Restoration of Connexin 40 (Cx40) in Renin-Producing Cells Reduces the Hypertension of Cx40 Null Mice

Loïc Le Gal,* Florian Alonso,* Charlotte Wagner, Stéphane Germain, Denise Nardelli Haefliger, Paolo Meda, Jacques-Antoine Haefliger

See Editorial Commentary, pp 1161–1162

Abstract—Connexin 40 (Cx40) is expressed by the renin-producing cells (RSCs) of the kidneys and the endothelial cells of blood vessels. Cx40 null mice (Cx40−/−) feature a much increased renin synthesis and secretion, which results in chronic hypertension, and also display an altered endothelium-dependent relaxation of the aorta because of reduced eNOS levels and nitric oxide production. To discriminate the effect of Cx40 in renin secretion and vascular signaling, we targeted Cx40 to either the RSCs or the endothelial cells of Cx40 null mice. When compared with Cx40−/− controls, the animals expressing Cx40 in RSCs were less hypertensive and featured reduced renin levels, still numerous RSCs outside the wall of the afferent arterioles. In contrast, mice expressing Cx40 in the endothelial cells were as hypertensive as Cx40−/− mice, in spite of control levels of Cx37 and eNOS. Our data show that blood pressure is improved by restoration of Cx40 expression in RSCs but not in endothelial cells, stressing the prominent role of renin in the mouse hypertension linked to loss of Cx40. (Hypertension. 2014;63:1198-1204.) • Online Data Supplement

Key Words: aorta • connexins • endothelial cells • hypertension • kidney • myocytes, smooth muscle • renin

The gap junction channels made by connexins (Cx) have many functions,1–3 including in the control of vascular function4 and the secretion of most hormones.5–7 A few Cx are shared by several types of vascular and endocrine cells.4–7 For example, in the kidney cortex, Cx40 and Cx37 interact within the endothelium of the afferent arterioles and also couple the endothelial cells (ECs) to the cells of the media layer, which produce renin (RSCs).5,6,8 Accordingly, the global invalidation of the Cx40 gene results in a sustained hypertension of mice,9,10 which has been accounted for by an increase in the synthesis and release of renin,9,10 as well as by peripheral vascular effects.11–13 The latter effects are associated with a decreased expression of the endothelial nitric oxide synthase (eNOS) in Cx40 null and Cx37 deficient mice,12,14 which impairs the release of NO whereby ECs usually relax the smooth muscle cells (SMCs) of arterioles. The SMC tone is further controlled by Ca2+ signaling in ECs, which is also modulated by both Cx40 and Cx37.15,16 The physiological relevance of these observations is supported by the findings that the renin-dependent hypertension increases the expression of Cx40 in ECs, as well as that of Cx37 and Cx43 in SMCs, via an angiotensin II–dependent mechanism.17

To assess the relative importance of Cx signaling on renin secretion and vasomotor function, a previous study has investigated the deletion of Cx40 in either RSCs or ECs, using the Cre-lox technology.11 Strikingly, the mice lacking Cx40 in RSCs were different from Cx40 null mice.8,11 Also, this study17 did not investigate the effects of the Cx40 deletion on other proteins of RSCs and ECs,12,13,17,18 thus complicating the identification of the effects specifically attributable to Cx40. To address these issues, we have now generated 2 novel lines of transgenic mice in which Cx40 has been restored in either the ECs or the RSCs of Cx40 null mice (Cx40−/−), under control of the mouse Tie2 promoter19–22 or the human renin promoter,23–25 respectively. Here, we show that Tie2-Cx40 mice, which express Cx40 in ECs, featured a restored expression of Cx37 and eNOS in the aortic endothelium, still remained as hypertensive as the parental Cx40−/− mice. In contrast, hRen-Cx40 mice, which express Cx40 in RSCs, featured a significant reduction in blood pressure. The data show that the prevalent cause of the hypertension linked to loss of Cx40 is an abnormal renin production.

Methods

Methods are available in the online-only Data Supplement.
Results

Cx40 Is Restored in the ECs of Tie2-Cx40 Mice and in the RSCs of hRen-Cx40 Mice

Immunolabeling showed that ECs, identified by CD31, expressed Cx40 in sections of lungs, kidney, and heart from wild-type (WT) mice (Figure 1A). In contrast, this Cx was not detectable in the corresponding organs of Cx40−/− mice (Figure 1A). These observations were confirmed in aortas (Figure 1A), at both Cx40 transcript and protein levels (Figure 1B and 1C).

The ECs of Tie2-Cx40 mice displayed Cx40 in many vessels (Figure 1A). The levels of both the Cx40 transcript and protein, as evaluated in aortas, were not statistically different from those of controls (Figure 1B and 1C). Immunostaining, quantitative polymerase chain reaction, and Western blots failed to detect Cx40 in the ECs of hRen-Cx40 mice (Figure 1A–1C). Cx40 was also expressed in the RSCs of WT and hRen-Cx40 mice but

Figure 1. The expression of connexin 40 (Cx40) is restored in the vascular endothelium of Tie2-Cx40 mice. A, Immunostaining for Cx40 (green) and CD31 (red) reveals the expression of Cx40 in the endothelial cells (ECs) of lung, kidney, and heart vessels of wild-type (WT) and Tie2-Cx40 mice. In contrast, no Cx40 is detected in the ECs of Cx40−/− and hRen-Cx40 mice. The insets show magnifications of the boxed areas. Bar, 50 μm. Immunostaining of aortas for Cx40 shows the spotted distribution of the Cx in the ECs of WT and Tie2-Cx40 mice but not in those of Cx40−/− and hRen-Cx40 mice. Bar, 30 μm. B and C, Comparable levels of the Cx40 transcript (B) and protein (C) are detected in the aortas of WT and Tie2-Cx40 mice but not in those of Cx40−/− and hRen-Cx40 mice. Results are mean±SEM. Number of mice is indicated above the bars. L indicates lumen; and M, media.
not of Cx40<sup>−/−</sup> and Tie2-Cx40 mice (Figure 2A). The expression of Cx40, which was restricted to the RSCs (Figure 2A; Figure S1 in the online-only Data Supplement), represented 20% of the WT levels (Figure 2B and 2C) and was significantly lower than that evaluated in Tie2-Cx40 mice (Figure 2B and 2C), which expressed Cx40 in many kidney vessels (Figures 1A and 2A).

**Blood Pressure and Cardiac Hypertrophy Are Improved in hRen-Cx40 Mice**

When compared with WT controls, Cx40<sup>−/−</sup> mice showed a significant increase in systolic blood pressure (Table), in heart weight, and in heart/body weight ratio (Table). Tie2-Cx40 mice featured similar alterations (Table). In contrast, hRen-Cx40 mice showed a systolic blood pressure that was reduced when compared with Cx40<sup>−/−</sup> mice, remaining significantly higher than WT controls (Table). hRen-Cx40 mice also featured a heart weight and a cardiac weight index intermediate between those of WT and Cx40<sup>−/−</sup> mice (Table).

**Restoration of Cx40 Does Not Modify the Altered Distribution of RSCs Still Decreases Renin Expression of hRen-Cx40 Mice**

In WT mice, all RSCs of WT mice were located within the juxta-glomerular portion of the afferent arterioles, identified by the green Cx40 staining (Figure 2A). The red staining for renin revealed the expression of Cx40 in the renin-producing cells of hRen-Cx40 mice. In Cx40<sup>−/−</sup> and Tie2-Cx40 mice, Cx40 was not detected in the kidneys of WT mice, less in those of Tie2-Cx40 mice, and even less in those of hRen-Cx40 animals. In contrast, no Cx40 mRNA was detectable in Cx40<sup>−/−</sup> mice. Results are mean±SEM. Number of mice is indicated above the bars. ***P<0.001 vs WT mice. a indicates arteriole; and gl, glomerulus.

![Figure 2.](image)

**Table.** Characteristics of the Control and Transgenic Mice

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<td>174.2±3.9*†</td>
<td>6.4±0.1*†</td>
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Values are expressed as mean±SEM. Cwi indicates cardiac weight index (Cwi=heart weight/body weight); Cx40, connexin 40; n, number of mice; SBP, systolic blood pressure; and WT, wild type.

*P<0.001 vs WT mice.
†P<0.001 vs Cx40<sup>−/−</sup> mice.
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by smooth muscle actin (Figure 3A). In contrast, Cx40−/− mice featured RSCs both within and outside these arterioles (Figure 3A). This abnormal distribution was also observed in hRen-Cx40 mice (Figure 3A). Cx40−/− and Tie2-Cx40 mice showed kidney levels of renin mRNA and protein 2- to 3-fold higher than those of WT controls (Figures 3B and 3C). In contrast, hRen-Cx40 mice featured a significantly lower renin expression, even though the kidney levels of the enzyme were higher than those of the control values (Figure 3B and 3C). Cx37 was expressed in the RSCs and ECs of all mice (Figure S2).

Restoration of Cx40 in ECs Improves the Expression of Cx37 and eNOS

Cx40 was expressed in the ECs of WT and Tie2-Cx40 mice but not in those of Cx40−/− and hRen-Cx40 mice (Figures 1, 2, and 4). EC-enriched samples revealed that ECs-lacking Cx40 also featured a decreased expression of Cx37 (Figure 4A and 4C), and eNOS (Figure S3A and S3B), 2 alterations that were corrected after restoration of Cx40 expression under control of the Tie2 promoter (Figure 4; Figure S3). An in situ assay revealed that eNOS and Cx40 directly interacted within the ECs of WT and Tie2-Cx40 mice (Figure S3A).

Cx37 and Cx43 Are Differentially Regulated in the Media of hRen-Cx40 Mice

The levels of Cx37 were increased over WT values in the aortas of the 3 transgenic lines we studied (Figure S4A). Immunolabeling of media samples showed that this difference was because of an increased expression of Cx37 by the SMCs (Figures S4B and S5). Cx43 was also increased in the aortic media of Cx40−/− and Tie2-Cx40 mice but not in that of hRen-Cx40 mice (Figure S4C and S4D).

Discussion

In rodents, loss of Cx40 causes a chronic hypertension, associated with an increase in both renin production and the tone of peripheral vasculature. To address which of these effects predominates in the regulation of blood pressure, a previous study investigated the deletion of Cx40 in either RSCs or ECs. However, the mice generated in this study differed from the previously reported Cx40 null

Figure 3. Restoration of connexin 40 (Cx40) does not modify the altered distribution of renin-producing cells (RSCs) in Cx40−/− mice and still decreases renin expression. A, Immunostaining of renin (green) and smooth muscle actin (SMA, blue) shows the presence of RSCs in the afferent arterioles of wild-type (WT) mice. In the absence of Cx40, the RSCs of Cx40−/− mice are often observed outside these vessels. In the kidneys of hRen-Cx40 mice, RSCs are seen both within the afferent arterioles (right) and outside these vessels (left). Bar, 50 μm. B and C. When compared with WT values, the levels of renin transcript (B) and protein (C) are markedly increased in the kidneys of Cx40−/− and Tie2-Cx40 mice. The expression of renin is significantly reduced in the kidney of hRen-Cx40 mice when compared with that observed in both Cx40−/− and Tie2-Cx40 mice. Still, it remains significantly higher than in controls. Results are mean±SEM. Number of mice is indicated above the bars. *P<0.05, **P<0.01, ***P<0.001 vs WT mice; °P<0.05 vs Cx40−/− mice.
mice, notably with regard to the expression and release of renin. These differences may be because of a partial, and possibly not fully cell-specific loss of Cx40, which in this study was solely supported by negative immunofluorescence observations, as sometimes observed with the Cre-lox methods. Also, this study did not investigate the effects of the Cx40 deletion on other proteins of RSCs and ECs, thus preventing the unambiguous identification of the biological effects specifically attributable to Cx40. To address these issues, we have generated 2 novel mouse lines in which Cx40 was selectively restored in either the ECs or the RSCs of Cx40−/− mice. Comparison of these animals showed that the expression of Cx40 in ECs restored control levels of endothelial Cx37 and eNOS but did not modify the hypertension observed in the parental Cx40−/− mice. In contrast, the expression of Cx40 in RSCs reduced this pressure, as well as renin production and cardiac hypertrophy. The data document that renin production plays a predominant role in the Cx40 control of blood pressure.

The decrease in blood pressure observed in hRen-Cx40 mice was not sufficient to normalize systolic values. Given that the activity of the human renin promoter is reduced in the presence of high AngII levels, such as those circulating in the Cx40−/− mice, it is possible that Cx40 expression in the RSCs of hRen-Cx40 mice was lower than that of control mice. At any rate, the data document that small changes in the overall renal expression of this connexin are sufficient to modulate the production of renin, strengthening the physiological relevance of the specific control Cx40 exerts on the secretion of renin.

Our data further document that the restoration of Cx40 failed to correct the abnormal distribution of RSCs observed in Cx40 null mice. Given that ectopic RSCs feature a less differentiated phenotype than the RSCs located in the afferent arterioles, it remains to be determined whether the renin...
expression of hRen-Cx40 mice is because of this altered distribution or to the loss of coupling between the RSCs and ECs of the afferent arterioles.

Previous studies have documented that Cx40−/− mice feature an improper reactivity of the peripheral vasculature, which is likely to be missed by present genetic analyses, may suffice to control human blood pressure, presumably because small changes of Cx40 expression, which are investigated in the present hypothesis, presumably because small changes of Cx40 expression, which are likely to be missed by present large-scale genetic analyses, may suffice to control human blood pressure, like we now report in the kidney of hRen-Cx40 mice.

Perspectives

The absence of Cx40 causes a form of hypertension associated with increased release of renin and enhanced tone of the peripheral vasculature. The generation of 2 novel lines of transgenic mice in which Cx40 was selectively restored in either the ECs or the RSCs of Cx40−/− animals now reveals for the first time that the primordial role of Cx40 in hypertension is via its physiological control of renin secretion by the kidney. Given that restoration of Cx40 in RSCs decreases but did not fully normalize blood pressure, more work is now required to establish whether and how this central scenario is modified by the other Cx isoforms that are expressed by the RSCs and ECs of the renal juxta-glomerular apparatus. Other studies should investigate the molecular mechanism whereby the expression of EC markers is linked to that of Cx40. In this perspective, the new Tie2-Cx40 line that we describe here will be instrumental also to unravel the physiological roles of connexins in ECs.

Sources of Funding

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Disclosures

None.

References


Novelty and Significance

What Is New?
• We developed 2 novel lines of transgenic mice, to document that the expression of connexin 40 (Cx40) in renin-producing cells improves renin production and hypertension, whereas the expression of Cx40 in ECs does not, in spite of full correction of several endothelial markers.

What Is Relevant?
• Cx40 channels form between renin-producing cells and ECs. Cx40 null mice feature an increased renin production, which results in chronic hypertension, and also display an altered vasomotor tone because of reduced nitric oxide production. We show that the selective expression of Cx40 in ECs restored control levels of Cx37 and eNOS but did not modify the hypertension of Cx40−/− mice. In contrast, the expression of Cx40 in renin-producing cells reduced this pressure, as well as renin production.

Summary
We document that the predominant role of Cx40 in the control of blood pressure is via a connexin-specific modulation of the renin production within the kidney.
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Restoration of Connexin40 in renin-producing cells reduces the hypertension of Cx40 null mice

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Running title: Cx40 and renin-producing cells

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METHODS

Generation of transgenic mice
The generation of Cx40-/- mice and their genotypic characterization have been described\(^1\),\(^2\). Breeding pairs of Cx40-/- mice were crossed with control C57/Bl6 partners. Cx40-/- and Cx40+/+ littermates were then identified by PCR of genomic DNA. Transgenic mice expressing Cx40 in an EC- and RSC-specific manner were obtained by associating either a 2 kb fragment of the mouse Tie2 gene promoter (mTie2)\(^3\)-\(^5\) or a 12 kb fragment of the human renin promoter (hRen), upstream to the 1,2 kb full length Cx40 cDNA\(^6\)-\(^8\), respectively. The cDNA constructs were purified and micro-injected into the pronucleus of fertilized oocytes from B6D2F1 mice, by the Transgenic Animal Facility of the Faculty of Biology and Medicine of Lausanne. Surviving embryos were transferred to recipient females according to standard protocols. Two independent lines featuring a stable integration of the transgene into the genome were established with each construct. The presence of the transgene was determined by PCR amplification of ear DNA, using a sense primer located in either the Tie2 or the hRen promoter and an antisense primer located in the Cx40 coding sequence. The sequence of these primers was as follows: for the hRen-Cx40 construct (343 bp amplicon), 5'-ATCAGCCTCTGCTGCTC-3' (sense) and 5'-CCGATGACTGTGGAGTGCTT-3' (antisense); for the Tie2-Cx40 construct (250 bp amplicon), 5'-CCTTGCCGCCAACTTGTAAAC-3' (sense) and 5'-AATGAACAGGACAGTGAGCCAGACATTTGTAAAC-3' (antisense). hRen-Cx40 and Tie2-Cx40 heterozygous mice were then backcrossed with C57BL/6-Cx40-/- partners to obtain hRen-Cx40+/-/Cx40-/- (hereafter referred to as hRen-Cx40 mice) or Tie2-Cx40+/-/Cx40-/- mice (hereafter referred to as Tie2-Cx40 mice). Identification of the wild type (WT; Cx40+/+), and Cx40 null mice (Cx40-/-) was performed by PCR of genomic DNA, using the following primers: for the Cx40 WT allele (614 bp amplicon), 5'-TGTCCGTCTGTCTGTCTGTGT-3' (sense) and 5'-GATGACTGTGGAGTGCTTGTG-3' (antisense); for the Cx40 null allele (512 bp amplicon), 5'-GGATCGGCCATTGAACAAGATGGATTGCAC-3' (sense) and 5'-CTGATGCTCTTCTGGAGGAGCTTGAG-3' (antisense). The 60 male mice used in these experiments were 3-5 month old, and were from litters obtained after at least 10 generations of backcrossing into the C57/Bl6-Cx40-/- background. All mice were housed in pathogen-free conditions as per our institutional guidelines. Mouse care, surgery and euthanasia procedures were all approved by our institutional committee for animal experiments, and the veterinary office (Lausanne, Switzerland; authorization 31.1.1008/1993/I). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, 2011).

Measurement of blood pressure
Systolic blood pressures were measured on conscious mice by a non-invasive, computerized tail-cuff method (BP-2000, Visitech Systems)\(^1\),\(^9\). Initially, mice were pre-trained during 7 days, and then pulse rate and blood pressure were recorded by the automatic system of cuff inflation and deflation. Measurements of systolic pressure were performed every day during 10 days.

RNA and protein analysis
Mice were sacrificed and immediately infused with 5 mL phosphate-buffered saline (PBS). Aortas and kidneys were carefully removed, rapidly placed into liquid nitrogen, reduced to
powder, and stored at -80°C. For RT-PCR analysis, this powder was homogenized in Tripure Isolation Reagent (Roche, Switzerland), and total RNA was extracted according to the manufacturer's instructions. RNA was analysed by real-time qPCR, using the primers given in Table S1. For western blots, the powder of aortas and kidneys was homogenized by sonication in lysis buffer (62.5 mM Tris HCl, 5% SDS, 10 mM EDTA, pH8). Protein content was measured using a detergent-compatible DC protein assay kit (Bio-Rad Laboratories, Reinach BL, Switzerland). Samples (30 µg) were loaded on a 10% polyacrylamide gel, separated by electrophoresis and transferred onto PVDF membranes (Immobilon-P; Millipore, Volketswil, Switzerland). Membranes were incubated for 1h in PBS or TBS containing 5% milk and 0.1% Tween 20 (blocking buffer). The membranes were then incubated overnight at 4°C with one of the following primary antibodies: rabbit polyclonal antibodies against Cx40 (Chemicon, AB1726; diluted 1:250), Cx37 (Biotrend Chemikalien, Cx37A11-A; diluted 1:500) or Cx43 (Cell Signaling, 3512S, 1:500); mouse monoclonal antibodies against renin (Swant; diluted 1:500), eNOS (BD Biosciences, 610297, 1:500) and α-tubulin (Sigma-Aldrich, T5168; diluted 1:2500). The secondary antibodies were horse radish peroxidase-conjugated goat anti-mouse immunoglobulins (Jackson Immuno research, 63343, diluted 1:20'000) or goat anti-rabbit immunoglobulins (Thermo Scientific, 31460, diluted 1:5'000), whichever adequate. Bands were developed for enhanced chemiluminescence (Millipore, Immunobilon Western Chemiluminescent HRP substrate), and visualized using a supercooled CCD camera (Chemidoc XRS, Bio-Rad Laboratories). Densitometric analysis was performed using an ImageLab Software (3.0.1 Bio-Rad Laboratories). To prepare samples enriched in ECs, thoracic aortas were longitudinally opened within 100 µl PBS, and pinned on a silicone plate, endothelium side up. A scalpel blade was then used to gently scrape off the ECs. Aliquots of ECs were homogenized in lysis buffer and processed for western blot analysis as described above.

Immunohistochemistry

For immunolabeling, freshly excised kidneys, aortas, lungs and hearts were rapidly frozen and processed for cryosectioning. Cryostat sections were stained using: one of the following antibodies: rabbit polyclonal against Cx40 (Chemicon, AB1726), Cx37 (Biotrend Chemikalien, Cx37A11-A) or Cx43 (Cell Signaling, 3512S); goat polyclonal antibody against renin (R&D, AF4277); biotinylated rat monoclonal against CD31 (BD Biosciences, 76026). All antibodies were diluted 1:100. Primary antibodies were detected using either anti-rabbit immunoglobulins labelled with AlexaFluor 488 (Invitrogen), anti-goat immunoglobulins labelled with AlexaFluor 350 (Invitrogen); or Streptavidin labelled with AlexaFluor 594 (Invitrogen), all diluted 1:500. To assess the distribution of RSCs, kidneys were fixed via an intracardiac injection of 4% paraformaldehyde (PFA), incubated overnight in 4% PFA, dehydrated, embedded in paraffin and sectioned at 5 µm thickness. After a 30 min exposure to 10% horse serum plus 1% bovine serum albumin in phosphate-buffered saline (PBS), the sections were incubated with either a chicken polyclonal antibody against renin (Davids Immunotechnologie, diluted 1:100) or a mouse monoclonal antibody against smooth muscle actin (Beckman Coulter, Immunotech) overnight at 4°C, followed by a 1 h incubation at room temperature in the presence of a relevant fluorescent secondary antibody (Dianova, diluted 1:500). For en-face immunostaining, thoracic aortas were removed, and opened longitudinally within a few drops of PBS warmed at 37°C. The vessels were then attached with micro pins on Sylgard-coated dishes, endothelium side up. Aortic strips were cut, fixed for 10 min in...
100% ethanol, cooled at -20°C, and incubated for 30 min in a blocking solution (PBS supplemented with 5% BSA and 0.2% Triton X-100). The strips were then labelled with either rabbit polyclonal antibodies against Cx37 (Biotrend Chemikalien, Cx37A11-A; diluted 1:50), or mouse monoclonal antibodies against Cx40 (Invitrogen; diluted 1:50). Primary antibodies were detected using either anti-rabbit immunoglobulins labelled with AlexaFluor 488 (Invitrogen) or anti-mouse immunoglobulins labelled with AlexaFluor 594 (Invitrogen).

**In situ proximity ligation assay**

A direct interaction between the eNOS and the Cx40 proteins was tested in the aortic endothelium using the Duolink in situ proximity ligation assay (OLINK Bioscience). Briefly, longitudinally opened aortas were fixed in 100% ethanol, and aortic strips prepared for the simultaneous incubation with rabbit polyclonal antibody against Cx40 (Chemicon AB1726) and mouse monoclonal antibody against eNOS (BD Biosciences 610297), both diluted 1:100. The incubation solution was then supplemented with oligonucleotide-conjugated secondary antibodies (diluted 1:5) and a ligation solution, consisting of two nucleotides and a ligase, to form a circular DNA when eNOS and Cx40 were in close proximity. The circular DNA was then amplified into a long, single-stranded concatemer by rolling circle amplification, using as primer an arm of one of the oligonucleotide-conjugated antibodies. The amplification product was collapsed into a DNA bundle, and detected by hybridizing fluorophore-labeled oligonucleotides (Duolink in situ detection reagent red) to the repeated sequences of the amplification product. Under a fluorescence microscope, the result was detected as red spots, which were localized by staining the EC nuclei with DAPI.

**Statistical analysis**

Values are given as means ± SEM. Mean values of different groups were compared by one-way ANOVA, using the post hoc Bonferroni test for multiple comparisons, as provided by The Statistical Package for the Social Science (SPSS 17.0, Chicago, IL). P values < 0.05 were considered as significant.

**REFERENCES**


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**Figure S1: Expression of Cx40 is exclusively restored in the renin-secreting cells of hRen-Cx40 mice.**

Kidney sections, immunostained for both Cx40 (green) and renin (red) and viewed at low magnification, revealed that Cx40 was selectively expressed in the RSCs of the afferent arterioles of wild type controls (WT) and hRen-Cx40 mice, but not in those of Cx40/- and Tie2-Cx40 animals. Bar = 50 µm. a = arteriole; gl = glomerulus.
Figure S2: Expression of Cx37 in the renin-secreting cells of hRen-Cx40 mice
Double immunostaining of kidney sections for Cx37 (green) and renin (red) reveals the expression of Cx37 in the RSCs of the afferent arterioles of wild type (WT) controls and of Cx40-/-, Tie2-Cx40 and hRen-Cx40 transgenic mice. Cx37 also labelled the ECs of various types of renal vessels. Bar = 30 µm. gl = glomerulus. The arrows in the images of the bottom line indicate the lumen of the kidney vessels.
Figure S3: Restoration of Cx40 in ECs prevents the down-regulation of eNOS expression

A. Cx40 and eNOS (red spots) closely interact in the ECs of WT and Tie2-Cx40 mice, as revealed by an *in situ* proximity ligation assay. This interaction is not detectable in the aorta of Cx40/-/- mice. DAPI staining (blue) reveals the EC nuclei. Bar = 30 µm. B. In EC-enriched extracts, eNOS protein is detected in the aortas WT mice, and in significantly lower levels in both Cx40/-/- and hRen-Cx40 mice. This down-regulation is prevented in the aortas of Tie2-Cx40 mice. Results are means ± SEM. Number of mice is indicated above the bars. *P < 0.05, vs WT mice; **P < 0.01 vs Cx40/-/- mice.
Figure S4: Cx37 and Cx43 are differentially regulated in the media layer of the aorta of hRen-Cx40 mice

A. As compared to control values (WT), the levels of Cx37 are similarly increased in whole extracts of aortas from Cx40-/-, Tie2-Cx40, and hRen-Cx40 mice. B. Section immunostaining shows that this increase is due to the induction of Cx37 in the SMCs of the media layer. The insets show enlargements of the boxed area to highlight the presence of sizable Cx37 dots. C. Compared to controls (WT), the levels of Cx43 were not statistically different in whole aorta extracts of hRen-Cx40 mice. In contrast, these levels were similarly increased in the whole aorta extracts of Tie2-Cx40 and Cx40-/- mice. D. Immunostaining reveals that this increase is due to the induction of Cx43 in the SMCs of the aortic media. The insets show enlargements of the boxed area to highlight the presence of Cx43 sizable dots. Bar = 30µm. M = Media; L = Lumen. Results are means + SEM. Number of mice is indicated above the bars. *P < 0.05, **P < 0.01 vs WT mice; °P < 0.05 vs Cx40-/- mice.
Figure S5: Cx37 is induced in the aortic media of the 3 transgenic mice line that we compared
As compared to control values (WT), the levels of Cx37 were similarly increased in aortic media extracts of Cx40-/-, Tie2-Cx40, and hRen-Cx40 mice. Results are means ± SEM. Number of mice is indicated above the bars. *P < 0.05, ***P < 0.001 vs WT mice.