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Transcriptional Control of Terminal Nephron Differentiation

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Abstract

Terminal differentiation of epithelial cells into more specialized cell types is a critical step of organogenesis. Throughout the process of terminal differentiation, epithelial progenitors acquire or up-regulate expression of renal function genes and cease to proliferate, while expression of embryonic genes is repressed. This exquisite coordination of gene expression is accomplished by signaling networks and transcription factors which couple the external environment with the new functional demands of the cell. While there has been much progress in understanding the early steps involved in renal epithelial cell differentiation, a major gap remains in our knowledge of the factors that control the steps of terminal differentiation. A number of signaling molecules and transcription factors have been recently implicated in determining segmental nephron identity and functional differentiation. While some of these factors (the p53 gene family, HNF1 β) promote the terminal epithelial differentiation fate, others (Notch, Brn-1, IRX, KLF4 and Foxi1) tend to regulate differentiation of specific nephron segments and individual cell types. This article summarizes current knowledge related to these transcription factors and discusses how diverse cellular signals are integrated to generate a transcriptional output during the process of terminal differentiation. Since these transcriptional processes are accompanied by profound changes in nuclear chromatin structure involving the genes responsible for creating and maintaining the differentiated cell phenotype, future studies should focus on identifying the nature of these epigenetic events and factors, how they are regulated temporally and spatially, and the chromatin environment they eventually reside in.

Introduction

Terminal differentiation is a key biological process characterized by cell cycle arrest and acquisition of specialized functions by each cell type. The genetic programs that couple cellular growth to functional differentiation are a subject of intense investigation (78). There is increasing appreciation that although formation of adequate numbers of nephrons (i.e., renal mass) is critical for achieving optimal renal function capacity, the signals that determine how, when, and which renal epithelial cells should acquire a given functional phenotype are equally essential. Aberrant terminal differentiation is a hallmark of dysplasia, cyst formation and cancer and therapeutic interventions to promote differentiation and cell cycle arrest (e.g., EGFR antagonists and histone deacetylase inhibitors) are effective measures in some models of polycystic kidney disease and cancer (17,26,72,73). Therefore, identification of factors that couple cell cycle arrest with functional differentiation is of great clinical importance.

Termination of nephrogenesis and differentiation are dynamic and coordinated processes

The metanephric kidney is derived from the intermediate mesoderm at embryonic (E) day 10.5 in mice and nephrogenesis continues postnatally (PN) in this species (24,58,68). While initial studies indicated that murine nephrogenesis ends between PN 7-10, recent molecular analysis has revealed that formation of nascent nephrons ceases by PN3 (20) and is accompanied by loss of ureteric bud (UB) ampullae and tip cell Wnt11 expression. Interestingly, the PN3 UB retains its ability to express the inductive molecule Wnt9b (at least in vitro) and to induce nascent nephrons (20).

The mechanisms and events leading to termination of nephrogenesis are not entirely clear. Hartman et al. (2007) proposed that the mechanism could involve depletion of the progenitor pool within the MM or UB cell lineages or a genetic switch in response to a physiologic sensor. It was found that there was no appreciable change in apoptosis that might explain the loss of metanephric mesenchyme and there was no evidence to support that capping mesenchyme had differentiated into another mesenchymal lineage. Accordingly, the loss of nephrogenic mesenchyme occurred because of conversion to nephrons without replenishment from the progenitor pool and not by cell death. The idea that onset of glomerular filtration provides an off-on switch to induce the terminal differentiation program in the tubular compartment is intuitively appealing. However, this hypothesis is challenged by activation and sustained expression of terminal differentiation genes in the non-filtering ex vivo cultured embryonic kidney (63). Nevertheless, tubular fluid flow is likely to provide important physiological clues by acting as a mechanosensory stimulus of the primary monocilium and downstream calcium-dependent signaling pathways (e.g., Stat-p21^{waf1}) (5, 41, 75).

Transcriptional networks in nephron patterning

Genetic and molecular studies in mice have shown that Wnt9b, which is strongly expressed in the UB branches, is essential for the induction of nephrogenesis (9). Wnt9b activates the canonical β -catenin pathway in surrounding MM and induces expression of early markers of nephron formation such as Pax8, FGF8, the LIM homeobox protein LIM1 (also known as LHX1), and Wnt4 (9). Wnt4 induces mesenchyme-epithelium transition and formation of the renal vesicle (11,33,34,57) (Fig. 1). Mice with a conditional deletion of FGF8 or LIM1 fail to express proximal and distal-patterning markers such as the Notch ligand, Delta-like 1 (DLL1) and BRN1 (11,34,67).

The mature nephron is subdivided into segments that are specialized for specific tasks. This specialization, which is acquired at terminal differentiation, is reflected in protein expression profiles of various segments that complement function. In several developmental systems, overlapping and differential expression of transcription factors generate combinations of interacting proteins that regulate cell-specific gene expression as well as developmental fate (4,15,30,56,60). With respect to the developing kidney, such interactions might also explain how segment identity and functional status are acquired. While there has been much progress in understanding the early steps involved in renal epithelial cell differentiation, a major gap remains in our knowledge of the factors that drive segmental nephron identity. Advances in studying model systems such as *Xenopus* and Zebrafish pronephros as well as in mice have been instrumental in understanding proximal-distal specification of the nephron (35,66,77).

LIM1 is required to induce the initial stages of patterning in the renal vesicle, by controlling the expression of the POU-domain transcription factor BRN1 and the Notch ligand DLL1 at

the pole of the vesicle that lies in close proximity to the ureter (11,34). DLL1 contributes via NOTCH2 in the specification of the proximal tubule fate (12,35). Under the control of transcription factors BRN1 and Iroquois-class homeodomain proteins IRX 1–3, distal segments further extend and differentiate towards distal tubule and the Henle's loop (46,49,76) (Fig. 1). *Brn1*^{−/−} mutant mice show disrupted development of the Henle's loop and formation of distal convoluted tubules (46). In *X. laevis*, a subset of Irx transcription factors (Irx1, Irx2 and Irx3) are specifically expressed in a medial zone of the developing pronephric anlagen, which will give rise to the intermediate tubule. Loss- and gain-of-function experiments in *X. laevis* revealed that *Irx3* (but not *Irx1* or *Irx2*) is required and sufficient to direct early distal tubule fate (49). In the mouse, expression of the same subset of Irx genes marks the future early distal tubule compartment in the S-shaped bodies of the developing metanephros. However, the function of Irx proteins in mouse kidney patterning remains to be defined and may be redundant, since Irx-2 null mutant are phenotypically normal (37).

The p53 family regulates both cell cycle progression and terminal differentiation markers

The *p53* gene encodes a sequence-specific DNA-binding protein/tumor suppressor that maintains genomic integrity via its ability to induce cell cycle arrest or apoptosis, depending on the type and magnitude of the stress and the cell type (47,71). Previous studies have demonstrated a developmental role for p53 in several organisms including *Xenopus* (2,23,64,65,69), and mouse (13,16,18,19,^{21,32},38,39,44,59). We recently reported that terminal nephron differentiation is accompanied by p53 phosphorylation and acetylation, protein stabilization, and enhanced DNA binding activity (54). Moreover, we identified several terminal differentiation genes, including the bradykinin B2 receptor (*Bdkrb2*), aquaporin-2 (*AQP2*), Na,K-ATPase α1, and angiotensin II type 1 receptor (*Agtr1*), as a novel group of p53-target genes (42,52,54) (Fig. 2). In contrast, the proliferating cell nuclear antigen (PCNA), which encodes a protein expressed in dividing cells and is necessary for cell cycle progression and DNA replication, is directly repressed by p53, and is therefore excluded from the differentiation zone of the developing renal cortex (55). In keeping with these findings, we found that p53-deficient newborn mice exhibit persistent renal cell proliferation, impaired cell cycle control, and disorganized spatial expression of nephron differentiation markers (17,24). Interestingly expression of terminal differentiation genes is attenuated but not abrogated in p53-null kidneys (52), raising the possibility of compensatory regulation by other developmentally regulated transcription factors with overlapping functions.

p53 cooperates with at least two other transcription factors, CREB (cyclic AMP response element-binding protein) and KLF4 (Kruppel-like factor 4), and several renal function genes (e.g. *Bdkrb2*, *AQP2*, *ENaC*) contain p53-CRE-KLF binding sites in their promoter regions. In one case, the promoter for the *Bdkrb2* gene (which encodes for the bradykinin B2 receptor) has contiguous binding sites for p53, CREB and KLF4 extending from position -44 to -82 bp, relative to the transcription start site. Disruption of function and/or binding of any of these factors impair the activity of the *Bdkrb2* promoter suggesting that the three factors act as an enhanceosome-like fashion (53). Chromatin immunoprecipitation confirmed that the p53/KLF4/CREB complex assembles in vivo on the mouse *Bdkrb2* gene in a developmentally regulated manner. Moreover, the complex is bridged together by a large co-factor, CBP (CREB-binding protein), which also has a histone acetyltransferase activity. Recruitment of CBP by the enhanceosome complex results in hyperacetylation of promoter-associated histones and thus further enhances gene transcription (53). Interestingly, angiotensin II, acting via AT1 receptor, stimulates a kinase cascade leading to phosphorylation of CREB on serine 133. p-CREB has a higher affinity to DNA as well as

to p53 and is capable of recruiting CBP as well. As a result, angiotensin II is a strong stimulus of *Bdkrb2* gene transcription (61) (Fig. 2B).

Recent studies have identified two homologues of p53, p63 and p73 (10,14,29,45,62). Both genes encode transcription factors with significant sequence homology to p53. Highest homology lies in the DNA-binding domain allowing p63 and p73 to bind and activate transcription of p53 target genes and to induce apoptosis and/or growth arrest. However, unlike the p53 gene, p73 and p63 are not induced by most DNA-damaging agents and are rarely mutated in tumors. On the other hand, there is strong genetic evidence supporting an important role for p63 and p73 in embryonic development. p63-null mice exhibit severe defects in limb, craniofacial, and epithelial development (36,40,43,45), whereas p73-null mice have central nervous system defects and pheromonal abnormalities (45,48,50,74). Unlike p53, transcription of the p73 gene yields multiple full-length (transactivation (TA) domain) and amino terminus-truncated (Δ N) isoforms. Δ Np73 acts in a dominant negative fashion to inhibit the actions of TAp73 and p53 on their target genes, promoting cell survival and proliferation and suppressing apoptosis (25). The balance between TAp73 and its negative regulator, Δ Np73, may therefore represent an important determinant of developmental cell fate.

Studies from our laboratory have shown that TAp73 and Δ Np73 exhibit reciprocal spatiotemporal expression and functions during nephrogenesis (51). TAp73 was predominantly expressed in the differentiation domain of the renal cortex in an overlapping manner with the vasopressin-sensitive water channel aquaporin-2 (AQP-2). Chromatin immunoprecipitation assays demonstrated that the endogenous AQP-2 promoter was occupied by TAp73 in a developmentally regulated manner. Furthermore TAp73 stimulated AQP-2 promoter-driven reporter expression. The transcriptional effects of TAp73 on AQP-2 were independent of p53. In marked contrast to TAp73, Δ Np73 isoforms were induced early in development and were preferentially expressed in proliferating nephron precursors. Moreover Δ Np73 was a potent repressor of AQP-2 gene transcription. We speculate that the spatiotemporal switch from Δ Np73 to TAp73 may play an important role in the terminal differentiation program of the developing nephron. These results led to the emergence of a new paradigm for terminal nephron differentiation in which the p53/p73 transcriptional network induces renal function gene expression while simultaneously inhibiting cell cycle progression. This contrasts to the role of the retinoblastoma gene product Rb, which is essential for enteroendocrine cells to undergo cell cycle arrest as they terminally differentiate but is not required for the expression of gastrointestinal hormones (70).

In the collecting duct, the forkhead transcription factor, *Foxi1*, mediates differentiation of intercalated cells from a precursor epithelial cell and is upstream of intercalated cell-specific genes (1,7) (Fig. 2C). *Foxi1*^{-/-} mice exhibit impaired expression of intercalated cell genes such as Pendrin, H-ATPase and AE1 but unaltered expression of principal cell markers. Preliminary results from our laboratory indicate that *Foxi1* is a target for p53-mediated transcriptional activation. Therefore, it appears that p53/p73 are capable of activating both principal and intercalated cell genes, arguing that a general function of the p53 gene family is to promote terminal differentiation rather than to specify a specific lineage or fate.

Hepatocyte nuclear factor 1 β (HNF1 β) controls a gene regulatory network necessary for epithelial differentiation

HNF1 β is a transcription factor that regulates tissue-specific gene expression in the kidney, liver, pancreas, and other organs (8). During embryonic development, HNF-1 β is expressed in the branching ureteric bud as well as in comma- and S-shaped bodies (28) and can regulate genes in various nephron segments. Mutations of HNF-1 β in humans produce

maturity onset diabetes of the young, type 5 (MODY5), a disorder that is frequently associated with congenital cystic abnormalities of the kidney (6).

HNF1 β is a direct regulator of at least two collecting duct genes: ksp-Cadherin (3), a kidney specific cadherin, and PKHD1, the gene mutated in autosomal recessive PKD (22,27). Kidney-specific inactivation of HNF-1 β uncovered a transcriptional network involving HNF-1 β -*Pkhd1-Umod-Pkd2* (28). In vivo chromatin immunoprecipitation experiments showed that HNF-1 β binds to several DNA elements in the 5'-flanking regions of the *Umod*, *Pkhd1*, and *Pkd2* genes. Similar to the mechanism by which p53 and p73 activate renal function genes, HNF-1 β activates *Pkhd1* transcription by recruiting coactivators that promote histone acetylation and chromatin remodeling at the promoter (28).

In summary, the formation of terminally differentiated cell types requires withdrawal from the cell cycle and repression of many genes involved in cell cycle control as well as activation of cell type-specific genes. A major question is how a cell can coordinate cell cycle control with functional differentiation? As discussed above, transcription factors such as p53 performs dual functions by inducing cell cycle arrest genes (e.g., p21^{Cip1}), repressing proliferation genes (e.g., PCNA and CDC2), and activating renal function genes (e.g., GPCRs and water channels). Cooperation with other segment-specific transcription factors and co-factors eventually ensures expression of segmental identity. As an example, the differential presence of Foxi1/p53 promotes intercalated cell identity, whereas KLF4/p53 favors principal cell gene expression. As recently discussed (31), these transcriptional processes are accompanied by profound changes in nuclear chromatin structure involving the transcription factors/co-factors needed to regulate the genes responsible for creating and maintaining the differentiated cell phenotype. Future studies should focus on identifying the nature of these epigenetic events and factors, how they are regulated temporally and spatially, and the chromatin environment they eventually reside in.

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Figure 1.

Transcriptional pathways which regulate the formation of the renal vesicle and its transition to the segmented early nephron. Wnt9b is synthesized and released from the ureteric bud branches. Wnt9b in cooperation with inducing factors (e.g., LIF, FGF2, TGFβ2) converge on β-catenin signaling to stimulate mesenchyme-epithelium transition and subsequently the conversion of the renal vesicle (blue) to comma- and S-shaped bodies (pink/green/grey). The transcription factor *Lim-1* promotes further patterning by stimulating expression *Brn1* (pink) and *Dll1* (green). *Brn1* in cooperation with Iroquois transcription factors *IRX* specify distal nephron fate, whereas *Dll1-Notch2* signaling specifies the proximal tubule fate. Data presented in this figure summarizes results published in the following articles: *Dev Cell* 9: 283-292, 2005; *Development* 132: 2809-2823, 2005; *Dev Biol* 297: 103-117, 2006; *Development* 134: 801-811, 2007; *Development* 130: 4751-4759, 2003; *Genes Dev* 21: 2358-2370, 2007.

Figure 2. Role of p53 family in terminal nephron differentiation

A. Terminal nephron differentiation is accompanied by p53 stabilization and enhanced DNA binding activity (*J Clin Invest* 109: 1021-1030, 2002). In the nephrogenic zone, p53 levels are kept low since *p53* is repressed transcriptionally by Pax2 (*EMBO J* 14: 5638-45, 1995). The promoter of proliferating cell nuclear antigen (PCNA), which encodes a protein expressed in dividing cells and is necessary for cell cycle progression and DNA replication, is directly repressed by p53, and is therefore excluded from the differentiation zone (*Am J Physiol Renal Physiol* 283: F727-733, 2002). The genes encoding the CDK inhibitor, p21 (Cip1/Waf1) and KLF4, are direct targets of p53 (*Cell* 75: 817-825, 1993; *J. Biol. Chem.* 278: 2101-2105, 2003). In the differentiating zone, p53 binds to the promoters of terminal differentiation genes (also called renal function genes), including the bradykinin B2 receptor, aquaporin-2, and Na,K-ATPase α 1. All of these genes have composite cis-regulatory elements for p53, KLF4 and CREB. **B.** Angiotensin II, acting via AT1 receptor-mediated signaling, stimulates the phosphorylation of CREB on Ser-133. The latter modification allows CREB to interact with p53 and the co-activator CBP. CBP acts as a bridging molecule with the basal transcriptional machinery as well as a histone acetyltransferase. The end result is enhanced *Bdkrb2* transcription (*J Am Soc Nephrol* 18: 1140-1149, 2007). **C.** p53 and its homologue p73 are capable of binding to the promoters of renal function genes and activating their expression. This function requires additional transcription factors including KLF4 or Foxo1. Fig. 2A is adapted with permission from *Am J Physiol Renal Physiol* 283: F727-733, 2002.