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A Novel Pig Cell Line as Model System for Studies of Calcineurin Inhibitors

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Cecilie Aas

Abstract

Organ transplantations may be necessary in situations of organ failure due to illness or injury. The total numbers of transplants are increasing; however, transplantations remain challenging.

High congruency of the immune system between recipient and donor is required to prevent the immune system to attack the graft organ and to reduce the risk of allograft rejection. Immunosuppressive agents like cyclosporine A and tacrolimus are used in transplantation medicine to prevent or inhibit allograft rejection. These immunosuppressive agents have serious side effects including nephrotoxicity, a damage of the kidney cells caused by an increase of the pressure in the kidney vessels. The occurrence of immunosuppressive agents induced nephrotoxicity may be studied in humans, animals, or cell models. Cellular models do not take into account changes in blood pressure, hormonal changes, or effects of other organs; however, cell models provide an opportunity to test direct toxicity of the drugs. Rodents are most frequently used for animal studies, but we have chosen a piglet kidney cell line, EFN-R obtained from Friedrich-Loeffler-Institut (FLI) in Germany, as a model system in our laboratory, as they exhibit properties that in some cases are closer to human than rodent models.

Flow cytometry of the cells indicated that the EFN-R consisted of one cell population. To further characterize the phenotype of the EFN-R cells, we investigated the distribution of protein markers including the proteins System N1 (SN1) amino acid transporter, Megalin (Lrp2), Tamm-Horsfall protein (THP), and Aquaporin 2 (Aqp2). SN1 is a transporter that regulates intracellular and extracellular concentration of glutamine, and are located in proximal tubule along with Lrp2. Lrp2 and THP are both glycoproteins, whereas THP is located in the Loop of Henle and distal convoluted tubule of the nephron. AQPs facilitate water transport into and out of the renal cell, whereas Aqp2 is located in the collecting duct system of the nephron. Western blotting detected the presence of SN1, THP, and Lrp2 in EFN-R, although, multiple protein fragments were detected for THP. Thus, Aqp2 results illustrated a protein fragment with lower SDS-PHAGE mobility than expected and we were not able to confirm its presence. Taking together, these findings indicate that the EFN-R cell line resembles proximal tubule cells.

Cyclosporine and tacrolimus are both dose-dependently reduced metabolism assessed by MTT. Morin protected against reduction in MTT cleavage indicating that toxicity exerted by tacrolimus was in part mediated by oxidative stress. Low and medium dose of cyclosporine A and tacrolimus did not significantly alter the expression of Bax, and Bcl2, but high dose results remain untested.

Multidrug resistance protein 4 (Mrp4) is a renal transporter central in drug distribution. A low dose of cyclosporine A, but not tacrolimus increases expression of Mrp4.

Conclusion: Our data reveal that the EFN-R cell lines consist of one cell population, with protein markers resembling that of proximal tubule cells. Morin protects indicating that oxidative stress may play a role in CNI toxicity in this cell line. A low dose of cyclosporine, but not tacrolimus, increases expression of Mrp4.

The next step in this project will be to investigate the presence of the protein markers THP and Aqp2 in EFN-R cell line by using western blot analysis and sequencing method. Our results show an increase of Mrp4 on mRNA level. To confirm this the next step would be to determine translation to protein using western blot analysis. SiRNA knockdown method can also be a value to obtain increased knowledge of Mrp4 regulation.

Sammendrag

Organtransplantasjon kan være nødvendig i situasjoner med organsvikt som følge av sykdom eller skade. Det totale antallet transplantasjoner øker, og transplantasjoner ansees fortsatt som utfordrende. Høy kongruens av immunsystemet mellom mottaker og donor er nødvendig for å hindre at immunsystemet angriper det transplanterte organet og dermed redusere risikoen for avstøtningsreaksjoner. Allogen transplantasjon krever ofte immunsupprimerende behandling for å hemme immunforsvaret. Immunsupprimerende behandling har alvorlige bivirkninger, inkludert nefrotoksisitet, en skade på nyreceller forårsaket av trykkøkning i nyre kar. Forekomsten av nefrotoksisitet grunnet immunsupprimerende midler kan bli studert i mennesker, dyr eller cellemodeller. Cyclosporin A og Tacrolimus er kjente immunsuppressiva som brukes innenfor transplantasjon for å hindre eller hemme avstøtningsreaksjon av transplantatet. Det er av interesse å studere toksisiteten og de metabolske effektene av disse medikamentene, og cellekulturer og dyremodeller kan være nyttig å bruke til dette. Cellulære modeller tar ikke hensyn til endringer i blodtrykk, hormonelle forandringer, eller effekten av andre organer, men de gir en mulighet til å undersøke direkte toksisitet av medikamenter. Gnagere er mye brukt i dyrestudier, men vi har valgt en nyrecellelinje fra gris, EFN-R, som er hentet fra Friedrich-Loeffler-Institut (FLI) i Tyskland. Griser viser egenskaper som i noen tilfeller er nærmere mennesket enn gnagermodeller og vi har derfor valgt å bruke denne cellelinjen som et modellsystem i vårt laboratorium.

Det var et ønske å karakterisere EFN-R celle linjen. Metoder som flowcytometri og immunoblotting ble brukt til dette. Flowcytometri indikerte at EFN-R besto av en cellepopulasjon. For ytterligere å karakterisere fenotypen av EFN-R-celler, undersøkte vi fordelingen av proteinmarkører inkludert System N1 (SN1) aminosyre transportør, Megalin (Lrp2), Tamm-Horsfall protein (THP) og Aquaporin 2 (Aqp2). SN1 er en glutamin transportør lokalisert i proksimale tubuli. Her finner vi også transmembranproteinet Lrp2. THP og Aqp2 er lokalisert i Henles sløyfe, distale tubuli og samlekanalen i nyrens nefroner. Immunoblotting fant tilstedeværelse av SN1, THP og Lrp2 i EFN-R. Aqp2 hadde lavere mobilitet enn forventet så vi kunne verken bekrefte eller avkrefte tilstedeværelse av dette proteinet. Disse funnene tyder på at EFN-R cellelinje ligner proksimale tubuli celler.

Dose responskurve av Cyklosporin A og Tacrolimus viste doseavhengighet og redusert metabolisme vurdert av MTT. Morin beskyttet mot reduksjon i MTT spaltning noe som indikerer at toksisiteten som utøves av Tacrolimus delvis var mediert av oksidativt stress. Middels og lav dose av Cyclosporin A og Tacrolimus endret ikke uttrykket av apoptose relaterte gener som Bax, og Bcl2. Høy dose er fremdeles ikke testet på cellene.

Multiresistens protein 4 (Mrp4) er en nyre transportør som finnes i proximale tubuli celler, og som deltar i medikament distribusjon. En lav dose Cyclosporin A økte ekspresjon av Mrp4. Mrp4 forble uendret etter tilsatt Tacrolimus.

Konklusjon: Våre data indikerer at EFN-R cellelinje består av en cellepopulasjon, med protein markører som minner om proksimale tubulære celler. Morin beskytter og indikerer at oksidativt stress kan spille en rolle i calcineurin hemmer (CNI) toksisitet i denne cellelinjen. En lav dose Cyclosporin A økte ekspresjon av Mrp4, hvorav Mrp4 forble uendret i celler tilsatt Tacrolimus.

Det neste trinnet i dette prosjektet vil være å undersøke tilstedeværelse av protein markørene THP og Aqp2 i EFN-R. Metoder som western blot og sekvensering kan være nyttige til dette. Våre resultater viser en økning av Mrp4 på mRNA nivå. Neste trinn vil være å undersøke om mRNA translateres til protein ved bruk av western blot. Ved tilstedeværelse av protein vil det være aktuelt å utføre siRNA for å få mer kunnskap om regulering av Mrp4 i proximale tubuli.

Abbreviations

Aqp2	Aquaporin 2
7AAD	7-Amino-Actinomycin
Bax	Bcl2 associated X protein
Bcl2	B-cell lymphoma
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Ct	Cycle threshold
CDCA	Chenodeoxycholic acid
CsA	Cyclosporine A
CNI	Calcineurin inhibitor
DCT	Distal convolute tubule
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FSC	Forward scatter
FXR	Farnesoid X receptor
FK506	Tacrolimus
FBS	Fetal bovine serum
FXRE	Farnesoid X receptor element
H2O2	Hydrogen peroxide
IL-2	Interleukin-2
LRP2	Megalin
MRP	Multi drug resistance protein
MTT	MTT 3-4(4,5-demethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NF-AT	Nuclear factor of activated T- cells
PCT	Proximal convolute tubule
PE	Phycoerythrin
PVDF	Polyvinylidene difluoride

Pen-Strep	Penicillin streptomycin
P-gp	P-glycoprotein
PS	Phosphatidylserin
qRT-PCR	Quantitative real time polymerase chain reaction
RxR	Retinoic acid receptor
RAS	Renin angiotensin system
RNA	Ribonukleinsyre
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SDS-PHAGE	Sodium dodecyl Sulphate polyacrylamide gel
SSC	Side Scatter
SN1	System N1 amino acid transporter
THP	Tamm Horsfall protein
Tm	Melting curve

Contents

Chapter 1

Introduction

1.1. Motivation: Challenges of human organ transplantation

The functionality of organs in humans may fail due to illness or injury, requiring organ transplantation, a process to transfer cells, tissues, or entire organs from a donor to a recipient. In 1954, Dr. Joseph E Murray carried out the first kidney transplantation between identical twins (Tullius 2013). Over the years, the transplantation of many different human organs has been performed, including heart, intestine, kidney, liver, lung, bone marrow, and pancreas, where liver and kidney transplantations are the most common ones (Slattry et al. 2012). Since the first Norwegian transplantation in 1969, a total of 10197 people have been transplanted (Organdonasjon 2013). The total number of transplantations is increasing; however, the process of transplantation remains challenging. The immune system represents an efficient mechanism to protect the body from substances identified as being foreign (Lea 2006). Donor organs are foreign tissue and thus the immune system has to be prevented from attacking the graft to avoid the risk of transplant rejection (Lea 2006). As a consequence, humans with transplanted organs undergo lifelong immunosuppressive therapy by taking immunosuppressive agents to prevent allograft rejection (Lea 2006; Slattry et al. 2012). Most immunosuppressive agents have severe side effects. To study the toxicity and the metabolic effects of immunosuppressive agents cell cultures and animal models are applied. This provides the possibility for an improved understanding of toxicity, to raise the effectiveness, and to counteract the side effects of various drugs, so that the process of transplantation can be improved in the future.

1.2. Immunosuppressive agents: Calcineurin inhibitors

In the 1980s and early 1990s the calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus (FK506) were introduced as immunosuppressive agents in transplantation medicine and the problem of organ rejection was significantly reduced (Groetzner et al. 2009; Slattry et al. 2012). CsA and FK506 differ in their chemical structure. CsA is a cyclic endocapeptide and FK506 is a macro cyclic lactone; however, both show similar working mechanisms (Slattry et al. 2012).

CNIs work by blocking the protein phosphates' calcineurin activating T-lymphocytes by binding with proteins known as immunophilins. CsA binds primarily to the immunophilin cyclophilin A, whereas FK506 is targeted to the immunophilin FK binding protein 12 in cytoplasm of the cell. Both immunophilins interact with calcineurin. Calcineurin is a calmodulin dependent phosphatase, which activates T-lymphocytes of the immune system by various events. Ones activated, it reacts with members of the nuclear factor of activated T-cells (NF-AT). NF-AT translocate to the nucleus, where it is associated with other transcription factors, and regulates transcription of T-cell growth factor interleukin-2 (IL-2) and other cytokines (Slattry et al. 2012). Consequently, inhibition of calcineurin prevents the ability to activate and dephosphorylate NF-AT affecting the transcription, which are important for the immune response. Progress in understanding these molecular mechanisms suggest that NF-AT pathway is unspecific. CNI can affect other cell types and de-phosphorylation of other substrates different from NF-AT may occur (Busauschina et al. 2004; Campistol & Sacks 2000; Naesens et al. 2009).

While CNIs are effective in immunosuppression, the side effects are a major drawback. Both FK506 and CsA provide similar effects, although patients need an FK506-concentration 50 to 100-fold lower than CsA (Lamoureux et al. 2011; Slattry et al. 2012). CNI provides several adverse effects, whereas nephrotoxicity is the most challenging. This involves both acute and chronic renal dysfunction. Acute nephrotoxicity is characterized by hemodynamic dysfunction (Slattry et al. 2012). This is often a reversible process although long exposure to toxic agents can cause irreversible damage (Xiao et al. 2011; Xiao et al. 2013). Hemodynamic disruption followed by activation of renin-angiotensin system (RAS) is observed in acute

nephrotoxicity (Slattry et al. 2012). This activation of RAS leads to production of vasoconstriction factors such as angiotensin II and endothelin (Slattry et al. 2012). Even though acute nephrotoxicity is reversible, long exposure to CNI can cause tubular damage and chronic nephrotoxicity (Slattry et al. 2012). Chronic CNI nephrotoxicity is often associated with histological properties such as renal fibrosis and tubular atrophy, whereas cell death can be the outcome (Slattry et al. 2012).

1.3. Kidney-structure and function

1.3.1. Kidney structure

The kidneys function is to maintain homeostasis in the body. Functional departments of the kidney are the nephrons, and each kidney is made up of about a million nephron units. Nephrons are divided into two parts, renal corpuscle and renal tubule (Taal et al. 2012). Renal corpuscle has two subunits, glomerulus, which is a network of capillaries, and Bowman's capsule, which encloses the glomerulus. The blood filters through glomerulus, which gives a filtrate consisting of water and solutes, so-called glomerular filtration.

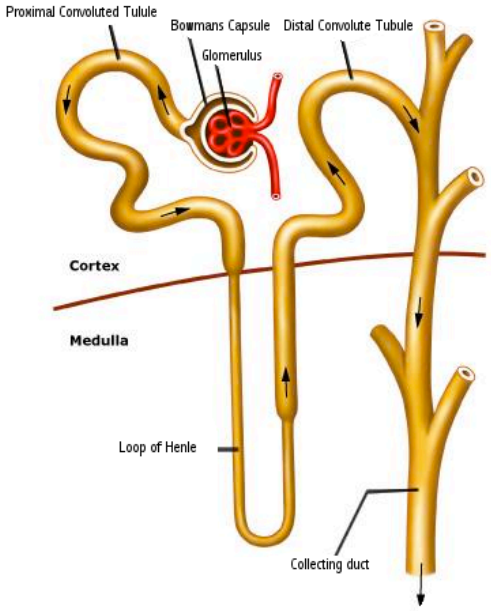


Figure 1.1.: **An illustration of the kidneys nephron filtering units**. Renal corpuscle has two subunits, glomerulus, which is a network of capillaries, and Bowman's capsule where the blood filters through. Renal tubule receives fluid from renal corpuscle and passes it through proximal tubule, Loop of Henle, distal tubule and collecting duct system. Figure modified from (Muskopf 2014).

This filtrate continues into the surrounding unit, Bowman's capsule, and continues through the tubules. Tubules modify the filtrate by reabsorption and secretion. The first part of the renal tubule is called the proximal convoluted tubule (PCT). Water and solutes, which has passed through PCT, enters the Loop of Henle consisting of two parts, the descending -and ascending limb of Henle. To pass the Loop of Henle the filtrate will pass from cortex to medulla and back to cortex again. Fluids passing the loop of Henle enter through the distal convolute tubule (DCT) and converge to the collecting duct (Taal et al. 2012).

1.3.2 Renal transporters and regulation

Transporters in the epithelial cells modify the filtrate produced by the nephrons. These transporters drive components into and out of the cell and play an important role in drug distribution, tissue-specific drug targeting, drug absorption, and elimination (Taal et al. 2012). Components can be reabsorbed from the fluid back into the cell or secreted with

the urine. Efflux transporters lead components from the cell to the lumen or the blood and influx transporters lead compounds from the blood into the cell (P.D. Ward 2013). Interaction between transporters located in basolateral and apical side of the membrane is crucial for renal clearance of a component (P.D. Ward 2013). Inhibition of basolateral transporters may result in increased exposure and circulation of a drug. Inhibition of apical transporters can lead to accumulation of the drug inside the cell and increase renal toxicity.

CNIs is in general metabolized by cytochrome P-450 (CYP) 3A4 in the gut and liver. In addition, CNIs are transported through an efflux pump P-glycoprotein (P-gp) which is found in the intestine, liver and kidneys (P.D. Ward 2013). CNI located in the cell membrane or cytoplasm is pumped over to the extracellular matrix by P-gp (Hesselink et al. 2010). Observations indicates that CNI induces P-gp in renal cells and thereby serve to protect the cell from toxicity (Hesselink et al. 2010).
As for the organic anion transporter (OAT) and the multi drug resistance protein (MRP) transporters they are localized in the basolateral and the apical side of the membrane in proximal tubule cells (Hauser et al. 1998). They contribute to tubular influx and efflux of drugs such as CNIs (Anzai & Endou 2007; El-Sheikh et al. 2013). However, drug transporters in tubular cells are essensial to nephrotoxic compounds as they modify drug transport and participate in the regulation of absorption and elimination (El-Sheikh et al. 2013).

1.4. Oxidative stress and apoptotic mechanisms in CNI nephrotoxicity

Apoptosis is a process that is required for normal development and maintenance of homeostasis in cells and tissues (Elmore 2007). Studies have shown that ROS and the resulting oxidative stress plays an important role in nomal function and apoptosis mechanisms (Anzai & Endou 2007). ROS are cytotoxic when it reaches a threshold in the cellular environment which is linked to the cells antioxidant level. This threshold can be lowered or raised by making the cell more or less exposed to oxidative stress by decrease or increase the level of the antioxidant defence (Kannan & Jain 2000). Flavonoids are known to influence this antioxidant defense (Kumar & Pandey 2013).

Nephrotoxicity is usually a reversible process although long exposure to toxic agents can cause irreversible damage to the renal structure and function. Long-term use of calcineurin inhibitors may cause kidney damage by activating the RAS and regulation of growth factors (Brunetti et al. 2013), leading to renal apoptosis and renal dysfunction. Research on apoptosis has been on-going for several years; however, the apoptotic mechanisms remain unclear (Xiao et al. 2011; Xiao et al. 2013). It has been suggested that there are at least five apoptotic pathways involved to mediate renal cell apoptosis, including FAS/FAS-L, mitochondrial, endoplasmatic reticulum, angiotensin II and hypertonic pathways (Xiao et al. 2011). In vitro studies showed that caspases might be the intersection of these pathways (Campistol & Sacks 2000; Xiao et al. 2013).

Mitochondrial pathway is the cornerstone of the apoptotic intrinsic pathway (Xiao et al. 2013). Mitochondrial injury leads to changes in the inner mitochondrial membrane, resulting in loss of transmembrane potential and subsequently release of pro-apoptotic proteins into the cytosol, which activates caspases and downstream pathways (Servais et al. 2008; Xiao et al. 2011; Xiao et al. 2013). *Bax* is a pro-apoptotic member of *Bcl2* family with the ability to activate mitochondrial permeability transition pore and affect mitochondria to release pro-apoptotic proteins to cytosol. The regulation of pro-apoptotic events occurs through activation of anti apoptotic *Bcl2* members (Alberts et al. 2008).

While some of the mechanisms of CNI associated nephrotoxicity are known, still a lot is unknown about clinically relevant modifying factors. Bile acids are frequently increased after liver transplantation. Biliary complications lead to accumulation of bile acids in plasma, and their only excretory route is through the kidneys. Different nuclear receptors are involved in the regulation. Farnesoid X receptor (FxR) is a ligand activated nuclear receptor expressed in the liver and kidney. In the liver it affects bile acid metabolism. Studies have observed that Mrp4 was stimulated in FxR knockdown mice liver (Lee et al. 2006). Resulting in increased levels of serum bile acids (Zelcer et al. 2003). FxR regulates organic solute transporters in the kidney (Zelcer et al. 2003), and can be activated by endogenous bile acids, such as chenodeoxy cholic acid (CDCA) (Lee et al. 2006). FxR activation leads to translocation of FxR into the nucleus, where it forms a heterodimer with Retinoic acid receptor (RXR), and this complex binds to the

FxR response elements (FXREs) on DNA, which leads to regulation of curtain genes (Zhang et al. 2014). Observations suggest a connection between FxR and Mrp4, but studies on this field are limited (Jiang et al. 2013).

1.5. Cell cultures and animal models

Culture of cell lines in the laboratory is a widely used technique in cellular and molecular biology. The technique was maintained for the first time in 1885, but was not successfully undertaken before the researcher Ross Harrison explanted tissue from frog embryo into frog lymph clots in 1907 (Zelcer et al. 2003). Cell cultures and animal models represent an important experimental set-up to study mechanisms of toxicity, since they provide the possibility to control and manipulate conditions. Different cell lines are available for laboratory experiments. To reduce the use of animal models and the risk assessment studies, toxicity caused by immunosuppressive drugs could be studied in in vitro models. Animal models have physiological and genetic characteristics similar and different from humans. A good model system should reflect an in vivo-like situation on the best possible way compared to human. In some cases pigs exhibit similarities closer to man compared to rodents models. Due to this we choose EFN-R cell line in our laboratory. This makes pigs as good models for research on complicated systems as in humans (Ryan 2014).

1.6. The aim of the project

The main goal of this project is to study CNI toxicity in a kidney cell-line model to reveal mechanisms of direct CNI toxicity to the kidney and to discover factors that may modify such mechanisms.
To achieve this, specific aims are:

1. To characterize the EFN-R kidney cell line with respect to culturing conditions and proteins markers

2. To examine the role of oxidative stress and apoptotic mechanisms in CNI toxicity in this model.
3. To examine whether CNI affects membrane transporters in the EFN-R kidney cell line.
4. To examine whether other factors may modify the effects of CNI mediated damage. Specifically we want to test the effect of the bile acid chenodeoxy cholic acid on CNI stimulated toxicity as bile acids are frequently increased after liver transplantation due to biliary complications.

Chapter 2

Materials and methods

2.1. Materials

2.1.1. Cell line

EFN-R fetal kidney cells were obtained from Friedrich- Loeffler- Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany.

2.2. Methods

2.2.1. Cell culture

The cells were cultured in sterile environment with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, California) supplemented with 10% fetal bovine serum (FBS) (BioWest) and 1% Penicillin-Streptomycin (Pen-Strep, Life Technologies, California) unless otherwise indicated. Serum (FBS) is of South America origin. Cells were rinsed with a Phosphate buffered saline (PBS), (BioWhittaker, Verviers), and detached by adding dissociation reagent, 0.25% Trypsin-Etylen-Diamin-Tetra-Acetat, (trypsin-EDTA, BioWittaker, Verviers) for 4 minutes. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Experiments were performed within passage six to twenty.

DMEM, RPMI-1640 and Williams -media was tested for physical properties as shown in chapter 3.1.1.

2.2.2. Counting cells

Cell concentration was determed by counting cells in a Burcher counting chamber, with a 1:10 dilution of 0,4% tryptan blue (Sigma-Aldrich, Missouri). Cells were visualized with a microscope (10X objective). Living cells were displayed as transparent dots against a clear background, whereas dead or damaged cells were indicated as distinct blue dots.

2.2.3. Cell stress parameters and oxidative agents

Cells were plated in a 12-well culture plate (Corning Life sciences, Massachusetts) with a density of 1 x 10^5 cells/ml in pre-warmed fresh medium supplemented with 10% FBS and 1% Pen-Strep. 24 h (hours) after plating the cells were treated with different concentrations of hydrogen peroxide (H_2O_2, Sigma-Aldrich, Missouri), ferulic (Sigma-Aldrich, Missouri), vanillic acid (Sigma-Aldrich, Missouri), morin (Sigma-Aldrich, Missouri), tacrolimus (FK506, Sigma-Aldrich, Missouri), cyclosporine A (CsA, Sigma-Aldrich, Missouri) and chenodeoxy cholic acid (CDCA, Sigma-Aldrich, Missouri) as indicated. Cells were incubated for 24 h in 37°C with 5% CO_2 prior to analysis.

2.3. Cell viability

2.3.1. MTT assay, determination of Cell Viability

The MTT assay is based on the conversion of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, Sigma-Aldrich, Missouri) into purple formazan crystals in viable cells (Delgado-Ortega et al. 2014; Jeon et al. 2013). MTT (5 mg/ml) was dissolved in PBS and 550 uM glucose (Sigma-Aldrich, Missouri), to make a MTT stock solution. Cell medium was removed and stock MTT solution was added (500 ul) to the wells. Incubation was carried out in humidified environment at 37°C with 5% CO_2. After 60 minutes, MTT solution was aspirated and 500 µl Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Missouri) was added to dissolve the crystals (formazan). After

15 minutes incubation in room temperature the measurements was carried out with enzyme-linked immunosorbent assay (Elisa) reader (Thermo Scientific, Massachusetts) by using Ascent Software and a wavelength at 570 nm to measure the released formazan, and 690 nm to subtract the background noise.

2.3.2. Flow Cytometry, determination of cell death

For analysis of cell death, cells were seeded in 12-well cell culture at a density of 2 x 10^5 cells per well and incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Cells were than treated with H_2O_2 (100 and 1000 μM) and morin (100 μM) and incubated for additional 24 h. After incubation, cells were stained with PE Annexin V Apoptosis Detection Kit I (BD Pharmingen, New Jersey) according to the manufactures instructions. Briefly, cells were detached with trypsin-EDTA solution, washed with PBS, resuspended in Annexin V binding buffer and incubated with phycoerythrin (PE) conjugated Annexin V and 7-Amino-Actinomycin (7-AAD) for 15 min at room temperature in the dark. Samples were analyzed on FACSCalibur (BD Pharmingen, New Jersey), and data were collected on 50.000 cells.

2.4. Gene expression studies

2.4.1. Total RNA extraction

Cells were seeded with a density at 1 x 10^5 cells/ml and incubated for 24 h before harvesting. Total RNA was isolated from EFN-R cells using E.Z.N.A.® Total RNA Kit from Omega following the manufactures instructions (http://omegabiotek.com/store/wp-content/uploads/2013/04/R6834.pdf). The quantity and purity of the RNA was measured spectrophotometric using NanoDrop ND-1000 (Saveen Werner, Malmö).

2.4.2. cDNA synthesis

cDNA was synthesized from mRNA with the enzyme revers transcriptase and was performed using the High-Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, California). cDNA master mix were manufactured as described in the kit. RNA samples were diluted with nuclease-free water (Life technologies, California), and cDNA master mix was added to a final concentration of 20 ng/µl. The samples were revers transcribed using the PTC-100 Thermal Cycler (California). Temperature program for this procedure are described in table 2.2.

Table 2.2. PCR program used to amplify cDNA

Step	Action	Temperature (°C)	Time (min)
1	Primer annealing	25	10
2	Revers transcription	37	120
3	Denaturation of the enzyme	85	0.5
4	Final extension Storage	4	∞

2.4.3. Quantitative Real Time PCR

The expression for selected genes (*Bcl2*, *Bax*, *FxR*, *Mrp4*) was analyzed by quantitative Real-Time PCR (qRT-PCR) using Power SYBR® Green (Applied Biosystems, California) dye–based PCR amplification and detection array, according to manufactures instruction. Primers used were;

Bcl2 forward: 5`- tggtgagtcggatcgcaact- 3` , revers 5`- agagttccacaaaagtgtcccag -3 ;

Bax forward: 5`- agcgagtgtctcaagcgcat - 3` , revers 5`- acacctctgcagctccatgttac - 3`;

FxR forward: 5`- cctgtgaggggtgtaaaggtttc - 3` , revers 5`- ccttagtcgacactcttgacactttc- 3`;

Mrp4 forward: 5`- ctgtgataggagatcgggga- 3` , revers 5`-ctgagaggatcgtccaggag- 3`;

PO forward: 5`- acaatgtgggctccaagca - 3`, revers: 5`- catcagcaccacggctttc - 3.

cDNA samples were diluted, and added to a master mix according to table 2.3. to a total

concentration of 10 ng.

Table 2.3.: **Master mix for qRT-PCR**. The volume indicates volume/sample.

Reagent	**Volume (ul)**
SYBR® Green	12,0
Forward primer (10 pmol/ul)	0,5
Revers primer (10 pmol/ul)	0,5
Nuclease-Free water	11,5
Total	25,0

Master mix and sample were added to each well (genes were run in triplicates) and centrifuged (Sorvall® RT6000B, Delevare), before the plate was preceded by SYBR-Green 96 well program on the qRT-PCR machine, Viia7 (Applied Biosystems, California) by a set-up according to table 2.4.

Table 2.4.: **Thermal cycle profile**

Temperature (°C)	**Time (min: sec)**
50	02:00
95	10:15
60	01:00
95	00:15
60	01:00

Reactions were run for 40 cycles before the melting curve (Tm) and cycle threshold (Ct) values were calculated. The Tm curve was determed to verify the specificity of the amplification. The instrument software calculated the detection threshold for each gene (Applied Biosystems, California), and the Ct value was calculated from threshold,

which corresponds to the cycle number and fluorescence signal in the exponential phase. The relative expression levels between the samples were calculated using the comparative delta delta Ct (threshold cycle number) method (van Meerloo et al. 2011). Ct values for target genes were normalized to Ct values for reference gene (*P0*), providing the ΔCt value ($Ct_{target\ gene} - Ct_{P0}$). Further, ΔΔCt was calculated for each gene by subtracting the ΔCt value of target sample from ΔCt value of control sample ($\Delta Ct_{target\ gene} - \Delta Ct_{ctl}$). This procedure provides to calculate relative gene expression between to samples, according to the formula $RE = 2^{-(_{\Delta\Delta}Ct)}$ (Schmittgen & Livak 2008).

2.5. Protein expression

2.5.1. Isolation of proteins from adherent cells

Cells were seeded as described earlier (Ch. 3.1.3.) and rinsed with cold PBS before they were collected with trypsin-EDTA solution after 10 minutes following incubation in humidified environment. Cold medium (DMEM) supplemented with 10% FBS was added to each well, before the samples were transferred to 15 ml tubes (Sarstedt, North Rhine-Westphalia) and centrifuged for 5 minutes, 1,100 g at 4°C. The supernatant was dissolved to a pellet, before the pellet was suspended in PBS and transferred to centrifuge tubes. The tubes were centrifuged at 3600 g for 5 min at 4°C. Supernatant was removed and 25 μl lysis buffer were added. The samples were boiled at 95°C, 2 x 5 min, vortexed, and centrifuged at 13.000 g for 5 minutes at 4°C. The supernatant was transferred to a new tube, and samples were stored at -20°C until further use.

2.5.2. Protein quantification with Bradford protein assay

Relative protein concentrations were measured using Bradford assay (Bio-Rad, California). Bovine Serum Albumin (BSA, Sigma-Aldrich, Missouri) was prepared by 0, 1, 2, 4, 6, 8 and 10 ul of 5 x 10^{-7} μg/l BSA, diluted in water to a total volume at 30 μl. Isolated protein from adherent cells were diluted to a 1:10 ratio, and isolated protein from cortex tissue was diluted 1:70. An aliquot of Bradford reagent was added to each sample, and was detected at 595 nm, after 5 minutes incubation in room temperature.

2.5.3. Western Blotting

Equal amounts (20 μg) of total protein were separated by 12% *Sodium dodecyl sulfate polyacrylamide gel electrophoresis* (SDS-PHAGE) (Bio-Rad, California) and transferred to a *polyvinylidendifluorid* (PVDF) membrane (Bio-Rad, California). Membranes were blocked with Tris buffer saline Tween 20 (TBS-Tween buffer) (Sigma-Aldrich, Missouri) supplied with 5% milk powder (Bio-Rad, California), and incubated with primary antibodies against Tamm Hosfall protein (THP, Sigma-Aldrich, Missouri), System N1 (SN1) amino acid transporter (Pierce, Massachusetts), Aquaporin 2 (Aqp2, Abcam, Massachusetts) or FxR (Pierce, Massachusetts) over night at 4 °C. The membranes were then washed and incubated with secondary antibody in room temperature for one hour. Protein bands were visualized after 5 minutes incubation with Horseradish Peroxidase (GE Healthcare, UK). Goat anti β-actin (Santa Cruz, Texas) was used as endogenous control. Pictures were captured with G:BOX imaging system (Syngene, UK), and data was normalized and calculated according to β-actin.

2.6. Statistics

Quantitative data are presented as mean and standard deviation. Two-tailed test is used and p-values less than 0.05 were considered significant. One-way analysis of variance with Bonferroni post-hoc test or Dennett's multiple comparison tests was used for the statistical analysis. Figures were generated using GraphPad Prism version 6.00 for Mac, GraphPad Software, La Jolla, CA.

Chapter 3

Results

3.1. Cell line characterization

3.1.1. General characteristics of EFN-R cell line; Morphology and growth conditions

EFN-R cells were thawed, grown, and split with a ratio 1:4 (figure 3.1.).

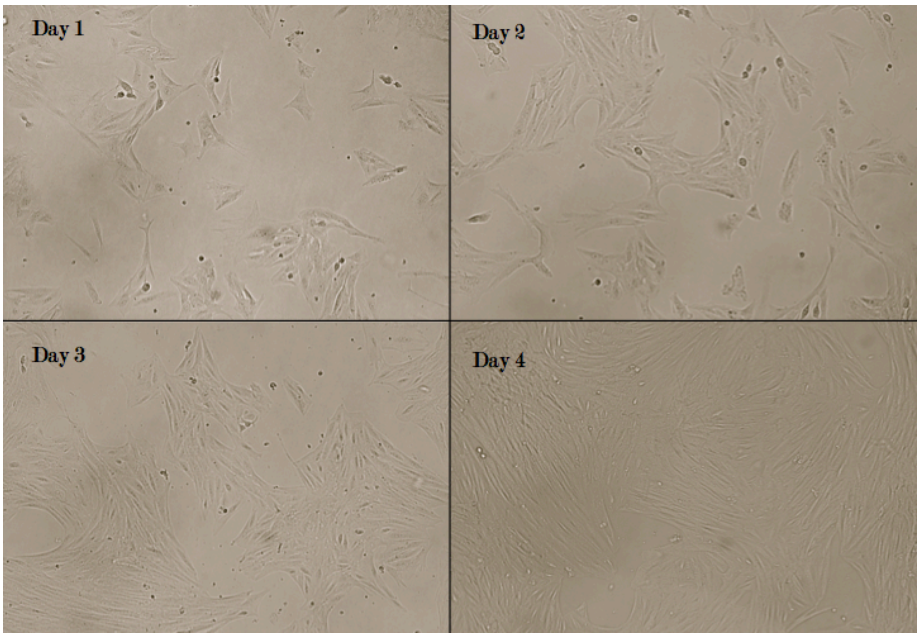


Figure 3.1.: **Piglet kidney cell line EFN-R at different states of confluence.** In the figure EFN-R cell cultures are shown at passage 10 throughout four days of culturing: (**A)** Day one after plating, cells have attached to the culture plate. **(B)** Day two, the cells have started to proliferate. **(C)** Day three, the cells have become semi-confluent. **(D)** Day four, the culture plate is completely covered with cells and shows almost 100% confluence.

The metabolic activity (measured by MTT assay, Ch. 2.3.1.) of EFN-R cells was tested by incubation with three different commercial cell media (table 3.1.), with and without

penicillin/streptomycin (figure 3.2.). No major difference in metabolic activity could be observed among the three tested cell culture media after 24 h. Further, 1% penicillin/streptomycin (Pen-Strep) did not affect metabolic activity (figure 3.2.).

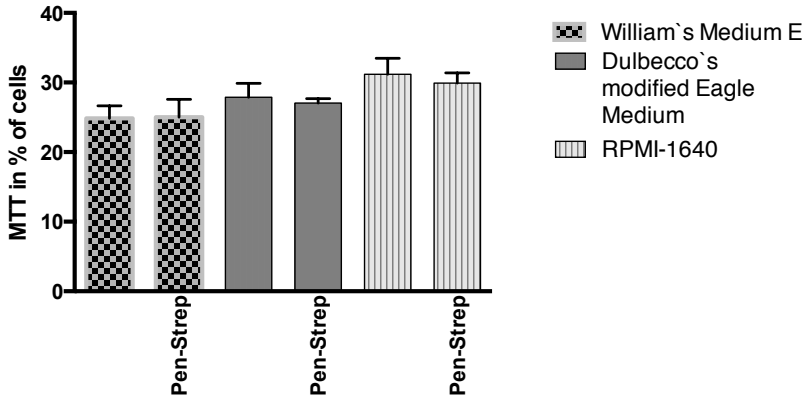


Figure 3.2.: **Effect of different growth mediums and Penicillin/Streptomycin (Pen-Strep)**. EFN-R cells were harvest in 12-well plates and incubated for 24 h in different growth medium diluted with 10% FBS and 1% Pen-Strep. X-axis illustrates different growth mediums with and without Pen-Strep, whereas y-axis shows percent of healthy cells after 24 h incubation. Mediums tested are Williams, DMEM and RPMI. Data are presented as mean and standard deviation with n=3 for all treatments.

Table 3.1. Characteristics of cell media

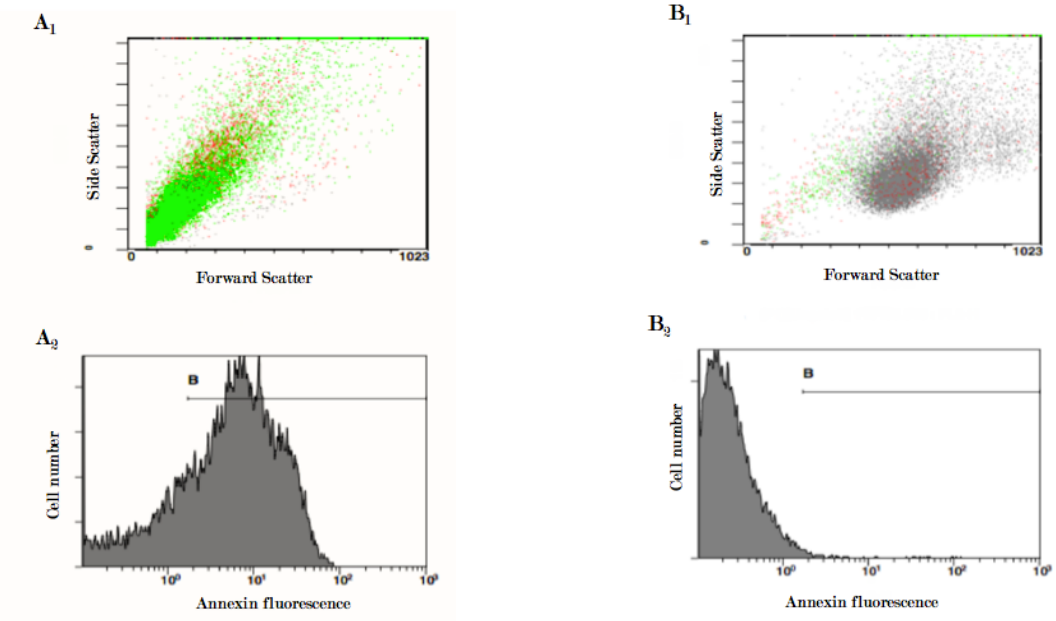
	Phenol Red (mg/L)	D-Glucose (mg/L)	Pyruvate (mg/L)	L-Glutamine (mg/L)	HEPES (mg/L)
William`s Medium E (Cat. N° 22551-022)	10.0	2000	25	–	–
Dulbecco`s Modified Eagle Medium (Cat. N° 31885-023)	15.0	1000	110	580	–
RPMI-1640 (Cat N° BE12-702F)	5.1	2000	–	300	–

3.1.2. Method testing: Cell harvesting

EFN-R cells were usually divided twice a week in a ratio (1:3-1:4). Some cell lines are very sensitive to treatment with dissociating enzymes like trypsin and are therefor harvested by scraping. Trypsin can cause degradation of surface antigens and thereby

change the flow characteristics (FCS and SSC) of the cells. To examine whether the EFN-R cells preferred harvested by mechanical cell scraping or enzymatic trypsinization we compared the two methods using PE Annexin V Apoptosis Detection Kit I (Ch. 2.3.2.). PE Annexin V and 7AAD differentiates between early and late apoptotic cells. Annexin has high affinity to phosphatidylserine (PS). 7AAD is an intercalator that only binds to DNA when the cell membrane is damaged. PS is primarily located on the endocellular side of the plasma membrane aside from when the cells undergo apoptosis, then PS will move to the extracellular side of the membrane marking the cell as an apoptotic body to the exterior environment. In a late phase of apoptosis an insertion of 7AAD occurs (Bouchier-Hayes et al. 2008; Zembruski et al. 2012).

200 000 cells/well were cultured in a 12-well plate for 24 hours and harvested with either careful mechanical scraping or treated with trypsin for 8 minutes. The cell suspension (50 000 cells) was passed through the flow cytometer and the physical properties for each cell was measured according to their fluorescence and plotted in a 2D plot. The appearance of early and late apoptotic cells in figure 3.3$A_{1\text{-}4}$ demonstrates the presence of cell death after harvesting by gently mechanical scraping. The 2D plots in figure 3.3$B_{1\text{-}4}$ illustrate undamaged and healthy cells treated by a standard trypsinization method.



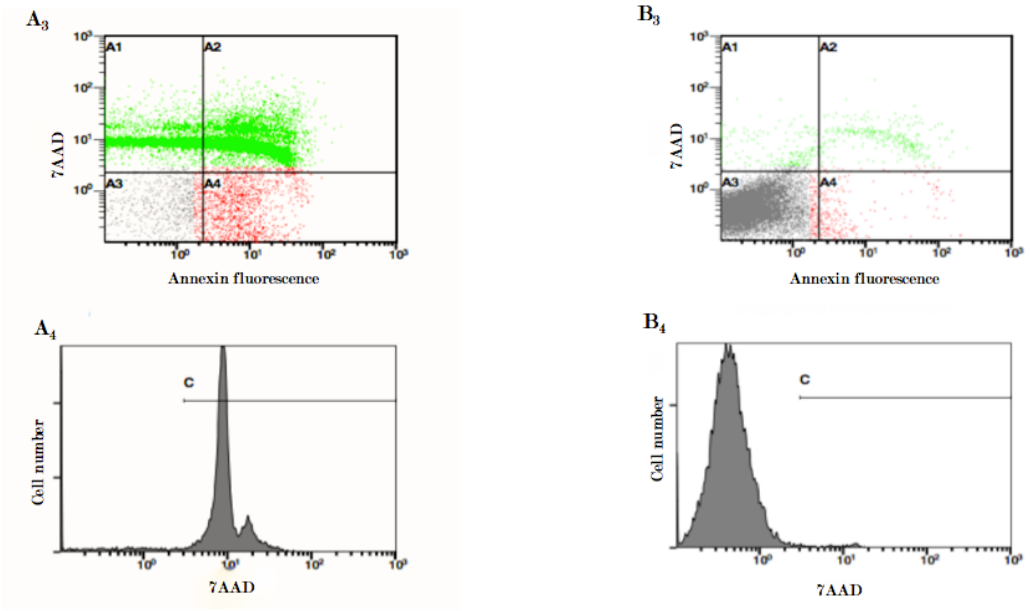


Figure 3.3.: **Effect of different harvesting methods on cell damage.** EFN-R cells were cultured in 24 h and detached by either by (**A**) mechanical scraping or (**B**) trypsinization. A_1 and B_1 demonstrated size (forward) and granularity (side) in the cell population. A_2 and B_2 show cells ability to extract Annexin/7AAD. A_3 and B_3 indicate cell number stained with Annexin. A_4 and B_4 illustrate cell number stained with 7AAD.

3.1.3. One or more cell populations in the EFN-R cell line?

To investigate whether the EFN-R cell line consists of one or more cell populations flow cytometry was performed. The cells are displayed in a scattered plot as one cell population (figure 3.4.). Forward scatter (FSC, x-axis) is proportional to the cells areal or size. Side scatter (SSC, y-axis) is proportional with the granularity and the complexity inside the cell. Results indicate that EFN-R cell line consist of one single cell population.

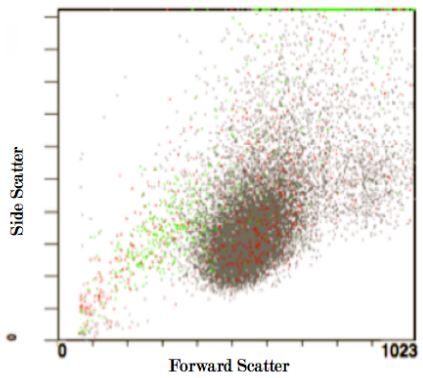


Figure 3.4.: **Flow cytometry to distinguish between different cell populations**. Cells were harvested and plated (200 000 cells/well) before flow cytometry analysis was performed. Cells are displayed as a single cell population in a scattered plot, where the relative fluorescence is measured against forward scatter (x-axis) and side scatter (y-axis) light. Forward scatter (FSC) is proportional with the cells areal or size. Side scatter (SSC) light was proportional with the granularity and the complexity inside the cell (n=3).

3.1.4. Characterization of protein markers in EFN-R cells

The distribution of proteins in the different sections of the nephron can be used as markers to distinguish between various cell types in the kidney. 24 h after plating the EFN-R cells were harvested (Ch. 2.2.1.), protein isolated (Ch. 2.5.1.), fractionated on a SDS-gel and analyzed by Western blotting (Ch. 2.5.3.). Western blotting detects the presence of SN1, THP and Lrp2. Aqp2 results illustrate a broad diffuse protein fragment slightly higher than expected and we were not finally able to neither confirm nor exclude its presence (figure 3.5.).

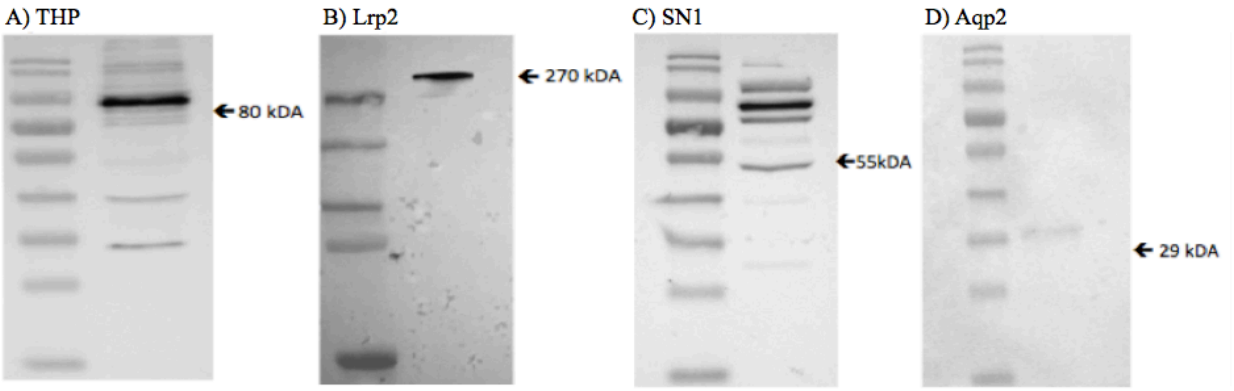


Figure 3.5.: **Detection of kidney specific proteins by Western Blotting.** Protein samples from cell-line EFN-R were separated by SDS-PHAGE and immunostained with antibody generated from different animals (Ch. 2.5.3.) Arrows mark the desired expression. (**A**) Anti-Tamm-Horsefall (THP) antibody detected THP at approximately 80 kDA, in addition to multiple bands of different conformations. (**B**) Megalin (Lrp2) was detected and revealed a clear band at 270 kDA. (**C**) System N glutamine transporter (SN1) was strongly expressed in more than one conformation (n=3). Additional fragments beside 55 kDA were unidentified. (**D**) Aquaporin 2 (Aqp2) exhibits a broad diffuse fragment with slightly increased size than expected. (n=3 for all targets, except for Aqp2 with n=5).

3.2. Effect of calcineurin inhibitors (CNI) on EFN-R cell line

3.2.1. Effect of calcineurin inhibitors regarding to cell viability

Cells were plated in 12-well plates (Ch. 2.2.3.) and exposed to different concentrations of CNI (0.1, 1.0, 10.0, 50.0 μM) for 24 h. The MTT assay (Ch. 2.3.1.) was performed to access cell viability after exposure. The CNIs dose-dependently decreased cell viability. 50 μM of FK506 and CsA reduces approximately 38,5% and 20,1%, respectively (figure 3.6. and 3.7.). Data represents group mean and standard deviation.

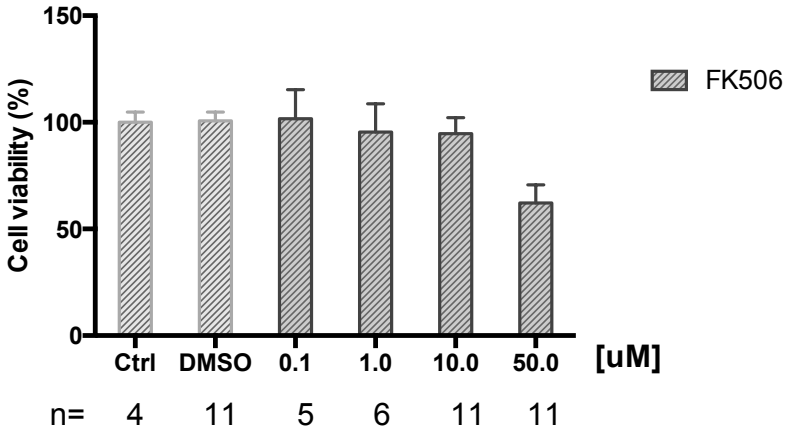


Figure 3.6.: **Effect of FK506 on metabolic activity in EFN-R cells.** EFN-R cells were treated with different concentrations of FK506 (0.1, 1.0, 10.0 and 50.0 μM) for 24 h, and measured with MTT assay. Data represents group mean and standard deviation with different replications (indicated by numbers below columns).

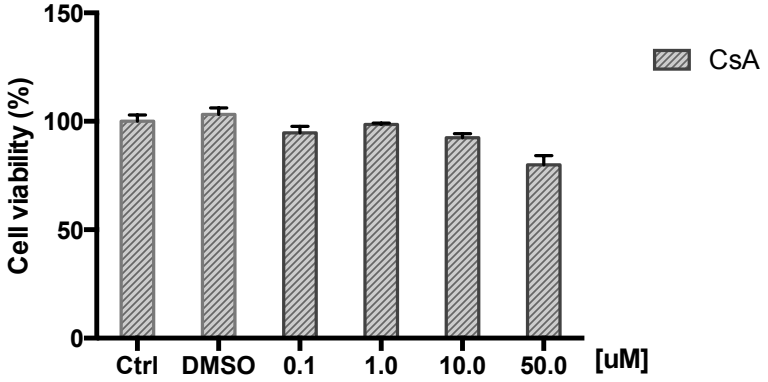


Figure 3.7.: **Effect of CsA on metabolic activity in EFN-R cells.** EFN-R cells were treated with different concentrations of CSA (0.1, 1.0, 10.0 and 50.0 μM) for 24 h, measured by MTT assay. Data represents group mean and standard deviation, n=4 for all groups.

3.2.2. Effect of oxidative stress and antioxidants on EFN-R cell-line

A task of this project was to establish a model of FK506 toxicity. To investigate whether oxidative stress could be a step in FK506 toxicity, we tested the effects of different antioxidants on hydrogen peroxide induced cell damage in order to find the most effective antioxidant in this system. 24 h after plating, cells were exposed to increasing concentrations of hydrogen peroxide (50, 100, 200 μM) for 24 h and MTT assay was conducted in order to assess cell viability. We observed a decrease in cell

viability in a dose-dependent manner (figure 3.8.). Data represents mean and standard deviation.

To find the most effective antioxidant on this cell line morin, ferulic and vanillic acid were tested according to MTT signals (Ch. 2.3.3.). One-way ANOVA and Dennett's multiple comparisons test ($P < 0.05$) was performed compared to group with H_2O_2. Significant difference was observed after exposure to morin (figure 3.9.).

EFN-R cells were grown and plated (Ch. 2.2.3.) prior to treatment with different consecrations of morin (100 and 200 μM) and hydrogen peroxide (100 μM) for 24 h. Morphological images illustrate higher cell viability after treatment of morin in a dose dependent manner (figure 3.10.).

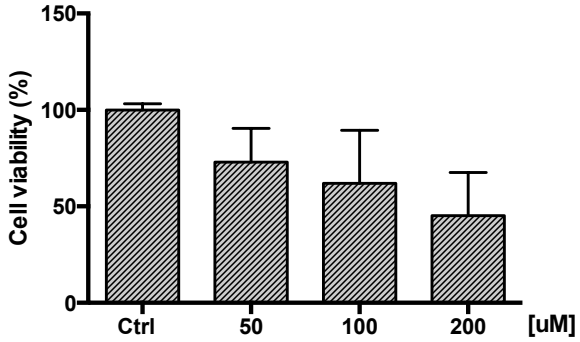


Figure 3.8.: **Hydrogen peroxide (H_2O_2) toxicity in EFN-R cells.** Cells were treated with different concentrations of H_2O_2 (50, 100, and 200 μM) for 24 h and measured with MTT assay. Treatments were performed with n=3 and data represents mean and standard deviation.

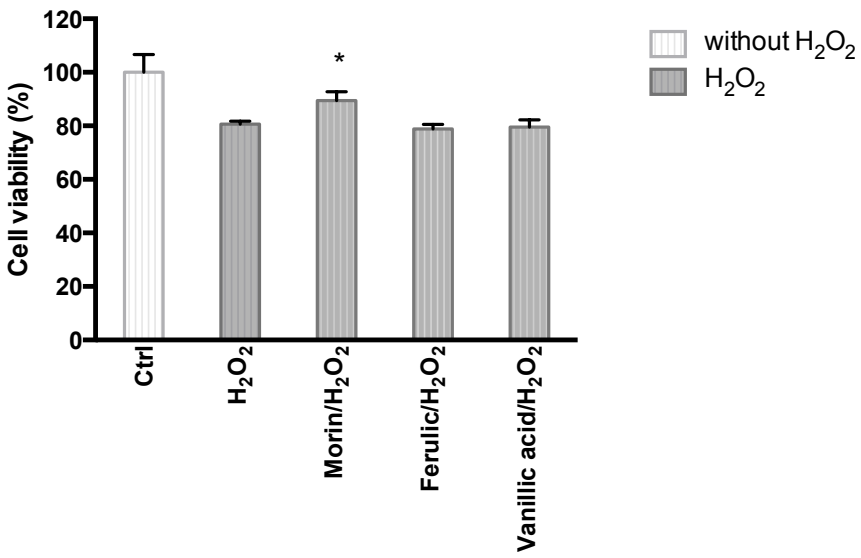


Figure 3.9.: **Effects of flavonoids on hydrogen peroxide toxicity in EFN-R cells.** Cells were exposed to 100 μM hydrogen peroxide and treated with different flavonoids (morin, ferulic or vanillic acid (100 μM)). MTT signal was measured after 24 h incubation (n=3). Data are presented as mean and standard deviation. One-way ANOVA and Dennett's multiple-comparisons test ($P < 0.05$) where performed and compared to the group with only H_2O_2.

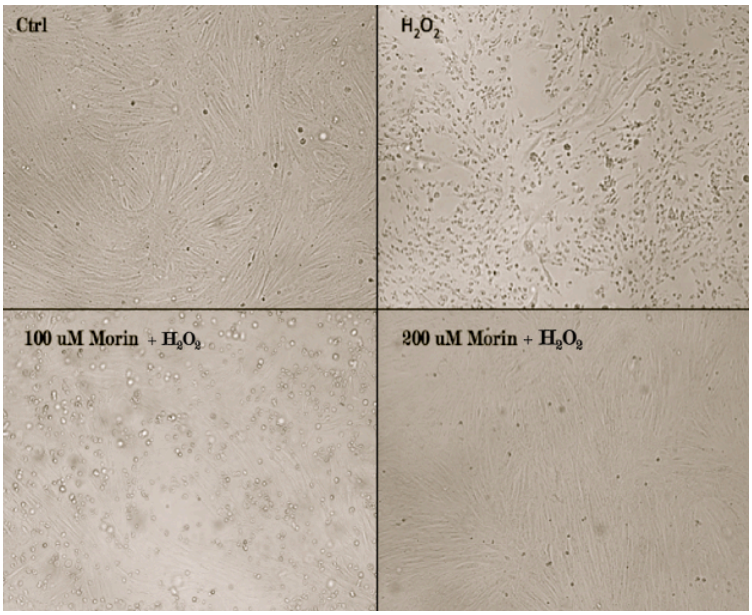
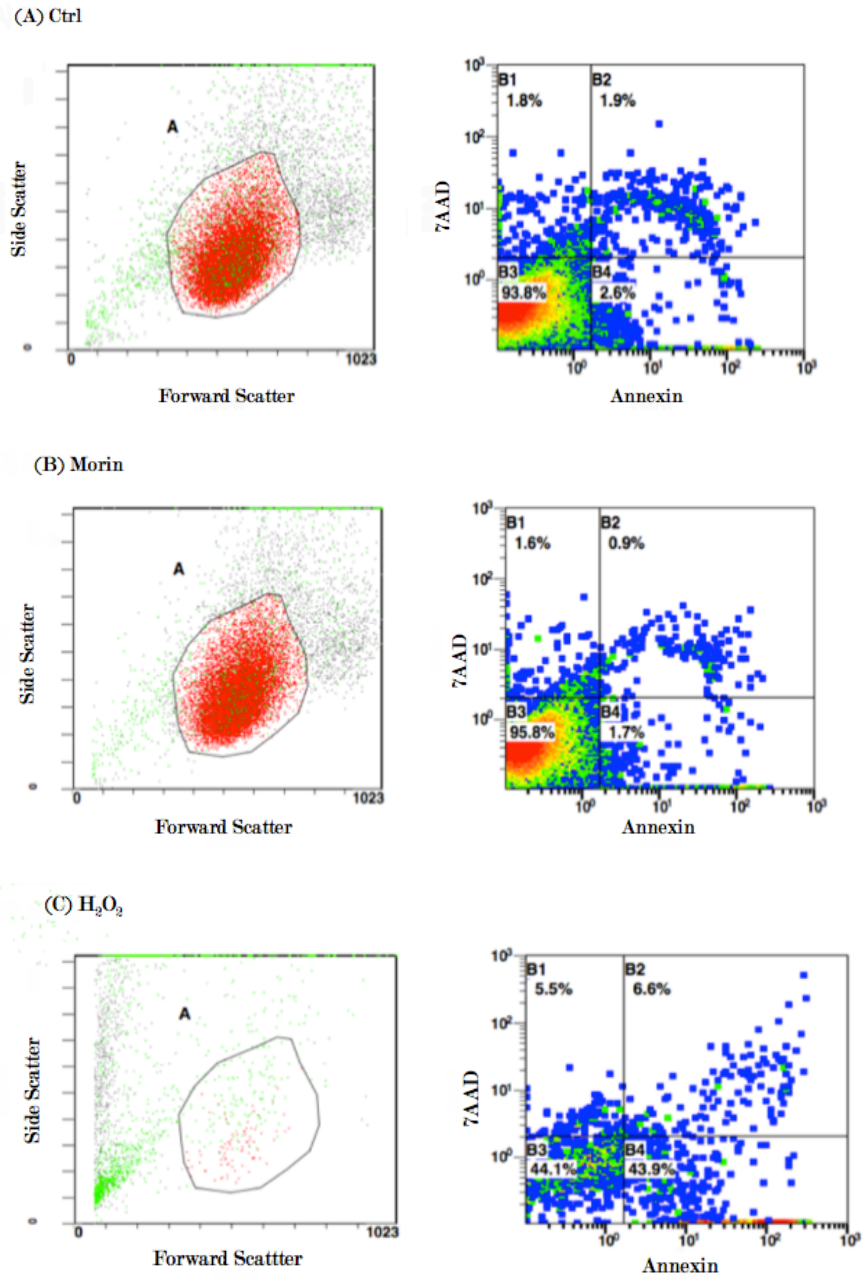


Figure 3.1.0.: **EFN-R cells exposed to hydrogen peroxide (100 μM) and Morin (100/200 μM).** EFN-R cells were grown and plated in 12-wells plates before treated with different consecrations of morin and hydrogen peroxide for 24 h. All samples except control sample contains 100 μM H_2O_2. Figures illustrate higher cell viability after treatment of morin in a dose-dependent manner.

By using enzymatic trypsinization method EFN-R cells were analyzed by flow cytometry (Ch. 2.3.2.) after exposure to morin (100 μM) and an extreme concentration

of hydrogen peroxide (1000 μM). Each particle appears in a scattered plot with intensity implied by a color. Where the red and blue color implies high and low intensity, respectively. Annexin stained (FL2 channel) cells were measured in against 7AAD-stained (FL3 channel) cells. Number of events scattered along the x-axis indicates Annexin stained cells, whereas number of events scattered along the y-axis indicates 7AAD-stained cells. The plot suggests early or late apoptotic cells. It should be noted that 1000 μM hydrogen peroxide caused extensive toxicity (<50 000 cells counted). Regarding the high concentration morin revealed a protective effect to the EFN-R cells (figure 3.11.).



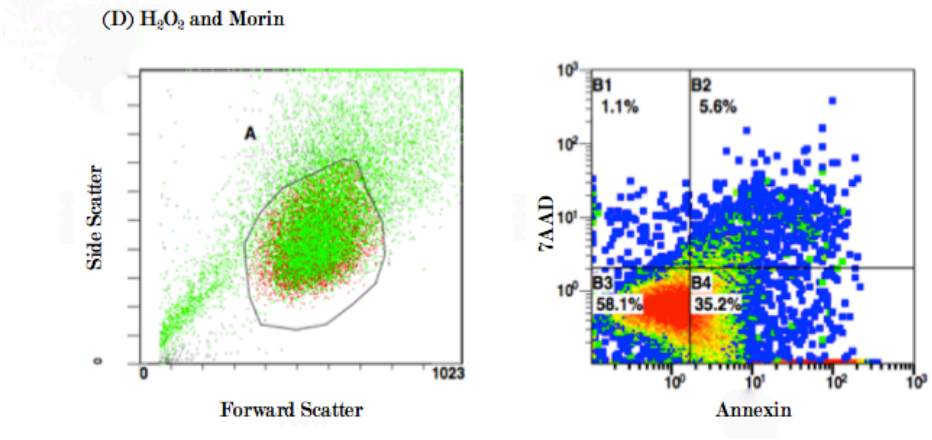


Figure 3.11.: **Flow cytometry analysis of Annexin/7AAD stained EFN-R cells exposed to morin (100 μM) and H_2O_2 (1000 μM).** Cells were plated and treated with morin and H_2O_2 for 24 h. The results are shown as fluorescence plotted against number of events for **(A)** control sample **(B)** sample treated with morin **(C)** sample exposed to hydrogen peroxide **(D)** sample treated with morin and hydrogen peroxide.

3.2.3. Is CNI associated nephrotoxicity caused by oxidative stress?

In order to investigate oxidative stress in relation to CNI we found the most effective antioxidant being morin (figure 3.9.). To investigate whether FK506 associated nephrotoxicity is related to oxidative stress we tested FK506 toxicity against the effective antioxidant. Cells were plated and exposed to morin (100 μM) and FK506 (50 μM) for 24 h. Cell viability was measured by MTT cleavage. Cells exposed to FK506 presented a significant increase in cell viability after treatment with morin (figure 3.12.). Data represents mean and standard deviation. The results were evaluated using one-way ANOVA with Bonferroni *post-hoc* test related to group with FK506.

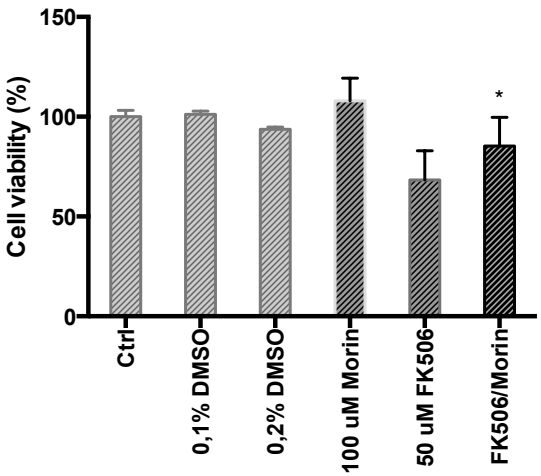


Figure 3.12.: **Cell viability of samples treated with morin and tacrolimus in 24 h.** Cells were plated and exposed to FK506 (50 μM) and morin (100 μM) for 24 h in humidified atmosphere. Cell viability was measured by MTT signaling (n=3). Data represents mean and standard deviation. The results were evaluated using one-way ANOVA with Bonferroni *post-hoc* test related to FK506 group.

3.3. Does chenodeoxycholic acids modify CNI toxicity?

3.3.1. Calcineurin inhibitor toxicity, bile acids and cell viability

In order to investigate if bile acids modify CNI toxicity in renal cells, CNI treated samples were exposed to 100 μM CDCA for 24 h. To measure their metabolic activity, the MTT assay was performed. CDCA did not modify CNI toxicity at the selected concentrations (figure 3.13.).

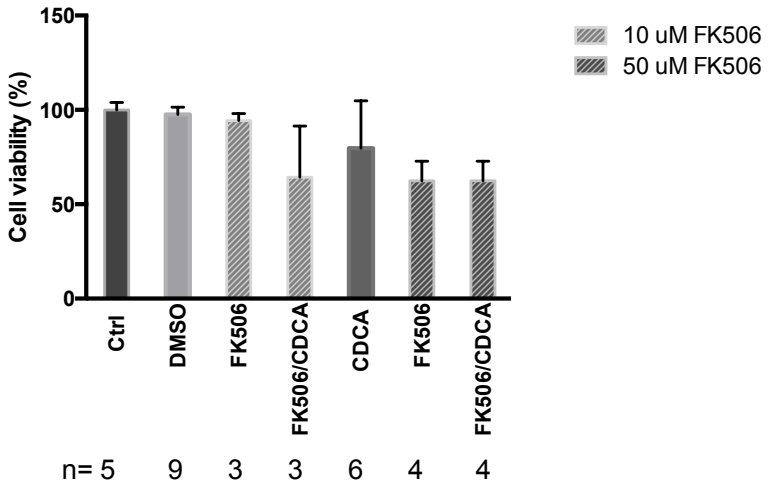


Figure 3.13.: **EFN-R cells exposed to chenodeoxycholic acid (CDCA) and FK506, measured by MTT assay.** Cells were plated and treated with different concentrations of FK506 (10, 50 μM) and CDCA (100 μM) for 24 h. Cell viability was measured by MTT method. Effect of 100 μM CDCA on CNI stimulated toxicity is not significant after 24 h exposure. Data represents group mean and standard deviation. Numbers below columns indicates replications.

3.3.2. Gene expression analysis of EFN-R cells

Relative gene expression of four different genes (*FxR, Mrp4, Bcl2, Bax*) was tested on EFN-R cells after exposure to different concentrations of CNI (0.1 and 10 μM). The p-value for *Mrp4* gene expression was considered significant after treatment with 0.1 μM CNI (figure 3.14.). There were no significant results of *FxR* gene expression after 24 h exposure of CNI at any of the concentrations (0.1 and 10 μM) selected (figure 3.14.). As for the apoptotic related genes, *Bax* and *Bcl2,* there were no significant results at the selected concentrations after 24 h.

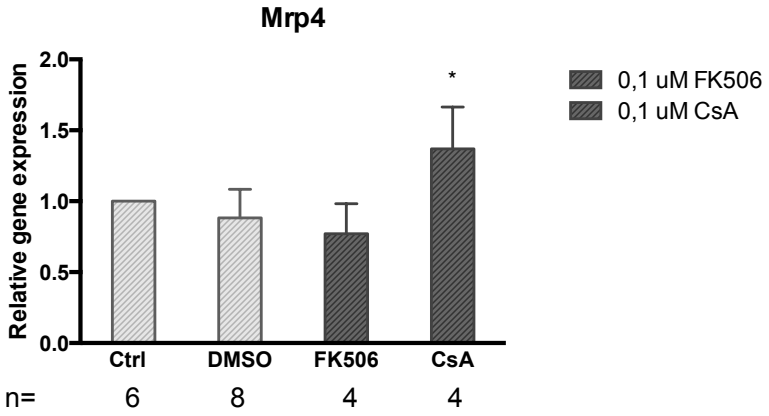


Figure 3.14.: **Gene expression analysis of samples treated with 0.1 μM CNI.** The relative expression between the treated samples is shown for the *Mrp4* gene. Samples were incubated for 24 h. Data represents group mean and standard deviation. The results were evaluated using one-way ANOVA with Bonferroni *post-hoc* test, whereas $p < 0.05$ was considered significant compared to DMSO group. Replications indicated by numbers below columns.

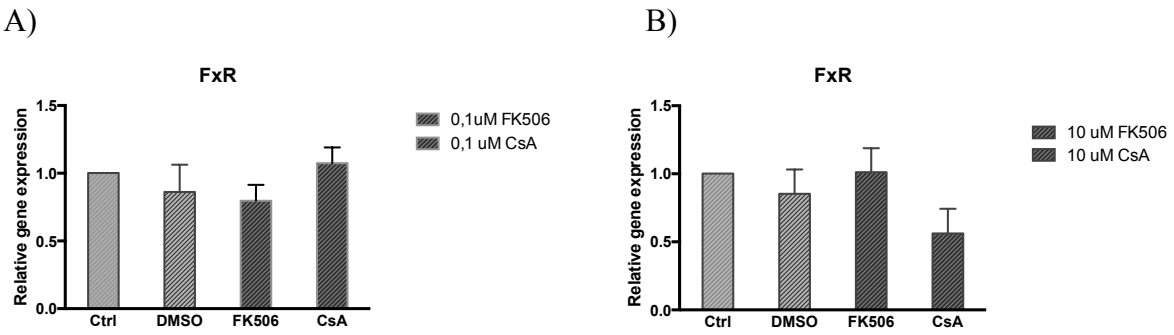


Figure 3.15.: **Gene expression analysis of samples treated with different concentrations of CNI.**
The relative expression between the treated samples is shown for the *FxR* gene. Samples were incubated for 24 h in humidified atmosphere. Figure shows *FxR* expression after exposure to (**A**) 0.1 μM CNI. (**B**) 10 μM CNI (n= 3 except for 10 μM CsA n=2). There were no significant results. Data represents group mean and standard deviation.

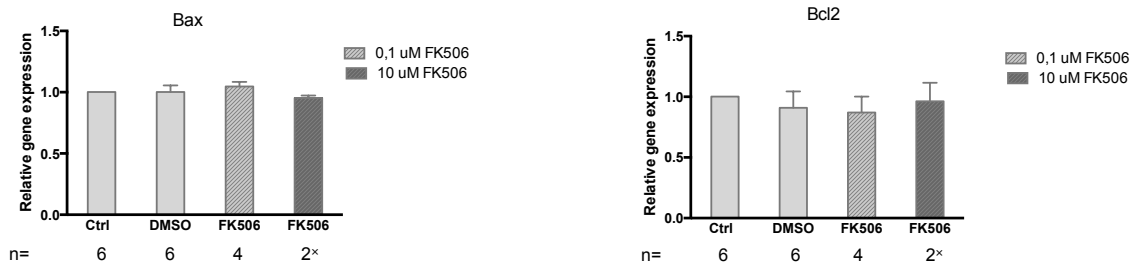


Figure 3.16.: **Gene expression analysis of samples treated with different concentrations of CNI.** The relative expression between the treated samples is shown for the *Bax* and *Bcl2* gene. Data represents group mean and standard deviation. [x] indicates results are limited to replications. Numbers below columns indicates replications.

3.4. Presence of Aquaporin 2 and Farnesoid X receptor on protein level

Cells were harvested (Ch. 2.2.3.), plated and treated with 10 μM FK506 for 24 h. Protein samples were isolated (Ch. 2.5.1.) and fractionated on a SDS-gel (Ch. 2.5.3.). Samples were immunostained with anti FxR and anti Aqp2 antibody and visualized by western blotting analysis (Ch. 2.5.3.). The results presented a clear fragment representative for FxR on protein level (figure 3.18.). Sample treated with 10 μM FK506 was decreased compared to control sample (figure 3.19.). Analysis is limited to replicates (n=1).

Figure 3.18.: **Detection of Farnesoid X receptor (FxR) in EFN-R cell line by Western blot analysis.** Protein samples were separated and immunostained with antibody raised from rabbit anti FxR antibody. Arrow marks the desired expression. The result was a clear fragment at 70 kDA specific for FxR protein (n=3).

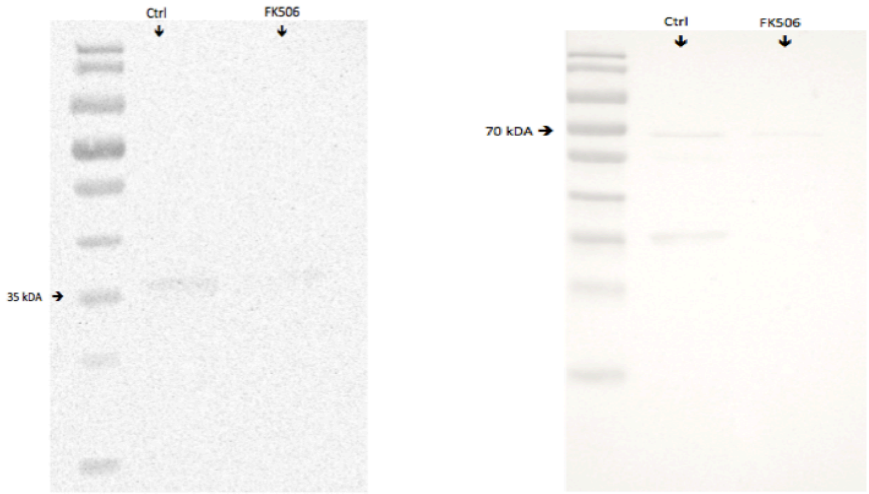


Figure 3.19.: **Western blot analysis of samples exposed to 10 μM FK506 for 24 h.** Results illustrates 75% down regulation of FxR and 36% down regulation of Aquaporin 2 (Aqp2) after exposure to FK506 (10 μM) for 24 h in humidified atmosphere. Samples were normalized to an endogen control (beta-actin).

Chapter 4

Discussion

4.1. General characterization of the kidney cell line EFN-R

4.1.1. Morphology and growth conditions

An epithelial-like piglet kidney cell line has been established in our laboratory and has been used as an alternative to a biological system in toxicology studies. Cell growth was satisfactory; cells were usually split when becoming semi confluent (figure 3.1.) and passaged from six to twenty with no visible morphological change.

4.1.2. The features of animal models

Animal models have physiological and genetic characteristics similar and divergent from humans. A good animal model system should reflect the vivo-like situation of the human counterpart in the best possible way (Schmittgen & Livak 2008). Rodents are most frequently used for animal studies because they are small, reproduce quickly, have a short lifetime (several generations can be observed in a relatively short period) and are often inbred. Due to their inbred origin they present less genetic variation than humans. However, pigs are anatomically, physically, immunological, metabolic and genetically more similar to humans making them a good model for research (Leist & Hartung 2013).

4.1.3 Cell line versus tissue

Compared to the complexity of a whole organism, in vitro models are simple. Experiments can be started and stopped continuously and results can be re producible and are generally less expensive in relation to animal experiments.

Human cell lines have similarity to "normal" human cells and are derived from cancer cells or have been transformed by the use of viral genes, DNA alkylation or oxidation reactions (Delgado-Ortega et al. 2014; Jeon et al. 2013). Human primary cells would be the most optimal solution; however, they are difficult to obtain in large quantities. Because of the pigs similarity to human there is a growing need for the development of new biomedical tools within this species (Gruber & Hartung 2002).
The great advantage of cell cultures is the immortality of cells. Repeated analyses with genetically identical cells along with clinical pharmacokinetic data may provide reliable results and reduce the need for further in vivo studies. However, the usage of cell cultures in the laboratory may not explore the role of epigenetics. Furthermore, the complex interplay between different cell types and the role of connective tissues are not taken into account.

4.1.4. Flow cytometry to distinguish between cell populations

The cell line EFN-R has undergone a permanent change in their phenotype through a spontaneous transformation (Lenk 2014). Genetic manipulation of cells may alter the native characteristics possibly influencing performed studies (Delgado-Ortega et al. 2014). Additionally, cell lines may have abnormal chromosomal content, unknown genetic mutations, abnormal protein expression, and modified metabolism (Skelin et al. 2010). To investigate these further, physiological properties of EFN-R cell line could be compared to different cell lines. Comparative methods to detect quantitative differences, such as the number and the morphology of the chromosomes and their evolvement after cell division should be investigated. To examine whether the EFN-R cell line consists of one or more cell populations flow cytometry was performed. Flow cytometry is a technic with the ability to separate cells according to intracellular and extracellular properties of the cells. Cells were displayed as one population in the scattered plot (figure 3.4.). This suggests that EFN-R cells have the same size and granularity. While these findings indicate that the EFN-R cell consist of one population of cells other methods such as fluorescence microscopy or fluorescence-activated cell sorting (FACS) have to be performed to finally conclude this.

4.1.5 Characterization of protein markers in EFN-R cells

To characterize the phenotype of the EFN-R cells we investigated the distribution of protein markers including the proteins SN1, Lrp2, THP and Aqp2 (figure 3.5.).

SN1 regulates intracellular and extracellular concentrations of glutamine by mediate electron-neutral and bidirectional transport of glutamine through the transporter localized in proximal tubule of the nephron (Skelin et al. 2010). We found that SN1 is expressed in EFN-R cell line, together with Megalin. Megalin (Lrp2) is a membrane glycoprotein that acts by binding to several physiologically relevant molecules, and ligands such as albumin, insulin, leptin, parathyroid hormone, and Angiotensin II have. Lrp2 have been implicated in pathological conditions including diabetes, hypertension and obesity, which can affect renal function (Nissen-Meyer & Chaudhry 2013; Solbu et al. 2005). We interpreted these findings as EFN-R expresses markers similar to proximal tubule cells. We also found that THP was expressed in the EFN-R cells. THP is the most abundant protein secreted in the urine under normal conditions; though its actual biological function is unexplained (Marzolo & Farfan 2011). THP has previously been located in Loop of Henle and distal tubule cells (Zacchia & Capasso 2014). It remains open if the observed changes in expression may be explained by the fetal origin of the cells, species-specific variations, or the remaining of a mixed cell population with very similar morphological characteristics.

Aquaporins (AQPs) are small membrane proteins regulating water intake and urinary excretion, whereas Aqp2 is localized in the collecting duct of the nephron (Cheng 1997). AQPs maintain water homeostasis by controlling the blood osmolality and blood volume essential for several physiological processes (Hoyer & Seiler 1979; Sikri et al. 1985). We did not finally confirm the presence of Aqp2 that previously has been demonstrated in collecting duct in lower molecular weight. Whether this is due to posttranslational modifications, such as glycosylation remains unknown.

To investigate this further there should be considered treatment with de-glycosylated enzymes to check for size shifts using western blot. A co-staining of THP and Aqp2 had been useful to see if they are expressed similar in these cells.

Taken together our results suggest that EFN-R have protein markers similar to proximal tubule cells.

4.2. Oxidative stress, drug transport and apoptotic mechanisms in CNI toxicity

4.2.1. Oxidative stress

Superoxide, hydroxyl radicals, and hydrogen peroxide are some of the most important reactive oxygen species (ROS) produced in different cellular activities. ROS are cytotoxic when it reaches a threshold in the cellular environment, which is linked to antioxidant level. This threshold can be lowered or raised by making the cell more or less exposed to oxidative stress by decrease or increase of the level of the antioxidant defense (Kortenoeven & Fenton 2014). Flavonoids are known to influence the antioxidant defense. Applying a hydrogen peroxide stress regime, we tested various antioxidative flavonoids. It turned out that the antioxidant morin was the most efficient inhibitor of hydrogen peroxide induced stress in EFN-R cells (figure 3.9.). Further, we determined if morin could protect against CNI associated toxicity. We found that morin had a protective effect against FK506 indicating that CNI toxicity is in part mediated by formation of reactive oxygen species (figure 3.12.). This findings is in line with previous findings by Han et al., (2006), they found increased oxygen free radical formation after exposure with CsA and FK506 in rat cells. Morin scavenges oxyradicals and inhibits xanthine oxidase, a free-radical generating enzyme (Remacle et al. 1995; Zeng et al. 1997). At concentrations of 75-100 micromolar, morin inhibits oxidation of low-density lipoprotein (LDL) by free radicals or Cu^{2+} (Kinuta et al. 1989; Linder et al. 1999). Morin is a powerful antioxidant; however, it also has other effects as inhibition of rat brain phosphatidyl-inositol-phosphate kinase activity in vitro and in vivo (Ghaffari & Mojab 2007; Safari & Sheikh 2003; Thompson et al. 1976). The findings of the protective effect of morin indicate a role for ROS formation; however, we cannot rule out other effects of morin. To investigate this further the effect of ROS formation after CNI exposure with and without morin should be measured and other antioxidants should be tested.

4.2.2. Apoptosis

A cause of chronic renal dysfunction is CNI nephrotoxicity. CNI nephrotoxicity is often a reversible process although long exposure to toxic agents can cause irreversible damage by activating RAS and regulate growth factors leading to renal apoptosis and renal dysfunction (Cheng 1997). To investigate the EFN-R cells response to CNI regards to nephrotoxicity we chose to evaluate the mRNA expression involved in apoptosis after exposure to different concentrations of FK506 (0.1 μM and 10 μM). We found that *Bcl2* and *Bax* gene expression was unaffected after 24 h exposure to FK506 at low and high concentration (figure 3.16.).

Jeon et al. (2010) illustrated activation of mitochondria pathway in FK506 (1.0 μM) treated dog cells after 24 h at protein level. They found *Bax* activation and *Bcl2* down regulation (Xiao et al. 2011; Xiao et al. 2013). As far as we know there is not much data on CNI toxicity related to *Bax* and Bcl2 genes. Data available on CNI toxicity is often related to CsA and it is expected that FK506 are based on similar patterns (Jeon et al. 2010). Xiao et al. (2011) reviled that 0.1 μM CsA has been reported to induce apoptosis in human cells after 24 h exposure, whereas *Bcl2* was down regulated. They summarize twelve experiments in his research, thus suggesting large differences between animals in exposure time and concentration (Slattry et al. 2012). However, our results differ from previous findings, possibly explained by the species differences in an exposure and time dependent manner. The therapeutic level of FK506 is 50-100 fold lower than CsA (Xiao et al. 2011; Xiao et al. 2013). This may suggest increase in concentration and exposure time of FK506 compared to CsA.

To investigate our findings further, other events, like mitochondrial permeability transition or mitochondrial fission and fusion associated with apoptotic pathway that could be determed. These events could also be monitored by time-lapse confocal microscopy.

4.2.2 Drug transport

To investigate differences in therapeutic outcomes it is crucial to understand the molecular mechanism for renal drug transport in terms of drug interactions and nephrotoxicity. To study the efflux of immunosuppressive drugs from proximal tubule

cells we have chosen to examine *Mrp4* transporter in response to CNI nephrotoxicity. Depending on the cell type, *Mrp4* can be located on either apical or basolateral side of the membrane (Urquhart et al. 2007). In chonoid plexus epithelium and hepatocytes Mrp4 is expressed at basolateral side of the cell, whereas in renal proximal tubule Mrp4 is identified in the apical membrane (Hoque et al. 2009). Mrp4 is known for its broad substrate specificity and its ability to transport a range of endogenous molecules that have a role in cellular communication (Hoque et al. 2009; Russel et al. 2008). Renal Mrp4 secretes organic anions from the cell into the lumen or back to the circulation system and play a role in the intracellular drug concentration (Russel et al. 2008).

We found that *Mrp4* expression was increased after exposure to 0.1 μM CsA for 24 h, in contrast to FK506, which was not affected at the selected concentration (figure 3.15.).

As far as we know there are few studies on this transporter regards to calcineurin inhibitors. Previously, El-Sheikh et.al (2013) found that in vitro studies in human kidney cells exposed to FK506 in interaction with methotrexsate inhibit Mrp2, an isoform of the MRP family (Anzai & Endou 2007; Foss 2012; Norris et al. 2005), and Mrp4, but CsA only inhibited Mrp2. El-Sheikh et.al (2013) used a very high concentration of FK506 (10 μM) whereas we selected a concentration closer to therapeutic level (0.1 μM), when testing efflux properties on this cell line. It remains unclear if the reason for these differences is based on the drug-drug interaction in their study or concentration differences. However, if this is due to concentration differences or if it is due to exposure time is undetermined, and we can neither role out the fact that it is a relation between drug concentration and exposure time. Further investigation on the effect of exposure time and different concentrations should be valued and tested on EFN-R and addition to another cell line.

4.2.3 Bile acids affect on CNI stimulated toxicity

Biliary complications lead to accumulation of bile acids in plasma, and their only excretory route is through the kidneys. It was our focus to investigate a possible connection between CNI nephrotoxicity and bile acids regards to cell viability. There was no significant effect on CNI treated cells exposed to CDCA regards to cell viability (figure 3.13.). As far as we no there is any previous reports in this subject.

Future aspects:

The focus of this thesis was to characterize the kidney cell line EFN-R by application of immunosuppressive agents.

EFN-R has protein markers similar to proximal convolute tubule; however, the presence of Aqp2 and THP was not ultimately confirmed. Further studies should investigate the expression of Aqp2 by treatment of EFN-R with de-glycosylated enzymes determining the differences in protein fragment using Western blot technology and for THP, protein-sequencing technology may be applied.

Mrp4 gene expression was increased after treatment to low dose CsA. Comprehensive experiments should include experiments with different concentrations, exposure times, and biological replicates of CsA. Furthermore, translation changes should be investigated applying western blotting. To study Mrp4 regulation, one may use siRNA-knockdown experiments.

Protein expression experiments are challenging. Reproducibility of the Aqp2 and FxR protein expression experiments should be increased by additional replicates.

Mitochondria are the major intracellular source of ROS. It may be interesting to investigate ROS formation after exposure to FK506 using a cytometric assay to measure the mitochondrial membrane potential.

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