Hormones and Cancer (2020) 11:52–62
https://doi.org/10.1007/s12672-019-00375-0

ORIGINAL PAPER

Molecular and Electrophysiological Analyses of *ATP2B4* Gene Variants in Bilateral Adrenal Hyperaldosteronism

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Received: 18 August 2019 / Accepted: 26 December 2019 / Published online: 30 January 2020

Abstract

Primary aldosteronism (PA) is the most common cause of secondary hypertension with a high prevalence among patients with resistant hypertension. Despite the recent discovery of somatic variants in aldosterone-producing adenoma (APA)-associated PA, causes for PA due to bilateral aldosterone production (bilateral hyperaldosteronism; BHA) remain unknown. Herein, we identified rare gene variants in *ATP2B4*, in a cohort of patients with BHA. *ATP2B4* belongs to the same family of Ca-ATPases as *ATP2B3*, which is involved in the pathogenesis of APA. Endogenous *ATP2B4* expression was characterized in adrenal tissue, and the gene variants were functionally analyzed for effects on aldosterone synthase (*CYP11B2*) expression, steroid production in basal and agonist-stimulated conditions, and for changes in biophysical properties of channel properties. Knockdown of *ATP2B4* in HAC15 exhibited reduced angiotensin II stimulation in one of four shRNA clones. Stable HAC15 cell lines with doxycycline (dox) – inducible wild-type and variant forms of *ATP2B4* – were generated, and dox-induced upregulation of *ATP2B4* mRNA and protein was confirmed. However, *ATP2B4* variants did not alter basal or agonist-stimulated *CYP11B2* expression. Whole-cell recordings in HAC15 cells indicated robust endogenous *ATP2B4* conductance in native cells but reduced conductance with overexpressed WT and variant *ATP2B4*. The previously defined PA-causing *ATP2B3* variant served as a positive control and exhibited elevated *CYP11B2* mRNA. In conclusion, while this study did not confirm a pathogenic role for *ATP2B4* variants in BHA, we describe the sequencing analysis for familial and sporadic BHA and outline a template for the thorough in vitro characterization of gene variants.

Keywords ATP2B4 · ATP2B3 · Ca-ATPases · Aldosterone · Primary aldosteronism · Doxycycline-inducible expression

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12672-019-00375-0) contains supplementary material, which is available to authorized users.

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Introduction

Primary aldosteronism (PA) is the most common form of endocrine hypertension and characterized by renin-independent aldosterone excess. PA can be categorized into two main subtypes: bilateral and unilateral disease. PA is most often sporadic, but familial forms have been described. Over the past decade, next-generation sequencing (NGS) has identified somatic variants in aldosterone-producing adenoma (APA)-related PA. Thus far, NGS has identified several genetic variants in APAs, including *KCNJ5*, *CACNA1H*, *CACNA1D*, *ATP2B3*, and *ATP1A1* [1–14], as the underlying mechanism of aldosterone excess in APA. While somatic variants in these genes are found in up to 90% of all APAs, and therefore the majority of cases of unilateral aldosterone excess, germline variants in these or other genes are rare, explaining likely less than 5% of all cases with bilateral aldosterone excess [15, 16].

The currently known germline variants cause a phenotype of bilateral disease, yet with significant variability in terms of severity, penetrance, age of onset, and associated symptoms. For instance, mutations in *KCNJ5* account for up to 40% of APA-related PA [17, 18]. However, while the germline variant $KCNJ5^{G151E}$ was associated with a phenotype of hypertension and negligible hyperplasia, a different germline variant at the same locus, $KCNJ5^{G151R}$, exhibited a severe phenotype with massive hyperplasia, requiring bilateral adrenalectomy [9, 14]. Familial PA due to germline variants in *CACNA1D* causes a novel phenotype of PA associated with seizures and other neurological abnormalities [11]. Finally, most recently, whole-exome sequencing revealed germline variants in the inward rectifying chloride channel *CLCN2* in a subgroup of patients with PA [19, 20]. These patients present with bilateral hyperaldosteronism (BHA), and this genetic cause of BHA is classified as familial hyperaldosteronism type II (FH-II). However, the *CLCN2* variants have a low prevalence, and the cause for aldosterone excess in the majority BHA cases remains unexplained.

The vast majority of patients with BHA present as apparently sporadic disease. The bilateral nature of the disease, however, suggests a germline genetic predisposition as one putative causative mechanism. Unfortunately, the availability of tissue for detailed analyses is limited as these patients are usually treated medically. Therefore, analysis is largely restricted to germline variants in families with multiple affected members with PA or patients with apparently sporadic PA, with the assumption that pathogenic variants in genes are shared in this population. Using this approach, we identified rare variants in *ATP2B4*, which encodes the calcium ATPase, isoform 4 (PMCA4).

PMCA4 belongs to the P-type family of calcium ATPases (PMCAs) [21–24] and is expressed on the cell surface in a variety of tissues, including the smooth muscle, liver, brain, heart, pancreas, and adrenal [25–30]. Within the adrenal, the *ATP2B4* gene is the highest expressed among all members of the calcium ATPase family [29]. PMCAs regulate intracellular calcium levels through their ability to hydrolyze one ATP molecule to actively pump a single intracellular calcium ion across the cell membrane. At low levels of cytoplasmic calcium, the calcium–calmodulin-binding domain interacts with other domains, blocking the access of calcium and/or ATP to the active site, thereby rendering the PMCA in an autoinhibited state [23–25, 31–33]. Increase in intracellular calcium levels causes the calcium-calmodulin complex to bind the carboxyl terminal, thus displacing it and enabling ATP-hydrolysis and pump activity. The *ATP2B4* gene is expressed as two transcript variants, 4a and 4b (PMCA4a and PMCA4b, respectively; NM_001001396 and NM_001684, respectively) [23–25, 34, 35]. The PMCA4b variants have a larger calcium–calmodulin-binding domain, lower basal activity, and greater responsiveness to stimulation by calmodulin [25, 36]. Altered interactions of PMCA4 in protein complexes or genetic mutations in *ATP2B4* or knockdown of the gene have been suggested to cause various pathologies including long QT syndrome, reduced sperm motility, and familial spastic paraplegia [23, 24, 37–39].

Since the *ATP2B4* variants observed in our patients are localized to the calcium-calmodulin domain, we hypothesized a loss of function, causing constitutive self-inhibition of PMCA4b and accumulation of cytosolic calcium ions, resulting in elevated *CYP11B2* expression and aldosterone production. We, therefore, analyzed these variants through an in vitro pipeline involving characterization of gene expression across tissues, analysis of effects of gene knockdown, as well as inducible overexpression of the gene variants on cellular mechanisms, LC-MS/MS measurement of steroids and electrophysiological characterization.

Materials and Methods

Next-Generation Sequencing

Informed consent was obtained from all individual participants included in the study. Patients were consented and analyzed through IRB-approved protocols (HUM0024461, HUM0151131). NGS was conducted in the UM sequencing core using the Illumina 2500. Paired-end reads (FASTQ file) were aligned to the hg19 assembly with Burrows-Wheeler alignment (BWA) 92. Then, picard tools were used for sorting and marking the duplicated reads in the resulting BAM files. Variant calling was performed with FreeBayes 93, and low-quality variants (QUAL < 20) were discarded. Common variants (MAF > 20) were filtered out in our in-house database. The resulting VCF files were then annotated with ANNOVAR 94 and KGGseq 95. The final list of candidate genes was generated after excluding variants that are common in the population (MAF > 1), non-coding and synonymous. From the resulting list of 502 variants, genes variants were selected if present only in the affected family members in each family, generating a list of 76 gene variants (Supplementary Table 1).

Generation of Vectors

The pMM2-hPMCA4b was a gift from John Penniston and Emanuel Strehler (Addgene plasmid # 47759; http://n2t.net/addgene:47759; RRID:Addgene_47,759). Wild-type PMCA4b was PCR amplified to include using restriction sites DraIII and XbaI at the 5′ and 3′ ends, respectively. The resultant amplicon was sub-cloned into the shuttle vector pENTR1A-GFP-N2 (FR1) [a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 19364; http://n2t.net/addgene:19364; RRID:Addgene_19,364)] [40]. The variants of PMCA4b were generated by site-directed mutagenesis

using the Q5® site-directed mutagenesis kit (New England Biolabs, Ipswich, MA). The resultant PMCA4b variants were inserted into the lentivector pCLX-pTF- R1-DEST-R2-EBR65 [a gift from Patrick Salmon (Addgene plasmid # 45952; http://n2t.net/addgene:45952; RRID:Addgene_45,952)] [41]. Lentiviruses ($\sim 1 \times 10^6$ TU/mL) were generated at the University of Michigan Biomedical Vector Core and used for transducing adrenal cells. Similarly, $ATP2B3^{WT}$ and the previously described pathogenic variant $ATP2B3^{\Delta Leu425\text{-}Val426}$ (kindly donated by Dr. Sasha Bandulik) [29] were also cloned in to the pCLX-pTF- R1-DEST-R2-EBR65 lentiviral system. These served as controls for the effect of the ATP2B family in adrenal cells.

Cell Culture and Viral Transduction of Cell Lines

The human adrenocortical carcinoma cell line, HAC15, was used as parental cells grown in DMEM-F12 containing 10% CCS, 1% ITS, and antibiotics (PenStrep and gentamicin), as previously described. Transductions were performed as previously described [42]. HAC15 cells were transduced with lentiviruses at a multiplicity of infection of 3, as follows: 2 million cells were plated in a T-75 flask for 24 h, after which they were incubated with fresh antibiotic-free growth media containing 10-μg/mL polybrene for 2 h. Cells were then spinoculated (centrifuged) at 1000 rpm with lentiviruses and antibiotic-free growth media with 8-μg/mL polybrene. After overnight incubation, the cells were recovered by the addition of twice the volume of growth media and incubated for additional 24 h. At the end of 48 h from transduction, the cells were incubated in regular growth media containing 5-μg/mL blasticidin (mammalian selection marker). A mixed population of blasticidin-selected HAC15 cells were used to analyze gene mutations. The generated cells carried doxycycline-inducible gene expression systems for $ATP2B4^{WT}$, $ATP2B4^{C3152A}$, $ATP2B4^{C3152T}$, and $ATP2B4^{G3346A}$. For experiments, HAC15-dox-ATP2B4 cells (wild-type or variant cells) were plated in 24-well plates at a density of 200,000 cells/well until 60% confluence. After 48 h, cells were starved in 0.1% CCS low serum media (LS) for 24 h and then treated with LS with/without doxycycline (1 μg/mL) for 72 h for maximal induction of the transgene. Agonists (10-nM Ang II and 18 mM K^+) were introduced for the last 12 h. For LC-MS/MS measurement of steroids, cells were incubated with LS with/without doxycycline and agonists for 72 h. Ang II was replenished every 24 h due.

For knockdown studies, HAC15-B2Luc cells (kindly donated by Dr. Celso Gomez-Sanchez) expressing a CYP11B2-driven-secreted luciferase were cultured and treated with dox and agonists as described for gene expression studies. These cells have been successfully used for functional analysis of the $KCNJ5^{T158A}$ *variant* in adrenal cells [43]. HAC15-B2Luc cells were transduced with four different clones of short hairpin RNA for ATP2B4 knockdown and selected with puromycin (10 μg/mL). As described elsewhere, 25 μL of the media were analyzed for luciferase activity using coelentrazine[43].

For electrophysiological experiments, circular coverslips were coated with 50-μg/mL poly-D-lysine for 2 h at room temperature, followed by coating with 1-μg/mL laminin (overnight at 4 °C). Coverslips were washed at least three times with 1X PBS and transferred to 24-well plates. At the time of patching, HAC15-dox-ATP2B4 cells incubated with doxycycline for 72 h were trypsinized and plated on the coated coverslips at a density of 100,000 cells/well in growth media with doxycycline and incubated for 1 h prior to patching.

Steroid Quantitation by Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)

A 150-μL aliquot of media from HAC15-dox-ATP2B4 cells, along with 50-μL internal standard of deuterated steroids, was deproteinated using acetonitrile as previously described [44]. Following deproteinization, extraction of steroids was performed using 1-mL methyl-t-butyl ether (MTBE). The organic phase was dried down and concentrated under nitrogen, reconstituted with 50 μL of 1:1 methanol-deionized water, and transferred to a 0.25-mL vial insert. The LC-MS/MS assay was performed as previously described [44].

Electrophysiology

Ion currents were recorded in whole-cell patch-clamp configuration using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA) as described earlier [42]. Patch pipettes had resistances of 5–8 MΩ when filled with intracellular pipette solution and placed in extracellular solution. External solution containing 137-mM NaCl, 5-mM KCl, 5-mM glucose, 1-mM $MgCl_2$, 1.2-mM $CaCl_2$, and 5-mM HEPES (pH 7.4) was perfused through the system. The internal pipette solution contained 95-mM K-gluconate, 30-mM KCl, 4.8-mM Na_2HPO_4, 1.2-mM NaH_2PO_4, 5 mM glucose, 2.38-mM $MgCl_2$, 0.726-mM $CaCl_2$, 1-mM EGTA, and 3-mM ATP (pH 7.2). Data acquisition and analysis were performed using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA). Normalization of current amplitudes to cell size was performed by dividing amplitudes by cell capacitance (Cm) to generate current densities (pA/pF) [42].

Immunofluorescence

Slides with 5-μ serial sections of the human adrenal cortex were deparaffinized. After antigen retrieval in pH 6 citrate buffer and pH 9 Tris buffer, respectively, the sections were incubated for 1 h in primary antibodies for CYP11B2 (mouse

Table 1 Characteristics of the ATP2B4 variants

(A) Rare gene variants in *ATP2B4*					
Gene Name	Nucleotide change	protein change	Frequency (EXAC)	Exon number	Transcript variant affected
ATP2B4	c.C3152A	p.T1051 N	0.0015	Exon 20	PMCA4a, PMCA4b
	cC3158T	p.S1053F	0.0081	Exon 20	PMCA4a, PMCA4b
	c.G3346A	p.E1116K	0.0004	Exon 21	PMCA4b only
(B). Clinical characteristic of BHA patients with rare *ATP2B4* variants					
Parameters	Patient 1	Patient 2	Patient 3	Patient 4	
ATP2B4 variant	c.C3152A	c.C3152A	c.G3346A	c.C3158T	
Serum K^+ (mEq/L)	3.2	4	3.4	3.4	
Aldosterone (ng/dL)	42.2	20.4	47	23.7	
PRA (ng/mL/h)	0.35	0.3	0.5	0.4	
ARR	120.5	68	94	59.3	
Confirmatory test	37 APR post captopril	10.9 ng/dL aldosterone post-saline load	11.2 ng/dL aldosterone post-saline load	12.1 ng/dL aldosterone post-saline load	
AVS	BHA	n/a	BHA	BHA	
Imaging					
Diagnosis	BHA	BHA	BHA	Micronodule 7 mm left adrenal	

monoclonal clone 41-17B; 1:100; kindly donated by Dr. Celso Gomez-Sanchez) and PMCA4b (mouse monoclonal clone JA9; Catalog# MA1–914, Millipore; 1:100). After three successive washes in 1X PBS-0.5% Tween (PBS-T), the sections were incubated Alexa Fluor® 647 goat anti-mouse antibody (1:100) for 1 h and with 100-nM DAPI for 5 mins. Following washes in 1X PBS-T, coverslips were mounted using the ProLong™ Gold Antifade Mountant without DAPI (Thermo Fisher Scientific). Sectioned treated with only secondary antibody served as negative controls. An H and E stained section of the adrenal was used to identify the zones.

RNA Isolation and Gene Expression Assays

RNA was extracted from tissue or cell lines using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), following manufacturer's recommendations for on-column DNase treatment (RNase-free DNase Kit, Qiagen). Using the High-Capacity cDNA Reverse Transcription Kit, 20 ng of RNA was reverse-transcribed (Thermo Fisher Scientific, Waltham, MA). Real-time quantitative PCR was performed for *ATP2B4* (Hs00608066_m1, Thermo Fisher Scientific) and CYP11B2 and normalized to PPIA (Hs99999904_m1, Thermo Fisher Scientific), as previously described [42]. Relative quantification of the data was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

All experiments were performed a minimum of three times. Results were expressed as mean ± S.E.M. Students t-tests or one-way ANOVA for multiple comparison was applied as required based on the specific groups being analyzed, using an alpha value of 5% and a power of 80%.

Results

Clinical Characteristics

Patient 1 presented with hypertension (Table 1), a serum K of 3.2 mEq/L, elevated aldosterone with suppressed renin (20.4 ng/dL aldosterone, plasma renin activity, i.e., PRA 0.35 ng/mL/h) and an aldosterone to renin ratio of 120.5. Diagnosis of PA was confirmed by the captopril test and bilateral aldosterone excess by adrenal vein sampling. Patient 2, the brother to Patient 1, was also diagnosed with BHA. Whole-exome sequencing identified a heterozygous germline variant c. 3152C > A (p.T1051 N) ($ATP2B4^{C3152A}$) in both patients, but not in unaffected family members (*Supplementary Figure, Panel 1A*). Additional rare variants ($ATP2B4^{C3158T}$ and $ATP2B4^{G3346A}$) in *ATP2B4* were identified in two patients with sporadic BHA (clinical details in Table 1). All variants occurred in the calmodulin (CaM)-binding domain of PMCA4. Additional characteristics of the observed variants including reported allelic frequencies and predicted effects of amino acid substitutions on protein function have been detailed in Supplementary Fig. 1, *Panel B.*

ATP2B4 Expression in Different Tissues

We initiated our studies by surveying for the differential expression of *ATP2B4* transcript levels across various

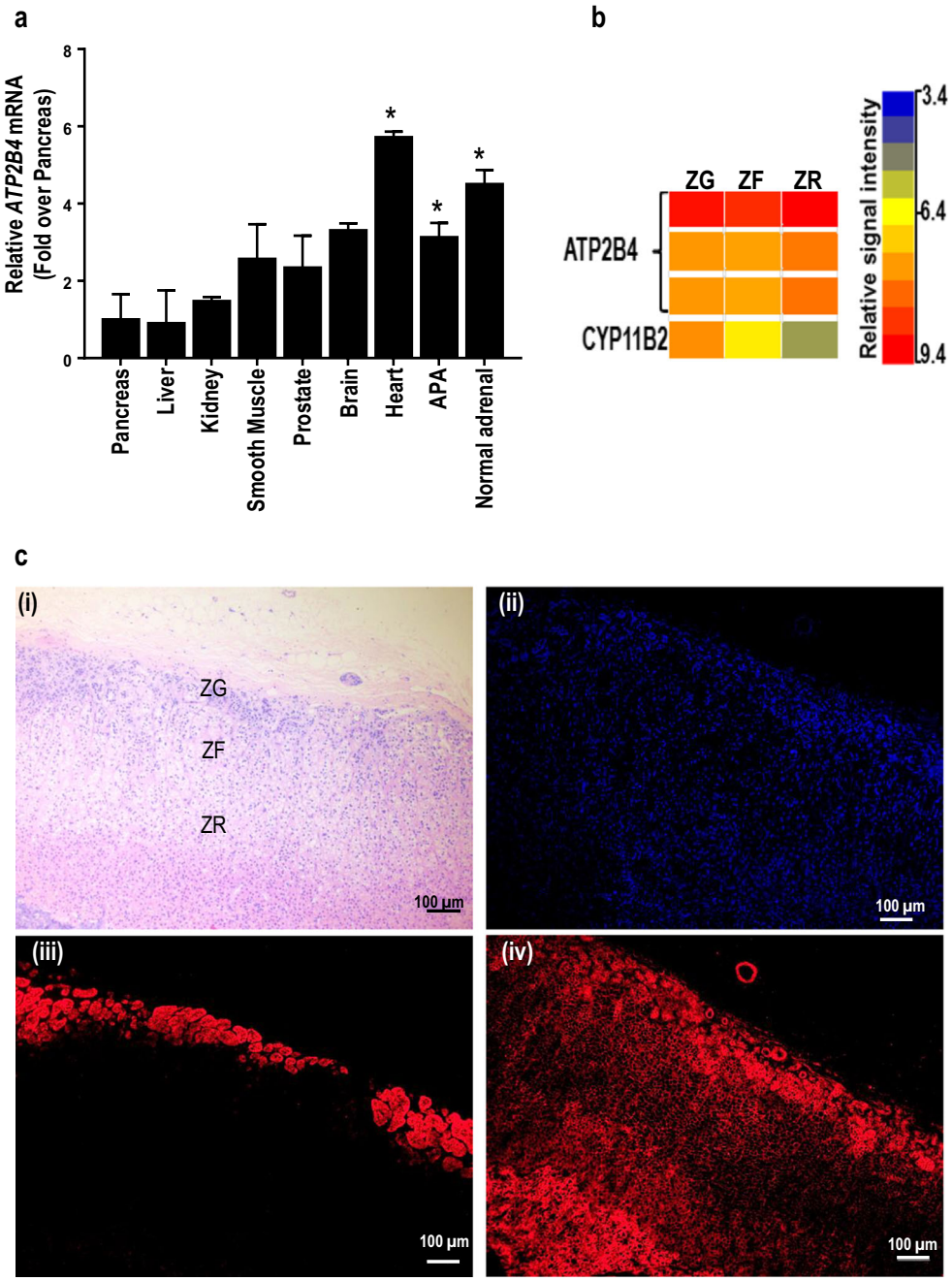

Fig. 1 Expression of *ATP2B4* in tissues. (**A**) Real-time analyses of *ATP2B4* transcript variant 2 in adrenal tissues in comparison with various tissues. $*p < 0.05$ versus pancreas. (**B**) Zonal expression of *ATP2B4* mRNA across the human adrenal using laser capture microdissection. The three probes correspond to 205410_s_at, 212136_at, 212135_s_at (top to bottom), which target the 3'UTR of both *ATP2B4* transcripts. (**C**) Immunofluorescence for PMCA4b protein on serial sections of the human adrenal cortex: (i) H&E, (ii) negative control incubated only with secondary antibody and DAPI, and (iii, iv) expression of CYP11B2 and PMCA4b, respectively

human tissues. Real-time quantitative PCR (RT-qPCR) analyses identified *ATP2B4* expression to be highest in the heart and healthy and pathological adrenal tissues (normal adrenals and APA) (Fig. 1, *Panel A*). Within the adrenal gland, the *ATP2B4* transcript was expressed throughout the cortex, without zone specificity (Fig. 1, *Panel B*). A similar pattern was observed for the ATP2B4 peptide, using immunofluorescence (Fig. 1, *Panel C*). Having confirmed the expression of *ATP2B4* in the adrenal and in aldosterone-producing adenomas, in vitro gene expression studies for functional analyses of the gene mutations were initiated.

Effect of *ATP2B4* Knockdown in HAC15 Cells

Since the variants in *ATP2B4* are clustered near the calcium-calmodulin domain involved in ATPase activity, we initiated studies that replicate null mutations through knockdown of the endogenous *ATP2B4* transcripts. Four clones of short hairpin RNA (shRNA) lentiviruses targeting *ATP2B4* were used to transduce the HAC15 cell line containing a CYP11B2 promoter-driven secreted Gaussian luciferase (HAC15-B2Luc) [43]. Non-targeted scrambled shRNA was used as a control. Real-time qPCR analyses confirmed significant, albeit not complete,

knockdown of endogenous *ATP2B4* transcript and protein levels (Fig. 2, *Panels A and B*). Knockdown of PMCA4b protein levels was also confirmed using western analysis. Among the four clones, only a single clone exhibited reduced angiotensin II (Ang II) stimulation of the *CYP11B2* promoter. *ATP2B4* knockdown did not affect basal or agonist (angiotensin II or K^+-stimulated *CYP11B2* promoter activity in the other clones (Fig. 2, *Panel C*).

Effect of *ATP2B4* Variants on Aldosterone Synthase (CYP11B2) Transcript Levels in HAC15 Cells

To determine the effects of the ATP2B4 variants in adrenal cell physiology in normal and agonist-induced conditions, the adrenocortical cell line HAC15 was transduced with doxycycline (dox)-inducible lentiviruses for wild-type (WT) or observed rare variants of *ATP2B4*. Dox treatment increased *ATP2B4* transcript levels by over 5-fold (Fig. 3, *Panel A*). Immunocytochemistry confirmed plasma membrane localization of the expressed protein (Fig. 3 *Panel B*). Expression of the variant $ATP2B4^{C3152A}$ did not increase basal CYP11B2 transcript levels. However, both WT and two of the rare variants ($ATP2B4^{C3158T}$ and $ATP2B4^{G3346A}$) did increase basal *CYP11B2* levels (Fig. 3, *Panel C*). Further detailed analyses including stimulation by agonists Ang II and K suggested a lack of effect of any of the two of the rare *ATP2B4* variants (Fig. 3, *Panel D*). On the other hand, cells expressing the $ATP2B4^{C3152A}$ variant displayed elevated CYP11B2 expression in K-stimulated conditions. As a positive control, dox-inducible wild-type *ATP2B3* ($ATP2B3^{WT}$) and a previously described pathogenic variant containing the deletion Leu425-Val426 ($ATP2B3^{\Delta425–426}$) [29] were expressed in HAC15 cells, using a similar strategy. This is the first ever study to develop and use a dox-inducible model for *ATP2B3* characterization. In this model system, incubation with dox induced *ATP2B3* transcript levels by ~fourfold (Fig. 3, *Panel E*). The $ATP2B3^{WT}$ variant decreased CYP11B2 transcript levels, in agreement with previous reports of reduced basal calcium levels in cells overexpressing the gene variant. Furthermore, $ATP2B3^{\Delta425–426}$ variant increased basal *CYP11B2* levels by ~15-fold, a more potent effect than earlier reports using transient transfection [29]. The stimulatory effects of agonists were also enhanced in cells expressing $ATP2B3^{\Delta425–426}$ compared to $ATP2B3^{WT}$ expressing adrenal cells (Fig. 3, *Panels F–H*).

Effect of *ATP2B4* Variants on Steroidogenesis

Following the observations of the effect of the *ATP2B4* variants on *CYP11B2* expression, we proceeded to evaluate downstream effects on steroid production in these cell lines. LC-MS/MS was used to measure the production of aldosterone and cortisol and that of hybrid steroids, 18-hydroxycortisol, and 18-oxocortisol. Basal aldosterone levels were slightly elevated (1.5 ± 0.12 fold, mean ± S.E.M, $p \leq 0.05$) in cells expressing

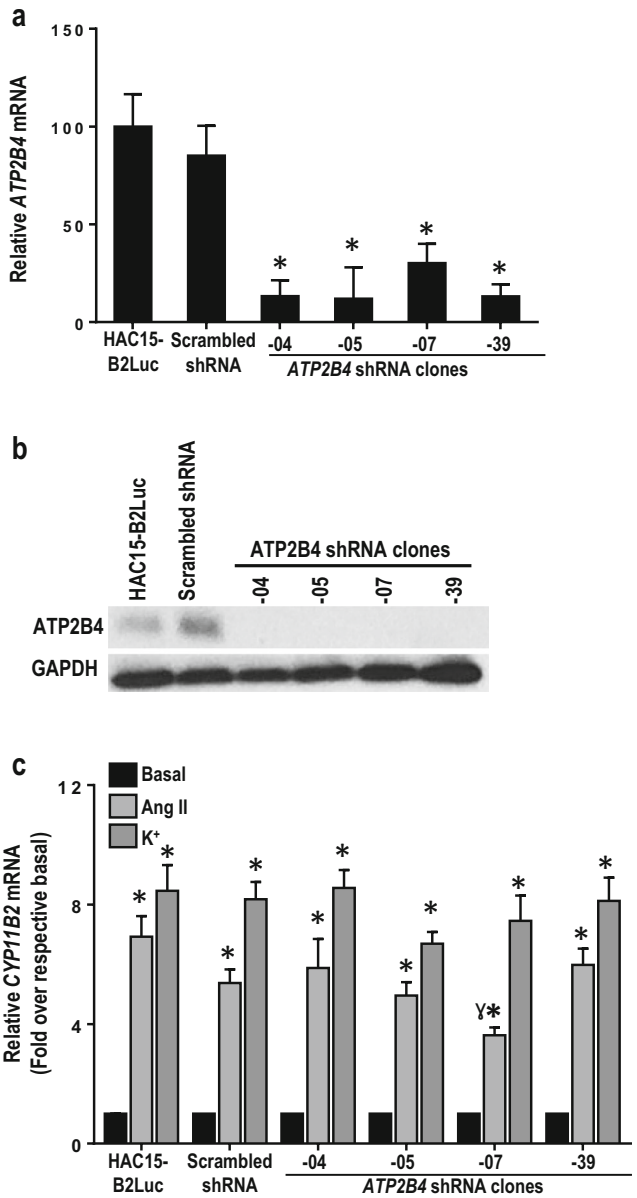

Fig. 2 Analysis of knockdown of *ATP2B4* in adrenal cells. (**A**, **B**) Real-time analysis demonstrating knockdown of *ATP2B4* mRNA and protein in four shRNA clones (− 04, − 05, − 07, and − 39) and controls (HAC15-B2Luc and scrambled shRNA). (**C**) Effect of *ATP2B4* shRNA on agonist stimulation. $^*p < 0.05$ versus basal; $^\gamma p < 0.05$ versus HAC15 Ang II stimulation

$ATP2B4^{WT}$, while they were unchanged for the variants $ATP2B4^{C3152A}$, $ATP2B4^{C3158T}$, and $ATP2B4^{G3346A}$ (Fig. 4, *Panel A*). Agonists Ang II and K^+, stimulated production of steroids (aldosterone, cortisol, 18-hydroxycortisol, and 18-oxocortisol), when compared to basal, remained unchanged in $ATP2B4^{WT}$ expressing cells or any of the cell lines expressing the rare *ATP2B4* variants (Fig. 4, *Panels B–D*). Basal and agonist-induced production of upstream Δ4 steroids, including progesterone, 17-hydroxyprogesterone, and corticosterone, was also evaluated and found to be unaltered (data not shown).

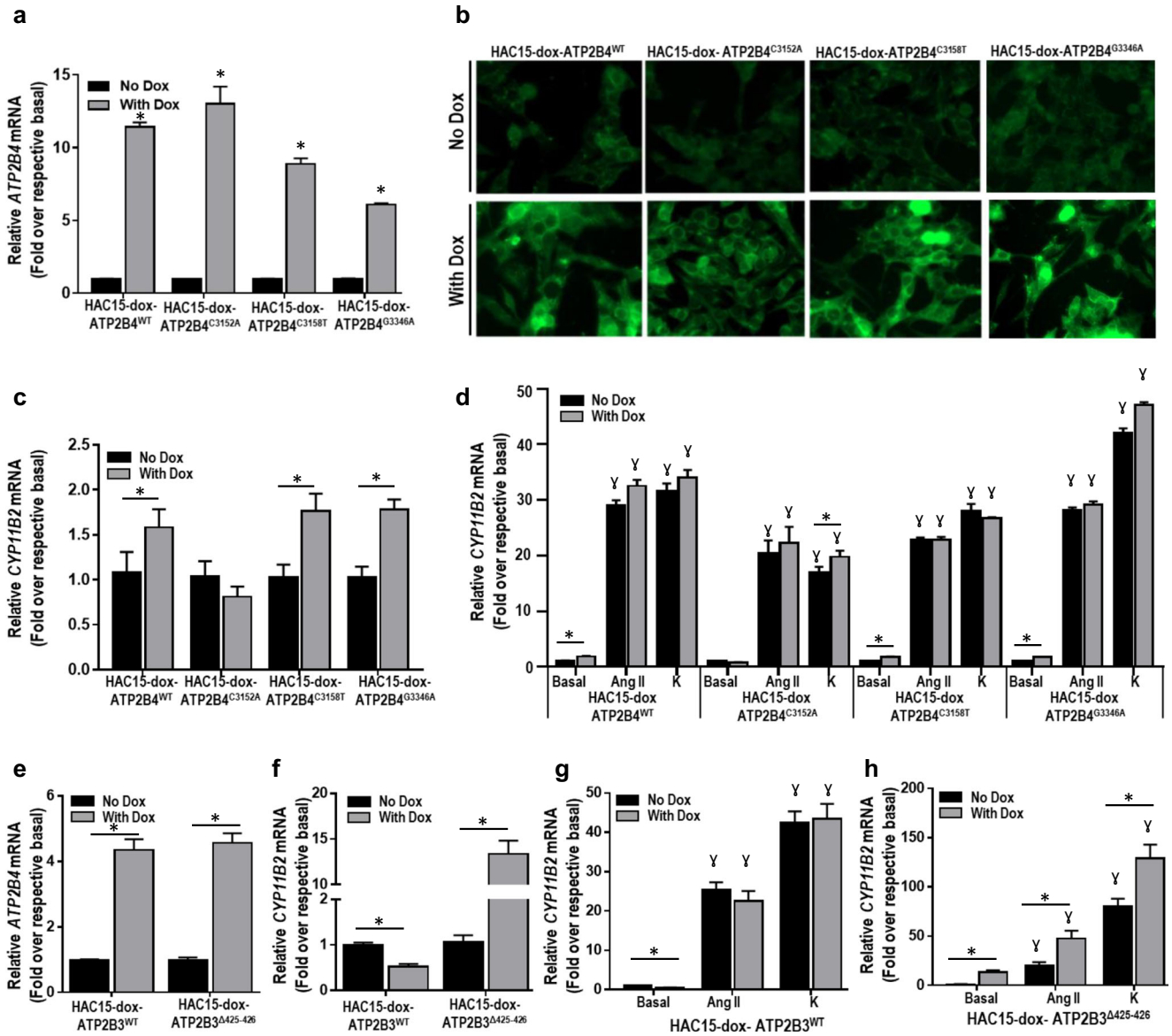

Fig. 3 Analysis of conditional expression of WT or variant *ATP2B4* in adrenal cells. (**A**, **B**) Real-time analysis demonstrating doxycycline-inducible expression of WT and variant *ATP2B4* forms (*ATP2B4*$^{WT/C3152A/C3158T/G3346A}$) mRNA and protein in the HAC15 adrenal cell line. (**C**, **D**) Comparison of the effect of *ATP2B4* variants (72 h incubation with 1-μg/mL dox) in basal and agonist-stimulated (12 h) conditions in HAC15 cells. (**E**) Real-time analysis demonstrating doxycycline-inducible expression of WT and mutant ATP2B3, mRNA as positive controls. (**F**–**H**) Comparison of the effect of ATP2B3^{WT} and ATP2B3$^{\Delta425–426}$ (72 h incubation with 1-μg/mL dox) in basal and agonist-stimulated (12 h) conditions in HAC15 cells. $^{*}p < 0.05$ versus respective treatment without dox; $^{\gamma}p < 0.05$ versus respective basal

Electrophysiological Analyses of *ATP2B4* Variants

Alongside of the effects of WT and variant forms of *ATP2B4* on steroidogenic processes, changes in the biophysical properties of *ATP2B4* variants were also determined. A voltage-step protocol was used on non-transduced HAC15 cells and those expressing the ATP2B4^{WT} or the ATP2B4 variants (Fig. 5, *Panels A and B)*. The endogenous ATP2B4 current in HAC15 cells was very robust, ensuring a functional endogenous *ATP2B4* activity. HAC15 cells expressing increased ATP2B4^{WT} had lower current density, similar to observations in cells expressing ATP2B3^{WT} [29]. Similarly, the density of the current rare variants was identical to that of ATP2B4^{WT}. The resting membrane potential of adrenal cells expressing the variant forms of ATP2B4 was unchanged.

Discussion

Over the last decade, our understanding of the contribution of somatic genetic changes to APA-related PA has significantly increased. Research on the causes of BHA, however, has been

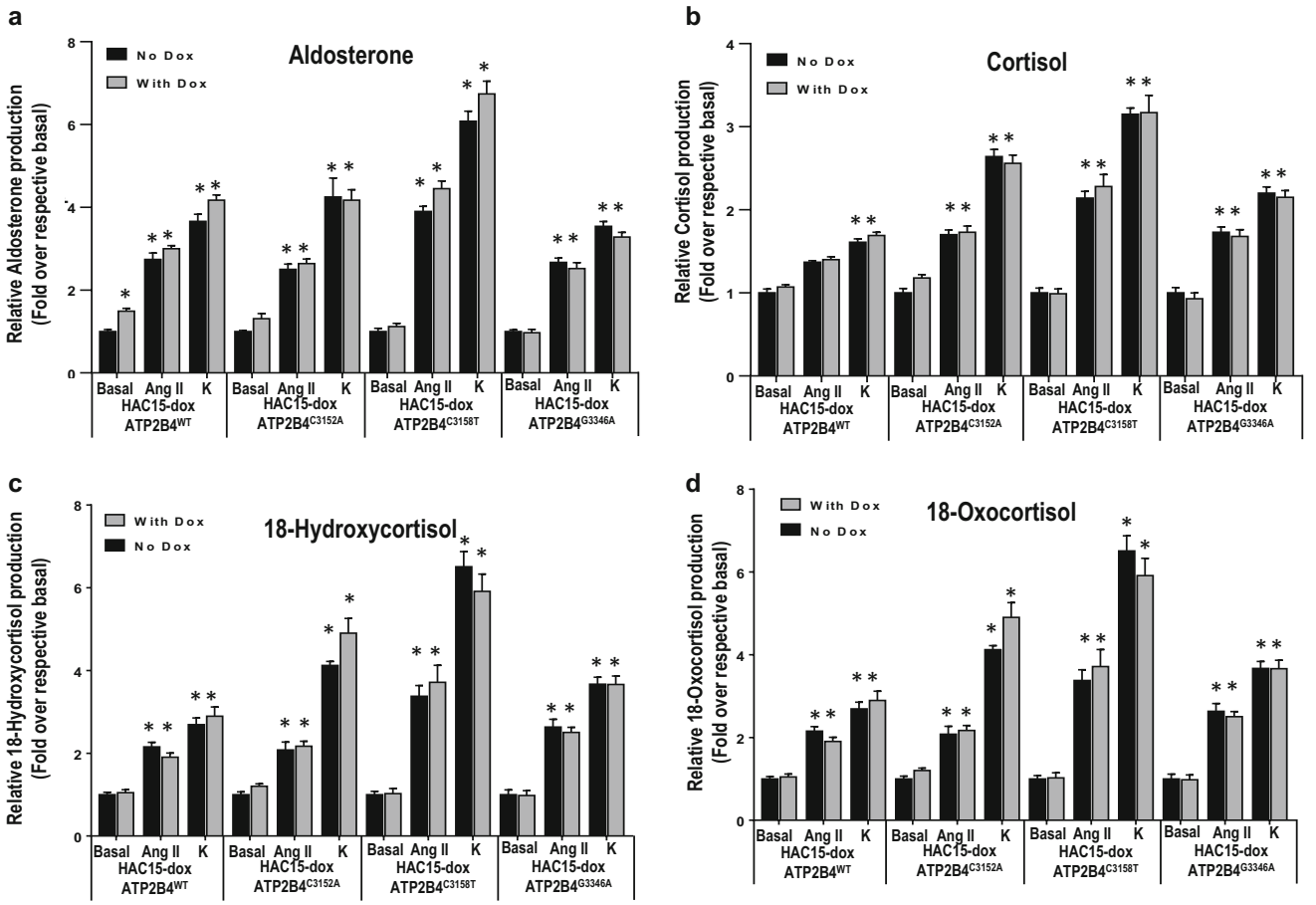

Fig. 4 Effect of WT and variant *ATP2B4* forms on steroid production on HAC15 cells. LC-MS/MS was used to determine the effect of elevated *ATP2B4* variants (*ATP2B4*$^{WT/C3152A/C3158T/G3346A}$) on steroid production in HAC15 adrenal cells. Amount of steroid was calculated in pg/mL and normalized to amount of protein per well. Means ± S.E.M were calculated as fold over respective basals. *$p < 0.05$ versus basals in respective cell lines

stymied by the very limited tissue availability due to mainly pharmacologic treatment of bilateral disease.

Herein, we used exome sequencing of germline DNA obtained from patients with BHA in an attempt to identify potential pathogenic gene variants. We focused on three rare variants in *ATP2B4*, which encodes the Ca^{2+} ATPase pump PMCA4. The mechanistic characterization of the variants involved the knockdown of endogenous *ATP2B4* as well as expression of wild-type and rare *ATP2B4* variants in basal and agonist-stimulated conditions in the human adrenocortical carcinoma cell line (HAC15). The *ATP2B4*^{C3152A} did not affect CYP11B2 transcript levels or the production of steroids, aldosterone, cortisol, 18-hydroxycortisol, or 18-oxocortisol, in basal conditions, although a minor increase in K^+-stimulated aldosterone has been observed. The *ATP2B4*^{C3158T} and *ATP2B4*^{G3346A} variants increased basal *CYP11B2* mRNA levels, although not more than the *ATP2B4*^{WT} transgene itself and far less than an *ATP2B3* variant observed in APAs that served as a positive control. No changes in current density were observed using electrophysiological analysis. Parallel analysis of a disease-causing variant in *ATP2B3* displayed dramatic increases of *CYP11B2* expression, making it unlikely that the subtle changes observed with *ATP2B4* variants are of pathophysiological relevance. Thus, overall, an overt pathogenic role with large increases in aldosterone production was excluded for the *ATP2B4* variants.

We cannot, however, exclude the possibility that the *ATP2B4* variants act in conjunction with other genes or environmental factors that affect aldosterone production. Indeed, the challenge in finding new genetic causes of PA is that any new gene harboring potential pathogenic variants might be associated with low disease penetrance. An example of this phenomenon can be seen in the recent study investigating the role of several unique hereditary *CLCN2* gene variants, where at least one large kindred (*CLCN2* ^{Arg172Gln}) showed incomplete penetrance [20]. Similarly, in vitro expression of several *CLCN2* variants (p.Gly24Asp, p.Tyr26Asn, p.Met22Lys, p.Ser865Arg, p.Arg172Gln) showed great variation of their effects on *CYP11B2* expression [19, 20]. Certainly, the incomplete penetrance could potentially be attributed, at least partially, to genomic and epigenomic changes acting as modifiers.

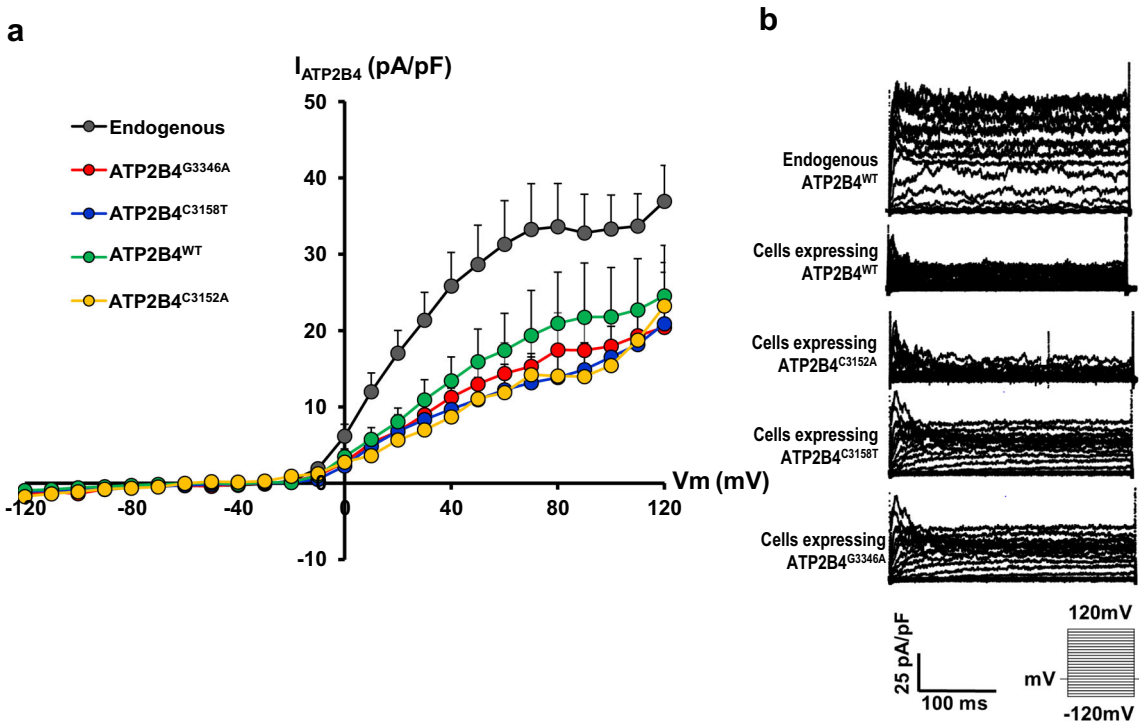

Fig. 5 Electrophysiological analyses of WT or variant *ATP2B4* in HAC15 cells. IV plots (**A**) and traces (**B**) for HAC15 cells expressing endogenous ATP2B4, *ATP2B4*^{WT}, *ATP2B4*^{C3152A}, *ATP2B4*^{C3158T}, and *ATP2B4*^{G3346A} ($n = 7–10$ for all variants). Internal and external solutions and protocols followed were as described in the Materials and Methods section

The hypothesis of a germline genetic predisposition to PA in BHA is based on the observation of bilateral renin-independent aldosterone production. However, there is also the possibility that a yet to be identified environmental or other pathogenic mechanism contributes to the phenomenon of BHA. To this extent, a recent study showed different rare variants in *CACNA1D* in different aldosterone-producing cell clusters found in the same adrenal gland from BHA patients [45]. It remains to be shown, whether these multiple somatic mutations could occur by chance or due to an underlying genetic predisposition.

In summary, while our study did not identify a pathogenic role of the *ATP2B4* variants, we focused on developing a pipeline for the detailed analyses of gene variants. We successfully demonstrated an efficient lentiviral approach for tight dox-inducible gene expression and the assessment of rare gene variants. Certainly, this system exhibited a remarkable improvement in the effects of the PA-causing *ATP2B3* mutation as compared to previous reports [29]. Further, we describe a thorough investigation of these variants including (1) characterization of gene expression across tissues, (2) knock-down analyses of an inactivating gene variant, (3) expression of the wild-type and rare gene variant to define their effects on *CYP11B2* transcript levels and steroidogenesis by LC-MS/MS, and (4) electrophysiological analyses of the biophysical properties of the variants in relation to the wild-type protein. Finally, we would like to emphasize the need to publish not only proven pathogenic genetic causes of BHA but also analysis of rare genetic variants, for which experimental data argues against an involvement in the pathogenesis of the disease, even if it is simply to avoid a double effort in the scientific community.

Acknowledgments We would like to acknowledge Dr. Celso Gomez-Sanchez for his advice on sub-cloning strategies and Dr. Sasha Bandulik for generously sharing expression vectors for ATP2B3^{WT} and ATP2B3$^{\Delta 425-426}$.

Funding Information This work was supported by the American Heart Association Postdoctoral Fellowship (16POST29900003) awarded to Namita G. Hattangady and a grant from the NHLBI (5R01HL130106) awarded to Dr. Tobias Else.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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