

Use of conditioned media (CM) and xeno-free serum substitute on human adipose-derived stem cells (ADSCs) differentiation into urothelial-like cells

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Abstract Background: Congenital abnormalities, cancers as well as injuries can cause irreversible damage to the urinary tract, which eventually requires reconstruction. Smooth muscle cells, endothelial cells, and urothelial cells are the major cell types required for the reconstruction of lower urinary tract. Adult stem cells represent an accessible source of unlimited repertoire of untransformed cells. **Aim:** Fetal bovine serum (FBS) is the most vital supplement in the culture media used for cellular proliferation and differentiation. However, due to the increasing interest in manufacturing xeno-free stem cell-based cellular products, optimizing the composition of the culture media and the serum-type used is of paramount importance. In this study, the effects of FBS and pooled human platelet (pHPL) lysate were assessed on the capacity of human adipose-derived stem cells (ADSCs) to differentiate into urothelial-like cells. **Methods:** ADSCs were isolated from human lipoaspirates and characterized by flow cytometry for their ability to express the most common mesenchymal stem cell (MSCs) markers. The differentiation potential was also assessed by differentiating them into osteogenic and adipogenic cell lineages. To evaluate the capacity of ADSCs to differentiate towards the urothelial-like lineage, cells were cultured with either conditioned (CM) or unconditioned urothelial cell media (UCM), supplemented with either 5% pooled human platelet lysate (pHPL), 2.5% pHPL or 10% FBS. After 14 days of induction, cells were utilized for gene expression and immunofluorescence analysis. **Results:** ADSCs cultured in CM and supplemented with FBS exhibited the highest upregulation levels of the urothelial cell markers; cytokeratin-18 (CK-18), cytokeratin-19 (CK-19), and Uroplakin-2 (UPK-2), with a 6.7, 4.2- and a 2-folds increase in gene expression, respectively. Meanwhile, the use of CM supplemented with either 5% pHPL or 2.5% pHPL, and UCM supplemented with either 5% pHPL or 2.5% pHPL showed low

expression levels of CK-18 and CK-19 and no upregulation of UPK-2 level was observed. In contrast, the use of UCM with FBS has increased the levels of CK-18 and CK-19, however to a lesser extent compared to CM. At the cellular level, CK-18 and UPK-2 were only detected in CM/FBS supplemented group. Growth factor analysis revealed an increase in the expression levels of EGF, VEGF and PDGF in all of the differentiated groups. **Conclusion:** Efficient ADSCs urothelial differentiation is dependent on the use of conditioned media. The presence of high concentrations of proliferation-inducing growth factors present in pHPL reduces the efficiency of ADSCs differentiation towards the urothelial lineage. Additionally, the increase in EGF, VEGF and PDGF during the differentiation implicates them in the mechanism of urothelial cell differentiation.

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Key words: adipose stem cells, urothelial cells, pooled human platelet lysate, cellular differentiation

Introduction

Congenital abnormalities, cancers as well as injuries may lead to irreversible damage to the urinary tract, which eventually requires reconstruction. Early attempts on the reconstruction of urinary tract have focused on the use of acellular matrix for bladder reconstruction. Acellular matrices are immunologically inert and act *in vivo* as scaffolds, to recruit the progenitor cells and infiltrate the matrix to produce bladder tissue (Howard et al., 2008). The two most commonly used acellular matrices for bladder and urethral reconstruction are the small intestinal submucosa (SIS) and the bladder acellular matrix (BAM) (Staack et al., 2005). In the year 2000, the FDA approved the use of porcine collagen matrix, derived from the small intestinal submucosa, in the reconstruction-based surgical procedures (Hodde et al., 2007). However, fibrosis, matrix shrinkage and the lack of the ability to perform urothelial anastomosis are the major hurdles that must be overcome before acellular matrices can be used in the bladder and urethral reconstruction (Portis et al., 2000; Campodonico et al., 2004; Azadzo et al., 1999; Horst et al., 2019).

Adult stem cells represent an accessible source of unlimited repertoire of untransformed cells. Early attempts to incorporate stem cells in urinary tract tissue engineering culminated in using stem cells without transdifferentiation and directly implanting them *in vivo* (Liao et al., 2013; Chung et al., 2005). Mesenchymal stem cells (MSCs) have been used in organ reconstruction by expanding them *in vitro* and then implanting them to induce their differentiation potential (Sharma et al., 2010; Sharma et al., 2009). On the other hand, stem cells can be expanded and differentiated

in vitro and then transplanted directly to the affected subject. Thus, direct differentiation of stem cells can reduce the time required for patient's recovery.

Smooth muscle cells, endothelial cells, and urothelial cells are the major cell types required for the reconstruction of lower urinary tract (Qin et al., 2014). Three induction protocols have been reported to induce the differentiation of stem cells towards the urothelial lineage: direct co-culture with urothelial cells, indirect co-culture system with urothelial cells, and culturing the stem cells in conditioned media (CM) derived from urothelial cell culture (Becker et al., 2007). The first protocol of direct co-culture system is inapplicable in cases of malignancies, infections, and inflammatory diseases (Liu et al., 2009; Shi et al., 2012). The indirect co-culturing is applicable in small systems such as the filter well insert (Liu et al., 2009). To the current time, the use of conditioned media represents the most favorable and easy-to-use method for induction of MSCs to urothelial cells. However, several problems still need to be addressed before using these cells in clinical therapy, including the limited differentiation capability (trans-differentiation efficiency ranges from 40-70%) and the presence of xenogeneic substances such as fetal bovine serum (FBS) in cell culture media (Zhang et al., 2014; Shi et al., 2012).

Fetal bovine serum is the most vital supplement used in cell culture media for cell proliferation and differentiation. However, due to its limited supply and the increased demand on manufacturing xeno-free stem cell-based cellular products, optimizing the composition of culture media and the type of serum used are of critical importance.

Human platelet lysate (HPL) is defined by Marx as "the volume of plasma fraction of autologous blood having a platelet concentration above baseline". It has been reported that HPL enhances the proliferation and differentiation of MSCs compared to xenogenic FBS (Kakudo, et al., 2008; Lucarelli, et al., 2003; Li, 2013; Mishra, et al., 2009; Cervellia, et al., 2012). These effects make

the HPL as an attractive alternative that can be used in MSCs culture with minimal adverse effects in clinical settings. In this study, the effects of FBS and pooled human platelet (pHPL) lysate were assessed on the capacity of huADSCs to differentiate into urothelial-like cells. Also, we aimed to compare the ability of both conditioned media (CM) and unconditioned urothelial cell media (UCM) to induce urothelial differentiation of ADCS.

Methods

Material and reagents:

Dulbecco's Modified Eagle's Medium DMEM (GIBCO, USA), collagenase type I (Worthington, Lakewood, NJ), FBS (GIBCO, USA), streptomycin and penicillin and 20 mM L-glutamine (Euroclone, Italy), 0.25% trypsin–0.04% EDTA (GIBCO, USA), (SV-HUC-1) ATCC (CRL-9520), StemPro Adipogenesis differentiation media (Invitrogen, USA), Trizol reagent (Invitrogen, USA), Human MSC Analysis Kit (BD, USA), iScript reverse transcription supermix (BioRad, USA), iQTM SYBR mix (BioRad, USA), cytokeratin-18 (Abcam, ab668, 1:200), uroplakin-2 (Santa Cruz, sc-15178, 1:50), ELISA kits (Abcam, UK).

Cell culture:

All experimental protocols involving human tissues were approved by the Ethics Committee at the King Abdullah Hospital, School of Medicine, Jordan University of Science and Technology (IRB No: IRB/7/2019). After obtaining signed informed consents, human adipose tissue aspirates were obtained from 3 individuals, aged 30, 31 and 35, who underwent liposuction procedures. ADSCs were isolated as previously described (Francis et al., 2010). Briefly, adipose tissue aspirates were digested with 0.1% collagenase type I (Worthington, Lakewood, NJ) in PBS, for 45 minutes at 37°C, with gentle shaking every five minutes. The enzyme was then diluted with an equal amount

of complete cell culture medium consisting of DMEM (GIBCO, USA) supplemented with 10% FBS (GIBCO, USA), 1% streptomycin and penicillin and 20 mM L-glutamine (Euroclone, Italy). The suspension was centrifuged at 1200 xg for 10 minutes and then the pellet was resuspended in 5 ml complete cell culture medium. After that, the cell suspension was passed through a 70 µm cell strainer and centrifuged at 500 xg for 10 minutes. The obtained cells were counted and seeded at a density of 2×10^5 cells/cm² in a tissue culture flask and incubated at 37 °C and 5% CO₂. The medium was changed every two to three days until the adherent ADSCs became 70-80% confluent. Cells were detached with 0.25% trypsin–0.04% EDTA (GIBCO, USA) solution, and the resulting ADSCs at passage 3-5 were used for further experiments. SV40 immortalized human ureter urothelium (SV-HUC-1) cell line was obtained from ATCC (CRL-9520). SV-HUC-1 cells were cultured in F-12K medium supplemented with 10% FBS and 1% streptomycin and penicillin. Conditioned medium derived from SV-HUC1 was collected and used to induce the ADSCs differentiation towards the urothelial-like cell lineage.

Flow cytometry

Cultured ADSCs at passage 3 and 70% confluency were utilized for cell surface marker assessment. Cells were detached using TrypLE (GIBCO, USA) and washed twice with FACS buffer (PBS, 1% FBS). Then, cells were counted and adjusted to 10⁶ cells/ml. Aliquots of 100µl were placed in test tubes and incubated with fluorochrome-conjugated antibodies using Human MSC Analysis Kit (BD, USA), which includes: CD-44, CD-105, CD-73, CD-90 and a negative cocktail includes: CD-34, CD-11b, CD-19, CD-45 and HLA-DR mix for 30 minutes in the dark, according to the manufacturer's instructions. Cells were then centrifuged at 300 xg for 5 minutes and resuspended in 500 µl FACS buffer. The analysis was performed using BD FACSCanto™ and the data were analyzed using Diva software.

Multilineage Differentiation

Adipogenic differentiation was performed using StemPro Adipogenesis differentiation media (Invitrogen, USA) for 14 days. After that, cells were washed twice with PBS, fixed in 4% formaldehyde for 15 minutes and stained with oil red O stain, to confirm the presence of adipocytes. StemPro Osteogenic differentiation kit (Invitrogen, USA) was used to induce ADSCs differentiation towards the osteogenic lineage. After 21 days in culture, differentiated cells were washed, fixed in 4% formaldehyde for 15 minutes, and stained with Alizarin Red S (ARS) stain, to verify the osteogenic differentiation. Cells under normal culture conditions were used as negative controls.

Preparation of Pooled Human Platelet Lysate (pHPL)

Platelet bags designated as platelet-rich plasma 1 (PRP1) were obtained from Jordan University Hospital/blood bank unit. Briefly, platelet bags from 17 donors were pooled in one container and centrifuged at 700xg for 17 minutes at 18 °C. After centrifugation, the platelets pellet was formed and the supernatant was designated as platelet-poor plasma (PPP). The latter was transferred into new sterile tubes, and platelets obtained from 1ml PRP1 were resuspended in 300µl of PPP, this was designated as PRP2. Following that, platelets concentration was adjusted to 2×10^6 platelets/µl with PPP, and lysed through two freeze/thaw cycles at -20°C and then at 37°C. Platelet fragments were removed by centrifugation at 3000 xg for 20 minutes at 18°C and filtrated through a 0.2 µm filter. The obtained supernatant is now called pHPL. The pHPL was aliquoted and stored at -20 °C. Upon supplementing the media with 10% pHPL, 2 IU/mL of heparin was added to prevent coagulation.

Differentiation of ADSCs into Urothelial-like Cells

When SV-HUC1 cells cultured in F-12K medium reached 80-90% confluency, medium was collected every 24 hours and changed into fresh medium for two sequential days. Next, collected medium was centrifuged at 3000 rpm for 5 minutes, pooled and filtered through a 0.22 μ m filter and stored at -20°C. For urothelial induction, ADSCs were seeded at a density of 500 cells/cm² and cultured with either conditioned or unconditioned medium (UCM) at a ratio of 1:4 F-12K: DMEM, supplemented with either 5% pHPL, 2.5% pHPL or 10% FBS. Uninduced controls were also included. On day 14 post-induction, cells were utilized for immunofluorescence staining and RNA extraction for relative gene expression analysis.

qRT-PCR Gene Expression Analysis of Urothelial Markers

Total RNA was isolated at day 14 of urothelial differentiation using Trizol reagent (Invitrogen, USA) and subsequently purified with RNeasy mini kit (Qiagen, USA). cDNA was synthesized using iScript reverse transcription supermix (BioRad, USA). Quantitative RT-PCR (qPCR) reactions were performed using iQTM SYBR mix (BioRad, USA) and 300nM of each forward and reverse primers. Primer sequence and product size are provided in Table 1.

Immunofluorescence staining of Urothelial Markers

After 14 days of induction, cells on coverslips were fixed in 4% formaldehyde for 15 minutes and permeabilized with PBS/0.1% Triton X-100 for 5 minutes. To prevent nonspecific binding, cells were incubated with blocking solution (3% BSA (wt/v) and 0.3% Triton X-100 (v/v) in PBS) for 60 minutes. Cells were then incubated with the primary antibodies against cytokeratin-18 (Abcam, ab668, 1:200) and uroplakin-2 (Santa Cruz, sc-15178, 1:50), diluted in blocking buffer overnight at 4°C in a humid chamber. Subsequently, cells were incubated with the appropriate secondary antibodies, either chicken anti-mouse IgG –FITC or donkey anti-Goat-IgG Cy3, for 1 hour at room

temperature followed by counterstaining with DAPI (4',6-diamidino-2-phenylindole) and mounting with mounting media (Invitrogen, USA).

Enzyme Linked Immunosorbent Assay (ELISA)

The secretion of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF-BB) in conditioned and unconditioned culture medium was measured using ELISA kits (Abcam, UK), according to the manufacturer's instructions. For growth factors measurement, fresh serum-free media was added on day 14 of urothelial induction and collected after 24 hours. Triplicate samples were run in 96-well plates coated with an antibody specific to a particular growth factor mentioned above. The absorbance was measured at 450 nm and within 30 minutes of completing the assay.

Statistical Analysis

All the experiments were performed at least three times, and statistical analysis was performed using SPSS 20.0. The data were represented as the mean \pm standard error of the mean (SEM) and tested for normality and equal variance before analysis using the Shapiro-Wilk test. Statistical differences were calculated using analysis of variance (ANOVA) and Post-hoc test for comparison between groups. The analysis of ELISA data was performed using Graphpad Prism for curve fitting and independent t-test for significance calculation. Differences were considered significant at $P < 0.05$.

Results

Isolation and Characterization of ADSCs

Cells with fibroblastic morphology were adhered to the tissue culture plate and reached the confluency within two weeks of the initial plating (Figure 1A). Flow cytometry staining of the most common MSC markers showed positive expression of the following markers: CD-44 (100%),

CD-105 (89.8%), CD-73 (99.9%), and CD-90 (100%) (Figure 1B-E). To confirm the purity of the isolated ADSCs from the hematopoietic stem cell contamination, flow cytometry staining was performed and demonstrated minimal expression levels of the negative cocktail markers (Figure 1F). To further validate the stemness of the isolated cells, ADSCs were transdifferentiated into the osteogenic and adipogenic cell lineages. Following 21 days of the osteogenic induction, cells exhibited flattened and more elongated morphology with extracellular calcium phosphate deposits as confirmed by Alizarin red S (ARS) staining. These deposits were absent in the uninduced ADSCs cultured in cell culture media (Figure 2 A-D). Additionally, adipogenic differentiation showed intracellular localization of lipid droplets. These droplets were positively stained with oil red O, and were absent in the uninduced negative control. Thus, the successful differentiation of ADSCs into osteoblasts and pre-adipocytes confirmed the multipotency of these cells (Figure 2 E-H).

Urothelial cell markers detection by qRT-PCR

To evaluate the capacity of ADSCs to differentiate towards the urothelial-like lineage, cells were cultured with either conditioned (CM) or unconditioned urothelial cell media (UCM), supplemented with either 5% pooled human platelet lysate (pHPL), 2.5% pHPL or 10% FBS. After 14 days of induction, cells were utilized for further experiments. To analyze the effect of induction CM and UCM media on the differentiation of ADSCs towards the urothelial lineage, we measured the gene expression levels of two cytokeratin proteins; CK-18 and CK-19 and one uroplakin protein (UPK-2), expressed by the urothelial cells (Figure 3). CK-18 and CK-19 are considered as early markers of urothelial cell specification, and uroplakin proteins representing the terminal maturation stage. The levels of CK-18 expression were not significantly altered in groups treated with CM supplemented with either 5% or 2.5% pHPL ($P > 0.05$). Meanwhile, a 6.7-fold

upregulation in CK-18 expression was observed in CM FBS treated group ($P=0.006$) (Figure 3A). Additionally, UCM increased the CK-18 expression of 3-fold in the presence of either FBS or pHPL (P-Value). On the other hand, culturing ADSCs in CM supplemented with FBS exhibited the highest upregulation level of CK-19 with a 4.2-fold increase compared to the uninduced control ($P= 0.01$). In contrast, CM 2.5% pHPL and CM 5% pHPL failed to upregulate the expression of CK-19. Whereas, UCM-FBS induced CK-19 expression, but to a lesser extent compared to CM-FBS. On the contrary, UCM supplemented with 5% and 2.5% pHPL failed to upregulate the expression of CK-19 (Figure 3B). Regarding the UPK-2 terminal differentiation marker, only CM-FBS and UCM-FBS cultures showed an increased level of expression of approximately 2-fold compared to control cells (Figure 3C).

Detection of Urothelial Cell Markers by Immunofluorescence

Since gene expression results suggest an enhanced cellular differentiation using CM culture conditions, we compared the expression of CK-18 (early differentiation marker) and UPK-2 (late differentiation marker) between pHPL and FBS supplemented cultures by immunofluorescence staining (Figure 4). Staining revealed that CM supplemented with FBS resulted in a 2.5-fold increase in the expression of CK-18 early differentiation marker and a 2-fold increase in UPK-2 expression compared to non-induced ADSCs control (Figure 4 A & B). Whereas groups treated with CM & 5% pHPL or 2.5% pHPL failed to elicit the same response (Figure 4 A & C).

Assessment of growth factor levels of induced ADSCs by ELISA

To assess changes in the growth factor levels produced by the induced cells, we measured the levels of EGF, PDGF-BB, and VEGF, the main growth factor proteins secreted by cells into the culture medium. Following 14 days of urothelial induction, measurement of the growth factors in

serum-free media collected after 24 hours resulted in the detection of higher levels of these factors in cells treated with CM compared to their counterparts cultured in UCM (Figure 5). Additionally, cells cultured in 5% pHPL produced higher levels of EGF, PDGF-BB, and VEGF. Levels of VEGF were significantly higher in CM-5% pHPL, CM-2.5% pHPL and UCM-pHPL ($P= 0.009, 0.023, 0.004$, respectively). Cells in FBS containing media showed the least amount of secretion of all three growth factors. Whereas, CM-FBS elicited higher levels of growth factors compared to UCM-FBS with 1.4-fold, 2.5-fold, and 2.6-fold difference for EGF, PDGF-BB, and VEGF, respectively (Figure 5).

Discussion

Currently, cell-based therapy and tissue engineering studies mostly rely on the *ex-vivo* expansion and differentiation of many cell types especially stem cells. The preferential use of MSCs over other types of stem cells is related to their ability to cross lineage commitment, they differentiate efficiently into many cell types, and their inability to form teratomas or tumors *in vivo* (Qin et al., 2014). Furthermore, MSCs exhibit low immunogenicity and possess immunosuppressive capabilities, facilitating their use in allogenic stem cell transplantation studies (Klyushnenkova et al., 2005; Le Blanc et al., 2004). However, the clinical use of MSCs is hampered by their limited availability, growth variability, invasive collection procedures, and the use of xenogeneic sources of serum such as FBS during the expansion and differentiation procedures. Conditioned medium is widely used to induce MSC differentiation into chondrocytes, osteocytes, dopaminergic neurons, cholinergic neurons as well as urothelial cells (Alves da Silva et al., 2015; Heino et al., 2004; Aliaghaei et al., 2016; Borkowska et al., 2015; Zhang et al., 2014).

Different types of MSCs have been differentiated efficiently towards urothelial cells using urothelial cell-derived CM or FBS, making them an attractive source for urinary tract tissue regeneration (Shi et al., 2012; Zhang et al., 2013). However, a cell line-derived CM might contain many undefined factors, which could influence the cells in a myriad of ways. In addition, the use of FBS carries the risk of infectious disease transmission and immunization, which results in restricting the use of such system in a clinical setting (Heiskanen et al., 2007; Sundin et al., 2007).

Thus, for the future translational purposes, we aimed to assess the ability of unconditioned urothelial cell media to substitute cell line-derived CM and pHPL as alternative to FBS, to induce ADSCs differentiation towards urothelial cells.

Our results indicate that although ADSCs are capable of expressing early differentiation markers of urothelial-like cells such as CK-18 and CK-19 when cultured in CM, this expression is minimal and could not be detected at the cellular level as observed by immunostaining. On the other hand, terminal differentiation phenotype associated with the expression of UPK-2 is only achievable in the presence of CM. This confirms the presence of certain signaling factors in the CM that act in a paracrine manner and are essential for the induction process. Zhang et al., reported an increased level of expression of a panel of cytokines and growth factors following 12 hours of induction of ADSC into urothelial cells (Zhang et al., 2013). However, after 21 days of induction, the levels normalized slowly until they reached a level similar to the baseline level seen before the induction process (Zhang et al., 2013). Thus, a critical timing window for the differentiation process is crucial during the early and intermediate stages of differentiation.

Since UCM by itself lead to a minimal increase in the urothelial markers, an addition of growth factors and cytokines during this critical timing window might increase the differentiation potential. In a previous study, the addition of FGF-10 to Warton jelly-derived MSC induced their differentiation towards urothelial cells, as evident by the co-expression of CK-8 and UPK-III (Chung & Koh, 2014). In a similar manner, discovering more of such inductive factors paves the way for the generation of a defined differentiation system.

Here we report increased levels of EGF, VEGF, and PDGF-BB growth factors when cells were cultured in urothelial cell-derived CM. Several other studies also showed elevated levels of secretion when either ADSCs or BMSCs are cultured under the same conditions (Tian et al., 2010; Shi et al., 2012; Zhang et al., 2014). EGF plays a crucial role in inducing early ESC differentiation towards the endodermal lineage (Cras-Méneur et al., 2001; Kumar et al., 2014). However, induction of terminal urothelial differentiation *in vitro* with PPAR γ activators requires the

inhibition of EGF pathway (Varley et al., 2004a, 2004b, 2006). Thus, the requirement of EGF might be essential during the early stages of induction, to induce a basal urothelial phenotype and it should be followed by inhibition stage in order to produce the terminal superficial urothelial phenotype.

It has been indicated that PDGF can induce proliferation and differentiation of cells originating from all three germ layers including lung, microvilli, gastrointestinal and endothelial cell development ~~based on the normal PDGF signaling~~ (Boström et al. 2002; Karlsson et al. 2000; Utoh et al. 2003; Ding et al. 2004; Calver et al. 1998). Additionally, PDGF is known to be secreted by endothelial and epithelial cells and it regulates the proliferation and differentiation of neighboring smooth muscle cells (Bostrom et al 2002, Barkauskas 2013).

On the other hand, VEGF mediates MSC differentiation towards the endothelial cell lineage via the Rho/MRTF-A pathway (Wang et al., 2013). Although the major role of VEGF is played during vasculoneogenesis and cardiac development, it also contributes significantly to the development of organs of endodermal origin including liver, lungs and pancreas (Carmeliet et al., 1999; Giordano et al., 2001; Lammert et al; 2001; Matsumoto et al., 2001; Compornolle et al., 2002).

Even though the role of these growth factors during the differentiation, development and organogenesis is well-established, a need arises to characterize the extent of involvement of each of these factors in the induction of urothelial cell differentiation. The general consensus on the mechanism of action of conditioned medium is the presence of undefined soluble factors released by the differentiated cells as a way of intercellular communications. These biologically active factors act in a paracrine manner and trigger an internal signaling pathways in MSCs, directing them to differentiate towards a certain lineage (Alves da Silva et al., 2015; Zhang et al., 2014). These differentiated cells in turn secrete bioactive factors, which act in an autocrine and paracrine

manner to maintain the differentiated state of the cells, as well as inducing the differentiation of neighboring cells (Alves da Silva et al., 2015; Zhang et al., 2014; Shi et al., 2012).

In terms of serum choice, we found that pHPL is not an efficient alternative for urothelial differentiation at a concentration of 5% and 2.5%. Substituting FBS with pHPL during the differentiation reduces the expression of urothelial related markers.

Several studies reported an increase in the differentiation potential of several types of MSCs towards the osteogenic, myofibroblastic, cardiomyogenic and adipogenic cell lineages in the presence of pHPL as a serum substitute (Karadjian et al., 2020; Samuel et al., 2017; Chignon-Sicard et al., 2017; Homayouni Moghadam et al., 2016). On the other hand, pHPL decreased the ability of certain types of stem cells to differentiate into osteoblasts, chondrocytes and adipocytes (Gruber et al., 2004; Lee et al., 2014; Chignon-Sicard et al., 2017). Thus, the response elicited towards the presence of pHPL in the culture media is affected by the stem cell type, the differentiation lineage, and the percentage of pHPL used in the culture media. MSC including ADSCs have an increased proliferative capacity in the presence of pHPL (Karadjian et al., 2020; Naaijken et al., 2012, K lle et al., 2013). As urothelial cells differentiate from the basal layer towards the superficial layer, they lose their ability to regenerate. Thus, the molecular circuitry governing differentiation depends on a skew towards differentiation by reducing proliferation, which probably could not be achieved in the presence of pHPL.

In conclusion, the induction of ADSCs towards the urothelial phenotype requires the presence of both CM and FBS. Substituting CM with UCM and FBS with pHPL significantly impacts the differentiation process. The levels of EGF, VEGF, and PDGF have increased during the differentiation process and thus might play an essential role in defining the mechanism of action of CM directed differentiation. Additionally, we found that pHPL at 5% and 2.5% concentrations

negatively influenced the differentiation process. This might be explained by a skew in cellular circuitry towards the proliferation rather than the differentiation process. Changing MSCs microenvironment to accommodate the urothelial cells microenvironment is a basic requirement for any successful differentiation protocol. This can be achieved by the addition of defined soluble factors, direct or indirect co-culture with differentiated cells, the use of conditioned media or direct implantation *in vivo*. Urothelial cell-derived CM represents a practical and efficient way for ADSCs differentiation into urothelial lineage. However, a demand arises to formulate a defined media that efficiently induces MSC differentiation towards the urothelial lineage for clinical purposes.

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Authors' contributions

Conceived and designed the experiments: BK and AA. Performed the experiments: BK and NA.
Analyzed the data: BK and SD. Wrote the manuscript: BK and NA.

Competing interests

The authors declare that they have no competing interests.

References

- Howard, D., Buttery, L. D., Shakesheff, K. M., Roberts, S. J. (2008). Tissue engineering: strategies, stem cells and scaffolds. *Journal of Anatomy*, 213(1), 66–72.
- Staack, A., Hayward, S. W., Baskin, L. S., Cunha, G. R. (2005). Molecular, cellular and developmental biology of urothelium as a basis of bladder regeneration. *Differentiation* , 73(4), 121-133.
- Hodde JP, Johnson CE.(2007). Extracellular matrix as a strategy for treating chronic wounds. *American Journal of Clinical Dermatology*, 8(2), 6-15.
- Portis, A. J., Shay Shalhav, A. L., Brewer, A., Humphrey, P., Mcdougall, E. M., Clayman, R. V. (2000). Laparoscopic augmentation cytoplasty with different biodegradable grafts in an animal model. *The journal of urology*, 164(3), 1405-1411.
- Campodonico, F., Benelli, R., Michelazzi, A., Ognio, E., Toncini, C., & Maffezzini, M. (2004). Bladder cell culture on small intestinal submucosa as bioscaffold: Experimental study on engineered urothelial grafts . *European Urology*, 46(4), 531-537.
- Azadzoi, K. M., Tarcan, T., Siroky, M. B., & Krane, R. J. (1999). Atherosclerosis-induced chronic ischemia causes bladder fibrosis and non-compliance in the rabbit. *The Journal of Urology*, 161(5), 1626-1635.
- Liao, W., Yang, S., Song, C., Li, X., Li, Y., & Xiong, Y. (2013). Construction of ureteral grafts by seeding bone marrow mesenchymal stem cells and smooth muscle cells into bladder acellular matrix . *Transplantation Proceedings*, 45(2), 730-734.
- Chung, S. Y., Krivorov, N. P., Rausei, V., Thomas, L., Frantzen, M., Landsittel, D., J.Fuchs, G. (2005). Bladder reconstruction with bone marrow derived stem cells seeded on small

intestinal submucosa improves morphological and molecular composition. *The Journal of Urology*, 174(1), 353-359.

Sharma, A., Fuller, N., Sullivan, R., Fulton, R., Hota, R., Harrington, D., Villano, J., Hagerty, J., Cheng, E. (2009). Defined populations of bone marrow derived mesenchymal stem and endothelial progenitor cells for bladder regeneration. *The Journal of Urology*, 182(2), 1898 – 1905.

Sharma, A., Hota, P., Matoka, D., Fuller, D., Jandali, D., Thaker, D., Ameer, G., Cheng, E. (2010). Urinary bladder smooth muscle regeneration utilizing bone marrow derived mesenchymal stem cell seeded elastomeric poly(1,8-octanediol-co-citrate) based thin films. *Biomaterials*, 31(1), 6207-6217.

Qin, D., Long, T., Deng, J., Zhang, Y. (2014). Urine-derived stem cells for potential use in bladder repair. *Stem Cell Research and Therapy*, 5(3), 69-79.

Becker, C., & Jakse, G. (2007). Stem cells for regeneration of urological structures. *European Urology*, 51(5), 1217-1228.

Liu, J., Huang, J., Lin, T., Zhang, C., & Yin, X. (2009). Cell-to-cell contact induces human adipose tissue-derived stromal cells to differentiate into urothelium-like cells *in vitro*. *Biochemical and Biophysical Research Communications*, 390(3), 931-936.

Shi, M., Zhang, Z., Xu, R., Lin, H., Fu, J., Zou, Z., Wang, F.S. (2012). Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients. *Stem Cells Translational Medicine*, 1(10), 725-731.

Zhang, M., Xu, M., Zhou, Z., Zhang, K., Zhou, J., Zhao, Y., Wang, Z., Lu, M. (2014) The differentiation of human adipose-derived stem cells towards a urothelium-like phenotype in vitro and the dynamic temporal changes of related cytokines by both paracrine and autocrine signal regulation. *PLoS One*. 9(4), e11786.

Francis, M. P., Sachs, P. C., Elmore, L. W., & E. Holt, S. (2010). Isolating adipose-derived mesenchymal stem cells from lipoaspirate blood and saline fraction. *Organogenesis*, 6(1), 11–14.

Klyushnenkova, E., Mosca, J. D., Zernetkina, V., Majumdar, M. K., Beggs, K. J., Simonetti, D. W., McIntosh, K. R. (2005). T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *Journal of Biomedical Sciences*, 12(1), 47-57.

Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S. E., Ringden, O. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scandinavian Journal of Immunology*, 57(1), 11-20.

Alves da Silva, M.L., Costa-Pinto, A.R., Martins, A., Correlo, V.M., Sol, P., Bhattacharya, M., Faria, S., Reis, R.L., Neves, N.M. (2015). Conditioned medium as a strategy for human

- 469 stem cells chondrogenic differentiation. *Journal of Tissue Engineering and Regenerative*
470 *Medicine*, 9(6), 714-23.
- 471 Heino, T.J., Hentunen, T.A., Väänänen, H.K. (2004). Conditioned medium from osteocytes
472 stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation
473 into osteoblasts. *Experimental Cell Research*, 294(2), 458-68.
- 474 Aliaghaei, A., Gardaneh, M., Maghsoudi, N., Salehinejad, P., Gharib, E. (2016). Dopaminergic
475 Induction of Umbilical Cord Mesenchymal Stem Cells by Conditioned Medium of Choroid
476 Plexus Epithelial Cells Reduces Apomorphine-Induced Rotation in Parkinsonian Rats.
477 *Archive of Iranian Medecine*, 19(8), 561-70.
- 478 Borkowska, P., Fila-Danilow, A., Paul-Samojedny, M., Kowalczyk, M., Hart, J., Ryszawy,
479 J., Kowalski, J. (2015). Differentiation of adult rat mesenchymal stem cells to GABAergic,
480 dopaminergic and cholinergic neurons. *Pharmacological Reports*, 67(2), 179-86.
- 481 Zhang, M., Peng, Y., Zhou, Z., Zhou, J., Wang, Z., Lu, M. (2013). Differentiation of human adipose-
482 derived stem cells co-cultured with urothelium cell line toward a urothelium-like phenotype
483 in a nude murine model . *Urology*, 81(2), 465-475.
- 484 Heiskanen, A., Satomaa, T., Tiitinen, S., Laitinen, A., Mannelin, S., Impola, U., Mikkola,
485 M., Olsson, C., Miller-Podraza, H., Blomqvist, M., Olonen, A., Salo, H., Lehenkari,
486 P., Tuuri, T., Otonkoski, T., Natunen, J., Saarinen, J., Laine, J. (2007). N-
487 glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal
488 stem cells is substantially reversible. *Stem Cells*, 25(1), 197-202.
- 489
490 Sundin, M., Ringdén, O., Sundberg, B., Nava, S., Götherström, C., Le Blanc, K. (2007). No
491 alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum
492 antibodies, after transplantation in allogeneic hematopoietic stem cell recipients.
493 *Haematologica*, 92(9), 1208-15.
- 494
495 Chung, S.S., Koh, C.J. (2013). Bladder cancer cell in co-culture induces human stem cell
496 differentiation to urothelial cells through paracrine FGF10 signaling. *In Vitro Cellular and*
497 *Developmental Biology. Animal*. 49(10), 746-51.
- 498
499 Tian, H., Bharadwaj, S., Liu, Y., Ma, P. X., Atala, A., & Zhang, Y. (2010). Differentiation of Human
500 Bone Marrow Mesenchymal Stem Cells into Bladder Cells: Potential for Urological Tissue
501 Engineering. *Tissue Engineering. Part A*, 16(5), 1769-79.
- 502 Cras-Méneur, C., Elghazi, L., Czernichow, P., Scharfmann, R. (2001). Epidermal growth factor
503 increases undifferentiated pancreatic embryonic cells in vitro: a balance between
504 proliferation and differentiation. *Diabetes*, 50(7),1571-9.
- 505 Kumar, S. S., Alarfaj, A. A., Munusamy, M. A., Singh, A. J. A. R., Peng, I.-C., Priya, S. P., Higuchi,
506 A. (2014). Recent Developments in β -Cell Differentiation of Pluripotent Stem Cells Induced
507 by Small and Large Molecules. *International Journal of Molecular Sciences*, 15(12),
508 23418-47.
- 509 Varley, C.L., Garthwaite, M.A., Cross, W., Hinley, J., Trejdosiewicz, L.K., southgate, J. (2006).
510 PPARgamma-regulated tight junction development during human urothelial
511 cytodifferentiation. *Journal of Cellular Physiology*, 208 (2), 407-417.

512

513 Varley, C.L., Stahlschmidt, J., Lee, W.C., Holder, J., Diggle, C., Selby, P.J., Southgate, J. (2004a).
514 Role of PPARgamma and EGFR signalling in the urothelial terminal differentiation
515 program. *Journal of Cell Science*, 117(10), 2029–36.

516

517 Varley, C.L., Stahlschmidt, J., Smith, B., Stower, M., Southgate, J. (2004b). Activation of
518 peroxisome proliferator-activated receptor-gamma reverses squamous metaplasia and
519 induces transitional differentiation in normal human urothelial cells. *The American Journal*
520 *of Pathology*, 164 (5), 1789–98.

521

522 Boström, H., Gritli-Linde, A., Betsholtz, C. (2002). PDGF-A/PDGF alpha-receptor signaling is
523 required for lung growth and the formation of alveoli but not for early lung branching
524 morphogenesis. *Developmental Dynamics*, 223(1), 155–62.

525

526 Karlsson L., Lindahl P., Heath J.K., Betsholtz C. (2000). Abnormal gastrointestinal development in
527 PDGF-A and PDGFR- α deficient mice implicates a novel mesenchymal structure with
528 putative instructive properties in villus morphogenesis. *Development*. 127(16), 3457–66.

529 Utoh R., Shigenaga S., Watanabe Y., Yoshizato K. (2003). Platelet-derived growth factor signaling
530 as a cue of the epithelial–mesenchymal interaction required for anuran skin
531 metamorphosis. *Developmental Dynamics*, 227(2), 157–169.

532 Ding H., Wu X., Boström H., Kim I., Wong N., Tsoi B., O'Rourke M., Koh G.Y., Soriano P.,
533 Betsholtz C. (2004). A specific requirement for PDGF-C in palate formation and PDGFR- α
534 signaling. *Nature Genetics*, 36(10), 1111–6.

535 Calver A.R., Hall A.C., Yu W.P., Walsh F.S., Heath J.K., Betsholtz C., Richardson W.D. (1998).
536 Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron*, 20(5), 869–
537 82.

538 Barkauskas, C.E., Crouce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B. R., Hogan,
539 B.L.M. (2013). Type 2 alveolar cells are stem cells in adult lung. *The Journal of Clinical*
540 *Investigation*, 123(7), 3025–36.

541 Wang, N., Zhang, R., Wang, S.J., Zhang, C.L., Mao, L.B., Zhuang, C.Y., Tang, Y.Y., Luo,
542 X.G., Zhou, H., Zhang, T.C. (2013). Vascular endothelial growth factor stimulates
543 endothelial differentiation from mesenchymal stem cells via Rho/myocardin-related
544 transcription factor--a signaling pathway. *The International Journal of Biochemistry and*
545 *Cell Biology*, 45(7), 1447–56.

546 Carmeliet P1, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig
547 M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen
548 D, Risau W, Nagy A. (1996). Abnormal blood vessel development and lethality in embryos
549 lacking a single VEGF allele. *Nature*, 380(6573), 435–9.

550 Giordano, F.J., Gerber, H.P., Williams, S.P., VanBruggen, N., Bunting, S., Ruiz-Lozano, P., Gu,
551 Y., Nath, A.K., Huang, Y., Hickey, R., Dalton, N., Peterson, K.L., Ross, J.J., Chien,
552 K.R., Ferrara, N. (2001). A cardiac myocyte vascular endothelial growth factor paracrine

553 pathway is required to maintain cardiac function. Proceeding of the National Academy of
554 Science USA, 98(10), 5780–5.

555 Lammert, E., Cleaver, O., Melton, D. (2001). Induction of pancreatic differentiation by signals from
556 blood vessels. *Science*, 294(5542), 564–7.

557 Matsumoto, K., Yoshitomi, H., Rossant, J., Zaret, K.S. (2001). Liver organogenesis promoted by
558 endothelial cells prior to vascular function. *Science*, 294(5542), 559–63.

559 Compernelle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor,
560 Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons,
561 L., Collen, D., Carmeliet, P. (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal
562 lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in
563 premature mice. *Nature Medicine*, 8(7), 702–10.

564 Samuel, S., Ahmad, R. E., Ramasamy, T. S., Karunanithi, P., Naveen, S. V., Murali, M. R., Kamarul,
565 T. (2016). Platelet-rich concentrate in serum free medium enhances osteogenic
566 differentiation of bone marrow-derived human mesenchymal stromal cells. *PeerJ*, 4, e2347.

567 Chignon-Sicard, B., Kouidhi, M., Yao, X., Delerue-Audegond, A., Villageois, P., Peraldi,
568 P., Ferrari, P., Rival, Y., Piwnica, D., Aubert, J., Dani, C. (2017).
569 Platelet-rich plasma respectively reduces and promotes adipogenic and myofibroblastic diffe
570 rentiation of human adipose-derived stromal cells via the TGFβ signalling pathway.
571 *Scientific Reports*, 7(1), 2954.

572 Homayouni Moghadam, F., Tayebi, T., Barzegar, K. (2016). Differentiation of Rat bone marrow
573 Mesenchymal stem cells into Adipocytes and Cardiomyocytes after treatment with platelet
574 lysate. *Int J Hematol Oncol Stem Cell Res*. 2016 Jan 1;10(1):21-9.

575 Lee JK1, Lee S, Han SA, Seong SC, Lee MC. The effect of platelet-rich plasma on the
576 differentiation of synovium-derived mesenchymal stem cells. *J Orthop Res*. 2014
577 Oct;32(10):1317-25. doi: 10.1002/jor.22668. Epub 2014 Jun 29.

578 Gruber R1, Karreth F, Kandler B, Fuerst G, Rot A, Fischer MB, Watzek G. (2004) Platelet-released
579 supernatants increase migration and proliferation, and decrease osteogenic differentiation of
580 bone marrow-derived mesenchymal progenitor cells under in vitro conditions. *Platelets*,
581 15(1), 29-35.

582 Naaijken, B.A., Niessen, H.W.M., Prins, H.J., Krijnen, P.A.J., Kokhuis, T.J.A., de Jong, N.,
583 Juffermans, L.J.M. (2012). Human platelet lysate as a fetal bovine serum substitute improves
584 human adipose-derived stromal cell culture for future cardiac repair applications. *Cell and*
585 *Tissue Research*, 348(1), 119–130.

586 Kakudo, N., Minakata, T., Mitsui, T., Kushida, S., Notodihardjo, F. Z., & Kusumoto, K. (2008).
587 Proliferation-Promoting Effect of Platelet-Rich Plasma on Human Adipose Derived Stem
588 Cells and Human Dermal Fibroblasts. *Plastic and Reconstructive Surgery*, 122(5), 2235-43.

589 Lucarelli, E., Beccheroni, A. D., Sangiorgi, L., Cenacchi, A., Del Vento, A. M., Meotti, C., Picci,
590 P. (2003). Platelet-derived growth factors enhance proliferation of human stromal stem cells.
591 *Biomaterials*, 24(3), 3094-3100.

Li, H., Usas, A., Poddar, M., Chen, C., Thompson, S., Ahani, B., Cummins, J., Lavasani, M., Huard, J. (2013). Platelet-rich plasma promotes the proliferation of human muscle derived progenitor cells and maintains their stemness. *PLoS ONE*, 8(6), e64923.

Mishra, A., Tummala, P., King, A., Lee, B., Kraus, M., Tse, V., & Jacobs, C. R. (2009). Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Engineering Part C: Methods*, 15(1), 431-435.

Cervellia, V., Sciolib, M. G., Gentile, P., Doldob, E., Bonannob, E., Spagnolib, L. G., & Orlandib, A. (2012). Platelet-rich plasma greatly potentiates insulin-induced adipogenic differentiation of human adipose-derived stem cells through a serine/threonine kinase Akt-dependent mechanism and promotes clinical fat graft maintenance. *Stem Cells Translational Medicine*, 1(5), 206-220.

Horst, M., Eberli, D., Gobet, R., & Salemi, S. (2019). Tissue Engineering in Pediatric Bladder Reconstruction-The Road to Success. *Frontiers in pediatrics*, 7, 91. <https://doi.org/10.3389/fped.2019.00091>

Karadjian, M.; Senger, A.-S.; Essers, C.; Wilkesmann, S.; Heller, R.; Fellenberg, J.; Simon, R.; Westhauser, F. Human Platelet Lysate Can Replace Fetal Calf Serum as a Protein Source to Promote Expansion and Osteogenic Differentiation of Human Bone-Marrow-Derived Mesenchymal Stromal Cells. *Cells* 2020, 9, 918.

Trojahn Køllef SF, Oliveri RS, Glovinski PV, et al. Pooled human platelet lysate versus fetal bovine serum-investigating the proliferation rate, chromosome stability and angiogenic potential of human adipose tissue-derived stem cells intended for clinical use. *Cytotherapy*. 2013;15(9):1086-1097. doi:10.1016/j.jcyt.2013.01.217

Figure 1: Characterization and differentiation potential of ADSCs. **Aa)** Primary ADSCs morphology after 14 days in culture under the inverted phase contrast microscope. Scale bar = 100µm. **B-F)** Flow cytometry staining of ADSC markers. Cells showed positive staining for

mesenchymal stem cells markers CD-44, CD-105, CD-73, CD-90 and negative for CD-34, CD-11b, CD-19, CD-45 and HLA-DR in the negative cocktail.

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Figure 2: Multilineage differentiation potential of ADSCs. **A-C)** Osteogenic mineral deposition was observed after 21 days of osteogenic induction and positively stained with Alizarin Red S stain (ARS). **B-D)** Uninduced ADSCs were used as a negative control and stained negatively with ARZ. **E-G)** Lipid droplets were observed after 14 days of adipogenic differentiation of ADSCs and positively stained with Oil Red O. **F-H)** ADSCs with normal culture media stained negatively for oil red O. Scale bar = 100µm. All differentiation experiments were repeated at least three times

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Figure 3. Relative gene expression of urothelial markers. Real time-PCR performed on urothelial-like cells differentiated from ADSCs, cultivated in either conditioned medium (CM) or unconditioned urothelial cell media (UCM) in the presence of either 5% pHPL, 2.5% pHPL or 10% FBS. Uninduced ADSCs were used as the calibrator sample. **A)** Relative expression of cytokeratin-18, **B)** cytokeratin-19 and **C)** uroplakin-2. * $P < 0.05$, ** $P < 0.01$. All experiments were repeated at least three times.

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Figure 4. Immunofluorescence staining of urothelial markers at the cellular level. **A)** ADSCs cultured in CM and different concentrations of pHPL (5% & 2.5%) or with FBS, were assessed for the expression of cytokeratin-18 (FITC, green) and uroplakin-2 (Cy3, red). SV-HUC cells were used as a positive control, meanwhile ADSCs were utilized as a negative control. Scale bar = 50µm. **B&C)** Semi-quantitative analysis of immunofluorescence representing percentage of positive cells relative to negative control. All experiments were repeated at least three times.

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Figure 5. Growth factor levels assessment in induced ADSCs. Enzyme linked immunosorbent assay (ELISA) was performed on conditioned media collected after 24 hours from cells induced for 14 days with urothelial cell derived conditioned medium (CM) or unconditioned urothelial cell media (UCM) with 5% pHPL, 2.5% pHPL or FBS. **A)** Measurement of epidermal growth factor levels (EGF). **B)** Platelet derived growth factor-BB (PDGF-BB). **C)** Vascular endothelial growth factor (VEGF). * $P < 0.05$, ** $P < 0.01$. All experiments were repeated at least three times.

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Comment [MOU1]:

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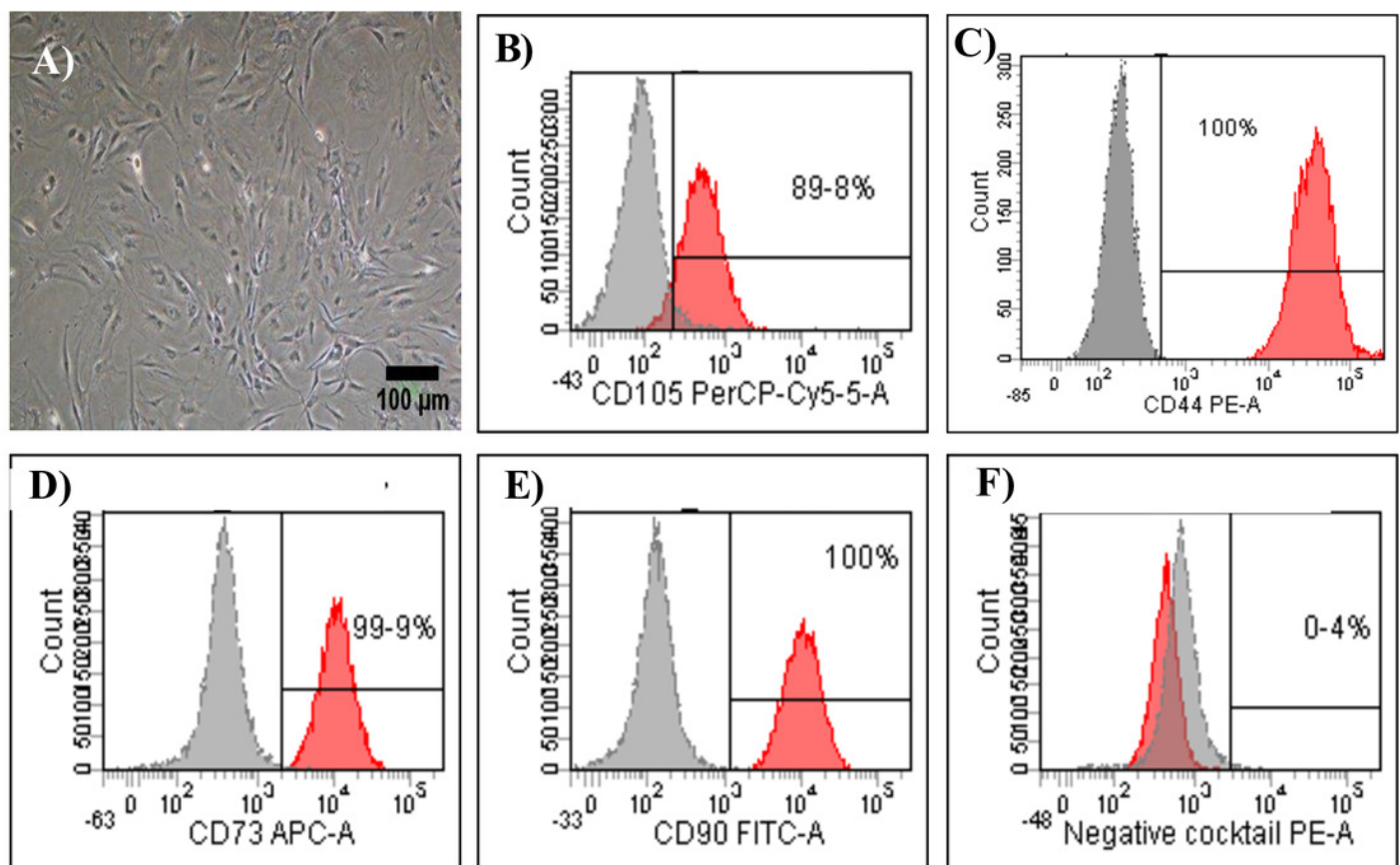


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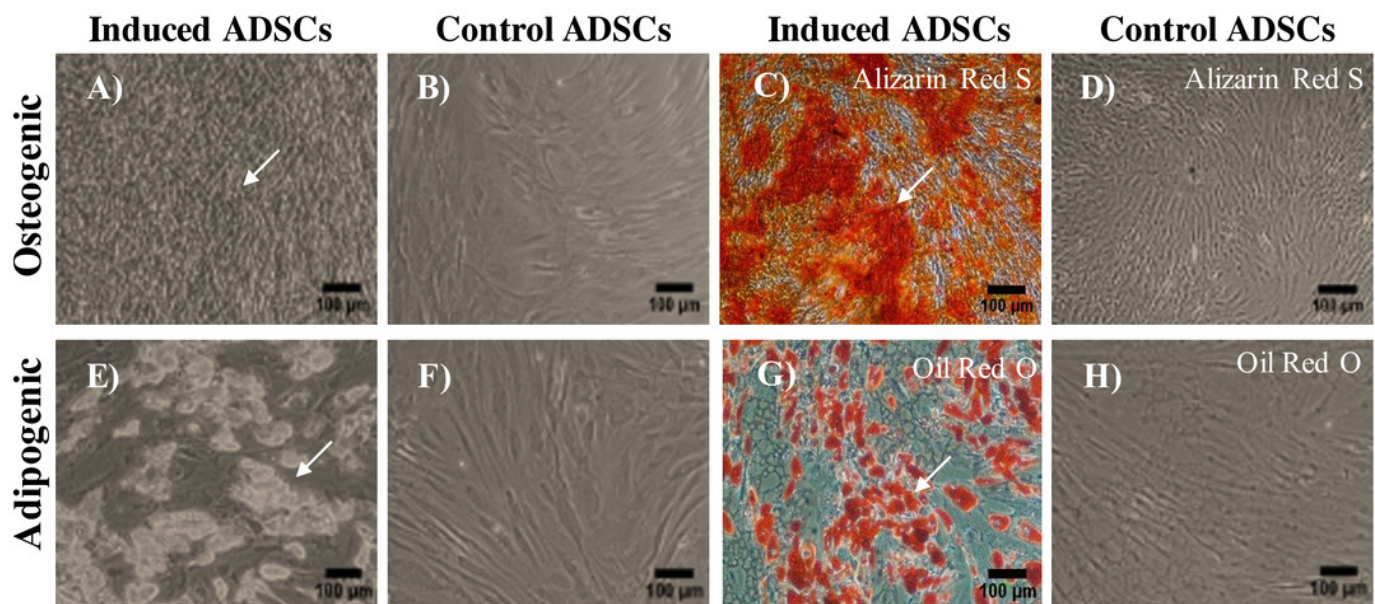


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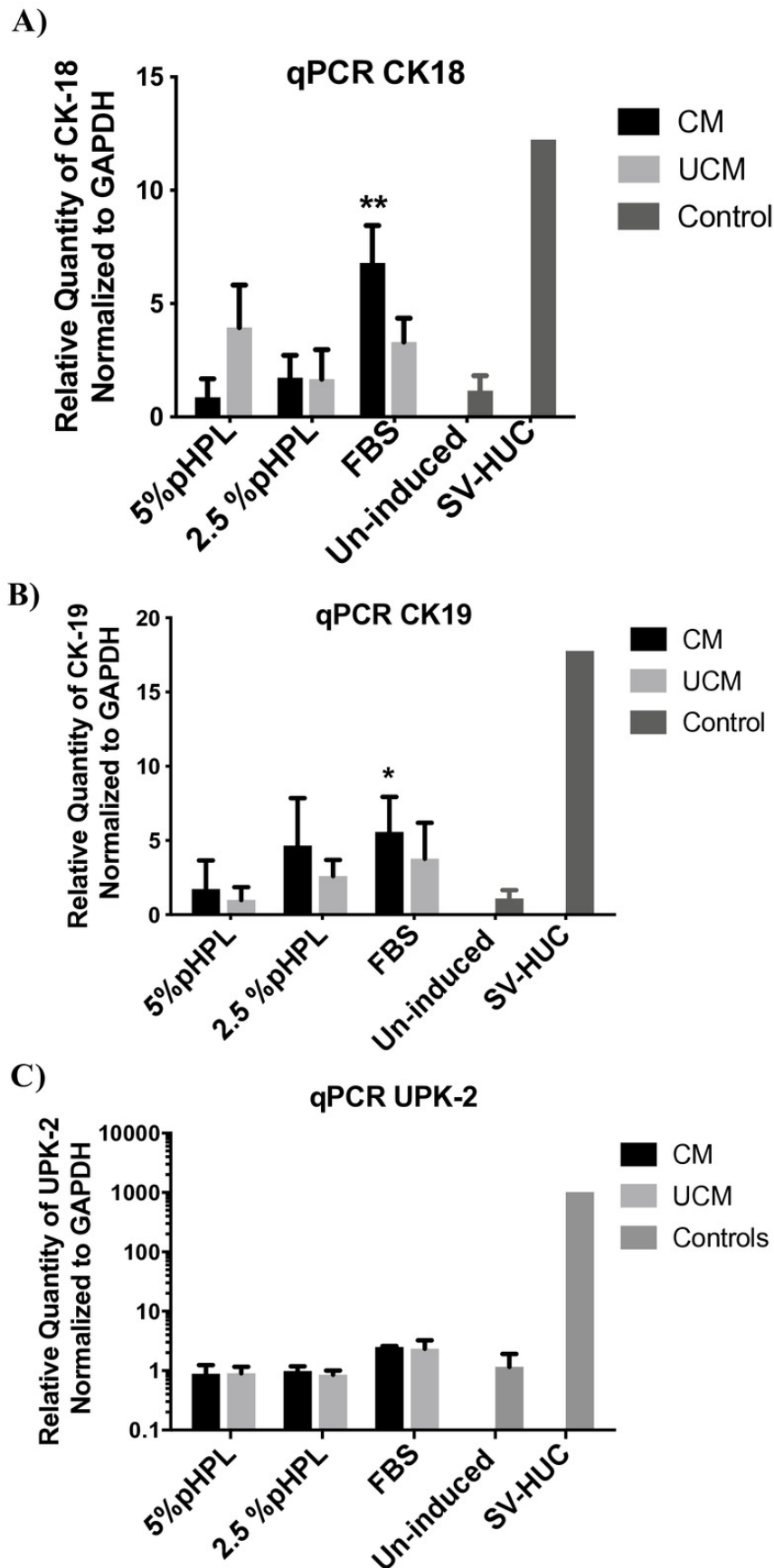


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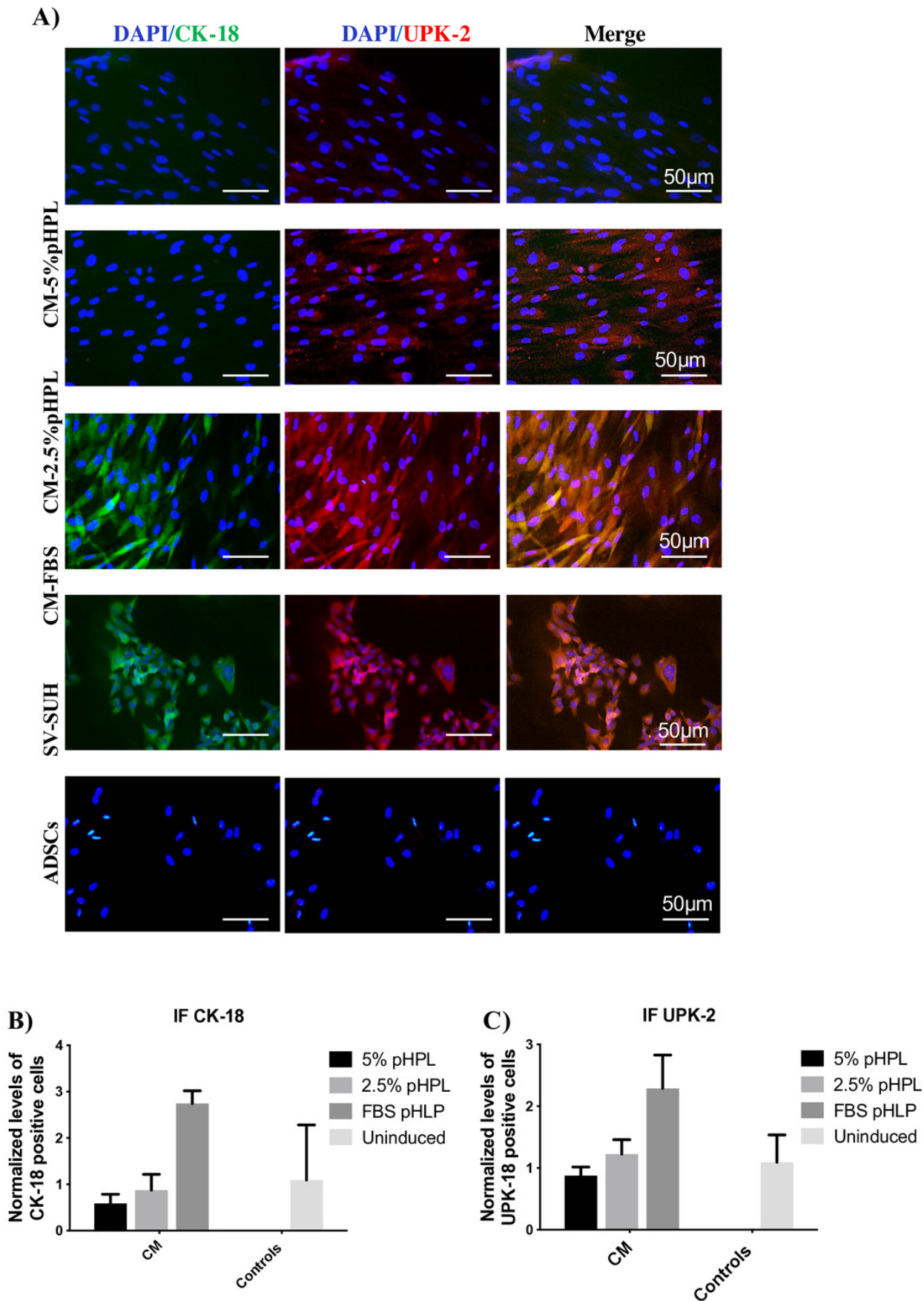
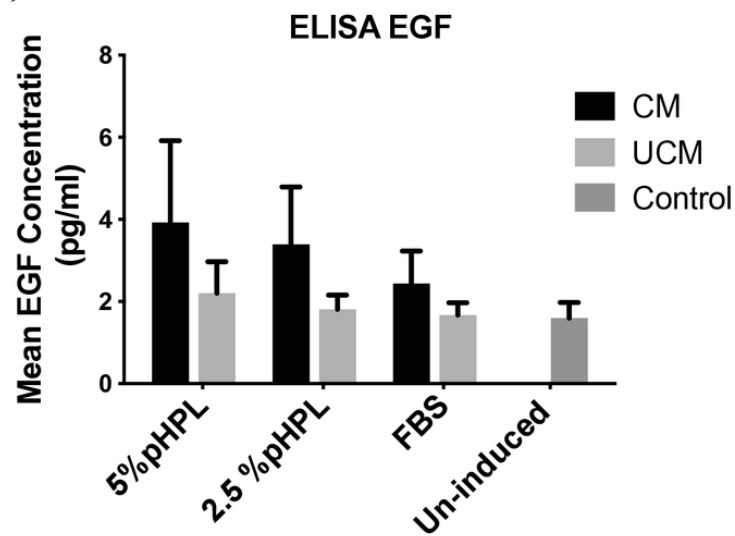


Figure 5

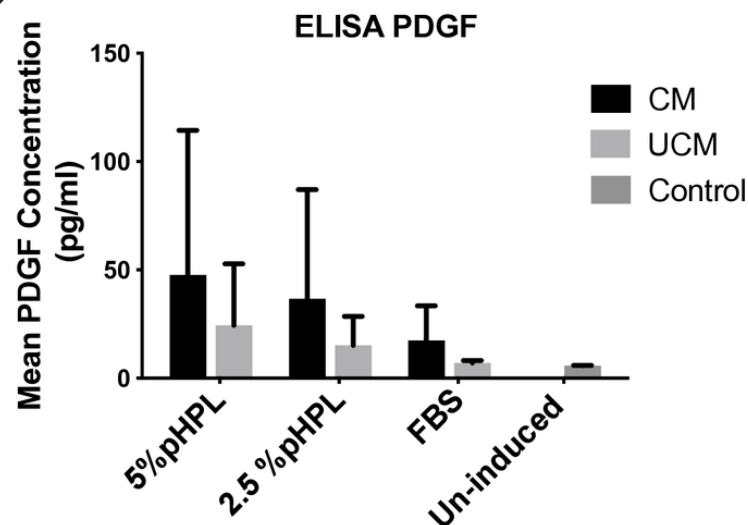
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A)



B)



C)

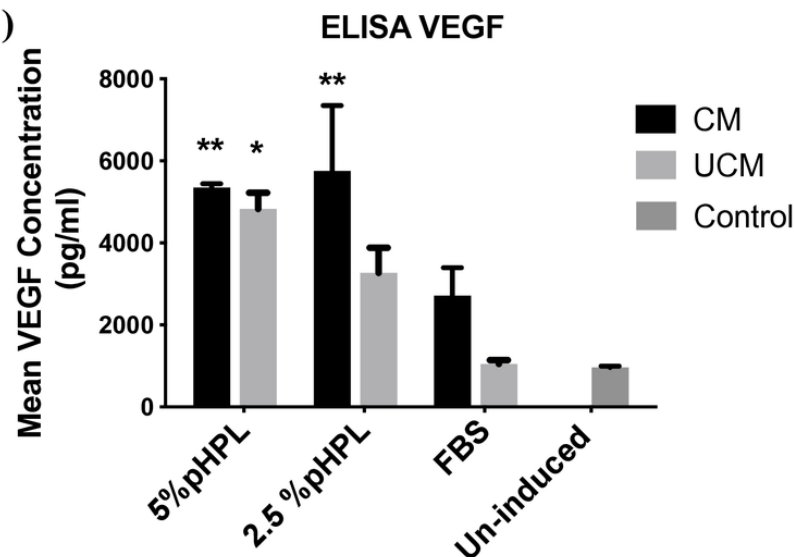


Table 1 (on next page)

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1 **Table 1.** qPCR primer sequences.

Gene	Forward Primer	Reverse Primer
Uroplakin-2	CGGAGAGCCGACAGCAAA C	ACTGAGCCGAGTGACTGTGAAG
Cytokeratin-18	GGTCAGAGACTGGAGCCA TTA	GGCATTGTCCACAGTATTTGC
Cytokeratin-19	CGGGACAAGATTCTTGGT	CCTTGATGTCGGCCTCCA
GAPDH	CAAGGTTGACAACTTTG	GGGCCATCCACAGTCTTCTG

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