



Combined transgene immortalized urothelial cells capable of reprogramming and hepatic differentiation

Matthias Weiland², Paula Ballmaier², Christoph Niemietz, Hartmut Schmidt¹, Andree Zibert^{*}

Medizinische Klinik B, Universitätsklinikum Münster, Münster, Germany

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ABSTRACT

Human primary cells, including urine-derived cells (UCs), are an excellent source for generation of pluripotent stem cells (iPSCs) to model disease. However, replicative senescence starts early and shortens the time window for generation of iPSCs. We addressed the question whether combinations of transgenes allows efficient immortalization of UCs, iPSC generation, and differentiation into hepatocyte-like cells (HLCs). Retroviral transfer of three gene cassettes HPVE6E7 (H), hTERT/p53DD (T), cyclinD1/CDK4R24C (C) encoding five genes was established in primary UCs. Long-term cell proliferation was observed in cells carrying transgenes H, HT, HC, and HCT, whereas cells carrying transgenes C, T and CT showed early senescence similar to UCs. iPSCs could be exclusively generated from immortalized UCs transduced with transgenes HCT and HC. iPSC colonies appeared however later and in smaller number as compared to UCs. Using an established hepatic differentiation protocol, HLCs were obtained with high efficacy. Of note, a high expression of individual transgenes was observed in immortalized UCs, which was down-regulated after reprogramming in four out of five genes. One transgene was re-expressed in HLCs as compared to iPSCs. Our data suggest that individual transgene combinations result in advanced growth rates of immortalized cells and do not prevent iPSC formation and HLC differentiation. Retroviral transgene expression is mostly silenced in iPSCs but can be rarely re-expressed after hepatic differentiation. An extended time window for iPSC establishment can be proposed that allows straightforward functional analyses of differentiated cells.

1. Introduction

Cellular models are frequently employed for study of disease. The gold standard are patient-derived primary cells, which have however a very limited proliferation capacity [1]. Urine-derived primary cells (UCs) represent a patient-friendly source since a noninvasive protocol can be used that is particularly suited for children and vulnerable patients [2,3]. In order to overcome senescence of UCs, expression of various genes, including human papillomavirus 16 (HPV16)-derived oncogenes E6 and E7 (HPVE6E7) [4], the catalytic subunit of human telomerase (hTERT) [5], and simian virus 40 large T antigen (SV40 TAG), were employed [6]. For non-urinary derived cells, transfer of genes including R24C mutant cyclin-dependent kinase 4 (CDK4R24C), human cell cycle regulator cyclin D1, and dominant negative tumor protein p53 deletion mutant (p53DD) was reported to result in long-term

cell proliferation [7,8]. However the cell type of immortalized UCs is confined to cells present in the urinary tract limiting the versatility of respective models. In recent years, reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) followed by a tissue-specific differentiation has tremendously broadened the application of tissue-specific cellular models. In fact, UC-derived iPSCs can encompass various tissues of all germ layers including neurons, cardiomyocytes and hepatocytes [2].

While most previous studies employed singular oncogene/tumor-suppressor genes for immortalization of UCs, we explored whether a combination of genes is favorable for immortalization. In order to extend our study to cell types not related to the urinary tract, we asked whether iPSCs can be derived from immortalized cells, a task that seems to be challenged by the general low reprogramming efficacy when aberrant oncogene/tumor-suppressor gene expression is present [9]. We

^{*} Corresponding author. Medizinische Klinik B (Gastroenterologie, Hepatologie, Endokrinologie, Klinische Infektiologie), Universitätsklinikum Münster, Albert-Schweitzer-Campus 1, Gebäude A14, D-48149, Münster, Germany.

E-mail address: andree.zibert@ukmuenster.de (A. Zibert).

¹ Present address: Klinik für Gastroenterologie und Hepatologie, Uniklinik Essen, Essen, Germany.

² Equal contribution.

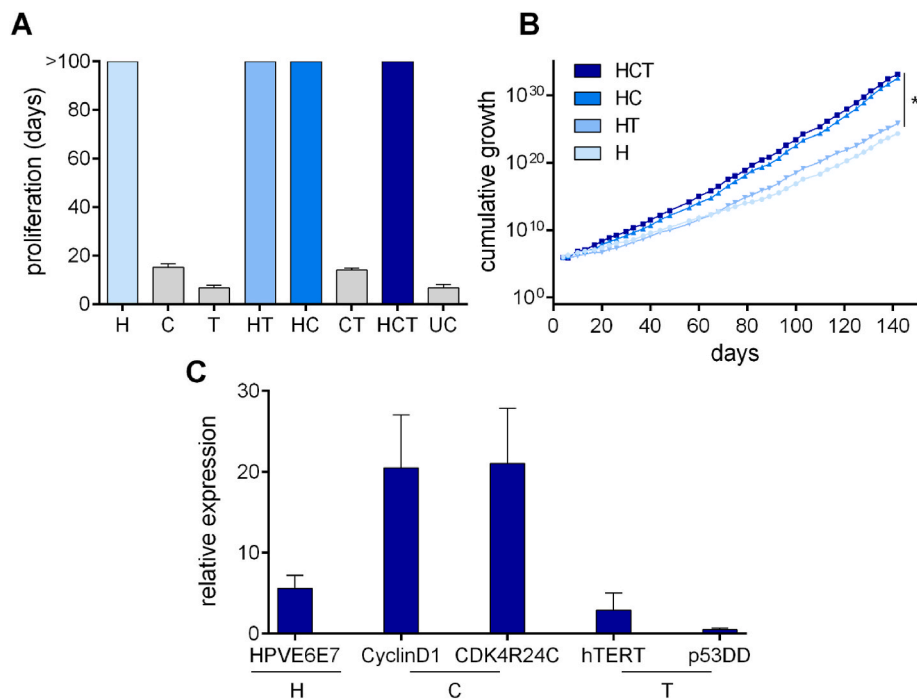


Fig. 1. Immortalization of urine-derived cells can be achieved by combined transgene expression encoded by retroviral vector. (A) Presence of cell proliferation is indicated following retroviral transduction of urine-derived cells obtained from three different donors up to day 100 (n = 3 independent experiments). (B) Cumulative growth curves of immortalized urine-derived cells. One typical out of three independent experiments is shown. Data of non-immortalized cells was omitted. Mann-Whitney *U* test was used to determine statistical significance. **P* < 0.05. (C) RT-qPCR analysis of transgenes expressed in immortalized urine-derived cells. Relative expression above negative values of primary UCs (ct > 40) was normalized to GAPDH (100%). Mean and SE of data (n = 5 independent experiments) is shown.

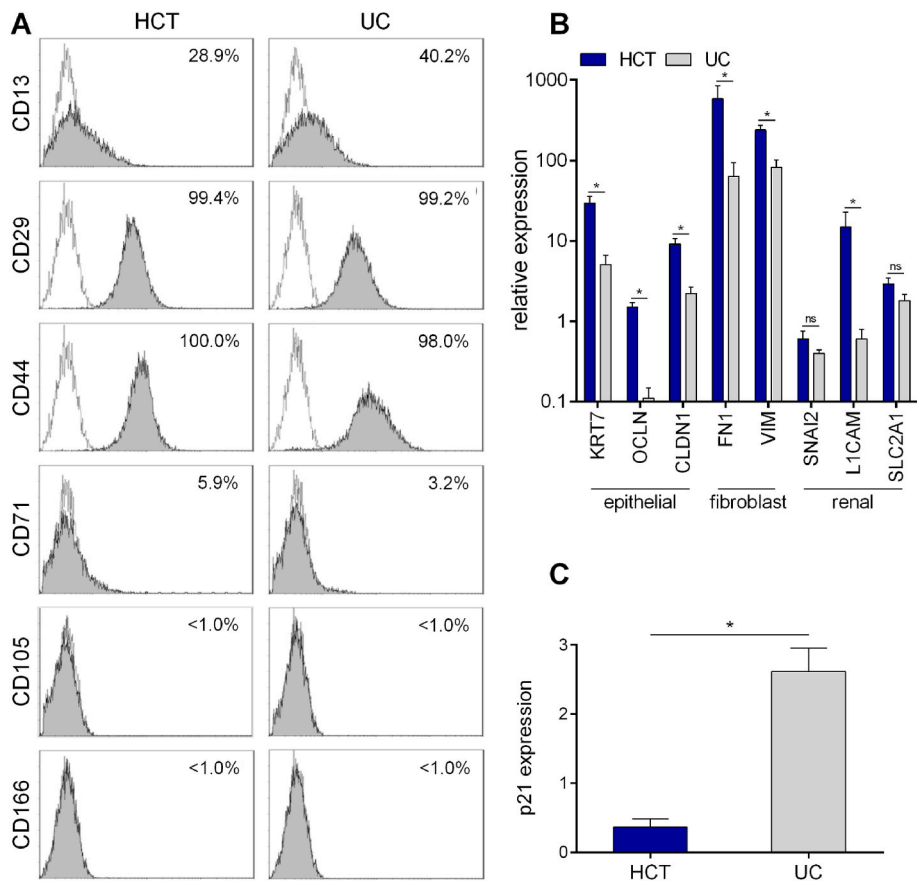


Fig. 2. Immortalized urine-derived cells show high urothelial marker expression. (A) Flow cytometry analysis of immortalized urine-derived cells (n = 3 independent experiments). Non-immortalized cells are shown as control. One experiment of three is shown. (B) Relative mRNA expression of epithelial, fibroblast and renal marker genes in transduced UCs (n = 7–9 independent experiments). Data from non-immortalized cells are shown as control. Data were normalized to GAPDH. Mean and SE is shown. Mann-Whitney *U* test was used to determine statistical significance. **P* < 0.05. (C) RT-qPCR gene expression analysis of p21 in immortalized cells and control (n = 3–5 independent experiments). Data were normalized to GAPDH. Mean and SE is shown. Mann-Whitney *U* test was used to determine statistical significance. **P* < 0.05.

Table 1
iPSC-like colonies derived from immortalized UCs.

	iPSC	No. colonies	Day colonies
H	0/4	0	–
HT	0/4	0	–
HC	3/4	9 ± 1	21, 24, 25
HCT	3/4	29 ± 4	21, 22, 24
primary UC	3/3	58 ± 4	14, 16, 21

also assessed whether hepatocyte-like cells (HLCs) can be differentiated from immortalized UCs to proof feasibility.

2. Materials and methods

2.1. Ethics statement

Urine samples were obtained in accordance with the ethics committee of the Universitätsklinikum Münster. Participants were informed regarding the use of data and written informed consent was obtained from all individuals enclosed in the study. Urine was derived from three healthy donors (Supplemental Table S1).

2.2. Isolation of urine-derived cells

Cells were harvested from urine samples as reported before [10]. Cultivation of cells was in DMEM/F-12 (Life Technologies), 10% FCS (Thermo Fisher Scientific), 1% penicilline-streptomycine, 1x MEM non-essential amino acid solution (NEAA, Sigma-Aldrich), 1x GlutaMAX-I, 0.1 mM 2-mercaptoethanol (Life Technologies), and SingleQuot Kit CC-4127 REGM (Lonza). Subconfluent primary cells were expanded for and frozen for experiments.

2.3. Retroviral transduction

Human embryonic kidney HEK293T cells were maintained in DMEM (Gibco) with 10% FCS and 1% penicilline-streptomycine. For generation of retroviral supernatants, HEK293T cells were transfected as described [11]. Plasmids pLXSN16E6E7, pbabe-cyclinD1+CDK4R24C and pbabe-hTERT + p53DD (Addgene plasmids #52394, #11129 and #11128, respectively) were used. GFP containing plasmid or empty vector were used as control.

2.4. Flow cytometry analysis

Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized by 0.5% Triton X-100 (Thermo Fisher Scientific). After blocking, a 1:100 dilution of antibodies directed to CD13, CD29, CD71, CD105, CD166 (all from Beckman Coulter) and CD44 (EuroBioScience) was added. Cells were analyzed by a Coulter Epics XL-MCL (Beckman Coulter).

2.5. Immunocytochemistry

Following fixation and permeabilization, cells were stained by antibodies directed to albumin antibody, transthyretin (both from Abcam), Serpina1 (R&D Systems), NANOG (Santa Cruz), Oct 3/4, SSEA-4 and TRA-1-60 (all from Stemcell Technologies). Photographs were taken using an Olympus CKX41-X10 microscope with CellSens Standard 1.11 imaging software.

2.6. Cumulative cell growth

Cells were seeded in a 6 well plate (Cellstar). The cell number was determined twice a week using trypan blue and 1.5×10^5 cells were reseeded. The cumulative cell number was calculated. Each cell count

was independently repeated at least three times. Growth rates were calculated by linear regression analysis.

2.7. Induced pluripotent stem cells

Establishment of iPSCs was performed as reported before [10]. Cells were transferred to Matrigel® matrix (Corning) coated 6-well cell culture plates (Greiner Bio-One) and cultivated in mTeSR-1 (Stemcell Technologies). iPSC colonies were subcultured every seven days using 1U/ml Dispase (Stemcell Technologies).

2.8. Differentiation of hepatocyte-like cells

Hepatic differentiation was induced as described previously [10]. Two clones of iPSCs were subjected to differentiation. At the end of differentiation at day 14, cells were subjected for further analysis.

2.9. PCR

Total RNA was prepared using RNeasy Mini Kit (Qiagen) and first strand synthesis was performed using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen). For real time PCR, Takyon ROX SYBR Master Mix blue (Eurogentec) and primers (Supplemental Table S2) were mixed. Analysis was on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Ct values were normalized to GAPDH and evaluated using comparative Ct method ($2^{-\Delta Ct}$).

2.10. Western blot

Lysates were prepared using RIPA buffer (60 mM tris-HCl, 150 mM NaCl, 2% Na-deoxycholate, 2% Triton X-10, 0.2% SDS and 15 mM EDTA) in the presence of protease inhibitors (Roche, Complete Mini, EDTA-free. Anti-β-actin and anti-p16 (both from Abcam) were used and applied to Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences). Blots were quantified by ImageJ 1.50i analysis software.

2.11. Statistical analyses

Statistical analyses were performed by Mann-Whitney U and Student's t-test using GraphPad Prism (v6.01, GraphPad Software) software. Data are given as mean ± standard error of mean (SE).

3. Results

3.1. Combined transgene expression results in immortalization and increased proliferation of urine-derived cells

Studies which involve a combination of oncogenes for immortalization of UCs are rare. The three gene cassettes HPVE6E7 (H), hTERT/p53DD (T) and cyclinD1/CDK4R24C (C) encoding five genes were selected [12,13] and delivered to UCs either alone (UC_H, UC_C, and UC_T, respectively) or in combination (UC_{HT}, UC_{HC}, UC_{CT} and UC_{HCT}). To examine the effect of the genes, proliferation was followed for several weeks. Within the group of singly transduced cells, only UC_H cells showed prolonged proliferation, whereas UC_C and UC_T stopped growth early (<17 days) which was in the range of primary UCs (Fig. 1A). However, UCs transduced with a combination including transgene H (UC_{HT}, UC_{HC} and UC_{HCT}) showed prolonged proliferation. Proliferation of these cell lines could be observed for more than 12 months without any signs of proliferation arrest (data not shown). In order to compare proliferation rates, cumulative growth curves were established (Fig. 1B). UC_{HCT} and UC_{HC} cells showed highest cumulative cell growth (growth rate $>0.33 \pm 0.07$), whereas cell lines UC_H and UC_{HT} had a lower growth rate (growth rate $<0.24 \pm 0.08$). An increased proliferation rate of transduced cells was observed when

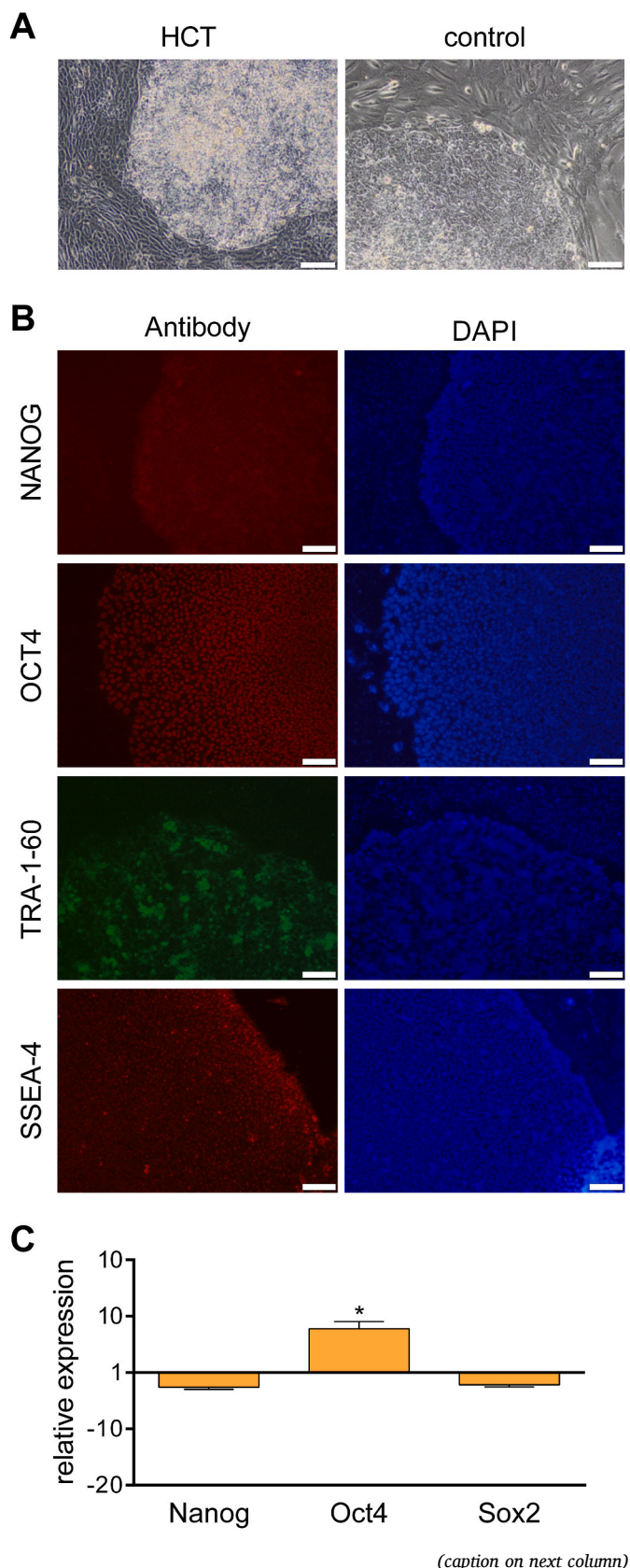


Fig. 3. Oncogene-expressing immortalized cells give rise to induced pluripotent stem cells.

Typical bright field image obtained at week three after reprogramming of immortalized urine-derived cells (left). iPSC colony from non-immortalized cells obtained at week two is shown as control (right). One typical colony is shown. Scale bars, 100 μ m. (B) Immunofluorescence staining of cells after reprogramming of immortalized urine-derived cells. DAPI was used for nuclear counterstaining. Scale bars, 100 μ m. (C) RT-qPCR gene expression analysis of pluripotency markers in reprogrammed cells obtained from immortalized urine-derived cells. Expression relative to iPSCs obtained from non-immortalized cells is shown ($n = 3-4$ independent experiments). GAPDH was used for normalization. Mann-Whitney U test was used to determine statistical significance. * $P < 0.05$.

transgene H was combined with transgene C, whereas transgene T had little or no effect on the proliferation rate (Supplemental Fig. S1). mRNA expression of transgenes was determined in immortalized cells suggesting that all transgenes were expressed, however at individual levels (Fig. 1C).

3.2. Urothelial marker expression of immortalized cells

The question was addressed whether combined transgene immortalization affects urothelial marker expression. Flow-cytometry analysis of proteins expressed at the cell surface membrane revealed expression levels at same range in immortalized and primary UCs, except for the renal proximal tubular cell marker CD13 (Fig. 2A). Analysis of mRNA levels indicated that *SNAI2* and *SLC2A1* expression was similar between immortalized and primary UCs, whereas a higher level was observed in immortalized UCs for other genes (*KRT7*, *OCN*, *CLDN1*, *FNI*, *VIM*, and *LICAM*) (Fig. 2B). As cell cycle regulators are frequently affected by immortalization [14], p21 (*CDKN1A*)-specific mRNA expression was determined in immortalized UCs indicating a lower expression level (factor ~ 9) as compared primary UCs (Fig. 2C). In addition, Western blot analysis of cell cycle regulator p16 (*CDKN2A*) revealed detectable expression in primary UCs, whereas an enhanced expression (factor ~ 6) was observed in immortalized UCs (Supplemental Fig. S2) suggesting that important cell cycle regulators are modulated by immortalization.

3.3. Onset of iPSC formation is delayed after reprogramming of transgene expressing cells

The question was addressed whether immortalized UCs can be reprogrammed to iPSCs. For reprogramming essentially the same protocol was used as reported previously by us that allowed robust iPSC formation of primary UCs [10,15,16]. The number and time point of iPSC-like colonies was recorded by daily inspection using light microscopy (Table 1). iPSC-like colonies were observed in three out of four experiments when UC_{HC} and UC_{HCT} cell lines were used which was slightly less efficient as observed for primary UCs. In contrast, iPSC-like colonies could not be observed in four experiments using cell lines UC_H and UC_{HT}. The number of iPSC-like colonies was highest for cell line UC_{HCT} which however had smaller sizes as compared to primary UCs (data not shown). The iPSC phenotype derived from immortalized UCs did not show significant differences as compared to primary UCs [10,15,16]. The iPSCs displayed a typical roundish cellular morphology (Fig. 3A). Protein expression was assessed by immunohistochemical stainings. A high presence of pluripotency associated markers NANOG, OCT4, TRA-60 and SSEA-4 were observed (Fig. 3B). A higher value of Oct4 mRNA was observed (Fig. 3C).

3.4. Hepatic differentiation of immortalized cells reveals typical marker expression and partly reverses transgene silencing

Having shown that immortalized UCs can be reprogrammed to iPSCs, the cells were subjected to a hepatic differentiation protocol giving rise

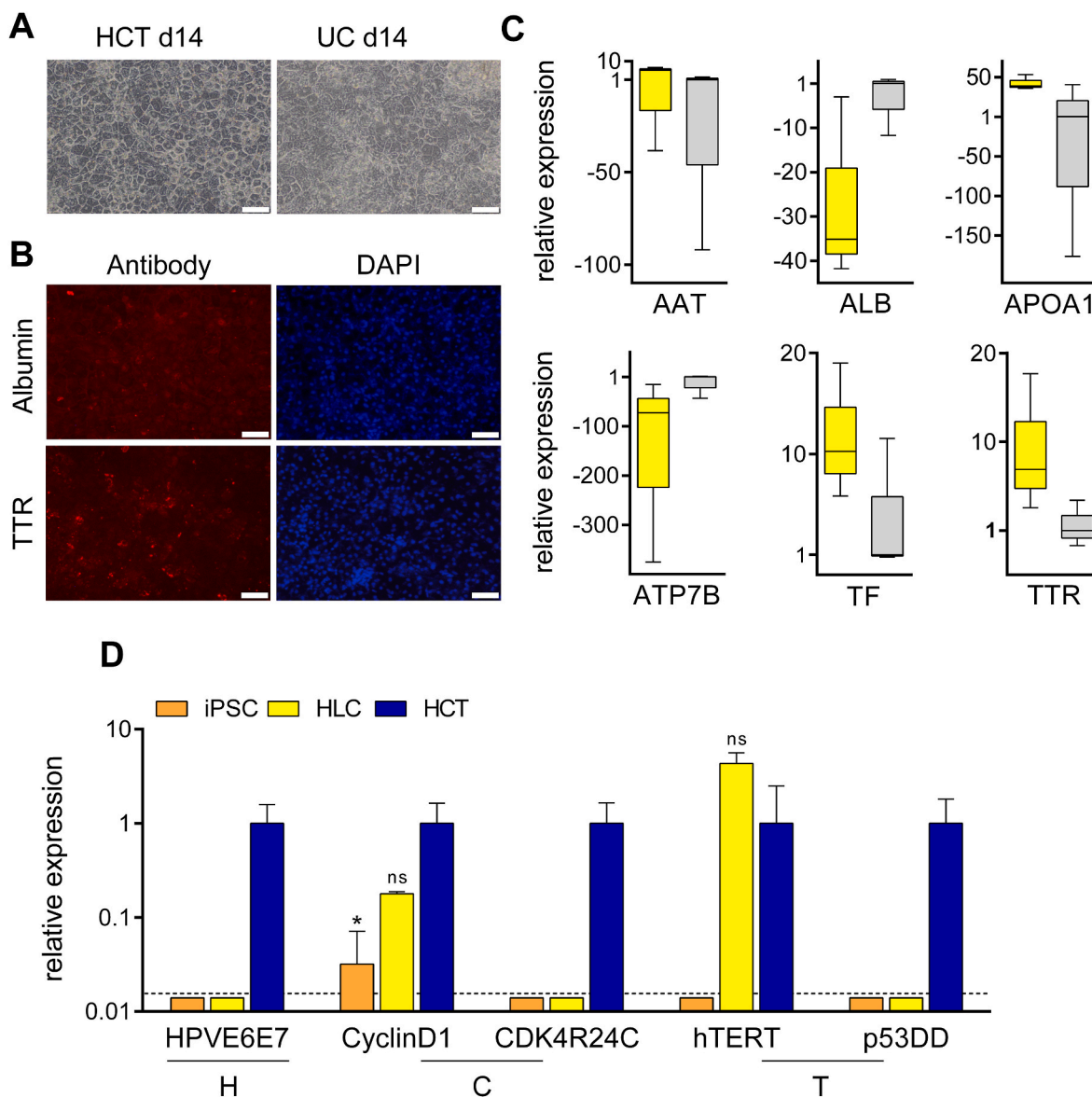


Fig. 4. Immortalization allows differentiation to HLCs which are subjected to reversal of transgene silencing.

(A) Bright field images of iPSCs obtained from immortalized urine-derived cells at day 14 of hepatic in vitro differentiation. Differentiation from non-immortalized cells served as control. One of three experiments is shown. Scale bars, 100 μ m. (B) Immunofluorescence staining of hepatic markers at day 14 of differentiation. DAPI was used for nuclear counterstaining. Scale bars, 100 μ m. (C) RT-qPCR analysis of HLCs derived from immortalized urine-derived cells (yellow) and primary UCs (grey) is shown. Box plot representation indicates expression levels relative to the median of control ($n = 3-4$ independent experiments). GAPDH was used for normalization. Mann-Whitney U test was used to determine statistical significance. * $P < 0.05$. (D) RT-qPCR analysis of transgene expression in iPSCs (orange) and HLCs (yellow) is shown. Immortalized cell line HCT (blue) was used for normalization. Mean and SE are given ($n = 3-5$ independent experiments). The dotted line indicates the detection limit of RT-qPCR analysis. Mann-Whitney U test was used to determine statistical significance. * indicates $P < 0.05$ versus HCT. ns, not significant versus HCT. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to HLCs [10]. Cells displayed a polygonal shape and a high staining of hepatic markers albumin and TTR at the end of the differentiation protocol (day 14) typical of hepatocytes [10] (Fig. 4A and B). mRNA expression of other hepatic markers, like *AAT*, *TF* and *TTR*, was almost in the same range (fold change < 10) for HLCs derived from immortalized and primary UCs (Fig. 4C). Expression of *APOA1* was increased, whereas *ALB* and *ATP7B* were decreased in HLCs derived from immortalized UCs. As expression of the transgenes could have been affected by the events of reprogramming and hepatic differentiation, a RT-qPCR analysis of iPSCs and HLCs was performed. Expression levels were compared to immortalized UCs (Fig. 4D). The analysis revealed that the transgenes except cyclinD1 were silenced in iPSCs below detection limits. After hepatic differentiation, hTERT was re-expressed to almost

same levels as compared to immortalized UCs.

4. Discussion

We established immortalized cell lines derived from primary UCs via transgenic co-expression of three gene cassettes which demonstrated high growth rates over many sequential passages, lack signs of senescence, and share markers of primary urothelial cells. Two immortalized cell lines, albeit expressing significant levels of transgenes, maintained the capability to be reprogrammed into iPSCs. Immortalized cells could be subjected to hepatic differentiation with high efficacy. Hepatic differentiation was accompanied by an infrequent reversal of transgene silencing observed in iPSCs.

Expression of p53DD either alone or in combination with CDK4R24C could not immortalize cells of the urinary tract supporting the observations of this study [17]. Our results suggest that expression of hTERT/p53DD was not sufficient to immortalize UCs and had little effect on proliferation rates when co-expressed with CDK4R24C/cyclinD1 and HPVE6E7. hTERT alone was previously shown to immortalize cells derived from the urinary tract [5]. In contrast to our protocol, the urothelial cells were obtained by biopsy rather than directly grown from urine samples suggesting that the origin of the cell type or co-expression of p53DD negatively modulate events of immortalization. Our results confirm that single transgene HPVE6E7 is sufficient to establish immortalization of UCs [4]. Of note, cells co-expressing HPVE6E7 and CDK4R24C/cyclinD1 showed improved proliferation rates, while CDK4R24C/cyclinD1 alone did not result in immortalization. Our data suggest a synergistic acceleration of cell proliferation in HPVE6E7-immortalized cells by co-expression of CDK4R24C/cyclinD1. The mechanism leading to synergy remains however to be studied further. HPVE6E7 is known to regulate the cell cycle by interfering with two major signal pathways orchestrated by tumor suppressor p53 and Rb [18]. HPVE6 can bind to p53 resulting in proteolytic degradation and HPVE7 inactivates Rb overall promoting cell-cycle progression [19]. CDK4R24C/cyclinD1, forming an activating complex, induces the phosphorylation/inactivation of Rb leading to a G1 to S phase transition. In line, we observed p16 overexpression in HCT immortalized cells which could be related to the relief of a negative feed-back transcriptional mechanism by inactivated Rb [20]. p16 induction is known to inhibit the activity of the CDK4/6/cyclinD1 complex by binding to CDK4 or CDK6, while CDK4R24C is a p16-resistant mutant suggesting that co-expression of CDK4R24C/cyclinD1 may enhance proliferation rates independent of the effects exerted by HPVE7 [21]. Interestingly, an increased level of p16 alone seems not to be sufficient to arrest cell proliferation as observed in HPVE6E7 expressing tumors. The effect of ectopically expressed HPVE6E7 is however complex due to the many targets of both HPV genes and the intermingled pathways regulating proliferation which were not analyzed here [22].

Senescence is known to represent a roadblock to iPSC reprogramming [23]. Despite overcoming senescence in four immortalized cell lines, we could establish iPSCs from two lines only at reduced efficacy suggesting that individual oncogenes used for immortalization might hamper reprogramming. Our observation seems to be in line with other reports of a reduced efficacy or even failure to reprogram cells having aberrant expression of oncogenes [9,24]. In contrast, SV40-TAg e.g. was shown to enhance iPSC formation of human fibroblasts when co-expressed with canonical reprogramming factors [25]. Establishment of iPSCs was not achieved in immortalized UCs expressing HPVE6E7 alone or co-expressed with hTERT/p53DD suggesting a general detrimental effect of HPVE6E7 that cannot be compensated by hTERT/p53DD. Of note, co-expression of CDK4R24C/cyclinD1 seems to rescue the iPSC refractory phenotype of HPVE6E7, while hTERT/p53DD may have a stimulatory effect. In summary, to our best knowledge, this is the first evidence of a successfully reprogramming using immortalized cells, with the exception of EBV infected B-cells [26].

Primary UCs used in this study showed morphological changes and proliferation arrest within a few weeks corroborating other reports using different isolation protocols [4–6]. The time window for characterization of cells before reprogramming is therefore highly limited and a time-consuming molecular characterization of subpopulations seems hardly achievable. Our findings thus indicate that the ease of reprogramming can be further improved, e.g. when heterogeneous cell sources like tumor tissues are used.

Expression of factors inducing pluripotency via retroviral vectors is silenced after establishment of iPSCs [27]. It was argued that such rapid gene silencing could be a prerequisite for reprogramming. We also observed silencing of most transgenes used in this study at the level of pluripotent cells. Hepatic differentiation released retroviral gene silencing of one transgene suggesting that a new rearrangement of the

genome-wide epigenetic remodeling may also occur [28]. It can however be hypothesized that an exceptional re-expression of a single transgene may not limit the straightforward functional analysis of differentiated cells. Taken together, our data indicate that immortalization, reprogramming and differentiation of cells expressing individual transgene combinations is highly feasible and significantly expands the generation of iPSC-based, patient-specific disease models.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2022.101308>.

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