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RESEARCH ARTICLE

MiR-4674 regulates angiogenesis in tissue injury by targeting p38K signaling in endothelial cells

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Icli B, Li H, Pérez-Cremades D, Wu W, Ozdemir D, Haemmig S, Guimaraes RB, Manica A, Marchini JF, Orgill DP, Feinberg MW. MiR-4674 regulates angiogenesis in tissue injury by targeting p38K signaling in endothelial cells. *Am J Physiol Cell Physiol* 318: C524–C535, 2020. First published January 8, 2020; doi:10.1152/ajpcell.00542.2019.—Neoangiogenesis is critical for tissue repair in response to injury such as myocardial ischemia or dermal wound healing. MicroRNAs are small noncoding RNAs and important regulators of angiogenesis under physiological and pathological disease states. Therefore, identification of microRNAs that may restore impaired angiogenesis in response to tissue injury may provide new targets for therapy. Using a microRNA microarray profiling approach, we identified a human-specific microRNA, miR-4674, that was significantly decreased in patients after myocardial tissue injury and had an endothelial cell (EC)-enriched expression pattern. Functionally, overexpression of miR-4674 markedly attenuated EC proliferation, migration, network tube formation, and spheroid sprouting, whereas blockade of miR-4674 had the opposite effects. Transcriptomic profiling, gene set enrichment analyses, bioinformatics, 3′-untranslated region (3'-UTR) reporter and microribonucleoprotein immunoprecipitation (miRNP-IP) assays, and small interfering RNA dependency studies revealed that miR-4674 regulates VEGF stimulated-p38 mitogen-activated protein kinase (MAPK) signaling and targets interleukin 1 receptor-associated kinase 1 (Irak1) and BICD cargo adaptor 2 (Bicd2) in ECs. Furthermore, Irak1 and Bicd2 were necessary for miR-4674-driven EC proliferation and migration. Finally, neutralization of miR-4674 increased angiogenesis, Irak1 and Bicd2 expression, and p38 phosphorylation in human skin organoids as a model of tissue injury. Collectively, targeting miR-4674 may provide a novel therapeutic target for tissue repair in pathological disease states associated with impaired angiogenesis.

angiogenesis; endothelial cells; microRNAs; p38 signaling; tissue repair

INTRODUCTION

In response to tissue injury, the initiation and orchestration of angiogenesis are critical for optimal tissue repair (45). Impaired proangiogenic signaling pathways can adversely impact endothelial cell (EC) proliferation and migration, and, in turn, the generation of a new vascular network. Indeed, diminished endothelial angiogenic responses have been linked to a range of ischemic cardiovascular diseases, such as myocardial infarction, peripheral artery disease, and dermal wound healing (45).

To improve perfusion of injured or ischemic tissues, therapeutic angiogenesis aims to increase the formation of new blood vessels, often using proangiogenic growth factors in various vectors, to promote reparative responses (8, 14, 17, 37, 38). Unfortunately, clinical trials using these growth factors to improve outcomes and tissue repair have failed so far to translate this approach into clinical benefits for patients (31, 33). While the lack of therapeutic success has been ascribed to diverse reasons, such as heterogeneity of patient cohorts, dosing, and delivery routes (31), accumulating studies indicate that these growth factors are not actually deficient, but rather their signaling pathways are impaired (4, 10, 25, 30, 32, 48).

For example, proangiogenic growth factors (VEGF-A, Tie2, or angiopoietin-2) were expressed higher in patients with coronary or peripheral artery disease (PAD) compared with healthy controls (10, 25, 44). Notably, subjects with critical limb ischemia had the highest levels of these proangiogenic circulating factors compared with those with intermittent claudication or without PAD (19). Therefore, identification of mediators that impact impaired downstream signaling may provide opportunities to overcome "angiogenic resistance" in an analogous manner as therapeutics that overcome defects in insulin signaling observed in subjects with insulin resistance and diabetes.

Activation of a broad range of canonical proangiogenic signaling pathways is achieved not only through traditional ligation of tyrosine kinase receptors (e.g., VEGFR1/2), but also via cross talk of downstream effectors that impact expression of signal transduction pathways, such as Akt/endothelial nitric oxide synthase (eNOS), or mitogen-activated protein kinase

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(MAPK)/extracellular signal-regulated kinases (ERK) pathways, that regulate proliferation, migration, and sprouting of endothelial cells (35). Indeed, endothelial-specific Akt/eNOS deficiency impaired blood recovery after hindlimb ischemia (40), and inhibition of p38 MAPK signaling reduced ischemia-reperfusion-induced coronary collateral growth (46), highlighting their proangiogenic roles. Thus, strategies to regulate specific downstream pathways of proangiogenic factors may improve maladaptive tissue repair and ischemic cardiovascular states.

MicroRNAs (miRNAs) are 20–22 nt long, single-strand noncoding RNAs involved in gene expression at the posttranscriptional level by base pairing with their target mRNAs and have been related to pathophysiological processes, including angiogenic responses. A growing number of miRNAs have been identified as regulators of angiogenesis through distinct mechanisms (13, 34). The ability of a few of these miRNAs, such as miR-26a, miR-135a-3p, miR-503, or miR-615-5p, to impede angiogenesis has been attributed to their ability to suppress downstream effectors of growth factor signaling in ECs (11, 12, 18, 20, 30, 55). Therefore, identification of new miRNAs that regulate angiogenic pathways by targeting downstream effectors of growth factor signaling in ECs may provide a novel therapeutic approach to improve tissue repair and ischemic disease states.

In this report, we identified the human-specific miR-4674 as a new regulator of angiogenesis by targeting a VEGF-interleukin 1 receptor-associated kinase 1 (IRAK1)/BICD cargo adaptor 2 (BICD2)-p38K signaling pathway. Collectively, these findings establish a new strategy to overcome impaired angiogenic signaling in response to tissue injury or ischemic disease states.

METHODS

Cell culture and transfection. Human umbilical vein endothelial cells (HUVECs) were cultured in growth media EGM-2 (Lonza, Walkersville, MD). Cells passaged fewer than five times were used for all experiments. For transfection studies, HUVECs were cultured overnight before being transfected with Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA). For reporter studies, HUVECs were transfected with 400 ng of the indicated reporter constructs. Each reading of luciferase activity was normalized to total protein read for the same lysate.

Real-time qPCR. HUVECs and human organoids were suspended in TRIzol reagent (Invitrogen) and total RNA and microRNA were isolated per manufacturers' instructions. Human skin and plasma were isolated using the Total RNA Purification Kit (Norgen Biotek) per the manufacturers' instructions. Reverse transcriptions were performed by using miScript reverse transcription kit from Qiagen (218061). Either QuantiTect SYBR Green RT-PCR Kit (204243) or miScript SYBR Green PCR Kit (218073) from Qiagen was used for quantitative real-time PCR analysis with the AriaMx real-time PCR system (Agilent Technologies) following the manufacturer's instructions. Gene-specific primers (Invitrogen) were used to detect human IRAK1 and BICD2. Samples were normalized to endogenous human GAPDH. To amplify mature miRNA sequences, miScript primer assays for Hs_miR-4674_1 (MS00045045) from Qiagen were used. Samples were normalized to endogenous U6 RNA (MS00033740). Fold changes were calculated by ΔΔCt method. Significance was determined by Student's two-tailed *t* test, $P < 0.05$.

Western blot analysis. HUVECs transfected with miR-4674 mimic, miR-4674 inhibitor, nonspecific miRNA controls, IRAK1 siRNA (L-004760-00-0005; Dharmacon), BICD2 siRNA (L-014060-00-0005, Dharmacon), or control siRNA were cultured for 72 h. Human organoids transfected with miR-4674 inhibitor or nonspecific miRNA controls were cultured for 72 h, treated with VEGF accordingly, and homogenized at 30/s for 5 min using Tissuelyser II (Qiagen). Total cellular protein was isolated by RIPA buffer (50 mM Tris·HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche). Cell or tissue debris was removed by centrifugation at 12,500 rpm for 15 min. Protein quantification was performed using the BCA kit (Thermo Scientific). Lysates were separated using 5–14% Mini-PROTEAN TGX Precast Gels (Bio-Rad), transferred to PVDF membranes, and subjected to Western blot analysis using antibodies against IRAK1 (MA515783, Invitrogen), BICD2 (MA523522, Invitrogen), P-AKT (4060L), Pan AKT (2920S), P-p44/42 MAPK (9101L), P44/42 MAPK (4696S), P-p38 MAPK (9215L), p38 MAPK (9212L), GADPH (2118L), and β-actin (3700S), all from Cell Signaling Technology (Danvers, MA). HRP-conjugated goat anti-rabbit or mouse antibody (Santa Cruz Biotechnology) was used at 1:2,000 dilution. ECL assay was performed per the manufacturer's instructions (RPN2132; GE Healthcare). Three or more biological replicates were performed for each experiment. Significance was determined by Student's two-tailed *t* test, $P < 0.05$.

BrdU assay. HUVECs transfected with miR-4674 mimic, miR-4674 inhibitor, or nonspecific negative controls were cultured for 5 days. Cell proliferation was measured using the BrdU ELISA assay as described by the manufacturer (Roche).

Cord formation on Matrigel (in vitro). Matrigel (BD Biosciences, San Jose, CA) basement membrane matrix was added to 96-well culture plates and incubated at 37°C until gelation occurred. Matrigel was not supplemented with additional growth factors. HUVECs transfected with miR-4674 mimic, miR-4674 inhibitor, and nonspecific negative controls were cultured at 20,000 cells/well. Network tube formation was assessed 6–10 h postplating and quantitated by counting the number of tubes formed per high-power field.

Chemotaxis assay. Migration assay was performed using ChemTX multiwell system (Neuro Probe) with 5-mm pore size and 96-well format. HUVECs transfected with miR-4674 mimic, miR-4674 inhibitor, or nonspecific negative controls were cultured for 72 h before being plated on the upper compartment of the multiwell plate to assess migration. Lower compartments were filled with EBM-2 medium containing VEGF (R&D Systems). The number of cells migrating to the lower chamber was counted using a hemocytometer after 16 h.

Scratch assay for EC migration. Scratch wound assay was performed using culture-insert 2 well 35-mm μ-dishes (ibidi). HUVECs transfected with miR-4674 mimic, miR-4674 inhibitor, nonspecific miRNA controls, IRAK1 siRNA, BICD2 siRNA, or control siRNA were cultured for 60 h in 12-well plates and plated at 21,000 cells per well into the μ-dishes. Inserts were lifted at 72 h after transfection, and cells were imaged using an Eclipse TE2000-U inverted microscope (Nikon) at ×2 and ×4 over time to assess for wound closure. Three technical replicates were performed per condition. Significance was determined by Student's two-tailed *t*-test, $P < 0.05$.

EC spheroid sprouting assay. Transfected HUVECs with miR-4674 mimic, miR-4674 inhibitor, or nonspecific miRNA controls were cultured overnight in hanging drops on nonadherent plastic dishes in EBM-2 (Lonza) medium with 0.2% methylcellulose (Sigma-Aldrich) using 1,000 cells/spheroid. Spheroids were embedded in a collagen matrix and incubated for 24 h. Number of sprouts and total sprout length of 10 spheroids per condition were used for data analysis by using NIH ImageJ software.

MiRNP immunoprecipitation. Myc-tagged Ago-2 (a kind gift from G. Hannon, Cold Spring Harbor, NY) was cotransfected with either miR-4674 mimic or miRNA negative control in HUVECs. Cells were washed in ice-cold PBS, released by scraping, and lysed in buffer (10 mM Tris·HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 100 U/mL of RNasin Plus (Promega) supplemented with 1× protease inhibitor (Roche). The lysed cell solution was adjusted to a

final NaCl concentration of 150 mM before centrifugation. One-twentieth of the supernatant volume was collected in TRIzol for use as an extract control. The remaining portion of the supernatant was precleared with Protein A/G UltraLink Resin (Pierce), to which 2 μg anti-c-myc antibody was added, and the mixture allowed to incubate overnight at 4°C; the following day protein A/G UltraLink resin was added. After 4 h of mechanical rotation at 4°C, the agarose beads were pelleted and washed four times in wash buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl, 0.05% and Triton X-100). Finally, 1 mL of TRIzol was added into the beads and RNA was isolated. Total RNA was reverse transcribed into cDNA for real-time qPCR analysis.

Microarray transcriptomic profiling and bioinformatics. For DNA microarray gene chip analysis, HUVECs were transfected with 30 nM miRNA negative control or miR-4674 mimics for 24 h. Cells were collected into RNeasy mini kit (Qiagen) and sent for two-color, 4 × 44 K format (Agilent Technologies) human whole genome oligo microarray service (ArrayStar). Differentially expressed genes that were identified as being at least 1.5-fold repressed ($P < 0.05$) were subjected to gene set enrichment analyses. Gene Ontology (GOs) pathways were explored to determine whether a known biological network, process, or molecular function was suppressed by overexpression of miR-4674 [Ingenuity Pathway Analysis (IPA; Qiagen)]. The top 10 GOs for molecular and cellular functions and the top 10 GOs for signaling networks were ranked using *P* value or score, respectively.

Circulating miR-4674 levels in patients with myocardial ischemia. Patient plasma samples were collected in EDTA-containing tubes as part of a prospectively enrolled cohort of patients that underwent either cardiac catheterization in accordance with the Institutional Review Board-approved protocols at Brigham and Women's Hospital or at the Ethics Committee in Research at the Instituto de Cardiologia, Fundação Universitária de Cardiologia, Porto Alegre, Brazil. Written informed consent was obtained from all participants or their appropriate surrogates. Control patients were defined as without clinically significant coronary atherosclerosis (<20% stenosis in any epicardial coronary artery determined by angiography) and had no elevation of cardiac biomarkers. Patients with myocardial ischemia were defined as acute atherothrombotic coronary artery occlusion resulting in either an NSTEMI (with >70% occlusion of an epicardial artery) or a STEMI (complete occlusion of an epicardial coronary artery determined by angiography) with elevation of cardiac biomarkers. Anonymized plasma samples were generated from blood collected in EDTA-containing tubes at the time of the procedure and stored at −80°C. Plasma was isolated from whole blood, and skin samples were homogenized for respective RT-quantitative PCR (qPCR) analyses. Plasma was isolated from whole blood at 1,500 *g* for 15 min at room temperature. 60% of the upper phase was collected. Samples that were red in color were excluded from the analyses, and only clear light yellow plasma samples were utilized for RNA extraction. Total RNA was isolated from plasma by using total RNA purification kit from Norgen Biotek (16, 52), and reverse transcription and real-time qPCR was performed as described in the section on "*Real-time qPCR*."

Generation of human skin organoids. Discarded human skin specimens were obtained after informed consent, in accordance with the Institutional Review Board-approved protocols at Brigham and Women's Hospital. Full-thickness circular (6-mm) human skin organoids were taken from surgical skin samples and 3-mm full-thickness wounds were created as described previously (6). Briefly, human skin organoids were then embedded in collagen I matrix and maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10-mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 50 μg/mL ascorbic acid, 100 μm adenine, 0.5 μm hydrocortisone, 0.1 nm cholera toxin, 100 μU/mL penicillin, and 10 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO). The ex vivo organ cultures were cultured at the air-liquid interface and maintained in the cell culture incubator at 37°C with 5% CO_2. The media were changed every other day. The viability of cultured explants was validated by histologic evaluation. Human skin organoids were transfected at the indicated time points using 30 nM miR-4674 mimics or nonspecific controls or 100 nM miR-4674 inhibitors or nonspecific controls. Neovascularization were measured at *days 3* and *7* to determine miR-4674 effects on wound healing.

Statistical analysis. Data are presented as means ± SE. All experiments are representative of three independent experiments unless indicated otherwise. Sample sizes for human organoid studies were chosen on the basis of pilot studies or similar well-characterized studies in the literature. For two group comparisons, data were subjected to unpaired two-sided Student's *t* test if it passed normality and equal variance tests. If data for either normality or variance tests failed, then nonparametric Mann-Whitney U test was used. For two or more group comparisons, if data passed normality and variance testing, one-way ANOVA with Bonferroni correction was used. If data failed to pass normality or equal variance tests, then nonparametric Kruskal-Wallis test with Dunn post hoc test was used. $P < 0.05$ was considered statistically significant.

RESULTS

To identify microRNAs (miRNAs) that regulate angiogenesis in response to tissue injury, microRNA (miRNA) microarray profiling studies (30) were undertaken using plasma from human subjects with myocardial ischemia or from individuals with normal coronary angiograms. Patients with myocardial ischemia were identified as individuals with coronary angiograms showing >70% stenotic lesions compared with nonmyocardial ischemia human subjects with coronary angiograms with lesions <20% stenosis. MiR-4674 was identified as one of the most differentially regulated miRNAs noted with myocardial ischemia (Supplemental Fig. S1; all Supplemental Material can be found at https://doi.org/10.6084/m9.figshare.11553339). We explored whether miR-4674 expression was regulated in a larger cohort of human subjects with myocardial ischemia by real-time PCR (RT-PCR) analysis. As shown in Fig. 1*A*, circulating levels of miR-4674 decreased by 45% in myocardial ischemia subjects (males and females) with coronary angiograms bearing >70% stenotic lesions compared with human subjects with coronary angiograms with lesions <20% stenosis. Similar differences were observed in the males alone (~47% decrease) and females alone (~51% decrease). Upon further investigation (http://mirbase.org and http://useast.ensembl.org), we found that miR-4674 was expressed in the human genome but lacked mouse or rat homologs. In addition, miR-4674 expression was significantly higher in ECs compared with fibroblasts and keratinocytes (Fig. 1*B*). In summary, these data suggest that miR-4674 is a human-specific miRNA highly expressed in ECs, and its expression may correlate with tissue injury.

Because miR-4674 expression was enriched in endothelial cells, we explored its functional role on EC growth by gain- and loss-of-function experiments. Overexpression of miR-4674 "mimics" (miR-4674$_m$) in HUVECs inhibited cell growth by 58%, whereas miR-4674 inhibitors (miR-4674$_i$, complementary antagonist) increased EC growth by 3.2-fold (Fig. 1*C*). Interestingly, miR-4674 expression was not regulated by VEGF stimulation in ECs (Supplemental Fig. S3*A*). MiR-4674 overexpression decreased EC migration by 19%, compared with the nonspecific (NS) control, whereas miR-4674 inhibition increased migration by 21% compared with the NS control (Fig. 2*A*). ECs overexpressing miR-4674 decreased wound closure in scratch assays by 58%, whereas inhibition of miR-4674 increased wound closure compared with the NS control

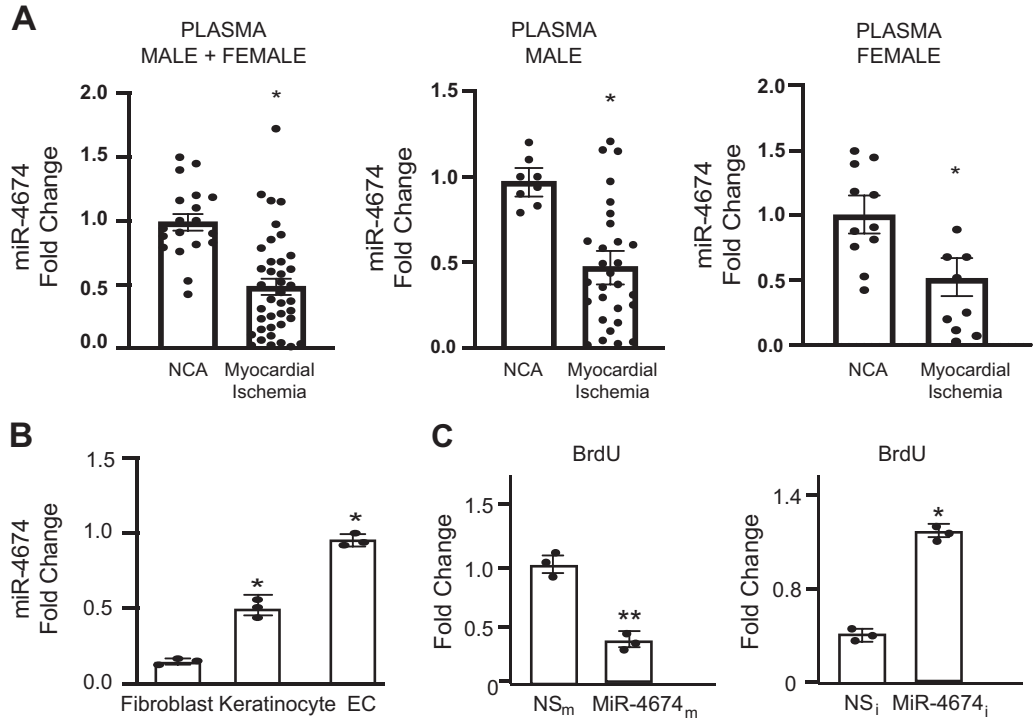

Fig. 1. Identification of miR-4674 as a regulator of endothelial cell growth. *A*: circulating miR-4674 levels are increased in plasma from human subjects with myocardial ischemia ($n = 40$; 28 male, 12 female) compared with subjects with normal coronary angiograms (NCA) ($n = 20$; 9 male, 11 female). $^*P < 0.05$ compared with normal coronary angiogram. *B*: expression of miR-4674 in endothelial cells (ECs), keratinocytes, and fibroblasts. *C*: human umbilical vein endothelial cells (HUVECs) transfected with miR negative control (NS_m), miR-4674 mimics (miR-4674_m), miR inhibitor negative control (NS_i), or miR-4674 inhibitor (miR-4674_i) were subjected to BrdU cell proliferation assay. $^*P < 0.05$, $^{**}P < 0.01$ compared with controls. Data are representative of $n = 3$ experiments. All data represent means ± SE.

by 36% (Fig. 2*B*). Ectopic miR-4674 expression at the time of the scratch assay remained comparable to the miR-4674 expression over the course of the scratch assay (Supplemental Fig. S3*B*). Vascular network formation assays in Matrigel revealed that overexpression of miR-4674 inhibited cord formation in Matrigel in vitro (Fig. 2*C*, *left*) by ~60%, whereas miR-4674 inhibition increased tube formation in Matrigel in vitro (Fig. 2*C*, *right*) by 38%. Finally, in accordance with its effects on EC growth and migration, overexpression of miR-4674 decreased EC sprouting by ~71%, whereas inhibition of miR4674 increased sprouting by ~23% (Fig. 2*D*). Taken together, these data indicate that miR-4674 inhibited EC angiogenic functions in vitro.

To identify potential target signaling pathways of miR-4674, we performed transcriptomic profiling from HUVECs overexpressing miR-4674 followed by gene ontology (GO) analyses. In accordance with our EC in vitro findings, cell cycle regulation was predicted to be one of the top biological pathways to be regulated by miR-4674 (Fig. 3*A*). In addition, the p38 MAPK signaling pathway is predicted to be among the top regulated signaling pathways by miR-4674 in ECs (Fig. 3, *B* and *C*). On the basis of the GO analyses, we first verified that p38 phosphorylation was significantly reduced by up to ~55% in response to 10 and 15 min of VEGF stimulation in HUVECs overexpressing miR-4674 (Fig. 4*A*), whereas miR-4674 inhibition increased p38 phosphorylation by approximately twofold in response to 10 min of VEGF stimulation (Fig. 4*B*). This regulation was specific to p38 signaling and not to other signaling pathways, including AKT and ERK1/2 (Supplemental Fig. S2).

To identify a direct target of miR-4674 and narrow down the potential targets from the microarray transcriptomic profiling data, we took a rigorous, systematic approach, using a combination of bioinformatics and prediction algorithms (e.g., miRWalk, Micro T4, miRNAMAP, RNAhybrid, and TargetScan) and validation by expression on the mRNA and protein levels (Fig. 5*A*). From 109 genes repressed by twofold, 67 genes contained at least 1 potential binding site in the 3′-UTR, and 26 genes showed significantly decreased mRNA expression in HUVECs overexpressing miR-4674 (Fig. 5*A* and data not shown). Overexpression of miR-4674 in HUVECs decreased the mRNA (Fig. 5*B*), as well as the protein expression (Fig. 5*C*) and 3′-UTR activity (Fig. 5*D*) of *IRAK1* and *BICD2*. In contrast, inhibition of miR-4674 increased IRAK1 and BICD2 expression (Fig. 5, *B* and *C*). Overexpression of miR-4674 in ECs inhibited the activity of luciferase reporter constructs containing the IRAK1 and BICD2 3′-UTR by 36% and 21%, respectively; in contrast, inhibition of miR-4674 increased IRAK1 and BICD2 3′-UTR reporter activity by 41% and 38%, respectively (Fig. 5*D*). Additionally, we performed Argonaute2 (AGO2) microribonucleoprotein IP (miRNP-IP) studies to assess whether IRAK1 and BICD2 mRNA are enriched in the RNA-induced silencing complex following miR-4674 overexpression in HUVECs. An approximately three-fold enrichment of IRAK1 and a 1.35-fold enrichment of BICD2 mRNA were observed after AGO2 miRNP-IP in the presence

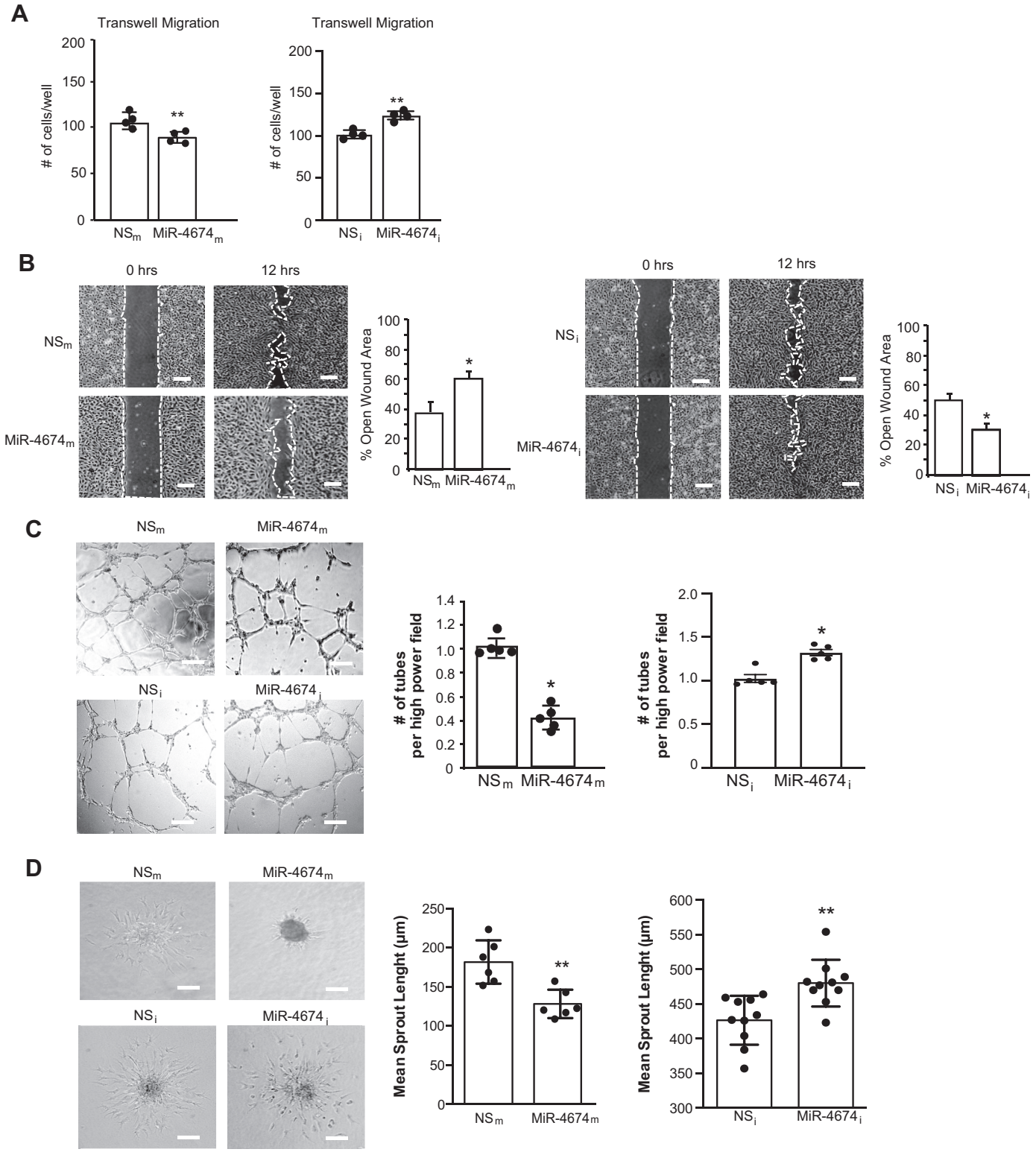

Fig. 2. MiR-4674 inhibits endothelial cell (EC) migration, network tube formation, and sprouting. HUVECs transfected with miR negative control (NS_m), miR-4674 mimics (miR-4674_m), miR inhibitor negative control (NS_i), or miR-4674 inhibitor (miR-4674_i) were subjected to EC migration in transwell Boyden chambers (*A*); scratch assay (*B*); tube-like network formation in Matrigel (*C*); and spheroid sprouting assay (*D*). $^*P < 0.05$, $^{**}P < 0.01$ compared with controls. Scale bars, 100 μm (*D*), 150 μm (*E*). Data are representative of $n = 3$ experiments. All data represent means ± SE.

A

Canonical Pathways	log(p-value)	# genes
Role of BRCA1 in DNA Damage Response	5.01	11
Role of CHK Proteins in Cell Cycle Checkpoint Control	4.69	9
Cell Cycle Regulation by BTG Family Proteins	3.36	6
Hereditary Breast Cancer Signalling	3.1	12
Ceramide Signaling	2.84	9
Acute Myeloid Leukemia Signaling	2.84	10
Cyclins and Cell Cycle Regulation	2.81	8
NFkB Signaling	2.75	13
Lymphotoxin B Receptor Signaling	2.66	7
Cell Cycle G1/S Checkpoint Regulation	2.66	7

B

Signaling Networks	score	# genes
ERK	51	32
Akt	48	31
FKRP	46	30
SWI/SNF	43	29
p38 MAPK	41	28
Jun	39	27
Smad	35	26
NFkB	33	25
MAPK	30	24
SMAD 1,5	27	23

C Top Regulated Signaling Network:p38

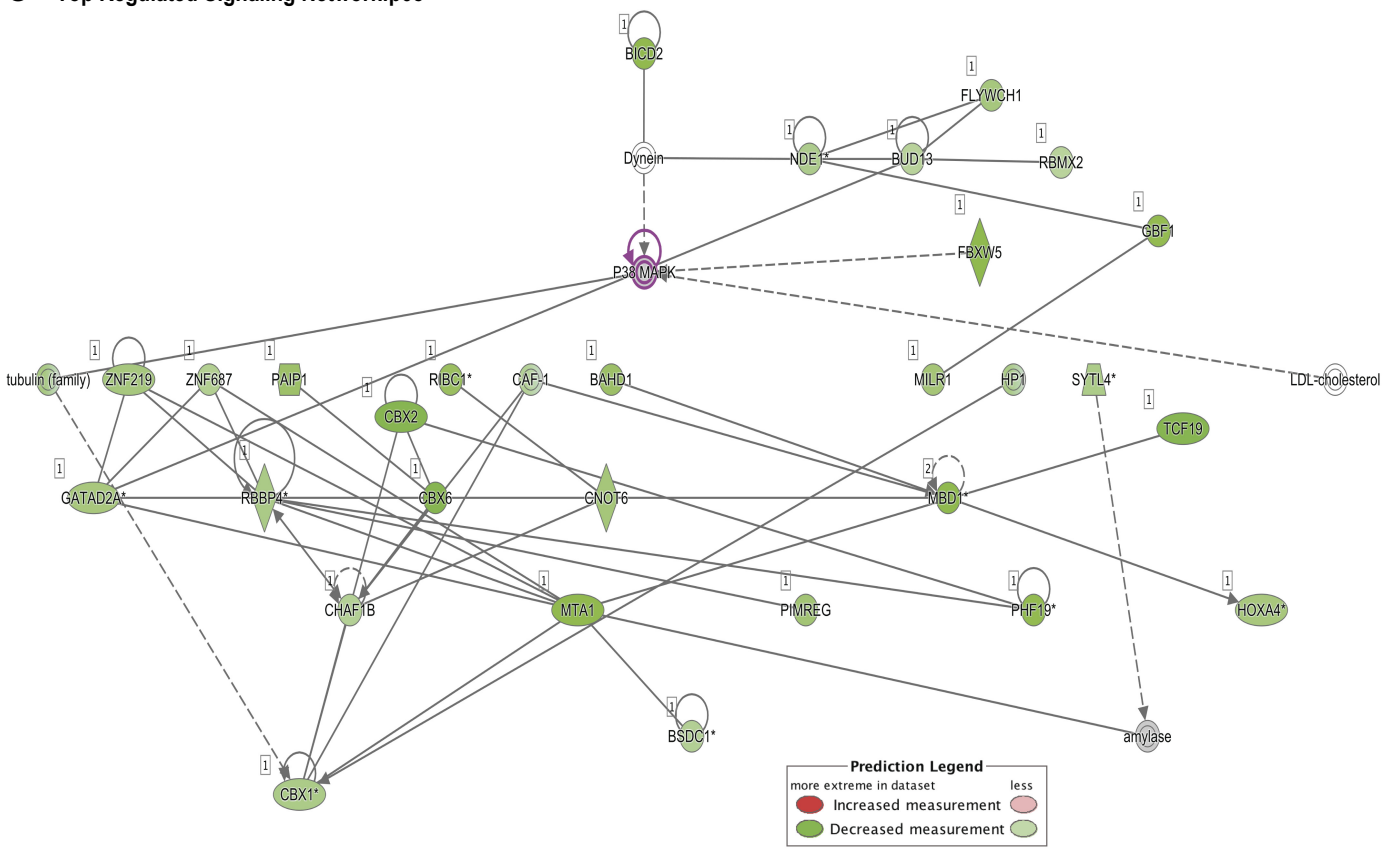

Fig. 3. Bioinformatics and miR-4674 gene profiling predict p38 as a top targeted signaling pathway. *A*: gene ontology analysis of 489 genes repressed by miR-4674 overexpression in endothelial cells (ECs) identified from transcriptomic profiling. *B* and *C*: the p38 signaling pathway is predicted to be among the top regulated signaling network regulated by miR-4674. GO, gene ontology.

of miR-4674, as compared with the miRNA-negative control. In contrast, AGO2 miRNP-IP did not enrich the mRNA for CBX6 or *SMAD1*, a gene that was not predicted to be a miR-4674 target (Fig. 5*E*).

To explore whether the targets IRAK1 and BICD2 "phenocopied" the angiogenic effects of miR-4674, we performed siRNA-mediated knockdown studies in ECs. siRNA knockdown of either IRAK1 (Fig. 6*A*) or BICD2 (Fig. 6*E*) significantly reduced EC proliferation in BrdU assays (Fig. 6, *B* and *F*) and scratch assays (Fig. 6, *C* and *G*). To explore whether the miR-4674-mediated inhibitory effects on EC proliferation were dependent on IRAK1 or BICD2, we performed siRNA knockdown studies using EC scratch assays and quantified EC wound closure. Functionally, in the absence of IRAK1 (Fig. 6*D*) or BICD2 (Fig. 6*H*), miR-4674 overexpression had markedly impaired ability to inhibit EC wound closure, showing that the miR-4674-mediated effects are dependent in part on IRAK1 and BICD2. Concurrent siRNA knockdown of IRAK1 and BICD2 did not show a cooperative effect of BrdU cell proliferation (Supplemental Fig. S4), suggesting that these may serve as redundant targets for miR-4674. Collectively, these data indicate that IRAK1 and BICD2 are bona fide targets of miR-4674 in ECs, and implicate that miR-4674 may serve as a molecular switch, whereby increased levels of miR-4674 reduce IRAK1 and BICD2 expression, thereby suppressing endothelial cell growth and angiogenesis.

Because miR-4674 is a human-specific miRNA, this precluded exploring its functional role in murine models of tissue injury. However, to evaluate whether neutralization of miR-4674 may regulate angiogenesis in human tissues, we developed a modified human skin organoid assay, in which a 6-mm circular full-thickness punch biopsy is generated. In the middle

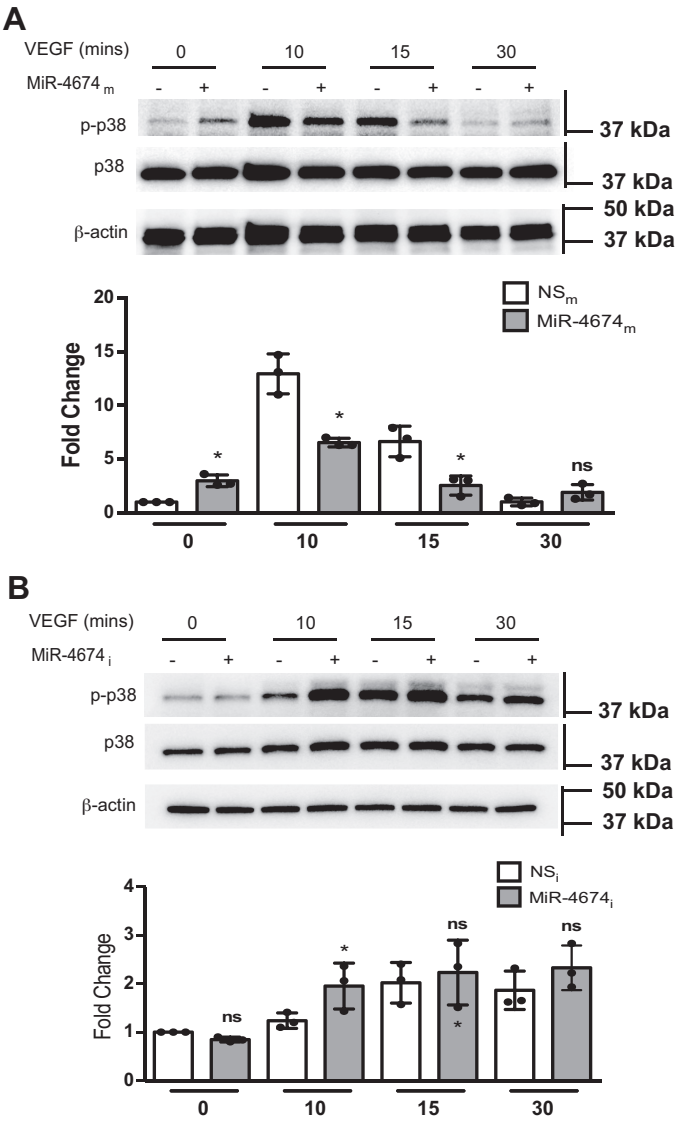

Fig. 4. MiR-4674 regulates the expression of downstream p38 signaling in endothelial cells (ECs). Human umbilical vein endothelial cells (HUVECs) transfected with miR negative control (NS_m) or miR-4674 mimics (miR-4674_m) (*A*) or miR inhibitor negative control (NS_i) or miR-4674 inhibitor (miR-4674_i) (*B*) and stimulated with vascular endothelial growth factor (VEGF) for indicated durations were subjected to Western analysis using antibodies to p-p38, p38, and β-actin ($n = 3$ to 5 experiments). P-p38 expression was normalized to both p38 and β-actin. All data represent means ± SE. *$P < 0.05$ compared with controls; ns, nonsignificant.

of each biopsy a 3-mm full-thickness wound is created using a 3-mm punch biopsy, which can be maintained at the air-liquid interface in culture for several days (Fig. 7*A*) (6). Human skin organoids can be transduced by microRNAs, as we have shown previously using a NS-Cy3 control (30). To explore whether miR-4674 also regulated angiogenesis in human skin organoids, we transduced them with anti-miR-4674 (miR-4674_i) or scrambled nonspecific control antimiRs (NS_i) (Fig. 7*A*). Remarkably, inhibition of miR-4674 induced angiogenesis, as measured by CD31 by ~1.9 fold (Fig. 7*B*). Additionally, we investigated the downstream effects of miR-4674 neutralization on IRAK1 or BICD2 in human skin organoids. In accordance with the in vitro findings in ECs, miR-4674 inhibitor significantly increased IRAK1 and BICD2 expression by 2.3- and 2.8-fold, respectively (Fig. 7*C*). Finally, inhibition of miR-4674 increased p38 phosphorylation by ~1.4-fold (Fig. 7*D*). Collectively, these data indicate that miR-4674 neutralization can promote angiogenesis in human tissues.

DISCUSSION

Angiogenesis is vital for many physiological processes and intimately involved in tissue repair and recovery following injury, resulting from pathological conditions, such as ischemic cardiovascular diseases and wound healing. Defects in this process can lead to maladaptive or delayed tissue recovery. Impaired EC function involving processes, such as EC proliferation, migration, lumen formation, and formation of new basement membranes, is a major obstacle in therapeutic angiogenesis (5, 21, 22, 45, 49). Delivery of recombinant growth factors, such as VEGF and a diverse set of molecules that can modestly or indirectly target angiogenic signaling pathways, such as free radical scavengers (22a, 23a, 24), receptors, such as angiotensin receptor (47), or angiopoetin 1 (Ang1) (36), and several others (9, 53), have shown limited efficacy with transitory effects in the attempt to promote angiogenesis in clinical settings (41). Studies of subjects with ischemic cardiovascular disease states support the notion of "angiogenic resistance" (25, 44). For example, human subjects with higher ischemic burden have higher circulating levels of proangiogenic growth factors (19), and there is markedly reduced activation of AKT phosphorylation (23) despite the presence of sufficient VEGF available to activate the downstream signaling pathways.

Accumulating studies demonstrate that targeting downstream effectors of VEGF signaling pathways may provide an alternative strategy to activate angiogenic functional responses. For example, the miRNAs miR-135a-3p or miR-615-5p targeted VEGF-p38 or VEGF-AKT/eNOS signaling pathways, respectively, resulting in improved angiogenesis in the dorsal skin wounds of diabetic mice and in human skin organoids (29, 30). Collectively, these studies highlight that defective angiogenesis may be improved by focusing on approaches to augment defects in downstream signaling of proangiogenic growth factors. Here, we identified a new miRNA, miR-4674, acting as a negative modulator of angiogenesis by regulating the VEGF-p38 signaling pathway through targeting the downstream genes *IRAK1* and *BICD2* (Supplemental Fig. S5). Interestingly, miR-4674 only has a human homolog, precluding its evaluation in experimental murine models. However, in human skin organoids as a model of tissue injury, inhibition of miR-4674 markedly increased angiogenesis and p38 signaling (Fig. 7). Endothelial cell proliferation and migration are critical steps in angiogenesis. The p38 signaling pathway has been implicated to play a role in EC proliferation, migration, and survival (30, 43, 54). Here, we show that miR-4674 regulates EC growth and angiogenesis through the selective regulation of VEGF-induced p38 pathway and not the AKT or ERK pathways (Figs. 3 and 4 and Supplemental Fig. S2) and is consistent with these previously documented findings.

We used an unbiased transcriptomic profiling approach in combination with bioinformatics to identify and validate miR-4674 target genes. After filtering 119 genes repressed by at least twofold by miR-4674 in transcriptomic profiling, only two target genes, *IRAK1* and *BICD2*, were verified by expres-

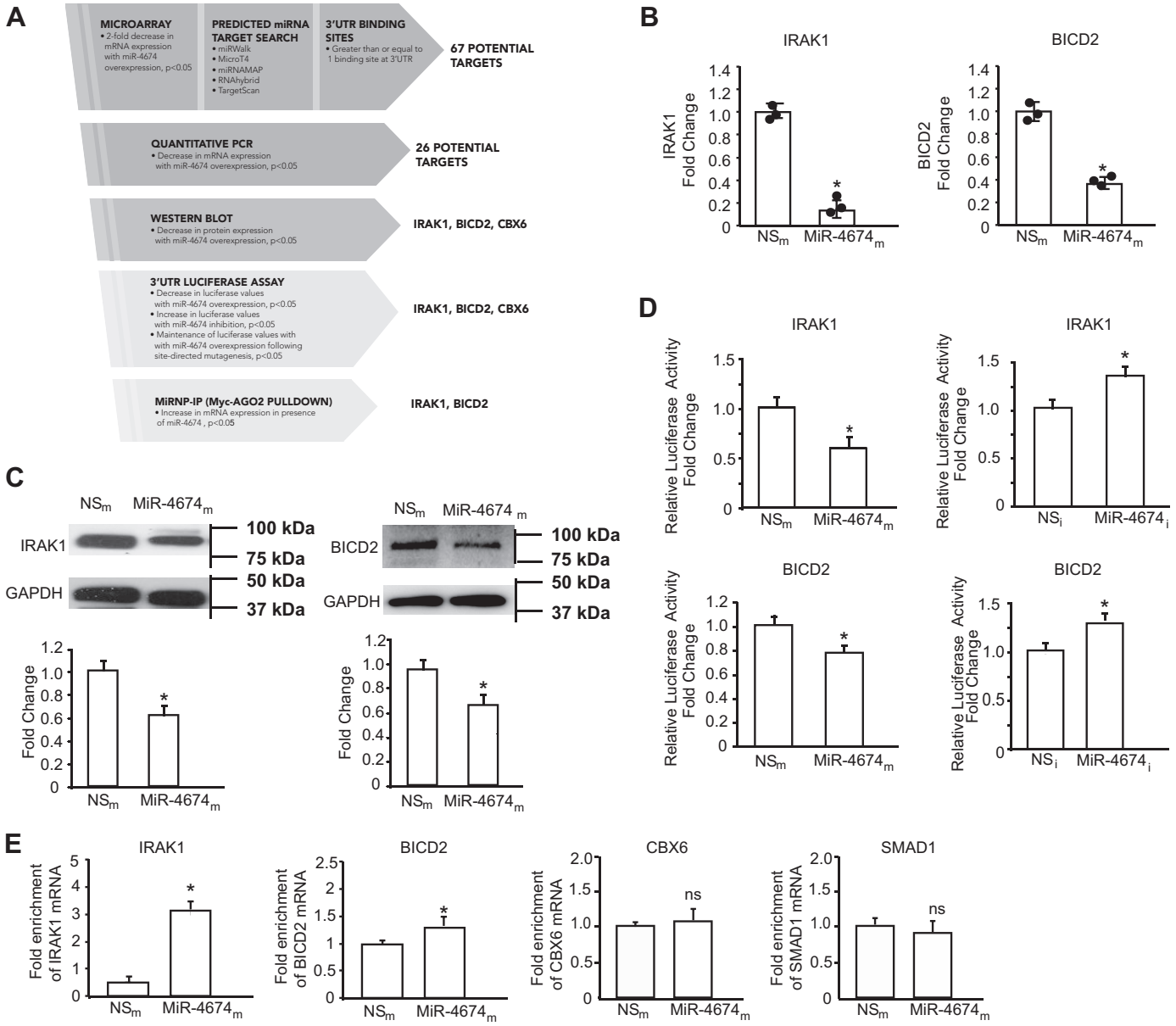

Fig. 5. Interleukin 1 receptor-associated kinase 1 (IRAK1) and BICD cargo adaptor 2 (BICD2) are bona fide targets of miR-4674 in endothelial cells (ECs). *A*: discovery and validation of miR-4674 target genes. Human umbilical vein endothelial cells (HUVECs) transfected with miR-negative control (NS_m) and miR-4674 mimics ($miR\text{-}4674_m$) were subjected to microarray gene profiling. Potential gene targets were further narrowed down by sequential use of bioinformatics and prediction algorithms, RT-quantitative PCR, Western blot analyses, 3′-untranslated region (UTR) reporter studies, and microribonucleoprotein immunoprecipitation (miRNP-IP) analysis. *B* and *C*: HUVECs transfected with NS_m or $miR\text{-}4674_m$ were subjected to RT-qPCR for IRAK1 and BICD2 expression (*B*) or Western blot analyses using antibodies to IRAK1, BICD2, and GAPDH ($n = 3$ experiments) (*C*). *D*: luciferase activity of IRAK1 3′-untranslated region (UTR) and BICD2 3′-UTR normalized to total protein was quantified in HUVECs transfected with NS_m, $miR\text{-}4674_m$, NS_i, or $miR\text{-}4674_i$ ($n = 3$ experiments). *E*: miRNP-IP analysis of enrichment of IRAK1 and BICD2 mRNA in HUVECs transfected with NS_m or $miR\text{-}4674_m$. $^*P < 0.01$. RT-qPCR was performed to detect IRAK1, BICD2, or SMAD1. Results are representative of $n = 3$ replicates per group and 2 independent experiments. $^*P < 0.01$. All data represent means ± SE.

sion, 3′-UTR reporter assays, miRNP-IP studies, and siRNA dependency (Figs. 5 and 6). In the absence of ribosome profiling studies, based on our data showing decreased mRNA levels of both IRAK1 and BICD2 in response to miR-4674 overexpression, we can speculate that destabilization of target mRNAs is likely one mechanism contributing to the decreased protein expression. Interestingly, while IRAK1 is known to mediate proinflammatory signaling (1) and LPS-mediated activation of proapoptotic signaling in ECs (7), to our knowledge, a direct assessment of IRAK1 in endothelial angiogenic assays has not been described. Similarly, the target bicaudal D2 (BICD2) has not been previously described as a direct regulator of endothelial cell angiogenic activity, although its downregulation has been linked to antitumor angiogenesis in xenograft models (15). Our findings demonstrate that IRAK1 or BICD2 deficiency, like miR-4674 overexpression, significantly impaired EC proliferation and migration. However, IRAK1 and BICD2 have no cooperative effect on EC proliferation

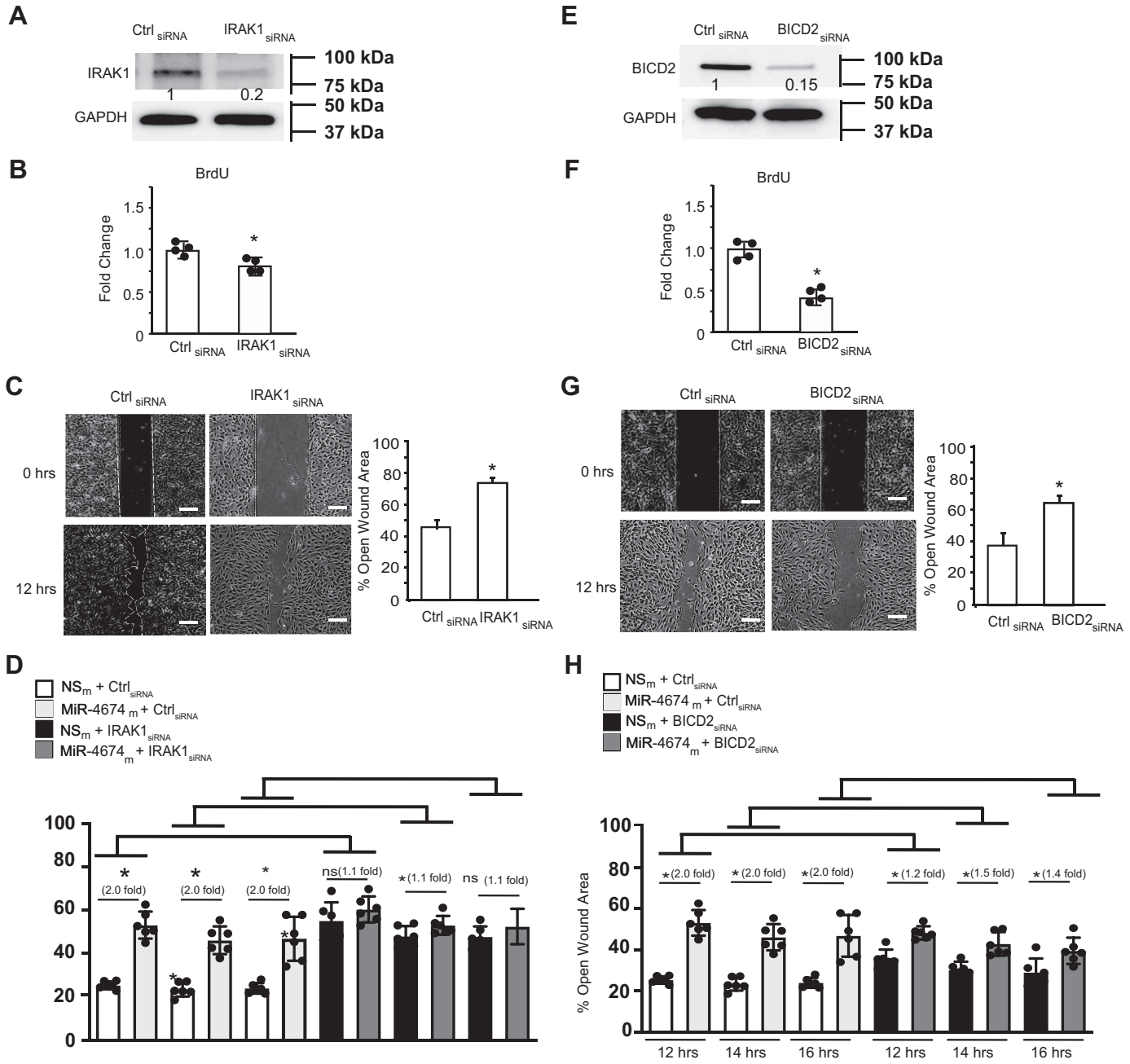

Fig. 6. SiRNA-mediated knockdown of interleukin 1 receptor-associated kinase 1 (IRAK1) and BICD cargo adaptor 2 (BICD2) recapitulates miR-4674 functional effects in endothelial cells (ECs). Human umbilical vein endothelial cells (HUVECs) were transfected with siRNA to IRAK1 (*A–D*), BICD2 (*E–H*), or scrambled control (ctrl) siRNA. Protein expression was determined by Western analysis (*A*, *D*) using antibodies to IRAK1, BICD2, and GAPDH ($n = 2$ experiments). $^*P < 0.01$. *B* and *F*: EC proliferation was quantified by BrdU assay. *C*, *D*, *G*, and *H*: migration of ECs was quantified by scratch assay. NS_m and miR-4674_m were also transfected as indicated in *D* and *H* to assess for dependency. $^*P < 0.01$; ns, nonsignificant. Results are representative of $n = 3$ replicates per group. All data represent means ± SE.

(Supplemental Fig. S3), suggesting that their effects may be redundant on EC growth. Alternatively, additional pathways that remain to be identified may be involved.

Dysregulation of miRNA expression has been linked to a range of pathophysiological disease states (39). MiRNAs are highly expressed in a diverse set of cell types, including ECs. For example, we previously identified that the expression of miR-26a, an antiangiogenic miRNA, was increased under acute myocardial infarction (MI) and in human subjects with MI (28) and in skin wounds of *db/db* mice, and that its neutralization could effectively promote angiogenesis by targeting Smad1, thereby promoting downstream effects of the bone morphogenic protein signaling pathway (27). Collectively, our findings have potential implications for utilization of microRNA-based therapeutics, as new approaches to address impaired neoangiogenesis observed in response to tissue injury, such as observed in diabetic wound healing or ischemic cardiovascular disease states.

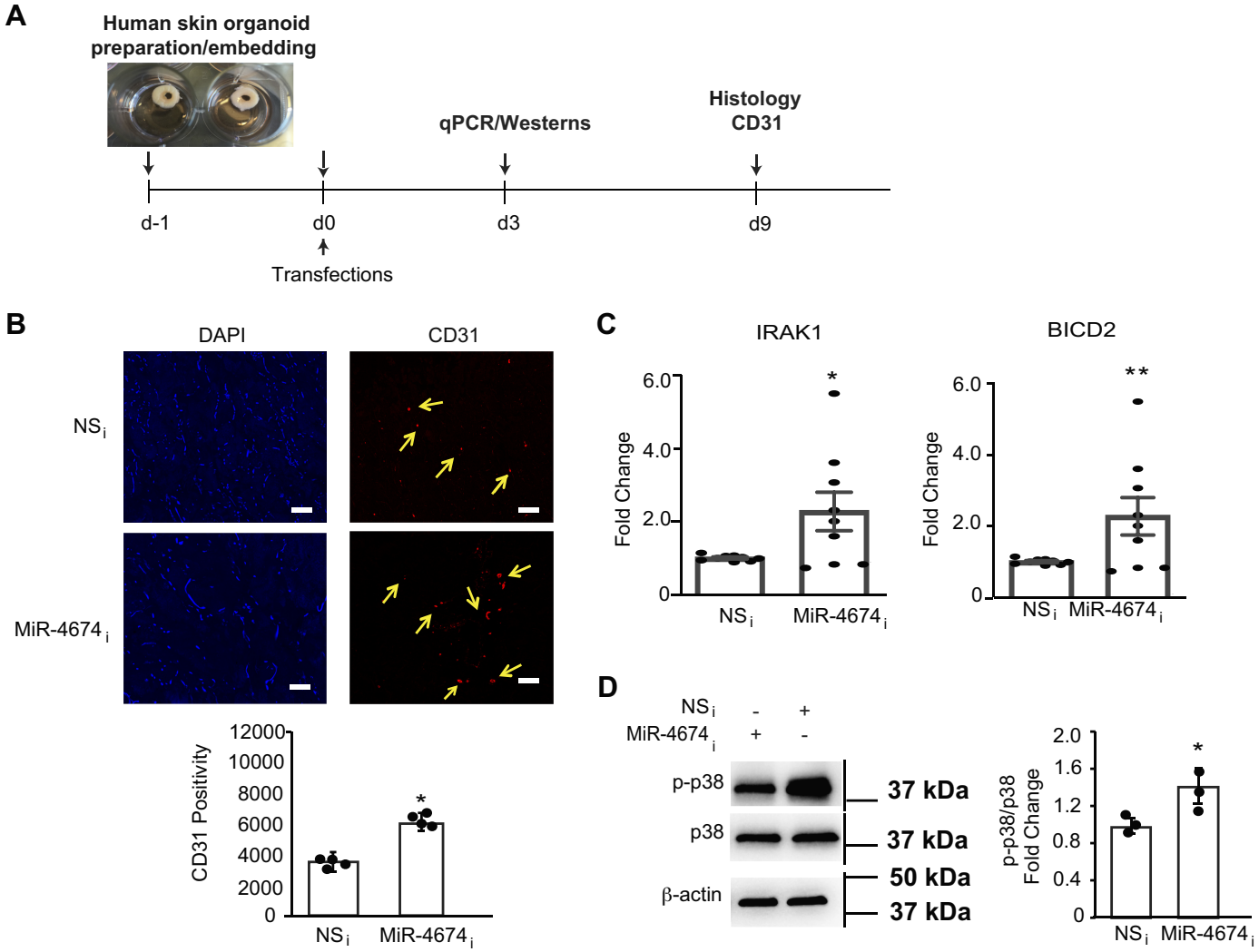

Fig. 7. Inhibition of miR-4674 promotes angiogenesis in human skin organoids. *A*: punch biopsies of human skin were embedded into a collagen matrix, transfected with miR inhibitor negative control (NS_i), or miR-4674 inhibitor ($miR\text{-}4674_i$) and cultured for the indicated number of days. *B*: human skin organoids were transduced with the indicated miRNAs and cultured for 9 days followed by confocal immunofluorescence staining for CD31 (arrows). CD31 was quantified at a fixed distance of 200 μm from the wound edge. *C* and *D*: human skin organoids ($n = 3–6$) were transduced with the indicated miRNAs and cultured for 3 days followed by RT-quantitative PCR analyses for interleukin 1 receptor-associated kinase 1 (IRAK1) and BICD Cargo Adaptor 2 (BICD2) (*C*) and Western blot analyses for p-p38, p38, and β-actin (*D*) at the indicated times. Scale bars, 50 μm (*B*). All data represent means ± SE. $*P < 0.05$. $**P < 0.01$.

In summary, we identified miR-4674 as a novel negative regulator of angiogenesis by regulating the p38 signaling pathway and direct targeting of *IRAK1* and *BICD2* genes. MiR-4674 mediates EC angiogenic functional responses by targeting IRAK1 and BICD2. However, additional targets may be involved in response to divergent pathophysiological stimuli, as we have observed for other microRNAs (42, 50, 51). Inhibition of miR-4674 markedly increased angiogenesis in human skin organoids. Therapies focusing on neutralization of miR-4674, or other miRNAs that regulate downstream angiogenic signaling pathways, may be beneficial for promoting neovascularization and tissue repair.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.W.F. conceived and designed research; B.I., H.L., D.P.C., W.W., D.O., S.H., R.B.G., A.M., J.F.M., and M.W.F. performed experiments; B.I., H.L.,

D.P.C., W.W., D.O., S.H., and M.W.F. analyzed data; B.I., H.L., D.P.C., W.W., D.O., S.H., and M.W.F. interpreted results of experiments; B.I., H.L., D.P.C., S.H., and M.W.F. prepared figures; B.I., D.P.C., and M.W.F. drafted manuscript; D.P.O. and M.W.F. edited and revised manuscript; B.I., H.L., D.P.C., W.W., D.O., S.H., R.B.G., A.M., J.F.M., D.P.O., and M.W.F. approved final version of manuscript.

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