ABSTRACT
Cancer-associated fibroblasts (CAF) are a major constituent of the tumor stroma, but little is known about how cancer cells transform normal fibroblasts into CAFs. microRNAs (miRNA) are small noncoding RNA molecules that negatively regulate gene expression at a posttranscriptional level. Although it is clearly established that miRNAs are deregulated in human cancers, it is not known whether miRNA expression in resident fibroblasts is affected by their interaction with cancer cells. We found that in ovarian CAFs, miR-31 and miR-214 were downregulated, whereas miR-155 was upregulated when compared with normal or tumor-adjacent fibroblasts. Mimicking this deregulation by transfecting miRNAs and miRNA inhibitors induced a functional conversion of normal fibroblasts into CAFs, and the reverse experiment resulted in the reversion of CAFs into normal fibroblasts. The miRNA-reprogrammed normal fibroblasts and patient-derived CAFs shared a large number of upregulated genes highly enriched in chemokines, which are known to be important for CAF function. The most highly upregulated chemokine, CCL5, (C-C motif ligand 5) was found to be a direct target of miR-214. These results indicate that ovarian cancer cells reprogram fibroblasts to become CAFs through the action of miRNAs. Targeting these miRNAs in stromal cells could have therapeutic benefit.

SIGNIFICANCE: The mechanism by which quiescent fibroblasts are converted into CAFs is unclear. The present study identifies a set of 3 miRNAs that reprogram normal fibroblasts to CAFs. These miRNAs may represent novel therapeutic targets in the tumor microenvironment. Cancer Discov; 2(12); 1100–8. © 2012 AACR.
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During invasion and metastasis, cancer cells change the normal stroma into a “reactive” environment, which promotes the growth and viability of tumor cells (1). Upon interaction with cancer cells, quiescent resident fibroblasts, which are the predominant cell type in normal stroma, are transformed into cancer-associated fibroblasts (CAF), which become a major component of the tumor stroma. CAFs promote cancer cell invasion, proliferation, and metastasis by secreting cytokines and chemokines, which stimulate receptor tyrosine kinase signaling and epithelial–mesenchymal transition (EMT) programs (1). Moreover, CAFs secrete a distinctive extracellular matrix that promotes the attachment and invasion of tumor cells (2). Several steps of the bidirectional signaling between cancer cells and fibroblasts have been elucidated. Neoplastic cells secrete cytokines such as interleukin (IL)-6, IL-8, and IL-1β to activate fibroblasts and stimulate their proliferation (3). The CAFs, in return, secrete cancer-activating chemokines such as SDF-1α, thereby stabilizing and promoting tumorogenesis (4). However, it is currently not clear how cancer cells reprogram quiescent fibroblasts to become CAFs.

MicroRNAs (miRNA) are small noncoding RNA molecules that negatively regulate gene expression at a post-transcriptional level (5). miRNAs are powerful regulators of cellular differentiation, as they affect the expression of many genes and are deregulated in cancer cells (6). It is not clear at present whether endogenous miRNAs are involved in the conversion of resident fibroblasts to CAFs. Several reports have shown that miRNAs can reprogram various somatic cells to become pluripotent stem cells (7). A combination of miR-124 and the transcription factors MYT1L and BRN2 reprograms primary human dermal fibroblasts into functional neurons that exhibit typical neural morphology, fire action potentials, and produce functional synapses (8). miR-15 and miR-16, are downregulated in fibroblasts surrounding prostate tumors (9), but their functional role in promoting tumor growth is unclear. Recently, miR-511-3p has been reported to prevent the tumor-promoting activity of tumor-associated macrophages (10).

Epithelial serous ovarian carcinoma has a unique pattern of metastasis that remains largely restricted to the abdominal cavity, in which the omentum, a large fat pad in front of the bowel, is a major site of colonization (11). Ovarian tumors and metastases have a substantial stromal component of which CAFs are an important constituent (3). Although the role of CAFs in ovarian cancer progression is well established, the mechanism of CAF formation remains unclear (12).

Most miRNA studies have focused on the tumor cell, but little is known about miRNA expression in the tumor microenvironment. Given the broad regulatory role of miRNAs, we have now investigated whether miRNAs are involved in the reprogramming of normal fibroblasts to CAFs, thereby promoting tumorigenesis. We report here that the combination of 3 miRNAs induces normal human omental fibroblasts to become CAFs, leading to the upregulation of chemokines important in invasion and metastasis.

RESULTS

Identification of miRNAs Deregulated in Cancer-Associated Fibroblasts

The miRNA expression in CAFs was compared with that in primary human normal omental fibroblasts (NOF) using 2 independent, unbiased approaches. In one approach, primary human CAFs were isolated from the omental metastases of patients with metastatic serous ovarian cancer and compared with tumor-adjacent NOFs extracted from a normal area of the omentum, at least 1 inch from the tumor, from the same patients. In the other approach, NOFs from the omentum of cancer-free patients operated on for benign gynecologic disease (e.g., fibroids) were compared with NOFs cocultured with HeyA8 ovarian cancer cells (Fig. 1A). While the former approach identifies the miRNA changes that occur during tumor progression, the latter detects the early changes induced exclusively by cancer cells. Confocal time-lapse microscopy revealed that CAFs were more migratory (Supplementary Fig. S1A and S1B) and induced the invasion of different ovarian cancer cell lines more efficiently than either NOFs or tumor-adjacent NOFs (Supplementary Fig. S2A). The induced CAFs, generated by a 7-day coculture of NOFs with ovarian cancer cells, had an enhanced ability to promote tumor cell invasiveness (Supplementary Fig. S2B), suggesting that cancer cells impart CAF-like properties to these cells.

Therefore, the miRNA expression of CAFs was compared with that of adjacent NOFs and the miRNA expression of NOFs was compared with that of induced CAFs (Fig. 1A). A miRNA array analysis of RNA isolated from these cells identified 19 significantly expressed miRNAs that were upregulated (P < 0.05) and 15 miRNAs that were downregulated in the cocultured, induced CAFs (Supplementary Table S1). Of these miRNAs, 1 was found to be upregulated and 4 downregulated in CAFs compared with matching adjacent NOFs from 6 patients with postmenopausal, advanced serous ovarian carcinoma (Fig. 1B and Supplementary Table S1). In both induced CAFs and primary patient-derived CAFs, the miRNAs that were most significantly up- and downregulated were miR-155 and miR-214, respectively, and therefore these were selected for further testing. miR-31 was included because it was the second most significantly downregulated miRNA in induced CAFs and because it was reported as downregulated in fibroblast lines derived from patients with endometrial cancer (13). Using quantitative real-time PCR (qRT-PCR), downregulation of miR-31 and miR-214 and upregulation of miR-155 was confirmed in all CAF and induced CAF samples (Supplementary Fig. S3A). In situ hybridization of an omental metastasis from a patient with ovarian cancer confirmed that the expression of miR-214 was lost in CAFs and high in adjacent NOFs; an opposite expression pattern was observed for miR-155 (Supplementary Fig. S3B).

Reversible Conversion of Normal Fibroblasts into CAFs

Triple transfection of NOFs with anti-miR-31, anti-miR-214, and pre-miR-155 (to generate miR-CAFs) enhanced fibroblast migration as well as the invasion and colony formation of cocultured HeyA8 and SKOV3ip1 cells (Fig. 1C–E), suggesting...
that deregulating the expression of the 3 miRNAs could convert NOFs to CAFs. The reverse was also true, as transfection of CAFs with pre-miR-31, pre-miR-214, and anti-miR-155 reduced their migration and also reduced the invasion and colony formation of cocultured ovarian cancer cells to levels similar to those of ovarian cancer cells cocultured with NOFs (Fig. 1C-E). miRNA transfection was validated by qRT-PCR (Supplementary Fig. S4A, B). To assess the contribution of each of the miRNAs to the functional effects of miR-CAFs on cancer cells (migration, invasion, and colony formation), NOFs or CAFs were transfected with individual miRNAs or their inhibitors. miR-214 was active in regulating fibroblast migration and cancer...
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To test the in vivo activity of the identified miRNAs, miR-CAFs or NOFs transfected with scrambled RNA were coinjected with luciferase-expressing HeyA8 cells subcutaneously into mice. Coinjected CAFs were used as a positive control. Both miR-CAFs and CAFs significantly enhanced the growth of tumor cells when compared with NOFs (Fig. 1F), which was also reflected in increased expression of the proliferation marker Ki-67 (Fig. 1G). This growth enhancement was a direct effect of fibroblasts on tumor cells because the number of infiltrating macrophages or neutrophilic granulocytes was similar in all experimental groups (Fig. 1G and data not shown).

Chemokines Are the Most Highly Deregulated Genes in CAFs, Induced CAFs, and miR-CAFs

To determine whether altering the expression of the 3 miRNAs in NOFs to generate miR-CAFs mimicked the differentiation of NOFs into CAFs in patients, tumor-adjacent NOFs and CAFs were subjected to gene array analysis. These data were compared with the gene expression profile obtained from miR-CAFs, which were reprogrammed NOFs derived from patients without cancer through transfection with anti-miR-214/31 and pre-miR-155. The successful transfection of the miRNAs/miRNA inhibitors and their functional effects on fibroblasts was confirmed using RT-PCR and invasion assays (data not shown). Supplementary Table S2 lists the genes that were upregulated more than 1.5-fold in CAFs (when compared with adjacent NOFs from the same patients) and in miR-CAFs (when compared with matching NOFs). Surprisingly, 7 of the 10 most highly upregulated genes were chemokines. Most of these chemokines were also found to be upregulated when CAFs were compared with NOFs and miR-CAFs to adjacent NOFs (Supplementary Table S3 and data not shown).

To determine the extent of the reprogramming by miRNAs, we compared gene expression changes between patient-derived CAFs, induced CAFs, and miR-CAFs and their respective

Figure 2. Chemokines are the most highly upregulated genes in CAFs, induced CAFs (iCAF), and miR-CAFs. A, comparison of genes upregulated (green) and downregulated (red) in patient-derived CAFs (compared with matched adjacent NOFs [aNOF]), induced CAFs derived from a 7-day coculture of NOFs with HeyA8 cells (compared with matched NOFs) and miR-CAFs 2 days after triple transfection (compared with matched NOFs). B, ranked list of genes found to be up (green) and downregulated (red) in CAFs, induced CAFs, and miR-CAFs. C, qRT-PCR to assess upregulation of the 3 highest upregulated chemokines for each array analysis. Error bars represent SD of a triplicate experiment.
normal fibroblasts (Fig. 2A). The overlap in induced gene changes between miR-CAFs and CAFs was 15.3% compared with 23.2% between induced CAFs and CAFs. Interestingly, only 13 genes were upregulated and 4 genes downregulated in the CAFs, induced CAFs, and miR-CAFs from all patients, and again, the top 3 upregulated genes were chemokines (Fig. 2B). The upregulation of these chemokines on the mRNA level was confirmed by qRT-PCR for each array analysis (Fig. 2C).

Although triple-transfected NOFs secreted most CCL5, anti-miR-214 was efficient in inducing expression of CCL5 when transfected alone (Fig. 3C), suggesting that CCL5 is a direct target of miR-214. Luciferase constructs were used with the CCL5 wild-type 3'-UTR and 2 different CCL5 3'-UTR mutants with substitutions in the miR-214 or the miR-31 seed matches (Fig. 3B). Cotransfection of miR-214 or miR-31 into 293T cells, together with the luciferase constructs, showed that miR-214 was more potent in targeting the CCL5 3'-UTR than miR-31 (Fig. 3D), and this activity was greatly diminished when the miR-214 seed match was mutated. This was confirmed for endogenous miRNAs by transfecting the reporter constructs into either primary CAFs or NOFs. Luciferase activity was significantly reduced in miR-214/miR-31 high-expressing NOFs when miR-CAFs and CAFs for possible miRNA seed matches using miRanda, TargetScan, and PicTar software. Only 1 chemokine was predicted to contain seed matches for any of the 3 miRNAs. Overlapping seed matches for miR-214 and miR-31 were found in the CCL5 3'-UTR (Fig. 3B).

Identification of CCL5 as a Target of miR-214

To determine whether miR-CAFs, generated by altering mRNA expression, secreted chemokines, a protein array was constructed for 10 of the most highly upregulated cytokines found on the gene array. In this assay, the array was constructed for 10 of the most highly upregulated secreted chemokines, a protein array. Although triple-transfected NOFs secreted most CCL5, anti-miR-214 was efficient in inducing expression of CCL5 when transfected alone (Fig. 3C), suggesting that CCL5 is a direct target of miR-214. Luciferase constructs were used with the CCL5 wild-type 3'-UTR and 2 different CCL5 3'-UTR mutants with substitutions in the miR-214 or the miR-31 seed matches (Fig. 3B). Cotransfection of miR-214 or miR-31 into 293T cells, together with the luciferase constructs, showed that miR-214 was more potent in targeting the CCL5 3'-UTR than miR-31 (Fig. 3D), and this activity was greatly diminished when the miR-214 seed match was mutated. This was confirmed for endogenous miRNAs by transfecting the reporter constructs into either primary CAFs or NOFs. Luciferase activity was significantly reduced in miR-214/miR-31 high-expressing NOFs when miR-CAFs and CAFs for possible miRNA seed matches using miRanda, TargetScan, and PicTar software. Only 1 chemokine was predicted to contain seed matches for any of the 3 miRNAs. Overlapping seed matches for miR-214 and miR-31 were found in the CCL5 3'-UTR (Fig. 3B).
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CCL5 Is Critical for the Activity of Both CAFs and miRNA Reprogrammed CAFs

To determine the in vivo relevance of CCL5 in the activity of miR-CAFs, miR-CAFs were generated by transiently transfecting NOFs with anti-miR-31, anti-miR-214, and pre-miR-155. Using an orthotopic ovarian cancer mouse model, luciferase-expressing HeyA8 cells were coincubated with the miR-CAFs into the ovaries of nude mice and tumor growth was monitored by bioluminescence (Fig. 4A and Supplementary Fig. S7A). Reprogrammed fibroblasts clearly increased the growth of co-injected HeyA8 cells, an effect that could be blocked by injections with a neutralizing anti-CCL5 antibody. This indicates that the CCL5 secreted by miR-CAFs is a key tumor-promoting factor. To determine the in vivo significance of CCL5 in the activity of patient isolated CAFs, luciferase-expressing HeyA8 cells were co-injected orthotopically with either NOFs or CAFs (Fig. 4B and Supplementary Fig. S7B). The neutralizing anti-CCL5 antibody again inhibited the augmented growth of HeyA8 promoted by co-injected CAFs. The histology of the ovaries from mice co-injected with either NOFs, miR-CAFs, or CAFs (Fig. 4C) showed that all tumors were invasive, high-grade, and were primarily composed of cancer cells. Tumor cells co-injected with either miR-CAFs or CAFs showed increased invasive growth replacing normal ovarian structures such as follicles and fallopian tube more efficiently as compared with co-injected NOFs. The cancer cells in both the miR-CAF IgG and
CAF IgG group had more Ki-67 staining (Supplementary Fig. S7C and S7D) indicating increased proliferation that could be inhibited by the anti-CCL5 antibody. Consistent with the histologic analysis of the subcutaneously injected tumors (Fig. 1G), there was no difference in infiltrating immune cells (data not shown).

Our data suggest that CCL5 is an important factor in the provision of a tumor-promoting environment for ovarian cancer cells. However, the in vivo experiments did not exclude the possibility that CCL5 acts by enhancing the conversion of NOFs to CAFs rather than by affecting the cancer cells. To test this possibility, a novel homing assay was developed in which Matrigel plugs containing the different fibroblasts were placed equidistantly in the same culture dish and overlaid with GFP-expressing HeyA8 cells. Half of the wells were treated with an IgG control antibody and half were treated with a neutralizing anti-CCL5 antibody (αCCL5). Consistent with the histologic analysis of the subcutaneously injected tumors (Fig. 1G), there was no difference in infiltrating immune cells (data not shown).

Our data suggest that CCL5 is an important factor in the provision of a tumor-promoting environment for ovarian cancer cells. However, the in vivo experiments did not exclude the possibility that CCL5 acts by enhancing the conversion of NOFs to CAFs rather than by affecting the cancer cells. To test this possibility, a novel homing assay was developed in which Matrigel plugs containing the different fibroblasts were placed equidistantly in the same culture dish and overlaid with media containing fluorescently labeled ovarian cancer cells. Two ovarian cancer cell lines were attracted by NOFs transfected with either anti-miR-31 or anti-miR-214 (Fig. 4D and E) as well as to induced CAFs (Fig. 4F), an effect that was inhibited by neutralizing CCL5 antibody. These data suggested that CCL5 acts by binding to one of its cognate receptors (CCR1, 3, and 5) on the surface of the cancer cells. Analysis of a data set comparing ovarian cancer samples and normal ovarian surface epithelial cells (14) revealed that CCR1 is upregulated in primary ovarian cancer (data not shown). Consistently, we found CCR1 to be expressed in both HeyA8 and SKOV3ip1 cells (Supplementary Fig. S8A), and treatment of the ovarian cancer cells with the CCR1/3 inhibitor J113863 blocked the homing of either HeyA8 or SKOV3ip1 cells to NOFs transfected with anti-miR-214 and anti-miR-31 (Fig. 4G and H). Finally, ectopic expression of CCL5 in NOFs (Supplementary Fig. S8B) significantly increased their ability to promote HeyA8 cell coinvasion, an effect blocked by a CCL5-neutralizing antibody (Fig. 4I).
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DISCUSSION

Although the cancer-promoting role of CAFs is unambiguously established, it is less clear how quiescent fibroblasts are transformed into CAFs (1). We tackled this question with 2 complementary approaches: comparing primary human fibroblasts from patients with and without ovarian cancer and a 3-dimensional organotypic coculture model. The results indicate that the downregulation of miR-214 and miR-31 and the upregulation of miR-155 can rapidly reprogram normal fibroblasts into CAFs. These 3 miRNAs combined are able to activate tumor-promoting functions, including migration, invasion, and colony formation, in normal fibroblasts. To the best of our knowledge, this is the first report that shows that 3 miRNAs cooperate in reprogramming quiescent fibroblasts into cancer growth–promoting active fibroblasts, suggesting that resting fibroblasts possess the plasticity to become CAFs.

Our data suggest that CCL5 is a miRNA-regulated candidate effector molecule in CAFs, contributing to tumor cell recruitment and growth. CCL5 is abundant in the serum of ovarian cancer patients with advanced, metastatic disease (15). It is also secreted by mesenchymal stem cells, which have similar functional properties to fibroblasts (16). Of note, the overexpression of CCL5 in fibroblasts was sufficient to promote the metastasis of admixed breast cancer cells (17) in a manner similar to our results, which showed that contact with CCL5-transfected NOFs increased the invasion of ovarian cancer cells.

CAFs at least partially maintain their characteristics when cultured in the absence of malignant cells, suggesting that genetic or epigenetic alterations have occurred. Although genetic alterations have been reported in CAFs from breast and ovarian cancer (18, 19), careful analysis showed that such genetic alterations are extremely rare (20). It is more likely that the stable phenotype of CAFs is regulated by epigenetic changes, as suggested by a genome-wide analysis of breast cancer stroma (21). Our results also lend support to a model in which tumor cells directly reprogram normal residential fibroblasts to become CAFs in the absence of permanently acquired mutations by changing miRNA expression in the fibroblasts (Fig. 4J). The tumor-promoting role of CAFs, together with their genetic stability, makes them an attractive therapeutic target in treatments aimed at removing tumor-supporting factors from the microenvironment. The model for miRNA-induced NOF → CAF transformation presented here opens the possibility of a treatment approach targeting the tumor stroma with miRNA and miRNA inhibitors.

METHODS

Additional methods are included in the Supplementary Data.

Fibroblast Isolation and Characterization

CAFs were isolated from the tumor-transformed omentum and adjacent NOFs from the normal part of the omentum. All patients had newly diagnosed advanced, metastatic high-grade serous ovarian carcinoma and were undergoing primary debulking surgery by a gynecologic oncologist at the University of Chicago (Chicago, IL). NOFs were from omentum of female patients undergoing surgery for benign reasons. CAFs, adjacent NOFs, and NOFs were isolated as described (22, 23). CAFs were characterized by the expression of α-smooth muscle actin (α-SMA), which was not expressed by the adjacent NOFs or NOFs (Supplementary Fig. S9A–C). The miR-CAFAs did not express α-SMA, which was expected, as it is not a target of the 3 miRNAs used to reprogram NOFs. Of note, α-SMA is not expressed in all CAFs even though these CAFs are functionally active (24). CAF activity was validated through functional experiments (increased invasion, migration, colony formation, and tumor growth when coinjected with ovarian cancer cells in vivo). Induced CAFs were generated by coculture of NOFs with ovarian cancer cells, and miR-CAFAs were generated by transfection with locked nucleic acid (LNA) anti-miR-214, anti-miR-31 (Exiqon), and pre-miR-155 (Ambion).

Cell Lines

Human ovarian cancer cell lines SKOV3ip1, OVCAR5, and HeyA8 were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifier kit (Applied Biosystems) and compared with known American Type Culture Collection fingerprints, the Cell Line Integrated Molecular Authentication database (CLIMA), and the University of Texas MD Anderson Cancer Center fingerprint database.

miRNA and Gene Array Analysis

Total RNA was isolated and subjected to both miRNA array (miRCURY LNA array, v. 10.0, Exiqon) and gene array analyses using GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). All gene array data are available through Gene Expression Omnibus (GEO) accession number GSE35364.

Invasion and Migration

Fibroblasts labeled with cell tracker red (CMTPX, Invitrogen) and GFP-expressing tumor cells were mixed and seeded in 96-well glass-bottom plates coated with growth factor–reduced Matrigel. Thereafter, upward invasion of cells through Matrigel towards Dulbecco’s modified Eagle’s medium with 10% FBS was followed with time-lapse confocal microscopy (Supplementary Fig. S2). The speed of the invading cancer cells was analyzed with Imaris Software (Bitplane Inc.). Migration was quantified using Transwell assays (23).

Plug-Homing Assay

NOFs transfected with anti-miR-214 and miR-31 or with equimolar scrambled controls were mixed with 20 μL of growth factor–reduced Matrigel. Plugs of miR-214 + miR-31 or control-transfected NOFs or Matrigel alone were placed equidistant in the same culture dish. Alternatively, plugs of induced CAFs or NOFs or Matrigel alone were used. Homing of GFP-labeled cancer cells towards the plugs was monitored using an Axio-observer A1 fluorescent microscope (Carl Zeiss) and quantified using Image J software (NIH).

 Xenograft Experiments

NOFs (1 × 10⁵ cells) transiently transfected with anti-miR-31, anti-miR-214, and pre-miR-155, or with the scrambled controls for each, were mixed with 0.5 × 10⁵ HeyA8-Luc cGFP cells and then injected subcutaneously into the flanks of female athymic nude mice. Alternatively, 50,000 CAFs, NOFs, anti-miR-214, anti-miR-31– and pre-miR-155–transfected NOFs, or scrambled control-transfected NOFs were coinjected with 25,000 HeyA8-Luc cGFP cells into the right ovary of nude mice with 2 μg/mL of CCL5-blocking antibody or nonspecific mouse IgG. Mice were subsequently injected with 1 mg/kg CCL5 antibody or IgG control intraperitoneally 3 and 6 days thereafter. Tumor growth was quantified using the Xenogen IVIS 200 Imaging System. Radiance was measured for tumors on each flank for the subcutaneous model. Total radiance from the tumors in each mouse was measured for the orthotopic, intra-abdominal model. Significance was calculated comparing all groups using 1-way ANOVA.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

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