMF-iPSCs introduced with *OSKM* (*OSKM*-iPSCs) and H9. Mild expression of endogenous Klf4 was detected in endometrium whereas strong expression of all stem cell markers was detected in both *OSKM*-iPSCs and H9 (Fig. 1B).

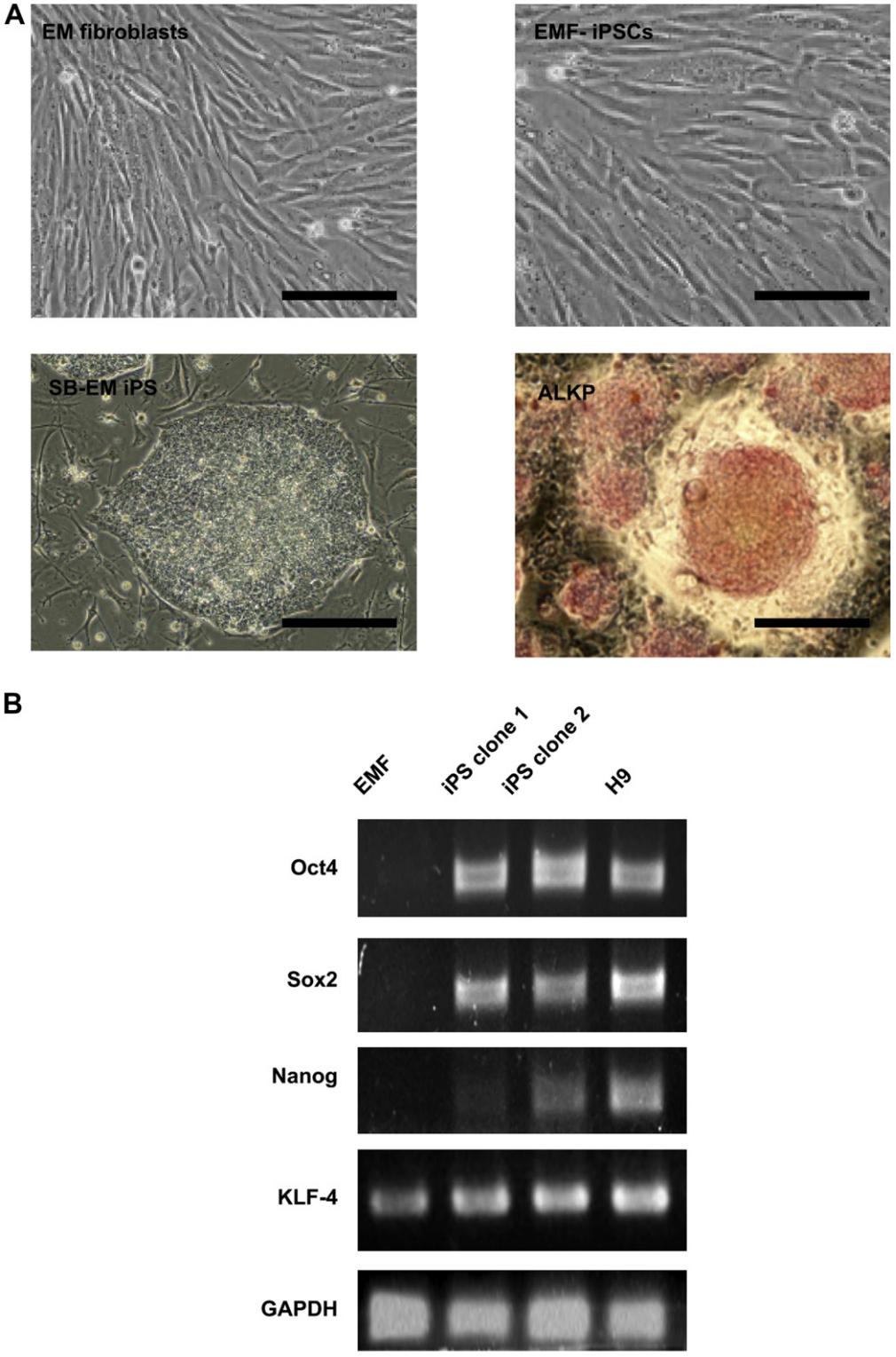

Fig 1. Characterization of endometrial fibroblasts (EMFs) and inducible pluripotent stem cells (iPSCs) derived from EMF (EMF-iPSCs). (A) Morphology of EMF isolated from tissue samples of normal endometrium. EMF-iPSCs developed a cluster-like morphology, and cell aggregates were observed at day 7. EMF-iPSCs formed 3D spheroid-bodies (SB-EMF-iPSCs) at day 14. Reprogramming plates stained with alkaline phosphate and relative reprogramming efficiencies of EMF-iPSCs. Bars: 50 μm. (B) Representative quantitative PCR analysis of EMFs and reprogrammed iPSCs for markers of pluripotency and reprogramming factors relative to factor levels in human embryonic stem cells (hESCs) (H9). Input EMFs express very low levels of most factors whereas EMF-iPSCs display levels similar to hESCs (H9).

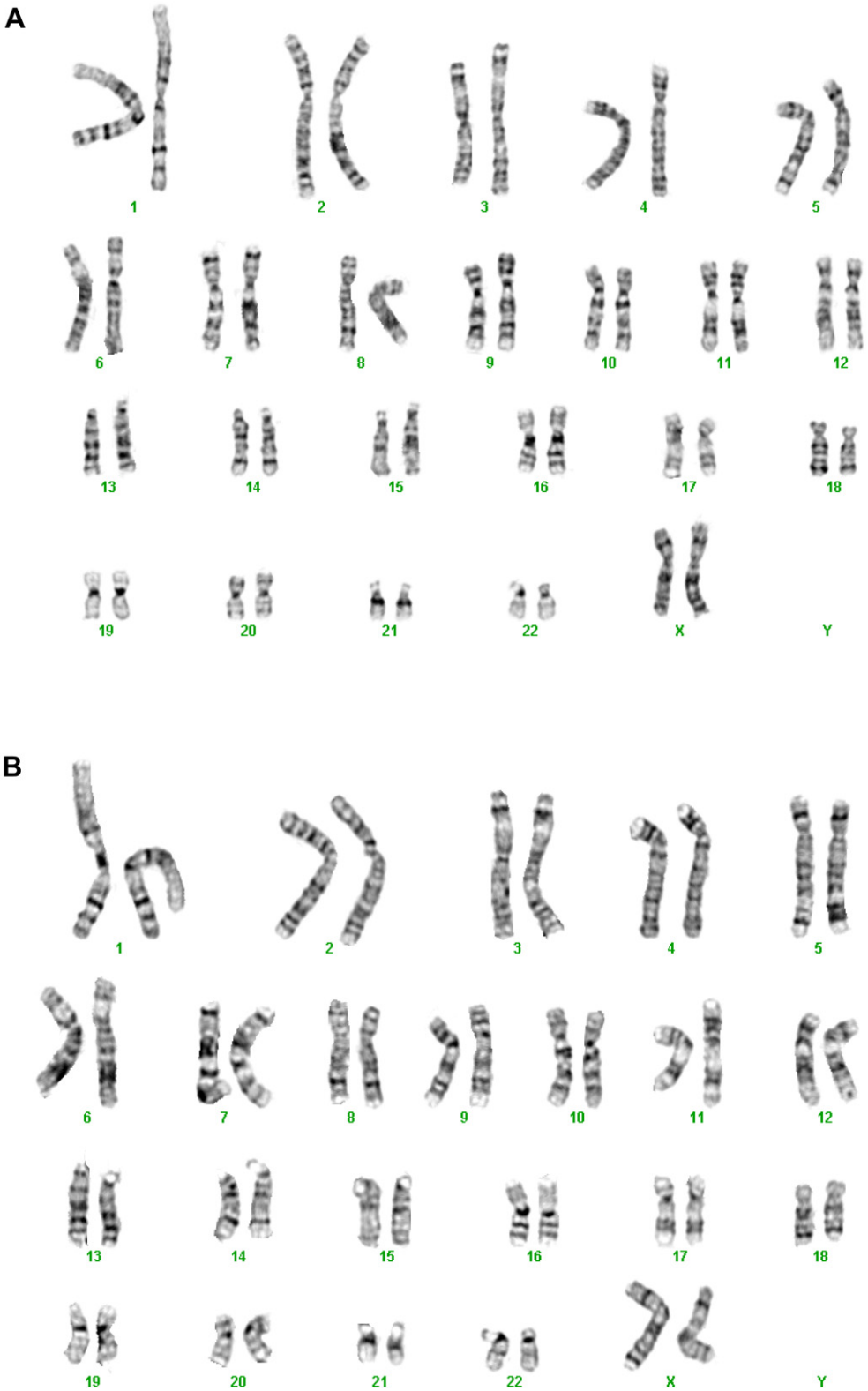

Fig 2. Karyotyping study. (A) Karyotype analysis of endometrial fibroblasts (EMFs) and (B) EMF-inducible pluripotent stem cells show normal karyotypes of 46, XX.

The utility of any stem cell population depends on its ability to expand without acquiring carcinogenic potential; to be useful, the cells must maintain a normal karyotype. Fig. 2A shows that EMFs have 23 pairs of chromosomes with no aneuploidy or other abnormalities, even after five cell passages. Even after EMFs introduced with *OSKM* were reprogrammed into EMF-iPSCs, there was no difference between EMFs and EMF-iPSCs in the karyotypic analysis (Fig. 2B).

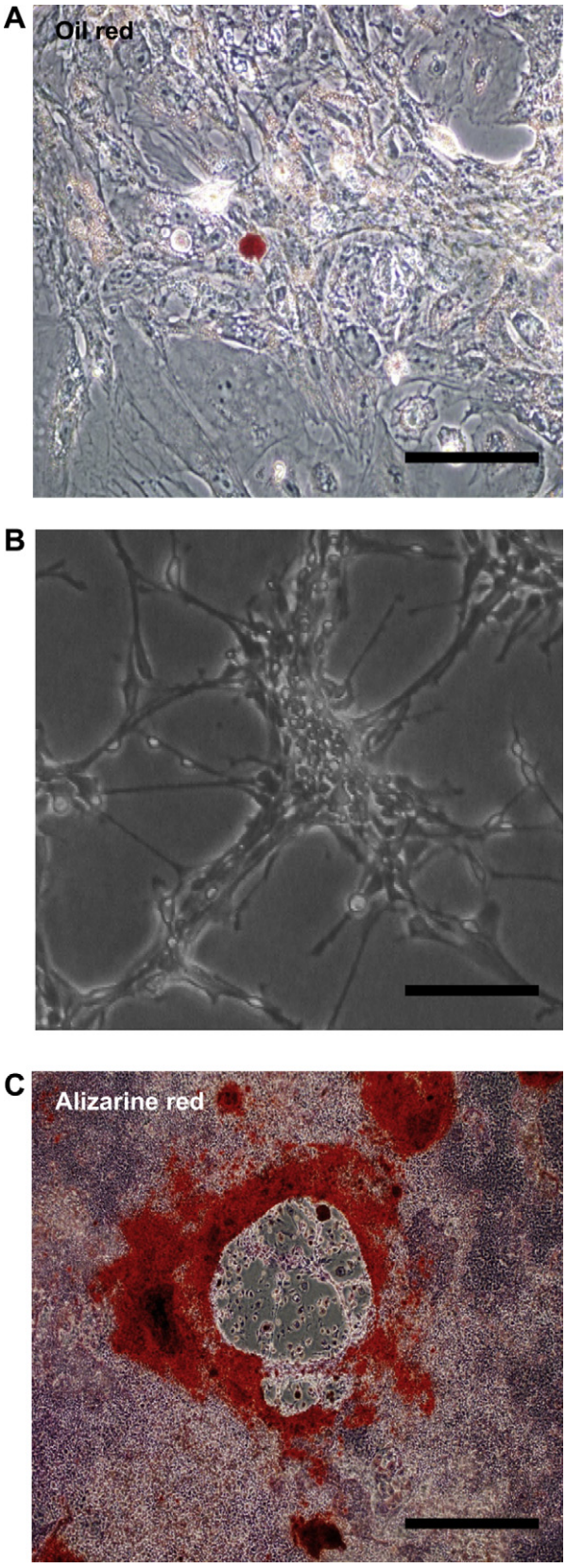

Fig 3. Potential for adipogenic, osteogenic and neuronal differentiation in endometrial fibroblast (EMF)-inducible pluripotent stem cells (iPSCs), but not in EMFs. (A) After 14 days of adipogenic induction, EMF-iPSCs were able to differentiate into adipocytes with positive oil-red O staining (an adipogenic gene). (B) Phase contrast imaging of the EMF inducible pluripotent stem-derived neuron-like cells. (C) Following 14 days of osteogenic differentiation, EMF-iPSCs differentiated into osteocyte-like cells, as detected by alizarine red staining. Bars: 50 μm.

The potential of osteogenic, adipogenic, and neurogenic differentiation from EMF-iPSCs

EMF-iPSCs have the capacity to differentiate in both mesodermal (osteocyte and adipocyte) and ectodermal (neuron) tissue lineages (Fig. 3), but EMF do not. These results are detailed below.

EMF-iPSCs successfully differentiated into adipocytes in 14 days when treated with adipogenic medium. These cells were positive for oil-red O staining (Fig. 3A). NPCs were generated from *OSKM*-EMF-iPSCs by employing the protocol of neurogenic differentiation. After 14 days, neuron-like cells with neurite formation were observed (Fig. 3B). To test the potential for osteogenic differentiation of EMF-iPSCs, fifth-passage cells were cultured in the presence of a high concentration of dexamethasone and β-glycerophosphate. These cells exhibited osteocyte phenotypes as evidenced by alizarin red staining (Fig. 3C). These results suggest that the EMF-iPSCs could be reprogrammed from EMF cells. Therefore, endometrial tissues and endometrium-derived cells could be an alternative source of cells that can produce iPSCs with cell reprogramming.

Discussion

Our results demonstrate that the endometrium could be a source of stem cells or fibroblasts to induce iPSCs. During the menstrual cycle, the endometrium undergoes periodic and dramatic changes involving tissue loss and regeneration. After menstruation, the stratum functionalis regenerates from the stratum basalis during the proliferative phase, reaching a thickness of up to 5–6 mm at the peak of the secretory phase. In the absence of implantation, the functionalis layer is shed, leaving the basalis, which is a structurally stable compartment of the uterus that is not degraded during menstruation [23]. In addition to its plasticity and regenerative capacity, the endometrium is also an easily accessible source of abundant cells that can be obtained through biopsy using minimally invasive techniques; additionally, the cells are easy to maintain and propagate *in vitro* with standard laboratory techniques [24]. Recently, Yamanaka and colleagues [1,19] demonstrated that induced iPSCs could be generated from MEFs as well as from adult human fibroblasts via the retrovirus-mediated transfection of four transcription factors, namely, *Oct-3/4, Sox2, c-Myc,* and *Klf4*. These studies further suggest that iPSCs are indistinguishable from ESCs with respect to morphology, proliferative abilities, surface antigens, gene expressions, epigenetic status of pluripotent cell-specific genes, and telomerase activity. In this study, we successfully isolated EMF cells from human endometrial tissues and reprogrammed the EMFs into EMF-iPSCs. The quantitative PCR results show that EMF-iPSCs preserved the genetic characteristics of a primitive embryonic stage as evidenced by the expression of the genes

Oct-4, Sox2, Nanog, and *Klf4*. We also showed that EMF-iPSCs have 23 pairs of chromosomes with no aneuploidy or other abnormalities. Moreover, as compared with EMFs, EMF-iPSCs can efficiently differentiate into adipogenic, osteogenic, and neuronal cells. Our results suggest that the EMF-iPSCs can be reprogrammed from EMFs.

Recently, Park et al. [16] showed that iPSCs could also be generated and reprogrammed from the endometrial cells that were transduced with *Oct-4, Sox2, Klf4,* and *Myc* [16]. Accordingly, the time for the derived iPSCs to be subcultured and propagated was 12 days, which is significantly earlier than the iPSCs derived from other cell types [16]. Moreover, the colony-forming efficiency of the endometrial cell-derived iPSCs was also higher than the skin fibroblast-derived iPSCs, indicating that endometrial cells are more efficient sources for human iPSC generation [16]. According to the report of Park et al [16], the embryonic bodies of the iPSCs derived from the endometrial cells expressed high levels of the markers for endoderm (e.g., AFP and GATA4), mesoderm (e.g., RUNX1 and brachyury) and ectoderm (e.g., nestin and NCAM1) than the endometrial iPSCs of origin [16]. In our study, we further found that EMF-iPSCs could be differentiated into neuronal-like cells, adipocytes and osteocyte-like cells, indicating that these EMF-iPSCs also have the potential for multi-lineage differentiation (Fig. 3) with intact genomes that were not interrupted by exogenous retroviral transduction (Fig. 2). Because of the physiological characteristics of the endometrium, which exhibit periodic macroscopic and microscopic tissue loss, regeneration, and differentiation, both our results and those from Park et al [16] suggest that the endometrium might be a good candidate source for iPSCs generation.

The advantages of iPSCs over ESCs are that iPSCs can be derived from the patient's somatic cells, thus avoiding the potential for immune rejection, and that they avoid the ethical concerns associated with ESCs. For example, a recent study demonstrated that patient-specific iPSCs from dermal fibroblasts of patients with long-QT syndrome can differentiate into functional cardiac myocytes but recapitulated the electrophysiological features of the disorder [8]. Safe iPSC clone-derived neural stem cell/NPCs could be an important source for transplantation therapy in stroke and neurodegenerative diseases [9,25]. The use of *L-Myc* to replace *c-Myc*, combined with *Oct-4/Sox2/Klf4*, promotes iPSCs generation without tumor formation [26]. We previously showed that the subdural transplantation of iPSCs mixed with fibrin glue did not result in tumor formation after 6 weeks [11]. Therefore, the major advantage of iPSCs over ESCs is that iPSCs can be derived from a patient's own somatic cells, they can be used for autologous transplantation without the risk of rejection, they avoid the ethical concerns associated with using ESCs, and they are regarded as candidates for cell therapy [1–4].

Among the four factors for conventional iPSCs reprogramming, the pro-oncogene *c-Myc* has a critical role in influencing reprogramming efficiency [10,26]. However, a previous report suggested that deregulated expression of *c-Myc* occurs in a wide range of human cancers; it is often associated with poor prognosis and may contribute to tumorigenesis by over-stimulating cell growth and metabolism and/or by causing genomic instability [27]. Thus, this oncogene may have a key role in tumor progression [27]. Therefore, the introduction of *c-Myc* could be a major obstacle in iPSC-based cell transplantation because of the tumorigenetic propensity and the risk of teratoma formation *in vivo* [11]. To eliminate teratoma formation, some alternative experimental approaches have been employed in several studies. One recent study demonstrated that replacing *c-Myc* with *L-Myc* in combination with *Oct-4, Sox2,* and *Klf4* promoted iPSC generation without tumor formation [26]. Tsuji et al [9] demonstrated that transplantation of neurospheres derived from safe iPSC clones into injured spinal cords promoted a functional recovery without any teratoma formation [9]. In one of our recent studies, we demonstrated that the oncogene *c-Myc* was expected to substantially contribute to tumor formation in acute hepatic failure (AHF) recipients but that the tumor formation could be prevented in the livers of mice that received 3-genes iPSCs and 3-genes iPSC-Heps 6 months after transplantation. Although the exclusion of *c-Myc* resulted in a less efficient induction of MEFs to iPSCs, we have shown that the 3-genes iPSC colonies were positive for Oct-4, SSEA-1 (ESC markers), and alkaline phosphatase, and that they were not affected by passage number; these results are identical to the results obtained from 4-genes iPSC colonies. Furthermore, the microarray gene expression between 3-genes IPSCs, 4-genes iPSCs, and ESCs were similar, and the 3-genes iPSC-derived embryonic bodies could also be differentiated into a neuroectodermal lineage (neuron-like cells), mesodermal lineages (osteocyte-like cells and adipocyte-like cells), and regular-beating cardiomyocyte-like cells. In addition, this mouse 3-genes iPSC (*Oct-4/Sox2/Klf4*) clone formed chimeric offspring through *in vitro* fertilization. This molecular, histological, and differentiation evidence implies a niche for 3-genes iPSCs in future cell therapy and demonstrates that iPSCs without *c-Myc*, especially under differentiated states, is a more feasible cell source for iPSC-based replacement therapy and *in vitro* screening.

In this study, the EMFs were reprogrammed into iPSCs through the cell reprogramming process by the introduction of four transcription factors. The sort of approach that employs the production of induced pluripotency as an intermediate for cell lineage switch is based on the landmark development of Takahashi and Yamanaka. Although several issues have been elucidated, such as the similarity between iPSCs and ESCs and the mechanism underlying the reprogramming of somatic cells to iPSCs, concerns regarding low reprogramming efficiency and the optimal strategy for pre-defined factor delivery remain to be clarified. Furthermore, the approach by transcription factor-based technology to induce cell reprogramming and pluripotency can be widely used in cell therapy, disease modeling, personalized medicine, and regenerative medicine [28]. In addition, several recent studies have shown an direct conversion from one to another type of cell or lineage and bypass pluripotency induction (e.g., from fibroblasts to excitatory neurons [29], cardiomyocytes [30], myeloid cells, and erythroid cells [31]). Therefore, both iPSC technology and direct reprogramming technology have the potential to become two important

therapeutic strategies for the future of regenerative medicine [28], and direct reprogramming of adult cells from one lineage of somatic cells to another combined with non-viral vector delivery should be further studied.

In conclusion, this is the first study to show that EMFs can be reprogrammed to iPSCs and that they are able to differentiate into multiple-lineage cells. In contrast to cell transplantation therapies, the application of stem cells can further provide a platform for drug discovery and small molecular testing. Therefore, EMFs are an excellent source of adult stem cells, and this approach to cell replacement therapy should overcome the ethical and immunologic concerns associated with the use of fetal tissues and ESCs.

Disclosures

None.

Acknowledegments

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