Supplementary Methods for

MicroRNAs Reprogram Normal Fibroblasts into Cancer-Associated Fibroblasts in Ovarian Cancer

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Glossary:

<table>
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<th>Term</th>
<th>Definition</th>
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<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
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<td>NOF</td>
<td>Normal omental fibroblast extracted from the omentum of patients operated on for a benign cause.</td>
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<tr>
<td>Adjacent NOF</td>
<td>Corresponding adjacent normal omental fibroblasts (NOFs) from histologically normal omentum at least one inch from the tumor used for CAF extraction.</td>
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<tr>
<td>iCAF</td>
<td>Induced CAF-like fibroblasts generated through co-culture of normal omental fibroblasts with ovarian cancer cell lines for two to seven days</td>
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<td>miR-CAF</td>
<td>Normal fibroblasts reprogrammed into CAFs by transfecting the normal omental fibroblasts with a combination of anti-miR-214 + anti-miR-31, and pre-miR-155.</td>
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Materials
CCL5 antibody (Cat# MAB278, Clone 21445) was purchased from R&D Systems (Minneapolis, MN) and nonspecific mouse IgG was from ChromePure, Jackson ImmunoResearch (West Grove, PA). Collagenase type 3 (Cat# LS004208) and hyaluronidase (Cat# LS002592) were from Worthington Biochemical Cooperation (NJ). Trypsin, Dulbecco's Modified Eagle Medium (DMEM), MEM vitamins, MEM nonessential amino acids and Penicillin-Streptomycin were purchased from Media Tech (Manassas, VA). TaqMan microRNA assays for hsa-miR-214, hsa-miR-31 and hsa-miR-155 and gene expression assays for CCL5, CCL20 and IL8 were obtained from Applied Biosystems (Foster City, CA). Low melting point agarose was from Research Products International Corporation (Mt. Prospect, IL). CMTPX (Cat# C34552) and CMFDA (Cat# C2925) were purchased from Invitrogen, growth factor reduced Matrigel from BD Biosciences (Rockville, MD), pre-miR-214, 31, 155 and scrambled pre-miR negative control were from Ambion (Austin, TX), and miRCURY LNA anti-miR-214, 31, 155 and scrambled anti-miR negative control were from Exiqon (Vedbaek, Denmark).

Plasmids
The lentivirus vector expressing copepod (c)GFP (CD511B-1), and the lentivirus packaging kit (LV500A-1) were purchased from System Biosciences (Mountain View, CA). Firefly luciferase 3’ UTR reporter construct with wild type human CCL5 3’-UTR was purchased from SwitchGear Genomics (Menlo Park, CA). Mutations at the seed sequence of hsa-miR-214 and hsa-miR-31 in the 3’-UTR of CCL5 were made using Quick Change Lightning mutagenesis kit (Stratagene) according to the manufacturer’s protocol. p-BABE-neo-hTERT, pWZL blastcidin human CCL5 and pWZL blast GFP were obtained through AddGene (Cambridge, MA) and is originally from Robert A. Weinberg, Whitehead Institute, MIT, Cambridge, MA.

Carcinoma Associated Fibroblasts (CAFs), Adjacent Normal Omental Fibroblasts (aNOFs), Normal Omental Fibroblast (NOFs), Co-culture Induced CAFs (iCAFs) and miRNA-reprogrammed CAFs (miR-CAFs)
aNOFs and CAFs were isolated (1, 2) from normal or tumor-transformed omentum of patients with newly diagnosed serous-papillary, grade III, ovarian cancer undergoing primary debulking surgery by a Gynecologic Oncologist at the University of Chicago Hospital, Department of Obstetrics and Gynecology, Section of Gynecologic Oncology.
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Normal omental fibroblasts were extracted from omental biopsy obtained from female patients undergoing surgery for a benign reason. Informed consent was obtained before surgery and the study was approved by the IRB of the University of Chicago where the studies were performed. In total, the study involved CAFs and NOFs from 59 patients (22 with serous ovarian cancer and 37 with benign disease). The fibroblasts were grown in DMEM with 10% FBS and used for experiments between passages 2 and 5. CAFs were characterized by the expression of α-SMA which was not expressed by the aNOFs or NOFs (Supplementary Fig. 9). The miR-CAFs did not express α-SMA, which was expected, since it is not a target of the three miRNAs used to reprogram NOFs. Of note, α-SMA is not expressed in all CAFs even though these CAFs are functionally active(3). Activity of CAFs was defined by functional experiments (increase in motility, invasiveness and colony formation of tumor cells mixed with fibroblasts and increased growth of co-injected tumor cells in \textit{in vivo} mouse experiments).

For \textit{in vivo} mouse experiments, fibroblasts were immortalized by hTERT (4). Induced CAFs (iCAFs) were generated by co-culture of NOFs with HeyA8 cells and miR-CAFs were generated by transfecting NOFs with (30 nM each) LNA anti-miR-214, anti-miR-31 (Exiqon), and pre-miR-155 (Ambion).

**Human Cell Lines**

Human ovarian cancer cell lines SKOV3ip1 and HeyA8 were from Dr. Gordon B. Mills (M.D. Anderson Cancer Center, Houston, TX) while OVCAR5, THP-1 and 293T cells were obtained from American Type Culture Collection. THP-1 cells were differentiated with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment for 3 days. HeyA8 cGFP and SKOV3ip1 cGFP cells (expressing copepod GFP) were generated by infecting HeyA8 and SKOV3ip1 cells with lentivirus generated from pCDH-CMV-MCS-EF1 vector (Cat# CD511B-1, SBI). All cell lines were validated by STR DNA fingerprinting using the AmpF/STR Identifier kit (Applied Biosystems). The STR profiles were compared to known ATCC fingerprints, to the Cell Line Integrated Molecular Authentication database (CLIMA), and to the MD Anderson fingerprint database.

**Co-culture of NOFs with HeyA8**

For miRNA micro array analysis, NOFs (7x10^5) were labeled with CMFDA (Cell tracker green, Invitrogen) and co-cultured with 1.5x10^6 HeyA8 cells for 2 days. For gene array analysis unlabeled NOFs (7x10^5) were co-cultured with 0.3x10^6 HeyA8 cGFP cells for 7 days to convert them into induced CAFs. Following co-culture, the induced CAFs were separated by FACS sorting (FACSAnia, BD Biosciences, Sparks, MD) for subsequent analysis.

**miRNA Array**

Total RNA was isolated using the miRNeasy mini kit (Qiagen) according to the manufacturer’s protocol and submitted to Exiqon for miRNA profiling using the miRCURY LNA array (v.10.0). All microRNAs with p-values \leq 0.05 for CAFs \textit{versus} adjacent NOFs (from 6 patients) were compared with those with p-values \leq 0.05 for induced CAFs \textit{versus} NOFs (from 3 patients) to identify miRNAs that were altered similarly in both. The p-values were measured by a one-sided paired t-test. In addition, all miRNAs with a fold change of less than 1.2 were eliminated. Finally, all miRNAs that were not significantly expressed were eliminated. Significance of expression was determined by ranking all average Hy3 signals after normalization across all slides. Signals ranged from 10 to about 13,000. All signals below 200 (~1.5% of the maximal median signal) were eliminated. The remaining miRNAs were ranked according to their significance of their expression in either CAFs or
miRNAs regulate CAFs and are shown in Table S1. Altered miRNA expression was confirmed by real time PCR as described (5).

miRNA *in situ* Hybridization

*miRNA in situ* hybridization on FFPE fixed samples was performed using the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) (Exiqon #90005) as described in the instruction manual v1.3. (Exiqon) which is based on a published protocol (6). The Roche DIG Wash and Block Buffer Set (Roche, Cat. No. 11 585 762 001) was used. Slides with tumor material and normal adjacent tumor-free omentum were processed in parallel. For the in situ hybridization, the LNA™ scrambled microRNA probe double-DIG labeled (5'- GTGTAACAGTCTATACCCCA -3') (Exiqon, Cat# 99004-15) was used as negative control. The hsa-miR-214 (5'-ACTGCCTGTCTGTGCCTGCTGT -3') (Exiqon, Cat# 38494-15), and hsa-miR-155 miRCURY (5'-ACCCCTATCACGATTTAGCATTAA-3') (Exiqon, Cat# 38537-15) LNA™ microRNA detection probes, both double-DIG labeled, were used to stain the FFPE fixed tissue. The miR-214 and miR-155 miRCURY LNA™ microRNA detection probes were used at a final concentration of 50 nM to 80 nM. 25 μl probe mixture was used for an 18x18 mm hybridization area. When the hybridization area was larger the volume was increased accordingly. The hybridization temperatures suggested by the vendor differed from those used due to the probe sequence and its respective LNA modifications and were, therefore, optimized for each probe individually. *In situ* hybridization and high stringency washes were performed at 55°C for the scrambled microRNA probe, at 51°C for miR-155, and 62°C for miR-214. The alkaline phosphatase reaction took place at 30°C for at least 120 min. Slides were placed at 4°C overnight to allow for more time flexibility during development of the dark-blue NBT-formazan precipitate. Prior to mounting, slides were counterstained with Nuclear Fast Red™ (Vector Laboratories, Burlingame, CA, Cat# H-3403) for 20 sec. Images were acquired on an Olympus BX41 microscope equipped with a digital camera and saved in 16 bit tiff format.

Transient Transfections

NOFs/CAFs were transfected (7) with 30 nM pre-miR-214, 155, or scrambled control oligo (Ambion) or with 30 nM miRCURY LNA anti-miR-214, 31, 155, or scrambled control LNA oligo (Exiqon) unless specified otherwise, using siPORT neoFX (Ambion). As a control for the triple transfection (pre-miR-214 and 31 together with anti-miR-155 or anti-miR-214 and 31 together with pre-miR-155) scrambled pre-miR and anti-miR controls were used in triple transfections at the same molar ratio as the respective pre-miRs and anti-miRs. The cells were used for experiments 48 h after transfection or as indicated. For individual transfections, each pre-miR, anti-miR or scrambled control was transfected similarly at 30 nM concentration.

Migration

Transwell migration assays were conducted using 8 μm pore size inserts (BD, Falcon). CAFs/NOFs (50,000 cells) transfected with pre- or anti-miRs were added to the upper chamber in 200 μl DMEM and allowed to migrate for 5 h at 37°C. DMEM with 10% FBS was used as a chemoattractant in the lower chamber. Cells were fixed in 4% paraformaldehyde, stained with Giemsa and cells in the upper chamber removed with cotton swabs to quantify the migrated cells.

Co-invasion – Confocal Imaging

Co-invasion of fibroblasts with ovarian cancer cells was assayed as outlined in Supplementary Fig2A. HeyA8 cGFP or SKOV3ip1 cGFP cells were used along with
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CMTPX (Cell Tracker red, Invitrogen) labeled CAFs/NOFs. Alternatively, ovarian cancer cells were labeled with CMTPX and CAFs/NOFs/aNOFs with CMFDA (Cell Tracker green, Invitrogen).

Ovarian cancer cells and fibroblasts (10,000 cells each) were mixed and seeded in a 96-well plate with a glass bottom (P96GC-1.5-5-F, MatTek, Ashland, MA) which was coated with growth factor reduced Matrigel. The cells were allowed to attach for 45 min and then covered with Matrigel which was allowed to polymerize for 45 min at 37°C. Thereafter, DMEM with 10% FBS was added and the upward invasion of the cells through Matrigel was monitored with time lapse confocal microscopy at 37°C, 5% CO2, and 95% humidity using a Carl Zeiss LSM 510 microscope as shown in the schematic in Figure S2A.

For the anti-CCL5 antibody experiments, 2μg/ml of the antibody or control IgG was added to both matrigel and medium. The path of each invading cancer cell was tracked and the speed of cells was quantified using the IMARIS 4D image analysis software (Bitplane Inc., Zürich, Switzerland).

Colony Formation

NOFs/CAFs were transfected with pre- or anti-miRs as indicated and used for the colony forming assay 24h later as previously described (8). Briefly, either 2000 HeyA8/HeyA8 cGFP cells or SKOV3ip1 cGFP cells and 40,000 CAFs/NOFs were mixed in medium with 0.35% low melting point agarose and plated in each well of a six well plate. Colonies were allowed to form for 15 days (HeyA8) or 30 days (SKOV3ip1), and imaged using the Syngene G-Box imaging system (Synoptics, Cambridge, UK) and quantified using Gene Tools software (Synoptics, Cambridge, UK).

Gene Array Analysis

RNA was isolated from three CAF/NOF comparisons using the miRNeasy mini kit (Qiagen) according to the manufacturer’s protocol.

1) Patient-derived CAFs and matched adjacent NOFs.

2) Induced CAFs derived from a 7-day co-culture of NOFs with HeyA8 cells and matched NOFs.

3) miR-CAFs 2 days after triple transfection and matched NOFs.

To generate miR-CAFs, NOFs were cotransfected with anti-miR-214, anti-miR-31, and pre-miR-155 (30 nM each). Controls were cotransfected with equimolar amounts of scrambled negative controls. Gene chip analysis was performed as described previously (9) using GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) in the University of Chicago Functional Genomics Facility. The probe level of all the Affymetrix gene array data were read into R software (http://www.r-project.org/), and were then pre-processed by the MAS 5.0 method (Affymetrix). Pre-processing included background correction, normalization, probe-specific background correction, and summarizing the probe set values into one expression measure. All gene array data are available through GEO (accession number GSE35364). The log2 ratio values of all genes were then calculated for all CAF/NOF pairs. All genes with upregulation > 1.5 (log2) both in CAFs and miR-CAFs are listed in Table S2. Array results for the top three genes were confirmed by qRT-PCR using TaqMan gene expression assays (Applied Biosystems) as described previously (10).

Chemokine Array

Based on the gene array results, the 10 most altered chemokines were included in a custom membrane based chemokine array (Ray Biotech). NOFs were transfected with anti-miR-214, anti-miR-31, and pre-miR-155 together (30 nM each) or with equimolar
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amounts of scrambled negative controls. The wells were washed with PBS 48h after transfection and DMEM without FBS was added. Conditioned medium was collected 24h later and used for the chemokine array as per the manufacturer’s protocol. The chemokine expression levels were detected by chemiluminescence (ECL, GE Healthcare) and quantified using Image J software (NIH).

CCL5 ELISA

NOFs were transfected with anti-miR-214, anti-miR-31, and pre-miR-155 individually or together (30 nM each) or with equimolar amounts of scrambled negative controls. Medium was replaced with serum free DMEM after 48h and conditioned medium was collected 24h later for ELISA. The CCL5 ELISA was done using a CCL5 ELISA kit (R&D) according to the manufacturer’s protocol.

Ectopic Expression of CCL5

NOFs were transfected with pWZL blasticidin human CCL5 (11) or control vector (4) obtained from Addgene (plasmid 21305 and 12269) and used 48 h later for co-invasion assay with HeyA8. Ectopic expression of human CCL5 was tested by qRT-PCR using Taqman probe (Applied Biosystems).

Plug-homing Assay

10,000 NOFs were transfected with both anti-miR-214 and anti-miR-31 (30 nM each) or with scrambled negative control. Agarose (3 ml) was layered in a 60 mm culture dish and 3 equidistant 5 mm diameter holes were punched in the Agarose. The transfected NOFs were mixed with 20 μl growth factor reduced Matrigel (1:1 diluted in DMEM) and added to the holes in the Agarose to form Matrigel plugs. Each dish consisted of a plug of anti-miR-214 and anti-miR-31 transfected NOFs, a plug of scrambled control NOFs, and a plug of Matrigel alone. HeyA8 cGFP or SKOV3ip1 cGFP cells (2x10^5 cells in 4 ml DMEM) were added to the plate 24 hour later with 2μg/ml CCL5 blocking antibody or mouse IgG and allowed to home to the plugs. Alternatively, 1nMJ113863 (Tocris Bioscience Cat.# 2595) a CCR1/3 inhibitor (12) or DMSO as a control was used instead of the CCL5 antibody and mouse IgG. To test the effect of induced CAFs, NOFs were co-cultured with HeyA8 for 4 days, sorted by FACS and embedded in matrigel plugs with plugs of parental NOF and matrigel alone as controls. Images of the plugs were taken on day 3 with Axio-observer A1 fluorescent microscope (Carl Zeiss) and quantified using Image J software (NIH).

Luciferase Assay

293T cells (50,000/well) were seeded in a 24-well plate and allowed to attach overnight. Cells were co-transfected with a) firefly luciferase construct (5 ng) with the wild type CCL5 3’-UTR (SwitchGear Genomics) or the CCL5 3’-UTR mutated at miR-214 or miR-31 seed sequences, and b) 0.5 ng pRL-SV40 (Renilla luciferase, Promega) along with 10 nM pre-miR-214/31/scrambled using Lipofectamine 2000 (Invitrogen). Dual Luciferase Assay (Promega) was done 24 h after transfection according to the manufacturer’s protocol. All experiments were performed in triplicate and normalized to Renilla luciferase activity. For each 3’-UTR construct, results for the different pre-miRs were normalized to the scrambled miR controls. To test the effect of endogenous miR-214 and miR-31 levels, 25,000 cells/well CAFs or NOFs were co-transfected with a) 2 μg firefly luciferase construct with the wild type CCL5 3’-UTR or the CCL5 3’-UTR mutated at miR-214 or miR-31 seed sequences along with b) 40 ng pRL-SV40 (Promega) using Lipofectamine 2000 (Invitrogen). A dual Luciferase Assay was performed 24 h after transfection (10). All experiments were performed in triplicate and normalized to Renilla luciferase activity.
alignment of the 3 CCL5 3'-UTRs in Figure S5 was done using the Megalign module of LaserGene 7.0 and the Cluster W function.

Xenograft Experiments
HeyA8 Luc cGFP cells stably expressing both firefly luciferase as well as copepod GFP were generated by infecting HeyA8 cells with a lentivirus expressing both luciferase and GFP. A pCDH-Luc1-cGFP vector was constructed by sub-cloning the firefly luciferase gene from the pGL3 vector (Promega) into the multiple cloning site of a pCDH-CMV-MCS-EF1 vector (SBI), so that both luciferase and cGFP are driven by the same CMV promoter. 1x10^5 NOFs, anti-miR-214, anti-miR-31 and pre-miR-155 transfected NOFs or CAFs were co-mixed with 0.5x10^5 HeyA8 Luc cGFP cells and then injected subcutaneously into the flanks of 6 week old female athymic nude mice. Tumor growth was imaged on day 14 using the Xenogen IVIS Spectrum Imaging System (Caliper Life Sciences, Hopkinton, MA) and tumor size quantified using Living Image software (Caliper Life Sciences). Mice were euthanized after imaging and the tumors were isolated, formalin fixed and paraffin embedded for subsequent immunohistochemical or hematoxylin and eosin staining. HeyA8 Luc cGFP cells (25,000 cells) were mixed with CAFs or NOFs or anti-miR-214, anti-miR-31 and pre-miR-155 transfected NOFs or scrambled control transfected NOFs (50,000 cells) in 5 μl growth factor reduced matrigel containing 2 μg/ml CCL5 blocking antibody or nonspecific mouse IgG. The cells were injected orthotopically into the right ovary of 6 week old female athymic nude mice using a Hamilton syringe as described (13). Thereafter, mice received 1 mg/kg CCL5 blocking antibody i.p. in 200 μl of PBS on days 3 and 6. Tumor growth was imaged on days 12 and 15 using the Xenogen IVIS Spectrum Imaging System and quantified using Living Image software. Mice were euthanized after last imaging and the tumors were isolated, formalin fixed and paraffin embedded for subsequent hematoxylin and eosin staining. Procedures involving animals were approved by the Institutional Committee on Animal Care, University of Chicago.

Immunohistochemistry
Immunohistochemistry and hematoxylin and eosin (H&E) staining was done as described previously (8). Briefly, tissue sections were deparaffinized and rehydrated for immunohistochemical staining for Ki-67 (1:300, Cat# RM-9106-s, Clone: SP6, Labvision, CA) and F4/80 (1:500, Cat# MCAP497, Clone: Cl:A3-1, AbD Serotec, NC). Detection was done with Envision+ system (DAKO, CA) or Elite kit (Vector Laboratories, CA) respectively. All H&E staining were evaluated by a gynecologic pathologist for stroma content, tumor cytology, infiltrating lymphocytes and granulocytes.

Immunofluorescence Microscopy
CAFs/NOFs/adjacent NOFs were probed with anti vimentin (Cat# V6630, Sigma, St. Louis, MO), α-smooth muscle actin (Cat# Ab7818, Abcam, Cambridge, MA), keratin antibodies (Cat# 4545, Cell Signaling, Danvers, MA) (1:200 dilution) and detected with anti-mouse Alexa Flour 488 conjugated secondary antibody (Cat# A11059, Invitrogen). Nuclei were stained with Hoechst 33342 (Invitrogen) and the cover slips were mounted with Prolong Gold (Invitrogen) and imaged with a fluorescence microscope (Carl Zeiss LSM 510).

Immunoblotting
CAF and adjacent NOF lysates were resolved on 4-20% SDS-PAGE and transferred to a nitrocellulose membrane, probed with α-smooth muscle actin (Cat# Ab7818, Abcam) and detected with HRP-conjugated anti-mouse secondary antibody (Cat#7076S, Cell Signaling) using ECL (GE Healthcare, Buckinghamshire, UK).
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**CCR1 RT-PCR**

Total RNA from HeyA8 and Skov3ip1 OvCa and the differentiated acute monocytic leukemia cells THP1 (positive control) cells were converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat# 4368814) followed by PCR for CCR1 or GAPDH using reported (14) primers and PCR conditions and the amplified product resolved on a 1% agarose gel and stained with ethidium bromide.

**SUPPLEMENTARY REFERENCES**
