

Chapter 2

Urine-Derived Stem Cells: Biological Characterization and Potential Clinical Applications

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Abstract A subpopulation of urine-derived cells, termed urine-derived stem cells (USCs), possess stem cell capabilities, such as self-renewal and multipotential differentiation. These cells can differentiate into mesodermal cell lineages, such as osteocytes, chondrocytes, adipocytes, endothelial cells, and myocytes, including smooth muscle cell differentiation and endodermal lineages (e.g., urothelial cells). These cells maintain high telomerase activity and possess long telomeres; further, they retain a normal karyotype in vitro even after several passages. Importantly, these cells do not form teratomas in vivo. USCs express cell surface markers associated with pericytes and mesenchymal stem cells. These cells can be isolated from regular voided urine from each individual via a noninvasive, simple, and low-cost approach. The USCs isolated from one single urine specimen can generate up to 100 million cells at early passage, sufficient numbers to use for cell-based therapy for tissue repair.

Keywords Stem cells • Urine • Cell differentiation • Urinary tract system • Tissue regeneration

Abbreviations

3-D	Three-dimension
ECs	Endothelial cells
EFM	Embryonic fibroblast medium

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EGF	Epidermal growth factor
FDA	Food and Drug Administration
HUVECs	Human umbilical venous endothelial cells
KSFM	Keratinocyte serum-free medium
MSC	Mesenchymal stem cells
PD	Population doublings
PDGF-r β	Platelet-derived growth factor-B and -receptor
RPM	Revolutions per minute
SIS	Small intestinal submucosa
SMCs	Smooth muscle cells
UCs	Urothelial cells
UPCs	Urine-derived progenitor cells
USCs	Urine-derived stem cells
uUSCs	Stem cells collected from upper urinary tract
VEGF	Vascular endothelial growth factor
vUSCs	Stem cells collected from voided urine samples
vWF	Von Willebrand factor
α -SM actin	Alpha-smooth muscle actin

2.1 Introduction

Each tissue and organ in the body has a population of stem/progenitor cells which are involved in tissue repair and regeneration after injury. When these cells are harvested and expanded in culture, their potential to differentiate into various cell lineages allows them to be used for cell-based therapies and tissue engineering. Currently, cells for use in tissue repair are usually obtained from biopsies of specific tissues. The utility of expanded cell populations from such biopsies has been demonstrated in the production of tissue-engineered bladders and urethral tissue. However, potential complications of the biopsy procedure include bladder or urethral trauma, local tissue bleeding, infection and patient discomfort. To eliminate these complications and decrease medical costs, a noninvasive procedure to obtain cells would be highly desirable.

We recently demonstrated that it is possible to isolate and expand stem/progenitor cells from human-voided urine (voided USCs) [1–3] and urine obtained from the upper urinary tract (uUSCs) [4]. Approximately 0.2 % of cells collected from voided urine express markers characteristic of mesenchymal stem cells (MSCs), and they can expand extensively in culture. USCs have self-renewal capability consistent with stem cells. These cells can grow up from a single cell clone to large amounts of cells with an average doubling time of 20–31 h, depending on the passage number, and these cultures can be maintained for up to 57 population doublings [4, 5]. Importantly, USC can differentiate toward multiple bladder cell lineages as identified by the expression of urothelial, smooth muscle, endothelial and interstitial cell markers. In recent experiments, our study indicated that

urine-derived cells can give rise to additional specialized types, including osteocytes, chondrocytes, and adipocytes. The benefits of employing USC's are that these cells can be obtained noninvasively, using a simple, low-cost technology to harvest cells with good quality and quantity [1]. Here, we review the biological characterization and the potential clinical applications of urine-derived stem cells based on our previous data.

2.2 Isolation of USC's

In our previous description of the isolation of USC's [1], mid- and last stream urine was collected, and these urine samples were centrifuged. The supernatant was removed. The cell pellet was gently resuspended in mixed media composed of embryonic fibroblast medium (EFM) and keratinocyte serum-free medium (KSFM) (1:1 ratio) and the cells were plated in 24-well plates (*p0*). Three types of living cells exist in urine: differentiated, differentiating, and urine-derived stem cells. About 99 % of the living cells in urine did not attach to culture plates and were removed when the culture medium was changed. Morphologically, these cells were large and flat epithelial cells, suggesting that they were terminally differentiated. About 0.1 % of cells in urine are differentiating cells. These cells attached to culture dishes, expanded to about 10^3 cells within 2–3 weeks and consisted of at least four cell types based on morphology and phenotype. Some cells had a cobblestone appearance under phase contrast microscopy and they expressed uroplakin on immunofluorescence staining, indicating that they were of urothelial origin, while other cells were spindle shaped and expressed desmin, suggesting that they were of muscle origin. A third cell type had a circular appearance and expressed Von Willebrand factor (vWF), indicating an endothelial origin. Finally, cells with an elongated appearance were found to express c-kit, and these were considered to be interstitial cells. However, the number of all four differentiating cell types in primary culture gradually decreased after 3–4 weeks and did not grow after subculture.

About 0.2 % of the cells in urine have a phenotype consistent with multipotent stem cells and we designated them as urine-derived stem cells. These cells are easily cultured, appear genetically stable after a number of passages, and maintain the ability to give rise to more differentiated progeny. USC's comprised an average of 5–10 cells per 100 ml urine. USC clones were obtained from almost all of the urine samples we tested. Fresh urine showed the highest rate of colony formation (67 %) and urine stored at 4 °C showed the lowest rate (30 %). Urine from 13 to 40-year-old volunteers provided the highest rate of clone recovery. Catheterization significantly enhanced the number of USC's in urine compared to spontaneously voided urine, possibly because more cells were scraped off the inner bladder wall by the catheterization procedure. Collecting triple urine samples also increased the rate of clone formation compared to using single urine samples. A few days after being placed in a well, a single cell formed a cluster of cells that appeared small, compact and uniform (Fig. 2.1). A consistently high yield of cells was achieved from each clonal line.

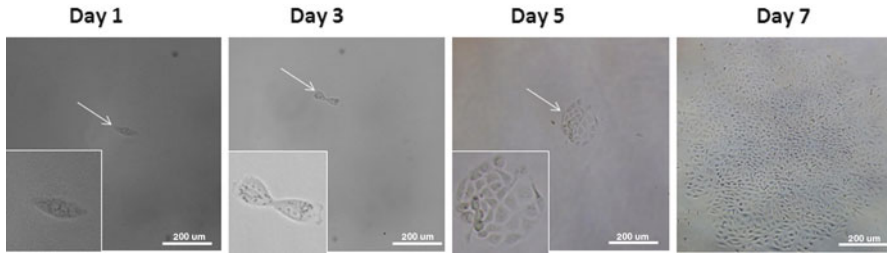


Fig. 2.1 One single USC (p0) that propagated into a clone. One single cell was founded at day 1 and it splited to two cells at day 3. A minor USC clone formed at day 5 and small, compact, and uniform “grain rice”-like clone was formed at day 7. Scale bar=200 μm

About 2 weeks were required for cells to attain confluence at passage 1 in a 3 cm diameter well, 3½ weeks to expand to approximately one million cells in a 10 cm culture dish at passage 2, and 6–7 weeks to expand to approximately 100 million cells at passage 4. These cells showed normal exponential cell growth patterns with a steady increase in number during a 10-day culture period. These urine-derived cells also showed the ability to differentiate into various cell lineages as described below, and were capable of growing for up to 19 passages in vitro.

2.3 Bio-characterization of USCs

Cells from human urine specimens can be consistently cultured long term using a medium that we originally developed for culture of rat urothelial cells (USc) [6]. However, we found that the phenotype of the cultured human urine-derived cells was not that of primary urothelial cells. The primary cultures from urine did not show expression of UC-specific markers such as uroplakin I/IIIa, and cytokeratins (CK7, CK13, and CK19/20). Instead, the cells that we have designated as USCs displayed a surface marker phenotype consistent with MSCs and pericytes, namely CD44, CD73, CD90 (Thy-1), CD105 (endoglin), CD133, CD146, NG2, and PDGF- $\text{r}\beta$. However, the percent of cells expressing these markers decreased with increasing passage number [7–11]. These cells were negative for the general hematopoietic cell marker CD45, hematopoietic stem cell markers, and other hematopoietic and endothelial lineage markers, including CD31 and CD34, indicating that these cells were not endothelial or hematopoietic progenitor cells.

USCs initially derived from a single cell are able to give rise to both UCs and SMCs [4]. After growth in medium containing epidermal growth factor (EGF, 30 ng/ml), the cells were efficiently induced to express the uroplakin and cytokeratin gene and protein markers [4]. After culture in a medium specialized for myogenic differentiation, the differentiated USCs expressed markers consistent with smooth muscle, including alpha-smooth muscle actin (α -SM actin), desmin, calponin, and myosin [1, 4]. We concluded that the urine-derived progenitors were at

least bipotential for the major bladder cell types. This result was surprising, because it was generally believed that muscle and uro-epithelial cells in bladder represent separate cell lineages derived from mesoderm and endoderm, respectively. We initially designated these cells as urine-derived progenitor cells, or UPCs, but we have recently observed that USCs can also differentiate to yield the characteristic cell lineages obtained from MSCs, such as osteocytes, adipocytes, and chondrocytes [12]. This led us to conclude that the urine-derived cells were stem cells capable of giving rise to both mesoderm and endoderm lineages.

The quality of cells obtained from urine is similar to that of the biopsy-derived cells described above. When differentiated, USCs express all proteins characteristic of the various bladder cell lineages. Karyotype analysis has demonstrated that these cells are genetically stable. Importantly, there is a major cost advantage to using USCs—it costs about US\$50 to obtain cells from urine, versus about US\$5,000 to isolate cells from a biopsy procedure. About 1.4×10^9 urothelial and SMCs are required for bladder tissue regeneration¹. We estimate that three to four urine samples (about 25–40 USC/800 ml urine) expanded for 4–5 weeks would yield a sufficient quantity of low passage, healthy cells for clinical tissue engineering applications. This time frame is comparable to that required for expansion from a tissue biopsy (7–8 weeks) [13]. USCs and the cells obtained through urological tissue biopsies come from the same urinary tract systems and have similar biological features. Therefore, collecting cells from urine could be an attractive alternative to the standard urological tissue biopsies currently used in cell therapy and tissue engineering.

2.4 Characterization of uUSCs

More recently, we found that some urine-derived cells from the upper urinary tract possessed characteristics similar to vUSCs, i.e., expansion capacity and bipotent differentiation to urothelium-like and SMC-like cells. The uUSCs can generate a large cell population from a single cell, like voided USC. We observed that the average expansion capacity of uUSCs is 46.5 ± 8.6 population doublings (PD) (range 35–57 PD, $n=4$). This implies that a single stem cell from the upper urinary tract, on average, can generate 1.0×10^{14} cells ($2^{46.5}$), within about 8 weeks. To retain good bipotent differentiation capacity, we typically use USC below expansion passage 5 (p5). Under our optimized culture conditions, one single cell of uUSC can generate $2^{28.6} = 4.0 \times 10^8$ cells within about 4 weeks [4], at p5. It is known that 1.4×10^9 cells are required for both SMCs and UCs to create a tissue-engineered bladder². Our recent data showed about 150 ml of urine obtained from the upper urinary tract via nephrostomy tube contains ten uUSC clones. Expansion of the stem cells from this volume of urine potentially can yield about 4×10^9 cells. Thus, assuming efficient differentiation, uUSCs can provide an adequate number of cells to engineer a neo-bladder. Importantly, uUSCs are a reliable cell source, as cell clones can be obtained from almost every urine sample [4]. It appears that uUSCs become voided USC when urine drains from the kidney to the bladder for storage.

In chronic bladder diseases or muscle-invasive bladder cancer, uUSCs might be a good cell source for bladder tissue regeneration because the cells from the upper urinary tract are usually normal. In addition, the risk of finding ureter, renal pelvic, or kidney cancer in bladder cancer patients could be eliminated with careful scanning by a series of examinations. These scanning examinations include urine cytology, imaging tests (such as intravenous pyelogram, bone scan, computed tomography scan, magnetic resonance imaging, and lung X-ray), and cystoscopy/nephro-ureteroscopy and tissue biopsy from upper urinary tract. In treatment of end-stage bladder diseases or bladder cancer, using engineered bladder tissue with uUSCs as the cell source would be superior to current surgical procedures, i.e., bladder reconstruction using intestinal segments. Risks of use of bowel segments include: (1) tumorigenicity, as intestinal segments appear to be at an increased risk for malignancy, particularly adenocarcinoma, because of histological changes in the intestinal mucosa after long-term exposure to urine; and (2) complications such as stone formation and excess mucous secretion. Harvesting uUSC from patients who already have a nephrostomy tube in place would be a simple and low-cost approach to obtaining cells for engineering bladder tissue. Therefore, cells derived from upper urinary tract urine might be a good source for bladder tissue engineering in patients with bladder cancer [4].

2.5 Interaction of USC and Biomaterials for Tissue Engineering

Combining autologous stem cells with natural or synthetic biomaterial scaffolds provides a promising strategy for cellular delivery and engineering tissues. When combined with appropriate scaffold materials, USCs could be effectively used in urological tissue engineering. We seeded USCs or urothelial and smooth muscle cells differentiated from USCs onto a porous bacterial cellulose scaffold or modified three dimension (3D) porous small intestinal submucosa (SIS) scaffold under dynamic culture conditions to generate a cell-based tissue-engineered urinary conduit or urethra [2, 14]. Porous bacterial cellulose and SIS provided a 3D cell growth environment *in vitro*. As a nondegradable material, bacterial cellulose is an attractive candidate for creating a tissue-engineered conduit because this Food and Drug Administration (FDA)-approved biomaterial [15] is highly hydrophilic and causes little fibrosis when implanted [16]. This polymer is biosynthesized as a network of nanofibrils. The fibril entanglement and hydrogen bonding within the cellulose network provides high mechanical strength and a large surface area [17]. When implanted subcutaneously in rats, bacterial cellulose does not elicit fibrosis or induce proliferation of giant cells [16]. Bacterial cellulose has been shown to remain intact for 90 days when implanted subcutaneously [16]. We chose to use the 300–500 μm pore size range for the USCs seeding experiment because this range would allow adequate space for cell growth and extracellular matrix secretion and

remodeling, as well as for ingrowth of blood vessels from the native tissue after implantation. Therefore, it would be an alternative for bladder replacement when USCs are seeded within the porous bacterial cellulose scaffold.

SIS, another commonly used natural collagen scaffold, possesses a unique property, in which its permeability is “sided,” or direction dependent. The mucosal to serosal direction is less permeable than the serosal to mucosal direction. When non-seeded SIS is used in urological applications, this “sidedness” property should be considered because it can assist in preventing urine leakage from the lumen of the urethra or bladder into surrounding tissues. However, in cell-based tissue engineering, this direction-dependent permeability appears less important in preventing urine leakage, because heavy cellular infiltration “fills up” the pores within the matrix to prevent leakage [18–24]. Therefore, USCs are able to form multilayered tissue structures and grow into the matrix as well when seeded on the more porous serosal side in vitro under dynamic culture conditions. The speed of dynamic culture also affects cell proliferation and multilayer formation on scaffold matrices. For example, it has been shown that when bladder cells are seeded on a collagen matrix such as decellularized bladder submucosa and cultured in dynamic conditions at 40 rpm (RPM), cell layer formation is enhanced compared to both 10 rpm and static culture conditions [25].

Additionally, epithelial–stromal cell interactions in cocultures play an important role in cell growth and are an efficient means of promoting cell growth, cell-matrix infiltration, and cell differentiation. The cell–cell communication present in coculture conditions facilitates cell signaling and thus promotes epithelialization [21]. Layered cocultures of urothelial and smooth muscle-differentiated USCs showed better cell growth and cell-matrix penetration and epithelialization compared to monoculture conditions [25]. The multilayered structure covered the entire surface of the polymer scaffold, with smooth muscle cells infiltrating the scaffold to a large extent.

2.6 Implantation of USCs In Vivo

To monitor the fate of differentiated USCs in vivo, cell-scaffolds were subcutaneously implanted into athymic mice and then tracked using immunohistochemical staining for human nuclear antigen. After USCs were induced to differentiate into urothelial and smooth muscle cells (SMCs), induced USCs (10^6 cells/cm²) were seeded onto scaffolds such as bacterial cellulose or SIS in a layered coculture fashion under static and 3D dynamic (10 or 40 rpm) conditions for 2 weeks. Following the in vitro culture, the cell-scaffold constructs were then implanted in vivo for 4 weeks. This revealed that the porous scaffolds allowed three-dimensional growth of the cells, leading to formation of a multilayered urothelium and SMC-I matrix infiltration [2]. USCs that were induced to differentiate also expressed UC markers (Uroplakin-III and AE1/AE3) or SMC markers (α -SM actin, desmin, and myosin) after implantation into athymic mice for 1 month, and the resulting tissues were similar to those formed when UCs and SMCs derived from native ureter were used [14].

We also evaluated the effects of vascular endothelial growth factor (VEGF) overexpression on urine-derived stem cell survival and myogenic differentiation to determine whether these cells could be used as a novel cell source for genitourinary reconstruction. USC_s were infected with an adenoviral vector containing the mouse VEGF gene (USC_s/Ad-VEGF). USC_s/Ad-VEGF was mixed with human endothelial cells (EC_s) (total, 5×10^6 cells) in a collagen-I gel. These cell containing gels were subcutaneously implanted in an athymic mouse model. USC_s expressed SMC markers after implantation *in vivo*, indicating that VEGF expression enhanced myogenic differentiation of USC_s and muscle regeneration *in vivo*. This result might be due to a direct effect of angiogenesis, an indirect effect mediated by an autocrine factor that promotes muscle cell differentiation or both [26–31]. Our recent study demonstrated that VEGF expression by VEGF-expressing USC_s, along with concurrent endothelial cell implantation, promoted angiogenesis, significantly improved *in vivo* cell survival and myogenic differentiation of USC_s, and enhanced nerve regeneration within the graft, which maintained its size [30]. The safety of using cells that gene overexpress VEGF remains a concern due to the fact that overexpression of VEGF has been associated with urothelial cancer. The optimal dosing of VEGF and long-term follow-up after implantation of cells expressing VEGF requires further investigation. Autologous VEGF-expressing USC_s combined with human umbilical venous endothelial cells (HUVEC_s) as an alternative cell source for urological cell therapy appears feasible and may be useful in genitourinary reconstruction, such as treating vesico-ureteral reflux and stress urinary incontinence with cell therapy or even in repairing urethral stricture and neuropathic bladder with tissue engineering technology.

2.7 Conclusion

There are several potential advantages to using USC_s as a cell source for urological tissue engineering, including the following: (1) the cells can be easily harvested by a noninvasive method and grown in culture, as USC_s do not require enzyme digestion or culture on a layer of feeder cells to support cell growth; (2) cells can be harvested from urine via noninvasive procedures rather than biopsies, and thus patient morbidity and potential complications such as urethral or bladder trauma and urinary tract infections are avoided; and (3) as USC_s are autologous somatic cells, no ethical issues are involved in their use for tissue reconstruction, and no immune reaction to engineered implants should occur. Therefore, obtaining and using cells from urine could be an attractive alternative to the standard urological tissue biopsies currently used in cell therapy and tissue engineering.

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