Hypertension

ORIGINAL ARTICLE

CCN2 (Cellular Communication Network Factor 2) Deletion Alters Vascular Integrity and Function Predisposing to Aneurysm Formation

Raúl R. Rodrigues-Díez,* Antonio Tejera-Muñoz,* Vanesa Esteban, Lasse B. Steffensen, Raquel Rodrigues-Díez, Macarena Orejudo, Sandra Rayego-Mateos, Lucas L. Falke, Pablo Cannata-Ortiz, Alberto Ortiz, Jesus Egido, Ziad Mallat, Ana M. Briones, M. Auxiliadora Bajo, Roel Goldschmeding, Marta Ruiz-Ortega

BACKGROUND: CCN2 (cellular communication network factor 2) is a matricellular protein involved in cell communication and microenvironmental signaling responses. CCN2 is known to be overexpressed in several cardiovascular diseases, but its role is not completely understood.

METHODS: Here, CCN2 involvement in aortic wall homeostasis and response to vascular injury was investigated in inducible *Ccn2*-deficient mice, with induction of vascular damage by infusion of Ang II (angiotensin II; 15 days), which is known to upregulate CCN2 expression in the aorta.

RESULTS: Ang II infusion in CCN2-silenced mice lead to 60% mortality within 10 days due to rapid development and rupture of aortic aneurysms, as evidenced by magnetic resonance imaging, echography, and histological examination. *Ccn2* deletion decreased systolic blood pressure and caused aortic structural and functional changes, including elastin layer disruption, smooth muscle cell alterations, augmented distensibility, and increased metalloproteinase activity, which were aggravated by Ang II administration. Gene ontology analysis of RNA sequencing data identified aldosterone biosynthesis as one of the most enriched terms in CCN2-deficient aortas. Consistently, treatment with the mineralocorticoid receptor antagonist spironolactone before and during Ang II infusion reduced aneurysm formation and mortality, underscoring the importance of the aldosterone pathway in Ang II–induced aorta pathology.

CONCLUSIONS: CCN2 is critically involved in the functional and structural homeostasis of the aorta and in maintenance of its integrity under Ang II–induced stress, at least, in part, by disruption of the aldosterone pathway. Thus, this study opens new avenues to future studies in disorders associated to vascular pathologies. **(*Hypertension*. 2022;79:e42–e55. DOI: 10.1161/HYPERTENSIONAHA.121.18201.)** • **Supplemental Material**

Key Words: aldosterone ■ aneurysm ■ aorta ■ extracellular matrix ■ hypertension

CCN2 (cellular communication network factor 2), previously known as CTGF (connective tissue growth factor), belongs to the CCN family,[1,2] including also by CCN1/Cyr61 (cysteine-rich protein), CCN3/Nov (nephroblastoma overexpressed protein), and 3 other matricellular proteins (CCN4-6), sharing a conserved tri- to tetramodular structure. These CCN proteins are important ECM (extracellular matrix) components involved in the regulation of different cellular functions.[1,3]

CCN2 exerts multiple context-dependent biological functions, including regulation of cell growth, differentiation, development, adhesion, inflammation, and ECM remodeling.[3,4] Regarding the cardiovascular system, CCN2 is highly expressed during development in

Correspondence to: Marta Ruiz-Ortega, Department of Medicine, Molecular and Cellular Biology in Renal and Vascular Pathology, UAM, IIS-FJD, Avda. Reyes Católicos, 2, 28040, Madrid, Spain, Email mruizo@fjd.es or Raquel Rodrigues-Díez, Department of Pharmacology, UAM, C/del Arzobispo Morcillo, 4, 28029, Madrid, Spain, Email rrodrigues@fjd.es
*R.R. Rodrigues-Díez and A. Tejera-Muñoz contributed equally.
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NOVELTY AND RELEVANCE

What Is New?

Ccn2 (cellular communication network factor 2) deletion in mice induces aortic structural modifications, including elastin layer disruption, smooth muscle cell alterations, increased metalloproteinase activity, and vascular function changes.

The vascular wall homeostasis disruption induced by *Ccn2* deletion leads to rapid aortic aneurysm generation and rupture after Ang II (angiotensin II) infusion in mice, suggesting that the relative resistance of C57Bl/6 mice to Ang II–induced aortic aneurysm is lost in the context of CCN2 deficiency.

Treatment of *Ccn2*-deleted mice with the mineralocorticoid receptor antagonist spironolactone before and during Ang II infusion reduced the incidence and lethal dissection and rupture of aneurysm without modifying blood pressure.

What Is Relevant?

The role of CCN2 in maintaining aortic wall homeostasis and preventing aortic aneurysm generation in a context of hypertension driven by Ang II is relevant for prevention of vascular complications of hypertension.

This preclincial study opens a new line of research to evaluate whether *CCN2* gene alterations could predispose to thoracic aortic disease in humans.

Spironolactone exerted beneficial effects in our experimental model of aneurysm generation in *Ccn2* deleted mice after Ang II infusion, encouraging future research targeting aldosterone for aneurysm-related pathologies.

Clinical/Pathophysiological Implications?

CCN2 contributes to aortic wall homeostasis and adaptive remodeling that prevents aneurysm generation, which might have important implications for further clinical exploration in patients at risk.

Nonstandard Abbreviations and Acronyms

ACTA2	alpha smooth muscle actin 2
Ang II	angiotensin II
CCN2	cellular communication network factor 2
CCN2-KO	CCN2 knockout
CTGF	connective tissue growth factor
ECM	extracellular matrix
MMP	matrix metalloproteinase
Nov	nephroblastoma overexpressed protein
RNA-seq	RNA sequencing
TGF	transforming growth factor
VSMC	vascular smooth muscle cell

heart, branchial arches, and in endothelium and vascular smooth muscle cells (VSMCs) of major blood vessels,[5,6] and it is overexpressed in experimental and human cardiovascular diseases, like heart failure, pulmonary hypertension, restenosis, and atherosclerosis.[5] CCN2 plays a relevant role in fibrogenesis, for which it is a well-established biomarker of fibrosis, and it has been considered the key downstream profibrotic mediator of TGF (transforming growth factor)-β and Ang II (angiotensin II).[3,7] Based on those studies, CCN2 was proposed as a growth factor and cytokine, but this concept has been recently extended to include also a role as matricellular ECM protein implicated in cell communication and coordination of responses to microenvironmental signaling.[2,4]

Preclinical studies suggest that the CCN2 blockade could be a potential therapeutic option for fibrotic diseases, based on the promising results in experimental liver, lung, and renal fibrosis,[8–10] as well as in pulmonary vascular remodeling and heart failure,[11,12] and anti-CCN2 therapy is in phase 2 or 3 clinical trials for Duchenne muscular dystrophy (NCT02606136), pancreatic adenocarcinoma (NCT04229004) and idiopathic pulmonary fibrosis (NCT03955146). However, CCN2 overexpression in cardiomyocytes protected mice from Ang II–induced pressure overload cardiomyopathy and ischemia-reperfusion injury.[13,14] Accordingly, CCN2 overexpression attenuated myocardial hypertrophy, cardiac dysfunction, and left ventricular remodeling in experimental pressure overload and stroke.[15] More recently, post-ischemic administration of recombinant CCN2 reduced infarct size and improved cardiac function recovery following ischemia-reperfusion injury.[16] CCN2 knockout (CCN2-KO) mice die shortly after birth by respiratory failure.[6] In inducible CCN2-KO mice, however, *Ccn2* deletion ameliorated renal fibrosis,[17] although it did not improve cardiac fibrosis and hypertrophy.[18] High circulating CCN2 levels have been proposed as a potential risk biomarker for cardiac dysfunction in patients with chronic heart failure and myocardial fibrosis.[19] Moreover, CCN2 mRNA expression was increased in human VSMCs from aneurysms and atherosclerotic plaques.[5,20] Nevertheless, scarce information is available about the impact of CCN2 modulation in these vascular diseases.

Our aim was to characterize the role of CCN2 in the regulation of vascular responses under normal and pathological conditions, using the inducible CCN2-deficient mouse strain (CCN2flox/floxROSA26-ERT/Cre; henceforth named CCN2-KO), and the well-known model of vascular damage induced by systemic Ang II

administration[21] in which aortic CCN2 expression is known to be upregulated.[22,23]

METHODS

The authors declare that all supporting data are available within the article (and its Supplemental Material). *Expanded methodology is available in the Supplemental Material.

Study Approval

Animal studies were performed at Instituto de Investigación Sanitaria Fundación Jiménez Díaz in accordance with the current European (Directive 2010/63/EU) and National (Real Decreto 53/2013) legislation for the Use and Care of Laboratory Animals and according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. The protocol was approved by the Instituto de Investigación Sanitaria Fundación Jiménez Díaz Animal Research Ethical Committee and by the Comunidad de Madrid Committee (PROEX 065/18).

Statistics

Data are expressed as mean±SEM. Normality distribution was tested with the Shapiro-Wilk test. Two-tailed Student *t* test was used to compare 2 groups, 1-way ANOVA (followed by the Tukey post hoc test) to compare one variable in multiple groups and 2-way ANOVA (followed by the Dunnett post hoc test) to compare 2 variables. For not-normally distributed data, nonparametric Mann-Whitney *U* test (to compare 2 groups) or Kruskal-Wallis test (to compare >2 groups, followed by the Dunn post hoc test) was used. The Kaplan-Meier method was used for survival curves, which were statistically analyzed by the log-rank test. Aneurysm incidence was analyzed by the Fisher exact test. Statistical analyses were performed on GraphPad Prism 8.0 (GrahPad Software, San Diego, CA). $P<0.05$ was considered statistically significant.

RESULTS

Ang II Induces Severe Aortic Aneurysm Formation and Dissection in CCN2-KO Mice

Ang II infusion in CCN2-KO mice dramatically decreased survival at 15 days (Figure 1A). Postmortem examination revealed aortic rupture as the cause of death. Visual aortic evaluation showed that 92% of Ang II–infused CCN2-KO mice developed thoracic and abdominal aorta aneurysms (thoracoabdominal aneurysms) of all types in the Crawford/Safi classification (Figure 1B and 1C). This phenotype could not be rescued by infusion of exogenous recombinant C-terminal fragment of CCN2 (CCN2-IV; Figure S1A and S1B).

Ang II administration increased systolic blood pressure in both CCN2-KO and control mice. In CCN2-KO mice, a slight and persistent decrease in systolic blood pressure was observed upon *Ccn2* deletion (Figure 1D). In ex vivo tension-extension studies, isolated thoracic aortic segments of CCN2-KO mice presented an increased vessel distensibility, as shown by a rightward shift in the tension-extension distance curve and increased 1/slope (Figure 1E and 1F), suggesting disruption of aortic homeostasis in the absence of CCN2.

Acquired CCN2 Deficiency Alters Aortic Structure and Increases Susceptibility to Ang II–Induced Aneurysm Formation

In vivo magnetic resonance imaging showed a large increase in aortic diameter in CCN2-KO mice as early as 48 hours after in response to Ang II administration (Figure 2A; Figure S2) indicative of aneurysm formation, and patchy reductions in aortic lumen associated with increased total aortic area suggestive of aortic wall dissection. In these mice, maximum total aortic area and minimum aortic lumen had significantly changed after 24 and 96 hours, respectively (Figure 2B through 2D). CCN2-KO mice had slightly but significantly increased maximal total and minimal luminal aortic areas compared with controls (Figure 2B through 2D), indicating aortic dilatation in CCN2-deficent mice already before Ang II administration, which was confirmed by an increased abdominal aorta diameter visualized by ultrasound (Figure 3A and 3B). Additionally, Ang II infusion in CCN2-KO mice dramatically increased the diameter of the thoracic descending aorta diameter and enhanced the abdominal aorta diameter increase observed in CCN2-KO mice (Figure 3A and 3C), consistent with thoracoabdominal aneurysm formation.

Acquired CCN2 Deficiency Alters Vascular Function

In CCN2-deficient mice, the ex vivo aortic vasoconstrictor response to phenylephrine was significantly increased compared with control mice. Moreover, the Ang II–induced increase of the vasoconstrictor response was further enhanced by CCN2 deficiency (Figure S3A). Endothelium-dependent relaxation induced by acetylcholine was not affected by *Ccn2* deletion alone, being similar in CCN2-KO and control aortic rings (Figure S3B). However, the decreased vasorelaxation response that characterizes the endothelial dysfunction induced by Ang II was aggravated in the absence of CCN2 (Figure S3B).

Ccn2 Deletion Reduces VSMC Migration and Proliferation

Scrape wounded monolayers of CCN2-KO aortic VSMCs showed a significant decreased spontaneous wound closure compared with control cells (Figure S4A through S4C), and also Ang II–induced migration was impaired in CCN2-deficient VSMCs (Figure S4D and S4E).

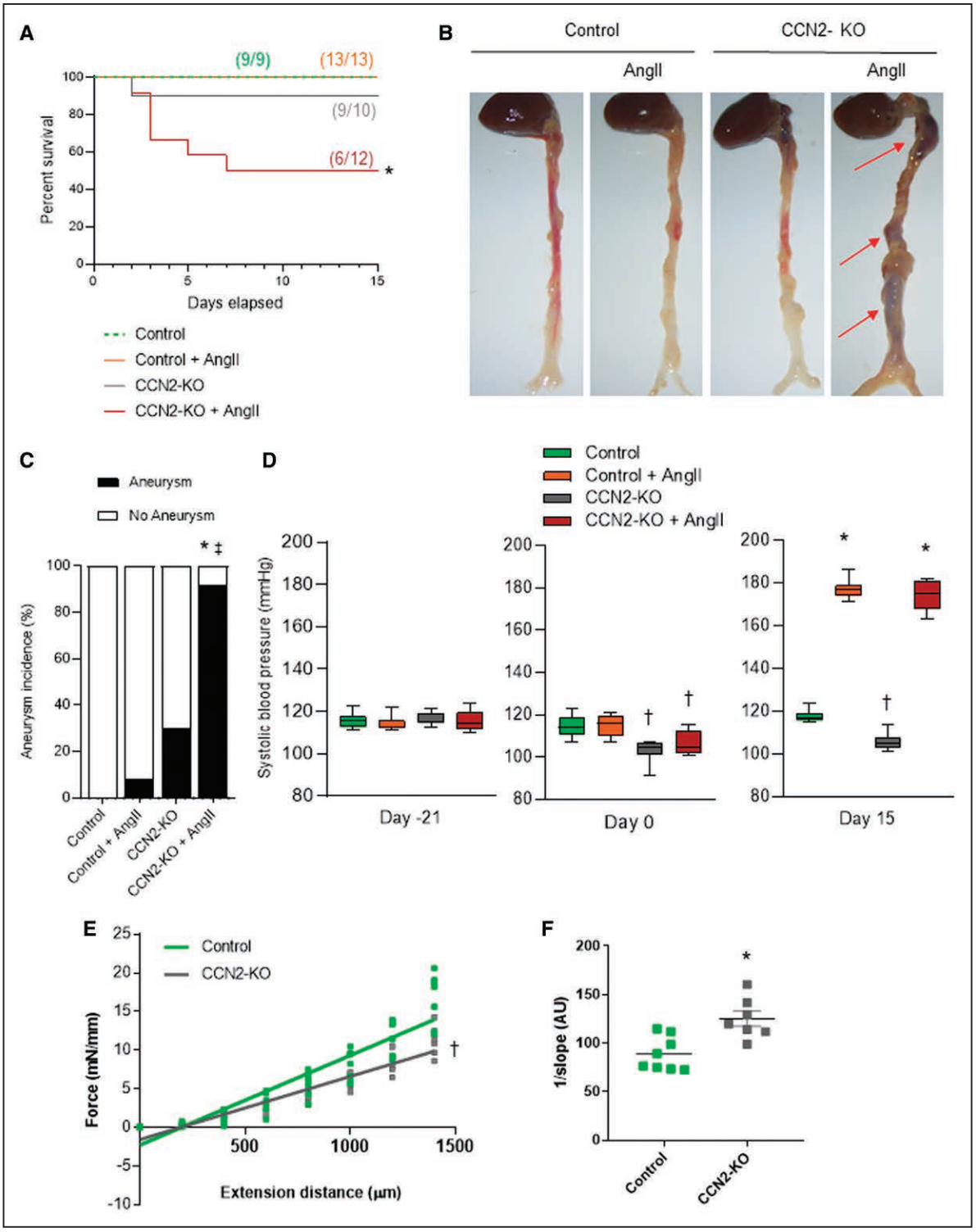

Figure 1. CCN2 (cellular communication network factor 2) deficiency decreases mice survival following Ang II (angiotensin II) administration as a result of aortic aneurysm development and rupture.

Control and CCN2 knockout (CCN2-KO) mice were infused with Ang II for 15 d. Survival, aneurysm generation, and systolic blood pressure (SBP) were analyzed. **A**, Kaplan-Meier survival curve: dramatic increase in mortality in Ang II–infused CCN2-KO mice. **B**, Clean whole aorta: thoracoabdominal aneurysm (TAAA) generation in Ang II–infused CCN2-KO mice for 15 d. **C**, Aneurysm appearance. Larger TAAA formation was found in 92% of Ang II–infused CCN2-KO mice and smaller abdominal aneurysm (AbA) in 30% and 8% of CCN2-KO and control+Ang II mice, respectively. **D**, Time course of SBP since the first tamoxifen injection (day −21): Ang II increased SBP in all groups, and CCN2 deficiency decreased SBP. Data shown as box and whisker plots, with 75th and 25th percentiles; bars represent maximal and minimal values. n=8 to 13 mice per group. **E**, Tension-extension distance relationship and (**F**) 1/slope of this relationship in aorta from control and CCN2-KO mice. n=7 mice per group. Data shown as mean±SEM. *P<0.05 increased vs control. †P<0.05 decreased vs control. ‡P<0.05 vs CCN2-KO.

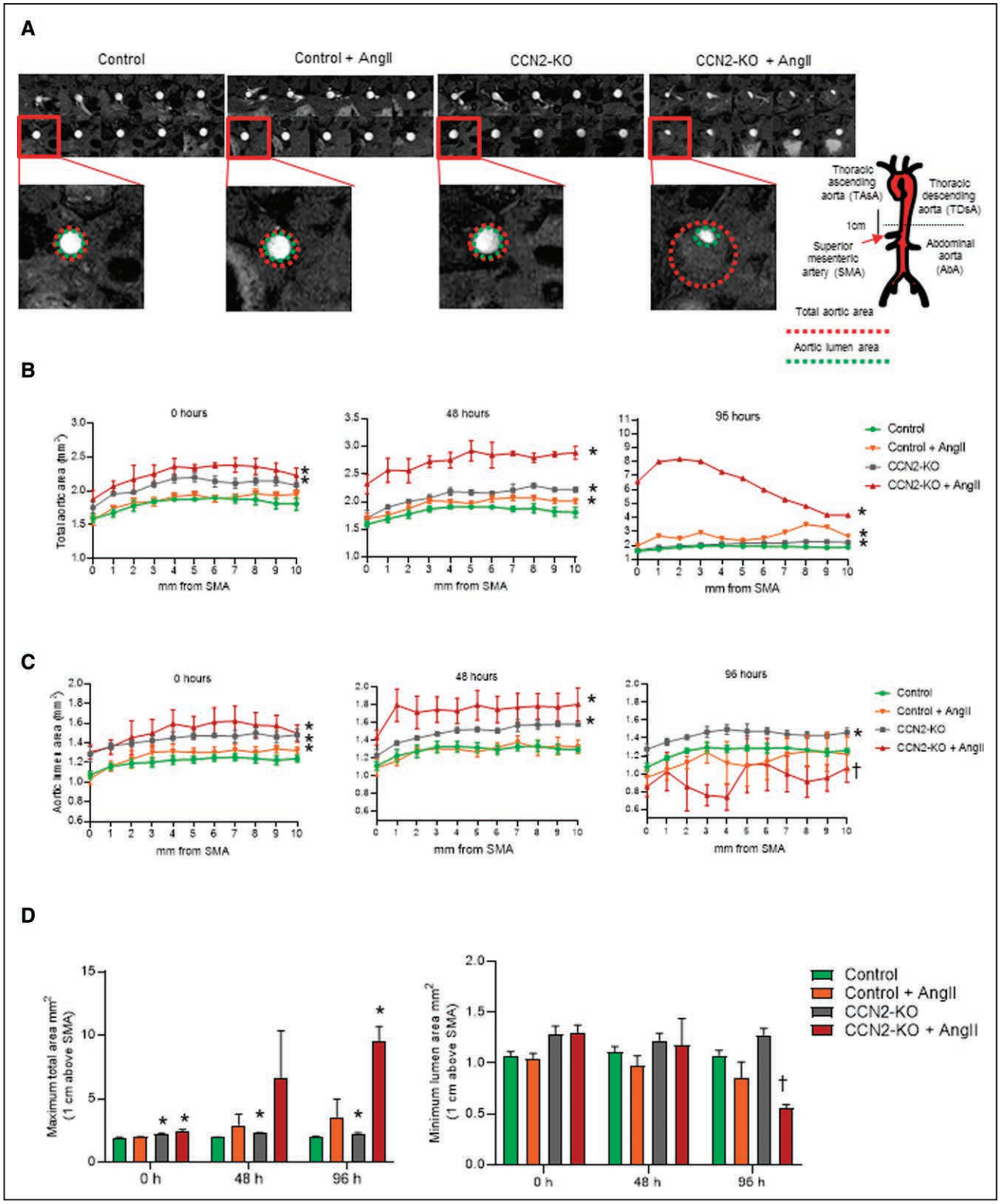

Figure 2. CCN2 (cellular communication network factor 2) deficiency induces early aneurysm formation in mice in response to Ang II (angiotensin II) administration.

Daily magnetic resonance imaging (MRI) assessments allowed a time course analysis showing early aneurysm formation in Ang II–infused CCN2 knockout (CCN2-KO) mice. MRI was followed only until 96 h to avoid excessive mortality. Studies were done in a 1-cm aortic section proximal to the superior mesenteric artery (SMA) origin. **A**, Axial MRI images: increased total aortic area and reduced minimal aortic lumen 48 h after Ang II administration in CCN2-KO mice. Time course quantification of (**B**) total aortic area (please note the different scale for the 96-h panel), (**C**) lumen area, (**D**) maximum total area, and (**E**) minimum lumen area of the 1-cm SMA analyzed. n=4 mice per group. Data are shown as mean±SEM. *P<0.05 increased vs control; †P<0.05 decreased vs control.

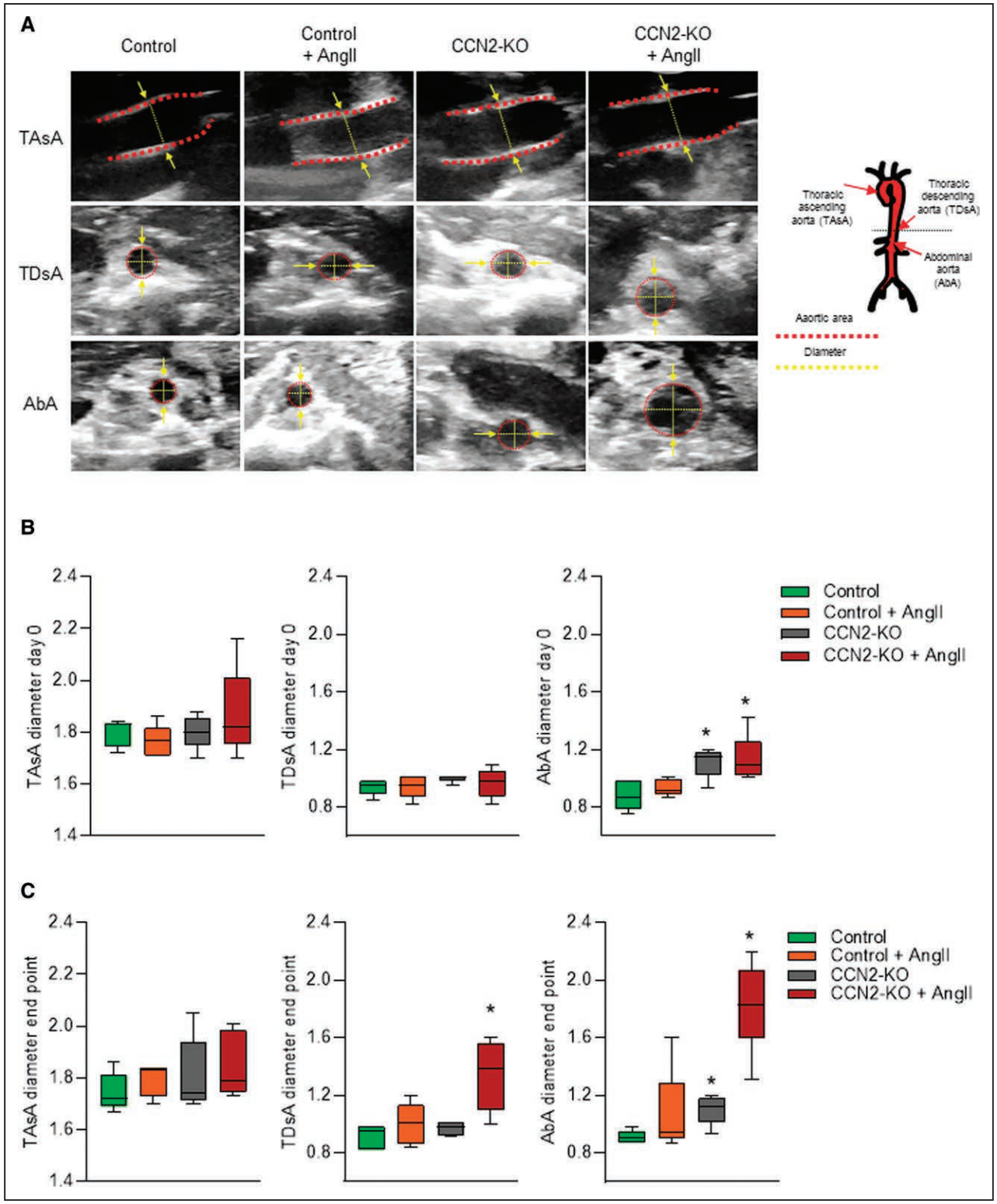

Figure 3. CCN2 (cellular communication network factor 2) deficiency predisposes to TAAA formation in response to Ang II (angiotensin II) administration in mice.

Ultrasound live was done at 0 and 15 d of Ang II administration. **A**, Ultrasound images. **B** and **C** Maximal diameters of TAsA, thoracic descending aorta (TDsA) and AbA at day 0 and endpoint, respectively. Red and yellow dashed lines indicate the lumen boundary and diameter, respectively. n=5 mice per group. Data shown as box and whisker plots, with 75th and 25th percentiles; bars represent maximal and minimal values. *$P<0.05$ increased vs control.

Acquired CCN2 Deficiency–Induced Aortic Elastic Layer Disruption and Protein Changes Are Increased by Ang II Infusion

In Ang II–infused CCN2-KO mice, aneurysmal lesions were characterized by elastic lamina rupture and aortic wall dissection with extravasation of red blood cells outside the muscle layer forming a neo-lumen. Aneurysms also presented inflammatory cell infiltration in the border of the dissected aortic wall, along with reduced muscular layer cellularity (Figure 4A). Van Gieson staining revealed areas with disrupted elastic layers in the aortic wall of CCN2-KO mice, both in untreated and Ang II–treated mice, as well as elastic layer thinning or absence in aortic aneurysm sections (Figure 4B, arrows). Electron microscopy showed structural abnormalities in the elastic lamina of CCN2-KO mouse aortas, such as variable width, discontinuities, and abnormal collagen distribution (Figure 4C). CCN2 protein was readily identified in the aortic vessel wall of control mice by multiple reaction monitoring mass spectrometry analysis (Table S1). Aortic CCN2 protein and gene levels were significantly reduced in CCN2-KO mice (Table S1; Figure S5), demonstrating the efficacy of gene targeting. ACTA2 (alpha smooth muscle actin 2) expression was significantly decreased (by ≈20%) while myosin heavy chain 9 was significantly increased in CCN2-KO mice (Table S1). Interestingly, CCN2 was the most Ang II–upregulated protein in the aortic wall of control mice (2.36-fold increase), suggesting that CCN2 plays an important role in Ang II–induced vascular responses, which might well include protective adaptive structural remodeling.

Acquired CCN2 Deficiency Increases Aortic Metalloproteinase Activity

Ccn2 deletion significantly increased MMP (matrix metalloproteinase) 2 and 9 (MMP-9) activities (Figure 5A) and relative MMP-8 concentrations of both latent and active protein (Figure 5B) compared with control mice. In addition, Ang II augmented MMP-2/9 activities and MMP-8 levels in the presence as well as in absence of CCN2 (Figure 5A and 5B). Furthermore, in situ zymography identified baseline MMP activity in thoracic descending aorta sections of control mice. Ang II administration and *Ccn2* deletion both increased local MMP activity, and the most activity was observed in the aneurysms of Ang II–treated CCN2-KO mice (Figure 5C).

Acquired CCN2 Deficiency Modifies the Ang II–Induced Aortic Gene Expression Pattern

A heat map based on 2-way hierarchical clustering of differentially expressed genes identified by RNA sequencing (RNA-seq) showed clear segregation of CCN2-KO+Ang II mice from the other 3 groups of mice. Additionally, CCN2-KO and control+Ang II mice also had markedly different gene expression patterns compared with vehicle-treated control mice (Figure S6A). Because RNA-seq analysis was performed on pooled samples per group, the power to determine statistical significance of differential gene expression was limited.[24] Results were, therefore, used for hypothesis generation. Thus, the most deregulated genes in response to Ang II in control mice (Table S2) were entered into a GO enrichment analysis[25] (Table S3), which identified myofibril assembly, immune system process, muscle contraction, and skeletal muscle contraction as the most enriched terms at the biological process level in response to Ang II. Hemoglobin complex, myofibril, Z disc, troponin complex, and extracellular space were among the most enriched terms in the cellular component category, while oxygen transporter activity, FATZ binding, Toll-like receptor 4 binding, and actin binding stood out in the molecular function category.

To further unravel the potential contribution of *Ccn2* deletion in the aortic response to Ang II, deregulated genes in response to Ang II in control mice were subdivided in 4 sets of genes according to their differential expression pattern in the absence of CCN2 (Table S4). Next, GO enrichment analysis was performed for each set of genes. Results for genes upregulated by Ang II in control but not in CCN2-KO were mainly related to muscle and myofibrils (Table S5), while those for gene sets 2 and 3 did not show statistically significant results for any GO category. Genes downregulated by Ang II and more decreased in the absence of CCN2 (gene set 4) were mainly related to inflammatory responses (Table S6).

Acquired CCN2 Deficiency Induces Changes in the Aortic Gene Expression Pattern

A new GO enrichment analysis was done using the most deregulated genes obtained from the RNA-seq studies comparing the control versus CCN2-KO groups (67 genes, Q value, <0.5; fold change, >0.6; Table S7). Biological process category results identified C21-steroid hormone, biosynthetic process, cholesterol metabolic process, steroid biosynthetic process, steroid metabolic process, or aldosterone biosynthetic process, as some of the most deregulated terms in the absence of CCN2. In the cellular component level, extracellular region, extracellular space, secretory granule, or mitochondrial crista were present, and in the molecular function category, Toll-like receptor 4 binding, scaffold protein binding, iron ion binding, and oxidoreductase activity (Table S8).

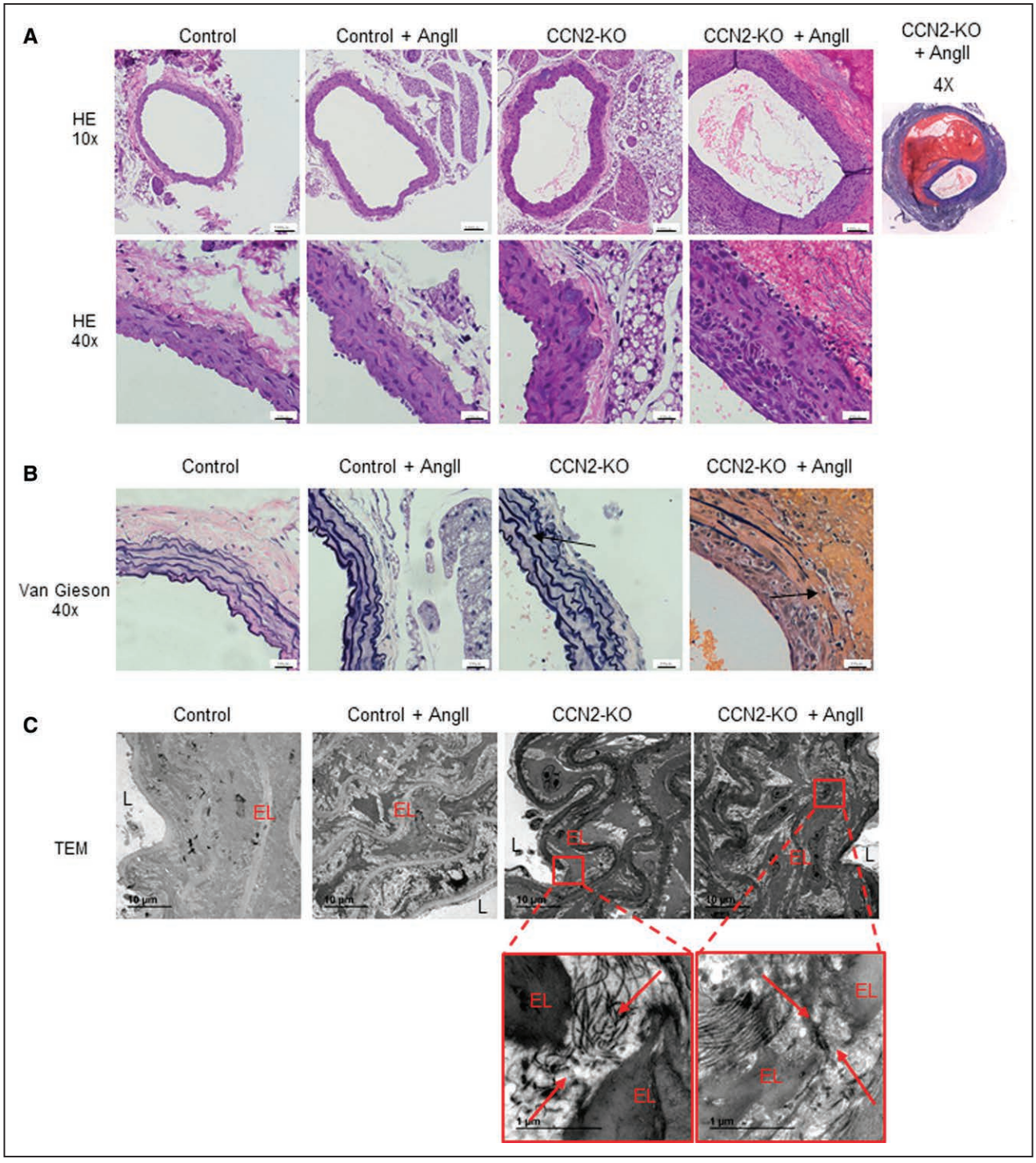

Figure 4. CCN2 (cellular communication network factor 2) deficiency promotes aortic structural and composition changes that are exacerbated in response to Ang II (angiotensin II) administration.
A, Hematoxylin/eosin (HE) at ×10 and their magnification at ×40. *Considering aneurysm size, a lower magnification (×4) image was included in the CCN2 knockout (CCN2-KO)+Ang II group. Aneurysms were characterized by elastic lamina rupture, aortic wall dissection, and extravasation of red blood cells outside the muscle layer. An inflammatory cell infiltration in the border of the dissected aortic wall and reduced cellularity of the muscular layer were also observed. **B**, Van Gieson staining: internal elastic lamina disrupted zones in CCN2-KO mice with or without Ang II infusion (black arrows). **C**, Transmission electron microscopy (TEM; ×2000): aortic elastic layer (EL) disruption in the absence of CCN2, boxed in red and magnified at ×15 000 below. Scale bars: 100 µm at ×10 objective and 20 µm at ×40 in histology; 10 µm at ×2000 and 1 µm at ×15 000 in TEM.

Quantitative real time polymerase chain reaction of key differentially expressed genes identified by RNA-seq analysis in the CCN2-KO group, that is, *S100a8*, *S100a9*, *Spp1*, *Saa3*, and *Ccl8* (Table S7), confirmed higher aortic mRNA expression levels in CCN2-KO than in control mice (Figure S6B).

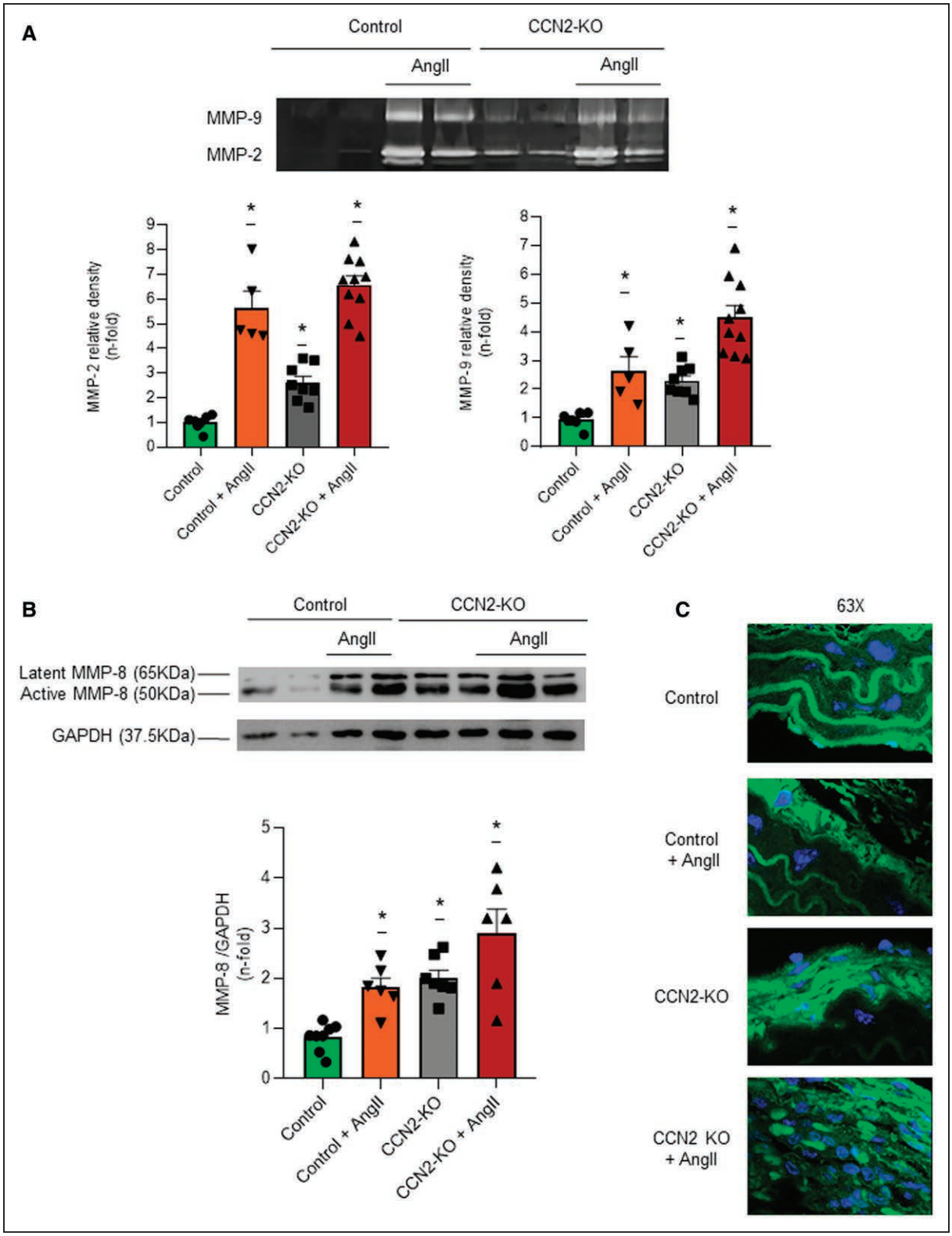

Figure 5. Ccn2 (cellular communication network factor 2) deletion increases vascular MMPs (matrix metalloproteinases). MMP activities or levels were evaluated in total aortic protein lysates. **A**, Representative MMP-2 and MMP-9 gel zymography and (**B**) MMP-8 Western blot (**top**) and corresponding quantification (**bottom**) showing elevated MMP activities and MMP8 levels in CCN2 knockout (CCN2-KO) group and increased following Ang II (angiotensin II) infusion. **C**, In situ MMP activity was evaluated in paraffin-preserved aortas by the DQ-Gelatin fluorogenic substrate assay. Aortas of CCN2-KO and Ang II–infused control mice displayed similar fluorescence intensity (green signal, observed between elastic layers), whereas there was a broadly distributed green signal in Ang II–infused CCN2-KO mice. n=5 to 10 mice per group. Data shown as mean±SEM. *P<0.05 increased vs control.

Mineralocorticoid Receptor Blockade Reduced Aneurysm Formation Induced by Ang II Administration in CCN2-KO

As aldosterone biosynthetic process was one of the most enriched biological process terms in the GO analysis of deregulated genes when comparing untreated CCN2-KO with control mice (Table S8), we investigated the effect of blocking mineralocorticoid receptor by spironolactone. Spironolactone administration did not prevent systolic blood pressure increase (Figure 6A) but tended to reduce mortality (Figure 6B), as well as aneurysm generation (92% versus 60%) in Ang II–infused CCN2-KO mice (Figure 6C). Importantly, spironolactone treatment significantly decreased both MMP-2 and MMP-9 activity (Figure 6D) and markedly improved both phenylephrine-vasoconstrictor and acetylcholine-vasorelaxant responses (Figure S7A and S7B).

DISCUSSION

The most important findings of this study are that near-total silencing of CCN2 expression disrupted the baseline aortic mechanical and structural homeostasis, as well as the adaptive response to Ang II–induced deleterious effects, culminating in life-threatening thoracoabdominal aneurysm formation and dissection. Our data point to a major physiological role of CCN2 as a key ECM component of the aortic wall that is essential for prevention of structural damage, especially in the context of hypertension and inflammation, which is well beyond its traditionally described role as growth factor.

Hypertension resulting from activation of the renin-angiotensin system has previously been associated with both abdominal and thoracic aortic aneurysms in certain strains of mice.[26] To this, we add that the resistance of young C57Bl/6 mice to Ang II–induced aortic aneurysm formation is lost upon silencing of CCN2 expression. In Ang II–infused wild-type mice, CCN2 is rapidly upregulated in the aorta, and this precedes collagen accumulation.[23] Our data confirm and extend this notion, showing that CCN2 is one of the most upregulated factors in the aorta. CCN2 inhibition is known to diminish Ang II–induced ECM overproduction in cultured VSMCs[23] and ameliorated experimental fibrosis.[8–10] However, data on the impact of CCN2 on cardiovascular pathology indicate that effects may vary with specific disease conditions.[15,16] As for the aortic wall, our observations regarding exacerbated aortic aneurysm formation and fatal dissection caused by Ang II in CCN2-deficient mice support a role of CCN2 in adaptive and protective responses essential to maintenance of aortic wall integrity.

Intriguingly, our results may seem to contrast with the recent description of reduced elastase-induced aneurysm formation in CCN2 haploinsufficient (*Ccn2*$^{+/-}$) compared with wild-type mice and with the involvement of CCN2/MMP2 pathway activation in Ang II–induced AAAs in double knockout (*IL12p40*$^{-/-}$; *ApoE*$^{-/-}$) mice.[27] The latter authors previously described that also deletion of only *IL12p40* promotes AAA induction by Ang II infusion. Unfortunately, CCN2 levels were not evaluated in that study, nor in a previous study showing that IL12p40 blockade mitigates elastase-induced AAA.[28,29] Therefore, it remains to be established how far apparent discrepancies between our results and previous studies might relate to differences between the models studied (elastase versus Ang II[30]) or differences between near complete silencing of CCN2 in adult mice and genetic CCN2 haploinsufficiency also during in utero development.

Other CCN family proteins are also involved in cardiovascular disease. Although CCN3 can negatively regulate CCN2 responses,[31] it was protective in experimental aneurysms induced by elastase or Ang II administration.[32] This might relate to the fact that CCN2/CCN3 heterodimers can also elicit cellular responses related to both CCN2 and CCN3 activity.[33] On the contrary, *Ccn4* deletion could mitigate aneurysm severity in *ApoE*$^{-/-}$ mice exposed to high-fat diet and Ang II infusion.[34] Thus, CCN proteins may play multiple and differential roles in maintaining the adult vascular wall architecture in health and disease.

Increased CCN2 expression has been associated with collagen deposition in human atherosclerotic lesions and in thoracic aortic dissection.[5,35] Interestingly, CCN2-KO mice had elastic layer disruption, which might relate to weakening of the meshwork of collagen fibers that should normally prevent rupture of elastin fibers by overextension of the vessel wall. Degradation or loss of elastin fibers significantly alters the mechanical properties of the aorta.[36] Enzymes involved in degradation of elastin and collagenous ECM components, such as MMPs and collagenases, are critically involved in aneurysm formation and rupture.[37] We found that acquired CCN2 deficiency increased MMP-2 and MMP-9 activity, as well as MMP-8 expression. The elevated MMP activity was located at sites of disruption and loss of elastin fibers. This strongly suggests that MMP dysregulation is also an important mechanism by which CCN2 loss, already prior Ang II infusion, led to aortic wall destabilization and susceptibility to aneurysm formation.

Another mechanism potentially involved in the detrimental effects induced by CCN2 deficiency might relate to changes in elastin-contractile unit proteins and altered VSMC properties. Both changes could negatively affect the ability of aortic VSMCs to respond to pulsatile blood flow in pathological conditions and increase aortic dissection susceptibility.[38] Acquired CCN2 deficiency interfered with the aortic expression of several cytoskeletal proteins, including ACTA2—a contractile element of VSMCs relevant to aneurysm formation. Heterozygous *Acta2* mutations cause familial thoracic aortic aneurysms.[38] ACTA2 knockout mice exhibited progressive aortic root dilation,

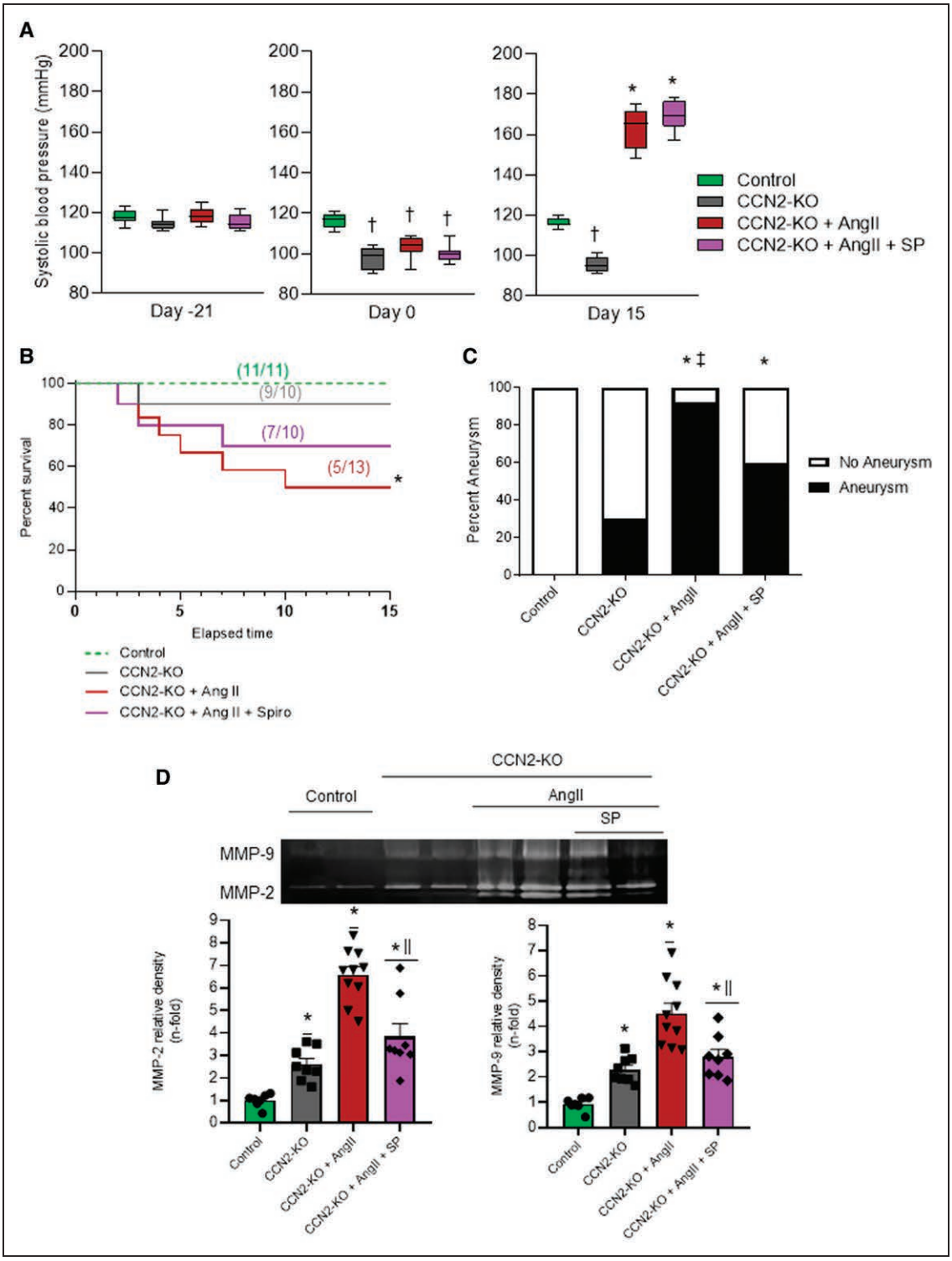

Figure 6. Mineralocorticoid receptor blockade by spironolactone (SP) improves survival, aneurysm appearance rates observed in Ang II (angiotensin II)–infused CCN2 knockout (CCN2-KO) mice.
SP (50 mg/kg per day, intraperitoneal in alternate days) treatment in CCN2-KO+Ang II group was started at the time of *Ccn2* deletion until the end of follow-up. **A**, Kaplan-Meier survival curve showed less mortality in SP-treated compared with untreated Ang II–infused CCN2-KO mice. **B**, SP treatment decreased the percentage of aneurysm appearance in the CCN2-KO+Ang II group. **C**, SP did not prevent Ang II–induced blood pressure level elevation. Data shown as box and whisker plots, with 75th and 25th percentiles; bars represent maximal and minimal values. n=8 to 10 mice per group. **D**, Representative MMP (matrix metalloproteinase) 2 and 9 gel zymography (**top**) and quantification (**bottom**) showing a significant MMP2 and MMP9 activity reduction in CCN2-KO+Ang II group treated with SP. n=7 to 10 mice per group. **P*<0.05 increased vs control. †*P*<0.05 decreased vs control. ‡*P*<0.05 vs CCN2-KO. ‖*P*<0.05 vs CCN2-KO+Ang II.

VSMC phenotype alterations, and aneurysm formation and dissection in response to Ang II.[39,40] Of note, our RNA-seq data revealed increased *Spp1* levels in the aorta of Ang II–infused *Ccn2*-deleted mice. *Spp1* encodes osteopontin—a key regulator of VSMC phenotype associated with (progression of) aneurysmatic aorta dilatation.[41] In agreement with previous observations that recombinant CCN2 stimulated migration and proliferation of rat VSMCs,[42] *Ccn2* deletion decreased wound healing and cell migration in cultured murine VSMCs. Our RNA-seq study showed that Ang II increased the aortic expression of *Saa3*, *S100a8*, *S100a9*, and *Ccl8*, which are all involved in vascular damage and inflammation,[43–46] and that CCN2 deficiency resulted in even higher expression of these genes, supporting a potential role in aneurysm formation. Elevated SAA and S100A8/S100A9 complexes were observed in human cardiovascular diseases including acute aortic dissection, aneurysms, or atherosclerosis.[46,47] Moreover, *Saa* deficiency protected *ApoE*$^{-/-}$ mice from Ang II–induced abdominal aortic aneurysm formation.[48] Interestingly, the systemic administration of a high dose of CCN2-IV failed in preventing aneurysm formation and rupture, suggesting that endogenous, local CCN2 expression is essential for the proper function, maintenance, and adaptive responses of the aortic vascular wall.

Several observational studies reported dysregulated CCN2 expression in both nonsyndromic[20] and syndromic human thoracic aortic aneurysms, as observed in several heritable connective tissue disorders such as Marfan, Loeys-Dietz, Ehlers-Danlos, aneurysms-osteoarthritis, and the arterial tortuosity syndrome.[49] These pathologies are characterized by altered connective tissue, resulting in perturbed ECM assembly, maintenance, and homeostasis in various organ systems.[49] In some of these syndromes, aneurysms have been linked to TGFβ pathway dysregulation. This is of particular interest since CCN2 is a direct transcriptional target and important mediator of TGFβ effects. Paradoxically, although increased TGFβ signaling was suggested to cause aortic aneurysm development, inhibition of TGFβ in experimental aneurysm models was not protective.[50–52] In fact, TGFβ blockade exacerbated aneurysm formation and dissection in several experimental models.[50,53,54] Also, although it has been suggested to consider Ang II receptor 1 blockade in patients with thoracic aortic aneurysms, based on its ability to inhibit TGFβ production and to attenuate aortic dilation in mice, clinical results are thus far controversial, with losartan not providing any benefit in Marfan patients, while irbesartan was associated with a reduced rate of aortic dilatation in children and young adults with this syndrome.[55,56] How these observations might relate to alteration of CCN2 expression and activity remains to be established.

Interestingly, human abdominal aortic aneurysms are associated with downregulation of transcripts encoded by a 16-Mbp segment between cytogenetic bands q22.1 and q23.2 of chromosome 6. Since CCN2 was found to be highly downregulated,[57] this could be directly related to aneurysm generation in these patients. The observed similarities between CCN2 deletion and TGFβ pathway disruption with respect to aortic aneurysm development may provide a novel framework for further exploration of the pathogenesis of aortic aneurysm formation and rupture.

Finally, RNA-seq analyses identified aldosterone as a potential contributor to aneurysm formation. Primary aldosteronism patients may develop aortic dissection,[58] and mineralocorticoid receptor blockers may slow aortic aneurysm progression.[59] Similarly, in mice fed a high-salt diet, a mineralocorticoid receptor agonist caused aortic aneurysm formation and rupture.[60] We observed that mineralocorticoid receptor inhibition by spironolactone slightly reduced aneurysm formation and death from aneurysm rupture following Ang II infusion in CCN2-KO mice, without preventing blood pressure elevation. Accordingly, another mineralocorticoid receptor antagonist, eplerenone, significantly reduced aneurysm development induced by Ang II and β-aminopropionitrile in mice, also without preventing blood pressure elevation.[59] Although spironolactone effects on regulation of other ECM components, such as collagen, have been previously described,[61] the present results strengthen specifically the relation between aldosterone and CCN2 and support the involvement of the aldosterone pathway in aortic aneurysm generation and the potential of mineralocorticoid receptor antagonists for prevention of arterial aneurysm formation and progression.

PERSPECTIVES

Our studies have revealed the crucial role of CCN2 in the maintenance of structural and functional aortic wall homeostasis and as an indispensable factor in adaptive remodeling for preservation of its integrity under (Ang II induced) stress conditions. The near-total absence of CCN2 affected the baseline condition of the aortic wall and predisposed the mice to a pathological response to Ang II, favoring aneurysm formation and rupture, which could not be rescued by exogenous CCN2 infusion. With respect to the lack of safety issues thus far reported for neutralizing anti-CCN2 antibodies currently in phase 2 and 3 clinical trials for various conditions, it should be noted that such treatment cannot be expected to reduce CCN2 availability in the aortic wall to levels near those obtained in our KO mice. Considering the well-established connection between TGFβ signaling and CCN2, our data further support a critical role for the TGFβ/CCN2 axis in the protection against the development and progression of aortic aneurysms and possibly other cardiovascular disease related to connective tissue disorders.

ARTICLE INFORMATION

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Affiliations

Molecular and Cellular Biology in Renal and Vascular Pathology, IIS-Fundación Jiménez Díaz-Universidad Autónoma Madrid, Spain (R.R.R.-D., A.T.-M., M.O., S.R.-M., M.R.-O.). Red de Investigación Renal, Instituto de Salud Carlos III, Madrid, Spain (R.R.R.-D., M.O., S.R.-M., P.C.-O., A.O., M.A.B., M.R.-O.). Department of Allergy and Immunology, FIIS-Fundación Jiménez Díaz, UAM, Asma, Reacciones Adversas y Alérgicas Network. Madrid, Spain (V.E.). Unit of Cardiovascular and Renal Research, Department of Molecular Medicine, University of Southern Denmark, Odense, Denmark (L.B.S). Department of Pharmacology, UAM, Instituto de Investigación-Hospital Universitario La Paz, IdiPaz, Ciber Cardiovascular, Madrid, Spain (R.R.-D., A.M.B.). Department of Pathology, University Medical Center Utrecht, the Netherlands (L.L.F.). IIS-Fundación Jiménez Díaz-Universidad Autónoma Madrid, Spain (P.C.-O., A.O., J.E., R.G.). Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Spain (J.E.). Department of Medicine, University of Cambridge, United Kingdom (Z.M.). Institut National de la Sante et de la Recherche Medicale, France, Cambridge, United Kingdom (Z.M.). Department of Nephrology, Instituto de Investigación-Hospital Universitario La Paz, Madrid, Spain (M.A.B.).

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Disclosures

None.

Supplemental Material

Figures S1–S9
Tables S1–S8

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