Identification of the Claudin-14 Promoter and Calcium Sensing Receptor (CaSR) Responsive Elements

by

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Abstract

One in ten Canadians will have a kidney stone during their life. Kidney stones cause significant pain and are expensive to treat due to recurrent emergency room visits and surgeries. The majority of kidney stones are composed of calcium and the greatest risk factor for kidney stones is hypercalciuria (i.e. urine with excess calcium), for which the etiology is unknown. A recent GWAS linked hypercalciuria to claudin-14, a gene we have shown increases expression in response to a calcium load, thereby inducing calciuria. Given this relationship we set out to 1) identify the claudin-14 promoter and 2) identify calcium sensing receptor (CaSR) sensitive signaling elements. To this end, we performed quantitative RT-PCR on the 5' untranslated regions of the 3 mouse claudin-14 variants and found that the expression of the first variant increases after CaSR activation. *In silico* studies comparing 5' of the 5' UTR of multiple species identified a highly homologous 25 bp region approximately 400 bp 5' of transcript variant-1, predicted to have promoter activity. We therefore cloned between 500-1500 bp 5' of the 5' UTR of the mouse variant 1 into the pGL3 Basic and Enhancer luciferase reporter constructs. As a positive control we cloned the SV40 promoter. We then expressed these constructs in both a HEK293 cell line and another renal tubular cell line over expressing the CaSR (OK-CaSR). Constructs containing 500, 700, 1000 or 1200 bp 5' of the 5' UTR of the mcldn-14 V1 didn't show any promoter activity when transfected into OK-CaSR cell lines. In contrast, the construct containing the 1500 bp fragment 5' of the 5' UTR of mcldn-14 V1 demonstrates promoter activity when transfected into both HEK293 and OK-CaSR cell lines and responds to CaSR activation in the OK-CaSR cell line. We therefore conclude that the claudin-14 promoter is located between 1200 (*exact length = 1243 bp*) – 1500 (*exact length = 1340 bp*) 5' to the 5'UTR of variant 1 and responds to CaSR activation. Future experiments will delineate the mechanism by which claudin-14 is regulated via this promoter.

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To my deeply missed grandmother, I dedicate my thesis.

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CHAPTER 1

Introduction

1.1 Kidney stones:

1.1.1 Incidence and Prevalence:

Kidney stones are a pandemic disease with increasing incidence and prevalence. The risk of getting, at some point in a person's life a kidney stone, is between 6-12% in the US population (Sakhaee, Maalouf & Sinnott 2012) . Moreover, the risk of developing kidney stones has increased in both genders and across ethnicity over the last thirty years (Sakhaee 2009). According to the Kidney Foundation of Canada 1 out of 10 Canadians will be diagnosed with a kidney stone at some point in their life; moreover, it tends to occur more commonly in males than females, in the middle ages of life and more frequently in places with a hot climate (Oreopoulos 2003) . In the United States, the prevalence has doubled from the period between 1964-1972 reaching a prevalence of around 5.2% in 1994 while in countries like Spain and Turkey the prevalence is 10% and 14.8% respectively (Romero, Akpinar & Assimos 2010) . They also showed that in cities like Thebes in Greece and Taiwan in China the prevalence of kidney stones is up to 15.2% and 9.6% in 2005 and 2002 respectively. More recent data on the incidence of kidney stone disease in the United States also demonstrates an increase in prevalence (Sayer 2011, Stamatelou et al. 2003).

In 1974, 208 cases per 100,000 were observed, this has increased to 1116 affected individuals between the ages of 18-65 per 100,000 in 2000. In general, an increased global incidence is also supported with data from: Germany, Japan, Spain and Sweden. Incidence is highest among Caucasian males, especially between the age of 40-60 and decreases after that. In females, the incidence is highest by the end of the 20's and declines after the age of 50 (Romero, Akpinar & Assimos 2010) .

Kidney stone disease is a serious condition and a systemic disorder that is associated with many medical sequelae such as chronic kidney disease and an increase risk of coronary artery disease (Alexander et al. 2014, Alexander et al. 2012). Furthermore, it has a huge economic burden, emphasized by the fact that the United States spends around $5 billion annually to treat this disease (Sakhaee, Maalouf & Sinnott 2012) .

1.1.2 Types

1.1.2.1 Calcium kidney stones

Calcium stones are the most common form of kidney stones globally (Sakhaee 2008). Around 85% of kidney stones are composed of calcium salts (Hess 2003) in which calcium oxalate comprises approximately 70-80% of the total (Oreopoulos 2003) . The remainder of calcium containing kidney stones is composed of calcium phosphate (15%) (Sakhaee, Maalouf & Sinnott 2012) .

An important factor promoting stone formation is urine supersaturation of solutes. This is the mechanism that drives a salt, such as calcium oxalate or phosphate from its dissolved face into a solid, forming a nidus upon which further salts can precipitate eventually forming a stone. The point where saturation is reached and crystals begin to form is called the thermodynamic solubility product, this is equal to the concentration of the solute at the point of saturation. Another concept relevant to crystal formation is the metastable zone. This is the zone at which the concentration of a salt is above its solubility and consequently precipitation will occur. The amount of solute (calcium and oxalate or phosphate) needed to form a calcium oxalate or calcium phosphate stone (solid

phase) is known as the upper limit of metastability. Thus, the upper limit of metastability tends to be lower in patients diagnosed with nephrolithiasis and this helps to explain their potential to form these stones (Miller, Evan & Lingeman 2007) .

1.1.2.2 Non- calcium kidney stones

1.1.2.2.1 Uric acid stones:

Approximately 10% of kidney stones in the United States are composed of uric acid. However, this percentage is larger in certain geographic regions; for example, the percentage is 40% in Israel, 25% in Germany and around 16% in Japan. This tells us that there is a huge global variation in the risk of having uric acid nephrolithiasis; although, the factors that cause this variation have not been identified (Cameron, Sakhaee 2007). Genetic, acquired or a combination of both could be the etiology for developing such stones? The metabolic syndrome is felt to be the most common cause of uric acid stone formation (Sakhaee, Maalouf & Sinnott 2012) . The metabolic syndrome is a cluster of conditions including dyslipidemia, obesity and hypertension that increase the risk of developing type 2 diabetes mellitus, and atherosclerosis (Sakhaee 2009). Consistent with an association between uric acid stones and the metabolic syndrome, a retrospective study in Dallas found that patients with idiopathic uric acid nephrolithiasis have many features of the metabolic syndrome (Taylor, Stampfer & Curhan 2005b, Taylor, Stampfer & Curhan 2005a) . Uric acid stones can exist in two forms, either pure or mixed with calcium containing stones. A small percentage of patients diagnosed with uric acid stones have hyperuricosuria; however, the majority of those patients have normal uric acid levels in the urine (Asplin 1996, Riese, Sakhaee 1992). A low pH of the urine (acidic

urine) is a common feature seen in all uric acid stone formers. Regardless, the pathophysiology of uric acid stone development likely varies between different groups of patients (Moe, Abate & Sakhaee 2002) .

1.1.2.2.2 Cystine stones:

Cystine nephrolithiasis represent a small percentage of kidney stones in adults; however, it is more common among children and adolescents. This disease is almost exclusively caused by cystinuria (Chillaron et al. 2010). Cystinuria can be inherited in an autosomal recessive fashion or an autosomal dominant manner. Children with the disease tend to display markedly increased urinary cystine excretion. This disease is generally due to a defect in renal tubular cysteine reabsorption secondary to an inherited defect in either SLC3A1 or SLC7A9. In both cases, due to the low solubility of cysteine in urine, cysteine stones form (Sakhaee 1996). Cystinuria is classified as type A if a mutation was in both alleles of SLC3A1, B if the mutations are only in SLC7A9 and AB if a single mutation is found in each gene (Sakhaee, Maalouf & Sinnott 2012, Dello Strologo et al. 2002) .

1.1.2.2.3 Infection related stones

Infection related stones may account for up to 10% of all urinary stones. These calculi, if left untreated, may cause significant morbidity and mortality. Thus, this type of stone needs to be treated aggressively by complete surgical removal of all stone fragments followed by good coverage with antibiotics (Flannigan et al. 2014). Infection related stones are composed of magnesium ammonium phosphate and are commonly

referred to as struvite stones. Calculi may form in the urinary tract in this case to protect against bacterial infection. Not only do bacteria participate in stone formation, but infection related stones also contribute to the acquisition of urinary tract infection by bacteria such as: *Proteus, Klebsiella, Staphylococcus Epidermidis, Pseudomonas,, Providencia, Ureaplasma urealyticum and Enterococcus* (Shortliffe, Spigelman 1986). These bacteria are urease-producing organisms, which leads to alkalization of the urine (pH > 7.2) and in the presence of supersaturated urine with magnesium and phosphate, the addition of ammonium leads to struvite stone formation. There are also other rare forms of kidney stones such as dihydroxyadanine, ammonium urate and stones resulting from protease inhibitors drugs (Sakhaee, Maalouf & Sinnott 2012) .

1.1.3 Pathophysiology

There are many theories that have been proposed for stone pathogenesis. One pathway suggests that as a result of tissue injury crystal retention happens. This is supported by tissue culture and animal model experiments (Khan 1997). When hyperoxaluria was induced in animal models it led to crystalluria and calcium oxalate crystal deposition in the kidney (Khan 1995). It is believed that hyperoxaluria increases the urinary excretion of enzymes such as N-acetyl-β-glucosaminidase, gamma-glutamyl transpeptidase, and alkaline phosphatase. These enzymes are associated with renal epithelial cell injury (Khan, Shevock & Hackett 1989) . Oxalate crystallization causes renal epithelial cell injury and the production of reactive oxygen species, which overwhelms the antioxidant system in renal cells causing oxidative stress and damage (Khan 2004, Khan 2006). Furthermore, oxalate increases the secretion of

macromolecules into the urine such as CD44, phosphatidylserine, hyaluronan and osteopontin. This in turn enhances the adherence of crystals to the renal epithelial cell surface (Asselman et al. 2003). In addition, tissue culture studies found that crystals favorably adhere to injured renal epithelial cells rather than healthy inner medullary collecting duct cells. Another study, which used injured MDCK-1 cells, has shown similar results where calcium oxalate crystals adhere only to the surface of injured cells (Asselman et al. 2005).

Other mechanisms have been proposed for calcium stone formation. One mechanism proposes that an increased concentration of stone forming salts leads to supersaturation followed by nucleation in the lumen of the nephron, which in turn causes crystal growth and obstruction of the distal nephron (Evan 2007). However, it has been observed that it is uncommon to have intraluminal plugging among kidney stone formers, calling into question the relevance of this proposal. Dr.Randall has argued that it is calcium phosphate deposits in the interstitium, which are the initial nidus, to which urinary crystals attach below cells of the renal papilla. He showed that these lesions are not intraluminal and are not associated with any inflammatory reaction; however, they are located in the interstitium. These lesions have subsequently been dubbed Randall's plaque. It has been shown that Randall's plaque is mainly localized to the basement membrane of the thin descending limb of the loop of Henle. This specific basement membrane is composed of collagen and mucopolysaccharides, which anchor calcium and phosphate ions to it, making it the most common place of stone formation. Once attached, the crystallization process begins. These crystals make their way through the urothelium

and become a nidus for calcium oxalate deposition ultimately forming kidney stones (Randall 1940).

There are three factors which contribute to the development of uric acid nephrolithiasis: hyperuricosuria, low urine volume and aciduria. Low urine pH is considered the most important factor for developing uric acid stones as uric acid becomes insoluble at low pH. High uric acid concentrations can be due to genetic or acquired causes; however, genetic causes are relatively rare. Acquired causes of hyperuricosuria may be secondary to chemotherapy treatment, malignancy or uricosuric medicines. Low urine volume (lower than 2 L/day) predisposes to the development of all kinds of kidney stones by relatively increasing the concentration of stone forming constituents. Patients at particularly increased risk of stone formation include those who are diagnosed with short bowel syndrome or have an ileostomy and are consequently suffering from chronic diarrhea. This is due to bicarbonate loss from the bowel, which will eventually lower urine pH and volume making the risk of developing uric acid stones even higher. Low urine pH, which is considered as the primary factor for developing uric acid stones, can be due to impaired ammoniagenesis, which causes protons to be buffered by titratable acids thereby lowering the urine pH or it can be due to increased net acid excretion (Kenny, Goldfarb 2010).

In summary, the pathogenesis of nephrolithiasis is complex and depends on the type of stone. Regardless, despite many studies having been performed to date on the topic of kidney stone pathogenesis, the exact mechanism(s) causing this disease remains elusive. Unfortunately, cell culture and animal models of kidney stone formation may apply only to a small group of patients for which the specific known defect is induced,

such as hyperoxaluria. Data from human cortical and papillary biopsies in patients with kidney stones has demonstrated a role for Randall's plaque in stone formation (Miller, Evan & Lingeman 2007) . More Studies are needed to assess the multiple theories put forth to explain the pathogenesis of kidney stones (Bagga et al. 2013).

1.1.4 Risk Factors

There are many causes and risk factors for developing kidney stones. In the case of calcium kidney stones, hypercalciuria is the main risk factor (Pak et al. 1980). There are a number of potential causes of hypercalciuria which can be subdivided into: vitamin D dependent (Broadus et al. 1984, Insogna et al. 1985), vitamin D independent (Pak et al. 1974), intrinsic renal calcium or phosphorus leak (Coe, Bushinsky 1984), or parathyroid hormone dependent, such as is seen in cases of primary hyperparathyroidism (Pak 1991). Another risk factor for developing uric acid kidney stones is hyperuricosuria secondary to purine rich food and urate overproduction (Coe 1978). Another risk factor is hypocitraturia, which can be secondary to distal renal tubular acidosis (Backman et al. 1980), treatment with carbonic anhydrase inhibitors (Gordon, Sheps 1957) , primary aldosteronism (Shey et al. 2004), increased salt intake (Sakhaee et al. 1993), angiotensin converting enzyme inhibitors (Melnick et al. 1998) or high protein consumption (Reddy et al. 2002). Hypocitraturia is a risk factor for calcium stone formation as citrate prevents the calcium-oxalate crystallization process. It does so by forming a soluble complex with calcium thereby decreasing the urinary saturation of calcium salts (Kok, Papapoulos & Bijvoet 1986) . Hyperoxaluria is another risk factor for calcium oxalate stone formation (Sakhaee 2009). It can be due to increased oxalate absorption from the intestine, diet

induced due to high oxalate intake or more rarely due to an inborn error of metabolism (Holmes, Goodman & Assimos 2001) . These genetic causes of hyperoxaluria have been divided into type I hyperoxaluria, which is due to a mutation in the hepatic enzyme, alanine glyoxylase transferase, type II primary hyperoxaluria which is due to a deficiency in glyoxylate reductase/hydroxypyruvate reductase or type III hperoxaluria that is caused by gain of function mutations in the mitochondrial 4-hydroxy-2-oxoglutarate aldolase (Danpure, Jennings 1986, Giafi, Rumsby 1998, Belostotsky et al. 2010). Finally, the last factor that might predispose patients to calcium kidney stone formation is a disturbance in urinary pH either very low (< 5.5) or very high (> 6.7) (Sakhaee, Maalouf & Sinnott 2012) . Given the tendency for calcium oxalate to precipitate at acidic pH and calcium phosphate to precipitate at alkaline pH, the specific stone type one is at increased risk for differs based on the alteration in urinary pH (Pak et al. 2003).

Another type of kidney stones is uric acid stone. The main causes of uric acid stone formation are low urine pH (as is seen with severe dehydration or chronic diarrhea), increase uric acid secretion and low urine volume (Hochreiter, Knoll & Hess 2003) . In the case of idiopathic uric acid nephrolithiasis (gouty diathesis), it has been proposed that it is either associated with or due to insulin resistance (Moe, Abate & Sakhaee 2002) .

To a lesser extent some patient might develop cysteine stones. Cystine stones are due to cystinuria, which is secondary to a defect in tubular reabsorption of cysteine. This solute has a low solubility in urine with a pH below 8, thereby promoting supersaturaion and stone formation (Hochreiter, Knoll & Hess 2003) .

Finally, the main factor for developing infection related stones is the presence of urease producing bacteria which splits urea, forming ammonia and raising the pH of

urine. This in turn promotes the crystallization of magnesium-ammonium-phosphate (struvite) which can grow very quickly into a staghorn calculi leading to the blockage of the collecting system of the kidney (Hochreiter, Knoll & Hess 2003) .

1.1.5 Treatment

The first line of treatment that must be considered in all stone formers is high oral fluid intake, up to 2.5 L/day (Borghi et al. 1996). Thiazide diuretics are commonly used for the treatment of patients with calcium containing kidney stones and hypercalciuria to prevent recurrence (Moe, Pearle & Sakhaee 2011) . Furthermore, potassium citrate can be used to alkalinize the urine and is hence used in the treatment of both calcium oxalate and uric acid stones (Sakhaee et al. 1983, Kang et al. 2007). Both osteopenia (decrease bone mineral density) and fractures are observed with increased frequency in patients with nephrolithiasis. Consequently both alkali and thiazides have been shown to increase bone mineral density (Sakhaee et al. 2011). However no data is available to support a decrease in the incidence of fractures by these therapies (Sakhaee, Maalouf & Sinnott 2012)

In patients with hyperuricosuric nephrolithiasis, allopuriniol can be employed as it blocks the enzyme xanthine oxidase thereby preventing the conversion of xanthine to uric acid. This decreases the uric acid concentration in urine (Sorensen, Chandhoke 2002). Treatment of uric acid nephrolithiasis is targeted towards correcting low urine volume by increasing fluid intake and lowering urine acidity by alkalinization of the urine with potassium citrate, which increases uric acid solubility (Pak, Sakhaee & Fuller 1986) Allopurinol is only used in cases of hyperuricemia or severe hyperuricosuria (> 800 mg/day) (Moe, Abate & Sakhaee 2002) Irrigation of uric acid stones in the bladder with

alkaline solution using a catheter may be employed when surgical intervention is contraindicated or as an adjuvant treatment to relieve obstruction caused by uric acid stone fragments (Sheldon, Smith 1982). For cystine stones the treatment depends on urine dilution and alkalinization (Hochreiter, Knoll & Hess 2003) In patients who suffer severe cystinuria, d-penicillamine and α-mercaptopropionylglycine are used to split the cysteine molecule into cysteines thereby producing a more soluble disulfide compound (Sakhaee, Maalouf & Sinnott 2012)

Finally, for treatment of a struvite stone, removal of all stones fragments is first required and then administration of a proper antibiotic to treat the infection based on stone culture or urine culture from the renal pelvis. Importantly a midstream urine culture should not be employed for this purpose as it often provides a false negative. The gold standard method that has been used to remove these types of stones is percutaneous nephrolithiotomy (PCNL); however, other modalities like extracorporeal shockwave lithiotripsy, ureteroscopy and anatrophic nephrolithotomy can also be used (Flannigan et al. 2014). In cases where surgical removal and treatment with antibiotics are not effective, acetohydroxamic acid can be used as treatment for infected stones. This compound inhibits the urease enzyme preventing increased urine pH and NH_4^+ (Sakhaee, Maalouf & Sinnott 2012)

1.2 Hypercalciuria

Given that the greatest risk factor for the most common kidney stones is the formation of urine with too much calcium in it, called hypercalciuria, it has been the focus of much research. Calcium filtered by the glomerulus is predominantly

(approximately 65%) absorbed from the proximal tubule, in a paracellular fashion down its concentration gradient (Ng, Rouse & Suki 1984, Pitts et al. 1988) The next greatest amount of calcium is absorbed, again in a paracellular fashion, from the thick ascending limb of the loop of Henle (Konrad et al. 2006). The driving force for the reabsorption of calcium from both these segments is the reabsorption of water which is itself driven by sodium reabsorption (Mount 2014). In the distal convolutions and the connecting tubule, approximately 5-10% of filtered calcium is reabsorbed by an active transcellular process (Loffing, Kaissling 2003) There is evidence supporting defective proximal tubular and thick ascending limb calcium reabsorption in patients with hypercalciuria (Dimke et al. 2013a, Pan et al. 2012)

A number of single gene disorders cause hypercalciuria including: Barter's syndrome, Dent's disease and distal renal tubular acidosis. However, they are associated with other abnormalities (Stechman, Loh & Thakker 2009) To date only three genes have been linked to isolated hypercalciuria: soluble adenylate cyclase (Reed et al. 2002) the vitamin D receptor (VDR) (Scott et al. 1999) and claudin-14 (Thorleifsson et al. 2009) The first two account for less than 5% of individuals with isolated hypercalciuria. Claudin-14 was only recently found to associate with hypercalciuria and kidney stones. The percentage of idiopathic stone formers with hypercalciuria due to altered claudin-14 expression/activity is unknown.

1.3 Claudins

The claudin family is a group of transmembrane proteins that localize to the tight junction (Krause et al. 2008, Angelow, Ahlstrom & Yu 2008, Piontek et al. 2008)

Remarkably, there are 27 human isoforms and their main function is to control paracellular ion fluxes (Anderson, Van Itallie 2009) A recent genome wide association study (GWAS) found an association between single nucleotide polymorphisms (SNPs) in the claudin-14 gene, encoding a member of the claudin family of tight junction proteins, and a risk of hypercalciuria and kidney stone formation (Thorleifsson et al. 2009) The identified linkage disequilibrium block contained only the coding exon of claudin-14 and no other genes. However, although the marker SNPs were in the coding region of claudin-14, they were synonymous (*i.e.* did not change the amino acid coded for). In addition, claudin-14 knockout mice (Wilcox et al. 2001, Ben-Yosef et al. 2003) and children with null mutations in claudin-14 are deaf (Bashir et al. 2013, Lee et al. 2012) and do not demonstrate alterations in calcium homeostasis. To understand the role of claudin-14 in renal calcium homeostasis, the Alexander laboratory and others discovered a novel pathway central to renal calcium handling (Dimke et al. 2013a, Gong et al. 2012) They called this pathway the CaSR-CLDN14 axis. Through manipulation of calcium homeostasis in mice they showed that claudin-14 expression is increased via activation of the CaSR. This was demonstrated by giving wild-type mice the CaSR agonist, Cinacalcet HCl. Remarkably this increased renal claudin-14 mRNA expression 40 fold (Dimke et al. 2013a) That claudin-14 expression induces calciuria was proven by feeding claudin-14 knockout mice a high calcium diet and observing less urinary calcium excretion than wild-type animals (Gong et al. 2012) A lacz reporter replacing the claudin-14 coding exon in the null mice was used to localize claudin-14 expression to the TAL (Gong et al. 2012) The functional role of claudin-14 in renal Ca^{2+} handling was deduced by over–expression studies in two renal tubular epithelial cell culture models (MDCK and OK

cells) (Dimke et al. 2013a) Consistent with previous cell culture work, claudin-14 is expressed predominantly in the tight junction where it increases transepithelial resistance (TER) and decreases paracellular Ca^{+2} and Na^{+} flux (Dimke et al. 2013a) Thus, claudin-14 is a paracellular cation blocker. These results lead to the proposal of the following model: increased circulating Ca^{2+} in the blood binds the basolaterally expressed CaSR in the thick ascending limb (Riccardi et al. 1996) , which increases claudin-14 expression. Newly synthesized claudin-14 traffics to the tight junction forming a paracellular barrier, preventing Ca^{2+} reabsorption and inducing calciuria (**Fig 1**).

Given the strong evidence that renal claudin-14 expression is transcriptionally regulated, and the central role of this gene in calcium homeostasis and the formation of kidney stones, we set out to deduce the promoter region of this gene, which may be sensitive to CaSR activation. To that end we performed *in silico* studies looking for regions of DNA with promoter characteristics 5' of the different splice variants, using the Eukaryotic Promoter Database(Dreos et al. 2014) and Softberry software (Solovyev, Shahmuradov & Salamov 2010, Solovyev, Shahmuradov 2003) We found the region -300 to -500 5' of the first splice variant of claudin-14 in human was highly predicated to contain the promoter.

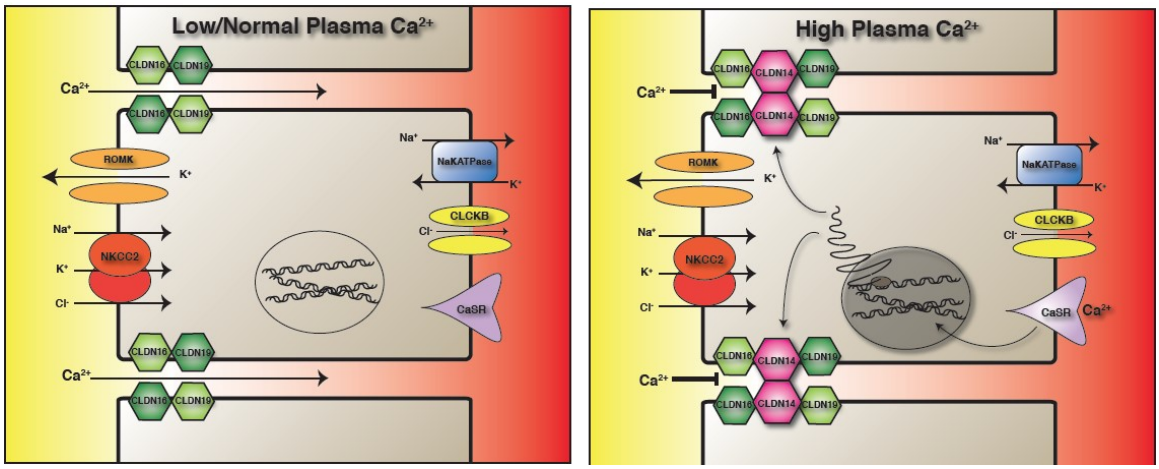

Figure 1. CaSR-CLDN14 axis. Schematic representation of the proposed CaSR-CLDN14 axis in the thick ascending limb (TAL) of the loop of Henle in the kidney. In the presence of low/normal plasma Ca^{2+} levels, Ca^{2+} flux across the TAL occurs passively down its concentration gradiant via claudin-16 (Cldn16) and claudin-19 (Cldn19). When plasma Ca^{2+} levels are high, they bind the CaSR on the basolateral membrane of the TAL activating the receptor, which results in increased expression of Cldn14 at the tight junction ultimately blocking Ca^{2+}reabsorption and inducing calciuria.

1.4 Hypothesis

Based on *in silico* studies we hypothesize that the claudin-14 promoter lies between: -35 to -500 bp 5' to the 5'UTR of mouse claudin-14 variant 1.

1.5 Objectives

- To identify and clone the claudin-14 promoter of the CaSR responsive variant.
- Understand the signaling pathway between CaSR activation and Claudin-14 expression using this tool.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of whole kidney

Wild type mice treated with either Cinacalcet HCl (Sensipar®, Amgen, CA, USA) in food at a dose of 1 mg/g body weight or vehicle (Dimke et al. 2013a) were euthanized by intraperitoneal injection with pentobarbital (50mg/kg) and the kidneys subsequently removed. Whole kidneys after mechanical disruption, were immediately transferred into RLT buffer from the RNeasy kit (Qiagen, Toronto,ON,Canada) and RNA was isolated as per the manufacturer's protocol. All experiments utilizing animals were performed in compliance with the animal ethics board at the University of Alberta, Health Sciences Section, protocol number 576.

2.2 Genomic DNA isolation

Whole kidney was digested overnight using digestion buffer (0.5M EDTA, 5M NaCl, 1M Tris pH and 20% SDS) and proteinase K (Invitrogen, Carlsbad, CA, USA) in a water bath at 56°C. The sample was mechanically disrupted, vortexed and left on ice for 5 mins. Then, it was mixed with 5M NaCl, chilled on ice for 5 mins and then centrifuged at 14,000 rpm at 4°C for 10 mins. Then, ice-cold isopropanol was used for DNA precipitation. Obtained pallet washed with ice-cold 70% ethanol and centrifuged at 14,000 rpm, 4°C, for 15 mins. The pellet was air dried and resuspended in autoclaved ddH_2O (Dimke et al. 2013a) .

2.3 Gel Electrophoresis

A 1.2% (1.08 g in 70 ml) agarose (Invitrogen, Carlsbad, CA, USA) gel was prepared in

1X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) and heated in a microwave for 1 mins. The solution was cooled for around 15 minutes and then 1.5 µl of ethidium bromide (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added, shaked for 30 seconds and poured into gel cast electrophoresis apparatus (Electrophoresis system, Fisher Scientific Company, Ottawa, ON, Canada) and allowed to solidify for 40 minutes. 3.5µl of 2X loading dye (Fermentas Canada Inc, Burlington, ON, Canada) was added to all the samples. All the samples in addition to 100 Kbp Plus DNA ladder (Generuler, Fermentas Canada Inc, Burlington, ON, Canada) were loaded on the gel and run at a current of 135 volts for 45 mins (Power Pca 200, BioRad, Mississauga, ON, Canada). The gel was viewed using a gel imager (Gel Doc EZ Imager, BioRad, Mississauga, ON, Canada).

2.4 Quantitative Real Time PCR

1.1.1 RNA Isolation

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate whole kidney mRNA as per the manufacturer's instructions. In brief, half a snap frozen kidney was homogenized in ice-cold TriZol; then, chloroform (Invitrogen, Carlsbad, CA, USA) was added to the supernatant. The aqueous phase was separated, mixed with isopropanol and then centrifuged at 4°C, 12000g for 15 mins. The pellet obtained was washed with 70% ethanol and then air-dried. The pellet was then dissolved in 10X DNase buffer and RNAse free water ;then, DNase added. RNA was precipitated using an ice-cold phenol:chloroform (1:1) mixture. 3M NaAC pH 5.2 and 100% ethanol were added to the

RNA and incubated overnight at -20°C, or for 2 hours at -80°C. The solution was centrifuged and the pellet obtained was washed with 70% ethanol and then air-dried. This pellet was dissolved in RNAse free water and the concentration measured using a spectrophotometer (Nanodrop 2000c, Thermo Scientific, Asheville, NC, USA) (Dimke et al. 2013a) .

1.1.2 Quantitative PCR

Isolated total mRNA was reverse transcribed into cDNA. To do so, 1 µg of RNA was reverse transcribed by Random Primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). 5µl (500 ng cDNA) was used as template to determine the gene expression of claudin-14 variants. A mixture consisting of TaqMan universal qPCR master mix (Applied Biosystems Inc, Foster City, CA, USA), primer, probe and RNAse free water was prepared and added to the cDNA in a 96-well plate (Sarstedt Inc, Montreal, QC, Canada). As an internal control mRNA levels of the housekeeping gene 18s ribosomal RNA were determined. Expression levels were quantified with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA). Primers and probes were made by IDT (Integrated DNA Technologies Inc, San Diego, CA, USA) or ABI (Applied Biosystems Inc, Foster City, CA, USA). The sequences of all primers and probes utilized are listed in **(Table 1)**.

Table 1. Real-Time PCR primers and probes

	Sequences
Cluaidn-14	Forward: TGGCATGAAGTTTGAAATCGG Reverse: CGGGTAGGGTCTGTAGGG Probe: TGAGAGACAGGGATGAGGAGATGAAGC
Variant 1	Forward: GGAATGGCATGTTCTGAAAGG Reverse: GCCTGAGGCGCACCTAGTT Probe: CAGCGTTGATAGCTG
Variant 2	Forward: TCCGTGGTCTACCTGAGAGCAT Reverse: AGCCACTCCACTCACATACAGAAC Probe: AAGGTGGATGGGACTGG
Variant 3	Forward: CAGCCACAGGGACCCATTAG Reverse: TTTGGAACAAGAATGCCAGAGA Probe: CGGCCGTGTGTAGAT

2.5 Cloning and sequencing

Gene fragments were amplified by PCR. The PCR reaction (94 °C for 1 min, 94 °C for 30 seconds, primer melting temperature (60-65 °C) for 30 seconds, 72 °C for 90 seconds, 39 cycles, 71°C for 10 mins and 4 °C forever) was carried out with a C1000 Thermal cycler (BioRad, Mississauga, ON, Canada) from mouse gDNA using cloning primers (IDT,oralville, IA, USA) with unique restriction enzyme sites – see **(Table 2)**. The PCR products were run out on agarose gel and visualized with ethidium bromide. PCR products were then digested with enzymes corresponding to the unique restriction sites KpnI and BglII (New England Biolabs, Ipswich, MA, USA)- see **(Table 3)** and ligated into the PGL3 Basic and Enhancer (Promega, Madison, WI, USA) vectors that were previously linearized using the same restriction enzymes. The fragment in each construct was sequenced by Eurofins MWG Operon (Huntsville, Alabama, USA) using both the forward and reverse cloning primer **(Table 2).**

Table 2. List of cloning primers

Construct	Forward Primer	Reverse Primer
1500	GGGGTACCCC -TGATGTAGGTGGCCTCATTTC	GAAGATCTTC-CTATCAACGCTGCCCTTTC
1200	GGGGTACCCC -CTTGAAGTCGAACCACACTATTG	
1000	GGGGTACCCC -GTCGAGCTCCGGAATTTGT	
750	GGGGTACCCC -CAAAGTGTCTTGTGCATGTGG	
500	GGGGTACCCC -CCCCTGGCAATAAAGTCAT	

* The restriction sites used for cloning into the PGL3 vectors are in red.

Table 3. Enzymes used for cloning and digestion site

Enzyme	Sequence
KpnI	Forward: GGGGTACCCC
BglII	Reverse: GAAGATCTTC

2.6 Cell Culture

All cell lines were originally obtained from ATCC (Rockville, MD, USA). OK cells were maintained in DMEM/F-12 medium, supplemented with 10% FBS and 5% penicillin streptomycin glutamine at 37°C in a 5% CO_2 incubator. HEK 293 cells were maintained in DMEM, containing 10% FBS and 5% penicillin streptomycin glutamine. OK cells overexpressing the calcium-sensing receptor (OK-CaSR) were generated by first transfecting into them the human CaSR (OriGene, Rockville, MD, USA) by electroporation with the Neon apparatus (Invitrogen Neon® Transfection System). They

were then selected with G418 to produce a polyclonal cell line. Finally monoclonal stably expressing cells were selected by limiting dilution (Pan et al. 2012) .

2.7 Immunocytochemistry

Cells were seeded on glass coverslips and allowed to reach confluence and then fixed using 4% paraformaldehyde (PFA). After being quenched with 5% glycine, the cells were permeabilized with 0.2% Triton X-100. Primary anti-Myc antibody (9B11, Cell Signaling Technology) was first applied. Secondary affiniPure donkey anti-rabbit cy3-conjugated antibody (Jackson ImmunoResearch Laboratories) and 4',6-diamidino-2-phenylindole (DAPI) were applied at a dilution of 1:500 for 1 h at room temperature. The cells were then mounted with Dako mounting medium. Specimens were analyzed using a spinning disc confocal microscope (WaveFx, Quorum Technologies, Guelph, Canada) (Dimke et al. 2013b) .

2.8 Dual Luciferase assay

The DNA fragments cloned into PGL3 Basic and PGL3 Enhancer vector (Promega, Madison, WI, USA) upstream of the luciferase gene (*luc*+) and the PRL-TK vector (a kind gift from Dr. Michael Walter), which was used as an internal control for vector expression, were transfected into OK or HEK cells. The above vectors and a construct containing the human CaSR (hCaSR-PCMV6-DDK-MYC) (OriGene, Rockville, MD, USA) were used for triple transfections into either the OK-CaSR or the HEK293 cell line. For this purpose, plasmid DNA for transfections had an A260/A280 ratio of 1.7–1.9. Purified DNA was prepared in sterile water. Transfection into OK cells was performed

with FuGENE® 6 (Promega, Madison, WI, USA). The optimal amount of DNA for transfection was determined to be 1000 ng (500 ng of our specific construct and 500 ng of the PRL-TK control) per well of a 12-well plate. A transfection reagent:DNA ratio of 3:1 was also determined to be optimal for expression. Transfected cells were incubated for 24 hours before assaying to allow time to express the transfected DNA. Cell extracts were prepared after incubation with 250 µl passive lysis buffer for 20 minutes (Promega, Madison, WI, USA) and analysed for luciferase activity. For analysis, we mixed 40 µl of cell lysate and 100 µl of Luciferase Assay Reagent and then measured the light produced using a luminometer (DLR Ready, TD- 20/20 Luminometer, Turner Design). When measuring light emitted by this protocol the first signal (number) is the firefly luciferase activity (i.e. our construct of interest) then we added 100 µl stop and glo buffer, mixed and measured light emitted again. This second measurement is the renilla luciferase activity (from out internal expression control, PRL-TK). As described in the manufacturers instructions (Promega, Madison, WI, USA), Firefly activity was normalized to Renilla activity (PRL-TK). Cinacalcet HCl (Sensipar, Amgen) was added 24 hrs after transfection in some cases. For these experiments luciferase activity was then measured either 24 or 48 hrs after transfection. Each reporter assay was done a minimum of two times with consistent results. In HEK293 cells, we used calcium phosphate for transfection. In this case we used 2950 ng of DNA of interest and 50 ng of PRL-TK per 100 mm petri dish. Transfected cells were incubated for 48 hours before assaying to allow time to express the transfected DNA.

2.9 Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons were made by one-way ANOVA with a Gesser-Greenhouse correction for multiple comparisons in experiments with 3 or more groups. In experiments with only 2 groups statistical comparisons were made with a Mann-Whitney test. A $p < 0.05$ was considered statistically significant. All analyses were performed using Graphpad Prism software (GraphPad Software Inc., San Diego, CA, USA).

CHAPTER 3

RESULTS

3.1 Identification of the claudin-14 variant that is regulated by the CaSR

A recent GWAS linked hypercalciuria to claudin-14 (Thorleifsson et al. 2009), a gene we have shown to have increased expression in response to CaSR activation, thereby inducing calciuria (Dimke et al. 2013a) . We therefore wanted to identify the signaling pathway by which claudin-14 is regulated. Looking at the mouse claudin-14 (mcldn14) gene in the NCBI database, we were able to identify three variants, which are depicted in (**Figure 2)**. (N.B. recently a fourth variant was identified, which was not included in our experiments). To identify the claudin-14 variant that is regulated by the CaSR**,** we performed quantitative real-time PCR employing primers and probes targeting the different 5' untranslated regions on cDNA isolated from whole kidney of mice treated with Cinacalcet HCl (a calcium sensing receptor agonist) or vehicle. As an internal positive control we also performed quantitative real-time PCR with primers for the coding region (**Figure 3)**.These experiments demonstrate that claudin-14 gene expression is increased by Cinacalcet HCl but not the control group (consistent with previous observations); moreover, it is the mcldn14 variant 1 (V1) that is regulated by activation of the CaSR with Cinacalcet HCl (**Figure 4)**.

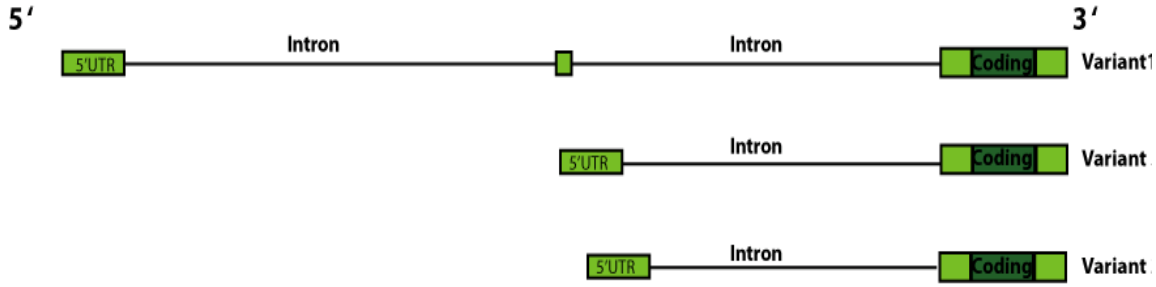

Figure 2. Pictorial representation of the mcldn14 variants. This is a schematic representation of the different mcldn14 variants as it appeared in the NCBI database, provided by RefSeq, Oct 2009. All of the variants have the same coding region, consequently they all code for the same protein, claudin-14. Variant 1 is the longest transcript and has 3 exons containing the 5'UTR, a coding region and a 3'UTR. The 3 variants are different from each other as they all have their own 5'UTR.

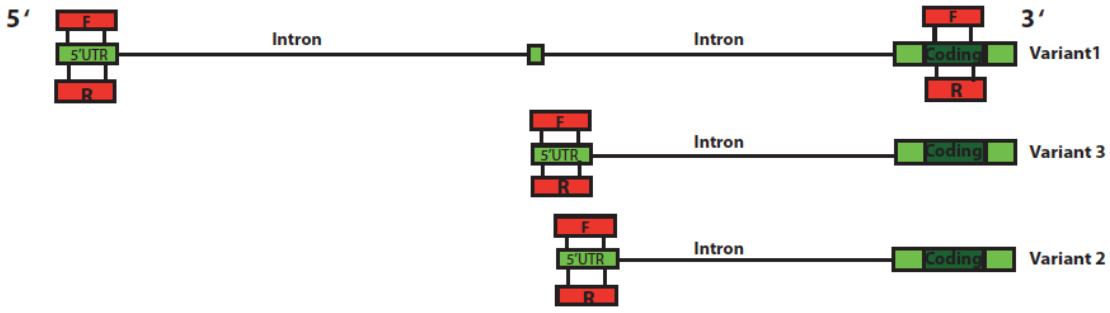

Figure 3. Forward and Reverse primers used to target the 5'UTR of each variant. For the 5'UTR of each variant, a forward and reverse primer and probe were designed to complement these unique areas. A forward (F) and reverse (R) primer was also designed to complement the coding region as a positive control since all three variants have the same coding region.

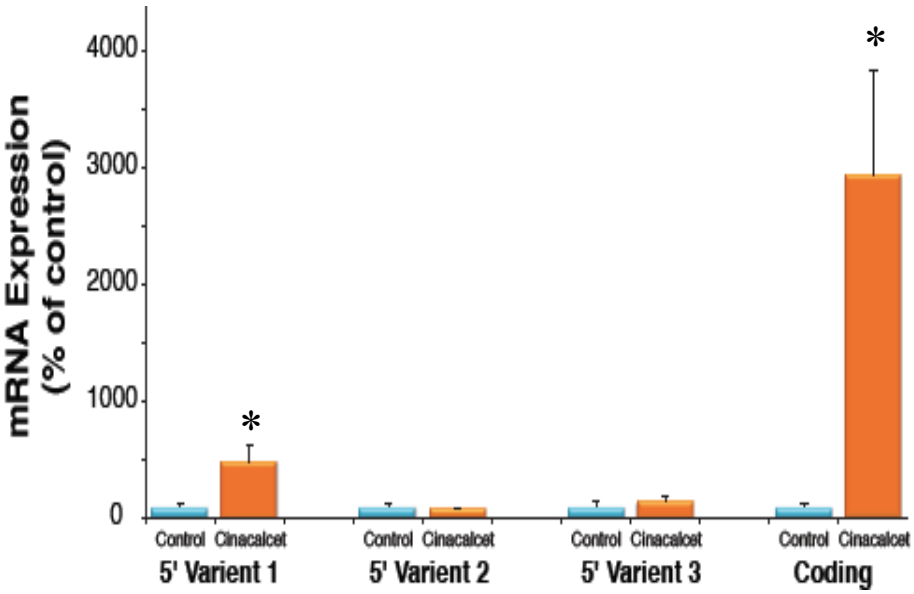

Figure 4. Quantitative real-time PCR on different mouse claudin-14 variants, (mcldn14). Quantitative real-time PCR was performed to compare the expression between the 3 mcldn14 variants. Expression was compared between two groups of mice, with one being treated with vehicle (blue bars) and the other treated with Cinacalcet HCl for 5 days (orange bars). As a positive control, primers for the coding region were employed. We found a statistically significant increase in mRNA expression of claudin-14 in the Cinacalcet HCl treated group vs. the control group (approximately a 30 x increase). Only mcldn14 V1 demonstrated a difference in expression between the two groups (5x higher in the Cinacalcet HCl treated vs. the control group). Out of the three mcldn14 variants, it is was only mcldn14 V1 that was upregulated by CaSR activation with Cinacalcet HCl. Expression was normalized to 18S, * represents $P < 0.001$.

3.2 Characterization of an OK cell line overexpressing the CaSR

In order to employ a cell line expressing the prerequisite components we thought necessary for claudin-14 gene expression and to hopefully increase the transfection efficiency of our constructs an opossum kidney cell line over expressing CaSR (OK-CaSR) was generated. Importantly the CaSR over-expressed in this cell line contains a myc tag at the C-terminus. To confirm that the CaSR was expressed, we first performed immunolocalization studies on confluent cultures of cells. We were able to detect significant CaSR expression (orange in **Figure 5A**) by immunostaining first with an anti-myc primary antibody and then a cy3 secondary antibody. We included the nuclear stain – dapi in this experiment. Consistent with significant CaSR receptor expression, we were able to detect a band of the appropriate molecular weight when we immunoblotted cell lysate from cells over-expressing the CaSR but not cell lysate from cells expressing the empty vector (**Figure 5B)**.

A **B**

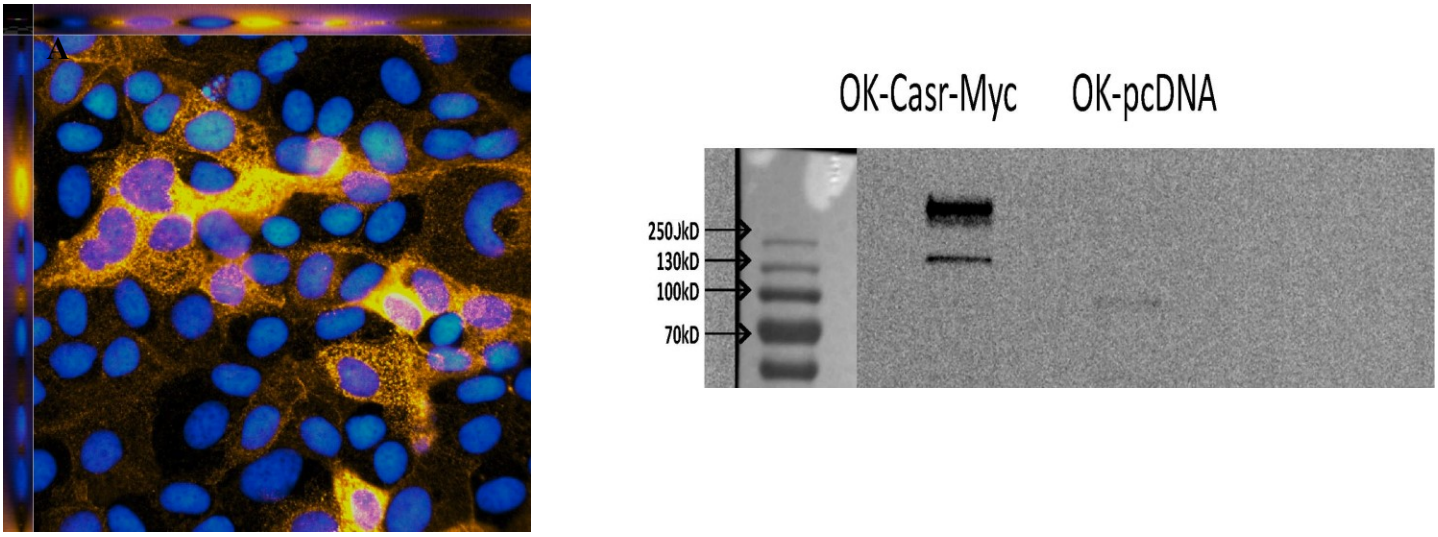

Figure 5. Characterization of the OK-CaSR cell line. A) Confluent monolayer of OK-CaSR cells immunostained for the CaSR (with an anti-myc antibody, red), and with dapi (a nuclear marker). **B)** Immunoblot of lysate form OK-CaSR cells or those expressing the Empty vector, showing a band with a size of 135KD, which corresponds to the appropriate size for the CaSR, and the absence of this band in the negative control.

3.3 Cloning 5' of the 5' UTR of mcldn-14 V1 into PGL3 basic

In Silico studies were performed to predict the promoter location by comparing 5' of the 5' UTR sequence from different species. This analysis predicted that the promoter lies between -35 – -500 bp 5' to the 5' UTR of mcldn14 V1. Therefore primers were designed to clone that area of mcldn14 V1 (529bp upstream of the 5'UTR) and ligate it into the PGL3 Basic vector upstream of the luciferase gene (**Figure 6)**. In order to examine the PGL3 Basic vector luciferase expression, the known SV40 promoter was ligated into that vector and we used this modified construct (PGL3 Basic containing the SV40 promoter) as a positive control. The three constructs were transfected into the OK-CaSR cell line and luciferase expression assay was done on the second day to measure the light signal from those constructs (i.e. the promoter activity). As shown in (**Figure 7)** there was no promoter activity detected with the 500 bp construct, but significant promoter activity was detected with the modified vector (PGL3 basic=SV40), which demonstrates that the PGL3 basic vector is able to detect promoter activity when a promoter sequence is ligated into it.

Subsequently, larger constructs, 700 bp, 1000 bp, 1200 bp, and 1340 bp 5' to the transcription start site were amplified by PCR and ligated into the same expression vector (PGL3 Basic), (**Figure 6)** and then transfected into the OK-CaSR cell line. The Dual luciferase assay was then performed the following day. The light signal generated by the 1500 bp construct was found to be significantly different from the negative control (PGL3 Basic alone). Moreover, no other construct showed significantly increased luciferase activity when compared to the negative control (**Figure 8)**.

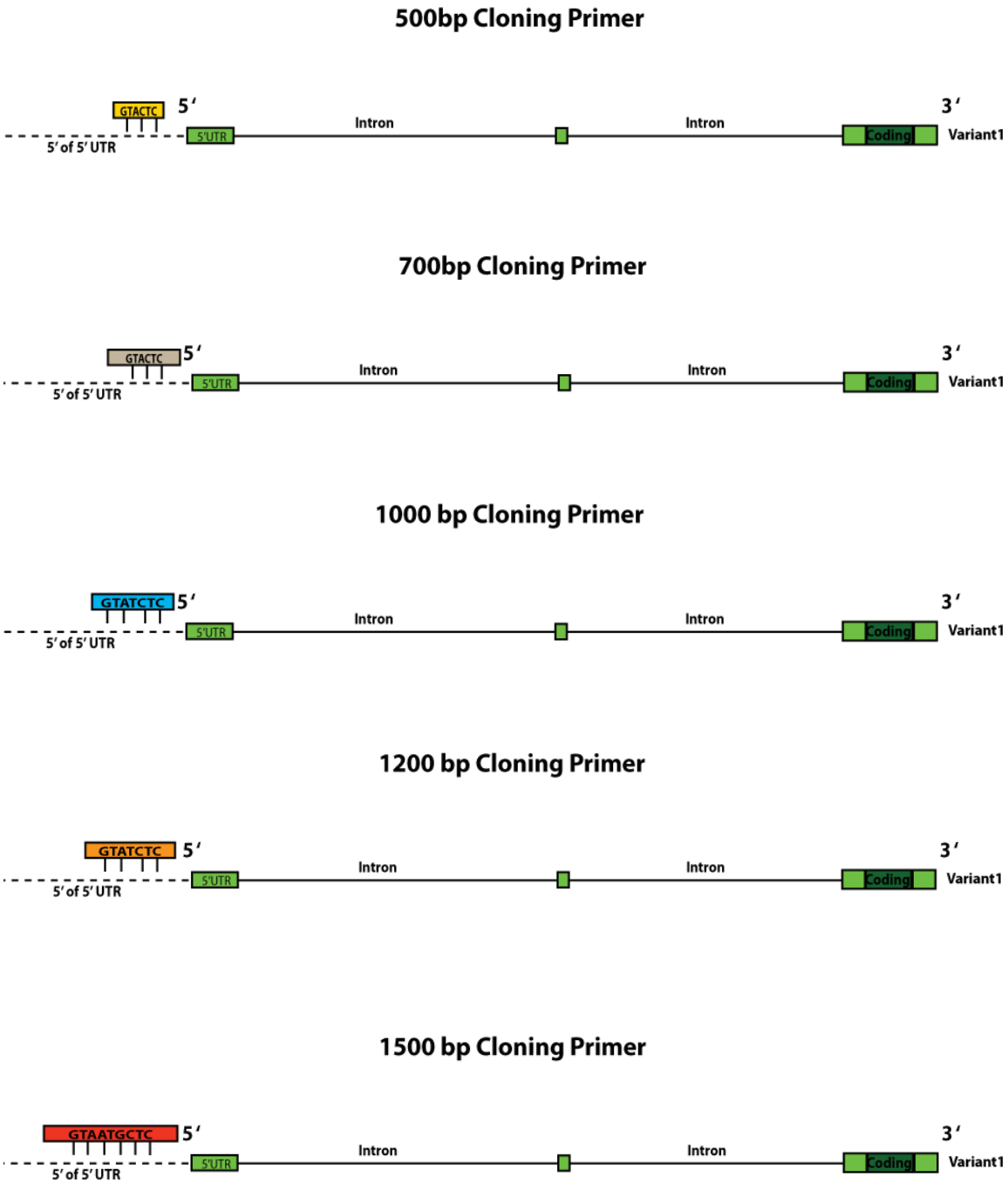

Figure 6. Schematic representation of mcldn14 V1 upstream cloned areas. Different lengths upstream of the putative transcription site of mcldn14 V1 were cloned into the PGL3 Basic vector. All 5 constructs share the same reverse primer, which sits just downstream of the putative transcription start site.

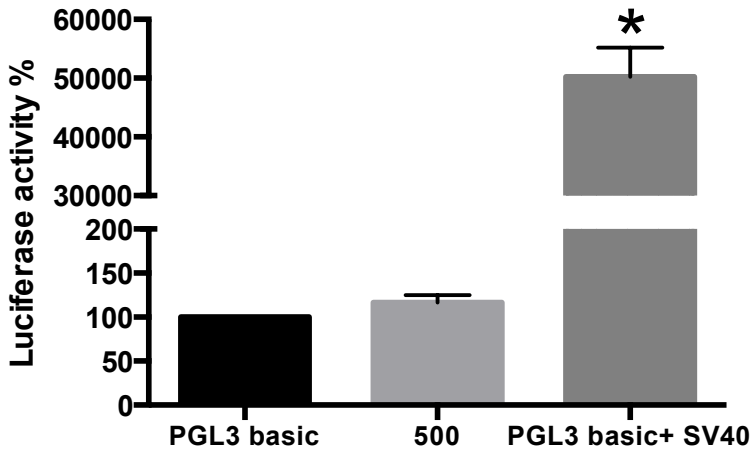

Figure 7. mcldn14 V1 500 Dual Luciferase assay. Analysis of luciferase signal produced by the 500 bp area upstream of the mcldn14 V1 gene cloned into the PGL3 Basic vector, which shows no significant increase in luciferase signal detected relative to the empty vector alone (PGL3 Basic, i.e. no promoter activity). However, the SV40 promoter when ligated into the PGL3 Basic vector, upstream of the firefly luciferase gene demonstrates that the PGL3 Basic vector is able to produce a luciferase signal when a promoter is ligated into it. Each of these constructs were transietly transfected with PRL-TK control vector into the OK-CaSR cell line. Firefly luciferase activity was measured and after normalization to Renilla luciferase activity, values were expressed as a percentage of the activity of the negative control, PGL3 Basic (set to a valued of 100%) (Mean ± SEM ; n=3). * Indicates a significant difference from the negative control ,PGL3 Basic: $p < 0.01$; one-way ANOVA with Gesser-Greenhouse correction.

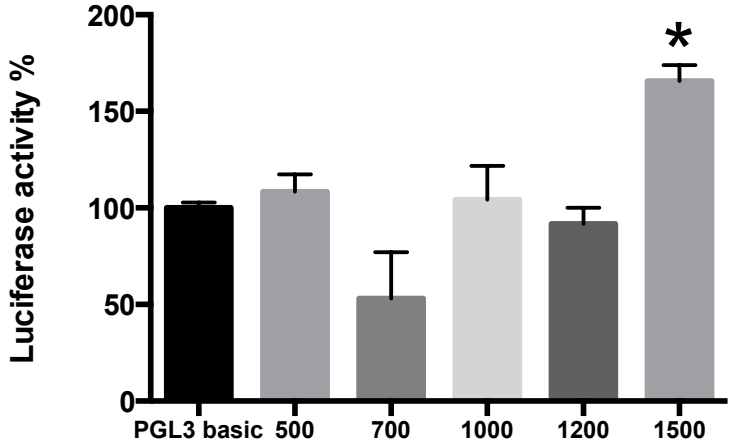

Figure 8. mcldn14 V1 1500 demonstrates a significantly increased luciferase (promoter) activity relative to empty vector. Analysis of the luciferase signal produced by different lengths upstream of the 5'UTR of mcldn14 V1 cloned into PGL3 Basic vector, upstream of the firefly luciferase gene. Each of these constructs was transiently transfected with the PRL-TK control vector into the OK-CaSR cell line. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, values were expressed as a percentage of the activity of the negative control, PGL3 Basic (valued as 100%). The mean ± SEM ; n=6 is displayed. * Indicates a significant difference from the negative control, PGL3 Basic: $p < 0.01$; a one-way ANOVA with Gesser-Greenhouse correction was used for comparison.

3.4 Activation of the CaSR using Cinacalcet HCl further increases the promoter activity of the 1500 bp construct

Expression of PGL3 Basic containing 1500 bp 5' to the 5' UTR of mcldn14 V1 produces greater luciferase activity than the negative control and the other shorter constructs. This is consistent with the promoter being present within this construct; however, whether this region contains an element sensitive to CaSR activation is not certain. To test whether this is the case, we transfected the 1500 bp containing construct into the OK-CaSR cell line and added 2 μM Cinacalcet HCl the day after transfection, then did the luciferase assay the following day (i.e. we assayed luciferase activity 48 hours after transfection). This experiment revealed that the luciferase signal from mcldn14 V1 1500 bp construct was significantly higher in the Cinacalcet HCl treated cells in comparison to non-treated cells (**Figure 9)**. Moreover, the addition of increasing doses of Cinacalcet HCl found a dose dependent increase in luciferase activity that was highest when 6 μM Cinacalcet HCl was administered (**Figure 10)**.

Next we sought to assess the time it took for Cinacalcet HCl to increase luciferase expression. We observed a slight, but statistically insignificant increase by 12 hours after adding Cinacaclet HCl, consistent with transcriptional activation of the regulatory region cloned in the PGL3 Basic vector **(Figure 11).** Despite there being a further increase in luciferase signal at 24 hours post incubation with Cinacalcet HCl, this difference did not reach statistical significance, (**Figure 11)**, which is in contrast to what we found in (**Figure 9)**. However, only one set of luciferase assays were performed for the timeline experiment, which may explain these non-significant results.

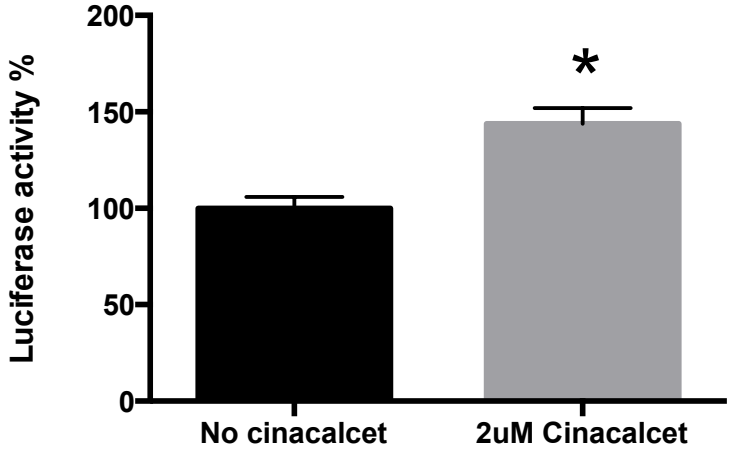

Figure 9. Cinacalcet HCl further increases mcldn14 V1 1500 luciferase activity. Analysis of the luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mcldn14 V1 cloned into PGL3 Basic vector treated with 2 μM Cinacalcet HCl. This construct was transiently transfected with the PRL-TK control vector into the OK-CaSR cell line. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, values are expressed as a percentage of the activity of the 1500 bp mcldn14 expression in the absence of Cinacalcet HCl treatment (this is set to 100%). Results are displayed as the mean ± SEM ; n=5. * Indicates a significant difference from the negative control (no treatment) :$p < 0.0025$; comparison made with a two-sample equal variance ttest with two-tailed distribution.

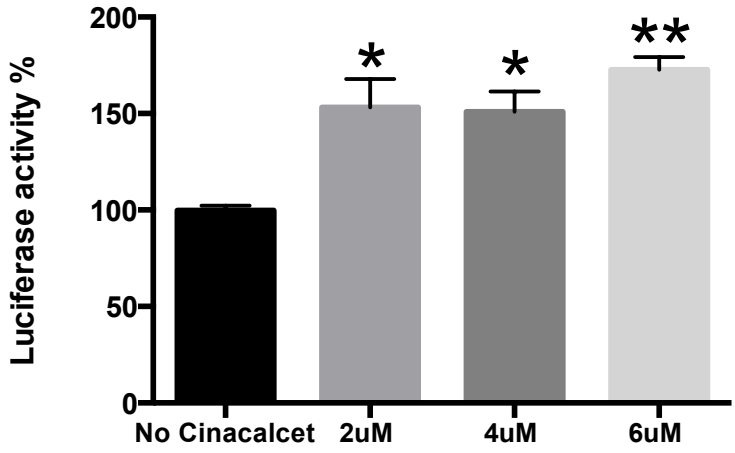

Figure 10. mcldn14 V1 1500 luciferase signal is Cinacalcet HCl dose dependent. Analysis of the luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mcldn14 V1 cloned into PGL3 Basic vector and treated with different cinacalcet doses. The construct was transiently transfected with the PRL-TK control vector into the OK-CaSR cell line. Firefly and luciferase activity were measured and normalized to Renilla luciferase activity. The data is expressed as a percentage of the activity of the 1500 bp mcldn14 V1 activity without Cinacalcet HCl treatment which was set to 100%. Displayed is the Mean ± SEM ; n=3. * Indicates a significant difference from the negative control; one-way ANOVA with Gesser-Greenhouse correction was performed for statistical analysis.

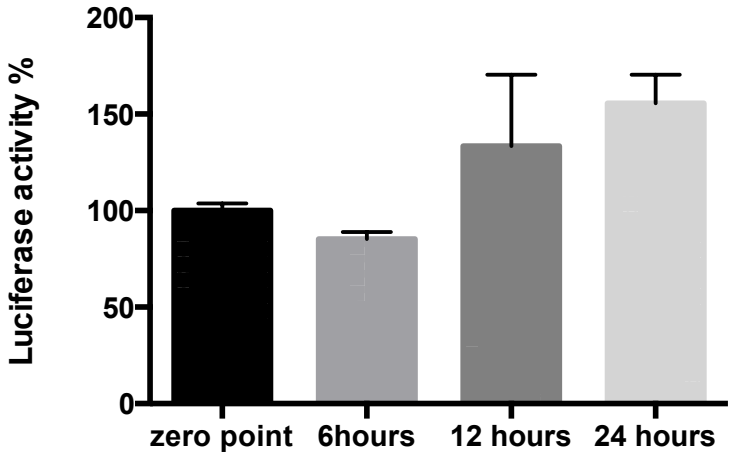

Figure 11. mcldn14 1500 luciferase signal at different time points after treatment with Cinacalcet HCl. Analysis of luciferase signal produced by the 1500 bp upstream fragment of the 5'UTR of the mcldn14 V1 cloned into the PGL3 Basic vector and treated with 2 μM Cinacalcet HCl for different periods of time. The construct was transiently transfected with PRL-TK control vector into the OK-CaSR cell line. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The data is expressed as a percentage of the activity of the 1500 bp mcldn14 expression without Cinacalcet HCl treatment, which is set to 100%. Data are expressed as the Mean ± SEM ; n=1. No significant difference from the negative control was found at any timepoint. A one-way ANOVA with Gesser-Greenhouse correction was performed to analyze the data.

3.5 Cloning 5' of the 5' UTR of mcldn-14 V1 into PGL3 enhancer

Next we subcloned the 1500 bp upstream fragment of the 5'UTR of the mcldn14 V1 (used for experiments displayed in Figures 7-10) into the PGL3 Enhancer vector. This vector has the same backbone as PGL3 Basic but also contains an SV40 enhancer downstream of the *luciferase gene*. We did this in order to confirm our observation that the mcldn14 V1 1500 bp fragment 5' of the 5'UTR has promoter activity and that the observed luciferase expression was not due to contamination or cryptogenic sequences within the vector that may give false positive results. We also felt that the addition of a promoter would provide greater expression than in the PLG3 Basic vector, as a repressor in addition to the promoter may be present. In other words, having a SV40 enhancer in the vector may cancel any effect of a repressor sequence present within the 1500 bp fragment. Experiments performed with the enhancer construct demonstrate that the mcldn14 V1 1500 bp fragment ligated into the PGL3 Enhancer vector shows a significant luciferase signal in comparison to the negative control, which was the PGL3 Enhancer vector alone (**Figure 12).** Interestingly, the magnitude of the mcldn14 V1 1500 bp fragment ligated into the PGL3 Enhancer vector is around 2.5x higher than the negative control (**Figure 12)** in comparison to 1.6 fold higher in the PGL3 Basic vector (**Figure 8)**.

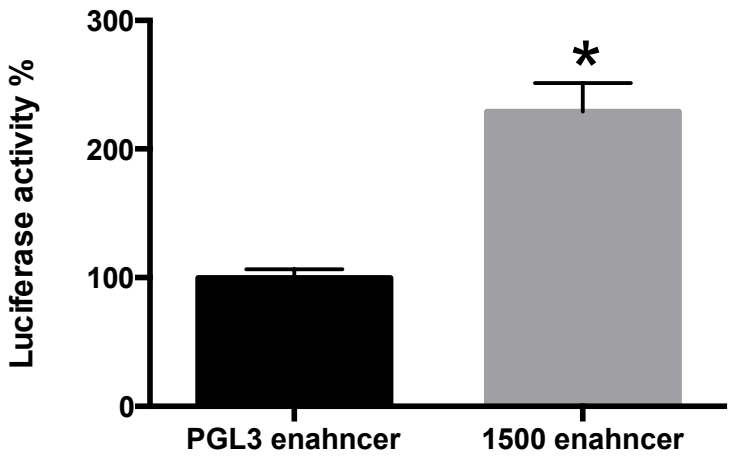

Figure 12. The mcldn14 V1 1500 bp fragment 5' of the 5' UTR ligated into the PGL3 Enhancer vector shows significant activity. Analysis of luciferase expression produced by the 1500 bp fragment upstream of the 5'UTR of mcldn14 V1 cloned into the PGL3 Enhancer vector. The construct was transiently transfected with the PRL-TK control vector into the OK-CaSR cell line. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The data are expressed as a percentage of the activity of the negative control, the PGL3 Enhancer vector, which was set to 100%. The data represent the mean ± SEM; n=6. * Indicates a significant difference from the negative control, PGL3 enhancer: $p < 0.0001$; a Two-sample equal variance ttest with two-tailed distribution was used for comparison.

3.6 Experiments in a cell line lacking the CaSR, HEK-293 cells

Next we repeated some key experiments in a cell line lacking the CaSR, HEK-293 cells (Kifor et al. 2004) . In this cell line we would expect that adding Cinacaclet HCl would not increase the luciferase signal in comparison to a non-Cinacalcet HCl treated group. First, we transfected either empty vector or the PGL3 Enhancer vector containing the 1500 bp fragment into HEK-293 cells and found significantly increased luciferase activity (**Figure 13)**.

Then we transfected the mcldn14 V1 1500 bp fragment in the PGL3 basic vector and treated the cells with either 2 μM cinacalcet on the second day or vehicle and then performed the dual luciferase assay on the third day. As expected there was no significant difference in the signal between the Cinacalcet HCl treated and non-Cinacalcet treated cells (**Figure 14).** Since the HEK-293 cell line lacks the CaSR (Kifor et al. 2004) , we attempted to complement its expression by transfecting the CaSR in the presence of the mcldn14 V1 1500 bp containing the PGL3 Basic vector and the PRL-TK vector. These experiments were performed in the presence or absence of Cinacalcet HCl. We found that the triple transfection with the addition of 2μM Cinacalcet HCl increased luciferase expression in comparison to the non-cinacalcet treated cells or the double transfection group; however, this was not significant ($p = 0.11$) (**Figure 15)**.

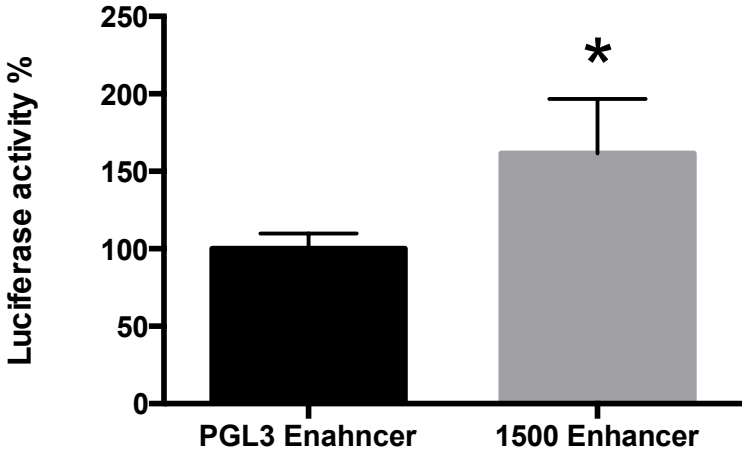

Figure 13. mcldn14 V1 1500 bp fragment ligated in the PGL3 Enhancer vector shows activity in the HEK-293 cell line. Analysis of the luciferase signal produced by the 1500 bp fragment 5' to the 5'UTR of mcldn14 V1 cloned into the PGL3 Enhancer vector. The construct was transiently transfected with the PRL-TK control vector into HEK-293 cells. Firefly luciferase activity were measured and normalized to Renilla luciferase activity. The data are expressed as a percentage of the activity of the negative control, PGL3 enhancer, which was set to 100%. The data are expressed as the mean ± SEM; n=4. * Indicates a significant difference from the negative control PGL3 Enhancer: $p < 0.015$; a two-sample equal variance ttest with two-tailed distribution was used for analysis.

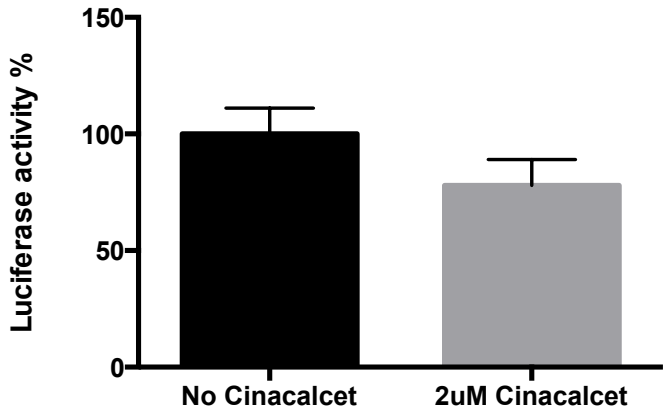

Figure 14. The addition of Cinacalcet HCl did not increase the luciferase activity of mcldn14 V1 1500 bp fragment when expressed in HEK-293 cells. Analysis of luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mcldn14 V1 cloned into the PGL3 basic vector. The construct was transiently transfected with PRL-TK control vector into HEK-293 cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The data are expressed as a percentage of the activity of the negative control, mcldn14 V1 1500 bp fragment in the PGL3 Basic vector without Cinacalcet HCl treatment, which was set to 100%. The data are presented as the Mean ± SEM; n=2, no significant difference was detected: P = 0.29; a two-sample equal variance ttest with two-tailed distribution was used for analysis.

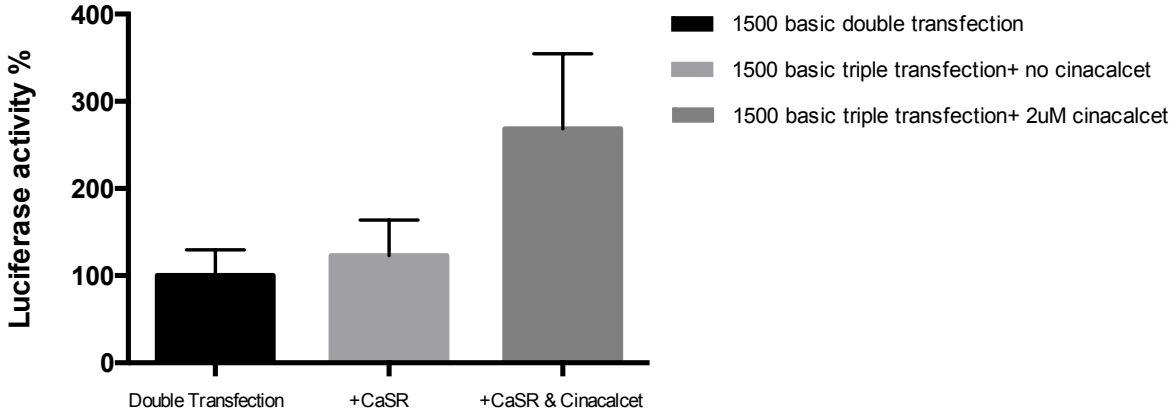

Figure 15. The effect of CaSR expression on the sensitivity of the 1500 bp fragment to cinacalcet. Analysis of luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mcldn14 V1 cloned into PGL3 Basic Vector. The construct was transiently transfected with the PRL-TK control vector into HEK293 cell line (double transfection) and with both the PRL-TK and CaSR (triple transfection). For the groups with the triple transfection, the cells were treated with either Cinacalcet HCl or the vehicle. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The data are expressed as a percentage of the activity of the negative control, mcldn14 V1 1500 double transfection. The data displayed are the mean ± SEM; n=6. * No significant difference between the groups was observed when analyzed by a one-way ANOVA.

CHAPTER 4

DISCUSSION

The objective of this study was to identify and clone the claudin-14 promoter of the CaSR responsive variant. It was our hope that this tool could be used to delineate the signaling pathway between CaSR activation and claudin-14 expression. In 2009, a Genomic Wide Association Study (GWAS) found that the claudin-14 gene associates with kidney stone formation. Interestingly the authors observed that the patients with the SNP, which most strongly associated with the development of kidney stones also display hypercalciuria (Thorleifsson et al. 2009). These studies strongly implicated claudin-14 in the pathogenesis of kidney stone formation. However the mechanisms by which this was occurring was not clear, as patients with null mutations in claudin-14 were deaf, as are mice genetically altered to lack claudin-14.

A number of groups set out to explore the role of claudin-14 in calcium homeostasis including the Alexander laboratory. Subsequently, clauidn-14 was identified as a tight junction protein present in the thick ascending limb of the loop of Henle (Gong et al. 2012). More recently, it was shown that activation of the CaSR with calcium or calcimimetics like Cinacalcet HCl increases the expression of clauidin-14 at the tight junction in the TAL. This localization was mediated through a physical interaction with clauidn-16 and -19. The function of claudin-14 was also explored. It was found to preferentially block paracellular cation movement and in particular the flow of calcium from the urine back into the blood. Consequently, increased claudin-14 expression via activation of the CaSR will increase the amount of calcium in the urine. Importantly, inappropriately increased claudin-14 expression in the kidney would cause hypercalciuria predisposing the patient to develop kidney stones

Identifying the molecular signaling between CaSR activation and increased claudin-14 expression will be the next challenge. (Gong et al. 2012) proposed that this regulation occurs at the level of micro-RNA, where miR-9 and miR-374 target the 3'UTR of the claudin-14 mRNA causing it to decay thereby preventing its translation. Consistent with this, when the CaSR is activated by Ca^{+2}, the level of miR-9 and miR-374 decrease, thereby allowing claudin-14 translation (Gong et al. 2012). Moreover, the same group also showed that treatment of mice with histone deacetylase (HDAC) inhibitors decreased the renal expression of claudin-14 mRNA and urinary calcium excretion in mice. They proposed that treatment of mice with HDAC inhibitors increased transcription of renal microRNA-9 (miR-9) and miR-374, which repressed CLDN14 mRNA expression allowing the paracellular reabsorption of calcium from urine back to blood.

In contrast to these observations, (Toka et al. 2012) found that the renal miR-9 and miR-374 expression level was not significantly different between CaSR deficient mice and control animals, when both where supplemented with calcium. This group went so far as to say that it is possible that microRNA doesn't play a role in claudin-14 regulation. Furthermore, a microarray performed by the Alexander laboratory on mRNA isolated from the kidney of mice treated with Cinacalcet HCl or vehicle did not demonstrate altered mir-9 expression. And perhaps most importantly, the SNPs identified in the GWAS study which showed an association between claudin-14 and kidney stones did not alter the micro RNA binding sites in the 3' untranslated region of claudin-14. Therefore the relevance of micro RNA down-regulation of claudin-14 to hypercalciuria and kidney stones is doubtful. Consequently we speculated that there might be another mechanism regulating claudin-14 expression. This led us to hypothesize that claudin-14

is regulated like most other genes, at the level of its promoter; however, this doesn't rule out the existence of other regulation mechanism.

Here we have shown that Cinacalcet HCl increases the expression of claudin-14 through activation of the CaSR. Furthermore, out of the three variants of mouse CLDN14 (mcldn14), it is mcldn14 V1 that's is regulated by the CaSR. The next step was to identify the mcldn14 V1 promoter. DNA regulatory sequences control the level of expression through interaction with *trans*-acting proteins (DNA binding proteins). The DNA binding site for these proteins can vary from a few base pairs to hundreds of base pairs. DNA regulatory sequences can be a promoter, enhancer, silencer, insulator or boundary element matrix attachment region (MAR) (Zhang, Liu & Liang 2003) . These regulatory sequences are generally located within a few kilobases of the exon or within an intronic region of the gene they control. However, other regulatory sequences like enhancers can control the gene from a longer distance. Thus, identifying these sequences is crucial to understanding gene regulation. Therefore strategies to find these sequences are needed. With the sequencing project success, methods such as ChIP-chip (chromatin immunoprecipitation-DNA microarray) can be efficiently used to map global binding sites for *trans*-acting elements (transcription factors) on a genomic-wide scale *in vivo*. This approach in combination with sophisticated software analysis may help to identify regulatory sequences (Wang et al. 2007)

The first strategy we employed to find the promoter was to do *in silico* studies whereby we scanned DNA sequences from different species including: human, gorilla, chimpanzee, rat and dog with the upstream area of the 5'UTR of mouse CLDN14 V1. The process predicts regulatory regions by looking for a conserved sequence between

species; in addition, we used softberry and eukaryotic promoter database as software analysis tools that predicts *cis*-regulatory elements based on transcription factor binding sites. These combined approaches predicted that the promoter sequence exists in the first 500 bp upstream of mcldn14 V1 5'UTR. However, cloning 500 bp 5' of the 5' UTR into the PGL3 Basic vector and transfecting it into OK-CaSR cells (opossum kidney proximal tubule cells that overexpress the CaSR) didn't show significantly increased luciferase activity, suggesting that the promoter is not contained within this 500 bp fragment.

We considered the possibility that our vector was corrupted in such a way that prevents it from reporting firefly luciferase activity. To address this possibility, a strong promoter element from SV40 was subcloned into the same vector and transfected into the same cell line. This experiment demonstrated significant luciferase signal. This experiment confirmed that our PGL3 Basic vector is able to express luciferase when a promoter sequence is present upstream of the luciferase gene. Since we demonstrated that our vector is functioning, we assumed that this sequence did not contain the promoter. We therefore proceeded to clone further upstream of the 5' UTR. We found that a fragment of approximately 1500 bp (exactly 1342bp) upstream of mcldn14 V1 displayed significantly more luciferase activity compared to the negative control, which was the empty vector (PGL3 Basic). However when we cloned shorter fragments of mcldn14 V1 5' to the 5' UTR, 1200 (exactly 1243bp), 1000 (1044bp) and 750 (747bp) there was no significant increase in luciferase signal detected, compared to the control empty vector. The fact that a luciferase signal was detected with the 1500 bp construct that was not present in the smaller fragments infers the promoter lies between 1340 bp and 1243 bp upstream of the 5'UTR of mcldn14 V1.

We next explored whether this 1500 bp fragment was regulated by the CaSR, since our laboratory has shown that activation of the CaSR increases the expression of claudin-14 in the tight junction of the TAL (Dimke et al. 2013a) . We sought to use an increase in the luciferase signal produced by the promoter construct mcldn14 V1 1500 as a readout of signalling mediated by CaSR activation. Therefore, this construct was transfected into the OK-CaSR cell line, treated with 2μM Cinacalcet HCl in the 2nd day post transfection for 18-24 hours and assayed the following day. This experiment demonstrated an increase in luciferase signal in the Cinacalcet HCl treated group relative to the control group (mcldn14 V1 1500 without Cinacalcet HCl treatment).

The data presented herein demonstrates that the promoter is within the mcldn14 V1 1500 construct. In line with that, it responds to CaSR activation with Cinacalcet HCl. However, the signal is relatively low when compared to the luciferase signal detected with the modified SV40 containing vector. The lack of a very large increase in luciferase expression in the 1500 bp construct relative to the SV40 (Simian vacuolating virus 40**)** containing construct is not surprising since the later promoter is from a DNA virus that has the potential to induce tumors, i.e a very strong promoter! Furthermore, there might be more than one regulation mechanism for claudin-14 or a repressor within the mcldn14 V11500 bp construct as well. This is why we decided to clone the same sequence into the PGL3 Enhancer vector, which has almost the same backbone as the PGL3 Basic vector but also contains an SV40 promoter downstream of the luciferase gene and the poly (A) signal. This may cancel any repressor effect if it is present thereby providing us with the relative promoter activity. This experiment shows a significantly greater luciferase signal. Furthermore this construct also responds to CaSR activation with 2 μM Cinacalcet HCl.

Using another cell line, HEK-293 cells, which lack the CaSR, we expressed the mcldn14 V1 1500 enhancer construct. We found a significant luciferase signal relative to the negative control. Moreover, when the mcldn14 1500 bp fragment in the PGL3 Basic vector was transfected into HEK-293 and treated with Ciancalcet HCl there was no significant difference from the control group, demonstrating that the lack of CaSR in this cell line prevents increased expression. However the addition of the CaSR back into the HEK cells was unable to reconstitute the system.

Conclusion:

The mouse claudin-14 gene promoter for variant 1 lies between 1200 (*exact length = 1243 bp*) and 1500 (*exact length = 1340 bp*) 5' upstream of the 5'UTR. Moreover, the 1500 bp 5' to the 5' UTR of mouse claudin-14 variant 1 is sensitive to CaSR activation.

Future Direction

The next step, after doing more dual luciferase assays using these constructs in the presence and absence of cinacalcet in both cell lines, OK-CaSR and HEK-293 cells, to make all N > 3 in the figures generated thus far, is to identify the signaling pathway involved after activation of the CaSR. To that end, the Alexander laboratory performed a microarray on mRNA isolated from kidneys of mice treated with vehicle or the calcium sensing receptor agonist Cinacalcet HCl. They observed significantly increased expression of Mitogen-Activated Protein Kinase Kinase Kinase 6 (MAPKKK6). This kinase is part of the ERK/MAP kinase pathway and could potentially activate the transcription factor SP1. SP1 mediates the increased expression of other molecules post CaSR activation (Gill et al. 1994, Li et al. 1991, Pugh, Tjian 1991, Su et al. 1991). Given this, we propose that CaSR activation will activate MAPKKK6 and then SP1 to increase claudin-14 expression. To test this we will both over express and knock-down these molecules in the cell culture model developed here-in, and repeat the luciferase activity assay in the presence of CaSR activation. We predict attenuated activity when these molecules are knocked down and augmented activity in their presence. Indeed it is likely that over expression of these signaling molecules may increase expression independently of the addition of Cinacalcet HCl (**Figure 16).**

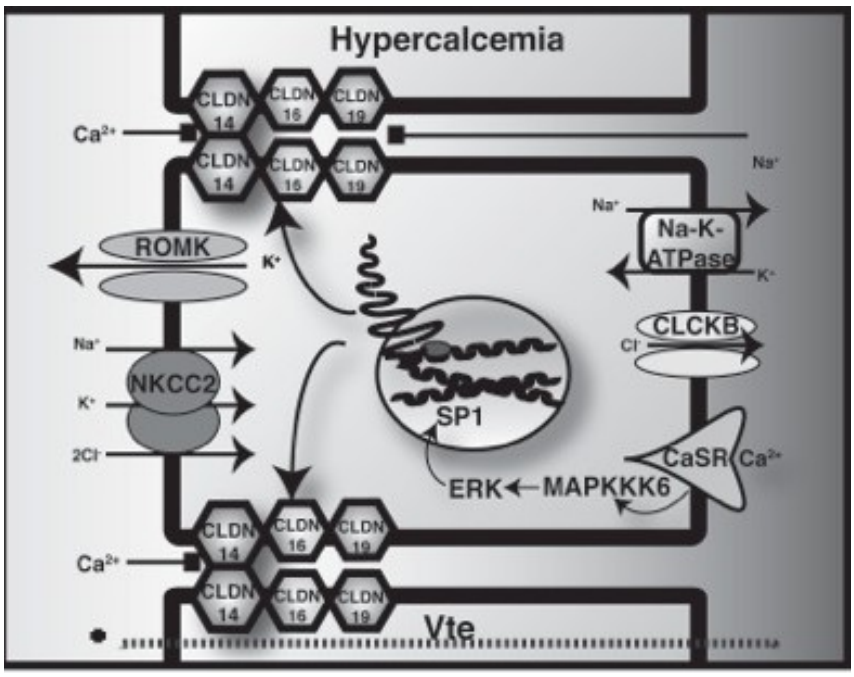

Figure 16. The Proposed CaSR-CLDN14 signaling pathway

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