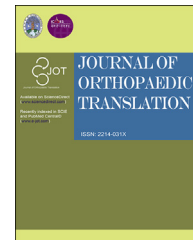




Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

ScienceDirect

journal homepage: <http://ees.elsevier.com/jot>



ORIGINAL ARTICLE

# A novel protocol for isolation and culture of multipotent progenitor cells from human urine

Weiping Lin <sup>a,b</sup>, Liangliang Xu <sup>c</sup>, Gang Li <sup>a,b,d,\*</sup>

<sup>a</sup> Department of Orthopaedics & Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, PR China

<sup>b</sup> Stem Cells and Regenerative Medicine Laboratory, Lui Che Woo Institute of Innovative Medicine, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, PR China

<sup>c</sup> Key Laboratory of Orthopaedics & Traumatology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, The First Clinical Medical College, Guangzhou University of Chinese Medicine, Guangzhou, PR China

<sup>d</sup> The CUHK-ACC Space Medicine Centre on Health Maintenance of Musculoskeletal System, The Chinese University of Hong Kong Shenzhen Research Institute, Shenzhen, PR China

Received 14 November 2018; received in revised form 10 February 2019; accepted 22 February 2019  
Available online 16 March 2019

## KEYWORDS

Cell therapy;  
Cloning cylinder;  
Differentiation;  
Progenitor cells;  
Protocol;  
Urine

**Summary** Cell therapy holds promise for treating a variety of diseases. Seeking available source of adult stem cells remains a great challenge in cell therapy. Urine is considered as an ideal source of adult stem cells which can be easily acquired by noninvasive methods. However, specific cell types in urine have not been well documented. Here, the aim of our study is to identify cell types in urine, and isolate and expand progenitor/stem cells from human urine and further evaluate their multipotency.

Urine samples were collected from healthy donors. The cell suspension was seeded and selected because of plastic adherence. Colonies with two different morphologies appeared 7 days later. One type of colony was spindle-shaped and fibroblast-like; the other cell type displayed rounder shape. Cells that displayed fibroblast-like shape were selectively enriched using a cloning cylinder. Then multidifferentiation induction assays and immunophenotyping assays were applied. Characterization assays indicated that adherent cells possessed potent trilineage differentiation capacity and expressed CXCR4 and Nanog, as well as some mesenchymal stem cell surface antigens (including CD90 and CD44). Taken together, at least two cell populations exist in human urine. A stem cell subpopulation with trilineage differentiation capacity from human urine can be selectively enriched using the cloning cylinder method.

\* Corresponding author. Department of Orthopaedics and Traumatology and Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR, PR China.  
E-mail address: [gangli@cuhk.edu.hk](mailto:gangli@cuhk.edu.hk) (G. Li).

Urine may become an ideal source of adult stem cells for cell therapy and further clinical implications.

© 2019 The Authors. Published by Elsevier (Singapore) Pte Ltd on behalf of Chinese Speaking Orthopaedic Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Cell therapy provides a novel approach for curing a series of diseases [1,2]. Cell therapy aims to restore injured tissues or organs by replacing lost or dysfunctional cells with functional cells to reestablish their normal functions [3]. Mesenchymal stem cells (MSCs) are multipotent stromal cells which are capable of self-renewal and differentiation into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [4]. Various populations of multipotent postnatal MSCs have been demonstrated to express pluripotency-associated markers, such as Nanog, which are essential for the maintenance of self-renewal and differentiation in MSCs [2].

Generally, functional cells for therapy are harvested from donor-derived somatic cells or stem cells. Usually, the collection process of donor-derived adult stem cells, such as adipose-derived stem cells or bone marrow-derived stem cells, requires needle insertion, or biopsy, which is highly invasive. Thus, it is necessary to seek an available source of adult stem cells. Strikingly, urine, which can be easily acquired noninvasively, has been considered as an ideal novel source of adult stem cells for personalized regenerative therapies [5–10]. Previous studies have indicated that urine-derived stem cells (USCs) possess characteristics of stem cells, including the capacity of plastic adherence, clonogenicity and multidifferentiation potentials [11].

However, few manuscripts have been published to describe the specific cell types in human urine. In addition, standard culture protocols of USCs have not been well documented. Here, we reported that at least two cell subpopulations in terms of different cell morphologies are present in human urine, including fibroblast-like cells and epithelial-like cells. Furthermore, characterization assays indicated that collected CXCR4- and Nanog-positive cells possessed multidifferentiation potential after selective expansion of fibroblast-like cell colonies using the cloning cylinder method.

## Materials and methods

### Collection of human urine samples

Urine samples were collected from healthy adult donors (age ranges from 25 to 40 years,  $n = 5$ ). The collection process was approved by the Clinical Research and Ethics Committee of the Chinese University of Hong Kong or the Guangzhou University of Chinese Medicine. Experimental procedures for urine samples and urine-derived cells were carried out according to the approved guidelines. All cells used in this study were harvested with the

informed consent of the donors for use in scientific research.

### Human urine-derived stem cell culturing

Urine samples (300 mL from each donor) were centrifuged at 300 g for 10 min and then washed with phosphate buffered saline (PBS). The cells collected were resuspended in complete culture media containing  $\alpha$ -minimum essential medium ( $\alpha$ -MEM; Gibco/Invitrogen, Thermo Fisher Scientific, USA) supplemented with 10% foetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin/neomycin. The cell suspension was seeded in 6-well plates at a density of  $0.3 \times 10^5$  cells/cm<sup>2</sup> and then incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The culture medium was refreshed every 3 days. Once the primary cultured cells reached a confluence of about 60%, cells of spindle shape (USCs) were selectively passaged using a cloning cylinder (Corning, USA).

### In vitro multilineage differentiation assays

#### Adipogenic differentiation

Human urine-derived cells were trypsinized and seeded in a 6-well plate with growth media at a concentration of  $1 \times 10^5$  cells per well. The cells were incubated in the  $\alpha$ -MEM complete medium until a confluence of 90% was reached. The medium was then replaced by an adipogenic induction medium containing 10% foetal bovine serum, 1  $\mu$ M dexamethasone, 10  $\mu$ g/mL insulin, 50  $\mu$ M indomethacin and 0.5 mM isobutylmethylxanthine. Adipogenic induction lasted for 2 weeks, and the medium was changed every 3 days. The cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) for 30 min. Oil Red O staining was applied, and the results were observed under a microscope.

#### Osteogenic differentiation

The cells were seeded and cultured until at a confluence of about 80% was reached. The medium was replaced by an osteogenic induction medium containing 100 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate. The cells exposed to complete culture media served as control groups. After 2 weeks of induction, Alizarin Red staining was applied to assess mineralization. The cell/matrix layer was stained with 0.5% Alizarin Red S (pH 4.1) for 5 min. The positive stain was viewed under a microscope.

#### Chondrogenic differentiation

Aliquots containing  $3 \times 10^5$  cells were centrifuged in polypropylene tubes to form a pelleted micromass. The pelleted cells were incubated in a chemically defined chondrogenic induction medium consisting of  $\alpha$ -MEM

supplemented with 10 ng/mL recombinant human transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), 100 mM dexamethasone, 1 mM sodium pyruvate, 0.2 mM ascorbic acid 2-phosphate (Sigma-Aldrich, USA) and ITS + Premix (BD Biosciences, San Jose, CA, USA). The cells cultured in a based medium of  $\alpha$ -MEM served as a negative control. The medium was changed every 3 days. The induction process lasted 3 weeks. Safranin O—fast green staining was applied, and the staining results were viewed under a phase-contrast microscope (Leica Microsystems Wetzlar Gesellschaft mit beschränkter Haftung, Wetzlar, Germany).

### Immunocytochemistry

The cells were seeded onto slides in a 12-well plate (3000 cells per well). The cells were incubated in  $\alpha$ -MEM complete culture media for 24 h. The cells were fixed with 4% paraformaldehyde for 30 min after washing with PBS. Then, the cells were blocked with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, USA) for 30 min at 37 °C followed by permeabilisation with 0.5% Triton X-100 (Sigma-Aldrich, USA). Anti-CXCR4 (1:300; R&D Systems, USA) and anti-Nanog (1:300; BD Biosciences, USA) antibodies were separately added and incubated at 4 °C overnight. Alexa Fluor 488-conjugated secondary antibodies (Life Technologies, USA) were incubated with the cells at 37 °C for 40 min. The cells were sealed using glycerin containing DAPI (4, 6-diamidino-2-phenylindole; Life Technologies, USA) after washing with PBS and then observed under a confocal microscope.

### Flow cytometry

The urine-derived cells were enzymatically digested with 0.25% trypsin—EDTA (Gibco, USA) and then resuspended in resuspension buffer containing 1% BSA (Sigma, USA) at  $10^6$  cells/mL. A volume of 300  $\mu$ L cells was transferred into a 5 mL flow cytometry tube. The cell aliquots were incubated with phycoerythrin (PE)-conjugated CD90, CD44, CD29 and CD73 or fluorescein isothiocyanate (FITC)-conjugated CD45 and CD34 or each corresponding isotype control antibody (BD Biosciences, USA) for 30 min at room temperature in the dark. The cells were resuspended in 1000  $\mu$ L resuspension buffer after washing with PBS. Then, the cell suspension was analysed by flow cytometric analysis using Cell Quest software. BD FACS Canto II was used for data acquisition by adjusting voltage and compensation using appropriate excitation and detection channels. Data analysis was performed using FlowJo software (FlowJo, USA).

### Statistical analysis

At least three sets of independent experiments were performed for each assay. All quantitative data were transferred to statistical spreadsheets and analysed by a commercially available statistical program SPSS version 16.0 (IBM SPSS Inc., Chicago, IL, USA). The student t test was used for comparisons of mean values between two groups with  $p < 0.05$  considered statistically significant. All data are presented as mean  $\pm$  standard deviation.

## Results

### Cell morphology observation

Adherent cells and colonies with two morphologies were observed 7 days after seeding (Fig. 1A). One exhibited spindle shape and fibroblast-like morphology; the other displayed rounder shape. Thus, urine may contain at least two cell populations, including MSC-like cells (Type A) and epithelial-like cells (Type B). Then, the spindle-shaped cells were selectively passaged and expanded using the cloning cylinder. The cells remained spindle-shaped and became homogeneous in morphology after passaging (Fig. 1B). However, the rounder-shaped cells soon disappeared after a few weeks of culture because epithelial-like cells proliferate much slowly in  $\alpha$ -MEM media, and their expansion requires epithelial cell—specific media [12,13].

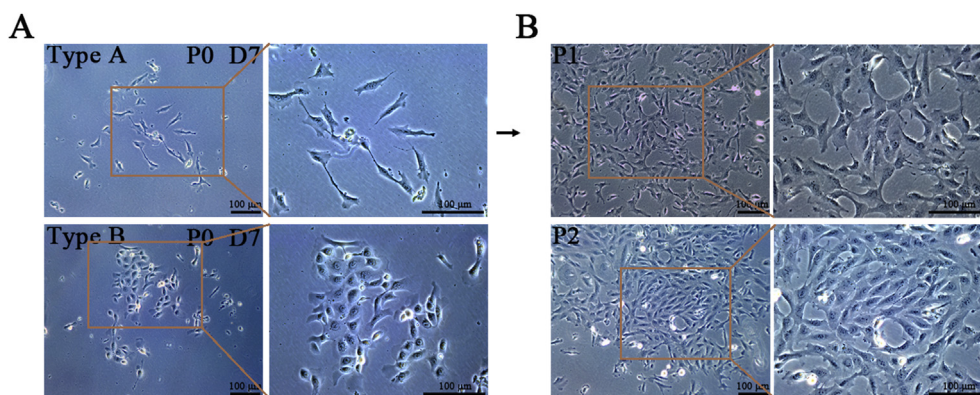
### Multidifferentiation induction and immunophenotyping assays

To determine the phenotype of the urine-derived cells cultured, immunocytochemistry was applied. The immunostaining results showed that spindle-shaped cells isolated from human urine were positive for CXCR4 and Nanog (Fig. 2). Simultaneously, the cells were exposed to various types of differentiation induction media. The results of trilineage differentiation induction assays (Fig. 4A—C) indicated that human urine—derived spindle-shaped cells were capable of osteogenic, adipogenic and chondrogenic differentiation under appropriate induction conditions. In addition, the results of real-time quantitative reverse transcription PCR (qRT-PCR) (Fig. 4D) further confirmed the increased expression of chondrogenic-associated markers (aggrecan, sox9 and collagen 2a) of the induction group compared with the noninduction group. In addition, flow cytometric analysis showed that USCs were positive for CD90 and CD44 (more than 85% expression rate), but lacked the expression of haematopoietic markers (CD45 and CD34), CD29 and CD73 (Fig. 3). Thus, in this study, USCs possessed trilineage differentiation capacity and expressed several MSC-specific markers. A CD90<sup>+</sup>/CD44<sup>+</sup>/CD29<sup>-</sup>/CD73<sup>-</sup>/CD45<sup>-</sup>/CD34<sup>-</sup> USC subpopulation could be selectively enriched through this protocol.

## Discussion

Urine, which could be collected noninvasively, seems to be an attractive available cell source for cell therapy. However, specific cell types in urine and an optimized culture protocol of urine-derived progenitor cells have not been well documented. To this end, this study was undertaken to study cell types in urine and develop a simplified and optimized culture protocol for urine-derived progenitor cells.

Consistent with previous studies, urine-derived cells express pluripotency markers at considerable levels, which may reduce the barrier to the generation of completely reprogrammed pluripotent cells. Therefore, urine has been considered a preferred source for generating induced pluripotent stem cells [14—16]. Furthermore, urinary



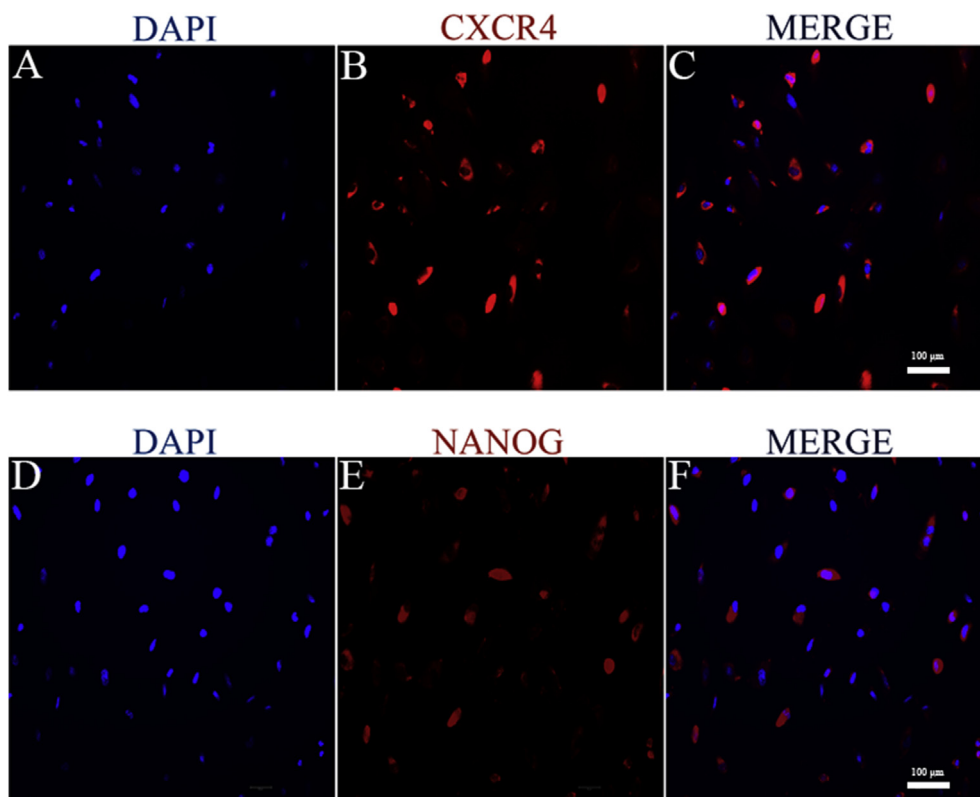
**Figure 1** Culture and morphology observation of human urine-derived samples. (A) Adherent cells with two different shapes appeared 7 days after seeding. One type displayed spindle shape, and the other exhibited epithelial-like cells. (B) Colonies of spindle-shaped cells were selectively expanded using the cloning cylinder method. The cells became homogeneous in morphology and remained spindle-shaped after passaging. Scale bar: 100  $\mu\text{m}$ .

induced pluripotent stem cells from urine samples of patients can also be used for disease modelling to study pathologic mechanisms and drug screening for rare genetic diseases [17–20].

In addition, USCs collected from human urine share the expression of some traditional MSC-surface markers, such as CD90 and CD44. On the other hand, however, there are some differences between the generally defined MSCs from the bone marrow and USCs in immunophenotype. In addition, in this study, the selectively enriched cells isolated from

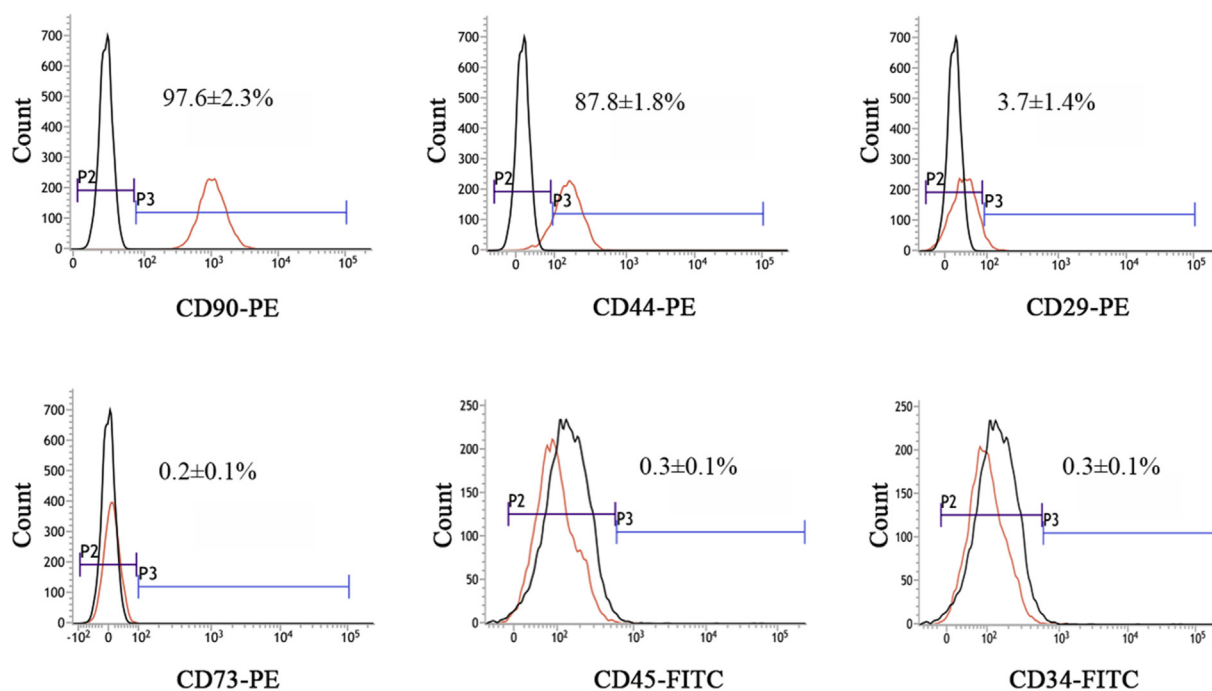
human urine were negative for CD73, a traditional MSC-specific surface antigen, which is not consistent with a previous study [5]. The difference in immunophenotype between USCs and generally defined MSCs or different USC subpopulations may be attributed to cell source-specific features between different tissue-derived adult stem cells and cell heterogeneity of USCs [21,22].

To conclude, our results indicate that MSC-like cell population exists in human urine and can be expanded rapidly in culture using this protocol. One of the

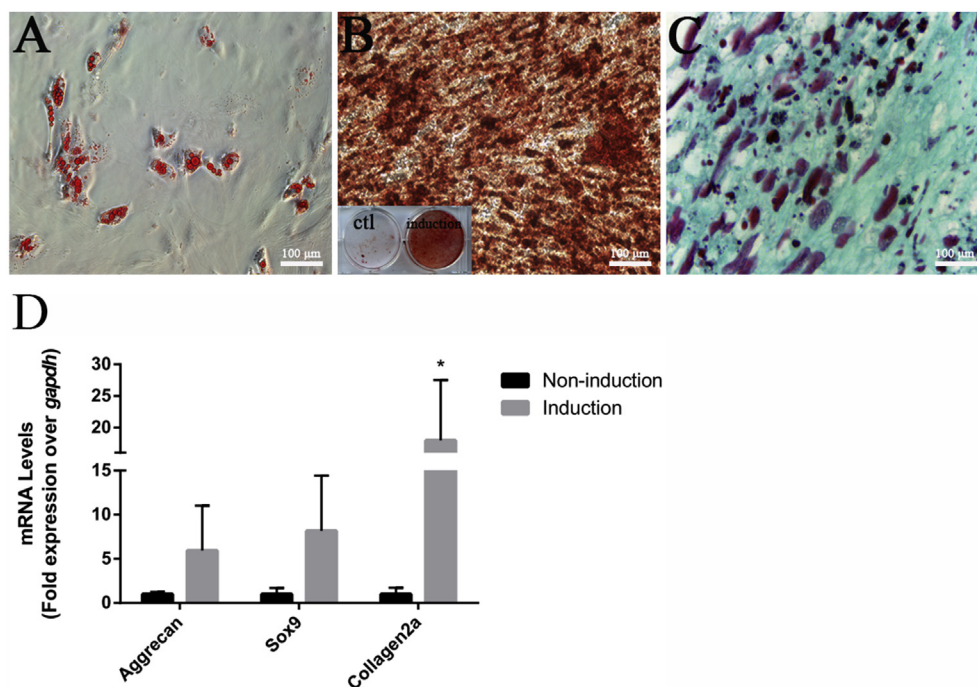


**Figure 2** Immunofluorescence staining evaluation of CXCR4 and Nanog. Fluorescence microscopy indicates CXCR4 (B)- and Nanog (E)-positive human urine-derived stem cells (USCs). (A and D) Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole; blue). (C) Merged images of CXCR4 and DAPI. (F) Merged images of Nanog and DAPI. Scale bar: 100  $\mu\text{m}$ .





**Figure 3** Immunophenotypic characterization of human urine-derived cells (USCs). The results of flow cytometric analysis showed that USCs of Passage 3 were positive for CD90 and CD44 (more than 85% expression rate). However, only 3.7% USCs were positive for CD29, and the cells cultured were negative for CD34, CD45 and CD73 (less than 1% expression rate). The expression of every surface antigen is shown together with their corresponding isotype control. PE = phycoerythrin; FITC = fluorescein isothiocyanate.



**Figure 4** Characterization by multidifferentiation induction assays. Urine-derived spindle-shaped cells were induced to differentiate into adipocytes (A), osteoblasts (B) and chondrocytes (C). (A) Adipogenesis was indicated by the presence of neutral lipid droplets that stained with Oil Red O staining. (B) Osteogenesis potential was identified with positive Alizarin Red staining. (C) Chondrogenic differentiation potential was indicated by positive safranin O-fast green staining. Scale bar: 100  $\mu$ m. (D) Chondrogenic-associated markers (aggrecan, sox9 and collagen 2a) were detected and compared through real-time quantitative reverse transcription PCR (qRT-PCR) between chondrogenic induction and negative control groups after 2-week induction.  $n = 3$ ; \*;  $p < 0.05$ .

urine-derived cell types display a fibroblast-like morphology, express some mesenchymal surface markers and embryonic markers and have the potential to multi-differentiate into osteoblasts, adipocytes and chondrocytes under appropriate induction conditions. This study provides an alternative protocol for selective expansion of a USC subpopulation. Urine-derived progenitor cells may serve as a cell bank for tissue engineering and cell therapy.

## Conflicts of interest

No competing financial interests exist.

## Acknowledgements

The work was partially supported by grants from the Hong Kong Government Research Grant Council, General Research Fund (14119115, 14160917, 9054014 N\_CityU102/15 and T13-402/17-N); National Natural Science Foundation of China (81430049 and 81772322) and Hong Kong Innovation Technology Commission Funds (ITS/UIM-305). This study was also supported in part by SMART program, Lui Che Woo Institute of Innovative Medicine, The Chinese University of Hong Kong and the research was made possible by resources donated by Lui Che Woo Foundation Limited.

## References

- [1] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci USA* 2002 Jun 25;99(13):8932–7.
- [2] Tsai CC, Su PF, Huang YF, Yew TL, Hung SC. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell* 2012 Jul 27;47(2):169–82.
- [3] Orlando G, Wood KJ, De Coppi P, Baptista PM, Binder KW, Bitar KN, et al. Regenerative medicine as applied to general surgery. *Ann Surg* 2012 May;255(5):867.
- [4] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999 Apr 2;284(5411):143–7.
- [5] Bharadwaj S, Liu G, Shi Y, Markert C, Andersson KE, Atala A, et al. Characterization of urine-derived stem cells obtained from upper urinary tract for use in cell-based urological tissue engineering. *Tissue Eng* 2011 Jul 20;17(15–16):2123–32.
- [6] Manaph NP, Al-Hawaas M, Bobrovska L, Coates PT, Zhou XF. Urine-derived cells for human cell therapy. *Stem Cell Res Ther* 2018 Dec;9(1):189.
- [7] Chen L, Li L, Xing F, Peng J, Peng K, Wang Y, et al. Human urine-derived stem cells: potential for cell-based therapy of cartilage defects. *Stem Cell Int* 2018;4686259.
- [8] Bharadwaj S, Wu S, Smith J, Atala A, Zhang Y, Winston-Salem NC. Skeletal muscle derived from human urine-derived cells: a potential source for injection therapy for the treatment of stress urinary incontinence. *J Urol* 2010 Apr 1;183(4):E681–2.
- [9] Wu S, Liu Y, Bharadwaj S, Atala A, Zhang Y. Human urine-derived stem cells seeded in a modified 3D porous small intestinal submucosa scaffold for urethral tissue engineering. *Biomaterials* 2011 Feb 1;32(5):1317–26.
- [10] Wu S, Liu G, Bharadwaj S, Hoagie S, Atala A, Zhang Y. 181 Cell therapy with autologous urine-derived stem cells for vesicoureteral reflux. *J Urol* 2011;4(185):e75.
- [11] Bodin A, Bharadwaj S, Wu S, Gatenholm P, Atala A, Zhang Y. Tissue-engineered conduit using urine-derived stem cells seeded bacterial cellulose polymer in urinary reconstruction and diversion. *Biomaterials* 2010 Dec 1;31(34):8889–901.
- [12] Kmit AH, Moreira J, Vinagre A, Bertuzzo CS, Ribeiro AF, Amaral MD. Isolation, production and culture of primary epithelial cells from patient's airways for the testing of new drugs to cystic fibrosis treatment: 001. *Pediatr Pulmonol* 2016 Apr 13;51:S7.
- [13] Kanoza RJ, Brunette DM, Sodek J. Isolation of epithelial-like cells from cultures of periodontal-ligament. *J Dent Res* 1977;56: A98–A98.
- [14] Zhou T, Benda C, Duzinger S, Huang Y, Li X, Li Y, et al. Generation of induced pluripotent stem cells from urine. *J Am Soc Nephrol* 2011 Jul 1;22(7):1221–8.
- [15] Zhou T, Benda C, Duzinger S, Huang Y, Ho JC, Yang J, et al. Generation of human induced pluripotent stem cells from urine samples. *Nat Protoc* 2012 Dec;7(12):2080.
- [16] Steichen C, Si-Tayeb K, Wulkan F, Crestani T, Rosas G, Dariolli R, et al. Human induced pluripotent stem (hiPS) cells from urine samples: a non-integrative and feeder-free reprogramming strategy. *Curr Protoc Hum Genet* 2017;92(21):21.7.1–21.7.22.
- [17] Ji X, Wang M, Chen F, Zhou J. Urine-derived stem cells: the present and the future. *Stem Cell Int* 2017;4378947.
- [18] Lee YM, Zampieri BL, Scott-McKean JJ, Johnson MW, Costa AC. Generation of integration-free induced pluripotent stem cells from urine-derived cells isolated from individuals with down syndrome. *Stem Cell Transl Med* 2017 Jun;6(6):1465–76.
- [19] Zhang SZ, Li HF, Ma LX, Qian WJ, Wang ZF, Wu ZY. Urine-derived induced pluripotent stem cells as a modeling tool for paroxysmal kinesigenic dyskinesia. *Biol Open* 2015 Dec 15;4(12):1744–52.
- [20] Zhang SZ, Ma LX, Qian WJ, Li HF, Wang ZF, Wang HX, et al. Modeling neurological disease by rapid conversion of human urine cells into functional neurons. *Stem Cell Int* 2016;2452985.
- [21] Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* 2005 Aug 1;52(8):2521–9.
- [22] Kaviani M, Azarpira N, Aghdaei MH, Esfandiari E, Geramizadeh B, Nikeghbalian S, et al. Comparison of human mesenchymal stem cells derived from various compartments of human fibrofatty and vascular tissues during organ donation. *Transpl Int* 2017;30:390–390.