Investigating Vitamin D Insufficiency in Claudin-2 and Claudin-12 Knockout Mice

by

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Abstract

Infants and children require a net positive calcium (Ca^{2+}) balance to achieve optimal bone mineral density by early adulthood. Vitamin D is a hormone that increases blood Ca^{2+} levels by increasing intestinal absorption and renal reabsorption of Ca^{2+} as well as bone remodelling. In both the kidney and intestine, Ca^{2+} is absorbed both paracellularly, through claudin proteins that make up the tight junction, and transcellularly, through Ca^{2+} specific transporters and channels. Claudins-2 and -12 are tight junction proteins that mediate Ca^{2+} permeability across renal and intestinal cell culture epithelial layers. In studying a claudin-2 and claudin-12 double knockout (DKO) mouse model, we observed a decreased Ca^{2+} balance that resulted in hypocalcemia, hypercalciuria and decreased bone mineralization. Interestingly, DKO mice did not have an increase in serum calcitriol levels or upregulation of the genes *CYP27B1* and *CYP24A1* regulating serum calcitriol, despite a significant increase in serum parathyroid hormone (PTH) levels and decreased serum Ca^{2+} levels compared to WT. We hypothesize that claudin-2 and/or claudin-12 are necessary for efficient signalling of *CYP24A1* and *CYP27B1*, and thus vitamin D production in the proximal tubule (PT). To address this, we have examined cell culture models in the PT, including HEK-293 and primary mouse proximal tubule cells. We have found that in HEK cells, PTH treatment results in increased expression of the CYP27B1 promoter, and that claudin-2 expression alone increases promoter expression to the same extent as PTH treated groups. Further, claudin-2 overexpression enhanced *CYP24A1* mRNA transcription with calcitriol treatment. In primary mouse proximal tubule cells, the treatment with PTH results in significantly increased *Cyp27b1* mRNA expression compared to controls, and notably, DKO cells have enhanced *Cyp27b1* mRNA expression with PTH treatment relative to WT cells. CaSR transfection into HEK-293 cells exposed to high extracellular calcium levels displayed an increase in *CYP24A1*

mRNA expression which was suppressed in claudin-12 transfected cells relative to EV and claudin-2 transfected cells, consistent with the CaSR having a role in the transcriptional regulation of *CYP27B1* and *CYP24A1* which is dependent on extracellular calcium levels but not on claudin expression. The results of this study are consistent with claudin-2 and/or claudin-12 playing a role in vitamin D metabolism in the proximal tubule via regulation of CYP27B1 and CYP24A1 transcription. Further work is needed to determine the mechanism behind this effect and what, if any, interactions exist between the claudins and the CaSR. This work will tease out the role of the proximal tubule tight junction proteins claudin-2 and -12 in regulating vitamin D levels.

Preface

This thesis is a part of a project; The role of transport proteins in Epithelial Sodium, Bicarbonate, Phosphate and Calcium Transport and Breeding Colonies, AUP00000213, which has received research ethics approval from the University of Alberta Research Ethics board.

A review of the items outlined in Chapter 1 of this thesis was published as **Young K, Beggs MR, Grimbly C, and Alexander RT**. Regulation of 1 and 24 hydroxylation of vitamin D metabolites in the proximal tubule. *Experimental Biology and Medicine*. 247: 1103-1111, 2022.

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List of Abbreviations

Adcy6	Adenylyl cyclase type 6
Calcitriol	Active vitamin D or 1,25-dihydroxyvitamin D
CaSR	Calcium Sensing Receptor
Cldn12	Claudin-12
Cldn2	Claudin-2
Cq	Quantification cycle
CRE	Cyclic AMP Response Element
CYP24A1	Human cytochrome P450 family 24 subfamily A member 1
Cyp24a1	Mouse cytochrome P450 family 24 subfamily A member 1
CYP27B1	Human cytochrome P450 family 27 subfamily B member 1
Cyp27b1	Mouse cytochrome P450 family 27 subfamily B member 1
DBP	Vitamin D Binding Protein
DKO	Double Knockout
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FBS	Fetal bovine serum
FGF23	Fibroblast Growth Factor 23
FGFR	Fibroblast Growth Factor 23 Receptor
gDNA	Genomic deoxyribonucleic acid
iCa	Ionized Ca^{2+}
KO	Knockout

MAPK	Mitogen-Activated Kinase
NaPi-IIb	Sodium Dependent Phospahte Transport Protein 2b
NHERF1	Sodium hydrogen exchanger regulatory factor 1
PBS	Phosphate buffered saline
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl fluoride
Prka	Protein kinase A
PSG	Penicillin-Streptomycin-Glutamine
PTH	Parathyroid Hormone
PTHR1	G-protein coupled type 1 Parathyroid hormone receptor
RXR	Retinoid X Receptor
VDR	Vitamin D Receptor
VDRE	Vitamin D Responsive Elements

CHAPTER 1: INTRODUCTION

Parts of this chapter have been previously published as **Young K, Beggs MR, Grimbly C, and Alexander RT**. Regulation of 1 and 24 hydroxylation of vitamin D metabolites in the proximal tubule. *Experimental Biology and Medicine*. 247: 1103-1111, 2022.

Calcium and Phosphate Physiological Functions

Calcium and phosphate are vital minerals found predominantly in the structural matrix of bone as hydroxyapatite. Calcium is also essential for a diversity of physiological functions including muscle contraction, neurotransmitter release, intracellular signal transduction, and blood clotting.[1,2] Phosphate also plays critical roles in the composition of the phospholipid bilayer and important nucleotide phosphates, such as ATP. Due to their critical importance in physiological and cellular processes, calcium and phosphate concentrations are tightly regulated within a narrow range in the circulation. Moreover, when the level of one ion becomes sufficiently elevated they can precipitate in the circulation forming extraosseous calcifications as seen in patients with renal insufficiency.[3] As such, the plasma levels of both minerals are regulated via the coordinated action of a group of hormones, referred to as phosphocalciotropic hormones, and the plasma concentration of phosphate and calcium are interdependent. This group of hormones include calcitriol (active vitamin D or 1,25-dihydroxyvitamin D), parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23).

Calcium and Phosphate Homeostasis Overview

Calcitriol

Calcitriol is a steroid hormone that acts by crossing the plasma membrane and binding the vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor (RXR). This complex then translocates to the nucleus where it transcriptionally regulates target genes via binding to vitamin D response elements (VDREs).[4–6] Through this process, calcitriol has a significant impact on calcium and phosphate homeostasis by indirectly influencing the handling

of these minerals in the intestine, kidney and bone.[4,6–12] The effects of calcitriol are primarily observed in the duodenum and colon, where transcellular calcium absorption is increased via increasing *TRPV6* expression.[8] Calcitriol likely also increases paracellular calcium absorption from the jejunum and ileum by increasing claudin 2 expression.[13] In the distal renal tubule calcitriol increases *TRPV5* expression thereby increasing the predominant transcellular reabsorption pathway for calcium.[14] Calcitriol also increases the reabsorption of phosphate from the proximal tubule and absorption of phosphate from the intestine, although the mechanism behind the former processes requires further elucidation.[15] In the intestine, calcitriol increases the expression and/or posttranscriptional modulation of the sodium-dependent phosphate transport protein 2b (NaPi-IIb), resulting in increased absorption of phosphate and increased serum phosphate concentrations.[16] In bone, high levels of calcitriol trigger resorption herby mobilizing calcium and phosphate stores into the circulation. However, calcitriol also supports bone mineralization by ensuring adequate levels of calcium and phosphate in plasma via stimulating intestinal absorption.[4,7,17–19]

Parathyroid Hormone (PTH)

Parathyroid hormone (PTH) is a peptide hormone secreted by the chief and oxyphil cells of the parathyroid gland. PTH acts to increase plasma calcium by increasing renal reabsorption and bone resorption while promoting excretion of phosphate. Its actions are summarized in Figure 1.1. The secretion of PTH is predominantly regulated by the extracellular calcium concentration, which is detected by the calcium-sensing receptor (CaSR) on the surface of chief and oxyphil cells of the parathyroid.[20,21] Calcium ions bind to the extracellular domain of the CaSR, which at high levels activate the receptor, thereby suppressing PTH secretion.[22,23] Phosphate also affects CaSR signaling by acting as a non-competitive inhibitor, whereas calcium is an agonist of the receptor.[23]

Elevated blood phosphate levels inhibit CaSR signaling, thus stimulating PTH secretion.[24] In the kidney, PTH inhibits phosphate reabsorption from the proximal tubule and increases renal calcium reabsorption from the distal nephron. These actions in the proximal tubule are the result of PTH binding to the G-protein coupled type 1 PTH receptor (PTHR1) on both the apical and basolateral surfaces of proximal tubule epithelial cells. The major effects of PTH are mediated by the coupling of PTHR1 to G_s- and $G_{q/11}$-proteins, stimulating the protein kinase A (PKA) and protein kinase C (PKC) pathways, respectively. These kinases phosphorylate the sodium hydrogen exchanger regulatory factor 1 (NHERF1) a PDZ domain-containing scaffold protein, which triggers degradation of sodium dependent phosphate transport protein 2A (NaPi-IIa), thereby attenuating phosphate reabsorption from the proximal tubule.[25] Likewise, PTH downregulates sodium dependent phosphate transport protein 2c (NaPi-IIc) in the kidney, targeting internalization of the protein, although the mechanism behind this is unknown.[25]

FGF23

FGF23 is a peptide hormone that is released in response to increased serum calcitriol and phosphate levels from osteocytes and osteoblasts of the bone.[25] FGF23 binds to the FGF receptor (FGFR) with the co-receptor αklotho in the proximal tubule of the nephron.[26] This binding results in ERK1/2 and serum/glucocorticoid-regulated kinase-1 signaling through WNK1/4 that downregulates membrane expression of the sodium-phosphate cotransporter NaPi-IIa.[26,27] This ultimately leads to less phosphate reabsorption in the kidney. Conversely, FGF23 acts in the distal nephron to increase the reabsorption of calcium. This occurs by ERK1/2 and serum/glucocorticoid-regulated kinase-1 signaling through WNK1/4 to increase transcription of the apical calcium channel TRPV5.[28,29]

Many of the phosphocalciotropic hormones, including those highlighted above, also regulate calcium and phosphate balance by increasing the production or inactivation of calcitriol (Figure 1.2). This is thought to occur primarily by regulating transcription of the 1- or 24-hydroxylating enzymes that activate and deactivate vitamin D, respectively.

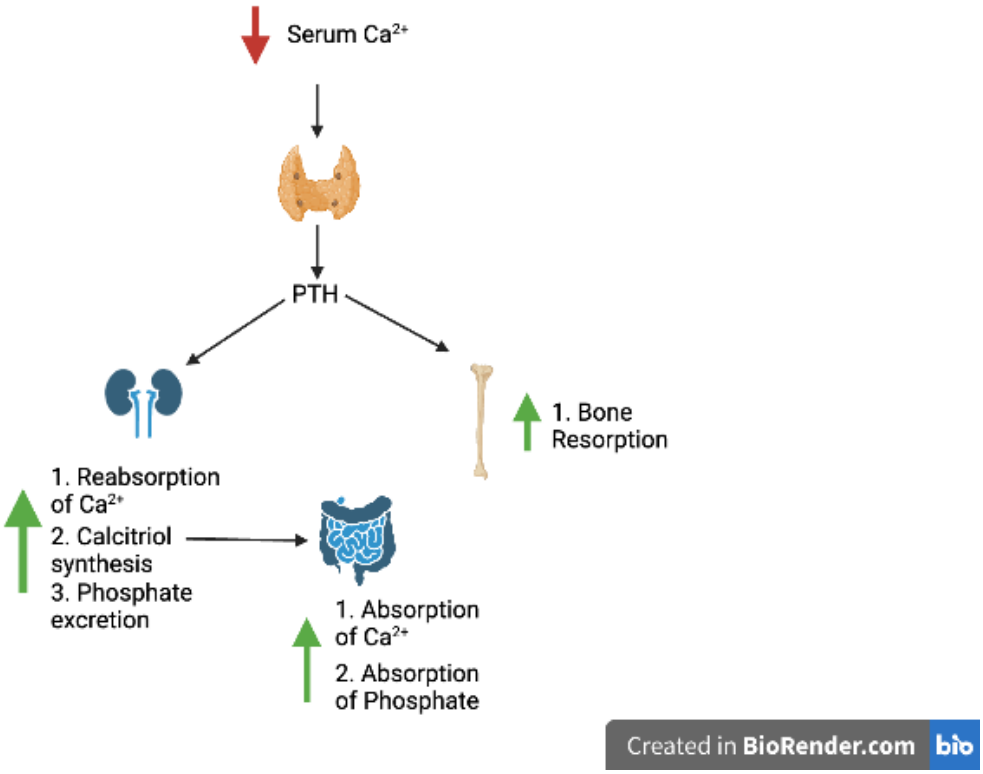

Figure 1.1. PTH regulation of calcium and phosphate homeostasis. PTH is released from the parathyroid gland in response to low serum calcium levels. PTH acts on the bone to promote resorption and thus the release of calcium into the circulation. Additionally, PTH acts in the kidney to increase reabsorption of calcium and excretion of phosphate. Additionally, PTH signals through its receptor in the proximal tubule to increase the transcription of CYP27B1, which in turn results in greater calcitriol activation, and consequently increased intestinal absorption of calcium and phosphate. (Created with BioRender.com)

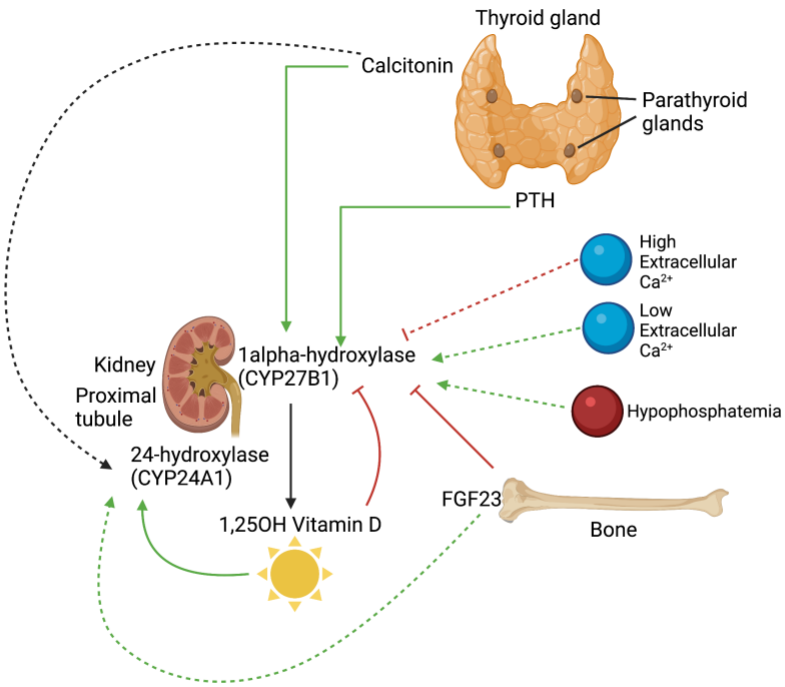

Figure 1.2. Regulation of *CYP27B1* and *CYP24A1* transcription by phosphocalciotropic hormones, calcium and phosphate. Calcitonin and PTH are secreted from the thyroid and parathyroid glands, respectively. Both act to upregulate the transcription of *CYP27B1*. Additionally, calcitonin affects *CYP24A1* by either upregulating or attenuating its transcription depending on the situation. High extracellular calcium has been associated with a decrease in *CYP27B1* transcription, while low extracellular calcium and hypophosphatemia are associated with increased *CYP27B1* transcription. FGF23 production in bone suppresses *CYP27B1* expression while enhancing *CYP24A1* production. Finally, calcitriol itself feedback inhibits its own production by inhibiting *CYP27B1* transcription and promoting its own inactivation by increasing *CYP24A1* transcription. (Created with BioRender.com)

CALCITRIOL SYNTHESIS AND TRANSPORT

Calcitriol can gain access to the circulation either from absorption from the diet or from endogenous production in the skin. The synthesis of calcitriol occurs in two steps in the skin. First, pre-vitamin D_3 is formed from 7-dehydrocholesterol following UV-B irradiation from the sun causing a break in the B-ring of cholesterol.[30,31] Subsequently a non-enzymatic, temperature dependent thermal isomerization occurs which produces vitamin D_3, or cholecalciferol, the final product of this reaction.[30] Vitamin D_3 is then readily taken up by the vitamin D binding protein (DBP), for which it has high affinity, into the circulation. This allows for a rightward shift in the equilibrium such that more vitamin D_3 production is continuously favoured.[30]

Vitamin D_3 can also be taken up into the circulation through intestinal absorption from the diet. While there has been some controversy surrounding the ways in which vitamin D is absorbed across the intestine and into the circulation, it is largely accepted that vitamin D is transported into enterocytes using apical cholesterol transporters.[32] It is proposed that vitamin D is then packaged into chylomicrons in the endoplasmic reticulum and Golgi apparatus before being released in exocytotic vesicles across the basolateral membrane of the enterocyte entering into the lymphatic system where it binds DBP prior to entering the systemic circulation. [33,34]

Once in the circulation, vitamin D_3 travels bound to DBP to the hepatocytes of the liver where it undergoes a first hydroxylation to form 25-hydroxyvitamin D by the enzyme 25-hydroxylase, encoded by CYP2R1.[9,30,31] This initial hydroxylation step is not tightly regulated and is only limited by the amount of vitamin D_3 in the circulation.[10] 25-hydroxyvitamin D_3 is then secreted from the hepatocyte and loaded onto DBP for further transport in the circulation, although the mechanism behind this is unknown.

The second hydroxylation step, which produces the active metabolite 1,25-dihydroxyvitamin D_3, or calcitriol, is the most regulated and occurs in the proximal tubule of the nephron. The enzyme responsible for this hydroxylation is 1-alpha hydroxylase, a mitochondrial protein belonging to the cytochrome P450 superfamily of enzymes.[35,36] Although there is evidence that 1-alpha hydroxylase can be phosphorylated, the effect of this on protein abundance and function is unclear due to a lack of adequate antibodies to study the enzyme and/or phosphoenzyme.[37,38] 1-alpha hydroxylase is a mixed function oxidase encoded by the gene *CYP27B1* within the nuclear genome.[39] This enzyme localizes to the inner membrane of mitochondria where it hydroxylates 25-hydroxyvitamin D at the 1α position to produce 1,25-dihydroxyvitamin D_3 (calcitriol), the biologically active form of the hormone.[35, 4,5,19,40] Calcitriol is also deactivated in the proximal tubule by the 25-hydroxyvitamin D-24-hydroxylase enzyme, encoded by the gene *CYP24A1*. This 24-hydroxylase is also a mitochondrial enzyme that catalyzes the hydroxylation of both calcitriol and its precursor 25-hydroxyvitamin D_3 to 1,24,25-trihydroxyvitamin D_3 or 24,25-dihydroxyvitamin D_3, respectively. 24-hydroxylation inactivates vitamin D, as these forms are unable to bind to VDR.[41] For these hydroxylation steps to occur, 25-hydroxyvitamin D_3 bound to the vitamin D-binding protein (DBP) is filtered at the glomerulus and subsequently endocytosed into the epithelial cells of the proximal tubule after binding to megalin and cubilin. Cubilin sequesters the 25-hydroxyvitamin D-DBP and megalin stimulates endocytosis of the complex. [42, 43] After trafficking to lysosomes, 25-hydroxytamin D_3 is liberated from DBP which is then degraded. [44] The vitamin D metabolite can then be shuttled to the mitochondria for further hydroxylation.[44] The hydroxylated product can then be exocytosed across the basolateral side of the epithelial cells into the circulation where it is again bound to DBP and delivered to target tissues.

Interestingly the proximal tubule is also the site of the majority (60-70%) of calcium reabsorption from the glomerular filtrate, via paracellular pathways.[45–47] The majority of tubular phosphate reabsorption also occurs in this segment but by a transcellular pathway.[48] There is also extra-renal expression of 1-alpha hydroxylase, though the effect on plasma calcitriol levels is negligible and thus these sites of calcitriol synthesis are mostly relevant for local, paracrine calcitriol action.[49–52] Consistent with this, a kidney-specific *Cyp27b1* pseudo-null mouse model displays a phenotype similar to the global knockout animal.[51] Further, extra-renal synthesis of 1-alpha hydroxylase appears to be regulated separately from the proximal tubule, as they do not contain the same regulatory module as the kidney gene.[51] Importantly, an increase in expression of *Cyp27b1* is closely tied to increased calcitriol production, consistent with transcription being the major mode of regulation in calcitriol production.[53]

Clinical Importance of Calcitriol

Abnormalities in calcitriol metabolism highlight the role of this hormone in the maintenance of calcium and phosphate homeostasis. Calcitriol deficiency results in hypocalcemia, hypophosphatemia, and vitamin D-dependent rickets. Rickets is a syndrome that has multiple features including bone weakness, fractures, pain, as well as abnormal bone bending and deformity of the tibiae and femora. Consistent with this, inactivating mutations in the *CYP27B1* gene results in vitamin D dependent rickets type I, an autosomal recessive disorder characterized by an inability to synthesize calcitriol which results in hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and rickets.[54] This disorder of 1-alpha hydroxylase deficiency is rare, but there is an unusually high frequency in the French-Canadian population due to a founder effect.

Fortunately, this condition responds to treatment with exogenous calcitriol.[55,56] However, calcitriol supplementation is more commonly prescribed to patients with chronic kidney disease to treat secondary hyperparathyroidism and hypocalcemia. Chronic kidney disease patients often have decreased calcitriol production as a result of lower levels of 1-alpha hydroxylase production due to decreasing renal mass.[57]

In contrast, loss of function mutations in the *CYP24A1* gene cause hypercalcemia due to the inability to inactivate calcitriol and can present as idiopathic infantile hypercalcemia or as a rare genetic cause of nephrolithiasis.[41,58–61] Granulomatous diseases such as subcutaneous fat necrosis, sarcoidosis, tuberculosis and lymphoma can contribute excess 1-alpha hydroxylase production due to macrophage activation. This results in increased calcitriol levels and hypercalcemia.[62–64] These diseases highlight the importance of *CYP27B1* and *CYP24A1* in maintaining appropriate concentrations of calcitriol in the circulation and the impact of dysregulated activity on calcium and phosphate homeostasis.

Classical Regulation of CYP27B1 and CYP24A1

Parathyroid hormone mediated regulation of CYP27B1 and CYP24A1

PTH acts to increase transcription of the rate limiting enzyme in calcitriol production, 1-alpha hydroxylase, in the proximal tubule (Figure 1.3) leading to greater synthesis of calcitriol.[65–67,68–75] This enables PTH to further increase serum calcium levels through the combined actions of PTH and calcitriol on the intestine and bone. This is the classical pathway through which PTH indirectly increases intestinal calcium absorption. Simultaneously, the half-life of *CYP24A1*

mRNA is reduced approximately 4-fold in the presence of PTH, thereby slowing the inactivation of calcitriol.[76]

Evidence of the important role PTH plays in regulating calcitriol production comes from animal models. When parathyroidectomized animals are fed a low calcium diet, they fail to increase circulating levels of calcitriol, yet have increased levels of the inactive hormone produced by 24-hydroxylase.[69] However, calcitriol production was able to be induced by administering either a parathyroid extract or PTH, consistent with PTH stimulating the production of calcitriol via increasing the expression of *Cyp27b1* and reducing *Cyp24a1*.[69]

Further studies on parathyroidectomized and calcitriol deficient rats implicated cAMP as an important intracellular signal involved in the stimulation of *Cyp27b1* transcription in response to PTH.[75] Notably, infusion of cAMP into parathyroidectomized rats mimicked the effect of PTH infusion, as it stimulated calcitriol production in a dose-dependent manner.[75] Moreover, renal adenylate cyclase activity was enhanced by PTH in rats made calcitriol-deficient by feeding them a calcitriol-deficient diet. In this study, the administration of PTH caused an immediate increase in renal cAMP levels.[75] Brenza et al. also provided evidence that cAMP is a second messenger involved in the induction of *CYP27B1* transcription in a pig cell line, AOK-B50 cells, as well as the human HCK-8 proximal tubule cell line.[71] In this cell culture work, forskolin, an adenylate cyclase activator that increases intracellular cAMP, increased the activity of a *CYP27B1* reporter.[71] However, direct application of PTH to these cell models resulted in a significantly greater response than forskolin treatment alone, suggesting that PTH is potentially activating more than one second-messenger pathway to increase transcription of *CYP27B1*.[71,72] Work done by Korkor et al. in mouse cortical kidney cell cultures (a model system composed predominantly of proximal tubule epithelial cells) further implicates cAMP as an important second messenger for PTH signaling.

They found that calcitriol synthesis was dependent on *de novo* production of 1-alpha hydroxylase, and that calcitriol synthesis was proportional to the amount of cellular cAMP.[77] Furthermore, addition of protein or mRNA synthesis inhibitors prevented cAMP-mediated stimulation of calcitriol production, leading to the conclusion that increased calcitriol production is dependent on an increase in novel protein synthesis.[77] Brenza et al. also found that the promoter for *CYP27B1* contains cyclic AMP response element (CRE) sites.[72] These are DNA binding sites for the CREB transcription factor, which is activated in response to phosphorylation through the PKA pathway.[78] These findings further implicate cAMP as the predominant second messenger mediating PTH-induced stimulation of *CYP27B1* transcription.[72]

Work by Zierold et al. in a porcine kidney cell line (AOK-B50) identified other specific transcription factors that upregulate *CYP27B1* gene production following exposure to PTH.[74] NR4A2 (Nurr1), a nuclear receptor that binds to DNA sequences in the promoter region of the genes it regulates, has been implicated in the upregulation of *CYP27B1* in response to PTH. NR4A2 over-expression increased *CYP27B1* mRNA by binding to the promoter and NR4A2 mRNA levels were increased in response to PTH.[74] C/EBPβ was also implicated in the regulation of *CYP27B1* transcription in these studies, acting directly on the promoter to decrease transcription and indirectly by reducing the amount of NR4A2 produced.[74] C/EBPβ is also known to upregulate *CYP24A1* transcription. The overall effect of C/EBPβ in this work was thus a decrease in calcitriol production. However, these results are in contrast to work interrogating the role of calcitonin in regulating calcitriol expression, which is discussed below.

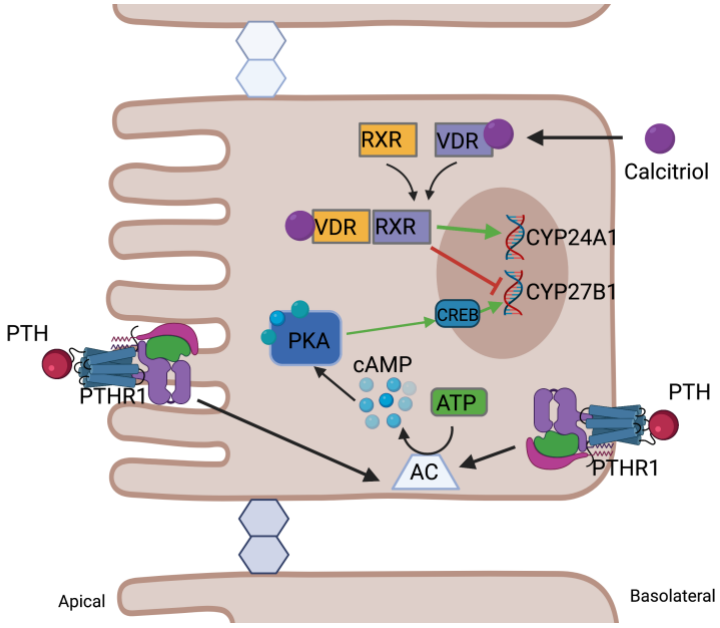

Figure 1.3. Transcriptional regulation of *CYP27B1* and *CYP24A1* by parathyroid hormone (PTH) and calcitriol in proximal tubular epithelial cells. PTH-induced transcriptional regulation occurs primarily by a PKA-mediated pathway. Upon binding to PTHR1, a G-protein coupled receptor in the apical or basolateral membrane, adenylate cyclase is activated producing cAMP, which in turn activates protein kinase A (PKA). PKA phosphorylates CREB, activating it and permitting binding to CRE sites in the promoter of *CYP27B1*. Calcitriol signalling occurs via binding to the intracellular vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor (RXR) prior to DNA binding. Together, liganded VDR-RXR enter the nucleus where it can bind to vitamin D response elements of target genes. This increases *CYP24A1* transcription, resulting in decreased calcitriol levels, and decreases *CYP27B1* transcription, reducing calcitriol production. (Created with BioRender.com)

FEEDBACK INHIBITION BY CALCITRIOL

Calcitriol has negative feedback effects on its own production by inhibiting *CYP27B1* transcription. This negative effect on *CYP27B1* expression occurs downstream of the binding of calcitriol to VDR. Calcitriol-bound VDR dimerizes with the retinoid X receptor (RXR), which in turn can bind to vitamin D response elements (VDREs) within DNA to either enhance or inhibit the transcription of responsive genes. Consistent with this concept of calcitriol feedback inhibition via nuclear signaling, inhibition of *Cyp27b1* expression is absent in VDR knockout mice, who have significantly higher calcitriol levels compared to wild-type animals.[4,5,7,19] Further, animals made calcitriol deficient by being fed a low vitamin D diet and receiving limited exposure to UV radiation had reduced *Cyp27b1* expression when given calcitriol supplementation.[5]

This is supported by work in rats. The administration of calcitriol to calcitriol-deficient rats reduced the transcription of *Cyp27b1* in both the absence and presence of PTH.[71] Calcitriol also inhibits parathyroid hormone gene transcription in the parathyroid to further suppress its own production.[71,79] This suggests that calcitriol can override PTH-mediated stimulation of *Cyp27b1* expression when the concentration of calcitriol is sufficiently high, although the exact level is currently unclear.[71] It is also unclear whether the ultimate effect of these two competing inputs depends on their relative concentrations or another input, such as the concentration of serum calcium or phosphate. Clearly more studies are needed to determine their roles in different conditions. Finally, calcitriol also upregulates C/EBPβ in rat kidney, which acts directly on the *Cyp27b1* promoter to inhibit its activity and reduce *CYP27B1* transcription.[74] C/EBPβ also inhibits the stimulatory action of NR4A2 on the promoter, thereby reducing the impact of NR4A2 in the presence of PTH and reducing *CYP27B1* transcription.[74]

Calcitriol also acts to limit its own production by increasing the expression of *CYP24A1*, which encodes the 24-hydroxylase that inactivates calcitriol.[39,71,80] When porcine AOK-B50 kidney cells were treated with calcitriol, *CYP24A1* gene expression was stimulated in concert with suppressed *CYP27B1* gene expression.[71] Transcription of *CYP24A1* was upregulated by calcitriol via VDR dependent control of VDREs in the *CYP24A1* promoter.[71,81] Calcitriol-induced upregulation of C/EBPβ in the kidney not only serves to repress *CYP27B1* transcription, but also to upregulate *CYP24A1* expression via C/EBPβ sites in the 24-hydroxylase promoter.[82] This is further supported by the observation that there is marked attenuation of *CYP24A1* transcription when this site is mutated.[82] Thus, not only is there negative feedback from calcitriol that inhibits further production of the hormone, there is also feedback to promote the inactivation of vitamin D metabolites.

Non-Classic Signaling Pathways

Calcitonin

Calcitonin is a hormone released from the C-cells of the thyroid gland in response to high blood calcium levels. It binds to the G-protein coupled calcitonin receptor, which then signals through both the PKC and PKA pathways.[83] Calcitonin protects against hypercalcemia largely by exerting a negative effect on osteoclasts in bone to prevent bone resorption and thus increases bone mineralization an effect potentially augmented by decreasing urinary calcium excretion.[84–86] In this way, calcitonin may redirect calcium from the blood and urine into bone mineralization. There is evidence, however, that calcitonin also plays a role in regulating calcitriol production by increasing *CYP27B1* transcription (summarized in figure 1.4). Under hypercalcemic conditions, calcitonin may be a significant regulator of *CYP27B1* expression, rather than PTH which is likely

more relevant in the hypocalcemic state.[53] While PTH signals through both the PKA and PKC pathways to induce *CYP27B1* transcription, calcitonin preferentially increases *CYP27B1* expression via the PKC pathway as demonstrated in renal porcine LLCPK cells.[83] Although administration of the PKA activator 8-bromo-cAMP had a stimulatory effect on *CYP27B1* mRNA expression, the PKA inhibitors Rp-cAMPS and H-89 had no effect on calcitonin-induced *CYP27B1* expression. In contrast, the PKC activator phorbol 12-myristate 13-acetate (PMA) increased *CYP27B1* mRNA levels to a similar extent as seen with calcitonin treatment, while administration of the PKC inhibitor staurosporine attenuated *CYP27B1* transcription induced by calcitonin in a dose-dependent manner.[83] This strongly implicates PKC in mediating the stimulatory effect of calcitonin on calcitriol production.

The transcription factor C/EBPβ is also a downstream effector of calcitonin (Figure 1.4). Expression of this transcription factor increased following exposure to calcitonin.[87] C/EBPβ can bind to the promoter of *CYP27B1* thereby increasing 1-alpha hydroxylase production. Further, the transfection with a dominant negative modulator of C/EBPs binding sites inhibited calcitonin induced *Cyp27b1* transcription in a dose dependent manner.[87] This is a potential mechanism by which calcium retention is increased despite a normal blood calcium concentration, thereby increasing calcium availability in the circulation in times of increased demand, such as pregnancy and lactation.[87] There is evidence therefore, supporting a role for C/EBPβ in both positive and negative regulatory pathways, as it is also associated with decreased *CYP27B1* expression after calcitriol exposure. These seemingly contradictory effects might be explained by other yet to be identified signaling events affecting C/EBP β gene modulation. This issue requires further investigation to elucidate the differing effects of C/EBPβ on *Cyp27b1* expression.

There is conflicting evidence surrounding the role of calcitonin on *CYP24A1* expression. Research in thyroparathyroidectomized rats, in which both PTH and calcitonin production are lost, fed a low calcium diet found 2-fold increased *Cyp24a1* expression, however calcitonin administration reduced *Cyp24a1* mRNA expression.[88] In contrast to this, *in vitro* work in human embryonic kidney (HEK-293) cells that were transfected with the calcitonin receptor found that calcitonin stimulates *CYP24A1* expression. Additionally, H89 and calphostin C, inhibitors of the PKA and PKC pathways respectively, reduced calcitonin-induced CYP24A1 expression by 60%.[89] It was proposed that calcitonin induced the PKA or PKC pathway which phosphorylates and activates the transcription factors Sp1 and NF-Y, which were both shown to increase expression of *CYP24A1*.[89] Although these studies are seemingly contradictory, there is a possibility that calcitonin can exert a different effect depending on calcium and calcitriol levels, promoting calcitriol degradation when blood calcium levels are high and suppressing degradation of calcitriol when calcium levels are low. Further work is required to determine the role of calcitonin in calcitriol metabolism.

FGF23/KLOTHO

As previously described, Fibroblast Growth Factor-23 (FGF23) is a peptide hormone produced in bone by osteoblasts and osteocytes that inhibits renal tubular phosphate reabsorption and calcitriol production to lower blood phosphate and increase blood calcium levels (summarized in Figure 1.4).[66,90]FGF23 binds to fibroblast growth factor receptor isoforms 3 and 4, thereby activating the receptors and inducing tyrosine autophosphorylation and the stimulation of its intrinsic tyrosine kinase activity.[91,92] This leads to the activation of the MAP kinase pathway and downstream phosphorylation of extracellular signal-regulated kinase-1 and -2 (ERK1/2).[93] The co-

receptor, klotho, is required for binding of FGF23 to the receptor and enabling its subsequent activation in the kidney.[93,94] FGF23 and klotho have a suppressive role in renal *CYP27B1* transcription through ERK1/2.[90,93,94] HEK-293 cells transfected with the *CYP27B1* promoter had suppressed *CYP27B1* promoter activity when exposed to FGF23. [49] The suppressive effect of FGF23 was blocked by a ERK1/2 inhibitor. Moreover, FGF23-null mice display 3-fold increased *CYP27B1* promoter activity in the kidney compared to WT mice with normal FGF23 levels, consistent with FGF23 suppressing *CYP27B1* expression.[49]

FGF23 also lowers blood calcitriol levels by promoting renal *CYP24A1* transcription, to promote calcitriol inactivation.[49,95] Consistent with this, FGF23-null mice display 63% lower *Cyp24a1* mRNA expression in the kidney compared to wild-type mice.[49] Further work is needed to elucidate the mechanism driving the FGF23-mediated increase in *CYP24A1* expression. Research into this area is of great interest because of potential implications in chronic kidney disease, where elevated FGF23 presents in the early phases and is accompanied by severe calcitriol deficiency.[96] Increased *CYP24A1* transcription, in response to FGF23, may exacerbate calcitriol deficiency and contribute to the progression of chronic kidney disease.[97,98]

Hypophosphatemia

Regulation of *CYP27B1* expression also occurs in response to alterations in serum phosphate concentration (summarized in Figure 1.4). Hypophosphatemia, as caused by a low phosphate containing diet, results in increased *Cyp27b1* expression independently of PTH and markedly elevated serum calcitriol levels.[99,100] This is supported by observations that phosphate-depleted mice exhibit 3-fold enhanced calcitriol production compared to control-fed mice.[101]

However, this effect is abolished in these mice post-hypophysectomy suggesting that the pituitary gland has a role in sensing circulating phosphate levels and altering serum calcitriol production in response.[100] Consistent with this is evidence that pituitary hormones may exert an effect on calcitriol production through transcriptional regulation of *Cyp27b1*.[100] Due to the previous association of growth hormone deficiency with hypovitaminosis D, growth hormone represents a potential candidate for this role.[102] However, the exact mechanism whereby extracellular phosphate sensing occurs, or how pituitary hormones affect *CYP27B1* expression in the kidney remains to be elucidated.

Calcium and the Calcium-sensing Receptor

In addition to plasma phosphate, plasma calcium concentration also modulates calcitriol production, independent of PTH or other calciotropic hormones (summarized in Figure 1.4). Thyroparathyroidectomized rats are unable to secrete PTH or calcitonin in response to altered blood calcium levels. However, the direct infusion of $CaCl_2$ into these animals resulted in the suppression of renal *Cyp27b1* expression.[67] Further, infusion of these rats with calcium and PTH simultaneously resulted in increased blood calcium levels, however *Cyp27b1* activity was suppressed compared to rats given PTH alone. Conversely, when PTH was administered with EGTA, a calcium chelator that prevents a rise in blood calcium levels, *Cyp27b1* expression was stimulated.[67] These results demonstrate that higher blood calcium levels counteract the effects of PTH to suppress production of calcitriol when blood calcium levels are high. Further work is needed to delineate the effect of increased blood calcium on *CYP24A1* expression.

The calcium-sensing receptor (CaSR) is a 7 transmembrane G-protein coupled receptor that classically senses the extracellular calcium concentration and negatively regulates PTH production and secretion from the parathyroid gland in response. The CaSR also has effects outside of the parathyroid gland, with expression in organs such as the brain, kidney and intestines where it has effects on neuro-pathological conditions and modulating renal calcium reabsorption and intestinal calcium absorption.[23,103–106] It acts to modulate the expression and activity of calcium and phosphate transporters, channels and pores, including tight junction proteins that regulate both paracellular and transcellular calcium (re)absorption across renal and intestinal epitehlia.[22,104,107–109] CaSR activity has also recently been linked to the modulation of *CYP27B1* expression in a HEK-293 cell model. CaSR expressing HEK-293 cells were transfected with a *CYP27B1* promoter reporter construct and exposed to increasing levels of extracellular calcium. Up to and including 3 mM extracellular calcium resulted in increased reporter activity.[110] This suggests that activation of the CaSR at higher calcium concentrations signals to the *CYP27B1* promoter to increase gene transcription. Interestingly, when the extracellular concentration of calcium was raised above 3 mM there was suppression of promoter activity.[110] This group further suggests that the CaSR signals through either a PKC or $ERK_{1/2}$ pathway to regulate *CYP27B1* expression, since administration of inhibitors of these pathways simultaneously blocked the effect of calcium on *CYP27B1* transcription, though administration of each inhibitor individually had little effect.[110] Further, whether CaSR alters *CYP24A1* expression is not known. Thus, further work is required to determine the exact effects of renal CaSR activation on *CYP27B1* and *CYP24A1* expression and the signaling pathways involved. Taken together with the *in vivo* experiments described above, this suggests that the CaSR might modulate the expression of *CYP27B1* in a dose-dependent

manner, such that further calcitriol production is not favoured when calcium levels are sufficient or elevated.

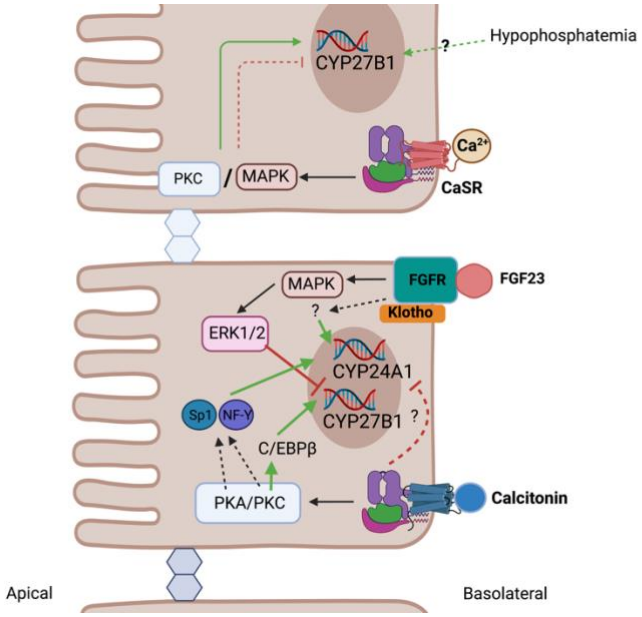

Figure 1.4. Transcriptional regulation of *CYP27B1* and *CYP24A1* by calcium, phosphate, FGF23 and calcitonin. Extracellular calcium activates the calcium sensing receptor, which in turn activates mitogen-activated protein kinase (MAPK) and PKC pathways. These pathways are proposed to interact with the *CYP27B1* promoter to increase its activity at low extracellular calcium concentrations and to decrease promoter activity at high extracellular calcium concentrations. Hypophosphatemia is linked to increased expression of *CYP27B1*. Fibroblast growth factor 23 (FGF23) binds to the FGF receptor (FGFR) and its cofactor klotho to activate the MAPK pathway, phosphorylating ERK1/2, which inhibits *CYP27B1* transcription. FGF23 signaling is also associated with increased *CYP24A1* levels. Calcitonin binds to its G-protein coupled receptor, activating both the PKA and PKC pathways. PKC increases the expression of the C/EBPβ transcription factor thereby promoting *CYP27B1* transcription. Calcitonin can also signal through the Sp1 and NF-Y transcription factors to upregulate *CYP24A1* transcription. (Created with BioRender.com)

CLAUDINS

Claudins are a family of tight junction proteins that confer selective permeability to epithelial cells enabling paracellular absorption or reabsorption of ions and small molecules. This family of proteins have four transmembrane domains and two extracellular loops that interact in the junction between two cells to form a selective pore or barrier, this interaction also mediates cell adhesion (Figure 1.5).[111,112] The proximal tubule is responsible for the reabsorption of approximately 65% of calcium that is filtered, the vast majority of which is reabsorbed via the paracellular pathway and mediated by claudins. It is known that two claudins mediate the permeability to calcium and other cations through the tight junction in the proximal tubule and the intestine, they are claudin-2 and claudin-12.[113,114]

Claudin-12 has been reported in a proximal tubule cell culture model in oppossum kidney (OK) cells, where it is likely to contribute to paracellular permeability.[115] Claudin-2 is expressed in various leaky epithelial tissues throughout the body, including the proximal tubule and intestine. It is a 230 amino acid, 24.5 kDa protein with a structure characteristic of claudin proteins as described above. The specificity of the pore is determined by amino acids D65 and Y67 within the first extracellular loop, which permits positive ions and water to pass through. There also exists a PDZ binding domain at the C-terminal end of the protein which associates with tight junction scaffold proteins that connect to the cellular cytoskeleton.[116] Claudin-2 is of particular interest in human diseases of the kidney and has been associated with nephrocalcinosis. Via the investigation of patients with nephrocalcinosis, or kidney stones, Curry et al. found several common genetic variants in the claudin-2 gene that associated with increased risk of developing kidney stones.[117]

Further, there is evidence that claudin-2 is involved in signal modulation that has effects on cell proliferation, migration and cell fate. However, the exact mechanisms mediating these effects remain to be further elucidated.[116]

Additionally, there is evidence for a role of calcitriol in regulating paracellular permeability through regulation of claudin gene transcription. VDR knockout mice have decreased mRNA and protein levels of claudin-2 and claudin-12. Further, administration of calcitriol results in increased claudin-2 and claudin-12 expression in an intestinal epithelial cell model.[11] As such, calcitriol facilitates increased intestinal absorption of calcium through both the transcellular pathway, by increasing *TRPV6* expression, and the paracellular pathway, by potentially increasing claudin-2 and claudin-12 expression. [116]

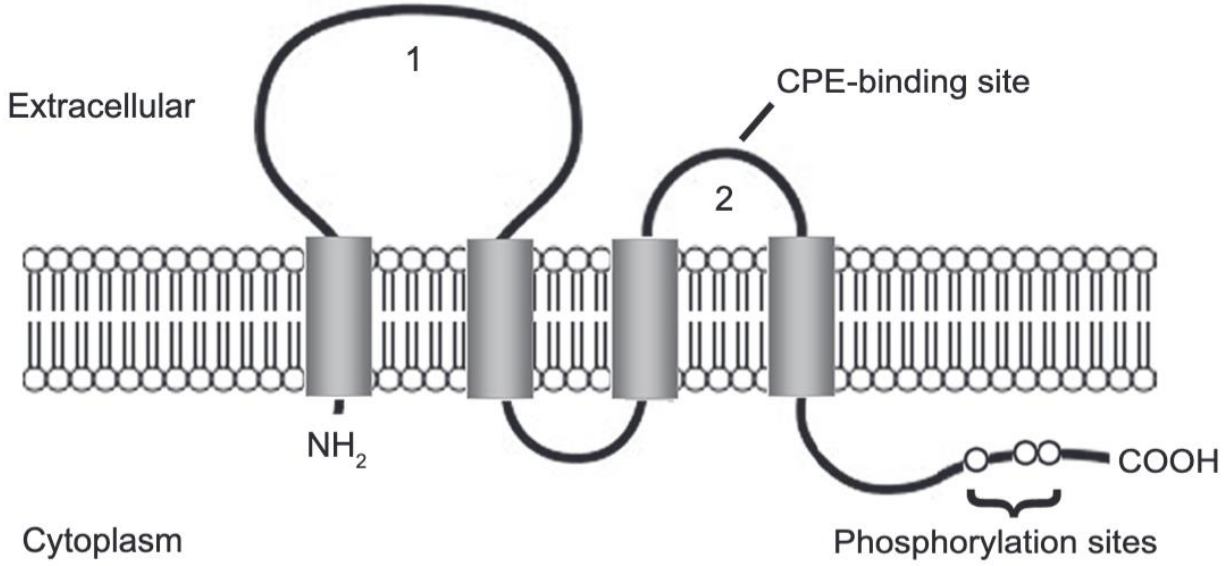

Figure 1.5. Structure of claudins. Figure from Ding et al..[133] Claudins are tetraspan membrane proteins with two extracellular loops that confer ion selectivity and permeability. The C-terminal end is involved in phosphorylation and interactions depending on the type.

Transgenic Claudin Mouse Models

Previous projects in the Alexander laboratory have investigated the roles of different claudins in calcium homeostasis through the generation of transgenic mouse models with a global knockout in claudin-2, claudin-12, or both claudin-2 and -12.

Claudin-2 Knockout

The genetic knockout of claudin-2 in mice resulted in a mild calcium phenotype. Claudin-2 KO mice showed no changes in serum calcium levels from WT mice and no difference in calciotropic hormone levels, calcitriol and PTH.[117] There was, however, a significantly greater excretion of calcium in the urine as a result of defective reabsorption of calcium from the proximal tubule of the nephron (although this was inferred as direct measurements of proximal tubule permeability were not made). KO mice also had papillary nephrocalcinosis, as evidenced by Von Kossa staining of kidney sections.[117] In order to compensate for the wasting of calcium in the urine, the KO mice had significantly greater absorption of calcium from the diet in the intestine. Further, a GWAS study linked the claudin-2 gene to altered risk of developing kidney stones.[117]

Claudin-12 Knockout

A claudin-12 knockout mouse model has also been generated to investigate the role of this protein in calcium homeostasis. It was found that claudin-12 KO mice had reduced permeability to calcium in the proximal tubule by *ex vivo* microperfusion of mouse proximal tubules relative to WT mouse proximal tubules.[114] Similar to the claudin-2 KO mice, claudin-12 KO mice had no changes in serum calcium, PTH, or vitamin D levels. However, unlike the claudin-2 KO, claudin-12 deficient

mice did not have alterations in urinary calcium excretion and was not different than WT mice.[114] The lack of a prominent phenotype led to the hypothesis that existing claudin-2 was compensating for the lack of claudin-12 in these mice, which led to the following experiment investigating the double knockout of both genes.

Claudin-2/12 Double Knockout

In order to elucidate the roles of claudin-2 and claudin-12 in calcium homeostasis, both genes were knocked out in a global claudin-2 and claudin-12 double knockout (DKO) model, which resulted in a significant calcium wasting phenotype. DKO mice were hypocalcemic, with significantly lower serum ionized calcium (iCa) compared to WT mice.[46] DKO mice also had significantly increased fractional excretion of calcium in the urine compared to WT mice and significantly reduced bioavailability of calcium from the diet and thus decreased absorption of calcium from the intestines. The bones of DKO mice had significantly lower trabecular and cortical bone mineral density relative to WT. Consequently, DKO mice had significant elevations in serum PTH levels compared to WT. As previously discussed, the increase in PTH levels in combination with hypocalcemia should result in an increase in serum calcitriol levels by promoting further activation of the hormone through transcriptional upregulation of the *CYP27B1* gene. The DKO mice did not, however, have a significant increase in serum calcitriol levels when compared to WT mice (Figure 1.6). This led us to investigate the transcription of *Cyp27b1* and *Cyp24a1*. To our surprise *Cyp27b1* transcription was not increased in the DKO mice and there was no difference in *Cyp24a1* transcription between groups.[46]

Hypothesis and Rationale

The absence of increased calcitriol and elevated renal *Cyp27b1* in the claudin-2 and claudin-12 DKO mouse model, led us to surmise there may be a role for these claudins in the regulation of

vitamin D metabolism in the proximal tubule. We therefore hypothesize that claudin-2 and/or claudin-12 are necessary for optimal signaling to increase *Cyp27b1* mRNA expression and thus calcitriol concentration in the proximal tubule of the nephron.

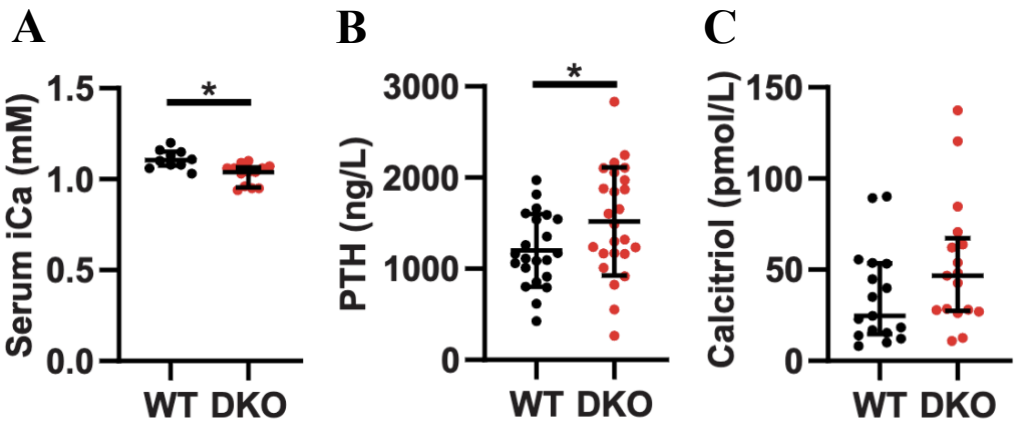

Figure 1.6. Serum ionized calcium, parathyroid hormone (PTH) and calcitriol levels in WT and DKO mice. (A) Serum iCa (median ± interquartile range [IQR], n = 10 WT, 13 DKO, Mann-Whitney U Test, and P = 0.002). (B) Serum PTH level (mean ± SD, n = 22 WT, 25 DKO, Student's t test, and P = 0.039). (C) Serum calcitriol (median ± IQR, n = 17 WT, 17 DKO, Mann-Whitney U Test, and P = 0.099). *P < 0.05.

CHAPTER 2 : MATERIALS AND METHODS

Ethics Approval and Animals

Animal ethics were obtained from the Animal care and Use Committee, Health Sciences Section (AUP00000213). Claudin-2 knockout, claudin-12 knockout and claudin-2/12 double knockout animals were maintained on a 12-hour light/dark cycle with water and chow *ad libitum* (Lab Diet Irradiated Rodent Diet 5053, 4% fat, 0.81% calcium). For all experiments, male and female mice were used and all data is presented combined as no sex-specific differences were found in any analysis.

Cldn2 global KO mice were from MMRRC at Univ. of California Davis. Cldn12 KO mice were made as described previously, as were claudin-2/12 double knockout mice.[46,114] Genotype was confirmed by real-time PCR and western blot where applicable.

Metabolic cage studies

WT and claudin-2/12 DKO mice aged 2-3 months were placed in metabolic cages for 72 hours as previously described.[118] Water and low-calcium chow (0.01% Calcium) were available *ad libitum*. Bodyweight was measured on day 0 and every 24 hours thereafter. Urine and feces as well as chow and water intake were measured and collected every 24 hours. Following a 72 hour acclimatization period, mice were euthanized with a lethal dose of sodium pentobarbital. Blood was collected in lithium heparin coated tubes and centrifuged at 3500 rpm for 20 minutes at 4 °C to collect serum that was stored at -80 °C.

Metabolic cage experiments with calcitriol administration were performed as above. After the initial 24 hours in the metabolic cages for baseline measurements, the mice were given an

intraperitoneal injection of 500 pg/g body weight $1,25(OH)_2D_3$ diluted in 5% PBS every 24 hours for three days.[119] Mice were then euthanized and samples collected as previously described.

URINE AND SERUM ANALYSIS

At the time of euthanasia, blood was also collected for blood gas analysis of electrolytes, ionized calcium (iCa), glucose, urea nitrogen (BUN), hematocrit (Hct) and hemoglobin (Hgb) using an iSTAT1 Analyzer (Abaxis, Union City, CA, USA) with a CHEM8+ cartridge. Serum creatinine was measured with the Diazyme creatinine kit (Diazyme Laboratories, CA, USA). Urine creatinine was measured with the Parameter creatinine kit (R&D systems, Minneapolis, USA). Urine electrolytes and serum electrolytes (NH_4^+, Ca^{2+}, Li^+, Mg^{2+}, K^+, Na^+, Cl^-, F^-, NO_3^-, PO_4^{2-}, SO_4^{2-}) were measured by ion chromatography (Dionex Aquion Ion Chromatography System, Thermo Fisher Scientific Inc., Mississauga, ON, Canada) with an autosampler. Urine samples were diluted 1:100 in ddH_2O and serum samples were diluted 1:500 in ddH_2O and carried in 4.5 mM Na_2CO_3/1.5 mM $NaHCO_3$ in ddH_2O for anion eluent, 20 mM methanesulfonic acid in ddH_2O for cation eluent. Calibration curves were created with serial dilutions of Dionex five anion and six cation-I standards (Dionex, Thermo Fisher Scientific Inc., Mississauga, ON, Canada). Results were analyzed using Chromeleon 7 Chromatography Data System software (Thermo Scientific). Urine cations were normalized to urine creatinine concentration. Serum PTH (Immutopics Mouse Intact PTH 1–84, San Clemente, CA, USA), FGF-23 (Cedarlane, Burlington, ON, Canada) and $1,25(OH)_2$-vitamin D were measured by ELISA (Immunodiagnostic Systems Limited, Boldon, UK).

REAL-TIME QUANTITATIVE PCR

Quantitative real-time PCR was performed as previously described.[120] Total RNA was isolated from frozen tissues stored at -80 °C using the TRIzol method (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and treated with DNAse (ThermoScientific, Vilnius, Lithuania). Alternatively, for collection of mRNA from cells in culture, the RNeasy kit (Qiagen) was used according to manufacturer's instructions. RNA quantity and purity were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was then reverse transcribed to make cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A pooled sample of RNA was used to create cDNA with serial dilutions for the standard curve for tissue samples. Quantitative RT-PCR was performed in triplicate on each sample using TaqMan Universal Master Mix II (Thermo Fisher Scientific, Carlsbad, CA, USA) with specific primers and probes on a QuantStudio 6 Pro Real Time PCR System (Thermo Fisher Scientific). Sequences for murine *Cyp27b1*, *Cyp24a1*, and *B-actin* have been published elsewhere.[121] The primers and probes utilized for RT-PCR are listed in Table 2.1. Specificity of primer sequences was assessed using NCBI Primer-BLAST. Samples from tissue were quantified using the standard curve method while samples from cells were quantified using the delta delta ct method. A Cq value greater than 35 was considered negligible.

Table 2.1. Primer and probe sequences for RT-PCR.

Gene	Forward Sequence 5' → 3'	Reverse Sequence 5' → 3'	Probe Sequence 5' → 3'
hCYP24A1	TTGTCTTCACTGGATCCCAAC	ACGCCGAGTGTACCATTTAC	56-FAM/TCACCCAGA/ZEN/ACTGTTGCCTTGTCA/31ABkFQ
hCYP27B1	CGGGTCTTGGGTCTAACTG	TCTCTTCCCTTTGGCTTTGG	56-FAM/CTCAGGCTG/ZEN/CACCTCAAAATGTGTT/31ABkFQ/
Prkaca	GTCATGGAGTATGTAGCTGGTG	CAGATACTCAAAGGTCAGGACG	/56-FAM/CGGATTGGA/ZEN/AGGTTCAGCGAGC/3IABkFQ/
PTHR1	ATGCTCTTCAACTCCTTCCAG	ACTCCCACTTCGTGCTTTAC	/56-FAM/CGGCTCCAA/ZEN/GACTTCCTAATCTCTGC/3IABkFQ/
Adcy6	GGTAGACATGATCGAAGCCATC	GACATCAAACTGCCATTTCCG	/56-FAM/CACACCTGT/ZEN/TACCTCACGCACCA/3IABkFQ/

IMMUNOBLOT

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X, 1% SDS, 1% NP-40, pH 7.4) with 1:100 PMSF (Thermo Scientific, Rockford, IL) and 1:100 protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein content was measured using Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA). 75 μg of protein was run on a 12% SDS-PAGE, electrotransferred to a PVDF membrane (Merck Millipore, Burlington, MA) and blocked overnight in TBST with 5% milk. The blots were then probed with primary antibody overnight at 4°C followed by secondary antibody for 1 hour at room temperature. Blots were then visualized using Immobilon Crescendo Western HRP substrate (Sigma-Aldrich, Canada) and a ChemiDoc Touch imaging system (Bio-Rad).

CELL CULTURE

Human Embryonic Kidney (HEK-293) cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were grown and maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FBS and 5% penicillin streptomycin glutamine at 37°C in 5% CO_2. OK cells (ATCC), LLCPK cells (ATCC), NRK cells (ATCC), HK-2 (ATCC) and HEK-293 cells were used in order to determine the best cell model for use in further experiments. For transfections, a calcium phosphate protocol was followed as previously described where 3.8 μg of total DNA was added.[122]

CLONING CYP27B1 PROMOTER

The human *CYP27B1* promoter was amplified from human genomic DNA (gDNA) by PCR. The PCR reaction was performed with a ProFlex PCR System Thermal cycler (Thermo Fisher Scientific) (94 °C for 1 min, 94 °C for 30 s, primer melting temperature (60-65 °C) for 30 s, 72 °C for 90 s, 39 cycles, 71 °C for 10 min and 4 °C forever). Cloning primers were made with unique restriction enzyme sites (Table 2.2), following the sequence previously published by Chanakul et al. in 2013.[123] The PCR products were run on an agarose gel and visualized to verify band size, then digested with restriction enzymes corresponding to the restriction sites KpnI and BglII (Thermo Fisher Scientific, Rockford, IL, USA). The digested DNA fragments were then ligated into the PGL3 Basic and Enhancer vectors (Promega, Madison, WI, USA) vectors that

were previously linearized using the same restriction enzymes. The ligated vector was then sent for sequencing to verify the correct sequence had been inserted before use in experiments.

Table 2.2. Primer sequences for CYP27B1 promoter cloning with KpnI (forward) and BglII (reverse) restriction enzyme sites

Promoter	Forward Sequence 5' → 3'	Reverse Sequence 5' → 3'
CYP27B1	CGG*GGTACC*CCGACTGACTAG	GGA*AGATCT*TCCCCAACTCGG

DUAL LUCIFERASE ASSAY

The assay was carried out per the manufacturer's directions (Promega Corp., Madison, WI, USA). Cells were transfected using a transient Ca^{2+} phosphate method 3-6 h following cell plating in a 6-well plate at a density of 3 X 10^5 cells per well. For use in the dual luciferase assay, HEK-293 cells were co-transfected with the PGL3 constructs, with the firefly luciferase gene downstream of either the *CYP27B1* or *CYP24A1* promoter, in addition to the pRL-TK vector containing renilla luciferase as an internal control. Subsequent experiments included the co-transfection of EV, CaSR, claudin-2, claudin-12 or both claudin-2 and claudin-12 DNA. Following a 48-h incubation period, the media was suctioned off and 3 mL of DMEM with 10% FBS and 5% PSG, with either 100 nM of PTH or control, were added to each well. Alternatively, in CaSR transfected experiments, after 48 hours the media was suctioned off and 3 mL of Ca^{2+} free DMEM with 10% FBS and 5% PSG supplemented with the appropriate amount of 2 M $CaCl_2$ to create either a low (0.1 mM) or high (5 mM) extracellular Ca^{2+} concentration was added. Following an additional 24 hours of incubation, the media was suctioned off and cells were washed twice with

PBS. Cells were then lysed with the manufacturer supplied lysis buffer and 5-15 μL of cell extract was used to determine luciferase activity in a GloMax 20/20 luminometer (Promega Corp.).

RATIOMETRIC CALCIUM IMAGING

Ratiometric calcium imaging was performed as previously described, where we measured Ca^{2+} uptake using the ratiometric calcium dye Fura-2.[124] HEK-293 cells were plated in 100-mm culture dishes on glass coverslips (no. 12-5451-102 25CIR-1, Fisher brand microscope coverglass) coated with poly-L lysine (Sigma Aldrich, St. Louis, MO, USA). Cells were seeded at a concentration of 1.5 X 10^6 cells/well and transfected by the Ca^{2+} phosphate transfection protocol as previously described. Cells were then incubated for 48 h prior to use for Ca^{2+} imaging. The glass coverslips were then transferred into a 10 mm culture dish and rinsed with PBS before being placed in 2 mL 0.5 mM $CaCl_2$-containing Ringer solution (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM HEPES, and 10 mM D-glucose, adjusted to pH 7.4 with Tris base) with 2 μL of Fura-2 AM (Molecular Probes, Eugene, OR) dissolved in DMSO with 0.1% Pluronic F-125 solution as well as 2 mM probenecid dissolved in NaOH (Sigma-Aldrich, Kenilworth, NJ) to inhibit efflux of the dye. Cells were incubated at 37°C for 1 h in the dark. Cells were then visualized using a Leica DMI6000B microscope with a Lambda DG4P-215 lamp supply and Quorum MAC6000 modular automation controller system. Throughout the experiment, cells were excited at 340- and 380-nm wavelengths and emitted light at 530 nm, which was captured using a Hamamatso Orca-Flash 4.0 digital camera (C11440). MetaFluor Fluorescence ratio imaging software was used to collect data as a ratio of 380 nm to 340 nm. All solutions and the microscope stage were kept at 37°C for the duration of the experiment.

Following the 1 h incubation time with Fura-2 and probenecid, cells were perfused with the first solution with a 0.5 mM $CaCl_2$ containing Ringer solution for 3 min. The solution was then immediately switched to the same Ringer solution, supplemented with 0.9 mM $CaCl_2$, for the following 3 min. The extracellular concentration of calcium was increased in all following solutions for 3 min each in the order 1.5 mM, 2.3 mM, 3.3 mM, 5.3 mM, 7.3 mM, 9.3 mM, and 11.3 mM to measure the response of cells to increasing extracellular calcium levels on intracellular calcium concentration. Calibration buffers were added during the final phase of the experiment to perform the calibration steps needed to calculate the intracellular calcium concentration. All calibrators were supplemented with the Ca^{2+} ionophore ionomycin at 2 μM (Sigma, St. Louis, MO). The first buffer was used to assess the minimum ratio (0 mM $CaCl_2$), the second was used to determine the maximum ratio (10 mM $CaCl_2$) and the third was used to quench the fluorescence of Fura-2 and measure the background fluorescence with the addition of 2 mM $MnCl_2$ to the solution. The equation derived by Grynkiewicz et al. was used to calculate the concentration of intracellular calcium over the course of the experiment.[125] The dissociation constant (K_d) of Fura-2 is 225 nM for intracellular conditions, R is the measured ratio, R_{min} is the ratio at 0 mM $CaCl_2$, R_{max} is the ratio at 10 mM $CaCl_2$, S_{f2} is the fluorescence intensity of 380-nm excitation at 0 mM $CaCl_2$, and S_{b2} is the fluorescence intensity of 380-nm excitation at 10 mM $CaCl_2$.

$$\left[Ca^{2+}\right]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

PRIMARY CELL CULTURE

Primary proximal tubule epithelial cells were isolated and cultured from mouse renal cortex. Mice were euthanized and placed in 70% ethanol prior to being transferred to the fume hood. Separate sets of sterile dissection tools were used to cut the epidermis, the peritoneum, and to remove the kidneys. Both kidneys were placed in a 100 mm culture dish with Hank's balanced salt solution (HBSS) (Life Technologies, Grand Island, NY, USA) supplemented with 5% PSG at 37°C. Using forceps, the capsule of the kidney was removed. The kidney was then dissected lengthwise into 3 sections, such that a clear distinction between the cortex and the medulla could be observed in the middle section with a magnifying lamp. Using a sterile scalpel, the outermost portion of the cortex of the kidney cross-section was dissected and then further cut into small pieces and placed in HBSS at 37°C. This was repeated with the second kidney and the tissue from both kidneys was combined for the remaining steps. The tissue suspended in HBSS was then spun at 200x g and HBSS was carefully suctioned off. The tissue fragments were washed in HBSS a further 2 times before being resuspended in 5 mL HBSS with 5 mg of collagenase (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C with shaking at 230 rpm for 45 min for the digestion. The tissue was then taken up by a syringe with a 18 G needle and passed through a 100 μm sieve to remove tubular fragments. The sample was then again taken up with a syringe and passed through a 40 μm sieve to remove glomeruli. Cells were then sedimented by centrifugation at 200x g for 5 min at room temperature and the supernatant then carefully suctioned off. The pellet was resuspended in 1 mL of serum-free media (DMEM-F12, Cytiva HyClone Laboratories, Logan, UT, USA) supplemented with 5% PSG, insulin (10 ug/mL), transferrin (5 ug/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL) and embryonic growth factor (EGF) (10 ng/mL). Cells were counted and plated in 6-well plates at a density of 3×10^5 cells/well for PTH treatment experiments and in 100 mm culture dishes at a density of 1.5×10^6 cells/plate

for collection of protein. Cells were incubated for 24 h, after which the media was changed. The media was changed thereafter every 48 h with hormone free media until the cells had grown for 7 days. At this point, the media was changed to DMEM-F12 supplemented with the addition of FBS and grown for a further 48 h. Then the cells were treated with either PTH (Sigma-Aldrich, St. Louis, MO, USA) at 100 nM or control and incubated for a further 24 h. The cells were then washed with 1X PBS and mRNA was collected for the synthesis of cDNA for real-time qPCR as previously described.

cAMP ELISA

For measurements of cellular cAMP concentration, HEK cells were plated in 6-well plates at a density of 3 X 10^5 cells/well and transfected with EV, claudin-2, claudin-12 or claudin-2 and -12 4-6 h later. After 48 h of incubation at 37°C, the media was carefully suctioned off and cells were rinsed with PBS before being treated with 100 nM PTH or control. After 15 min, media was again suctioned off and rinsed with PBS. Then 0.1 M HCl was added to each well for 10 min to lyse the cells which were then pelleted by centrifugation to remove cellular debris. The cAMP determination protocol was then followed according to the manufacturer's instructions to measure cAMP concentration via colorimetric detection (Enzo Life Sciences, Farmingdale).

CHAPTER 3: RESULTS

CLAUDIN-2 AND -12 DOUBLE KNOCKOUT MICE DISPLAY PERSISTENT HYPOCALCEMIA ON A LOW CALCIUM DIET BUT INCREASE PLASMA CALCIUM WHEN ADMINISTERED CALCITRIOL

It was surprising that claudin-2 and -12 DKO mice did not increase plasma calcitriol levels, given their calcium-wasting phenotype with increased urinary calcium excretion, hypocalcemia and decreased intestinal absorption.[46] This led us to enquire whether they could increase calcitriol in response to a low calcium diet challenge or if calcitriol itself could increase plasma calcium in these mice. We therefore challenged the DKO mice and their wild-type littermates with a low Ca^{2+} diet to see if the phenotype worsened or if the DKO mice would be able to compensate. Further, experiments conducted by Debbie O'Neill sought to investigate whether DKO mice could respond to calcitriol by intraperitoneal injection and rescue the hypocalcemia observed.

Consistent with our prior investigation of the claudin-2/12 DKO mice, when placed on a low Ca^{2+} diet (0.01% Ca^{2+}) they had significantly lower serum ionized Ca^{2+} levels (iCa) compared to WT animals on the same diet (Figure 3.1 A). The serum iCa levels in DKO mice on a low Ca^{2+} diet was not, however, significantly lower than DKO mice on a normal chow diet. Notably, when mice were given an intraperitoneal injection of calcitriol (500 pg/g body weight daily for 3 days), both groups responded to the hormone appropriately by increasing their serum iCa levels, however, DKO mice were not able to increase their iCa levels to the same extent as WT (Figure 3.1A).

Analysis of urine calcium content showed increased urinary calcium levels, normalized to creatinine, in the DKO mice compared to WT on both the regular (0.6% Ca^{2+}) and low Ca^{2+} diets (Figure 3.1 B). Mice on a low Ca^{2+} diet excreted significantly more Ca^{2+} in their urine when compared to mice fed a normal chow diet in both WT and DKO groups. Mice injected with

calcitriol showed a modest increase in urinary Ca^{2+} excretion relative to WT and DKO mice on a regular chow diet, however this difference was not significant. There was no difference between WT and DKO urinary Ca^{2+} excretion in the calcitriol injected mice.

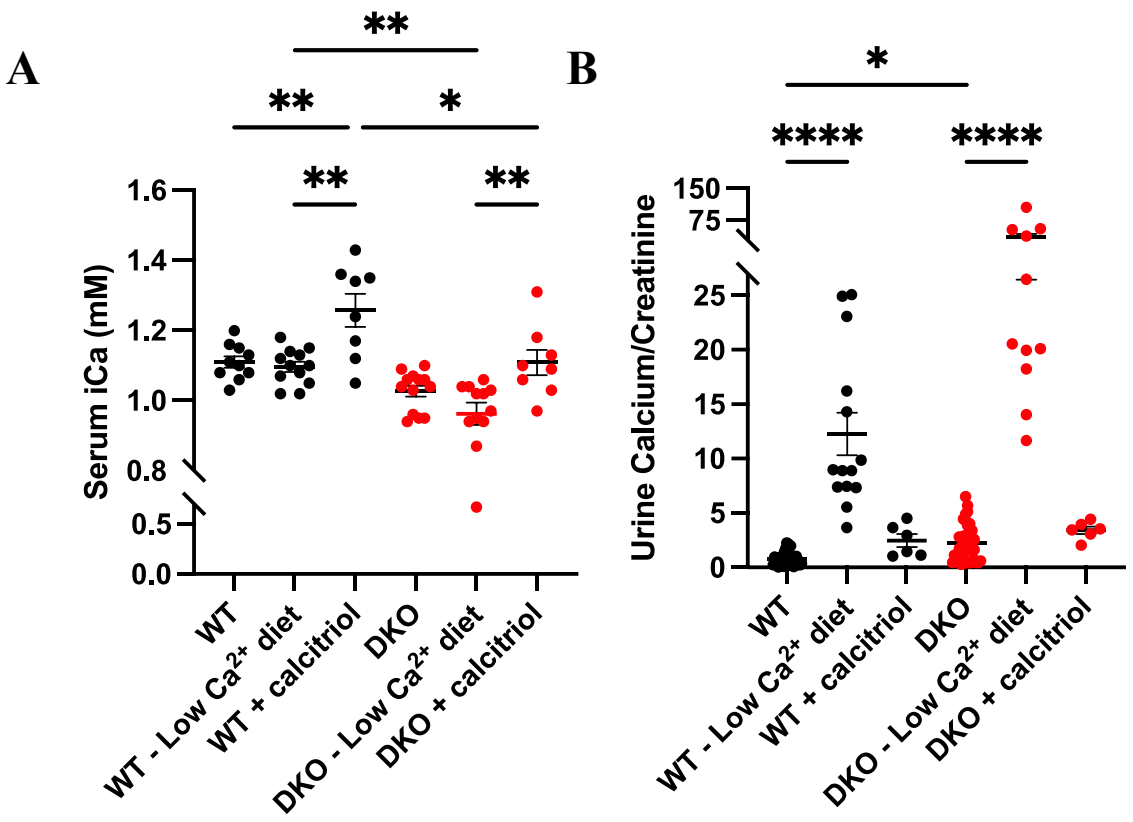

Figure 3.1. Serum and urinary calcium levels in DKO mice fed a normal chow diet, low Ca^{2+} diet, or after receiving IP calcitriol. A) Serum ionized Ca^{2+} (mean ± SEM, n=10 WT, n=12 WT Low Ca^{2+}, n=8 WT calcitriol, n=13 DKO, n=12 DKO Low Ca^{2+}, n=8 DKO calcitriol, one-way ANOVA); B) Urinary calcium excretion normalized to urine creatinine concentration (mean ± SEM, n=31 WT, n=14 WT Low Ca^{2+}, n=6 WT calcitriol, n=38 DKO, n=11 DKO Low Ca^{2+}, n=6 DKO calcitriol, Kruskal-Wallis test). (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

SERUM PTH AND CALCITRIOL OF WT AND DKO MICE ON A LOW-Ca^{2+} DIET AND ADMINISTERED CALCITRIOL

Given the alterations in calcium homeostasis observed in the DKO mice under all conditions, we sought to determine whether there were any differences in the serum levels of PTH and calcitriol in these mice. DKO mice on a normal chow diet and low Ca^{2+} diet had reduced serum iCa levels, which should in turn increase serum PTH and consequently serum calcitriol levels. As expected, DKO mice on both a regular diet and the low Ca^{2+} diet had significantly increased serum PTH compared to WT (Figure 3.2A). Additionally, DKO mice injected with calcitriol had a modest but significant increase in serum PTH relative to WT. It was expected that we would observe an increase in serum calcitriol levels in both the regular and low Ca^{2+} diet DKO animals. Although we observed a trend for increased calcitriol in the DKO of mice fed a regular diet, the difference was not significant. The DKO mice fed a low Ca^{2+} diet however, showed a significant increase in serum calcitriol relative to WT, and both groups had significantly greater levels of the hormone when compared to mice fed a regular diet (Figure 3.2 B). Serum calcitriol levels were not measured in calcitriol injected mice as they were receiving equal amounts of the hormone by IP injection.

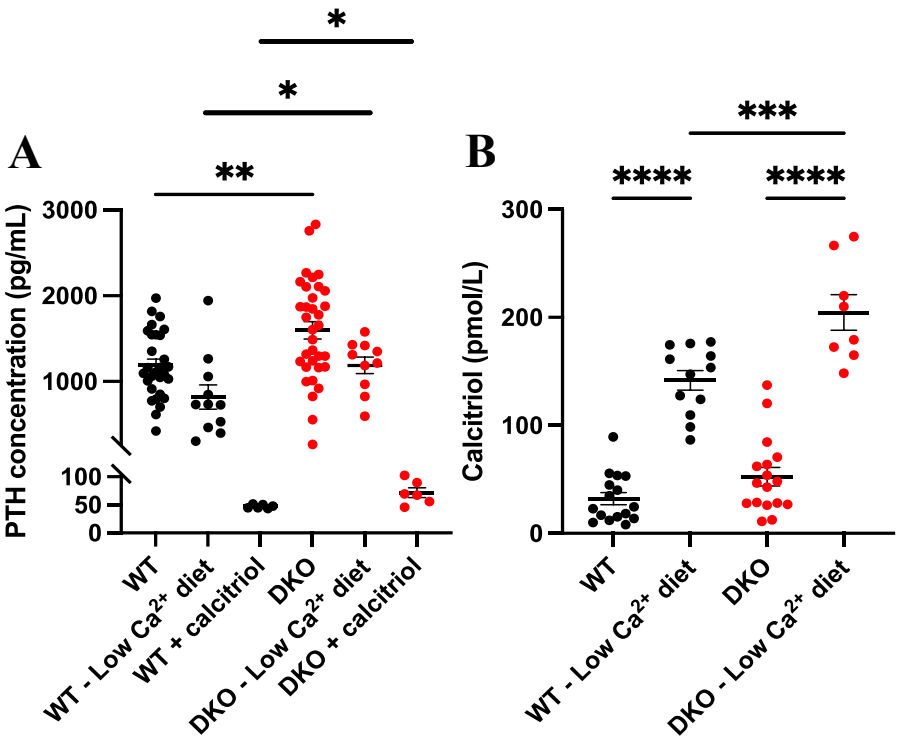

Figure 3.2. Serum PTH and calcitriol levels in WT and DKO mice fed a normal chow diet, low Ca^{2+} diet, or after receiving IP injections of calcitriol. A) Serum PTH (mean ± SEM, n=29 WT, n=11 WT Low Ca^{2+}, n=6 WT calcitriol, n=34 DKO, n=10 DKO Low Ca^{2+}, n=6 DKO calcitriol, Kruskal-Wallis); B) Serum calcitriol (mean ± SEM, n=16 WT, n=12 WT Low Ca^{2+}, n=17 DKO, n=8 DKO Low Ca^{2+}, Kruskal-Wallis test). (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

Renal *Cyp27b1* and *Cyp24a1* Expression of WT and DKO Mice is Not Different

To determine if the DKO mice were failing to increase their production of calcitriol in the proximal tubule of the kidney due to a failure in PTH signalling to stimulate its production, we looked at the expression of PTH-responsive genes which regulate the metabolism of vitamin D in the kidney. *Cyp27b1* and *Cyp24a1*, as previously described, are genes which encode the enzymes that activate and deactivate vitamin D, respectively. As previously shown, the expression of *Cyp27b1* in the DKO mice on a regular diet showed no significant increase in expression relative to WT (Figure 3.3 A).[46] Interestingly, although we were able to detect a significant increase in serum calcitriol levels in the DKO mice fed a low Ca^{2+} diet, we did not see a significant increase in *Cyp27b1* mRNA expression relative to WT (Figure 3.3 C). Further, there was no significant difference between groups in the calcitriol injected mice for *Cyp27b1* (Figure 3.3 E) and there was no difference in Cyp24a1 mRNA expression between groups under all conditions investigated (Figure 3.3 B, D, F).

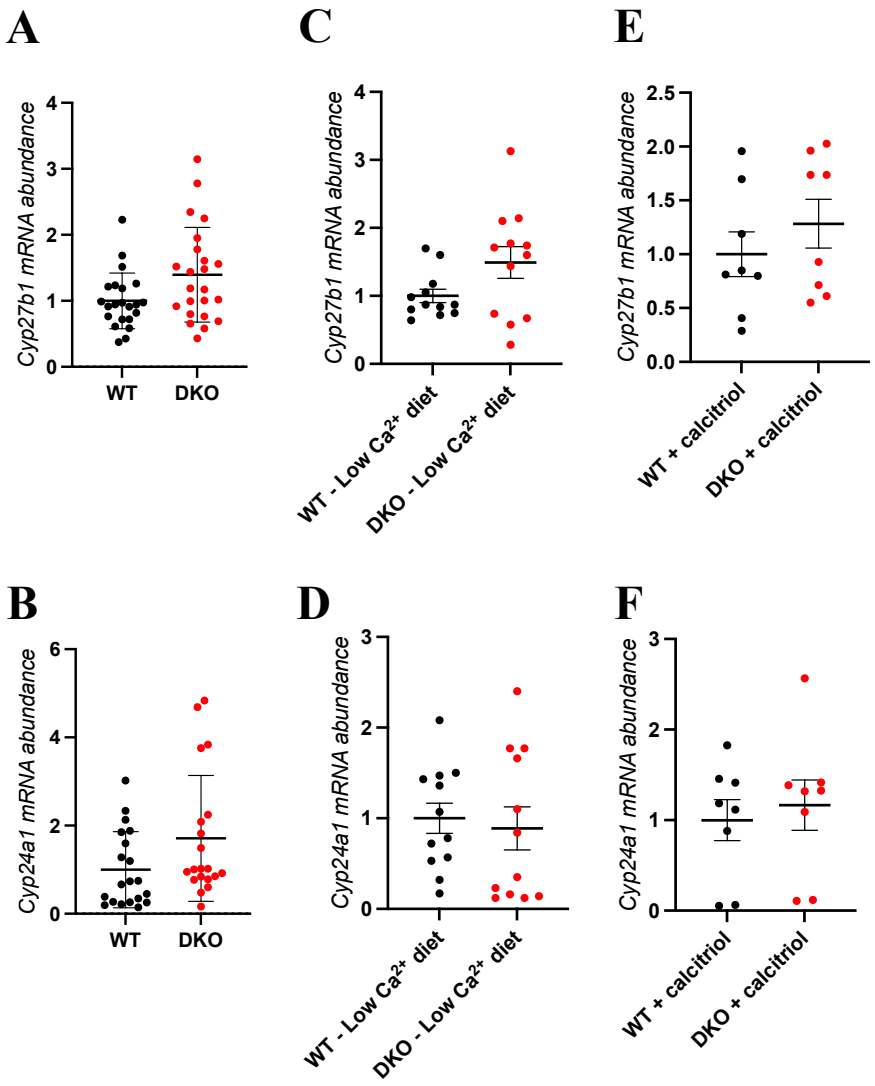

Figure 3.3. *Cyp27b1* and *Cyp24a1* expression of WT and DKO mice fed a normal chow diet, low Ca^{2+} diet, or that received IP calcitriol. mRNA abundance of renal *Cyp27b1* (A, C, E) and Cyp24a1 (B, D, F) in WT and DKO kidney tissue. Results are normalized to *β-Acti*n and expressed relative to WT. Data is presented as mean ± SEM and compared using a Mann-Whitney test.

HEK-293 Cells Express *CYP27B1* and *CYP24A1* and only *CYP24A1* is Appropriately Regulated by Calcitriol

In order to interrogate why *Cyp27b1* mRNA expression was not upregulated in DKO mice in response to hypocalcemia and increased serum PTH, we sought a cell culture model to interrogate what role, if any, claudins may play in the metabolism of vitamin D. To this end, we examined a variety of cell types to identify a suitable model system to investigate this phenomenon. We cultured NRK cells, HK-2 and HEK-293 cells until confluent and then collected mRNA and performed quantitative real time PCR for the key genes involved in vitamin D metabolism. The results are summarized in Table 3.1 and show the expression levels of *PTHR1, CaSR, Adcy6, Prka, CYP24A1* and *CYP27B1*. We *a priori* considered a Cq value greater than 35 as insignificant gene expression.

We found that *CYP27B1* and *CYP24A1* are endogenously expressed in HEK-293 and HK-2 cells as well as all parts of the primary PTH signalling pathway. HEK-293 and HK-2 cells do not, however, respond appropriately to PTH treatment as there was no increase in *CYP27B1* mRNA expression after 24 h (Figure 3.4A). Additionally, there was no change in *CYP24A1* mRNA expression with PTH treatment (Figure 3.4B). There was, however, a robust response to calcitriol treatment in both HEK-293 and HK-2 cells with significant increases in *CYP24A1* mRNA expression in response to calcitriol (Figure 3.4D). Because both cell lines express the necessary genes, we decided to pursue further experiments with the HEK-293 cells due to their greater transfection efficiency and ease of use.

Table 3.1. Summary of cell model gene expression.

Gene	NRK cell	HEK-293 cell	HK-2 cell
PTHR1	No	Yes	Yes
CaSR	No	No	No
Adcy6	Yes	Yes	Yes
Prka	No	Yes	Yes
CYP27B1	No	Yes	Yes
CYP24A1	No	Yes	Yes

NRK, normal rat kidney epithelial cell line; HEK-293, human embryonic kidney cell line; HK-2, human kidney cell line. Murine primers were used for NRK cells, which did not lead to amplification of the genes shown. Human primers were designed and used for HEK-293 and HK-2 cells.

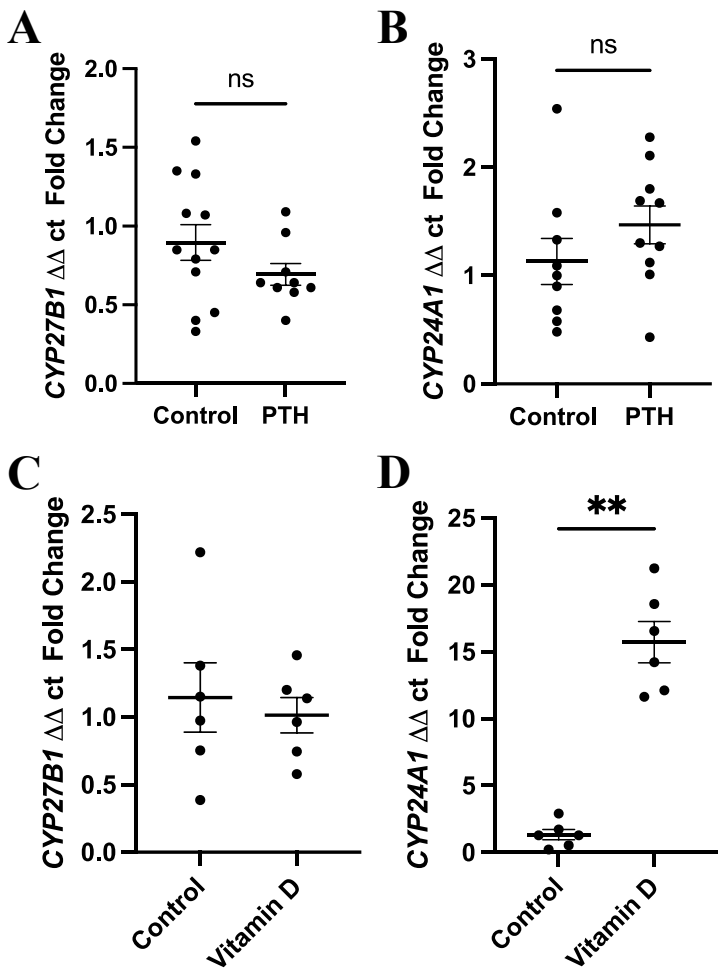

Figure 3.4. HEK-293 mRNA abundance of CYP27B1 and CYP24A1 in response to PTH and calcitriol treatment. HEK-293 cells were treated with either control, PTH or calcitriol at 100 nM and mRNA was collected for RT-PCR analysis of gene expression. *CYP27B1* (A and C) and *CYP24A1* (B and D) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Data presented as mean ± SEM and compared using Mann-Whitney test. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

CLAUDIN-2 ENHANCES CYP24A1 MRNA EXPRESSION WITH CALCITRIOL TREATMENT

We next decided to investigate the role of claudins on *CYP27B1* and *CYP24A1* mRNA expression after cells were treated with calcitriol. HEK-293 cells transfected with either empty vector (EV), claudin-2, claudin-12 or both claudin-2 and -12 were treated with either calcitriol or vehicle. Calcitriol treatment resulted in significantly reduced *CYP27B1* mRNA expression in EV transfected cells (Figure 3.5A). Surprisingly, cells transfected with either claudin-2 or claudin-12 and treated with vehicle had reduced *CYP27B1* mRNA expression compared to EV transfected control. Moreover, the claudin-2 or claudin-12 transfected cells did not display further suppression of *CYP27B1* mRNA expression when treated with calcitriol. Cells transfected with both claudin-2 and -12 did not show the same suppression of *CYP27B1* mRNA expression when treated with vehicle, i.e. the level of *CYP27B1* expression was not different to EV control. However, when claudin-2 and -12 transfected cells were treated with calcitriol, there was no suppression in *CYP27B1* mRNA expression as was seen in EV transfected cells. Together this data is consistent with claudin-2 and claudin-12 inhibiting calcitriol mediated feedback inhibition of *CYP27B1*.

We also examined *CYP24A1* mRNA expression to see how claudins might affect its regulation in response to calcitriol. Across all groups we observed an increase in *CYP24A1* mRNA expression with calcitriol treatment (Figure 3.5B). This effect was enhanced relative to EV cells treated with calcitriol in claudin-12 transfected cells and claudin-2 and -12 transfected cells to the same extent. Interestingly, claudin-2 transfected cells treated with calcitriol had the greatest increase in *CYP24A1* mRNA expression, significantly greater than all other calcitriol treated groups.

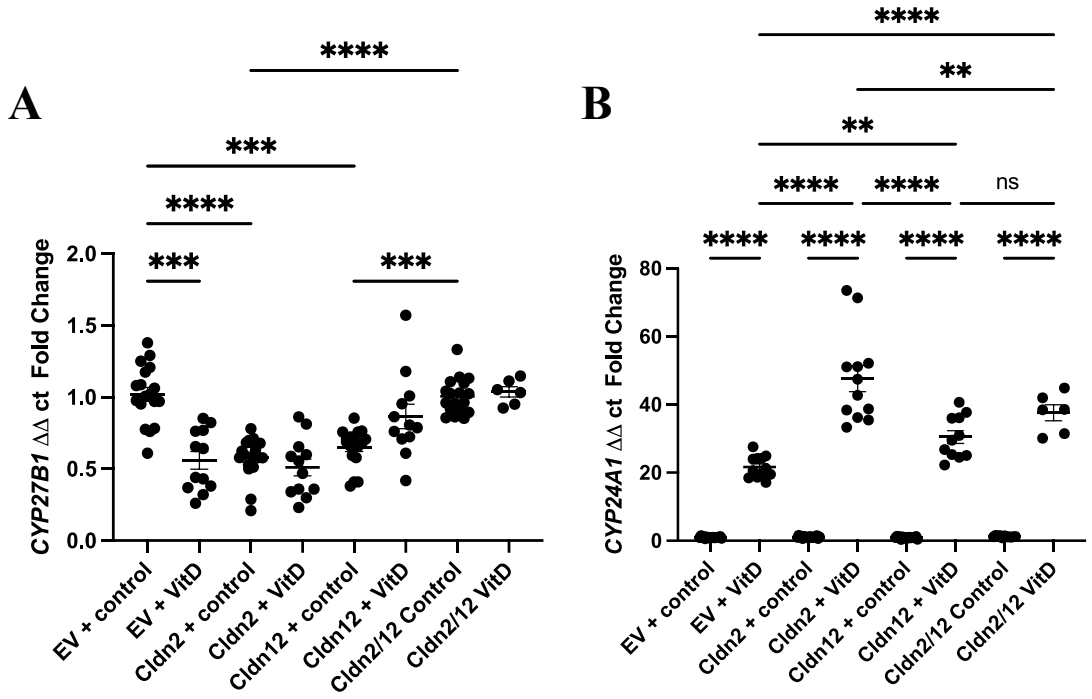

Figure 3.5. HEK-293 mRNA abundance in claudin transfected cells treated with calcitriol. HEK-293 cells were transfected with EV, claudin-2, claudin-12 or both claudin-2 and -12 and then treated with either vehicle or 100 nM calcitriol. mRNA was collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Data presented as mean ± SEM and compared using one-way ANOVA. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

CLAUDIN-2 ENHANCES CYP27B1 PROMOTER ACTIVITY IN HEK-293 CELLS TO A SIMILAR EXTENT AS PTH

We used the CYP27B1 promoter construct as described by Chanakul et al., which contains the firefly luciferase gene downstream of the promoter, to measure the luciferase activity of cells transfected with this plasmid and treated with either vehicle, PTH, or calcitriol.[123] The promoter was regulated appropriately. Treatment with 100 nM PTH, resulted in increased luciferase activity, and treatment with 100 nM calcitriol, lead to a significant reduction in luciferase activity (Figure 3.6A and B). Given the appropriate response to the traditional hormonal inputs, we proceeded to use this system to interrogate the roles of the claudins on CYP27B1 expression in HEK-293 cells.

We determined the effect of claudins on PTH and calcitriol treatment by measuring luciferase activity in cells co-transfected with the *CYP27B1* promoter construct and EV, claudin-2, claudin-12 or both claudin-2 and -12. We observed a significant increase in luciferase activity in EV and claudin-12 transfected cells treated with PTH relative to vehicle treated cells (Figure 3.7A). Interestingly, claudin-2 transfected cells treated with vehicle showed increased luciferase activity to the same extent as EV and claudin-12 transfected cells treated with PTH. Moreover when claudin-2 transfected cells were treated with PTH, there was no further increase in luciferase activity observed. Further, claudin-2 and -12 transfected cells treated with PTH did not show a significant increase in luciferase activity relative to control treated cells, pointing to a potential suppressive role of claudin-2 and -12 together on increasing *CYP27B1* promoter activity.

This experiment was repeated but with calcitriol treatment instead of PTH (Figure 3.7B). EV transfected cells treated with calcitriol had a greater than 2-fold decrease in luciferase activity,

however this difference was not significant by one-way ANOVA. Similarly, claudin-12 transfected cells treated with calcitriol had a greater than 3-fold decrease in luciferase activity, although the difference was not significant by one-way ANOVA. Cells transfected with claudin-2 and treated with vehicle again showed significantly increased luciferase activity relative to either cells transfected with EV, claudin-12 or claudin-2 and -12. Claudin-2 transfected cells did display a significant decrease in luciferase activity with calcitriol treatment. Interestingly, claudin-2 and -12 transfected cells did not display decreased luciferase activity with calcitriol treatment, but instead displayed a modest increase in luciferase activity that was not significantly different than vehicle treated cells transfected with both claudins.

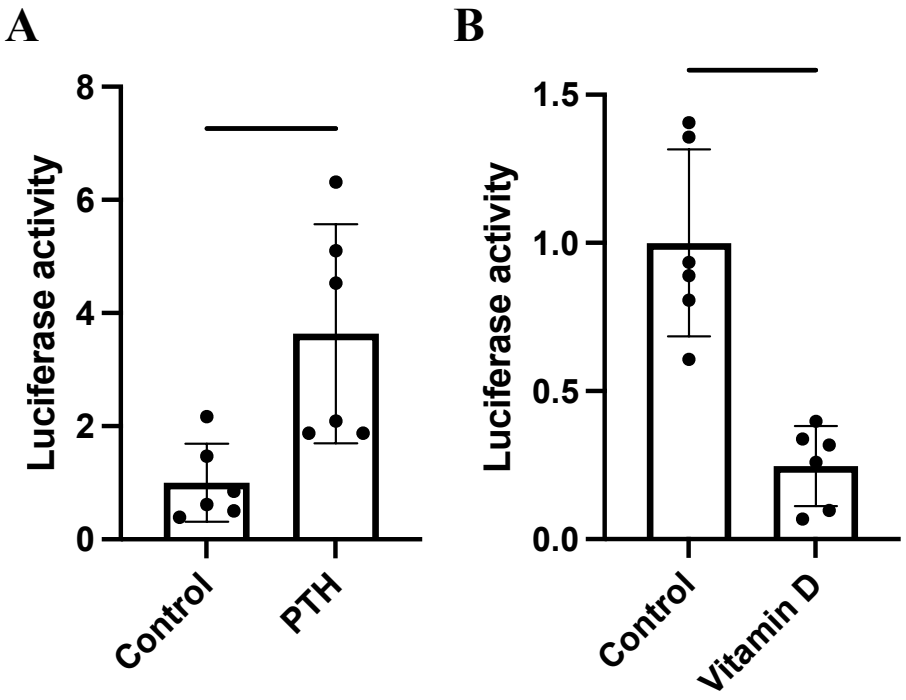

Figure 3.6. Luciferase activity of HEK-293 cells transfected with the CYP27B1 promoter construct and then treated with PTH or calcitriol. Cell lysate from HEK-293 cells was collected and luciferase activity measured. Cells were treated with either control, 100 nM PTH (A) or 100 nM calcitriol (B). Data presented as mean ± SEM and compared using Mann-Whitney test. (*P < 0.05; ***P < 0.001;).

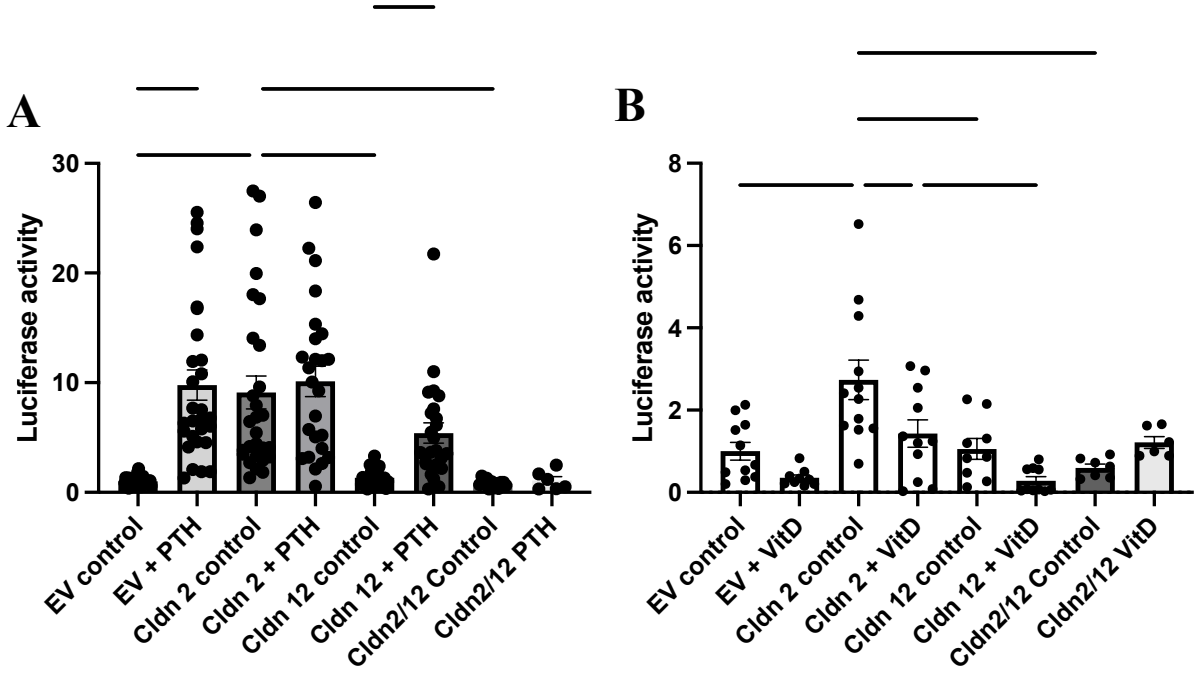

Figure 3.7. Luciferase activity of HEK-293 cells transfected with the CYP27B1 promoter construct and claudins and then treated with PTH or calcitriol. Cell lysate from HEK-293 cells was collected and luciferase activity measured Cells were treated with either control, 100 nM PTH (A) or 100 nM calcitriol (B). Data presented as mean ± SEM and compared using Kruskal-Wallis test. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

CALCIUM-SENSING RECEPTOR TRANSFECTED CELLS SHOW AN INCREASE IN *CYP24A1* MRNA EXPRESSION WITH HIGH EXTRACELLULAR CALCIUM LEVELS AND AN INCREASE IN *CYP27B1* PROMOTER ACTIVITY WITH LOW EXTRACELLULAR CALCIUM LEVELS

Calcitriol synthesis may be regulated by extracellular calcium independently of PTH or calcitriol.[67,110] To assess whether *CYP27B1* and *CYP24A1* expression were altered by extracellular calcium, HEK-293 cells were initially transfected with EV or the calcium-sensing receptor (CaSR) and incubated with medium containing either low (0.1 mM $CaCl_2$) or high (5 mM $CaCl_2$) extracellular calcium levels. There was no effect observed on *CYP27B1* mRNA expression with differing calcium concentrations, with and without transfection of the CaSR (Figure 3.8A). There was however a significant decrease in *CYP24A1* mRNA expression when the CaSR was transfected and the cells incubated with low extracellular calcium levels compared to EV transfected cells and CaSR transfected cells incubated with high extracellular calcium (Figure 3.8B). This effect was not observed in the absence of the CaSR. Consequently, the following experiments all included the co-transfection of the CaSR with either EV, claudin-2, or claudin-12.

Surprisingly, cells transfected with the CaSR and EV had a significant increase in *CYP27B1* mRNA expression when incubated with a high extracellular calcium containing medium relative to a low extracellular calcium containing medium (Figure 3.9A). There were no other differences observed in *CYP27B1* mRNA expression among the groups. *CYP24A1* mRNA expression was also measured and across all groups there was an increase in *CYP24A1* mRNA expression in the high extracellular calcium treated groups compared to the low calcium treated groups (Figure 3.9B). EV and claudin-2 transfected cells displayed increased *CYP24A1* mRNA

expression to the same extent. Claudin-12 transfected cells did not increase CYP24A1 mRNA to the same extent as claudin-2 transfected cells, displaying slightly reduced but still increased expression.

Due to the fact that *CYP27B1* mRNA expression was not regulated as expected, we again decided to employ the *CYP27B1* promoter construct to investigate the role of the claudins on CaSR signaling to altered *CYP27B1* activity. We first co-transfected cells with the promoter construct and EV or CaSR, then incubated them with either low or high extracellular calcium containing medium as previously. As expected, CaSR-transfected cells incubated with low extracellular calcium containing medium displayed increased luciferase activity compared to EV transfected cells, while those incubated with high extracellular calcium were not different than EV transfected cells (Figure 3.10A). As a result, the following experiments were performed as co-transfections with the CaSR and the CYP27B1 promoter construct, as well as either EV, claudin-2 or claudin-12. Across all groups, there was a significant decrease in luciferase activity with high extracellular calcium treatment (Figure 3.10B). There was no statistically significant differences between groups transfected with EV, claudin-2 or claudin-12 treated with low extracellular calcium, however there was a trend toward decreasing luciferase when claudin-2 or claudin-12 were transfected. Together this data suggests that claudins-2 and -12 do not alter calcitriol levels by altering CaSR signalling in the proximal tubule.

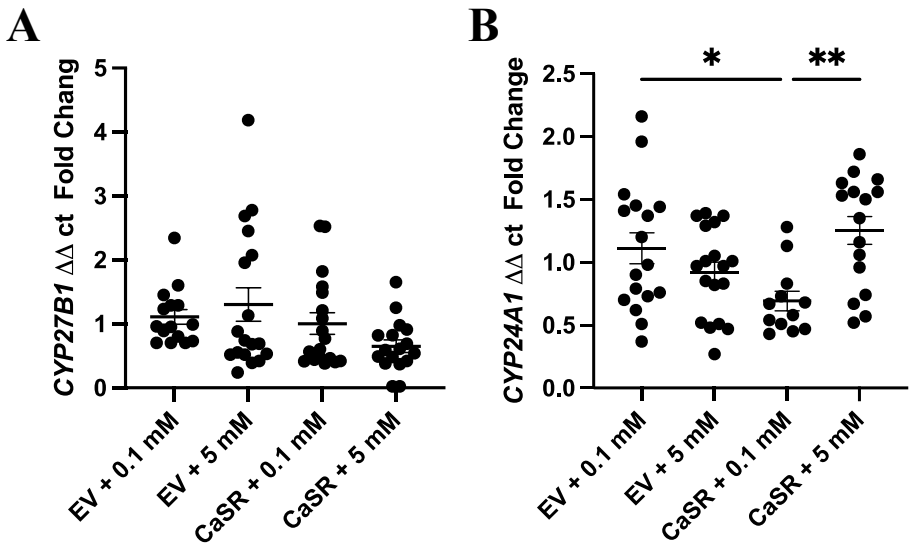

Figure 3.8. HEK-293 mRNA abundance of *CYP27B1* and *CYP24A1* in response to low and high extracellular calcium. HEK cells were transfected with EV or CaSR and then treated with either low (0.1 mM $CaCl_2$) or high extracellular (5 mM $CaCl_2$) Ca^{2+} and then mRNA was collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Data presented as mean ± SEM and compared using one-way ANOVA. (*P < 0.05; **P < 0.01).

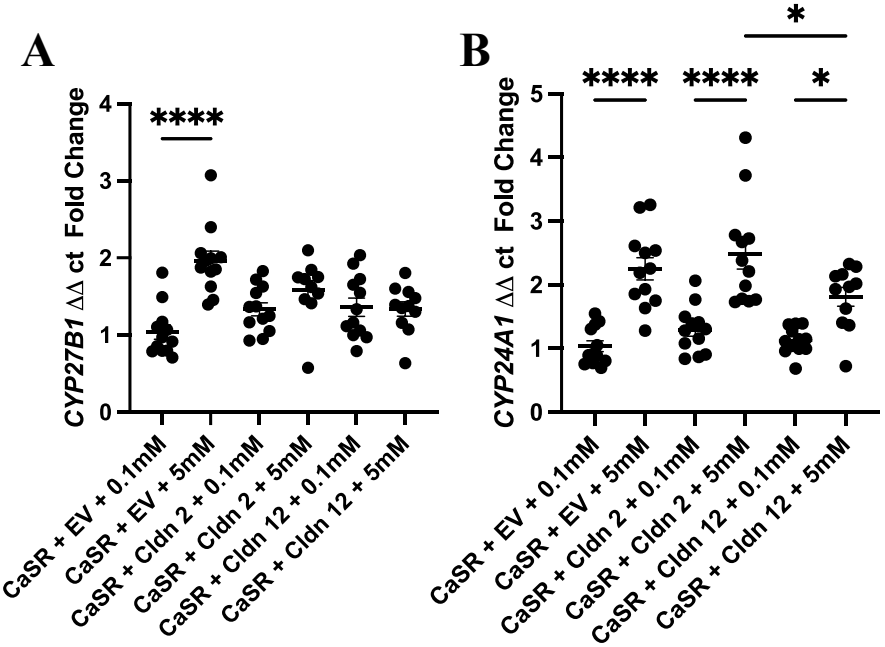

Figure 3.9. HEK-293 mRNA abundance of *CYP27B1* and *CYP24A1* in response to low and high extracellular calcium treatment with claudins. HEK cells were transfected with CaSR and EV, claudin-2 or claudin-12 and treated with either low (0.1 mM $CaCl_2$) or high extracellular (5 mM $CaCl_2$) Ca^{2+} and mRNA was collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Data presented as mean ± SEM and compared using one-way ANOVA. (*P < 0.05; ****P < 0.0001).

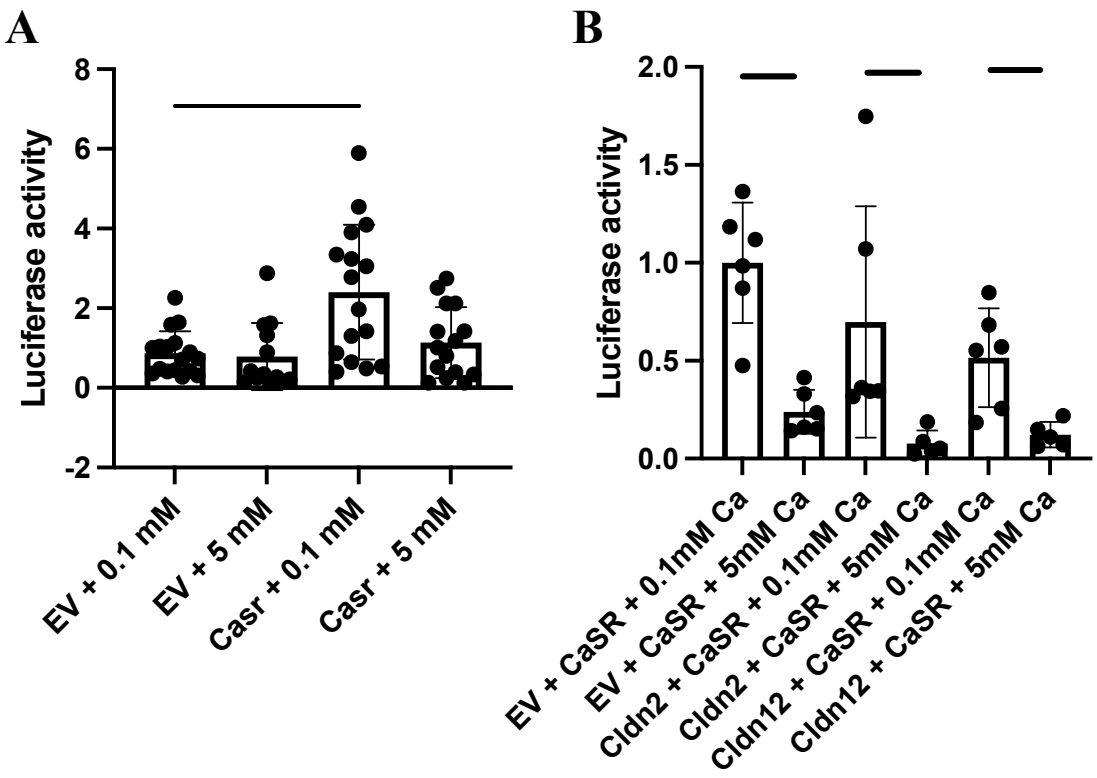

Figure 3.10. Luciferase activity in HEK-293 cells transfected with the CYP27B1 promoter construct in response to low and high extracellular calcium. HEK cells were transfected with the CaSR and the CYP27B1 promoter construct as well as EV, claudin-2 or claudin-12 and then incubated with either low (0.1 mM $CaCl_2$) or high extracellular (5 mM $CaCl_2$) Ca^{2+} and luciferase activity measured. EV and CaSR transfected treated cells (A) and CaSR with EV, claudin-2 or claudin-12 transfected treated cells (B) are shown and normalized to EV cells treated with low extracellular calcium. Data presented as mean ± SEM and compared using one-way ANOVA. (**P < 0.01)

Claudin-2 and Claudin-12 Suppress CaSR-mediated increase In Intracellular Calcium Levels

To further interrogate whether claudins alter CaSR signaling, we examined the increase in intracellular calcium levels induced by CaSR signaling with ratiometric calcium imaging. We used HEK-293 cells transfected with either EV, CaSR, claudin-2, claudin-12, or the CaSR in conjunction with claudin-2 or with claudin-12. The increase in intracellular calcium induced by increasing the extracellular calcium concentration was plotted over the course of the experiment (Figure 3.11A and B). As expected, a robust increase in intracellular calcium was observed in the CaSR transfected cells as extracellular calcium was increased, which was approximately 2-fold greater than EV transfected cells. Claudin-2 and claudin-12 transfected cells were not different than cells transfected with EV. Interestingly, cells co-transfected with the CaSR and either claudin-2 or claudin-12 demonstrated a reduced response to even the EV, and an approximately 4-fold decrease relative to CaSR transfected cells alone. Together this data supports that claudin-2 and claudin-12 attenuate the CaSR signaling which, under normal conditions, increases intracellular calcium levels.

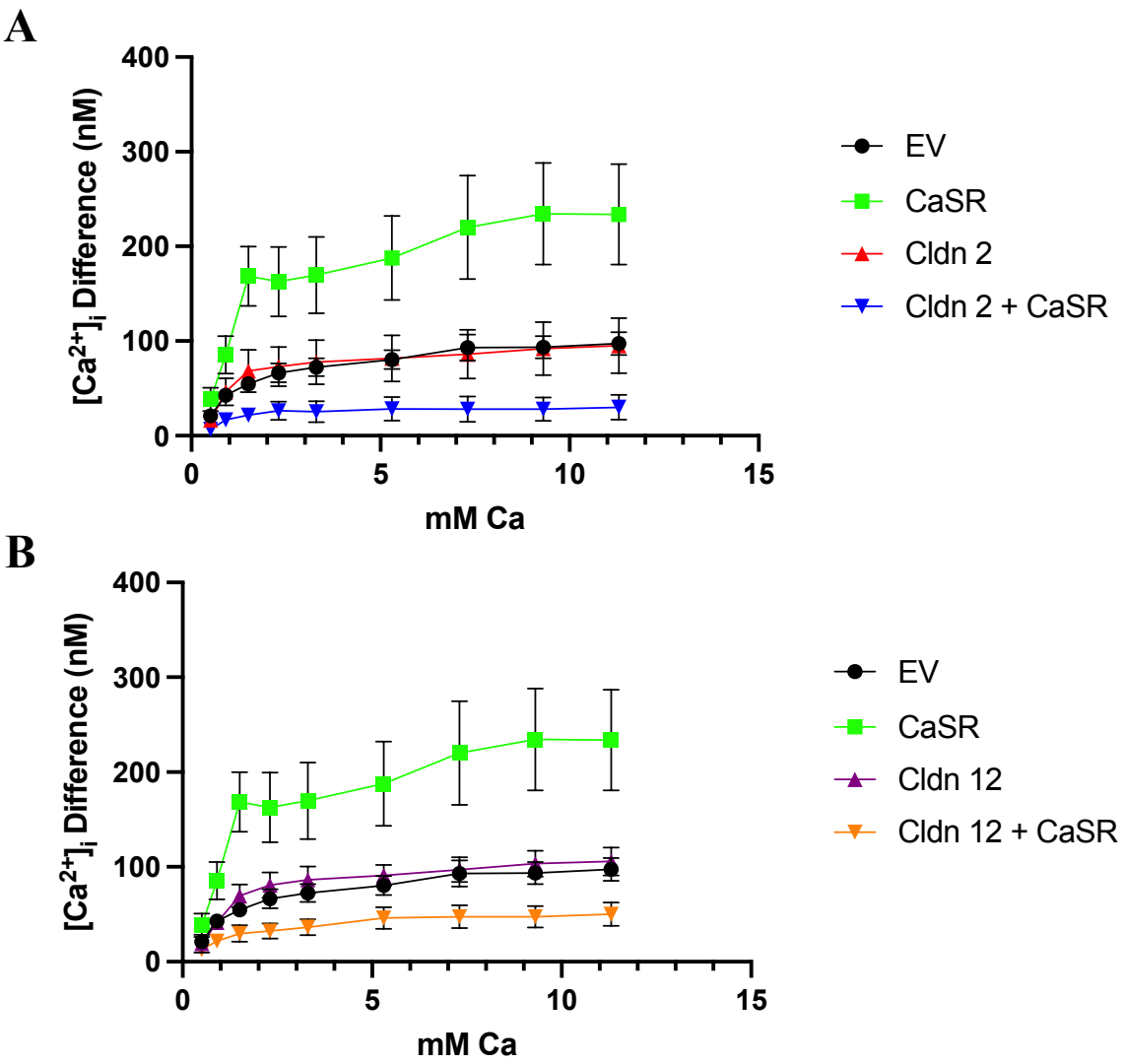

Figure 3.11. Relative increase (ie difference) in intracellular calcium concentration after exposure to increasing levels of extracellular calcium. HEK-293 cells were transfected with EV (n=6), CaSR (n=4), claudin-2 (n=5), claudin-12 (n=4), CaSR and claudin-2 (n=2) or CaSR with claudin-12 (n=3). Claudin-2 transfected cells (A) and claudin-12 transfected cells (B) are shown. Data presented as mean ± SEM.

PRIMARY CELL CULTURE: DKO PRIMARY CELLS HAVE ENHANCED RESPONSE TO PTH

The HEK-293 cells failed to appropriately respond to PTH treatment by increasing *CYP27B1* mRNA expression (Figure 3.4A). Moreover, our cell culture data supported the opposite conclusion to what we observed *in vivo*, *i.e.* the DKO mice had attenuated PTH signaling while in HEK-293 cells over-expressing claudin-2 and claudin-12 also displayed reduced PTH signaling. We therefore sought another model system to interrogate the effects of PTH on *CYP27B1* expression. The Alexander laboratory breeds inhouse claudin-2, claudin-12 and claudin-2 and -12 DKO mice. We therefore isolated primary proximal tubule cells from these mice and their WT counterparts and then treated these cells with vehicle or PTH to evaluate the response in *Cyp27b1* and *Cyp24a1* mRNA abundance.

Claudin-2 KO and WT primary cells showed significant increases in *Cyp27b1* mRNA expression with PTH treatment, relative to vehicle treated cells (Figure 3.12A). This difference was not, however significantly different between PTH treated groups. There was no difference in *Cyp24a1* mRNA expression with PTH treatment in either group, however there was significantly lower *Cyp24a1* mRNA expression in the KO cells compared to WT (Figure 3.12B). The genotype of these cells was confirmed by RT-PCR, where claudin-2 KO cells lacked mRNA expression of claudin-2 (Figure 3.12C).

Next, claudin-12 KO and WT primary cells were isolated and employed for the same purpose. PTH treatment resulted in a significant increase in *Cyp27b1* mRNA expression in WT cells and KO cells, however the increase in expression in KO cells was significantly lower than WT cells (figure 3.13A). There was no difference in *Cyp24a1* mRNA expression with PTH

treatment and no differences between groups (Figure 3.13B). The genotype of these cells was confirmed by RT-PCR, where claudin-12 KO cells have a lack of claudin-12 mRNA expression as well as ZENUB1 mRNA expression (the transgene knocked in), as expected (Figure 3.13C and D).

Finally, claudin-2 and -12 DKO and WT primary proximal tubule cells were isolated and treated with PTH or vehicle and the analysis of mRNA expression performed. Both WT and DKO cells displayed increased *Cyp27b1* mRNA expression with 100 nM PTH treatment. However, interestingly DKO cells treated with PTH had significantly greater increased *Cyp27b1* mRNA expression than WT cells treated with PTH (Figure 3.14A). There was no significant difference between groups for *Cyp24a1* mRNA expression (Figure 3.14B). The genotype of these cells was confirmed by both western blot (Figure 3.14C) and RT-PCR, where DKO cells lacked mRNA expression of claudin-2 and claudin-12 and had significant expression of the transgene inserted, ZENUB1 (Figure 3.14D, E, F).

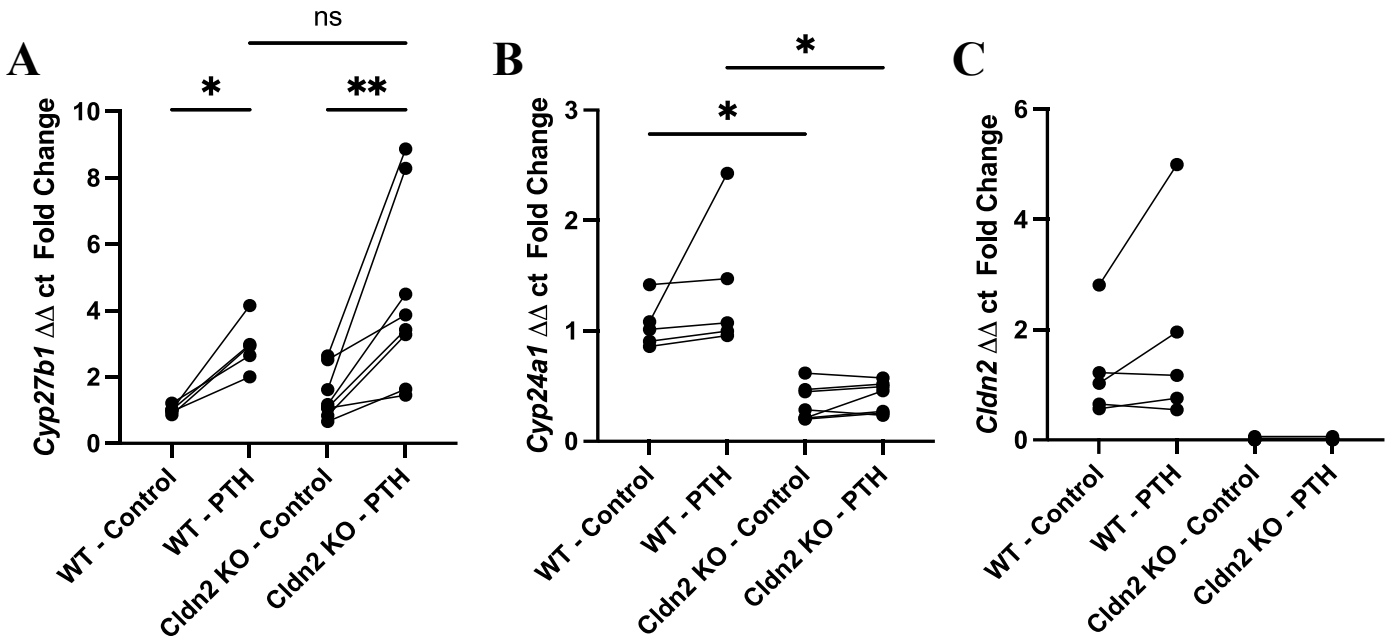

Figure 3.12. Claudin-2 KO and WT primary proximal tubule cells treated with PTH or control. Cells were treated with either control or 100 nM PTH and mRNA then collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. The absence of claudin-2 mRNA expression confirms the genotype of claudin-2 KO cells (C). Data presented as mean ± SEM and compared using a paired t-test and Kruskal-Wallis test. (*P < 0.05; **P < 0.01).

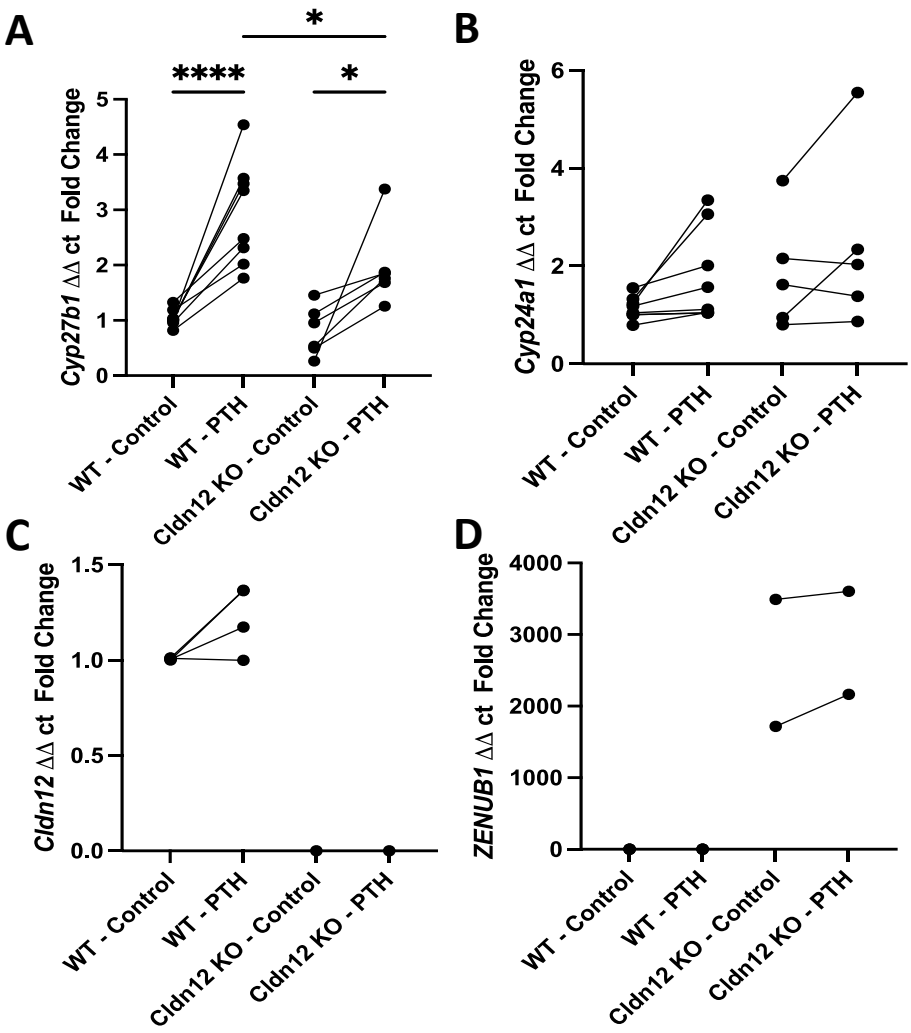

Figure 3.13. Claudin-12 KO and WT primary proximal tubule cells treated with PTH or control. Cells were treated with either control or 100 nM PTH and mRNA then collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Claudin-12 (C) and ZENUB1 (D) mRNA expression confirms the genotype of claudin-12 KO cells. Data presented as mean ± SEM and compared using a paired t-test and Kruskal-Wallis test. (**P < 0.01)

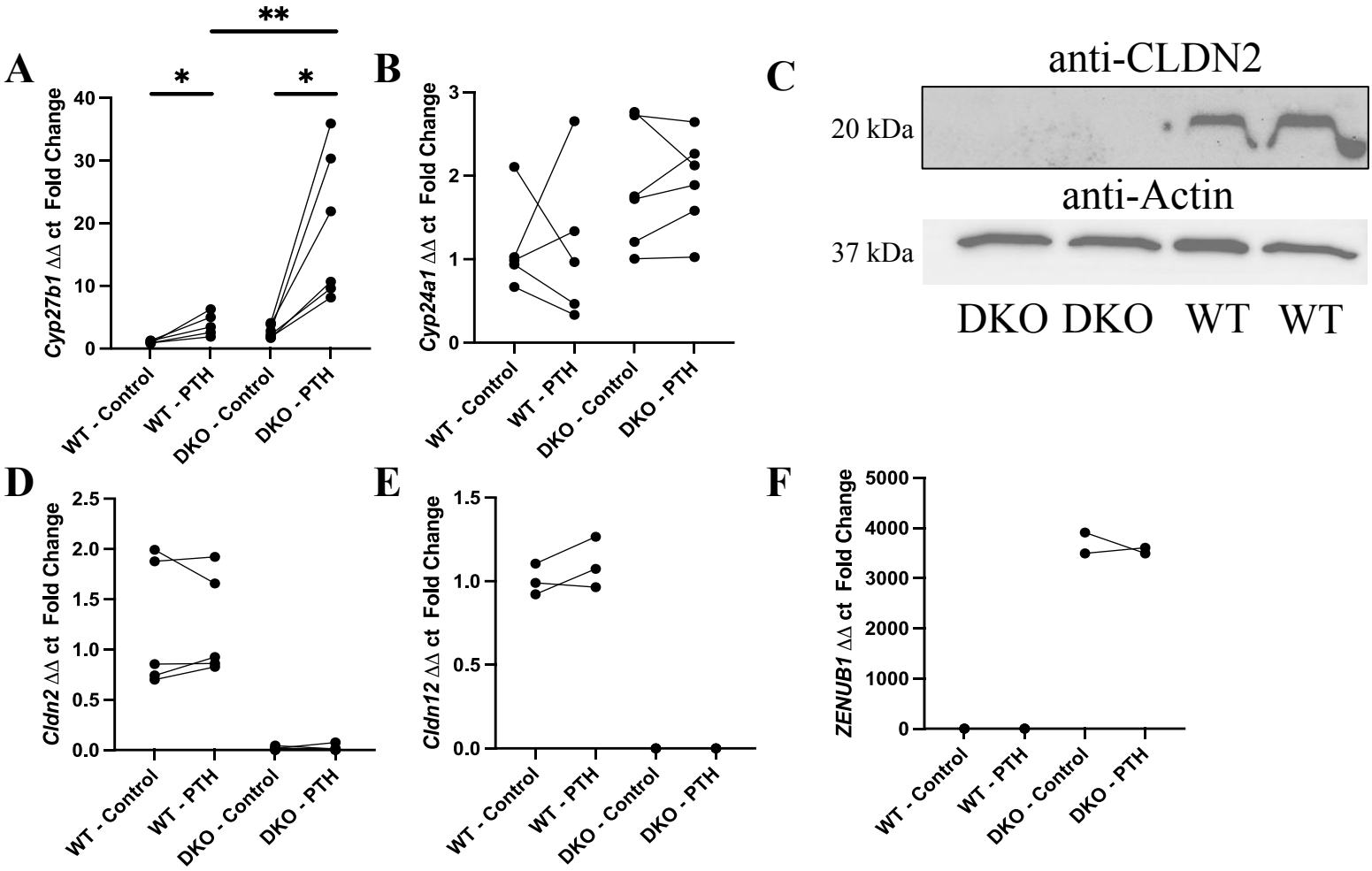

Figure 3.14. Claudin-2 and -12 DKO and WT primary proximal tubule cells treated with PTH or control. Cells were treated with either control or 100 nM PTH and mRNA was then collected for RT-PCR analysis of gene expression. CYP27B1 (A) and CYP24A1 (B) mRNA abundance measured by the delta delta ct method is shown normalized to *B-actin*. Western blot confirms the absence of claudin-2 in DKO cells (C). Claudin-2 (D), claudin-12 (E) and ZENUB1 (F) mRNA expression confirms the genotype of the DKO cells. Data presented as mean ± SEM and compared using a paired t-test and Kruskal-Wallis test. (*P < 0.05; **P < 0.01).

HEK-293 Cells Show no Change in cAMP Concentration Following PTH Treatment

Due to the alterations in PTH signalling observed in cells transfected with claudins and treated with PTH in our dual luciferase experiments and the findings with our primary cells, we sought to investigate whether cAMP, the compound produced downstream of PTH receptor activation, is altered in HEK-293 cells treated with PTH or vehicle. We used HEK-293 cells transfected with EV, claudin-2, claudin-12 or claudin-2 and -12 treated with PTH or control and then lysed the cells and measured cAMP concentration after 15 minutes of treatment. There was no difference in cAMP concentration in EV, claudin-2 or claudin-12 transfected cells treated with PTH relative to vehicle (Figure 3.15). Interestingly, there was a suppression in cAMP concentration in claudin-2 and -12 transfected cells treated with PTH, relative to vehicle. These claudin-2 and -12 transfected cells had significantly less cAMP than both claudin-2 and claudin-12 transfected cells as well. Overall this data suggests that the lack of an increase in CYP27B1 in response to PTH in HEK-293 cells is likely due to the absence on CAMP signaling. Moreover, it appears that the expression of claudin-2 and claudin-12, but not of either claudin individually, supresses CAMP.

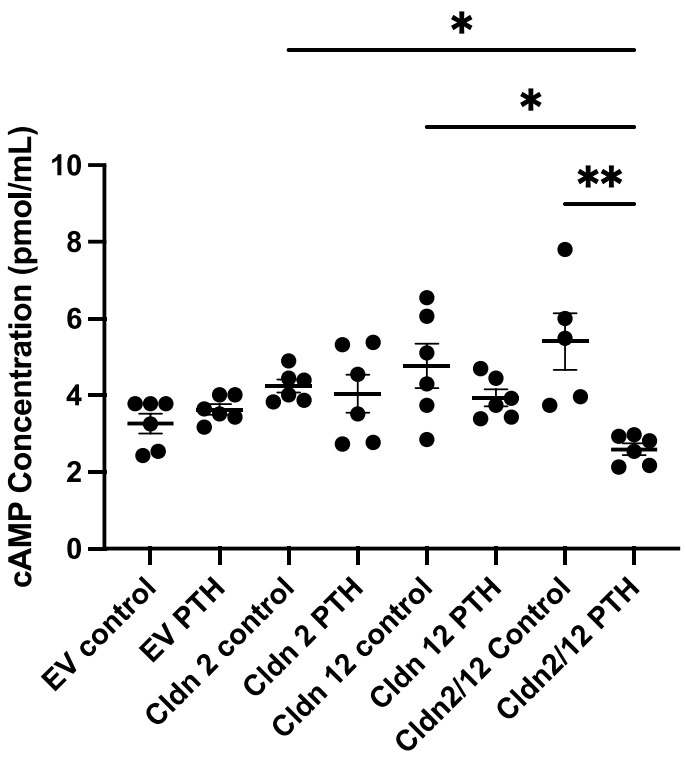

Figure 3.15. cAMP concentration in transfected HEK cells treated with PTH. Cells were transfected with EV, claudin-2, claudin-12 or claudin-2 and -12 and treated with PTH or control for 15 minutes. cAMP concentration was measured following this. Data presented as mean ± SEM and compared using a Kruskal-Wallis test. (*P < 0.05; **P < 0.01).

Blood and Urine Phosphate Levels of WT and DKO Mice on a Low-Ca^{2+} diet and after Calcitriol Treatment

We have conflicting findings between our cell culture experiments and our previous *in vivo* results. *In vitro* we observe that claudin-2 and claudin-12 overexpression results in a suppression of *CYP27B1* expression stimulation while *in vivo* we observe that the absence of claudin-2 and claudin-12 appear to suppress *CYP27B1* expression. During cell culture experiments we were able to limit the variable that was altered to one, i.e. just increased PTH. However, *in vivo* the DKO mice likely had several parameters that were different, such as low plasma calcium and increased PTH levels. We therefore hypothesized that another factor other than PTH might be supressing *Cyp27b1* mRNA expression in the DKO mice. Importantly FGF23 and phosphate itself is known to affect *Cyp27b1* expression.[90,93,94,99,100] We therefore decided to investigate phosphate homeostasis in the DKO mice.

Serum phosphate concentration was measured by IC across all groups. Serum phosphate was significantly increased in the DKO mice compared to WT fed both the regular and the low Ca^{2+} diet (Figure 3.16A). There was no significant difference in plasma phosphate between WT and DKO mice that had been injected with calcitriol.

Urinary phosphate concentration was also measured by IC and normalized to creatinine concentration. Urinary phosphate levels were not different between WT and DKO fed both the regular diet and when injected with calcitriol (Figure 3.16B). WT mice on a low Ca^{2+} diet had significantly increased urinary phosphate excretion compared to WT mice on a regular diet and WT mice injected with calcitriol. Similarly, DKO mice on a low Ca^{2+} diet had significantly increased urinary phosphate excretion relative to calcitriol injected mice. There was no significant

difference in urinary phosphate excretion between WT and DKO mice on a low Ca^{2+} diet however, the DKO mice excreted approximately 2-fold more phosphate in the urine compared to WT animals.

Following the observation that DKO mice fed either a regular or low Ca^{2+} diet have increased serum phosphate levels, we sought to determine if serum Fibroblast Growth Factor-23 (FGF23) concentration was different between groups. There was no difference observed when comparing WT and DKO mice fed a regular chow diet. DKO mice fed a low Ca2+ diet, however, had a significant increase in serum FGF23 levels compared to WT (Figure 3.17). Mice injected with calcitriol had FGF23 levels above the detectable limits for this assay, consistent with FGF23 production being stimulated by calcitriol in the bone. Together this data suggests that increased plasma phosphate, but not FGF23, is attenuating *CYP27B1* expression and thus calcitriol levels in the claudin-2 and claudin-12 DKO mice.

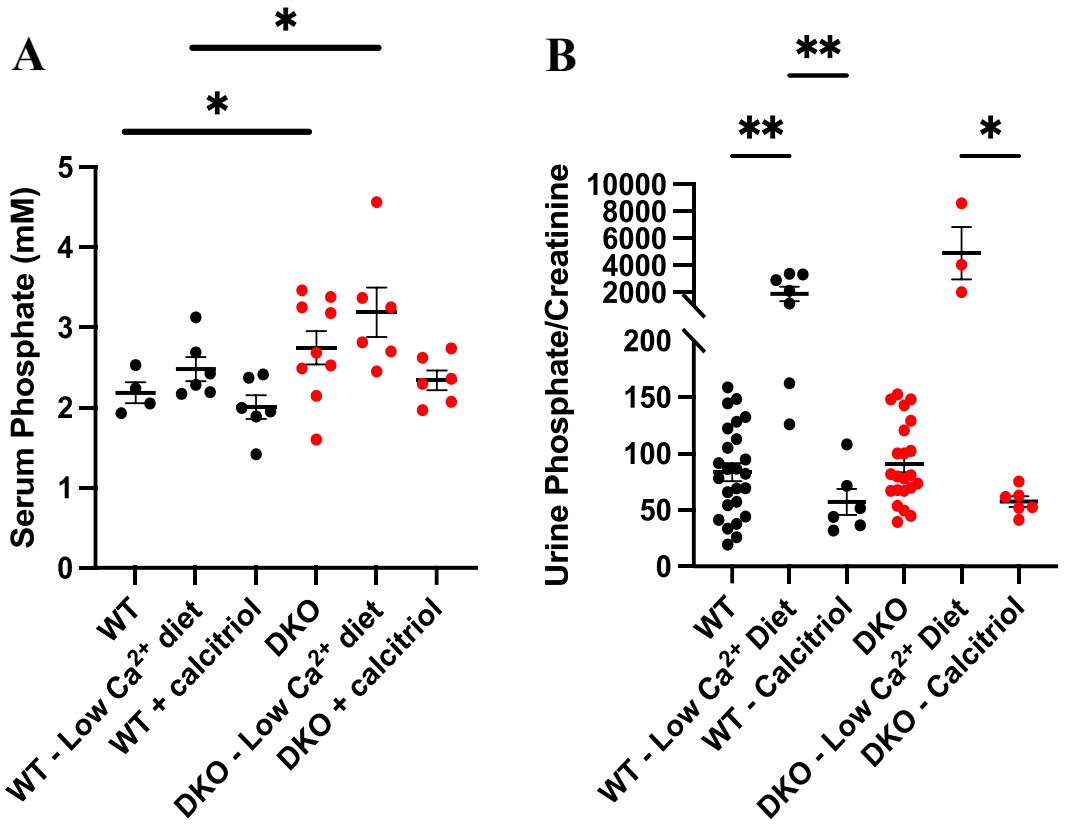

Figure 3.16. Serum phosphate and urinary phosphate excretion in WT and DKO mice on a regular chow diet, low Ca^{2+} diet or after calcitriol treatment. A) Serum phosphate (mean ± SEM, n=4 WT, n=6 WT Low Ca^{2+}, n=6 WT calcitriol, n=9 DKO, n=6 DKO Low Ca^{2+}, n=6 DKO calcitriol, one-way ANOVA); B) Urinary phosphate excretion normalized to urine creatinine concentration (mean ± SEM, n=25 WT, n=7 WT Low Ca^{2+}, n=6 WT calcitriol, n=22 DKO, n=3 DKO Low Ca^{2+}, n=6 DKO calcitriol, Kruskal-Wallis test). (*P < 0.05; **P < 0.01).

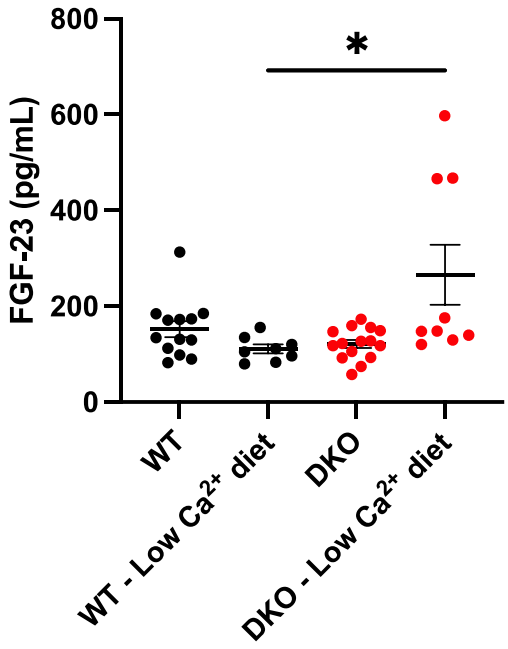

Figure 3.17. Serum FGF23 levels in WT and DKO mice fed a normal chow diet or low Ca^{2+} diet. Serum FGF23 (mean ± SEM, n=13 WT, n=8 WT Low Ca^{2+}, n=15 DKO, n=9 DKO Low Ca^{2+}, Kruskal-Wallis). (*P < 0.05).

CHAPTER 4: DISCUSSION

This thesis investigated the effects of claudin-2 and claudin-12 on renal vitamin D metabolism. Given the abnormal regulation of calcitriol in claudin-2 and claudin-12 DKO mice, which appears secondary to their inability to sufficiently increase the transcription of *CYP27B1* and consequently significantly increase calcitriol in their blood in response to the appropriate stimuli, it is likely that claudin-2 and/or claudin-12 play a role in the regulation of this process. It is only when challenged with a low Ca^{2+} diet that DKO mice are able to increase blood calcitriol levels, yet they are still hypocalcemic to a greater degree than WT and DKO mice on a regular chow diet, thus calcium homeostatic mechanisms are perturbed in these mice.

We found that HEK-293 cells express the *CYP27B1* and *CYP24A1* genes endogenously and that *CYP24A1* could be regulated appropriately by calcitriol. *CYP27B1* transcriptional regulation by PTH, however, was inconsistent with the known regulation of this gene by PTH not reliably increasing mRNA expression. When performing a dual luciferase assay, a claudin-2 transfection stimulated *CYP27B1* promoter activity to the same extent as PTH treatment. When using a primary cell model to investigate the effect of PTH treatment, we found that claudin-2 and claudin-12 KO cells increase their *CYP27B1* mRNA expression to the same extent as WT cells when treated with PTH. Interestingly, claudin-2 and -12 DKO cells showed enhanced *CYP27B1* mRNA expression relative to WT. This is in contrast with our published findings that DKO mice fail to increase *CYP27B1* mRNA expression and as a result do not have increased circulating calcitriol levels in response to increased PTH and hypocalcemia. To explain these findings we measured and observed increased phosphate levels in the claudin-2 and claudin-12 DKO mice, which likely inhibit *Cyp27b1* expression and thus calcitriol synthesis *in vivo*. Nonetheless our cell culture studies clearly implicate these claudins in the regulation of *Cyp27b1* transcription.

Further, CaSR transfection into HEK-293 cells exposed to high extracellular calcium levels displayed an increase in *CYP24A1* mRNA expression which was suppressed in claudin-12 transfected cells relative to EV and claudin-2 transfected cells. In accordance with this, *CYP27B1* promoter activity was suppressed in response to high extracellular calcium levels. Through ratiometric calcium imaging experiments on transfected HEK-293 cells, we found that claudin-2 and claudin-12 suppress the CaSR-mediated increase in intracellular calcium levels, which are consistently below EV and claudin-2 or claudin-12 transfected cells alone. Taken together, these results suggest that claudin-2 and claudin-12 interact in some way with the CaSR to suppress its intracellular signalling and are consistent with the CaSR having a role in the transcriptional regulation of *CYP27B1* and *CYP24A1* which is dependent on extracellular calcium levels but not on claudin expression.

Calcium Sensing Receptor

Given the dysregulated calcium homeostasis in DKO mice, we sought to investigate the effects of differing extracellular calcium concentration and claudins on *CYP27B1* and *CYP24A1* mRNA expression in HEK-293 cells because of the known effect of calcium on *CYP27B1* expression independent of PTH. [67,110] Our data is consistent with previous studies by Huang et al. that have looked at luciferase activity with a CYP27B1 promoter construct at different extracellular calcium levels, where luciferase activity is suppressed at high calcium concentrations.[110] It has been proposed that through the CaSR in the proximal tubule, Ca^{2+} can prevent the inhibition of the phosphaturic action of PTH.[126,127] The mechanism behind this is not known, though it could be a means through which high calcium concentrations target *CYP24A1* to increase calcitriol

inactivation or inhibit CYP27B1 transcription. Although controversial, it has been proposed that the CaSR is expressed on the apical and basolateral membranes of the proximal tubule.[126] With an increased luminal concentration of calcium in the proximal tubule due to a reduction in reabsorption or increased filtration, it is possible that the CaSR could be activated to stimulate *CYP24A1* and inhibit *CYP27B1* transcription. Further, our data does not support that claudin-2 and claudin-12 alter this pathway directly, as overexpression of either protein does not result in a significant change in expression pattern. However, our calcium imaging studies infer a role for these claudins in modulating CaSR signaling that does not alter *CYP27B1* or *CYP24A1* expression (Figure 3.11). NHE3, the major proximal tubule sodium proton exchanger responsible for transcellular sodium and thus consequently calcium reabsorption is regulated by altered intracellular calcium levels.[128] Perhaps CaSR signalling through altered cytosolic calcium levels is attenuated by proximal tubule claudins so as to prevent the stimulation of NHE3 activity and thus further proximal tubule calcium reabsorption.

CLAUDIN-2 MODULATES CYP27B1 AND CYP24A1 TRANSCRIPTION

The proximal tubule is the site of 60-70% of calcium reabsorption as well as the site of calcitriol synthesis and PTH action.[45–47] PTH binds to its receptor expressed in either the basolateral or apical side of the proximal tubule epithelial cells and increases the transcription of *CYP27B1*, primarily through a PKA and cAMP dependent pathway.[75] Our initial experiments in HEK-293 cells investigating the mRNA expression of *CYP27B1* did not produce the expected results; PTH did not increase its expression when cells were treated. However, when using a PGL3 construct system with the *CYP27B1* promoter, we see a significant increase in luciferase activity with PTH treatment in all groups except the claudin-2 and -12 transfected cells. Interestingly, claudin-2 overexpression alone resulted in significantly increased luciferase activity. To see if

these effects were being modulated through cAMP production as a result of PTHR1 activation, we measured cAMP concentrations in the cell following 15 min of PTH treatment. Similar to our mRNA expression experiments, we did not observe the appropriate increase that should be seen with PTH treatment. In fact, we observed a significant decrease in cAMP concentration in claudin-2 and -12 transfected cells treated with PTH, which did correlate with the decrease in luciferase activity of claudin-2 and -12 transfected cells. Together these studies suggest that HEK-293 cells lack sufficient PTHR1 and/or other machinery to illicit increased cAMP and thus *CYP27B1* expression in response to extracellular PTH.

HEK-293 cells were unable to respond appropriately and regulate *CYP27B1* mRNA expression and so a mouse primary proximal tubule cell culture was pursued. Our primary cell experiments were very interesting and produced results that conflict with our previous *in vivo* studies. We observed that DKO cells had enhanced *CYP27B1* mRNA expression in response to PTH treatment relative to WT cells. This could be due to the lack of competing inputs in this model, where PTH is the only factor that is being altered. Further, the molecular explanation for this observation may be a loss of intracellular signalling pathways in the primary cells, such as the RhoA-Rho Kinase signalling pathway as discussed below, that could be altering signalling from what is observed *in vivo.* Future experiments should seek to investigate if this pathway is present and functioning.

Calcitriol itself has a negative feedback effect on its own production, inhibiting *CYP27B1* and stimulating *CYP24A1* transcription. [71,39,71,80] Our data is consistent with the latter, where calcitriol treatment of HEK-293 cells induced greater mRNA expression of *CYP24A1*. Interestingly, we observed enhanced *CYP24A1* mRNA expression when claudin-2 was overexpressed in cells treated with calcitriol for 24 h.

Our investigation of HEK-293 cells with claudin-2 overexpression yielded interesting results. Apart from its permeability functions, claudin-2 has been associated with several cellular functions, such as cell proliferation, migration and signal-modulation.[116] In particular, the cytoplasmic domain of the protein has been shown to interact with multiple proteins and seems to be of interest for its signal modulating functions, although the interactions and the exact function of this remain to be determined.[116] One of the observed effects of claudin-2 is that it suppresses RhoA activation in LLC-PK cells as RhoA activation was significantly higher when claudin-2 was silenced.[129] Other studies have shown that RhoA and its downstream target Rho kinase have a negative effect on *CYP27B1* transcription and that when RhoA-Rho Kinase signalling is inhibited, there is greater *CYP27B1* mRNA expression in the kidney.[130] This may be the mechanism by which claudin-2 overexpressing cells stimulate *CYP27B1* promoter activity, which is somehow lost when claudin-12 is co-transfected. Additionally, this would explain the phenotype observed in DKO mice, where when claudin-2 is lost there is a potential overexpression of the RhoA-Rho Kinase signalling pathway which results in decreased *CYP27B1* transcription. This is not, however, the case in our DKO primary proximal tubule cell experiments, which have enhanced *CYP27B1* mRNA expression with PTH treatment. These results will need to be the subject of future investigation to determine the mechanism behind this phenomena.

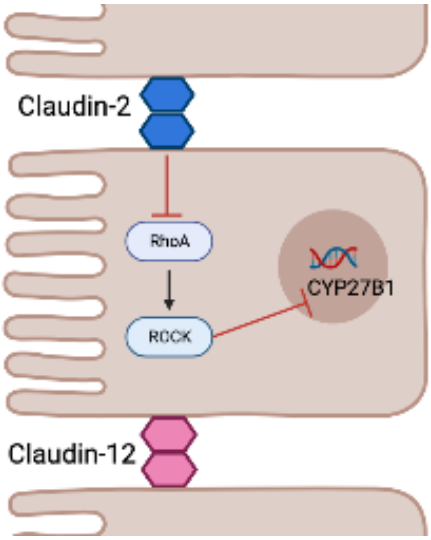

Figure 4.1. Downstream signalling of RhoA-ROCK with claudin-2 expression and the effect on CYP27B1 expression in LLCPK cells. Claudin-2 has a negative effect on RhoA-ROCK activation. RhoA-ROCK activation in turn has a negative effect on CYP27B1 transcription.

CLAUDIN-2 AND CLAUDIN-12 DKO MICE HAVE DYSREGULATED VITAMIN D METABOLISM

We found that when fed a low calcium diet, DKO mice are able to significantly increase their serum calcitriol levels compared to DKO mice fed a normal chow diet and to a greater extent than WT mice fed a low calcium diet. Interestingly, DKO mice on a low calcium diet fail to significantly increase renal *CYP27B1* mRNA expression despite significantly elevated serum PTH levels and hypocalcemia relative to WT animals fed the same diet. These findings support our hypothesis that claudin-2 and -12 DKO mice have dysregulated vitamin D metabolism in the proximal tubule. Despite this, however, these mice can significantly increase calcitriol levels beyond that of WT mice fed a low Ca^{2+} diet. As such, it is possible that under the challenged condition, the DKO mice might be more reliant on extra-renal synthesis of calcitriol. It has been suggested that extra-renal synthesis of calcitriol becomes more physiologically relevant for systemic concentrations of the hormone when there is reduced renal mass and/or renal failure. [52,131] Individuals in these circumstances can increase serum calcitriol levels with the administration of 25-hydroxyvitamin D, despite being unable to produce it in the kidneys.[52,131] Potentially, with the DKO mice under this challenged condition of severe hypocalcemia, hypercalciuria and reduced calcium absorption from the diet, the extra-renal pathway becomes heavily upregulated and used to increase the synthesis of circulating calcitriol levels in an attempt to rescue serum calcium levels.

Importantly, DKO mice are able to respond to calcitriol, indicating that the hypocalcemia observed in DKO mice fed a normal or low calcium diet is not a result of vitamin D resistance but rather is likely the result of an inability to produce adequate amounts of the hormone. As such, production of calcitriol in the proximal tubule is insufficient to rescue the calcium wasting

phenotype observed in these animals fed a normal or low calcium diet. There is likely a dysfunction in vitamin D metabolism in the kidney which is leading to this phenotype.

Interestingly, urinary calcium excretion was significantly higher in the mice fed a low calcium diet compared to mice of the same genotype fed a regular chow diet. Given the significant increases in serum calcitriol, PTH and FGF23 in these mice, which all stimulate calcium reabsorption in the kidney, it is surprising that serum calcium remains low relative to WT and that the DKO mice are more hypercalciuric on a low Ca^{2+} diet. This indicates that while these DKO mice are able to significantly increase their serum calcitriol levels, any increase in serum calcium that would result is being lost in the urine. DKO mice injected with calcitriol, however, do not have this same elevation in calcium excretion in the urine as those mice fed a low calcium diet, and so it is likely that there is another input contributing to the reduction in calcium reabsorption in the kidney of low calcium-fed animals. Alternatively, it would be interesting to investigate the urinary 25-hydroxyvitamin D and calcitriol concentrations in mice on a low Ca^{2+} diet and injected with calcitriol to determine if the phenotype is a result of calcitriol wasting in the urine. The precursor, 25-hydroxyvitamin D, is freely filtered at the glomerulus and must be taken up by the proximal tubule epithelial cells in order to undergo its final hydroxylation.[42,43] If there is a dysfunction in this process, DBP-bound vitamin D metabolites might remain in the lumen of the nephron and get excreted in urine.

PHOSPHATE AS A NEGATIVE REGULATOR OF *CYP27B1* TRANSCRIPTION

As a result of the above observations and the conflicting data from our cell culture studies, we sought to investigate if the effect we were seeing on *CYP27B1* mRNA expression *in vivo* was

due to a competing input to downregulate its transcription. This could be what is occurring within DKO mice on a regular diet and low Ca^{2+} diet, where serum phosphate is elevated and may be signalling through another pathway in the proximal tubule to inhibit *CYP27B1* transcription. There is evidence that with low serum phosphate levels there is greater synthesis of calcitriol, while when there is high serum phosphate the inactive 24,25-dihydroxyvitamin D form is predominant.[99] This work is consistent with low serum phosphate stimulating *CYP27B1* transcription independently of PTH, as well as high serum phosphate stimulating *CYP24A1* transcription and/or inhibiting *CYP27B1* transcription.[99] This would explain our findings that claudin-2 and -12 DKO mice fed a regular or low calcium diet, which have high serum phosphate levels compared to WT, have suppressed *CYP27B1* mRNA transcription. Phosphate may compete with and override the effect of PTH signalling on *CYP27B1* mRNA expression. It has previously been shown that when calcitriol and PTH are given together, calcitriol wins as the competing input and *CYP27B1* mRNA is decreased and *CYP24A1* mRNA increased, which may be occurring with phosphate as well.[71]

Future Directions

While it is clear that claudin-2 and claudin-12 have an effect on vitamin D metabolism, much more work is needed to determine the mechanisms that underlie this effect. In particular, the role of RhoA-Rho Kinase in *CYP27B1* transcription is interesting and future experiments should investigate the effects of inhibitors of this pathway on increasing *CYP27B1* expression as a means to rescue calcitriol production in disease states.

Our study of mRNA expression in HEK-293 cells had a 24 h treatment that was potentially too long to see any effect since the mRNA at this time point might be already degraded or

translated. Future studies should seek to investigate the appropriate time course for this experiment, incubating the cells for varying amounts of time to determine if the window is missed at 24 h. Similarly, cAMP levels did not increase as expected within a 15 min PTH treatment, however further investigation at different time points could be done to determine an ideal treatment interval, as previous studies have observed changes in intracellular cAMP concentration in as early as 2 min in enterocytes.[132]

Future experiments with the DKO mouse model could employ RNA-seq on known extra-renal sites of *CYP27B1* expression, such as the thyroid, appendix, and adrenal glands, among others, to see if vitamin D metabolizing genes are differentially regulated in DKO mice compared to WT animals. We should also determine the role of phosphate in this process, using cell culture with different concentrations of extracellular phosphate in order to determine if there is any change in *CYP27B1* mRNA expression. This could be further elucidated with the co-treatment of cells with PTH to see if the same effect is observed in this system as in the mouse models. Additionally, it remains to be seen whether a phosphate-sensor exists which could be mediating these effects and what mechanism is being used to exert these effects on *CYP27B1* and *CYP24A1* transcription.

While we have provided some investigation into the roles of claudin-2 and claudin-12 on vitamin D metabolism, there remains much more information to be gained from further studies. The intracellular signalling mechanism behind claudin-2 increasing CYP27B1 mRNA expression, as well as the role of phosphate in this process, are two prominent questions that remain to be answered. Claudins, in particular claudin-2, appear to have a role in signaling and modulating *CYP27B1* and *CYP24A1* transcription.

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