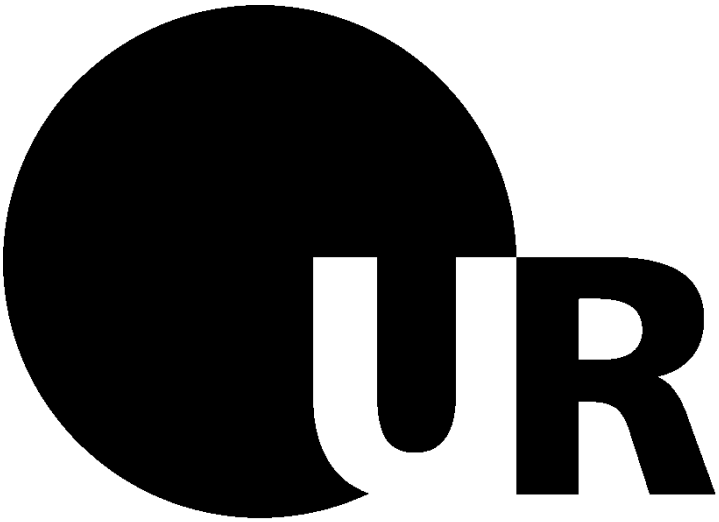
The endothelin-A and endothelin-B receptor as potential factors to control synthesis and secretion of renin



DISSERTATION

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„Bones can break, muscles atrophy, glands can loaf, even the brain can go to sleep, without immediately endangering our survival, but when the kidneys fail to manufacture the proper kind of blood neither bone, muscle, gland or brain can carry on."

Homer W. Smith, From Fish to Philosopher

To my grandfather Edmund

Contents

Abstract

The secretion and synthesis of renin is regulated by several systemic and local factors. Besides salt balance, extracellular volume and blood pressure, different hormones such as ANG II, arginine vasopressin and norepinephrine were examined as renin mediators. Moreover, endothelins have been hypothesized as negative regulators of renin secretion and synthesis *in vitro* whereas they have been suggested to primarily inhibit the release of renin through a high cytosolic Ca^{2+} concentration in JG cells. The endothelin system consists of three peptide hormones Endothelin-1, Endothelin-2 and Endothelin-3 and their G-protein coupled receptors ETA- and ETB-receptor. ET-1 has been determined as a strong vasoconstrictor in the renal vasculature mainly mediated by ETAR, whereas ETB-receptor, at least initially, rather promotes vasodilatation.

Present thesis aimed to investigate either, if endothelins bind on the ETA- and/or ETB-receptors following a Ca^{2+}-dependent direct inhibiting effect on renin cells or if different systemic factors, e.g. extracellular volume, are affected by endothelins resulting in the modulation of the renin system *in vivo*. In order to characterize the role of endothelins on the renin system further, each single ET-receptor could be addressed as general markers for renin producing cells. Therefore experiments were performed with renin cell-specific ETAR or ETBR isoform knockout animal model ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$, $Ren^{1d+/Cre}$-$ETBR^{fl/fl}$) and a renin cell-specific ETAR and ETBR knockout animal model ($Ren^{1d+/Cre}$-$ETBR^{fl/fl}$-$ETAR^{fl/fl}$) to assess a potential direct effect *in vivo*. Afterwards, possible indirect effects of ET-receptors based on vascular actions of endothelins were examined. Therefore several mouse models were generated additionally: ETAR and/or ETBR knockout model to study systemic effects (SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$,SMMHC-Cre-ER^{T2}-$ETBR^{fl/fl}$); stroma derived cell-specific effects ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$, $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$, $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$) and general effects *in vivo* (CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$).

All mentioned Cre^{+}-animals, revealed normal developed renin producing cells, renin mRNA abundance, plasma renin concentration levels, unchanged systolic blood pressure indicating that each ET receptor isoform and the combination of both ET receptors located on mentioned cells are not of major relevance for renin synthesis and secretion. In addition, all Cre^{+} animals, except of $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ displayed via the isolated perfused kidney model an inhibition of renin secretion rate through ET-1 in increasing concentrations mainly mediated by a decline of the renal blood flow as in controls. Conversely all $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals showed no decline of renin secretion rate and an abolished decline of the renal blood flow indicating that these results appeared due to absence of systemic effects in this ex vivo situation.

However, present findings indicate for the very first time that ETA-and/or ETB-receptor isoform located on the renin cell lineage and on stroma derived cells seem to be under normal conditions of less relevance for the renin synthesis and secretion *in vivo* and *in vitro* whereas not yet addressed tubular effects or possible systemic effects are more responsible for inhibition of the renin system through endothelins in order to equilibrate general homeostasis. In addition, the role of ET-receptors located on renin producing cells in terms of renal pathophysiology e.g. renal fibrosis or diabetic nephropathy need to be evaluated prospectively further.

1 Introduction

1.1 Renin-Angiotensin-Aldosterone System (RAAS) in the kidney

The Renin-Angiotensin-Aldosterone system (RAAS) is a key regulator in homeostatic processes such as arterial pressure and fluid volume control in the human body.

Since the discovery of the protease renin over 100 years ago *(Tigerstedt and Bergman, 1898),* RAAS became an important target of many studies. Drugs that modulate different parts of this system were discovered as pharmacological tools in order to combat high blood pressure, myocardial infarctions and diabetic nephropathy (Cagnoni *et al.*, 2010). Renin is a proteolytic enzyme which is mainly synthesized in the kidney. It has the ability to catalyze the hydrolysis of Angiotensinogen, a glycoprotein which is secreted from the liver into the blood, to Angiotensin I (ANG I) and is therefore initiator of a complex cascade of enzymes (Hackenthal *et al.*, 1990; Hall, 2003; Persson *et al.*, 2004) (see Fig.1).

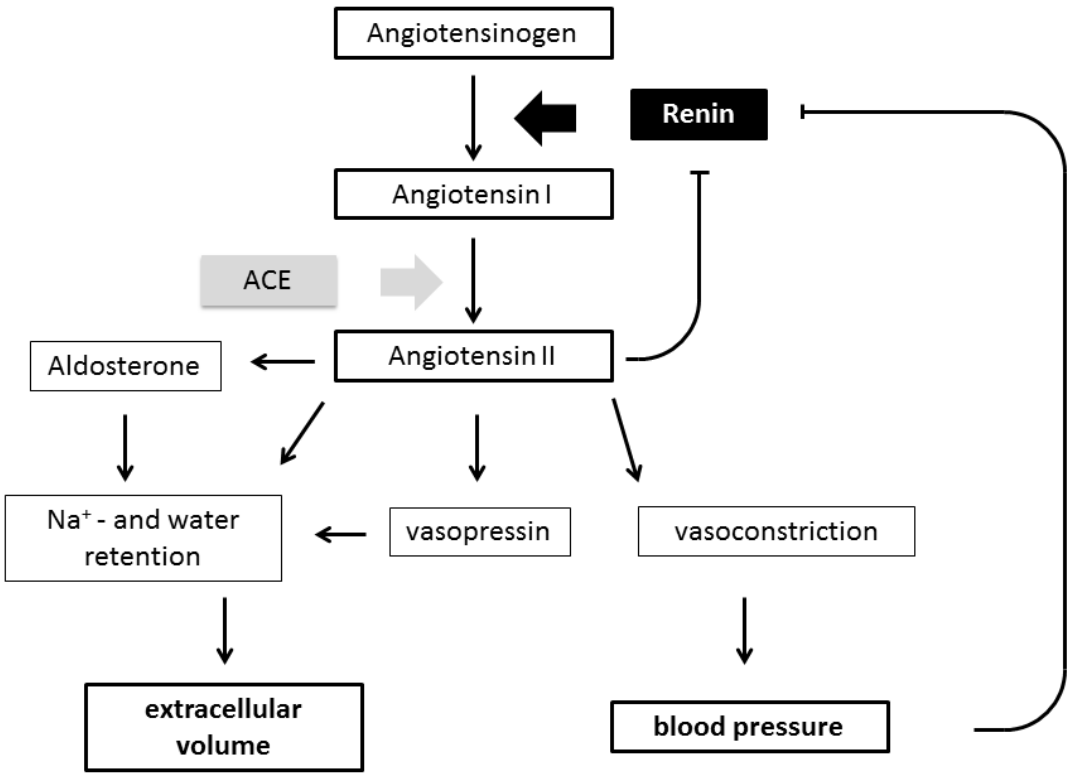

Fig. 1: Schematic representation of Renin-Angiotensin-Aldosterone system (explanation see text)

The Angiotensin-converting enzyme (ACE) is a metalloproteinase, which derives from the lung epithelium and transforms the biological inactive decapeptide ANG I into an octapeptide termed as Angiotensin II (ANG II). This biological active hormone is the main mediator of the system and acts via binding to angiotensin receptors which are located on cells of the cardiovascular, endothelial and endocrine system. ANG II acts as a vasoconstrictor and causes an increase in vascular resistance and by association rise of systemic blood pressure. In addition, ANG II stimulates the release of vasopressin in the posterior pituitary of the hypothalamus in order to increase water reabsorption. Furthermore, it mediates the intrarenal sodium and water reabsorption in the tubules via angiotensin receptors and by increased aldosterone synthesis in the adrenal gland (Hall, 2003; Quinn and Williams, 1988; Hackenthal *et al.*, 1990).

In humans, the activity of RAAS depends on plasma renin concentration (PRC) because the hydrolysis of ANG I into ANG II is limited by renin whereas Angiotensinogen and ACE are secreted in abundance under healthy conditions. Therefore, synthesis and secretion rate of renin are key targets of several negative feedback loops to regulate RAAS. It has been shown that increased systemic blood and renal perfusion pressure, increased tubular sodium concentration and also increasing amount of ANG II concentration suppresses synthesis and secretion of renin (Hackenthal *et al.*, 1990; Kurtz *et al.*, 1986b; Kurtz and Wagner, 1999; Vander and Geelhoed, 1965).

1.2 Synthesis and secretion of renin in the juxtaglomerular epitheloid cell apparatus

In the adult kidney, renin is synthesized in cuboid structured-like cells which are localized in the media layer of afferent arterioles close to the vascular pole of glomeruli (Ganten *et al.*, 1976; Hackenthal *et al.*, 1990; Taugner *et al.*, 1979; Sequeira-Lopez *et al.*, 2015). Due to their almost exclusive localization cells are termed as juxtaglomerular (JG) cells (see Fig. 2). In humans, unlike in mice, renin is encoded by one single gene. Rodents which are used for scientific research can be divided into two groups. Mouse lines which possess one renin gene (Ren-1c) (i.e. C57/Bl6, BALB/C) and mouse lines (129 SV, Swiss) which possess two genes (Ren-1d and Ren-2). It is assumed that Ren-2 is established through gene duplication out of Ren-1 (Abel and Gross, 1990; Dickinson *et al.*, 1984). All renin genes are encoded for proteins which are almost homologue on the level of amino acids but differ in glycolization (Sigmund and Gross, 1991). Ren-1-proteins can be glycolized to three asparagine residues whereas Ren-2 proteins are not known to possess binding sites for glycolization.

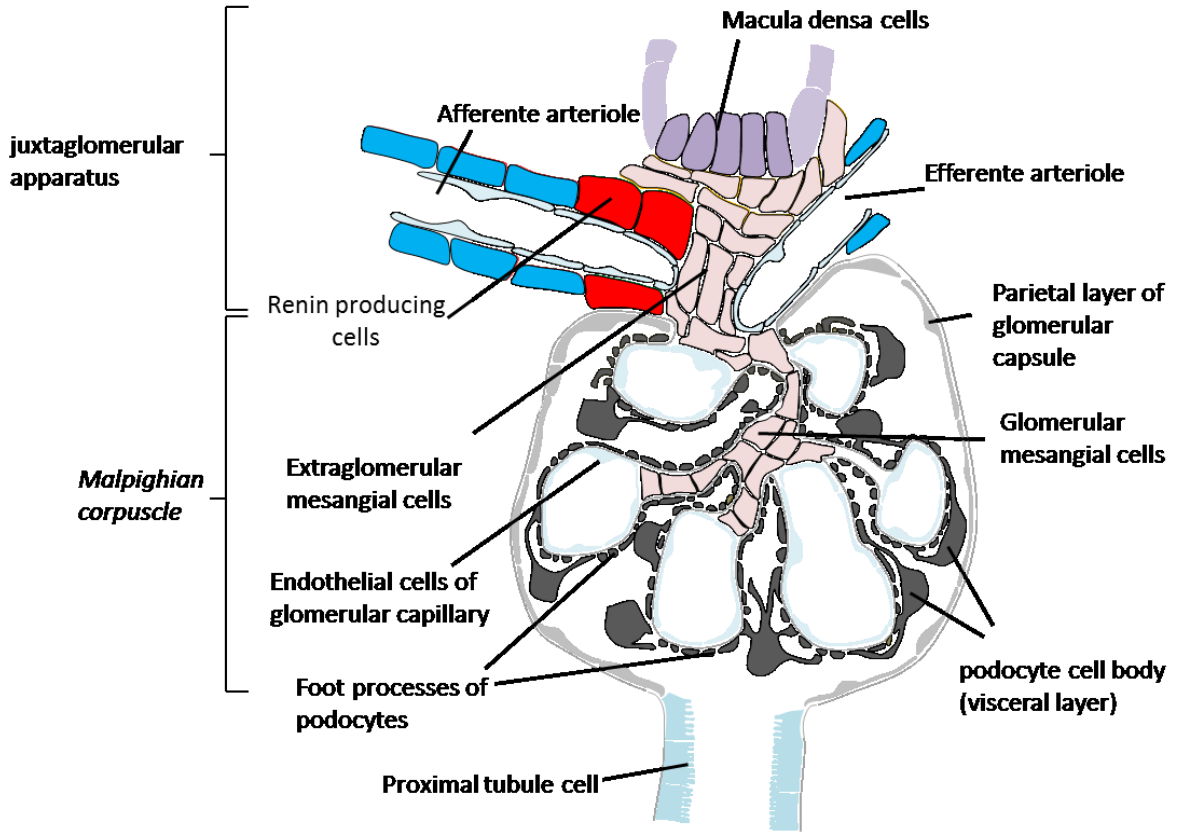

Fig. 2: Schematic representation of glomerulus with detailed description of juxtaglomerular apparatus (explanation see text) modified from Dr.Karger

Renin is first synthesized as preprorenin with a molecular mass of 48 kDa. After transportation into the *endoplasmatic reticulum* (ER), a pre-sequence is catalyzed and given prorenin passes in the *Golgi apparatus.* There, it can enter one of two further processing pathways. Prorenin either can be glycolized via mannose-6-phosphate (M6P) residues, transported into tight, lysosomal-like dense core vesicles (DCV) and stored for regulated exocytosis or prorenin is not glycolized and secreted constitutively into *clear* vesicles. Glycolization is therefore prerequisite for regulated exocytosis (Morris, 1992; Mullins *et al.*, 2000). Therefore Ren-2 is exclusively secreted constitutively because it is free from binding sites for glycolization and has no function for regulated exocytosis. Only Ren-1 is stored in DCV (Sharp *et al.*, 1996; Clark *et al.*, 1997). In those vesicles the 43 amino acid long N-terminal pro-segment of the protein is catalyzed by a proteolytic mechanism which is mainly unknown. Besides prorenin, several proteases such as cathepsin B (Neves *et al.*, 1996), proprotein convertase PC5 (Mercure *et al.*, 1996) and kallikrein (Kikkawa *et al.*, 1998) are meant to be important mediators for processing of this pathway. The pH-value in vesicles (4-6) is preferential for proteolysis. Active renin is stored in DCV until controlled release.

1.3 Regulation of renin synthesis and secretion

1.3.1 Regulation through local and systemic factors

As mentioned above, the release of active renin leads to high amount of ANG II, which is determining for regulation of blood pressure and sodium homeostasis. Therefore, the release of renin from JG cells itself is regulated by various local and systemic factors.

1.3.1.1 Systemic blood pressure

The concept of an intrarenal, pressure dependent mechanism which regulates the release of renin, was first postulated by Skinner *et al.* (SKINNER *et al.*, 1964). An increase of arterial blood pressure leads to an inhibition of synthesis and secretion of renin via a negative feedback-loop. Whereas a decrease of blood pressure results into a stimulation of renin release (Wagner and Kurtz, 1998a). This effect has also been shown in isolated perfused kidneys (Scholz and Kurtz, 1993) and its most presumably a renal baroreceptor involved in this mechanism. This physiological key regulator is assumed to be localized in the renin producing cells themselves but the identification of this receptor is mostly unknown (Gomez and Sequeira Lopez, 2009). Isolation of JG cells causes a pressure dependent regulation of renin secretion. In order to stimulate an increased blood pressure, cells were mechanically stretched. This caused a reduced renin secretion (Ryan *et al.*, 2000; Carey *et al.*, 1997). The pathway itself is mostly unknown but it is hypothesized that a stretched cell membrane lead to a higher amount of calcium influx in JG cells through stretch-dependent calcium channels and activation of phospholipase C (Ryan *et al.*, 2000). The absence of extracellular calcium prevents pressure dependent inhibition of renin secretion (Ichihara *et al.*, 1999). The postulation of a calcium dependency in the baroreceptor mechanism was confirmed in isolated perfused mice and rat kidneys (Scholz *et al.*, 1994a; Wagner *et al.*, 2007).

1.3.1.2 Salt balance

The NaCl-balance of the human body and the salt intake which comes with daily nutrition are well-known regulators of RAAS activity and by association of renin synthesis and secretion. High concentration of oral salt intake mediates an inhibition of renin secretion and *vice versa* (Wagner and Kurtz, 1998b). Vander and Miller described present chloride concentration in the distal tubular structures as an important link in this mechanism (Vander and MILLER, 1964). In addition, a cluster of specialized endothelial cells of the distal tubulus close to renin producing cells termed as macula densa, displays a central function in the regulation of renin activity. The macula densa is known to be the sensor of chloride concentration in the distal tubulus and therefore leads a current low ion concentration to an increase in renin secretion and *vice versa* (Skott and Briggs, 1987; Schnermann, 1998). Furthermore, specific chloride concentration modulates vascular resistance. More precisely, a high concentration of chloride concentration results in a vasoconstriction of the afferent arteriole and *vice versa*.

This effect is also termed as the tubuloglomerular feedback (Castrop, 2007; Schnermann and Levine, 2003). This feedback prevents loss of salt and contributes to sodium homoeostasis in the body. Yet, the precise mechanism remains unknown but there were findings which detects the apical based transporter Na^+-K^+-$2Cl^-$ (NKCC2) to be the sensor for tubular Cl^--ion concentration (Lorenz *et al.*, 1991; Bell and Lapointe, 1997). The macula densa cells release ATP when a high Cl^--concentration is present and can therefore modulate renin secretion. In contrast, a low ion concentration leads to an increase release of prostaglandin E2 (PGE_2) via COX-2 and nitric oxide (NO) via neuronal NO-synthases (nNOS) (Bell *et al.*, 2003; Kurtz and Wagner, 1998; Peti-Peterdi *et al.*, 2003; Yang *et al.*, 2000). PGE_2 and NO are also known to stimulate renin secretion for a short-term basis but recent studies identifies, both not essential for salt-dependent regulation of renin secretion (Sallstrom *et al.*, 2008; Kim *et al.*, 2007; Castrop *et al.*, 2004; Hoecherl *et al.*, 2002). In conclusion, the macula densa mechanism as mentioned above is important for detecting spontaneous alterations of tubular Cl^- concentration associated by modulation of renin secretion. However it still remains unknown if the macula densa cells or which factors do play a key role in the long-term mechanism of salt-dependent regulation of renin secretion.

1.3.1.3 Sympathetic nervous system

The tubular system, the renal vascular system and the juxtaglomerular apparatus are innervated by the sympathetic nervous system (Hackenthal *et al.*, 1990). Boivin et al. could localize the β1 adrenoreceptor on JG cells (Boivin *et al.*, 2001), which leads to an increase in cAMP production in JG cells when activated through renal nervous system. A higher amount of cAMP production is known to stimulate renin secretion (DiBona and Kopp, 1997). In addition, a vasoconstrictive effect of afferent arterioles via activation of α adrenoreceptors triggers secretion of renin (Ehmke *et al.*, 1989; Kirchheim *et al.*, 1985). But sympathetic input of local nerve endings is not essential for modulation of renin secretion while reacting on various stimuli. Renin secretion was altered even at complete renal denervation as well as at pharmacological inhibition of β adrenoreceptors (Golin *et al.*, 2001; Holmer *et al.*, 1994; Holmer *et al.*, 1993). Therefore, β1 adrenoreceptors seem to be essential for providing renin synthesis rate, which allows an availability of ready-to-use renin vesicles. This high amount of renin vesicles enables the reaction of external stimuli.

1.3.1.4 Angiotensin II

Angiotensin II is the effector hormone in the RAAS and mediates its physiological function mainly via angiotensin type-1 receptors (AT1 receptors). When circulating in the bloodstream, ANG II inhibits renin synthesis and secretion via negative feedback loop (Hackenthal *et al.*, 1990; Muller *et al.*, 2002). Experiments display an increase in renin synthesis and rate, after inhibiting or blocking AT1 receptor (Castrop *et al.*, 2003; Hoecherl *et al.*, 2001) whereas exogenic infusion of ANG II in rats and mice lowers them (Schunkert *et*

al., 1992). The effect of ANG II on the renin secretion is independent of renal perfusion pressure or salt concentration in tubular cells leading to hypothesis of a direct effect on the level of renin producing cells. In addition, the expression of AT1 receptors on renin producing cells supports this theory (Harrison-Bernard *et al.*, 1997; Kimura *et al.*, 1997). ANG II mediates an increase of intracellular concentration of calcium which inhibits the release of renin. However, recent experiments could negate the theory of a direct effect. A renin cell-specific AT1a receptor animal model was generated where no difference either in renin mRNA abundance or in plasma renin concentration could be obtained compared to control littermates (unpublished data).

In addition, ANG II is a vasoconstrictor and inhibits renin secretion indirectly through enhanced blood pressure where it promotes aldosterone and adrenaline release in the adrenal gland and triggers sensation of thirst via stimulation of AT1 receptors in the hypothalamus (Hackenthal *et al.*, 1990; Crowley *et al.*, 2005).

1.3.1.5 Controlling of renin release on cellular level

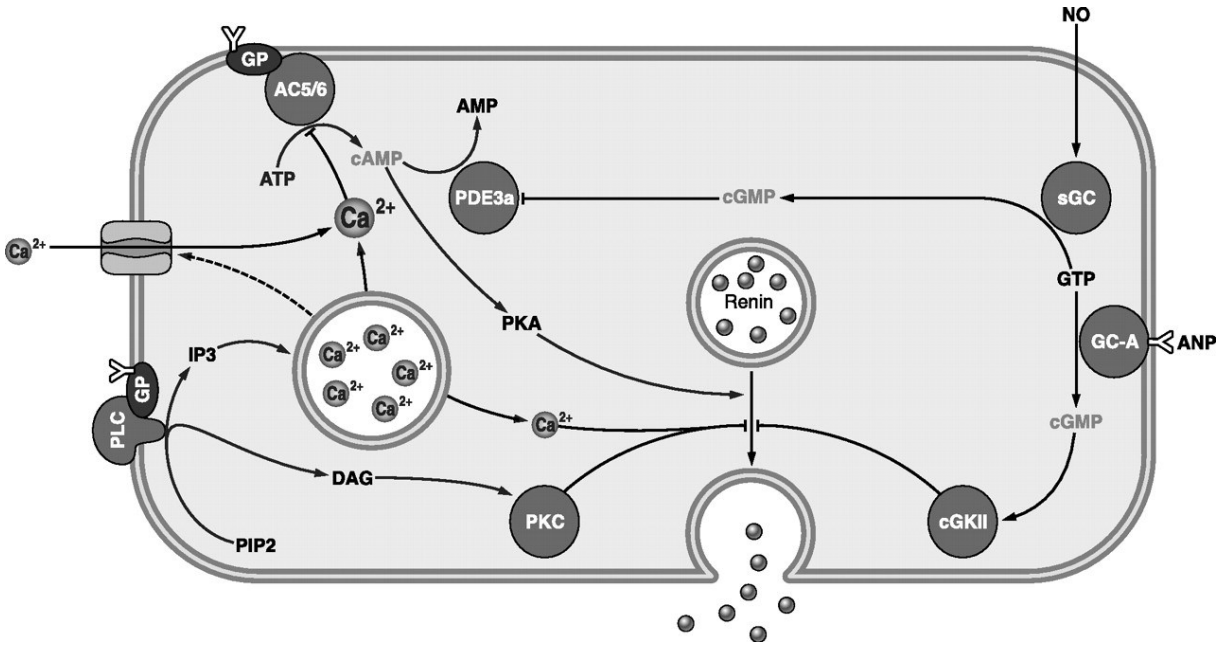

Fig. 3: Intracellular signaling pathways controlling renin exocytosis (Castrop *et al.*, 2010)
(explanation see text)

The systemic and local factors mentioned above which modulate renin secretion and synthesis all result in three main intracellular second messenger systems with following signaling molecules: cyclic nucleotides cAMP and cGMP and the intracellular free calcium concentration (Schweda and Kurtz, 2004; Castrop *et al.*, 2010; Grünberger *et al.*, 2006).

Due to a variety of indirect and direct evidences, cAMP is postulated as the central intracellular stimulator of renin release. An increase in cAMP stimulates protein kinase A (PKA) which induces renin exocytosis. All mentioned extracellular stimulating factors mediate

their effect by stimulating intracellular cAMP concentration. The amount of cAMP concentration is triggered by either receptor induced activation of adenylyl cyclase or inhibition of cAMP phosphodiesterase (PDE).

Pharmacological stimulation of β adrenoreceptors stimulates cAMP concentration via activation of adenylyl cyclase which in turn increases renin secretion (Keeton and Campbell, 1980; Vandongen *et al.*, 1973; Weinberger *et al.*, 1975). In addition, the direct activation of adenylyl cyclase activity by forskolin, a labdane diterpene, increases cAMP levels and renin release in JG cells (Castrop, 2007; Kurtz *et al.*, 1984; Grünberger *et al.*, 2006). For cAMP production in renin juxtaglomerular cells one of the main adenylyl cyclases isoforms are AC-5 and AC-6 (Grünberger *et al.*, 2006; Ortiz-Capisano *et al.*, 2007b, 2007c).

As mentioned above, intracellular cAMP levels are also determined by cAMP hydrolysis to 5'-AMP through the activity of cAMP PDEs. A nonselective inhibition of PDE activity using 3-isobutyl-1-methylxanthine (IBMX) stimulates renin release. Studies with selective blockers of PDE-1,-3 and -4 isoforms displayed similar results (Chiu *et al.*, 1996; Chiu and Reid, 1996; Chiu *et al.*, 1999; Castrop, 2007) (see Fig.3).

Although, a variety of studies could indicate that cAMP is the main stimulator of renin release, the complete mechanisms how cAMP mediate renin exocytosis remains unknown. The release of renin by cAMP involves a PKA-dependent step, but phosphorylation targets of PKA in JG cells are still unknown (Friis *et al.*, 2002; Castrop, 2007; Kurtz *et al.*, 1998b).

In contrary to cAMP, the effects of cGMP are able to either stimulate or inhibit renin secretion in JG cells. Low cGMP concentration in those cells seems to stimulate renin secretion whereas high concentration rather mediates an inhibiting effect. Whereas stimulating and inhibiting cGMP signaling pathways seem to be localized in different parts of the cell.

It has been shown that decreased concentration of cGMP mediates an inhibiting effect on PDE-3 synthesis which leads to an increased cAMP concentration and a strong renin exocytosis (Kurtz *et al.*, 1998b; Kurtz and Wagner, 1998; Beierwaltes, 2006). This so called cAMP-cGMP interaction was confirmed in both patch-clamp experiments on single JG cells and *in vivo* (Friis *et al.*, 2002; Beierwaltes, 2006). Additionally, cGMP-inhibited PDE-3 plays a mediator role in the stimulation of renin release by nitric oxide (NO). The activated soluble guanylate cyclase (sGC) which is activated by NO as well as PDE-3 is localized in the cytosol. Therefore, cGMP produced by sGC inhibits PDE-3 which could explain the stimulating effect of NO on renin secretion (Kurtz *et al.*, 1988; Kurtz *et al.*, 1998a). In contrast to sGC, the membrane-bound particulate guanylate cyclase (pGC) is located next to vesicles which are associated with cGMP-dependent proteinkinase II (cGKII) and it has been shown to be activated by atrial natriuretic peptide (ANP). ANP is known to inhibit renin exocytosis,

which could be explained by produced cGMP from pGC which in turn inhibits cGKII and by implication renin release (Vandongen *et al.*, 1973; Kurtz *et al.*, 1986a; Wagner and Kurtz, 1998b) (see Fig.3).

In contrast to cAMP and cGMP signaling control on renin release, free cytosolic Ca^{2+} concentration is considered as the primary inhibitor (Castrop *et al.*, 2010). Therefore, an increased intracellular Ca^{2+} concentration in JG cells mediates an inhibition of renin secretion and a low concentration enhances renin secretion out of the vesicles. But in all other secretory cells, except parathyroid gland cells, an increased cytosolic Ca^{2+} concentration initiates and supports renin exocytosis (Cohen *et al.*, 1997). This phenomenon in JG cells has been termed as the “calcium paradoxon” of renin release. Many hormones, for example, ANG II and arginine vasopressin are suggested to mediate their inhibiting effect in JG cells via an increase of cytosolic Ca^{2+} concentration (Ichihara *et al.*, 1995; Kurtz *et al.*, 1986b; van Dongen and Peart, 1974). Further, the usage of BAPTA, an intracellular Ca^{2+} chelator, reduces cytosolic Ca^{2+} concentration and stimulates renin secretion (Ortiz-Capisano *et al.*, 2007a; Ortiz-Capisano *et al.*, 2007c).

Not only intracellular Ca^{2+} concentration, but also extracellular concentration of Ca^{2+} is known to modulate renin secretion. Several studies on isolated JG cells, glomeruli, kidneys and also on single kidney sections demonstrated an increased renin secretion after reducing extracellular Ca^{2+} concentration (Moe *et al.*, 1991; Scholz *et al.*, 1994b; Baumbach *et al.*, 1976; Ortiz-Capisano *et al.*, 2007c; Kurtz and Wagner, 1999). Presumably, low extracellular Ca^{2+} -concentration turns into low transmembrane Ca^{2+} influx which results in menial amount of Ca^{2+} ions in the cytosolic area. In addition, these parallel changes in the extra- and intracellular Ca^{2+} concentrations, the Ca^{2+}-sensing receptor, which also controls the release of the parathyroid hormone in a Ca^{2+}-dependent manner, may participate in the regulation of renin release by the extracellular ion concentration (Ortiz-Capisano *et al.*, 2007a; Kurtz and Wagner, 1999; Castrop *et al.*, 2010).

However, the specific mechanisms behind the suppression of renin release in response to an increasing concentration of cytosolic Ca^{2+} remain unknown and need to be investigated further. Recent studies focused on a further downstream target of Ca^{2+}, specifically, the Ca^{2+}-inhibited adenylyl cyclases AC5 and AC6 (Kurtz and Penner, 1989; Hackenthal *et al.*, 1990; Castrop *et al.*, 2010; Schweda and Kurtz, 2004; Grünberger *et al.*, 2006; Ortiz-Capisano *et al.*, 2007c). High Ca^{2+} concentration inhibits both cyclases which turns into decreased cAMP concentration and suppression of renin secretion (Ortiz-Capisano *et al.*, 2007c; Grünberger *et al.*, 2006).

Further, evidences suggest that the suppression of renin release by Ca^{2+} involves Ca^{2+}/calmodulin-dependent processes or activation of proteinkinase C (Castrop, 2007; Hackenthal *et al.*, 1990; Kurtz and Wagner, 1999; Schweda and Kurtz, 2004).

1.3.2 Endothelins as potential regulators of RAAS

A variety of *in vitro* studies and one *in vivo* study using dogs have examined endothelins (ETs) as negative regulators of renin synthesis and secretion out of the cell, mediating their direct effect through an increase in cytosolic Ca^{2+} concentration (Berthold *et al.*, 1999; Scholz *et al.*, 1995; Ritthaler *et al.*, 1995; Ritthaler *et al.*, 1996). However it still remains unknown to what extend the inhibiting direct effect becomes relevant *in vivo.*

1.3.2.1 General characterization of the endothelin system

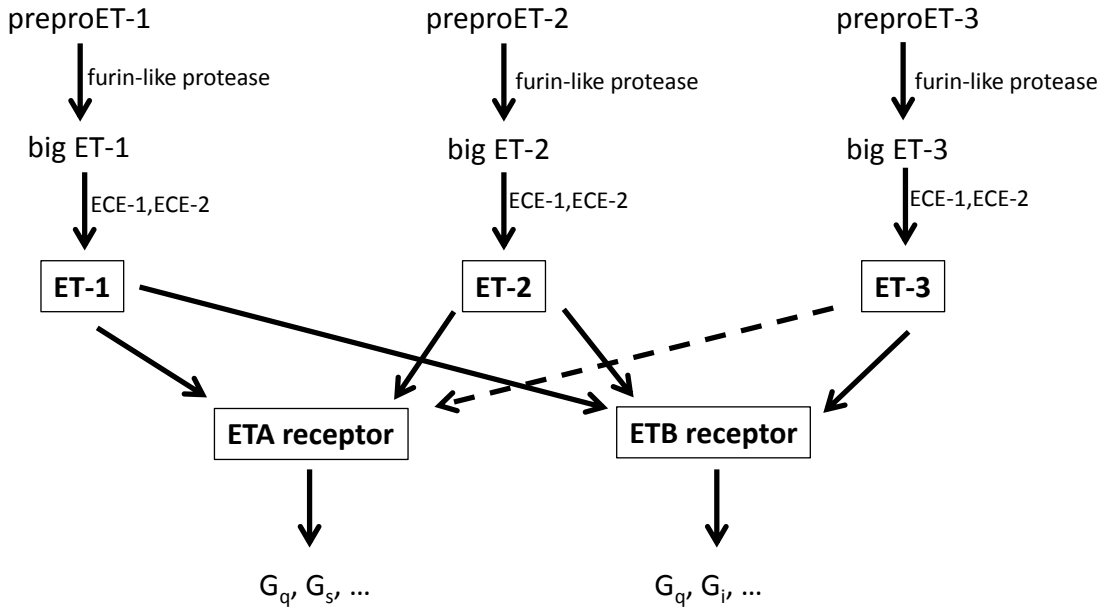

Fig. 4: Schematic representation of the endothelin pathway (adapted from Kedzierski and Yanagisawa, 2001) preproET: preproendothelins; ECEs: endothelin-converting enzymes; G_x: various G proteins

In 1988, a 21-amino acid vasoconstricting factor termed endothelin was isolated from cultured porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). ETs are a family of naturally occurring peptides with well-established growth-promoting, vasoactive and nociceptive properties that affect the function of a number of tissues and systems. The endothelin system consists of endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3).

ET-1 is a 21-amino-acid peptide with a hydrophobic C-terminus and two cysteine bridges at the N-terminus. In addition, two structurally related peptides differing by two and six amino acids were identified and termed as ET-2 and ET-3 shortly after (Yanagisawa and Masaki, 1989). In humans, endothelin represents the most potent and long-lasting vasoconstrictor known, being 100 times more potent than noradrenaline (Yanagisawa *et al.*, 1988; Luscher

and Barton, 2000; Maguire and Davenport, 2002; Hillier *et al.*, 2001; Davenport *et al.*, 2016). ET-1 is suggested as the predominant isoform and the biologically most relevant (Luscher and Barton, 2000; Kedzierski and Yanagisawa, 2001) and is produced by smooth muscle cells, vascular endothelial cells, macrophages, fibroblasts and brain neurons among others (Ortmann *et al.*, 2005; Luscher and Barton, 2000; Kedzierski and Yanagisawa, 2001). ET-2 expression is found in the ovary and in intestinal epithelial cells such as in lung alveolarization. ET-3 can be determined in endothelial cells, brain neurons, and renal tubular epithelial cells rather mediating the release of vasodilators, including NO and prostacyclin (Kedzierski and Yanagisawa, 2001). In the kidney, ET-1 is synthesized by endothelial cells of vessels and ET-1 such as ET-3 are exclusively expressed in epithelial cells of medullary and cortical collecting ducts (Kohan, 1991; Karet and Davenport, 1996).

The endothelin precursors are processed by two proteases to create the mature active form. The so called preproendothelins are cleaved at dibasic sites via furin-like proteases and form physiological inactive 37-41-amino acid long peptides termed big endothelins. Big ETs are cutted via endothelin-converting enzymes (ECEs) at the Trp-Val of ET-1 and ET-2 at the Trp-Ile of ET-3 to build the final peptides (Inoue *et al.*, 1989; Kedzierski and Yanagisawa, 2001).

In mammals, all three peptides mediate their actions via two 7-transmembrane domain, G-protein coupled receptors. They have been identified and termed as Endothelin-A and Endothelin-B (ETAR,ETBR) receptor (Masaki *et al.*, 1994; Davenport, 2002; Arai *et al.*, 1990; Sakurai *et al.*, 1990). Whereas ETAR has been determined to display similar affinities for ET-1 and ET-2 signaling and 100-fold lower affinity for ET-3 (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). ETBR receptor shows equal affinities for all ETs (Barton and Yanagisawa, 2008). (see Fig.4)

1.3.2.2 Role of endothelin system in the kidney

In the kidney, all ETs have been shown to equipotently inhibit cAMP-stimulated renin secretion in a Ca^{2+}-dependent fashion from cultured renal JG cells *in vitro* but it still remains unknown wether ETAR and/or ETBR is involved in this mechanism (Ritthaler *et al.*, 1995; Ritthaler *et al.*, 1996) and if the effects are reproducible *in vivo*. ETAR and ETBR are widely distributed in the kidney whereas one single cell can express one or both receptor isoforms (Kohan *et al.*, 2011a). In general, ETAR has been shown to be localized in vascular smooth muscle, mesangial cells, pericytes, collecting duct and thick ascending limb, while ETBR predominates on endothelial cells and renal tubules (Kohan *et al.*, 2011a). ET receptors activate a host of signaling systems that vary on the cell type and mediate therefore often different biological effects (Kohan, 2010). ET receptors connect to different members of the G protein family (see Fig. 4) where actions result in a variety of signaling cascades, including adenylyl cyclases, cyclooxygenases (COX), nitric oxide synthase (NOS) and others (Kohan

et al., 2011b). The ETAR receptor has been considered as the primary vasoconstrictor and growth promoter whereas ETBR rather has been suggested, at least initially, to cause vasodilatation and inhibit cell growth (Kohan *et al.*, 2011a; Kohan, 2010; Kohan *et al.*, 2011b; Barton and Yanagisawa, 2008; Yanagisawa *et al.*, 1988). In addition, various studies hypothesized endothelins as regulators of several aspects of kidney physiology since ET-1 could be determined to be synthesized in almost every cell type within the organ (Kohan *et al.*, 2011b). Further, ETAR and ETBR could be localized in kidney tissue, as mentioned above, in abundance, the endothelin system has been postulated to be a potential regulator of renal blood flow, glomerular filtration rate and transport of sodium, water, protons and bicarbonate (Kohan *et al.*, 2011a). Administration of ET-1 in anesthetized rabbits reduced cortical perfusion, urinary flow, sodium excretion, cortical perfusion and glomerular filtration rate (Evans *et al.*, 1998). Further, ET system has emerged as being of importance in mediating renal injury and/or disease progression in a variety of pathological conditions including hypertension, ischemia, congestive heart failure, stroke, diabetic retinopathy and nephropathy as acute and chronic renal failure (Rubanyi and Polokoff, 1994; Kohan, 2010). ETs derived from individual renal cells act primarily in an autocrine or paracrine fashion; thus the renal ET system must be viewed within the context of the local microenvironment. Several studies administered key ET agonists and antagonists to get a closer focus on the role of renal ET in the regulation of blood pressure and sodium homeostasis (Maguire and Davenport, 2015). The cyclic pentapeptide BQ-123 *(D-Asp-L-Pro-D-Val-Leu-D-Trp-)* and a linear tripeptide termed as FR139317 *(N*-[*N*-[*N*-[(*Hexahydro-1*H-*azepin-1-yl)carbonyl]-L-leucyl]-1-methyl-D-tryptophyl]-3-(2-pyridinyl)-D-alanine)* are well-known highly selective ETA-receptor antagonists in mammals which have been used in Research and Clinical Studies (Ihara *et al.*, 1992; Aramori *et al.*, 1993; Maguire and Davenport, 2015). Thereby, ET-1 was shown to mediate renal vasoconstriction most presumably via ETA receptor signaling pathway, although it is not apparent from the studies, if ETA-receptor antagonists are able to prevent ET-1 induced vasoconstriction (Schmetterer *et al.*, 1998). Still, medical studies examined that BQ123 inhibited the ET-1 mediated increase in renal vascular resistance of human patients promoting further ETAR as being the main vasoconstrictor (Kaasjager *et al.*, 1997; Bohm *et al.*, 2003). The usage of BQ788, a highly selective ETBR antagonist, supports hypothesis of ET-1 actions in renal vascular system (Bohm *et al.*, 2003; Maguire and Davenport, 2015). In addition, the ET receptor subtype B has been shown to rather play an important role in the clearance of exogenous ET-1, which could be shown in administration of BQ788 in endothelial cell-specific knockout rats (Kelland *et al.*, 2010; Fukuroda *et al.*, 1994).

In summary, endothelin is an important proven inhibiting factor of the renin system *in vitro* and has emerged as a crucial regulator in a variety of renal pathologies (Kedzierski and Yanagisawa, 2001; Davenport *et al.*, 2016; Palmer, 2009; Patel and McKeage, 2014;

Sidharta *et al.*, 2015). Regarding the renin system, endothelin is thought to exert its effect through vasoconstriction in the renal vascular system and/or a potential direct inhibitory effect on renin producing JG cells. Among the two present ET receptor subtypes, it remains unknown if ETAR and/or ETBR are localized on renin producing JG cells and if the inhibiting effect of endothelins on renin synthesis and secretion is a direct effect on the level of those cells *in vivo.*

1.4. Aim of thesis

The protease renin is considered as a central regulatory factor of the renin-angiotensin-aldosterone system. It is synthesized primarily from juxtaglomerular epitheloid cells in the kidney and stored until release in secretory vesicles. Exocytosis and synthesis of renin from JG cells itself is regulated by different systemic and local factors.

Synthesis and secretion of renin is stimulated through cAMP signaling pathway and is, while focusing on local factors, primarily inhibited by an increase cytosolic concentration of Ca^{2+} in JG cells. A large number of vasoconstrictor hormones such as ANG II and arginine vasopressin have been shown to inhibit renin release from juxtaglomerular epithelial cells in the kidney.

In addition, endothelins have been suggested as negative regulators of renin secretion and synthesis. The endothelin system consists of three peptide hormones Endothelin-1, Endothelin-2 and Endothelin-3 and their G-protein coupled receptors ETA- and ETB-receptor. ET-1 has been determined as a strong vasoconstrictor in the renal vascular system mainly mediated by ETAR, whereas ETB-receptor rather promotes vasodilatation. Concerning the contribution of endothelins on the regulation of the inhibition of renin exocytosis and synthesis, it still remains unknown either if endothelins bind on their receptors following a Ca^{2+}-dependent direct inhibiting effect on renin cells or if different systemic factors, e.g. extracellular volume, are affected by endothelins resulting in modulating RAAS. Endothelins can modify blood pressure through their vasoconstrictive and vasodilatory characteristic or through effects located on the tubular system which regulates for example the extracellular volume.

Previous *in vitro* studies have shown that endothelins, focusing on ET-1 inhibit the renin synthesis and secretion (Ritthaler *et al.*, 1995; Ritthaler *et al.*, 1996; Berthold *et al.*, 1999; Scholz *et al.*, 1995; Ackermann *et al.*, 1995) The work at hand concentrates on *in vivo* investigations which mechanisms mentioned above play a central role and moreover which ET-receptors are relevant.

In order to characterize the role of endothelins on the renin system, the first part of present thesis, focusses on the localization of each ET-receptor isoform in the adult murine kidney. With the help of immunohistochemistry and in situ hybridization assay both ET-receptors were determined in different localization sites of the kidney.

The second part of this study concentrates on the relevance of renal ETA-receptor isoform as well as ETBR receptor isoform for renin synthesis and secretion *in vivo* and *in vitro.*

In the beginning, it was ineluctable to investigate a potential direct effect on the level of renin producing cells. Therefore, the generation of a renin cell-specific ETAR as well as an ETBR knockout model was necessary ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$, $Ren^{1d+/Cre}$-$ETBR^{fl/fl}$).

Afterwards, possible indirect effects of ET-receptor isoforms based on vascular actions of endothelins were examined. Thereby the generation of additional cell-specific animal models of single ET-receptors were provided: α-sma-ETAR or ETBR knockout model to study systemic effects on the renin system (SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$, SMMHC-Cre-ER^{T2}-$ETBR^{fl/fl}$); a stroma derived ETAR or ETBR knockout model in order to study if ET-receptors located on stroma derived cells are involved in the renin synthesis and secretion ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$, $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$) and a conditional ETAR knockout model (CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$) to investigate if general ETAR expression is relevant for the regulation of the inhibition of renin synthesis and secretion.

In the last few years, theories of potential ET-receptor interactions have been discussed. Therefore, third part of present thesis focusses on the importance of both renal ET-receptors for the renin synthesis and secretion *in vivo* and *in vitro*. To investigate if both ET-receptors mediate the renin system through a direct effect *in vivo and in vitro*, a renin cell-specific ET-receptor "double-knockout" model ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$) was used for further experiments. In addition, to answer the question if both ET-receptors located on stroma derived cells are relevant for the regulation of the renin system, a stroma derived ET-receptors "double knockout" animal model was provided ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$).

Altogether, by means of different parts of investigations this work contributes to research on possible factors which modulate renin synthesis and secretion out of secretory vesicles, concentrating on negative regulators of renin release. The ET system, primarily ET-1 has been shown to inhibit renin release *in vitro* whereas the characterization if this inhibition is controlled by a possible direct effect on renin producing cells or a potential indirect effect through ET-1/ETA and/or ETBR has yet to be resolved and has been considered as one of the main approaches of present thesis.

2 Material and Methods

2.1 Material

2.1.1 Appliances

APPLIANCE	COMPANY
agarose gel electrophoresis	Compact M, Biometra, Göttingen
autoclave	DX-23, Systec, Linden
blood pressure measuring monitor	blood pressure measuring Monitor, 9001-series; TSE Systems, Bad Homburg
camera	AxioCam MRm, Zeiss, Jena Axiocam 105 color, Zeiss, Jena
centrifuges	Labofuge 400, Heraeus, Hanau centrifuge 5415C, Eppendorf, Hamburg
computer	Precision 690, Dell, Frankfurt am Main
cryostat	CM 3050S, Leica, Wetzlar
filter sets **TRITC-Filter:** excitation 533-558 nm emission 570-640 nm **Cy2-Filter:** excitation 450-490 nm emission 500-550 nm	filter set 43 DsRed, Zeiss, Jena filter set 38 HE, Zeiss, Jena

Cy5-Filter: excitation 625-655 nm emission 665-715 nm **DAPI-Filter:** excitation 335-383 nm emission 420-470 nm	filter set 50, Zeiss, Jena filter set 49, Zeiss, Jena
fluorescent lamp	Colibri.2, Zeiss
heat block	Thermomixer, Eppendorf, Hamburg Thermomixer 5436, Eppendorf, Hamburg
heat chamber	Memmert, Schwabach
heat plate	HI 1220, Leica, Wetzlar
heating bath	Modell W13, Haake, Karlsruhe 1083, GFL, Burgwedel
homogenizer	Ultra-Turrax T25, Janke & Kunkel, Staufen
ice machine	Ziegra Eismaschinen, Isernhagen
incubator	Model B6200, Heraeus, Hanau Modell 300, Memmert, Schwabach
magnetic mixer	MR 80, Heidolph, Schwabach MR 3001 K, Heidolph, Schwabach
microscope	Axio Observer Z1, Zeiss, Jena
microtome	rotary microtome RM2165, Leica, Wetzlar

microwave	Sharp, Osaka
PCR-cycler	Labcycler, Sensoquest, Göttingen Lightcycler LC480, Roche, Mannheim
perfusion pump	323, Watson Marlow, Wilmington, USA
pH electrode	Hanna Instruments, Vöhringen
photometer	NanoDrop 1000, Peqlab, Erlangen
pipettes	Pipetman P10, P20, P100, P200, P1000, Gilson, Middleton, USA
pure water system	MilliQ Plus PF, Millipore, Schwalbach
refrigerator/freezer	Santo refrigerator, AEG, Nürnberg Comfortplus freezer, Liebherr, Ochsenhausen Ultra-low-freezer -85°C, New Brunswick Scientific
RNAscope® heat oven	HybEZ Oven, Advanced Cell Diagnostics (ACD), Hayward, USA
scales	Scale ABT 120-5DM, kern, Balingen-Frommern EMS, Kern, Balingen-Frommern
scan table	Marzhäuser Sensotech GmbH, WTzlar
shaker	GFL, Burgwedel Rotamax, Heidolph, Schwabach
UV dark chamber	Fusion FX7, Vilber, Eberhardzell
UV light table	fluorescent tables, Renner, Dannstadt

vortex mixer	USA REAX1, Heidolph, Schwabach
water bath	Aqualine, AL12, Lauda, Lauda-Königshofen 1083, GFL, Burgwedel

2.1.2 Consumable material

PRODUCT	COMPANY
cover slips	Roth, Karlsruhe
dissecting set	Hammacher, Solingen
falcon 15 ml, 50 ml	Sarstedt, Nümbrecht
filter	Schleicher & Schuell, Dassel
glassware	Roth, Karlsruhe Schott, Mainz
gloves	neoLab Migge GmbH, Heidelberg
hematocrit capillaries	Sanguis Counting, Nürnbrecht
hematocrit sealing kit	Brand, Wertheim
light cycler multiwell plates 96	Sarstedt, Nürnbrecht
liquid blocker pen	Super PAP-Pen, Science Services, Munich ImmEdge Pen, Vector Laboratories, Burlingame, USA
low salt chow 0.02 % NaCl **high salt chow, 5 % NaCl**	Ssniff, Soest

microscope slides, Superfrost Plus	Menzel-Gläser, Braunschweig
mold (silicone rubber)	Roth, Karlsruhe
needles	Becton Dickinson, Franklin Lakes, USA
paraffin wax	Sarstedt, Nümbrecht
parafilm	Bemis, Neenah, USA
pasteur pipettes	VWR, Darmstadt
pipette tip (filter or no filter)	Sarstedt, Nümbrecht Biozym Scientific, Hessisch Oldendorf neoLab Migge GmbH, Heidelberg
serological 5 ml, 10 ml, 25 ml pipettes	Sarstedt, Nürnbrecht
surgical blade	Feather, Köln
tissue embedding cassettes	Roth, Karlsruhe
tubes, 0.5 ml, 1.5 ml, 2.0 ml	Sarstedt, Nümbrecht

2.1.3 Chemicals, Enzymes, Kits

PRODUCT	COMPANY
agarose	Biozym, Oldendorf
ammonium hydroxide solution	Sigma-Aldrich, Munich
bovine serum albumin (BSA)	Sigma-Aldrich, Munich
Dulbecco's PBS	Sigma-Aldrich, Munich

chloroform	Merck, Darmstadt
diethyl pyrocarbonate (DEPC)	Fluka, Neu-Ulm
DNA ladder: gene ruler	Gene Ruler™ 100bp plus DNA ladder, Thermo Scientific, Waltham, USA
ELISA-kit (ANG I) PRA	IBL International, Hamburg
enalapril	Sigma, Deisenhofen
ethanol p.a.	Honeywell, Morris Plains, USA
Ethylendiamintetraacetate (EDTA)	Merck, Darmstadt
formaldehyde solution (37 %)	Merck, Damrstadt
glycergel Mounting Medium (IHC)	Dako Cytomation, Glostrup, Dänemark
glycerol 87 %	AppliChem, Darmstadt
goTaq DNA Polymerase, 5 U/µl	Promega, Mannheim
GoTaq Reaction Buffer Green, 5x bzw. Colorless, 5x	Promega, Mannheim
HCl 1N	Merck, Darmstadt
hematoxylin	Gill Nr.1, Sigma Aldrich, Munich
heparin Liquemin® 25000 (5000 I.E./ml)	Roche, Mannheim
horse serum	Gibco, Life technologies, Grand Island, USA
isopropyl alcohol	Merck, Darmstadt

isopropyl alcohol (p.a.)	AnalaR Normapur, VWR, Radnor, USA
isotonic NaCl solution 0.9 %	B. Braun, Melsungen
K_2HPO_4 x 3 H_2O	Merck, Darmstadt
KCl	Merck, Darmstadt
ketamine 10 %	Bela-pharm, Vechta
KH_2PO_4	Merck, Darmstadt
methanol	Merck, Darmstadt
$MgCl_2$	Merck, Darmstadt
M-MLV *reverse transcriptase*, 200 u/µl	Invitrogen, Karlsruhe
Na_2HPO_4	Sigma-Aldrich, Munich
Na_2HPO_4 x 2 H_2O	Merck, Darmstadt
NaCl	Merck, Darmstadt
NaH_2PO_4	Sigma-Aldrich, Munich
NaOH 1N	Merck, Darmstadt
nonfat dry milk	Biorad, Munich
nuclease-free water	GibcoBRL, Eggenstein
Oligo$(dT)_{15}$ Primer, 0.5 µg/µl	Thermo Scientifix, Waltham, USA
paraformaldehyde	Roth, Karlsruhe
Paraplast-Plus paraffin	Sherwood, St. Louis, USA
PCR nucleotide mix	Promega, Mannheim

(dATP, dCTP, dGTP, dTTP, je 10 mM)	
RNAscope® 2.5 HD detection reagents	ACD, Hayward, USA
RNAscope® H2O2 & protease plus reagents	ACD, Hayward, USA
RNAscope® Target retrieval reagents	ACD, Hayward, USA
RNAscope® washing buffer	ACD, Hayward, USA
Roti®-safe gel stain	Roth, Karlsruhe
SYBR® Green PCR Kit	Roche, Mannheim
tamoxifen chow (400 mg tamoxifen citrate/kg)	Harlan Laboratories, NM Horst, Niederlande
Tissue-TeK®	Sakura, NL
Tris(hydroxymethyl)aminomethane (TRIS)	Affymetrix, Cleveland, USA
PeqGold TriFast™	Peqlab, Erlangen
VectaMount™	Vector, Laboratories
xylazine, 2 %	Serumwerk, bernburg
xylol **xylol (p.a) for ISH**	Merck, Darmstadt AppliChem, Darmstadt

2.1.4 Buffer and solutions

So far as not mentioned further, chemicals for buffer and solutions were dissolved in $H_2O_{bidest.}$.

2.1.4.1 Immunohistochemistry and in situ hybridization

Fixation for perfusion (IHC), pH 7,4

PBS (Phosphate Buffered Saline) buffer Dulbecco	
Paraformaldeyde	3 %

Fixation for perfusion (RNAscope®/BASEscope® ISH) pH 7

Formaldehyde solution	25 ml
Na_2HPO_4	1.625 g/l
NaH_2PO_4	1 g/l
H_2O Millipore	225 ml

PBS-(Phosphate Buffered Saline) Buffer

NaCl	136 mM
KCl	2.7 mM
$Na_2HPO_4 \times 2H_2O$	10.1 mM
KH_2PO_4	1.8 mM

PBS-Otto-Buffer, pH 7,4

$K_2HPO_4 \times 3H_2O$	10 mM
NaCl	140 mM
KH_2PO_4	10 mM

Blocking solution A

dry milk powder	5 %
PBS buffer	

Blocking solution B

BSA (Bovine Serum Albumin)	1 %
HS (Horse Serum)	10 %
PBS buffer	

2.1.4.2 Molecular biology

Agarose gel

TAE	
agarose	2 %
Roti®-gel stain	

DEPC-H2O

1/1000 Vol. DEPC diluted in ddH20, agitate,

leave open under hood overnight, autoclave afterwards

NaOH for gDNA-Extraction

NaOH	25mM

10x TAE (Tris-acetic-EDTA), pH 8,5

Tris	40 M
Acetic acid	20 M
EDTA	1 M

Tris HCl for gDNA-Extraction, pH8,0

Tris HCl	1 M, pH 8

2.1.5 Oligonucleotides

Oligonucleotide-Primer were synthesized and sent in a lyophilized manner by Eurofins MWG®. After adding nuclease-free water a concentration of 100 pmol/µl was calculated.

name	species	orientation	sequence from 5‘ to 3‘
genotyping			
ETAR	mus musculus	fw	CCT CAG GAA GGA AGT AGC AAG
		rev	ACA CAA CCA TGG TGT CGA
ETBR WT	mus musculus	fw	CTG AGG AGA GCC TGA TTG TGC CAC
		rev	CGA CTC CAA GAA GCA ACA GCT CG

ETBR KO	mus musculus	fw	TGG AAT GTG TGC GAG GCC
		rev	CAG CCA GAA CCA CAG AGA CCA CCC
RenCre	mus musculus	653 Ren1d	GAA GGA GAG CAA AAG GTA AGA G
		468 Ren1d	GTA GTA GAA GGG GGA GTT GTG
		400 Cre	TTG GTG TAC GGT CAG TAA ATT GGA C
SMMHC-Cre-ERT^2	mus musculus	SMWT1	TGA CCC CAT CTC TTC ACT CC
		SMWT2	AAC TCC ACG ACC ACC TCA TC
		phCREAS1	AGT CCC TCA CAT CCT CAG GTT
FoxD1-Cre	mus musculus	FOXD1-Cre s	TCT GGT CCA AGA ATC CGA AG
		FOXD1-Cre as	GGG AGG ATT GGG AAG ACA AT
		FOX-D1-Cre WT s	CTC CTC CGT GTC CTC GTC
CAGG-Cre-ER^{T2}	mus musculus	mCAGG s geno	CTC TAG AGC CTC TGC TAA CC
		mCAGG as geno	CGC CGC ATA ACC AGT GAA AC
mRNA-studies			
GAPDH	mus musculus	GAPDH fw	ATG CCA TCA CTG CCA CCC AGA AG
		GAPDH rev	ACT TGG CAG GTT TCT CCA

			GGC
renin	mus musculus	Renin fw	ATG AAG GGG GTG TCT GTG GGG TC
		Renin rev	ATG CGG GGA GGG TGG GCA CCT G
ETAR	mus musculus	fw	AGG AAC GGC AGC TTG CGG AT
		rev	AGC AAC AGA GGC AGG ACT GA
ETBR	mus musculus	fw	GAA GAG CGG TAT GCA GAT TG
		rev	TAT TGC TGG ACC GGA AGT TG

Table 1: Oligonucleotide sequence for PCR and quantitative real-time RT-PCR; fw = forward, rev = reverse;

2.1.6 Antibodies and hybridization probes (ISH)

first antibody	clonality	company	dilution
chicken anti-**renin**-IgG	polyclonal	Davids Biotech, Regensburg	1:400
mouse-anti-**smooth-muscle actin**-IgG	polyclonal	Abcam, Cambridge, UK	1:400
rabbit-anti-**ETAR**-IgG	polyclonal	Alomone Labs, Israel	1:100
mouse-anti-**calbindin-D28K**-IgG	monoclonal	SWANT, Marly	1:200

goat-anti-**α-integrin8**-IgG	polyclonal	R&D systems, Wiesbaden	1:200
goat-anti-**aquaporine2**-IgG (C-17)	polyclonal	Santa Cruz, Heidelberg	1:200
goat-anti-**CD31**-IgG	polyclonal	R&D systems, Wiesbaden	1:200
second antibody	**conjugation**	**company**	**dilution**
donkey **anti-chicken**-IgY	rhodamin-(TRITC)	Dianova, Hamburg	1:400
donkey **anti-chicken**-IgY	Cy2	Dianova, Hamburg	1:400
donkey **anti-mouse**-IgG	Cy2	Dianova, Hamburg	1:400
donkey **anti-mouse**-IgG	Cy5	Dianova, Hamburg	1:400
donkey **anti-rabbit**-IgG	rhodamin-(TRITC)	Dianova, Hamburg	1:400
probes (ISH)		**company**	
RNAscope® negative control probe –**DapB**		ACD, Hayward, USA	
RNAscope® positive control probe –mM-**PPIB**		ACD, Hayward, USA	

RNAscope® - mM-**ETBR**-C1		ACD, Hayward, USA	
BASEscope®- mM-**ETBR**-C1		ACD, Hayward, USA	

2.2 Methods

2.2.1 Animals

All Animals were treated in conformity with *'National Institute of Health guidelines for the care and use of animal research'* and were approved by the local commission of ethics (temperature 23°C ± 2°C, relative humidity 55% ± 5%, light/dark interval 12h, food and water *ad libitum*).

In order to generate a renin cell-specific ETAR-deleted animal model, the established Cre/loxP-recombinase system was used for present experiments. $ETAR^{flox/flox}$ animals (with kind permission of Mr. Yanagisawa, University of Texas Southwestern medical Center at Dallas) were initially paired with Cre^{Ren1d} (with kind permission of *Ariel Gomez, University of Virginia*). The offspring was genetically determined either as $ETAR^{flox/flox}$/RenCreHet or $ETAR^{flox/flox}$ and $ETAR^{flox/flox}$/RenRen. Whereas 'RenCreHet' signifies, that animals possess both an active Ren1d-gene and additionally activated Cre^{Ren1d}-recombinase: $Ren1d^{+/Cre}$-$ETAR^{fl/fl}$. $ETAR^{flfl}$/RenRen represents the control littermates where no Cre-recombinase is active ($ETAR^{fl/fl}$).

$ETBR^{flox/flox}$ animals (with kind permission of Miles L. Epstein, University of Wisconsin, Madison) were initially paired with Cre^{Ren1d}. Therefore $Ren1d^{+/Cre}$-$ETAR^{fl/fl}$ was genetically determined as knockout mouse with active Cre-recombinase as well as active Ren1d-gene and $ETBR^{fl/fl}$ signifies control littermates.

Further $ETAR^{flox/flox}$ and $ETBR^{flox/flox}$ animals were also paired with FoxD1-Cre to activate recombinase in stroma-derived cells (commercially acquirable at The Jackson Laboratory, Maine USA). In addition both ET-receptors loxP strains were paired, in addition with SMMHC-$CreER^{T2}$ (with kindly permission of Stefan Offermanns, University of Heidelberg) where a fusion protein is expressed under the control of the SMMHC-promotor consisting of of the Cre-recombinase and a modified estrogen binding site: SMMHC-$CreER^{T2}$- $ETAR^{fl/fl}$ and SMMHC-$CreER^{T2}$- $ETBR^{fl/fl}$

$ETAR^{flox/flox}$ animals were also paired with CAGG-Cre-ER^{T2} (commercially acquirable at The Jackson Laboratory, Maine USA) where recombinase is active through induction with

tamoxifen chow ubiquitous under the control of β-actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer (CAGG-Cre-ER^{T2}): CAGG-Cre-ER^{T2}-ETAR$^{fl/fl}$-mice, in order to study if general ETAR expression is involved in the regulation of renin synthesis and secretion. All animals possessed a mixed genetic background of 129Sv and C57Bl/6.

Animal strains used for present thesis:

strain	genetic background	origin
C57/Bl6	C57/Bl6	Charles River, Sulzfeld
CAGG-Cre-ER^{T2}	C57/Bl6	Jackson Laboratories
ETAR loxP	129/SV	Masashi Yanagisawa
ETBR loxP	129/SV	Miles L. Epstein, University of Wisconsin
FoxD1-Cre	Bl6/129SV	Jackson Laboratories
Ren-Cre	Bl6/129SV	R. Ariel Gomez, University of Virginia School of Medicine
SMMHC-Cre-ER^{T2}	Bl6/129SV	Stefan Offermanns, University of Heidelberg

2.2.2 *In vivo* studies

Mice used for molecular biological and histological experiments were 90-120 d of age.

High salt and low salt diet

In order to modulate RAAS, animals were treated for 14 d with either a high-salt diet (4 % NaCl; Ssniff) or a low salt diet (0.02 % NaCl; Ssniff). Low salt diet was in combination with the ACE-inhibitor enalapril (10 mg kg^{-1} day^{-1}; Sigma-Aldrich, St. Louis, MO) dissolved in drinking water bottle. Controls were maintained on standard rodent chow (0.4 % NaCl; Ssniff)

Extraction of kidneys for molecular and histological experiments

One kidney was used for histological work and therefore a retrograde arterial perfusion was performed with 3 % paraformaldehyde in PBS in order to fixate the kidney. Second kidney was used for mRNA-studies and subsequently stored immediately at -80° C after cording up a*rteriae renales* with a polyglactin suture (Vicryl) to avoid flushing kidney with fixing medium.

Retrograde arterial perfusion for IHC

For this procedure mice were first anesthetized with a solution of ketamine and xylazine (80 mg/kg BW; i.p.). After opening abdomen, displaced abdominal aorta was clamped underneath the outlet of *arteriae renales* in order to avoid disturbances of blood circulation of the kidney. On the next step, catheter of perfusion was inserted distal to the clamp and fixed in this position. For pressure equalization, *vena cava inferior* was opened with the help of a scissor. Fixed clamp was then removed and via catheter, 20 ml of isotonic NaCl-solution with heparin were perfused retrograde. Afterwards, fixation was performed with 3 % Paraformaldehyde in PBS with a constant flow of 50 ml/3 min. Dissected and perfused kidneys were stored at 4°C in 70% methanol until paraffin embedding for histological experiments.

Retrograde arterial perfusion for in situ hybridization (RNAscope®):

Procedure of perfusion for ISH is similar to retrograde arterial perfusion for IHC except solution for fixation. Instead of 3 % Paraformaldehyde in PBS a solution based on formalin was used for this experiment. Animals were fixated with a constant flow of 50 ml/3 min. After dissection of kidneys, organs were stored in this fixation solution for 24 h before proceeding ISH (RNAscope®) protocol.

Solution for fixation (3 animals):

- 25 ml formalin
- 225 ml H_20
- 1 g/l NaH_2PO_4
- 1,625 g/l Na_2HPO_4

2.2.3 *In vitro*/*Ex vivo* studies

Isolated perfused mouse kidney model (IPMK)

The isolated perfused mouse kidney is an important tool for experimental nephrology (Schweda *et al.*, 2003; Czogalla *et al.*, 2016) where one can study organ function in the absence of systemic influences such as blood pressure, autonomic nervous system or several hormones . The combination of *in vitro* and *in vivo (ex vivo)* has the ability to examine physiological, pharmacology or biochemical aspects of renal function. Therefore kidneys are dissected and perfused in a heated moist chamber through renal artery with a constant pressure (80-100 mmHg). The perfusate solution is collected through the renal vein for measuring renin activity. The perfusate solution is a modified Krebs-Henseleit solution containing BSA (6 g/100ml) and human erythrocytes (10 % hematocrit). In order to

investigate the effects of ET-1 on the renin secretion rate with different ET-receptor animal models, the IPMK is a perfect tool.

Protocol (after Czogalla *et al.*, 2016):

1. Buffer Preparation:
    - Prepare the dialysis buffer:

Solution 1 (10x concentrated):

NaCl	126 g	107.8 mM
$NaHCO_3$	46 g	27.4 mM
KCl	6.6 g	4.42 mM
Urea	7.3 g	6 mM
Creatinine	0.5 g	0.132 mM
Ampicillin	0.3 g	
$MgCl_2 * 6H_20$	4 g	1 mM

add dH_20 to a final volume of 2l

Solution 2 (10x concentrated):

Glucose	15 g	8.32 mM

Add dH_20 to a final volume of 2l

Solution 3 (10x concentrated):

$CaCl_2 * 6H_20$	2.6 g	0.935 mM

Add dH_20 to a final volume of 1l

Solution 4 (10x concentrated):

NaH_2PO_4	0.4 g	2.88 mM
Na_2HPO_4	1.2 g	0.66 mM

Add dH_20 to a final volume of 1l

Antidiuretic hormone (ADH) solution:

1µg

Dialysis buffer (composition)

Solution1	500 ml
Solution2	500 ml
Solution3	500 ml
Solution4	500 ml

Aminoplasmal	83 ml	
ADH solution	0.5 ml	
Na-Pyruvate	165 mg	0.3 mM
Na-Glutamate	262 mg	0.31 mM
Na-Malate	281 mg	1.15 mM
α-ketoglutarate	1133 mg	1.2 mM
Na-Lactate	1176 mg	2.1 mM
FITC-Inulin	25 mg	

Add dH_20 to a final volume of 5l: adjust pH 7.7

Perfusion buffer for one mouse:

Dialysis buffer	400 ml

Human erythrocytes to hct of 10 %

- Erythrocyte preparation: dilute 250 ml of human erythrocyte concentration (tested material obtained from the local blood bank) to 500 ml with dialysis buffer. Centrifuge at 2.000 x g for 8 min. Remove the buffer, being careful not to remove any erythrocytes. Repeat 3x
- Prepare the albumin (BSA) buffer: in 200 ml of dialysis buffer, dissolve 44 g of BSA using a stir bar. Filter the solution with filter paper
- Prepare the perfusate: filter the erythrocytes through filter paper into the BSA buffer. Fill up to a total volume of 800 ml with dialysis buffer: Note: the hematocrit should be now between 8-12 %. It can be stored for 12hr at 4 °C.

2. Initiating dialysis and oxygenation: (Fig. 5)
    - Turn on the water bath surrounding the larger buffer reservoir, smaller buffer reservoir and the moist chamber to 37 °C.
    - Fill the larger buffer reservoir with the dialysis buffer and the smaller reservoir with the perfusate
    - Turn on 5% CO_2/95% O_2 gas inflow to the dialysis buffer
    - Switch on continuous dialysis of the perfusate against the dialysis buffer.

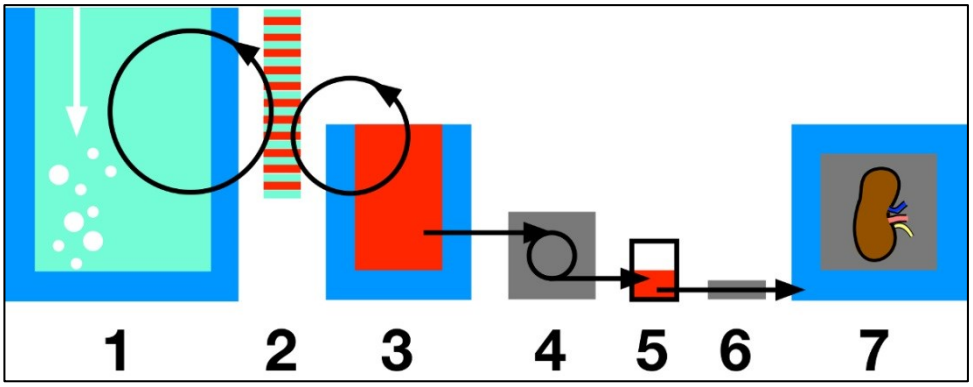

Fig. 5: Schematic drawing of the perfusion circuit and the direction of buffer flow (Czogalla *et al.*, 2016):all components surrounded by blue are kept at 37 °C with a water bath (1) dialysis buffer with continuously 95% O_2/5% CO_2 (2) dialysis tube with roller pump to continuously dialyze dialysis buffer and perfusion buffer against each other (3) perfusion buffer is enriched with 9% O_2/5% CO_2 and electrolyte levels are kept constant throughout perfusion (4) a roller pump connects perfusion buffer towards the kidney (5) windkessel removes peristaltic waves and traps bubbles (6) pressure transducer (7) kidney remains in the moist chamber

3. Surgical procedure (part 1) (see Fig.6)
    - Anesthetize mouse with 10 µl/g of body weight, 20mg/ml ketamine and 1 mg/ml xylazine dissolved in 0.9 % NaCl
    - Fix mouse in the moist chamber. Place a 1 ml syringe below the spine to elevate the lumbar vessels
    - Perform a median laparotomy from the pubic crest to the sternum opening first the skin, then the abdominal muscles, with scissors
    - Remove the intestine and place it on the left side of the mouse lateral from the abdomen
    - Free the bladder from connective tissue and explore both ureters and urethra
    - Place and close ligature I
    - Place and close ligature II
    - Place ligature III
    - Incise the bladder (1 mm)
    - Cannulate the opening with 2 cm PE 50 tubing
    - Close ligature III around the tubing
    - Cut the left ureter and urethra distal from the ligatures. The bladder is now attached to the right ureter only and freely moving
    - Clear the abdominal aorta of connective tissue and fat
    - Place an abdominal mid-aorta ligature (ligature IV)
    - Place a ligature around the aorta below the diaphragm between the superior mesenteric artery and the coeliac trunk (lig.V)
    - Place a ligature around the superior mesenteric artery (lig.VI)
    - Place an aortic ligature directly below the right and above the left renal artery (lig.VII)

- Place a ligature around the caudal vein package (lig.VIII)

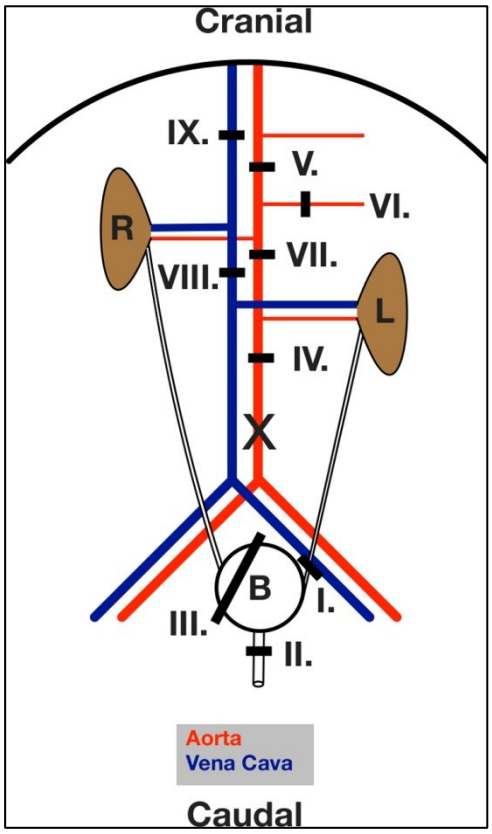

Fig. 6: Schematic drawing of the ligatures placed during surgery (Czogalla *et al.*, 2016)

4. Priming of the perfusion circuit
    - Start the rotary pump and fill the tubing with perfusate. Remove all air bubbles
    - fill the windkessel device to approximately mid-level with perfusate
    - Calibrate the pressure transducer to 0 mmHg when all tubing is filled and flow is 0. Keep the perfusion needle at the kidney level during this time
    - Keep flow at constant minimal level (0.6 ml/min) and proceed further
5. Surgical procedure (part 2)
    - Place a clamp between lig.IV and the branching of the left renal artery
    - Make a small incision in the aorta caudal of lig.IV, do not cut the dorsal wall
    - Dilate the opening in the aorta with a vessel dilator
    - Cannulate the aorta with a needle (ca. 2 cm long, pulled PE 50), pushing the tip just to the clamp
    - Open the clamp
    - Push the tip of the needle cranially until it reaches the junction of the right kidney artery and the aorta
    - Close lig.VII

- Close lig. IV
- Open the chest with scissors by dissecting the diaphragm. With a single cut, separate the aorta, vena cava, heart and vegetative nerves.
- Start pressure control of the perfusion pump. Maintain the mean pressure between 80-100 mmHg
- Close lig.V
- Close lig.VI
- Close lig.VIII
- Free the right kidney from connective tissue and its embedding into the adipose capsule with scissors
- Cut the aorta proximally to lig.V
- Cut the superior mesenteric artery distally to lig.VI
- Cut the kidney-supporting vessel bundle out, do not cut into the vessels themselves
- Cut the liver at the connection to the kidney. Take care to free the kidney, but leave a small part of the liver adherent to it, so that the vena cava is kept open by it
- Take the kidney bundle out of the mouse. Remove the mouse from the moist chamber
- Place ligature around the connection of liver and kidney (lig.IX)
- Cannulate the vena cava with a venous line (2 cm PE 50)
- Clos lig.IX. venous outflow through the venous line should immediately start
- Close the moist chamber

6. Downstream analysis:
   During the following hour, monitor blood flow and intravascular pressure. Collect venous outflow which is used for renal renin release

The renal function is therefore sustainable for at least 1h:

General results:

- Vascular resistance, perfusate flow remain steady
- e.g. glomerular filtration rate: 130 µl/min*g kidney weight
- fractional sodium and potassium resorption are unchanged
- all segments of the kidney show no signs of organ damage

For present study, this tool allows studying changes regarding renin secretion as reaction to ET-1 in increasing concentrations (30,100,300 pM and 1 nM). Every 2 min samples are collected for evaluation of renin secretion rate and the activity of renin was measured via ANG I ELISA assay afterwards (2.4.4). The rate of renin secretion (ng ANG I/ml*h^{-1}) is defined as the activity of renin (ng ANG I/h x min x g) multiplied by renal blood flow (ml/g*organ weight*min^{-1}).

2.3 Histological methods

2.3.1 Immunohistochemically staining of paraffin embedded kidney sections

In order to avoid autocatalytic mechanisms and to have a better quality of the sample, kidney tissue was fixed before dissection through PFA-retrograde arterial perfusion (see. 2.2.2).

For the beginning of tissue embedding with paraffin, perfused kidneys were dehydrated with an ascending methanol series [2x (70 %, 80 %, 90 % and absolute) for 30 min]. Afterwards, kidneys were washed in absolute isopropyl alcohol 2x 30 min at room temperature and additionally stored in an isopropyl alcohol/paraffin- composite (1:1; 55°C) for another 30 min. Subsequently, kidneys were incubated for 2x24 h at 60°C in paraffin and then transferred into silicon-rubber- embedding forms where they cured overnight.

With the help of a rotation microtome, paraffin slices were cut (5 µm), transferred in a 40 °C water bath and taken up by a microscope slide. In the end, paraffin slides were dried for 12 h in a dry chamber (40°C).

When performing immunofluorescent staining, deparaffining steps were necessary after protocol in advance:

- 2 x xylol 10 min
- 2 x isopropyl alcohol absolute; 5 min
- isopropyl alcohol 96 %; 5 min
- isopropyl alcohol 80 %; 5 min
- isopropyl alcohol 70 %; min

2.3.2 Immunofluorescent staining

After paraffin embedding of the tissue, kidney slides were stained with the help of indirect immunofluorescence. This method uses the specificity of antibodies to their antigen to target fluorescent dyes to specific targets, and therefore allows visualization of the target molecule. An unlabeled first antibody is used for binding specifically to the target molecule and the second antibody carries the fluorophore, recognizing the first antibody and binds to it. It

should be noted the second antibody complies with antigen sequences of host from first antibody.

Protocol for immunofluorescent staining of tissue (paraffin):

- wash slides with kidney sections 3 x for 5 min with PBS
- according to requirements of antibody: unmasking tissue while incubating with Tris/EDTA for 45 min at 97 °C in water bath
- 2 x washing with PBS for 5 min each
- Incubate with blocking solution 4 % milk powder in PBS for weakening unspecific binding: 1 h at RT
- Incubate with first antibody (diluted in blocking solution) in a moist chamber over night at 4°C
- 3 x washing with 4 % milk powder in PBS for 5 min each
- Incubate with second antibody (flurophore conjugated antibody; 1:400): 90 min at RT in a moist chamber (dark surrounding)
- 3 x washing with PBS for 5 min each
- Embedding sections with glycergel mounting medium
- Overview:

staining	**ETAR, renin, α-sma**
tissue	paraffin sections 5 µm
demasking	TrisEDTA, pH8.5, 1h, 95°C
blocking solution	5% Milk powder in PBS, 1h, RT
first antibodies (ON)	rabbit anti-ETAR (1:100) chicken anti-renin (1:400) mouse anti-α-sma (1:400)
diluted solution	5% milk powder in PBS
second antibodies (90 min)	Cy2 donkey anti-rabbit TRITC donkey anti-chicken Cy5 donkey anti mouse (1:400)

2.3.2 Microscopy

Acquisition of fluorescent stainings was provided with Colibri.2 and Axio Observer Z1 microscope with motorizing object holder from Zeiss. With the help of Zen software from Zeiss (Zen 2012), pictures were digitalized and edited. In order to avoid weakening of quality, TIFF format was used for present thesis.

2.4 Molecular biology

2.4.1 Polymerase chain reaction assays for genotyping

With the help of the polymerase chain reaction used mice were tested for positive transgenical allele. Therefore 0.5 cm of the mouse tail were digested for 1 h with 100 µl 25 mM NaOH solution and deactivated with 10 µl of 1 M of Tris-HCl solution

PCR-approach (25 µl)	
10x buffer	5 µl
Tag Polymerase	0.3 µl
dNTPS	2 µl
sense primer (10pmol/µl)	1 µl
antisense primer (10pmol/µl)	1 µl
dd H_20	13.7 µl
DNA-template	2 µl
∑	**25 µl**

Table 2: PCR approach (25 µl)

program for cycler			
step	**temperature**	**duration**	**repeat**
1. Initial Denaturation	94 °C	5 min	

2. Denaturation	94 °C	30 sec	36 x
3. Annealing	variable	30 sec	
4. Elongation	72 °C	1 min	
5. Final Elongation	72 °C	5 sec	
6. Hold	15 °C	∞	

Table 3: PCR program

PCR: Annealing temperature for different genotype PCR	
ETAR loxP	60 °C
ETBR loxP	66 °C
FoxD1-Cre	58 °C
Ren-Cre	56 °C
SMMHC-Cre	62 °C
CAGG-Cre (TamCre)	65 °C/ 55 °C

Table 4: Annealing temperature of different PCR approaches

2.4.2 Gel electrophoreses

After running the polymerase chain reaction (PCR) the size of the resulting amplicon could be determined through gel electrophoresis.

Therefore 1 g of agarose was completely dissolved in 100 ml 1x TAE-Buffer through cooking in a microwave for 3 min. After cooling down to 60 °C, 3 µl of a non-toxic stain solution (Roti®-GelStain) were added to the approach. The electrophoresis chamber was filled up with 1x TAE-Buffer after gel was cured. Present samples were applied on the gel and electrophoreses could be performed for 45 min on 130 V. After fractionation of DNA, gel was removed out of the chamber and monitored with a camera system under UV-light irradiation in a trans illuminator.

2.4.3 Expression studies

In order to examine the potential role of ETAR and/or ETBR through ET-1 on the renin system *in vivo*, expression studies were implemented via quantitative real-time RT-PCR-analyses of kidneys from different animal models. After harvesting kidneys from animals,

next step was to isolate whole RNA from tissue first. In addition, transformation into cDNA and subsequently quantitative real-time RT-PCR was performed.

2.4.3.1 Isolation of RNA from tissue

After harvesting the kidneys out of the rodents, organs were stored in peqGOLD TriFast™-reagent (Trizol) in order to macerate tissue. With the help of a homogenizer, organs were homogenized. Trizol is a monophasic solution with guanidinium thiocynate and phenol. By the use of chloroform, present homogenized suspension is separated into three parts: A red phenol-chloroform-part on the bottom of the cup, a white interphase and a colorless aqueous part on the top. The RNA appears exclusively in the top part und can be precipitate with isopropyl alcohol.

Protocol for RNA-Isolation with TriFast™-reagent

- 1000 µl (for ½ kidney) TriFast™ reagent
- homogenize tissue for 30 sec
- adding 200 µl chloroform, shake gently
- 10 min incubate at room temperature
- centrifuge samples for 15 min at 12.000 g (4°C)
- transfer colorless aqueous part on the top into new cup
- adding 500 µl isopropyl alcohol, vortex
- centrifuge samples for 10 min at 12.000 g (4°C); remove supernatant
- washing pellet with 1 ml 75 % ethanol (2 x)
- centrifuge samples for 10 min at 12.000 g (4°C)
- dry pellet for 20 min under hood
- solve pellet in 300 µl DEPC-water (for ½ kidney)
- 2 min on shaker, 65 °C
- measure concentrations with NanoDrop (1 µl); storage at -80°C

The concentration of isolated RNA was measured with a photometer. To determine the clearance of each sample, extinction value at 260 and 280 nm wavelengths against RNAase free water was ascertained. The reference range from $E_{260}/_{280}$ was defined between 1.80 and 2.00.

2.4.3.2 cDNA synthesis

approach	
1 µg RNA	var.
oligo-dT	1 µl
nuclease-free water	add to 10 µl
5 min, 65 °C thermocycler	
approach	12 µl/sample
dNTP (2.5 mM)	4 µl
5x Buffer	4 µl
reverse transcriptase	1 µl
nuclease-free water	3 µl
1 h 37 °C, 2 min 95 °C, ice	

Table 5: RT-PCR approach

RNA can be transformed into cDNA with the help of the enzyme *reverse transcriptase*. In order to transcribe primarily mRNA with a polyA-tail, oligo-dT-chains were used for this procedure.

The cDNA-synthesis proceeds at 37 °C. Inactivation of *reverse transcriptase* expires at 85 °C. Present cDNA was stored at -20 °C for quantitative real-time RT-PCR-analyses.

2.4.3.3 Quantitative real-time RT-PCR

The complementary DNA (cDNA) serves as a raw material for established quantitative real-time RT-PCR. It is first synthesized over r*everse transcriptase* out of mRNA. In order to amplify a prospecting transcript, normal PCR-program with specific primers is implemented. To quantify the expression of certain transcripts, the exponential enhancement of the product is measured continuously and fluorescent based. A fluorescent dye (SYBR-Green) is used, which can intercalate into the PCR-products and is innervated by a laser. The intensity of the emitted fluorescence is ascertained by a detector. This so-called fluorescent intensity is

adequate to the present amount of DNA because the fluorescent dye embeds exclusively into double-stranded DNA and when uncombined it achieves a weak fluorescent signal. Therefore the amount of products can be traced during running of the program.

The c_T-value (*cycle threshold*) states the number of cycles where fluorescence exceeds above the background. At this point the exponential increase of the product is able to be monitored. In addition the c_T-value is dependent on applied cDNA concentration of the examined gene. A quantitative evaluation of the original mRNA amount is therefore possible. After amplification, a melting curve is created to delimit possible primer dimers from specific product.

The target gene is set on ratio to a reference gene in terms of the relative quantification. Typical constitutive 'housekeeping genes' are expressed in all cells of an organism and relatively constant in their level of expression.

2.4.3.4 Running the quantitative real-time RT-PCR

Expression studies were performed with different kidneys from existing animal models via quantitative real-time RT-PCR. The analysis was carried out by the Lightcycler LC480, Roche Detection System with common 96-well-plate system. In order to detect dsDNA from each specific transcript, a fluorescent-dye (SYBR Green) was used for samples.

For relative quantification and therefore normalizing the amount of cDNA, GAPDH and RPL32 were used as reference genes.

Master-Mix approach per well	
SYBR Green MM	5 µl
Primer Forward (10mM)	0.5 µl
Primer reverse	0.5 µl
H_20 (RNase free)	3 µl
cDNA	1 µl
	∑ 10 µl

Table 6: Approach quantitative RT-PCR

The 96-well-plate was sealed with a special film and centrifuged for 2 min with 2500 g before running RT-PCR.

Step	Number of cycles	Temperature	Time
Activation	11	95 °C	15 min
Denaturation Annealing Elongation	40	95 °C 58 °C 72 °C	15 s 20 s 20 s

Table 7: Program for quantitative RT-PCR

Data was collected with LightCycler 480 SW 1.5 software and evaluated with Microsoft Office Excel. Relative cDNA levels X regarding the Target gene were normalized to the c_T-value of the reference gene and to the standard conditions.

2.4.4 ELISA assay for plasma renin concentration (PRC)

The transformation of Angiotensinogen into ANG I is catalyzed by renin. The amount of renin is proportional to emerging amount of ANG I. Therefore a direct Enzyme-linked Immunosorbent Assay (ELISA-kit) of ANG I was performed to determine quantitatively the plasma renin concentration.

The kit measures plasma renin activity (PRA) in terms of mass of Ang-I generated per volume of mouse plasma in unit time (ng/ml). All solutions needed for present methods are included in given kit.

Before running assay procedure, required amount of blood need to be collected from animals in plastic-hematocrit-capillaries coated with EDTA in order to avoid hemostasis.

ELISA-method in general involves at least one antibody with specificity for a particular antigen. Each sample with an unknown amount of antigen is transferred on the surface of a polypropylene test tube. During the first incubation unlabeled ANG-I compete with biotinylated Ang-I to bind to the anti-Ang-I antibody. In the second incubation the labelled Streptavidin-HRP conjugate, binds to immonilized Ang-Biotin. Unbound material is removed with various washing steps. The colorimetric HRP substrate is added and after stopping the color development reaction, the light absorbance (OD) is measured with a microtiter plate reader. The absorbance values are inversely proportional to the concentration of Ang-I in the sample. In order to plot a standard curve a set of calibrators is used.

Protocol for Ang-I ELISA (PRA):

- collect blood in plastic-hematocrit-capillaries coated with EDTA
- centrifuge capillaries for 4 min at room temperature at 8000 rpm
- transfer plasma sample to cup at room temperature
- dilute samples in Maleate buffer 1:50

- add PMSF (1:100), renin substrate and generation buffer (1:10)
- divide samples into cold- and warm value:
  warm value: 90 min at 37°C
  cold value: 90 min on ice (4°C)
- 45 µl of each sample and standard solutions transfer into 96-well plate
- add 100 µl of Biotin-conjugate solution into each well, incubate 1 h at RT (200 rpm with shaker)
- 5 x washing step with washing buffer
- add 150 µl of Streptavidin-HRP in each well, incubate 30 min at RT (200 rpm)
- 5 x washing step with washing buffer
- add 150 µl of TMB-substrate in each well, incubate for 15 min at RT
- add 50 µl of stop solution, measurement of samples at 450 nm

2.4.5 In situ hybridization RNAscope® and BASEscope®

In situ hybridization was realized by RNAscope® Reagent Kit (brown) and BASEscope® Detection Reagent Kit (red) and RNAscope® 2.5 Duplex Detection kit from Advanced Cell Diagnostics. Therefore a retrograde arterial perfusion was performed after protocol (see 2.2.2) and kidneys were dissected afterwards. ISH-assays are able to detect nucleic acids on the tissue. Due to lack of working antibody for ETBR, we could detect ETBR mRNA signal in kidney slides of different animal models.

Solution for Fixation (FFPE sample preparation):

- 25 ml formalin
- 225 ml H_20
- 1 g/l NaH_2PO_4
- 1.625 g/l Na_2HPO_4

Paraffin embedding procedure of kidneys after following protocol:

- 2x 70 % EtOH p.a.
- 2x 80 % EtOH p.a.
- 2x 90 % EtOH p.a.
- 1x 100 % EtOH p.a.
- 1x 100 % isopropyl alcohol

→ 30 min on shaker

- 1x 100 % isopropyl alcohol (45 °C); 45 min
- 1x 1:1 Isopropyl alcohol/paraffin-composite (60 °C; 45 min)
- 2x 24 h incubate in paraffin (60 °C)

With the help of a rotation microtome, paraffin slices were cut (5/7 µm), transferred in a 40 °C water bath and taken up by a microscope slide. In the end, paraffin slides were dried for 12 h at RT.

Deparaffine and preparation for ISH RNAscope® and BaseScope® method:

- Microscope slides with paraffin slices for 1 h in dry oven at 60 °C
- 2 x 10 min in Xylol at RT
- 2 x 2 min in EtOH p.a. at RT
- air dry slides for 5 min at RT
- prepare 1 x RNAscope® Target Retrieval Reagents
- apply RNAscope® Hydrogen Peroxide on slides (10 min)
- 2 x H_20 RNase free washing step
- apply RNAscope® Target Retrieval Reagents (15 min)
- 2 x H_20 RNase free washing step
- 1 x 2 min in EtOH p.a. at RT
- air dry slides ON at RT
- draw barrier around each section with hydrophobic barrier pen

RNAscope® 2.5 assay

- prepare all materials after manual guideline (oven, water bath, target probes,washing buffer)
- apply RNAscope® Protease Plus (30 min) at 40°C in oven
- wash with H_20 RNase free
- apply RNAscope® Target probe (mM-ETAR, mM-ETBR) for 2 h at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 1**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 2**) for 15 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 3**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 4**) for 45 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 5**) for 30 min at RT
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 6**) for 30 min at RT

- Detection of signal with DAB-A and DAB-B solution after manual guidelines (1:1; 10 min)
- 2 x 2 min 50 % hematoxylin staining solution (Gill's hematoxylin)
- 3 x washing step with H_20 RNase free until slides are clear
- 1x 10 sec 0.02 % ammonium hydroxide-solution
- 3-5 times with H_20 RNase free washing step
- mount slides and place coverslip over the section (air dry > 5 min)

BASEscope® Detection Regant Kit-Red

- prepare all materials after manual guideline (oven, water bath, target probes,washing buffer)
- apply BASEscope® Protease III (30 min) at 40°C in oven
- wash with H_20 RNase free
- apply BASEscope® Target probe (mM-ETBR) for 2 h at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 0**) for 30 min at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 1**) for 15 min at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 2**) for 30 min at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 3**) for 30 min at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 4**) for 15 min at 40°C
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 5**) for 30 min at RT
- 2 x 2 min washing step with BASEscope® washing buffer
- Hybridize signal amplification molecule (**AMP 6**) for 15 min at RT
- Detection of signal with RED-A and RED-B solution after manual guidelines (1:60;10 min)
- 2 x 2 min 50 % hematoxylin staining solution (Gill's hematoxylin)
- 3 x washing step with H_20 RNase free until slides are clear
- 1x 10 sec 0.02 % ammonium hydroxide-solution
- 3-5 times with H_20 RNase free washing step
- Incubate for 15 min in dry oven at 60 °C
- Mount slides and place coverslip over the section (air dry > 5 min)

RNAscope® 2.5 Duplex Detection kit:

- prepare all materials after manual guideline (oven, water bath, target probes, washing buffer)
- apply RNAscope® Protease Plus (30 min) at 40°C in oven
- warm probes for 10 min at 40°C; mix 1:50 ratio of C2 (PDGFRβ) probe to C1 (ETBR) probe
- put mixture for 2 h at 40°C in the oven
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 1**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 2**) for 15 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 3**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 4**) for 15 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 5**) for 30 min at RT
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 6**) for 15 min at RT
- 2 x 2 min washing step with RNAscope® washing buffer
- Detection of RED Signal (1:60 Red-B to Red-A); 10 min at RT on slides
- Hybridize signal amplification molecule (**AMP 7**) for 15 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 8**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 9**) for 30 min at 40°C
- 2 x 2 min washing step with RNAscope® washing buffer
- Hybridize signal amplification molecule (**AMP 10**) for 15 min at RT
- 2 x 2 min washing step with RNAscope® washing buffer
- Detection of Green Signal (1:50 Green-B to Green-A); 10 min at RT on slides
- 2 x 2 min 50 % hematoxylin staining solution (Gill's Hematoxylin)
- 3 x washing step with H_20 RNase free until slides are clear
- 1x 10 sec 0.02 % ammonium hydroxide-solution
- 3-5 times with H_20 RNase free washing step
- incubate for 60 min in dry oven at 60 °C

- mount slides and place coverslip over the section (air dry > 5 min)

For additional staining (IHC) with antibodies after RNAscope® 2.5 assay and BASEscope® assay

- after last washing step with H_20 RNase free; slides were incubated for 3 x 10 min in PBS
- incubate with blocking solution (10 % horse serum in 1 % BSA/PBS-Otto) for 1 h at RT
- incubate with first antibody (diluted in blocking solution) in a moist chamber over night at 4°C
- 3 x washing with 1 % BSA/PBS-Otto for 5 min each
- incubate with second antibody (fluorophore conjugated antibody; 1:400): 90 min at RT in a moist chamber (dark surrounding)
- 3 x washing with PBS-Otto for 5 min each
- embedding sections with glycergel mounting medium

2.5 Analyzing and statistics

The program Microsoft® Excel (Microsoft® Corporation, Redmond, USA) was used for analyzing numeric data. General ‘Students T-Test’ was performed for statistical analysis whereas significant differences were presumed as $p < 0.05$ (*) and highly significant differences as $p < 0.01$ (**). Statistical difference compared to controls was marked directly above SEM (standard error of the mean)-bar. The software GraphPad Prism 5 was used for all presented graphs.

3 Results

3.1 Localization of ETA- and ETB-receptor in the adult murine kidney

In order to study the potential role of ETA- and/or ETB-receptor for the renin synthesis and secretion *in vivo* and *in vitro* localization of ET receptors in the mouse kidney was provided by immunofluorescence staining for ETAR and due to the lack of a working antibody by in situ hybridization RNAscope/BASEscope® assay for the ETBR (protocol 2.3 and 2.4.5).

ETAR expression was localized on renin producing cells (Fig.7A) regardless of whether renin cells were localized in classical juxtaglomerular or in recruited or ectopic position (Fig.7B,C). These findings indicate ETAR as a general marker of renin producing cells in the kidney. Furthermore, slight ETAR expression could be detected on mesangial cells (Fig.8B) whereas smooth muscle cells (Fig.8A), distal tubular structures (Fig.8C) and collecting duct cells (Fig.8D) (Table 8) show strong expression for ETAR.

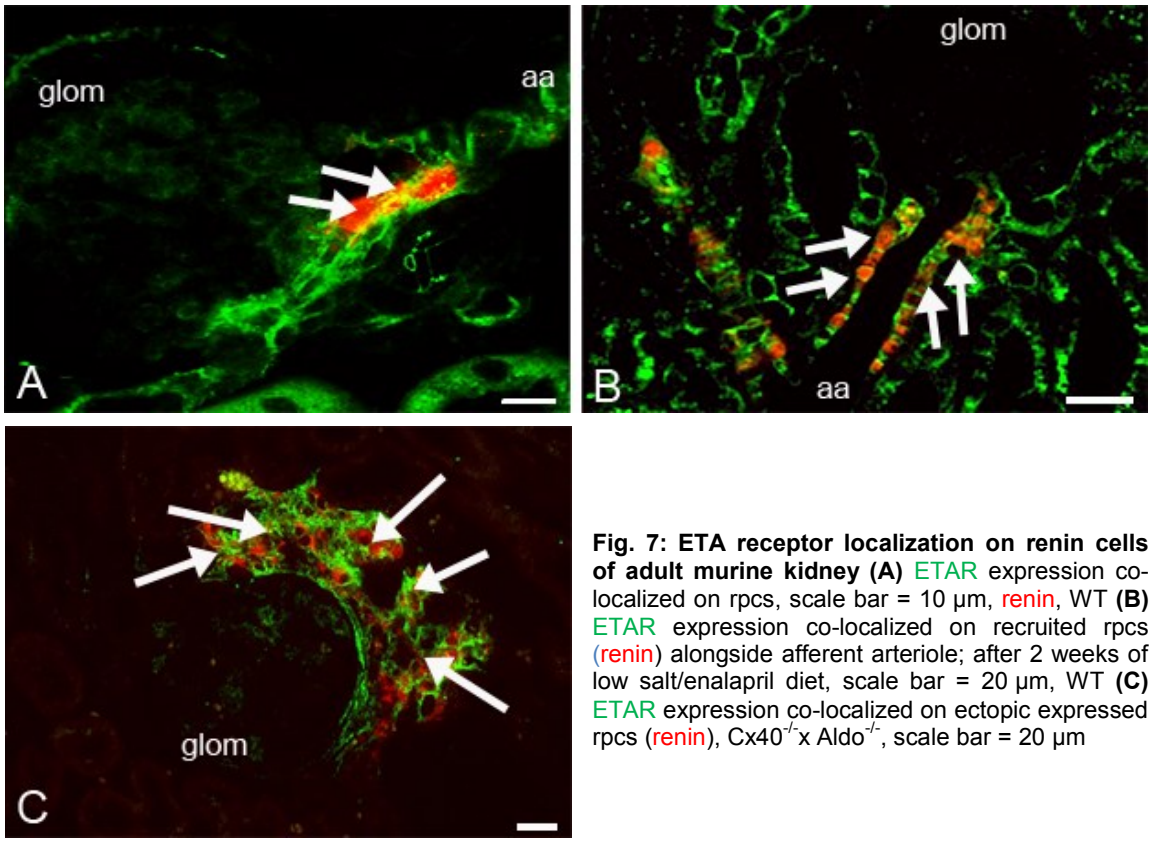

Fig. 7: ETA receptor localization on renin cells of adult murine kidney (A) ETAR expression co-localized on rpcs, scale bar = 10 µm, renin, WT **(B)** ETAR expression co-localized on recruited rpcs (renin) alongside afferent arteriole; after 2 weeks of low salt/enalapril diet, scale bar = 20 µm, WT **(C)** ETAR expression co-localized on ectopic expressed rpcs (renin), Cx40$^{-/-}$x Aldo$^{-/-}$, scale bar = 20 µm

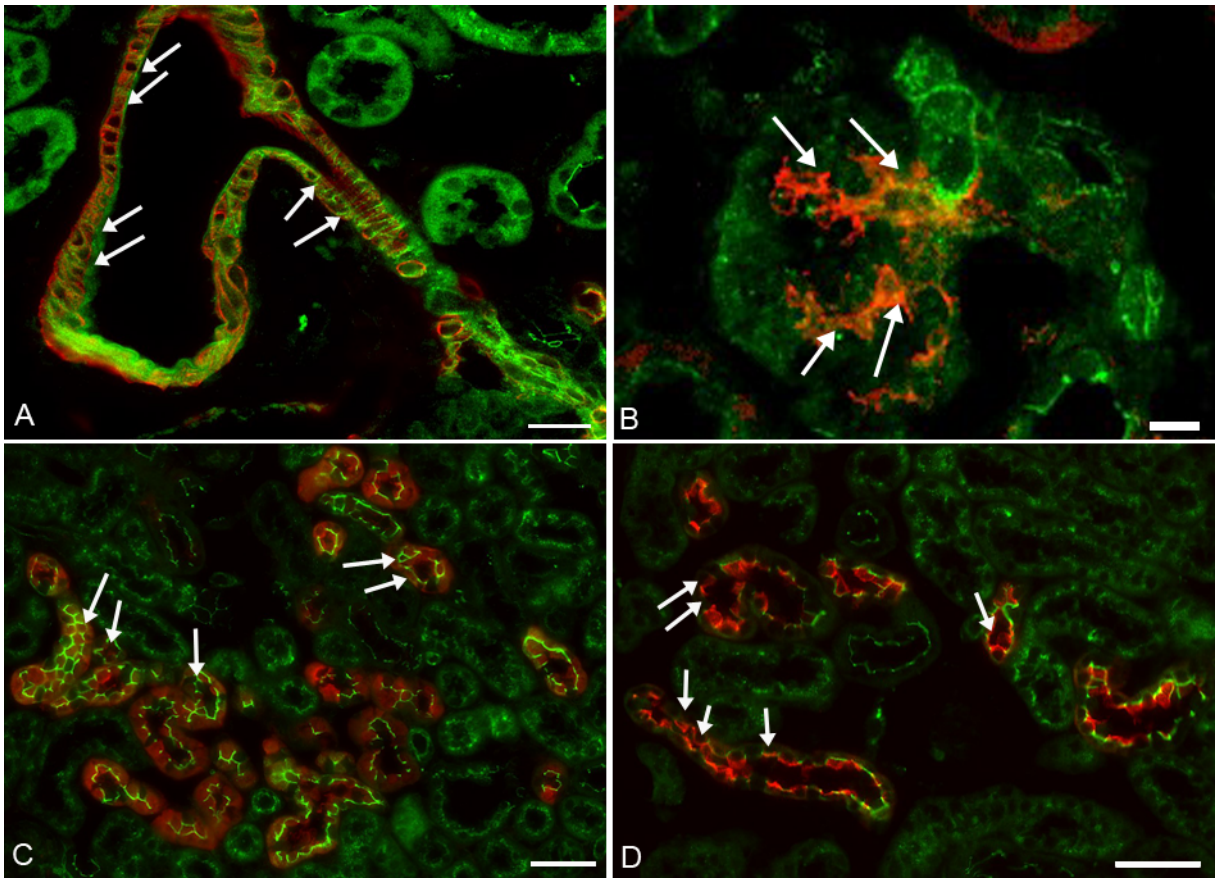

Fig. 8: ETA receptor localization in the adult murine kidney (A) ETAR expression co-localized on smooth muscle cells (α-sma), scale bar = 20 µm **(B)** ETAR expr. co-localized on mesangial cells (α-integrin-8), scale bar = 10 µm, WT**)** ETAR expression co-localized on distal tubular structures (calbindin), scale bar = 50 µm, WT **(D)** ETAR expr. co-localized on collecting duct cells (AQP2), scale bar = 50 µm, WT

In order to localize the expression of ETBR in the adult murine kidney, experiments with ISH RNAScope/BASEscope® assay from ACD were performed (Fig.9,10). ETBR mRNA signals could be localized not only on renin producing JG cells (Fig.9A), but additionally on recruited renin producing cells as shown in Fig.9B. Similar to ETAR, the ETB-receptor seems to be a general marker for renin positive cells, too.

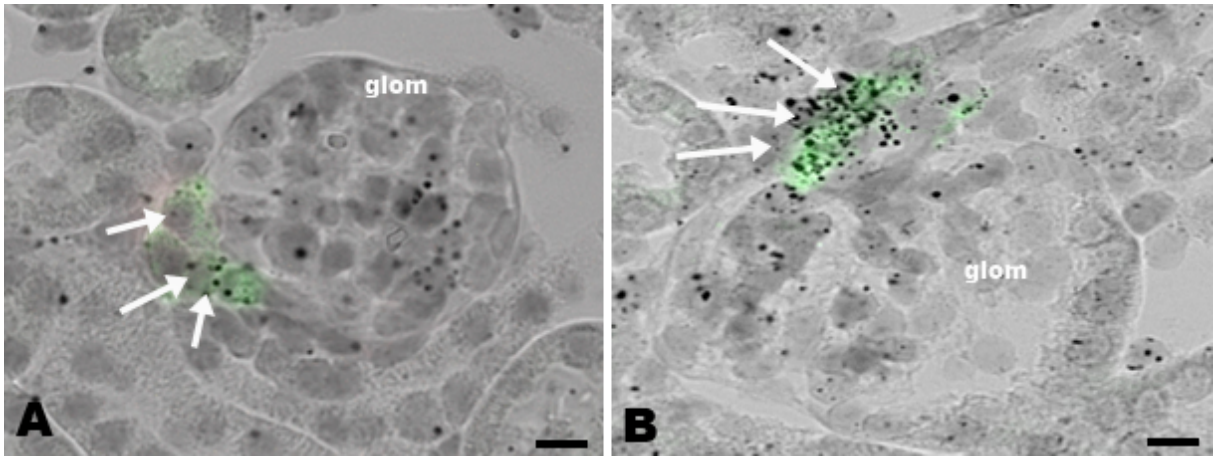

Fig. 9: Localization of ETBR on renin cells of adult murine kidney of WT kidney sections with ISH technique RNAscope® and Basescope®; ETBR mRNA signal detection co-localized on **(A)** rpcs (renin), scale bar = 10 µm **(B)** recruited renin positive cells (renin) after 2 weeks of low salt/enalapril diet; scale bar = 10 µm

Moreover, ETB-receptor is known to be widely distributed in the kidney. ETB receptor mRNA signal could be detected on mesangial cells (Fig.10A), distal tubular cells (Table 8), proximal tubular cells (Table 8), cortical and medullary endothelial cells (Fig.10C,D), cortical and medullary collecting duct cells (Table8), smooth muscle cells (Table 8) and interstitial cells (Fig.10B) on mouse kidney sections.

As mentioned above, ETBR mRNA signal could also be detected in cortical and medullary interstitial cells. Co-localization analysis with RNAscope® DUPLEX assay could detect ETBR expression widely distributed on PDGFRβ$^+$ cells, a marker for pericytes (Fig.10B). However, it should be noted at this juncture, that not all renal PDGFRβ$^+$ cells express ETBR signal and *vice versa.*

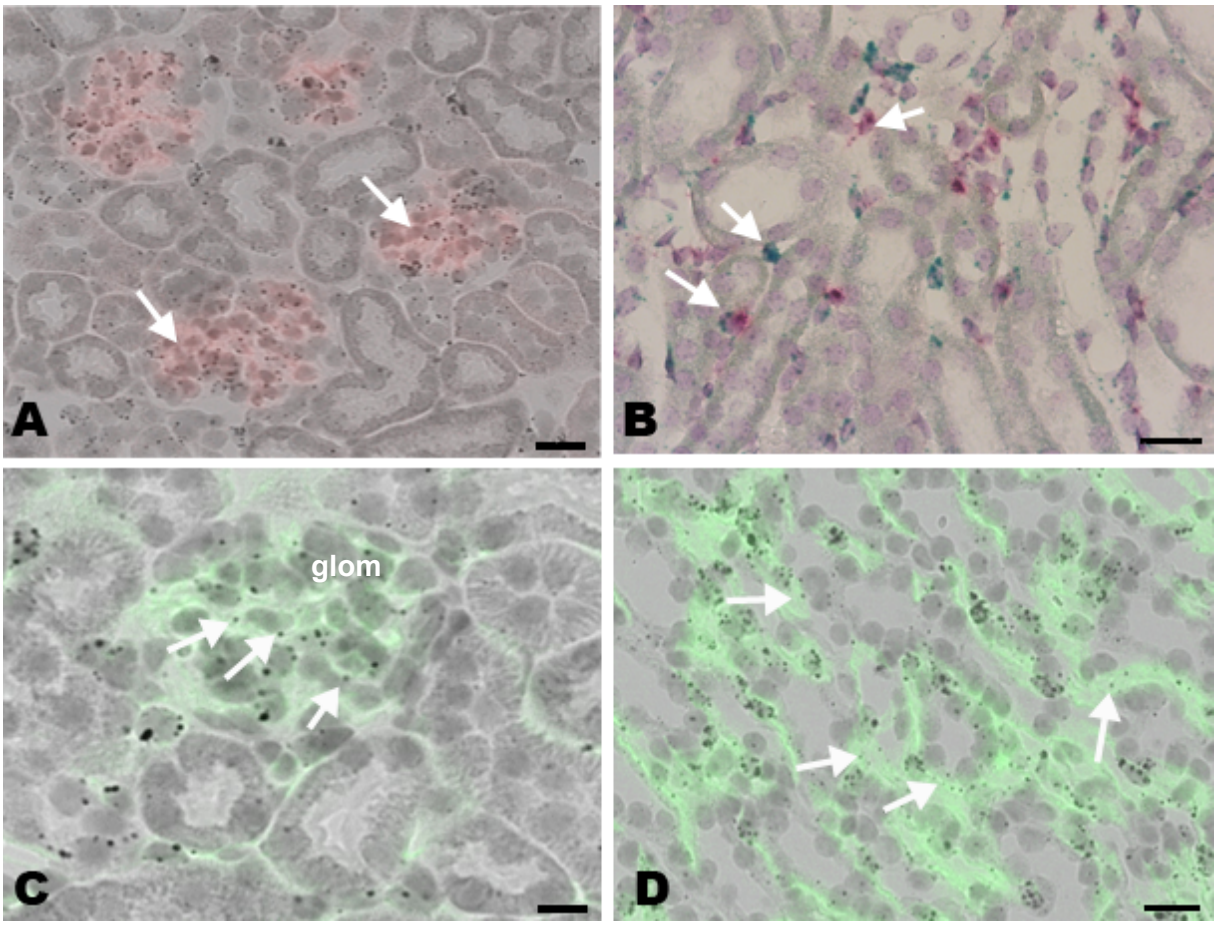

Fig.10: Localization of ETBR in the adult murine kidney of WT kidney sections with ISH technique RNAscope® and Basescope®; ETBR mRNA signal detection co-localized on **(A)** mesangial cells (integrin-8), scale bar = 20 µm **(B)** Duplex Assay RNAscope® ISH; ETBR mRNA signal on interstitial cells (PDGFRβ), **scale bar =** 20 µm **(C)** cortical endothelial cells (CD31), scale bar = 10 µm **(D)** medullary endothelial cells (CD31), scale bar = 20 µm

The location as well as the quantity of ETA- and ETB-receptor expression in the murine kidney is summarized in Table 8.

renal cells		ETA-receptor	ETB-receptor
renin producing JG cells		++	++
smooth muscle cells		++	+
mesangial cells	extraglomerular	+	-
	intraglomerular	+	++
endothelial cells		-	++
proximal tubular cells		-	+
distal tubular cells		++	+
collecting duct	cortical	++	++
	medullary	+	+
interstitial cells	cortical	-	+
	medullary	-	++

Table 8: Localization and quantity of ETA- and ETB-receptor expression in the adult murine kidney after analysis; immunohistochemistry and in situ hybridization RNAscope/BASEscope technique assay; ++ strong expression; + weak expression

3.2 Role of ETA-receptor isoform on renin synthesis and secretion *in vivo*

3.2.1 Verification of renin cell-specific deletion of ETAR ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$)

In order to study the potential direct effect of ET-1/ETA-receptor pathway for the renin synthesis and secretion it was necessary to generate a renin cell-specific ETAR animal model with the help of the Cre/loxP system (ETAR loxP animals used for present study with kind permission of Yanagisawa M. from Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas USA; $Ren^{1d+/Cre}$ animals were used with kind permission of Gomez RA from University of Virginia, Charlottesville, USA). Due to the strong expression of ETA-receptor on renin producing JG cells and the fact that ET-1 inhibits renin in a Ca^{2+}-dependent manner, it was inevitable to hypothesize a potential direct inhibiting role of ETAR on renin synthesis and secretion. Therefore, mice genotyped as $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$ were considered as knockout animals with loss of ETAR exclusively on renin producing cells. Whereas mice with no active Cre-recombinase were determined as control animals ($Ren^{1d+/+}$-

ETAR$^{fl/fl}$ or ETAR$^{fl/fl}$). In order to proof present deletion on renin producing cells, IHC with given antibodies was performed on paraffin embedded kidney sections (Fig.11). The efficacy of deletion could be verified after analyzing the section with the fluorescent microscope. The ETAR expression only lacked on renin producing juxtaglomerular cells in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ animals compared to control littermates (ETAR$^{fl/fl}$) (Fig.9), whereas it was maintained on smooth muscle cells, mesangial cells and tubular structures.

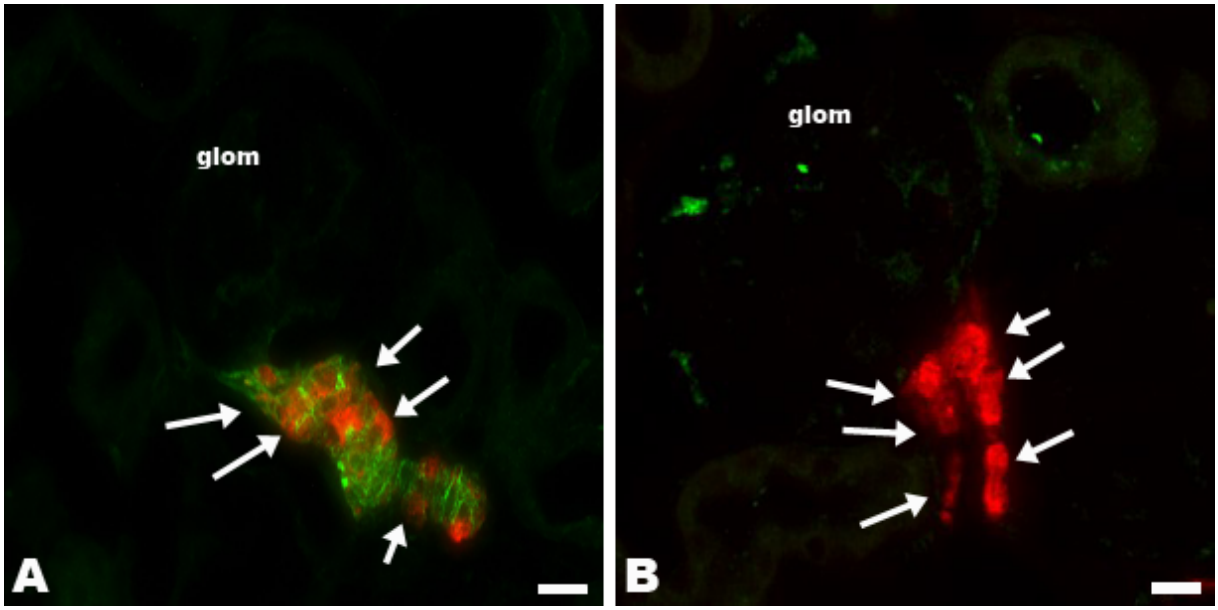

Fig. 11: Verification of renin cell-specific deletion of ETAR on kidney sections (A) ETAR$^{fl/fl}$ as controls; with ETAR, renin, scale bar = 10 µm **(B)** Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$;ETAR, renin, scale bar = 10 µm after a two week low salt + enalapril diet**;** ETAR expression only lacks on rpcs

3.2.2 Characterization of potential direct effect of ETAR on renin synthesis and secretion *in vivo*

In order to study a potential direct effect of ETAR on the level of renin producing cells on the renin synthesis and secretion *in vivo,* relative mRNA abundance, plasma renin concentration levels and systolic blood pressure was detected under basal conditions first. The results showed that relative renin mRNA abundance (Fig.12A) and plasma renin concentration (Fig.12B) were unchanged in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ compared to control littermates. In addition, Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ had normal systolic blood pressure values (144 ± 1.920) relative to ETAR$^{fl/fl}$ animals (139.1 ± 1.818) (Fig.10C) and as mentioned in 3.2.1 normal developed juxtaglomerular cells under basal conditions after performing immunohistochemistry on kidney sections.

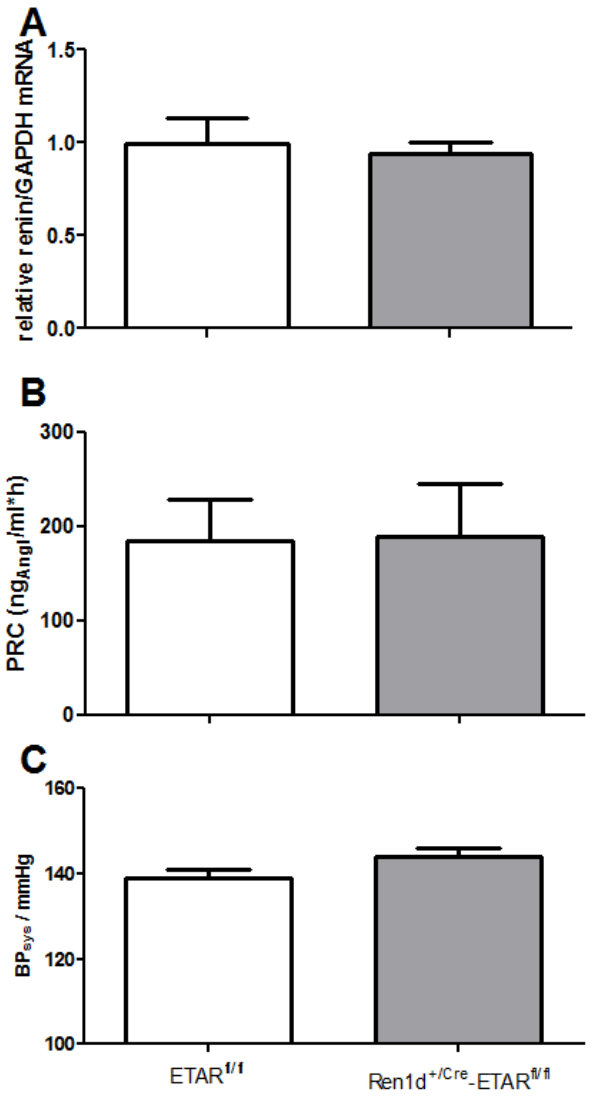

Fig.12: Characterization of potential role of ETAR isoform expressed on rpcs on renin system *in vivo* **(A)** relative renin mRNA abundance**, (B)** plasma renin concentration and **(C)** blood pressure measurement via tail cuff method was unchanged in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ compared to controls (ETAR$^{fl/fl}$) under basal conditions; n=5

In order to investigate the role of ETA-receptor on the physiological regulation of the renin system, Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ animals got a two week low salt diet (0.02 % NaCl) with ongoing enalapril intake to stimulate renin expression and therefore RAAS. A diet with low concentration of sodium leads to a stimulation of RAAS and therefore to a retrograde recruitment of renin producing cells along the afferent arteriole and an increased number of renin positive cells. In order to study the role of ETA-receptor on the suppression of RAAS, Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ mice got a 2 week high salt diet (4 % NaCl).The chronic modulation of the renin system (Fig.13 A,B) , especially feeding animals with a low salt diet increased renin mRNA abundance 4-fold and PRC 12-fold, without any difference between controls and Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$. Whereas feeding a high salt diet lowered PRC values by 69% without any

difference between the two genotypes (Fig.13 A,B). Untreated adult Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ mice had similar renin mRNA and PRC levels compared to controls.

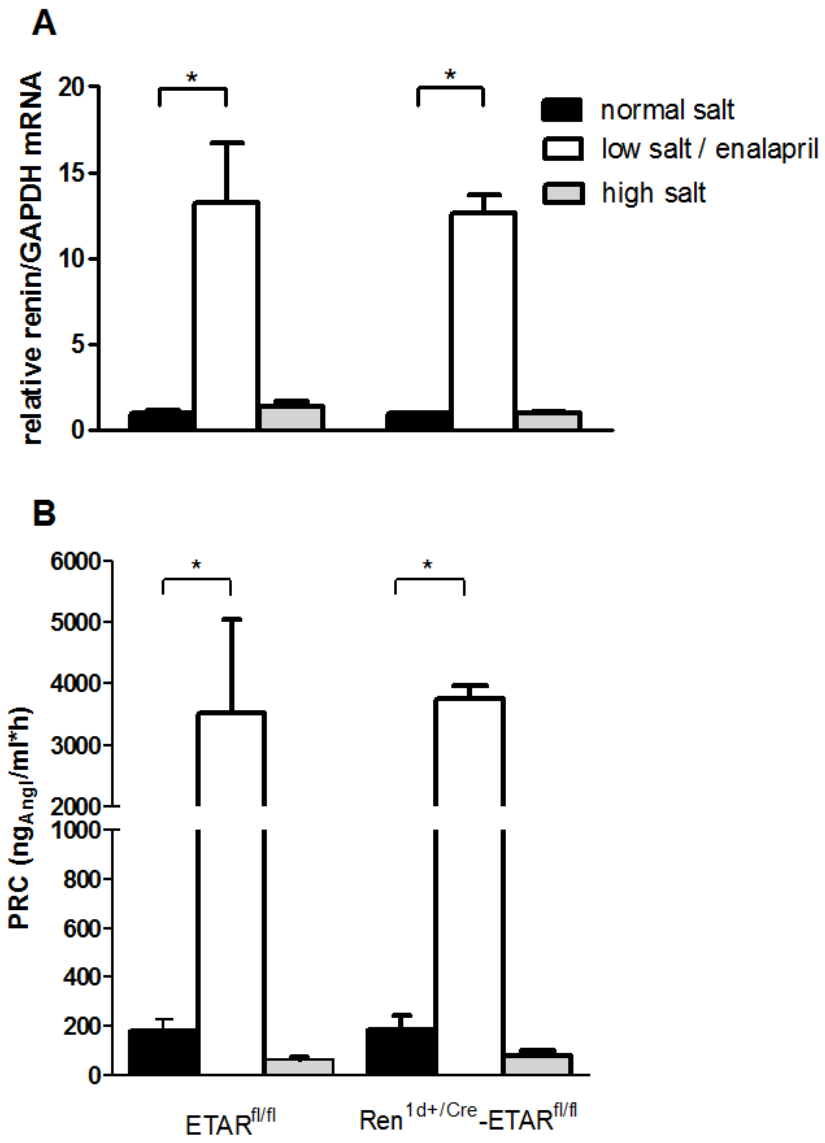

Fig. 13: Characterization of potential role of ETAR isoform expressed on rpcs on stimulated and suppressed renin system *in vivo* (A) relative renin mRNA abundance**, (B)** plasma renin concentration and was unchanged in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ compared to controls (ETAR$^{fl/fl}$) under basal conditions and under low and high salt diet for two weeks; a low salt diet lead to an significantly increase of renin mRNA abundance and PRC levels with no difference in both groups; whereas a high salt diet leads to an slightly decrease of renin mRNA and PRC level in both genotypes; $p<0.05$;n=5

3.2.3 Role of ETA-receptor on the renin cell lineage for renin secretion *in vitro*

With respect to previous findings, the ETA-receptor has no essential role on the renin synthesis *in vivo*. Therefore it was inevitable to further investigate the role of ETAR located on renin producing cells for renin secretion *in vitro.*

In the isolated perfused kidney model (2.2.3) of control animals (ETAR$_{fl/fl}$) ET-1 was added to the *ex situ* system with increasing concentrations (30 pM, 100 pM, 300 pM and 1 nM ET-1),

resulting in an inhibition of renin secretion (Fig.14A). It should be mentioned at this juncture that the renin secretion is the product of renal blood flow and renin concentration (data not shown).The inhibition of renin secretion is mainly mediated through a reduction of renal blood flow (ml/g*organ weight*min^{-1}) (Fig.14B). In adult Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ animals, similar effects could be determined. The deletion of ETAR on renin producing JG cells did not markedly influence the inhibition of renin secretion through ET-1. Whereas renin secretion rate was declined to 80 % in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ compared to a decrease of 90 % in controls. Further, the inhibition of renin secretion was mainly mediated by a decline of the renal blood flow. The flow decreases through ET-1 from nearly 10 ml/min*g to 2 ml/min*g organ weight. Whereas renin concentration (ANG I/ml*h) was unchanged in both genotypes after adding ET-1 to the system (data not shown).

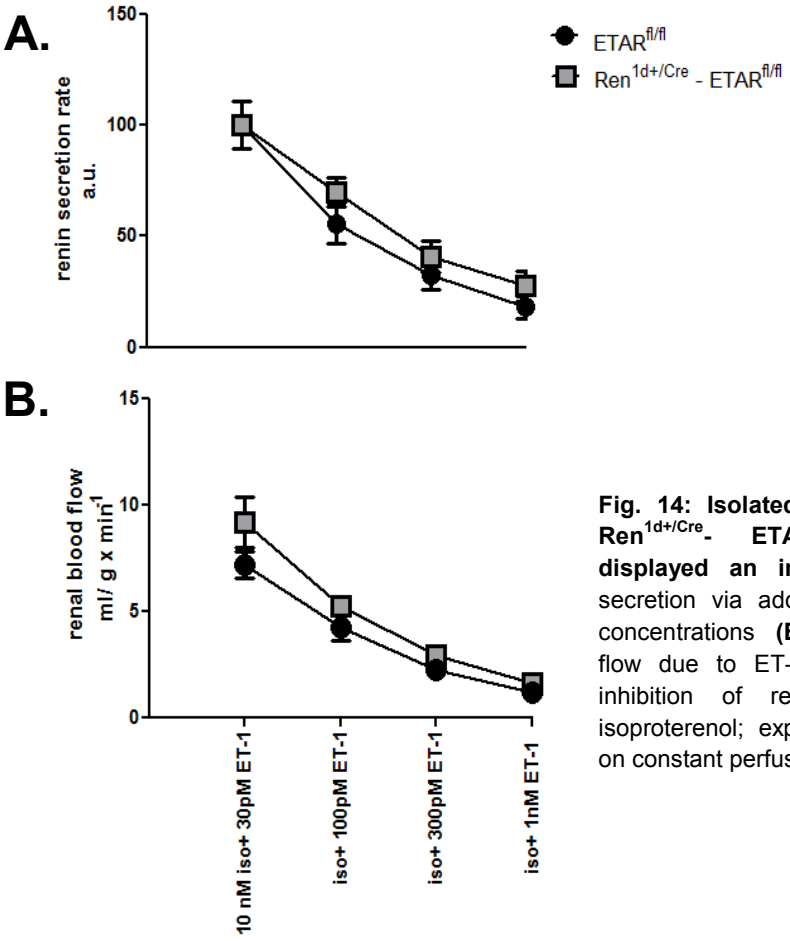

Fig. 14: Isolated perfused kidneys of Ren$^{1d+/Cre}$- ETAR$^{fl/fl}$ and controls displayed an inhibition of (A) renin secretion via adding ET-1 in increasing concentrations **(B)** decline of renal blood flow due to ET-1;flow mainly mediates inhibition of renin secretion; iso = isoproterenol; experiment was performed on constant perfusion pressure

3.2.4 Characterization of renal ETA-receptor isoform and its potential indirect effects on renin synthesis and secretion *in vivo*

All measured *in vivo* parameters mentioned in 3.2 indicate for the first time that ETAR seems to be of less relevance for the physiological relevance of renin synthesis and secretion *in vivo*. Furthermore, this study suggests rather no essential direct effect of ETAR isoform on

the level of renin positive cells *in vivo* and due to results in the isolated perfused kidney *in vitro.* Therefore, it was necessary to further study a potential indirect effect of renal ETA receptor isoform for renin synthesis and secretion *in vivo.*

Besides the renin producing JG cell area at the vascular pole of the glomerulus, the ETAR is suggested to be also expressed on smooth muscle cells, distal tubular cells, collecting duct cells and mesangial cells in the kidney (as already mentioned in 3.1.1). In order to study a potential general indirect effect of ETAR on the renin system *in vivo*, additional mouse models were generated *a priori* to further understand the crucial role of renal ETAR expression on the renin system.

3.2.4.1 The potential indirect role of renal ETA-receptor isoform on renin synthesis and secretion *in vivo*

To investigate, if the potential role of ETAR for the control of renin synthesis and secretion is based on stroma derived cells in the kidney, ETAR loxP mice were paired with animals holding Cre activation under the control of FoxD1 promotor. All stroma derived cells possess this specific promotor including renin positive JG cells, fibroblasts, pericytes, smooth muscle cells and mesangial cells. Due to renal ETA-receptor isoform expression, $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$ kidneys immunoreactivity of ETAR was lacking on renin producing cells, smooth muscle cells and mesangial cells only compared to controls ($ETAR^{fl/fl}$) (Fig. 15A).

In order to examine, if the potential role of ETAR expressed in the vasculature system for renin synthesis and secretion is regulated by systemic factors such as the blood pressure, mice with inducible Cre-ER^{T2} activation under the control of smooth muscle myosin (*Myh11*) promotor were paired with ETAR loxP animals in order to generate SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$ mice. All Cre-ER^{T2} kidneys showed ETAR immunoreactivity lacking on smooth muscle cells only (Fig. 15B)

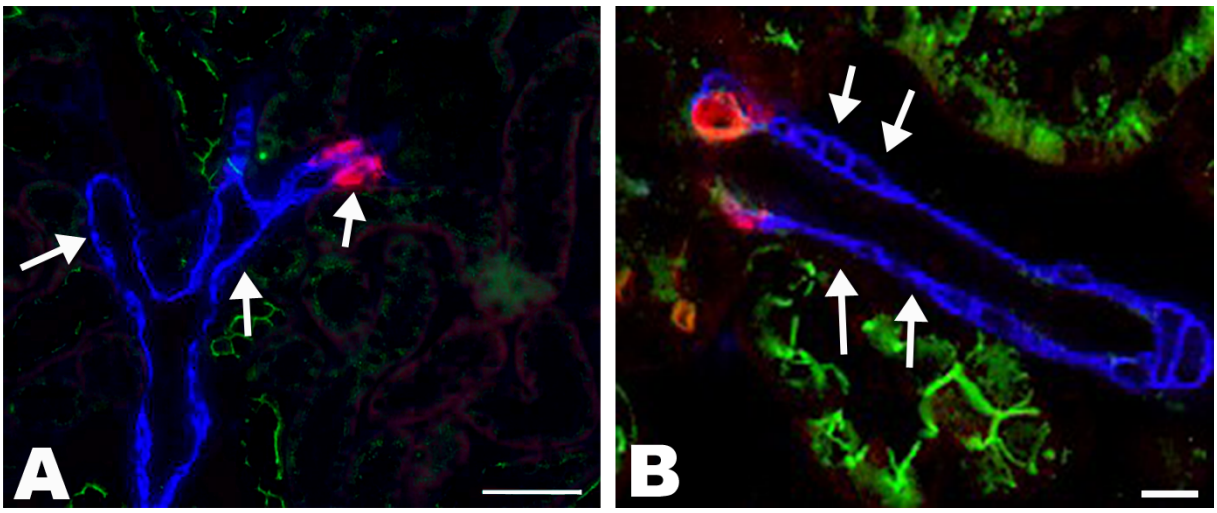

Fig. 15: Verification of cell-specific deletion of ETAR on (A) FoxD1-stroma derived cells (**FOX$^{D1Cre/+}$-ETAR$^{fl/fl}$**); scale bar = 50 µm, ETAR, renin actin **(B)** vascular smooth muscle cells (**SMMHC-Cre-ER^{T2}-ETAR$^{fl/fl}$**);scale bar = 10 µm;ETAR, renin actin

All FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$ animals displayed normal developed renin producing JG cells, unchanged renin mRNA abundance, PRC levels as well as similar systolic blood pressure values compared to controls (Fig. 16). These given results indicate, that deletion of ETAR on renin producing cells, mesangial cells, smooth muscle cells has no significant indirect effect on the regulation of renin synthesis and secretion.

All SMMHC-Cre-ER^{T2}- ETAR$^{fl/fl}$ kidneys showed ETAR immunoreactivity lacking in smooth muscle cells only (Fig. 15B). In addition, all SMMHC-Cre-ER^{T2}- ETAR$^{fl/fl}$ animals showed normal developed JG cells, slightly decreased renin mRNA abundance and unchanged systolic blood pressure values and PRC levels (Fig. 16) compared to controls (ETAR$^{fl/fl}$).

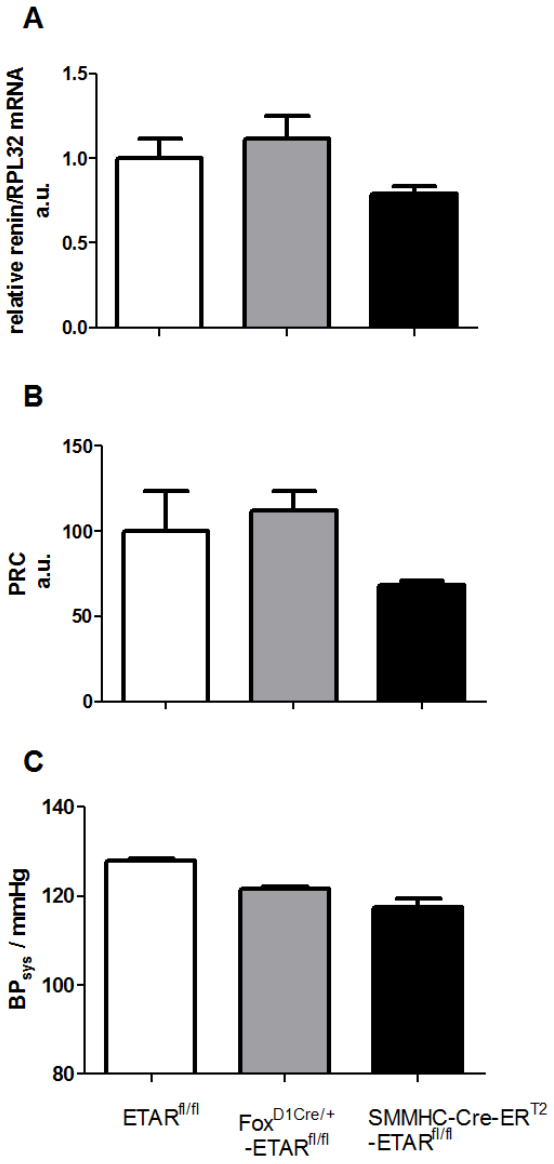

Fig. 16: Characterization of ETA receptor isoform expressed on FOXD1 stroma derived cells and on vascular smooth muscle cells for renin synthesis and secretion (A) relative renin mRNA abundance**, (B)** plasma renin concentration **(C)** and blood pressure measurement via tail cuff method was not essentially changed in FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$ and in SMMHC-Cre-ER^{T2}-ETAR$^{fl/fl}$ compared to controls; n=10 except bpm n=3 each group

To investigate if further ETA-receptor expression locations have a crucial effect on the renin system in general, mice with conditional CAGG-Cre-ER^{T2} recombinase were paired with ETAR loxP animals in order to decrease ETAR expression under the control of β-actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer. All CAGG-Cre-ER^{T2}-ETAR$^{fl/fl}$ kidneys showed a reduction of ETA-receptor expression of 62 % after 3 weeks of tamoxifen chow induction (Fig.17A) compared to controls (ETAR$^{fl/fl}$). In addition, IHC studies on kidney paraffin sections showed a decrease of ETAR expression widely distributed in the kidney compared to controls (Fig 17B).

Moreover, all CAGG-Cre-ER^{T2}- ETAR$^{fl/fl}$ displayed normal developed renin positive JG cells as well as unchanged renin mRNA levels, PRC values and systolic blood pressure levels compared to controls (Fig.18A,B,C).

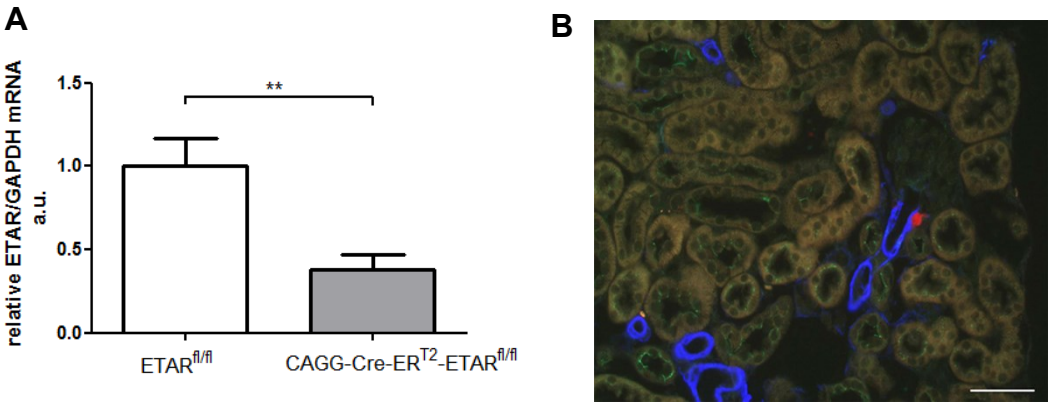

Fig. 17: Verification of reduced expression of ETAR in the kidney due to conditional deletion (A) decline of ETAR expression after tamoxifen induction diet for 3 weeks of 62%; n= 8 each group; p= 0.0044 (**) **(B)** IHC of **CAGG-Cre-ER^{T2}-ETAR$^{fl/fl}$** kidney section, ETAR, renin actin,scale bar = 50 µm, ETAR expression staining with antibody was remarkably reduced on kidney sections

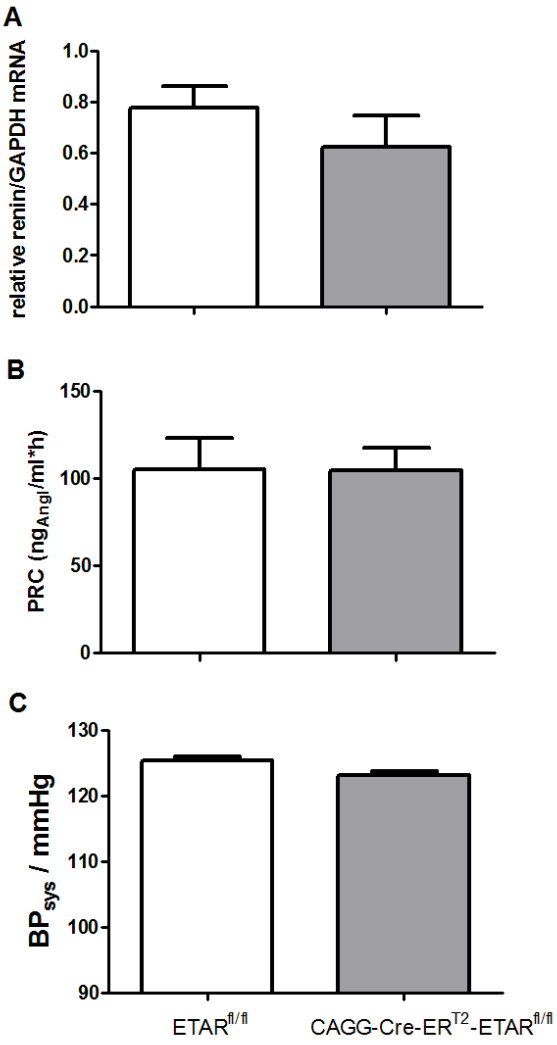

Fig. 18: Characterization of ETA receptor isoform on the renin system in CAGG-Cre-ER^{T2}-ETAR$^{fl/fl}$ (A) relative renin mRNA abundance, n=10 **(B)** plasma renin concentration, n=10 **(C)** blood pressure measurement via tail cuff method was unchanged in CAGG-Cre-ER^{T2} compared to controls; n=3 each group

3.2.4.2 The role of ET-1 after cell specific deletion of ETAR on the renin secretion *in vitro*

After studying the direct effect of ET-1 through ETAR expressed on renin producing JG cells for the renin synthesis and secretion (3.2.3) it was necessary to examine potential indirect effects of ET-1/ETAR signaling pathway on RS in addition. All Cre^{+} strains mentioned and verified in 3.2.4 displayed in the isolated perfused kidney model an inhibition of renin secretion via ET-1 whereas it should be noted that differences occurred between groups with no essential changes compared to controls (Fig.19A). All groups resulted in an inhibition of nearly 80-90 % at 1 nM ET-1.The inhibition is mainly determined through a reduction of renal blood flow (Fig.17B; ~5 ml/g x min^{-1} or below at 1 nM ET-1) and not due to a changes in renin concentration (data not shown). All experiments were performed under same conditions with constant pressure and all animals had similar bodyweights (20 g – 25 g). After adding an increasing concentrations (10 pM, 30, 100, 300 pM and 1nM) of ET-1 to the system, all groups showed a decrease in renin secretion rate. There was no significant change to the controls ($ETAR^{fl/fl}$).

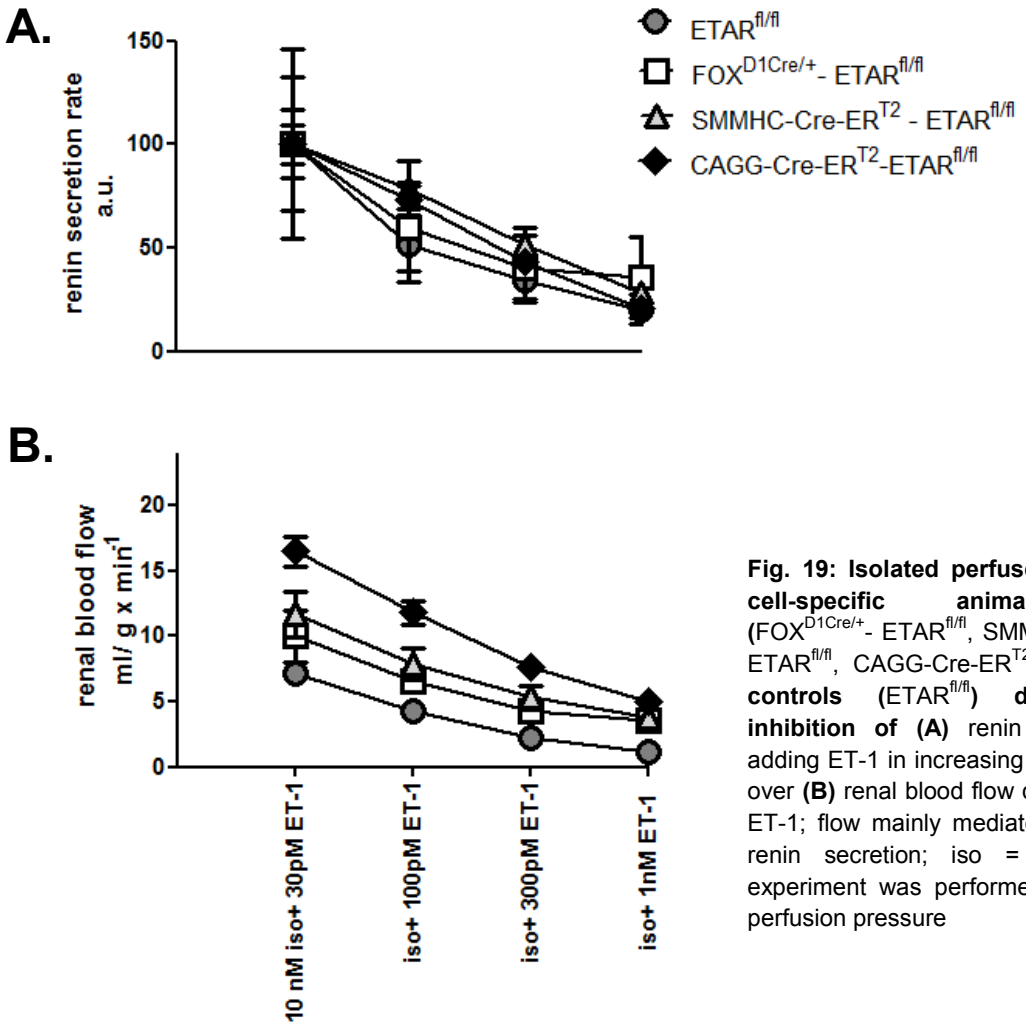

Fig. 19: Isolated perfused kidneys of cell-specific animal models ($FOX^{D1Cre/+}$- $ETAR^{fl/fl}$, SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$, CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$**) and controls** ($ETAR^{fl/fl}$) **displayed an inhibition of (A)** renin secretion via adding ET-1 in increasing concentrations over **(B)** renal blood flow declined due to ET-1; flow mainly mediates inhibition of renin secretion; iso = isoproterenol; experiment was performed on constant perfusion pressure

To summarize the first chapter of the present study, given results show no significant indices for a direct inhibiting effect of ET-1/ETAR signaling pathway on the level of renin positive cells for renin synthesis and secretion *in vivo.* In addition, all adult Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ animals showed normal developed renin positive JG cells, similar systolic blood pressure and PRC values as well as no essential change in renin mRNA abundance compared to controls. Moreover, first results suggest that the deletion of ETAR isoform on smooth muscle cells, stroma derived cells as well as the significant reduction of ETAR expression (SMMHC-Cre-ER^{T2}- ETAR$^{fl/fl}$, FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$, CAGG-Cre-ER^{T2}-ETAR^{flf}) seem to have no essential effect on the renin mRNA abundance, systolic blood pressure values and PRC-levels. The renal ETA-receptor isoform is suggested to be of less relevance for renin synthesis and secretion *in vivo* and *in vitro*.

3.3 Role of ETB-receptor isoform on renin synthesis and secretion *in vivo*

3.3.1 Verification of renin cell-specific deletion of ETBR (Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$)

Previous shown results indicate that ETA-receptor isoform localized on renin producing cells has no essential direct effect on the level for renin and synthesis. The following chapter concentrates on the potential role of ETB receptor isoform located on renin producing cells for renin synthesis and secretion. It should be mentioned at this juncture ETBR mRNA signal could also be detected on renin producing cells as shown in 3.1. Therefore a renin cell-specific ETBR animal model via Cre/loxP system (ETBR loxP animals with kindly permission of Miles L. Epstein from Department of Anatomy, School of medicine and Public Health, University of Wisconsin, Madison, USA) was generated. Mice which were genotyped as Ren$^{1d+/Cre}$- ETBR$^{fl/fl}$, were considered as knockout animals with a loss of ETBR exclusively on renin producing cells, whereas mice, analyzed as Ren$^{1d+/+}$-ETBR$^{fl/fl}$ (or ETBR$^{fl/fl}$) with no active Cre-recombinase were determined as control animals. The efficacy of ETBR deletion was verified by ETB-receptor in situ hybridization BaseScope® assay from ACD on adult kidney sections due to lack of working antibody. In control kidneys (ETBR$^{fl/fl}$) ETB-receptor mRNA signal was found on vascular smooth muscle cells, endothelial cells, proximal tubular cells, collecting duct, mesangial cells, interstitial cells and renin producing JG cells (Table8 and Fig.9,10). In Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ kidneys ETBR mRNA signal was lacking on renin producing cells only (Fig.20B) compared to ETBR$^{fl/fl}$ mice (Fig.20A).

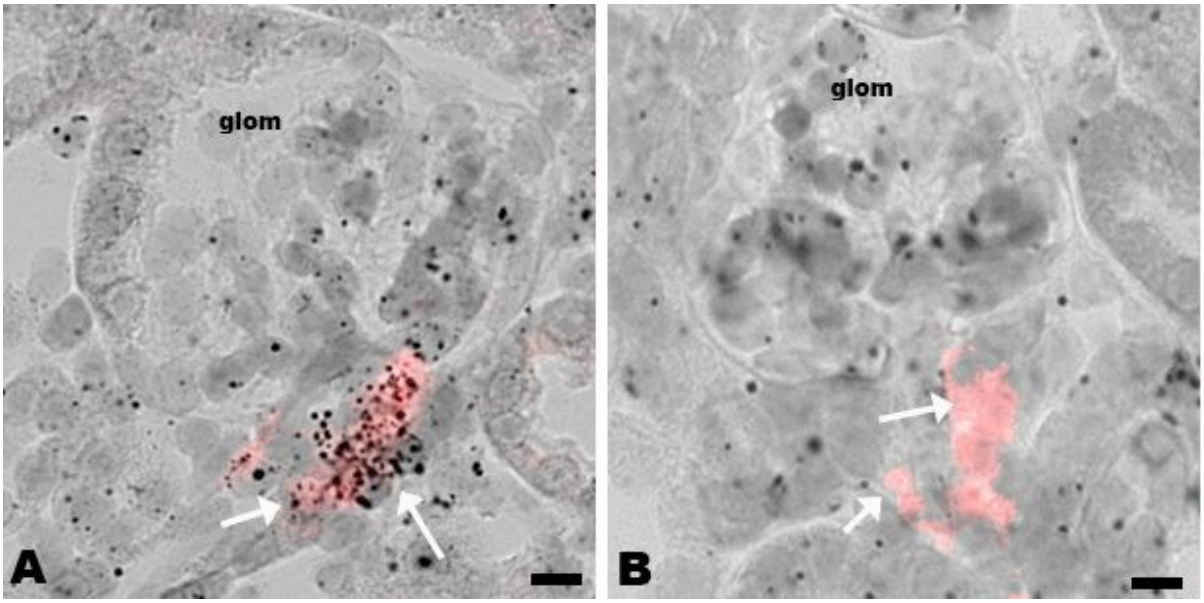

Fig. 20: Verification of renin cell-specific deletion of ETBR via BaseScope® ISH assay with (A) kidney section of ETBR$^{fl/fl}$ after chronic stimulation with low salt diet/enalapril intake for three weeks; ETBR mRNA signal with renin; scale bar = 10 µm **(B)** kidney section of **Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$** after chronic stimulation with low salt diet/enalapril intake for three weeks; ETBR mRNA signal lacked on renin producing cells only; renin; scale bar = 10 µm

3.3.2 Characterization of renal ETB-receptor isoform and its potential direct effect on renin synthesis and secretion *in vivo*

In order to study a potential direct effect of ETBR pathway on the level of renin producing cells on the renin system, more precisely on renin synthesis and secretion *in vivo,* it was necessary to study relative renin mRNA abundance, plasma renin concentration as well as systolic blood pressure under basal conditions first.

All Ren$^{1d+/Cre}$- ETBR$^{fl/fl}$ animals showed unchanged relative renin mRNA abundance and plasma renin concentration compared to controls. In addition, Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ mice had normal systolic blood pressure values (139.8 ±4.897) relative to ETBR$^{fl/fl}$ animals (134.8 ± 1.001) (Fig.21) and normal developed juxtaglomerular renin positive cells after performing immunohistochemistry on kidney sections. These results indicate that ETB-receptor located on renin producing cells seems to be of less relevance for renin synthesis and secretion *in vivo*.

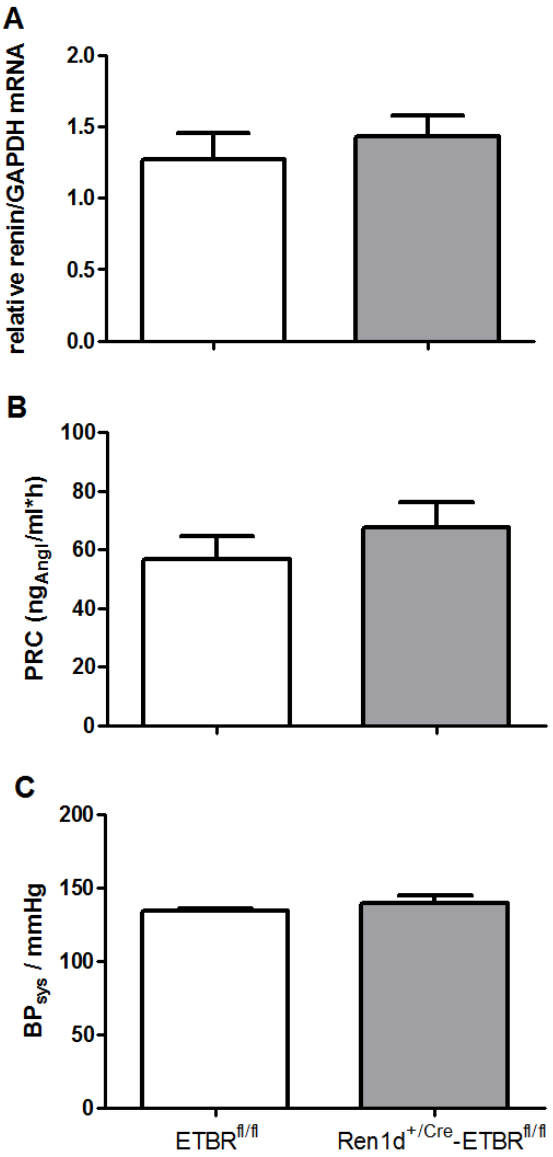

Fig.21: Characterization of potential role of ETBR on renin system *in vivo* (A) relative renin mRNA abundance was unchanged compared to controls under basal conditions and **(B)** plasma renin concentration was unchanged in both models, n=5; **(C)** systolic blood pressure measurement was unchanged in both groups (ETBR$^{fl/fl}$: 134.8 ± 1.001; **Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$:** 139.8 ±4.897); n=4

In order to study if ETBR expressed on renin producing cells is directly involved on the regulation of the renin system, Ren$^{1d+/Cre}$- ETBR$^{fl/fl}$ were treated with either a high salt (4 % NaCl) or low salt (0.02 % NaCl) diet with ongoing enalapril intake for three weeks. (Fig.22). Feeding a low salt/enalapril diet increased renin mRNA abundance 16-fold and PRC 62-fold, without differences between controls and Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$. However, feeding a high salt diet reduced renin mRNA abundance slightly in controls whereas renin levels were significantly decreased in Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ mice compared to ETBR$^{fl/fl}$ after normal salt (p=0.0012) and high salt diet (p=0.006) (Fig.22A). Moreover, Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ showed unchanged PRC-levels after high salt diet compared to controls (Fig.22B).

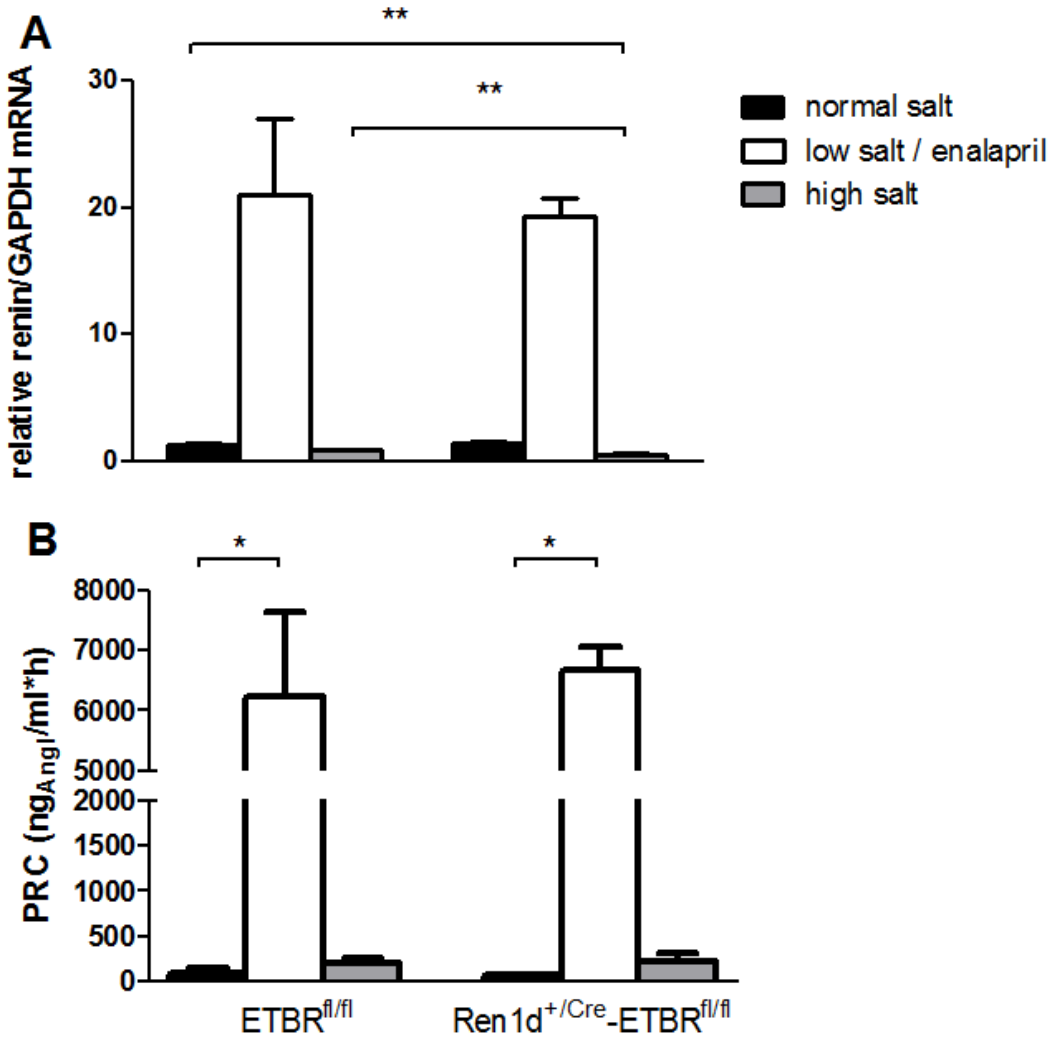

Fig. 22: Characterization of potential role of ETBR on the physiological control of the renin system *in vivo* (A) relative renin mRNA abundance was unchanged compared to controls under basal conditions and after 3 week of LSE in both genotype groups; relative renin mRNA levels were significantly decreased in **Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$** after high salt diet compared to NS-controls (p=0.0012) and HS-controls (p=0.006), n=5 **(B)** plasma renin concentration was unchanged in both models under basal conditions and after 3 week of LSE and HS relative renin mRNA levels were significantly increased after LSE (p<0.05); n=5;

3.3.3 Role of ETB-receptor isoform on the renin cell lineage for renin secretion *in vitro*

To further study the potential role of the ET-1/ETBR pathway on renin secretion, isolated perfused kidney experiments were performed in adult Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ animals (2.2.3 and for protocol and setup). The renin cell-specific deletion of ETBR did not influence the inhibition of renin secretion through ET-1 (Fig.23). After adding ET-1 (30 pM, 100 pM, 300 pM and 1 nM ET-1) in increasing concentrations to the *ex situ* system, Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ kidneys displayed an inhibition of renin secretion. (Fig.23A) mainly mediated through a reduction of renal blood flow (ml/ g x min^{-1}) as in controls (Fig.23B; <5 ml/min x g). All

experiments were performed under same condition with constant pressure and all animals had similar bodyweights (20 g – 25 g).

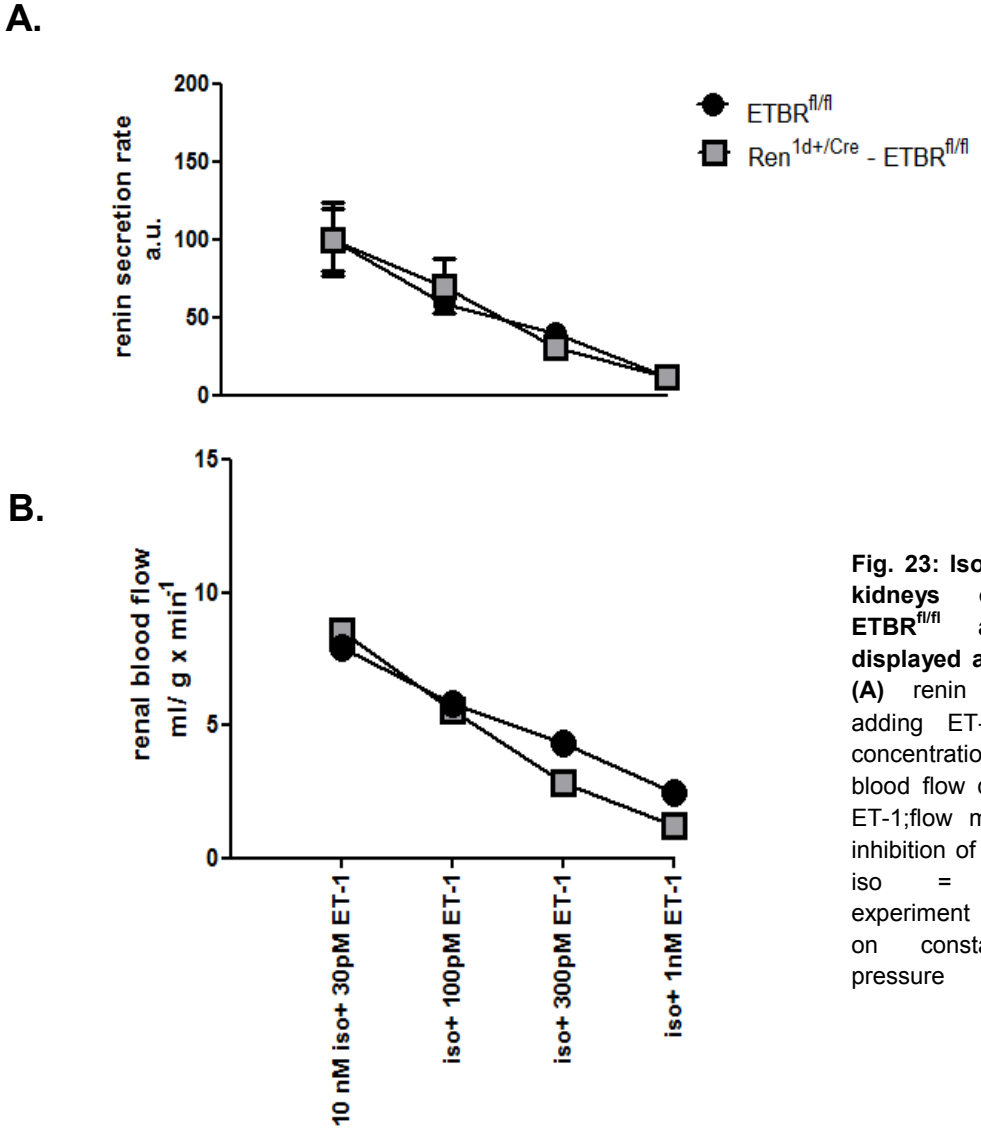

Fig. 23: Isolated perfused kidneys of Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ and controls displayed an inhibition of (A) renin secretion via adding ET-1 in different concentrations **(B)** renal blood flow declined due to ET-1;flow mainly mediates inhibition of renin secretion; iso = isoproterenol; experiment was performed on constant perfusion pressure

With respect to results of the second chapter of present study, it can be concluded that there are no essential indices for a direct involvement of ETBR isoform located on the level of renin positive cells for renin synthesis or secretion and in addition on the physiological regulation of the renin system *in vivo.* Moreover, all adult Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ animals showed normal developed renin positive JG cells, similar systolic blood pressure and PRC values as well as no essential change in renin mRNA abundance compared to controls. The inhibition of renin secretion in Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$ isolated perfused kidneys is primarily mediated by the renal blood flow as shown in Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$ experiments (3.2.3).

3.4 Role of ETA and ETB-receptors on renin synthesis and secretion *in vivo*

Previous shown results indicate that ETA- and ETB-receptor isoforms located on renin positive JG cells, are of less relevance for the physiological control of renin synthesis and secretion *in vivo*. In order to exclude possible ET-receptors interactions, it was necessary to further study a potential direct influence of both ET-receptors for renin synthesis and secretion *in vivo*.

3.4.1 Verification of renin cell-specific deletion of both ET receptors (Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$)

In order to study the potential role of both ET-receptors on the level of renin producing cells for renin synthesis and secretion, it was necessary to generate a renin cell-specific ETAR and ETBR animal model via Cre/loxP system. Mice which were genotyped as Ren$^{1d+/Cre}$-ETBR$^{fl/fl}$-ETAR$^{fl/fl}$, were considered as knockout animals with a loss of ETAR and ETBR exclusively on renin producing cells. Whereas mice, analyzed as Ren$^{1d+/+}$-ETBR$^{fl/fl}$-ETAR$^{fl/fl}$ (or ETBR$^{fl/fl}$-ETAR$^{fl/fl}$) with no active Cre-recombinase were determined as control animals. The efficacy of ETAR and ETBR deletion was verified by either ETAR immunohistochemistry (Fig.24A) or ETB-receptor in situ hybridization BaseScope® assay from ACD (Fig.24B) on adult kidney sections. In control kidneys both receptors were present on renin producing JG cells.

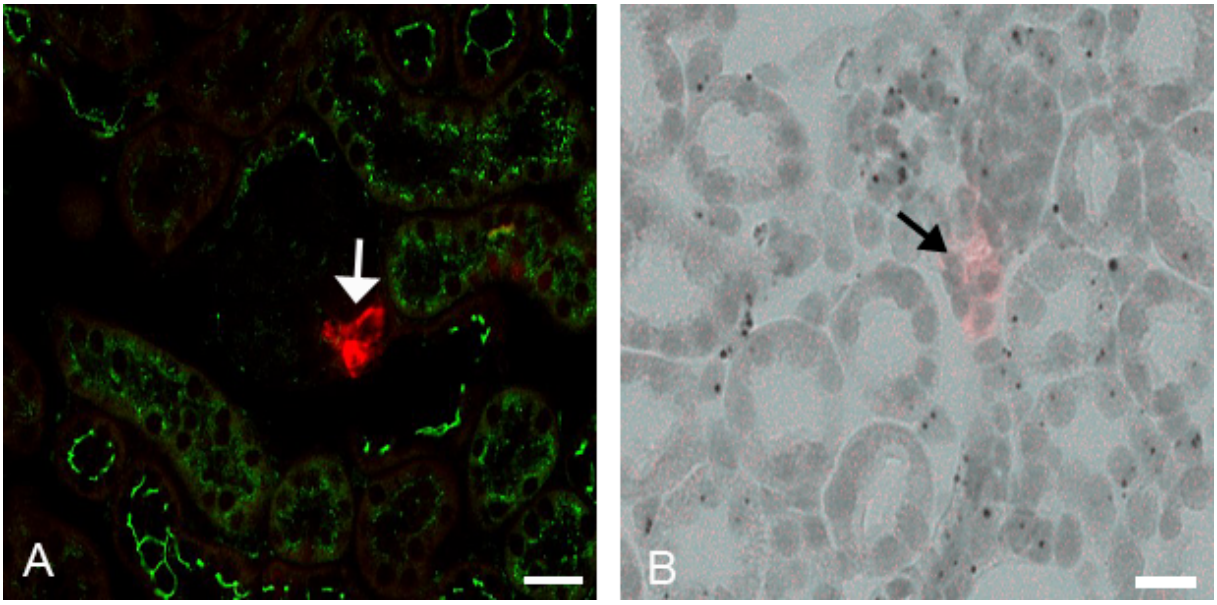

Fig. 24: Verification of renin cell-specific deletion of (A) ETAR and (B) ETBR of Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$; (A) IHC with ETAR, renin ,scale bar = 20 µm; ETAR lacked exclusively on rpcs of Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$ **(B)** ETBR BASEscope® assay of **Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$** with additional IHC with renin; scale bar = 20 µm; ETBR lacked on rpcs of Ren$^{1d+/Cre}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$

3.4.2 Investigations to study a potential direct effect of ETA and ETB receptors expressed on renin producing juxtaglomerular cells for renin synthesis and secretion *in vivo* and *in vitro*

After verification of ETA- and ETB-receptor renin cell-specific knockout animal model with in situ hybridization BASEscope® from ACD and IHC (Fig.24), it was necessary to study the potential effect on the renin system further. Mice which were genotyped as $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ were considered as knockout animals with a complete loss of ET-receptors on renin producing cells. Whereas mice, analyzed as $Ren^{1d+/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ (or $ETAR^{fl/fl}$-$ETBR^{fl/fl}$) with no active Cre-recombinase were determined as control animals. All $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals displayed normal renin mRNA abundance (Fig.25A) and normal systolic blood pressure values (Fig.25C) as in controls under basal conditions. In addition, plasma renin concentration levels were reduced in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals compared to control littermates (Fig.25B) but showed no significant difference in both groups. All *in vivo* experiments were performed with adult mice only. Histological staining on kidney sections revealed normal developed renin positive cells at the juxtaglomerular cell apparatus in the kidney (Fig.24).

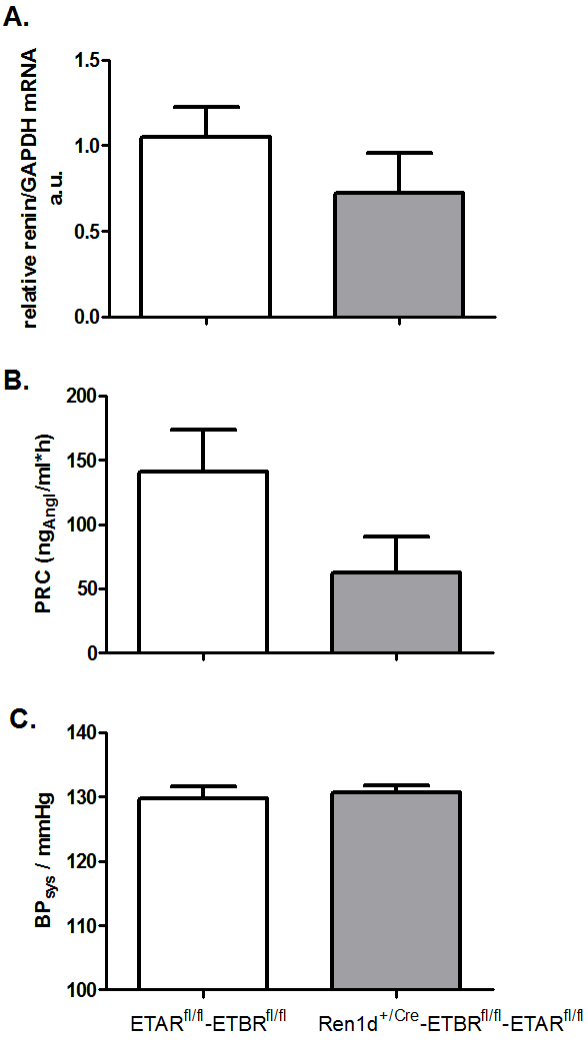

Fig. 25: Characterization of potential effect of both ET receptors on the renin synthesis and secretion (A) relative renin mRNA abundance was unchanged in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ compared to controls ($ETAR^{fl/fl}$-$ETBR^{fl/fl}$),**(B)** plasma renin concentration was not significantly changed in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ compared to controls **(C)** no difference in systolic blood pressure values of both groups; (A),(B) n=6 each group; (C) n=3 for controls, n=4 for "double knockout"

In order to study the potential role of ET-1/ET-receptors pathway on renin secretion further, isolated perfused kidney was performed in adult $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ mice (2.2.3 for protocol and setup). The renin cell-specific deletion of both ET-receptors did not influence the inhibition of renin secretion through ET-1 (Fig.26). After adding ET-1 (30 pM, 100 pM, 300 pM and 1 nM ET-1) in increasing concentrations to the *ex situ* system, $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ kidneys displayed an inhibition of renin secretion (Fig.26A) mainly mediated through a reduction of renal blood flow (ml/g x min^{-1}) (Fig.26B) as in controls. All experiments were performed under same condition with constant pressure and all animals had similar body weights (20 g – 25 g).

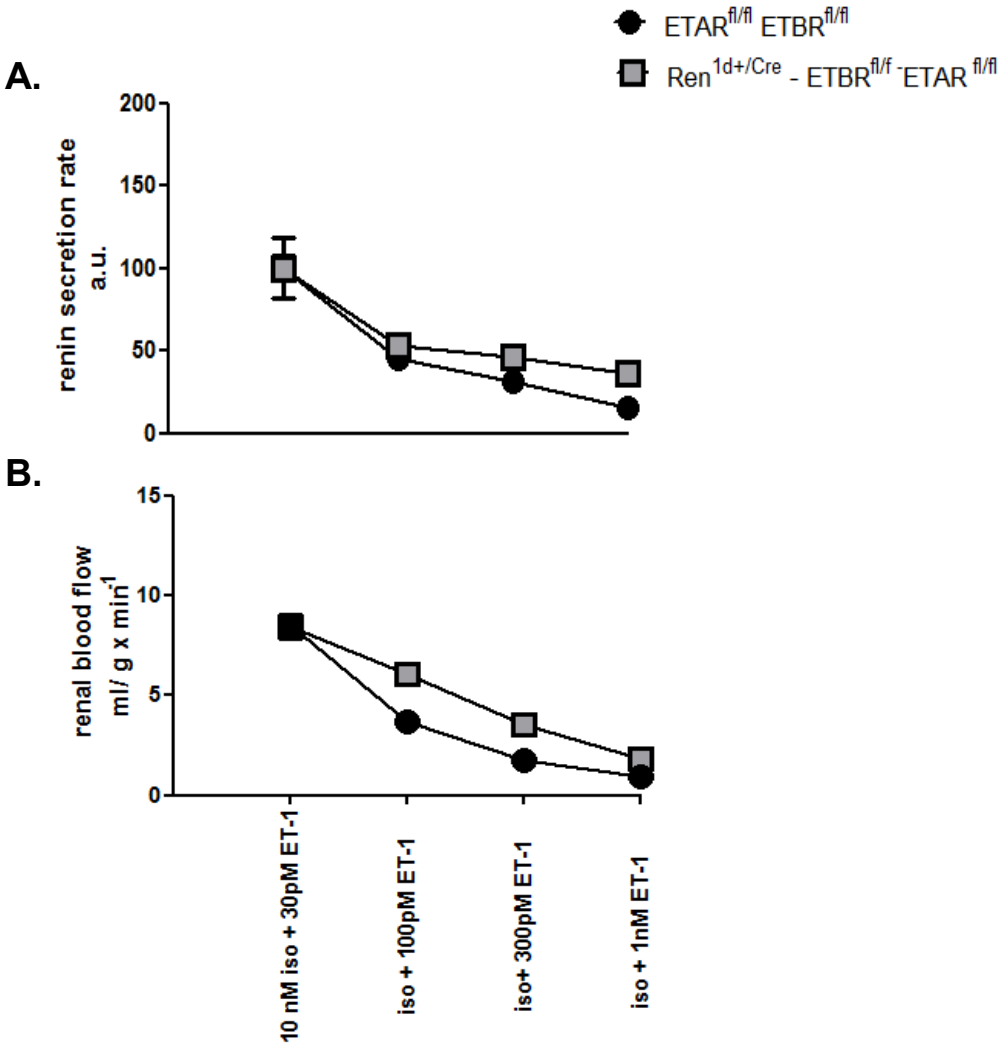

Fig. 26: Isolated perfused kidneys of $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ and controls displayed an inhibition of (A) renin secretion via adding ET-1 in different concentrations over **(B)** renal blood flow decline due to ET-1;flow mainly mediates inhibition of renin secretion; iso=isoproterenol; experiment was performed on constant perfusion pressure

Thus, the results of present chapter suggests, that renin cell-specific deletion of both ET receptors has no significant direct effect on renin synthesis and secretion, moreover on the renin system *in vivo* and *in vitro*.

3.4.3 Characterization of renal ETA- and ETB-receptors expressed on stroma derived cells on renin synthesis and secretion *in vivo* and *in vitro*

Previous shown results indicate that the crucial role of ET-receptors expressed on renin producing cells seems to be of less relevance for the renin synthesis and secretion *in vivo*. In addition, neither ETA-receptor nor ETB-receptor isoform expressed on stroma derived cells seem to have no effect on the renin system. Further experiments should investigate if there is an effect of both ET receptors based on stroma derived cells on the renin synthesis and secretion.

Therefore it was necessary to generate an ET-receptor stroma derived cell-specific "double knockout" animal model ($FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$). The efficacy of deletion was verified by IHC for ETAR (Fig. 27) and ISH BASEscope® assay for ETBR (Fig.28). In $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals, both ET-receptors expression lacked exclusively on renin producing cells, mesangial cells, vascular smooth muscle cells as well as interstitial cells.

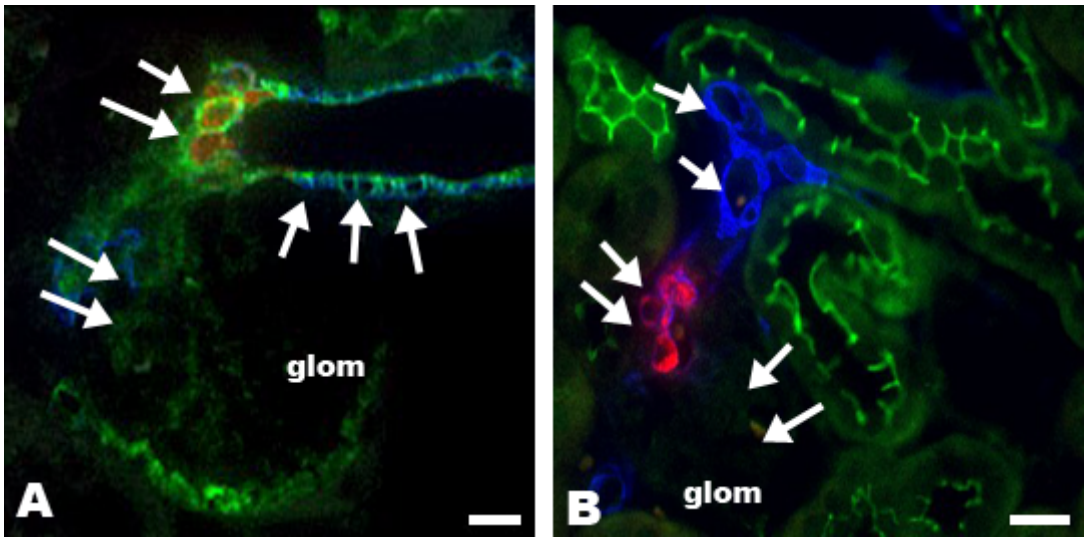

Fig. 27: Verification of stroma derived cell-specific deletion of ETAR in $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ (A) WT kidney section, IHC with ETAR, renin and α-sma, scale bar = 10 µm, ETAR was expressed on rpcs, smooth muscle cells and mesangial cells (stroma derived cells) of $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ **(B)** IHC of $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ kidney section with ETAR, renin and α-sma scale bar = 20 µm; ETAR lacked on rpcs, smooth muscle cells and mesangial cells

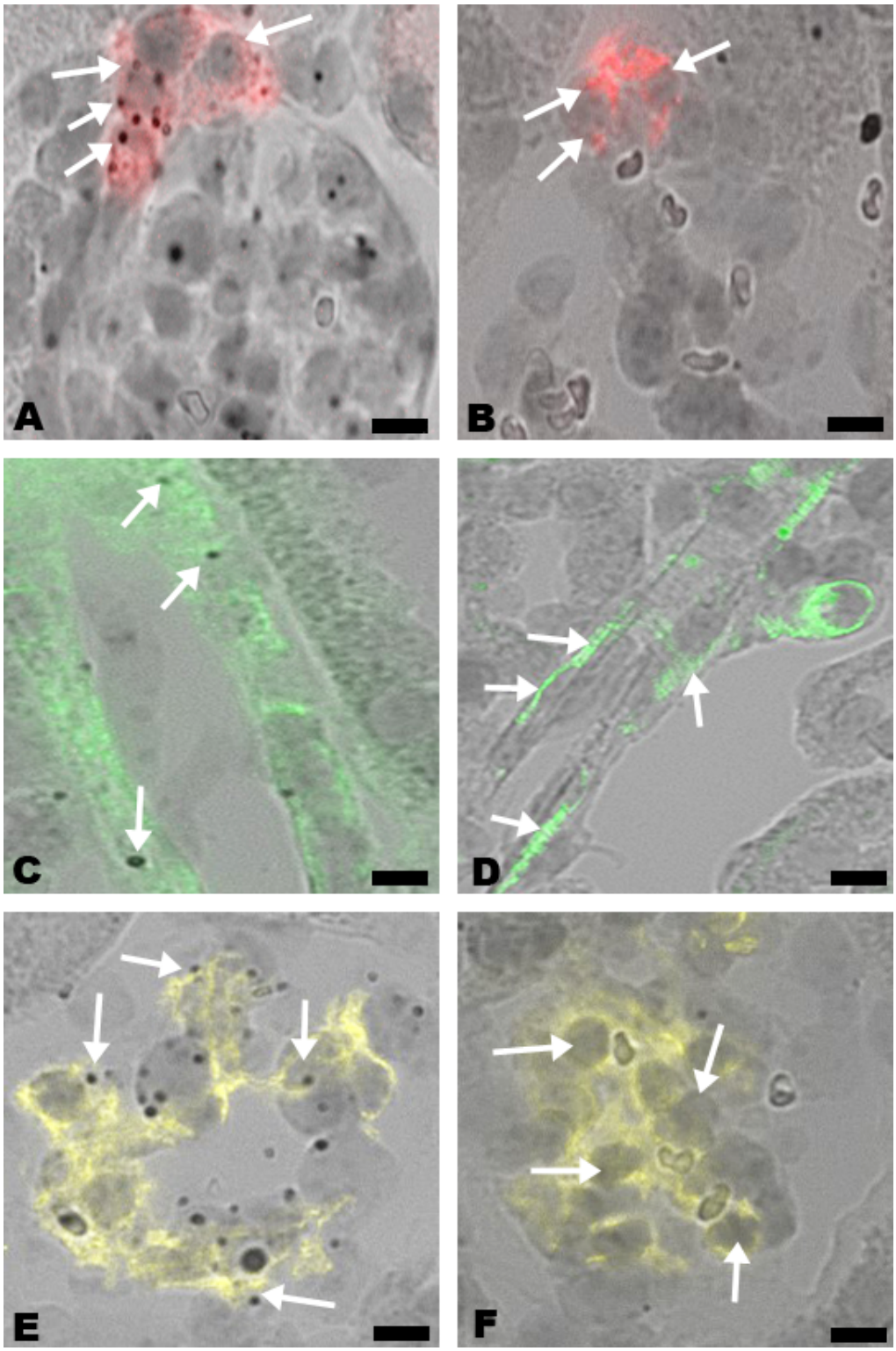

Fig. 28: Verification of stroma derived cell-specific deletion of ETBR in FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$-ETBR$^{fl/fl}$ with ISH BaseScope® assay and IHC (A) WT (ETAR$^{fl/fl}$-ETBR$^{fl/fl}$) kidney section, ISH with ETBR and additional IHC with renin, ETBR is expressed on rpcs **(B)** in **FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$-ETBR$^{fl/fl}$** kidneys ETBR lacks on renin producing cells, ISH BaseScope with IHC renin **(C) WT** kidney section, ISH and IHC with α-sma, ETBR is expressed on smooth muscle cells **(D)** in **FOX$^{D1Cre/+}$- ETAR$^{fl/fl}$-ETBR$^{fl/fl}$** kidneys ETBR lacks on smooth muscle cells, ISH with IHC with α-sma, **(E) WT** kidney section, ISH with EBTR and α-integrin-8, ETBR is epressed on mesangial cells **(F)** in **FOX$^{D1Cre/+}$-ETAR$^{fl/fl}$-ETBR$^{fl/fl}$** kidneys ETBR lacks on mesangial cells, ISH with IHC α-integrin-8, (A)-(F) scale bar = 5 µm

In order to study a potential stroma derived cell-specific indirect effect of renal ET-receptors on the renin system more precisely on renin synthesis and secretion *in vivo,* it was necessary to study relative renin mRNA abundance, plasma renin concentration as well as systolic blood pressure under basal conditions prospectively after verification of deletion.

All $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals showed no significant difference in relative renin mRNA abundance and plasma renin concentration compared to controls under basal conditions (Fig.29). PRC level were slightly diminished in $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ compared to controls ($ETAR^{fl/fl}$-$ETBR^{fl/fl}$) In addition, $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ mice had normal systolic blood pressure values (128.5 ±1.109) relative to $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ control littermates (130 ± 1.048) (Fig.29) and as shown in Fig.27/28 normal developed juxtaglomerular renin positive cells after performing immunohistochemistry and in situ hybridization (BASEScope® assay) on kidney sections. These results indicate that both ET receptors expressed on stroma derived cells in the kidney seem to be of less relevance for renin synthesis and secretion *in vivo.*

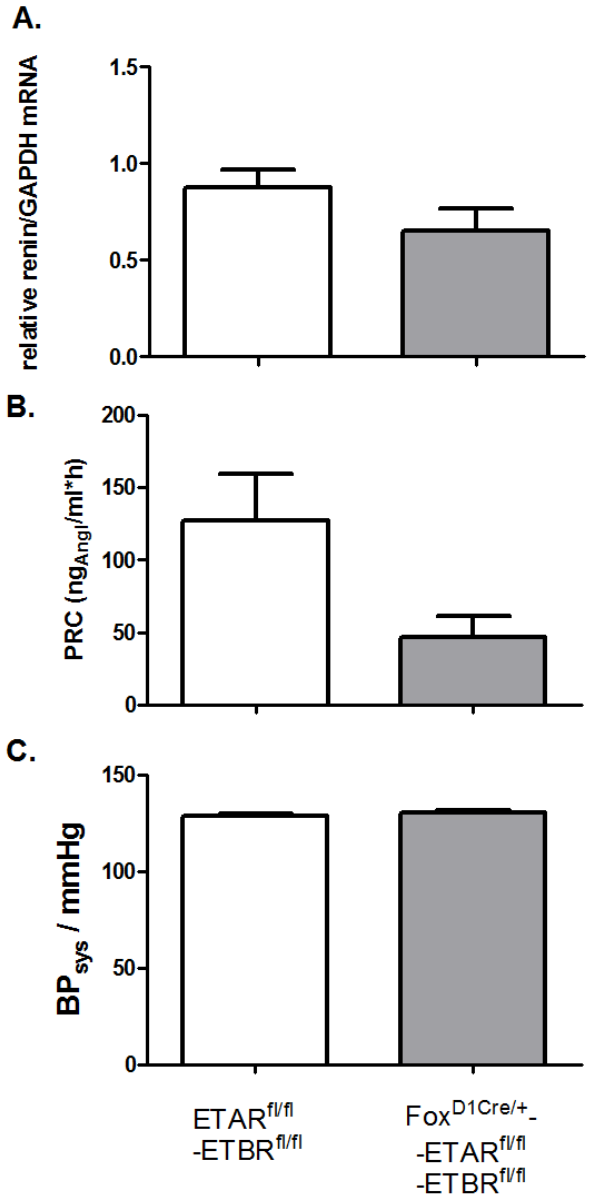

Fig. 29: Characterization of potential indirect kidney effect of both ET receptors on renin synthesis and secretion *in vivo* **(A)** relative renin mRNA abundance was not significantly changed in **$FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$** compared to controls ($ETAR^{fl/fl}$-$ETBR^{fl/fl}$) n=4,**(B)** plasma renin concentration was not significantly changed in **$FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$** compared to controls n=2 **(C)** no difference in systolic blood pressure values of **$FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$** (n=2) compared to controls n=3

In the isolated perfused kidney (setup and protocol 2.2.3) all $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ and $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$ displayed an inhibition of renin secretion mainly mediated by the decline of renal blood flow via ET-1. However renin secretion did not show an inhibition in $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ through ET-1 (Fig 30A). Moreover, in $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$ renin secretion showed inhibition down to 60 % whereas $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ isolated kidneys declined down to 92 %.

The effect of $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ and $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ was mainly mediated by a reduction of the renal blood flow (<5 ml/g x min^{-1}) whereas decline of renal blood flow in $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ was attenuated rapidly (20 ml/g x min^{-1}) by 10 pM ET-1 to 14 ml/g x min^{-1} by 1 nM ET-1) (Fig.30B). The renal blood flow of $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ was nearly in a steady state compared to ET-receptor isoform knockout kidneys. In summary, the deletion of both ET-receptors in stroma derived cells did not inhibit renin secretion compared to single receptor isoform deletion whereas it seems not to be mediated by a decline of the renal blood flow because the flow could be detected nearly in a steady state in $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$.

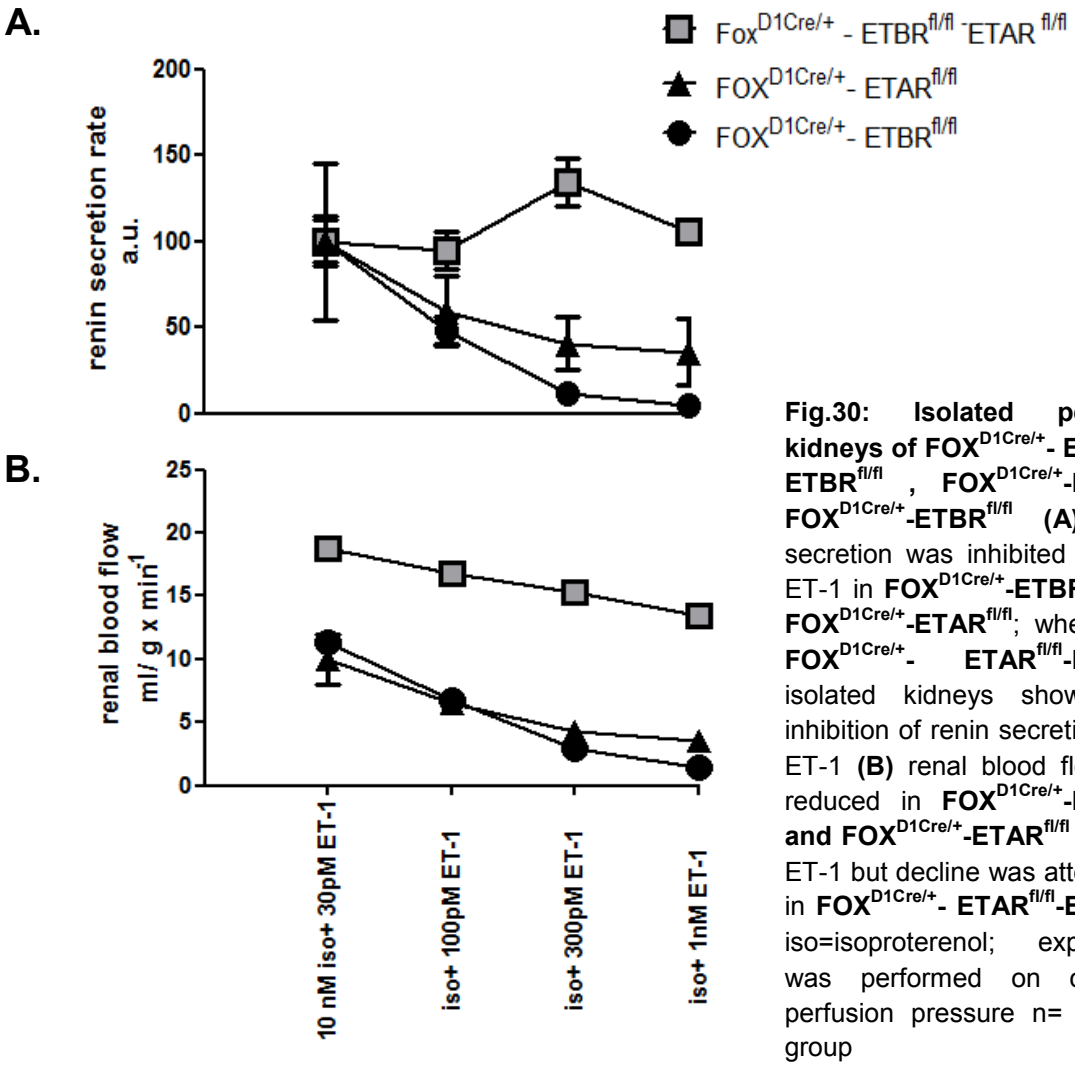

Fig.30: Isolated perfused kidneys of $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$, $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$ $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ (A) renin secretion was inhibited through ET-1 in **$FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ and $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$**; whereas in **$FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$** isolated kidneys showed no inhibition of renin secretion after ET-1 **(B)** renal blood flow was reduced in **$FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ and $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$** through ET-1 but decline was attenuated in **$FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$**; iso=isoproterenol; experiment was performed on constant perfusion pressure n= 2 each group

4 Discussion

Renin is the central regulatory factor of the Renin-Angiotensin-Aldosterone system. It is mainly synthesized in the kidney from juxtaglomerular epitheloid cells and is stored in secretory vesicles until release. The release of renin is stimulated by cAMP signaling pathway and inhibited by an increase in cytosolic Ca^{2+} concentration. Renin synthesis and its release from JG cells are regulated by various local and systemic factors. Several vasoconstrictor hormones such as ANG II and arginine vasopressin have been shown to inhibit renin release from juxtaglomerular cells. In addition, different *in vitro* studies and one *in vivo* study on dogs have suggested endothelins as negative regulators of renin expression and secretion (Berthold *et al.*, 1999; Scholz *et al.*, 1995; Ritthaler *et al.*, 1995; Ritthaler *et al.*, 1996). The endothelin system consists of three peptide hormones Endothelin-1, Endothelin-2 and Endothelin-3 and their G-protein coupled receptors Endothelin-A and Endothelin-B receptor. Previous studies displayed Endothelin-1 as a potent vasoconstrictor in the renal vascular system and as an inhibitor of the synthesis and secretion of renin from juxtaglomerular epitheloid cells *in vitro* whereas ETAR is suggested to act as the main receptor (Kaasjager *et al.*, 1997; Bohm *et al.*, 2003). The work at hand aimed to examine the relevance of endothelins focusing on ET-1/ETA-receptor and/or ETB-receptor signaling pathway for the physiological regulation of renin synthesis and secretion *in vivo*. Moreover, we tried to investigate if the proven inhibiting effect on the renin system via ETAR and/or ETBR signaling pathway is accompanied by a direct effect on the level of renin producing juxtaglomerular cells in the kidney or if the effect of renal ET receptors on the renin system is systemic, general or regulated by ET receptors expressed on stroma derived cells in the kidney.

4.1 ET-receptor localization in the adult murine kidney

It was inevitable to study the expression pattern of both receptors in the adult murine kidney first. The ETA- and ETB-receptor isoforms were localized on mouse kidney sections with co-localization (IHC, ISH) analysis on renin producing JG cells for present study. Moreover, both ET-receptors are suggested as general markers for renin producing cells because they could be located on recruited, ectopic expressed and fetal renin producing cells, too.

In addition, both receptors could be detected on smooth muscle cells, but only ETBR expression was found on endothelial cells of vascular structures. Similar results could be demonstrated by Wendel *et al*. in the rat kidney, whereas they noted, a weak immunostaining for ETB receptor on endothelial cells (Kohan *et al.*, 2011a; Wendel *et al.*, 2006).The ETA receptor could also be detected on collecting duct cells, mesangial cells and distal tubular structures showing no signal on podocytes and proximal tubular structures whereby Garvin

and Sanders and Wendel *et. al*. published similar results using rats in their experimental approaches (Garvin and Sanders, 1991; Wendel *et al.*, 2006). With the help of ISH technique, expression of ETB-receptor mRNA signal was detected further on interstitial cells, mesangial cells, proximal tubular cells and collecting duct cells (cortical and medullary) which was also shown by Wendel et al. whereas their study did not focus on ETBR expression in renal interstitial cells (Wendel *et al.*, 2006).

It has been shown, that ETA and ETB receptors are widely expressed in the kidney and some specific cells are able to express both receptor isoforms (Kohan *et al.*, 2011b). Taken together, both ET-receptor isoforms are co-expressed on renin producing cells, smooth muscle cells, mesangial cells, distal tubular structures and collecting duct cells. The renal ETBR is suggested as the predominant isoform in the kidney (Kedzierski and Yanagisawa 2001) which could be affirmed in present thesis.

Endothelin receptors are known to be present on plasma membrane of each cell where ETs can bind to its receptors causing a variety of different but often similar biological effects based on various signaling cascades, including cyclooxygenases, cytochrome P-450, nitric oxide synthase and adenylyl cyclases and others (Kohan *et al.*, 2011b; Sorokin and Kohan, 2003; Kohan, 2010). It should also be mentioned that the relative expression pattern of both receptors differs between animal models and humans, possibly reflecting regional differences within the kidney (Kohan *et al.*, 2011b). With respect to present findings, all experiments were performed with adult mice.

4.2 The ET receptor isoforms and its potential role on the renin synthesis and secretion *in vivo*

Focusing on the localization of both ET receptors on renin producing JG cells to study a potential role on the renin system it was necessary to generate a renin cell-specific model in which either ETA- ($Ren^{1d+/Cre}$- $ETAR^{fl/fl}$) or ETB-receptor ($Ren^{1d+/Cre}$- $ETBR^{fl/fl}$) isoform was deleted. The efficacy of deletion was determined by IHC or ISH technique, whereby in all Cre^{+} kidney sections ET-receptor immunoreactivity or mRNA detection signal was lacking on renin producing cells only. Therefore all used Cre^{+}-models were distinct to study the role of ET receptors further.

4.2.1 The potential role of ETAR located on the renin cell lineage for renin synthesis and secretion *in vivo*

Besides all three peptides, ET-1 is the most potent and has been shown to primarily act as a vasoconstrictor via ETA-receptor in the kidney (Kaasjager *et al.*, 1997; Bohm *et al.*, 2003; Maguire and Davenport 2011). In addition, *in vitro* studies have shown that ET-1 is able to inhibit renin release from juxtaglomerular cells through a Ca^{2+} dependent mechanism

(Matsumura *et al.*, 1989; Rakugi *et al.*, 1988; Takagi *et al.*, 1988; Kohan *et al.*, 2011b). The ETAR expressed in the kidney has been shown to play a crucial role in a variety of renal physiological and pathological functions *in vivo*, whereas its role, primarily through ET-1, focusing on the level of renin producing cells and the potential direct effects on the renin synthesis and exocytosis are still unknown.

$Ren^{1d+/Cre}$-$ETAR^{fl/fl}$ animals showed normal renin mRNA levels, PRC values and normal systolic blood pressure compared to controls ($ETAR^{fl/fl}$). In addition, all animals developed normal renin producing JG cells as in controls. During nephrogenesis renin producing cells have been shown to originate from the metanephric mesenchyme and accumulate afterwards at the vascular wall (Sequeira Lopez *et al.*, 2001). Although, we could detect ETAR on mesenchymal renin producing cells during kidney development (data not shown), the receptor seems to be not of major relevance for the development of renin producing cells.

During further procedure one tried to understand the potential role of ETAR on the physiological regulation of the renin system. $Ren^{1d+/Cre}$- $ETAR^{fl/fl}$ mice displayed after a low salt/enalapril diet a significant increase in renin mRNA levels and PRC with no change to controls. Conversely, feeding high salt diet lowered renin mRNA abundance and PRC values, again without difference between the two genotypes. Although, renin producing cells and recruited renin positive cells of the kidney express ETAR isoform, it appears not to be of major relevance for the physiological regulation of renin synthesis by the rate of salt intake.

The isolated perfused kidney of $Ren^{1d+/Cre}$- $ETAR^{fl/fl}$ displayed an inhibition of renin secretion after ET-1 was given to the *ex situ* system in increasing concentrations, whereas this effect is mainly mediated through a reduction of the renal blood flow. These results indicate that the ETA receptor isoform located on renin producing cells has no essential direct effect on the inhibition of renin secretion and synthesis.

It should be noted, that the main concept concerning renal ET biology is based on the context of local microenvironment (Schneider *et al.*, 2007; Kohan *et al.*, 2011b). ET receptors localized in different parts within the kidney are meant to show opposing actions, e.g. in the vasculature, where ETAR activation causes primarily vasoconstriction whereas ETBR activation causes, at least initially, vasodilatation mediated by nitric oxide (NO) produced from endothelial cells (Kohan *et al.*, 2011b). However it has also been shown, that ETB shows vasoconstrictive effects mediated on vascular smooth muscle cells (Schneider *et al.*, 2007) primarily on afferent arterioles whereas ETBR provide a vasodilatory influence on normal efferent arterial vascular tone (Kohan *et al.*, 2011b). In addition, under normal conditions i.e. low concentrations of ET-1 most of the vasoconstriction is ETA-dependent. However it has been shown after additional infusion of ET-1 in rats that ETA-independent

vasoconstriction was present (Pollock and Opgenorth, 1993, 1994b; Kohan *et al.*, 2011a, 2011b), suggesting these results rather promote ETB-mediated actions in situations where concentration of ET-1 is strongly increased (e.g. chronic salt diet, renal fibrosis).

With respect to present deletion of ETAR on renin cells, our results rather promote theory of potential compensational effects of ETB-receptor and possible ET-receptor interaction. Besides binding to ETAR, ET-1 has also been investigated to display vasoconstrictor effects through binding to ETBR (Davenport, 2002) showing a potential compensatory mechanism equilibrating physiological homeostasis. In addition, the treatment of selective ETAR antagonist BQ-123 did not change arterial blood pressure as well (Kaasjager 1997, Maguire Davenport 2015). In the last few years, several studies promote a potential ETA-ETB receptor crosstalk in the mediation of responses to ET-1 (Rapoport and Zuccarello, 2011; Harada *et al.*, 2002). For example, ETB receptors in the anterior pituitary gland appeared to bind only ET-1 during blockade of ETA receptors *in vitro* (Harada *et al.*, 2002; Schneider *et al.*, 2007).

These results seem to support the theory, that both receptors are able to function in a similar manner depending on which receptor is activated (Harada *et al.*, 2002).

If expression of ETAR is declined or the activation is somehow ceased on renin producing cells, ET-1 appears to bind primarily on ETBR. There are several findings in the work at hand which support hypothesis of ETBR activation while ETA receptor isoform expression is decreased in different renal cells. All used $Ren^{1d+/Cre}$- $ETAR^{fl/fl}$ and $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$ kidneys displayed for instance an increase in ETBR mRNA abundance compared to control animals (data not shown).

With respect to present findings in the isolated perfused kidney model, the inhibition of renin secretion via ET-1 is not essential mediated via ETAR isoform but rather ETB-receptor seems to be able to compensate present deletion or “inactivation” of ETAR on renin producing JG cells while displaying similar vasoconstrictive effects as in controls. To support or exclude this hypothesis it was necessary to generate further an ETBR renin cell-specific animal model and in addition an ETAR and ETBR double knockout renin cell-specific animal model.

However it should be concluded at this juncture that the role of ETA receptor isoform expressed on renin producing JG cells due to present findings is of less relevance for the physiological regulation of renin synthesis and secretion *in vivo* and seems to become more relevant in terms of pathophysiology.

Recent studies suggest that the ET system, i.e. ETAR, plays an active role in progression of renal failure (Kedzierski and Yanagisawa, 2001). Several ETAR antagonist administration studies support this hypothesis. The usage of BQ-123 in rats with acute renal failure improved for example glomerular filtration rate (GFR) and net tubular reabsorption, two important parameters for kidney function (Chan *et al.*, 1994). In addition, treatment with FR139317 in partially nephrectomized rats lowered proteinuria and prolonged animal survival (Benigni *et al.*, 1993).

4.2.2 The potential role of ETBR located on the renin cell lineage for renin synthesis and secretion *in vivo*

As mentioned above, ETBR has similar affinities for all three endothelin peptides (Davenport, 2002) and has been shown to display crucial interplays regarding renal function (Kohan *et al.*, 2011a, Kohan *et al.*, 2011b) e.g. the role in renal sodium excretion, vasopressin regulation and acid base status (Ohuchi *et al.*, 1999; Gariepy *et al.*, 1998; Edwards *et al.*, 1993). However, its potential role on renin producing cells for renin synthesis and secretion *in vivo* has yet to be resolved.

All $Ren^{1d+/Cre}$- $ETBR^{fl/fl}$ kidneys showed normal renin mRNA and PRC levels under basal conditions. Moreover, animal kidney sections displayed normal developed renin cells at the juxtaglomerular cell apparatus with ISH assay and had normal systolic blood pressure compared to controls. These results indicate that ETB receptor isoform expressed on renin positive cells is suggested to mediate rather no crucial direct effect on the renin synthesis and secretion. Moreover, experiments with $Ren^{1d+/Cre}$- $ETBR^{fl/fl}$ kidneys in isolated perfused kidney model reveal an inhibition of renin secretion while adding ET-1 in increasing concentrations whereas effect is primarily dependent on the decline of the renal blood flow suggesting rather ETA-mediated constrictor effects through ET-1.

Compare to investigations discussed in 4.2.1, both groups ($Ren^{1d+/Cre}$- $ETAR^{fl/fl}$, $Ren^{1d+/Cre}$- $ETBR^{fl/fl}$) displayed similar results regarding effects on renin system compared to control littermates regardless of which receptor isoform is deleted on renin JG cells. These results promote hypothesis that both receptor isoforms can show similar biological function (Kohan, 2010; Kohan *et al.*, 2011a) and in addition, they support theory mentioned above of potential ETA-ETB receptor cross talk (Kohan *et al.*, 2011b; Kohan *et al.*, 2011a; Yoon *et al.*, 2016)

Under basal conditions ETBR is suggested to mediate vasodilatation by the release of endothelin derived factors (NO, prostacyclin and/or endothelium-derived hyperpolarizing factor) acting as a feedback mechanism to limit the vasoconstrictive effect of ET-1 (Maguire and Davenport, 2015).

Regarding the role of ETB receptor on the physiological regulation of the renin system, $Ren^{1d+/Cre}$- $ETBR^{fl/fl}$ mice showed a significant increase in renin mRNA abundance and PRC with no change to control animals after a low salt/enalapril diet. In contrast, feeding high salt lowered renin mRNA significantly compared to controls. With respect to present deletion of ETBR on renin cells, ET-1 seems to solely bind to ETAR rather promoting the inhibition of renin secretion and synthesis *in vivo*, as already concluded *in vitro* (Scholz et al., 1995; Ritthaler et al., 1995; Ritthaler et al., 1996).

In theory, it is suggested that renin release is inhibited by high Na^+-intake due to high renal ET-1 production, whereas Na^+-transport mechanisms are inhibited in the proximal tubules, thick ascending limb and collecting duct cells in order to reduce Na^+ reabsorption (Kohan *et al.*, 2011a). Johnston et al., could determine ET-1 as a crucial regulator of sodium balance by promoting natriuresis through ETB-receptor in response to chronic salt intake (Johnston *et al.*, 2016). Activated renal ETBR appears to promote these effects, at least in the medulla, via NO release (Plato *et al.*, 2000; Jesus Ferreira and Bailly, 1997; Schneider *et al.*, 2007). Moreover, our results showed high renal ET-1 expression in control kidneys after a low salt diet with ongoing enalapril intake as well as increased expression of ETBR (data not shown) in contrast ET-1 and ETBR production was unaffected by high salt diet (data not shown). Therefore, present results rather indicate an ETB-receptor mediated regulation during low salt diet whereas the involvement of ETB-receptor isoform during a high salt diet seems to be not of major relevance *in vivo.*

Concentrating on the inhibition of renin release regulated by Ca^{2+}-dependent mechanism, it should be noted that ET-1 maybe also potent for reducing renin release stimulated by different factors such as isoproterenol or cAMP (Kurtz *et al.*, 1991; Moe *et al.*, 1991; Kohan *et al.*, 2011a), most likely via ETBR-dependent NO release (Ackermann *et al.*, 1995; Ritthaler *et al.*, 1995; Scholz *et al.*, 1995; Kohan *et al.*, 2011a, 2011b). However, the physiological connection between ET-1 and NO to control renin release need to be clarified first because opposing roles proposed for NO have been published. NO has been demonstrated to directly stimulate the release of renin from isolated JG preparations, although NO-dependent vascular relaxation can increase the pressure reaching the glomerulus, which would inhibit the exocytosis of renin through the intrarenal baroreceptor (Schweda *et al.*, 2007; Kohan *et al.*, 2011a, 2011b) whereas the inhibition of NO synthase reduces renin release in isolated afferent arterioles (Tharaux *et al.*, 1997)

With respect to present findings, ETB receptor isoform located on renin producing cells seems to be of less relevance for the physiological regulation of the renin system moreover for renin synthesis and secretion. The ETBR is suggested to be of crucial relevance for additional renal parameters mentioned above located on other renal cells. In contrast, in

order to examine, if the effect of ET-receptor isoform and the combination of both ET-receptors for renin synthesis and secretion is based on systemic factors or stroma derived cells, additional experiments were performed.

4.2.3 The potential indirect effects of ETA and ETB-receptor on renin synthesis and secretion *in vivo*

Besides renin producing cells, both ET receptors are widely expressed in the kidney. After IHC and ISH co-localization analyzing studies, both ET-receptors could be determined on renin JG-cells indicating the functional role of each specific isoform seem to have no essential direct role for renin synthesis and secretion *in vivo*. Therefore, it was inevitable to further characterize potential indirect effects of renal ETA or ETB receptor isoforms expressed on additional renal cells than the renin cell lineage on the renin system *in vivo*.

As mentioned in 4.1 renal ETA-receptor could be additionally localized on vascular smooth muscle cells, mesangial cells, collecting duct and distal tubular structures. Therefore different animal models were generated, where ETAR was cell-specific deleted in order to examine if the functional role on RAAS is regulated by ETAR expression on stroma derived cells or if the effect is systemic ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$, SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$). Moreover, in order to characterize the general role of ETAR expression for renin synthesis and secretion, ETAR loxP animals were paired with conditional knockout CAGG-Cre-ER^{T2} animals (CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$). After activation of Cre recombinase through induction with tamoxifen chow for three weeks, ETAR expression was significantly decreased in the kidney down to 38 % compared to controls. The efficacy of each single deletion ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$, SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$, CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$) was verified by IHC staining whereby ETAR exclusively lacked on specific cell types only.

After *in vivo* investigation studies, all Cre^{+} animals mentioned above showed normal developed renin positive JG cells, renin mRNA abundance, PRC levels and systolic blood pressure values compared to controls. Only in CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$ mice renin mRNA abundance was slightly diminished. However these results indicate that general renal ETA receptor isoform seems to be of less relevance for renin synthesis and secretion. Moreover, it should be mentioned that induced CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$ mice showed no specific phenotype, had the ability of fertility and developed apparently completely normal. In contrast, different studies postulated that general deletion of either ETA-receptor isoform or ET-1 knockout mice were neonatal lethal due to developmental defects in cardiac and craniofacial structures (Clouthier *et al.*, 1998; Kurihara *et al.*, 1994) rather promoting that both ETAR and ET-1 are crucial for embryonic development whereas the inducible decline of receptor isoform expression showed no severe phenotype in the adulthood. In addition, general ETBR knockout mice die before adulthood because they develop aganglionic

megacolon and white spotting on the coat suggesting ETBR signaling is crucial for generating enteric neurons and melanocytes (Akashi *et al.*, 2016; Baynash *et al.*, 1994).

In addition, isolated perfused kidney experiments were performed with all Cre^{+}-genotypes, resulting in an inhibition of renin secretion after adding ET-1 to the *ex situ* system as shown in control littermates. It should be noted that the inhibition is primarily mediated by the reduction of the renal blood flow and not by an alteration of the renin concentration. With respect to present renal ETA receptor isoform deletion in different cell types, one can suggest that ETB-mediated effects are responsible for displaying mentioned results. It should be noted, that all Cre^{+} animal models showed increased ETBR mRNA abundance compare to controls supporting present hypothesis (data not shown).

Focusing on ET receptors on smooth muscle cells, ETA receptor has been shown to be mainly expressed on those cells (Davenport and Maguire, 2011), which support present findings. However, recent studies confirm that the usage of ETAR-selective antagonists fully block these vasoconstrictor responses (Davenport and Maguire, 2011), which could not be confirmed in present thesis. All used SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$, and in addition all $FOX^{D1Cre/+}$-$ETAR^{fl/fl}$ as well as CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$ animals displayed an inhibition of renin secretion in the isolated perfused kidney through ET-1 and therefore suggesting ETB-mediated vasoconstrictor effects.

It has been shown that ET-1 via ETB-receptor is able to demonstrate vasoconstriction in the vascular system (Maguire and Davenport, 2015) especially in the afferent arteriole (Kohan *et al.*, 2011a). Moreover, in humans, results of ET-1 infusion in the brachial artery show at low concentration an ETB-mediated vasodilatation. However, as the concentration increases to higher pathological concentrations, vasodilatation is overwhelmed by ETAR-mediated constrictor responses or involve non-ETAR receptor mediation (Maguire and Davenport, 2015; Pollock and Opgenorth, 1994a). If ETAR expression is deleted on vascular smooth muscle cells, it is suggested, that our findings perchance support theory of ETB-mediated vasoconstrictor effect and hypothesis of ETA-ETB receptor cross link.

Similar results were determined in SMMHC-Cre-ER^{T2}-$ETBR^{fl/fl}$ (data not shown) and $FOX^{D1Cre/+}$-$ETBR^{fl/fl}$ animals in the isolated perfused kidney model. As mentioned in 4.1 ETB-receptor isoform is additionally expressed on interstitial cells, mesangial cells and vascular smooth muscle cells. All Cre^{+} animals displayed an inhibition of renin secretion via ET-1 mainly mediated by renal blood flow. Therefore, it is rather ETAR-mediated vasoconstrictor effects through ET-1 during present ETBR-isoform deletion.

In summary these results support theory that each receptor isoform seems to be able to compensate function of the other receptor isoform. Therefore, ETA and ETB-receptor

isoforms expressed in the kidney seem to have solely no indirect effect on the renin system and to be of less relevance for the physiological regulation of renin synthesis and secretion. The deletion of each single receptor is suggested to be compensated by the other receptor isoform to maintain physiological homeostasis. Both receptors' function in terms of pathophysiology is suggested to be of more importance.

4.3 The role of both ET-receptors expressed on the renin cell lineage for renin synthesis and secretion *in vivo*

Expression of a single ET-receptor isoform on renin producing JG cells seems apparently not to be involved in mediating renin synthesis or secretion. In order to characterize potential compensational receptor effects or possible ET receptor interaction, it was necessary to further study the functional role of both ET receptors expressed on renin positive cells via ET-1. Although a great number of pharmacological agents have been used to analyze ET receptor isoform function, recent studies suggest an ET-receptor dimerization leading to substantial uncertainty about single receptor function (Boesen, 2008; Kohan *et al.*, 2011a; Schneider *et al.*, 2007).

Perhaps characterization of single ET receptor function in the field of endothelin system is limited because *in vitro* studies showed ET receptors are able to form homo- and heterodimers (Gregan *et al.*, 2004a; Gregan *et al.*, 2004b) suggesting the process is regulated by a PDZ finger (Evans and Walker, 2008). Evans *et al.*, could demonstrate that mutation of PDZ domain leads to delayed ET receptor internalization and prolonged increase in intracellular Ca^{2+}-concentration in response to ET-1, suggesting that ET receptor heterodimerization affects receptor function (Evans and Walker, 2008; Kohan *et al.*, 2011a). In order to investigate potential effects of receptor dimers on ET receptor isoform function on the renin system and to exclude potential compensational effects of ET receptor isoforms, it was inevitable to generate an animal model where ETA- and ETB-receptor are both deleted exclusively on renin producing cells ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$).

All $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals showed normal renin mRNA abundance and unchanged systolic blood pressure values compared to control littermates. Only PRC levels were slightly diminished in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ as in controls. These results were surprising because one would rather assume an increase of PRC through inhibitory effects of ET-1 in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ mice.

However, present results support theory that both ET receptors seem to show no important direct effect via ET-1 on mediating renin synthesis and secretion expressed on renin producing cells *in vivo*.

Moreover, it has been shown, that alongside ET-1 other vasoconstrictive hormones such as ANG II, arginine vasopressin are also known as negative regulators of renin synthesis. The work at hand promote theories that these hormones mediate renin inhibition independently of ET system or ET-receptors located on other cells than renin cell lineage are more involved in regulating renin synthesis and secretion *in vivo*. The isolated perfused kidney experiments with $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals showed an inhibition of renin secretion mainly mediated by the reduction of renal blood flow after adding ET-1 to the *ex situ* system as in controls. After comparing current *in vivo* and *in vitro* results, one could assume that the ET-system maybe more involved in short-term regulation of renin secretion and synthesis but seems of less relevance on a long-term basis.

These results indicate the hypothesis that additional renal ET receptors than ETAR or ETBR are present on renin producing cells, although Maguire and Davenport concluded in 2015 that there are still no evidences for this theory (Maguire and Davenport, 2015).

However, it should be mentioned at this juncture that potential ET receptor heterodimers are still present in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals unaffected by present Cre-recombinase activation. Perhaps, these heterodimers are able to bind ET-1 resulting in present *in vivo* data. In addition, Inscho *et al*., postulated in 2005, that either an ETA receptor selective or ETB-selective antagonist was able to abolish afferent arteriole vasoconstrictor responses to low concentrations of ET-1, which support given results of isolated perfused kidney data (Inscho *et al.*, 2005; Boesen, 2008). Several findings promote given hypothesis of heterodimerization but the functional consequences *in vivo* remains an open field of future inquiry (Boesen, 2008).

Nonetheless, present findings support previous results on ET receptors isoforms. Both receptors expressed on renin producing cells suggest no essential direct effect on renin synthesis and secretion. The role of both receptors expressed on renin cell lineage is additionally rather more important in terms of pathophysiology. Bosentan and macitentan are both approved mixed antagonists in the clinic (Davenport *et al.*, 2016; Palmer, 2009; Patel and McKeage, 2014; Sidharta *et al.*, 2015) and used for a variety of diseases including scleroderma renal crisis and pulmonary hypertension.

However, present results indicate that both ET-receptors located on renin cell lineage are not able to promote this inhibiting effect on renin synthesis and secretion by themselves. Following chapter discusses if a combination of several renal cell types more likely display the inhibiting effect of endothelins through ETAR and ETBR.

4.4 The role of both ET-receptors expressed on stroma derived cells on renin synthesis and secretion *in vivo*

Previous shown results indicate no essential direct effect of ET receptors localized on the renin cell lineage for renin synthesis and secretion *in vivo*. Since the RenCre-promotor is active in other renal cell types e.g. collecting duct cells during nephrogenesis (Castrop *et al.*, 2006) the question arises, if the renin cell lineage is not able to promote this effect by itself. Therefore the question must be, if other renal cell types, expressing both receptors, regulate the inhibiting effect of endothelins on renin synthesis and secretion *in vivo.* In order to characterize the role of ET-receptor on stroma derived cell lineage, animals with Cre activation under the control of FoxD1 promotor were paired with ETAR and ETBR loxP mice.

In $Fox^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals both receptor isoforms were deleted not only on renin producing cells but also on mesangial cells, smooth muscle cells and interstitial cells (Sequeira-Lopez *et al.*, 2015; Kobayashi *et al.*, 2014).

All Cre^{+} animals showed normal renin mRNA levels as in control littermates and developed normal renin producing cells at the juxtaglomerular cell apparatus. In addition, the plasma renin concentration was not significantly changed in $Fox^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ than in control littermates. Although levels were slightly diminished in knockout mice compared to controls but it should be noted that present results were not expected. One would estimate an increase during inhibitory effects of ET-1.

Moreover, these results were supported by systolic blood pressure measurements. All $Fox^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals as well as the ET receptor isoform knockouts ($Fox^{D1Cre/+}$-$ETAR^{fl/fl}$ [as shown in 3.2.4] and $Fox^{D1Cre/+}$-$ETBR^{fl/fl}$ [data not shown]) revealed normal blood pressure values as in control animals. These results indicate that the potential indirect stroma derived cell-specific effect of endothelins via both ET-receptors on the renin system seems of less relevance for renin synthesis and secretion *in vivo*.

In contrast, isolated perfused kidney experiments with $Fox^{D1Cre/+}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ revealed no inhibition of renin secretion rate compared to controls ($Fox^{D1Cre/+}$-$ETAR^{fl/fl}$, $Fox^{D1Cre/+}$-$ETBR^{fl/fl}$) through ET-1. Moreover, the decline of the renal blood flow was abolished and revealed no clear drop as in controls after adding ET-1 in increasing concentrations.

With respect to previous results regarding renin cell lineage, the isolated perfused mouse kidney technique is an *ex vivo/in vitro* model where one can study kidney function in the absence of systemic influences such as the blood pressure, autonomic nervous system or different hormones. After comparing obtained *in vivo* and *ex vivo*/*in vitro* data, one can assume that systemic effects might explain current *in vivo* results.

It should also be noted, that changes in the renal blood flow are mainly based on the vasoconstriction of the renal vasculature (i.e. afferent arteriole) but also on the vasoconstriction of mesangial cells. Besides renin producing cells, both receptors are present on vascular smooth muscle cells but also on mesangial cells in the stroma derived cell lineage. Therefore, additional studies in the future need to distinguish between those cell types to investigate further the inhibiting effect of endothelins on renin synthesis and secretion *in vivo*.

4.5. Outlook

As mentioned above present results indicate that both ET receptors located on renin producing cell lineage seem to be of less relevance for the renin synthesis and secretion moreover for the physiological regulation of the renin system *in vivo.* The ETA and ETB-receptor are also expressed on tubular structures (distal tubular structures and collecting duct cells) and maybe these cells are somehow involved in homeostasis of the extracellular volume and perhaps in ET-1 mediated inhibition of renin secretion and synthesis. Therefore, it would be a stringent approach to investigate the role of ET-receptors in mice with ETAR and/or ETBR deletion especially in tubular renal segments.

ET-1 is suggested to primarily act through its ET-receptors in a Ca^{2+} dependent mechanism to inhibit renin release *in vitro* whereas studies indicate that the action is rather ETB-mediated (Ritthaler *et al.*, 1995). Recent experiments showed an increase in Ca^{2+} concentration when ET-1 was added to isolated renin positive cells of $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$ kidneys loaded with Fura-2 suggesting that ETBR is able to compensate function when ETAR is deleted on renin producing cells (data not shown). More experiments need to be performed further to better understand the mechanism beyond. Moreover, the specific role of both ET receptors on the Ca^{2+}-dependent mechanism of renin inhibition located on renin producing cells needs to be evaluated prospectively ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$).

Over the years, a number of studies focused on renal interstitial cells and their possible physiological functions. With respect to present findings, it is ETBR as part of the endothelin system which could be localized on renal interstitial cells. With the help of co-localization studies, one could reveal that $PDGFR\beta^{+}$ cells also express ETBR. However this phenomenon does not occur continuously in the kidney. In order to study a potential role of renal ETBR expressed on interstitial cells for renin synthesis and secretion *in vivo*, additional experiments are planned for the future ($PDGFR\beta^{+}$-Cre-ER^{T2}-$ETBR^{fl/fl}$).

In terms of possible ET receptor dimerization and its effects on the renin system, Zeng and colleagues reported a possible ETB-Angiotensin type 1-receptor interaction *in vitro* (Zeng et al., 2005). Further *in vivo* investigations can examine possible renal physiological consequences located on renin producing cells with the help of an animal model ($Ren^{1d+/Cre}$-$ETBR^{fl/fl}$-$AT1a^{fl/fl}$). Riggelmann et al., concluded that ET receptors antagonists are able to abolish acute and chronic effects of ANG II (Riggleman *et al.*, 2001). Perhaps this receptor interaction can reveal more details about the influence of endothelin system on renal physiology and RAAS in general.

The question arises, what is the function of the strong ET-receptors expression on renin producing cells. It is therefore possible to consider that both ET receptors seem to become

more relevant in terms of pathophysiology. Different studies indicate an involvement of the ET-system in sepsis, chronic kidney disease or cardiovascular diseases (Barton and Yanagisawa, 2008). In order to get a better understanding how and to what extend renal ET-receptors located on renin producing cells are involved in situations of pathophysiology, it would be interesting to investigate the role of ET-receptors in renal disease models. In this context, recent data of our group showed a dramatic increase of ET-1 and ET-receptor mRNA levels during the progression of renal fibrosis. A future approach is to perform unilateral ureter obstruction (UUO) inducing renal fibrosis in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$, $Ren^{1d+/Cre}$-$ETBR^{fl/fl}$ as well as in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals.

5 Summary

The protease renin is known as the central regulatory factor of the Renin-Angiotensin-Aldosterone system. It is primarily synthesized in the kidney from juxtaglomerular epitheloid cells and is stored in secretory vesicles until release. The release of renin has been shown to be stimulated by cAMP signaling pathway and inhibited by an increase of cytosolic Ca^{2+} concentration. Several local and systemic factors including different vasoconstrictor hormones such as ANG II and arginine vasopressin have been shown to inhibit renin release from juxtaglomerular cells in the kidney.

Moreover, endothelins have been demonstrated to attribute as inhibiting regulators of renin synthesis and secretion *in vitro* (Berthold et al., 1999; Scholz et al., 1995; Ritthaler et al., 1995; Ritthaler et al., 1996; Ackermann et al., 1995). The endothelin system consists of three peptide hormones Endothelin-1, Endothelin-2 and Endothelin-3 and their G-protein coupled receptors Endothelin-A and Endothelin-B receptor. The endothelin system could be detected in different parts of the human body, including lung, heart, central nervous system and the kidney. Previous studies displayed Endothelin-1 as one of the potent vasoconstrictors in the renal vasculature whereas ET-1/ETAR signaling pathway is suggested to act as the main vasoconstrictor (Kaasjager *et al.*, 1997; Bohm *et al.*, 2003) while ETB-receptor, at least initially, causes vasodilation (Kohan *et al.*, 2011a).

The work at hand aimed to characterize the relevance of endothelins focusing on ET-1/ETA-receptor and/or ETB-receptor signaling pathway for the physiological regulation of renin synthesis and secretion *in vivo*. Moreover, we addressed the question if the proven inhibiting effect on the renin system via ETAR and/or ETBR signaling pathway is accompanied by a direct effect located on the level of renin producing juxtaglomerular cells or through indirect effects based on renal stroma derived cells or on systemic or general effects. The first part concentrates on the potential effect of ET-1/ETAR signaling pathway on the renin system *in vivo*. For this purpose, a renin cell-specific ETAR animal model was generated ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$). More precisely, with the help of the Cre/loxP system, ETA-receptor could be deleted exclusively on renin producing cells to examine further its possible effects on the level of JG cells for renin synthesis and secretion *in vivo*. Due to a widely distribution of ETAR receptor expression in the kidney, it was ineluctable to further investigate if ETA-receptors located on other renal cells display a potential indirect effect on the renin synthesis and secretion. In order to study, if the effect is based on stroma derived cells including renin producing cells, mesangial cells, vascular smooth muscle cells and interstitial cells ($FOX^{D1Cre/+}$-$ETAR^{fl/fl}$) or through systemic (SMMHC-Cre-ER^{T2}-$ETAR^{fl/fl}$) or general effects (CAGG-Cre-ER^{T2}-$ETAR^{fl/fl}$) additional animal models were provided.

The second part of present thesis concentrates on the potential effects of ET-1/ETBR isoform signaling pathway on the renin system *in vivo*. Therefore, the generation of a renin cell-specific ETBR animal model was necessary ($Ren^{1d+/Cre}$-$ETBR^{fl/fl}$) which was verified by in situ hybridization assay. In addition, further experiments aimed to characterize, if the possible inhibitory effect of ETBR isoform on renin synthesis and secretion is regulated by stroma derived cells ($FOX^{D1Cre/+}$-$ETBR^{fl/fl}$).

With respect to present results of the first and second chapter, the renal ET-receptor isoforms seem to be of less relevance for the renin synthesis and secretion *in vivo*. All used animals showed normal developed renin positive cells, renin mRNA abundance, PRC values and unchanged systolic blood pressures compared to controls. In addition, all animals displayed an inhibition of renin secretion mainly mediated by a decline of the renal blood flow in the isolated perfused kidney model after adding ET-1 in increasing concentration to the *ex vivo* system.

In order to exclude potential ET-receptors interactions on renin positive cells *in vivo*, a renin cell-specific ETAR and ETBR "double knockout" animal model ($Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$) was generated in the last part of the present study. Several functional *in vivo* experiments were administered subsequently. All Cre^{+} animals showed normal renin mRNA abundance and unchanged systolic blood pressure values compared to controls. Only plasma renin concentration was slightly diminished in $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$ compared to controls. In addition, all animals developed normal renin positive cells at the juxtaglomerular cell apparatus as in controls. The isolated perfused kidneys of $Ren^{1d+/Cre}$-$ETAR^{fl/fl}$-$ETBR^{fl/fl}$-mice revealed an inhibition of renin secretion rate mediated by the decline of the renal blood flow through ET-1 similar to control animals. All mentioned results indicate that both ET-receptors on renin producing cells seem to be of less importance for the renin synthesis and secretion moreover for the renin system *in vivo* and *in vitro*. It was assumed that renal ET-receptors expressed on other renal specific cells maybe more important for this regulation. Therefore, to further examine a potential indirect effect of ET-receptors on the renin system a stroma derived cell-specific "double knockout" animal model was generated ($FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$).

All $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ animals displayed normal levels of renin mRNA and no significant changes in plasma renin concentrations compared to controls. These results were supported by systolic blood pressure measurements. Via the tail cuff method, one could detect that $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ mice had normal levels of blood pressure as shown in control littermates.

In the absence of systemic influences, the isolated perfused kidney model displayed in $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ no inhibition of renin secretion and a nearly steady renal blood flow compared to controls. These results support theory that systemic effects such as the blood pressure, the autonomic nervous system or different hormones are maybe involved in present situation. Moreover, those effects seem to interact in current ET receptor deletion equilibrating homeostasis.

In summary, present thesis indicate for the very first time that both ET-receptors are markers for renin producing cells. Moreover both ET-receptor isoforms seem to be not of major relevance for renin synthesis and secretion. There are findings which support theory of receptor interaction and that each single receptor isoform is able to take over functional role of the other receptor. Besides the potential direct effect, present thesis also focused on hypothesis that the inhibiting effect of endothelins on renin synthesis and secretion is regulated by ET receptors located on stroma derived cells ($FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$). However after investigation, present results seem to refute given postulation because there were no significant changes in $FOX^{D1Cre/+}$- $ETAR^{fl/fl}$-$ETBR^{fl/fl}$ mice regarding *in vivo* parameters compared to controls. All animals developed normal renin producing cells as in controls.

The inhibiting effect of endothelins on renin synthesis and secretion is therefore not primarily mediated by either renin cell lineage or stroma derived cells. Present results indicate that systemic effects are more involved in the regulation of the renin system or other expression sites as for example mesangial cells or tubular structures are possible to regulate the inhibition of renin secretion and synthesis through endothelins. Further investigations need to evaluate prospectively, if ET-receptors located on renin producing cells are more involved in terms of situations of pathophysiology.

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7 Supplement

7.1 List of abbreviations

°C	degree Celsius
µl	microliter
µm	micrometer
∞	unlimited
A	ampere
a	adenine
aa	afferent arteriole
ANG-I	angiotensin-I
ANG-II	angiotensin-II
AQP2	aquaporine-2
ca.	circa
CAGG	(C) cytomegalovirus (CMV) early enhancer element (A) the promoter, first exon and the intron of chicken beta-actin (G) the splice acceptor of the rabbot beta globin gene
CD31	cluster of differentiation 31 (PECAM-1)
cDNA	complementary DNA
ch	chicken
Cre/loxP	
Cre-ER^{T2}	fusion protein consisting of Cre-recombinase and modified estrogen binding site
d	days
dH2O	distilled water
dk	donkey
DMEM	Dulbecco's Modified Eagle Medium
ECE	endothelin-converting enzyme
ECE	endothelin-converting enzymes
ELISA	enzyme-linked immunosorbent assay
et al.	and others
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
ETAR	endothelin-A receptor
ETBR	endothelin-B receptor
Fig.	Figure
FOXD1	forkhead box D1
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GFP	green fluorescent protein
gt	goat

h	hours, human
i.e	for example
IPMK	isolated perfused kidney model
ISH	in situ hybridization
iso	isoproterenol
JG	juxtaglomerular
L	liter
LD	loading dye
M	molar (mol/L)
mA	milliampere
min	minute
ml	milliliter
mRNA	messenger ribonucleic acid
ms	mouse
ng	nanogram
nm	nanometer
ON	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	polyethylene
PFA	paraformaldehyde
prepro-ET	prepro-endothelins
RAAS	Renin-Angiotensin-Aldosterone system
rb	rabbit
rpcs	renin producing cells
RPL32	ribosomal protein L32
rpm	revolutions per minute
RS	renin system
RT	reverse transcription, room temperature
SMMHC	Smooth muscle myosin, heavy chain (myosin-11 or SMMHC)
TAE	Tris acetate EDTA buffer
Taq Polymerase	thermostable DNA polymerase named after *Thermus aquaticus*
Tris	tris hydroxymethyl aminomethan
TRITC	Tetramethylrhodamine isothiocyanate
V	volt
W	watt
WT	wildtype
α	anti or alpha
α-sma	α-smooth muscle actin
β	beta
µ	micro (10^-6)

The knowledge of the chemical elements and SI units as well as usually used abbreviations in science or medicine was assumed.

7.2 Congress contributions

95th Annual Meeting of the German Physiological Society, 02.03.-05.03.2016, Lübeck, Germany – poster

“The endothelin-A receptor is expressed by renin cells of the kidney but is of less relevance for the physiological control of renin synthesis and secretion”

8th Annual Meeting of the “Deutschen Gesellschaft für Nephrologie”, 10.09.-13.09.2016, Berlin, Germany – poster

“Die Rolle des Endothelin-1/Endothelin-A-Rezeptor Signalweges für die Regulation des Reninsystems in der Mausniere”

96th Annual Meeting of the German Physiological Society, 16.03.-18.03.2017, Greifswald, Germany – poster

“The endothelin-A and endothelin-B receptor are expressed by renin cells of the kidney but seem to be of less relevance for the physiological control of renin synthesis and secretion *in vivo*

The Fifteenth International Conference on Endothelin, 4.10.-7.10.2017, Prague, Czech Republic - talk

“Is the inhibiting effect of endothelins on renin synthesis and secretion a direct effect on renin producing cells?”

7.3 Publications

Role of ET-receptors in the renin cell lineage for renin synthesis and secretion *in vivo* and *in vitro*

Neder TH, Neubauer B, Epstein M, Gomez RA, Yanagisawa M, Kurtz A, Wagner C: (in prep)

7.4 Declaration

I herewith declare in lieu of oath that I have composed this thesis without any inadmissible help of a third party and without the use of aids other than those listed. The data and concepts that have been taken directly or indirectly from other sources have been acknowledged and referenced.

Other persons have not helped to produce this work as regards to its content or making. In particular, I have not used the services of any professional agencies in return for payment or those of other persons. Nobody has received payment in kind – neither directly nor indirectly – from me for any work that is connected with the content of this doctoral thesis.

This thesis has not been submitted, wholly or substantially, neither in this country nor abroad for another degree or diploma at any university or institute.

Regensburg, der 27.10.2017

Thomas Neder

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