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Kidney versus Liver Specification of SLC and ABC Drug Transporters, Tight Junction Molecules, and Biomarkers

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ABSTRACT

The hepatocyte nuclear factors, Hnf1a and Hnf4a, in addition to playing key roles in determining hepatocyte fate, have been implicated as candidate lineage-determining transcription factors in the kidney proximal tubule (PT) [Martovetsky et. al., (2012) Mol Pharmacol 84:808], implying an additional level of regulation that is potentially important in developmental and/or tissue-engineering contexts. Mouse embryonic fibroblasts (MEFs) transduced with Hnf1a and Hnf4a form tight junctions and express multiple PT drug transporters (e.g., Slc22a6/Oat1, Slc22a12/Urat1, Abcg2/Scrp, Abcc2/Scrp2, Abcc4/Scrp4), nutrient transporters (e.g., Slc34a1/Npi-2, Slc1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2). In contrast, the coexpression (with Slc34a1/NaPi-2, Slco1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2). In contrast, the coexpression (with Slc34a1/NaPi-2, Slco1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2). In contrast, the coexpression (with Slc34a1/NaPi-2, Slco1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2). In contrast, the coexpression (with Slc34a1/NaPi-2, Slco1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2). In contrast, the coexpression (with Slc34a1/NaPi-2, Slco1a6), and tight junction proteins (occludin, claudin 6, ZO-1/Tjp1, ZO-2/Tjp2).

Several hepatocyte markers, including albumin, apolipoprotein, and transferrin. A similar result was obtained with primary mouse PT cells. Thus, the presence of Gat4 and Foxa2/Foxa3 appears to alter the effect of Hnf1a and Hnf4a by an as-yet unidentified mechanism, leading toward the generation of more hepatocyte-like cells as opposed to cells exhibiting PT characteristics. The different roles of Hnf4a in the kidney and liver was further supported by reanalysis of ChIP-seq data, which revealed Hnf4a colocalization in the kidney near PT-enriched genes compared with those genes enriched in the liver. These findings provide valuable insight, not only into the developmental, and perhaps organotypic, regulation of drug transporters, drug-metabolizing enzymes, and tight junctions, but also for regenerative medicine strategies aimed at restoring the function of the liver and/or kidney (acute kidney injury, AKI; chronic kidney disease, CKD).

Introduction

Because of the large number of pediatric and adult patients with kidney and liver disease, there is a great need to devise tissue engineering and regenerative medicine strategies to supplement and/or replace the function of both of these organs. Moreover, since neonates and preterm infants are routinely treated with a wide variety of drugs (i.e., antibiotics, nonsteroidal anti-inflammatory drugs, antivirals, and antihypertensives) whose absorption, disposition, and elimination (and therefore efficacy), as well as potential toxicity, are dependent upon functionally immature kidneys and livers (Kearns et al., 2003), it may be useful to devise strategies to enhance kidney and/or liver function, including transport capacity. For these purposes, it is important to establish cells with characteristics that can partially or fully attain the properties of mature cell types from a cultured cell source. This may require achieving the desired characteristics of the target cell type while silencing/excluding those of other cell types.

Since the advent of induced pluripotent stem cells, differentiating stem cells toward desired cell fates has become a promising strategy for future clinical application (Graf and Enver, 2009). However, due to the tumorigenic potential of stem cells (Lu and Zhao, 2013; Okano et al., 2013; Harding and Mirochnitchenko, 2014), there is an advantage to being able to generate desired cell fates without having to undergo a pluripotent state. There have recently been a number of advances in establishing hepatocyte-like cells from embryonic or mature fibroblasts. In these cases, ectopic expression of lineage-determining transcription factors was used to achieve trans Differentiation (Huang et al., 2011, 2014; Sekiya and Suzuki, 2011; Du et al., 2014; Simeonov and Uppal, 2014). Although the derivation of proximal tubule (PT)-like cells from stem cells has been reported (Narayan et al., 2013), the establishment of PT cell characteristics starting with nonpluripotent cells, or by inducing a defined transcriptional program, is not well understood. Defining how sets of transcription factors guide the differentiation of cell ABBREVIATIONS: ABC, ATP-binding cassette; AKI, acute kidney injury; Alb, albumin; ApoA1, apolipoprotein A; Cdh, Cadherin; ChIP-seq, chip immunoprecipitation followed by high throughput sequencing; CKD, chronic kidney disease; DMEM, Dulbecco’s modified Eagle’s medium; Dsp, desmplakin; FBS, fetal bovine serum; Fox, forkhead box protein; GFP, green fluorescent protein; IHC, immunohistochemistry; MEF, mouse embryonic fibroblast; NEAA, nonessential amino acid supplement; OcIln, occludin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PT, proximal tubule; SLC, solute carrier; Trf, transferrin; Trt, transthyretin; UCSD, University of California, San Diego.

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types that may share some characteristics but ultimately perform very
different essential functions in different organs (e.g., kidney, liver) is
crucial for further refining regenerative medicine and tissue engineering
strategies.

Here, we show that, although Hnf1a and Hnf4a alone are insufficient
to completely transdifferentiate mouse embryonic fibroblasts (MEFs)
toward a proximal tubule cell–like fate, they are capable of inducing the
mRNA expression of a number of genes important for proximal tubule
identity and function, including solute carrier (SLC) and ATP-binding
cassette (ABC) drug transporters, as well as the establishment of tight
junctions. Remarkably, coexpression of either Gata4, a combination of
forkhead box protein A2 and A3 (Foxa2/3), or all three along with Hnf1a
and Hnf4a in MEFs largely eliminated the induction of proximal tubule
markers and, instead, strongly induced the expression of hepatocyte
markers. In a similar manner, Gata4 and Foxa3 downregulated PT
markers and induced expression of hepatocyte markers in primary
PT cells (which endogenously express low levels of Hnf1a and Hnf4a).
Together, these findings outline a foundation for transdifferentiation
between proximal tubule-like cells and help to clarify the involvement
of Hnf1a and Hnf4a in transdifferentiation toward hepatocyte cellular
identity. The results may be relevant to our understanding of proximal
tubule and hepatocyte development and terminal differentiation as well
as to regenerative medicine contexts.

Materials and Methods

Animals, MEF Isolation, and Cell Culture. All animal procedures were
approved by the University of California, San Diego (UCSD) Institutional
Animal Care and Use Committee. E16.5 MEFs were prepared as previously
described (Martovetsky et al., 2013), whereas E13.5 and E15.5 MEFs were
made using a modified procedure. In brief, wild-type pregnant female mice on a
129-C57Bl/6 mixed background that were housed under basal conditions
with a 12-hour light/dark cycle with ad libitum access to food/water were
sacrificed at 13.5, 15.5, and 16.5 days of gestation (day 0 of gestation corre-
sponds to visualization of the vaginal plug). Uteri were removed. Unsexed
embryos were isolated, and tissues (minus head and viscera) were minced
in phosphate-buffered saline (PBS) in a culture dish. The PBS was then
replaced with 0.25% Trypsin/EDTA containing DNase and incubated at 37°C
for 10 minutes, triturated, and then incubated another 10 minutes. Dulbecco’s
modified Eagle’s medium (DMEM/F-12 medium with 10% fetal bovine serum
(FBS), nonessential amino acid supplement (NEAA), and penicillin/streptomycin
(media A) was then added to quench the reaction (this was the same medium later
used for cell culture), and the suspension was transferred to a 50-ml conical tube
and gravity pellet for 1 minute to allow the undigested pieces to settle. All of the
suspension was then plated into collagen I–coated six-well plates. After 2 days, medium was replaced with medium B, cells were infected with lentivirus for 8 hours, and medium was replaced with fresh medium B. Because primary cells can be difficult to transduce,
the number of different transducing factors was kept to a minimum; thus, since
these primary PT cells can produce some level of endogenous Hnf1a and Hnf4
(see Resul), and since studies have shown that either Foxa2 or Foxa3 alone can
induce hepatocyte-like characteristics in the presence of Hnf4a (with Foxa3
appears to be slightly more robust) (Huang et al., 2011; Sekiya and Suzuki, 2011;
Yu et al., 2013), these cells were transduced with either Gata4, Foxa3, or a
combination of these two factors. Medium was changed again 3 days after
infection. RNA was collected for quantitative reverse-transcription polymerase
chain reaction (PCR) analysis 6 days after infection.

Quantitative Reverse-Transcription PCR. RNA extraction, cDNA prepa-
ration, and quantitative reverse-transcription PCR was carried out as previously
described (Martovetsky et al., 2013). The list of primer sequences is included
(Supplemental Table 1). Significance was determined using the raw values with
GraphPad Prism 6 (GraphPad Software, La Jolla, CA) using a one-way analysis
of variance with default settings (α value set to 0.05) followed by the Tukey post-hoc
analysis.

Immunohistochemistry/Microscopy. Phase microscopy was conducted us-
ing a Nikon Eclipse TE3000 microscope (Nikon Instruments, Melville, NY) with
an attached Nikon DS50 camera. Fluorescent microscopy of GFP-positive cells
and immunostained cells was carried out using a Zeiss Axio Observer A1
microscope (Zeiss USA, Thornwood, NY). Immunostaining was carried out as
previously described, with slight modifications (Martovetsky et al., 2013). Cells
were fixed in 4% formaldehyde in PBS overnight at 4°C. Fixation was quenched
with 50 mM glycine in PBS for 30 minutes at room temperature. Cells were then
blocked with 10% bovine serum albumin in PBS containing 0.1% Tween 20 and
0.05% Triton X-100 for 1 hour at room temperature. Next, cells were incubated
with a 1:250 dilution of anti–ZO-1 (TJP1) antibody (Invitrogen; 33-9100)
overnight at 4°C in PBS containing 0.1% Tween 20 and 0.05% Triton X-100 with
2% bovine serum albumin [immunohistochemistry (IHC) buffer]. Cells were then
washed with IHC buffer three times for at least 1 hour per wash at room
temperature, and then incubated with secondary antibody (anti-mouse Alexa
Fluor 594; Life Technologies, Carlsbad, CA) overnight at 4°C. Cells were then
washed three times for at least 1 hour per wash with IHC buffer at room
temperature, and then covered with a coverslip within the tissue culture plate
using Fluoromount-G (Electron Microscopy Services, Hatfield, PA). ImageJ software
(National Institutes of Health, Bethesda, MD) was used for image processing.

Microarray analysis. For the analysis of expression in embryonic and adult
isolated proximal tubules and liver tissue, publicly available mRNA expression
data were used (Supplemental Table 2): GSE6290–GSM144594–144595 (E15.5
CD1 mouse PT), GSM6889–GSM152247–152249 (E15.5 CD1 mouse PT),
GSE10162–GSM256959–256961 (adult C57Bl/6 mouse PT), GSM10162–
GSM256959-256961 (adult C57bl/6 mouse PT), GSE7342–GSM177040-177042 (E15.5 C57bl/6 mouse liver), GSE11899–GSM300676-300680, GSE8969–GSM227410-227412, GSE32354–GSM801178-801182 (adult C57bl/6 mouse liver). To facilitate comparisons between samples generated by different laboratories, different mouse strains, and at different ages, the samples were normalized using the robust multi-array average algorithm. Probes that did not have a present flag in more than half of the samples in at least one of the four conditions as determined by the MA55 algorithm were discarded. A moderated t test with a Benjamini-Hochberg multiple test correction was used to identify genes that are differentially expressed by at least 100-fold (P < 0.05) between either E15.5 PT and E15.5 liver, adult PT and adult liver, or in both E15.5 and adult tissues. The combined list of resulting genes was used to perform hierarchical clustering using default settings in GeneSpring (Agilent Technologies, Santa Clara, CA).

Chromatin Immunoprecipitation Sequencing. Chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) for Hnf4a and p300 (GSE50815) has been previously published (Martovetsky et al., 2013), and relevant reanalyses were performed here. In that study, the chromatin was prepared from adult Sprague-Dawley rat whole kidneys and kidney cortex, and the ChIP analyses were performed in duplicate using either 4 μg of anti-HNF4a antibody (sc-8987; Santa Cruz Biotechnology, Dallas, TX) or 10 μg of anti-p300 antibody (sc-585; Santa Cruz Biotechnology). The antibody-bound complexes were then recovered using a mix of preblocked protein A and protein G beads that were washed and eluted with SDS-containing buffer. DNA was purified and libraries were prepared with the ChIP-Seq DNA Sample Prep Kit (Illumina, Inc., San Diego, CA) using either pooled duplicates (the ChIP samples) or 50 ng for the inputs. The HiSeq. 2000 instrument (Illumina, Inc.) was used to sequence amplified DNA fragments (200–400 bp long) which were aligned to the m4 genome by BIOGEM (Genomics Data Analysis Services, UCSD) according to the standard Illumina pipeline.

The HOMER v3.13 software package (UCSD; Heinz et al., 2010) was used for further analysis. Clonal reads were removed, and default settings designed for ChIP-seq analysis were used to define and annotate peaks and calculate measures for quality control. The UCSC genome browser was used to generate screenshots of Hnf4a and p300 binding at specific genes of interest. To quantify Hnf4a peaks in adult rat kidneys associated with either PT-enriched or hepatocyte-enriched genes, peaks were assigned to genes if their transcription start site was the nearest annotated transcription start site as opposed to any other protein-coding gene. Total number of peaks per gene were then quantified and graphed with a box-whisker plot in GraphPad Prism 6. Significance was calculated with a two-tailed t test.

Results

Using developmental transcriptomic and ChiP-seq data as a guide (discussed later), as well as the literature on stem cell differentiation, we sought to define the transcriptional program that leads to the expression of kidney proximal tubule versus hepatocyte SLC and ABC drug/solute transporters, tight and other junction molecules, as well as biomarkers considered selective for one cell type or the other.

Ectopic expression of Hnf1a and Hnf4a in MEFs leads to the mRNA expression of many PT-expressed SLC and ABC transporters and junctional component genes with differential contributions of the two transcription factors. We have previously shown that hepatocyte nuclear factors Hnf1a and Hnf4a play a role in regulating drug-metabolizing enzymes and transporters in the kidney (Martovetsky et al., 2013). However, Hnf1a and Hnf4a, without additional factors, have also both been used in reprogramming fibroblasts toward a hepatocyte-like phenotype (Huang et al., 2011, 2014; Sekiya and Suzuki, 2011; Du et al., 2014; Simeonov and Uppal, 2014). With this in mind, we set out to further examine the potential of Hnf1a and Hnf4a to induce expression of proximal tubule–enriched versus liver-enriched genes (Fig. 1).

To better gauge the extent of transdifferentiation toward PT cell fate by Hnf1a and Hnf4a overexpression, a cohort of genes involved in influx/efflux transport of drugs/solutes and junction formation (Denker...

Fig. 1. Lentiviral transduction of MEFs followed by quantitative reverse-transcription PCR (RT-qPCR) determination of changes in gene expression for selected PT markers. (Left panel) MEFs isolated from different days of gestation (i.e., E13.5, E15.5, and E16.5) were transduced without (uninfected) or with lentivirus expressing either GFP or Hnf1a and Hnf4a (either singly or in combination). (Right panel) The expression of several markers of the kidney proximal tubule [i.e., transmembrane transporters, Slc22a6 (Oat1), Slc34a1 (NaPi-2a), and Slc47a1 (Mate1), as well as the PT brush border marker Ggt1] was determined by quantitative reverse-transcription PCR. The graph on the bottom right shows the induction of several of these markers upon transduction of the MEFs with Hnf1a and Hnf4a; mean ± S.E.M. (n = 3) (Supplemental Fig. 1). Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
and Nigam, 1998), both defining characteristics of PT cells and relevant to their physiologic function, were examined. In all instances, when MEFs were transduced with both Hnf1a and Hnf4a, the transcription of several prominent proximal tubule markers [selected based on literature and various expression databases and later experimentally confirmed (discussed later)], including Slc22a6 (also known as Oat1 or NKT), Slc34a1, Slc47a1 (Mate1), and γ-glutamyltransferase 1 (Ggt1—a PT brush border marker), were markedly upregulated compared with MEFs that were either uninfected or transduced with GFP alone (Fig. 1). Interestingly, in two of the populations (E13.5 and E15.5, which were prepared slightly differently from the E16.5 MEFs, as described in Materials and Methods), ectopic expression of Hnf4a induced the expression of endogenous Hnf1a, albeit at lower levels than when transduced with Hnf1a (Supplemental Fig. 1). This level of Hnf1a appeared to be sufficient to complement exogenously expressed Hnf4a in inducing the expression of the PT-enriched markers. Nonetheless, the results are consistent with the finding that Hnf1a and Hnf4a act synergistically in the induction of several key proximal tubule genes upon transduction into MEFs (Martovetsky et al., 2013). Because of the opportunity to analyze the roles of Hnf1a and Hnf4a separately as well as together, we further characterized the response in the E16.5 MEFs, in which Hnf4a did not induce endogenous Hnf1a expression.

The transport of drugs, metabolites, and waste products by the postnatal, juvenile, and adult proximal tubule relies on a system of influx and efflux transporters on the basolateral (interacting with
Induction of multiple intercellular junctional components in MEFs transduced with Hnf1a and Hnf4a. To test induction of junctional components, the same MEFs were used as in Fig. 2. (A) Simplified schematic shows which junctional features correspond to the tested genes. (B) Expression of three cadherin genes (Cdhd1, Cdhd6, and Cdhd26) associated with adherens junctions upon transduction. (C) Expression levels of three desmosomal genes—desmocolin 2 (Dsc2), desmoglein 2, and desmoplakin (Dsp)—upon transduction. (D) Expression of several types of genes involved in tight junction formation—Cldn6, Cldn9, Cldn12, Ocln, tight junction proteins (Tjp1 and Tjp2, which are also known as ZO-1 and ZO-2), and epithelial polarity regulator Par6βb. The mean ± S.E.M. for each tested gene is depicted (n ≥ 3); significance of differential expression is summarized in Table 1. For convenience, results for genes within similar families are shown on a single graph. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
genes, along with their fold change in expression in MEFs upon Hnf1a and Hnf4a transduction as well as their levels of endogenous expression in embryonic and adult proximal tubules and liver tissue, is included (Supplemental Table 3). In other words, whereas MEFs transduced with Hnf1a and Hnf4a began expressing PT-enriched genes, many of the other transcriptionally responsive genes are shared between PT cells and hepatocytes. Furthermore, some of the tested genes that are endogenously expressed in both PT cells and hepatocytes in adult mouse kidney cortex and liver tissue were examined at embryonic and adult time points. When we limited to genes that exhibit more than a 100-fold change in expression between either embryonic or adult PT and liver samples, or both, we derived a list of candidate markers that could be used to distinguish between the two tissues, which included multiple widely used markers for both PT cells and hepatocytes (Fig. 5A). We validated a number of selected markers by testing their expression in adult mouse kidney cortex and liver tissue (Fig. 5B). As predicted, Mate1, Oat1, NaPi-2a, and Ggt1 had a much higher expression in the kidney cortex compared with the liver, whereas markers such as transferrin (Tf), transfthretin (Ttr), apolipoprotein A (Apoa1), albumin (Alb), Fabp1, and Serpin1 had a much higher expression in liver compared with the kidney cortex.

**Transduction of Hnf1a and Hnf4a alters the morphology of MEFs and induces formation of tight junctions.** Whereas MEFs transduced with Hnf1a and Hnf4a began expressing proximal tubule markers, another distinct property of mature proximal tubule cells is their epithelial phenotype. We had previously shown that transduction of Hnf1a and Hnf4a leads to upregulation of mRNA expression for Cdh1, an adherens junction marker, and Tjp1 (also known as ZO-1 or zonula occludens-1), a tight junction marker, both of which are present in mature PT cells (Martovetsky et al., 2013). However, when the E13.5 or E15.5 MEFs were in culture for a week or more after transduction, we achieved substantially higher levels of expression (Fig. 4C). Furthermore, additional components of intercellular junctions were upregulated, including Ocnl, Tjp2, and Cldn6 (Fig. 4C). Whereas other claudins tested also exhibited some transcriptional response, Cldn6 (which is endogenously expressed in developing and postnatal PTs but downregulated in mature PTs) was the most upregulated (Supplemental Table 3); this result suggests that, although MEFs transduced with Hnf1a and Hnf4a have not acquired mature PT-like cell properties, they do resemble immature PT-like cells to some extent. In addition to the upregulation of multiple junctional markers, the mesenchymal marker vimentin was downregulated in response to transduction (Fig. 4D). When an extracellular matrix was provided, the transduced MEFs that were cultured on collagen-coated plates for 3 weeks had extensive formation of epithelial sheets with Tjp1 localized to the cellular junctions, indicative of tight junction formation in MEFs transduced with Hnf1a and Hnf4a, but not control MEFs (Fig. 4, E–H).

**Liver lineage-determining factors Gata4 and Foxa2 and/or Foxa3 act as a transcriptional switch to redirect Hnf1a and Hnf4a activity from regulating PT-enriched genes to liver-enriched genes.** Although Hnf1a and Hnf4a might not be sufficient to fully transdifferentiate MEFs toward a proximal tubule fate, our results suggested that they are indeed “lineage-determining TFs” (Heinz et al., 2010) for proximal tubule cells, at least in the context of the SLC and ABC drug transporters and junctional molecules examined. To gain a deeper perspective of the shared and defining characteristics of PT cells and hepatocytes, the transcriptomes of isolated proximal tubules and liver tissue were examined at embryonic and adult time points. When we limited to genes that exhibit more than a 100-fold change in expression between either embryonic or adult PT and liver samples, or both, we derived a list of candidate markers that could be used to distinguish between the two tissues, which included multiple widely used markers for both PT cells and hepatocytes (Fig. 5A). We validated a number of selected markers by testing their expression in adult mouse kidney cortex and liver tissue (Fig. 5B). As predicted, Mate1, Oat1, NaPi-2a, and Ggt1 had a much higher expression in the kidney cortex compared with the liver, whereas markers such as transferrin (Tf), transfthretin (Ttr), apolipoprotein A (Apoa1), albumin (Alb), Fabp1, and Serpin1 had a much higher expression in liver compared with the kidney cortex. We also examined the genes deriving from the analysis shown in Fig. 5A in the context of existing Hnf4a ChIP-seq data (Martovetsky et al., 2013). Importantly, we found that in samples of adult rat kidney, the selected PT marker genes were highly bound by Hnf4a, whereas the selected hepatocyte markers were almost completely devoid of Hnf4a binding in this tissue (Fig. 6). In addition, comparison of p300 binding in adult rat kidney cortex was found to be highly similar to that seen for Hnf4a in adult kidney for all of the selected kidney and liver marker genes (Fig. 6).

We then set out to determine what effect coexpression of Gata4, Foxa2, and Foxa3 with Hnf1a and Hnf4a would have on proximal tubule and hepatocyte transcriptional signatures. Because previous reports have shown that both Foxa2 and Foxa3 had the strongest effects in
transdifferentiation protocols compared with Foxa1, we decided to use them in combination to activate transcription mediated by the Foxa family. We found that coexpression of either Gata4 or Foxa2/3 significantly downregulated the proximal tubule markers Slc22a6, Slc34a1, and Slc47a1, while dramatically upregulating hepatocyte markers Alb, Apoa1, Fabp1, Serpina1, Trf, and Ttr (Fig. 7, B and C; Table 3). While Gata4 coexpression had a strong inhibitory effect on proximal tubule marker expression, it was insufficient to upregulate liver markers regardless of Hnf4a and Hnf1a presence (Fig. 7, B and C; Table 3). In contrast, whereas Foxa2 and Foxa3 were capable of downregulating PT marker expression, they also appeared to be the main drivers of hepatocyte marker expression in the presence of Hnf1a and Hnf4a (Fig. 7, B and C; Table 3).

Finally, we tested the ability of Gata4 and Foxa3 to induce the expression of hepatocyte markers in primary mouse proximal tubule cells (Fig. 8; Table 4). These cells expressed some level of endogenous Hnf1a and Hnf4a, as well as multiple PT markers. Upon Gata4 transduction, several PT markers were downregulated (Slc34a1, Slc47a1, kidney androgen-regulated protein, and Ggt1) (Fig. 8B). Of the tested hepatocyte markers, only Trf expression was modestly induced (Fig. 8C). In contrast, Foxa3 overexpression not only had a repressive effect on kidney androgen-regulated protein but also strongly induced the expression of a number of hepatocyte markers (Alb, Apoa1, Trf, Serpina1, and Ttr), with Alb and Trf appearing to respond synergistically to Gata4 and Foxa3 coexpression (Fig. 8C). Although the transcriptional response in primary PT cells was more modest in scale compared with those observed in MEFs (Fig. 7, B and C), this might be due to the reduced plasticity of terminally differentiated cell types compared with embryonic fibroblasts.

Nevertheless, these findings further supported the idea that Hnf1a and Hnf4a serve as a foundation for proximal tubule and hepatocyte transcriptomes, but require additional inputs to establish tissue-specific expression. In the absence of additional hepatocyte lineage-determining factors, Hnf1a and Hnf4a induce expression of genes common to both PT cells and hepatocytes, with an apparent bias toward well described PT-specific genes. In cells expressing Hnf1a and Hnf4a, Gata4, Foxa2, and Foxa3 downregulate key PT genes (with Gata4 having the stronger repressive effect) and cooperate with
Hnf1a and Hnf4a to induce hepatocyte-specific gene expression (with Foxa2/3 playing the major role in gene induction, synergized by Gata4 in some cases).

**Discussion**

Kidney proximal tubule cells and hepatocytes are both involved in the transport and metabolism of many drugs and toxins as well as metabolites. This requires establishment of a permeability barrier (mediated by tight junctions), expression of appropriate ABC and SLC drug and solute transporters, as well as drug-metabolizing enzymes. For example, the major transporter of many organic anion drugs, toxins, and metabolites, OAT1 (originally identified as NKT) must be expressed on the basolateral surface of the proximal tubule cell (Lopez-Nieto et al., 1997; Nigam et al., 2015b; Wu et al., 2015; Zhu et al., 2015); when this gene is deleted, there is considerable loss of renal transport of many organic anion drugs, toxins, and metabolites (Eraly et al., 2006; Truong et al., 2008; Nagle et al., 2011; Torres et al., 2011; Wikoff et al., 2011). Although there is some commonality of expressed genes, they are often differentially expressed. Other genes appear to be largely expressed in the kidney or in liver, and some of these are considered signature genes.

The transcriptional program regulating the development and differentiation of cells toward the proximal tubule as opposed to hepatocytes is only beginning to be defined; understanding this in detail is of relevance to organ development and maturation as well as regenerative medicine. Our experimental results seem generally consistent with developmental

![Fig. 5](image-url). Systems-level analysis of PT and liver expression aid in selection of differential markers. (A) Hierarchical clustering of genes with at least a 100-fold change between either embryonic isolated proximal tubules compared with embryonic liver or adult isolated proximal tubules compared with adult liver (all mouse tissues). This analysis identified most (but not all) of the markers used for further experiments to differentiate between PT-like and hepatocyte-like cellular identity. (B) The PT markers used in this study—Slc47a1 (Mate1), Slc22a6 (Oat1), and Slc34a1 (NaPi-2a)—are highly enriched in the kidney cortex, where proximal tubule cells comprise more than half of the cellular content. Conversely, hepatocyte markers Fabp1, Trf, Tr, Apoa1, Serpina1, and Alb are highly expressed in the liver and negligibly expressed in the PT. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

![Fig. 6](image-url). Hnf4a binding events revealed by ChIP-seq colocalize near PT-enriched genes in the kidney while rarely binding near liver-enriched genes. (A) When Hnf4a peaks in adult rat kidney are assigned to genes based on the nearest transcription start site, on average there are more than 3 times as many peaks associated with highly PT-enriched genes compared with highly liver-enriched genes (derived from analysis depicted in Fig. 5A comparing embryonic and adult liver versus embryonic and adult isolated PTs). Whiskers set at 10th and 90th percentiles. (B) Previous p300 and HNF4a ChIP-seq data (Martovetsky et al., 2013) were re-examined for determining the binding of these transcription factors to selected kidney and liver marker genes in adult rat kidney cortex or proximal tubules. Shown are examples for the PT marker, Slc34a1 (NaPi-2a), and the liver marker, Alb. Patterns similar to Slc34a1 were also seen for the PT markers Slc22a6 (Oat1) and Slc47a1 (Mate1) (Martovetsky et al., 2013), whereas patterns similar to Alb were also seen for the liver markers fatty acid binding protein 1 (Fabp1), serpin peptidase inhibitor clade 1 (Serpina1), Trf, and Trt.
analyses of gene expression (Fig. 5) and ChIP-seq data (Fig. 6). To summarize, we have shown that, despite the use in the literature of Hnf1a and Hnf4a along with other factors in transdifferentiation toward hepatocyte-like cells, these two transcription factors are also at the core of proximal tubule gene expression. Overexpression of Hnf1a and Hnf4a in MEFs induced the expression of several key markers of proximal tubule cellular identity (Fig. 1). We also found that Hnf1a and Hnf4a induced the expression of a number of genes essential to intercellular junctions (tight, adherens, desmosomal), as well as apical and basolateral transporters of small solutes, such as drugs and metabolites (Figs. 2 and 3), which would be expected to be necessary for vectorial transport in the PT in vivo. MEFs transduced with these transcription factors and cultured on collagen I for 3 weeks revealed immunocytochemical evidence of tight junction formation around the full perimeter of cells in epithelial sheets (Fig. 4). Crucially, our studies indicate that, without the coexpression of additional hepatocyte lineage–determining transcription factors, such as Gata4, Foxa2, and Foxa3, the transactivation specificity of Hnf1a and Hnf4a is insufficiently defined toward hepatocytes and, indeed, may lean toward a proximal tubule cell expression signature (Fig. 7; Table 2). Thus, these additional transcription factors (Gata4, Foxa2/3) may be viewed as altering the direction of transdifferentiation from a cell expressing some PT markers to a more hepatocyte-like cell.

Apart from their potential relevance to kidney and liver development and maturation, our results may have translational importance, as the kidney and liver are major targets for a variety of cell-based tissue engineering and regenerative medicine approaches. These approaches

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<th>Gene Symbol</th>
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<th>Hnf1a + Hnf4 + Foxa2/3 versus</th>
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The statistical significance of the differential expression as determined by one-way ANOVA and Tukey’s post-hoc analysis of the various genes is shown (*No significant difference in expression; $^{*}P \leq 0.05; ^{**}P \leq 0.01; ^{***}P \leq 0.005$).

Column 1 is uninfected MEFs versus those expressing Hnf1 + Hnf4; column 2 is uninfected MEFs versus those expressing Hnf1 + Hnf4 + Gata4; column 3 is uninfected MEFs versus those expressing Hnf1 + Hnf4 + Foxa2/3; column 4 is uninfected MEFs versus those expressing Hnf1 + Hnf4 + Gata4 + Foxa2/3; column 5 is MEFs expressing Hnf1 + Hnf4 versus those expressing Hnf1 + Hnf4 + Gata4; column 6 is MEFs expressing Hnf1 + Hnf4 versus those expressing Hnf1 + Hnf4 + Foxa2/3; column 7 is MEFs expressing Hnf1 + Hnf4 versus those expressing Hnf1 + Hnf4 + Gata4 + Foxa2/3; column 8 is MEFs expressing Hnf1 + Hnf4 + Gata4 + Foxa2/3 versus those expressing Hnf1 + Hnf4 + Foxa2/3; column 9 is MEFs expressing Hnf1 + Hnf4 + Gata4 + Foxa2/3 versus Hnf1 + Hnf4 + Gata4 + Foxa2/3; and column 10 is MEFs expressing Hnf1 + Hnf4 + Foxa2/3 versus those expressing Hnf1 + Hnf4 + Gata4 + Foxa2/3.
Table 4: Significance (P value) of differential expression in Fig. 8 determined by one-way analysis of variance and Tukey’s post-hoc analysis.

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The statistical significance of the differential expression as determined by one-way ANOVA and Tukey’s post-hoc analysis of the various genes is shown (No significant difference in expression; *P ≥ 0.05; **P ≥ 0.01; ***P ≥ 0.005).

Fig. 8. Overexpression of Gata4 and Foxa3 in primary proximal tubule cells downregulates PT marker expression and induces expression of liver marker genes. (A) Experimental design of primary proximal tubule cell transduction. (B and C) Transduction of primary PT cells with Gata4 results in downregulation of PT marker expression. Transduction of these cells with Foxa3 results in weaker downregulation of PT marker expression compared with Gata4, but leads to pronounced transcriptional induction of liver markers, an effect sometimes increased by coexpression of Gata4 (C). The mean ± S.E.M. for each tested gene is depicted (n ≥ 3); significance of differential expression is summarized in Table 4. For convenience, results for marker genes for the proximal tubule or hepatocytes are each shown on a single graph. Gapdh, glyceraldehyde-3-phosphate dehydrogenase. RT-qPCR, quantitative reverse-transcription PCR.
genes compared with hepatocyte signature genes (Fig. 6). This implies tissue-specific activity of Hnf4a directed at regulating PT-enriched genes in the kidney. Nevertheless, our lentiviral transduction data indicated that Hnf4a, even together with Hnf1a, was insufficient to fully specify a proximal tubule phenotype. For example, whereas certain PT transcripts were strongly increased (e.g., Oat1 or SLC22a6) and tight junctions were formed (Fig. 4), other genes that are considered to be indicators of the PT were not expressed or were induced at very low levels. This indicates that Hnf1a and Hnf4a likely require additional factors to fully specify PT cell fate. This is consistent with published studies that Foxa and Gata4 transcription factor binding often colocalizes with Hnf4a in hepatocytes, suggesting that their presence might partially determine Hnf4a binding sites. Indeed, it has been reported that both Gata and Foxa transcription factors are “pioneering transcription factors” (Zaret and Carroll, 2011), meaning that they can access condensed chromatin and establish binding sites de novo; thus, they may alter Hnf4a specificity not only by altering the functionality of enhancers established by Hnf4a through recruitment of coregulators and transcriptional machinery, but also by establishing new binding sites that are otherwise inaccessible to Hnf4a alone.

Thus, we have shown that, whereas Hnf1a and Hnf4a are lineage-determining factors for both proximal tubule cells and hepatocytes, the specificity toward either lineage appears to be affected by coexpression of Gata4 and Foxa2/3 (and possibly other factors that were not tested). Future studies should aim at identifying additional coregulators that may need to be added or silenced to achieve complete transdifferentiation toward one cell lineage or the other. Together, these findings advance the understanding of the transcriptional basis of proximal tubule cell differentiation and function, and clarify how two transcription factors central to both hepatocytes and PT cell fate can be guided toward divergent specificity by other coregulators. The results seem generally compatible with transcriptomic and ChIP-seq data during organ development (Figs. 5 and 6). Our results may also contribute to the future development of therapeutic strategies to enhance PT function and regeneration (Figs. 5 and 6) as well as tissue engineering (reviewed in Nigam, 2013; Martovetsky and Nigam, 2014).

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Authorship Contributions
Participated in research design: Martovetsky, Nigam.
Conducted experiments: Martovetsky.
Performed data analysis: Martovetsky.
Wrote or contributed to the writing of the manuscript: Martovetsky, Bush, Nigam.

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