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Urine-derived stem cells serve as a robust platform for generating native or engineered extracellular vesicles

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Abstract

Background Mesenchymal stromal cell (MSC) therapy holds great potential yet efficacy and safety concerns with cell therapy persist. The beneficial effects of MSCs are often attributed to their secretome that includes extracellular vesicles (EVs). EVs carry biologically active molecules, protected by a lipid bilayer. However, several barriers hinder large-scale MSC EV production. A serum-free culturing approach is preferred for producing clinical-grade MSC-derived EVs but this can affect both yield and purity. Consequently, new strategies have been explored, including genetically engineering MSCs to alter EV compositions to enhance potency, increase circulation time or mediate targeting. However, efficient transfection of MSCs is challenging. Typical sources of MSC include adipose tissue and bone marrow, which both require invasive extraction procedures. Here, we investigate the use of urine-derived stem cells (USCs) as a non-invasive and inexhaustible source of MSCs for EV production.

Methods We isolated, expanded, and characterized urine-derived stem cells (USCs) harvested from eight healthy donors at three different time points during the day. We evaluated the number of clones per urination, proliferation capacity and conducted flow cytometry to establish expression of surface markers. EVs were produced in chemically defined media and characterized. PEI/DNA transfection was used to genetically engineer USCs using transposon technology.

Results There were no differences between time points for clone number, doubling time or viability. USCs showed immunophenotypic characteristics of MSCs, such as expression of CD73, CD90 and CD105, with no difference at the assessed time points, however, male donors had reduced CD73 + cells. Expanded USCs were incubated without growth factors or serum for 72 h without a loss in viability and EVs were isolated. USCs were transfected with high efficiency and after 10 days of selection, pure engineered cell cultures were established.

Conclusions Isolation and expansion of MSCs from urine is non-invasive, robust, and without apparent sex-related differences. The sampling time point did not affect any measured markers or USC isolation potential. USCs offer an attractive production platform for EVs, both native and engineered.

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Introduction

Mesenchymal stromal cells (MSCs) are an attractive cell-based treatment, based on their regenerative and immunomodulatory properties [1–3]. Autologous and allogeneic MSC therapies have been used and are accepted as immunologically privileged or immune evasive respectively [4, 5]. However, the production of donor-specific antibodies has been observed for allogeneic therapies, which could cause MSC clearance by host recipient immune cells and long-term effects remain to be investigated [6]. Other risks, such as uncontrollable growth of transplanted cells and chromosomal instability, have also been reported [7, 8]. The therapeutic effects of MSCs, when treating immune-mediated diseases, are often attributed to the secretome [9]. MSCs secrete molecules such as prostaglandin E2, various cytokines, and extracellular vesicles (EVs), which collectively contribute to the immunomodulatory effects [9, 10]. As such, a secretome-based and therefore cell-free therapy could circumvent some of the risks associated with MSC therapy. EVs have gained extensive attention in this context, as they are complex entities interacting with multiple modulatory pathways. EVs serve as a physiological drug delivery system that can alter the immune system more subtly and sustainably than individual molecules as they contain several bioactive molecules such as lipids, regulatory RNAs and proteins [11, 12]. MSCs can be isolated from various tissues and fluids of either neonatal-associated or adult origin [13]. EVs derived from the most investigated MSCs, bone marrow-, neonatal-associated, and adipose-derived MSCs (AD-MSCs), have been assessed for their modulatory effects in both pre-clinical and clinical settings [14, 15]. EVs from MSCs derived from less investigated sources such as dental pulp, central nervous system, menstrual blood and urine-derived stem cells (USCs) have mostly been investigated in preclinical trials but are gaining traction as alternative sources of MSCs [16–19]. USCs, derived from the same donor, have been shown to possess superior immunosuppressive functions and different differentiation preferences when compared to AD-MSCs highlighting the untapped potential of alternative MSC sources as therapeutics [20].

Altering the properties of EVs through engineering has been extensively conducted with potency being enhanced by the incorporation of new recombinant cargo or interfering RNAs into EVs [21, 22]. Organ-specific targeting can also be achieved by modulating proteins displayed on the EV surface [23]. Further, as the half-life of EVs in vivo is short, engineering has been used to increase circulation time [24]. Some of these endeavors have translated into clinical trials but predominantly as cancer treatments [25]. For convenience, EV-engineering is most often performed using cell lines that are simple to transfect and are immortalized for scalability purposes [26]. Primary MSCs are conversely more difficult to transfect, have a limited cell doubling before they enter senescence, and can enter a state of cell cycle arrest during selection, which can interfere with upstream EV production [22]. Efficient methods of genetic engineering such as lentiviral transduction have been used, however, downstream challenges may arise as the absence of residual infectious viral particles has to be validated [27]. Several other methods exist to introduce genes into cells, either transiently or with permanent integration into the genomes, that rely on chemical or mechanical delivery into the cell [28]. DNA plasmids and mRNA can be delivered into the cells, whilst mRNA will be directly translated into protein, DNA has to enter the nucleus where it will be transcribed into mRNA before it translation [29]. Non-viral genome integration can be achieved using methods like zinc-finger nucleases, CRISPR-cas9, and transposons [30]. The major advantage of these systems are the reduced safety concerns, ability to work in BSL1 laboratories and ability to transfect large inserts (+100 kb) [31]. A drawback of these non-viral transfection systems is that they do not enter the cell easily and need a transfection method. Many commercial DNA condensing methods exist that aid in the delivery of plasmids into cells, with the most cost-effective being polyethyleneimine (PEI) [32]. While DNA condensing methods work well for cell lines, they are not efficient at transfecting primary cells including MSCs and more specialized and higher-cost transfection methods such as electroporation are often required [33]. It is therefore crucial to refine transfection methods as high transfection success is needed for EV production as MSCs have a finite doubling potential. Conversely, USCs and menstrual blood-derived MSCs could be preferential for genetic engineering applications due to superior doubling capabilities compared to MSCs from other sources [34, 35].

The majority of EV treatments entering clinical trials have native MSCs as a cell source [15], yet production of clinical-grade EVs is not trivial, as contaminating factors, including serum, are often required in vitro to ensure high-performing cells as well as sufficient EV purity, yield and to limit the presence of apoptotic bodies [36]. MSCs are predominantly isolated using invasive procedures, such as bone marrow or lipid aspiration, leading to potential donor or patient discomfort [37]. As a noninvasive alternative, urine-derived stem cells (USCs) can easily be procured from urine, are simple to isolate and expand ex vivo, and the immunomodulatory capabilities

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of USC-derived EVs point to a high therapeutic potential [38]. As such, this study aimed to investigate the isolation of USCs from healthy donors at different time points, to obtain USCs for ex vivo expansion and EV production under serum-free conditions. Cellular characteristics including surface markers and EV engineering potential were investigated. Combined, this work presents USCs as a novel and viable source of stem cell EVs, which show potential as a production platform for therapeutic applications.

Materials and methods

USC production and culture conditions

Male donors briefly disinfected the external urethral orifice with 70% ethanol, whereas female donors used baby wipes. The first approximate third of the urination was discarded to minimize microbiological contamination from the urethra. Urine, with the addition of 50 U/ml pen/strep (Biowest), was collected in sterile containers and all processing was hereafter conducted aseptically. Urine was aliquoted into conical 50 ml tubes and centrifuged at 400 x g (acceleration 9, deceleration 6) for ten minutes. Pellets were resuspended and pooled in a total volume of 50 ml PBS 1X pen/strep and subjected to another centrifuge step at the same conditions. The final pellet was resuspended in 24 ml USC media and seeded in a 24 well, Cell+, F plate (Sarstedt) and incubated at 37 °C and 5% CO2 with saturating humidity. USC media consisted of 50% DMEM/F12 (Thermo Fisher) and 50% keratinocyte SFM supplemented with 2.5 μg/500 ml human recombinant epidermal growth factor and 25 mg/500 ml bovine pituitary extract (Thermo Fisher), to the final mix was added 5% FBS and 50 U/ml pen/strep and sterile filtered (0.2 μm). Media was exchanged after 24 h and every 48 h subsequently. Colonies were counted on day 7 post-seeding and transferred into a T175 Cell+flask using TrypLE (Thermo Fisher) as a disassociation reagent. On day 14–16 post-seeding, cells were harvested for flow cytometry. Cell enumeration and viability were determined by the count and viability protocol, using a NucleoCounter NC-202 (Chemometec). Doubling time was determined from colony-forming cells at seeding from colony count and the viable cells at harvest for flow cytometry as T1 and T2.

Flow cytometric characterization of USCs

Harvested cells were resuspended in 2% FBS in PBS at a concentration of approximately 1×10^6 cells/mL. To 100 μl of cell suspension, the following antibodies were added (all from BioLegend): 1.5 μl anti-CD73-PE, 1.5 μl anti-CD90-APC-Cy7, 5 μl anti-CD105-APC, 0.5 μl anti-CD14-FITC, 0.5 μl anti-CD19-FITC, 0.5 μl anti-CD31-FITC, 1.5 μl anti-CD45-FITC, 0.5 μl anti-HLA-DR-FITC. For the identification of non-viable cells, 1 μl of 1 mg/mL 7-Aminoactinomycin D (7-AAD, AAT Bioquest) was also added. For analyses including CD146, 5 μl anti-CD146-BV421 (BD Biosciences) was added. Cells were stained for 30 min at room temperature, followed by one wash with PBS/0.1% BSA and centrifugation at 440 x g for 5 min. The cell pellet was resuspended in 400 μl PBS/0.1% BSA followed by immediate flow cytometric acquisition on a NovoCyte 3000 (Agilent), using the NovoExpress Software (version 1.5.0). Calibration of the cytometers was performed daily, using the NovoCyte QC particles (Agilent). Compensation was performed monthly, using the CompBeads, (Anti-Mouse Ig, K and Negative Control, BD Biosciences). The gating strategy included an initial removal of doublet events, debris, and dead cells (7AAD+) after which the remaining gating on marker expression was based on fluorescence Minus One (FMO) controls. Adipose-derived MSC (AD-MSC), used for comparative reasons, were produced as previously described [39] and stained as described above.

EV engineering of USCs

We inserted mCherry fused to a *686-truncated PTG-FRN scaffold with a 3x SGGGG linker into the genome of USCs using transposon technology. 8 μg donor (mCherry-*686-truncated PTGFRN scaffold) and 2 μg hyperactive piggyBac plasmid DNA (VectorBuilder) was complexed with 30 μg PEI max and incubated for 30 min at room temperature prior to addition to cells. USCs for transfection were harvested at passage 2 (P2) and 1,000,000 cells were resuspended into 1 ml Optimem (Thermo Fisher) followed by the addition of PEI complexed DNA. Cells were then incubated in a tube revolver (Thermo Fisher) at 20 revolutions per minute for 30 min at room temperature and thereafter seeded into a T25 Cell+flask and incubated at 37 °C and 5% CO2. After 24 h, media was exchanged with selection media containing 250 μg/ml hygromycin B and incubated without media change for 3 days followed by two further days without selection. A secondary round of selection was performed for 4 days after which a pure engineered population was expanded.

Cell imaging

Live cells were directly imaged using a ZOE Fluorescent Cell Imager (Biorad). USCs were imaged with brightfield and red (excitation: 556/20 nm and emission: 615/61 nm) fluorescent channel. Images were processed and merged using ImageJ (1.53T NIH).

EV enrichment

Cells were grown until 90–100% confluent, after which USC media was removed and cells were washed with PBS, followed by the addition of Optimem in the absence of serum and incubated for 72 h. At harvest

cell-conditioned media (CCM) was differentially centrifuged at 500 x g for 10 min, 2,000 x g for 15 min, and 10,000 x g for 30 min. At CCM harvest, cell viability was determined as described above. The supernatant from differential centrifugation was concentrated 10 times on a 300 kDa MWCO filter, using an Amicon stirred cell (Sigma Aldrich) followed by a further concentration step to 500 µl on 100 kDa MWCO spin filters (Thermo Fisher). Size exclusion chromatography (SEC) with a pore size of 70 nm (Izon) was utilized to reduce protein contaminants in the concentrated CCM, and non-retained/non-chromatographed fractions [7–9] were pooled as EV-containing fractions and stored at 5 °C until characterization by NTA. The relative fluorescence intensity of EV preparations from engineered cells was measured on a DS-11 FX Fluorometer (DeNovix).

NTA
EVs were analyzed using NTA within 24 h after enrichment by SEC. Three videos of 60 s were recorded for each sample at camera level 16, fixed temperature at 24 °C, a flow at 10 µl/minute and analyzed at a detection threshold set to 5 (NTA 3.4 Build 3.4.003). Light scattering was produced by a 405 nm laser on a Nanosight NS300 system (Malvern Panalytical). System validation was performed using 97 nm polystyrene beads. Samples were diluted to a concentration of 40–100 particles per frame with 0.2 µm filtered PBS.

Dot blot
Dot blots were performed by spotting 3 µl of samples on nitrocellulose membranes (Thermo Fisher) with a 0.45 µm pore size. Membranes were dried for 30 min before blocking in 5% BSA in PBS for one hour. Blots were then incubated with a monoclonal antibody targeting the tetraspanin CD63 (Clone-10628D: Thermo Fisher) for one hour with shaking. Blots were then washed 3x in PBS then incubated with HRP-conjugated anti-mouse secondary antibody (Thermo Fisher) for one hour. Blots were washed 3x in PBS prior to development

using Clarity Western ECL (BioRad) and visualization using a Chemidoc Imager (BioRad).

Statistical analysis
Mixed-effect analysis followed by Tukey's multiple comparisons test was performed, using GraphPad Prism version 10.2.0 for Windows (GraphPad Software). The mixed-effect model was applied instead of repeated measures ANOVA, to accommodate missing values in the data set. A p-value < 0.05 was considered statistically significant. Results are displayed as mean ± SEM unless otherwise described.

Results
Urine sampling time does not affect USC yield or expansion capabilities
To identify optimal collection conditions, we first evaluated the effect of sex and urination time points. We selected morning, noon and afternoon but omitted evening collection as this time point is inconvenient for patients and personnel. We included four female donors and four male donors. All donors were healthy, BMI between 20 and 25 and a mean age of 32.6 (24–50) years. One male morning urination was lost due to a missed visit and one female donor was discarded due to contamination. After initial preparation, cells were seeded, and adherent and proliferating cells were regarded as USCs. The sample to growing clone success rate was 100%. One sample only produced a single clone that stopped proliferating after three divisions and was not possible to characterize further. Of the samples collected, we had a success rate of 95% for growing highly proliferative USCs. Clear histological differences were initially observed between female and male samples (Fig. S1) with samples from female donors containing an abundant number of squamous cells. However, after the first media replenishment, no differences were observed between cultures derived from male or female donors.

Colonies were counted 7 days post seeding and imaged. Three distinct phenotypes were observed (Fig. 1). The

Fig. 1 Clones grew into three phenotypical morphologies. (**A**) The most abundant growth morphology grew in close parameters to each other. (**B**) Another phenotype grew with swarming and migrating cells. (**C**) The third phenotype grew with very confined growth borders in an almost tissue-like manner

most abundant phenotype (Fig. 1A) produced distinct colonies with small to no distance between the cells with an occasional few that migrated away from the colony. The second phenotype (Fig. 1B) consisted of swarming cells, originating from the same clone, yet they were highly active and migrated extensively. The third phenotype (Fig. 1C) grew in a confined manner, resembling tissue-like growth with clear borders.

We recorded urine volume, and clones per urination and determined clones per 100 ml (Fig. 2A, B and C). The time point of urination did not affect the number of clones generated or the number of clones per 100 ml of urine. However, there were large variations in how many clones were present in individual donor urinations at various time points.

At harvest (day 14–16 post isolation) characterization by flow-cytometry, viability, and doubling time were determined. Viability ranged from 73.9 to 99.7% (Fig. 2D) with all but one donor above 90%. Doubling time ranged from 19.0 to 28.7 h (Fig. 2E). There were no significant differences in urination time points regarding viability or doubling time. Pooled viability had a mean of 94.6% (95%

CI: 91.7–97.6) and doubling time had a mean of 23.9 h (95% CI:22.6–25.1 h).

USCs display canonical MSC markers

Flow cytometric characterization was performed on pooled colonies at the three time points of urination for each donor. In general, all USCs exhibited a consistent immunophenotype, with negligible expression (<1%) of negative MSC markers (CD14, CD19, CD31, CD45, and HLA-DR). We analyzed the USCs for MSC-related markers CD73, CD90, and CD105. Expanded USCs had a mean expression above 99.5% and 98.0%, for CD73 and CD90, respectively, at all three time points (Fig. 3A). The mean expression of CD105 varied slightly more and ranged from 80.9 to 95.3% between the measured sampling times. However, no significant differences were observed between time points or sexes, with the exception of % CD73+, which was significantly differently expressed between sexes with fewer CD73+USCs from male donors ($p=0.0147$, Fig. S2). Moreover, there were no differences in the detected levels for each marker, expressed as the median fluorescence intensity (MFI)
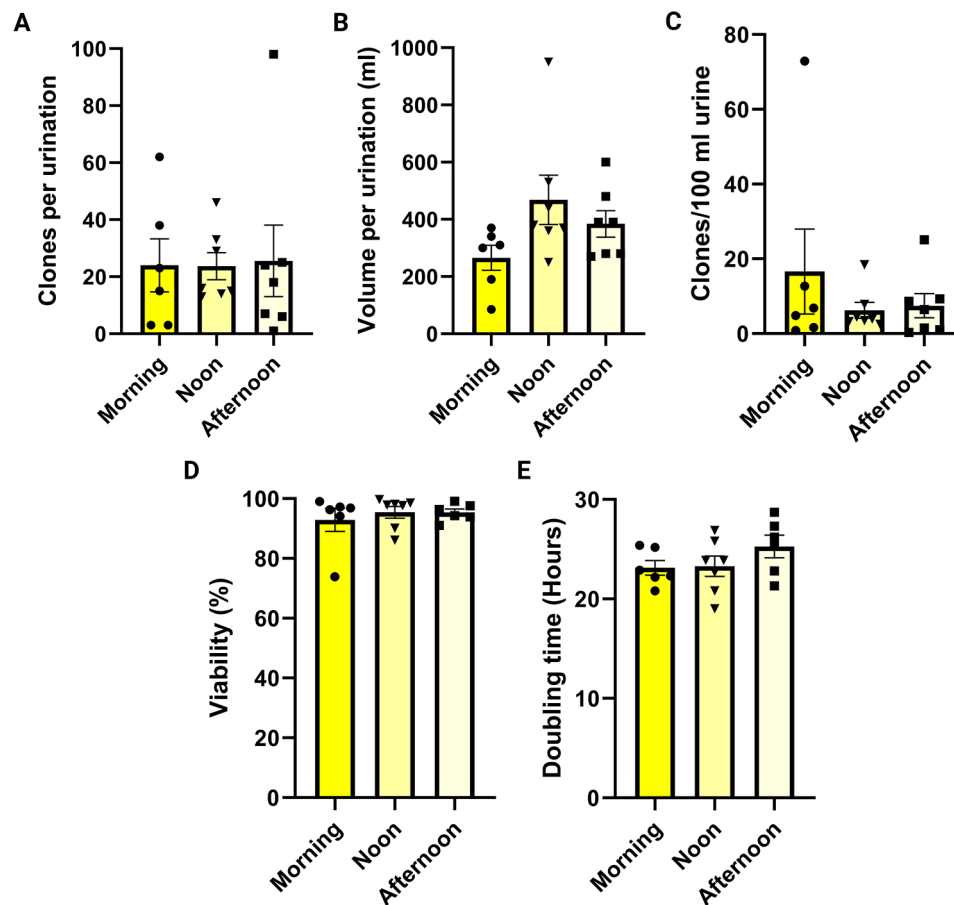

Fig. 2 Pooled data from both sexes. (**A**) Clones per urination were counted, (**B**) volume per urination was measured and (**C**) clones per 100 ml urine were calculated. At harvest (day 14–16) (**D**) viability and (**E**) doubling time was determined for the expanded USCs. No differences were seen for any of the investigated parameters between time points of urinations
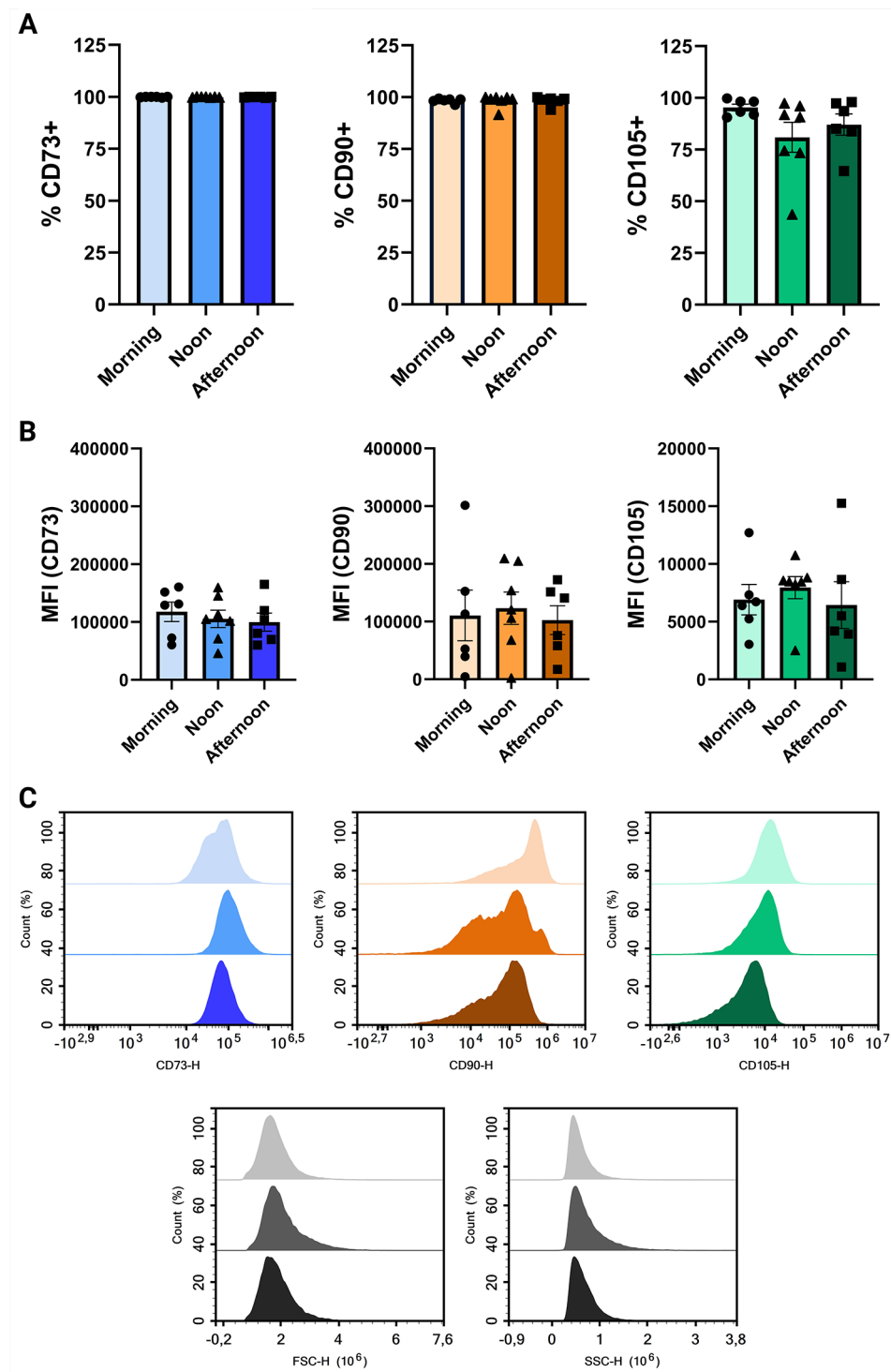
Fig. 3 Expanded USC express MSC-associated markers CD73, CD90, and CD105. After culturing, the expression of selected surface markers on clones collected at different time points was evaluated by flow cytometry. Stratification for gender is not included in the displayed results. The percentwise expression (**A**) and median fluorescence intensity (MFI) (**B**) for each surface marker, are provided as mean±SEM for all included donors. (**C**) Representative histograms for the investigated surface marker as well as forward scatter (FSC) and side scatter (SSC)

(Fig. 3B and C). Although varying intensities of all three MSC markers were detected across time points (Fig. 3C), the intensities were well-separated from background (Fig. S3). Interestingly, USCs showed a similar expression pattern of CD73, CD90, and CD105 as primary adipose-derived MSC (AD-MSC) (Fig. S4) and also expressed CD146 (Fig. S3A). Finally, the detected light scatter parameters (Fig. 3C), translating to cellular size and granularity, did not change significantly over time or with sexes at the time of harvest.

USCs enter an arrested proliferating state, with no loss in viability, under EV production using basal media devoid of growth factors

Two random male donors were selected for EV production, and both donated in the afternoon. Briefly, USCs were expanded to 200 million cells and media was exchanged to chemically defined and growth factor-free basal Optimem, after 72 h media was harvested, concentrated and EVs were enriched using SEC.

Dot blot identified the classical EV marker CD63 in the EV-enriched preparations but not in the protein fractions (Fig. 4A). Particles in the EV fraction had a hydrodynamic diameter modal size ranging from 93.9 to 98.2 nm and a size profile consistent with small EVs derived from mammalian cells [40] (Fig. 4B) with a concentration of $1.1–1.4 \times 10^{11}$ particles per ml. At harvest, cell viability was determined to be 95.3–97.1% ater three days in Optimem

in the absence of supplements, which was comparable to viability at seeding. For comparison, we cultured AD-MSCs at the same conditions and length which showed viability of 83.1% (5% C:78 -88.1) at harvest.

To evaluate the ease of engineering USCs we inserted a PTGFRN construct into the genome as overexpressed PTGFRN fused proteins are selectively sorted into EVs. We added mCherry to the construct as a reporter gene for both genome insertion and EV incorporation. Isolated USCs were genetically engineered to express a construct of mCherry fused to a truncated PTGFRN scaffold to enable display of mCherry on the surface of secreted EVs using the cost-efficient and simple PEI/DNA complexing method. After two rounds of selection over 10 days, a pure engineered USC population was achieved (Fig. 5) expressing the construct (top engineered, bottom native USC). 45-second graphic interchange format timelapse (Fig. S5) of engineered USCs showed the rapid movement of recombinantly expressed mCherry scaffold, presumably in endosomes. EVs derived from mCherry-engineered USCs had similar size profiles (Fig. S6) as non-engineered. In contrast to EVs derived from HEK293T, engineered using the same construct, relative fluorescence units were low (Table S1) at similar particle concentrations, suggesting reduced incorporation of PTGFRN-scaffold in MSCs compared to HEK293T cells.
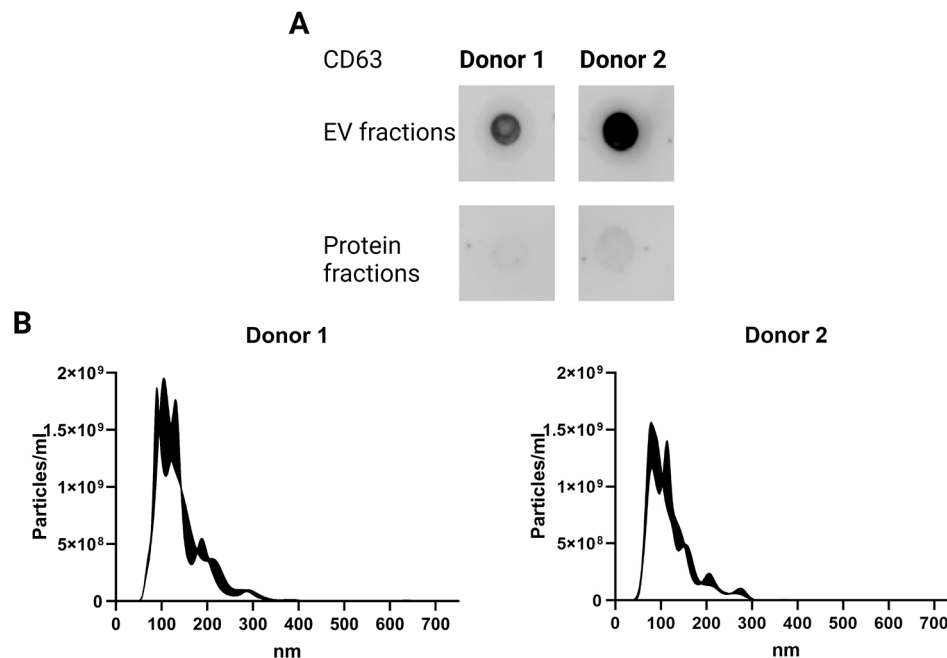

Fig. 4 EVs were enriched from two male donors and briefly characterized for small EV marker and size and concentration. (**A**) Immuno dot blot of EV fractions and protein fractions, 5 μl of each was added to the blot and antibodies against the canonical small EV marker CD63 was added. This showed clear EV enrichment in the expected EV fraction. (**B**) NTA size profile of EV fractions from each donor showed an expected size profile comparable to EVs derived from other sources enriched with the same procedure
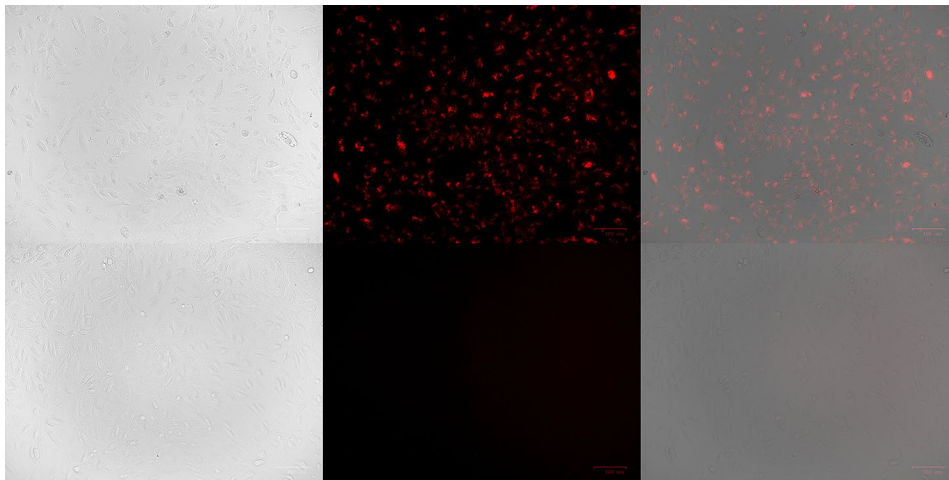
Fig. 5 Left brightfield, middle red channel, right merged images. Top mCherry-PTGFRN-engineered USCs. Bottom native USCs. Images were taken ten days after the engineering during which two cycles of selections had been conducted. After this, a pure engineering population was achieved

Discussion

To our knowledge, the robustness of USCs as an EV production platform and their ability to maintain high viability over 72 h at low nutrient conditions has not been previously investigated. Whilst xeno-free EV-production alternatives have been made [41], the absence of serum or growth factors significantly reduces barriers to up and downstream EV production. Based on the viability of our comparison of USCs and AD-MSCs grown without growth factors, and that it is known that MSCs can enter apoptosis if serum-starved [42, 43], it presents USCs as an ideal low-cost MSC platform easily adaptable to large-scale GMP production [44]. We find the particle size and concentrations comparable to MSC EVs reported by others [45]. The dot blot showed enrichment of EV marker CD63 in the EV-enriched SEC fractions, validating the presence of EVs.

The therapeutic potential of stem cells has been under immense attention, yet the development of cell-free advanced therapies, such as EVs, presents an attractive next-generation alternative. The use of EVs as a therapeutic entity offers possibilities of producing engineered drug vehicles with enhanced capabilities and reduced health risks as they are non-replicative. We therefore investigated the feasibility of genetically engineering USC derived EVs. A truncated PTGFRN scaffold has previously been reported to be efficient in either displaying or internalizing recombinant protein in HEK293T cells [46], we, therefore, utilized this scaffold to display mCherry on secreted EVs from engineered USCs. Whilst we saw a high amount of EV-coupled mCherry secreted by HEK293T cells, fluorescence analysis on secreted EVs from the engineered USCs was limited (Table S1). Whilst USCs were readily engineerable, and the recombinant protein was abundant in the regions where PTGFRN is most enriched (Fig. S5), such as the Golgi apparatus, ER

membrane and endosomes, it was not secreted at high levels which have been previously reported for stem cells [47]. We did not validate the expression of PTGFRN in USCs, and this pathway might not be predominant during the biogenesis of EVs in USCs as compared to HEK293 cells; other scaffolds may therefore be better suited to USCs. Genetic engineering of stem cells is not without challenges as the required culturing time and passages are increased compared to native MSCs, which could lead to chromosomal alterations, phenotypic changes and senescence [48]. However, we demonstrated a robust and efficient transfection method and observed a rapid expansion under selection conditions compared to other stem cells types we have engineered in the laboratory (personal observation). The PiggyBac transposon integrates donor DNA into inverse terminal repeats of the genome which are considered safe harbours and the PiggyBac system has been used in vivo and in clinical trials [49, 50], as such it would not be expected that oncogenes are activated or otherwise deleterious mutations that could affect EV payloads. The engineered USCs expanded to quantities required for therapeutic EV production at a similar rate as non-engineered USCs. Hence, USCs could serve as a unique platform for engineered MSCs for both cell-based and secretome-based therapy.

As USCs are relevant to several potential clinical applications, we aimed to evaluate some of the factors critical for the suitability and translational aspects of USC in this context. This includes sex-specific differences and an optimal collection time, which could affect the resulting colonies morphology, proliferation rate, and immunophenotype. Although our sample size is not large enough to determine this, we find indications that the aseptic sampling stringency between sexes seems to have an impact on potential contamination. Sex differences in urinary tracts are well described with females

have a more complex microbiota, and cellular differences are also apparent [51]. It could be presumed that the risk of contamination is higher when sampling women. The increased microbiological complexity and the shorter urethra could explain the increased turnover of squamous cells (Fig. S1) released into the urine of women. If autologous cells are a necessity, optimization could therefore be needed to ensure consistency of the quality between donors of different sexes.

Single clones have often been selected in prior works with USCs [52, 53]. While this strategy could potentially yield very homogeneous phenotypes and secretomes, it could lead to reduced genetic stability as more doublings are needed before reaching EV production cell counts [54]. Nonetheless, it has been reported that there were no chromosomal variations present at passage 7 for USCs [20]. We pooled all clones to maintain the potential positive effects of heterogeneity and reduce the number of cell doublings before EV production. Our observations of three distinct phenotypes (Fig. 1) merit further investigation into whether potential modulatory effects on recipient cells differ, but that is beyond the scope of this study. The success rate for urination to large expansions and clones per ml of urine was in line with what has been described, albeit our rates and incidents were somewhat higher than previously reported [53, 55]. Our data does not suggest that there would be any time during the day that is better to deliver a urine sample when harvesting USCs, nor do they show that there could be an increased risk of contamination in morning urine.

Within the investigated cohort, a relatively homogenous immunophenotype of USCs was demonstrated. Expression of CD73, CD90, and CD105 constitutes a common and shared phenotypic trait of MSCs from different tissue sources and is recommended for minimal characterization of MSC from adipose tissue [56]. In this study, CD73 was found to be slightly but significantly upregulated on USC from female donors, compared to the male donors (Fig. S2). In a therapeutic setting, CD73 has been demonstrated to identify cells with pro-angiogenic features [57], as well as displaying anti-inflammatory [58] and anti-fibrotic properties [59]. Hence, choosing clones from female donors, with the highest CD73 expression, could potentially identify those with the most potent therapeutic effect. In a wider context, donor age and sex have been proposed to affect the therapeutic potential of MSC-based therapies, however, this subject has not been systematically addressed yet [60, 61]. Whether these features impart a heterogeneity in USC remains to be established in a larger cohort, to allow for adjustment for both sex and age. Nevertheless, the investigated phenotypic attributes are interesting within the scope of clinical translation, as the expression profile of CD73, CD90, and CD105 is accepted by regulatory authorities as a quality parameter for AD-MSCs used in clinical application when combined with the absence of several negative markers (CD14, CD19, CD31, CD45, HLA-DR) [62] which is in line with previously observed analysis [20] although our analysis showed higher expression of CD90 and CD105. In particular, the absence of HLA-DR on USCs in this study may point to an expected low immunogenicity of these cells, which could support usage in an allogeneic therapeutic strategy. The presence of CD146 on USCs suggests shared characteristics with other MSC types, as this marker is also expressed by other types of MSC. CD146-positive MSCs have been shown to have high proliferating capabilities and maintain multipotency regarding differentiation potential into the three classical lineages [63] and also neurological cells [64]. CD146-positive MSCs have also been linked to having higher modulatory and migratory capabilities and the secreted EVs were more potent in immunomodulation and regeneration [65, 66].

Autologous MSCs have been reported to be a superior therapeutic option for certain clinical indications compared to allogeneic cells. Cell transplants with autologous MSCs have shown higher success rates for engraftment and are not expected to lead to alloimmunity [67, 68]. MSCs derived from diseased and older individuals could potentially be less potent, but Zhang et al. showed that USCs derived from various end-stage liver disease patients had the same potency in reducing acute and chronic liver injury in a murine model [69]. On the contrary to cell transplantation, treatment with non-self EVs does not seem to be as immunogenic [70], yet prolonged treatment with allogenic EVs could lead to immune clearance, reduced circulation time, and lowered therapeutic effects, as previously reported [71]. As such, the ease of isolating, growing autologous and the engineering of USCs as we have shown could potentially outweigh the burden from using autologous cells, and lead to a potent and indefinite treatment form with minimal discomfort for the donors.

In summary, the presented culture strategy is independent of sampling time or sex and yields USCs with a stable proliferation capacity and viability, whilst also producing cells that display a shared stem cell immunophenotype with MSCs derived from other tissue types. These results warrant further investigation to discover if these traits extrapolate into comparable regenerative and immunomodulatory potential. As USC isolation is non-invasive, we see USCs as a unique source of primary MSCs, readily available for all researchers. Lastly, we believe that USCs could serve as a bridge from research to clinical application as they are easily manipulated and are even more robust than cell lines during EV production.

Conclusion

USCs are simple to isolate and expand with a high success rate from both men and women. Our findings suggest that USCs will be of key interest to the EV field in the future as they can maintain high viability and secrete EVs at unprecedentedly low nutritional/serum-free conditions giving both up and downstream advantages for large-scale and GMP production. Finally, USCs can be engineered very easily with large plasmids, using PEI, and can be readily expanded to a pure engineered culture under selection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03903-0.

Supplementary Material 1

Data availability
All data is presented in this publication, either as primary or supplementary data.

Declarations

Ethical approval and consent to participate
Written informed consent was obtained from all the participants before inclusion. The study was conducted under the ethically approved project: Cellebaseret behandling af patienter med multipel sklerose. Approval was granted by The South Denmark Region Committee on Biomedical Research Ethics and the Danish Data Protection Agency (no. 23/55327). The project number is S-20220041 and has an amendment with the number 106489. Ethics approval was granted on the 5. August 2022 and the amendment was approved 5. December 2023.

Consent for publication
No individual information that could identify the donors was gathered.

Competing interests
The authors declare that they have no competing interests.

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