Laminin and Platelet-Derived Growth Factor-BB Promote Neuronal Differentiation of Human Urine-Derived Stem Cells

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Abstract Urine-derived stem cells (USCs) are considered as a promising cell source capable of neuronal differentiation. In addition, specific growth factors and extracellular matrix are essential for enhancing their neuronal differentiation efficiency. In this study, we investigated the possibility of neuronal differentiation of USCs and the role of laminin and platelet-derived growth factor BB (PDGF-BB) as promoting factors. USCs were isolated from fresh urine of healthy donors. Cultured USCs were adherent to the plate and their morphology was similar to the cobblestone. In addition, they showed chromosome stability, rapid proliferation rate, colony forming capacity, and mesenchymal stem cell characteristics. For inducing the neuronal differentiation, USCs were cultured for 14 days in neuronal differentiation media supplemented with/without laminin and/or PDGF-BB. To identify the expression of neuronal markers, RT-PCR, flow cytometry analysis and immunocytochemistry were used. After neuronal induction, the cells showed neuron-like morphological change and high expression level of neuronal markers. In addition, laminin and PDGF-BB respectively promoted the neuronal differentiation of USCs and the combination of laminin and PDGF-BB showed a synergistic effect for the neuronal differentiation of USCs. In conclusion, USCs are noteworthy cell source in the field of neuronal regeneration and laminin and PDGF-BB promote their neuronal differentiation efficiency.

Keywords Urine-derived stem cells · Laminin · Platelet-derived growth factor-BB · Neuronal differentiation · Mesenchymal stem cell source

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1 Introduction

Loss or injuries of the nervous system can cause functional disorders on the damaged organ. As damaged neuronal cells are generally known to be incapable of proliferation, identification of cell populations capable of neuronal differentiation has generated significant interest in the field of regenerative medicine [1, 2]. Although neuronal tissue engineering provides a potential solution for various neuronal diseases [3], there are several limitations to its application owing to the shortage of suitable cell sources. The clinical applications of autologous stem cells require genetic stability, tolerability to immune response, and legitimacy of ethic controversies. In addition, the multi-lineage differentiation potential of stem cells is the basis for considering their use in treating diseases and tissue

regeneration. A number of studies have investigated the capacity of stem cells to differentiate into neuronal cells. Recently, several studies have been conducted to determine the differentiation capacity of mesenchymal stem cells (MSCs) to neuronal cells, and they showed that MSCs can be differentiated into neuronal cells under specific induction protocols [4–6]. To regulate self-renewal and differentiation capacity, stem cells require a specific microenvironment surrounding the cells, and it is known as stem cell niche. The stem cell microenvironment consist of cells, soluble molecules, and extracellular matrix (ECM) [7]. The soluble molecules and ECM are important factors for the cell survival and neuronal differentiation [8]. The neuronal differentiation protocols included cytokines [9, 10], growth factors [11] often combined with ECM, chemical reagents [12], and mixture of these components [13, 14] in MSCs studies.

Literatures demonstrated that urine-derived stem cells (USCs) have self-renewal and differentiation abilities [15]. USCs can be harvested by less invasive procedures and low-costs; they also exhibit multipotent differentiation capacities. In the presence of certain growth factors, USCs can be induced to different cell types that include the osteogenic, chondrogenic, adipogenic, and neurogenic lineages [16, 17]. Recent studies revealed that platelet-derived growth factor BB (PDGF-BB) acts as a neurotrophic factor and it is involved in the proliferation and differentiation of neuronal progenitor cells [18–20]. PDGF-BB was observed in the embryonic ventricular zone and in post-natal brain development. PDGF-BB has also been found to play in the development of glial cells in the central nervous system (CNS). And, PDGF involved in differentiation of human neuronal stem/precursor cells (NSPCs) and also involved in NSPCs expansion [21]. A previous study demonstrated that laminin to be an effective in promoting neuronal differentiation and neurite outgrowth in bone marrow-mesenchymal stem cells (BMSC) [22]. Mruthyunjaya et al. [23] reported that laminin-1-induced neurite outgrowth and neuronal morphology of MSCs. And they demonstrated that laminin-1-induced neurite outgrowth in human MSCs is mediated through $\alpha 6 \beta 1$ integrin and involves enhanced activation of FAK-MEK/ERK pathway. Flanagan et al. [24] reported that laminin and its integrin receptors act as key regulators of NSPCs behaviour, including growth, differentiation, and migration in cell culture. Laminin matrices enhanced NSPCs migration, expansion, differentiation into neurons and astrocytes, and elongation of neurites from NSPCs-derived neurons.

In terms of potency and accessibility, USCs are considered as an alternative cell source capable of neuronal differentiation. In addition, specific growth factors and ECM are essential for enhancing their neuronal differentiation efficiency. In this study, we investigated the

possibility of neuronal differentiation of USCs and the role of laminin and PDGF-BB as promoting factors.

2 Materials and methods

2.1 Isolation of urine-derived cells

This study was approved by the Ethics Committee of our institution. All patients gave informed consent before providing urine samples. The urine samples (100 mL) were freshly obtained via urethral catheterization without urine stasis in the urinary bladder after washing out the initially drained urine from 8 healthy individuals, who underwent live kidney donor nephrectomy (mean age 49.5 years, range 19–66 years; male to female ratio = 5:3).

2.2 Primary culture of USCs

The urine samples (100 mL) were centrifuged, and the cell pellets were washed with phosphate-buffered saline (PBS). The cells were resuspended and plated in 100-mm tissue culture plates with a mixed medium composed of Keratinocyte serum-free medium (Invitrogen, Carlsbad, CA, USA), DMEM (high glucose) and DMEM/Hamm's F12 (Thermo Scientific, Rockford, IL, USA) in a 2:1:1 ratio, 5% fetal bovine serum, 25 ng/ml bovine pituitary extract, 7.5 ng/ml epidermal growth factor, 0.2 ng/ml hydrocortisone, 2.5 ng/ml insulin, 2.5 μ g/ml transferrin, 1×10^{-4} M 3,3',5'-triiodo-L-thyronine, 0.9×10^{-4} M adenine, 19.2 ng/ml cholera toxin, and 1% penicillin–streptomycin. After 2 days of incubation, the non-adherent cells were removed, followed by a medium change, and only the cells that were attached to the culture plates were used further. When the cells reached about 85% confluence, they were detached using 0.25% Trypsin/EDTA (Gibco, Grand Island, NY, USA) and were reseeded at a dilution of 1:4. The cultured cells at passage 4 were used for further analysis.

2.3 Characterization of USCs

2.3.1 Karyotyping

Karyotyping was performed to identify the chromosomal stability of the cultured cells at passage 4. The cultured cells were treated overnight with 0.02 μ g/mL Colcemid (Life Technologies, Carlsbad, CA, USA), incubated in hypotonic solution (0.075 M KCl) for 1 h at 37 °C, and then fixed in Carnoy's fixative (3:1 methanol/acetic acid, 4 °C). Metaphase spreads were prepared on glass slides, digested with 0.01% trypsin, and then stained with Giemsa stain to generate G bands. Chromosome image capture and

karyotyping were performed using CytoVision, version 3.7 (Applied Imaging, San Jose, CA, USA). The experiment was repeated thrice independently.

2.3.2 Cell proliferation assay

To generate a growth curve, USCs (cell passage 4) were seeded at a density of 500 cells/cm². The cell proliferation rates were analyzed using the cell counting kit-8 (Dojin-do, Kumamoto, Japan) according to the manufacturer's instructions at 3, 5, 7, and 14 days after plating.

2.3.3 Colony forming assay

For the colony forming assay, USCs were seeded at a density of 2×10^2 cells/well in a 6-well plate. After incubation for 14 days, the cells were fixed with 10% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The cells were then washed with distilled water and the plate was scanned after drying.

2.3.4 Flow cytometry

Flow cytometric evaluation of USCs (passage 4) was performed with phycoerythrin (PE)-conjugated mouse monoclonal antibodies specific for embryonic/mesenchymal stem cell marker (SSEA4), mesenchymal stem cell markers (CD44, CD73, CD90, and CD105), hematopoietic stem cell markers (CD34 and CD45), and an immunological marker (HLA-DR) (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. An isotype control was included in each experiment and the antibody dilution used was 1:20. Approximately 1×10^4 cells were analyzed using a fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, USA) system equipped with the CellQuest program.

2.3.5 Immunocytochemical analysis

The cells were cultured on 4-well chambered slides (Thermo Scientific, Waltham, MA, USA) for staining with embryonic/mesenchymal stem cell marker (SSEA4), mesenchymal stem cell marker (CD73), hematopoietic stem cell marker (CD34), and immunologic marker (HLA-DR). We purchased the primary antibodies for SSEA4 from Cell Signaling (Danvers, MA, USA), CD73 from NOVUS (Littleton, CO, USA), CD34 from GeneTex (Irvine, CA, USA), HLA-DR from Abcam (Cambridge, MA, USA). When the cells reached 80% confluence, they were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and washed thrice with PBS. After blocking with 5% bovine serum albumin, the

cells were incubated overnight with primary antibodies. After removal of primary antibodies, the cells were washed thrice with PBS and incubated with the fluorescence-conjugated secondary antibody for 1 h. The cells were then washed thrice with PBS and mounted in a medium containing DAPI (4'-6-diamidino-2-phenylindole) to stain the nuclei (VectaShield, Vector Labs, Burlingame, CA, USA). The fluorescent cells were visualized with a fluorescence microscope (Eclipse 8Oi, Nikon, Tokyo, Japan).

2.4 Neuronal differentiation of USCs

To induce USCs differentiation into neurons, we used the neuronal differentiation media (DM) composed of DMEM/F-12 containing 2% B27, 1% L-glutamine, 1% nonessential amino acids, and 10 mM all-trans retinoic acid (RA). At 80% confluence, the culture medium was replaced with the specific differentiation medium. The medium was changed every 3 days, and the experiments were terminated at day 14 for neuronal differentiation. To evaluate the effects of laminin and PDGF-BB on the differentiation of USCs into neuron, we cultured USCs in 4 different conditions. The cell differentiation was characterized before and 14 days after culture using real-time PCR, flow cytometry, and immunocytochemical analysis. We used 5 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN, USA) for treatment and used laminin (Corning, NY, USA) coated culture dishes at 5 µg/cm².

Induced condition I: DM

Induced condition II: DM plus PDGF-BB

Induced condition III: DM plus laminin

Induced condition IV: DM plus PDGF-BB plus laminin (DM plus both)

2.4.1 Quantitative real-time polymerase chain reaction analysis for marker gene expression

Total RNA was extracted using an RNeasy-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In total, 2 µg of RNA was used for cDNA synthesis using reverse transcription kits (Applied Biosystems, Warrington, UK). Primers were designed using Primer Express Software (Applied Biosystems, Warrington, UK). The assay was performed using the ABI Prism Sequence Detection System 7500 with the SYBR Green Polymerase Chain Reaction Master Mix (Applied Biosystems, Warrington, UK). To analyze the data, the $2^{-\Delta\Delta C_t}$ method of relative quantification was adapted to estimate the copy numbers.

2.4.2 Flow cytometry

Flow cytometric evaluation of differentiated USCs for 14 days was performed using phycoerythrin (PE)-conjugated mouse monoclonal antibodies specific to mesenchymal stem cell markers (CD44, and CD90) (BD Biosciences, San Jose, CA, USA), neuron specific markers (Nestin, MAP2, and Neurofilament-M) (Novus, Littleton, CO, USA) according to the manufacturer's instructions. We used CD44, CD90, Nestin obtained from Approximately 1×10^4 cells were analyzed using a fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, USA) system equipped with the CellQuest program.

2.4.3 Immunocytochemical analysis

The cells were cultured on 4-well chambered slides (Thermo Scientific, Waltham, MA) for staining with neuron-specific markers (Nestin, MAP2, β -TUBULIN-III, and NF-M). We purchased the primary antibodies for Nestin from Santa Cruz Biotechnology (Santa Cruz, CA, USA), MAP2 and β -TUBULIN-III from Abcam (Cambridge, MA, USA), NF-M from Thermo (Waltham, MA, USA). The cells were stained after culture under differentiation conditions for 14 days. The fluorescent cells were visualized with a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan).

2.5 Statistical analysis

All the data are reported as the sample mean $\pm$ the standard deviation. Pairwise comparisons between means of different groups were performed using a Student *t* test (two tailed, unpaired, unpaired) where, for each couple of normally distributed populations, the null hypothesis that the means are equal were verified. Everywhere in the text the difference between two subsets of data is considered statistically significant if the Student *t*-test gives a significance level *P* (*P* value) < 0.05 .

3 Results

3.1 Isolation and characterization of USCs

Isolated USCs from 8 healthy individuals were attached to the culture plates after 2 days of initial seeding. These cells were small, oval-shaped and exhibited cobblestone morphology (Fig. 1A–H). To identify the chromosomal stability of the cultured cells, we performed a karyotype investigation of the USCs from 8 healthy individuals. The analysis involved comparing chromosome numbers, lengths, placement of the centromere, and the G-banding

pattern. Our data showed normal diploid complement of autosomes and sex chromosomes in these. Chromosomal aberrations were not found in any cell (Fig. 1I–P). We determined cell proliferation on days 3, 5, and 7 after plating (Fig. 2A). After day 3 of seeding, the cells were well adherent to the culture dishes and then rapidly proliferated until confluent, followed by a plateau after 8 days of culture. Colony formation analysis could be performed after incubation for 14 days. Colony forming capacity indicated the greatest replicative potential (Fig. 2B). The USCs from 8 donors showed a greatly increased number of colony-forming cells. Although sample variance was observed, all the USCs showed colony forming capacity. The USCs were then subjected to flow cytometry analysis. The USCs were stained with the anti-embryonic/mesenchymal stem cell marker (SSEA4), the anti-mesenchymal stem cell markers (CD44, CD73, CD90 and CD105), and the anti-hematopoietic lineage and immunogenic markers (CD34, CD45 and HLA-DR) (Fig. 3A–I, Table 1). USCs revealed strongly positive expression for SSEA4, CD44, CD73, and CD90; whereas CD105 expression was lower than that of other mesenchymal stem cell markers. The hematopoietic and immunogenic markers showed extremely low expression in USCs. Immunohistochemical staining with anti-SSEA4 (Fig. 4A–H), anti-CD73 (Fig. 4I–P), anti-CD34 (Fig. 5A–H), and HLA-DR (Fig. 5I–P) showed an expression pattern very similar to that shown by flow cytometry.

3.2 Neuronal differentiation of USCs

Neuronal differentiated USCs were obtained using the protocol depicted in Fig. 6A. Neuronal induction conditions consist of DM with/without 5 ng/ml PDGF-BB and with/without 5 $\mu\text{g}/\text{cm}^2$ laminin coating. After culture in the neuronal induction conditions for 14 days, the USCs exhibited neuron-like morphological changes, including a pyramidal cell body, axon like structure, and dendrites (Fig. 6B–E). Neuron-like cells were observed in all the four induction conditions, and among these more neuronal cells were found in the group treated with both laminin and PDGF-BB group than other groups. The cell body became typically neuron-like with a shiny nucleus, a cell soma having many dendrites, and an axon-like structure. The laminin and PDGF-BB exposure can give rise to distinct neuronal populations with neuronal phenotypes. These morphological changes suggest that laminin and PDGF-BB stimulate the trans-differentiation of USCs into neuronal cells.

Real time PCR analysis showed that expression of neuronal marker genes (*Nestin*, *MAP2*, β -*TubulinIII*, *NF-M*, and *NeuN*) was increased in DM plus PDGF-BB plus laminin (condition IV) compared to that in the other

Fig. 1 Morphology of USCs and G-banded karyotypes for USCs. **A–H** The USCs at passage 4 were expanded and were cultured in growth medium. USCs show the cobblestone-shaped morphology. Images are shown at $\times 200$ magnification. **I–P** Chromosome analysis of USCs at

passage 4. Karyotype analysis showed that USCs had a normal size, shape, and number of chromosomes. USCs did not show any chromosomal aberrations

conditions (Fig. 6F–J). All the induced conditions expressed neuronal gene markers, but condition IV showed the highest neuronal gene expression levels. As shown in Fig. 6F–J, Nestin ($p = 0.00002$), MAP2 ($p = 0.00000002$), β -TubulinIII ($p = 0.0012$), NF-M ($p = 0.0004$) and NeuN ($p = 0.00002$) genes were expressed significantly higher in condition IV than in condition I (DM). Nestin ($p = 0.0003$), MAP2

($p = 0.0376$), β -TubulinIII ($p = 0.0013$), NF-M ($p = 0.0154$) and NeuN ($p = 0.0042$) genes were expressed significantly higher in condition IV compared with condition II (DM plus PDGF-BB). MAP2 ($p = 0.0068$) and NeuN ($p = 0.0213$) genes were expressed significantly higher in condition IV compared with condition III (DM plus laminin). MAP2 ($p = 0.0389$) and NeuN ($p = 0.0370$) genes were expressed significantly higher in

Fig. 2 Analysis of stem cell proliferation. **A** Comparison of cell proliferation rates at day 3, 5, and 7. USC lines showed a time-dependent increase in growth rate. **B** Quantity of USC colonies at passage 4. The number of stained colonies from the 8 USC lines was compared. There was variation between the samples, but the number of colonies increased in all the samples

Fig. 3 Comparison of stem cell marker expression in USC lines. **A–I** Specific cell surface markers were assessed by flow cytometry in passage 4 of USC lines. USC lines were strongly positive for mesenchymal stem cell markers and negative for hematopoietic lineage and immunogenic markers

condition II compared with condition I. Nestin ($p = 0.0049$), MAP2 ($p = 0.0003$), β -TubulinIII ($p = 0.0328$), NF-M ($p = 0.0001$) and NeuN ($p = 0.0242$) genes were expressed significantly higher in condition III compared with condition I. β -TubulinIII ($p = 0.0397$) gene was expressed significantly higher in condition III compared with condition II. In real time PCR, condition IV expressed the highest expression level than other conditions and condition II and III expressed higher than condition I.

Neuronal markers expressed on USC lines were analyzed by flow cytometry (Fig. 7A–Y, Table 2). USC lines differentiated for 14 days express neuronal markers (Nestin, MAP2, and NF-M), while retaining the MSCs markers (CD44 and CD90). In the induced condition IV, CD44 and CD90 expression was lower than that in other conditions. When comparing all the four methods, the differentiated USC lines showed similar strongly positive expression of neuronal markers such as Nestin (97.57, 97.55, 97.46, 96.13%), MAP2 (86.55, 83.71, 83.34, 74.83%), and NF-M (97.58, 97.77, 97.91, 94.03%) respectively in conditions I, II, III, and IV. Undifferentiated USC lines were demonstrated to express MSCs markers; however, a small subpopulation of USC lines expressed neuronal markers. We probed the cellular proteins at 14 days after neuronal induction by immunocytochemical staining. Cells from the four induced conditions were stained with specific antibodies against the neuronal marker proteins—Nestin, MAP2, β -TubulinIII, and NF-M. All the induced conditions exhibited positive cytoplasmic staining for all antibodies, except for the undifferentiated USC lines (Fig. 8A–T).

4 Discussion

Among stem cells, USC lines have a relatively superior accessibility for clinical utility because they can be obtained through minimally invasive procedures [17]. USC lines possess MSCs-like features and it can differentiate into neuronal cells upon supplementing with different growth factors and ECM [15]. Previous studies have demonstrated that laminin and PDGF-BB are involved in neuronal growth and differentiation [8, 20]. However, they did not show the result with the usage of laminin and PDGF-BB in combination to promote neuronal differentiation in USC lines. The novelty of our study is that we examined the synergistic neuronal differentiation effect of the laminin and PDGF-BB combination in USC lines. We demonstrated an increased expression of the neuronal markers in the PDGF-BB treated USC lines cultured on the laminin coated plate. Our result showed a promising result when laminin and PDGF-BB are combined and it can

Table 1 The percentage of stem cell marker expression in USCs

Cell line	Mesenchymal stem cell marker				Hematopoietic stem cell marker		Immunogenic marker	
	SSEA4	CD44	CD73	CD90	CD105	CD34	CD45	HLA-DR
USC_JY#1	93.16%	98.78%	95.93%	98.53%	87.87%	0.08%	0.09%	0.09%
USC_JY#2	78.81%	99.95%	97.79%	70.34%	31.58%	0.11%	0.13%	0.02%
USC_JY#3	92.01%	89.77%	88.18%	61.74%	63.15%	0.06%	0.10%	0.25%
USC_JY#4	94.76%	97.58%	91.37%	91.05%	85.66%	0.33%	0.04%	0.03%
USC_JY#5	85.83%	99.86%	93.61%	93.46%	54.30%	0.01%	0.06%	0.07%
USC_JY#6	87.82%	99.39%	97.51%	94.49%	73.29%	0.20%	0.03%	0.02%
USC_JY#7	81.21%	92.18%	93.09%	91.13%	25.63%	4.36%	3.71%	3.52%
USC_JY#8	96.05%	95.44%	95.97%	96.80%	46.56%	3.31%	3.44%	3.31%

eventually provide a valuable tool for generating neuron like cells.

Many researchers have demonstrated that cells with MSCs-like characteristics can be isolated from different tissues [25–27]. In accordance to other studies, cultured MSCs have been demonstrated to differentiate into cells of multiple germ layers, including osteocytes, chondrocytes, adipocytes, cardiomyocytes, and neuron-like cells [28, 29]. Various studies investigated the stem cell differentiation into neuron because the mammalian nervous system has a limited potential to generate new nerve cells after cell damage [30]. Although adult MSCs have a limited capacity to differentiate into fully mature neurons, these cells do not elicit graft versus host disease [31]. For this reason, they are regarded as important candidate for the stem cell therapy and have been investigated in a considerable number of trials and diverse research groups have described different protocols for the induction of neuronal cells [4]. Studies have investigated the effects of growth factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and basic fibroblast growth factor (bFGF) on differentiation of MSCs into neuronal cells [11, 32]. Other researchers have reported that ECM proteins such as fibronectin, collagen-1, and laminin induce a neuronal phenotype in MSCs [33, 34]. Recently, Yang et al. [20] showed that the PDGF-BB promotes the proliferation and differentiation of neuronal progenitor cells. di Summa et al. [35] demonstrated that adipose-derived stem cells grown on laminin enhanced neurite length outgrowth. However, these researches were unable to show clear evidence that MSCs are capable of differentiating into

functional neuronal cells *in vitro*. To overcome the technical challenges of generating neuronal cells from MSCs, it is vital to organize a more rapid and efficient method of differentiation.

Although human urine is known to contain a heterogeneous cell population, most cells in urine do not attach to culture plates [15–17]. Stem cells derived from urine easily attach to culture dishes and show ability to differentiate into multiple types of lineages, similar to BMSC [12, 13]. USCs clearly have an advantage compared to MSCs, as these cells can be easily obtained through less invasive procedure with tolerable discomfort. The common features of MSCs, despite the variety of their sources, are expression of CD73, CD90, and CD105 and the lack of CD34, CD45, and HLA-DR [36]. By flow cytometry, USCs showed representative MSCs marker profiles. The USCs population of about 88.71% showed positive staining for SSEA-4, embryonic/mesenchymal stem cell marker. Regarding the four MSC markers CD44, CD73, CD90, and CD105, USCs show expression on about 96.62, 94.18, 87.19, and 58.51% positivity, respectively. The CD105 expression on USCs was lower than that of other markers; CD105 positive cells are known to express diversely according to the culture medium [37]. USCs were negative for the hematopoietic stem cell markers, CD34 and CD45 [38]. The immunogenic marker, HLA-DR (Class II MHC), was expressed in less than 3% of the cells indicating a low immune rejection [39]. To confirm our data, we used immunocytochemistry [40]. The immunocytochemistry showed significantly enhanced SSEA4 and CD73 protein expression, whereas CD34 and HLA-DR (Class II MHC)

Fig. 4 Comparison of stem cell marker expression in USCs. **A–P** Immunofluorescence staining of USCs at passage 4. USCs were labeled with **A–H** embryonic/mesenchymal stem cell marker (SSEA4), **I–P** mesenchymal stem cell marker (CD73)

were not detectable. The USCs proliferation rates were measured by cck-8 and cck-8 increased over time. Our result reflects that USCs can maintain their proliferation ability. We also observed colony forming units, suggesting that USCs maintain their stem cell characteristics. The colony forming ability depends on the donor condition. The cultured USCs possessed stem cells characteristics including clonogenicity, proliferation potential, and chromosomal stability. These findings mirrors that the isolated

USCs show typical MSCs characteristics and also possess the capacity to differentiate towards the neuronal lineage.

In the past decade, MSCs derived from multiple tissue types have been differentiated into neuronal cells *in vitro* by means of a variety of inducing chemicals. The differentiation of MSCs resides in a specific microenvironment, commonly referred to as the niche [41]. The fate of MSCs is regulated by various signaling cues in the stem cell niche [42]. The stem cell niche consists of various cell types,

Fig. 5 Comparison of stem cell marker expression in USCs. **A–P** Immunofluorescence staining of USCs at passage 4. USCs were labeled with **A–H** hematopoietic stem cell marker (CD34), and **I–**

P immunogenic marker (HLA-DR). No visible labeled cells were observed for CD34 and HLA-DR

ECM, and soluble factors. Soluble factors such as growth factors, trophic factors, and cytokines are the most powerful epigenetic effectors that act at various levels, in the process of neuronal and glial formation during neurogenesis. Although *in vivo* function of PDGF-BB is not well known, recent studies showed that PDGF-BB treatment of cell cultures leads to neuronal growth and survival [18, 19, 43]. It has been found to have a role in the neurotrophic effect of stem cells. Other groups have reported

that PDGF-BB is required for the differentiation of progenitor cells into different neuronal cells. Yang et al. [20] demonstrated that exposure of neuronal progenitor cells to PDGF-BB resulted in increased expression of miR-9. The miR-9/MCP1P1 in PDGF-BB-mediated regulates neuronal progenitor cells proliferation, differentiation as well as migration via the suppression of miR-9 target, MCP1P1. PDGF-BB plays a crucial role in neuronal survival and cell differentiation during embryonic development and in the

Fig. 6 Neurogenic *in vitro* differentiation. **A** Experimental design for the induction of neurons. Four different kinds of neural differentiation conditions (differentiation medium with/without 5 ng/ml PDGF-BB and with/without 5 $\mu\text{g}/\text{cm}^2$ laminin coating) induced USCs into neural like cells in 2 weeks. **B–E** Morphology of USCs after differentiation with neural differentiation media for 2 weeks. Images shown at $\times 200$ magnification. **F–J** Determination of neural gene expression in differentiated USCs by RT-PCR. Quantitative real time PCR was performed on total RNA using neuron-specific primers (*Nestin*, *MAP2*, β -tubulinIII, *NF-M*, and *NeuN*). The values were calculated with reference to GAPDH and the samples from 8 USC lines were run in duplicate. The neural markers, *Nestin*, *MAP2*, β -tubulinIII, *NF-M*, and *NeuN* were significantly increased in condition IV compared to condition I. Compared with condition II, condition IV showed significantly higher expression of *Nestin* ($p = 0.0003$), *MAP2* ($p = 0.0376$), β -TubulinIII ($p = 0.0013$), *NF-M* ($p = 0.0154$) and *NeuN* ($p = 0.0042$). Compared with condition III, condition IV showed significantly higher expression of *MAP2* ($p = 0.0068$) and *NeuN* ($p = 0.0213$). Compared with condition I, condition II showed significantly higher expression of *MAP2* ($p = 0.0389$) and *NeuN* ($p = 0.0370$). Compared with condition I, condition III showed significantly higher expression of *Nestin* ($p = 0.0049$), *MAP2* ($p = 0.0003$), β -TubulinIII ($p = 0.0328$), *NF-M* ($p = 0.0001$) and *NeuN* ($p = 0.0242$). Compared with condition II, condition III showed significantly higher expression of β -TubulinIII ($p = 0.0397$). Student t-test was used for gene expression analysis using real-time PCR data. Values are mean $\pm$ SD (* $p < 0.05$), (** $p < 0.005$), and (***) $p < 0.0005$). Abbreviations: I, DM; II, DM plus PDGF-BB; III, DM plus Laminin; IV, DM plus PDGF-BB plus Laminin

adulthood. The multiple roles and mechanisms of PDGF-BB are still not known. However, in a previous study, PDGF-BB promoted proliferation of neuronal progenitor cells in the subventricular zone in an animal model of Parkinson's disease [44].

The ECM as an essential niche constituent directly or indirectly regulates stem cells; for example, the ECM provides regulatory signals to support stem cell proliferation, self-renewal, migration, and fate decision [45]. Therefore, adhesion to ECM could affect cell survival, differentiation, and cell migration [46]. Laminin is widely regarded as an enhancer of neuronal progenitor expansion and neuronal differentiation. Mruthyunjaya et al. [23] reported that laminin-1 promoted neurite outgrowth of MSCs in the absence of growth factors/differentiating agents. Flanagan et al. [24] demonstrated that migration, expansion, differentiation, and neurite outgrowth of NSPCs-derived neurons were enhanced on laminin-containing matrices. Laminin is expressed in the NSPCs niche of the developing cortex. Advanced studies have demonstrated the benefits of using various growth factors and ECM for neuronal differentiation [47]. However, they have not used the combination of laminin and PDGF-BB, and the efficiency of differentiation using them has not yet been investigated.

In this study, we successfully isolated USCs from human urine and confirmed their MSCs characteristics. In addition, we were able to show neuronal differentiation of

USCs and the promoting effects of laminin and PDGF-BB. The USCs were cultured in neuronal DM supplemented with/without laminin and PDGF-BB. We also examined the effects of either laminin, PDGF-BB, or a combination of these factors on the neuronal differentiation of USCs grown under neuronal DM. The experimental groups were DM (condition I), DM plus PDGF-BB (condition II), DM plus laminin (condition III), and DM plus PDGF-BB plus laminin (condition IV). Cultures in condition IV showed the greatest enhancement of neuronal-like morphology and expression of the neuronal markers. These morphological changes were accompanied by changes in the total RNA and protein expression of neuron-specific markers (*Nestin*, microtubule-associated protein 2 (*MAP2*), β -TubulinIII, neurofilament M (*NF-M*), and *NeuN*) [12]. The gene expressions of *Nestin*, *MAP2*, β -TubulinIII, *NF-M*, and *NeuN*, were significantly increased in condition IV. We compared PDGF-BB with laminin for analyzing the each effect of laminin and PDGF-BB. The laminin treated group showed better potential for neurogenic differentiation compared to that of the PDGF-BB treated group. The neurogenic induction of USCs also resulted in increasing expression of neuronal specific markers as analyzed by flow cytometry analysis. The neuronal markers were confirmed by immunocytochemical staining analysis, and upregulation of these proteins were observed for all groups. Although a significant increase of neuronal markers were observed, MSCs markers such as CD44 and CD90 retained their expression in all groups of neuronal induction. Furthermore the undifferentiated USCs expressed neuronal proteins such as *Nestin* (51.68%), *MAP2* (36.67%), *NF-M* (60.14%). This observation may be explained by their plasticity to differentiate easily with in neuronal tissues [48].

There were several limitations in this study. As to the concentration of laminin and PDGF-BB, Erlandsson et al. used 10 ng/ml PDGF-BB and Hall et al. used 10 $\mu\text{g}/\text{cm}^2$ laminin in their studies [21, 49]. However, we used half levels of laminin and PDGF-BB compared to the previously reported concentration, because we expected a synergistic effect of combined factors on the neuronal differentiation of USCs. Further studies are needed to elucidate the optimal concentrations of laminin and PDGF-BB and to examine on the stage-dependent expression pattern of neuronal differentiation markers and to elucidate the maturation pattern of neuronal differentiation. In addition, further studies are essential to confirm neural functionality that is required to enable its transplantation into clinical application.

The functional assessment including synaptic transmission and electrophysiological properties of these differentiated USCs would be of interest for future studies.

Fig. 7 Determination of neural protein expression in differentiated USC using flow cytometry. Immunofluorescence of differentiated USC at passage 4. Upon neural differentiation, USC were stained for specific markers. **A–Y** Flow cytometric analysis. USC were

labeled with mesenchymal stem cell markers (CD44 and CD90), neuron specific markers (Nestin, MAP2, and NF-M). Differentiated USC were strongly positive for neural cell markers and still expressed mesenchymal stem cell markers

We evaluated that the possibility of USC as an alternative cell source of neuronal regeneration and the role of laminin and PDGF-BB as promoting factors in the process. USC possess MSCs-like features and have a high potential to differentiate the neuronal cells. In addition, laminin and

PDGF-BB promote the neuronal differentiation of USC and the combination of laminin and PDGF-BB seems to have a synergistic effect for the neuronal differentiation of USC, providing a promising approach that may be modified and utilized in the near future stem cell therapy.

Table 2 The percentage of neuronal protein expression in differentiated USCs

Induced condition	Mesenchymal stem cell marker		Neuronal marker		
	CD44	CD90	Nestin	MAP2	NF-M
Undifferentiated USCs	91.99%	78.23%	51.68%	36.67%	60.14%
DM	90.79%	49.06%	97.57%	86.55%	97.58%
DM+PDGF-BB	92.33%	47.95%	97.55%	83.71%	97.77%
DM+Laminin	85.20%	42.90%	97.46%	83.34%	97.91%
DM+PDGF-BB+Laminin	77.90%	41.24%	96.13%	74.83%	94.03%

Fig. 8 Determination of neural protein expression in differentiated USCs using immunocytochemistry. Immunofluorescence of differentiated USCs at passage 4. Upon neural differentiation, USCs were stained for specific markers. **A–T** Immunocytochemistry. USCs were

labeled with neuron specific markers (Nestin, MAP2, β -tubulin, and NF-M). Un-differentiated USCs do not express neural markers. Images are shown at x 200 magnification

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical Statement This study was approved by the Ethics Committee of the Kyungpook National University School of Medicine (No. KNUMC 2016-05-021). This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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