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# KRIT1 loss-mediated upregulation of NOX1 in stromal cells promotes paracrine pro-angiogenic responses

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ABSTRACT

Cerebral cavernous malformation (CCM) is a cerebrovascular disorder of proven genetic origin characterized by abnormally dilated and leaky capillaries occurring mainly in the central nervous system, with a prevalence of 0.3–0.5% in the general population. Genetic studies have identified causative mutations in three genes, *CCM1/KRIT1*, *CCM2* and *CCM3*, which are involved in the maintenance of vascular homeostasis. However, distinct studies in animal models have clearly shown that CCM gene mutations alone are not sufficient to cause CCM disease, but require additional contributing factors, including stochastic events of increased oxidative stress and inflammation. Consistently, previous studies have shown that up-regulation of NADPH oxidase-mediated production of reactive oxygen species (ROS) in KRIT1 deficient endothelium contributes to the loss of microvessel barrier function.

In this study, we demonstrate that KRIT1 loss-of-function in stromal cells, such as fibroblasts, causes the up-regulation of NADPH oxidase isoform 1 (NOX1) and the activation of inflammatory pathways, which in turn promote an enhanced production of proangiogenic factors, including vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2). Furthermore and importantly, we show that conditioned media from KRIT1 null fibroblasts induce proliferation, migration, matrix metalloproteinase 2 (MMP2) activation and VE-cadherin re-distribution in wild type human endothelial cells.

Taken together, our results demonstrate that KRIT1 loss-of-function in stromal cells affects the surrounding microenvironment through a NOX1-mediated induction and release of angiogenic factors that are able to promote paracrine proangiogenic responses in human endothelial cells, thus pointing to a novel role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM pathogenesis, and opening new perspectives for disease prevention and treatment.

## 1. Introduction

Cerebral cavernous malformations (CCMs), also known as cavernous angioma or cavernoma, are vascular anomalies typically found in the brain and spinal cord with a prevalence of 0.3%–0.5%. CCMs are mulberry-like, thin-walled sinusoidal capillaries lacking normal vessel structural components, including pericytes and astrocytes, and often surrounded by hemosiderin deposits and gliosis [1–3]. These vascular lesions can develop anywhere in the body, but signs and symptoms generally appear only when they occur in brain and spinal cord, where they account for 5–15% of all vascular malformations.

CCMs can occur as single or multiple lesions (even in the hundreds order), with size ranging from a few millimeters to a few centimeters. Despite the high prevalence of CCM lesions, approximately only 30% of

*Abbreviations:* CCM, Cerebral Cavernous Malformation; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase 2; ICH, intracerebral hemorrhage; fCCM, familial cerebral cavernous malformation; sCCM, sporadic cerebral cavernous malformation; CNS, central nervous system; NVU, neurovascular unit; ROS, reactive oxygen species; SOD, superoxide dismutase; FoxO1, Forkhead box protein O1; GKT, GKT137831; ML, ML171; NS, NS398; COX-2, cyclooxygenase 2; PGE2, Prostaglandin E2

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affected people develop clinical symptoms, including recurrent headaches, neurological deficits, seizures, stroke, and intracerebral hemorrhage (ICH); however, the majority of CCM lesions remain clinically silent during most of the host's lifetime [3].

CCM is a disease of proven genetic origin that can arise sporadically or may be inherited as an autosomal dominant condition with incomplete penetrance and variable clinical expressivity [4]. The sporadic form (sCCM) accounts for up to 80% of cases, whereas the familial form (fCCM) accounts for at least 20% of cases. Genetic studies have identified three genes associated to CCMs: *KRIT1* (*CCM1*), *MGC4607* (*CCM2*) and *PDCD10* (*CCM3*), which account for about 50%, 20% and 10% of the cases, respectively. The remaining 20% are likely associated to undetected genetic alterations of CCM genes [5]. Many different CCM mutations have been identified, most of which typical of a single family. A different clinical penetrance between the CCM genes (60–88% for *KRIT1,* up to 100% for *CCM2*, and 63% for *PDCD10*) [6], and a large variability of severity of CCM lesions even among family members carrying the same germline mutation have been observed. However, distinct studies in animal models have clearly shown that mutations of CCM genes are not sufficient to cause CCM disease, suggesting that additional factors can contribute to CCM disease pathogenesis [5,7]. Consistently, DNA sequencing analysis of surgically-resected CCM lesions from autosomal dominant CCM patients have identified somatic mutations at very low frequencies, suggesting that the minority of cells in the mature CCM harbor these mutations [8,9].

In the central nervous system (CNS) the endothelium is part of complex units, called neurovascular units (NVU), where it is in close contact with other cell types (pericytes, astrocytes and neurons). All components of this unit interact with each other in a multidimensional process in which mediators released from multiple cells engage distinct signaling pathways and effector systems in a highly orchestrated manner and safeguard the integrity of the structure itself by regulating immune response, angiogenesis, vasculogenesis, oligodendrogenesis, neuroprotection and neuroplasticity [10–12].

To date, the effect of the KRIT1 loss on NVU is not known. However, recent observations outline the importance of microenvironment in the development of vascular lesions observed in fCCM. For instance, Louvi et al., demonstrated in an animal model that *CCM3* neural deletion has cell nonautonomous effects resulting in the formation of multiple vascular lesions that closely resemble human cavernomas. Consistently, in a very recent paper Malinverno et al., showed that vascular lesions originate from clonal expansion of few *CCM3* KO endothelial cells that attract sourrounding wild-type endothelial cells thus contributing to cavernoma growth [3,13,14].

Although the effective mechanisms through which loss of CCM proteins leads to vascular malformations remain to be comprehensively defined, in recent years it has been demonstrated that these proteins exert pleiotropic effects, related to their role in the regulation of multiple molecules and mechanisms involved in angiogenesis, cellular response to oxidative stress, inflammation, cell-cell and cell-matrix adhesion, and cytoskeleton dynamics [3,15].

Reactive oxygen species (ROS) are produced by the activity of a wide array of cellular enzymes, including NADPH oxidases (NOX), enzymes of the mitochondrial respiratory chain, xanthine oxidases, cytochrome *p*450 monooxygenases, lipoxygenases and cyclooxygenases, which can be induced by a variety of endogenous and exogenous chemical and physical stimuli [16]. The NOX family of enzymes produces ROS as their sole function, and are becoming recognized as key modulators of signal transduction pathways with a physiological role under acute stress and a pathological role after excessive activation under chronic stress. ROS produced by NOX proteins are now recognized to play essential roles in the regulation of cytoskeletal remodeling, gene expression, proliferation, differentiation, migration, and cell death. The NOX isoforms (NOX1–5, and DUOX1/2) differ in their regulation, tissue and subcellular localization and even ROS products [17,18]. NOX1, NOX2, NOX4, and NOX5 are expressed in endothelium, vascular smooth muscle cells, fibroblasts, or perivascular adipocytes. While NOX1/NOX2 promote the development of endothelial dysfunction, hypertension, and inflammation, NOX4 may play a role in protecting the vasculature during stress; however, when its activity is increased, it may be detrimental [19]. Recently NOX1 has been involved in several brain diseases [20], and has been also described to play a role in cancer by inducing tumor progression and angiogenesis through the regulation of vascular endothelial growth factor (VEGF) expression [21–23].

Previously, we demonstrated that KRIT1 loss affects the intracellular redox homeostasis and results in increased ROS production through distinct mechanisms, including Forkhead box protein O1 (FoxO1) and superoxide dismutase (SOD) downregulation, NOX4 upregulation, and abnormal antioxidant responses, suggesting a novel pathogenetic mechanism whereby CCM disease may result from impaired endothelial cell defenses to microenvironmental oxidative stress events [4,16,24–30].

Herein, we show that KRIT1 loss-of-function in fibroblasts induces the upregulation of NOX1, which in turn can trigger a paracrine proangiogenic response in wild type endothelial cells through increased production and release of angiogenic growth factors, suggesting a novel important role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM disease pathogenesis, and pointing to NOX1 as a major regulator of these effects and as a new potential therapeutic target.

## 2. Material and methods

### 2.1. Cell culture

Wild-type (K+/+) and KRIT1 knock-out (K−/−) mouse embryonic fibroblast (MEF) cell lines were established from KRIT1+/+ and KRIT1−/− E8.5 mouse embryos, respectively [28]. KRIT1−/− MEFs re-expressing KRIT1 (K9/6) were obtained as previously reported [28]. Cells were cultured at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), with 4500 mg/l glucose and 100 U/ml penicillin/streptomycin (Euroclone, Milan, Italy).

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Lonza, Basel, Switzerlans) and Human Cerebral Microvascular Endothelial Cells (HCMEC/d3) were from MERK (MERK, Darmstadt, Germany). All experiments were performed on low passage cell cultures. Cells were grown on gelatin-coated dishes in Endothelial Growth Medium (EGM-2) (EBM-2, FBS 10%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000) (Lonza) at 37 °C and 5% $CO_2$.

### 2.2. Conditioned media (CMs) preparation

MEFs were seeded on 24-well plates ($4 \times 10^4$ cells/well) in DMEM 10% FBS. After 24 h the medium was replaced with DMEM 0.5% serum and collected after 48 h. When reported, cells were treated for 24 h with NOX1/4 inhibitor GKT137831 (GKT, 5 μM) (Cayman Chemical, Ann Arbor, MI, USA), NOX1 inhibitor ML171 (ML, 5 μM) (EMD Millipore, Darmstadt, Germany) or COX-2 inhibitor NS398 (NS, 5 μM) (Cayman Chemical). The medium was then replaced with DMEM, 0.5% serum, and conditioned media (CM) were collected after 48 h and stored at −80 °C for further analysis. For the measurement of MMPs activity (gelatin zymography) CM were prepared without serum.

### 2.3. Proliferation assay

HUVEC and HCMEC/d3 cells were plated at density of $1.5 \times 10^3$ cells/well in 96-well microplates in EGM-2 with 10% serum for 24 h. After incubation for 24 h with medium added with 0.1% serum, cells were treated with MEF CMs for 72 h and then fixed and stained with

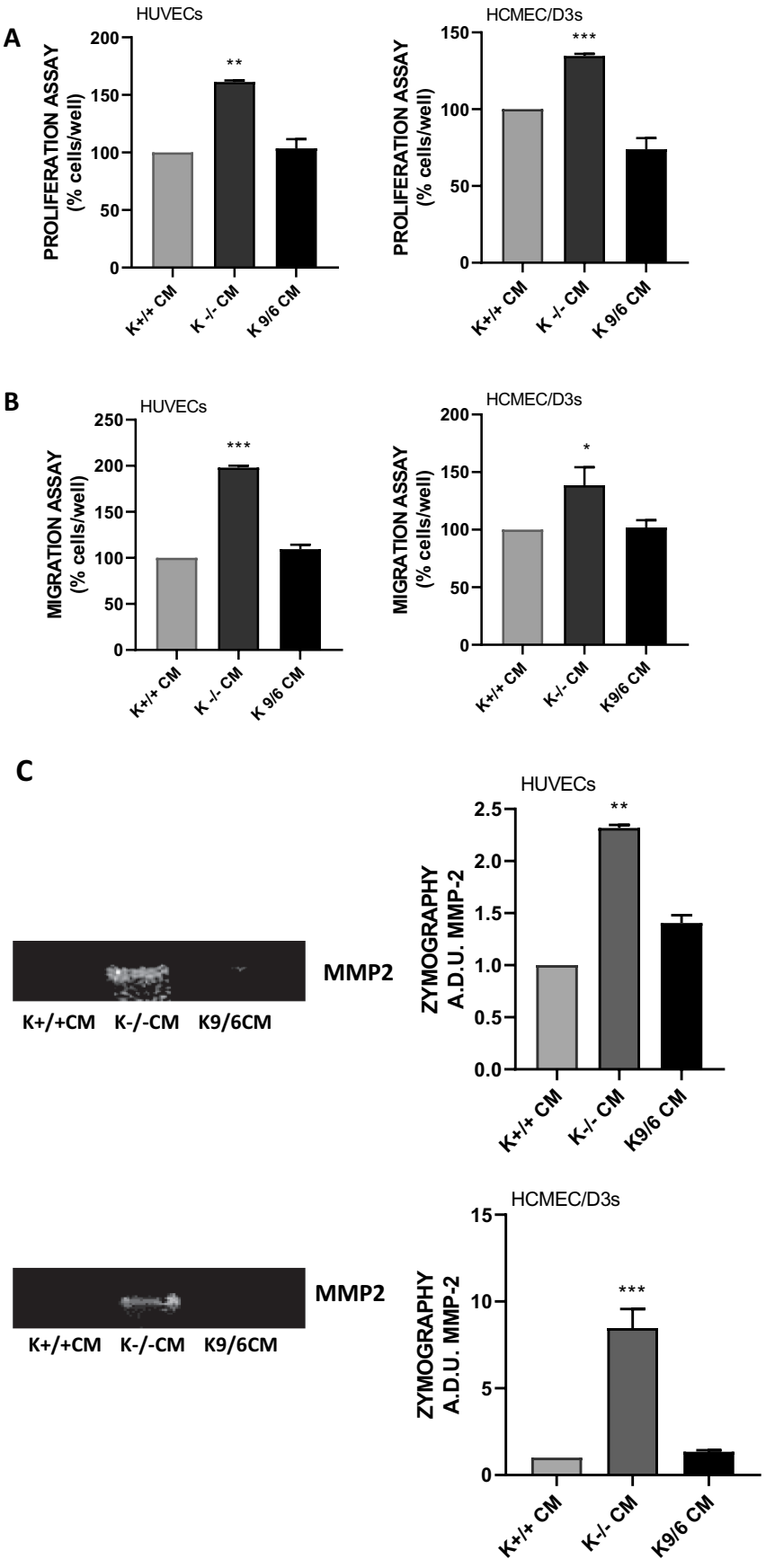


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**Fig. 1.** Conditioned media from KRIT1 K−/− MEFs induce a pro-angiogenic response in HUVEC cells. (A) HUVEC and HCMEC/d3 proliferation. Starved endothelial cells were incubated with conditioned media (CM) obtained from K+/+, K−/− and K9/6 MEFs. Cells were then fixed, stained with PanReac and counted; results are expressed as percentage of proliferating cells and are representative of three independent experiments (** $p < .01$ vs K+/+ MEF). (B) HUVEC and HCMEC/d3 migration. The migration assay was performed by using the Boyden chamber as detailed in Materials and Methods. Data are reported as percentage of migrated cells per well and are representative of three independent experiments (*** $p < .001$ vs K+/+ MEF). (C) MMP-2 enzymatic activity through gelatin zymography. Endothelial cells were treated for 24 h with serum-free CM obtained from K+/+, K−/− and K9/6 MEF cells. MMP-2 band was evaluated with quantitative densitometry and normalized to number of cells/well. Data are representative of three independent experiments (A.D.U.: arbitrary densitometry units) (*** $p < .001$ vs K+/+ MEF). (D) Immunofluorescence analysis of VE-cadherin and β-catenin localization in HUVEC cells. HUVECs were cultured on glass cover-slips, starved and treated with CM from K+/+ or K−/− cells. HUVECs were then fixed and stained with anti-VE-cadherin or anti-β-catenin. Images are representative of three different experiments.

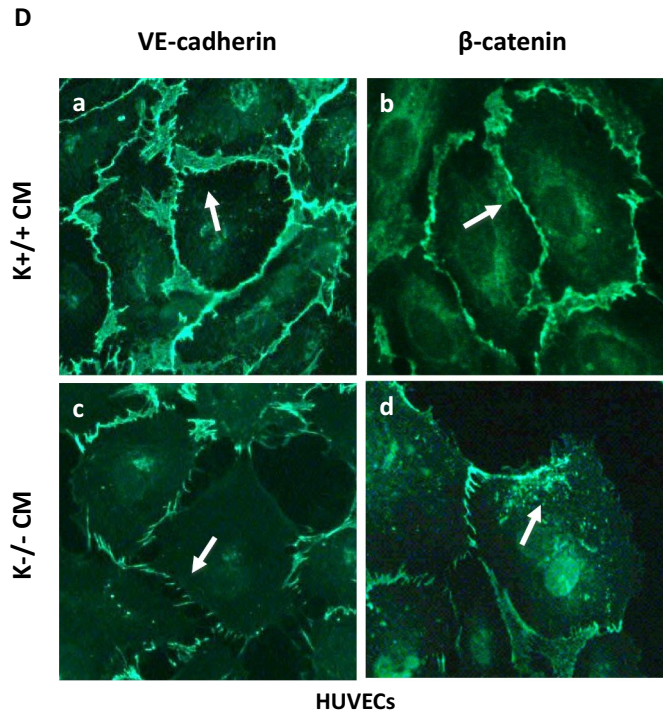


**Fig. 1.** (*continued*)

PanReac (Biomap, Applichem Gmbh, Darmstadt, Germany). The number of proliferated cells present in five fields/well was counted at 10× magnification. Data were reported as percentage of proliferating cells [31].

### 2.4. Migration assay

Chemotaxis experiments were performed using the Boyden chamber technique (Neuroprobe 48-well microchemotaxis chamber), with the filter coated with 1% gelatin (Sigma–Aldrich, Milan, Italy) [31,32]. HUVEC or HCMEC/d3 were added to the upper wells of the chamber ($1.25 \times 10^4$ cells/well) suspended in 0.1% serum, and chemoattractant (0.5% serum) was placed in the lower wells. After 6 h incubation the cells were fixed and stained with PanReac. The number of migrated cells present in five fields/well was counted at 40× magnification. Data were reported as percentage of migrating cell.

### 2.5. Western blotting

K+/+, K−/− and K9/6 MEFs ($1.5 \times 10^5$ cells/well) were seeded in 6-well multiplates in DMEM added with 10% serum. Cells were then treated with GKT (5 μM), ML (5 μM) or NS (5 μM) in DMEM with 0.5% serum. After 48 h, extraction of total proteins was performed by lysing cells in precooled radioimmunoprecipitation assay (RIPA) lysis buffer.

HUVECs ($1.5 \times 10^5$ cells/well) were seeded in 6-well multiplates in EGM-2 added with 10% serum for 24 h. After starvation with EBM-2 (0.1% serum), cells were treated with CMs for 15 min.

Protein concentration of cell extracts was determined spectrophotometrically using the BCA protein assay kit (Euroclone S.P.A., Pero, MI, Italy). For western blotting analysis, aliquots of cell extract supernatants containing an equal amount of proteins (50 μg) were treated with Laemmli buffer, boiled for 10 min, resolved on 4–20% stain-free gel and then blotted onto a nitrocellulose membrane using Novablot Semidry System (GE Healthcare s.r.l., Milan, Italy). The blots were blocked with 5% nonfat dry milk (Euroclone) in Tris-buffered saline (TBS) containing 0.5% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with appropriate dilutions of primary antibodies. Subsequently membranes were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies. Proteins were then visualized by an enhanced chemiluminescence detection system (EMD Millipore). The primary antibodies used in the present study included anti-COX-2 (Cell Signaling Technology, Leiden, The Netherlands), anti-GAPDH (EMD Millipore), anti-NOX1 (GeneTex, Irvine, CA, USA), anti-VEGF (EMD Millipore), anti-β-catenin and anti-P-β-catenin (Cell Signaling Technology). Affinity-purified HRP-conjugated secondary antibodies were from Sigma-Aldrich. Protein bands from western blots were quantified by densitometry using the ImageJ software, and their relative amounts were normalized to the levels of housekeeping proteins serving as internal loading controls.

### 2.6. Gelatin zymography

$5 \times 10^3$ cells/well (HUVECs and HCMEC/d3) were cultured in 96-well cell culture plates in 10% FBS medium. After adhesion, cells were incubated with 100 μl of serum-free MEF CMs for 48 h. The CMs were substituted with 50 μl of EBM and, after 48 h, media were collected, clarified by centrifugation and assayed for zymography. Media were subjected to electrophoresis in 8% SDS-PAGE containing 1 mg/ml gelatin under non-denaturing conditions, by using Sample Buffer w/o β-ME and sample boiling. After electrophoresis, gels were washed with 2.5% Triton X-100 to remove SDS and incubated for 48 h at 37 °C in 50 mM Tris buffer containing 200 mM NaCl and 20 mM $CaCl_2$, pH 7.4. Gels were stained with 0.05% Coomassie brilliant blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 45% methanol. Bands of gelatinase activity appeared as transparent areas against a blue background. Gelatinase activity was then evaluated by quantitative densitometry [32].

### 2.7. Immunofluorescence analysis

HUVEC cells ($1 \times 10^5$ cells/well) were seeded on glass cover-slips pre-coated with 1% gelatin. After starvation, cells were treated with MEF conditioned media for 24 h.

Cells were fixed with acetone for 5 min, incubated with 3% BSA for 40 min and stained overnight at 4 °C with primary antibody. Slips were washed three times with PBS and then incubated 1 h at room temperature with Alexa Fluor 488 or 555 secondary antibody (ThermoFisher Scientific, Waltham, MA, USA). The anti β-catenin and anti VE-cadherin primary antibodies were from Cell Signaling Technology. Microscopy imaging was performed on Axio Lab A1 microscope (Carl Zeiss S.P.A., Milan, Italy) using a 40× objective.

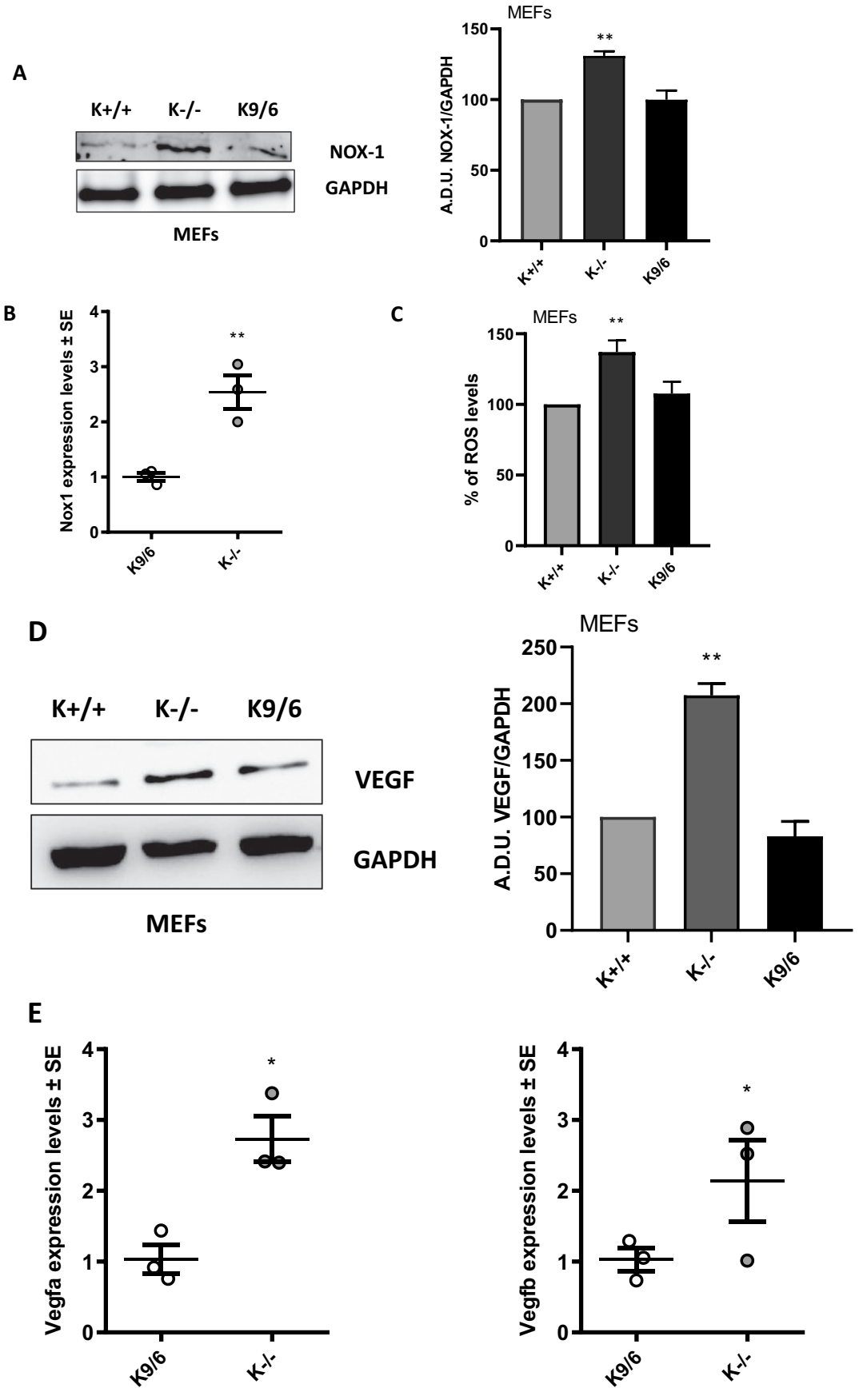


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**Fig. 2.** KRIT1 loss in stromal cells affects NOX1 and COX-2 expression. (A, D, F) Representative images and quantification of western blot analysis of NOX1, VEGF and COX-2 expression in K+/+, K−/− and K9/6 MEFs. The gels are representative of three independent experiments (A.D.U.: arbitrary densitometry units). (B, E, G) mRNA levels of Nox1, Vegf a and b and Cox-2 in K+/+, K−/− and K9/6 MEFs measured by RT-PCR (C) ROS measurement after 24 h of incubation in DMEM containing 0.5% serum. Data are expressed % of ROS levels. (H) PGE2 levels of K+/+, K−/− and K9/6 MEFs measured by EIA assay. Data are expressed as pg/ml. (***p < .001,** p < .01 and *p < .05 vs K+/+ MEF).

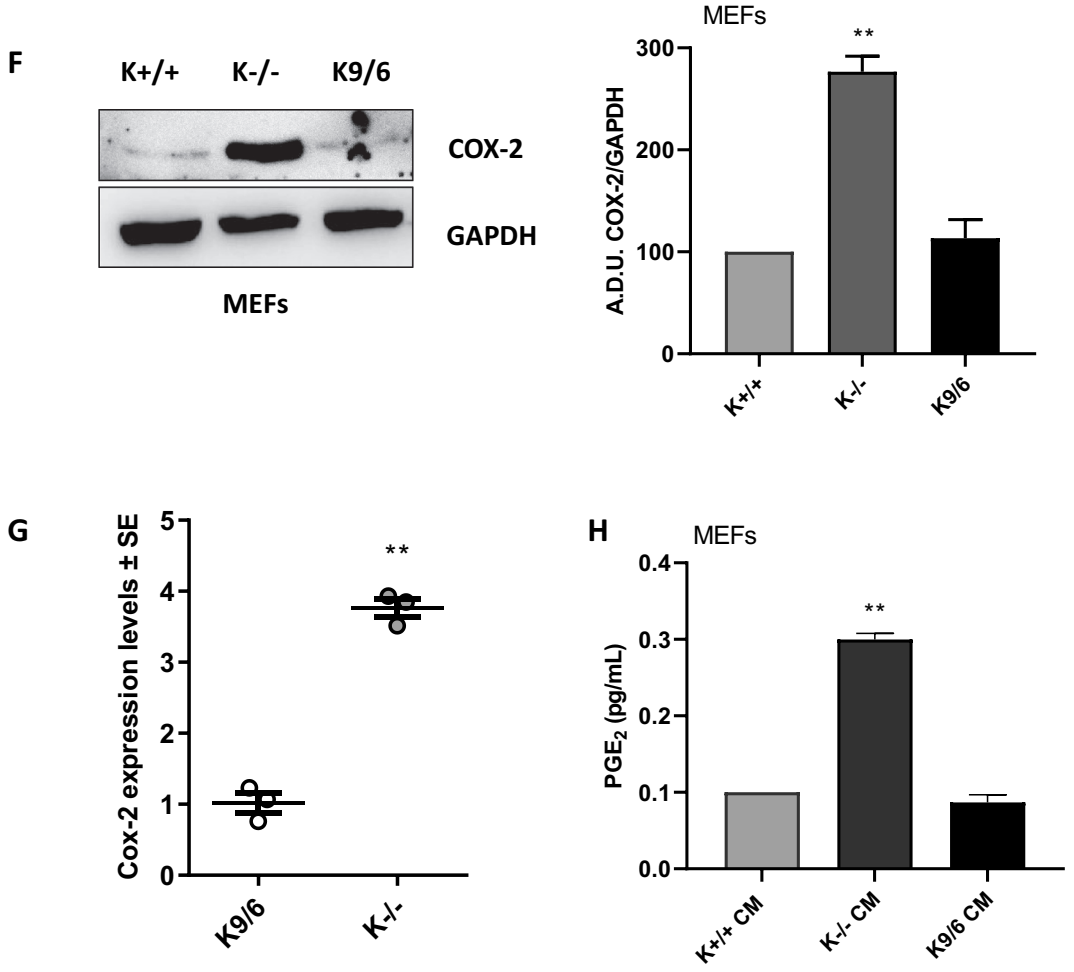


**Fig. 2.** (*continued*)

### 2.8. PGE2 immuno-assay

PGE2 was measured by an enzyme immunoassay (EIA) kit (Prostaglandin E2 EIA kit-Monoclonal, Cayman Chemical). MEFs were seeded on 24-well plates ($4 \times 10^4$ cells/well) in DMEM 10% FBS. After 24 h the medium was replaced with DMEM without serum and, when reported, cells were treated with NOX1 inhibitor, ML (5 μM), or COX-2 inhibitor, NS (5 μM). Media were collected after 48 h and stored at −80 °C.

### 2.9. Quantitative RT-PCR

Total RNA was extracted with the RNeasy micro kit (Qiagen GmbH, Hilden, DE), and reverse transcribed to cDNA with the High-Capacity cDNA Archive kit (Applied Biosystems, Thermofisher, Waltham, USA). Quantitative Real Time RT-PCR was performed as described [33], either with predeveloped Taqman assays (Applied Biosystems, Thermofisher, Waltham, USA) or by combining the RealTime Ready Universal Probe Library (UPL, Roche Diagnostics, Monza, Italy) with the primers listed below. A relative quantification approach was used, according to the 2-ddCT method [34]. To normalize expression levels, GAPDH was used for quantifications in MEFs.

List of the predeveloped Taqman assays or combinations of primers + UPL probes used in the present study:

| *Gene* | *Taqman assay (Applied Biosystems)* | *Primers + UPL probe (Roche Diagnostics)* |
|---|---|---|
| Ptgs2 (Cox2) - mouse | | FW: gatgctcttccgagctgtg<br>RV: ggattggaacagcaaggattt<br>Probe #45 |
| Vegfa - mouse | Cat. N: #Mm0437304_m1 | |
| Vegfb - mouse | | FW: gctcaacccagacacctgtag<br>RV: aggaggttcgcctgtgct<br>Probe #7 |
| Nox1 - mouse | | FW: atccttgcaccgattgctt<br>RV: cattagatgggtgcatgacaa<br>Probe #3 |
| GAPDH - mouse | Cat. N: # Mm99999915_g1 | |

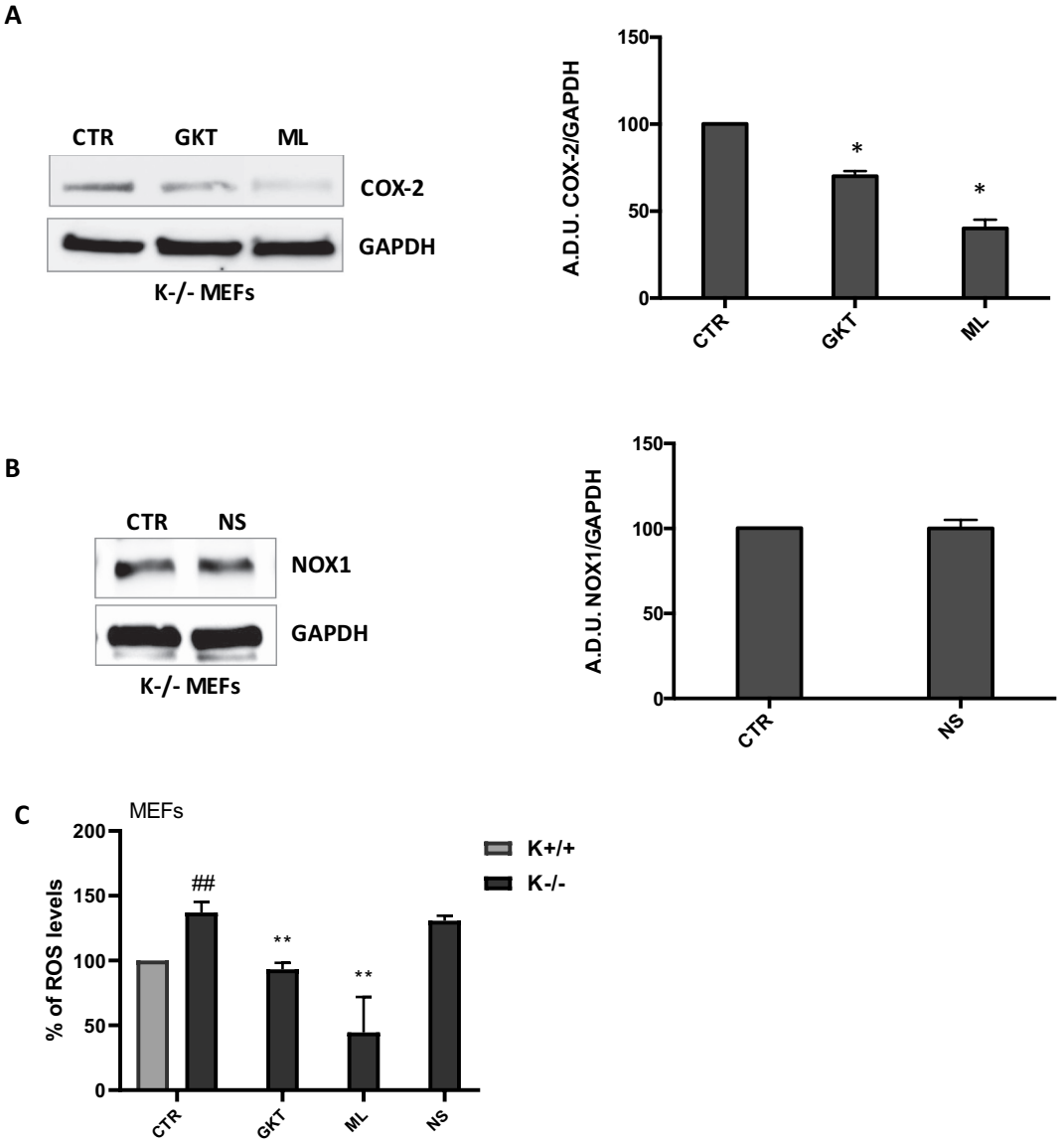


**Fig. 3.** NOX1 controls COX-2 expression in K−/− MEFs. (A) Western blot analysis of COX-2 expression in K−/− MEFs incubated for 48 h with a NOX1/NOX4 inhibitor (GKT, 5 μM) or a selective NOX1 inhibitor (ML, 5 μM) (B) Western blot analysis of NOX1 expression in K−/− MEFs incubated for 48 h with a COX-2 specific inhibitor (NS, 5 μM). Untreated K−/− MEFs (CTR) were analyzed as controls. The gels shown in the figure are representative of three independent experiments (A.D.U.: arbitrary densitometry units). (C) (*p < .05 vs CTR).

### 2.10. ROS measurement

ROS levels were evaluated as previously reported [35]. 1.5 × $10^3$ cells (K+/+, K−/− or K9/6 MEFs) were seeded in 96-multiwell plates and, after adherence, were maintained for 24 h in medium without phenol red (0.5% serum) or treated with NOX1/4 inhibitor GKT137831 (GKT, 5 μM), NOX1 inhibitor ML171 (ML, 5 μM) or COX-2 inhibitor NS398 (NS, 5 μM) for 24 h. DCFH2-DA (2,-7-dichlorodihydrofluorescein diacetate) (Invitrogen, Milan, Italy) was added (10 μM, 30 min) and intracellular levels of ROS were evaluated photometrically with a microplate reader (excitation/emission 495/527) (EnVision, PerkinElmer).

### 2.11. Statistical analysis

Data were generated from three independent experiments and expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test for unpaired data; $p < .05$ was considered statistically significant.

## 3. Results

### 3.1. Conditioned medium from KRIT1−/− fibroblasts induces pro-angiogenic responses in wild type human endothelial cells

Accumulated evidences demonstrate that KRIT1 protein plays an important role in modulating different molecular pathways involved in the angiogenic process, whereas KRIT1 loss induces alteration of endothelial cell-cell and cell-extracellular matrix (ECM) adhesion, and enhanced vascular permeability [3,36,37]. However, recent evidence in conditional knockout (cKO) animal models has clearly shown that loss-of-function mutations in CCM genes alone are not sufficient for the development of CCM lesions, suggesting the potential contribution of endothelial cell-nonautonomous mechanisms and paracrine cues that

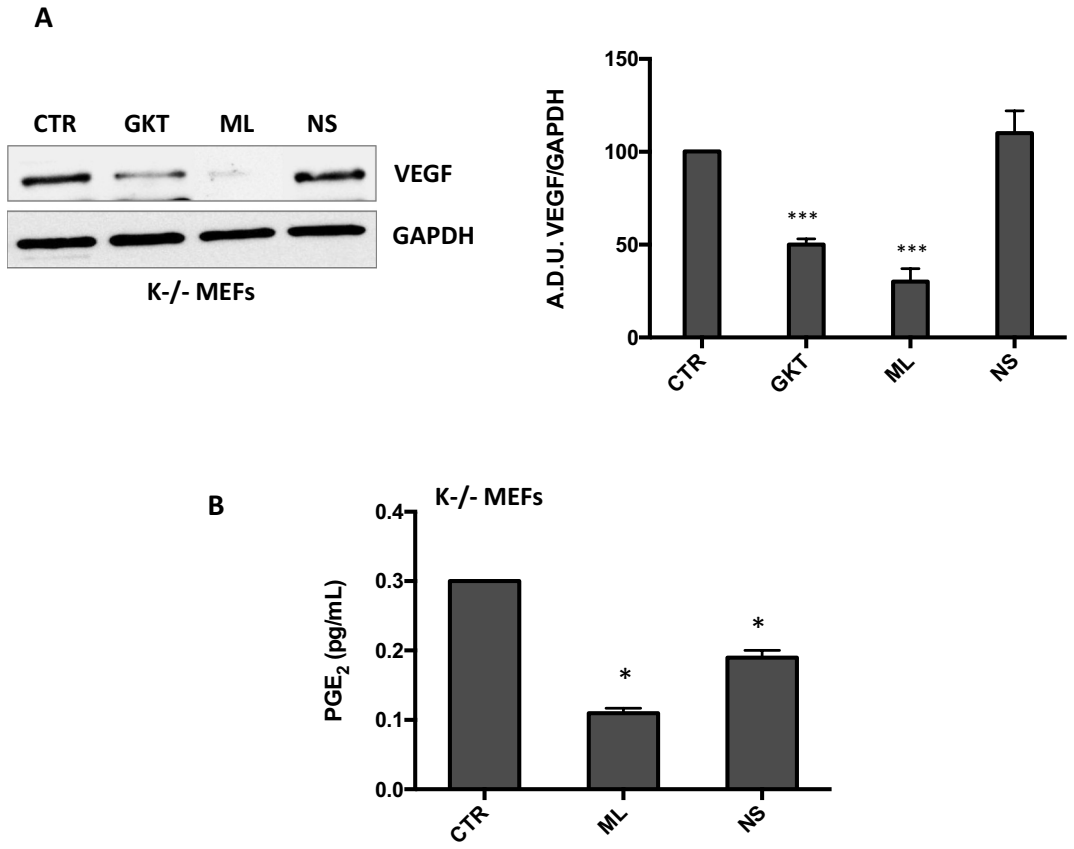


**Fig. 4.** NOX1 regulates VEGF expression and PGE2 production in K−/− MEFs. (A) Western blot analysis of VEGF expression in K−/− MEFs treated for 48 h with 5 μM NOX1/NOX4 inhibitor (GKT) or selective NOX1 inhibitor (ML). Untreated K−/− MEFs (CTR) were analyzed as controls. The gels shown are representative of three independent experiments (A.D.U.: arbitrary densitometry units) (*p < .05 and ***p < .001 vs CTR). (B) PGE2 levels measured in K−/− MEFs treated for 48 h with 5 μM NOX1 inhibitor (ML) and in untreated K−/− MEFs (CTR) by EIA assay. K−/− MEFs treated with the COX-2 inhibitor (NS), were used as internal control. Data are expressed as pg/ml (*p < .05 vs CTR).

affect the vascular microenvironment [3].

To address this possibility, we investigated the ability of conditioned media from KRIT1−/− fibroblasts to induce a proangiogenic response in wild type human endothelial cells. To this end, we took advantage of wild-type (K+/+) and KRIT1 knock-out (K−/−) Mouse Embryonic Fibroblasts (MEFs), a well-established cellular model of CCM disease [4,22,24,26,28,37–40], to produce conditioned media (CM) and test their effects on Human Umbilical Vein Endothelial Cells (HUVECs) and Human Cerebral Microvascular Endothelial Cells (HCMEC/d3). First we compared proliferation and migration of HUVECs and HCMEC/d3 treated for 48 h with CM from either K+/+ or K−/− MEFs, observing that CM from K−/− MEFs (K−/− CM) increased proliferation (Fig. 1A) and migration (Fig. 1B) of both endothelial cell lines compared to CM from K+/+ MEFs (K+/+ CM). In order to exclude that the differences observed could be caused by the different genetic background of K+/+ and K−/− MEF, we performed the same experiments by using KRIT1−/− MEFs re-expressing KRIT1 (K9/6 MEF) [28]. As reported in Fig. 1A and B, K9/6 CM was not able to induce neither proliferation or migration of both endothelial cell lines, suggesting that the activation of endothelial cells is specifically dependent to the levels of KRIT1 expression in stromal cells.

It is known that endothelial cell migration during angiogenesis is an invasive process that involves proteolytic activities required for the degradation of the endothelial basement membrane [41]. To investigate whether the increased migration of HUVECs cultured with K−/− CM was related to the activation of specific metalloproteinases (MMPs), we measured MMP-2 activity by gelatin zymography. As shown in Fig. 1C, MMP-2 resulted significantly activated in HUVECs and HCMEC/d3 treated with K−/− CM as compared to controls or K9/6 MEF. Accordingly, the mRNA levels of MMP2 in HUVEC and HCMEC/d3 treated with K−/− CM resulted higher compared to endothelial cells treated with K+/+ or K9/6 MEF (data not shown).

These data clearly indicate that CM derived from K−/− MEFs promotes endothelial cell proliferation and migration, suggesting a switch of endothelial cells from quiescent to active state.

Finally, in order to evaluate whether KRIT1 loss in fibroblasts could affect endothelial cell-cell interactions, we treated confluent HUVECs with either K+/+ or K−/− CM, and analyzed VE-cadherin and β-catenin expression by immunofluorescent staining. As showed in Fig. 1D, in HUVECs treated with K+/+ CM VE-cadherin expression was detected at junctional regions with a continuous zigzag pattern (panel a), while in cells cultured with K−/− CM VE-cadherin staining appeared intermittent, showing frequent gaps (panel c). Similarly, we detected a different pattern of β-catenin expression in HUVECs treated with K−/− CM, including increased protein internalization (panel d), as compared to cells treated with K+/+ CM (panel b). These results show that the CM generated by KRIT1-null fibroblasts is able to modulate VE-cadherin and β-catenin localization in wild type human endothelial cells, suggesting that KRIT1 loss in stromal cells may modulate cell-cell interactions in normal endothelium via paracrine mechanisms.

### *3.2. KRIT1 loss-of-function in stromal cells affects NOX1 and COX-2 expression*

In recent years, it has been demonstrated that KRIT1 plays an important role in the manteinance of cellular redox homeostasis by

modulating master regulators of intracellular ROS levels and cell responses to oxidative stress, including SOD2, FOXO1 and NOX4 [26,28,29]. Conversely, KRIT1 loss-of-function has been clearly associated with the alteration of distinct redox-sensitive mechanisms, including the upregulation of major pro-oxidant and proinflammatory proteins such as c-Jun and cyclooxygenase 2 (COX-2) [26]. On the other hand, consistent with a possible significant involvement in CCM pathogenesis, oxidative stress and inflammation have been clearly implicated in vascular remodeling and endothelial dysfunction associated with cerebrovascular diseases [28]. Indeed, whereas it is known that NADPH oxidases are major enzymes responsable for ROS production [19,42,43], we previously demostrated that NOX4 is involved in CCM disease [29].

To address the putative mechanisms involved in the paracrine signaling between KRIT1-null fibroblasts and wild type human endothelial cells, we considered the possible involvement of NADPH oxidases, as these enzymes are known to play major regulatory roles both in ROS production and in the modulation of proinflammatory and proangiogenic responses [19,29,42,43]. In particular, very recent papers showed that NOX1 upregulation in tumor cells is able to induce the production of angiogenic factors, which in turn activate endothelial cells and trigger an angiogenic response [21–23]. To investigate the potential effects of KRIT1 loss-of-function on NOX1, we evaluated NOX1 expression levels in K+/+, K−/− and K9/6 MEFs both by western blot analysis and RT-PCR. As showed in Fig. 2A and B, NOX1 levels were significantly higher in K−/− cells compared to wild type cells (K+/+) and KRIT1−/− MEFs re-expressing KRIT1 (K9/6), suggesting a functional relathionship. In addition K−/− MEF expressed higher levels of ROS when compared to K+/+ and K 9/6 cells (Fig. 2C). Moreover, to address the putative correlation between NOX1 overexpression in KRIT1-null stromal cells and the observed paracrine effects on wild type human endothelial cells, we firstly investigated the expression levels of VEGF, a major angiogenic growth factor whose expression and signaling have been previously shown to be modulated by NADPH oxidase-derived ROS [44–46]. Western blot and RT-PCR analysis of total cell extracts showed that K−/− MEF cells expressed significantly higher levels of VEGF when compared to K+/+ and K9/6 cells (Fig. 2D and E). Furthermore, consistently with our previous findings that KRIT1 loss is associated with increased activation of inflammatory pathways [26], as well as that the main prostaglandin produced by COX-2, prostaglandin E2 (PGE2), exerts a potent proangiogenic activity [32,47,48], we found higher COX-2 expression levels (Fig. 2F and G) and PGE2 production (Fig. 2H) in K−/− MEF compared to K+/+ and K9/6 MEF.

In the attempt to determine if there was any hierarchy among major pro-oxidant and pro-inflammatory proteins affected by KRIT1 loss-of-function, including NOX1 and COX-2, we inhibited alternatively their activity with specific pharmacological inhibitors, namely ML (NOX1 inhibitor), GKT (NOX1/NOX4 inhibitor) and NS (COX-2 inhibitor). Interestingly, NOX1 inhibition in K−/− MEFs resulted in reduced COX-2 expression levels (Fig. 3A), whereas COX-2 inhibition did not affect NOX1 expression (Fig. 3B) or ROS production (Fig. 3C), suggesting that COX-2 activation induced by KRIT1 loss-of-function occurs via NOX1. Moreover, since both oxidative stress and inflammation can induce the upregulation of VEGF [45], we analyzed its expression levels in K−/− cells after either NOX1 or COX-2 inhibition. As reported in Fig. 4A, we observed a significant reduction of VEGF expression in K−/− cells treated with NOX1 inhibitor, while VEGF expression was not impaired by treatment with the COX-2 inhibitor. As expected, both NOX1 and COX-2 inhibition reduced PGE2 production (Fig. 4B). These data clearly indicate that NOX1 upregulation in K−/− MEFs controls both VEGF and COX-2/PGE2 production.

### 3.3. NOX1 upregulation in stromal cells regulates the paracrine activation of endothelial cells

Combining our findings that treatment of HUVECs with K−/− CM induces a pro-angiogenic phenotype and that NOX1 upregulation in K−/− MEFs induces overproduction of pro-angiogenic factors, we speculated that NOX1 inhibition in K−/− fibroblasts could revert the activation of endothelial cells triggered by K−/− CM. To test this

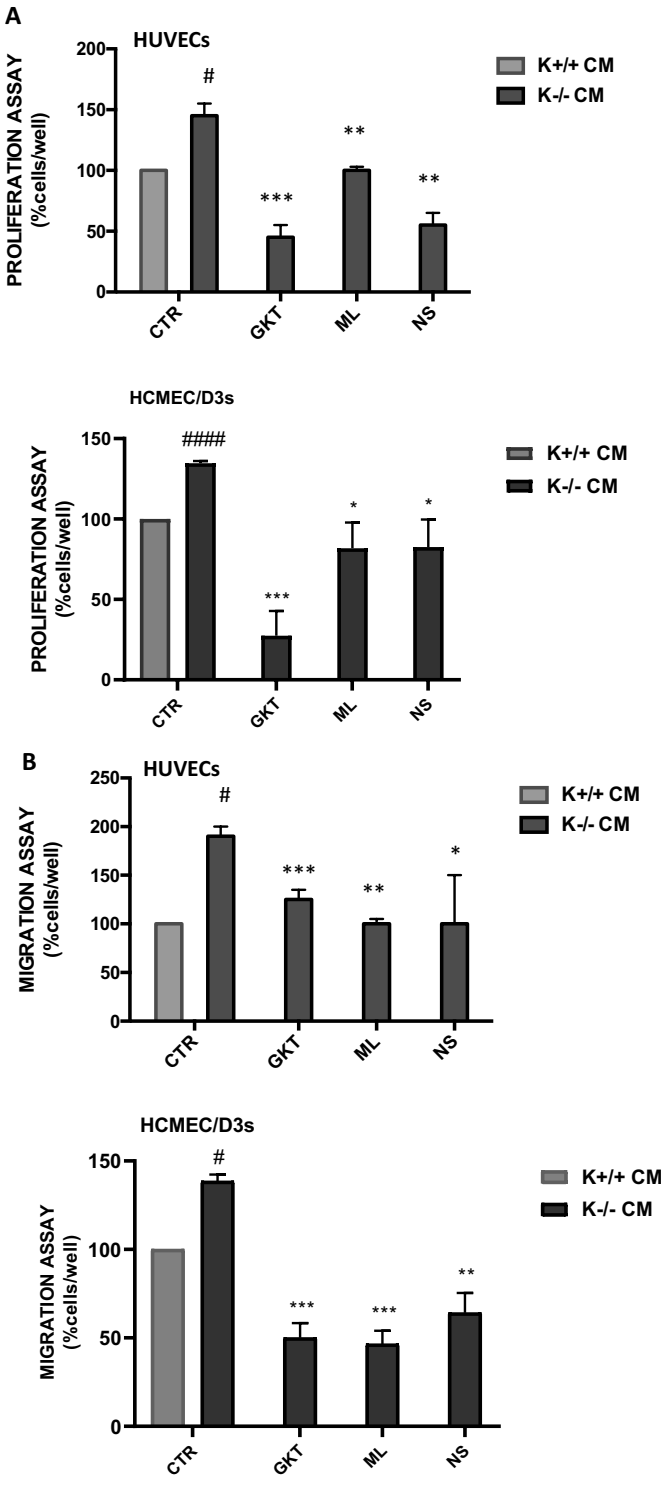


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**Fig. 5.** NOX1 overexpression in stromal cells controls proliferation, migration and MMP-2 activation in endothelial cells. (A) Proliferation assay. HUVEC and HCMEC/d3 cells were cultured for 48 h with CM isolated from K+/+ or K−/− MEFs pre-treated with NOX1 or COX-2 inhibitor as previously described. Data are reported as percentage of number of cells per well (#p < .05 vs K+/+ MEF; *** p < .001 and **p < .01 vs K−/− MEF). (B) Migration assay. HUVEC and HCMEC/d3 cells were treated with CM isolated from K+/+ MEFs or K−/− MEF cells pre-treated with NOX1 or COX-2 inhibitor. The migration assay was performed by using the Boyden chamber as detailed in Materials and Methods. Data are reported as percentage of number of cells per well (#p < .05 vs K+/+ MEF; *** p < .001, **p < .01 and *p < .05 vs K−/− MEF). (C) Determination of MMP-2 enzymatic activity by gelatin zymography. Representative image of MMP-2 activity of HUVECs and HCMEC/d3 cultured with CM from K−/− MEFs pre-treated with NOX1 or COX-2 inhibitor. MMP-2 bands were evaluated by quantitative densitometry and normalized to the number of cells/well (A.D.U.: arbitrary densitometry units). Data are representative of three independent experiments (*p < .05 vs CTR).

possibility, we performed proliferation and migration assays on HUVECs and HCMEC/d3 treated with CM from K−/− MEFs pre-treated with NOX1 inhibitors. Moreover, as COX-2 has been implicated in PGE2-dependent neovascularization, we also pre-treated K−/− MEFs with the COX-2 inhibitor in order to assess whether either NOX1 or COX-2 inactivation could revert the HUVEC angiogenic phenotype triggered by K−/− CM. Interestingly, we observed a significant reduction in proliferation (Fig. 5A) and migration (Fig. 5B) when endothelial cells were cultured with CM from K−/− MEFs pre-treated with either NOX1 (GKT and ML) or COX-2 (NS) inhibitors. Consistently, MMP-2 activity decreased in HUVEC and HCMEC/d3 cells incubated with CM from K−/− pre-treated with ML or NS (Fig. 5C). These results indicate that NOX1 and COX-2 overexpressed in K−/− fibroblasts are able to induce paracrine effects that regulate proliferation and migration of endothelial cells, suggesting that NOX1 upregulation and activation of downstream pro-oxidative and proinflammatory pathways in stromal cells play a key role in the paracrine modulation of angiogenic phenotypes of HUVEC cells.

In addition, we performed immunofluorescence analysis of VE-cadherin and β-catenin to assess whether NOX1 and COX-2 inhibition could rescue also the altered cell-cell adhesion phenotype detected in HUVECs treated with CM from K−/− MEFs. As shown in Fig. 6A and B, HUVECs cultured with CM from K−/− MEFs pre-treated with either GKT, ML or NS (panels c, d and e respectively) exhibited a VE-cadherin (Fig. 6A) and β-catenin (Fig. 6B) distribution comparable to cells cultured with K+/+ CM (panels a), suggesting that inhibition of NOX1 and COX-2 rescues the altered cell-cell adhesion phenotype induced by KRIT1 loss (panels b). To further confirm these observations, we analyzed the phosphorylation levels of β-catenin in HUVECs treated for 15 min with CM from K+/+ or K−/− MEFs pre-treated or not with inhibitors. We observed consistent β-catenin phosphorylation in HUVECs treated with CM from K−/− MEFs, which was reverted by K−/− MEF pre-treatment with NOX1 and COX-2 inhibitors (Fig. 6C).

These results suggest that either NOX1 or COX-2 inhibition in K−/− MEFs can rescue the altered cell-cell adhesion phenotype observed in HUVECs treated with K−/− CM.

## 4. Discussion

CCM lesions are typically found in the CNS and are characterized by dilated and leaky capillaries that are devoid of normal vessel structural components.

Several lines of evidence show that the KRIT1 protein is involved in different physiological aspects of endothelial biology, including vascular development, modulation of different redox-sensitive signaling pathways, and maintenance of endothelial barrier homeostasis, as well as that the absence of KRIT1 in endothelial cells induces an angiogenic

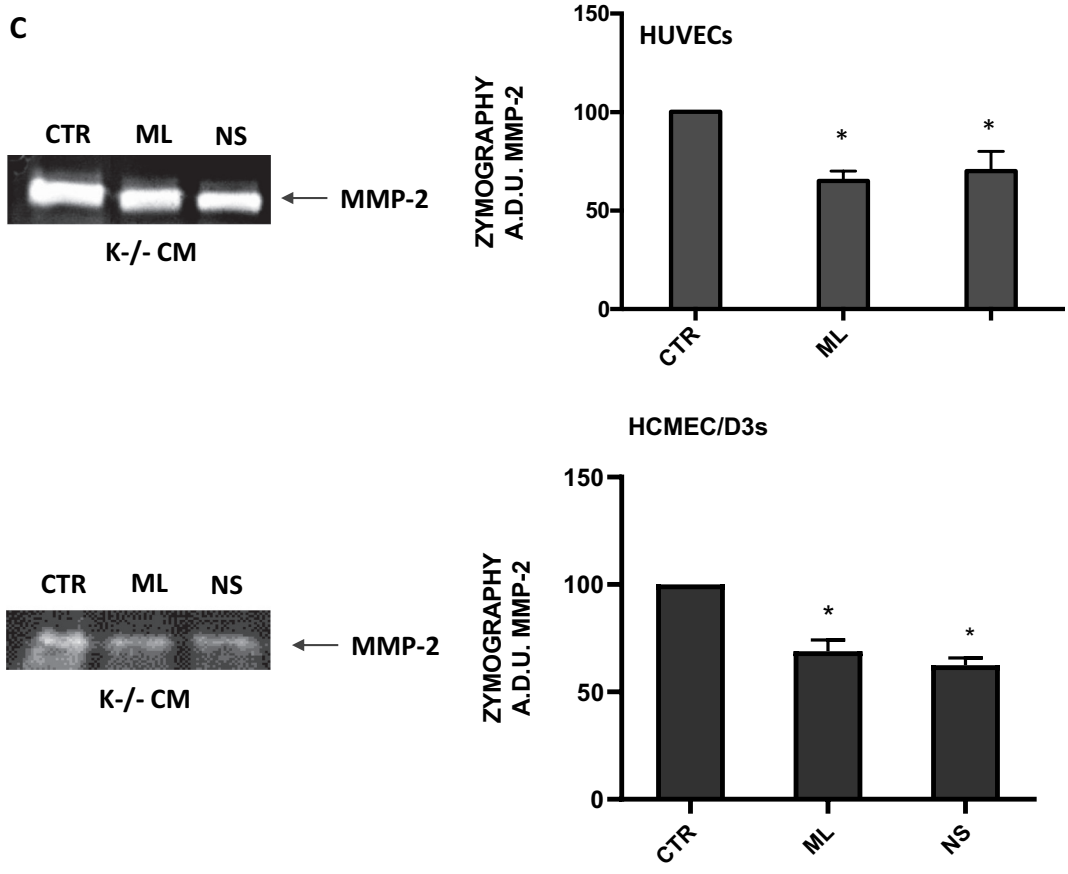


**Fig. 5.** (*continued*)

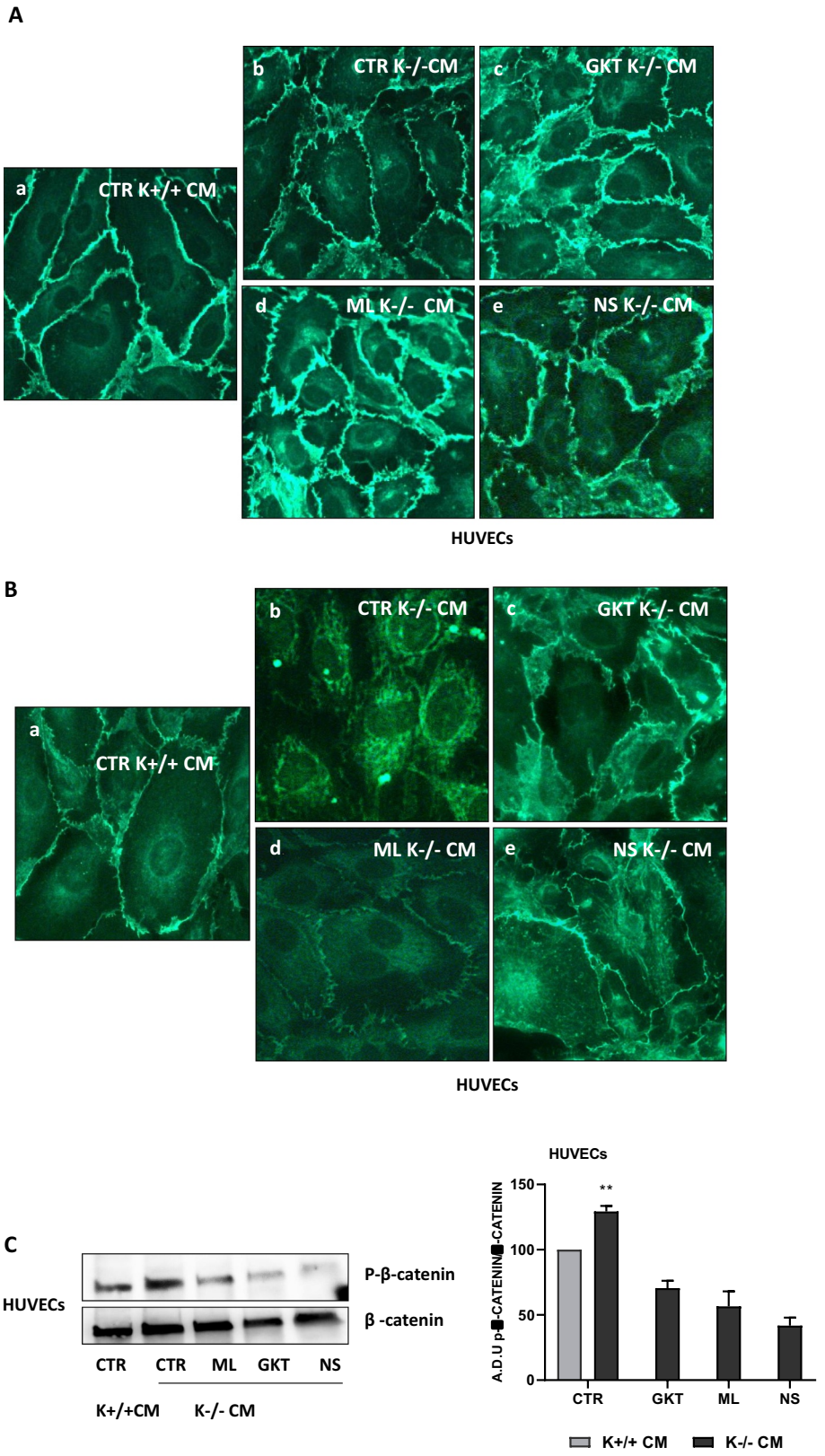


**Fig. 6.** NOX1 overexpression in stromal cells regulates VE-cadherin distribution and β-catenin activation in endothelial cells. (A) VE-cadherin and (B) β-catenin expression in HUVEC cells cultured with CM isolated from K+/+ or K−/− MEFs pre-treated or not with NOX1 or COX-2 inhibitor. HUVECs were grown for 24 h with (a) K+/+ CM; (b) K−/− CM; (c) GKT K−/− CM; (d) ML K−/− CM or (e) NS K−/− CM and then stained with (A) anti-VE-cadherin or (B) anti-β-catenin primary antibody. Images are representative of three different experiments. (C) Representative images and quantification of β-catenin phosphorylation in HUVEC cells. Protein extracts of HUVECs grown with K+/+ CM; K−/− CM; GKT K−/− CM; ML K−/− CM or NS K−/− CM were analyzed by western blot with anti-β-catenin and anti-P-β-catenin primary antibodies. The gel shown is representative of three independent experiments (A.D.U.: arbitrary densitometry units).

phenotype with increased proliferation, migration, ability to form pseudocapillaries, and activation of angiogenic pathways [3,36,37]. However, while it is established that KRIT1 loss-of-function predisposes to the development of CCM lesions by affecting endothelial cell-autonomous mechanisms, emerging evidence suggests that in the central nervous system (CNS) the endothelium is part of complex units, called neurovascular units (NVU), where it is in close contact with other cell types including pericytes, astrocytes and neurons. All components of this unit interact with each other by releasing signals that safeguard the integrity of the structure itself [49]. However, until now the effect of

the absence of CCM genes on NVU integrity and functionality is poorly investigated. By using neural specific conditional mouse mutants, Louvi et al. demonstrated that in the familial form of CCM, the CCM3 protein has both neural cell autonomous and nonautonomous functions. CCM3 neural deletion leads to increased proliferation, increased survival, and activation of astrocytes through cell autonomous mechanisms. In addition, loss of neural CCM3 results in the formation of multiple vascular lesions that closely resemble human cavernomas, probably through cell nonautonomous mechanisms, indicating that the primary defects in CCM disease need not be endothelial specific [13]. In addition, in recent papers, the authors hypothesized that CCM lesions develop as a consequence of clonal expansion of a single mutant cell, proposing a mechanism in which the first clonal expansion of somatically mutated endothelial cells is followed by the incorporation of normal non mutated endothelium that leads to the formation of the abnormal vessels [14,50], indicating a possible autocrine/paracrine mechanism of endothelial cell recruitment. In our model, conditioned media from KRIT1 null stromal cells induce endothelial cell proliferation, migration, MMP activation and loss of endothelial junctions, recapitulating endothelial cells behavior described in CCM lesions. These data indicate that the anomalous microenvironment derived from KRIT1 loss is able to induce a proangiogenic response in quiescent endothelial cells, and strongly suggest that for the formation of the abnormal vessels present in CCM lesions is sufficient a mutation in any of the cell components of NVU, which in turn may act on neighboring endothelial cells via a paracrine mechanism.

It is well known that the angiogenic process is tightly regulated by a balance between pro- and anti- angiogenic factors; this balance results altered in CCM vascular malformations as a consequence of a sort of threshold overcoming of environmental angiogenic signaling. For instance, alteration of VEGF, PDGF, TGF-β and NOTCH pro-angiogenic signaling pathways in CCM models has already been described [3], supporting the idea that KRIT1 loss of function could lead to a proangiogenic microenvironment. Here we showed that in addition to inducing overexpression of VEGF, stromal cells lacking KRIT1 (K−/−) are able to produce increased amounts of PGE2, a potent inflammatory and proangiogenic product of arachidonic acid pathway [32,48] that contributes to the proangiogenic threshold overcoming.

It is known that a failure in the ability of NVU cells to maintain the proper balance between ROS production and their neutralization causes the disruption of NVU itself, and that this event is associated with many CNS diseases. In this context, NADPH oxidases play a major role in the maintenance of the ROS levels, and NOX family over-activation/expression causes the disruption of vascular homeostasis, which can underlie the development of CNS diseases [51]. In a recent paper, we reported that NOX4 is upregulated in KRIT1 silenced endothelial cells and in KRIT1−/− mice, and controls vascular permeability [29]. However, while NOX4 have been described to play a central role in the control of ROS homeostasis in endothelial cells, very recent papers showed that NOX1 overexpression drives the angiogenic switch in in vitro and in vivo models of tumor [21–23], indicating that this isoform of NADPH oxidase may regulate the release of vasoactive and proangiogenic factors. Here, we demonstrated that K−/− MEFs exhibited higher levels of NOX1 and that COX-2/PGE2 and VEGF expression levels were reduced in K−/− cells treated with NOX1 inhibitors. Consistently, proliferation, migration, MMP2 activation and junction alteration of endothelial cells induced by CMs from K−/− MEFs were significantly affected when endothelial cells were cultured with CMs from K−/− MEFs treated with NOX1 and COX-2 selective inhibitors. These data indicate that oxidative stress consequent to KRIT1 loss mediated upregulation of NOX1 plays a key role in the modulation of microenvironment composition that leads to the acquisition of an angiogenic phenotype by endothelial cells.

It has been extensively reported that CCM onset and progression are linked to increased oxidative stress and inflammation subsequent to CCM genes loss [3]. Here we further outlined the central role of ROS increase in this disease by describing NOX1 as the major player of the angiogenic process induced by KRIT1 loss, and as the regulator of COX-2 expression and PGE2 production, suggesting a sort of hierarchy between oxidative stress and inflammation in CCM pathogenesis. In particular, as our data show that NOX1 inhibition is able to revert the COX-2 upregulation observed in K−/− cells, while COX-2 inhibition did not prevent NOX1 upregulation, but completely reverted the activation of endothelial cells, we suggest the prevalent role of NOX1/COX-2/PGE2 axis rather than NOX1/VEGF in the angiogenic process induced by stromal KRIT1 loss.

## 5. Conclusions

Our findings provide novel insights into CCM pathogenesis, and suggest novel promising therapeutic options for CCM prevention and treatment.

Currently, the only therapy available for CCM is surgical excision or radiological destruction of the lesions. While several compounds are being investigated in preclinical studies, only a few agents have reached clinical testing and to date CCM has no pharmacological options [52]. Several studies have been conducted to identify new CCM-related cellular mechanisms and some progresses to link basic and translational science have been made allowing to hypothesize new putative treatment targets, including angiogenesis. At the present, the potential efficacy of targeting angiogenesis in CCM is only supported by case reports where bevacizumab, the anti–VEGF A antibody, and propranolol, a β-adrenergic blocker and antiangiogenic agent used to treat hypertension and infantile hemangioma, are described to induce lesion regression or resolution [53,54]. Despite these encouraging observations, to our knowledge, the efficacy of antiangiogenic drugs in CCM has not been confirmed and further clinical studies are necessary.

The novel findings reported here show that KRIT1 loss-of-function in stromal cells affects the surrounding microenvironment through a NOX1-mediated induction and release of angiogenic factors that are able to promote paracrine proangiogenic responses in endothelial cells. These data strongly suggest a novel role for endothelial cell-nonautonomous effects of KRIT1 mutations in CCM pathogenesis, and point to NOX1 as a major regulator of these effects thus opening new perspectives for the identification of novel potential therapeutic targets for disease prevention and treatment.

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