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The Role of Hepatocyte Nuclear Factors 1a and 4a (Hnf1a and Hnf4a) in the Specification and Transcriptional Regulation of the Kidney Proximal Tubule

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The Role of Hepatocyte Nuclear Factors 1a and 4a (Hnf1a and Hnf4a) in the Specification and Transcriptional Regulation of the Kidney Proximal Tubule

A dissertation submitted in partial satisfaction of the Requirements for the degree Doctor of Philosophy in Biomedical Sciences with a Specialization in Multi-Scale Biology by Gleb Nicolai Martovetsky

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2016
The Dissertation of Gleb Nicolai Martovetsky is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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Kevin T. Bush and Sanjay K. Nigam in Drug Metabolism and Disposition, 2016. The dissertation author is the primary author, and Sanjay K. Nigam is the senior author.
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ABSTRACT OF THE DISSERTATION

The Role of Hepatocyte Nuclear Factors 1a and 4a (Hnf1a and Hnf4a) in the Specification and Transcriptional Regulation of the Kidney Proximal Tubule

by

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Doctor of Philosophy in Biomedical Sciences with a Specialization in Multi-Scale Biology

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The proximal tubule (PT) is responsible for more than half of the water reabsorption, recovery of organic solutes, and practically all of the clearance of drugs and metabolites within the kidney. Understanding the transcriptional regulation of PT cells is of major biological and clinical importance, yet much remains to be known. This dissertation attempts to address this problem using multiple molecular, cellular and
systems biological approaches. Chapter 2 explores the regulation of drug metabolizing enzyme and transporter (collectively referred to as DMEs) expression in the PT, predominantly focusing on the role of hepatocyte nuclear factor 4a (Hnf4a). Systems analysis revealed hepatocyte nuclear factors Hnf1a and Hnf4a as being potential regulators of DME expression in the PT. Examining genomic localization of Hnf4a and enhancer-associated protein E1A binding protein p300 (p300) by ChIP-sequencing provided additional evidence that Hnf1a and Hnf4a are candidate lineage-determining transcription factors for the proximal tubule cellular identity. A small molecule Hnf4a antagonist was used to show that Hnf4a is required for the expression of multiple DMEs in the kidney proximal tubule in an ex vivo kidney organ culture model. Finally, ectopic expression of Hnf1a and Hnf4a in mouse embryonic fibroblasts (MEFs) was used to show that Hnf1a and Hnf4a can induce the transcription of a number of important DMEs, and established functional organic anion transport capacity – a specific property of the PT. Chapter 3 further explores the capacity of Hnf1a and Hnf4a to establish a PT cell-like identity. We show that while transducing MEFs with Hnf1a and Hnf4a does not completely transdifferentiate them to PT-like cells, they do form tight-junctions in culture and express a broad array of transporters and junctional components, both important characteristics of PT cells. We then show that three hepatocyte lineage-determining factors Gata4, Foxa2 and Foxa3 repress the induction of PT signature genes by Hnf1a and Hnf4a, and in turn upregulate the expression of hepatocyte signature genes, thus identifying a transcriptional switch that appears to help confer tissue-specific roles of Hnf1a and Hnf4a.
CHAPTER 1:

Introduction

1.1 THE PROXIMAL TUBULE

1.1.1 Role in Solute Transport

The kidney is a vital organ involved in maintaining homeostasis. The functional unit of the kidney is considered to be the nephron, which is composed of the glomerulus, proximal tubule, loop of Henle, and distal tubule. The proximal tubule (PT), while unique as a single entity, is composed of the convoluted and straight tubules, which can further be partitioned into the S1, S2, and S3 segments based on gradients of metabolic and transport activity. The proximal tubule is integral to many processes of the nephron, including the majority of organic solute reabsorption, and more than half of the water and electrolyte reabsorption. The PT is also capable of endocytosis of albumin-bound molecules and secretion of signaling molecules, namely erythropoietin. In addition, the proximal tubule plays an essential role in clearing many widely-administered pharmaceuticals (e.g. NSAIDs, diuretics, antivirals, antibiotics), exogenous toxins and harmful endogenous metabolites from surrounding capillaries, which cannot pass through the slit diaphragm of the glomerulus.

These PT transport functions rely on an extensive system of apical (side of the PT cell interacting with glomerular filtrate in the lumen of the nephron) and basolateral (side of the PT cell interacting with the blood via peritubular capillaries) transmembrane transporters, many of which in the kidney are exclusively expressed in the proximal
tubule. One example of a transporter key to PT function is the organic anion transporter 1 (Oat1 or Slc22a6), which is one of several basolateral transporters that are responsible for the uptake of small anions, including common drugs, metabolic byproducts and signaling molecules, into PT cells. The negatively charged intracellular environment makes organic anion import energetically unfavorable, and thus relies on a dicarboxylate counterion gradient established by cotransport with Na+, which ultimately relies on Na+/K+ ATPase activity. Because import of Oat1 substrates is typically followed by efficient apical secretion by other ATP binding cassette (ABC) and solute carrier family (SLC) transporters, the basolateral uptake step is considered to be rate-limiting, thus dictating the clearance of its substrates.

Due to the indispensable role of proximal tubules in maintaining homeostasis, proximal tubule functions remain under constant regulation through intracellular and extracellular signaling pathways during development and throughout the lifetime of the organism, many of which exert their effect through changes in expression. However, the unique capability to import a wide variety of substrates does not come without a price; proximal tubules are constantly exposed to toxic substances, and are therefore highly susceptible to nephrotoxicity. Many conditions have been reported to cause proximal tubule-specific toxicity (eg. HgCl₂, ochratoxin A, cisplatin, ischemia/reperfusion). Pathophysiological regulation of specific proximal tubule transport activity can exacerbate clinical outcome: downregulation can lead to systemic toxicity due to accumulation of harmful substrates in the bloodstream, while upregulation can also lead PT injury by increasing intracellular toxin concentrations. Significant proximal tubule damage can progress to acute kidney injury, chronic renal disease, and renal failure. In
less severe cases, the proximal tubule has the unique capacity to regenerate and partially restore function – a very clinically relevant phenomenon that remains poorly defined, but is thought to be mechanistically related to normal development.

1.1.2 Role in Drug Metabolism

When concerning drug metabolism, the process involving uptake and excretion of drugs by transporters is referred to as Phase III (sometimes characterized as Phase 0 for uptake into the cellular compartment and Phase III for efflux into waste compartments – urine, bile and feces), where Phase I is enzymatic modification (e.g. oxidation, reduction, hydrolysis) and Phase II is the conjugation of various moieties (e.g. glucoronidation, sulfation, acetylation), which alters solubility and can consequently alter pharmacokinetics. The PT and the hepatocytes of the liver both play major roles in Phase III drug excretion. However, while the majority of Phase I and Phase II metabolism is typically attributed to the liver, especially because it is first in line to be exposed to blood containing substances absorbed from the intestine (referred to as first-pass metabolism), the PT also expresses a large number of Phase I and II enzymes. This is important because it may allow for the PT to functionally compensate in the case of liver injury or declining liver function. Furthermore, it is important to consider the repertoire of Phase I and II activity in PT cells, because once substrates enter the cell, in some cases they can be modified by Phase I and II reactions, thereby altering their toxicity and specificity for various efflux transporters; this can lead to accumulation of toxic levels of substrates and tubular injury, which can lead to further complications.
1.2 KIDNEY DEVELOPMENT

1.2.1 Nephrogenesis

In vivo, kidney development is considered to begin when the ureteric bud (UB) grows out from an epithelial tubule called the Wolffian duct (WD) into a population of cells referred to as the metanephric mesenchyme (MM), which originates from the intermediate mesoderm. The UB and MM then go through mutually inductive interactions, where the MM supports the dichotomous branching of the UB to form the collecting duct system, while the UB induces the MM to undergo nephrogenesis, which gives rise to the proximal (including the epithelial cells of the glomerulus) through distal portions of nephrons. Upon initiation, nephrogenesis goes through well-defined, distinct morphological stages – condensed mesenchyme, pretubular aggregate, renal vesicle, comma-shaped body, s-shaped body, followed by the formation of a premature tubular structure; this process gives rise to a single nephron. During this iterative process, vasculature and innervation originating from the base of the UB are also established. The first appearance of defined proximal tubules markers is detected during early stages of kidney development, several days after the induction of the first nephrons; however, functional maturity is not reached until adulthood. Once fully developed, the kidney contains more than two dozen distinct cell types which must be spatially organized to carry out their functions; this complexity has made it difficult to develop tissue engineering strategies to replace kidney function.

1.2.2 Systems view of transcriptional regulation of embryonic and postnatal kidney development
Before the advent of microarrays and massively parallel sequencing, most studies aimed at understanding transcriptional regulation focused on individual genes and mechanisms. In the last couple of decades, however, there have been huge advances in the ability to examine the transcriptome and epigenome on a genome-wide scale. While this has greatly advanced our understanding of how cell behavior is controlled at the level of transcription, it has revealed how incredibly complex and dynamic this process really is.

Transcription factors are still considered to play a critical role by binding cis-regulatory elements and recruiting transcriptional machinery, chromatin modifying complexes, or proteins that repress transcription [1, 2]. In addition to defining gene expression programs during development, transcription factors can respond to numerous extracellular and intracellular signals, thus adding additional layers of complexity [3-5]. However, recent advances in genomic technologies have greatly advanced the understanding of how various other mechanisms help refine cell-specific expression. For example, the epigenome, mainly defined by histone modifications and DNA methylation, appears to help determine which genomic regions are accessible to being bound by transcriptional regulators and machinery, as well as which specific proteins are recruited. Yet another level of regulation is carried out by noncoding RNA (ncRNA), such as microRNA (miRNA), short interfering RNA (siRNA), Piwi-interacting RNA (piRNA), small nucleolar RNA (snoRNA), enhancer RNA (eRNA), long interspersed noncoding RNA (lincRNA), and other less-defined species. For example, microRNAs repress protein-coding mRNA, and appear to function as a type of stabilizer of gene expression, while also reducing levels of unwanted transcripts. LincRNAs, on the other hand, are
thought to play important roles in recruiting transcriptional regulators to specific loci. Together, all of these mechanisms allow for an incredible number of configurations, which helps explain how a single genome can lead to such diverse and precise transcriptional programs in the various cell types found within a given organism.

The two dimensional model of transcription has become obsolete, and replaced with a 3-D or even 4-D (3-D changing in time) model, where not only the genomic sequence and epigenetic landscape matters, but also the arrangement of the genome in space, which determines which regulatory elements, and their corresponding protein complexes, have access to various target genes. Recent advances in chromatin conformation capture techniques have revealed that the genome is organized into topological domains, with the median size being slightly less than one megabase, and that most interactions between enhancers and promoters are contained within these domains, and rarely cross these boundaries [6]. While these domains are relatively stable, and help dictate not only the transcriptome during homeostasis but also various responses upon perturbation, they have recently been found to be dynamic throughout differentiation and development [7]. Therefore, while a static depiction of the transcriptome or epigenome serves as a useful foundation for explaining the phenotype, it is important to consider the limitations of such a depiction.

To gain a deeper understanding of kidney development, mouse and rat kidneys have been analyzed by expression microarrays at multiple timepoints throughout development, beginning with a tiny structure consisting of only a ureteric bud and surrounding metanephric mesenchyme, all the way through to adult kidneys [8-10]. This revealed the dynamics of the kidney transcriptome, and suggested that transcriptional
changes do not occur steadily along a continuum, but rather in stages, likely driven by distinct stimuli that result in noticeable transitions. However, due to the large number of different cell types in the kidney, it is difficult to infer cell-specific phenomena from analyzing the organ as a whole. In-situ hybridization or immunohistochemistry can be used to examine tissue-specific expression in kidney cross-sections, however, this approach is limited to a one-by-one approach (though progress is being made to create an atlas of tissue-specific expression in the kidney by automating this process). To address the question of tissue-specific expression in rodent kidneys on a global scale, various substrucutres were excised from frozen embryonic kidney sections using laser capture microdissection, followed by analysis with expression microarrays[11]. This helped define the transcriptome of various renal constituents, including developing proximal tubules, and revealed which genes are broadly expressed versus which ones define the unique properties of the various cell types. Furthermore, this information helped contextualized transcriptome data gathered from whole kidney tissue. While this knowledge is critical for better understanding kidney development and function at a transcriptional level, much more work remains to be done before we can generate a blueprint for how the kidney is formed and regulated.

1.3 TISSUE-SPECIFIC TRANSCRIPTIONAL REGULATION

1.3.1 Knockout mouse models relevant to the proximal tubule development

Due to the high level of functional and genetic homology between rodent and human kidneys, transgenic mouse lines have been indispensable in advancing the understanding of kidney development. Many transgenic lines have been created that
result in embryonic lethality, kidney agenesis, or severe kidney dysplasia, which are documented in The Kidney Development Database. However, most of the progress has been made in defining genetic requirements of early kidney patterning events upstream of proximal tubule development, which fail to reveal the contribution to terminal differentiation events.

Current understanding of proximal tubule-specific genetic requirement is limited to the knowledge that conditional deletion of Notch 2 or downstream effector Rbp-j in the mesenchyme results in loss of proximal fate during development (including Bowman’s capsule), while markers for distal nephron fate are present [12]. Early events in nephrogenesis require Wnt and growth factor signaling pathways (such as Fgf8), which may be involved in establishing general tubular epithelium functions [12-14], but specific contributions to proximal tubule fate and function are undetermined. Interestingly, deletion of Notch2 downstream targets Hes1 and Hes5 led to no discernible proximal tubule phenotype, despite proximal tubule-specific expression of Hes1 in the kidney. Expression of Notch ligands and downstream targets Jagged1 and Dll1 is also thought to be relevant to proximal tubule fate[15]. However, Jag1 and Dll1 exert their effect by activating Notch, which only serves to highlight the importance of Notch signaling. Hepatocyte nuclear factor 1 beta (Hnf1b) was also found to be required for proximal tubule fate specification; however, this appeared to be due to the regulation of Notch pathway signaling components by Hnf1b, thus providing insight regarding upstream regulators of this critical pathway [16].

Activation of Notch2 in the mesenchyme led to severe kidney dysgenesis, and suggested that Notch2 has a stabilizing rather than inductive role in establishing proximal
fate by depleting undifferentiated precursors[17]. Inhibition of gamma-secretase at different stages of kidney development revealed that blocking Notch activity at the s-shaped body stage did not prevent proximal tubule formation, suggesting that proximal cell fate is already specified at that stage; however, gamma secretase inactivation in formed nephrons revealed a role in regulation of tubule diameter by regulating the plane of division[12]. While Notch1 signaling is detected in proximal tubules during development, Notch1 deletion led to no discernible effect on proximal tubule formation. Ironically, ectopic activation of the Notch1 intracellular domain drove proximal tubule formation, while inhibiting formation of distal fates. Inactivation of Notch1 in a sensitized Notch2 background revealed a potential role for Notch1, yet it was found to converge on Rbp-j, and did not provide new candidate effectors [18].

1.3.2 Transcriptional regulation of mature proximal tubule function

Despite evidence for changes in proximal tubule function [19], morphology[20] and expression from initial specification during embryonic development to adulthood, the transcriptional mechanisms that regulate proximal tubule functional maturation are also not well defined. Hormone regulation is known to be involved, as parathyroid hormone regulates proximal transport activity through cAMP production[21], sex hormones are known to result in increased organic anion transport expression in males[22], and glucocorticoid hormone has an effect on sodium and phosphate transport[23]. Furthermore, activation of nuclear receptors, including Vdr [24], Ppara [25], Lxr [26] and Fxr [27], is known to be involved in integrating signals in proximal tubules, and several others are also known to be highly or moderately expressed [28], and are also likely to play a regulatory role. However, while nuclear receptors are thought to act by repressing
or activating expression[4], characterization of transcriptional effects has been largely limited to individual genes, and has not revealed master regulators of proximal tubule cell identity. While many transcription factors are expressed in proximal tubules, Hnf1a is the only transcription factor with a defined role in proximal tubules [29]. Mice with homozygous inactivation of Hnf1a have proximal tubule dysfunction, termed Fanconi syndrome, with a pronounced defects in glucose reabsorption and phosphate uptake in morphologically normal proximal-tubules [30], which is attributed to decreased expression of sodium/glucose transporter Sglt2 and sodium/phosphate transporter Npt1-Npt4[31]. Transactivation studies have reinforced the role of Hnf1a in regulating specific transporters in the proximal tubule [32-34].

1.3.3 Differentiation and Transdifferentiation

A major goal in the field of renal tissue engineering and regenerative medicine is to gain the ability to establish a renewable source of patient-specific cells for every renal cell type. Most of the early studies that have differentiated cells towards renal lineages have been aimed at creating a progenitor population. Recently, mature cell types have become a target of interest as well. While the field is far from being able to engineer all renal cell types, there are reports of establishing proximal tubule-like cells [32], podocyte-like cells [33], and uterine bud-like cells [35] from human pluripotent stem cells. Interestingly, in the case of the PT-like and podocyte-like cells, while the endpoints appear to be morphologically and functionally different, both studies used retinoic acid, BMP7, and activin A to achieve the final phenotype; BMP2 was also included to differentiate towards a proximal tubule-like phenotype. Furthermore, these factors have also been used to program renal progenitor populations. It will be important to establish
clear criteria to characterize the endpoint, as well as to test the specific capacity for which the cells are designed, such as function and integration, in an injury model or population of a scaffold.

While signaling factors have often been used to differentiate stem cells into various lineages, transcription factors are often considered to be mechanistically critical for cell lineage determination. Furthermore, while there are a number of reports of using soluble signaling factors to differentiate stem cells to a more restricted fate, most reports of changing cellular fate from one differentiated state to another (transdifferentiation) have involved the ectopic expression of transcription factors [31, 34, 35]. With the advent of induced pluripotent stem cells (iPSCs), it should theoretically be possible to establish every cell type with the use of the appropriate spatiotemporal signaling cues as in natural development. However, even though iPSCs can now be generated without altering the genome by transient expression of reprogramming factors, the use of pluripotent cells as a cell source for clinical applications poses the risk of tumorigenicity. Largely for this reason, transdifferentiation, which bypasses pluripotency (presumably lowering the risk of tumorigenicity) and may be accomplished without altering the genome [36], should still be explored as a strategy for generating mature cell types. Finally, establishing differentiation protocols with soluble signaling factors (which often affect many pathways and may cause unwanted changes along with the desired ones) largely relies on adjusting the recipe until certain readout criteria are met, while ectopically expressing transcription factors provides the ability to directly fine-tune the transcriptional landscape; however, this applies to both differentiation and transdifferentiation, as stem cells can also be differentiated with ectopic expression of lineage-determining TFs.
The starting material (iPSCs, differentiated somatic cells, or adult stem cells) for reprogramming is also worth considering because the epigenetic memory of the initial cell state has been demonstrated to play a role in determining the endpoint cell phenotype. Also, the epigenetic landscape influences developmental competence [37], which can cause various cell types to have a different response to the same cues. This is especially important for the proximal tubule, because PT progenitor cells (metanephric mesenchyme) must go through multiple highly-coordinated spatiotemporal signaling events before PT cells are specified, during which cells destined to become PT cells undergo sequential epigenetic changes that prime them for the next stage of development. It may be difficult to accurately jump from one cell type to another without undergoing intermediate stages that occur during in utero development. Nevertheless, in addition to the aforementioned studies, there are multiple reports of the successful establishment of functional differentiated cell types of multiple organs from various cell sources, indicating that this should be achievable for most lineages.

1.4 HEPATOCYTE NUCLEAR FACTORS 1A AND 4A (HNF1A AND HNF4A)

Hnf1a and Hnf4a were named hepatocyte nuclear factors because they were initially discovered in either rat liver [38] or rat hepatocyte [39] extracts, respectively. However, they are now known to be strongly expressed in other epithelial tissues with metabolic functions, including the proximal tubule of the kidney, intestine, colon, choroid plexus, cholangiocytes and B-islet cells of the pancreas, and are attributed to tissue-specific expression in some of these tissues. Some of the earlier studies have suggested regulation of these two factors by themselves and by each other, suggesting
interdependence of these two factors. Furthermore, studies have revealed that these factors exhibit a high level of co-localization at genomic regions [40], and regulated some genes in a synergistic manner [41]. Hnf1a and Hnf4a are known to be lineage-determining transcription factors for the hepatocyte cell fate, meaning that they have been used in various combination with other factors to establish cells with some hepatocyte-like properties [36, 42-45]. However, it is important to understand that the same transcription factors can be lineage-determining transcription factors for different cell types. For example, it has been shown that Hnf4a has different binding patterns in different tissues and regulates different genes. The specificity likely comes from cooperation with other transcriptional co-regulators, as well as developmental competence, which determines which regulatory elements are activated by a factor in a given cell type.

1.4.1 Hnf1a

In the rodent kidney Hnf1a is specifically expressed in the proximal tubule [30]. As already mentioned in section 1.3.3, Hnf1a deletion in mice resulted in renal Fanconi syndrome, which is caused by proximal tubule dysfunction; a liver phenotype was also reported, supporting the notion that Hnf1a is essential for regulating both metabolic cell types. Interestingly, a developmental phenotype was not reported in these animals, suggesting that it may be more important in establishing functions associated with terminal differentiation rather than early cell fate specification.

1.4.2 Hnf4a

While Hnf4a expression in the adult kidney is restricted to the proximal tubule, as is Hnf1a, Hnf4a expression in the developing kidney is detected throughout
nephrogenesis possibly as early as the condensed mesenchyme stage [46] and definitely by the time the comma-shaped body is formed [47]. While two alternate promoters regulate Hnf4a transcription, P1 and P2, which result in different regulation properties[48], P2-driven transcription is lost after the s-shaped body stage[47]. Interestingly, while the P2 promoter is typically associated with embryonic expression, it is the primary promoter used in mature B-islets, which may account for some of its tissue-specific properties.

Unlike Hnf1a, Hnf4a is classified as a nuclear receptor – Nr2a1 (nuclear receptor subfamily 2, group A, member 1). Hnf4a typically functions as a homodimer (but also has the ability to heterodimerize with closely related Hnf4g), and can act as a constitutive or possibly ligand-dependent transcriptional activator; Hnf4a can also directly interact with small heterodimer protein (SHP) or co-repressor SMRT [49] to act as a transcriptional repressor. Hnf4a is still classified as an orphan nuclear receptor; while the endogenous ligand (at least in liver) has been identified as linoleic acid, how and whether it modulates Hnf4a activity remains unclear.

Hnf4a inactivation leads to embryonic lethality prior to kidney development [50] due to defects in gastrulation, which has prompted the creation of multiple tissue-specific knockout models, including the pancreas, small intestine, colon and liver. In general, these studies have revealed that while the degree of regulation by Hnf4a varies from tissue to tissue, with the liver being one of the most affected by Hnf4a deletion, the transcription factor/nuclear receptor plays important roles in regulation of epithelialization, transport and metabolism. Ironically, while the proximal tubule expresses high levels of Hnf4a, a kidney-specific Hnf4a knockout model has yet to be
reported. However, knockdown of Hnf4a by siRNA in kidney explant cultures lead to apoptosis in the condensed mesenchyme [46]. While this did not provide further insight into the role of Hnf4a in proximal tubules, it does suggest the possibility that Hnf4a may be important in proximal tubule development.

1.4.3 Human relevance

Interestingly, there is an extensive amount of literature describing multiple mutations in HNF1A and HNF4A in humans that cause maturity onset diabetes of the young (MODY), but very few other phenotypes are described in these patients. However, there is at least one HNF4A mutation that resulted in Fanconi syndrome (proximal tubule functional defect) along with a B-islet phenotype (pancreatic cell responsible for insulin production which causes diabetes when dysregulated) [51]. While in rodents it is known that Hnf1a plays an important role in PT function and evidence is mounting that Hnf4a plays an important role in the development and mature function of the PT, it remains to be seen to what extent this relates to regulation of human proximal tubule cells. However, HNF1A and HNF4A are known to be critical for hepatocyte specification and function, and have been used along with FOXA transcription factors to establish hepatocyte-like cells from human fibroblasts [36, 44, 45]; thus, the fact that most MODY mutations have no associated liver or kidney phenotype in humans appears not to be a predictor of the role of HNF1A and HNF4A in the proximal tubule.

1.5 CELLULAR STRATEGIES AIMED AT REPLACING KIDNEY FUNCTION

Over 80% of people on the waiting list for an organ transplant are waiting for a kidney (OPTN). While dialysis is commonly used to help alleviate the symptoms of
kidney disease, it cannot fully replace the metabolic and detoxifying functions of the kidney, and organ transplantation eventually becomes necessary. From a tissue-engineering perspective, while the ultimate endpoint is to create an organoid in a laboratory setting that can replace the function of a healthy kidney, the most feasible means of achieving this goal is not yet clear. A number of approaches have been proposed. With the advent of decellularized organ scaffolds, one conceivable option is to reintroduce the necessary types of differentiated patient-specific kidney cells into a full-size scaffold rather than following a strictly developmental program. This will require safe and efficient methods to program the distinct kidney cell types from patient cells. An alternative strategy is to mimic endogenous development, which consequently reduces the requirement of starting materials to a small set of progenitor cell populations, but this may necessitate a more intricate culture process. Finally, until it becomes possible to culture replacement organs suitable for transplantation, it is worthwhile to also consider developing cell-based bio-cartridges that can perform most of the functions of the proximal tubule as a supplement to standard osmosis-based dialysis.

1.6 SCOPE OF DISSERTATION

At the beginning of this thesis work, relatively little was known about the transcriptional regulation of proximal tubule cells, and what was known was limited to regulation of individual genes and mainly examined in vitro within immortalized cell lines. The overall objective of the studies described in this thesis was to gain a broader understanding of transcription factors that are integral to establishing proximal tubule cell identity and function.
Chapter 2 describes the roles of Hnf1a and Hnf4a as candidate PT lineage-determining transcription factors, and that they likely play major roles in establishing the tissue-specific properties of the PT transcriptome. Because the proximal tubule plays a large role in drug clearance, which is of great clinical importance, we chose to focus on the regulation of drug metabolizing enzymes and drug transporters by Hnf4a. This work revealed that Hnf4a is likely to regulate a large number of tissue-specific genes in the proximal tubule, at least some of which also require coordinate regulation by Hnf1a. However, additional PT candidate lineage-determining transcription factors were not identified, thus bringing into question what determines Hnf1a and Hnf4a specificity in different tissues, and to what extent these two factors alone actually contribute to the tissue-specific versus the more ubiquitous components of the PT transcriptome.

Chapter 3 attempts to further explore the uncertainties from the work described in chapter 2. This chapter first addresses whether developmental competence (or cellular background) contributes to differential effects of Hnf1a and Hnf4a overexpression. Then, we addressed whether Hnf1a and Hnf4a induce morphological changes, explore the induction of transporters and junctional genes on a broader scale, and attempt to reconcile whether Hnf1a and Hnf4a induces genes indicative of a bias towards a specific cell fate. Finally, chapter 3 describes a transcriptional mechanism which appears to account for the differences in expression and function between proximal tubule cells and hepatocytes, despite Hnf1a and Hnf4a playing important roles in both cell types.

In the final chapter, we discuss the implications of this work, and future experiments that might further elucidate the mechanisms involved in the transcriptional specification and regulation of the proximal tubule, as well as transcriptional mechanisms
that confer specificity to transcription factors that play different but related roles in various tissues.
CHAPTER 2:

Hepatocyte Nuclear Factors 4a and 1a Regulate Kidney Developmental Expression of Drug-Metabolizing Enzymes and Drug Transporters

2.1 ABSTRACT

The transcriptional regulation of drug-metabolizing enzymes and transporters (here collectively referred to as DMEs) in the developing proximal tubule is not well understood. As in the liver, DME regulation in the PT may be mediated through nuclear receptors which are thought to “sense” deviations from homeostasis by being activated by ligands, some of which are handled by DMEs, including drug transporters. Systems analysis of transcriptomic data during kidney development predicted a set of upstream transcription factors, including Hnf4a and Hnf1a, as well as Nr3c1 (Gr), Nfe2l2 (Nrf2), Ppara, and Tp53. Motif analysis of cis-regulatory further suggested that Hnf4a and Hnf1a are the main transcriptional regulators in the PT. Available expression data from tissue-specific Hnf4a KO tissues revealed that distinct subsets of DMEs were regulated by Hnf4a in a tissue-specific manner. ChIP-seq was performed to characterize the PT-specific binding sites of Hnf4a in rat kidneys at three developmental stages (prenatal, immature, adult), which further supported a major role for Hnf4a in regulating PT gene expression, including DMEs. In ex vivo kidney organ culture, an antagonist of Hnf4a (but not a similar inactive compound) led to predicted changes in DME expression, including among others Fmo1, Cyp2d2, Cyp2d4, Nqo2, as well as organic cation transporters and organic anion transporters Slc22a1(Oct1), Slc22a2 (Oct2), Slc22a6 (Oat1),
Slc22a8(Oat3), and Slc47a1(Mate1). Conversely, overexpression of Hnf1a and Hnf4a in primary mouse embryonic fibroblasts (MEFs), sometimes considered a surrogate for mesenchymal stem cells, induced expression of several of these proximal tubule DMEs, as well as epithelial markers and a PT-specific brush border marker Ggt1. These cells had organic anion transporter function. Taken together, the data strongly supports a critical role for HNF4a and Hnf1a in the tissue-specific regulation of drug handling and differentiation toward a PT-like cellular identity. We discuss our data in the context of the Remote Sensing and Signaling Hypothesis [52, 53].

2.2 INTRODUCTION

The kidney proximal tubule is involved in reabsorption of water, electrolytes, and organic solutes, tubular secretion and other processes. Some PT transporters play vital roles in the clearance of many substrates, including some metabolic intermediates, xenobiotics and environmental toxins. Importantly, they are also responsible for excretion of many commonly administered pharmaceuticals or their metabolites generated by the cohorts of Phase I and Phase II drug-metabolizing enzymes. Hence, these transporters, which belong to the ABC and SLC gene families, are commonly grouped as Phase III drug transporters.

The Phase I and II processes of drug metabolism, which result in chemical modification and conjugation of drugs, respectively, have been largely studied in hepatocytes. However, a significant number of genes associated with Phase I and II reactions are expressed in the kidney, some of which have been shown to serve important functional roles [54, 55]. While much remains to be understood regarding the
contribution of genes involved in proximal tubule cell systemic and Phase I, Phase II, and Phase III metabolism, even less is known about the transcriptional regulation of these genes.

Little is understood about how DME expression is coordinated in the developing and postnatal PT, in part because many knockout models aimed at studying the kidney experience developmental defects prior to PT formation. The best characterized transcriptional regulator of physiologically relevant transporters in the PT in vivo is Hepatocyte Nuclear Factor 1 alpha (Hnf1a). While still able to form upon complete Hnf1a ablation, the proximal tubule exhibits several transport deficiencies, similar to the characteristics of Fanconi syndrome in humans [30]. It is possible that expression of Phase I and Phase II genes was altered as well, but this remains to be studied in detail.

While regulation of the DME repertoire in the PT remains to be explored, we have previously performed a focused study on the regulation of the organic cation transporter Slc22a1 (Oct1), and organic anion transporters Slc22a6 (Oat1) and Slc22a8 (Oat3), in cultured kidney tissues. These transporters are highly enriched in the proximal tubule, where they mediate the rate-limiting uptake step of many drugs and toxins [56-59]. They have also been hypothesized to function as part of a larger “remote sensing and signaling” system in whole organism homeostasis [52, 53]. We found multiple lines of evidence suggesting that the nuclear receptor Hepatocyte Nuclear Factor 4a (Hnf4a) may be involved in their regulation, which was further supported by detection of Hnf4a binding in rat kidneys at all three promoters in vivo [60].

Nevertheless, more direct and functional evidence is lacking. In this study, we sought to identify transcriptional regulators involved in the initiation and maturation of
DME expression at distinct stages of prenatal and postnatal PT development. Systems analysis of previously published microarray expression data suggested a large role for Hnf4a in regulating Phase I and Phase II drug-metabolizing enzymes and Phase III drug transporters. Based on the important role of genomic enhancer elements in establishing cell-specific expression [61-63], which is in part defined by expression of specific Phase I, II and III genes in the PT, we set out to characterize the genome-wide localization of p300 in adult rat kidney cortex, where proximal tubules make up the dominant cell fraction. Motif analysis of enhancer elements identified Hnf4a and Hnf1a as the major “lineage-determining” factors of the PT.

To gain more insight into Hnf4a-dependent transcriptional regulation during development, we analyzed publically-available microarray expression data from five different WT and Hnf4a KO tissues: embryonic liver, embryonic and adult colon, adult small intestine and adult B-islet cells. All tissues exhibited some degree of differential DME expression as a result of Hnf4a ablation, with liver exhibiting the most severe effects. Hence, to better understand PT-specific role of Hnf4a, we used ChIP-seq to determine its binding profile in rat PTs at three progressive stages of PT development: E20, 2 weeks and 8 weeks. Hnf4a binding was found to be correlated to levels of DME expression in PTs. A small molecule antagonist was used to show that Hnf4a regulates key representative Phase I, Phase II and Phase III genes in ex vivo rat kidney cultures. Finally, lentiviral-mediated transduction of Hnf1a and Hnf4a into mouse embryonic fibroblasts (MEFS) induced the expression of proximal tubule Phase I, II and III genes. Together, these findings reveal the pivotal role of Hnf4a and Hnf1a in coordinating DME expression in the developing and postnatal proximal tubule.
2.3 MATERIALS AND METHODS

2.3.1 Microarray Expression Analysis

Seven separate analyses were performed: a comparison of embryonic and adult mouse proximal tubule cell expression, a time series of expression in whole rat kidneys, and five comparisons of wildtype and Hnf4a knockout mouse tissues. To compare expression in prenatal and postnatal proximal tubules, we analyzed publicly available mRNA expression data from proximal tubules isolated from E15.5 (GSM144594-144595, GSM152247-152249) [11] and adult mouse kidneys (GSM256959-256961 [64], GSM490067-490069). Time series data was obtained from a previously published study [10], and restricted to genes determined to be enriched in the proximal tubule[11]. To investigate the tissue-specific changes in DME expression upon Hnf4a deletion, the following datasets were analyzed: GSE3126 (E18.5 liver [65]), GSE3116 (E18.5 colon [66]), GSE11759 (adult colon [67]), GSE3124 (adult small intestine), and E-MEXP-1729 (adult isolated B-islet cells [41]). Microarrays for each of the seven analyses were prepared separately with Genespring 12.5 software using the RMA algorithm. Probes were discarded within each analysis group unless they had present flags in more than half of the samples in at least one sample group (E15.5/Adult PT, Hnf4a WT/KO tissue) or time-point (E13-E21, P0, 1WK, 4WK, Adult), as determined by the MAS5 algorithm. For the whole rat kidney data, sex chromosome-linked genes were excluded as well. Many genes are represented by more than one probeset; Figure 2.1A, 2.2A, and 2.4A depict differential probeset signal intensities, while Figure 2.1B, 2.1C and 2.4B consider the average of corresponding probe intensities for each gene. In the comparison of expression in pre and postnatal proximal tubules, differential expression was defined as
having more than a 2-fold change (FC) with p<0.05 with the Benjamini Hochberg correction. For pairwise comparisons of WT and Hnf4a +/- tissue, it was important to be as inclusive as possible in order to consider changes from tissues with weaker phenotypes. Thus, the cutoff was lowered to FC>1.3 with p<0.05, and no multiple testing correction was applied to increase the solution space. It should be noted that even though all of the five experiments were conducted in mice, two different array platforms were used: Affymetrix mouse 430_2 (45,101 probe sets) and mouse 430A (22,690 probe sets). Overall, the 430_2 array is more thorough and has a higher probe/gene ratio. Embryonic colon and small intestine were analyzed using the 430A array, therefore, the differential expression may be underrepresented relative to the other three tissues. To define proximal tubule-enriched gene expression in the kidney during development, a conversion algorithm integrated within GX 12.5 was used to translate probes determined to be enriched in the early mouse proximal-tubule to the rat 230 2.0 platform. For network analysis, probe IDs were imported into the Ingenuity Pathway Analysis software suite (IPA). Core analysis was conducted using the default settings. Upstream regulators were selected based on molecule type and prediction z-score above 2.0.

2.3.2 Chromatin Immunoprecipitation

ChIP was performed as previously described [60] with some modifications. Isolating proximal tubules from adult kidneys requires enzymatic and mechanical manipulation – processes which can alter the native state of proximal tubule cells before the ChIP part of the technique is performed. To preserve the endogenous chromatin landscape of cells, freshly-isolated kidneys from unsexed E20, P13 or male adult Sprague Dawley rats – corresponding to the three states of DME regulation in the proximal tubule
identified by the developmental time series analysis in the context of proximal-tubule genes – were collected as previously described [10] and immediately frozen in liquid nitrogen. To isolate kidney cortex, a frozen whole adult kidney was kept frozen within a ceramic mortar surrounded by dry ice while the outer ~1mm was shaved off with a razor. Whole kidneys or isolated cortex were thawed and minced in 1% formaldehyde in PBS on ice, followed by rotation for 15 minutes at room temperature. Fixation was quenched with glycine for 5 minutes. Fixed samples were then homogenized with a tissue grinder, washed twice with cold 0.5% IGEPAL CA-630 in PBS, and further disrupted in the same buffer using a type A glass dounce homogenizer. Nuclei were pelleted and sonicated on ice (three 5 minute cycles of 30 seconds on/30 seconds off) in ChIP buffer using a Cole Palmer handheld sonicator. To quantify the chromatin and for use as control samples, “input” samples were prepared from the chromatin by treatment with first RNAse and then Proteinase K, further de-crosslinking overnight at 65C, phenol extraction and ethanol precipitation. Concentrations were determined using a NanoDrop. ChIP for Hnf4a was performed in duplicate using 20ug of chromatin from E20, P13 and adult whole kidneys and 4ug anbitody (Santa Cruz, sc-8987); for p300, 2ug of chromatin from adult cortex and 10ug antibody (Santa Cruz, sc-585) was used. A mix of pre-blocked protein A/G beads was used to recover antibody-bound complexes, which were subsequently washed and eluted with SDS-containing buffer. The DNA was purified from the enriched samples as described above for the input samples.

2.3.3 Massively Parallel Sequencing and Analysis

Libraries were prepared using the Illumina ChIP-seq DNA Sample Prep Kit, using either the pooled duplicates for the ChIP samples or 50ng for the inputs. Amplified DNA
fragments within 200-400bp long were sequenced using the HiSeq 2000 instrument and aligned to the rn4 genome by BIOGEM (Genomics Data Analysis Services, UCSD) according to the standard Illumina pipeline. All further analysis was performed using the HOMERv3.13 software package [61]. 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 raw reads and peak files have been deposited in NCBI’s Gene Expression Omnibus database under the GEO series accession number GSE50815 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50815).

HOMER software was also used to generate files for peak visualization in the UCSC Genome Browser, as well as to calculate overlapping and differentially bound peaks between the 4 samples. Motif enrichment within promoter-distal peaks was calculated with default settings in HOMER, except the number of background sequences was increased to be over five times higher than the number of peaks.

2.3.4 Organ and Cell Culture

Kidneys were dissected from E13.5 Sprague Dawley rat embryos and cultured on Transwell filters in DMEM/F-12 media supplemented with 10% FBS and 1% Pen/Strep, as previously described [68]. Media was changed every three days. After 6 days, cultures were treated in triplicate with either 1:1000 DMSO, 2.5uM and 5uM Hnf4a antagonist BI6015 [2-Methyl-1-(2-methyl-5-nitrophenylsulfonyl)-1H-benzo[d]imidazole [69]], or 10uM of the analogous inactive compound BI6018 for 12 hours (both kindly provided by Dr. Fred Levine, Sanford Burnham Research Institute). Samples were then stored in RNAlater (Ambion) until further processing for RT-qPCR.
MEFs were prepared from E16.5 mouse embryos using a modified version of a previously described method [43]. Embryonic tissue (excluding CNS and visceral organs) was minced and digested in 0.25% Trypsin in DMEM containing DNase I at 37C in a shaker, periodically being agitated with a pipettor. The resulting suspension was gravity pelleted for 2 minutes to allow large undigested pieces of tissue to settle. The supernatant was collected, pelleted, and resuspended in L-15 media with 1% Pen/Strep, filtered through several layers of sterile gauze, and then twice through 40 micron cell strainer. Resulting cells were again pelleted and frozen (in 45% DMEM, 45% FBS, 10% DMSO) for future use. All animal procedures were approved by IACUC.

2.3.5 Immunofluorescence

Kidney organ cultures were fixed in 4% formaldehyde in PBS at 4C overnight, and then kept in PBS at 4C until staining. The filter membrane was cut out from the Transwell, residual glycine was blocked with 50mM Glycine in PBS, followed by blocking in PBS with 10% BSA, 0.1% Tween 20 and 0.05% Triton-X 100 for one hour at room temperature. Samples were then incubated with mouse E-cadherin antibody (Zymed) in IHC buffer (2% BSA, 0.1% Tween 20, 0.05% Triton-X 100) overnight at 4C. After three-one hour washes in IHC buffer at room temperature, samples were incubated with anti-mouse Cy5 (Jackson ImmunoResearch), rhodamine-conjugated Dolichos Biflorus (Vector Labs) and DAPI at 4C overnight. After three-one hour washes with 0.1% Tween 20 in PBS, samples were mounted with Fluormount, sealed, and imaged using the Olympus FluoView FV1000 confocal microscope.

2.3.6 Lentiviral Transduction
Lentiviral plasmids pWPI-mHnf4a and pWPI-mHnf1a [42] were kindly provided by Dr. Lijian Hui, Shanghai Institute of Biochemistry and Cell Biology. To produce lentiviral vector, Fugene HD (Promega) was used to co-transfect HEK293T cells at ~50% confluency with pWPI-Hnf4a or pWPI-Hnf1a, psPAX2 (Addgene) and pCMV-VSVG[70] in DMEM/F-12 media with 10% FBS and 1% Pen/Strep. After 24hr, media was replaced, then collected at 48 and 72 hours, and kept at -80°C until use. MEFs were plated the day before infection, and infected overnight at ~40% confluency with media mixed 1:1 with viral supernatant, or 2:1:1 when infecting with both Hnf1a and Hnf4a, in the presence of 8ug/mL Polybrene (Sigma-Aldrich). On the next day, MEF media was replaced with DMEM/F-12 containing 1% FBS, 1% Pen/Strep, 1X Insulin/Transferrin/Selenium (Invitrogen), 20ng/mL EGF (R&D Systems), 4ng/mL Triiodothyronine (Sigma-Aldrich), 20ng/mL Dexamethasone (Sigma-Aldrich), 10ng/mL Cholera Toxin (Sigma-Aldrich) for both infected and uninfected cells. For all assays, cells were sampled 3 days after switching the media. Because pWPI includes EGFP downstream of the insert, we used FACS to quantify transduction efficiency. Of the live cells, based on forward and side scatter, ~70-80% were positive for EGFP.

2.3.7 Real Time qPCR

RNA was extracted from cultured embryonic kidneys and MEF cultures using the RNeasy Micro Kit (Ambion), and cDNA was made using SuperScript III First Strand cDNA Synthesis Kit (Thermo Scientific). Real Time PCR was carried out using a 3-step cycle on the 7900HT Fast Real-Time PCR instrument (Applied Biosystems) with Power SYBR Green Master Mix (Applied Biosystems). Melt curves were examined to confirm primer specificity. The list of primer sequences is included in Supplemental Table 1.
2.3.8 6-Carboxyfluorescein Uptake Assay

Uninfected and transduced MEFs were washed with PBS, and incubated in either PBS, PBS with 10uM 6-CF, or PBS with 10uM 6-CF and 1mM Probenecid in triplicate at room temperature for 10 minutes. Cells were then washed three times with cold PBS, and levels of fluorescence were measured using a model 1420 Multilabel Counter (PerkinElmer). Assays were performed as previously described [68, 71].

2.4 RESULTS

2.4.1 Curating an extensive list of Phase I, II, and III genes

To begin our analysis of DME transcriptional regulation in the proximal tubule, it was first necessary to define a list of genes potentially involved in Phase I, II or III drug-metabolism. We employed several different sources for the purpose of curating such a list (Table 2.1). For so-called Phase I and II enzymes, we first included all of the gene families containing classically accepted DMEs (Aldh, Cyp, Gsta, Sult, Ugt, etc.) [72]. In order to work with the broadest possible list (that may also be relevant to the handling of toxins and endogenous metabolites), additional enzymes were then added either based on their ability to catalyze the same class of reactions that are carried out by known DMEs, or previous studies [73, 74] suggesting the gene’s involvement in Phase I or II reactions. Paralogs and orthologs of known DMEs were included as well. For phase III transporters, we included all members of Abc and Slc transporter subfamilies that include known drug transporters, as classified by the UCSF-FDA TransPortal [75] (Abcb, Abcc, Abcg, Slc10, Slc21, Slc22, Slc47, Slc51). Some of these transporter family members are associated with metabolite rather than drug handling. However, transporters can typically transport a
range of chemical moieties, metabolite handling is commonly reported for classical drug transporters, and many metabolic transporters can bind or transport drugs. The list of “clinically-relevant” DMEs continues to evolve, particularly for non-hepatic tissues; so, at this first stage, we aimed for inclusivity. However, data was collected from multiple species; some gene IDs chosen based on human data may or may not exist as identically-named homologs in rodents, and vice versa. Others might exist only in rat or mouse, but not both. The resulting list is summarized in Table 2.1.

2.4.2 Analysis of changes in DME expression during pre and postnatal kidney development identifies a set of potential transcriptional regulators

We sought to determine how the DMEs were transcriptionally regulated throughout pre and postnatal renal development. Of 455 DMEs (201 Phase I, 183 Phase II, 71 Phase III) annotated on the Mouse 430 2.0 microarray platform, 297 were expressed in either embryonic or adult proximal tubules, of which 159 were significantly changing (p<0.05) at least 2-fold (Fig. 2.1A). Of those 159, 66 belonged to Phase I, 71 belonged to Phase II, and 22 belonged to Phase III. Interestingly, 37 of the 159 changing DMEs were found to be downregulated in adult proximal tubules compared to embryonic kidney, indicating selective regulation of DMEs opposed to a general increase in expression of “terminal differentiation” genes, as is sometimes assumed to be the case.

The list of 159 significantly changing genes, along with corresponding log ratios, was then analyzed using the Ingenuity Pathway Analysis software suite (IPA). Upon analyzing changes of DME expression in proximal tubules from E15.5 to adulthood, 7 upstream transcriptional regulators were predicted to be activated: Hnf4a, Nr3c1 (Gr), Nfe2l2 (Nrf2), Ppara, Hnf1a, Tp53 and Nr1i3 (Car). However, the IPA knowledge base
compiles information from multiple tissues, species and experimental models, which can introduce error when looking for the most likely regulators of gene expression in particular tissue. To help resolve this, we examined the expression of the predicted regulators in E15.5 and adult proximal tubules, shown in Figure 2.1B. Multiple studies have reported expression of Hnf1a in the proximal tubule, and deletion of Hnf1a leads to broad defects in proximal tubule transport function in mature animals [30]. Figure 2.1C displays these transcription factors in the predicted regulatory network, with their corresponding predicted targets. While the Car nuclear receptor is heavily associated with regulating drug metabolism in the liver, it was omitted from the network because it is not significantly expressed in the kidney. The remaining predicted transcriptional regulators – Tp53, Hnf4a, Nfe2l2, Ppar alpha, Hnf1a, and Gr – are highly expressed in prenatal and adult proximal tubules. If one further examines the level of connectivity, it is noteworthy that Gr, which is thought to regulate proximal tubule maturation in vivo, and Hnf4a, were both connected to 33 predicted targets; this was followed by a decline in connectivity to 26, 22, 18 and 17 for Nfe2l2, Ppara, Hnf1a and Tp53, respectively.

2.4.3 Analysis of the proximal tubule transcriptome during developmental time points reveals a dominant contribution of DMEs

Nevertheless, sampling proximal tubule expression at two extremes along the developmental spectrum does not provide sufficient resolution to determine the dynamics of this transition. While proximal tubule expression profiles have not been collected from other time points, a thorough time series of genome-wide expression data has previously been collected from whole rat kidneys at various stages of development [10]. Although physiological studies clearly indicate that the proximal tubule is an important site of drug
metabolism and transport, due to the many cell types that make up the kidney, it is difficult to attribute the expression profile of widely-expressed genes in the whole developing and adult organ to any single cell type. Therefore, we restricted our analysis to proximal tubule-enriched genes, as previously defined [11]. When focusing on DMEs that are enriched in the proximal tubule in the kidney, it became apparent that they are a major contribution to proximal tubule cell gene expression. Even though those DMEs in our classification account for less than 3% of all protein-coding genes, they make up approximately 12% of the total PT-enriched kidney transcriptome (Fig. 2.2). Furthermore, the dynamics of DME regulation were revealed. Initial induction of DME expression can be seen in late embryonic stages, followed by a surge shortly after birth, and continues to increase into puberty. While it is conceivable that a fraction of the observed increases in transcription might be attributed to a rising fraction of proximal tubule cells, it is important to emphasize that many accepted proximal tubule markers do not change. This suggests regulatory mechanisms independent of cell number. Based on the expression profile of PT-enriched genes, we concluded that three distinct stages minimally describe the transitions in proximal tubule transcription: late embryonic development, postnatal maturation, and adulthood. In mature proximal tubule cells, DMEs are some of the highest expressed genes. This suggests that transcriptional regulatory mechanisms involved in proximal tubule maturation likely include those playing a key role in the regulation of DMEs.

2.4.4 Motif analysis of Ep300-bound cis-regulatory elements in the developing kidney suggests Hnf4a and Hnf1a as key regulators
While we had already established a list of top candidate transcriptional regulators of DMEs, we sought to isolate the dominant contributor(s). Recent data from the ENCODE project and other published work indicates that cis-regulatory enhancer elements are key determinants of cell-specific expression, and they are enriched in binding motifs and binding events of transcription factors responsible for lineage determination [61, 63, 76, 77]. Thus, we set out to characterize the co-localization of a known enhancer marker, Ep300, in adult rat proximal tubules using ChIP-sequencing. We obtained high quality data in terms of signal-to-noise and number of peaks; the ChIP-seq yielded 11,726,046 unique mappable positions for p300, and 7,785,098 for the control input sample in the rn4 genome build. 18% of the mapped reads were contained within highly enriched regions, resulting in 42,537 peaks (Fig. 2.5A). Of those 20,590 were located in intergenic regions, 14,252 in introns, 906 in exons, and 7,439 in promoters [-1000 to +100bp relative to transcription start site (TSS)]. Based on the literature [78], this distribution is consistent with expectations of specific p300 marks. Screenshots of representative peaks are displayed in Figure 2.3A.

Ep300 cannot directly bind DNA; rather, it enhances transcription by interacting with DNA-bound transcription factors. Thus, the detected binding sites are contingent on the presence of recruiting transcription factors. We used the HOMER software, which has previously been effectively used to identify functional motifs [61], to perform motif analysis on 34,034 “distal” enhancers, defined by peaks located more than 1Kb upstream and 500bp downstream of annotated TSSs. Consistent with our earlier pathway analysis, the HOMER software determined that the highest enriched de novo motif best matched the known binding motif for Hnf4a (Fig. 2.3B). The second most enriched motif was the
target sequence for Ctcf, an insulator protein that helps establish chromatin architecture. This finding of Ctcf agrees with previous studies, which found CTCF enrichment at enhancers [63] and DNaseI hypersensitive sites [79]. Interestingly, the third most enriched motif matched the known Hnf1a motif, a known regulator of proximal tubule cell identity. As already mentioned, the contribution of Hnf1a to mature proximal tubule function has been previously examined in a mouse knockout model, but the role of Hnf4a and Hnf1a in the developing proximal tubule, especially with respect to DME regulation, is not well understood.

2.4.5 Transcriptomic analysis of microarray data from Hnf4a tissue-specific knockouts revealed that distinct subsets of Phase I, Phase II and Phase III genes were affected in developing liver, colon, intestine and pancreas.

There is no mouse model with a kidney-specific deletion of Hnf4a; this may reflect a role very early in kidney development (prior to proximal tubule morphogenesis) [46], in addition to the later postnatal role in proximal tubule DME regulation that we have focused on here. Nevertheless, this nuclear receptor has been deleted in five other developing and mature tissues; the knockout tissue has been subjected to microarray analysis. Together, these tissues express most of the DMEs from our original curated list. Therefore, it is possible to determine which of the DMEs has direct or indirect regulation of its expression by Hnf4a; to the extent that these DMEs overlap with the set identified above in the developing proximal tubule of the kidney, it may be possible to infer a high probability of transcriptional regulation by Hnf4a in the kidney.

To this end, we analyzed the published microarray expression data from five tissues with specific Hnf4a knockout models: E18.5 liver [65], E18.5 colon [66], adult
colon [67], adult small intestine, and adult isolated B-islet cells [41]. Of note, the consequence of deletion of Hnf4a has varying effects on the different tissues. Hnf4a is required for epithelialization and functional differentiation of hepatocytes; as a result, the consequences of removing this gene in the liver are severe, leading to embryonic lethality. The differentiation and function of the colonic epithelium is altered, but this does not prevent the animal from reaching adulthood. While the small intestine also expresses Hnf4a, its deletion apparently has minimal morphological consequences [80]. Finally, B-islet cells lacking Hnf4a exhibit mild phenotypic and functional consequences, despite marked differences in the transcriptome.

We compared Hnf4a /- tissues to matched wild type samples and identified significantly changing (p<0.05, FC>1.3) probe sets in each tissue (Fig. 2.4A). We then assembled a list of DMEs that were differentially-expressed as a result of Hnf4a deletion in at least one tissue. The list includes 203 regulated DMEs (96 Phase I enzymes, 79 Phase II enzymes, and 28 Phase III transporters). Liver experienced the most changes in DME expression; followed by adult colon, B-islets, small intestine and embryonic colon, respectively. Of note, 156 of the 203 DMEs downstream of Hnf4a regulation in at least one knockout tissue are expressed in either embryonic or adult mouse proximal tubules, and they thus represent a group of DMEs potentially regulated in the proximal tubule by Hnf4a under either basal or stimulated conditions (Fig. 2.4B).

Interestingly, not a single DME gene was significantly changing in more than three tissues. In most cases, genes affected in more than one tissue were changing in the same direction. However, there are examples of the same gene being regulated in opposite directions in different tissues. Of the 203 affected DMEs, 12 changed in three
tissues, 47 in two, and 144 were specifically regulated in a single tissue. On the other hand, expression of related gene family members in different tissues was frequently observed. These findings further supported that Hnf4a plays an important role in transcriptional regulation of DMEs in specific tissues during development, as well as in mature tissues. However, based on the large divergence of affected downstream targets, Hnf4a-mediated regulation appears to highly depend on cellular context.

2.4.6 Hepatocyte nuclear factor 4a plays a major role in establishing and maintaining transcriptional enhancer elements that regulate DME genes in the proximal tubule

To gain more insight into proximal tubule-specific regulation, we used ChIP-seq to determine the co-localization of Hnf4a in rat proximal tubule cells at the three earlier-identified stages – pre-natal differentiation, maturation and adulthood. To represent these transitions, we selected the following three time points: 20 days post coitum (E20), 2 weeks old (P13) and adult (8 weeks). By E20, late differentiation events are occurring, although new nephrons are still being formed; importantly, at this time point, the kidney is naïve to the influences of birth and the extra-uterine environment. At P13, new nephrons are no longer being formed [20], but the transcriptional profile or functional capacity has not yet reached mature levels [19]. Finally, the proximal tubule reaches maturity after puberty, which occurs around 4-6 weeks in rodents. In the kidney, Hnf4a expression has been reported in condensed mesenchyme [46], and in segments of nephron progenitor structures throughout all of nephrogenesis [47], though ultimately its expression becomes restricted to the proximal tubule. Thus, even when using chromatin prepared from the entire population of kidney cells, Hnf4a-binding can be attributed to Hnf4a-expressing cells.
Figure 5A quantifies general results of Hnf4a ChIP-seq, along with the p300 data. At E20, Hnf4a occupancy was detected at 38,145 sites, revealing robust activity during late embryogenesis. During maturation, at P13, Hnf4a was detected at 52,541 locations. In mature proximal tubules, 79,871 sites containing Hnf4a were found (Fig. 2.5A). Thus, the number of Hnf4a binding locations in proximal tubules more than doubles from initial differentiation until reaching maturity. Subsequently, Hnf4a-mediated transcriptional regulation is expected to change as a result of gained or lost binding events. Figure 6 shows representative screenshots of binding events and peak overlap from all four ChIP-seq experiments. Perhaps one of the most striking findings was the high level Hnf4a and p300 co-localization (Fig. 2.5B). At E20, 14,966 Hnf4a peaks, 39% of all peaks, were at locations that are occupied by p300 in mature proximal tubules. At P13, the number goes up to 20,209, or 38% of total Hnf4a peaks at this age. Finally, in the adult, 25,171 Hnf4a binding locations, or 32% of all peaks, are co-occupied by p300. This suggests that Hnf4a might directly recruit p300, thus establishing cis-regulatory enhancer elements. Furthermore, the high level of Hnf4a-containing enhancer elements in adult proximal tubules implies a large role for Hnf4a in establishing gene expression profiles in these cells, exemplified by a specific DME repertoire.

We also examined differential binding of Hnf4a at the three developmental stages (prenatal, postnatal, adult). Peak overlap is one possible approach; however, this fails to differentiate between significant changes and borderline differences that affect peak calling. Instead, we identified peaks with at least 4-fold changes in tag density at different time points (Fig. 2.5B). Of 38,145 peaks present at E20, 6,285 were significantly downregulated or completely lost by P13. Conversely, only 1,856 of 52,541 peaks
present at P13 were downregulated/absent in adult proximal tubules. Both transitions experienced comparable numbers of upregulated Hnf4a occupancy, with 13,432 upregulated sites from E20 to P13, and 17,706 upregulated sites from P13 to Adult (Fig. 2.5B). Interestingly, motif analysis of Hnf4a peaks revealed that Hnf1a binding motifs were enriched at Hnf4a binding sites in P13 and adult proximal tubules, but not at E20 (data not shown).

Thus, there are substantial changes in Hnf4a binding during pre and postnatal development. Based on Hnf4a occupancy throughout proximal tubule development and maturation, it is clear that it plays important roles in regulation of the proximal tubule transcriptome. We then focused in on peaks near or within DMEs, which are likely to regulate that locus. We found that most of the expressed DMEs were bound by Hnf4a near or within the genes, many of which contained peaks near the TSS. While there was a notable degree of differential binding, it was more common to see peaks that were present at E20 become more enriched and formation of new peaks as proximal tubule cells mature.

Part of Hnf4a-mediated regulation of DMEs in the proximal tubule may depend on exerting specific effects on other DME transcriptional regulators, rather than directly targeting DMEs. It appears that this is plausible – based on Hnf4a binding at relevant nuclear receptors. Very high Hnf4a enrichment was observed along the Ppara gene locus, a nuclear receptor predicted earlier (Fig. 2.1A) to regulate a subset of DMEs in the PT. In contrast, Pparg had a very low number of binding events, consistent with lack of expression (Fig. 2.6A). An interesting observation was a modest presence of Hnf4a co-localization around genes that are not basally expressed in the proximal tubule,
sometimes even directly at the promoter. Conversely, all predicted DME transcriptional regulators included in Figure 1 had high Hnf4a enrichment at multiple sites, often including the promoter.

2.4.7 Administration of an Hnf4a small molecule antagonist in an ex vivo kidney organ culture model markedly attenuated the expression of representative Phase I, II and III DMEs

Our findings support the view that Hnf4a plays an important role in transcriptional regulation of DMEs during kidney differentiation and maturation. There is currently no reported mouse model with kidney-specific ablation of Hnf4a to test this in vivo. Instead, we utilized a recently developed small molecule compound that specifically antagonizes Hnf4a activity [69]. With this compound, we tested the effect of Hnf4a downregulation in embryonic kidney organ culture (grown for 6.5 days to allow nascent PT formation), which recapitulates many characteristics of proximal tubule differentiation, including the acquisition of organic anion transport mediated by Oat1 and Oat3 [68]. Based on in vitro and in vivo knockout data, this is generally believed to be the main pathway for kidney elimination of many common drugs (e.g. antibiotics, antivirals, diuretics, nonsteroidal anti-inflammatory drugs) and toxins (e.g. mercury conjugates) [81, 82]. This pathway has received increased attention given new FDA guidelines, and its role in neonatal drug handling is of considerable interest.

Hnf4a inhibition in kidney culture caused differential expression, mainly (but not exclusively) downregulation, of many representative Phase I, II and III drug-metabolizing genes (Fig. 2.7). Targets were selected based on exhibiting PT-specific expression in the kidney (Fig. 2.2), known expression in embryonic proximal tubules, presence of Hnf4a
binding near or within the gene, and in some cases because they were regulated by Hnf4a in other tissues (Fig. 2.4). Some genes exhibited a dose-dependent effect in downregulation, while expression of others was maximally downregulated with the lower antagonist concentration, without further decrease at a higher concentration. Of the genes tested, several did not change or were upregulated, indicating that the antagonist had a selective effect on only a certain subset of PT DMEs, presumably those regulated by Hnf4a. This role of Hnf4a was further explored below by lentiviral transduction into cells.

2.4.8 Overexpression of Hnf1a and Hnf4a in mouse embryonic fibroblasts induces expression of proximal tubule Phase I, II and III DMEs

To further explore the role of Hnf4a, and the highest associated co-regulator Hnf1a, we examined the capacity of these factors to induce expression of DMEs highly expressed in the proximal tubule in MEFs. Using lentiviral transduction, mouse Hnf1a and Hnf4a cDNA with downstream EGFP was introduced either individually or in combination into primary fibroblasts derived from E16.5 mouse embryos. Approximately 75% transduction efficiency was routinely achieved (Fig. 2.8A). As shown in Figure 2.8B, transduction of Hnf1a did not induce expression of endogenous Hnf4a, or vice versa; however, both genes are expressed within the cell population upon transduction of both factors. Unaltered expression of p53 suggests the lack of apoptotic response from the genomic integrations or overexpressed proteins. As can be seen in Figures 2.8C and 2.8D, expression of a limited number of DMEs and epithelial markers [E-cadherin (Cdh1) and Tight junction protein 1 (Tjp1)] was induced to different extents by Hnf1a or Hnf4a alone. Importantly, expression of many genes was dependent on the presence of
both Hnf4a and Hnf1a. We then sought to determine if the function of some genes relevant to drug metabolism by the proximal tubule upon transduction of Hnf1a and Hnf4a was consistent with their mRNA expression. To this end, we examined the ability of transduced MEFs to take up 6-Carboxyfluorescein in a probenecid-sensitive manner, which is a classical indicator of Slc22a6 (Oat1) function. Oat1 is one of the major drug, toxin and metabolite transporters in the developing and mature proximal tubule [52, 53, 83-85]. As shown in Figure 8E, upon expression of Hnf1a and Hnf4a, transduced MEFs gained the ability to accumulate 6-CF through a functional specific transport mechanism (via Oat1). Although the bulk of the evidence suggests that the MEFs differentiated toward PT-like cells, without an exhaustive analysis of other tissue markers we cannot exclude the possibility of characteristics overlapping with other cell types expressing Hnf4a and Hnf1a.

2.5 DISCUSSION

Even in such a well-studied organ as the liver, the developmental maturation of drug handling and metabolism—which depends on Phase I and Phase II enzymes as well as Phase III transporters – remains incompletely understood. Even less is known about other developing organs, including the kidney. In order to properly dose neonates and children, and to diminish adverse effects from drugs and environmental toxins, it is important to understand the molecular basis of drug and toxin handling. In this study, we have combined systems, molecular and cellular biology approaches to show that Hnf1a and Hnf4a cooperate to play a major role in the transcriptional initiation and maturation
of genes in the developing and postnatal proximal tubule involved in Phase I, II and III drug metabolism.

In order to approach the problem of prenatal and postnatal proximal tubule maturation, we began with a broad approach, employing microarray data to study a broadly-defined set of DMEs (Table 2.1) in the context of proximal tubule expression. Network analysis of transcriptional profiles of DMEs in nascent and mature mouse proximal tubules suggested roles for a small set of transcription factors which may be orchestrating the expression of Phase I, II and III genes in the proximal tubule (Fig. 2.1). Further analysis of a time-series of expression in rat kidneys not only defined the dynamics of DME expression but revealed a significant role of DMEs in defining the proximal tubule-specific transcriptome in the kidney. This finding suggested that lineage-determining factors associated with PT-specific enhancers on a global scale would likely regulate expression of DMEs.

In our studies, transcription factor motifs present in enhancer regions marked by p300 suggested that Hnf4a, along with Hnf1a – a known transcriptional regulator of proximal tubule function [30], might be the major regulators of PT expression of DMEs. We chose p300 over histone modifications to characterize enhancers due to two main advantages: 1) p300 cannot bind DNA directly, thus peaks represent presence of DNA-binding factors that recruited p300; 2) p300 peaks are more localized than histone modification, facilitating recognition of binding motifs of responsible targeting factors. Nevertheless, it is possible that additional information could be gained by examining other enhancer elements that lack p300 co-localization in future studies, which may offer
additional clues regarding the contribution of other transcription factors, potentially including those identified in Figure 2.1C.

Although there is good evidence that HNF4a can regulate transcription of a number of DMEs in the liver, there has been little direct evidence of this in the PT. A kidney-specific knockout has not been reported thus far, possibly because of an early defect in nephrogenesis that precedes PT differentiation [46]. Nevertheless, by restricting the list of targets downstream of Hnf4a in other tissues to those expressed in the prenatal or mature PT, we were able to suggest a subset of DMEs that are potentially basally or constitutively regulated by HNF4a in the proximal tubule (Fig. 2.4B). This subset had many Phase I, II and III genes known to carry out important reactions involved in drug metabolism. However, while many of these genes are likely to be regulated by Hnf4a in the PT, Figure 2.4A shows that there is a substantial portion of targets regulated in only a single tissue. Furthermore, it is important to note that some very important DMEs expressed in the PT fail to be detected by expression microarrays in these other tissues, such as Oat1 and Oat3.

Given the evidence strongly suggesting that Hnf4a might be playing a critical role in DME regulation in the proximal tubule, and the tissue-specific nature of this regulation, we decided to characterize the genome wide co-localization of Hnf4a in the developing kidney in-vivo at three distinct developmental stages – prenatal differentiation, postnatal maturation and adulthood. The time points chosen to represent these stages (E20, P13 and Adult) were chosen mainly based on analysis shown in Figure 2.2. However, the selection of time points was also supported by prior analysis of time series microarray expression data from developing and adult kidneys [10], as well as
functional correlations such as the in vivo ability to transport classical organic anion substrates such as para-aminohippurate, which may be viewed as a surrogate for the capacity of the kidney to eliminate drugs and toxins by the Oat1 transporter [19].

As mentioned, it has been previously shown that Hnf4a plays a key role in morphogenesis during kidney development – cap mesenchyme survival [46] – which occurs far before the differentiation and development of the PT; the role of Hnf4a in the maturation of the proximal tubule during late prenatal and postnatal development remained poorly defined. By using a recently developed small molecule antagonist which has been shown to selectively inhibit Hnf4a [69], we were able to explore the consequences of Hnf4a downregulation in early proximal tubules in 7 day organ culture, which serves as a model for kidney development. These have been shown to express Slc22 transporters such as Oat1 and Oat3 and eventually become capable of organic anion transport function [68]. As predicted, Hnf4a inhibition by the compound (but not a structurally similar inactive compound) resulted in markedly diminished expression of important Phase I, II and III DMEs, including Oat1 and Oat3, suggesting that Hnf4a is required to maintain basal expression of many functionally important DMEs in the PT. Expression of a small group of DMEs, however, was unchanged or upregulated in response to the Hnf4a antagonist, potentially indicating reversal of Hnf4a-mediated repression. These experiments supported a key role for DME regulation by Hnf4a in the whole organ culture. Nevertheless, we also sought to prove this directly by lentiviral transduction of Hnf4a and Hnf1a into MEFs.

Overexpression of Hnf1a and Hnf4a in MEFs provided the final clues to help postulate a working model for the role of Hnf4a in the transcriptional regulation of PT
development and function. For all of the tested genes aside from Gsta1 and Slc47a1 (Mate1), Hnf4a alone failed to induce expression of PT DME genes. Since we have also presented substantial data suggesting the involvement of Hnf1a (Fig. 2.1B,C and Fig. 2.3B), we also tested Hnf1a by itself. Hnf1a alone also showed little potential to induce expression of PT DMEs in MEFs. We were surprised to find that neither factor was able to induce the expression of the other one by itself, considering multiple lines of evidence for cross-regulation, and the fact that a set of clinically-relevant Phase III transporters – Slc22a6, Slc22a7, Slc22a8 and Slco1a1 – are significantly downregulated in Hnf1a-/- mature kidneys [86]. Remarkably, when introduced in combination, Hnf4a and Hnf1a were able to induce robust expression of many predicted DME targets in the PT (Fig. 2.8C).

Taking all of our findings into consideration, we suggest the following model. The primary role of Hnf4a is to set up basal and constitutive cis-regulatory enhancer elements, which then become accessible to other co-regulators. Considering the fact that Hnf4a plays important but varying roles in the liver and other tissues, chromatin state and other factors are likely to be important in helping establish tissue-specific binding profiles of Hnf4a. Hnf1a and other co-regulators may establish an additional layer of specificity in a combinatorial fashion, leading to the appropriate expression profiles in different tissues. Other co-regulators might involve other members of the nuclear receptor family, which are known to play important roles in regulating metabolic function in the proximal tubule, as well as transcriptional repressors. Of note, a generic nuclear receptor binding motif was highly abundant and enriched within Hnf4a peaks (data not shown). It might be possible that nuclear receptors regulate target genes via Hnf4a-established enhancers,
either by binding nearby or by competing for lower-affinity binding sites. In this context, it is worth pointing out that there were Hnf4a peaks at non-expressed genes that might be important in later proximal tubule function (eg. Car (Nr1i3), Pxr (Nr1i2)); some of these may be poised enhancers, perhaps requiring stimulation by other transcription factors (including those implicated in Figure 2.1C) in later life or during periods of stress. These nuclear receptors are thought to act as ligand-dependent “sensors” [4]. According to the Remote Sensing and Signaling Hypothesis, “drug” transporters such as those regulated by nuclear receptors play a role in regulating inter-organ communication in normal physiology, after perturbation of homeostasis and possibly during development; in this context, it is conceivable that HNF4a and other nuclear receptors play a key role in the sensing specific metabolic alterations, leading to the necessary changes in transporter and DME expression.

In summary, we have shown that Hnf4a is required for basal expression of DMEs in the proximal tubule. Furthermore, we demonstrated that, together, Hnf1a and Hnf4a are sufficient to induce the expression of representative proximal tubule Phase I, II and III drug-metabolizing genes, further supporting their roles in DME regulation in the developing PT. The data from this study should prove helpful in defining the steps involved in the transcriptional maturation of the proximal tubule that involve Hnf4a, Hnf1a and other transcription factors as they relate to the expression of drug transporters and Phase I/II drug-metabolizing enzymes.
2.6 ACKNOWLEDGEMENTS

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The work presented in Chapter 2, in full, is a reprint of the material as it appears in “Hepatocyte Nuclear Factors 4a and 1a Regulate Kidney Developmental Expression of Drug-Metabolizing Enzymes and Drug Transporters” by Gleb Martovetsky, James B. Tee and Sanjay K. Nigam in Molecular Pharmacology, 2013, 84(6): 808-823. The dissertation author is the primary author, and Sanjay K. Nigam is the senior author.

2.7 AUTHOR CONTRIBUTIONS

Participated in research design: Martovetsky, Tee, Nigam

Conducted experiments: Martovetsky

Contributed new reagents or analytic tools: Martovetsky, Tee, Nigam

Performed data analysis: Martovetsky

Wrote or contributed to the writing of the manuscript: Martovetsky, Nigam
Table 2.1. List of Drug-Metabolizing Enzymes and Transporters

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<th>Additional Genes</th>
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</tbody>
</table>
Figure 2.1. DMEs are dynamically regulated in proximal tubule development. (A) Of 297 DMEs expressed in either embryonic (E15.5) or adult mouse proximal tubules, 170 were significantly changing (P < 0.05), at least 2-fold. (B) Expression of IPA-predicted upstream regulators of DMEs in mouse PTs. *Although Hnf1a expression in the proximal tubule is well documented (Lazzaro et al., 1992; Pontoglio et al., 1996; Coffinier et al., 1999), there appeared to be issues with probe sets on the mouse chip; therefore, an expression value (not to scale) has been adopted from another publically available source – the Rat Proximal Tubule Transcriptome Database http://dir.nhlbi.nih.gov/papers/lkem/pttr/). Car expression was not detected in any of the datasets. (C) Ingenuity Pathway Analysis–predicted network of DME transcriptional regulation in proximal tubule development and maturation.
Figure 2.2. DMEs contribute to proximal tubule gene expression and cellular identity. While DMEs make up less than 3% of the total annotated genes, DMEs account for over 12% of proximal tubule–enriched gene expression in the rat kidney throughout development (subset of published data; Tsigelny et al., 2008), based on lists of enriched genes in E15.5 mouse proximal tubules, which were adapted from a published study (Brunskill et al., 2008). DMEs are highlighted in green (35/280 genes).
Figure 2.3. P300 ChIP-seq in adult rat kidney cortex reveals Hnf4a as the top transcriptional regulator in proximal tubules. (A) Screenshots of p300 peaks (tags per bp normalized to input). P300 is highly enriched at the Oat1, Oat3, and Mate1 loci, three highly expressed drug transporters specific to the proximal tubule in the kidney. Aldh3a2, which is expressed in the PT, had a strong peak at the promoter; conversely, Aldh3a1 and Mate2, which are not expressed in the PT, exhibited minimal p300 colocalization. There were multiple peaks at the Hnf1a locus, but none at Nr1i3, which is consistent with the finding that Hnf1a but not Car is expressed in the PT (Fig. 2.1B). (B) Motif analysis of promoter-distal p300 peaks revealed that the two most highly enriched transcription factor binding motifs matched those of Hnf4a and Hnf1a. The motif for the ubiquitous insulator CTCF (CCCTC-binding factor) was also enriched around p300 binding sites, which is consistent with previous studies.
Figure 2.4. Hnf4a is highly involved in tissue-specific expression of DMEs. (A) Comparison of significantly changing (FC > 1.3, P < 0.05 using uncorrected t-test) probe sets in four tissues (E18.5 liver, E18.5 colon, adult colon, adult B-islet cells) with conditional Hnf4a deletion. (B) Heatmap of significantly changing DME genes from same four tissues in A, in addition to small intestine, matched to expression levels in embryonic and adult mouse proximal tubules. Rows are color-coded for phase I, II, and III with yellow, green, and blue, respectively, and split into those expressed in the pre- and/or postnatal PT (top) and those that are not expressed in the PT (bottom). Their corresponding expression in E15.5 and adult PTs is shown on the left (red, high expression; light, low expression; white, not detected). Genes are sorted within phase I, II, and III groups by expression levels in adult proximal tubules. To the right, differential expression in corresponding tissues is depicted. Of 203 differentially expressed DMEs 156 were found to be expressed in proximal tubules.
Figure 2.5. Quantitation and characterization of ChIP-seq reads. (A) General measures of ChIP-seq data. (B) Pairwise quantitation of overlapping peaks between all four ChIP experiments (top), as well as differentially bound Hnf4a peaks with higher signal (without input normalization) in column relative to row (bottom)
Figure 2.6. Hnf4a regulates PT gene expression throughout kidney development and maturation. (A) Screenshots of peaks from all four ChIP-seq experiments. Oat1 and Oat3, two major drug transporters in the proximal tubule, accumulate Hnf4a binding events throughout development. While there was minimal colocalization of Hnf4a and p300 at the Pparg locus, there were multiple binding events associated with Ppara, which was implicated in Fig. 2.1C as a regulator of DME expression in the PT. (B) Schematic representing the overlap of all possible combinations of peaks. (C) Proportionally-scaled heatmap of tag densities of five representative subcategories highlighted in Fig. 5B.
Figure 2.7. Antagonism of Hnf4a in embryonic rat kidney culture leads to differential effects on phase I, II, and III DMEs. E13.5 rat kidneys were cultured on Transwell filters for 6.5 days, and treated with a small-molecule antagonist of Hnf4a or an analogous inactivated compound for 12 hours. (A) Schematic of embryonic kidney culture on Transwell filters. (B) Example of embryonic kidney cultured for 7 days (red, D. biflorus; green, E-cadherin; blue, DAPI). (C) Changes in expression in whole rat embryonic kidneys in response to Hnf4a inhibition with a small molecule antagonist, relative to treatment with carrier alone (dimethylsulfoxide).
Figure 2.8. Hnf4a and Hnf1a induce expression of proximal tubule DMEs. (A) Flow cytometry was used to quantify transduction efficiency, which was routinely around 70–80% as judged by the fraction of green fluorescent protein (GFP+) live cells. (B) Uninfected cells do not express Hnf1a or Hnf4a, while lentiviral transduction leads to expression of the respective genes. Unaltered p53 expression levels indicate lack of toxicity and apoptotic response. (C) Phase I, II, and III genes expressed specifically in the proximal tubule are strongly induced upon expression of Hnf1a and Hnf4a in combination. (D) Hnf1a and Hnf4a upregulate expression of epithelial markers Cdh1 and Tjp1. (E) Slc22a6 exhibits function in transduced MEFs, as indicated by probenecid-sensitive uptake of organic anion 6-carboxyfluorescein.
CHAPTER 3:

Transcriptional Switch Modulates Hnf1a and Hnf4a Specificity Toward Kidney Proximal Tubule-like or Hepatocyte-like Fate

3.1 ABSTRACT

Defining how sets of transcription factors guide the differentiation of cell types that may share some characteristics but ultimately perform very different essential functions in different organs (eg. kidney, liver) is crucial for further refining regenerative medicine and tissue engineering strategies. Although hepatocyte nuclear factors Hnf1a and Hnf4a have been identified as candidate lineage-determining transcription factors (TFs) for the kidney proximal tubule (PT), multiple studies have shown that these TFs also play key roles in determining hepatocyte cellular fate. This implies an additional important level of regulation, which we attempt to define here. We show that Hnf1a and Hnf4a induce expression of PT signature genes in mouse embryonic fibroblasts (MEFs) and cells derived from the metanephric mesenchyme (a PT precursor tissue). MEFs transduced with Hnf1a and Hnf4a form tight junctions and express the mRNA of multiple PT drug (eg. Slc22a6 or Oat1 and Slc47a1 or Mate1) and nutrient transporters (eg. Slc34a1) and constituents of intercellular junctions (eg. Tjp1 or ZO-1, cadherins, claudins, occludin). Importantly, the co-expression of GATA binding protein 4 (Gata4), and forkhead box TFs Foxa2 and Foxa3 downregulates PT markers and drastically upregulates hepatocyte markers, including albumin (Alb), apolipoprotein (Apoa1), and transferrin (Trf). A similar result was obtained with primary mouse PT cells. Thus, we
define a transcriptional switch by which Gata4 and Foxa2/Foxa3 modulates transdifferentiation toward PT cell-like versus hepatocyte-like fates induced by Hnf1a and Hnf4a. These findings should help advance various strategies aimed at restoring kidney and liver function.

3.2 INTRODUCTION

The kidney is a complex organ, containing over two dozen cell types with highly specialized functions that are spatially organized into segments, such as the proximal tubule, to carry out its functions. Along with reabsorption of organic solutes and water, as well as endocrine functions, PT cells play an important role in handling of drugs, toxins, gut microbiome products and endogenous metabolites. Many drugs excreted by the PT are first metabolized in the liver by Phase 1 and Phase 2 drug metabolizing enzymes (DMEs) before uptake and elimination by Phase 3 drug transporters in the PT; this paradigm also holds true for many nondrug substrates [87, 88]. Thus, the roles of these two organs are partially interdependent and must be highly coordinated for efficient detoxification and elimination. When these organs begin to functionally deteriorate, there are major disturbances in metabolism, drug handling and toxin elimination.

Because of the large number of patients with kidney and liver failure, and the long waiting lists for organ transplantation, there is a great need to devise tissue engineering and regenerative medicine strategies to replace kidney function. For this purpose, it is important to establish cells with characteristics that can partially or fully recapitulate the properties of mature cell types from a cultured cell source, which involves 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 (iPSCs), differentiating stem cells toward desired cell fates has become a promising strategy for future clinical application. However, due to the tumorigenic potential of stem cells, 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 transdifferentiation [36, 42-45]. While the derivation of proximal tubule-like cells from stem cells has been described [89], the establishment of PT cell characteristics starting with non-pluripotent cells or by inducing a defined transcriptional program has yet to be reported.

Here, we show that while Hnf1a and Hnf4a alone are insufficient to completely transdifferentiate various cell types towards a proximal tubule cellular fate, they are capable of inducing the mRNA expression of a number of genes important for proximal tubule identity and function, and they lead to establishment of tight junctions. Remarkably, co-expression of either Gata4, a combination of Foxa2 and Foxa3 (Fova2/3) 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. Furthermore, Gata4 and Foxa2/3 downregulated PT markers and induced expression of hepatocyte markers in primary PT cells. Together, these findings define a foundation for transdifferentiation towards proximal tubule cells by inducing a defined
transcriptional program, and clarify the involvement of Hnf1a and Hnf4a in transdifferentiation towards hepatocyte cellular identity.

3.3 EXPERIMENTAL PROCEDURES

All animal procedures were approved by IACUC.

3.3.1 Cell Culture

E16.5 MEFs were prepared as previously described [90]. E13.5 and E15.5 MEFs were prepared using a modified procedure. Briefly, after the head and visceral organs were removed from embryos, remaining tissue was minced in PBS in a culture dish. The PBS was then replaced with 0.25% Trypsin/EDTA containing DNAse, and incubated at 37ºC for 10 min, triturated, and then incubated another 10 min. DMEM/F-12 media with 10% FBS, non-essential amino acid supplement (NEAA) and Penicillin/Streptomycin (media A) was then added to quench the reaction (this was the same media later used for cell culture), and the suspension was transferred to a 50 mL conical tube and gravity pelleted to let undigested pieces settle. The suspension was then plated in tissue culture flasks with additional media. In some cases, an aggregate composed of lysed cells and DNA formed, which was aspirated from the culture. The cells were then expanded, trypsinized, pelleted, and frozen in cryovials in 45% media, 45% FBS and 10% DMSO for future use. The origin of BSN [91] and IMCD-3 [92] cells has been previously described and they were cultured in the same media as MEFs. For investigating junctional formation, rat type I collagen-coated plates were used.

3.3.2 Lentiviral Transduction
Lentivirus was produced using as previously described [90] with slight modifications. Briefly, the same plasmids were used as previously described. In addition, the following plasmids were used to make the corresponding lentiviral preparations: pWPI control plasmid, pWPI-Gata4, pWPI-Foxa2 and pWPI-Foxa3 (gift of Dr. Lijian Hui). HEK 293T cells were cultured in “media A”. On the day of HEK 293T transfection, media was replaced with DMEM/F-12 containing 10% FBS and NEAA, without antibiotics. The following day, the media was replaced with “media A”, discarded and replaced the next day. On the second day after transfection, the media was collected, replaced with fresh media, and collected again the next day. The supernatant from the first collection was kept at 4°C until the second collection, then pooled, filtered through a 0.45uM syringe filter, and at 23,000 x g at 4°C for 24 hours. One-one hundredth of initial volume of PBS containing magnesium and chloride was then added to the pellet, and allowed to incubate at 4°C overnight. Then, the pellet was resuspended, and aliquoted, and stored at -80C. Viral titer was then tested by infecting MEFs with serial dilutions of viral preps in the presence of 8 ug/mL polybrene (Sigma-Aldrich) and measuring GFP+ viable cell fractions using an Accuri C6 flow cytometer. After overnight infection, media was switched to DMEM-F12 containing 1% FBS, Penicillin/Streptomycin, 1x NEAA, 1x Insulin-Transferrin-Selenium (Invitrogen), 20ng/mL epidermal growth factor (R&D Systems), 4ng/mL triiodothyronine (Sigma-Aldrich), and 20ng/mL dexamethasone (Sigma-Aldrich) (media B). For expression analysis, RNA was collected one week after transduction; immunohistochemistry was performed 3 weeks after transduction.

3.3.3 Primary Proximal Tubule Cell Culture
Adult mouse kidneys were placed in ice-cold PBS and decapsulated. The cortex was then separated from the medulla, minced into small pieces, and transferred to a 50 mL conical tube. The PBS was aspirated, and replaced with L-15 media containing 1 mg/mL Collagenase Type IV, 10 units/mL DNAs and 1% Penicillin/Streptomycin, and placed in a shaker at 37°C for 15 minutes. The suspension was then triturated, placed back in the shaker for an additional 15 minutes, triturated again, and allowed to gravity pellet for 1 minute to allow the undigested pieces to settle. All following procedures were carried out in a sterile environment. The suspension was passed through a 100 uM mesh cell strainer. Remaining tubules on the mesh were washed with “media A”, and then the cell strainer was inverted and the tubules were rinsed from the mesh with a 1:1 mix of “media A” and “media B” with the addition of 1x antibiotic/antimycotic into a tissue culture plate, and inspected under a microscope to confirm enrichment of proximal tubule segments and depletion of glomeruli. The resulting tubular suspension was then plated into Collagen I-coated 6-well plates. After two days, media was replaced with “media B,” cells were infected with lentivirus for 8 hours, and media was replaced with fresh “media B.” Media was changed again 3 days after infection. RNA was collected for qRT-PCR analysis 6 days after infection.

3.3.4 Quantitative Reverse Transcription PCR

RNA extraction, cDNA preparation, and RT-qPCR was carried out as previously described [90]. The list of primer sequences is included in Table 3.1.

3.3.5 Immunohistochemistry/Microscopy

Phase microscopy was conducted using a Nikon Eclipse TE3000 microscope with an attached Nikon D50 camera. Fluorescent microscopy of GFP+ cells and
immunostained cells was carried out using a Zeiss Axio Observer A1 microscope. Immunostaining was carried out as previously described with slight modifications [90]. Cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS) overnight at 4°C. Fixation was quenched with 50mM glycine in PBS for 30min at room temperature. Cells were then blocked with 10% BSA in PBS containing 0.1% Tween-20 and 0.05% Triton X-100 for 1hr 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 .1% Tween-20 and .05% Triton X-100 with 2% BSA (IHC buffer). Cells were then washed with “IHC buffer” 3 times for at least 1 hour per wash at room temperature, and then incubated with secondary antibody (anti-mouse Alexa fluoro 594, Life Technologies) overnight at 4°C. Cells were then washed 3 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 Fluormount-G. ImageJ software was used for image processing.

3.3.6 Microarray analysis

For analysis of expression in embryonic and adult isolated proximal tubules and liver tissue, we used Agilent GeneSpring software and publically available data: GSM144594-144595, GSM152247-152249 (E15.5 mouse PT); GSM256959-256961, GSM490067-490069 (adult mouse PT); (E15.5 mouse liver); and (adult mouse liver). Samples were normalized using the RMA 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 MAS5 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<.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.

3.3.7 ChIP-sequencing

Hnf4a and p300 ChIP-seq and analysis has been previously published [90]. The UCSC genome browser was used to generate screenshots of Hnf4a and p300 binding at specific genes of interest. ChIP-seq data of H3K4me1 and H3K4me3 enrichment in human kidney tissue was obtained from NCBI (GSM773001 and GSM773005). Enhancers and enriched motifs were identified using HOMER software [61]. Briefly, the “analyzeChIP-Seq.pl” script was used to analyze H3K4me1 with H3K4me3 as the control (input) file (with the global options -enhancer and -style histone).

3.4 RESULTS

3.4.1 Potential of Hnf1a and Hnf4a to induce a proximal tubule mRNA expression signature is dependent on cellular context

While hepatocyte nuclear factors Hnf1a and Hnf4a are known to play a role in regulating DMEs and transporters in the kidney PT [90], in the context of transdifferentiation, this may vary depending on which transcriptional regulators are expressed in the starting cell type, as well as the epigenetic landscape and chromatin organization. Thus, to evaluate the robustness of Hnf1a and Hnf4a overexpression in inducing expression of proximal tubule markers, we examined their effect in multiple cell types. First, we compared three independently prepared MEF populations from E13.5, E15.5 and E16.5 embryos. In all instances, when the MEFs were transduced with both Hnf1a and Hnf4a, the transcription of prominent proximal tubule markers Slc22a6 (Oat1)
and Slc34a, in addition to Slc47a1 (Mate1), which are highly expressed in the proximal tubule, were significantly upregulated compared to corresponding MEFs that were either uninfected or transduced with GFP alone (Fig. 3.1). Interestingly, in two of the populations (E13.5 and E15.5, which were prepared slightly differently from the E16.5 MEFs, as described in Methods), ectopic expression of Hnf4a induced the expression of endogenous Hnf1a, albeit at lower levels than when transduced with Hnf1a (data not shown). This level of Hnf1a was sufficient to complement exogenously expressed Hnf4a in inducing expression of the four PT markers. Nonetheless, the results are consistent with our finding that Hnf1a and Hnf4a are synergistic in inducing the mRNA expression of several key proximal tubule genes upon transduction into MEFs [90].

While MEFs are a convenient cell culture model of relatively plastic and highly proliferative somatic cells, they originate from the ectoderm germ layer whereas the kidney originates from the mesoderm; thus, MEFs are “developmentally distant” from proximal tubule cells. We therefore set out to determine how a kidney-derived cell line which contains some properties of kidney progenitor cells – the BSN cell line [91] – would respond to Hnf1a and Hnf4a transduction. This cell line was derived from the metanephric mesenchyme (mass of progenitor cells that is the source of nephrons and stroma of the kidney) and shares multiple characteristics with pre-proximal tubule cells [93-95]. Consistent with this view, BSN cells responded to transduction of Hnf1a and Hnf4a with the transcriptional activation of proximal tubule markers such as Slc22a6, Slc34a1 and Slc47a1 (Fig. 3.1).

In light of the findings that a kidney progenitor tissue-derived cell line responded to Hnf1a and Hnf4a overexpression very similarly to MEFs, we decided to test the effect
of Hnf1a and Hnf4a in another kidney-derived cell line that is not derived from the MM – mIMCD-3 cells [92], which were derived from mature inner medullary collecting ducts. While BSN and mIMCD-3 cells can both be traced back to the intermediate mesoderm (IM), their fates diverge upon the formation of the epithelial nephric duct (Wolffian duct) and the eventual condensation of IM cells to form the MM [96, 97]; furthermore, mIMCD-3 cells were derived from terminally differentiated adult tissue. When transduced with Hnf1a and Hnf4a, none of the three tested “PT signature genes” were induced at appreciable levels (Fig. 3.1). Thus, compared to MEFs and the BSN cell line, IMCD cells were poorly responsive to overexpression of Hnf1a and Hnf4a (as measured by PT signature gene expression). Together, these findings indicated that Hnf1a and Hnf4a overexpression consistently result in the transcriptional activation of a proximal tubule expression profile in non-terminally differentiated cells. However, while Hnf1a and Hnf4a transcriptionally activated a number of proximal tubule markers, complete transdifferentiation was not achieved under tested conditions. Several other proximal tubule markers were tested, including Slc5a2 (Sglt2), Slc22a8 (Oat3) and Kap (kidney androgen regulated protein); while their expression was noticeably upregulated upon Hnf1a and Hnf4a overexpression (data not shown), they were expressed at two orders of magnitude below the genes shown in Figure 3.1.

In addition to investigating the effect of Hnf1a and Hnf4a expression in cell types with different developmental origins, in our preliminary studies we also examined their effect in human cells. Because we first identified Hnf1a and Hnf4a as candidate PT lineage-determining transcription factors by motif analysis of p300-bound distal enhancers in rat kidney cortex [90], we performed a similar analysis on publically
available H3K4me1 and H3K4me3 ChIP-seq data obtained from normal donor kidney tissue. We defined distal cis-regulatory enhancers as regions with H3K4me1 enrichment lacking H3K4me3 enrichment outside of proximal promoter regions; this strategy has previously been utilized to identify cis-regulatory elements highly enriched for lineage-determining transcription factor binding motifs for multiple tissues and cell types [61]. Motif analysis of these distal enhancer elements in human kidney tissue revealed motifs for HNF1a and/or HNF4a among the most enriched binding motifs, suggesting that they likely play key regulatory roles in the human proximal tubule. These results further supported the relevance of HNF1a and HNF4a in regulating gene expression in human cells. However, we chose to focus our experiments on MEFs due to their practicality as a model system for transdifferentiation and their generally more robust response in the experiments described below.

3.4.2 Transduction of Hnf1a and Hnf4a alters the morphology of MEFs and induces formation of tight junctions

While 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 of E-cadherin (Cdh1), an adherence junction marker, and tight junction protein 1 (Tjp1; also known as ZO-1 or zonula occludens-1), a tight junction marker, both of which are present in mature PT cells [90]. 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. 3.2A). Furthermore, additional components
of intercellular junctions were upregulated, including occludin (Ocln), tight junction protein 2 (Tjp2) and claudin 6 (Cldn6) (Fig. 3.2A). While 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 (Table S1); this result suggests that while 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. 3.2B). When the transduced MEFs were cultured on collagen-coated plates for three weeks, we observed 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. 3.3B-E).

3.4.3 More extensive characterization of the mRNA expression of transporters and junctional component genes reveals differential and synergistic regulation by Hnf1a and Hnf4a

To better gauge the extent of transdifferentiation towards PT cell fate by Hnf1a and Hnf4a overexpression, we chose to examine a cohort of genes involved in influx/efflux transport and junction formation, both defining characteristics of PT cells and relevant to their physiological function (Fig 3.4, Table 3.2). This provided a more extensive representation of the dynamic range of gene expression changes caused by Hnf1a and Hnf4a; in addition, it examined the potential complexity of changes induced by these two factors when overexpressed alone or together.
The larger sample size revealed that target genes have quite different responses, so we grouped the genes based on their transcriptional responses, which resulted in 4 predominant modes of regulation: 1) synergistic, often with Hnf1a and/or Hnf4a having some effect on its own (Fig. 3.4A); 2-3) regulated by Hnf1a independent of Hnf4a (Fig. 3.4B) and vice versa (Fig. 3.4C); and 4) downregulated by Hnf4a (Fig. 3.4D). Several genes did not fall into these categories and were either upregulated by one of the factors while downregulated by the other or, in a single instance, upregulated by either without synergism (Fig. 3.4E); additional genes differentially regulated in one of the ways described above but expressed at comparably lower levels are shown in Figure 3.4F.

The most prevalent trend was transcriptional upregulation by Hnf1a and Hnf4a in a synergistic fashion, including the “PT signature genes” Slc22a6, Slc34a1 and Slc47a1, as well as important drug-transporters such as Abcc2 (Mrp2), Abcc5 (Mrp5) and Abcg2 (Bcrp). (Fig. 3.4A). Interestingly, Cdh6 (Cadherin 6), which in the kidney is PT-specific, was most upregulated by Hnf1a transduction without Hnf4a (Fig. 3.4B); in contrast, Dsg2 (Desmoglein 2) and Dsc2 (Desmocolin 2), both important for desmosomal junction formation, were most upregulated upon Hnf4a transduction without Hnf1a (Fig. 3.4C). Finally, a number of genes that had a moderate to high level of expression in uninfected MEFs were unaffected by Hnf1a but noticeably downregulated upon Hnf4a overexpression, such as drug transporter Abcc1 (Mrp1) or cell polarity regulator Pard3; for the most part, co-expression of Hnf1a with Hnf4a counteracted the repression by Hnf4a. Detailed descriptions of all of the genes shown in Figure 3.4, along with their relative expression in embryonic and adult proximal tubule and liver tissue are included in Table 3.2.
Nevertheless, many of the genes involved in transport, junction formation and polarization that were transcriptionally upregulated in response to Hnf1a and Hnf4a did not reveal a bias towards a proximal tubule transcriptome versus hepatocytes (Table 3.2). In other words, while MEFs transduced with Hnf1a and Hnf4a began expressing PT markers, many of the other transcriptionally responsive genes are shared between PT cells and hepatocytes. Furthermore, some of the tested genes that are endogenously expressed at very high levels in proximal tubules exhibited a relatively weak transcriptional response to Hnf1a and Hnf4a transduction.

3.4.4 Liver lineage determining factors Gata4 and Foxa2/Foxa3 act as a transcriptional switch to redirect Hnf1a and Hnf4a-mediated transcriptional regulation from proximal tubule to hepatocyte cellular identity

While Hnf1a and Hnf4a might not be sufficient to fully transdifferentiate MEFs towards a proximal tubule fate, our results suggested that they are indeed lineage-determining TFs for proximal tubule cells. However, Hnf1a and Hnf4a, although never without additional factors, have both been used in reprogramming MEFs towards a hepatocyte-like phenotype [36, 42-45]. To gain a deeper perspective of the shared and defining characteristics of PT cells and hepatocytes, we analyzed the transcriptomes of isolated proximal tubules and liver tissue 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. 3.5A). We examined these
and other genes in the context of existing Hnf4a ChIP-seq data [90]. Indeed, we found that the PT marker genes that we selected were highly bound by Hnf4a in the late prenatal, postnatal and adult kidney (and p300 in adult kidney cortex, which is highly enriched in PTs), while the hepatocyte markers were almost completely devoid of Hnf4a and p300 binding in the kidney, with representative examples (Slc34a1, albumin) depicted in Figure 3.5B.

Next, we validated a number of selected markers by testing their expression in adult mouse kidney cortex and liver tissue (Fig. 3.6A,B). As predicted, Mate1, Oat1 and NaPi-2a were expressed much higher in the kidney cortex compared to the liver, while markers such as Transferrin (Trf), Transthyretin (Ttr), Apolipoprotein A (Apoa1), Albumin (Alb), Fabp1 and Serpina1 were expressed much higher in liver compared to cortex. 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 to Foxa1, we decided to use them in combination to activate transcription mediated by the Foxa family. We found that co-expression 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. 3.6C, D). While Gata4 co-expression had the strongest inhibitory effect on proximal tubule marker expression, it was insufficient to upregulate liver markers regardless of Hnf4a and Hnf1a presence. In contrast, while Foxa2/3 were also 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.

Finally, we tested the ability of Gata4 and Foxa3 to transdifferentiate primary mouse proximal tubule cells towards a hepatocellular fate (Fig. 3.7). 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, Kap, and Cdh16). Of the tested hepatocyte markers, only Trf expression was modestly induced (Fig. 3.7B). In contrast, Foxa3 overexpression not only had a repressive effect on Kap but also strongly induced the expression of a number of hepatocyte markers (Alb, Apoa1, Trf, Serpina1, and Ttr), with Alb and Trf responding synergistically to Gata4 and Foxa3 coexpression (Fig. 3.7D). While the transcriptional response in primary PT cells was more modest in scale compared to those observed in MEFs (Fig. 3.6C,D), this might be due to the reduced plasticity of terminally differentiated cell types compared to embryonic fibroblasts.

Nevertheless, these findings further supported our working model regarding the role of Hnf1a and Hnf4a in the transcriptional regulation of PT and hepatocyte cellular fate, which is presented in Fig. 3.8. We propose 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).

3.5 DISCUSSION

We have shown that, despite the use of Hnf1a and Hnf4a along with other factors in transdifferentiation towards hepatocyte-like cells, these two transcription factors are also at the core of transdifferentiation towards a proximal tubule-like cellular identity. In MEFs as well as BSN cells (which are derived from metanephric mesenchyme, the embryonic precursor of the PT), overexpression of Hnf1a and Hnf4a induced the expression of several key markers of proximal tubule cellular identity (Fig. 3.1). 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 metabolites and drugs (Fig. 3.4), 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. 3.3). Crucially, our studies indicate that without the co-expression of additional hepatocyte lineage-determining transcription factors, such as Gata4, Foxa2 and Foxa3, the transactivation specificity of Hnf1a and Hnf4a is insufficiently defined towards hepatocytes, and, indeed, may lean towards a proximal tubule cell expression signature (Fig. 3.6). Thus, these additional transcription factors (Gata4, Foxa2/3) may be viewed as “switching” the direction of transdifferentiation from
a cell expressing some PT markers to a more hepatocyte-like cell. The working model is presented in Figure 3.8.

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 often require the ability to generate large amounts of patient-specific cells in vitro. While induced pluripotent stem cells (iPSCs) have opened up a whole field of research focused on establishing various patient-specific cell types, it might be advantageous to circumvent the requirement for pluripotency and the threat of oncogenicity by using somatic cells as a cell source. If so, it may be crucial that the cells be differentiated as specifically as possible toward a fate reflective of mature organ function. This study – which clarifies how Hnf4a/1a expressing cells can be shifted from cells expressing some PT-specific genes to a hepatocyte-like fate – should be valuable in this regard. In light of the results in this study and others, it appears that while Hnf4a has been called a “master regulator,” and is necessary for the transcriptional regulation of a large number of genes, much of its function and specificity is dependent on the presence or absence of other co-regulators. This could provide flexibility of transporter expression that may be physiologically important. Injury to the liver or the kidney alters drug transporter expression in the injured organ and sometimes in the other tissue, which has been hypothesized to facilitate remote communication via small molecules to reestablish homeostasis (Nigam 2015, Nigam et al 2015, Ahn and Nigam 2009, Wu 2011). In light of our results, it is conceivable that Hnf4a/1a-centered regulation, modulated by co-regulators, might provide the flexibility for the kidney or liver to temporarily take on a subset of functions of another injured organ.
While MEFs transduced with Hnf1a and Hnf4a expressed a number of PT signature genes, as well as a number of other genes that are highly expressed in the PT but are also expressed in other tissues, some key PT genes were expressed at very low levels or not detected. Thus, it is likely that additional transcription factors are required to establish a fully PT cell fate; these additional factors might help refine specificity and/or suppress differentiation toward non-PT cell fates. Based on our results showing that the presence of either Gata4 or Foxa2/3 alters the effects of Hnf1a and Hnf4a transduction, it is also conceivable that other transcription factors that are not endogenously expressed in the proximal tubule might be expressed to some extent in MEFs and BSN cells – and thus be interfering with the ability of Hnf1a and Hnf4a to further induce a PT cell-like transcriptome. All of these factors are further complicated by the pre-existing epigenetic landscape of starting cells prior to transdifferentiation, which may contain some features that may be difficult to remove or that may make it difficult to establish new regulatory elements [98, 99]. The very different responses of MEFs and BSN cells compared to IMCD (ureteric bud-derived) cells to Hnf1a and Hnf4a transduction (Fig. 3.1) support these notions. In the future, it will be important to consider various cell types that are available in the clinical setting, as some may require different/additional inputs for achieving transdifferentiation.

In addition to providing evidence that either proximal tubule signature genes or hepatocyte signature genes are induced depending on absence or presence of Gata4 and Foxa2/3, we also demonstrated that Hnf4a binding in the kidney is enriched at PT signature genes and depleted at hepatocyte signature genes (Fig. 3.6B). 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 TFs are “pioneering transcription factors” [100], 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 co-regulators and transcriptional machinery, but also by establishing new binding sites that are otherwise inaccessible to Hnf4a alone.

In summary, we have shown that while Hnf1a and Hnf4a are lineage-determining factors for both proximal tubule cells and hepatocytes, the specificity towards either lineage is determined by coexpression of Gata4 and Foxa2/3, and possibly other factors that were not tested. Thus, we are able to suggest a model for a “transcriptional switch” between PT and hepatocyte fate, at least from the perspective of transdifferentiation (Fig. 3.8). Future studies should be aimed at identifying additional co-regulators that may need to be added or silenced to achieve complete transdifferentiation. Together, these findings advance the understanding of the transcriptional basis of proximal tubule cellular identity and function, and clarify how two transcription factors central to both hepatocytes and PT cell fate can be guided towards divergent specificity by other co-regulators. This knowledge may contribute toward the future development therapeutic strategies to enhance PT function and regenerative capacity as well as tissue engineering (reviewed in [101, 102]).
3.6 ACKNOWLEDGEMENTS

We thank Drs. Bing Ren, Chris Glass, Karl Willert and Scott Thomson for advice throughout this project. We would also like to thank Dr. Lijian Hui for providing the lentiviral plasmids used in this study. This work was supported by NIH grants GM098449 and U54 HD07160.

The work presented in Chapter 3, has been published in the study “Transcription factor-mediated regulation of kidney versus liver specification of Slc and Abc drug transporters, tight junction molecules and signature biomarkers” by Gleb Martovetsky, Kevin T. Bush and Sanjay K. Nigam in Drug Metabolism and Disposition, 2016. The dissertation author is the primary author, and Sanjay K. Nigam is the senior author.
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Table 3.2. Expression of transporters and junction-related genes in transduced MEFs, isolated PTs and liver tissue.

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<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Category</th>
<th>Expression in Uninfected MEFs (Relative to Gapdh)</th>
<th>Basal junction (fold change)</th>
<th>Mediation junction (fold change)</th>
<th>Delta (fold change)</th>
<th>Delta (Relative to Basal)</th>
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* Fourth column colored based on basal expression level in MEFs. Next three columns colored by relative fold-change compared to basal expression; decrease (blue) and strongest increase (red). Final four columns are colored based on expression (light – low, dark green – high).
Overexpression of Hnf1a and Hnf4a leads to PT marker expression in MEFs and BSN cells but not IMCD-3 cells. Lentiviral transduction of E13.5, E15.5 and E16.5 MEFs with Hnf1a and Hnf4a induces transcription of PT markers Slc22a6 (Oat1), Slc34a1 (NaPi-2a), and Slc47a1 (Mate1). Transduction of BSN cells with Hnf1a and Hnf4a, which were derived from mouse metanephric mesenchyme, induced the transcription of the 4 PT markers, as in MEFs. Slc22a6 and Slc34a1 were not expressed at appreciable levels in IMCD-3 cells (derived from mature mouse collecting duct tissue) upon Hnf1a and Hnf4a transduction, and Slc47a1 expression was induced at much lower levels than in MEFs or BSN cells. Error bars depict SEM for MEFs and standard deviation BSN and IMCD cells.
Figure 3.2. Transduction of MEFs with Hnf1a and Hnf4a leads to upregulation of intercellular junction marker expression and downregulation of mesenchymal marker. Tight junction markers occluding (Ocln), tight junction proteins 1 and 2 (Tjp1 or ZO-1 and Tjp2 or ZO-2), and claudin 6 (Cldn6), as well as adherens junction marker E-Cadherin (Cdh1) and were markedly upregulated at the mRNA level in MEFs transduced with Hnf1a and Hnf4a compared to a GFP control one week after transduction (A). Conversely, mesenchymal marker vimentin (Vim) was downregulated (B).
Figure 3.3. Transduction of MEFs with Hnf1a and Hnf4a leads to tight junction formation. A) E15.5 MEFs prior to infection. B) MEFs 3 days after transduction with Hnf1a and Hnf4a were nearly all GFP-positive, indicating very efficient transgene expression. C-D) Control MEFs transduced with only GFP showed no junctional formation after 3 weeks of culture on Col I(E), while those transduced with Hnf1a and Hnf4a showed extensive tight junction formation (D) (Blue – DAPI, Red – Tjp1). A digitally-enhanced view of Tjp1 staining from panels E and F is shown in greyscale in panels E and F, respectively.
Figure 3.4. MEFs transduced with Hnf1a and/or Hnf4a express a number of relevant transporters and genes involved in establishing intercellular junctions. In one of our tested MEF populations (E16.5), Hnf4a overexpression did not induce expression of Hnf1a (data not shown). This allowed us to examine the differential capacity of Hnf1a and Hnf4a individually and together to induce the transcription of a cohort of genes involved in transport, junctional formation and polarity, all properties integral to the identity of proximal tubule cells. A) Genes synergistically upregulated by Hnf1a and Hnf4a. B) Genes induced solely by Hnf1a. C) Genes induced solely by Hnf4a. D) Genes downregulated solely by Hnf4a. E) Less common modes of differential regulation by Hnf1a and Hnf4a include downregulation by Hnf1a but upregulation by Hnf4a and vice versa, and non-synergistic upregulation by Hnf1a and Hnf4a. F) Genes that fall into one of the five aforementioned categories (A-E) but expressed at lower levels.
Figure 3.5. Systems level analysis of PT and liver expression identify a panel of selective differential markers. A) Hierarchical clustering of genes with at least a 100 fold-change between either embryonic or adult liver and isolated proximal tubules. 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) Hnf4a in rat kidneys throughout development, as well as p300 binding in the adult cortex, is generally enriched at PT marker genes and depleted at hepatocyte-specific gene markers; two representative examples are shown (Slc34a1, Albumin).
Figure 3.6. Expression of PT and hepatocyte markers in adult mouse kidney cortex, adult mouse liver, and transduced MEFs. A,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, Ttr, Apoa1, Serpina1, and Alb are highly expressed in the liver and negligibly expressed in the PT. C,D) Expression of PT and hepatocyte markers upon transduction of E15.5 MEFs. Hnf1a and Hnf4a activate expression of PT markers, which is downregulated or silenced by Gata4 and Foxa2/3 (C). Conversely, hepatocyte marker expression is induced when Foxa2/3 is co-expressed with Hnf1a and Hnf4a, some of which are further upregulated by Gata4 (Fabp1, Trf) (D).
Figure 3.7. Overexpression of Gata4 and Foxa3 in primary proximal tubule cells downregulates PT marker expression and induces expression of hepatocyte marker genes. A) Photograph of primary proximal tubule cells migrating out of tubules 2 days after plating. B) Primary proximal tubules cells at the time of RNA collection. 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 to Gata4, but leads to pronounced transcriptional induction of hepatocyte markers, an effect sometimes synergizing with co-expression of Gata4 (D).
Figure 3.8. Schematic of working model. Based on aggregate data from experimental work in MEFs, BSN cells and primary PT cells, expression of Hnf1a and Hnf4a lead to PT marker expression in the absence of Gata4, Foxa2/3, and possibly other liver lineage-determining factors. Co-expression of Gata4 and Foxa2/3 leads to downregulation of PT markers and strong induction of hepatocyte marker expression. While Gata4 is insufficient to upregulate liver marker expression, Foxa(s) are capable of inducing liver factors when co-expressed with Hnf1a and Hnf4a independently of Gata4. Importantly, for some genes co-expression of Gata4 increases hepatocyte marker expression levels above those with Hnf1a, Hnf4a and Foxa2/3.
CHAPTER 4:

Conclusion

Acute and chronic kidney disease has an enormous economic and health impact, which has fueled research aimed at improving outcomes. While there is a long way to go before it is possible to culture a kidney “from scratch” in vitro that can entirely replace the functions and properties of a healthy human kidney, including regenerative capacity and monitoring by the immune system, progress is being made towards recapitulating kidney functions with cell-based constructs. Two promising strategies being explored involve either: 1) the recellularization of allogenic or xenogenic extracellular matrix scaffolds derived from human or animal kidneys; and 2) engineering bioartificial filtration devices containing cells that can carry out functions of the proximal tubule. One of the many obstacles integral to this mission is the ability establish a renewable source of cells in vitro that can be used for these purposes.

Though life-extended primary cells from donor kidneys have been proposed as a potential cell source, multiple tissue engineering strategies might benefit from the ability to generate unlimited amounts of patient-specific proximal tubule-like cells in vitro. The advent of induced pluripotent stem cells (iPSCs) has opened up a whole field of research focused on the potential of establishing various patient-specific cell types. However, it might be advantageous to circumvent the requirement for pluripotency and the threat of oncogenicity by using somatic cells as a cell source. In any case, understanding the underlying transcriptional mechanisms that establish proximal tubule cellular identity and
function would be helpful for refining and possibly enhancing proximal tubule cellular function, whether with tissue engineering applications or through therapeutic intervention.

4.1 DISCUSSION

Central to systems biology is the concept of signaling hubs, which are basically pathways where individual perturbations can lead to a large number of changes. Hubs exist at all levels of signaling, including extracellular receptors, kinases and transcription factors, though the effector of the response to perturbations is often at the level of changes in gene transcription. Based on the work described in this dissertation, we believe that Hnf1a and Hnf4a serve as a “transcriptional hub” in proximal tubule cells. However, in light of the results in this study and others, it appears that while Hnf4a has been called a “master regulator,” and is necessary for the transcriptional regulation of a vast array of genes, much of its function and specificity depends on the presence or absence of other co-regulators. Identifying additional transcriptional co-regulators required specifying PT cellular fate, as well as transcription factors that must be absent in order to not interfere with establishing a PT-specific transcriptome, should be a focus of future studies. This is relevant both for better understanding transcriptional networks within PT cells as well as establishing a protocol to differentiate multipotent/pluripotent stem cells or transdifferentiate somatic cells towards a functional PT-like cellular fate.

Hnf1a knockout mice generated almost two decades ago revealed its contribution to adult rodent proximal tubule function; however, it now seems that Hnf1a often relies on co-regulation with Hnf4a, and it is unclear to what extent Hnf4a can compensate for
Hnf1a absence or downregulation, especially after stable transcriptional landscapes are established via epigenetic modifications and chromatin spatial organization. This is important to determine in order to consider potential effects of changes in Hnf1a levels upon regulation by various signaling pathways upon perturbations to homeostasis; this is also true for Hnf4a. A kidney-specific Hnf4a knockout model remains to be generated, and this dissertation may provide the best currently available evidence that Hnf4a plays a large role in PT cellular regulation (and likely specification); however, because Hnf4a expression in the kidney is initiated during early stages of nephrogenesis, it is possible that Hnf4a deletion during early kidney development might interfere with proximal tubule formation (also supported by previously published work [46]), thus a proximal tubule-specific knockout model might be necessary to examine the effects of Hnf4a in this differentiated cell type. For now, it remains undetermined whether Hnf4a is required for PT specification, or if the phenotype will be relatively mild.

In the experiments described in chapter 2, most of the genes that responded to ectopic expression of Hnf1a and Hnf4a required the presence of both factors. However, in the experiments described in chapter 3 it was revealed that the regulation is not always synergistic, and is quite more complex. One possible explanation for the upregulated genes that were observed to respond to Hnf1a alone is that they are regulated by Hnf1a via non-Hnf4a bound enhancers, or by binding the promoter directly. Similarly, genes that responded to Hnf4a alone might be regulated by Hnf4a-bound enhancers that do not contain Hnf1a binding sites, or by direct promoter binding. Furthermore, recent genome-wide studies have revealed that while enhancers are highly involved in establishing tissue-specific transcriptional programs, promoters are less enriched with tissue-specific
transcription factor binding motifs and promoter-bound transcription factors are more ubiquitously expressed, despite being crucial for modulation by enhancers. Thus, it is important to also consider the possibility that in some cases either of these two transcription factors directly interact with other transcriptional regulators found at promotors of target genes to initiate transcription, or that they bind gene promoters and interact with other factors at cis-regulatory enhancers. Another surprising finding was the number of genes downregulated by the overexpression of Hnf4a, despite many early studies suggesting that Hnf4a mainly acts as a transcriptional activator. This might be caused by the establishment of enhancer elements by Hnf4a that then recruit transcriptional repressors. In addition to all of these potential mechanisms, it is also possible that observed changes in transcription of tested genes are indirectly regulated by Hnf1a or Hnf4a by affecting expression levels of other transcriptional regulators.

While MEFs transduced with Hnf1a and Hnf4a expressed a number of PT signature genes, as well as a number of other genes that are highly expressed in the PT but are more widely expressed in other tissues, some key PT genes were either very lowly expressed, or were not detected. In future attempts to establish a PT cell-like fate via transcriptional reprogramming, it will be important to identify additional inputs that might help refine specificity and enhance the robustness of the response. Based on our results showing that the presence of either Gata4 or Foxa2/3 alters the effects of Hnf1a and Hnf4a transduction, it is possible that other transcription factors known to play important roles in hepatocytes (e.g. Foxa1, Hnf6, Gata6, Hex, Cebpα) or other Hnf4a-expressing tissues (e.g. Cdx2 in colon or Nkx6-1 in B-islet cells) might be expressed to
some extent in MEFs and thus interfering with the ability of Hnf1a and Hnf4a to further induce a PT cell-like transcriptome.

In addition to providing evidence that either proximal tubule signature genes or hepatocyte signature genes are induced depending on absence or presence of Gata4 and Foxa2/3, we also showed that Hnf4a binding in the kidney is enriched at PT signature genes and depleted at hepatocyte signature genes. 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; however, other mechanisms are also likely to be involved.

In utero, the transition from a pluripotent state to a highly specialized, terminally differentiated cell type involves a myriad of spatiotemporal events. While perfectly recreating the endogenous developmental environment in vitro would seem ideal, this may be unrealistic. On the other hand, many studies have demonstrated that a small set of cues, whether it is via soluble signaling factors, reprogramming transcription factors, or other methods, are often sufficient to drive cells directly towards various cell lineages [31]. In some cases differentiation protocols involve several sequential steps attempting to mimic progressive developmental stages, but they do not fully correspond to the complexity of normal development. Nevertheless, these protocols have been able to achieve promising results, especially for non-renal tissues such as the pancreas, liver, and cardiomyocytes, where cells programmed from either stem cells or somatic cells have been used to restore some functionality in injury/disease models. This provides hope that, with a deeper understanding of the lineage-determining traits of the different types of
renal cells, it may become possible to develop methods to differentiate stem cells or transdifferentiate somatic cells towards cells capable of reconstituting a kidney.

The ability to transdifferentiate from one cell identity to another can be highly influenced by the epigenetic landscape and expression profile of the starting cell type. It has been shown that while the epigenome is largely remodeled during transdifferentiation to reflect the target cell type, some features from the starting cell type that do not belong in the target cell type remain. It is likely that superfluous new features are established as well. It is important to consider the fact that transcription factors can bind to many variations of their preferred binding motif “consensus sequence,” albeit with different affinities, and that there are always more binding motifs in the genome for any given factor than actual binding sites in any given cell type. Furthermore, considering the mechanism of action of lineage determining transcription factors, which largely act in a combinatorial fashion at cis-regulatory enhancer elements and depend on physical interactions with transcriptional complexes at gene promoters, the specific array of expressed transcriptional regulators prior to transdifferentiation are bound to have a large impact on the final outcome.

4.2 FUTURE DIRECTIONS

Based on the findings in this work, Hnf1α and Hnf4α induce the expression of a number of proximal tubule signature genes, at least in the setting of transdifferentiation. However, even with the more extensive query of transporter and junctional component gene expression, we still lack a comprehensive genome-wide understanding of the effects of Hnf1α and Hnf4α. RNA-sequencing after Hnf1α and Hnf4α transduction would provide
a thorough representation of which genes are induced by these two transcription factors, and provide insight into what is missing and what is superfluous compared to endogenous proximal tubule expression. This might also reveal what other TFs are required, as well as which TFs are likely to be interfering. In the latter case, it may be possible to incorporate the use siRNA or shRNA to downregulate interfering TFs during transdifferentiation. This might also be done by introducing miRNA mimics if some superfluous genes thought to be critical for correct cell fate determination coincide with miRNA that are highly expressed in kidney cortex. While miRNA-seq data exists for the kidney cortex, it would be important to also perform miRNA-seq on uninfected MEFs to know initial levels, as well as MEFs transduced with Hnf1a and Hnf4a. This may reveal not only which miRNA are missing and could be supplemented to help improve specificity, but also which existing miRNA might be repressing desired transcripts.

Another approach that might yield additional information about how to improve transdifferentiation towards a PT-cell like fate would be to examine Hnf1a and Hnf4a binding in transduced cells, and compare it to binding in vivo. Motif analysis of differential sites between transduced cells and native proximal tubule cells may yield insight regarding which transcription factors might be required to prime chromatin for Hnf1a and Hnf4a recruitment as well as TFs that might be helping establish sites which do not belong in the PT. We conducted Hnf4a ChIP-seq in rat kidneys while the transductions were done in mouse embryonic fibroblasts; it would be advantageous to compare binding in vivo and in vitro in the same species, which would likely entail repeating the Hnf4a ChIP-seq in mouse kidneys, or using publically available data of Hnf4a binding in mouse kidneys (though it would be ideal to prepare libraries from
endogenous tissue and transduced cells side-by-side). Furthermore, bisulfite sequencing, ChIP-seq of various chromatin modifications (e.g. H3K4me1, H3K4me3, H3K27me3), GRO-seq and Hi-C of endogenous PTs (ideally throughout development) and cells before and after transduction would also help refine the understanding of the transdifferentiation processes as well as its shortcomings.

Apart from expressing additional transcription factors and repressing others, other strategies should be explored to enhance transdifferentiation towards a proximal tubule cellular identity. In the beginning of chapter 3 it was shown that the choice of starting cell type had a significant impact on PT signature gene expression. It may be possible to prime cells with signaling molecules prior to ectopic expression of Hnf1a and Hnf4a to enhance the response. It is known that activation of the Wnt signaling pathway initiates nephrogenesis, and is required along with the Fgf signaling pathway for this process to occur; thus, conditioning cells with recombinant proteins (or small molecule Gsk3 inhibitors in the case of Wnt) prior to transduction may be a worthwhile strategy to explore. Additionally, since Bmp4, Bmp7, retinoic acid and activin A have been used in the sole report of differentiating stem cells to a PT-like fate [89], using these signaling molecules in the latter stages of culture post-transduction may be worth considering.

The work in this dissertation focused on targeted approach to identifying PT lineage-determining transcription factors and examining their potential to transdifferentiate cells toward a PT-like cellular identity. However, now that Hnf1a and Hnf4a have been identified as essential but insufficient regulators of PT cell fate, an untargeted approach may be appropriate. By using cells transduced with Hnf1a and Hnf4a, along with a readout that is amenable to high throughput screening, it would be
worthwhile to test various combinations of culture substrates, media conditions and signaling molecules to help optimize transdifferentiation. The readout would likely need to be fluorescent to increase screening efficiency, such as 6-CF uptake or expression of a fluorescent protein driven by the promoter of a PT-specific gene (eg. Oat1, Slc34a1, Oat3, Sglt2). In fact, if GFP is removed from the constructs used to generated lentiviral vectors for transduction, multiple markers could be investigated at once, increasing the chances of a robust response.

The objective of this dissertation was to investigate transcriptional mechanisms regulating PT cell identity; thus using mRNA levels as the readout was sufficient. However, apart from showing that Tjp1 protein levels and localization were concordant with mRNA levels, and 6-CF uptake which indicated functional Oat1 transport capacity, protein levels remained largely unexamined. This could be done by western blot, immunofluorescence microscopy or flow cytometry. Furthermore, staining and flow cytometry could also address the level of heterogeneity in the cellular responses to ectopic Hnf1a and Hnf4a expression. One potential issue is the fact that induction of some of the most functionally significant PT signature genes was relatively low, either due to a weak response in a large population of cells or due to only a small fraction of responsive cells. Thus, if this is caused by a generally weak response, it might be beneficial to first attempt various strategies to enhance induction of key PT genes. If it turns out that there is a substantial response but only in a small fraction of cells, it would be beneficial to develop an enrichment strategy, possibly by cell sorting. However, it may be difficult to expand a subpopulation in this case, especially because proximal tubule cells are considered to be terminally differentiated. If possible, it would be beneficial to
develop an intermediate state when cells are primed to become proximal tubule cells, but retain some proliferative capacity.

In this dissertation we have shown that Gata4, Foxa2 and Foxa3 can suppress the PT-specific effects of Hnf1a and Hnf4a, while inducing hepatocyte marker expression. However, the mechanism of this phenomenon remains unresolved. It is very likely that Gata4 and Foxa transcription factors open up closed chromatin, thus exposing Hnf1a and Hnf4a binding sites. However, this fails to explain the loss of expression that is induced by Hnf1a and Hnf4a alone. It is also possible that Gata4 and Foxa2/3 by themselves, or together with Hnf1a and Hnf4a, induce the transcriptional repressors or induce miRNAs that silence some PT-specific expression. Performing ChIP-seq for these factors using transduced cells with the various permutations of these transcription factors could yield valuable insight regarding the mechanism of this transcriptional switch. As described above for cells transduced with Hnf1a and Hnf4a, other sequencing-based assays – bisulfite sequencing, ChIP-seq for histone modifications, GRO-seq and Hi-C – would contribute additional layers of information. It may turn out that some of the transcriptional effects occur without changes in Hnf1a and Hnf4a localization, which would indicate regulation at the level of transcriptional machinery recruitment or transcriptional elongation. In this case, Hnf1a and Hnf4a co-IP followed by mass-spec using nuclei extracts from primary PT cells and hepatocytes (or transduced cells in order to resolve differences relevant to transdifferentiation) might help identify co-regulators, especially those that do not have DNA-binding properties, that drive specificity in the two different tissues.
Finally, because the long term goal of this research is to contribute towards translational medicine, an obvious future direction is to transfer this insight established in rodent systems to human cells. The most likely clinically-relevant cell sources from human patients would likely be either skin fibroblasts, mesenchymal stem cells (derived from blood or adipocytes) or iPSCs. It remains to be seen whether iPSCs differentiated towards PT-like cells can be safely used in the clinic, or whether transdifferentiation of non-pluripotent cells turns out the be the preferable approach. At this point, it is worth developing both of these strategies.

As a final consideration, it is important to establish a system to test cell performance. For example, if transdifferentiated cells are meant to recellularize decellularized kidney scaffolds, and specifically repopulate the PTs, then they should be tested in this system as the transdifferentiation protocol is being developed. Alternatively, if their purpose is for populating a biofilter, then they can be initially cultured on transwell filters to test capacity of vectorial transport, and later seeded on the biofilter membranes and tested for metabolic and transport activity using synthetic or real blood. While there is a long way to go before we are able to culture artificial organs suitable for transplantation, the work presented in this dissertation aimed at understanding the transcriptional basis of proximal tubule cell identity hopefully makes a small, yet important, contribution to this end.
REFERENCES


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