Differentiation Capacity of Human Urine-Derived Stem Cells to Retain Telomerase Activity

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Telomerase activity is essential for the self-renewal and potential of embryonic, induced pluripotent, and cancer stem cells, as well as a few somatic stem cells, such as human urine-derived stem cells (USCs). However, it remains unclear how telomerase activity affects the regeneration potential of somatic stem cells. The objective of this study was to determine the regenerative significance of telomerase activity, particularly to retain cell surface marker expression, multipotent differentiation capability, chromosomal stability, and in vivo tumorigenic transformation, in each clonal population of human primary USCs. In total, 117 USC specimens from 10 healthy male adults (25–57 years of age) were obtained. Polymerase chain reaction amplification of a telomeric repeat was used to detect USCs with positive telomerase activity (USCsTA+). A total of 80 USCsTA+ (70.2%) were identified from 117 USC clones, but they were not detected in the paired normal bladder smooth muscle cell and bone marrow stromal cell specimens. In the 20–40 years age group, approximately 75% of USC clones displayed positive telomerase activity, whereas in the 50 years age group, 59.2% of the USC clones expressed positive telomerase activity. USCsTA+ extended to passage 16, underwent 62.0 ± 4.8 population doublings, produced more cells, and were superior for osteogenic, myogenic, and uroepithelial differentiation compared to USCsTA−. Importantly, USCs displayed normal chromosome and no oncological transformation after being implanted in vivo. Overall, as a safe cell source, telomerase-positive USCs have a robust regenerative potential in cell proliferation and multipotent differentiation capacity.

Keywords: telomerase, urine-derived stem cells, longevity, tissue regeneration, differentiation

1 INTRODUCTION

Telomerase activity (TA) is closely related to the longevity of pluripotent stem cells (Huang et al., 2014; Li et al., 2020), embryonic stem cells (ESCs) (Hiyama and Hiyama, 2007), induced pluripotent stem cells (iPSCs) (Wang et al., 2012), and tumor cells (Hiyama and Hiyama, 2007). In normal somatic cells, the activity of telomerase extends telomeric repeats and is usually reduced after birth.
Interestingly, TA often cannot be detected in most human mesenchymal stem cells (MSCs) (Zimmermann et al., 2003; Hiyama and Hiyama, 2007), whereas low levels of telomerase are identified in some somatic stem cells from the hematopoietic system (Thongon et al., 2021), intestinal mucosa, and epidermal basal layers (Hiyama and Hiyama, 2007). Human MSCs, such as bone marrow-derived stem cells (BMSCs) (Bernardo et al., 2007; Hiyama and Hiyama, 2007), adipose-derived stem cells (ASCs) (Nava et al., 2015b), and skeletal muscle progenitor cells (SMPCs), often display telomerase negativity, although these stem cells have the MSC phenotype (SH2+, SH3+, SH4–, CD29+, CD44+, CD14–, CD14+, CD34–, and CD45+) and can differentiate into adipocytes, chondrocytes, and osteoblasts (Zimmermann et al., 2003). A possible reason for the negative telomerase result is the occurrence of alternative lengthening of telomeres (ALT) in MSCs, which is an alternative telomere length-maintaining mechanism (Lafferty-Whyte et al., 2009).

Our previous study was the first to demonstrate that progenitor/stem cells exist in the urine, thus we proposed the name urine-derived stem cells (USCs) (Zhang et al., 2008; Bodin et al., 2010; Wu et al., 2011; Bharadwaj et al., 2013). These cells can be easily isolated from urine samples via a non-invasive approach (Kang et al., 2015), which offers clear advantages over the stem cells harvested from other sources, like bone marrow or adipose aspirates. Clonal USC populations can be readily generated from a single cell by limiting dilution of the starting mixed culture. Each micro-colony will proliferate into a clonal population with many cells (Bharadwaj et al., 2013) whereas low levels of telomerase were detected in some somatic stem cells from the hematopoietic system (Thongon et al., 2021), intestinal mucosa, and epidermal basal layers (Hiyama and Hiyama, 2007). The aim of this study was to determine the role of TA in multiple differentiation potential is controversial. The aim of this study was to determine the role of TA in maintaining stemness with cell longevity, proliferation capacity, multipotent differentiation potential, cell surface marker expression, karyotype stability, and the risk of in vivo teratoma formation in human primary urine-derived stem cells (USCs).

### 2 MATERIALS AND METHODS

#### 2.1 Collection and Culture of Urine-Derived Stem Cells

This study was approval by the Wake Forest University Health Sciences Institutional Review Board. A total of 50 urine samples were collected from 10 healthy male individuals ranging from 25 to 57 years of age and divided into four age groups (20, 30, 40, 50 years of age). A total of 117 USC clones were isolated, expanded, and characterized as previously described (Zhang et al., 2008; Bodin et al., 2010; Wu et al., 2011). Briefly, USC clones were grown in culture media composed of keratinocyte serum-free medium (KSFM) and embryonic fibroblast medium (EFM) mixed at a ratio of 1:1 (Zhang et al., 2008). Only wells in 24 plates that contained single cell were scored and used for further experimentation.

#### 2.2 Telomerase Activity Assay

Telomerase activity levels were measured with the Telo TAGGG Telomerase PCR ELISA plus kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer’s recommendations. HEK 293 cells were used as a positive control, and human BMSC and smooth muscle cells (SMC) were used as negative controls. Briefly, 2 × 10⁶ cells at passage two were collected after being trypsinized and washed with cold PBS. Telomerase added the telomeric repeats (TTAGGG) in the kit to the 3′ end of the biotin-labeled synthetic P1-TS primer. These elongated products, and the internal standard (IS) included in the same reaction vessel, were amplified by PCR using the primers P1-TS and the anchor-primer P2. The PCR products were divided into two aliquots, denatured, and hybridized separately to digoxigenin (DIG) labeled detection probes specific for the telomeric repeats and for IS (P3-Std). The resulting products were immobilized via the biotin label onto a streptavidin-coated microplate. Immobilized amplicons were detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB. Absorbance values were measured as the A₄₅₀nm reading against a blank (reference wavelength A₆₉₀nm) by using a spectrophotometer. Relative telomerase activity (RTA) within different samples in an experiment were obtained using the following formula (Kim et al., 1994; Kim and Wu, 1997):

\[
\text{RTA} = \frac{[\text{AS} - \text{AS}, \text{IS}]/[\text{ATS}, 8\text{IS}, 0]/\text{ATS}, 8\text{IS}, 0]}{\times 100}
\]

AS: absorbance of sample; AS, 0: absorbance of heat-treated sample; AS, IS: absorbance of Internal standard (IS) of the sample; ATS8: absorbance of control template (TS8); ATS8, 0: absorbance of lysis buffer; AT8, IS: absorbance of Internal standard (IS) of the control template (TS8). The kit included the IS and TS8.

To further determine the influence of time on telomerase activity, the RTA of two pairs of USC (age group 20–50 years), telomerase activity positive (TA⁺) and telomerase activity negative (TA⁻), after every five passages or the end passage were measured by the above protocol (Kim et al., 1994).

#### 2.3 Cell Proliferation

The USC in passage three were seeded in 96-well plates at a density of 2,500 cells/well. The culture medium was replaced every second day. Cell proliferation was determined on days 1, 3, 5, and 7 using an MTS cell proliferation assay kit (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer’s instructions. Briefly, the MTS reagent was incubated with the cells in the dark for 1 h at 37°C.
Following incubation, the absorbance was measured at 490 nm using a spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, United States). Triplicate measurements were conducted for each time point. The population doubling (PD) and doubling time (DT) calculations were determined based on single USCs at p0 up to the maximum passage (p16) for each clone. The USCs were trypsinized when they reached 70–80% confluence, and the cells were counted manually using a hemocytometer. The PD and DT were calculated using the following formula (Bharadwaj et al., 2011; Bharadwaj et al., 2013):

\[ PD = \ln (N_f/N_i)/\ln (2) \]  
\[ DT = Ct/PD \]

where \( PD \) is the number of cell doublings, \( DT \) is the culture time, \( N_f \) is the final number of cells, \( N_i \) is the initial number of cells, and \( C_t \) is the culture time.

### 2.4 Flow Cytometry

Flow cytometry analysis for the USCs involved staining the USCs with specific labeled anti-human antibodies: CD25-PE, CD31-FITC, CD34-FITC, CD44-FITC, CD45-FITC, CD73-PE, CD90-PE, and/or histochemical staining for specific components were recorded.

#### 2.4.1 Flow cytometry of USCs

To determine the differentiation capacity difference between USCs TA+/TA− and USCs TA−, cells were subjected to the following induction described below, and their changes in morphology and/or histochemical staining for specific components were recorded.

- **Smooth muscle cell induction:** Three pairs of USCs TA+ and USCs TA− (p3) from different age groups were plated at 2,000 cells/cm² in smooth muscle differentiation media containing equal amounts of DMEM (high glucose) and EFM with 10% fetal bovine serum containing 1% bovine serum albumin (BSA). The fluorescence conjugated antibodies listed above were incubated with USCs on ice for 30 min in the dark. IgG1-PE, IgG1-FITC, IgG2b-FITC, and IgG1-PerCP-Cy5.5-conjugated isotype control antibodies were used to determine background fluorescence. Cells were washed twice with wash buffer, passed through a 70 µm filter, and analyzed using FACS Calibur™ flow cytometry (BD Biosciences, Franklin Lakes, NJ, United States).

- **Osteogenic induction:** USCs TA− (p3) were seeded at a density of 4,000 cells/cm² and cultured in serum containing DMEM low-glucose medium with 100 mM dexamethasone, 10 mM β-glycerophosphate and 50 mM ascorbic acid-2-phosphate (Wako Chemicals, Richmond, VA, United States) for 28 days. The induced cells were harvested after 28 days and fixed in 95% ethanol before histochemical staining. For detection of calcium secreted by the osteogenic-differentiated cells, Alizarin Red S staining was conducted. Briefly, the fixed cells were incubated with 0.5% Alizarin Red S dye (pH 4.1) to sufficiently cover the cell layer for 3–5 mins. Excess dye was removed with distilled water before photo documentation.

- **Uroepithelial induction:** Three pairs of USCs TA+ and USCs TA− were induced with 0.25% Trypsin-EDTA solution, resuspended in hypotonic solution (0.075 M KCl) and then fixed with methanol/acetic acid solution in a 3:1 proportion. The metaphase spread on glass slides was digested by trypsin and then stained with Giemsa stain to generate G bands along each chromosome. Standard cytogenetic analysis was performed under microscopy. Chromosomal image capture and karyotyping were performed using CytoVision®, version 3.7.

- **2.8.1 Soft Agar Assay in vitro**

Agar assays are often used to distinguish tumor cells from non-transformed or normal cells because normal cells cannot undergo anchorage-independent growth or thrive on an agar substrate. To evaluate whether both USCs TA+ and USCs TA− induce tumorigenicity, USCs were tested on agar gels. HeLa cells and SMC were used as positive and negative controls, respectively.
Briefly, 0.35% upper agar layer and 4% base agar layer were prepared in 35 mm tissue-treated dishes. Cells were seeded at the top of the upper agar at a density of 5,000 cells/well. Culture medium was changed twice a week. Cell morphology, proliferation rate, anchorage-independent growth, and cell colony formation were observed under a phase contrast microscope. After culturing for 2 weeks, all the samples were stained with 1 mL of 0.05% nitroblue tetrazolium (NBT) prepared in PBS and sterilized (0.2 micron). This was incubated overnight at 37°C. The cells that took up NBT and showed a violet color were determined to be live cells.

2.10 Spontaneous Transformation In Vivo Assay
Experiments using nude mice were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee. To further determine the non-tumorigenicity of USCsTA+ in vivo, two USCsTA+ (p5) were implanted in the kidney subcapsular region of NSG (NOD.Cg-Prkdcsid-Il2rtnm1Wjl/SzJ) mice. H9 (human embryonic stem cell line) was used as a positive control. A total of 12 six-week-old female NSG mice (Jackson Labs, Bar Harbor) were used and divided into two groups: USCTA+ (3 mice/clone) and H9 groups (6 mice/group). In total, 2 x 10^5 cells in 20 µl of PBS were injected into the subcapsular region of the right kidney. After 8 weeks, the mice were sacrificed and bilateral kidneys were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. For evaluation of general graft histology and teratoma formation, routine hematoxylin and eosin (H&E) staining was performed.

3 RESULTS
3.1 Telomerase Activity Detected in USCs at Early Passage
A total of 80/117 USC clones (p2) from 10 individuals showed detectable TA (70.2% were USCsTA+). There were no significant differences in the positive rate for TA in the 20–40 years age group: 20 s: 30/39 (76.9%), 30 s: 14/19 (73.7%), and 40 s: 16/22 (72.7%) represented USC clones, respectively, but the RTA notably decreased to 16/27 (59.2%) in the 50 years age group, as shown in Figure 1 and Table 1. TA could not be detected in human BMSCs at p2.

To determine if the TA in human USCs decreased as the passage progressed, we measured the TA of two pairs of USCsTA+ and USCsTA− at early passage (p2) and late passages (p7, p11 or the last passage for USCS TA−) from the 20s and 50s groups. The strength of RTA of USCTA+ clones decreased gradually with passage (Figure 2), which seems to make a formerly TA+ clone equivalent to a TA− clone. The RTA remained at undetectable levels through all the passages of USCTA− clones.

3.2 Self-Renewal Ability of USCsTA+ Generated More Cells
The USC TA+ and USCsTA− paired clones from a single donor were tested in parallel to prevent other factors from affecting the results of the comparison. The USCTA+ clone A35 which was isolated from a 27-year-old male donor, could be passed more than the USCsTA− clone A42 which was isolated from the same donor, and consistently maintained the original “rice-grain like” morphology until they reached cell senescence at p16. Similarly, the USCTA− clones steadily displayed the similar cell morphologic appearances and finally displayed a larger, flattened, and the typical “fried egg” morphology of quiescent cells at p9 (Figure 3).

According to the cell growth curve data (Figure 4), the USCTA+ clones grew more rapidly than the USC TA− clones. Consequently, the PD of USCTA+ clones increased significantly compared to that of the USCTA− clones, regardless of the individual’s age (p < 0.001). The mean DTs of USC TA+ clones were significantly shorter than those of the USCTA− clones (Table 2; Figure 5) (p < 0.001).

However, the relative TA of USCs was not completely coordinated with the cell proliferation capacity (PD, DT, and passage) (Table 2).
TABLE 1 | The rate, mean, and highest telomerase activity of the isolated USCs\textsuperscript{TA+} clones for individual donors (p2) in the four different age groups.

| Donors’ ages (no. of donors) | Donors (no. of clones) | No. of USCs\textsuperscript{TA+} clones (%) in total USC | Mean TA ± SD (%) | Highest RTA |
|-----------------------------|------------------------|--------------------------------------------------------|-----------------|------------|
| 20s (n = 3)                 | A (n = 19)             | 16 (84.2%)                                             | 24.38 ± 14.41   | 53.25      |
|                             | B (n = 10)             | 6 (90.0%)                                              | 14.94 ± 7.93    | 25.94      |
|                             | C (n = 10)             | 8 (80.0%)/76.9%                                        | 16.85 ± 10.86   | 32.24      |
| 30s (n = 2)                 | D (n = 12)             | 9 (75.0%)                                              | 17.41 ± 10.99   | 34.50      |
|                             | E (n = 10)             | 7 (70.0%)/72.7%                                        | 21.69 ± 16.03   | 53.75      |
|                             | F (n = 10)             | 9 (90.0%)                                              | 18.55 ± 12.37   | 41.26      |
| 50s (n = 3)                 | G (n = 10)             | 14 (93.3%)                                             | 26.67 ± 13.84   | 47.49      |
|                             | H (n = 10)             | 16 (90.0%)                                             | 16.03 ± 14.50   | 37.12      |
|                             | J (n = 10)             | 8 (80.0%)/76.9%                                        | 12.98 ± 5.28    | 20.56      |
|                             | K (n = 10)             | 8 (80.0%)/76.9%                                        | 19.94 ± 12.05   | 44.93      |

Notes: Telomerase activity was expressed as a percentage of the RTA, of USCs, to the RTA, of TS8. The suffixes A, B, C, H, D, F, J, K represent the ten healthy individuals who participated in the study.

3.3 Both USCT\textsuperscript{TA+} and USCT\textsuperscript{TA−} Clones Showed Similar Cell Surface Markers

To identify the cell surface markers of USC clones, six pairs of USCS\textsuperscript{TA+} and USCS\textsuperscript{TA−} were subjected to flow cytometry analysis. All the USC clones showed strong positive MSCs markers including CD44, CD90, CD73, CD105, and CD146 and were negative for hematopoietic stem cell markers including CD25, CD31, CD34, CD45, and CD117 (Figure 6; Table 3). However, there was no significant difference in the CD105 expression between USCT\textsuperscript{TA+} and USCT\textsuperscript{TA−}.

3.4 Potent Differentiation Capacity of USCS\textsuperscript{TA+}

The USC\textsuperscript{TA+} and USC\textsuperscript{TA−} paired clones from a single donor were tested in parallel to prevent other factors from affecting the results of the comparison. To test the difference in differentiation capability between USCS\textsuperscript{TA+} and USCS\textsuperscript{TA−}, both USCS\textsuperscript{TA+} and USCS\textsuperscript{TA−} (p3) were induced to osteogenic, myogenic, and uroepithelial differentiation (Table 4). Both the USCS\textsuperscript{TA+} clone A35 that was isolated from a 27-year-old male donor, and the USCS\textsuperscript{TA−} clone A42 that was isolated from the same donor (p3) differentiated into the smooth muscle lineage and the urothelial lineage (Figures 7A,B). Urothelial-differentiated cells developed a cobblestone-like morphology (Figure 7Ai) and expressed the urothelial-specific proteins uroplakin-Ia and uroplakin-III and the generic epithelial cell markers CK7, CK13, and AE1/AE3 (Figure 7Aii). Furthermore, the expression of these proteins was significantly higher in the UC-induced USCS\textsuperscript{TA+} clones [A35 and J29 (from another male aged 55 years)] than in the UC-induced USCS\textsuperscript{TA−} clones (A42 and J18 [from the same male aged 55 years as clone J29]) and uninduced USC clones. In an assay of cellular barrier function, the UC-induced USCS\textsuperscript{TA+} clone A35 showed increased expression of specific tight junction protein markers (E-cadherin) compared to the UC-induced USCS\textsuperscript{TA−} clone A42 and uninduced USC clones (Figure 7Ai). In barrier function assays, both the urothelial-differentiated USCs (USCS\textsuperscript{TA+} 41.0% ± 1.7% and USCS\textsuperscript{TA−} 44.8% ± 0.4%) showed significant reduction in leakage of fluorescent tracer through the insert in vitro (p < 0.01 and p < 0.001, respectively), which means lower permeability and higher tight junction property, compared to the non-differentiated USCs (USCS\textsuperscript{TA+} 63.1% ± 4.3%, USCS\textsuperscript{TA−} 62.2% ± 1.1%) (Figure 7Aiiii) at day 3; and similar results received at day 7 (p < 0.01), the leakage percentage of urothelial-differentiated USCs (USCS\textsuperscript{TA+} 35.5% ± 1.2% and USCS\textsuperscript{TA−} 42 38.9% ± 4.3%), less than the non-differentiated USCs (USCS\textsuperscript{TA+} 54.8% ± 2.6%, USCS\textsuperscript{TA−} 59.6% ± 0.5%). However, there was only a slight difference in leakage protection at day 3 between the UC-induced USCS\textsuperscript{TA+}-A35 and USCS\textsuperscript{TA−}-A42 clones (p < 0.05). In addition, no significant differences between them, although reduce in leakage, were noted in UC-induced USCS\textsuperscript{TA+}-A35 clone on day 7.

Both myogenic differentiated USCS\textsuperscript{TA+} and USCS\textsuperscript{TA−} became elongated and spindle-shaped (Figure 7Bi) and expressed smoothelin, a smooth muscle-specific protein marker, and calponin (Figure 7Bii). Moreover, the expression of
smoothelin and calponin of SMC-differentiated USCsTA+ were higher than the SMC-differentiated USCsTA−A42 confirmed by western blotting (Figure 7Bii). Finally, both USCsTA+ and USCsTA−A42 were induced to differentiate into the osteogenic lineage using our previous protocols. USCsTA+ could be induced to osteocytes but not USCsTA−A42, as evidenced by Alizarin S Red staining (Figure 7C). Moreover, both USCsTA+ and USCsTA−A42 were difficult to differentiate into adipocytes, as evidenced by Oil Red-O staining (data not shown).

3.5 Karyotype Remains Stable in USCsTA+
To investigate the potential susceptibility of USCsTA+ to malignant transformation, cells were tested via cytogenetic analysis, agar culture in vitro, and teratoma formation in vivo, and the results were compared to those of USCsTA− and controls.
TABLE 2 | Population doubling and doubling time of USCTA+ vs USCTA− in the 20 and 50 years age groups in early and late passages to determine if there was a difference in telomerase activity with increasing age.

| Individual clones (s) | Age (yrs) | RTA (%) | Population Doubling | Doubling Time |
|----------------------|-----------|---------|---------------------|---------------|
|                      |           |         | PD M ± SD           | DT (hrs.) M ± SD |
| 20s USCTA+ (n = 3)   |           |         |                     |               |
| A35                  | 27        | 45.6    | 67.5 ± 6.0          | 27.1 ± 1.6**  |
| G1                   | 25        | 25.9    | 59.2 ± 6.7          | 24.3          |
| B36                  | 28        | 32.2    | 59.2 ± 6.7          | 27.0          |
| USCTA− (n = 3)       |           |         |                     |               |
| A42                  | 27        | —       | 42.0 ± 6.0          | 37.3 ± 2.4    |
| G12                  | 25        | —       | 36.8 ± 6.0          | 35.7          |
| B31                  | 28        | —       | 40.1 ± 6.0          | 32.7          |
| 50s USCTA+ (n = 3)   |           |         |                     |               |
| E37                  | 50        | 41.4    | 55.5 ± 6.0          | 29.2 ± 1.3**  |
| J29                  | 55        | 46.6    | 59.2 ± 6.0          | 27.0          |
| K5                   | 57        | 37.1    | 55.2 ± 6.0          | 29.3          |
| USCTA− (n = 3)       |           |         |                     |               |
| E34                  | 50        | —       | 35.6 ± 6.0          | 36.6 ± 2.5    |
| J18                  | 55        | —       | 36.2 ± 6.0          | 34.2          |
| K6                   | 57        | —       | 37.2 ± 6.0          | 39.1          |

*p < 0.001: USCTA+ vs USCTA− in population doubling at age of 20s, and 50s groups, respectively.

**p < 0.01: USCTA+ vs USCTA− in doubling time at age of 20s, and 50s groups, respectively. The suffixes A, B, G, C, H, D, E, F, J, and K represent the ten healthy individuals who participated in the study.

FIGURE 5 | Population doubling, doubling time, and in vitro survival time course of USCsTA+ vs USCsTA−. (A) Population doubling significantly increased for USCsTA+ compared to USCsTA−. (B) Doubling time was significantly shorter in USCsTA+ than in USCsTA−. (C) Cellular senescence in USCsTA− occurred earlier than that in USCsTA+. These data indicate that USCsTA+ proliferates more rapidly, generates more cells, and survives longer than USCsTA−. USCs from healthy individuals (n = 12) were cultured following plating at a single cell/well. Six individual USCTA+ clones (n = 6, p3, A35, B36, G1, E37, J29, and K5) generated significantly more cells and grew faster than USCTA− clones (n = 6, p3, A42, B31, G12, E34, J18, and K6). USCTA+ clones survived longer and had longer population doubling times. The suffixes A, B, G, C, H, D, E, F, J, and K represent the ten healthy individuals who participated in the study.
USCs expressing mesenchymal stem cell surface markers. USCTA+ clones (A35, B36, G1, E37, J29, and K5) and USCTA− clones (A42, B31, G12, E34, J18, and K6) both displayed sets of mesenchymal stem cell (MSC) surface markers (CD44, CD90, CD73, CD105, and CD146), positive at similar levels, whereas both USCTA+ and USCTA− did not express sets of haematopoietic stem cell markers (i.e., CD31, CD34, CD45, CD25, and CD117). However, three of five USCTA+ displayed CD105 expression, but no or weak expression in all the four USCTA− cell clones. MSC surface markers of USCs at p4 were detected via flow cytometry.

| TABLE 3 | Cell surface markers of USCTA+ and USCTA− clones at passage four detected using fluorescence-activated cell sorting. |
| CLONES (s) | Cell surface markers (%) |
| | CD25 | CD31 | CD34 | CD44 | CD73 | CD105 | CD146 | CD140b | CD146 | CD117 | CD140b | NG2 | HLA-a,b,c | HLA-DR/DQ | HLA-G |
| 20s | |
| TA+ A35 | 0.36 | 0.51 | 0.40 | 99.9 | 0.34 | 99.8 | 93.4 | 82.2 | 0.5 | 3.9 | 99.89 | 3.05 | 99.97 | 0.81 | 4.53 |
| TA− A42 | 0.69 | 0.81 | 0.98 | 100 | 0.54 | 99.9 | 95.1 | 5.3 | 0.6 | 7.7 | 99.86 | 29.34 | 99.98 | 1.18 | 0.30 |
| TA+ G1 | 0.37 | 0.09 | 0.07 | 100 | 0.09 | 99.86 | 90.5 | 90.7 | 0.4 | 5.0 | 99.96 | 88.96 | — | — | — |
| TA− G12 | 0.04 | 0.39 | 0.20 | 99.7 | 0.02 | 97.70 | 99.8 | 99.0 | 0.9 | 0.1 | 99.67 | 0.44 | — | — | — |
| TA+ B36 | 0.21 | 0.52 | 0.60 | 96.8 | 0.77 | 99.73 | 99.8 | 33.0 | 0.8 | 12.0 | 99.08 | 15.48 | 99.69 | 0.67 | 0.86 |
| TA− B31 | 0.60 | 0.61 | 0.55 | 99.8 | 0.93 | 99.91 | 99.8 | 1.7 | 0.5 | 9.3 | 99.68 | 29.56 | 99.90 | 0.64 | 0.84 |
| 50s | |
| TA+ E37 | 1.37 | 0.86 | 0.52 | 99.8 | 0.98 | 99.97 | 98.9 | 66.1 | 0.93 | 0.1 | 99.26 | 5.00 | — | — | — |
| TA− E34 | 3.45 | 0.77 | 0.99 | 100 | 0.70 | 99.98 | 99.7 | 91.4 | 1.41 | 0.2 | 99.76 | 25.20 | — | — | — |
| TA+ J29 | 0.41 | 0.33 | 0.16 | 99.94 | 0.37 | 100 | 92.12 | 55.44 | 0.31 | 5.98 | 98.12 | 3.06 | 99.97 | 0.13 | 1.09 |
| TA− J18 | 0.45 | 0.55 | 0.53 | 98.36 | 0.41 | 95.61 | 95.61 | 3.50 | 0.44 | 0.62 | 96.51 | 59.35 | 98.40 | 1.06 | 1.73 |
| TA+ K5 | 0.46 | 0.33 | 0.26 | 99.85 | 0.35 | 99.90 | 83.20 | 48.67 | 0.41 | 8.71 | 99.14 | 64.55 | 99.70 | 0.48 | 1.90 |
| TA− K6 | 0.71 | 0.47 | 0.26 | 99.57 | 0.52 | 98.84 | 49.13 | 15.25 | 0.70 | 8.39 | 99.41 | 71.18 | 99.20 | 0.37 | 1.42 |

| TABLE 4 | Induced and non-induced multipotent differentiation potential of USCs. |
| Positive control | Induced USCTA+ | Non-induced USCTA+ | Induced USCTA− | Non-induced USCTA− |
| Myogenic differentiation | (4+) SMC | (3+) SMC | 0/1 | 0/2 | 0/1 |
| Urothelial differentiation | (4+) UC | (3–4+) UC | 0/1 | 0/2 | 0/1 |
| Osteogenic differentiation | (4+) ASC | (2+–3+) ASC | N/A | 0/2 | N/A |

Abbreviation: SMC, smooth muscle cells; UC, urothelial cells; ASC, adipose-derived stem cells. Notes: 1+, 1–25% differentiated cells; 2+, 26–50% differentiated cells; 3+, 51–75% differentiated cells; 4+, >75% differentiated cells.
Both TA+ and TA- USC clones in the early (p4) and late passage (USCsTA+-A35, J29 clone in p12 and USCsTA−-A42, J18 clone at p8) displayed a normal karyotype of 1 X and 1 Y chromosome, as expected for a male donor, and a normal diploid (2n = 44) complement of autosomes and a pair of sex chromosomes (Figure 8A; Table 5). No multiploidy or obvious chromosomal rearrangements in metaphase were detected by Giemsa bandings at p4 or late passage of both USC clones.

### 3.6 No Tumorigenic Transformation of USCsTA+ Was Observed

USCTA+ clones-A35, J29 remained the same size on day 14 as that on day 1, after being cultured in soft agar, like colonies of SMCs. However, HeLa cancer cells formed large colonies on day 14 (Figure 8Bi). These single cells (SMC and USC) or colonies (HeLa cancer cells) were viable, which was confirmed by NBT staining (Figure 8Bii). Furthermore, no teratoma was formed when all USCsTA+-A35 were implanted in the subcapsular region of the kidney of NSG mice after 8 weeks. All human ES cell lines (H9) formed derivatives of the three embryonic germ layers (Figure 8C).

### 4 DISCUSSION

We characterized the stemness features of human USCs, including long-term survival with self-renewal capacity, multilineage differentiation, MSCs surface markers, expression of telomere maintenance mechanisms (TA) in in vitro culture time frames, and capacity to form teratomas. USC clones in the same individual urine sample displayed telomere heterogeneity, which could be due to USCs at different stages of the telomerase activation processes.

Stemness refers to the molecular processes underlying the fundamental stem cell properties of self-renewal and the
generation of differentiated daughter cells. The stemness properties of adult-derived stem cells decline after birth, compared to those of ESCs (Hofmeister et al., 2015). Most human somatic or stem cells do not express OCT4/SOX2/KLF4/MYC. Forced expression of OCT4/SOX2/KLF4/MYC in somatic cells such as fibroblasts can reprogram cells to a pluripotent stem cell fate. USCs are multipotent, rather than pluripotent, and express low levels of OCT4/SOX2/KLF4/MYC (Bharadwaj et al., 2013). Our previous studies demonstrated that USCs possess limited stemness properties including self-renewal and multiple differentiation capacity but do not induce teratoma formation. This is different from iPSC that have higher expression levels of OCT4/SOX2/KLF4/MYC and form teratoma. In summary, USCs are multipotent and thus do not express the higher levels of OCT4/SOX2/KLF4/MYC that are observed in pluripotent stem cells.

Two methods are predominantly used to track the in vitro age of a cell culture or cell proliferation capacity. 1) The passage number implies the number of times a cell has been passaged, which is most commonly used in the laboratory. However, the cell passage number is imprecise because different laboratories may use different initial cell seeding densities, which affects the number of times cells divide in culture. 2) The PD indicate the number of cell generations the cell lineage has undergone—the number of times the cell population has doubled. PD of primary cells is a better practice for reporting cellular age in vitro, which is often used to set an acceptable upper limit for cell production, or the maximum number of cells generated in culture. In this study, we used both terms (passage number and PD number) to present cell lifespan, and there was good agreement between the two measures. USCsTA+ with higher TA could reach higher PD number or passage.

**FIGURE 8** | Spontaneous transformation assays of USCTA+ clones. (A) Karyotypes of USCTA+ vs. USCTA- clones. Both USCTA+ (n = 2, A35, RTA:45.6; and J29, RTA:46.6) and USCTA- (n = 2, A42 and J18) clones in the early passage (p4) and late passage (TA + clone in p12, TA-clone at p8) displayed normal complement of diploid (2n = 46). (B) In vitro agar assay of USCs. The size of the cell clones (seeded at a density of 5,000 cells/well) of USCTA+ clones (A35 and J29) and USCTA- clones (A42 and J18) remained the same between days 1 and 14 after plating on soft agar gel. However, clone size of the cancer cell line HeLa cells, as the positive control, at 14 days were significantly increased compared to that at the first day. Normal bladder smooth muscle cell clones were used as the negative control. Images were captured using a phase contrast microscope. Cell clones were stained with nitro blue tetrazolium and photographed using bright field soft agar assays for anchorage-independent cell growth of USCTA+ in vitro. (C) In vitro transformation assay of USCs. No teratoma formation was observed under the microscope 2 months after USCTA+ (two million cells/graft, n = 2, white arrow) were implanted into the capsules of the kidneys of NSG mice. There was no teratoma observable grossly (i, ii) or microscopically (iii, iv). In contrast, H9 cells as the positive control formed derivatives of all the three embryonic germ layers (v, vi). A35-clone (USCsTA+, RTA: 45.60) and A42 clone (USCsTA-, RTA: negative) were both isolated from one donor (male, 27 year-old); J29 (USCsTA+, RTA:46.6) and J18 (USCsTA-, RTA: negative) were isolated from another donor (male, 55 year-old).

**TABLE 5** | Conventional karyotypes of USCTA+ and USCTA- clones in the 20- and 50-years age groups at early and late passages.

| Age at 20s group | Age at 50s group |
|-----------------|-----------------|
| **USC clones**  | **Karyotypes**  | **USC clones**  | **Karyotypes**  |
| USCTA+          | A35 (p4)        | 46, XY          | J29 (p4)        | 46, XY          |
|                 | A35 (p12)       | 46, XY          | J29 (p12)       | 46, XY          |
| USCTA-          | A42 (p4)        | 46, XY          | J18 (p4)        | 46, XY          |
|                 | A42 (p8)        | 46, XY          | J18 (p8)        | 46, XY          |

Notes: A35-clone (USCsTA+, RTA: 45.60) and A42 clone (USCsTA-, RTA: negative) were both isolated from one donor (male, 27-year-old); J29 (USCsTA+, RTA:46.6) and J18 (USCsTA-, RTA: negative) were isolated from another donor (male, 55 year-old).
Telomerase is activated and maintains cellular immortality in ESCs or iPSCs, which plays an import role such as to protect the genome from nucleolytic degradation, unnecessary recombination, repair, and intrachromosomal fusion (Hiyama and Hiyama, 2007); however, the level of TA is low or absent in most MSCs and ASCs (Zimmermann et al., 2003; Hiyama and Hiyama, 2007) regardless of their proliferative capacity. Numbers of BMSCs are low in bone marrow nucleated cells with a frequency of colony-forming unit-fibroblasts (CFU-F) of 1: 35,700 (Lu et al., 2006). In addition, small amounts of stem cells are mixed with a large amount of stromal cells in the bone marrow, which makes it challenging to isolate true stem cells and measure their levels of TA. In contrast, USCs start with a single stem cell that forms cell clones and expands to a large number of stem cells of which most display TA. There are a couple of studies comparing hUSC to hBMSC (Sun et al., 2021) and ASC (Kang et al., 2015). In in vitro experiments, hUSC presented with better capacity for proliferation than hBMSC, while hBMSC had greater chondrogenic ability than hUSC. However, hUSC and hBMSC had similar cartilage repair effects in vivo. Results indicated that hUSC can be a stem cell alternative for cartilage regeneration, provide a powerful platform for cartilage tissue engineering, and clinical transformation (Sun et al., 2021). Similar outcomes are achieved in studies of comparison between hUSC and hASC (Kang et al., 2015). TA levels can be detected or consistently expressed in most human USCs (>70%) obtained from healthy middle-aged donors, although levels of this enzyme were lower than those of ESC. Most USCs express TA however, along with the aging process in individuals, a reduction in USC regenerative capacity occurs in the 50s age group, which also means a decrease in cell proliferation capacity along with the reduced number of USCsTA+ with passage; thus, the proliferation potential with PD and DT of USCs gradually decreased, with the cells finally reaching senescence within 8 weeks in 2D culture and 10 weeks in 3D culture models (data not shown).

Telomerase and telomeres are strongly associated with cell renewal and proliferation, but it is controversial whether telomerase is associated with cell differentiation in MSCs (Zimmermann et al., 2003; Hiyama and Hiyama, 2007). The epigenetic nature of telomeres appears to depend on different human cell linages (Dogan and Forsyth, 2021). In adult human stem cells, both BMSCs and ASCs lack telomerase, but can retain their functional characteristics and multiple differentiation potential (Zimmermann et al., 2003; Hiyama and Hiyama, 2007). The latter is associated with cell differentiation in MSCs (Simonsen et al., 2002). However, increased TA enhanced self-renewal ability, proliferation, and differentiation efficiency in TERT-overexpressing ES cells (Armstrong et al., 2005). High TA or the expression of TERT can therefore be regarded as a marker of undifferentiated ES cells. Downregulation of TA in differentiating EC cells was reported to be closely correlated with histone deacetylation and DNA methylation of the TERT gene (Lopatina et al., 2003). In this study, USCsTA+ performed better in terms of multiple differentiation capacity in osteogenic, myogenic, and uroepithelial differentiation than USCsTA−, indicating that telomerase is required for not only cell proliferation but also multiple differentiation in human USCs, which is a guarantee for future studies to determine whether USCsTA+ induce better in vivo tissue regeneration than USCsTA−.
As telomerase is related to both normal stem cells and tumor stem cells, it is necessary to determine the alteration of karyotypes, with the in vitro agar assay and the in vivo risk of tumor formation of telomerase-positive cells for their safe transplantation application. Giemsa-based chromosomal banding and staining techniques are important for cytogenetics. USCs maintain a normal diploid chromosome recognized as 46 during long-term culture or overexpansion for up to p16 or 68 PD (268). Cytogenetic analyses showed USCs can safely expand in vitro (p4, p8, and p16) with no sign of immortalization or development of chromosomal abnormalities.

Cloning techniques with semisolid medium, such as agar gel for evaluating cell growth, are commonly used to study the biology of cancer cells due to their anchorage-independent growth requirements. The ability of cancer cells to proliferate without firm attachment (i.e., anchorage independence) is one of the best in vitro indicators of tumorigenicity. Importantly, USCsTA+ do not present a propensity for spontaneous oncogenic transformation 60 days after in vivo implantation.

The tumorigenic potential of USCsTA+ was not found in vitro or in vivo. Thus, USCs as a new source of seed cells, which are non-invasive, highly proliferative, and abundant, can be used for tissue engineering and regenerative medicine.

TA appears to be related to the stemness of USCs (Table 6). USCsTA+ survived for a significantly longer period with intact morphological appearance, rapid proliferation, and ample cell generation, and possessed more potent differentiation capacity than USCsTA−. Both human USCsTA+ and USCsTA− can be safely expanded in vitro maintaining normal karyotype and showed tumor-free formation after in vivo implantation, which makes them appropriate sources for cell-based therapies. Thus, TA could be an independent predictive factor for the regenerative capacity of USCs. In addition, human USCsTA+ provides sufficient cell numbers for drug testing. TA is a good indicator of stemness (cell renewal and differentiation potential) of human adult stem cells, but a collection of primary cultured cells is required, which is cumbersome. Therefore, simpler and low-cost methods of measuring TA and telomeres in stem cells are highly desired.

5 CONCLUSION

This study demonstrated that human primary urinary stem cells with positive TA act as a distinct subpopulation with potential regeneration capacity in both cell proliferation and multiple differentiation. USCsTA+ can more efficiently give rise to osteogenic, skeletal myogenic, smooth muscle, and urothelial cell lines than USCsTA−. Importantly, despite that USCs display TA, they do not form teratoma, which provides a safe cell source for clinical application. In addition, the number of USCsTA+ decline with increasing age. Future investigations should focus on understanding the role that physiological factors play in regulating both the temporal pattern of USCsTA+ and their influence on the ability of these cells to participate in better tissue repair. Determining the requirements for the effect of TA on the paracrine effect of USC has important implications for understanding the anti-inflammatory, fibrosis inhibition, and redox effect of USCsTA+. It will be beneficial to better understand alterations in this cell subpopulation throughout the human lifespan, and how they translate into, aging, renal dysfunction, drug-induced nephrotoxicity, or cancer, among others.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material,
further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the All human tissue samples were approved Human urine-derived stem cells (USC): All human tissue samples were approved for acquisition by the Wake Forest University Institutional Review Board. Participants provided their written informed consent to participate in this study. The research was reviewed and approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

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AUTHOR CONTRIBUTIONS

YZ supervision; YS and GL analyzed the data; XS, GL, RW, DM, XS, JM, XG, and AA contributed materials, reagents, analytic tools; YS, GL, and YZ wrote the original manuscript. All authors have read, edited, and approved the manuscript for publication.

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