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ORGAN PHYSIOLOGY

Connexin 40 is dispensable for vascular renin cell recruitment but is indispensable for vascular baroreceptor control of renin secretion

Katharina Machura · Bjoern Neubauer · Hanna Müller · Philipp Tauber · Armin Kurtz · Lisa Kurtz

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Abstract Defects of the gap junction protein connexin 40 (Cx40) in renin-secreting cells (RSCs) of the kidney lead to a shift of the localization of RSCs from the media layer of afferent arterioles to the periglomerular interstitium. The dislocation of RSCs goes in parallel with elevated plasma renin levels, impaired pressure control of renin secretion, and hypertension. The reasons for the extravascular shift of RSCs and the blunted pressure regulation of renin secretion caused by the absence of Cx40 are still unclear. We have therefore addressed the question if Cx40 is essential for the metaplastic transformation of preglomerular vascular smooth muscle cells (SMCs) into RSCs and if Cx40 is essential for the pressure control of renin secretion from RSCs located in the media layer of afferent arterioles. For our study, we used mice lacking the angiotensin II type 1A (AT_{1A}) receptors, which display a prominent and reversible salt-sensitive metaplastic transformation of SMCs into RSCs. This mouse line was crossed with Cx40-deficient mice to obtain AT_{1A} and Cx40 double deleted mice. The kidneys of $AT_{1A}^{-/-}Cx40^{-/-}$ mice kept on normal salt (0.3 %) displayed RSCs both in the inner media layer of preglomerular vessels and in the periglomerular interstitium. In contrast to hypotensive $AT_{1A}^{-/-}$ (mean bp syst 112 mmHg) and hypertensive $Cx40^{-/-}$ (mean bp syst 160 mmHg) mice $AT_{1A}^{-/-}Cx40^{-/-}$ mice were normotensive(mean bp syst 130 mmHg). Pressure regulation of renin secretion from isolated kidneys was normal in $AT_{1A}^{-/-}$ mice, but was absent in $AT_{1A}^{-/-}Cx40^{-/-}$ mice alike in $Cx40^{-/-}$ mice. Low-salt diet (0.02 %) increased RSC numbers in the media layer, whilst high-salt diet (4 %) caused disappearance of RSCs in the media layer but not in the periglomerular interstitium. Blood pressure was clearly salt sensitive both in $AT_{1A}^{-/-}$ and in $AT_{1A}^{-/-}Cx40^{-/-}$ mice but was shifted to higher pressure values in the latter genotype. Our data indicate that Cx40 is not a requirement for intramural vascular localization of RSCs nor for reversible metaplastic transformation of SMCs into RSCs. Therefore, the ectopic localization of RSCs in $Cx40^{-/-}$ kidneys is more likely due to a disturbed intercellular communication rather than being the result of chronic overactivation of the renin-angiotensin-aldosterone system or hypertension. Moreover, our findings suggest that Cx40 is a requirement for the pressure control of renin secretion irrespective of the localization of RSCs.

Keywords Connexin 40 · Renin secretion · Baroreceptor control · Renin-angiotensin-aldosterone system

K. Machura (✉) · B. Neubauer · H. Müller · P. Tauber · A. Kurtz
Institut für Physiologie, Universität Regensburg, 93053 Regensburg, Germany
e-mail: katharina.machura@vkl.uni-regensburg.de

L. Kurtz
Department of Internal Medicine, University of Regensburg, Regensburg, Germany

Introduction

Renin-secreting cells (RSCs) of the kidneys are typically located in the terminal portions of afferent arterioles at juxtaglomerular position, and they regulate renin secretion in response to blood pressure, adrenergic neurotransmitters, and a variety of hormones and autacoids [14]. They display a strong expression of the gap junction protein connexin 40 (Cx40) [15, 16]. In states of renin cell recruitment as it occurs in chronic threats of volume and of blood pressure homeostasis, additional smooth muscle cells (SMCs) of preglomerular arterioles transform into RSCs. This metaplastic transformation of SMCs is associated with an induction of Cx40 expression and downregulation of connexin 45 [13]. Cx40-gap junctions couple RSCs among each other and their neighbors, such as endothelial, mesangial, and SMCs [28]. Loss of function defects of Cx40 in RSCs leads to a translocation of RSCs

from the media layer of afferent arterioles into the periglomerular interstitium [17] and an enhancement of renin secretion due to interruption of the negative pressure control leading to hypertension [3, 12, 30]. The reasons for this particular relevance of Cx40 for the localization and function of RSCs are yet unknown.

The disappearance of RSCs from the smooth muscle cell layer may reflect an essential requirement of Cx40 for RSCs to integrate into the vessel wall. It may also be secondarily caused by the persistent hypertension and/or chronically increased levels of angiotensin II in states of defective Cx40. The missing pressure control of renin secretion in the absence of Cx40, leading to renin hypersecretion and hypertension, may simply result from the inability of extramural cells to sense the intravascular pressure.

We were therefore interested to see if preglomerular SMCs can transform into RSCs and exist in the media layer in the absence of Cx40. If so, we further aimed to find out if the cells located in the media layer of preglomerular vessels but lacking Cx40 show a normal or an altered secretory response to the perfusion pressure.

To address these questions, we aimed to examine mice lacking angiotensin II type 1A ($AT_{1A}^{-/-}$) receptor [22] with intact or with deleted Cx40 ($Cx40^{-/-}$). There were several reasons to use $AT_{1A}^{-/-}$ mice for this study. Firstly, they show a prominent vascular recruitment of RSCs, which can be triggered by low-salt intake [21]. Secondly, they have an ineffective renin-angiotensin-aldosterone system (RAAS), which should counteract effects of renin hypersecretion on the RAAS seen in Cx40-defective mice [21, 28, 29]. Thirdly, $AT_{1A}^{-/-}Cx40^{-/-}$ mice are expected to have lower blood pressure than Cx40-deficient mice, if the hypertension induced by Cx40 deletion is causally mediated by activation of the RAAS.

Materials and methods

Animals AT_{1A} and Cx40 double knockout ($AT_{1A}^{-/-}Cx40^{-/-}$) mice were generated by crossing Cx40-deficient ($Cx40^{-/-}$) mice of C57BL/6 genetic background which are originally generated by Kirchhoff et al. [10] and obtained from K. Willecke (Institute of Genetics, University of Bonn, Germany) and angiotensin II-AT_{1A} receptor-deficient ($AT_{1A}^{-/-}$) mice of 129/Sv background, provided by T. M. Coffman [8]. Wild-type (wt) mice were obtained from the F2 generations of $AT_{1A}^{-/-}Cx40^{-/-}$ mice and C57BL/6-129/Sv breedings. For all experiments, female 16- to 24-weeks-old mice were used. Genotyping was performed by PCR on DNA isolated from tail biopsies using the following primers: Cx40-1 5′-gggagatgagcaggccgacttccggtgcg-3′; Cx40-2 5′-gtagggtgccctggacaatcttccc-3′; Cx40-3 5′-ggatcggccattgaacaagatggattgcac-3′; Cx40-4 5′-ctgatgctcttcgtccagatcatcctgatcg-3′. Primers 1 and 2 amplify a 494-bp fragment from the Cx40 knockout allele. Primers 3 and 4 amplify a 314-bp fragment from the Cx40 wt allele. AT_{1A}-1 5′-tgagaacaccaatctcactg-3′; AT_{1A}-2 5′-ttcgtagacaggcttgag-3′; AT_{1A}-3 5′-ccttctatcgccttcttgacg-3′ (wild-type band, 483 bp; mutant band, 520 bp). All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. For changes in oral salt intake, a group of six female mice received a low-salt diet (0.02 % *w*/*w* NaCl; Ssniff Spezialdiäten, Soest, Germany) or a high-salt diet (4 % *w*/*w* NaCl) for 10 days, while a control group (n=10) obtained a standard rodent chow (0.3 % *w*/*w* NaCl).

Blood pressure measurements Measurements of the systolic arterial pressure were performed noninvasively by tail cuff manometry (TSE Systems, Germany). Therefore, the blood pressure of conscious mice was measured in a 37 °C warm box at the same time of the day. Before the first blood pressure determination, the animals were habituated to the experiment procedure by placing them into the holding device on 5–7 subsequent days for 15 min per day. On 10 sequential days, 5 measures per day were analyzed. The mean value per day and mouse was used for calculation.

Determination of renin mRNA expression by real-time PCR Animals were anesthetized by i.p. injection of ketamine (80 mg/kg body weight; bela-pharm, Germany) and xylazine (12 mg/kg body weight; Serumwerk, Germany). After ligation of the left renal artery, the kidney was removed, frozen in liquid N_2, and stored for mRNA analysis. Total RNA was isolated from frozen kidneys as described by Chomczynski and Sacchi [2] and quantified by a photometer. The cDNA was synthesized by MMLV reverse transcriptase (Superscript; Invitrogen). For quantification of renin mRNA expression, real-time PCR was performed using a LightCycler 480 Instrument (Roche Diagnostics Corp.) and the LightCycler SYBR Green I Master kit (Roche Diagnostics Corp.) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. For amplification of mouse renin and GAPDH cDNAs, the following primers were used: renin-1 5′-atgaaggggtgtctgtggggtc-3′; renin-2 5′-atgcggggagggtgggcacctg-3′; GAPDH-1 5′-atgccatcactgccacccagaag-3′; GAPDH-2 5′-acttggcaggtttctccaggcgg-3′. The primers used to analyze the Cx37, Cx40, Cx43, and Cx45 mRNA expression were previously described [15].

Immunohistochemistry and 3D reconstruction For immunohistochemistry and 3D reconstruction, the right kidney was perfused with 40 ml of 3 % paraformaldehyde solution in phosphate buffered saline (pH 7, 4) at a constant perfusion

pressure of 120 mmHg through the abdominal aorta. After dehydration and embedding in paraffin, 5-μm-thick serial slices were stained for renin and α-SMA. The deparaffinized kidney sections were blocked with 10 % horse serum, 1 % BSA in PBS, incubated with anti-renin (1:400, generated by Davids Biotechnologie, Germany) and anti-α-SMA (1:600, Abcam, Cambridge) antibodies overnight at 4 °C, followed by incubation with fluorescent secondary antibodies. Slices were mounted with DakoCytomation Glycergel mounting medium. The digitalization of the antibody-stained serial sections (40–60) was performed using an AxioCam MR3 camera mounted on an Axiovert 200M microscope with fluorescence filters (TRITC, filter set 43; Cy2, filter set 38; Zeiss, Germany). The resulting image sequence was converted to an image stack with constant picture size with the graphics software ImageJ (Wayne Rasband; NIH, Bethesda, MD). The equalized data were imported into Amira 5.6 software (FEI Visualization Group, Berlin, Germany) for subsequent generation of aligned grayscale stacks of renin and α-SMA. After correct scaling and alignment, renin and α-SMA were labeled and assigned to different defined materials. Finally, the surfaces were computed from the material data, resulting in a 3D model of α-SMA and renin. All shown surfaces base upon these segments.

Volume calculations Renin-immunoreactive volume per glomerulus was calculated by Amira 5.6. from 3D kidney reconstructions of three animals per experimental group containing seven to ten glomeruli each. Data is expressed in cubic micrometers.

Isolated perfused mouse kidney The isolated perfused mouse kidney model has been described in detail elsewhere [7]. Briefly, the kidneys were perfused ex situ with a modified Krebs-Henseleit solution [25] supplemented with 6 g/100 ml bovine serum albumin and human red blood cells (10 % hematocrit) at a constant pressure of 90 mmHg. Using an electronic feedback control, perfusion pressure could be changed stepwise in a pressure range between 40 and 140 mmHg to assess the pressure control of renin secretion. Chemicals to be tested for their effects on renin secretion were prepared as stock solutions and infused into the arterial limb of the system. For the determination of renin secretion rates (RSR), three samples of the venous effluent were taken every 2 min. Renin activity in the venous effluent was measured by radioimmunoassay (RENCTK; DiaSorin, Germany) as described [5, 7]. RSR is denoted as perfusate flow ($ml/g_{organ\ weight} \times min^{-1}$) multiplied by renin activity ($ng_{ANG\ I}/ml \times h^{-1}$) of the perfusate.

Aldosterone and renin measurements in plasma samples Blood samples from mice were taken by facial vein puncture and collected in EDTA-coated capillaries. Plasma was separated by centrifugation in a hematocrit centrifuge and stored at −20 °C until measurements. A specific ELISA from IBL (Hamburg, Germany) was used to determine the concentration of aldosterone in appropriate dilutions of plasma. For determination of plasma renin concentration, plasma samples were incubated for 1.5 h at 37 °C with plasma from bilaterally nephrectomized male rats as renin substrate. The generated angiotensin I ($ng/ml \times h^{-1}$) was determined by radioimmunoassay (RENCTK; DiaSorin, Germany).

Statistical analysis All values are presented as mean±SE. Differences between experimental groups were calculated by analysis of variance (ANOVA) and Bonferroni's adjustment for multiple comparisons. *P* values less than 0.05 were considered statistically significant unless otherwise specified. Prism 5.0 software (GraphPad, San Diego, CA) was used for all calculations.

Results

Basic characterization of mice

Basic data of the four genotypes of mice used in this study are summarized in Table 1. Kidney weights were not different between the genotypes. Whilst heart weights were increased in Cx40-deficient mice, heart weights were normal in $AT_{1A}^{-/-}Cx40^{-/-}$ mice. Plasma renin concentration was increased in $Cx40^{-/-}$ mice and even more in $AT_{1A}^{-/-}$ mice. The plasma renin concentration of $AT_{1A}^{-/-}Cx40^{-/-}$ mice was between those of $Cx40^{-/-}$ and $AT_{1A}^{-/-}$ mice. Plasma aldosterone concentration was increased in $Cx40^{-/-}$ mice only among the four genotypes under investigation. Renal connexin 40 mRNA was increased in $AT_{1A}^{-/-}$ mice (Table 2). Renal connexin 37, 43, and 45 mRNA levels were not different between the four genotypes under investigation (Table 2).

Intrarenal localization of RSCs in $AT_{1A}^{-/-}Cx40^{-/-}$ mice on a normal salt diet

In wild-type mice kept on a normal salt diet, RSCs were restricted to the classical juxtaglomerular position, where RSCs continued the media layer of afferent arterioles into the glomeruli (Fig. 1a). Virtually every glomerulus had a few cubic-shaped RSCs at its vascular pole (Fig. 2a). In Cx40-deficient kidneys, RSCs showed a more variable distribution pattern with regard to localization and numbers (Fig. 1b). RSCs were localized outside the arteriolar vessel wall and showed a more irregular circumference. Some of the cells extended into the peritubular/periglomerular interstitium. Virtually all RSCs were associated with glomeruli, however, with interglomerular differences in the number of RSCs per glomerulus (Fig. 2b). The kidneys from $AT_{1A}^{-/-}$ mice showed

Table 1 Weight index of kidneys and hearts ($mg_{organ\ weight}$ per $g_{body\ weight}$), plasma renin concentration (PRC), and plasma aldosterone concentration (PAC) of wild-type (wt), $Cx40^{-/-}$, $AT_{1A}^{-/-}$, and $AT_{1A}^{-/-}Cx40^{-/-}$ mice

Group	*n*	Kidney weight (mg/g)	Cardiac weight (mg/g)	PRC ($ng_{ANGI}/ml \times h^{-1}$)	PAC (pg/ml)
wt	6	5.4±0.2	5.0±0.2	136.9±17.8	111.3±14.9
$Cx40^{-/-}$	5	5.8±0.3	7.8±0.8*	551.1±86.5	261.0±68.2*
$AT_{1A}^{-/-}$	6	5.3±0.2	4.6±0.2	1683.0±430.2*	143.3±20.9
$AT_{1A}^{-/-}Cx40^{-/-}$	7	5.4±0.4	5.6±0.5	839.2±133.9	116.9±23.8

Values are expressed as mean±SE

n number of mice

*$P<0.01$ vs wt control

an increased number of RSCs, which were almost exclusively localized in the vascular walls (Fig. 1c). RSCs were found over the whole length of afferent arterioles, sometimes also in the walls of interlobular arteries (Fig. 2c). In the kidneys of $AT_{1A}^{-/-}Cx40^{-/-}$ mice, a mixed distribution pattern of RSCs was found. It was an assembly of RSCs which were localized inside the vessel wall in the media layer of afferent arterioles as in $AT_{1A}^{-/-}$ mice and cells of a more irregular shape which were situated outside the vessel wall, similar to the findings in $Cx40^{-/-}$ kidneys (Fig. 1d). Under normal salt diet, RSCs were localized ectopically as well as in correct intramural position. In $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, RSCs were more restricted to the juxta/periglomerular areas and did not spread over the whole length of afferent arterioles such as those in $AT_{1A}^{-/-}$ kidneys (Fig. 2d).

The quantitative impression of renin expression derived from the 3D reconstructions (Fig. 2) was corroborated by calculations of renin-immunoreactive volumes from the 3D reconstructions. Those calculations revealed 5-fold and 3-fold increases of renin-immunoreactive volumes in $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice, respectively (Fig. 3a), whilst the renin-immunoreactive volume in $Cx40^{-/-}$ mice was not significantly changed. Renin mRNA abundance was doubled in $Cx40^{-/-}$ mice and was increased 6- and 3-fold in $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice, respectively (Fig. 3b). Plasma renin concentrations paralleled the changes of renin mRNA (Table 1).

Table 2 Renal expression of Cx37, Cx40, Cx43, and Cx45 (Cx-mRNA/GAPDH-mRNA) of wild-type (wt), $Cx40^{-/-}$, $AT_{1A}^{-/-}$, and $AT_{1A}^{-/-}Cx40^{-/-}$ mice

Group	*n*	Cx37	Cx40	Cx43	Cx45
wt	10	1.00±0.07	1.00±0.11	1.00±0.05	1.00±0.08
$Cx40^{-/-}$	7	0.94±0.09	ND	1.03±0.09	1.34±0.14
$AT_{1A}^{-/-}$	7	1.03±0.06	3.87±0.57*	1.29±0.12	1.16±0.05
$AT_{1A}^{-/-}Cx40^{-/-}$	10	0.95±0.06	ND	0.94±0.15	1.04±0.14

Values are shown as x-folds of wt control and are expressed as mean±SE

ND not detectable, *n* number of mice

*$P<0.01$ vs wt control

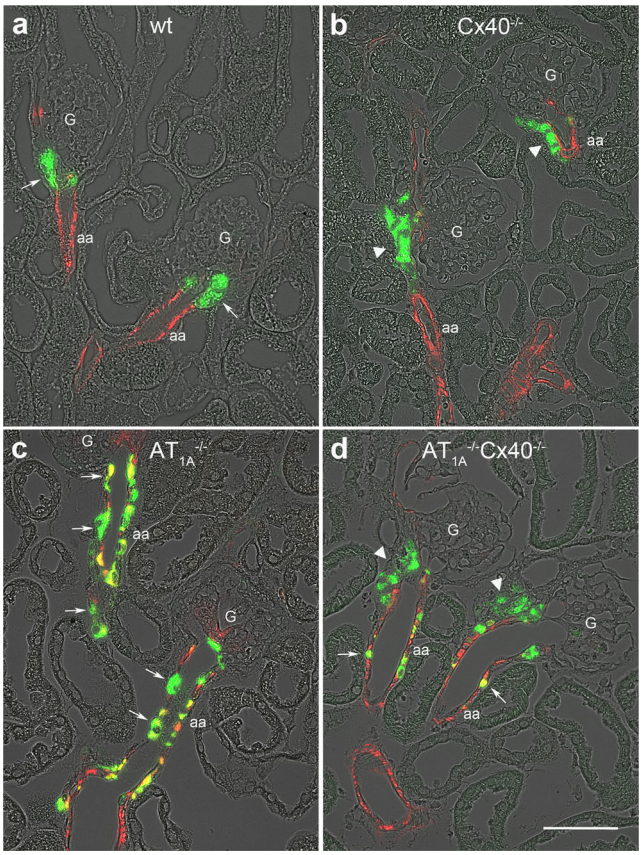

Fig. 1 Representative immunohistochemical stainings of renin (*green*) and α-smooth muscle actin (α-SMA, *red*) in kidneys under normal salt conditions of wild-type (*wt*) (**a**), $Cx40^{-/-}$ (**b**), $AT_{1A}^{-/-}$ (**c**), and $AT_{1A}^{-/-}Cx40^{-/-}$ (**d**) mice. All figures show afferent arterioles (*aa*), RSCs, glomeruli (*G*), and tubules (*not marked*). In wt kidneys, RSCs continue the media layer (α-SMA positive) of afferent arterioles at the glomerular vascular pole (*a*, *arrows*). In $Cx40^{-/-}$ kidneys, RSCs are located outside the media layer of afferent arterioles in the periglomerular area (*b*, *arrowheads*). In $AT_{1A}^{-/-}$ kidneys, RSCs are located in the media layer of afferent arterioles (*c*, *arrows*). *Yellow color* indicates overlay of renin and α-SMA immunoreactivity. In $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, RSCs are located both within the media layer of afferent arterioles (*d*, *arrows*) and outside in the periglomerular interstitium (*d*, *arrowheads*). *Scale bar*=50 μm

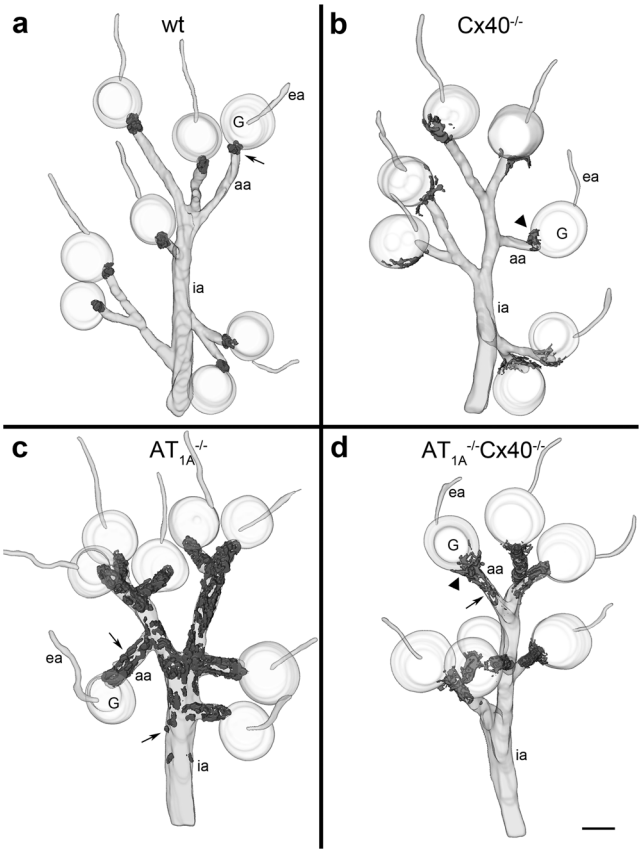

Fig. 2 3D reconstructions of renin-immunoreactive areas (*dark gray*) and α-smooth muscle actin (α-SMA, *light gray*) in kidneys under normal salt conditions of wild-type (*wt*) (**a**), $Cx40^{-/-}$ (**b**), $AT_{1a}^{-/-}$ (**c**), and $AT_{1A}^{-/-}Cx40^{-/-}$ (**d**) mice. 3D reconstructions show interlobular arteries (*ia*), afferent arterioles (*aa*), glomeruli (*G*), and efferent arterioles (*ea*). The pictures indicate the classic distribution pattern with RSCs conferred to the juxtaglomerular apparatus in wt mice (*a*, *arrow*) and dislocation of RSCs to the periglomerular interstitium in $Cx40^{-/-}$ mice (*b*, *arrowhead*). $AT_{1A}^{-/-}$ mice displayed a marked increase in the number of RSCs in juxtaglomerular areas and along afferent arterioles and interlobular arteries (*c*, *arrows*), whereas $AT_{1A}^{-/-}Cx40^{-/-}$ mice showed RSCs along afferent arterioles (*d*, *arrow*) and in the periglomerular interstitium (*d*, *arrowhead*). *Scale bar*=50 μm

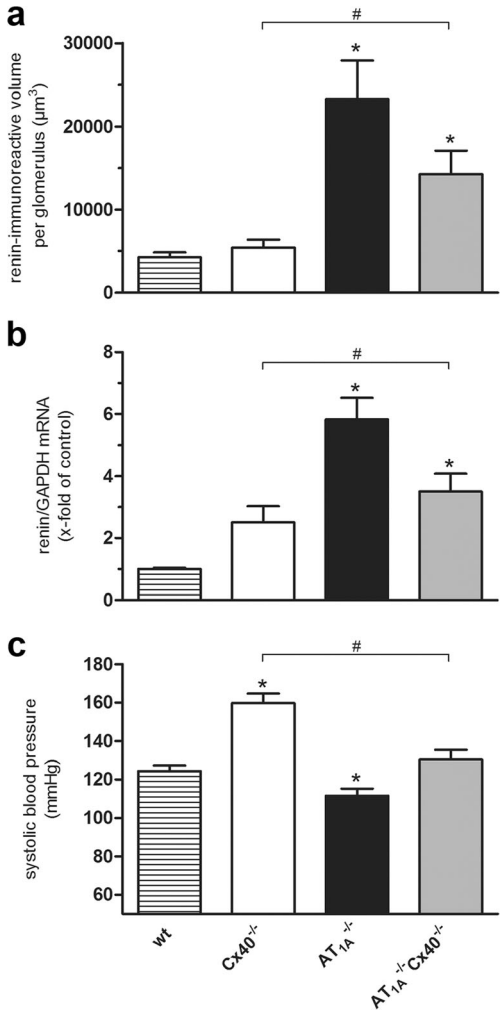

Fig. 3 **a** Renin-immunoreactive volume per glomerulus of wild-type (wt), $Cx40^{-/-}$, $AT_{1A}^{-/-}$, and $AT_{1A}^{-/-}Cx40^{-/-}$ mice under normal salt conditions calculated from 3D reconstructions (7 to 10 glomeruli analyzed from 3 kidneys per group). **b** Renin mRNA abundance in the kidneys of $Cx40^{-/-}$ (n=7), $AT_{1A}^{-/-}$ (n=7), and $AT_{1A}^{-/-}Cx40^{-/-}$ (n=10) mice under normal salt conditions compared to wild-type (wt; n=10) mice. **c** Systolic blood pressure of wild-type (wt), $Cx40^{-/-}$, $AT_{1A}^{-/-}$, and $AT_{1A}^{-/-}Cx40^{-/-}$ mice (5 animals per group). All data are mean±SE. *P<0.05 vs wt control, #P<0.05 (ANOVA) between the indicated groups

Recruitment of RSCs in $AT_{1A}^{-/-}Cx40^{-/-}$ mice by variation of oral salt intake

Next, we determined if in the absence of Cx40, mural cells of preglomerular vessels can undergo reversible metaplastic transformation into RSCs and vice versa. For this aim, $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice were fed low- (0.02 %) and high- (4 %) salt diets for 10 days, and renin cell distribution and renin mRNA abundance were analyzed thereafter. In $AT_{1A}^{-/-}$ kidneys, low-salt diet further increased and high-salt diet markedly reduced the number of preglomerular mural cells expressing cells (Figs. 4a, 5a, and 6a, c). During low-salt diet, mural RSCs almost completely covered the entire afferent arterioles and in part also interlobular arteries. During high-salt diet, RSCs were more restricted to the walls of terminal parts of afferent arterioles. In $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, low-salt diet also further increased and high-salt diet reduced the number of RSCs (Figs. 4b–d, 5b, and 6b, d). The increase of the number of RSCs during low-salt diet resulted from a clear recruitment of renin expression in the vessel walls of the afferent arterioles on the one hand (Figs. 4c and 6b), which, however, was less prominent than in the $AT_{1A}^{-/-}$ kidneys (Figs. 4a and 6a). In $AT_{1A}^{-/-}Cx40^{-/-}$ mice kept on high-salt diet, mural RSCs had disappeared and only ectopic periglomerular RSCs remained visible (Figs. 5b and 6d). The quantitative impression of renin expression derived from the 3D reconstructions (Fig. 6) was corroborated by calculations of renin-immunoreactive volumes from the 3D reconstructions (Fig. 7a). Those calculations revealed 6- and 4-fold difference increases of renin-immunoreactive volumes

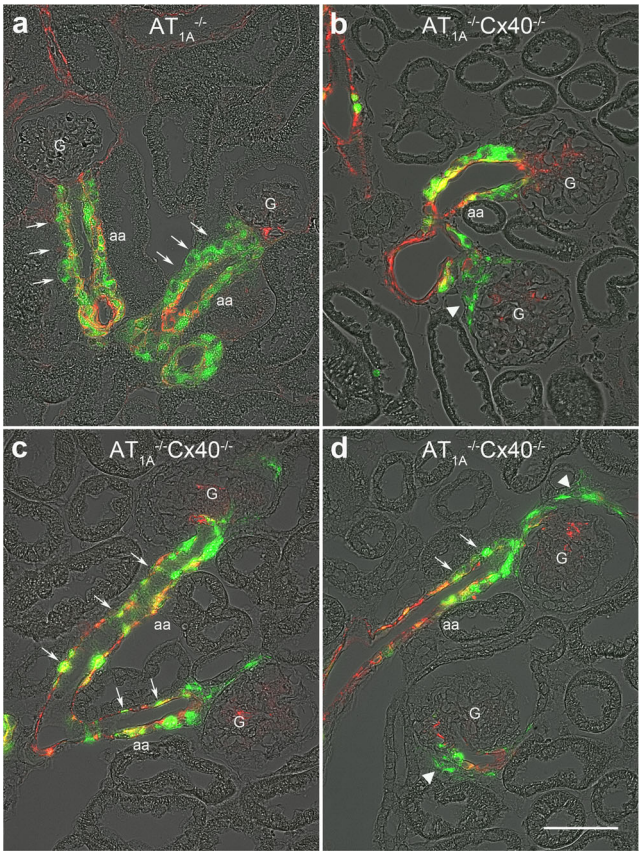

Fig. 4 Representative immunohistochemical stainings of renin (*green*) and α-smooth muscle actin (α-SMA, *red*) in kidneys of $AT_{1A}^{-/-}$ (**a**) and $AT_{1A}^{-/-}Cx40^{-/-}$ (**b**–**d**) mice kept on low-salt diet for 10 days. In $AT_{1A}^{-/-}$ kidneys, there is a further increase of RSCs in the media layer of preglomerular vessels (*arrows*). In $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, there was also an increase of RSCs in the media layer of preglomerular vessels (*arrows*) and also an increase of ectopically located RSCs (*arrowheads*). *Scale bar*=50 μm

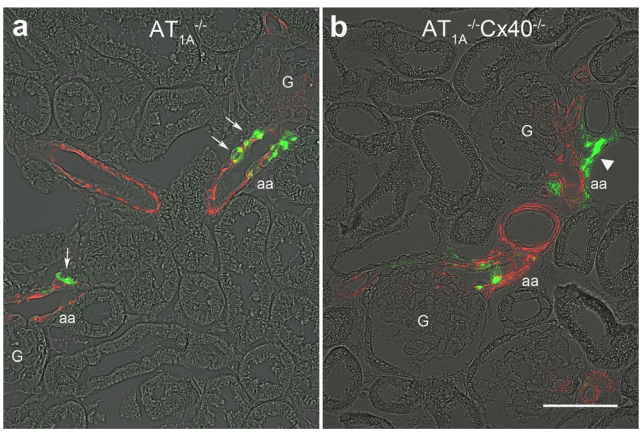

Fig. 5 Representative immunohistochemical stainings of renin (*green*) and α-smooth muscle actin (α-SMA, *red*) in kidneys of $AT_{1A}^{-/-}$ (**a**) and $AT_{1A}^{-/-}Cx40^{-/-}$ (**b**) mice kept on high-salt diet for 10 days. In $AT_{1A}^{-/-}$ kidneys, there is a reduction of RSCs in the media layer of preglomerular vessels (*arrows*) but no ectopic localization of RSCs. In $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, RSCs in the media layer almost disappeared and mainly ectopically located RSCs remained visible (*arrowheads*). *Scale bar*=50 μm

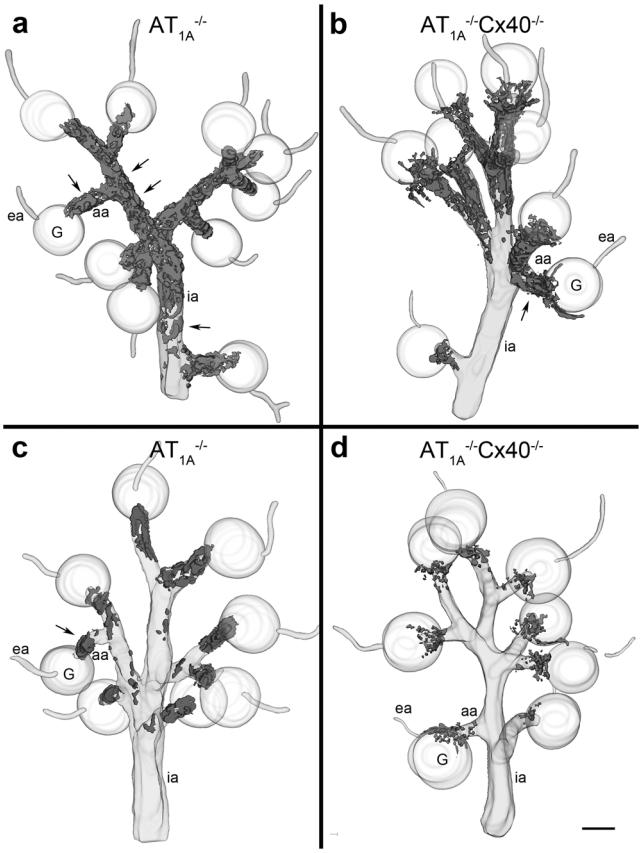

Fig. 6 3D reconstructions of renin-immunoreactive areas (*dark gray*) and α-smooth muscle actin (α-SMA, *light gray*) in kidneys of $AT_{1A}^{-/-}$ (**a**, **c**) and $AT_{1A}^{-/-}Cx40^{-/-}$ (**b**, **d**) mice, kept on low- (**a**, **b**) and high- (**c**, **d**) salt diets, respectively. 3D reconstructions show interlobular arteries (*ia*), afferent arterioles (*aa*), glomeruli (*G*) and efferent arterioles (*ea*). The pictures indicate an increased number of RSCs along preglomrerular vessels in $AT_{1A}^{-/-}$ mice on low-salt diet (*a*, *arrows*). Also in $AT_{1A}^{-/-}Cx40^{-/-}$ mice kept on low-salt diet, the number of RSCs in the walls of afferent arterioles increased (*b*, *arrow*). In $AT_{1A}^{-/-}$ mice kept on high-salt diet, the number of RSCs in the walls of preglomerular vessels was clearly reduced (*c*). In $AT_{1A}^{-/-}Cx40^{-/-}$ mice on high-salt diet, RSCs were not found anymore along the vessel walls of afferent arterioles. *Scale bar*=50 μm

between high- and low-salt intake in $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice, respectively (Fig. 7a). Renin mRNA levels mirrored the salt-dependent changes of renin-immunoreactive volumes in the two genotypes (Fig. 7b).

Again, it turned out that overall renin expression at any level during normal and low-salt intake was lower in kidneys of $AT_{1A}^{-/-}Cx40^{-/-}$ mice than in kidneys of $AT_{1a}^{-/-}$ mice (Figs. 6 and 7a). The difference in renin mRNA between the two genotypes decreased with increasing salt intake (Fig. 7b).

Blood pressure in $AT_{1A}^{-/-}Cx40^{-/-}$ mice

Hypertension in Cx40-defective mice is hypothesized to be triggered by RAAS activity [12]. To estimate the contribution of ANGII-AT_{1A} receptor-mediated effects for hypertension in Cx40-deficient mice, we measured

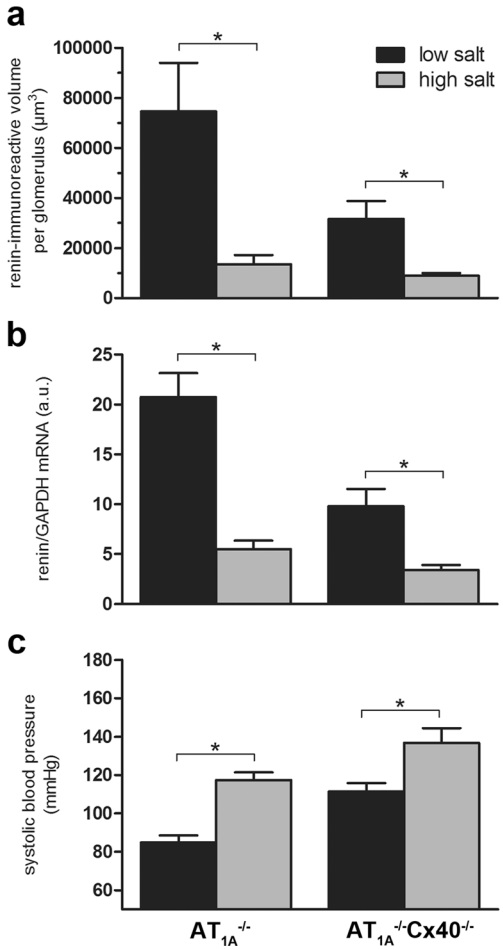

Fig. 7 **a** Renin-immunoreactive volume per glomerulus of $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice under low- and high-salt intakes calculated from 3D reconstructions. Seven to ten glomeruli were analyzed from three kidneys per group. **b** Renin mRNA abundance in the kidneys of $AT_{1A}^{-/-}$ (n=6) and $AT_{1A}^{-/-}Cx40^{-/-}$ (n=6) mice. **c** Systolic blood pressure of $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice kept on low-salt or on high-salt diet (five animals per group). All values are mean±SE. *P<0.05 (ANOVA) between the low- and high-salt group

blood pressure in wt, $Cx40^{-/-}$, $AT_{1A}^{-/-}$, and $AT_{1A}^{-/-}Cx40^{-/-}$ mice on standard chow (Fig. 3c). Mean systolic blood pressure of $AT_{1A}^{-/-}$ mice on normal salt diet was on average 12 mmHg lower than in wild-type controls. The mean systolic blood pressure of $AT_{1A}^{-/-}Cx40^{-/-}$ mice on normal salt diet was on average 30 mmHg lower than that of $Cx40^{-/-}$ controls, but was still higher by 18 mmHg compared to $AT_{1A}^{-/-}$ mice. In both genotypes, low-salt diet further reduced blood pressure by 20–30 mmHg, with pressure values remaining higher in $AT_{1A}^{-/-}Cx40^{-/-}$ compared to $AT_{1A}^{-/-}$ mice (Fig. 7c). Conversely, switching salt diet from normal to high increased systolic blood pressure by about 10 mmHg in both genotypes. Again, blood pressure values in $AT_{1A}^{-/-}Cx40^{-/-}$ mice remained higher than those in $AT_{1A}^{-/-}$ controls (Fig. 7c) during high-salt intake.

In vitro control of renin secretion from $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys

The considerable amount of mural RSCs found in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys raised the question if those cells secrete renin in a pressure-controlled fashion unlike RSCs of $Cx40^{-/-}$ kidneys, in which the pressure control of renin secretion is blunted [29]. Measuring perfusion pressure-dependent secretion of renin from isolated kidneys revealed that the typical inverse relationship between renin secretion and perfusion pressure was maintained in $AT_{1A}^{-/-}$ kidneys, but was completely absent in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, alike in $Cx40^{-/-}$ kidneys (Fig. 8a). The pressure control of renin secretion involves calcium by a yet unknown mechanism. Calcium per se exerts an usual effect on renin secretion in the way that lowering of the calcium concentration strongly enhances renin secretion [4]. This special effect of low extracellular calcium on renin secretion was conserved in $AT_{1A}^{-/-}$ kidneys, but was absent in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys similar to $Cx40^{-/-}$ kidneys (Fig. 8b) [30].

Discussion

A main finding of the present study is that in the adult kidney RSCs can exist in the inner media layer of afferent arterioles in the absence of Cx40 and that SMCs of the afferent arteriole can transform reversibly into RSCs in the absence of Cx40. This finding is relevant since SMCs switch their connexin expression from Cx45 to Cx40 during metaplastic transformation into RSCs [13] and since RSCs have been observed to be ectopically located in the periglomerular interstitium if Cx40 function in RSCs is impaired [17, 19, 29]. Apparently, Cx40 in RSCs and their precursors are not essentially required for induction and maintenance of RSCs in the preglomerular vasculature. We speculate therefore that the disappearance of RSCs in the preglomerular vessels and their ectopic appearance in the renocortical interstitium as typically seen in adult mice with functional defects of Cx40 in RSCs are distinct processes. Since recruitment and existence of RSCs in the walls of preglomerular vessels are considered to be dependent on the intrarenal blood pressure [1, 6, 21], in the way that low pressure favors and high pressure suppresses mural renin expression, the long-lasting hypertension found in all animal models with defective Cx40 function may have prevented mural renin expression by short-term maneuvers of blood pressure lowering. In contrast to $Cx40^{-/-}$ mice, $AT_{1A}^{-/-}Cx40^{-/-}$ mice are normotensive that may allow mural renin expression. The functional consequence of the lower blood pressure in $AT_{1A}^{-/-}Cx40^{-/-}$ mice compared with hypertensive $Cx40^{-/-}$ mice is also reflected by prevention of cardiac hypertrophy seen in $Cx40^{-/-}$ mice (Table 1). Actually, renin

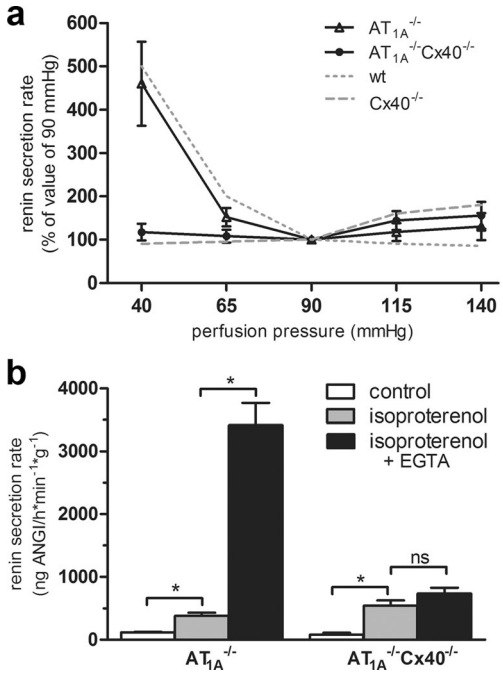

Fig. 8 **a** Perfusion pressure-related renin secretion rates in isolated perfused kidneys of $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice. Kidneys were perfused with pressures ranging from 40 to 140 mmHg in the presence of the β-adrenergic agonist isoproterenol (3nM). Renin secretion rates are normalized to those measured at 90 mmHg. Data are mean±SE of five kidneys each. For comparison, also schematic curves for wild-type (wt) kidneys and $Cx40^{-/-}$ kidneys have been included in the figure. Those curves were adapted from previous publications [13, 20, 30]. **b** Effects of isoproterenol (3nM) and of reduction of the extracellular calcium concentration by adding the calcium chelator EGTA (3.1 mM) to the perfusate on renin secretion from the kidneys of $AT_{1A}^{-/-}$ and $AT_{1A}^{-/-}Cx40^{-/-}$ mice. Kidneys were perfused at a constant pressure of 90 mmHg. Data are mean±SE of five kidneys each. *$P<0.05$ (ANOVA) between the indicated groups

expression and blood pressure in $AT_{1A}^{-/-}Cx40^{-/-}$ mice are salt sensitive alike in $AT_{1A}^{-/-}$ mice [21] with the difference that blood pressure values are shifted to higher and renin expression to lower levels. The higher blood pressure values in $AT_{1A}^{-/-}Cx40^{-/-}$ mice therefore could explain the lower mural renin expression, which completely ceases when blood pressure values go beyond 140 mmHg.

Nonetheless, also in normotensive and in hypotensive $AT_{1A}^{-/-}Cx40^{-/-}$ mice, a substantial part of RSCs were located outside the preglomerular vessels as well known from Cx40-defective mice [17, 19, 29]. The appearance of RSCs in the interstitium is therefore unlikely to result from hypertension or chronically RAAS overactivation since biologic RAAS activity should be low in $AT_{1A}^{-/-}Cx40^{-/-}$ mice. Ectopic renin expression both in $AT_{1A}^{-/-}Cx40^{-/-}$ mice and in $Cx40^{-/-}$ mice shows a greater heterogeneity than the juxtaglomerular and vascular renin expression seen in wild-type or in $AT_{1A}^{-/-}$ mice. It will be a task for future research to define the origin and the development of the cells that are located in the interstitium and that express renin if Cx40 is lacking in the cells. The localization of the ectopic RSCs would support the speculation that they derive from extraglomerular mesangial cells which strongly express Cx40 [15, 31] and which are susceptible for renin gene induction under stress conditions [26].

The second main finding of this study was that the pressure control of renin secretion from the kidneys is lacking if Cx40 is defective, irrespective of the intrarenal localization of RSCs. Thus, in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys, in which RSCs were located both within the vessel wall as well as in the periglomerular interstitium, the pressure control of renin secretion was completely absent as in $Cx40^{-/-}$ kidneys, whilst it was preserved in $AT_{1A}^{-/-}$ kidneys. Apparently, Cx40 function in RSCs is essential for the control of secretion by the perfusion pressure. This conclusion is in accordance with the observation that the nonselective gap junction inhibitor 18αGA impairs pressure control of renin secretion [30] and that peptidic gap junction blockers acutely increase renin secretion from isolated perfused normal adult kidneys [27]. It is known that the pressure control of renin secretion depends on extracellular calcium, in the way that lowering of the extracellular calcium concentration typically stimulates renin secretion and at the same time blunts the pressure control [23]. In line, also the stimulatory effect of low extracellular calcium on renin secretion was blunted in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys as in $Cx40^{-/-}$ kidneys [30], whilst it was preserved in $AT_{1A}^{-/-}$ kidneys. Altogether, it appears as if Cx40 is required for the physiological control of renin secretion, irrespective of the localization of the RSCs.

Loss of baroreceptor control of renin secretion is considered as a reason for the characteristic hypertension developing in mice with defective Cx40 function [29]. This conclusion is supported by the observation that hypertension is absent if both Cx40- and AT_{1A}-receptors are lacking. On the other hand, introducing the Cx40 deletion into AT_{1A}-deficient mice clearly increased blood pressure from hypo- to normotension. One may argue that this residual blood pressure-increasing effect may result from an incomplete inhibition of RAAS activity, eventually mediated by ANGII-AT_{1B} receptors. However, also experiments with pharmacological RAAS blockers revealed that angiotensin I converting enzyme inhibitors reduced blood pressure in $Cx40^{-/-}$ mice to normotensive values but did not completely abolish the blood pressure difference between wild-types and Cx40-deficient mice [3, 11, 12, 30]. In line, transgenic reintroduction of Cx40 in RSCs of mice with a general Cx40 knockout attenuated but did not abolish hypertension [18]. We considered the possibility that aldosterone could contribute to the residual elevation of blood pressure. Our data in fact confirm that aldosterone is elevated in Cx40-deficient mice, but they also show that the increase of plasma aldosterone is absent in $AT_{1A}^{-/-}Cx40^{-/-}$ kidneys (Table 1). Therefore, the possibility has to be considered that apart from activation of the renin-

angiotensin system also additional mechanisms contribute to hypertension in states of Cx40 deficiency. Since selective deletion of Cx40 in the endothelium has no influence on blood pressure [9], at least an endothelial contribution to hypertension in states of Cx40 deficiency appears less likely in this context. Similarly, changes of the renal expression pattern of other vascular connexins appear unlikely to explain the residual increase of blood pressure because we, like others [24], found no difference in Cx37, Cx43, and Cx45 expression levels in $Cx40^{-/-}$ and in $AT_{1A}{}^{-/-}Cx40^{-/-}$ kidneys.

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Conflict of interest The authors declared no competing interest.

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