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Deregulated MicroRNAs in Cancer-Associated Fibroblasts from Front Tumor Tissues of Lung Adenocarcinoma as Potential Predictors of Tumor Promotion

María Cristina Negrete-Garcia,[1,2] Sandra Lizbeth Ramírez-Rodriguez,[1] Claudia Rangel-Escareño,[3] Said Muñoz-Montero,[3] Javier Kelly-García,[4] María Eugenia Vázquez-Manríquez,[5] Patricio Santillán,[6] Martha Montaño Ramírez,[7] Gustavo Ramírez-Martínez,[8] Alejandra Ramírez-Venegas[9] and Blanca Ortiz-Quintero[1]

[1]Research Unit, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico
[2]Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina del Instituto Politécnico Nacional, Mexico City, Mexico
[3]Instituto Nacional de Medicina Genómica, Mexico City, Mexico
[4]Oncology Service, Centro Médico Siglo XXI, Mexico City, Mexico
[5]Department of Pathology, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico
[6]Department of Thoracic Surgery, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico
[7]Department of Cellular Biology, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico
[8]Department of Immunobiology and Genetics, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico
[9]Smoking and COPD Research Department, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas," Mexico City, Mexico

Cancer-associated fibroblasts (CAFs) are the main component of the tumor stroma and promote tumor progression through several mechanisms. Recent evidence indicates that small noncoding RNAs, microRNAs (miRNAs), play key roles in CAF tumor-promoting properties; however, the role of miRNAs in lung cancer-associated fibroblasts remains poorly defined. We characterized the differential miRNA expression profile of fibroblasts isolated from matched tumor front (F-CAFs), inner tumor (In-CAFs), and normal adjacent (NFs) tissues from four lung adenocarcinoma patients (ADs) using microarray analysis. Proliferation and invasion assays of A549 human lung cancer cells in the presence of conditioned medium from F-CAFs, In-CAFs or NFs were performed to assess tumorigenic properties. Ten identified candidate miRNAs in F-CAFs, In-CAFs and NFs from 12 ADs were then validated by RT-PCR. Both F-CAFs and In-CAFs enhanced the proliferation and invasion of A549 cells compared with NFs; moreover, F-CAFs showed a significantly stronger effect than In-CAFs. RT-PCR validation demonstrated three downregulated miRNAs in F-CAFs compared with NFs (miR-145-3p, miR-299-3p, and miR-505-3p), two in F-CAFs compared with In-CAFs (miR-410-3p and miR-485-5p), but no differentially expressed miRNAs between In-CAFs and NFs. Further target-gene prediction and pathway enrichment analysis indicated that deregulated miRNAs in F-CAFs showed significant associations with "pathways in cancer" (miR-145-3p, miR-299-3p and miR-410-3p), "Wnt signaling pathway" (miR-410-3p and miR-505-3p), and "TGF-beta signaling pathway" (miR-410-3p). Importantly, a tumor-promoting growth factor targeted by those miRNAs, VEGFA, was upregulated in F-CAFs compared with NFs, as judged by RT-PCR. In conclusion, deregulated miRNAs in F-CAFs are potentially associated with CAF tumor-promoting properties.

Keywords: cancer-associated fibroblast; lung adenocarcinoma; microenvironment heterogeneity; microRNA; tumor stroma
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Correspondence: Blanca Ortiz-Quintero, Ph.D., Research Unit. Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas". Calzada de Tlalpan 4502, Colonia Sección XVI, Mexico City 14080, Mexico.
e-mail: boq@iner.gob.mx

Introduction

Lung cancer is the main cause of cancer death worldwide, with the lowest 5-year survival rate (18.6%) among other leading cancers (Noone et al. 2018; Siegel et al. 2018). The low survival rate is attributed to metastasis and the ineffectiveness of available therapies (Ferlay et al. 2010; Valastyan and Weinberg 2011). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, and lung adenocarcinoma is the most common histological subtype of NSCLC. Notably, the high mortality rate of NSCLC has remained unchanged despite current knowledge and the introduction of new therapies. Therefore, a better understanding of the molecular mechanisms related to lung carcinogenesis and tumor progression is needed.

Tumor progression leads to metastasis and involves both intrinsic alterations of cancer cells and dynamic interactions with tumor stromal components (Joyce and Pollard 2009; Hanahan and Weinberg 2011; Quail and Joyce 2013). These cellular and non-cellular components of the tumor comprise the tumor microenvironment. The tumor microenvironment consists of cancer cells and non-transformed stromal cells, including fibroblasts, endothelial cells, and immune cells, extracellular matrix, nerves, and vessels. It has been suggested that this microenvironment is heterogeneous, with apparent variations between the tumor center and edge (Halama et al. 2011; Quail and Joyce 2013).

During tumor progression, cells at the invasive tumor front respond to extrinsic stimuli that influence their motility and metastatic capacity (Khalil and Friedl 2010; van Zijl et al. 2011; Quail and Joyce 2013). In addition, the stroma is characterized by increased matrix protein deposition, angiogenesis, immune cell infiltration, and activation of stromal fibroblasts to cancer-associated fibroblasts (CAFs) (Quail and Joyce 2013). CAFs are the major cellular component of the tumor stroma and contribute to tumor proliferation, invasion, metastasis and angiogenesis in several types of cancer (Bremnes et al. 2011; Shiga et al. 2015). The known mechanisms of tumorigenesis include the secretion of various growth factors, cytokines, and chemokines and degradation of extracellular matrix (ECM) proteins (Ishii et al. 2016). In NSCLC, CAFs enhance invasiveness and motility and induce epithelial-mesenchymal transition of lung cancer cells (Kim et al. 2013; Shintani et al. 2013, 2016; Yu et al. 2016). In addition, CAFs isolated from NSCLC specimens express specific gene signatures associated with their tumor-induced properties (Navab et al. 2011; Vicent et al. 2012).

Recent evidence indicates that microRNAs (miRNAs) are involved in the carcinogenic changes that occur in the tumor microenvironment. In particular, miRNAs have been shown to regulate the capacity of CAFs to promote tumor progression of oral, gastric, colorectal, breast, ovarian, and bladder cancers (Enkelmann et al. 2011; Zhao et al. 2012; Bullock et al. 2013; Yang et al. 2014; Kurashige et al. 2015; Li et al. 2015; Min et al. 2016). However, the role of miRNAs in the mechanisms underlying the tumor-promoting effect of CAFs in NSCLC remains poorly defined.

MicroRNAs (miRNAs) are small noncoding RNAs (~ 22 nucleotides) of endogenous origin that posttranscriptionally regulate the expression of genes by preventing the translation of specific target mRNAs (Petersen et al. 2006; Carthew and Sontheimer 2009; Martinez-Rivera et al. 2018). MiRNAs recognize specific complementary sequences of target mRNAs and induce their degradation or inhibit their translation (Lewis et al. 2005; Petersen et al. 2006). Furthermore, miRNAs are involved in gene regulatory pathways for all cellular processes, including essential processes such as proliferation, development, apoptosis, cell cycle, differentiation and angiogenesis (Reinhart et al. 2000; Brennecke et al. 2003; Avila-Moreno et al. 2011). It should be noted that these cellular processes are altered in malignant neoplasms (Hanahan and Weinberg 2011).

MiRNAs are expressed in normal physiological conditions in a cell-, tissue- and body fluid-specific manner (Ortiz-Quintero 2016; Martinez-Rivera et al. 2018). However, their expression was found to be aberrant in several cancers. This aberrant expression is involved in the initiation, progression and metastasis of several human cancers (Ortiz-Quintero 2016).

Although it is now accepted that miRNAs are altered in human cancers and are involved in carcinogenesis, our understanding of how miRNAs regulate the tumor-promoting mechanisms of CAFs in the tumor microenvironment is incipient. Notably, few studies have been published regarding the role of miRNAs in CAF tumor properties in NSCLC (Shen et al. 2016). Moreover, no previous studies have analyzed CAFs from different NSCLC tumor zones, despite evidence that the tumor microenvironment is heterogeneous.

To better understand the molecular mechanisms of CAF and tumor microenvironment interactions in lung cancer progression, we analyzed the miRNA expression profiles of fibroblasts isolated from matched tumor front (F-CAFs) and inner tumor (In-CAFs) tissues as well as from normal adjacent tissue (NFs) obtained from patients with lung adenocarcinoma using Affymetrix microarray technology at the screening phase. A quantitative real-time polymerase chain reaction (qPCR) assay was used to validate the microarray results. Target gene prediction and pathway enrichment analysis were conducted to identify functional pathways regulated by candidate miRNAs. Finally, the expression levels of three cancer-promoting target genes were tested by qPCR in F-CAFs and NFs to analyze the potential effect of differentially expressed miRNAs in those predicted target genes.

Our findings suggest that the differentially expressed miRNAs in F-CAFs, which showed the strongest effect on the invasive and proliferative capacity of cancer cells, are predictive of CAF tumor-promoting properties. To the best of our knowledge, this is the first study to describe the miRNA expression patterns of CAFs isolated from specific

topological tumor mass locations in NSCLC.

Materials and Methods

Study design

To elucidate the role of miRNAs in the tumor-promoting properties of CAFs from different lung tumor areas, we characterized the differential miRNA expression profile of fibroblasts isolated from tumor front (F-CAFs), inner tumor (In-CAFs), and normal adjacent (NFs) tissue in lung adenocarcinoma using a five-step approach analysis (Fig. 1). In step 1, we established primary cultures of F-CAFs, In-CAFs and NFs (triples) from 12 lung adenocarcinoma patients. In step 2, four matched triples of those fibroblasts were randomly selected for the microarray analysis (screening phase). Proliferation and invasion assays of A549 human NSCLC cells grown in conditioned medium from the fibroblasts were performed to assess CAF tumor-promoting properties. In step 3, ten candidate miRNAs were validated by qPCR in 12 triples of F-CAFs, In-CAFs and NFs (validation phase). In step 4, target gene prediction and pathway enrichment analysis were performed for validated miRNAs using predictive bioinformatics tools. In step 4, miRNAs associated with carcinogenesis mechanisms and their potential target genes were identified. Finally, in step 5, the expression levels of three cancer-promoting target genes were tested by qPCR in F-CAFs and NFs from 12 adenocarcinoma patients to analyze the potential effect of differentially expressed miRNAs in those predicted target genes.

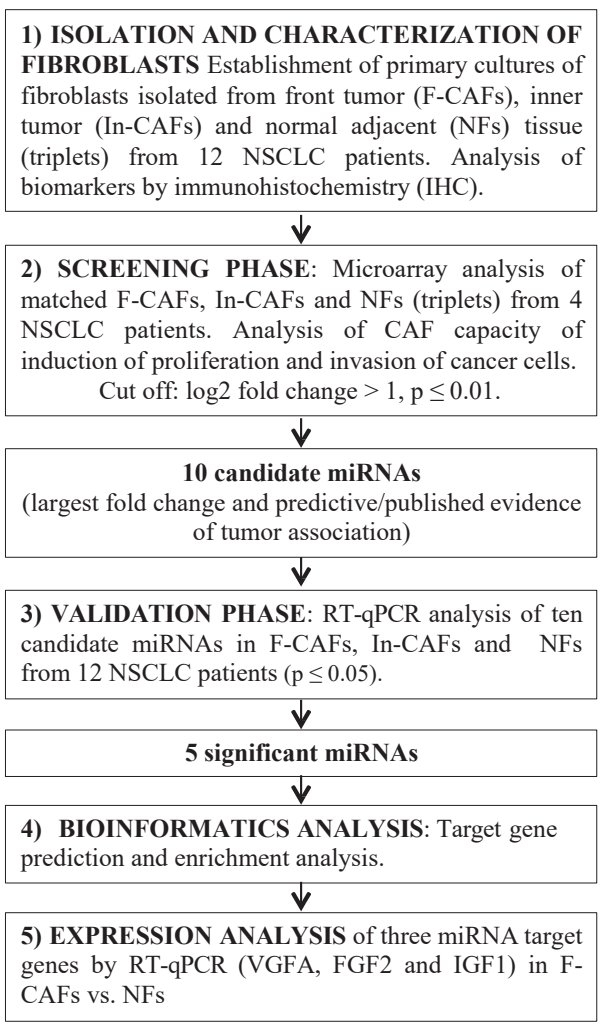

Fig. 1. An overview of the study design strategy.

Biological samples and patients

Tumor front tissue samples, matched inner tumor tissue samples, and matched adjacent normal lung tissue samples from each patient with primary NSCLC subtype adenocarcinoma (tumor stage 1A) were obtained by surgical resection at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas, Mexico City. Topographic identification of the center (inner tumor) and edge (tumor front) of the tumor mass was performed by the attending surgeon based on macroscopic features. An independent pathologist histopathologically confirmed malignancy in all tumor tissue samples and the absence of cancer and inflammatory cells in the adjacent normal tissue samples. None of the patients received adjuvant therapy before surgery. Tissue samples were harvested within 30 min of resection and kept in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco) and penicillin–streptomycin on ice for immediate transport to the laboratory. Sections of formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin and used to confirm malignancy and histological subtype by an independent pathologist. The Scientific and Ethics committees of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas approved the use of specimens in this study. Informed consent was obtained from all individual participants.

Patients

Twelve patients were included in this study. Four patients were included in the microarray analysis, and twelve patients were included in the validation phase of the microRNA analysis (RT-qPCR). All patients were diagnosed with NSCLC subtype adenocarcinoma and had T1N0M0 Stage IA. Nine patients were female and three patients were male with a mean age of 61 ± 10.4 years. Seven were smokers, and five were nonsmokers.

Isolation and primary culture of fibroblasts

Tumor tissues from the tumor front, matched inner tumor tissues, and matched adjacent normal lung tissues were washed three times with sterile phosphate-buffered saline (PBS). The tissues were minced with sterile scissors in a culture dish and digested for 30 min at 37°C in digestion buffer containing 0.25% collagenase type II and IV (Worthington Biochemical Corporation, Lakewood, NJ, USA). The cell suspension was washed with DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin (DMEM-S) at 500 × *g* for 10 min. After washing, the pellet was resuspended in fresh DMEM-S and plated onto a 25-cm^2 tissue culture flask at 37°C in a 5% CO_2 atmosphere for 2 days. Fibroblast-like adherent cells were maintained in DMEM-S. The third- or fourth-passage primary fibroblasts were used in all experiments. The primary fibroblasts isolated from the tumor front, inner tumor, and adjacent normal lung tissue were identified as F-CAFs, In-CAFs, and NFs, respectively.

Characterization of fibroblasts by immunocytochemistry

Primary CAFs and NFs were grown on coverslips, previously treated with 1:1 HCl:H_2SO_4 for 48 h and then fixed with cold acetone for 10 min on ice. After several washes with cold PBS, the coverslips were incubated with 3% hydrogen peroxide for 60 min and then with

1% bovine serum albumin for 60 min at room temperature to block nonspecific interactions. Fibroblasts were identified by immunocytochemistry using the following antibodies: anti-α-SMA (1:12,000), anti-PDGFR-β (1:600), anti-vimentin (1:4,000), and anti-pan-cytokeratin (1:6,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each antibody was incubated at 4°C overnight. After washing with PBS, the coverslips were incubated with biotinylated universal secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. After washing with PBS, the reaction was amplified and revealed with the Vectastain Elite ABC kit (Vector Laboratories).

Preparation of conditioned medium (CM)

F-CAFs, In-CAFs, and NFs were cultured in DMEM-S to 80% confluence. Then, the medium was changed to DMEM without FBS, and the cells were cultured for another 48 h. The CM was collected from the supernatant and centrifuged at 15,000 × *g* for 15 min to remove cell debris and stored at -80°C until further use.

Proliferation assay of A549 NSCLC cells

A549 human NSCLC cells (*American Type Culture Collection,* Manassas, VA, USA) were cultured in DMEM-S to 80% confluence and then in serum-free DMEM for 24 h at 37°C in a 5% CO_2 atmosphere. Next, the tumor cells were cultured in 96-well plates (5,000 cells per well) in the presence of CM from CAFs or NFs for 72 h at 37°C in a 5% CO_2 atmosphere. Then, 15 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Promega Corporation, Madison, WI, USA) was added to each well, and the plate was incubated for 4 h at 37°C. Finally, 100 μL of solubilization/stop solution (Promega) was added, and the absorbance was measured at 570 nm. Tumor cells were also cultured in serum-free DMEM as controls.

Cell migration and invasion assay of A549 NSCLC cells

Invasion and migration assays were performed with Corning BioCoat Matrigel Invasion Chambers with Falcon cell culture inserts containing an 8-μm pore size p*olyethylene terephthalate* (PET) membranes, following the manufacturer's instructions. Briefly, 2.5×10^4 A549 cells were seeded in each upper chamber in serum-free DMEM and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. The lower chamber was filled with serum-free DMEM containing CM from F-CAFs, In-CAFs, or NFs as chemoattractants, and the cells were incubated for an additional 24 h. Serum-free DMEM was used as a negative control. After incubation, the non-invading cells were removed from the upper surface of the membrane by scrubbing twice. The membranes were fixed in 70% ethanol and stained with 0.2% crystal violet. The total number of cells that migrated onto the underside of the membrane was counted using an optical microscope at 40 × magnification. Data were expressed as the percent invasion through the Corning® Matrigel® matrix and membrane relative to the migration of cells through the control membrane using the following formula: % invasion = mean number of cells invading through the Matrigel insert membrane / mean number of cells migrating through the control insert membrane × 100. Data are presented as the mean ± SD of four triplets of F-CAFs, In-CAFs and NFs (four patients) and represent at least two independent experiments performed in triplicate.

Proliferation and invasion assay of fibroblasts

Isolated CAFs and NFs were cultured in DMEM-S in 96-well plates (3,000 cells per well in quadruplicate) for 24 h, 48 h, 72 h and 96 h at 37°C in a 5% CO_2 atmosphere. Then, cell proliferation was detected by the MTT assay using the CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega Co.) following the manufacturer's instructions. For the migration and invasion assays, fibroblasts (4×10^4 cells) were cultured in serum-free DMEM in the upper chambers of 24-well Falcon™ Cell Culture inserts with an 8-μm PET membrane (Corning BioCoat Matrigel Invasion Chambers). DMEM-S was added to the lower chambers to serve as a chemoattractant. After 24 h, cells remaining on the upper filter were removed, while cells that passed through the inserts and Matrigel membrane were fixed and stained as described above.

MicroRNA microarray analysis

Total RNA was isolated from early passages (passages 3 to 4) primary cultures of matched F-CAFs, In-CAFs, and NFs (triplets) from four adenocarcinoma patients using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The total RNA concentration was assessed using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was validated by electrophoresis on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara CA, USA). Only RNA samples with an integrity number ≥ 7.5 were used for microarray analysis. Microarray studies were performed by the Instituto Nacional de Medicina Genomica (INMEGEN) using the Affymetrix Genechip miRNA 4.0 (Affimetrix, Santa Clara, CA, USA), which provides 100% miRBase v20 coverage, 2,578 human mature miRNA probe sets, 3,770 pre-miRNA hairpin sequence probe sets, and 1,996 human snoRNA and scaRNA probe sets. Briefly, total RNA (150 ng) was prepared for labeling with a FlashTag Biotin HSR RNA Labeling Kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's instructions (http://www.affymetrix.com/support/technical/manual.affx). Next, the samples were hybridized on the high-density array miRNA 4.0 (Affymetrix). Only one microarray screening was performed using RNA obtained from matched F-CAF, In-CAF and NF specimens. The expression signals were corrected with robust multiarray averaging (Irizarry et al. 2003) and normalized using quantile normalization (Bolstad et al. 2003). Differential expression was determined using statistical linear models, and contrasts of interest were analyzed using the Bioconductor library limma (Ritchie et al. 2015). Differentially expressed miRNAs were selected based on a log-fold change ≥ ±1, and $p \leq 0.05$ indicated statistical significance. The miRNA expression data are available in the Gene Expression Omnibus (GSE113805).

RT-qPCR assay for miRNAs

TaqMan Advanced miRNA Assays (Thermo Fisher Scientific, Waltham, MA, USA) were used to quantify the expression of candidate miRNAs. Total RNA (10 ng) from F-CAFs, In-CAFs, and NFs (triplets) from twelve patients diagnosed with primary lung adenocarcinoma patients were reverse transcribed using a TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems), according to the manufacturer's instructions. Relative quantification was performed with TaqMan Advanced miRNA assays on a StepOnePlus Real-Time PCR System (Applied Biosystems) in a reaction volume of 10 μL, containing 1× TaqMan Advanced Master Mix (Applied Biosystems) and 1× of the following specific probes: hsa-miR-505-3p (ID

478145_mir), hsa-miR-299 (ID 478792_mir), hsa-miR-145-3p (ID 477915_mir), hsa-miR-485-5p (ID 478126_mir), hsa-miR-410-3p (ID 478085_mir), hsa-miR-6875-3p (ID 480500_mir), hsa-miR-6735-5p (ID 480249_mir), hsa-miR-181c-5p (ID 477934_mir), hsa-miR-6867-5p (ID 480488_mir) and hsa-miR-5006-5p (ID 480081_mir) (all from Applied Biosystems). The thermal profile was as follows: 50°C for 2 min, 95°C for 20 s, and 40 cycles at 95°C for 1 s and 60°C for 20 s. All amplification reactions were performed in triplicate, and the relative quantification of miRNA expression was calculated using the comparative threshold cycle method ($\Delta\Delta$Ct) (Schmittgen and Livak 2008). MiRNAs with raw Ct values ≥ 35 were considered undetected. The levels of miRNA expression were assessed after normalization using the hsa-miR-23b-3p (ID 478602_mir) probe as an endogenous control. The hsa-miR-23b-3p probe was chosen from three candidate normalizers (miRNAs) that were identified from the microarray data, and it was later validated by RT-qPCR using the NormFinder algorithm (http://moma.dk/normfinder-software) (Andersen et al. 2004). qRT-PCR analysis was performed for three independent experiments. Statistically significant changes between groups were assessed by the Mann-Whitney U test. Data are presented as the mean ± standard error of the mean, and $p \leq 0.05$ indicated statistical significance. Statistical analysis was performed using GraphPad Prism Software (San Diego, CA, USA).

Target gene and pathway enrichment analysis

Target mRNAs of the miRNAs were predicted using TargetScan v7.1. The filtering of predicted target genes was set by a cumulative weighted context++ score (CWCS) ≤ –0.01. The enrichment pathway analysis was conducted using the Database for Annotation Visualization and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov). The pathway information was generated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Enriched pathways showing statistical significance ($p \leq 0.05$) were subjected to further molecular analysis and interactome network construction. Interactome networks were constructed to connect miRNAs to their putative target genes within the selected enriched pathways; the resulting networks were exported to Cytoscape v3.1.0 (https://cytoscape.org/) for visualization.

Quantitative Real-Time PCR for VEGFA, FGF2 and IGF1

Total RNA was extracted from F-CAF, In-CAF and NF primary cultures from 12 adenocarcinoma patients using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quantity and purity were measured using a NanoDrop 2000 (Thermo Scientific, Waltham, and Mass). Total RNA (1 μg) was reverse transcribed with a cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Amplification of insulin-like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) templates was performed by quantitative reverse transcriptase real-time PCR (RT-qPCR) using a StepOne system (Thermo Fisher Scientific, USA) with a thermal program including initial denaturation (10 minutes at 95°C) followed by 40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 40 sec at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The primer sequences were as follows: 5′-GCA ATG GGA AAA ATC AGC AG-3′ (Forward) and 5′-GAG GAG GAC ATG GTG TGC A-3′ (Reverse) for IGF1; 5′-GTG TGT GCT AAC CGT TAC CT-3′ (Forward) and 5′-GCT CTT AGC AGA CAT TGG AAG-3′ (Reverse) for FGF2; 5′-GCC TTG CTG CTC TAC CTC CA-3′ (Forward) and 5′-CAA GGC CCA CAG GGA TTT T-3′ (Reverse) for VEGFA and 5′-GAC CAC TTT GTC AAG CTC ATT TTC-3′ (Forward) and 5′- GTG AGG GTC TCT CTC TTC CTC TTG T-3′ (Reverse) for GAPDH.

Results

To characterize the differential miRNA expression profiles of fibroblasts isolated from matched tumor front (F-CAFs), inner tumor (In-CAFs), and normal adjacent (NFs) tissue in lung adenocarcinoma, we initially performed a microarray analysis of the global miRNA profiles in the screening phase, and the results were subsequently validated by qPCR. Fig. 1 shows an overview of the study design strategy.

Isolation and characterization of CAFs and NFs

Primary cultures of F-CAFs, In-CAFs, and NFs were successfully established from 12 patients diagnosed with primary lung adenocarcinoma. All primary fibroblasts showed a spindle-like morphology (Fig. 2A). CAF and NF purity were identified by immunocytochemical staining with the following antibodies: anti-pan-cytokeratin, anti-α-SMA, anti-PDGFR-β, and anti-vimentin. All fibroblasts expressed the fibroblast biomarkers vimentin and PDGFR-β. The absence of epithelial cancer cells was verified by a negative immunocytochemistry reaction to the specific epithelial cell marker pan-cytokeratin. In addition, α-SMA (a biomarker for active fibroblasts) was highly expressed in F-CAFs, less expressed in In-CAFs, and weakly expressed in NFs (Fig. 2A).

F-CAFs and In-CAFs differentially enhance the proliferation and invasion of lung adenocarcinoma cells

CAFs have been proposed to promote tumor growth through direct interaction or secreted factors (Orimo et al. 2005; Erez et al. 2010), but previous studies have not analyzed CAFs from different tumor zones or microenvironments in NSCLC. To determine whether F-CAFs and In-CAFs enhance tumor cell proliferation and invasion compared with NFs, A549 lung tumor cells were cultured *in vitro* with CM from four triplets of matched F-CAFs, In-CAFs, or NFs. The proliferation of A549 cells was increased significantly after culture with CM from F-CAFs or In-CAFs compared with CM from NFs (Fig. 2B). The enhanced proliferation of F-CAFs was more significant than that of In-CAFs when compared with NFs ($p = 0.0002$ and $p = 0.003$, respectively). Nevertheless, F-CAFs induced more significant proliferation of A549 cells than In-CAFs ($p = 0.0003$), suggesting a greater capacity of F-CAFs in CM to stimulate tumor growth.

To further explore whether CAFs from the tumor front and inner tumor tissues differentially enhance the metastatic potential of tumor cells, we performed an *in vitro* invasion assay using invasion chambers coated with Matrigel. The invasion capacity of A549 cells was significantly increased by CM from F-CAFs and In-CAFs compared with CM

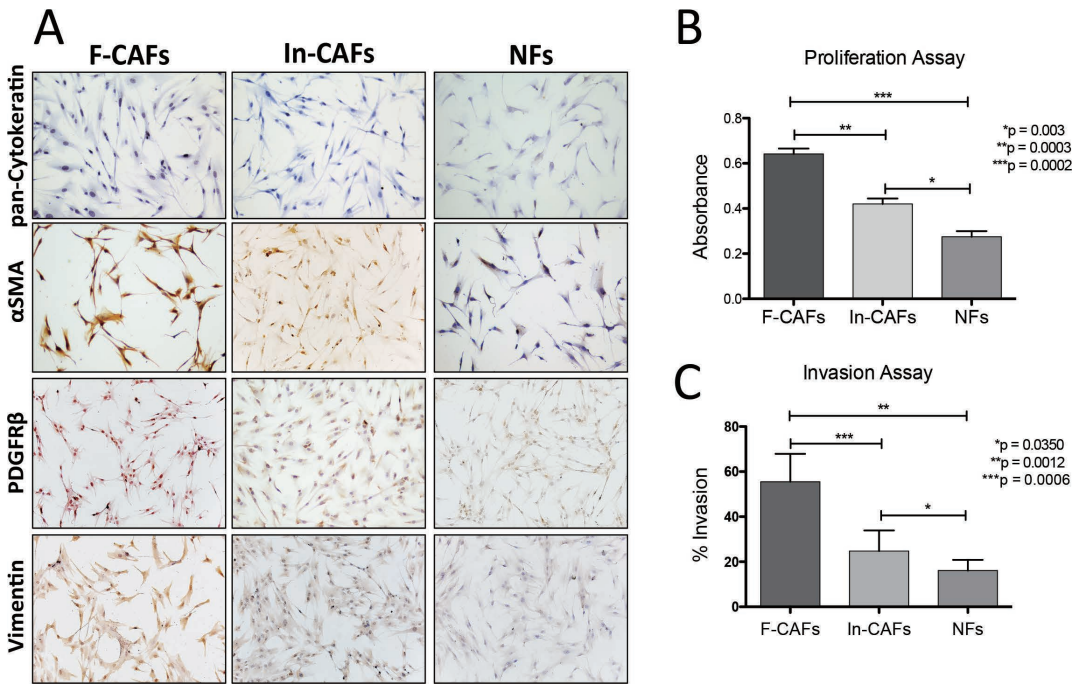

Fig. 2. Characterization of cancer-associated fibroblasts (CAFs) from the tumor front (F-CAFs) and the inner tumor (In-CAFs) and normal fibroblasts (NFs).
A) Representative immunocytochemical staining with anti-pan-cytokeratin, α-SMA, PDGFR-β, and vimentin antibodies of fibroblast primary cultures from 12 adenocarcinoma patients. All fibroblasts are negative for pan-cytokeratin (a biomarker for epithelial cells) and positive for vimentin and PDGFR-β (both biomarkers for fibroblasts). α-SMA, a biomarker for active fibroblasts, is highly expressed in F-CAFs, followed by In-CAFs, and weakly expressed in NFs (magnification, 20 ×). Brown color indicates positive staining, and blue background is indicative of negative staining. In the first row (pan-cytokeratin staining), a negative blue background is shown among all the cells. The second, third, and fourth rows show stained cells with a brownish color. B) Proliferation of A549 cells (MTT assay, 72 h) and C) Matrigel invasion ability of A549 cells cultured with conditioned media from F-CAFs, In-CAFs and NFs from four adenocarcinoma patients. Significance was tested with the two-tailed Mann-Whitney U test. Data are shown as the mean ± SD (n = 4).

from NFs (Fig. 2C), but F-CAFs enhanced invasion to a greater degree than In-CAFs when compared with NFs ($p = 0.0012$ and $p = 0.0350$, respectively). Furthermore, F-CAFs induced a more significant increase in the invasion capacity of A549 cells than In-CAFs ($p = 0.0006$).

In addition, we conducted proliferation and invasion assays with isolated fibroblasts. The data showed that F-CAFs, In-CAFs and NFs independently have different proliferation and invasion capacities. Fig. 3 shows that the proliferation capacity of F-CAFs was highest, followed by that of In-CAFs and NFs, and the invasion capacity of F-CAFs was greater than that of NFs.

Identification of differentially expressed miRNAs in CAFs

To determine whether CAFs from different tumor areas express a specific miRNA signature, we evaluated the global miRNA expression of CAFs from matching inner tumor, tumor front and normal adjacent tissues of four adenocarcinoma patients using a miRNA microarray screen. The miRNA expression data are available in the Gene Expression Omnibus (GSE113805). The results showed 13 upregulated and 7 downregulated miRNAs in F-CAFs compared with NFs, while three downregulated miRNAs were identified in In-CAFs compared with NFs. Additional analysis showed 12 upregulated and five downregulated miRNAs in F-CAFs compared with In-CAFs (Table 1). Among the miRNAs identified as downregulation in this study, miR-145-3p and miR-505-3p (downregulated in F-CAFs vs. NFs), miR-181c (downregulated in In-CAFs vs. NFs) and miR-410-3p and miR-485-5p (downregulated in F-CAFs vs. In-CAFs) have previously been shown to function as tumor suppressor genes and are downregulated in various tumor tissues (Yamamoto et al. 2011; Guo et al. 2015a; Guo et al. 2015b; He et al. 2016; Matsushita et al. 2016; Zhang et al. 2016).

Next, we selected ten different candidate miRNAs for RT-qPCR validation of the microarray results using matched F-CAFs, In-CAFs and NFs (triplets) from twelve patients diagnosed with primary lung adenocarcinoma. We chose these candidate miRNAs from the microarray results because these miRNAs exhibited the largest fold change or/and were associated with previously published evidence of relationships with tumors (Yamamoto et al. 2011; Shiah et al. 2014; Chen et al. 2015; Guo et al. 2015a; Guo et al. 2015b; Mussnich et al. 2015; He et al. 2016; Matsushita et al. 2016). The selected candidate miRNAs were miR-

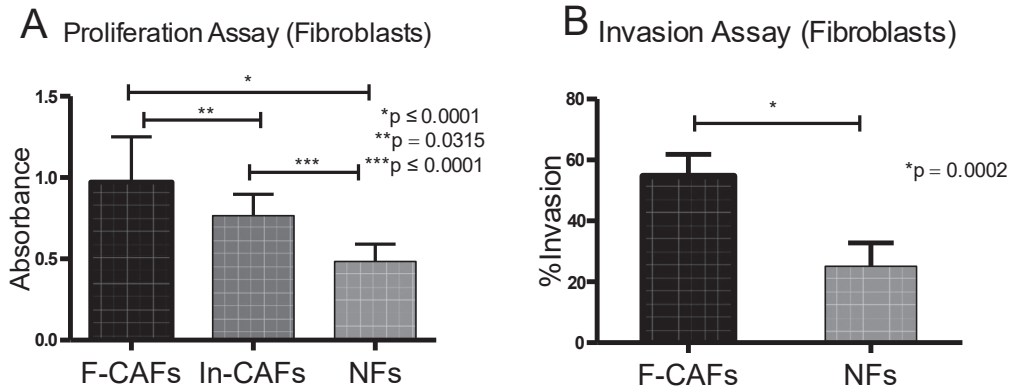

Fig. 3. Data of the proliferation and invasion capabilities of isolated fibroblasts.
A) Proliferation of isolated fibroblasts by MTT assay (72 h), and B) Matrigel invasion ability of isolated fibroblasts from 12 adenocarcinoma patients. Data are shown as the mean ± SD (n = 12).

Table 1. Differentially expressed miRNAs in CAFs identified by miRNA microarray analysis.

A) F-CAFs vs. NFs			B) In-CAFs vs. NFs			C) F-CAFs vs. In-CAFs		
miRNA name	**LogFCh**	**p value**	**miRNA name**	**LogFCh**	**p value**	**miRNA name**	**LogFCh**	**p value**
hsa-miR-6846-5p	2.093	0.0078	**hsa-miR-6867-5p**	–1.4053	0.00463	**hsa-miR-6875-3p**	1.85864	0.00307
hsa-miR-6875-3p	2.035	0.0016	**hsa-miR-5006-5p**	–1.4247	0.00761	**hsa-miR-6735-5p**	1.85167	0.00077
hsa-miR-1238-3p	1.864	0.0086	**hsa-miR-181c-5p**	–1.6099	0.00701	hsa-miR-2277-5p	1.66803	0.00929
hsa-miR-6797-5p	1.8581	0.0014				hsa-miR-6797-5p	1.51584	0.00565
hsa-miR-2392	1.5595	0.0082				hsa-miR-5006-5p	1.50211	0.00554
hsa-miR-3622a-3p	1.387	0.0033				hsa-miR-7846-3p	1.4855	0.003232
hsa-miR-6894-3p	1.342	0.0061				hsa-miR-550b-2-5p	1.4008	0.00937
hsa-miR-6735-5p	1.3123	0.0078				hsa-miR-6894-3p	1.2632	0.00884
hsa-miR-4472	1.292	0.0072				hsa-miR-640	1.2024	0.00456
hsa-miR-6877-3p	1.206	0.009				hsa-miR-4310	1.16143	0.00226
hsa-miR-3151-5p	1.094	0.0039				hsa-miR-939-3p	1.09312	0.00682
hsa-miR-939-3p	1.078	0.0073				hsa-miR-3151-5p	1.01181	0.00643
hsa-miR-659-3p	1.07	0.0073				**hsa-miR-485-5p**	–1.3157	0.00407
hsa-mir-520f	–1.0964	0.0078				hsa-miR-411-5p	–1.3525	0.00802
hsa-miR-505-3p	–1.2679	0.0041				hsa-miR-539-5p	–1.80719	0.00265
hsa-mir-299-3p	–1.2993	0.0041				hsa-miR-770-5p	–1.8076	0.00375
hsa-miR-411-5p	–1.3439	0.0083				**hsa-miR-410-3p**	–2.2362	0.00155
hsa-miR-4306	–1.6105	0.0006						
hsa-miR-6867-5p	–1.7415	0.001						
hsa-miR-145-3p	–2.0365	0.0036						

(A) Tumor front CAFs (F-CAFs) compared with normal fibroblasts (NFs), (B) inner tumor CAFs (In-CAFs) compared with NFs, and (C) F-CAFs compared with In-CAFs.
Matched primary cultures (F-CAFs, In-CAFs and NFs) from four adenocarcioma patients were used for microarray analysis.
Cut-off: log2 FCh > 1 and p value ≤ 0.01; FCh, fold change; Bold miRs: Chosen for RT-qPCR validation; Shadowed box: Reported as tumor suppressor and/or downregulated in tumor tissue in literature.

145-3p, miR-299-3p, and miR-505-5p (downregulated in F-CAFs vs. NFs); miR-6875-3p and miR-6735-5p (upregulated in F-CAFs vs. NFs); miR-181c-5p, miR-5006-5p, and miR-6867-5p (downregulated in In-CAFs vs. NFs); miR-410-3p and miR-485-5p (downregulated in F-CAFs vs. In-CAFs); and miR-6875-3p and miR-6735-5p (upregulated in F-CAFs vs. In-CAFs) (Table 1). Notice that two miRNAs (miR-6875-3p and miR-6735-5p) showed both upregulation in F-CAFs compared with NFs and upregulation in F-CAFs compared with In-CAFs.

Of these ten candidate miRNAs, expression levels were significantly downregulated for miR-145-3p, miR-299-3p and miR-505-3p (F-CAFs vs. NFs) and for miR-410-3p and miR-485-5p (F-CAFs vs. In-CAFs) (Fig. 4). However, no significant differences were detected in miR-181c-5p or miR-5006-5p with respect to In-CAF vs. NF

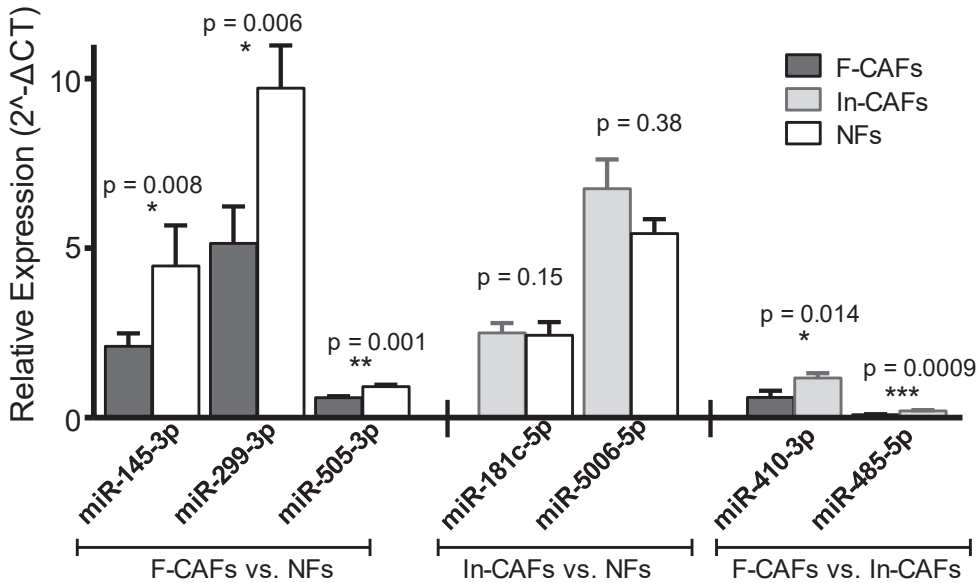

Fig. 4. Quantification of the expression levels of candidate miRNAs in F-CAFs, In-CAFs and NFs by qPCR. The expression levels of 10 candidate miRNAs in F-CAFs, In-CAFs and NFs from 12 adenocarcinoma patients were analyzed by qPCR. Of those miRNAs, seven were detectable in the samples. The expression levels of miR-145-3p, miR-299-3p and miR-505-3p (F-CAFs vs. NFs) and miR-410-3p and miR-485-5p (F-CAFs vs. In-CAFs) were significantly downregulated in F-CAFs. No significant differences were detected in miR-181c-5p and miR-5006-5p (In-CAFs vs. NFs) expression levels. Data are shown as relative expression (2^-ΔCT). $p \leq 0.05$ indicates statistical significance (n = 12).

expression. The levels of the other three miRNAs were undetectable in the tested samples.

Pathway enrichment analysis indicates that miRNAs differentially expressed in F-CAFs are associated with mechanisms that promote carcinogenesis

To determine the potential biologic relevance of the five validated miRNAs, a target gene prediction and pathway enrichment analysis was conducted. First, target genes from validated miRNAs were predicted using TargetScan v7.1. These predicted target genes were used for subsequent pathway enrichment analysis using DAVID v6.8, as indicated in the Materials and Methods section.

The top pathways enriched by miR-145-3p, miR-299-3p and miR-505-3p putative target genes (F-CAFs vs. NFs) are shown in Table 2. The top pathways enriched by miR-410-3p and miR-485-5p putative target genes (F-CAFs vs. In-CAFs) are shown in Table 2. Notably, "pathways in cancer" was the third most significantly enriched pathway in F-CAFs vs. NFs (p = 8.3E-5) and the 11th most enriched pathway in F-CAFs vs. In-CAFs (p = 2.0E-3). Other relevant enriched pathways were "Wnt signaling pathway" (p = 6.6E-3 in F-CAFs vs. NFs and p = 1.8E-4 in F-CAFs vs. In-CAFs) and "transforming growth factor beta (TGF-beta) signaling pathway" (p = 1.4E-2 in F-CAFs vs. In-CAFs). These pathways have key roles in carcinogenesis (Lamouille et al. 2014). The TGF-beta signaling pathway is also associated with tumor promotion by CAF-secreted factors (Ishii et al. 2016).

We focused subsequent analyses on "pathways in cancer", "Wnt signaling pathway" and "TGF-beta signaling pathway" to clarify major mechanisms potentially involved in carcinogenesis and metastasis. First, we determined which deregulated miRNAs and their predicted target genes were associated with each pathway by analyzing the individual miRNAs using DAVID v6.8. Next, we generated networks of the miRNAs and their predicted target genes that were significantly associated with those pathways.

We found that miR-145-3p and miR-299 were significantly associated with "pathways in cancer", while miR-505-3p was associated with "Wnt signaling pathway" (Fig. 5A). On the other hand, miR-410-3p was significantly associated with "pathways in cancer," "Wnt signaling pathway," and "TGF-beta signaling pathway"; however, miR-485-5p was not associated with those pathways (Fig. 5B). Notably, miR-299-3p targeted three growth factors, namely, IGF1, VEGFA and FGF2 (Fig. 5A), which are secreted by CAFs as a mechanism of tumor progression (Ishii et al. 2016). Additionally, miR-145-3p targeted IGF1 receptors (IGF1Rs) and endothelial growth factor receptors (Fig. 5A), whereas miR-410-3p targeted IGF1 (Fig. 5B).

VEGFA is upregulated in F-CAFs compared with NFs

To investigate whether the expression levels of three miRNA targets are upregulated, we quantified the expression of VEGFA, FGF2 and IGF1 by RT-qPCR. Fig. 6 shows the expression levels of VEGFA and FGF2. IGF1 expression was not consistently detected in most of the samples analyzed and is, therefore, not shown in the figure. The results showed that the level of VEGFA was upregu-

Table 2. The top KEGG pathways enriched by hsa-miR-145-3p, miR-299-3p and hsa-miR-505-3p putative target genes (F-CAFs vs. NFs) and by hsa-miR-410-3p and hsa-miR-485-5p putative target genes (F-CAFs vs. In-CAFs).

F−CAFs vs. NFs

KEGG_PATHWAY	Genes Count	p value
Proteoglycans in cancer	52	5,7E-6
Rap1 signaling pathway	53	1,1E-5
Pathways in cancer	82	8,3E-5
cAMP signaling pathway	47	1,9E-4
Oxytocin signaling pathway	39	3,2E-4
PI3K-Akt signaling pathway	70	6,9E-4
TNF signaling pathway	28	9,1E-4
Hepatitis B	35	1,1E-3
Neuroactive ligand-receptor interaction	57	1,7E-3
Focal adhesion	45	1,7E-3
AMPK signaling pathway	30	1,9E-3
Viral carcinogenesis	44	2,7E-3
Prolactin signaling pathway	20	2,7E-3
Acute myeloid leukemia	17	2,8E-3
ErbB signaling pathway	23	2,9E-3
Neurotrophin signaling pathway	29	3,0E-3
cGMP-PKG signaling pathway	37	3,3E-3
Cholinergic synapse	27	4,0E-3
Hippo signaling pathway	34	4,2E-3
FoxO signaling pathway	31	4,2E-3
Insulin resistance	26	5,5E-3
Wnt signaling pathway	31	6,6E-3

F−CAFs vs. In−CAFs

KEGG_PATHWAY	Genes Count	p value
Hippo signaling pathway	27	3,6E-9
Signaling pathways regulating pluripotency of stem cells	22	1,3E-6
Axon guidance	19	1,7E-5
Basal cell carcinoma	12	3,1E-5
Proteoglycans in cancer	24	3,9E-5
Melanogenesis	16	4,4E-5
Wnt signaling pathway	18	1,8E-4
Thyroid hormone signaling pathway	16	2,0E-4
Rap1 signaling pathway	22	6,0E-4
Adherens junction	11	1,4E-3
Pathways in cancer	32	2,0E-3
Insulin resistance	13	3,8E-3
Glycosaminoglycan biosynthesis	6	4,1E-3
Transcriptional misregulation in cancer	17	4,3E-3
ErbB signaling pathway	11	6,2E-3
Ras signaling pathway	20	7,6E-3
Neurotrophin signaling pathway	13	8,7E-3
cAMP signaling pathway	18	9,2E-3
TGF-beta signaling pathway	10	1,4E-2

p value of ≤ 0.05 was used to select significant pathways. TargetScan v7.1 was used for prediction of target genes. DAVID 6.8 was used for enrichment analysis.

lated in F-CAFs compared with NFs ($p = 0.03$). There was no significant difference in the expression level of FGF2 among the fibroblasts.

Discussion

Tumor progression involves complex and dynamic interactions of cancer cells and stromal components within the tumor microenvironment. CAFs are the main component of the tumor stroma and play a crucial role in tumor progression. Recent evidence indicates that miRNAs are involved in the capacity of CAFs to promote tumor progression in several types of cancer, but their role in NSCLC remains poorly defined. Additionally, no research has been performed to date analyzing CAFs from different tumor zones in lung cancer, despite evidence of tumor microenvironment heterogeneity.

In this study, we analyzed the expression pattern of miRNAs from CAFs isolated from different topological areas of lung adenocarcinoma. We were able to isolate fibroblasts from the center of the tumor mass (In-CAFs) despite speculation that CAFs are located primarily at the tumor margins. We found that both F-CAFs and In-CAFs expressed CAF markers and enhanced proliferation and invasion of lung cancer cells compared with NFs. However, F-CAFs showed the strongest biological effect on cancer cells, suggesting that these fibroblasts differentially affect tumor promotion compared with In-CAFs (Fig. 1).

According to these findings, we expected that F-CAFs and In-CAFs would show distinct miRNA expression patterns compared with NFs. However, qPCR analysis showed only miRNAs differentially expressed in F-CAFs compared with both NFs and In-CAFs. This finding may indicate that the observed biological effect of In-CAFs on cancer cells may not be regulated primarily by miRNAs. In contrast, several significantly downregulated miRNAs were identified in F-CAFs compared with both NFs (miR-145-3p, miR-299-3p and miR-505-3p) and In-CAFs (miR-410-3p and miR-485-5p) (Fig. 2).

As reported previously, miR-145-3p and miR-505-3p (Yamamoto et al. 2011; Matsushita et al. 2016) as well as miR-410-3p and miR-485-5p (Shiah et al. 2014; Chen et al. 2015; Mussnich et al. 2015) are downregulated in tumor tissues from different cancers and appear to function as tumor suppressor genes. Consistent with these reports, the pathway enrichment analysis in this study showed that miR-145-3p, miR-299-3p (F-CAFs vs. NFs) and miR-410-3p (F-CAFs vs. In-CAFs) were significantly associated with “pathways in cancer”, whereas miR-505-3p (F-CAFs

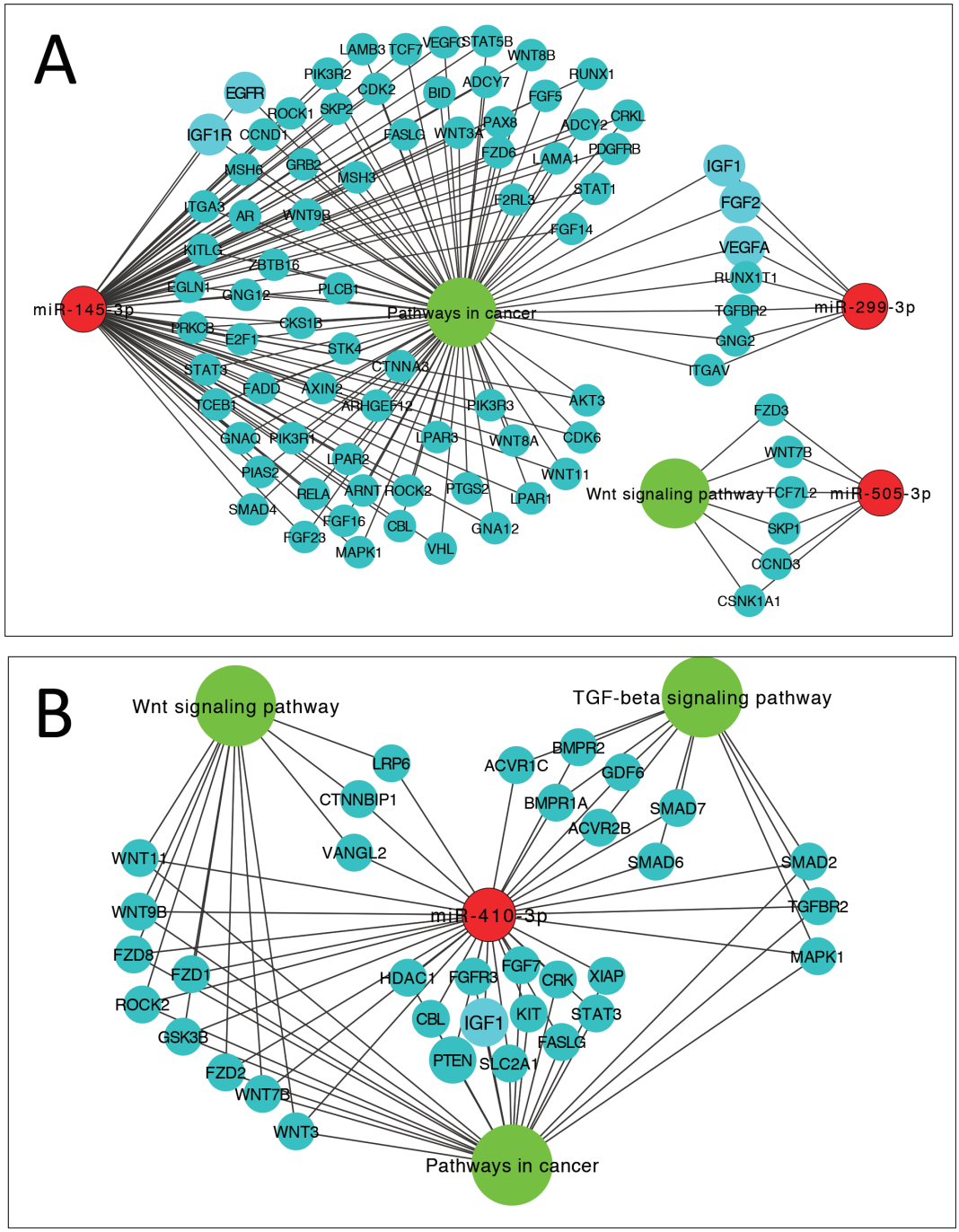

Fig. 5. Networks of miRNAs and their putative target genes significantly associated with "pathways in cancer," "Wnt signaling pathway," and "TGF-beta signaling pathway."
A) Network generated with downregulated miR-145-3p, miR-299-3p and miR-505-3p from F-CAFs vs. NFs. B) Network generated with downregulated miR-410-3p from F-CAFs vs. In-CAFs. Red circles represent miRNAs. Blue circles represent predicted target genes. Light blue circles represent molecules involved in CAF secretory mechanisms associated with tumor progression according to the literature.

vs. NFs) was associated with the "Wnt signaling pathway". Wnt has been shown to contribute to cancer progression (Zhan et al. 2017), but only one study of fibroblasts and the Wnt pathway (fibroblast-derived Wnt-ligand Wnt3a) evaluating breast cancer promotion and inhibition has been conducted thus far (Green et al. 2013). Notably, miR-410-3p is also associated with the "Wnt signaling pathway" and the "TGF-beta signaling pathway". The TGF-beta signaling pathway is an important pathway that has been implicated in metastasis (Padua and Massague 2009) and plays a key role in CAF mechanisms that promote tumor progression (Ishii et al. 2016).

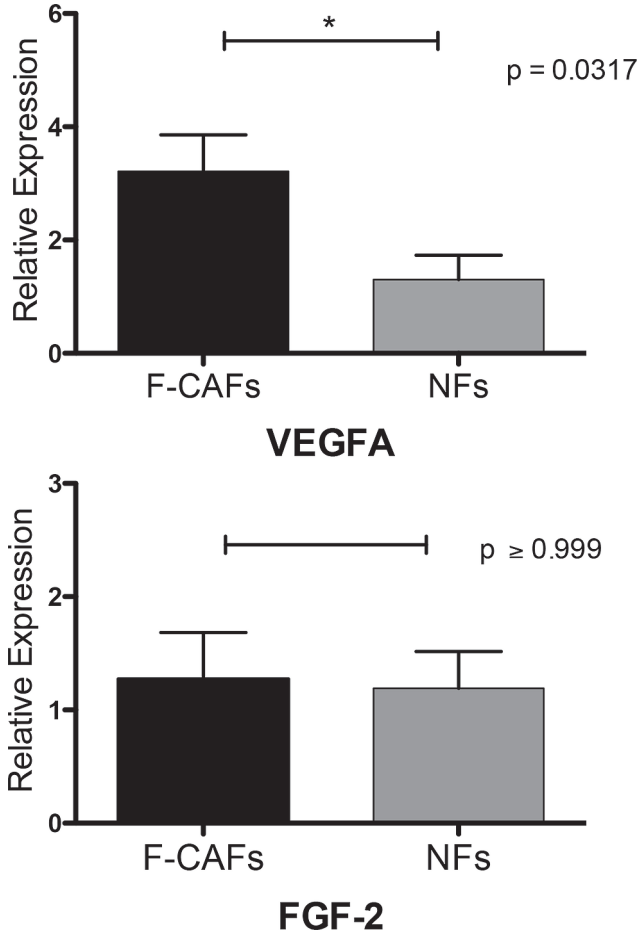

Fig. 6. Expression levels of VEGFA and FGF2 in F-CAFs compared with NFs by qPCR. The relative expression levels of VEGFA and FGF2 were quantified in F-CAFs and NFs from 12 adenocarcinoma patients by qPCR. $p \leq 0.05$ indicates statistical significance (n = 12).

Further analysis of the network comprising miRNAs and their putative target genes enriched in "pathways in cancer" point to three growth factors: IGF1 (mir-299-3p), FGF2 and VEGFA (miR-299 and mir-410-3p). It is widely accepted that CAFs promote tumor progression via the secretion of several factors, including IGF1, FGF2 and VEGF (Shiga et al. 2015; Ishii et al. 2016). Direct evidence indicates that IGF1 produced by stromal fibroblasts induces survival and migration of melanoma cells (Satyamoorthy et al. 2001, 2002), whereas FGF2 produced by CAFs induces tumor growth in hormone-independent mouse mammary tumors (Giulianelli et al. 2008). Furthermore, increased secretion of VEGFA by stromal fibroblasts stimulated by IL-6 may induce angiogenesis in colon cancer (Nagasaki et al. 2014).

Additionally, predictive data also indicate that type-2 TGF-beta receptors (TGFBR2) are targeted by downregulated miR-410-3p, suggesting that this receptor could be upregulated in F-CAFs. Although CAF secretion of TGF-beta and overexpression of type-1 TGF-beta receptors have been associated with tumor progression (Forrester et al. 2005; Ishii et al. 2016), TGFBR2 is downregulated in CAFs in breast cancer and oral carcinoma. Moreover, loss of TGFBR2 expression in mouse CAFs is linked to tumor initiation and metastasis (Meng et al. 2011; Busch et al. 2015). Because clinical and experimental data already demonstrated that this pathway could have opposing roles in tumor progression even within the same tumor type (Padua and Massague 2009; Smith et al. 2012), this discrepancy is not unexpected and indicates that a more in-depth analysis of this pathway in CAFs of NSCLC is still needed.

We also tested the expression levels of three of the predicted target genes that encode growth factors (VEGFA, FGF2 and IGF1), and we found evidence that VEGFA is upregulated in F-CAFs compared with NFs. This is consistent with the hypothesis that downregulated miRNAs may regulate key mechanisms of CAFs to promote tumor progression in NSCLC through regulation of this growth factor. This result provides an avenue for further investigations; however, the roles of FGF2 and IGF1 should not be ruled out because these growth factors were not quantified in culture supernatant from the fibroblasts. Additionally, multiple factors may be involved in the mechanisms of CAFs to promote tumor progression. Other predictive pathways, such as TGF-beta, may be involved and should be further investigated.

Alternatively, the potential role of miRNAs secreted by exosomes in CAFs in the ability of cancer cells to migrate and invade cannot be ruled out. Exosomal miRNAs can be taken up by bystander cells and functionally delivered as a mechanism of cell-to-cell communication (Bach et al. 2017; Bayraktar et al. 2017; Yang et al. 2017). However, it has been demonstrated that the secreted miRNA expression pattern does not always match that of endogenous miRNAs (Valadi et al. 2007; Pigati et al. 2010). Interestingly, relatively little information is available about the potential role of exosomal miRNAs released by human primary CAFs (Yang et al. 2017). Nevertheless, a recent study showed that miRNAs released by CAFs affect cancer cell migration, invasion and metastasis in hepatocellular carcinoma (Zhang et al. 2017).

Although there was essentially no overlap between the differentially expressed miRNAs discovered in this study and those described in CAFs from other types of tumors, it is known that each tissue type has a specific miRNA signature, which could explain this discrepancy (Chang et al. 2002; Rinn et al. 2006). The only other recent study that performed miRNA profiling of paired CAFs and matched NFs from lung adenocarcinoma (from three patients) did not provide localization details about CAF isolation (Shen et al. 2016). Tumor microenvironment heterogeneity (Facciabene et al. 2011; Halama et al. 2011; Quail and Joyce 2013) might explain the lack of commonly altered miRNAs between that study and ours. In this regard, one limitation of our study was the macroscopic method used to identify the center and the edge of the tumor mass. However, our data showed that F-CAFs and In-CAFs were

distinguishable by their capacity to promote the proliferation and invasion of A549 tumor cells, even when a specific miRNA signature could not be validated in In-CAFs. To standardize research data on solid tumors, we posit that the components of the tumor microenvironment should be analyzed with respect to their topological location within the tumor tissue using more precise methods such as laser microdissection. Interestingly, a recent study provided indirect evidence that CAFs from lung adenocarcinoma induced stronger expression of integrins and Bcl2 on invasive tumor front cancer cells than in inner tumor cancer cells (An et al. 2013).

Remarkably, no previous studies have analyzed CAFs from different NSCLC tumor zones, despite evidence that the tumor microenvironment is heterogeneous. Interestingly, there are two studies that reported evidence of the biological heterogeneity of CAFs within the same tumor by establishing single cell-derived clones from parental CAFs isolated from three (Neri et al. 2016) and two (Hashimoto et al. 2017) different lung adenocarcinoma patients. The authors of the previous study initially did not find significant differences in the invasion capacity of parental CAFs among the different patients; however, among the single cell-derived clones from each parental CAF, the invasion abilities of the CAF clones and the cocultured cancer cells varied greatly (Neri et al. 2016). In addition, the expression levels of two specific genes associated with CAF carcinogenesis mechanisms were different among the clones (Hashimoto et al. 2017). Therefore, this heterogeneity among CAFs within a tumor can be detected only when single cell-derived clones are obtained. In these studies, there is no indication that parenteral CAFs were obtained from a specific topographical tumor area from each patient, which highlights the relevance of the different properties observed in CAFs isolated from two distinct topographical areas within the same tumor in each patient in our study. Indeed, both those studies and the current study provide evidence of biological heterogeneity of CAFs within the same tumor in lung adenocarcinoma.

Overall, our results provide new insights into the potential molecular mechanisms involved in the tumor-promoting effect of CAFs in lung cancer; however, further experiments should be performed to validate the biological relevance of these findings. Transfection of miRNAs in CAFs or experimental downregulation of miRNAs in normal fibroblasts could provide experimental evidence of the role of miRNAs in CAF tumor-related properties.

In conclusion, our findings suggest that differentially expressed miRNAs in CAFs from the tumor front, which most strongly affect cancer cell invasion and proliferation capacity, have potential predictive associations with CAF tumor-promoting properties. This study provides new clues for further studies aimed at understanding the molecular mechanisms of CAFs and tumor microenvironment interactions in NSCLC progression.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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