FKBP12 ALONG THE NEPHRON IS ESSENTIAL FOR TACROLIMUS-INDUCED HYPERTENSION AND ELECTROLYTE IMBALANCE

By

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List of Abbreviations

$BaCl_2$	Barium Chloride
Ca^{2+}	Calcium
CD/CT	Collecting Duct/Connecting Tubule
CO	Cardiac Output
CaN	Calcineurin
DCT	Distal Convoluted Tubule
ENaC	Epithelial Na^+ Channel
FHHt	Familial Hyperkalemic Hypertension
FK-506	Tacrolimus
FKBP12	FK-506 Binding Protein, 12 kDa
Glom	Glomerulus
K^+	Potassium
MAP	Mean Arterial Pressure
Mg^{2+}	Magnesium
Na^+	Sodium
NCC	Sodium Chloride Cotransporter

NFAT	Nuclear Factor of Activated T-Cells
OxSR1	Oxidative Stress Responsive 1
SPAK	STE20/SPS1-related proline/alanine-rich kinase
ROMK	Renal Outer Medullary Potassium Channel
TAL	Thick Ascending Limb
TRPV5	Transient Receptor Potential cation channel, subfamily V, Member 5
TRPM6	Transient Receptor Potential cation channel, subfamily M, Member 6
WNK	With-No-Lysine (family of kinases)

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Graduate school has been a transformative journey for me. Along the way I have gained confidence and become more comfortable with the uncertainty inherent in both science and life. While there are many people who have helped me along this path there are a few that have been so critical to my successes in science and development as a person that I would like to mention them here.

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Abstract

Tacrolimus (FK-506) is an immunosuppressive drug often used to prevent graft rejection in organ transplant patients. It inhibits the phosphatase calcineurin, when bound to an endogenous protein FKBP12. In T-cells this leads to immunosuppression. While effective, tacrolimus also causes side effects including hypertension, diurnal dipping disruption, hyperkalemia, hypomagnesemia and hypercalciuria. In an effort to develop safer immunosuppressive drugs and more effectively treat patients, unraveling these pathological mechanisms has become a priority.

Tacrolimus leads to hypertension by pleiotropic effects in the vasculature, central nervous system, renin-angiotensin/aldosterone axis and the kidney. We recently found, however, that the renal sodium chloride cotransporter (NCC) is necessary for the development of tacrolimus-induced hypertension. Furthermore, tacrolimus increases NCC phosphorylation, a mark of activity. The essential nature of NCC in this multi-faceted pathology, suggests that understanding, and avoiding, NCC activation may be the key to developing safer immunosuppressive drugs. It is currently unclear, however, whether tacrolimus is activating NCC directly, or by affecting extra- or intra-renal targets upstream of NCC activation. It is also unknown whether calcineurin or FKBP12 inhibition is involved.

I hypothesize that calcineurin is responsible for dephosphorylating NCC. Thus, its inhibition in the DCT leads to an increase in NCC phosphorylation, which is essential for the development of tacrolimus-induced hypertension and electrolyte disorders. To test this I generated a mouse model in which FKBP12, the necessary binding partner of tacrolimus, can be inducibly deleted along the nephron (KS-FKBP12$^{-/-}$). I tested *in vivo* 1) whether FKBP12 disruption along the nephron contributes to NCC phosphorylation, hypertension or electrolyte

disorders and 2) whether protecting calcineurin from inhibition along the nephron is *sufficient* to ameliorate these side effects.

I found that KS-FKBP12$^{-/-}$ mice were phenotypically normal at baseline, suggesting that FKBP12 disruption along the nephron does not underlie tacrolimus-induced hypertension or electrolyte disorders. This also implies that immunophilin-sparing calcineurin inhibitors will not alleviate the renal-component of these side effects.

Conversely, KS-FKBP12$^{-/-}$ mice treated with tacrolimus had lower blood pressures and more effectively recovered their diurnal dipping patterns than tacrolimus-treated control mice. They also had lower levels of phosphorylated NCC (pNCC), maintained a normal pNCC: plasma [K^+] relationship (suggesting protection from K^+ dysregulation), and were protected from hypomagnesemia and hypercalciuria. Additionally, *in vitro* I found that tacrolimus inhibits NCC dephosphorylation. Taken together, this suggests that calcineurin plays a role in NCC dephosphorylation and that its inhibition in DCT cells leads to an increase in NCC activity, hypertension and hyperkalemia. An FKBP12-dependent event along the nephron, such as calcineurin inhibition, also causes hypomagnesemia, likely by reducing TRPM6 mRNA abundance, and hypercalciuria by reducing calcium transport across the DCT.

Collectively this supports the idea that tacrolimus-induced dysregulation of the DCT contributes to a host of pathologies including an increase in NCC activity. Calcineurin inhibitors which can't access the nephron may be substantially safer therapeutics. Until then, thiazide diuretics, which directly inhibit NCC activity, will likely be particularly effective at treating hypertensive or hyperkalemic tacrolimus patients.

Chapter 1 -Introduction

Organ Transplantation

On December 23, 1954, Dr. Joseph Murray and Dr. David Hume performed the first successful human solid-organ transplant.[1] A kidney donated by 23 year-old Ronald Herrick (who unprecedentedly was still alive at the time) was grafted into his dying identical twin brother, Richard. The surgery was a success. Richard returned to health and went on to live for 8 more years, ultimately dying of a heart attack; a fate unrelated to his organ transplant.[2] His brother, Ronald, went on to live another 56 years. He ultimately died at the age of 79 from complications following heart surgery.[3]

Figure 1.1 Photograph of Richard and Ronald Herrick.

Richard (left) and Ronald Herrick one year after organ transplantation. Picture courtesy of the Boston Globe.[3]

This was a landmark case in many ways, earning Murray the 1990 Nobel Prize in Medicine.[†] This success established the feasibility of long term-graft survival in humans and opened a new chapter in biomedical ethics. Importantly though, this case underscored one of the biggest hurdles to successful organ transplantation; preventing graft rejection by the recipient's immune system. A complication neatly side stepped in the case of identical twins.

In the wake of this surgery, attempts to artificially suppress organ recipient's immune systems and make organ transplantation possible for the masses were attacked with renewed vigor. Initial trials with whole-body irradiation[4,5] ultimately gave way to the development of one of the first immunosuppressive drugs, azathioprine (trade name Imuran). Though successful in dogs, azathioprine proved to be toxic to humans at the doses required for the immunosuppression levels seen in canines.[6] Lower doses were used with some success,[7] but attention turned towards finding a new pharmacologic solution to the immunosuppression problem.

Calcineurin Inhibitors

Cyclosporine

In 1970 a major breakthrough came with the discovery of cyclosporine,[8] the first of the class of drugs now known as calcineurin inhibitors. Cyclosporine, a natural byproduct of the soil fungus *Tolypocladium inflatum* was discovered by Sandoz pharmaceuticals (now Novartis) during an immunosuppressive drug screen.[8]

[†] Nobel Prizes are only awarded to the living. Hume, Murray's fellow surgeon, had died in an airplane accident by the time their work was recognized. Merrill, the lead investigator on the project and in many ways a founding father of nephrology, had also died in a boating accident.[4]

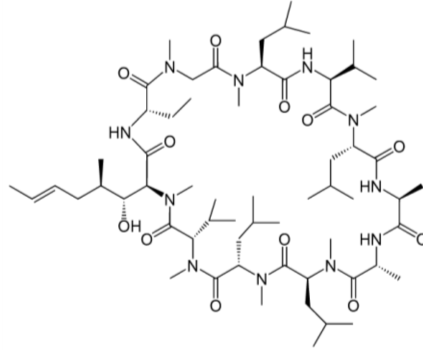

Figure 1.2-Molecular structure of cyclosporine.

Image courtesy of Wikimedia.[9]

Initial reports that cyclosporine was an effective immunosuppressant in animals[10] gave way to clinical use. Cyclosporine was extremely effective. One year graft survival soared from 47% on azathioprine (Imuran) to 87% with cyclosporine,[11] making organ transplantation a viable option.

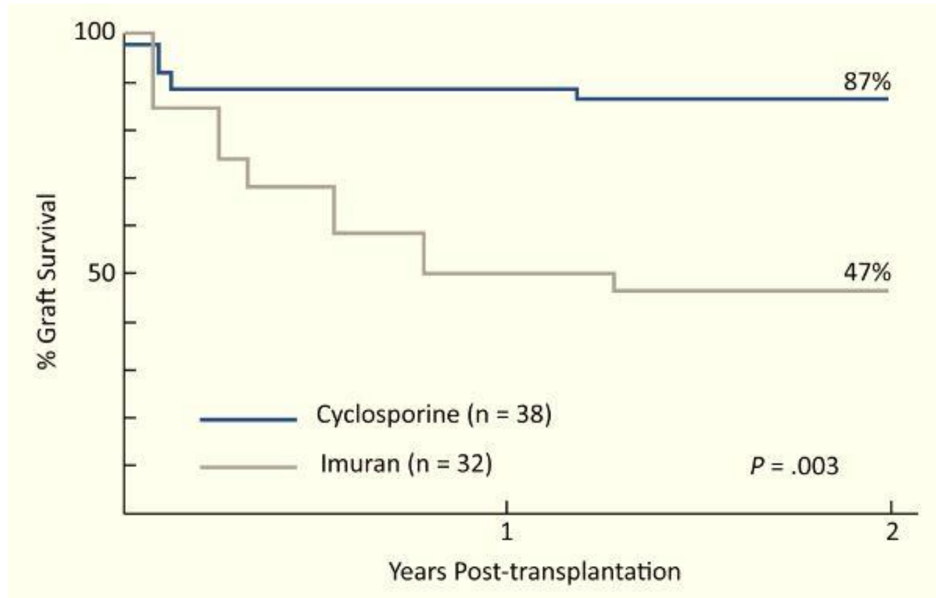

Figure 1.3 Cyclosporine vs Imuran kidney allograft survival rates.

Kidney allograft survival rates for all primary deceased-donor transplants performed in 1981 with at least 15 months of follow-up.[11]

Unfortunately, cyclosporine also has some serious side effects including nephrotoxicity, hypertension and electrolyte imbalance. Though using lower doses of cyclosporine in combination with steroids helped[11,12] the need for a less toxic immunosuppressive was apparent.

Tacrolimus

Recognizing this, Fujisawa pharmaceuticals began screening compounds for a more potent immunosuppressant. In 1987 and 1988 they published a slew of reports describing FK-506, a compound isolated from the bacteria *Streptomyces tsukubaensis*, native to the soil of Japan.[13-18]

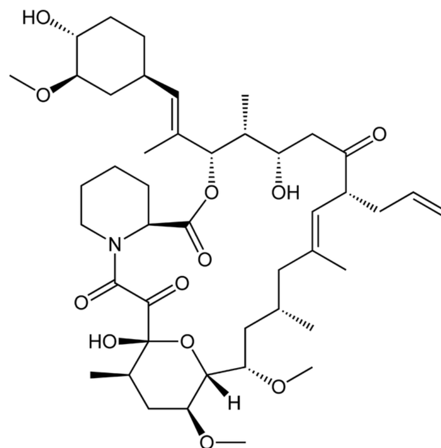

Figure 1.4-Molecular structure of tacrolimus (FK-506).

Image courtesy of Wikimedia.[19]

This lactone macrolide is now also known as tacrolimus and under the trade name Prograf. Tacrolimus turned out to be 10x more potent than cyclosporine,[20] generating excitement that off-target effects would be minimized while maintaining adequate immunosuppression. Tacrolimus did prove to be somewhat more effective than cyclosporine,[21] and today, in combination with

other immunosuppressive agents and steroids it is one of the most commonly prescribed chronic immunosuppressive therapies.[22]

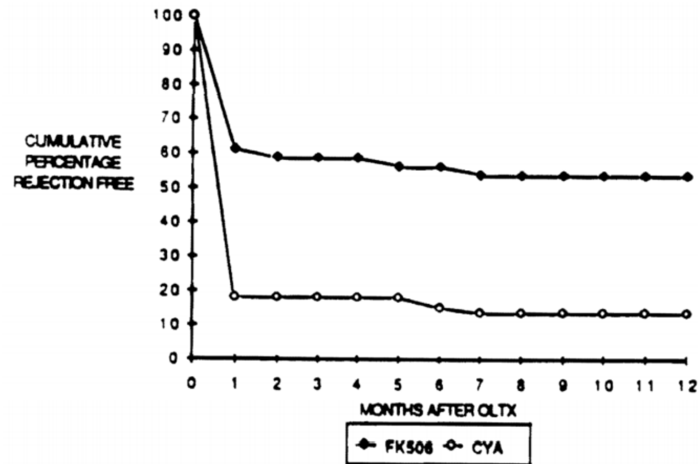

Figure 1.5 Tacrolimus vs. cyclosporine liver rejection rates.

Percentage of rejection free liver transplant patients in the 1990 tacrolimus vs. cyclosporine clinical trials plotted against months post-tranplantation.[21]

Tacrolimus is recommended by the National Kidney Foundation as a first line immunosuppressive treatment for organ transplant patients.[22] As of 2006 79% of transplant patients were initially treated with tacrolimus and 99% take some type of calcineurin inhibitor one year post-transplant.[22] Therefore, understanding tacrolimus mediated pathologies and developing ways to ameliorate or avoid them is extremely clinically relevant.

Demand for Immunosuppressive Drugs

Immunosuppressive drugs, such as tacrolimus, are a large part of the reason that today patient survival rates one year after organ transplantation are around 95% and one year graft-survival rates are over 90% globally.[23] Organ transplantation now occurs in 80 countries,[23] has led to the

creation of organ donation programs and has become the preferred method for treating end-stage renal disease.[24] When compared to dialysis, kidney transplantation has been shown to improve the quality of life, and lengthen it, reducing long term mortality by 68%.[25] For 20-39 year old patients, this means adding an estimated 17 years with loved ones.[25] In addition, kidney transplantation leads to an estimated savings of $200,000/year/transplant in Medicare dollars in the United States.[26] Thus, the importance of tools such as tacrolimus which make these operations a success cannot be understated.

Though experimental 50 years ago, organ transplantation has evolved from transplantation of kidneys between living donors, to include the use of organs from deceased donors, and tissues ranging from livers, hearts and lungs to faces and limbs. In addition to organ transplant patients, tacrolimus is also being used to treat chronic autoimmune diseases such as systemic lupus erythematosus.[27] It has been proposed as a preventive measure for atherosclerotic patients at risk for myocardial infarctions and stroke[28] and may improve outcomes after acute myocardial infarctions.[29-31] As the scope of transplantation and tacrolimus use expands, so do the number of patients living under chronic immunosuppression.

Yet, tacrolimus also causes several serious side effects. Ironically, given the number of kidney transplant patients on tacrolimus, these include chronic kidney disease,[32] as well as hypertension,[33] disruption of diurnal blood pressure dipping,[34] and electrolyte disorders such as hyperkalemia,[33] hypercalciuria[35] and hypomagnesemia.[36] These problems are compounded as, unlike antibiotics, tacrolimus is often used for the remainder of a patients' life.

As the organ transplant patient population grows and new uses for tacrolimus are discovered the need to more effectively treat these side effects and ultimately design safer therapeutics is

pressing. For that we need to understand the pathological mechanisms that underlie tacrolimus-induced toxicities, which is the focus of this thesis.

Tacrolimus side effects

Hypertension

Hypertension, dubbed "The Silent Killer", is a well-recognized risk-factor for premature mortality today. It is the leading contributing factor to premature death worldwide,[37] a risk factor for cardiovascular disease and stroke,[38] and cost an estimated 73.4 billion US dollars in 2009.[38] Only 100 years ago though, it was mired in controversy, and with limited therapeutics available, treating-hypertension was considered a risk in itself.[39]

The first to come down hard on the deleterious consequences of high blood pressure were the life insurance agents.[39] The numbers most often associated with hypertension, 140/90, are derived from their actuarial tables.[39] In the early 1900s they recognized that the risk of death, and a claims payout, became unacceptably high over those values and began requiring blood pressure measurements with life insurance applications.[39]

At the time there were not a lot of treatments for hypertension, but by the mid 1950's safe and effective antihypertensive drugs, including thiazide diuretics,[40] were developed. With tools in hand Ed Freis led the VA cooperative clinical trials to determine whether treating people with hypertension was beneficial.[41,42] The results were so overwhelmingly positive, that his initial study was stopped early because it was deemed unethical to withhold drugs from the placebo-treated patients.[39]

Today treating hypertension is highly recommended.[43] Decades of research have revealed that above systolic pressures of 120 mmHg or diastolic pressures of 80 mmHg, an increase in blood

pressure is associated with a continuous graded increase in risk for adverse events including death.[43] Although extremely low blood pressures are also associated with mortality,[44] hypertension, the more common pathology in the western world is the focus of this thesis. The current blood pressure classifications put out by the American Heart Association are presented below.

Table 1.1-The American Heart Association guidelines for classifying hypertension.[45]

Blood Pressure Category	Systolic mm Hg (Upper #)		Diastolic mm Hg (Lower #)
Normal	Less than 120	And	Less than 80
Prehypertension	120-139	Or	80-89
High Blood Pressure (Hypertension) Stage 1	140-159	Or	90-99
High Blood Pressure (Hypertension) Stage 2	160 or higher	Or	100 or higher
Hypertensive Crisis (Emergency care needed)	Higher than 180	Or	Higher than 110

In addition to these dangers, organ transplant patients are also at risk for organ graft loss, which increases with increasing blood pressures.

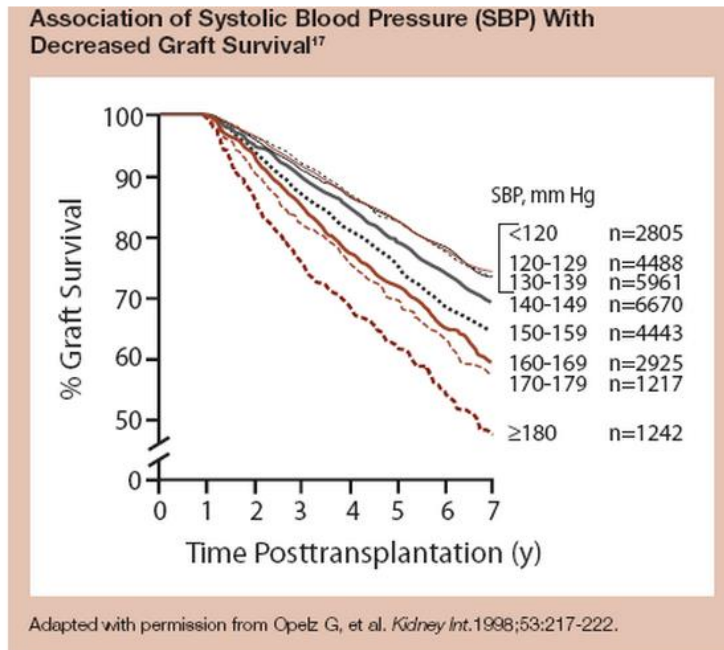

Figure 1.6-Decrease in graft survival with increasing systolic blood pressure.

Graph courtesy of Medscape,[46] data from Opelz et al.[47]

Unfortunately, 70-90% of organ transplant patients on calcineurin inhibitors develop hypertension.[48] Despite the use of anti-hypertensive drugs 60% of patients have uncontrolled hypertension 1 year post-transplant.[49] While the Kidney Disease: Improving Global Outcomes (KDIGO) workgroup recommends maintaining transplant patient blood pressures below 130/80[50] they make no recommendations regarding which class of anti-hypertensive agents to use.[50] The most effective strategies for maintaining post-transplant blood pressures below guidelines are controversial, although it is generally agreed that two or more drugs are necessary.[49]

Ideally, a thorough understanding of the pathological mechanisms behind tacrolimus-induced hypertension would lead to the development of immunosuppressive drugs that avoided this complication. In the meantime mechanistic insight would allow for the selection of antihypertensive agents that directly targeted the underlying problem. In this vein a considerable

amount of research has been poured into elucidating the causes of calcineurin inhibitor-induced hypertension. The results, which will be discussed in detail later, have implicated the vasculature,[51-53] nervous system,[54,55] renin-angiotensin-aldosterone system[56,57] and kidney in this pathology.[57,58]

Diurnal blood pressure dipping

Diurnal blood pressure dipping refers to the drop in blood pressure during the sleep cycle. In humans there is typically a 10-20% reduction in MAP at night.[59] Loss of diurnal blood pressure dipping is associated with an increased risk for myocardial infarctions, stroke, congestive failure and overall mortality.[60] Tacrolimus is known to cause loss of blood pressure dipping.[34] Though the exact etiology of this is unknown the implications for long term patient health are concerning.

We do know that diurnal blood pressure dipping loss is associated with Na^+ retention.[59] This is a phenomenon seen in patients with chronic kidney disease and salt-sensitive hypertension as well as patients taking tacrolimus.[57] Dipping patterns have been successfully restored in salt-sensitive hypertensive patients by restricting sodium intake or treating with diuretics.[59]

Hyperkalemia

Hyperkalemia, or high plasma [K^+], is often defined as plasma K^+ levels above 5.5 mmol/L.[61] As plasma [K^+] plays a key role in generating the electrical potential across cellular membranes, perturbations in plasma [K^+] concentrations can have significant consequences for excitable cells, including the heart and nervous system. As a result hyperkalemia can cause potentially fatal heart arrhythmias.[61]

Although hyperkalemia is somewhat variable in tacrolimus-treated patients, affecting about 40% of the patient population,[21] the potential consequences are serious enough to warrant

consideration here. Several classes of anti-hypertensive drugs are known to exacerbate hyperkalemia, such as ACE inhibitors and mineralocorticoid receptor blockers.[62] The work in this thesis points to utilizing thiazide-diuretics which both reduce blood pressure and plasma [K^+].

Hypomagnesemia

Hypomagnesemia, which is defined as plasma [Mg^{2+}] below 1.8 mg/dL,[63] is so ubiquitous amongst tacrolimus-treated patients that in some studies 100% of patients are affected.[36] Mg^{2+} is a co-factor for over 300 enzymes, including all those that utilize ATP. Therefore, Mg^{2+} wasting can lead to serious consequences including tetany, heart arrhythmias, calcium and potassium dysregulation and general malaise.[64] In addition, tacrolimus-induced hypomagnesemia has been identified as an independent predictor of new onset diabetes.[65] This is a complication which affects ~25% of tacrolimus-treated patients[65] and appears to be more prevalent in patients treated with tacrolimus than those treated with cyclosporine.[66]

In light of this, along with the general dysfunction caused by hypomagnesemia, prevention or treatment seems beneficial. The current treatment for hypomagnesemia, which is variably applied at different transplant institutions, is dietary Mg^{2+} supplementation, usually with milk of magnesia. Unfortunately, milk of magnesia also causes diarrhea reducing intestinal reabsorption of Mg^{2+} and causing dysregulation of other nutrients and ions as well as discomfort. A problem exacerbated by the chronic nature of the drug regimen.

Hypercalciuria

Solid organ transplant patients, have a five-fold increased risk for bone fracture and osteoporosis.[67] Although calcium wasting is not usually recognized as a clinical sign of calcineurin inhibitor use, it is a hallmark of experimental animal models.[35,57,58] Calcium is a key element of many important signaling cascades and plasma [Ca^{2+}] is tightly regulated by a variety

of hormones. In addition calcium wasting can often be compensated for by the large calcium reserves found in bone. Therefore, plasma [Ca^{2+}], even in experimental models, does not fluctuate with tacrolimus use.[35,57] As a result calcium wasting can only be detected in urine samples. Given this and the laundry list of life threatening derangements facing organ transplant patients, it is not surprising that hypercalciuria is rarely reported in the clinical setting. As tacrolimus is usually taken for life, however, ameliorating calcium wasting side effects could be beneficial to bone health in the long term.

Immunosuppressive vs Pathological Pathways

As tacrolimus use is typically a lifelong affair, the side effects accompanying it are all the more serious due to their unrelenting nature. Elucidating the mechanisms behind these pathologies is crucial for developing drug design strategies that side step these side effects.

Of primary importance is determining whether the immunosuppressive and pathological pathways of tacrolimus are identical. If so, more nuanced approaches which exploit subtle differences between the pathways are justified. Alternatively, if these pathways are mechanistically distinct, calcineurin inhibitors with fewer (or different) off-target effects would be the goal.

The Immunosuppressive Mechanisms of Calcineurin inhibitors

In 1991, years after cyclosporine and tacrolimus had been on the market, Liu et al. elucidated what is now the canonical mechanism of action of cyclosporine and tacrolimus.[68] They found that both cyclosporine and tacrolimus act as prodrugs, first binding to an endogenous protein, dubbed an immunophilin, before inhibiting the serine/threonine phosphatase calcineurin. Cyclosporine binds to the immunophilin cyclophilin A[69] and tacrolimus (FK-506) binds to FKBP (FK-506 binding protein, now known as FKBP12)[70] before inhibiting calcineurin.

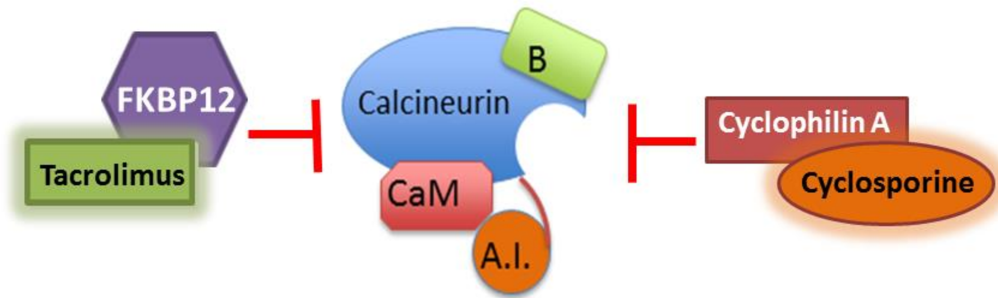

Figure 1.7-Cartoon of tacrolimus and cyclosporine calcineurin inhibition.

Both of these drugs require an immunophilin binding partner to facilitate calcineurin inhibition.

The uncanny similarity between these two drugs was somewhat surprising. Though cyclosporine and tacrolimus are both large (by drug standards) hydrophobic molecules they are structurally unrelated. They are products of a Norwegian soil fungus and Japanese bacteria respectively. Yet, they both employ a similar two step strategy to inhibit calcineurin. Schreiber and Crabtree hypothesized in their 1996 review,[71] that this is actually the discovery of an ancient microbial mechanism for inhibiting the growth of competing microorganisms.[‡]

Regardless of primordial intentions, when these drugs are given to humans they inhibit T-cell growth and proliferation remarkably well, leading to immunosuppression. In T-cells, an immune response is triggered by antigen presentation. This leads to calcium release, which activates the phosphatase calcineurin. Calcineurin is then able to dephosphorylate the transcription factor

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[‡] Crabtree and Schreiber argue that secreting hydrophobic substances, which would permeate the cellular membranes of nearby microbes, is the first step in a drug delivery system. Hydrophobic drugs tend to lack specificity, but bound to an immunophilin both tacrolimus and cyclosporine gain incredible specificity, in large part due to the increased surface area created by the drug-immunophilin interaction. This also hints at the possibility that the host could avoid self-inhibition if it lacked the prerequisite immunophilin.

Nuclear Factor of Activated T-cells (NFAT). When dephosphorylated a nuclear localization signal is unveiled and NFAT is translocated to the nucleus where it transcribes a variety of pro-inflammatory transcripts. Both tacrolimus and cyclosporine interfere with this process by binding to immunophilins and then inhibiting calcineurin's ability to dephosphorylate NFAT.[72]

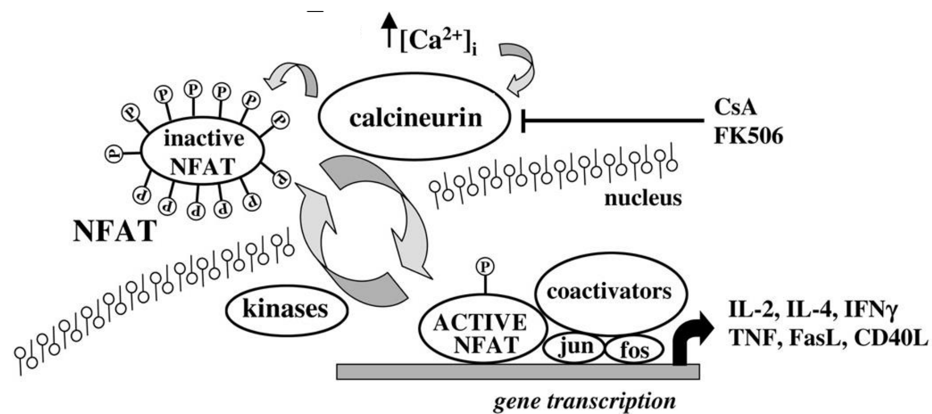

Figure 1.8-Calcineurin Inhibitors inhibit NFAT translocation.

The immunosuppressive effects of calcineurin inhibitors are the result of inhibiting NFAT dephosphorylation and nuclear translocation.[73]

Calcineurin

Calcineurin, however, has other functions throughout the body. As one of a handful of serine/threonine phosphatases, calcineurin is responsible for removing phosphate groups from a variety of proteins, which often turns them "on" or "off". Unlike other known phosphatases, however, calcineurin requires calcium for activation.[74] This puts it in a unique position to respond to acute stimuli, such as antigen presentation.

Calcineurin is comprised of two subunits, a catalytic (A) subunit and a regulatory (B) subunit. At rest an autoinhibitory domain fills the catalytic pocket. In the presence of calcium, calmodulin binds to calcineurin which causes conformational changes that remove the autoinhibitory domain

from the catalytic pocket.[75] This allows substrates to bind and dephosphorylation to occur. Both tacrolimus and cyclosporine (when bound to their respective immunophilins) bind to calcineurin at a hydrophobic groove at the junction of the catalytic A and regulatory B subunits.[76] This blocks the catalytic pocket, inhibiting dephosphorylation.

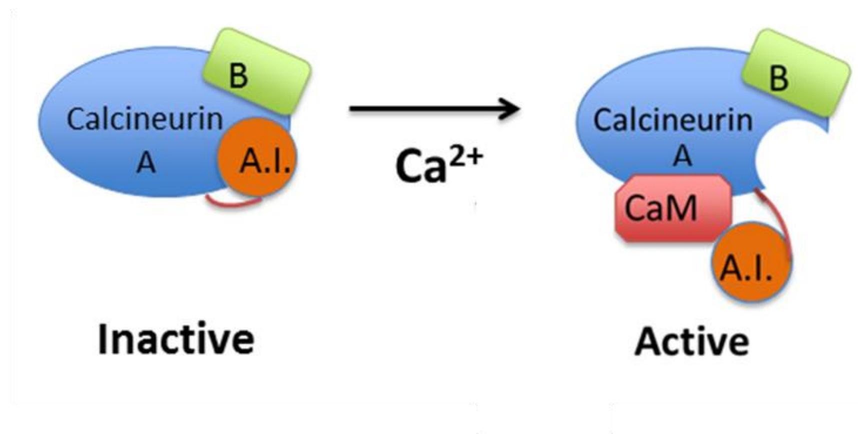

Figure 1.9-Calcineurin activation by Ca^{2+}.

At rest calcineurin is inhibited by an autoinhibitory domain (A.I.). Calcium release allows calmodulin (CAM) to bind both Ca^{2+} and calcineurin, inducing changes which remove the autoinhibitory domain from the catalytic pocket.

Tacrolimus is a more potent inhibitor of calcineurin than cyclosporine,[20] and is therefore used at lower doses. Yet it causes similar rates of toxicity.[36] This has led to the theory that calcineurin inhibition in off-target tissues is the cause of tacrolimus-induced pathologies, as calcineurin inhibition seems to track with side effects as well as immunosuppression. This is important because if true, future calcineurin inhibitors will be plagued by similar problems, unless differences between the immunosuppressive and pathological mechanisms can be exploited. This is the focus of Chapter 4.

FKBP12

In the process of unraveling the immunosuppressive mechanism of tacrolimus and cyclosporine it was discovered that both FKBP12[70,77] and cyclophilin A,[78,79] which facilitate immunosuppression by tacrolimus and cyclosporine respectively, are cis-trans peptidyl prolyl isomerases. In other words these molecules help isomerize x-proline bonds, an often rate-limiting step in protein folding. This common function has led to the hypothesis that immunophilin disruption is at the heart of calcineurin-inhibitor pathologies.

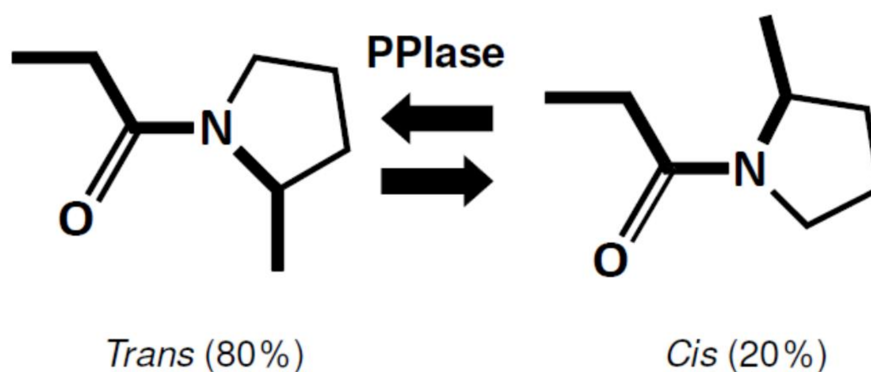

Figure 1.10-Peptidyl-prolyl isomerase action.

FKBP12 is a peptidyl-prolyl isomerase which increases the rate at which cis bonds before proline residues are converted to the trans configuration. This is important for proper protein folding as this conversion can be a rate limiting step. Illustration from Budiman et al.[80]

Additionally, FKBP12 stabilizes other molecules in either “on” or “off” states, including TGF-β receptors[81] and calcium channels.[82] Disruption of these functions could also have deleterious effects.

In line with this hypothesis systemic FKBP12 deletion in mice is embryonic lethal, owing to severe cardiac developmental abnormalities,[83] and tissue specific FKBP12 deletion in endothelial and hematopoietic cells has been reported to cause hypertension.[81] Therefore, the possibility that

FKBP12 disruption is the cause of tacrolimus-induced hypertension has been raised. For this reason, efforts are underway to design immunophilin sparing calcineurin inhibitors, with the hope that they will be safer therapeutics. Chapter 3 explores the possibility that FKBP12 disruption along the nephron is involved in tacrolimus-induced hypertension and electrolyte imbalance.

Blood Pressure Regulation

Although calcineurin inhibition in off-target tissues is often hypothesized to be involved in tacrolimus-induced hypertension, *where* this occurs has become an increasingly complicated question. This is because blood pressure is regulated by a complex network of organs, many of which utilize calcineurin or FKBP12, and many of which are dysregulated by calcineurin inhibitors, including tacrolimus.

Facing similarly complicated questions, Arthur Guyton, a giant in the field of hypertension research, developed a computer model to synthesize experimental data into a comprehensive circuit diagram detailing the factors involved in blood pressure regulation. The results are, at first blush, overwhelming.

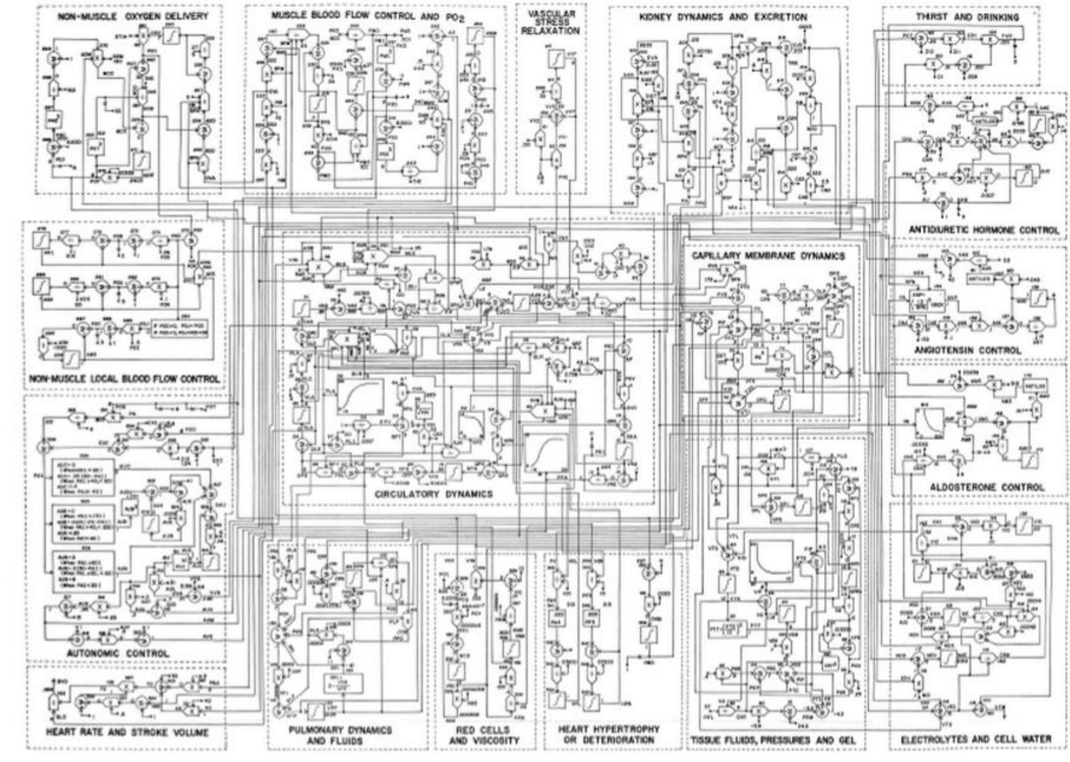

Figure 1.11-Guytonian blood pressure circuit diagram.[84]

Given the usually spare efficiency of biological organisms, such a network speaks to the importance of blood pressure homeostasis. Multicellular organisms rely on a circulatory system to deliver nutrients, including oxygen, to cells and remove waste products. Keeping blood pressure high enough to facilitate circulation and adequately perfuse tissues and low enough to avoid damaging delicate cells is a process that all organs of the body have a vested interest in. Therefore, it is tightly regulated by multiple layers of inputs and compensatory mechanisms.

At its core though, there are three basic variables that are usually modulated to maintain blood pressure; fluid volume, vascular resistance and heart rate. If we think of the circulatory system in terms of plumbing we can intuitively see that pressure will increase if we increase the volume of fluid flowing through the pipes, decrease the diameter of those pipes or increase the

rate at which fluid is pumped through. Changing the body's fluid volume, vascular resistance or heart rate has the same effect. This can also be illustrated mathematically:

Mean arterial pressure=Cardiac output * resistance

Cardiac output=stroke volume* heart rate

Stroke volume = End diastolic volume-End systolic volume

Therefore, Mean arterial pressure=heart rate*(End diastolic volume-End systolic volume)* resistance

These three primary variables act in concert, communicating via the nervous system and hormonal peptides to keep blood pressure fairly constant. What complicates this picture is the variety of *types* of blood pressure disturbance that the body must respond to. Although it seems like there are only two possible deviations, too high or too low, both of these situations are nuanced. An acute drop in blood pressure from standing up suddenly is addressed by different mechanisms than the acute drop in blood pressure due to hemorrhage.

Moreover, different regulatory mechanisms act on different time scales. In general, acute changes in blood pressure are usually attributed to changes in heart rate, the vasculature and signaling by the nervous system. Hormones such as the renin-angiotensin-aldosterone system come into play at an intermediate timescale, while the kidney is implicated in more chronic cases of blood pressure regulation.[85]

Tacrolimus causes both acute and chronic hypertension. This is consistent with findings that calcineurin inhibitors disrupt the nervous system,[54,55] vasculature,[51-53] renin-angiotensin-aldosterone system[56,57] and the kidney.[57,58]

The Sodium Chloride Cotransporter is *Essential* for tacrolimus-induced hypertension

Although it can be difficult to trace hypertension to a specific organ, electrolyte disorders, are often a kidney problem. In 2010 Ewout Hoorn, a visiting scientist in our lab, observed that patients on calcineurin inhibitors phenocopy patients with the monogenic disorder Familial Hyperkalemic Hypertension (FHHt). FHHt is a disorder which is attributed to an increase in the activity of the renal sodium chloride cotransporter (NCC). Hoorn went on to show that the NCC was activated (phosphorylated) by tacrolimus and that NCC was essential for the development of tacrolimus-induced hypertension. **This suggested that although tacrolimus-induced hypertension is a multi-faceted pathology, ameliorating the effects on NCC activity might be sufficient to protect patients from the hypertensive effects of tacrolimus.** The work in this thesis tests this hypothesis.

The Kidney

One of the major roles of the kidney is to act as the body's filtration system. The kidneys filter approximately 180 Liters of blood a day, reabsorbing the valuable components and excreting the waste products which leave the body as urine.

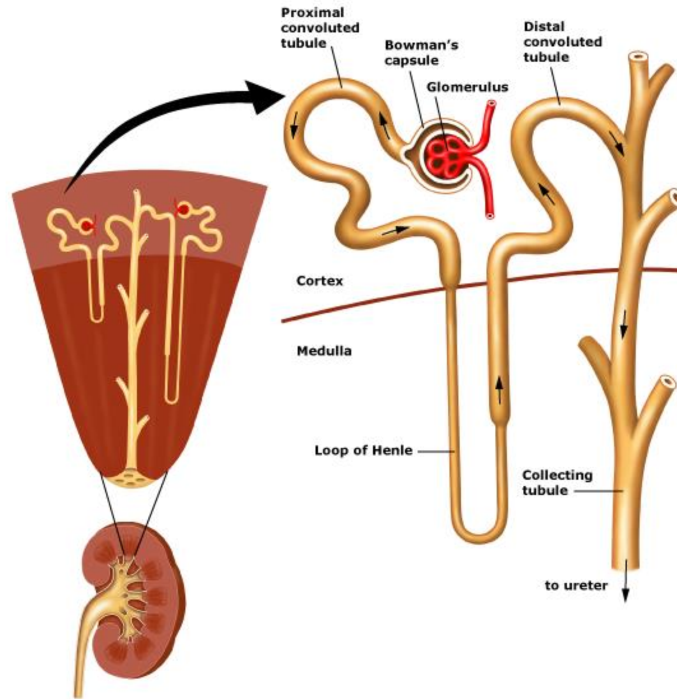

Figure 1.12-The kidney and nephrons.

Image courtesy of Biology Corner.[86]

Each of the two human kidneys has approximately 1 million nephrons. These are long tubules that stretch behind a "filter" also known as the glomerulus. Blood passing through the leaky tuft of blood vessels that make up the glomerulus is filtered into the nephron. This ultrafiltrate runs the course of the nephron which ultimately empties into the bladder. Along the way the filtered ions, small solutes and water that the body wants to keep, are reabsorbed, making their way across the nephron, into the interstitial fluid and eventually into the blood vessels that run parallel to the nephrons. Anything that is not reclaimed by the time the filtrate reaches the end of the nephron is emptied into the bladder where it is excreted as urine. In addition, waste products and toxic molecules which are too large to be filtered (or bound to proteins) can be secreted from the blood into the nephron lumen and ultimately excreted in the urine. This is also

true of substances such as K^+, which at times need to be excreted more rapidly than filtration rates alone would allow for.

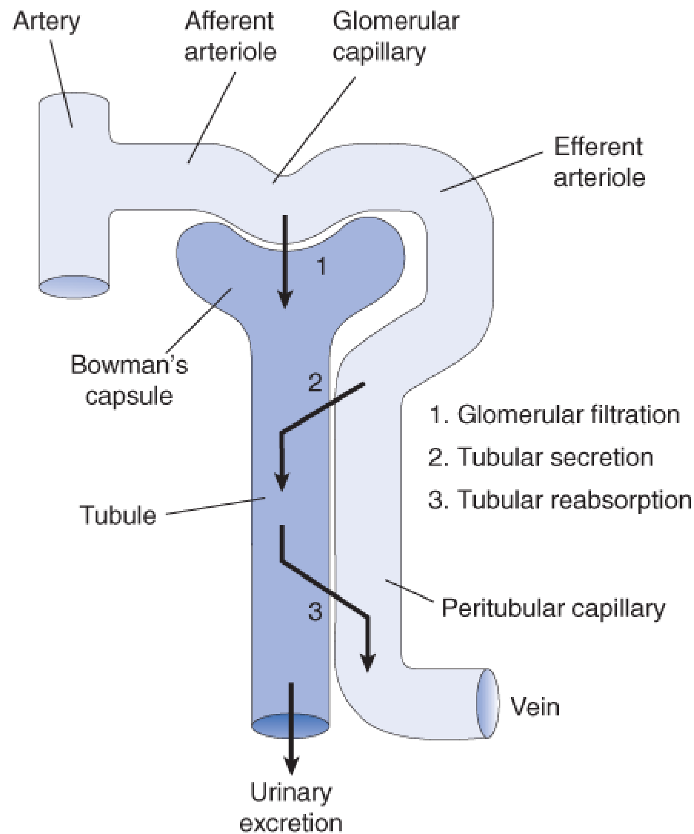

Figure 1.13-Pathways through the nephron.

Solutes can be 1) filtered, 2) secreted or 3) reabsorbed. The combination of these events determines how much of a substance remains in the body and how much is excreted in the urine. Image from Vander's Renal Physiology.[87]

Although the kidneys effectively filter about 180 Liters of blood a day, the average 150 pound person has only ~5.5 Liters of blood. Part of this apparent discrepancy reflects the large volume of fluid that resides outside of the bloodstream. This includes the intracellular and extracellular fluid, which is in a constant state of exchange with the bloodstream. This brings the

fluid volume of the body up to 42 L. Filtering the body's fluid volume about 4.5 times a day seems somewhat more appropriate.

This herculean effort, however, could easily deplete the body of essential resources if not done efficiently. The kidney is extraordinarily good at reclaiming the parts of the ultrafiltrate that it wants. Valuable molecules are reabsorbed paracellularly, being pulled between "leaky epithelial cells", or transported transcellularly, through cells by specialized transporters. The kidney is also adept at adjusting reabsorption to reflect the needs of the body, which are constantly changing. This requires an inordinate amount of communication with the rest of the body which we will discuss later. However, it is this mechanism that enables your body to maintain homeostasis despite changes in eating and exercise habits.

Table 1.2-Renal Reabsorption of Common Solutes.

This table (adapted from Vander's renal physiology[87]) illustrates the efficiency with which the kidneys can reabsorb important molecules such as Na^+ and glucose.

Substance	Amount Filtered/Day	Amount Excreted	% Reabsorbed
Water (L)	180	1.8	99.0
Sodium (g)	630	3.2	99.5
Glucose (g)	180	0	100
Urea (g)	56	28	50

To accomplish this, the nephron has become extremely specialized, and can be broken down into different segments based on both structure and function. For our purposes this includes the Proximal tubule (PT), Thick Ascending Limb (TAL), Distal Convoluted Tubule (DCT) and Connecting Tubule/Collecting Duct (CT/CD).

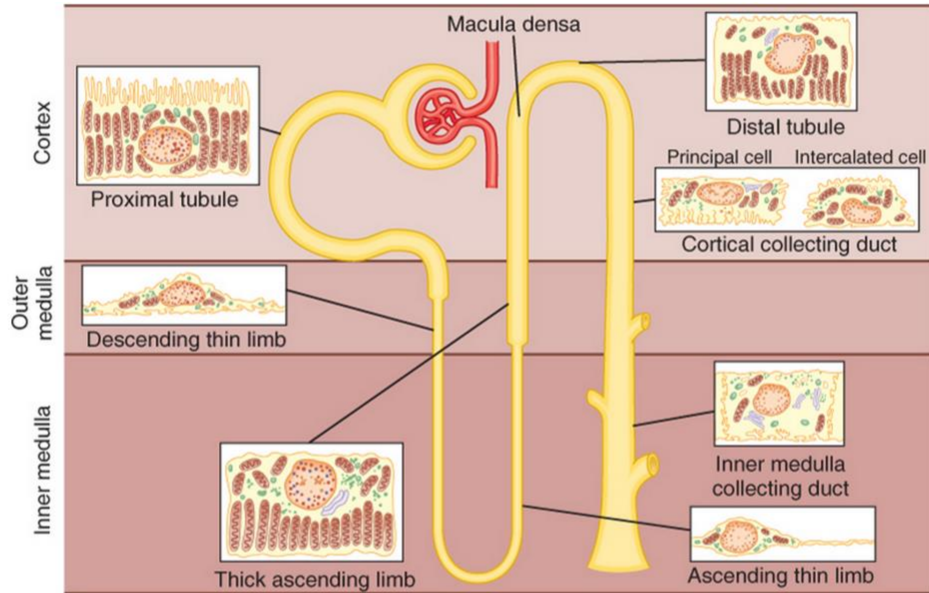

Figure 1.14-Nephron Segments.

Figure from Koepen & Stanton.[88]

Although each ion is handled differently, as a general rule, large amounts of both water and ion reabsorption occurs in the PT, solute reabsorption occurs in the TAL, solute reabsorption is fine tuned in the DCT and water reabsorption is balanced in the collecting duct. NCC, which is the focus of this thesis, is found in the DCT, and will be discussed in more detail later.

The Kidney's Role in Blood Pressure Regulation

Although filtering and "cleaning" the blood seems like a reasonable housekeeping measure, it is not immediately obvious what this has to do with blood pressure. The short answer is, reabsorbing water to modulate fluid volume. Counterintuitively, the kidney regulates water balance by altering Na^+ transport. Water is then reabsorbed to adjust the concentration of ions in the blood to appropriate levels. The body has a fairly severe Goldilocks complex when it comes to blood composition and maintains solutes at the concentrations listed below.

Table 1.3- Plasma composition.

The kidneys play a crucial role in keeping plasma composition within narrow windows, which is critical for cellular function (generating electrical potentials, keeping membrane proteins folded, etc.). Figure from Vander's Renal Physiology.[87]

Sodium	140 ± 5 mEq/L
Potassium	4.1 ± 0.8 mEq/L
Calcium (free fraction)	1.0 ± 0.1 mmol/L
Magnesium	0.9 ± 0.1 mmol/L
Chloride	105 ± 6 mEq/L
Bicarbonate	25 ± 5 mEq/L
Phosphate	1.1 ± 0.1 mmol/L
Glucose	5 ± 1 mmol/L
Urea	5 ± 1 mmol/L
Creatinine	1 ± 0.2 mg/dL
Protein (total)	7 ± 1 g/L

Of the ions found in blood the most abundant is Na^+. Therefore, by regulating Na^+ and then reabsorbing or excreting water to obtain the appropriate Na^+ concentration, the kidney regulates fluid volume.

So how do the kidneys "know" how much Na^+ to reabsorb? It turns out that Na^+ (and water) excretion increase with increasing pressures, and vice versa. This is a phenomenon known as pressure natriuresis. *Ex vivo* the kidney is capable of doing this in direct response to variations in filtration pressure.[89] The renal function curve, depicted below, illustrates an *ex vivo* experiment, in which an isolated kidney was subjected to different perfusion pressures[89] (this was done experimentally but you could imagine a change in vascular resistance having a similar effect). As

pressure increases so does Na^+ (and water excretion) putting the body in negative Na^+ balance, reducing ECF volume, and in the context of a whole system, reducing pressure. Over time this process settles at an equilibrium point at which Na^+ excretion matches input (steady state).[90]

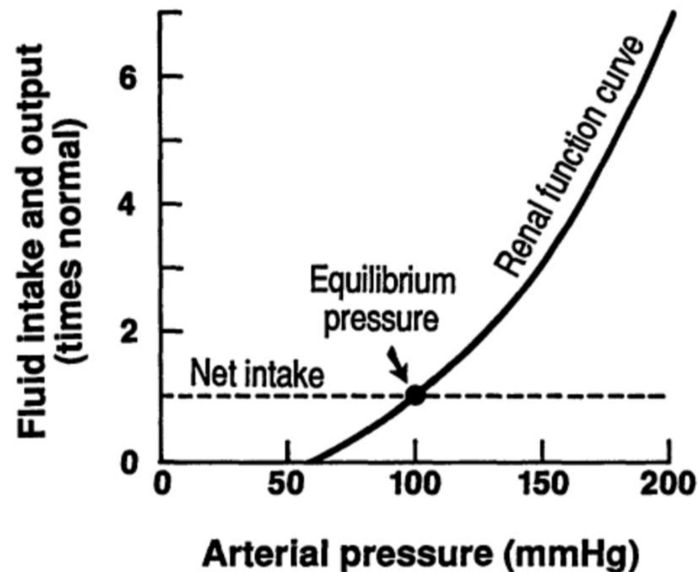

Figure 1.15-*Ex Vivo* Renal Function Curve.

Figure and legend from Guyton et al.[85]

This, however, would suggest that blood pressure varies dramatically with Na^+ intake. Yet in healthy individuals that is usually not the case. That is because *in vivo*, the shape of the renal-function curve changes (figure below). With layers of extra-renal regulation feeding the kidneys information about changes in pressure sensed elsewhere, the kidneys fine tune Na^+ reabsorption. This keeps blood pressure relatively constant over a wide range of NaCl intakes.

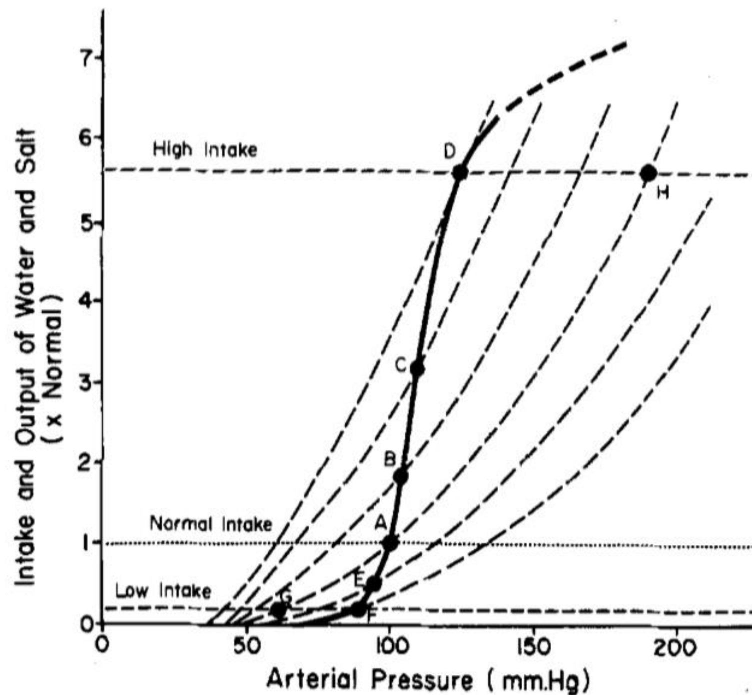

Figure 7. ***Relationship between renal function curves for output of water and salt as measured in isolated kidneys (the dashed curves) and the approximate renal function curve observed in the normal human being (the very dark solid curve).***

Figure 1.16-*In vivo* Renal Function Curve.

Figure and legend from Guyton et al.[85]

Taken together, this makes the kidneys a powerful but slow way of modulating blood pressure. A 5% increase in Na^+ reabsorption will ultimately add up to a large difference when you take into account the massive amount of fluid being filtered daily. Over days to weeks this makes a substantial impact on fluid volume and blood pressure. This is why an increase in the activity of a Na^+ transporter, such as NCC (which reabsorbs 5% of the filtered load), can become such a problem.

When the activity of a Na^+ transporter, such as NCC, is chronically and pathologically increased the slope of the renal function curve flattens out (becoming more like the dashed lines in Fig 1.17). This means that at any given Na^+ intake, steady state blood pressure will be higher. This is the case for people with salt-sensitive hypertension. In these situations, reducing dietary salt (NaCl) intake is particularly effective at reducing blood pressure because it lowers the bar at which Na^+ intake hits the renal function curve.

Therefore, in the long term, the kidneys are a key arbitrator of Blood Pressure. Although they are a powerful regulator of fluid volume in their own right, the kidneys are influenced by almost every other system in the blood pressure circuit. For this reason while *ex vivo* kidneys respond to changes in pressure, *in vivo* kidneys are able to maintain blood pressure around a relatively constant value over a wide range of conditions. This also means that inappropriate signaling from any of these input systems can lead to an increase in renal Na^+ reabsorption and an increase in blood pressure. Therefore, problems that originate in many different organ systems can ultimately manifest as an increase in renal Na^+ reabsorption and hypertension.

The Kidney's role in Electrolyte Homeostasis

In addition to blood pressure regulation the kidneys also play an important role in electrolyte homeostasis. As mentioned earlier even small differences in ion reabsorption can lead to major losses, given the rate at which the kidneys filter fluid. Although Na^+ is the most abundant ion in the extracellular fluid it is not the only one. K^+, Ca^{2+}, Mg^{2+} and their balancing anions Cl^-, bicarbonate and phosphate also need to be appropriately modulated. Interestingly, instead of regulating the balance of each ion individually, most ion transport is coupled to other ions, making use of electrical and chemical gradients which are ultimately fueled by the Na^+/K^+/ATPase. In addition, upstream events affect the availability of ions for downstream

transporters and changes in electrical gradients (due to depolarization or hyperpolarization) affect entire cells which house multiple transport pathways that all rely on the same electrical gradient.

It gets more complicated. Most ions are ingested independently of one another. It is not simply a matter of increasing or decreasing all ion reabsorption in lock step to accommodate fluctuations in fluid volume. Meals heavy in K^+ require excreting (and secreting) K^+ while the same meal can be low in Na^+, which requires retaining Na^+. The combinations of ions available and the needs of the body at any given moment can get complicated fast. For the most part the body is quite adept at navigating conflicting needs. It also appears to have a hierarchy of priorities. For example, we are beginning to realize that when pressed the kidneys will address K^+ homeostasis at the expense of blood pressure.[91,92]

This interrelated nature, however, means that when a single transporter is over or under active, it often manifests with an "electrolyte fingerprint"; a unique signature of electrolyte disorders which point to a pathology in a specific region. In the case of patients on tacrolimus the combination of hypertension, hyperkalemia and hypercalciuria resembles patients with FHHt (an increase in NCC activity) and is the mirror image of those with Gitelman's syndrome (NCC loss). This is the observation that inspired Hoorn's work, which found that tacrolimus increases NCC activity and that this is necessary for the development of tacrolimus-induced hypertension.

Table 1.4-Summary of Patient Symptoms.

Data for Gitelman's syndrome and FHHt excerpted from McCormick et al.[93] for tacrolimus data is taken from Jain et al.[33] (Blood Pressure and K^+) and Hoorn et al.[57] (Mg^{2+} and Ca^{2+}).

	Gitelman's (NCC loss)	**FHHt (NCC activation)**	**Tacrolimus**
Blood Pressure	↓ or ↔	↑	↑
Plasma K^+	↓	↑	↑
Plasma Mg^{2+}	↓	↔	↓
Urinary Ca^{2+}	↓	↑ or ↔	↑

The Distal Convoluted Tubule

The Distal Convoluted tubule is a short segment of the nephron, just beyond the macula densa. It is traditionally thought of as a region for fine tuning Na^+ reabsorption and blood pressure; however, emerging evidence suggests that its role modulating ions such as K^+ is even more critical.[91,92] In fact the DCT is also responsible for fine tuning Mg^{2+} and Ca^{2+} balance as well.

The DCT can be further broken down into two segments, one which is insensitive to the hormone aldosterone, the DCT1, and one that is, the DCT2. The transporters found in these segments vary a bit, but they both express the sodium chloride cotransporter (NCC), which is so exclusive to this region of the kidney that it is usually used to identify the segment. Loss of NCC by either genetic ablation[94] or chronic drug inibition[95] leads to atrophy of the DCT1. While an increase in Na^+ transport leads to hypertrophy and hyperplasia of this segment.[96] The DCT2, however, does not appear to be morphologically sensitive to these changes.

The DCT also expresses transporters that reabsorb calcium and magnesium. TRPM6, which is a magnesium transporter, is found in both segments of the DCT.[93] TRPV5, and the proteins responsible for sequestering intracellular calcium and trafficking it across the cell, are found in the DCT2 as well as the connecting tubule[93] (since Ca^{2+} is an important intracellular messenger Ca^{2+} is “escorted” across cells by other proteins). The DCT is considered to be a crucial region for finalizing both fluid volume as well as K^+, Ca^{2+} and Mg^{2+} balance. To do this effectively it must integrate information from around the body. Thus pathologies of the DCT may be due to intracellular dysregulation or inappropriate signaling to the DCT. The focus of this thesis is to try and tease apart which is occurring to lead to the tacrolimus-induced increase in NCC phosphorylation and activity.

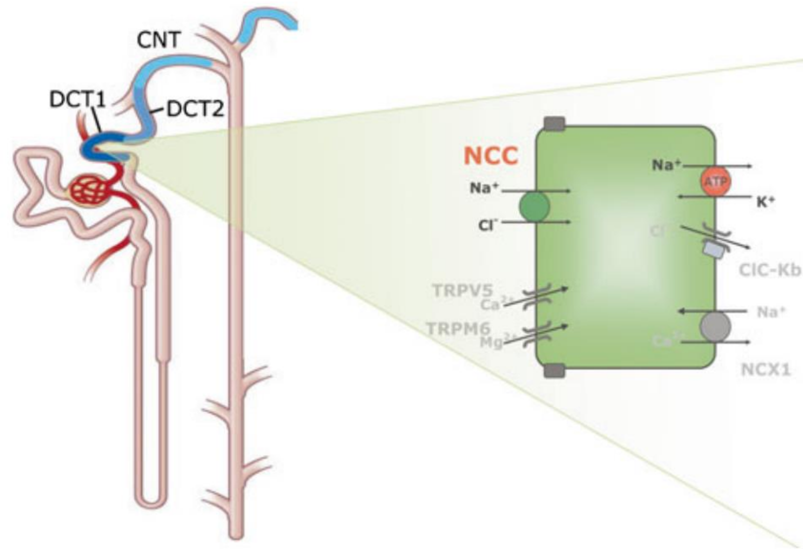

Fig. 1 Model of transcellular transport in the early distal convoluted tubule (DCT). A kidney tubule is shown schematically on the *left* indicating the locations of DCT type 1 and type 2 (*DCT1*, *DCT2*) and the connecting tubule (*CNT*). The sodium chloride cotransporter (*NCC*) is primarily expressed in DCT1. A model of transcellular transport in DCT1 is shown on the *right*, including the apical transporters NCC and transient receptor potential channels TRPV5 (a calcium channel) and TRPM6 (a magnesium channel). On the basolateral side, the sodium potassium ATPase pump is shown as well as the chloride channel ClC-Kb and the sodium–calcium channel NCX1. This figure was adapted from [5, 59]

Figure 1.17-Transporters of interest in the DCT.

Figure and legend from Moes et al.[97]

The Sodium Chloride Cotransporter (NCC)

NCC, or SLC12A3, is an electroneutral cotransporter found in the apical membrane of the DCT.[98] Structurally, NCC is a 12 transmembrane spanning protein with long cytosolic N and C-terminal domains.[97] Its predicted molecular weight is about 110 kDa, but it often runs higher on gels owing to post-translational modifications.[93] NCC is glycosylated at two sites (ASN 404 & 424), both of which are necessary for full function.[99] This appears to be primarily due to a reduction in NCC trafficking to the membrane in their absence.[99] NCC is also phosphorylated at key serine/threonine residue on its N-terminal tail which is necessary for activity.[100]

When active NCC transports Na^+ and Cl^- from the kidney ultrafiltrate into DCT cells which is then extruded from the cells by the Na/K^+/ATPase and CLC-Kb channels respectively. In order to maintain fluid osmolarity, this influx of Na^+ and Cl^- leads to an increase in water reabsorption and ultimately fluid volume. Thus, increasing NCC activity raises blood pressure and drugs that block its activity such as thiazide diuretics are powerful anti-hypertensive agents.

Thiazide diuretics have become a critical tool for studying the electrically silent NCC. They were serendipitously created in an attempt to improve the diuretic properties of the anti-microbial para-amino-benzene-sulfonamide.[101] Chlorothiazide, one of the resulting derivatives, proved to be remarkably effective.[40] How it caused diuresis was unclear at the time, but we now know that it is a selective inhibitor of NCC.

This was discovered via a long line of elegant micropuncture studies in the 1970s and ‘80s which suggested the existence of an electroneutral NaCl cotransporter along the distal tubule,[102] which was sensitive to thiazide diuretics.[103] Ellison and colleagues showed that this electroneutral thiazide-sensitive pathway was located in the DCT.[98] The discovery that thiazide diuretics also blocked electroneutral Na^+ and Cl^- transport in the bladder of Winter Flounders[104] led Gerardo Gamba to first clone NCC from these fish in 1993.[105]

Since then a great deal of work has gone into elucidating the cellular mechanisms that regulate NCC. This was particularly true after the discovery by Richard Lifton that mutations in two kinases belonging to the With-no-lysine (WNK) family, led to FHHt.[106] Given the efficacy of thiazide diuretics in ameliorating FHHt,[107] the race began to link WNK1 and WNK4 to NCC regulation. Since then the WNK story has gotten increasingly complicated and is still thick with controversy. Though somewhat peripheral to this story an excellent review of the WNK saga can be found at McCormick et al.[108] More recently, mutations in Cullin3 and Kelch-like 3, proteins

involved in ubiquitination, have also been found to cause FHHt[109,110] Understanding how these proteins fit into NCC regulation is a hot topic of research at the moment.

What is clear is that NCC is activated by phosphorylation at key serine/threonine residues at its N-terminal tail.[100] This appears to be directly mediated by two related kinases STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Oxidative Stress Responsive 1 (OxSR1).[111] Though a variety of knockout mouse models[112,113] have led to the hypothesis that SPAK primarily regulates NCC while OxSR1 predominates NKCC1 regulation (a homologous protein found just upstream of NCC in the TAL) it is clear that in certain contexts OxSR1 can also regulate NCC.[113,114] Both SPAK and OxSR1 are activated by the WNK family,[115] which also interacts with itself and other family members to ultimately affect both phosphorylation of NCC and its abundance at the apical membrane.[108,116] Both of which are necessary for ion transport.

In addition to intracellular regulation NCC is the target of a stunning amount of extracellular regulation.

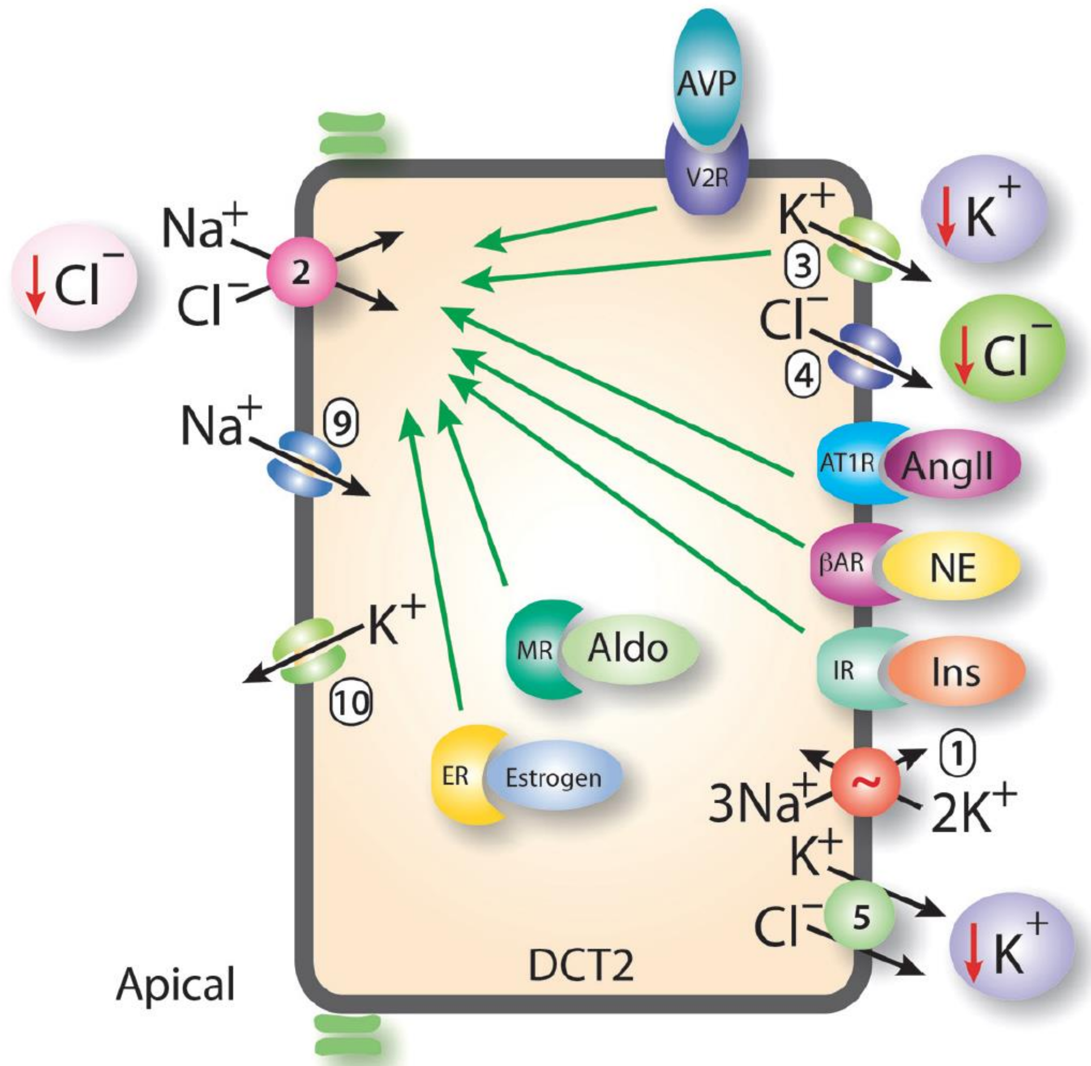

Figure 4 Regulation of sodium and chloride transport along the DCT. Panel A: Major factors known to regulate NCC along the DCT are shown. Note that regulation of ENaC and ROMK is omitted, as it is similar to regulation along the CNT and CD. Various peptide (AngII, insulin, and AVP) and steroid (aldosterone and estrogen) hormones, as well as norepinephrine (NE), stimulate NaCl transport primarily by enhancing NCC activity, although effects on other transporters are also likely. NCC is stimulated by low paracellular [K^+], perhaps directly via Kir4.1/5.1 channels, but also perhaps by stimulating cell chloride depletion via KCC4. Transporters are identified by numbers shown in Table 1. Panel B: highly simplified scheme

Figure 1.18-Extracellular regulation of NCC.

Figure and legend from McCormick et al.[93]

This is consistent with its role in the fine tuning of not only blood pressure but ion homeostasis, illustrated by the K^+, Mg^{2+} and Ca^{2+} derangements that accompany the loss or gain of NCC activity. To appropriately adjust these parameters NCC needs information from around

the body, which it then integrates to keep blood pressure and electrolytes in balance. As NCC responds to signals such as Angiotensin II, aldosterone, norepinephrine, AVP, estrogen, insulin, nervous system stimulation and is affected by changes in renal vasoconstriction that alter renal blood flow,[93] a pathological increase in any of these pathways can lead to a pathological increase in NCC activity. In addition, intracellular dysregulation could affect NCC directly or indirectly via activating or inactivating proteins.

Tacrolimus is known to dysregulate almost all of these extracellular and many intracellular factors. Thus the goal of this project is to determine if NCC, which is pathologically increased with tacrolimus treatment, is responding to aberrant signaling from extracellular sources, or if it is primarily an intracellular pathology. This is important because if it is an intracellular problem then protecting the DCT from either FKBP12 disruption or calcineurin inhibition may lead to safer therapies. If it is extracellular, directly inhibiting those pathways may lead to more effective treatment options and avoiding their dysregulation may improve the safety of future therapeutics.

An alternative view is that tacrolimus causes an increase in pNCC abundance because *dephosphorylation is inhibited*. This seems particularly likely as tacrolimus is a phosphatase inhibitor. Calcineurin, the target of tacrolimus is expressed in the DCT[117] but whether or not it dephosphorylates NCC is unclear. Indeed, although NCC is presumably inactivated by dephosphorylation at its key serine/threonine residues, the mechanism and players are currently unclear. There is some evidence to support a role for the phosphatases PP1,[118] calcineurin[57,58] and PP4.[119] Indeed, all may be involved either in complex cascades or in response to different stimuli.

Relationship Between NCC Regulation & Other Ions

Potassium Homeostasis

Potassium (K^+) plays a key role in determining the membrane potential, which has important consequences for electrically excitable tissues such as the heart, nervous system and muscle tissues. While intracellular [K+] are high, plasma K^+ levels are maintained at low levels. To facilitate this the kidney has a mechanism for secreting K^+ into the distal tubule. This allows K^+ to be purged quickly, in excess of what is filtered by the glomerulus. There are two pathways usually considered important for K^+ secretion. One is flow mediated and involves Big K^+ (BK) channels. The other is electrically driven by Na^+ reabsorption. The effects of NCC activity on K^+ homeostasis have largely focused on this secondary pathway. The emerging picture suggests that NCC may be predominantly a mechanism for regulating potassium as opposed to blood pressure.[92,120]

The current model is that NCC, by modulating Na^+ reabsorption, alters Na^+ delivery to the distal epithelial Na^+ channel (ENaC). Unlike NCC, ENaC is electrogenic and is coupled to the potassium secreting channel Renal Outer Medullary Potassium channel (ROMK). Therefore, when NCC activity is low more Na^+ is delivered to downstream ENaC which reabsorbs more Na^+ thus driving K^+ excretion via ROMK. Conversely, if NCC is activated more Na^+ is reabsorbed before reaching ENaC resulting in less Na^+ driven K^+ excretion.

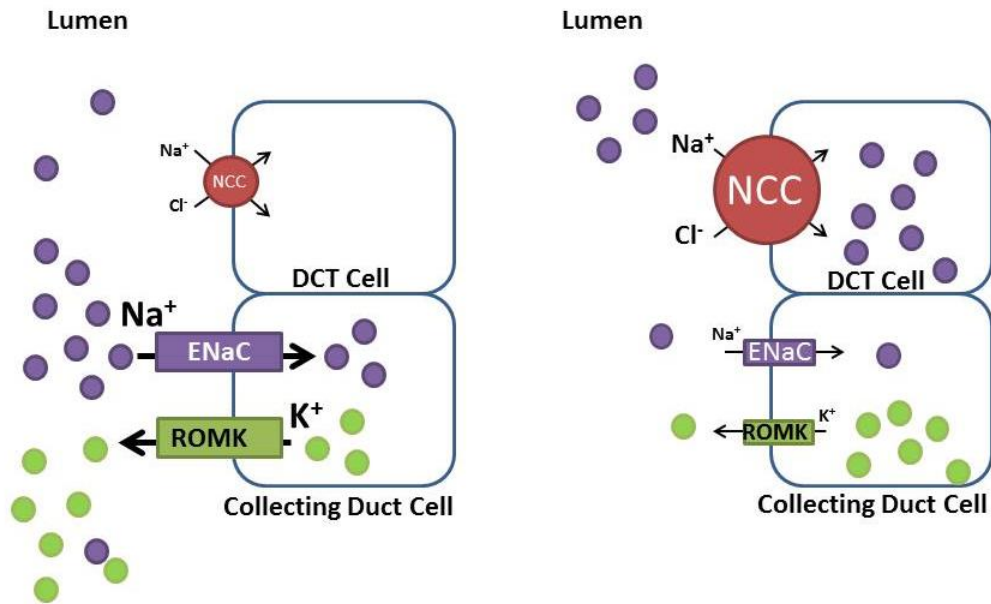

Figure 1.19-Model of NCC's role in K^+ regulation.

In this model NCC's role in K^+ homeostasis is to siphon off Na^+ which would otherwise be available to drive K^+ secretion.

This model requires that NCC "knows" the state of potassium balance at any given time. The canonical mechanism for this is the hormone Aldosterone. Aldosterone is produced in the adrenal glands and is released by increases in plasma K^+ which depolarize the cells. Paradoxically, it is also released by angiotensin II in response to fluid contraction and in this context serves to increase NCC activity and Na^+ reabsorption.

An alternative mechanism for sensing plasma K^+ is that, like in the adrenal glands, plasma K^+ is sensed directly by depolarization. In this model Na^+, Cl^- and K^+ are the determinants of membrane potential. The ratio of the intracellular: extracellular concentrations of all of these ions are of particular importance in accordance with the Goldman–Hodgkin–Katz voltage equation (below). Plasma $[K^+]$ plays a particularly prominent role in cellular depolarization owing to its

high intracellular and low extracellular concentrations. Of the 3 ions thought to govern membrane potential, both Na^+ and Cl- are found at high extracellular and low intracellular concentrations. Slight fluctuations in extracellular Na^+ and Cl^- do not alter the fold change between intracellular and extracellular concentrations dramatically, whereas even minor differences in plasma K^+ levels do.

$$V = \frac{RT}{F} \ln\left(\frac{p_K[\mathrm{K}]_{out} + p_{Na}[\mathrm{Na}]_{out} + p_{Cl}[\mathrm{Cl}]_{in}}{p_K[\mathrm{K}]_{in} + p_{Na}[\mathrm{Na}]_{in} + p_{Cl}[\mathrm{Cl}]_{out}} \right)$$

Figure 1.20 The Goldman–Hodgkin–Katz voltage equation.

In accordance with this model, Sorensen et al. showed *in vivo* that K^+ loading decreases the abundance of pNCC acutely;[121] they suggested that this is due to NCC dephosphorylation. This effect is likely triggered by cell depolarization, brought on by the high plasma [K^+].[92] Interestingly, depolarization has also been shown, in other tissues, to activate calcineurin.[122] This suggests that calcineurin may be responsible for dephosphorylating NCC in response to elevated potassium levels. Work regarding this is presented in Chapter 4.

Magnesium Homeostasis

Hypomagnesemia is one of the defining traits of Gitelman's syndrome[123] (loss of NCC) and is also caused by thiazide diuretics.[93] This suggests a relationship between NCC activity and magnesium balance. Paradoxically, calcineurin inhibitors also cause hypomagnesemia,[36] although in every other way patients on calcineurin inhibitors phenocopy patients with FHHt[57] (increase in NCC activity), the mirror image of Gitelman's syndrome.

This is a mystery. A plausible explanation may lie with the magnesium transporter TRPM6, which is expressed in the DCT1 as well as the DCT2.[93] NCC loss, whether genetic or due to chronic inhibition, results in atrophy of the DCT1.[94,95] This may affect Mg^{2+} balance by reducing the number of cells in which Mg^{2+} transport occurs. How calcineurin inhibitors cause hypomagnesemia is somewhat less obvious and is the subject of chapter 5.

Calcium Homeostasis

Although calcium regulation is complex, and is tightly governed by a network of hormones, changes in NCC activity appear to affect its balance. Patients with Gitelman's syndrome (loss of NCC function) often present with hypocalciuria.[124] An effect that is recapitulated by thiazide diuretics.[124] This is somewhat of an advantage to prescribing thiazides to the elderly who are often fighting both high blood pressures and bone loss.

Conversely, FHHt patients in which WNK4 has been mutated (Q565E) present with hypercalciuria, while patients with mutations in WNK1 that lead to FHHt do not.[107,125] This has inspired work at the molecular level to try and elucidate the relationship between WNK4 and the resident calcium transporter in the DCT, TRPV5.[126] Although there is evidence that NCC and TRPV5 are reciprocally regulated, the mechanisms governing this are still unclear.

It has also been suggested that the effects of alterations in NCC activity on renal calcium handling are secondary to changes in Extracellular Fluid Volume status.[124] In this model an increase in NCC activity leads to volume expansion an increase in blood pressure and a reduction in Ca^{2+} reabsorption by the proximal tubule. A decrease in activity has the opposite effect. This may also play a role, however, it has been shown that acute injections of hydrochlorothiazide increase TRPV5 abundance.[127] Therefore, it seems likely that there is also a local effect of changes in NCC that influence TRPV5 regulation. This is explored in chapter 5.

Hypothesis

Though previous work in the lab implicated an increase in NCC phosphorylation and activity in tacrolimus mediated hypertension and electrolyte disorders it is unclear whether this is due to direct effects of tacrolimus on the DCT. Calcineurin inhibitors such as tacrolimus have well documented effects in the vasculature, renin-angiotensin/aldosterone system and nervous system all of which could ultimately increase NCC phosphorylation and activity. Moreover, it is known that tacrolimus, must first bind to FKBP12, presumably disrupting its endogenous function, before inhibiting Calcineurin. When this occurs in T-Cells it leads to immunosuppression. I am testing the hypothesis that tacrolimus acts by this same mechanism, directly along the nephron, to increase NCC phosphorylation and lead to hypertension and electrolyte disorders.

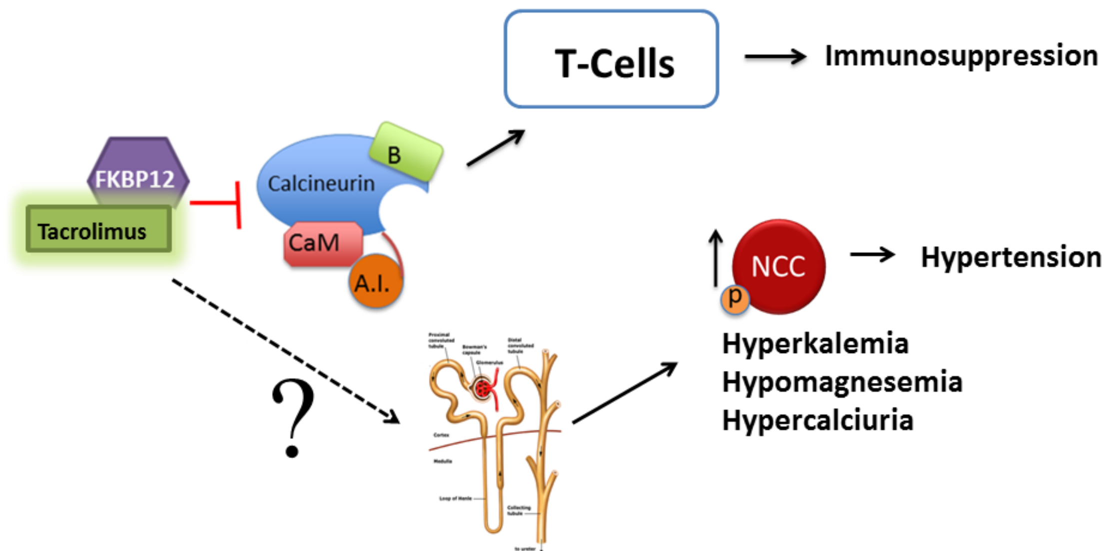

Figure 1.21-Overarching Hypothesis.

My overarching hypothesis is that tacrolimus acts directly in the epithelial cells of the nephrons to inhibit calcineurin, by way of FKBP12, which increases NCC phosphorylation, causes hypertension and electrolyte disorders. Nephron image courtesy of Biology Corner.[86]

To this end I generated a unique mouse model in which FKBP12, the necessary binding partner of tacrolimus, is deleted selectively along the nephron in response to doxycycline exposure (KS-FKBP12$^{-/-}$ mice); circumventing any developmental complications. I tested the effects of FKBP12 disruption along the nephron to determine whether or not FKBP12-disruption along the nephron plays a role in tacrolimus-induced hypertension and electrolyte disorders. Information which can be used to predict whether or not proposed immunophilin-sparing calcineurin inhibitors will have improved safety profiles.

I also treated Control and KS-FKBP12$^{-/-}$ mice with tacrolimus. In KS-FKBP12$^{-/-}$ mice the nephron is protected from FKBP12-dependent events such as calcineurin inhibition, allowing me to tease apart the extent to which tacrolimus-induced pathologies are due to FKBP12-dependent events, such as calcineurin inhibition, directly along the nephron. This information is relevant to the development of safer immunosuppressive drugs, and also adds to our understanding of renal physiology.

Chapter 2 -Methods

In Vivo

Choice of *in vivo* model system

There are many animal models which have been used to study the kidney. These include such diverse species as drosophila, zebrafish, mice, rats and rabbits, to name a few. My work follows on the heels of a ground breaking finding which was made in mice.[57] As there are notable differences between animal models it made sense to continue my study in mice. Additionally, mice compare favorably to other animal models with regard to the following factors:

1) Ability to measure both blood pressure and levels of phosphorylated NCC
2) Genetic tractability
3) Similarity to human renal physiology
4) Our laboratories expertise in using the model organism.

Although these advantages far outweighed the disadvantages, there are certainly some drawbacks worth noting. For starters mice are small. To effectively measure subtle blood pressure differences in mice radiotelemetry, a challenging and expensive technique, is necessary. Ultimately, we purchased a radiotelemetry system and I learned how to surgically implant the probes. Secondarily, unlike rats, mice do not develop overt hyperkalemia in response to tacrolimus-treatment, which makes this side effect difficult to study. I was able to address this by using less direct, mathematical approaches (chapter 4). The third drawback worth noting here is that there is considerable variability between mouse strains with regard to hypertension, renal damage and as it turns out tacrolimus-induced side effects.[57] The previous study used mice on a Balb/C background, which appear more susceptible to tacrolimus-induced hypertension (though less so to tacrolimus-induced hypercalciuria).[57] In this study, I used mice on a primarily Black/6 background (see animal section for details) because the mice expressing the necessary genetic

components (FKBP12$^{fl/fl}$ mice and mice expressing the Pax8-rtTA/TRE-LC1 system) were already on a Black/6 background.

The Pax8-rtTA/TRE-LC1 system enables the selective and robust deletion of FKBP12 along the nephron and is also inducible, offering temporal selectivity. As FKBP12 systemic deletion is embryonic lethal and its role in the development of the kidney is unknown, this was critical. To effectively breed mice with all 3 of these genetic components on to a Balb/C background, however, would have taken multiple years. Thus, I used a higher dose of tacrolimus to induce tacrolimus-mediated hypertension, instead.

Animals

All animal studies were approved by OHSU's animal care and use committee (protocol IS918). Control and Kidney-specific FKBP12 knockout mice (KS-FKBP12$^{-/-}$) were generated by breeding FKBP12 $^{fl/fl}$ mice, with Pax8-rtTA/TRE-LC1 mice. Dr. Susan Hamilton graciously supplied the FKBP12$^{fl/fl}$ mice, which were generated using AB2.1 ES cells[128] and were crossed with C57bl/6 mice (personal correspondence) to produce chimeric offspring. Dr. Jim McCormick generously supplied the CRE Pax8-rtTA/TRE-LC mice,[129] which are on a C57bl/6 background (personal correspondence). Mice homozygous for floxed FKBP12 and possessing at least one copy of Pax8-rtTA and TRE-LC1 were identified by PCR genotyping of tail clipping DNA. Genetically identical mice were treated with either Doxycycline (2g/L, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) at 4-7 weeks of age for 2 weeks (water changed every 3 days). Male mice from 10-20 weeks old were used for experiments.

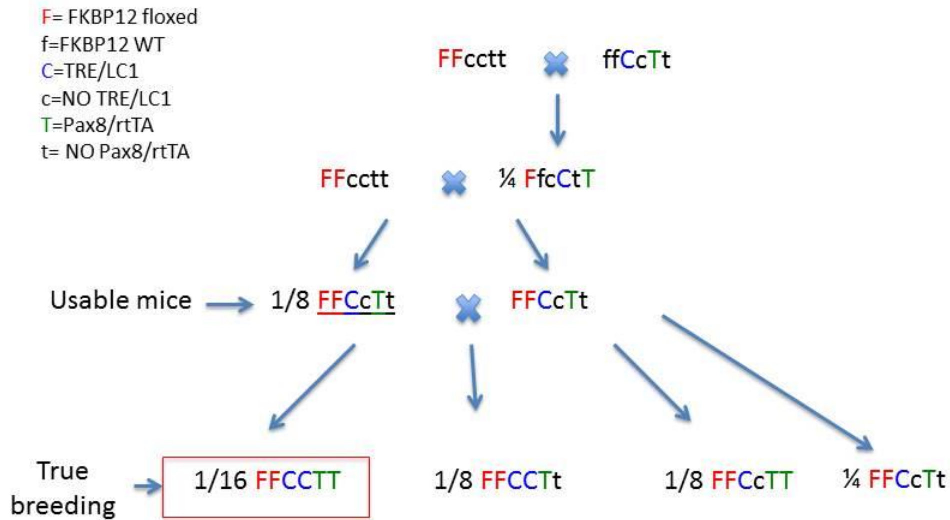

Figure 2.1-KS-FKBP12$^{-/-}$ Mouse Breeding Scheme.

Mice homozygous for floxed FKBP12 and possessing at least one copy of CRE/LC1 and Pax-8/rtTA were identified by PCR, using DNA extracted from tail clippings. Genetically identical mice were treated with either Doxycycline (2g/L, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) in their drinking water for 2 weeks (changed every 2-3 days) from 3-7 weeks of age. Male mice from 12-20 weeks were used in all experiments, with the exception of aging studies in which the mice were 8 months old.

Use of Male Mice

I restricted experiments to male mice, as this work builds on research conducted in male mice. Further, there are gender differences in tacrolimus metabolism that would have made the results from mixed gender studies challenging to interpret.[130]

Power Calculations

I did power calculations to determine the number of mice needed for radiotelemetry (blood pressure) and Western blot studies (molecular data). To detect a 5 mmHg difference in blood

pressure, using an alpha value of 0.05 power of 0.8, and a delta of 3 mmHg (reported by the manufacturer, DSI international) n=6 is required. Previously, we found that tacrolimus induces a 40% increase in NCC phosphorylation.[57] To detect this, assuming a standard deviation of 27 (previous work[57]) and using an alpha of 0.05 and a power of 0.8, an n=7 is needed to detect such differences by Western Blot.

Tacrolimus

Tacrolimus stock solutions of 30 mg/mL were prepared by dissolving 10 mg of dry powdered tacrolimus (pure) (Cayman Chemicals https://www.caymanchem.com) into 333.3 uL of solvent (sterile DMSO and sterile filtered Tween-20 in a 3:1 ratio). Working solutions of tacrolimus (15 ug/mL) were prepared fresh, daily, by diluting the stock solution with sterile PBS. Mice were injected subcutaneously with 3 mg/Kg tacrolimus daily, for 18 days.

Telemetry

Mice were given analgesic, 0.3mg/kg Buprenorphine and antibiotic,10 mg/Kg Ciprofloxacin, subcutaneously prior to surgery. Under anesthesia (isoflurane in O_2; 5%-loading 1.5-2% maintenance) radiotelemetry probes (TA11PA-C20; Data Sciences International) were surgically implanted into the aortic arch via the left carotid artery. Recordings began after 6-10 days of recovery. Data was collected for 20 seconds every 10 minutes.

Tail Cuff

Tail pressures were taken using the CODA 6 from Kent scientific. Experiments were begun daily at 9:30 AM. Mice were maintained at 32-36 degrees C with heating pads and acclimated for one week prior to data collection. After mice were warm, VPR tail cuffs were moved up the mouse's tail until resistance was felt. After 10 acclimation cycles, 15 data cycles were completed. Data was averaged daily for each mouse and as a group for each week.

Metabolic cages

Mice were housed individually in metabolic cages (Hatteras instruments). After two days, to acclimate, urine was collected for 24 hours in glass jars containing a layer of water saturated mineral oil (to prevent evaporation). Food was provided in gel form. A Na^+ deficient diet, from Harlan laboratories, was brought up to 0.49% NaCl (normal chow). Additional water was provided ad libitum.

Gel diet for metabolic cages

Gel diets consisted of 5 g of powdered salt deficient Harlan diet, 8 mL of water, 0.0225 g of bacterial agar and 0.49% NaCl per serving.

Euthanasia

Mice were euthanized using two of the following procedures:

1) Drug overdose with mouse cocktail (600 uL/mouse)

Mouse Cocktail		
	[stock] mg/mL	**mLs**
Ketamine	50	1.5
Xylazine	20	0.75
Acepromazine	10	0.25
sterile water		7.5

2) Cervical dislocation

3) Cutting diaphragm

Ex Vivo

Genotyping

Genomic DNA from mouse tail clippings were heated in 75 uL NaOH (pH 12.0) at 95 C for 45 min and neutralized with 75 uL Tris-HCl (pH 5.0). Genotypes were determined by PCR using 4 uL of crude genomic lysate, 21 uL of Invitrogen TaqDNA Polymerase native master mix (cat # 18038-018) using the following primers:

FKBP12	F:5’ AGAACTTGCCCTTCAGTATT 3’
	R:5’AGGCTTGTACCACTATTTTCT3’
PAX8-rtTA	F:5’CCATGTCTAGACTGGACAAGA 3’
	R:5’CAGAAAGTCTTGCCATGACT 3’
TRE-LC1 (CRE)	F:5’TTTCCCGCAGAACCTGAAGATG 3'
	R:5’TCACCGGCATCAACGTTTTCTT 3’

and cycling conditions:

Step	Temp (Celsius)	Time	# of cycles
1	98	30 sec	1
2	98	10 sec	40
3	72	10 sec	40
4	72	8 sec	40
5	**Go to step 2 (39 times)**		
6	72	5 min	1
7	16	forever	1

PCR products were run on 1% agarose gels for Pax-8 and TRE-LC1 (CRE) and 2% for FKBP12, to achieve adequate separation.

Genotypes were confirmed by immunoblotting for FKBP12 at the conclusion of experiments.

Genomic DNA semi-quantitative PCR

Genomic DNA was extracted from snap frozen tissues using Qiagen DNAeasy kit and following the manufacturer's protocol. Semi-quantitative PCR was performed using the following primers:

FKBP12	F 5' GTCCTCTTTCTCACGGT 3'
	R:5'AGGCTTGTACCACTATTTTCT 3'

and cycling conditions:

Step	Temp (Celsius)	Time	# of cycles
1	95	3 min	1
2	95	30 sec	35
3	62	30 sec	35
4	72	1 min	35
5	**Go to step 2 (34 times)**		
6	72	5 min	1
7	12	forever	1

mRNA PCR

Tissue was preserved at the time of collection in RNALater, snap frozen and stored at -80C. mRNA was extracted using Oligotex Direct mRNA mini kit, following the manufacturer's protocol. cDNA was transcribed using Finnzymes Phusion Kit and amplified by PCR using the following exon spanning primers:

FKBP12	F: 5'GAGTGCAGGTGGAGACCATCTCTC 3'
	R: 5'CATGGCAGATCCACGTGCAGAG 3'

and cycling conditions:

Step	Temp (Celsius)	Time	# of cycles
1	98	30 sec	1
2	98	10 sec	40
3	72	10 sec	40
4	72	8 sec	40
5	**Go to step 2 (39 times)**		
6	72	5 min	1
7	16	forever	1

Quantitative real-time PCR

Nina Himmerkus and Katharina Blankenstien did the nephron segment experiments (Chapter 4). Nina isolated mouse renal tubule segments by microdissection. Katharina extracted RNA from the segments using a RNA extraction kit (Invitek) and following the manufacturer´s protocol. cDNA was synthesized by reverse transcription (Tetro Reverse Transcriptase, Promega). Quantitative PCR was performed using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne) and primers specific for:

FKBP12	F:5’ACTACACGGGGATGCTTGAA 3’
	R:5’GCTCTCTGACCCACACTCAT 3’
Calcineurin-α	F:5'CCAACACTCGCTACCTCTTC 3'
	R:5' GTGCCTACATTCATGGTTTCC 3'
Calcineurin-β	F:5' GCAACCATGAATGCAGACACC 3'
	R:5'CAAGGGGCAAGCTGTCAAAAG 3'

and the following cycling conditions:

Step	Temp (Celsius)	Time	# of cycles
1	50	2 min	1
2	95	10 min	1
3	95	15 sec	40
4	**Go to step 3 (39 times)**		
5	60	1 min	1

Gene expression analysis was performed applying the ΔCt method and normalized against β-Actin.

Sabina Jelen (Dutch collaborator) did the qRT-PCR in chapter 5. Total RNA was isolated from mouse kidney with Trizol Reagent (Life Technologies, Grand Island, NY, USA) The obtained RNA was subjected to DNAse treatment to prevent genomic DNA contamination. Thereafter, 1.5 ug of RNA was reverse transcribed by M-MLV reverse transcriptase (Invitrogen, Grand Island, NY, USA). The following primers were used:

Gene	Primer
TRPV5	F:5' CTGGAGCTTGTGGTTTCCTC 3'
	R:5' TCCACTTCAGGCTCACCAG 3'
TRPM6	F:5' CTTACGGGTTGAACACCACCA 3'
	R:5' TTGCAGAACCACAGAGCCTCTA 3'
NCC	F:5' CTTCGGCCACTGGCATTCTG 3'
	R:5' GATGGCAAGGTAGGAGATGG 3'
CLDN16	F:5' GTTGCAGGGACCACATTAC 3'
	R:5' GAGGAGCGTTCGACGTAAAC 3'
CLDN19	F:5' GGTTCCTTTCTCTGCTGCAC 3'
	R:5' CGGGCAACTTAACAACAGG 3'
NCX1.3	F:5' CTCCCTTGTGCTTGAGGAAC 3'
	R:5' CAGTGGCTGCTTGTCATCAT 3'
Calbindin 28K	F:5' GACGGAAGTGGTTACCTGGA 3'
	R:5' ATTTCCGGTGATAGCTCCAA 3'
GAPDH	F:5' TAACATCAAATGGGGTGAGG 3'
	R:5' GGTTCACACCCATCACAAAC 3'

And cycling conditions:

Step	Temp (Celsius)	Time
1	95	7 min
2	95	1 min
3	60	15 sec
4	60	10 sec
5	**Go to step 2**	
6	60-95	5 sec/0.5 degrees C

QRT-PCR was performed with iQ SYBR Green supermix system (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. The Livak method[131] was used to quantify gene expression.

Immunoblotting

Tissue lysates were prepared by dounce homogenizing snap frozen samples, on ice, in 1 mL chilled Homogenization Buffer:

Homogenization buffer

	Per 10ml	final	stock
sucrose	1.03 g	0.3M	
tris-HCl pH 7.5	500 µl	50mM	1M
EDTA	20 µl	1mM	0.5M
EGTA	20µl	1mM	0.5M
aprotinin	0.92 µl	1ug/ml	21.6mg/ml
leupeptin	3.6 µl	8.5µM	10mg/ml
Na^+ orthovanadate	100 µl	1mM	100mM
sodium fluoride	500 µl	50mM	1M
DTT	5 µl	1mM	2M
PMSF	30 µl	1mM	0.3M (17.4mg in 300ul EtOH)
PhosStop	1 tablet	1x	10x (Roche Life Sciences)

Samples were spun down at 6000 RPM for 15 min at 4℃, proteins were separated on 4-12% Bis-Tris gels (Invitrogen) transferred overnight at 4℃ and immunoblotted with antibodies described in the table below. Densitometry was quantitated using image J software (**http://rsbweb.nih.gov/ij/**) and protein expression was normalized to β-actin.

Antibodies

Antibodies used	Source	[primary]	[secondary]
pNCC	Ellison laboratory	1:4000	1:4000 anti-rabbit
tNCC	Ellison laboratory	1:10000	1:5000 anti-rabbit
β-actin	abcam ab8227	1:10000	1:10000 anti-rabbit
WNK 4	Ellison laboratory	1:4000	1:5000 anti-rabbit
SPAK	C8 Delpire laboratory	1:5000	1:5000 anti-rabbit
OSR1	C8 Delpire laboratory	1:5000	1:5000 anti-rabbit
PNKCC2	Bachmann Laboratory	1:14000	1:7500 anti-rabbit
tNKCC2	Bachmann laboratory	1:3000	1:5000 anti-guinea pig
FKBP12	Abcam 2918	1:5000	1:5000 anti-rabbit

Blood sample Analysis (electrolytes and tacrolimus)

Blood was obtained by cardiac puncture (under anesthesia) and put into heparinized tubes. 100 uL of blood was immediately pipetted into an I-STAT Chem 8^+ cartridge (Abbott Pointe of Care) for analysis. 30 uL of blood was pipetted onto dried blood spot cards for analysis of tacrolimus and creatinine by the pharmacokinetics core using tandem Mass Spec.[132] The remainder of the blood sample was spun down at 2000 RPM for 5 min at room temperature and the plasma was used to determine Mg^{2+} concentrations by the Xylidyl Blue colorimetric assay (Pointe Scientific HM929).

Urinary electrolyte analysis

Na^+ and K^+ urinary concentrations were measured by dual flame photometry (Cole-Parmer 2655-10), using the reference mode and Li^{2+} internal standards. 10 uL of sample was used with 3.990 mL of molecular grade water. Urinary Ca^{2+} was measured by a colorimetric assay from Pointe Scientific (catalog #12-C7529-120)

In Vitro

HEK-293 FLP-In cells

HEK-293 cells stably expressing a single copy of mouse NCC in response to tetracycline were generated previously using Invitrogen's FLP-In 293 cells. They are described by Hoorn et al.[57] in detail as follows:

"cDNA encoding full-length mouse NCC was subcloned into the pcDNA5/FRT/TO vector (Invitrogen). Flp-In 293 host cells (Invitrogen) were co-transfected with the pcDNA5/FRT/TO-NCC construct and pOG44, a plasmid expressing Flp recombinase, resulting in a homologous recombination event. Transfected cells were screened for hygromycin resistance and lack of β –galactosidase activity. The Flp-In T-REx 293 NCC cell line was maintained in high-glucose DMEM containing 10% v/v FBS, 200 μg ml^{-1} hygromycin, 15 μg ml^{-1} blasticidin, and penicillin/streptomycin. NCC induction was confirmed by incubating the cells with or without tetracycline (1 μg ml^{-1}), followed by cell lysis and immunoblotting with antibodies against NCC and phosphorylated NCC."

NCC dephosphorylation assay

HEK-293 cells expressing NCC (described above) and transiently transfected (lipofectamine) with constitutively active SPAK (T243E/S383D) were treated for 25 minutes with 10 mM $BaCl_2$ (dissolved in DMEM), before collecting cells in chilled lysis buffer for immunoblotting.

Cell collection

Cell lysis buffer, 5ml	amount	final conc.	stock soln.
triton x-100	25ul (dissolve in 4.5 mL water)	0.50%	
Tris–HCl, pH 8.0	50ul	10 mM	1M
EGTA, pH 8.0	50ul	5 mM	0.5M
MgCl2	12.5ul	2.5 mM	1M
Add reagents above first, chill on ice.			
protease inhibitor cocktail set II (Calbiochem)	50ul		
PhosStop Phosphatase Inhibitor cocktail (Roche)	1mL		
leupeptin	4.75ul	20uM	21.03mM (10mg/ml)
aprotinin (box in fridge)	17.3ul	800nM	

100ul of lysis buffer was added to each well. Cells were transferred to microcentrifuge tubes and centrifuged at 9100 rpm (8000 g) for 15 minutes at 4 degrees C. The supernatant was used in all future steps. The protein concentration was quantified by a modified colorimetric Bradford assay (Bio-Rad Protein Assay Kit I 500000). Proteins were separated on 4-12% Bis-Tris gels (Invitrogen) in MOPs buffer transferred overnight at 4 C and immunoblotted with antibodies described in the table below. Densitometry was quantitated using image J software (**http://rsbweb.nih.gov/ij/**).

Antibodies Used	Source	[Primary]	[Secondary]
pNCC #40	Ellison laboratory	1:6000	1:4000 anti-rabbit
tNCC	Ellison laboratory	1:10000	1:5000 anti-rabbit
β-actin	abcam ab8227	1:10000	1:10000 anti-rabbit
FKBP12	Abcam	1:5000	1:5000 anti-rabbit

Calculations

pNCC: plasma [K^+] ratio: The absolute difference between predicted pNCC values, using the best fit line for previous data $y=6.593^{(-0.5383*x)}-0.01761$ and actual pNCC values (pNCC densitometry was normalized to untreated control mice on a normal diet run on the same western blot) were averaged.

Statistics: Nephron tubule segment specific qRT-PCR and deviations from the predicted pNCC: plasma [K^+] relationship were analyzed by one way ANOVA with Tukey's post-hoc test, blood pressure changes were analyzed by linear regression. Student T-tests were used for all other analyses. For all analyses a $p<0.05$ was considered significant.

Chapter 3 -Development and Characterization of Kidney-Specific FKBP12 Knockout Mice

Abstract

The immunosuppressive drug tacrolimus inhibits its canonical target, the phosphatase calcineurin, only after binding to an endogenous protein FKBP12. Previous work has shed light on the importance of the kidney in the development of tacrolimus-mediated pathologies such as hypertension and electrolyte disorders. Specifically, we have found that tacrolimus increases the activity of the thiazide-sensitive renal sodium chloride cotransporter (NCC) and that this is necessary for the development of tacrolimus-induced hypertension.

It is currently unclear, however, whether tacrolimus leads to pathology by binding to and disrupting FKBP12's endogenous functions or by inhibiting its canonical target calcineurin. Here we test the hypothesis that tacrolimus-induced pathologies are due to FKBP12 disruption along the nephron. To do this, we developed a murine model in which FKBP12 is inducibly deleted from the nephrons of adult mice (KS-FKBP12$^{-/-}$). We find that, relative to their untreated control littermates KS-FKBP12$^{-/-}$ mice are normotensive, with normal electrolyte balance and normal levels of both total and phosphorylated (active) NCC.

Introduction

Tacrolimus is a widely used immunosuppressive drug, which requires a binding partner, FKBP12 to inhibit its canonical target calcineurin. FKBP12, however, has its own important physiologic functions, which are presumably disrupted by binding to tacrolimus. Thus, tacrolimus side effects, including hypertension, hyperkalemia, hypomagnesemia, and hypercalciuria, may be due to FKBP12 disruption. If this is the case drugs which target calcineurin without interfering with FKBP12 may be substantially safer immunosuppressive agents.

FKBP12 is the canonical member of the FKBP family of proteins. This large family with diverse functions has, as a common denominator, an FKBP binding motif.[133] Though FKBP12 is most famous for binding to the immunosuppressive agents tacrolimus and rapamycin, it is also involved in protein folding,[134] TGF-beta signaling,[81] and calcium receptor stabilization.[133] Both FKBP12 and cyclophilin A (the binding partner of the calcineurin inhibitor cyclosporine) are peptidyl prolyl isomerases.[134] These are molecules that assist proteins in converting cis x-proline bonds into the trans conformation. This common function has fueled arguments that disruption of FKBP12 and cyclophilin A (dubbed immunophilins) contribute to calcineurin inhibitor pathologies.

FKBP12 is essential, as systemic deletion of FKBP12 in mice is embryonic lethal, owing to developmental heart defects.[83] Moreover, disruption of FKBP12 in the vasculature and hematopoietic cells has been reported to cause hypertension.[81] Thus, FKBP12 appears to play an important role in the cardiovascular system and potentially blood pressure regulation.

Our recent work has identified the NCC as a necessary player in tacrolimus-induced hypertension. NCC, found in the Distal Convoluted Tubule (DCT) of the kidney, is activated by phosphorylation. Tacrolimus increases NCC phosphorylation, (pNCC) and blockade or deletion

of NCC ameliorates tacrolimus-induced hypertension. Whether FKBP12 disruption, by tacrolimus, is involved in this or related pathologies is currently unclear.

Though FKBP12 is thought to be ubiquitous, its role in the kidney has not been described. To this end I developed an inducible KS-FKBP12$^{-/-}$ mouse model specific to the epithelial cells of the nephrons to test the hypothesis that the increase in NCC phosphorylation, hypertension and electrolyte imbalance caused by tacrolimus is due to FKBP12 disruption along the nephrons.

Methods

Animals: All animal studies were approved by OHSU's animal care and use committee (protocol IS918). Kidney-specific FKBP12 knockout mice (KS-FKBP12$^{-/-}$) were generated by breeding FKBP12 $^{fl/fl}$ mice, generously donated by Dr. Susan Hamilton,[83] with Pax8-rtTA/TRE-LC1[129] mice, generously donated by Dr. James McCormick. Mice homozygous for floxed FKBP12 and possessing at least one copy of Pax8-rtTA and TRE-LC1 were identified by PCR genotyping of tail clipping DNA. Genetically identical mice were treated with either Doxycycline (2g/L, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) at 4-7 weeks of age for 2 weeks (water changed every 3 days). Male mice from 10-20 weeks old were used for experiments.

Genotyping: Genomic DNA from mouse tail clippings were heated in 75 uL NaOH (pH 12.0) in a hot block at 95 C for 45 min and neutralized with 75 uL Tris-HCl (pH 5.0). Genotypes were determined by PCR using the following primers:

FKBP12	F:5' AGAACTTGCCCTTCAGTATT 3'
	R:5'AGGCTTGTACCACTATTTTCT3'
CRE/LC1	F:5'TTTCCCGCAGAACCTGAAGATG 3'
	F: 5'TCACCGGCATCAACGTTTTCTT 3'
Pax8-rtTA	F-5'CCATGTCTAGACTGGACAAGA 3'
	R:-5'CAGAAAGTCTTGCCATGACT 3'

Genotypes were confirmed by immunoblotting for FKBP12 (abcam 2918, 1:5000, 1:5000 anti-rabbit) at the conclusion of experiments.

Genomic DNA semi-quantitative PCR: Genomic DNA was extracted from snap frozen tissues using Qiagen DNAeasy kit, as per the manufacturer's protocol. Semi-quantitative PCR was performed using the following primers:

FKBP12	F 5' GTCCTCTTTTCTCACGGT 3'
	R:5'AGGCTTGTACCACTATTTTCT 3'

mRNA PCR: Tissue was preserved at the time of collection in RNALater, snap frozen and stored at -80C. mRNA was extracted using Oligotex Direct mRNA mini kit. cDNA was transcribed using Finnzymes Phusion Kit and amplified by PCR using the following exon spanning primers:

FKBP12	F: 5'GAGTGCAGGTGGAGACCATCTCTC 3'
	R: 5'CATGGCAGATCCACGTGCAGAG 3'

Immunoblotting: Tissue lysates were prepared by dounce homogenizing snap frozen samples, on ice, in 1 mL chilled lysis buffer as previously described by McCormick et al.[112] Samples were spun down at 6000 RPM for 15 min at 4°C, proteins were separated on 4-12% Bis-Tris gels (Invitrogen) transferred overnight at 4°C and immunoblotted with antibodies described in the antibody table. Densitometry was quantitated using image J software (**http://rsbweb.nih.gov/ij/**) and proteins were normalized to actin.

Blood sample Analysis: Blood was obtained by cardiac puncture (under anesthesia) and put into heparinized tubes. 100 uL of blood was immediately pipetted into an I-STAT Chem 8^{+} cartridge (Abbott Pointe of Care) for analysis. The remaining sample was spun down at 2000

RPM for 5 min and the plasma was used to determine Mg^{2+} concentrations (Pointe Scientific, Xylidyl Blue assay).

Blood Pressure by Tail Cuff: Tail pressures were taken using the CODA 6 from Kent scientific. Experiments were begun daily at 9:30 AM. Mice were maintained at 32-36 degrees C with heating pads and acclimated for one week prior to data collection. After mice were warm, VPR tail cuffs were moved up the mouse's tail until resistance was felt. After 10 acclimation cycles, 15 data cycles were completed. Data was averaged daily for each mouse and as a group for each week.

Blood Pressure by Radiotelemetry: Mice were given 0.3mg/kg Buprenorphine (analgesic) and 10 mg/Kg Ciprofloxacin (antibiotic) prior to surgery. Under anesthesia (isoflurane in O_2; 5%-loading 1.5-2% maintenance) TA11PA-C20 radiotelemetry probes (Data Sciences International) were implanted into the left carotid artery of mice. Data collection began after 6-10 days of recovery and was collected for 20 seconds every 10 minutes for the length of experiments.

Metabolic cages: Mice were housed individually in metabolic cages (Hatteras instruments). After two days, to acclimate, urine was collected for 24 hours in glass jars containing a layer of water saturated mineral oil (to prevent evaporation). Food was provided in gel form and water was provided ad libitum.

Gel diet for metabolic cages: Gel diets consisted of 5 g of powdered sodium deficient diet (Harlan) 8 mL of water, 0.0225 g of bacterial agar and were reconstituted to 0.49% NaCl per serving.

Urinary electrolyte Analysis: Na^+ and K^+ urinary concentrations were measured by dual flame photometry (Cole-Parmer 2655-10), using reference mode and Li^{2+} internal standards. 10 uL of sample was used with 3.990 mL of molecular grade water. Urinary Ca^{2+} was measured by a colorimetric assay from Pointe Scientific (catalog #12-C7529-120).

Results

Generating KS-FKBP12$^{-/-}$ mice.

To study the effects of FKBP12 disruption in the kidney, I generated mice in which FKBP12 could be deleted along the nephron in response to doxycycline. I bred mice with loxP sites surrounding exon 3 of FKBP12[83] (FKBP12$^{fl/fl}$, generously donated by Susan Hamilton) to mice carrying Pax8-rtTA and TRE-LC1[129] (generously donated by Dr. Jim McCormick).

The resulting offspring were screened and selected to be homozygous for FKBP12$^{fl/fl}$ and have at least one copy of both Pax8-rtTA and TRE-LC1. These mice were born at the expected rate and appeared normal at birth and throughout development. At 4-7 weeks of age, the mice were given doxycycline to induce FKBP12 recombination and deletion. Genetically identical mice were treated with vehicle and serve as control mice in these experiments (see Methods). A cartoon schematic can be found in (Fig 3.1).

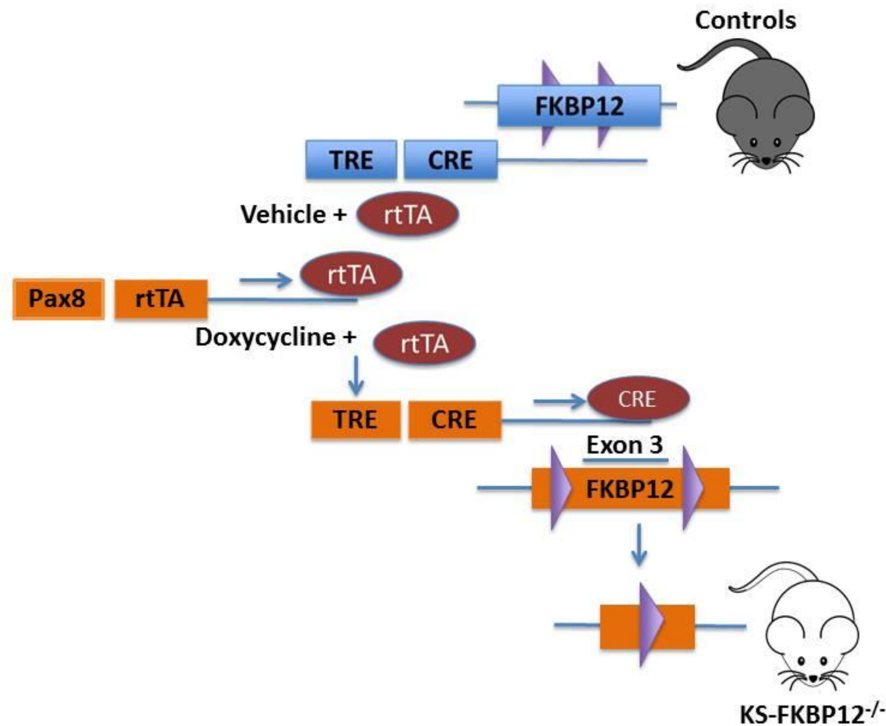

Figure 3.1-KS-FKBP12$^{-/-}$ Cartoon Schematic.

Control and KS-FKBP12$^{-/-}$ mice are genetically identical. Treatment with either vehicle (control) or Doxycycline (KS-FKBP12$^{-/-}$) determines experimental group.

Verifying FKBP12 recombination and deletion.

Doxycycline treatment was tolerated well and resulted in FKBP12 gene recombination that was detected in the kidney, but not heart, brain or muscle (Fig 3.2A). Recombination was also detected in the liver, consistent with prior reports using this system.[135] RT-PCR of mRNA from mice treated with doxycycline showed that the expected full-length FKBP12 transcript was absent in kidney. Instead, there was a shorter band, consistent with a transcript missing the excised exon

(Fig 3.2B). Western blot showed greater than 90% reduction in FKBP12 abundance in the kidney of KS-FKBP12$^{-/-}$ mice (Fig 3.2C), which was relatively exclusive to the kidney but also occurred in the liver, as reported by others (Fig 3.2D).

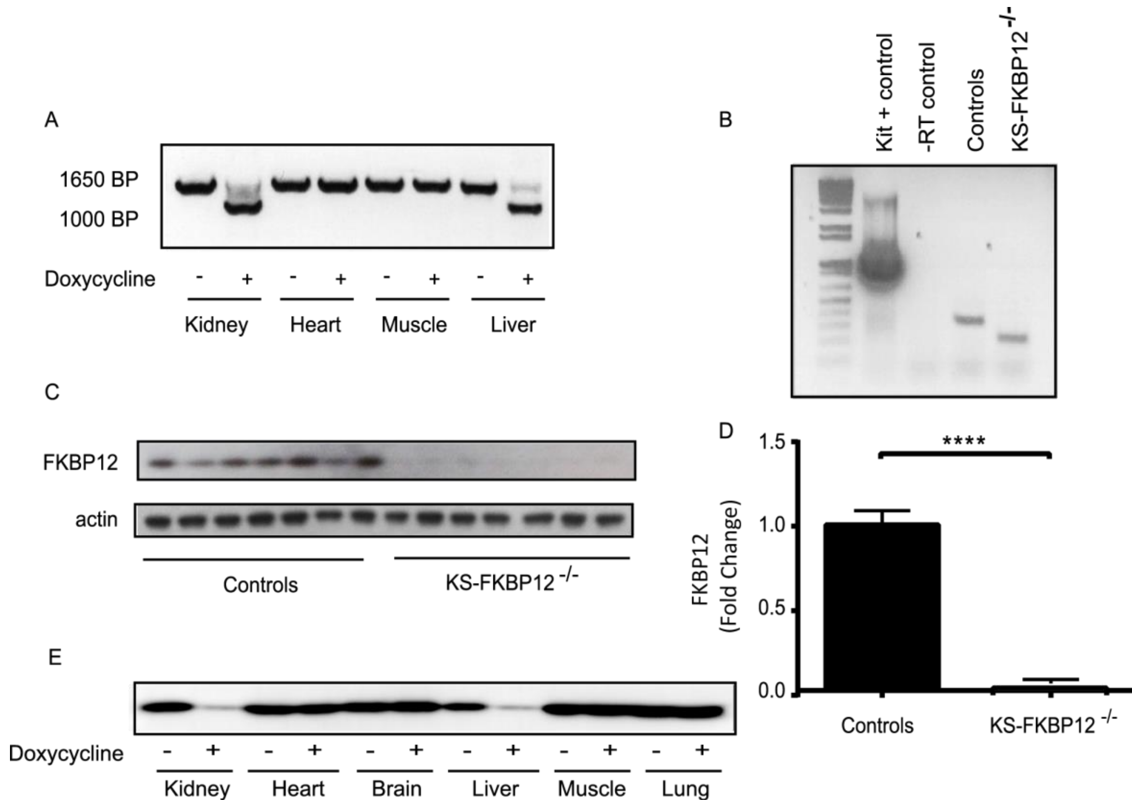

Figure 3.2-FKBP12 is effectively deleted in KS-FKBP12$^{-/-}$ mice.

A) Semi-quantitative PCR of Genomic DNA collected from control (-Dox) and KS-FKBP12$^{-/-}$ mice (+ Dox), demonstrating genomic recombination of FKBP12. B) Semi-quantitative PCR products derived from control and KS-FKBP12$^{-/-}$ mouse renal mRNA. A stable FKBP12 transcript is detected in KS-FKBP12$^{-/-}$ mice at the smaller, predicted size for FKBP12 missing floxed exon 3. C) Western blot of homogenized control and KS-FKBP12$^{-/-}$ kidneys. D) Quantification of panel C, showing significant reduction in FKBP12 renal protein expression (student's *t*-test, n=7, **** $p<0.0001$, $^{+}/-$ SEM). E) Western blot of FKBP12 across a panel of tissues showing recombination specificity at the protein level.

KS-FKBP12$^{-/-}$ mice appear healthy at baseline.

Control and KS-FKBP12$^{-/-}$ mice had similar body weights, urine volumes and plasma creatinine levels. Suggesting that FKBP12 deletion along the nephron does not lead to an overt renal phenotype (Table 3.1).

KS-FKBP12$^{-/-}$ Blood Pressure is the same as controls.

Tacrolimus causes hypertension, as does FKBP12 deletion in the endothelial and hematopoietic cells of mice. Therefore, I investigated whether FKBP12 disruption along the nephron also leads to an increase in blood pressure. The systolic blood pressure of control and KS-FKBP12$^{-/-}$ mice were comparable by measurement with the VPR tail cuff, (Fig 3.3A) a method which has been successfully validated against radiotelemetry.[136] This did not rule out the possibility that KS-FKBP12$^{-/-}$ have a very subtle blood pressure phenotype or determine if they exhibited disruption of their diurnal blood pressure dipping pattern, another side effect of tacrolimus. To address this I measured blood pressure in both groups by radiotelemetry, a more sensitive technique that also allows for non-invasive 24 hour blood pressure monitoring. Both the mean arterial pressure and diurnal dipping patterns of KS-FKBP12$^{-/-}$ mice were comparable to control mice (3.3B). As was the diastolic and systolic pressures, and heart rates of the mice (Fig 3.3C-E).

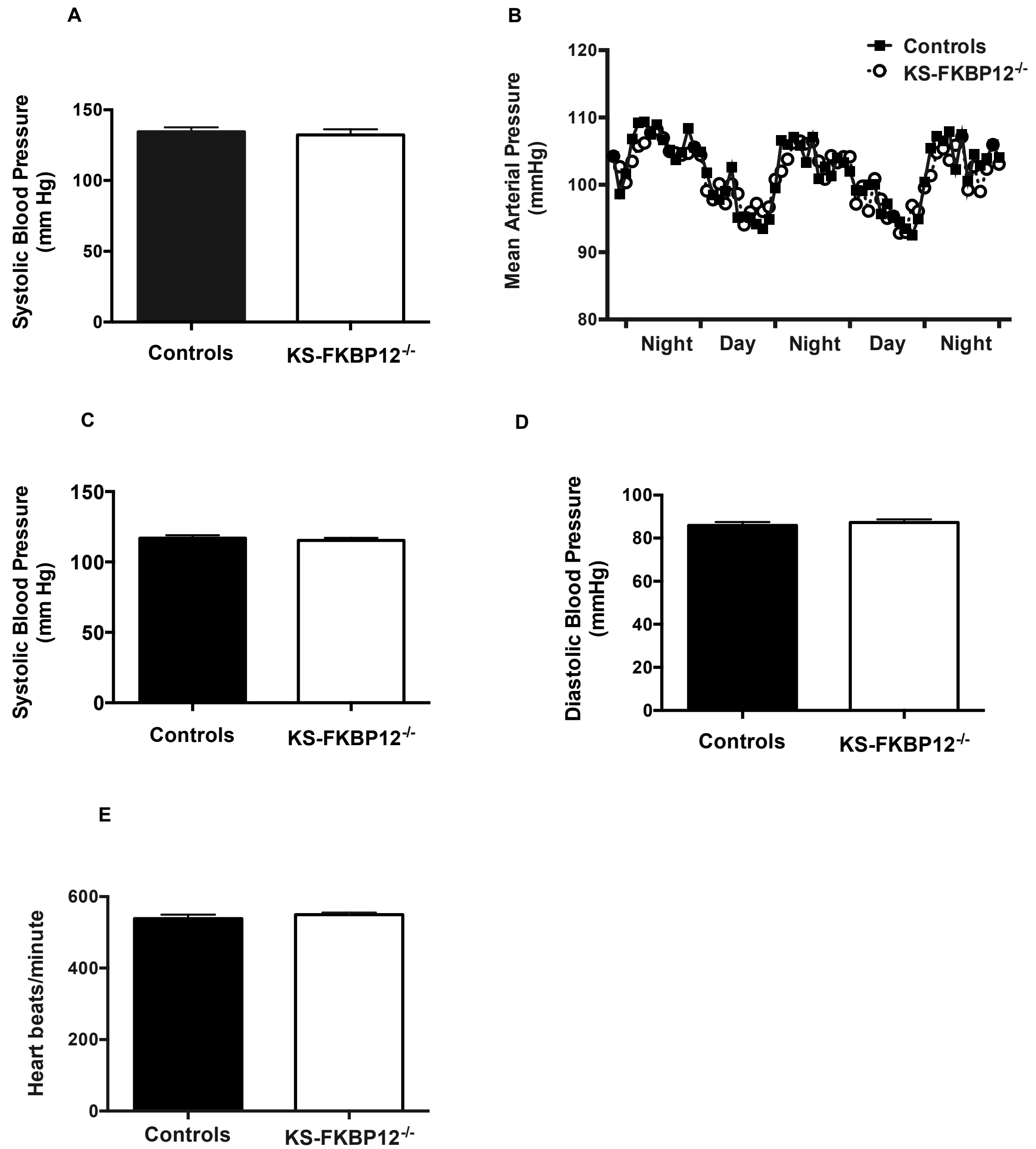

Figure 3.3-Blood pressure and heart rate are the same between Control and KS-FKBP12$^{-/-}$ mice.

A) By VPR tail cuff no difference was detected in systolic blood pressure between the two groups (N=14) B) By the more sensitive radiotelemetry no differences were detected in Mean Arterial Pressure or diurnal dipping patterns (n=9) c) Systolic blood pressures were the same by radiotelemetry (n=6), though lower than measured by tail cuff. This is in line with the increased sensitivity and accuracy of radiotelemetry, the effects of averaging

blood pressures over the day and night cycle and refraining from handling the mice, as is necessitated daily of VPR tail cuff. D) Diastolic blood pressure by radiotelemetry are no different between groups (n=6) E) Neither are heart rates measured by radiotelemetry (n=6). (*t*-test, P<0.05, +/-SEM).

Plasma electrolytes of KS-FKBP12$^{-/-}$ mice are comparable to controls.

Plasma electrolytes of KS-FKBP12$^{-/-}$ mice were normal, relative to control mice, with the exception of plasma Cl^-. Although plasma Cl^- and the anion gap were significantly lower in KS-FKBP12$^{-/-}$ mice (Table 3.1), these differences were very small and of questionable physiological significance. Further, when electrolyte values were determined 6 months after deletion of FKBP12, the phenotype remained normal relative to controls, including plasma Cl^- and anion gap (Table 3.2).

Table 3.1-Baseline Phenotype of KS-FKBP12$^{-/-}$ mice.

(t-test, p<0.05)

	units	Controls	KS-FKBP12$^{-/-}$	P-value	n
Body Weight	g	28	27.56	0.50	14
Plasma Values					
Na^+	mmol/L	145.29	144.43	0.12	7
K^+	mmol/L	3.94	3.96	0.93	7
Cl^-	mmol/L	107.14	105.29	0.03 *	7
Ca^{2+}	mmol/L	1.24	1.23	0.37	7
Mg^{2+}	mmol/L	2.33	2.33	0.99	7-8
TCO_2	mmol/L	25.71	27.57	0.07	7
BUN	mg/dL	18.14	16.57	0.19	7
Creatinine	mg/dL	0.30	0.30	1.00	7
Hematocrit	%PCV	40.71	42.71	0.23	7
Hemaglobin	g/dL	13.84	14.54	0.22	7
Anion Gap	mmol/L	17.57	16.29	0.03*	7
Urinary Values					
urine volume	mL	4.14	4.81	0.14	13
Na^+	µmol/day/g bodyweight	3.433	4.278	0.43	13
K^+	µmol/day/g bodyweight	9.04	9.999	0.30	13
Ca^{2+}	mg/g bodyweight/24 hours	0.0297	0.0297	1.00	7

Urinary Electrolytes are the same between groups.

Urinary Na^+, K^+ and Ca^{2+} excretion were similar between control and KS-FKBP12$^{-/-}$ mice (Table 3.1).

Table 3.2-Baseline Electrolytes of Aged Mice.

Electrolytes of mice 6 months after doxycycline-induced FKBP12 recombination (t-test, $p<0.05$).

Plasma values	units	Control	KS-FKBP12$^{-/-}$	*p*-value	n
Na^+	mmol/L	142.50	142.00	0.67	4
K^+	mmol/L	4.58	4.35	0.31	4
Cl^-	mmol/L	102.25	102.50	1	4
Ca^{2+}	mmol/L	1.27	1.27	1.00	4
TCO_2	mmol/L	27.25	28.25	0.65	4
BUN	mg/dL	24.25	21.25	0.09	4
Creatinine	mg/dL	0.50	0.48	0.75	4
Hematocrit	%PCV	38.00	36.00	0.11	4
Hemaglobin	g/dL	12.93	12.23	0.10	4
Anion Gap	mmol/L	18.50	17.00	0.47	4

NCC is the same between groups.

Our previous work implicated an increase in NCC phosphorylation, a marker of activity, in tacrolimus-induced pathologies.[57] At baseline, levels of phosphorylated NCC (pNCC, active form) and total NCC (tNCC, both active and inactive) were similar between control and KS-FKBP12$^{-/-}$ mice (Fig 3.4).

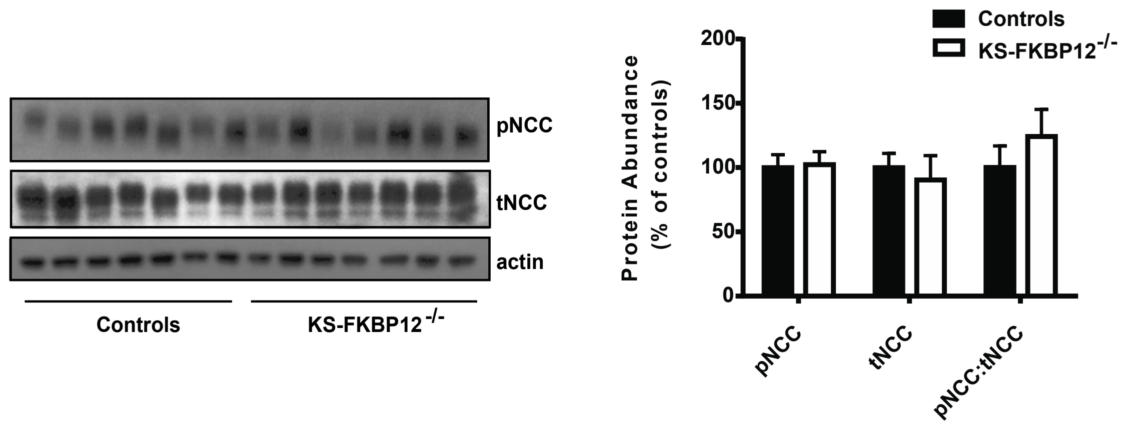

Figure 3.4 –Control and KS-FKBP12$^{-/-}$ mice have similar levels of pNCC and tNCC at baseline.

A) Western Blot of homogenized kidneys from control and KS-FKBP12$^{-/-}$ mice probed for pNCC and tNCC at baseline. B) Quantification of panel A, levels of pNCC and tNCC are comparable (n=7, *t*-test, $p<0.05$, $^{+}$/-SEM).

Discussion

Tacrolimus is a widely used immunosuppressive drug that binds to an endogenous protein, FKBP12, before inhibiting its target the phosphatase calcineurin. Unfortunately, like other calcineurin inhibitors, tacrolimus often causes hypertension, hyperkalemia, hypomagnesemia and hypercalciuria. We recently discovered that tacrolimus causes an increase in the abundance of phosphorylated NCC (active) and that functional NCC is necessary for the development of tacrolimus-mediated hypertension. It remained unclear, however, whether FKBP12 disruption along the nephron was playing a role in the increase in pNCC, hypertension and electrolyte imbalance.

Though FKBP12 is found in the kidney its role there is unknown. I generated KS-FKBP12$^{-/-}$ mice in which FKBP12 is deleted along the nephron of adult mice in response to Doxycycline treatment. As the floxed FKBP12 mice used in my breeding strategy were previously used to produce an embryonic lethal strain of FKBP12$^{-/-}$ mice and doxycycline treatment resulted in greater than 90% reduction in FKBP12 protein expression and was confirmed by multiple techniques I am confident that KS-FKBP12$^{-/-}$ mice lack functional FKBP12 along the nephron.

Despite this, KS-FKBP12$^{-/-}$ mice, do not have an apparent phenotype at baseline. Suggesting that along the nephron, FKBP12, though present, does not play a critical role in NCC, blood pressure or electrolyte regulation. As FKBP12 is a member of a larger family of related proteins it is possible that a related protein may be able to compensate for loss of FKBP12 function along the nephron.

My results suggest, however, that immunophilin sparing calcineurin inhibitors will not ameliorate the renal component of tacrolimus-induced hypertension and electrolyte disorders.

Acknowledgements

I thank N. Desmarais for technical assistance (OHSU) and Dr. Susan Hamilton and Dr. Jim McCormick for the mice used to generate the KS-FKBP12$^{-/-}$ line. This work was supported by the NIH (DK 095841 to D.H.E. and C.L.Y.) and the AHA (predoctoral award to R.A.L. 14PRE18330021). This work was performed by R.A.L. in partial fulfillment for a PhD in Pharmacology and Physiology from Oregon Health and Science University. Portions of this work have been presented at scientific meetings[137-140] and have been accepted for publication at JASN under the title *Renal FKBP12 Deletion Attenuates Tacrolimus-Induced Hypertension.*

Chapter 4 -Tacrolimus-induced Hypertension and Hyperkalemia

Abstract

Tacrolimus is a widely used immunosuppressive drug that inhibits the phosphatase calcineurin when bound to the endogenous protein FKBP12. When this occurs in T-cells, it leads to immunosuppression. Tacrolimus also causes side effects, however, such as hypertension and hyperkalemia. Previously, we reported that tacrolimus stimulates the renal thiazide-sensitive sodium chloride cotransporter (NCC) which is necessary for the development of hypertension. It was unclear if this was due to effects of tacrolimus directly on calcineurin in renal epithelial cells, or in extra-renal tissues. To test this I developed a mouse model in which FKBP12 could be deleted along the nephron. FKBP12 disruption itself did not cause phenotypic effects. When treated with tacrolimus, however, mice lacking FKBP12 along the nephron had lower blood pressures and less of the phosphorylated, active, form of the sodium chloride cotransporter than control mice. They also maintained a normal relationship between plasma potassium and the abundance of the phosphorylated form of the sodium chloride cotransporter. In cultured cells, tacrolimus inhibited dephosphorylation of the sodium chloride transporter. Together, these results suggest that tacrolimus causes hypertension largely by inhibiting calcineurin directly in cells expressing the sodium chloride cotransporter. They suggest that thiazide diuretics will be particularly effective for lowering blood pressure in tacrolimus-treated hypertensive patients.

Introduction

Tacrolimus, a widely prescribed calcineurin inhibitor, is an immunosuppressive drug often used to prevent the rejection of transplanted organs.[141] Its use, however, is frequently complicated by side effects, including hypertension, hyperkalemia, and chronic kidney disease.[32] We reported previously that tacrolimus causes hypertension and increases the abundance of the phosphorylated, active, form of the renal sodium chloride cotransporter (NCC) in mice. We showed that NCC activity is essential for the full development of hypertension, and confirmed that tacrolimus increases the abundance of phosphorylated NCC (pNCC) in humans, as well.[57] The molecular mechanisms, however, remain largely unknown.

To inhibit calcineurin, a serine/threonine phosphatase, tacrolimus must bind to an endogenous protein, FKBP12.[68] When this occurs in T-cells, cytokine production is inhibited and immunosuppression results. Calcineurin and FKBP12, however, are not expressed exclusively in T-cells. Both proteins are widely distributed, so it is possible that calcineurin inhibition in the vasculature, the nervous system, or even T cells themselves, contributes to tacrolimus-induced hypertension. Alternatively, calcineurin inhibition in the distal convoluted tubule (DCT), where NCC is expressed may activate NCC directly, leading to hypertension.

Here, I tested the hypothesis that tacrolimus activates NCC and causes systemic hypertension by inhibiting calcineurin in the DCT. I used a mouse model in which FKBP12 can be deleted inducibly along the nephron to test this hypothesis *in vivo*, and corroborated the findings in cultured cells. The results confirm that FKBP12-dependent effects of tacrolimus, such as calcineurin inhibition, along the nephron are largely responsible for its actions on blood pressure and potassium homeostasis.

Methods

Nephron Segment qRT-PCR: mRNA was extracted from microdissected nephron segments using RNA extraction kit (Invitek). cDNA was synthesized by reverse transcription (Tetro Reverse Transcriptase, Promega). Quantitative PCR was performed using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). Gene expression analysis was performed applying the ΔCt method and normalized against β-Actin.

Animals: Studies were approved by OHSU's animal care and use committee (protocol IS918) and adhered to the NIH Guide for the Care and Use of Laboratory Animals. KS-FKBP12$^{-/-}$ were generated by breeding FKBP12 $^{fl/fl}$ mice with Pax8-rtTA/TRE-LC1 mice. Mice homozygous for floxed FKBP12 and possessing at least one copy of Pax8-rtTA and TRE-LC1 were identified by PCR genotyping of tail clipping DNA. Genetically identical mice were treated with either Doxycycline (2g/L, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) at 4-7 weeks of age for 2 weeks. Male mice from 10-20 weeks old were used for experiments.

Genomic DNA semi-quantitative PCR: Genomic DNA was extracted from snap frozen tissues using Qiagen DNAeasy kit and amplified by semi-quantitative PCR.

mRNA PCR: Tissue was preserved in RNALater, mRNA was extracted with Oligotex Direct mRNA mini kit, cDNA was transcribed using Finnzymes Phusion Kit and amplified by PCR using exon spanning primers.

Immunoblotting: Tissue lysates were prepared by dounce homogenizing snap frozen samples, in chilled lysis buffer as described .[112] Samples were spun and separated on 4-12% Bis-Tris gels (Invitrogen) and immunoblotted with antibodies described in the Supplement. Blots were quantified using image J software(**http://rsbweb.nih.gov/ij/**)

Blood sample Analysis: Blood was obtained by cardiac puncture in heparinized tubes. 100 uL was used for analysis by I-STAT (Chem 8^+ cartridge, Abbott Pointe of Care). The rest was spun and the plasma used for a colorimetric Mg^{2+} assay (Pointe Scientific, Xylidyl Blue assay).

Blood Pressure: Mice were given 0.3mg/kg Buprenorphine (analgesic) and 10 mg/Kg Ciprofloxacin (antibiotic) prior to surgery. Under isoflurane anesthesia TA11PA-C20 radiotelemetry probes (Data Sciences International) were implanted into the left carotid artery of mice. Data collection began after 6-10 days of recovery and was collected for 20 seconds every 10 minutes for the length of experiments.

Tacrolimus: Powdered tacrolimus was dissolved in a 3:1 solution of DMSO:tween-20 to 30 mg/mL were. This was diluted with PBS to 15 ug/mL, mice were injected subcutaneously with 3 mg/Kg for 18 days.

pNCC: plasma [K^+] ratio: The absolute difference between predicted pNCC values, using the best fit line for previous data $y=6.593^{(-0.5383*x)}-0.01761$ and actual pNCC values (pNCC densitometry was normalized to untreated control mice on a normal diet run on the same western blot) were averaged.

***In vitro* NCC dephosphorylation assay:** HEK-293 cells expressing NCC (described in Hoorn 2011[57]) and transiently transfected with SPAK (T243E/S383D) were treated for 25 minutes with 10 mM $BaCl_2$. Cells were pre-treated with 30 ug/mL of tacrolimus for 25 min before treating with $BaCl_2$.

Statistics: Nephron tubule segment specific qRT-PCR and deviations from the predicted pNCC: plasma [K^+] relationship were analyzed by one way ANOVA with Tukey's post-hoc test,

blood pressure changes were analyzed by linear regression. Student T-tests were used for all other analyses. For all analyses a $p<0.05$ was considered significant.

Supplementary methods

Nephron Segment qRT-PCR Primers:

FKBP12	F:5'ACTACACGGGGATGCTTGAA 3'
	R:5'GCTCTCTGACCCACACTCAT 3'
Calcineurin-α	F:5'CCAACACTCGCTACCTCTTC 3'
	R:5' GTGCCTACATTCATGGTTTCC 3'
Calcineurin-β	F:5' GCAACCATGAATGCAGACACC 3'
	R:5'CAAGGGGCAAGCTGTCAAAAG 3'

Animals: Control and FKBP12$^{-/-}$ mice were generated by crossing FKBP12$^{fl/fl}$ mice with Pax8-rtTA/TRE-LC1 mice. Dr. Susan Hamilton graciously supplied the FKBP12$^{-/-}$ mice, which were generated using AB2.1 ES cells[128] and were crossed with C57bl/6 mice (personal correspondence) to produce chimeric offspring. Dr. Jim McCormick generously supplied the CRE Pax8-rtTA/TRE-LC mice, which are on a C57bl/6 background (personal correspondence).

Tacrolimus: Tacrolimus stock solutions of 30 mg/mL were prepared by dissolving powdered tacrolimus in a 3:1 solution of DMSO:tween-20. Working solutions were prepared fresh, daily, by diluting with PBS to 15 ug/mL. Mice were injected subcutaneously with 3 mg/Kg tacrolimus daily, for 18 days at 9 AM and tissues were collected at 4 PM.

Use of Male Mice: I restricted the analysis to male mice, as this is a follow on paper, extending prior results obtained in males. Further, there are gender differences in tacrolimus metabolism that would have made the feasibility of these difficult studies difficult.[130]

Power Calculations: We did power calculations to determine the number of mice needed. To detect a 5 mmHg difference in blood pressure, using an alpha value of 0.05 power of 0.8, and a delta of 3 mmHg (reported by the manufacturer, DSI international) n=6 is required. Previously, we found that tacrolimus induces a 40% increase in NCC phosphorylation.[57] To detect this, assuming a standard deviation of 27 (previous work[57]) and using an alpha of 0.05 and a power of 0.8, an n=7 is needed to detect such differences by Western Blot.

Genotyping: Genomic DNA from mouse tail clippings were heated in 75 uL NaOH (pH 12.0) at 95 C for 45 min and neutralized with 75 uL Tris-HCl (pH 5.0). Genotypes were determined by PCR using 4 uL of crude genomic lysate, 21 uL of Invitrogen TaqDNA Polymerase native master mix and the following primers:

FKBP12:F:5' AGAACTTGCCCTTCAGTATT 3' R:5'AGGCTTGTACCACTATTTTCT3'**CRE:**F:5'TTTCCCGCAGAACCTGAAGATG 3'R:5'TCACCGGCATCAACGTTTTCTT 3'**Pax8:**F-5'CCATGTCTAGACTGGACAAGA 3'R:-5'CAGAAAGTCTTGCCATGACT 3'. Genotypes were confirmed by immunoblotting for FKBP12 (abcam 2918, 1:5000) at the conclusion of experiments.

Immunoblotting: Tissue lysates were prepared by dounce homogenizing snap frozen samples, on ice, in 1 mL chilled lysis buffer as previously described by McCormick et al.[112] Samples were spun down at 6000 RPM for 15 min at 4 C, proteins were separated on 4-12% Bis-Tris gels (Invitrogen) transferred overnight at 4 C and immunoblotted.

Antibodies: The following specific antibodies were used:

pNCC (1:4000) (1:6000 anti-rabbit)[142]

tNCC (1:10,000) (1:5000 anti-rabbit)[143]

tNKCC2 (1:3000)(1:5000)[144]

β-actin abcam ab8227 (1:10,000) (1:10,000 anti-rabbit)

FKBP12 abcam ab2918 (1:5000) (1:5000 anti-rabbit)

WNK4 (1:4000) (1:5000 anti-rabbit)[145]

SPAK (1:5000 overnight) (1:5000 anti-rabbit)-generous gift from Eric Delpire

OxSR1 (1:5000 overnight) (1:5000 anti-rabbit)-generous gift from Eric Delpire

pNKCC2 (1:7000) (1:75000)-Generous gift from Sebastian Bachmann

Blood sample Analysis: Blood was obtained by cardiac puncture (under anesthesia) and put into heparinized tubes. 100 uL of blood was immediately pipetted into an I-STAT Chem 8^{+} cartridge (Abbott Pointe of Care) for analysis. The remaining sample was spun down at 2000 RPM for 5 min and the plasma was used to determine Mg^{2+} concentrations (Pointe Scientific, Xylidyl Blue assay).

Blood Pressure: Mice were given 0.3mg/kg Buprenorphine (analgesic) and 10 mg/Kg Ciprofloxacin (antibiotic) prior to surgery. Under anesthesia (isoflurane in O_2; 5%-loading 1.5-2% maintenance) TA11PA-C20 radiotelemetry probes (Data Sciences International) were

implanted into the left carotid artery of mice. Data collection began after 6-10 days of recovery and was collected for 20 seconds every 10 minutes for the length of experiments.

Results

FKBP12 and calcineurin are found along the nephron.

To determine whether tacrolimus could exert direct effects in the DCT, Katharina Blankenstein used nephron-segment quantitative RT-PCR to identify sites along the nephron where FKBP12 and calcineurin are expressed. FKBP12 was expressed by all nephron segments and within glomeruli (Fig 4.1A). Confirming previous results, the α and β isoforms of calcineurin were found along the nephron and glomeruli (Fig 4.1B). As CaN-α and CaN-β are 81% homologous[146] they confirmed that the results were isoform specific by using tissue from CaN-$\beta^{-/-}$ mice as a control (Fig 4.1C).

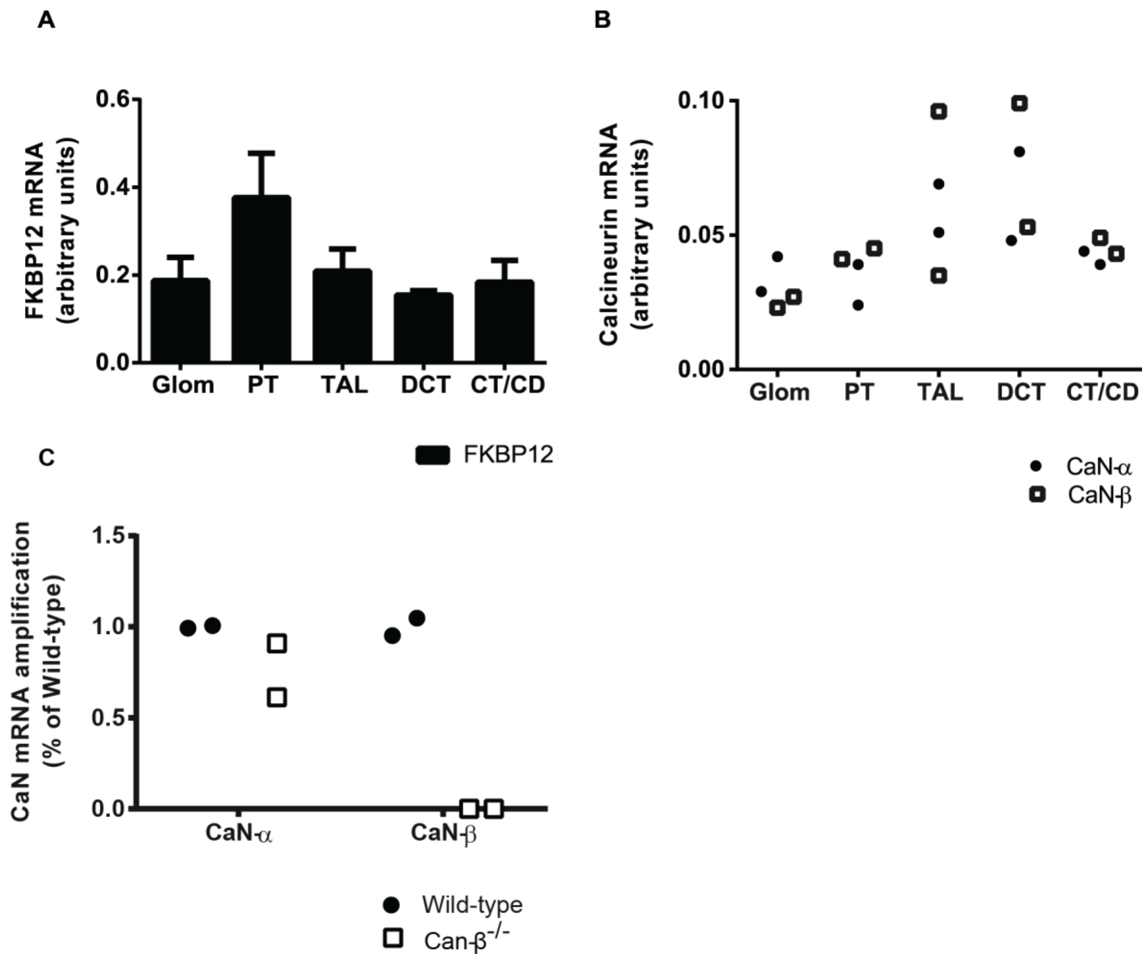

Figure 4.1-FKBP12 and calcineurin mRNA are present along the nephron.

qRT-PCR of mRNA, extracted from microdissected nephron segments (Glomerulus (Glom) Proximal Tubule (PT) Thick Ascending Limb (TAL) Distal Convoluted Tubule (DCT) Connecting Tubule/Collecting Duct (CT/CD)). A) FKBP12 mRNA is distributed evenly across nephron segments (one-way ANOVA, $p<0.05$, n=3, $^{+}$/-SEM). B) Confirming prior results, Calcineurin is found in the DCT. Both the Calcineurin-α and β isoform are present along the nephron (n=2). *(segmental controls published in JCI,[147] Primer controls in supplemental figure 1)* C) To verify primer specificity Primers specific to Calcineurin-α (CaN-α) and Calcineurin-β (CaN-β) were used to amplify Wild type and CaN-$\beta^{-/-}$ kidney samples. CaN-α primers were able to generate a signal in CaN-$\beta^{-/-}$ tissues while CaN-β primers did not, confirming primer specificity (n=2).

Tacrolimus concentrations are similar in control and KS-FKBP12$^{-/-}$ mice.

Tacrolimus is known to be metabolized by the liver. Though KS-FKBP12$^{-/-}$ mice have relatively specific reduction of FKBP12 in the kidney, there is some recombination in the liver (See chapter 3 Fig 2). As its function there is unknown, I wanted to be sure that KS-FKBP12$^{-/-}$ mice are still capable of properly metabolizing tacrolimus. Therefore, I submitted plasma blood spot samples from control and KS-FKBP12$^{-/-}$ mice treated with tacrolimus for 18 days to the Pharmacokinetics core at OHSU. Dr. Dennis Koop and Lisa Bleyle analyzed the samples by liquid chromatography tandem Mass Spectrometry. A new method which they developed, detailed in their recent publication.[132] There was not a statistically significant difference in plasma [tacrolimus] between the genotypes (Fig 4.2).

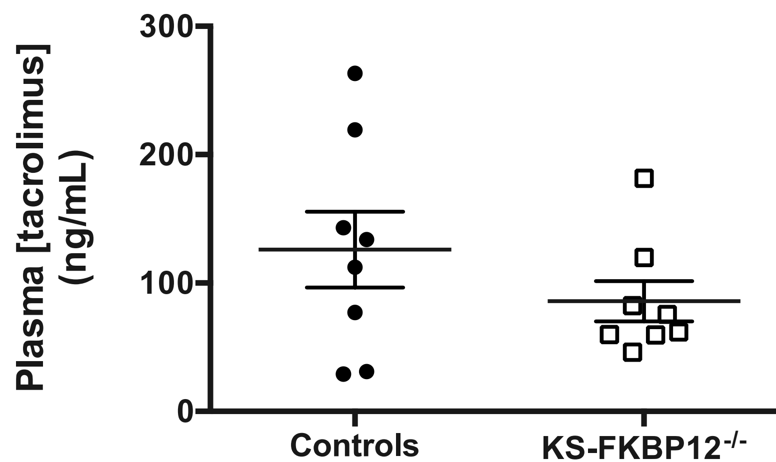

Figure 4.2-The plasma concentration of tacrolimus is similar between control and KS-FKBP12$^{-/-}$ mice. (n=8, t-test $p<0.05$, $^{+}$/- SEM).

Effects of tacrolimus on arterial pressure are attenuated in KS-FKBP12$^{-/-}$ mice.

To inhibit calcineurin, tacrolimus must bind to FKBP12. Thus, KS-FKBP12$^{-/-}$ mice are vulnerable to the effects of tacrolimus outside of the kidney, but protected from FKBP12-dependent events, along the nephron. Thus, I tested if tacrolimus had different effects in control and KS-FKBP12$^{-/-}$ mice. Tacrolimus treatment caused an initial increase in mean arterial pressure in both groups, but blood pressure continued to rise in the control mice, whereas it remained stable in the KS-FKBP12$^{-/-}$ mice (Fig 4.3). The difference between the slopes of the blood pressure curves was significant (Fig 4.4A). After 14 days of tacrolimus treatment, although MAP remained significantly higher in control mice relative to KS-FKBP12$^{-/-}$ mice, blood pressures in both groups began to decline, suggesting some adaptation (data not shown).

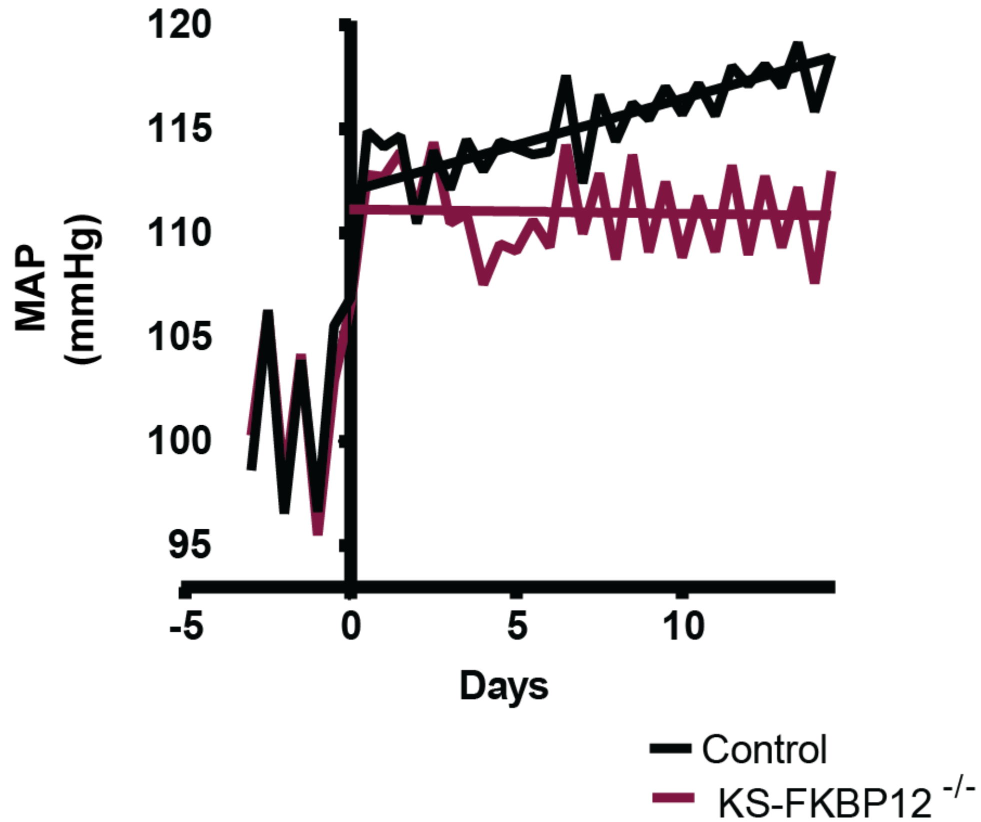

Figure 4.3-KS-FKBP12$^{-/-}$ mice are protected from a tacrolimus-mediated increase in blood pressure and maintain diurnal dipping patterns.

Average 12 hour MAP for light and dark cycles in control (-Dox +Tac) and KS-FKBP12$^{-/-}$ mice (+Dox +Tac) treated with 3 mg/Kg of tacrolimus for 18 days (n=6).

In rodents and in humans, blood pressure typically exhibits a diurnal rhythm, with a decline, called 'dipping', during the inactive period of the day. Calcineurin inhibitors have been reported to attenuate diurnal blood pressure dipping in humans.[34] In the current experiments, dipping was initially disrupted by tacrolimus injections in both groups. Dipping, however, appeared to recover better in KS-FKBP12$^{-/-}$ mice than in controls (Fig 4.4B & C).

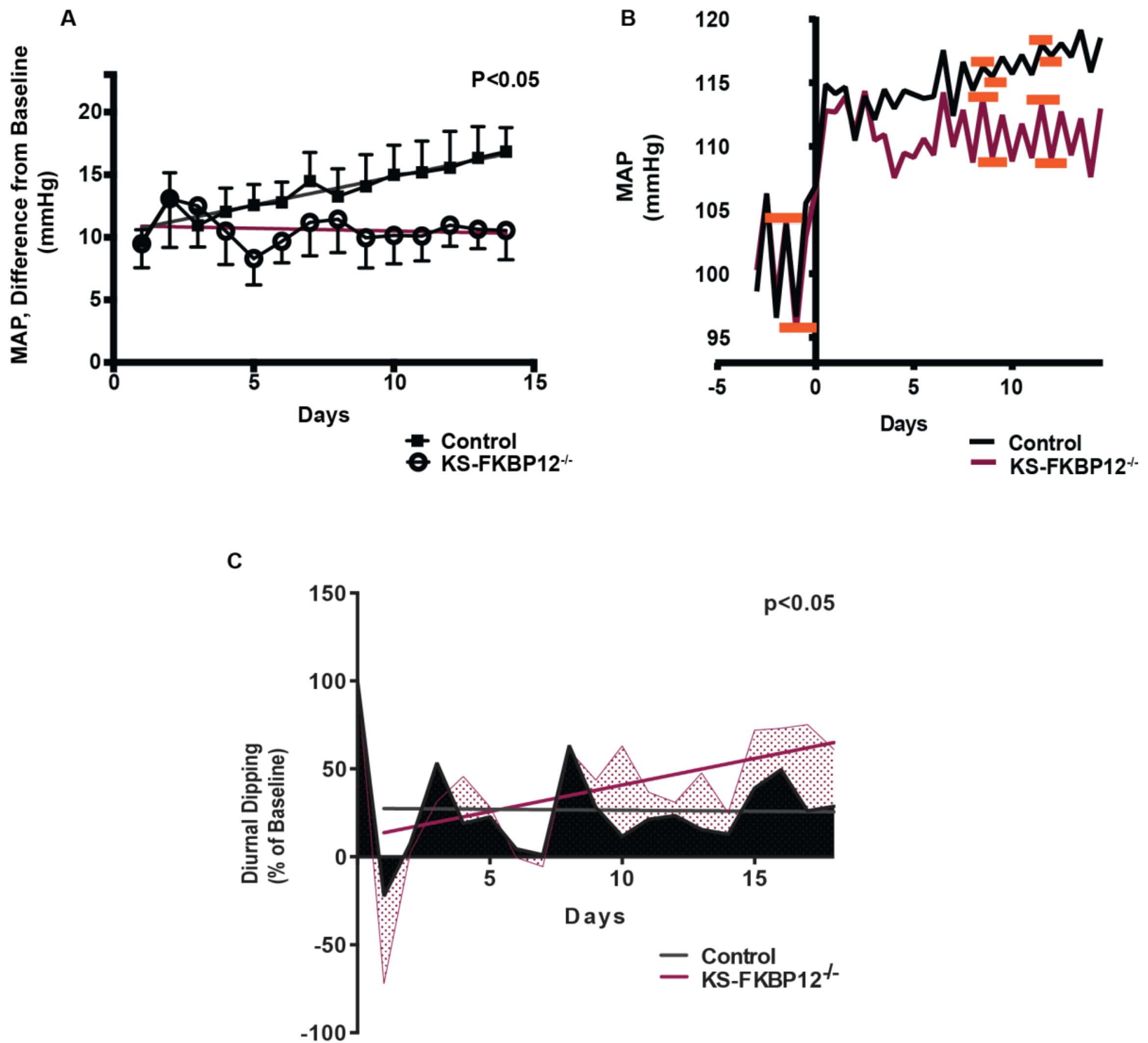

Figure 4.4-Quantification of KS-FKBP12$^{-/-}$ protection from tacrolimus-induced blood pressure abnormalities.

A) Differences from baseline in average 24 hour MAP in control (-Dox) and KS-FKBP12$^{-/-}$ (+Dox) mice treated with 3 mg/Kg subcutaneously for 18 days (difference in slopes of linear regression, $p<0.05$, n=6). B) Illustration of maximal and minimal values used to calculate diurnal dipping patterns. C) The average diurnal-dipping pattern (difference between average MAP during 12 hour dark cycle and 12 hour light cycle) at baseline (average of values from pretreatment days) is set at 100%. Daily diurnal dipping patterns with tacrolimus treatment are represented as a percentage of baseline dipping. Linear regression illustrates that though there is initial disruption

in both groups KS-FKBP12$^{-/-}$ mice begin to recover their diurnal dipping patterns more effectively than control mice (difference in slopes of linear regression, p<0.05, n=6).

pNCC is lower in tacrolimus-treated KS-FKBP12$^{-/-}$ mice than tacrolimus-treated controls..

Our previous work illustrated the importance of NCC in tacrolimus-induced hypertension and showed that tacrolimus treatment increased pNCC abundance without affecting tNCC abundance.[57] Here, the abundance of pNCC was significantly higher in the kidneys of tacrolimus-treated control mice than in the kidneys of tacrolimus-treated KS-FKBP12$^{-/-}$ mice (Fig 4.5), suggesting that tacrolimus increases pNCC by inhibiting calcineurin along the nephron. tNCC was slightly lower in tacrolimus-treated control mice than tacrolimus-treated KS-FKBP12$^{-/-}$ mice (Fig 4.5).

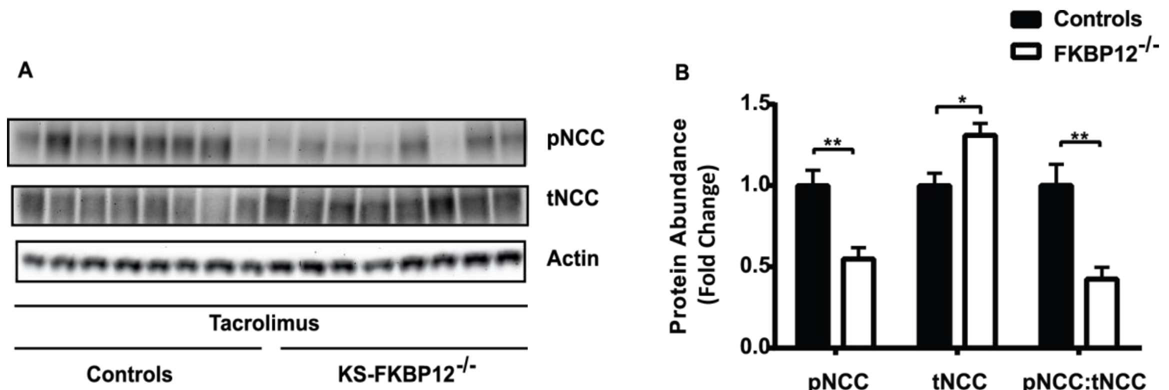

Figure 4.5-pNCC is lower in tacrolimus treated KS-FKBP12$^{-/-}$ mice than controls.

A) Western Blot of kidney lysates from tacrolimus-treated control (-Dox +Tac) and KS-FKBP12$^{-/-}$ (+Dox +Tac) mice probed for pNCC (T53), tNCC and the loading control actin. B) Quantification of pNCC and tNCC normalized to actin as well as the pNCC: tNCC ratio in tacrolimus-treated control and KS-FKBP12$^{-/-}$ animals (*t*-test, *p<0.05, **p<0.01, n=8, $^{+}$/- SEM).

Tacrolimus-treated KS-FKBP12$^{-/-}$ mice also tended to excrete more Na^+ and K^+ than tacrolimus treated control mice although these differences did not quite reach statistical significance (Fig 4.6).

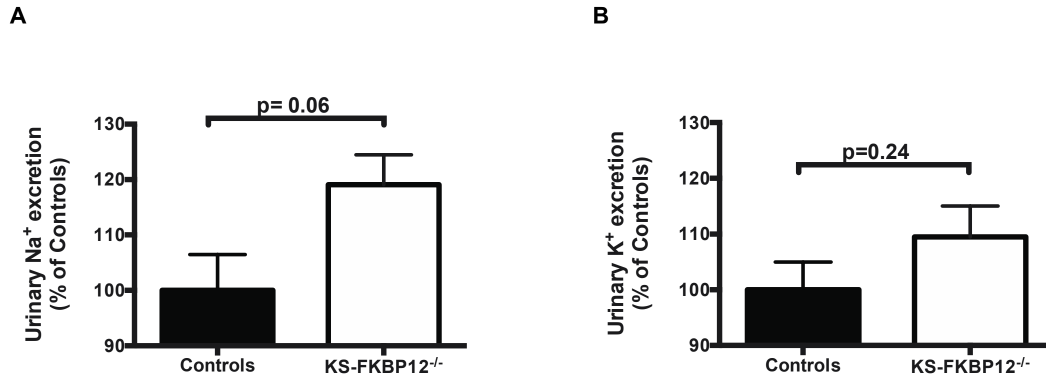

Figure 4.6-Tacrolimus-treated KS-FKBP12$^{-/-}$ mice have a tendency to excrete more Na^+ and K^+ than tacrolimus-treated controls.

Ion excretion was normalized to the tacrolimus-treated controls in each of the 2 experimental replicates. A) Na^+ excretion (mmol/24 hours/g bodyweight, % of controls) B) K^+ excretion (mmol/24 hours/g bodyweight, %of controls) (t-test, $P>0.05$, n=7-5, $^+$/- SEM).

Effects of tacrolimus on plasma [K^+] homeostasis.

Plasma K^+ concentration was not significantly higher in tacrolimus-treated control mice than in tacrolimus-treated KS-FKBP12$^{-/-}$ mice (Fig 4.7A). As there was a trend towards lower K^+ concentration in the KS-FKBP12$^{-/-}$ mice, and as our prior report documented subtle potassium dysregulation in tacrolimus-treated wild type mice,[57] I explored this issue further. We recently

found that pNCC abundance exhibits a strong curvilinear relationship with plasma [K^+] under many different conditions.[114] This suggests that pNCC abundance is driven strongly by plasma K^+ concentration, an effect that impacts downstream K^+ excretion and homeostasis. Here, I compared plasma K^+ concentration and pNCC abundance (see Methods). Control mice treated with tacrolimus had higher pNCC values than predicted (Fig 4.7C). They deviated significantly more from predicted values than both untreated control mice and KS-FKBP12$^{-/-}$ mice (Fig 4.7E). As activation of NCC tends to reduce potassium excretion, this provides evidence that dysregulated NCC may contribute to the hyperkalemia that can result from tacrolimus administration. In contrast to this, tacrolimus treated KS-FKBP12$^{-/-}$ mice exhibit pNCC values that fall close to the predicted normal range (Fig 4.7D).

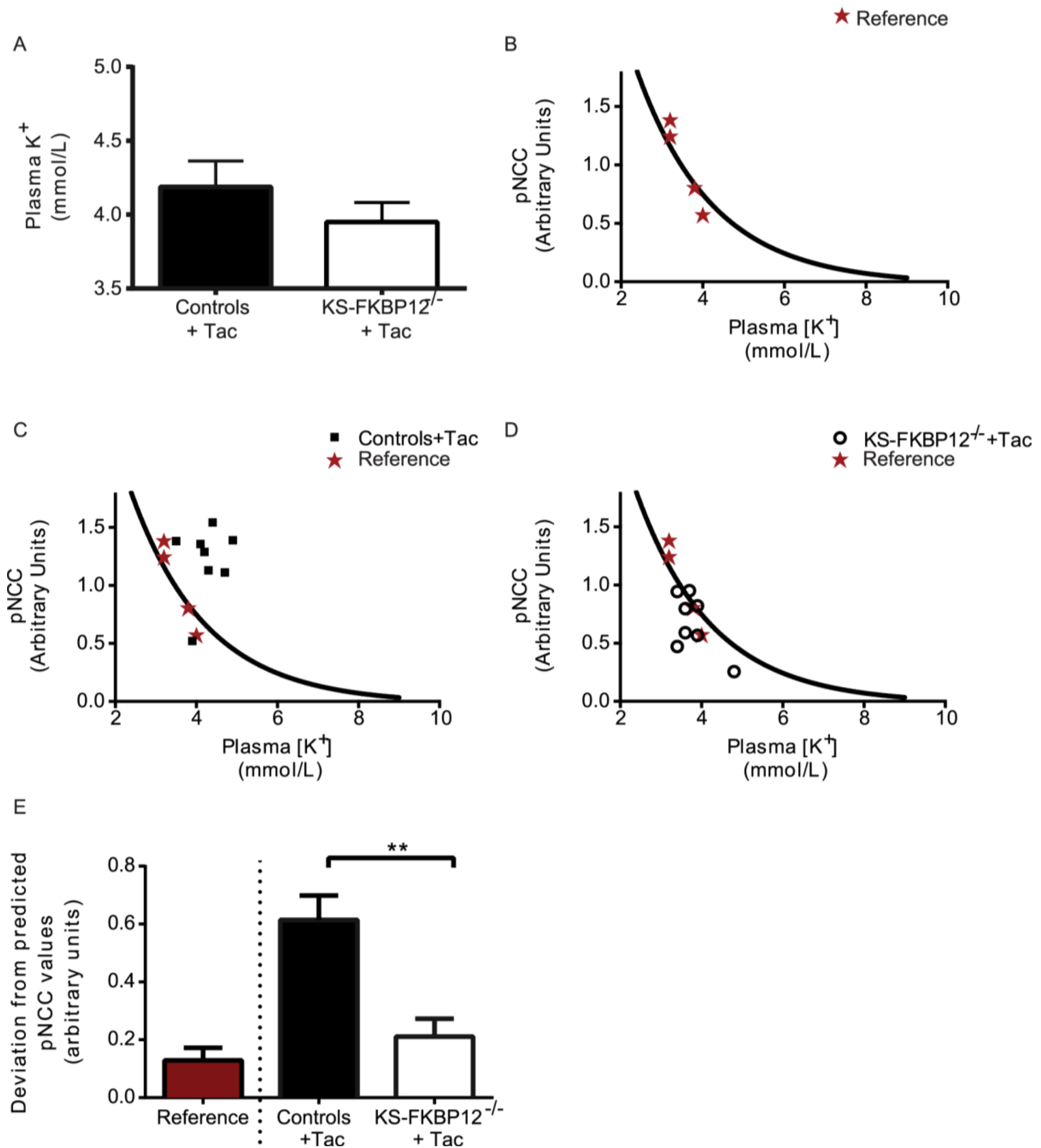

Figure 4.7-Tacrolimus-treated KS-FKBP12$^{-/-}$ mice maintain their pNCC: plasma K^{+} relationship.

A) Plasma K^{+} levels are not statistically different between tacrolimus treated control (-Dox +Tac) and KS-FKBP12$^{-/-}$ mice (+Dox +Tac) (*t*-test, P<0.05, n=14-16, $^{+}$/- SEM) B) The relationship between pNCC and plasma K^{+} levels has been established by others[137] and is shown as a line graph. C) In our hands reference mice (control mice that are not treated with tacrolimus or experimentally matched to others in this figure) adhere to this

relationship well. D) Tacrolimus-treated control mice do not. E) tacrolimus-treated KS-FKBP12$^{-/-}$ mice do maintain this relationship. E) Quantification of B-D. The absolute difference between the values predicted by the pNCC: plasma K^+ curve and actual values were taken and averaged for each group (n=4-8, *t*-test **P<0.01 $^+$/-SEM).

NCC activation.

As FKBP12 deletion along the nephron alone is without phenotypic effect, and yet FEBP12 deletion along the nephron largely prevents tacrolimus effects on blood pressure and pNCC, this suggests that calcineurin is involved in NCC regulation. Although calcineurin is a phosphatase and might be expected to regulate NCC by dephosphorylating it, previous work suggested that tacrolimus might also affect the abundance of NCC-activating kinases. I determined the abundance of two previously implicated kinases, WNK4 and SPAK.[57] Following tacrolimus treatment, the abundance of the two kinases was similar in control and KS-FKBP12$^{-/-}$ mice (Fig 4.8A&B). Although SPAK is believed to be the predominant kinase that activates NCC, a homologous kinase, OxSR1 is also able to activate NCC, and appears to play an especially important role in some circumstances.[113,114] The abundance of OxSR1 was lower in KS-FKBP12$^{-/-}$ mice treated with tacrolimus than in controls treated with tacrolimus. A smaller molecular weight band obtained using the same antibody, however, was similar between the two groups (Fig 4.8C&D). Though NKCC2, a NCC homologue, is regulated by OxSR1, neither total NKCC2 nor pNKCC2 was different between tacrolimus treated control and KS-FKBP12$^{-/-}$ mice (Fig 4.8C & D).

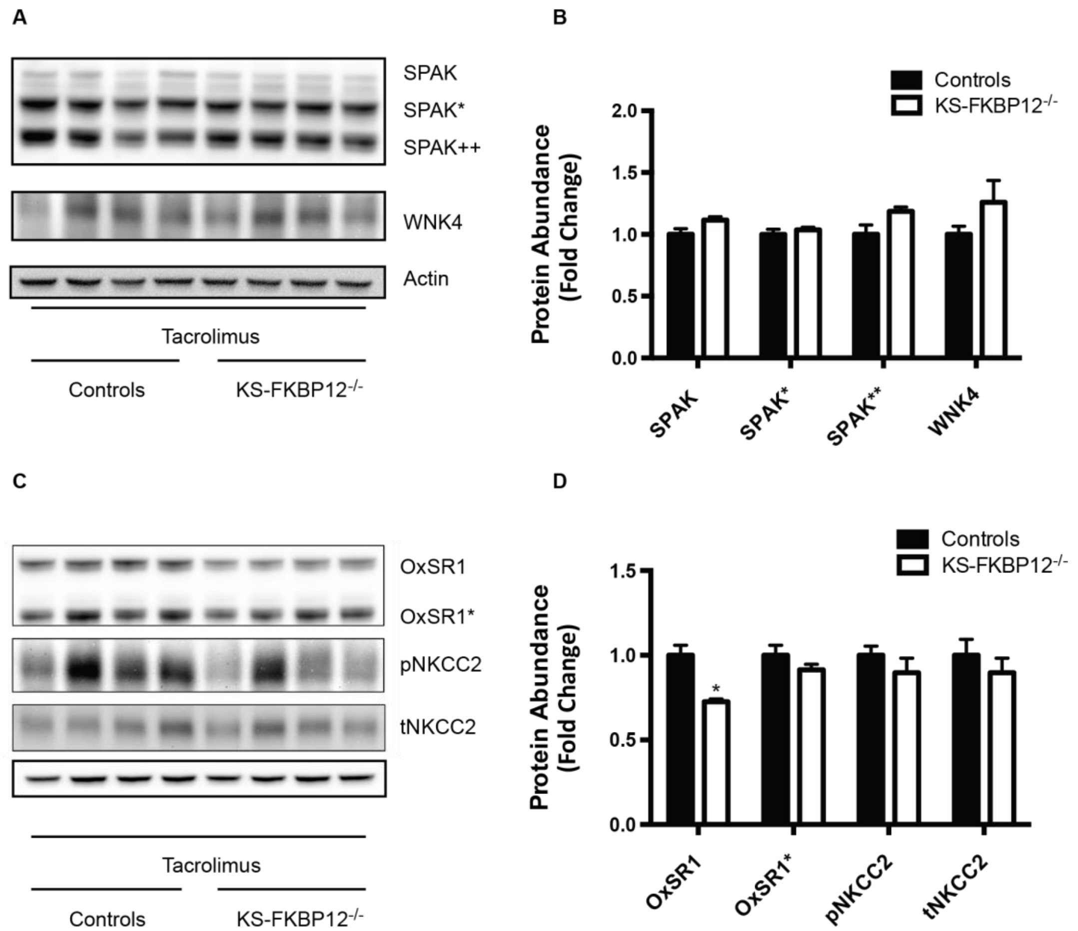

Figure 4.8- Abundance of known regulators of NCC.

Representative Western blots of tacrolimus-treated control (-Dox +Tac) and KS-FKBP12$^{-/-}$ (+Dox +Tac) kidney lysate A) probed for SPAK, WNK4 and actin as a loading control . B) Quantification of panel A. C) probed for OxSR1, pNKCC2, NKCC2 and actin. D) Quantification of panel C (Quantifications for all panels in Figure 8 are normalized to actin: *t*-test, p<0.05, n=4-15, +/-SEM).

Tacrolimus inhibits pNCC dephosphorylation in cells.

The results described above suggested that tacrolimus inhibits calcineurin along the nephron to activate NCC, but these effects could still be indirect. To provide additional support for a direct mechanism, I tested whether tacrolimus could inhibit NCC dephosphorylation in cells. I modified an assay developed by my lab mate, Dr. Andrew Terker, to develop a dephosphorylation assay system in HEK-293 cells using the K^+ channel blocker $BaCl_2$ to depolarize cells.[92] Before inducing dephosphorylation, NCC phosphorylation was stimulated by transfecting constitutively active SPAK (T243E/S383D). Acute treatment with $BaCl_2$ led to a significant reduction in pNCC, whereas tNCC levels remained unchanged, suggesting dephosphorylation (Fig 4.9A&B). Short term (25 minute) pre-treatment with tacrolimus significantly attenuated this response to $BaCl_2$, abrogating NCC dephosphorylation (Fig 4.9C&D).

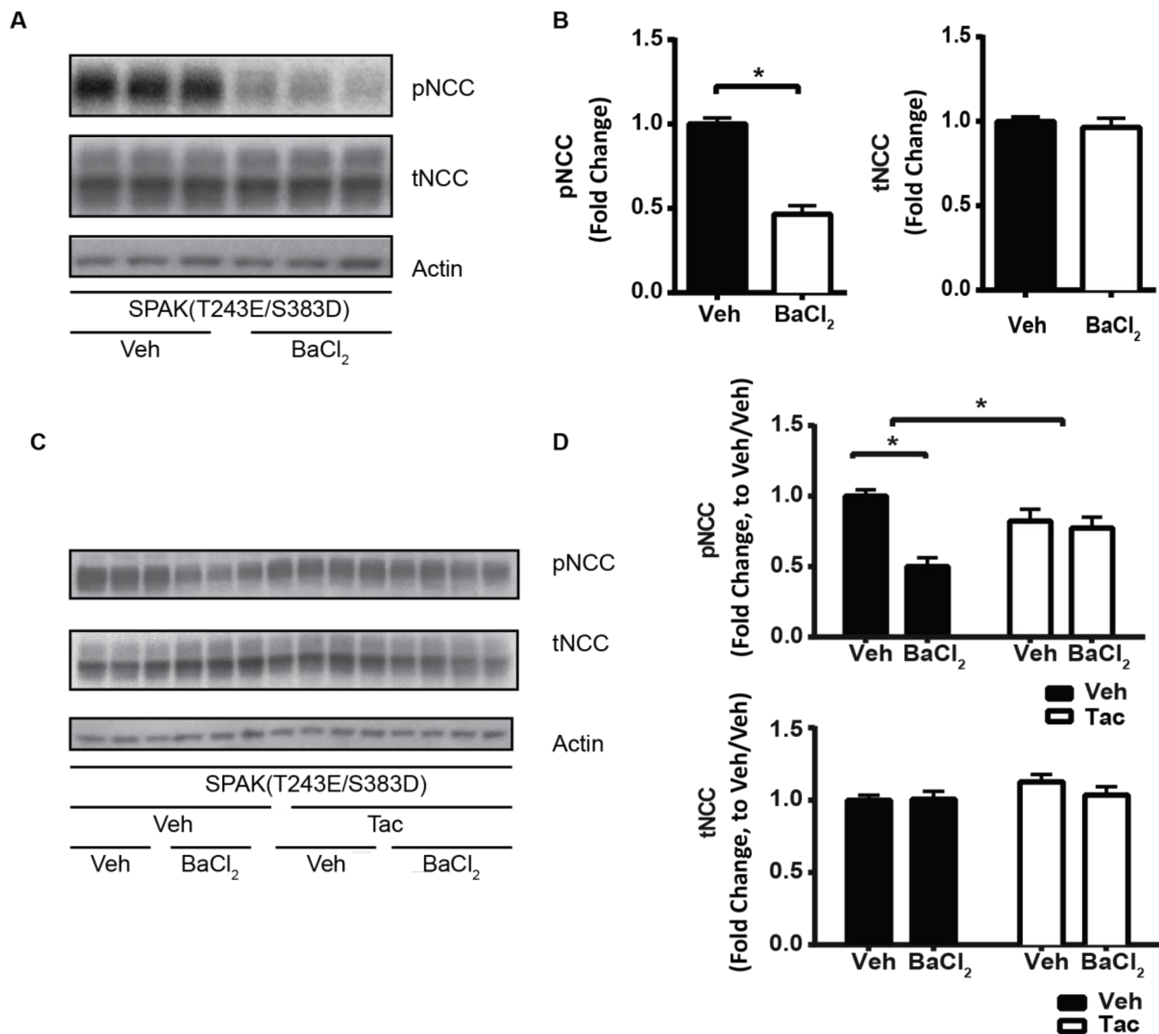

Figure 4.9-Tacrolimus inhibits NCC dephosphorylation in cell culture.

A) Western blot of HEK-293 cells expressing NCC, stimulated with SPAK (T243E/S383D), depolarized with $BaCl_2$, probed for pNCC (T53) and tNCC. B) Quantification of panel A (Quantifications normalized to actin, *t*-test, $p<0.05$, n=3 wells per experiment, 3 experiments, $^{+}$/- SEM). C) Western blot of cells pretreated with vehicle or tacrolimus before $BaCl_2$ depolarization. D) Quantification of panel C (2 way ANOVA, $P<0.05$, n=3-4 well per experiment, 3 experiments, $^{+}$/- SEM).

Discussion

Tacrolimus is a widely used immunosuppressive drug that inhibits its canonical target, calcineurin, when bound to FKBP12. Our previous work showed that tacrolimus increased pNCC, and that NCC is necessary for tacrolimus-induced hypertension to develop. While those results suggested that tacrolimus might be stimulating NCC through direct effects on calcineurin in DCT cells, this remained speculative. Here, I tested the hypothesis that tacrolimus acts directly on kidney tubule cells to cause hypertension, and that inhibition of calcineurin is required. To do this, I generated a new mouse model, KS-FKBP12$^{-/-}$, in which FKBP12 can be deleted inducibly along the nephron.

The structurally dissimilar calcineurin inhibitors, cyclosporine and tacrolimus, both cause hypertension, suggesting that calcineurin inhibition contributes importantly to this pathology. One possibility is that effects of tacrolimus in tissues other than the kidney are involved in the hypertension and NCC activation. Tacrolimus causes vasoconstriction,[53] increases aldosterone[57] and activates the sympathetic nervous system,[55] all of which can contribute to hypertension. These systems also ultimately lead to an increase in NCC activity.[114,148] As arterial pressure rose in both control and KS-FKBP12$^{-/-}$ mice, extra-renal effects likely contribute to tacrolimus-induced hypertension as well, although we can't rule out an effect from the stress of injections themselves. Nevertheless, deletion of FKBP12 along the nephron significantly attenuated the hypertensive response to tacrolimus, suggesting that effects in kidney cells are essential.

The current results concord with our previous study, which showed an essential role for NCC in mediating tacrolimus-induced hypertension. The finding in the current study that KS-FKBP12$^{-/-}$ mice are not completely protected from the tacrolimus-induced increase in blood pressure, however, does contrast with the complete protection in NCC$^{-/-}$ mice, observed

previously.[57] Both mechanistic and technical factors may account for this difference. In the current studies, tacrolimus effects on regulatory pathways outside of the kidney, such as the nervous system and the renin/angiotensin/aldosterone axis, may have stimulated NCC, despite deletion of FKBP12 from kidney cells. As such upstream effects would be retained in KS-FKBP12$^{-/-}$ mice, blood pressure could still rise through secondary activation of NCC. In NCC$^{-/-}$ mice, however, the final common pathway, NCC activation, is absent, and the sympathetic nervous system, and aldosterone, cannot enhance NCC activity.

In the previous study, VPR tail cuff monitoring, which is less sensitive than radiotelemetry, was used to assess systolic blood pressure. Additionally, the dose of tacrolimus used in the previous study was lower than that used here. Though I observed both an increase in blood pressure and a statistically significant difference in pNCC using the lower dose in control and KS-FKBP12$^{-/-}$ mice, in order to ensure that I could detect partial protection in these mice I increased the dose which magnified both the blood pressure and pNCC phenotype. The higher dose of tacrolimus may have increased extra-renal effects, contributing to an increase in blood pressure that is both dependent on NCC and independent of it. Nevertheless, the qualitative effects of tacrolimus on both arterial pressure and on pNCC abundance were similar in the two studies.

One advantage of telemetric pressure monitoring is that pressures can be monitored throughout the day and night. Surprisingly, despite being woken up for daily tacrolimus injections, nocturnal KS-FKBP12$^{-/-}$ mice largely recovered their diurnal blood pressure dipping patterns. Non-dipping patterns, which are associated with several adverse outcomes,[149] are also correlated with salt-sensitive hypertension, like that which results from tacrolimus administraton.[59] Phosphorylation of NCC has recently been shown to oscillate in a diurnal

rhythm,[150] with lower levels of pNCC preceding the dip in blood pressure associated with the inactive period. This suggests that disruption of diurnal NCC regulation by tacrolimus may contribute to the attenuation in diurnal blood pressure dipping.

The abundance of pNCC in tacrolimus-treated control mice was higher than in tacrolimus-treated KS-FKBP12$^{-/-}$ mice. This is in line with previous work and suggests a role for calcineurin in NCC regulation. Earlier work described an increase in the abundance of kinases that activate NCC, WNK4 and SPAK,[57] as well an electric mobility shift in the later, when control mice were treated with tacrolimus. An increased abundance of WNK4 was also implicated in an increase in pNCC during administration of the calcineurin inhibitor cyclosporine.[58] As calcineurin is involved in regulating transcription factors, such as NFAT, differences in protein abundance are in line with calcineurin activity. While differences between tacrolimus treated control and KS-FKBP12$^{-/-}$ mice were not observed in the current studies, both groups in this study were treated with tacrolimus. Only FKBP12-dependent effects along the nephron would result in detectable differences in this study. Thus, WNK4 and SPAK abundance or activation may have changed in response to extra-renal signals or FKBP12/calcineurin independent events, but I would not be able to detect such differences here. This does suggest, however, that if WNK4 and SPAK dysregulation are playing a role in tacrolimus-induced pathologies, they do not account for the full effect, as tacrolimus treated KS-FKBP12$^{-/-}$ mice did exhibit lower levels of pNCC and an attenuated blood pressure response.

The most straightforward interpretation of the current results is that calcineurin dephosphorylates NCC and that by inhibiting this process in the DCT, tacrolimus leads to an increase in pNCC and hypertension. To test this directly I developed an NCC dephosphorylation assay in HEK-293 cells. Sorensen et al. showed *in vivo* that K^+ loading decreases the abundance

of pNCC acutely;[121] they suggested that this is due to NCC dephosphorylation. This effect is likely triggered by cell depolarization, brought on by the high plasma [K^+].[92] Interestingly, depolarization has also been shown, in other tissues, to activate calcineurin.[122]

Here, I mimicked the depolarization triggered by high plasma K^+ with $BaCl_2$, which depolarizes cells and increases pNCC in cells exposed for 24 hours.[92] Instead of overnight treatment, however, I used a short-term $BaCl_2$ exposure, and included constitutively active SPAK (T243E/S383D) to ensure that NCC was fully activated. As tacrolimus inhibited NCC dephosphorylation in this model, it provides evidence that calcineurin plays a direct role in NCC dephosphorylation. As these effects were observed in the presence of constitutive activation of the proximate NCC-phosphorylating kinase (SPAK), they strongly suggest that dephosphorylation is involved. These results contrast with those of Glover et al., in which Xenopus oocytes co-injected with NCC and the catalytic subunit of calcineurin showed no difference in $^{22}Na^+$ transport relative to controls.[119] Calcineurin, however, is not active at baseline. Activation by depolarization, a physiologically relevant stimulus, may explain differences between our studies. Calcineurin may also have an indirect role in reducing NCC phosphorylation and critical intermediaries that are present in HEK-293 cells may be absent in oocytes.

Deletion of FKBP12 did not significantly affect the plasma [K^+] during tacrolimus treatment. Even control mice treated with tacrolimus had physiologic plasma K^+ levels. This is in line with our previous findings that mice are somewhat resistant to tacrolimus-induced hyperkalemia.[57] Yet there was a trend for plasma [K^+] to be lower in the KS-FKBP12$^{-/-}$ mice. As the abundance of pNCC has recently been shown to be strongly dependent on plasma [K^+] across a wide range of conditions, and as this relationship appears crucial for normal K^+ homeostasis, we tested whether the relationship between pNCC and plasma [K^+] was altered by tacrolimus

treatment. The results clearly show that NCC is less susceptible to suppression by $[K^+]$ during tacrolimus treatment, and that this effect is dependent on renal FKBP12. This suggests that, though tacrolimus-treated mice do not become frankly hyperkalemic, a subtle K^+ regulatory defect is present.

My data suggest that calcineurin is involved in NCC dephosphorylation. Tacrolimus, which inhibits calcineurin, would increase pNCC by inhibiting its dephosphorylation, contributing to hypertension and hyperkalemia. While these results confirm an important role of the kidney in tacrolimus-induced hypertension, they also suggest an important role for external effects, as the KS-FKBP12$^{-/-}$ mice did experience a rise in arterial pressure during tacrolimus treatment. The results suggest that the therapeutic and toxic effects of tacrolimus both arise from calcineurin inhibition; thus, calcineurin inhibitors may need to be targeted to immune cells or specific calcineurin isoforms,[32] to avoid the toxic effects that occur frequently. Until such drugs are available, the use of thiazide diuretics may prove especially useful in patients who develop tacrolimus-related hypertension.

Acknowledgements

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Chapter 5 -Tacrolimus-Induced Calcium and Magnesium Wasting

Abstract

Tacrolimus is an immunosuppressive drug that inhibits the phosphatase calcineurin by way of a binding protein, FKBP12. Along with immunosuppression, tacrolimus also causes side effects including hypomagnesemia and hypercalciuria. A decrease in renal reabsorption of these minerals is thought to be involved, but the molecular mechanisms are unclear. Here, I utilized KS-FKBP12$^{-/-}$ mice to determine *in vivo* if either FKBP12 disruption or FKBP12-dependent events, such as calcineurin inhibition, along the nephron cause hypomagnesemia or hypercalciuria.

I found that KS-FKBP12$^{-/-}$ mice, in which FKBP12 along the nephron is inducibly deleted, are phenotypically normal. Suggesting that FKBP12 disruption along the nephron is insufficient to cause hypomagnesemia or hypercalciuria. Conversely, I found that unlike tacrolimus-treated control mice, tacrolimus-treated KS-FKBP12$^{-/-}$ mice are protected from both of these disorders. They are also protected from the decrease in mRNA encoding the magnesium transporter TRPM6 and the calcium transport proteins Calbindin-28K and the Na^{+}/Ca^{2+} exchanger-1, which occurs in tacrolimus-treated control mice.

All of these transport proteins are located in the Distal Convoluted Tubule (DCT), where we previously identified dysregulation of the resident sodium chloride cotransporter (NCC), which has been implicated in tacrolimus-induced hypertension and hyperkalemia. In contrast, mRNA expression of Claudin 16 and 19, Thick Ascending Limb (TAL) proteins involved in Mg^{2+} and Ca^{2+} which are often associated with hypercalciuria, remain unaffected in both groups.

Collectively, this suggests that an FKBP12-dependent event, such as calcineurin inhibition, along the nephron is essential for the development of tacrolimus-induced hypomagnesemia and

hypercalciuria. The results also suggest that the DCT is playing a critical role in these pathologies.

Introduction

Tacrolimus is a widely used immunosuppressive drug often used to prevent graft rejection in organ transplant patients.[151] While effective it leads to several side effects including hypomagnesemia[36] and hypercalciuria.[35,57,58] Both of which are exacerbated by the chronic nature of tacrolimus treatment. Hypomagnesemia, which is extremely common in tacrolimus-treated patients,[36] is often refractory to treatment and can lead to a host of complications including tetany, heart arrhythmias, calcium and potassium dysregulation and general malaise.[65] It is also an independent predictor of new onset diabetes, a pathology that afflicts ~25% of patients on tacrolimus.[65] Tacrolimus-induced hypercalciuria, on the other hand, often goes unrecognized, as plasma Ca^{2+} levels remain normal.[57] Calcium, an important intracellular messenger, is tightly regulated by hormonal control and buffered by large Ca^{2+} reserves stored in bone. Chronic calcium wasting, however, eventually taxes these reserves, leading to bone health complications. The five-fold increased risk for bone fracture and osteoporosis, in the solid-organ transplant population,[67] suggests that calcium wasting is more common than we think.

In order to design safer therapeutics and more effectively treat patients taking tacrolimus we must understand the molecular underpinnings of these pathologies. We know that both Mg^{2+} and Ca^{2+} are regulated largely by the intestines, bone and the kidneys. Previous work, in both humans and rats, however, has suggested that renal ion wasting plays a primary role in tacrolimus-induced hypomagnesemia and hypercalciuria.[35]

Of the 70% of plasma magnesium that is freely filterable, 10-20 % is reabsorbed passively by the proximal tubule; a smaller fraction than most other electrolytes. The TAL is the primary site of magnesium reabsorption. Here, 65-70% of the filtered load is passively reclaimed by paracellular reabsorption, driven by a positive lumen electrical gradient. The tight junction

proteins Claudin 16 and 19, which complex with one another, play an important role in this process, illustrated by mutations in either protein which lead to the hereditary magnesium wasting disorder familial hypomagnesemia with hypercalciuria and nephrocalcinosis.[152,153] Magnesium reabsorption also occurs in the DCT, where it is fine-tuned by an active process. TRPM6 is thought to be the apical transporter responsible for reclaiming Mg^{2+} from the ultrafiltrate.[154] Previous reports have implicated a reduction in TRPM6 mRNA abundance in calcineurin-inhibitor hypomagnesemia.[35]

In contrast to magnesium, about 60% of filtered calcium is reabsorbed passively by the leaky epithelia of the Proximal Tubule. The TAL reabsorbs another 30% paracellularly, which is also modulated largely by intraluminal electrical potential and the tight junction proteins Claudin 16 and 19. Like magnesium, calcium is also reabsorbed in the DCT by an active process. Calcium enters the DCT via the apical transporter TRPV5. Given calcium's role as a potent intracellular messenger, intracellular calcium is quickly sequestered, binding to Calbindin-28K and Calbindin-9. It is then shuttled to the basolateral side of the cell where it is extruded largely by the Sodium Calcium Exchanger (NCX-1) but also by the Ca^{2+}/ATPase (PMCA1b).[93] Although the DCT only reabsorbs a fraction of the filtered calcium load its importance is highlighted by the severe calcium wasting and bone derangements in TRPV5 $^{-/-}$ mice.[155]

How, tacrolimus alters renal ion handling and where along the nephron this occurs is currently unclear. I hypothesize that tacrolimus acts directly along the nephron by its immunosuppressive mechanism (tacrolimus binding to FKBP12 and inhibiting calcineurin) which leads to hypomagnesemia and hypercalciuria. Whether FKBP12 disruption or calcineurin inhibition underlies these pathologies is unclear. As other drugs which bind to FKBP12, such as rapamycin, also lead to hypomagnesemia,[156] it is possible that FKBP12 disruption is involved. In

which case, FKBP12-sapring calcineurin inhibitors may ameliorate this side effect. On the other hand, the structurally unrelated calcineurin inhibitor cyclosporine also causes these side effects[36] suggesting that calcineurin inhibition is involved.

To clarify this I utilized KS-FBP12$^{-/-}$ mice, which lack FKBP12 along the nephron, to determine 1) whether FKBP12 disruption along the nephron is involved in causing hypomagnesemia and hypercalciuria 2) whether protecting the nephron from FKBP12-dependent events, such as calcineurin inhibition, is sufficient to protect tacrolimus-treated mice from hypomagnesemia and hypercalciuria and 3) to identify the nephron segments and renal ion transport proteins involved in this pathology.

Methods

Animals: Studies were approved by OHSU's animal care and use committee (protocol IS918) and adhered to the NIH Guide for the Care and Use of Laboratory Animals. KS-FKBP12$^{-/-}$ were generated by breeding FKBP12 $^{fl/fl}$ mice with Pax8-rtTA/TRE-LC1 mice. Mice homozygous for floxed FKBP12 and possessing at least one copy of Pax8-rtTA and TRE-LC1 were identified by PCR genotyping of tail clipping DNA. Genetically identical mice were treated with either Doxycycline (2g/L, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) at 4-7 weeks of age for 2 weeks. Male mice from 10-20 weeks old were used for experiments.

Metabolic cages: Mice were housed individually in metabolic cages (Hatteras Instruments). After two days of acclimation, urine was collected for 24 hours in glass jars containing a layer of water saturated mineral oil (to prevent evaporation). Food was provided in gel form and water was provided ad libitum.

Gel diet for metabolic cages: Gel diets consisted of 5 g of powdered salt deficient Harlan diet, 8 mL of water, 0.0225 g of bacterial agar and 0.49% NaCl per serving.

Tacrolimus: Powdered tacrolimus was dissolved in a 3:1 solution of DMSO:tween-20 to 30 mg/mL were. This was diluted with PBS to 15 ug/mL, mice were injected subcutaneously with 3 mg/Kg for 18 days.

Blood sample Analysis: Blood was obtained by cardiac puncture in heparinized tubes. 100 uL was used to detect plasma [Ca^{2+}] using an I-STAT analyzer (Chem 8^{+} cartridge, Abbott Pointe of Care). The remainder was spun down at 2000 RPM for 5 minutes to separate the plasma which was removed and stored at -80C until used for a colorimetric Mg^{2+} assay (Pointe Scientific, Xylidyl Blue assay).

Urine analysis: Urinary calcium was measured using the colorimetric cresolphthalein complexone method (Pointe Scientific).

qRT-PCR: Kidneys were preserved in RNAlater and stored at -80C and then shipped to our Dutch collaborators for qRT-PCR.

In the Netherlands, total RNA was isolated from mouse kidney with Trizol Reagent (Life Technologies, Grand Island, NY, USA) The obtained RNA was subjected to DNAse treatment to prevent genomic DNA contamination. Thereafter, 1.5 ug of RNA was reverse transcribed by M-MLV reverse transcriptase (Invitrogen, Grand Island, NY, USA).

Following primers were used:

Gene	Primer
TRPV5	F:5' CTGGAGCTTGTGGTTTCCTC 3'
	R:5' TCCACTTCAGGCTCACCAG 3'
TRPM6	F:5' CTTACGGGTTGAACACCACCA 3'
	R:5' TTGCAGAACCACAGAGCCTCTA 3'
NCC	F:5' CTTCGGCCACTGGCATTCTG 3'
	R:5' GATGGCAAGGTAGGAGATGG 3'
CLDN16	F:5' GTTGCAGGGACCACATTAC 3'
	R:5' GAGGAGCGTTCGACGTAAAC 3'
CLDN19	F:5' GGTTCCTTTCTCTGCTGCAC 3'
	R:5' CGGGCAACTTAACAACAGG 3'
NCX1.3	F:5' CTCCCTTGTGCTTGAGGAAC 3'
	R:5' CAGTGGCTGCTTGTCATCAT 3'
Calbindin 28K	F:5' GACGGAAGTGGTTACCTGGA 3'
	R:5' ATTTCCGGTGATAGCTCCAA 3'
GAPDH	F:5' TAACATCAAATGGGGTGAGG 3'
	R:5' GGTTCACACCCATCACAAAC 3'

Q-PCR was performed with iQ SYBR Green supermix system (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Livak method was use in order to quantify gene expression.

Immunoblotting: Tissue lysates were prepared by dounce homogenizing snap frozen samples, in chilled lysis buffer as described .[112] Samples were spun and separated on 4-12% Bis-Tris gels (Invitrogen) and immunoblotted with antibodies described in the Supplement. Blots were quantified using image J software(**http://rsbweb.nih.gov/ij/**)

Statistics: All analyses were carried out by 2-Way-ANOVA with a p-value of <0.05 considered significant.

Results

KS-FKBP12$^{-/-}$ mice are protected from tacrolimus induced hypomagnesemia.

I previously generated mice in which FKBP12 could be inducibly deleted along the nephron in response to doxycycline exposure. If FKBP12 disruption along the nephron leads to tacrolimus-induced hypomagnesemia and hypercalciuria these mice should have a phenotype at baseline. As FKBP12 is required for tacrolimus to inhibit calcineurin differences between tacrolimus-treated control and KS-FKBP12$^{-/-}$ mice can be attributed to an FKBP12-dependent event along the nephron, such as calcineurin inhibition. To test the direct effects of tacrolimus on the nephron *in vivo* I treated control and KS-FKBP12$^{-/-}$ mice with either vehicle or tacrolimus for 18 days.

Control and KS-FKBP12$^{-/-}$ mice have similar plasma Mg^{2+} levels. Control mice treated with tacrolimus, however, develop hypomagnesemia, an effect which tacrolimus treated KS-FKBP12$^{-/-}$ mice are protected from (Fig 5.1).

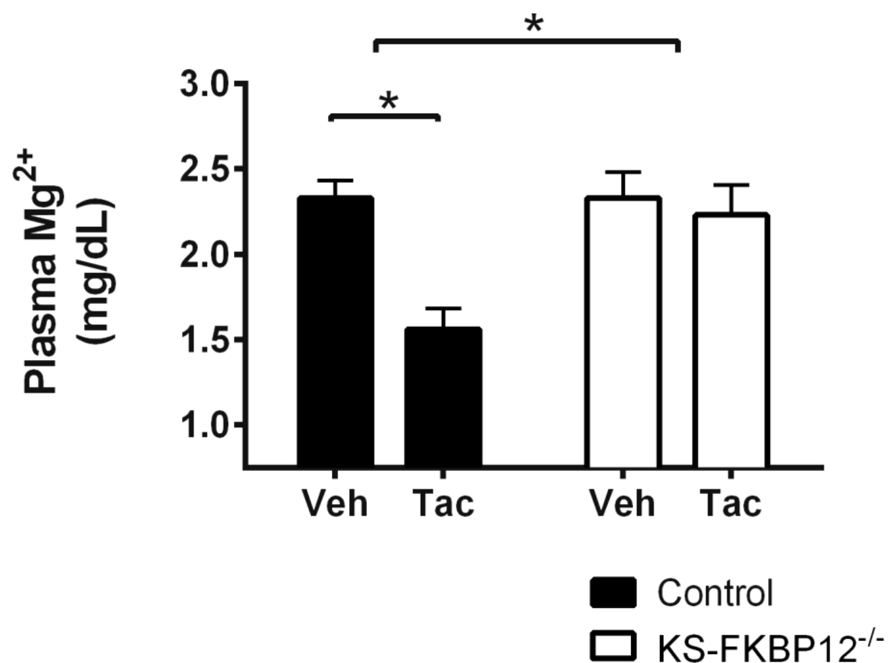

Figure 5.1- KS-FKBP12$^{-/-}$ Mice are Resistant to Tacrolimus-Induced Hypercalciuria.

(2-WAY ANOVA, *p<0.05, n=5-7, $^{+}$/-SEM)

As previous work in rats found that tacrolimus treatment led to a decrease in mRNA levels of TRPM6,[35] a magnesium transporter found in the DCT, my Dutch collaborators assessed TRPM6 mRNA levels. Indeed, control mice treated with tacrolimus have less TRPM6 mRNA relative to vehicle treated control mice, an effect which was absent in KS-FKBP12$^{-/-}$ mice (Fig 5.2A).

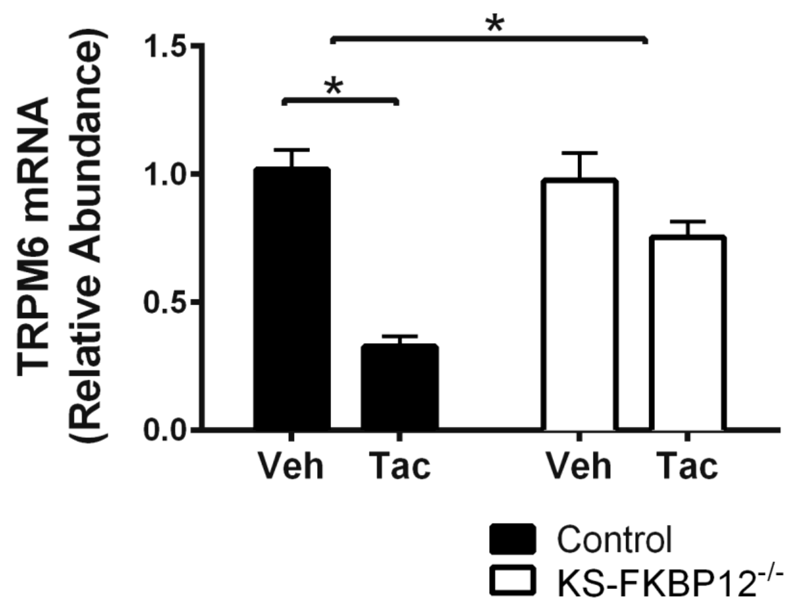

Figure 5.2- TRPM6 mRNA is protected in tacrolimus-treated KS-FKBP12$^{-/-}$ mice.

(2-way ANOVA, * $p<0.05$, n=5-7, $^{+}/-$ SEM)

Tacrolimus is also known to create a negative calcium balance in patients, which leads to bone health complications.[67] Similar to tacrolimus-treated patients, both tacrolimus-treated control and KS-FKBP12$^{-/-}$ mice were able to maintain normal plasma Ca^{2+} levels (Fig 5.3A). Tacrolimus-treated control mice, however, developed hypercalciuria, an effect that was absent in KS-FKBP12$^{-/-}$ mice (Fig 5.3B). Interestingly, the calcium wasting phenotype observed in tacrolimus-treated control mice take several days to develop (Fig 5.3C).

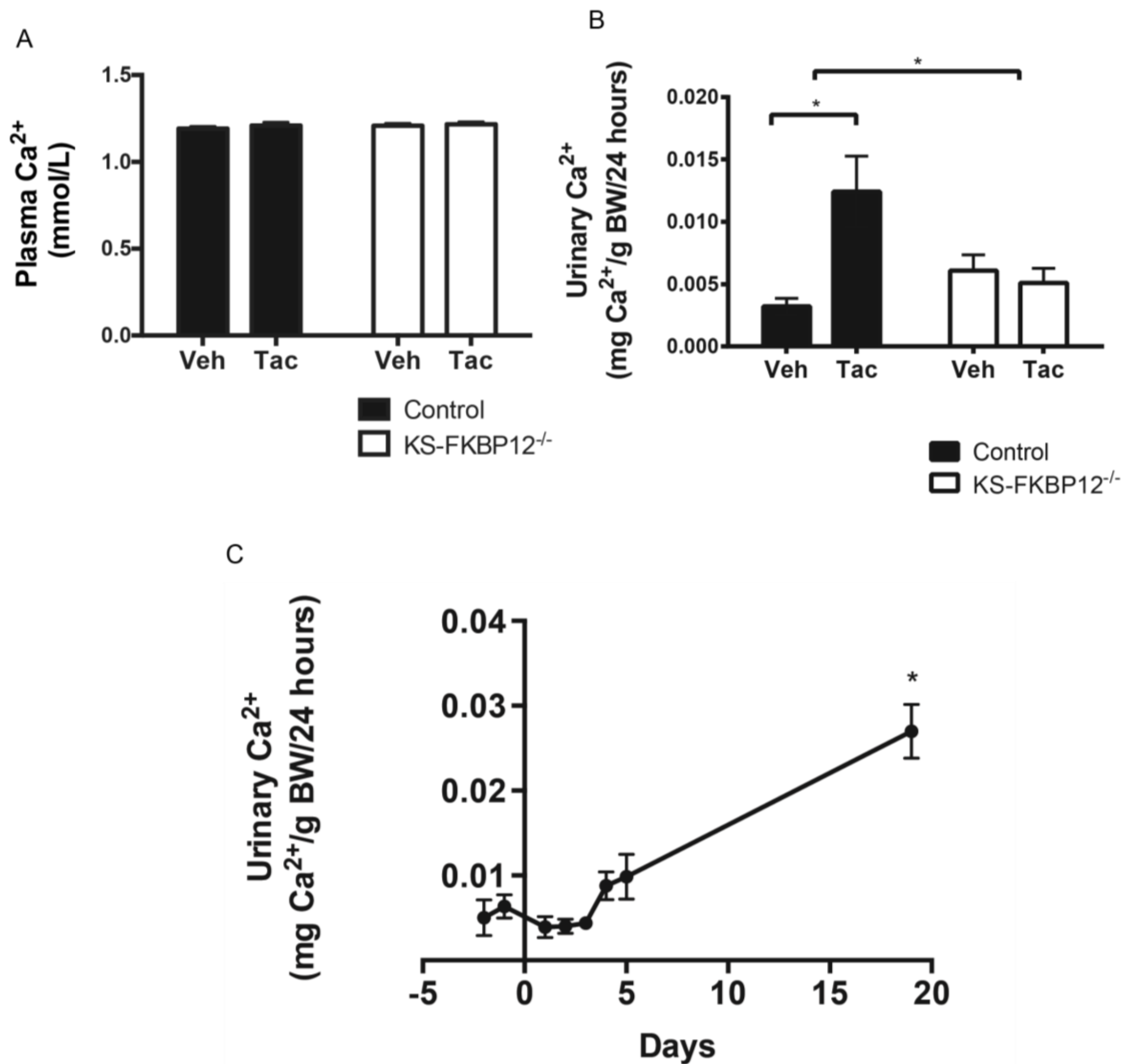

Figure 5.3-KS-FKBP12$^{-/-}$ mice are protected from tacrolimus-induced hypercalciuria.

A) Plasma [Ca^{2+}] remains normal in both control and KS-FKBP12$^{-/-}$ mice treated with tacrolimus. B) KS-FKBP12$^{-/-}$ mice are protected from the tacrolimus-induced increase in urinary Ca^{2+} excretion. (For A&B 2-Way ANOVA, $p<0.05$, n=5-7, $^{+}$/-SEM) C) In control mice the increase in urinary Ca^{2+} excretion caused by tacrolimus is gradual, increasing with time (One Way-ANOVA, $p<0.05$,n=3, $^{+}$/-SEM).

TRPV5 mRNA.

As TRPV5, a calcium channel in the DCT, was previously implicated in tacrolimus-induced calcium wasting,[35] my Dutch collaborators determined TRPV5 mRNA abundance. In contrast to previous reports in rats,[35] they found no difference in TRPV5 abundance at the mRNA level in these mice (Fig 5.4A). In the tacrolimus-treated control mice, however, they did see a dramatic decrease in the mRNA abundance of Calbindin-28k and the Sodium-Calcium Exchanger (NaCX-1). These are the proteins necessary for transporting Ca^{2+}, which enters the DCT through TRPV5, across DCT cells and out the basolateral membrane. Tacrolimus-treated KS-FKBP12$^{-/-}$ mice, on the other hand, had similar levels of Calbindin-28K and NaCX-1 to vehicle-treated KS-FKBP12$^{-/-}$ mice, suggesting that in these mice Ca^{2+} is still effectively reabsorbed at the DCT.

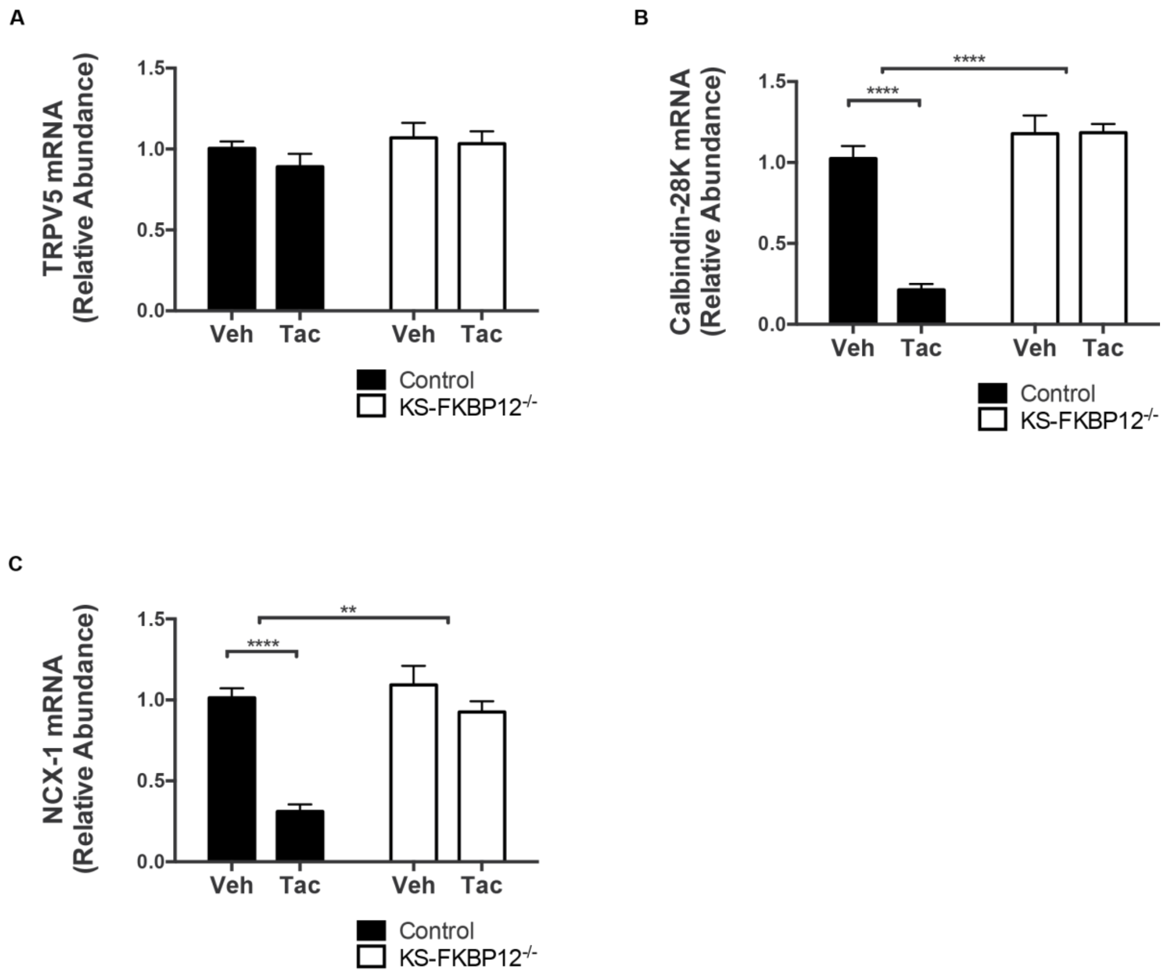

Figure 5.4-Tacrolimus-treated KS-FKBP12-/- mice are protected from a decrease in mRNA expression of DCT calcium transport proteins.

A)TRPV5 mRNA abundance (apical transporter) is unaffected across groups. B) mRNA abundance of Calbindin 28K (transports Ca^{2+} across the DCT) is reduced in tacrolimus treated control, but not KS-FKBP12$^{-/-}$ mice. C) This is also true of NCX-1 mRNA abundance (extrudes Ca^{2+}). (2-Way ANOVA, $p<0.05$, n=5-7, $^{+}$/-SEM)

mRNA levels of Claudin 16 and 19 remain unchanged in tacrolimus-treated mice.

Calcium is also reabsorbed paracellularly in the TAL of the nephron. Tight junction proteins such as Claudin 16 and 19 play a particularly important role in allowing Ca^{2+} to selectively pass through the barriers they form between cells. The genetic disorder Familial

hypomagnesemia with hypercalciuria and nephrocalcinosis which resembles tacrolimus-induced hypomagnesemia and hypercalciuria, is caused by disruptions in these genes.[157] The mRNA abundance of Claudin 16 and 19, however, were unchanged with tacrolimus treatment in both control and KS-FKBP12$^{-/-}$ mice, suggesting that the hypomagnesemia and hypercalciuria may indeed be due to disruption in the DCT.

Discussion

I found that KS-FKBP12$^{-/-}$ mice are protected from tacrolimus-induced hypomagnesemia and hypercalciuria. These side effects are the result of an FKBP2 dependent-event, such as calcineurin inhibition, along the nephron. This suggests that calcineurin inhibitors which can't access the nephron may be safer therapeutics. Conversely, as KS-FKBP12$^{-/-}$ mice did not exhibit a phenotype at baseline, FKBP12 disruption along the nephron is insufficient to cause hypomagnesemia or hypercalciuria. Therefore, it is unlikely that immunophilin-sparing calcineurin inhibitors will ameliorate these side effects.

The qRT-PCR data generated by my colleagues, as well as work discussed in Chapter 4, point to a tacrolimus-induced pathology of the DCT. This is counterintuitive, as tacrolimus-induced Ca^{2+} and Mg^{2+} wasting resembles the genetic disorder hypomagnesemia with hypercalciuria and nephrocalcinosis, which has been traced to mutations in the TAL proteins Claudin 16 and 19.[152,153] Yet, we found no difference in mRNA expression levels of these proteins. While this data does not preclude derangements in calcium reabsorption at the TAL, we found no evidence for that here.

Instead, we found dramatic differences in mRNA transcripts encoding DCT transport proteins. The reduction in TRPM6 mRNA in tacrolimus-treated control mice combined with the protection from this phenomenon seen in tacrolimus-treated KS-FKBP12$^{-/-}$ mice, suggest that TRPM6 downregulation is responsible for tacrolimus-induced hypomagnesemia. Given calcineurin's role in transcriptional regulation, a reduction in transcription seems likely. Our results, corroborate previous findings which implicated the kidneys in tacrolimus-induced hypomagnesemia.[35]

Our data also suggests that tacrolimus-induced hypercalciuria is a DCT phenomenon. Although our collaborators did not see a decrease in TRPV5 mRNA, which they previously observed in a study using rats, and which has also been reported in mice,[158] the reduction in Calbindin-28K and NCX-1 support a model in which calcium reabsorption at the DCT is reduced. While previous results implicated a decrease in TRPV5 mRNA expression, it is also possible that tacrolimus reduces TRPV5 activity, which leads to a decrease in calcium reabsorption and the proteins involved in transporting calcium across the DCT. Further work is needed to untangle these possibilities.

In summary, tacrolimus causes hypomagnesemia and hypercalciuria by an FKBP12-dependent mechanism, along the nephron. Our data support a model in which this occurs largely by reducing Mg^{2+} and Ca^{2+} transport across the DCT. This work suggests that FKBP12-sparing calcineurin inhibitors are unlikely to lead to safer immunosuppressive drugs with regard to magnesium and calcium wasting. Calcineurin inhibitors which do not penetrate the nephrons, however, may be significantly safer therapeutics.

Acknowledgements

I thank Sabina Jelen for carrying out qRT-PCR experiments, and Kayla Erspamer for assisting with metabolic cage experiments.

Chapter 6 –Discussion

As the demand for tacrolimus grows, so does the need to improve its safety profile. The unparalleled success of calcineurin inhibitors and the well-defined pathway by which they lead to immunosuppression make refining calcineurin inhibition an attractive approach to developing safer immunosuppressive drugs. The inherent hope being that a few key modifications will lead to equally efficacious, safer therapeutics.

To this end, a similar level of mechanistic detail regarding the pathologies caused by calcineurin-inhibitors is needed to exploit differences between their immunosuppressive and pathological mechanisms. A breakthrough in this regard came with the discovery, by our lab, that tacrolimus increases NCC activity and that NCC is essential for the development of tacrolimus-induced hypertension.[57] This implied that understanding the mechanism by which tacrolimus dysregulates NCC may provide the key to side stepping these pathologies all together.

It is worth noting that this also suggested that thiazide diuretics, which inhibit NCC activity, may be a promising short term solution to ameliorating tacrolimus-induced hypertension. Indeed this may be true. As with all drugs, however, thiazides also cause side effects including frequent urination and sexual dysfunction.[159] Like calcineurin inhibitors, thiazides would also need to be taken chronically (for life). As they cause tangible consequences in the process of ameliorating hypertension, which though life shortening is painless, it is worth improving the safety profile of the initial immunosuppressive drugs themselves, instead of layering medications, which come with their own complications, on top of one another.

In order to design safer drugs, which circumvent these disorders altogether, we need to know how tacrolimus causes an increase in NCC phosphorylation. The work in this thesis begins to tease apart the components of tacrolimus induced hypertension and electrolyte disorders. Collectively my work points to an essential role of the nephron in the full-development of

tacrolimus-induced hypertension and electrolyte disorders by an FKBP12-dependent mechanism, such as calcineurin inhibition. These findings and their implications for future drug development are discussed below.

FKBP12 along the nephron and Immunophilin (FKBP12)-Sparing Calcineurin Inhibitors

As both tacrolimus and cyclosporine inhibit calcineurin only after binding to an immunophilin (FKBP12 or Cyclophillin A respectively) it has long been speculated that immunophilin inhibition was at the heart of some tacrolimus-mediated pathologies. To this end immunophilin-sparing drugs (which inhibit calcineurin directly) have been proposed as safer alternatives to the calcineurin-inhibitors currently on the market.[160] A notion supported by the embryonic lethality of systemic FKBP12 knockout mice[83] and the hypertensive phenotype of FKBP12 endothelial and hematopoietic knockout mice.[81]

FKBP12 is found along the nephron, but its role there is unknown. I tested the possibility that FKBP12 disruption along the nephron contributes to tacrolimus induced NCC dysregulation, hypertension and electrolyte disorders by generating an inducible kidney-specific knockout mouse model (KS-FKBP12$^{-/-}$). As I found that KS-FKBP12$^{-/-}$ mice do not have a phenotype at baseline (Chapters 3 and 5), we now know that FKBP12 deletion along the nephron is not sufficient to cause NCC dysregulation, hypertension or electrolyte disorders in mice. A model often used to extrapolate human consequences.

My findings are in contrast to work by Chiasson and colleagues in which FKBP12 disruption in hematopoietic and endothelial cells was sufficient to cause hypertension.[81] This offers one explanation for the incomplete protection from hypertension afforded tacrolimus-treated KS-FKBP12$^{-/-}$ mice. The acute increase in blood pressure after injections could indeed be due to

FKBP12 disruption in the endothelial and hematopoietic cells. Therefore my work suggests that immunophilin-sparing calcineurin inhibitors will not ameliorate the *renal-component* of tacrolimus-induced hypertension or electrolyte disorders. It also illustrates that there is an extra-renal or FKBP12-indpendent renal component to tacrolimus-induced hypertension.

While the renal-component of tacrolimus-induced hypertension does not account for the entire hypertensive effect, it is significant, as tacrolimus-treated KS-FKBP12$^{-/-}$ mice fare substantially better than their tacrolimus-treated controls with regard to hypertension and diurnal dipping dysregulation. They are also completely protected from tacrolimus-induced hypomagnesemia and hypercalciuria and appear to maintain their ability to regulate K^+. If the extra-renal component of tacrolimus-induced hypertension is due to FKBP12 disruption in other tissues, as put forth by Chiasson and colleagues,[81] then immunophilin-sparing drugs may reduce the severity of tacrolimus-induced hypertension somewhat. The electrolyte disorders and the renal component of tacrolimus-induced hypertension, however, would be predicted to remain problematic.

My work suggests that disruption of FKBP12's endogenous function, along the nephron by tacrolimus, does not underlie tacrolimus-induced NCC dysregulation, hypertension or electrolyte disorders. This interpretation rests on the assumption that when tacrolimus binds to FKBP12 it disrupts the endogenous function of FKBP12, contributing to pathologies by a *loss-of-function* mechanism. An idea supported by the marked phenotype of other FKBP12 knockout mouse models[81,83] and *in vitro* data in which tacrolimus reduces the peptidyl prolyl isomerase activity of FKBP12,[161] one of the best known endogenous functions of FKBP12.

Another possibility, which is not tested here, is that FKBP12, along the nephron, may be involved in tacrolimus-mediated pathologies by a *gain-of-function* mechanism. FKBP12 is most

famous for its ability to facilitate inhibition of calcineurin[68] and mTOR[162] (by tacrolimus and rapamycin respectively) by a *gain-of-function* mechanism. It is not outside the realm of possibility that in the presence of tacrolimus FKBP12 acquires abilities in addition to calcineurin inhibition, which contribute pathologies. Such a mechanism would not be detected in Chapter 3, but would appear in Chapter 4.

Calcineurin Inhibition along the Nephron

My data suggests that calcineurin inhibition along the nephron is involved in tacrolimus-induced NCC dysregulation, hypertension and electrolyte disorders. However, I never tested this directly. This is because my true goal was not to determine if calcineurin inhibition in the kidney was *sufficient* to cause side effects, but if it was *necessary*. If so, developing immunosuppressive agents that avoid NCC dysregulation could prove to be safer therapeutics. My approach, more directly addressed the clinically pressing question, *how do we design safer immunosuppressive drugs?* It also addressed the possibility that tacrolimus causes side effects by a yet unidentified FKBP12-mediated gain-of-function, that is independent of calcineurin inhibition.

Using this strategy I found that an FKBP12-dependent mechanism along the nephron is essential for the full development of tacrolimus-induced hypertension and electrolyte disorders. This is critical information as extra-renal dysfunction could also explain the increase in NCC activity and ensuing pathologies. If so future calcineurin inhibitors designed to avoid the kidney would do little good. Indeed, as tacrolimus-treated KS-FKBP12$^{-/-}$ mice were only partially protected from tacrolimus-induced hypertension, tacrolimus-induced hypertension may be caused by a combination of extra-and intra-renal affects. In contrast, tacrolimus-induced electrolyte disorders appear to be of intra-renal origin.

This suggests that renal-sparing calcineurin inhibitors may resolve the renal-component of tacrolimus-induced pathologies. Although this may not afford complete protection from tacrolimus-induced hypertension, it may improve blood pressure and diurnal dipping patterns of patients on calcineurin inhibitors. Moreover, it will likely ameliorate tacrolimus-induced hypomagnesemia and hypercalciuria and may improve potassium handling.

Another possibility is that a FKBP12-dependent event along the nephron, other than calcineurin inhibition, is responsible for tacrolimus-induced pathologies. This harkens back to the idea that tacrolimus may be causing problems by another, yet unknown, *gain-of-function* mechanism. In which case, FKBP12-sparing calcineurin inhibitors should be sufficient to avoid complications. Further work is needed to clarify calcineurin's role in these pathologies more directly.

The DCT appears to be a particularly good place to start. My data shows that in cell culture tacrolimus can inhibit NCC dephosphorylation by acting directly on NCC-expressing cells. This, in tandem with my results regarding tacrolimus-induced hypomagnesemia and hypercalciuria, point to a pathology of the DCT. It is certainly tempting to speculate that calcineurin inhibition in the DCT is the cause of these side effects, though the evidence for this is indirect. This does suggest, however, that the role of calcineurin in the DCT may be a particularly fruitful place to begin to unravel both the pathophysiology of tacrolimus and elucidate the molecular details of the physiologic regulation of the DCT.

Future Calcineurin Inhibitors

Exploring the relationship between calcineurin and NCC, as well as the DCT at large, may also shed light on some intriguing drug design strategies. Two ideas which would benefit from an intimate understanding of calcineurin's relationship with NCC are illustrated below.

Targeting the beta isoform

There are two isoforms of calcineurin expressed along the nephron, including the DCT, (See chapter 4 Fig 1). Tacrolimus and cyclosporine inhibit both isoforms.[163] Constitutive knockout mice have also been developed for both isoforms.[164,165] CaN-$\beta^{-/-}$ mice develop an immunocompromised phenotype,[164] but appear to develop normal functional kidneys.[166] This led to the conclusion that the immunosuppressive properties of calcineurin inhibitors hinge on suppressing CaN-β in T-cells.

Constitutive systemic CaN-α knockout mice, on the other hand, have a pronounced renal phenotype[166] and are not immunocompromised.[165] In fact they remain susceptible to immunosuppression by tacrolimus and cyclosporine.[165] This was originally considered strong evidence that calcineurin inhibitors were causing renal pathology by inhibiting CaN-α. Further work, however, revealed that CaN-α plays a critical role in the production of salivary enzymes.[167] CaN-$\alpha^{-/-}$ mice have a reduced absorption of nutrients and exhibit a failure to thrive phenotype including renal developmental abnormalities. Rescue of the salivary defect with a special diet still leaves CaN-$\alpha^{-/-}$ mice with a renal phenotype suggesting that CaN-α may indeed play a critical role in kidney function.[168] The true test will come with the characterization of tissue specific inducible CaN-$\alpha^{-/-}$ mice, avoiding developmental and extra-renal complications, which is underway.

Collectively, however, this suggests that CaN-α and CaN-β may play different roles in the kidney. Intriguingly while CaN-β inhibition is of importance for immunosuppression, CaN-α inhibition, at this point, appears more likely to cause renal pathologies. Determining which calcineurin isoform is involved in NCC dephosphorylation may justify developing isoform specific calcineurin inhibitors.

PxIxIT Inhibitors

Alternatively, my cell culture data suggests that tacrolimus causes NCC-related pathologies by preventing acute dephosphorylation of NCC. The rapid nature of this mechanism points to a transcriptionally-independent pathology. This is of relevance as the immunosuppressive effects of calcineurin inhibitors are due to the blockade of NFAT dephosphorylation, a transcription factor which plays a critical role in mounting an immune response. When calcineurin dephosphorylates NFAT it translocates to the nucleus where it transcribes a variety of pro-cytokine transcripts.

Interestingly, calcineurin must rapidly dephosphorylate NFAT at 13 phosphorylation sites for translocation to occur.[169] This is feasible because, unlike most calcineurin substrates, NFAT binds to calcineurin at two binding sites.[170] The LxVP motif,[171] is the docking site for both tacrolimus and cyclosporine A.[76] Blocking this site inhibits the phosphatase activity of calcineurin.[172] The second motif, PxIxIT, serves as a secondary binding site for NFAT.[173] Unlike the LxVP site, PxIxIT inhibitory peptides do not inhibit phosphatase activity.[172] NFAT uses these two sites in combination, effectively stringing itself across the catalytic jaw of calcineurin, facilitating the rapid removal of all 13 phosphate groups.

If interaction between calcineurin and NCC involves the primary LxVP site, where most phosphatase activity is thought to occur, while interaction between calcineurin and NFAT requires both, selective inhibitors to the secondary PxIxIT motif should inhibit calcineurin's ability to inhibit NFAT dephosphorylation while maintaining its ability to regulate proteins such as NCC. In theory such drugs should ameliorate the renal component of tacrolimus-induced hypertension and may improve electrolyte disorders. If such drugs also avoided FKBP12 disruption in the vasculature and hematopoietic cells they may afford even more complete protection.

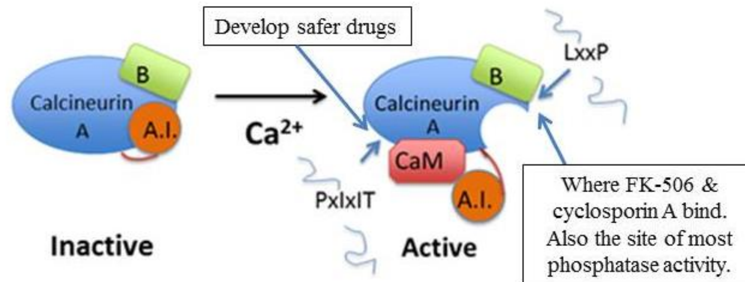

Figure 6.1-Illustration of exploitable calcineurin binding sites.

Blocking the PxIxIT motif may selectively inhibit calcineurin's ability to dephosphorylate NFAT, leading to immunosuppression, while retaining its ability to dephosphorylate substrates such as NCC, alleviating NCC related side effects.

Renal Physiology

In addition to gaining insight into future drug design this project also shed light on some finer points of renal physiology.

My data points to a model in which calcineurin is involved in NCC dephosphorylation. As the phosphatase responsible for dephosphorylating NCC is currently unknown this is an important finding. It seems quite likely to me that calcineurin is one of several phosphatases involved in NCC regulation. Its requirement for Ca^{2+} activation uniquely positions it to respond to stimuli such as high plasma [K^+], but may render it ineffective with regard to housekeeping roles. Like the kinase cascades that activate NCC dephosphorylation, dephosphorylation cascades or branches, triggered by different stimuli, would allow NCC to integrate a variety of informational inputs.

Although NCC has been traditionally thought to be involved in blood pressure regulation, we are beginning to realize that it also plays an important role in potassium homeostasis. Work from our lab and others suggest that NCC is regulated preferentially in response to potassium needs at

the expense of volume regulation.[91,92] The data presented here, suggests that calcineurin is involved in dephosphorylating NCC in response to an acute rise in plasma K^+. A phenomenon which was recently demonstrated *in vivo* by Loffing and colleagues, but was mechanistically unclear.[121] My data, in conjunction with the results of others,[92] suggest that NCC can respond directly to changes in membrane potential generated by small fluctuations in plasma $[K^+]$. This hormonally-independent response to plasma K^+ provides a key to unraveling the long-standing Aldosterone paradox.

The Aldosterone Paradox is a confusing situation in which Aldosterone, when released in response to hypovolemia leads to an increase in NCC activity and conversely, when released in response to hyperkalemia leads to a reduction in NCC activity. This contradiction can be resolved if NCC is not responding to Aldosterone directly, which is becoming more clear,[174] but instead detects hypovolemia and hyperkalemia by independent mechanisms. Responding directly to plasma $[K^+]$ fluctuations provides a direct mechanism by which NCC could respond to $[K^+]$ changes independently of Aldosterone.

Summary

In summary, while the work presented in this thesis provides support for the use of thiazide diuretics in hypertensive tacrolimus-treated patients, it also points towards strategies for developing calcineurin inhibitors which are devoid of these side effects entirely. While there is still much work to be done, this data suggests that safer chronic immunosuppressive drugs may be possible.

Chapter 7 -Conclusions

In conclusion, my work illustrates the importance of the nephron in tacrolimus-induced hypertension and electrolyte disorders. I have found that disruption of FKBP12 along the nephron is insufficient to cause hypertension, diurnal dipping dysregulation, hyperkalemia, hypomagnesemia, hypercalciuria or an increase in NCC phosphorylation in mice. Conversely, FKBP12-dependent events, such as calcineurin inhibition, are necessary for the development of these pathologies as tacrolimus treated KS-FKBP12$^{-/-}$ mice are protected from these side effects.

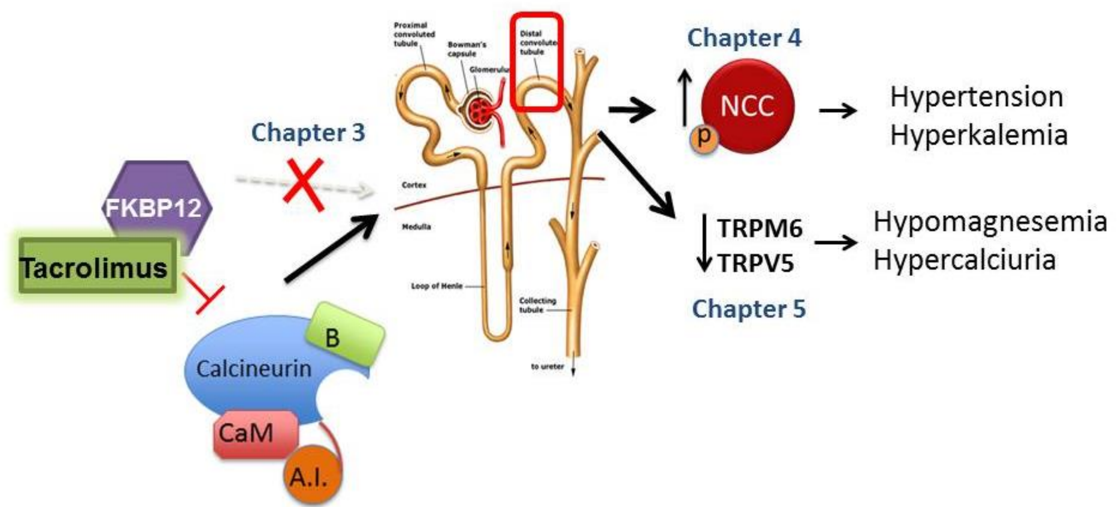

Figure 7.1-Conclusion Cartoon.

FKBP12 disruption along the nephron is not sufficient to cause tacrolimus-induced hypertension or electrolyte disorders. Conversely, FKBP12-dependent events along the nephron, such as calcineurin inhibition, are necessary for the full development of these disorders.

My data support a model in which calcineurin in the DCT is responsible for dephosphorylating NCC in response to depolarizing stimuli such as high plasma [K^+]. Inhibition of this process by tacrolimus would lead to an increase in phosphorylated NCC which increases Na^+ retention, contributing to hypertension, and impairs downstream K^+ excretion, contributing to hyperkalemia.

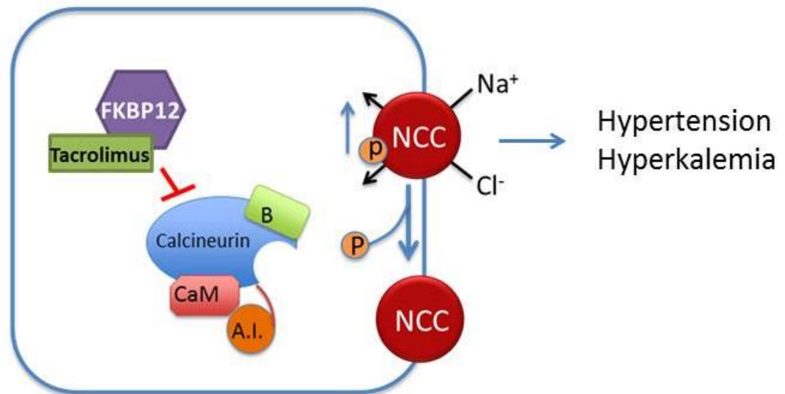

Figure 7.2-Working model.

My data supports a model in which calcineurin, in the DCT, is involved in NCC dephosphorylation. Inhibition of this process by tacrolimus would increase pNCC contributing to hypertension and hyperkalemia.

I have also shown that tacrolimus-induced hypomagnesemia and hypercalciuria are the result of a FKBP12-dependent event, such as calcineurin inhibition, directly along the nephron. Again, the DCT appears to play an important role in these pathologies. The abundance of mRNA encoding TRPM6, a magnesium transporter in the DCT, is decreased. This is also true for the mRNA levels of the calcium transport proteins Calbindin-28K and the NaCX-1, which are essential to transporting calcium across the DCT. Together this suggests that calcineurin inhibition along the nephron leads to a decrease in Mg^{2+} and Ca^{2+} reabsorption in the DCT.

Collectively, the work in this thesis points to a central role of the DCT in tacrolimus-induced pathologies and suggests a role for calcineurin in NCC dephosphorylation. The mechanics of which, should be taken into consideration in the development of future calcineurin inhibitors. It also suggests that immunophilin-sparing calcineurin inhibitors will not ameliorate the renal component of tacrolimus-induced hypertension and electrolyte disorders. Conversely, thiazide

diuretics which inhibit the sodium chloride cotransporter directly and lead to a reduction in blood pressure, plasma $[K^+]$ and an increase in Ca^{2+} reabsorption may prove to be a particularly safe, effective and inexpensive option for treating tacrolimus-induced hypertension, hyperkalemia and hypercalciuria.

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