Prorenin, its maturation and the (pro)renin receptor

Thesis submitted for the degree of

Doctor of Philosophy

at

The University of Edinburgh

Nathalie L'Huillier, BSc, MPhil
Molecular Physiology Laboratory
University of Edinburgh

February 2006

I declare that this thesis was compiled by myself and that all the work presented in this thesis is my own, except where otherwise stated.

Nathalie L'Huillier

Abbreviations

The following abbreviations have been used throughout this thesis and are described in full in brackets after their first use in the text.

AA : Afferent arteriole
ACE : Angiotensin converting enzyme
AMP : Adenine monophosphate
ANF : Atrial natriuretic factor
Ang : Angiotensin
AOGEN : Angiotensinogen
AT : Angiotensin II receptor
ATCC : American Tissue culture Collection
ATP : Adenosine triphosphate
BAC : Bacterial artificial chromosome
BP : Blood pressure
BSA : Bovine serum albumin
COD1 : X-linked cone-rod dystrophy
CTP : Cytidine triphosphate
CVD : Cardiovascular disease
DAB : Diaminobenzidine
DEPC : Diethyl pyrocarbonate
DMEM : Dulbecco's modified Eagle's medium
DNA : Deoxyribonucleic acid
DOCA : Deoxycorticosterone acetate
dpc : Days post-coitum
DTT : Dithioerythritol
EA : Efferent arteriole
ECM : Extracellular matrix
EDTA : Ethylenediaminetetraacetic acid
eNOS : endothelial nitric oxide synthase
ERK : Extracellular signal-regulated kinase
ET-1 : Endothelin-1
ET_A : Endothelin receptor A
GFP : Green fluorescent protein
GMEM : Glasgow's modified Eagle's medium
GTP : Guanosine triphosphate
hAOGEN : Human angiotensinogen
HPLC : High performance liquid chromatography
hRen : Human renin
HRP : Horseradish peroxidase
I3C : Indole-3-carbinol
iNOS : Inducible nitric oxide synthase
IPTG : Isopropyl β-D-thiogalactoside
JG : Juxtaglomerular
JGA : Juxtaglomerular apparatus
JGC : Juxtaglomerular cell
LVH : Left ventricle hypertrophy
M6Pr : Mannose-6-phosphate receptor
MAP : Mean arterial pressure
MAP kinase : Mitogen-activated protein kinase
MD : Macula densa
MH : Malignant hypertension
mRNA : Messenger RNA
NO : Nitric oxide
NTP : Nucleic acid triphosphate
PBS : Phosphate-buffered saline
PCR : Polymerase chain reaction
PP : Pulse pressure
PRC : Plasma renin concentration
QTL : Quantitative trait loci
RAAS : Renin-angiotensin-aldosterone system
RAS : Renin-angiotensin system
RNA : Ribonucleic acid
RT-PCR : Reverse transcriptase polymerase chain reaction
SBP : Systolic blood pressure
s.d. : Standard deviation
SDS : Sodium dodecyl sulphate
SHR : Spontaneous hypertensive rat
SMC : Smooth muscle cell
SMG : Submandibular/submaxilliary gland
SPSHR : Stroke-prone SHR
SSC : Sodium- chloride/citrate buffer
TBS : Tris-buffered saline
TEM : Transmission electron microscope
TGF : Transforming growth factor
TGR : Transgenic rat
tRNA : Transfer ribonucleic acid
TTP : Thymidine triphosphate
UTP : Uridine triphosphate
UTR : Untranslated region
V-ATPase : Vacuolar ATPase
X-Gal : 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
WHO : World Health Organisation

Acknowledgements

Firstly, I am grateful for the Wellcome Trust generous support during my Ph.D project and for the award of a Prize Studentship. I am also thankful for the award of a Marie Curie Fellowship from the European Union.

I would like to thank my supervisors, Prof. John Mullins, Dr. Matt Sharp, Dr. Janice Paterson and Dr. Linda Mullins for their support throughout my studies. I am grateful for their enthusiasm, knowledge and constructive criticism and for the training I received while under their supervision. I am also thankful to Dr. Gillian Gray and Dr. Paddy Hadoke for their help, suggestions and encouragements.

I am indebted to all the members of the Molecular Physiology laboratory for providing endless supply of technical help at the bench and with computing issues, a stimulating environment in the laboratory and for their support at difficult times. I am also grateful to Lynn Meikle for administrative support and for her enthusiastic attitude.

I am also thankful to Prof. Jos Smits for allowing me to work in his laboratory in Maastricht and to his staff, for making me feel welcome, for giving me invaluable training and for teaching me survival Dutch.

I would also like to thank, Prof. Stewart Fleming (University of Dundee) for processing and analysis of electron microscopy samples, Dr. David Browstein for histopathology support and Lynne Ramage for her technical advice on and teaching of *in situ* hybridisation. I would also like to acknowledge the excellent assistance of Gill Brooker, Christine Marshall and Keith Chalmers with animal issues.

I would like to thank my parents, J-F and Marie-Thé, my sister, Cécile and my brother, Philippe for their support and for always managing to make me smile. I must also thank the Mather clan for being there for me when most needed, all my "Edinburgh" friends for helping me with the social aspect of my PhD studies and the "Maastricht" crowd for a wonderful time in the Netherlands and a lasting friendship.

Last, but not least, my deepest appreciation goes to Mark for many years of support and encouragement during happy, frustrating and successful times throughout my studies.

To Mark and to my parents, Jean-Francois and Marie-Thérèse for sticking with me through thick and thin and for their everlasting optimism.

Pour Mark et pour mes parents, Jean-Francois et Marie-Thérèse qui sont toujours à mes cotés. Merci pour votre éternel optimisme.

"Of Mice and *Ren*"

"It is demonstrable [...] that things cannot be otherwise than as they are; for all being created for an end, all is necessarily for the best end". Pangloss, Professor of metaphysico-theologico-cosmolo-nigology, *Candide, Voltaire.*

"Il est demonstrable [...] que les choses ne peuvent être autrement: car tout étant fait pour une fin, tout est necessairement pour la meilleure des fins". Pangloss, Professeur de metaphysico-theologico-cosmolo-nigologie, *Candide, Voltaire.*

Abstract

The renin-angiotensin system (RAS) is a key biochemical pathway controlling homeostasis and blood pressure. The initial step of the RAS is carried out by renin to produce angiotensin (Ang) I which is converted to AngII by the angiotensin-converting enzyme (ACE). Renin is synthesized as a pro-enzyme which can be activated in dense core granules of the renal juxtaglomerular cells or released as prorenin. There is evidence that prorenin can, in addition, be activated in a reversible manner, via a prorenin receptor. Recently, a specific (pro)renin receptor was identified in human tissues. Binding of this receptor to (pro)renin caused increased cleavage of angiotensinogen and stimulation of an intracellular signalling pathway. The aim of this thesis is to investigate the biology of prorenin, its maturation and the (pro)renin receptor.

Expression of the mouse putative (pro)renin receptor (RR) was detected by RT-PCR in all tissues and cell lines examined including human mesangial cells previously reported to be negative for RR. Mouse RR was also present during development from E9.5. The mouse and rat RR cDNA were found to be highly homologous (92% and 91%, respectively) to the human cDNA. Surprisingly, the translated human, mouse and rat cDNAs exhibited sequence identity with a small protein co-purifying with a bovine vacuolar-ATPase called M8-9 which had not been reported previously. V-ATPases are critical for cell survival. Phylogenic studies revealed RR is highly conserved between species and likely to be important physiologically. The role of RR was investigated in a high circulating prorenin rat model [TGR(*Cyp1a1-Ren2*)], in which (pro)renin triggers malignant hypertension (MH).

Uptake of prorenin by the heart previously demonstrated in this transgenic model may be mediated by RR. In the present study, the animals, from a new colony, had a gradual hypertensive response. Cardiovascular stiffening was measured using echo(cardio)graphy. Despite an obvious hypertrophic remodeling and longer exposure to the inducer, no signs of microinfarctions or inflammatory infiltration cells were observed in the heart. Fibrinoid necrosis of small intra-renal vessels with glomerulosclerosis and mesenteric artery remodeling were also observed. The phenotype differs from the original work. Surprisingly, RR was not up-regulated. The reasons for the phenotypic differences between TGR(*Cyp1a1-Ren2*) colonies were examined. Two main observations were made: dietary sodium levels appear to correlate with the severity of MH and TGR(*Cyp1a1-Ren2*) animals reported in this thesis had a lower pathogen load.

To investigate the possible role of RR, a prorenin decoy peptide was used to attempt to ameliorate the MH phenotype in TGR(*Cyp1a1-Ren2*) animals. This peptide which competes with prorenin for binding to RR, has been showed to improve vascular injuries in diabetic nephropathy. In TGR(*Cyp1a1-Ren2*), however, no changes in the MH phenotype could be observed, except in the mesentery in which less severe fibrinoid necrosis developed.

To complement work on RR, prorenin maturation and renin storage were studied during development. The data showed the complete absence of renin granules in mouse kidneys before birth. This indicates that renin could not be stored and may not be processed through the regulated pathway as observed in the adult. Low sodium diet and ACE inhibition triggered (pro)renin granules to be produced in the foetal kidney. Two ACE inhibitors differing in their ability to cross the placenta were used. The data suggest that foetal renin granule formation is under dual control from both foetal and maternal RAS.

Although the (pro)renin receptor may be important physiologically, the data presented in this thesis suggest a more fundamental role in cell biology than had previously been recognised. The lack of evidence for regulation of RR in a model of high prorenin and malignant hypertension suggests that the function of this protein may need to be re-assessed.

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Publication arising for the work presented in this thesis:

L'Huillier N., Dunbar D., Sharp M., Mullins J.J., On the relationship between the renin receptor and the vacuolar proton-ATPase membrane sector-associated protein M8-9 in: *The local cardiac renin-angiotensin-aldosterone system*, E.D. Frolich and R.N. Re Editors. 2005, Springer, New York, USA. p. 17-34.

Figures and Tables

Chapter 1
General Introduction

The World Health Organisation (WHO) reported that, in 2001, 33.5 million people were affected by so-called "non-communicable diseases", namely cancer, stroke and cardiovascular diseases (CVD), worldwide. Half of these individuals were diagnosed as having CVD making these conditions the most important cause of death after smoking. The healthcare and social costs in the United States of America alone for the treatment of CVD and lost man hours were estimated to $368.4 billion by the American Heart Association (2004). Stroke and CVD have become the two leading causes of deaths in Western society. Although CVD were originally thought to be "Western world conditions", there has been a marked increase in the number of affected people in "developing" countries (World Health Organisation 2003). This prompted the WHO to make prevention and research in CVD a new priority. Many cardiovascular conditions, namely cardiac hypertrophy, arrhythmias, coronary heart disease and myocardial infarction have been linked to high blood pressure. The increase in CVD risk can be attributed to physiological and structural changes to target organs, in particular to the heart and the kidneys.

1.1 Hypertension

Hypertension (or increased blood pressure) is the chronic physiological state in which arterial blood pressure is elevated and may potentially cause harm to patients if untreated. The European Society of Hypertension and the European Society of Cardiology guidelines on the management of hypertension 2003 recommended that blood pressure-lowering treatment be prescribed to patients with systolic pressure above 140mmHg and diastolic pressure above 90mmHg, although additional factors such as diabetes must be taken into account in the evaluation of the blood pressure level threshold (European Society Cardiology 2003). Hypertension is well known to be a complex multi-factorial disease making assessment, screening and choice of treatment difficult. Physiological mechanisms regulating blood pressure are themselves numerous and interlinked (Figure 1.1).

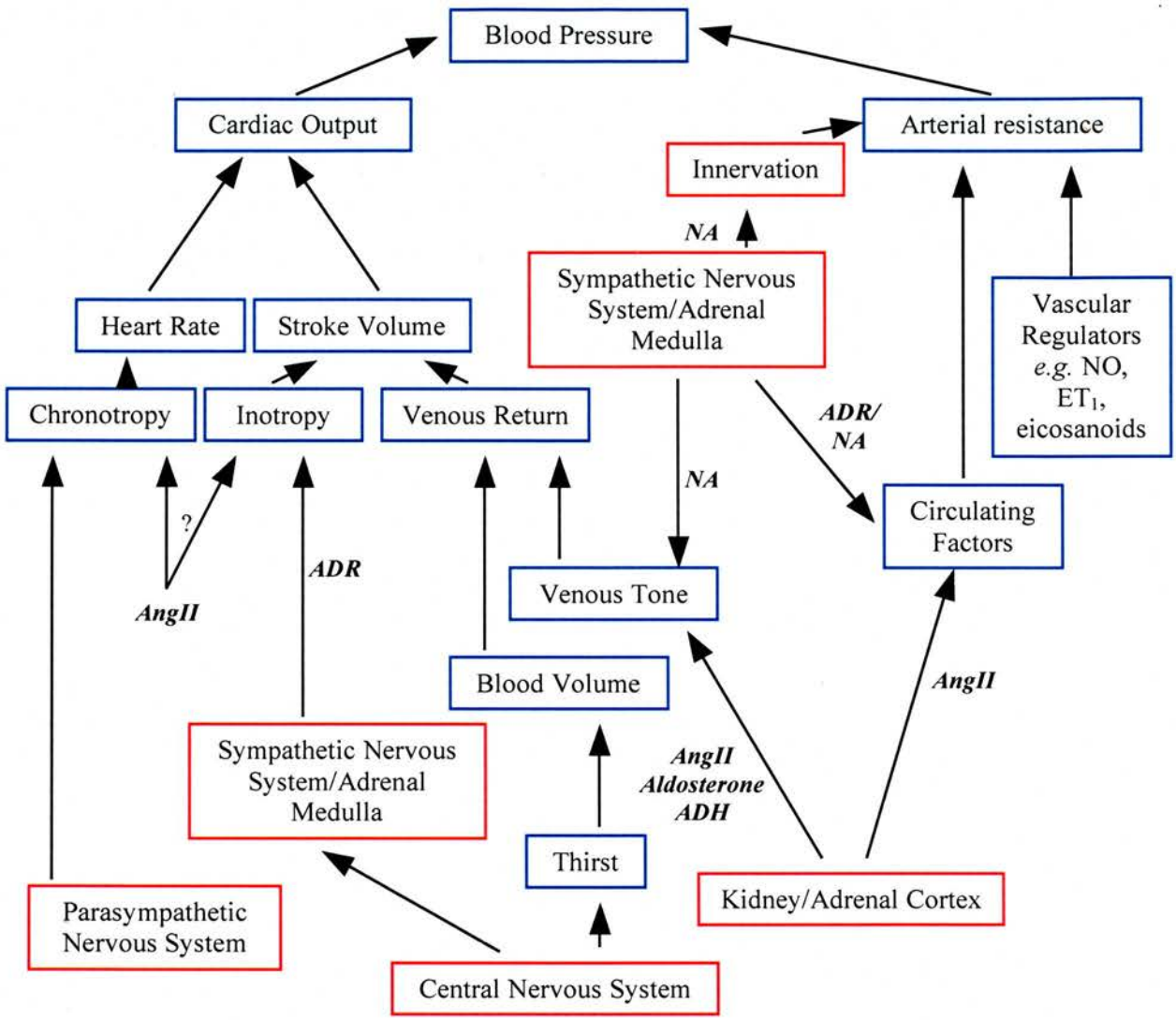


Figure 1.1: Mechanisms and pathways involved in the regulation of normal blood pressure. The two main physiological influences on blood pressure are cardiac output and peripheral resistance which are themselves under the control of several systems such as sympathetic nervous system or/and the renin-angiotensin system. ADH = vasopressin, ADR = adrenaline, AngII = angiotensin II, ET = endothelin, NA = noradrenaline. NO = nitric oxide. Blue boxes represent physiological responses while red boxes indicate organs or anatomical structures. Adapted from Mayet and Hughes (2003).

Treatments available for hypertension are of five classes (Genest 2001; Ramsay *et al.* 1999): diuretics (the most commonly used), β-blockers, calcium-channel blockers, angiotensin-converting enzyme (ACE; see below) inhibitors and antagonists of angiotensin receptors (see below). The choice of therapy is dependent on age, level of blood pressure, progression/complications such as renal diseases and environmental factors. In more than 90% of hypertensive cases, the cause is unknown. This is termed essential (or primary) hypertension. Remaining cases called secondary hypertension are the results of an underlying condition such as: chronic kidney disease, chronic alcohol abuse, hormonal disturbances and endocrine tumours (Wales 1997). The prevalence of hypertension is increased by several factors such as poor diet, high sodium intake, obesity, smoking, diabetes, family tendency to high blood pressure and certain medicines such as corticosteroids (Kannel 1990; Kannel 2000; Kannel *et al.* 2002; Kannel *et al.* 1970; Sowers and Haffner 2002).

Hypertension is a polygenic trait in which the effects may be, in addition, modulated by gene-environment (as described above) interactions. Monogenic disorders of hypertension although uncommon, also exist (Lifton *et al.* 2001). For example, individuals with apparent mineralocorticoid excess syndrome display high blood pressure due to a deficiency in 11-β-hydroxysteroid dehydrogenase type II. This allows an erroneous activation of the mineralocorticoid receptor by glucocorticoid which leads to increased renal sodium and water reabsorption. (Stewart 1999). In Liddle's syndrome, a mutation in the epithelial sodium channel was found to be responsible for a similar phenotype to the mineralocorticoid excess syndrome (O'Shaughnessy 2001). These single-gene forms of hypertension contributed to the advances in the understanding of the systems involved in blood pressure regulation. Both syndromes are characterised by low or suppressed plasma renin and aldosterone levels. This suggested that the renin-angiotensin-aldosterone system (RAAS) and sodium homeostasis are important systems in maintenance of volume homeostasis, electrolyte balance and therefore, the regulation of blood pressure.

Malignant hypertension (MH) is a severe complication of hypertension resulting in a characteristic lesion. Fibrinoid arterial or arteriolar necrosis affects the small arteries of the kidney. Small arteries of the brain, gut, heart, adrenal and retina are also susceptible to this lesion (Robertson 1997).

The most severe outcomes of MH are myocardial infarction, heart and kidney failure and strokes. Left ventricular hypertrophy (LVH) and microalbuminuria precede the development

of the cardiac and renal injuries, respectively. These can be used as markers of hypertensive complications in humans and also in animal models (Abdi and Johns 1996; Griffin and Bidani 2004; Mayet and Hughes 2003). The physiological changes involved in LVH have been shown to be linked to both haemodynamic factors such as shear stress (Hudlicka 1994) and non-haemodynamic events such as the renin-angiotensin (Sadoshima *et al.* 1993; Yamazaki *et al.* 1995) or the adrenergic system. The precise mechanisms causing LVH are unclear and it may be that LVH is responsible for the increased blood pressure. However, increased blood pressure leading to increased mechanical stretch is initially involved in the development of this phenotype (Morgan *et al.* 1998). The involvement of the RAS in the hypertensive pathophysiology of cardiac and vascular damage is discussed in more detail in *Chapter 1.4.4.*

1.2 Animal models of hypertension

Animal models of hypertension are widely available. They allow the study of a variety of mechanisms and markers involved in the pathogenesis of hypertension which may be applicable to human disease. The importance of genetic background of the strains used in hypertension research is now well known. As well as being a major determinant in animal studies, this aspect has also been utilised to identify quantitative trait loci (QTL) associated with hypertension (and cardiovascular associated phenotypes) in both genetic and transgenic models and more recently to generate congenic lines. To date, evidence is available showing that at least one blood pressure QTL has been identified on each rat chromosome, except for chromosomes 6, 11 and 15 (Rapp 2000).

1.2.1 Genetic models

Genetic models such as the spontaneous hypertensive rat (SHR), the spontaneous hypertensive stroke-prone rat (SPSHR) or the Dahl salt-sensitive rat are useful tools to study the effects of blood pressure and of genetic predisposition to hypertension. The following paragraphs highlight the main characteristics and regulatory systems and factors involved in the pathogenesis of hypertension in these models.

1.2.1.1 Spontaneous hypertensive rat

The spontaneous hypertensive rats (SHR) were obtained by inbreeding Wistar-Kyoto rats with the highest blood pressure. They develop hypertension (systolic blood pressure up to 180-200mmHg) from the age of 4-6 weeks, largely independently of dietary salt content and display cardiac hypertrophy and renal dysfunction. All animals have impaired endothelium-dependent relaxation (Pinto *et al.* 1998) without gross apparent vascular complications and many SHR will develop heart failure if untreated (Pinto *et al.* 1998). End-organ damage was shown to be dissociated from high blood pressure since diuretics and vasodilators reduce blood pressure but do not reduce cardiac hypertrophy compared with ACE inhibitors (Fukui *et al.* 1989; Limas *et al.* 1984). However, both ACE inhibition (captopril treatment) and vasodilation (hydralazine treatment) improved aortic remodelling, namely increased intimal/medial thickness and intimal collagen deposition, demonstrating that these processes are linked to hypertension rather than being dissociated from it (Limas *et al.* 1984).
The atrial natruretic factor (released by cardiac tissue in response to increased mechanical stress) is significantly reduced by ACE inhibition whereas a lesser effect was obtained using the vasodilator hydralazine (Fukui *et al.* 1989). Although, the RAS is associated with hypertension in cardiovascular remodelling events, a direct involvement of the RAS in the cardiac end-organ damage observed in the SHR has been demonstrated independently of blood pressure.

The RAS is, however, not the sole system involved in the pathophysiology of SHR. Strong evidence is available suggesting that oxidative stress in the vasculature is an important determinant in the pathophysiology of SHR. Development of arterial stiffness has been reported in SHR and it appears that in the early phase of hypertension in this model, changes in arterial structure and endothelial response to increased pressure are involved whereas in older SHR loss of elastic properties is observed (Safar *et al.* 2001).
It is known that oxidative stress occurs prior to blood pressure rising (Nabha *et al.* 2005; Vaziri *et al.* 1998). While nitric oxide (NO) production by endothelial NO synthase (eNOS) is well known for its vasorelaxing properties, excessive NO production through the inducible NO synthase (iNOS) may have cytotoxic effects and contribute to the endothelial dysfunction seen in models of hypertension (Hong *et al.* 2000). Vascular eNOS is increased in SHR before and after the onset of hypertension, possibly as a protective mechanism but appears to be offset by increased expression of detrimental iNOS in the renal vasculature and this may be a potential mechanism for renal end-organ damage. Inhibition of iNOS (by

aminoguanadine treatment) decreases blood pressure and improves vascular function (Hong *et al.* 2000). Inhibition of the transcription factor NF-κB can, in addition, reduces the extent of cardiac hypertrophy in SHR independently of blood pressure reduction (Gupta *et al.* 2005). Treatment with Tempol, a superoxide dismutase mimetic, abolished the rise in systolic blood pressure normally observed in SHR (Nabha *et al.* 2005). This was further confirmed by a significant over-expression of iNOS in the proximal tubule of the SHR's kidney and a reduction in the antioxidant enzyme glutathione dismutase (Kumar *et al.* 2005).

Although endothelium dysfunction may play an important role in the pathogenesis of SHR, endothelin-1 (a potent vasoconstrictor released by endothelial cells) is not increased in SHR (Schiffrin *et al.* 1995). While the mechanisms of the development of hypertension and of end-organ damage are still being investigated, the SHR has been one of the most widely used genetic models that has contributed to the elucidation of the aetiology of essential hypertension (Pinto *et al.* 1998). This model is currently being used for the identification of quantitative trait loci (QTL) associated with blood pressure regulation (Dominiczak *et al.* 2000). SHR is also used for the generation of congenic lines. Congenic lines are a subset of animals in which a chromosomal region has been substituted by the homologous region of another strain (McBride *et al.* 2004). Linkage with large chromosomal regions is the first step to identify candidate genes and congenic lines allow the identification of more refined chromosomal areas associated with the phenotypic trait of interest (Dominiczak *et al.* 2000). Further investigation of the blood pressure status of these lines reveals information concerning more precise chromosomal region of interest and the identification of QTLs (Dominiczak *et al.* 2000; McBride *et al.* 2004).

A subgroup of SHR is characterised by higher levels of hypertension and their tendency to die of stroke and is termed the stroke-prone SHR (SPSHR) (Sun and Zhang 2005). The phenotype can be accelerated by administration of a high-fat/high-salt diet (Abrahamsen *et al.* 2002). The SPSHR is characterised by hypertension and weight loss. In the kidney, increased levels of the pro-inflammatory cytokine TGF-β1 which up-regulates fibronectin, collagen and plasminogen-activator inhibitor-1, and causes fibrosis in the kidney (Abrahamsen *et al.* 2002) were detected. All these detrimental factors were improved or abolished by concomitant ACE inhibitor or AT_1 antagonist treatment suggesting that the RAS is promoting pro-inflammatory, pro-thrombotic and fibrotic events in the SPSHR. In their studies using the SHR and the SPSHR (crossed with their respective control strains), Dzau *et al.* (1995) used congenic lines and reported a locus for the angiotensin-converting

enzyme linked to salt-sensitive hypertension. Additional loci that co-segregate with hypertension using different crosses of genetically hypertensive strains have been reported [reviewed in (Dzau *et al.* 1995)]. Using the SPSHR and normotensive controls, glutathione-S-transferase (involved in oxidative stress) was identified as phenotypic candidate after congenic lines were produced for linkage analysis followed by genome-wide mRNA expression studies (McBride *et al.* 2004)

1.2.1.2 Lyon Hypertensive rats and Milan Hypertensive rats

Other inbred genetically hypertensive rats have been produced on different strain backgrounds such as the Lyon hypertensive rats (Sassard *et al.* 2003) from Sprague –Dawley stocks and the Milan hypertensive rats (Bianchi *et al.* 1975) strains from Wistar stocks. These two rat models display similar features such as hypertension, cardiac hypertrophy and increased renal vascular resistance. Each strain has a low, normal or high blood pressure sub-group allowing phenotypic comparison.

Lyon rats display salt-sensitive hypertension, decreased lifespan, increased body weight and heart weight, dyslipidemia and increased insulin/glucose ratio (Bilusic *et al.* 2004) which are features common to the metabolic syndrome in humans. Although renal and plasma renin and prorenin levels are reduced in this model compared to normotensive controls, angiotensin-converting enzyme (ACE) inhibition abolishes established hypertension, cardiac hypertrophy and restores the heart to normal physiology (Sassard *et al.* 2003). Early ACE inhibition prevented hypertension and cardiac hypertrophy suggesting that an activated RAS is needed in the developing hypertension phase (Lantelme *et al.* 1997). It has, indeed, been hypothesised that a short-term rise in plasma renin may be involved in early stages of hypertension in the Lyon rats (Sassard *et al.* 2003). Concomitant angiotensin (Ang) II infusion with an ACE inhibitor causes a rise in blood pressure suggesting an increased sensitivity to AngII (Lantelme *et al.* 1997).

Other mechanisms controlling blood pressure in the Lyon and Milan models may be due to activation of local RAS notably in the brain as demonstrated for the deoxycorticosterone acetate -salt model (Itaya *et al.* 1986) or the "Goldblatt" model (Kagiyama *et al.* 2001). Comparison between two strains, the Japanese SHR (and normotensive controls WKY) and the Lyon strains showed that they possess intrinsic differences independent of blood pressure. The SHR/WKY were found to have higher carotid wall stiffness, increased aortic

hypertrophy and levels of collagen and an exaggerated affinity of aortic smooth muscle α-receptors than the Lyon strain (Safar *et al.* 2001). This suggested that genetic factors also play a role in the phenotypes observed.

Post-weaning Milan hypertensive rats (MHR) displayed blood pressure levels 40 to 50 mm Hg higher than in their normotensive controls. Plasma renin activity was lower in MHR. The most striking feature in this model was the renal phenotype. Urine output in MHR was 50% to 100% greater possibly due to sodium retention which was greatly increased compared to control animals during the period when the blood pressure difference developed (Bianchi *et al.* 1975). The authors suggested that the kidney may play a crucial role in the pathogenesis of hypertension in MHR. Further studies revealed that an intrinsic inability of the proximal tubule cells to reabsorb fluid was present as well as a reduced of calcium ATPase activity in the kidney of MHR and may be the cause of the abnormal fluid and sodium handling (Salvati *et al.* 1987). This cellular anomaly was also found in red blood cells which were characterised by smaller volume and lower sodium contents. This impaired mechanism is genetically modified at the stem cell level and, in humans, correlated with a renal phenotype similar to that of MHR (Bianchi *et al.* 1986). The same research group later identified modifications in a cytoskeleton protein, adducin, consisting of three sub-units encoded by three separate genes.

A polymorphism in the α-sub-unit was identified in MHR which correlated with increased blood pressure and a linkage analysis revealed an association between the adducin α-sub-unit polymorphism and hypertension in Caucasian populations (Barlassina *et al.* 1997; Bianchi *et al.* 1994). This polymorphism is thought to influence kidney function through the regulation of ion handling by epithelial tubular cells. More recently, plasma levels of ouabain, a hypothalamic hormone which modulates Na(+)/K(+)-ATPase activity in renal tubular cells, were found to correlate with the number of copies of the mutated adducin α-sub-unit allele in cultured renal cells (Staessen and Bianchi 2005). A competitive inhibitor of ouabain was shown to lower blood pressure in MHR and humans and is being considered in clinical trials.

1.2.1.3 Dahl salt-sensitive rat

Another genetic model of hypertension is the Dahl salt-sensitive rat which was inbred from Sprague-Dawley stocks on the basis of their developing high blood pressure with salt diet (Pinto *et al.* 1998). The rats develop hypertension on normal sodium diet and displayed

increased mortality on high sodium diet. As for the SHR, cardiac hypertrophy (and cardiac failure) and endothelial impairment were present but with more severe renal damage. A close relationship between polymorphism in the atrial natruretic factor (ANF) and angiotensin-converting enzyme (ACE) genes and hypertension in these rats was reported (Deng and Rapp 1992) and links in part the RAS to the pathophysiology of this model. Congenic strains produced with the Dahl salt-sensitive rat have shown that the QTL marked by the inducible form (but not the constitutive form) of nitric oxide synthase (iNOS) is segregating with alleles relating to blood pressure (Deng and Rapp 1995), thus identifying iNOS as a potential candidate influencing blood pressure in the Dahl salt-sensitive rat.

1.2.2 DOCA-salt model

The "pharmacological model" of deoxycorticosterone acetate (DOCA)-salt (known as a salt-retaining hormone and to cause renal tubular enlargement) treatment is another model of hypertension. It was first performed in chicks which were treated with DOCA-salt for 20 days and develop high blood pressure and end-organ damage after chronic administration of DOCA (Selye 1942). The model was characterised by increased water intake, generalised oedema, cardiac hypertrophy, aortic/large vessel thickening due to collagen deposition and increased renal weight. It was demonstrated that, in the DOCA-salt treated rat, plasma renin activity is low (lower than controls), hence constituting a low-renin model of hypertension (Wilson *et al.* 1987). Altered regulation of central sympathetic and pressor systems as well as the brain RAS are involved in the development of hypertension (Gomez-Sanchez *et al.* 1996). In phases of development of hypertension and maintenance of hypertension, intracerebroventricular administration of the ACE inhibitor, captopril, causes suppression of drinking response to hypertension as well as inhibition of vasopressin release and restoration of impaired baroreceptors reflexes (Itaya *et al.* 1986). Central RAS inhibition appears therefore to be involved in the amelioration of blood pressure via action on sodium metabolism and the baroreceptors (Itaya *et al.* 1986). Another hypertensive mechanism in this model and the genetic models described above is endothelial dysfunction. It was shown that in DOCA-salt treated rats, the levels of circulating endothelin-1 are normal but endothelial cells of aortic and mesenteric vessels overexpress endothelin 1 and when SHR are treated with DOCA-salt exacerbation of the phenotype was observed (Schiffrin *et al.* 1995).

1.2.3 Surgical models

The Goldblatt model (Goldblatt *et al.* 1934) is perhaps the oldest model of hypertension. The original model was applied to dogs in which the renal artery was constricted ("clipped") on one side, hence reducing renal blood flow while the other kidney is either left intact (2-kidney/1-clip) or removed surgically (1-kidney/1-clip). The initial "clipping" causes the kidney to respond to the reduced blood flow by producing excessive amounts of renin causing systemic increase in renin levels (Goldblatt *et al.* 1934). This, in turn, leads to an increase in blood pressure mediated by AngII vasoconstriction and fluid retention (Goldblatt *et al.* 1934). The intact kidney is then involved in regulating salt and fluid balance as a result of the unilateral renal artery stenosis. This surgical model has since been applied to various species such as the rat (Abdi and Johns 1996; Morgan *et al.* 2001), the monkey (Panek *et al.* 1991) and the mouse (Wiesel *et al.* 1997). The increased blood pressure and dysregulation of homeostasis is even more marked in the 1-kidney/1-clip model since the intact kidney is not present to compensate for the "clipped" kidney. Berg *et al.* reported that the blood pressure, renin-angiotensin system (RAS) activation and fluid homeostasis is returned to normal when the constricted renal artery is "unclipped" (in the 2-kidney/1-clip) in rats (Berg *et al.* 1979). It therefore provides a reversible (although surgical) model of high renin and renovascular hypertension which mimics the equivalent human condition of renal artery stenosis.

The 2-kidney/1-clip animals are characterised by increased renin release in the circulation and increased plasma renin activity leading to increased AngII production. This causes vasoconstriction, cardiac hypertrophy and, in the kidney, increased natriuresis, hence this model constitutes, not only a high renin model but also a volume depleted state (Pinto *et al.* 1998). Administration of a renin inhibitor returns mean arterial pressure and plasma renin activity to normal (Panek *et al.* 1991). Moreover, the effects of AngII were shown to be attenuated by injection of the RNA anti-sense to the angiotensin I receptor type 1 (Galli and Phillips 2001), confirming the involvement of the RAS in the pathology of the 2-kidney/1-clip animals. It is worth noting that, after the initial phase of RAS activation and hypertension in the dog, the blood pressure can decrease due to collateral vessel development (Watkins *et al.* 1976). In the 2-kidney/1-clip monkeys, the blood pressure remains elevated after 24 weeks (Panek *et al.* 1991) and in the 2-kidney/1-clip rat, it has been shown that, although the peripheral (circulating) RAS returns to normal, the blood pressure remains elevated for several months (Kagiyama *et al.* 2001). This is dependent on activation of a central RAS which was inhibited by intracerebroventricular injection of anti-

sense RNA for angiotensin I receptor type 1 (Kagiyama *et al.* 2001). The 2-kidney/1-clip model is known to be sensitive to sodium diet modulation, *i.e.* blood pressure decreases on low sodium diet (Leenen and de Jong 1975) which may be the result of renal or central control over sodium balance.

1.2.4 Transgenic models

Transgenic models have become widely used in the field of cardiovascular science and several rat lines have been generated in order to study the involvement of renin-angiotensin system (RAS) genes and pathways in the pathophysiology of hypertension.

1.2.4.1 Human AOGEN x human renin transgenic rats

Two normotensive transgenic rat lines were produced, one carrying the human angiotensinogen gene (*hAOGEN*) and the other harbouring the human renin gene (*hRen*) (Ganten *et al.* 1992). Plasma human AOGEN was increased in line TGR(*hAOGEN*) with expression of *hAOGEN* in liver, kidney, brain, lung, heart, and the gastrointestinal tract. The TGR(*hRen*) line had increased human renin in the kidney after sodium depletion. In addition, human renin infusion in TGR(*hAOGEN*) also triggered an increased in blood pressure which was inhibited by a human renin inhibitor. When the same renin inhibitor was added to plasma samples of sodium depleted double transgenic animals, the human renin activity was inhibited but not the rat renin activity and rat renin infusion in TGR(*hAOGEN*) triggered an increased in blood pressure which was not inhibited by the hRen inhibitor. This study demonstrated the species specificity of renin for its substrate angiotensinogen. However, the double transgenic animals did not develop hypertension. A second double transgenic line was produced using the original *hAOGEN* animals and a new *hRen* line (Bohlender *et al.* 1997) but no information has been published regarding this new line. The new double transgenic line has also been shown to succumb to end-organ damage affecting mainly the heart and the kidney (Luft *et al.* 1999). Infiltration of inflammatory cells in the vascular wall precedes fibrinoid injury. The authors demonstrated that blood pressure reduction was achieved with both ACE inhibitors and angiotensin receptor inhibitors but only partly with a renin inhibitor. The latter, however, prevented the development of vascular injury as did endothelin receptor blockers.

Using blood pressure lowering drugs that do not interfere with the RAS (such as the vasodilator hydralazine), it was later demonstrated that the role of the RAS in the inflammatory pathways responsible for end-organ damage in this model is independent of hypertension (Mervaala *et al.* 2000). This double transgenic model was also used to study the effects of endothelin-1 (ET_1) and AngII in the phenotype of this model (Gerbaulet *et al.* 2005). Sub-pressor doses of angiotensin receptor type I (AT_1) inhibitor and endothelin receptor A (ET_A) blocker did not affect the hypertension in these rats but a combination of both agents did. Similarly, coronary artery remodelling, cell proliferation and inflammation were reduced by both treatments and in combination showing that AngII is involved in all these processes and there is a synergistic action of both AngII and ET_1 in the pathogenesis of malignant hypertension. This model has also been proposed as a model of gestational hypertension as pregnant TGR(*hAGT*) developed hypertension in late gestation when mated with TGR(*hRen*) males (Bohlender *et al.* 2000). Blood pressure returned to normal after delivery but the offspring developed the phenotype described above. The data suggested that placental tissue of foetal origin was capable of expressing human renin and of converting human AOGEN of maternal or foetal origin.

1.2.4.2 TGR(mRen2)27

TGR(*mRen2*)27 are transgenic rats carrying a transgene containing the mouse *Ren2* gene (Mullins *et al.* 1990). They display fulminant hypertension from 5 weeks of age (230mmHg) and the animals suffer from malignant hypertension (MH) injuries leading to heart failure unless treated with ACE inhibitors.

The TGR(*mRen2*)27 animals displayed suppressed concentrations of active plasma and renal renin, low or unchanged levels of plasma AngII but increased levels of circulating prorenin compared to controls (Lee *et al.* 1995). The highest expression of the transgene was found in the adrenal gland as demonstrated by adrenalectomy which reduces plasma prorenin by 80% and returns blood pressure to normal (Tokita *et al.* 1994). These findings underline the importance of the RAS and local RAS and, in particular, of prorenin in the development of hypertension and hypertensive injuries in these animals. As discussed in the double transgenic rat model (Luft *et al.* 1999), some treatments such as hydralazine (a vasodilator) have been shown to reduce the blood pressure without preventing/attenuating the vascular and organ injuries present in these models suggesting other mechanisms such as activation of

the RAS plays an important role in the pathology observed (Pinto *et al.* 1998). In TGR(*mRen2*)27, ACE inhibitors (*e.g.* quinapril) and angiotensin receptor antagonists (*e.g.* losartan) have hypotensive effects accompanied by a decrease in the severity of end-organ damage (Teisman *et al.* 1998). Furthermore, there is an indication that these hypertensive agents can prevent end-organ damage when administered in sub-pressor doses, again showing dissociation of hypertension from end-organ damage (Linz *et al.* 1989). In addition, Jacinto *et al* demonstrated that impaired peripheral and intrarenal responses to endothelium-dependent vasodilators may be responsible for an exaggerated peripheral and renal response to the vasoconstriction effects of AngII (Jacinto *et al.* 1999). Increased sensitivity to AngII was also demonstrated in the genetic low-renin Lyon hypertensive rat model (Lantelme *et al.* 1997).

Crosses of TGR(*mRen2*)27 to other rat strains have showed that malignant hypertension is linked to the strain genetic background, TGR(*mRen2*)27 rats on a Fischer (F344) background displaying the most extensive injuries whereas TGR(*mRen2*)27 rats on a Lewis background had the least damaging lesions (Mullins and Mullins 2003). Linkage analysis has shown that factors on chromosome 10 and 17 close to the ACE and AT_1 loci are linked with the malignant hypertension phenotype in the TGR(*mRen2*)27 animals (Kantachuvesiri *et al.* 1999).

1.2.4.3 Other "prorenin" rat transgenic models

Another transgenic rat model was generated to study the effects of exaggerated circulating rat prorenin by directing expression of a rat prorenin-containing transgene to the liver (Veniant *et al.* 1996). Plasma prorenin levels were similar to the previous model but plasma renin levels were normal. The animals, however, did not display elevated blood pressure until after the renal and cardiac lesions were well established, indicating that prorenin may be involved in the MH lesions independently of blood pressure. An inducible rat model of hypertension TGR(*Cyp1a1-Ren2*) was recently developed in this laboratory based on *Ren-2* transgene used in TGR(*mRen2*)27 animals. In this instance, the transgene was under the control of cytochrome P450 *Cyp1a1* promoter (Kantachuvesiri *et al.* 2001). The promoter is induced when the animals are fed a diet containing the natural xenobiotic indole-3-carbinol (I3C) which allows discreet and precise control over the onset of hypertension. The expression of the transgene is detected 24h after induction (0.3% I3C in food) in the liver. Upon chronic induction, TGR(*Cyp1a1-Ren2)* animals displayed a rapid increase in BP

accompanied by weight loss and polyuria, together with an activated circulating RAS (in particular prorenin) and malignant hypertension lesions. This model has been further characterised haemodynamically and is described with all the other "prorenin" transgenic animals in *Chapter 4*. Using the advantages of this inducible model, congenic lines carrying the *Ren2* transgene and the region of chromosome 10 associated with blood pressure derived either from the Lewis and the Fischer (F344) are currently being developed (Mullins and Mullins 2003).

The renin-angiotensin-aldosterone system plays an important role in regulating blood volume, arterial pressure, and cardiac and vascular function. The RAS is a pivotal circulating component of homeostatic regulation and in addition pathways for the RAS have been found in a number of tissues. In addition, AngII has been shown to be a reproductive and growth hormone. Hence, the RAS plays a crucial role in normal physiology and is involved in the pathogenesis of hypertensive disease which explains why a large body of work, including this thesis has focussed on the study of its regulation in a variety of biological systems.

1.3 The Renin-Angiotensin-Aldosterone System

AngII, the resulting peptide and effector molecule of the RAS cascade, possesses a variety of actions such as resistance vessels constriction thereby increasing systemic vascular resistance and arterial pressure, adrenal cortex stimulation to release aldosterone, which in turn acts upon the kidneys to increase sodium and fluid retention, triggering the release of anti-diuretic hormone from the posterior pituitary which acts upon the kidneys to increase fluid retention, stimulation of thirst response within the brain, facilitation of norepinephrine release from sympathetic nerve endings and inhibits norepinephrine re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function and causing cardiac and vascular hypertrophy.

AngII, a potent vasoconstrictor peptide, is produced from the initial cleavage of angiotensinogen following its release from the liver by renin in the circulation. Renin and its secretion are therefore major determinants (and limiting factors in humans) of the RAS. The product, angiotensin (Ang) I is, in turn, cleaved by the angiotensin-converting enzyme (ACE - located on the luminal surface of the vascular endothelium in the lungs) forming AngII (Figure 1.2). AngI can also be converted to AngII by ACE-independent mechanisms such as tissue plasminogen activator, cathepsin G or chymase (Johnston and Risvanis 1997). AngII,

itself, plays an important role in the regulation of blood pressure, volume homeostasis, salt balance and also tissue remodelling. The effects of AngII occur *via* binding to its receptors, named angiotensin receptor-type 1 (AT_1) and angiotensin receptor-type 2 (AT_2) to cause increased sodium and water retention in the proximal renal tubule, increased aldosterone release from the zona glomerulosa of the adrenal gland, itself causing increased sodium and water retention in the distal renal tubule and vasoconstriction (Campbell 1987). Embryonic development, notably in the vasculature and the kidney, is known to be dependent, in part, on the renin-angiotensin system (RAS). During development all components of the RAS are present, although appearing at different stages of development. Angiotensin (Ang) II is a well-recognised reproductive (Speth *et al.* 1999) and development hormone (Tebbs *et al.* 1999). The respective contribution of the maternal RAS and the foetal RAS to embryonic development is not fully understood.

Knock-out models of components of the RAS, namely angiotensinogen (AOGEN), renin, angiotensin-converting enzyme (ACE) and angiotensin receptors have provided useful information regarding the importance of the RAS in development. The kidney appears to be a primary target for abnormalities. These could be caused by a variety of mechanisms relating to maternal and/or foetal blood pressure regulation, the absence of renin-expressing cells in the foetus or the decreased availability of AngII in the mother, the foetus and/or the uteroplacental complex. Although the deformities in the RAS knock-out animals are very severe, the results from these animals have revealed hidden roles for the RAS in embryogenesis. It raises the possibility that, during gestation, modulations in the RAS by water and electrolyte imbalances (Ross *et al.* 2005), dietary modifications such as maternal undernutrition (Kwong *et al.* 2000; Langley-Evans and Jackson 1996), salt intake (Lawlor and Smith 2005) and increased maternal blood pressure may have lasting effects on the progeny, notably in terms of predisposition to hypertension (Vehaskari *et al.* 2004; Woods *et al.* 2001).

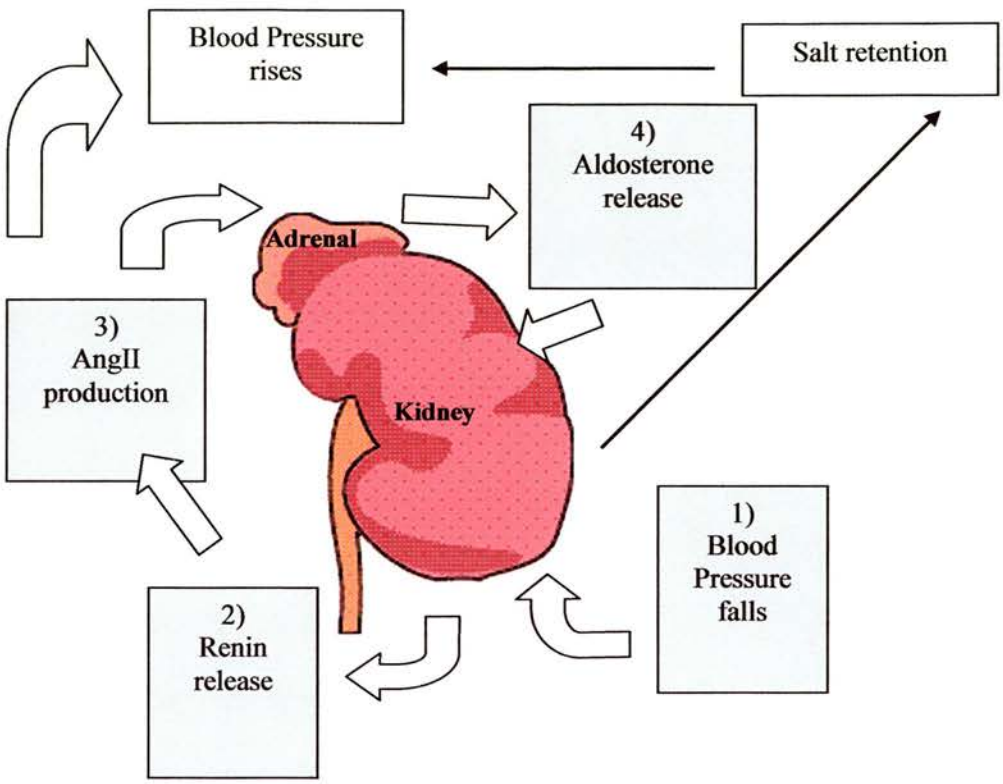


Figure 1.2: The renin-angiotensin-aldosterone system. The diagram depicts the classical roles of the circulating RAS in blood pressure regulation (adapted from Campbell *et al.*, 1987)

1.3.1 Renin and prorenin

1.3.1.1 Synthesis

Renin is an aspartyl protease that functions to convert its only known substrate, angiotensinogen (AOGEN) producing the decapeptide AngI by proteolytic cleavage at the N-terminus (Poulsen and Jacobsen 1993). It was first reported, in 1971, that plasma and amniotic fluid contained more renin after dialysis to pH 3.3-3.6 (Lumbers 1971) and after treatment with proteolytic enzymes (Morris and Lumbers 1972), respectively. The authors postulated that renin may therefore first exist as an inactive pre-pro-enzyme activable by low pH and proteolysis. Poulsen *et al.* reported that renin is synthesized as a pre-pro-enzyme of 50kDa (Poulsen *et al.* 1979) using a cell-free

translation system whereas renin of 40kDa size was purified from the submandibular gland (SMG) (Nielsen *et al.* 1979). This confirmed the idea that renin was first produced in a precursor form. In addition, Nielsen *et al.* reported that renin from the submandibular gland is not stored nor secreted in an inactive or high molecular weight and that the only form of renin in this organ is the fully active 40kDa enzyme. High molecular weight "renins" have also been reported in the plasma and may represent renin bound to plasma lipoproteins or serine protease inhibitors such as pepstatin (Nielsen *et al.* 1979). The reasons for the discrepancy in molecular size compared to Poulsen *et al.* and Nielsen *et al.* are unclear and may be due to the methodological differences.

Further work on the biosynthesis of the mouse renin was performed by Catanzaro and colleagues who reported that mouse renin translated from mRNA (selected using a renin cDNA) was a 45kDa protein (Catanzaro *et al.* 1983). Similarly, the presence of a translation product from SMG of 45kDa in size, in mice characterised by high renin levels was reported (Piccini *et al.* 1982). The authors showed that renin from the SMG was produced in amounts between 50- to 100-fold higher than in the kidney in "high renin" animals, that it was under testosterone-control and that it is produced from a second renin gene (Piccini *et al.* 1982). Preprorenin was converted to the 43kDa prorenin which, in turn, was converted rapidly to a 38kDa single chain renin (Catanzaro *et al.* 1983).

This renin form could be hydrolysed to 33kDa and 5kDa chains linked by disulphide bonds (Figure 1.3). This is in accordance with the estimation of Misono *et al.* based on the amino-acid sequence (Misono *et al.* 1982). In addition, the light chain (5kDa) was found to provide enzymatic stability (Pratt *et al.* 1983). A similar pathway is present in humans but the heavy and light chains have molecular weights of 22kDa and 18kDa, respectively (Do *et al.* 1987). It was also reported that only the one- and the two-chain renin (but not prorenin) have enzymatic activity (as quantified by binding to pepstatin) (Pratt *et al.* 1983). This is in contradiction with previous publications [reviewed by Skinner (Skinner 1987)] reporting a "renin-like" activity of prorenin, albeit slower.

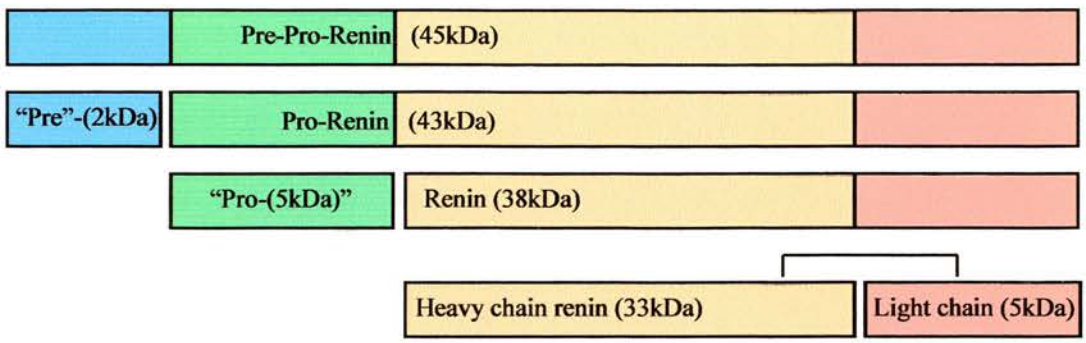


Figure 1.3: Processing of mouse pre-prorenin. As described in text, the "pre" signal is removed to give rise to prorenin which in turn can be proteolytically resulting in removal of the prosegment. The resulting renin is 38kDa in molecular weight and can be slowly hydrolysed to 2-chain renin in which the 2 sub-units are linked by a disulphide bridge.

1.3.1.2 Maturation of prorenin and processing of renin

Using anti-sera corresponding each to the N-terminus, the C-terminus and the middle portion of the prosegment, Taugner *et al.* (1987) determined the fate of human prorenin during granule secretion. The prosegment was demonstrated to be cleaved off step by step: the N-terminus is removed in the Golgi whereas the middle portion and C-terminus are cleaved off in the endoplasmic reticulum (Taugner *et al.* 1987). Both in humans and mice, the resulting prorenin can then either be: a) secreted constitutively, b) packaged into low density granules ("juvenile") or, c) converted intracellularly to active renin (40kDa) and stored in dense core/mature granules before secretion when required (Friis *et al.* 2000). Compared with mature granules, juvenile granules were found to have a lower fusion ability and therefore be less likely to undergo exocytosis (Taugner and Metz 1986).

After prorenin secretion, the mechanisms of downstream prorenin activation/conversion are, to date, unclear. In the plasma, in the presence of protease inhibitors, the conversion of prorenin is much slower than in the kidney (Leckie and McGhee 1980) but candidate enzymes for prorenin activation include cathepsin D and G, acid protease and kallikrein. Cathepsin B, which co-localises with renin into storage granules may be responsible for the conversion of prorenin (Taugner *et al.* 1985) but the mechanisms are still unclear.

Evidence is accumulating in favour of an additional pathway of non-proteolytic activation of prorenin. Prorenin is now known to be activated without changes in molecular weight and with the ability to return to an inactive conformation by trypsin or acid treatment (Derkx *et al.* 1987). Non-proteolytic activation of prorenin (Figure 1.4) may play an important role in the normal physiology, notably during gestation since high levels of prorenin are detected in the amniotic fluid, the role of which is unknown. It may be a crucial element in the pathophysiology of high prorenin states such diabetes and in animal models characterised with high circulating and local prorenin such as TGR(*mRen2*)27 transgenic animals and the diabetic streptozotocin-treated rats.

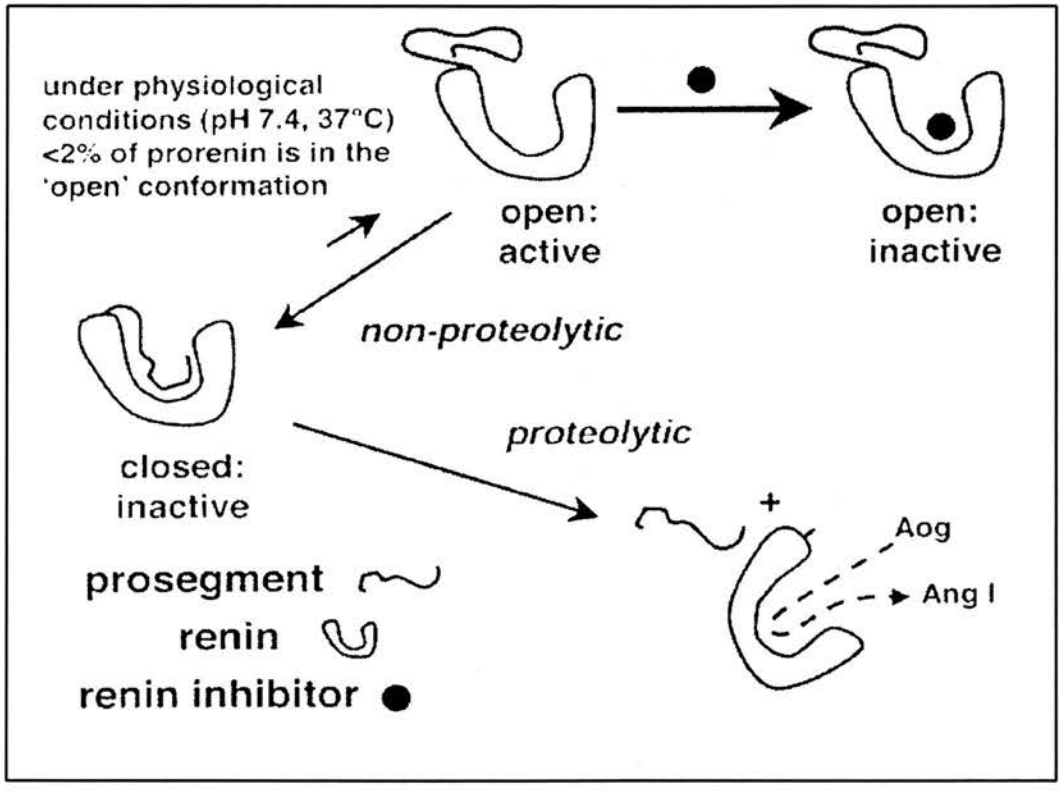


Figure 1.4: Schematic representation of prorenin activation pathway. The predominant pathway of prorenin activation is by the removal of the prosegment as found in immature and juvenile secretory granules of the juxtaglomerular cells leading to the formation of renin. Other pathways may be responsible for proteolytic activation of prorenin in the plasma but these are, to date, unclear. A new non-proteolytic activation of prorenin has been identified and may involve binding to a receptor. Although not depicted in this cartoon, non-proteolytically activated prorenin may be able to convert AOGEN into AngI. Aog = angiotensinogen. Reproduced from (Danser and Deinum 2005).

The physiological mechanisms of prorenin non-proteolytic activation, however, remain unclear but it has been shown that an antibody corresponding to the N-terminal of prorenin induces activation (Suzuki *et al.* 1999) and the rate of prorenin activation is determined by this sequence (Suzuki *et al.* 2000). In addition, Suzuki *et al.* identified two key regions in the prosegment for non-proteolytic conversion using two antibodies: the first one blocked the re-inactivation of acid-activated prorenin and the other non-proteotycally activated prorenin (Suzuki *et al.* 2003).

1.3.1.3 Juxtaglomerular cells

Renin is mainly released by a discreet renin-secreting population of cells of the juxtaglomerular apparatus (Figure 1.5). The smooth muscle cells of the afferent arterioles are capable, under stimulation (*e.g.* sodium concentration decrease sensed by the macula densa) to undergo reversible differentiation (Taugner *et al.* 1984). The renin-secreting population extends to the pre-glomerular portion of the afferent arteriole (Hackenthal *et al.* 1990). These cells, called epitheloid cells, situated in the media of the afferent arterioles extend in varying length depending on renin production/secretion stimulation.

In addition, there is heterogeneity between arterioles, not all of them containing granulated renin-producing cells. Furthermore, Hackenthal *et al.* (1990) reviewed the mechanisms of renin secretion and reported that, along the afferent arterioles, smooth muscle cells at varying stages of metaplastic differentiation can be observed. Therefore, close to the macula densa the cells are "plump" in shape with many dense core granules and have lost their contractility and downstream from the macula densa, the cells become gradually more spindle-shaped, have less or no dense core granules (and only "immature renin granules") and are more contractile (Hackenthal *et al.* 1990).

The formation of renin granules in JG cells is described in *Chapter 7*. Renin release from granules in JG cells can be stimulated or inhibited by a number of stimuli. Although, an increase in intracellular calcium has been shown to inhibit renin secretion from JG cells, it is generally a triggering factor for the release of other enzymes contained in secretory vesicles. Lysosomal enzymes such as cathepsins are present in secretory granules and co-localise with renin (Taugner *et al.* 1985). Although renin granules are acidic, they may however lack the necessary machinery for calcium-associated exocytosis. This is why renin granules are often referred to as "modified lysosomes".

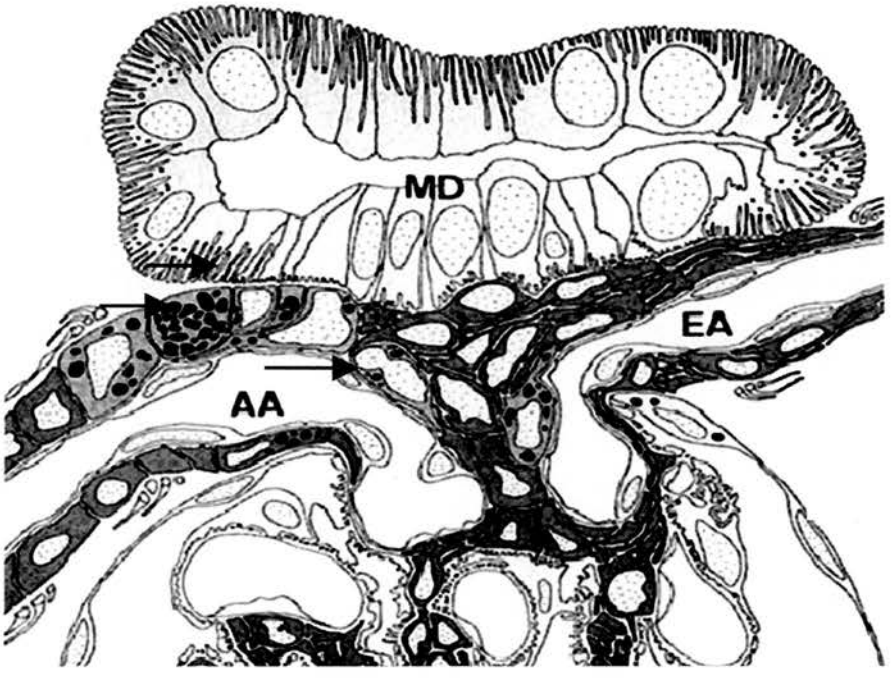


Figure 1.5: Juxtaglomerular apparatus. The juxtaglomerular apparatus is made up of the afferent arteriole (AA), efferent arteriole (EA) and macula densa (MD). JG cells are located in the media of the afferent arteriole (arrows) and contain dense core renin granules. Reproduced and adapted from (Hackenthal *et al.* 1990).

The developmental occurrence of renin-expressing cells has been shown to start at E13 in the mouse (Kon 1999). During embryogenesis, some renin-expressing cells are present prior to vessel formation in the loose renal mesenchyme (Gomez *et al.* 1990b). Renal granulated cells, possibly containing renin have been found in species of fish which do not possess a macula densa such as chondrichtyes (Wilson 1984). This is also observed in the zebrafish where mesonephric cells are present before macula densa cells appear (Liang *et al.* 2004). These findings suggest that renin has a physiological role in development separate from blood pressure regulation. Renin expression in the developing mouse kidney, however, essentially follows the formation of the arcuate and interlobar arteries [(Mullins *et al.* 2000), Figure 1.6] suggesting a physiological role of renin in renal development notably in vascular branching before blood pressure regulation. At embryonic day 17.5 (E17.5), renin expressing cells are found within the afferent arteriole wall and then become a discreet

population localised to the JG location in the adult as described and illustrated above. A similar pattern is observed in the rat (Gomez and Norwood 1995) and in both rats and mice, renin mRNA is developmentally regulated, its expression being higher in the foetus and the newborn than in the adult (Gomez and Norwood 1995).

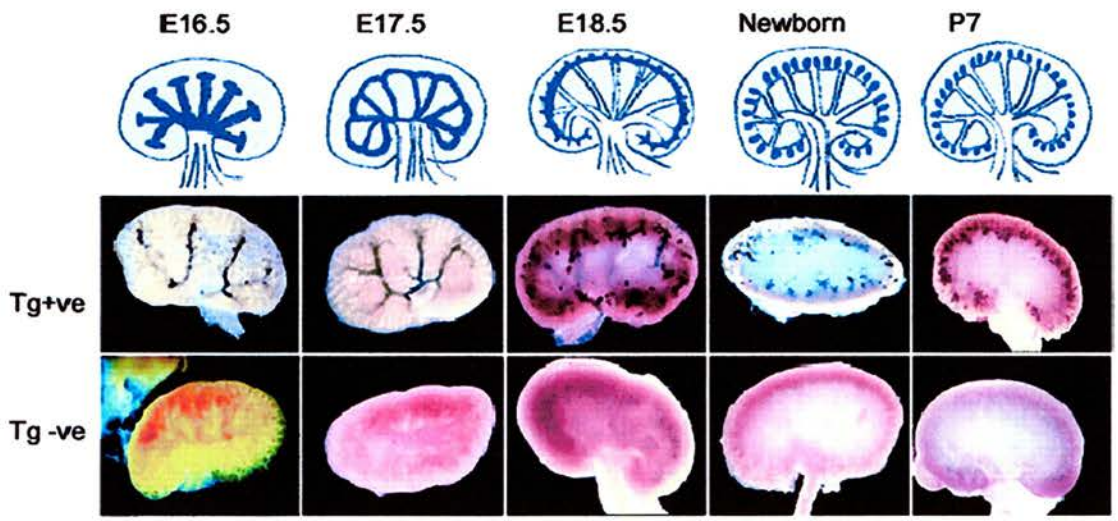


Figure 1.6: Development pattern of renin expression using a transgenic LacZ-expressing mouse line. Renin is first expressed in the large arcuate arteries and interlobar arteries and is gradually shifted outwards during the peri-natal period to reach a discreet localisation in the juxtaglomerular apparatus. Reproduced and adapted from (Mullins *et al.* 2000).

JG cells are considered to be terminally differentiated from SMC since they possess SMC characteristics and produce the hormone renin. It has been shown that when homeostasis is threatened (as it may happened during haemorrhage or hypotension), smooth muscle cells of the renal arterioles can be recruited and they undergo reversible metaplastic differentiation (Gomez *et al.* 1990b; Pentz *et al.* 2001). Reversible metaplastic transformation allows production of renin in response to those physiological changes (Figure 1.7). Hence, the number of renin-positive cells along the afferent arterioles varies. This allows "extra" renin-producing cells to be present as to trigger the RAS cascade leading to the production of AngII and restoration of normal blood pressure. Whether these cells return to their original smooth muscle cell-only characteristic is unclear. Using a GFP-*Ren1* reporter system, Sequeira-Lopez *et al.* (2004) followed the fate of

renin-expressing cells from their embryonic location to their recruitment under salt depletion conditions. The authors demonstrated that it was the same renin-producing cells which line the afferent and efferent arterioles during nephrogenesis which become SMC but retain the "memory" of becoming renin producing when needed (Figure 1.7). Specific ablation of renin-expressing cells by placing the diphteria toxin A chain under the control of the *Ren1*^{d} promoter in two renin-gene mice (Pentz *et al.* 2004) showed that renin cells *per se* are involved in renal development. Although renin-expression was normal in the submandibular gland (due to *Ren2* expression), renin expressing cells were not seen in the kidney. Homozygous animals displayed phenotypic renal abnormalities mainly associated with tubular (dilation and atrophy) and glomerular (hyperplasia or atrophy) morphology.

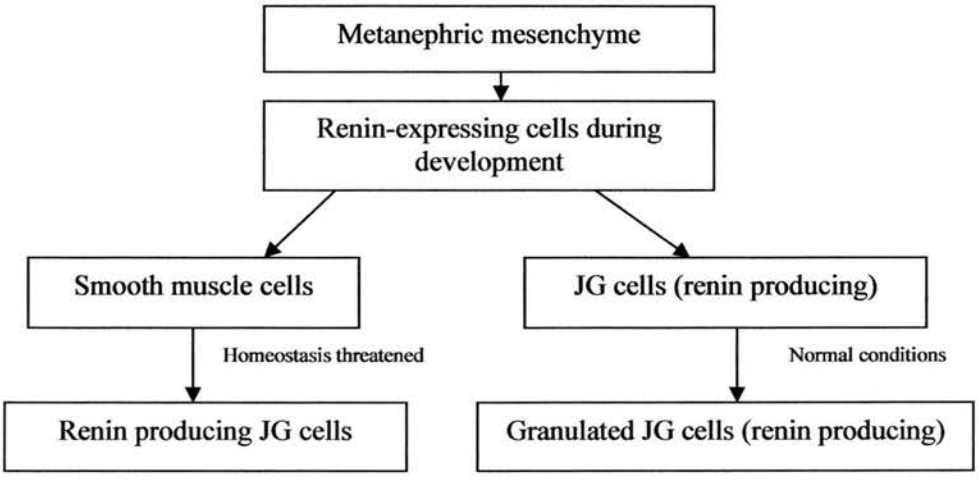


Figure 1.7: Fate of renin producing cells. Schematic representation of the fate of JGC and SMC during development and during episodes of RAS activation.

The presence of renin granules pre-natally is still a matter of debate. Post-natally, the presence of renin granules is unequivocal (Bruhl *et al.* 1974; Egerer *et al.* 1984). However, few reports demonstrate the presence of renin granules in JG cells during renal development. Considering renin dense core granules only contain active renin, it may be of interest to clarify whether renin granulation (and foetal active renin production) indeed occurs pre-natally in the mouse.

Could it be that renal prorenin has a direct role in early renal development? Would pharmacological or dietary changes which modulate renin and prorenin expression/processing

have an effect on embryonic development? The stimuli involved in the formation of granules were studied using ACE inhibition and dietary sodium modifications during pregnancy in *Chapter 7*. Is their a role for a (pro)renin receptor/non-proteolytic conversion of prorenin in these events?

1.3.1.4 Renin Secretion

The release of active renin from JG cells is controlled by several distinct mechanisms:
a) intra-renal receptors, namely baroreceptors, which sense changes in arteriolar pressure and cell volume and the macula densa which senses changes in sodium chloride delivered to the distal nephron (Schnermann 1998). This process appears to be a new mechanism in evolutionary terms. Species of fish (*e.g.* teleost fish) which have plasma and kidney renin activity do not possess a macula densa suggesting a fairly recent acquirement of tubulo-glomerular feedback regulation (Wilson 1984).
b) *via* sympathetic nervous system action. The β-adrenoreceptor activation under decreased blood pressure conditions, for example, triggers the stimulation of sympathetic nerves or the release of catecholamines inducing JG renin release (Hackenthal *et al.* 1990),
c) humoral factors such as prostaglandins and catecholamines can stimulate the release of renin whereas AngII, aldosterone and ANF (in part due to a negative feedback loop) inhibit it (Hackenthal *et al.* 1990),
d) intracellular second messengers are released as a response to all the above stimuli and either induce (*e.g.* cyclic AMP) or suppress (*e.g.* Ca^{2+}) the release of renin (Kurtz and Wagner 1999).
Increased blood pressure and AngII can increase intracellular calcium secretion, an event known to trigger the release of material stored in secretory granules. This, however, does not apply to the release of renin from JG cells since it is prevented by increased levels of circulating AngII as part of a negative feedback loop. This phenomenon has been referred to as the "calcium paradox". Adrenocortical cells of TGR(*mRen2*)27 rats which express the mouse *Ren2* gene in the adrenal gland (and in tissues normally expressing *Ren2* in mice) can be stimulated by the calcium ionophore A23187 (Peters and Clausmeyer 2002). This demonstrates that adrenal renin secretion is under differential regulation than that of the JG cells. The exact cellular events governing the release of renin from JG cells are not fully understood but electrophysiological studies have shown that exocytosis involved in this process (Friis *et al.* 2000).

1.3.1.5 Expression and localisation in extra-renal tissues

As mentioned above, the main site of renin release is the kidney. In humans, the kidney is the only known organ to release active renin and extra-renal sites of renin production secrete prorenin exclusively (Sealey and Rubattu 1989). However, some non-renal renin producing tumours have been described (Atlas *et al.* 1984). In addition, in the mouse, active renin has been found to be released by the submandibular gland (Catanzaro *et al.* 1983) and by the adrenal gland (Berka *et al.* 1996; Deschepper *et al.* 1986; Inagami *et al.* 1989). It is the detection of high levels of renin in the mouse sub-maxilliary gland (SMG) which allowed the purification of the aspartyl protease (Cohen *et al.* 1972). Deschepper *et al.* (1986) used a combination of immunocytochemistry and *in situ* hybridisation (ISH) to determine renin expression and protein location in several mouse tissues: in the kidney, both renin protein and mRNA were present at the vascular pole of the glomerulus, in the adrenal the outer layer of the cortex showed the strongest antibody and riboprobe signal suggesting that in these tissues transcriptional events and protein processing occur at the same sites and in the testis, renin mRNA and protein were localised to the cytoplasm of the Leydig cells. In the pituitary, the ISH signal was identified in both the intermediate and anterior lobes while the protein was exclusively found in the anterior lobe. Although controversial, this suggests that renin may be taken up/trafficked to the anterior lobe from the intermediate lobe (Deschepper *et al.* 1986).

In addition to the kidney, sites of prorenin production include the adrenal, the pituitary, the reproductive organs and the uteroplacental complex, namely myometrium, placenta, and chorio-decidua (Berka *et al.* 1996; Carretero *et al.* 1972; Lumbers 1993; Nakayama *et al.* 1989; Poisner 1998; Skinner 1987). These tissues have been shown to contain renin and prorenin but it is unclear whether prorenin produced at those sites is converted to renin and released in the circulation or taken up as prorenin prior to conversion in tissues. The direct actions of prorenin have been a recent area of research and there has been a controversy about the existence of a (pro)renin receptor. This may help to elucidate the functional role of prorenin and why prorenin is the only "renin species" present in several extrarenal tissues. Another example of prorenin localisation is in the amniotic fluid which contains high amounts of prorenin (Broughton-Pipkin 1993; Lumbers 1993). Several possibilities for the origin of amniotic fluid prorenin have been put forward such as the chorion, the foetal kidney/urine, maternal circulation or the maternal ovaries but the source of amniotic fluid prorenin and its role in pregnancy/embryonic development is still unclear.

1.3.1.6 Renin genes

Phylogenetic studies identified two groups of mice, the first group produced low levels of "thermostable" renin (*i.e.* active renin) in the submandibular gland (SMG) and the second group secreted high levels of "thermolabile" renin [(*i.e.* prorenin); (Wilson and Taylor 1982)]. This was originally thought to be arising from a single renin gene with different regulatory elements. However, the identification and cloning of two distinct renin genes were performed by Southern blotting using a renin cDNA from DBA/2 mouse submandibular gland as a probe (Mullins *et al.* 1982). It was also discovered that SMG renin levels were genetically controlled and that high and low SMG renin levels correlated with the number of renin genes (Piccini *et al.* 1982; Wilson and Taylor 1982). Whereas humans, rats and most strains of mice possess only one renin gene (denoted $Ren1^c$ for mice), some strains of mice carry two renin genes ($Ren1^d$ and *Ren2*). This was found to be unique to mouse and due to a gene duplication on chromosome 1 and not due to chromosomal rearrangement. It has been estimated that the *Ren1* gene duplication event occurred 2.75-5.5 million years ago but it cannot be ruled out that $Ren1^c$ mice were the result of an ancestral duplication followed by gene deletion (Dickinson *et al.* 1984). It was also demonstrated that there is a sexual dimorphism and that renin from SMG is synthesized in greater amounts in male and testosterone-treated female mice carrying two renin genes (Catanzaro *et al.* 1983; Piccini *et al.* 1982).

Although only renin activity derived from *Ren2* has been detected in the SMG of two-renin gene strains of mice, $Ren1^c$ transcript and isozyme activity has been measured (although at lower levels than *Ren2* in two-renin gene strains) in the SMG of one-renin gene strains of mice (Wilson and Taylor 1982). After mouse kidney cDNA library screening, *Ren1* was first found to be present at higher levels that *Ren2* in the kidney (Field *et al.* 1984). However, subsequent work showed that, in the kidney, both *Ren2* and $Ren1^d$ transcripts were present in equivalent amounts and when the transcript abundance was normalised to the number of renin-producing cells, *Ren2* mRNA expression was found to be equivalent in kidney JG cells and in SMG granular convoluted tubule cells (Field and Gross 1985). The authors hypothesized that elevated expression in the SMG of two-renin gene mice may not be due to gene upregulation but to the inability to repress *Ren2* gene expression. The mouse renin genes span 13kB containing 9 exons and 8 introns and all three murine renins share 97% homology at the protein levels (Figure 1.8).

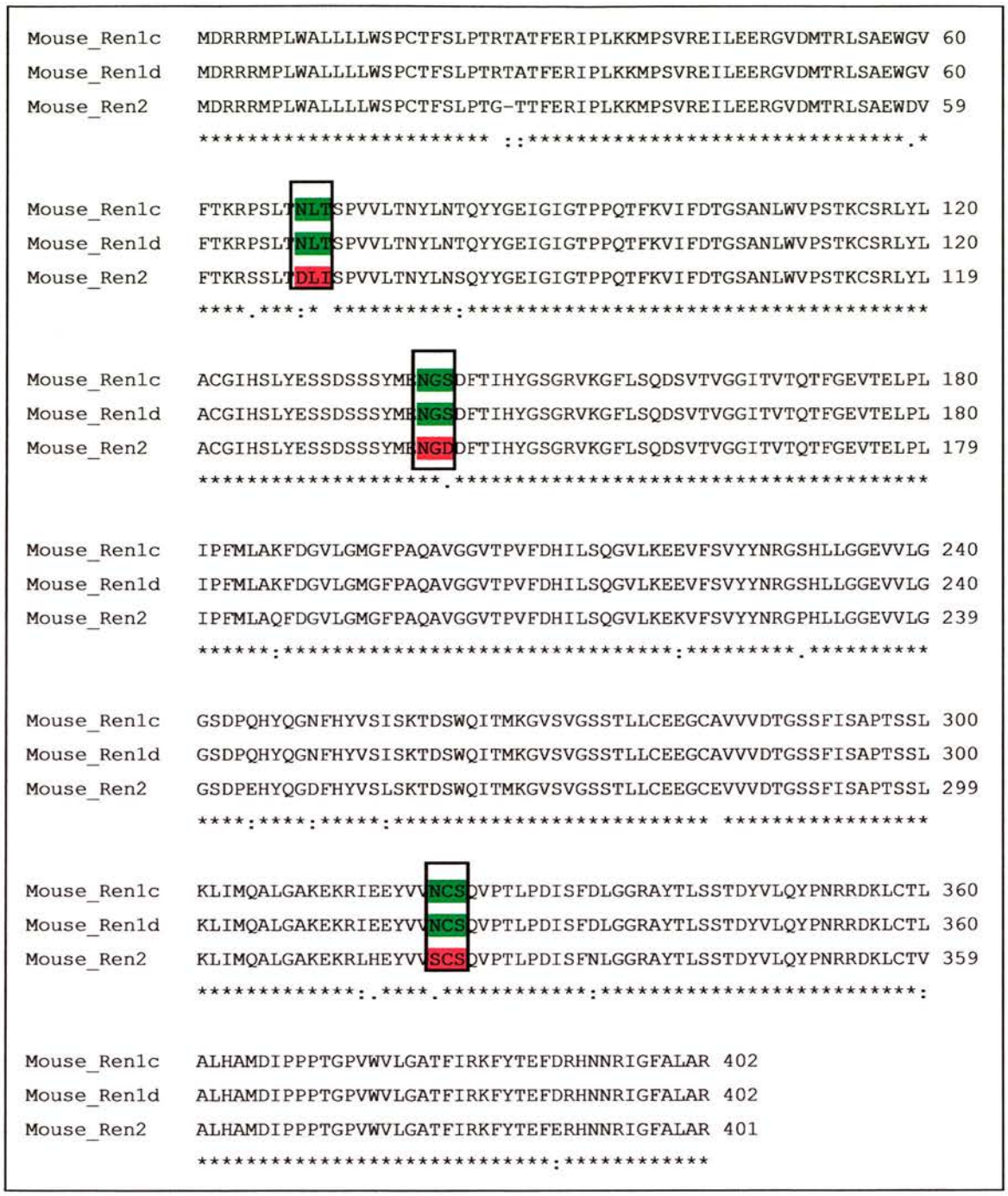


Figure 1.8: CLUSTAL alignment of amino acid sequence for all three murine renin protein from renin genes *Ren1*^{c}, *Ren1*^{d} and *Ren2*. N-linked glycosylation sites are boxed and are only present in "*Ren1*" proteins. Note the high degree of homology between the three amino-acid sequences. "*" = identity, ":" = conserved substitution, "." = semi-conserved substitution.

Renin proteins show differences at 3 asparagine-linked glycosylation sites found exclusively in proteins arising from the "*Ren1*" genes. The modifications at the gene level were found to be lacking in a wild type strain of mice which carries two renin genes suggesting that the duplication of the *Ren* locus occurred prior to glycosylation site insertions (Sigmund and Gross 1991). Genotypic differences between "*Ren1*" genes and the *Ren2* gene may be involved in the sorting of prorenin into the regulated pathway. *Ren1*^{d} and *Ren2* are separated by 21Kb and share the same transcription direction with *Ren2* upstream of *Ren1*^{d} (Sigmund and Gross 1991). While *null* mutants for the *Ren2* gene displayed no histopathological injuries and reduced plasma prorenin (Sharp *et al.* 1996), deletion of the *Ren1*^{d} gene in two-renin gene mice revealed important but subtle phenotypic changes, namely absence of renin granules in JG cells, macula densa hyperplasia and sexually dysmorphic hypotension (Clark *et al.* 1997). In contrast, one-renin gene mice in which *Ren1*^{c} has been deleted, had severe kidney defects associated with tubules and macula densa and required salt injection to prevent neonatal death (Yanai *et al.* 2000). Increased fluid intake and urine output were observed. These animals were completely devoid of renin granules in JG cells, displayed hypotension and absence of plasma AngI.

1.3.2 Angiotensinogen

The precursor of angiotensin peptides, angiotensinogen (AOGEN), is a large protein composed of 452 amino acids. It is released constitutively from the liver where it does not appear to be stored (Menard *et al.* 1993). Unlike renin in the kidney, AOGEN expression in the liver does not increase when homeostasis is threatened (Ingelfinger *et al.* 1986). AOGEN expression does, however, increase in the kidney re-enforcing the idea of a locally regulated RAS (Ingelfinger *et al.* 1986). The expression of AOGEN is up-regulated by several other factors such as exogenous AngII administration. It has been shown that renin and angiotensin do not compartmentalise into the same secretory granules suggesting that they are produced by different cells in tissues (Menard *et al.* 1993). Because the association constant of AOGEN with renin is high compared to the catalytic constant, it has been suggested that AOGEN may be a renin inhibitor rather than a substrate for the formation of AngI (Poulsen and Jacobsen 1986) but no experimental data have proved or disproved this hypothesis.

Angiotensinogen (AOGEN) is first detected in the mouse foetus from E13dpc in the liver and from E18dpc in the kidney (Niimura *et al.* 1997). Although, AOGEN expression is first

detected in the rat foetus from E11.5dpc (in the yolk sac) (Lee *et al.* 1987), expression of AOGEN is very low in the liver during gestation and increases dramatically post-natally (Niimura *et al.* 1997), possibly as a result of stimuli associated with birth or preparation for extra-uterine life. It is interesting that, in the kidney, renin expression precedes that of its substrate, AOGEN,while it is unclear whether renin is secreted as an active enzyme during development. In addition, AOGEN is detected in foetal fat suggesting a role in temperature regulation and also in the brain and in the kidney where it may be involved in organ development (Gomez and Norwood 1995). Angiotensinogen *null* mice (Tanimoto *et al.* 1994) display phenotypic changes in the vascular structure of the kidney, notably a striking thickening of the renal arterioles. It is possibly associated with a negative feedback on renin secretion and hence, deregulated renin-expressing cell recruitment. In contrast with angiotensinogen *null* mice or the ACE *null* mice (Esther *et al.* 1996), the abnormalities reported in the "renin cell ablation" model (Pentz *et al.* 2004) do not affect the vasculature which is normal whereas dilated and atrophic tubules were observed. It appears therefore that renin-expressing cells (which may not contain active renin granules during development) are needed for the formation of the tubular network while the RAS cascade appears to be required for vasculogenesis.

1.3.3 Angiotensin-converting enzyme

The angiotensin-converting enzyme (ACE) is a zinc metalloproteinase which metabolises many polypeptides. There are two forms of ACE, a somatic (140-160kDa) and a germinal (80kDa) form, both originating from the same gene but transcribed through the involvement of two distinct promoters. The main role of somatic ACE is the conversion of AngI to AngII (Figure 1.9). However, additional functions for this metalloproteinase include the inactivation of bradykinin and the hydrolysis of substance P, both known as vasodilators (Turner and Hooper 2002). The germinal form of ACE is found exclusively in the testes and is thought to be important in fertility. A small proportion (10%) of ACE is found in the plasma but the majority is located in tissues notably, lungs, vasculature, heart, kidney and the adrenal gland. Somatic ACE is mainly made by the endothelium and contains two extracellular catalytic sites and a transmembrane domain. ACE is also produced by smooth muscle cells and by macrophages. Hence, in the vasculature, ACE appears to mediate many different actions such as vasoconstriction, inflammation, vascular remodelling and also regulation of thrombotic response through the formation of AngII and the degradation of

bradykinin (Dzau *et al.* 2001). Very little ACE is detected in the heart under normal physiological conditions and most of cardiac ACE is found in the endothelium of arteries and arterioles (Yamada *et al.* 1991). Cardiomyocytes are known to express ACE *in vitro* and possess the ability to produce AngII, only when stretch-induced (Sadoshima *et al.* 1993). Hence, ACE has a pivotal role in normal physiology and in the patholophysiogy of cardiovascular disease. In humans, an insertion/deletion (I/D) polymorphism at the *ACE* locus has been found to be responsible for variations in blood pressure. Blood pressure and serum and cardiac ACE levels are increased in individuals with the D allele and have been found to be more susceptible to LV hypertrophy (van Berlo and Pinto 2003). Factors mapped to chromosome 10 close to the ACE locus have also been linked to the malignant hypertension phenotype in the TGR(*mRen2*)27 animals (Kantachuvesiri *et al.* 1999).

Tissue ACE activity and mRNA are highest in the lung during embryonic development but are also prominent in the kidney, heart and aorta (Yosipiv and El-Dahr 1998). ACE is first detected in the foetal rat from E18dpc in the lungs and E16dpc in the kidney (Gomez and Norwood 1995), suggesting, once more, the importance of a potential renal RAS during development. In addition, ACE expression has been located in the proximal tubule mediating the formation of locally produced AngII which may influence growth and establishment of tubular structures in late gestation (Jung *et al.* 1993). The presence of ACE in the developing cardiovascular structures is also of importance since AngII is know to promote cellular growth of neonatal rat cardiomyocyte (Schorb *et al.* 1993) and bradykinin was found to have anti-proliferative effects in blood vessels, mechanism which may be inhibited by ACE during development to promote vasculogenesis (Linz and Scholkens 1992). The severe hypotension observed in ACE *null* mutants could be due to the absence of AngII but also to the accumulation of bradykinin. In addition, in these animals, the development of the renal arterial tree is blunted (Esther *et al.* 1996). AngII therefore appears to have a distinct role in renal vasculogenesis. Similarly to AOGEN *null* mutants, papillary and tubular atrophy were observed which suggests that it is the absence of AngII which is responsible for this phenotype.

1.3.4 Angiotensin II and its receptors

Most of AngII cardiovascular effects are mediated by the binding to its highly specific receptor located on the cell membrane primarily in the adrenal glands, vascular smooth

muscle cells, the kidney, the brain and the heart (Unger 2002). Ang II receptors (AT) exist in two distinct forms, type I and type II, which are expressed in different tissues (Timmermans *et al.* 1993). They are both 7-transmembrane domain receptor coupled to a G-protein associated with pathways involving phospholipases, calcium channels and kinases (Kim and Iwao 2000; Timmermans *et al.* 1993; Unger 2002). The two receptors display only 34% amino acid sequence homology overall (Timmermans *et al.* 1993).

In addition to its vasoconstrictor effects, AngII has growth factor properties which are involved in cell growth and differentiation during development. These properties may also play a role in the pathogenesis of cardiac hypertrophy, arterial stiffness and vascular injuries seen in hypertension and diabetes. Ruiz-Ortega *et al.* have reviewed the mechanisms of AngII action and they reported that the AT_1 receptor promotes inflammation, growth and proliferation while the AT_2 receptor counteracts these actions. Indeed, after myocardial infarction in rats, the expression of AT_1 receptor (Tan *et al.* 2004) as well as levels of angiotensinogen and renin proteins (Passier *et al.* 1996) was increased in cardiomyocytes and renin has been detected in the heart at the site of infarct after a similar surgical intervention in the rat (Sun *et al.* 2001). These findings suggest local Ang II formation and a role in cardiac/vascular injury.

Other functions and actions of AngII are exemplified by the fact that AT_1 receptor in the brain has been associated with central actions on homeostasis such as pressor and drinking responses, vasopressin release and salt appetite (Ganong 1984; Phillips 1987). This receptor is also known to be involved in cell growth and proliferation in a variety of cardiovascular-associated cells implicating it in pathologies such as left ventricular hypertrophy, atherosclerosis and glomerulosclerosis (Lucius *et al.* 1999; Unger *et al.* 1996). AngII infusion in rats can induce cardiac hypertrophy (Yamazaki *et al.* 1999) and glomerulosclerosis (Miller *et al.* 1991) without elevation of blood pressure. AngII (*via* the AT_1 receptor) also has similar hypertrophic effects on cultured vascular smooth muscle cells (Unger *et al.* 1996). The mechanisms by which AngII triggers these injuries are not fully understood but excessive cell growth and induction of extracellular matrix, as well as intracellular pathways (*e.g.* MAP kinases) may play an important role (Kim and Iwao 2000). AngII is well known to have angiogenic effects as well as promoting cell growth and proliferation through its AT_1 receptor, mechanisms which may be important in embryonic development. AT_1 inhibition and angiotensin receptor blockade are also known to cause similar abnormalities, notably disrupting the vascular branching and glomerular development in the kidney (Gomez and Norwood 1995).

Recently, two isoforms of the AT_1 receptor, AT_{1A} and AT_{1B}, have been identified in the mouse (Sasamura *et al.* 1992). These receptors are the products of two distinct, highly homologous genes. AT_{1A} is highly expressed in the kidney, liver, adrenal gland, brain, heart, lung and the reproductive organs whereas the localisation of AT_{1B} is less widespread and high expression levels confined to the adrenal gland, the brain and testes (Burson *et al.* 1994). In double *null* mutants for AT_{1A} and AT_{1B}, ACE inhibition has the effect of increasing mean arterial pressure suggesting that the AT_2 receptor signalling may be diminished by the treatment (Oliverio *et al.* 1998). This agrees with the hypothesis that AT_2 may oppose detrimental effects of AngII (*via* the AT_1 receptor). Mice lacking the AT_{1B} receptor subtype displayed normal growth (Oliverio *et al.* 1997) whereas mice lacking both isoforms had decreased body weight and renal abnormalities resembling that of the *ACE* or *AOGEN null* mutants, namely papillary and tubular atrophy and thickening of the vascular wall (Oliverio *et al.* 1998). The authors showed that the severity of the abnormalities is greater in the double knockout mice showing that both AT_1 receptor subtypes are needed for normal renal development. Hence, each subtype can partly compensate for the absence of the other (Oliverio *et al.* 1998).

AT_2 receptors are expressed at high density throughout development in all tissues from E11 until E19, when its expression begins to decrease and in the adult, is only present in the adrenal gland, the ovary, the uterus and some areas of the brain (Dzau 1995). The receptor also occurs as a feature of certain conditions such as heart failure where it may be involved in tissue repair (Lucius *et al.* 1999; Unger *et al.* 1996). It may be that cells that express AT_2 in adult life revert to a foetal phenotype of angiotensin-receptor expression in these conditions. Many reports suggest that AT_2 is therefore involved in cell differentiation, tissue growth, wound healing and apoptosis (Lucius *et al.* 1999; Nakajima *et al.* 1995; Unger *et al.* 1996). It has been demonstrated that AngII has direct growth promoting effects through AT_2 during organogenesis in an *in vitro* cultured whole rat embryo model and that this effect could be blocked via specific inhibition with an AT_2 receptor inhibitor (Tebbs *et al.* 1999). This differs from the work of Norwood *et al.* who reported that AT_2 being expressed in early undifferentiated mesenchyme may have a role in early nephrogenesis rather than late foetal development (Norwood *et al.* 1997). The earlier results could, however, be due to the culture conditions. AT_2 receptor *null* mice revealed a role for this receptor in arteriogenesis during development since the expression of calponin (an actin binding protein responsible for actin-myosin interactions) is lowered in the aorta compared to wild-type animals (Yamada *et al.*

1999). It may, therefore, be that the AT_1 and AT_2 receptor functions differ from one organ to the other and between the adult and the embryo.

Although AngII may contribute to tubulogenesis, the data suggest that Ang II plays a lesser role in tubular framework development. This is further confirmed by Nagata *et al.* who reported that AngII added to a ureteric bud culture did not trigger any advance in tubular growth (Nagata *et al.* 1996). The antagonising role of AT_2 receptor to AT_1 functions has been reported and it may inhibit cell proliferation and promote cell differentiation. This is suggested by the presence of AT_2 receptors in the ureteric bud during early differentiation of the nephron in the developing kidney (Aguilera *et al.* 1994), although the level of AT_2 expression in this localization has been reported to precede glomeruli proliferation (Gimonet *et al.* 1998). The renal abnormalities displayed by AT_2 *null* mutants were less severe than that of the AT_1 *null* mutants (Miyazaki and Ichikawa 2001) in which vascular and tubular components of the kidney were affected.

1.3.5 Angiotensin III and IV

Other AngII derived peptides have been found to have biological activities. AngII has a very short half-life in the circulation and it can be converted to a 7-amino acid peptide termed AngIII by removal of the N-terminal asparagine residue by aminopeptidase A. AngIII appears to have central actions notably triggering vasopressin release after intra-cerebroventricular injection (Zini *et al.* 1996). However, the mechanisms remain unknown and it is not clear through which receptor it carries out its actions.

AngIII can also be metabolised to a hexapeptide termed Ang(3-8) or AngIV by aminopeptidase B [(Wright *et al.* 1995); Figure 1.9]. AngIV, which can be found in the plasma displays a lower affinity for AT_1 and AT_2 than AngII (de Gasparo *et al.* 2000) and recently, a receptor for AngIV (AT_4) was purified (Albiston *et al.* 2001). AT_4 is widely distributed in tissues, namely kidney, heart, brain, lung, prostate and small intestine (Wright *et al.* 1995). AngIV has also been shown to block the increase of RNA and protein synthesis caused by AngII in cardiomyocytes and therefore may be involved in counteracting hypertrophic effects of AngII (Baker and Aceto 1990).

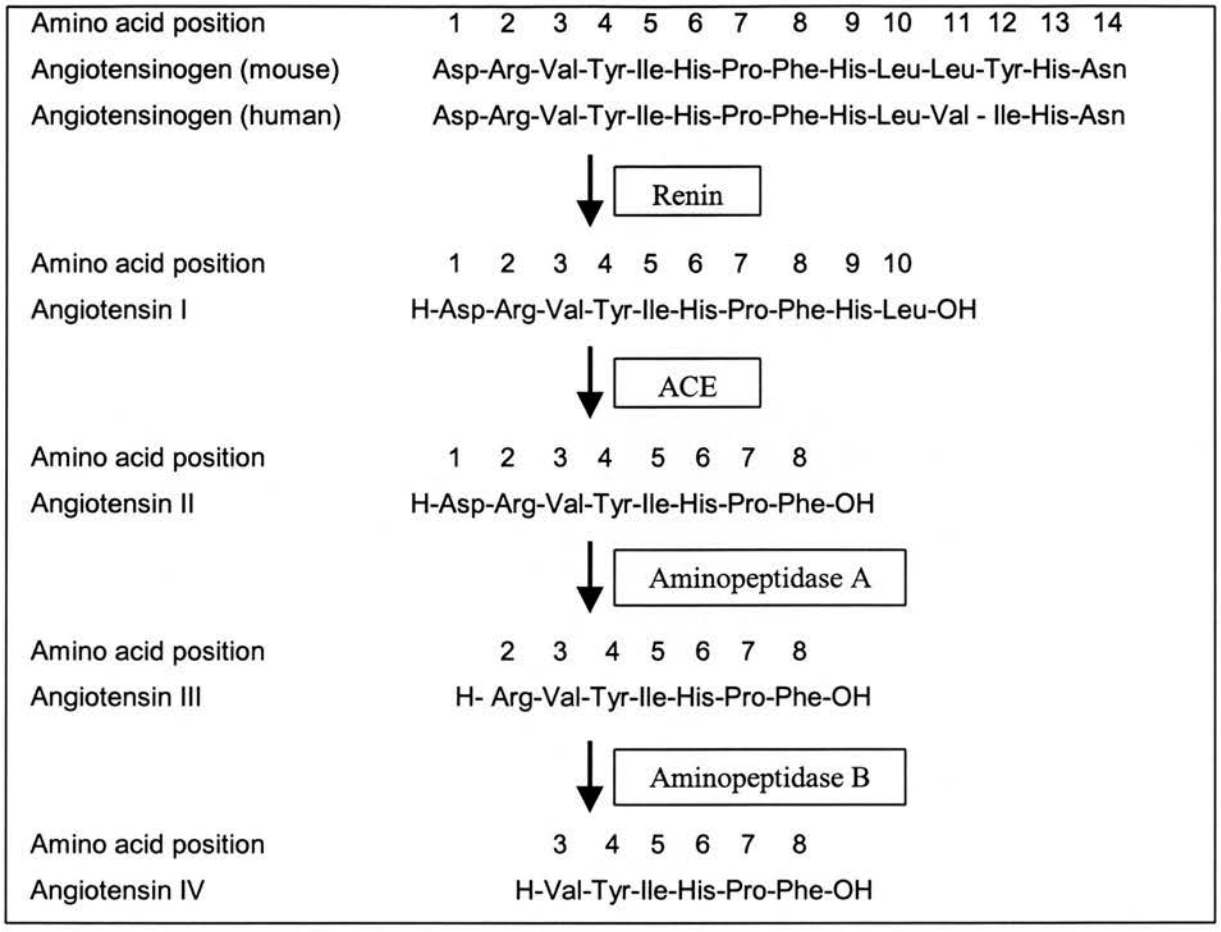


Figure 1.9: Formation of angiotensin peptides. The first step of step is catalysed by renin resulting in the conversion of the 452 amino-acid AOGEN (shown truncated in the above figure) to the 10 amino-acid AngI. AngI is then converted to AngII, the effector molecule of the RAS by ACE. AngII is further metabolised to the heptapeptide AngIII and the hexapeptide AngIV by aminopeptidase A and aminopeptidase B, respectively. Adapted from Robertson (1997).

In the brain, AT_4 expression overlaps with expression of AT_1 and AT_2 but it is also uniquely expressed in areas such as the cerebral cortex, the hippocampus and the cerebellum which are associated with memory and cognitive processes as well as sensory /motor functions (Wright *et al.* 1995). In addition, AngIV is involved in facilitating blood flow in the brain after intra-cerebroventricular injection and in the kidney after injection in the renal artery (Harding *et al.* 1994) suggesting that AngIV is also part of a local RAS. Interestingly, the findings of Kakinuma *et al*, showed that infusion of AngII and Ang IV inhibited the tubular atrophy observed in the AOGEN *null* mice (Kakinuma *et al.* 1999).

1.4 The Renin-Angiotensin System in Hypertensive Disease

1.4.1 Cardiac Hypertrophy

Hypertension is linked to cardiac hypertrophy which is characterised by an increase in cardiac muscle mass (Figure 1.10). Tissue culture studies have shown that cardiomyocytes and fibroblasts are activated by AngII via the AT_1 receptor (Sadoshima and Izumo 1993a).

In cardiomyocytes, AngII activates second messenger pathways via phospholipases C, D, and A2 and protein kinase C which are linked to the transcription factor *c-fos* (Booz *et al.* 1999; Sadoshima and Izumo 1993b). AngII also increases the tyrosine phosphorylation of cytosolic proteins and up-regulates MAP kinases (ERK1 and 2) which may cause hypertrophic responses (Booz *et al.* 1999). The signalling pathways involved in the cardiomyocyte hypertrophy are complex and it is now well known that cardiomyocyte growth (and notably protein synthesis) stimuli such as MAP kinases and second messengers are activated by stretch (Yamazaki *et al.* 1999). Stretching of cardiomyocyte cultures *in vitro* triggers the release of Ang II which acts directly on the cells as a growth factor (Sadoshima *et al.* 1993; Yamazaki *et al.* 1995). *In vivo*, this response to stretching may cause hypertrophy but may be adaptative to improve cardiac contraction compensating for increased wall tension.

Aikawa *et al.* showed that integrins were activated in response to mechanical stress in cardiomyocytes again via a MAP kinase (p38) pathway (Aikawa *et al.* 2002). The cardiac interstitium is made up of non-myocyte cells namely, fibroblasts and endothelial cells. It is the fibroblast population which is responsible for the production of extracellular matrix (ECM) proteins. Studies in humans and animal models have implicated AngII as a growth promoter of both cardiomyocytes and fibroblasts during the remodelling events which occur to trigger hypertension and myocardial infarction (De Mello and Danser 2000; Dostal *et al.* 1996; Kenchaiah and Pfeffer 2004; Kim and Iwao 2000; Mayet and Hughes 2003).

The abnormal accumulation of fibrillar collagen plays a role in such pathology (Baker *et al.* 1992). In addition, cardiac fibroblast culture medium was shown to induce protein synthesis and signal transduction events rapidly in neonatal rat ventricular myocytes and to increase angiotensinogen messenger RNA (mRNA) levels (Booz *et al.* 1999). Cardiac fibroblasts have been shown, in turn, to respond to AngII with increased expression of integrins (transmembrane receptors involved in cell attachment to the ECM) and α-actinin in SHR (Kawano *et al.* 2000). The expression of these genes was inhibited by the AT_1 receptor antagonist losartan but not by diuretic treatment.

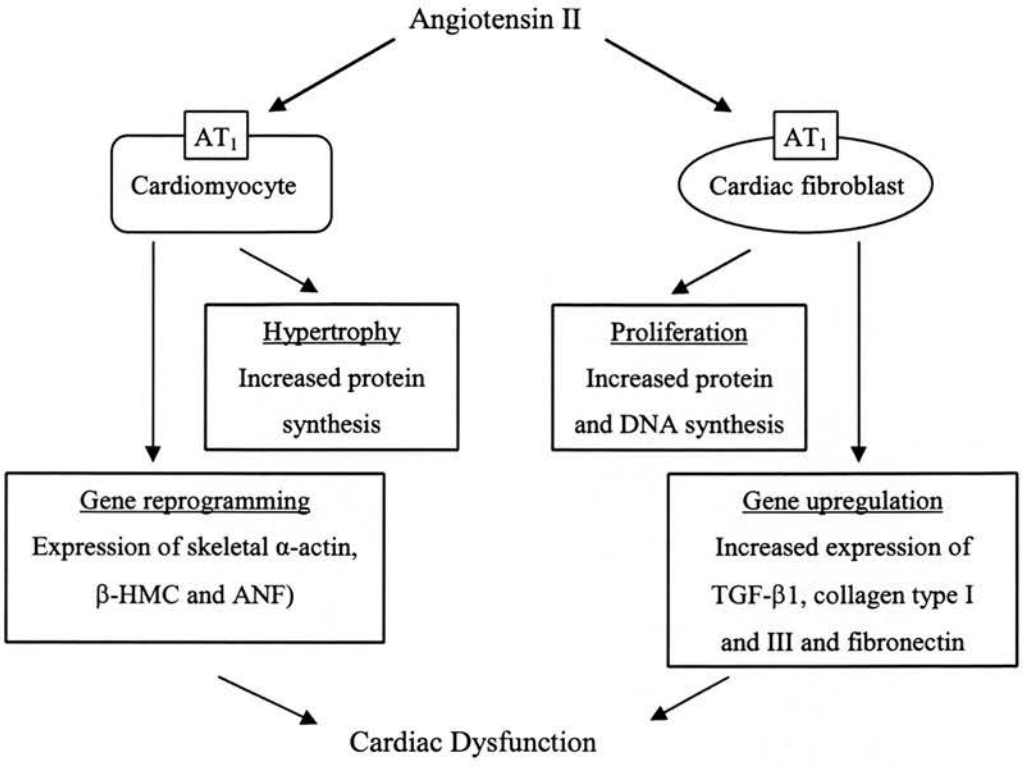


Figure 1.10: Actions of AngII on cardiac cells. The effects of AngII via AT_1 on both cardiomyocytes and cardiac fibroblast contribute towards the hypertrophic response of the heart leading to cardiac dysfunction.

Angiotensin-receptor inhibition in rats with pressure-overload hypertrophy was also effective in reducing left ventricular wall thickness and mass (Iwai *et al.* 1995). In addition, Kojima *et al.* showed that it is not just a reduction of blood pressure which may resolve cardiac hypertrophy in rats with malignant hypertension since angiotensin-receptor inhibition was beneficial but a classic diuretic was not (Kojima *et al.* 1994). In addition, it was demonstrated that sodium intake plays an important role in the cardiac hypertrophic response to angiotensin II but not the BP response to Ang II (Morgan *et al.* 1998). Interactions between sodium intake and AngII and left ventricular hypertrophy was also reported in Goldblatt rat models (Morgan *et al.* 2001).

1.4.2 Renal and Vascular Fibrosis

In addition to cardiac hypertrophy, renal and vascular fibrosis are some of the most important complications of hypertension (Abdi and Johns 1996; Griffin and Bidani 2004; Mayet and

Hughes 2003). The mechanisms described above for cardiac hypertrophy, notably mechanical stress (Hudlicka 1994), ECM proteins adhesion molecules (Ignotz and Massague 1986) and RAS activation (Navar *et al.* 2002; Ruiz-Ortega and Egido 1997) have also been shown to cause vascular remodelling in large and peripheral vessels. Large arteries exhibit two functions: 1) a conduit function which determines mean arterial blood pressure and blood flow. It enables appropriate delivery of oxygen-rich blood to tissues and alterations in the conduit properties of large arteries such as those caused by atherosclerosis result in stenosis or ischaemia in brain, heart or kidney; 2) a buffering function which determines pulsatile pressure and flow. It enables the pulsatile blood flow from the heart to be turned into continuous flow at the peripheral level. It is dependent on the visco-elastic properties of the aorta and large arteries and can be modulated by vascular remodelling. Pulse pressure (PP) in determined by both properties and in ageing /hypertensive states, increased PP in a result of increased in 1) and decrease in 2). It is therefore an important determinant on blood pressure status and is sensitive to the mechanisms described below.

In addition, resistance arteries are also affected by remodelling in hypertensive disease. These changes involve media thickening, reduced lumen diameter and consequent increased media:lumen ratio. The mechanisms triggering these structural changes involve hypertrophy and hyperplasia of vascular smooth muscle cell (VSMC) and increased extracellular matrix abundance. Inflammation including macrophage infiltration, fibrosis and increased expression of pro-inflammatory genes (such as interleukins) is also pivotal to vascular remodelling (Touyz, 2006). Hence vascular remodelling of large and small arteries has many implications during hypertensive states.

Mechanical stimuli can act on the blood vessels by increasing vascular tone (Hudlicka 1994) and an increase in vascular resistance to blood flow is characteristic of hypertensive states. It appears that very small changes in arterial diameter and thus in the blood flow have an impact on the heart in humans (Hughes *et al.* 1993). As for cardiac hypertrophy, hypertensive vascular/renal dysfunction may be the result of a similar involvement and of pro-fibrotic and pro-inflammatory factors. In addition, renal damage has been used as a phenotype in QTL analysis on the basis of the association between hypertension and renal damage. Two QTLs for renal sclerosis and proteinuria have been found, the former co-localising with blood pressure QTL on rat chromosome 1 (Rapp 2000). This section underlines the complexity of the mechanisms involved in cardiac, vascular and renal disease associated with hypertension (and diabetes or atherosclerosis) and highlights the pivotal role of the RAS in the hypertensive pathogenesis. More recently, the adaptive role of kallikrein in

vascular shear stress was highlighted in humans with a loss-of function polymorphism in the human tissue kallikrein gene (resulting in a decrease in the enzyme activity) (Azizi *et al.* 2005).

Although many interlinked additional factors modulate vascular remodelling, AngII alone is a powerful mediator of events regulating vascular structures:

1) Tyrosine kinases (e.g.: Src or JAK) cause phosphorylation of cytosolic proteins such as MMP2 in endothelial cells or IP3K in VSMC following AngII binding to AT_1. PI3K, in turn, triggers signal transduction cascade which may cause increased cell survival and proliferation augmenting muscle cell contraction.
2) MAP kinase activation results in a variety of responses in VSMC population such as hyperplasia, hypertrophy, inflammation and collagen synthesis. Activation of AT_1 receptors promotes tyrosine phosphorylation and stimulates mitogen-activated protein kinases and proliferation. Following AngII binding to AT_1, insulin-like growth factor 1 (IGF-1) receptor, epidermal-derived growth factor (EDGF) receptor and platelet-derived growth factor (PDGF) receptors become "transactivated" resulting in further MAP kinases activation. The process of receptor transactivation by AngII is poorly understood but is thought to involve intermediate intracellular mediators such as increased intracellular calcium (Laurette *et al.* 2005).
3) NADPH oxidase is involved in the formation reactive oxygen species (ROS) and has an important role on oxidative stress. Oxidative stress results from an imbalance between the formation of deleterious reactive oxygen species and the production of antioxidants (Touyz and Schiffrin 2004). NADPH oxidase activation leads to the formation of superoxide and hydrogen peroxide which cause cellular damage and activation of NF-κB and AP-1 resulting in increased adhesion molecules, chemokines and cytokines but also in a decrease of bio-availability of the vasodilator nitric oxide. Superoxide also inhibit the formation of the vasodilating molecule endothelial-derived relaxing factor while hydrogen peroxide stimulates the release of endothelial-derived contracting factor. ROS in turn activate MAP kinases triggering the cascade of events leading to fibrotic and inflammatory responses. iNOS expression together with NF-κB (by which the iNOS promoter is regulated) were also increased in the renal cortex of SHR compared to control (Kumar *et al.* 2005). In cultured rat aortic smooth muscle cells, interleukin-6 was able to induce an increase in ROS, notably superoxide and this was accompanied by an up-regulation of AT_1 receptor expression (Wassmann *et al.* 2004). In addition, the authors reported an impaired endothelium-dependent vasodilation triggered by interleukin-6 in mouse cultured aortic

rings although this was not observed in AT_1 receptor knock out mice. Similarly, AT_1 antagonism and inhibition of NF-κB in rats with unilateral ureteral obstruction (a model of obstructive nephropathy) had beneficial effects on the renal formation of reactive oxygen species (Nakatani *et al.* 2002).

4) Intracellular calcium. AngII binding to its G protein-coupled receptor results in phosholipase C-mediated formation of inositol triphosphate which triggers release of calcium from sarcoplasmic reticulum stores. Transactivation of calcium channel also increases the influx of calcium ions from extracellular locations. These events, together with inhibition of myosin light chain phosphatase mediate actin-myosin interaction and cellular contraction in VSMC increasing vascular tone.

TGF-β receptors are single-pass transmembrane proteins. A role for TGF-β has been reported (Ignotz and Massague 1986; Tharaux *et al.* 2000; Yamamoto *et al.* 1993) in hypertensive renal disease and TGF-β is known to be up-regulated by AngII and also by glucose (hence its major role in diabetic nephropathy). Using a transgenic mouse model expressing the reporter gene luciferase under the control of the collagen I α2-chain, Fakhouri *et al.*, showed that administration of exogenous AngII, ET-1 and TGF-β activates the collagen-1 gene in aortic and renal cortical tissues (Fakhouri *et al.* 2001). The mechanisms underlying this response have been attributed to activation of MAP/ERK pathway and TGF-β (Tharaux *et al.* 2000). Using specific ET receptor antagonist, it was shown that the AngII-mediated up-regulation of collagen-1 expression also involves ET-1. Additional *in vitro* studies have shown that both AngII and ET-1 may promote the synthesis of fibronectin and collagen in a dose-dependent manner (Fakhouri *et al.* 2001). ET-1 is also up-regulated by superoxide. Other factors involved in hypertensive fibrotic/inflammatory events are the plasminogen activator inhibitor-1 (Dong *et al.* 2003), chymase (Miyazaki and Takai 2001); or increased cell adhesion (Okamoto *et al.* 2004).

1.5 Local versus Circulating Renin-Angiotensin System

1.5.1 Local RAS – Tissue and intracellular RAS

It is now well established that the renin-angiotensin system is not just an endocrine (*i.e.* circulating) system since AngII can be produced locally in a number of tissues such as the vascular beds, the brain or the heart. The idea of the existence of a local RAS (paracrine or

autocrine) arose from the following findings: anephric patients have a plasma level of renin which does not reach zero, continue to have high levels of plasma prorenin and retain the ability to produce AngII suggesting that AngII can be produced independently of renal renin production (Sealey *et al.* 1977; Weinberger *et al.* 1977). Similar findings were obtained in animals having undergone bilateral nephrectomy (Danser *et al.* 1994). Secondly, recent reports have demonstrated that all the components of the renin-angiotensin system (RAS) are present in several tissues suggesting the existence of a local paracrine or autocrine.

1.5.1.1 Cardiac RAS

AngII can be readily detected in the heart but it is still a matter of debate whether AngII is made intracellularly by cardiomyocytes or taken up by cardiac tissue. In order to allow cardiac synthesis, the presence of renin, AOGEN and ACE is required. It is unclear whether cardiac renin comes from uptake or *de novo* synthesis. Renin protein was detected in rat cardiomyocytes cultured in serum-free conditions [reviewed by (Schuijt and Danser 2002)]. This may be explained by sequestration prior to serum removal or due to the recently identified "Renin1A", an intracellular form of the enzyme arising form an alternatively spliced transcript lacking exon 1 (hence the "PRE" segment of the precursor enzyme which act as a signal for secretion) which has been replaced by exon1A (Clausmeyer *et al.* 2000). Another reason for the presence of renin in the heart is uptake of prorenin following binding to the mannose-6-phosphate receptor (M6Pr) (Saris *et al.* 2001a) to the more recently identified human (pro)renin receptor ((Nguyen *et al.* 2002) and *Chapter 3*) or to an as yet unidentified receptor. In the case of non-glycosylated renin (e.g. from *Ren2* gene) a role for mannose-6-phosphate receptors in renin uptake can be excluded.

If recombinant prorenin and AOGEN are added to cultured rat neonatal cardiomyocytes, total cellular protein content increases as well as leucine and thymidine incorporation and this was inhibited by AT_1 antagonist, but not AT_2 antagonist or mannose-6-phosphate (Saris *et al.* 2002). The cell growth promoting effects were not observed if prorenin or AOGEN alone were used separately. Furthermore, the absence of intracellular AngI in these cells is in agreement with other reports (de Lannoy *et al.* 1997) showing that AOGEN is neither present in cardiomyocytes or taken up by these cells. The conversion of AOGEN by prorenin must, however, be the result of non-proteolytic/reversible activation of prorenin following binding to receptors other than M6Pr (Saris *et al.* 2002). van Kesteren *et al.* (1999) also

showed that cardiomyocytes did not release either renin or angiotensinogen in a stretch-induced hypertrophy experiment suggesting hypertrophy was AngII-independent.

Using the isolated perfused rat heart model, de Lannoy *et al.* collected coronary effluent and interstitial transudate separately. They reported that infusion of renin or AOGEN in this model did not result in the formation of AngI suggesting that *in vivo*, circulating AOGEN- and circulating (pro)renin-independent AngII formation in cardiac tissues is unlikely to occur. However, if AOGEN and renin were infused concomitantly, AngI could be detected in the interstitial fluid which may be explained by enzymatic action of renin on its substrate in the liquid phase. In addition, the authors showed that the AngI concentration in coronary effluent was higher than in the interstitial fluid suggesting that there might be an additional site of AngI production at the luminal vascular location where renin may be receptor-bound. Transfection of rat hepatoma cells with a mutated AOGEN cDNA results in a non-secreted protein which enhanced growth through upregulation of platelet-derived growth factor expression. (Cook *et al.* 2001). This was inhibited by losartan but not candesartan The latter is known to bind to surface AT_1 without further internalisation of the receptor and pre-treatment with candesartan prevented cellular growth inhibition by losartan. This suggests that there are two distinct types of AT_1 receptor on the cell surface and in a nuclear location but this may depend on the cell type.

ACE mRNA was found in the heart, by autoradiography (Yamada *et al.* 1991). ACE was, more precisely, found on the cell membrane where its active domain is extracellular and no ACE could be detected in the cytosol (Beldent *et al.* 1995) while ACE inhibition had no effect on release of intracellular AngII in stretched cardiomyocytes suggesting that intracellular AngII in these cells is not dependent on membrane-bound ACE (Sadoshima *et al.* 1993). All the evidence points towards a "partial cardiac RAS" and dismisses the hypothesis that, under normal physiological circumstances, AngII may be produced intracellularly by cardiomyocytes. AngII may, nonetheless, be produced in the interstitial fluid or on the luminal surface of the cardiac vasculature and subsequently internalised through a AT_1-mediated endocytosis. The lack of intracellular AngII synthesis does not mean that AngII does not have intracellular actions. Indeed, AngII has been shown to be present in nuclear fractions and to be involved in the regulation transcription via binding to chromatin (Re and Parab 1984), process which is dependent on binding to AT_1 since this is inhibited by losartan treatment. If AngII is present in the heart, what is its function in cardiac physiology? Is AngII solely triggering processes during pathophysiological events such as myocardial infarction? In cases of hypotension, AngII and activation of the RAS will

contribute to the homeostatic response and its vasoconstricting actions will participate in the re-establishment of normal blood pressure.

In normal physiological situations, AngII is involved in vasoconstriction of intra-cardiac vessels and possibly in the regulation of cardiac inotropy. However, excess AngII in the heart has detrimental effects. Mice over-expressing AT_1 specifically in the heart were normotensive but displayed ventricular hypertrophy and fibrosis accompanied with increased ANF expression leading to heart failure at 4-5 months of age (Paradis *et al.* 2000). Another cardiac specific AT_1 over-expressor mouse model resulted in a more severe phenotype. The animals died a few weeks after birth due to cardiomyocyte hyperplasia, bradychardia and heart block (Hein *et al.* 1997). Differences between the two models may be due to the mouse strain used but both models demonstrate that AngII promotes cell growth *in vivo* consistent with previous *in vitro* studies showing mitogenic effects of AngII (Kojima *et al.* 1994; Yamazaki *et al.* 1995). In addition, in AT1 *null* mutants, the extent of the ventricular remodelling was improved after experimental myocardial infarction (Harada *et al.* 1999).

1.5.1.2 Intrarenal RAS

The kidney is responsible for the majority of the production and release of plasma renin and the presence of a renal RAS has been thoroughly evaluated since locally produced AngII may exert vasoconstricting effects on the intra-renal vasculature, hence affecting vascular tone, glomerular filtration pressure and reabsorption of sodium (Figure 1.11). Zou *et al.* showed that, following infusion of Val^5-AngII for two weeks in rats, two-third of the renal AngII was of plasma origin, a process mediated by AngII via binding to AT_1 (Zou *et al.* 1998). However, total kidney AngI and AngII contents are consistently higher than plasma levels indicating that AngI and AngII are produced in the kidney and are not solely a result of uptake from the circulation (Navar *et al.* 2002). Celio and Inagami studied the presence of the RAS components in the kidney by immunocytochemistry. Renin was found in the JG cells at the vascular pole of the afferent arterioles, ACE was bound to the luminal surface of the endothelial cells of the renal arteries and arterioles in addition to the capillary loops of the glomueruli whereas AngII was exclusively located in the cytoplasm of the JG cells (Celio and Inagami 1981). In addition, AngII is found in the interstitial fluid, and AT_1 is detected in SMC or afferent and efferent arterioles and mesangial cells as well as in all the nephron segments (Navar *et al.* 2002). AOGEN mRNA and protein, renin and all the other components of the RAS have been detected in the proximal tubule (Moe *et al.* 1993). It is not

known how much AngII is produced in the JG cells, the interstitial fluid and in the tubular fluid. The arguments against JG cell internalisation of AngII through AT_1 are that AngII is not present in SMC although AT_1 receptors are present on the surface of these cells and that pre-treatment of kidney sections with AngII does not increase AT_1 immunodetection (Celio and Inagami 1981). AngII located in the JG cells may constitute a link connecting distal tubular fluid to resistance of glomerular arterioles, hence regulating tubulo-glomerular feedback in addition to a macula dense-driven mechanism. Furthermore, locally produced AngII by a proximal tubule RAS may provide a rapid response able to modulate fluid and salt reabsorption preceding that of the circulating RAS in response to homeostatic changes.

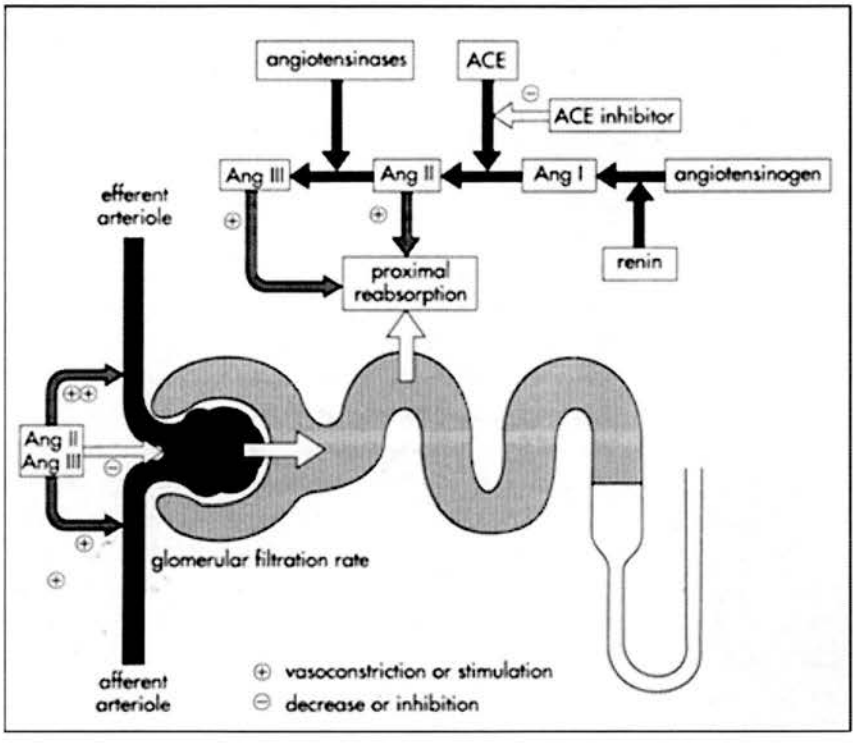


Figure 1.11: The intra-renal RAS. Diagram depicting the actions of a local RAS in the kidney, mainly renal blood flow by constriction of the efferent and afferent arteriole, glomerular filtration rate by constriction of the afferent arteriole and on proximal reabsorption of sodium chloride and bicarbonate. Note that the origin of renin and AOGEN is not detailed on the schematic representation. However, they have been detected in the proximal tubule and renin and ACE have observed in the afferent and efferent arterioles. Reproduced from (Zhuo and Mendelsohn 1993).

1.5.1.3 Adrenal RAS

All the genes of the RAS are indeed expressed in the adrenal and renin is localised to zona glomerulosa cells which are the aldosterone-producing cells and in the inner adrenal cortex

(Deschepper *et al.* 1986). This does not, however, mean that all adrenal AngII is locally produced. ^{125}I-Ang I intravenous infusion resulted in accumulation of ^{125}I-AngII but not ^{125}I-AngI in the adrenal gland and this was reduced by 80% after administration of the AT_1 antagonist, eprosartan showing that a major proportion of AngII in the adrenal gland is the result of accumulation after uptake from the circulation (van Kats *et al.* 2005). In addition, this report suggested that involvement of a recently identified truncated prorenin found in the adrenal mitochondria (Clausmeyer *et al.* 1999) in the intracellular generation of AngII is unlikely. The remaining angiotensin II found in the adrenal after eprosartan treatment may be the result of incompletely blocked AT_1 receptors or due to (pro)renin uptake in by the adrenal as part of a local RAS.

When nephrectomised rats (*i.e.* with low levels of circulating AngII and with levels of renin near zero), aldosterone release is markedly increased as is the number of adrenal cells expressing renin (Peters *et al.* 1999). AngII infusion to nephrectomised rats prevented the increase in aldosterone release suggesting that an intra-adrenal RAS is present but inhibited under normal conditions when AngII is present in the plasma. This action of AngII may have implications for the pathogenesis of the hypertensive rat model TGR(*mRen2*)27 in which a mouse *Ren2* transgene is highly expressed in the adrenal gland (Peters *et al.* 1993). Due to increased level of plasma prorenin, plasma and renal renin levels are suppressed whereas circulating AngII levels are unchanged or low. Uninhibited adrenal RAS may result and contribute towards the hypertensive phenotype. This did not apply to TGR(*mRen2*)27 in which adrenal renin and prorenin (of transgene origin) release can be triggered by AngII in cell preparations (Peters *et al.* 1993). This effect was only observed for prorenin after prolonged exposure to AngII whereas renin release was a rapid and sustained response. Although these results suggest that stimulation of renin and prorenin release occurs through different intracellular pathways as previously shown for the pituitary cell line AtT-20 and CHO cells (Paul *et al.* 1992), the stimulation of renin release by AngII may represent epigenetic effects on the phenotype of this transgenic model.

1.5.1.4 Other tissue RAS

Renin activity has long been reported in the brain (Ganten *et al.* 1971). AOGEN, ACE and AngI/II are also found in the brain suggesting that AngII may be produced locally (McKinley *et al.* 2003). AT_1 and AT_2 receptors have also been detected in many parts of the brain where AngII is thought to function as a neurotransmitter involved in sympathetic

control of thirst, sodium excretion and renin release (McKinley *et al.* 2003). All RAS components have also been found in reproductive tissues such as the ovary and the utero-placental unit where locally produced AngII may regulate smooth muscle cell contraction, steroid synthesis and cell growth/differentiation (Nielsen *et al.* 1995). There may be a direct role for renin and prorenin independently of AngII production via binding to the newly identified human renin receptor (Nguyen *et al.* 2002) or to another unknown receptor to trigger non-proteolytic activation of prorenin and/or downstream intracellular signalling.

1.5.2 Renin Uptake

von Lutterotti *et al.* (1994) concluded that, although controversial, renin synthesis does not occur in the heart under normal physiological conditions (von Lutterotti *et al.* 1994). There is, in addition, evidence of an uptake of prorenin by the heart. Müller *et al.* (1998) showed that isolated hearts from transgenic rats over-expressing the human angiotensinogen gene, perfused with human renin, generated AngII (Muller *et al.* 1998). This continued after renin infusion was stopped, suggesting that renin was taken up and sequestered in cardiac tissues and this effect was abolished by the administration of the specific human renin inhibitor, remikiren. In their review, De Mello and Danser (2000) concluded that AngII synthesis in the heart depends on renin and angiotensinogen being taken up by cardiac tissues. Additional reports of prorenin uptake come from transgenic rat studies (Kantachuvesiri *et al.* 2001; Prescott *et al.* 2000). Firstly, a transgenic mouse model was obtained by crossing animals expressing human renin in the liver with animals expressing human angiotensinogen in the heart (Prescott *et al.* 2000). The double transgenic progeny display AngI presence in the heart suggesting uptake of (pro)renin by cardiac tissues. Similarly, in the inducible rat model TGR(*Cyp1a1-Ren2*), the uptake of prorenin by cardiac tissues is demonstrated by a high level of circulating prorenin which precedes cardiac prorenin detection which, in turn, precedes cardiac renin presence (Kantachuvesiri *et al.* 2001). The mechanisms of prorenin uptake remain, however, unclear.

Several molecules have been identified as renin-binding molecules. The mannose-6-phosphate receptors (M6Pr) are involved in the internalisation of prorenin in endothelial cells (van den Eijnden *et al.* 2001) and cardiac cells (Saris *et al.* 2001a; Saris *et al.* 2001b; van Kesteren *et al.* 1997) leading to rapid proteolytic conversion of prorenin to renin. This only applies to glycosylated renin forms since a mannose-6-phosphate group is needed for binding to this receptor. In addition, cardiomyocytes *in vitro* were shown to bind to prorenin

from amniotic fluid and from plasma of anephric patients suggesting that these prorenin species are unglycosylated (Saris *et al.* 2001b). Prorenin uptake and/or conversion to renin could be of significance in the heart for the local formation of AngII and may play a role in cardiac hypertrophy and tissue repair. The uptake process is, however, not unique to prorenin since M6Pr also bind thyroglobulin. The pathway of prorenin uptake followed by proteolytic processing to renin (Saris *et al.* 2001a; Saris *et al.* 2001b) is now accepted as a clearance pathway for prorenin (Schuijt and Danser 2002). This is further supported by the fact that myocyte proliferation in response to stretch is independent on intracellular AngII formation although prorenin is taken up by cardiomyocytes in this study (Saris *et al.* 2002).

The renin-binding protein (RnBp) binds avidly and specifically to renin (as observed in kidney homogenates) and masks its protease activity. It was, however, shown to be identical to an epimerase (Maru *et al.* 1996) and it is thought to be involved in the modulation of the release of active renin from renin-producing cells (Inoue *et al.* 1990). In addition, RnBp *null* mouse mutants displayed no phenotypic changes in renin synthesis, secretion or activity (and other RAS components) or in blood pressure (Schmitz *et al.* 2000) indicating that RnBp has no effect on the RAS cascade.

It has been shown that prorenin through AngII formation can induce cardiomyocyte proliferation but this process was independent of M6Pr and more likely to be due-non proteolytic activation of prorenin (Saris *et al.* 2002). Studies with the inducible hypertensive rat model have shown that uptake of prorenin also occurs in the heart preceding the formation of renin (Kantachuvesiri *et al.* 2001). The source of prorenin in this model is the transgene carrying the mouse *Ren2* gene which gives rise to a non-glycosylated form of prorenin suggesting once again that mechanisms independent of M6Pr are involved (Kantachuvesiri *et al.* 2001).

Recently, Nguyen *et al.* (2002) described a novel renin receptor (in human kidney). It is a 45kDa transmembrane protein. Transfection of the cloned cDNA into non-expressing cells resulted in expression of a protein which specifically binds to renin and prorenin but not to other aspartyl proteases. Binding of this receptor to renin and also prorenin resulted in an increased cleavage of angiotensinogen and the stimulation of an intracellular signalling pathway involving MAP kinases. The discovery of a renin receptor, would not only strengthen the argument that (pro)renin is taken up in tissues but it also implies a direct involvement of prorenin in cardiovascular injuries which may be mediated by uptake or by non-proteolytic/reversible activation.

1.6 Pathophysiology of prorenin

It has been proposed that prorenin/renin may not solely be upstream components of the RAS cascade with the endpoint of generating AngII for blood pressure regulation (Sealey and Rubattu 1989). The arguments for a direct involvement of prorenin are:

1) prorenin possesses a 'renin-like' activity. Although this activity is lower compared to renin, the levels of plasma prorenin are several-fold higher than renin conferring it a substantial capacity for angiotensin production (Skinner 1987);

2) prorenin is present in very high levels in the feto-placental tissues where, unlike in the kidney, it is not converted to renin before secretion and released as prorenin (Poisner 1998). Renin activity can, however, be measured at high levels suggesting an activation of prorenin, possibly via receptor binding or other non-proteolytic pathways (Poisner 1998);

3) the RAS is well known to be hyper-stimulated during development where it participates in angiogenesis (Walther *et al.* 2003) and in cell growth/differentiation (Tebbs *et al.* 1999). In addition, an intact RAS is essential for normal renal development (Guron and Friberg 2000);

4) there is a strong relationship between renal foetal renin concentration and gestational age (Gomez and Norwood 1995). In addition, high levels of prorenin in the amniotic fluid again suggest a role in feto-placental development (Lumbers 1993). High levels of circulating prorenin have been also reported in humans and animal models displaying a variety of pathologies such as diabetes with nephropathy and retinopathy, pre-eclampsia/pregnancy-induced hypertension and hypertension-induced vascular injuries.

1.6.1 Prorenin and Diabetes

Despite renin plasma concentrations being normal of even low in human patients with insulin dependent diabetes mellitus, prorenin values are significantly correlated to a microvascular pathophysiology (Deinum *et al.* 1999). Franken *et al.* had previously reported that increased plasma prorenin levels are a more important determinant in retinopathy than nephropathy (Franken *et al.* 1990). The reasons for increased prorenin levels are unknown and the mechanisms involved remain unclear. It was shown that deregulation of renal renin secretion was not involved since the ratio of arterial to venous renal prorenin was not elevated (Franken *et al.* 1990). This could be due to increased secretion of prorenin from extra-renal sites or decreased degradation of renin in the circulation.

The production of TGF-β1 was demonstrated to be up-regulated in humans with diabetic nephropathy (Yamamoto *et al.* 1993). Furthermore, transgenic mice over-expressing TGF-β1 in JG cells were characterised by increased plasma prorenin concentration (Krag *et al.* 2000). It is possible that prorenin (which has some "renin activity") contributes towards an elevated AngII level since ACE inhibitors and AT_1 receptor antagonists decrease the incidence of vascular complications associated with diabetes (Cooper 2004). Similarly, these treatments have been known to reduce the development of type 2 diabetes in patients with hypertension (Scheen 2004). More recently, Ichihara *et al.* (2004) reported a marked improvement of end-organ damage (notably nephropathy) in streptozotocin-induced diabetic animals after administration of a peptide consisting of the handle region of prorenin. This was accompanied by a decrease in renal AngII. The authors speculated that this "decoy peptide" may competitively bind the putative prorenin/renin receptor and hence, block the non-proteolytic conversion of prorenin with downstream AngII production. The results again suggest a role for prorenin and the prorenin receptor (Nguyen *et al.* 2002) in the onset of vascular diabetic injuries.

1.6.2 Prorenin in Pre-eclampsia and in Pregnancy-Induced Hypertension

Prorenin and renin levels are elevated during pregnancy in humans (de Leon *et al.* 2001) and in the mouse (Xia *et al.* 2002). It was shown that in women with pregnancy-induced hypertension plasma renin concentrations (PRC) are elevated whereas no changes in PRC in women with pre-eclampsia. Conversely, a recent study in which prorenin was measured after acid activation revealed that both renin and prorenin were elevated in pre-eclamptic amnion, chorion and placenta (Singh *et al.* 2004). This is in disagreement with other studies which measured it with different methods and this may be the reason for the discrepancy. Another explanation could be the stage of gestation at which the samples where taken. In the study, the tissues were collected at term whereas in other studies the gestational stage is not mentioned. Increased levels of renin and prorenin only in the chorion have been found in pregnancy-induced patients (Kalenga *et al.* 1996). The fact that the patients displayed pregnancy-induced hypertension rather than pre-eclampsia may explain this difference. It would therefore appear that elevated prorenin in feto-placental tissues might be associated with another form of vascular pathology.

1.6.3 Prorenin and Hypertension

In human patients with hypertension, however, plasma prorenin levels vary widely and it appears that only a sub-group of hypertensive patients display elevated plasma prorenin levels (Atlas *et al.* 1977). In the TGR(*mRen2*)27 rat transgenic model of hypertension, animals displayed suppressed concentrations of active plasma and renal renin, low or unchanged levels of plasma AngII but increased levels of circulating prorenin compared to controls (Lee *et al.* 1995). This and other studies (Bohm *et al.* 1995; Gardiner *et al.* 1993; Jacinto *et al.* 1999) have demonstrated that the hypertension occurring in these rats is caused by activation of AT_1 receptors by AngII in addition to a local RAS stimulation. It has been shown that the expression of the *Ren2* transgene was detected at extra-renal sites (see below) whereas the kidney renin was decreased or unchanged. In the adrenal gland, increased levels of *Ren2* mRNA (*i.e.* from the transgene) correlated with increased steroid excretion (Langheinrich *et al.* 1996). Despite these findings, mineralocorticoid excess did not appear to play a role in the hypertensive pathology since the aldosterone antagonist, spironolactone, did not have anti-hypertensive effects, confirming the involvement of a local adrenal RAS. In addition, bilateral adrenalectomy resulted in a significant decrease in plasma prorenin demonstrating that the adrenal gland is the major source of circulating prorenin in this model (Bachmann *et al.* 1992). However, significant levels of active plasma renin remained after nephrectomy with adrenalectomy (Tokita *et al.* 1994). These results suggest that extra-renal sites of AngII generation and the high levels of circulating prorenin may be potential candidates for the triggering of the injuries observed in the heart, kidney and vasculature of these animals. This is further confirmed by the fact that transgene expression precedes the onset of hypertension (Zhao *et al.* 1993).

Evidence for a local vascular RAS component was given in these transgenic rats in which expression of *Ren2* mRNA was observed in mesenteric/aortic tissues (Hilgers *et al.* 1992). Peters *et al.* reported that plasma prorenin was elevated in those animals which have not yet developed hypertension (Peters *et al.* 1996). Reduction in BP alone was not enough to prevent the vascular and cardiac pathology in TGR(*mRen2*)27 rats and AngII was shown to play a vital role in hypertrophic responses in the vasculature since ACE inhibitors and AT_1 receptor blockers were more effective than diuretics and/or vasodilators in reducing the MH-associated lesions (Brosnan *et al.* 1999). It was suggested by the authors that a local RAS may be involved and that aortic expression of the *Ren2* transgene may lead to increased AngII levels in this tissue causing hypertrophic vascular growth. Similarly, the fact that ACE

inhibitors and AT_1 receptor blockers can ameliorate hypertension without improving cardiac function may be due to locally formed AngII from a local RAS independently of its generation in the circulation. Left ventricular hypertrophy was also present in TGR(*mRen2*)27 rats and they displayed increased wall (media) thickness in the heart and the aorta with focal perivascular fibrosis due to increased myocyte and vascular myocyte size as well as increased synthesis of extracellular matrix proteins (Lee *et al.* 1995). The kidneys of these rats were also subject to pathological alterations, mainly increased thickness of the arcuate and interlobular arteries (Langheinrich *et al.* 1996). Some glomerular damage was also present (Bachmann *et al.* 1992) without affecting renal function in adult rats. The severe increase in blood pressure may also cause endothelial damage and proliferation (also potentially triggered by AngII) with endothelial swelling and fibrinoid necrosis as demonstrated by Kincaid-Smith *et al.* in their model. Renal ischaemia due to vascular alterations can also trigger RAS activation causing a further increase in blood pressure (Kantachuvesiri *et al.* 1999). Due to the susceptibility of this model to MH, the animals had to be kept under blood pressure-lowering treatment.

Another rat model carrying the *Ren2* transgene was generated to study the effects of high levels of circulating rat prorenin by directing the expression of the transgene exclusively in the liver (Veniant *et al.* 1996). Endogenous renin expression in the kidney was similar to wild type unlike in the previous model in which renal renin was decreased but the animals displayed characteristic MH lesions in the heart and in the kidney suggesting a direct effect of circulating prorenin in the pathophysiology of hypertension and that prorenin caused MH lesions independently of blood pressure. The advantages of an on-off switch for the *Ren2* transgene are evident in terms of control over the duration and intensity of the hypertension. The inducible rat model of hypertension, TGR(*Cyp1a1-Ren2*) displayed a rapid increase in BP accompanied by weight loss and polyuria, as a result of salt and water depletion (Kantachuvesiri *et al.* 2001). The animals exhibited hypertensive injuries in several vascular beds and organs. There was a distinct difference in the response to injury, the heart and the mesentery exhibiting greater sensitivity whereas the kidneys appeared to be affected to a lesser extent (Collidge *et al.* 2004). Prorenin was detected in cardiac tissues following transgene induction which preceded the detection of renin suggesting an uptake of prorenin. Prorenin produced in treated TGR(*Cyp1a1-Ren2*) is derived from the mouse *Ren2* gene and does not possess glycosylation sites preventing its binding to mannose-6 phosphate receptors.

Taken together, these results indicate that (pro)renin uptake results from binding and

internalisation through an additional receptor-mediated mechanism. The recent report of a (pro)renin receptor in human tissues demonstrated an increase in AngII formation following binding of prorenin (and renin) to this receptor. The existence of a (pro)renin receptor links the malignant hypertension injuries of TGR(*Cyp1a1-Ren2*) to a locally activated renin-angiotensin-system in the heart (and perhaps also in the vasculature), in particular prorenin and/or inflammation may play an important role in the mechanisms underlying vascular and end-organ injury due to hypertension.

1.7 Aims of the thesis

Considering the potential importance of prorenin and renin in the development of vascular and cardiac injuries in animal models of hypertension and diabetes and in patients exhibiting hypertension, diabetes and pre-eclampsia, the general aim of this thesis is to examine the pathophysiology of (pro)renin and its putative receptor in hypertension. For this purpose, identification of potential mouse homologue(s) to human (pro)renin receptor and cloning the full length cDNA was performed. Expression profile of the putative (pro)renin receptor at different embryonic stages tissues and localisation of this receptor in adult tissues and cell lines was undertaken. Conservation of the putative (pro)renin receptor between species was assessed (*Chapter 3*). In order to establish the potential role of the putative (pro)renin receptor in the pathophysiology of malignant hypertension, an inducible rat model of hypertension characterised by high circulating prorenin, TGR(*Cyp1a1-Ren2*), was used. Detailed cardiovascular phenotyping using echocardio- and echography methods and histopathology of this model was undertaken as well as the regulation of the putative (pro)renin receptor in this high circulating prorenin model (*Chapter 4*). The modulation of the phenotype due to environmental and experimental factors and genetic background was evaluated through survey questionnaires and extensive microsatellite marker screening (*Chapter 5*). The direct involvement of this putative (pro)renin receptor in the cardiovascular injuries of this transgenic model was studied through inhibition of the receptor by a decoy peptide (*Chapter 6*). To complement my studies on the putative (pro)renin receptor, prorenin maturation and renin storage during development was examined. The role of foetal (pro)renin/RAS regulation in foetal renin granulation independently of blood pressure regulation was also be investigated. A separate, parallel study investigated the absence or presence of renin granules in the renal juxtaglomerular cells during the peri-natal period and at identifying when these granules can first be detected in the kidney and provided insight in

the process of prorenin maturation and may help identify the pathways of renin secretion during embryogenesis. Finally, the stimuli involved in the regulation of (pro)renin granulation during peri-natal kidney development were studied and the differential roles of the maternal and the foetal RAS on foetal/neonatal granulation investigated (*Chapter 7*).

Chapter 2

Material and Methods

2.1 Materials

All chemicals and solutions were purchased from Sigma, (UK) unless otherwise stated.

2.2 Common Laboratory Solutions

The following common buffers and solutions were used in this thesis.

10x tris-borate-EDTA buffer
Tris Base: 108g
Orthoboric acid: 55g
Sodium EDTA: 9.3g
Distilled water to final volume of 1L

DNA gel loading buffer
0.025% (w/v) bromophenol Blue
0.025% (w/v) xylene cyanol FF
30% (v/v) glycerol

20x SSC
Sodium chloride: 175.3g
Sodium citrate: 88.2g
Distilled water to final volume of 1L
Adjust to pH 7.4

MOPS solution
MOPS: 20.6g
DEPC-treated 50mM sodium acetate to final volume of 800ml

RNA gel running buffer
MOPS solution: 800ml
0.5M Sodium EDTA: 10ml
Adjust to pH 7
DEPC-treated water to final volume of 1L

RNA gel loading buffer
95% (v/v) deionised formamide
0.025% (w/v) bromophenol Blue
0.025% (w/v) xylene cyanol FF
5mM sodium EDTA (pH:8)
0.025% (w/v) SDS

50x tris-acetate-EDTA buffer
Tris-base: 242 g
Glacial acetic acid: 57.1 ml
0.5M sodium EDTA: 100ml.
Adjust to pH 8.5
Distilled water to final volume of 1L

HEPES buffered saline
Sodium chloride: 0.8g
Sodium phosphate: 27mg
HEPES: 1.2g
Adjust to pH 7.05
Distilled water to final volume of 200ml

Tail Buffer
1M Tris HCl (pH 8): 12.5ml
0.5M sodium EDTA (pH 8): 50ml
5M sodium chloride: 5ml
20% SDS: 12.5ml
Distilled water to final volume of 250ml

2.3 *Cell Lines*

Cell lines, AS4.1 (ATCC no: CRL2193, a mouse kidney tumour cell line), E14TG2a (ATCC no: CRL 1821, a mouse embryonic stem cell line) were kind gifts of Dr P. Lawlor and Dr M. Sharp (University of Edinburgh, UK) and HMC (a human mesangial cell line) was a kind gift from Dr B. Banas (University of Regensburg, Germany). AS4.1 and HMC were grown in monolayers in DMEM/F12 medium (Invitrogen, UK) supplemented with 10% foetal calf serum and 2mM L-glutamine and passaged every 3 days or when 70% confluent with trypsin/EDTA. E14TG2a were cultured in monolayer on gelatin coated-cell culture flasks in GMEM supplemented with 2mM L-Glutamine, 10% foetal calf serum, 100μM sodium pyruvate, 1% non-essential amino-acids, 100μM 2-mercapto-ethanol and 10^3 units of leukaemia inhibitory factor. The cells were passaged every three days with trypsin/EDTA/chicken serum.

2.4 *Animals*

All experimental procedures for this thesis were performed at the University of Edinburgh with the exception of the echographic study on TGR(*Cyp1a1-Ren2*) inducible transgenic rat model (*Chapter 4*) which was performed at the University of Maastricht (The Netherlands) by myself.

2.4.1 Mice – General procedures

The mice were purchased from Charles River (Margate, UK) and Harlan (Oxon, UK). The breeding, maintenance and study of animals were performed according to Home Office regulations (in pairs in standard cages and 12h light-dark cycles). Prior to and during any experimental procedures, mice were given free access to water. For control animals and prior to any experimental procedures, mice were given free access to standard commercial rodent chow containing 0.32% sodium and 0.395% chloride (CRM, Special Dietary Services, Witham, UK). For foetal tissue collection, female mice were paired with males in the afternoon before plug examination and were examined for vaginal plugs the following morning. The time of the plug detection was termed embryonic day 0.5 (E0.5).

2.4.2 Tissue collection from mice – Foetal and adult tissues

For studies described in *Chapter 3*, on the selected time point, the embryos were dissected out of the uterus rapidly under a dissecting microscope. One placental cone was saved and treated as described below for adult tissues. Using fine forceps, the heart and lungs, liver and gut of the foetus were removed to expose the dorsal part of the embryos' abdomen. Gender was determined visually for embryos older than E15.5. The kidneys were removed, placed in RNA*later* (Ambion, Texas, USA) and kept at 4°C until needed. In the case of newborn or young pups, decapitation was performed prior to removing the kidneys.

Cervical dislocation or carbon dioxide asphyxiation was used to collect tissues from the mother such as heart, lungs, submandibular gland, liver, kidneys, adrenal glands, ovary, mesenteric fat, muscle and brain in that order. The adult tissues were also placed in RNA*later* and kept at 4°C until processed for RNA extraction. Tissues for *in situ* hybridisation (whole embryos or adult tissues) were dissected out rapidly and placed on crushed dry ice until frozen and kept at -80°C until required.

2.4.3 Mouse renin granulation studies – Set up and endpoints

Mice were purchased from Charles River (Margate, UK), except for the 1-renin/2-renin gene comparison study for which the FVB/N mice were bought in from Harlan (Oxon, UK). The breeding, maintenance and study of animals were performed according to Home Office regulations (in pairs in standard cages and 12h light-dark cycles). Prior to and during any experimental procedures, mice were given free access to water. For control animals and prior to any experimental procedures, mice were given free access to standard commercial rodent chow containing 0.27% sodium and 0.395% chloride (CRM, Special Dietary Services, Witham, UK). Female C57/Bl6 and FVB/N mice were paired with males of respective strains (in the afternoon before plug examination) and were examined for vaginal plugs the following morning. The time of the plug detection was termed E0.5.

Control C57/Bl6 and FVB/N mice were housed in pairs and the embryos collected after maternal cervical dislocation at E15.5, E16.5, E18.5, P1 and P28. Only mice of C57/Bl6 strain were used for the experimental groups. For treatment groups 1 and 2, mice were housed individually and placed on a low sodium (0.03%) or high sodium (3%) diet, respectively and received the modified diet from E12.5 as to not interfere with implantation and/or intrauterine early development. The modified diets were given in powder form.

For treatment groups 3 and 4, mice were housed individually and received captopril (30mg/kg/day) or enalapril (30mg/kg/day), respectively, by gavage. Due to previous reports of foetal toxicity during maternal exposure, captopril and enalapril gavage was started at E15.5. For the treatment groups 1-4, the embryos were collected at E18.5 and P1. For all treatment and control groups, maternal plasma and foetal plasma was collected in EDTA containing tubes, kept on ice, centrifuged at 9,300g for 10mins at 4°C and stored at -80°C until needed. Maternal plasma was collected by cardiac puncture whereas foetal or newborn plasma collection was performed by decapitation.

For captopril- and enalapril treated-mice, HPLC was performed to detect captopril and enalaprilat (the metabolite of enalapril) in the plasma and whole embryo and to demonstrate placental transfer. Enalaprilat was obtained from QMX laboratories (Thaxted, UK). Only deuterium-labelled enalaprilat was available commercially and could therefore only be used to construct a standard curve for HPLC and not mass spectrophotometry due to difference in mass from enalapril (derived from enalapril given to mice).

2.4.4 Rats – General procedures

2.4.4.1 TGR(Cyp1a1-Ren2) transgenic rats - Maastricht

TGR(*Cyp1a1-Ren2*) animals were bred from a single breeding pair originally obtained from the Molecular Physiology laboratory at the University of Edinburgh and identical to those reported by Kantachuverisi *et al.* (2001).

The breeding, maintenance and study of the TGR(*Cyp1a1-Ren2*) animals at the University of Maastricht (The Netherlands) were performed according to in-house established animal handling guidelines. All animals were housed in pairs in standard cages except when placed in metabolic cages (singly housed) with free access to tap water and standard commercial rodent chow containing 0.25% sodium prior to experimental procedures (Ssniff, Germany) and 12h light-dark cycles. The number of animals used was 10 per group except for the metabolic cages where it was 2 and in the arteriograph where the number of controls was 9.

2.4.4.2 TGR(Cyp1a1-Ren2) transgenic rats - Edinburgh

For experiments performed in *Chapter 6*, the TGR(*Cyp1a1-Ren2*) inducible hypertensive rats were inbred at the University of Edinburgh. The breeding, maintenance and study of animals were performed according to Home Office regulations (in pairs in standard cages and 12h light-dark cycles). Prior to and during any experimental procedures, rats were given free access to water. Rats were given free access to standard commercial rodent chow containing 0.32% sodium and 0.395% chloride (CRM, Special Dietary Services, Witham, UK) which was replaced by diet in powdered form containing 0.3% indole-3-carbinol (see *Chapter 2.3.11*) for induction of the transgene.

2.4.5 Induction of transgene in TGR(*Cyp1a1-Ren2*) rats - Maastricht

For *Chapter 4*, TGR(*Cyp1a1 Ren2*) animals were fed either normal chow with (treated group – 10 animals) or without (control group – 10 animals) 0.3% indole-3-carbinol (I3C) for 5 weeks. The inducer (I3C) was mixed in peanut oil, added to normal chow and reduced to complete powder which was given to the animals *ad libitum* as a thick paste (mixed with water) to avoid food wastage. The animals were housed in pairs for the duration of the study, except for a period of 24h when the animals were placed in metabolic cages.

2.4.6 Blood pressure measurement in TGR(*Cyp1a1-Ren2*) rats – Maastricht and Edinburgh

Systolic blood pressure (SBP) was measured in conscious restrained animals by an indirect tail-cuff method using a tail cuff inflating unit linked to a blood pressure transducer (IICT Life Sciences, USA – *Cf. Annex 4*). The animals were trained for one week prior to the start of the experiments. Training consisted of placing the animals in the heated (30°C) measuring chamber for 15 minutes and then inflating the cuff to take 3-4 mock measurements. SBP was then measured on day 0-3, 7-8, 14, 21, 28, 35. Three measurements were taken for each animal on each recording day and then averaged. Blood pressure was also measured in lightly anaesthesised animals during wall track procedures (described below) under isofluorane anaesthesia in week 5 of treatment.

2.4.7 Food consumption, fluid handling, body weight in TGR(*Cyp1a1-Ren2*) rats - Maastricht

Food consumption was measured per cage at regular intervals throughout induction. The data was calculated and plotted per rat. Body weight was measured prior to the start of the induction and on day 1, 2, 7, 8, 14, 21, 28 and 35. The animals were individually caged in metabolic cages for 12 hours and water intake and urine output were measured. Urine was collected from 2 control and 2 treated animals house individually in metabolic cages. Dipstick tests were carried out using Multistix 10SG (Bayer, Germany) to measure glucose (mmol/L), ketones (mmol/L), urine density, blood trace, proteins (mg/dl) and the presence of leukocytes.

2.4.8 Echocardiography in TGR(*Cyp1a1-Ren2*) rats - Maastricht

Echocardiography was performed under light isofluorane anaesthesia prior to the start of the treatment, during week 1, 2 and 4 of induction. The animals were kept warm on a heat pad (37°C) connected to a rectal probe. The left ventricle (LV) measurements were taken using a 15-Mhz linear array transducer (Hewlett-Packard, USA) interfaced with a Sonos 5500 echocardiography system (Philips, The Netherlands).

Two-dimensional B-mode echocardiograms were acquired from parasternal long axis views and from mid-papillary short-axis views. Digital recordings were analysed off-line using three cardiac cycles as to obtain measurements on both the long and short axis in three systoles and three diastoles per animal per time point. Using long-axis images, left ventricular area, aortic diameter, internal ventricular length and cardiac length were directly measured whereas using short-axis images, wall and lumen thickness were measured directly. Cardiac output, stroke volume and ejection fraction were calculated from the above values.

2.4.9 Aorta wall track in TGR(*Cyp1a1-Ren2*) rats - Maastricht

Aorta wall track was performed under isofluorane anaesthesia during week 5 of induction. Measurements were taken using a 10-Mhz linear array transducer linked to a AU4 echography system (Esaote, Italy) in combination with local aortic pressure measurements (2F Millar probe). Images were sent to a software analysis which permitted manual detection

of the aortic wall and automatic calculations of distension, compliance and pulse wave velocity. Mechanical parameters were calculated at end-systolic blood pressure. An average of 10 heart beats (and an average of 5 repeats) was used per animal. Heart beats during breathing were discarded as the ultrasound signal was distorted due to diaphragm movements.

2.4.10 Organ collection in TGR(*Cyp1a1-Ren2*) rats - Maastricht

At the end of week 5 of induction, the animals were anaesthesised with pentobarbital (intraperitonally) and exsanguinated for blood collection through the abdominal aorta. The blood samples were treated in the same manner described in *Chapter 2.3.6*. The heart was retro-perfused with saline to remove residual blood. The following tissues were collected, rinsed free of blood, blotted dry, weighed and stored as described in Table 2.1.

Organ/vessel	Measurement	Snap frozen	Fixed for histology in formaldehyde
Whole Heart	Weight	N/A	N/A
Atria	Weight	✓	x
Left ventricle	Weight	Cut longitudinally, 1 part ✓	Cut longitudinally, 1 part ✓
Right ventricle	Weight	✓	x
Lungs	Weight	x	x
Liver	None	✓	x
Adrenals (x2)	Weight	1 adrenal ✓	1 adrenal ✓
Right kidney	Weight	Cut longitudinally, 2 parts ✓	x
Left kidney	Weight	Cut longitudinally, 1 part ✓	Cut longitudinally, 1 part ✓
Abdominal aorta	None	x	✓
Thoracic aorta	None	x	✓
Left renal artery	None	x	✓
Mesentery	None	x	Small portion ✓
Mesentery artery	None	x	✓ and used for arteriograph
Tibia	Length	x	x

Table 2.1: Organ collection, processing and storage for Maastricht TGR(*Cyp1a1-Ren2*) animals.

2.4.11 *In vitro* mesenteric artery arteriograph in TGR(*Cyp1a1-Ren2*) rats - Maastricht

Mesenteric resistance arteries were isolated and cannulated in an arteriograph, filled with calcium-free HEPES buffer containing 10μmol/L sodium nitroprusside to insure maximal dilatation. A pressure diameter curve was constructed by recording the lumen diameter stepwise (10mmHg) in intraluminal pressure from 20 to 140mmHg.

2.4.12 Pilot decoy peptide study in TGR(*Cyp1a1-Ren2*) rats, Edinburgh

The animals were fed as described previously (*Chapter 2.3.5*) without addition of peanut oil and weighed prior to the start of treatment, everyday for the first 3 days and every second day thereafter. Three days before the start of the study, the diet was changed from pellet form to powder form (RM1, Special Dietary Services, Witham, UK) and on the day of the start of the dietary induction with 0.3% I3C, an osmotic mini-pump (Model 2002, 14-day use, 0.5μl/hour, Charles River, Margate, UK) was implanted sub-cutaneously under isofluorane anaesthesia with analgesia. The mini-pump was filled with a custom-made peptide (Sigma-Genosys, UK) in saline, (NH_2-RILLKKMPSV-COOH), at a concentration of 0.4mg/kg/day in saline or saline alone. Two animals were given powdered diet mixed with 0.3% I3C in addition to saline-filled osmotic mini-pump and two animals were given 0.3% I3C in addition to the decoy peptide-filled mini-pump.

Systolic blood pressure (SBP) was measured in conscious restrained animals by an indirect tail-cuff method using a tail cuff inflating unit linked to a blood pressure transducer (IICT Life Science, USA). The animals were trained for 5 days prior to the start of the experiments. Training consisted of placing the animals in the restraining tube in a warm (30°C) chamber for 15 minutes and then inflating the cuff to take 3-4 mock measurements. Immediately before starting the induction diet of 0.3% I3C and during the 14 day experiment, 3 measurements were taken for each animal on each recording day and then averaged. SBP and weight were measured on day 0, 1, 2, 3, 5, 7, 9, 11 and 13.

The animals were sacrificed 14 days after mini-pump implantation and dissected for pathological analysis. The tissues taken for pathology were: heart, kidney, and mesentery which were fixed in formaldehyde. Due to the small sample group in this pilot study, only qualitative analysis was performed and no gene expression investigation could be performed.

The pathologist was blinded to the treatment received by the animals as to remove biased analysis.

2.5 RNA Preparations

2.5.1 Tissues Homogenization

For both rat and mouse tissues, a range of 60mg to 100mg of tissue was used with the exception of embryonic kidneys, adrenal glands and ovaries where the amount of tissue was in the range of 5 to 10mg. Placed in Trizol™ (Invitrogen, UK) according to manufacturer's instructions, the samples were homogenized with a bead mill (Mixer Mill MM 300, Retsch, UK) previously optimised for each tissue type to achieve complete disruption (assessed visually).

Subsequently, the samples were incubated at room temperature for 5 minutes after homogenisation. 0.2 ml of chloroform (BDH, UK) per ml of Trizol was added, mixed vigorously, then incubated at room temperature for 2-3 minutes. The mixes were then centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous layer was transferred to a fresh tube. At this stage, 5-10μg of glycogen was added as a carrier to tissue samples of 1-10mg. The aqueous phase was added to 0.5ml of isopropanol (BDH, UK) per millilitre of Trizol, mixed, incubated at room temperature and centrifuge at 12,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 1ml of 70% ethanol. The samples were mixed and centrifuged at 7,500g for 5 minutes at 4°C. The pellet was allowed to dry for 5 minutes at room temperature and then was re-suspended in 50μl of DEPC-treated water.

On some occasions, it was necessary to incubate the RNAs for 10 minutes at 55°C to complete dissolution. Table 2.2 summarises the conditions used for the extracted tissues listed above. The RNA samples were also DNase-treated using an RNase-free DNase kit (Ambion, Texas, USA), then RNA concentration was determined by spectrophotometry at wavelengths 260nm and 280nm. The RNAs were then stored at –80°C.

Tissue Type	Bead Size (mm)	Homogenisation Time (s)	Agitation Speed (Hz)	Volume of Trizol (ml)
Embryonic kidney (x2)	3	2x 120	30	0.1
Pre-wean kidney (x2)	3	2x 120	30	0.1
Ovary (x2)	3	2x 120	30	0.1
Adrenal gland (x2)	3	2x 60	30	0.1
Liver	6	2x 120	30	1
Brain	6	2x 120	30	1
Adult kidney	6	2x180	30	1
Heart	6	2x180	30	1
Lung	6	2x180	30	1
Placenta	6	2x180	30	1
Muscle	6	2x180	30	1

Table 2.2: Homogenisation conditions for RNA extraction from tissues.

2.5.2 Cultured Cells

RNA was extracted from cultured cells using an RNA preparation kit (RNeasy, Midi kit, Qiagen, UK). The cells were grown to confluency, rinsed with PBS before being collected using the lysis buffer provided and the RNA extracted as per manufacturer's instructions. RNA samples were also DNase-treated using an RNase-free DNase kit (Ambion, Texas, USA), then RNA concentration was determined by spectrophotometry at wavelengths 260nm and 280nm. The RNAs were then stored at –80°C.

2.6 Reverse Transcription

Two micrograms of RNA were used in a reaction containing random octamers (1pMol) and dNTPs (1mM). This was incubated at 65°C for 5 minutes. Forty units of RNase Inhibitor (Sigma, UK), 10mM DTT and 25 units of Superscript II Reverse Transcriptase (Invitrogen, UK) were added and the samples were incubated under the following conditions: 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes. Negative controls were also made by replacing the enzyme by 0.5µl of reaction buffer.

2.7 *Mouse (Pro)Renin Receptor Polymerase Chain Reaction*

Polymerase chain reaction (PCR) was performed on the reverse transcribed samples using the primers (MGW Biotech, Germany) described in Annex I.

The PCR conditions were as follows: Taq polymerase activation for 10 minutes denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C for mouse (pro)renin receptor coding sequence primers or 61°C for mouse (pro)renin receptor whole cDNA primers and polymerisation/extension for 2 minutes at 72°C. Using rat (pro)renin receptor primers, the annealing temperature was 58°C for 45 seconds. Optimisation data for the mouse primers are shown in *Chapter 3*. Two and a half units of a Taq polymerase (HotStartaq, AbGene, UK) was used with 5pMol of each primer, 1.5mM$MgCl_2$ and 10mM dNTP. The samples were amplified for 30 cycles and analysed by agarose gel electrophoresis in 1x Tris-acetate-EDTA buffer.

2.8 *Sequence Identity Analysis*

A sequence identity search was conducted using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/) which is a set of similarity search programs designed to explore all of the available sequence databases with the human (pro)renin receptor cDNA clone, N14F (Nguyen *et al.* 2002) (GenBank: AF291814). The resulting mouse sequences were located in the mouse genome sequence using ENSEMBL (http://www.ensembl.org/Mus_musculus/). Vector NTI 5.3.0 was used to translate cDNAs into amino-acid sequence and ClustalW was used for sequence alignments (Chenna *et al.* 2003). Hydrophobicity prediction plots were obtained using TMHMM (Krogh *et al.* 2001). A bacterial artificial chromosome (BAC - 12J18) containing the putative mouse (pro)renin receptor gene of strain C57/Bl6 was purchased from Invitrogen, UK.

2.9 *cDNA purification and TA cloning*

PCR products were excised from a 0.8% agarose gel containing ethidium bromide under UV illumination, and purified using a gel purification kit (QiaQuick, Qiagen, UK). Where needed, the product was cloned using a TA cloning kit (pGEM-t easy kit, Promega, UK) using the protocol described in Table 2.2. 1µl of the resulting DNA was electroporated into

electrocompetent *E.coli* DH5α and plated onto ampicillin (100μg/ml) agar plates containing X-Gal (80μg/ml) and IPTG (0.5mM). Successful cloning of a PCR product using the aforementioned kit interrupts the coding sequence of β-galactosidase, producing white colonies. However, it remains possible that the PCR product may integrate in-frame with the lacZ gene retaining β-galactosidase activity and producing blue colonies. Positive white colonies were selected, grown in 25ml cultures and the DNA extracted with a Midi plasmid preparation kit (Sigma, UK).

	Standard Reaction	**Positive Control**	**Background Control**
10x T4 ligase Buffer	1μl	1μl	1μl
pGEM-T Easy Vector (50ng)	1μl	1μl	1μl
PCR Product	2μl	-	-
Control Insert DNA	-	2μl	-
T4 DNA Ligase (3 units/μl)	1μl	1μl	1μl
Deionised water to a volume of	10μl	10μl	10μl

Table 2.3: TA cloning protocol.

The plasmids were sequenced on an ABI Prism 377 DNA sequencer using BigDye 2 sequencing kit by N. Wrobel (Molecular Physiology, University of Edinburgh) and the orientation and sequence of the PCR product examined by restriction mapping.

2.10 Real-time quantitative PCR

Quantitative PCR analysis in real time was performed using an Applied Biosystems 7000 sequence detector using SYBR green fluorescence with primers for the rat renin receptor described in Annex I. The SYBR green results were normalised using a human 18S Taqman assay (Applied Biosystems, UK). Reactions were performed in 25μl and cycling was for 2 minutes at 50°C, 15 minutes at 95°C and product amplification by 40 cycles of 95°C (15s) and 60°C (1 minute). The tested samples were plated in quadruplicates and each five point of the standard curve was plated in triplicates. The tested samples were read against their respective standard curves and normalised with 18S. Standard curves for 18S with a slope of -3.03 (representing optimal doubling efficiency of product being amplified) were used. An n number of 5 or 6 (as detailed on each graph) was used per tissue/treatment and a Student t-test was performed between respective treatment and control groups.

2.11 Microsatellite marker PCR

Genomic DNA was extracted from liver samples (animals sampled in Maastricht) or ear/tail biopsies (Animals sampled in Edinburgh) using phenol-chloroform organic phase extraction. Polymerase chain reaction (PCR) was performed on genomic DNA. Using microsatellite marker primers (See Annex I), PCR conditions were as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C and polymerisation/extension for 1 min at 72°C. Half unit of a Taq polymerase (AbGene, UK) was used with 5pMol of each primer, 1.5mM$MgCl_2$ and 10mM dNTP. Samples were amplified for 30 cycles, added Cresol red dye for visualisation and analysed by acrylamide gel electrophoresis in 1x tris-borate-EDTA buffer (TBE). Gels were then stained in a solution of ethidium bromide (1mg/ml) in 1xTBE.

2.12 Northern Blotting and Hybridisation

2.12.1 Agarose/formaldehyde RNA gel

400mls of agarose/formaldehyde gel mixture was prepared by melting 4g of agarose in 250ml of DEPC-treated water. 1.1 part of 5x running buffer and 1 part of formaldehyde were added to the agarose. The gel was cast in an electrophoresis tray and allowed to set for at least 30min.

2.12.2 Sample preparation

Up to 30µg of RNA in 4.5µl was mixed with 2µl of 5x gel running buffer, 3.5µl of formaldehyde and 10µl of formamide and incubated at 65°C for 15 minutes. 2µl of DEPC-treated gel loading buffer were then added to the samples. The samples were placed on ice while the gel was pre-run 5V/cm for 5 minutes. The samples were loaded and separated on the agarose gel at a voltage of 3-4V/cm until bromophenol blue had migrated 8cm. Half way through electrophoresis, the running buffer was collected, mixed and returned to the tank.

2.12.3 Blot

The gel was placed up side down on a 20x SSC –soaked blotting paper and overlaid successively with one sheet of nylon membrane (Hybond XL, Amersham-Pharmacia, UK), 3

pre-soaked sheets of blotting paper and blotting paper towels. The blotting procedure was performed overnight. The membrane was then baked at 80°C for 2 hours and stored between two layers of blotting paper until needed.

2.12.4 Pre-hybridisation and hybridisation

Na_2HPO_4 (1M) and Modified Church and Gilbert buffer (0.25M Na_2HPO_4, 7% SDS. 1mm EDTA) were pre-warmed to 65°C. The membrane was placed in a roller bottle in a hybridisation over and incubated in Na_2HPO_4 (1M) and Modified Church and Gilbert buffer at 65°C for 30mins each. While the membrane was pre-hybridising, the ^{32}P labelled-probe was prepared using Ready-to-use labelling beads (Amersham-Pharmacia, UK). 50ng of PCR product (made with primers JJM515-516) was used. The probe was purified and eluted in Tris-EDTA buffer. Church and Gilbert buffer was replaced by fresh pre-warmed buffer and the probe added in the hybridisation buffer (not directly on the membrane) and incubated overnight at 65°C.

2.12.5 Post hybridisation Washes

In the roller bottle, the nylon membrane was washed twice with pre-warmed 2xSSC, 0.1% SDS for 20 minutes and twice with 0.2xSSC, 0.1% SDS for 20 minutes at 65°C. Higher stringency (0.02% SSC) was performed if the membrane was still "hot" (*i.e.* counts per second above 10). The membrane was pat dried, wrapped in cling film and exposed to X-ray film with an intensifying screen at -80°C overnight.

2.13 In situ hybridisation on frozen tissue sections

2.13.1 ^{35}S-UTP RNA probes

Reagents were added to an RNase-free tube in the following order: 5x transcription buffer (Promega, UK), 1mM ATP, CTP and GTP, 10mM dithiothreitol, the linearised DNA template [for the mouse putative (pro)renin receptor probes, the plasmid constructed by TA cloning of RT-PCR product from mouse kidney with primers JJM 515-516 was restriction digested with *SpeI* for the anti-sense probe or with *ApaI* for the sense probe], 40 units of

RNase inhibitor (Promega, UK), RNA polymerase (20 units T7 RNA polymerase for the anti-sense probe and 15 units SP6 RNA polymerase for the sense probe) and 160μCi ^{35}S-labelled UTP. The reaction was carefully mixed and incubated for 1 hour at 37°C for the T7 RNA polymerase or 40°C for the SP6 RNA polymerase. DNase treatment was then performed using RQ1 DNase (Promega, UK) at 37°C for 10 minutes. The RNA probes were then purified on a Nick column (Amersham-Pharmacia, UK) and eluted in 400μl of 1M Tris-EDTA buffer and kept at -20°C.

2.13.2 Tissue sections

7mm frozen tissue sections were prepared in a cryostat microtome (Bright 5040 OTF, UK) at -20°C on commercially purchased polylysine–coated slides (BDH, UK) and kept at -80°C until needed.

2.13.3 *In situ* hybridisation procedure

The sections were fixed and dehydrated at room temperature in successful incubations of the following solutions: 4% cold paraformaldehyde (10 minutes), 1x phosphate buffered solution (5 minutes, twice), 0.1M triethanolamine with 0.25% acetic anhydride (10 minutes), 1x phosphate buffered solution (3 minutes), 70% ethanol (2 minutes), 80% ethanol (2 minutes) and 95% ethanol (2 minutes). The slides were then left to air dry for a minimum of 30 minutes.

Pre-hybridisation was performed in sealed containers lined with blotting paper soaked in 4x SSC with 50% deionised formamide. The slides were placed in this environment covered with 200μl of the following buffer: 0.12M NaCl, 2mM Tris (pH: 7.5), 0.2x Dehnardt's, 200μM EDTA, 100μg salmon sperm DNA, 2mg yeast tRNA and 50% deionised formamide and incubated at 50°C for 2 hours. The probe buffer was made of the same mix containing the probe (40 million counts/minute) and 50% deionised formamide. This was heated up to 80°C for 10 minutes, cooled down on ice and DTT added to 10mM. The pre-hybridisation buffer was replaced by the probe-containing buffer and incubated at 50°C for 16 hours.

The slides were drained and rinsed in three 2x SSC washes for five minutes each. 200µl RNase containing buffer (0.5M NaCl, 10mM Tris, 1mM EDTA, 30µg RNase A) was added to the sections and incubated at 37°C for 1 hour to remove unbound probe.
The slides were then washed through the following treatment: 2xSSC at room temperature for 30 minutes, 0.1xSSC at 60°C for 1 hour, 0.1xSSC at 60°C (and cooled at room temperature) for 1 hour. Dehydration was performed in successive washes of 50% ethanol in 0.3M ammonium acetate, 70% ethanol in 0.3M ammonium acetate and 90% ethanol in 0.3M ammonium acetate. The slides were left to dry completely at room temperature and exposed to X-ray autoradiography for 48 hours. When quantitative measurement was needed, the slides were dipped into liquid silver chloride emulsion, exposed for two to three weeks in the dark at 4°C and the image analysed for silver grain deposition.

2.14 Histology

These techniques were performed at the University of Edinburgh, Core pathology facilities and the pathology assessment was performed by Dr. David Browstein.

2.14.1 Paraffin embedding and paraffin sections

After initial fixation in formalin (overnight for large adult tissues), the tissues were processed using an automated paraffin wax embedder (Citadel 2000, Shandonn, UK). Firstly, the dehydration steps were done at 35°C, each for 1 hour: 50% ethanol, 70% ethanol, 95% ethanol, 99% ethanol (twice), 100% ethanol (twice), xylene (thrice).

The paraffin steps were then carried out at 60°C: wax (1 hour, 4 times). The tissues were then placed in the correct orientation into moulds and covered in paraffin wax. The paraffin blocks were kept at 4°C until sectioned. The tissues were sectioned with a microtome (Bright 5040, Bright, UK) and the resulting 3µm-sections were bound to pre-coated polylysine slides (BDH, UK) before drying them at 37°C overnight.

2.14.2 Haematoxylin-Eosin staining of paraffin sections

All steps were performed at room temperature. The tissue sections were de-paraffinised and re-hydrated by successive incubations in xylene (5 minutes), absolute ethanol (2 minutes),

99% ethanol (2 minutes), 70% ethanol (2 minutes) and tap water (2 minutes). Staining was performed by placing the sections into haematoxylin for 5mins, tap water (5 seconds), acid alcohol (0.12M hydrochloric acid in 99% ethanol; 5 seconds), tap water (5 seconds) and eosin for 2 minutes before a final rinse in tap water. The sections were then dehydrated in 95% ethanol, 99% ethanol and twice in absolute ethanol, each for 5 seconds. The sections were stored in xylene until mounted with DPX (BDH,UK) and sealed with a glass coverslip.

2.14.3 Sirius Red staining of paraffin sections for collagen fibres

Sections were de-paraffinised and re-hydrated by a series of 5 minute-incubations as follows: xylene (twice), 100% ethanol, 98% ethanol, 70% ethanol, 30% ethanol and distilled water (twice). Slides were then incubated in saturated picric acid (aqueous solution of saturated picric acid containing 0.1% Fast green FCF and 0.1% direct red 80) for 2 hours. A wash step in distilled water was performed for 6 minutes before dehydrating the sections using a reverse ethanol series. Slides were mounted using Permount and a glass coverslip.

2.14.4 Gomori's stain on paraffin sections for reticulin fibres

The sections were de-paraffinised and re-hydrated by a series of incubations as follows: xylene (5 minutes – twice and 2 minutes once), absolute ethanol (5 minutes - twice), 90% ethanol (2 minutes), 70% ethanol (2 minutes), 50% ethanol (2 minutes) and tap water (5 minutes). The sections were then treated as follows with 5 minute-washes in tap water between each step: oxidisation using acidified potassium permanganate (1% in hydrochloric acid), incubation in oxalic acid (1%), sensitisation in iron alum (2.5%), reduction in 10% formalin, staining in gold chloride (0.2%), fixation in sodium thiosulphate (5%) and nuclei counterstaining with safranin (1%). The section were then de-hydrated in the reverse ethanol-xylene series and mounted in DPX and a glass coverslip.

2.15 Electron Microscopy

Embryonic kidneys were carefully isolated and one kidney per embryo was used for electron microscopy examination. The rest of the procedure was performed at the Department of

Pathology, University of Dundee and the presence or absence of renin granules was assessed by Prof. Stewart Fleming.

The samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C, for a minimum of 4 hours. Tissues were cut into 1mm cubes for fixing, washed in 0.1M buffer overnight at 4°C, post fixed in osmium tetroxide in 0.2M buffer for 1 hour and rinsed in 0.2M buffer before being dehydrated in an ethanol and propylene oxide series. The samples were then gradually processed into epoxy resin and allowed to polymerise at 60°C for 48 hours. Several 1μm sections were cut using glass knives and an ultramicrotome, then dried onto a glass slide on a hotplate at 80°C to ensure adhesion. Staining with 1% toluidine blue in 1% borax solution for 1 minute at 80°C was performed before mounting the sections with a coverslip. Target areas of the sections were identified before thin sectioning was performed using a diamond knife at around 100nm. The sections were picked up onto 300 mesh (300 squares), thin-bar, copper grid.

2.16 Statistical analysis

All graphs show mean ± standard error. *: $p>0.05$; **: $p>0.01$; ***: $p>0.001$. Statistical analysis consisted of Student's t-test for individual variables.

In the events of several time points (or pressure in the case of arteriography) being recorded for the same animals, two batteries of statistical tests were used:

1) Repeated measure two-way Analysis of Variance (ANOVA) was employed.
2) However, if data points were missing due to experimental procedures (such as animals being restless during blood pressure measurements), Prism statistical analysis software did not allow repeated measure two-way Analysis of Variance (ANOVA). For this reason, a standard two-way Analysis of Variance (ANOVA) was undertaken as stated in the text.

Bonferroni Post-test was deemed too stringent if a high number of repeated measurements were recorded. It was used solely to examine differences at individual time points for measurements such as cardiac output and stroke volume recorded on 4 different occasions. Least significant difference Post test was employed if a high number of repeated measurements, for example, blood pressure measured food consumption measured on 15 different occasions.

Due to weight loss in TGR(*Cyp1a1-Ren2*) animals, organ weights were not expressed over body weight but normalised to tibial length which was judged to remain constant throughout the experimental period.

Chapter 3
The Putative (Pro)Renin Receptor in the Mouse

3.1 Introduction

Nguyen *et al.* (2002) recently described the cloning of a human (pro)renin receptor that was found to be expressed in multiple tissues and was localised to the mesangium of glomeruli, the coronary artery and the vascular bed of the placenta. Transfection of the cDNA into human foetal mesangial cells (HMC) resulted in expression of a membrane-associated protein that specifically bound to renin and prorenin but not other aspartyl proteases. Binding resulted in a 5-fold increase in angiotensinogen cleavage (compared with renin or prorenin in solution), the rapid phosphorylation of the receptor and the activation of ERK1 and ERK2 (MAP kinases).

A (pro)renin receptor has important implications in RAS biology in normal physiology and in pathological situations and there are several ways in which it may play a pivotal role. Firstly, a (pro)renin receptor may be involved in (pro)renin uptake. Mannose-6-phopshate receptors are well known to play a role in this event in cardiac and vascular tissues (Peters *et al.* 2002; Saris *et al.* 2002; van den Eijnden *et al.* 2001; van Kesteren *et al.* 1997) but this process is limited to the glycosylated form of renin. The role of mannose-6-phosphate receptors in cardiac and vascular hypertrophy has been extensively investigated (Saris *et al.* 2001a; Saris *et al.* 2001b; Saris *et al.* 2002). The data suggest that although this receptor can bind and internalise prorenin (Saris *et al.* 2001b), this pathway may be a clearance pathway for prorenin since proteolytic activation of prorenin was observed following receptor binding (Saris *et al.* 2001a).

It was previously demonstrated in TGR(*Cyp1a1-mRen2*) that prorenin resulting from the mouse *Ren2* gene (hence non-glycosylated) is present in the heart prior to mouse renin detection suggesting that a (pro)renin receptor may be involved in uptake and activation of prorenin. Secondly, non-proteolytic reversible activation of prorenin has been shown to play

a role in diabetic nephropathy in a diabetic rat model and it was demonstrated that a rat (pro)renin receptor was involved (Ichihara *et al.* 2004).

In addition, putative (pro)renin receptor over-expression in smooth muscle cells of a transgenic rat model resulted in increased heart rate and in progressive elevation of systolic blood pressure (Burckle *et al.* 2006). It is also possible that a (pro)renin receptor may play additional roles in normal (such as during gestation or in ovarian biology) or abnormal (such as hypertension) physiology associated with prorenin secretion and non-proteolytic activation of prorenin.

The aim of this chapter is to identify potential mouse homologues of the human (pro)renin receptor reported by Nguyen *et al.* (2002) using bioinformatics tools and to clone the cDNA(s) for future work. Sequence comparison of the human (pro)renin receptor with a vacuolar proton-ATPase membrane sector-associated protein (M8-9 (Ludwig *et al.* 1998)) in a variety of species including mouse, chicken and *C.elegans* is also reported. The findings suggest that the (pro)renin receptor is conserved between these species. The protein identified as the (pro)renin receptor may also function as part of a vacuolar ATPase protein complex and is the product of a single gene. In addition, we studied the mouse homologue and found that at the cDNA and protein levels, mouse and human sequences are highly similar. Transcripts were found (using RT-PCR) in all adult tissues examined and in the kidney from E9.5dpc indicating that the molecule is ubiquitously expressed in the mouse. It was not possible to identify a tissue or cell line negative for the mouse putative (pro)renin receptor and expression was also detected in embryonic stem cells.

Once the existence of a murine homologue of the human (pro)renin receptor was established, the mRNA expression patterns in mouse adult and foetal tissues were investigated, as well as the cellular localisation of this putative receptor. In all other tissues, the receptor expression was ubiquitous. In the kidney, results suggest that the receptor is expressed differentially and that it is present in the renal cortex but not the medulla and more precisely in smooth muscle cells of the arterioles.

3.2 Results

3.2.1 Genomic structure and homology searches

Three mouse cDNA sequences were found by BLAST using the human (pro)renin receptor cDNA sequence N14F [(Nguyen *et al.* 2002), GenBank accession no: AF291814)]. The first

was from a mammary tumour library generated from a mouse of FVB/N strain. The other two clones were from an 8-day mouse embryo whole body and from a mouse neonatal head library, both of C57/Bl6 origin (GenBank: AK017482 and AK029405, respectively). The cDNAs were all approximately 2Kb in length and had a stop codon located within exon 9 producing a long 3' untranslated region of approximately 800bp (Figure 3.1).

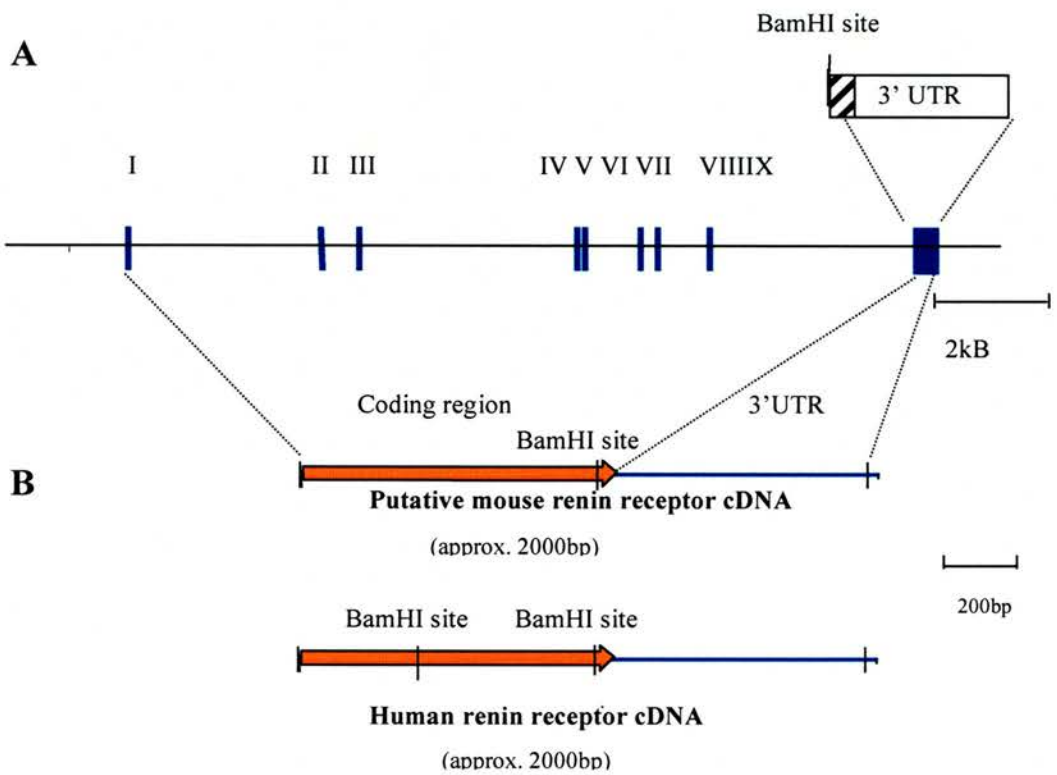


Figure 3.1: (Pro)renin receptor gene and cDNA structure. A) Genomic structure of the putative mouse (pro)renin receptor. Roman numerals represent the exons. The striped area indicates the coding region of exon 9 while the plain area indicates the 3' untranslated region of exon 9. B) Alignment of mouse putative (pro)renin receptor with human rennin receptor cDNAs. The orange arrow denotes the coding region of the cDNA while the blue line denotes the 3' untranslated region. Note the position of the conserved *BamHI* restriction site at the 3' end of the coding region between human and mouse cDNAs.

The mouse cDNAs mapped to chromosome X in the mouse genome, more precisely at locus A1.2 (Figure 3.2). From this locus, a C57/Bl6 BAC clone (12J18) was identified using ENSEMBL.

The BAC sequence by comparison with cDNA sequence predicts that the mouse putative (pro)renin receptor gene is 25Kb in length and has 9 exons and 8 introns (Figure 3.1). Another genomic match was obtained by ENSEMBL on chromosome 1. However, no identified gene, cDNA or ESTs are present in this region. This may be evidence for a pseudogene, the function of which is unknown.

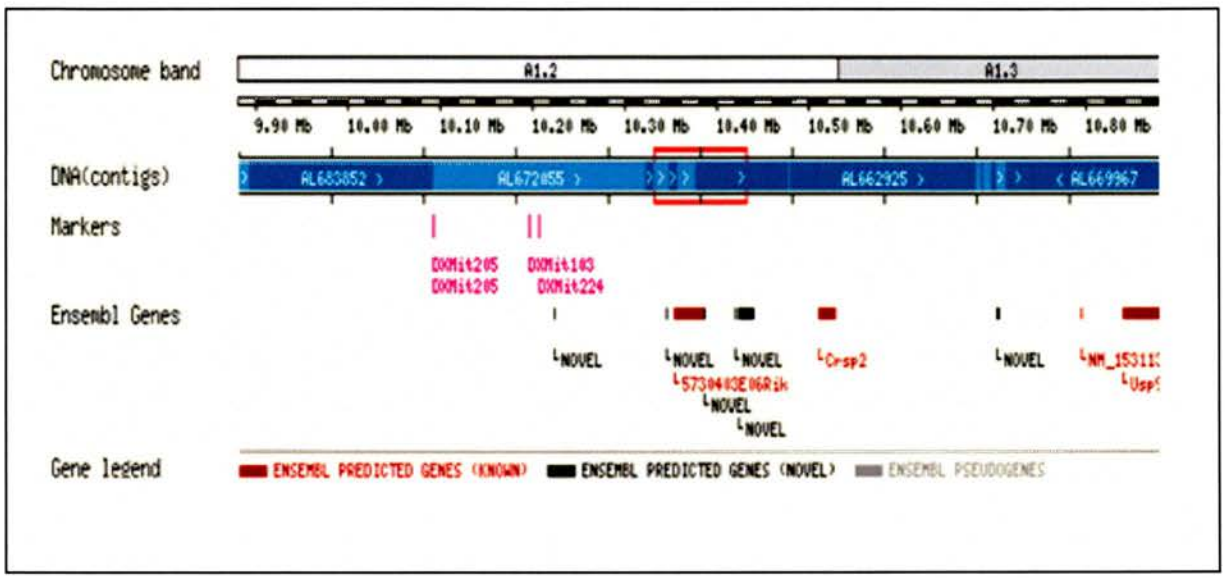


Figure 3.2: Putative (pro)renin receptor Contig from Ensembl. The sequence was from Chromosome X of the mouse genome. The cDNA clone BC014706 (57304E06Rik) is found within this contig.

3.2.2 Sequence alignments

DNA sequences of the three murine putative (pro)renin receptor cDNA clones found by BLAST were aligned and compared with the human (pro)renin receptor clone (Figure 3.3). Very few differences in sequence were observed between mouse strains with an average of 83% sequence identity between all 3 cDNAs. The mouse putative (pro)renin receptor protein has two hydrophobic regions located at amino acid position 219-235 and amino acid position 336-350.

Mouse cDNA	*Whole cDNA*	*Coding Region*	*3' Untranslated Region*
BC014706	82.9%	88.8%	75%
AK017482	81.6%	88.2%	73.2%
AK029405	82.8%	88.1%	80.2%

Figure 3.3: Nucleotide sequence alignment matching of putative (pro)renin receptor. Three mouse cDNA clones, BC014706, AK017482 and AK029405 were aligned against the human (pro)renin receptor nucleotide sequence. Results are expressed as a percentage of nucleotides matching the human (pro)renin receptor within the whole cDNA, the coding region only and the 3' untranslated region.

```
Mouse MAVLVVLLFFLVAGALGNEFSILRSPGSVVFRNGNWPIPGDRIPDVAALSMGFSVKEDLSWPG
Human  MAVFVVLL-ALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSWPG

Mouse  LAVGNLFHRPRATIMVMVKGVDKLALPAGSVISYPLENAVPFSLDSVANSIHSLFSEETPVVL
Human  LAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEETPVVL

Mouse  QLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSLLNSLPLNSLSRNNEVDLLFLSELQ
Human  QLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVDLLFLSELQ

Mouse  VLHDISSLLSRHKHLAKDHSPDLYSLELAGLDELGKRYGEDSEQFRDASKILVDALQKFADD
Human  VLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKILVDALQKFADD

Mouse  MYSLYGGNAVVELVTVKSFDTSLVRKSRTILEAKQ-ENTQSPYNLAYKYNLEYSVVFNLVLW
Human  MYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYNFEYSVVFNMVLW

Mouse  IMIGLALAVIITSYNIWNMDPGYDSIIYRMTNQKIRID
Human  IMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD
```

Figure 3.4: Alignment of amino acid sequences between human (pro)renin receptor and mouse putative (pro)renin receptor. Shaded sequences indicate identity. Bold amino acids indicate conservative substitution while unshaded amino acids denotes a non-conservative substitution.

The data clearly show that the mouse sequences are highly similar to the human sequence reported by Nguyen *et al.* (2002). At the amino acid level, human and mouse proteins also displayed sequence identity. Only 22 of the total 350 amino acids differed between the two species (Figure 3.4), 10 of them being conservative substitutions. The 3' untranslated region of the (pro)renin receptor showed the lowest identity sequence between mouse and human suggesting it may not be as important for gene function.

When carrying out the BLAST search, a truncated vacuolar ATPase protein called M8-9 (Ludwig *et al.* 1998) from adrenal chromaffin cells (co-purifying with V-ATPases) was found to have homology with the human and mouse (pro)renin receptor cDNA and also the mouse cDNA. Amino acid alignment between the bovine M8-9 V-ATPase sub-unit (30 amino acids) and the human (pro)renin receptor translation showed a high degree of sequence homology (Figure 3.5A). Eight out of the 30 amino acids differed between the two sequences, 3 of which were conservative substitutions. Similar analyses revealed that 10 amino acids differed between the murine and the bovine sequences (Figure 3.5B), 3 of which were conservative substitutions.

Nucleotide sequence identity was also demonstrated between the human (pro)renin receptor and M8-9 (Figure 3.6).

A

```
Bovine M8-9          (1)   -ETKQVKDPSTTYNLAYKYNFEYPVVFNLVL----
Human (N14F)         (281) LEAKQAKNPASPYNLAYKYNFEYSVVFNMVLWIMI
```

B

```
Bovine M8-9          (1)   --ETKQVKDPSTTYNLAYKYNFEYPVVFNLVL---
Mouse (BC014706)     (281) ILEAKQENTQ-SPYNLAYKYNLEYSVVFNLVLWIM
```

Figure 3.5: M8-9 alignments. A) Alignment of amino acid sequences between human (pro)renin receptor and bovine M8-9 vacuolar ATPase sub-unit B) Alignment of amino acid sequences between mouse (pro)renin receptor and bovine M8-9 vacuolar ATPase sub-unit. Shaded sequences indicate homology. Bold amino acids within the shaded sequence indicate conservative substitution while unshaded amino acids denote a non-conservative substitution. Numbers in brackets indicate the amino acid number.

```
Renin_receptor  MAVFVVLLALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSW 60
M8-9            MAVFVVLLALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSW 60
                ************************************************************

Renin_receptor PGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEE 120
M8-9           PGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEE 120
               ************************************************************

Renin_receptor TPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVD 180
M8-9           TPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVD 180
               ************************************************************

Renin_receptor LLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKIL 240
M8-9           LLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKIL 240
               ************************************************************

Renin_receptor VDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 300
M8-9           VDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 300
               ************************************************************
VATN                      MYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 50

Renin_receptor FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 350
M8-9           FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 350
               ************************************************
VATN           FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 100
```

Figure 3.6: CLUSTAL alignment of human amino acid sequences. (Pro)renin receptor (GenBank accession AF291814) and M8-9 (GenBank accession NM_005765). In addition, the shorter sequence described as M8-9 (VATN; Swissprot accession 075787) was aligned manually to show extent of its identity with the other sequences. Figure compiled with the help of Dr D. Dunbar.

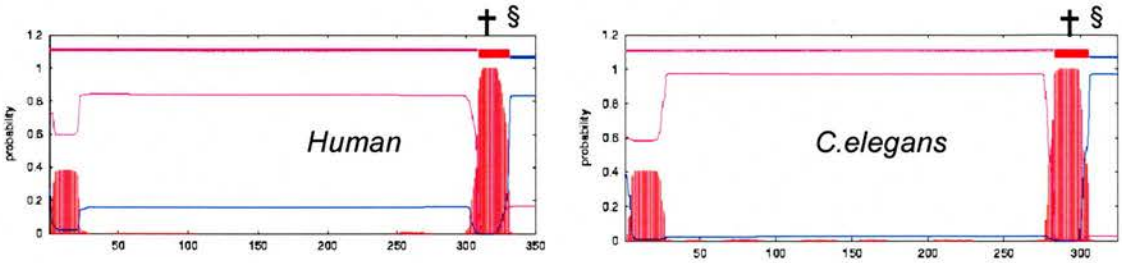
3.2.3 (Pro)renin receptor phylogenic conservation

Conservation of the putative (pro)renin receptor in several species was studied using the homologous sequences available in the ENSEMBL database. A multi-species protein sequence comparison (Figure 3.7A) revealed homologues to the human receptor in a variety of species including rat, mouse (as already mentioned), chicken, frog, zebrafish, mosquito or drosophila suggesting that the putative (pro)renin receptor gene is highly conserved between a large number of species. The multi-species sequence alignment (Figure 3.7B) shows homologues in species as remotely related to humans as *C.elegans*.

A

	Mouse	Human	Rat	Chicken	Frog	Zebrafish	Drosophila	Anopheles	C.elegans
Mouse	100	92	96	79	68	67	27	23	20
Human		100	91	79	69	68	26	23	20
Rat			100	78	68	67	26	22	21
Chicken				100	69	67	24	23	22
Frog					100	63	26	22	21
Zebrafish						100	23	22	22
Drosophila							100	36	21
Anopheles								100	20
C.elegans									100

B



C

```
Human       MAVFVVLLALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSWPGLA 64
C_elegans   MKFVLLVSALVAACAASSLEIIAAPASLKFPDADATLKTTLISTLNENILGLSARQVDGFS--V 64
            * ..::: ****.  ...:.*: :*.*: * :.: .:    *. :    :*:*.::  .:.  .

Human       VGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEETPVVLQLA 128
C_elegans   SVDMFSRPRALAVVTVIGADNLNLG-----------------------GKTYSTKSDGVENVG 102
          : :* **** .:* * *.::* *                          .:* ::     * ::.

Human       PSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVDLLFLSELQVLHD 192
C_elegans   FDQDMALIFGADR---ENVQVSAGGITGSMLALAAQQEAVDATVIKTKR---STLVKELEAVYQ 160
            .:: . :.*  .   *::.*:   : . : :   :  .::  . :. :.     ::.**:.:::

Human       VDALQKFADDMYSLYGGISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKI 256
C_elegans   KAAIEALTASINNAYGGLAAAIKSAG-VQMDNNADVFRVSITGLVGI-----TESAQKEEAVAD 218
          *:: :: .: . ***::: :.    :  *:..*:: :.:::*  *      :*  * . *:   *

Human       LNAVVELVTVKSFDTSLIRKTR-----TILEAKQAKNPASPYNLAYK-------YNFEYSVVFN 308
C_elegans   VQAVIELLAFEAESGAAEKSTESREIPNHNIQKRDTKSKTPTQLARENLQVTVPVSSDYPAIFA 302
            ::**:**::.:: . :  :.*.     .    *: .:. :* :* * :       . :*..:*

            Human       MVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 348
            C_elegans   IFLGLVVILVVALIYIVVGMASIDPEKDSIIYRMTTTRMKKD 322
                        :.* ::: *.:*:*    .: .:**  ********. ::: *
```

Figure 3.7: Conservation of amino acid sequence of (pro)renin receptor/M8-9. A) Sequence identity matrix showing percentage identity of amino acid sequence between nine species. B) Hydrophobicity plots for human (pro)renin receptor and C.elegans M8-9 generated by TMHMM program. "†" denotes transmembrane regions and "§" denotes highly conserved intracellular/vacuolar domains. C) CLUSTAL alignment of amino acid sequence for human (pro)renin receptor and *C.elegans* M8-9. "*" = identity, ":" = conserved substitution, "." = semi-conserved substitution. Figure compiled with the help of Dr D. Dunbar.

It is worth noting that the regions showing greater homology between the human sequence and *C.elegans* encompass the transmembrane region of the protein and the predicted intracellular/intravacuolar portion of the molecule as shown on the hydrophobic prediction plot (Figure 3.7C). Analysis of the database showed that the genomic structure described above (Figure 3.1) is the only sequence that encodes a functional gene and therefore this gene must encode both the (pro)renin receptor and the truncation products (M8-9) which are associated with the V-ATPase.

3.2.4 Reverse Transcriptase Polymerase Chain Reaction

3.2.4.1 Optimisation of RT-PCR reaction

Optimisation of the RT-PCR reactions with primer pairs encompassing the coding region and the complete cDNA of the mouse putative (pro)renin receptor were performed on kidney and AS4.1 cell RNA. Figure 3-8A&B shows the effects of an annealing temperature gradient (59-64°C) and of magnesium chloride concentration on the efficiency of the RT-PCR on kidney RNA with primers designed to amplify the coding region of the cDNA (JJM 515-516). Figure 3-8C shows the effects of the same temperature gradient with primers designed to amplify the whole cDNA (JJM 515-517).

Both PCR reactions were found to be efficient along the temperature gradient (only up to 62°C for JJM 515-517 primers). An annealing temperature of 60°C for JJM 515-516 primers and 61°C for JJM 515-517 primers was used thereafter. The product band was sharper at a concentration of 1.5mM $MgCl_2$ than at a concentration of 2.5mM and this condition was adopted as standard for further RT-PCR reactions of the mouse putative (pro)renin receptor.

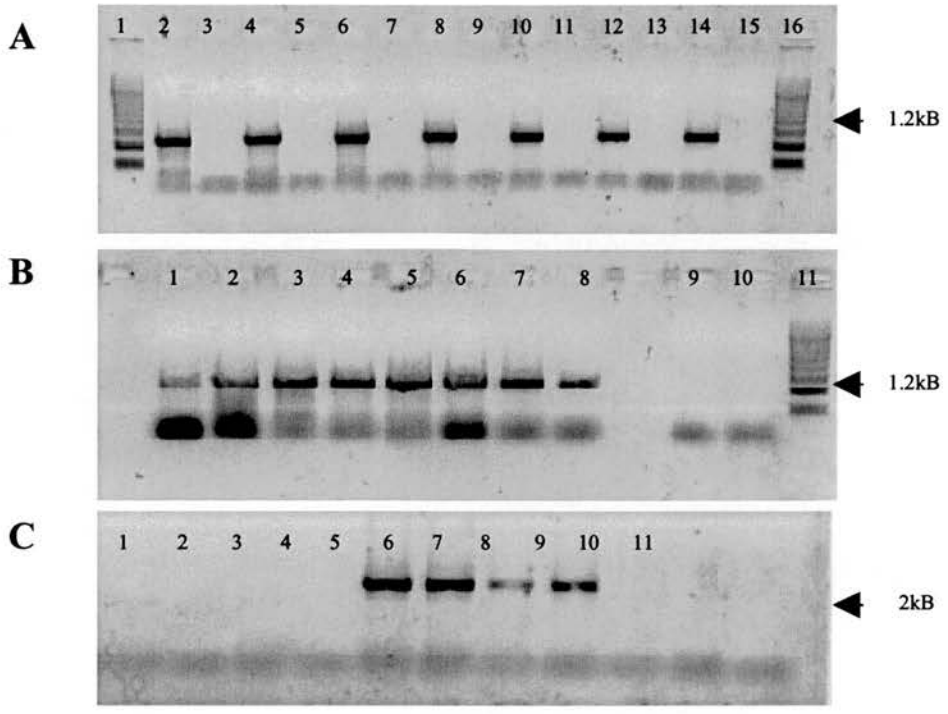


Figure 3.8: Optimisation of reverse transcriptase (RT) PCR primers for mouse putative (pro)renin receptor. Electrophoresis on agarose gels.

A) Coding region primers, Temperature gradient (59-64°C) and magnesium chloride concentration 1.5mM. Lanes 2, 4, 6, 8, 10, 12, 14: mouse putative (pro)renin receptor PCR product of 1.2kB. The PCR reaction is efficient at all temperatures tested. An additional band was observed in lane 2 representing an unspecific product due to low annealing temperature. Lanes 3, 5, 7, 9, 11, 13, 15: negative PCR reaction with RT reaction lacking the reverse transcriptase enzyme. Lanes 1 and 16: DNA ladder of 500bp increments.

B) Coding region primers, Temperature gradient (59-64°C) and magnesium chloride concentration 2.5mM. Lanes 1-8: mouse putative (pro)renin receptor PCR product of 1.2kB. The PCR reaction resulted in a product of correct size at all temperatures tested. The reaction appears less efficient at both ends of the temperature range. Lanes 9-10: negative PCR reaction with RT reaction lacking the reverse transcriptase enzyme. Lane 11: DNA ladder of 500bp increments.

C) Whole cDNA primers, Temperature gradient (59-64°C) and magnesium chloride concentration 2.5mM. Lanes 1-4: negative PCR reaction with RT reaction lacking the reverse transcriptase enzyme. Lanes 5-11: mouse putative (pro)renin receptor PCR product of 2kB, the PCR reaction resulted in a product of correct size only at lowest temperature of tested range.

For all three gels the small "fuzzy" band at the bottom of the gel represents unincorporated oligonucleotides.

3.2.4.2 TA cloning of the mouse putative (pro)renin receptor PCR products

The same reactions were performed with RNA extracted from AS4.1 cells and from kidney tissue. Products from AS4.1 RNA were named AS516 if amplified with JJM 515-516 and AS517 if amplified with JJM 515-517. Products from kidney RNA were named K516 if amplified with JJM 515-516 and K517 if amplified with JJM 515-517. The products were cloned into pGEM-T easy vectors as described in *Chapter 2.8* on the day of purification (after electrophoresis and size check) so as not to risk loss of the 3' poly-A tail addition by Taq polymerase. The cloning procedure used does not allow for directional cloning. The resulting plasmids were named pGRenRec (and the name of PCR product), sequenced using universal primers recognising T7 and SP6 promoter sequences to confirm identity, checked for orientation and used in further experiments including Northern blotting and *in situ* hybridisation.

3.2.5 Adult and foetal tissue expression and cell line screening

RT-PCR was performed using optimised conditions reported above. Murine RNA was processed to examine the expression of the putative mouse putative (pro)renin receptor transcripts in a variety of tissues and embryonic/postnatal stages (Figure 3.10). Primers encompassing only the coding region were used for RT-PCR and the PCR product was found in two strains of mice: C57-Bl6 which possess one-renin gene (data not shown) and FVB/N which harbour two-renin genes (Figure 3.9A). Putative (pro)renin receptor expression was detected in all tissues examined, namely, brain, heart, kidney, liver, lung, submandibular gland, adrenal gland, muscle, mesenteric fat, ovary, suggesting the molecule may be expressed ubiquitously (Figure 3.9A). The PCR reaction was highly efficient and specific as no additional bands were obtained in the positive reactions and no bands were produced in the negative reactions in which PCR was performed on reverse transcriptase mixes in which no reverse transcriptase was added. Using the same RT-PCR protocol as for adult tissues, (pro)renin receptor was detected in kidneys of mouse pups of post-natal day 1 stage (Figure 3.9B). Similarly, embryonic expression of the putative (pro)renin receptor was examined in mouse embryonic kidneys (Figure 3.9C). RT-PCR analysis was performed on RNA extracted from embryos from E9.5dpc to E18.5dpc. Putative (pro)renin receptor expression was detected at all stages examined (Figure 3.9C) suggesting that this receptor may be important in early embryogenesis.

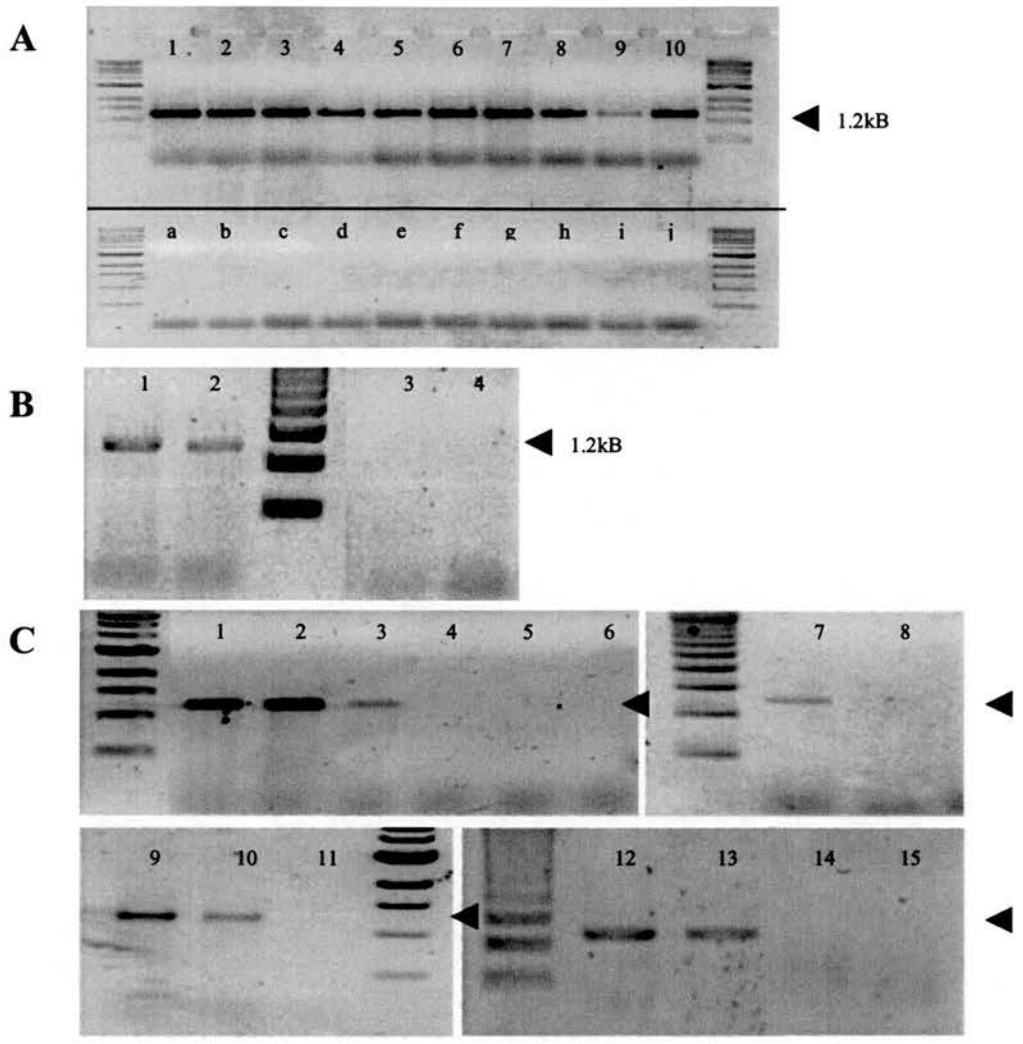


Figure 3.9: Agarose gel electrophoresis of mouse putative (pro)renin receptor reverse transcriptase PCR. A) Female mouse tissue of FVB/N strain. (Pro)renin receptor product (1.2kB) is present in all tissues. Lane 1: brain, Lane 2: heart, Lane 3: kidney, Lane 4: liver, Lane 5: lung, Lane 6: submandibular gland, Lane 7: adrenal gland, Lane 8: muscle, Lane 9: mesenteric fat, Lane 10: ovary; Lanes a-j = corresponding negative PCR with RT reaction lacking the reverse transcriptase enzyme. B) Post-natal day 1 (P1) kidney from mouse of FVB/N strain. (Pro)renin receptor product of 1.2kB size is present only in positive reactions. Lanes 1-2: P1 kidney, Lanes 3-4: negative PCR with RT reaction lacking the reverse transcriptase enzyme. C) Developmental profile of (pro)renin receptor expression of embryonic kidneys from mouse of FVB/N strain. (Pro)renin receptor product of 1.2kB size (arrowhead) is present at all stages examined but only in positive reactions. Lanes 1: E9.5, Lane 2: E11.5, Lane 3: E12.5, Lane 7: E14.5, Lanes 9-10: E16.5, Lanes 12-13: E18.5; Lanes 4-6, 8, 11, 14-15: negative PCR with RT reaction lacking the reverse transcriptase enzyme. For all gels, the second band at the bottom of the gels is unincorporated oligonucleotides. The DNA marker is a 1kB ladder.

Additional analysis showed that the mouse putative (pro)renin receptor was expressed in two murine cell lines: AS4.1 (from mouse kidney) and E14TG2a (an embryonic stem cell line) and that the human (pro)renin receptor was expressed in HMC by RT-PCR and Northern blot analysis (Figure 3.10). The results with the HMC are in disagreement with previous published data (Nguyen *et al.* 2002) which reported that HMC did not express the human (pro)renin receptor.

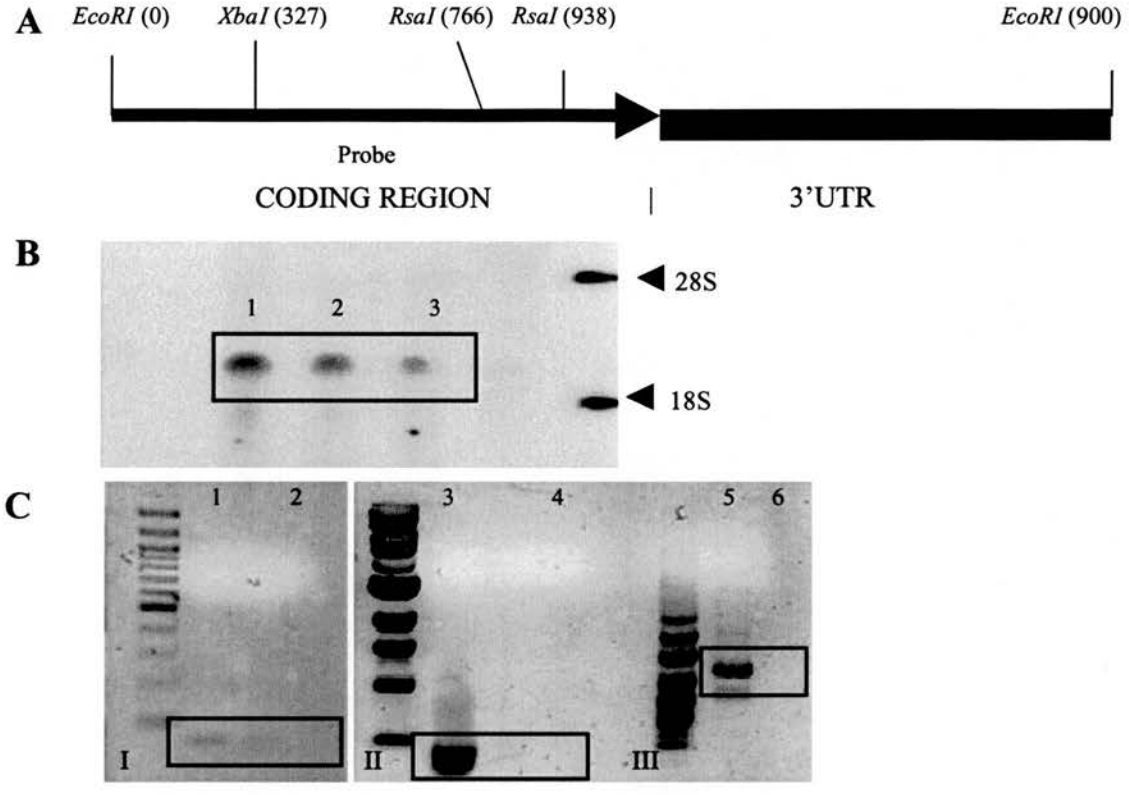


Figure 3.10: Cell line expression of the putative (pro)renin receptor. A) Schematic representation of the mouse (pro)renin receptor cDNA as cloned from kidney preparations highlighting the Northern blot probe (fragment *XbaI-RsaI*). B) Autoradiography of a Northern blot with *XbaI-RsaI* fragment of the (pro)renin receptor as a probe. The highlighted bands (boxed area) represent the putative (pro)renin receptor (approx. 2kB). Lane 1: AS4.1 cells ; Lane 2: ES cells; Lane 3: human mesangial cell line. The ribosomal bands are indicated by the arrows, 28S and 18S accordingly and used as size markers. C) (Pro)renin receptor RT-PCR on human mesangial cell line RNA: with primers designed on the human renin receptor (N14F) sequence, Lanes 1, 3 and 5: positive RT reactions; lanes 2, 4 and 6: negative RT-PCR reactions. (I), expected band size 65bp, DNA ladder = 100bp; with primers designed for housekeeping gene GAPDH (II), expected band size 480bp, DNA ladder = 1kB; with primers designed on the human (pro)renin receptor sequence (III), expected band size 1200bp, with primers designed on the putative mouse (pro)renin receptor sequence; DNA ladder = 500bp.

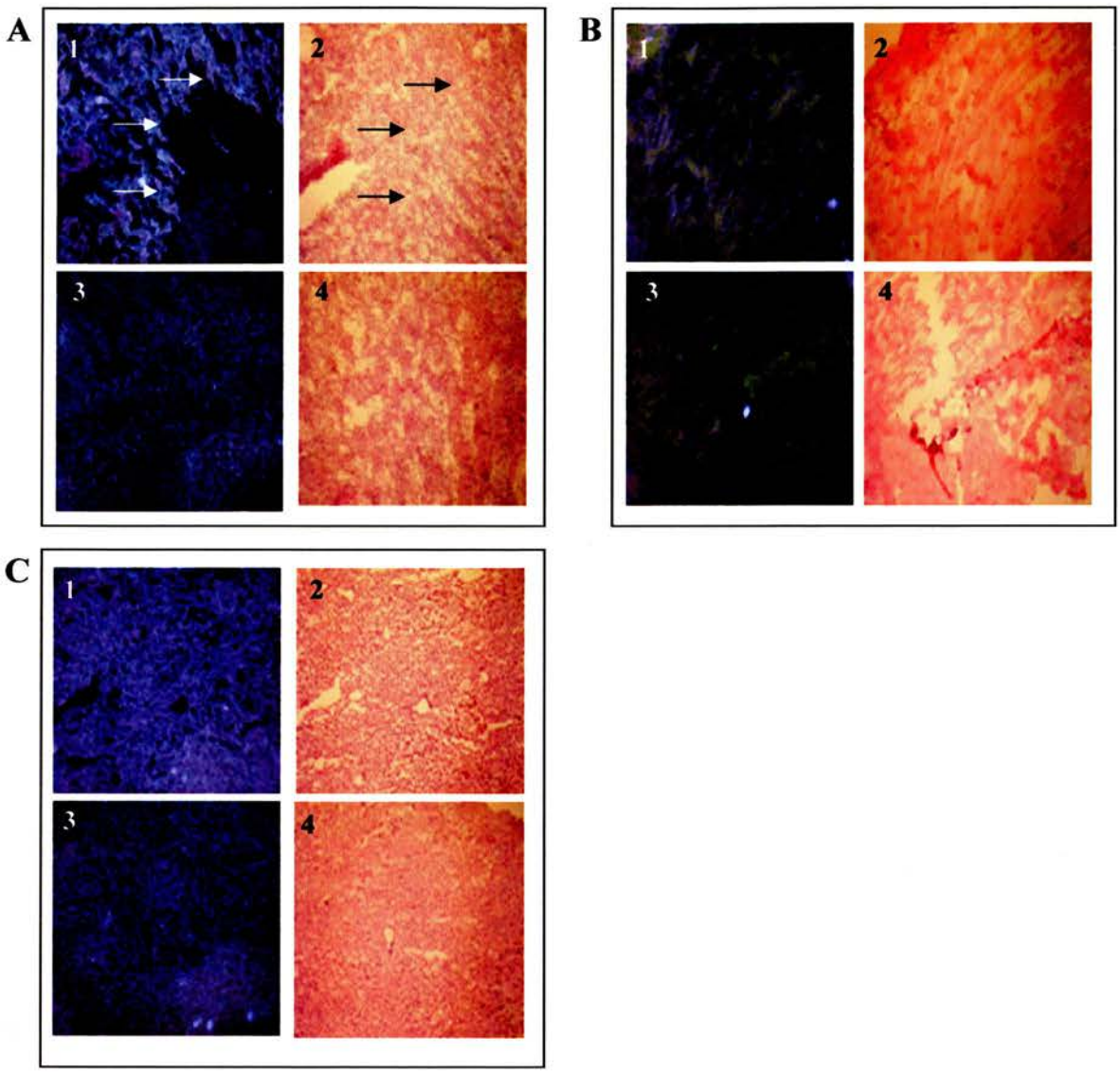


Figure 3.11. *In situ* ^{35}S hybridisation of mouse (C57/Bl6) tissues with (pro)renin receptor probes. Dark and light field photographs of frozen tissue sections (4μm).

Photographs no 1 = Dark field photographs of sections hybridised with anti-sense probe, Photographs no 2 = Corresponding haematoxylin and eosin (H&E) staining photograph of sections hybridised with anti-sense probe, Photographs no 3 = Dark field photographs of sections hybridised with sense probe (background control), Photographs no 4 = Corresponding (H&E) staining photograph of sections hybridised with sense probe. A) Longitudinal sections of kidney. arrows indicate the cortex-medulla delimitation; note the staining solely in the cortical region. This pattern was specific to anti-sense probe hybridisation. B) Sections of skeletal muscle note the overall increased staining with the anti-sense probe (B-1) compared with sense probe (B-3). C) Sections of liver; note the overall increased staining with the anti-sense probe (C-1) compared with sense probe (C-3).

In situ hybridisation (ISH) showed that the mouse putative (pro)renin receptor is not expressed uniformly within the kidney. Higher levels of expression were found in the cortex (Figure 3.12) which suggests that the putative (pro)renin receptor is expressed in the vasculature rather than in tubular structures. Although, no expression could be detected in the renal medulla, it is possible that mRNA levels are low and undetectable by ISH. Gross examination of liver and skeletal muscle sections revealed no particular pattern and the hybridisation appears to be widespread throughout these two tissues. Hybridisation of the (pro)renin receptor riboprobe to heart and placenta did not reveal any signal above background. This may be due to low mRNA amounts which could not be detected by *in situ* hybridisation. Hence, prorenin/renin uptake may not be mediated through the (pro)renin receptor in the heart under normal physiological conditions but may be up-regulated during stimulated conditions.

Additional ISH assays were performed at the College de France in Paris on paraffin embedded embryonic kidney, heart and lung sections provided by myself. The results were provided and interpreted by Dr Gasc and Dr NGuyen. These revealed that the staining was strongest in the kidney at E15.5 compared to later embryonic stages of E16.5 and E18.5. This indicates that the mouse putative (pro)renin receptor may not play a role in late nephrogenesis. The riboprobe labelling showed a similar pattern to the adult in post-natal (P1) kidneys with an intense signal in the external and internal cortices, (stronger in the external cortex). In the heart, labelling was only evident at E16.5, other embryonic stages, namely E15.5 and E18.5 showed very faint staining. At these stages, the heart and the kidney are structurally fully developed suggesting that the mouse putative (pro)renin receptor may play another role than establishing cardiac and renal organogenesis. It may indeed function to mediate prorenin actions independent or dependent on downstream AngII production such as homeostatic balance. In accordance with the results obtained with adult cardiac tissues, very weak labelling was detected in the heart of post-natal mouse pups. The (pro)renin receptor may again be expressed at low levels, not detectable by *in situ* hybridisation. Indeed, in the adult, mouse putative (pro)renin receptor can be detected by RT-PCR, a more sensitive technique.

3.3 Discussion and conclusions

3.3.1 Cloning of the mouse putative (pro)renin receptor

This chapter describes the existence of a putative homologue of the human (pro)renin receptor in the mouse. Three complete putative (pro)renin receptor cDNA clones were identified by BLAST search using a human cDNA sequence recently reported to be the (pro)renin receptor as template

[GenBank number: AF291814 (Nguyen *et al.* 2002)]. The cDNA sequences of these three mouse putative (pro)renin receptor clones are highly similar, two of the clones being from the C57/Bl6 strain and one being from the FVB/N strain. Alignment with the human (pro)renin receptor sequence indicated an average of 83% identity at the nucleotide level. At the protein level, only 21 out of the 351 amino acids of the predicted sequence differed between the mouse and the human proteins and of those 21 amino acids, 10 were predicted to be conservative substitutions which may imply functional homology. Phylogenic studies revealed that the (pro)renin receptor is highly conserved between species, including *D.rerio* (67% identity with mouse sequence) and *C.elegans* (21% identity with mouse sequence). Rat (pro)renin receptor nucleotide sequence displayed 91% and 96% identity with the human and mouse sequence respectively. This mouse putative (pro)renin receptor was then cloned, sequenced and used for further study. The cDNAs obtained from kidney and AS4.1 cells (a mouse kidney tumour cell line) were identical to the nucleotide sequences of the cDNA identified by BLAST and were homologous to the human cDNA identified by Nguyen *et al*(Nguyen et al. 2002). A BAC clone (12J18) was identified containing the putative (pro)renin receptor gene of mouse strain C57/Bl6. The gene was localised to chromosome X, locus A1.2 and predicted to possess 8 introns and 9 exons. However, additional exons may be present since expressed sequence tags were obtained during the BLAST search aforementioned that differed significantly at the 3' end.

3.3.2 Homology of the putative (pro)renin receptor to a membrane sector associated subunit

Surprisingly, however, the translated human cDNA (GenBank accession no AF291814) as well as the translated mouse cDNAs (BC014706, AK017482 and AK029405) exhibited sequence identity with a small protein that co-purifies with a bovine vacuolar ATPase (V-ATPase) called M8-9 (Ludwig *et al.* 1998). M8-9 is a membrane sector associated portion of V-ATPase. Human M8-9 has been mapped to the same chromosomal region as the human (pro)renin receptor (locus: Xp11.4) which also contains the critical region of a rare progressive visual disease called X-linked cone-rod dystrophy (COD1) (Demirci *et al.* 2001). The authors reported that the M8-9 gene is not responsible for the disease. This gene may, however, undergo alternative splicing and/or have multiple sites of transcription start. It is worth noticing that the amino acid sequence identified as the human M8-9 is 316 amino acid in length compared with the bovine form which is 30 amino acid in length. This may be the

result of post-transcriptional processing, possible alternative splicing, post-translational modifications or proteolytic cleavage occurring within the cell or when M8-9 was purified. It is not known whether the human M8-9 cDNA is the same length as the human (pro)renin receptor or if it is indicative of a cDNA with an alternative transcription start. It may suggest that M8-9 and the (pro)renin receptor are the same gene or that two proteins are produced from the same gene. V-ATPases contain 6-10 subunits divided into two domains: a transmembrane proton-conducting sector and an extramembrane catalytic sector responsible for ATP hydrolysis (Harvey and Wieczorek 1997; Stevens and Forgac 1997). Moreover, individual V-ATPase subunit null mutants are lethal and yeast models have to be used.

In mammalian cells, V-ATPases play important roles in energy conservation and secondary active transport, acidification of intracellular compartments and cellular pH homeostasis (Nelson 2003; Nelson and Harvey 1999). The gene encoding the (pro)renin receptor was conserved between species including organisms from lower orders such as *C.elegans*. Phylogenic conservation indicates that it is physiologically important and a second protein arising from the same gene would explain the homology with the vacuolar ATPase subunit, M8-9. Indeed, the human (pro)renin receptor described by Nguyen *et al* and the vacuolar ATPase subunit M8-9 must the product of the same gene since only one gene was found identified in ENSEMBL database. Additional investigations designed to elucidate whether these proteins are functionally related remains to be undertaken.

3.3.3 Developmental and tissue expression profile

The RT-PCR results reported in this chapter demonstrate the robustness of the PCR reaction developed to amplify the mouse putative (pro)renin receptor and optimised in *Chapter 3*. The PCR reaction was performed successfully on a variety of tissues and it was demonstrated that the mouse putative (pro)renin receptor is expressed in all tissues examined. No negative tissues or cell lines have been identified to date. This could be explained if the mouse putative (pro)renin receptor was expressed in the microvasculature since the blood vessels were not microdissected out. In the present work, the mouse putative (pro)renin receptor was also expressed throughout development from E9.5. This raises the question of the role of a mouse (pro)renin receptor in embryonic stages independently of renin/prorenin. As mentioned in *Chapter 1*, components of the RAS are present in foetal tissues and AngII is known to be involved in cell growth/differentiation (Unger *et al.* 1996). It is therefore possible that uptake of renin or prorenin occurs in these tissues to initiate the

RAS cascade and the production of AngII. Whether, renin or prorenin themselves have a direct role in development is, however, not known.

The function of the putative (pro)renin receptor in kidney and heart development remains unclear but expression of this gene is evident throughout embryogenesis in these tissues with highest expression detected at E15.5 and E16.5, respectively. The mouse putative (pro)renin receptor is expressed in early embryogenesis, *i.e.* before the need for and the establishment of a functional RAS. It suggests that it may have a role independently of renin/prorenin and of AngII generation and may have functions relating to its homology with a V-ATPase sub-unit. It is worth noting that ES cells (which are not smooth muscle cells) express the mouse putative (pro)renin receptor which are not smooth muscle cells and that one of the cDNA identified was from an E8.5dpc embryo library (when blood vessels are at a blood island stage. Taken together, this suggests that the protein identified as a (pro)renin receptor may have "housekeeping" functions and have a more basic biological role than thought.

The results above demonstrate messenger RNA (mRNA) expression and it cannot be excluded that protein expression pattern may differ from that of mRNA due to post-transcriptional modification. In an attempt to rectify this, a mouse (pro)renin receptor antibody was made from two mouse peptides homologous to those used by Nguyen *et al.* in their publication on the human (pro)renin receptor. Chicken was the species chosen for immunisation with these peptides (performed by a commercial manufacturer). The rationale behind this was that an anti-mouse renin antibody raised in rabbit was available in the laboratory. This would allow for double labelling without species cross-reactivity between the two antibodies. The aim was to use the antibody in binding and immuno-precipitation studies to confirm the results of Nguyen *et al.* using mouse cell/tissues. The titer of the anti-sera collected after the different immunisations was very low and no difference were observed between pre-immune serum and post-immunisation sera when tested on AS4.1 cell lysate on a dot blot. This is possibly due to the high homology of the mouse peptides with the chicken corresponding protein resulting in low/no production of anti-bodies against the injected peptides.

3.3.4 Comparison of putative (pro)renin receptor expression between two mouse strains

The use of two different strains of mice, one-carrying one renin gene (C57/Bl6) and the other carrying two renin genes (FVB/N) highlighted the fact that there is no differences in

expression of the mouse putative (pro)renin receptor in the tissues examined. It is not possible, however, from the data shown to say whether levels of expression are similar in both strains. Quantitative investigations may help identify those tissues that express the receptor abundantly, *e.g.* tissues which do not express renin but show renin activity (such as the heart) require uptake of renin or prorenin

3.3.5 On the relationship between M8-9 and the (pro)renin receptor

A mouse putative (pro)renin receptor could therefore be a very important tool to understand (pro)renin uptake in the heart/vascular tissues and the direct roles of (pro)renin independently of angiotensin II formation. However, it is easy to be confused when trying to disentangle the available information relating to M8-9 and the (pro)renin receptor. It is important to remember that the name 'M8-9' refers to a group of short peptides of overlapping sequence, believed to be the truncation products of a larger, as yet unpurified, protein. In 2001, the encoded cDNA sequence and genomic structure of a gene encoding the M8-9 peptides was reported [24]. Although there is no biochemical evidence to prove that the M8-9 peptides are derived from the protein encoded by this cDNA, the fact that there are no other such sequences in the genome suggests that this must indeed be the precursor. It is not known whether the encoded protein is itself associated with the V-ATPase, or how its truncation products, which do associate with the V-ATPase, are formed. What seems clear is that this gene, termed the 'M8-9' gene, encodes sequences that co-purify with the V-ATPase. Independently, in 2002, the sequence of the human (pro)renin receptor was reported (Nguyen *et al.* 2002). The authors commented on the homology between the peptide sequence encoded by the (pro)renin receptor cDNA and the M8-9 peptides. However, on further comparison it is clear that the (pro)renin receptor cDNA sequence must the product of the gene referred to in the database as the 'M8-9' gene. Perhaps until we understand more fully the relationship between the (pro)renin receptor and the V-ATPase-associated peptides this gene should be referred to as the 'M8-9/(pro)renin receptor gene. There is clearly much work to do in elucidating the role(s) of the product(s) of this gene and clarification of the function(s) of the encoded protein(s) has important implications for cell function (via the V-ATPase function) and the cardiovascular system (via its role as a renin receptor).

The (pro)renin receptor was recently described as a trigger for pro-fibrotic genes and hypertrophic response of mesangial cells exposed to stimulus (Huang *et al.* 2006). The authors used the technique of siRNA inhibition against the (pro)renin receptor. This may

result in inhibition of the M8-9 portion of the (pro)renin receptor and the results may therefore not be representative of the receptor involvement in the phenotype. It is worth noting, however, that the stimulus used was renin rather than prorenin. One may question the validity of this study since 1) renin does not have a prosegment which is recognised as the binding region, 2) the authors did not exclude that mesangial cells may produce AOGEN and ACE and hence the effects seen reflects a RAS stimulation, 3) M8-9 may be involved. Similarly, the work of Nguyen *et al.* describes binding to the described human (pro)renin receptor to both renin and prorenin. No common sequence between the two proteins was identified as a binding region to the receptor and it is important to consider how in the absence of "pro" sequence renin may interact with the putative (pro)renin receptor. More studies are required to shed the light on the biology on the putative (pro)renin receptor and to remove any controversy concerning its importance in several pathophysiological events. The results described in this chapter (and the subsequent chapter) cast doubts over the role of this molecule as "the" (pro)renin receptor. It cannot be excluded that other molecules may act as "(pro)renin receptor" and this discussed in more detail in Chapter 4.

In addition, since the role of this gene may prove to be critical for cell survival and considering that my data differs results from Nguyen *et al.* (2002) it was felt more reasonable not to attempt to produce mice lacking this gene through the use of gene targeting. For the present thesis, it was, therefore, decided to investigate whether this molecule has indeed any pathophysiological role in a transgenic model of high circulating prorenin which is characterised by malignant hypertension (Kantachuvesiri *et al.* 2001) (See *Chapter 4 and 5*). Two studies looking at inhibition of the (pro)renin receptor by a prorenin decoy peptide (Ichihara *et al.* 2004) and at overexpression of the (pro)renin receptor (Burckle *et al.* 2006) have demonstrated a physiological role for the (pro)renin receptor/M8-9 and for non-proteolytic activation of prorenin.

Chapter 4

Vascular Injury in an Inducible Rat Model of Hypertension Characterised by High Prorenin

4.1 Introduction

Transgenic models have become widely used in the field of cardiovascular science and three different transgenic models characterised by high prorenin have been produced to study the involvement of circulating and local renin-angiotensin system (RAS) genes and pathways in the pathophysiology of hypertension. TGR(*mRen2*)27 animals were the first transgenic rats used for cardiovascular and hypertension research. These animals carry the mouse *Ren2* gene (Mullins *et al.* 1990) and they display fulminant hypertension from 5 weeks of age (230mmHg). The animals suffer from malignant hypertension (MH) injuries leading to heart failure unless treated with ACE inhibitors (Mullins *et al.* 1990). The TGR(*mRen2*)27 animals displayed suppressed concentrations of active plasma and renal renin, low or unchanged levels of plasma AngII but increased levels of circulating prorenin compared to controls (Lee *et al.* 1995). A second rat model carrying a "*Ren2* transgene" was generated to study the effects of high levels of circulating rat prorenin by directing the expression of the transgene exclusively to the liver (Veniant *et al.* 1996). Endogenous renin expression in the kidney was similar to wild type, unlike in the previous model in which renal renin was decreased, but the animals displayed characteristic MH lesions in the heart and in the kidney suggesting a direct effect of circulating prorenin in the pathophysiology of hypertension. A third and inducible rat model of hypertension was recently developed in this laboratory. It carries an *mRen2* transgene which is under the control of cytochrome P450 *Cyp1a1* promoter (Kantachuvesiri *et al.* 2001). Expression of the prorenin transgene is restricted to the liver and small intestine where no malignant hypertension lesions occur. The promoter is induced when the animals are fed a diet containing the natural xenobiotic indole-3-carbinol (I3C). Upon chronic induction, TGR(*Cyp1a1-Ren2*) animals displayed a rapid increase in blood pressure

accompanied by weight loss and polyuria, as a result of salt and water depletion. The animals exhibited hypertensive injuries in several vascular beds and organs. Microinfarctions, inflammatory cell infiltration and fibrinoid necrosis were observed in the heart. The latter was also present in the mesentery. In the kidney, distal tubule hyperplasia was consistent with polyuria, intrarenal arteries displayed a thickening of the media layer but the glomeruli were unaffected. There was a distinct difference in the response to injury, the heart and the mesentery exhibiting greater sensitivity whereas the kidneys appeared to be affected to a lesser extent. Involvement of a locally activated renin-angiotensin-system, in particular prorenin and/or inflammation may play an important role in the mechanisms underlying vascular and end-organ injury due to hypertension (Collidge *et al.* 2004). The aims of this chapter were to characterise the pathological and haemodynamic changes to the heart and to the vasculature in this rat model using echography techniques and correlating these findings with histological studies. Presence and expression of the putative (pro)renin receptor in rat tissues was studied using PCR. Potential regulation of the (pro)renin receptor in the inflammatory/remodeling response and malignant hypertension were investigated using quantitative PCR techniques

4.2 Results

4.2.1 Physiological measurements

4.2.1.1 Blood pressure and heart rate

Systolic blood pressure and heart rate were measured using a photoelectric sensor for pulse detection in rat tail. Following tail cuff inflation to a pressure superior to the systolic blood pressure, the pulse waveform becomes indistinguishable from baseline. Observation of the return of the pulse signal during progressive (automated) deflation of the tail-cuff blood pressure could be done visually from the trace as exemplified below in Figure 4.1. The tail cuff pressure at which the pulse wave form returns after maximal inflation identifies systolic blood pressure.

Transgene induction resulted in a distinct but gradual increase in blood pressure (Figure 4.2) in treated TGR(*Cyp1a1-Ren2)* animals from 24h after treatment started. Statistical analysis showed that blood pressure from treated animals was significantly increased compared to controls throughout the experimental period starting at day 1. This difference in blood

pressure between the groups was also found to increase over time indicating that the phenotype is worsening.

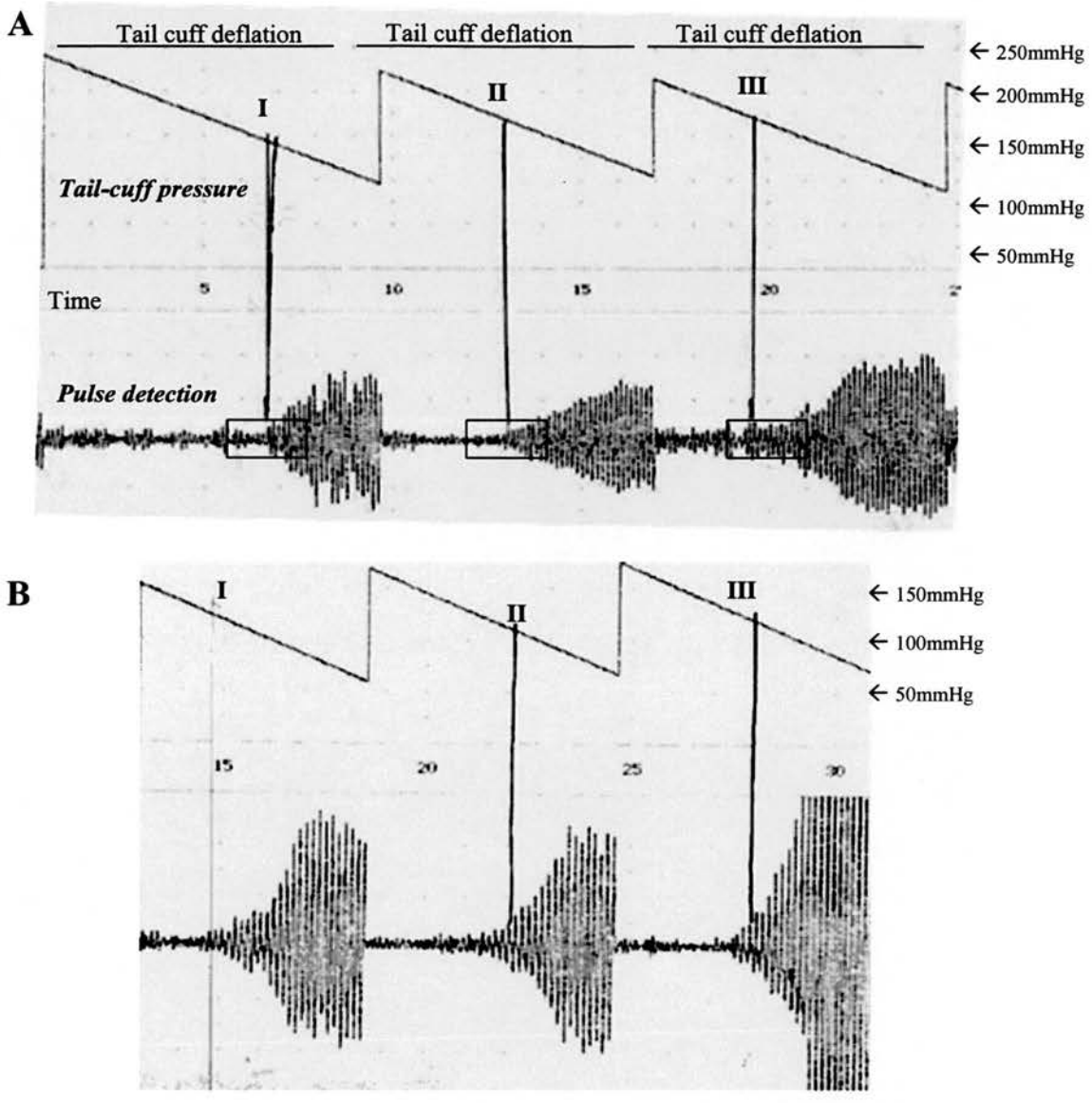


Figure 4.1: Examples of tail cuff plethysmography recordings. A) From hypertensive animals, systolic blood pressure measurements were: 150mmHg (I), 165mmHg (II) and 170mmHg (III). Tail-cuff pressure scale in shown on the right of the tracing while an example of a pressure point at which the pulse waveform return is highlighted in boxed area. B) From normotensive animal, systolic blood pressure measurements were: 115mmHg (I), 110mmHg (II) and 115mmHg (III).

Two days before transgene induction and at day 0, the blood pressure was slightly elevated in both groups compared to day 1 in the control group (data not shown) which may reflect a stress effect of the methodology. This did not, however, reach significance and blood pressure remained in the normal range for the control group throughout the experimental period.

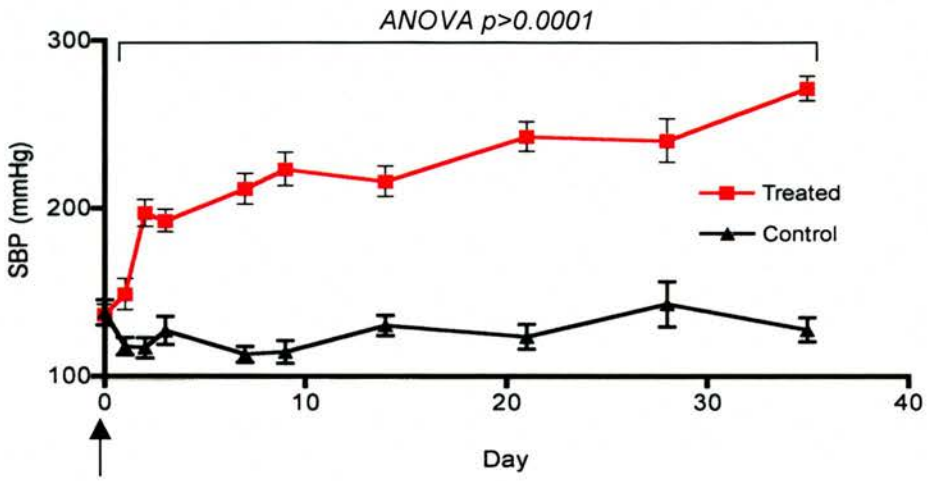


Figure 4.2: Systolic blood pressure (mmHg) in conscious TGR(*Cyp1a1-Ren2*) rats. The arrow indicates the start of the 0.3% indole-3-carbinol-containing diet for the treated group. Standard two-way ANOVA with post test showed that systolic blood pressure is significantly elevated compared to control from 24h after starting the induction.

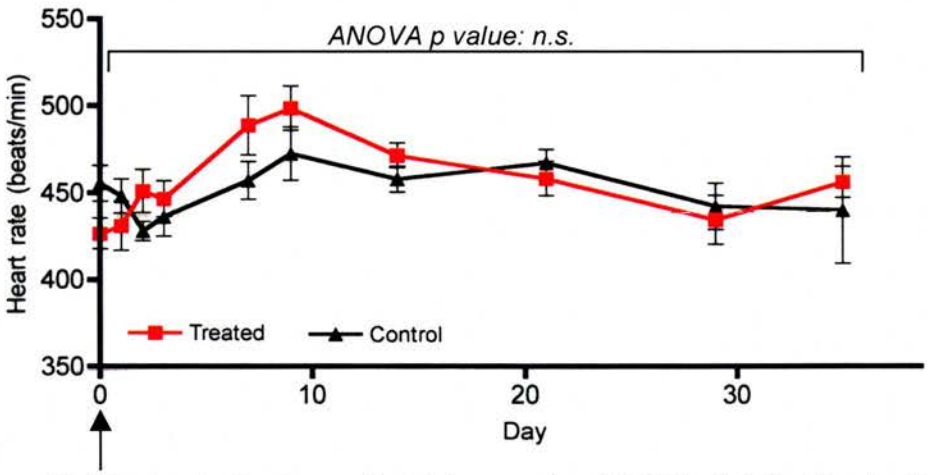


Figure 4.3: Heart rate (beat per minute) in conscious TGR(*Cyp1a1-Ren2*) rats. Standard two-way ANOVA confirmed the heart rate was unaffected by treatment N=10; n.s = non-significant. The arrow indicates the start of the 0.3% indole-3-carbinol-containing diet for the treated group.

The heart rate, however, remained unchanged in the treated animals compared to controls throughout the treatment period (Figure 4.3).

4.2.1.2 Body weight, food consumption, fluid intake

Increased blood pressure was accompanied by gradual weight loss from day 1 of the experiment in the treated animals which only became evident from controls from day 14 (Figure 4.4). As a result, repeated measure two-way ANOVA revealed that the difference in body weight between the two groups was almost significant (p= 0.052) over the entire experimental period.

The total average weight loss for the treated group at the end of the experiment was 12.1g. However, considering that the animals were young and that the control animals gained approximately 44g over the experimental period, the weight loss can be regarded as fairly severe.

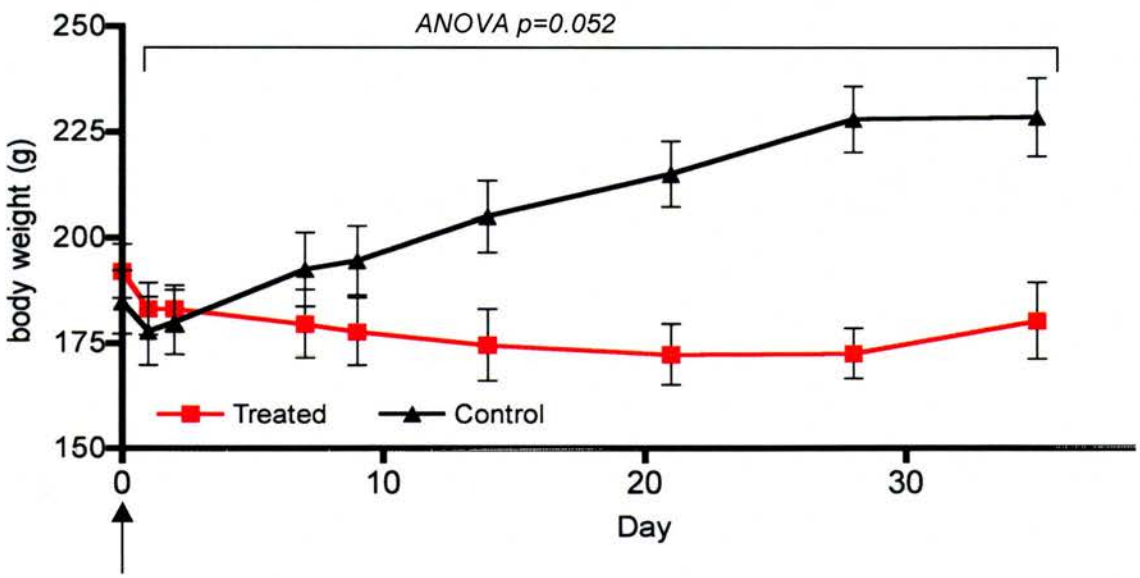


Figure 4.4: Body weight (g) in TGR(*Cyp1a1-Ren2*) rats. The arrow indicates the start of the 0.3% indole-3-carbinol-containing diet for the treated group. Note the weight gain in the control group due to growth while the effect was absent in the treated group due to body mass loss.

Over the entire experimental period, increased food consumption was observed in the treated animals compared to the control group (Figure 4.5). Repeated measure two-way ANOVA also

identified a significant effect over time suggesting that the treated animals are increasingly consuming more food than the control animals. Polyuria and polydypsia (Figure 4.6) were also noted but due to technical and ethical protocol restrictions, fluid intake and urine production measurements were only taken from two animals at a unique time point during week 4 of treatment. There was no indication of diabetes as demonstrated by the absence of glucose in the urine of treated animals (Table 4.1). The reasons for the presence of ketones, the increased detection of proteins (in two animals) and nitrite (in one animal only) in the urine of the control animals are unknown. It is possible that the increased fluid loss through urine output diluted this parameters in the treated animals and therefore does not represent an accurate reading. It is worth noting that the dipstick method is a rapid but crude method. However, leucocytes were detected in the urine of treated TGR(*Cyp1a1-Ren2*) animals suggesting renal inflammation potentially linked to hypertensive injury.

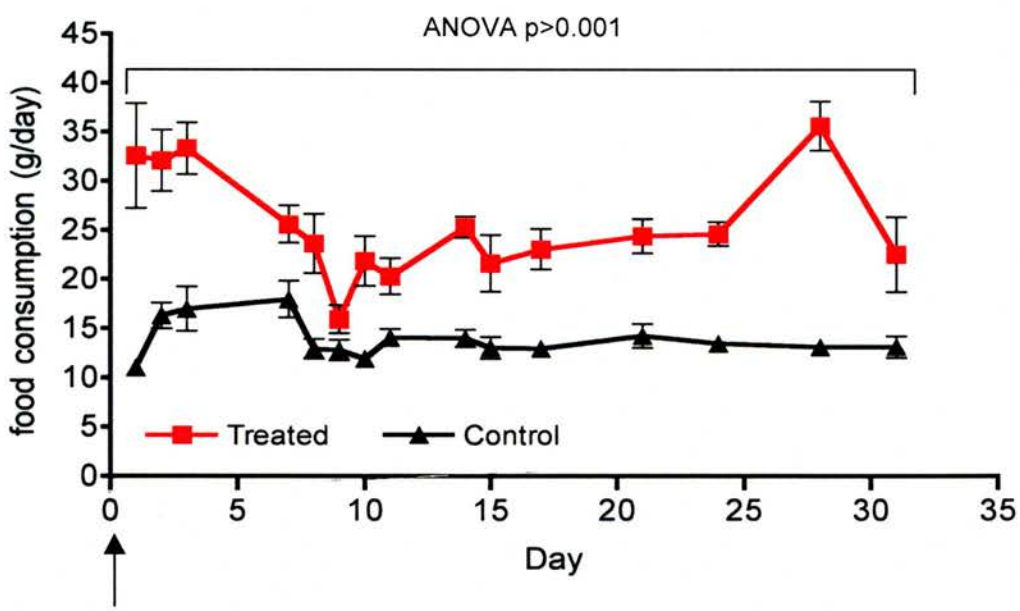


Figure 4.5: Food consumption (g/day) per TGR(*Cyp1a1-Ren2*) rats. N=10; Repeated measure two-way ANOVA analysis showed an increased food consumption of the animals in the treated group throughout the experimental period. The arrow indicates the start of the 0.3% indole-3-carbinol-containing diet for the treated group.

A similar procedure was repeated using urine samples collected from a previous experiment with TGR(*Cyp1a1-Ren2*) animals at the University of Maastricht (data not shown). When blinded to the treatment, it was possible to identify those animals which received control diet, those animals that received the inducer, and those in which the transgene had been induced for several weeks and subsequently allowed to recover, simply on the basis on the leucocyte detection system on the dipstick.

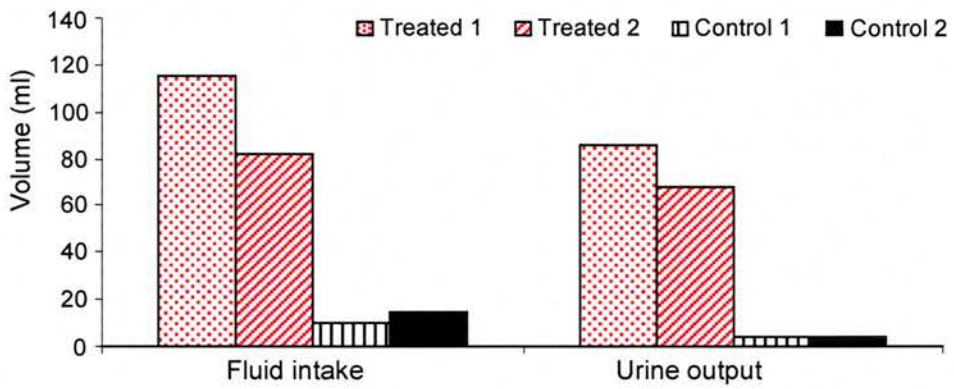


Figure 4.6: Urine output and fluid consumption (ml) in 2 TGR(*Cyp1a1-Ren2*) rats. Measurement were performed over 12h during week 4 of the study. Only two treated (Red bars) and two control (Black bars) animals were used and for clarity, results are presented per animal.

	Treated 1	Treated 2	Control 1	Control 2
Glucose (mmol/L)	ND	ND	ND	ND
Ketones (mmol/L)	ND	ND	++	++
Blood	ND	ND	ND	ND
Protein (mg/dL)	++	++	+++	+++
Nitrite	ND	ND	ND	+
Leucocytes	+	+	ND	ND

Table 4.1: Urine dipstick results. Readings were obtained visually against standard colorimetric measurement provided by the manufacturer (Bayer, Germany). ND : Not detected. + : indicates low amounts ++ : indicates moderate amounts and +++ : indicates high amounts. Leucocyte detection was based on esterase activity and given arbitrary units.

4.2.2 Echocardiography and aorta wall track

Echocardiography data shows an increase in left ventricular area suggesting development of ventricular hypertrophy in treated TGR(*Cyp1a1-Ren2)* animals (data not shown). This was confirmed by an increase in left ventricle weight (Figure 4.9).

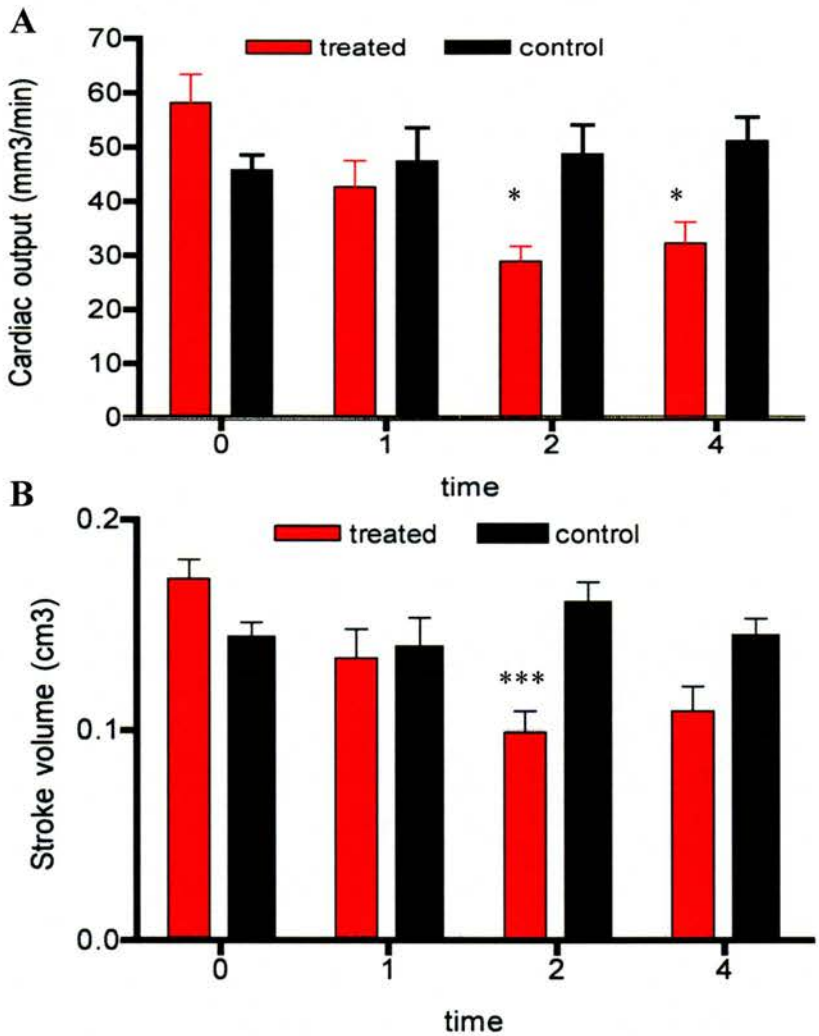


Figure 4.7: Echocardiographic measurements of TGR(*Cyp1a1-Ren2)* rats. Results were calculated from parasternal long axis B-mode echocardiograms A) Cardiac output (in cm^3/min) B) Stroke volume (in cm^3). Red bars represent the treated animals and solid black bars represent the control animals. Standard two-way ANOVA analysis with Bonferroni post-test showed a decrease in cardiac function from 2 weeks after start of induction. N=10, *: p>0.05, **: p>0.01, ***: p>0.001.

Cardiac output and stroke volume (Figure 4.7A&B) were markedly reduced compared to control, which may be indicative of heart failure. This event was only significant from two weeks after the start of the treatment with indole-3-carbinol. Since blood pressure is a function of cardiac output and peripheral resistance, a decrease in cardiac output would represent an adaptative response from the heart.

Aorta wall track echography demonstrated a stiffening (calculated as compliance and distensibility – Figure 4.8) of the abdominal aorta. This effect is likely to be a result of blood pressure increase and vascular remodeling.

	MAP (mmHg)	***α***	***Compliance (mm2/kPa)***	***Distensibility (Pa-1)***	***Aorta Diameter (mm)***	***Pulse wave velocity (m/s)***	***PP (mmHg)***
Mean Treated	99.512	2.424	0.106	34.582	1.989	5.473	43.093
S.d Treated	13.306	0.473	0.022	6.990	0.156	0.665	11.552
Mean Control	83.007	1.524	0.142	60.126	1.746	4.183	35.711
S.d Control	8.547	0.334	0.027	7.767	0.128	0.301	3.494
p value	**	***	**	***	**	***	*

Figure 4.8: Aorta wall track echography in anaesthetized TGR(*Cyp1a1-Ren2)* animals. Direct (MAP, PP and diameter) and calculated measurements (α, compliance, distensibility, PWV). α is a pressure independent measurement of aorta wall compliance. N=10, *: p>0.05, **: p>0.01, ***: p>0.001.

The lumen diameter (Figure 4.9) of mesenteric arteries of TGR(*Cyp1a1-Ren2)* animals was significantly smaller (inward remodeling) as compared to controls. Response to intraluminal pressure was significantly reduced in the vessels isolated from the treated animals even at low pressure (20mmHg). Whether this inward remodeling is hyper/hypo-eutrophic depends on the cross-sectional area of the tunica media.

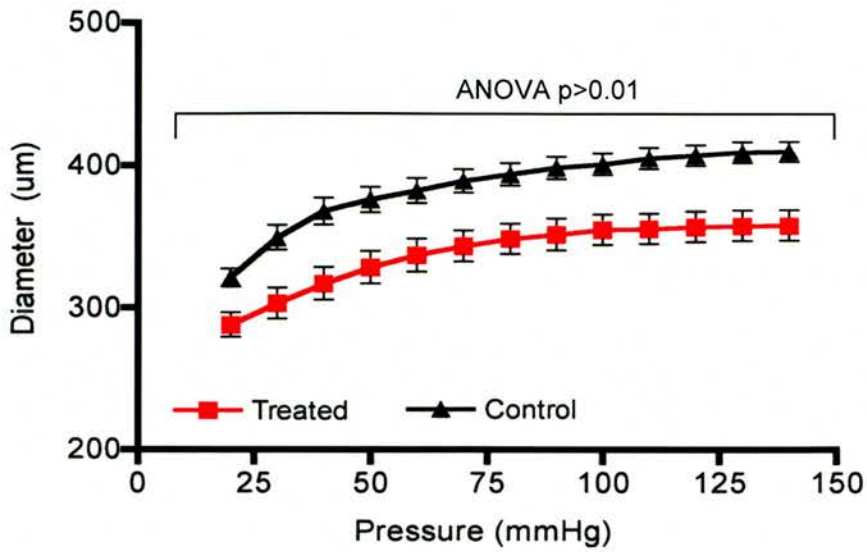


Figure 4.9: Pressure (mmHg)/diameter (mm) curve of first order mesentery arteries. Arteries from treated and control TGR(*Cyp1a1-Ren2* rats) were mounted on an arteriograph in relaxed state in a physiological buffer with nitroprusside after week 5 of the study. N = 10 for treated animals. N = 9 for control animals *: p>0.05, **: p>0.01, ***: p>0.001.

4.2.3 Organ weights and Histopathology

Histology processing was carried out at the Core Pathology unit. Histopathological analysis and photographs were supplied by Dr David Brownstein.

In accordance with the echocardiography and echography data, the left ventricle (expressed as weight/tibial length) was consistently larger in the treated animals than in the control (Figure 4.10). The weight of kidneys from treated animals was higher than in control animals which may be caused by several factors such, hyperplasia enlargement of capillaries and tubules or as increase fibrotic tissue formation. The latter was observed after macroscopic examination of sections stained for collagen and also in after microscopic analysis which revealed fibrinoid necrosis. The adrenal gland of induced animals only displayed a slight increase in weight compared to control (data not shown) which may indicate hyperplasia. No histological changes were, however, detected in this tissue.

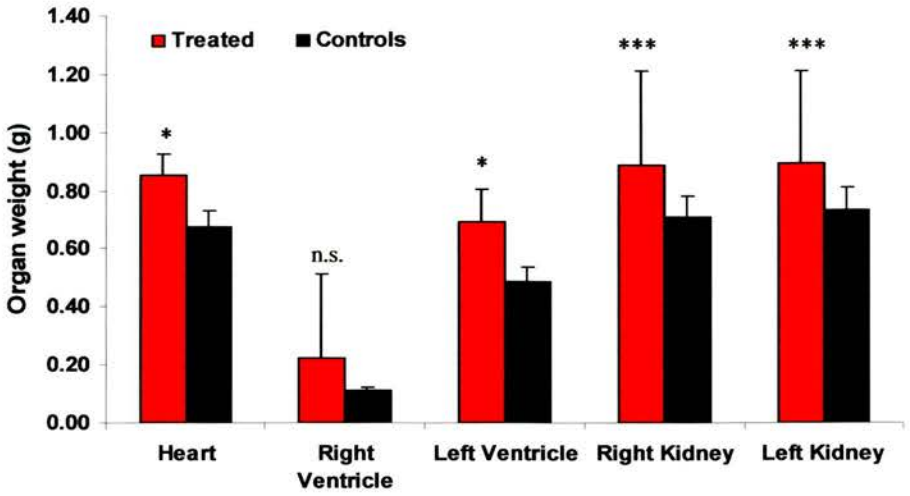


Figure 4.10: Organ weights normalised to tibial length of TGR(*Cyp1a1-Ren2*) rats. LK = Left Kidney, RK = Right Kidney, TL = Tibial Length, LV = Left Ventricle, RV = Right Ventricle. Treated animals (dotted bar); control animals (striped bar). N=10, n.s = not significant, *: p>0.05, **: p>0.01, ***: p>0.001.

4.2.3.1 Heart

Left ventricular hypertrophy was also evident under histopathological investigation (Figure 4.11). Cardiac hypertrophy was observed which correlated with the echocardiography data. No vascular injury was present although dilation of the coronary arteries was prominent (Figure4.12).

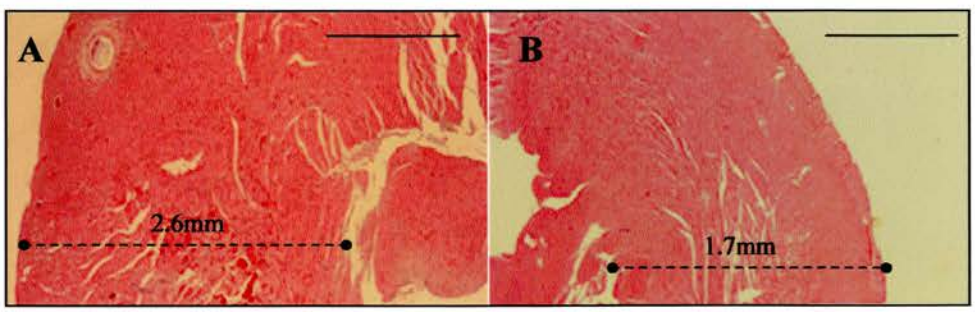


Figure 4.11: H&E stained sections of paraffin embedded hearts. A) Treated TGR(*Cyp1a1-Ren2*) rat left ventricle wall. B) Control TGR(*Cyp1a1-Ren2*) rat left ventricle wall. Bars: 1mm. Dashed lines indicate the thickness of the ventricular wall. Sections: 4μm.

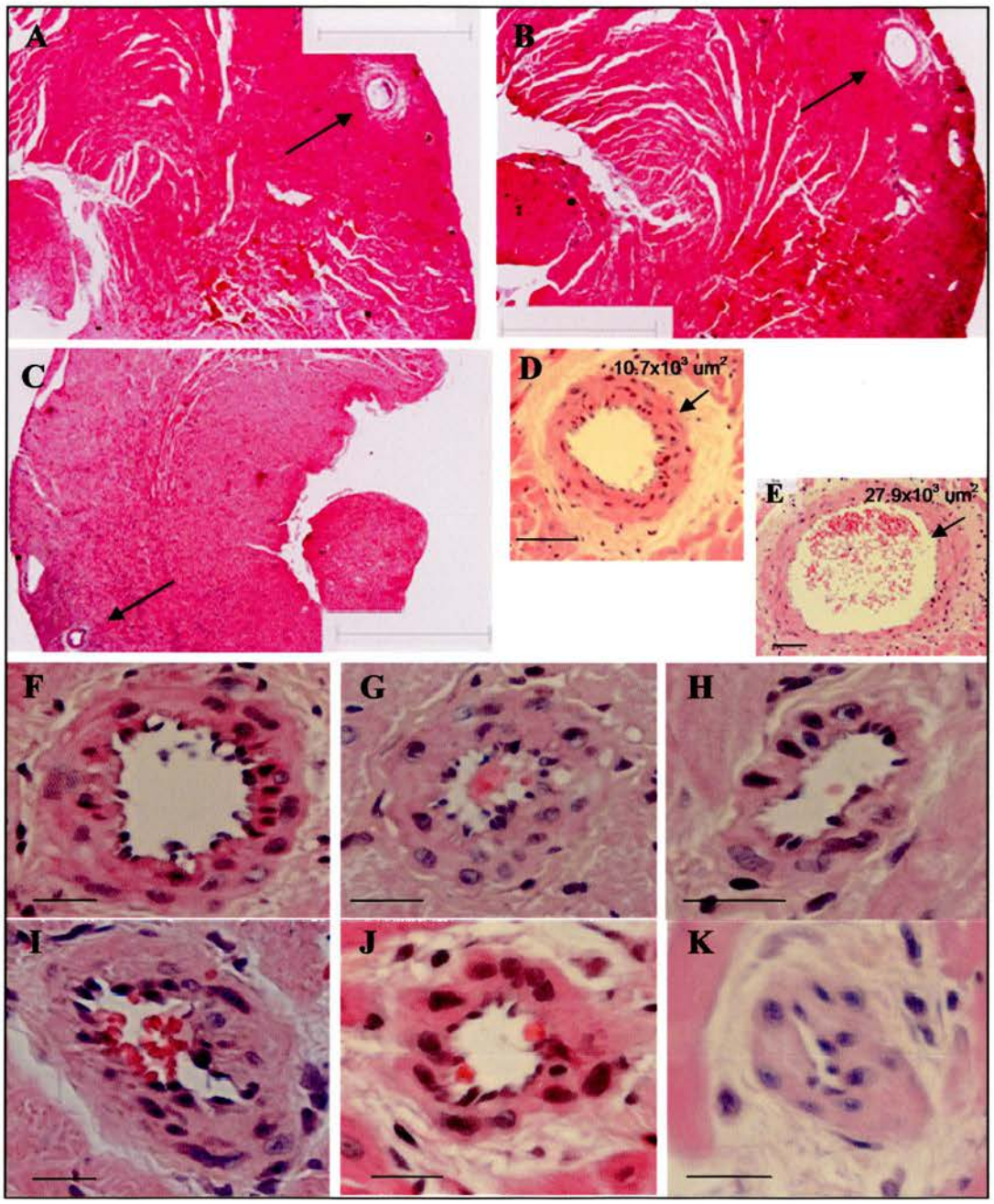


Figure 4.12: H&E stained sections of paraffin embedded hearts of TGR(*Cyp1a1-Ren2)* rats. Left ventricle: A-B) Treated heart, C) Control heart. Septal branch of the left coronary artery (arrow). Bar: 1mm. Arterial lumen area size: D) Control and E) Treated left coronary artery, Area measured = lumen area. Bar: 50μm. Coronary arteries size 80-10μm: F-H): Control hearts, I-K): Treated hearts, Bar: 10μm, sections: 4μm.

4.2.3.2 Kidneys

Kidneys were undergoing hypertrophy as demonstrated by the increased weight to tibial length ratio in treated animals. This is not obvious histopathologically. The kidney however displayed glomerular sclerotic areas which may be consistent with the increase weight (Figure 4.13).

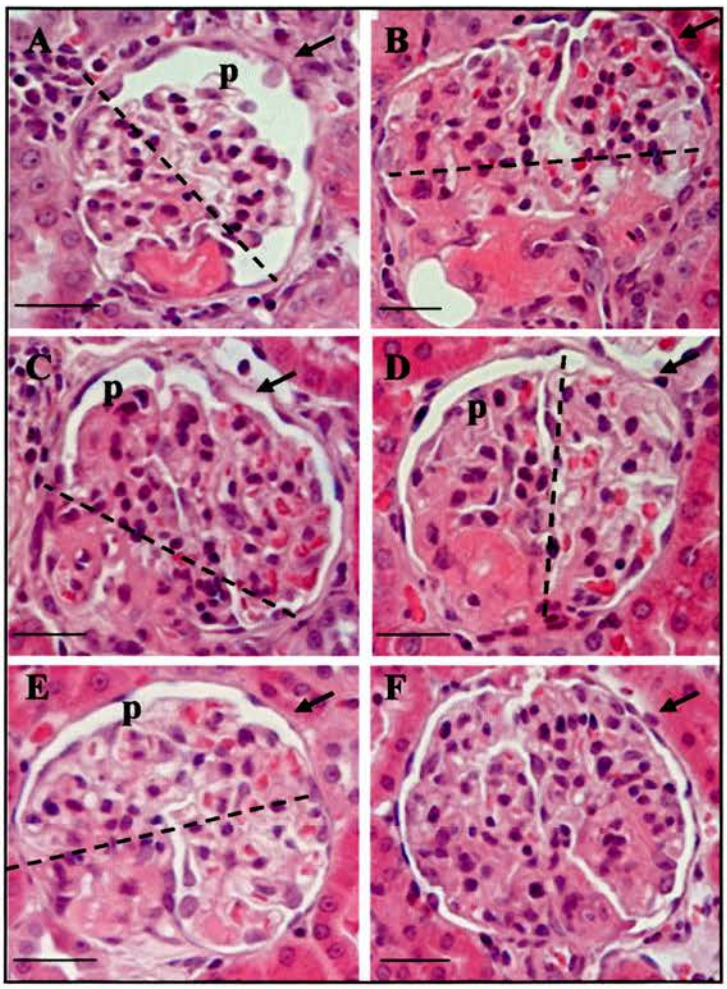


Figure 4.13: H&E stained section of paraffin embedded kidneys of TGR(*Cyp1a1-Ren2)* rats. Glomeruli of treated animals. A) Severe phenotype due to loss of podocyte attachment (p) to the Bowman's capsule. A-E) Note the segmental appearance of the injuries (indicated by the dashed line) B) No loss of podocyte attachment, presence of inflammatory infiltrating cells. C-E) Mild loss of podocyte attachment, presence of inflammatory infiltrating cells. F) Normal glomerulus. Bar represents 25μm, sections: 4μm, the arrow indicates the Bowman's capsule.

In 50% of glomeruli in the treated group, fibrinoid necrosis affected the afferent arteriole Segmental glomerulosclerosis was present (*i.e.* only parts of the glomerulus were affected) characterised in some glomeruli by a loss of podocytes causing adhesion to the Bowman's capsule (Figure 4.13). Increased collagen deposition was present over the whole kidney (Figure 4.14).

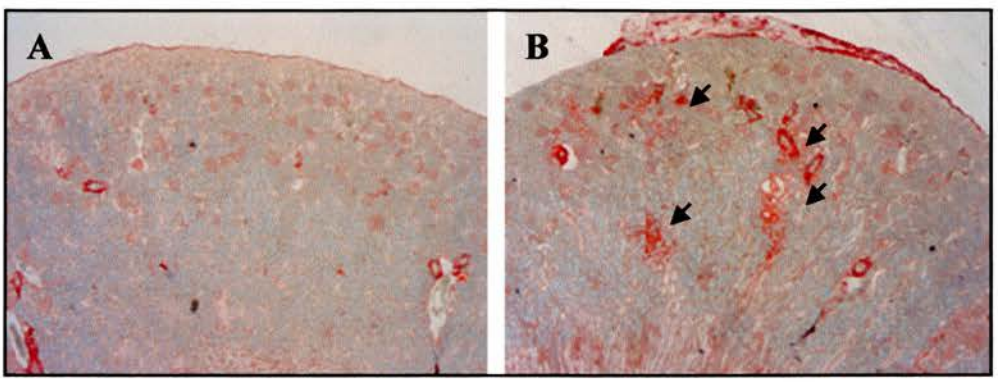


Figure 4.14: Sirius red staining for collagen staining of renal cortices. A) Control TGR(*Cyp1a1-Ren2)* rat, B) Treated TGR(*Cyp1a1-Ren2)* rat. Note the increased collagen deposition (arrows). sections: 4μm.

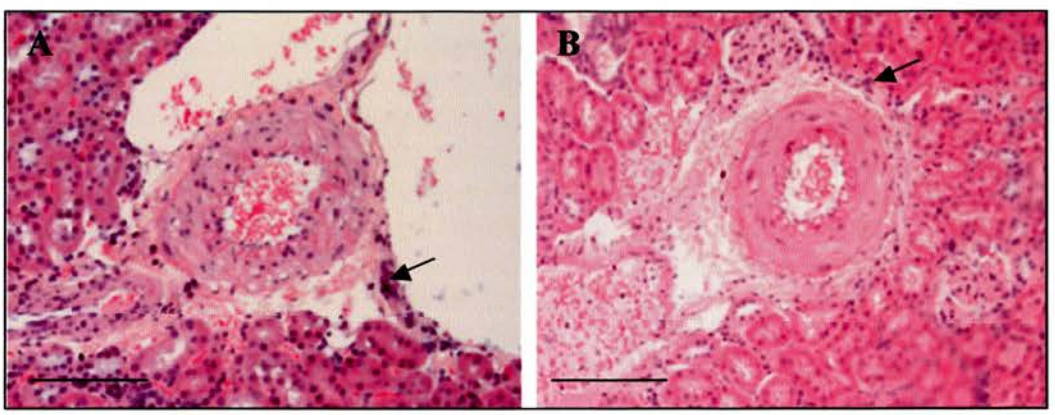


Figure 4.15: H&E stained sections of paraffin embedded kidneys. Arcuate arteries. A) Control TGR(*Cyp1a1-Ren2)* rat, B) Treated (*Cyp1a1-Ren2)* rat. Bar represents 100μm, sections: 4μm, arrow indicates the infiltrating inflammatory cells.

Minimal changes were observed in the interlobar-arcuate arteries (size: 120-250μm – Figure 4.15) finding which again differs from previous reports. Profound changes become apparent in the interlobular arteries, more markedly in the peripheral vessels (Figure 4.16 B&D).

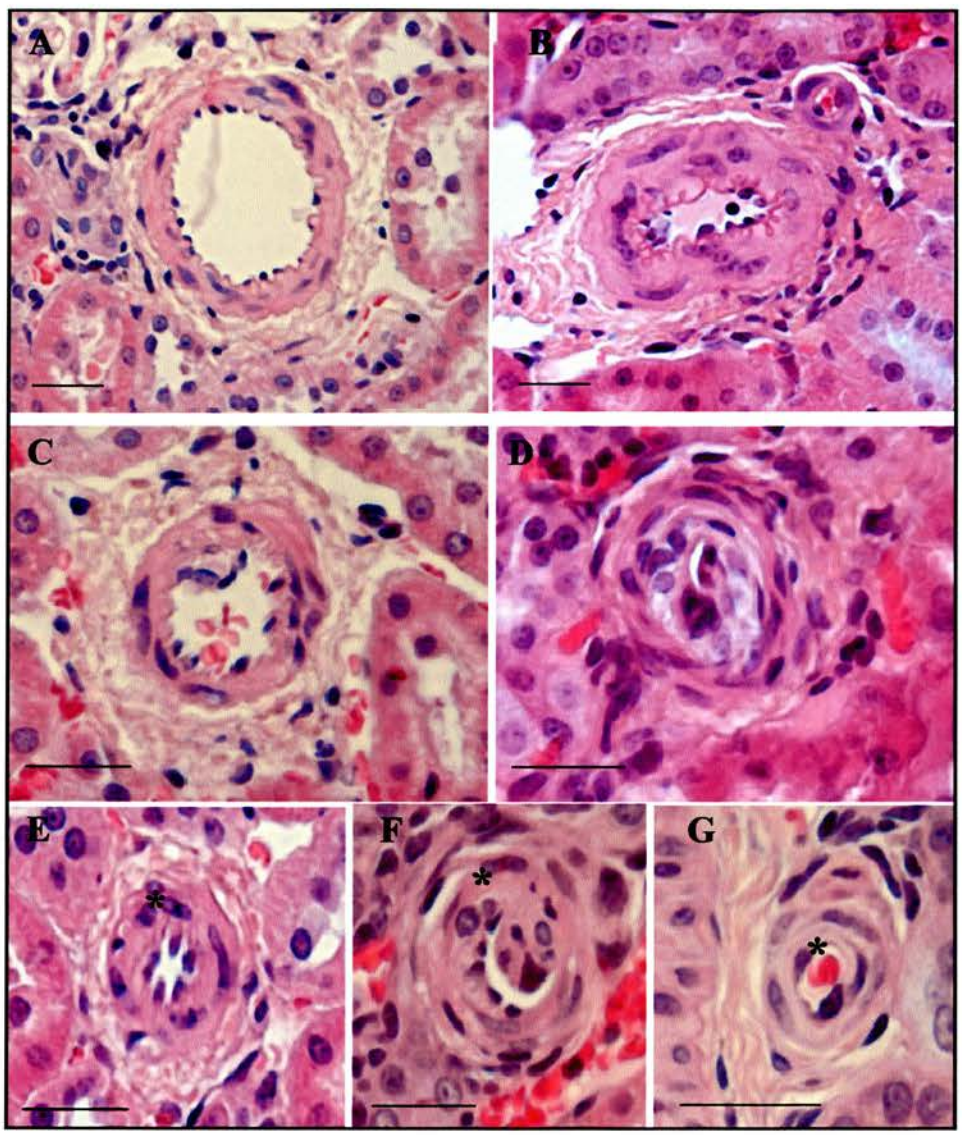


Figure 4.16: H&E stained sections of paraffin embedded kidneys TGR(*Cyp1a1-Ren2)* rats. Intra-renal vasculature. A) Control interlobular artery, vessel size approx 80μm. B) Treated interlobular artery, vessel size approx 80μm. Note the thickening of the adventitia indicated by the arrow. C) Control interlobular artery, vessel size approx 40μm, D) Treated interlobular artery, vessel size approx 40μm. Note the "onion skin" appearance indicated by the asterisk, E) Control (*Cyp1a1-Ren2)* rat interlobular artery, vessel size < 40μm, F-G) Treated TGR(*Cyp1a1-Ren2)* rat interlobular artery, vessel size < 40μm. Note the marked "onion skinning" and loss of intimal space indicated by an asterisk. Sections: 4μm.

The media was thickened; the lumen decreased in thickness and collagen deposition was increased in adventitia. The cross-sectional area was increased due to smooth muscle cells hypertrophy and the nuclei of the smooth muscle cells appeared to be more numerous in the treated animals, which may be due to diploidy. In smaller vessels (60-40μm), intimal proliferation (as demonstrated by the presence of proliferative endarteritis) was present (Figure 4.16). The demarcation of the vascular layers was lost and the vessels had an "onion skinning" appearance which is associated with malignant hypertension. In the smallest vessels (<40μm), this was even more marked (Figure 4.16 F&G) with near complete loss of luminal space.

4.2.3.3 Mesentery

Mesentery artery pressure-diameter data are in accordance with gross and microscopic findings:

The morphology of the isolated first order mesenteric arteries may be different from ante-mortem because the vessels were used for constructing a pressure-diameter curve and were therefore pressure-inflated (up to 220mmHg). They displayed an apparent increased medial area and a decreased vascular lumen (Figure 4.17). This was common to all calibres of mesentery arteries. Despite the pressure diameter curve, the cross-sectional area would not have changed with pressure. The vessels also had a thickened adventitial layer due to increased collagen deposition. It was estimated than an increase of 25% in cross-sectional area was present. This event is most likely due to hypertrophy and/or an increased extracellular matrix production.

Histopathological stains for elastin, collagen and reticulin were performed and only collagen staining was higher in treated animals than in controls notably in the media (data not shown). Proliferative endarteritis was seen in mesentery arteries of size 40μm or lower (Figure 4.17 E&F). Inflammatory cell infiltration was not present in this vascular bed.

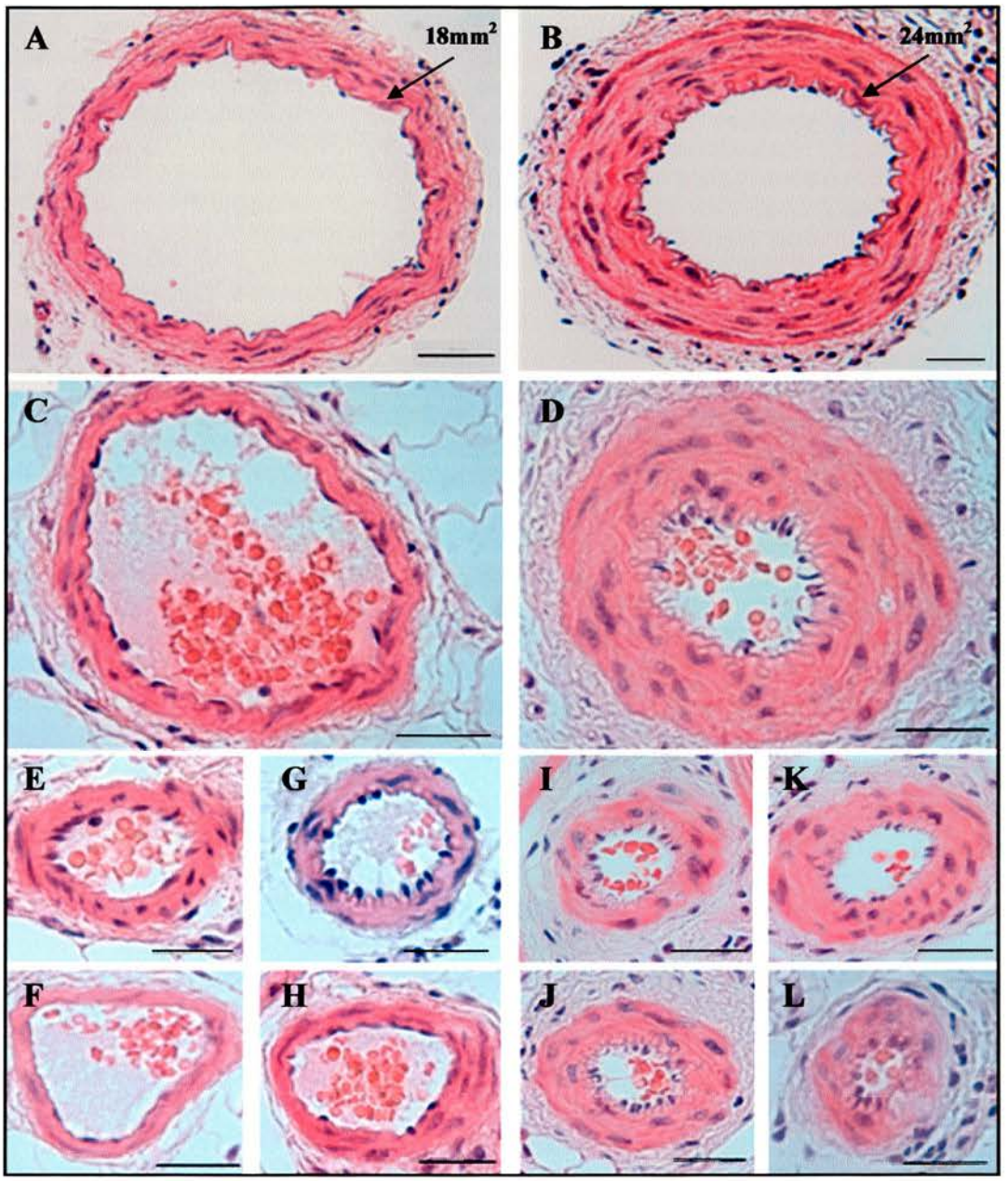


Figure 4.17: H&E stained sections of paraffin embedded mesenteric vessels of TGR(*Cyp1a1-Ren2)* rats. A) Control and B) treated first order artery, vessel size 250-120μm, Area measured represents the areterial wall surface areabar represents 50μm. C) Control and D) Treated second order artery, vessel size 120-100μm, measured area = lumen area, bar represents 50μm. E-H) Control and I-L) treated third order arteries, vessel size 100-40μm, bar represents 25μm. Note the thickening of the adventitia and the reduced luminal area in all treated vessels. Sections: 4μm.

4.2.4 (Pro)renin receptor expression in wild type rat tissues and regulation during induction of transgene in TGR(*Cyp1a1-Ren2*) animals

Considering that, in TGR(*Cyp1a1-Ren2*) rats, prorenin is derived from the mouse gene *Ren2* and therefore unglycosylated, the mannose-6-phosphate receptor is an unlikely candidate for prorenin uptake. An alternative pathway may be through the (pro)renin receptor described by Nguyen *et al.* The upregulation of the rat putative (pro)renin receptor was examined in TGR(*Cyp1a1-Ren2*) tissues. For this reason, expression of the homologous (pro)renin receptor in rats was first verified in a wide range of tissues.

4.2.4.1 RT-PCR on Fischer (F344) female adult tissues

Standard RT-PCR was used to study the expression pattern of the putative (pro)renin receptor in Fischer (F344) adult rat and compare it to the pattern obtained in the mouse (*Chapter 3.2.4*). As for the mouse, the (pro)renin receptor was detected in all tissues examined showing that, in this species, the (pro)renin receptor is ubiquitously expressed (Figure 4.18).

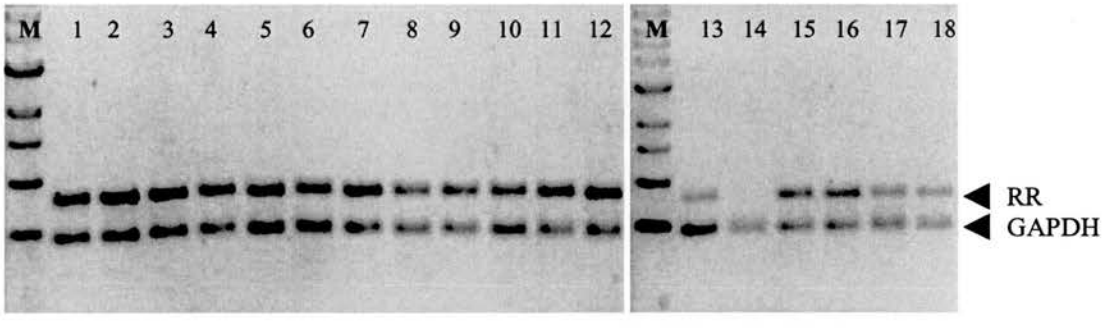
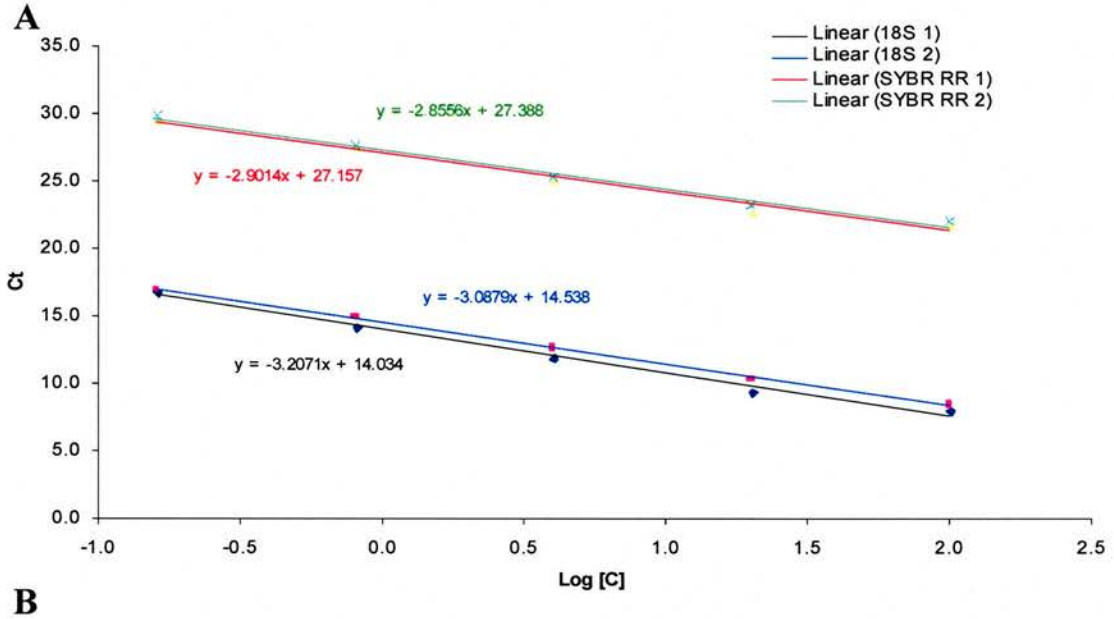


Figure 4.18: Reverse Transcriptase PCR of putative rat (pro)renin receptor (RR) and GAPDH. Agarose gel electrophoresis. Female adult tissue samples from two rats of Fischer (F344) strain. Bands of the correct sizes (RR : 900bp; GAPDH : 480bp) were obtained in all tissues except muscle for which only one repeat reaction was positive. The two PCR reactions were performed independently but electrophoresis was done simultaneously. DNA marker (M) : 1kB ladder. Lane 1-2 = Brain, Lanes 3-4 = Mesenteric Fat, Lanes 5-6 = Heart, lanes 7-8 = Kidney, Lanes 9-10= Liver, Lanes 11-12 = Lung, Lanes 13-14 = Muscle, SMG = Submandibular Gland, Sp = Spleen.

4.2.4.2 *Regulation of expression of the (pro)renin receptor in TGR(Cyp1a1-Ren2) rats*

A SYBR green real-time RT-PCR assay was designed to measure differences in (pro)renin receptor expression in treated and control TGR(*Cyp1a1-Ren2)* animals. Optimisation of the method was performed to obtain the optimal primer concentrations. Three concentrations of primers, 300nM, 600nM and 900nM, were tested with a cDNA mastermix (made up of all RNAs to be tested) at a concentration of 100ng/µl to obtain the lowest CT with the lowest primer concentration pair. The results showed that a concentration of 300nM for the forward primer and 600nM for the reverse primer were optimal (data not shown).

A



B

Template concentration (ng/ml)	Log concentration	18S plate 1 Mean Ct	18S Plate 2 Mean Ct	SYBR RR Plate 1 (Mean Ct)	SYBR RR Plate 2 (Mean Ct)
100	2.0	8.1	8.5	21.8	22.1
20	1.3	9.4	10.4	22.9	23.3
4	0.6	12.0	12.6	25.1	25.4
0.8	-0.1	14.2	14.9	27.6	27.7
0.16	-0.8	16.9	17.0	29.6	29.9

Figure 4.19: Standard curves for rat (pro)renin (SYBR green) and 18S (VIC) quantitative PCR measurements. A) A standard curve was incorporated to each sample plate to avoid plate to plate variation. The standard curve for each gene was reproducible and the efficiency of the each assay was high (slope = 2.85 and 2.9 for SYBR green; 3.08 and 3.2 for VIC). B) Table of individual standard curve value points.

For future investigations, the highest value of the standard curve was selected to be 100ng/μl as used for the optimisation step. Using the SYBR green optimised assay, a standard curve was constructed with the cDNA mastermix described above at 100, 20, 4, 0.8, 0.16 and 0ng/μl (Figure 4.18). Three animals per group were used for each tissue analysed, namely heart (left ventricle), kidney, adrenal gland. In order to normalise the data and to account for pipetting errors, an endogenous gene assay (Taqman 18S-VIC) was also performed using the same standard curve and the same samples. Each plate with samples contained its own standard curve to account for plate to plate variation. Hence, due to the number of samples used in the present experiment two plates for each assay were needed to accommodate PCR reactions on left ventricle, adrenal gland and kidney samples from TGR(*Cyp1a1-Ren2*) animals. Standard curves for each assay, Taqman with 18S primers and SYBR green with rat putative (pro)renin receptor primers were reproducible from plate to plate. The slopes of the standard curves showed that the assay performed with 18S primers was more efficient than with rat putative (pro)renin receptor primers, although the slope was close to optimum (3.03) for each primer set (Figure 4.19). It was found that no differences between the treated and the control groups could be observed in terms of prorenin receptor expression (Figure 4.20) in all three tissues examined, namely left ventricle, kidney and adrenal gland. Expression of the rat putative (pro)renin receptor was higher in the left ventricle than in the kidney and the adrenal gland, although this was not statistically significant.

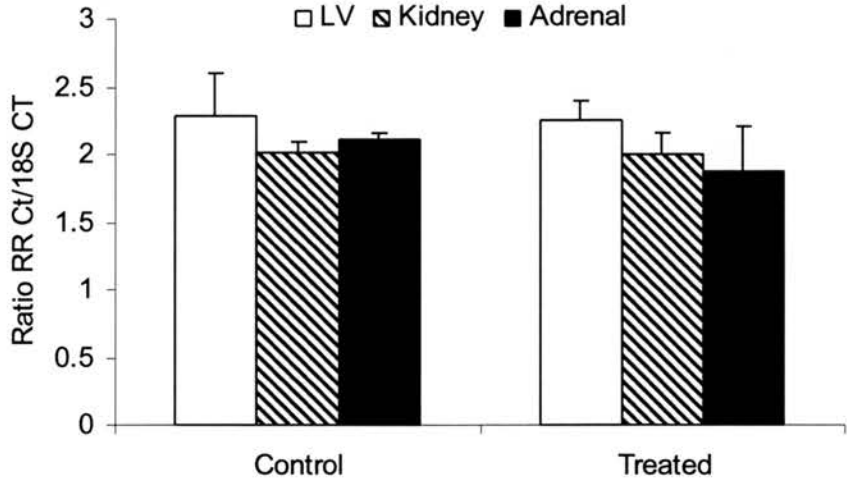


Figure 4.20: Analysis of the putative (pro)renin receptor expression in TGR(*Cyp1a1-Ren2*) rats by real-time RT-PCR. Data is expressed as a ratio of CT for (pro)renin receptor (RR) with SYBR green dye and of CT for 18S (Taqman probe labelled with VIC). P value was determined by Student's t-test. LV = left ventricle.

4.3 Discussion and conclusions

This study demonstrates the advantages of this recently developed transgenic rat model of hypertension, notably the fact that the severity and length of exposure to high blood pressure can be modulated at will. This allows the study of chronic as well as acute effects and mechanisms of hypertension but also to investigate recovery mechanisms when treatment has ceased. Echocardiography techniques used in this report has been shown to be powerful non invasive tools to evaluate the extent of cardiovascular damages in hypertensive animals. Transgene induction resulted in a rapid increase in blood pressure in treated TGR(*Cyp1a1-Ren2*) animals. This was accompanied by weight loss, polyphagia, polyuria and polydypsia without indication of diabetes. Leucocytes were detected in the urine of treated TGR(*Cyp1a1-Ren2*) animals suggesting renal inflammation potentially linked to hypertensive injury.

4.3.1 Cardiac and aortic phenotype

Echocardiography data shows an increase in left ventricular area suggesting development of ventricular hypertrophy in treated TGR(*Cyp1a1-Ren2*) animals. This was confirmed by an increase in left ventricle weight. The increase in blood pressure may also be responsible for a stiffening (calculated as compliance and distensibility) of the abdominal aorta. It is possible that, in turn, aortic stiffening may aggravate vascular and organ remodeling by increasing shear stress. The molecular mechanisms triggering this process remain unknown but histological investigations (namely collagen and elastin staining) have revealed that an increase in the number of collagen fibres as well as hypertrophy in smooth muscle cells which is likely to be responsible for the aortic stiffening. Whether this response is causative of or caused by hypertension is unclear. However, due to the rapid increase in blood pressure after two days of transgene induction, one may speculate that arterial stiffness is the result of increased blood pressure and shear stress. In turn, a decrease in the compliant, blood pressure-dampening properties of the aorta could contribute towards the gradual blood pressure elevation after the initial period of hypertension due to the transgene activation.
Cardiac output (CO) and stroke volume were markedly reduced compared to control after two weeks of treatment which may also be an adaptative response to increased blood pressure (BP) and increased peripheral resistance (PR) since blood pressure is dependent on both factors (BP=CO*PR). Indeed, it was shown that mesenteric arteries developed

increased stiffness and this could be the case in other vascular beds. Stroke volume was reduced from week 2 in the treated group. Although a clear reduction in stroke volume was also observed in week 4, this was not significantly significant compared to controls. Decreased cardiac function was, however, not observed in older (24 weeks) treated TGR(*Cyp1a1-Ren2*) animals (B. Janssen, personal communication). Hence, the timing and onset of the hypertension are important determinants of hypertrophy and cardiac failure. Dilation of the coronary arteries was obvious which is likely to be an compensatory response in order to decrease local blood pressure and to prevent increased shear stress-mediated injury to the cardiac tissues. In contrast with the results published in (Kantachuvesiri *et al.* 2001), no inflammatory injury to cardiac tissues could be observed. It may explain why the rats survive/handle exposure to increased blood pressure for such a prolonged period of time compared with rats available in Edinburgh (as investigated in *Chapter 5*).

4.3.2 Renal phenotype

The renal phenotype consisted of general but subtle fibrosis in the cortex as evidenced by a small increase in collagen staining, segmental glomerulosclerosis, profound changes to the interlobular arteries and fibrinoid necrosis of smaller renal vessels and afferent arterioles. The "onion skinning" appearance was gradually more marked with decreasing vessel size. There are two forms of hypertensive injury to the kidney. Firstly, the afferent arterioles constrict until the high blood pressure is not carried on to the glomerulus, hence preventing exposure to high blood pressure in the glomerulus and the efferent arteriole which can become ischaemic. This causes severe arteriolar nephrosclerosis. Secondly, increased arterial pressure is transmitted through the afferent arteriole to the glomerulus which is the most affected. In most rat models and in the present one, no global sclerosis was observed suggesting that the afferent arterioles had recently lost control of high blood pressure dampening to the glomerulus leading to fibrinoid necrosis and segmental glomerulosclerosis. This, however, differs from the original publication (Kantachuvesiri *et al.* 2001) which presented this model in which normal glomerular histology was reported. The renal arteries were unaffected by treatment and minimal changes to interlobar-arcuate vessels was noted. Mild inflammatory cell infiltration was observed in the latter which may explain the leucocyte detection in the urine. The absence of injuries to these vessels suggest, however, that inflammation at earlier stages of transgene induction did not occur.

4.3.3 Mesenteric phenotype

A reduction in the lumen diameter was observed in mesenteric resistance arteries (inward remodelling) of TGR(*Cyp1a1-Ren2*) animals compared to controls. Whether this inward remodelling is hyper/hypo-eutrophic depends on the cross-sectional area of the tunica media. Inward eutrophic remodelling and hypertrophic remodelling are observed in small and large arteries in humans, respectively (P. Schiffers, Personal communication). Indeed, in this study, histopathology revealed that both a reduction in lumen diameter and an increase in vascular wall thickness are present. The wall thickness of the mesentery arteries was increased due to remodelling of the tunica media and the tunica adventitia, the latter displaying a small increase in collagen deposition. As for the aorta, vascular remodelling involving the production of extracellular matrix is likely to be the mechanism involved in the stiffening of small arteries since collagen was increased in the mesenteric bed. No changes in the amount of elastin or reticulin were also observed. In contrast to the aorta, changes in smooth muscle cells were not detected in the mesenteric arteries.

4.3.4 (Pro)renin receptor

Increased local and circulating levels of prorenin may have triggered these remodeling events. Binding of prorenin to the human (pro)renin receptor was shown to trigger intracellular signaling cascades linked to MAP kinases ERK1 and ERK2. Although ERK1 and ERK2 have been demonstrated to initiate hypertrophic events in cardiomyocytes, the involvement of the (pro)renin receptor in hypertrophic responses has not been reported. The remodeling events observed in TGR(*Cyp1a1-Ren2*) animals are, in fact, not due to increased expression of (pro)renin receptor in left ventricle, kidney or adrenal since quantitative PCR revealed no differences in (pro)renin expression between treated and control rats.

It is possible that increased expression of (pro)renin receptor in the vasculature plays a role in haemodynamics as demonstrated in a (pro)renin receptor over-expressing transgenic rat model by Burckle *et al.* Quantitative expression of the (pro)renin receptor in isolated vessels was not measured in the present study. Basal levels of the receptor may be normally high and increased prorenin may result in increased binding to the receptor followed by non-proteolytic activation. It is worth noting that the vasculature was not microdissected out of the tissues examined in the present study. However, an increase in the expression of the (pro)renin receptor solely in the vasculature is unlikely to be the major contributor in the

pathogenesis of TGR(*Cyp1a1-ren2*) animals since overexpression of the receptor did not trigger malignant hypertensive injuries (Burckle *et al.* 2006). The author also used a anti-human (pro)renin receptor antibody(kindly provided by Dr Nguyen) in an attempt to localise the protein in several tissues such as the heart, the kidney and the brain. Paraffin sections from TGR(*Cyp1a1-Ren2*) animals were tested for efficiency of antibody binding to rat tissues. However, this proved to be unsuccessful and resulted in non-specific binding and high background levels. It also made impossible the comparison of protein levels by Western blotting which would have complemented the real-time PCR data. It cannot be excluded that, although the putative (pro)renin receptor mRNA levels were not different between control and transgenic animals, post-transcriptional up-regulation is occurring.

Overexpression of the putative (pro)renin receptor in transgenic rats (Burckle *et al.* 2006) results in increased blood pressure and heart rate but does not trigger any end-organ damage or inflammatory events, arguing for a role of this molecule in blood pressure –dependent mechanisms. The prorenin pathway of non-proteolytical activation is, however, thought to be linked to hypertrophic and inflammatory responses independently of blood pressure. This is also in contradiction with the work of Ichihara *et al.* on the involvement of the putative (pro)renin receptor in diabetic nephropathy. In addition, the increased levels of aldosterone reported by Burckle *et al.* may be related to the M8-9 function of the putative (pro)renin receptor. The work of Nguyen *et al.* reports binding of prorenin, non-proteolytic activation and intracellular signalling cascade upon binding to the human (pro)renin receptor. However, the mechanism of prorenin uptake is neither addressed nor discussed in these studies. This is of great importance, in particular for the transgenic rat model *Cyp1a1-Ren2* and this is further investigated using a decoy peptide as inhibitor of the putative (pro)renin receptor.

In order to clarify the involvement of the putative (pro)renin in the malignant hypertension phenotype, inhibition of the putative (pro)renin receptor in TGR(*Cyp1a1-ren2*) animals was studied in a pilot study (*Chapter 6*). A decoy peptide showed to competitively bind the putative (pro)renin receptor in a rat model of diabetic nephropathy (characterised by an elevated concentration of circulating prorenin; (Ichihara *et al.* 2004)) was employed and histopathology data analysed.

Chapter 5

Genetic, Environmental and Experimental Factors in the Phenotype of TGR(*Cyp1a1-Ren2*) animals

5.1 Introduction

For this chapter, the phenotype of TGR(*Cyp1a1-Ren2)* rats studied at the University of Maastricht was compared with the phenotype described in the original publication (Kantachuvesiri *et al.* 2001) which described the TGR(*Cyp1a1-Ren2)* produced at the University of Edinburgh. Differences in phenotype were noted from the previous data as highlighted in *Chapter 4.2.3*. Although, infiltrating inflammatory cells were noted in the large arteries of the kidney (arcuate arteries) and in the glomerulus, the expected significant inflammatory response in the kidney and in the heart was absent which differed from the previously published data (Kantachuvesiri *et al.* 2001). Glomerulosclerosis was observed in the treated "Maastricht" TGR(*Cyp1a1-Ren2)* after 5 weeks of transgene induction which was not observed previously in the "Edinburgh" TGR(*Cyp1a1-Ren2)* after 2 weeks of treatment. Glomerulosclerosis may be an adaptative response to the prolonged exposure to high blood pressure although it would be expected to be a rapid, early rather than a chronic phenotype. In addition, no signs of cardiac tissue infarctions were observed despite an obvious hypertrophic effects in the left ventricle of induced TGR(*Cyp1a1-Ren2)* rats.

Another striking feature of the "Maastricht" TGR(*Cyp1a1-Ren2)* is their apparent reduced sensitivity to the transgene inducer (indole-3-carbinol) as observed by the very low mortality rate in this colony. Except for the excess micturition and increased food and water intake, the animals did not display any signs of illness such as reduced activity, cessation of grooming, hunch back or unkempt appearance and they appear to thrive as illustrated by the lack of weight loss showing that the growth of the young animals was not inhibited.

The reasons for these discrepancies were examined, namely genotypic and environmental differences. In order to address these issues, a questionnaire was designed to compare dietary, experimental and general animal care in all 5 populations of TGR(*Cyp1a1-Ren2)* rats existing round the world. In addition, extensive genotyping of the two "Edinburgh"

populations of TGR(*Cyp1a1-Ren2)* and of the "Maastricht colony" was undertaken using microsatellite markers to determine if strain background has a role in these phenotypic differences. Other TGR(*Cyp1a1-Ren2)* were not examined in terms of genetic background since they were not re-derived and are believed to be identical from the original strain. It may, however, be useful to undertake genotypic screening of other lines since the genetic background could account for differences in the sensitivity to hypertensive injuries.

The aim of this chapter was, therefore, to identify potential contamination with rat strains other than Fischer (F344) which may account for the phenotypic differences observed during the experiments carried out in Maastricht and described in *Chapter 4*. Genetic background is of great importance considering the hypertensive rat model TGR(*mRen2*)27, which expresses the mouse *Ren2* gene and displays severe malignant hypertension accompanied by high circulating prorenin concentrations and by elevated tissue RAS levels (Mullins *et al.* 1990). Crosses of TGR(*mRen2*)27 to different rat strains have showed that malignant hypertension is linked to the strain genetic background, TGR(*mRen2*)27 rats on a Fischer (F344) background displaying the most extensive injuries whereas TGR(*mRen2*)27 rats on a Lewis background exhibited less striking lesions (Mullins and Mullins 2003).

5.2 Results

The re-derivation, environmental, dietary and experimental information was compiled by myself for the two Edinburgh colonies and by myself from the colony held in Maastricht University (The Netherlands) with the help of Dr Ben Janssen (University of Maastricht) who had performed earlier work on the animals. Dr. Kenneth Mitchell and Prof. Jörg Peters kindly provided information relating the transgenic animals held in Tulane University (New Orleans, USA) and in the University of Greifswald (Germany), respectively.

5.2.1 Transfer and re-derivation of different TGR(*Cyp1a1-Ren2)* rat colonies

The "Maastricht" TGR(*Cyp1a1-Ren2)* rats were re-derived by caesarian section from the animals examined in this publication. As part of a department re-structuring in 2004, the "Edinburgh" TGR(*Cyp1a1-Ren2)* rats were also re-derived by embryo transfer to a separate animal facility (Table 5.1).

Re-derivation	Edinburgh-WGH	Edinburgh-LF	Maastricht	New Orleans	Greifswald
Were the animals re-derived from the original Edinburgh stock after reception?	No – original stock	Yes	Yes	No	No
If, yes. Were they re-derived on a Fischer F344 background?		Yes	Yes. From a single male animal		
How were they re-derived? Caesarian section or embryo transfer?		Embryo transfer	Caeserian section		

Table 5.1: Comparison of re-derivation procedures used to establish TGR(*Cyp1a1-Ren2*) colonies at the University of Edinburgh and in collaborating laboratories.

The only other colony which was re-derived was the colony established at the University of Maastricht. This was performed by for the caesarian section procedure and the rationale for re-derivation was that the original animals from Edinburgh were found to have pinworms, and, in addition, *S.aureus* which is not routinely screened as part of Home Office regulations in the UK but required in the Netherlands.

5.2.2 Indole-3-Carbinol and diet preparation

Indole-3-carbinol is the compound used to induce the cytochrome P450 1A1 (*Cyp1a1*) promoter under which control the mouse *Ren2* gene is. It is therefore an important factor in the transgene activation and the downstream production and release of prorenin in the plasma. Preparation of the diet and its basal sodium contents may also have an effect on the phenotype of TGR(*Cyp1a1-Ren2*).

Indole-3-carbinol	Edinburgh WGH	Edinburgh LF	Maastricht	New Orleans	Greifswald
Which dose of Indole-3-carbinol (I3C) do you use?	0.3% (Have used 0.15%)	0.3%	0.3% (Have tried 0.6% and 1%)	0.15% 0.3%	Testing optimal range. 0.03-0.15 %
What is your supplier of I3C?	Sigma	Sigma	Sigma	ICN	Sigma
How do you mix the I3C with the diet?	Manually with a little water	Manually with a little water	Manually with a little water	ICN-made dry pellets (0.15%)	Ssniff-made dry pellets (=0.1666% or 0.075% I3C)
Is I3C pre-mixed with peanut/sesame oil?	No	No	Yes- peanut oil	No	No but the pellets are banana-chocolate flavoured
Do you sterilise the prepared I3C diet?	No	No	No	No	No
Diet	Edinburgh WGH	Edinburgh LF	Maastricht	New Orleans	Greifswald
What is the sodium content of the diet used to prepare I3C?	0.32%	0.32%	0.25%	0.5%	1%
Is the I3C diet given to the animals as a paste or powder?	Paste to avoid food wastage	Paste to avoid food wastage	Wet powder	Pre-made pellets	Pre-made pellets
Are food and water given *ad libitum*?	Yes	Yes	Yes	Yes	Yes

Table 5.2: Comparison of indole-3-carbinol (I3C) preparation used in TGR(*Cyp1a1-Ren2*) colonies at the University of Edinburgh and in collaborating laboratories. I3C = Indole-3-carbinol.

The concentrations of I3C used routinely for transgene induction were consistent between the laboratories, namely 0.3% although other I3C concentrations have been tested (Table 5.2). The exception is in Greifswald where the standard concentration used is 0.15%. In Maastricht, due to a low number of animals developing cardiac failure, higher concentrations of 1% (which was discarded as a working dose due to the pungent smell of I3C preventing the rats from eating the diet) and 0.6% (which did not yield significant results from 0.3% in terms of mortality or phenotype) . In Edinburgh, a lower dose of 0.15% was used previously resulting in a phenotype which developed more slowly and which was less severe than with 0.3% (A. Ryding, University of Edinburgh, 2005, PhD thesis). The amount of vegetable derivatives i.e.: potentially containing I3C may vary between batches/supplies of normal chow.

In addition, it was noted that peanut oil was used to mix I3C before adding it to the diet in Maastricht. Another striking difference was the sodium contents between the rodent chow used in all different animal holding facilities, the highest being in Greifswald and New Orleans.

5.2.3 Transgene induction duration and protocol

The exposure time to the inducer varied greatly between laboratories due to a variable mortality (Table 5.3). The shortest routine length of exposure is 12 days at Tulane University (New Orleans) if the animals are exposed to 0.3% due to the animals showing signs of ill health.

Transgene induction	Edinburgh WGH	Edinburgh LF	Maastricht	New Orleans	Greifswald
How long do you usually carry out the induction for?	14 days	14 days	5 weeks but 2-20 weeks has been done	Never more than 12 days due to high mortality	2 weeks to 3 months
What is the lethality rate?	High mortality rate after 14 days on 0.3%, lower on 0.15%.	Not determined.	Very low (*e.g.* one animal found dead in cage after 20 weeks)	Animals unwell from day 12, similar to Edinburgh animals	With 0.1%; high mortality rate at 6 weeks
During induction, are the animals housed individually or in groups?	Individually	Individually	Two per cage	Individually for telemetry. Normally in groups.	Individually for telemetry. Normally in groups.

Table 5.3: Comparison of transgene induction procedures used to establish TGR(*Cyp1a1-Ren2*) colonies at the University of Edinburgh and in collaborating laboratories.

However, the "Greifswald" animals displayed high mortality when fed a diet containing a low concentration of 0.1% for 6 weeks. It is worth noting that the diet used at Tulane University and at the University of Greifswald contain the highest amount of sodium.

The animals from Maastricht appear to be the least sensitive to the transgene induction as reflected by very few (if any at all) animals dying although their blood pressure is increased to a level comparable to the treated animals from Edinburgh and from the other colonies. In

addition, the amount of sodium in the diet purchased in Maastricht was the lowest compared with all the other animal holding facilities (Table 5.3). If the results are extrapolated, it appears that dietary sodium contents are correlated with the severity of the phenotype, the time course of phenotype progression and with the mortality rate.

5.2.4 Blood pressure measurement

The blood pressure measurements are similar between all the laboratories when measured by higher, except in Greifswald where the I3C doses were lower and one would expect the transgene induction to be reduced compared to that of animals from other colonies (Table 5.4). The values obtained in New Orleans differ depending on the blood pressure sampling method, namely, tail-cuff or radiotelemetry. This is thought to be due to the restraining procedure during the tail-cuff procedure.

Physiology	Edinburgh WGH	Edinburgh LF	Maastricht	New Orleans	Greifswald
What is the blood pressure of induced animals?	<200mmHg after 7 days (Telemetry)	<200mmHg after 14 days (Tail cuff)	<200mmHg after 14 days (Tail cuff)	<200mmHg (T.C.) <160mmHg (Telemetry)	On 0.075%, 160/130 mmHg after 3 weeks
What is the method of blood pressure measurement used?	Tail cuff or telemetry	Tail cuff	Tail-cuff	Tail cuff or telemetry	Telemetry

Table 5.4: Blood pressure measurements obtained with TGR(*Cyp1a1-Ren2*) animals from all the different holding facilities. Blood pressure was measured in for all animal population after induction of the transgene with indole-3-carbinol. T.C. = Tail cuff.

5.2.5 Histopathology

It is difficult to assess whether histopathology data is comparable between the TGR(*Cyp1a1-Ren2)* colonies since different length of exposure, dosage are used and the organ of interest/investigations may vary. General phenotypic similarities were, however, observed.

A *Pathology*	Edinburgh WGH	Edinburgh LF	Maastricht	New Orleans	Greifswald
When during induction are signs of malignant hypertension first observed?	BP: 1 day Pathology: 3 days	BP: 1 day Pathology: 3 days	BP: 2 days but blood pressure response much more progressive Pathology: 7 days	BP: 1 day Pathology: 7 days	Not applicable since lower dose
Do you observe infiltration of inflammatory cells?	Yes	Yes	Very little	Yes	Some but not prominent
Do you have evidence of *in vivo* vascular stiffening?	Not done	Not done	Yes	Not done	Not done

B	Is the observed pathology correlating with the original results published in Kantachuvesiri *et al.* (2001) ?
Edinburgh WGH	N/A – original work
Edinburgh LF	Microinfarctions, fibrinoid necrosis, inflammatory cells in heart and similar injuries to kidneys: intrarenal vascular thickening without glomerulosclerosis
Maastricht	No, reduced inflammatory component. No microinfarction, fibrinoid necrosis in heart. Fibrinoid necrosis of small intra-renal vessels with glomerulosclerosis.
New Orleans	In kidney: vascular and interstitial cell proliferation. Segmental glomerulosclerosis and tubular cell proliferation.
Greifswald	Lower doses were used so less severe phenotype. However, hypertrophy and fibrosis present. Inflammatory cell infiltration in the heart.

Table 5.5: Table comparing establishment of phenotype, inflammatory response and histopathology between TGR(*Cyp1a1-Ren2*) animals from all the collaborative laboratories and from the "Edinburgh" facility. A) Description of the histopathological phenotype, B) Comparison of the phenotype of each colony with the previously published results (Kantachuvesiri *et al.* 2001) N/A = not applicable, MH = malignant hypertension.

The comparison of the phenotype between treated animals from all 5 existing colonies is summarised in Table 5.5. The rise in blood pressure was rapid in all treated TGR(*Cyp1a1-Ren2)* rats except for "Maastricht" animals for which it was more progressive, did not reach a plateau and continued to increase throughout the 5-week experiment (*Cf. Chapter 4.2.1.1*). The common phenotype between all the animals was cardiac hypertrophy accompanied by fibrosis although the latter may be a very subtle response as observed in the "Maastricht" rats, despite longer exposure to inducer. Inflammatory cell infiltration was not a significant feature of the heart, mesentery or kidney, except around the large intra-renal arteries in the

"Maastricht". This was also less prominent in the "Greifswald" which may be due to a lower I3C dose. All the other treated animals displayed infiltration of inflammatory cells. No information was obtained about renal histopathology for the "Greifswald" animals. The overall phenotype (renal and cardiac) was considered to be less severe with these animals due to the lower dosage used but to follow a similar pattern of end-organ damage (J. Peters, personal communication).

Segmental glomerulosclerosis was demonstrated in the "New Orleans" and the "Maastricht" animals although, in the latter, the severity of the glomerular injuries was not homogeneous and normal glomeruli were found when examining the same section. Vascular remodeling of the intra-renal vessels was common to the treated animals from all colonies. However, only the "Maastricht" animals displayed fibrinoid necrosis in interlobular arteries and the afferent arterioles which may be due to longer exposure to the transgene induction (five weeks). The "Edinburgh" and the "Maastricht" animals differed again in terms of the lesions in mesenteric bed which were shown to be more severe in the "Edinburgh" animals which displayed vascular remodeling characterised by fibrinoid necrosis (*Cf. Chapter 4*). The mesenteric phenotype consisted of apparent increased medial area and a decreased vascular lumen without fibrinoid necrosis or presence of inflammatory cells in the "Maastricht" rats. What is clear from this questionnaire is that there is a multitude of factors to consider when attempting to evaluate endpoints of the same models used by different experimenters.

5.2.6 Genetic screening

Another important factor in hypertensive phenotypic differences is the genetic background of the animals. Forty-nine microsatellite markers were used and PCR analysis was performed. The original TGR(*Cyp1a1-Ren2)* rats were produced on a Fischer (F344) background and all the re-derived animals (namely Edinburgh-LF and Maastricht) were re-derived on this background. It was, however, decided to verify the genetic strain as it is known to affect cardiovascular phenotypes.

Two animals from Maastricht. two animals from Edinburgh-WGH and 3 animals (from three different breeding pairs) from Edinburgh-LF were analysed using microsatellite markers. The results are summarised in Table 5.6 and examples of genotyping and evidence of genetic contamination are shown in Figure 5.1.

Animal *Marker*	*WGH* *6015*	*WGH* *6031*	*Maastricht* *4*	*Maastricht* *6*	*LF* *182*	*LF* *170*	*LF* *199*
D1Mgh2	FF	FF	FF	FF	FF	FF	**LL**
MT1PA	FF	FF	FF	FF	FF	FF	FF
D1Rat51	FF	FF	FF	FF	FF	FF	FF
D2Mgh14	FF	FF	FF	FF	FF	FF	FF
D2Mit6	FF	FF	FF	FF	FF	FF	FF
D2Rat83	**FL**	**FL**	FF	FF	FF	**FL**	FF
D3Mit10	FF	FF	FF	FF	FF	FF	FF
D4Mit2	FF	FF	FF	FF	FF	FF	FF
D4Mit17	FF	FF	FF	FF	FF	**FL**	FF
D5Mgh13	FF	FF	FF	FF	FF	**FL**	FF
D5Mit4	FF	FF	FF	FF	FF	FF	**LL**
D5Mit13	FF	FF	FF	FF	FF	**FL**	FF
D6Rat24	FF	FF	FF	FF	FF	FF	**FL**
D7Mit4	FF	FF	FF	FF	FF	FF	FF
D7Rat24	FF	FF	FF	FF	FF	FF	FF
D8Rat16	FF	FF	FF	FF	FF	FF	FF
D8Rat77	**FL**	**FL**	FF	FF	FF	FF	FF
D9Mit2	**FL**	**FL**	FF	FF	FF	**FL**	FF
D9Rat81	**FL**	**FL**	FF	FF	FF	FF	FF
D10Mit9	**LL**	**LL**	FF	FF	**LL**	FF	**FL**
PPY	FF	FF	FF	FF	FF	FF	FF
D10Rat142	FF	FF	FF	FF	**FL**	FF	FF
D10Rat32	FF	FF	FF	FF	FF	FF	FF
735-736	FF	FF	FF	FF	FF	FF	FF
D11Mgh4	FF	FF	FF	FF	FF	FF	FF
D11Rat52	FF	FF	FF	FF	FF	FF	FF
D12Mit4	FF	FF	FF	FF	FF	FF	FF
D12Rat59	FF	FF	FF	FF	FF	FF	FF
D13Mit1	FF	FF	FF	FF	FF	FF	FF
D13Uwm1	FF	FF	FF	FF	FF	FF	FF
D14Mgh2	FF	FF	FF	FF	FF	FF	FF
D14Mit2	FF	FF	FF	FF	FF	FF	FF
D15Mgh2	FF	FF	FF	FF	FF	FF	FF
D15Rat71	FF	FF	FF	FF	FF	FF	FF
D15Rat123	FF	FF	FF	FF	**FL**	FF	FF
D16Rat53	FF	FF	FF	FF	FF	FF	FF
D16Rat90	FF	FF	FF	FF	FF	FF	FF
D17Mit4	**FL**	**FL**	FF	FF	**FL**	**FL**	FF
D17Rat85	FF	FF	FF	FF	**FL**	**FL**	FF
D17Rat89	**FL**	**FL**	FF	FF	**FL**	**FL**	FF
D18Mgh2	FF	FF	FF	FF	FF	FF	FF
D18Mit1	**FL**	**FL**	FF	FF	FF	**FL**	FF
D18Mit3	**FL**	**FL**	FF	FF	FF	FF	FF

Table 5.6: continues overleaf

D19Rat30	FF	FF	FF	FF	FF	FF	FF
TAT	FF	FF	FF	FF	FF	FF	FF
D20Mgh1	FF	FF	FF	FF	FF	FF	FF
D20Rat21	FF	FF	FF	FF	FF	FF	FF
DXRat15	FF	FF	FF	FF	FF	FF	FF
DXRat31	FF	FF	FF	FF	FF	FF	ND

Table 5.6: Table summarising the genotype of TGR(*Cyp1a1-Ren2)* rats from animal facilities in Edinburgh (WGH, LF) and Maastricht. Animal numbers were generated as a unique identifier. Each animal is from a different litter or breeder pair. FF = homozygous Fischer for the named marker, FL = heterozygous Fischer for the named marker, LL = homozygous for another strain for the named marker. The number following "D" indicates chromosome number. MT1PA: chromosome 1 marker. PPY and 735/736: chromosome 10 markers. TAT: chromosome 19 marker. ND = not determined.

The results revealed that the Maastricht rat colony was of pure inbred Fischer (F344) origin which shows that the strain of the population from which they were derived was indeed pure Fischer (F344). It can therefore not account for the phenotypic differences between the Maastricht rat colony and the Edinburgh rat colony and other factors, unidentified to date, must be involved. Surprisingly, however, the Edinburgh-WGH (the descendants of the original TGR(*Cyp1a1-Ren2)* rat population) animals displayed contamination with what appeared to be of Lewis strain origin although this is difficult to ascertain as information about band size for other strains with the markers used was not available.

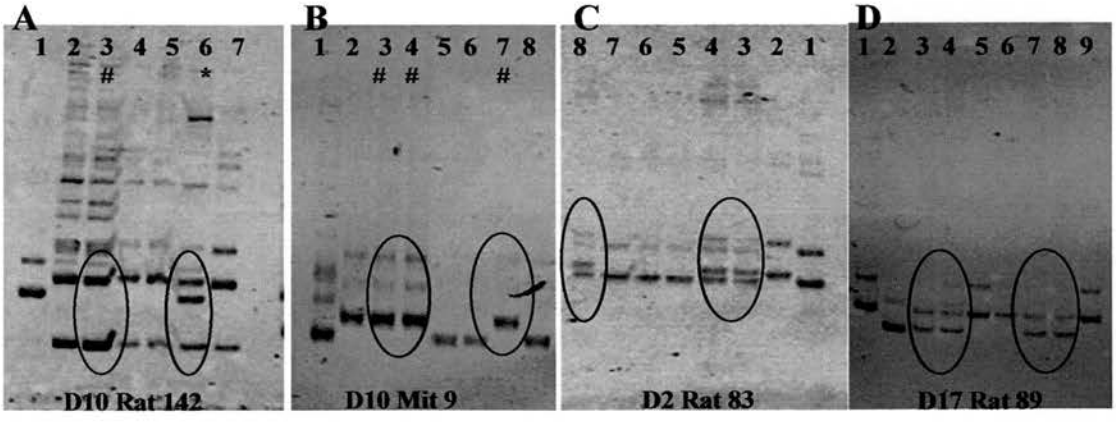


Figure 5.1: Acrylamide gel electrophoresis of microsatellite markers genotyping on genomic rat DNA. A) Microsatellite marker D10Rat142 on chromosome 10, B) Microsatellite marker D10Mit9 on chromosome 10, C) Microsatellite marker D2Rat83 on chromosome 2, D) Microsatellite marker D17Rat89 on chromosome 17. Lane 1 is from Fischer DNA, lane 2 is from Lewis DNA, lane 3 is from animal WGH 6015, lane 4 is from animal WGH 6031, lane 5 is from animal Maastricht 4, lane 6 is from animal Maastricht 6, lane 7 is from animal LF 182, lane 8 is from animal LF 170 and lane 9 is from animal LF 199. '#' indicates homozygosity for a loci of strain different from Fischer, '*' indicates heterozygosity, circles show the genotypic contamination.

As a result of this contamination, the TGR(*Cyp1a1-Ren2)* rat population from the new animal holding facility which was re-derived by embryo transfer from Edinburgh-WGH animals was also contaminated. Studies evaluating the correlation between dietary sodium and the severity of the phenotype were infeasible and will require further analysis.

5.3 Discussion and Conclusions

As presented in *Chapter 4*, histopathological data using TGR(*Cyp1a1-Ren2*) showed a reduced inflammatory response normally associated with the vascular injuries compared with the original work in this model (Kantachuvesiri *et al.* 2001). Despite an obvious hypertrophic remodeling and a longer length of exposure to the inducer, no signs of microinfarctions or infiltration of inflammatory cells were observed in the heart, which differs from the original publication (Kantachuvesiri *et al.* 2001). Although the direct comparison between this study and the original data is difficult to assess due to the different exposure length, two main observations were made regarding response of the "Maastricht" TGR(*Cyp1a1-Ren2*) to transgene induction following indole-3-carbinol (I3C) administration. Firstly, it appears that the "Maastricht" rats are less sensitive to the I3C treatment and although a five-week exposure resulted in a distinct histopathological and physiological phenotype, the mortality rate was not increased which is in contrast with the "Edinburgh" TGR(*Cyp1a1-Ren2*) rats (A. Ryding, personal communication), with the "New Orleans" and the "Greifswald" animals. Secondly, in the present study, the arcuate-interlobar arteries were unaffected and it would appear that no severe overall renosclerosis was observed. Segmental glomerulosclerosis suggests that the afferent arterioles had recently lost control of high blood pressure dampening to the glomerulus leading to fibrinoid necrosis which again differs from the original publication (Kantachuvesiri *et al.* 2001) which reported normal glomerular histology. The mesentery displayed similar hypertensive injuries, albeit less severe and no infiltration of inflammatory cells was noted in the Maastricht rats.

Due to these findings, investigations were carried out in order to identify the factors involved in the different phenotypes between the "Maastricht" rats described in (*Chapter 4)* and the "Edinburgh" rats studied in (Kantachuvesiri *et al.* 2001). Re-derivation, environmental, dietary and genetic information was collected for all the TGR(*Cyp1a1-Ren2*) rat colonies existing in the world. The reasons for the discrepancies may be multiple: different investigators, diet preparations and housing may be involved. The "Maastricht" animals were

re-derived by caesarian section after veterinary screening which revealed a dual commensal infection (pinworms and *S.aureus*). Although a carry over of certain pathogens during this procedure cannot be excluded, the immunologically stimulated state of the "Edinburgh" TGR(*Cyp1a1-Ren2)* rats may be a factor in the phenotypic differences. The testing of this hypothesis was hampered by a genetic contamination in the "Edinburgh-LF" animals. These animals had recently been re-derived by embryo transfer but no histopathological comparison was possible since these were not of pure Fischer (F344) origin. A difference in genetic background may have been responsible since we know that the Fischer (F344) and Lewis strains differ in ACE activity levels associated with a quantitative trait locus on chromosome 10 in the vicinity of the *ACE* gene. Genetic background was investigated and was shown, as for the original publication that the "Maastricht" animals were on a pure Fischer (F344) background. While it is not impossible that untested animals in the "Maastricht" colony may also display genetic contamination, the animals used for the present study were from the same breeding pair and can therefore be considered to be of pure Fischer (F344) origin. Hence, the discovery of the genetic contamination in the "Edinburgh" animals was not the key factor accounting for the differences in phenotype between the "Maastricht" animals and the original "Edinburgh" TGR(*Cyp1a1-Ren2)*. The reasons for a genetic contamination were traced to the commercially purchased stock. It demonstrates that regular in-house genetic screening may be necessary to insure the strain purity of transgenic lines and in particular, when producing congenic lines.

Another explanation could be the levels of dietary sodium given to the animals. TGR(*Cyp1a1-Ren2)* animals have been shown to develop salt-sensitive hypertension following a two-week exposure to the inducer (Howard *et al.* 2005). The present study revealed that the "Maastricht" rats were fed the lowest sodium amounts compared with animals from the other laboratories holding this model. It is unlikely that a 0.07% difference in sodium levels with the "Edinburgh" diet is responsible for the phenotypic divergences. It has been previously reported that the induced TGR(*Cyp1a1-Ren2)* animals fed a high salt diet (4%) or offered saline solution did not display exacerbated end-organ pathology to the kidney, heart or mesentery although cerebrovascular injuries were detected (Collidge *et al.* 2004). It could be that the levels of dietary sodium in the laboratory in Maastricht are below a threshold which is suggested by the increased mortality rate at 12 days in the "New Orleans" population (greater than that of the "Edinburgh"), which are fed a diet with higher sodium contents (K. Mitchell, Personal communication). The shape of the blood pressure curve may have been affected by dietary sodium levels which would explain why the

"Maastricht" animals develop a more gradual hypertension rather than a rapid, severe increase in blood pressure. Adaptative mechanisms to progressive blood pressure elevation may be likely to play a physiological role and to prevent mortality amongst treated "Maastricht" TGR(*Cyp1a1-Ren2)* animals. Furthermore, the lack of inflammatory cell infiltration in the "Maastricht" rats correlates with a possible decreased pathogen load in these animals following caesarian section re-derivation. Studies designed to investigate the effects of dietary sodium in different strains and pathogen load including the recently re-derived "Edinburgh-LF" TGR(*Cyp1a1-Ren2)* animals are required but further experiments were hampered by the discovery that these animals were no longer of pure Fischer (F344) origin. Careful diet content verification should be undertaken as to enable comparison between publications addressing the same model and to ensure an accurate interpretation of the data.

Another consideration to address phenotypic differences between different TGR(*Cyp1a1-Ren2)* colonies housed in several laboratories may be to measure the level of transgene induction following induction with I3C treatment. Prorenin plasma concentrations are in direct correlation with transgene activation as a result of the mouse *Ren2* gene expression. This measurement could be used to avoid variations from diet and to exclude an effect from sodium contents in the diet as well as from possible pathogen load. Plasma prorenin levels were not measured for the present thesis due to the lack of prorenin substrate availability (i.e.: plasma containing angiotensinogen and devoid of renin/prorenin. Home office license restriction prevented the preparation of nephrectomised mouse or rat plasma and the prorenin assay could not be performed. This and the other environmental/genetic factors described above are of considerable importance for further studies TGR(*Cyp1a1-Ren2)* and for future research dealing with animal models of hypertension/cardiovascular diseases.

Chapter 6
Inhibition of Cardiovascular Injuries in TGR(*Cyp1a1-Ren2*) using a Prorenin Decoy Peptide

6.1 Introduction

After prorenin secretion, the mechanisms of downstream prorenin activation/conversion are, to date, unclear. Evidence is accumulating in favour of a non-proteolytic activation of prorenin. An alternative pathway for prorenin conversion is now known resulting in the pro enzyme to be activated without changes in molecular weight and with the ability to return to an inactive conformation by heat treatment or following binding a (pro)renin receptor (Derkx *et al.* 1987; Leckie and McGhee 1980). The physiological regulation and relevance of non-proteolytic prorenin activation remain unclear in malignant hypertension.

The aim of the present chapter is to assess whether the prorenin handle region peptide described by Ichihara *et al.*, (2005) abolishes the malignant hypertension cardiovascular lesions in the TGR(*Cyp1a1-Ren2*) model. This will evaluate the involvement of non-proteolytic activation of prorenin and of its receptor in the pathophysiology of malignant hypertension. Due to the genetic contamination uncovered in the animals held at the University of Edinburgh, a full scale study including renin gene expression, plasma prorenin/renin levels and tissue prorenin/renin amounts was unachievable. A pilot study was therefore undertaken concentrating on histopathology in key organs, namely heart, kidney and mesentery, having been shown to exhibit malignant hypertension end-organ damage in the original publication on TGR(*Cyp1a1-Ren2*) animals (Kantachuvesiri *et al.* 2001). Animals from the Edinburgh-LF colonies were used, despite their mixed genetic background. As a consequence and due to the small sample size, only qualitative analysis was performed.

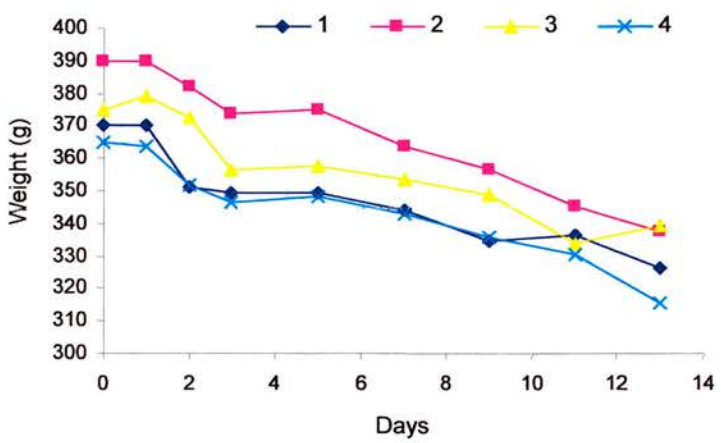
6.2 *Results*

Previous research (Ichihara *et al.* 2004) reported the use of the peptide (NH_2-RILLKKMPSV-COOH) which abolished diabetic nephropathy in streptozotocin-treated rats. The dose used was 0.1mg/kg. The peptide was used as a competitive inhibitor of prorenin for its receptor. Comparing the levels of prorenin in the plasma between the streptozotocin-treated rats and the TGR(*Cyp1a1-Ren2*) rats, it was decided to use a higher dose (0.4mg/kg) of decoy peptide for the present experiment.

6.2.1 Body weight and blood pressure

Animals 1 and 2 received the inducer and the decoy peptide in saline whereas animals 3 and 4 received the inducer and saline only. The animals were weighed prior to the start of transgene induction with 0.3% I3C, everyday for the first 3 days and every second day thereafter. All 4 animals displayed weight loss which was progressive over 14 days (Figure 6.1). The total weight loss was between 36.1g and 52.4g (Figure 6.1). The highest and smallest weight loss did not correspond with either receiving the decoy peptide or saline in the osmotic mini-pumps.

A



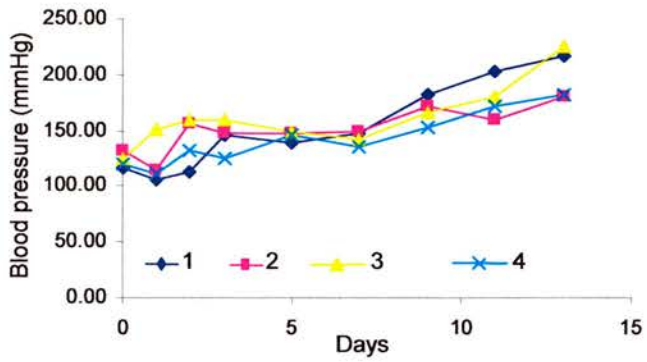
B

	Total weight loss (g)
1	44.1
2	52.4
3	36.1
4	49.5

Figure 6.1: Body weight and weight loss in TGR(*Cyp1a1-Ren2*) animals. A) Body weight (g) of individual animals, B) Total weight loss over 14 day-experiment of each animal.

The blood pressure was measured in conscious restrained animals and was shown to increase progressively over the experimental time from day 3 (Figure 6.2). The range of blood pressure values was more widespread between animals in the first 5 days and may be due to difference in adapting to the restrain conditions between each individual animal. At the end of the study, the increase in blood pressure was between 100mmHg and 48.3mmHg (Figure 6.2). These differences in blood pressure increase may be due to the methodology which can trigger stress in the animals. However, the blood pressure changes do not appear to be different with decoy peptide treatment compared with animals receiving saline only and do not correspond to weight loss. The decoy peptide overall does not seem to affect or ameliorate the weight loss or increase in blood pressure in transgenic animals receiving the inducer.

A



B

	Total BP increase (mmHg)
1	99.58
2	48.33
3	100
3	62.5

Figure 6.2: Blood pressure measurement in conscious restrained TGR(*Cyp1a1-Ren2*). A) Blood pressure (mmHg) of individual animals measured by indirect tail-cuff method. B) Total blood pressure increase over 14 day study.

6.2.2 Histopathology

Considering the histopathology data reported by Kantachuverisi *et al.* (2001) and in this thesis gross and microscopic examination of the key tissues affected by end-organ damage was undertaken to identify the effects of the prorenin decoy peptide of end-organ damage in TGR(*Cyp1a1-Ren2*) animals.The results presented represents data from each of the four rats sampled during this study. All four animals displayed homogeneous increase in left ventricular (LV) wall thickness indicative of concentric LV hypertrophy (Figure 6.3). No difference was noted between the animals on I3C alone or the induced rats which were implanted with mini-pumps containing the prorenin decoy

peptide. Another striking feature similar to all the animals was the presence of infarction in the right ventricle (RV), including in the RV free wall (Figure 6.3 A-D)

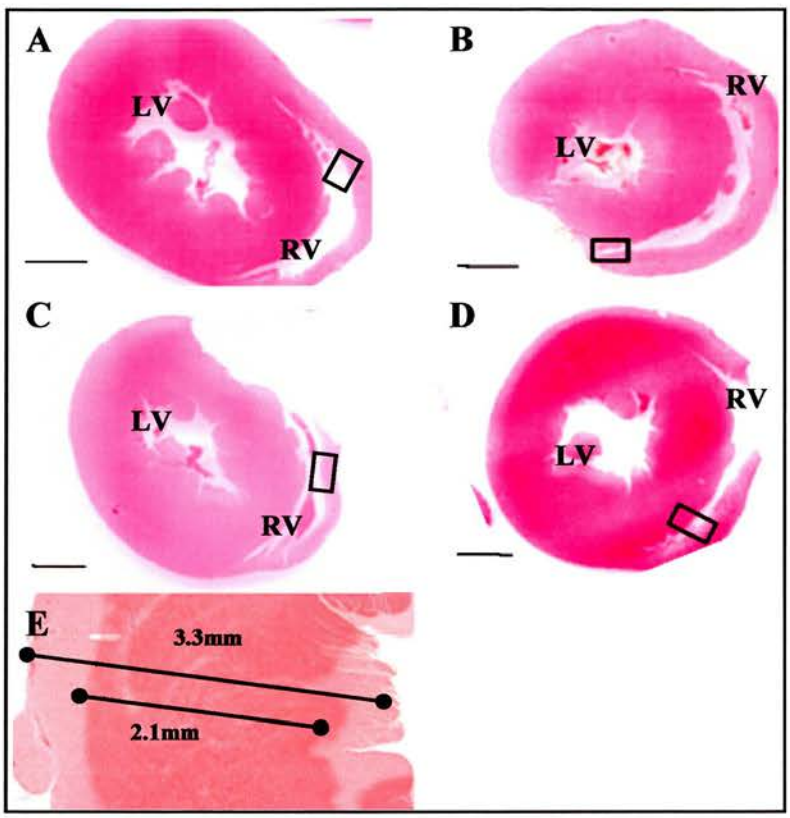


Figure 6.3: Scanned images of H&E stained cross sectioned hearts of TGR(*Cyp1a1-Ren2*). A) Rat 1 (I3C and decoy), B) Rat 2 (I3C and decoy), C) Rat 3 (I3C alone), D) Rat 4 (I3C alone). All animals displayed the same degree of concentric LV hypertrophy, boxed areas indicate sites of infarct. Bar = 2mm, LV = left ventricle, RV = right ventricle. E) Superimposed intraventricular septa of rat 4 (width 3.3mm) and of control rat from *Chapter 4* (wall width 2.1mm).

Infarction in the RV wall accompanied by fibrotic lesions in the right coronary artery was seen in all four rats (Figure 6.4). Reduction in lumen size and fibrinoid necrosis of intramural right coronary arteries generally found in or near areas of infarction (Figure 6.5) was observed. No other coronary vascular lesions were found.

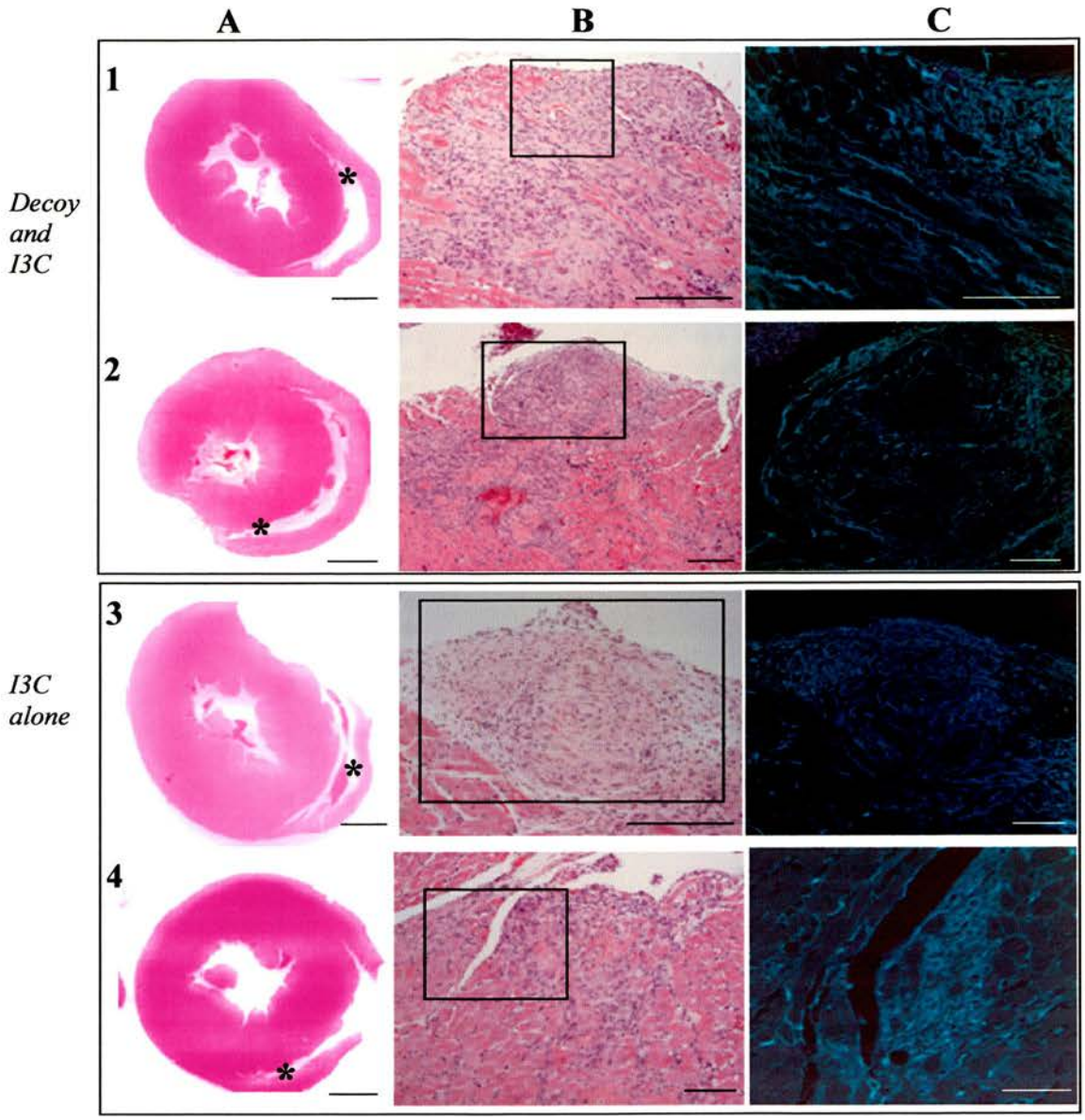


Figure 6.4: Localisation of infarcts and microscopic examination of heart sections stained with H&E and Sirius Red (for collagen) of TGR(*Cyp1a1-Ren2*). Each horizontal panel groups photographs from individual animals. A) Whole heart sections as shown in Figure 6.3. (*) indicates the position of the RV infarct, Bar = 2mm. B) H&E staining of the right ventricular infarcts showing inflammatory cell infiltration and fibrotic areas. Boxed areas represent regions shown in (C). Bar = 200μm. C) Reverse photographs of Sirius Red stained RV infarcts showing collagen deposition (white staining) indicative of fibrosis and tissue scaring. 1-C and 4-C: bar = 50μm, 2-C and 3-C: bar = 100μm.

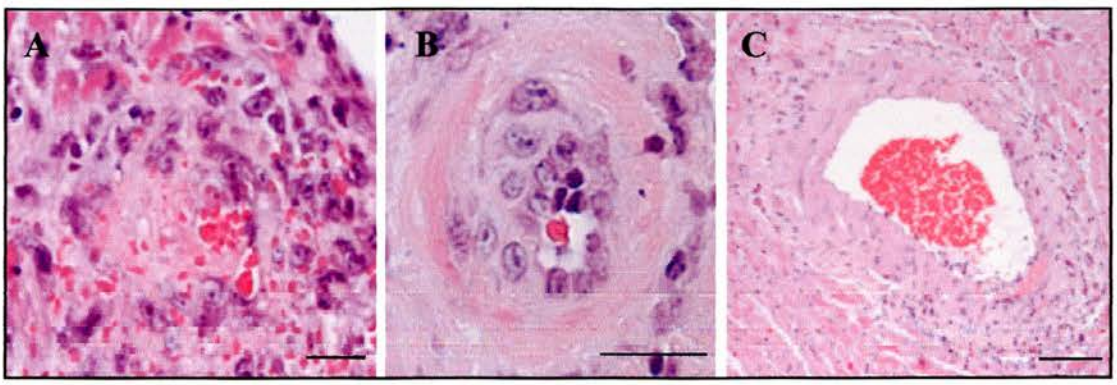


Figure 6.5: Right coronary artery branches from TGR(*Cyp1a1-Ren2*) animals. A) Rat 4 (I3C alone), Bar = 25μm, B) Rat 2 (I3C and decoy), Bar = 25μm, C) Rat 3 (I3C alone), Bar = 50μm. All calibres of right coronary artery branches were affected by fibrinoid necrosis.

In the kidney, hypertrophy of the tunica media and a decrease in lumen size suggested hypertrophic remodelling of the interlobular arteries of all calibres (Figure 6.5). Segmental fibrinoid necrosis as indicated by unilateral loss of smooth muscle cells was observed in some vessels. There are no discernible differences between the treated rats which received the decoy peptide (rats 1 and 2) and the ones which did not (rats 3 and 4).

All four animals showed glomerular enlargement and fibrinoid necrosis of the vascular pedicle (Figure 6.7). Glomerular enlargement appears to result primarily from capillary distension. Glomerular cellularity did not appear to be increased nor was sclerosis evident. Again, this phenotypic characteristics were common to all four rats and no improvement or inhibition of these injuries were noted as a result of decoy peptide administration.

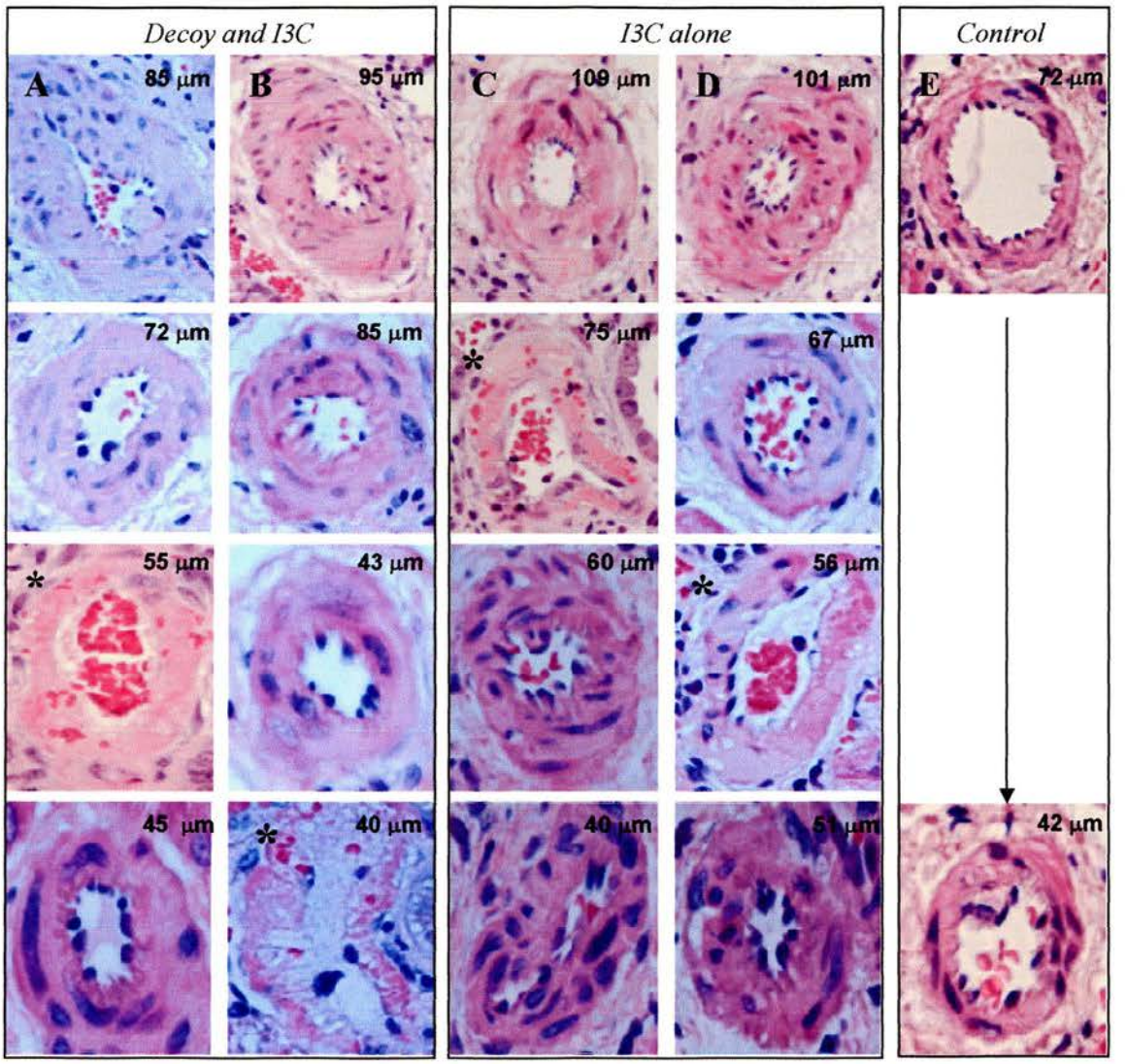


Figure 6.6: H&E stained sections of paraffin embedded kidneys TGR(*Cyp1a1-Ren2*) rats. Intra-renal vasculature. For each of the 5 photograph series presented interlobular arteries of decreasing calibre are shown from top to bottom. A) Rat 1, B) Rat 2, C) Rat 3, D) Rat 4, E) Untreated TGR(*Cyp1a1-Ren2*) rat from study presented in *Chapter 4*. (*) indicates vessels affected by segmental fibrinoid necrosis. Medial hypertrophy and decreased lumen size are present in the vessels of all rats.

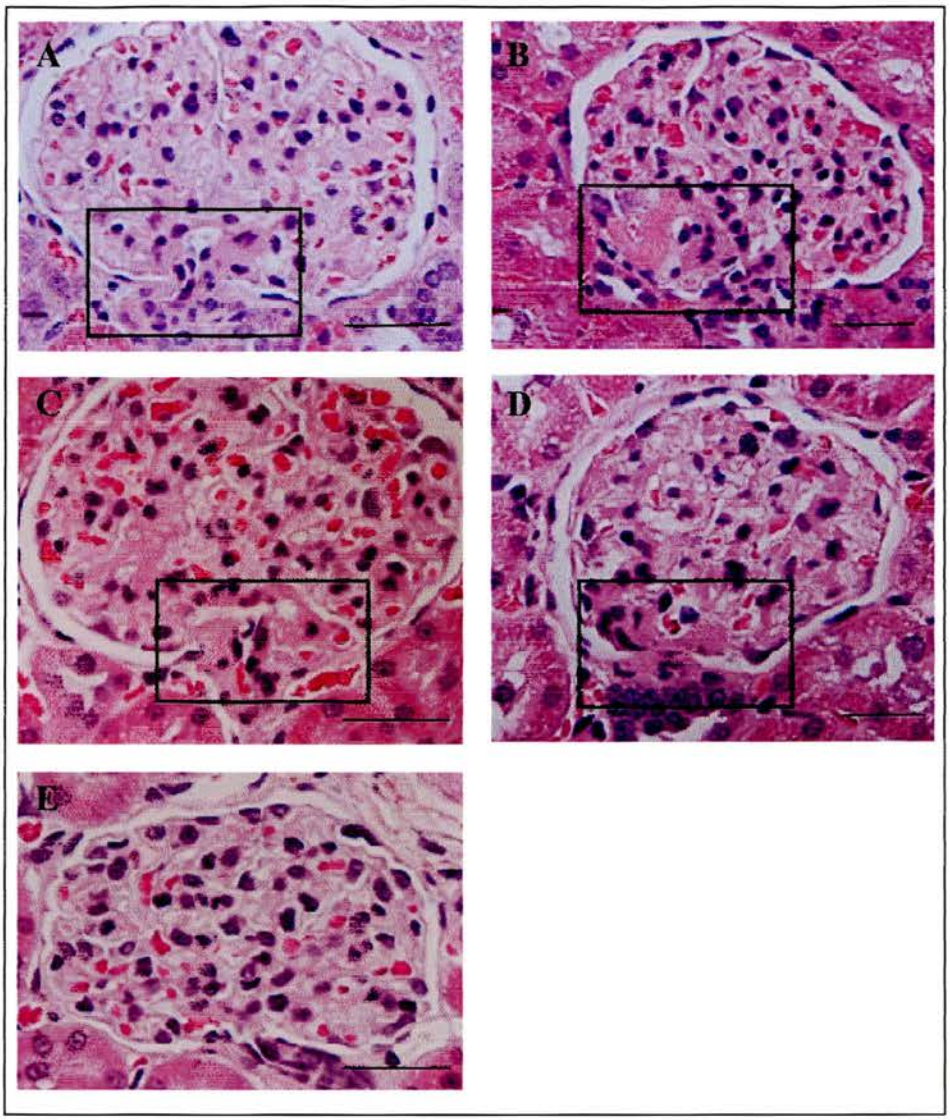


Figure 6.7: H&E stained sections of paraffin embedded kidneys TGR(*Cyp1a1-Ren2*) rats. Glomerular phenotype. Glomerulus from A) Rat 1 (I3C and decoy peptide), B) Rat 2 (I3C and decoy peptide), C) Rat 3 (I3C alone), D) Rat 4 (I3C alone), E) Control rat from study presented in *Chapter 4*. The boxed area highlights the vascular pedicle of the glomerulus affected by fibrinoid necrosis. Note the glomerular enlargement compared to control. Bar = 25μm.

Mesenteric vessels from all four animals showed medial hypertrophy and small lumens suggestive of hypertrophic remodelling (Figure 6.8). Only vessels from rats 3 and 4 which had not been administered with the prorenin decoy peptide show segmental fibrinoid necrosis or hyperplastic arteriosclerosis (onion skinning).

This was not seen in rats 1 and 2 which had received the decoy peptide suggesting that this peptide may have prevented development of vascular remodelling and injuries. Histopathology analysis was performed by an independent pathologist who was blinded to the treatment. It is worth noting that the animals were grouped correctly by the pathologist.

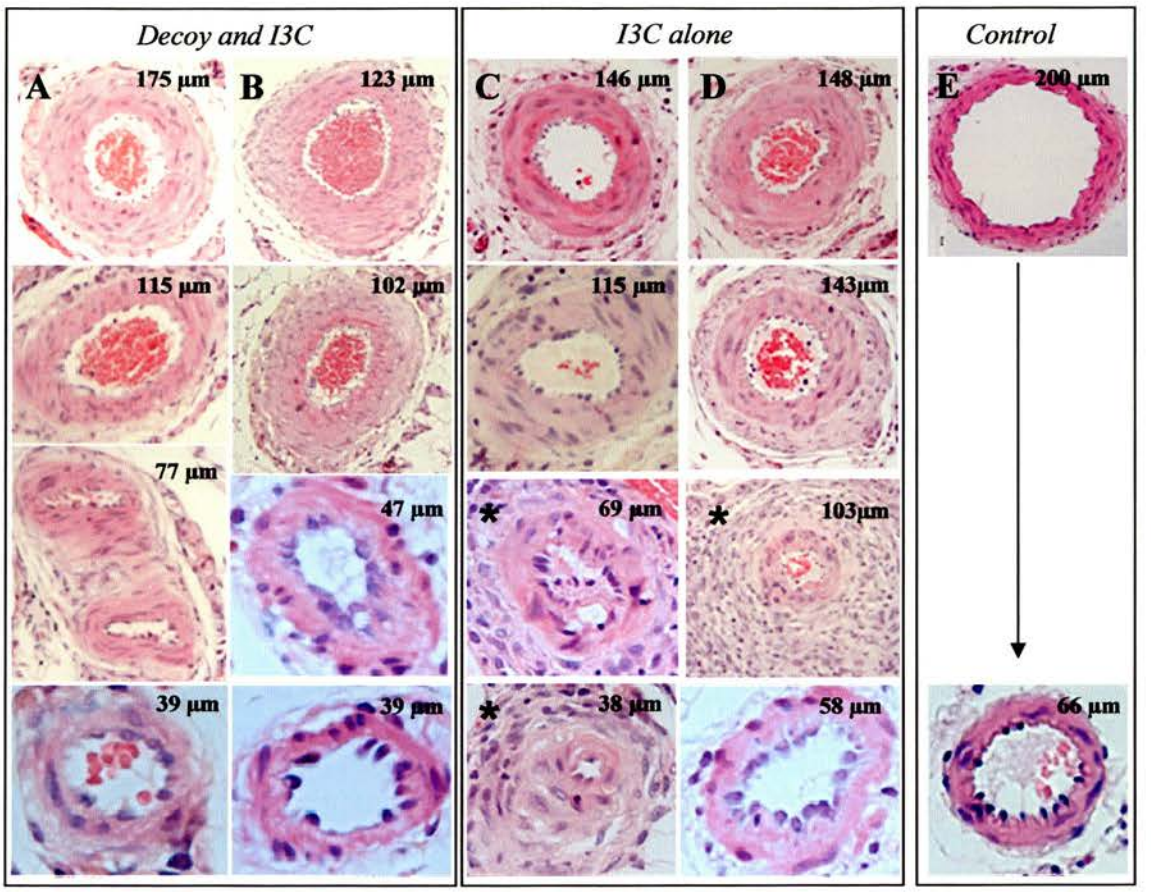


Figure 6.8: H&E stained sections of paraffin embedded mesentery TGR(*Cyp1a1-Ren2*) rats. For each of the 5 photograph series presented mesenteric arteries of decreasing calibre are shown from proximal at the top to distal at the bottom. A) Rat 1 (I3C and decoy peptide), B) Rat 2 (I3C and decoy peptide), C) Rat 3 (I3C alone), D) Rat 4 (I3C alone), E) Untreated TGR(*Cyp1a1-Ren2*) rat from study presented in *Chapter 4*. (*) indicates vessels affected by fibrinoid necrosis. Note that fibrinoid necrosis was only present in vessels of animals treated with I3C alone.

6.3 Discussion and conclusions

All four animals used in this pilot study displayed weight loss starting 2 days after transgene induction and increase in blood pressure beginning 3 days after transgene induction. The initial weight loss may have been due to the change of diet from pellet to powder form, although the animals were given powdered food prior to the addition of the inducer to the diet. The weight loss was progressive throughout the experimental period and all animals followed the same trend regardless of the administration of the decoy peptide. The blood pressure elevation was also gradual which, surprisingly, corresponded to the hypertensive profile in the animals described in *Chapter 4* rather than in the original report on the TGR(*Cyp1a1-Ren2*) model (Kantachuvesiri *et al.* 2001).

Histopathological analysis revealed the classical injuries associated with malignant hypertension. In the kidney, hypertrophic remodelling of the interlobular arteries of all calibres with occasional segmental fibrinoid necrosis, glomerular enlargement and fibrinoid necrosis of the vascular pedicle were observed. Vascular remodelling was present in all three tissues examined. The prorenin decoy peptide did not prevent the development of the renal lesions. Left ventricular hypertrophy was present without, however, the presence of microinfarctions. Unexpectedly, infarcts were found in the right ventricle of all animals, including in the right ventricular free wall. This shows again that, in this study, progression of cardiac remodeling was not alleviated by the decoy peptide. In humans, right ventricular (RV) infarction occurs when there is an occlusion of the right coronary artery proximal to the acute marginal branches. Isolated RV infarction is extremely rare representing 2% of autopsies. In animal models RV hypertrophy and infarcts are generally associated with surgical ligation of the right coronary artery. Indeed, luminal size decrease was noted in all calibres of right coronary artery branches found in the vicinity of the infarct. The mechanisms by which right ventricular infarction developed in TGR(*Cyp1a1-Ren2*) animals are unknown. It cannot be excluded that it may be a consequence of the mixed genetic background identified in *Chapter 5*. Further investigations would be required to understand the factors involved in this particular phenotype.

An interesting result was the amelioration of the lesions in mesenteric vasculature following treatment with the prorenin handle region peptide. Although mesenteric vessels from all four rats developed medial hypertrophy and reduction of lumen size, there was a distinct absence

of fibrinoid necrosis in the animals receiving the peptide. This isolated effect in the mesentery may reflect differential access of the peptide to this tissue and hence, an enhanced protective outcome. What is not known from this study is whether the injuries developed and their progression was subsequently delayed or if the lesions were prevented from developing. In contrast with the work of, Ichihara *et al.* (2004) my experimental procedures did not include administration of the decoy peptide several weeks prior to the expected vascular phenotypic changes to occur. Using TGR(*Cyp1a1-Ren2*) animals, the inhibitor was applied at the same time as the transgene, indole-3-carbinol, which triggers malignant hypertension injuries from day 3 of exposure. Hence my study represents an attempt to ameliorate the lesions as they are developing. This differential dosage strategy may explain why little improvement in the histopathology findings was detected. A dose of decoy peptide four-fold higher that used by Ichihara *et al.* (2004) was expected to be sufficient to counterbalance the shorter length of treatment. This was, however, not observed.

The "handle" region of prorenin, i.e.: the portion of the protein thought to interact with binding molecules and/or receptors to trigger non-proteolytic/reversible activation of prorenin has been previously been used and described as a "decoy" peptide (Ichihara *et al.* 2004). It may be more accurate to describe it as a competitive inhibitor of prorenin, preventing this molecule from binding to potential receptor(s). While this peptide has been shown to bind the (pro)renin receptor reported by Nguyen *et al.*, it cannot be excluded that it may bind additional receptors or converting enzymes. Hence the effects obtained by Ichihara *et al.* may reflect these direct or indirect mechanisms which have not been described. It is, in addition, possible that the peptide acts as a chaperone rather than having a true effect on the putative rat (pro)renin receptor.

Huang *et al.* (2006) showed that expression of pro-fibrotic genes such as TGF-β induced by renin in cultured mesangial cells could be inhibited by short interfering RNA (siRNA) corresponding to the putative (pro)renin receptor mRNA. Although recent evidence suggesting a strong interaction between components of the renin-angiotensin system and fibrosis is available, it is attractive to hypothesize that renin may be carrying out its effects, independent of its enzymatic action to produce angiotensin II. However, the data arising from siRNA inhibition of the (pro)renin receptor mRNA in mesangial cells (Huang *et al.* 2006) may reflect a more general effect on these cells which have altered pro-fibrotic proteins synthesis. As noted in chapter 3, the mesangial cell cultures were exposed to renin rather than prorenin to trigger the fibrotic response, hence raising the question of an involvement of the putative (pro)renin receptor in the process. Data presented in *Chapter 3*

showed that the (pro)renin receptor is homologous with a sub-unit (M8-9) of a vacuolar-ATPase. It cannot be excluded that inhibition of (pro)renin receptor mRNA translation may also result in interferences with this system involved in cell survival. In contrast with the above results, competitive inhibition for the prorenin binding site of the (pro)renin receptor did not inhibit fibrotic injuries in interlobular arteries and in the vascular pole on glomeruli. In addition, inhibition of the (pro)renin receptor binding to prorenin for several weeks in control animals by Ichihara *et al.* did not have any effect on blood pressure or body weight (Ichihara *et al.* 2004) suggesting that the protein does not have an important role in normal physiological situations.

"Three studies have used the prorenin handle region of prorenin to reduce vascular/cardiac injuries. Firstly, Ichihara *et al.* (2004) showed amelioration of vascular injuries in a model of diabetic nephropathy and this study is discussed above. Secondly, reduction of cardiac fibrosis and blood pressure upon "decoy peptide" administration was noted in the very severe model of SPSHR (Ichihara et al. 2006a). Thirdly, prevention of the development of glomerulosclerosis was achieved in diabetic AT1a *null* mice and MAPK activation was abolished (Ichihara et al. 2006b). All three systems used have a common pathway linked to disturbances in sodium and/or glucose homeostasis. In addition, TGF-β activation is involved in the pathophysiology of these all models and the "decoy" peptide effects on the putative (pro)renin receptor may be linked to this pathway rather than having a direct inhibitory effect on prorenin. This could explain why minimum effects were observed in TGR(*Cyp1a1-Ren2*) animals when administered with the peptide. The "decoy" peptide used in the present study was identical to that used by Ichihara *et al.* and therefore the amino-acid sequence was from rat. Prorenin expressed by TGR(*Cyp1a1-Ren2*) animals is of murine origin. Rat and mouse prorenin are, however, highly homologous and it is unlikely that species differences can account for the lack of effect in this study. Additionally, since the decoy peptide administration in TGR(*Cyp1a1-Ren2*) did not result a significant amelioration of the phenotype, it can be concluded that the putative (pro)renin receptor does not play a role in this high prorenin model of malignant hypertension. No evidence has been published to suggest that the (pro)renin receptor is involved in uptake of prorenin, a major component of the phenotype in TGR(*Cyp1a1-Ren2*) rats. Therefore, it could be hypothesised that additional, unknown receptors and/or binding proteins may be involved in this pathogenesis.

A separate protein (*i.e.* M8-9) originating from the same gene than the putative (pro)renin receptor may be produced to carry on basic cell biology functions. The lack of evidence for regulation of the (pro)renin receptor in TGR(*Cyp1a1-Ren2*) animals suggests that the

function of this protein may need to be re-assessed. The work reported in the present chapter indicates that non-proteolytic activation of prorenin through the recently published (pro)renin receptor does not play a crucial role in malignant hypertension in the inducible transgenic rat model of high prorenin. Further studies are needed to understand alternative physiological roles for this newly identified gene.

Chapter 7
Prorenin Maturation and the Regulation of Renin Granulation during Mouse Development

7.1 Introduction

Pro-hormones such as pre-pro-renin are synthesized in the rough endoplasmic reticulum and sorted into the constitutive pathway or the regulated secretory pathway from which they are released after extracellular stimulation. The regulation of granule biogenesis is poorly understood but Kim *et al.* demonstrated that chromogranin A is essential for dense core granule formation and for storage of hormones directed to the regulated secretory pathway (Kim *et al.* 2001). The formation of epitheloid renin granules has been described although some information remains hypothetical (Hackenthal *et al.* 1990). Schematic representation of all stages of granule formation is shown in Figure 7.1. In summary, immature granules first appear as the result of "budding" of the Golgi resulting in small spindle-shaped granules which may round off to form rhomboid granules. These granules may become larger when coalescing with other immature granules resulting in intermediate granules. It has been hypothesized that immature granules may be more fusogenic than other types of renin granules (Taugner *et al.* 1987; Taugner and Metz 1986). Immature granules (containing prorenin) may be trafficked to the cell membrane to release inactive renin as part of the constitutive secretory pathway. Intermediate granules are also produced when the material contained in them is condensed into a para-crystalline structure and then appears as an electron dense product. As a result polymorphous intermediate granules can be found in different size and shape but often contain (pro)renin material which can be para-crystalline in appearance. The final form of these storage cisterns contain dense renin material (the so-called dense core mature renin granules) and have the ability to release renin upon calcium influx (Hackenthal *et al.* 1990). Secreting granules and intermediate polymorphous granules can be difficult to differentiate as the outside of the secretory granule may appear "fuzzy" if releasing renin resembling either late stages of immature/intermediate granule coalescence or

non-homogeneous material contained within intermediate granules. Fusion of granules appears to be present only in higher vertebrates suggesting a relatively recent evolutionary physiological adaptation (Kon 1999). In Cyprinidae (*e.g.* carps), only three types of granules can be detected since no coalescence occurred resulting in granules smaller in size (approximately 200nm) compared with mouse granules which can be 1μm or greater in diameter (Kon 1999). Examples of the main types of granules in the mouse are shown in the result section of this chapter. Renin granules can be mistaken from lysosomes and stains such as cresyl violet can detect both types of secretory granules. Electron microscopy is, to date, the most reliable technique for identifying dense core renin granules and for isolating the different types of juvenile and immature (pro)renin granules.

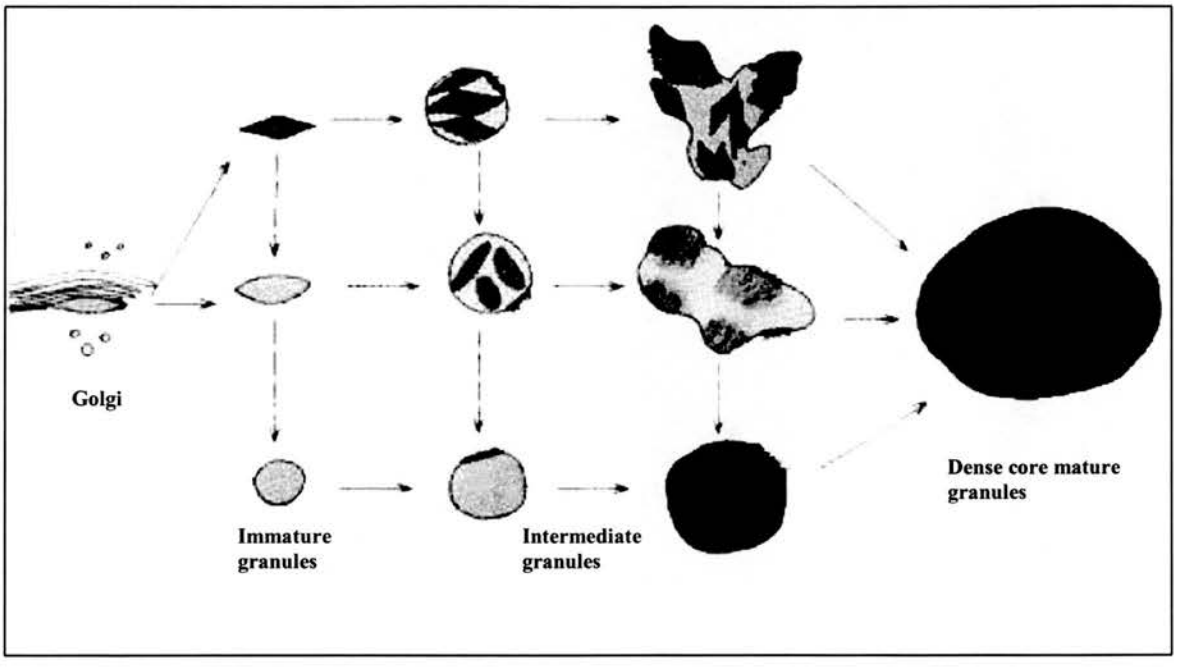


Figure 7.1: Diagram representing renin granulogenesis in the regulated secretory pathway from Golgi to mature granule formation. Reproduced and adapted from Hackenthal *et al.* (Hackenthal *et al.* 1990).

As discussed in *Chapter 1.3.1.1*, renin synthesized as the pro-enzyme, prorenin, by the JG cells in the kidney, can then either be, a) packaged into low density granules ("juvenile") or b) converted intracellularly to active renin (40kDa) and stored in dense core/mature granules before secretion when required (Friis *et al.* 2000), both in humans and mice. In humans, the kidney is the only known organ to release active renin, other extra-renal sites of renin production secreting prorenin exclusively (Sealey and Rubattu 1989). After secretion, the

mechanisms of downstream prorenin activation/conversion are, to date, unclear. In the plasma, in the presence of protease inhibitors, the conversion of pro-renin is much slower than in the kidney (Leckie 1981) and prorenin is known to have "renin-like" enzymatic activity (Skinner 1987).

Renin expression in the foeto-placental tissues namely, placenta, amnion, chorion and foetus has been reported previously (Carretero *et al.* 1972; Poisner 1998) in humans and in mice. During renal development, renin expression in the developing mouse kidney essentially follows the formation of the arcuate and interlobar arteries (Gomez *et al.* 1990a) suggesting a physiological role for renin in nephrogenesis before blood pressure regulation. Renin expression then becomes localised to the JG cells. In addition, the smooth muscle cells (SMC) of the afferent arterioles are capable, under stimulation to undergo reversible metaplastic transformation (Taugner *et al.* 1984). Renin secretion from granules may be triggered by a variety of stimuli such as a decrease in blood pressure or sodium depletion (Atlas *et al.* 1977). JG cells are considered to be terminally differentiated from SMC since they possess SMC characteristics and produce the hormone renin. Renin expression has been detected in the kidney from E14 (Kon 1999).

Although, post-natally the presence of renin granules is unequivocal (Bruhl *et al.* 1974), few reports demonstrate the presence of renin granules in JG cells during renal development. In the pig, studies showed the presence of granules in the mesonephros and metanephros (precursors of the mature kidney) (Tiedemann and Egerer 1984). In the sheep, granulated (putatively with renin granules) peripolar cells are the most numerous at the newborn stage but the identity of the enzymes contained in this granules and their role in the juxtaglomerular apparatus is unclear. In addition, the presence of juxtaglomerular renin granules was observed in E18 Wistar rats (Bruhl *et al.* 1974). Minuth *et al.*, reported the presence of renal renin granules in NMRI mice (Minuth *et al.* 1981). In the latter, it is worth noting that the transmission electron microscopy data was not shown. In human foetuses, a juxtaglomerular index (the number of granules/number of cells) of zero or below one was recorded (Ljungvist and Wagermark 1966). It appears, therefore, important to clarify whether renin dense core granules are present pre-natally. The presence of renin JG granules in pre- and post-natal stages in the mouse was investigated by electron microscopy. In addition, the potential involvement of the presence of two renin genes in different foetal renin granulation profiles was addressed. This allowed the phenotypic comparison of the granulation phenotype in the adult and in the foetus. Some of the stimuli involved in the

control of renin granulation were assessed using maternal dietary manipulations and late gestation angiotensin-converting enzyme (ACE) inhibition.

7.2 Results

Embryos from control C57/Bl6 and FVB/N mice were sampled at gestational age E15.5, E16.5, E18.5, P1 and P28. For dietary manipulation and ACE inhibition experiments only mice of C57/Bl6 strain were used. The mice were divided into 4 treatment groups. For treatment groups 1 and 2, mice were placed on a low sodium (0.03%) or high sodium (3%) diet, respectively and received the modified diet from E12.5. For treatment groups 3 and 4, mice received captopril (30mg/kg/day) or enalapril (30mg/kg/day), respectively, by gavage. Due to previous reports of foetal toxicity during maternal exposure, captopril and enalapril gavage was started at E15.5. For the treatment groups 1-4, the embryos were collected at E18.5 and P1.

7.2.1 Peri-natal renin granulation in mice.

Because the kidney vasculature develops centrifugally, both deep and peripheral arteries were studied at stages where the renal vasculature may not be fully developed. No granules were detected pre-natally at embryonic day E15.5 (data not shown), E16.5 (Figure 7.2) or E18.5 (Figure 7.3). Post-natally, the presence of granules was clearly observed in JG cells (Figure 7.4). At post-natal day 1 (P1), each SMC contained approximately 5-6 granules whereas at weaning (P28), 20-25 granules could be detected showing that the kidneys are still maturing after birth (Figure 7.5). Renin expression (which starts prior to vessel formation in the kidney - [19]) preceded the appearance of renin granules and it appears that renin granulation does not overlap with renin expression along the vasculature.

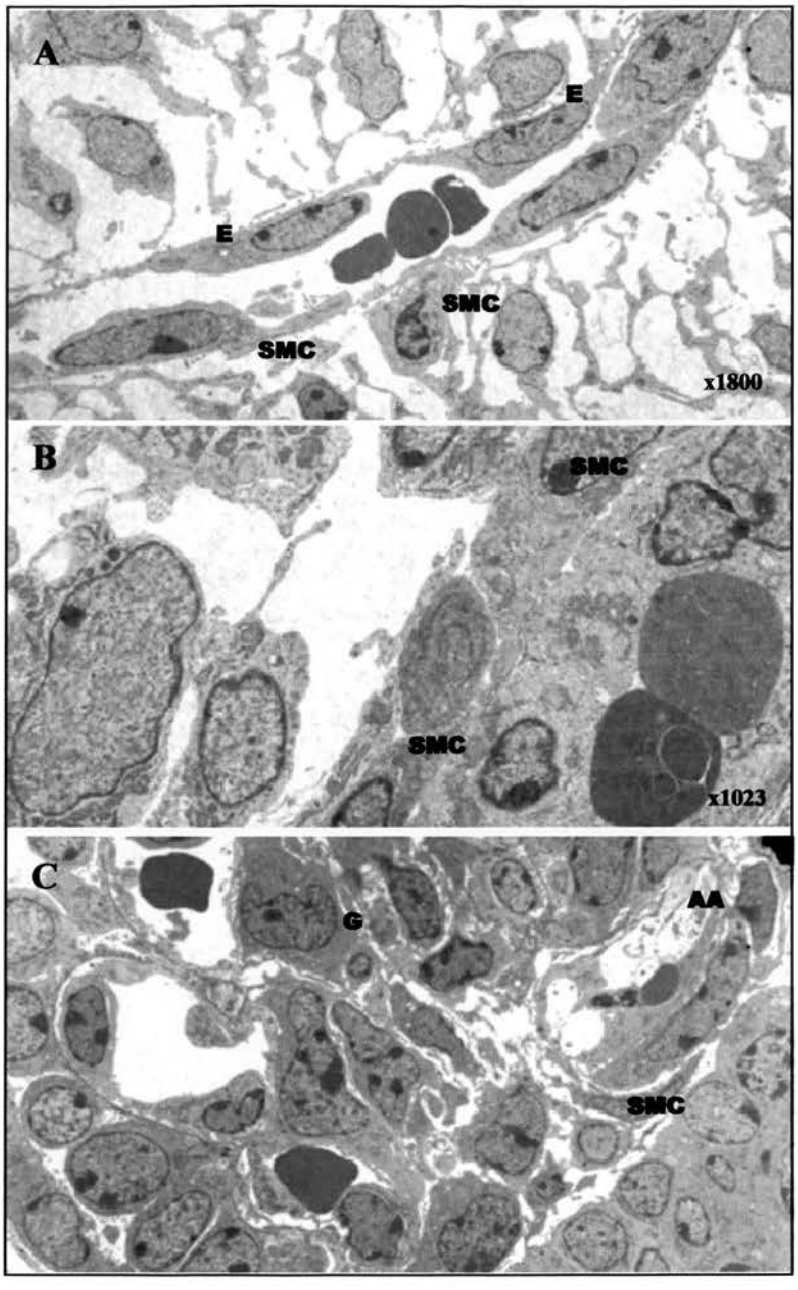


Figure 7.2: TEM photographs of embryonic pre-natal mouse kidneys (C57/Bl6) at embryonic day 16.5. A) Peripheral interlobular artery, B) Deep interlobular artery, C) Vascular pole of JGA including afferent arteriole. Note the absence of renin dense core granules in SMC of the interlobular arteries and afferent arterioles of the juxtaglomerular apparatus. "E" denotes the embryonic day. AA = afferent arteriole, E = endothelial cells, G = glomerulus, MD = macula densa, SMC = smooth muscle cell.

7.2.2 Comparison between one renin- and two renin gene mouse strains.

Considering the differences in processing between renin from *Ren1*^{d} and *Ren2*, we investigated whether differences in granulation could be detected between one renin- (C57/Bl6) and two renin (FVB/N) gene mice. An identical pattern of renin granule formation was observed in FVB/N (a 2-renin gene mouse strain) embryonic and neonatal kidneys (Figure 7.6). Juvenile (also called "rhomboid") granules containing para-crystalline material characteristic of renin as well as intermediate and mature granules were all observed post-natally (Figure 7.6) but not pre-natally.

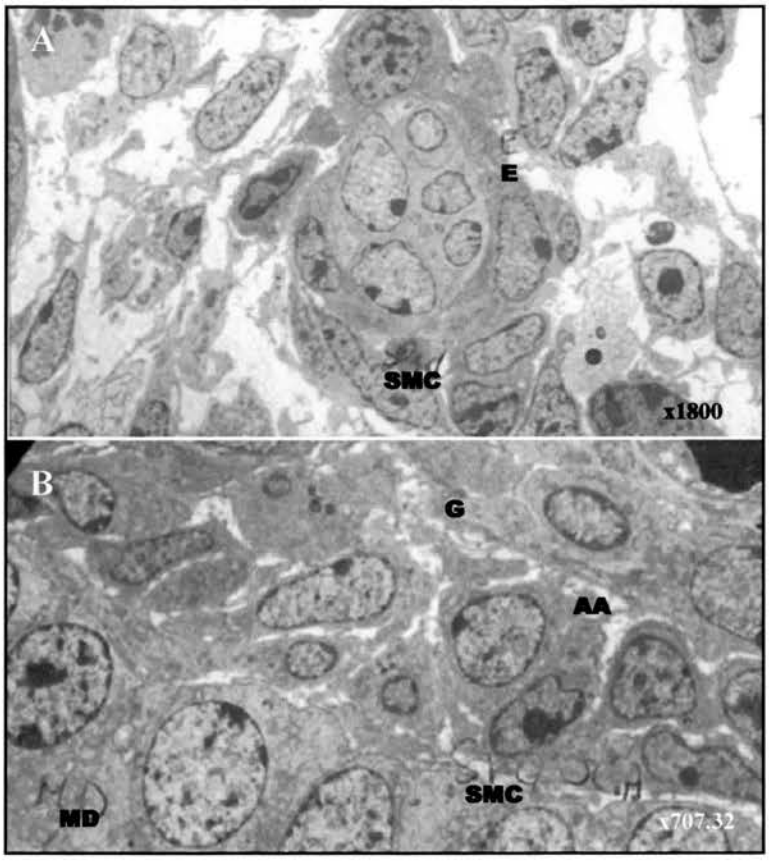


Figure 7.3: TEM photographs of embryonic pre-natal mouse kidneys (C57/Bl6) at embryonic day 18.5. A) Interlobular artery, B) Afferent arteriole. Note the absence of renin dense core granules in SMC of the interlobular arteries and afferent arterioles of the juxtaglomerular apparatus. AA = afferent arteriole, E = endothelial cells, G = glomerulus, MD = macula densa, SMC = smooth muscle cell.

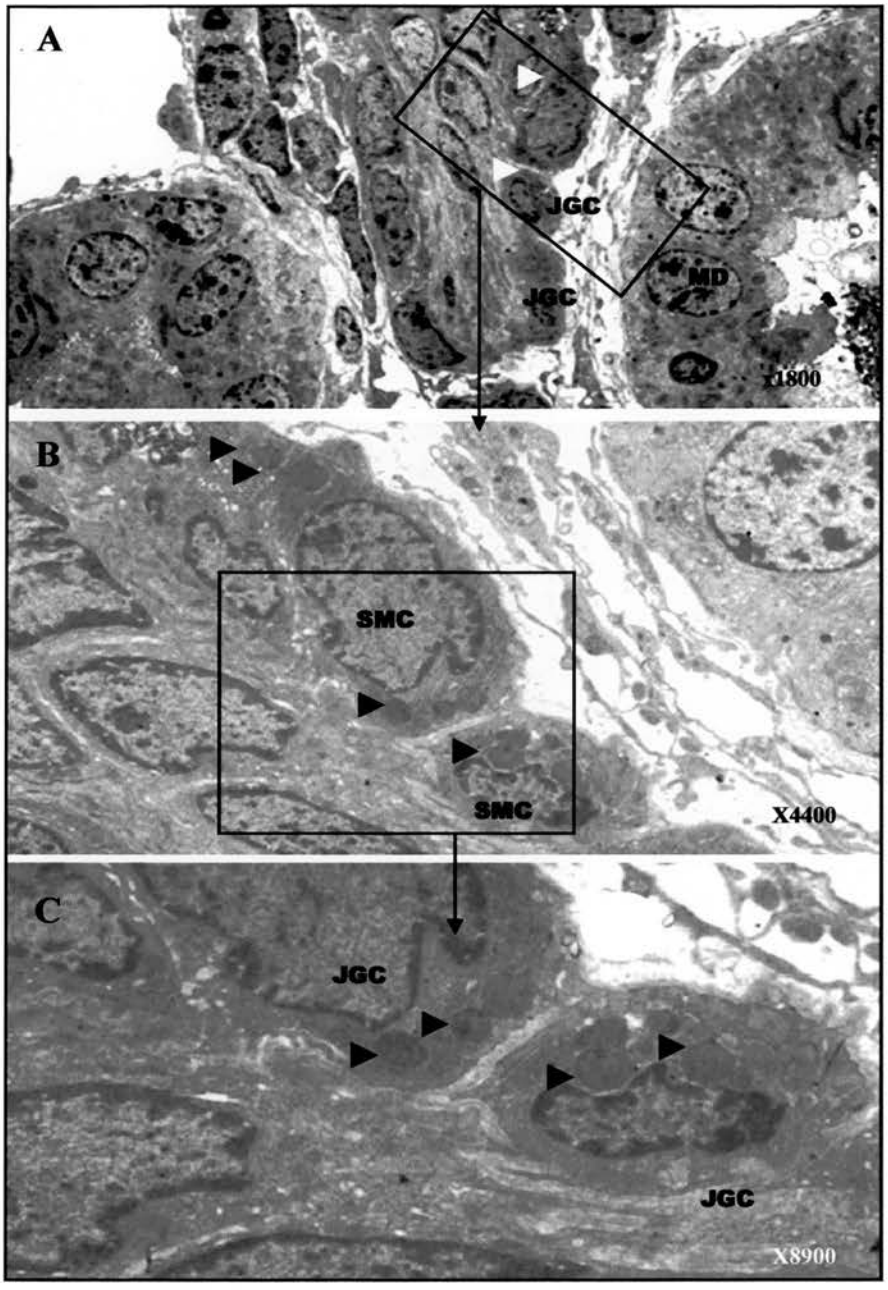


Figure 7.4: TEM photographs of post-natal P1 mouse (C57/Bl6) kidneys. A) Vascular pole of JGA showing afferent arteriole. B) Higher magnification of (A) showing the renin granule-containing cells along the afferent arteriole. C) Higher magnification of (B) showing individual dense core granules in JGC. The presence of renin granules is clearly observed. Arrowheads indicate the dense core renin granules. MD = macula densa, SMC = smooth muscle cell, JGC = juxtaglomerular cell.

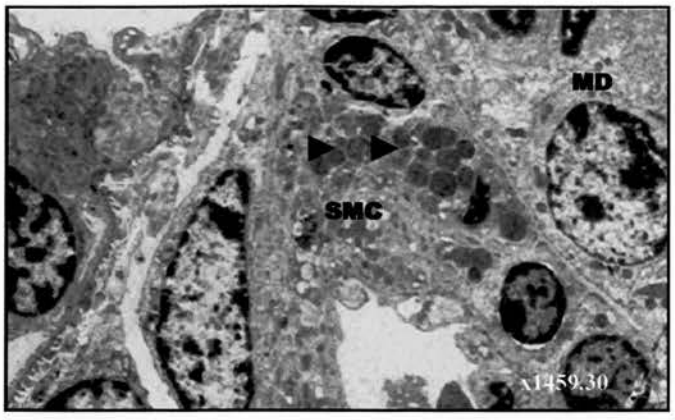


Figure 7.5: TEM photograph of post-natal P28 mouse (C57/Bl6) kidneys. Note the presence of renin granules in SMC of the afferent arterioles. Arrowheads indicate the dense core renin granules. MD = macula densa, SMC = smooth muscle cell.

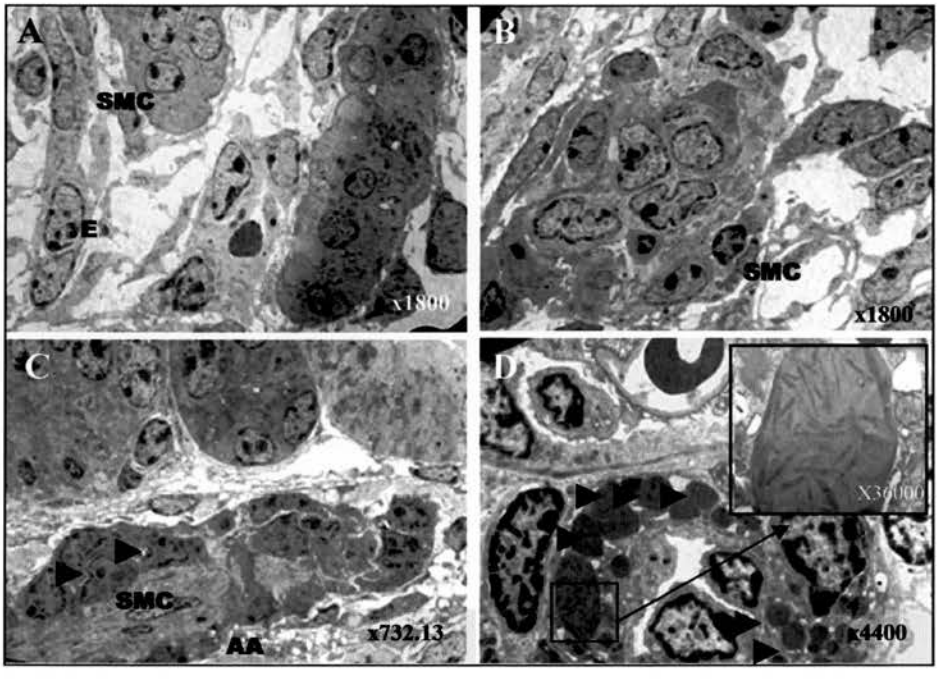


Figure 7.6: TEM photographs of mouse FVB/N kidneys during peri-natal period. A) Peripheral interlobular artery, embryonic day 16.5. B) Interlobular artery, embryonic day 18.5, C) Afferent arteriole, post-natal day 1, D) Afferent arteriole, post-natal day 28, inset: intermediate granule showing para-crystalline renin material. Arrowheads indicate the dense core renin granules. SMC = smooth muscle cell, E = endothelial cell, G = glomerulus, AA = afferent arteriole, MD = macula densa.

7.2.3 Effect of sodium depletion on granulation (Experimental group 1).

Contrary to the absence of prenatal granulation in control animals, renin granulation was detected at E18.5 when the mothers were fed a low sodium (0.03%) diet until giving birth. (Figure 7.7). Low sodium diet is well known to up-regulate renin expression and production in the adult and it is demonstrated here that it also has an effect on the foetal RAS regulation. The number of granules was approximately 5-6 granules per JG cell.

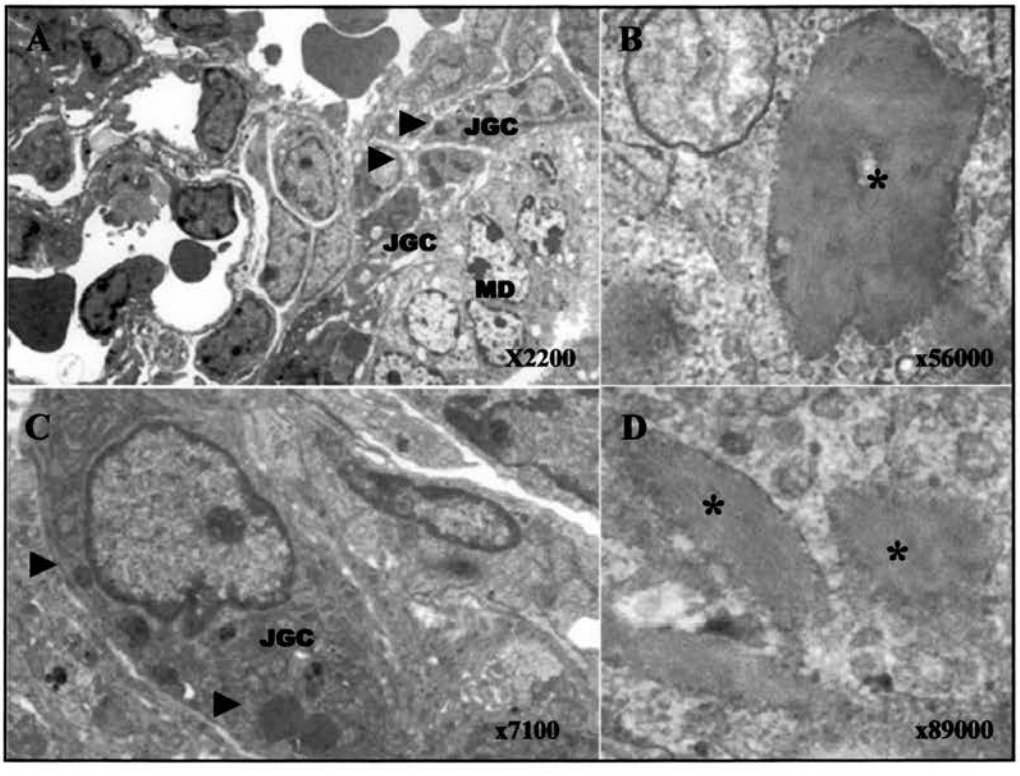


Figure 7.7: TEM photographs of pre-natal mouse (C57/Bl6) kidneys at embryonic day 18.5 after maternal low sodium diet. A) Vascular pole of JGA including renin-containing juxtaglomerular cells (JGC), B) Immature amorphous (pro)renin granule in JGC containing para-crystalline material, C) Afferent arteriole containing JGC, D) Immature spindle-shaped (pro)renin granules in JGC containing para-crystalline material. Note the presence of renin dense core granules in JGC of the afferent arterioles. arrowheads indicate the dense core renin granules. JGC = Juxtaglomerular cell. (*) immature (pro)renin granules.

7.2.4 Effect of sodium loading on granulation (Experimental group 2).

To reinforce the results obtained with the low sodium, we studied the effects of salt loading on foetal renin granulation. High sodium diet (3%) is known to inhibit the RAS and in particular renin release. As expected high sodium diet did not stimulate any renin granule formation pre-natally (Figure 7.8) and may have suppressed it further and this may need to be investigated in more detail.

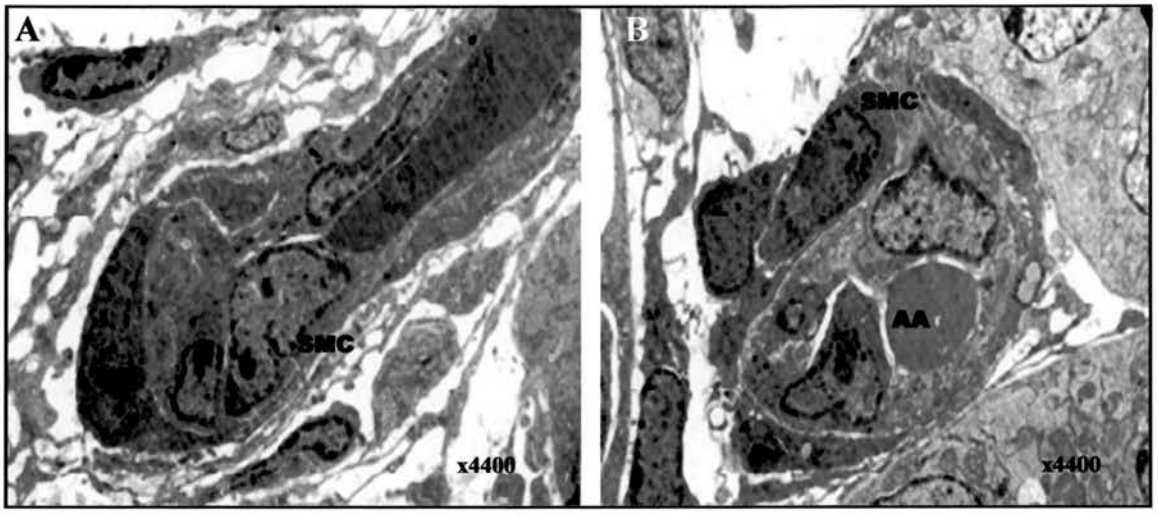


Figure 7.8: TEM photographs of pre- and post-natal mouse (C57/Bl6) kidneys at embryonic day 18.5 after maternal high sodium diet. A) Interlobular artery, B) Afferent arteriole. Note the absence of renin dense core granules in SMC of the interlobular artery and in the afferent arterioles of the juxtaglomerular apparatus. AA = afferent arteriole, SMC = smooth muscle cell.

7.2.5 Effects of ACE inhibition on granulation.

7.2.5.1 Captopril (Experimental group 3)

In the adult, pharmacological angiotensin-converting enzyme (ACE) inhibition up-regulates renin expression and production [34] as part of a feedback loop response. The physiological regulation of pre-natal granule absence was therefore studied by inhibiting maternal ACE.

Captopril (30mg/kg/day) was administered to pregnant C57/bl6 mice from day 15 of gestation until birth. As demonstrated with salt depletion, captopril ACE inhibition was found to be a stimulus for foetal renin granulation. It is worth noting that the number of renin granules in the foetus were elevated (30 granules per JG cell) after captopril ACE treatment compared with low sodium diet. The number of dense core granules compared to immature granules appeared to be higher after captopril ACE inhibition than after maternal sodium depletion. The data underline that maternal captopril ACE inhibition (and hence a reduction in circulating AngII), similar to low sodium diet, promotes the formation of renin granules in the foetal kidney before birth (Figure 7.9).

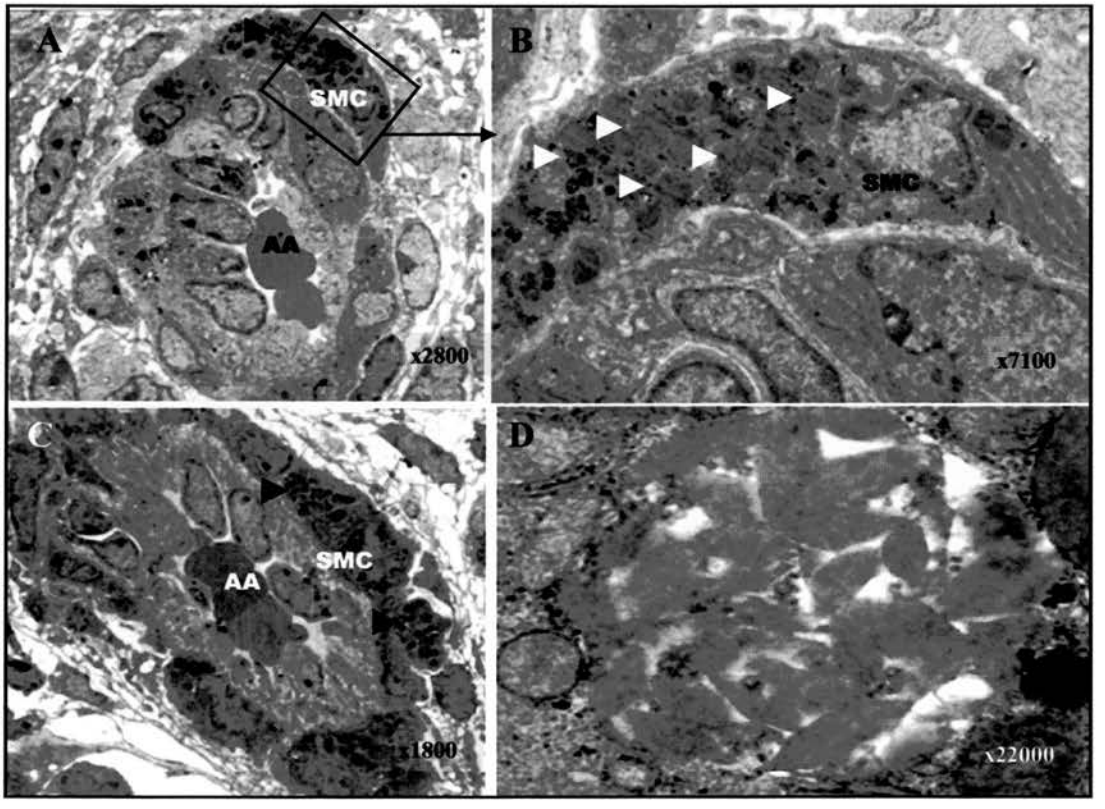


Figure 7.9: TEM photographs of C57/Bl6 embryonic and post-natal kidneys after maternal captopril treatment. A) Afferent arteriole with renin granule-containing smooth muscle cell, embryonic day 18.5, B) Higher magnification of (A) showing numerous dense core granules in smooth muscle cell, C) Afferent arteriole showing smooth cells containing dense core granules, post-natal day 1, D) Immature granule containing para-crystalline material. Arrowheads indicate the dense core renin granules. AA = afferent arteriole, SMC = smooth muscle cell.

7.2.5.2 Enalapril (Experimental group 3)

Due to potential cross-placental crossing of captopril, another ACE inhibitor, enalapril, was used to study the control of foetal kidney renin granulation. Pregnant C57/Bl6 mice were treated with enalapril (30mg/kg/day) from day 18 of gestation until birth. It has been shown that enalapril does not cross the placental barrier in the sheep and it may therefore enable differentiation between direct maternal or combined foetal/maternal effects of ACE inhibition when comparing the consequences of captopril and enalapril treatment on prenatal renin granulation.

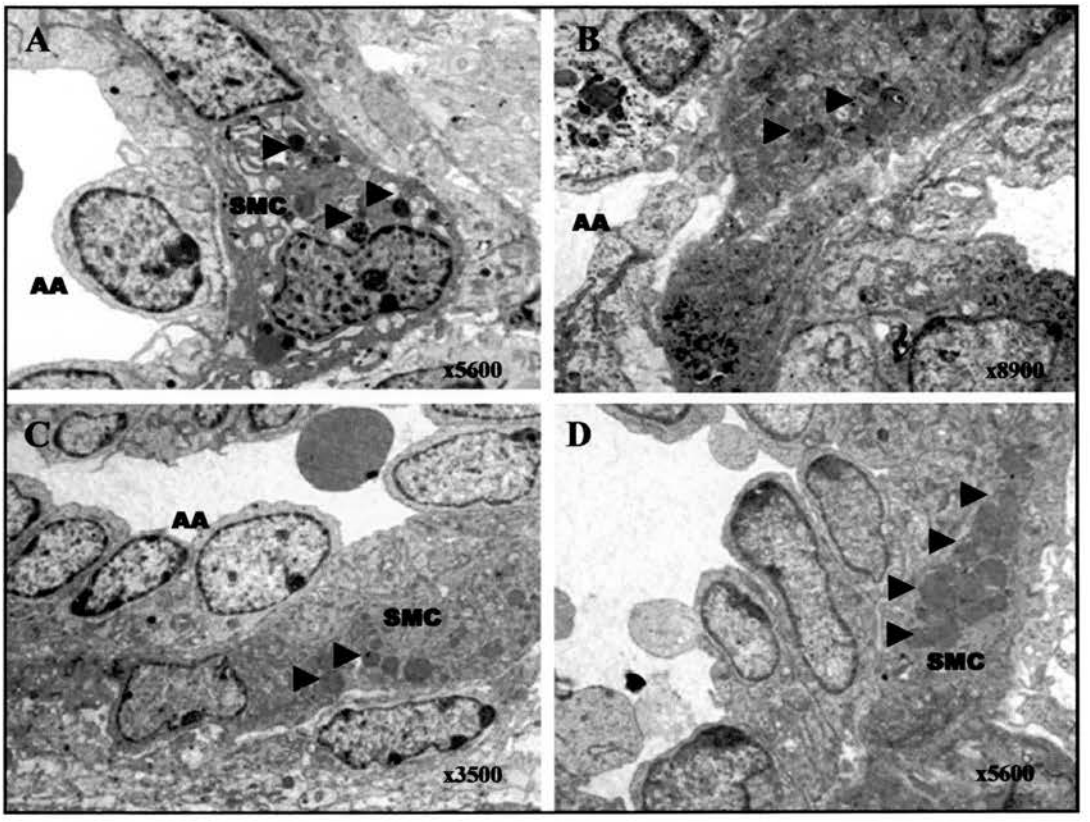


Figure 7.10: TEM photographs of pre- and post-natal mouse (C57/Bl6) kidneys after maternal captopril treatment. A-C) Afferent arterioles with smooth muscle cells containing dense core renin granules, embryonic day E18.5. D) Afferent arterioles with smooth muscle cells containing dense core renin granules, post-natal day 1. Arrowheads indicate the dense core renin granules. AA = afferent arteriole, SMC = smooth muscle cell.

As with captopril treatment, enalapril stimulated the formation of renin granules in the foetal kidneys (Figure 7.10) which do normally contain renin secretory granules before birth. Smooth muscle and JG cells contained fewer dense core granules than after captopril treatment and had more apparent immature granules resembling the granulation profile caused by maternal low sodium diet.

7.3 Discussion

7.3.1 Absence of renin dense core granules in foetal kidney

The data presented in this chapter clearly shows the complete absence of dense core renin granules in mouse kidneys before birth suggesting that renin was neither stored or packaged as observed in the adult. Prorenin maturation in the kidney results in packaging of the pro-hormone in secretory granules which are processed into dense core granules following the conversion of prorenin to renin and condensation of renin material within these granules. If prorenin is the only "renin species" in mouse foetal kidney, its role in kidney development may be essential. Measurement of foetal plasma and foetal kidney renin/prorenin contents would confirm the electron microscopy results and ascertain prorenin as a pivotal protein in nephrogenesis independently or as part of a local RAS system. This assay was not carried out in this thesis due to the unavailability of nephrectomised plasma to be used as a substrate for prorenin/renin. Similarly, the identification of renin granules could be confirmed by immunogold procedures. However, this was deemed unnecessary since the electron micrographs were processed and analysed by Prof. Stewart Fleming (University of Dundee) an expert in this area. The alternative pathway by-passes dense core granulogenesis and results in the release of the protease in its "inactive" form via the constitutive pathway. Surprisingly, this is in contradiction with Minuth *et al.* who reported the presence of renal renin granules in NMRI mice (Minuth *et al.* 1981). In this study, it is worth noting that the transmission electron microscopy data were not shown and that the mouse strain differences may be an important factor as well as the renin granule detection methodology. In addition, other electron dense micro-vesicles may have been observed and mistaken for dense core renin granules. Our results also differ from reports in the pig which showed the presence of granules in the mesonephros and metanephros (Tiedemann and Egerer 1984) and studies in Wistar rats (Bruhl *et al.* 1974). These differences may reflect the fact that renal maturation in mice continues after birth. Renin granules appeared post-natally in their "adult" location, the

juxtaglomerular cells. The number of renin granules was showed to increase from birth to weaning indicating that the kidneys were still maturing after birth and gradually adapting to independent feeding and RAS/blood pressure or salt balance regulation.

No differences could be observed between the two mouse strains used, FVB/N possessing 2-renin genes (*Ren1*^{d} and *Ren2*) while C57/Bl6 have only one renin gene (*Ren1*^{c}). All three murine renins share 97% homology at the protein levels and show differences, at the nucleotide levels, at 3 asparagine-linked glycosylation sites found exclusively in the "*Ren-1*" genes (Sigmund and Gross 1991). *Ren-1*^{d} null mutants (Clark *et al.* 1997) displayed a distinct phenotype in which no dense core granulation was observed in the juxtaglomerular (JG) cells, indicative of absence of storage vesicles for renin and the inability to package renin originating from *Ren2*. These mice also have a modified macula densa morphology (the cells appear more columnar and numerous). The granulation and macula densa phenotype were rescued by crossing the *Ren-1*^{d} knock-out animals with a bacterial artificial chromosome (BAC) clone encompassing both *Ren-2* and *Ren-1* genes (BACN10). This was not observed when the mice were crossed with transgenic mice carrying BACN10 in which *Ren-1*^{d} has been replaced by a gene conferring β-galactosidase activity. This confirms that *Ren-1*^{d} is important in the granulation process. Over-expression of *Ren-2* (from BACN10) did not compensate for the renal phenotype but did participate in the regulation of blood pressure as male *Ren-1*^{d} null mutants have normal pressure (Mullins *et al.* 2000).

In the adult, the number of renin-positive cells along the afferent arterioles varies. It has been shown that when homeostasis is threatened (as it may happen during haemorrhage or hypotension), smooth muscle cells of the renal arterioles can be recruited and they undergo the metaplastic differentiation events mentioned above (Gomez *et al.* 1990b; Pentz *et al.* 2001). Using a GFP-*Ren1* reporter system, Sequeira-Lopez *et al.* followed the fate of renin-expressing cells from their embryonic location in normal physiological conditions to their location under salt depletion conditions in the adult (Sequeira Lopez *et al.* 2004). They demonstrated that it was the same renin-producing cells which line the afferent and efferent arterioles during nephrogenesis which become SMC in the adult but retain the "memory" of becoming renin producing when needed. My studies, however, revealed that the embryonic phenotype for JG cells in the mouse was characterised by a complete absence of renin granules suggesting that the adult and embryonic juxtaglomerular cells differ in their prorenin/renin storage capacity (Figure 7.11).

Prorenin is now known to be activated without changes in molecular weight through an alternative activation mechanism. This process possibly involves a (pro)renin receptor which gives prorenin the ability to return to an inactive conformation after unfolding of the prosegment to reveal the catalytic site (Derkx *et al.* 1987; Leckie and McGhee 1980)

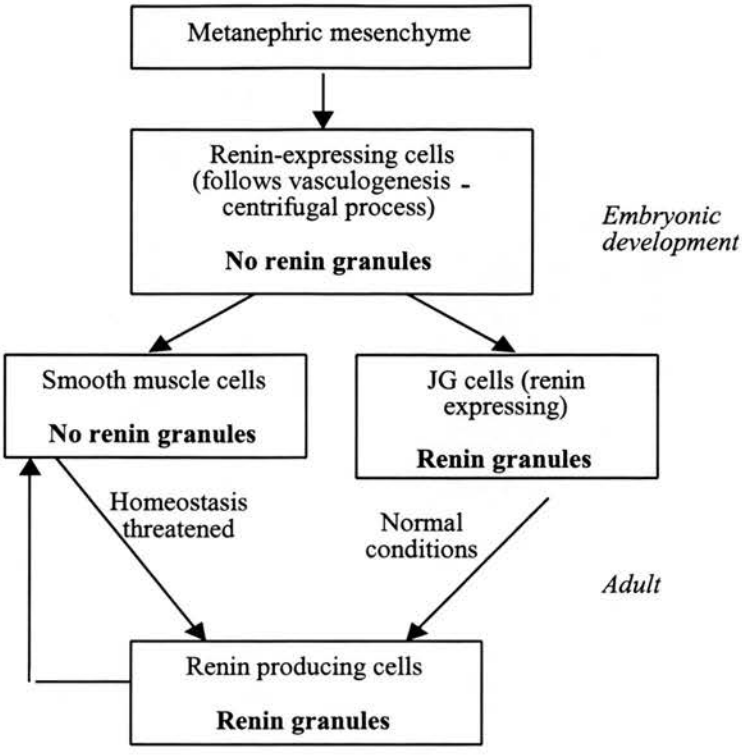


Figure 7.11: Schematic representation of the fate of JGC and SMC during development and during episodes of RAS activation in terms of granulation according to the results presented.

7.3.2 Stimulation of foetal renin granulation

Two different ACE inhibitors, captopril and enalapril, were used to stimulate the foetal kidney to generate renin granules. Both ACE inhibition and low salt diet triggered renin granule production in the foetal kidney at embryonic day E18.5 (E18.5). It is not known if it is the distinct result of maternal homeostasis stimulation or if it is a combined effect on both the foetal and the maternal RAS. The response to captopril treatment appears to be enhanced compared with sodium depletion in terms of the number of granules produced. This could be due to the difference in administration route or to a difference in the degree of action which these two conditions have on the foetal kidney. Similarly, the number of renin granules in the

prenatal kidney was higher during captopril treatment than enalapril treatment. This may reflect a different drug metabolism for enalapril. It may also be that both low salt treatment and enalapril have isolated effects on maternal homeostasis resulting in a less widespread stimulation of the foetal kidney. Captopril has been shown to cross the placental barrier in the sheep whereas enalapril did not (Table 7.1).

Compound	*Species*	*Placental Transfer*	*Blood-Brain Barrier Transfer*	*Reference*
Enalapril	Rats (SHR)	-	No	(Jouquey *et al.* 1995)
Enalapril	Sheep	No	-	(Broughton Pipkin and Wallace 1986)
Enalapril	Sheep	No	-	(Stevenson *et al.* 1995)
Enalapril	Human (*in vitro*)	Yes	-	(Reisenberger *et al.* 1996)
Captopril	Rat	-	No	(Barry *et al.* 1984)
Captopril	Sheep	Yes	-	(Broughton Pipkin *et al.* 1982)
Captopril	Rabbit	Yes	-	(Broughton Pipkin *et al.* 1982)
Captopril	Sheep	Yes	-	(Stevenson *et al.* 1995)
Trandolapril (lipophilic)	Rat	-	Yes	(Tan *et al.* 2005)
Lisinopril (hydrophilic)	Rat	-	Yes (but higher dose than trandopril)	(Tan *et al.* 2005)

Table 7.1: Placental and blood-brain barrier transfer of ACE inhibitors.

The differences in foetal granulation after captopril and enalapril treatment may reside in the transplacental transfer of one drug and not the other. It may, indeed, not reflect what occurs in the mouse due to different placental structures. However, foetal renin granule stimulation may be accounted for as a response to maternal ACE inhibition but also as a direct result of foetal RAS modulation. This is illustrated by the detection of granules with both drug treatments and salt depletion. In addition, enalapril is not the active drug and after intestinal absorption needs to be hydrolysed to its active metabolite, enalaprilat. Captopril, on the other hand, does not require activation to exert its effects. Considering both drugs were administered at the same dose, the available active inhibitor may differ in amount between the two treatments and may have a different influence on renin granulation.

Quantitative analysis of the number of granules in foetal kidneys from captopril- and enalapril-treated mothers as well as from animals receiving a low sodium diet may be useful to compare the different regimen. The manner in which the sections were obtained and the micrographs examined does not allow statistical significance. This considered for future studies and work.

In summary, the present study demonstrated that the processes involved in prorenin maturation are different in the embryo and in the adult. Renin expressing cells during nephrogenesis differ from adult JG cells in their ability to package prorenin/renin in secretory granules although they retain the "memory" of renin production in the adult. The mechanisms governing the processing of renin granules appear to be under a dual protective system of maternal and of foetal origin. This study suggests that the maternal and foetal RAS may have very separate roles during foetal development pertaining to blood pressure regulation and to nephrogenesis, respectively. Further investigations are required to dissect the different actions of the RAS during pregnancy and embryogenesis.

Chapter 8
Conclusions and Perspectives

The results presented and discussed in this thesis contribute to a large body of work pertaining to newly identified functions of the renin-angiotensin system (RAS) within tissues. Recent reports have demonstrated that prorenin, rather than being solely an inactive precursor of renin, is itself part of the RAS enzymatic cascade and may also carry out functions independently of angiotensin (Ang) II production. Considering the potential importance of prorenin and renin in the development of vascular and cardiac injuries in animal models of hypertension and diabetes and in patients exhibiting hypertension, diabetes and pre-eclampsia, the general aim of this thesis was to examine the pathophysiology of prorenin and renin in malignant hypertension and to investigate (pro)renin maturation during embryogenesis as well as its regulation.

For this purpose, the identification, characterisation and localisation of a prorenin/renin receptor homologous to the human (pro)renin receptor in mouse and rat tissues as well as extensive phylogenic studies and foetal expression profiles were performed. Expression of the mouse putative (pro)renin receptor was detected in all tissues and cell lines examined including human mesangial cells previously reported to be negative for (pro)renin receptor. Mouse putative (pro)renin receptor was also present during development from E9.5, hence before renin expression begins. The mouse and rat (pro)renin receptor cDNA were found to be highly homologous (92% and 91%, respectively) to the human cDNA. Surprisingly, the translated human, mouse and rat cDNAs exhibited sequence identity with a small protein co-purifying with a bovine vacuolar-ATPase called M8-9 which had not been reported previously. V-ATPases are critical for cell survival. Phylogenic studies revealed (pro)renin receptor is highly conserved between species, including those as distant as *C.elegans*. This molecule is, therefore, likely to be important physiologically. The role of (pro)renin receptor was investigated in a rat model in which (pro)renin triggers malignant hypertension (MH). TGR(*Cyp1a1-Ren2*) animals carry a mouse renin gene under the control of the *Cyp1a1* promoter inducible by dietary indole-3-carbinol. A rapid rise in blood pressure was accompanied by weight loss and polyuria. The animals exhibited microinfarctions, inflammatory cell infiltration and fibrinoid necrosis in the heart. In the kidney, distal tubule

hyperplasia and thickening of intra-renal arterial wall without glomerulosclerosis were observed. High prorenin plasma concentrations due to expression of the mouse *Ren2* transgene were reported and uptake of this non-glycosylated prorenin by the heart, previously demonstrated in this model, may be mediated by (pro)renin receptor. In the present study, the animals, from a separate colony, developed hypertension in a more progressive manner. Cardiac remodeling and vascular stiffening were measured using echo(cardio)graphy techniques. Despite an obvious hypertrophic response of the heart and longer exposure to the inducer, no signs of microinfarctions or inflammatory infiltration cells were observed. Fibrinoid necrosis of small intra-renal vessels with glomerulosclerosis and mesenteric artery remodeling were also observed. The phenotype, therefore, differs from the original work. Surprisingly, the (pro)renin receptor expression was not up-regulated in this model. Phenotypic differences between TGR(*Cyp1a1-Ren2*) colonies were examined and two main observations were made: dietary sodium levels appear to correlate with the severity of MH and TGR(*Cyp1a1-Ren2*) animals reported in this thesis had a lower pathogen load.
To investigate the possible role of the putative (pro)renin receptor, a prorenin decoy peptide was used to attempt to ameliorate the MH phenotype in TGR(*Cyp1a1-Ren2*) animals. This peptide which competes with prorenin for binding to the (pro)renin receptor has been showed to improve vascular injuries in diabetic nephropathy. In TGR(*Cyp1a1-Ren2*), however, no changes in the MH phenotype could be observed, except in the mesentery in which less severe fibrinoid necrosis developed. To complement work on the putative (pro)renin receptor, prorenin maturation and renin storage were studied during development. The data showed the complete absence of renin granules in mouse kidneys before birth. This indicates that renin was not stored and may not be processed through the regulated pathway as observed in the adult. Low sodium diet and ACE inhibition triggered (pro)renin granules to be produced in the foetal kidney. Two ACE inhibitors differing in their ability to cross the placenta were used. The data suggest that foetal renin granule formation is under the control of both foetal and maternal RAS.

8.1 Local renin-angiotensin system

The renin-angiotensin system (RAS) is not just an endocrine/circulating system and two decades of research have provided strong evidence for the presence of a tissue RAS and its functional role in health and disease. It is only in the late 1970s that the term "local RAS" first appeared and that the notion of independent RAS systems was brought forward. In

support of this hypothesis, all components of the RAS have been found in tissues such as kidney, brain, heart and feto-placental tissues. However, it remains, to date, unclear whether these tissues produce RAS components such as renin and angiotensinogen *de novo* or whether these are taken up by the tissues. The role of the local RAS has been strongly linked to localised effects of AngII on blood pressure, salt and electrolyte balance and vasoconstriction as a result of local production. Indeed, in the kidney, AngII and AngI levels are found are higher amounts than expected from equilibration with plasma levels and are not solely a result of uptake from the circulation (Navar *et al.* 2002). AngII located in the juxtaglomerular cells may constitute a link connecting distal tubular fluid to resistance of glomerular arterioles, hence regulating tubulo-glomerular feedback in addition to a macula densa-driven mechanisms. Furthermore, locally produced AngII through a proximal tubule RAS may provide a rapid response able to modulate fluid and salt reabsorption preceding that of the circulating RAS in response to homeostatic changes. In the heart, what would AngII function be in cardiac physiology? Locally produced AngII and activated RAS may contribute to the homeostatic response and vasoconstricting actions may participate in the re-establishment of normal blood pressure. In normal physiological situations, AngII may be involved in vasoconstriction of intra-cardiac vessels (and hence the blood supply to cardiac tissues) and possibly in the regulation of cardiac inotropy.

8.2 Prorenin

Direct and separate actions of the enzyme catalyzing the first rate-limiting step of the RAS cascade, renin, have been hypothesized together with a role for prorenin, the "inactive" form, of the protease. The rationale behind this hypothesis originated from the fact that: 1) prorenin is present at high concentrations in the plasma where they remain elevated after bilateral nephrectomy although plasma renin almost but not completely disappears (Sealey and Rubattu 1989; Sealey *et al.* 1977; Weinberger *et al.* 1977). 2) Elevated plasma prorenin levels have been found during pregnancy and in conditions such as diabetic nephropathy. 3) Prorenin is found in large amounts in the amniotic fluid and in the ovary. Additional sites of prorenin production include the adrenal, the pituitary and the uteroplacental complex, namely myometrium, placenta, and chorio-decidua (Carretero *et al.* 1972; Lumbers 1993; Poisner 1998; Skinner 1987). These tissues have been shown to produce renin and prorenin but it is unclear whether prorenin produced at those sites is converted to renin and released in the circulation or taken up as prorenin prior to conversion in tissues. Several possibilities for

the origin of amniotic fluid prorenin have been proposed such as the chorion, the foetal kidney/urine, maternal circulation or the maternal ovaries.

Since prorenin can be found in these tissues and in the plasma, one question which remains to be elucidated is the potential role for prorenin and its mode of action. *Null* mutants for the renin gene *Ren1d* produced in a two-renin gene strain of mice displayed normal blood pressure in males only (Clark *et al.* 1997). Females animals were characterised by hypotension. This is explained by the fact that a second renin gene *Ren2* is expressed in the sub-maxilliary gland (SMG) in a sexually dysmorphic fashion. In males, renin and prorenin are released by the SMG under testosterone control. This suggests that *Ren2* does participate in the maintenance of blood pressure in the absence of *Ren1d* in two renin gene-mice. The gene *Ren2* is known to give rise predominantly to prorenin and a decrease in prorenin in *Ren2* null mutants (Sharp *et al.* 1996) was demonstrated suggesting that prorenin can play a role in blood pressure maintenance. This process is likely to occur through the formation of AngII. Hence, prorenin may result in localised production of AngII leading to AngII-associated physiological events such as inflammation as observed in the ovary, vascular remodelling as seen in the heart and blood vessels and steroid secretion from the adrenal gland.

Are tissue and plasma prorenin converted to renin by removal of the prosegment? This process occurs normally during prorenin maturation through the regulated pathway and other mechanisms must therefore be involved in tissues or in the plasma. Prorenin conversion to renin was identified experimentally as a two-step process, most likely to be associated with an acidified microenvironment such as that of secretory granules (Derkx *et al.* 1987). Proteases are also present in the plasma and in tissues such as the utero-placental complex and may be responsible for enzymatic removal of the prosegment. However, Brechler *et al*, identified a sequence signal in the prorenin associated with protease processing of prorenin occurring during the regulated pathway, excluding a contribution to the conversion of secreted prorenin. Prorenin is taken up by cardiac tissues in the inducible high prorenin transgenic model (Kantachuvesiri *et al.* 2001). Since prorenin is originating from the mouse *Ren2* gene and therefore unglycosylated, uptake must result from binding to another receptor which may activate prorenin. Similar to the glycosylation states of renin from mouse *Ren1* and *Ren2* genes, differences exist in humans between prorenin of renal and extrarenal origin [Hosoi *et al*, 1992]. Although the existence of a (pro)renin receptor had not been demonstrated and was still a matter of debate, binding to this receptor was hypothesized to trigger unfolding of the prosegment to expose the catalytic site and conferring it the ability to

convert angiotensinogen. [(Sealey *et al.* 1987; Sealey and Rubattu 1989); Figure 8.1]. This was proposed to happen without removal of the prosegment and to be reversible. Indeed, Suzuki and colleagues demonstrated that prorenin possessed a "gate region" necessary for the unfolding of the prosegment and a "handle region" which binds to a putative (pro)renin receptor resulting in reversible non-proteolytic activation of prorenin (Suzuki *et al.* 1999; Suzuki *et al.* 2003).

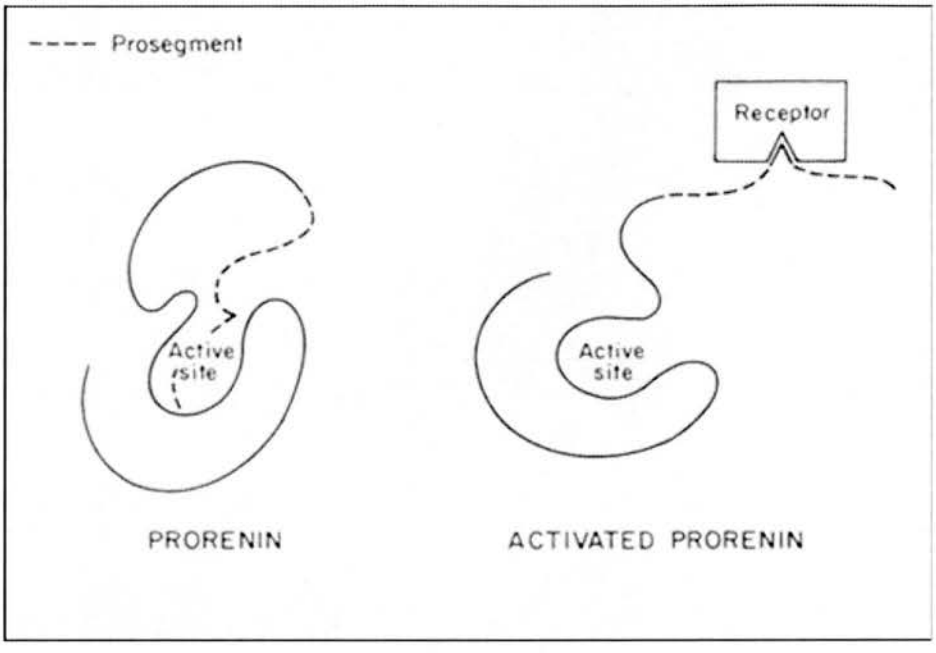


Figure 8.1: Schematic representation of prorenin and binding to a putative receptor. Binding of prorenin results in unfolding of the protein without removal of the prosegment. This process is similar to the non-proteolytic activation described by Suzuki *et al.* (2003). Figure reproduced from (Sealey and Rubattu 1989).

8.3 Putative (pro)renin receptor

The present report was first aimed at studying the biology of prorenin through its putative (pro)renin receptor. Recently, a specific (pro)renin receptor was identified in human tissues (Nguyen *et al.* 2002). Binding of this receptor to (pro)renin caused increased cleavage of angiotensinogen and stimulation of an intracellular signalling pathway (Nguyen *et al.* 2002). This is of importance for a physiological role of prorenin since prorenin may participate in AngII formation or have direct actions independent of AngII. This putative receptor was

identified, cloned and shown to be expressed in all mouse tissues examined. Although, expression in the microvasculature of these tissues may explain this widespread presence, it was also detected in cell lines including human mesangial cells previously reported to be negative for (pro)renin receptor (Nguyen *et al.* 2002). The conclusion from these findings was that this gene was ubiquitously expressed and not differentially regulated in different tissues. Phylogenic studies revealed that the mouse and rat putative (pro)renin receptor cDNAs are highly homologous (92% and 91%, respectively) to the human cDNA and that the receptor is highly conserved between species as unrelated as *D.rerio* and *C.elegans*. Surprisingly, the translated human, mouse and rat cDNAs exhibited sequence identity with a small protein co-purifying with a bovine vacuolar-ATPase called M8-9 which had been previously overlooked (*Chapter 3*). Considering that binding of renin to the receptor also resulted in increased AOGEN conversion to AngI, it appears that the receptor identified by Nguyen *et al.* does not match the previously proposed model described in Figure 8.1 and one may wonder how activated renin *i.e.* without a prosegment may bind this receptor since it does not possess a "handle region". Although the (pro)renin receptor may be important physiologically, the data and in particular homology with a vacuolar ATPase subunit presented in this thesis suggest a more fundamental role in cell biology than had previously been recognised.

In mammalian cells, V-ATPases play important roles in energy conservation and secondary active transport, acidification of intracellular compartments and cellular pH homeostasis (Nelson 2003; Nelson and Harvey 1999).The functions of both the (pro)renin receptor and M8-9 subunit are therefore not incompatible. However, my studies have shown that the human (pro)renin receptor described by Nguyen *et al* (2002) and the vacuolar ATPase subunit M8-9 must be the product of the same gene since only one gene was found in the database. Whether these proteins are functionally related remains to be investigated. For example as a transporter of renin/prorenin from the circulation into tissues, an activator of prorenin, an initiator of a intracellular pathway upon binding to (pro)renin and secretory granules acidification, the latter *via* potential differential exon usage and post-translational modifications which give rise to the M8-9 sub-unit of a V-ATPase. In the kidney, notably, plasma membrane V-ATPases play a major role in homeostatic processes of several nephron segments (Schoonderwoert and Martens 2001). Furthermore, V-ATPases are closely involved in the process of dissociation of receptor to ligand and receptor recycling by creating an acidic environment after internalization of the complex (Nelson 2003) suggesting that V-ATPases and a (pro)renin receptor may interact in the cell. It is attractive to speculate

that renin may be transported/taken up [by a (pro)renin receptor mediated mechanism] in the mitochondria where V-ATPases are responsible for cytoplasmic acidification (Nelson and Harvey 1999). A truncated non-secreted form of prorenin termed renin 1A has, now, been identified in the mitochondria and uptake of prorenin through a receptor may therefore not be needed for an interaction with V-ATPase in the mitochondria.

While intracellular pathway activation following binding of prorenin to the receptor is an important mechanism for cardiac hypertrophy and vascular remodelling in hypertension and diabetes, it is worth noting that only one publication (Nguyen *et al.* 2002) reported up-regulation of second messenger pathways. This publication contained two major discrepancies: no expression of the human (pro)renin receptor in human mesangial cells which I found to be erroneous (and is now recognised by the author as an error) and homology with the V-ATPase subunit which was previously overlooked and was described in this thesis. The latter is of considerable importance in terms of a putative role for this protein in basic cell function maintenance. Due to these findings, it was decided that attempts at producing *null* mutants of this receptor would potentially be unfeasible. The expression of a (pro)renin receptor in murine embryonic tissues may be linked to its putative functional homology with the V-ATPase subunit as discussed in *Chapter 3*.

In mammalian cells, V-ATPases play important roles in energy conservation and secondary active transport, acidification of intracellular compartments and cellular pH homeostasis (Nelson and Harvey 1999). These V-ATPase functions are likely to be essential in development. A gene trap cell line was available commercially in ES cells of female origin (http://baygenomics.ucsf.edu/cgi-bin/BaySearch.py, Genetrap reference RST307) in which it caused no lethality. The position of the gene trap between exon 8-9 (upstream of the transmembrane region) suggests that the transmembrane region is essential for protein function and that a soluble form of the (pro)renin receptor is unlikely to be functional. It has come to my attention that attempts at creating (pro)renin receptor/M8-9 *null* mutants in laboratories of collaborators using this gene trap were indeed unsuccessful (Nguyen G., Personal communication).

8.4 TGR(Cyp1a1-Ren2) – a high prorenin transgenic inducible model of hypertension

Several rat models of hypertension are available for investigations of hypertensive end-organ damage, genetic influences and pharmacological intervention benefits in this pathological condition. Genetic rat models (SHR, Dahl, Milan) and transgenic models such as TGR(mRen2)27 and dTGR(*hAOGEN*x*hRen*) have allowed researchers to study pathophysiological factors involved in hypertension, notably the (local) renin-angiotensin system or oxidative stress and inflammation. However, these animals do not provide a controllable system for the timing of the development or severity of hypertension. The importance of having a non-surgical inducible model is further highlighted by the fact early events in malignant hypertension are largely unknown. Indeed, a large group of patients with malignant hypertension are diagnosed once the injuries have developed which prevents investigations of early hypertensive mechanisms. It is important to be able to trigger hypertension at different stages of the lifespan in animal models since foetal and neonatal programming may play a crucial role in the pathogenesis. In addition, hypertension in young animals may not represent a relevant physiological situation to that seen in humans. Hence, the inducible hypertension model TGR(*Cyp1a1-Ren2*) provides researchers with a flexible and adaptable model. Previous publications (Collidge *et al.* 2004; Howard *et al.* 2005; Kantachuvesiri *et al.* 2001) and the present thesis highlight the fact that can be used for separate areas of research and shows that this model is of use and in use in several laboratories all studying aspects of hypertension biology. Unique endpoints and assays are used but careful interpretation of the data when comparing previous published work must be taken as found in my experiments using a separate transgenic colony.

The experiments described in this thesis provided extensive study of the haemodynamic response in this strain (*Chapter 4*). Echocardiography which is a non-invasive and powerful technique allowed me to take repeat measurements of cardiac function. Although the blood pressure and histopathological phenotype was found to be less severe than previously reported (see below), it was revealed that ventricular hypertrophy in treated TGR(*Cyp1a1-Ren2)* animals does not develop until two weeks after blood pressure elevation showing that this event is downstream consequence of RAS activation and/or hypertension. In addition, adaptative mechanisms were clearly observed in heart since cardiac output was reduced in parallel with cardiac hypertrophy development.

Peripheral resistance was increased as measured in mesenteric vessels *in vitro*. The lumen diameter of mesenteric arteries of TGR(*Cyp1a1-Ren2)* animals were affected by inward remodeling as compared to controls. Response to intraluminal pressure was significantly reduced in the vessels isolated from the treated animals even at low pressure (20mmHg). Histopathology results showed that this inward remodeling is dependent on an increase in the cross-sectional area of the tunica media. Since blood pressure is a function of cardiac output and peripheral resistance, it appears that this increase is an important factor. In addition, a decrease in cardiac output would represent an adaptative response from the heart. Aorta wall track echography demonstrated a stiffening (calculated as compliance and distensibility) of the abdominal aorta. This effect is likely to be a result of blood pressure increase and remodeling. However, a decrease in the compliant, blood pressure-dampening properties of the aorta could contribute towards the gradual blood pressure elevation after the initial period of hypertension due to the transgene activation. Because aorta wall track echography is a semi-invasive procedure requiring cannulation and subsequent ligation of the femoral artery, only two measurements can be done. In the present work, aorta wall track echography was only employed in the final week of treatment due to equipment use restriction but it would be informative to collect measurements at the start of the experimental period.

Increased collagen deposition was present over the whole kidney. In the aorta, the number and size of elastic fibres was increased and changes in smooth muscle cell morphology were associated with hypertrophic events. Surprisingly, no cardiac fibrosis was observed. The remodelling events which lead to increased ventricular mass have been shown to be linked to increased cell size and/or number but also inappropriately excessive production of extracellular matrix (ECM). This is confirmed in studies of myocardial infarction in which the inhibition of DNA synthesis and collagen deposition in myocardial interstitium by ACE inhibitor suggests a role for AngII in interstitial and perivascular fibrosis (Dostal *et al.* 1996). Clearly, ECM deposition in the heart is not the causative mechanism for ventricular hypertrophy in the present study. Histopathological stains in the mesenteric bed for elastin, collagen and reticulin were performed and only collagen staining was higher in treated animals than in controls. Accumulation of ECM proteins within the renal interstitium and atrophy of the tubules have been identified in chronic renal disease (Klahr and Morrissey 2003). *In vitro* studies have shown that both AngII and endothelin 1 (ET-1) promotes the synthesis of fibronectin and collagen in a dose-dependent manner in vascular smooth muscle cells (Grainger *et al.* 1994). It is therefore possible that these mechanisms are independent of blood pressure increase and are a result of RAS activation either through local AngII

production or through prorenin actions which remain to be determined. The (pro)renin receptor does not, however, appear to be involved. The regulation of this putative (pro)renin was further investigated in TGR(*Cyp1a1-Ren2)* animals. Inhibition of the (pro)renin receptor was also performed using a decoy peptide spanning the handle region of the prorenin protein.

8.5 Phenotypic differences in colonies of TGR(Cyp1a1-Ren2) rats

Experiments using these animals from a separate re-derived colony at the University of Maastricht, however, revealed a different phenotype from the original work (Kantachuvesiri *et al.* 2001), notably an inflammatory tissue response which was reduced, an increased resistance to malignant hypertension injuries and a dissimilar pattern of the vascular and tissue injuries. Indole-3-carbinol is a natural substances occurring in cruciferous vegetables. Most rodent chows are made from vegetal matter and therefore may contain varying amounts of this chemicals as well as other xenobiotics able to activate the transgene and this may explain differences in phenotype reported in *Chapter 4*. This was not tested.

Investigations designed to identify the factors responsible for the phenotypic differences showed that two main influences may be involved: lower dietary sodium levels and lower pathogen load. Comparison of the phenotype of TGR(*Cyp1a1-Ren2)* between collaborative laboratories is not sufficient to correlate results and clearly several environmental factors need to be considered when linking results obtained with this model. It has been previously reported that the induced TGR(*Cyp1a1-Ren2)* animals fed a high salt diet (4%) or offered saline solution did not display exacerbated end-organ pathology to the kidney, heart or mesentery (although cerebrovascular injuries were detected) (Collidge *et al.* 2004). In addition, the animals were found to develop salt sensitive hypertension after treatment (for 14 days) with inducer was ceased (Howard *et al.* 2005). Hence, dietary salt levels may exacerbate the phenotype observed in this rats. It could be that the levels of dietary sodium in the laboratory in Maastricht are below a threshold which is suggested by the increased mortality rate at 12 days in the "New Orleans" population (greater than that of the "Edinburgh"), which are fed a diet with higher sodium contents (K. Mitchell, Personal communication). This phenomenon is also reflected in the shape of the blood pressure curve which rose slowly in the "Maastricht" animals.

Microsatellite marker genotyping confirmed that the animals used in the original publication and those used for this thesis at the University of Maastricht were of the same strain and that the phenotype was not modified due to different genetic background. Unfortunately, this procedure also provided evidence for a genetic contamination in the new re-derived colony of TGR(*Cyp1a1-Ren2*) animals held at the University of Edinburgh and prevented further experiments to perform salt diet modulation or to study the importance of a pathogen-induced phenotype. Another important aspect is the inducer dose which is associated with a severity of the hypertensive response both in terms of blood pressure and end-organ damage [J. Peters, Personal communication and (Kantachuvesiri *et al.* 2001)].

8.6 Putative (pro)renin receptor inhibition using a decoy peptide

In the TGR(*Cyp1a1-Ren2*) rat model, inhibition of the putative (pro)renin receptor was performed using a decoy peptide spanning the handle region of the prorenin protein (*Chapter 6*). This peptide had been previously associated with alleviation of inflammatory vascular injuries in a model of diabetic nephropathy (Ichihara *et al.* 2004) through competitive binding to the (pro)renin receptor. In my studies using TGR(*Cyp1a1-Ren2*) animals, however, the decoy peptide caused no improvement in malignant hypertensive injuries observed in the heart. Indeed, left ventricular hypertrophy and fibrinoid necrosis of intramural right coronary arteries was observed in all animals. Prorenin decoy peptide administration did not alleviate glomerular enlargement or fibrinoid necrosis of intrarenal vessels. A small effect was, however, seen in the mesenteric vessels which showed hypertrophic remodelling but did not develop segmental fibrinoid after administration of the decoy peptide. The animals used for this pilot study originated from the TGR(*Cyp1a1-Ren2*) colony held in Edinburgh and were of mixed genetic background. Right ventricular infarcts which have not been previously observed in this model may be a consequence of the mixed strain origin and one may wonder what would be the factors involved. Very few studies have been focussing on this isolated cardiac complication and no studies have been done to correlate right ventricular infarction to genetic components.

Ichihara *et al*, (2004) applied the inhibitor to a model of diabetic rats prior to the appearance of vascular lesions. In my study, the decoy peptide was administered concurrently to transgene inducer treatment for a period of only 14 days rather than over a period starting prior to the development of vascular lesions or hypertension which may explain the lack of improvement in the phenotype. This work therefore indicates that non-proteolytic activation

of prorenin through the recently published (pro)renin receptor does not play a crucial role in malignant hypertension in the inducible transgenic rat model of high prorenin. However, it would be interesting to study the effects of the same inhibitor peptide on diabetic nephropathy animal models which already display renal vascular lesions. The role of non-proteolytic activation of prorenin by the putative (pro)renin receptor in the development of the lesions could therefore be separated from a role in the maintenance of the injuries. A larger scale study with TGR(*Cyp1a1-Ren2*) would be useful to find out whether uptake of prorenin in the heart was decreased or inhibited. If this was the case, it would provide proof of prorenin uptake through the (pro)renin receptor. This is unlikely unless prorenin and prorenin uptake has no role in the phenotype of these rats. If prorenin is still detected in the heart, it would indicate that a separate, yet unidentified, uptake pathway for unglycosylated prorenin is involved.

8.7 Foetal renin granulation – a system regulated by maternal or foetal RAS?

As part of my studies on prorenin, I investigated prorenin synthesis and maturation, and in particular renin granulogenesis during embryogenesis when prorenin may play a role in development, foetal RAS regulation and salt homeostasis. *Chapter 7* described studies of the presence of renin granules in the kidney during embryogenesis and if, so at what stages these granules are first detectable. The data indeed showed that dense core and immature renin granules are completely absent for juxtaglomerular (JG) cells and smooth muscle cells before birth. The data raises the question of whether embryonic kidneys possess active renin at all. Are embryonic kidneys able to produce active renin? It may be that the foetal RAS and prorenin carry out other functions than blood pressure regulation such as organogenesis. The data indicate that mouse foetal smooth muscle cells are not able to package and process prorenin into renin through the regulated pathway. Hence, the possibility of a lack of prorenin maturation highlights the fact that prorenin may be released solely through the constitutive during embryonic development.

Additional experiments may be useful to confirm three important aspects of this work. 1) Measurements of prorenin and renin (which were not performed due to technical restrictions in both foetal plasma and kidney samples may provide unequivocal evidence of a pivotal role of prorenin during development, either indirectly though the RAS or directly via

putative binding proteins or receptors. 2) Immunogold techniques to confirm morphological identification of renin granules may be useful to remove any bias or false positive in the analysis, This should be considered in all cases when an expert pathologist is not available for the analysis. 3) Quantitative evaluation of renin granules in samples from different treatments may be useful to compare the effects of different regimen, notably when comparing a downstream effect on foetal kidneys from treated dams. The experiments described in this thesis were, however, not set up to provide statistical meaningfulness.

If renin granules are absent in the mouse foetus, does renal prorenin which is released constitutively possess a direct role in early renal development? Prorenin is now known to be activated without changes in molecular weight through an alternative activation mechanism. In contrast, foetal kidneys from rats and sheep have been shown to possess renin dense core granules. Although my results are in contradiction with results obtained with NMRI mice (Minuth *et al.* 1981), this report did not include electron microscopy data and it is possible that other structures such as lysosomes were mistaken for renin granules. In conclusion, the mouse appears to be unique for this foetal granulation phenotype. Information about the presence of granulation in humans seems to be dogmatic and it is believed that human foetal kidneys do possess dense core renin granules. The literature, however, does not support this theory. Only one publication relating to perinatal human renin granulation reveals that the juxtaglomerular index was zero (or one) which suggests that the human JG cell is agranular. The methodology used (secretory granule staining) at times when renin JG granulation research was most active, may not provide an accurate technique and again other secretory vesicles may have been mistaken for renin granules. It may be informative to investigate human renin granules in archived foetal kidney electron microscopy samples. The mouse may provide an appropriate model to study this aspect of kidney and RAS physiology.

Renin granule production could be stimulated by maternal sodium depletion and maternal treatment with two ACE inhibitors shown to differ in their ability to cross the placental barrier. Hence, it appears that the prorenin maturation is under the control of maternal and the foetal RAS. They may act as redundant systems when homeostasis is threatened in the foetus or/and could play separate roles in blood pressure regulation and establishment of embryonic structures during normal physiological processes. In addition, the present study revealed that foetuses from mothers fed a high sodium diet had no renal renin granules pre-natally. It is possible that a high salt diet during pregnancy may suppress the foetal RAS further and compromise nephrogenesis. The effects of high salt during pregnancy on blood pressure in adulthood has been a matter of debate but it is possible that foetal programming

by high (or low) salt resets the renin-angiotensin system or the tubulo-glomerular feedback loop to a lower level and triggers an inadequate sodium handling in the adult. It is known that perinatal AngII decrease interferes with renal development and has long-term consequences in the adult in terms of blood pressure (Woods *et al.* 2001). To reinforce this concept, Ross *et al.* showed that AngII infusions in the peri-natal period triggers an increase in blood pressure in the foetus (Ross *et al.* 2005) demonstrating a crucial influence of AngII in foetal RAS regulation. The ACE inhibitor, captopril, has been shown to cross placenta in the sheep (Stevenson *et al.* 1995) and rabbit (Broughton Pipkin *et al.* 1982) while enalapril does not (Stevenson *et al.* 1995). It may not reflect what happens in the mouse but if captopril does indeed gain access to foetal circulation while enalapril does not, the results can be interpreted to show that foetal granulation is under both the maternal and foetal control since granules were detected prenatally with both drug treatment and with salt depletion.

This redundant system may represent a protective mechanisms during the perinatal period while the foetal system is preparing for life *ex-utero* but only when maternal homeostasis is threatened to preserve adequate blood pressure levels. Foetal renin may therefore not be essential for haemodynamics purposes at this developmental stage and may solely be involved in embryonic growth. Although at E18.5 the main architecture (tubular and vascular) of the kidney is complete, kidney maturation is still on-going. Since the placenta contains proteases, AngII might not cross placenta from the maternal circulation and locally produced AngII is likely to be involved in nephrogenesis.

8.8 Future work

As highlighted in the "Discussion and conclusions" sections at the end of Chapters 3, 4, 5, 6 and 7, the work presented in this thesis may benefit from the following additional experiments. These experiments were not undertaken for the present work due to time or technical restrictions.

- Localisation of the (pro)renin receptor protein in embryonic and adult tissue sections would confirm the mRNA data. In addition, an anti-mouse putative (pro)renin receptor antibody would allow the repetition of binding and immuno-precipitation studies done with human material by Nguyen *et al.* using mouse cell/tissues. It would also make possible the comparison of the putative (pro)renin receptor protein

levels by Western blotting in TGR(*Cyp1a1-Ren2*) which would have complemented the real-time PCR data. It cannot be excluded, although the (pro)renin receptor mRNA levels were not different between control and transgenic animals, that post-transcriptional up-regulation is occurring. An up- or down-regulation of the putative (pro)renin receptor would have demonstrated an involvement of this molecule in the malignant hypertension injuries observed in this model.

- It appears also essential when the Edinburgh colony is re-established to repeat, on a larger scale, the "prorenin handle region" experiment to confirm that the putative (pro)renin receptor is not involved in the malignant hypertension of TGR(*Cyp1a1-Ren2*) rats.
- A full study using the TGR(*Cyp1a1-Ren2*) animals from the colony housed in Maastricht and varying the dietary sodium levels would provide useful information in the sensitivity of this model to this physiological stimulus. Similarly, when the colony is re-established in Edinburgh (due to genotypic contamination), a comparison between re-derived animals and non-re-derived animals (for example, those housed in Greiswald) would give insight in the influence of the pathogen load in the pathophysiology of malignant hypertension. In addition, measurements of plasma and tissue prorenin levels as a routine screen when using these animals may be a powerful tool to explain phenotypic differences.
- To complete studies described in Chapter 7 on renin granulation during embryonic development, measurement of foetal plasma and foetal kidney renin/prorenin contents could be undertaken. This would confirm the electron microscopy results and ascertain prorenin as a pivotal protein in nephrogenesis independently or as part of a local RAS system. Immunogold techniques would remove bias and ascertain the identity of renin granules. The differences in transplacental crossing of the two ACE inhibitors used would also strengthen the result presented and foetal plasma and kidney analysis could be analysed by HPLC for this purpose.
- The results described throughout this thesis and the publications discussed have provided a range of evidence for the existence of additional receptors and/or molecules which may interact with prorenin. This interactions may be involved in the uptake and activation of prorenin in tissues such as the heart, notably in the rat model, TGR(*Cyp1a1-Ren2*), studied in the present body of work. One technique, termed "yeast two-hybrid" may reveal new biological "partners" or chaperones for prorenin. In this assay, two fusion proteins are created: the protein of interest, which

is constructed to have a DNA binding domain attached to its N-terminus, and its potential binding partner, which is fused to an activation domain. If both proteins have an interaction, the binding of these two will form an intact and functional transcriptional activator. This newly formed transcriptional activator will then trigger a reporter gene transcription, which is simply can be easily detected and measured. In this way, the amount of the reporter produced can be used as a measure of interaction between the protein of interest and its potential partner. Hence The yeast two-hybrid assay would be an elegant means of investigating prorenin interactions with other proteins. It is expected that the putative (pro)renin receptor and other "described prorenin binding proteins" would be identified by such procedure. It would be interesting to investigate whether M8-9 would have interactions with prorenin and whether additional targets would be obtained. Prorenin sequence from several species could be used. In addition, the advantages of the system is that, following identification of new proteins interacting with prorenin, mutations can be introduced in the sequence to highlight regions important in the interaction.

8.9 Final remark

The widespread expression of the putative (pro)renin receptor, its phylogenic conservation in lower species, its homology with M8-9 and the lack of evidence for regulation of the (pro)renin receptor in TGR(*Cyp1a1-Ren2*) animals suggests that the function of this protein may need to be re-assessed. The work reported in the present thesis indicates that non-proteolytic activation of prorenin through the recently published (pro)renin receptor does not play a crucial role in malignant hypertension in the inducible transgenic rat model of high prorenin. Further studies are needed to understand alternative physiological roles including during embryogenesis for this newly identified gene. While the role of prorenin in the pathogenesis of malignant hypertension of TGR(*Cyp1a1-Ren2*) is unquestionable, it is still unclear how prorenin gains access to the heart and whether uptake through an alternative receptor occurs. The data presented in this thesis suggest that prorenin may be the only "renin species" present in the developing mouse kidney and indicate that renin was not stored or processed through the regulated pathway as observed in the adult. Hence, prorenin may play a crucial role during foetal development which may involve the synthesis of AngII

or direct actions in embryonic homeostasis through potential receptor binding and/or non-proteolytic activation.

Chapter 9
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Chapter 10
Additional Information

10.1 ANNEX 1: PCR Primers

Reverse Transcriptase PCR primers

Name	Sequence	Target
Mouse renin receptor (forward) JJM 515	GGAAGATCTGGCACCATGGCTGTGCT (underlined = BglII site)	Coding region and whole cDNA of mouse renin receptor
Mouse renin receptor (reverse 1) JJM 516	GAATTCCTCAACTTGTCAACACTATA AATCACTCT (underlined = EcoRI site)	Coding region of mouse renin receptor
Mouse renin receptor (reverse 2) JJM 517	GAATTCTTCATGTGCAAATGGACCAA TATC (underlined = EcoRI site)	Whole cDNA of mouse renin receptor
Mouse RR sequencing (forward primer 1) JJM 518	TGGCTCCCAGCGAGGAGA	Mouse renin receptor whole cDNA
Mouse RR sequencing (reverse primer 1) JJM 519	ATGTGACTGAAAGGTCTT	Mouse renin receptor whole cDNA
Mouse RR sequencing (forward primer 2) JJM 520	CAAATCATTCGACACATC	Mouse renin receptor whole cDNA
Mouse RR sequencing (reverse primer 2) JJM 521	TTTGGGTGTTCTCTTGTT	Mouse renin receptor whole cDNA
Mouse RR sequencing (forward primer 3) JJM 522	AGAGTGATTTATAGTGTT	Mouse renin receptor whole cDNA
Mouse RR sequencing (reverse primer 3) JJM 523	ACGGATCACCAAACTTGC	Mouse renin receptor whole cDNA
Mouse RR sequencing (forward primer 4) JJM 524	TGTATATGATTAGCAAGC	Mouse renin receptor whole cDNA
Rat renin receptor (forward) JJM 938	ATGGCTGTGCTTGTCGTTCT	Rat renin receptor coding region
Rat renin receptor (reverse) JJM 939	CTCTACCACTGCATTCCCAC	Rat renin receptor coding region
Rat renin receptor (Real time – forward) JJM 954	GGGCTTCTCTGTGAAGGAAGAC	Rat renin receptor cDNA
Rat renin receptor (Real time – reverse) JJM 955	GGGCCGGTGGAATAGGTT	Rat renin receptor cDNA

Real-time Quantitative PCR primers

Name	Sequence	Target
Rat renin receptor (Real time – forward) JJM 954	GGGCTTCTCTGTGAAGGAAGAC	Rat renin receptor cDNA
Rat renin receptor (Real time – reverse) JJM 955	GGGCCGGTGGAATAGGTT	Rat renin receptor cDNA

Microsatellite marker primers

Name	Forward	Reverse
D1Mgh2	ACCTGGATGGGCCTCTGT	TGCATCAATAAAACCCTAACTGG
MT1PA	TGTAATGGAATCTGATGCCC	GGGCTCTATAGATAGGAGGTTTTAT
D1Rat51	CCCTCAGTTCAAGAGTAACCTCA	TTGCCTGAGAGACTGTGCC
D2Mgh14	ACCGCCCCAGGGAATTAT	GCCCTCGATGTGGACTTTTA
D2Mit6	TGTCAAAGGCAGGAATCAAC	ACCCCTTTTGAGATAGCGCT
D2Rat83	TCTGCTTGTCCATGTGTGTG	GGTCATAGTTGCATACCTACATCC
D3Mit10	CATACACAGGCAGGGCTTCT	CCTGAAACTGAGCGTGAACA
D4Mit2	TTCTGTATTAACCACAGAAAGAAGC	AAGCCAGCCCAAAGTAAATG
D4Mit17	GATGTTGGTAAGGTATATCTGGGG	AAGACAAGAGAGACCTTGCTTCA
D5Mgh13	TCACATGTGGACAGAAAAAAGG	ATCCATGACAAGAAAGACTTCATG
D5Mit4	CCAGCTCATGTGCACAGG	GTTGTTGATGTTGTTGTTGTTGG
D5Mit13	CTTTGAGAGAAGTAAATTGTTGAGG	TGGTTAGTGTTAATCCATTGTCA
D6Rat24	CAGCAGGAGTCATACTCACCC	GTCCTTCTTCCTCTCCACCC
D7Mit4	CTCCAAACTTTCCAGGGGAT	GATATTGAAAACAACCTACCTTGC
D7Rat24	CATAATCAGAGCAAGCCAAGG	TGTGTCAGAGAAAAATGGCAG
D8Rat16	TTTTAAATTGCTGTTTGATTGGTT	TCTGCACTTTAACAAGGTGTGA
D8Rat77	ACCTGTGCGTAATCACACCT	TGCAGATTATTGAGGTAGCATTG
D9Mit2	TGACCAGTTAGCCCTTTCCA	GGGAGCAGGGTTCTACACAG
D9Rat81	ACATACGTGTGTGTGCTGCA	TGTGTCTTAAAGGGGTTTCTTTAAA
D10Mit9	CTCTTTGGGATGAACCGGTA	AATGGGAAGCAACAGCATTC
PPY	ATCAACACACTGACCAGGCC	CGTCGTTCACAGGTGTCAGC
D10Rat142	CTCCCACGGTCTATGGTCTA	AAAAAGGGCCAGCAAGATG
D10Rat32	GGATGAACCTTTCCTGGGTC	CTCTGACTTCAACACATGAGGTG
735-736	GCTCCTTTCTCTCGGGTTCT	CAGAGTGAGGGCTTCACAGA
D11Mgh4	TGTTCTCAGGTGGGACTGAA	TGTGAGCACAGGAACACACA
D11Rat52	CCCCGACACATACATACATACA	CTAAGACACAGGCTTTGGGC
D12Mit4	TGTGTGAGACTGTATGCATGTG	GGCTCCAGAGGAATGACATC
D12Rat59	CAGCAATTTTCAGACCTTTCC	TGGGGTCCTGGTCTTTGATA
D13Mit1	TCATTCAACATTCTGTCAATCG	CACAACAAGGTTAACCTCTAGACA
D13Uwm1	AAGAAAACACTGTCCTCCACTG	GGCTATGGGTAGTATGATTAGAAGA
D14Mgh2	CTTTAACCTGCCCTCAGTTCC	ATTTAGCACAGCGCAATATGG
D14Mit2	TGCTCCTCTTGGCTTAGGTG	CGCAAGGCTAAATGTCTGGT
D15Mgh2	TTCAGATTAGTATAGGCAGGGTCC	GAGACCCTCAACCTGTGCAT
D15Rat71	CCTCTGCGGTCACTCACATA	GCCATGGAGCAGATCTGTTT

Name	Forward	Reverse
D15Rat123	CCCCTACATCCATGGCATAC	TTCCCCCTCTTGTTCTTCCT
D16Rat53	TTCAGTGCTACTGGACTGCC	TGCCTTTCCATCTCCCATTA
D16Rat90	GGAGTCAAGGCAGGAAAACA	AAGGTGGAAGGAGAGAACCA
D17Mit4	GATTGAGACCCTCCCTCAGA	GTTTGGTATGCTCTTTGGGC
D17Rat85	TTGCAGGTGAGTTCTCAGACA	CCCTAGGTTTAATCTCCTGTACCA
D17Rat89	TGAGGTTTTCATCACACAAAGG	TCCTTGAAGACGTTCAGGGT
D18Mgh2	GTGTTGAAGTGGGGACGC	ACCTCAAGTGTCTCCTCTGCT
D18Mit1	GCGGTACAGAAAGAAAGAGAGA	AGAGTGTTGGCCATAAAAGACA
D18Mit3	AGATGAACACATAAGATTGACAT CG	AAAAGAGTCACATGAGGACTGG
D19Rat30	GCCAGGCTCTACCTTCCTTC	CACACGAACTCTCTCATACACTCA
TAT	GTACCTTATGGTAAGCGTGCG	CACCCATGACGATCCTGAC
D20Mgh1	CTCTCCCTTCAGTCCCATTG	TCCTAGAGCCCCTTTTCACA
D20Rat21	CCCCCTTAAACTACATGACACTG	GGGGCCAAGAATTCTGAACT
DXRat15	TCAGTTGGGGTATCCAGAGG	ATTCGTGGACCTTCTGTTGG
DXRat31	GCCTTCTTTCCAGGGAGAAT	GAGGCTACTGGAGGTCCACA

10.2 ANNEX 2 : CLUSTAL Classification of amino-acid substitutions

The conservation line output in the CLUSTAL format alignment file is made up of three commonly used characters.

'*' indicates positions which have a single, fully conserved residue

':' indicates that one of the following 'strong' groups is fully conserved:-

STA
NEQK
NHQK
NDEQ
QHRK
MILV
MILF
HY
FYW

'.' indicates that one of the following 'weaker' groups is fully conserved:-

CSA
ATV
SAG
STNK
STPA
SGND
SNDEQK
NDEQHK
NEQHRK
FVLIM
HFY

These are all the positively scoring groups that occur in the Gonnet Pam250 matrix. The strong and weak groups are defined as strong score >0.5 and weak score =<0.5 respectively.

10.3 ANNEX 3: Certificate of analysis of decoy peptide

SIGMA
GENOSYS

Customer Number:	101721
Peptide #:	17857-1
Lot #:	L9849-006
Sequence:	RILLKKMPSV
Length:	10
Theoretical Mass:	1,184.57
Observed Mass:	1,184
Amount Shipped:	32.3 Mgs
Final Purity (%):	96
Peptide Content %:	
Appearance:	Lyophilized Powder
Solubility:	1mg/ml in acidic solution pH<7.5 (basic peptide)
Conjugation:	
Peptide Name:	RILL
Terminal Modifications	N-Term:[H] C-Term:[OH]
Chemist's Notes:	

Column:	Discovery Bio Wide Pore C-18, 250mmX4.6mm, 5µm
Mobile Phase:	A=0.1% TFA/Water
	B=0.1% TFA/Acetronitrile
Gradient:	0 to 2 min.: 100% A
	2 to 20 min.: 0 to 67.5% B
Detection:	214nm
Storage Conditions:	-20 C for Long Term
	4 C for Short Term

Released By: D. Thumes **Date Released:** 10/19/05

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Sigma-Genosys
1442 Lake Front Circle
The Woodlands, Texas 77380-3600
800 234 5362

Sigma-Genosys Ltd.
Homefield Road Business Park
Homefield Road, Haverhill CB9 8QP, UK
44 0 1440 767000

Sigma-Genosys Japan K.K.
777-13 Nishi-I, Shin-Ko, Ishikari
Hokkaido, Japan
81 133 73 5005

Sigma-Aldrich Pty Ltd.
Unit 2, 14 Anella Avenue
Castle Hill NSW 2154
Australia
800 800 097

Cashmere Scientific Company
No. 2-1, 2F, Lane 40
Chang-An W. Road
Taipei, Taiwan 104
886-2-25416188

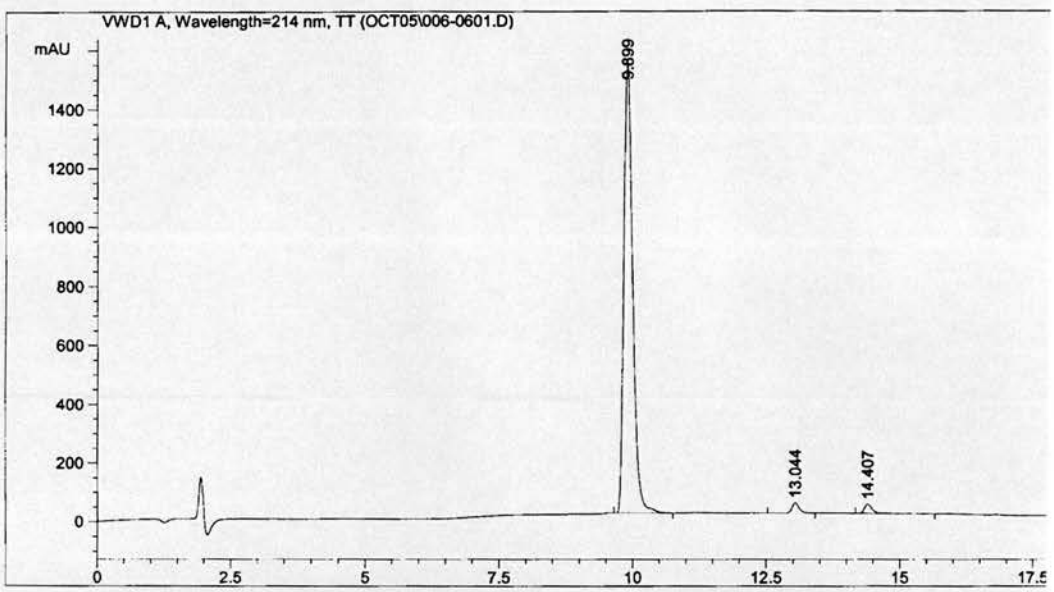
Sigma-Aldrich Korea
Samhan Camus Annex, 10th Floor
17-26 Yoido-dong, Yungdeungpo-ku
Seoul, Korea
82 2 783 5211

Sigma-Aldrich Canada Ltd.
2149 Winston Park Drive
Oakville, Ontario L6H 6J8
CANADA 905 829 9500

Sigma-Aldrich Pte. Ltd.
102E Pasir Panjong Road
#08-01 Citilink Warehouse
118529, Republic of Singapo
65 271 1089

Data File C:\CHEM32\1\DATA\ANALYTICAL\OCT05\006-0601.D
Sample Name: 17857-1-1fqc16-24

```
Acq. Operator   : AD                                  Seq. Line :   6
Acq. Instrument : Latasha                             Location : Vial 6
Injection Date  : 10/19/2005 7:32:16 AM                   Inj :   1
                                                    Inj Volume : 10 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 5 µl
Method          : C:\CHEM32\1\METHODS\ANALYTICAL_LC.M
Last changed    : 10/4/2005 2:42:49 PM by ad
Method Info     : LC runs for peptide analysis
```



Area Percent Report

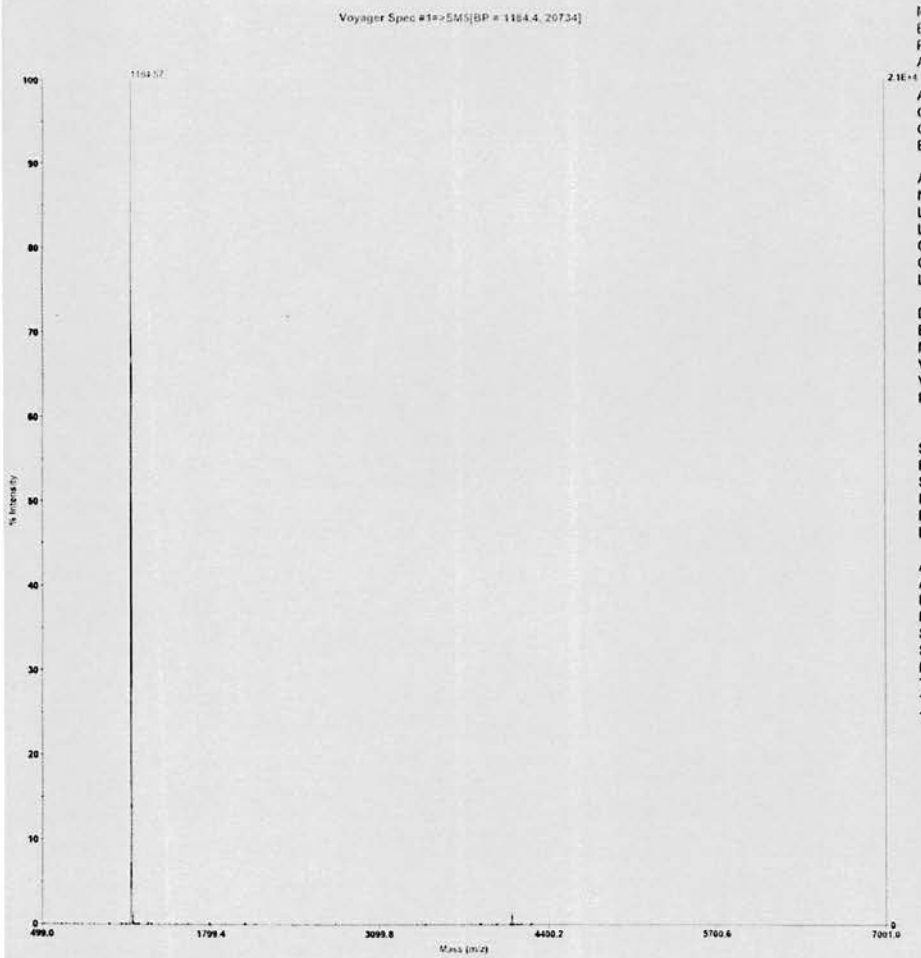
```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: VWD1 A, Wavelength=214 nm, TT

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	9.899	BB	0.1831	1.78489e4	1525.02637	96.5721
2	13.044	BB	0.1390	310.64813	34.81441	1.6808
3	14.407	BB	0.1572	322.90814	31.56318	1.7471
Totals :				1.84825e4	1591.40396	

*** End of Report ***

SIGMA



Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	94.5%
Guide wire 0:	0.04%
Extraction delay time:	400 nsec
Acquisition mass range:	500 -- 7000 Da
Number of laser shots:	60/spectrum
Laser intensity:	2189
Laser Rep Rate:	3.0 Hz
Calibration type:	External -- D:\Voyager\Calibrate\SEP05-LO.cal
Calibration matrix:	a-Cyano-4-hydroxycinnamic acid
Low mass gate:	Off
Digitizer start time:	14.664
Bin size:	2 nsec
Number of data points:	19886
Vertical scale:	200 mV
Vertical offset:	-1%
Input bandwidth:	150 MHz
Sample well:	15
Plate ID:	100 WELL PLATE
Serial number:	1105
Instrument name:	Voyager-DE
Plate type filename:	C:\VOYAGER\100 well plate.plt
Lab name:	PE Biosystems
Absolute x-position:	21244.9
Absolute y-position:	42109.4
Relative x-position:	-662.612
Relative y-posititon:	-118.062
Shots in spectrum:	3
Source pressure:	3.712e-007
Mirror pressure:	0
TC2 pressure:	0.005516
TIS gate width:	30
TIS flight length:	940

Acquired: 01:44:00, October 19, 2005
Sample Description: 17857-1-1FQC16-24
D:\Voyager\Data\Peptides\OCT05\19OCT05_0013.dat

Printed: 01:44, October 19, 2005