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Human Turbinate-derived Mesenchymal Stem Cells Differentiated into Keratocyte Progenitor Cells

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Abstract

Objective: Though keratoplasty is used to treat corneal blindness, donor shortage, tendency of stimulated keratocyte transformed to fibroblast and immunological rejection are still big problems. As a solution, cornea tissue engineering using non-corneal tissue sourced cells become emerging issue. Thus, this study was designed to find novel material for keratoplasty.

Methods: Human turbinate-derived mesenchymal stem cells (hTMSCs) were obtained from patients and cultured with differentiation medium for 14 days. The keratocyte markers, stem cell markers, early corneal stromal stem cell (CSSC) markers, were measured by real time-PCR. The MSC markers were detected by FACS.

Results: After 14 days of differentiation medium exposure, hTMSCs expressed markers of keratocyte such as keratocan sulfate proteoglycan (KERA) and aldehydrogenase (ALDH). As the hTMSCs became keratocytes, the expression of embryonic ocular precursor markers ABCG2 and PAX6 decreased but were still measurable. Early CSSC markers including SIX2, SIX3, BMI expression was elevated after 7 d and reduced after 14 d of KDM treatment. The stem cell markers such as SOX2, Notch were decreased. After 14 d of differentiation, the hTMSCs expressed MSC markers CD73, CD90, and CD105, but not hematopoietic markers CD14, CD19, CD34, HLA-DR; these changes indicate development of a characteristic MSC phenotype. hTMSCs inhibited the tube-formation ability of human microvessel endothelial cells. hTMSCs derived from neural crest could differentiate into keratocyte progenitors.

Conclusions: This study first reveals that the hTMSCs have potential to be differentiated into keratocyte progenitor-like cells. Use of hTMSCs derived from neural crest in cell-based therapeutics as source for corneal tissue engineering may overcome the problems of keratoplasty such as immunological rejection and limiting supply of human donor corneas.

Introduction

The cornea is a transparent structure that consists of a central stroma that constitutes 90% of the corneal depth, covered anteriorly with epithelium and posteriorly with endothelium [1]. The neural crest derived keratocytes maintain the corneal stroma, which has stem cell traits [2]. Collagens and proteoglycans interact in the cornea stroma, thereby helping to maintain corneal structure and the function of extracellular matrices [3]. Normally, human corneal stroma contains mainly type I and type V collagens which are arranged into hybrid fibrils of regular diameter. The quantities of these collagens increase during several pathological conditions, such as wound healing and inflammation [4,5]. Proteoglycans are characterized by a protein core, covalently linked glycosaminoglycan side chains [6]. The main components of cornea-specific glycosaminoglycans are keratocan sulfate, dermatan sulfate and keratan sulfate, with smaller amounts of heparan sulfate [7,8].

Corneal keratocytes originate from the cranial neural crest; they are not terminally differentiated, but maintain plasticity and multipotentiality, so they can heal corneal damage [9]. Corneal diseases such as stromal opacity are a main cause of blindness, from which more than million people are suffered in the world [10]. Bilateral corneal blindness is mainly caused by trauma, infection or genetic disease [10]. The only effective therapy for corneal opacity is transplantation of corneal allografts. The transplantation of allograft has a high success rate, but still has problems, including shortage of donor corneas, and immunological rejection [11]. And CSSC derived from neural crest spontaneously differentiate into keratocyte *in vivo* or *in vitro* but stromal population of CSSC is less than 1% [12]. Thus, novel strategy for stromal replacement is needed.

Several types of stem cells can differentiate into functional keratocyte and generate extracellular matrix like native stromal tissue. Examples include human mesenchymal stem cells [13], human embryonic stem cells [14], human corneal stroma stem cells [15,16], adipose-derived stem cells [17,18], and dental pulp stem cells [2].

The fact that cornea develops from cranial neural crest suggests that other stem cells that have the same origin could also differentiate into keratocytes. Mesenchymal stem cells (MSCs) are adult mesenchymal progenitor cells that can differentiate into cells of various connective tissue lineages, including bone [19], cartilage [20], adipocyte [21] and keratocyte [11]. MSCs are good materials for cell therapy because of their accessibility, easy isolation, capability of preservation, and failure to induce immunological rejection [22].

hTMSCs are located in the interior of turbinate; they are easy to reach, and have high proliferation competence, relatively high yield and prolonged efficacy [23]. hTMSCs have been used in cell therapy for chondrogenic [23,24], osteogenic [23-26], adipogenic [24], neurogenic[24] and tracheal epithelial differentiation [27]. Characteristics of hTMSCs do not differ significantly between patients under 20 years old and patients in their 40s or 50s [23], but the ability of hTMSCs to differentiate into keratocyte progenitor like cells has never been demonstrated. Thus, this study first reveal the differentiation of hTMSCs into keratocyte progenitor like cells.

Methods

Isolation and culture of hTMSCs

Fresh turbinate mesenchymal stem cells were obtained from patients who had undergone partial turbinectomy at the Catholic University of Korea, St. Mary's Hospital [23,24]. The protocols of this study were approved by the Internal Review Board for Human Subjects Research and Ethics Committee (KC08TISS0341), and informed consent was acquired from each patient before enrollment in this experiment. hTMSC were cultured in normal medium containing high-glucose Dulbecco's Modified Eagle's medium (DMEM high glucose, GenDEPOT) containing 10% fetal bovine serum (FBS), 10,000 U/ml streptomycin/penicillin (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2. Cell seed confluence reach 80~90% after 1 d, the culture medium was replaced with Differentiation Medium containing 10 ng/ml KGF/EGF. Recombinant Human keratocyte growth factor (Peprotech, Rocky Hill, USA), 10 ng/ml EGF (Peprotech, Rocky Hill, USA), 1% Horse serum, 10,000 U/ml streptomycin/penicillin (Sigma-Aldrich, USA). The culture medium was changed every 3 d. At 1 d, 7 d and 14 d, cells were harvested for qPCR and FACS.

Real time-polymerase chain reaction (RT-PCR)

Total RNA was isolated from control hTMSCs and differentiated hTMSCs pellets in six-well plates by using TRI-reagent (Invitrogen, USA). The amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, USA) was used to reverse-transcribe 50 ng total RNA (per 20 µl reaction final volume) into cDNA. The mixture was incubated as follows: 1 min at 60°C, 5 min at 25°C, 30~60 min at (37~55°C), 1 min at 85°C, 5 min at 4°C in a thermal cycler. Qualitative polymerase chain reaction (qPCR) was performed using SYBR Green premix ex Taq (Takara, Otsu, Shiga, Japan). Cornea-specific gene primers (Table 1) were used for KERA, ALDH, PAX6, ABCG2, SIX2, SIX3, BMI1, SOX2, Notch and GAPDH. Samples were initially incubated at 95°C for 10 min, then. PCR was performed for 40 cycles of 15 s at 95°C and 1 min at 60°C. Changes in content were calculated using the standard C^T method with GAPDH as the housekeeping gene. Three individual gene-specific values thus calculated were averaged to obtain mean ± SD.

Tube formation assay

hTMSCs and HMVECs (human microvessel endothelial cells) were seeded with 1×10^5 cells in six-well Matrigel-coated dish (BD). EGM2

Human gene Primer sequence (5'-3')	
KERA	F: GCC TCC AAG ATT ACC AGC CAA
	R: ACG GAG GTA GCG AAG ATG AGG T
ALDH	F: CGC TCC TGA TGC AAG CAT GGA AGC
	R: CTC CCA ACA ACC TCC TCT ATG GCT
BMI1	F: CTC CAC CTC TTC CTG TTT GC
	R: CCA GAT GAA GTT GCT GAC GA
PAX-6	F: AGA TGA GGC TCA AAT GCG AC
	R: GTT GGT AGA CAC TGG TGC TG
ABCG2	F: TGC AAC ATG TAC TGG CGA AGA
	R: TCT TCC ACA GCC CCC AGG
SIX2	F:CTC TCT TCC TTT GCC CTC CT
	R:CCG AGA AAC ACT GAG GGG TA
SIX3	F:ACC ATC AAC AAC TCC CAA CC
	R:AGC GGT GCT TGT CCT AGA AA
Sox 2	F: GGC AGC TAC AGC ATG ATG C
	R: TCG GAC TTG ACC ACC GAA C
Notch	F: GTC GGA CTG GTG AGG ACT G
	R: AGC CCT CGT TAC AGG GGT T

Table1: Primer sequences used for RT-PCR.

(Lonza, Muenchensteinerstrasse, Switzerland) with 1% FBS and 1% P/S and DMEM high glucose was added to hTMSCs and HMVECs. After 1 d, optical images were obtained using a phase contrast microscope (AE 2000 Series, Motic, Wetzlar, Germany).

Proliferation assay (CCK assay)

Cell proliferation was assayed using a Cell Counting Kit-8 (CCK-8, Dojindo, and Kumamoto, Japan). At days 1, 7 and 14 after seeding, the cell culture medium in each well was discarded and 100 µl of fresh medium containing 10 µl CCK-8 was added and the cultures were incubated for 2 h. The absorbance at 450 nm was measured for each well. Cell proliferation was examined 1 d, 7 d, and 14 d after incubation, in three exposures of each sample under the same conditions.

Seahorse analyzer (XF-24) assays

Mitochondria respiration and extracellular acidification rates of adherent cells were measured. hTMSCs were seeded (1×10^4 cell/well) in XF 24-well plates, then assayed using a Seahorse XF-24 analyzer (Seahorse Bioscience, North Billerica, MA, USA) 3 d, 7 d, or 14d later. Normal medium was added to hTMSCs on the first day; the next day, the medium was changed to keratocyte differentiation medium (KDM). Culture medium was replaced every 3 d. hTMSCs (1×10^4 cell/well) were maintained with Differentiation Medium (containing KGF/EGF), and proliferation medium 10% FBS for 14 d; was replaced every 3 d. Cells were preconditioned by washing twice and filling with XF base medium containing 5% glucose (Sigma, USA). Oxygen consumption values were assayed from 3-min measurement cycles using XF Reader software Version 1.4 updated with a recent correction. Acidification rates were measured as the mean rate of the second and third baselines.

FACS

The hematopoietic markers (CD14, CD19, CD34, and HLA-DR) and MSC markers (CD73, CD90, and CD105) of hTMSCs and differentiated hTMSCs were measured using flow cytometry. The

hTMSCs were divided to two groups: the normal group was supplied with normal medium; the keratocyte-like differentiation group was supplied with KDM. After 1, 7, or 14 d, hTMSCs were harvested and placed into a test tube (BD, Franklin Lakes, NJ, USA) at 1×10^5 cells/ml and treated three times with wash buffer (PBS and 3% FBS). The cells were incubated for 40 min with flourishing concentration of primary monoclonal antibodies against CD14, CD19, CD34, CD73, CD90, CD105 and HLA-DR. The cells were washed three times in buffer and centrifuged at 1,200 rpm for 5 min, then re-suspended in ice-cold PBS and incubated with a FITC-or PE-labeled secondary antibody for 30 min in darkness at 40°C. Cell fluorescence was evaluated using fluorescence-activated cell sorting (FACS) with a Caliber instrument (BD): the analysis was done using Cell Quest software (BD).

Cytology and immunostaining

Morphology of the cells was visualized by phase contrast microscopy (AE 2000 Series, Motic, Wetzlar, Germany) at 400 × magnification. To identify differentiation into functional kertocyte and keratocyte progenitor-like cells, expression of keratocan PAX6 were measured using an immunostaining assay. Cells were fixed at room temperature in 4% paraformaldehyde and double-stained with Alexa 488 and DAPI prepared by rehydration in PBS followed by incubation in 0.2% Triton X-100 for 10 min. For PAX6 staining, cells were treated with H_2O_2 to permeate nuclei membranes for nucleus staining. The samples were rinsed with PBS three times for 5 min each, then exposed to permeabilization solution. The cells were preincubated with PBST 1% BSA containing horse serum to block nonspecific staining, then coordinated labeling with anti-Keratocan (Santa Cruz, sc-33244, Texas, USA) labeled with donkey anti-goat IgG-FITC (Santa Cruz, sc-2024, Texas, USA). Anti-PAX6 (Abcam, ab5790, Cambridge, England) were observed, counterstained with DAPI and Alexa Fluore 488-conjugated secondary antibody for 60 min, and then observed under a fluorescence microscope (LMS 510 Meta, Zeiss, Germany).

Statistical analysis

All the experiments were independently repeated at least three times. Data are presented as mean ± standard error of mean (SEM). The statistical analyzes were performed using Student's t-test or a one-way repeated-measure Analysis of Variation test. To compare multiple data groups, post-hoc test was used. Values of $p<0.05$ were considered statically significant.

Results

hTMSCs Differentiated into keratocyte progenitor cells

To test whether the hTMSCs could differentiate into keratocyte or keratocyte progenitor like cells, qRT-PCR was conducted for keratocyte markers (KERA and ALDH) and CSSC-specific embryonic ocular precursor markers PAX6 and ABCG2.

The expressions of KERA (Figure 1A) and ALDH (Figure 1B) was weekly detected until 7 days of differentiation and was increased more than 10,000 times in KDM treated group. The similar tendency was observed in human embryonic cells and corneal stromal stem [11,28]. The expressions of embryonic ocular precursor markers, PAX6 (Figure
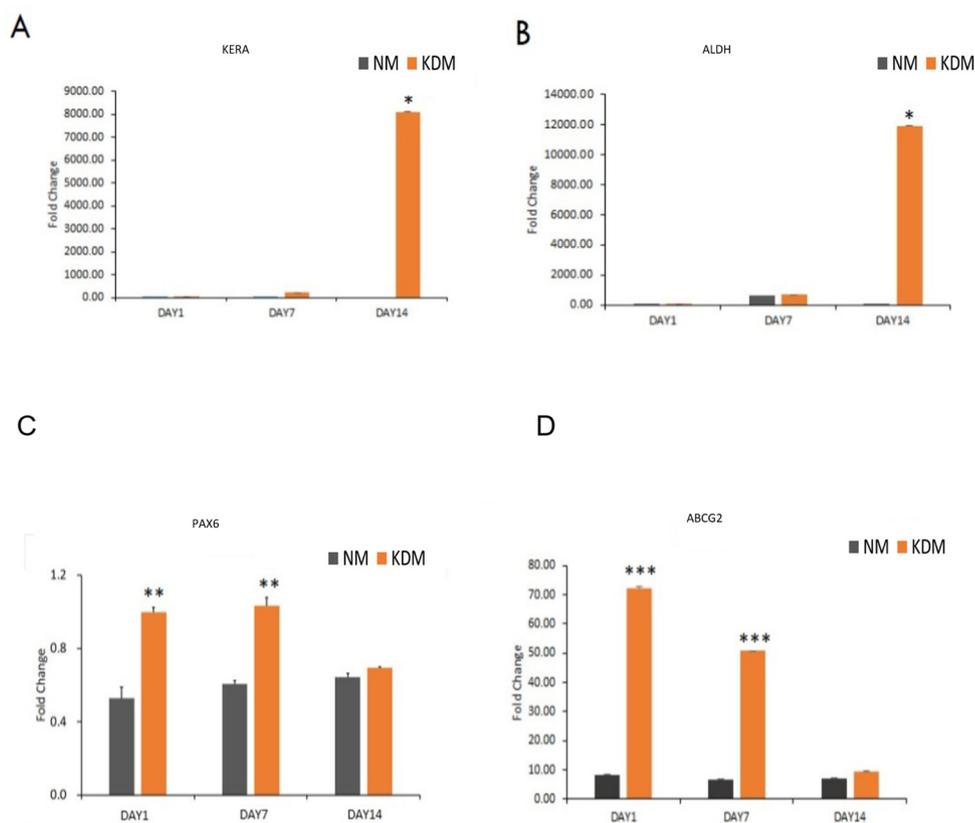
Figure 1: Expressions of keratocyte markers and keratocyte progenitor markers of hTMSCs in KDM. hTMSCs were seeded (1×10^5 cell/well) in 6-well culture dishes in triplicate. The next day and every 3 d, NM was replaced with fresh KDM and the cells were incubated for 7 or 14 d. KDM: Keratocyte Differentiation Medium; NM: Medium containing 10% FBS. Cells were harvested and prepared for qRT-PCR with primers of KERA **(A)**, ALDH **(B)**, PAX-6 **(C)**, and ABCG2 **(D)**.

1C), ABCG-2 (Figure 1D), which are not detected in adult stem cell or fibroblast, decreased but were still detectable after 14 d in KDM. SIX2, SIX3 and BMI1 were reported to be an early corneal markers [12,29-31]. Thus, the expression of SIX2, SIX3, BMI1 were measured (Figures 2A-2C). The results showed that the expression of early corneal markers were increased after 7 days and decreased after 14 days of KDM treatment. Embryonic stem cell markers SOX2 (Figure 2D) and Notch (Figure 2E) were also measured. The expression of SOX2 and Notch were significantly decreased over time after treatment of KDM containing KGF/EGF.

hTMSCs possessed multipotency after differentiation

To identify the multipotent ability of hTMSCs, cell surface markers were assessed by FACS analysis. hTMSCs were negative for hematopoietic markers CD14, CD19, CD34 and HLA-DR, and positive for MSC markers CD73, CD90 and CD105. This result indicates that 14 d of differentiation does not affect multipotent ability of hTMSCs (Figure 3) [23].

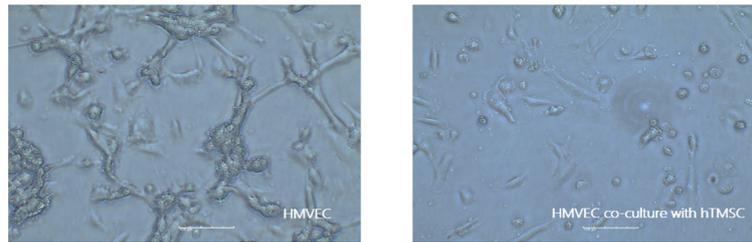### hTMSCs inhibited tube formation of HMVECs

The major factor of immune rejection of keratoplasty includes neovascularization in corneal stroma [32]. To examine the anti-neovascularization effect of hTMSC, tube formation assay was conducted. *In vitro* neovascularization related experiment is recommended by tube formation. As shown in Figure 4, hTMSCs inhibited tube formation morphology of HMVECs.
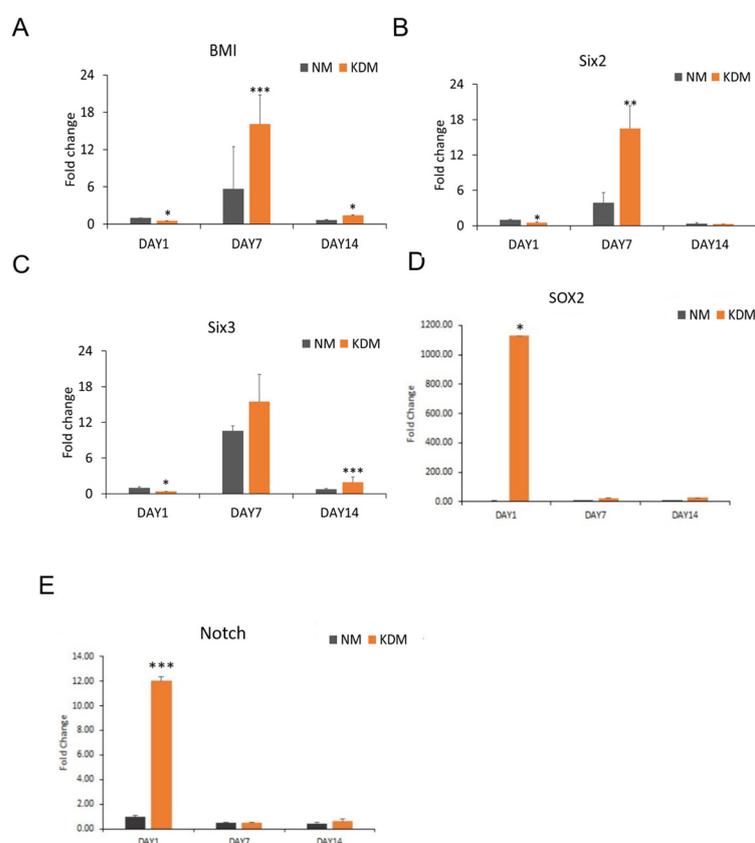

Figure 2: Expressions of early corneal stromal stem cell markers and stem cell markers of hTMSCs in KDM. hTMSCs were seeded (1 × 10⁵ cell/well) onto 6-well culture dishes in triplicate. The next day and every 3 d, medium was replaced with fresh KDM and the cells were incubated for 7 or 14 d. KDM: Keratocyte Differentiation Medium; NM: Medium containing 10% FBS. Cells were harvested and prepared for qRT-PCR with primers of BMI1 **(A)**, SIX2 **(B)**, SIX3 **(C)**, SOX2 **(D)** and Notch **(E)**.

	CD14	CD19	CD34	CD73	CD90	CD105	HLA-DR
1NM	0.4 %	0.4%	0.5%	100%	100%	99.6%	0.3%
7NM	1.1%	0.8%	2.2%	100%	100%	99.9%	0.8%
14NM	0.1%	0.1%	0.1%	99.7%	100%	98.4%	0.1%
1KDM	0.2%	0.1%	0.1%	100%	100%	98%	0.1%
7KDM	1.1%	0.9%	1.1%	99.9%	99.9%	98.8%	0.9%
14KDM	0.5%	0.5%	0.15%	99%	99.9%	82.9%	0.2%

Figure 3: Expressions of hematopoietic markers and MSC markers of hTMSCs in KDM. Cells were seeded (1 × 10⁵ cell/ml) in 6-well culture dishes. NM and KDM were added to the cultures for 1, 7, or 14 d. Cells were incubated with primary antibodies to CD14, CD19, CD34, CD73, CD90, CD105 and HLA-DR for 40 min and with FITC or PE–labeled secondary antibodies for 30 min in darkness. Cells were assayed using fluorescence-activated cell sorting.

Figure 4: Inhibitory anti-neovascularization effect of hTMSCs on tube formation of HMVECs. The hTMSCs and HMVECs were mixed and seeded medium in Matrigel coated culture dish at a concentration of 1×10^5 cells/well. Optical images were obtained next day.
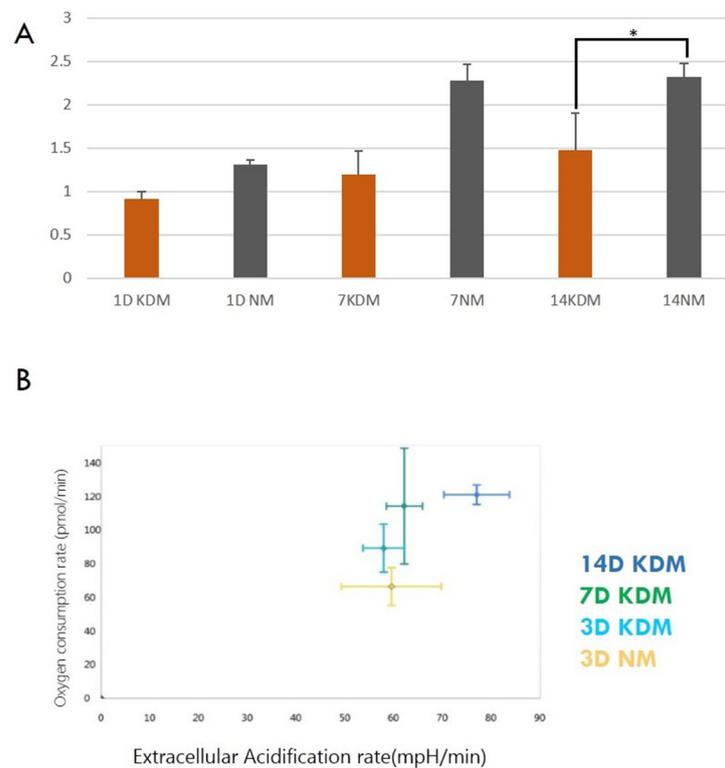
Figure 5: Changes of proliferation and energy metabolism of differentiated hTMSCs. **(A)** hTMSCs were seeded (1×10^4 cell/well) in 96-well culture dish, then incubated with NM or KDM for the indicated times, then CCK assay was used to measure cell proliferation. **(B)** Cells were seeded (5×10^4 cells/24 well) on an XF plate, then subjected to XF assay.

The proliferation of differentiated hTMSCs was suppressed by differentiation

To determine whether the proliferation competence of differentiated hTMSCs is affected by differentiation, a CCK assay was performed. After 14 ds of differentiation, the proliferation ability was suppressed compared to the group in the normal medium (Figure 5A). To verify differentiation at the intracellular energy metabolism level, an XF assay was performed. The hTMSCs became increasingly energetic over time (Figure 5B).
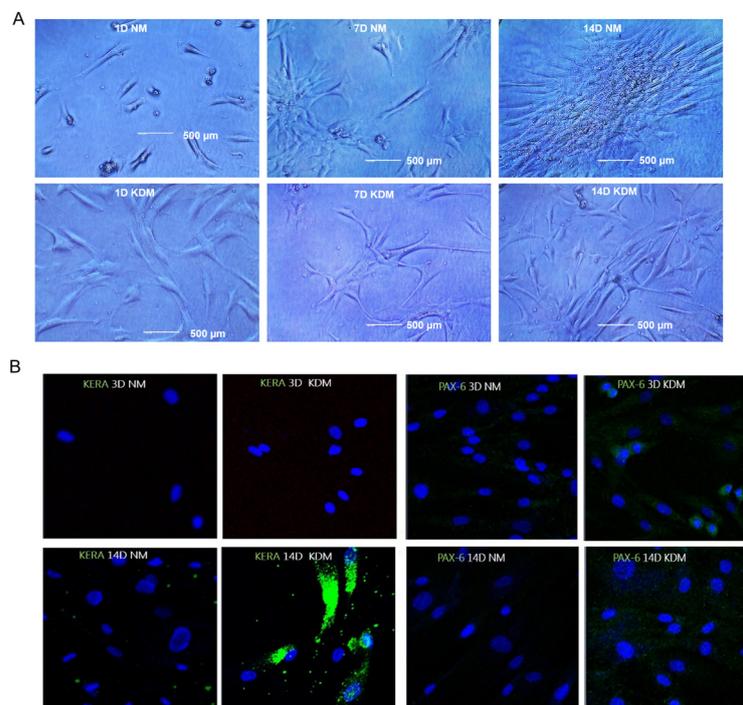
Morphological changes of differentiated hTMSCs

To confirm the change of hTMSCs morphology after differentiation, phase contrast microscopy was used. Morphology of hTMSCs supplied with normal medium is characterized by typical self-renewal behavior such as clonal growth that is analogous to aggregate formation caused mainly by expansion that close associated with CSSC phenotype.

However, hTMSCs in KDM achieved the keratocyte-like dendritic within 14 d (Figure 6A). To identify the expression of Keratocan and translocation of PAX6, immunostaining was performed. After differentiation, the expression of Keratocan was significantly increased in cytosol of hTMSCs (Figure 6B). PAX6 was expressed in the nucleus and cytosol in hTMSCs that had been exposed to KDM for 3 d. After 14 d of KDM treatment, PAX6 expression decreased (Figure 6C). Figure 7 shows brief schematic diagram of differentiation process of hTMSC into keratocyte progenitor/ CSSC like cell.

Discussion

MSCs are widely used in cell-based therapy due to their facile isolation and multipotent capacity including proliferation compared to other adult stem cell [23,33]. However, cell-based therapy for keratocyte has severe limitations because keratocytes easily transform to fibroblast that secrete metalloproteinase that induces extracellular remodeling

Figure 6: Morphological change and expression of KERA and PAX-6 of differentiated hTMSCs. **(A)** The hTMSCs were treated with KDM for indicated days and the morphology of the cells were visualized with phase contrast microscopy (400×). Scale bar: 500 µm **(B, C)** Cells were seed 2×10^5 cells/well in 6 well culture dish. Primary antibodies of KERA (green) and PAX6 (green) was detected after 3, 7 and 14 days of KDM treatment. Cells were incubated with primary antibodies over night at 4℃ and then secondary antibodies. Cells were counter stained with DAPI (blue) for 60 min. Scale bar: 100 µm.
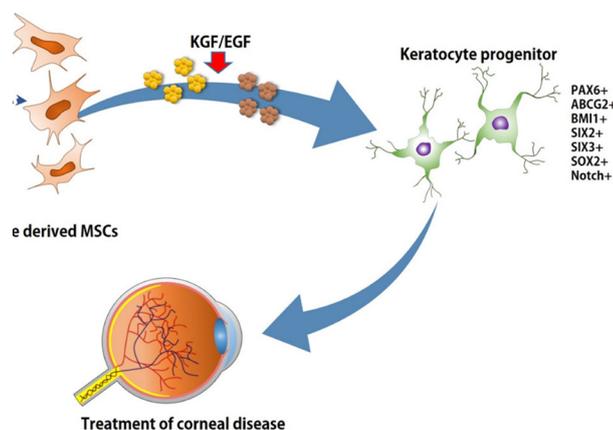
Figure 7: Schematic diagram of differentiation of hTMSC into keratocyte progenitor-like cells.

in vivo and *in vitro*. Recent observations demonstrate that keratocyte progenitor cells derived from neural crest can differentiate into keratocytes without changing to fibroblasts [12,34-36]. Progenitor cells derived from neural crest migrate precisely to the targeted tissue origin pathway. Various MSCs derived from cranial neural crest are involved in corneal stromal stem cells, dental pulp, periodontal ligament, skin and hair follicles, but these MSCs have the disadvantages that their detachment requires difficult surgery, and that they have restricted life span [31]. In contrast, surgery to obtain hTMSC is minimally invasive and causes minimal physical pain to the patient; it also provides the possibility of autologous therapy [23,37]. The present paper presents the possibility of hTMSCs to differentiate into keratocyte progenitor

cells after 7D of KDM treatment. hTMSCs are candidates for cell based therapy for corneal regeneration, without causing ethical conflicts that embryonic stem research causes [23]. hTMSC have excellent proliferation ability and higher yield at the time of tissue culturing than do bone marrow-derived MSCs (5 times) and adipose-derived MSCs (30 times) [23]. Additionally hTMSCs can differentiate into neurogenic, chondrogenic, osteogenic and adipogenic cells due to multipotency, so hTMSCs also might differentiate into CSSC from the same origin, neural crest [23,24]. This study is the first demonstration that MSCs derived from non-corneal tissue can differentiate into keratocyte progenitor as well as CSSC. After 14 days of differentiation, the expressions of keratocyte markers KERA and ALDH in hTMSCs were remarkably

increased, indicating that hTMSCs differentiated into keratocytes. Also, BMI, SIX2, SIX3 were upregulated after 7 days of differentiation, which are CSSC-specific embryonic ocular markers and DNA-binding transcription factors, essential factors for the development of eye [38]. The critical expressions of keratocytes progenitor markers, ABCG2 and PAX6 reduced but still detectable after 14 days of KDM treatement because of differentiation of hTMSCs. SOX2 is an embryonic stem cell marker and the Notch pathway has an important function in stem-derived neural/neural crest cell biology [39]. The suppressed expression of embroyonic stem cell markers, SOX2 and Notch, indicating that KDM treated hTMSC differentiated into keratocyte progenitor cells.

We hypothesized that the hTMSCs differentiate into keratocyte progenitor cells, which have multipotent ability, similar to CSSC character [40]. To identify the multipotent ability of hTMSCs that had been treated with KDM, hematopoietic and MSC markers were measured. During the 14 d of experiments, hTMSCs produced MSC markers CD73, CD90 and CD105, but not hematopoietic markers CD14, CD19, CD34 and HLA-DR; these results indicate that 14 d of differentiation did not affect the multipotent ability of hTMSCs. During differentiation, the proliferative potential of stem cells is suppressed [41]. After 14 d of differentiation, the proliferation ability was suppressed compared to normal medium group. To determine whether this decreased proliferation is related to differentiation, an XF assay was performed. After differentiation, hTMSCs tended to become more energetic state by using oxidative phosphorylation to produce ATP by mitochondrial respiration rather than by glycolysis; this change increased the extracellular acidification rate. Keratocytes are typical elongated and spindle-shaped [42]. The hTMSCs showed this morphology after KDM treatment; this observation suggests that hTMSCs were well-differentiated to keratocytes. Also, 14 d after KDM exposure, expression of PAX6 was decreased.

The purpose of this study was to investigate whether hTMSCs can differentiate into keratocyte progenitor cells including remaining multipotency of CSSC; the ultimate goal is to resolve the current limitations of corneal xenotransplantation. Further animal studies are needed to evaluate the feasibility of using keratocyte progenitor cells in corneal tissue engineering.

Acknowledgements

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