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Physiological and pathophysiological regulation of aldosterone secretion

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Introduction

Arterial hypertension represents one of the most common diseases in developed countries affecting 30–45% of the adult European population (Mancia et al., 2013). Primary aldosteronism is the most frequent cause of endocrine hypertension with a prevalence of 7–10% among unselected hypertensive patients (Rossi et al., 2006, Hannemann et al., 2012).

Primary aldosteronism is characterized by inadequately high aldosterone values, suppressed plasma renin concentrations, hypertension and hypokalemia. It develops from autonomous hypersecretion of the mineralocorticoid aldosterone, independent from angiotensin II stimulation and non-suppressible by sodium load. Aldosterone is rapidly synthesized in the zona glomerulosa cells upon stimulation of the renin-angiotensin-aldosterone system (RAAS). Several enzymes are involved in this steroidogenic process. Early studies identified steroidogenic acute regulatory protein (StAR) to initiate the adrenal pathway of steroidogenesis with the translocation of cholesterol from the outer to the inner mitochondrial membrane (Black et al., 1994, Clark et al., 1994, Spät and Hunyady 2004). StAR (STARD1) expression is stimulated by ACTH and Ca^{2+} mediated stimuli such as angiotensin II and potassium (Spät and Hunyady 2004). The cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone at the matrix side of the inner mitochondrial membrane (Hum and Miller 1993). Pregnenolone exits the mitochondria and 3-b-hydroxysteroid dehydrogenase of the microsomal compartment transforms it to progesterone. Subsequently, progesterone undergoes 21-hydroxylation to form 11-desoxycorticosterone (Otis and Gallo-Payet 2007). The aldosterone synthase (CYP11B2) is involved in the last steps of aldosterone biosynthesis catalyzing the 11-β-hydroxylation and 18-oxidation of 11-desoxycorticosterone to corticosterone and furthermore the 18-hydroxylation of corticosterone and 18-oxidation of 18-OH-corticosterone, finally resulting in the production of aldosterone (Curnow et al., 1991). *CYP11B2* is transcriptionally regulated

by the nuclear transcription factors NGF1-B (NR4A1) and NURR1 (NR4A2), whose expression is in turn stimulated by angiotensin II (Bassett et al., 2004).

There are two major causes of primary aldosteronism: aldosterone-producing adrenal adenomas and bilateral adrenal hyperplasia (Mulatero et al., 2004, Rossi et al., 2006). Despite its high prevalence, so far, the genetic background of primary aldosteronism due to bilateral hyperplasia has been elucidated only in a small subgroup of cases: Familial hyperaldosteronism type I (FH-1, glucocorticoid remediable hyperaldosteronism) is an autosomal dominant disorder, caused by unequal crossing-over of the *CYP11B1* and *CYP11B2* and the formation of a hybrid gene (Sutherland et al., 1966, Lifton et al., 1992). FH-1 patients present an ACTH-dependent production of aldosterone, independent from angiotensin II regulation. Familial hyperaldosteronism type II (FH-2) also appears to be inherited as an autosomal dominant trait. Linkage studies performed in two Australian, one South American and two Italian families demonstrated association with a locus on chromosome 7p22 (Gordon et al., 1991, So et al., 2005), but the linkage area could not be correlated to any causative mutation. Familial hyperaldosteronism type III (FH-3), characterized by early-onset hypertension, profound hypokalemia and significant overproduction of 18-oxocortisol and 18-hydroxycortisol (Geller et al., 2008), however, has been recently associated to gain-of-function mutation in the *KCNJ5* gene, encoding an inwardly rectifying potassium channel (Choi et al., 2011). *KCNJ5* mutations lead to a loss in channel selectivity and by that to continuous membrane depolarization and increased intracellular Ca^{2+} concentrations resulting in aldosterone excess. These mutations are more prevalent in younger patients and in women and correlate with higher plasma aldosterone levels (Boulkroun et al., 2012). Additionally, somatic mutations in *ATP1A1* and *ATP2A3*, two members of the P-type ATPase gene family, were identified by exome sequencing in aldosterone producing adrenal adenomas (Beuschlein et al., 2013, Fernandes-Rosa et al., 2014). Finally, germline mutations in the Ca^{2+}-channel genes *CACNA1D* (Scholl et al., 2013)

and *CACNA1H* (Scholl et al., 2015) were recently identified as a rare cause of early onset primary aldosteronism. Taken together, genetics of the vast majority of cases of bilateral adrenal hyperplasia remain enigmatic.

Mice provide a valuable model for the investigation of mechanisms involved in blood pressure regulation and specifically for the study of the RAAS. Several animal models with targeted genetic alterations such as Task1 and Task3 knockout mice (Davies et al., 2008, Heitzmann et al., 2008) and a circadian clock-deficient cry-null mouse model showing an upregulation of the adrenal Hsd3b6 (Doi et al., 2010) have been presented to date.

A method for development of new mouse models with a specific phenotype is random mutagenesis of the mouse genome using the mutagenic substance *N*-ethyl-*N*-nitrosourea (ENU) (Clark et al., 2004). ENU is an alkylating agent inducing ethylation of nucleic acids, which finally results in point mutations. This enables the production of many F1 founder animals from a single treated male, reducing the number of animals receiving ENU-handling. Approximately 1 in 1000 F1 animals is heterozygous for a functionally relevant mutation of interest, according to the specific-locus test (Russell et al., 1979). Specific screening tools should then be applied to distinguish a sometimes-mild effect (Rossant and Scherer 2003). In such a phenotype-driven genetic approach the genomic association is finally achieved either by linkage analysis using single nucleotide polymorphisms (SNPs) or exome sequencing. Inbred mouse strains, which are progenitors of chromosome substitution strains (CSS) and recombinant inbred strains (RIS), can be utilized for mapping the position of a gene effect. Linkage analysis upon crossbreeding of mutant animals from one strain to wild-types from another can associate the phenotype with a small locus on the genome, hosting the causative gene for the investigated trait. However, C57BL/6J and C3H/HeJ inbred strains, which constitute the genetic background of the available CSS and one of the RIS panels, present variations in several physiological and behavioral traits (Singer et al., 2004) and such

differences of the respective investigated phenotype need to be defined to prevent from misleading genetic associations.

Genome-wide association studies (GWAS) are important tools for the identification of common genetic susceptibility loci in human heredity and disease. This method uses genotyping arrays of a dense set of common single-nucleotide polymorphisms to generate genotypes in large populations of cases and controls (Frazer et al., 2007). GWAS improved our knowledge on hereditary characteristics leading to predisposition to diseases influenced from both genetic and environmental factors (Hirschhorn and Daly, 2005). In fact, in two recent GWAS, both systolic and diastolic blood pressure could be associated with 16 and 107 independent loci, respectively, containing genes that regulate blood pressure (Ehret et al., 2011, Warren et al., 2017). As in investigations utilizing inbred mouse strains, in population-based studies was demonstrated that the aldosterone to renin ratio (ARR) is an inherited characteristic and can be correlated with the risk of blood pressure progression and incident hypertension (Newton-Cheh et al., 2007).

Objectives

First aim of the work present herein was to examine the physiological regulation of parameters of the adrenal aldosterone secretion by defining immediate transcriptional changes that take place in the adrenal gland upon specific stimulation and suppression experiments modulating aldosterone secretion *in vivo*. As the basis for upcoming exploration of potential genetic mechanisms involved in the modulation of aldosterone secretion it was further necessary to determine the differences in aldosterone values and the molecular differences in C3HeB/FeJ and C57BL/6J inbred mouse strains. Subsequently, in an attempt to define novel genetic loci involved in the pathophysiology of primary aldosteronism, an ENU-mutagenesis screen for the parameter aldosterone had to be established, followed by the phenotypic and genetic characterization of identified mouse lines. A parallel approach for the detailed molecular and functional characterization of genes involved in the regulation of the RAAS was to examine candidate genes identified by GWAS in a population-based study.

Studies carried out and main findings

1. Determinants of baseline aldosterone secretion in mice

Spyroglou A, Sabrautzki S, Rathkolb B, Bozoglu T, Hrabé de Angelis M, Reincke M, Bidlingmaier M, Beuschlein F. Gender-, strain-, and inheritance-dependent variation in aldosterone secretion in mice. J Endocrinol. 2012 Dec;215(3):375-81.

Aim of this study was to determine the differences in regulation of aldosterone production in C3HeB/FeJ and C57BL/6J inbred mouse strains as the basis for future exploration of potential genetic mechanisms involved in the modulation of aldosterone secretion. Therefore, aldosterone and renin levels as well as the adrenal steroidogenic profile were defined in male and female mice of two inbred strains, C3HeB/FeJ and C57BL/6J, and their offspring of the F1 and F2 generation (for nomenclature refer to Table 1).

Measurement of baseline aldosterone levels in male and female mice of the strain C3HeB/FeJ (CCCC) revealed baseline aldosterone values of 134±11 and 78±5 pg/ml respectively, which resulted in a statistically significant difference between the genders ($P<0.001$, Table 2). Similarly, male C57BL/6J (BBBB) had significantly higher aldosterone values than their female littermates ($P<0.01$, Table 2). Additionally, BBBB animals presented significantly higher values than CCCCs for both genders. Upon mating of male BBBB to female CCCC or male CCCC to female BBBB mice, all F1 offspring were tested for their aldosterone values. Also in these F1 mice, significant gender differences in aldosterone values were documented (BBCC males vs. females $P<0.05$; CCBB males vs. females $P<0.05$). Furthermore, animals carrying C3HeB/FeJ genetic background presented a tendency toward lower aldosterone values. Further mating of those male and female F1 offspring to inbred BBBBs of both genders gave rise to F2 animals with a mixed genetic background. Depending on the extent of C57BL/6J genetic background on the paternal side, plasma aldosterone values showed a gradual reduction, with male animals having in all cases higher aldosterone than their female littermates (Table 2).

Generation	Paternal×maternal origin	Abbreviation
F0	C3HeB/FeJ	CCCC
	C57BL/6J	BBBB
F1	C57BL/6J×C3HeB/FeJ	BBCC
	C3HeB/FeJ×C57BL/6J	CCBB
F2	(C57BL/6J×C57BL/6J)×(C57BL/6J×C3HeB/FeJ)	BBBC
	(C57BL/6J×C57BL/6J)×(C3HeB/FeJ×C57BL/6J)	BBCB
	(C57BL/6J×C3HeB/FeJ)×(C57BL/6J×C57BL/6J)	BCBB
	(C3HeB/FeJ×C57BL/6J)×(C57BL/6J×C57BL/6J)	CBBB

Table 1: Nomenclature for C3HeB/FeJ and C57BL/6J mice and their F1 and F2 offspring. Reproduced from Spyroglou et al., 2012.

Strains Aldo	Males (pg/ml)	Females (pg/ml)	*P*
BBBB	237±13	210±15	<0·01
BBBC	225±15	147±11	<0·01
BBCB	177±16	140±16	0·12
BCBB	125±18	80±7	0·07
BBCC	120±2	81±8	<0·05
CCBB	80±1	70±4	<0·05
CBBB	74±15	75±13	0·69
CCCC	134±11	78±5	<0·001
P	<0·0001	<0·0001	

Table 2: Aldosterone values (pg/ml) of male and female C3HeB/FeJ, C57BL/6J mice and their F1 and F2 offspring. Adapted from Spyroglou et al., 2012.

Subsequently, focus was put on the F2 animals and a more detailed phenotypical characterization concerning their RAAS was performed. No differences in renin values could be observed among the groups of both genders (males P=0.27, females P=0.96). Likewise, calculation of the aldosterone to renin ratio (ARR) showed no differences within the groups for both male (P=0.99) and female (P=0.27) animals (Table 3). The potassium values did not indicate any specific tendency within the examined groups (males, P=0.37; females, P=0.10) and overall remained within the normal range. Kidney parameters creatinine and urea were also defined in the F2 offspring of both genders and in all instances remained within the normal range without evident differences among the examined groups (creatinine: males, P=0.55 and females, P=0.36; urea: males, P=0.18 and females, P=0.86, Table 3).

On the molecular level, quantification of the adrenal expression levels of steroidogenic enzymes involved in aldosterone synthesis displayed the same trend as the aldosterone values themselves, with animals hosting C57BL/6J background in their paternal origin having also the highest expression levels for StAR, cyp11a1, and cyp11b2 enzymes. In details, StAR showed the highest expression in BBBC male and female animals and the lowest in CBBB animals (males P<0.05; females P<0.01). Similar was the tendency for cyp11a1 expression which was highest in BBBCs, with a gradual reduction in BBCBs and BCBBs and the lowest levels in CBBBs (males P<0.001; females P<0.001). The same observation was obtained for the cyp11b2 (P<0.05 for both genders, Table 3).

Taken together, these data reveal gender- and strain-dependent differences in the baseline aldosterone levels in age-matched mice and demonstrate that the genetic background of the animals plays a significant role modulating their plasma aldosterone levels without distinct impact on other parameters of the RAAS.

Males	**Aldo**	**Renin**	**ARR**	**K+**	**Crea**	**Urea**	**StAR**	**cyp11a1**	**cyp11b2**
	(pg/ml)	(ng/ml/hr)	(pg/ml/ ng/ml/hr)	(mmol/l)	(mg/ml)	(mg/ml)	(%)	(%)	(%)
BBBC	225±19	93.7±22	3.8±0.9	5.1±0.13	0.35±0.01	61,4±1	100±11	100±10	100±14
BBCB	213±12	73.3±9	4.0±1.0	4.7±0.11	0.35±0.01	58.5±2	81.8±3	81.4±5	79.8±5
BCBB	180±12	60.7±10	3.8±0.9	4.9±0.11	0.36±0.01	57.3±4	84.7±8	67.6±6	65.3±8
CBBB	175±14	42.5±8	3.8±0.6	4.8±0.13	0.34±0.01	56.3±2	61.9±6	52.1±2	65.9±8
P value	<0.05	0.2719	0.9958	0.3737	0.5562	0.1881	<0.05	<0.001	<0.05
Females	**Aldo**	**Renin**	**ARR**	**K+**	**Crea**	**Urea**	**StAR**	**cyp11a1**	**cyp11b2**
	(pg/ml)	(ng/ml/hr)	(pg/ml/ ng/ml/hr)	(mmol/l)	(mg/ml)	(mg/ml)	(%)	(%)	(%)
BBBC	172±12	51.5±7	3.8±0.4	4.6±0.16	0.33±0.01	59.5±2	100±7	100±6	100±13
BBCB	169±12	53±13	3.5±0.6	4.3±0.11	0.35±0.01	50.2±3	78.4±4	88±5	62.1±6
BCBB	140±15	52.2±10	3.2±0.9	4.6±0.12	0.35±0.01	58.2±2	83.3±6	86.3±7	58.4±7
CBBB	115±15	53.1±4	2.6±0.2	4.2±0.07	0.34±0.01	49.6±3	73.7±3	58.4±3	56.9±5
P value	<0.05	0.9603	0.2660	0.1054	0.3592	0.8599	<0.01	<0.001	<0.05

Table 3: Aldosterone (pg/ml), renin (ng/ml per h), ARR (pg/ml per ng per ml per h), potassium (mmol/l), creatinine (mg/ml), urea (mg/ml), and levels of expression of the enzymes StAR, cyp11a1 and cyp11b2 for the groups BBBC, BBCB, BCBB, and CBBB of both genders. Adapted from Spyroglou et al., 2012.

2. *In vivo* regulation of aldosterone secretion

Spyroglou A, Manolopoulou J, Wagner S, Bidlingmaier M, Reincke M, Beuschlein F. Short term regulation of aldosterone secretion after stimulation and suppression experiments in mice. J Mol Endocrinol. 2009 May;42(5):407-13.

In this study, the murine adrenal steroidogenic response and aldosterone secretion was investigated upon specific functional tests. More specifically, one group of 35 mice received an i.p. angiotensin II injection, the second an i.p. sodium load and the third a sham injection. In each one of three groups 5 animals were euthanized at baseline and at following time points (10, 20, 30, 40, 60 and 120 min) upon injection. Their plasma aldosterone levels and the adrenal expression of enzymes involved in steroidogenesis were subsequently defined.

In the control group, aldosterone secretion in animals 10 min after sham injection was significantly higher than under baseline conditions ($P<0.005$) but already at 30 min and thereafter dropped back to levels comparable with baseline values (Figure 1). qPCR analysis revealed an increase of StAR expression over the baseline values only 20 min after the sham injection ($P<0.05$), sustained until 60 min later ($P<0.05$). Similarly, Cyp11a1 expression started increasing 20 min after the sham injection ($P<0.001$) and did not normalize within the observation period ($P<0.05$). By contrast, Cyp11b2 expression showed an increase already 10 min after the injection ($P<0.05$) and rapidly normalized in the 20-min group ($P=0.93$) and thereafter (Figure 2A). NGF1-B expression was significantly upregulated already after 10 min ($P<0.001$) whereas Nurr1 expression showed a transient decrease up to 20 min ($P<0.05$) and subsequently a slight increase ($P<0.05$, Figure 3A).

Ten minutes after the angiotensin II injection, the aldosterone concentration was significantly elevated in comparison to baseline ($P<0.001$). The subsequent measurements showed a stepwise reduction while aldosterone secretion remained significantly higher in comparison with the baseline values ($P<0.01$, Figure 1). A parallel increase in StAR and Cyp11a1 expression could be observed already 10 min upon injection (StAR $P<0.05$, Cyp11a1 $P<0.01$)

with a peak of StAR mRNA levels at 20 min (P<0.05) and sustained elevation of both enzymes until 120 min later (StAR P<0.05, Cyp11a1 P<0.01). Furthermore, angiotensin II injection resulted in a significant increase in Cyp11b2 expression after 10 min (P<0.005) which was continued up to 60 min later (P<0.01, Figure 2B). Upregulation of Cyp11b2 expression was significantly higher in the angiotensin II group in comparison with the sham injection group (P<0.01 for time points 10–60 min). A pronounced increase in the expression of both NGF1-B and Nurr1 could be documented already 20 min after injection (Nurr1 P<0.01, NGF1-B P<0.001) which lasted up to 60 min (Nurr1 P<0.005, NGF1-B P<0.001, Figure 3B).

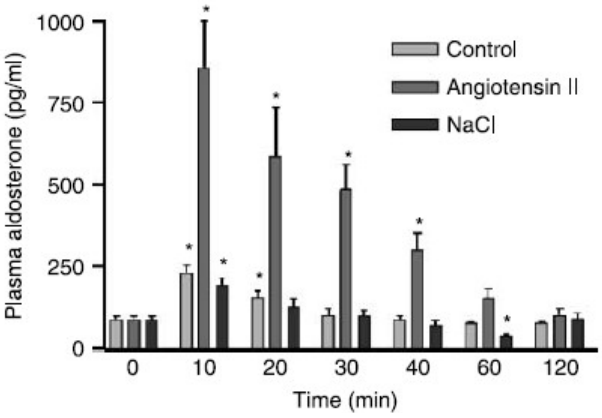

Figure 1: Aldosterone values in control animals after sham injection, after angiotensin II stimulation and upon NaCl suppression. Stars denote significant changes over baseline values (i.e., 0 min). Reproduced from Spyroglou et al., 2009.

After i.p. injection of 1 ml NaCl 0.9%, aldosterone secretion showed a short-term increase (P<0.005) comparable with that seen after sham injection. This initial peak was followed by a slow decrease with the lowest aldosterone levels 60 min after the injection, significantly lower in comparison with baseline values (P<0.005, Figure 1). Both StAR and Cyp11a1 expressions were reduced upon sodium load between 20 min (StAR P<0.001, Cyp11a1 P<0.001) and 60 min (StAR P<0.05, Cyp11a1 P<0.05). Furthermore, sodium load had significant effects on Cyp11b2 expression levels that remained suppressed between 20 min (P<0.05) and 60 min (P<0.01, Figure 2C). Thirty min after the sodium load, Nurr1 mRNA displayed a transient increase (P<0.05) and later a progressive decrease with a significant drop under baseline parameters after 120 min (P<0.005). NGF1-B expression, however, displayed a constant and significant increase during the first hour (10 min: P<0.05, 40 min: P=0.01, Figure 3C) as after

sham and angiotensin II injection. While increases in NGF1-B mRNA levels were similar between the treatment groups, Nurr1 expression levels were induced only upon angiotensin II administration.

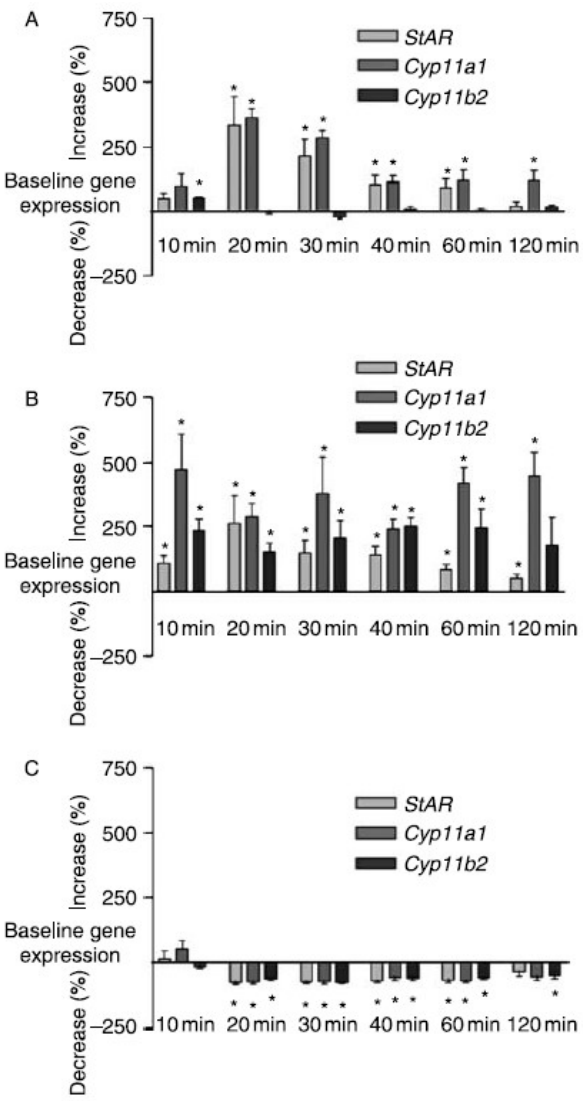

Figure 2: (A) Increase or decrease of the mRNA expression levels of StAR, Cyp11a1, and Cyp11b2 genes in the control group upon sham injection, (B) after angiotensin II stimulation, and (C) after NaCl challenge. Stars denote significant changes for each gene over baseline expression levels which are set as 100% for each gene. Reproduced from Spyroglou et al., 2009.

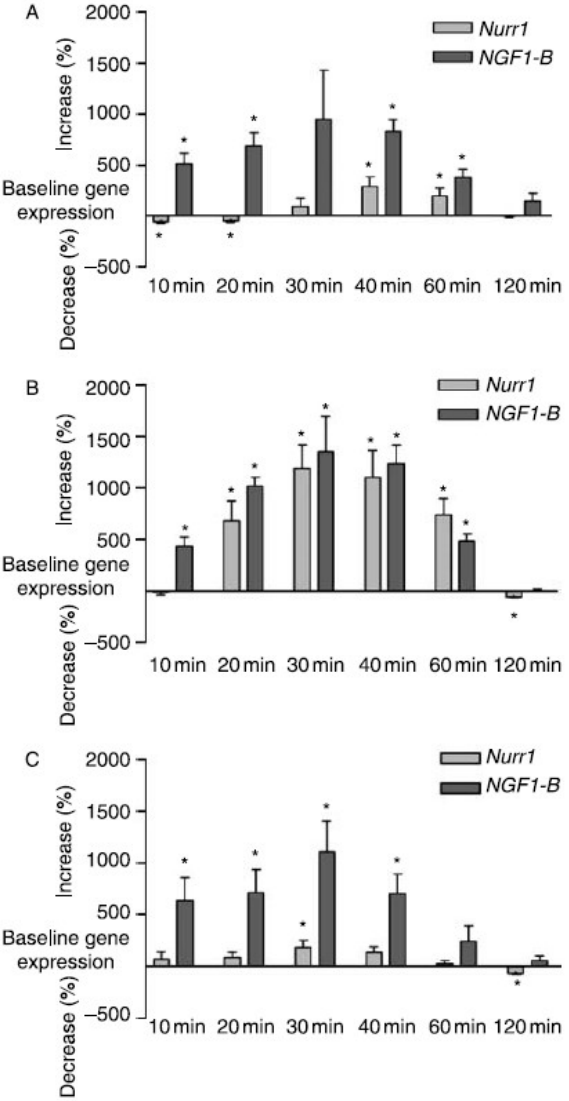

Figure 3: (A) Increase or decrease of the mRNA expression levels of Nurr1 and NGF1-B in the control group upon sham injection, (B) after angiotensin II stimulation, and (C) after NaCl challenge. Stars denote significant changes for each gene over baseline expression levels which are set as 100% for each gene. Reproduced from Spyroglou et al., 2009.

Taken together, these data demonstrate that regulation of aldosterone secretion upon specific stimulation and suppression paradigms *in vivo* is achieved by short term transcriptional

changes of key steroidogenic enzymes and nuclear transcription factors. From the investigated parameters on the mRNA level StAR, Cyp11a1 and NGF1-B appear to respond more to unspecific stress-related effects, whereas Cyp11b2 and Nurr1 are preferentially regulated by specific stimulation of the RAAS.

3. Mutagenesis screen for primary aldosteronism

Spyroglou A, Wagner S, Gomez-Sanchez C, Rathkolb B, Wolf E, Manolopoulou J, Reincke M, Bidlingmaier M, Hrabé de Angelis M, Beuschlein F. Utilization of a mutagenesis screen to generate mouse models of hyperaldosteronism. Endocrinology. 2011 Jan;152(1):326-31.

The ENU-mutagenesis screen was utilized for the phenotype hyperaldosteronism and the aldosterone values of all 12-weeks-old F1 offspring from ENU-treated males and wild-type females were measured. The normal range of aldosterone values was defined as mean + 3 SD of a wild-type age and gender matched population. In the initial screen 2864 mice took part: 1450 male and 1414 female animals. Of this cohort 83 animals initially presented aldosterone values above the normal range (18 males and 65 females; Figure 4, left panel). All animals that showed once pathological aldosterone levels were retested at the age of 16 weeks and upon confirmation measurement, only 11 mice had constantly high aldosterone values (two males and nine females; Figure 4 right panel). These were then mated to wild-type animals of the opposite gender. Two mice with abnormal aldosterone levels died due to undefined reasons, before further breeding could take place and one mouse gave rise solely to unaffected offspring, so that finally only eight lines with affected offspring could be generated. Taken together, 2.9% of all offspring tested for hyperaldosteronism showed initially high aldosterone values. From these 13.2% had sustained hyperaldosteronism (females: 81.8%, males:18.2%). In total, 0.38% of all mice screened had confirmed hyperaldosteronism.

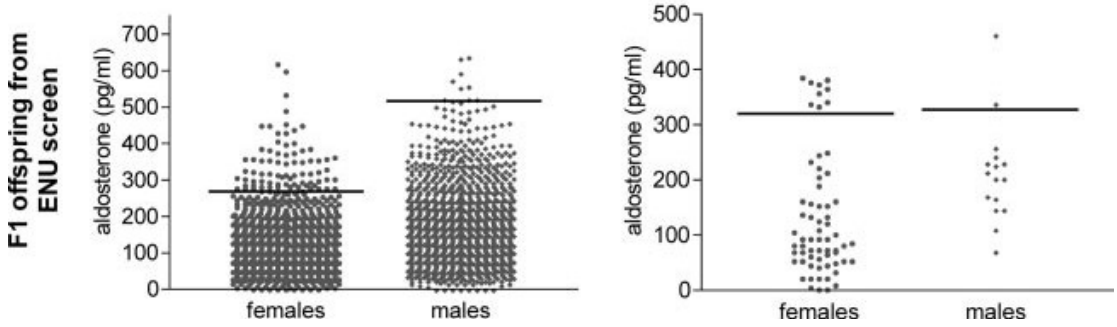

Figure 4: Aldosterone values of F1 offspring of ENU-treated mice at 12 weeks (females, n=1414; males, n=1450; left panel) and at the age of 16 weeks (females, n=65; males, n=18; right panel), respectively. Horizontal bars represent cut-off levels defined as 3 SDs above the mean of gender- and age-matched controls. Adapted from Spyroglou et al., 2011.

Affected F2 animals of the eight established lines were then further bred by mating to wild-type mice of the opposite gender. From the pedigrees obtained we could gain first evidence for possible patterns of inheritance of the trait of hyperaldosteronism. In all pedigrees, the founder animal originated from a different ENU-treated male parent, so that it is very probable that different mutations are the genetic cause of the investigated phenotype in each case. Accordingly, different patterns of inheritance between individual pedigrees were observed (Figure 5 and Table 4): In line I, male and female animals were both affected in approximately 35% of the cases. In line III the pathological phenotype was present in both male and female mice in 27% and 22% of animals, respectively, with total male to female ratio of 1.5:1. In line V only male animals were affected at a rate of 38%, with total male to female ratio 4:3. From line VIII 30% of the males and 28% of the females showed hyperaldosteronism. However, in this mouse line the total male to female ratio was 2:1, possibly because of excess mortality in female offspring.

A penetrance of the investigated phenotype above 50% is considered as suitable for an ENU screen (Aigner et al., 2008). In our cohort, all but one mouse lines showed a suitable penetrance in either one or both genders (Table 4). In mouse line IV, where penetrance is 40 and 45%, in females and males, respectively, breeding over two generations showed preservation of the abnormal phenotype. Further aldosterone measurements in older animals (20 weeks old) revealed a trend to higher penetrance with increasing age. In all eight lines, the numbers of animals displaying abnormal plasma aldosterone levels (using as cut-off: mean + 3 SD) were significantly ($P<0.001$) higher than the expected 0.3% of the C3HeB/FeJ mouse population.

Taken together, in this study we have demonstrated the utility of a phenotype-driven mutagenesis screen for the identification of mouse lines with chronic hyperaldosteronism.

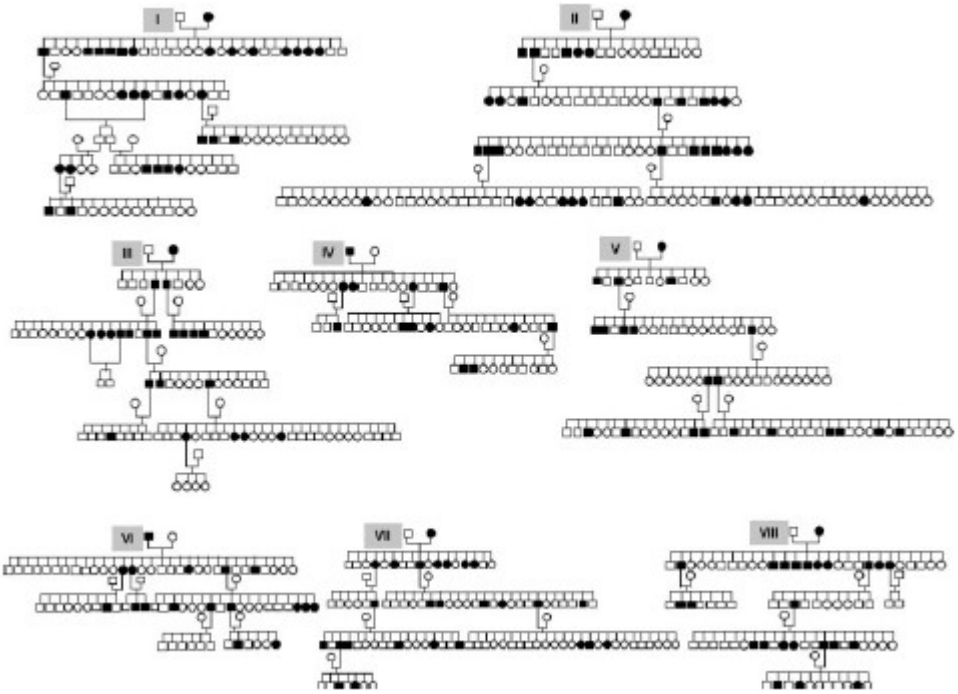

Figure 5: Pedigrees with hyperaldosteronism generated from the ENU screen. Black symbols denote affected animals. Reproduced from Spyroglou et al., 2011.

Mouse line	Males affected	Females affected	Penetrance males	Penetrance females
I	34.9% (15/43)	34.7% (16/46)	70%	70%
II	25.4% (16/63)	28.1% (18/64)	51%	56%
III	27.5% (14/51)	21.9% (7/32)	55%	44%
IV	22.6% (7/31)	20.0% (5/25)	45%	40%
V	38.3% (23/60)	0.0% (0/46)	77%	0%
VI	20.0% (9/45)	25.0% (7/28)	40%	50%
VII	26.9% (14/52)	25.0% (11/44)	54%	50%
VIII	30.6% (15/49)	27.6% (8/29)	62%	55%

Table 4: Percentages of affected male and female mice and their respective penetrances. Phenotypic penetrance (100%) is defined in the case of the appearance of 50% offspring exhibiting hyperaldosteronism after mating phenotypic mutants to wild-type mice. The observed penetrance in individual mouse lines was calculated by number of affected animals: (all animals: 2) and expressed as percent. Adapted from Spyroglou et al., 2011.

4. Reverse genetics for primary aldosteronism

Perez-Rivas LG, Rhayem Y, Sabrautzki S, Hantel C, Rathkolb B, Hrabé de Angelis M, Reincke M, Beuschlein F, Spyroglou A. Genetic characterization of a mouse line with primary aldosteronism. J Mol Endocrinol. 2017 Feb;58(2):67-78.

One of the previously described ENU-generated mouse lines with hyperaldosteronism was genetically characterized. Upon next-generation sequencing (NGS) applied on two different affected animals of this mouse line, a list with 31 single-nucleotide variations (SNV) was generated; eight of those were common for both investigated animals: SCO-spondin (*Sspo*), deoxyguanosine kinase (*Dguok*), 5730446D14Rik (Hoxa cluster antisense RNA 2 – *Hoxaas2*), calsyntenin 3 (*Clstn3*), all four on chromosome 6, integrator complex subunit 10 (*Ints10*), on chromosome 8, ataxia telangiectasia mutated (*Atm*), timeless interacting protein (*Tipin*) and mitogen-activated protein kinase 6 (*Mapk6*), the latest three on chromosome 9. These were subsequently sequenced in a large cohort (n = 126, 63 females, 63 males) of phenotypically 'affected' and 'unaffected' animals of this mouse line. *Ints10* was not found mutated in the examined cohort of animals and was excluded from further analyses.

All animals of this cohort were stratified into wild-type and mutant according to their genotypes for each of the candidate genes to enable association of each identified candidate gene with the phenotype of hyperaldosteronism. Thereby, we aimed at isolating a potential effect of one mutation on the investigated phenotype even though the random presence of mutant variants of the other candidate genes might have further modulating effects. We correlated the genotype with the respective aldosterone, urea and creatinine values as well as steroidogenic enzyme expression levels. We observed significantly increased plasma aldosterone values in animals with *Sspo* ($P<0.01$), *Dguok* ($P<0.01$), *Hoxaas2* ($P<0.01$) and *Clstn3* ($P<0.001$) mutation (Figure 6). Urea and creatinine levels did not differ significantly among wild-type and mutated animals.

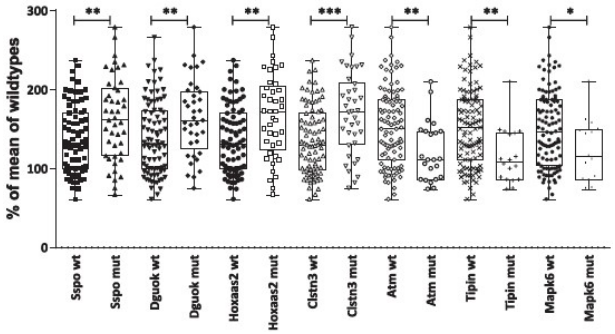

Figure 6: Aldosterone values in wild-type or mutant animals (n = 126) for each of the following genes: *Sspo*, *Dguok*, *Hoxaas2*, *Clstn3*, *Atm*, *Tipin* and *Mapk6*. Values are expressed as % of the mean aldosterone levels of wild-type male or female mice, respectively. Reproduced from Perez-Rivas et al., 2017.

StAR expression levels were significantly lower in *Clstn3*-mutants (P<0.05, Figure 7A) and *Cyp11a1* expression was reduced in *Sspo* (P<0.05), *Dguok* (P<0.01), *Hoxaas2* (P<0.01), *Clstn3* (P<0.001) and *Atm* (P<0.05) mutants (Figure 7B). *Hsd3b6* levels were lower in *Clstn3* (P<0.05), *Atm* (*P*<0.01), *Tipin* (P<0.05) and *Mapk6* (P<0.05) mutated animals (Figure 7C). No differences could be found for *Cyp11b1* expression levels for any of the mutations investigated (Figure 7D). In *Sspo*-mutated animals, *Cyp11b2* expression was significantly higher (P<0.05), whereas *Dguok*-mutated animals tended to present higher *Cyp11b2* levels, without reaching statistical significance (P=0.07, Figure 7E). The orphan nuclear factor *Nurr1*, showed higher expression levels in *Sspo*- (P<0.05) and *Hoxaas2*- (P<0.05) mutated mice (Figure 7F).

From the results presented in Figure 6, it is apparent that *Atm*, *Tipin* and *Mapk6* do not associate with the phenotype of primary aldosteronism since aldosterone values of wild-types were significantly higher than those of animals carrying one, two or all three above mentioned SNVs. To further narrow down the list to one causative SNV from the remaining four genes (*Sspo*, *Dguok*, *Hoxaas2* and *Clstn3*), aldosterone levels of animals excluding mice carrying *Atm* and/or *Tipin* and/or *Mapk6* mutations were compared. Animals carrying *Sspo* and/or *Dguok* and/or *Hoxaas2* and/or *Clstn3* SNVs demonstrated significantly higher aldosterone values than wild-type only animals and the mutant-to-wild-type ratio of aldosterone levels was in all cases higher in this restricted analysis (Table 5).

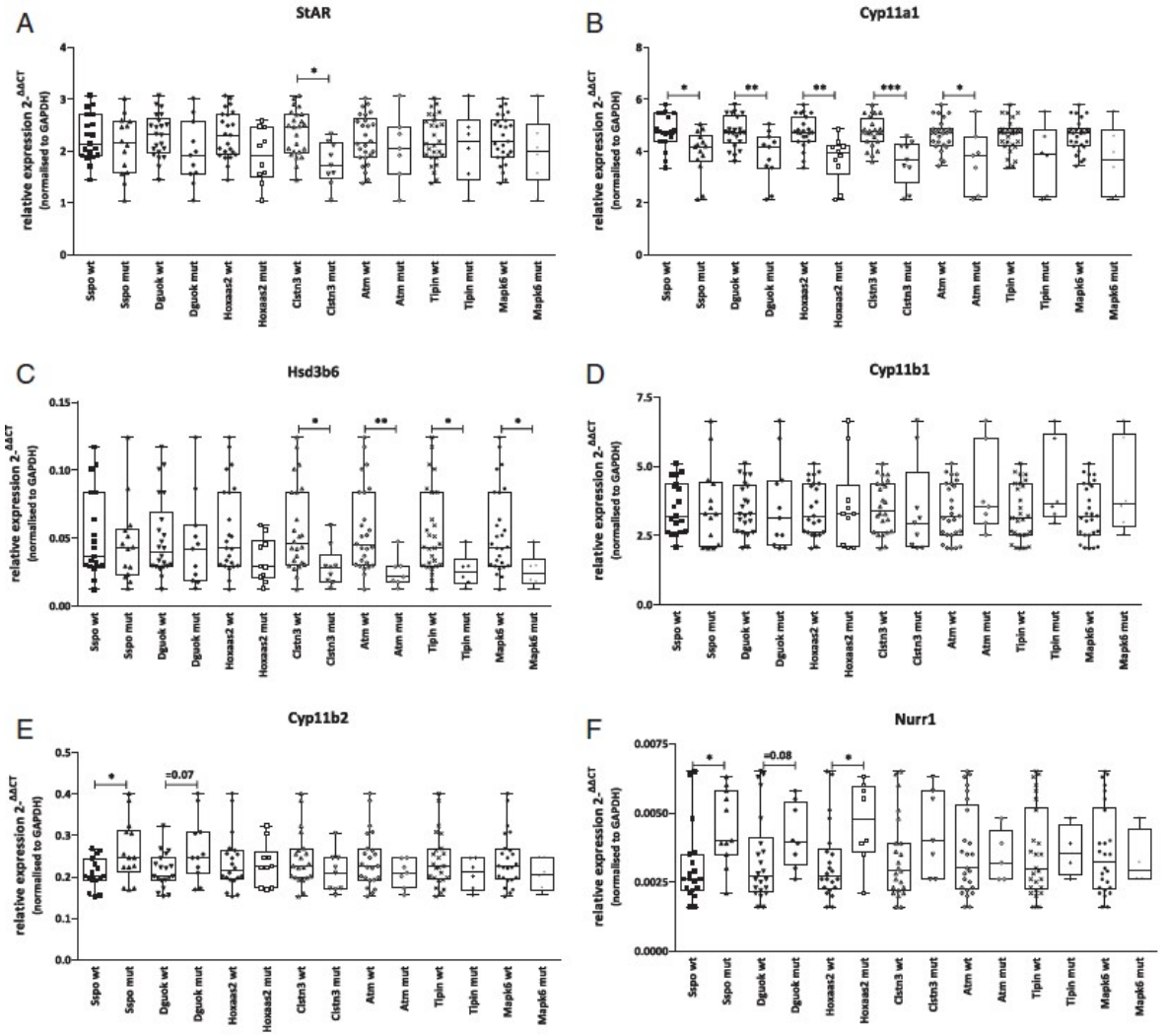

Figure 7: Expression levels of steroidogenic enzymes involved in aldosterone biosynthesis in wild-type or mutant animals (n = 33) for each of the following genes: *Sspo*, *Dguok*, *Hoxaas2*, *Clstn3*, *Atm*, *Tipin* and *Mapk6*. Values are relatively expressed to the housekeeping gene *Gapdh*. Reproduced from Perez-Rivas et al., 2017.

cohort	**Sspo**				**Dguok**			
	wt	mut	fold	p	wt	mut	fold	p
All (n=126)	351±120	405±132	1.15	<0.05	354±127	408±120	1.15	<0.05
Restricted (n=99)	349±107	426±128	1.22	<0.01	354±117	431±114	1.22	<0.01
cohort	**Hoxaas2**				**Clstn3**			
	wt	mut	fold	p	wt	mut	fold	p
All (n=126)	350±119	413±132	1.18	<0.01	346±116	428±132	1.23	<0.001
Restricted (n=99)	346±106	436±126	1.26	<0.001	341±101	459±123	1.34	<0.0001

Table 5: Aldosterone plasma levels (pg/mL, mean ± s.d.) in animals with the respective genotype in the whole cohort (all) and excluding animals with mutations in Atm, Tipin and/or Makp6 (restricted). Adapted from Perez-Rivas et al., 2017.

Aldosterone to Renin Ratio (ARR)			
		mean ± sd (% of mean of wt)	*P*
Sspo	wt (n=24)	100 ± 43	<0.05
	mut (n=17)	142 ± 71	
Dguok	wt (n=27)	100 ± 49	=0.09
	mut (n=14)	130 ± 60	
Hoxaas2	wt (n=27)	100 ± 47	=0.10
	mut (n=14)	130 ± 65	
Clstn3	wt (n=26)	100 ± 46	=0.80
	mut (n=15)	96 ± 55	

Table 6: Aldosterone-to-renin ratio of all experimental animals. In each line 'mut' denotes those mice which carry at least the respective mutant gene, whereas 'wt' combine those with the absence of the mutant gene. Adapted from Perez-Rivas et al., 2017.

Plasma renin activity did not show any significant differences between animals with wild-type and mutant genotype for the above four SNVs. Calculation of the aldosterone-to-renin ratios (ARR) demonstrated a significant ARR increase only in mice carrying the *Sspo* SNV (Table 6). Mice with the *Dguok* and/or *Hoxaas2* SNV also presented a trend toward increased ARRs. Histological examination of the adrenal glands of animals carrying all four SNVs (*Sspo*, *Dguok*, *Hoxaas2* and *Clstn3*) did not reveal any structural abnormalities such as adrenocortical adenomas. Moreover, there was no sign of adrenocortical hyperplasia as the cell count of the outer, middle and inner cortical area of these animals did not differ significantly from the wild-types (P=0.45, P=0.80 and P=0.29, respectively). However, upon immunohistochemical staining with murine CYP11B2 antibody, significantly more positively stained cells were present in the adrenal cortex of mutant animals in comparison to the wild-types (P<0.001, Figure 8A, B and C). Furthermore, CYP11B2 staining of the middle (P<0.001) and inner (P<0.001) cortical area was significantly more pronounced in mutant mice, suggesting alternated zonation pattern in animals with the four SNVs (Figure 8D–F).

Taken together, the characterization of this mouse line led to the identification of seven candidate genes for the investigated phenotype of primary aldosteronism. From the identified candidates *Atm*, *Tipin* and *Mapk6* appear to be irrelevant to the investigated phenotype, whereas no definite conclusion can be reached in the case of the role of *Sspo*, *Dguok*, *Hoxaas2* and *Clstn3* in primary aldosteronism. Thus, further efforts will be necessary to rule in or out the role of these four genes in the pathogenesis of primary aldosteronism.

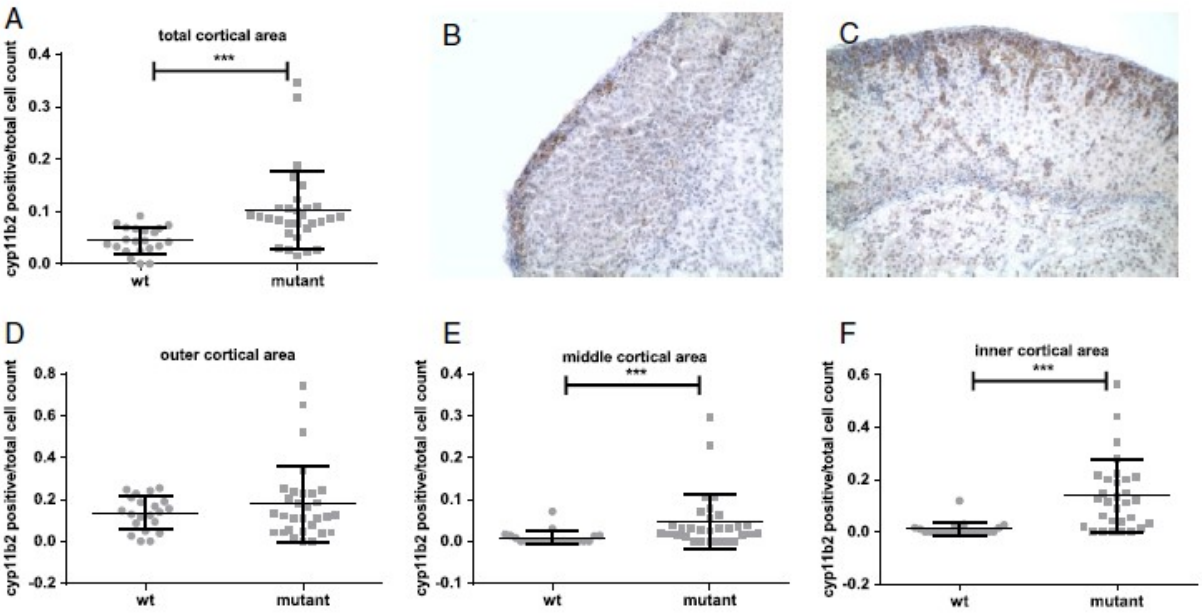

Figure 8: Histological examination of the adrenal glands of wild-type animals and mutants for all four following genes: *Sspo*, *Dguok*, *Hoxaas2* and *Clstn3*. Cell count of CYP11B2 positively stained cells per standardized area in the adrenal cortex of wild-type and mutant animals to the total cell count per area (A). Representative immunohistochemical staining of the adrenal cortex of wild-type (B) and mutant (C) mice. Ratios of CYP11B2 positively stained cells to the total cell count in the outer (D), middle (E) and inner (F) area of the adrenal cortex of wild-type and mutant mice (*** for $P<0.001$). Reproduced from Perez-Rivas et al., 2017.

5. Genome-wide association studies for primary aldosteronism

Spyroglou A, Bozoglu T, Rawal R, De Leonardis F, Sterner C, Boulkroun S, Benecke AG, Monti L, Zennaro MC, Petersen AK, Döring A, Rossi A, Bidlingmaier M, Warth R, Gieger C, Reincke M, Beuschlein F. Diastrophic dysplasia sulfate transporter (SLC26A2) is expressed in the adrenal cortex and regulates aldosterone secretion. Hypertension. 2014 May;63(5):1102-9.

A parallel approach for the detailed molecular and functional characterization of genes involved in the regulation of the RAAS was to examine candidate genes identified by genome-wide association study (GWAS) in a population-based study. The GWAS was performed on 1786 subjects of the Cooperative Health Research in the Region of Augsburg F4 cohort. A strong linkage of the ARR to chromosome 5 reaching genome-wide significance (P=6.78X10^{-11}; Figure 9A) could be demonstrated. The identified locus harbored four genes *SLC26A2*, *HMGXB3* (HMG box domain containing 3), *TIGD6* (tigger transposable element derived 6), and *CSF1R* (colony-stimulating factor 1 receptor) (Figure 9B). There was no evidence that the CSF1-CSF1R system has a functional effect in aldosterone secretion. On the contrary, SLC26A2 has a known function as transporter and showed an interesting expression pattern upon initial expression studies. Thus, SLC26A2 was chosen for further investigation.

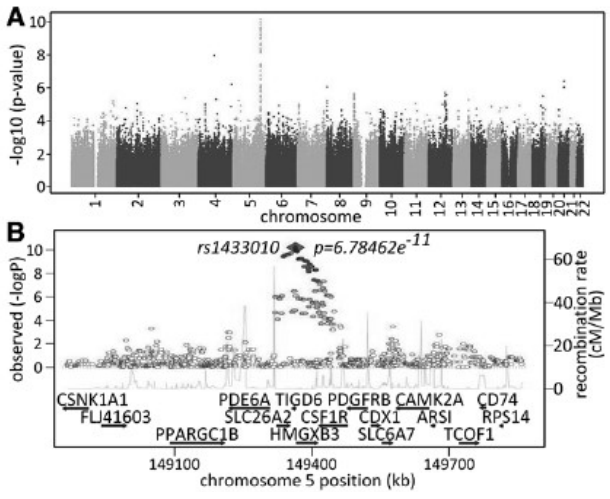

Figure 9: Genome-wide association study for aldosterone to renin ratio. (A) GWAS of 1786 subjects from the Cooperative Health Research in the Region of Augsburg F4 population using 1000 K Affymetrix chip, and the ARR are depicted as a Manhattan plot, demonstrating a genome-wide significant locus (P=6.78X10^{-11}) within chromosome 5. (B) Zooming into the specific locus indicates position of genes within this region including SLC26A2, HMGXB3, TIGD6, and CSF1R. Adapted from Spyroglou et al., 2014.

After real-time PCR analysis in wild-type mice, highest levels of *slc26a2* were identified in murine adrenal glands in comparison with kidney (P<0.001), lung (P<0.001), heart (P<0.001),

liver (P<0.001), and spleen (P<0.001) among other organs (Figure 10A). Immunohistochemically, adrenocortical expression of SLC26A2 was clearly documented in normal human adrenal glands, however, less intensive in aldosterone producing adrenal adenomas (APAs). Likewise, expression of *SLC26A2* was readily detectable in the human adrenocortical cell line NCI-H295R (Figure 12A). Furthermore, APAs displayed significantly lower *SLC26A2* levels than normal adrenal tissues (P<0.0001; Figure 10B), independently of sex (Figure 10C) or any underlying somatic mutation in *KCNJ5* or ATPase (*ATP1A1* and *ATP2B3*) in these adenomas (Figure 10D).

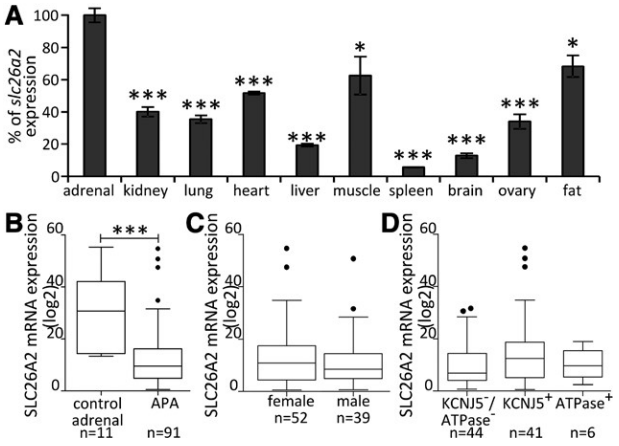

Figure 10: Expression of *SLC26A2* in murine tissues and human aldosterone producing adenomas. (A) Investigation of tissue distribution in wild-type mice demonstrates slc26a2 expression to be highest in adrenal glands in comparison with a variety of other tissues. (B–D) *SLC26A2* expression is significantly lower in APAs (n=91) in comparison with control adrenal glands (normal adrenal, n=11; B). Within the groups of APAs, expression was independent of sex (C) or underlying somatic mutation in KCNJ5 (KCNJ5+), ATP1A1, and ATP2B3 (ATPase+; D). Adapted from Spyroglou et al., 2014.

We next investigated *slc26a2* expression in wild-type mice treated with angiotensin II and potassium, known stimulators of the aldosterone secretion. Short-term stimulation with angiotensin II caused a significant downregulation of adrenal *slc26a2* expression at 120 minutes (P<0.05; Figure 11A). Similarly, after potassium supplementation in the drinking water for 1 week, adrenal *slc26a2* expression was significantly downregulated at day 1 (P<0.05) and day 4 (P<0.05) while this effect was less pronounced at the end of the experiment (Figure 11B). Thus, these findings demonstrated relevant adrenal *slc26a2* expression changes upon experiments affecting the RAAS system, suggesting a potential role of this transporter on adrenal aldosterone output.

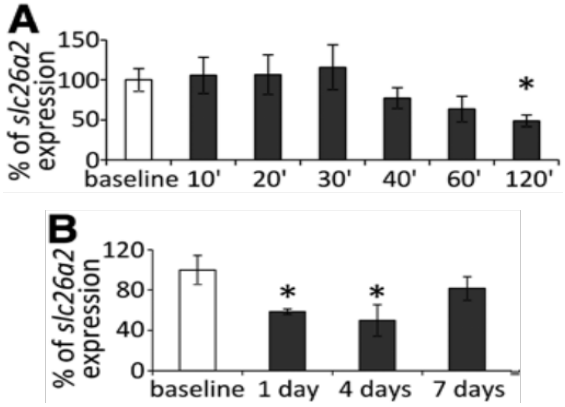

Figure 11: Transcriptional regulation of solute carrier family 26 member 2 (slc26a2) in vivo in mice. *SLC26A2* expression is significantly downregulated in adrenals from wild-type animals on angiotensin II stimulation (A) and after a high potassium diet (B). Adapted from Spyroglou et al., 2014.

To evaluate whether SLC26A2 function in adrenocortical cells would directly influence aldosterone secretion, we used a lentiviral based knockdown system in adrenocortical NCI-H295R cells and achieved knockdown efficacy in the range of 20% of untreated cells ($P<0.001$; Figure 12A). In comparison with an unspecific, nontargeting shRNA construct which had no effect on aldosterone secretion specific knockdown of *SLC26A2* caused a significant increase of aldosterone levels in the supernatant ($P<0.001$). Accordingly, aldosterone production after potassium and angiotensin II stimulation was further enhanced by *SLC26A2* knockdown (Figure 12B). *HSD3B2* and *CYP11B2* expression were upregulated in knockdown cells under baseline conditions (*HSD3B2:* $P<0.001$; *CYP11B2*: $P<0.001$) and even more upon potassium and angiotensin II stimulation (Figure 12E and 13F). Similar, but overall less pronounced changes were observed for *StAR* and *CYP11A1* expression (Figure 12C and D).

The role of adrenal *SLC26A2* expression on aldosterone output was also investigated with a *slc26a2* knock-in mouse model (p.A386V). The mutation induced here was initially identified in a patient with a mild phenotype of diastrophic dysplasia and provoked a partial loss of function of this sulfate transporter. Additionally, in this mouse model, the intronic cassette used caused splicing impairment of the mutant allele, resulting not only in structural protein changes but also in reduced expression levels of the mutant *slc26a2* mRNA. The adrenal glands of mutant animals presented morphologically no changes in comparison to wild-type controls (Figure 13A–13D). However, as, expected, adrenal *slc26a2* expression was

significantly lower in animals of both sexes (males: P<0.001; females: P<0.01; Figure 13E). Interestingly, aldosterone output was significantly increased only in female animals (P<0.01; Figure 13F), whereas male mice displayed only slight differences in their plasma aldosterone levels (P=0.27). Steroidogenic enzymes with a non-specific expression pattern (*star*, *cyp11a1*) or a zona fasciculata–specific distribution (*hsd3b1*) showed downregulation in the mutant mice (Figure 13G–13H). In contrast, adrenal enzymes with a specific role in aldosterone synthesis and preferential expression in the zona glomerulosa including *hsd3b6* (Figure 13I) and *cyp11b2* (Figure 13J) showed higher expression levels in mutant animals. Although some sex-specific changes were present, the steroidogenic enzymes' expression profiles showed similar trends in *slc26a2* mutant animals of both genders.

In summary, these findings delineate a possible effect of SLC26A2 in the regulation of aldosterone secretion potentially involved in the pathogenesis of primary aldosteronism.

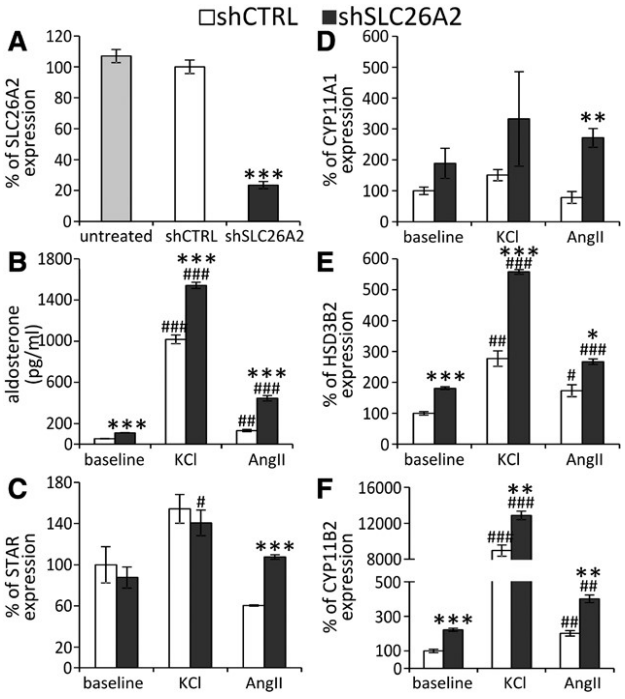

Figure 12: Steroidogenesis is upregulated in adrenocortical cells on solute carrier family 26 member 2 (SLC26A2) knockdown. (A) *SLC26A2* expression in untreated NCI-H295R cells, in NCI-H295R cells transfected with nontargeting shRNA (shCTRL), and in cells transfected with a specific SLC26A2 shRNA (shSLC26A2). (B) Aldosterone production and mRNA expression of (C) *StAR*, (D) *CYP11A1*, (E) *HSD3B2*, and (F) *CYP11B2* in control cells and specific knockdown cells at baseline conditions, on KCl and angiotensin II stimulation, respectively. *Significant differences between shCTRL and shSLC26A2 cells within an experimental condition, whereas # depict significant differences between baseline and stimulated cells: Adapted from Spyroglou et al., 2014.

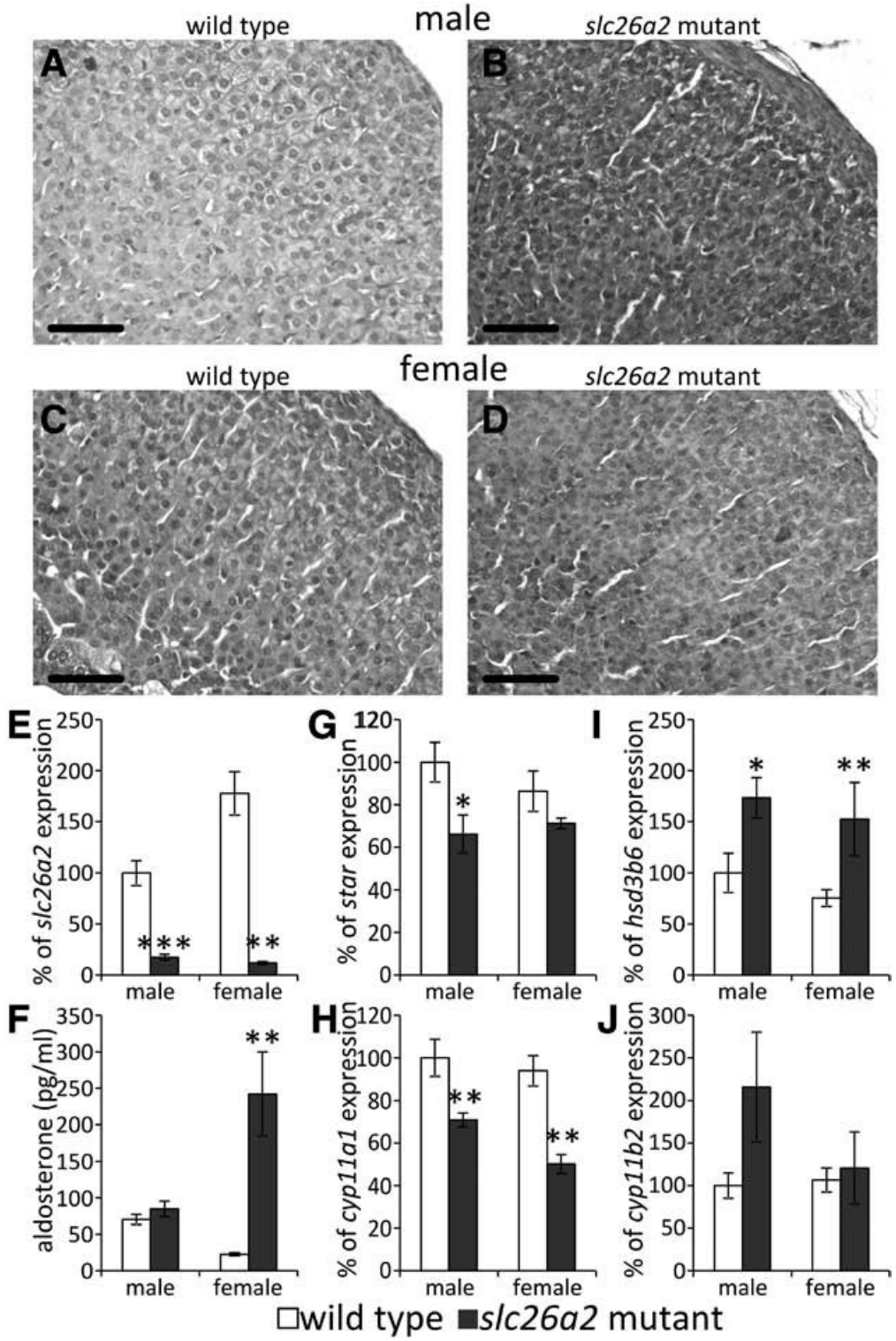

Figure 13: Solute carrier family 26 member 2 (Slc26a2) mutant animals display higher aldosterone output in a sex-dependent manner. A–D: No gross alterations in adrenal morphology and zonation are evident in slc26a2 mutant animal in comparison with wild-type controls. E: As expected, adrenal *slc26a2* mRNA expression is significantly lower in knockout animals. F: This difference is associated with higher serum aldosterone concentrations in female mice, lower expression of zone unspecific or zona fasciculata–specific steroidogenic enzymes including *Star* (G) and *cyp11a1* (H), and higher expression of glomerulosa-specific enzymes such as *hsd3b6* (I) and *cyp11b2* (J). Bars in A–D represent 50 μm. Adapted from Spyroglou et al., 2014.

Discussion

The studies presented herein had as initiating point the need to further elucidate mechanisms regulating the aldosterone secretion under physiological conditions and thereby to approach genetic alterations leading to aberrant aldosterone production in primary aldosteronism.

Aldosterone values present strong gender dependency in C3HeB/FeJ mice, with male animals presenting significantly higher plasma aldosterone levels than females (Spyroglou et al., 2012). Similarly, it could be documented that the corticosterone output of the murine adrenal gland is gender dependent (Spyroglou et al., 2015). In a small observational study investigating aldosterone levels in another strain (CD-1 mice) only small gender differences could be identified (Sun et al., 2010). Differences in the regulation of blood pressure between genders have already been described in human, with both pre- and postmenopausal women having lower blood pressure values than men (Burt et al., 1995) and women displaying lower aldosterone levels than men (Miller et al., 1999). Moreover, angiotensin II receptor levels were downregulated by estrogen replacement therapy in ovariectomized rats (Roesch et al., 2000), all suggesting that the RAAS is estrogen dependent.

Besides from gender differences, murine aldosterone synthesis varies between two inbred strains, with C57BL/6J animals displaying higher levels in comparison to C3HeB/FeJ mice (Spyroglou et al., 2012). Interestingly, in this study, plasma aldosterone levels were higher if the C57BL/6J strain was on the paternal side and were accompanied by respective expression pattern of steroidogenic enzymes in the adrenal glands. In population-based studies has been observed that a continuous spectrum of disease activity exists from normal renin–angiotensin–aldosterone function in essential hypertension to low renin hypertension and primary aldosteronism with its normokalemic and hypokalemic variance (Hannemann et al., 2012). Furthermore, in a haplotype analysis of the human CYP11B2 gene, an association of a polymorphism with plasma aldosterone levels could be documented (Russo et al., 2002). Different inbred mouse strains could represent such examples of aldosterone variations.

Transcriptional activation and post-transcriptional modification of steroidogenic enzymes are the major regulatory mechanisms for acute and chronic modulation of adrenocortical steroid production and release (Christenson and Strauss 2001). Angiotensin II and extracellular K^+ trigger the aldosterone response from glomerulosa cells and long-term stimulation results in increased synthesis of steroidogenic enzymes (Spät and Hunyadi 2004). Furthermore, short term K^+ stimulation induces a significant increase in adrenal *Cyp11b2* expression levels *in vivo* (Dierks et al., 2010). It was also shown, that the aldosterone response occurs already within minutes upon stimulation with angiotensin II, accompanied by fast changes in adrenal mRNA expression levels of steroidogenic enzymes, which were evident already 10 min after the stimulatory event (Spyroglou et al., 2009). In contrast to angiotensin-II-related changes of *StAR* and *Cyp11a1* mRNA levels which were in a similar range than those of sham treated animals, probably due to endogenous ACTH stimulation (Stier et al., 2004), angiotensin II-induced upregulation of *Cyp11b2* was much more pronounced as compared with sham-injection-induced changes. Regulation of *Cyp11b2* expression within such a short period of time had not been reported previously. As elongation rate of mRNAs has been determined in the range of 18 nucleotides per second (Schafer et al., 1991), detectable upregulation of the investigated steroidogenic enzymes with a transcript size in the range of 855-1581 bp (for *StAR*, *Cyp11a1* and *Cyp11b2*) is realistic.

NGF1-B and Nurr1 have been identified as two transcription factors suggested to be involved in transcriptional regulation of *Cyp11b2* expression (Bassett et al., 2004). Interestingly, we observed robust overexpression only of *Nurr1* upon short-term angiotensin II stimulation, correlating also with the *Cyp11b2* expression levels (Spyroglou et al., 2009). Thus, these results provide evidence that from both transcription factors which have been considered to be involved in aldosterone secretion, only Nurr1 is likely to have impact in short term aldosterone regulation. Accordingly, higher Nurr1 immunoreactivity was observed in Conn

adenomas in comparison with Cushing adenomas, whereas NGF1-B immunoreactivity showed no significant differences between the two groups (Lu et al., 2004).

Utilization of an ENU mutagenesis screen for the parameter aldosterone led to the identification of significantly more female animals displaying the pathological phenotype of hyperaldosteronism (Spyroglou et al., 2011). Such a gender-specific phenomenon has already been observed in mice carrying a targeted deletion of TASK1 or both TASK1 and TASK3, where only female mice presented the phenotype of primary aldosteronism, and which was reversible upon testosterone treatment (Heitzmann et al., 2008, Davies et al., 2008). In other studies, the percentage of established mutant lines varied from 0.04-3% of all F1 animals screened (Mohan et al., 2008, Reijmers et al., 2006). In the present mutagenesis screen the percentage was 0.28% of all screened animals probably due to the strict selection criteria applied here (Spyroglou et al., 2011). Interestingly, in our screen the eight established lines displayed different patterns of inheritance of the pathological trait and since they derived from distinct ENU-treated founder males, various mutations are likely to contribute to the phenotype. In all established mouse lines the increased aldosterone levels were preserved in animals of at least three generations, indicating heritability of this phenotype (Spyroglou et al., 2011). Thereby, an ENU mutagenesis screen is an applicable approach for the investigation of genetic causes of hyperaldosteronism.

Upon characterization of one of the established mouse lines seven candidate genes were recognized by exome sequencing (Perez-Rivas et al., 2017). However, none of those had a known function in aldosterone regulation and only *Clstn3* had a pronounced expression in the murine adrenal gland. Although *Clstn3* has not an known functional role in the RAAS, a hypothesis-free approach such as the ENU screen has the power of evoking unpredictable findings. Exome sequencing from patient samples (Beuschlein et al., 2013, 2014) and population-based genetic studies (Spyroglou et al., 2014) provide examples for the power of these strategies. From the seven genes identified in this mouse line, *Atm*, *Tipin* and *Mapk6*, all

located on chromosome 9 and apparently inherited in parallel, due to genetic linkage, seem not to be causative for the hyperaldosteronism phenotype. In contrast, SNVs on *Sspo*, *Dguok*, *Hoxaas2* and *Clstn3*, all located on chromosome 6, were associated with a significant increase in aldosterone values of the respective mutant animals, suggesting a role of these genes in the phenotype of primary aldosteronism. Upon calculation of the aldosterone-to-renin ratio, only animals with the *Sspo* SNV showed higher ARRs in comparison to their wild-type littermates. Similarly, *Sspo* mutants also had significantly higher *Cyp11b2* and *Nurr1* expression levels, suggesting possibly a particular role of this gene in the described phenotype. However, *Dguok* mutants also presented relatively high *Cyp11b2* and *Nurr1* levels, *Hoxaas2* mutants had significantly higher *Nurr1* levels and *Clstn3* mutants displayed the highest aldosterone values with the highest mutant-to-wild-type ratio. Furthermore, the increased adrenocortical CYP11B2 expression of mutant animals (carrying all four SNVs: *Sspo, Dguok, Hoxaas2* and *Clstn3*) seems to be based on the presence of CYP11B2 expression in the middle and inner adrenocortical area (Perez-Rivas et al., 2017). Thereby, no safe conclusion on the effect of each of the four SNVs on the increased aldosterone production and altered adrenocortical zonation can be made. An interaction of more than one mutation, although not likely is still conceivable (Moreno et al., 2007, Tayo et al., 2016, Wei et al., 2015).

The clinical hallmark of classical primary aldosteronism is hypokalemic hypertension with low plasma renin and elevated aldosterone. Thus, ARR is the standard screening method for this disease (Funder et al., 2008). Furthermore, a continuous gradient of higher possibility of blood pressure progression with increasing ARR levels has been documented (Newton-Cheh et al., 2007). Recently, several GWAS were applied for quantitative traits (Ehret et al., 2011, Köttgen et al., 2013, Scott et al., 2012, Kaplan et al., 2011). In a GWAS of a population-based sample, a highly significant correlation of ARR to a locus containing the *SLC26A2* gene was identified (Spyroglou et al., 2014). SLC26A2 is present in many epithelial and connective tissues (Haila et al., 2001) and functions as $SO4^{2-}/Cl^{-}$ exchanger (Satoh et al., 1998)

providing $SO4^{2-}$ for the sulphation of proteoglucans in cartilage (Forlino et al., 2005). *SLC26A2* mutations cause diastrophic dysplasia, a phenotype of cartilage and bone malformations (Forlino et al., 2005). It was now demonstrated that *SLC26A2* is highly expressed in the adrenal gland and is downregulated upon physiological aldosterone stimulation and autonomous aldosterone secretion (Spyroglou et al., 2014). No somatic or germline mutations of *SLC26A2* were documented so far in aldosterone producing adenomas (Beuschlein et al., 2013, Scholl et al., 2013, Azizan et al., 2013). Thus, possibly epigenetic mechanisms lead to expression changes of *SLC26A2* with functional effect. Only female slc26a2 mutant animals displayed elevated plasma aldosterone values, suggesting also in this case a potentially sex-dependent phenotype, similarly to previously described gender dependent aldosterone differences (Heitzmann et al., 2008, El Wakil et al., 2012, Spyroglou et al., 2012). However, since no significant differences of *SLC26A2* expression could be documented in APAs from both male and female patients, this hypothesis cannot be confirmed so far.

Summary

Summarizing, the studies described herein have contributed to the understanding of the regulation of aldosterone secretion. Murine plasma aldosterone levels are gender dependent. Additionally, the genetic background of the animals plays a significant role modulating their aldosterone levels without clear interference of other parameters in the RAAS. Acute regulation of aldosterone synthesis is accompanied by fast transcriptional modulation of steroidogenic enzymes and transcription factors that are likely to be involved in aldosterone secretion. The different approaches used for the identification of genes involved in aberrant aldosterone regulation validate the described methods as applicable in the investigation of such an endocrine phenotype. A phenotype driven mutagenesis screen has been proven as a feasible method to detect and establish mutant mouse lines with a phenotype of chronic hyperaldosteronism. A detailed phenotypic and genetic characterization of one the established mouse lines revealed *Sspo, Hoxaas2, Dguok* and *Clstn3* as new candidate genes involved in this trait. Based on a GWAS an effect of *SLC26A2* in the regulation of aldosterone secretion and potential involvement in the pathogenesis of primary aldosteronism could be demonstrated *in vitro* and *in vivo*. The above described mouse lines can additionally serve in the future for pharmacological intervention studies, and thus in the development of new drug treatments for patients with hyperaldosteronism.

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