Genomic Mechanisms Governing Mineral Homeostasis and the Regulation and Maintenance of Vitamin D Metabolism

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ABSTRACT

Our recent genomic studies identified a complex kidney-specific enhancer module located within the introns of adjacent Mettl1 (M1) and Mettl21b (M21) genes that mediate basal and PTH induction of Cyp27b1, as well as suppression by FGF23 and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]. The tissue specificity for this regulatory module appears to be localized exclusively to renal proximal tubules. Gross deletion of these segments in mice has severe consequences on skeletal health, and directly affects Cyp27b1 expression in the kidney. Deletion of both the M1 and M21 submodules together almost completely eliminates basal Cyp27b1 expression in the kidney, creating a renal specific pseudo-null mouse, resulting in a systemic and skeletal phenotype similar to that of the Cyp27b1-KO mouse caused by high levels of both 25-hydroxyvitamin D3 [25(OH)D3] and PTH and depletion of 1,25(OH)2D3. Cyp24a1 levels in the double KO mouse also decrease because of compensatory downregulation of the gene by elevated PTH and reduced FGF23 that is mediated by an intergenic module located downstream of the Cyp24a1 gene. Outside of the kidney in nonrenal target cells (NRTCs), expression of Cyp27b1 in these mutant mice was unaffected. Dietary normalization of calcium, phosphate, PTH, and FGF23 rescues the aberrant phenotype of this mouse and normalizes the skeleton. In addition, both the high levels of 25(OH)D3 were reduced and the low levels of 1,25(OH)2D3 were fully eliminated in these mutant mice as a result of the rescue-induced normalization of renal Cyp24a1. Thus, these hormone-regulated enhancers for both Cyp27b1 and Cyp24a1 in the kidney are responsible for the circulating levels of 1,25(OH)2D3 in the blood. The retention of Cyp27b1 and Cyp24a1 expression in NRTCs of these endocrine 1,25(OH)2D3-deficient mice suggests that this Cyp27b1 pseudo-null mouse will provide a model for the future exploration of the role of NRTC-produced 1,25(OH)2D3 in the hormone’s diverse noncalcemic actions in both health and disease. © 2020 The Authors. JBMR Plus published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research.

KEY WORDS: CYTOCHROME P450; CRISPR/Cas9; ChIP-seq; VITAMIN D; GENE REGULATION; 1,25(OH)2D3; Cyp27b1-KO; Cyp24a1; FIBROBLAST GROWTH FACTOR 23; PARATHYROID HORMONE; PTH/Vit D/FGF23; CYTOKINES; TRANSCRIPTIONAL REGULATION; GENETIC ANIMAL MODELS

Introduction

Biological processes integral to the maintenance of mineral homeostasis are highly complex, and likely represent one of the most exquisite regulatory systems that can be defined in higher vertebrates. The need for this regulation is quite clear: Appropriate levels of calcium (Ca) and phosphorus (P), as well as other rare nutrients, are essential for the unique functioning of many if not most life processes. Thus, aberrant levels of these elements can lead to an astounding array of human diseases. Ca and P levels, in particular, are regulated by vitamin D, PTH, and FGF23, the three primary mineralotropic hormones whose independent actions in the intestine, bone, and kidney orchestrate mineral absorption, resorption, and reabsorption, respectively. Interestingly, aside from their unique and frequently overlapping functions in these key tissues, as seen in Fig. 1, each hormone also coordinately regulates the production, processing, and/or activity of the other two. An additional target is the parathyroid gland (PTG) because this organ is the sole producer of PTH. Indeed, PTH is subject to positive regulation by low Ca and negative regulation by FGF23 and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] under a variety of physiological states. From a mineral homeostasis perspective, however, the dominant of the three hormones may be vitamin D, given the intricate nature of its metabolic activation, its striking regulation of both PTH and FGF23, and its broad activity profile across tissues. Thus, though each of the three hormones displays novel activities at the kidney and skeleton, and at other nonmineralizing tissues, vitamin D is alone in its capacity to induce dietary Ca and P absorption from the gut.
Vitamin D is derived through sunlight photo-conversion from cutaneous 7-dehydrocholesterol in the skin. Nevertheless, it must be converted via two sequential hydroxylation reactions to the active hormone, first in the liver by CYP2R1 to 25-hydroxyvitamin D3 (25(OH)D3), though other enzymes and tissues convert smaller amounts, and then in the kidney by CYP27B1 to 1,25(OH)2D3, the active hormone, whereupon it is released as an endocrine principle into the circulation. Of significance, the levels of this hormone are also governed via the regulated expression of Cyp24a1, which initiates the eventual degradation of 1,25(OH)2D3 to calcitroic and calcioic acids via less active 24,25(OH)2D3 or 23,25(OH)2D3 intermediates. Thus, 1,25(OH)2D3 levels are determined by the coordinated expression and actions of two gene products; the synthesis and degradation of PTH and FGF23 also involve actions by several different gene products. Recent studies suggest that CYP24A1 also contributes to the regulation of 25(OH)D3 via its actions to maintain appropriate vitamin D substrate concentrations, not through the control of synthesis, but rather through catabolism to the less active metabolites 24,25(OH)2D3 and 23,25(OH)2D3. Thus, the biological function of CYP24A1 may be largely to prevent inappropriate increase in both 25(OH)D3 and 1,25(OH)2D3 under conditions where these vitamin D metabolites could reach toxic levels, thereby provoking potentially lethal hypercalcemia.

Much of the interregulatory nature of the three mineralotropic hormones as depicted in Fig. 1 has been defined over the past several decades. Thus, it is well known that PTH is a primary inducer of the renal expression of the Cyp27b1 gene encoding CYP27B1 in the kidney, whereas both FGF23 and 1,25(OH)2D3 itself are strong suppressors of Cyp27b1 expression. More recent studies have shown that the renal Cyp24a1 gene is transcriptionally regulated reciprocally by these same hormones, driven by homeostatic responses that occur as a result of changes in Cyp27b1 expression that link the actions of both enzymes to the maintenance of appropriate 1,25(OH)2D3 levels. 1,25(OH)2D3 feedback, in turn, downregulates PTH expression/secretion from the PTGs, while simultaneously inducing FGF23 expression from osteocytes in bone. Like 1,25(OH)2D3, however, FGF23 feedback suppresses the production of PTH, thus providing additional transcriptional control. Importantly, PTH and FGF23 provide direct links to both Ca and P homeostasis, respectively, via the ability of Ca to control PTH and P to control FGF23 levels.

Despite observations over decades documenting the above complex regulatory phenomena, the genomic and molecular mechanisms that mediate these activities in vivo are only now emerging. We turned our attention several years ago toward understanding each of the molecular regulatory events that govern the expression of renal Cyp27b1 and Cyp24a1 and thus the production and maintenance of endocrine 1,25(OH)2D3. The goal was to define the genomic sites of action of each hormone, which we hypothesized would first reveal important initial insights and then provide an entrée into the molecular mechanisms involved. Although we began similar studies of the regulation of PTH and FGF23 genes by these mineralotropic hormones, we summarize in this article our recent efforts to define the genomic mechanisms through which Cyp27b1 and Cyp24a1 expression are regulated by PTH, FGF23, and 1,25(OH)2D3. We took advantage of newly established techniques that enabled an unbiased study of gene regulation entirely in the mouse. Accordingly, we first employed ChIP-seq analysis of the kidney cortex and other tissues to identify potential sites of genomic action of key transcription factors, to characterize the epigenetic histone environment that surrounded these genomic sites and to determine chromatin/DNA sequence accessibility.

We also extended our findings at Cyp27b1 and Cyp24a1 loci using several genetic mouse models wherein the overall expression of these two genes was strikingly enhanced because of highly elevated PTH and lowered FGF23 levels. Finally, we assessed the functions of these regulatory regions to alter Cyp27b1 expression in vivo by using a CRISPR/Cas9 gene-editing approach wherein key segments of both genes were deleted individually from the mouse genome and the regulatory, systemic, and skeletal phenotypes evaluated. Utilizing these techniques, we discovered several complex distal regulatory modules that control the expression of Cyp27b1 and Cyp24a1 uniquely in the kidney that modulate, in turn, the blood levels of endocrine 1,25(OH)2D3.

### Regulation and Maintenance of Vitamin D Metabolism

Identifying the complex tissue-specific regulatory module that controls renal Cyp27b1 expression and the endocrine production of 1,25(OH)2D3

We commenced our study of the regulation of Cyp27b1 and Cyp24a1 in the kidney by first establishing regulatory responses to exogenous administration of PTH, FGF23, and 1,25(OH)2D3 in vivo. These studies confirmed the reciprocal nature of the response of these two genes to PTH, FGF23, and 1,25(OH)2D3 as previously identified. We then confirmed through gain of function transgene experiments that segments controlling these features of the transcriptional regulation of Cyp27b1 and Cyp24a1 were indeed present and located within the extended
genomic regions contained within the transgenes. Accordingly, we introduced large genetically marked BAC clone-derived segments of DNA into the mouse genome using traditional methods (Fig. 2A), and selected gene positive mouse strains that were then explored for their level of basal expression and regulation by PTH, FGF23, and 1,25(OH)2D3. We measured transgene-derived RNA transcripts using novel probes that required the presence of unique sequences located within the transgenes themselves. As shown in Fig. 2B, examination of the output of both Cyp27b1 and Cyp24a1 genes confirmed appropriate and reciprocal hormonal regulation as previously observed for the same endogenous mouse genes in vivo. The expression of the Cyp24a1 transgene was also assessed in a Cyp24a1-null mouse following transgenic rescue of Cyp24a1 expression in this Cyp24a1-null mouse via a genetic cross. Transgenic expression of Cyp24a1 in the kidneys of the rescued mouse resulted in the appearance of 24,25(OH)2D3 in the blood at levels slightly higher than those seen in normal animals. These levels were caused by a below-normal expression of the Cyp24a1 transgene in the kidneys that resulted in a paradoxical rise in 24,25(OH)2D3 as previously discussed. This transgenic confirmation of regulation within defined Cyp27b1 and Cyp24a1 loci narrowed the potential location of regulatory elements and was necessary in light of recent genomic discoveries indicating that regulatory regions for genes can occur frequently many kilobases or even megabases distal to their genetic targets. Our transgenic results provided the rationale for a more-focused search for genomic elements within the surrounding loci for both Cyp27b1 and Cyp24a1 that could regulate the expression of both genes in the kidney.

Identifying the sites of action of PTH, FGF23, and 1,25 (OH)2D at the Cyp27b1 gene in the kidney

As indicated above, we utilized ChIP-seq analysis of the kidney to identify the potential sites of action of each hormone at the Cyp27b1 and Cyp24a1 loci. An initial examination following injection of 1,25(OH)2D3 or PTH revealed the presence of four novel vitamin D receptor (VDR)-bound sites located within the introns of the immediately upstream genes Mettl1 and Mettl21b (now termed Eef1akmt3), sites that we designated M1 and M21 as identified in Fig. 3. A known mediator of PTH action via the PKA pathway, p133-CREB (pCREB) also colocalized to these sites. Exploration of the histone environment surrounding these sites also revealed the presence of histone marks consistent with epigenetic characteristics of regulatory elements. These modifications included H3K4 methylation (me1), H3K9 acetylation, and H3K36 methylation (me3). Importantly, ChIP-seq analyses of the changes that occurred to these histone marks upon injection of PTH, 1,25(OH)2D3, and FGF23 were indicative of altered gene expression, strongly indicating that these regulatory regions were active. PTH mediated an upregulation of Cyp27b1, whereas FGF23 and 1,25(OH)2D3 mediated suppression. Thus, although PTH is known to activate a number of

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**Fig 2.** Extended bacterial artificial chromosome (BAC) clone transgenes that contain mouse Cyp27b1 and Cyp24a1 genetic loci recapitulate the hormonal regulation by exogenous PTH, FGF23, and 1,25(OH)2D regulation seen for endogenous genes in mice. (A) Schematic depiction of mouse transgene structures. (B) Hormonal regulation. Transgenic mice were prepared and selected as recently reported. Animals were injected with PTH (230 ng/g body weight (BW; blue), FGF23 (50 ng/g BW; green), or 1,25(OH)2D3 (10 ng/g BW; black) and tissues harvested at 1, 3, and 6 hours, respectively. Transgene-derived Cyp27b1 and Cyp24a1 transcripts were quantitated using probes that required the presence of the internal ribosome entry-site module incorporated into the BAC clones. Data are derived from four to six mice per group, and presented as the means ± SEM, *p < 0.05.
transcription factors in addition to pCREB, the presence of the VDR and pCREB suggested that 1,25(OH)₂D₃ and PTH were active at these four sites. In the case of FGF23, however, because the transcription factor pathways for this hormone at Cyp27b1 and Cyp24a1 are currently unknown, only increased epigenetic histone activity pointed to where this hormone might act. We also discovered that each of these novel sites contained an open chromatin configuration, which is essential to the functional operation of genomic control elements, an experimental result conducted in the kidney by the ENCODE (Encyclopedia of DNA Elements) Consortium via DNase hypersensitivity sequencing (DHS)-based analysis. Interestingly, none of these features was present within the Mettl2 and Mettl21b genes in any nonrenal tissues. This finding supports our conclusion that the regulatory module we identified is likely specific to the kidney and represents the sole determinant of unique Cyp27b1 response to PTH, FGF23, and 1,25(OH)₂D₃ that links the endocrine production of 1,25(OH)₂D₃ to Ca and P homeostasis.

Identifying the sites of action of PTH, FGF23, and 1,25(OH)₂D₃ at the Cyp24a1 gene in the kidney

With regard to the reciprocal regulation of Cyp24a1 by PTH, FGF23, and 1,25(OH)₂D₃, as depicted in Fig. 3, ChIP-seq analysis of kidney DNA surrounding this gene’s locus in mice revealed a similar overall organization. Thus, two extended, but separate regions downstream of the gene were observed, in addition to well-known promoter-proximal sites, bound occupied clusters of either VDR or pCREB following either 1,25(OH)₂D₃ or PTH injection. Our earlier studies of nonrenal cells in vitro had indicated that one of these downstream regions bound the VDR and contained vitamin D response elements that were transcriptionally active at the Cyp24a1 promoter. Further analysis of the kidney revealed that the chromatin state and epigenetic histone environment across these downstream regions in the kidney were also characteristic of regulatory regions and that the appropriate reciprocal regulation of epigenetic histone density was exerted by PTH and 1,25(OH)₂D₃ as well, indicating that these regions were transcriptionally active. Importantly, regulation of the density of these histone marks by FGF23 exclusively within the region that also bound pCREB provided unique support for a direct role for this hormone’s induction of Cyp24a1 expression, thereby providing a potential mechanistic linkage between the opposing regulatory actions of PTH and FGF23 at this gene. Interestingly, analogous to Cyp27b1, this downstream chromatin feature present at the PTH and FGF23 sensitive region in Cyp24a1 locus is absent in nonrenal tissues. This provides a mechanistic explanation for why the actions of PTH and FGF23 at the Cyp24a1 gene appear to be limited to the kidney, whereas those of 1,25(OH)₂D₃ span all tissues that express the VDR.

Characterizing the regulatory phenotypes of mice with mutations in the Cyp27b1 regulatory module

The identification of potential sites of action of PTH, FGF23, and 1,25(OH)₂D₃ at the Cyp27b1 and Cyp24a1 genes prompted a series of loss of function studies to characterize the specific activities of these regulatory modules at Cyp27b1 and Cyp24a1 in vivo and to confirm whether these modules were indeed functionally specific for the kidney. CRISPR/Cas9-mediated gene-editing techniques in mice provided the essential tool through which we could examine the potential functional features of these regulatory regions across multiple tissues, including the kidney. The editing technique also provided the opportunity to evaluate the phenotypic consequences that emerged following the alteration of Cyp27b1 and Cyp24a1 expression via these regulatory deletions, including an assessment of consequences on the production of endocrine 1,25(OH)₂D₃ itself. As shown in Fig. 3, we utilized pairs of RNA guides to direct Cas9 digestions in oocytes and created mice whose genomes contained an approximate 400-bp deletion (M1) within the intron of Mettl1 (termed M1-IKO for M1-intronic knockout), a 5-kb deletion of the PTH, FGF23, and 1,25(OH)₂D₃ that links the endocrine production of 1,25(OH)₂D₃ to Ca and P homeostasis.

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Fig 3. Schematic representation of the genomic enhancers for Cyp27b1 and Cyp24a1. (A) The locations of enhancers for Cyp27b1 are shown in purple and designated M1 and M21 with the gene dense region of the Cyp27b1 locus, and mediated by PTH (M1; blue), FGF23 (M21; green), and 1,25(OH)₂D₃ (M1 and M21; yellow). (B) The locations of enhancers for Cyp24a1 are shown in purple and designated PP1, DS1, and DS2 within the Cyp24a1 gene locus, and mediated by PTH and FGF23 (DS1; blue and green) and 1,25(OH)₂D₃ (PP1 and DS2; yellow). Figure modified from Meyer and Pike. (26)
(M21) within the extended intron of Mettl21b (termed M21-IKO) and a double deletion at both M1 and M21 (termed M1/M21-DIKO). Although all three strains were indistinguishable from WT littermates at weaning, M1-IKO and M1/M21-DIKO mice began to exhibit retarded growth patterns early on that resulted in reduced body weight and smaller stature, which by 8 weeks reflected the physical appearance of Cyp27b1-null mice. In contrast, the growth pattern and the physical appearance of the M21-IKO mice were unremarkable relative to their WT littermate counterparts. Systemic measurements of Ca, P, PTH, and FGF23 revealed that like those of the Cyp27b1-null mouse, both M1-IKO and M1/M21-DIKO mice exhibited hypocalcemia, hypophosphatemia, hyperparathyroidism, and very low levels of FGF23, all indicative of a potential reduction or the absence of circulating 1,25(OH)2D3. Indeed, measurements of the vitamin D hormone in the blood revealed substantially lower, but not absent levels in M1-IKO mice and even lower levels in the M1/M21-DIKO mice; these levels were undetectable in Cyp27b1-null mice. As with the latter mice, however, both the M1 and M1/M21 deleted strains also exhibited striking changes in skeletal morphology and low BMD as previously identified. These additional systemic, hormonal, or skeletal features were absent in the M21-IKO mice.

The underlying molecular basis for these phenotypic differences between M1-IKO and M1/M21-DIKO mice and M21-IKO and WT mice emerged upon analysis of the expression patterns of Cyp27b1 and Cyp24a1 in the kidney. Accordingly, Cyp27b1 expression was reduced dramatically in M1-IKO and M1/M21-DIKO mice; the latter strain retained a 99% reduction. A similar reduction in the level of renal Cyp24a1 expression was also noted, a decrease that correlated directly with the absence of circulating 24,25(OH)2D3. Interestingly, although M21-IKO mice also exhibited reductions in renal Cyp27b1 and Cyp24a1 expression, these decreases were not as profound. Unexpectedly, however, circulating 1,25(OH)2D3 and 24,25(OH)2D3 were either normal or slightly above normal, respectively. The striking reduction in Cyp24a1 expression in M1-IKO and M1/M21-DIKO mice was also accompanied by extremely high levels of 25(OH)D3 relative to WT and M21-IKO mice as well. Finally, despite the overall impact of these deletions on Cyp27b1 and Cyp24a1 expression in the kidney and on the phenotype of each of these mutant mouse strains, there was no effect observed on the basal expression of these two genes in nonrenal tissues such as skin, bone, intestine, spleen, or immune cells.

Characterizing the regulatory phenotypes of mice with mutations in the Cyp24a1 regulatory module

We also utilized the CRISPR/Cas9 approach to delete the separate regulatory regions located downstream of the Cyp24a1 gene that bound clusters of either VDR or CREB, as illustrated in Fig. 3 and discussed above, and that appeared to mediate the expression of Cyp24a1 in the kidney.86 Deletion of the DS1 region that mediated both the downregulation of Cyp24a1 by PTH and its upregulation by FGF23 resulted in a significant reduction in basal Cyp24a1 expression and complete loss of response to both hormones in the kidney, but no reduction in renal response to 1,25(OH)2D3. 1,25(OH)2D3 activity was also retained in nonrenal tissues where, as expected, PTH or FGF23 were inactive. Interestingly, deletion of the DS2 region that mediated the downstream actions of 1,25(OH)2D3 on Cyp24a1 expression had no effect on the gene’s basal expression or on its suppression by PTH in the kidney or its induction by either FGF23 or 1,25(OH)2D3. Surprisingly, however, deletion of this region decreased the efficacy of response to 1,25(OH)2D3 in nonrenal tissues such as intestine and bone. No striking phenotypic alterations were identified in either DS1-KO or DS2-KO mice with the exception that the loss of basal expression of Cyp24a1 in the DS1 strain resulted in modest homeostatic compensatory changes in PTH and FGF23 levels and a reduction in the renal expression of Cyp27b1. This feature reinforces the idea of the reciprocal coregulation of Cyp27b1 and Cyp24a1 in the kidney that serves to maintain levels of circulating 1,25(OH)2D3. Therefore, our results support the observation at chromatin-, epigenetic-, and now gene-regulatory levels that the downstream PTH/FGF23 regulatory module is active only in the kidney, thereby defining an underlying mechanism through which homeostatic control of Cyp24a1 is restricted to this organ. However, it also illuminates a novel finding that the vitamin D-regulated module downstream of the gene is also dispensable in the kidney, providing a focus on promoter-proximal vitamin D regulatory elements, but enhances the sensitivity of induction of Cyp24a1 by 1,25(OH)2D3 in nonrenal tissues. Thus, the coordinated expression of both Cyp27b1 and Cyp24a1 to maintain circulating 25(OH)D3 and 1,25(OH)2D3 levels in vivo is determined by common structural features within chromatin that facilitate the differential expression and regulation of Cyp27b1 and Cyp24a1 in either the kidney or in nonrenal tissues.

Cyp27b1 and Cyp24a1 genes are regulated and functional in renal proximal tubules

Although early studies suggested that Cyp27b1 and Cyp24a1 were expressed selectively in the proximal tubules of the kidney, respectively, more recent studies using immunocytochemical analyses have indicated that these genes, and especially Cyp27b1, could be produced in additional renal cell types, as well as other nonrenal cell types.27–31 This uncertainty drove our initial decision to explore the entire kidney as above, yet represented a potential complexity relevant to the interpretation of our initial genomic studies of Cyp27b1 expression. This issue also raised additional biological questions relative to the linkage between Cyp27b1 and Cyp24a1. Fortunately, however, recent genomic studies have been conducted by Cusanovich and colleagues on individual kidney cell isolates, as well as numerous nonrenal cell types from C57BL/6 mice using ATAC-seq analysis.32 This approach, like DHS, reveals the presence of open chromatin sites across genomes.33–35 Several genomic data tracks from these analyses at the Cyp27b1 and Cyp24a1 gene loci are documented in Fig. 4. As can be seen, these analyses reveal that the regulatory sites with open chromatin features that we defined within the introns of Mettl1 and Mettl21b in total kidney tissues are also evident exclusively in cells of proximal, but not distal tubule origin, or indeed in other cells of either renal or nonrenal origin. These results confirm that proximal tubules are the principle sites of Cyp27b1 expression and the dominant source for the regulated production of endocrine 1,25(OH)2D3. Indeed, they confirm that the original identification of proximal tubules as the sources of Cyp27b1 expression and the production of 1,25(OH)2D3 was likely correct. These data also indicate that the regulatory module we identified previously is not just kidney-specific, but rather proximal renal tubule-specific. Whether Cyp27b1 expression occurs that is insensitive to PTH and FGF23 regulation in additional kidney cell types, consistent with immunocytochemical identification, remains to be resolved. Interestingly, the open chromatin states as seen in Fig. 4 that define
the regulatory expression of *Cyp24a1* in the kidney are also present exclusively in proximal, but not distal tubules or the other renal cell types, as previously suggested. In conclusion, *Cyp27b1* and *Cyp24a1* are both expressed in the same key endocrine cell type in kidney, and thus capable of coordinating in real time the coregulation of endocrine 1,25(OH)2D3 production that is subsequently secreted into the blood. This has profound implications for the importance of both genes as determinants of vitamin D activation and maintenance, illustrating the power of genomic approaches for illuminating key physiological principles.

**Creation of a kidney-specific Cyp27b1 pseudo-null mouse deficient in endocrine 1,25(OH)2D3**

Research over the past several decades has suggested that the conversion of 25(OH)D3 to 1,25(OH)2D3 occurs not only in the kidney, as above, but also in a myriad of nonrenal tissues/cells (NRTCs) that include the skin, parathyroid glands, bone cells, both cardiovascular and immune cells, and many others. This idea stems from early immunocytochemical observations suggesting that CYP27B1 expression is also present in NRTCs, albeit at very low levels relative to the primary renal source. It also became evident that the regulation of *Cyp27b1* expression in NRTCs is different from that in the kidney. Accordingly, though renal *Cyp27b1* expression is tightly modulated by PTH, 1,25(OH)2D3, and FGF23, and now known to occur through the proximal tubule-specific renal regulatory module described earlier, these mineralotropic hormones are generally inactive at *Cyp27b1* in NRTCs, where inflammatory mediators such as IL-1β, TNFα, LPS, and certainly others function to induce this gene. Some insight has emerged with regard to the pathways that are involved in Cyp27b1 induction by these inflammatory modulators, although the sites of action of these regulators at *Cyp27b1* itself have not been identified in vivo. Moreover, although it is now clear why Cyp27b1 is regulated in the kidney, but not in NRTCs by the three mineralotropic hormones, the molecular basis for the differential basal expression of Cyp27b1 expression in the kidney and NRTCs, although under investigation, remains unknown.
Elucidation of this feature is important because it is likely integral to the evolutionary development and maintenance of the vitamin D endocrine system.

Advancing the features of Cyp27b1 expression and activity in NRTCs

Aside from the concept of local production of 1,25(OH)₂D₃, it is noteworthy that although the synthesis of 1,25(OH)₂D₃ in NRTCs has gained wide acceptance, fundamental insights supporting the mechanism, relevance, and biological consequence of this cellular source of 1,25(OH)₂D₃ production remain outstanding. Importantly, although attempts have been made to selectively delete Cyp27b1 from specific cell types such as chondrocytes, and to assess the phenotypic consequences of these gene deletions on specific tissues, this approach has met with only modest success, perhaps because fundamental questions regarding NRTC production of 1,25(OH)₂D₃ in the absence of renal production remain at issue.⁵⁻⁹ Key issues pertinent to the nature of local production are as follows: (i) Does 1,25(OH)₂D₃ production in NRTCs occur in healthy as well as in diseased subjects in vivo, and does this local synthesis exert a measurable impact on the mechanisms of vitamin D receptor activation that both selectively alter gene expression and uniquely modify the functions of the individual cell types involved; (ii) is locally produced 1,25(OH)₂D₃ routinely secreted into the blood in health as has been amply demonstrated in certain human disease states; (iii) is the overall activity of locally produced 1,25(OH)₂D₃ influenced by or dependent upon the circulating levels of endocrine-derived hormone; and (iv) does vitamin D supplementation and/or circulating concentrations of substrate 25(OH)D₃ differentially impact the local versus kidney production of 1,25(OH)₂D₃. Resolution of these and other issues may lie at the heart of a successful vitamin D supplementation regimen capable of achieving effective therapeutic efficacy in the prevention or treatment of disease. It is noteworthy, however, that precedent has been firmly established in humans for both the production and secretion of 1,25(OH)₂D₃ from macrophages derived from patients with a diverse set of granulomatous diseases.⁺⁻¹¹ The elevated levels of 1,25(OH)₂D₃ are indeed active in these patients and exaggerate the disease by accelerating the uptake of calcium from the gut that results in hypercalcemia. Nevertheless, the overall relationship between this specific NRTC activity to produce 1,25(OH)₂D₃ relative to that derived from the kidney remains to be fully understood. Indeed, not all patients with inflammatory diseases present with elevated blood levels of 1,25(OH)₂D₃ and hypercalcemia.

Utility of animal models to explore NRTC expression and regulation of Cyp27b1

It is clear that unique animal models selectively deficient in the endocrine production of 1,25(OH)₂D₃ will be essential for advancing our understanding of whether and how the local production of 1,25(OH)₂D₃ is achieved and how it contributes to biology. These models will have to be amenable to exploration into the specific issues outlined above and, in particular, to the genetic imposition of inflammatory disease states such as CKD, IBD, atherosclerosis, or perhaps even infectious diseases such as COVID-19. Models such as the latter will enable an evaluation of the hypothesis that 1,25(OH)₂D₃ production is accelerated through an inflammation-induced upregulation of Cyp27b1 expression in the immune system, but not in kidney, for example, that will reduce the overall state of inflammation. Unfortunately, global Cyp27b1-null mice are inappropriate, and efforts to create a CRE-generated kidney-selective Cyp27b1-null mouse have thus far been unsuccessful largely because of the complexity of distinct cell types that comprise even renal proximal tubules. Regardless of whether this is achieved, however, the linkage between renal Cyp27b1 and Cyp24a1 expression suggests that any downregulation of Cyp27b1 will be accompanied by a similar suppression of Cyp24a1 expression, thereby preserving even small amounts of 1,25(OH)₂D₃ that might be secreted into the blood, although not sufficiently active.⁸⁻¹⁹,²⁰ Indeed, it is clear that Cyp27b1 expression is unlikely to be genetically modified and/or suppressed without the homeostatic downregulation of Cyp24a1 by PTH and FGF23.

The Cyp27b1 pseudo-null M1/M21-DIKO mouse as a model for studying NRTC production of 1,25(OH)₂D₃

Interestingly, the M1/M21-DIKO mouse described above exhibits just such a Cyp27b1 pseudo-null phenotype wherein the production of 1,25(OH)₂D₃ is strongly downregulated and incapable of maintaining normal PTH and FGF23 balance and mineral homeostasis.²⁰ This state profoundly disrupts skeletal development and integrity, and mimics the overall Cyp27b1-null mouse phenotype. Given the suppression and loss of regulation of Cyp27b1 expression, it was surprising that even low detectable levels of 1,25(OH)₂D₃ were evident in the blood. Our interpretation of this finding is that though well below normal, the circulating levels of the kidney-derived hormone still remain “inappropriately high” because of the striking suppression of renal Cyp24a1 expression and 24,25(OH)₂D₃ production that is evident as a result in high PTH and low FGF23 levels. This linkage to Cyp24a1 expression as suggested earlier, however, resulted in the absence of both 1,25(OH)₂D₃ and 25(OH)D₃ catabolism in the kidney, thus raising both of these vitamin D metabolites to higher than expected levels in the blood. Given the inability of the mutated Cyp27b1 gene in these mice to respond to PTH, FGF23, or 1,25(OH)₂D₃ regulation, we hypothesized that renal Cyp24a1 levels under dietary conditions of high Ca and P exposure should be upregulated and restored as a result of the normalization of PTH and FGF23 levels, analogous to that seen in Cyp27b1-null mice. Indeed, the application of this “rescue” diet to these M1/M21-DIKO mice fully normalized the aberrant levels of high PTH and low FGF23, appropriately raised Cyp24a1 expression without an effect on Cyp27b1, dramatically reduced 25(OH)D₃ levels, and fully eliminated 1,25(OH)₂D₃ in the blood, as measured in the latter two cases by liquid chromatography–tandem mass spectrometry analysis. This diet also restored all the systemic parameters of normal mineral metabolism, induced genes essential for Ca and P uptake in the intestine, and rescued the skeletal phenotype as well. Cyp27b1 and Cyp24a1 expression in all NRTCs was unperturbed, as were genes that might be expressed as a result of the local production of 1,25(OH)₂D₃. These observations did not support the alternative explanation that the modest amounts of circulating 1,25(OH)₂D₃ in the M1/M21-DIKO mouse were caused by the induced secretion of 1,25(OH)₂D₃ from NRTCs. We conclude that this rescued mouse strain, amenable to additional dietary and genetic as well as disease-inducing manipulations, will likely prove useful in exploring key details of the NRTC production of 1,25(OH)₂D₃.
**Summary and Conclusions**

Here we have summarized our recent work utilizing a series of genomic approaches coupled with loss-of-function studies, which has identified novel distal regulatory modules that mediate the reciprocal expression of Cyp27b1 and Cyp24a1 in the kidneys of mice. This dual regulation is essential for the control of endocrine 1,25(OH)2D3 in the circulation. Additional studies suggest that the regulated expression of both genes by these modules occurs exclusively in proximal tubules. The renal module for Cyp27b1 is dispersed and located within specific introns in the adjacent Mettl1 and Mettl21b genes in the kidney and is absent in all NRTCs. The submodule in Mettl1 controls the upregulation of Cyp27b1 by PTH, whereas three separate submodules in the Mettl21b gene control suppression by FGF23. All four submodules mediate downregulation by 1,25(OH)2D3. Thus, 1,25(OH)2D3 functions to reinforce the suppressive regulatory actions of FGF23, while opposing the inducing actions of PTH at the Cyp27b1 gene. Of course, 1,25(OH)2D3 also induces Cyp24a1 as well as Fgf23.

The renal module for Cyp24a1 regulation is located intergenically downstream of the gene and is comprised of one segment that mediates opposing regulation by PTH and FGF23. Although this module is absent in NRTCs, a second module, which controls positive regulation by 1,25(OH)2D3, is present and active in all cell types that are targets of 1,25(OH)2D3 activity except the kidney. Deletion of the PTH sensitive component (M1) in the Cyp27b1 gene or both components simultaneously (M1/M21-DIKO) lead to a decrease in basal expression of Cyp27b1 of up to 99%, and strongly reduces circulating levels of endocrine 1,25(OH)2D3. Lowered basal levels of 1,25(OH)2D3 in M1-IKO and M1/M21-DIKO mice lead, in turn, to reduced intestinal absorption of Ca and P, which causes hypocalcemia and hypophosphatemia that promotes a rise in PTH and a reduction in FGF23 levels, and a broad Cyp27b1 null-like skeletal phenotype. Cyp24a1, on the other hand, is downregulated by these high PTH and low FGF23 levels, which leads to high levels of 25(OH)D3 and a loss of 24,25(OH)2D3. Both features are responsible for the low, residual levels of 1,25(OH)2D3 in the M1-IKO and M1/M21-DIKO mice.

Rescue of these mice with high Ca and P diets, particularly the M1/M21-DIKO mice, raises systemic Ca, P, and FGF23; suppresses PTH; and normalizes the expression of Cyp24a1. This homeostatic increase reduces 25(OH)D3 levels and eliminates circulating 1,25(OH)2D3. These observations provide supportive evidence for our conclusion that Cyp27b1 and Cyp24a1 are coregulated in the kidney, and that the residual source of 1,25(OH)2D3 in the blood of M1/M21-DIKO mice on a normal mineral diet is not derived from NRTC sources, but rather from the kidney. Diet-rescued M1/M21-DIKO mice, devoid of Cyp27b1 expression and circulating endocrine 1,25(OH)2D3, but with intact expression and regulation of Cyp27b1 in NRTC, represent an appropriate model with which to explore features of Cyp27b1 expression in NRTC. These include its mechanisms, substrate dependencies, role in NRTC production of 1,25(OH)2D3, and impact on noncalcemic actions in multiple tissues in both healthy subjects and in disease. Rescued M1/M21 mutant mice will be useful for exploring supplementation, the impact of 25(OH)D3 on Cyp27b1 expression in renal and nonrenal tissues, and the influence of endocrine 1,25(OH)2D3 on local 1,25(OH)2D3 activation of gene expression.

A collection of previous gene deletion models has enhanced our understanding of both vitamin D metabolism and mineral homeostasis. These individual models have been placed strategically in the context of the actions of the three mineralotropic hormones or their mediators in Fig. 5. We have now added at the appropriate interaction nodes, our newly defined mouse regulatory deletion models, providing an additional level of mechanistic complexity to the interactions that occur between the three hormones to regulate vitamin D metabolism and ultimately mineral homeostasis. Our current studies are now focused on the molecular details of the renal regulation of Cyp27b1 and Cyp24a1 and the genomic mechanisms through which the nonrenal expression of Cyp27b1 activity is achieved. They are also aimed at exploiting the Cyp27b1 pseudo-null mouse to study the nonrenal regulation of Cyp27b1 expression and to determine the underlying genomic mechanisms through which this is achieved.

**Disclosures**

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

J. Pike: Conceptualization; funding acquisition; project administration; supervision; writing-original draft; writing-review and editing. Seong min Lee: Data curation; investigation; methodology. Nancy Benkusky: Data curation; investigation; methodology. Mark Meyer: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; writing-original draft; writing-review and editing.

Authors’ roles

Study design: JWP and MBM. Data collection: MBM, SML, and NAB. Data analysis: MBM, SML, and NAB. Manuscript drafting and revisions: JWP and MBM. Approving final manuscript: JWP, SML, NAB, and MBM.

PEER REVIEW

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