Bachelor Thesis

Gene expression profiling in healthy and diseased kidney glomeruli isolated from transgenic mice induced by TGF-β receptor signaling

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Abstract

English

A time course study of a transgenic mouse model with doxycycline induced constitutive TGF-β receptor I expression revealed that through TGF-β signaling increased glomerular cell density, glomerulosclerosis and severe proteinuria is caused. Earlier studies also indicated that as the disease progresses mesangial cells start to expand. We tried to identify the factor stimulating that proliferation and selected 20 candidate genes based on microarray and qRT-PCR studies. Further investigation using immunohistochemistry showed that Axl and Pdgfc play an important role as they get highly expressed in the glomeruli of diseased animals shortly after doxycycline introduction.

Also the effects of the drugs Enalapril and Mitotempo on disease progression were examined in an intervention study which showed that both drugs were able to prevent proteinuria and therefore inhibit disease progression.

German

Eine Studie des zeitlichen Krankheitsveralufs mit einem transgenen Maus-Model mit durch Doxycycline induzierter konstitutiver Expression des TGF-β Rezeptors I zeigt, dass es durch TGF-β Signalisierung zu erhhter glomuerulrer Zellularitt, Glomerulosklerosis und Proteinuria kommt. Frher Studien haben ergeben, dass sich im Krankheitsverlauf mesangiale Zellen vermehren. Wir versuchten den dieser Proliferierung zugrunde liegenden Faktor zu identifizieren und whlten 20 Gen Kandidaten basierend auf Microarray und qRT-PCR Studien. Weitere Untersuchungen mit immunohistochemischen Methoden zeigten, dass Axl und Pdgfc eine wichtige Rolle spielen, da diese kurz nach Introduktion von Doxycycline in den Glomueruli erkrankter Tiere stark exprimiert werden.

Zustzlich wurden die Auswirkungen der Medikamente Enalapril und Mitotempo auf den Krankheitsverlauf in einer Interventionsstudie untersucht welche ergab, dass beide Medikamente Proteinuria verhindern und damit den Fortschritt der Krankheit hemmen.

Contents

Chapter 1

Introduction

1.1 The kidney

1.1.1 Kidney physiology

The main functional unit of the kidney is the nephron which filters the blood by absorbing and further secretion of water, salts and other minerals of your body. There about one million nephrons in the cortex of the kidney and the consist of a renal corpuscle and a renal tubule which are responsible for conducting the function of the kidney. The renal corpuscle includes the glomerulus and bowman's capsule which encloses the glomerulus. The glomerulus is the main filter device of the nephron and consists of tiny tubes which the blood passes. The blood enters the glomerulus through an afferent arteriole. In the glomerulus water and soluble metabolic wasted products are passed through a semipermeable layer consisting of endothelial cells, glomerular basement membrane and podocytes by ultrafiltration. The

innermost of that ultrafiltration layer consists of endothelial cells which has pores, so called fenestrae that in comparison to other fenestrated capillaries do not have a diaphragm spanned are just big enough to let anything smaller than a red blood cell pass through. The endothelial cells follows the glomerular basement membrane (GBM) which is exceptionally thick and contains many glycosaminoglycans resulting in a negative overall charge that repels negatively charged proteins from the blood preventing them from entering Bowman's space. The podocytes build the outermost layer of the ultrafiltration barrier and build a network of foot processes (pericels) spanned by slit diaphragms which lines part of Bowman's space. This enables them to control filtration from the capillary lumen to Bowman's space. The glomerulus also contains mesangial cells (intraglomerular mesangial cells) which are located in the interstitium between endothelial cells. They are specialized pericytes not considered part of the filtration barrier but are able to contract and reduce the glomerular surface area which affects the glomerulus filtration rate. The ultrafiltrate that enters Bowman's lumen is also known as primary urine and is then passed through the proximal tubule, the loop of Henle, the distal convolute tubule and several collecting ducts. There water and sodium gets reabsorbed because of an osmotic pressure gradient. Between the vascular pole of the renal corpuscle and the returning distal convoluted tubule lies the juxtaglomerular apparatus. It is essential for regulating renal blood flow and the glomerular filtration rate and consists of the macula densa, extraglomerular mesangial cells and juxtaglomerular cells. Together

with other important structures the renal corpuscles, renal tubules and the juxtaglomerular apparatus the kidney plays an important role in the urinary system and maintains essentials homeostatic functions.

1.1.2   Kidney failure

Renal failure refers to the status when the kidney is unable to filter the blood adequately. It is divided into two forms, acute kidney injury and chronic kidney disease. It is detected by an elevated serum creatine level which results from a decreased glomerular filtration rate.

Acute kidney injury

Acute kidney injury is characterized by a rapid loss of renal function which results into decreased urine production, body water disturbances and electrolyte derangement. Is can be caused by low blood volume, toxin exposure or prostate enlargement but can be reversed by treating the underlying cause [Lameire et al., 2005].

Chronic kidney disease

Approximately 26 millions in the US only suffer from chronic kidney disease (CKD) and is mainly caused by diabetes and increased blood pressure. In contrast to acute kidney injury CKD is the loss of renal functions over months or even years. CDK can be divided into five stages whereas stage 5 is also known as established chronic kidney disease or end-stage renal disease

(ESRD). In that stage only 15 % or less of the normal kidney function is left. That corresponds to glomerular filtration rate of $15mL/min/1.73m^2$. In that stage patients either have to rely on dialysis or get a kidney transplant. Currently there are specific treatments to efficiently slow down disease progression. For an adequate treatment or intervention it therefore becomes crucial to understand the early mechanisms of the disease.

1.2 Transforming Growth Factor β (TGF-β)

1.2.1 Introduction

The first members of the TGF-β superfamily have been recognized in the 1980s and nowadays it includes more than 100 proteins like different TGF-β isoforms, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) [Kingsley, 1994]. The three most abundant in in mammalian tissues are TGF-β 1,2 and 3 which are highly homologous isoforms. They are believed to be responsible for the regulation of a broad array of growth- and development events in the organism like cell proliferation, differentiation and immunosuppression and act via autocrine, paracrine and endocrine signaling. Also they are known to be an important factor for fibrotic- , parasitic- and autoimmune diseases as well as for carcinogenesis [Massagu, 1990, Roberts, 1998].

1.2.2 TGF-β structure and activation

The precursors of the different TGF-β isoforms are 392 amino acids long polypeptides that are synthesized as dimeric propeptides held together by disulfide bonds. During secretion they are cleaved to two 112 amino acids long identical chains so mature cytokines are produced [Massagu, 1990,Gleizes et al., 1997]. However the C-Terminal fragment remains associated with the N-terminal of the propeptide, the so called latency-associated peptide (LAP). This C-Terminal which forms the TGF-β1 must still be released from LAP to carry out its biological activity. The activation of TGF-β1 can be triggered by several factors like retinoic acid, fibroblast growth factor-2, endotoxin or vitamin D3. Additionally to LAP TGF-β is often found associated with another protein, the latent TGF-β binding protein (LTBP). It is a 120-140 kDa long glycoprotein and binds to LAP through disulfide bonds during secretion. Different forms of LTBP exist in different cell types which is accomplished through alternative splicing and proteolytic cleavage. LTBP addresses the latent TGF-β to the extracellular matrix (ECM) and thus helps to regulates its bioavailability [Gleizes et al., 1997].

1.2.3 TGF-β receptors

The main receptors involved in TGF-β signal transduction are receptors I (TβRI) and II (TβRII). They both consist of a small extracellular -, a single transmembrane - and cytoplasmic region which includes a serine/threonine

kinase domain. Receptor I has a highly conserved serince, glycine enriched domain, the so called GS domain, a juxtamembrane segment next to the kinase domains N-terminal [Wrana et al., 1994, Huse et al., 2001]. The signaling cascade is triggered by the binding of TGF-β to TβRII which then recruits the TβRI to form a heteromeric complex. The TβRI then gets activated by phosphorylation of its GS domain through TβRII. Upon phosphorylation downstream signaling gets turned on [Wrana, 1998].

1.2.4   TGF-β signal transduction

SMAD pathway

The most significant pathway that is turned on by TGF-β signaling is the SMAD pathway. The SMADs proteins are basically divided into three functional groups. The receptor regulated SMADs (R-SMAD), the collaborating SMADs (CO-SMADs) and the inhibiting SMADs (I-SMAD). The R-SMADs include the proteins SMAD1, 2, 3, 5 and 8 where as only SMAD2 and SMAD3 are significant in the TGF-β signaling pathway and get phosphorylated by the type I receptor [Macas-Silva et al., 1996, Kretzschmar et al., 1997]. The CO-SMADs only have one member, the SMAD4 protein which cannot be phosphorylated itself but binds to the phosphorylated R-SMADs. SMAD6 and SMAD7 count to the I-SMADs which have as the name suggests inhibiting functions by blocking signaling from active R-SMADs and CO-SMADs [Imamura et al., 1997, Nakao et al., 1997, Hayashi et al., 1997].
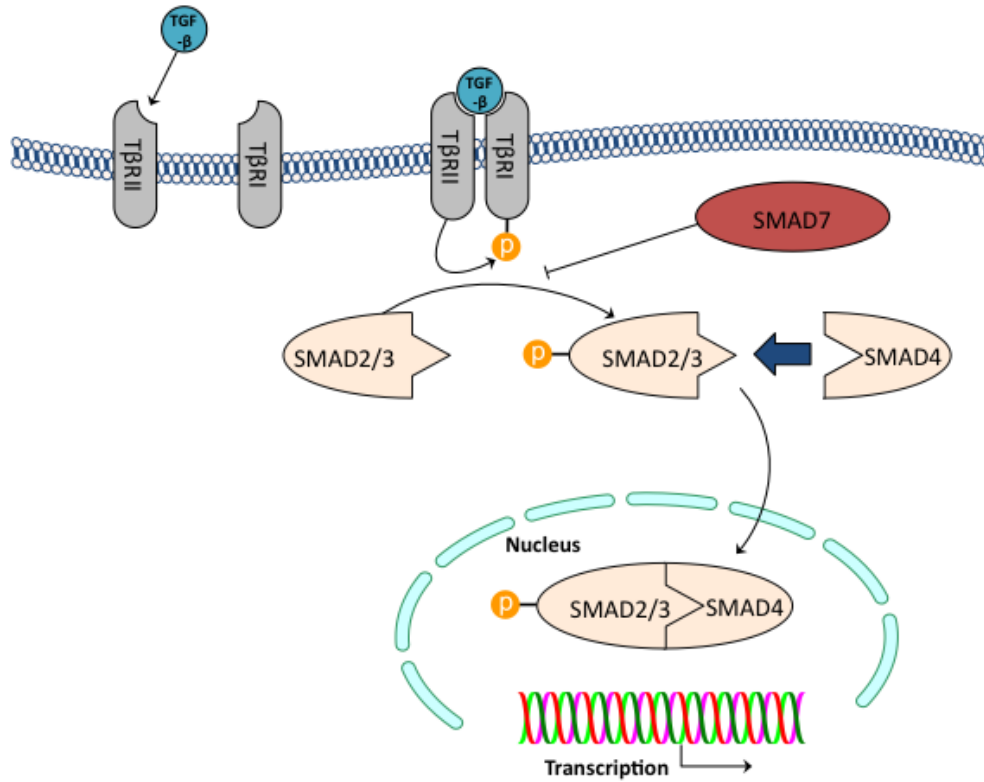
Figure 1.1: SMAD pathway

The SMAD protein generally consists of the N terminal MH1 domain, the C terminal MH2 domain and an in between linker region. If not bound to any ligand the protein is in its basal state in which the MH1 and MH2 domain block each others function. The MH2 domain of the R-SMADs is essential for the binding of TβRI. Once phosphorylated the R-SMADs bind to the CO-SMADs and then go into the nuclei to turn on transcription of specific target genes [Kretzschmar et al., 1997].

SMAD independent pathways

SMAD independent pathways include the Notch - and the Mitogen-activated protein kinase (MAPK) pathway [Moustakas and Heldin, 2005].

Chapter 2

Methods

2.1   Mouse line

The used mice had all been maintained in the lab on a FVB/NJ background
and they all adhered to the National Institutes of Health Guide for the Care
and Use of Laboratory Animals. All experiments were conducted according
to approved protocols of the Institutional Animal Care and Use Committee
(IACUC). The used Doxycycline chow was obtained from Research Diets,
INC (New Brunswick, NJ) (order # D07053002).

2.2   Transgenic mouse with constitutive active TGF-β receptor

In order to study the effects of TGF-β on podocytes in vivo a transgenic
mouse model with a constitutive active TGF- β receptor had to be intro-
duced. A tetO-TβR(AAD) x NPHS2-rtTA + Doxycycline system was cre-

ated for this purpose.

2.2.1 TetO-TβR(AAD) mouse line (R-Line)

In a wild-type mouse the binding of TGF-β to TGF-β receptor II (TβRII)
recruits the receptor to form a heteromeric complex with TGF-β receptor I
(TβRI). The receptors both have serine / threonine kinases embedded in their
C-terminal which are constitutively active and due to the close proximity
when forming a heterocomplex the GS domain of TβRI gets phosphorylated
by the serine / threonine kinase of TβRII and this signal is transducted on-
wards to SMAD proteins by further phosphorylation or other SMAD indepen-
dent pathways. The GS domain is located between the transmembrane and
the kinase domain of the TβRI and is a highly conserved repetitive glycine-
serine motif that contains a TTSGSGSGLP (185 - 194) sequence [Wieser
et al., 1995, Altschul et al., 1997]. In order to activate TβRI three to five
serine / threonine residues need to be phosphorylated. The immunophilin
FKBP12 is able to bind to TβRI at position 193 (leucine) and 194 (proline)
and works as a negative regulator for the TGF-β signal transduction because
the GS domain cannot be phosphorylated any longer. Studies showed that
TGF-β receptor internalization was enhanced when FKBP12 was prevented
from binding which was achieved by mutating the FKBP12 binding residues
on TβRI into alanine [Yao et al., 2000]. The signaling can be increased
further by also mutating the threonine at position 204 to aspartic acid re-
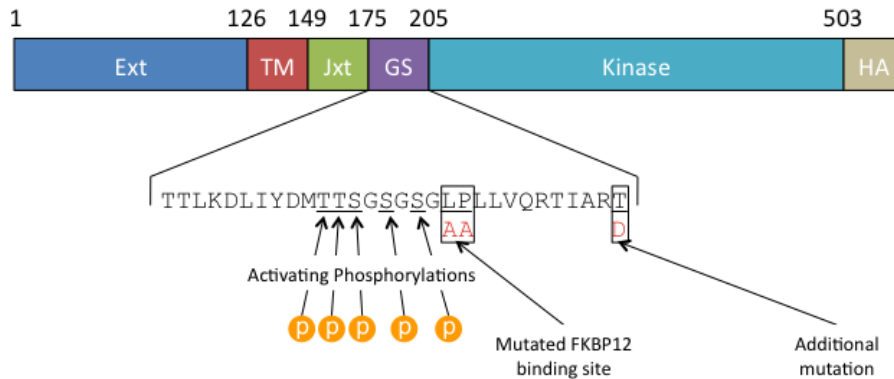sulting into a charge substitution which further lead to autophosphorylation

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Figure 2.1: Mutated TGF-β Receptor I

of TβRI [Charng et al., 1996]. To facilitate the detection of this mutant TβRI(AAD) a hemagglutinin tag was added to its C-terminal which does not effect the TGF-β signaling pathway (Figure 2.1).

The mutant TβRI(AAD) was then linked downstream of a tetracycline responsive element (TRE) consisting of seven tet operator sequences (tetO) joined to minimal promoters derived from the cytomegalovirus IE promoter $P_{min-CMV}$. TetO is a regulatory DNA sequence from the bacterial tet operon and by binding of the reverse tetracycline controlled transactivator protein (rtTA) and doxycycline the $P_{min-CMV}$ reaches the strength of its parent promoter and the transcription of the mutant TβRI(AAD) is induced. [Baron et al., 1995, Baron et al., 1997].

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2.2.2   NPHS2-rtTA mouse line (P-Line)

The NPHS2 gene encodes the protein podocin which is almost exclusively expressed in podocytes [Boute et al., 2000]. Therefore the NPHS2 promoter can be used to induce tetracycline controlled transactivator protein (rtTA) expression.

RtTA is a fusion protein of the engineered reverse tet repressor protein (rTetR) and the herpes simplex virus protein 16 (VP16). The normal tet repressor protein (tetR) cannot bind to the tet operon in the presence of doxycycline but if there is no doxycycline present it is enabled to bind and consequently activate the transcription of its downstream gene. This is called the Tet-Off system. In the Tet-On system on the other hand the rTetR protein is used which turns on transcription in the presence of doxycycline. [Baron et al., 1995, Baron et al., 1997]. At its activation domain (AD) the VP16 is fused to the rTetR protein and reduces off-targets effects as well as toxicity.

2.2.3   TetO-TβR(AAD) x NPHS2-rtTA + Doxycycline system (PR+D)

The P- and the R-line can now be crossed and the mice with a double transgenic genotype are selected (PR-mice). In these PR-mice the rtTA is exclusively expressed in podocytes because of the NPHS2 promoter which is inducing its expression. When they are now treated with doxycycline it binds

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to rtTA which then subsequently binds to the tet operators of the tetracycline responsive element (TRE). These then activate the transcription at the $P_{min-CMV}$ region and the downstream linked mutant TβRI(AAD) gene is transcribed. As a result the mutant TβRI(AAD) is expressed in podocytes exclusively and the TGF-β signaling pathway is now turned on constitutive (Figure 2.2).

As the concentration of doxycycline determines the expression level of the TGF-β signaling this system provides a great scalable model to study the function of TGF-β in podocytes in vivo.

2.2.4   TetO-TβR(AAD) transgene construct

To induce the mutant TβRI(AAD) gene a pCMV5 vector plasmid including the TβRI(AAD) construct (pCMV5-TβRI(AAD)) was provided by Dr. Joan Massague. In order to release the TβRI(AAD) fragment the plasmid was treated with restriction enzymes HindIII and BamHI and eventually Klenow fragment of DNA polymerase I was used to remove the overhangs and create blunt ends. The products were then gel purified.

The accepting vector plasmid pDNR-1r which is a component of the BD Creator Cloning KIt was digested using the restriction enzyme SmaI and as well gel purified. To prevent self ligation shrimp alkaline phosphatase was used. The products were then heat inactivated and again gel purified. For
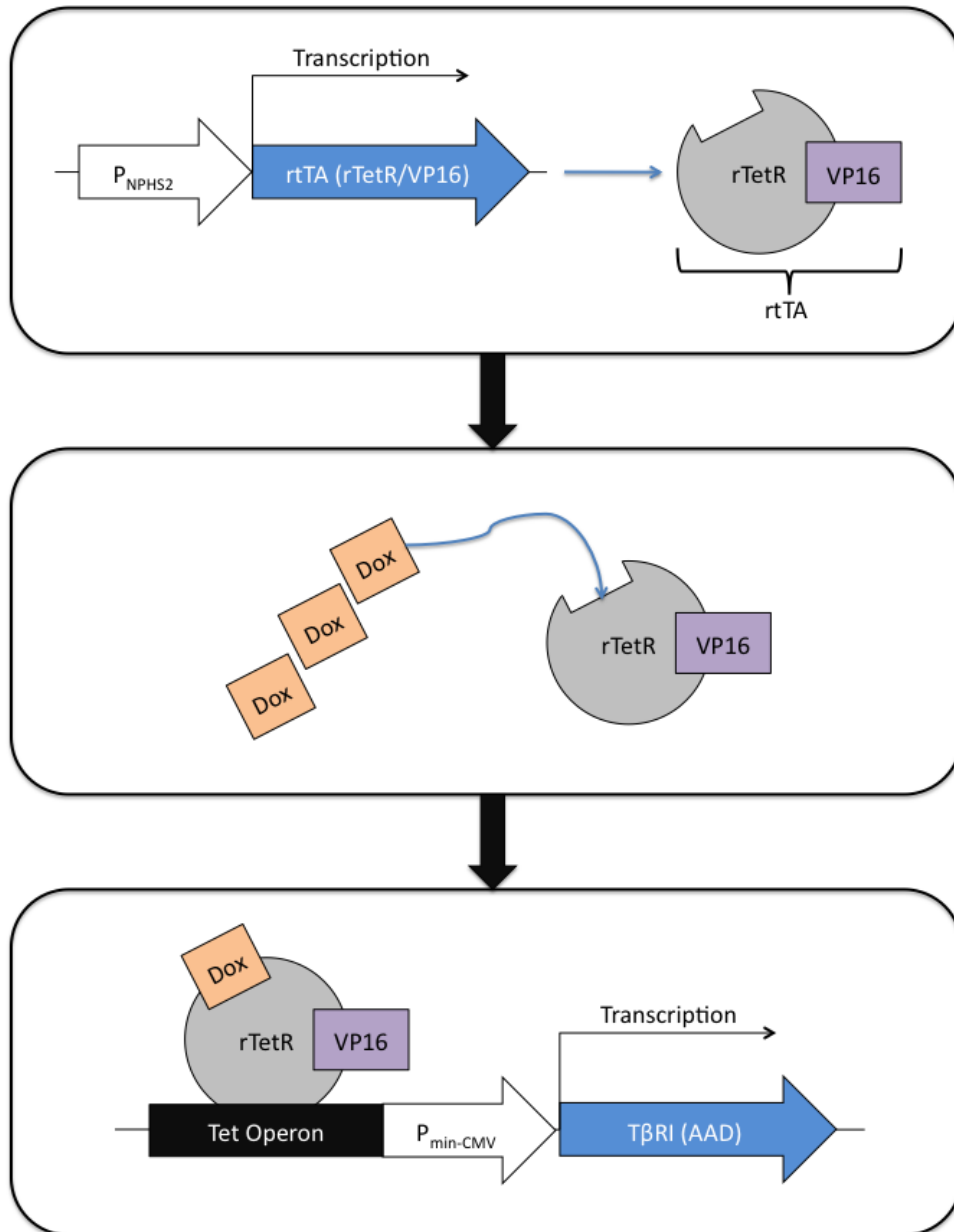
Figure 2.2: TGF-β Signaling Activation

the ligation process the insert/vector ratio was 3:1 and the chloramphenicol resistant (CM^r) were selected to culture. To confirm the correct insertion and insertion direction of the fragment the plasmid was again restricted. Finally a pDNR-1r-TβRI(AAD) plasmid was obtained. That was then used as donor to transfer the TβRI(AAD) fragment to the pLP-TRE2 accepting vector to generate a tetracycline-regulated expression construct for the inducible expression of TβRI(AAD). This was could now be done using the BD Creator Cloning Kit. Chloramphenicol and sucrose selected clones were then verified and eventually the obtained pLP-TRE2-TβRI(AAD) plasmid was sequenced to confirm a complete and correct linkage.

2.3   Genotyping

2.3.1   Tail digestion

2 mm mouse tail was digested in a 80 μl digestion buffer. The digestion buffer consisted of 8 μl 10x MGB buffer, 4 μl 10% Triton-X, 0.8 μl 2-Mercaptoethanol and 67.2 μl ddH2O. The digestion buffer including 2 mm tail pieces was first heated to 93° C for 3 min. Then 2 μl of Proteinase K were added to each tube. After making sure that the tail pieces are suspended in buffer the mixture was put at 55° C for 1 hour. Then after vortexing the mixture it was again put to 55° C for another 10 min. Proteinase K was heat inactivated by heating buffer to 95° C for 10 min and then everything spun down at 10 000 rpm for 10 min. 1 μl of the supernatant was used for each

PCR reaction

2.3.2 Polymerase Chain Reaction

Each PCR with the NPHS2-rtTA primer pair contained 4 μl Q Solution 5x, 2.5 μl Qiagen Buffer 10x, 0.19 μl 10 mM DNTP, 2 μl 25 mM $MgCl_2$, 0.1 μl of each the 100 μM Forward Primer, the 100 μM Backward Primer and the Taq Polymerase, 15.01 μl ddH2O and 1 μl genomic cDNA. For the TetO-TβR(AAD) primer pair it consisted of 3 μl Q Solution 5x, 1.5 μl Qiagen Buffer 10x, 0.15 μl 10 mM DNTP, 0.6 μl 25 mM $MgCl_2$, 0.075 μl of each the 100 μM Forward Primer, the 100 μM Backward Primer and the Taq Polymerase, 8.525 μl ddH2O and 1 μl genomic cDNA. The PCR conditions used were 95° C for 2 minutes, 35 cycles of 95° C for 30 seconds, 55° C for 1 minute and 72° C for 1 minute, then 72° C for 5 minutes.

Amplified Gene	Forward Primer	Backward Primer
TetO-TβR(AAD)	AAAGTCATCACCTGGCCTTG	TTCTCCAAATCGACCTTTGC
NPHS2-rtTA	CCTGCTCCATTCTTGGAATG	GCTTATGCCTGATGTTGATGATGC

Table 2.1: Gene specific primer for Genotyping PCR reactions.

2.4 Intervention drugs

Enalapril was administered to the animals in drinking water at a concentration of 60 mg/l.

Mitotempo was given via daily injections with saline as vehicle. An experimental mouse received 0.2 mg of Mitotempo dissolved in 100 μl saline a day. Controls got 100 μl saline injections only.

2.5 Albuminuria measurement

The albumin amount in the urine was briefly examined with Albustix from Bayer (Elkhart, IN). For more detailed analysis of the albumin / creatinine ration the Albuwell M ELISA Test Kit and the Creatinine Companion ELISA Test Kit from Exocell (Philadelphia, PA) have been used. The experiment was conducted according to the protocol of the manufacturer.

2.6 RNA Extraction and cDNA Synthesis

The RNA was extracted using the mirVana miRNA isolation kit obtained from Ambion (order # 1560 1561). In a 20 μl reaction containing 500 ng oligo Dt12-18 primer (Invitrogen, Carlsbad, CA) and 200 U supertranscript II Rnase H negative reverse transcriptase (Invitrogen, Carlsbad, CA) 0.5 - 1 μg RNA was reverse transcribed. The mixture was heated to 65° C for 5 min and then chilled quickly on ice. Afterwards it was incubated at 42° C for 50 min and then heat inactivated by heating to 70° C for 15 minutes. The resultant cDNA was diluted 1:5 for qRT-PCR.

2.7 Real Time Quantitative Polymerase Chain Reacion

The cDNA was amplified in 10 μl reactions that contained 4 μl 2X SYBR-Green PCR master mix (Applied biosystems, Foster City, CA), 2 μl cDNA and 4 μl of the gene specific primer pair at a concentration of 5 μM. The primers were designed using the qRT-PCR assay design tool of Integrated DNA Technologies which can be found under

http://biotools.idtdna.com/Scitools/Applications/RealTimePCR/. The used conditions for the qRT-PCR were Stage 1: 95.0° C, 10 min; Stage 2: 95.0° C, 15 sec followed by 60.0° C, 30 sec, 40 repeats; Stage 3: 95.0° C, 15 sec, followed by 60.0° C, 15 sec, followed by 95.0° C, 15 sec

The used device was ABI-Prism 7900 HYT Sequence detection system (Applied Biosystems, Foster City, CA). The results were evaluated with the software SDS 2.0 (Invitrogen, Carlsbad, CA). Normalization was done using the house-keeping gene GAPDH.

2.8 Kidney perfusion, isolation and fixation

A mouse was first anesthetized by injection 2.5 % Avertin (15 μl/g body weight). Once fully anesthetized 70 % alcohol was sprayed on the mouse and the abdominal skin was cut open by a longitudinal incision. Skin was removed and the left kidney was exposed and its renal artery clamped. After

Amplified Gene	Forward Primer	Backward Primer
Cdk19	GGATCTGTTTGAGTACGAAGGG	ACAAGCCGACATAGATATTCCTG
Bmpr1b	GAATACCAGCTTCCCTATCACG	TGTCATAAGCTTCCCCATCTG
Fgfr4	TGTCAAATTCCGCTGTCCAG	ACCACACTTTCCATCACCAG
Fosl2	CATCAGACTCCTTGAACTCCC	GGCCCCTTATTCCCTCAAG
Gas1	AACACCCGCCTTTACCAG	GAAAAGTTTGTCCGAGTTCCG
Ntrk3	AGGGAACAGCAATGGGAAC	CTTGATGGTCAGCTTCTGGAG
Axl	GTGGAAAGAGGTGAACTGGTAG	GTCTCGTAGTTTCTCCTTCAGC
Cdk6	GTTCAGACGTGGATCAACTAGG	TGTCACAAACTTCTCGATGGG
Cdkn1b	TGGACCAAATGCCTGACTC	GGGAACCGTCTGAAACATTTTC
Fgfr2	AGGAGTTTAAGCAGGAGCATC	GTGGTAGGTGTGGTTGATGG
Ednra	GTCTTGAACCTCTGTGCTCTC	GATCCCGATTCCTTGAACTCG
Ncf2	GCTGTTCCGTCCAAATGAGAG	GCGAAGCCAGAAAAGTTGTC
Tie1	CCAGTGCCAGTGTCAAAATG	CCTATGTTGAACTCCACCTCTG
Angptl2	CAATGAGATCCAGAGTGACCAG	TGCTGTGACCATCTTCCAG
Tyro3	AAGGAGAGTTTGGATCAGTGC	CAAACTCCTTCATGCAAGCTG
Pdgfc	AGCGACAAGGAACAGAACG	AACTAATCTCCACACCAGCAC
Nek1	AGATGAAGCGGCAAGAGAAG	GAGAACACGGTGATGGAGAG
Frs2	GCTCGCTTACCTTCAGTCG	ATTAGAAACCCTTGCCTCTGG
Pros1	CCTACCGATGGATTTCTGGAC	TGGCACCTAAAGCTCCTTG
TβRI(AAD)	GCAGCATTGCTTGTTCAGAG	ATCTCTGCCTCACGGAACC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Table 2.2: Gene specific primer for qRT-PCR reactions.

taking out the kidney its capsule was removed and it was then cut into three equally sized pieces which were then put in tubes and snap frozen in liquid nitrogen and then stored at -80° C. These three pieces were later used for genomic DNA, mRNA and protein analysis.

In order to perfuse the right kidney the beating heart was first exposed by cutting the sternum and the diaphragmatic muscle. A perfusion pump (Miniplus3) and perfusion tube from Gilson were used to perfuse the kidney with 4 % paraformaldehyde (PFA, pH=7.4) and 18 % sucrose (pH=7). The pump was turned on at a perfusion speed of 11.5 rpm and a butterfly needle was inserted into the left ventricle. Immediately afterwards the vena cava was cut in order to allow a rapid drain of blood and the perfusion solutions.

After perfusing 3 minutes with 4 % PFA, 18 % sucrose was perfused for 5 minutes. Then the kidney was taken out and again its capsule was removed. It was then cut in half by a longitudinal cut. One half was formalin fixed for 24 hours and then stored at 70 % ethanol to produce paraffin-embedded sections from it while the other half was embedded in OCT and snap frozen and then stored at -80° C until sectioning.

2.9   Periodic Acid Schiff Staining

PAS Staining was performed on paraffin embedded kidney sections. To soften the paraffin the sections were first heated to 55° C in an oven for half an hour. Then the were put into 100 % xylene for 10 minutes three times. Afterwards 5 minutes each into 100 % ethanol twice, 95 % ethanol, 70% ethanol. Then for another 5 minutes into 1x PBS. The slides were then put into 0.2 % periodic acid for 5 minutes and then 2 minutes into ddH2O which was changed once. After that they were stained with Schiff reagent for 7 minutes and rinsed with running tap water for 5 minutes. The nuclei staining was done by putting the sections into Mayer's Hematoxylin for 30 seconds and again they were rinsed with running tap water for 5 minutes. After rinsing they were put 5 minutes each into 70 % ethanol, 95 % ethanol, 10 % ethanol twice. Then again into 100 % xylene for 10 minutes three times. Eventually the slides were mounted and examined under the microscope.

2.10 Immunohistochemistry Paraffin Embedded Sections

Immunohistochemistry of paraffin embedded sections was performed using the ABC method. To soften the paraffin the sections were first heated to 55° C in an oven for half an hour. Then the were put into 100 % xylene for 10 minutes three times. Afterwards 5 minutes each into 100 % ethanol twice, 95 % ethanol, 70% ethanol. Then for another 5 minutes into ddH2O. Then heat induced epitope retrieval was performed by putting by putting the slides into 2 % sodium citrate (pH=6.0) and heating them in a microwave oven 3 minutes three times and letting them cool down 1 minute in between. After the third time the slides were left to cool down to room temperature for 1 hour. Then the slides were washed with 1x PBS for 5 minutes three times. They were then put into 3 % hydrogen peroxide for 5 minutes. After another washing step serum of the animal in which the secondary antibody was made in was applied to the sections for 1 hour at room temperature to block unspecific binding. Afterwards they were incubated with Avidin and then Biotin, each 15 minutes at room temperature. After another washing step the primary antibody was applied and left overnight at 4° C. Then the slides were washed again the secondary antibody was applied at room temperature for 1 hour. Again the slides were washed and the A- and B-reagent of the Vectastain ABC kit (Vector Laboratories) were mixed to an biotinylated enzyme complex and which the sections were then incubated

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with for 30 minutes. After another washing step the DAB substrate for peroxidase from the DAB substrate for peroxidase kit (Vector Laboratories) was applied until the desired color ha been stained. The slides were then rinsed with running tap water for 5 minutes. The nuclei staining was done by putting the sections into Mayer's Hematoxylin for 30 seconds and again they were rinsed with running tap water for 5 minutes. After rinsing they were put 5 minutes each into 70 % ethanol, 95 % ethanol, 10 % ethanol twice. Then again into 100 % xylene for 10 minutes three times. Eventually the slides were mounted and examined under the microscope.

Chapter 3

Time course studies

3.1 Design of experiment

Two experiments were set up to evaluate the constitutive expression of TβRI after doxycycline introduction for different periods of time. One experiment with 4 weeks old mice lasted for 21 days while another one with 8 weeks old mice was taken up to 7 days only. Also an earlier independent experiment conducted by Taoran Zhang was partially analyzed. Figure 3.1 gives an overview of the experimental groups in which mice were put on doxycycline up to 21 days.
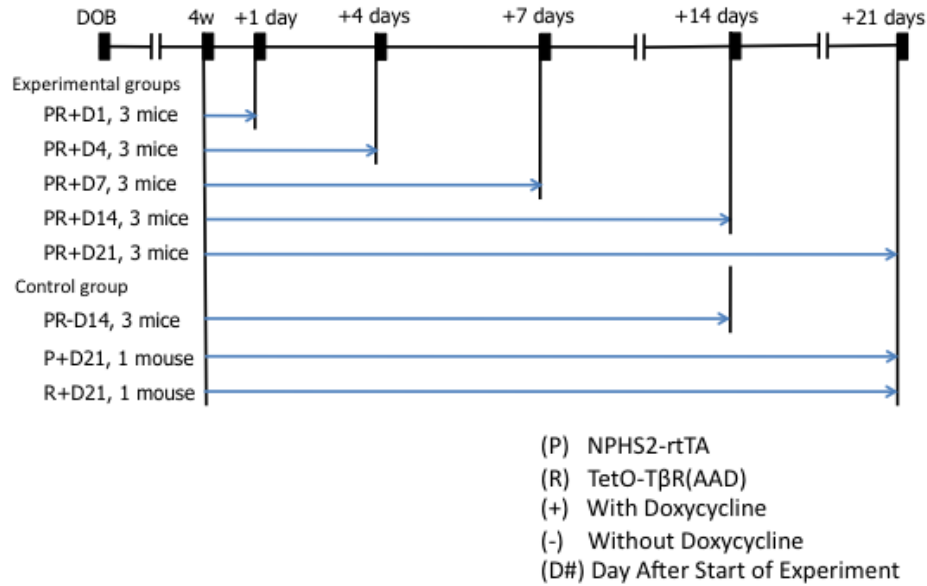
Figure 3.1: Overview of the doxycycline timecourse experiment. Mice were sacrificed on 1, 4, 7, 14 and 21 days after Doxycycline introduction respectively. Animals in the control group that did not get Doxycycline were sacrificed after 14 days. Single transgenic animals (P or R only) received Doxycycline and were sacrificed after 21 days and were also used as controls.

3.2   TGF-β Receptor I (TβRI) expression

In order to measure whether the TβRI really gets expressed after doxycycline introduction the expression of TβRI(AAD) was evaluated using qRT-PCR. Figure 3.2 and 3.3 show how its expression increases the longer the animals have been on doxycycline.
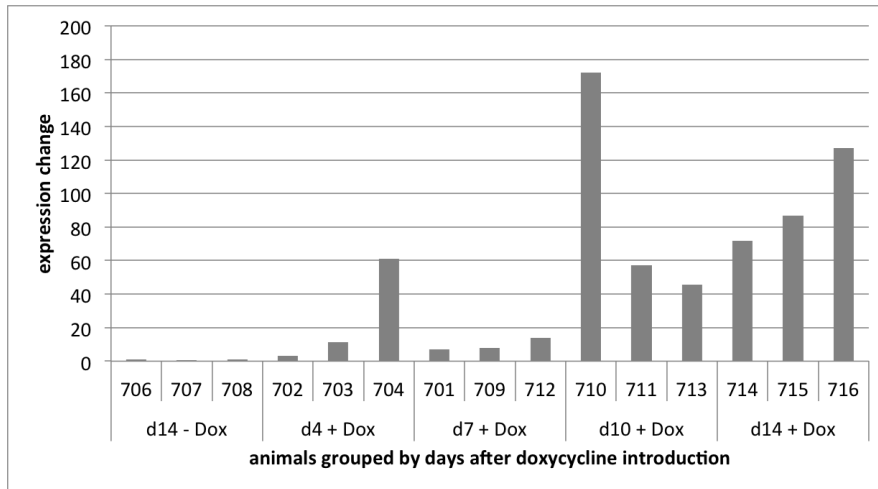
Figure 3.2: TβRI expression in animals on doxycycline up to 14 days. The figure confirms that mice on doxycycline express TβRI and the expression goes up the longer they are on doxycycline
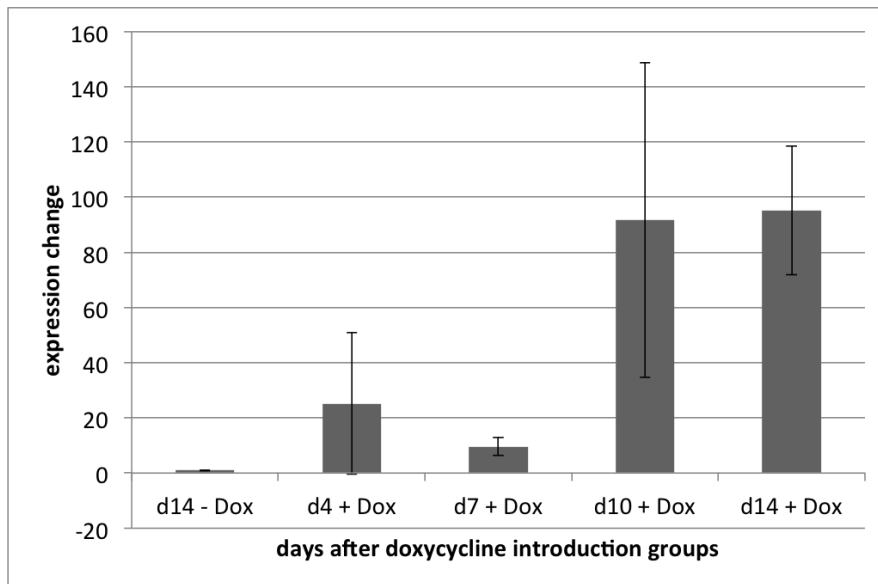
Figure 3.3: Average TβRI expression in animals on doxycycline up to 14 days.

3.3   Urine albumin to creatinine ratio

To confirm that animals on doxycycline that express TβRI really got sick too their urine albumin/creatinine ratio (UACR) was measured since a high ratio is an indicator of proteinuria. Figure 3.4 and 3.5 show the UACR of mice on doxycycline up to 14 days and the ratios of the experiment with mice on doxycycline up to 7 days are shown in figure 3.6 and 3.7.
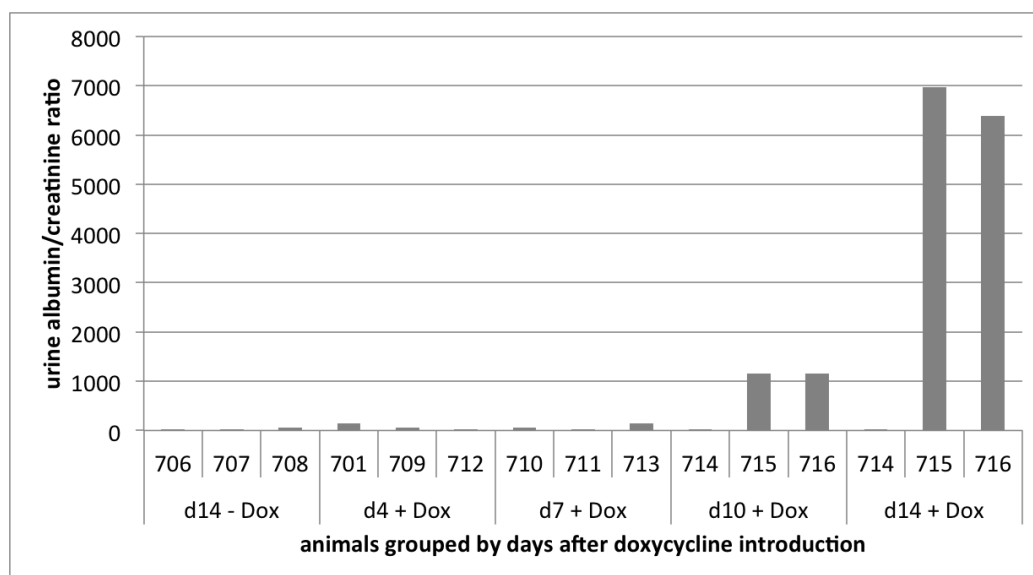
Figure 3.4: Urine albumin to creatine ratio of of animals on doxycycline up to 14 days. Generally the figure shows that mice had a higher urine albumin/creatinine ratio the longer they were on doxycycline which confirms that they really became sick. Mouse 714 is an exception and for some reason did not get sick.
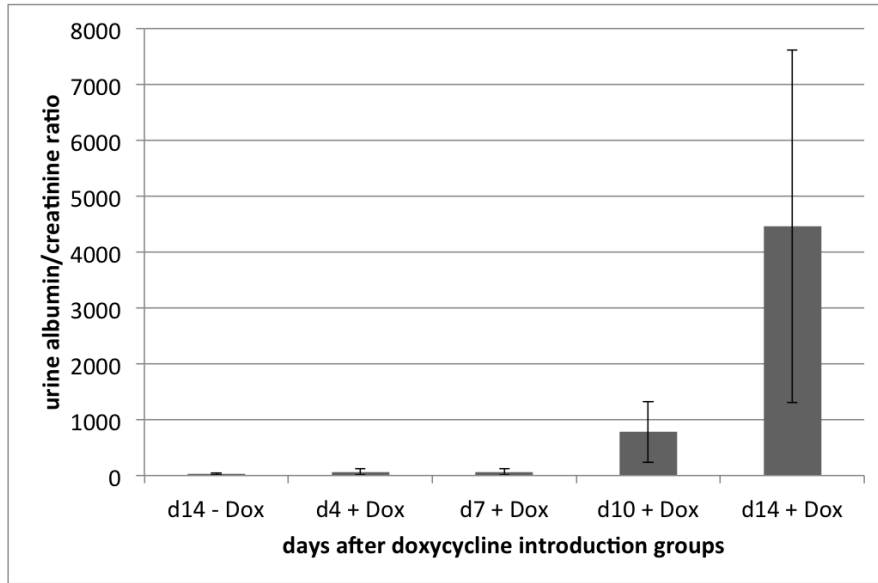
Figure 3.5: Average urine albumin to creatine ratio of of animals on doxy-cycline up to 14 days.
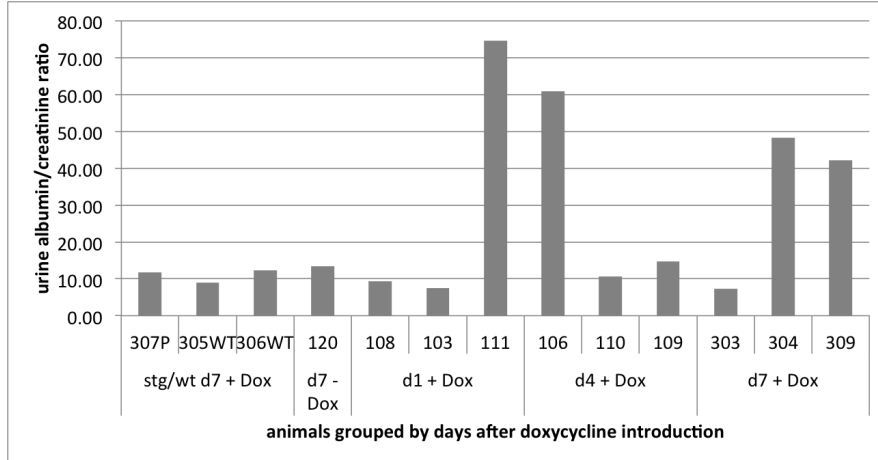
Figure 3.6: Urine albumin to creatine ratio of of animals on doxycycline up to 7 days. Animals on doxycycline only up to seven days did not have a high urine albumin/creatinine ratio. Compared to the high UACR values of mice that have been on doxycycline for 14 days these values are not significant.
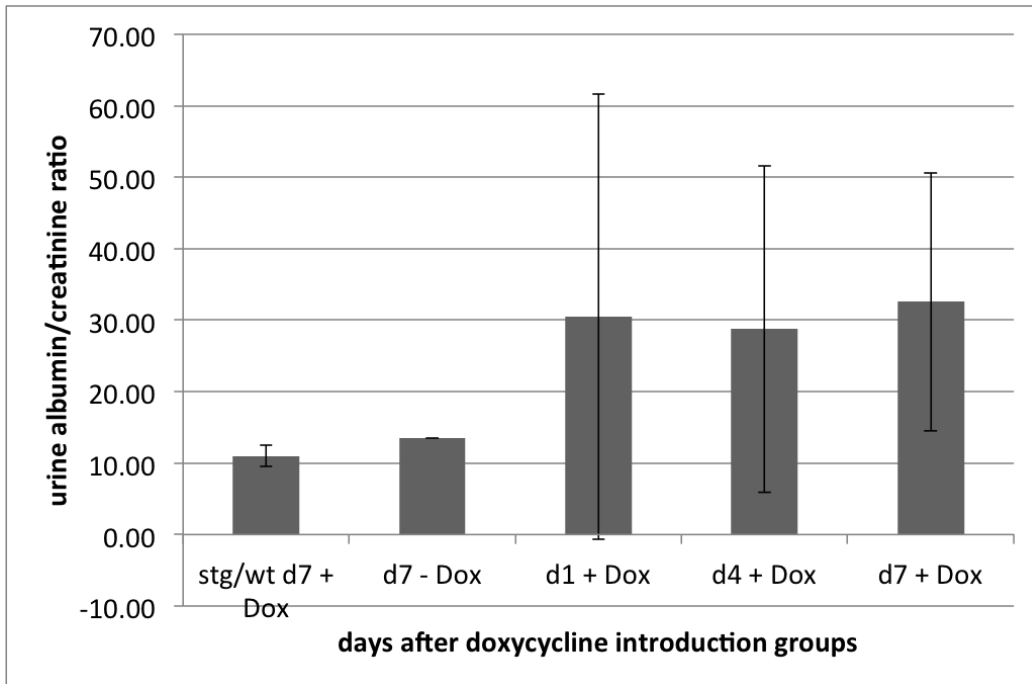
Figure 3.7: Average urine albumin to creatine ratio of of animals on doxycycline up to 7 days.

3.4 20 potential genes involved in mesangial and endothelial cell proliferation

Earlier studies showed that three days after doxycycline introduction mesangial cells start to proliferate. In order to find the responsible factor stimulating growth of mesangial cells, genes that might be involved in proliferation were selected from a list which was derived from a microarray. The selection was based on importance, relevance, novelty and expression changes over the

time course. The selected genes were mainly cyclin-dependent kinases, tyrosine kinase and its receptors and growth factors. Table 3.1 summarizes the genes selected from the microarray data and their function. Figure 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, 3.14 show the expression change of the selected genes obtained from microarray and qRT-PCR data. Most gene expressions measured by qRT-PCR showed the same pattern as the microarray and therefore validated the data. Data obtained from microarray was based on cDNA from 3-4 mice per day and qRT-PCR data on 1-2 mouse cDNAs per day.

Abl1	c-abl oncogene 1, receptor tyrosine kinase
Angptl2	angiopoietin-like 2
Axl	AXL receptor tyrosine kinase
Bmpr1b	bone morphogenetic protein receptor, type 1
Cdk6	cyclin-dependent kinase 6
Cdkn1b	cyclin-dependent kinase inhibitor 1B
Ednra	endothelin receptor type A
Fgfr2	fibroblast growth factor receptor 2
Fgfr4	fibroblast growth factor receptor 4
Fosl2	fos-like antigen 2
Frs2	fibroblast growth factor receptor substrate 2
Gas1	growth arrest specific 1
Ghr	growth hormone receptor
Ncf2	neutrophil cytosolic factor 2
Nek1	NIMA (never in mitosis gene a)-related expressed kinase 1
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3
Pdgfc	platelet-derived growth factor, C polypeptide
Tie1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
Tyro3	TYRO3 protein tyrosine kinase 3

Table 3.1: Selected genes from microarray involved in cell proliferation and therefore might trigger mesangial cell growth.
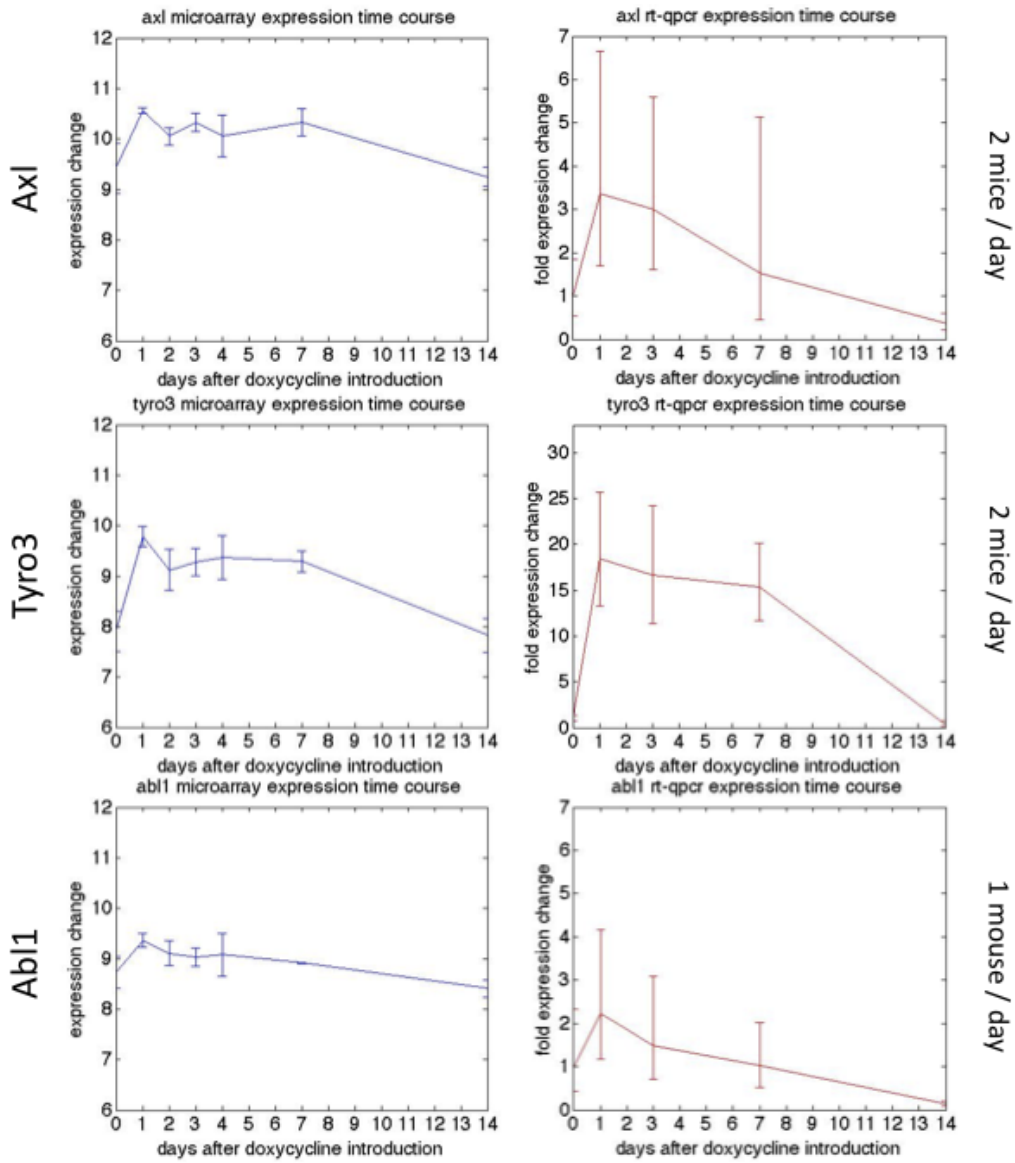
Figure 3.8: Microarray and qRT-PCR derived expression of Axl, Tyro3 and Abl1
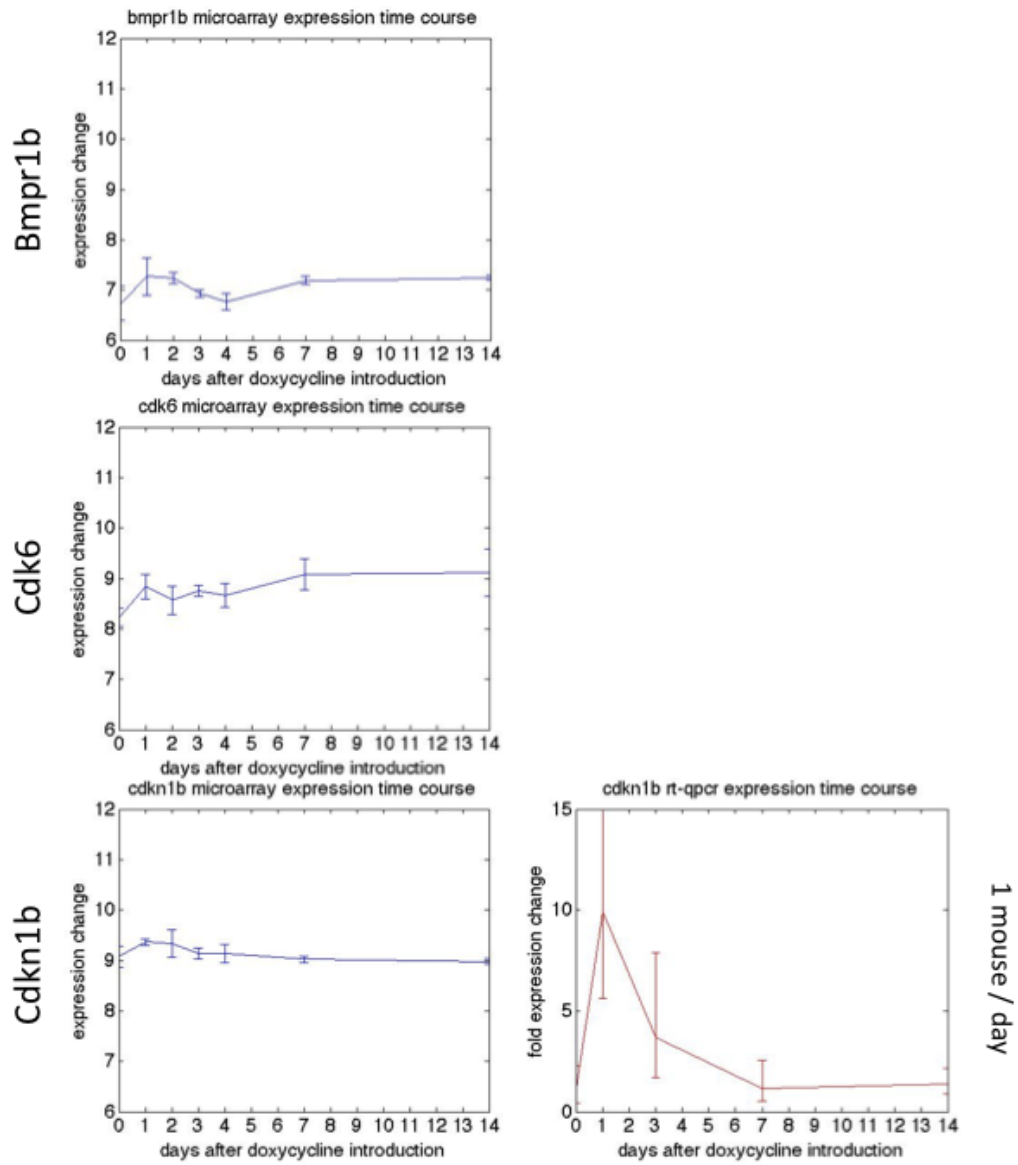
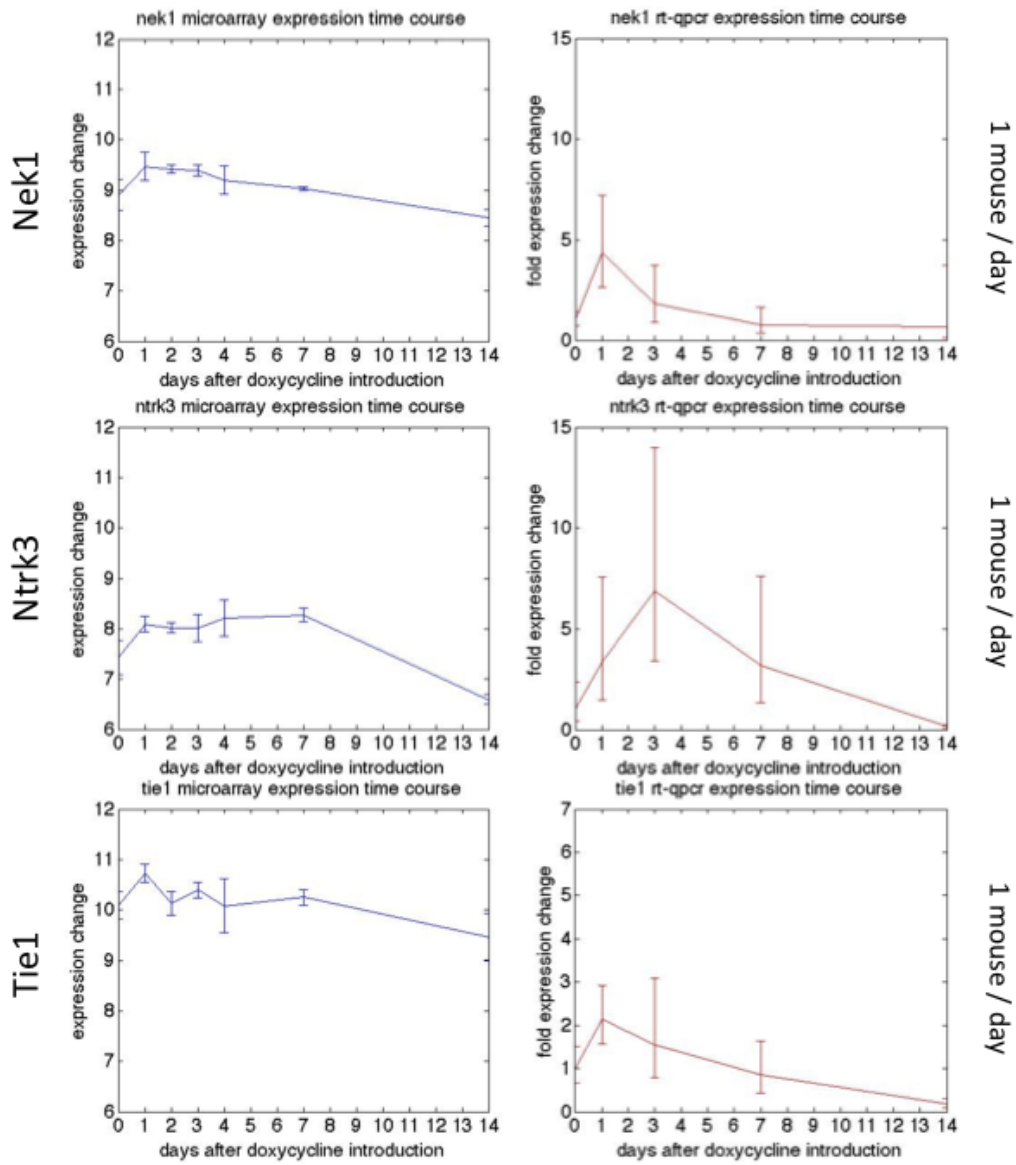Figure 3.9: Microarray and qRT-PCR derived expression of Bmpr1b, Cdk6 and Cdkn1b

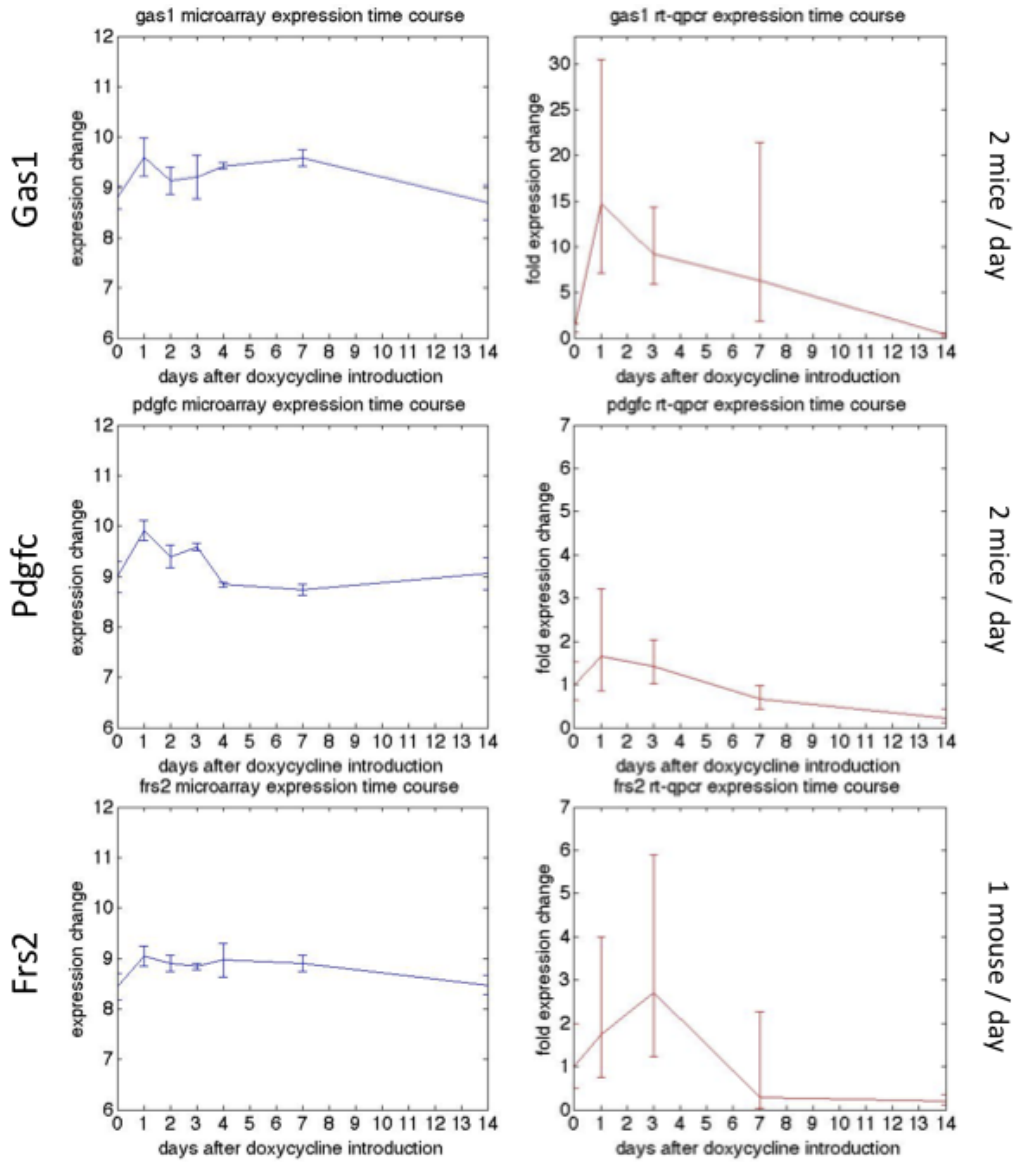Figure 3.10: Microarray and qRT-PCR derived expression of Nek1, Ntrk3 and Tie1

Figure 3.11: Microarray and qRT-PCR derived expression of Gas1, Pdgfc and Frs2
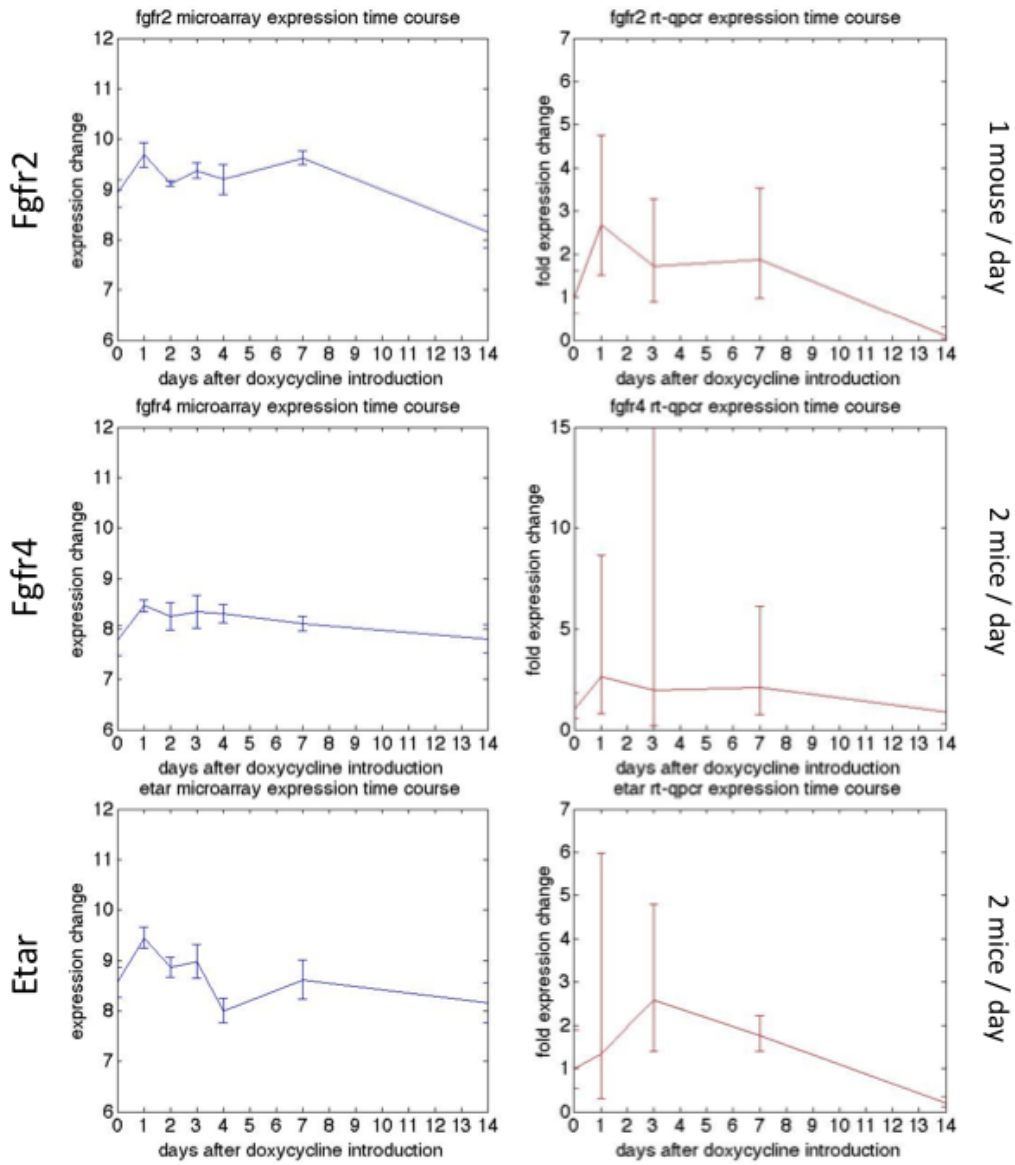
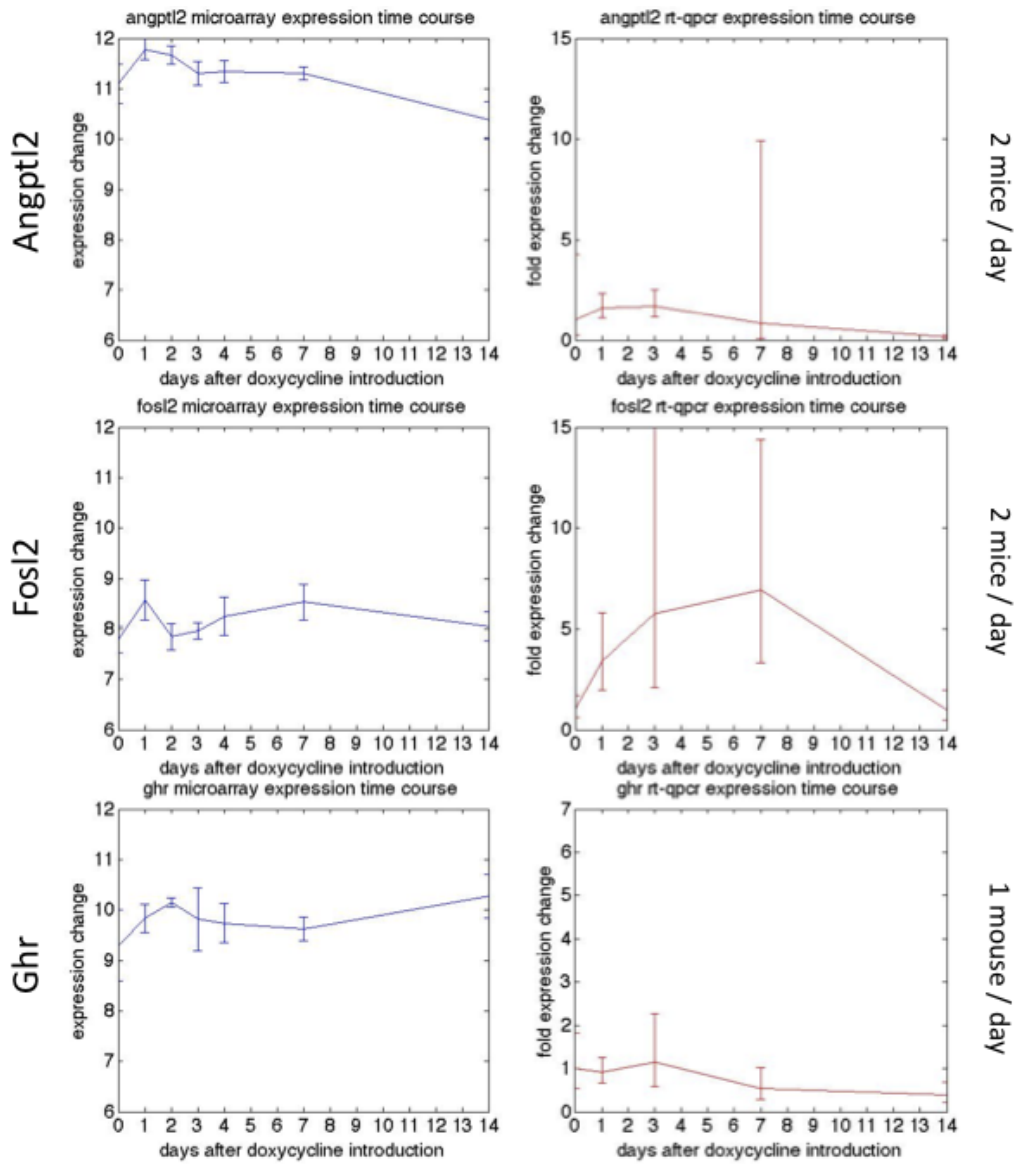Figure 3.12: Microarray and qRT-PCR derived expression of Fgfr2, Fgfr4 and Etar

Figure 3.13: Microarray and qRT-PCR derived expression of Angptl2, Fosl2 and Ghr
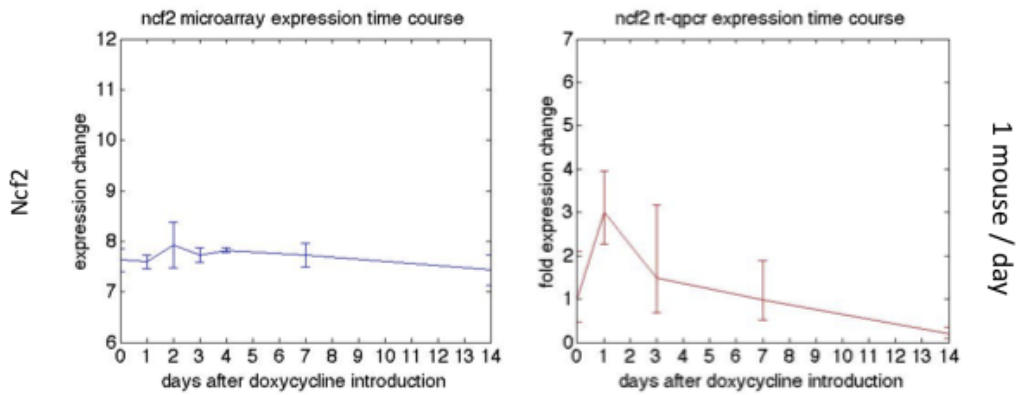
Figure 3.14: Microarray and qRT-PCR derived expression of Ncf2

3.5 Periodic Acid Schiff Stain

A periodic acid schiff (PAS) stain was performed on the kidney sections of animals that were on doxycycline up to 21 days. Figure 3.15 shows the result of that stain. An increasing cell density can be observed during disease progression as well as glomerulosclerosis after 14 days becoming very severe after 21 days of doxycycline introduction.
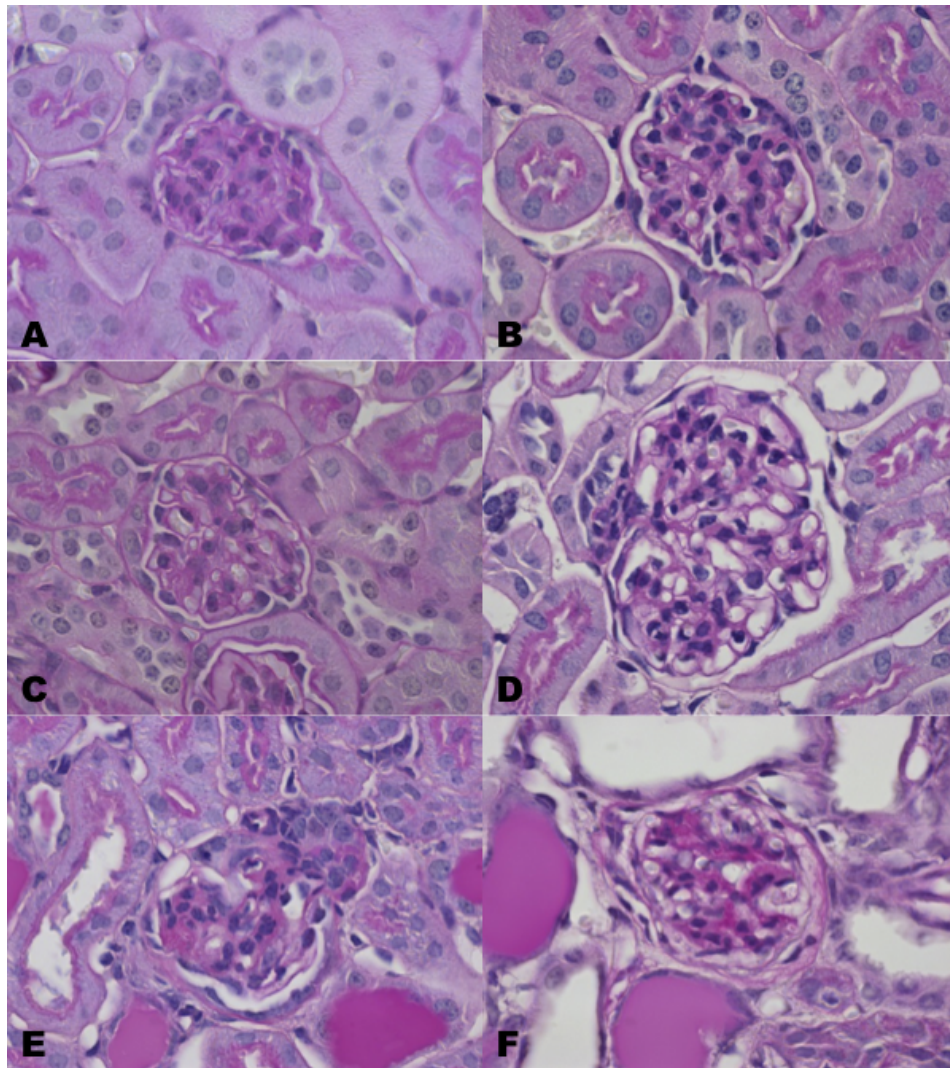
Figure 3.15: Periodic Acid Schiff Stain of the 21 days doxycycline time course.
A) no doxycycline, B) 1 day after doxycycline introduction, C) 4 days after doxycycline introduction, D) 7 days after doxycycline introduction, E) 14 days after doxycycline introduction, F) 21 days after doxycycline introduction. Cellularity increases after doxycycline introduction which can be observed in particular in picture B, D and E. 14 and 21 days after doxycycline introduction glomerulosclerosis is observable and urine spots which are stained intensively form around the glomeruli.

3.6 Phosphorylated Axl expression

Axl is of the receptor tyrosine kinase family and conveys signals from the extracellular matrix into the cytoplasm. This is triggered by binding to growth factors like Gas6 (growth-arrest-specific gene 6). It is known to trigger cell proliferation and therefore is a candidate gene that is involved in mesangial cell growth what is also indicated by the microarray obtained data.

In order to observe the location of phosphorylated Axl in the kidney, paraffin embedded sections from the experiment of Taoran Zhang were stained for phosphorylated Axl expression. The stained sections were from mice without doxycycline and 1, 3, 7 and 14 days on doxycycline respectively. Figure 3.16 shows microscopy obtained pictures of this stain. It seems that phosphorylated Axl is strongly expressed in the glomeruli one day after doxycycline introduction. Then expression gets lower until day 7 and by day 14 almost no expression can be detected in the glomeruli any longer. This change of expression during disease progression suggests that Axl plays an important role in kidney diseases and might trigger mesangial growth because the expression pattern over time correlates with mesangial cell proliferation studied in earlier experiments.
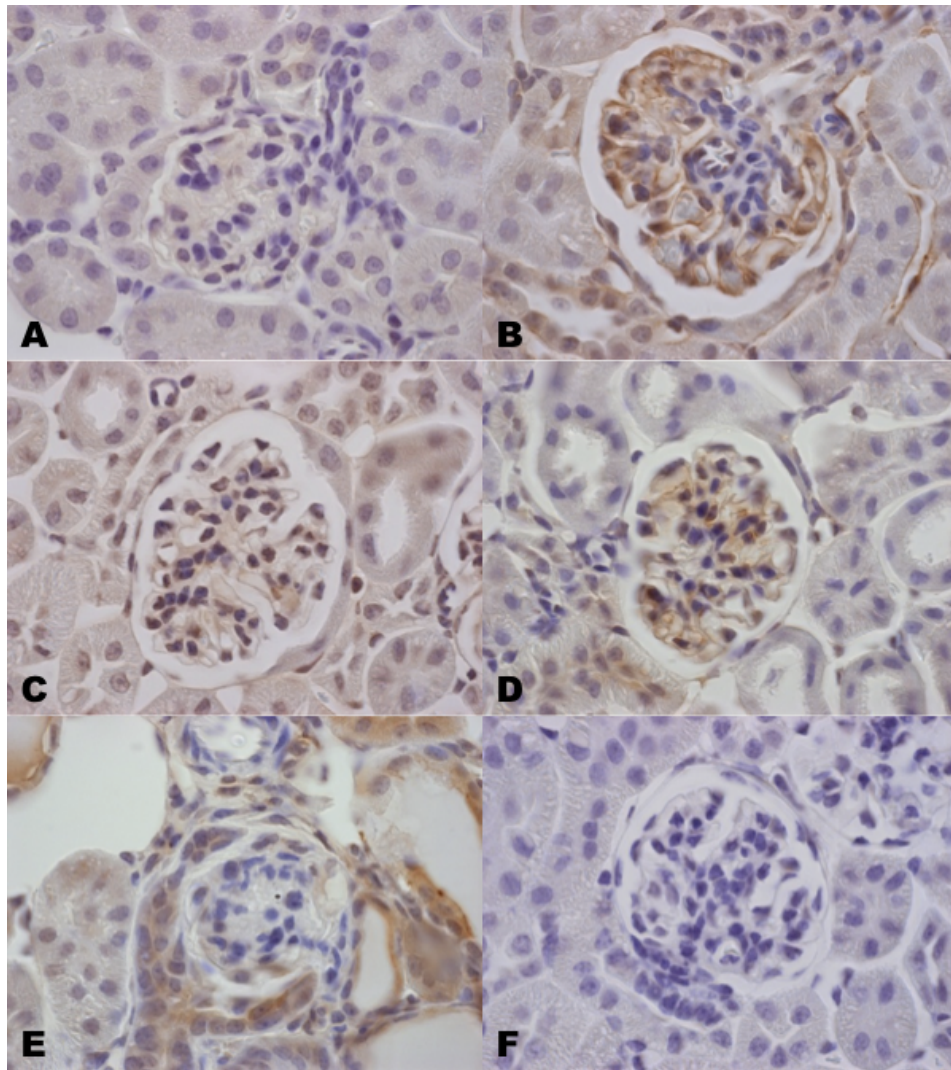
Figure 3.16: Phosphorylated Axl Stain of doxycycline time course. A) no doxycycline, B) 1 day after doxycycline introduction, C) 3 days after doxycycline introduction, D) 7 days after doxycycline introduction, E) 14 days after doxycycline introduction, F) control without primary antibody. Phosphorylated Axl is clearly expressed in the glomeruli one day after doxycycline introduction and then expression goes down and is out of the glomeruli by day 14. It appears to be expressed not only in podocytes but also in mesangial or endothelial cells.

3.7 Pdgfc expression

Pdgfc is a platelet derived growth factor that is mitogenic for cells of mesenchymal origin. It has been identified as a mitogen for rat mesangial cells and also plays a role in various kidney diseases in humans [Eitner et al., 2003]. The paraffin embedded sections of mice on doxycycline up to 14 days were stained for Pdgfc. Figure 3.17 shows that the expression in glomeruli is up between four and seven days after doxycycline introduction and that after 14 days there is no more Pdgfc expressed. These results and the fact that Pdgfc is known to be mitogenic for cells of mesenchymal origin also make Pdgfc a very possible candidate for triggering mesangial proliferation.
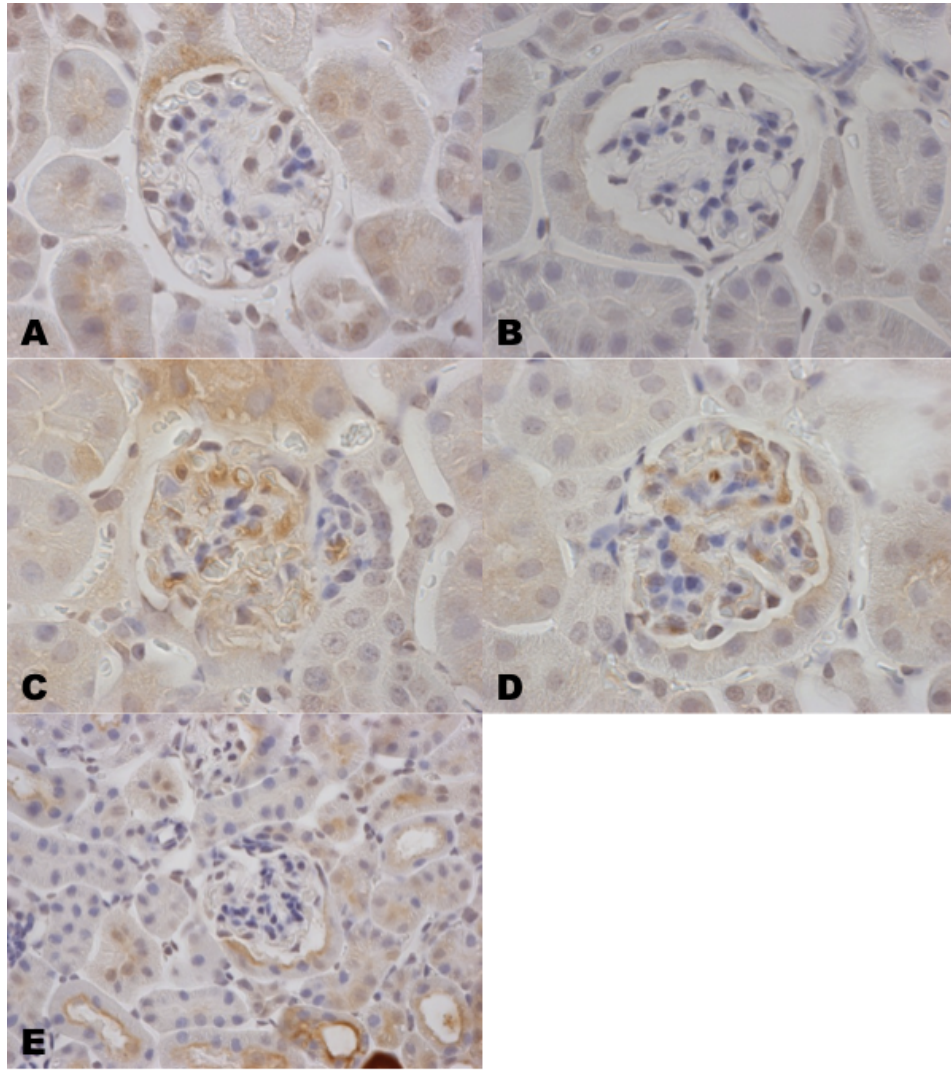
Figure 3.17: Immunohistochemistry staining of paraffin embedded sections of doxycycline time course for Pdgfc. A) no doxycycline, B) 1 day after doxycycline introduction, C) 4 days after doxycycline introduction, D) 7 days after doxycycline introduction, E) 14 days after doxycycline introduction. While there is not much Pdgfc expressed in the glomeruli of the of no doxycycline or 1 day after doxycycline introduction mice, the expression goes up on day 4 and day 7 and is down again after 14 days. It appears to be expressed in podocytes but also in mesangial or endothelial cells.

Chapter 4

Intervention studies

4.1   Design of Experiment

Mice were grouped to receive different treatments in order to evaluate the effects of the drugs Enalapril and Mitotempo on disease progression. The experimental groups are summarized in table 4.1.

4.1.1   Enalapril

Enalapril is an angiotensin converting enzyme (ACE) inhibitor and is used to treat hypertension and also chronic heart failure. It lowers the blood pressure by preventing ACE from constricting blood vessels.

4.1.2 Mitotempo

Mitotempo is mitochondria-targeted antioxidant and therefore inhibits superoxide production in mitochondria.

Number of mice	Doxycycline	Enalapril	Mitotempo
5	+	-	-
5	+	+	-
3	-	+	-
5	+	-	+
5	-	-	+

Table 4.1: Different treatments for intervention studies.

4.2 Urine albumin to creatinine ratio

To determine how sick the mice with the different treatments became their urine albumin/creatinine ratio was measured. The effect of Enalapril is shown in figure 4.1 and 4.2 while figure 4.3 and 4.4 demonstrate the effects of Mitotempo. Proteinuria was completely prevented in the animals that received the drug. Unfortunately just one of the five animals that received Doxycycline only got sick. So it is not certain that proteinuria in the mice with double treatment was actually prevented by the tested drug.
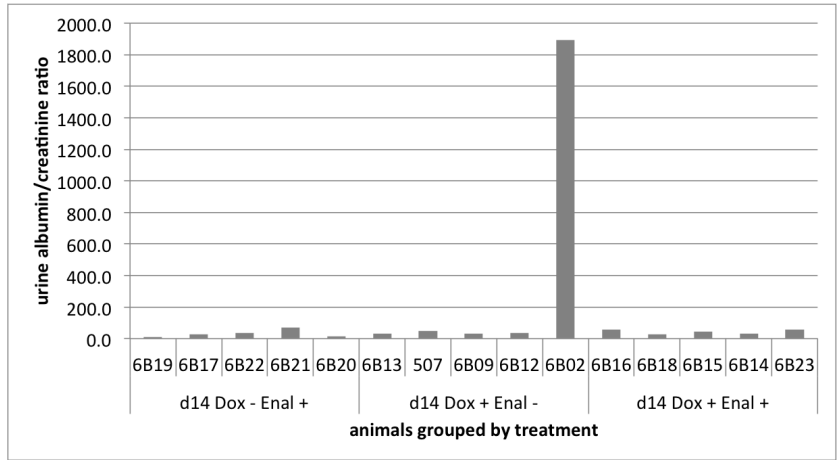
Figure 4.1: Urine albumin to creatine ratio of animals treated with Doxycycline and/or Enalparil. No animal in the group that was treated with Doxycycline and Enalapril showed any proteinuria. But since only one animal in the Doxycycline only group became sick it is not certain if Enalapril is responsible for the prevention.
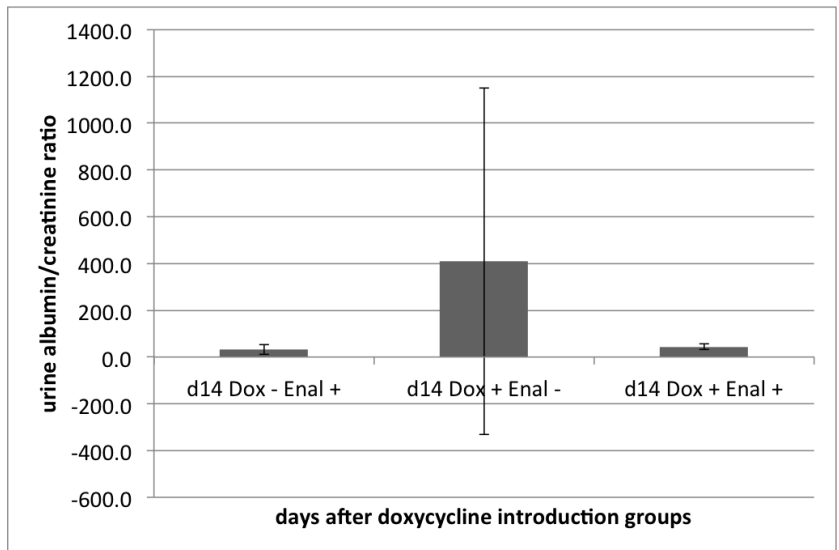

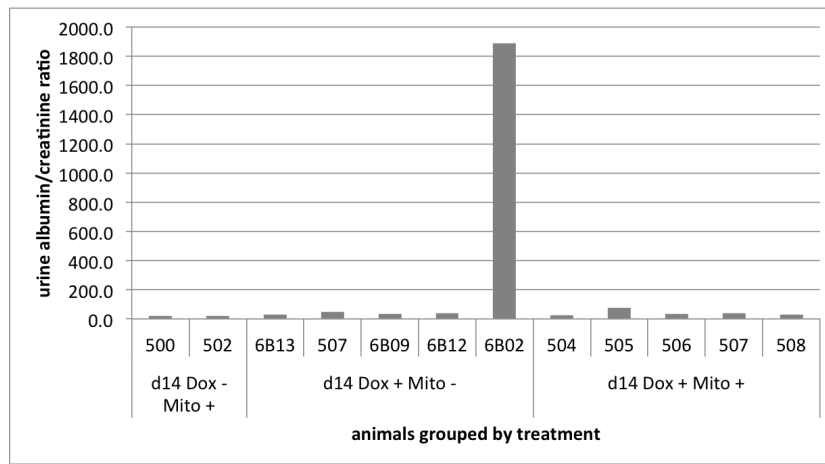Figure 4.2: Average urine albumin to creatine ratio of animals treated with Doxycycline and/or Enalparil

Figure 4.3: Urine albumin to creatine ratio of animals treated with Doxycycline and/or Mitotempo. Also here animals treated with Doxycycline and Mitotempo did not become sick. But as the same Doxycycline only group in which only one animal became sick as in the Enalapril experiment was it is uncertain if the proteinuria is prevented because of Mitotempo.
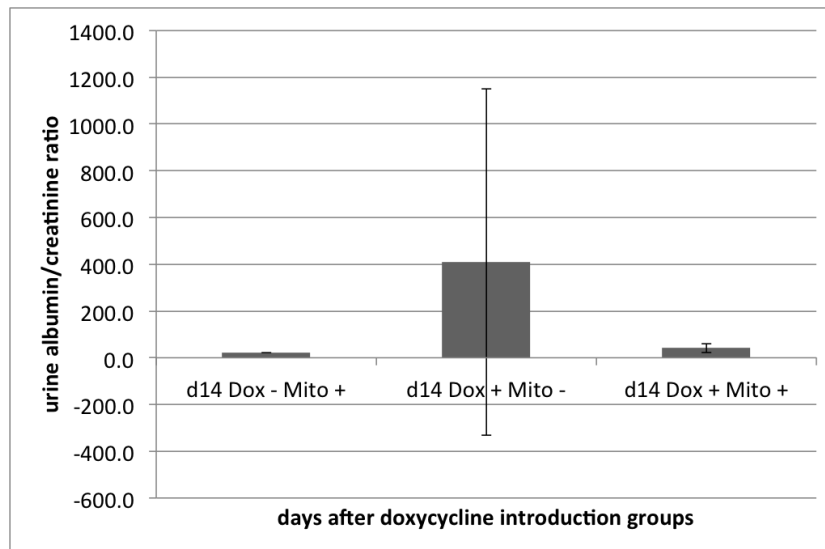

Figure 4.4: Average urine albumin to creatine ratio of animals treated with Doxycycline and/or Mitotempo

4.3   Periodic Acid Schiff Stain

The PAS stain of the mice and differently treated groups is shown in figure 4.5. The figure shows that the glomeruli of the mice that received a double treatment did not show any damage whereas the glomeruli of at least one mouse (the one shown in the figure) of the Doxycycline only group developed glomerulosclerosis. Although it is unsure if the glomerulosclerosis was prevented because of the drugs the doublet treated mice received or if they would not have become sick anyway this is a promising result which suggests that Enalapirl ad Mitotempo can inhibit disease progression and prevent glomerulosclerosis.
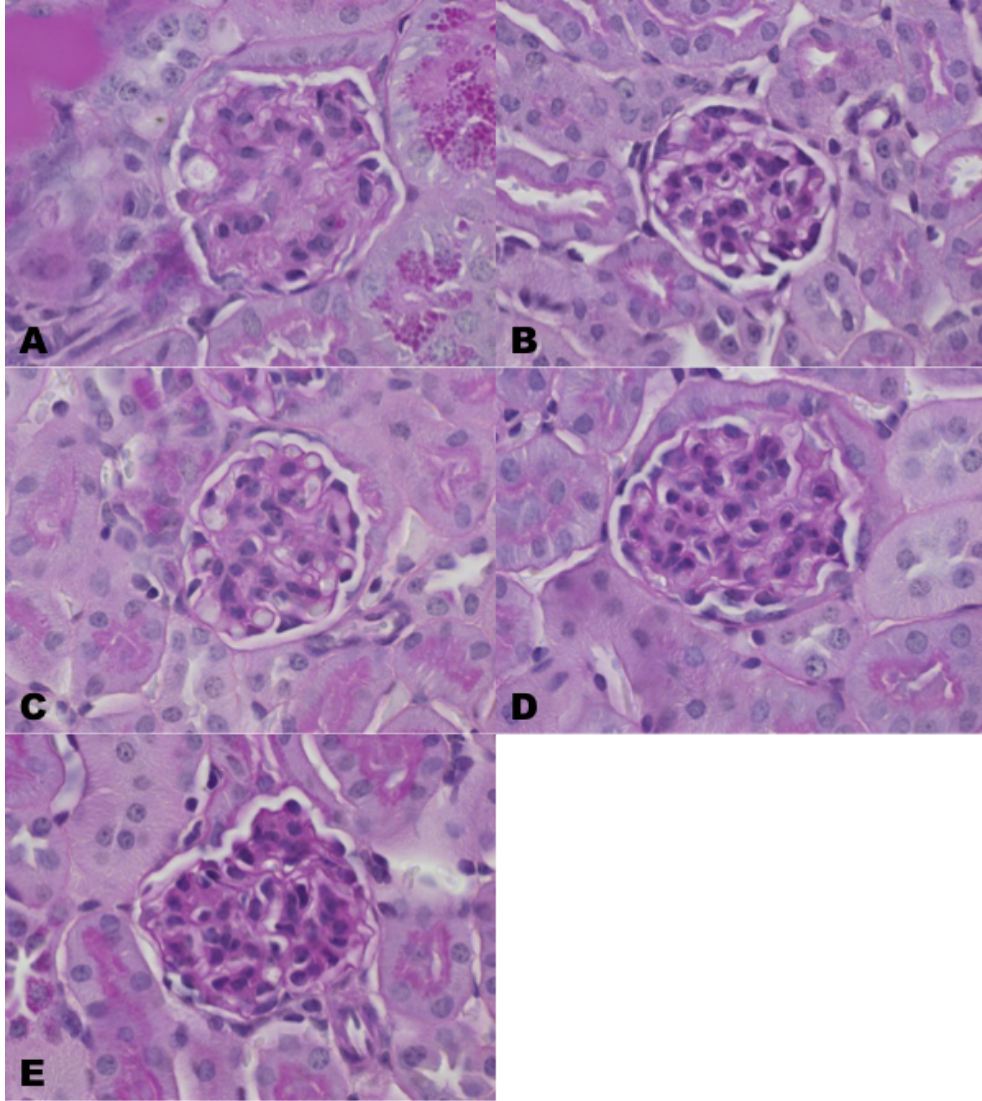
Figure 4.5: Periodic Acid Schiff Stain of Enalapril and Mitotempo intervention studies. Mice got different drug treatments for a period of 14 days. A) doxycycline only, B) doxycycline and enalapril, C) enalapril only, D) doxycycline and mitotempo, E) mitotempo only. While in the mouse that got doxycycline only glomerulosclerosis is observable it was completely prevented in the mice that got doxycycline and either enalapril or mitotempo. The drugs administered alone without doxycycline did not cause any changes.

Chapter 5

Discussion

The transgenic mouse with constitutive active TGF-β receptor provides a great model to study the disease progression in kidneys since the disease can be induced arbitrarily. With the time course studies we first of all were able to identify candidate genes that might be responsible for stimulating the mesangial cell expansion during early disease stage. In particular Axl, a receptor tyrosine kinase seems to play an important role in mesangial cell proliferation as it becomes highly expressed in glomeruli, maybe even in mesangial cells, right after doxycycline introduction. Immunohistochemistry also revealed that Pdgfc, a platelet derived growth factor that is mitogenic for cells of mesenchymal origin might have some thing to do with it. Also here obtained pictures show that expression in glomeruli is upregulated about four days after doxycycline introduction. Further investigation in coculture is required to identify the factor simulating mesangial expansion.

The intervention studies show that Enalapril and Mitotempo are both promising drugs that restrict disease progression in the transgenic mouse model with constitutive active TGF-β receptor. Proteinuria could be completely prevented when treating animals with these drugs. Future investigations include a long time study of 21 days where Mitotempo will get administered in a physiological dosis via osmotic minipumps.

Chapter 6

Acknowledgements

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