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ORIGINAL ARTICLE

TERM

The Efficacy and Safety of Collagen-I and Hypoxic Conditions in Urine-Derived Stem Cell *Ex Vivo* Culture

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Upper urinary tract-derived urine stem cells (USCs) are considered a valuable mesenchymal stem cell source for autologous cell therapy. However, the reported culture condition for USCs is not appropriate for large-quantity production, because cells can show limited replicativity, senescence, and undesirable differentiation during cultivation. These drawbacks led us to reconstitute a culture condition that mimics the natural stem cell niche. We selected extracellular matrix protein and oxygen tension to optimize the *ex vivo* expansion of USCs, and compared cell adhesion, proliferation, gene expression, chromosomal stability, differentiation capacity, immunity and safety. Culture on collagen type I (ColI) supported highly enhanced USC proliferation and retention of stem cell properties. In the oxygen tension analysis (with ColI), 5% O_2 hypoxia showed a higher cell proliferation rate, a greater proportion of cells in the S phase of the cell cycle, and normal stem cell properties compared to those observed in cells cultured under 20% O_2 normoxia. The established reconstituted condition (ColI/hypoxia, $USCs^{recon}$) was compared to the control condition. The expanded $USCs^{recon}$ showed highly increased cell proliferation and colony forming ability, maintained transcription factors, chromosomal stability, and multi-lineage differentiation capacity (neuron, osteoblast, and adipocyte) compared to the control. In addition, $USCs^{recon}$ retained their immune-privileged potential and non-tumorigenicity with *in vivo* testing at week 8. Therefore, reconstituted condition allows for expanded uUSC cell preparations that are safe and useful for application in stem cell therapy.

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Key Words: Urine-derived stem cells; Collagen-I; Hypoxia; Culture condition; Mesenchymal stem cell source

INTRODUCTION

Urine-derived stem cells were first described as epithelial cells [1] with high proliferation efficiency [2]. Then, voided urine-derived stem cells were investigated as multipotent progenitor cells with the capacity to differentiate into lineage-specific cells [3,4]. Recently, upper urinary tract-derived urine stem cells (USCs) were reported to be the most effective urine stem cells based on their high therapeutic utility [5,6]. In addition, USCs can be obtained from bladder cancer patients, because the upper urinary tract is pathologically normal even in these patients. Hence, there is increasing interest in USCs as a useful autologous stem cell source.

The therapeutic application of USCs is dependent on their potential for large quantity *ex vivo* expansion. However, the reported culture conditions of USCs (USC^{ctrl}) are hard to achieve, because cells can show limited replicability, senescence [5], and undesirable differentiation during cultivation [7]. Replicative senescence is indicated by proliferation arrest, morphological abnormality, and attenuated expression of specific surface markers [8]. The USCs must be expanded *in vitro* to maintain their multi-lineage potential before differentiation into a mesenchymal lineage for application in regeneration therapy. In order to obtain optimal *ex vivo* expansion conditions, we designed a culture condition based on the natural stem cell niche.

Stem cell expansion is mainly controlled by ECM protein and oxygen tension [9,10]. Our first optimizing factor was ECM protein. To retain stem cell properties, coating of plates with a

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substrate can be an important step [11], because surface modification with ECM can mimic the stem cell niche [12]. A second critical factor is oxygen tension. Cells are usually maintained under atmospheric oxygen pressure, which is around 150 mm Hg (21% O_2), whereas physiological oxygen pressure ranges from 5 to 50 mm Hg (0.7–7% O_2) [13]. Therefore, a hypoxic rather than normoxic environment in cell culture better represents the physiological stem cell niche.

Here, we present culture conditions that allow for the efficient expansion of USCs. We selected ECM protein and oxygen tension to optimize the *ex vivo* expansion of USCs, and compared cell adhesion, proliferation, gene expression, chromosomal stability, differentiation capacity, immunity, and safety between our conditions and those previously reported.

MATERIALS AND METHODS

Collection of upper urinary tract urine sample

The Ethics Committee of Kyungpook University School of Medicine approved this study and 7 bladder cancer patients (Table 1) gave informed consent to participate. To obtain the upper urinary tract urine sample, a ureteral catheter was inserted from the ureteral orifice to the renal pelvis with a cytoscope guide, and the urine was collected into a drainage bag. The urine sample (100 mL) was centrifuged and the cell pellet was washed with phosphate buffered saline (PBS).

Isolation of stem cells from urine contained cell

About 500 cells were plated in each well of 24-well plates and cultured on a medium composed of keratinocyte-serum-free medium and progenitor cell medium (Gibco-Invitrogen, Grand Island, NY, USA) in a 1:1 ratio [4]. According to a time-gradient attachment method [14], cells were cultured in a 24-well plate for 24 hr, and then the non-adherent cells were transferred into another 24-well plate for another 48 hr of culture. The non-adherent cells were transferred again for another 72 hr of culture, and the cells attached at 72 hr were cultured for 14 days. Cells at passage 4 were used in the following analysis.

Table 1. Patients' information

Case No.	Age	Gender
1	45	M
2	54	F
3	66	M
4	72	M
5	59	F
6	49	M
7	62	M

ECM selection

ECM proteins that were used included collagen-I (ColI), -IV, fibrinogen, fibronectin, and laminin (Cell Biolabs, San Diego, CA, USA). After an analysis of cell proliferation and gene expression, the optimal ECM was selected. For the control, non-coated tissue culture plates were used.

Oxygen tension

With the selected ECM, the effects of hypoxic conditions on maintaining USC properties were evaluated. Cultures were placed in humidified portable isolation chambers (Billups-Rothenberg, Del Mar, CA, USA), flushed daily with a gas mixture of 5% O_2/5% CO_2/90% N_2. The entire chamber was housed in an incubator to maintain a temperature of 37°C. To minimize oxygen fluctuation at the time of medium changes, media were pre-equilibrated to a 5% O_2 concentration in a separate chamber for 12 hr in the incubator. For the control, 20% O_2 and non-coated culture dishes were used. After an analysis of cell proliferation, cell cycle phase gene expression, and apoptosis, the optimal oxygen content was selected.

Comparisons of control and reconstituted culture conditions

The USCs from 7 different donors were expanded in control ($USCs^{ctrl}$) or reconstituted culture conditions ($USCs^{recon}$) with 3 independent preparations. The control conditions involved using a plastic culture plate and 20% O_2. The reconstituted conditions involved using ColI pre-coated dishes and 5% O_2. The influences of culture conditions on cell proliferation, cell cycle, colony formation, gene expression, immunophenotype, chromosomal stability, multi-lineage differentiation, immunogenicity, and tumorigenicity over an expansion were compared.

Cell proliferation analysis

For the cell proliferation analyses, cells were seeded in triplicate on 96-well plates at a density of 2×10^3 cells/well. Cell growth was measured using the Cell Counting Kit-8 (CCK-8) by following the manufacturer's instructions (Dojindo, Gaithersburg, MD, USA) at 1, 3, 5, and 7 days. For the cell cycle phase analysis, cells in the log phase were harvested and washed twice with ice-cold PBS. Cell pellets were fixed in 70% ethanol, treated with RNase A (Boehringer Mannheim, Indianapolis, IN, USA) and stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). DNA contents were measured with a flow cytometer. For the colony formation analysis, single cell suspensions were plated in triplicate on 6-well plates, at a density of 50 cells/plate in 1 mL of medium. Cells were grown for 7 days and stained with crystal violet [5% (w/v) in 95% ethanol] to visualize colonies. Cell clusters containing >50 cells were sco-

red as colonies. The colony count and size measurements were performed under a microscope.

Gene expression analysis

For the gene expression analysis, total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription reactions were performed with cDNA reverse transcription kits (Applied Biosystems, Paisley, UK). The primers for mesenchyme, muscle, neuron, osteoblast, adipocyte, and epithelial cell detection markers were designed with Primer Express Software (Applied Biosystems, Paisley, UK); the sequences are listed in Table 2. The assay was carried out using the ABI Prism Sequence Detection System 7500 with SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK). PCR conditions were as follows: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. To analyze the data, the $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy numbers.

Immunophenotype

Cell phenotyping was performed with phycoerythrin (PE)- or fluorescein isothiocyanate-conjugated mouse monoclonal antibodies specific for mesenchymal (CD44, CD90) and hematopoietic (CD34, CD45) cells (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Approximately 1×10^5 cells were evaluated using a FACSCalibur (BD Biosciences, San Jose, CA, USA) equipped with CellQuest software. An isotype control was included in each experiment and the antibody dilution used was 1:20.

Chromosomal stability

Karyotyping of expanded USCs was carried out using a standard Giemsa staining procedure. At least 20 metaphase spreads were analyzed and images were acquired using CytoVision version 3.7 (Applied Imaging, San Jose, CA, USA). For a numerical and structural chromosome abnormality study, cell numbers, translocations, inversions, deletions, and duplications were evaluated.

Multi-lineage differentiation of USCs^{recon} *in vitro*

For the differentiation of USCs into neurons, conditioned medium (CM) was used. The primary cells were kindly provided by Dr. B. S. Kim (Kyungpook National University Hospital, Daegu, Korea). The CM was collected every 3 days from a primary cell culture medium (ScienCell, Carlsbad, CA, USA). The USCs were treated for 7 days in CM for differentiation and genotypic conversion into each lineage, and then real-time PCR and immunocytochemical staining were performed. For osteogenic differentiation, cells were expanded in the presence of fibroblast growth factor 2 and dexamethasone (DEX) for 5 days, and then were replated on 48-well plates at a density of 1×10^4 cells per well. On the following day, osteoblastic differentiation was induced by treatment with osteogenic medium (α-MEM supplemented with 10% FBS, 50 μg/mL α-ascorbic acid, 10 mM β-glycerophosphate, and 10 nM DEX) for 14 days. Osteoblastic

Table 2. Primer sequences for real-time PCR

Markers	Target gene	Forward	Reverse
Stem cell	*OCT4*	ggagaatttgttcctgcagtgc	agaaccacactcggaccacatc
	SOX2	tcacgcaaaaaccgcgat	tatacaaggtccattcccccg
	NANOG	gcatccgactgtaaagaatcttca	catctcagcagaagacatttgca
	ALKALINE PHOSPHATASE	acgtggctaagaatgtcatc	ctggtaggcgatgtcctta
	HIF-1α	gtagtgctgaccctgcactcaa	tccatcggaaggactaggtgtc
Muscle	*PAX7*	gcaaattgctgtcctgctca	tgaaaactggtcacatctgcct
Neuron	*NESTIN*	ggcagcgttggaacagaggttgga	ctctaaactggagtggtcagggct
	MAP2	ctgaaaggtgaacaagagaaa	gatcaaggcggagcaggggaa
	β-TUBULIN III	aacacggatgagacctactgcat	gggtgcggaagcagatgt
Osteoblast	*BONE SIALOPROTEIN*	gcgagacacagatgctcagac	tgggttttccttccattgct
	COLLAGEN I	tgacgagaccaagaactg	ccatccaaaccactgaaacc
	OSTEOCALCIN	catgagagccctcaca	agagcgacaccctagac
	OSTEONECTIN	ttgcagccttctcagccaa	caaaagcaaatcactgcaattctc
Adipocyte	*PPAR-γ2*	attgacccagaaagcgattc	caaaggagtgggagtggtct
	ADIPONECTIN	atcaccactaactcagag	agtgggcacaaaatagcact
	LEPTIN	agttcaaatagaggt ccaaatca	ttctgaggttgtgtcactggca
Epithelium	*UROPLAKIN Iα*	cgctggtgcctggattg	ggcacccacaccaaaactg
Control	*β-ACTIN*	atcgtccaccgcaaatgct	aagccatgccaatctcatcttg

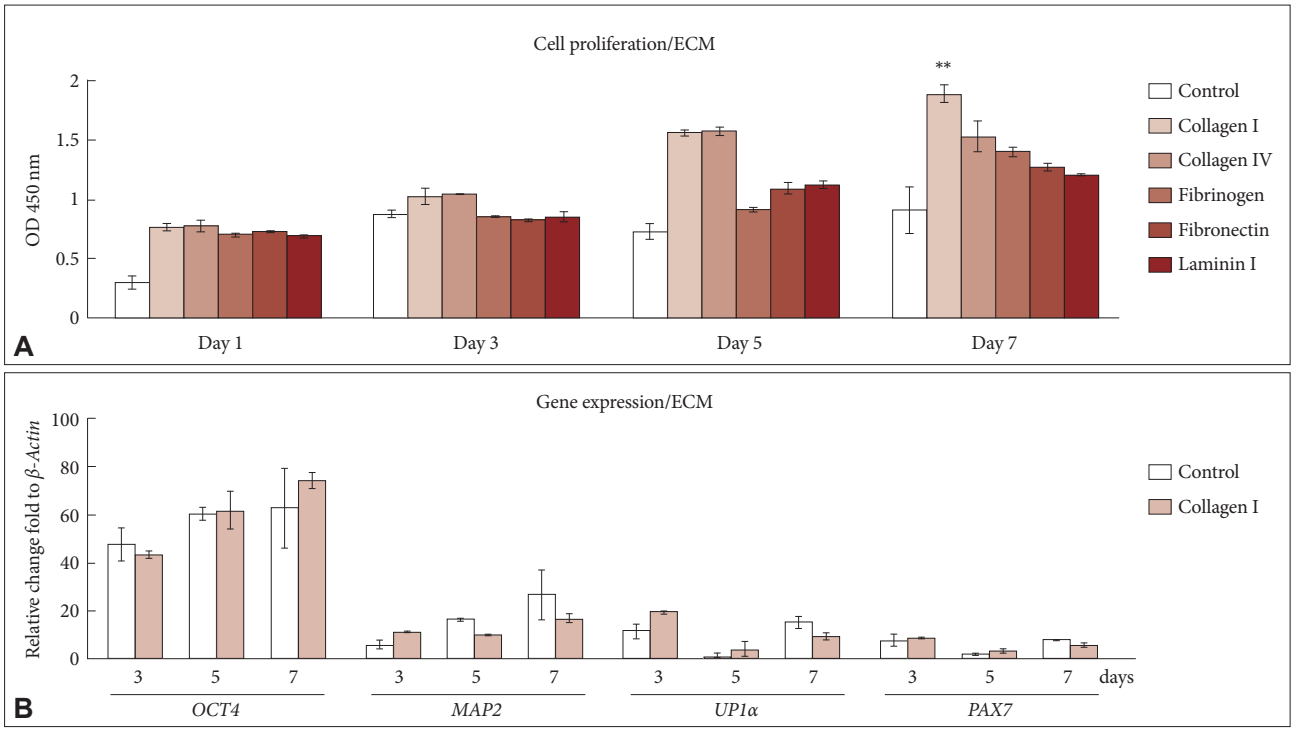

Figure 1. Influence of ECM protein on cell proliferation and gene expression. (A) ECM-mediated uUSC proliferation assay. (B) Effect of collagen-I on the retention of stem cell properties and suppression of spontaneous differentiation. **p<0.01. ECM: extracellular matrix, OD: optical density.

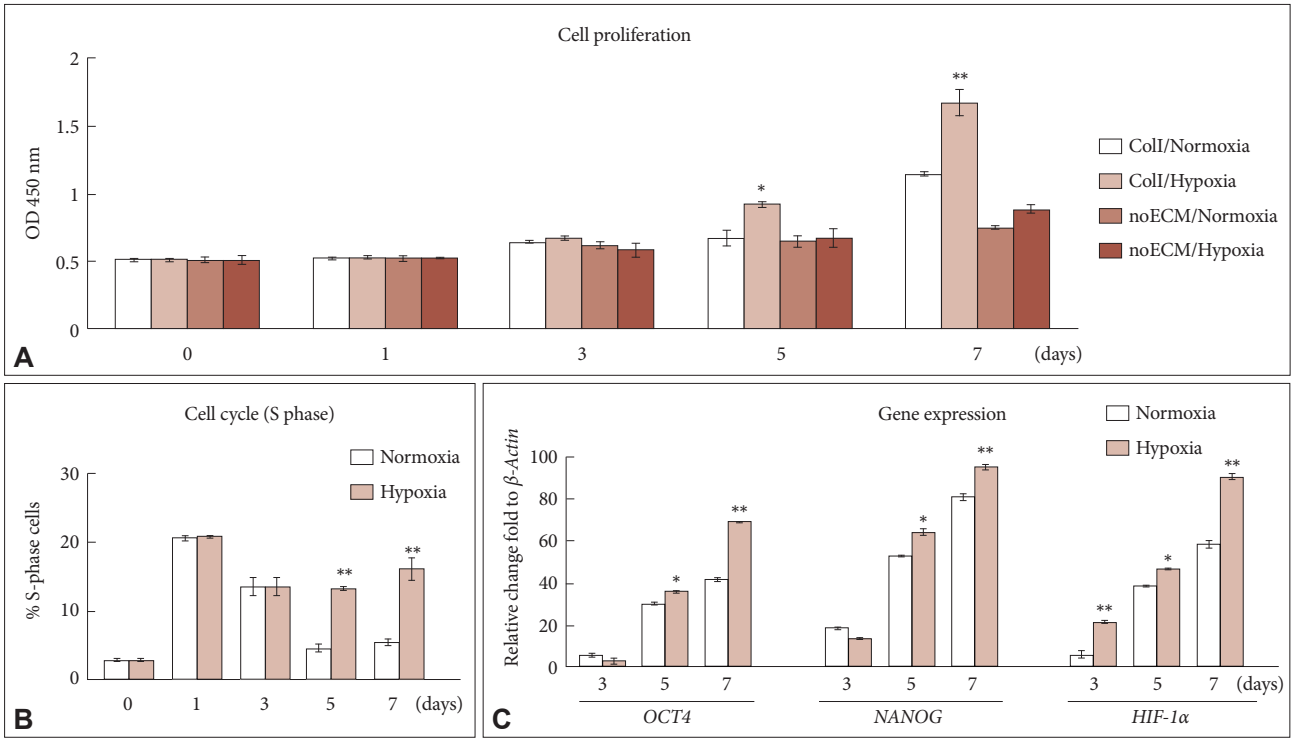

Figure 2. Influence of 5% O_2 hypoxia on cell proliferation, cell cycle distribution, and gene expression. (A) Cell proliferative effect under hypoxic conditions. (B) Analysis of hypoxic/collagen-I (Col1) condition-mediated cell cycle distribution. (C) Effect of the hypoxic/collagen-I condition on retention of stem cell properties. *p<0.05, **p<0.01. ECM: extracellular matrix, noECM: notreated ECM, OD: optical density.

differentiation was assessed using alizarin red S (AR-S) staining. AR-S deposits were recovered by incubating the cells in a solution of 10% cetylpyridinium chloride (Sigma-Aldrich, St. Louis, MO, USA) in 10 mM sodium phosphate, pH 7.0, for 15 min at room temperature. The amount of AR-S was determined by measuring the absorbance at 570 nm. Adipogenic differentiation was induced by treatment with adipogenic medium (α-MEM supplemented with 10% FBS, 1 μM DEX, 111 μg/mL isobutylmethylxanthine, 0.2 mM indomethacin, and 10 μg/mL insulin) for 21 days. Adipogenesis was assessed using Oil Red O staining. In order to obtain quantitative data, 300 μL of isopropyl alcohol was added to the stained cells, and the amount of extracted dye was determined by measuring absorbance at 540 nm using an ELISA plate reader.

Immunogenicity and tumorigenicity *in vivo*

The immunogenicity of USCs, determined by the expression of MHC class II cell surface receptors (HLA-DR, BD Biosciences, San Jose, CA, USA), was evaluated by flow cytometry. For the analysis of tumorigenicity, 1×10^6 USCs were injected into the subcapsular space of kidneys in mice (n=9). The mice were sacrificed 8 weeks later and kidneys were harvested for histological confirmation.

Statistical analysis

The data are presented as mean±SD. The statistical analysis was conducted by Kruskal-Wallis test and one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant. When the value was found to be significant after assessment using the ANOVA statistical test, Tukey's post-hoc comparison was used.

RESULTS

Selection of the optimal ECM

The optimal ECM for uUSC expansion was determined on the basis of cell proliferation rate and gene expression. The USCs cultured on ECM-coated plates showed higher prolifera-

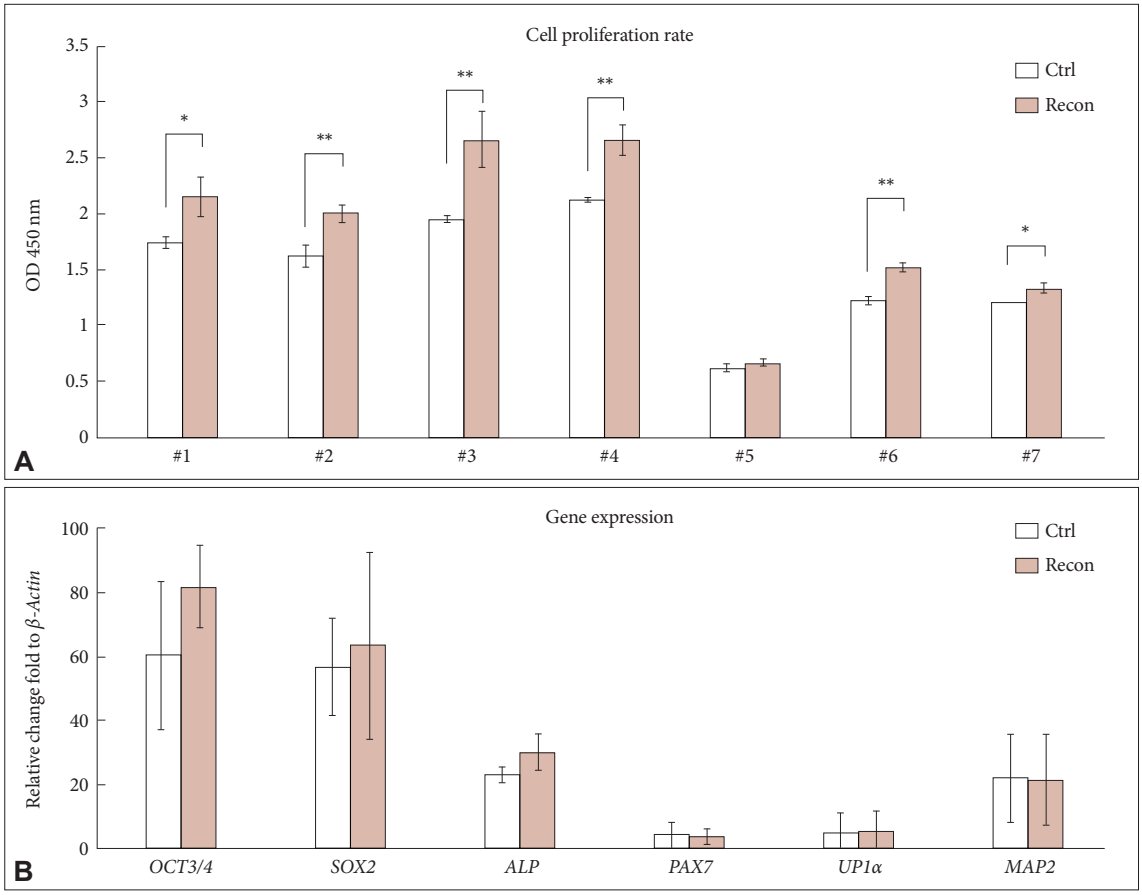

Figure 3. Comparison of USCs between the control and reconstituted culture conditions. (A) Effect of the reconstituted culture condition on the proliferation rate of cells from the samples obtained from 7 patients. (B) Effect of the reconstituted culture condition on retention of stem cell properties and suppression of spontaneous differentiation. *p<0.05, **p<0.01. USC: urine-drived stem cell, OD: optical density.
(Continued to the next page)

tion rates than cells cultured on regular culture plates from day 1 (p<0.0001). After 3 days of culture, enriched populations of USCs emerged on collagen, with a higher number of cells that that obtained on the control, fibrinogen, fibronectin, and laminin (p<0.05). The mean optical densities of USCs at day 7 on control, ColI, ColIV, fibrinogen, fibronectin, and laminin were 0.91±0.2, 1.89±0.07, 1.53±0.13, 1.40±0.04, 1.27±0.03, and 1.21±0.01, respectively (p<0.01) (Fig. 1A); ColI-coated surfaces were associated with enhanced cell proliferation. To detect undesirable differentiation induced by the ECM treatment, stem cell differentiation markers were analyzed with real-time PCR. The ColI-coated condition showed high expression of stem cell marker *OCT4* and low expression of differentiation markers for muscle, neuron, and epithelium progenitors, *PAX7*, *MAP2*, and *UP1a*, respectively (Fig. 1B). Based on these results, ColI was selected as the optimum ECM protein for *ex vivo* expansion of USCs.

Effect of hypoxia and selection of reconstituted culture conditions

Next, the oxygen tension effect was analyzed based on the cell proliferation rate, cell cycle, and stem cell marker expression. When USCs were cultured in hypoxic or normoxic conditions with ColI or without, such as ColI/hypoxia, ColI/normoxia, noECM/hypoxia, and noECM/normoxia, the proliferation rate was the highest in the ColI/hypoxic condition at day 7. The mean optical density of each condition was 1.67±0.10, 1.15±0.02, 0.89±0.03, and 0.75±0.01, respectively (p<0.01) (Fig. 2A).

Based on this result, the effect of advanced oxygen tension was analyzed in a ColI-coated condition. To analyze the cell proliferation stimulation mechanisms with hypoxia, cell cycle distribution was analyzed. The USCs under hypoxic conditions showed a significantly increased percentage of cells in the S phase from day 5, while the USCs under normoxic conditions displayed a significantly decreased percentage of cells in the S phase at the same time point (Fig. 2B). The differences in genetic expression of expanded USCs in a hypoxic condition were d by real-time PCR for the retention of stem cell and hypoxia markers (*OCT4*, *NANOG*, and *HIF-1α*). The expression levels of these markers under hypoxic conditions were significantly higher than those under normoxic conditions from day 5 (p< 0.01) (Fig. 2C). These results indicate that hypoxic conditions

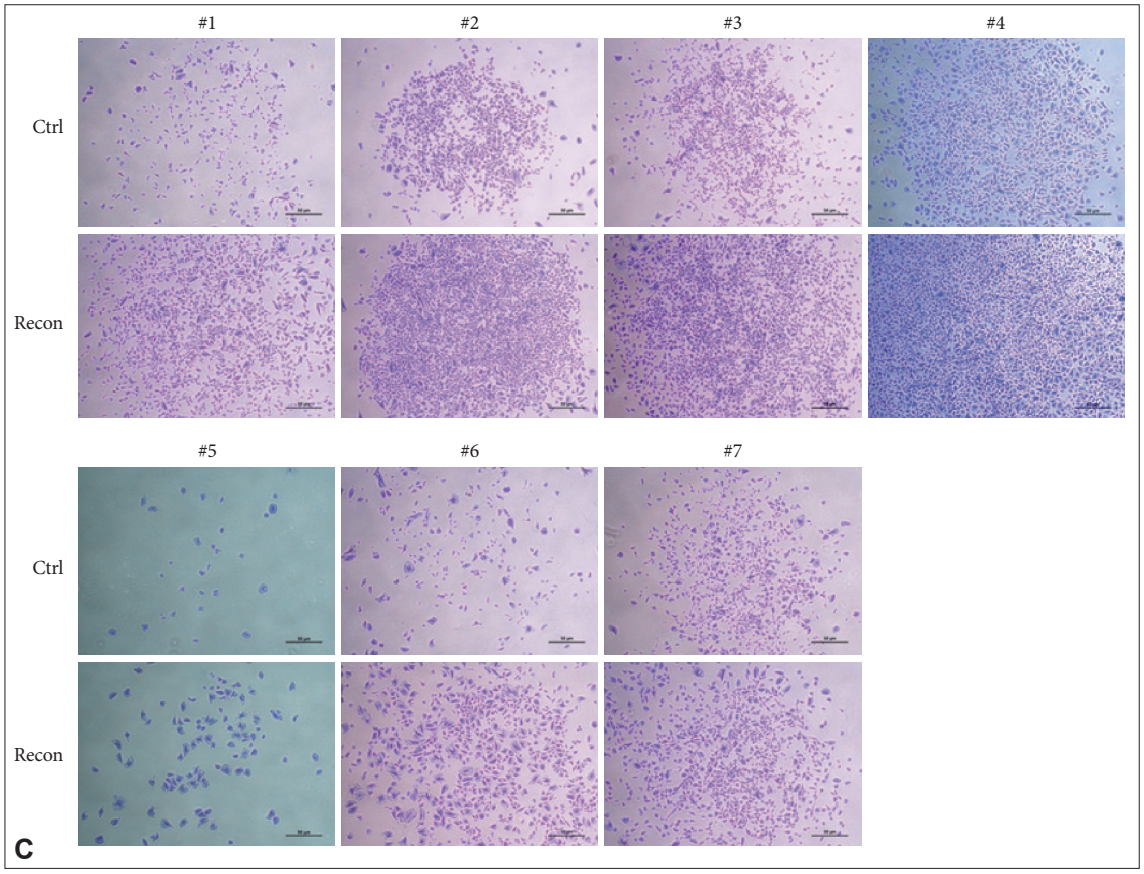

Figure 3. (Continued from the previous page) (C) Comparison of the colony forming ability of cells cultured under the control and reconstituted conditions. Crystal violet stain 40×.
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could accelerate expansion of USCs. Therefore, ColI/hypoxia was determined to be the optimal reconstituted culture condition.

Comparisons of cells under control and reconstituted culture conditions

To determine whether the reconstituted culture condition has an effect on the cell proliferation rate, colony forming ability, immunophenotypes, stem cell transcription factor expression, differentiation, immunogenicity, and tumorigenicity, the previously reported culture condition (no ECM/normoxia) was used as a control in our comparisons.

In the cell proliferation analysis after 7 days of culture, 6 of 7 cases showed significantly increased proliferation rates in USCs^{recon} compared to USCs^{ctrl}. In the cases of #1 and #7, the *p*-values for this difference were <0.05, and in #2, #3, #4, and #6, these values were <0.01. In contrast, case #5 did not show an effect on cell proliferation with the reconstituted condition (Fig. 3A). Whether USCs^{recon} can retain stem cell properties and whether they undergo spontaneous differentiations were analyzed by real-time PCR of cells from experimental plates (n=7). Stem cell markers were highly expressed in USCs^{recon} compared to USCs^{ctrl}. Spontaneous differentiations were effectively prevented in the reconstituted condition (Fig. 3B). The assessment of the ability to form colonies revealed that the size of the colonies of USCs^{recon} were considerably larger than those of USCs^{ctrl}. The mean colony numbers in the reconstituted condition and the control at day 7 were 28.33±6.23 and 15.32±4.08, and colony sizes were 196.5±1.99 μm^2 and 115.5±1.35 μm^2

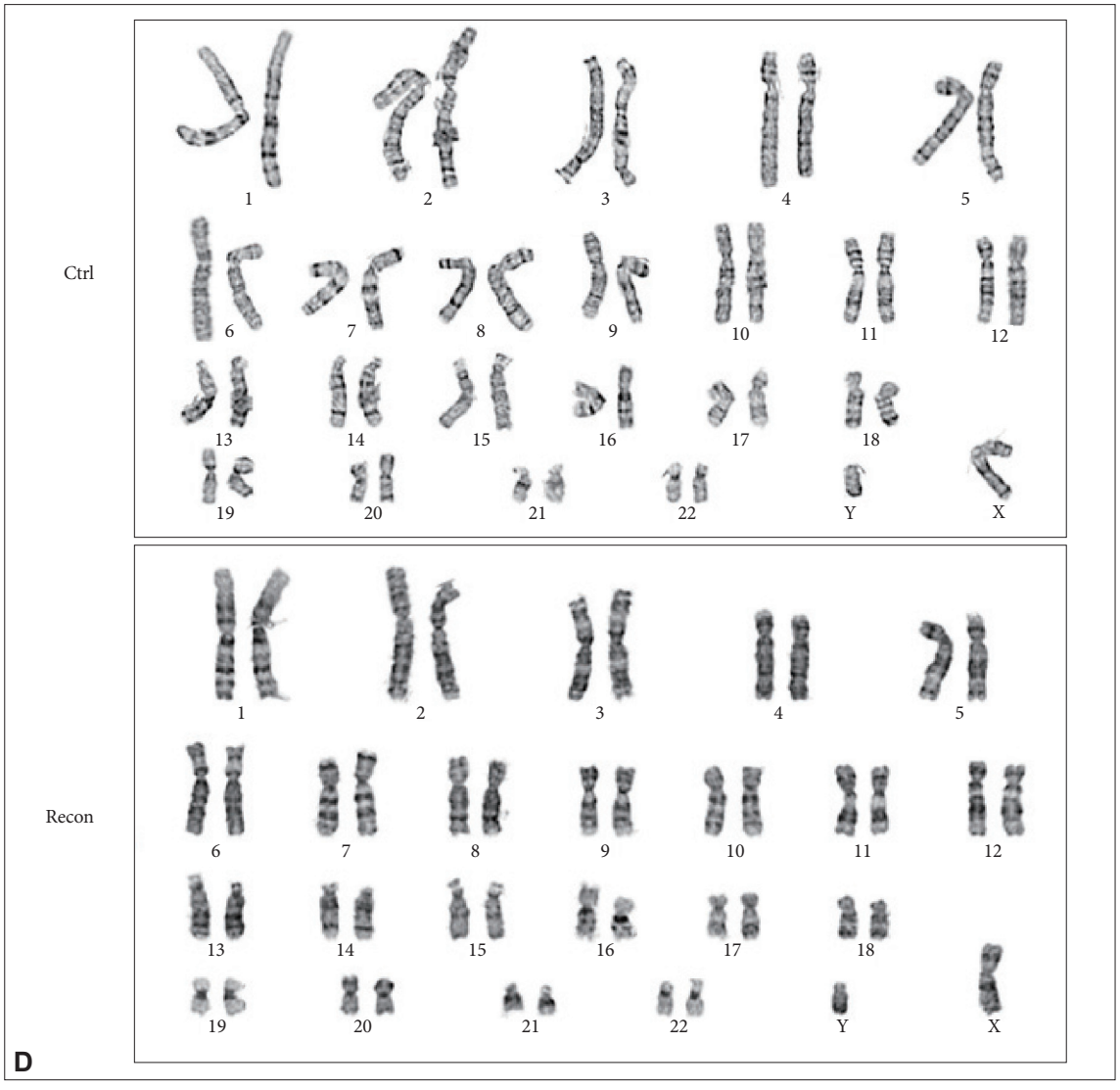

Figure 3. (Continued from the previous page) (D) G-banded karyotype of expanded USCs under the control and reconstituted culture conditions at passage 6 (representative image from #1 patient). USC: urine-derived urine stem cell. (Continued to the next page)

(Fig. 3C). In flow cytometry, the expected immunophenotypes such as CD34$^-$, CD44$^+$, CD45$^-$, and CD90$^+$ were retained in USCs^{recon} (Table 3). A karyotype analysis was performed to evaluate the chromosomal stability of expanded USCs^{recon}. Expansion of USCs^{recon} for 4 passages resulted in cells with normal karyotypes in all examples. A normal diploid complement of autosomes (22 pairs) and 2 sex chromosomes (X and/or Y) suggest that the reconstituted condition for uUSC expansion is a safe approach (Fig. 3D, representative image).

To ensure maintenance of the differentiation capacity of cells under the reconstituted condition, cells were treated with differentiation medium for 7–21 days to differentiate cells into neurons, osteoblasts, and adipocytes. In neurogenic differentiation, 68% of the USC^{srecon} acquired the morphologic and phenotypic characteristics of neurons (*NESTIN*, *MAP2*, *β-TUBULIN III*), as shown by immunocytochemistry. On day 7, *NESTIN*, *MAP2*, and *β-TUBULIN III* mRNA increased 1.5–2 fold over the control (Fig. 3E). In osteogenic differentiation, approximately 60% of the USCs^{recon} acquired an osteogenic morphology and 30% of the cells showed calcium deposits. The USCs^{recon} showed significantly enhanced expressions of osteogenic markers for bone sialoprotein, ColI, osteocalcin, and osteonectin at day 14 (Fig. 3F). In adipogenic differentiation, more than 70% of the USCs^{recon} acquired an adipogenic phenotype and expressed PPARγ2, adiponectin, and leptin. On day 21, adiponectin and leptin mRNA were upregulated 1.3–1.8 fold over the control, and about 30% of cells were visualized in oil droplets Oil Red O staining (Fig. 3G). Therefore, the reconstituted condition showed morphologic, phenotypic, and multi-lineage characteristics indicating the potential for therapeutic applicability.

The immunogenicity of cells was analyzed with HLA-DR (Fig. 4A). The USCs^{recon} cultured for 4 passages were negative for HLA-DR, as determined by flow cytometry. The mean value of HLA-DR in the control was 1.54±0.78 and in the reconstituted condition was 1.25±0.88. In the *in vivo* tumorigenicity analysis, no teratoma formations were observed in tissues re-

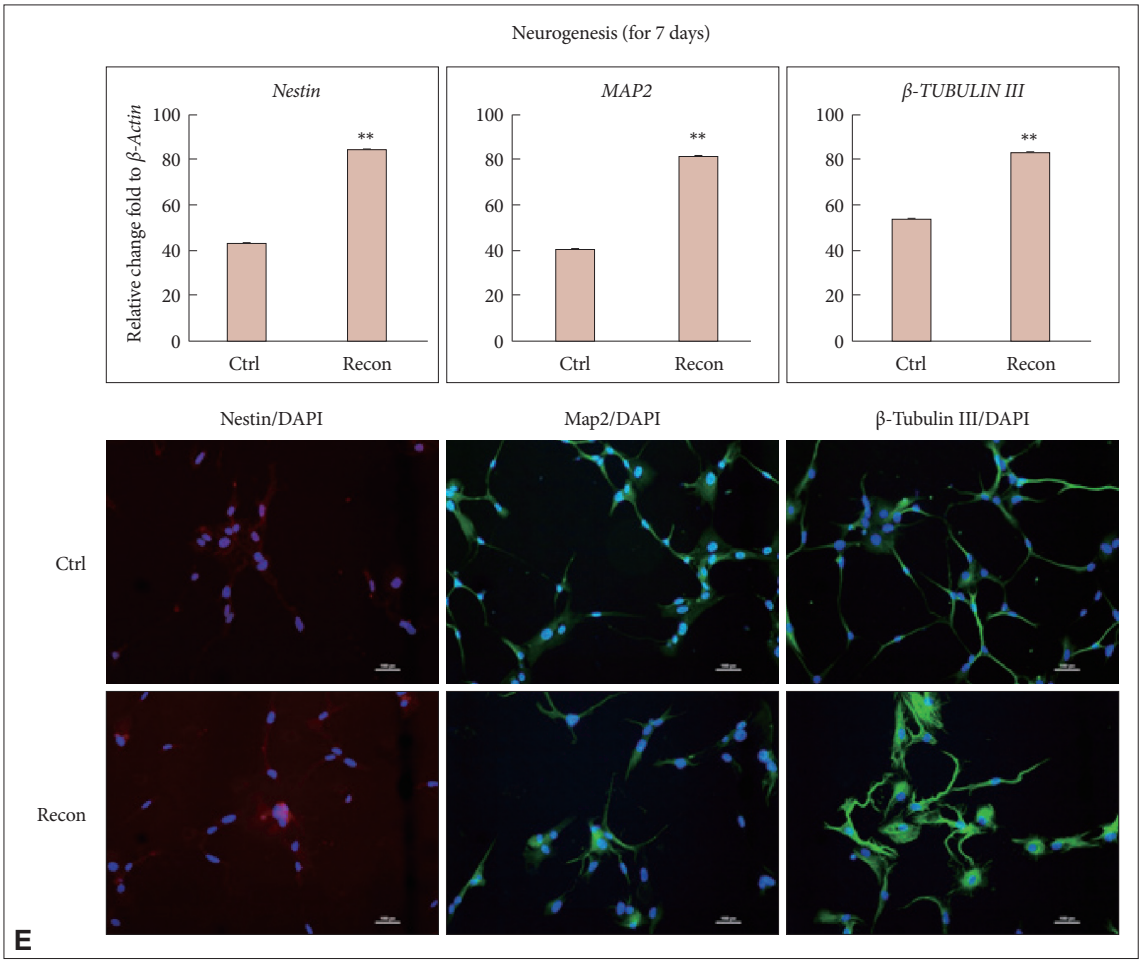

Figure 3. (Continued from the previous page) (E) *In vitro* neuronal differentiation capacity of USCs after expansion under the control and reconstituted culture condition. Immunocyto chemical staining 200×. **p<0.01. USC: urine-derived urine stem cell.

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trieved 8 weeks after renal subcapsular injection of USCs^{recon} (Fig. 4B).

DISCUSSION

In order to achieve the fast expansion of USCs, we established a reconstituted culture condition that was optimized using ColI and hypoxia.

The USCs were cultured on ECM protein-coated plates, and the effects of ECM on cell proliferation and stem cell properties were investigated. The tested ECM types are selected based on the Gordon report [15], which showed that cultured marrow stromal cells made ColI, -IV, fibronectin, laminin, and other adhesive proteins. The reconstitution of the ECM provides the benefit of precise control of uUSC functions, because cell-matrix interactions play critical roles in controlling stem cell properties [16]. Specifically, the ECM and integrins on the cell surface lead to intracellular signals that control critical cell functions [9]. Culture on ColI-coated surfaces showed the greatest enhancement of uUSC proliferation. This effect may be related to the P-15 cell-binding domain of ColI. Incorporation of this peptide has been shown to significantly promote cell adhesion and proliferation [17]. The USCs cultured on ColI may be expected to have clinical benefits because ColI is the main component of the ECM, and pre-transplant exposure may increase the adoption and survival of stem cells *in vivo* [18]. Gene expression profiles showed that increased mRNA levels of genes related to stem cell properties, and the downregulation of differentiation genes, supporting the observation of accelerated uUSC expansion on ColI without loss of stem cell properties. Therefore, ColI can be a valuable choice for creating a stem cell niche.

Conventional *in vitro* culture does not mimic normal physiological conditions. In the usual cell culture in an incubator, the oxygen tension is 20%, which is higher than the stem cell niche *in vivo* (0.7–7% O_2) [9], and exposure to high levels of oxygen can cause side effects in cells [13]. Hypoxic culture conditions represent a physiologically relevant oxygen pressure and can

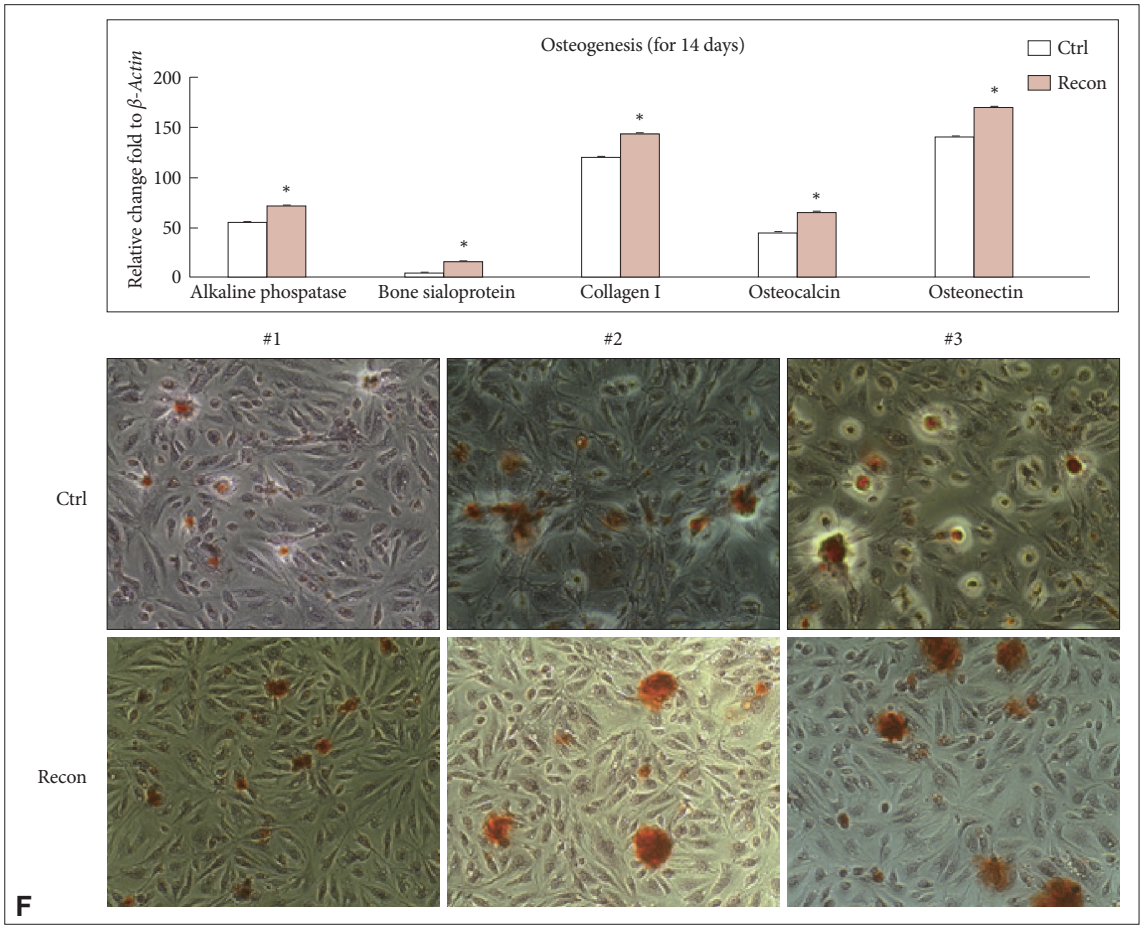

Figure 3. (Continued from the previous page) (F) *In vitro* osteogenic differentiation capacity of USCs after expansion under the control and reconstituted culture condition. Alizarin red S staining 200×. *p<0.05. USC: urine-derived urine stem cell.

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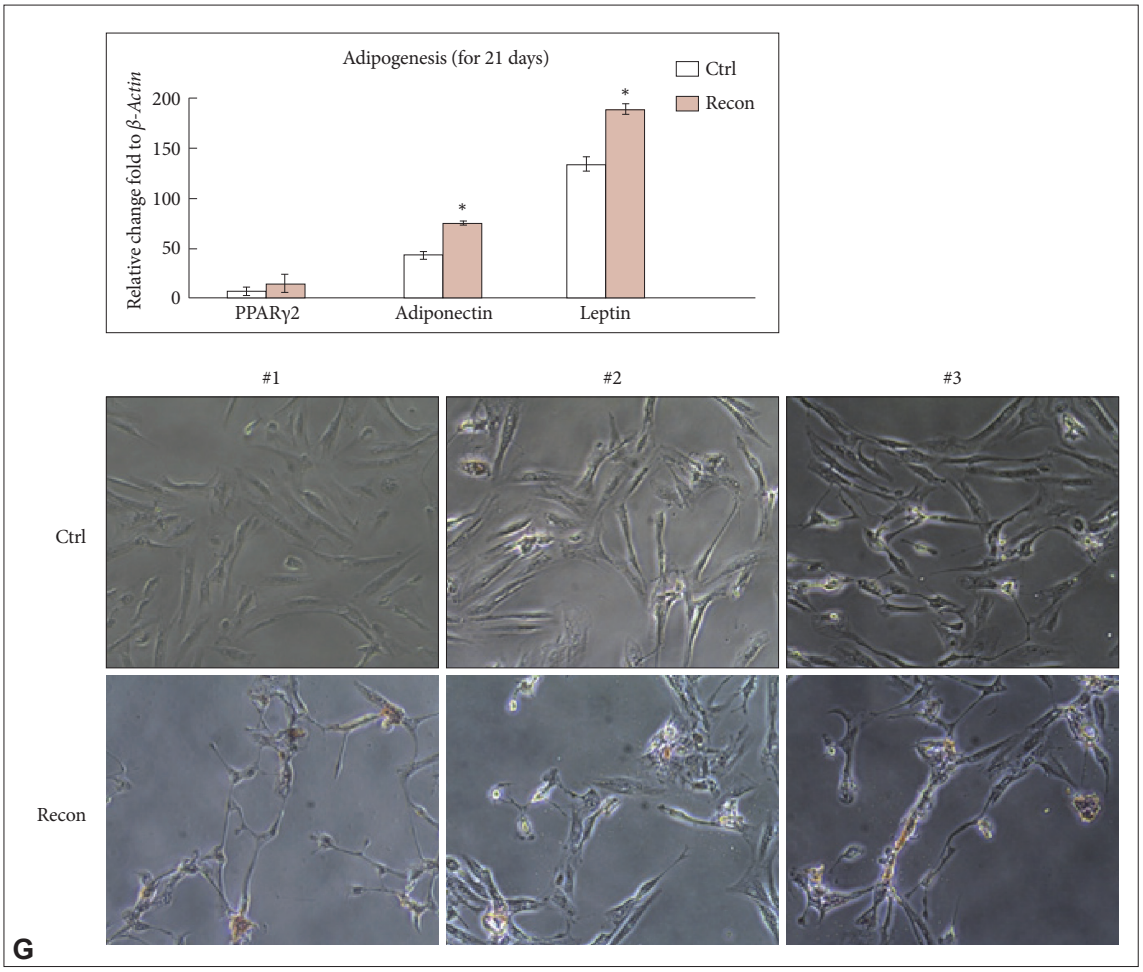

Figure 3. (Continued from the previous page) (G) *In vitro* adipogeric differentiation capacity of USCs after expansion under the control and reconstituted culture condition. Oil Red S staining 200×. *p<0.05. USC: urine-derived urine stem cell.

Table 3. Flow cytometry analysis

Case No.	Group	FACS (normalized % by isotype, at 7 day)			
		CD34	CD44	CD45	CD90
1	Ctrl	0	98.83	0.07	95.84
	Recon.	0	98.79	0.21	95.87
2	Ctrl	0.02	97.89	0	98.53
	Recon.	0.04	98.77	0.17	96.09
3	Ctrl	0.08	98.09	0.02	94.65
	Recon.	0.12	94.61	0.07	94.60
4	Ctrl	0	92.38	0	46.82
	Recon.	0	95.28	0.03	50.99
5	Ctrl	1.07	89.05	1.49	45.61
	Recon.	0.09	93.45	0.01	23.10
6	Ctrl	0.23	80.92	0.02	90.54
	Recon.	0.27	89.39	0	93.63
7	Ctrl	0.32	99.13	0.07	25.88
	Recon.	0.20	99.47	0.09	12.11

mimic the *in vivo* environment. In addition, exposure to hypoxia before cell transplantation is a useful pre-conditioning technique to enhance stem cell efficacy. The adopted cells cultured under hypoxic conditions showed a highly potent phenotype for regeneration [18]. Culture under hypoxic conditions (with ColI) showed significantly enhanced uUSC proliferation from day 5, and the cell proliferation rate was significantly increased at day 7. In a low oxygen condition, stem cell expansion is increased and differentiation is reduced, because the hypoxia-inducible transcription factor (HIF)-1α inhibits the LIF-STAT3 pathway [19]. In addition, the number of cells in the S phase of the cell cycle can be another indicator for the cell proliferation rate, because cell proliferation is related to DNA replication [20]. Gene expression profiles showed increased mRNA levels of genes associated with stem cell properties (*OCT4*, *NANOG*, and *HIF1α*). These results support the view that a low oxygen environment is an essential condition for the retention of stem cell properties of USCs.

For future clinical applications, the reconstituted condition (ColI and 5% O_2 hypoxia) was compared to the previously reported culture condition (control) by analyzing cell proliferation, gene expression, colony forming ability, surface markers, chromosome stability, and multi-lineage differentiation capabilities.

In the cell proliferation analysis, 6/7 cases of USCs^{recon} showed significantly enhanced cell proliferation at day 7 compared to USCs^{ctrl}. In the gene expression assay with real-time PCR, stem cell markers, such as *OCT4*, *SOX2*, and *ALP*, were highly expressed in USCs^{recon}, while differentiation markers for myoblasts (*PAX7*), epithelial cells (*UROPLAKIN 1α*, *UP1α*), and neurons (*MAP2*) were reduced in USCs^{recon}. We observed a more than 1.85-fold increase in colony forming units in our culture when compared to the control. By flow cytometry, both cells showed similar, representative mesenchymal marker profiles. The USCs^{recon} were uniformly positive for CD44 and CD90, and negative for the hematopoietic lineage markers, including CD34 and CD45. No phenotypic differences were observed when comparing USCs^{recon} and USCs^{ctrl}. These results indicate that the reconstitut-

A

HLA-DR	#1	#2	#3	#4	#5	#6	#7	Average
Ctrl	0.67	0.93	2.43	1.55	2.1	0.54	2.53	1.54±0.78
Recon	0.82	1.81	2.1	1.07	2.6	0.06	0.27	1.25±0.88

B

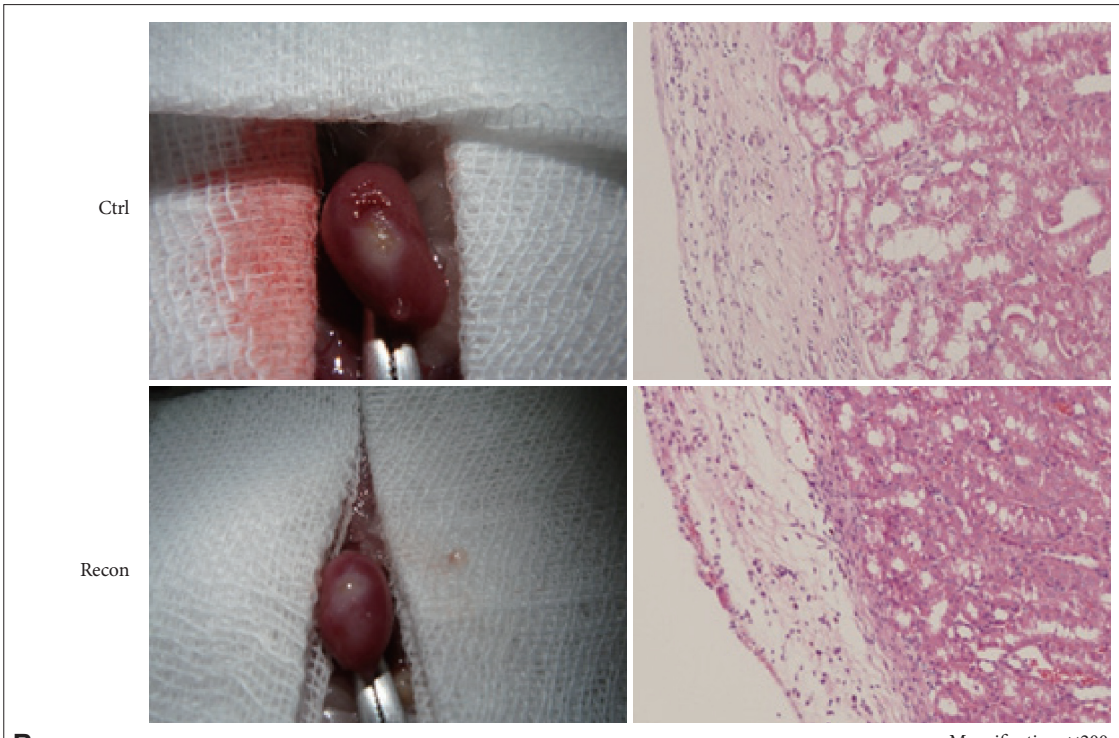

Figure 4. Free immunogenicity and tumorigenicity of USCs under the control and reconstituted culture conditions. (A) Immunogenicity analysis with HLA-DR expression. (B) Tumorigenicity analysis 8 weeks after renal subcapsular injection of cells. Ctrl: control culture condition, Recon: reconstituted culture condition, USC: urine-derived urine stem cell.

ed condition can stimulate cell proliferation, retain stem cell properties, and inhibit cell differentiation.

Genomic stability is an important concern when cells are expanded *in vitro*, because *in vitro* expansion of the mesenchyme leads to spontaneous transformations [21]. To determine the effect on genomic stability of the reconstituted condition, a cytogenetic study was performed. Karyotype analysis over 4 passages revealed that USCs^{recon} displayed normal G-banding patterns without numeral or structural abnormalities.

Differentiations into neurons, osteoblasts, and adipocytes were used to demonstrate the multi-potency of USCs^{recon}. Each differentiation was induced in the expanded USCs^{recon} by treatment with a conditioned or commercial medium. Induction was apparent by the expression of lineage-specific markers and cell morphology. The differentiation of cells into neurons was evident by the expression of *NESTIN*, *MAP2*, *β-TUBULLIN III* mRNA. Differentiated osteoblasts expressed bone sialoprotein, ColI, osteocalcin, and osteonectin. Differentiated adipocytes expressed PPARγ2, adiponectin, and leptin. All of the genes, except PPARγ2, showed significantly greater expression in the reconstituted condition compared to control, indicating that *ex vivo* expanded USCs conserved their multi-lineage differentiation capability. This result represents a major benefit to the utilization of USCs^{recon} for autologous stem cell therapy.

One of the most interesting properties of USCs^{recon} is their immunomodulatory function. Their quality of non- or hypo-immunogenicity is supported by the lack of expression of the cell surface marker for HLA-DR (a MHC class II cell surface receptor), meaning that USCs^{recon} could not elicit lymphocyte proliferative responses. In addition, when USCs^{recon} were injected into the subcapsular space of kidneys, they did not develop tumors but rather formed an osteoid-like tissue mass. Similarly, pellets of mesenchymal stem cells form hard tissue when the three-dimensional culture conditions provide the proper microenvironment for osteo/chondrogenic differentiation [22]. Taken together, our reconstituted condition allows for expanded uUSC cell preparations that are safe and useful for application in stem cell therapy.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Ethics Committee of the Kyungpook National University School of Medicine (IRB no. KNUH 2012-10-018).

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