Diabetic Complications Consortium – PROGRESS REPORT

Application Title: **Direct reprogramming of human adult kidney cells**

Principal Investigator: **Benjamin Dekel MD PhD**

1. Project Accomplishments:

Epithelial stemness has been recently shown not to be an intrinsic feature of a specific cell population but rather an induced state. This is especially true in organs like the adult kidney which lack a distinct stem cell hierarchy and for which there is limited ability to long-term propagate true clonal populations of precursor cells ex-vivo. Towards this goal we tested the paradigm of shifting the balance between differentiation and stemness in the kidney by introduction of pluripotency factors. Here we show that human adult kidney epithelial cells (hKEpC) grown in adhesion cultures can readily proliferate, clonally emerge and long-term expand *in vitro* as unipotent stem cells by ectopic expression of *Oct4*. The expanded cells show normal karyotype and constant doubling-time with no evidence for tumorigenicity or teratoma growth. Clonal progeny generates functional proximal tubular cells and sustains *in vivo* tubulogenic capacity and integration potential into human fetal kidney - induced tubules over many generations. Gene expression analysis and interrogation of global methylation patterns revealed that reestablishment of stemness in hKEpC activates multiple pathways including Sonic Hedgehog, Notch and Akt/mTOR pathways. Altogether, clonal long-term expansion of primary adult renal precursors opens up new vistas for drug discovery, toxicology studies and regenerative medicine.

2. Specific Aims:

Specific Aim 1: To direct reprogramming of human adult kidney cells into expandable iNF cells in vitro by pluripotent factors

Results: We ectopically expressed human OCT4 gene in human primery adukt kidney cultured cells (human kidney epithelial cells – hKEpC). Cells were analyzed after 2-3 weeks. *Oct4*-hKEpC underwent profound morphological changes, appearing as homogenous clusters of small round cells (Figure. 1A). The proliferative capacities of *Oct4*-hKEpC were then monitored *in vitro*. Remarkably, *Oct4*-hKEpC were proliferative in adhesion culture and could be expanded to high passages (P15) in the presence of serum, while maintaining their homogenous small round morphology, indicative of long-term self-renewal (Figure 1B). This is in sharp contrast to mock-infected controls, which proliferate to adopt a fibroblastic-like appearance upon passages and fail to generate colonies (Figure 1B). In contrast to control cells, which demonstrated an increase in doubling time along passages, *Oct4*-hKEpC maintained a stable proliferation rate, indicating a stable phenotype (Figure 1C). Chromosomal G-band analyses showed that *Oct4*-hKEpC retained normal diploid karyotype, indicating that the cells did not undergo neoplastic transformation (Figure 1D). To demonstrate that the *Oct4*-hKEpC small round cell aggregates emerge from a single cell upon culture passage we labeled cells with a red fluorescent protein using a lentivirus-based vector directing constitutive expression of mCherry. This construct did not include any type of antibiotic selection enabling the formation of two cell populations, $mCherry^{POS}$- *Oct4*-hKEpC and $mCherry^{NEG}$- *Oct4*-hKEpC. We observed red or colorless but no mixed colonies indicating that clonal expansion underlies cell growth (Figure 1E). In addition, colony-forming-unit (CFU) and single cell clonal assays (limiting dilution) demonstrated that control cells formed colonies only rarely, while *Oct4*-hKEpC showed significant colony formation capacity, including at the single cell level (Figure 1F). Thus, induction of OCT4 in hKEpC generated a clonal culture allowing cell expansion and preservation of clonal properties.

Next, *Oct4*-hKEpC were subjected to microarray analysis and compared to control kidney epithelial cells. Hierarchical clustering separated *Oct4*-hKEpC

from control samples indicating distinct biological entities (Figure 2A). OCT4 induction led to a significant increase in epithelial to mesenchymal **(**EMT**)** induction markers such as *SNAI2, THY-1,* Cadherin11 and Desmuslin, the expression of regulators of DNA transcription including key effectors of early renal mesenchymal progenitors and metanephric kidney development (*OSR1, SIX1, HOXD11,10, 8, 4, 3*) (Di-Poi et al., 2007; Rosenblum, 2008; Reidy and Rosenblum, 2009). Thus, while in pluripotent stem cells OCT4 is a well-known suppressor of lineage differentiation markers we observed induction of such markers in hKEpC. In addition, *Oct4*-hKEpC displayed significant elevation of genes associated with the Wnt signaling pathway (*Fzd7, Fzd5, APCDD, Tle4, NDP, TCFL1, TCFl2, NLK, Ror2, PTK7*) (Table 1), a developmental pathway that has been linked to the effects of progenitor cell induction and renewal by OCT4 in the mouse model (Hochedlinger et al., 2005). Finally, genes associated with cell cycle progression and DNA replication were remarkably unaffected indicating lack of tumorigenicity or aberrant growth advantage of *Oct4*-hKEpC compared to the mock-infected hKEpC (or parental control hKEpC). We then verified with qPCR some of the transcriptional changes revealed by microarrays, demonstrating de-differentiation/EMT (*E-cadherin*, *Vimentin*) and induction of early renal mesenchymal markers (*OSR1, SIX2)*. Clonal *Oct4*-hKEpC showed induction in *OSR1* transcripts and *SIX2* (Figure 2B). Moreover, similar to microarray data, no evidence of additional pluripotency gene induction (*NANOG*, *KLF4*) was apparent in *Oct4*-hKEpC (Figure 2C). We also verified transcriptional changes at the protein level; immunofluorescent staining of *Oct4*-hKEpC for Vimentin and pan-Cytokeratin revealed that in contrast to control-hKEpC that express both mesenchymal Vimentin and epithelial Cytokeratin, *Oct4*-hKEpC showed reduced Cytokeratin expression (Figure 2D). Altogether, *Oct4*-hKEpCs demonstrate a de-differentiated phenotype. Since *Oct4*-hKEpCs show clonal behavior over many passages, we determined whether a similar gene pattern is maintained across multiple single cell clones that emerged from *Oct4*-hKEpC. Multi-gene expression analysis of six different clones demonstrated a similar gene profile in respect with EMT, cell cycle and kidney-segment related genes, suggestive of clonal homogeneity. Importantly, single cell clones were also reminiscent to the total culture of *Oct4*-hKEpC and distinct from naïve hKEpC (Figure 2E).

To provide further molecular insight into epithelial stemness of *Oct4*-hKEpC we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) analysis for the activity-related histone modification H3K4me3, comparing similar passage *Oct4*-hKEpC and naïve hKEpCs. We identified 5405 genes demonstrating enhanced H3K4 methylation of their promoter regions in *Oct4*-hKEpCs, compared to control cells (Figure 3A, B). Enhanced promoter H3K4me3 levels represent genes that underwent chromatin activation usually resulting in either transcriptional activity or transcriptional potential, but with little or no expression. Promoter regions which are marked by H3K4me3 but are not active are generally also associated with the suppressive histone mark H3K27me3. The double H3K4me3/H3K4me27 signature, known as the 'bivalent' mark (Harikumar and Meshorer, 2015) keeps genes poised for activation, but inactive. We found, as expected from proliferating cells, a significant increase in the number of 'chromatin active' genes in the *Oct4*-hKEpC cells. The great majority (87%) of these genes are not overlapping with the control genes, indicating a broad difference between the two cell types. We further looked for distinct chromatin signatures which dictate active pathways which distinguish the *Oct4*-hKEpC cells. Using HOMER (Heinz et al., 2010), we identified several key cellular pathways that exhibit differential activity in *Oct4*-hKEpCs (Figure 3C). These include the sonic hedgehog pathway, which has been shown to regulate stem cell self-renewal (Liu et al., 2006; Xiao et al., 2015) and participate in renal morphogenesis and embryonic kidney differentiation (Yu et al., 2002; Hu et al., 2006), c-MYC, which controls self-renewal across several types of stem cells (Wilson et al., 2004; Kerosuo et al., 2008; Kwan et al., 2015), including the embryonic pool of renal progenitors (Couillard and Trudel, 2009; Xu et al., 2014) and mTOR, which has been implicated in the self-renewal of pluripotent stem cells (Zhou et al., 2009). Interestingly, *Oct4*-hKEpC also demonstrated differential activity of the p53 pathway. While classically regarded as a negative regulator of cell proliferation, p53 has recently been shown to enable the self-renewal of kidney progenitors (Li et al., 2015).

To determine whether high-passage *Oct4*-hKEpC retain renal identity we carried out multi-gene expression analysis comparing the latter with early passage naïve

hKEpC that harbors the multiple cell phenotypes of the adult kidney*Oct4*-hKEpC showed a decrease in Notch pathway-related genes and elevation of EMT genes, indicative of dedifferentiation (Figure 4A), while cell cycle genes demonstrated similar expression levels in both cell types (Figure 4A). Importantly, a decrease in the expression of different segment-specific genes of the adult kidney, including distal tubules, loop of Henle and podocytes, but not the proximal tubule marker CD13. These observations lead us to explore proximal identity of *Oct4*-hKEpC. We observed uniform expression of proximal tubule markers: LTA, LRP2 and CD13, but not distal tubule markers: DBA or EMA (Figure 4B and C). Moreover, while *Oct4*-hKEpC show induction of mesenchymal genes and skewing towards a less differentiated epithelial state placing these cells in various media that induces mesenchymal lineage failed to do so, in contrast to control hKEpCs. Moreover, when high-passage *Oct4*-hKEpC were placed in epithelial differentiation medium we observed re-differentiation, strong induction of cytokeratin and tubular organization, in sharp contrast to control hKEpCs, which acquire a disorganized phenotype (Figure 4D). Taken together, these results indicate that *Oct4*-hKEpC are locked to retain a proximal renal phenotype. Next, we were interested in finding out whether *Oct4*-hKEpCs retain functional capacity of the proximal tubule lineage. Towards this aim, we evaluated the activity of two proximal tubule-specific transporters of the MDR family in *Oct4*-hKEpC and control-hKEpC. For this purpose, cells were incubated with varying concentrations of two specific transporter inhibitors, MK571 and PSC833, in the presence of the fluorescent dye CMFDA. As the two transporters function in proximal cells as efflux pumps for CMFDA, their inhibition results in intra-cellular accumulation of dye. In line with the above results, *Oct4*-hKEpC demonstrated significantly higher fluorescence when exposed to both inhibitors compared to control-hKEpC indicating the presence of the proximal transporters (figure 4E). Moreover, we detected diminishing transporter activity in control cells along passages. Remarkably, high passage *Oct4*-hKEpC demonstrated higher transporter activity even when compared to low passage control hKEpC (figure 4E). In conclusion, alongside their proximal precursor phenotype, *Oct4*-hKEpC harbor the functional traits of proximal tubule transporters.

We next analyzed whether *Oct4*-hKEpC retain renal identity upon in vivo grafting experiments. To that end we performed a series of grafting experiments of high-

passage clonal *Oct4*-hKEpC in NOD/SCID mice to determine their *in vivo* fate. *Oct4*-hKEpC and control-hKEpC were analyzed immediately after grafting within matrigel (T0) and at 2 weeks post-transplant (T14). At T14, a human cell origin, indicated by HLA expression, was observed exclusively in *Oct4*-hKEpC-derived grafts in which human-specific tubular structures appeared (Figure 5A). Importantly, OCT4 was not detected in tubule-like structures (Figure 5A), indicating that once differentiation occurred OCT4 was down-regulated. On close inspection, the T14 *Oct4*-hKEpC-derived grafts were seen to harbor numerous blood vessels, whereas T14 control grafts were devoid of such vessels (Figure 5B). These results suggest that *Oct4*-hKEpC have the capacity to promote vasculogenesis. Moreover, while the progenitor state of *Oct4*-hKEpC in culture was associated with down-regulation of cytokeratin and de-differentiation (EMT) we observed that parallel to the loss of Oct4, cells within tubular structures acquire cytokeratin indicating re-differentiation through mesenchymal to epithelial transition (MET) (Figure 5C). We then interrogated renal segment identity of grafted *Oct4*-hKEpC using CD13 and EMA. At T14, tubular structures were positive for CD13 and LTA, indicative of proximal nature with no evidence for transdifferentiation to other kidney epithelial lineages (Figure 5D and 5E). We then developed an assay to analyze the fate of OCT4 induced adult cells in a milieu of human fetal kidney cells (hFKC). Clonal *Oct4*-hKEpCs were labeled with a fluorescent marker cmDil and co-grafted with hFKC at a ratio of 1:2 (respectively) cells in NOD/SCID mice. Analysis at T14 showed that hFKC generated a tubular network in which *Oct4*-hKEpC either generated whole tubules adjacent to hFKC-derived tubules (Figure 5F) or alternatively integrated into hFKC-derived tubules as single cells or extended tubular portions (Figure 5F). Importantly control high-passage mock infected cells and parental cells failed to generate any renal structures or integrate to hFKC-derived tubules (0/45). Moreover, we noticed that the number of hFKC-derived structures were higher when hFKC were co-grafted with *Oct4*-hKEpC compared to co-grafting with control-hKEpC or when grafted alone, indicating a role for a microenvironment generated by *Oct4*-hKEpC (Figure 5G).

Figure 1: ***Ectopic expression of* OCT4 *in hKEpC results in long-term clonal expansion***

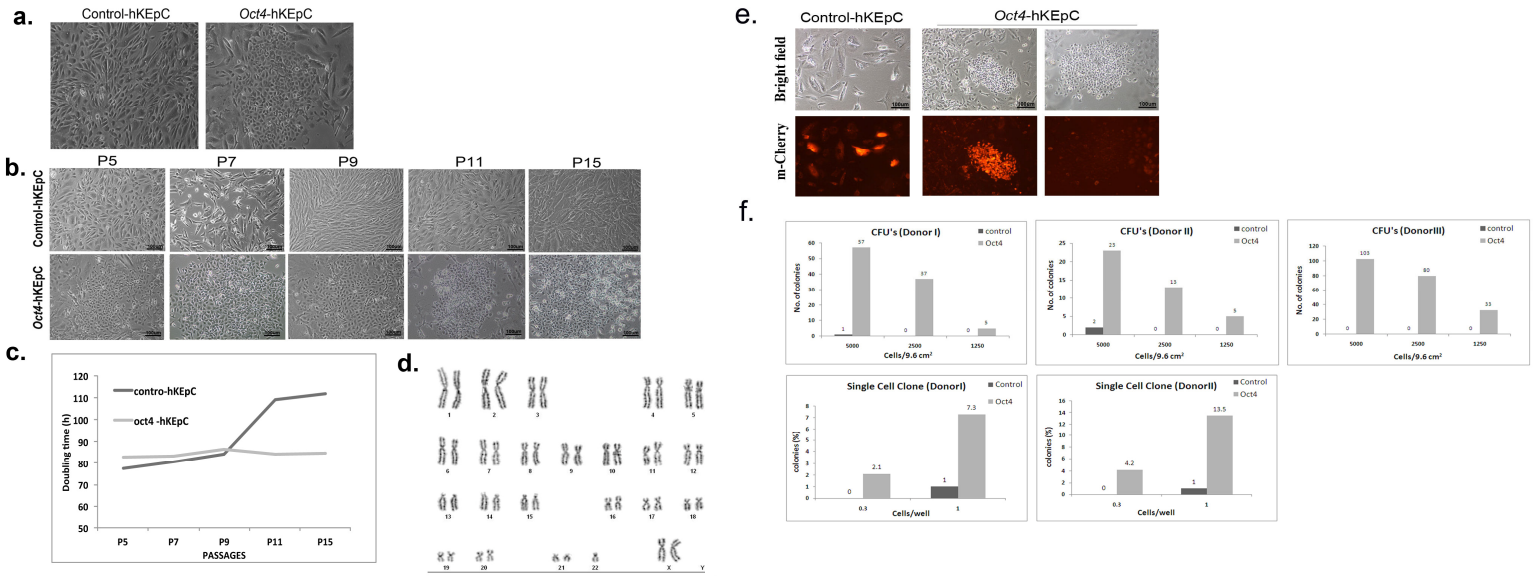


Figure1: OCT4 overexpression in hKEpC was achieved via lentiviral infection; OCT4 expressing cells (*Oct4*-hKEpC), carrying a puromycin-resistance cassette, were selected by puromycin. Cells carrying an empty vector and a puromycin-resistance cassette served as control (control-hKEpC). (a) *Oct4*-hKEpC (right) undergo a phenotypic switch, appearing as a homogenous cluster of small round cells. (b) *Oct4*-hKEpC maintained their round morphology through 15 passages, compared to control cells that spindle-shaped morphology. (c) *Oct4*-hKEpC demonstrate a stable proliferation rate, whereas control cells exhibit an increased doubling time along passages. (d) *Oct4*-hKEpC harbor a normal karyotype 46XX. (e) *Oct4*-hKEpC demonstrate clonal expansion, as manifested by the formation of two types of colonies: mCherry^{POS}-*Oct4*-hKEpC and mCherry^{NEG}- *Oct4*-hKEpC. (f) *Oct4*-hKEpCs are clonogenic at high passages (P5-P7), as seen in the colony forming unit (CFU) assay. At the same passages, *Oct4*-hKEpCs are capable of generating single cell-derived colonies. While control cells formed colonies only rarely, *Oct4*-hKEpC demonstrated significant and consistent colony formation capacity. Shown are results from independent samples.

Figure 2: **OCT4** ***alters hKEpC differentiation of and induces stemness genes***

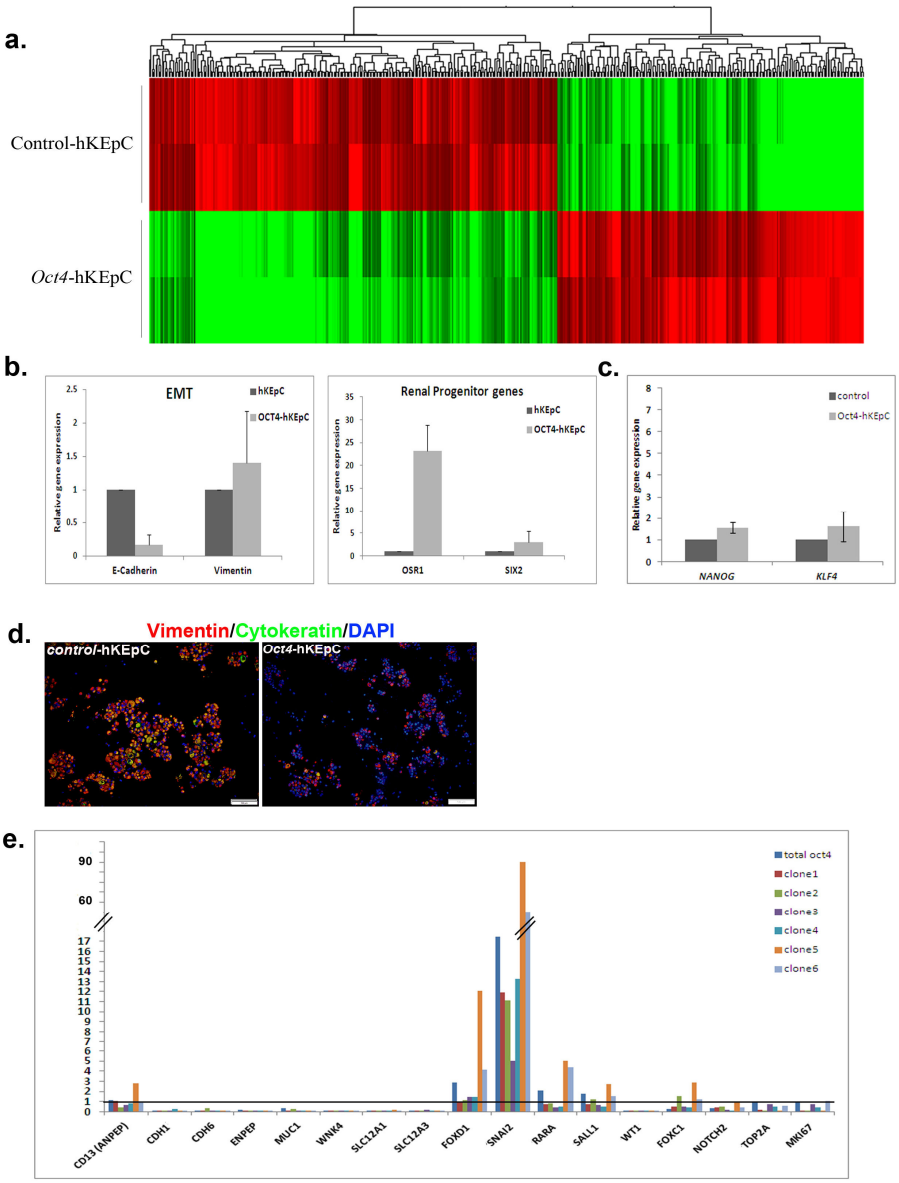
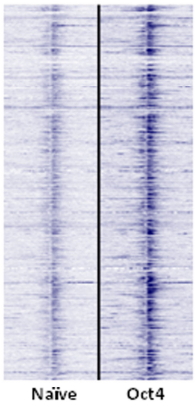


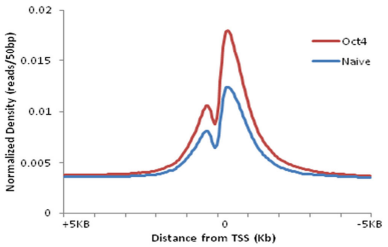
Figure 2: (a) Microarray analysis of *Oct4*-hKEpC. Heat map of *Oct4*-hKEpC compared to control-hKEpC yielding hierarchical clustering, separated *Oct4*-hKEpC from control samples, indicating distinct biological entities. (b) qRT-PCR analysis of control-hKEpC and *Oct4*-hKEpC. Shown are mesenchymal-epithelial transition (MET) genes (*Vimentin, E-Cadherin)* and nephric-progenitor (*SIX2, OSR1*). (c) qRT-PCR analysis demonstrating similar expression levels of the pluripotency genes *KLF4 and NANOG* in control-hKEpC and *Oct4*-hKEpC. The values for untreated control-hKEpC were used to normalize data. Data represents three independent experiments, all yielding similar results. Data was calculated as average ± SD. *, $p < 0.05$. (d) control-hKEpC express both cytokeratin and vimentin, whereas *Oct4*-hKEpC are completely devoid of cytokeratin expression, indicating a de-differentiated phenotype. (e) *Oct4*-hKEpC-derived colonies showed a similar genetic profile to *Oct4*-hKEpC, as demonstrate by multi-gene expression analysis

Figure 3: ***ChIP-seq analysis of Oct4-hKEpCs further delineates stemness pathways***

a.



b.



c.

Term	p-Value	# Genes	Gene Symbols
Validated targets of C-MYC transcriptional activation	7.2561E-06	20	PMAIP1, SLC2A1, TAF12, NME1-NME2, KAT2A, NME2, RUVBL1, CREBBP GAPDH, BAX, HSP90AA1, CCNB1, SHMT1, HSPD1, MAX, HMGA1, LDHA EIF4A1, ENO1, MTA1
p53 pathway	0.00018709	14	YY1,PRMT5,CSNK1G3,CCNA2,CSNK1E,PPP2R4,CREBBP,SMYD2,TRIM28, RFWD2,USP7,SETD7,CSNK1A1,CSNK1G2
Hedgehog signaling events mediated by Gli proteins	0.00188536	10	CREBBP,PRKACA,CSNK1G2,CSNK1A1,IFT172,CSNK1G3,CSNK1E,STK36,SIN3B,GLI3
Regulation of Telomerase	0.00731494	13	XRCC6,CDKN1B,RAD9A,PTGES3,TERF2IP,XRCC5,SIN3B,HSP90AA1,SMG6, POT1,MAX,ACD,PINX1
Signaling events regulated by Ret tyrosine kinase	0.01225982	8	GAB1,CREB1,PIK3R1,SOS1,DOK5,PRKACA,PXN,GDNF
TGF-beta receptor signaling	0.01504593	10	AXIN1,YAP1,PML,EIF2A,FKBP1A,PARD6A,SKIL,SOS1,ITCH,SMURF1
mTOR signaling pathway	0.03051399	11	YY1,YWHAB,SREBF1,YWHAZ,PXN,ATG13,TSC1,MLST8,RB1CC1,EIF4A1, PML
BMP receptor signaling	0.03266363	7	SKI,PPM1A,NUP214,SMURF1,CTDSP1,SMAD1,CTDSP2
Canonical Wnt signaling pathway	0.03421251	6	RANBP3,CSNK1A1,CSNK1E,APC,SKP1,AXIN1

Figure 3: (a) Mapped H3K4me3 read densities from hKEpC (left) and *Oct4*-hKEpC (right) cells in a window of 10 kb centered around the TSS of UCSC genes (± 5Kb). Data was generated using SeqMiner (Ye et al., 2011). (b) Normalized mapped read density profiles of H3K4me3 ±5 kb around the TSS in hKEpC (blue) and *Oct4*-hKEpC (red) cells. (c) Significant pathway interactions ($P < 0.05$) relevant to kidney development in UCSC genes showing H3K4me3 promoter peaks exclusively in the *Oct4*-hKEpC cells compared with the hKEpC controls. GO term analysis and motif discovery was performed using HOMER (Heinz et al., 2010).

Figure 4: ***Clonal Oct4-hKEpC maintain renal identity***.

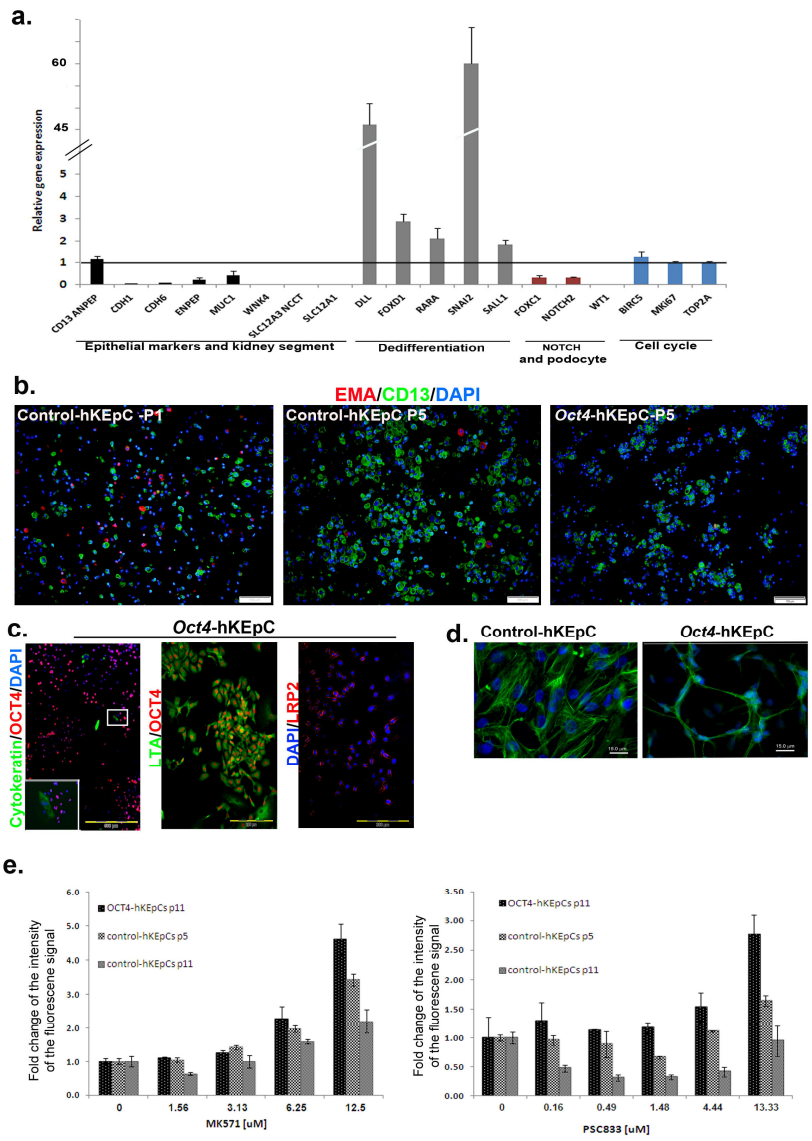
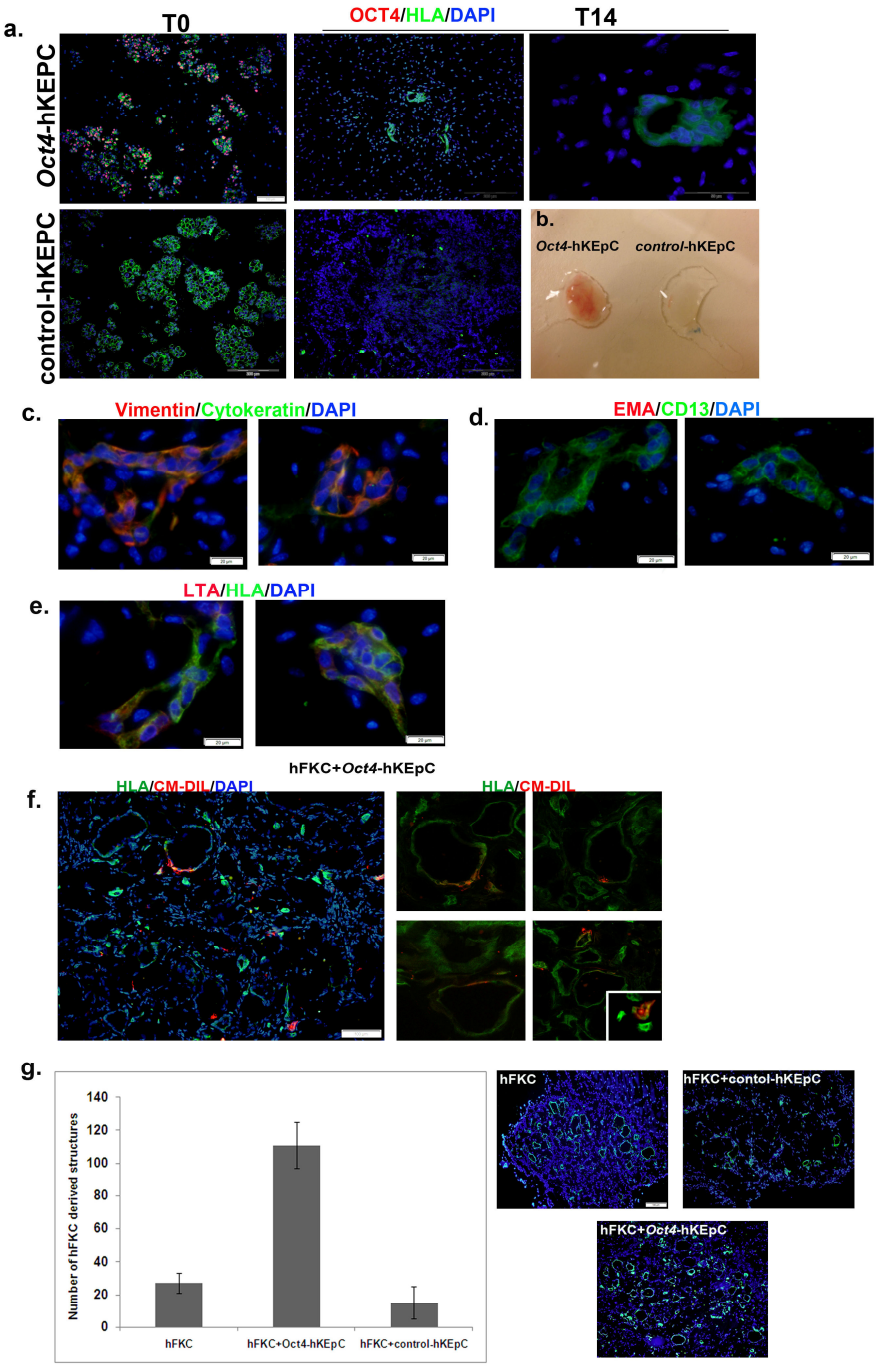


Figure 4: (a) Multi-gene expression reveal down regulation in kidney segment genes and elevation in EMT genes support dedifferentiation of *Oct4*-hKEpC. (b) Control-hKEpC demonstrate lower expression levels of the distal tubule marker EMA along passages (shown are passage 1 vs. passage 5 cells). *Oct4*-hKEpC uniformly express the proximal tubule marker CD13 but are completely devoid of EMA expression. (c) Cultured *Oct4*-hKEpC do not express cytokeratin and demonstrate positive staining for the proximal tubule markers LTA and LRP2. Notably, rare cytokeratin-expressing cells do not express OCT4. (d) Cultured *Oct4*-hKEpC form tubule-like structures. Following 21 days of growth in the presence of epithelial differentiation medium, both cultured control-hKEpC and *Oct4*-hKEpC stain strongly for cytokeratin. However, while *Oct4*-hKEpC organize into tubule-like structures, control-hKEpC remain scattered, demonstrating spindle-shaped morphology. (e) *Oct4*-hKEpCs and control-hKEpCs were incubated simultaneously with fluorescent substrates and different concentrations of MK571 - an inhibitor for the efflux transporters of the Multidrug Resistant-associated Protein (MRP), which are expressed in the apical membrane of proximal tubules and control the drugs intracellular concentration(left panel), or PSC833 - an inhibitor for the transmembrane efflux pumps from the Multiple Drug Resistance proteins (MDRs) family - also referred as P-glycoprotein (Pgp), which are expressed in the luminal membrane of proximal tubule cells in the kidneys and pumps drugs into the urine (right panel). Increasing concentration of the inhibitor diminished the activity of the MRP's or MDRs and caused the accumulation of the fluorescent substrate inside the cells.

Figure 5: ***Oct4*-hKEpC maintain proximal identity in vivo.**



Oct4-hKEpC were injected subcutaneously within matrigel into NOD/SCID mice. Cell grafts were removed and analyzed immediately after injection (T0) and following 14 days (T14). (a) At T0, Oct4-hKEpC positively stained for both OCT4 and HLA. At T14, Oct4-hKEpC, but not control-hKEpC organized into tubule-like structures, while down-regulating OCT4 expression. (b) T14 Oct4-hKEpC-, but not control-hKEpC-derived grafts harbor blood vessels. (c) At T14, the Oct4-hKEpC-derived structures demonstrate combined expression of vimentin and cytokeratin, consistent with epithelial re-differentiation. (d and e) At T14, Oct4-hKEpC demonstrate a proximal tubule phenotype, as manifested by the expression of CD13 and LTA. (f) Oct4-hKEpC both integrated into hFKC-derived tubular structures, seen as red cells within green (HLA+) structures and also formed structures composed solely of Oct4-hKEpC (all cells are red). (g) The number of hFKC-derived structures was higher when hFKC were co-grafted with Oct4-hKEpC compared to co-grafting with control-hKEpC or when grafted alone. Right panel shows representative areas of grafts derived from each cell combination stained for HLA (green).

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