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Original Article

Diastrophic Dysplasia Sulfate Transporter (SLC26A2) Is Expressed in the Adrenal Cortex and Regulates Aldosterone Secretion

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Abstract—Elucidation of the molecular mechanisms leading to autonomous aldosterone secretion is a prerequisite to define potential targets and biomarkers in the context of primary aldosteronism. After a genome-wide association study with subjects from the population-based Cooperative Health Research in the Region of Augsburg F4 survey, we observed a highly significant association (P=6.78×10^{-11}) between the aldosterone to renin ratio and a locus at 5q32. Hypothesizing that this locus may contain genes of relevance for the pathogenesis of primary aldosteronism, we investigated solute carrier family 26 member 2 (SLC26A2), a protein with known transport activity for sulfate and other cations. Within murine tissues, adrenal glands showed the highest expression levels for SLC26A2, which was significantly downregulated on in vivo stimulation with angiotensin II and potassium. SLC26A2 expression was found to be significantly lower in aldosterone-producing adenomas in comparison with normal adrenal glands. In adrenocortical NCI-H295R cells, specific knockdown of SLC26A2 resulted in a highly significant increase in aldosterone secretion. Concomitantly, expression of steroidogenic enzymes, as well as upstream effectors including transcription factors such as NR4A1, CAMK1, and intracellular Ca^{2+} content, was upregulated in knockdown cells. To substantiate further these findings in an SLC26A2 mutant mouse model, aldosterone output proved to be increased in a sex-specific manner. In summary, these findings point toward a possible effect of SLC26A2 in the regulation of aldosterone secretion potentially involved in the pathogenesis of primary aldosteronism. (***Hypertension***. **2014;63:00-00.)** ● **Online Data Supplement**

Key Words: aldosterone to renin ratio ■ calcium signaling ■ genome-wide association study ■ knockout mice ■ primary hyperaldosteronism ■ solute carrier family 26 (sulfate transporter), member 2 protein, human

Arterial hypertension is a major cardiovascular risk factor that affects ≈30% to 60% of the population depending on the age group concerned.[1] Pathogenic mechanisms underlying so-called essential hypertension are complex and include genetic and environmental, and vascular and hormonal factors. In community-based samples, increased aldosterone levels within the physiological range predispose to the development of high blood pressure.[2,3] On the far side of the spectrum, primary aldosteronism (PA), a form of endocrine hypertension, is characterized by inappropriately high aldosterone levels suppressing plasma renin concentrations, elevated blood pressure, and hypokalemia. PA is now recognized as the most common cause of secondary hypertension.[4,5] Aldosterone excess in the context of PA is relatively autonomous from angiotensin II stimulation and, thus, nonsuppressible by sodium loading. Aldosterone-producing adrenal adenomas or bilateral adrenal hyperplasia are responsible for the vast majority of cases, adenomas presenting more prominent renin system independency of aldosterone secretion.[5,6]

Despite its high prevalence, to date, the genetic causes of PA have been elucidated mainly in rare familial forms of the disease. Few families have been identified with familial hyperaldosteronism type 1, also known as glucocorticoid remediable hyperaldosteronism. This disease is caused by unequal crossing over of the CYP11B1 (11β hydroxylase) and CYP11B2 (aldosterone synthase) and the formation of a

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hybrid gene, which results in adrenocorticotropic hormone-dependent production of aldosterone.[7] Only recently, mutations in KCNJ5 have been determined as the cause of familial hyperaldosteronism type 3.[8] Interestingly, somatic mutations in the potassium channel KCNJ5 have also been identified in a large proportion of sporadic aldosterone-producing adenomas (APAs), confirming the effect of this gene on autonomous aldosterone secretion.[9] Familial hyperaldosteronism type 2 summarizes several forms of genetically determined PA. Likewise, the morphological and functional phenotype of familial hyperaldosteronism type 2 is variable from APAs to bilateral hyperplasia. A locus has been mapped on chromosome 7p22 in some but not all families,[10] but the linkage area has not been resolved to any causative mutation.

Similar to investigations in inbred mouse strains,[11] in population-based studies, the aldosterone to renin ratio (ARR) has been recognized as an inherited trait and predictor of increased blood pressure.[12,13] Technological advances in human genomics now allow studying a large entity of the allelic spectrum from the frequent to the rare inherited or de novo genetic variants. Genome-wide association studies (GWAS) are powerful tools to identify common genetic susceptibility loci in human heredity and disease. GWAS use genotyping arrays of a dense set of common single-nucleotide polymorphisms to generate genotypes in large populations of cases and controls.[14] This method has greatly improved our knowledge about human genetics, especially for common diseases, where genetic and environmental factors both contribute to disease susceptibility.[15] In fact, in a recent GWAS, systolic and diastolic blood pressure has been correlated with 16 novel loci.[16]

The specific aim of this study was the detailed molecular and functional characterization of genes involved in the regulation of the renin–angiotensin–aldosterone pathway identified by GWAS in a population-based study. We speculated that by combining genetic techniques with molecular in vitro and in vivo studies, novel genes could be identified with particular relevance for the pathophysiology of hypertension and its associated sequelae.

Materials and Methods

Genome-Wide Association Study

The Cooperative Health Research in the Region of Augsburg cohort consists of all German residents of this region[17] from which a 2-stage cluster sample of 6640 subjects aged 24 to 75 years was obtained. From all participants of the Cooperative Health Research in the Region of Augsburg F4 cohort, a fasting venous blood sample was obtained while in a sitting position. The study protocol had been accepted by the local ethics committee, and all participants provided written informed consent. Genome-wide association of the ARR with genetic markers was applied on 1814 subjects from this cohort (for the methods applied, see ref 18) using a 1000 K chip (Affymetrix 6.0, genotyping at the Helmholtz Zentrum Munich, Neuherberg, Germany). Twenty-eight patients on hypertensive treatment were excluded, leaving 1786 subjects for final analysis. In total, ≈2.7 million of directly genotyped or imputed single-nucleotide polymorphisms were analyzed.

Hormone Measurements

Aldosterone was determined with an in-house time-resolved fluorescent immunoassay as described in detail before.[19] Cortisol concentrations were determined using an automated chemiluminescence assay (Liaison, Diasorin, Italy). Corticosterone levels were measured by Corticosterone EIA kit (Immunodiagnostic Systems, United Kingdom).

Microarray Analysis

mRNA expression data for 91 APA samples with genotype data and 11 control adrenals were retrieved from a pangenomic transcriptome analysis performed on 123 samples collected through the COrtico et MEdullosurrénale: les Tumeurs Endocrines network from patients operated on APA between 1994 and 2008 in the Hypertension Unit at the Hôpital Européen Georges Pompidou in Paris. Procedures for data acquisition and calculation have been described in details elsewhere.[9]

Animal Experiments

All animal studies were performed according to protocols examined and approved by the Animal Care and Use Committee of the University of Pavia or the Regierung von Oberbayern according to the German Animal Protection Law, respectively. Dtd (slc26a2 mutant and wild-type control) and C3HeB/FeJ mice were kept in a specific pathogen-free animal facility area at an ambient temperature of 25±2°C (relative humidity, 60±5%) on a 12/12 hour light/dark cycle. Animals were fed with standard pelleted food no. 2018 (Harlan Laboratories, Udine, Italy) or chow no. 1314 (Altromin, Lage, Germany), respectively, ad libitum with free access to tap water. Specific methods of performed experiments are described in the online-only Data Supplement information.

Quantitative Real-Time Polymerase Chain Reaction

Both adrenals from each individual animal were combined and homogenized in lysis buffer while still frozen. NCI-H295R cells were lysed directly by addition of lysis buffer. RNA extraction from lysates was performed using the The RNeasy Plus Mini Kit according to the instructions of manufacturer (Qiagen, Hilden, Germany). RNA quality was verified on a 1.5% agarose gel. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the reverse transcription system (Fermentas, St Leon-Rot, Germany). Gene expression of slc26a2 and selected genes involved in steroidogenesis were investigated using real-time polymerase chain reaction. For primer sequences, annealing temperatures, and polymerase chain reaction conditions, see the online-only Data Supplement information.

Cell Culture

NCI-H295R (CRL-2128; American Type Culture Collection, Manassas, VA) cells were cultured in a RPMI 1640 medium (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum, 1% insulin–transferrin–selenium supplement, 1% penicillin/streptomycin, and 100 nmol/L hydrocortisone in a humidified atmosphere (95% CO_2). For stimulation and inhibition experiments, cells were seeded in 24-well plates at 200 000 cells per well and grown for 48 hours. Cells were then treated with 10 mmol/L KCl, 100 nmol/L angiotensin II (Sigma-Aldrich, Taufkirchen, Germany), or 10 µmol/L Forskolin (Sigma-Aldrich) for stimulation of aldosterone synthesis. For suppression experiments, 3 µmol/L calmidazolium or KN-93 (Sigma-Aldrich) was included in the treatment. After incubation for 48 hours, supernatant and cells from each well were harvested for aldosterone measurement and RNA extraction.

Knockdown Experiments

Knockdown of solute carrier family 26 member 2 (SLC26A2) expression in NCI-H295R cells was achieved using MISSION shRNA lentiviral transduction particles from Sigma-Aldrich. The shRNA sequences were validated by The RNAi Consortium and were inserted into The RNAi Consortium 2 pLKO-puro plasmid backbone. For the lentiviral infections, protocol of manufacturer was followed with modifications (refer to the online-only Data Supplement information for expanded description of protocol).

Quantification of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} was measured with a filter wheel–based imaging system (Universal Imaging Corporation, Downingtown, PA) mounted on an inverted microscope (Axiovert 200, Zeiss, Jena, Germany) using the ratiometric Ca^{2+} dye fura-2-AM (Invitrogen; excitation at 340 nm and 380 nm; emission, 490–530 nm). NCI-H295R cells grown on glass cover slips were loaded with fura-2-AM (50 µmol/L) for 30 minutes at 37°C. The extracellular Ringer-type solution contained (in mmol/L) 137 NaCl, 5 glucose, 1 $MgCl_2$, 1.3 $CaCl_2$, 5 HEPES, and 5 KCl (pH 7.4). For high Ca^{2+} and K^+ solutions, Na^+ was replaced by the respective amounts of Ca^{2+} and K^+. Measurements were performed at 37°C. Mean fluorescence ratios were calculated for single cells using the Metafluor software (Universal Imaging, West Chester, PA).

Statistical Analysis

Statistical analysis was performed with the Prism3.02 (GraphPad Software, La Jolla, CA). All results are expressed as mean±SEM. Statistical significance was determined using the Student *t* test, whereas for expression array analyses, the Mann–Whitney test was applied. Statistical significance was denoted by asterisks in the figures as **P*<0.05, ***P*<0.01, and ****P*<0.001.

Results

GWAS for Aldosterone to Renin Ratio

The GWAS performed on 1786 subjects of the Cooperative Health Research in the Region of Augsburg F4 cohort demonstrated strong linkage of the ARR to chromosome 5 reaching genome-wide significance ($P=6.78\times10^{-11}$; Figure 1A). The identified locus included 4 known genes (Figure 1B): *SLC26A2*, *HMGXB3* (HMG box domain containing 3), *TIGD6* (tigger transposable element derived 6), and *CSF1R* (colony-stimulating factor 1 receptor). Because there was no evidence for functional effect of the CSF1 system in aldosterone production (Figure S1 in the online-only Data Supplement) and because of its known function as a transporter and based on initial expression studies, SLC26A2 was chosen for further investigation.

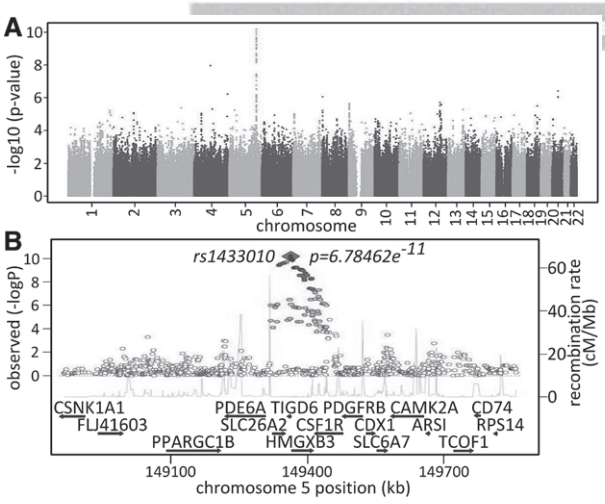

Figure 1. Genome-wide association study (GWAS) for aldosterone to renin ratio (ARR). **A**, GWAS of 1786 subjects from the Cooperative Health Research in the Region of Augsburg F4 population using 1000 K Affymetrix chip, and the ARR are depicted as a Manhattan plot, demonstrating a genome-wide significant locus ($P=6.78\times10^{-11}$) within chromosome 5. **B**, Zooming into the specific locus indicates position of genes within this region including SLC26A2 (solute carrier family 26 member 2), HMGXB3 (HMG box domain containing 3), TIGD6 (tigger transposable element derived 6), and CSF1R (colony-stimulating factor 1 receptor).

Expression of SLC26A2 in Murine Tissues and Human APA

After real-time polymerase chain reaction analysis in wild-type mice, highest levels of *slc26a2* mRNA were identified in murine adrenal glands (100±4%) in comparison with kidney (40±3%; *P*<0.001), lung (35±2%; *P*<0.001), heart (51±1%; *P*<0.001), liver (19±1%; *P*<0.001), and spleen (5±0.1%; *P*<0.001) among other organs (Figure 2A). Similar results were obtained by immunohistochemistry, demonstrating adrenocortical expression in human tissue samples, where SLC26A2 protein expression was detected in the adrenal cortex with less intensity in APA samples (Figure S2). Likewise, expression of SLC26A2 was readily detectable in the human adrenocortical cell line NCI-H295R (Figure 4A). Furthermore, *SLC26A2* expression was investigated in a series of human surgical tumor samples. Thereby, APAs displayed significantly lower *SLC26A2* mRNA levels than normal adrenal tissues (12.4±1.1 versus 31.2±4.2; *P*<0.0001; Figure 2B). This finding was independent of sex (Figure 2C) or any underlying somatic mutation in *KCNJ5* or ATPase (*ATP1A1* and *ATP2B3*) in the adenomas (Figure 2D).

Transcriptional Regulation of slc26a2 In Vivo

We next investigated *slc26a2* expression in wild-type mice that were treated with different stimulators of aldosterone secretion such as angiotensin II and potassium. Short-term stimulation with angiotensin II resulted in a significant downregulation of adrenal *slc26a2* expression at 120 minutes

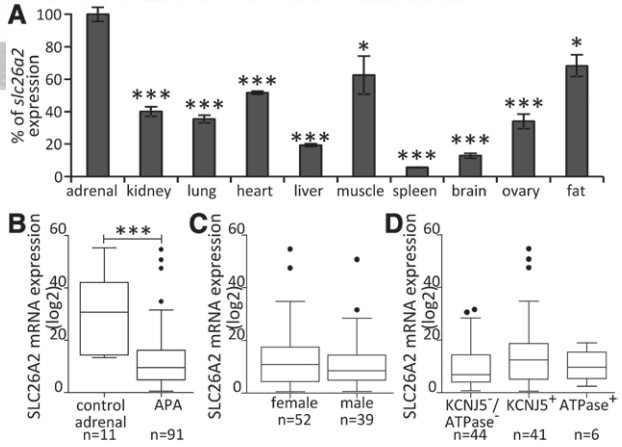

Figure 2. Expression of solute carrier family 26 member 2 (SLC26A2) mRNA in murine tissues and human aldosterone-producing adenomas (APAs). **A**, Investigation of tissue distribution in wild-type mice demonstrates slc26a2 expression to be highest in adrenal glands in comparison with a variety of other tissues. *Significant differences to adrenal gland expression. **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**). Whisker plots depicting first, second, and third quartiles, with whiskers for 1.5 interquartile range of the lower and upper quartile and dots for outliers are shown. *Statistical significance: **P*<0.05, ***P*<0.01, and ****P*<0.001.

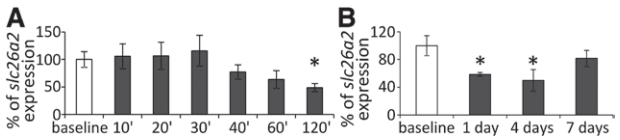

Figure 3. Transcriptional regulation of solute carrier family 26 member 2 (slc26a2) in vivo in mice. SLC26A2 expression is significantly downregulated in adrenals from wild-type animals on angiotensin II stimulation (**A**) and after a high potassium diet (**B**). *Significant differences in comparison with baseline values.

(49±7% versus 100±14%; *P*<0.05; Figure 3A). Similarly, after potassium supplementation in the drinking water for 1 week, adrenal *slc26a2* expression was significantly downregulated at day 1 (58±2%; *P*<0.05) and day 4 (49±15%; *P*<0.05) in comparison with baseline values (100±14%) while this effect was less pronounced at the end of the experiment (Figure 3B). Taken together, these findings demonstrated relevant expression changes of adrenal *slc26a2* on stimulation experiments, suggesting a potential role of this transporter on adrenal aldosterone output.

Aldosterone Output and Steroidogenesis on SLC26A2 Knockdown in NCI-H295R Cells

We next aimed at the evaluation whether SLC26A2 function in adrenocortical cells would effect directly on aldosterone secretion. To answer this question, we used a lentiviral based knockdown system in adrenocortical NCI-H295R cells. After this approach, we achieved knockdown efficacy in the range of 20% of untreated cells (untreated versus shSLC26A2; *P*<0.001; Figure 4A). In contrast to an unspecific, nontargeting shRNA construct that did not affect aldosterone secretion compared with untreated NCI-H295R cells, specific knockdown of *SLC26A2* resulted in a significant increase in aldosterone levels in the supernatant (111±1 versus 54±3 pg/mL; *P*<0.001). Accordingly, aldosterone biosynthesis after stimulation with potassium and angiotensin II was enhanced further by *SLC26A2* knockdown (Figure 4B). On a molecular level, *HSD3B2* and *CYP11B2* expression was upregulated in knockdown cells under baseline conditions (*HSD3B2*: 181±4% versus 100±5%, *P*<0.001; *CYP11B2*: 222±9% versus 100±10%, *P*<0.001) and was enhanced further on stimulation by potassium and angiotensin II (Figure 4E and 4F). Similar, but overall less relevant changes were observable for *StAR* and *CYP11A1* expression (on angiotensin II stimulation, *StAR*, 107±2% versus 60±1%, *P*<0.001 and *CYP11A1*, 271±30% versus 78±19%, *P*<0.01; Figure 4C and 4D). In addition, only minor changes were seen for *CYP11B1* expression (Figure S3). Accordingly, in serum-starved cells, differences in cortisol output was much less prominent for cortisol (baseline, 3-fold increase) as for aldosterone (baseline, 12-fold increase; Figure S4).

Molecular Pathways Involved in slc26a2-Dependent Aldosterone Secretion

Because Ca^{2+}-dependent signaling represents the best characterized pathway involved in aldosterone regulation,[20] we investigated potential changes in the Ca^{2+}/calmodulin-dependent protein kinase signaling pathway in the context of *SLC26A2* knockdown. In fact, pharmacological blockage of calmodulin

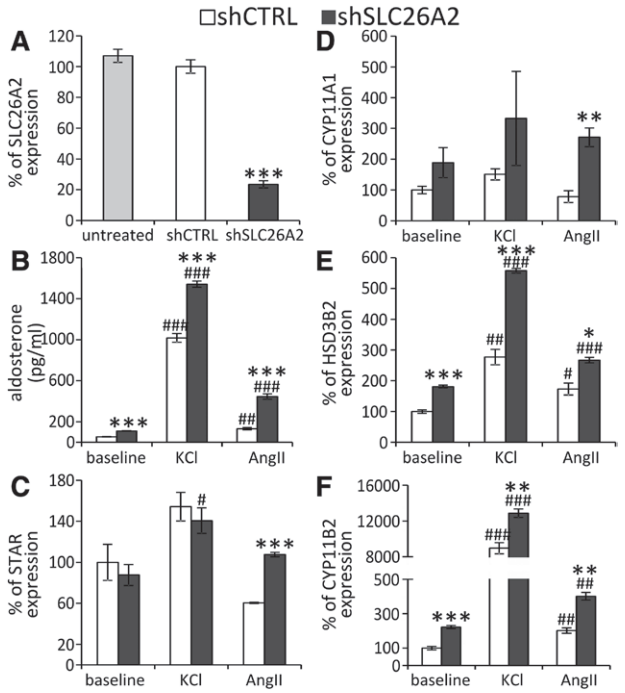

Figure 4. Steroidogenesis is upregulated in adrenocortical cells on solute carrier family 26 member 2 (SLC26A2) knockdown. **A**, SLC26A2 expression in untreated NCI-H295R cells, in NCI-H295R cells transfected with nontargeting shRNA (shCTRL), and in cells transfected with a specific SLC26A2 shRNA (shSLC26A2). **B**, Aldosterone production and mRNA expression of (**C**) StAR, (**D**) CYP11A1, (**E**) HSD3B, 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: */#*P*<0.05, **/##*P*<0.01, and ***/###*P*<0.001.

by calmidazolium and CaM kinase by KN-93 in *SLC26A2* knockdown cells caused a significant reduction of aldosterone output (calmidazolium, –2.06±0.10-fold, *P*<0.001; KN-93, –3.37±0.17 fold, *P*<0.0001). In contrast, these differences were less evident in control cells (calmidazolium, –1.26±0.05-fold, *P*<0.03; KN-93, –1.37±0.10-fold, *P*<0.03; Figure 5A). Along the same line, intracellular Ca^{2+} content was significantly higher in knockdown cells, independent of the applied experimental conditions (Figure 5B). Furthermore, expression levels of *CAMK1* at baseline conditions and on potassium and angiotensin stimulation were elevated (baseline, 180±4% versus 100±2%; *P*<0.001; Figure 5C), as well as further downstream effectors such as the transcription factors *NR4A1* (baseline, 131±3% versus 100±2%; *P*<0.01) and *NR4A2* (baseline, 154±6% versus 100±6%; *P*<0.01; Figure 5D) providing further evidence for an involvement of Ca^{2+} signaling in the mediation of *SLC26A2*-dependent effects on aldosterone output.

Aldosterone Output and Steroidogenesis in a slc26a2 Knockin Mouse Model

To substantiate further these findings of the role of adrenal *SLC26A2* expression on aldosterone output, we went on to an in vivo system. Specifically, we made usage of a *slc26a2* knockin model in which a mutation as detected in a patient

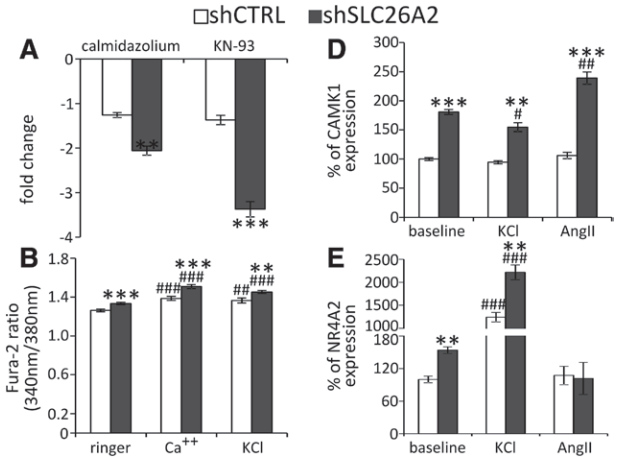

Figure 5. Induction of Ca^{2+}-dependent signaling in adrenocortical cells on solute carrier family 26 member 2 (SLC26A2) knockdown. **A**, Pharmacological blockage of calmodulin by calmidazolium and CaM kinase by KN-93 causes a more pronounced reduction in aldosterone biosynthesis in SLC26A2 knockdown cells in comparison with control cells. Knockdown of SLC26A2 is associated with higher intracellular Ca^{2+} levels (**B**) and mRNA expression of CAMK1 (**C**) and NR4A2 (**D**) at baseline conditions and on potassium and angiotensin stimulation. *Significant differences between shCTRL and shSLC26A2 cells within an experimental condition, whereas # depict significant differences between baseline and stimulated cells: */#*P*<0.05, **/##*P*<0.01, and ***/###*P*<0.001.

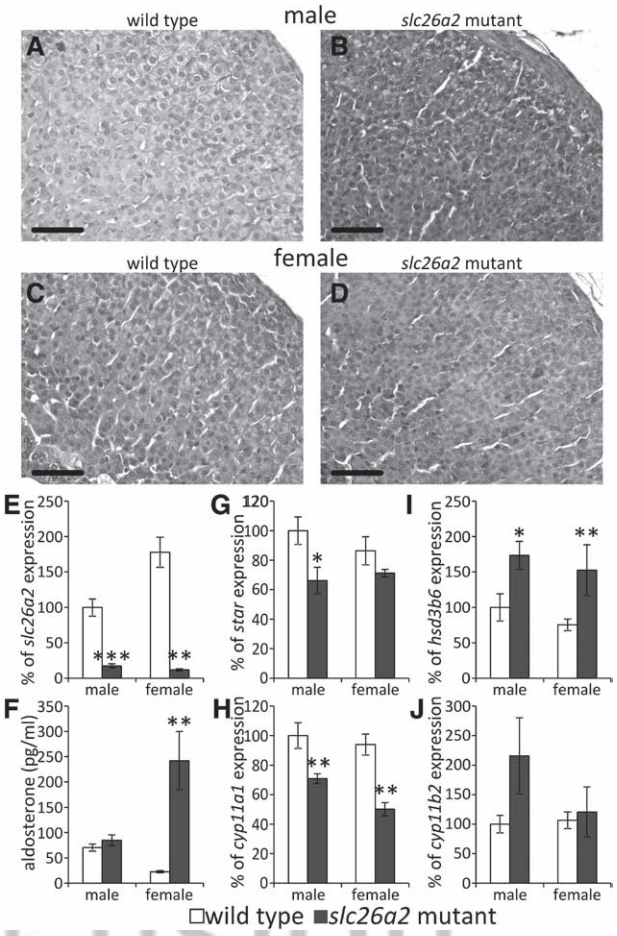

Figure 6. Solute carrier family 26 member 2 (Slc26a2) mutant animals display higher aldosterone output in a sex-dependent manner. **A**–**D**, No gross alterations in adrenal morphology and zonation are evident in slc26a2 mutant animal in comparison with wild-type controls. **E**, As expected, adrenal 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. *Significant differences between mutant and wild-type animals within the same sex group.

with mild phenotype of diastrophic dysplasia (p.A386V) had been introduced. In this model, a further splicing impairment is present, resulting in both structural protein changes, as well as reduced expression levels of mutation carrying *slc26a2* mRNA.[21] Although no morphological alterations were observed in adrenals of mutant animals compared with wild-type controls (Figure 6A–6D), as expected adrenal slc26a2 expression was significantly reduced in animals of both sexes (males: 17±3% versus 100±12%, *P*<0.001; females: 12±1% versus 177±21%, *P*<0.01; Figure 6E). Interestingly, aldosterone output was significantly increased in female animals (242±57 versus 22±2 pg/mL; *P*<0.01; Figure 6F), whereas only slight differences were observed in male mice (84±10 versus 70±7 pg/mL; *P*=0.27). In addition, although there were significant differences for corticosterone levels between wild-type and mutant animals, no such sex difference was evident for animals of the same genotype (Figure S5). Steroidogenic enzymes with a less specified expression pattern (*star*, *cyp11a1*) or a more zona fasciculata–specific distribution (*Hsd3b1*) showed a tendency or significant downregulation in the mutant mice (Figure 6G–6H and *Hsd3b1*, males: 90.3±7.9% versus 100±8.4%, *P*=0.431; females: 81.9±1.4% versus 132.9±11.4%, *P*<0.05). In contrast, adrenal enzymes with a more specific role in aldosterone synthesis and preferential expression in the zona glomerulosa including *hsd3b6* (males: 173±20% versus 100±19%, *P*<0.03; females: 153±36% versus 75±8%, *P*<0.009; Figure 6I) and *cyp11b2* (males: 215±64% versus 100±14%, *P*=0.2; females: 120±42% versus 107±14%, *P*=0.7; Figure 6J) showed a significantly higher expression levels or a trend toward higher expression levels in mutant animals. Although some sex-specific changes were evident, trends for differences between wild-type and slc26a2 mutant animals were comparable.

Discussion

The clinical hallmark of classical PA is hypokalemic hypertension with low plasma renin and elevated aldosterone, which occurs as a result of a dysregulated adrenal aldosterone production. In addition to these effects of aldosterone excess, a continuous gradient of increasing risk of blood pressure progression across ARR levels has been recognized in non-hypertensive individuals.[12] According to twin studies, a strong familial pattern of biochemical markers of glucocorticoid and mineralocorticoid secretion has been demonstrated.[22] Along the same line, the heritability for ARR was found to be 40% after multivariable adjustment in the Framingham study[12] and 38% in a UK study.[13] Herein, we were able to identify a locus

that contained only a small number of genes including that of *SLC26A2*, with a highly significant correlation to the ARR in a large population-based sample.

Elevation of ARR is predominantly a low renin indicator,[23] and low renin levels can result in false-positive ARR even when plasma aldosterone levels are inappropriately low to suggest PA. Along the same line, the cutoff between PA and low renin hypertension is arbitrary.[24] However, despite these limitations, application of the ARR for the screening of PA is widely accepted and part of international guidelines.[25] Importantly, the starting point for the current investigation was a population-based cohort. Although it is to be expected that a small percentage of patients with PA are among hypertensive individuals,[18] we did not concentrate on this disease entity but rather included subjects within the whole spectrum of distribution of the renin–angiotensin–aldosterone system. Similar approaches in other GWAS have been applied recently for quantitative traits such as urate,[26] glucose,[27] and adiponectin[28] but also systolic or diastolic blood pressure.[16] This, however, does not weaken the conclusion that a correlation between a genetic locus and a phenotypic trait exist that has a high probability for a functional effect. In fact, from both the in vitro and in vivo experiments conducted in this study, we could provide evidence for a direct link between SLC26A2 function and regulation of adrenal aldosterone output.

The solute carrier 26 transporters represent anion transporters with diverse substrate specificity. Several members are ubiquitously expressed, whereas others show tissue restricted distribution. They are present in epithelia and, to the extent known, play a central role in anion secretion and absorption.[29] As evident in the literature, SLC26A2 is abundant in many epithelia, as well as in connective tissues.[30] It has been proposed that SLC26A2 functions as a SO_4^{2-}/Cl^- but not as a SO_4^{2-}/HCO_3^- exchanger.[31] Through its ability to transport SO_4^{2-}, SLC26A2 provides the substrate for proteoglycan sulphation, which is required for cartilage development.[21] Accordingly, mutations in the *SLC26A2* gene cause diastrophic dysplasia, a disease characterized by severe cartilage and bone malformation.[32] In contrast, to date, no data had indicated a potential role of SLC26A2 in adrenal function or a contribution to hyperaldosteronism. We herein demonstrate that SLC26A2 is highly expressed in the adrenal gland and that it is downregulated in the context of physiological aldosterone stimulation and in aldosterone autonomy. We further show that decrease in *SLC26A2* expression in vitro results in induction of Ca^{2+}-dependent signaling and amplification of aldosterone output.

The mechanisms involved in the regulation of aldosterone secretion only recently have started to be unraveled with the help of mouse models, as well as high throughput genetic techniques. Several murine models with targeted genetic alterations including Task1 and Task3 knockout animals[33,34] have been found to display a phenotype of autonomous aldosterone secretion. Similarly, a circadian clock–deficient cry-null mouse model showing an upregulation of adrenal Hsd3b6 expression has also been demonstrated to display salt-sensitive hypertension.[35] After a complementary approach, we have recently used a phenotype-driven mutagenesis screen to generate further mouse models and potentially identify genes with functional effect on aldosterone secretion.[36]

Using exome sequencing mutations in the selectivity filter of a specific potassium channel, *KCNJ5*, have been demonstrated to cause autonomous aldosterone secretion.[8] Interestingly, this mechanism not only contributes to rare familial cases[8,37] but can also be found as somatic mutations in more than one third of APAs.[9] Similarly, somatic mutations in ATPase family members[38]—*ATP1A1* and *ATP2B3* coding for Na^+, K^+-ATPase and the plasma membrane calcium-transporting ATPase 3—and in voltage gated calcium channels[39,40] can be regarded as additional causes of aldosterone excess. In contrast, on the basis of published exome sequencing data, no mutation in *SLC26A2* has been found in adenomas or germline DNA.[38–40] Thereby, we conclude that mutations in *SLC26A2* are at least no common genetic contributors to develop PA. This does not, however, exclude the possibility that epigenetic mechanisms could result in expression changes with functional effect. In all instances of acquired somatic mutations, these affect mechanisms that converge in the elevation of intracellular Ca^{2+} levels. Cytoplasmic Ca^{2+} can be increased through liberation of intracellular stores or through opening of membranous calcium channels, which triggers Ca^{2+} influx.[41,42] Similar effects seem to be in place when aldosterone release is associated with *SLC26A2* function because we can demonstrate induction of Ca^{2+}-dependent signaling in our specific in vitro system under baseline conditions, on stimulation experiments, as well as pharmacological inhibition. However, the exact mechanism of how SLC26A2 affects intracellular Ca^{2+} levels in adrenal cells remains to be elucidated in future experiments, and it is possible that broader changes in other pathways could be evoked by *SLC26A2* knockdown. In addition, *SLC26A2*-dependent mechanism in the kidney could affect the renin–angiotensin–aldosterone system. However, in vitro experiments inducing *SLC26A2* knockdown in human kidney cortical collecting duct cells provided no evidence on *SLC26A2* dependent changes in the epithelial Na^+ channel expression as one of the prime examples of hormone-dependent sodium and fluid absorption (Figure S6).[43]

Based on our in vitro studies and on the expression analyses, we hypothesized that slc26a2 mutant animals should present with higher aldosterone levels in comparison with their wild-type littermates. This hypothesis could be verified for female animals, whereas male mice were mainly unaffected with regard to aldosterone output. Accordingly, adrenal expression levels of various steroidogenic enzymes were altered, pointing toward an upregulation of enzymes specifically required for aldosterone synthesis, whereas those with a less glomerulosa-restricted distribution were rather downregulated. It is possible that sex-dependent differences in aldosterone metabolism might have contributed to the observed changes in plasma aldosterone in mutated female animals. Furthermore, changes in salt intake might have been necessary to bring about a more distinct phenotype of the genetically altered animals. Investigation of the endocrine phenotype of these animals was, however, complicated by their small size and consecutive small blood volume of mutant mice. Although mutant animals present a nonlethal phenotype, they only survive for a few weeks with a mortality of ≈50% from birth to D21 and are characterized by a reduced growth and body weight and limited motor

activity.[21] Sex differences have been observed for several PA animal models with female mice carrying the more severe phenotype in most instances.[33,34] Notably, also in patients with genetically defined subgroups of APAs, sex differences have been recognized with KCNJ5 mutations being more abundant in females[9] and ATPase mutations predominating in males.[38] It is possible that this observation could represent a selection bias introduced by differences in sex-dependent phenotypic penetrance. For genetically modified mice, it has been proposed that sex steroid–induced differences in TASK3[33] and dickkopf-3–dependent signaling could be responsible for these effects.[44] The observation that there is no significant difference in *SLC26A2* expression levels in APAs from male and female patients, however, argues against a direct sex-dependent transcriptional regulation in the adenoma tissue.

Perspectives

We provide evidence for a functional effect of SLC26A2 on aldosterone secretion. As evident from a population-based GWAS study and expression analysis, it can be concluded that any impediment to the SLC26A2 function may contribute to development of secondary hypertension in the context of autonomous aldosterone production in PA. Whether this gene might be involved in rare familial cases of PA or as a modifier of sporadic forms of the disease will require further genetic and epidemiological studies.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Our study demonstrates a significant correlation of aldosterone to renin ration with a genetic locus on chromosome 5 using a population-based genome-wide association study.

What Is Relevant?

- Starting from this genetic finding, we provide evidence from in vitro and in vivo models that a sulfate transporter from this locus, SLC26A2, has functional links via Ca^{2+}-dependent signaling to aldosterone biosynthesis.

Summary

Primary aldosteronism that is caused by unopposed secretion of aldosterone is recognized as the most prevalent form of secondary hypertension. Aside from few familial types, genetic causes of this disease are not well understood. Our findings extend insight into genetic contributors which may potentially be evaluated as therapeutic targets in the treatment of primary aldosteronism.

ONLINE SUPPLEMENT

THE DIASTROPHIC DYSPLASIA SULFATE TRANSPORTER (SLC26A2) IS EXPRESSED IN THE ADRENAL CORTEX AND REGULATES ALDOSTERONE SECRETION

Short title: SLC26A2 and primary aldosteronism

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Supplementary Materials and Methods

Animal experiments

All animal studies were performed according to protocols examined and approved by the Animal Care and Use Committee of the University of Pavia or the Regierung von Oberbayern according to the German Animal Protection Law, respectively. Dtd (slc26a2 mutant and wild type control) and C3HeB/FeJ mice were kept in a specific pathogen-free animal facility area at an ambient temperature of 25±2°C (relative humidity 60±5%) on a 12h-12h light-dark circle. Animals were fed with standard pelleted food #2018 (Harlan Laboratories, Udine, Italy) or chow #1314 (Altromin, Lage, Germany), respectively, *ad libitum* with free access to tap water. The experiments were performed on 8 weeks old female and male mice, which were maintained in groups of 6-8 individuals. 4-8 animals were used for each experiment per gender and genotype. For the investigation of the phenotype of those animals under baseline conditions, animals were euthanized by decapitation for collection of trunk blood and adrenal glands and/or kidneys, lungs, heart, lean muscles, spleen, brain, fat and ovaries without previous treatment. All adrenals were immediately cleaned from adjacent tissue using a stereo microscope, and all organs were snap frozen in liquid nitrogen and stored at -80°C until further processing or stored in 4% paraformaldehyde and subsequently embedded in paraffin blocks. From each animal 0.25 ml of blood were collected in Li-heparin coated tubes to avoid coagulation. After centrifugation at 10000 x g for 10min plasma was separated and kept at -20°C until measurement. Slc26a2 expression in the adrenal glands of C3HeB/FeJ animals was investigated upon angiotensin II stimulation [1] or following a high potassium diet [2], as described previously.

Quantitative Real-time PCR

Both adrenals from each individual animal were combined and homogenized in lysis buffer while still frozen. NCI-H295R cells were lysed directly by addition of lysis buffer. RNA extraction from lysates was performed using the The RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality was verified on a 1.5% agarose gel. For cDNA synthesis, 1 µg of total RNA were reverse transcribed utilizing the reverse transcription system (Fermentas, St Leon-Rot, Germany).

Gene expression of slc26a2 and selected genes involved in steroidogenesis were investigated (for primer sequences and annealing temperatures see Supplementary Table S1). For quantification of the investigated genes the Sso Fast EVA Green Supermix (Biorad Laboratories Berkley, CA) in the Mx3000P QPCR System (Stratagene, La Jolla, CA) was used. Real-time PCR conditions in the Mx3000P were pre-incubation at 95°C for 10 min followed by amplification of 40 cycles at 95°C for 10 sec at the annealing temperature for 5 sec (primer dependent, see table S1), and extension at 72°C. The melting curve analysis was performed between 65 and 95°C (0.1° C/sec). Furthermore, the products were run on a 1% agarose gel to verify the correct size of the amplified product. Quantification was adjusted using the housekeeping genes *HPRT1* for human samples and *Actb* for mouse samples. To facilitate overall comparison of individual

real-time experiments, expression levels of the particular genes were set as 100% for control animals or cells.

Immunohistochemistry

For SLC26A2 immunohistochemistry in human tissues a polyclonal mouse anti human primary antibody (HPA041957, Sigma-Aldrich, Taufkirchen, Germany) was utilized. CYP11B2 antibody was obtained from Dr. Celso Gomez-Sanchez (University of Mississippi, Jackson, MS)[3]. Staining procedure was followed as described in detail before [4].

Cell culture

NCI-H295R (CRL-2128; American Type Culture Collection, Manassas, VA) cells were cultured in a RPMI 1640 medium (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum, 1% insulin–transferrin–selenium supplement, 1% penicillin/streptomycin, and 100 nM hydrocortisone in a humidified atmosphere (95% CO_2). For simultaneous measurement of cortisol and aldosterone production of knockdown cells under baseline and stimulated conditions, cells were seeded in 24 well plates at 200000 cells per well and grown for 48 hours and then incubated in serum-free media for 24 hours. Cells were then treated with 10 mM KCl, 100nM Angiotensin II (Sigma-Aldrich, Taufkirchen, Germany), 10 µM Forskolin (Sigma-Aldrich) or vehicle in serum-free media for stimulation of steroid production. Following incubation for 48 hours, supernatant and cells from each well were harvested for aldosterone measurement and RNA extraction. For stimulation of CSF1R expression, cells were seeded and grown as above, then treated with 0,016-50 ng/ml CSF1 (Novus Biologicals, UK) for 48 hours in media with additives followed by harvesting of supernatant and cells for aldosterone measurement and RNA extraction.

Human principal cortical collecting duct cells were obtained from Dr. Wolfgang Neuhofer (University Clinic Munich) and maintained in DMEM/F-12 (31330, Gibco) supplemented with 2% fetal bovine serum, 1% insulin–transferrin–selenium supplement, 1% penicillin/streptomycin, and 50 nM dexamethasone. Cells were seeded in 24 well plates at 50000 cells per well and grown for 48 hours. After serum starvation for 18 hours with serum-free DMEM/F-12, cell were stimulated with 1µM aldosterone for 6 hours and harvested.

Knock-down experiments

Human SLC26A2-targeting and non-mammalian control MISSION® shRNA lentiviral transduction particles were obtained from Sigma-Aldrich. The shRNA sequences were validated by The RNAi Consortium (TRC) and were inserted into TRC2 pLKO-puro plasmid backbone. For the lentiviral infections, manufacturer's protocol was followed with modifications. In brief, H295R cells were seeded in 96-well plates with $1x10^4$ cells per well. 24 hours later, medium was renewed and supplemented with 2 µg/ml polybrene (Sigma). Lentivirus particles were added to the cells at a MOI of 10 and proceeded with spin infection at 700x g for 30 minutes at 37°C. Medium was

changed after overnight incubation. For selection, 7 μg/ml puromycin (Sigma) was given to the cells after 48 hours, with medium changes every two days, for the first 3 passages. After this point RNA was extracted for assaying knockdown efficacy and cell lines were kept under selective pressure by puromycin containing medium every third passage.

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Table S1: Primer sequences and experimental conditions used for real-time PCR analysis.

gene (accession)	species		sequence (5' > 3')	annealing temperature
CAMK1 (NM_003656.4)	human	fwd	CATCGCCTACATCTTGCTCTG	60°C
		rev	TTCTTCTTGATCTGCTCACTCAC	
CYP11B1 (NM_000497.3)	human	fwd	GGGTGGCCTACAGACAACATC	60°C
		rev	GGCGACAGCACTTCTGGATT	
CYP11B2 (NM_000498.3)	human	fwd	ACTCGCTGGGTCGCAATG	60°C
		rev	AGTGTCTCCACCAGGAAGTGC	
HPRT1 (NM_000194.2)	human	fwd	TGCTGACCTGCTGGATTACA	60°C
		rev	CCTGACCAAGGAAAGCAAAG	
HSD3B1 (NM_000862.2)	human	fwd	AGAAGAGCCTCTGGAAAACACATG	60°C
		rev	TAAGGCACAAGTGTACAGGGTGC	
HSD3B2 (NM_000198.3)	human	fwd	AGAAGAGCCTCTGGAAAACACATG	60°C
		rev	CGCACAAGTGTACAAGGTATCACCA	
NR4A1 (NM_002135.4)	human	fwd	TCGGGGATACTGGATACACC	60°C
		rev	TGTTCGGACAACTTCCTTCA	
NR4A2 (NM_006186.3)	human	fwd	AGTCTGATCAGTGCCCTCGT	60°C
		rev	CTGGGTTGGACCTGTATGCT	
SCNN1A (NM_001038.5)	human	fwd	CAACCAGGTCTCCTGCAAC	60°C
		rev	GGGTTTCCTTCCTCATGCT	
SLC26A2 (NM_000112.3)	human	fwd	CAATGCCCATAGTGCTCCTT	60°C
		rev	ATCCACTCAGCAAGGCATCT	
Actb (NM_007393.3)	mouse	fwd	ACCCGCGAGCACAGCTTCTT	60°C
		rev	TCTGGGCCTCGTCACCCACATA	
Cyp11a1 (NM_019779.3)	mouse	fwd	GCTGGAAGGTGTAGCTCAGG	60°C
		rev	CACTGGTGTGGAACATCTGG	
Cyp11b2 (NM_009991.3)	mouse	fwd	CAGGGCCAAGAAAACCTACA	60°C
		rev	ACGAGCATTTTGAAGCACCT	
Hsd3b1 (NM_008293.3)	mouse	fwd	AAGGAGGAATTCTCCAAGCTG	60°C
		rev	GAGCTGCAGAAGATGAAGGC	
Hsd3b6 (NM_013821.3)	mouse	fwd	ATCAGAACCAGCCATTCCAA	60°C
		rev	AAAACCCTCCTGCTCCAGTT	
Slc26a2 (NM_007885.2)	mouse	fwd	CTGCCCTGACACTGATGCTA	60°C
		rev	ACGTGAGGATGGTGAAGGAG	
Star (NM_011485.4)	mouse	fwd	GACCTTGAAAGGCTCAGGAAGAAC	63°C
		rev	TAGCTGAAGATGGACAGACTTGC	

S1

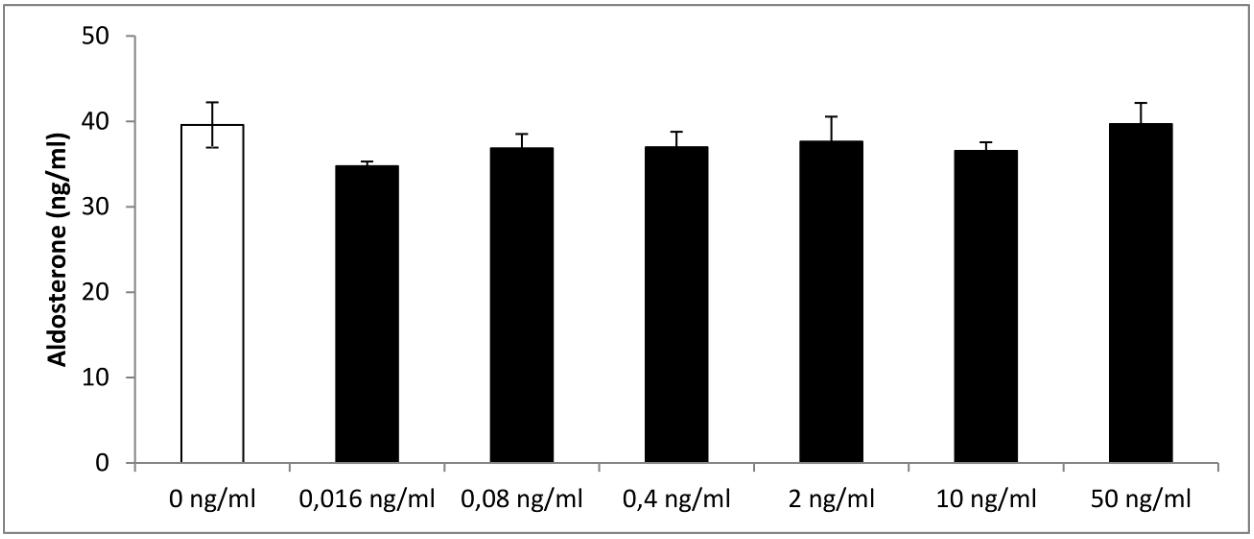

Figure S1: Production of aldosterone by NCI-H295R cells treated with various concentrations of colony stimulating factor 1 (CSF1). Stimulation of CSF1R by its ligand, CSF1, does not affect aldosterone production by NCI-H295R cells.

S2

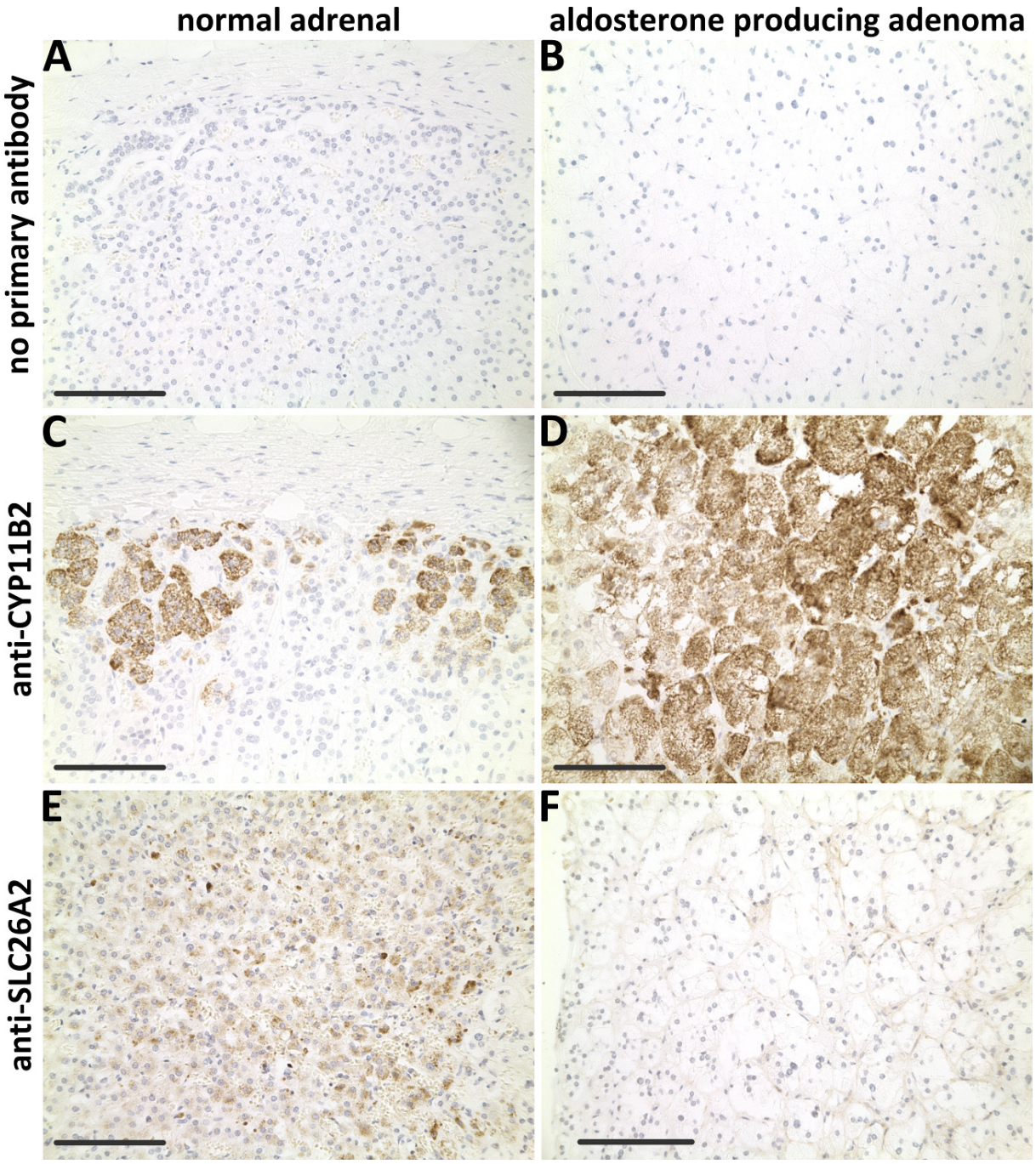

Figure S2: CYP11B2 and SLC26A2 immunohistochemistry of normal human adrenal glands (A, C, E) and aldosterone producing adenomas (B, D, F). No specific staining is detectable in samples excluding the primary antibody (A, B). CYP11B2 staining shows specific immunopositivity in the zona glomerulosa (C) and intense staining in the adenoma (D). In contrast, SLC26A2 staining was weaker in adenoma tissue (F) in comparison to normal adrenals which did furthermore not display zonal specificity (E). Bars represent 125µm.

S3

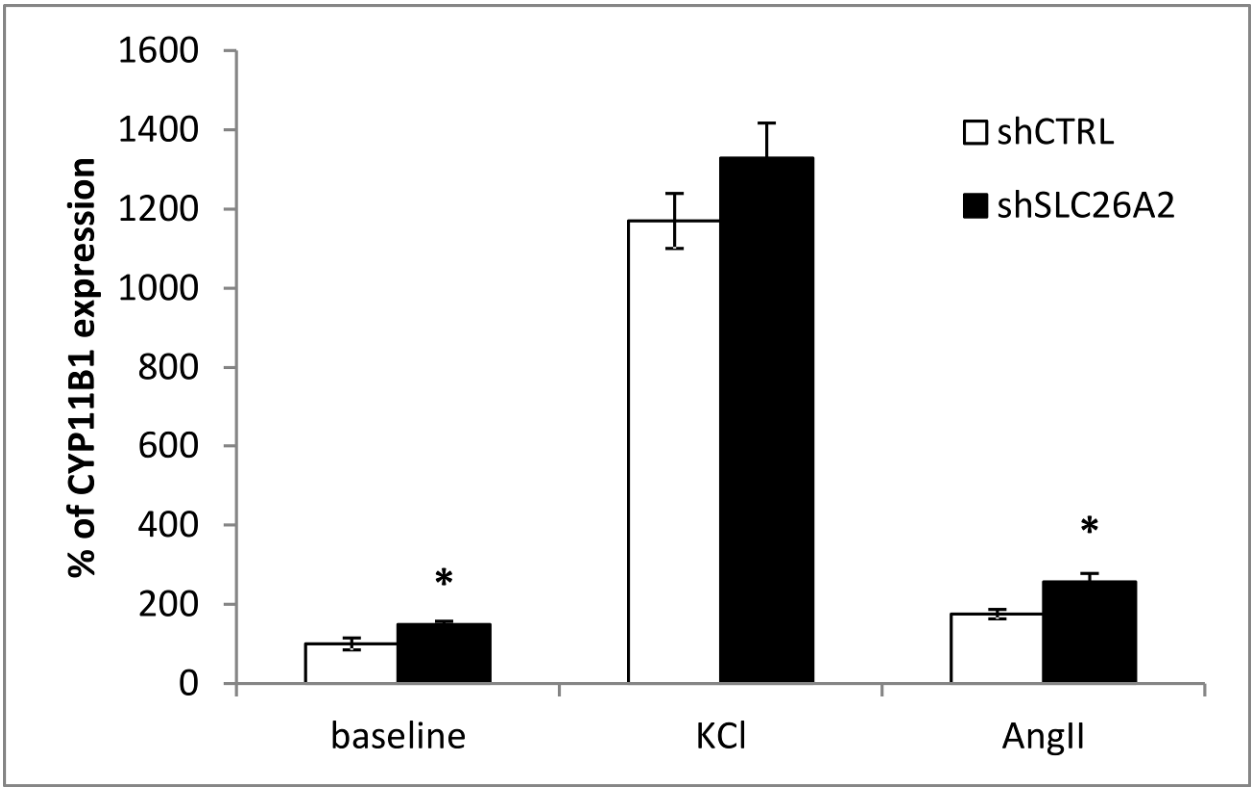

Figure S3: Expression of *CYP11B1* in *SLC26A2* knockdown NCI-H295R cells. *CYP11B1* was inducible by 10 mM potassium (KCl) and only weakly by 100 µM angiotensin II (AngII). Upregulation by *SLC26A2* knockdown was evident at baseline and upon AngII stimulation. This increase in *CYP11B1* expression was found to be much less extensive in comparison to that of *CYP11B2* (Figure 4F).

S4

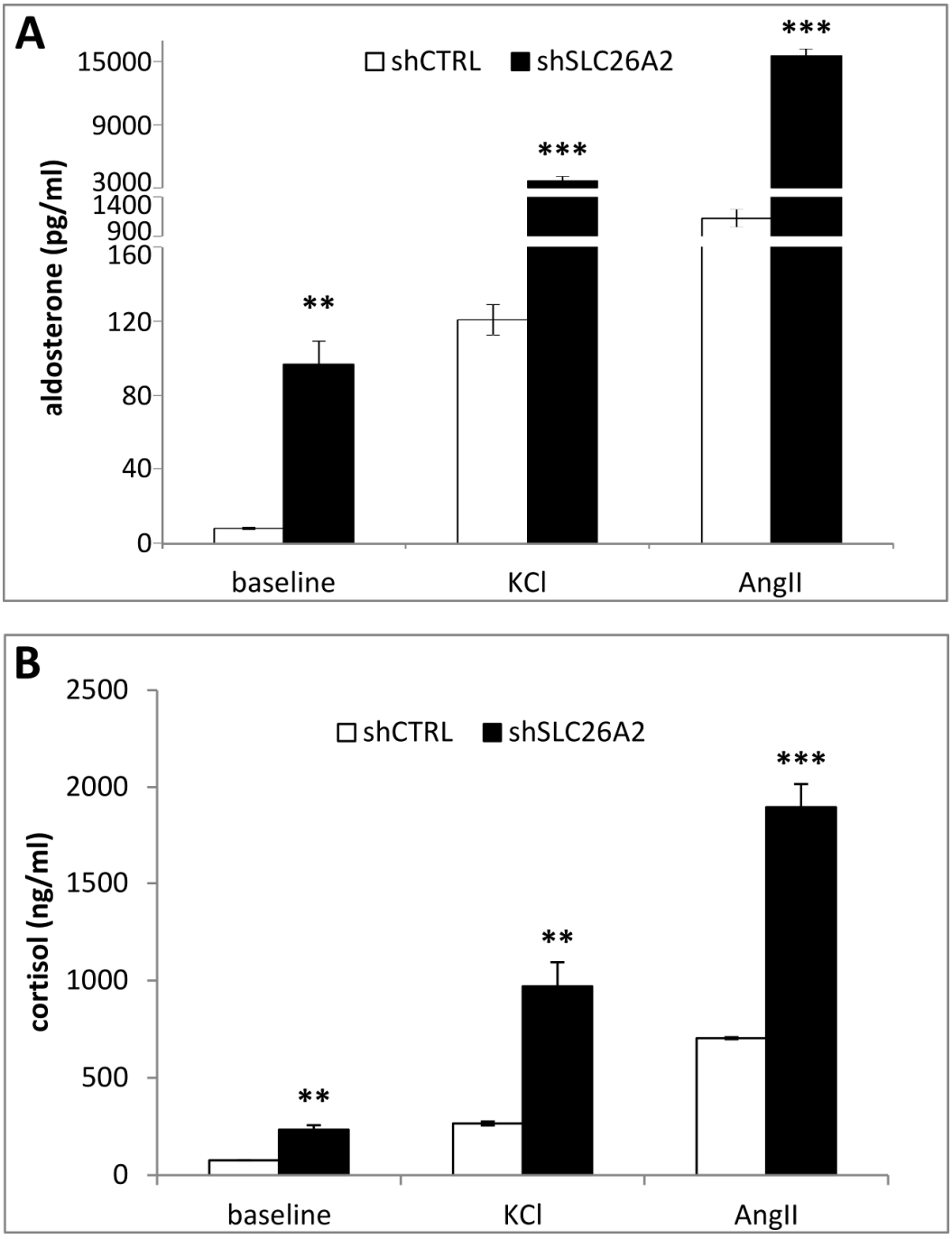

Figure S4: Production of aldosterone (A) and cortisol (B) by SLC26A2 knockdown NCI-H295R cells after 24 hours of serum starvation. Cortisol secretion increased by threefold under baseline conditions, the effect being comparable in the presence of 10 mM KCl (3.6-fold in comparison to control cells) or 100 µM angiotensin II (2.7-fold) (A). In contrast, increase in the production of aldosterone by the same cells was more profound, 12-fold (over control cells) under baseline conditions, 30-fold with KCl and 14-fold with Angiotensin II.

S5

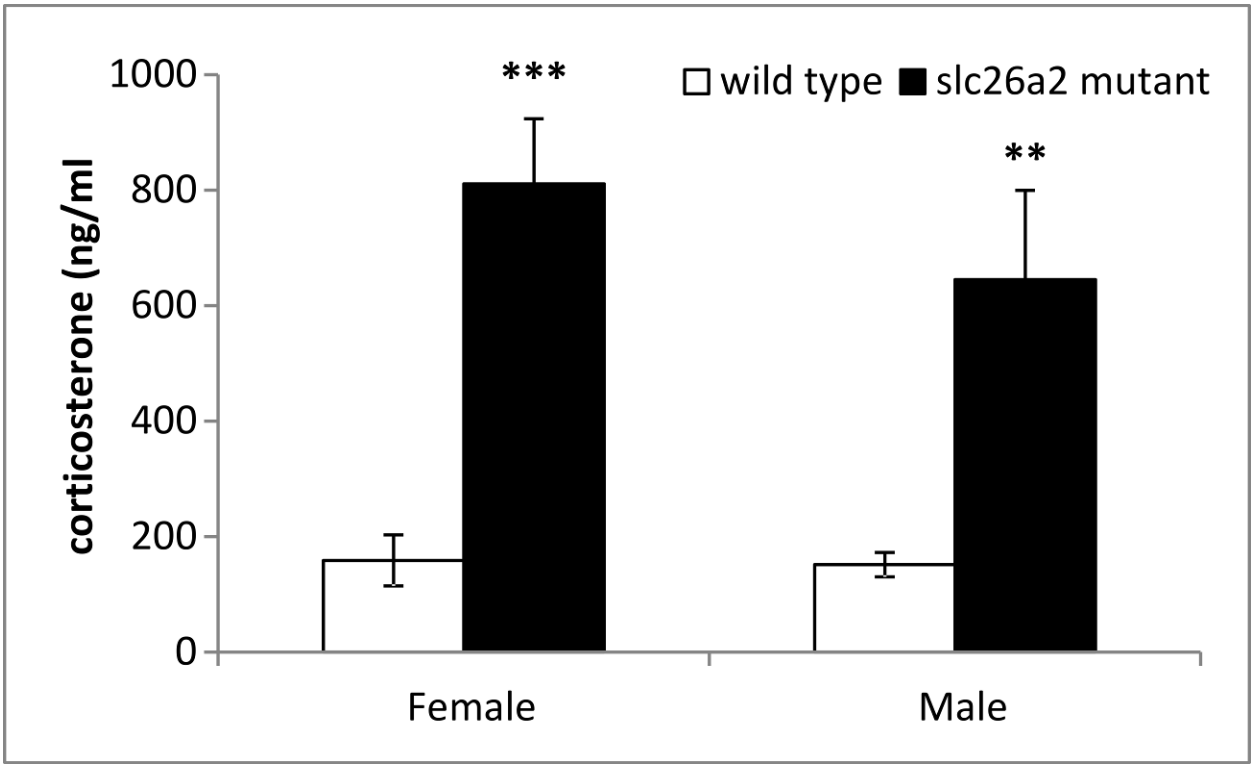

Figure S5: Plasma corticosterone levels of wild-type and *Slc26a2*-knockout mice. Mutant animals of both genders were found to have higher levels of corticosterone, whereas no such significant difference was observed between genders of either genotype.

S6

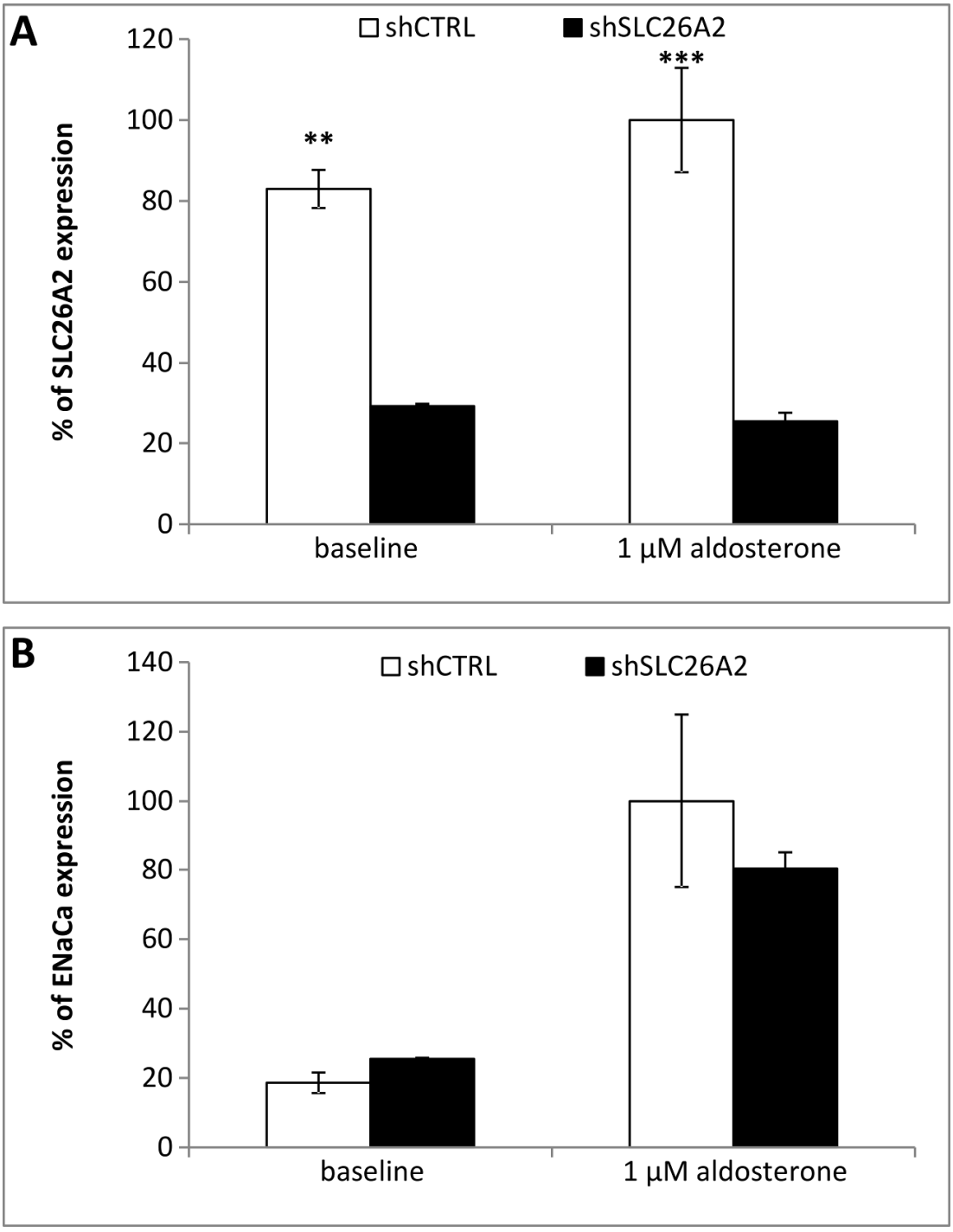

Figure S6: Knockdown of SLC26A2 in human kidney cortical collecting duct cells. A knockdown efficacy of 30 % of control cells was achieved under baseline and aldosterone stimulated conditions (A). The upregulation of ENaCa by aldosterone stimulation was not significantly changed by SLC26A2 knockdown (B).