

Relaxin receptor deficiency promotes vascular inflammation and impairs outward remodeling in arteriovenous fistulas

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ABSTRACT: The pathophysiology of arteriovenous fistula (AVF) maturation failure is not completely understood but impaired outward remodeling (OR) and intimal hyperplasia are thought to be contributors. This adverse vascular response after AVF surgery results from interplay between vascular smooth muscle cells (VSMCs), the extracellular matrix (ECM), and inflammatory cells. Relaxin (RLN) is a hormone that acts on the vasculature *via* interaction with RLN/insulin-like peptide family receptor 1 (RXFP1), resulting in vasodilatation, ECM remodeling, and decreased inflammation. In the present study, we evaluated the consequences of RXFP1 knockout (*Rxfp1*^{-/-}) on AVF maturation in a murine model of AVF failure. *Rxfp1*^{-/-} mice showed a 22% decrease in vessel size at the venous outflow tract 14 d after AVF surgery. Furthermore, a 43% increase in elastin content was observed in the lesions of *Rxfp1*^{-/-} mice and coincided with a 41% reduction in elastase activity. In addition, *Rxfp1*^{-/-} mice displayed a 6-fold increase in CD45⁺ leukocytes, along with a 2-fold increase in monocyte chemoattractant protein 1 (MCP1) levels, when compared with wild-type mice. *In vitro*, VSMCs from *Rxfp1*^{-/-} mice exhibited a synthetic phenotype, as illustrated by augmentation of collagen, fibronectin, TGF- β , and platelet-derived growth factor mRNA. In addition, VSMCs derived from *Rxfp1*^{-/-} mice showed a 5-fold increase in cell migration. Finally, RXFP1 and RLN expression levels were increased in human AVFs when compared with unoperated cephalic veins. In conclusion, RXFP1 deficiency hampers elastin degradation and results in induced vascular inflammation after AVF surgery. These processes impair OR in murine AVF, suggesting that the RLN axis could be a potential therapeutic target for promoting AVF maturation.—Bezhaeva, T., de Vries, M. R., Geelhoed, W. J., van der Veer, E. P., Versteeg, S., van Alem, C. M. A., Voorzaat, B. M., Eijkelkamp, N., van der Bogt, K. E., Agoulnik, A. I., van Zonneveld, A.-J., Quax, P. H. A., Rotmans, J. I. Relaxin receptor deficiency promotes vascular inflammation and impairs outward remodeling in arteriovenous fistulas. *FASEB J.* 32, 6293–6304 (2018). www.fasebj.org

KEY WORDS: hemodialysis vascular access • RXFP1 • macrophages • VSMC • extracellular matrix

ABBREVIATIONS: α SMA, SM- α -actin; AVF, arteriovenous fistula; CCR2, chemokine receptor type 2; DAB, 3,3'-diaminobenzidine; ECM, extracellular matrix; FBS, fetal bovine serum; GR1, granulocytic marker; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IEL, internal elastic lamina; IH, intimal hyperplasia; MAC3, macrophage marker; MCP1, monocyte chemoattractant protein 1; NHS, normal human serum; OR, outward remodeling; RLN, relaxin; RXFP1, relaxin/insulin-like peptide family receptor 1; VSMC, vascular smooth muscle cell; WT, wild type

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A properly functioning vascular access site is a crucial lifeline for patients required to undergo hemodialysis. Although an arteriovenous fistula (AVF) remains the vascular access of choice, the frequency of primary AVF failure is ~40% (1). Moreover, the primary patency rates 1 yr after creation do not exceed 60% (2), making vascular access-related complications one of the most common causes of hospitalization and morbidity in hemodialysis patients.

Although the pathophysiology of AVF maturation failure is incompletely understood, it is well established that both intimal hyperplasia (IH) and outward remodeling

cava was transected followed by a fixation with 4% formalin through an intracardiac perfusion. The tissue was embedded in paraffin and 5 μm sections of the venous outflow tract were cut perpendicular to the vein at intervals of 150 μm .

Morphometric and histologic analysis

All immunohistochemical and histochemical staining and measurements were performed on the first 3 venous cross-sections

downstream from the anastomosis. Weigert's elastin stain was used for the morphometric measurements. The area of the internal elastic lamina (IEL) was measured to assess the degree of OR (Fig. 1A). IH was calculated by subtracting the luminal area from the area within the IEL. Composition of the AVF lesions was further evaluated by staining for total leukocytes (CD45, 1:200; 550539; BD Biosciences, Franklin Lakes, NJ, USA) and macrophages [macrophage marker (MAC3), 1:200, 550292; BD Biosciences] in combination with CCR2 (chemokine receptor type 2) for a proinflammatory phenotype (1:400; ab32144; Abcam,

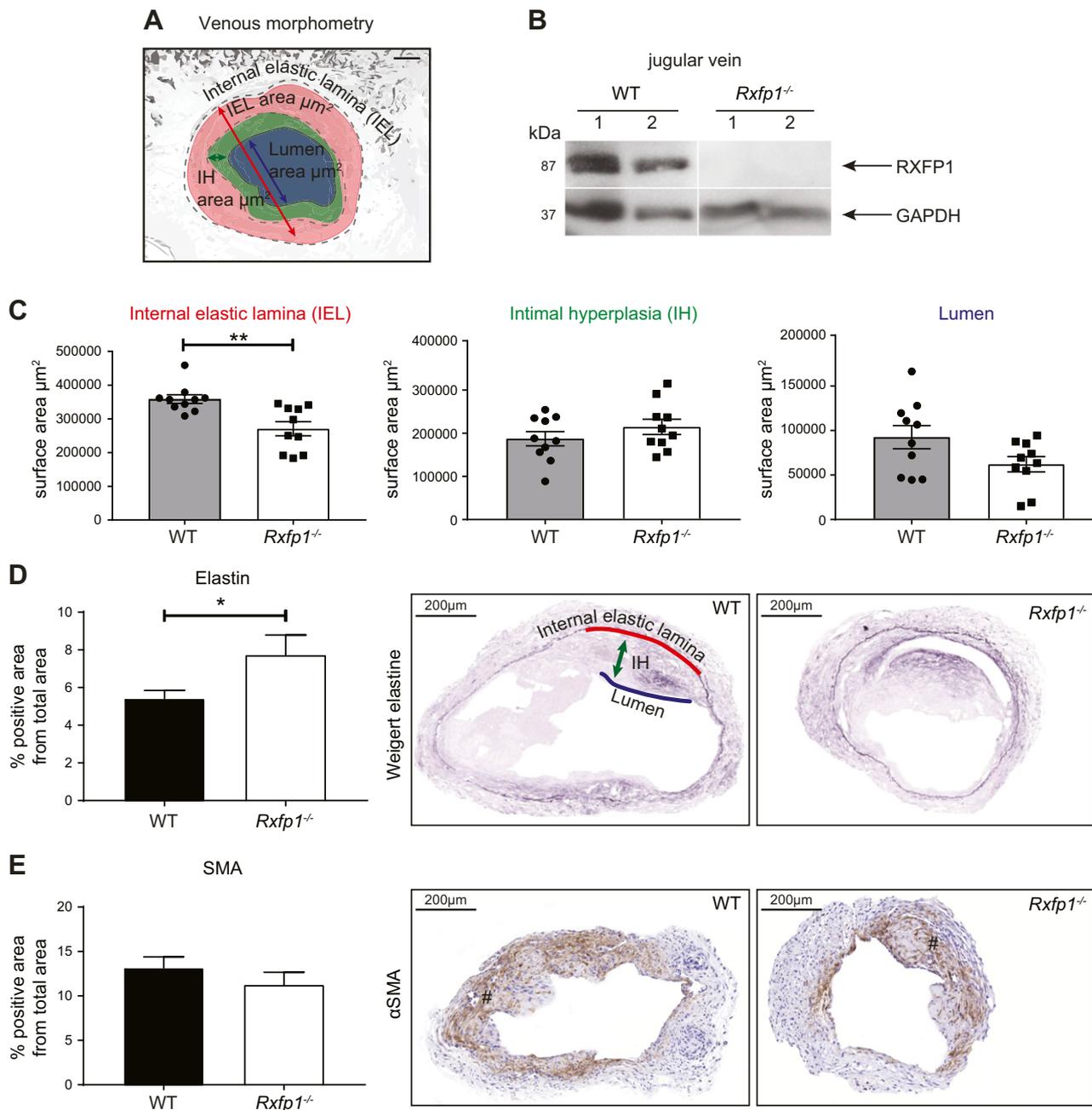


Figure 1. Morphometry and histology of the venous outflow tract of the AVF in *Rxfp1*^{-/-} and WT mice 14 d after surgery. **A**) The area within the IEL (red) reflects the degree of OR of the venous outflow tract. IH (green) was calculated by subtracting the luminal area (blue) from the area within the IEL. **B**) Western blot of the jugular vein confirms the absence of RXFP1 protein in RXFP1-deficient mice. GAPDH was used as a loading control. **C**) Quantification of histomorphometry. RXFP1-deficient mice displayed decrease in the area of IEL (22%, $P = 0.002$) and lumen (31%, $P = 0.14$), whereas IH was comparable to that of WT mice. **D**) Increase in elastin deposition in the AVF lesions from *Rxfp1*^{-/-} mice. **E**) α SMA staining shows area of IH which did not differ between the groups. Weigert elastine staining was used to determine histomorphometrical parameters of the vessel. * $P < 0.05$; ** $P < 0.005$ ($n = 10/\text{group}$).

Cambridge, United Kingdom) or CD206 (1:1000, ab64693; Abcam) for an anti-inflammatory phenotype, neutrophils [granulocytic marker (GR1), 1:300; from G. Kraal, VU University Medical Center, Amsterdam, The Netherlands], T-lymphocytes (CD3, 1:300, ab32144; Abcam), and monocyte chemoattractant protein 1 (MCP1, 1:300, sc-1784; Santa Cruz Biotechnology, Dallas, TX, USA). VSMCs [SM- α -actin (α SMA), 1:1000, M0851; Agilent Technologies, Santa Clara, CA, USA] were stained in a combination with Ki67 (1:200, 550609; BD Biosciences) to detect proliferating VSMCs.

All slides were digitized using an automated slide scanner (Pannoramic MIDI; 3DHistech, Budapest, Hungary). For the immunohistochemical analysis of the MAC3/CD206, MAC3/CCR2, and α SMA/Ki67 staining, the number of positive cells was counted in 3 random fields of view at $\times 400$ magnification, from which the mean was calculated. Quantification of CD45⁺, GR1⁺, CD3⁺, and MCP1 staining was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) by calculating percentage of 3,3'-diaminobenzidine (DAB) positive area from the total vessel area.

Cell culture

Primary arterial and venous VSMCs were isolated from the carotid artery and vena cava of C57BL/6 and *Rxfp1*^{-/-} mice ($n = 4$ /group), respectively. Connective tissues were removed and vessels cut open. The endothelial monolayer was detached by gently scraping with sterile surgical forceps. The carotid artery and caval vein were dissected into small pieces and plated onto fibronectin-coated Petri dishes (0.1 mg/ml) 100 or 60 mm in diameter. After a 14 d culture in DMEM supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, cells were trypsinized and replated onto 6- or 12-well plates and cultured for 7 d. At 80–90% confluence, VSMCs were trypsinized and seeded at the required density for further functional assays.

Western blot

Proteins from tissue lysates were harvested in RIPA buffer and subjected to SDS-PAGE. A bicinchronic acid assay was performed to ensure equal protein loads in each sample. RXFP1 was detected using primary antibodies (1:500, AP23446SU-S; OriGene Technologies, Rockville, MD, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000, 5174S, Cell Signaling Technology, Danvers, MA, USA) was used as a loading control. All tests used Bio-Rad TGX precast gels and were blotted on nitrocellulose 0.2 μ m using the Bio-Rad TurboBlot system (Hercules, CA, USA).

RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA was extracted from VSMCs using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was reverse transcribed using the M-MLV First-Strand Synthesis System (Thermo Fisher Scientific) and used for quantitative analysis of mouse genes (Supplemental Table 1) with SYBR Green Master Mix (Thermo Fisher Scientific). Murine β -actin was used as the standard housekeeping gene. The relative mRNA expression levels were determined using 2^{- $\Delta\Delta$ Ct} method.

VSMC migration and haptotaxis assays

Primary arterial and venous VSMCs from control and *Rxfp1*^{-/-} mice were grown to confluence and made quiescent in cultured

medium supplemented with 1% FBS for 24 h. Cells were detached from the surface and suspended at a concentration of 100,000 cells/ml in culture medium supplemented with 1% FBS. Migration was assayed with inserts having 8 μ m pores in 24-well chemotaxis chambers using the CytoSelect Cell Migration Assay Kit (Cell Biolabs, San Diego, CA, USA). Haptotaxis was assayed by plating cells into Transwell inserts with collagen I-coated inserts (CytoSelect Cell Haptotaxis Assay; Cell Biolabs). After 16 h, migratory VSMCs were lysed and labeled with fluorescent dye according to the manufacturer's instructions. A gradient of 20% FBS was used. Quantification was performed by reading fluorescence at 480/520 nm.

VSMC proliferation assay

Murine VSMCs, explanted from aortas and veins of control or *Rxfp1*^{-/-} mice, were cultured as described in the previous section, divided into aliquots, and plated on a 96-well plate. Cells were quantified 24 h later using the CyQuant Direct Cell Proliferation Assay according to the manufacturer's instructions (Thermo Fisher Scientific).

Elastase activity assay

Elastase activity was measured on total tissue lysates isolated from *Rxfp1*^{-/-} and WT mice ($n = 10$ per group) using the Enz-Chek Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 10 μ m sections of AVF were lysed in RIPA buffer and a bicinchronic acid assay was performed to ensure equal loading of protein in each sample. DQ elastin, a soluble bovine neck ligament elastin labeled with BODIPY FL dye (Thermo Fisher Scientific), was used as a substrate to quench the conjugate's fluorescence. In the presence of elastase, the nonfluorescent substrate is digested, yielding highly fluorescent fragments, which were measured with a microplate reader at an excitation wavelength of 480 \pm 20 nm and an emission wavelength of 528 \pm 20 nm.

Collagen assay

Collagen content was analyzed using the QuickZyme Total Collagen Assay Kit (QuickZyme Biosciences, Leiden, The Netherlands) on samples of formaldehyde-fixed, paraffin-embedded venous tissues isolated from *Rxfp1*^{-/-} and WT mice ($n = 10$ per group). In brief, 5–10 tissue sections (10 μ m long) were hydrolyzed by overnight incubation at 95°C in a heat block. Upon hydrolysis, without any pretreatment, 35 μ l was used for collagen quantification using the QuickZyme Total Collagen Assay Kit (assay time 90 min). The assay measured the total amount of hydroxyproline, a representative for all collagen types, present in the sample. The assay results in a chromogen with a maximum absorbance of 570 nm.

Human tissue specimens

Human cephalic veins before AVF surgery ($n = 3$) and during surgical revisions of AVF ($n = 3$) were obtained at the Leiden University Medical Center in accordance with guidelines set out by the Code for Proper Secondary Use of Human Tissue enforced by the Dutch Federation of Biomedical Scientific Societies, which conform to the principles outlined in the Declaration of Helsinki. Specimens were fixed with formalin, embedded in paraffin, and sectioned. The human AVF sections were stained with α SMA (1:1000; M0851; Agilent Technologies), RLX2 (RLN2, 1:1000, ab183505; Abcam), and RXFP1 (1:4000, AP23446SU-S; OriGene Technologies).

For RXFP1 antibody validation, 3 human fistula samples were incubated with the primary antibody or isotype control, both at 1:4000 dilution. Human endometrium was used as a positive control. Briefly, after deparaffinization step endogenous peroxidase was suppressed by incubation in 1% H₂O₂ for 15 min at room temperature. Sections were then blocked with 5% PBSA (NaPO₄, NaCl, NaAz and 1% bovine serum albumin) and 5% normal human serum (NHS) for 30 min. Next, sections were incubated overnight at room temperature with the primary antibody or isotype control, diluted at 1:4000, in 1% PBS buffer without calcium and magnesium, supplemented with 5% NHS. After rinsing in PBS for 3 cycles of 5 min each, sections were incubated with rabbit Evison (Agilent) and 5% NHS for 30 min at room temperature. Specific signals were detected using DAB (MilliporeSigma, Burlington, MA, USA) as chromogen. The reaction was halted by rapidly rinsing the sections in tap water before subjecting tissue samples to conventional hemalaun counterstaining.

For immunofluorescent staining of double-positive α SMA⁺ and RXFP1⁺ cells, after exposure to primary antibodies, tissue sections were counterstained with Alexa 568-conjugated goat antimouse IgG2a (1:250; A21134; Molecular Probes, Eugene, OR, USA) for α SMA, and Alexa 488-conjugated secondary goat antirabbit IgG (1:250; A11008; Molecular Probes) for RXFP1. Secondary antibodies were diluted in 1% PBSA; 5% NHS was added to prevent nonspecific binding. Nuclei were visualized with ProLong Gold Antifade Mountant with DAPI (P369; Thermo Fisher Scientific) (Supplemental Fig. 6).

Statistical analysis

Results are expressed as means \pm SEM and values of $P < 0.05$ were considered statistically significant. Student's *t* tests and the Mann-Whitney *U* tests for parametric and nonparametric data, respectively, were used as appropriate. For all *in vitro* experiments, 4 samples from different animals were used (biological $n = 4$). Every experiment within each animal was performed in triplicates (experimental triplicate $n = 3$).

RESULTS

Surgical outcome and patency

In total, AVF surgery was successfully performed on 23 mice (WT: $n = 12$; $Rxfp1^{-/-}$: $n = 11$), of which 2 (17%) from the WT group and 1 (9%) from the $Rxfp1^{-/-}$ group were occluded 14 d after surgery. The occluded AVFs were excluded from morphometric analysis, yielding 10 animals/group. The body weight of the mice was similar in both groups (29 ± 3 g) and remained stable until the end of experiment.

Effect of RXFP1 deficiency on AVF maturation

To study the effect of RXFP1 deficiency on AVF maturation, we created AVFs in WT and $Rxfp1^{-/-}$ mice in an end-to-side fashion between the jugular vein and carotid artery as previously described (34). Given that most of the stenotic lesions in human AVFs occur in the venous outflow tract, we harvested the first 3 consecutive venous sections downstream of the anastomotic area for morphometric analysis. The impact of RXFP1 deficiency on vessel morphology was evaluated by assessing both intimal and

luminal area as well as the area within the IEL, reflecting the degree of OR of the venous outflow tract (Fig. 1A). The absence of the RXFP1 protein in $Rxfp1^{-/-}$ mice was confirmed by using Western blot to analyze total tissue lysates isolated from jugular veins (Fig. 1B). Mice deficient in RXFP1 showed a 22% decrease of the IEL area at the venous outflow tract when compared with WT mice ($P = 0.002$). The luminal area of the venous outflow tract in $Rxfp1^{-/-}$ mice was 31% smaller when compared with control mice, although this difference was not significant ($P = 0.14$). The intimal area did not differ between groups (Fig. 1C). Arterial blood pressure and compliance did not significantly differ between $Rxfp1^{-/-}$ and WT mice (Supplemental Fig. 1).

In terms of ECM remodeling, we observed a 43% increase in elastin content in $Rxfp1^{-/-}$ mice over that of WT mice ($P = 0.04$) (Fig. 1D), whereas collagen production was similar in both groups (Supplemental Fig. 2). In both WT and $Rxfp1^{-/-}$ mice, the majority of intimal cells expressed α SMA, indicating that these cells are primarily of VSMC or fibroblastic origin (Fig. 1E).

RXFP1 deficiency resulted in increased inflammation of AVF lesions

Immunohistochemical analysis of sections obtained from the venous outflow tract showed a 6-fold increase in the number of CD45⁺ leukocytes in lesions of $Rxfp1^{-/-}$ mice when compared with WT mice ($P = 0.02$) (Fig. 2A). As shown in Fig. 2B, C, further characterization of the leukocyte subpopulations revealed a 4-fold increase in the number of proinflammatory Mac3⁺/CCR2⁺ macrophages ($P = 0.02$) and a 43% increase in the Mac3⁺/CD206⁺ anti-inflammatory macrophages in the lesions from $Rxfp1^{-/-}$ mice ($P = 0.04$). The number of GR1⁺ neutrophils was 3-fold higher in $Rxfp1^{-/-}$ mice than in WT mice ($P = 0.05$) (Fig. 2D). The number of CD3⁺ T-lymphocytes in the venous outflow tract was not affected by RXFP1 deficiency (Supplemental Fig. 3). At the level of cytokine production within the lesions, MCP1 expression was 2-fold greater in $Rxfp1^{-/-}$ mice than in WT mice ($P = 0.01$) (Fig. 3).

In vitro, activated macrophages that were differentiated from monocytes derived from $Rxfp1^{-/-}$ mice produced a similar amount of cytokines as WT mice did; ELISA analysis demonstrated comparable expression of MCP1, IL12, and IL10 proteins (data not shown).

Phenotypic switch of arterial and venous VSMCs upon RXFP1 deletion

The impact of RXFP1 deficiency on the VSMC phenotype was studied *in vitro* using primary arterial and venous VSMCs isolated from the aorta and caval vein, respectively, of $Rxfp1^{-/-}$ or WT mice. Phenotypic difference of arterial and venous VSMCs was confirmed by mRNA levels of ephrin B2, an established embryological marker of arterial origin (35). EphrinB2 was increased in arterial VSMCs from both WT and $Rxfp1^{-/-}$ mice following 2 wk of culture, but not in venous VSMCs (Fig. 4A). Further characterization of these VSMCs confirmed that the cells

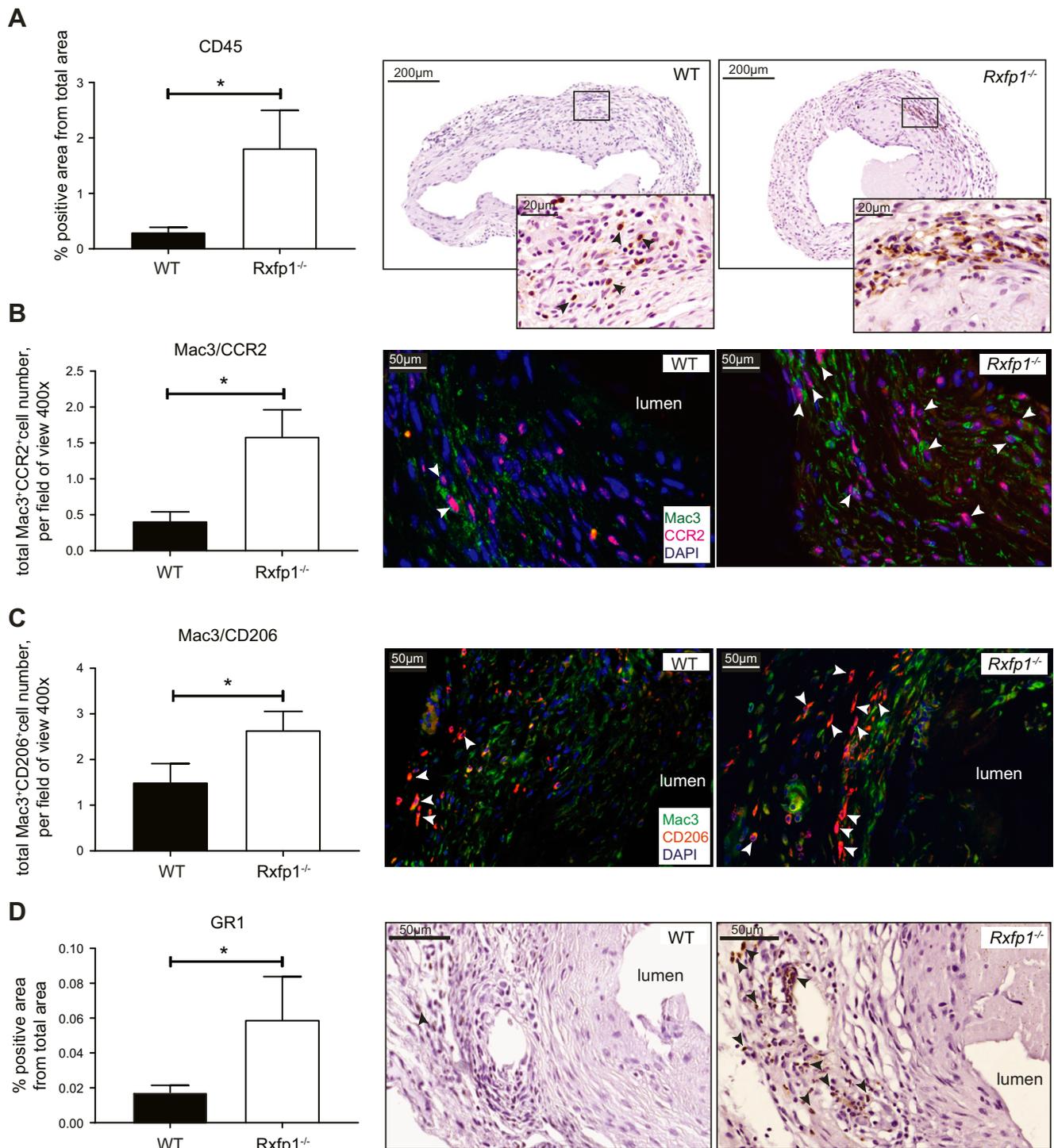


Figure 2. Effects of RXFP1 deficiency on inflammatory status *in vivo*. Quantification and immunohistochemical staining of CD45⁺ leukocytes (black arrows) (A), MAC3⁺/CCR2⁺ macrophages (B), MAC3⁺/CD206⁺ macrophages (white arrows) (C), and GR1⁺ neutrophils (black arrows) (D) in the AVF lesions 14 d after surgery. All cell populations were higher in the lesions from *Rxfp1*^{-/-} mice compared with those in the WT. **P* < 0.05 (*n* = 10/group).

maintained a highly differentiated state, as evidenced by the maintenance of myosin heavy chain, calponin, and caldesmon expression at 2 wk of culture (Fig. 4B).

Because vascular injury and remodeling trigger the phenotypic switch of VSMCs from a contractile to a synthetic state, mRNA expression levels of several genes associated with this switch were measured (36, 37). Arterial VSMCs from *Rxfp1*^{-/-} mice expressed markedly higher

levels of the synthetic VSMC markers type I collagen (38) and fibronectin (39) than did VSMCs derived from WT mice [5-fold (*P* = 0.03) and 10-fold (*P* = 0.04), respectively]. Similarly, the mRNA expression levels of both type I collagen and fibronectin were higher in venous VSMCs harvested from *Rxfp1*^{-/-} mice [3-fold (*P* = 0.05) and 4-fold (*P* = 0.04), respectively]. Furthermore, genes linked with cellular proliferation, such as TGF-β1 (40) and platelet-derived

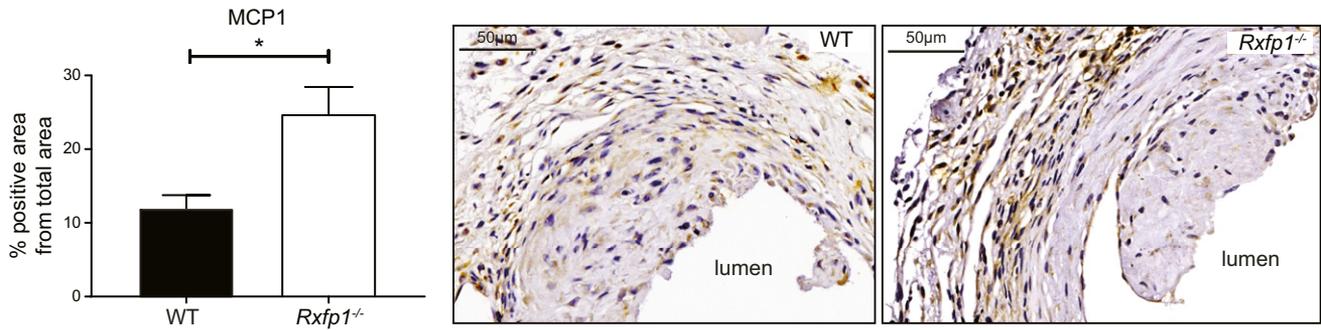


Figure 3. Accumulation of MCP1 in the AVF lesions from *Rxfp1*^{-/-} and WT mice 14 d after surgery. RXFP1 deficiency resulted in greater MCP1 expression (brown DAB signal) than that found in WT mice. **P* < 0.05 (*n* = 10/group).

growth factor (41), displayed on average 3-fold higher expression levels in both arterial and venous VSMCs isolated from *Rxfp1*^{-/-} mice than did WT mice (Fig. 4C).

To further examine whether RLN deficiency impacts VSMC function, we performed cellular migration assays. These studies revealed that arterial VSMCs derived from

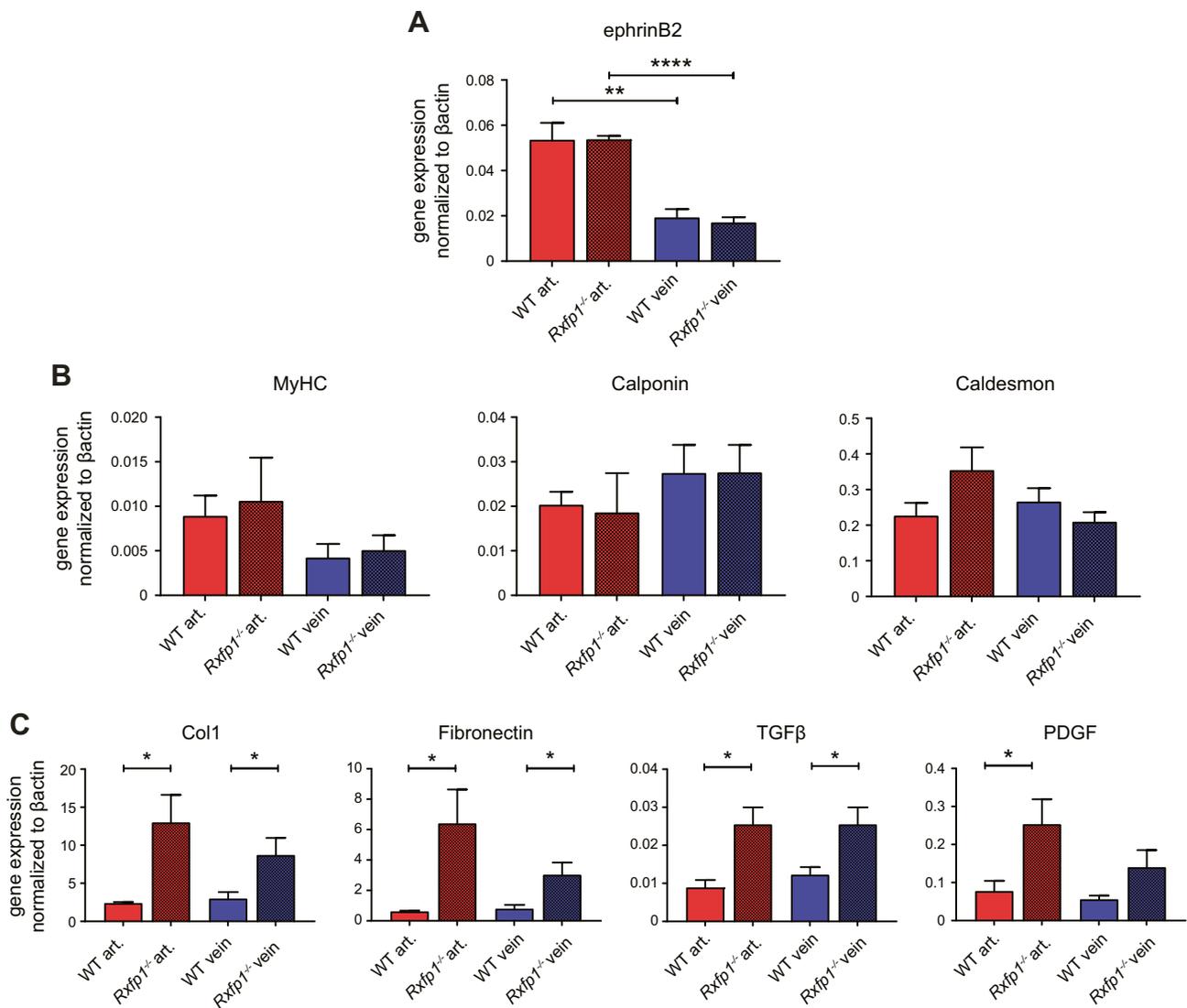


Figure 4. *In vitro* phenotypic difference between primary arterial and venous VSMCs isolated from *Rxfp1*^{-/-} and WT mice. A) Stable increase in ephrinB2 gene expression was detected in arterial VSMCs isolated from WT and *Rxfp1*^{-/-} mice. B) Both arterial and venous VSMCs isolated from *Rxfp1*^{-/-} and WT displayed characteristics of mature VSMCs as confirmed by a stable expression of myosin heavy chain, calponin, and caldesmon genes. C) RXFP1 deficiency resulted in a switch of arterial and venous VSMCs to a synthetic phenotype as confirmed by increased collagen I (Col1), fibronectine, TGF- β , and platelet-derived growth factor mRNA expression levels. VSMCs were maintained in culture for 14 d. **P* < 0.05, ***P* < 0.005, *****P* < 0.0001 (*n* = 4).

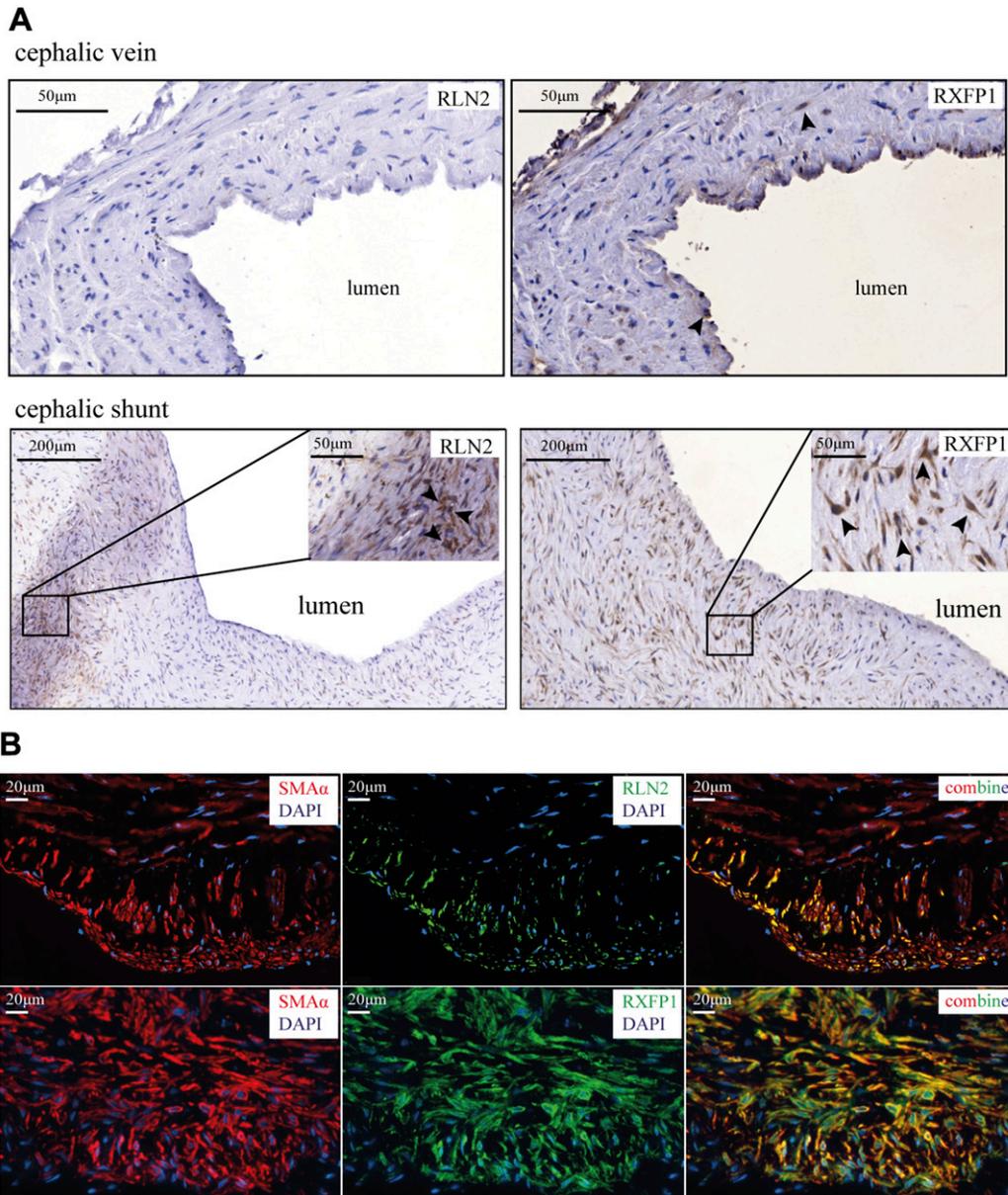


Figure 7. Expression of RLN2 and RXFP1 proteins by α SMA⁺ cells in dialysis patients. *A*) Representative images of RLN2 and RXFP1 proteins (black arrows) in human cephalic veins prior to AVF surgery and cephalic venous outflow tract from AVF. *B*) Cells positive for RLN2 and RXFP1 (green) were mainly located in the intimal region and were coexpressing α SMA (red); $n = 3$.

potential cause for this phenotype. As such, impaired elastin degradation in combination with enhanced vascular inflammation after AVF surgery likely triggers adverse vascular response in humans following the placement of an AVF.

Impaired elastin degradation and vascular remodeling in RXFP1-deficient mice

Expansion of the venous lumen and an initial vasodilatory response are prerequisites for successful AVF maturation and patency. In particular, the process of OR plays a pivotal role in this process. Although past research has focused mainly on the development of strategies to reduce IH, the current view on AVF maturation underscores the link between impaired OR and AVF failure (42, 43). To promote

OR, matrix metalloproteinase expression must be augmented to degrade and restructure the vascular matrix (44), which is largely composed of elastic fibers. Interestingly, periaortitis application of recombinant elastase has been shown to stimulate OR in a rabbit model of AVF (45). Interestingly, this concept of pharmacological elastin degradation to promote AVF maturation is now being evaluated in phase III clinical trials (46).

Enhanced expression of RLN is critical for ECM remodeling in the cervix and myometrium during pregnancy (47, 48). Administration of recombinant RLN increases the activity of elastase in human myometrial cells (26). Our discovery that RXFP1 deficiency significantly reduced elastase activity, resulting in elastic fiber accumulation in AVF lesions, suggests that RXFP1 is an important regulator of elastin degradation during AVF maturation. Furthermore, it supports the concept that

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