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Direct reprogramming to human nephron progenitor-like cells using inducible *piggyBac* transposon expression of *SNAI2-EYA1-SIX1*

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Abstract

All nephrons in the mammalian kidney arise from a transient nephron progenitor population that is lost close to the time of birth. The generation of new nephron progenitors and their maintenance in culture are central to the success of kidney regenerative strategies. Using a lentiviral screening approach, we previously generated a human induced nephron progenitor-like state *in vitro* using a pool of six transcription factors. Here, we sought to develop a more efficient approach for direct reprogramming of human cells that could be applied *in vivo*. PiggyBac transposons are a non-viral integrating gene delivery system that is suitable for *in vivo* use and allows for simultaneous delivery of multiple genes. Using an inducible piggyBac transposon system, we optimized a protocol for the direct reprogramming of HK2 cells to induced nephron progenitor-like cells with expression of only 3 transcription factors (*SNAI2*, *EYA1*, and *SIX1*). Culture in conditions

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Author contributions

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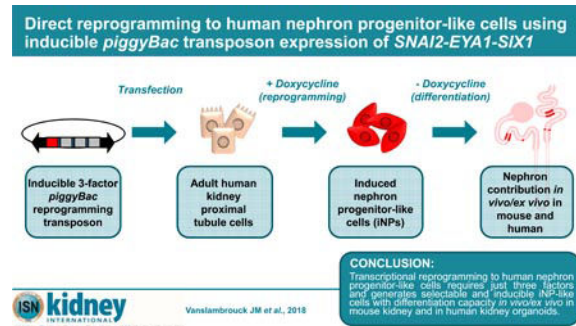
Disclosure

M.H.L. has a research contract with and has consulted for Organovo Inc.

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supportive of the nephron progenitor state further increased the expression of nephron progenitor genes. The refined protocol was then applied to primary human renal epithelial cells, which integrated into developing nephron structures *in vitro* and *in vivo*. Such inducible reprogramming to nephron progenitor-like cells could facilitate direct cellular reprogramming for kidney regeneration.

Graphical Abstract



Keywords

Reprogramming; direct reprogramming; transposon; nephron progenitor; cap mesenchyme

Introduction

The continued rise in the global incidence of chronic kidney disease (CKD)¹ is spurring efforts to develop novel approaches that may benefit future regenerative or cellular therapies. However, progress in this field has been hindered by the complex cellular structure of the mature human kidney. During mammalian development, these multiple kidney cell types are derived from two main progenitor pools: the ureteric bud (UB) and the mesenchymal nephron progenitors (NPs). Through a series of reciprocal inductive signals between these populations, the UB branches to form an intricate collecting duct system for drainage of urine, whilst the NPs undergo an epithelial-to-mesenchymal transition (EMT) to form nephrons (reviewed in Saxen L *et al.*²). Despite the critical role played by the NP population, these cells exist only transiently during mammalian development, with nephrogenesis ceasing near the time of birth following a final burst of nephron formation.^{3–6}

As such, human NPs are an ideal candidate cell type for kidney regenerative therapies due to their capacity to give rise to all segments of the mammalian nephron. Whilst isolation of putative NPs from human fetal kidneys has been reported,^{7,8} availability and access to such tissues remains limited. Recently, there has been great interest around the generation of human NPs from pluripotent cell sources,^{9–14} including our own studies showing the generation of kidney organoids.^{11,15} While such protocols generate cells possessing phenotypic characteristics of *bona fide* human NPs, including a capacity to form segmented nephrons, a self-renewing NP population required for continued nephrogenesis is lacking. The long-term maintenance and expansion of these multipotent cells in culture, regardless of source, also continues to be a challenge in the field. We have previously demonstrated the

feasibility of direct reprogramming to induced nephron progenitor-like cells (iNP-like cells) using a lentivirus-mediated screen that identified a pool of six transcription factors (*SIX1*, *SIX2*, *OSR1*, *HOXA11*, *EYA1* and *SNAI2*).¹⁶ The resulting iNP-like cells demonstrated appropriate NP gene and protein expression together with a selective capacity to integrate into the endogenous NP population within *ex vivo* embryonic mouse kidney cultures. However, this screening approach relied on transduction of individual viral constructs, lacking transcription factor inducibility and a selective marker, thus hampering the evaluation of subsequent nephron differentiation capacity.

Here we report the development of a novel *piggyBac* transposon system for direct transcriptional reprogramming to iNP-like cells. Incorporation of an inducible promoter, selectable marker for cell enrichment and 2A-peptide cleavage signals enabled inducible, simultaneous expression of all six iNP reprogramming factors. Clonal selection and analysis of transposon integration events revealed a requirement for only three of these genes, *SNAI2*, *EYA1* and *SIX1*, to generate an iNP-like phenotype. The combination of inducible expression of these genes with culture in a previously reported NP-supportive medium, NPSR, proved to be the most effective method to generate human iNP-like cells and also enabled reprogramming of primary proximal tubule cells.¹⁷ Such cells were able to contribute to nephron formation *in vivo* and *ex vivo*, in neonatal mouse kidney and human iPSC-derived kidney organoids. Not only does this approach represent a significant step towards larger-scale production of NPs, whereby reprogrammable cells may be generated to facilitate bioengineering or drug screening applications, but may also be of significance to *in vivo* reprogramming approaches in the future.

Results

Development of a *piggyBac* transposon-mediated reprogramming system

PiggyBac transposons are a non-viral integrating gene delivery system offering several advantages over traditional methods, including a large cargo capacity,¹⁸ multiplexed gene delivery,¹⁹ flexibility of target cell type²⁰, suitability for *in vivo* applications^{21,22}, and ability to be excised from the genome.²³ To generate a reprogramming system that would provide greater control over reprogramming factor expression compared to lentiviral transduction of individual transcription factors,¹⁶ a three-component system was designed that comprised two *piggyBac* transposons for reprogramming factor delivery (pT-SOH [*SIX2*, *OSR1* and *HOXA11*] and pT-SES [*SNAI2*, *EYA1* and *SIX1*]), a separate *piggyBac* transposon conferring doxycycline inducibility (pT-TetON)²¹ and a hyperactive *piggyBac* transposase plasmid (p-EF-1 α -HA-m7pB) for efficient mammalian genomic integration (Figure 1 and Table S1).^{22,24} The multicistronic *piggyBac* reprogramming transposons were each engineered under the control of a TightTRE doxycycline-inducible promoter, driving the mCherry fluorescent reporter and three reprogramming factors separated by unique 2A peptides (Figure 1 and Table S2). Precise excision of the transposon plasmids from the genome was confirmed (Figure S1A), as was the efficient cleavage of all six intervening 2A-peptides (Figure S1B) and the expression and correct localisation of reprogramming factor proteins for which functional commercial antibodies were available (Figure S1C and Table S3). A transposon with the mCherry reporter alone (pT-mCherry) under the control of the

TightTRE promoter was constructed as a control for the transfection and integration process itself (Figure 1). Expression of this reporter confirmed functionality and stringency of the TightTRE promoter (Figure S1D).

***In vitro* reprogramming of human kidney epithelial cells to a NP-like phenotype**

To confirm successful reprogramming to an iNP-like state using the *piggyBac* transposon system, we utilized the same adult human kidney epithelial cell line (HK2) that was used in our original lentiviral screening approach.¹⁶ Following co-transfection of the reprogramming and TetON transposons (pT-SOH, pT-SES and pT-TetON) along with the m7pB hyperactive *piggyBac* transposase, HK2 cells were exposed to reprogramming conditions with doxycycline and gene expression assessed over a 10 day time course using primers that detect only endogenous gene expression (Figure 2A). A number of NP and EMT markers, including *SALL1*, *SIX2*, *PAX2*, *OSR1*, *MMP2* and *MMP9*, were found to increase in expression over time. Despite *CITED1* RNA levels decreasing dramatically after 4 days of doxycycline exposure, robust *CITED1* protein expression was observed between days 6–8 of reprogramming. This data, combined with the spike in stromal marker *FOXD1* by day 10, suggested 8 days of doxycycline exposure to be optimal.

By day 8 of reprogramming, the formation of raised structures co-expressing the NP marker *CITED1* and mCherry reporter were observed in cultures transfected with the reprogramming transposons (Figure 2 B, C), but were absent from control cells (Figure 2C). Further analysis revealed expression of a range of NP marker proteins in these structures in addition to *CITED1*, including *PAX2*, *SIX2* and *EYA1* (Figure 2D), with co-expression of NP proteins including *EYA* and *SIX2* being confirmed where permitted by antibody species cross-reactivity (Figure S2A). Furthermore, these structures lacked epithelial E-CADHERIN protein expression (Figure S2B) which, along with the increase in *MMP2* and *MMP9* gene expression, was suggestive of an EMT event required to transform HK2 cells to a mesenchymal state. Taken together, these data suggested the presence of iNP-like cells after reprogramming.

While this represented a polyclonal reprogrammed population with individual cells having different levels and locations of transposon integration, the capacity of these iNP-like cells to form nephrons was assessed using a mouse neonatal kidney injection model which exploits the persistence of the mouse NP population to postnatal day 2 (PND2) (Figure 3A).²⁵ Injections into the cortical renal parenchyma of PND1 mouse kidneys were performed using reprogrammed iNP-like cells (test) or control HK2-mCherry cells (control; HK2 cells transfected with a transposon carrying mCherry only and subjected to the same reprogramming protocol), each combined with fluorescent microspheres for confirmation of injection site. At 3 days post-injection, surviving cells were seen in both test and control kidneys, with a proportion of these cells integrated into developing tubules defined by laminin-bound basement membranes (Figure 3Bi). However, at 7 days post-injection no HK2-mCherry cells persisted in control kidneys. The presence of fluorescent microspheres confirmed that the suspension was delivered successfully and is targeted to the cortical region using this technique (Figure 3C). In contrast, abundant reprogrammed iNP-like cells were integrated into LTL^+ proximal and LTL^- distal nephron segments (Figure 3Bii).

Notable was the formation of extended tubule segments by the iNP-like cells and their binding of LTL on the apical brush border membrane as would be expected for a mature proximal tubule. No reprogrammed cells were observed within the interstitium. Taken together, these data indicated the acquisition of a NP-like phenotype by the reprogrammed HK2 cells and the presence of a subset of cells with nephrogenic differentiation capacity.

Reprogramming is achieved with three factors and is improved with NP-supportive maintenance media

Isolation of individual reprogrammed subclones was performed to examine the relationship between pT-SOH, pT-SES and pT-TetON expression and the presence of a NP-like phenotype. The clone possessing the highest level of endogenous *SIX2* and *CITED1* expression following reprogramming (Clone 8, Figure S3A) also displayed pronounced mesenchymal morphology from day 5 of reprogramming (Figure S3B). While transposon copy number analysis revealed very low levels of pT-SOH integration in all clones, Clone 8 possessed the highest copy number of pT-SES (Figure S3C). The clone with the highest pT-SOH integration (Clone 12) lacked evidence of NP gene induction. Our previous combinatorial screen for reprogramming factors¹⁶ indicated that the three pT-SES genes transduced individually, similar to *SIX1* and *SNAI2* in combination (Figure S3D), were unable to induce *CITED1* expression. This suggests that co-expression of all three genes, *SNAI2*, *EYA1* and *SIX1*, is sufficient to induce endogenous *SIX2* and *CITED1* gene expression in HK2 cells and reprogram to an iNP-like state. All additional experiments were performed using this optimally reprogrammable clone.

To examine whether culture of iNP-like cells in recently defined NP maintenance media was feasible, we cultured Clone 8 in three previously described media (CDBLY²⁶, NPEM²⁷ and NPSR²⁵) during reprogramming. Complete replacement of the original basal media for all 8 days of the reprogramming protocol was found to have a negative effect on *SIX2* expression (data not shown). Instead, NP-supportive media was added from the time of *CITED1* protein expression (approximately day 5 post-doxycycline addition; refer to Figure 2). The addition of NP-supportive media had a significant effect on the expression profile of Clone 8 iNP-like cells ($P = 0.008$ by two-way ANOVA on normalized [- Ct] values), improving the expression of a range of NP genes compared to HK2 growth media (HGM) with slightly different outcomes for each media formulation (Figure 4A). Culture in NPEM improved *SIX1*, *PAX2* and *MEOX2*, while CDBLY culture improved *SIX1*, *PAX2*, *MEOX2* and *CITED1*. NPSR resulted in a greater improvement in global NP gene expression, with a larger number of NP markers showing increased average fold change expression above control compared to HGM, CDBLY and NPEM conditions, including *SALL1*, *SIX1*, *SIX2*, *EYA1*, *MEOX2* and *OSR*. Importantly, control cells lacking the reprogramming transposon but cultured in NPEM or CDBLY exhibited little induction in NP markers, highlighting the importance of having the reprogramming transposons present (Figure S4A). While NPSR alone increased the expression of some NP genes, this induction was lower than in Clone 8 iNPs-like cells for several key NP markers (Figure S4B), suggesting a complementary effect between the media and transposon.

When developing the NPSR media for NP maintenance, Li *et al.*¹⁷ showed that isolated NPs have the capacity to be cultured as 3D aggregates, a format that was optimal for the maintenance of NP identity in their hands. To investigate the response of reprogrammed cells to this culture system, Clone 8 iNP-like cells were transferred to low attachment 96-well plates and cultured in NPSR. Within 3 days, these cells formed tight spheres while continuing to proliferate, in some cases forming a single floating aggregate by 5 days of culture onwards (Figure S4C). In contrast, Clone-8 iNP-like cells cultured in control media (HGM) and HK2-mCherry control cells cultured in NPSR could not be maintained in this fashion, highlighting the cooperation between the transposon reprogramming and the optimal media conditions.

Induction of a nephron progenitor-like transcriptional profile

In order to further characterize the Clone 8 iNP-like cells at a transcriptional level, RNAseq profiling was performed on parental Clone 8 cells without induction (no doxycycline, HGM media) in comparison to the same lines after 8 days doxycycline induction cultured in either control (HGM) or NP maintenance (NPSR) media. Profiling was performed in triplicate and a PCA analysis showed tight correlation of triplicates for each sample (Figure S4Di). Genes differentially expressed (\log_2 threshold > 1 , FDR < 0.05) between control uninduced Clone 8 and both induced conditions were selected (Table S4). An unbiased clustering revealed strong alignment in gene expression between the induced NPs compared with the uninduced parental clone (Figure 4Bi). GO analysis (<https://toppgene.cchmc.org/>) using these genes revealed upregulation of a signature of 36 genes from within the top 500 genes enriched in isolated murine NPs (www.gudmap.org.au; P4 KidCapMesRenVes_Crym_top-relative-expression-ranked_500) (Figure 4Bii). This signature also included genes within the reprogramming transposons. While a specific analysis of these 6 transcription factors revealed evidence that all had been induced (Figure S4Dii), expression was significantly higher for SIX1, EYA1 and SNAI2 (Figure S4Diii).

Despite a lack of global RNAseq data from pure populations of embryonic human NPs, to gain insight into the similarities between direct reprogramming-derived iNP-like cells and endogenous human NPs, RNASeq data from the uninduced and induced Clone 8 cells were compared to datasets from NP-enriched cell populations derived from the outer nephrogenic zone of embryonic human kidneys (Figure 4Biii-v).^{28,29} Using genes from a differential expression comparison between uninduced Clone 8 samples with MARIS/RNA-Seq data from human SIX2-enriched NPs and human SIX2/MEIS1-enriched interstitial progenitor cells (IPCs), induced Clone 8 iNP-like cells were observed to segregate with human endogenous NP populations and showed similar upregulation to human SIX2-enriched cells for multiple gene groups (Figure 4Biii). In subsequent analyses focusing on these gene groups, gene expression was seen to progressively increase from uninduced to induced Clone 8 samples, with overall higher expression in the induced Clone 8 iNP-like cells cultured in NPSR (Figure 4Biv). When comparing to a Single Cell Sequencing dataset obtained from the outer nephrogenic zone of human fetal kidney, iNP-like cells again segregated with the endogenous human progenitor cells rather than the uninduced samples and were enriched for a number of markers that were identified to be differentially expressed in the human NP cluster (Figure 4Bv).

Clone 8 iNP-like cell show nephrogenic potential in mouse and human organoids

While *in vitro* data supported the role of NP-maintenance media for sustained iNP-like cell culture, it was important to confirm the nephron forming capacity of the resulting cells. To do this, we used two *ex vivo* differentiation assays; a gold-standard embryonic mouse kidney organoid assay^{30,31} (Figure 5Ai) and a novel human iPSC-derived kidney organoid assay utilizing our directed differentiation protocol. Of importance, the latter approach enabled the examination of iNP-like cell behavior in a more human context compared to mouse organoids (Figure 5B). Prior to harvesting, organoids were briefly exposed to doxycycline to initiate the re-expression of mCherry by either Clone 8 iNP-like cells or control cells. In mouse kidney organoids, reprogrammed cells were found to integrate into both LTL⁺ proximal nephron segments as well as LTL⁻ distal nephron segments (co-stained with the epithelial marker E-CADHERIN) when reprogramming was performed in either HGM, CDBLY, NPEM and NPSR (Figure 5Aii). Consistent with the results of gene expression analysis, Clone 8 iNP-like cells cultured in NPSR showed the greatest level of contribution, with extended tubule segments composed of reprogrammed cells, predominately in proximal tubules. Within tubules, these integrated cells also showed evidence of mature segment-specific functional proteins, including the distal tubule solute transporter, Slc12a1, and the proximal tubule endocytic receptor, Megalin (Figure S5A). In contrast, HK2-mCherry control cells failed to contribute to nephrons, in some instances clustering together and causing disruption to organoid structure (Figures S5B and S5C). The integration capacity of uninduced Clone 8 control cells (cultured in HGM without doxycycline induction) was similarly evaluated. These cells did not integrate or survive the duration of the experiment (Figure S5C).

Our original lentiviral-mediated screen, via quantification of integration into the endogenous nephron progenitor compartment of mouse organoids, estimated reprogramming efficiency to be 0.875%.¹⁶ We sought to perform a similar quantification in the current study to determine differentiation efficiency of Clone 8 iNP-like generated using the optimal reprogramming condition in NPSR. Whilst variation existed between organoids, quantification of 6 organoids across 3 independent experiments revealed that of the cells subjected to reprogramming, approximately 39% possessed differentiation efficiency and thus were efficiently reprogrammed (Figure S5D).

In order to further investigate the survival and differentiation capacity of NPSR-cultured Clone 8 iNP-like in a more human setting, these cells were subjected to a human iPSC-derived kidney organoid assay (Figure 5Bi). Similar to mouse kidney organoids, Clone 8 iNP-like cells showed the capacity to differentiate and integrate into developing LTL⁺ proximal and LTL⁻ distal nephron segments (Figure 5Bii). As would be expected for *bona fide* NPs, Clone 8 iNP-like cells contributed to NPHS1⁺ glomeruli but not GATA3⁺ collecting duct (Figure 5Bii). The use of MAFB^{mTagBFP} iPSC-derived organoids, which report the expression of MAFB-expressing podocytes of the glomeruli (blue),³² enabled further confirmation of the contribution of iNP-like cells to glomeruli and epithelial via endogenous (unstained) expression of mCherry and blue fluorescent protein (BFP), as well as phase contrast imaging showing epithelium (Figure 5Biii). Once again, the ability of uninduced Clone 8 control cells without doxycycline exposure to integrate into developing

nephrons was assessed and revealed no integration (Figure S5E). These control cells were seen to cluster together within the interstitium at early time points and either did not survive until the experimental endpoint of 10 days organoid culture or remained clustered within the interstitium.

Refined reprogramming conditions generated iNP-like cells from primary human proximal tubule cells

Primary human renal epithelial cells represent an accessible and reprogrammable cell source that can be isolated from patients through surgery or biopsy or via non-invasive urine collection methods.³³³⁴ We have previously demonstrated that normal human proximal tubule epithelial cells (hRPTECs) transduced with the six iNP reprogramming factors, *SIX1*, *SIX2*, *OSR1*, *HOXA11*, *EYA1* and *SNAI2*, showed an increase in the expression of NP genes.¹⁶ However, these cells did not integrate into endogenous kidney sub-compartments when assessed for nephron progenitor potential in mouse kidney organoid assays (data not shown). Having now developed a refined inducible reprogramming system, hRPTEC cells were subjected to this novel protocol and functionally assessed in the mouse kidney organoid assay (Figure 6).

Following electroporation with pT-SES, pT-TetON and hyperactive *piggyBac* transposase plasmids, hRPTECs were exposed to the optimised reprogramming protocol and media conditions (HGM followed by NPSR) then harvested at day 8 of reprogramming for the mouse kidney organoid assay. These cells showed a marked change in morphology and an increase in the expression of all NP markers except for *PAX2* compared to parental hRPTECs (Figure 6A and B). We would note that we have previously noted high endogenous *PAX2* expression in parental hRPTECs.¹⁶ In functional assays, transfected hRPTECs were observed in three separate kidney recombinations in a variety of locations, including one organoid with abundant hRPTEC iNP-like cells integrated within developing nephron structures (Figure 6C). As anticipated, they were absent from collecting duct (Figure S6A). hRPTEC-mCherry control cells completely failed to integrate into developing nephron structures (Figure S6B). Taken together these data are evidence of the transferability of transposon-mediated reprogramming to hRPTECs, with such cells representing a feasible primary cell source for iNP-like cell derivation.

Discussion

Here we report the use of a *piggyBac* transposon-mediated system of reprogramming cells to a nephron progenitor-like state with nephrogenic potential. The resulting iNP-like cells showed a capacity to form nephrons both *in vivo* and *in vitro* within mouse kidney, as well as integrate into tubules within human kidney organoids. Transcriptional profiling demonstrated the adoption of a NP-like transcriptional profile comparable to the developing human NP population and clonal analysis clarified the requirement for only three transcription factors, *SNAI2*, *EYA1* and *SIX1*, to recreate an iNP-like phenotype.

The ability to reprogram to iNPs in the absence of *SIX2* was surprising given the critical role played by this gene in NP self-renewal and maintenance as shown in mouse kidney,^{35–37} as well as previous reports associating *SIX2* mutation with renal dysplasia and Wilms tumour.

38–40 However, it has been recently reported that the NPs of the human fetal kidney express both *SIX1* and *SIX2*, whereas the Six1 protein is not evident in the same region in the mouse.⁴¹ Interestingly, *SIX1* was found to be one of the most highly regulated targets of *SIX2* in human kidney, with both proteins likely to regulate a common set of NP genes through recognition of the same DNA binding motifs. Whilst both proteins complex with EYA1, they are likely to form independent regulatory complexes *in vivo*. Taken together, successful reprogramming to human iNPs in the absence of *SIX2* may be attributed to the functional similarity between these proteins in human kidney and the fact that *SIX1* expression may be at least partially activated by *SIX2*. Genetic loss of *Six1* in mouse results in a marked reduction of *Six2*, *Pax2* and *Sall1*, but not *Eya1*, leading to apoptosis of the metanephric mesenchyme (MM).^{42–44} Indeed, data from mouse models suggest that *Six1* and *Eya1* act upstream of these factors and form a transcriptionally active complex necessary for normal kidney development. This confirms conserved regulatory mechanisms between *Six1* and *Eya1*, also demonstrated by developmental studies of their homologs in drosophila eye^{45,46} and human fetal kidney,⁴¹ as well as co-transfection studies *in vitro*.⁴⁷

Expression of *Snai2* (*Slug*) in the endogenous mouse NP population is weak, instead showing strong expression in the cortical stroma.⁴⁸ *SNAI2* was included in the original lentiviral screen due to its activity as an EMT regulatory factor.⁴⁹ While HK2 cells transduced with *SNAI2* alone underwent a marked EMT, they did not adopt a NP-like phenotype,¹⁶ suggesting *SNAI2*'s role the within the reprogramming pool relates to inducing EMT. In support of this concept, studies of murine NP maintenance *in vitro* highlighted the favorable increase in *Snai2* expression when isolated MM cells were treated with low levels of LIF (activating JAK/STAT signaling and maintaining nuclear *SIX2*) and the Rho kinase inhibitor Y27632 (inhibiting MET through the JNK-mediated differentiation pathway), a combination that is utilized in both CDBLY and NPSR.⁵⁰ Nevertheless, while inclusion of *SNAI2* in the reprogramming cocktail may improve EMT, this factor may not be required for a non-epithelial starting cell population. Similarly, whilst having refined the gene set to just three factors for NP induction, it is well-accepted that reprogramming can be imprecise (reviewed in Hendry CE *et al.*⁵¹), hence there may be several ways to reach a desired endpoint. It will now be possible to use this approach to further characterize and improve reprogramming of primary kidney cells and additional starting cell types.

Inducible transposon approaches are of relevance for transdifferentiation to a range of cellular endpoints, as has been shown with the use of transposons for reprogramming to pluripotency.⁵² The current study, which is the first demonstration of inducible *piggyBac*-mediated reprogramming to iNP-like cells with differentiation capacity, represents an incremental yet important advance, potentially inching the field closer to the development of reprogramming strategies as novel disease therapies or for understanding disease pathogenesis. Importantly, this approach may facilitate the large-scale generation of reprogrammable cells that avoids long term culture and expansion of the NPs themselves while retaining their unique phenotype, an issue that continues to be problematic the field. Furthermore, owing to its versatility, this approach not only has the potential to be extended to additional starting cell types, but may also represent a method of precisely generating specific individual cell types of the nephron or achieve direct cellular reprogramming *in vivo* for tissue regeneration.

Methods

Reprogramming constructs, transfection and clone generation

Reprogramming (pT-SOH and pT-SES) and control (pT-mCherry) transposons were generated using a combination of commercial gene synthesis (GENEWIZ, South Plainfield, New Jersey) and standard cloning procedures. Plasmids pT-TetON²¹ and pEF-1 α -m7pB⁵³ have been described previously. Plasmid DNA transfection was performed with FuGENE (Promega, Madison, Wisconsin) or via electroporation (Neon Transfection system; ThermoFisher Scientific, Waltham, Massachusetts), according to manufacturer's directions. Additional information can be found in the Supplementary Materials and Methods section. For reprogramming experiments using stably-transfected HK2 cells, transfected cells were cultured for 2 weeks prior to brief exposure to doxycycline and FACS isolation of mCherry-expressing stably transfected cells. Stably transfected clones were generated through serial dilution and expansion of the FACS isolated population.

Cell culture and reprogramming

HK2 cells (ATCC; CRL-2190) were maintained in HK2 growth media (HGM) as described previously.^{16,54} hRPTECs (Lonza, Walkersville, Maryland; CC-2553) were maintained in Renal Epithelial Cell Growth Media (REGM; Lonza) according to manufacturer's instructions. For reprogramming, stably transfected cells were seeded at a density of 4000 cells/cm². After 48 hours, media was replaced with HGM containing 2 mM valproic acid (VPA; Sigma Aldrich) and 2 μ g/mL doxycycline (Sigma Aldrich) for 48 hours. Cells were then exposed to media containing doxycycline without VPA for the duration of reprogramming, refreshing every 24 hours. For NP media experiments, HGM media was removed and replaced with either CDBLY, NPEM or NPSR^{17,26,27} containing doxycycline from day 5 onwards.

Immunofluorescence

Cells on coverslips, organoids and tissues were fixed in ice cold 2–4% paraformaldehyde (PFA; Sigma Aldrich). Tissues for sectioning were cryoprotected in 30% sucrose solution (Sigma Aldrich) overnight prior to embedding. Immunofluorescence was performed as previously described,¹⁵ with shortened (1 hour) incubation times for cells and tissue sections. Additional information can be found in the Supplementary Materials and Methods section.

Quantitative RT-PCR

RNA was extracted from cell cultures using RNeasy Mini/Micro Kits (Qiagen) or Bioline Isolate II Mini/Micro Kits (Bioline, New South Wales, Australia) as per manufacturer's instructions. Synthesis of cDNA and qRT-PCR was performed and analysed as described previously and using the same qRT-PCR primers.¹⁶ Each qRT-PCR reaction was performed in triplicate for each biological replicate, with means expressed as \pm SEM. Data was graphed and analysed in GraphPad Prism 7.

RNA-Seq

RNA was sequenced and genes analysed for differential expression (see Supplementary Materials and Methods section). Differential expression was tested between four groups: (1) Clone 8 uninduced and Clone 8 iNP-like cells in HGM, (2) Clone 8 uninduced and Clone 8 iNP-like cells in NPSR, (3) Clone 8 uninduced and both Clone 8 iNP-like cells in HGM and NPSR combined, (4) Clone 8 iNP-like cells in HGM and Clone 8 iNP-like cells in NPSR. The datasets were deposited in the Gene Expression Omnibus(GEO)⁵⁵ (accession GSE107410). Additional comparisons between Clone 8 cells and human SIX2-enriched NPs and SIX2/MEIS1 IPCs was calculated by adding samples from GEO accession GSE102596 and using the same methods to perform differential expression tests between the uninduced Clone 8 cells and human SIX-enriched cells as a group.

Neonatal injection assay

Neonatal injections were performed under ultrasound guidance into the renal cortex as described previously²⁵ in accordance with Institutional animal ethics guidelines (IMB/132/13/NHMRC). Injections were performed in triplicate for control and test cells (approximately 4.5×10^4 cells per injection) and kidneys were harvested at 3 and 7 days post-injection. Reprogramming of the injected test cell population was confirmed via qRT-PCR.

Mouse kidney organoid assay

Assays were performed as previously described,^{30,31} with 5% exogenous (control or test) cells added to dissociated kidneys prior to aggregation and culture as organoids on 6-well Transwell filter plates (Corning, Corning, New York) for 7 days. A minimum of 3 mouse organoids were generated for each cell type and media condition. Prior to harvesting, organoids were briefly exposed to doxycycline (<6 hours) to re-express mCherry in the exogenous cells.

Human iPSC-derived kidney organoid assay

The CRL1502 clone C32 hiPSC⁵⁶ and the MAFB^{mTagBFP} hiPSC³² lines were differentiated to renal progenitors as described previously.¹⁵ At day 7 differentiation, the iPS-derived progenitor cells were combined with test or control cells to form suspensions (95% iPS-derived progenitors and 5% exogenous cells). Suspensions were then aggregated and cultured as described previously.¹⁵ At least 3 organoids were generated for each cell type and media condition. Prior to harvesting, organoids were briefly exposed to doxycycline (<6 hours) to re-express mCherry in the exogenous cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Statement:

The detrimental global impact of rising rates of chronic kidney disease and limited patient treatment options is propelling efforts to explore new approaches that may benefit future therapies. In the current study, we identify a combination of three reprogramming factors (SIX1, EYA1 and SNAI2) that, when delivered using a novel inducible piggyBac transposon system, are sufficient to induce reprogramming of adult kidney cells to nephron progenitor-like cells that possess differentiation capacity. It is our hope that advance such as this in the reprogramming field will be of value to the development of novel, regenerative and patient-specific treatment options, as well as studies of disease pathogenesis and drug screening applications, in the future.

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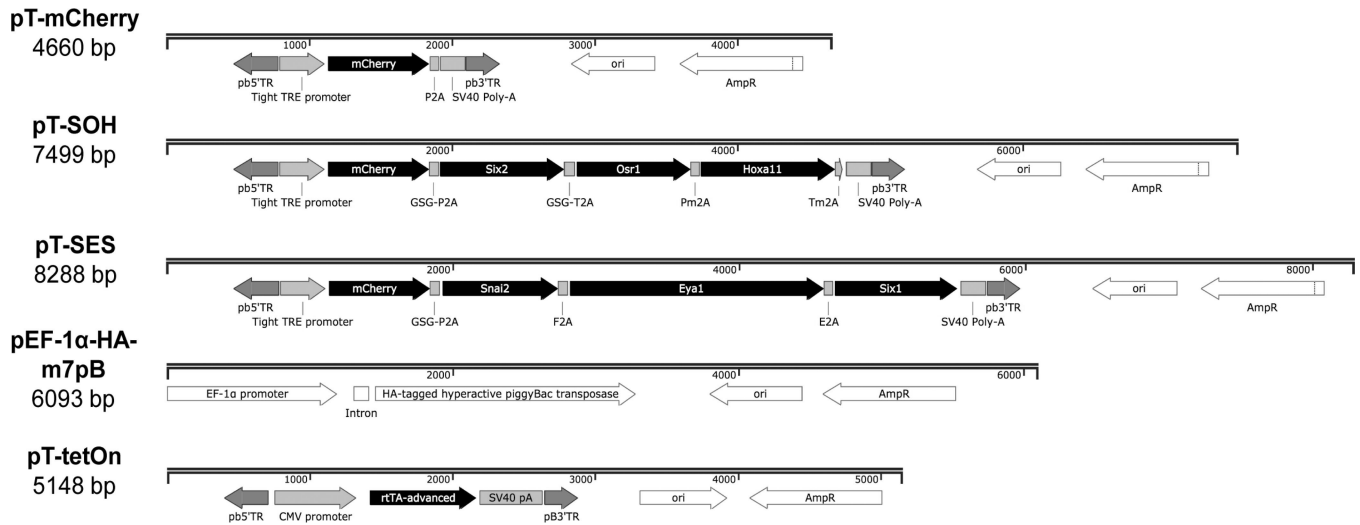


Figure 1: piggyBac transposon and transposase plasmid maps.

Transposon plasmids contain the *piggyBac* 3' and 5' terminal repeats (pb3' TR, pb5' TR) flanking the transposon sequence to be integrated permanently into the genome.

Abbreviations are as follows: CMV (cytomegalovirus, constitutive viral promoter); rtTA-advanced (advanced reverse tetracycline transactivator protein); SV40 pA (SV40 virus polyadenylation signal); ori (origin of replication); AmpR (ampicillin resistance gene); Tight TRE promoter (tight tetracycline response element promoter); EF-1 α promoter (Elongation Factor-1 α , constitutive endogenous promoter); HA (N-terminal hemagglutinin tag). White colouring depicts non-transposon portions of the vectors, while black and grey depicts integrating portions of the transposons. Refer to Table S1 for reprogramming factor accession numbers.

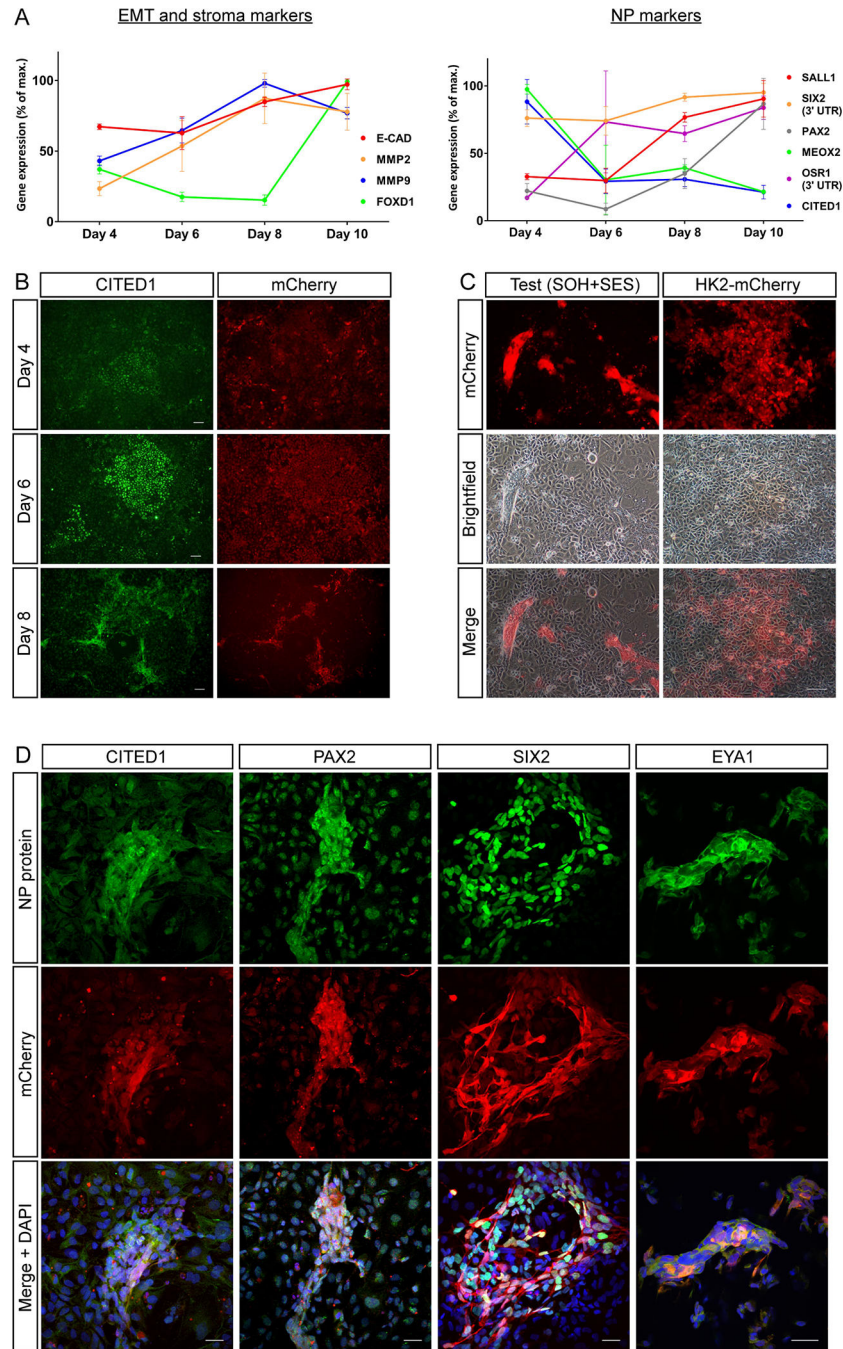


Figure 2: HK2 cells show evidence of reprogramming *in vitro* when transfected with the reprogramming transposons, pT-SOH and pT-SES.

(A) Quantitative RT-PCR (qRT-PCR) time course analysis of EMT, stoma and NP genes in HK2 cells undergoing reprogramming for 4, 6, 8 and 10 days. Normalised gene expression at each time point is presented as a percentage of the maximum expression observed across the experiment duration. Error bars represent \pm SD of the mean. (B) CITED1 immunofluorescence time course analysis of HK2 cells from (A), depicting CITED1 and mCherry expression at days 4, 6 and 8 of reprogramming. Scale bars represent 500 μ m. (C) Brightfield and fluorescence images of test (transfected with reprogramming transposons)

and control (expressing mCherry only) HK2 cells after 8 days of reprogramming. Scale bars represent 500 μm . (D) Immunofluorescence of NP proteins in reprogramming transposon-transfected cells following 8 days of reprogramming. Scale bars represent 30 μm .

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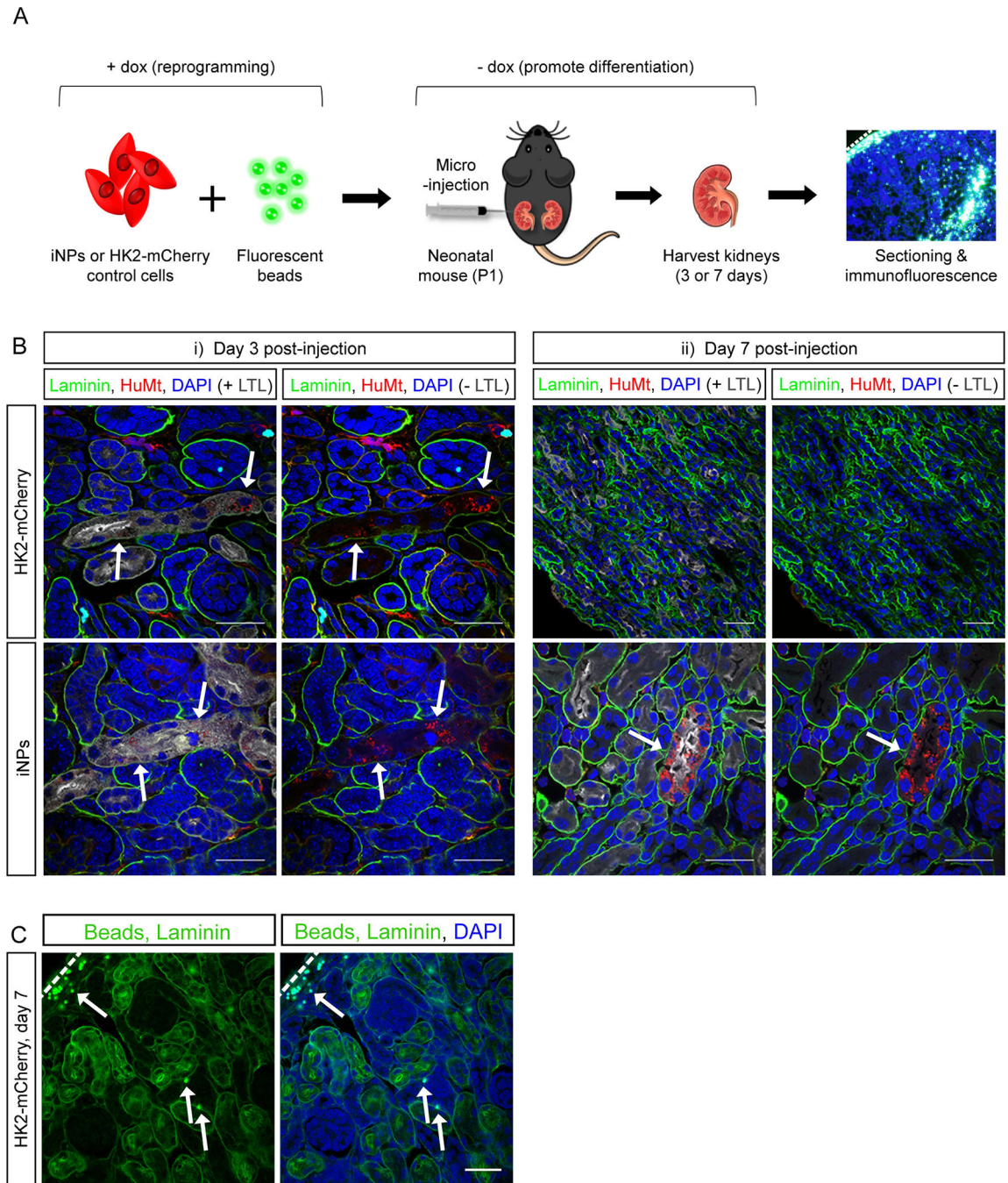


Figure 3: iNP-like cells contribute to nephrons in mouse kidney *in vivo*.

(A) Schematic of the neonatal injection assay used to determine iNP differentiation capacity.

(B) Immunofluorescence of sections through mouse kidneys at 3 and 7 days post-injection with iNP-like cells or control HK2-mCherry cells (stained with HuMt, red). Arrows depict examples of integrated HuMt+ cells or HuMt+ tubules. Scale bars represent 30 μ m.

(C) Immunofluorescence analysis and detection of fluorescence microsphere auto-fluorescence in neonatal kidneys injected with HK2-mCherry control cells and harvested 7 days post-

injection. Arrows depict examples of fluorescent microspheres (green). Scale bars represent 30 μm . Dashed lines in A and C indicate the outer edge of the kidney cortex.

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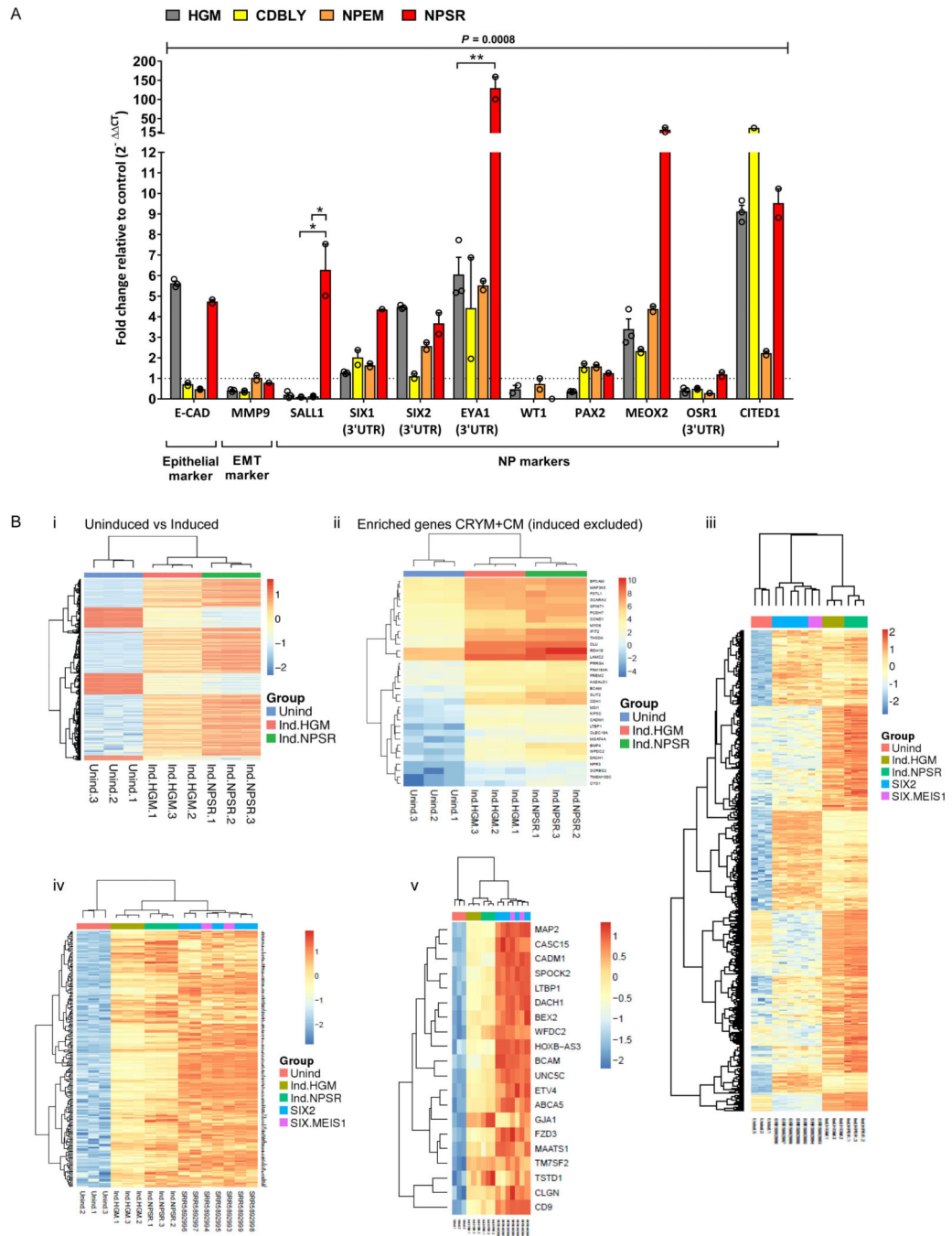


Figure 4: Reprogramming to iNP-like cells is supported by NP-specific maintenance media. (A) qRT-PCR analysis of NP markers in Clone 8-derived iNP-like cells relative to HK2-mCherry control cells in HGM. Data are presented as mean \pm SEM. Statistical significance for each gene across treatment groups was determined using two-way ANOVA and Tukey’s post-test analysis of normalized (C_t) values ($*P$ 0.05, $**P$ 0.01). (B) RNA-Seq analyses comparing uninduced Clone 8 control cells (blue) with induced Clone 8 iNP-like cells cultured in HGM (red) and NPSR (green), with $n = 3$ for each condition, showing (i) TREAT analysis heatmap showing relative expression levels of genes identified as significantly

differentially expressed between the uninduced and induced conditions (\log_2 threshold > 1 , FDR < 0.05), with expression row scaled to a mean of 0 and standard deviation of 1, (ii) heatmap of gene expression (\log_2 counts per million) for cap mesenchyme / Crymenriched cap mesenchyme markers, excluding genes present in the reprogramming transposon (*SIX1*, *EYA1* and *SNAI2*), (iii) heatmap of row scaled gene expression of genes differentially upregulated in human SIX2-enriched NPs and SIX2/MEIS1 interstitial progenitor cells compared to uninduced Clone 8 cells (adjusted p-value < 0.001), and clustered with expression of induced Clone 8 cells, (iv) heatmap of row scaled gene expression of subset of genes that are upregulated in human SIX2-enriched cells compared to uninduced Clone 8 cells that cluster human cells with Clone 8 induced cells, (v) heatmap of row scaled gene expression of genes found in both the subset of genes that cluster human with induced cells, and NP marker genes from single cell analysis of cells from the nephrogenic zone of human fetal kidney.

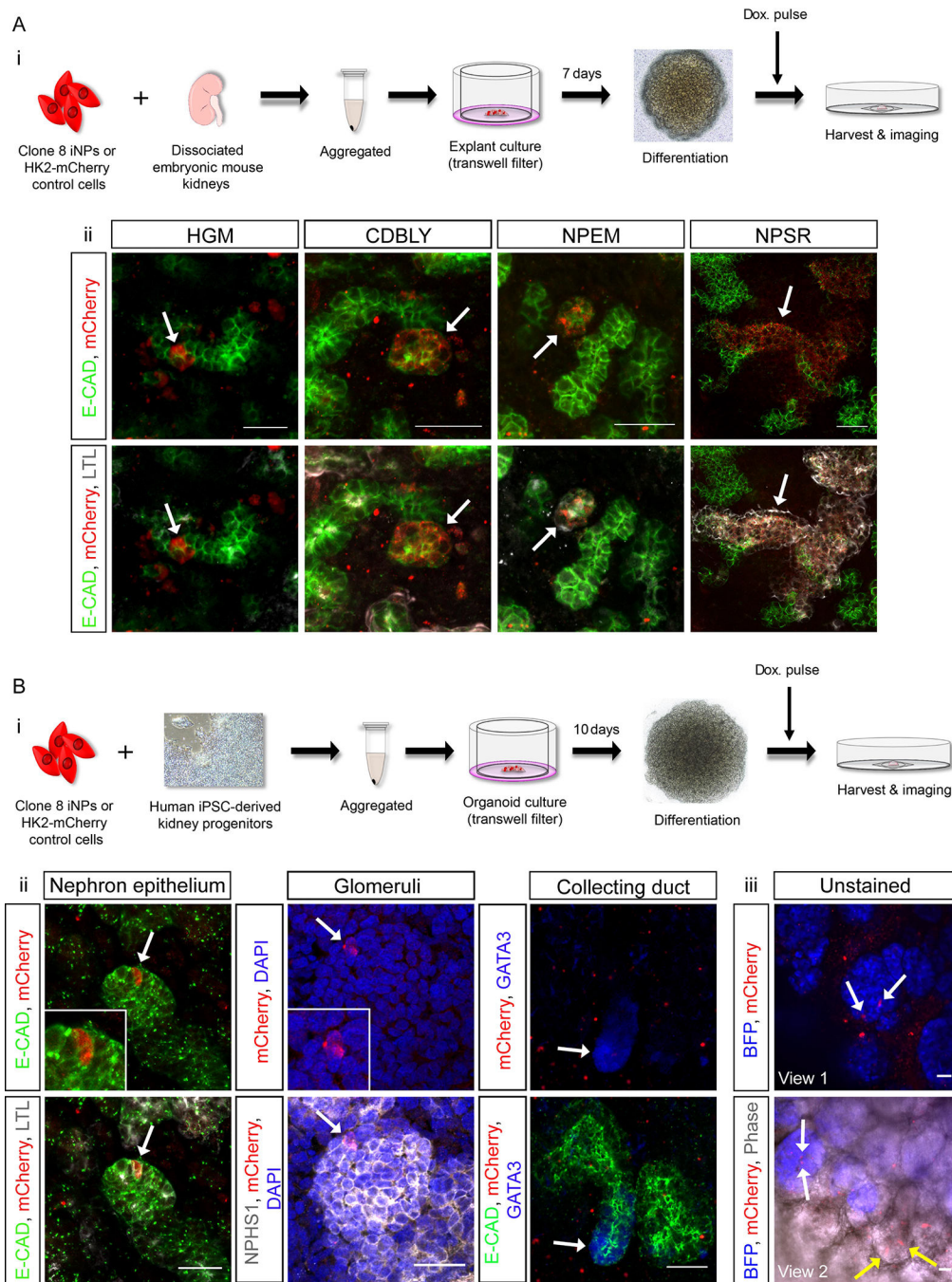


Figure 5: iNP-like cells reprogrammed in HGM or NP-supportive media show evidence of nephron-forming capacity in mouse and human organoids.

(Ai) Schematic of the embryonic mouse kidney organoid assay and (Aii) immunofluorescence demonstrating the contribution of Clone 8 iNP-like cells to developing nephrons. Arrows in images depict examples of integrated iNP-like cells (red). (Bi) Schematic of the human iPSC-derived kidney organoid assay and (Bii) immunofluorescence demonstrating the contribution of NPSR-cultured Clone 8 iNP-like cells (red) to developing ECAD⁺ nephron epithelium (green), NPHS1⁺ glomeruli (grey) and absence from GATA3⁺ collecting duct (blue, arrow). Arrows in left and middle panels depict examples of

integrated iNP-like cells (red; magnified in insets). (Biii) Two fields of view from unstained MAFB^{mTagBFP} iPSC-derived organoids demonstrating integration of iNP-like cells (red) into MAFB⁺ glomeruli (blue) and epithelial structures using endogenous fluorescence of the reporters and phase contrast imaging. Arrows depict examples of iNP-like cells integrated into glomeruli (white arrows) and epithelium (yellow arrows). Scale bars in all panels represent 30 μm .

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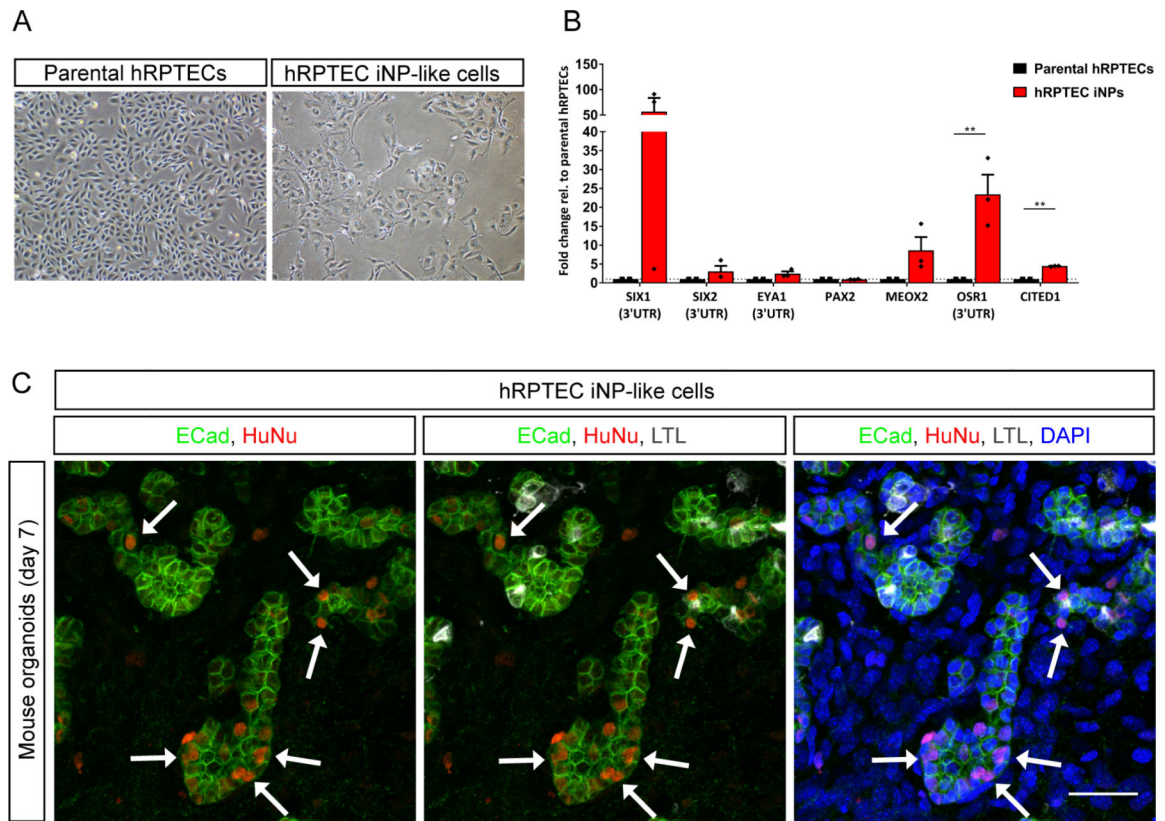


Figure 6: Refinement of reprogramming factors and conditions enables the generation of iNPlike cells from primary human proximal tubule cells (hRPTECs).

(A) Brightfield images of parental (untransfected) hRPTECs and hRPTEC iNPs that have undergone transfection with pT-SES, pT-TetON and pEF1-HA-m7pB followed by reprogramming. (B) qRT-PCR analysis of NP gene expression in hRPTEC iNP-like cells relative to parental hRPTECs. Data are presented as mean \pm SEM. Statistical significance was determined by Student's t-test (** P 0.01, n = 3 biological replicates). (C)

Immunofluorescence of the mouse kidney organoid assay demonstrating the contribution of hRPTEC iNP-like cells to developing nephrons marked by E-Cadherin (ECad; 692 green). Arrows depict examples of integrated hRPTEC iNP-like cells marked by human nuclear 693 antigen (HuNu; red) and DAPI (nuclei; blue). Scale bar represents 30 μ m.