Genetic characterization of a mouse line with primary aldosteronism

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Abstract

In an attempt to define novel genetic loci involved in the pathophysiology of primary aldosteronism, a mutagenesis screen after treatment with the alkylating agent N-ethyl-N-nitrosourea was established for the parameter aldosterone. One of the generated mouse lines with hyperaldosteronism was phenotypically and genetically characterized. This mouse line had high aldosterone levels but normal creatinine and urea values. The steroidogenic enzyme expression levels in the adrenal gland did not differ significantly among phenotypically affected and unaffected mice. Upon exome sequencing, point mutations were identified in seven candidate genes (Sspo, Dguok, Hoxas2, Clstn3, Atm, Tipin and Mapk6). Subsequently, animals were stratified into wild-type and mutated groups according to their genotype for each of these candidate genes. A correlation of their genotypes with the respective aldosterone, aldosterone-to-renin ratio (ARR), urea and creatinine values as well as steroidogenic enzyme expression levels was performed. Aldosterone values were significantly higher in animals carrying mutations in four different genes (Sspo, Dguok, Hoxas2 and Clstn3) and associated statistically significant adrenal Cyp11b2 overexpression as well as increased ARR was present only in mice with Sspo mutation. In contrast, mutations of the remaining candidate genes (Atm, Tipin and Mapk6) were associated with lower aldosterone values and lower Hsd3b6 expression levels. In summary, these data demonstrate association between the genes Sspo, Dguok, Hoxas2 and Clstn3 and hyperaldosteronism. Final proofs for the causative nature of the mutations have to come from knock-out and knock-in experiments.

Introduction

Arterial hypertension is a major cardiovascular risk factor affecting 30–45% of the adult European population (Mancia et al. 2013) with primary aldosteronism being the most frequent cause of endocrine hypertension with a prevalence of 7–10% in unselected hypertensive patients (Rossi et al. 2006, Hannemann et al. 2012). The large

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Key Words
- mutagenesis screen
- primary aldosteronism
- mouse model
- steroidogenic enzymes

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majority of primary aldosteronism (PA) is caused either by an aldosterone-producing adrenal adenoma or by bilateral adrenal hyperplasia (Mulatero et al. 2004, Rossi et al. 2006). Despite its high prevalence, so far, the genetic causes of primary aldosteronism due to bilateral hyperplasia have been elucidated only in a very small subgroup of cases: Familial hyperaldosteronism type I is 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), responsible for ACTH-dependent production of aldosterone instead of being regulated by angiotensin II. Familial hyperaldosteronism type II (FH-2) also seems to be inherited as an autosomal dominant trait. A locus has been mapped on chromosome 7p22 in some but not all families (Gordon et al. 1991, So et al. 2005), but the linkage area has not been resolved to any causative mutation. Familial hyperaldosteronism type III (FH-3) (Geller et al. 2008), however, has been recently linked to gain-of-function mutation in the KCNJ5 gene, encoding an inwardly rectifying potassium channel (Choi et al. 2011). These mutations induce a loss in channel selectivity, depolarization and increased intracellular Ca²⁺ concentrations leading to aldosterone excess. In a recent genetic analysis in 46 patients from 21 European families, a new germline G151E mutation was identified (Mulatero et al. 2012). KCNJ5 mutations are more prevalent in women and younger patients, and correlate with higher plasma aldosterone levels (Boulkroun et al. 2012). Furthermore, exome sequencing in aldosterone producing adrenal adenomas revealed somatic mutations in ATP1A1 and ATP2A3, two members of the P-type ATPase gene family (Beuschlein et al. 2013, Fernandes-Rosa et al. 2014). Finally, germline mutations in the Ca²⁺-channel genes CACNA1D (Scholl et al. 2013) and CACNA1H (Scholl et al. 2015) have been found as a rare cause of PA of early onset with CACNA1D mutations additionally causing neuromuscular abnormalities and partial or generalized seizures (Scholl et al. 2013). CACNA1H mutations were documented in patients with different phenotypic presentations of primary aldosteronism, such as early-onset primary aldosteronism, familial hyperaldosteronism or aldosterone-producing adrenal adenoma (Daniil et al. 2016). All in all, genetics of the vast majority of cases of bilateral adrenal hyperplasia remain enigmatic.

Animal models can play an important role in gaining functional insights into the physiology of aldosterone regulation and on the pathophysiology of autonomous aldosterone secretion. Furthermore, they can be used for the investigation of cardiovascular effects of autonomous aldosterone synthesis on several organs and can serve for pharmacologic intervention studies. So far, though, only a few mouse models have been described with a phenotype reminiscent of primary aldosteronism. A mouse with TASK channel deletion (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) belong to this group. An approach to develop new mouse models for a specific phenotype is induction of genetic variation by random mutagenesis of the mouse genome using the mutagenic substance N-ethyl-N-nitrosourea (ENU) (Hrabe de Angelis et al. 2000, Hagge-Greenberg et al. 2001, Clark et al. 2004). ENU is an alkylating agent that causes ethylation of nucleic acids and thereby point mutations in pre-meiotic spermatogonial stem cells (Russell et al. 1979, Rinchik 1991). The mutations derived from ENU handling may, therefore, be hypomorphic (partial loss of function). Mutants with gain-of-function as well as complete loss-of-function mutations are also possible (Justice et al. 2000, Noveroske et al. 2000). In the case of phenotype-driven genetics, the genomic association is accomplished utilizing common single-nucleotide polymorphisms (SNPs) or more recently exome sequencing.

Objective of the present study was to phenotypically characterize a mouse line generated using this mutagenesis screen and to identify candidate genes correlating with the phenotype of primary aldosteronism.

Materials and methods

Animals and housing conditions

All animal studies were performed according to protocols examined and approved by the Regierung von Oberbayern and according to the German Animal Protection Law. Mice were kept in a specific pathogen-free animal facility area at an ambient temperature of 22±2°C, (relative humidity 60±5%) and on a 12h–12h light–darkness cycle. Animals were fed with chow #1314 (Altromin/Lage, Germany) ad libitum and had free access to tap water. C3HeB/FeJ mice (Jackson Laboratory) involved in the experiments were maintained in groups of three or less individuals per cage. The generation of this mouse line with hyperaldosteronism has been described previously (Spyroglou et al. 2011). In brief, in the Munich ENU project performed at the Institute of Experimental Genetics of the Helmholtz Center Munich, C3HeB/FeJ male mice were treated with three weekly injections of 90mg/kg ENU at approximately 10–14 weeks of age. Recovery of fertility upon treatment could be observed approx. 80 days later. All F1 offspring from ENU-treated males and wild-type females born at
least after two cycles of spermatogenesis following the last injection were checked for their aldosterone levels in plasma at the age of 12 and 16 weeks. Out of this cohort of 2800 offspring, 83 displayed high aldosterone values at the age of 12 weeks, and upon confirmation measurement at the age of 16 weeks, only 11 mice had sustained high aldosterone values (2 males and 9 females). Upon mating to wild-type mice, eight lines with affected offspring in the heterozygous state had been established. Here, we describe the phenotypical and genetic characterization of one of these mouse lines.

**Blood sampling**

To avoid any influence of the examined parameters through hypothalamic–pituitary–adrenal (HPA) axis activation, handling of the animals until blood sampling was kept to an interval of less than one minute. Blood sampling took place between 08:00 and 11:00h for all animals. After effective inhalation anesthesia, the retrobulbar vein plexus of the mouse was punctured with a glass capillary (external diameter 0.8 mm), and from each animal, blood was collected in Li-Heparin-coated tubes to avoid coagulation. After centrifugation (10,000g, 10 min), plasma was separated and kept at −20°C until measurement.

**Biochemical and endocrine profiling**

Urea and creatinine values were measured in the clinical chemical laboratory of the German Mouse Clinic (Fuchs et al. 2011) using an AU480 Autoanalyser (Beckman-Coulter/Krefeld, Germany) and adapted reagent kits provided by the manufacturer (urea: OSR6134; creatinine (Jaffe): OSR6178) (Rathkolb et al. 2013).

Aldosterone was determined in 50µL of mouse plasma samples with an in-house time resolved fluorescent immunoassay as described in detail elsewhere (Manolopoulos et al. 2008).

After 1.5h of incubation of two equal fractions of each mouse plasma sample with plasma of bilaterally nephrectomized male rats as renin substrate at 0°C and 37°C, respectively (pH=6, with addition of the protease inhibitor PMSF), angiotensin I was generated. Subsequently, angiotensin I concentration of each fraction was measured by a commercially available angiotensin I (PRA) ELISA Kit (IBL International, Hamburg, Germany) according to the manufacturer’s instructions. Plasma renin activity in each sample was calculated using the following equation: \( PRA = \frac{((\text{AngI } 37°C - \text{AngI } 0°C)/\text{incubation time (h)})}{1.11} \). Aldosterone-to-renin ratios (ARR) were calculated for each mouse sample and expressed as % of the mean of male or female wild types, respectively.

Corticosterone levels were measured with the Corticosterone HS EIA Immunoassay (IDS, Boldon, Tyne and Wear, United Kingdom) according to the manufacturers’ instructions.

**Organ sampling**

After anesthesia, mice were killed, and the abdominal cavity was opened. Both adrenal glands of each mouse, as well as the spleen were identified and removed. Directly after collection, adrenals were cleaned from surrounding fat tissue using a stereoscope. All organs were snap-frozen in liquid nitrogen and stored at −80°C or stored in 4% paraformaldehyde and subsequently embedded in paraffin blocks.

**Capture and sequencing of murine exomes**

In-solution targeted enrichment of exonic sequences was performed by DNA extracted from the spleens of two different affected animals from the first and third generation, respectively, using the SureSelectXT Mouse All Exon 50Mb kit from Agilent as already described (Diener et al. 2016). The generated libraries were indexed, pooled and sequenced as 100bp paired-end runs on a HiSeq2000 system (Illumina, San Diego, CA, USA).

**Mapping and variant calling**

Read alignment to the mouse genome assembly 9 mm was done with Burrows–Wheeler Aligner (BWA, version 0.6.2) and yielded 12.1 and 11.5Gb of mapped sequence data corresponding to an average coverage of 143x and 137x, respectively. Single-nucleotide variants (SNVs) and small insertions and deletions (indels) were detected with SAMtools (version 0.1.18). SNVs were only lightly filtered on quality scores (SNV quality >40, mapping quality >50) because we preferred false positives to negatives. After subtracting variants from dbSNP128 and from 158 other mice in our in-house database, 31 unique coding variants specific to the mutant mouse line remained.

**Validation of candidate variants by capillary Sanger sequencing**

Candidate SNVs were further investigated by amplifying DNA of mutant and control mice with intronic primers (Supplementary Table 1, see section
on supplementary data given at the end of this article). Bidirectional Sanger sequencing was then performed using the ABI Prism BigDye Terminator, v.3.1 Cycle Sequencing Kit on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific). A genotype-phenotype correlation was then performed.

**Real-time PCR**

Both adrenals from each individual animal were combined and homogenized in extraction buffer while still frozen. RNA extraction was performed using the Maxwell 16 LEV simplyRNA according to the manufacturer’s instructions (Promega). RNA quantification was performed with the NanoDrop spectrophotometer. The 260/280-absorbance ratio was used to assess RNA purity. Additionally, RNA integrity was assessed on denaturing agarose gel stained with ethidium bromide. For cDNA synthesis, 500ng of total RNA were reverse transcribed according to the manufacturers’ instructions (M-MLV Reverse Transcriptase, dNTP Mix, Recombinant RNasin Ribonuclease Inhibitor all from Promega). The expression of selected genes involved in steroidogenesis was investigated: *STAR* (steroidogenic acute regulatory protein), *Cyp11a1* (cholesterol side-chain cleavage enzyme), *Hsd3b6* (3β-hydroxysteroid dehydrogenase), *Cyp11b1* (11β-hydroxylase), *Cyp11b2* (aldosterone synthase), *Nurr1*, *Nur77* and *Kim1* (kidney injury molecule-1, Havcr1). *Gapdh* was used as reference gene. For primer sequences, see Supplementary Table 1. The SsoFast EVAGreen Supermix (Biorad Laboratories) in the Mx3000P QPCR System (Stratagene) was used for the quantification of the investigated genes. Real-time PCR conditions in the Mx3000P are as indicated in Supplementary Table 1. To verify the presence of a unique amplicon with the correct size, a melting curve analysis was performed between 55°Cand 95°C (0.1°C/s) and the products were run on a 1% agarose gel. To proof the specificity of the amplification product for each primer pair, we performed sequencing of the different genes with the primers used for the qPCR experiments. Quantification was adjusted using the mouse *Gapdh* expression as reference. With a coefficient of variation (CV) below 5%, *Gapdh* was considered to have a stable expression in the adrenal gland and therefore was chosen to be used as housekeeping gene in this experimental setting.

**Histological examination**

Tissues remained in 4% paraformaldehyde overnight and then were dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin following standard protocols. Hematoxylin and eosin-stained adrenal sections were examined with a light microscope using magnifications of ×40 and ×400.

For CYP11B2 immunohistochemistry, paraffin-embedded sections were rehydrated, blocked with 0.3% H₂O₂ in methanol for 10min and incubated with blocking buffer (0.1M Tris–HCl pH = 7.4 (Sigma-Aldrich), 20% goat serum (Jackson Immunoresearch Laboratories) and 0.05% SDS (Sigma-Aldrich)) for 1h. CYP11B2 was immunodetected overnight at 4°C by means of a rabbit-anti-mouse-CYP11B2 antibody (kindly provided by Prof Celso Gomez-Sanchez, University of Mississippi, USA) in a dilution of 1:1000 in an antibody buffer containing 0.1M Tris–HCl, 20% goat serum and 0.1% Tween 20 (Sigma-Aldrich). After rinsing for 15min in PBS, secondary antibody (goat-anti-rabbit-biotinylated IgG (Vector Laboratories, Burlingame, CA, USA)) in a dilution of 1:2000 in antibody buffer was applied for 30min at room temperature. For the visualization of the bound CYP11B2 antibody, Vectastain Elite ABC system (Vector Laboratories) and Sigma Fast diaminobenzidine (Sigma-Aldrich) were used, and slides were counterstained with Harry’s hematoxylin.

All captured images were color-calibrated to the negative control. A randomly selected rectangle area of the adrenal cortex of each animal extending vertically from the outer border of the adrenal medulla up to the surface of the adrenal gland was divided into three subareas (outer (1/4), middle (2/4) and inner area (1/4)). The total counterstained cell population of the selected area was analyzed according to the color of the cell cytoplasm; cells were categorized as positive or negative. Positively and negatively CYP11B2 – stained cells were counted on three independent sections per animal by two independent investigators under blinded conditions. Results are presented as means of ratios of positive to total cell counts per standardized area.

**Statistical analysis**

Statistical analysis was carried out with the Prism 3.02 (GraphPad Software). Statistical significance was determined using the unpaired *t*-test for normally distributed parameters and Mann–Whitney test for non-normally distributed parameters. Statistical significance was denoted by asterisks in the figures as *P*<0.05, **P*<0.01 and ***P*<0.001.
Results

Screening parameters

All offspring of the founder animal (n=158, Fig. 1) were tested for their aldosterone values at the age of 12 and 16 weeks. Animals repeatedly showing increased values were grouped to the ‘affected group’ and those with normal values (normal rage: mean ± 3 s.d., defined previously (Spyroglou et al. 2011)) to the ‘unaffected group’. Out of this cohort, 33 animals (17 unaffected and 16 affected) from generations F3–5 were further analyzed. As expected, affected animals of both genders had significantly higher aldosterone values than their unaffected littermates (P < 0.001, Fig. 2A, 33% of all offspring of this mouse line were affected and the affected male:female ratio was 4:3).

Corticosterone levels of affected and unaffected animals of both genders did not differ significantly (unaffected: 2.64 ± 1.5 ng/mL, affected 2.36 ± 1.79 ng/mL, P = 0.75), and no correlation of aldosterone to corticosterone values could be observed (R = 0.16, P = 0.52), excluding increased stress as confounding factor for the elevated aldosterone values. Urea and creatinine, as parameters of kidney function, showed no differences between affected animals and their unaffected littermates, suggesting that secondary hyperaldosteronism due to overt renal disease can be excluded in this mouse line (Fig. 2B and C). Consistently, the expression of KIM1, as a biomarker of renal tubule injury, did not differ in the two groups (relative expression normalized to Gapdh: unaffected: 0.08884 ± 0.01548, affected: 0.1163 ± 0.03992, P = 0.46).

Expression levels of several genes involved in steroidogenesis were quantified in the adrenal glands of these animals. Unexpectedly, the expression levels of all investigated steroidalogenic enzymes (StAR, Cyp11a1, Hsd3b6, Cyp11b1 and Cyp11b2) and orphan nuclear factors (Nurr1 and Nur77-data not shown) did not display any statistically significant differences between unaffected and affected animals (Fig. 2D, E, F, G, H and I).

Identified candidate genes

For the genotypic characterization of the mouse line, we made use of next-generation sequencing (NGS) exome analysis (Institute of Human Genetics, Helmholtz Center, Munich, Germany (Diener et al. 2016)). After exome sequencing performed on two different affected animals from the first and third generation, respectively, a list with induced single-nucleotide variations (SNV), filtered on the basis of technical quality criteria (SNV quality >40, mapping quality >50), was generated (Supplementary Table 2). Exome sequencing identified SNVs on 31 different genes; eight of those were common for both investigated animals: SCO-spondin (Sspo), deoxyguanosine kinase (Dguk), 5730446D14Rik (Hoxa cluster antisense RNA 2 – Hoxaas2), calysntenin 3 (Clstn3), integrator complex subunit 10 (Ints10), ataxia telangiectasia mutated (Atm), timeless interacting protein (Tipin) and mitogen-activated protein kinase 6 (Mapk6). Sspo, Dguk, Hoxaas2 and Clstn3 are located on mouse chromosome 6, Ints10 is located on chromosome 8 and Atm, Tipin and Mapk6 are located 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. As ENU is likely to induce more coding SNVs in the first generations than the one responsible for the ‘high aldosterone’ phenotype, we again investigated the potential function of those genes and their expression levels in the adrenal gland. Based on a detailed database search, none of the genes had a known function in the adrenal gland and/or aldosterone biosynthesis. Increased adrenal expression according to the BioGPS database could be documented only for the murine Clstn3 gene.

Figure 1
Pedigree of the investigated mouse line with primary aldosteronism. Increased aldosterone values (+3 s.d. from the mean) observed in five successive generations, in animals of both genders. Affected animals 33%, males: females ratio 4:3. Squares: males, circles: females, black: affected animals.
Genotype/phenotype correlation

In an attempt to correlate the identified candidate genes with the phenotype of interest, animals were stratified into wild-type and mutated groups according to their genotypes for each of the candidate genes. We correlated the genotype with the respective aldosterone, urea and creatinine values as well as steroidalogenic enzyme expression levels. To uniformly assess the differences in the aldosterone values in mice of both genders, these were expressed as percentage of the mean of the respective gender. Due to the fact that the majority of investigated animals carried more than one of the seven SNVs identified during the study, for the results depicted in Figs 3 and 4, animals carrying two wild-type copies for the respective gene were considered as ‘wild types’. Thus, in the majority of cases, these animals were carriers of mutant variants of one or several genes of the panel. Along the same line, ‘mutants’ for the specific gene also often carried in parallel further mutations. Thereby we aimed at isolating a potential effect of one mutation on the investigated phenotype even though the random presence of other variants might have further modulating effects. We observed significantly increased plasma aldosterone values in animals carrying an Sspo (P < 0.01), Dguok (P < 0.01), Hoxaas2 (P < 0.01) and Clstn3 (P < 0.001) mutation. In contrast, animals carrying the wild-type gene for Atm, Tipin and Mapk6 displayed significantly higher aldosterone values than their mutant littermates (Fig. 3). Urea and creatinine levels did not differ significantly among wild-type and mutated animals.

The correlation of the expression levels of steroidalogenic enzymes to the genotypes was less clear-
A mouse line with primary aldosteronism

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Figure 4
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.

Cut with large variation among wild-type and mutated animals in most of the cases. StAR expression levels, regulating the initial step in steroidogenesis, were significantly lower in Clstn3-mutated animals (P<0.05, Fig. 4A), whereas Cyp11a1 expression was lowered in Sspo (P<0.05), Dguok (P<0.01), Hoxaas2 (P<0.01), Clstn3 (P<0.001) and Atm (P<0.05) mutants (Fig. 4B). Significantly lower were also the Hsd3b6 levels in Clstn3 (P<0.05), Atm (P<0.01), Tipin (P<0.05) and Mapk6 (P<0.05) mutated animals (Fig. 4C). No such differences could be found for Cyp11b1 expression levels for any of the mutations investigated (Fig. 4D).

Sspo-mutated animals, Cyp11b2 expression, as marker of the rate-limiting step of aldosterone biosynthesis, was significantly higher (P<0.05). Dguok-mutated animals showed a tendency to higher Cyp11b2 levels, without reaching statistical significance (P=0.07) (Fig. 4E).

The orphan nuclear factor Nurr1, also involved in the aldosterone synthesis was found significantly higher in Sspo- (P<0.05) and Hoxaas2- (P<0.05) mutated mice. Dguok mutants also tended to display higher Nurr1 levels (P=0.08) (Fig. 4F).

From the results depicted in Fig. 3, we conclude that Atm, Tipin and Mapk6 do not correlate with the phenotype.
of primary aldosteronism as aldosterone values in wild-type animals were found to be significantly higher than those in animals carrying one, two or all three SNVs. To further narrow down our list to one causative SNV from the remaining four genes (Sspo, Dguok, Hoxaas2 and Clstn3), we compared the aldosterone levels of animals excluding mice carrying Atm and/or Tipin and/or Mapk6 mutations. Animals carrying Sspo and/or Dguok and/or Hoxaas2 and/or Clstn3 SNVs displayed significantly higher aldosterone values than wild-type only animals and the mutant-to-wild-type ratio was in all cases higher in this restricted analysis (Table 1, for detailed combination of the variants see Supplementary Table 3). A combination of SNVs in both chromosomes 6 and 9 (all seven mutations) leads to weakening of the phenotype of hyperaldosteronism (data not shown).

Quantification of plasma renin activity in these animals did not present any significant differences between animals with wild-type and mutant genotype for the above four SNVs. Calculation of the aldosterone-to-renin ratios (ARR) showed a significant ARR increase only in mice carrying the Sspo SNV (Table 2). Mice with the Dguok and/or Hoxaas2 SNV presented a tendency toward increased ARRs.

Interestingly, when comparing corticosterone levels of animals carrying the four different mutations to their wild-type littermates, it could be observed that mutant animals displayed slightly lower corticosterone values than the wild types without reaching statistical significance (mutants: 2.36±0.53 ng/mL vs wild types: 4.06±0.95 ng/mL, P=0.14).

Histological examination of the adrenal glands of animals carrying all four SNVs (Sspo, Dguok, Hoxaas2 and Clstn3) did not reveal any adrenocortical adenomas. Moreover, there was no indication of 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, immunohistochemical staining with murine CYP11B2 antibody showed significantly more positively stained cells in the adrenal cortex of mutant animals in comparison to the wild types (P<0.001, Fig. 5A, B and C). Additionally, in the adrenal cortex of mutant animals, CYP11B2 staining was significantly more pronounced in the middle (P<0.001) and inner (P<0.001) area when compared to wild types, suggesting an alternated zonation pattern in animals carrying the four SNVs (Fig. 5D, E and F). Thereby the presence of aldosterone synthase expression in the middle and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>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 Mapk6 (restricted).</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>wt</td>
</tr>
<tr>
<td>Cohort</td>
<td></td>
</tr>
<tr>
<td>All (n=126)</td>
<td>351±120</td>
</tr>
<tr>
<td>Restricted (n=99)</td>
<td>349±107</td>
</tr>
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...
inner cortical area of the mutant animals seems to exclusively account for the increased total expression of aldosterone synthase.

Discussion

In this study, an ENU mutagenesis–derived mouse line with hyperaldosteronism was phenotypically and genetically characterized. The trait of increased aldosterone values above 3 s.d. from the mean was preserved for more than five generations in a considerable proportion of animals of both genders. Specifically, one-third of all investigated animals were affected, with the ratio of males to females being 4:3. Animals with high aldosterone values showed in all cases normal kidney retention parameters and Kim1 expression excluding a relevant renal disorder as the cause for the described phenotype. Furthermore, corticosterone levels, mirroring stress levels of affected and unaffected animals, did not differ in the present study. However, the adrenal expression of steroidogenic enzymes and especially Cyp11b2, the rate-limiting step of aldosterone biosynthesis was not concordant with the significantly increased plasma aldosterone levels. Although blood pressure measurement would have been a clinically relevant endpoint in the investigation of this mouse model in the context of the current study, we focused on the adrenal phenotype of these animals. Future investigations with state-of-the-art analytic methods will be required to investigate in depth the cardiovascular effects of the animal model.

Table 2  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.

<table>
<thead>
<tr>
<th>Aldosterone-to-renin ratio (ARR)</th>
<th>Mean ± S.D. (% of mean of wild types)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sspo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt (n = 24)</td>
<td>100 ± 43</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>mut (n = 17)</td>
<td>142 ± 71</td>
<td></td>
</tr>
<tr>
<td>Dguok</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt (n = 27)</td>
<td>100 ± 49</td>
<td>0.09</td>
</tr>
<tr>
<td>mut (n = 14)</td>
<td>130 ± 60</td>
<td></td>
</tr>
<tr>
<td>Hoxaas2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt (n = 27)</td>
<td>100 ± 47</td>
<td>0.10</td>
</tr>
<tr>
<td>mut (n = 14)</td>
<td>130 ± 65</td>
<td></td>
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<tr>
<td>Clstn3</td>
<td></td>
<td></td>
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<tr>
<td>wt (n = 26)</td>
<td>100 ± 46</td>
<td>0.80</td>
</tr>
<tr>
<td>mut (n = 15)</td>
<td>96 ± 55</td>
<td></td>
</tr>
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</table>

Figure 5

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).
Following the described approach, we identified seven candidate genes for the phenotype hyperaldosteronism. Out of the identified genes, none had a known function in aldosterone secretion or regulation and only Clstn3 had a pronounced expression in the mouse adrenal gland as well as in the pituitary and brown adipose tissue according to the BioGPS database. Clstn3 codes for a postsynaptic Ca\(^{2+}\)-binding membrane protein, a member of the cadherin superfamily involved in the development of synapses (Lu et al. 2014), with the highest expression levels in GABAergic neurons (Hintsch et al. 2002). It is also present in endocrine secretive granules of gonadotrope, somatotrope and thyrotrhopo cells of the anterior pituitary, whereas CLSTN1, another member of the superfamily, was found to be localized in glucagon-containing secretory granules of pancreatic a-cells (Rindler et al. 2008). Thus, it is hypothesized that soluble Clstns, released locally, could potentially serve as modulators of endocrine function (Rindler et al. 2008). Although Clstn3 has not been appreciated to play a functional role in adrenal physiology or disease, it is the strength of a hypothesis-free approach such as the ENU screen that potentially surprising findings can be generated. 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.

When aldosterone values were displayed with mice grouped according to the presence or absence of specific mutations, an interesting phenomenon was observed: Animals carrying part of the identified mutations displayed significantly lower aldosterone levels compared to the wild types for the specific mutation. An explanation for this paradoxical finding could be that animals were misleadingly grouped into ‘mutant’ and ‘wild types’ for the specific genes as these genotypes were not responsible for the phenotype of interest. Accordingly, Atm, Tipin and Mapk6, all located on chromosome 9 and apparently inherited in parallel, probably due to genetic linkage, seem not to be causative for the hyperaldosteronism phenotype as aldosterone values of ‘wild-type’ animals were in these three cases higher than those in their ‘mutant’ littersmates. In contrast, it was reassuring that 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 littersmates, suggesting possibly a particular role of this gene in the described phenotype.

Furthermore, it can be speculated that downregulation of the initial enzymes of steroidogenesis, such as StAR, Cyp11a1 and Hsd3b6 as displayed on Fig. 4, was due to a negative feedback loop by parallel autonomous aldosterone secretion. Consistent with the unchanged Cyp11b1 expression, no differences in corticosterone values could be observed in the study. Additionally, we observe a discrepancy between the moderate adrenal Cyp11b2 RNA expression and the significantly higher aldosterone values, aldosterone-to-renin ratios and number of CYP11B2 positively stained adrenocortical cells in mutant animals. The interpretation of the only mild Cyp11b2 upregulation with the presence of SNVs on some of the investigated genes (Sspo and much less Dguok) or the complete absence of upregulation in other cases (Hoxaas2, Clstn3) appears thereby more challenging. However, it is possible that even moderate increased Cyp11b2 RNA expression could result in a pronounced difference in protein expression possibly due to increased stability of this enzyme. This explanation would require further analysis. Another interesting observation in mutant animals (all four Sspo, Dguok, Hoxaas2 and Clstn3) is that the increase in aldosterone synthase expression seems to be based on the presence of aldosterone synthase expression in the middle and inner adrenocortical area. This alteration of the functional zonation of the adrenal cortex also needs further elucidation.

Taken together, from the identified candidate genes Atm, Tipin and Mapk6 seem to not be responsible for the investigated phenotype of primary aldosteronism, whereas no safe conclusion can be reached in the case of Sspo, Dguok, Hoxaas2 and Clstn3 and their potential role in PA. Ideally, to distinguish among those four genes, further breeding should take place to genetically separate them in strains carrying only one candidate SNV. Practically, this was not yet achieved after seven generations of breeding after embryo transfer (F1–F7), probably as all four genes are located in near regions of the same chromosome and thereby inherited together. Thus, further efforts will be necessary to rule in or out the role of these four genes in the pathogenesis of primary aldosteronism.

Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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