An Endothelial Cell Genetic Screen Identifies the GTPase Rem2 as a Suppressor of p19\textsuperscript{ARF} Expression That Promotes Endothelial Cell Proliferation and Angiogenesis*\textsuperscript{S}

Received for publication, September 5, 2007; revised form, November 29, 2007 Published, JBC Papers in Press, December 3, 2007; DOI 10.1074/jbc.M707438200

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Angiogenesis requires an increase in endothelial cell proliferation to support an increase in mass of blood vessels. We designed an in vitro endothelial cell model to functionally screen for genes that regulate endothelial cell proliferation. A gain of function screen for genes that bypass p53 endothelial cell arrest identified Rem2, a Ras-like GTPase. We show that ectopic Rem2 suppresses p14\textsuperscript{ARF} (human) or p19\textsuperscript{ARF} (mouse) expression that leads to increased endothelial cell proliferation. Conversely, loss of ectopic Rem2 by RNA interference restores p19\textsuperscript{ARF} expression in endothelial cells. We further show that Rem2-interacting 14-3-3 proteins are involved in the cell localization of Rem2, regulation of p19\textsuperscript{ARF} expression, and endothelial cell proliferation. Finally, we demonstrate using the RIP1 tag2 mouse model of pancreatic disease that Rem2 is up-regulated in endothelial cells of stage IV disease. The data unravel a possible molecular mechanism for Rem2-induced angiogenesis and suggests Rem2 as a potential novel target for treating pathological angiogenesis.

Angiogenesis, the formation of new blood vessels, is a complex process that involves many steps including endothelial cell proliferation, migration and invasion, tube formation, and vessel maturation. Angiogenesis is involved in disease states such as arthritis and atherosclerosis, and is particularly important for the growth, invasion, and metastatic spread of tumors (1, 2). Although the regulation of endothelial cell proliferation is therefore of obvious importance, very little is known about cell cycle control in endothelial cells.

The Ink4a-Arf locus encodes two tumor suppressor proteins, p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} (p14\textsuperscript{ARF} in humans) that up-regulate the activities of the retinoblastoma protein (Rb)\textsuperscript{3} and the p53 transcription factor, respectively (3). The p16\textsuperscript{INK4a} protein inhibits the activity of cyclin D-dependent kinases, thereby maintaining Rb in its hypophosphorylated, growth-suppressive state (3). p19\textsuperscript{ARF} antagonizes Mdm2 activity, resulting in a p53 transcriptional response that leads to cell cycle arrest or apoptosis. Loss of p16\textsuperscript{INK4a} or p19\textsuperscript{ARF} function is a critical event for tumor promotion as evidenced by extinguished expression of the p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} protein in a variety of tumors (4, 5). Interestingly, p19\textsuperscript{ARF} loss affects the normal development of the eyes of newborn mice, where persistence of the hyaloid vasculature in the vitreous results in destruction of both the lens and neuroretina, directly implicating p19\textsuperscript{ARF} in regulation of pathological angiogenesis (6, 7).

Rem2 is a recently identified member of the Rem/Rad/Gem/Kir (RGK) family of Ras-related GTPases that share structural features that are different from other Ras-related proteins (8, 9). These include non-conservative amino acid substitutions within regions known to be involved in guanine nucleotide binding and hydrolysis, extended N and C termini, and a conserved C-terminal motif thought to mediate membrane association but lacking a prenylation site present in other Ras-like molecules (10). Two important functions of RGK proteins are the regulation of voltage-gated Ca\textsuperscript{2+} channel activity and cell shape associated with angiogenesis (11–14). RGK proteins interact with 14-3-3 and calmodulin and Gem regulates endothelial shape changes required for angiogenesis (12, 13). Recently it has been shown that 14-3-3, together with calmodulin, regulates the subcellular distribution of Rem2 between the cytoplasm and the nucleus and this distribution has been correlated to cell shape changes (12, 13, 15). However, given past research efforts, little is known about the cell function of the RGK family of GTPases, particularly Rem2.

Here we show a novel function for Rem2 as a suppressor of p19\textsuperscript{ARF} transcription, partially dependent on 14-3-3 protein binding to promote accelerated endothelial cell proliferation. Furthermore, we show that ectopic Rem2 promotes angiogenesis in vitro and is expressed in endothelial cells of the RIP1 tag2 mouse model of pancreatic disease.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Infection**—Large T SV40 temperature-sensitive endothelial cells (ts T endothelial cells) microvascular endothelial cell; GFP, green fluorescent protein; RNAi, RNA interference; HA, hemagglutinin; WT, wild type; shRNA, short hairpin RNA; CMV, cytomegalovirus; CaM, calmodulin.
were subcloned from a polyclonal population of brain capillary endothelial cell line, derived from H-2Kb-tsA58 transgenic mice, and display endothelial cell-specific characters, such as expression of von Willebrand factor and uptake of acetylated low density lipoprotein (16). Endothelial cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Twenty clones were expanded and 3 clones selected based on the lowest number of background colonies that grew at 39 °C. Experiments were performed with clones 5 and 12 (LTEC5 and LTEC12: large T endothelial Cell 5 and 12) that have virtually no background growth at 39 °C (see Fig. 1). The functional screen was carried out with clone LTEC5, which showed lowest background in repetitive experiments, and genes discovered were later retested in mouse ts T endothelial cells LTEC5 and LTEC12. All results are shown with LTEC5 cell line.

Ecotropic retroviral supernatants were produced by transfection of genes into Phoenix packaging cells by calcium-phosphate precipitation. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum at 37 °C. Forty-eight hours post-transfection, the tissue culture medium Eagle’s medium supplemented with 8% fetal calf serum at 37 °C. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% low density lipoprotein (16). Endothelial cells were cultured in RPMI 1640 and M199 (1:1), supplemented with 10% fetal calf serum. Twenty clones were expanded and 3 clones (LTEC5 and LTEC12: large T endothelial Cell 5 and 12) were subcloned from a polyclonal population of brain capillary endothelial cell line, derived from H-2Kb-tsA58 transgenic mice, and display endothelial cell-specific characters, such as expression of von Willebrand factor and uptake of acetylated low density lipoprotein (16). Endothelial cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Twenty clones were expanded and 3 clones selected based on the lowest number of background colonies that grew at 39 °C. Experiments were performed with clones 5 and 12 (LTEC5 and LTEC12: large T endothelial Cell 5 and 12) that have virtually no background growth at 39 °C (see Fig. 1). The functional screen was carried out with clone LTEC5, which showed lowest background in repetitive experiments, and genes discovered were later retested in mouse ts T endothelial cells LTEC5 and LTEC12. All results are shown with LTEC5 cell line.

Ecotropic retroviral supernatants were produced by transfection of genes into Phoenix packaging cells by calcium-phosphate precipitation. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum at 37 °C. Forty-eight hours post-transfection, the tissue culture medium was filtered through a 0.45-μm filter and added to target cells. High titer retroviral library supernatants derived from mouse whole brain (Clontech) were used to infect 2 × 10^6 ts T endothelial cells. Twenty-four hours after infection, cells were plated at a density of ~1 × 10^5 cells per 10-cm dish and 48 h after infection the cells were shifted to 39 °C (the non-permissive temperature for the transforming SV40 T antigen). Colonies of cells appeared only in the library-infected populations. These colonies were picked, expanded, and pro-viral inserts identified by re-cloning into library vector (pLIB) and identified by sequencing. To analyze whether the rescue was due to expression of a retroviral library-derived cDNA, a second round with re-cloned library cDNA was performed.

Primary HUVECs were isolated from umbilical veins and cultured in medium containing RPMI 1640 and M199 (1:1), 20% human serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 33 mg/ml glutamine. 500,000 HUVECs at passage 2–3 were electroporated with 9 μg of RGK expressing vector by a previously described method (17). Cells were allowed to recover and 200,000 cells plated on fibronectin-coated plates to determine growth rate after 3 days. Immunohistochemistry was performed concurrently as described below.

Human microvascular endothelial cells (HMEC-1) were cultured in Dulbecco’s modified Eagle’s medium with 10% serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 33 mg/ml glutamine. 500,000 HUVECs at passage 2–3 were electroporated with 9 μg of RGK expressing vector by a previously described method (17). Cells were allowed to recover and 200,000 cells plated on fibronectin-coated plates to determine growth rate after 3 days. Immunohistochemistry was performed concurrently as described below.

Human microvascular endothelial cells (HMEC-1) were cultured in Dulbecco’s modified Eagle’s medium with 10% serum on gelatin-coated plates. Ecotropic receptor was electroporated into HMEC-1 cells following a described method and clones selected with Zeomycin. Stable HMEC-1 ecotropic receptor expressing cells were then infected with high titer GFP, Rem2HA, or Ras v12 virus and injected into mice for Matrigel plug angiogenesis assay (see below).

cDNA and RNAi Constructs—For overexpression studies we used the TBX3, pBabe Zeo Ecotropic Receptor, LZRS-p19ARF (Red), pBabe-Ras v12, and GFP constructs as previously described (18). Following identification of Rem2 from the functional screen, wild type mouse Rem2 was constructed by PCR from the same library used in the screen (whole brain pLib-cDNA retroviral library from Clontech). Primers for Rem2 with a N-terminal HA tag where designed and cloned back into pLib vector (same as cDNA library) using EcoRI and NotI sites (forward, 5’-gatcAAATTCGGATCCGGTACCATCACATGACCTGGA-3’ and reverse, 5’-gatcGGGGCCGCTACAGAGCGAAGGTCGTGACATGACCTGGA-3’).

We reasoned that wild type (WT) Rem2 was more biologically relevant than a constitutively active mutant because WT Rem2 would be dependent on GDP-GTP cycling and specific GEFs or GAPs in the cell. For transient expression studies, wild type Rem2 cloned into Myc-tagged pME18S constructs were a kind gift from Walter Hunziker and Pascal Beguin (13, 15). Rem2 14-3-3 and CaM point binding mutants (Myc tagged) were also provided by Walter Hunziker and Pascal Beguin.

For mouse p53 shRNAi the sequence is published elsewhere (19). For mouse p19ARF we targeted the following sequence: GTCGTGCGATCCCGGAGA. RNAi directed against 14-3-3-y was cloned into pSUPER and was a gift from Dr. T. Suzuki (20). For mouse Rem2 we targeted the following sequences: 1) GCGGGCGGGCCCAAGCTGTA, 2) GCCCGGCTCCCAGGAGGTAT, and 3) CGGGGGATGCCTTTCTCAT.

Western Blotting, Immunohistochemistry, and Fluorescence-activated Cell Sorter—Western blots were probed with antibodies against: 14-3-3-y (Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin (Sigma), p53 (Santa Cruz), p21 (Santa Cruz), p14ARF (Neomarker), p19ARF (Abcam), CDK4 (Santa Cruz), HA tag (Santa Cruz), Myc tag (4AE Roche), Cyclin A (Santa Cruz), Cyclin D (Santa Cruz), p107 (Santa Cruz), and p27 (Santa Cruz). A polyclonal antibody for Rem2 was developed against the following peptide sequence CVPRNAKFFKQRSRS. The day 28 bleed was used at 1:750 dilution in 1% milk TBS, Tween 20. Western blots were developed using enhanced chemiluminescence (Amersham Biosciences) following the instructions of the manufacturer.

For visualization of actin, we used BODIPY 650/665-phalloidin (Molecular Probes, Leiden, Netherlands). For detection of trimethylated histone H3 at lysine 9 a polyclonal anti-H3K9-Me antibody (Cell Signaling Technology) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were used. 300 cells displaying only high levels of signal were selected and the number of cells containing trimethylated histone H3 lysine 9 were counted by light microscopy (equivalent expression levels to cells at 39 °C). Myc-tagged Rem2 was detected using 9E10 and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) as a secondary antibody. Nuclei were stained using TOPRO-3 (Molecular Probes). Cells were embedded in Vectashield mounting medium (Vector Laboratories, Burlington, VT) and analyzed by confocal microscopy using a Zeiss LSM510 with the appropriate filter settings.

For the quantification of nuclear exclusion of Rem2 and its mutants, 60–70 Rem2 expressing cells from three independent experiments were randomly selected and a single confocal xy-plane was imaged through the middle of the cell. Rem2 exclusion was arbitrarily scored as none, partial, or complete based on its colocalization with the nuclear TOPRO-3.

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Dr. John Collard, personal communication.
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Reverse Transcriptase-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) and a subsequent clean-up RNeasy protocol (Qiagen Inc., Valencia, CA). 250 ng of total RNA was reverse transcribed into cDNA following instructions from the manufacturers and the resulting cDNA was analyzed by PCR.

Primers for p19ARF used were: Exon 1 forward: 5'-GTCGGACGTTTGTCTTGCAGTGA-3 and Exon 2 of p16 reverse: 5'-TGCTTCGACGTTCACTTCG-3. Primers for mouse Rem2 were: forward: 5'-CGTGGGGGAGATGGCCTGGG-3' and reverse: 5'-ACGAGTGTTGTGCTGAGGCC-3 producing a 445-bp fragment. Primers were annealed at 60 °C at 27–35 cycles as described in the figures.

Colony Formation Assay and β-Galactosidase Staining—Cells were plated at low density after retroviral infection with genes of interest and left at 32 °C overnight. The following day, plates were shifted to 39 °C and left for 2–6 weeks, depending on the gene being investigated. Plates were then washed, fixed in methanol, and stained with Coomassie Blue for 1 h, washed, and air-dried. β-Galactosidase staining was performed as described before and 300 cells counted for blue signal per condition described (21). Statistical analysis of cell numbers of colony formation assay was performed using Student’s t test with Excel software.

Luciferase Assays—Equal numbers of HEK cells were transfected with 1–3 mg of CMV-RGK, 2 mg of luciferase construct, and 1 mg of CMV-β-galactosidase. Total protein was measured and 4 mg measured for luciferase activity and 8 mg for β-galactosidase activity. Luciferase counts were corrected to β-galactosidase levels.

RESULTS

Functional Screen in Endothelial Cells Identifies Rem2—To study the genes involved in the p53/Rb pathway in mouse endothelial cells, we subcloned a mouse endothelial cell line expressing a temperature-sensitive mutant of SV40 T antigen (ts T) to identify a clone with no background in repetitive experiments (16). These cells were given the acronym LTEC5 for Large T endothelial cell, clone number 5. This mutant binds and inactivates both pRb and p53 at 32 °C allowing the cells to proliferate indefinitely at this temperature. At 39 °C, however, the mutant T antigen is degraded, so that p53 and pRb are released, resulting in cell cycle arrest (see Fig. 1A, inset i). Acidic β-galactosidase staining of endothelial cells and their flattened morphology suggests that 91% of cells undergo a senescence-like arrest at 39 °C (Fig. 1A, insets i and ii). To further characterize the cell cycle arrest we assessed the state of trimethylation of histone H3 lysine 9, an established marker of cell senescence (22). We found that 99.6% of cells express high levels of trimethylated histone H3 lysine 9 at 39 °C, and only 1.3% of cells at 32 °C express similar levels to the non-permissive temperature (Fig. 1A, insets iii and iv). Furthermore, we found that the endothelial cell cycle arrest is reversible supporting other data that rodent senescence is p53 dependent and reversible (see supple-
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Rem2 Suppresses p19ARF/p14ARF Expression in Endothelial Cells—To elucidate the mechanism of the Rem2-induced bypass of the p53-dependent endothelial cell cycle arrest, endothelial cell clones that overexpress Rem2 at 39 °C were picked and expanded for further analysis. A total of 13 clones were first analyzed for SV40 Large T antigen (T) and Rem2 expression levels (see supplementary information, Fig. S2, panel A). Next we determined the p53 status of those endothelial clones that expressed various levels of Rem2 and not T (see supplementary information, Fig. S2 panel B). Cisplatin causes activation of wild type p53 and subsequently one of its targets, p21CIP1, making it a useful tool to determine whether p53 is wild type and functional. Following treatment with cisplatin, p53 and p21CIP1 were both activated in all endothelial cell clones, demonstrating that ectopic Rem2 expression was able to bypass a wild type p53-induced cell cycle arrest and that this response was uniform for all clones. We continued to investigate those endothelial clones that expressed a range from low to high levels of Rem2 protein expression with a wild type p53.

Because p53/Rb regulate the G1 phase of the cell cycle (see supplementary information, Fig. S1, panel B), we next performed Western blot analyses of genes regulating the G1-S phase of the endothelial cell cycle (see supplementary information, Fig. S2, panel C) and found that the most significant change caused by ectopic Rem2 was the suppression of p19ARF protein levels (Fig. 3A).

To verify that ectopic Rem2 specifically regulated the p19ARF protein levels, we designed three individual shRNA constructs to knock down Rem2, resulting in ~70% knockdown (Fig. 3B, inset i). The use of three shRNAi constructs reduces the chances of off target effects to nil. Loss of ectopic Rem2 by shRNAi resulted in restoration of the p53 pathway, with increased p19ARF and p53 protein levels following Rem2 RNAi treatment with any of the three individual constructs (Fig. 3B, inset ii). Moreover, functional analyses demonstrated that loss of ectopic Rem2 resulted in reduced colony formation at 39 °C, not seen in p53 and Rem2 RNAi double-treated cells (Fig. 3B, inset iii, first 4 panels). This data supports the idea that ectopic Rem2 suppresses p19ARF expression leading to increased proliferation. Loss of endogenous Rem2 in p53 null-treated endothelial cells had no effect on colony formation assay suggesting that activation of Rem2 is needed before suppressing p19ARF expression (Fig. 3B, inset iii, fourth panel).

To further establish that the Rem2-mediated effects on p19ARF are needed for proliferation we overexpressed p19ARF in Rem2 expressing mouse endothelial cell clones by retroviral infection (70% infection levels) and performed colony formation assay. Re-introduction of p19ARF expression inhibited Rem2-induced proliferation demonstrating that p19ARF is essential for the proliferative effects of ectopic expression of Rem2 (Fig. 3B, inset iii, bottom left panel). The use of a GFP RNAi construct demonstrates that the effects of the Rem2 RNAi on proliferation are specific (Fig. 3B, inset iii, bottom right panel). Quantification of the colony formation assay demonstrates the clear differences between treatments (Fig. 3B, inset iv).

To further investigate the mechanism of regulation of p19ARF by Rem2, we assessed the mRNA levels following Rem2

Additional text:

mentary information, Fig. S1, panel A) (19). Finally, fluorescence-activated cell sorter analysis of the mouse endothelial cells demonstrates that they arrest in the G1 phase of the cell cycle (see supplementary information, Fig. S1, panel B).

To investigate whether cell cycle arrest in these endothelial cells is dependent on the p53 pathway, a main regulator of the G1 phase of the cell cycle, we used shRNA-mediated persistent RNA interference to knock down the p53 and p19ARF tumor suppressor genes. Fig. 1B shows that reduced levels of p53 and p19ARF proteins rescue the temperature shift-induced cell cycle arrest and thus causes immortalization at 39 °C. The results indicate that the endothelial cell arrest at 39 °C is dependent on the p53 pathway in endothelial cells (Fig. 1B). Given that loss of p19ARF or p53 resulted in rescue of the cell cycle arrest, we propose an in vitro genetic model to screen for genes that can modulate the p53-induced senescence-like cell cycle arrest in mouse endothelial cells (Fig. 1, A and B).

A functional genomic screen was performed in the ts T mouse endothelial cells using a mouse whole brain cDNA library (Clontech). Sequencing genomic DNA recovered from a rare colony that proliferated at 39 °C identified Rem2. Rem2 is a new member of the RGK family of Ras-like GTPases (Rad, Gem (Kir), Rem and Rem2), which have a previously described role in aspects of the angiogenic cascade (12, 14). Consequently, we focused on Rem2.

Re-cloning wild type Rem2 into the same vector from the library, we found that ectopic Rem2 rescued the p53 cell cycle arrest to immortalize endothelial cells (Fig. 2A). As a control already known to regulate the p53 pathway via p19ARF suppression (18), we overexpressed TBX3 and found that the endothelial cell cycle arrest was rescued (Fig. 2, A and B). Oncogenic Ras (Ras v12) expression caused endothelial cell cycle arrest as expected (Fig. 2, A and B). Analysis of expression of the ectopic Rem2 protein after 2 weeks reveals that it is not degraded at 39 °C and therefore was sufficient to rescue a p53 cell cycle arrest (Fig. 2C). Therefore, we focused on the role of Rem2 in endothelial cells.
ectopic expression. Using semiquantitative reverse transcriptase-PCR we found that the endogenous levels of Rem2 mRNA are low in mouse brain capillary endothelial cells (Fig. 3C, last lane). We found that in mouse endothelial cell clones, which at 39 °C expressed low to moderate levels of Rem2 based on protein expression (see supplementary information, Fig. S1, panel B), p19ARF protein levels in clone 8 treated with the three RNAis against Rem2. Inset ii, analysis of p53, and p19ARF protein levels in clone 8 untreated or an RNAi for Rem2 (top panels); p53 null cells expressing an RNAi untreated or an RNAi for Rem2 (middle panels); and clone 8 cells overexpressing p19ARF (bottom left panel) or an RNAi for GFP (bottom right panel). Inset iii, graph of quantification of number of cells from colony formation assay. Panel C, reverse transcriptase-PCR (27, 32, and 35 cycles) of four Rem2 endothelial cell clones for p19ARF mRNA compared with cell cycle-arrested endothelial cells expressing GFP at 39 °C. GAPDH mRNA is used as a loading control and endogenous Rem2 mRNA levels are shown in the bottom panel. The gel for Rem2 mRNA level is overexposed to demonstrate the low levels of expression of endogenous Rem2 in control (GFP) endothelial cells. This experiment was performed three times. Panel D, cell localization studies for HA-Rem2 in mouse endothelial cell clones overexpressing HA-Rem2 demonstrate cytoplasmic location, which were immortalized at 39 °C, compared with p53 null cells. Actin is used as a co-stain and overlaid. The experiment was performed three times. Panel E, inset i, effect of ectopic expression of WT Rem2, on primary HUVEC proliferation. Inset ii, Western blot for the Myc tag and p14ARF expression in control HUVEC expressing GFP, and in HUVEC expressing Rem2. Tubulin is used as loading control. The experiment was performed three times in duplicate.

To functionally assess if Rem2 can accelerate primary HUVEC proliferation rates and to rule out any effects of SV40 large T antigen that may have been undetected in the mouse endothelial cells (see supplemental Fig. 2B), we overexpressed wild type Rem2 and measured primary HUVEC proliferation. Ectopic WT Rem2 increased HUVEC proliferation rates above controls, demonstrating that ectopic Rem2 induces endothelial cell proliferation (Fig. 3E, inset i). Importantly we found that ectopic Rem2 completely suppressed p14ARF protein expression in HUVEC, supporting the data in mouse endothelial cells that overexpressed Rem2 suppresses p14ARF and p19ARF expression (Fig. 2E, inset ii).

14-3-3 Protein Binding Is Involved in Cell Localization of Rem2 and Suppression of p19ARF Expression—We found Rem2 in the cytoplasm and so investigated a role for 14-3-3 proteins
in mediating the Rem2 effects for three reasons. First, Rem2 and 14-3-3 proteins have been shown to physically interact in the cytoplasm (13, 15). Second, 14-3-3 proteins have been found to be associated with actively expressed gene promoters, suggesting a role for 14-3-3 proteins in gene regulation (23). Third, a small chemical inhibitor, LAF 389 (Novartis), inhibits 14-3-3/H9253, resulting in inhibition of HUVEC proliferation (24).

To investigate the cell localization of Rem2 further, we confirmed the work of others that 14-3-3/H9253 colocalizes with Rem2 in the cytoplasm of HUVEC cells (data not shown) (13). Next we semi-quantitatively assessed the intracellular localization of ectopic Rem2 wild type (WT), a Rem2 point mutant construct that does not bind 14-3-3 proteins or calmodulin (S58A/S323A/L306G), or a calmodulin (CaM) Rem2 point mutant (L306G) alone (Fig. 4A, inset i). These Rem2 point binding mutants have been demonstrated previously not to bind either 14-3-3 or CaM (kind gift from Walter Hunziker) (15). We found that 100% of HUVEC with ectopic WT Rem2 was cytoplasmic, where it is thought to be active, and that with the loss of binding to 14-3-3 proteins less than 25% of cells had exclusively cytoplasmic localization of Rem2, which had moved nuclear (Fig. 4A, inset ii). This data suggests that binding of Rem2 to 14-3-3 proteins is involved with the cytoplasmic localization of Rem2 in endothelial cells.

To assess if 14-3-3 proteins are important in the regulation of p19ARF by Rem2 we used the same 14-3-3 Rem2 point binding mutants used in the cell localization study described above, in a p19ARF luciferase promoter assay. We included a Rem2 14-3-3 point binding mutant alone (S58A/S323G), which we were unable to overexpress in primary HUVEC. We found that wild type Rem2 suppress the p19ARF luciferase promoter ~6-fold (Fig. 4B). The Rem2 14-3-3 point mutant partially released the suppressive ability of Rem2 on p19ARF expression (Fig. 4B) to 50% of WT Rem2, suggesting a role for 14-3-3 protein binding in mediating the effects of Rem2 on transcription of p19ARF in the nucleus. The CaM mutant had no effect compared with WT Rem2. We repeated this experiment in primary HUVEC with similar results (data not shown).
ing of 14-3-3 proteins to Rem2 is not only important in the cell localization of Rem2 but on the ability of Rem2 to suppress p19ARF expression.

To further assess the role of 14-3-3 expression in the Rem2 effects on proliferation, we used shRNAi methodology, and knocked down 14-3-3γ in a Rem2-expressing mouse endothelial cell clone (Fig. 3C). Loss of 14-3-3γ in Rem2 expressing endothelial cells resulted in reduction of Rem2-induced proliferation and, importantly, return of p19ARF protein levels (Fig. 4C, insets i and ii). This data demonstrates that the availability of 14-3-3γ to Rem2 plays an important role in the suppression of p19ARF expression and subsequent endothelial cell proliferation.

Rem2 Promotes Angiogenesis and Is Up-regulated in Endothelial Cells of RIP1 Tag2 Disease—To test whether Rem2 ectopic expression regulates angiogenesis we tested a number of known models of angiogenesis. First, we used an in vitro Matrigel tube formation assay. Ectopic expression of Rem2 was found to increase tube formation 2-fold over control p53 null endothelial cells (Fig. 5A). As we found that ectopic Rem2 can promote tube formation, we tested the ability of Rem2 to promote migration of endothelial cells, another important step in tube formation. We found by scratch assay that Rem2 expressing mouse endothelial cell clones promoted migration of endothelial cells into the wound compared with controls (Fig. 5B). Moreover, overexpression of p19ARF in a Rem2 mouse endothelial cell clone reduced the migration into the wound, underscoring a role for p19ARF in regulating the migration effects of ectopic Rem2 (data not shown). This suggests that Rem2 may have multiple functions in promoting angiogenesis by promoting proliferation and migration of endothelial cells.
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To further test the question of Rem2 expression in vivo, we developed an antibody to Rem2 (see supplementary information, Fig. S4). We examined the physiological and pathological expression of Rem2 in an established mouse model of tumor angiogenesis, the RIP1 tag2 mouse model of pancreatic disease (25). Remarkably, we found that Rem2 is not expressed or lowly expressed in endothelial cells of blood vessels of normal stage 1 pancreas and is up-regulated in the cytoplasm of endothelial cells of disease-associated vessels (Fig. 5C). We found that ~2.6% of CD31 positive vessels are Rem2 positive (low levels of expression) in normal pancreatic β islet tissue (Fig. 5C, inset ii). However, in stage IV disease this increased 3-fold to 8.4% Rem2 positive vessels (high levels of expression). This was statistically significant (p = 0.012).

DISCUSSION

We have reasoned that an increase in blood vessel mass (angiogenesis) requires an increase in endothelial cell proliferation and to this we screened genome-wide for genes that regulate endothelial cell proliferation. We identified Rem2, a new family of Ras-like GTPases, which suppresses the expression of p19ARF in mouse endothelial cells (p14ARF in humans) to promote endothelial cell proliferation. Interestingly, we found that Rem2 also causes an increase in endothelial cell migration, suggesting that Rem2 has multiple roles in the formation of new blood vessels from a pre-existing blood supply. Indeed a role for Ras-like GTPases in angiogenesis has been found before in which another family member, Gem, regulates endothelial cell shape and promotes angiogenesis (14). This data suggests that the Ras-like family of RGK GTPases may function in regulation of angiogenesis.

A previous role for 14-3-3 proteins in regulation of p53 has been demonstrated by regulation of MDMX proteins and may place Rem2 at another level of regulation within the p53 pathway (26). We have found that ectopic Rem2 is mainly cytoplasmic and needs to bind 14-3-3 proteins to remain cytoplasmic or moves back to the nucleus, where it is presumed Rem2 is inactive. We have seen using an antibody to Rem2 that, in normal cycling HUVEC in vitro, endogenous Rem2 is lowly expressed and is almost exclusively nuclear (data not shown). In mouse endothelial cells, we also found very low levels of Rem2 protein in cycling cells (Fig. S4, p53 null cells). The loss of binding of Rem2 to 14-3-3 and subsequent re-location of Rem2 to the nucleus supports the idea that interaction between Rem2 and 14-3-3 proteins in the cytoplasm are involved in mediating the effects of Rem2 on transcription of p14ARF/p19ARF in the nucleus, we have observed (Fig. 4, A and B). To support this novel idea, we show that loss of 14-3-3γ expression by RNAi in Rem2 expressing endothelial cells results in return of p19ARF expression, further underscoring a role for 14-3-3γ in mediating the effects of Rem2 on p19ARF expression (Fig. 4C).

Taken together, this data supports the notion that 14-3-3γ proteins play a role in mediating the effects of ectopic Rem2 on p19ARF expression. However, it is important to note that the effects of the Rem2 point mutant that does not bind 14-3-3γ on the p19ARF promoter seen in our study are not fully released and so cannot rule out other possible mechanisms. Another plausible mechanism of Rem2 suppression of p14ARF/p19ARF expression could be the GTPase activity of Rem2 and this remains to be tested.

We aimed to test if Rem2 has a role in angiogenesis. Using two established assays, we found that Rem2 can promote angiogenesis and suggests that Rem2 has multiple roles, such as migration, aside to proliferation alone. Further tests to see if Rem2 affects other steps in the cascade of events leading to new vessel formation, such as apoptosis, vessel maturation, and pericyte recruitment needs to be investigated. Moreover, it is possible that the role of Rem2 in angiogenesis is via the p53-independent functions of p19ARF, which has been previously suggested in the literature (6, 7). The use of other angiogenesis models such as the CAM assay, aortic ring assay, or mouse models is warranted to fully understand the role of Rem2 in physiological and pathological angiogenesis.

Work in this direction that we have performed, using the RIP1 tag2 model of pancreatic disease, suggests that increased levels of Rem2 has a role in pathological angiogenesis (Fig. 5C). The low levels of endogenous Rem2 that we have detected (Fig. 3C, last panel, and supplemental Fig. S4, p53 null cells) in normal cycling or resting endothelial cells (in vitro) compared with the up-regulation of Rem2 protein in endothelial cells of diseased vessels (Fig. 5C) suggested that angiogenic growth factors regulate Rem2 to activate aspects of the angiogenic cascade to form new vessels from a pre-existing circulation. Our preliminary work indicates that Rem2 is regulated by angiogenic growth factors suggesting that Rem2 is an inducible gene (data not shown). Endogenous Rem2 levels may play some role in maintaining normal...
Small G Protein Rem2 Suppresses p19ARF Expression

cell cycle; however, overexpression of Rem2 (possibly by angiogenic growth factors) activates Rem2 to accelerate endothelial cell proliferation, migration, and angiogenesis (Fig. 6). The development and fine-tuning of Rem2 antibodies may provide further insights into the prognostic role of Rem2 in pathological angiogenesis as well as further molecular insights at the chromatin level of gene expression.

We propose that ectopic Rem2 suppresses p19ARF gene expression, involving the binding of 14-3-3ε proteins, leading to increased endothelial cell proliferation. The subsequent increase in proliferation and migration induced by Rem2 then supports an angiogenic phenotype (Fig. 6).

In conclusion, this work has defined a new functional role for Rem2 GTPase. The suppression of p19ARF by Rem2 places it upstream in the p53 pathway. p53 induced cell cycle arrest leading to cellular senescence may play a role in some vascular diseases. Bypass of senescence of endothelial cells may therefore have a clinical significance. Future development of this novel finding through the development of specific chemical inhibitors of Rem2 activity may pave the way for better treatment strategies for disease dependent on accelerated endothelial cell proliferation.

Acknowledgments—We thank Walter Hunziker and Pascal Beguin for reagents and John Collard for useful discussions and advice; Beleña Silva for the 14-3-3ε construct. Assistance; and Imma Hernandez-Munoz for the shRNAi GFP construct.

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