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miR-20a represses endothelial cell migration by targeting MKK3 and inhibiting p38 MAP kinase activation in response to VEGF

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Abstract Endothelial cell migration induced in response to vascular endothelial growth factor (VEGF) is a crucial step of angiogenesis and it depends on the activation of the p38 MAP-kinase pathway downstream of VEGFR2. In this study, we investigated the role of microRNAs (miRNAs) in regulating these processes. We found that the VEGF-induced p38 activation and cell migration are modulated by overexpression of Argonaute 2, a key protein in the functioning of miRNAs. Thereafter, we found that miR-20a expression is increased by VEGF and that its ectopic expression inhibits VEGF-induced actin remodeling and cell migration. Moreover, the expression of miR-20a impairs the formation of branched capillaries in a tissue-engineered model of angiogenesis. In addition, the lenti-virus-mediated expression of miR-20a precursor (pmiR-20a) is associated with a decrease in the VEGF-induced activation of p38. In contrast, these processes are increased by inhibiting miR-20a with a specific antagomir. Interestingly, miR-20a does not modulate VEGFR2 or p38 protein expression level. miR-20a does not affect either the expression of other known actors of the p38 MAP kinase pathway except MKK3. Indeed, by using quantitative PCR and Western Blot analysis, we found that pmiR-20a decreases the expression of MKK3 and we obtained evidence indicating that miR-20a specifically binds to the 3′UTR region of MKK3 mRNA. In accordance, the VEGF-induced activation of p38 and cell migration are impaired when the MKK3 expression is knocked down by siRNA. We conclude that miR-20a acts in a feedback loop to repress the expression of MKK3 and to negatively regulate the p38 pathway-mediated VEGF-induced endothelial cell migration and angiogenesis.

Keywords miR-20a · VEGF · p38 · Cell migration · Angiogenesis

Introduction

Angiogenesis, the sprouting of new capillaries from pre-existing ones, contributes to the expansion of the vascular network in a number of physiological and pathological situations [1]. Physiological angiogenesis is a fundamental process that is highly regulated during development and wound repair [2]. Persistent dysregulated angiogenesis is a common denominator and often a causal factor of several diseases, including proliferative retinopathy, rheumatoid arthritis, tumor progression, and metastasis [3–6]. Angiogenesis is a multistep process that involves proteolytic degradation of the extracellular matrix, followed by migration and proliferation of capillary endothelial cells, pericytes recruitment, and assembly of the mature vessel [7]. Vascular Endothelial Growth Factor (VEGF) is the major promoter of both physiological and pathological angiogenesis [7].

Vascular endothelial growth factors encompass a family of six structurally related proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) [8, 9]. Human VEGF-A monomers exist as 5 different isoforms, of which VEGF165, herein referred to as

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VEGF, is the most abundant and biological active form [7, 8]. VEGF binds two tyrosine kinase receptors on blood vessel endothelial cells, VEGF receptor-1 (VEGFR1/Flt-1) and VEGF receptor-2 (VEGFR2/KDR/Flk-1). In adult, VEGFR-2 is the major signaling endothelial cell receptor for VEGF on blood vessels [10].

By activating different kinase pathways, VEGFR2 regulates all the major steps of angiogenesis including endothelial cell proliferation and migration [7, 8, 10, 11]. Notably, the activation of VEGFR2 triggers the activation of several motogenic pathways including the phosphatidylinositol-3 kinase (PI3 K-Akt) pathway, the ERK pathway, the p38α and p38γ pathways and the Rho GTPases and Focal Adhesion Kinase (FAK) pathways [8, 12–14]. By contributing to the phosphorylation of heat-shock protein 27 (HSP27) and annexin A1 (ANXA1), we have provided evidence that the p38α (herein called p38) pathway mediates actin-based motility by regulating actin remodeling and cell contractility [15–18]. Activation of p38 requires an association between VEGFR2 and integrin $\alpha_v\beta_3$ and the phosphorylation of Y1214 within the cytoplasmic domain of VEGFR2. In turn, this triggers the recruitment of non-catalytic region of tyrosine kinase adaptor protein 1 (Nck) to P$\sim$Y1214 and the sequential activation of Fyn and of the small GTPase Cdc42, upstream of the p38 MAPK module [17, 19–21]. Within this module, mitogen-activated protein kinase kinases 3/6 (MKK3/6) are the major upstream activators of p38 and appear to have redundant functions during development, as MKK3 or MKK6 knockout mice are viable and healthy whereas the double knockout mice died in midgestation with defects in the placenta and embryonic vasculature [22, 23]. However, it has been shown that MKK3 but not MKK6 is required for VEGF-induced endothelial cell migration downstream of p38α and p38γ [12]. In contrast to the p38 cascade activation process, the mechanisms involved in shutting-off the activation of the p38 pathway are still ill defined except that they implicate phosphatases and receptor internalization [24].

MicroRNAs (miRNAs) are an evolutionarily conserved group of small RNAs (21–24 nucleotides) that inhibit gene expression and thus may participate in repressing the VEGF signal to the p38 pathway. microRNAs are transcribed first as pri-miRNAs (pri-miRs) from mainly intergenic or intronic regions by the RNA polymerase II. The double-stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8 protein) and the RNase III enzyme Drosha, that form the microprocessor complex, process long primary miRNAs (pri-miRNAs) into short hairpins called precursor miRNAs (pre-miRNAs) [25–27]. The resulting hairpins are exported into the cytoplasm [28, 29] where they are processed by the RNAse III Dicer into mature miRNAs [30, 31]. The miRNA duplex generated is rapidly loaded to the RNA-induced silencing complex (RISC) where selection of the more stable strand occurs [25]. It is as a part of the RISC that these small RNAs modulate gene expression by partially base-pairing with target mRNA sequences generally in its 3′-untranslated region (3′UTR), thereby repressing translation and/or degrading mRNA [32–35]. Functional miRNAs should be associated with several proteins including members of the Argonaute (Agos) family that are part of an active RISC. These proteins are rate-limiting and are considered as the actual mediators of silencing [36, 37]. Studies in endothelial cells reveal that disruption in the miRNA pathway functioning impairs the angiogenic potential of endothelial cells in vitro and in vivo [38–41]. In particular, the miR-17 ∼ 92 cluster is involved in the regulation of tumor angiogenesis in a mouse model by downregulating anti-angiogenic factors such as thrombospondin-1 (TSP1) and connective tissue growth factor (CTGF) in the tumorigenic cells [42]. miR-17 ∼ 92 is a highly conserved cluster of miRNAs consisting of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1) within a non-coding RNA encoded by the *c13orf25* host gene localized on chromosome 13. The identity or function of each member of the cluster in tumorigenesis is not clearly established.

In the present study, we investigated whether miRNA regulates the p38-mediated endothelial cell migration. We found that miR-20a, a member of the miR-17 ∼ 92 cluster, regulates the p38 activation and functions in response to VEGF. We provide evidence indicating that miR-20a represses the activation of the p38 pathway at the level of MKK3, which ultimately represses cell migration and, as a consequence, impairs capillary formation in a human engineered model of angiogenesis.

Materials and methods

Reagents and antibodies

All the informations about reagents and antibodies are available as Supplementary material.

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords [43]. The cords were obtained after approbation of the CRCHUQ Ethical Committee. Subcultures were maintained in EGM2 media (LONZA, Allendale, NJ, USA). Replicated cultures were obtained by trypsination and were used at passages < 5. Treatments were done on HUVECs cultivated on gelatin and made quiescent by serum-starvation using M199 media containing 5 % heat-inactivated fetal bovine serum (FBS), L-glutamine, and antibiotics. Normal Human Dermal

Fibroblasts (NHDFs) were obtained from LONZA (Allendale, NJ, USA) and maintained in DMEM media supplemented with 10 % FBS and antibiotics. Human Embryonic Kidney cells (HEK293T) were cultivated in DMEM containing 10 % FBS and antibiotics.

Treatments

All treatments were done using recombinant human VEGF-A165 (herein named VEGF) produced by R&D Systems (Minneapolis, MN, USA) and kindly provided by the NCI Biological Resources Branch (Rockville, MD, USA).

Lentiviral particles preparation

HEK293T were plated at 3×10^6 per 100 mm Petri dish in 10 ml DMEM supplemented with 10 % FBS. Co-transfection was perfomed by Ca^{2+} phosphate-mediated transfection using 2.5 μg of pRSV-Rev, 6.5 μg of pMDLg-pRRE, 3.5 μg of pMD2.G together with 10 μg of transgene expressing vectors (pmiR-20a or pmiR-empty vectors). After overnight incubation, media were replaced with 5 ml lentiviral particle collection media (M199 medium containing 20 % heat-inactivated FBS, ECGS (60 μg/ml) (Sigma-Aldrich, Oakville, ON, Canada), L-glutamine, heparin and antiobiotics. After 24 h, the media were collected and centrifuged at 4 °C, 1,200 rpm, for 5 min. The supernatant was stored in aliquots at −80 °C. The Multiplicity of Infection (MOI) known as the ratio of infectious virus particles per cell, was determined by a 48 h transduction of HUVECs in a cascade dilution experiment. The percentage of GFP positive cells was determined by FACS using EPICS-XL-MCL flow cytometer (Beckman-Coulter, Ramsey, MN, USA).

Transfection and transduction

Human umbilical vein endothelial cells were transfected with expression vectors using X-tremeGene HP Transfection Reagent obtained from Roche (Laval, QC, Canada) according to the manufacturer's protocol. HUVECs were transduced with miR-20a precursor expressing vector (pmiR-20a) or with empty vector (pmiR-empty) using lentivirus-mediated infection (System Biosciences, Mountain View CA, USA).

Plasmids and miRNA mimics

The pmiR-20a expressing plasmid was obtained from System Biosciences (Mountain View, CA, USA). GFP expressing vector (pmiR-empty) and plasmids necessary for lentiviral particles production were a kind gift of Dr. Manuel Caruso (Laval University, QC, Canada). Human Ago2 expressing plasmid was obtained from OriGene (Rockville, MD, USA). Mature miR-20a mimic and controls were obtained from Dharmacon (Lafayette, CO, USA). psiCHECK-2 vector was obtained from Promega (Madison, WI). Antago-miRNAs were purchased from Dharmacon and siRNAs were purchased from Qiagen (Valencia, CA) and In Vitrogen (Carlsbad, CA). Additional informations are available in Supplementary materials.

RNA extraction, RT-qPCR

RNA extraction

Total RNAs were extracted using Mirvana Isolation kit (Ambion Applied Biosystems Streetville On Canada). Total RNA concentration was determined using Nanodrop 1000 spectophometer (Thermo Fisher Scientific, Lafayette, CO, USA) and quality was assessed using a 1 % agarose gel before its utilization for cDNA production.

SYBR green RT-qPCR

For gene expression, reverse transcription was performed to obtain cDNA using omniscript RT-kit (QIAGEN, Mississauga, Canada). Specific primers were designed using Primer blast-free software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) for MKK3, p38 and 3 housekeeping genes HMBS, GAPDH and YWHAZ. All primers were obtained from Sigma Aldrich (Mississauga, ON, Canada). Gene expression was quantified using Fast SYBR Green Master Mix for real-time qPCR and following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA, AB 7900). The geNorm program was used to select the best housekeeping gene as a normalizer (medgen.ugent.be/genorm). The $2^{\Delta\Delta Ct}$ method was used to determine the Fold Changes (FC).

Primers used:

Amplicon	Forward primer 5′–3′	Reverse primer 5′–3′
MKK3	AGGAAGAACCCCGCAGAGCGTA	TCACGAAGGCAGCAATGTCCGT
p38	TCCAGACCATTTCAGTCCATCA	CGTCCAACAGACCAATCACATT
HMBS	CAGCCTGGCCAACTTGTTG	CATCTGTGCCCCACAAACC
GAPDH	TTGACGCTGGGGGCTGGCATT	AGGTCCACCACCCTGTTGCTGT
YWHAZ	CAGCCTGCATGAAGTCTGTAACTG	CCTACGGGCTCCTACAACATTT

TaqMan RT-qPCR

For miRNA expression quantification, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit following the manufacturer's procedures and using specific microRNAs primers for reverse transcription from microRNA assays (hsa-miR-20a : hsa-miR-17, and the housekeeping short non-coding RNA U6 snRNA) (Life Technologies, Carlsbad, CA, USA). microRNA level of expression was determined using Universal PCR Master Mix, No AmpErase UNG according to the manufacturer protocol (Life Technologies, Carlsbad, CA, USA, AB 7900). The $2^{\Delta\Delta Ct}$ method was used to determine the Fold Changes (FC).

Western blotting

After treatments, cells were lysed using SDS-PAGE loading buffer. Equal amounts per well of total proteins were separated by SDS-PAGE, and the gels were transferred onto nitrocellulose membranes for Western blotting. After incubating nitrocellulose membranes with the appropriate primary antibodies, antigen–antibody complexes were detected with an anti-IgG antibody coupled to horseradish peroxidase and then revealed using an enhanced chemiluminescence kit. Alternatively, antigen–antibody complexes were detected with an anti-IgG antibody coupled to IR-Dye800 or IRDye680 and revealed using an infrared imaging system (Li-Cor, Lincoln, NE). Quantification of the immunoreactive bands was done by densitometric scanning using the Image J software. All antibodies and reagents are listed in Supplemental Tables 1–3.

Luciferase reporter assay

MKK3 and ANXA1 mRNA 3′UTR regions were amplified from HUVEC total RNA following cDNA production using Omniscript RT-kit (QIAGEN, Mississauga, Canada) before amplification using KOD Hot Start DNA polymerase according to the manufacturer protocol (EMD, Philadelphia, PA, USA). Then, MKK3 or ANXA1 mRNA 3′UTR were cloned just after the Renilla luciferase stop codon in psiCHECK-2 vector using XhoI/NotI restriction sites using the following primers : MKK3 Forward primer 5′-CACTCGAGACTCCGGCCCTCCAGAGC-3′ and Reverse primer 5′-CTGCGGCCGCCCAGGTAACTGCCC CAACCCATCA-3′, and for ANXA1 Forward primer 5′-C ACTCGAGACATTCCCTTGATGGTCTC-3′; Reverse primer 5′-CTGCGGCCGCTCATTTTATTTTCAGCTAC ATAG-3′. After sequencing, the resultant vector psi-CHECK-2-ANXA1-3′UTR-wt and psiCHECK-2-MKK3-3′UTR-wt containing Renilla Luciferase under the control of ANXA1 3′UTR or MKK3 3′UTR and Firefly Luciferase

as a control were obtained. psiCHECK-2-MKK3-3′UTR-wt was used to produce mutant psiCHECK-2-MKK3-3′UTR (A, B, C: see supplementary Fig. 2) by directed mutagenesis using specific primers. All vectors were used in reporter luciferase assays as described below. HUVECs (2.5×10^5 cells) were transduced with pmiR-20a or pmiR-empty (MOI of 65 for 72 h) and then transfected with psiCHECK-2-ANXA1-3′UTR-wt, psiCHECK-2-MKK3-3′UTR-wt or psiCHECK-MKK3-3′UTR-mutated on three potential miR-20a binding sites (A, B, C: see supplementary Fig. 3). The following day, cells were lysed and luciferase activity was evaluated using Dual-Luciferase Reporter Assay System following manufacturer's protocol (Promega, Madison, WI). Renilla luciferase activity, which is under the control 3′UTR insert, was evaluated and Firefly luciferase was measured as a loading control in each condition using a Glomax luminometer 20/20 (Promega, Madison, WI, USA).

Cell migration assays

Boyden chamber assay

Endothelial cell migration in Boyden chambers was assayed as previously reported [18]. Briefly, 48 h-transfected or 96 h-transduced cells were serum-starved overnight using M199 media supplemented with 5 % heat-inactivated fetal bovine serum, L-glutamine and antibiotics. Then, cells were harvested with trypsin, counted, centrifuged, and resuspended at 1.5×10^6 cells/ml in migration buffer (199 medium, 10 mM HEPES pH 7.4, 1 mM MgCl$_2$, and 0.5 % bovine serum albumin). Cells (1.5×10^5) were added on the upper part of 8.0-μm pore size gelatin-coated migration chambers (Corning, NY, USA) separating the upper and lower chambers of a 6.5-mm transwell. Cells were left to adhere for 1 h. Then, 10 ng/ml VEGF were added in the lower chamber for 4 h. In transfection experiments, pEGFP was co-transfected to visualize and count transfected cells that crossed the membrane of the Boyden chamber. In some experiments, cells were fixed using cold methanol and the cells in the upper part of the chamber were removed with a cotton swab. Then, cells that crossed the membrane were stained with Hoechst. In both cases, the cells were counted manually in five fields using a 20 × lens (numerical aperture of 0.45) on a Nikon-TE300 inverted microscope equipped with a Metavue (7.7.5 version) imaging system a Photometrics CoolSNAP HQ camera. In transduction experiments, 4 h after VEGF treatment, the number of fluorescent transduced cells that crossed the membrane of the Boyden chamber was manually counted from five fields using the same microscopic imaging facilities as described above. All experiments were performed three times at least

in duplicates. In each condition, the mean of the sum of migrating cells from five different fields is represented. Student t test was performed and a p value lower than 0.05 was considered as significant.

Wound closure assay

Human umbilical vein endothelial cells were plated in a 12-wells plate at 100,000 cells/wells. The day after, cells were transfected using X-tremeGENE HP DNA Transfection Reagent. For Ago2 experiments, they were transfected with piRES (control vector) or pAGO2 (vector expressing human Ago2) (EIF2C2, NM_012154.2) together with of pEGFP (eGFP expressing vector). For miR-20a studies, cells were transfected with a mature miR-20a mimic or a control mimic. Forty-eight hours later, cells were serum-starved for 4 h. Then, a scratch was applied manually to the confluent cell monolayer. After three washes to remove the detached cells, the remaining cells were placed in serum-starved medium with or without VEGF treatment (10 ng/ml). The plate was then incubated in the living chamber of a Nikon-TE2000 inverted microscope equipped $10\times$ lens (numerical aperture of 0.30), with a Metamorph (7.7.5 version) imaging system and with a Photometrics Cool-SNAP HQ2 camera. The pictures were captured using both transmitted light together or not with fluorescence. For miR-20a studies, the total number of cells that filled the scratch in each condition was manually counted in 3–4 different fields at 8, 12 and 18 h. For each time-point, the number of cells in VEGF-treated fields was normalized against the mean of corresponding untreated fields to represent the relative VEGF-induced migration.

Angiogenesis assay

Human umbilical vein endothelial cells were transduced with pmiR-20a containing miR-20a precursor and GFP or with an empty vector containing only GFP (pmiR-empty) using lentivirus-mediated infection (MOI of 65). After 96 h, 50,000 transduced cells were plated on a 12-days monolayer of NHDFs. NHDFs were grown and maintained in DMEM supplemented with 10 % fetal bovine serum and 50 μg/ml sodium L-ascorbate to facilitate the formation of a sheet (60,000 cells per well of a 12-well plate). Co-cultures were maintained for 6 days in DMEM supplemented with 10 % fetal bovine serum, M199 supplemented with 5 % fetal bovine serum and L-glutamine (v:v) and 50 μg/ml sodium L-ascorbate [44]. Media was replaced every 48 h with or without VEGF treatment (10 ng/ml) together or not with Z-VAD (OMe)-FMK (annoted Z-VAD, 50 μM) or with Q-VD-Oph Non-O-methylated (annoted QVD, 10 μM) to inhibit caspase mediated-apoptosis. This was performed for a total of 3 treatments (D0, D2, D4).

Capillary-like structures were observed at day 6 using a $4\times$ lens (numerical aperture of 0.13) mounted on a Nikon-TE300 inverted fluorescent microscope equipted with a Metavue (7.7.5 version) imaging system and a Photometrics CoolSNAP HQ camera. Fifteen pictures per well from different fields were captured. Raw images were processed with ImageJ software using the following pipeline: images were made binary using a threshold value of 50 applied on 8 bit images. Thereafter, they were despeckled, converted to mask and skeletonized. We considered the number of remaining white pixels as the length of the capillary structures per field. Also the plugin AnalyseSkeleton in ImageJ was used to obtain the number of branches (http://fiji.sc/wiki/index.php/AnalyzeSkeleton). The total length of capillary like structures and the number of branches were expressed as the mean of sums from 15 fields per replicate.

Fluorescence microscopy

After treatments, transduced cells (pmiR-20a or pmiR-empty, MOI of 65) were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % saponin in phosphate buffer, pH 7.5. F-actin was detected using Rhodamine-phalloidin (Invitrogen, Carlsbad, CA) diluted 1:400 in phosphate buffer. The cells were examined by confocal microscopy using Olympus-FV1000 imaging system equipped with a $40\times$ objective lens (numerical aperture of 0.90).

Proliferation assay

Human umbilical vein endothelial cells were transduced in EGM2 media supplemented with hexadimethrine bromide 8 μg/ml with pmiR-20a or pmiR-empty (MOI of 65). Fourty-eight hours later, they were plated on a cover slip coated with gelatin (110,000 cells per 6 plate well). Forty-eight hours later, cells were fixed and immunostaining of nuclear KI67 antigen was performed using a specific rabbit polyclonal antibody. The binding of the primary antibody into the proliferative cells expressing KI67 was revealed by a secondary anti-rabbit Alexa 568 antibody. DAPI staining was included in the mounting buffer. Pictures were captured from 12 different fields (at least 150 cells per replicate) using (numerical aperture of 0.75) mounted on a Nikon-E600 inverted microscope equipted with a Metavue (7.7.5 version) imaging system and a Photometrics cool snap FX camera. The percentage of proliferating cells was determined using the ratio KI67/DAPI positive cells.

Statistical analysis

Values are expressed as fold changes or mean ± SD. Unpaired Student t tests were used for comparison between

two means. A *p* value <0.05 was considered as statistically significant.

Results

Overexpression of Ago2 impairs VEGF-induced activation of p38 and endothelial cell migration

We first attempted to verify whether the VEGF-mediated activation and migratory functions of the p38 pathway were under the control of miRNAs. Given that Argonautes (Agos) are rate-limiting step in the functioning of miRNA pathways [36, 37], we hypothesized that if the p38 pathway was controlled by miRNAs, the increased activity of a miRNA pathway conferred by Agos overexpression should affect the activation of one or several members of the p38 MAP-kinase cascade following exposure to VEGF. In accordance, we overexpressed Ago2, a prototype member of the Ago family, and ascertained its role in VEGF-induced cell migration using wound closure healing and Boyden chamber assays and determined p38 activation in Western blotting. As shown in Fig. 1a–c, the endothelial cell migration was impaired in VEGF-treated cells in which Ago2 was expressed. Moreover, the VEGF-induced activation of p38 was reduced in cells expressing Ago2 (Fig. 1d). We thus concluded that at least one miRNA modulates p38 activity and the resultant endothelial cell migration in response to VEGF.

miR-20a represses the p38 pathway activity by targeting MKK3

By using qPCR TaqMan miRNA assays, we next verified whether VEGF induced the expression of miRNAs. In accordance with the work of Suarez et al., we found that VEGF induced by 2- and 1.4-fold the expression of miR-17 and miR-20a, respectively ([45] and Supplementary Fig. 1). We also found that both miRNAs are constitutively expressed at high levels in several lines of HUVECs and HMEC-1, which is consistent with previous findings indicating that these miRNAs are part of the endothelial signature ([46] and data not shown). Both miR-17 and miR-20a are members of the miR-17 ∼ 92 cluster that has been reported to have both opposite pro- and anti-angiogenic properties [47, 48]. In particular, miR-92a increases the angiogenic potential of endothelial cells whereas miR-20a represses it [47, 48]. This suggests that the angiogenic properties of the cluster are under complex but still misunderstood mechanisms of regulation. Notably, miR-20a has been proposed to target Jak1 but the role of JAK1 in VEGF signaling is unclear [47].

p38 activation is a major pathway that modulates endothelial cell migration and angiogenesis in response to VEGF and shear stress-induced activation of VEGFR2 [7, 49]. In order to better understand the anti-angiogenic properties of miR-20a, we thus verified whether miR-20a could affect the p38 pathway in response to VEGF. We express miR-20a in HUVECs via lentiviral-mediated infection of a plasmid expressing pre-miR-20a (pmiR-20a). We found that p38 activation in response to VEGF was inhibited by pmiR-20a expression (Fig. 2a). Accordingly, the kinase activity of p38 was diminished and the phosphorylation of its downstream substrate, MAPKAP-K2 was reduced (Fig. 2a). Similarly, HSP27, a typical MAPKAP-K2 substrate downstream of p38, was less phosphorylated in cells expressing pmiR-20a (Fig. 2a). In contrast the VEGF-induced activation of p38 was increased in cells in which miR-20a was inhibited by a specific antagomir (Fig. 2b). On the other hand, the activation of ERK by VEGF was not affected by pmiR-20a, which suggests that the repressing effect of miR-20a might be selective for the p38 pathway (Fig. 2c).

Importantly, we found that the total level of p38 was not affected by miR-20a, which indicated that p38 was not a direct target of miR-20a in endothelial cells (Fig. 2a and Supplementary Fig. 2a). Moreover, the total level of VEGFR2 and its autophosphorylation at Y1214 that is necessary to the activation of p38 pathway in response to VEGF were not affected by the expression of miR-20a (Fig. 2d and Supplementary Fig. 2b). Based on these findings, we thus concluded that the miR-20a target should be located between the receptor and p38. We previously identified key proteins that were required for p38 activation in response to VEGF-mediated VEGFR2 autophosphorylation at Y1214 [17, 21]. As shown in supplementary Fig. 2b,c,d, neither Nck, Fyn, Cdc42, Pak2 or MKK6 were affected at the protein level by miR-20a. However, we found that the MKK3 level was reduced in a dose-dependent manner by pmiR-20a and also by expressing the miR-20a mimic (Fig. 3a, b). At the mRNA level, we also found that the MKK3 mRNA, but not that of p38, was decreased by the expression of miR-20a (Fig. 3c). Moreover, we observed that Luciferase reporter carrying wild type human MKK3 3′-UTR region was significantly sensitive to miR-20a ectopic expression. In contrast, mutations of 3 potential miR-20a complementary sites within MKK3 3′-UTR relieve miR-20a-dependent repression (Fig. 3d and Supplementary Fig. 3). Along these lines, a Luciferase reporter carrying Annexin A1 mRNA 3′-UTR region, taken as a negative control, was insensitive to miR-20a expression. This suggests that miR-20a represses the expression of MKK3 by its specific binding within the 3′UTR region of its mRNA. Consistent with the fact that miR-20a targeted the expression of MKK3, a direct activator of p38, we found that p38 activation and HSP27 phosphorylation were reduced by siRNA-mediated knockdown of MKK3 (Fig. 3e).
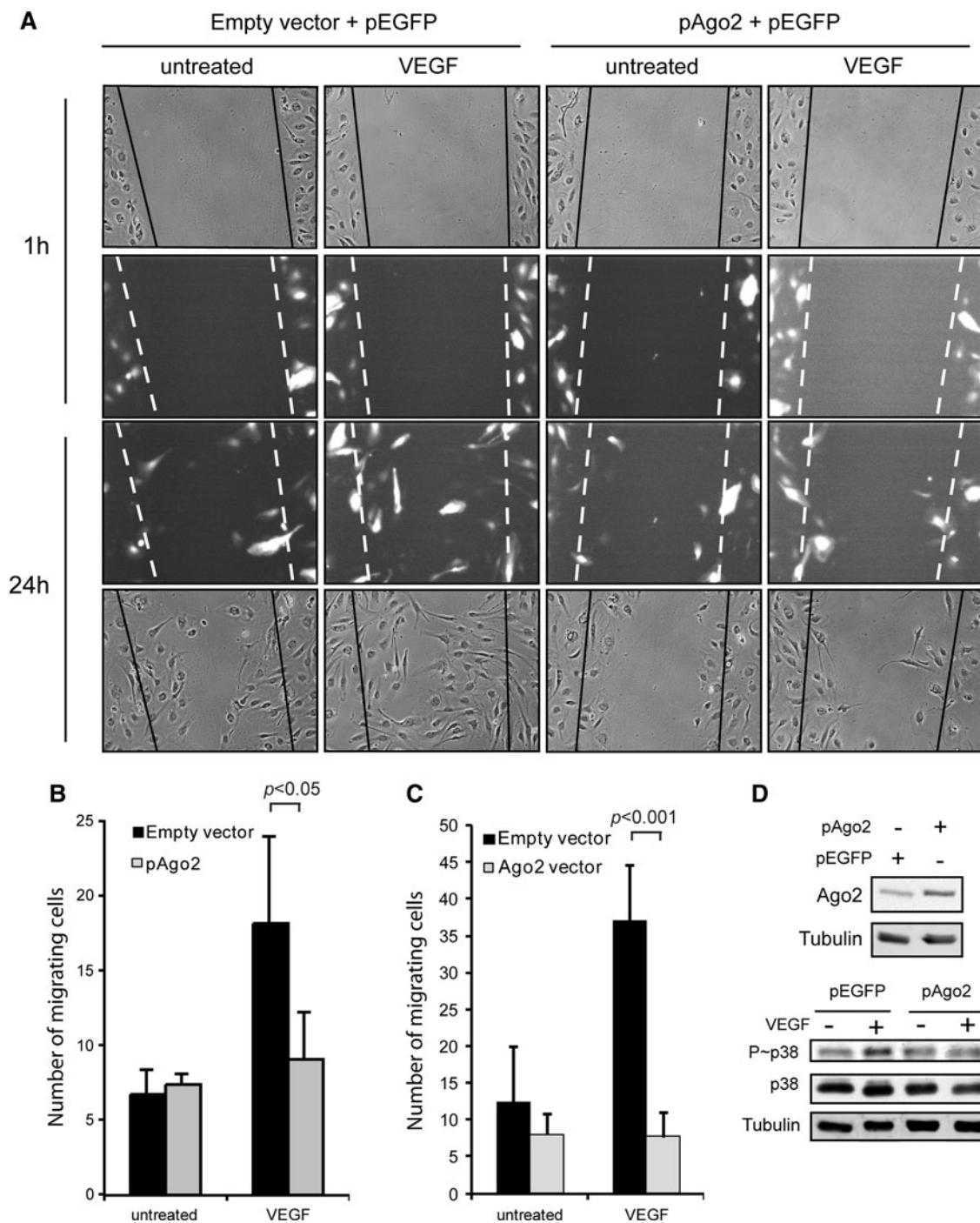
Fig. 1 Ago2 modulates the activation of the p38 cascade and endothelial cell migration in response to VEGF. **a** HUVECs were co-transfected with pAgo2 or empty vector together with pEGFP to visualize transfected cells. After 48 h, cells were serum-starved for 4 h before being evaluated in wound healing assay. A wound was manually applied to the confluent cell monolayer, before live cell evaluation of wound closure with or without VEGF treatment (10 ng/ml). Images were captured using an inverted microscope (×10) after 1 and 24 h. In each image, the *black* and *dot lines* represent the initial wound. **b** Cells expressing GFP that have crossed the border of the initial wound were counted in three different fields after 24 h of treatment. **c** HUVECs were transfected with pAgo2 or empty vector together with pEGFP to visualize transfected cells. Overnight-serum-starved cells were evaluated for cell migration in a transwell migration assay using VEGF (10 ng/ml for 4 h) as chemoattractant. **d** HUVECs were transfected with pEGFP or pAgo2. After 48 h, proteins were extracted from overnight-serum-starved cells after VEGF treatment (10 ng/ml for 5 min). After protein separation by SDS-PAGE and transfer onto nitrocellulose membrane, Ago2 over-expression, phosphorylation level of P $\sim$ p38 (T180, Y182), total p38 and tubulin levels were revealed by Western Blot using specific antibodies
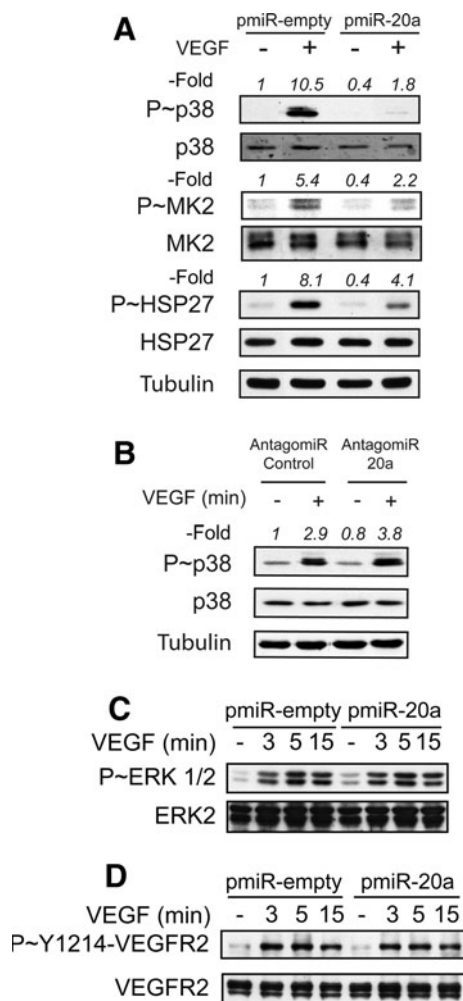
Fig. 2 miR-20a modulates VEGF-mediated activation of the p38 pathway. **a** HUVECs were transduced with miR-20a precursor (pmiR-20a) or with an empty vector (pmiR-empty) using lentivirus-mediated infection (MOI of 65). After 96 h, overnight-serum-starved cells were treated or not with VEGF (10 ng/ml) for 15 min. The proteins were extracted, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Phosphorylation levels of P $\sim$ p38 (T180, Y182), P $\sim$ MK2 (T222), P $\sim$ HSP27 (S78, S82) and their total levels of together with Tubulin α were revealed by Western Blot using specific antibodies. **b** Untransfected HUVECs or HUVECs transfected for 48 h with an antago-miR-20a or a control antagomiR (100 nM) were serum-starved overnight and then treated or not with VEGF (10 ng/ml) for 5 min. Western blots against P $\sim$ p38 (T180, Y182) and p38 total levels together with Tubulin a were processed on the extracted proteins. **c**, **d** HUVECs were transduced as in (**a**). After 96 h, overnight-serum-starved cells were treated or not with VEGF (10 ng/ml) for increasing periods of time as indicated. The proteins were extracted, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Phosphorylation levels of P $\sim$ ERK1/2 (T202, Y204) and P $\sim$ VEGFR2 (Y1214) and their total levels were revealed by Western Blot using specific antibodies

Overall, these findings indicate that miR-20a inhibits the expression of MKK3 to repress the p38 pathway activation induced by VEGF/VEGFR2.

miR-20a inhibits actin remodeling and endothelial cell migration induced by VEGF

p38 activation, downstream of VEGF-induced stimulation of VEGFR2, is a major signaling pathway that regulates actin remodeling and endothelial cell migration [10, 15]. Consistent with these findings, we found that miR-20a-mediated inhibition of the p38 pathway was associated with an inhibition of endothelial cell migration in response to VEGF either in wound healing closure or Boyden chamber assays (Figs. 4a, b and 5a, b). In contrast, the inhibition of miR-20a was associated with both p38 activation and increased cell migration (Figs. 2b and 5c). Additionally, in accordance with the fact that miR-20a reduced the expression of MKK3 upstream of p38, we found that the knockdown of MKK3 was associated with an inhibition of VEGF-induced cell migration (Supplementary Fig. 4).

The VEGF induced-endothelial cell migration requires actin cytoskeleton remodeling into stress fibers as a result of HSP27 phosphorylation downstream of the VEGFR2-p38-MAPKAP K2 axis [10, 15]. Along these lines, we found that the decreased migratory potential of HUVECs expressing miR-20a was associated with a reduced formation of stress fibers as compared to control cells (Fig. 6a and b).

Altogether, these results suggest that miR-20a, via its modulation of the p38 pathway, is an important regulator of actin remodeling and endothelial cell migration in response to VEGF.

miR-20a expression inhibits the formation of capillary-like structures in a simplified engineered tissue model of angiogenesis

Endothelial cell migration is an essential step of angiogenesis [7, 10]. As miR-20a blocks endothelial cell migration, we investigated next, whether it repressed angiogenesis using a newly-developed simplified human reconstructed tissue culture model of neovascularization in vitro [44]. Briefly, HUVECs expressing miR-20a precursor or an empty vector were seeded on a monolayer of Normal Human Dermal Fibroblasts (NHDF) and were treated or not with VEGF. In this model, the addition of VEGF induced a well-developed network of capillary-like structures (Fig. 7a and c). As expected, miR-20a inhibited the formation of capillary-like structures induced by VEGF treatment (Fig. 7c and d). In fact, we noted a marked reduced length and number of VEGF-induced capillary-like structures in HUVECs expressing miR-20a (Fig. 7e and f). Of note, the miR-20a-dependent inhibition of capillary formation in vitro could not be attributed to increased
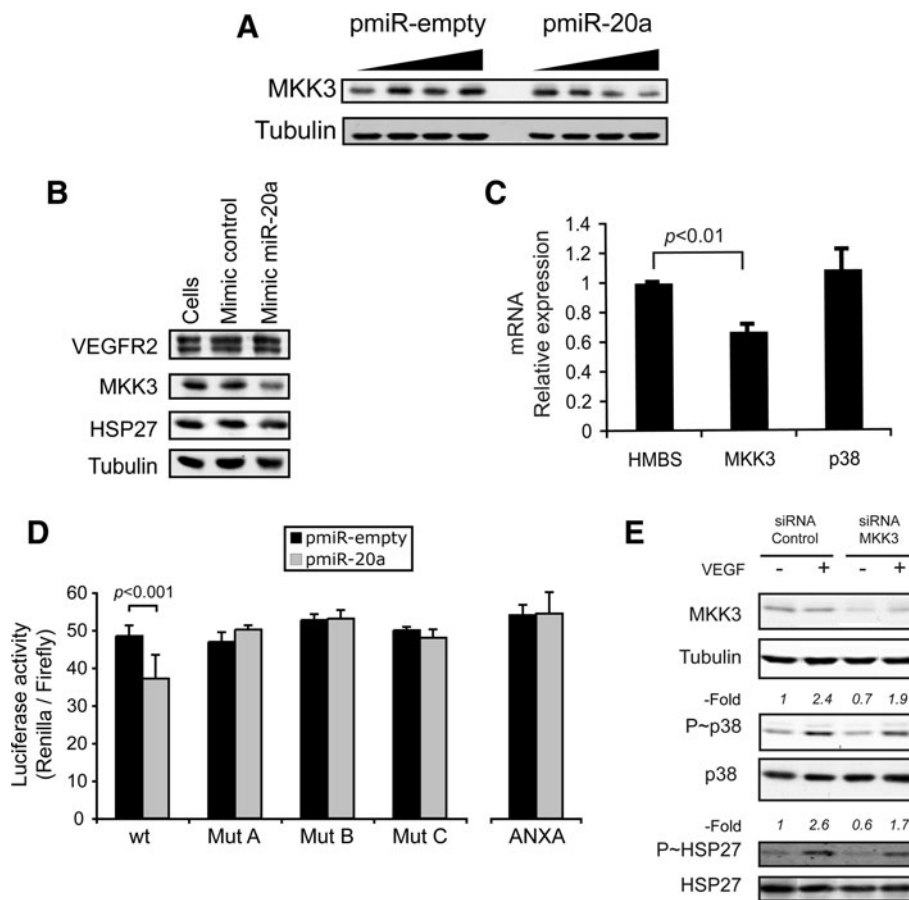
Fig. 3 miR-20a impairs p38 pathway activation in response to VEGF by targeting MKK3. **a** HUVECs were transduced with miR-20a precursor (pmiR-20a) or with an empty vector (pmiR-empty) using lentiviral-mediated infection for 96 h (A: MOI of 7, 14, 65 or 130). The proteins were extracted, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Total levels of MKK3 and Tubulin were revealed by Western Blot using specific antibodies. **b** HUVECs were transfected or not (Cells) with miR-20a mimic or mimic control (100 nM) for 48 h. Extracted proteins were processed for Western blots to detected total levels of VEGFR2, MKK3, HSP27 and Tubulin a using specifics antibodies. **c** Total RNA was extracted from transduced HUVECs (as in (**a**) but using : MOI of 65), reverse transcribed and the relative mRNA levels of MKK3, p38, and HMBS were quantified by SYBR Green Fast real time qPCR. HMBS was selected as the housekeeping gene normalizer using Genorm program. Fold changes were calculated using $2^{\Delta\Delta Ct}$ method. **d** Transduced

HUVECs (as in **c**) were transfected with psiCHECK-2 vectors carrying MKK3 mRNA 3′UTR wt, MKK3 mRNA 3′UTR mutated on three potential binding sites (Mut A, B and C, see Supplementary Fig. 3 for sequences) or ANXA1 mRNA 3′UTR wt as a negative control. Twenty-four hours later, the activity of Renilla luciferase (which expression was under the control of the 3′UTR insert) was measured and normalized against Firefly Luciferase activity as control. **e** HUVECs were transfected for 48 h with control siRNA or siRNA targeting MKK3. Overnight-serum-starved cells were treated or not with VEGF (10 ng/ml for 5 min). Then the proteins were extracted, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Phosphorylation levels of P ∼ p38 (T180, Y182), P ∼ HSP27 (S78, S82) and their total levels together with total levels of MKK3 and Tubulin a were revealed by Western Blot using specific antibodies

apoptosis or reduced proliferation. Indeed, inhibition of caspase-mediated apoptosis using either Z-VAD or QVD in this model of angiogenesis did not restore the miR-20a-associated inhibition of VEGF-induced formation of capillary-like structures (Supplementary Fig. 5a and 5b). Moreover, we found that there was no change in HUVEC rate of proliferation in the presence of miR-20a (Supplementary Fig. 6a and 6b). Hence, miR-20a does not affect apoptosis or proliferation of endothelial cells.

Overall, these results indicate that miR-20a by repressing the activity of the p38 pathway at the level of MKK3

can be a major repressor of angiogenesis induced by VEGF.

Discussion

Endothelial cell migration induced by VEGF is an essential step of angiogenesis. It requires p38-mediated actin remodeling whose major role is to allow cell contraction enabling traction of the cell from the rear toward the front [7]. This actin remodeling requires the integrated activation

Fig. 4 miR-20a inhibits VEGF-mediated endothelial cell migration in a wound closure assay. **a** HUVECs were transfected with a miR-20a mimic or a mimic control (100 nM, for a total of 48 h). Cells were serum-starved for 4 h before being evaluated in wound healing assay. Images were captured using an inverted microscope (×10) after 1, 8, 12 and 18 h. The *black line* represents the initial wound. **b** Cells that cross the line were manually counted from four different fields. The VEGF-induced relative migration is expressed at each time point by the ratio VEGF-treated cells/untreated cells
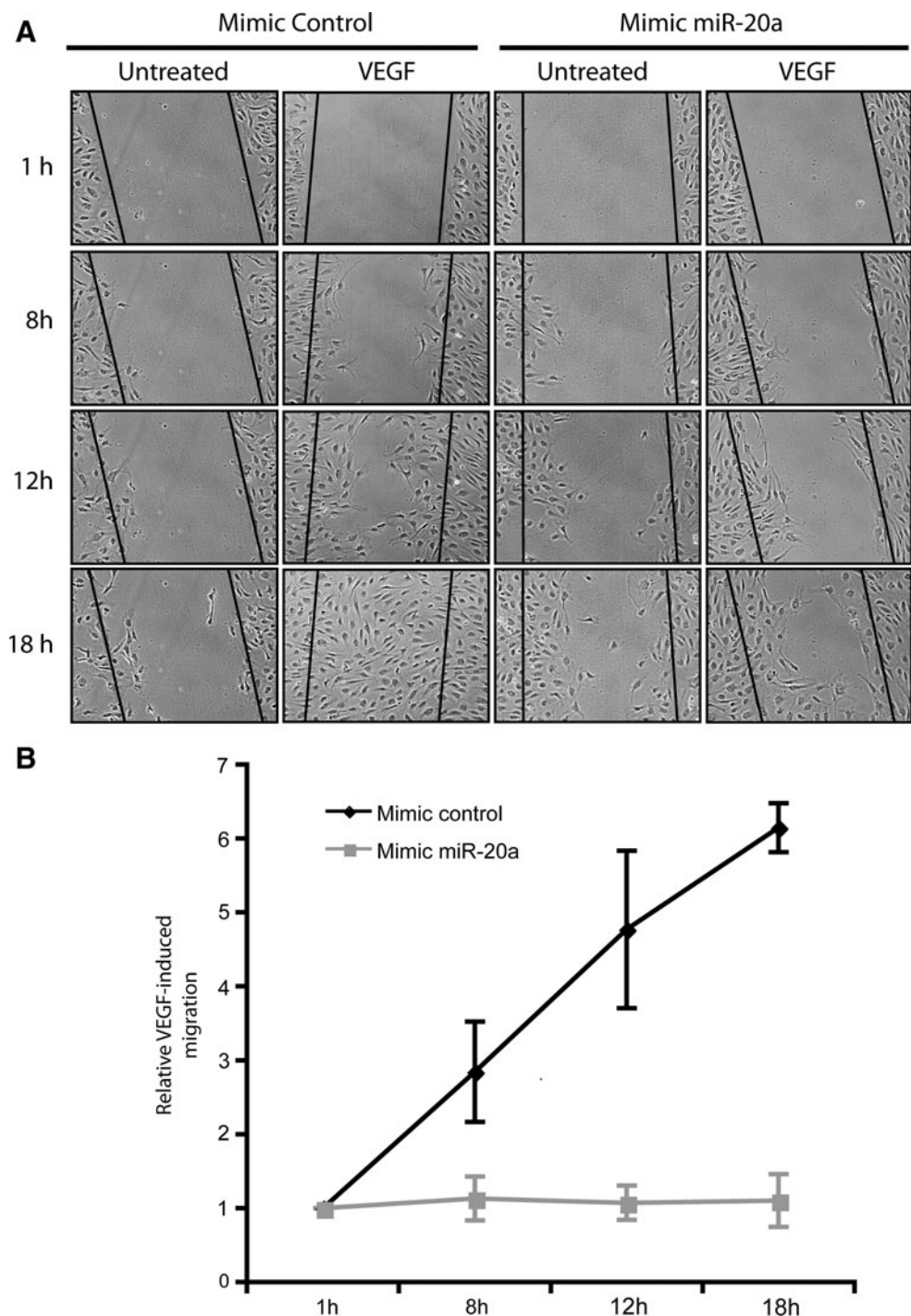
of several signaling pathways that hierarchically and tightly regulate the motile process including actin reorganization into stress fibers and lamellipodia [7, 15]. Here, we obtained the first evidence suggesting that the p38-mediated endothelial cell migration is under the regulation of miRNAs. This is supported by the fact that the ectopic Ago2 expression inhibits the VEGF-induced endothelial
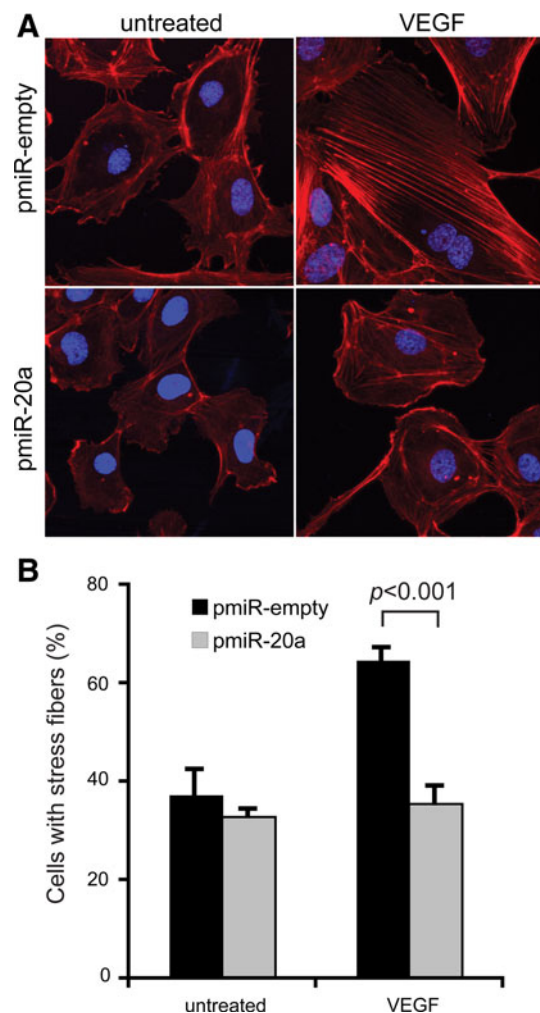
cell migration as well as p38 activation. Indeed, Ago2 is an essential component of the RNA-induced silencing complex and is considered along with other Ago proteins as actual mediators of miRNA-mediated gene silencing [36, 37, 50]. Based on this finding, we systematically investigated the expression level of individual miRNAs in response to VEGF and we found, as reported by Suarez and
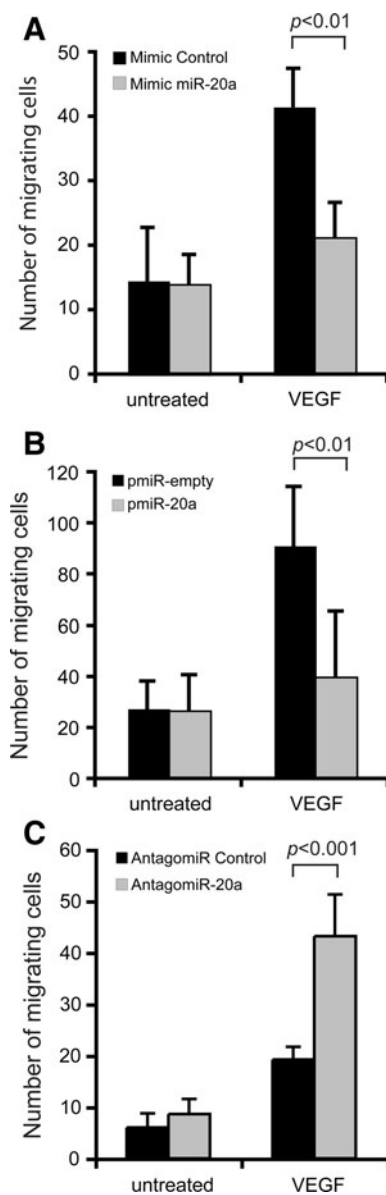
Fig. 5 miR-20a inhibits VEGF-induced endothelial cell migration in Boyden chamber chemotactic assay. **a** HUVECs were transfected with a miR-20a mimic or a mimic control (100 nM, for a total of 48 h) together with pEGFP to visualize and count transfected cells. Overnight-serum-starved cells were evaluated for cell migration in a transwell migration assay using VEGF (10 ng/ml for 4 h) as chemoattractant. **b** HUVECs were transduced with miR-20a precursor (pmiR-20a) or with an empty vector (pmiR-empty) both expressing GFP using lentiviral-mediated infection (MOI of 65) for 96 h. Overnight-serum-starved cells were processed for cell migration assay as in (**a**). **c** Untransfected HUVECs or HUVECs transfected 48 h with an antagomiR-20a or a control antagomiR (100 nM) together with pEGFP, to visualize and count transfected cells, were serum-starved overnight and then were processed for cell migration assay as in (**a**)

Fig. 6 miR-20a inhibits stress fibers formation. **a** HUVECs were transduced with miR-20a precursor (pmiR-20a) or with an empty vector (pmiR-empty) both expressing GFP using lentiviral-mediated infection (MOI of 65). Ninety-six hours later, overnight serum-starved cells were treated or not with VEGF (10 ng/ml) for 15 min and thereafter fixed, permeabilized and stained with Rhodamine-phalloïdin and DAPI to detect F-actin and nucleus, respectively. A representative field for each condition was captured using a confocal microscope (×40) and a merged picture is shown for each condition. **b** Twelve independent fields from A were captured and total cells were counted using DAPI staining with a minimum of 200 cells per condition for each experiment. Cells containing stress fibers were evaluated using F-actin staining. The percentage of cells containing stress fibers is shown (mean ± SD). The experiments were performed at least in triplicates

collaborators [45], that the expression of miR-17 and miR-20a were both increased by VEGF.

miR-17 and miR-20a are members of the miR-17 ∼ 92 cluster which is encoded by *C13orf25* gene [51].

Interestingly, the activation of signal transducer and activator of transcription-3 (STAT3) following IL-6 treatment is an inducer of this cluster in endothelial cells [51]. Here, we found that VEGF, as IL-6, induces the phosphorylation of STAT3 on the activating residue Y705 (Supplementary Fig. 7). This suggests that VEGF-mediated increased expression of miR-17 and miR-20a could result from
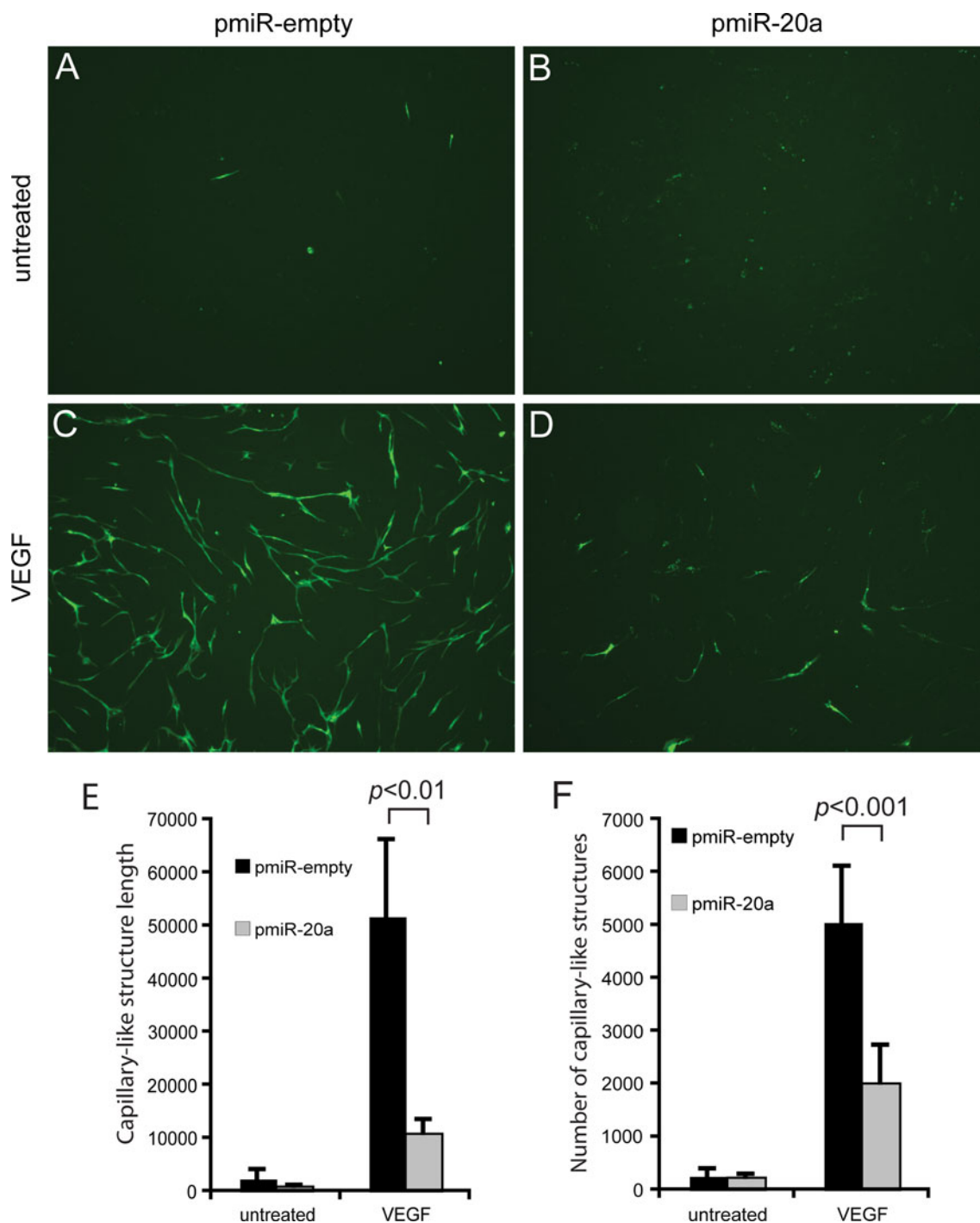
Fig. 7 miR-20a modulates VEGF-mediated formation of capillaries. **a–f** HUVECs were transduced with miR-20a precursor (pmiR-20a) or with an empty vector (pmiR-empty) both expressing GFP using lentiviral-mediated infection (MOI of 65). After 96 h, 50,000 transduced cells were plated on a 12 days Normal Human Dermal Fibroblasts (NHDF) monolayer. Co-cultures were maintained for 6 days and media was replaced every 48 h with or without VEGF treatment (10 ng/ml) for a total of 3 treatments. Capillary-like structures were observed at day 6 using an inverted fluorescent microscope (×4). Representative fields are shown for each condition (**a–d**). **e** Fifteen pictures per condition from different fields were captured to measure the length of the capillaries using ImageJ software. **f** The same approach as in (**e**) was used to calculate the number of capillaries but by using AnalyzeSkeleton plugin from ImageJ. Results are expressed as the mean ± SD of capillary length or number of capillaries. The experiments were performed at least in triplicates

STAT3-dependent activation of *C13orf25*. It is difficult at the present time to explain how STAT3 is activated by VEGF. One possibility, initially proposed by Kari Alitalo is that STAT3 is activated following its recruitment to P∼Y1175 or P∼Y801 (in mouse, numbered Y1173 and Y799) within VEGFR2 [52]. Considering that both Y1175 and Y801 have perfect match with STAT3 consensus YXXQ, it is possible that STAT3 binds directly to these sites and then become phosphorylated on Tyr by activated VEGFR2 [52]. Intriguingly, it has been reported that c-Myc, E2F transcription factors or p53 can also regulate *C13orf25* [53–56]. Our results do not allow us to exclude the participation of these actors in contributing to activate *C13orf25* in response to VEGF.

The observation that the expression of both miR-17 and miR-20a was increased by VEGF raises our interest. Indeed, the miR-17 ∼ 92 cluster has previously been shown to regulate angiogenesis [42, 47, 48]. However, the members of the cluster possess different and even opposite effects on angiogenesis that are difficult to explain at the present time. Notably, in pathological angiogenesis such as during cancer, miR-18 and miR-19 are responsible for the repression of the anti-angiogenic factors thrombospondin-1 and CTGF produced by tumor cells and are classified as pro-angiogenic miRNAs [42]. In endothelial cells, miR-92a and miR-20a respectively promote and repress the angiogenic potential of these cells [47, 48]. Moreover, the level of expression of only three miRNA components of the entire cluster, miR-17, miR-18a, and miR-20a were identified to be elevated by VEGF suggesting a selective miR-17 ∼ 92 cluster members biogenesis under given biological contexts [45]. It is possible that the different and opposing roles played by the members of the miRNA cluster miR-17 ∼ 92 rely on specific maturation or stabilization processes that may be cell type-, agonist- and context-dependent.

An important contribution of our study is to have highlighted the signaling mechanisms underlying the role of miR-20a in VEGF-dependent angiogenesis. In particular, we found that the ectopic expression of miR-20a almost completely impairs endothelial cell migration either in Boyden chamber or in wound closure assays in response to VEGF. More interestingly, we also found that the inhibition of miR-20a by a specific antagomir is associated with an increased in both VEGF-induced activation of p38 and cell migration. These findings are strong indications that the endogenous level of miR-20a is a key regulator of the p38 pathway activated in response to VEGF. Moreover, consistent with the fact that endothelial cell migration is an essential step of angiogenesis, we further found that the ectopic expression of miR-20a completely abolishes the formation of capillary-like structures induced by VEGF in a modified tissue-engineered model of angiogenesis [44]. In this assay, miR-20a does not affect cell proliferation or

apoptosis suggesting a direct role on the mechanisms affecting actin-based motility. Along these lines, we previously reported that endothelial cell migration induced by VEGF relies in part on the phosphorylation of HSP27 that governs the formation of actin stress fibers downstream of the VEGFR2/p38/MAPKAP K2 axis [7, 15]. Consistent with these findings, we found that miR-20a-mediated inhibition of endothelial cell migration is associated with a decreased phosphorylation of HSP27 and an impaired formation of actin stress fibers in response to VEGF. Overall, these findings strongly suggest that miR-20a represses angiogenesis by inhibiting the p38 pathway. Interestingly, Ago2 is activated by MAPKAP K2, downstream of p38, suggesting that the activation of p38 by VEGF could contribute to increase the activity of the miRNA pathway [57]. In turn, this supports the point that VEGF-induced expression of miR-20a may act in a feedback loop to repress the activation of the pathway.

Another important novel finding of our study is that we discovered that miR-20a represses the activation of p38 by decreasing the level of MKK3, a direct upstream activator. This is supported by our results showing: (1) that the decreased level of MKK3 protein is proportional to an increase of miR-20a, (2) that the transcript level of MKK3 is also reduced by miR-20a, (3) that miR-20a decreases the expression of MKK3 in response to VEGF by specifically targeting the 3′UTR of MKK3 mRNA, (4) that the decreased level of MKK3 is associated with a reduction of HSP27 phosphorylation, an effector of the p38 pathway, and (5) that the siRNA-mediated knockdown of MKK3 decreases the activation of p38 and endothelial cell migration induced by VEGF. Interestingly, we found that no other major proteins known to be involved in p38 activation induced by VEGF were negatively regulated by miR-20a, including VEGFR2, Nck, Fyn, Pak2, Cdc42 and MKK6 [7]. Incidentally, the fact that miR-20 does not target MKK6 is in agreement with a previous report showing that this kinase is not involved in regulating p38-mediated endothelial cell migration by VEGF [12]. These results also suggest that MKK6 does not rescue the miR-20a repression of cell migration in response to VEGF. Of note, members of the miR-17 ∼ 92 cluster that share seed region sequence homology with miR-17, including miR-20a, were reported to target directly p38 (MAPK14) in murine epithelial cell [58]. However, we did not observe any modulation of p38 protein or transcript levels when miR-20a was expressed in human endothelial cells. These contrasting results are in accordance with the possibility that miRNA-dependent functions may be cell type specific. Hence, it seems that the miR-20a-induced decreased activation of p38 results mainly from an inhibition of MKK3 expression in endothelial cells. Along these lines, the siRNA-mediated knockdown of MKK3 completely

Fig. 8 miR-20a targets the expression of MKK3 to regulate p38-mediated endothelial cell migration. The binding of VEGF to VEGFR2 triggers the phosphorylation of Tyr1214 within the receptor. In turn, this elicits the activation of the p38 cascade leading to phosphorylation of HSP27, actin remodeling and endothelial cell migration [21]. From the present study, we propose that VEGF-induced cell migration is regulated by the miRNA pathway being affected by Ago2. More specifically, we present evidence in support that VEGF induces the expression of miR-20a, via activation of STAT3. In turn, miR-20a binds to the 3′-UTR region of MKK3 mRNA, which decreases the expression of MKK3 and thereby p38 activation, endothelial cell migration and angiogenesis
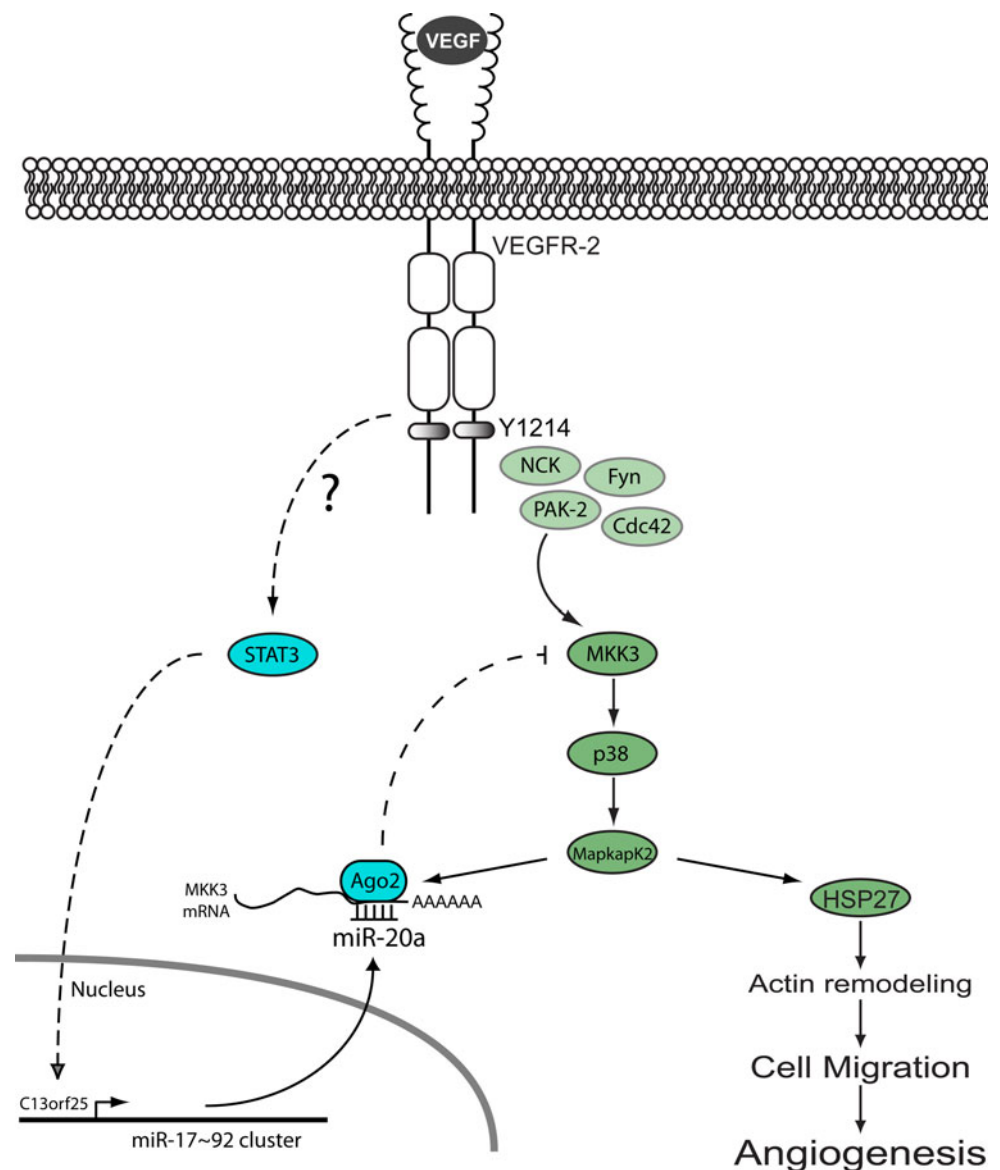
abolishes cell migration, which is in agreement with the findings that MKK3 targets both p38α (herein p38) and p38γ the two p38 isoforms that mediate endothelial cell migration [[7], [12]]. In corollary, this may also explain why the knocking down of MKK3 totally inhibits cell migration without totally blocking VEGF-induced activation of p38α. Intriguingly, Bonauer and collaborators propose that MKK4 could be a target of miR-92a [48], suggesting that MKKs might be primary targets of the miR-17 ∼ 92 cluster. Yet, miR-20a does not target MEK1/MEK2, the upstream activators of ERK1/ERK2 [59], since the ERK activation in response to VEGF is not affected by miR-20a. Given that ERK regulates endothelial cell proliferation in response to VEGF [15], the finding is also in accordance with our observation that miR-20a does affect endothelial cell proliferation. In corollary, this

finding suggests that miR-20a selectively targets the p38 pathway. However, this does not exclude the possibility that miR-20a may affect other VEGF-or non-VEGF-dependent motile pathways, given that miRNAs may have different targets and thereby may affect a same cellular process via different pathways. Notably, miR-20a has been proposed to target Jak1 but the role of JAK1 in VEGF signaling is unclear [47].

Given that angiogenesis should be sustained during cancer, we believe that the miR-20a-mediated repressing loop of p38 activation is not functional during cancer angiogenesis. We hypothesize that cancer cells or their microenvironment release factors that impair the functioning of the miR-20a regulated pathway in surrounding endothelial cells and thus promote anarchical angiogenesis. In this context, inhibition of miR-17 and miR-20a after IV

injection of antagomiR-17/20 does not modulate tumor angiogenesis in a tumorigenic mouse model [47]. This suggests that these miRNAs have already been inhibited by the release of inhibitory factors. Interestingly, members of the cluster including miR-20a are down-regulated by hypoxia mimetic treatment of cancer cells [60, 61]. Given that miR-20a directly targets HIF1α and thereby VEGF expression, its down regulation by hypoxia would further contribute to increase angiogenesis in cancer [60, 62].

From our study, we thus conclude that miR-20a acts in a feedback loop to repress the expression of MKK3, which contributes to impair p38-mediated endothelial cell migration and physiological angiogenesis in response to VEGF (see the model in Fig. 8). Our findings yield new insights in better understanding therapeutic angiogenesis.

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