

miR-139 and miR-200c regulate pancreatic cancer endothelial cell migration and angiogenesis

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Abstract. Pancreatic cancer remains the fourth deadliest cancer worldwide with a 5-year survival rate of only 4%. The present study tested the hypothesis that dysregulated microRNA (miRNA) expression by pancreatic cancer endothelial cells (CECs) may regulate angiogenesis. Primary EC cultures were established from the pancreatic tumor and adjacent normal tissues of three pancreatic cancer patients. A miRNA microarray was used to identify miRNAs that were differentially expressed. The expression patterns of four highly expressed miRNAs in CECs were confirmed by qPCR analysis. The effects of dysregulated miRNA expression on CEC proliferation, migration and tube formation were determined after transfection with specific miRNA inhibitors. The expression of 14 miRNAs was increased by >20-fold in the CECs of all three pancreatic patients; the increased expression of miR-200c and miR-139 in CECs was confirmed. miR-1, miR-139 and miR-200c inhibitors significantly reduced CEC migration (all $P < 0.05$), yet not proliferation. The average tube length and total loop number were also significantly decreased upon miR-139 and miR-200c inhibition in all three CEC cultures (all $P < 0.05$). Upregulation of miR-139 and miR-200c expression may increase CEC migration and tube formation, which suggests that these miRNAs may regulate pancreatic tumor angiogenesis.

Introduction

Despite treatment advances in other types of cancer, pancreatic cancer remains the fourth deadliest cancer worldwide with a 5-year survival rate of only 4% (1) in part since it is rarely detected at an early stage. For patients with inoperable locally advanced pancreatic cancer, standard treatment with chemo-

therapy or chemoradiotherapy results in a median survival time of 6-9 months (2) with combined radiochemotherapy resulting in improved 6-, 12- and 18-month survival rates (3). Therefore, current efforts have focused on identifying novel therapeutic targets for the treatment of pancreatic cancer.

Angiogenesis is crucial for the growth and progression of tumors. In isolated perfused thyroid glands, transplanted melanoma cells grew into tumors of only 1-2 mm in diameter and were not vascularized, suggesting that neovascularization is required for tumor growth (4). Tumors may become necrotic or even apoptotic in the absence of vascular support (5,6); therefore, angiogenesis inhibition has become an important therapeutic strategy in the prevention of tumor expansion and metastasis (7). Evidence suggests that specific microRNAs (miRNAs) can regulate angiogenesis through the downregulation of angiogenesis-related genes by interacting with their 3' untranslated region (UTR) (8). Recent studies that disrupted the function of Dicer and Drosha have revealed the important roles of certain miRNAs, known as angiomiRs, in regulating angiogenesis and modulating endothelial cells (ECs) (9-11). *Dicer* hypomorphic mouse lines have defects in vascular remodeling during development and ovarian angiogenesis (9,10). In addition, *in vitro* knockdown of Dicer or Drosha in human ECs decreased angiogenesis (11).

AngiomiRs regulate angiogenesis either cell-autonomously or non-cell-autonomously (12). miR-296 targets hepatocyte growth factor-regulated tyrosine kinase substrate mRNA, thereby suppressing the degradation of vascular endothelial growth factor receptor 2 (VEGFR2) and platelet-derived growth factor receptor- β (PDGFR- β) (13). In addition, miR-296 inhibition decreased tumor xenograft angiogenesis *in vivo* (13). Conversely, ectopic expression of miR-18a inhibited gastric cancer cell xenograft growth *in vivo* by reducing tumor angiogenesis (14). miRNAs also modulate the angiogenic properties of human umbilical vein ECs (HUVECs) (15); suppression of miR200a expression inhibited HUVEC viability and migration (16). Ectopic expression of miR-199a suppressed EC migration and reduced the expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (17). miR-149 regulated fibroblast growth factor 2 (FGF2)-induced EC proliferation and migration (18).

Given the importance of angiogenesis in tumor progression, the present study was undertaken to test the hypothesis that

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certain miRNAs expressed in cancer-associated ECs (CECs) may regulate angiogenesis. We identified miRNAs that were differentially expressed in primary EC cultures derived from three pancreatic cancer tissues as compared to those from adjacent normal tissues. Inhibition of miR-139 or miR-200c significantly reduced cancer endothelial cell (CEC) migration, the average tube length, and total loop number, suggesting that they play a role in tumor angiogenesis.

Materials and methods

Patient tissue samples. Pancreatic tumor and normal adjacent tissues were obtained from three pancreatic cancer patients who underwent surgery in the Department of General Surgery, Shanghai First People's Hospital, Shanghai Jiaotong University from October 2012 to May 2013. According to the tumor-node-metastasis (TNM) staging system (7th edition) of the American Joint Commission on Cancer (AJCC), all patients had stage I or II (T1-3, N0-1, M0) pancreatic cancer. None of the patients had received chemotherapy or cytotoxic agents within the last 12 months prior to the inclusion into this study. None of the patients showed clinical signs for active infectious diseases. Patients with stage III and IV pancreatic cancer with locally advanced unresectable foci (T4) and/or systemic metastases (M1) were excluded. Informed consent was obtained from all study participants. The present study was approved by the Ethics Committee of Shanghai First People's Hospital, Shanghai Jiaotong University, Shanghai, China.

Establishment of primary endothelial cell cultures from human pancreatic tumor samples. Primary endothelial cell cultures derived from human pancreatic tumor masses or adjacent normal tissue were prepared according to the method reported by Naschberger *et al.* (19). Briefly, after tissues were cut into 1-mm³ blocks, they were digested with collagenase II (17,100 U/g) in 5 ml of endothelial basal medium (EBM-2) supplemented with 0.5% fetal bovine serum (FBS) (EBM-low; both from Lonza, Cologne, Germany) at 37°C with 5% CO₂ for 1 h under constant agitation. Following digestion, cells were filtered (cell strainer 100 µm; BD Biosciences, Franklin Lakes, NJ, USA) and isolated by centrifugation at 500 x g for 5 min at 20°C. The cell pellet was resuspended in 5 ml of EGM-2-MV (EGM-2-MV BulletKit; Lonza), and the cell suspension was added to flasks pre-coated with 1.5% gelatin. The growth medium was refreshed every 2 days for 5-7 days until the cell confluence reached 70-80%. After the cells were detached with 1-2 ml of Accutase (Life Technologies, Carlsbad, CA, USA), they were washed with EGM-2-MV. The cells were resuspended in 60 µl of MACS buffer (1X PBS pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), and then were incubated with 20 µl of CD31 beads (CD31 MicroBead kit; Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 min. After washing with MACS buffer, the cells were resuspended in 1 ml MACS buffer, which was applied to a MACS separation column (Miltenyi Biotec), and the endothelial cells were isolated following the manufacturer's instructions. The cell concentration was adjusted to 1-2x10⁴/cm² and cultured on 25 T flasks pre-coated with 0.5% gelatin. Cells were cultured until complete confluency was reached, and the medium was refreshed every 2 days.

miRNA extraction. Total RNA was isolated using the mirVana™ microRNA isolation kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). In brief, the sample was first lysed in a denaturing lysis solution, and the lysate was then subjected to phenol-chloroform extraction. After purification over a glass-fiber filter, the total RNA integrity was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

miRNA microarray and data analysis. The miRNA expression profiles in CECs were compared with those of normal endothelial cells (NECs) derived from the same patient. Affymetrix FlashTag® Biotin HSR was used for miRNA labeling and hybridization onto the Affymetrix GeneChip® MicroRNA 3.0 Array (both from Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. The miRNA expression profile was scanned through a GeneChip® Scanner 3000 (cat. #00-00212, Affymetrix), and primary data were analyzed using GeneChip-compatible™, Command Console Software 3.1 and Expression Console Software. The CEC and NEC miRNA expression profiles of the same patient were compared to identify differentially expressed mRNAs. In addition, the array results were also compared against the miRNA databases, microrna.org and TargetScan, to identify candidate miRNAs that are relevant to angiogenesis.

Candidate miRNA validation. The differential expression of miR-200c, miR-182, miR-139-5p and miR-200b was validated by qPCR analysis using the stem-loop TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Briefly, mature candidate miRNAs were reverse transcribed into cDNA from 10 ng of total RNA with mature miRNA-specific looped RT primers from the TaqMan MicroRNA assays kit and reagents from the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. Real-time PCR was performed on the 5'-extended cDNA with Applied Biosystems TaqMan 2X Universal PCR Master Mix and the appropriate 5X TaqMan MicroRNA Assay Mix (both from Applied Biosystems) for each miRNA of interest. For each sample, the threshold cycle (Ct) was calculated by the ABI 7500 Sequence Detection System software. Standard curves were used to determine miRNA concentration in the samples, which were then normalized to small nuclear RNA U (RNU) RNA.

miRNA knockdown in CECs. The specific inhibitors for miR-139 or miR-200c (MiScript microRNA Inhibitor; Qiagen, Hilden, Germany) were added into CEC cultures to evaluate the effect of these miRNAs on CEC proliferation. In addition, since miR-1 regulates angiogenesis (i.e., enhanced tube formation and migration of human-derived cardiomyocyte progenitor cells) (20), a miR-1 inhibitor (Qiagen) was used as a positive control. An inhibitor with scrambled sequence that has no homology to any known mammalian gene (Qiagen) was used as a negative control. Briefly, cells were seeded in a 24-well plate at a density of 0.4-1.6x10⁵ cells/well in 500 µl of culture medium containing serum and antibiotics. After 4 h, cells were incubated with 50 nM miRNA inhibitor diluted in 50 µl of culture medium without serum and with 1.5 µl

Table I. Differentially expressed miRNAs with a >20-fold increased expression in CECs relative to NECs (N=3).

miRNA	Fold-change in expression (CEC/NEC) ^a
hsa-miR-25*	63.47±17.96
hsa-miR-27a*	86.33±32.15
hsa-miR-92a-1*	62.63±22.31
hsa-miR-139-5p	356.53±181.85
hsa-miR-182	951.97±86.51
hsa-miR-183	99.9±57.27
hsa-miR-192	47.77±20.83
hsa-miR-194	55.2±15.14
hsa-miR-200a	46±15.83
hsa-miR-200b*	93±39.66
hsa-miR-200b	151.07±67.69
hsa-miR-200c	4,574.97±2,209.62
hsa-miR-203	77.87±37.57
hsa-miR-375	58.2±33.34

^aMean ± SD miRNA expression in three pancreatic cancer patients. CECs, cancer endothelial cells; NECs, normal endothelial cells.

HiPerfect transfection reagent (Qiagen). The cells were incubated with the transfection complexes under normal growth conditions, and gene expression was monitored after 12 h by qPCR. qRT-PCR data were calculated as $2^{-\Delta\Delta C_t}$ after normalizing to the control.

Cell proliferation assay. Cell proliferation was assessed using the MTT assay (Sigma, St. Louis, MO, USA) 48 h after siRNA inhibition following the manufacturer's instructions.

Cell migration assay. Transwell migration assay were conducted 48 h after siRNA inhibition using a fluorometric cell migration assay kit with polycarbonate membrane inserts (5- μ m pore size; Cell Biolabs, San Diego, CA, USA) using a modified protocol described by Chim *et al* (21). Briefly, cells were serum-starved overnight in Dulbecco's modified Eagle's medium (DMEM) prior to initiation of the experiment. The lower chambers were filled with 1 ml of medium containing 10% serum. Cells (4×10^4) were resuspended in 200 μ l of Opti-MEM and added to the upper chamber. After 24 h at 37°C, migrating cells were counted after staining with crystal violet.

Tube formation assay. After cells were serum-starved overnight in DMEM, they were seeded in 24-well plates that were coated with Geltrex™ reduced growth factor basement membrane matrix (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C for 30 min with Opti-MEM. Medium was then removed and replaced with the medium containing 10% serum and incubated at 37°C for 24 h. Four randomly selected fields of view were analyzed, and tube formation was quantified by measuring the length of tube-like structures and the number of branching points. Tube length was assessed by drawing lines along the tube-like structure and measuring the length

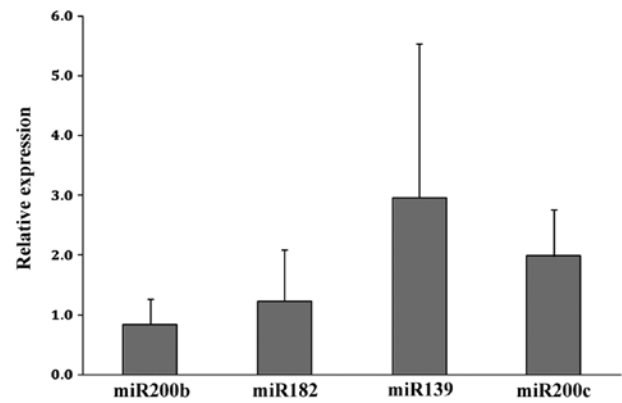


Figure 1. Validation of increased miR-139 and miR-200c expression in CECs relative to NECs. miRNA levels in three pancreatic cancer patients were determined by qPCR. Data are presented as mean ± SD of the relative miRNA expression as calculated by the $2^{-\Delta\Delta C_t}$ method. CECs, cancer endothelial cells; NECs, normal endothelial cells.

of the line in pixels using a modified protocol as previously described (21).

Statistical analysis. For cell proliferation and migration as well as tube length and total loops, data are presented as mean ± standard deviation (SD). One-sample t-tests were performed to evaluate the cell proliferation with the mean of the control group set to 1. Differences among the negative control, miR-1, miR-139 and miR-200c inhibitor groups were also assessed by one-way ANOVA with a *post-hoc* LSD test as for pair-wise comparisons. One-sample t-tests were also performed to evaluate the mean tube length or total loops set as 1. Moreover, differences between the negative control and miR-1 inhibitor groups were compared using the two-sample t-test. Statistical assessments were two-tailed, and P-values <0.05 were considered to indicate statistically significant results. SPSS 18.0 statistics software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

miRNA expression profiles in CECs and NECs. The miRNA expression profiles of CECs were compared with those of the NECs in three pancreatic patients. As shown in Table I, 14 miRNAs were upregulated by >20-fold in the CECs as compared to NECs, including hsa-miR-139-5p, hsa-miR-182, hsa-miR-183, hsa-miR-192, hsa-miR-194, hsa-miR-200a, hsa-miR-200b*, hsa-miR-200b, hsa-miR-200c, hsa-miR-203, hsa-miR-25*, hsa-miR-27a*, hsa-miR-375 and hsa-miR-92a-1*.

Validation of differential expression of candidate angiomiRs in CECs and NECs. We compared the primary miRNA data with available miRNA databases (<http://www.microrna.org>; www.targetscan.org) to identify candidate angiomiRs.

Of the miRNAs with >20-fold increased expression, miR-182, miR-183, miR-192, miR-194, the miR-200 family, miR-203, miR-27a*, miR-375 and miR-92a-1* have been implicated in regulating tumor angiogenesis (16,22-32). We chose to validate the upregulated expression of miR-200b, miR-182, miR-139 and miR-200c using qPCR analysis as

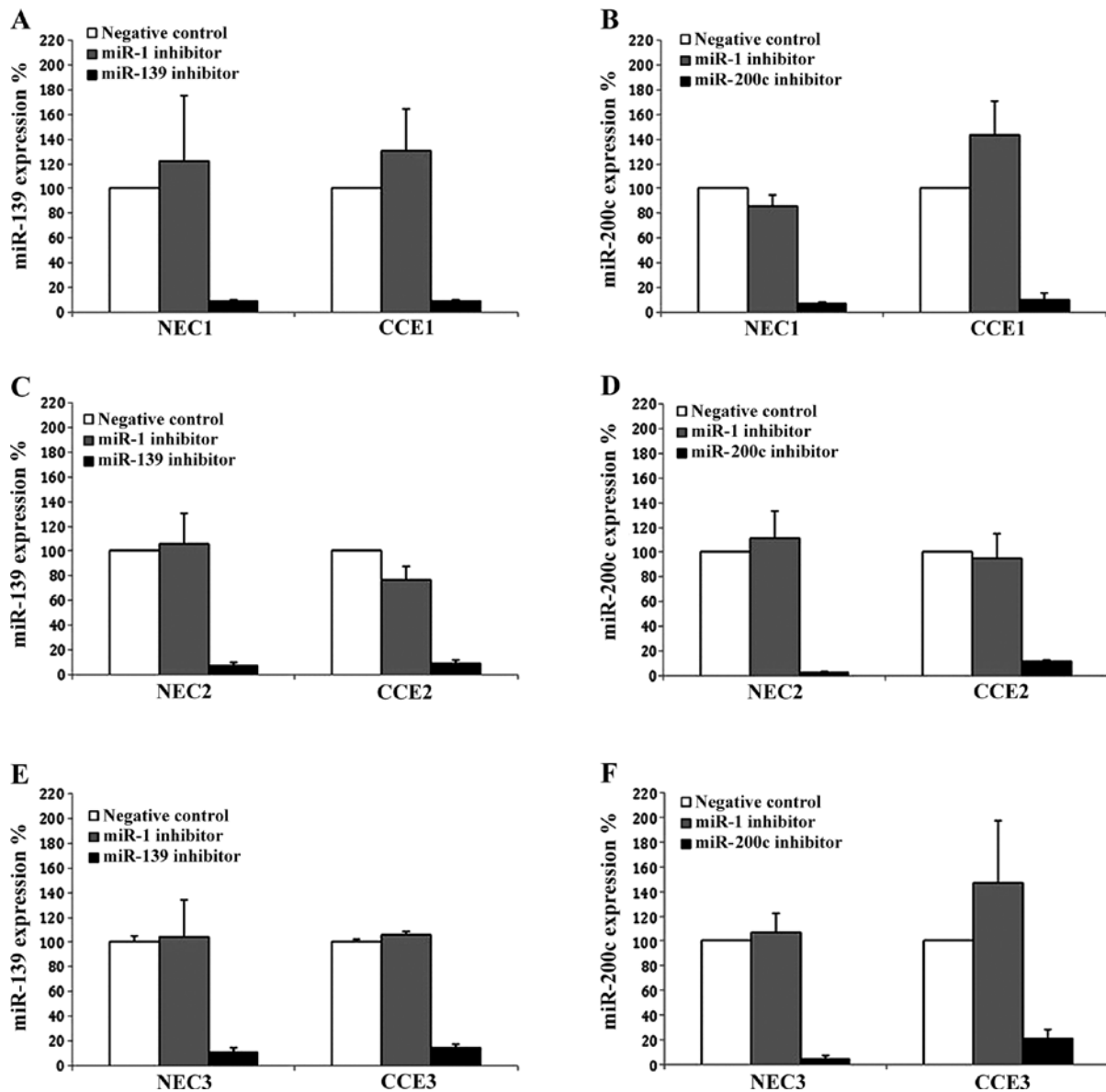


Figure 2. Efficiency of miRNA knockdown. NEC and CEC cultures were obtained from patients 1 (A and B), 2 (C and D) and 3 (E and F) and were transfected with vector alone or inhibitors of miR-1, miR-139 or miR-220c. The expression of miR-139 (A, C and E) and miR-220c (B, D and F) was determined by qPCR. Data are presented as mean \pm SD of the relative expression levels for each subject as determined using the $2^{-\Delta\Delta Ct}$ method. (n=3 for each pancreatic patient). NEC1-3, normal endothelial cells from patients 1-3; CEC1-3, cancer endothelial cells from patients 1-3.

these candidate angiomiRs had the greatest increase in fold-expression in the CECs (Table I). As shown in Fig. 1, the upregulated expression of miR-139 and miR-200c was confirmed in CECs relative to NECs.

Effect of miRNA on CEC proliferation. To determine whether either miR-139 or miR-200c influences CEC proliferation, specific inhibitors against them were utilized. As shown in Fig. 2, the efficiency of the miRNA inhibition was confirmed in three different pairs of NEC and CEC cultures. Each miRNA inhibitor suppressed the expression of their target miRNA by at least 80%. We next determined whether inhibition of either miR-139 or miR-200c could influence CEC proliferation using MTT assays. As shown in Fig. 3, cell proliferation was not altered upon inhibition of miR-139 or miR-200c as compared to the negative control group. Similar

results were obtained after inhibition of miR-1, which is a known regulator of angiogenesis (20). However, cell proliferation was significantly decreased in all groups as compared to the untransfected control cells (all $P < 0.05$). These data suggest that neither miR-139 nor miR-200c influences pancreatic CEC proliferation.

Effect of miR-139 and miR-200c on CEC migration. The effects of miR-139 and miR-200c on CEC migration were next evaluated using their specific inhibitors. As compared to the untransfected cells, cell migration in all the samples was significantly decreased (all $P < 0.05$, Fig. 4). Notably, significantly reduced CEC migration was observed after transfection with the miR-1, miR-139 and miR-200c inhibitors compared to the negative control group (all $P < 0.05$). These data suggest that miR-139 and miR-200c regulate CEC migration.

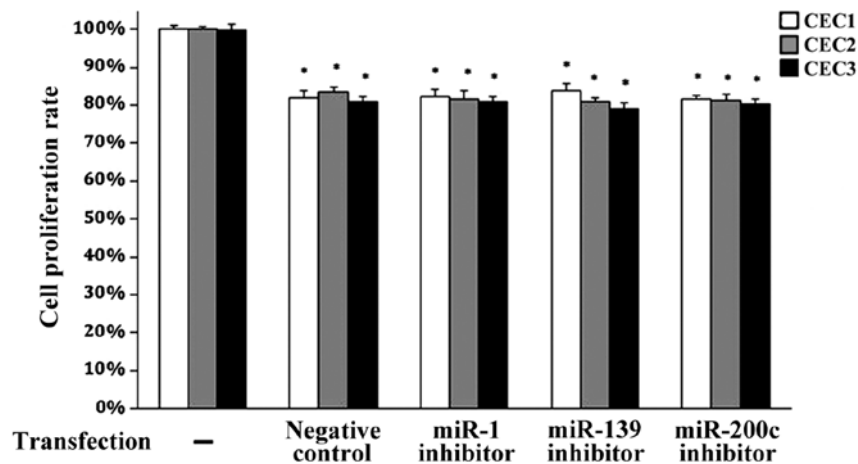


Figure 3. miR-139 and miR-220c do not alter CEC proliferation. CEC proliferation was determined using the MTT assay after transfection with the indicated inhibitors. Data are presented as mean \pm SD for each condition (n=3/condition). *P<0.05, indicates a significant difference as compared to the untransfected controls. CEC1-3, cancer endothelial cells from patients 1-3.

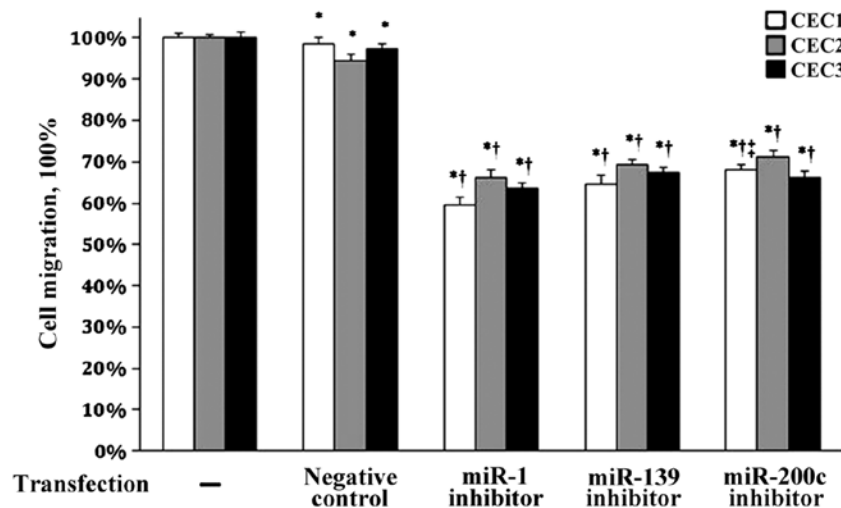


Figure 4. Cell migration was inhibited by miR-139 and miR-220c. After transfection with the indicated inhibitors, CEC migration was determined using Transwell migration assays. Data are presented as mean \pm SD for each condition (n=3/condition). *P<0.05, indicates a significant difference as compared with the untransfected controls. †P<0.05, indicates a significant difference as compared to the negative control and ‡miR-1 inhibitor groups. CEC1-3, cancer endothelial cells from patients 1-3.

Effect of miR-139 and miR-200c on angiogenesis in CECs. The effects of miR-139 and miR-200c on CEC tube formation were next evaluated as an *in vitro* measure of their effects on angiogenesis. Representative images of the cells from all three CEC cultures are shown in Fig. 5A-C. As shown in Fig. 5D and E, the average tube length and total loop number were significantly decreased with miR-139 and miR-200c inhibition in all three CEC cultures compared to those of the negative control group (all P<0.05). No such changes were observed with miR-1 inhibition. Thus, miR-139 and miR-200c may regulate vasculature formation during angiogenesis.

Discussion

Inhibition of angiogenesis to suppress tumor expansion and metastasis in pancreatic cancer has become a promising therapeutic strategy for many types of cancer. Although the

importance of miRNAs in vasculogenesis was illustrated in Dicer-null mice (10) and several angiomiRs have been identified (9-11,13), there is limited information regarding their role in pancreatic carcinoma. Therefore, the present study aimed to identify angiomiRs in pancreatic CECs. Fourteen miRNAs were differentially expressed by >20-fold in the CECs of all three patients analyzed. Subsequent inhibition studies revealed that miR-139 and miR-200c may regulate CEC migration and tube formation but not proliferation.

In laryngeal squamous cell carcinoma, miR-139 expression decreased with disease progression, and *in vitro* and *in vivo* studies suggest that it inhibits proliferation, migration and metastasis (33). Similar tumor-suppressive functions have been reported in glioblastomas, hepatocellular carcinomas and gliomas (34-36). In contrast, miR-139 expression was upregulated in CECs as compared to NECs in the present study, which may be due to cell type-specific differences.

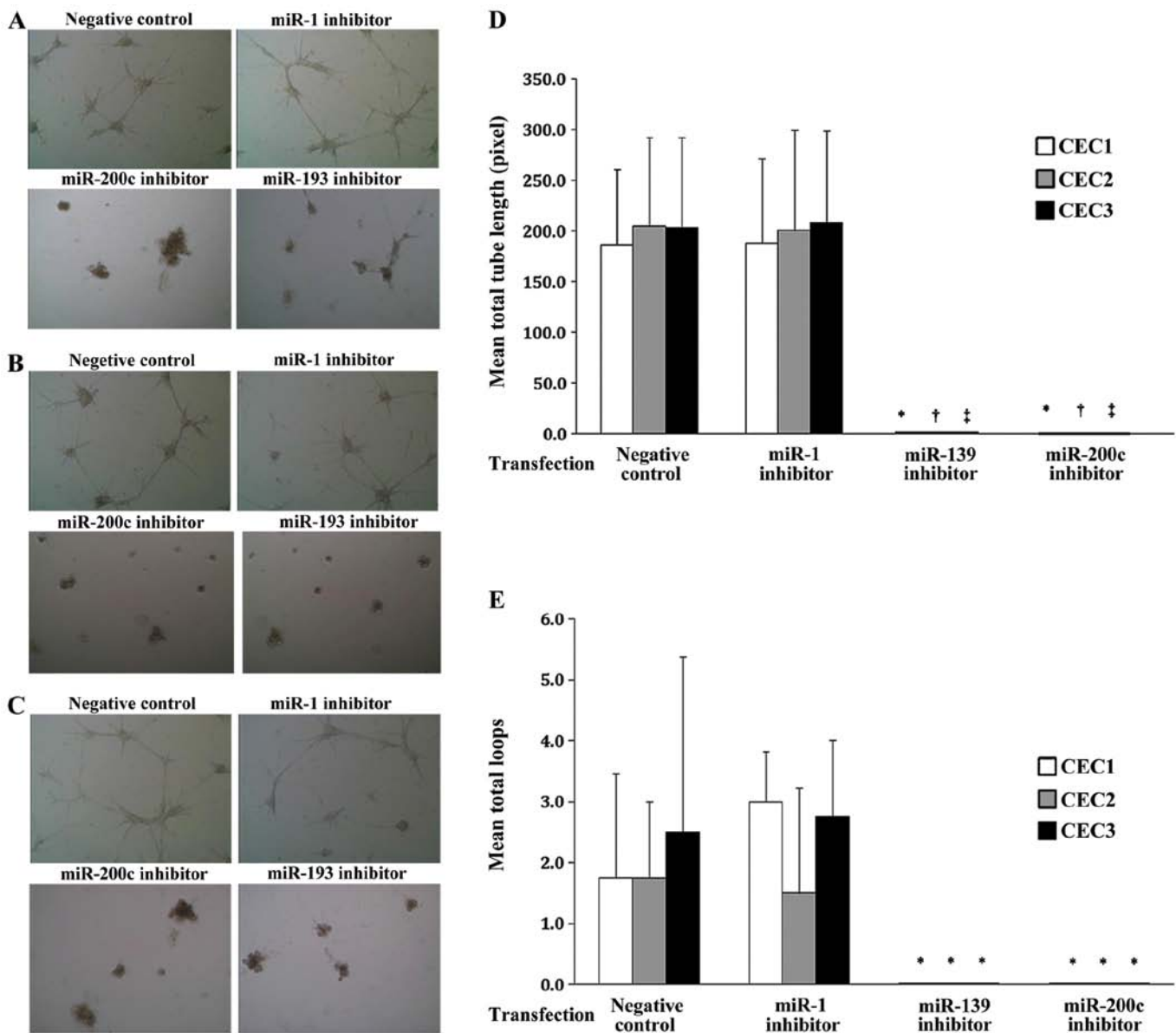


Figure 5. miR-139 and miR-200c regulate cancer endothelial cell (CEC) tube formation. Representative images from (A) CEC1, (B) CEC2 and (C) CEC3 are shown after transfection with the vector control or the miR-1, miR-200c or miR-193 inhibitors. (D) Mean tube length and (E) total loop number formation in the indicated groups. Data are presented as mean \pm SD for each condition (total of four randomly selected fields were calculated). The tube length and total loop number in cells treated with miR-139 and miR-200c inhibitors were set as 1. One-sample t-tests were performed to evaluate the mean tube length or total loops, and differences between negative control and miR-1 inhibitor were compared using two-sample t-test. $^{**}P < 0.05$, indicates a significant difference as compared with the negative control in * CEC1, † CEC2 and ‡ CEC3. CEC1-3, cancer endothelial cells from patients 1-3.

In human embryonic stem cells, miR-200c was necessary for EC differentiation and *in vivo* vasculogenesis through inhibition of the transcription repressor, zinc finger E-box-binding homeobox (ZEB1) (37). Reduced expression of miR-200c in leiomyomas, which are benign, fibrotic uterine tumors, altered ZEB1/ZEB2, VEGFA, FBLN5 and TIMP2 expression (38). In contrast, miR-200c expression is increased in endometrial cancer (39), and ectopic expression increased Ishikawa cell proliferation (40). Although we also observed increased miR-200c expression in the CECs as compared to NECs, its inhibition did not influence cell proliferation. However, inhibition of miR-200c reduced CEC migration and tube formation, which is similar to that reported for miR-200a (15). Given the role of the putative miR-200c target genes

in EMT, angiogenesis and matrix remodeling, further studies will evaluate the effects of miR-200c on tumor growth and metastasis.

Fourteen miRNAs were differentially expressed in the CECs of all three patients analyzed, among which the roles for miR-182, miR-183, miR-192, miR-194, the miR-200 family, miR-203, miR-27a*, miR-375 and miR-92a-1* in regulating tumor angiogenesis have been reported (16,22-32). Subsequent qPCR analysis of miR-200b, miR-182, miR-139 and miR-200c confirmed that miR-139 and miR-200c levels were increased in the CECs relative to the NECs. Such differences between the microarray results and qPCR validation may be attributed to the distance between the PCR primers and microarray probes for a specific gene (41) as well as spot intensity (42)

and microarray data filtering (i.e., p-value) (43). Although both miR139 and miR-200c were selected for further analysis on the basis of their high expression and validation by qPCR, further studies will analyze the roles of the other miRNAs in angiogenesis.

In zebrafish embryos, inhibition of miR-1 inhibited angiogenesis and reduced EC levels (44). It also regulates cardiomyocyte progenitor cells (20). Although knockdown of miR-1 influenced CEC migration in the present study, no effects were observed on tube length and loop numbers. These differential effects may be due to tissue-specific regulation by miR-1.

The present study is limited in that the mechanism by which miR-139 and miR-200c influence cell migration and tube formation were not explored. Previous studies have reported that miR-139 targets chemokine receptor 4 (CXCR4) (33) and miR200c targets ZEBs, which regulate EMT during cancer development by repressing E-cadherin (45,46), as well as VEGFA, FLT1, IKK β , KLF9, FBLN5 and TIMP2 (38,40). Therefore, these putative miRNA targets will be explored further. In addition, the expression of CEC miRNAs was analyzed in only three pancreatic cancer patients; therefore, larger studies are necessary to determine the full significance of altered miRNA expression in pancreatic CECs. Furthermore, the differential miRNA expression observed in the present study may only be applicable for Asian patients as differential expression of miR-200c was noted in the leiomyomas of African-Americans vs. Caucasians (38). Finally, the effects of miR-139 and miR-200c on the proliferation, migration and vasculogenesis of NECs also need to be determined to fully explore the therapeutic potential of these miRNAs.

In summary, the present study identifies two miRNAs, miR-139 and miR-200c, that were upregulated in CECs derived from pancreatic tumors and that regulate CEC migration and tube formation. The therapeutic value of targeting these miRNAs in pancreatic cancer will be assessed in further *in vivo* studies.

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