Cancer-associated fibroblasts activated by miR-196a promote the migration and invasion of lung cancer cells

Sieun Lee\textsuperscript{a,b,1}, Ji Hyung Hong\textsuperscript{c,1}, Jeong Seon Kim\textsuperscript{a,b}, Jung Sook Yoon\textsuperscript{c}, Sang Hoon Chun\textsuperscript{c}, Soon Auck Hong\textsuperscript{d}, Eun Ju Kim\textsuperscript{a,b}, Keunsoo Kang\textsuperscript{e}, Jihee Lee Kang\textsuperscript{b,f}, Yoon Ho Ko\textsuperscript{c,g,2}, Young-Ho Ahn\textsuperscript{a,b,*}

\textsuperscript{a} Department of Molecular Medicine, College of Medicine, Ewha Womans University, Seoul, 07804, South Korea
\textsuperscript{b} Inflammation-Cancer Microenvironment Research Center, College of Medicine, Ewha Womans University, Seoul, 07804, South Korea
\textsuperscript{c} Division of Oncology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, 06591, South Korea
\textsuperscript{d} Department of Pathology, College of Medicine, Chung-Ang University, Seoul, 06974, South Korea
\textsuperscript{e} Department of Microbiology, College of Science & Technology, Dankook University, Cheonan, 31116, South Korea
\textsuperscript{f} Department of Physiology, College of Medicine, Ewha Womans University, Seoul, 07804, South Korea
\textsuperscript{g} Cancer Research Institute, College of Medicine, The Catholic University of Korea, Seoul, 06591, South Korea

\textsuperscript{1} Sieun Lee and Ji Hyung Hong contributed equally to this work.

\textsuperscript{*} Corresponding author. Department of Molecular Medicine, College of Medicine, Ewha Womans University, 25 Magokdong-ro 2-gil, Gangseo-gu, Seoul, 07804, South Korea.
\textsuperscript{2} Corresponding author. Division of Oncology, Department of Internal Medicine, Eunpyeong St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, 1021 Tongil-ro, Eunpyeong-gu, Seoul, 03312, South Korea.

E-mail addresses: koyoonho@catholic.ac.kr (Y.H. Ko), yahn@ewha.ac.kr (Y.-H. Ahn).

\textbf{ARTICLE INFO}

\textbf{Keywords:}
Cancer-associated fibroblasts
miR-196a
Tumor microenvironment
Invasion
Lung adenocarcinomas

\textbf{ABSTRACT}

Fibroblasts in the tumor microenvironment, known as cancer-associated fibroblasts (CAFs), promote the migration, invasion, and metastasis of cancer cells when they are activated through diverse processes, including post-transcriptional regulation by microRNAs (miRNAs). To identify the miRNAs that regulate CAF activation, we used NanoString to profile miRNA expression within normal mouse lung fibroblasts (LFs) and CAFs. Based on NanoString profiling, miR-196a was selected as a candidate that was up-regulated in CAFs. miR-196a-overexpressed LFs (LF-196a) promoted the migration and invasion of lung cancer cells in co-culture systems (Transwell migration and spheroid invasion assays). ANXA1 was confirmed as a direct target of miR-196a, and adding back ANXA1 to LF-196a restored the cancer cell invasion promoted by miR-196a. miR-196a increased CCL2 secretion in fibroblasts, and that was suppressed by ANXA1. Furthermore, blocking CCL2 impeded cancer spheroid invasion. In lung adenocarcinoma patients, high miR-196a expression was associated with poor prognosis. Collectively, our results suggest that CAF-specific miR-196a promotes lung cancer progression in the tumor microenvironment via ANXA1 and CCL2 and that miR-196a will be a good therapeutic target or biomarker in lung adenocarcinoma.

1. Introduction

Lung cancer is a major cause of cancer-related death worldwide [1]. Although diverse therapies against lung cancer have been developed, the 5-year survival rate of lung cancer patients is still much lower than that of other cancer patients [2]. The poor prognosis of lung cancer patients is mostly due to the high prevalence of metastasis [3]. The metastatic potential of cancer cells is determined by their direct or indirect interactions with the multiple types of surrounding cells, such as immune cells, blood vessel cells, and fibroblasts, that compose the tumor microenvironment along with secretory signaling factors and the extracellular matrix. The tumor microenvironment associated with lung cancer progression has been attracting attention in investigations of effective prognostic and therapeutic targets [4]. To achieve effective patient treatment and improve patient survival rates, novel anti-cancer strategies targeting both cancer cells and the tumor microenvironment are urgently required.

Fibroblasts in the tumor microenvironment promote the
proliferation, epithelial-mesenchymal transition (EMT), migration, and invasion of cancer cells. Cancer-associated fibroblasts (CAFs) promote EMT and tumor progression by secreting cytokines and growth factors such as IL-6, VEGF-A, EGF, and TGF-β in lung cancer [5,6] and endometrial cancer [7]. Moreover, CAFs regulate cancer invasion through direct physical interactions with cancer cells [8]. This direct interaction enhances cancer stemness in pancreas cancer [9]. Through both paracrine signaling and physical contact, CAFs facilitate cancer development and metastasis.

The influence of CAFs on cancer exacerbation can be modulated by microRNAs (miRNAs). miRNAs are endogenous inhibitors of gene expression that silence target mRNAs by inducing mRNA cleavage or blocking protein translation [10]. As a form of paracrine signaling, metastasis via exosome-mediated miRNA delivery [11,12]. Cancer cells expression that silence target mRNAs by inducing mRNA cleavage or exosomes in the tumor microenvironment [13].

Obviously, intracellular miRNAs can also modulate the functions of CAFs in the tumor microenvironment. During the process of CAF activation, global miRNA expression changes occur; however, the functions and roles of CAF-specific miRNAs have not yet been studied in detail. Therefore, in this study, we sought to identify CAF-specific miRNAs and demonstrate their roles in the tumor microenvironment. We performed miRNA expression profiling in CAFs and normal lung fibroblasts (LFs) using the NanoString method and identified numerous miRNAs that were up- or down-regulated in CAFs, for further study. CAFs with high miR-196a expression enhanced cancer cell migration, invasion, and metastasis. In lung adenocarcinoma patients, high miR-196a expression was found to be associated with poor prognosis. These findings suggest that CAF-specific miR-196a can be considered as a cancer biomarker and a new therapeutic target in lung adenocarcinoma.

2. Materials and methods

2.1. Cell culture

344SQ, 344LN, 531LN2 (murine) and A549 (human) lung cancer cells were cultured in RPMI 1640 (Welgene, Gyeongsan, Korea) with 10% fetal bovine serum (FBS; HyClone, Logan, UT) at 37 °C in the presence of 5% CO2. To authenticate A549 cell line, short tandem repeat profiling was performed using Powerplex 18D kit (Promega, Madison, WI). Normal murine LFs were isolated from the lungs of 129/Sv wild type mice, and CAFs were isolated from the lung tumors of Kras-mutant (KrasLA1) mice using magnetic-activated cell sorting with a fibroblast-specific marker, THY-1, as described previously [6]. Human lung fibroblasts were isolated from normal lung tissues surrounding surgically resected lung tumors. Fibroblasts were then cultured in alpha-MEM (Welgene, Gyeongsan, Korea) containing 20% METHOCEL (Sigma-Aldrich) and 1% Matrigel (BD Biosciences, Franklin Lakes, NJ) were hung on the lids of 150-mm dishes dividing the total invaded area by the central spheroid area, as measured at 90 °C until required. ANXA1 neutralizing antibody (anti-human/mouse/rat Annexin A1 polyclonal goat IgG, #AF-3770), CCL2 neutralizing antibody (anti-mouse CCL2/JE/MCP-1 polyclonal goat IgG, #AF-479), and recombinant mouse CCL2 protein (#479-JE) were from R&D Systems (Minneapolis, MN).

2.2. miRNA expression profiling by NanoString

Total RNA was isolated from LFs and CAFs in triplicate using an AccuPrep Universal RNA Extraction Kit (Bioneer). After assessing RNA quantity and quality, miRNA expression profiling was performed by PhiloKorea (Seoul, Korea) using a nCounter Mouse v1.5 Assay kit (NanoString Technologies, Seattle, WA). miRNAs with oligonucleotide tags were hybridized with the miRNA code sets and processed according to the manufacturer’s instructions. miRNA data were collected and quantified using the nCounter Digital Analyzer and analyzed using nSolver software.

2.3. Transwell migration assay

Fibroblasts (1 × 10^5 cells per well) were seeded in 24-well plates one day before the experiment. 344SQ cells (1 × 10^5 cells per well) were then seeded in hanging inserts (SPL-Inert Hanging PET membrane, 8 μm pore; SPL Life Sciences, Pocheon, Korea) in serum-free medium. Cells were incubated at 37 °C for 24 h, and the 344SQ cells that migrated were stained with 0.1% crystal violet. Three randomly selected microscopic fields (×100 magnification) per chamber were photographed and counted manually using the ImageJ (http://imagej.nih.gov/ij) Cell Counter plug-in.

2.4. Spheroid invasion assay

To create spheroids, mcherry-labeled 344SQ cells (5 × 10^4 cells) and GFP-labeled fibroblasts (1 × 10^5 cells) in 5 mL of complete medium containing 20% METHOCEL (Sigma-Aldrich) and 1% Matrigel (BD Biosciences, Franklin Lakes, NJ) were hung on the lids of 150-mm dishes and incubated at 37 °C for 2 days. A spheroid mixture (spheroids in 0.5 × PBS, 0.01 N NaOH, and 3 mg/mL collagen) was then implanted in the center of each well of a 12-well plate. After the gels were polymerized, the wells were filled with cell culture medium. In addition, 344SQ-alone spheroids were overlaid on a confluent fibroblast feeder layer. After 1–2 days, phase-contrast and fluorescence microscopy were used to capture images of the invading cells. The invasion ratio was calculated by dividing the total invaded area by the central spheroid area, as measured using ImageJ.
2.5. Matrigel 3-D culture

Cells were grown in 3-D culture on Matrigel in 8-well chamber sides, as described previously [14]. In brief, mCherry-labeled 344SQ cells (5 × 10^5 cells) prepared as a single-cell suspension were plated onto a layer of growth factor–reduced Matrigel and grown in complete growth medium with 2% Matrigel. After 7 days, GFP-labeled fibroblasts (1 × 10^6 cells) were overlaid on the acini of 344SQ cells. After another 2-3 days, fluorescence and confocal microscopy (LSM800; Zeiss, Oberkochen, Germany) were used to capture cellular images. The percentages of cancer cell spheres with an intact central lumen in total spheres were presented.

2.6. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from cells using WelPrep Total RNA Isolation Reagent (Welgene) according to the manufacturer’s protocol. To analyze mRNA levels, qRT-PCR assays were performed using a BioFACT A-Star Real-time PCR Kit with SFCgreen1 (BioFACT, Daejeon, Korea) after reverse transcription with an ELPS RT Prime Kit (Elsips-Biotech, Daejeon, Korea). mRNA levels were normalized to Rpl32 mRNA. The sequences of the qRT-PCR primers used in this paper are presented in Supplementary Table 1, miRNA levels were quantified using an HB miR Multi Assay Kit (Heimbiotek, Seongnam, Korea) according to the manufacturer’s protocol, and they were normalized to the level of RNU6B snoRNA.

2.7. 3′-UTR reporter assay

The ANXA1 3′-untranslated region (UTR, 286 bp) was amplified by PCR from 293T cell genomic DNA and cloned into the psiCHECK-2 vector (Promega). 293T cells (1 × 10^5 cells/well) were seeded on 24-well plates one day prior to transfection and then transfected with an ANXA1 3′-UTR reporter (500 ng) using the TransIT-X2 reagent (Mirus Bio) in the presence or absence of a miR-196a mimic (20 nM; Bioneer). Two days after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega).

2.8. Cytokine array

Conditioned media from LFs transduced with miR-196a (LF-196a) or empty lentiviral vector (LF-vec) were prepared as described above. Using a Proteome Profiler Mouse XL Cytokine Array Kit (R&D Systems), array membranes were incubated overnight with conditioned medium (4 mL), and membrane-bound cytokines were detected using biotinylated detection antibodies and streptavidin-horseradish peroxidase. The pixel densities of the cytokine spots were analyzed using Quantity One software (Bio-Rad, Hercules, CA).

2.9. Mouse experiments

Before initiation, all proposed mouse studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University College of Medicine (EUM19-0451). Mice were cared for and euthanized according to the standards set forth by the IACUC. 344SQ cells (1 × 10^6 cells in 100 μL of PBS per mouse) were subcutaneously injected into syngeneic (129/Sv) mice in the right flank. Beginning two days later, conditioned medium (100 μL per mouse) derived from LF-196a or LF-vec was administered via intratumoral injection three times a week. The mice were euthanized five weeks after 344SQ cell injection. They were necropsied to isolate the primary tumors and normal tissues, as explained above.

2.10. RNA sequencing

Total RNAs were isolated in triplicate from LF-vec and LF-196a using an AccuPrep Universal RNA Extraction Kit (Bioneer) and sent to Macrogen (Seoul, Korea) for RNA sequencing. After checking the quantity and integrity of the total RNA, an RNA library was constructed from 1 μg of total RNA using an Illumina TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA). Paired-end (2 × 100 bp) sequencing of the indexed library was performed using Illumina NovaSeq (Illumina), and RNA sequencing data were analyzed using the Octopus-toolkit [15].

2.11. RNA extraction from formalin-fixed paraffin-embedded (FFPE) tumors

This study was approved by the Institutional Review Board of Catholic Medical Center (No. UCI17SESI0073) and was performed following the guideline for human research. Patients with lung adenocarcinoma (n = 140) or head and neck squamous cell carcinoma (n = 27) were retrospectively selected from among those who underwent surgical resection with a curative aim at Seoul St. Mary’s Hospital, Yeouido St. Mary’s Hospital, Bucheon St. Mary’s Hospital, or Uijeongbu St. Mary’s Hospital of the Catholic Medical Center (Seoul, Korea). From the FFPE tumors and surrounding normal tissues of those patients, we extracted RNA using a mirNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. miR-196a expression was measured using NanoString (lung adenocarcinoma samples, n = 115; nCounter Human v3 Assay kit) or qRT-PCR (tumors and surrounding normal tissues), as explained above.

2.12. Statistical analysis

Data were analyzed with the Student’s t-test, Spearman’s rank correlation test, Wilcoxon-Mann-Whitney test, and log-rank test using GraphPad Prism (La Jolla, CA), unless otherwise noted.

3. Results

3.1. CAFs promote migratory and invasive activity of lung cancer cells

To investigate the effects of CAF-specific miRNAs on the activity of lung cancer cells, we first isolated murine LFs and CAFs using magnetic-activated cell sorting. LFs were isolated from the lungs of 129/Sv wild type mice, and CAFs were isolated from the lung tumors of Kras-mutant (KrasL599H) mice [6] using THY-1, a fibroblast-specific marker [16]. The isolated LFs and CAFs showed morphological differences in 2-D cultures. To observe the morphology more clearly, actin cytoskeletons of LFs and CAFs were stained with Alexa Fluor 594-conjugated phalloidin (Supplementary Fig. 1D and 2). Spheroids composed of lung cancer cells (Supplementary Fig. 1D and 2). Next, we performed spheroid invasion assays to examine the effects of LFs and CAFs on the invasiveness of lung cancer cells (Supplementary Fig. 1D and 2). Spheroids composed of 344SQ cells and fibroblasts were generated using the hanging-drop method and embedded in collagen gels. After 48 h, cancer spheroids co-cultured with CAFs invaded the surrounding collagen gels much more effectively than those co-cultured with LFs. These results demonstrate that CAFs facilitate the migration and invasion of lung cancer cells more effectively than LFs.
3.2. miR-196a mediates CAF activity in promoting lung cancer cell migration and invasion

To identify the miRNAs that mediate the pro-tumorigenic activity of CAFs, we used the NanoString method to profile the miRNAs in LFs and CAFs (Fig. 1A). Many miRNAs were differentially expressed in CAFs and LFs: 139 miRNAs were up-regulated, and 307 miRNAs were down-regulated significantly in CAFs (fold-change ≥ 2, P ≤ 0.05) compared with LFs. miRNAs with high fold-changes were selected, and their expression levels were reconfirmed by qRT-PCR, which showed expression patterns similar to the NanoString data (Fig. 1B). Among those miRNAs, miR-196a, one of the miRNAs highly expressed in CAFs, was previously reported to promote the proliferation and invasion of lung cancer cells [19]. To investigate the link between miR-196a and cancer more closely, we checked the miR-196a expression levels in public databases using dbDMEC 2.0 [https://www.pcb.ac.cn/dbDMEC] [20]. In 45 The Cancer Genome Atlas (TCGA) and NCBI Gene Expression Omnibus (GEO) datasets containing lung cancer data, 39 experimental datasets (86.7%) indicated that miR-196a expression levels were up-regulated in cancer tissues compared with normal tissues (Fig. 1C); therefore, we selected miR-196a for further study.

To study the function of miR-196a in CAFs, we first overexpressed miR-196a in LFs through lentiviral transduction (Fig. 1D). Using qRT-PCR, we measured the expression levels of well-known CAF activation markers (Twy1, Fip1, α-Sma, and Fap) in LFs transduced with miR-196a (LF-196a) or an empty vector (LF-vec) (Fig. 1E). α-Sma and Fap were more highly expressed in LF-196a than in LF-vec, suggesting that miR-196a partially activates CAFs. To investigate the effect of miR-196a on fibroblast activity in promoting cancer cell migration, we performed Transwell migration assays. LF-196a or LF-vec were seeded on the bottom wells, and 344SQ murine lung cancer cells were seeded on the upper wells of Transwell plates (Fig. 1F). Cancer cells co-cultured with LF-196a migrated more actively than those co-cultured with LF-vec. Next, we performed spheroid invasion assays to examine the role of miR-196a in CAF-activated cancer cell invasion. Spheroids composed of murine lung cancer cells (344SQ, 344LN, or 531LN2) and LF-196a invaded the collagen matrix better than those composed of cancer cells and LF-vec (Fig. 1G and Supplementary Fig. 3). LFs transiently transfected with the miR-196a mimic were able to promote cancer spheroid invasion as well as those stably transfected with the miR-196a lentivirus (Fig. 1H), whereas a miR-196a inhibitor attenuated the ability of CAFs to promote cancer spheroid invasion (Fig. 1I). We also isolated and cultured human lung fibroblasts taken from the normal lung tissue surrounding a tumor after surgical resection. Just like the murine LFs, human LFs transiently transfected with the miR-196a mimic enhanced the invasion of cancer spheroids composed of A549 human lung cancer cells (Fig. 1J).

344SQ cells form well-polarized spheres with an acinar structure on Matrigel 3-D culture [21]. When stimulated with external cues that induce EMT, 344SQ 3-D spheres lose their polarity and form invasive protrusions. Interestingly, this conversion to the invasive phenotype was also induced by overlaying LF-196a or CAFs on 344SQ 3-D spheres (Fig. 2A and B). When 344SQ spheroids were seeded on fibroblast feeder layers [22], the cancer spheroids on the LF-196a feeder layers invaded and expanded into the feeder cells better than those seeded on LF-vec feeder layers (Fig. 2C). All these results suggest that CAF-specific miR-196a activates fibroblasts, and that, in turn, promotes the migration and invasion of lung cancer cells.

To clarify whether the effects of miR-196a-activated fibroblasts on cancer cells occur through direct or indirect interactions, we repeated the experiments just described using conditioned media derived from various fibroblasts. When conditioned medium from LF-196a was added to the bottom wells instead of fibroblasts, the migration activity of the 344SQ cells was also enhanced (Fig. 2D). Conditioned medium from LF-196a and CAFs stimulated the invasiveness of 344SQ spheroids (Fig. 2E), implying that secretory factors from LF-196a or CAFs activated the cancer cells in our system. To investigate the effect of miR-196a on tumor growth and metastasis, 344SQ cells were subcutaneously injected into syngeneic mice, and conditioned medium derived from LF-vec or LF-196a was injected intratumorally three times per week. Five weeks after the original injections, mice treated with LF-196a conditioned medium showed bigger primary tumors at the injection sites (Fig. 2F) and more metastatic nodules on the surfaces of their lungs than mice injected with LF-vec conditioned medium (Fig. 2G). This mouse experiment suggests that CAF-specific miR-196a promotes tumorigenesis and the metastasis of lung cancer cells in the tumor microenvironment.

3.3. ANXA1 is a direct target gene of miR-196a

To determine which genes are directly targeted by miR-196a during CAF activation, we predicted 10 candidate target genes for miR-196a using TargetScan and previous reports [23,24]. The expression levels of candidate genes were then measured in LFs and CAFs, and in LF-vec and LF-196a using qRT-PCR. Target genes of miR-196a would be down-regulated in the CAFs and LF-196a compared with the LFs and LF-vec, respectively. In the qRT-PCR results, Anxa1, Mryn, and Kr5 were among the top five genes suppressed in both CAFs and LF-196a (Fig. 3A). To identify more reliable target genes of miR-196a, we analyzed TCGA lung adenocarcinoma data. Target gene expression would correlate negatively with miR-196a expression. Among the three genes listed above, only ANXA1 showed a negative correlation with miR-196a (Fig. 3B and Supplementary Fig. 4A). In addition, ANXA1 has been already reported as a target of miR-196a mediating proliferation, migration, and invasion of cancer cells in esophageal squamous carcinoma, head and neck squamous cell carcinoma, and breast cancer [25–27]; therefore, ANXA1 was selected as a putative target gene of miR-196a in this study.

As mentioned above, Anxa1 expression decreased in LFs after miR-196a overexpression (Fig. 3A). In the TCGA data, miR-196a expression levels were higher in tumors than in normal tissues, whereas ANXA1 expression levels were lower in tumors than in normal tissues (Supplementary Fig. 4B). To confirm whether ANXA1 is a direct target of miR-196a, we performed a 3′-UTR reporter assay (Fig. 3C). As reported previously [25,26,28,29], the luciferase activity of an ANXA1 3′-UTR reporter was decreased by the miR-196a mimic, suggesting that ANXA1 is a direct target of miR-196a. Moreover, adding back ANXA1 to LF-196a (Fig. 3D) decreased the enhanced spheroid invasion seen with miR-196a (Fig. 3E). When Anxa1 was knocked down in LFs using siRNAs (Fig. 3F), spheroid invasion was stimulated (Fig. 3G). Treatment of ANXA1 neutralizing antibody also promoted cancer spheroid invasion, implying that extracellular ANXA1 is involved in this process (Fig. 3H). In contrast, ANXA1 overexpression in CAFs (Fig. 3I) inhibited cancer cell invasion in the spheroid invasion assay (Fig. 3J). All these results suggest that ANXA1 is a target gene of miR-196a that mediates the regulation of cancer cell invasion by CAFs.

3.4. CAF-specific miR-196a stimulates the secretion of CCL2, which promotes cancer cell invasion

Recently, it was reported that CAFs comprise two distinct subpopulations: myofibroblastic CAFs and inflammatory CAFs [30]. Myofibroblastic CAFs interact directly with cancer cells and activate invasion by remodeling the extracellular matrix. Inflammatory CAFs contribute to the formation of an inflammatory tumor microenvironment that promotes tumor progression through the secretion of paracrine cytokines. To check whether miR-196a helps CAFs acquire inflammatory features, we measured diverse cytokine levels using a cytokine array and found that Serpin E1 and IGFBP-6 decreased and CCL2, MMP3, and proliferin increased in LF-196a compared with LF-vec (Fig. 4A). Among those cytokines, CCL2 was the most highly induced by miR-196a at both the protein and mRNA levels (Fig. 4B), and it has been reported to mediate crosstalk between cancer cells and fibroblasts in tumor
Fig. 1. miR-196a activates fibroblasts to promote the migration and invasion of lung cancer cells. A. Heatmap of NanoString data showing miRNAs differentially expressed between LFs and CAFs. Yellow: increased expression; blue: decreased expression. B. qRT-PCR of selected miRNAs from NanoString profiling. Log2 fold-change values (CAFs vs LFs) are presented in the graph. Mean ± SD (n = 3). *P < 0.05, **P < 0.01, two-tailed Student’s t-test. C. miR-196a expression levels in 45 TCGA and NCBI GEO datasets obtained from the dbDEMC 2.0 database. Log2 fold-change (cancer vs normal) values are presented in the graph. Six lung cancer datasets are highlighted (orange). D. qRT-PCR of miR-196a in LFs transduced with empty (LF-vec) or miR-196a lentiviral vector (LF-196a). miR-196a levels were normalized to the RNU6B snoRNA level, and values relative to those of LF-vec (set at 1.0) are presented. Mean ± SD (n = 3). P, two-tailed Student’s t-test. E. qRT-PCR of CAF markers in LF-vec and LF-196a. Expression levels were normalized to the Rpl32 mRNA level, and values relative to those of LF-vec (set at 1.0) are presented. Mean ± SD (n = 3). *P < 0.05, **P < 0.01; two-tailed Student’s t-test. F. Transwell migration assay in 344SQ cells co-cultured with LF-vec (+vec) and LF-196a (+196a). 344SQ cells were seeded in the upper wells, and fibroblasts were seeded in the bottom wells. After 24 h, the migrated 344SQ cells were photographed and counted. Mean ± SD (n = 3). P, two-tailed Student’s t-test. G. Spheroid invasion assay in 344SQ cells co-cultured with LF-vec (+vec) or LF-196a (+196a). 344SQ cells were seeded in the upper wells, and fibroblasts were seeded in the bottom wells. After 24 h, the migrated 344SQ cells were photographed and counted. Spheroid invasion ratios (ratio of whole cell area to central spheroid area) were measured using ImageJ. Mean ± SD (+vec, n = 27; +196a, n = 22). P, two-tailed Student’s t-test. H. Spheroid invasion assay in 344SQ cells co-cultured with LFs transfected with control (+con) or miR-196a mimic (+196a mimic). Mean ± SD (+con, n = 22; +196a mimic, n = 25). P, two-tailed Student’s t-test. I. Spheroid invasion assay in 344SQ cells co-cultured with CAFs transfected with control (+con) or miR-196a inhibitor (+196a inhibitor). Mean ± SD (+con, n = 22; +196a inhibitor, n = 28). P, two-tailed Student’s t-test. J. Spheroid invasion assay in A549 cells (labeled with mCherry) co-cultured with human LFs transfected with the control (+con) or miR-196a mimic (+196a mimic) vector. Mean ± SD (+con, n = 7; +196a mimic, n = 14). P, two-tailed Student’s t-test.
In Transwell migration assays, CCL2-neutralizing antibody diminished cancer cell migration toward fibroblasts (Fig. 4C). In spheroid invasion assays using co-cultures of cancer cells and fibroblasts, CCL2-neutralizing antibody attenuated the activity of the LF-196a and inhibited spheroid invasion (Fig. 4D). On the contrary, CCL2 recombinant protein promoted cancer cell migration and invasion in both mono- and co-culture conditions (Fig. 4E–G), suggesting that CCL2 mediates the effects of miR-196a on cancer cell migration and invasion. Interestingly, it has been reported that ANXA1 is an anti-inflammatory protein that suppresses the secretion of multiple cytokines [32–35]. In our system, ANXA1 overexpression in CAFs suppressed Ccl2 mRNA expression (Fig. 4H), and conversely Anxa1 knockdown in LFs enhanced the expression of multiple cytokines, including CCL2 (Fig. 4I and Supplementary Fig. 5), suggesting that miR-196a regulates the secretion of inflammatory cytokines, including CCL2, via ANXA1 (Fig. 4J and K) and reinforces the inflammatory features of CAFs. Given that miR-196a induces myofibroblastic markers such as αSMA and FAP (Fig. 1E) and inflammatory cytokines, we assume that miR-196a can elicit both myofibroblastic and inflammatory features from CAFs.

3.5. miR-196a causes global transcriptomic changes that facilitate CAF activation

To gain insight into the global transcriptomic changes influenced by miR-196a, we performed an RNA-sequencing analysis on CAFs, LFs, and LF-196a. Our analysis of differentially expressed genes (fold change ≥2, adjusted P ≤ 0.05, and average transcripts per million ≥1) revealed that many genes were commonly up-regulated (n = 742) or down-regulated (n = 840) in both LF-196a and CAFs compared with LFs (Fig. 5A). A rank–rank hypergeometric overlap analysis [36] showed that two pairs of transcriptomic profiles (LF-196a vs. LFs and CAFs vs. LFs) correlated...
significantly (Fig. 5B), suggesting that forced expression of miR-196a made the global transcriptome of LFs similar to that of CAFs. We then performed a gene ontology (GO) enrichment analysis of the genes up-regulated in LF-196a. Among the enriched GO terms were “actin filament-based process” (adjusted $P = 7.42E-11$), “muscle system process” ($P = 5.92E-10$), “positive regulation of cell migration” ($P = 2.08E-08$), “focal adhesion” ($P = 1.45E-06$), “extracellular matrix organization” ($P = 2.79E-06$), and “regulation of cell adhesion” ($P = 2.08E-08$).
Gene signatures in LF-196a included LF-196a. Similar to the GO analysis, the enriched or over-represented signaling pathways affected by miR-196a, we performed a gene set enrichment analysis (GSEA) on the RNA-sequencing profiles of LFs and LFs. We then chose several genes within those signatures and measured their mRNA expression levels in LFs and LF-196a (Fig. 5 E). Most of the signature genes were increased by miR-196a overexpression in LFs, providing a molecular transcriptomic basis for miR-196a-mediated CAF activation, which promotes the migration and invasion of cancer cells.

1.63E-05 (Fig. 5C), which could be the cellular processes that underlie the effects of miR-196a on CAF activation. To further analyze the signaling pathways affected by miR-196a, we performed a gene set enrichment analysis (GSEA) on the RNA-sequencing profiles of LFs and LF-196a. Similar to the GO analysis, the enriched or over-represented genes signatures in LF-196a included “extracellular matrix,” “actin cytoskeleton,” “epithelial-mesenchymal transition,” and “myogenesis” (Fig. 5D). We then chose several genes within those signatures and measured their mRNA expression levels in LFs and LF-196a (Fig. 5E).

3.6. miR-196a is a poor prognosis marker in lung adenocarcinoma patients

In the tumor microenvironment, CAFs activated by miR-196a promoted the migration, invasion, and metastasis of lung cancer cells. To determine the clinical relevance of these in vitro and in vivo results, we measured miR-196a levels by qRT-PCR in FFPE tumor tissues and matched adjacent normal tissues from lung adenocarcinoma (LUAD, n = 25) and head and neck squamous cell carcinoma (HNSCC, n = 27) patients. As observed in the public databases, miR-196a levels were significantly higher in tumors than in normal tissues (Fig. 6A and B). To examine the effect of miR-196a on cancer patient survival, we used the NanoString method to measure the miR-196a levels in 115 FFPE tumor samples from LUAD patients. We then divided the patients into two groups based on their miR-196a expression levels (miR-196a_high and miR-196a_low) and analyzed their survival rates. The overall and
recurrence-free survival times of the miR-196a_high group were significantly shorter than those of the miR-196a_low group (Fig. 6C). In the TCGA and GEO datasets, LUAD patients with higher miR-196a showed poorer survival rates than those with lower miR-196a, but statistical significance was not very high ($P = 0.026$; Supplementary Fig. 6). Thus, through in-depth follow-up research, miR-196a could be used as an efficient marker predicting poor prognosis in LUAD patients.
4. Discussion

In tumor microenvironments, stromal CAFs and cancer cells interact through diverse direct and indirect mechanisms in which miRNAs can function as key mediators [37,38]. miRNAs can directly regulate CAFs’ typical features by intervening in differentiation and reprogramming processes. In this work, we used spheroid 3-D cultures composed of lung cancer cells and CAFs to show that miR-196a induces the reprogramming of stromal fibroblasts in ways that facilitate cancer cell invasion. We also confirmed that miR-196a is strongly expressed in the stroma of tumor tissues (data not shown) and is significantly associated with poor prognosis in lung adenocarcinoma patients.

miR-196a functions as an oncogenic miRNA in diverse types of cancer. miR-196a is up-regulated in breast cancer and enhances cancer cell proliferation, migration, and tumor growth by targeting SPREDI [39]. In gastric cancer, miR-196a promotes cell growth and tumor formation by down-regulating p27 [40]. In non-small cell lung cancer, miR-196a is a tumor promoter that increases drug resistance and the proliferation and invasion of cancer cells [12,19,41]. Moreover, miR-196a derived from CAFs is reported to be transferred to head and neck cancer cells via exosomes to increase their proliferation, survival, and drug resistance [11]. These previous results restrictively describe the functions and effects of miR-196a because they focus only on cancer cells. In this paper, however, we first identified miR-196a as a CAF-specific miRNA in a mouse lung cancer model and then investigated its specialized roles during CAF reprogramming and activation. Heightened miR-196a in LFs accelerates cancer cell migration and invasion, which is mediated by ANXA1, a direct target of miR-196a. Heightened miR-196a in LFs also elevates the expression of inflammatory cytokines, including CCL2.

Similar to cancer cells, CAFs have been reported to be heterogeneous [42,43]. In pancreatic cancer, two subtypes of CAFs, myofibroblastic and inflammatory, have been identified and shown to differ in their functional mechanisms and relative distance from neighboring tumor cells [30]. Myofibroblastic CAFs promote the proliferation of adjoining epithelial cancer cells through direct contact, whereas inflammatory CAFs secrete cytokines that activate cancer cells via paracrine signaling. In the lung tumor microenvironment, CAFs also have pro-tumorigenic functions with both myofibroblastic and inflammatory features [30]. In this study, the forced expression of miR-196a stimulated LFs to express myofibroblastic markers such as αSMA and FAP and promoted cancer cell invasion in 3-D co-culture models through direct interaction. In addition, compared with control LFs, LFs transduced with miR-196a secreted more inflammatory cytokines and thus indirectly attracted more cancer cells in Transwell migration assays. Collectively, miR-196a encourages CAFs to exhibit both myofibroblastic and inflammatory features.

We predicted the target genes of miR-196a by searching the literature and TargetScan database. Among the predicted target genes, we selected those that correlated negatively with miR-196a in LUAD data in TCGA. In that way, we identified ANXA1 as a target gene of miR-196a, confirming previous research [25,26,28,29]. miR-196a promotes the proliferation, migration, and invasion of breast [25], esophageal [26], and head and neck cancer cells [28] by directly targeting ANXA1. In this study, we also found that miR-196a inhibited ANXA1 expression through direct 3’-UTR targeting. Adding back ANXA1 restored the effect that miR-196a has on cancer spheroid invasion. Knocking down of ANXA1 in LFs has a pro-invasive effect on cancer spheroids, compared to that of miR-196a overexpression. Therefore, we conclude that the effect of miR-196a on CAF activation is mediated by the direct targeting of ANXA1. ANXA1 is a calcium-dependent phospholipid-binding protein localized in the plasma membrane, the cytoplasm, and the nucleus, and was first identified as a negative regulator of cytosolic phospholipase A2 (PLA2) [44]. ANXA1 exerts anti-inflammatory activity by inhibiting PLA2, cyclooxygenase-2, and inducible nitric oxide synthase [45,46]. ANXA1 inhibits leukocyte transmigration, promotes M2-macrophage differentiation, accelerates apoptotic cell removal, and thus resolves inflammation [44]. ANXA1 also negatively regulates the production of neuroendocrine hormones (adrenocorticotropic hormone and corticotrophin-releasing hormone) [47] and attenuates the insulin response [48]. In addition, ANXA1 helps maintain blood-brain barrier integrity and provides a protective effect against neurodegenerative diseases such as Alzheimer’s disease [49]. In terms of cancer development and progression, ANXA1 has contradictory effects depending on the types of cancer [50]. ANXA1 is up-regulated in gastrointestinal cancer and is associated with enhanced invasion/metastasis and poor prognosis [51]. ANXA1 is also up-regulated in triple-negative breast cancer and is associated with aggressive phenotypes such as immune cell infiltration and angiogenesis [52]. On the contrary, ANXA1 is down-regulated in oral squamous-cell carcinoma and is associated with poor clinicopathologic features [53]. In invasive prostate cancer, ANXA1 expression is reduced compared with benign prostatic epithelium [54]. These contradictory effects can be attributed to diverse cellular and physiologic functions and differential sub-cellular localization of ANXA1.

ANXA1 has been known as an anti-inflammatory protein which suppresses the expression of inflammatory cytokines including CCL2 [35,55]. The inhibition mechanism has not been clearly identified yet, but it can be mediated through the binding of extracellular ANXA1 to cell surface formyl peptide receptors, FRP1 and FRP2 [35,56,57]. ANXA1-FRP axis has been known to inhibit cyclooxygenase-2 [45,46,57], a key activator of pro-inflammatory cytokines including CCL2 [58,59]. In this study, anti-ANXA1 neutralizing antibody promoted cancer cell invasion, and ANXA1 overexpression suppressed CCL2 expression in CAFs, which might provide a mechanistic explanation for how miR-196a-ANXA1 axis regulates the inflammatory features of CAFs.

Cytokine profiling revealed that miR-196a induces changes in the expression and secretion of diverse inflammation-related proteins: Serpin E1 and IGFBP-6 were down-regulated, and CCL2, MMP3, and proliferin were up-regulated. A CCL2 neutralizing antibody blocked the enhanced cancer cell migration and invasion found in LFs over-expressing miR-196a, suggesting that CCL2 is a mediator of miR-196a. It is unclear how miR-196a and ANXA1 regulate CCL2 secretion; further studies are needed. In addition, Serpin E1 and MMP3 could be functional mediators of miR-196a in tumor microenvironments. MMP3 enzymatically cleaves and inactivates Serpin E1 (plasminogen activator inhibitor-1) and thereby promotes cellular adhesion and migration [60]. Inversely, Serpin E1 inhibits the processing of plasminogen into plasmin by blocking the tissue-type or urokinase plasminogen activator, which subsequently disrupts MMP-3 activation [61]. Intriguingly, our TargetScan prediction shows that Serpin E1 could be a miR-196a target (data not shown). These findings imply that miR-196a, Serpin E1, and MMP-3 could form another regulatory network in tumor microenvironments.

In this work, we showed that a synthetic miR-196a inhibitor effectively blocked the invasion of spheroids composed of cancer cells and fibroblasts. Therefore, miR-196a and its target genes could be putative therapeutic targets in lung cancer patients. In fact, blocking CCL2 with anti-CCL2 antibodies or CCR2 chemical antagonists has been in clinical trials against liver fibrosis, pancreatic cancer, and prostate cancer [62]. A blockade of the CCL2/CCR2 axis also enhances the anti-cancer effects of immunotherapies [63,64]. Therefore, a combination treatment of miR-196a inhibitors and CCL2/CCR2 blockers or conventional anticancer drugs could be a successful therapeutic option for targeting both cancer cells and stromal cells in the tumor microenvironment simultaneously.

Authors’ contributions

SL, JHH, JSK, JSY, SHC, EJK, JLK and Y-HA performed the experiments. SL, YHK and Y-HA designed the study. SL, JHH, SHC, SAH, KK and Y-HA analyzed the data. SL and Y-HA wrote the manuscript.
Declaration of competing interest
The authors declare no potential conflicts of interest.

Acknowledgements
The authors thank Dr. Jonathan M. Kurie (University of Texas MD Anderson Cancer Center) for his valuable suggestions. This research was supported by grants of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI17C0666 to Y-HA; HI20C0461 to SL), and by National Research Foundation of Korea (NRF) grants funded by the Korean government (MSIT) (NRF-2020R1A502191210 to Y-HA; NRF-2018R1D1A2A02085738 to YHK; 2017R1C1B2011702 to JHH). This work was also supported by the LG Yonam Foundation of Korea (to Y-HA) and by an Ewha Womans University scholarship in 2019 (to SL).

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2021.03.021.

References


