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Research paper

A major role of TWEAK/Fn14 axis as a therapeutic target for post-angioplasty restenosis

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ABSTRACT

Background: Tumor necrosis factor-like weak inducer of apoptosis (*Tnfsf12*; TWEAK) and its receptor Fibroblast growth factor-inducible 14 (*Tnfrsf12a*; Fn14) participate in the inflammatory response associated with vascular remodeling. However, the functional effect of TWEAK on vascular smooth muscle cells (VSMCs) is not completely elucidated.

Methods: Next generation sequencing-based methods were performed to identify genes and pathways regulated by TWEAK in VSMCs. Flow-citometry, wound-healing scratch experiments and transwell migration assays were used to analyze VSMCs proliferation and migration. Mouse wire injury model was done to evaluate the role of TWEAK/Fn14 during neointimal hyperplasia.

Findings: TWEAK up-regulated 1611 and down-regulated 1091 genes in VSMCs. Using a gene-set enrichment method, we found a functional module involved in cell proliferation defined as the minimal network connecting top TWEAK up-regulated genes. In vitro experiments in wild-type or *Tnfrsf12a* deficient VSMCs demonstrated that TWEAK increased cell proliferation, VSMCs motility and migration. Mechanistically, TWEAK increased cyclins (cyclinD1), cyclin-dependent kinases (CDK4, CDK6) and decreased cyclin-dependent kinase inhibitors ($p15^{INK4B}$) mRNA and protein expression. Downregulation of $p15^{INK4B}$ induced by TWEAK was mediated by mitogen-activated protein kinase ERK and Akt activation.

Tnfrsf12a or *Tnfsf12* genetic depletion and pharmacological intervention with TWEAK blocking antibody reduced neointimal formation, decreasing cell proliferation, cyclin D1 and CDK4/6 expression, and increasing $p15^{INK4B}$ expression compared with wild type or IgG-treated mice in wire-injured femoral arteries. Finally, immunohistochemistry in human coronary arteries with stenosis or in-stent restenosis revealed high levels of Fn14, TWEAK and PCNA in VSMCs enriched areas of the neointima as compared with healthy coronary arteries.

Interpretation: Our data define a major role of TWEAK/Fn14 in the control of VSMCs proliferation and migration during neointimal hyperplasia after wire injury in mice, and identify TWEAK/Fn14 as a potential target for treating in-stent restenosis.

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1. Introduction

Pathological vascular wall remodeling is a critical feature of vascular diseases such as atherosclerosis, post-angioplasty restenosis, vein graft stenosis, abdominal aortic aneurysm, and vasculopathy after transplantation [1]. Vascular smooth muscle cells (VSMCs) are key players in adult vascular remodeling due to their remarkable phenotypic plasticity [2]. Under physiological conditions, VSMCs are quiescent, contractile and non-migratory in the vessel wall. However, in response to vascular injury the resident medial VSMCs are activated proliferating and migrating into the intima, where they accumulate and subsequently produce proinflammatory cytokines and chemokines and abundant

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extracellular matrix proteins to form the neointima [3,4]. Intimal hyperplasia formation and restenosis can be controlled by the use of drug-eluting stents. However, the in-stent restenosis still occur in 10% patients and remained a significant clinical problem to be solved [5].

Several studies have shed light on some of the pathophysiological mechanisms that are involved in VSMCs proliferation and migration. Nevertheless the identification of molecular mediators that link these coordinated responses of VSMCs to injury could help to design new and selective treatment strategies to prevent VSMCs activation [6].

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, *Tnfsf12*) and its cognate receptor fibroblast growth factor-inducible 14 (Fn14, *Tnfrsf12a*) belong to the tumor necrosis factor superfamily of proteins [7]. Several physiological and pathological processes are induced by TWEAK depending of the cell type and environment [8]. In the vasculature, TWEAK is expressed in both the healthy and the pathological arterial wall [9]. Whereas Fn14 is low or absent in normal arteries, it is highly abundant under pathological conditions including atherosclerosis and abdominal aortic aneurysm [9–11]. Several stimuli such as endothelial growth factor, Interferon-γ, Interleukin-1β, thrombin and angiotensin stimulate Fn14 expression in cultured VSMCs [7,9]. During pathological vascular wall remodeling, TWEAK/Fn14 axis participates in regulating endothelial dysfunction, inflammation, angiogenesis and thrombosis [12]. Thus, TWEAK injection aggravates atherosclerotic plaque development in hyperlipidemic apolipoprotein-E deficient mice by boosting the inflammatory response, among other detrimental processes [13]. Conversely, lack of *Tnfsf12* gene or in vivo neutralizing using Fn14 and TWEAK antibodies reduces atherosclerotic lesion size and increases plaque stability [11,13,14]. In addition, loss-of function approaches have demonstrated that TWEAK/Fn14 axis participates in the development of abdominal aortic aneurysms in mice through the regulation of the proinflammatory and metalloproteinase environment [10].

Thus, the TWEAK/Fn14 axis exerts a key regulatory role during vascular remodeling, nonetheless the TWEAK-Fn14 downstream mechanisms are yet to be defined. Therefore, we performed a genome-wide RNA sequencing screen in cultured VSMCs aiming to identify the molecular mediators of the TWEAK/Fn14 axis. The *in-silico* analysis of the RNA-seq data using network biology software demonstrates that one of the main biological processes modulated by TWEAK is associated to cell proliferation. In line with this analysis, we mechanistically demonstrate that TWEAK in VSMCs increases cyclin D1 and CDK4/6 and decreases $p15^{INK4B}$ expression, which in turn leads to activate VSMCs proliferation and migration. In addition, we validated our results in vivo since we show in this work that TWEAK accelerates neointimal formation after wire injury in femoral arteries. Finally, we demonstrate that pharmacological intervention with anti-TWEAK antibody reduced neointimal hyperplasia after wire injury in mice. These findings indicate that the TWEAK/Fn14 system may represent an attractive potential therapeutic target for treating vascular remodeling, including restenosis after angioplasty.

2. Materials and methods

2.1. Cell culture

Aortic VSMCs were isolated from aorta of wild-type (WT) and *Tnfrsf12a* deficient mice [15]. Briefly, adhering fat and connective tissue were removed by blunt dissection from the thoracic aorta. Aortas were minced into small ring and preincubated in DMEM (Whitaker) containing 1 mg/mL collagenase (type II, 290 U/mg), penicillin (100 U/mL), streptomycin (100 lg/mL), and glutamine (2 mmol/L) (Sigma) for 15 to 20 min at 37 °C in 95% air/5% CO_2 and then explants were seeded in DMEM with 10% FBS. Cells were harvested for passaging at 2- to 3-day intervals and used between the second and seventh passages. VSMCs were stained with specific markers of VSMC α-SMA (A2547; Sigma) and calponin (ab46794; Abcam), endothelial cells (CD31, ab28364; Abcam) and fibroblast (S100A4, ab93283; Abcam) to ensure the purity of VSMC isolation methods (Fig. S1). For experimental analysis, cells were made quiescent by 24-h incubation in medium with 0–0.5% FBS.

2.2. RNA-Seq library construction and sequencing

RNA-Seq libraries were prepared using the Illumina TruSeq Stranded Total RNA library prep, after ribodepletion with the Epicenter Ribozero Gold kit (cat# RZE1224) starting from 500 ng of DNAse I treated total RNA, following the manufacturer's protocol, with the exception that 14 cycles of PCR were performed to amplify the libraries, to keep the duplication rate lower than with the recommended 15 cycles. The amplified libraries were purified using AMPure beads, quantified by Qubit and QPCR, and visualized in an Agilent Bioanalyzer. The libraries were pooled equimolarly, and loaded on an Illumina HiSeq 2500 flow cell, v4 chemistry as paired end 50. The R statistical software environment was used to run the Bioconductor package, DESeq2 to analyze the RNA-Seq data set for differential expression between groups (Applied Bioinformatics Laboratory, NYU School of Medicine, New York, USA).

2.3. Gene set enrichment

A method for gene set enrichment analysis based on logistic regression [16] implemented in the Babelomics suite [17] was used to extract the GO terms (biological processes, molecular functions and cellular components) and KEGG pathways (annotation extracted using KEGG rest service) over-represented in each of the conditions. A threshold of adjusted *p*-value by FDR < 0.05 was used to select the significant GO terms and KEGG pathways.

2.4. Functional module extraction and functional enrichment

The NetworkMiner [18] web-tool implemented in the Babelomics suite [17] was used to extract the relevant protein-protein interaction networks associated to each of the two conditions compared. The input for NetworkMiner was the list of genes identified in the RNASeq experiment ranked by the stat parameter of the DeSeq2 analysis. We used the "All ppis" interactome (genes version) and allowed one external intermediate protein.

The FatiGO algorithm [19] was applied to perform the functional enrichment analysis of the proteins in each of the networks (excluding orphan nodes). We run FatiGO using GO terms (biological processes) as annotation and the interactome used for the NetworkMiner analysis as the reference list of genes. A value of *p*-value adjusted by FDR <0.05 was considered for statistical significance.

2.5. Flow cytometry

Cells were harvested by trypsinization, fixed overnight in 70% ethanol, washed and incubated for 1 h in PBS containing 100 μg/mL RNAse A, 10 μg/mL propidium iodide (PI) and 0.05 Nonidet P-40%. The total cell number and the percentage of cells in G0-G1, S or G2-M phases was counted using standard flow cytometry methods and a BD FACSCanto II flow cytometer (BD Biosciences).

2.6. Wound closure assay

Wild type or $Tnfrsf12a^{-/-}$ VSMCs were grown to confluence in growth medium and then serum deprived for 24 h. A single scrape wound was made, and cells were then incubated with or without rTWEAK (100 ng/mL). 10% FBS was used as a positive control. Migration into the denuded area was monitored by photomicroscopy. To inhibit VSMCs proliferation, cells were preincubated 30 min with an antiproliferative dose of Actinomycin D (0.01 μg/mL; Sigma) [20] before rTWEAK stimulation.

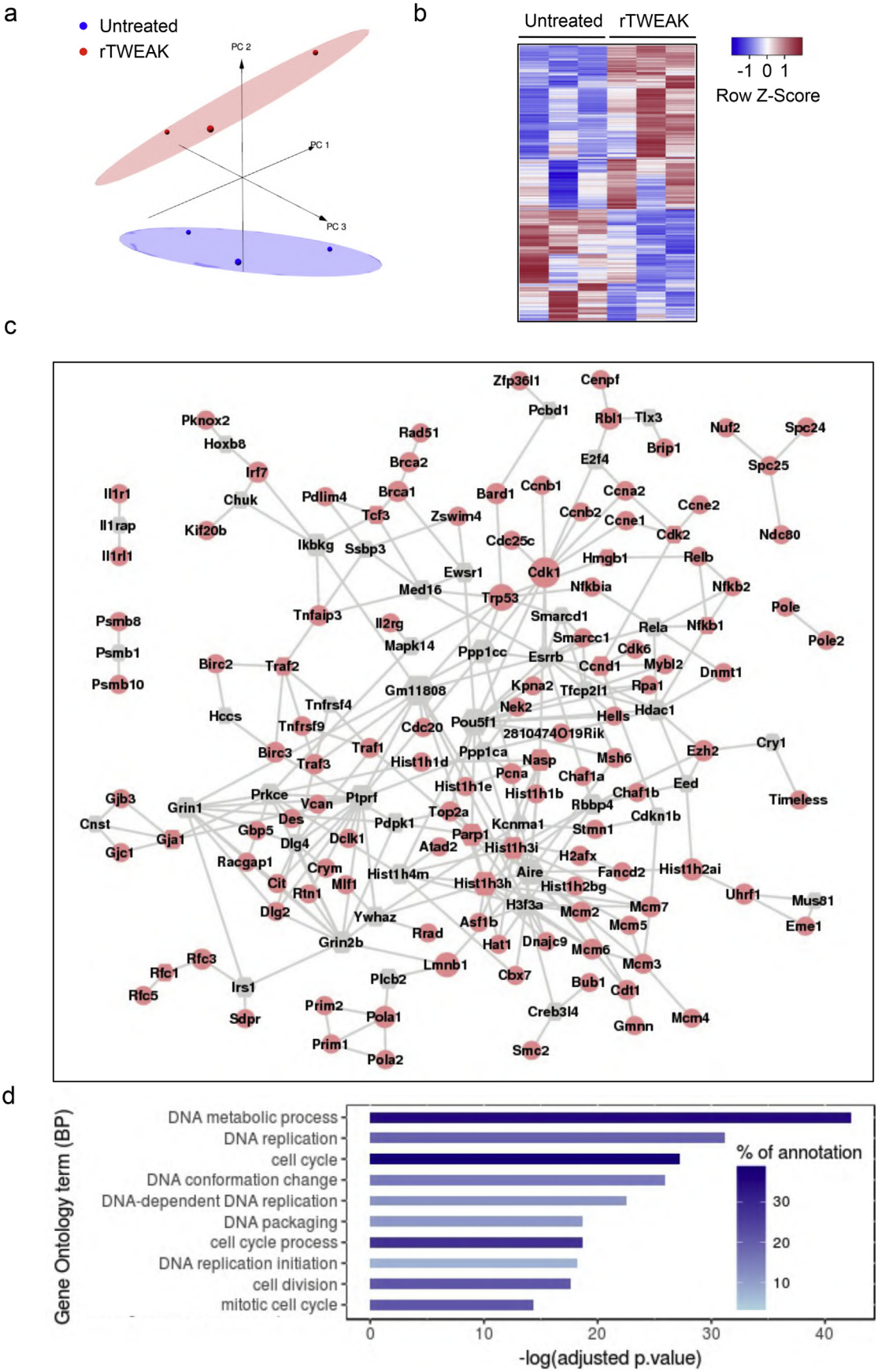


Fig. 1. RNA-Seq analysis of differentially expressed genes between TWEAK-treated and untreated VSMCs. A) Principal-component analysis (PCA) of VSMCs incubated 24 h in the presence or absence of rTWEAK ($N = 3$) based on RNA-sequencing gene expression levels. Color coded according the group of mice. B) Heat map representing differentially expressed genes between TWEAK-treated and untreated VSMCs (only genes with adjusted p-value by FDR < 0.05). C = control; TW = rTWEAK. C) Network linked to upregulated genes by rTWEAK in VSMCs. D) Top ten Gene Ontology (biological processes) terms significantly over-represented (adjusted p-value by FDR < 0.05) in the set of proteins from the network associated to TWEAK upregulated genes.

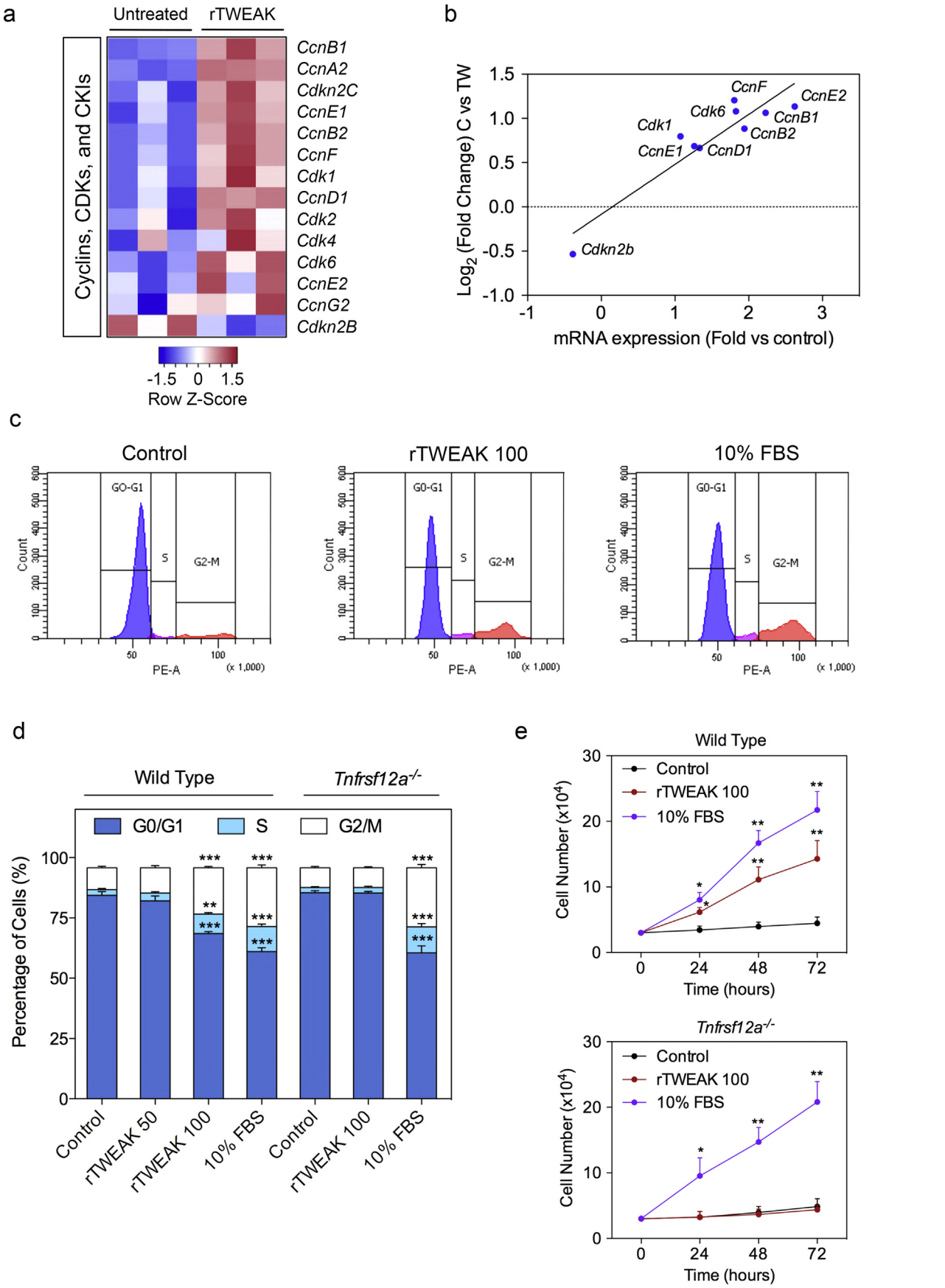


Fig. 2. TWEAK increases VSMCs proliferation. A) Heat map shows cyclins (Ccn), cyclin-dependent kinases (Cdk) and cyclin-dependent kinase inhibitors (Cdkn) regulated by TWEAK in VSMCs (adjusted $p < .05$). B) Validation by RT-qPCR of select genes identified by RNASeq. RT-qPCR data represented as fold vs unstimulated cells of three biological replicates run in experimental duplicate and normalized to *GADPH* expression. Scatter plot showing the significant positive relationship between selected mRNA gene expression obtained by RNA-Seq and real-time PCR. Pearson correlation. $r = 0.80$; $p = .006$. C) Cell cycle analysis of VSMCs by propidium iodide staining and flow cytometry after 18 h of treatment. Control (0% FBS), rTWEAK (100 ng/mL rTWEAK) and 10% FBS (positive control). D) The percentage of VSMCs in G0/G1, S or G2/M phase of the cell cycle after 18 h of incubation with 0%FBS (Control), rTWEAK (50–100 ng/mL) or 10%FBS. Data represent mean ± SEM of 4 independent experiments (Student's *t*-test). $^{**}p < .01$ vs Control and $^{***}p < .001$ vs Control). E) Proliferative curve of wild type or *Tnfrsf12a*$^{-/-}$ VSMCs cultured in the presence of 0%FBS (Control), rTWEAK (100 ng/mL) or 10%FBS from 0 to 72 h after serum starvation. Data represent mean ± SEM of 3 independent experiments (Student's *t*-test) $^{*}p < .05$ vs Control and $^{**}p < .01$ vs Control).

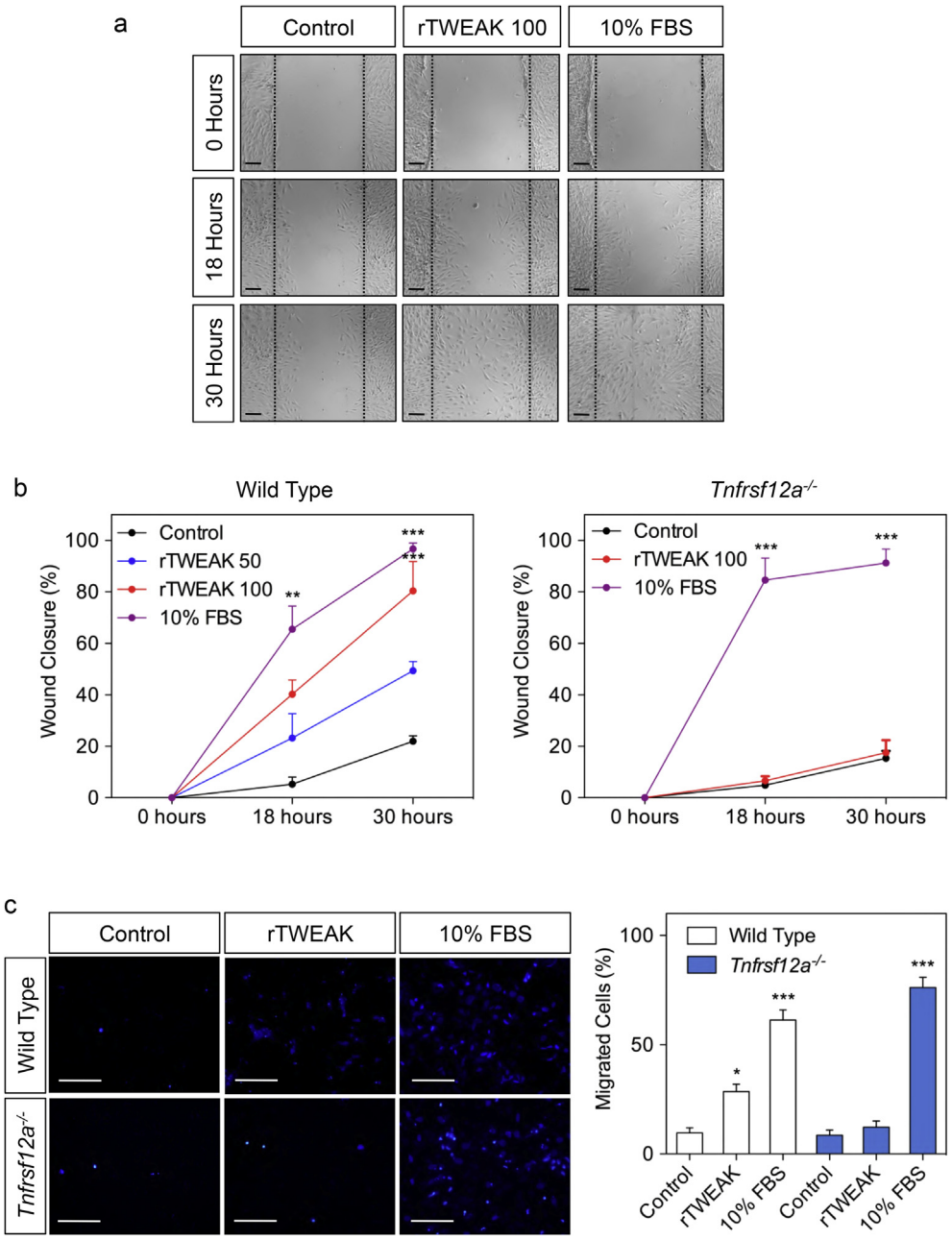


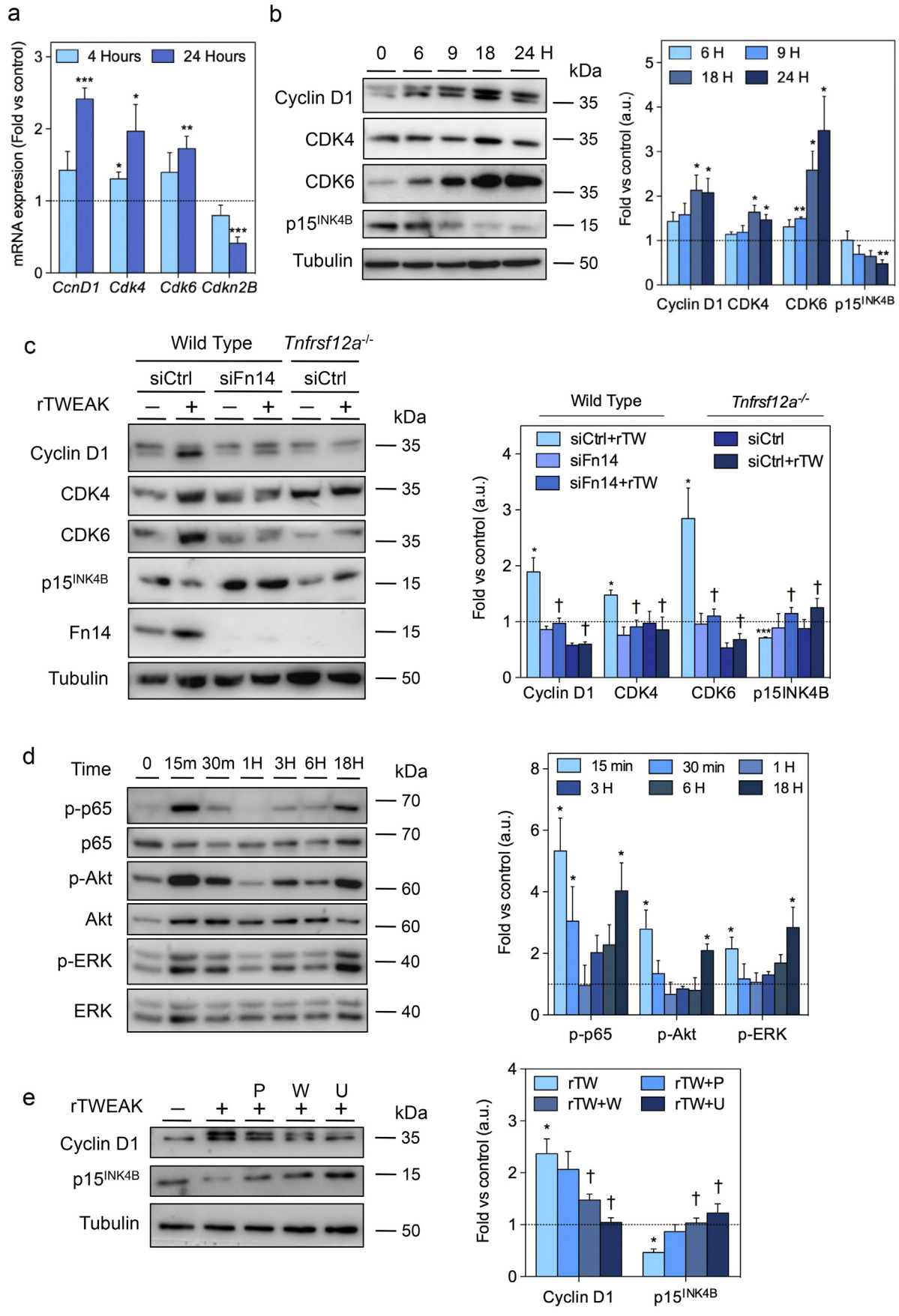
Fig. 3. TWEAK increases migration of cultured VSMCs. A) Representative photograph of VSMC migration in the wound-healing assay. A single scrape wound was made on confluently plated wild type or *Tnfrsf12a*$^{-/-}$ VSMCs. Cells were then incubated for 18 to 30 h in the presence of 0.5% FBS (control), rTWEAK (50–100 ng/mL + 0.5% FBS) or 10% FBS. Wound closure images were captured and analyzed using an inverted microscope. Scale bars 200 μm. B) VSMCs migration was quantified by percentage of wound closure along time. Data represent mean ± SEM of 4 independent experiments (Student's *t*-test) $^{**}p < .01$ vs Control and $^{***}p < .001$ vs Control. C) WT or *Tnfrsf12a*$^{-/-}$ cells were seeded in the upper surface of chemotaxis chambers and stimulated with 0.5% FBS (control), rTWEAK (100 ng/mL + 0.5% FBS) or 10% FBS. Quantification of migrated cells in ten fields per condition. Data represent the mean ± SEM of 4 independent experiments (Student's *t*-test) $^{*}p < .05$ vs Control and $^{***}p < .001$ vs Control. Scale bars 20 μm.

2.7. Migration assay

Migration of Wild type or Tnfrsf12a$^{-/-}$ VSMCs was measured in 8 μm pore transwell 24-well cell culture inserts (Costar). Cells containing 0.2% BSA in DMEM were seeded (70×10^4 per well) into the migration chamber. The lower wells of the chemotaxis chambers were filled with rTWEAK (50 or 100 ng/mL) plus 0.5% of FBS. 10% FBS was used as positive control of migration. After 4 h of nonmigrating cells were removed from the upper part of the chamber and, the nuclei of migrated cells was fixated and stained with DAPI. The number of migrated cells was counted in 10-randomly selected fields by Nikon Eclipse E400 fluorescence microscope.

2.8. RNA extraction and real-time PCR

Femoral tissues were snap frozen in N2 liquid and homogenates were resuspended for mRNA analysis. Total RNA from VSMC or femoral arteries was obtained by TRIzol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. 2μg of total RNA was reverse transcribed according to the manufacturer's protocol (Applied Biosystems). Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using specific TaqMan probe for 18S (VIC-TAMRA 431089E), *Tnfrsf12a* (Mm_00489103_m1) and *Tnfsf12* (Mm 02583406_s1) or SYBR Green (Takara Biotechnology) depend of the studied gene. PCR primers for SYBR Green are available in the data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Cdk6
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p15INK4B
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18 H
24 H
Fold vs control (a.u.)
Cyclin D1
CDK4
CDK6
p15INK4B
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Wild Type
Tnfrsf12a-/-
siCtrl
siFn14
siCtrl
rTWEAK
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Cyclin D1
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rTW+P
rTW+W
rTW+U
Fold vs control (a.u.)
Cyclin D1
p15INK4B

supplemental (Supp. Table 1). 18S rRNA served as housekeeping gene for TaqMan studies and *Gapdh* for SYBR. Housekeeping genes were amplified in parallel with the genes of interest. All measurements were performed in triplicate. The amount of target mRNA in samples was estimated by the 2ΔCT relative quantification method. Values of each sample were obtained as multiples of their baseline values.

2.9. Western-blot

Cultured murine VSMCs from WT or *Tnfrsf12a*$^{-/-}$ mice in the different experimental conditions were collected in ice-cold buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.2 mM PMSF, 0.2 mM Na3VO4 and 10 μL/mL of phosphatase inhibitor cocktail (P0044 Sigma) and pelleted. After normalizing for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS-PAGE. Following transfer of the proteins onto nitrocellulose membranes and probed using the following antibodies: anti-Fn14 antibody (1:1000; EPR3179, Abcam), anti-p15^{INK4B} (1:500; AV03047, Sigma), anti-Cdk4 (1:1000; ab137675, Abcam), anti-Cdk6 (1:1000; sc53638, Santa Cruz Biotechnology), anti-Cyclin D1 (1:1000; s18396, Santa Cruz Biotechnology) and anti-alpha-tubulin (1:10000; T5168, Sigma). After incubation with appropriate HRP-conjugated secondary antibody (Jackson Laboratory), proteins were visualized by ECL Western Blotting Detection Reagents (Amersham Biosciences) according to manufacturer instructions. Densitometry analysis of the gels was carried out using ImageJ software from the NIH (http://rsbweb.nih.gov/ij/).

2.10. Human samples

Stented [21] and non-stented coronary arteries [22] were isolated from hearts of cardiac transplant recipients. Coronary arteries were classified according to their localization, atheromatous status, nature of the donor, and then stored in a biobank (INSERM U1148, Bichat hospital, Paris, France) [23]. The Institutional Review Board, IRB 0006477 of Hôpitaux Universitaires Paris-Nord Val de Seine, Paris7 University, and Assistance Publique-Hôpitaux de Paris approved the use of explanted hearts for research. Human healthy coronary arteries were collected from freshly excised hearts removed during transplant operations at the Hospital de la Santa Creu I Sant Pau (Barcelona, Spain). The local ethics committee approved the use of healthy coronary arteries.

2.11. Wire injury model

Animal procedures were strictly in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee of IIS-Fundación Jiménez Díaz. *Tnfrsf12a*-knockout mice, *Tnfsf12* knockout mice, and wild-type (WT) counterparts (generously provided by Biogen, Inc.) have been reported previously and backcrossed onto the C57BL/6 strain [24,25].

Male mice aged 10 weeks were anesthetized by ketamine/xylazine and endoluminal injury to the common femoral artery was performed by three passages of a 0.25-mm diameter angioplasty guidewire (ev3™, Medtronic) as previously described [26]. To study the effect of anti-TWEAK treatment, 10 weeks-old wild type mice were divided in two groups: Mice injected i.p. with anti-TWEAK mAb (10 mg/kg twice a week) or an irrelevant isotype matched control IgG specific for Hen egg lysozyme (10 mg/kg twice a week). Biogen, Inc. generously provided both anti-TWEAK mAb (clone P2D10; mIgG2a generated by immunizing TWEAK-deficient mice on C57BL/6 background with recombinant human TWEAK) and irrelevant isotype matched control IgG specific for Hen egg lysozyme.

2.12. Morphometric analysis

The femoral artery was excised from the inguinal ligament to the branching of the profundal femoris artery and stored in paraformaldehyde for 24 h and later in ethanol until paraffin embedded. Femoral arteries were cut into 5-μm serial sections, and 6 cross sections taken at regular intervals throughout the artery were stained with hematoxylin and eosin. For the morphometric analyses, Leica DMD 108 microscopy was used to measure the external elastic lamina, internal elastic lamina, and lumen circumference to calculate the medial- and neointimal area and the neointima/media ratio 14 days after dilation.

2.13. Immunohistochemistry

Immunohistochemical analysis was carried out as previously described [13]. For mice tissues, primary antibodies were the anti-smooth muscle cell markers alpha smooth muscle actin (clone 1A4, F3777, Sigma) and calponin (1:100; ab46794 Abcam), anti-TWEAK (1:50; NBP1-6774 Novus), anti-Fn14 (1:50; 4403S Cell Signalling), anti-cyclin D1 (1:50; 92G2 Cell Signalling), anti-CDK4 (1:100; ab137675 Abcam), anti-CDK6 (1:100; GTX103992 GeneTex), anti-p15^{INK4B} (1:100; AV03047 Sigma), the proliferation marker anti-PCNA (1:200; sc-7907, Santa Cruz Biotechnology), T-lymphocyte marker anti-CD3 (1:500; A0452 Dako), macrophage marker anti-CD68 (1:200; ab53444 Abcam), endothelial cell marker anti-CD31 (1:50; ab28364 Abcam) and fibroblast marker anti-S100A4 (1:50; ab93283 Abcam). Donkey anti-goat biotin, donkey anti-rabbit biotin and, goat anti-rat biotin (Amersham) was used as secondary antibodies, ABComplex/HRP was then added and sections. Immunohistochemistry color was developed with DAB (Dako), and section were counterstained with hematoxylin, and mounted in DPX (Millipore). Incubation without primary antibodies and/or irrelevant species and isotype-matched immunoglobulins was performed as a negative control for all immunostaining studies. Computer-assisted morphometric analysis was performed with the Image-Pro Plus software (version 4.5.0 for Windows) in a blinder manner. The threshold setting for area measurement was equal for all images. Results were expressed as % positive area of, Cyclin D1, CDK4, CDK6 and, p15^{INK4B} in total artery, and as % of PCNA positive cells versus total cells.

For human tissues, primary antibodies were anti-α-SMA (1:500; ab5694 Abcam), anti-calponin (1:100; ab46794 Abcam), anti-TWEAK (1:100; ab37170 Abcam), anti-Fn14 (1:50; ab109365 Abcam) and anti-PCNA (1:200; sc-7907, Santa Cruz Biotechnology).

2.14. Statistical analysis

Animal sample size for each study was chosen based on literature documentation of similar well-characterized experiments. The number of animals in each study is listed in the figure legends. Values are expressed as mean ± SEM (n is noted in the figure legends). In vitro experiments were replicated at least 3 times unless otherwise noted. Statistical differences were measured using two-sided Student's test or a one-way ANOVA followed by a post hoc Bonferroni pairwise comparison test. A nonparametric test (Mann-Whitney) was used when data

Fig. 4. TWEAK regulates cyclin D1, CDK4, CDK6 and p15^{INK4B} expression in VSMCs. A) Quantitative real-time PCR analysis of *CcnD1*, *Cdk4*, *Cdk6* and *Cdkn2B* mRNA expression in VSMCs upon rTWEAK (100 ng/mL) stimulation. Data represent the mean ± SEM of 4 independent experiments (Student's *t*-test) $^{*}p < .05$ vs 0 h, $^{**}p < .01$ vs 0 h and $^{***}p < .001$ vs 0 h). B) Western-blot analysis of cyclin D1, CDK4, CDK6 and p15^{INK4B} in WT VSMCs. Data represent the mean ± SEM of 4 independent experiments (Student's *t*-test) $^{*}p < .05$ vs 0 h, $^{**}p < .01$ vs 0 h). C) Western-blot analysis of CcnD1, CDK4, CDK6, p15^{INK4B} and Fn14 in WT or *Tnfrsf12a*$^{-/-}$ VSMCs transfected with negative control siRNA (siCtrl) or Fn14 siRNA after 18 h of rTWEAK (100 ng/mL) stimulation. Data represent the mean ± SEM of 4 independent experiments (Student's *t*-test) $^{*}p < .05$ vs siControl, $^{***}p < .001$ vs siControl. $^{\dagger}p < .05$ vs siControl + rTW). D) Western-blot analysis of ERK, Akt and p65 phosphorylation in WT VSMCs upon rTWEAK (100 ng/mL) stimulation. Data represent the mean ± SEM of 3 independent experiments (Student's *t*-test) $^{*}p < .05$ vs 0 h. E) Western-blot analysis showing the effect of parthenolide (P), wormanin (W) and UO126 (U) on cyclin D1 and p15^{INK4B} expression. Data represent the mean ± SEM of 3 independent experiments (Student's *t*-test) $^{*}p < .05$ vs control; $^{\dagger}p < .05$ vs rTW.

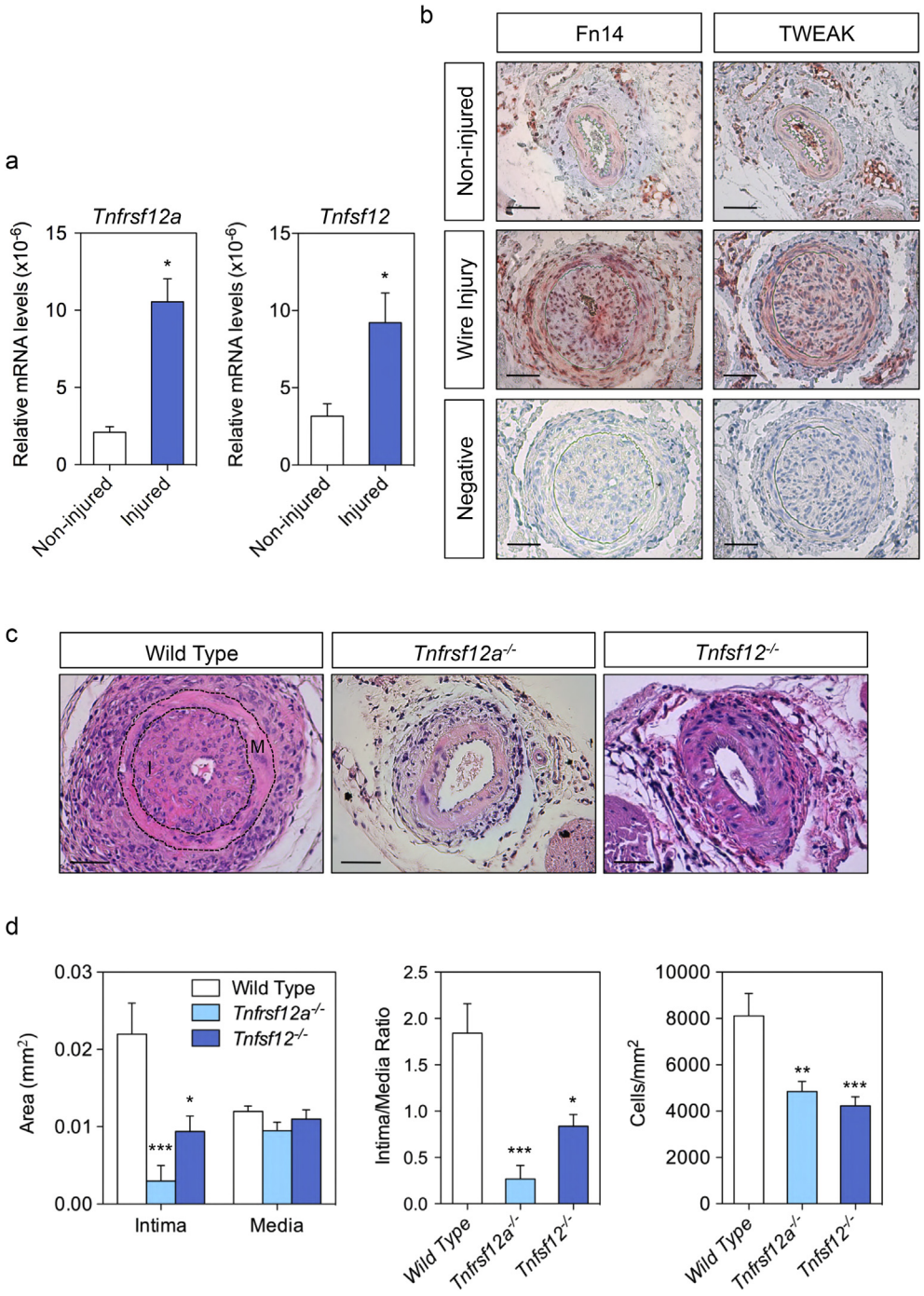


Fig. 5. TWEAK participates in neointimal formation after wire injury. A) Relative *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ expression levels normalized to 18S rRNA of non-injured ($N = 4$) or injured ($N = 5$) femoral artery from wild type mice. Data represent the mean ± SEM (Student´s t test) *$p < .05$ vs non-injured artery. B) Representative images of non-injured and injured femoral artery cross-sections immunostained with anti-Fn14 or anti-TWEAK antibodies. Negative controls were incubated with non-specific IgG. Scale bars 50 μm. C) Representative images of injured femoral artery cross sections stained with hematoxylin and eosin from wild type, *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ mice. Scale bars 50 μm. M = media; I = intima. D) Cell number quantification per mm^2, intima and media area, and intima/media ratio of cross-sections of injured femoral arteries from wild type ($N = 11$), *Tnfrsf12a*$^{-/-}$ (N = 11) or *Tnfsf12*$^{-/-}$ ($N = 10$) mice. Data represent the mean ± SEM (One-way ANOVA with Bonferroni's post-test) *$p < .05$ vs WT, **$p < .01$ vs WT and ***$p < .001$ vs WT.

did not pass the normality test. A value of $p < .05$ was considered statistically significant. Data analysis was performed using GraphPad Prism Software Version 7 (GraphPad, San Diego, CA, USA).

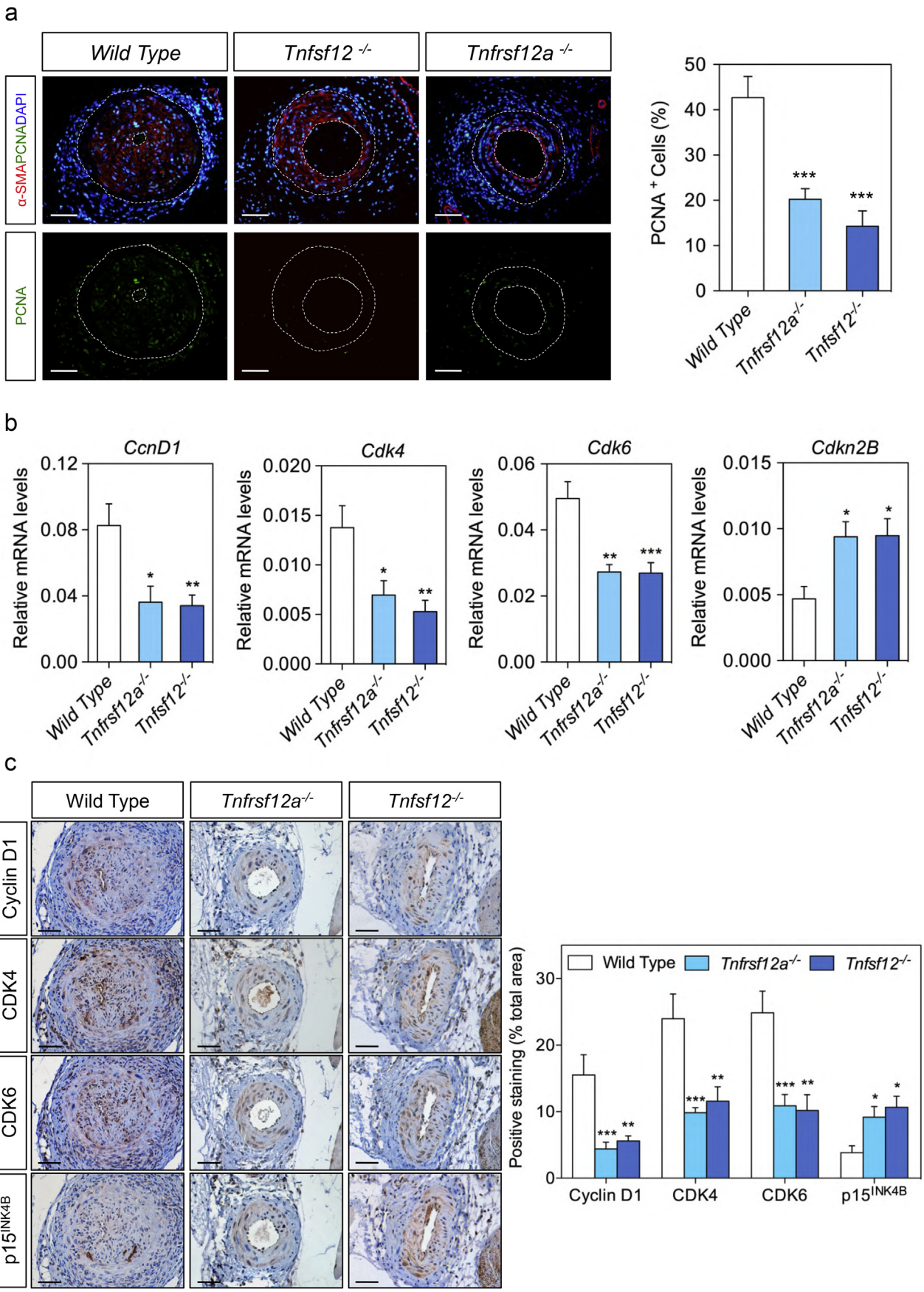
2.15. Data statement

Datasets have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO series number GSE114116 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114166).

3. Results

3.1. TWEAK modulates the expression of genes associated with cell proliferation in VSMCs

We initially aimed to identify genes that are modulated by TWEAK in VSMCs. To this end, murine aortic VSMCs were incubated in the presence or absence of murine recombinant TWEAK (rTWEAK; 100 ng/mL) for 24 h. The dose of rTWEAK was selected based in our


a
Wild Type
Tnfsf12 -/-
Tnfrsf12a -/-
α-SMAPCNADAPI
PCNA
PCNA + Cells (%)
50
40
30
20
10
0
***
***
Wild Type
Tnfrsf12a-/-
Tnfsf12-/-
b
CcnD1
Cdk4
Cdk6
Cdkn2B
Relative mRNA levels
0.12
0.08
0.04
0.00
0.020
0.015
0.010
0.005
0.000
0.06
0.02
0.015
*
**
***
Wild Type
Tnfrsf12a-/-
Tnfsf12-/-
c
Wild Type
Tnfrsf12a-/-
Tnfsf12-/-
Cyclin D1
CDK4
CDK6
p15INK4B
Positive staining (% total area)
30
20
10
0
Cyclin D1
CDK4
CDK6
p15INK4B

previous studies [10,13]. The RNA Sequencing (RNA-Seq) transcriptome profiling analysis revealed 2702 genes differentially expressed in VSMCs under rTWEAK stimulation, 1611 up-and 1091 down-regulated (Fig. 1). The number of significant up and down-regulated genes exceeds the range where a classical functional enrichment method can characterize gene signatures. Instead, we performed a gene set enrichment analysis that reported 984/1032 biological processes, 10/10 molecular functions, 6/6 cellular components and 62/92 KEGG pathways over-represented in rTWEAK stimulated versus control conditions (Suppl. File 1). We performed a gene set-like network enrichment analysis using NetworMiner algorithm in order to capture the most relevant functional modules, that are up/down regulated by rTWEAK in VSMCs [18] . This method extracts functional modules associated to a phenotype by evaluating both, the topological robustness of a protein-protein interaction network and the link of its components to a condition. The modules are presented as protein networks and the level of significance is obtained from a comparison to networks generated from random lists of genes. Remarkably, the resultant network may contain proteins not necessarily labeled as associated to the phenotype by the expression analysis but important in the internal structure of the functional module.

We obtained two significant protein networks linked to genes up and down-regulated by TWEAK. The network associated to the up-regulation by rTWEAK in VSMCs (Fig. 1C) contains 159 connected proteins (116 significantly up-regulated in the expression analysis) that are enriched in Gene Ontology terms related to cell proliferation (Fig. 1D and Suppl. Table 2). We also found a smaller but significant network of down-regulated genes associated with several biological over-represented processes related to DNA binding and histone regulation, development processes and cell cycle (Fig. S2 and Suppl. Table 3).

G_1 to S cell cycle progression is modulated by the balance between positive cell cycle regulators [cyclins and cyclin-dependent kinases (CDK)] on one hand and CDK inhibitors (CDKI) on the other [27]. Fig. 2A shows a heatmap of differentially expressed cyclins, CDK and CDKI in VSMCs incubated in the presence or absence of rTWEAK. To confirm the results from the RNA-Seq, a subset of different genes associated with cell proliferation (*Ccn B1*, *B2*, *D1*, *E1*, *E2*, and *F*, *Cdk1*, *Cdk4* and *Cdk6*, and *Cdkn2b*) were independently validated using RT-qPCR (Suppl. Table 4). We observed an excellent correlation between RNA-Seq and RT-PCR findings ($r = 0.80$; $p = .006$; Pearson correlation; Fig. 2B). Interestingly, *Cdkn2b*, a highly conserved cell-cycle regulator and tumor suppressor gene, was consistently downregulated by TWEAK in VSMCs (Fig. 2A–B). Collectively, these data suggest that TWEAK signaling contributes to VSMCs proliferation. Therefore, we decided to analyze the role of TWEAK on cell proliferation both in vitro and in vivo.

3.2. TWEAK increases VSMCs proliferation and migration

We analyzed Fn14 expression in VSMCs and we observed that α-SMA positive VSMCs express Fn14 (Supp. Fig. S1B–C). To determine whether TWEAK regulates VSMCs proliferation, we performed cell cycle analysis of rTWEAK-stimulated VSMCs by measuring the DNA content of cells stained with propidium iodide. Cells growing in 10% FBS were used as a positive control of cell proliferation. There was a decrease of cells in G0/G1 phase and an increase of cells in the S and G2/M phase in rTWEAK-stimulated VSMCs at 18 h compared with control cells (Fig. 2C–D). However, rTWEAK was unable to induce the transition of cells from G0/G1 to S phase in VSMCs from *Tnfrsf12a* deficient mice (*Tnfrsf12a*$^{-/-}$) (Fig. 2C–D), indicating that TWEAK activates cell proliferation through its functional receptor Fn14. Consistently, cell number increased over the time when VSMCs were incubated in the presence of rTWEAK (Fig. 2E). No effect was observed in VSMCs lacking the Fn14 receptor (Fig. 2E).

We next performed scratch wound healing assays in VSMCs from wild type (WT) and *Tnfrsf12a*$^{-/-}$ mice to analyze whether TWEAK plays a role in cell migration. rTWEAK-stimulated VSMCs from WT mice efficiently reoccupied the scratched area, whereas *Tnfrsf12a*$^{-/-}$ VSMCs did not migrate in response to rTWEAK (Fig. 3A–B). To confirm that TWEAK induces VSMCs migration, cells were preincubated 30 min in the presence or absence of anti-proliferative and non-apoptotic concentration of actinomycin D (ActD) [20]. rTWEAK-stimulated VSMCs also reoccupied the scratched area in presence of ActD indicating that TWEAK induces VSMCs migration (Fig. S3). Moreover, WT VSMCs efficiently migrated in transwell assays whereas similarly treated *Tnfrsf12a*$^{-/-}$ VSMCs did not (Fig. 3C). Altogether, our results demonstrate that TWEAK-induced VSMCs proliferation and migration is dependent on its binding to Fn14.

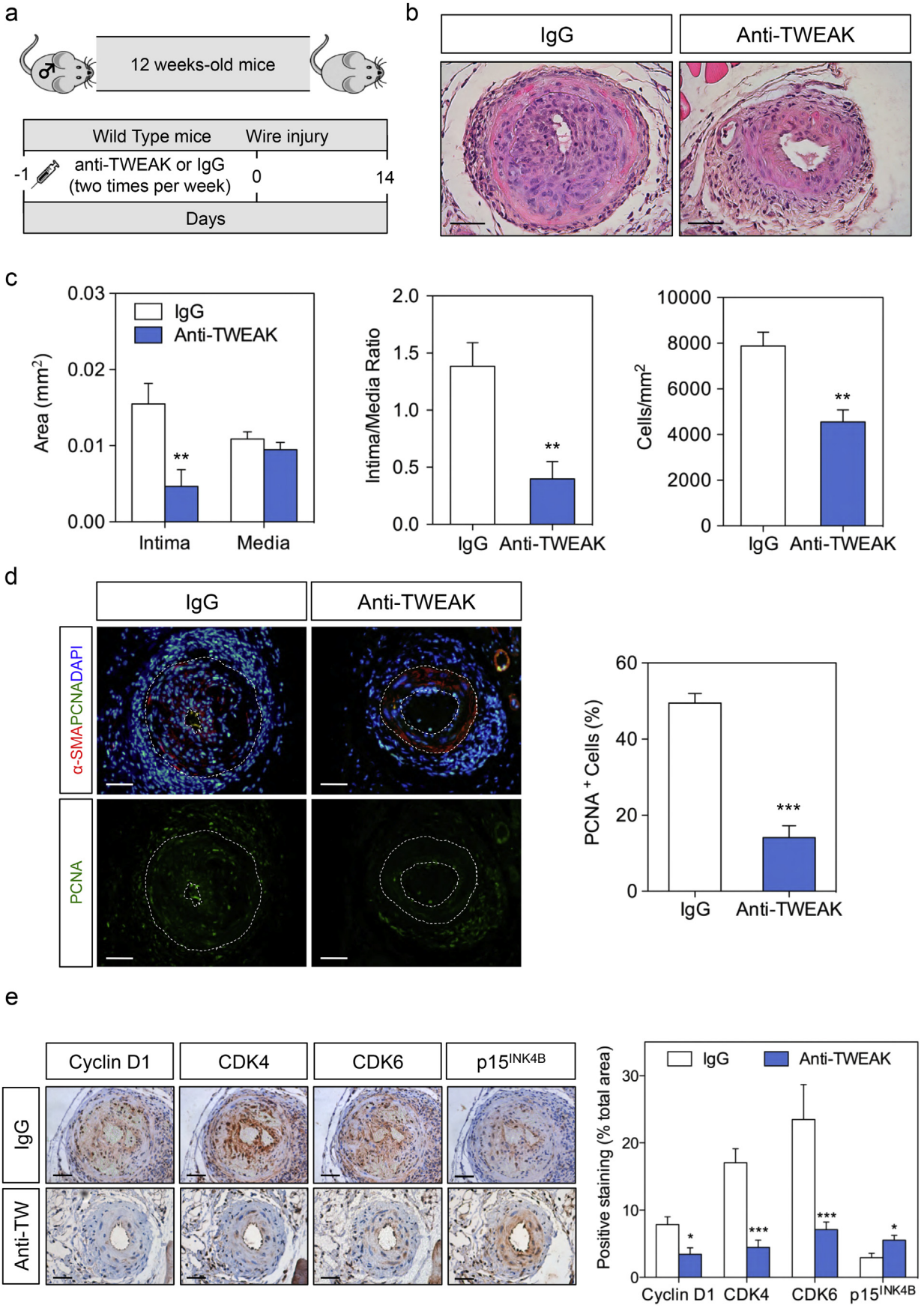
We subsequently screened the expression of TWEAK-regulated genes identified by RNA-seq to gain more mechanistic insight into the proliferative effects of TWEAK. As commented above, *Cdkn2b* ($p15^{INK4B}$), a highly conserved cell-cycle regulator and tumor suppressor gene, was consistently downregulated by TWEAK in the RNA-Seq and qRT-PCR studies. Time-kinetic studies assessed in WT VSMCs showed that rTWEAK downregulated *Cdkn2B* mRNA expression and $p15^{INK4B}$ protein level in a time-dependent manner (Fig. 4A–B). In addition, the downregulation of $p15^{INK4B}$ was dependent of the presence of Fn14, since TWEAK failed to downregulate $p15^{INK4B}$ protein level in siRNA Fn14-transfected VSMCs or *Tnfrsf12a*$^{-/-}$ VSMCs (Fig. 4C).

The transition of G_1 to S phase is regulated by D-type cyclins and it has been demonstrated that $p15^{INK4B}$ is an inhibitor of CDK4/CDK6 and cyclin D1 expression in VSMCs [28]. Therefore, we have analyzed *CcnD1* (cyclin D1), *Cdk4* and *Cdk6* mRNA expression in rTWEAK-stimulated VSMCs. In line with Mathew et al. [28], rTWEAK increased *CcnD1*, *Cdk4* and *Cdk6* mRNA expression (Fig. 4A). In addition, rTWEAK increased cyclin D1, CDK4 and CDK6 proteins levels in a time-dependent manner (Fig. 4B). The upregulation of cyclin D1, CDK4 and CDK6 was also dependent of Fn14 since TWEAK failed to upregulate these proteins in *Tnfrsf12a*$^{-/-}$ VSMCs or wild type cells transfected with siRNA against Fn14 (Fig. 4C).

3.3. $p15^{INK4B}$ and cyclin D1 expression is regulated by ERK1/2 and Akt in VSMCs

To analyze the mechanism/s by which TWEAK regulates $p15^{INK4B}$ and cyclin D1 expression in VSMCs, we studied signal pathways that control cell proliferation. TWEAK induced phosphorylation of ERK1/2, Akt and p65, a subunit of nuclear factor kappa B (NF-kB), in a time-dependent manner, peaking at 15 min and 18 h (Fig. 4D). To analyze the role of ERK1/2, Akt and NF-kB on $p15^{INK4B}$ and cyclin D1 expression, we used the specific inhibitor UO126 (ERK1/2), wortmannin (PI3K) and partthenolide (NF-kB). The inhibitors of ERK1/2 (UO126) and Akt (wortmannin) prevented $p15^{INK4B}$ downregulation and cyclin D1

Fig. 6. TWEAK increases proliferation in vivo. A) Representative staining and quantification of positive PCNA cells in injured femoral artery cross-sections from Wild type ($N = 9$), *Tnfrsf12a*$^{-/-}$ ($N = 11$) or *Tnfsf12*$^{-/-}$ ($N = 10$) mice. Data represent the mean ± SEM (One-way ANOVA with Bonferroni's post-test) ***p < .001 vs WT. Scale bars 50 μm. B) Relative *CcnD1*, *Cdk4*, *Cdk6* and *Cdkn2B* mRNA expression levels normalized to *GAPDH* mRNA expression of WT, *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ femoral arteries after wire injury. Data represent the mean ± SEM ($N = 8$ per group) (One-way ANOVA with Bonferroni's post-test) *$p < .05$ vs WT, **$p < .01$ vs WT and ***$p < .001$ vs WT. C) Representative images of anti-Cyclin D1, CDK6, CDK4 and $p15^{INK4B}$ staining of cross-sections of injured femoral arteries from WT, *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ mice. Quantification of intimal and medial percentage of Cyclin D1, CDK6, CDK4 and $p15^{INK4B}$ staining respectively, in injured femoral artery cross-sections of WT ($N = 11$), *Tnfrsf12a*$^{-/-}$ ($N = 11$) or *Tnfsf12*$^{-/-}$ ($N = 8$) Scale bars 50 μm. Data represent the mean ± SEM (One-way ANOVA with Bonferroni's post-test) *$p < .05$ vs WT, **$p < .01$ vs WT and ***$p < .001$ vs WT.


a
12 weeks-old mice
Wild Type mice
Wire injury
-1
anti-TWEAK or IgG
(two times per week)
0
14
Days
b
IgG
Anti-TWEAK
c
Area (mm2)
IgG
Anti-TWEAK
Intima
Media
Intima/Media Ratio
Cells/mm2
d
α-SMAPCNADAPI
PCNA
PCNA+ Cells (%)
e
Cyclin D1
CDK4
CDK6
p15INK4B
IgG
Anti-TW
Positive staining (% total area)

upregulation induced by TWEAK (Fig. 4E). No effect was observed with NF-kB inhibitor parthenolide (Fig. 4E).

3.4. Loss of Tnfrsf12a or Tnfsf12 decreases neointimal formation in femoral artery after wire injury

VSMCs migration from media to intima and their subsequent proliferation is a pathological manifestation of restenosis after angioplasty [29]. To corroborate the in vitro findings, we next tested the functional role of the TWEAK/Fn14 axis in VSMCs-rich lesion formation in vivo performing a guidewire injury in femoral arteries of mice. This experimental model that mimics the damage caused by angioplasty in humans [30]. The gene and protein expression levels of TWEAK and Fn14 were increased in femoral artery after 14 days of wire injury in WT mice compared with non-injured arteries (Fig. 5A–B). Immunohistochemistry analysis also showed a predominant localization of both TWEAK and Fn14 protein in the medial and neointimal layers (Fig. 5B). The majority of cells present in the neointima were positive for markers of VSMCs such as α-SMA and calponin, and also for TWEAK and Fn14 (Fig. S4). These data should indicate that the in vivo phenotype observed in our animal model is mainly dependent of VSMC activity. We next performed a guide-wire injury in femoral arteries of *Tnfrsf12a* and *Tnfsf12* deficient mice to analyze the role of TWEAK and Fn14 in vascular remodeling. Wire injury-mediated neointimal formation determined by intima area and intima to media ratio was significantly smaller in *Tnfrsf12a*$^{-/-}$ and *Tnfsf12*$^{-/-}$ compared with WT mice (Fig. 5C–D). In addition, the number of cells per mm^2 was also decreased in the femoral sections of *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ compared with WT mice (Fig. 5D).

Proliferating cell nuclear antigen (PCNA) positive cells in wire-injured femoral arteries were significantly reduced in either *Tnfrsf12a*$^{-/-}$ or *Tnfsf12*$^{-/-}$ femoral cross-sections compared with WT mice (Fig. 6A). We next tested how the TWEAK or Fn14 absence impacts on the expression of cell cycle regulators. We found that lack of *Tnfrsf12a* or *Tnfsf12* increased *Cdkn2B* and decreased *Cdk4*, *Cdk6* and *CcnD1* mRNA expression in injured femoral arteries with respect to WT mice (Fig. 6B). Consistently, cyclin D1, CDK4 and CDK6 protein levels were markedly reduced and p15^{INK4B} increased in the injured femoral arteries of *Tnfrsf12a* or *Tnfsf12* deficient mice compared with WT mice (Fig. 6C). These results indicate that TWEAK/Fn14 axis play a key role in neointimal formation through the regulation of the cell cycle by reduction of p15^{INK4B} expression and increase of CDK4/CDK6 and cyclin D1 expression.

3.5. Treatment with anti-TWEAK antibody reduces neointimal formation after wire injury in mice

To analyze the protective effect of TWEAK-based therapy against injury-induced neointimal hyperplasia, WT mice were treated with anti-TWEAK mAb or control IgG (10 mg/kg/twice a week). Mice were treated the day before the wire injury was performed and during 14 days after this procedure (Fig. 7A). Neointimal formation determined by intima area and intima to media ratio was significantly diminished in anti-TWEAK-treated compared to IgG-treated mice (Fig. 7B–C). In addition, the number of cells/mm^2 was also reduced in femoral sections from anti-TWEAK treated mice compared with IgG treated mice (Fig. 7B–C). Consistent with data obtained from *Tnfsf12*$^{-/-}$ mice, femoral cross-sections from anti-TWEAK treated mice showed a 71% reduction in PCNA$^+$ cells compared with IgG-treated mice (Fig. 7D). Finally, we observed reduced protein expression of cyclin D1 and CDK4/CDK6 alongside with increased p15^{INK4B} levels in injured femoral arteries of mice treated with anti-TWEAK compared with those treated with IgG (Fig. 7E). Collectively, these data provide evidence that anti-TWEAK mAb treatment ameliorates restenosis after angioplasty.

3.6. TWEAK/Fn14 axis in human intimal thickening

Finally, in order to translate the results obtained from our in vivo model to the human context, we have performed α-SMA, calponin, Fn14, TWEAK and PCNA immunohistochemistry in human healthy and stenotic coronary arteries. TWEAK was expressed in healthy coronary arteries while Fn14 expression was absent. In addition, we observed that both, TWEAK and Fn14 are expressed in human coronary arteries with stenosis (Fig. 8) as well as in-stent restenosis (Fig. 8) colocalizing with markers of VSMCs such as α-SMA and calponin. PCNA$^+$ cells are also present in the neointima of both types of human injured coronary arteries, but not in healthy artery (Fig. 8).

4. Discussion

Percutaneous transluminal coronary angioplasty has been widely used to open up blocked coronary arteries [6]. However, many patients undergoing coronary angioplasty experience postangioplasty restenosis, which is a major obstacle in the long-term outcome of angioplasty interventions. Restenosis is defined as the healing response of the arterial wall to mechanical injury and implicates neointimal hyperplasia (VSMCs proliferation and migration) and vessel remodeling. It has been previously demonstrated that TWEAK participates in several pathologies that course with vascular remodeling. In this sense, TWEAK increases the inflammatory response associated with atherosclerotic plaque development in mice [13,14]. In addition, TWEAK and Fn14 participate in matrix degradation and induce angiogenesis in experimental abdominal aortic aneurysm [10]. Although it is known that VSMCs are key cells implicated in vascular remodeling, there are so far no studies focusing on the TWEAK-regulated signaling pathways in VSMCs. Here, we provide the first evidence for a pivotal role of TWEAK/Fn14 axis in neointimal formation after angioplasty. We used RNA-Seq to systematically investigate the global transcriptome of cultured VSMCs incubated in the presence or absence of TWEAK. In this manner we generated a useful resource for understanding the effect of TWEAK/Fn14 interaction on VSMCs biology. RNA-Seq and network analyses unveiled that one of the main actions of TWEAK in VSMCs is cell proliferation regulation. Our in vivo findings indicate that: i) both TWEAK and Fn14 mRNA and protein expression are induced in vascular wall after injury in wild type mice; ii) gene deletion of TWEAK and Fn14 in *Tnfsf12* or *Tnfrsf12a* deficient mice protects from cell proliferation and neointimal formation; iii) anti-TWEAK treatment prevents cell proliferation and neointimal formation in injured arteries; iv) TWEAK and Fn14 are expressed in human coronary in-stent restenosis. Furthermore, our mechanistic studies demonstrate that TWEAK interaction with its cognate receptor Fn14 induces the proliferation and migration of VSMCs through the concomitant activation of CDK4/6 and cyclin D1 and inhibition of p15^{INK4B} expression. Moreover, p15^{INK4B} and cyclin D1 expression are regulated by ERK1/2 and Akt kinases. Therefore, this work defines a hitherto unknown role of TWEAK/Fn14 axis during the development of restenosis after angioplasty (Fig. 9).

In vitro experiments in VSMCs confirmed that TWEAK, through its receptor Fn14, promotes VSMCs proliferation. The proliferative action

Fig. 7. Anti-TWEAK treatment inhibits neointimal formation after wire injury. A) Experimental mouse design. Briefly, femoral arteries of 12-week-old male wild type mice were injured and treated with anti-TWEAK or IgG (10 mg/kg two times per week) for 14 days. B) Representative images of injured femoral artery cross sections from IgG or anti-TWEAK treated mice stained with H&E. Scale bars 50 μm. C) Quantification of number of cells per mm^2, intima and media area, and intima/media ratio of cross-sections in injured conditions shown in B. IgG ($N = 10$), anti-TWEAK ($N = 14$). Data represent the mean ± SEM (Mann-Whitney *U* test) $^{**}p < .01$ vs IgG. D) Representative images of anti-Cyclin D1, CDK6, CDK4 and p15^{INK4B} staining of cross-sections of injured femoral arteries from IgG and anti-TWEAK treated mice. Quantification of intimal and medial percentage of Cyclin D1, CDK6, CDK4 and p15^{INK4B} staining respectively, in the injured femoral artery of IgG (N = 10), *anti-TWEAK* ($N = 11$). Data represent the mean ± SEM (Mann-Whitney U test) $^{**}p < .01$ vs IgG.

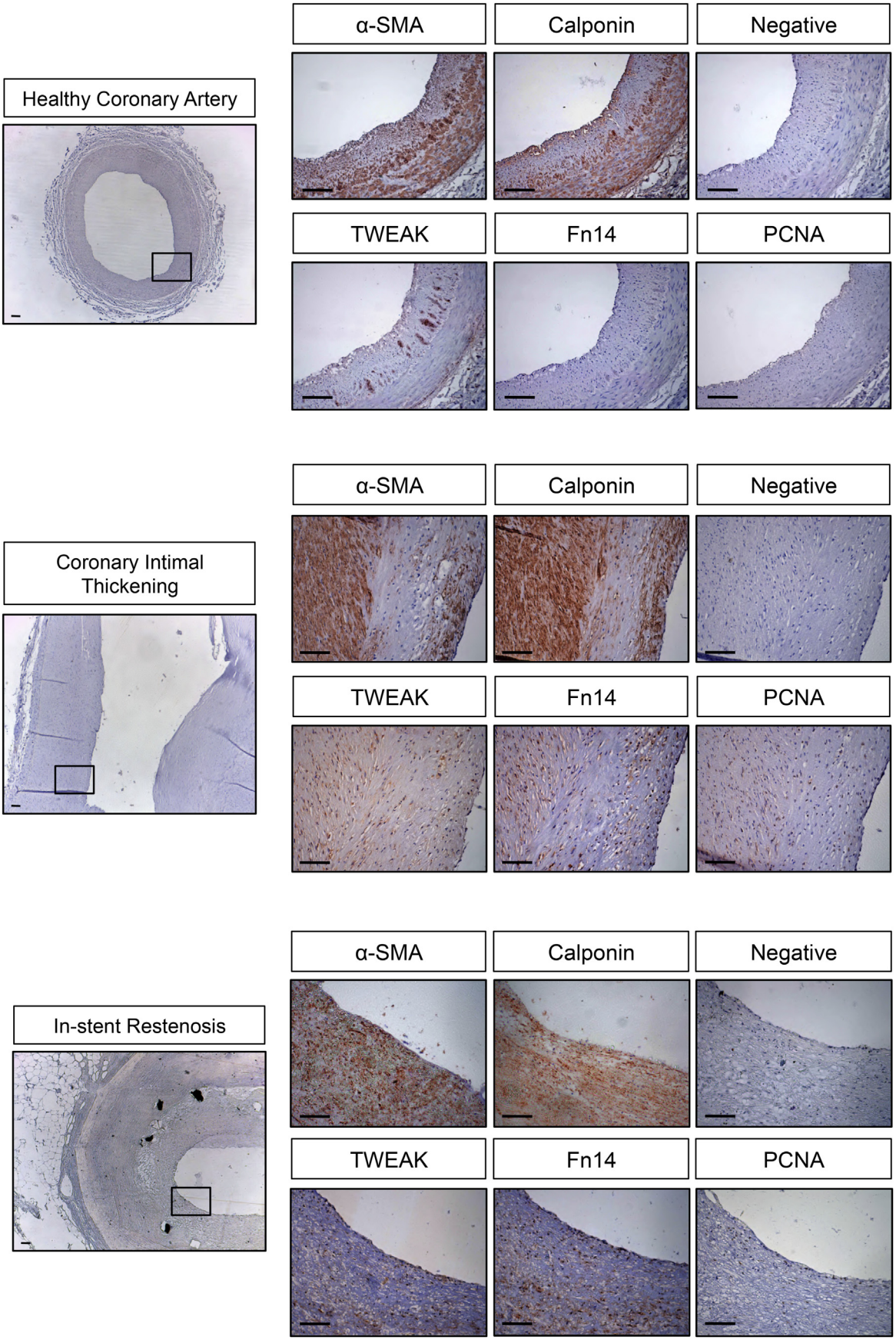


Fig. 8. TWEAK and Fn14 are highly expressed in the neointima of human coronary arteries. Representative images of immunostaining for markers of VSMCs (α-SMA and calponin), TWEAK, Fn14 or PCNA in serial section from human coronary artery with or without stenosis. Negative controls were stained with non-specific IgG. Scale bars 100 μm.

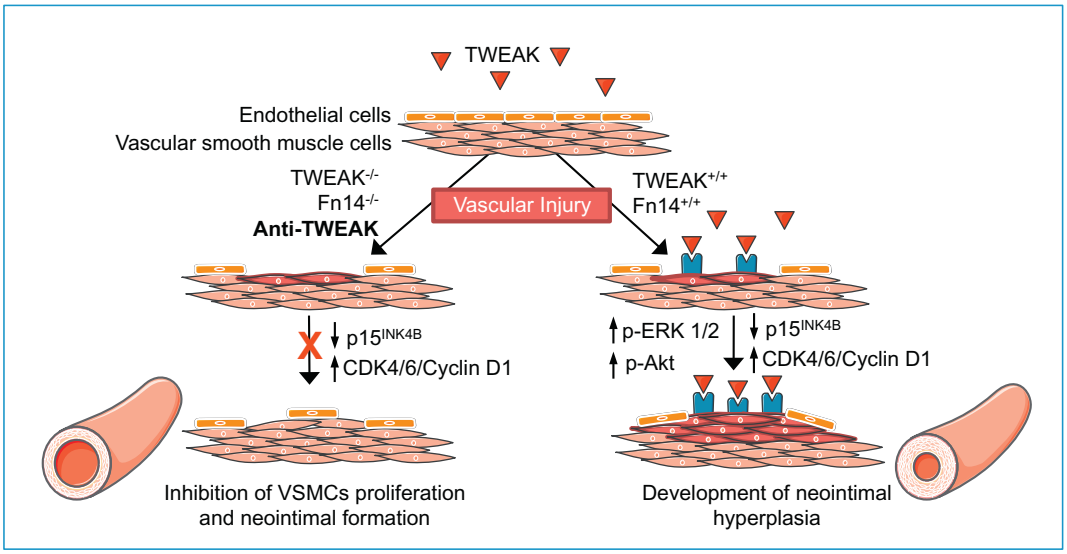


Fig. 9. Model illustrating the potential mechanism of TWEAK/Fn 14 axis in the development of neointimal formation after endovascular injury. Cartoon depicting TWEAK/Fn14 function in neointimal formation after wire injury. The interaction of TWEAK with its receptor Fn14 diminishes $p15^{INK4B}$, increases cyclin D1, CDK4 and CDK6 expression and ERK1/2 and Akt activation in VSMCs, leading to an increase in VSMCs proliferation and migration. Therapeutic intervention with anti-TWEAK antibodies reduces neointimal formation.

of TWEAK has been previously reported in several cell types including endothelial cells, cardiomyocytes, cardiac fibroblast, keratinocytes, hepatic cells and several tumoral cell lines [7,31–33]. Although the mechanisms by which TWEAK induces cell proliferation are poorly known, it has been previously shown that TWEAK induces cyclin D2 upregulation and $p27^{kip1}$ downregulation in cardiomyocytes, effect dependent of ERK and PI3K signaling [32]. Now, we demonstrate for the first time that TWEAK decreases the expression of $p15^{INK4B}$, a cyclin-dependent kinase inhibitor implicated in cell cycle regulation. TWEAK-mediated $p15^{INK4B}$ downregulation was related to the interaction with its sole receptor Fn14. Thus, VSMCs from *Tnfrsf12a*$^{-/-}$ mice or siRNA against Fn14 prevented the downregulation of $p15^{INK4B}$ induced by TWEAK.

The $p15^{INK4B}$ protein participates in the maintenance of the quiescent state in different cells. In VSMCs, $p15^{INK4B}$ upregulation results in G1 phase arrest and inhibition of pRb phosphorylation [34]. In addition, overexpression of $p15^{INK4B}$ in vivo inhibits in-stent intimal hyperplasia in rabbits [34]. However, although genetic deletion of *Cdkn2B* in mice accelerates VSMCs proliferation, paradoxically it leads to smaller neointimal lesions [35]. Now, we observed a downregulation of $p15^{INK4B}$ expression in TWEAK-treated VSMC and, an increase of $p15^{INK4B}$ levels in *Tnfrsf12a* and *Tnfsf12* deficient mice, an effect associated with a decrease in cell proliferation. Based on these results, it is conceivable to think that the effect of $p15^{INK4B}$ in neointimal formation is dependent on its expression level within the arterial wall. The precise mechanism by which TWEAK decreases $p15^{INK4B}$ should be related with the activation of different pathways. In endothelial cells, TWEAK activates ERK1/2, JNK 1/2 and NF-kB, but not p38 MAPK [31]. In addition, TWEAK activates PI3K/Akt in osteoblastic cells [36] and ERK1/2 in human VSMCs [37]. Now, we observed that TWEAK induces ERK1/2, Akt and p65 phosphorylation in VSMCs. Accordingly, ERK activation seems to be essential for cell cycle progression in VSMCs [38]. Using specific inhibitors, we observed that inhibition of ERK1/2 and Akt activation prevented $p15^{INK4B}$ downregulation, indicating that the mechanism by which TWEAK decreases $p15^{INK4B}$ expression it may be related to ERK1/2 and Akt activation. We have also observed that TWEAK induces a rapid p65, Akt and ERK phosphorylation, followed by a secondary and delayed phosphorylation of these proteins. The mechanisms ruling the bimodal activation of p65, Akt and ERK are unknown. This could be due by to the fact that TWEAK upregulates several molecules such as cytokines, chemokines and other members of the TNF superfamily that could be responsible for the secondary signal observed.

$p15^{INK4B}$ is a known inhibitor of CDK4/CDK6 and cyclin D1 expression in VSMCs [28]. Accordingly, we observed an increase in CDK4/CDK6 and cyclin D1 mRNA and protein expression under TWEAK stimulation in VSMCs. The increase of these cyclin-dependent kinases and cyclin D1 was accompanied by an augmentation in the number of cells in S phase and total number of cells, and a decrease of cells in G0/G1. Very importantly, we also provide evidence that our in vitro observations are applicable in vivo by demonstrating that *Tnfrsf12a* or *Tnfsf12* deletion decreased the number of proliferative cells, CDK4/6 and cyclin D1 expression, and increased $p15^{INK4B}$ expression, ending in less neointimal formation in a murine model of vascular injury.

VSMCs proliferation is associated with their plasticity [39]. VSMCs have the ability to change from a differentiated and quiescent contractile state to a proliferative and migratory synthetic phenotype in response to surrounding stimulus. In this sense, we have previously demonstrated that TWEAK-stimulated VSMCs differentiate from a contractile to a synthetic phenotype [15]. Thus, TWEAK decreases markers of contractile phenotype such as α-SMA and calponin, and increases markers of synthetic phenotype such as osteopontin and metalloproteinase 9 in cultured VSMCs [10,15]. Change in the phenotype induced by TWEAK is in agreement with the increase in VSMCs proliferation observed in our study. However, it is important to note that many expanded VSMC-derived cells in the injured-induced neointima maintain α-SMA expression [40]. In this context, it has been previously demonstrated that TWEAK/Fn14 axis increases α-SMA expression during myofibroblasts differentiation [41]. VSMCs migration is also an important component that contributes to neointimal formation [4]. Although it has been reported that TWEAK increases cell migration in a variety of cell types such as endothelial cells [42], data regarding the effect of TWEAK in VSMCs migration were lacking. Here we demonstrate that TWEAK induces VSMCs motility. This effect is directly related with the expression of Fn14 since cells lacking *Tnfrsf12a* gene failed to migrate in response to TWEAK. Therefore, the induction of cell migration by TWEAK/Fn14 axis can also contribute to increase lesion formation in vivo.

The most important finding in our study is the potential translationality of our results obtained from the use of anti-TWEAK therapy to limit neointimal formation after wire injury. Although the introduction of drug-eluting stents has improved the prevention and treatment of restenosis [5], neointimal obstruction persists in most cases [43]. In addition, the cytostatic agents such as rapamycin are

relatively nonspecific and are associated with late-stent thrombosis [5]. Our finding demonstrated that blockade of TWEAK by specific antibodies can recapitulate the genetic phenotype obtained in *Tnfsf12*$^{-/-}$ mice preventing cell proliferation after injury by increasing p15^{INK4B} expression and reducing CDK4/6 and cyclin D1 expression levels in injured vessel. However, it is important to note that VSMCs proliferation could impact other vascular remodeling processes such as atherosclerosis. In fact, while VSMCs proliferation is detrimental in the early stages of atherosclerotic plaque developing, it is protective in advanced atherosclerotic lesions, preventing fibrous cap from rupturing and promoting plaque repair [44]. For that reason, a local administration of TWEAK/Fn14 blockers through drug-eluting stents could be a better option to lower the restenosis rate.

In conclusion, our findings provide novel and important insights into the role of TWEAK/Fn14 axis in neointimal formation after wire injury. TWEAK increases VSMCs proliferation and migration through p15^{INK4B} downregulation and CDK4/6 and cyclin D1 upregulation. Therapies aimed to block TWEAK/Fn14 interaction could protect from restenosis after angioplasty.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.072.

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Author contributions statement

The study was conceived by JE, JLMV and LMBC. NMB and LMBC designed the study and analyzed the data. NMB, CGM and VE performed the experiments. JMM and PM performed RNA-Seq and analyzed the obtained data. JBM performed human samples studies. NMB and LMBC wrote the manuscript with contributions of JLMV and VE. All authors read and approved the manuscript.

Declaration of Competing Interest

None.

Acknowledgments

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