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ORIGINAL PAPER

Transcription Factor Networks in Invasion-Promoting Breast Carcinoma-Associated Fibroblasts

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Abstract Carcinoma-associated fibroblasts (CAFs) contribute to both tumor growth and cancer progression. In this report, we applied an emerging transcription factor (TF) activity array to fibroblasts to capture the activity of the intracellular signaling network and to define a signature that distinguishes mammary CAFs from normal mammary fibroblasts. Normal fibroblasts that restrained cancer cell invasion developed into an invasion-promoting CAF phenotype through exposure to conditioned medium from MDA-MB-231 breast cancer cells. A myofibroblast-like CAF cell line expressing high levels of smooth muscle actin was compared to normal mammary fibroblasts before and after induction. Comparison of TF activity profiles for all three fibroblast types identified a TF activity signature common to CAFs which included activation of reporters for TFs ELK1, GATA1, retinoic acid receptor (RAR), serum response factor (SRF), and vitamin D receptor (VDR). Additionally, CAFs resembling myofibroblasts, relative to normal fibroblasts, had elevated activation corresponding to NF-kappaB, RUNX2, and YY1, and distinct activity patterns for several differentiation-related TF reporters. Induction of CAFs by exposure of normal fibroblasts to conditioned medium from MDA-MB-231 cells resulted in increased activation of reporters for HIF1, several STAT TFs, and proliferation-

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related TFs such as AP1. Myofibroblast-like CAFs and induced normal mammary fibroblasts promoted invasion of breast cancer cells by distinct mechanisms, consistent with their distinct patterns of TF activation. The TF activity profiles of CAF subtypes provide an overview of intracellular signaling associated with the induction of a pro-invasive stroma, and provide a mechanistic link between the microenvironmental stimuli and phenotypic response.

Keywords Carcinoma-associated fibroblasts · Breast cancer · Transcription factors · Gene regulation · Induction

Introduction

Cancer is a disease of dysfunctional organogenesis that cannot be fully understood by considering cancer cells in isolation from other cell types [1–5]. The importance of noncancerous, supportive cell types in tumor development is reflected by the value of stromal characteristics in prediction of disease progression and treatment response [6–12]. In addition to impacting response, stromal cells such as carcinoma-associated fibroblasts (CAFs) are attractive therapeutic targets, as these cells are relatively stable genetically and less prone than cancer cells to developing resistance [2, 5, 13]. The important contribution of fibroblasts to cancer progression, in combination with the potential to target these cells and curtail therapeutic resistance, has fueled interest in these supportive tumor cells.

Normal fibroblasts restrain tumor growth and help to guide normal tissue homeostasis [14–18]. CAFs, which resemble activated fibroblasts in wound healing and fibrosis, contribute to cancer progression by directly stimulating cancer cell proliferation and invasion, as well as by promoting angiogenesis, lymphangiogenesis, and immune evasion [5, 18–28]. Myofibroblasts are increased in cancer stroma, wounds and fibrotic tissue and can contribute to cancer progression [5, 22, 26]. Thus, the myofibroblast protein α-smooth muscle actin (α-SMA) is frequently used as a marker for CAFs [21, 26, 29, 30]. However, some reports indicate that less than half of fibroblasts in mammary tumor stroma are α-SMA-positive [31, 32]. Additionally, α-SMA does not always correlate with the growth- and invasion-promoting properties of CAFs, and fibroblasts can assume a tumor-supporting role without upregulating α-SMA [31, 33, 34]. The analysis of CAFs has identified considerable heterogeneity that has complicated the study of CAFs with tumor-supporting properties [13, 21, 32, 33, 35–37]. Fibroblasts in noncancerous tissues with phenotypic and functional heterogeneity represent distinct and stably-differentiated fibroblast subtypes [38, 39], and we hypothesize that this is also true of CAFs [13].

The heterogeneity of CAFs and their function can be investigated using a systems biology approach, as complex effects on tumor progression likely result from alterations in multiple cellular processes [40]. Stromal gene expression profiles captured by microarrays have prognostic significance in several cancers [36, 41, 42]. Furthermore, expression profiles have shown that fibroblasts exposed to tumor cells [43] or serum [38] maintain distinctly differentiated phenotypes. While mRNA expression profiles have provided a means to characterize these cells, additional techniques that identify active cellular processes could facilitate a more complete view of fibroblast activation and the promotion of tumor invasion.

In this study, we applied an emerging transcription factor (TF) activity array [44, 45] to fibroblasts to capture the activity of the intracellular signaling network associated with distinct cellular phenotypes. TFs are powerful effectors of cellular responses, as indicated by their ability to turn fibroblasts into induced pluripotent stem cells [46] yet their activity is not well captured by microarrays due to the potential for post-translational regulation and cellular compartmentalization. The novel TF activity array allows for large-scale analysis of multiple TFs simultaneously, and was applied to normal fibroblasts and CAFs. The array was also applied to analyze the activation of normal fibroblasts by cancer cells. These CAFs were subsequently analyzed for the mechanisms involved in promotion of invasion by breast cancer cells, with the goal of connecting the pattern of TF activity and marker expression with the cell phenotype. The TF activity networks in CAFs provide a broad view of the intracellular signaling associated with the induction of a pro-invasive stroma, and may ultimately identify potential targets that will aid in the reversion of CAFs to a tumor-suppressive role.

Materials and Methods

Cell lines and Cell Culture Normal mammary fibroblast (NMF pBabe p53/NMFp) and carcinoma-associated fibroblast (CAF pBabe p53/CAFp) parent lines were a gift of Dr Vincent Cryns. NMF pBabe p53 had been originally isolated from reduction mammoplasty and CAF pBabe p53 had been isolated from an invasive ductal carcinoma with enzymatic digestion and differential centrifugation according to an IRB-approved protocol [26, 47]. Both lines had been immortalized by retroviral infection with pBABEp53DN followed by puromycin (1 μg/mL) selection. BJ HFF were obtained from American Type Culture Collection (ATCC). Cells were maintained in DMEM/F12 supplemented with 10 % fetal bovine serum (FBS) (Benchmark), penicillin/streptomycin, non-essential amino acids, and insulin-transferrin-selenium supplement (Sigma-Aldrich) ("Fibroblast medium"). Fibroblasts

were used before passage 15 for all experiments. MDA-MB-231 cells were obtained from ATCC and maintained in DMEM/F12 supplemented with 10 % FBS, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate ("MEC medium"). Immortalized human mammary luminal epithelial (HMLE) cells were a gift of Dr Robert Weinberg and were maintained in mammary epithelial growth complete medium (Lonza) supplemented with bovine pituitary extract per the manufacturer's instructions (Lonza).

Conditioned Media Experiments Fibroblast-conditioned media were collected for treatment of mammary epithelial cells (MECs). Fibroblasts were allowed to proliferate until they covered approximately 30 % of the culture flask surface area. Medium was then replaced with fresh MEC medium, which was conditioned for 48 h and then filtered (0.2 μm, Millipore) and mixed in a 1:1 ratio with fresh MEC medium. The mixture was stored at −20 °C until use.

For culture of MECs in fibroblast-conditioned media, MDA-MB-231 or HMLE cells were plated at a density of 2×10^4 cells/cm^2 in NMFp-, CAFp,- iNMFp-conditioned, or control medium. Cells were allowed to proliferate for 1 week with complete media changes every 2–3 days. After 7 days, cells were either collected by trypsinization and snap frozen in liquid nitrogen for later quantitative PCR analysis (HMLE cells), or serum starved in MEC media without FBS overnight for invasion assays (MDA-MB-231 cells).

To generate cancer cell-conditioned medium, fresh fibroblast medium was conditioned by MDA-MB-231 cells covering approximately 30 % of the culture flask surface area for 48 h before collection and filtering through a 0.2 μm filter (Millipore). MDA-MB-231-conditioned medium was mixed in a 1:1 ratio with fresh medium and stored at −20 °C until use. To generate iNMFp and iCAFp (induced fibroblasts), NMFp and CAFp were grown in the 1:1 mixture of conditioned:fresh media for 14 days. Following induction, iNMFp and iCAFp were grown in fresh fibroblast medium. Phenotypes were stable for several weeks. For all conditioned media experiments, control medium was generated by filtering fresh medium through a 0.2 μm filter, mixing 1:1 with unfiltered medium, and frozen as described for conditioned medium, until use.

Direct Co-culture Experiments One day prior to plating in co-culture with fibroblasts, MDA-MB-231 cells were stained with the fluorescent vital dye CellTrace CFSE (Invitrogen). The HMLE line expressed green fluorescent protein at high levels so staining was not necessary to distinguish HMLEs from co-cultured fibroblasts. For co-culture, cells were plated at a density of 2×10^4 cells/cm^2 (MECs): 1×10^4 cells/cm^2 (fibroblasts) for a total of 3×10^4 cells/cm^2. Control MECs cultured alone were plated at 3×10^4 cells/cm^2. Co-culture proceeded for 5 days before cells were trypsinized, filtered (100 μm, Millipore), resuspended in 0.5 % bovine serum albumin in phosphate-buffered saline, and MEC populations isolated by fluorescence activated cell sorting (FACS) using a Beckmann Coulter MoFlo High Speed Cell Sorter. A small amount of each isolated MEC sample was plated in a 96-well plate following sorting and inspected to confirm that sorting was effective in removing the fibroblasts from MEC samples. Following sorting, HMLE cells were pelleted by centrifugation, snap frozen in minimal medium and stored at −80 °C for qPCR. MDA-MB-231 cells were re-plated in MEC medium for 8 h to allow attachment to the plate. Medium was then changed to MEC medium without FBS and cells were incubated overnight prior to invasion assays. In a pilot study, growing fibroblasts in conditioned medium from the aggressive breast adenocarcinoma MDA-MB-231 cell line produced similar gene expression changes in fibroblasts as direct co-culture with cancer cells. Consequently, large-scale experiments were performed using conditioned medium for experimental simplicity.

Invasion Assays MDA-MB-231 cells were isolated by FACS following direct co-culture so that changes in their intrinsic invasive properties induced by contact with fibroblasts could be studied without the confounding effect of increased matrix degradation in the presence of fibroblasts. Serum-starved MDA-MB-231 cells were collected using a cell scraper and plated at a density of 2×10^4 cells per chamber in modified Boyden chambers (BD Biosciences) in serum-free medium. Medium containing 10 % FBS was used as a chemoattractant in the bottom chamber. Negative control wells contained serum-free medium rather than FBS-containing medium. Each well contained cells from an independent co-culture or conditioned medium experiment; for experiments involving iNMFs, each well contained MDA-MB-231s cultured with independently-induced iNMFs. Invasion chambers were incubated at 37 °C for approximately 72 h, after which total cells visible in 4 separate fields per chamber were counted and averages for each well calculated. Cells remaining in the top chamber (i.e., cells that did not invade) were then removed with a cotton swab and chambers were fixed and stained in 0.5 % crystal violet solution in 60 % ethanol/40 % phosphate-buffered saline for 1 h. Remaining (invaded) cells in 4 separate fields per well were then counted and averaged for each well. Average invaded cells divided by average total cells for each well was interpreted as the invasive percentage of cells for that well. Invasion assays were repeated 2–6 times for each condition.

Quantitative PCR RNA was extracted using the RNeasy kit (Qiagen) and included DNAseI incubation (Qiagen) to

remove genomic DNA. Total RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific) and an equal amount of RNA was added to each cDNA reaction. cDNA was reverse transcribed using the Accuscript First-rand cDNA synthesis kit (Agilent) and random hexamer primers. For qPCR, the following Taqman assays were used (all from Applied Biosystems, Foster City, CA; assay ID number follows gene name): 18 s, HS99999901_s1; α-SMA, Hs00426835_g1; Caveolin 1, Hs00971716_m1; E-cadherin, Hs01023894_m1; N-cadherin, H200983056; Fibroblast activating protein, Hs00990806_m1; GAPDH, Hs03929-97_g1; Keratin 5, H300361185_m1; MMP1, Hs00899658_m1; MMP3, Hs00968305_m1; MMP14, Hs00237119_m1; Vimentin, Hs01549976. Primer sequences are referenced on the Applied Biosystems website. qPCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). Fold change in gene expression following treatment was calculated using the ΔΔCt method using GAPDH (for fibroblasts) and 18 s (for MECs) as endogenous controls after validating that expression of these genes did not vary with experimental conditions for each cell type. Relative gene expression in different fibroblast cell lines was calculated as 2^-ΔCt and reported relative to the level in NMFp. At least three independent samples collected on different days were run for each comparison.

Immunoassays Medium was collected from 3 to 6 independent cultures of each fibroblast cell line, centrifuged to remove particulates, and frozen at −80 °C until use. Prostaglandin E2 was quantified using the Parameter PGE_2 kit (R&D Systems); transforming growth factor β1 and stromal cell-derived factor 1α were quantified using Duokit enzyme-linked immunosorbent assays (R&D Systems) according to the manufacturer's instructions.

Collagen Staining Each fibroblast cell line was plated into 5–6 wells of chamber slides and cultured until cells covered approximately 50 % of each well. Medium was then removed and cells were fixed in 4 % paraformaldehyde for 2 h. Cultures were then stained with picrosirius red (Polysciences) which stains collagen I and III [48] according to the manufacturer's instructions. Following wash, slides were photographed and retention of picrosirius red stain quantified using ImageJ (National Instituites of Health). Staining intensity values for each well were normalized to the average level for corresponding NMFp wells within each experiment.

TF Activity Reporter Arrays The TF activity array, like traditional luciferase assays, uses TF-specific binding sites driving expression of firefly luciferase to quantify TF-dependent transactivation. The TF activity array consists of multiplexed single transcription factor reporters with each TF reporter expressed in parallel in a different well of a 384-well plate. TF reporters consist of a specific TF response element (Supplementary Table 1) cloned upstream of a TA promoter driving the gene for firefly luciferase (*FLUC*) and are packaged in self-inactivating lentiviral vectors. The reporters are referred to using italicized letters to indicate that data are readouts from DNA sequences (reporter constructs) known to be transactivated by the specific TFs for which they are named (Weiss et al., in preparation; Siletz, unpublished results; additional reporter specificity and sensitivity studies are referenced on the TRANSFAC database [49] and Promega website, www.panomics.com). The suffix *–r* is added to indicate that the DNA sequence is a transactivation reporter, and not the gene encoding the TF (Table 1). To fabricate arrays, fibroblasts were removed from culture flasks by trypsinization, spun down and resuspended in complete medium. Fibroblasts were transduced with lentiviral vectors at a multiplicity of infection of approximately 50, and plated at 3×10^4 cells/cm^2 in black 384-well plates (Greiner Bio-One). Plated arrays were incubated for 5 days to allow full reporter expression. On the 5th day after plating, D-Luciferin (Caliper) was added to all wells to a final concentration of 1 mM and allowed to equilibrate for 10 min. Arrays were imaged using a Lumina LTE Xenogen IVIS camera (Caliper) and photon flux (photons/second) for each well was measured. A basal *TA-FLUC* construct without specific TF binding sequences was included in all arrays to control for differences in basal transcription rate. To interpret luminescent signal, luminescence for each well was normalized to the average luminescence from corresponding *TA-FLUC* control wells to account for differences in basal TA activity between cell types. Normalized values were expressed as levels relative to levels for the NMFp cell line to account for differences in viral vector transduction efficiency between experimental replicates. Fibroblasts that were not transduced served as controls for non-enzymatic D-luciferin breakdown in the array. Transduced wells with luminescent signal that were not significantly increased relative to non-transduced control wells were not included in analysis; corresponding reporters were interpreted as having insufficient data to draw conclusions on TF activity and were listed as "Insufficient Data" in Table 2. Each array had 4 repeats per TF reporter and complete arrays were repeated at least 3 times on different days.

Statistical Analysis For analyses comparing more than 2 groups, one-way analysis of variance with Tukey post-test was used to determine the significance of differences. For analyses comparing 2 groups, a two-tailed unpaired t-test was used.

Table 1 List of TF reporters broadly divided into categories of biological function of associated TFs. Note many TFs have well-characterized roles in multiple categories; assignment to a given category of broad biological functions facilitates discussion of results. General biological functions are referenced on the TRANSFAC database (Matys et al. 2003, Reference #49 in text) and the Online Mendelian Inheritance in Man database (http://omim.org). Reporter TF binding sequences and references are listed in Supplementary Table 1

Category	Reporter name	General biological functions of associated TF
Apoptosis and DNA repair	E2F1-r	Cell cycle arrest, apoptosis
	FOXO3A-r	Apoptosis, DNA repair
	SP1-r	Apoptosis, differentiation
Canonical pathways	β-CATENIN-r	Wnt response, Cell cycle, differentiation
	CREB-r	cAMP response
	ELK1-r	MAPK response, proliferation, apoptosis
	GLI1-r	Sonic hedgehog pathway response, transformation
	GR-r	Glucocorticoid pathway response
	RAR-r	Retinoic Acid response pathway, differentiation, apoptosis
	SMAD3-r	TGF-β pathway response
	VDR-r	Cholecalciferol response, differentiation, immune modulation
Cell cycle and proliferation	AP1-r	Cell cycle/Proliferation
	AP3-r	Cell cycle/Proliferation
	C-MYC-r	Proliferation, transformation
	ETS1-r	Proliferation, differentiation, migration, invasion
	PTTG-r	Proliferation, Transformation
	WT1-r	Proliferation, differentiation
	YY1-r	Proliferation, differentiation
Differentiation and development	AP2-r	Development, transformation
	AP4-r	Proliferation, differentiation
	FOXA-r	Differentiation, development
	GATA1-r	Hematopoeitic differentiation
	GATA2-r	Endothelial and adipocyte differentiation, angiogenesis
	GATA3-r	Adipocyte differentiation, T-cell differentiation
	MNX1-r	Differentiation
	RUNX1-r	Hematopoietic differentiation
	RUNX2-r	Osteogenesis, transformation
Inflammatory response	NFAT-r	Inflammatory response, differentiation
	NFκB-r	Inflammation, transformation, metastasis
	STAT1-r	Interferon response
	STAT3-r	Acute phase response
	STAT4-r	IL-12 response
Pluripotency	KLF4-r	Pluripotency, differentiation
Hypoxia response	HIF1-r	Hypoxia response, angiogenesis
Wound response	SRF-r	Serum response, proliferation, differentiation

Results

Activated Fibroblast Cell Lines Relate to Differing Reports of Carcinoma-Associated Fibroblasts in the Literature

A schematic of experiments to assess fibroblast subtypes is presented in Fig. 1. Two immortalized human mammary fibroblast lines were obtained, one isolated from normal mammary tissue (NMF pBabe p53, abbreviated NMFp) and the other from invasive ductal carcinoma (CAF pBabe p53, abbreviated CAFp). The two fibroblast cell lines were noted to have morphological differences consistent with previous reports [23, 29] (Fig. 2a). NMFp morphology was similar to that of other normal fibroblasts with spindle-shaped cell morphology and alignment of long axes of multiple cells in one direction as the cells became confluent. CAFp fibroblasts were broader, more pleiomorphic, and had disorganized orientation, consistent with the phenotype of activated myofibroblasts known to promote tumor growth and spread [22, 26, 29]. Both NMFp and CAFp

Table 2 Summary of transcription factor reporters with altered activity in myofibroblast-like CAFp and cancer cell conditioned medium-induced iNMFp fibroblasts

	Common to CAFp and iNMFp	CAFp only	iNMFp only	No significant difference in either	Insufficient data
Increased	ELK1-r GATA1-r RAR-r SRF-r VDR-r	NFAT-r NFkB-r RUNX2-r YY1-r	AP1-r AP4-r E2F1-r HIF1-r STAT1-r STAT3-r STAT4-r	AP2-r AP3-r CRE-r FOXA-r FOXO3A-r GATA3-r SMAD3-r SP1-r	β-CATENIN-r GL1-r GR-r KLF4-r PTTG-r
TOTAL (percent of all reporters)	5 (14 %)	4 (11 %)	7 (20 %)		
Decreased		C-MYC-r E2F1-r GATA2-r MNX1-r RUNX1-r WT1-r	ETS1-r		
Total (percent of all reporters)	0	6 (17 %)	1 (3 %)	8 (23 %)	1 (3 %) *undetectable in both cell lines* 4 (11 %) *nonsignificant in one cell line, undetectable in the other*

expressed the fibroblast marker vimentin at levels comparable to the well-characterized dermal fibroblast cell line, BJ HFF. Additionally, none of the 3 fibroblast lines expressed the myoepithelial marker keratin 5 [23, 30] (Fig. 2b).

The exposure of normal fibroblasts to cancer cells or cancer cell-secreted factors induced the normal fibroblasts to adopt a CAF phenotype, consistent with previous reports [23, 29, 33]. NMFp fibroblasts cultured in MDA-MB-231 conditioned medium were designated iNMFp to indicate induction by cancer cell secreted factors. Following treatment with conditioned medium, iNMFp cells displayed a phenotype intermediate between NMFp and CAFp, with broadening of cell bodies and some loss of organization of long axes (Fig. 2a). These changes were consistent with previous reports of cancer cell exposure inducing a CAF phenotype in normal fibroblasts [29] and the cellular changes were stable for several passages following removal of conditioned medium.

Gene expression changes associated with growth- and invasion-promoting properties of CAFs were observed in CAFp and iNMFp. Expression levels of α-SMA, matrix metalloproteinases (MMPs), caveolin-1, and fibroblast activation protein (FAP) were investigated, as expression of these factors in breast tumor stroma have demonstrated prognostic significance (Fig. 2c and d) [10, 12, 50–52]. Relative to NMFp, CAFp expressed high levels of α-SMA message, consistent with the activated myofibroblast phenotype that contributes to cancer progression in the desmoplastic stroma around many carcinomas [5, 22, 26]. No difference in mRNA levels for FAP, caveolin-1, MMP1, MMP3, and MMP14 was observed between CAFp and NMFp. In contrast, iNMFp had decreased expression of α-SMA message compared to parental NMFp, and had strong induction of all matrix metalloproteinases assayed, a modest increase in FAP message, and a reduction in caveolin-1 message. Taken together, both CAFp and iNMFp displayed known properties of CAFs, yet the two cell lines had distinct gene expression profiles.

Factors implicated in CAF paracrine function were subsequently measured, including stromal cell-derived factor 1 (SDF-1) [26]; transforming growth factor β1 (TGF-β1) [53]; and prostaglandin E2 (PGE_2) [34]. Quantification of SDF-1α showed increased expression in α–SMA-overexpressing CAFp but not in iNMFp (Fig. 3a), consistent with previous reports of myofibroblast function in tumors [26]. In contrast, TGF-β1 and PGE_2 were not increased in CAFp and iNMFp relative to NMFp (Fig. 3b and c). Increased deposition of collagen by CAFs is a hallmark of the desmoplastic reaction that characterizes the stroma of many breast tumors, and increased collagen in tumor stroma has been associated with poor prognosis [6, 30]. Collagen accumulation assayed by picrosirius red stain was greater in both CAFp and iNMFp cultures compared to NMFp cultures (Fig. 3d). In summary, the CAFp line expresses α–SMA at high levels and has increased production of SDF-1 and collagen, indicative of an activated myofibroblast phenotype. In contrast, NMFp expressed PGE_2 and contact with MDA-MB-231 cells induced expression of MMPs, FAP, collagen, and decreased caveolin-1 expression in iNMFp, suggesting a non-myofibroblast CAF phenotype.

Distinct Mechanisms of Inducing Cancer Invasion by CAF Subtypes

The mechanisms by which the fibroblast subtypes alter the invasive properties of breast cancer cells was subsequently investigated by culturing fibroblast subtypes with MDA-MB-231 cells. Relative to MDA-MB-231 cells cultured alone, co-culture with CAFp or iNMFp significantly increased the invasiveness of the MDA-MB-231 cells, while NMFp did not significantly impact invasion (Fig. 4b). The effect of paracrine factors secreted by each fibroblast type

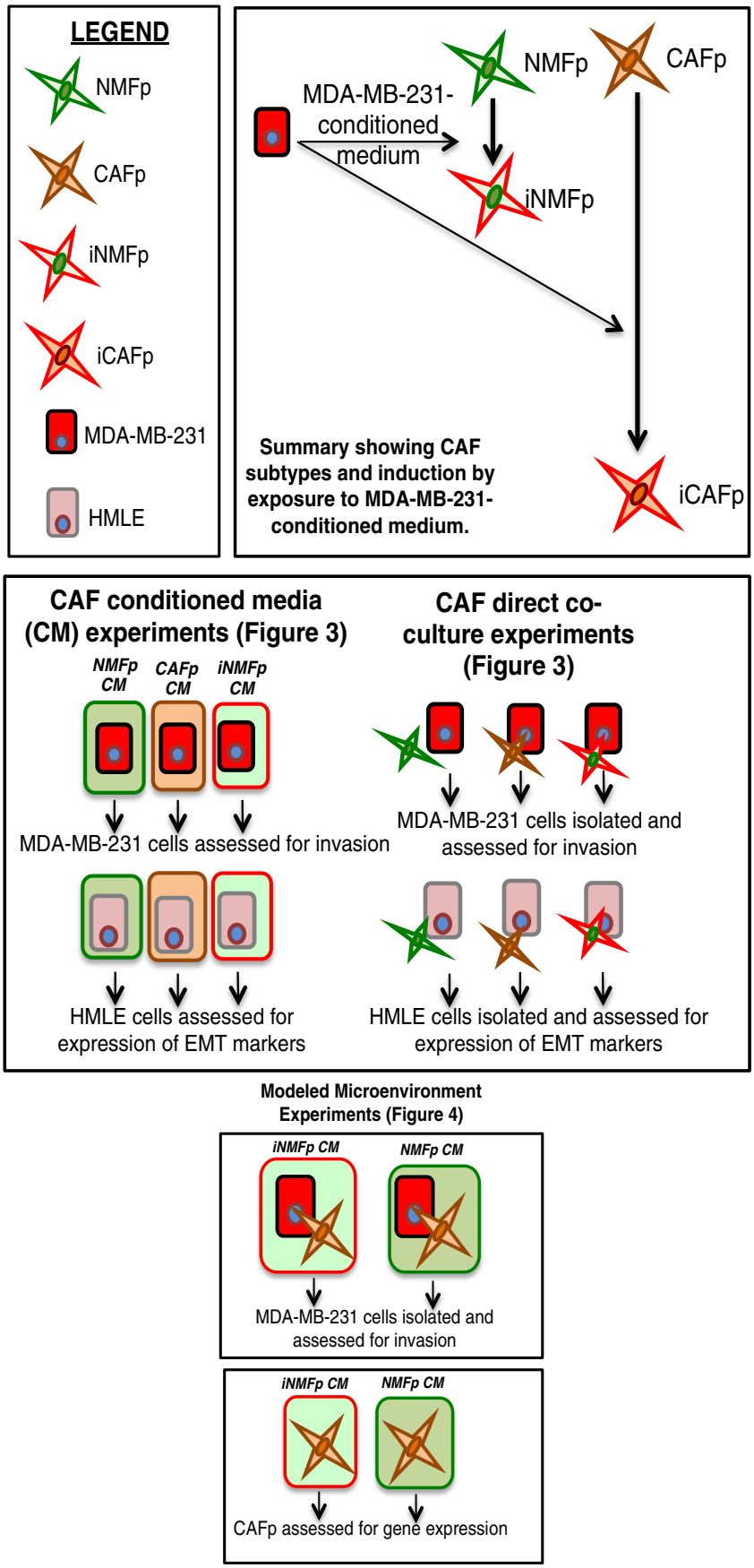

Fig. 1 Schematic of experiments in this study to assess CAF subtypes and normal fibroblasts

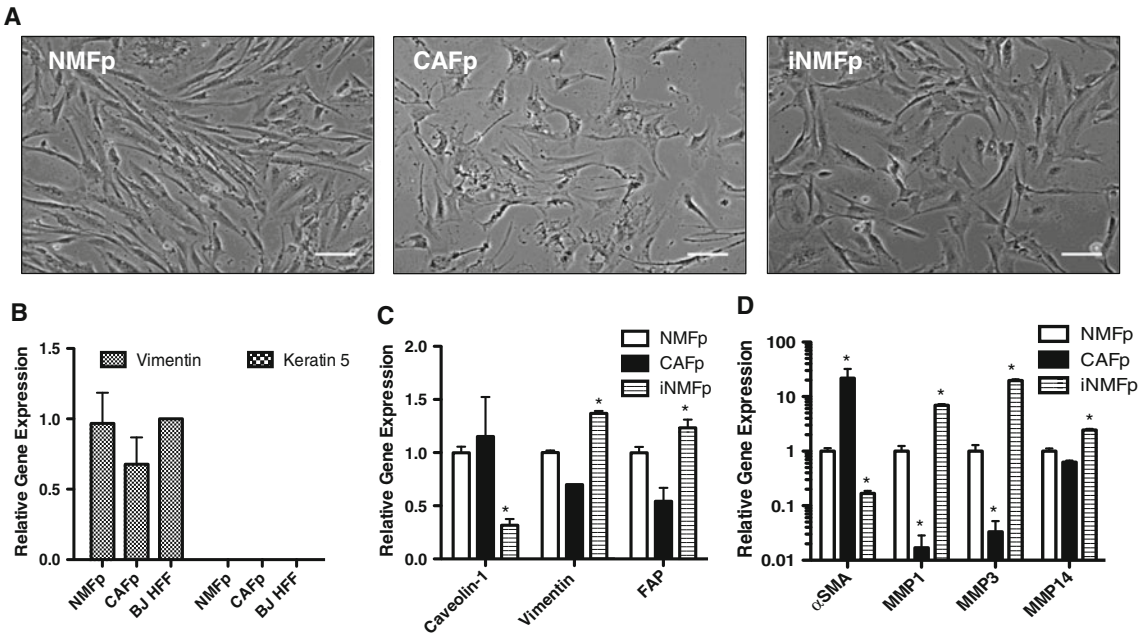

Fig. 2 Phenotypic differences between normal and carcinoma-associated fibroblasts. **a** Morphology of immortalized normal and carcinoma-associated mammary fibroblasts. NMF pBabe p53 (NMFp) have elongated spindle morphology and orient with long axes in the same direction. CAF pBabe p53 (CAFp) are broader, more pleiomorphic, and do not display alignment in the same direction. NMFp exposed to conditioned medium from cancer cells for 14 days to induce fibroblast activation (iNMFp) have an intermediate phenotype with broader cell bodies than NMFp and some loss of orientation of long axes. **b** Expression of fibroblast marker vimentin and myoepithelial marker keratin 5 in NMFp and CAFp relative to the dermal fibroblast line BJ HFF. Keratin 5 was undetectable in all fibroblast samples. **c** and **d** Gene expression of cancer-associated fibroblast markers in NMFp, CAFp, and iNMFp expressed relative to NMFp levels. Note: levels of expression of alpha smooth muscle actin in CAFp varied from 5-fold to 200-fold in all samples assayed. Values shown are representative cultures with intermediate expression. Note log scale in **d.** Scale bar: 100 μm. *Error bars* indicate standard error of the mean. *Asterisk* indicates significantly different from NMFp ($p \leq 0.05$). $n \geq 3$ for all experiments

was then examined by exposing the breast cancer cells to conditioned media from the fibroblasts (Fig. 4c). Conditioned medium from CAFp had no significant effect on cancer cell invasiveness. Conditioned medium from iNMFp significantly increased invasion, although to a lesser extent than the effect of direct co-culture, implicating induction of invasion by both paracrine and direct cell-cell or cell-matrix interactions. Interestingly, conditioned medium from NMFp restrained invasion relative to cancer cells cultured alone. Taken together, CAFp induced invasiveness in the MDA-MB-231 cell line by either direct cell-cell contact or deposition of factors such as matrix components, while iNMFp induced invasiveness by a mechanism that included secretion of paracrine factors and this invasiveness was augmented by direct co-culture.

The ability of each fibroblast line to induce epithelial-mesenchymal transition (EMT) in co-cultured immortalized human mammary luminal epithelial cells (HMLE) was also assessed. EMT is a mechanism by which epithelial-derived breast carcinoma cells become invasive. Hallmarks of EMT include decreased expression of E-cadherin, as well as increased expression of mesenchymal markers such as N-cadherin, fibronectin, vimentin, and matrix metalloproteinases. We investigated whether fibroblasts could induce invasiveness in a well-differentiated, non-tumorigenic mammary epithelial line representing the earliest stages of carcinogenesis. CAFp induced a downward trend in E-cadherin mRNA expression and a significant upregulation of mesenchymal markers in HMLEs in direct co-culture (Fig. 4d). Conditioned medium from CAFp induced variable expression, with a significant decrease in E-cadherin expression but induction of fewer mesenchymal genes than in direct co-culture, paralleling the findings in MDA-MB-231 cells (Fig. 4e). iNMFp also induced expression of mesenchymal genes, but did not repress E-cadherin in direct co-culture and conditioned medium studies. NMFp fibroblasts in direct co-culture had a variable expression pattern, with induction of some mesenchymal genes and repression of others, consistent with the net insignificant effect on invasion seen in experiments with MDA-MB-231 cells. Conditioned medium from NMFp induced expression of some mesenchymal genes, repressed others, and had a trend of increased expression of E-cadherin that was not statistically significant. Taken together, these results indicate that the direct cell-cell contact and paracrine effects of

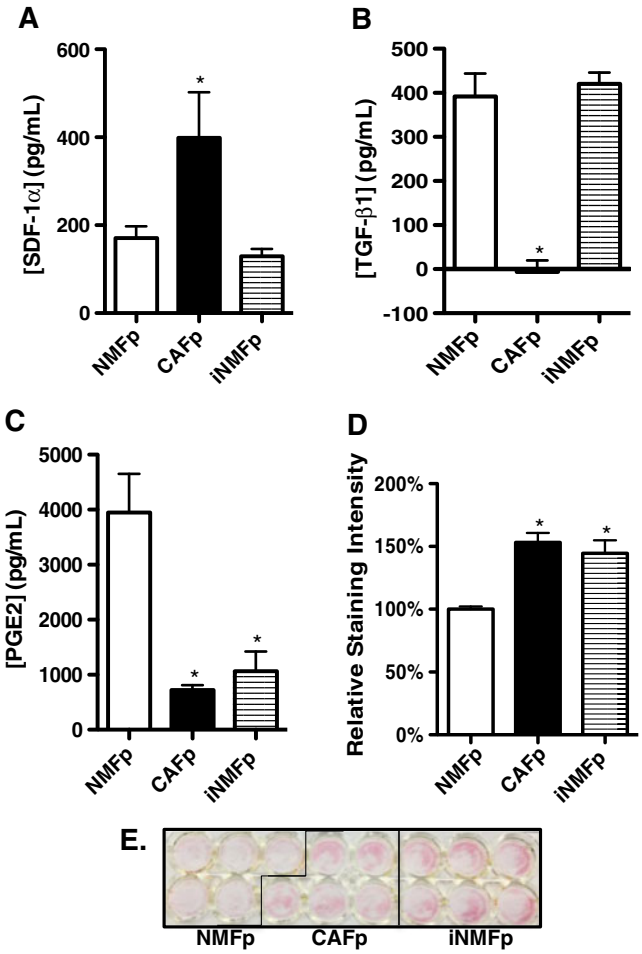

Fig. 3 Quantification of CAF secreted factors. Levels of stromal cell-derived factor 1α (SDF1α) (**a**) and transforming growth factor β1 (TGF-β1) (**b**) in medium from fibroblasts measured by enzyme-linked immunosorbent assay after subtraction of background level from fetal bovine serum in growth medium. **c** Levels of prostaglandin E_2 (PGE_2) in medium from fibroblasts measured by competitive immunoassay after subtraction of background level from fetal bovine serum in growth medium and non-specific binding effect**. d** Quantification of collagen I/III accumulation in fibroblast cultures measured by retention of picrosirius red dye. Staining intensity is shown as a percentage of NMFp average intensity. Representative picrosirius red staining in culture wells is shown in **e.** *Asterisk* indicates significantly different from NMFp with $p \leq 0.05$. $n \geq 3$ for all experiments

fibroblast lines on EMT in the nontumorigenic HMLE cell line did not completely parallel the effects on invasion in an advanced adenocarcinoma cell line.

Mimicking Fibroblast Crosstalk in Cancer Invasion

Stromal fibroblasts in tumors have significant heterogeneity [31, 32], so consequences of fibroblast heterogeneity in the tumor microenvironment were modeled by incorporating both activated fibroblast subtypes. As CAFp primarily increased invasion in direct co-culture, while iNMFp increased invasion by paracrine factors, MDA-MB-231 cells were cultured in direct contact with CAFp while treating with conditioned medium from iNMFp to combine effects from both subtypes. Following isolation, MDA-MB-231 cells displayed an additive increase in invasion compared to the individual effects of direct CAFp culture and iNMFp conditioned medium (Fig. 5a).

Apart from cancer cell-stromal interactions, tumor progression also involves numerous stromal interactions [5]. The effect of fibroblast interactions was further assessed by exposing CAFp to media conditioned by NMFp and iNMFp. Expression of α-SMA was significantly divergent when CAFp fibroblasts were exposed to medium conditioned by fibroblasts, with normal fibroblasts decreasing and activated fibroblasts increasing expression of α-SMA (Fig. 5b). Accumulation of collagen was not significantly altered upon exposure to iNMFp medium; however, collagen accumulation was significantly decreased upon exposure to NMFp medium (Fig. 5c). This result suggests that normal mammary fibroblasts can maintain tissue homeostasis by restraining the abnormal behavior of both mammary epithelial cells (Fig. 3) and other resident fibroblasts (Fig. 5b and c). The functional consequences of fibroblast heterogeneity observed in this simplified model underscore the need for high-resolution studies to elucidate CAF biology.

Distinct TF Circuitry Underlies Carcinoma-Associated Fibroblast Phenotypes

The TF activity array was applied to stromal fibroblasts to identify active pathways and processes associated with fibroblast activation. The array was applied to NMFp, CAFp, and iNMFp fibroblasts, with TF activity derived from specific binding sequences driving expression of firefly luciferase to quantify TF-dependent activity. Reporters are named for the TF that is known to bind each sequence during transactivation (Table 1, Supplementary Table 1), with the suffix *–r* indicating the measured quantity is DNA reporter transactivation from which activity of the TF can be inferred. The 35 TF reporters assayed included those for TFs that act in inflammatory, wound healing, the hypoxia response, pluripotency, and cellular differentiation processes as well as downstream mediators of canonical signaling (Table 1). Of the 35 TF reporters, 15 (43 %) had altered TF activity in CAFp compared to the normal mammary fibroblast line (Table 2, Supplementary Table 2). Nineteen (54 %) had no significant difference and luminescence from one reporter (3 %) was too low to be analyzed in both cell lines. Thirteen of the 35 (37 %) TF reporters had altered TF activity in iNMFp compared to untreated normal mammary fibroblasts; 17 (49 %) showed no significant difference and luminescence from 5 reporters (14 %) was too low for reliable analysis in iNMFp (Table 2, Supplementary Table 3). The TF reporters with insufficient activity for

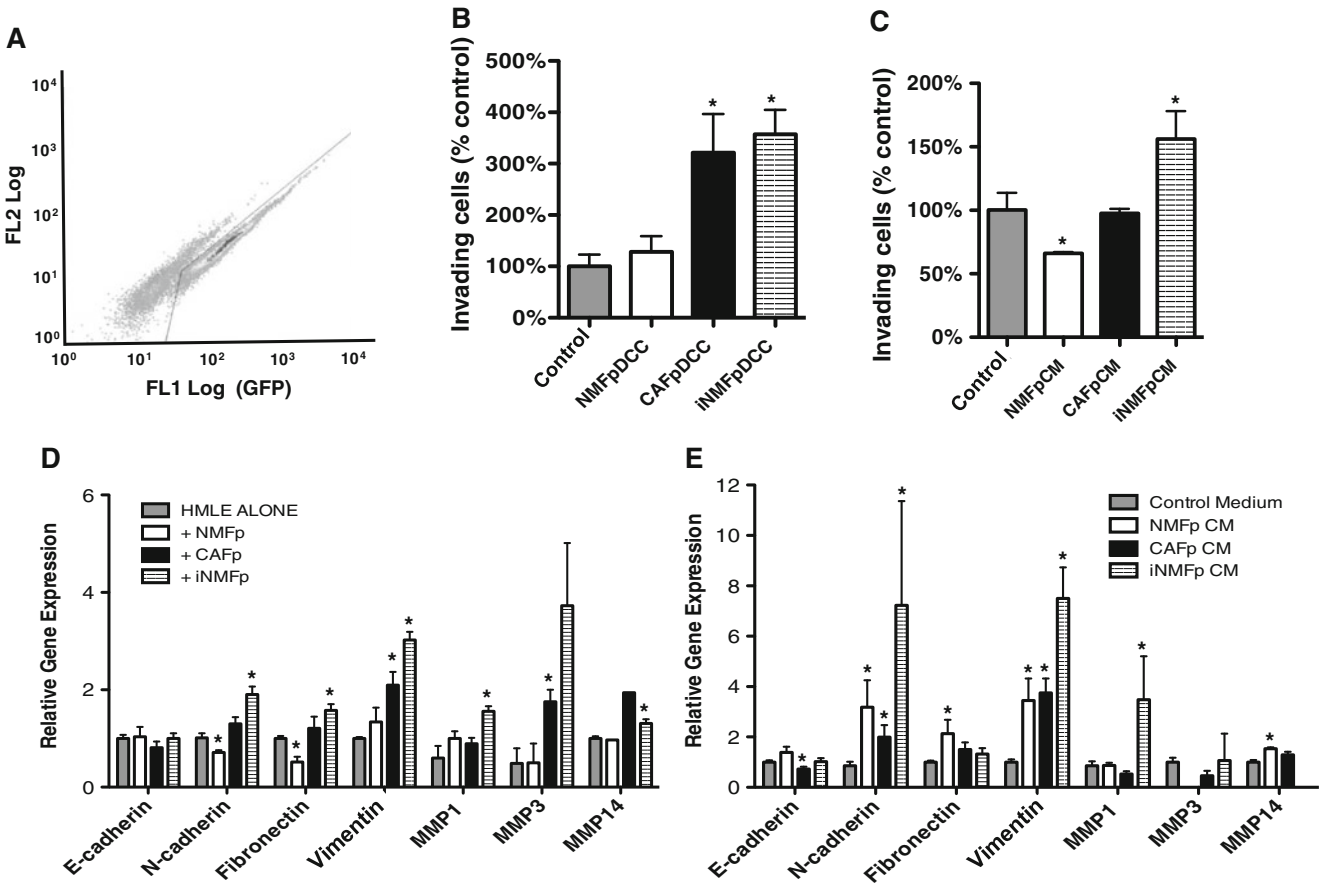

Fig. 4 CAFp and iNMFp fibroblasts increase invasion of mammary epithelial cells by different mechanisms while NMFp fibroblasts restrain invasion by a paracrine mechanism. MECs were co-cultured directly with fibroblasts and then isolated by FACS before assaying, or cultured in conditioned media for 5 days. **a** Representative FACS plot showing isolation of GFP+ mammary epithelial cells from fibroblast populations. Image shown is CAFp/MDA-MB-231 co-culture with 70 % GFP+ MDA-MB-231 cells; because of the higher growth rate of mammary epithelial cells relative to fibroblasts, by the end of culture typically 70–90 % of cells in fibroblast/mammary epithelial cell co-cultures were GFP+ epithelial cells. Note typical high autofluorescence of fibroblast population (left gate) (FL1 is non-GFP fluorescence). For direct co-culture (**b** and **d**), controls were MECs cultured alone. **b** Relative invasion of MDA-MB-231 adenocarcinoma cells in direct co-culture (abbreviated DCC) with fibroblasts after isolation from fibroblasts using FACS. **c** Relative invasion of MDA-MB-231 adenocarcinoma cells cultured in conditioned media (abbreviated CM) from fibroblasts. **d** Expression of epithelial and mesenchymal markers by quantitative PCR in immortalized mammary epithelial (HMLE) cells after direct co-culture with fibroblasts. **e** Expression of epithelial and mesenchymal markers by quantitative PCR in HMLE cells exposed to conditioned media from fibroblasts. *Error bars* indicate standard error of the mean. *Asterisk* indicates significantly different relative to control with $p \leq 0.05$. $n \geq 3$ for all experiments

measurement in iNMFp were not significantly different between CAFp and NMFp.

The TF activity array identified distinctive patterns within the two CAF subtypes. Both CAFp and iNMFp had increased transactivation of *ELK1-r, GATA1-r, RAR-r, SRF-r,* and *VDR*-r (Fig. 6b, Table 2). This result suggests a set of TFs involved in cell growth and differentiation relate to the activation of invasion-promoting fibroblasts. CAFp had elevated transactivation of inflammatory reporters (*NFAT-r, NFkB-r*), as well as divergent transactivation of various reporters relating to differentiation (*GATA2-r, MNX1-r, RUNX1-r, RUNX2-r,*) and cell proliferation (*C-MYC-r , E2F1-r, WT1-r, YY1-r*) compared to NMFp (Fig. 6c, Table 2, Supplementary Table 2). By contrast, iNMFp had mostly increased transactivation of proliferation-related reporters (*AP1-r, AP4-r, E2F1-r, ETS1-r*) as well as an increase in inflammatory (*STAT1-r, STAT3-r*, and *STAT4-r*) and hypoxia response (*HIF1-r*) reporters (Fig. 6d, Table 2, Supplementary Table 3) relative to NMFp. Interestingly, many of the TF activities that were significant in only one activated fibroblast type trended in the opposite direction in the other activated fibroblast type. Ten of the 15 TF activity alterations observed in CAFp relative to NMFp (67 %) and eight of the 13 TF activity alterations in iNMFp relative to NMFp (62 %) were unique to that cell line. Thus the TF regulatory circuitry in CAFp and iNMFp fibroblasts appeared distinctive, which is consistent with the observation of multiple CAF phenotypes.

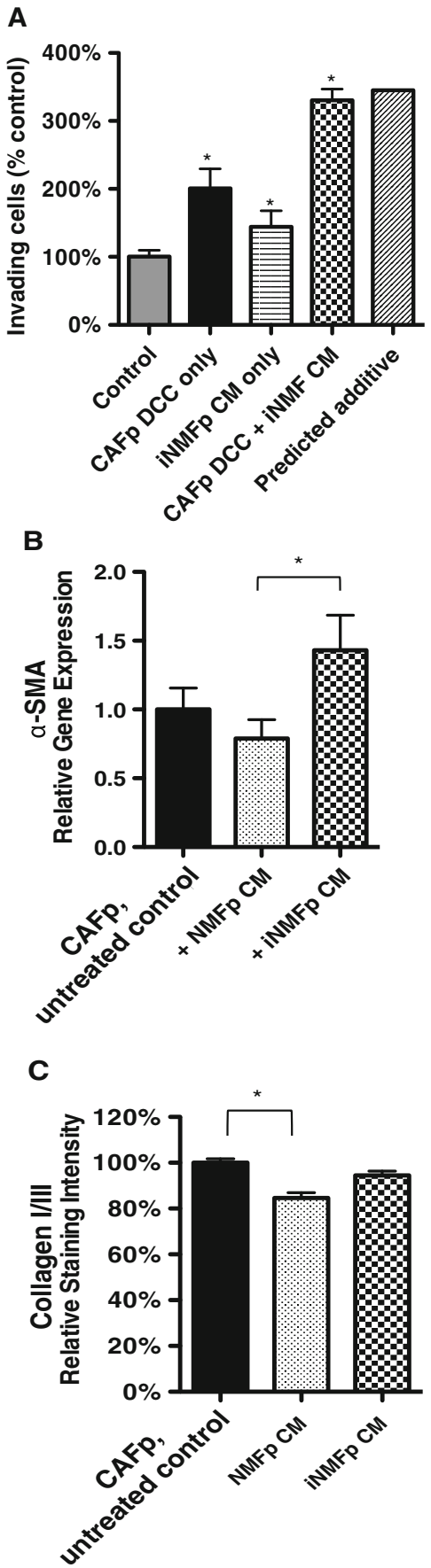

Fig. 5 Combined effects of distinct CAF subtypes in a model of the tumor microenvironment. **a** Effect on invasiveness of MDA-MB-231 cells in direct co-culture with CAFp fibroblasts with and without addition of conditioned medium from iNMFp fibroblasts. Predicted additive effect is the sum of effects of CAFp direct co-culture alone and conditioned medium from iNMFp alone. **b** Changes in α-SMA mRNA expression in CAFp fibroblasts upon treatment with conditioned media from either NMFp or iNMFp fibroblasts. **c** Relative picrosirius red staining intensity indicative of collagen accumulation in CAFp fibroblast cultures treated with conditioned media from NMFp or iNMFp fibroblasts. *Error bars* indicate standard error of the mean. *Asterisk* indicates significantly different from control (CAFp cultured in unconditioned medium) with $p \leq 0.05$. $n \geq 3$ for all experiments

Fibroblasts have Distinct Responses to Cancer Cell Conditioned Medium

The observation of gene regulatory differences in CAFp and iNMFp motivated studies on CAFp exposed to MDA-MB-231-conditioned medium in order to distinguish induction responses that are specific to the conditioned medium from those that are due to the intrinsic properties of the fibroblasts. CAFp exposed to conditioned medium, designated iCAFp, maintained a similar phenotype to parental CAFp (data not shown). In contrast to the response of NMFp to conditioned medium, iCAFp further upregulated α-SMA expression and decreased matrix metalloproteinase expression, increasing the disparity between CAFp and iNMFp (Fig. 7a). Thus the specific response to cancer cell secreted factors was intrinsic to fibroblasts, based on the gene expression markers shown in Fig. 7a.

TF activities were analyzed in iCAFp to identify key processes and pathways associated with cancer cell-stromal crosstalk. Increased activation of *NFκB-r, RUNX2-r,* and *YY1-r* was observed in CAFp and iCAFp but not iNMFp (Figs. 7b and 8, Supplementary Table 4). These results indicate a stable subtype-specific set of TFs whose activity is not altered by exposure to cancer cells. Similarly, although both iNMFp and iCAFp were exposed to the same cancer cell conditioned medium, the increased activation of *AP1-r, AP4-r, E2F1-r,* and *STAT4-r* observed in iNMFp was not observed in iCAFp. Thus, these reporters captured TF activity that was distinct from normal fibroblasts, and could distinguish CAF subtypes regardless of the specific activating signals from cancer cells. The iCAFp TF activity profile also shared some features with the iNMFp profile that were not seen in parental CAFp fibroblasts. iCAFp displayed increased activation of *HIF1-r, STAT1-r* and *STAT3-r* and decreased activation of *ETS1-r*, consistent with iNMFp and indicating a common response in fibroblasts of dissimilar background to specific cancer cell signals. TF reporters with significantly increased activation in both CAFp and iNMFp were also increased in iCAFp (Fig. 8, Supplementary Table 4). These TFs may represent a fundamental activated fibroblast regulatory profile, as they are elevated

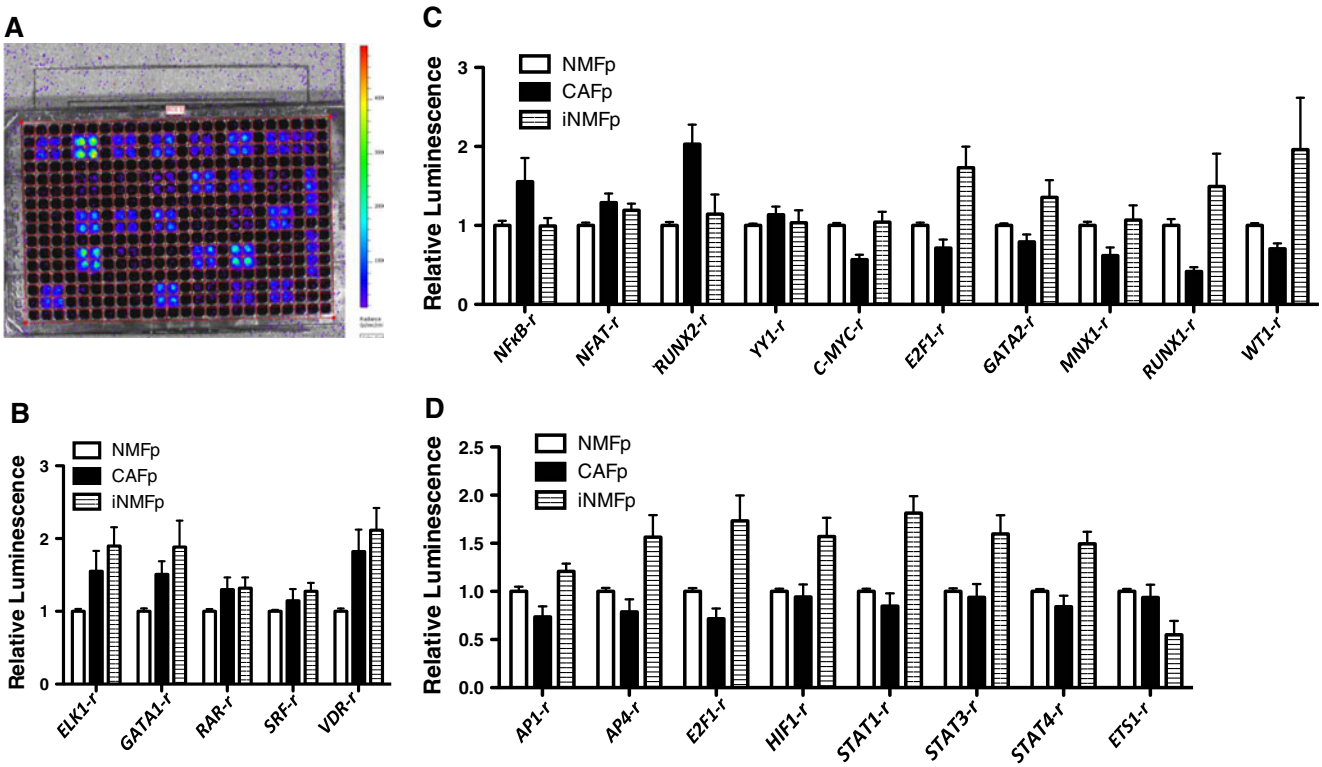

Fig. 6 Transcription factor activity differences in invasion-promoting fibroblast subtypes versus invasion-restraining normal mammary fibroblasts. **a** False color image of a TF reporter array showing luminescent signal from cells transduced with TF reporters with addition of D-luciferin substrate. **b** Transcription factor reporters displaying significantly ($p \leq 0.05$) altered activity relative to normal mammary fibroblasts in both CAFp and iNMFp. **c** Transcription factor reporters displaying significantly ($p \leq 0.05$) altered activity relative to normal mammary fibroblasts in CAFp not observed in iNMFp. Reporters with increased activity in CAFp are shown in alphabetical order followed by reporters with decreased activity in alphabetical order. **d** Transcription factor reporters displaying significantly ($p \leq 0.05$) altered activity relative to normal mammary fibroblasts in iNMfp not observed in CAFp. Normalized luminescence proportional to transcription factor-dependent translation of firefly luciferase is shown on y-axes. Note *E2F1-r* activity was significantly different from NMFp in both CAFp and iNMFp but changes were in opposite directions as shown in **c** and **d**. $n \geq 3$ for all experiments

in CAFs of different origin and inductive treatment. Taken together, these results illustrate TF activity changes that depend on intrinsic fibroblast biology, or intrinsic cancer cell biology, respectively.

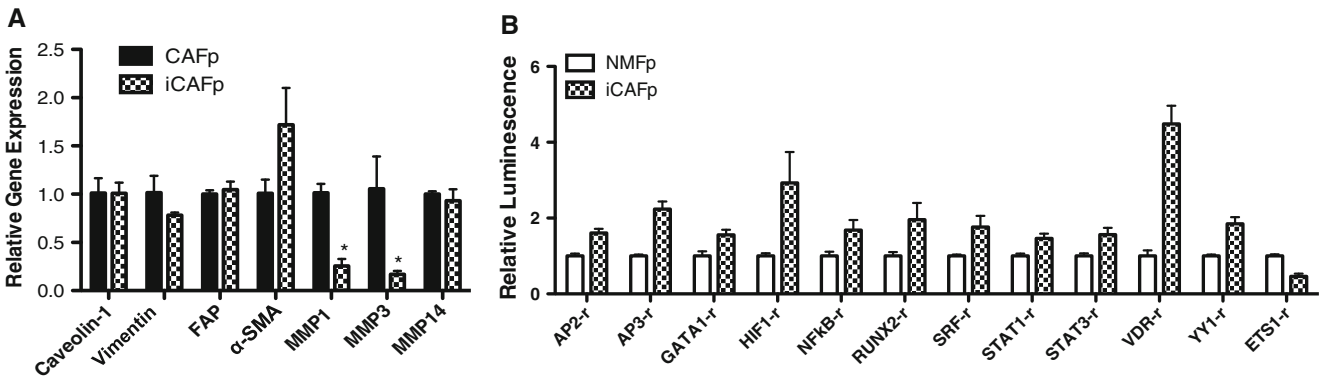

Fig. 7 Gene expression changes and TF activity in CAFp fibroblasts exposed to cancer cell-conditioned medium. **a** Quantitative PCR for CAFp fibroblasts treated with medium conditioned by MDA-MB-231 breast adenocarcinoma cells (iCAFp) compared to untreated CAFp. Compare with Fig. 2c and d. *Error bars* indicate standard error of the mean. *Asterisk* indicates $p \leq 0.05$. $n=3$. **b** Transcription factor reporters displaying significantly ($p \leq 0.05$) altered activity relative to normal mammary fibroblasts in iCAFp. Normalized luminescence proportional to transcription factor-dependent translation of firefly luciferase is shown on y-axis. $n=3$

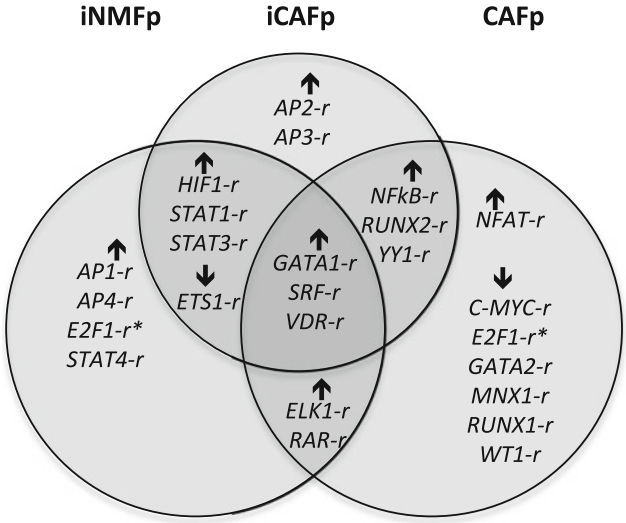

Fig. 8 Venn diagram summarizing TF reporter activation compared to NMFp in CAF subtypes. **E2F1-r* activity was significantly elevated in iNMFp compared to NMFp fibroblasts. The increased activity seen in iCAFp was not significantly different from NMFp levels, but was significantly greater than parental CAFp levels. Significant elevation of *E2F1-r* activity relative to baseline is therefore a common response of iNMFp and iCAFp fibroblasts to MDA-MB-231 conditioned medium, and the opposite is seen in CAFp. Note *ELK1-r and RAR-r* activities were nonsignificantly elevated relative to NMFp in iCAFp fibroblasts; for *ELK1-r* $p=0.06$ and for *RAR-r* $p=0.08$. For all other TF reporters shown $p \leq 0.05$ for all comparisons

Discussion

Tumor invasion is a property critical to cancer progression that arises from interactions between cancer cells and the surrounding stroma [20]. In this report, we investigated two stromal fibroblast populations (CAFp, iNMFp), which had distinct invasion-promoting actions. These fibroblasts differentially expressed gene products implicated in tumor-supportive function, which would indicate distinct roles for CAF subtypes pending confirmation with human tumors. CAFp fibroblasts, similar to previous reports on CAFs, resemble myofibroblasts in their increased expression of α-SMA, SDF-1, and collagen, relative to normal fibroblasts [5, 26, 30, 54]. CAFp fibroblasts induced an invasive phenotype from cancer cells when grown in direct co-culture, which could not be recapitulated with conditioned medium, suggesting an effect mediated by direct cell-cell contact or interactions of cancer cells with extracellular matrix produced by fibroblasts. Conversely, iNMFp did not have high expression of α-SMA but nevertheless supported cancer invasion by both direct co-culture and paracrine mechanisms. The NMFp cell line expressed high levels of PGE_2 and increased expression of MMPs upon induction to iNMFps. High levels of MMP1 [33] and PGE_2 [34] have been previously described in CAFs that lack high α-SMA expression. Taken together, our results demonstrate that the distinctly differentiated phenotypes of CAFp and iNMFp influence cancer invasion by unique mechanisms. Our results (Fig. 5) also suggest that in addition to fibroblast-cancer cell communication, crosstalk between fibroblast subpopulations may influence the malignant potential of the microenvironment. Characterization of these active processes may ultimately have prognostic and therapeutic significance for human breast tumors and other malignancies [13, 19, 34, 36].

TFs are the downstream targets of signaling pathways, and the TF array reflects the activity within the intracellular signaling network. These measurements complement the gene expression profiles provided by microarray technology, by identifying TFs associated with the phenotypes of normal and activated fibroblasts. Significant differences in TF activity within activated fibroblasts relative to normal fibroblasts were detected by nearly two-thirds (63 %) of the 35 TF reporters included in this study. Of these, five reporter activity changes (14 % of total) were common to CAFp and iNMFp, with similar findings in iCAFp although *ELK1-r* and *RAR-r* elevation did not quite reach significance in iCAFp (Fig. 8). Three additional reporters (9 % of total) had significantly altered activity in CAFp and iCAFp, and four additional reporters (11 % of total) had altered activity in iNMFp and iCAFp (Fig. 8). The 5 reporter constructs that had increased activity in all CAFs relative to normal fibroblasts were *ELK1-r, GATA1-r, RAR-r, SRF-r* and *VDR-r*. ELK1 and SRF direct the gene expression response to serum, which has prognostic significance in breast cancer [8, 41]. Increased transactivation of *SRF-r* echoes the growing body of evidence suggesting that tumor biology has a pathologic similarity to wound biology [8, 30, 55]. Furthermore, ELK1 function is increased in activated fibroblasts from the pathologically-exaggerated wound healing response of keloid scars [56]. Elevated *VDR-r* activity has also been connected to a wound-like response, with gene expression induced by serum [57]. GATA1 and RAR are important drivers of cellular differentiation in a number of cell systems, such as hematopoietic differentiation [58, 59]. Fibroblasts in normal tissues progress through stages of differentiation with distinct levels of mitotic activity and patterns of protein synthesis [39]. The common transactivation differences induced by the tumor microenvironment in biologically-distinct CAF subtypes also suggest a conserved set of factors associated with fibroblast activation.

The array captured differences in CAFp and iNMFp associated with distinct effects of each fibroblast line on cancer cell invasion. Relative to normal fibroblasts, CAFp had significantly altered activation of reporters associated with cell proliferation (MYC, E2F1 [decreased], and YY1), differentiation (RUNX2, GATA2, MNX1, RUNX1, and YY1), and inflammation (NFκB, NFAT). In contrast, iNMFp had significantly altered activity for reporters associated with proliferation

(AP1, E2F1 [increased], ETS1); differentiation (AP4), hypoxia (HIF1); and inflammation (STAT1, STAT3, STAT4). While processes such as proliferation, differentiation, and inflammation are common to the TF activity profiles, the specific factors associated with these processes differ. Activity of the inflammation-associated reporters is consistent with an inflammatory microenvironment [28, 60, 61], yet, increased activity of *NFκB-r* was stable for CAFp/iCAFp but not observed in the NMFp/iNMFp line. Both cell lines had increased activation of *STAT1-r* and *STAT3-r* in response to MDA-MB-231 cells (Figs. 6d and 7b, iNMFp and iCAFp), but iNMFp was distinguished by the ability to increase *STAT4-r* activity. Differential activation of NFκB and STAT4 links alternative maturation signals to discrete functional features in dendritic cell populations [62]. CAFp had an increased activity of *RUNX2-r,* which is a master regulator of osteogenic differentiation and is upregulated in breast tumors that metastasize to bone [63]. An osteogenic gene expression signature induced in breast cancer cells and breast stroma has been proposed as the origin of the breast cancer cell/bone stromal interactions that allow for the development of bone metastases [64]. CAFp also had increased transactiviation of *YY1-r* and *NFκB-r*, which together control the differentiation of myoblasts, and are persistently activated in rhabdomyosarcomas [65]. A YY1 binding site is present in the SDF-1 promoter [49] which was upregulated in CAFp but not iNMFp and can endow myofibroblasts with proangiogenic properties [26]. Finally, we note that a striking feature of iNMF gene expression studies was the greatly increased expression of matrix metalloproteinases 1 and 3 (Fig. 2). A TRANSFAC database analysis of sequences within and proximal to the promoters of MMP1 and MMP3 [49] revealed binding sites for AP1 and STAT factors, which were not highly active in CAFp. The TF activity data that is specific to either CAFp or iNMFp suggests these factors may contribute to a fibroblast subtype-specific differentiation program.

The comparison of iNMFp to NMFp and iCAFp to CAFp can identify conserved and differential TF activation during induction by MDA-MB-231 cells. Breast cancer cells likely induce inflammatory and wound-healing responses in their surroundings through specific mechanisms driven by molecular subtype [66]. Conditioned medium from MDA-MB-231 cells contains numerous growth factors and mediators of inflammation, notably IL-6, which canonically activates STAT3, and PDGF, TGF-β, and FGF factors, which are linked to CAF activation by cancer cells [5, 67]. Accordingly, inflammatory (*STAT1-r, STAT3-r)* and growth-related (*ETS1-r*) TF reporters had altered activity in iNMFp and iCAFp relative to normal and CAFp fibroblasts (Fig. 8). *E2F1-r* and *HIF1-r* activities in iCAFp and iNMFp were significantly increased relative to parental CAFp and NMFp levels, which is also consistent with a common response to MDA-MB-231 paracrine signaling. The response to conditioned medium between fibroblast subtypes differed in proliferation and inflammation related reporters. *AP1-r, AP4-r,* and *STAT4-r* activities were elevated in iNMFp, while *AP2-r* and *AP3-r* (an alternate ETS family reporter) were elevated in iCAFp. Activation of proliferation-related reporters is consistent with the markedly increased proliferation of activated fibroblasts [5] and variations in specific proliferation-related activities likely reflects distinct cellular signaling states that integrate paracrine input from the cancer cells to direct fibroblast phenotype. Importantly, all experiments with iNMFs and iCAFs were performed days to weeks after removal of MDA-MB-231 conditioned medium, with stable activity profiles, mRNA expression, and morphology observed over time.

In conclusion, this report investigated TF activity profiles in invasion-promoting mammary fibroblasts. TF activity signatures were identified in multiple CAF functional phenotypes, which promoted invasion through distinct mechanisms. Comparison of TF activity profiles for all three fibroblast types identified a TF activity signature common to CAFs, which may represent TF regulatory events common to activated fibroblast phenotypes. Additionally, CAFs resembling myofibroblasts had distinct TF reporter activity relative to normal fibroblasts that were induced to an activated phenotype by cancer cells. These TF activity profiles complement DNA microarray studies, and may provide a mechanistic link between tumor microenvironmental stimuli and phenotypic response. The systems biology approach described in this study links intracellular (TF) and intercellular (cancer cell-fibroblast and fibroblast-fibroblast) networks in breast cancer invasion, and may ultimately identify potential targets to facilitate return of CAFs to a tumor-suppressive role. Further studies will be needed that assess human tumors for the multiple CAF subtypes at the functional, marker expression, and TF activity levels.

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Conflict of interest The authors declare they have no conflict of interest.

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