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ORIGINAL ARTICLE

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Cancer-associated fibroblasts show heterogeneous gene expression and induce vascular endothelial growth factor A (*VEGFA*) in response to environmental stimuli

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Abstract

Aim: Cancer-associated fibroblasts (CAF) play a crucial role in angiogenesis in the complex tumor microenvironment. However, fibroblasts show extensive heterogeneity and their dynamic functions against stressors remain largely unknown.

Methods: We collected patient-derived CAF and carried out perturbation-based monitoring of the dynamic functions. Clinically relevant experimental stimuli were defined as follows: hypoxia, cisplatin, fluorouracil, coculture with cancer spheroids (interaction through paracrine signals). We selected 18 marker genes that encode components for fibroblast activation, intracellular communication, and extracellular matrix remodeling. Quantitative reverse transcription polymerase chain reaction was carried out for data collection and statistical analyses were carried out using SPSS software.

Results: Kruskal-Wallis multivariate analysis of variance showed that variations in expression of 11 marker genes were explained, in part, by a difference in tissue of origin. Friedman and two-sided Wilcoxon signed rank tests detected significant perturbations in expression of marker genes. Paracrine signal from cancer spheroids induced vascular endothelial growth factor A (*VEGFA*) in CAF but not in fetal lung fibroblasts.

Conclusion: We have established perturbation-based monitoring of patients' CAF. Further data collection and individual patient follow up is ongoing to identify critical determinants of disease outcome.

KEYWORDS

cancer-associated fibroblast, complex system, heterogeneity, perturbation, VEGF-A

1 | INTRODUCTION

Genomic mutations of cancer cells are considered the driving force of tumor development and progression. However, cancers develop in complex tissue microenvironments which contain genetically normal stromal cells.[1,2] Pathologists have observed that not only the mutation profile of cancer cells, but also stromal activation is a predictor of patients' prognosis.[3–6] Among several stromal cell

types, cancer-associated fibroblasts (CAF) play a crucial role in angiogenesis and are attractive therapeutic targets.[7] However, specifically inhibiting CAF is a challenging task. Fibroblasts and their derivatives show great heterogeneity, depending on their tissue of origin[8] and their association with various environmental contexts.[7] Overall, patient outcomes are difficult to predict even when the genomic alterations are fully grasped through sequencing.[9] Genome biologists are becoming aware of the importance of "perturbation-based functional monitoring"[9,10] for precision medicine to achieve its goal. In systems biology, "perturbation" is defined as an alteration of the function of biological systems by external or internal means such as environmental stimuli.[11] Clinical regimens change tumor microenvironments and monitoring the responses of CAF is equally important in the monitoring of cancer cells. In this study, we collected and cultured patient-derived CAF and carried out perturbation-based monitoring of 18 marker genes.

2 | METHODS

2.1 | Patients and biospecimens

This study was approved by the Institutional Review Board of Dokkyo Medical University (ID: 28019) and Ibaraki Medical Center of Tokyo Medical University (ID: 16-43) on the basis of the Ethical Guidelines for Clinical Research of the Ministry of Health, Labour and Welfare, Japan. Patients who were diagnosed as having malignancy at Ibaraki Medical Center agreed to donate the surgically resected tumor specimens for research purposes. Written informed consents have been obtained from all patients. Tumor specimens from 27 patients were used in this study (Table 1).

2.2 | Cell culture

Tumor specimens were washed vigorously with saline and transported from Ibaraki Medical Center to Dokkyo Medical University

TABLE 1 Case description of tumor specimens used in the present study

Patient ID	Age (y)	Gender	Tumor location	Tumor histology	TNM stage
1	61	M	Hepatocellular	Moderate	I
2	62	M	Hepatocellular	Moderate	II
3	80	M	Cholangiocellular	Poor	IVa
4	69	M	Gastric	Well	Ia
5	60	F	Gastric	Poor	IV
6	83	F	Gastric	Poor	IIIb
7	67	M	Gastric	ND	ND
8	49	F	Sigmoid colon	Moderate	IIIb
9	66	F	Ascending colon	Moderate	IIa
10	84	F	Rectum	Papillary adeno	IIa
11	72	M	Transverse colon	Moderate	IIa
12	68	M	Cecum	Moderate	II
13	48	F	Rectum	Moderate	IIIb
14	68	F	Descending colon	Moderate	IIIa
15	79	F	Rectal	Moderate	IIa
16	75	M	Liver meta (rectum)	Moderate	IVb
17	44	M	Hepatocellular	Moderate	I
18	76	F	Pancreatic	Moderate	IIa
19	74	M	Liver meta (sigmoid)	Moderate	IIb
20	79	F	Transverse colon	Moderate	IIa
21	59	M	Liver meta (rectum)	Moderate	IVb
22	65	M	Vater's ampulla	Moderate	IVb
23	75	F	Liver meta (rectum)	Moderate	IVb
24	78	M	Gallbladder	Moderate	IIIb
25	70	M	Ascending colon	Moderate	IIIb
26	60	M	Pancreatic	Moderate	IVb
27	68	F	Liver meta (rectum)	Mucinous	IVb

adeno, adenocarcinoma; meta, metastasis; ND, not determined.
Cancer-associated fibroblasts were derived from tumor samples (ID1-15). Cancer tissue-originated spheroids were derived from tumor samples (ID1-27) and the latter half (ID16-27) were used for coculture with fetal lung fibroblasts.

TABLE 2 Kruskal-Wallis multivariate analysis of variance

Dependent variable	Kruskal-Wallis *H*	Significance
ACTA2	1.073	0.554
CXCL8	5.020	0.066
CXCL12	10.402	0.009*
FN1	18.039	0.000*
HGF	13.433	0.002*
IL6	5.027	0.077
LOX	6.021	0.139
MMP2	3.412	0.230
MMP9	11.110	0.002*
PDGFA	36.027	0.000*
POSTN	20.315	0.001*
PTGES	28.326	0.000*
S100A4	20.007	0.000*
TGFB1	5.345	0.050
TNC	31.156	0.000*
TNF	16.000	0.000*
VEGFA	0.002	0.997
VIM	9.109	0.004*

Three levels were defined as: cancer-associated fibroblasts from colorectal, gastric or liver cancers (hepatocellular or cholangiocarcinoma) so that the degree of freedom is two.
*The null hypothesis was rejected ($P < 0.01$, there exists main effect by a difference in tissue of origin).

by refrigerated courier service. We first confirmed that both the CAF and the cancer tissue-originated spheroids (CTOS) were mostly viable after transportation. Neoadjuvant chemotherapies could affect the viability of both CAF and CTOS. However, only a few patients fulfilled the condition in this study (Table 1, patients with ID21 and ID27), making the statistical determination difficult. Dissociation of primary culture of specimens was modified from a previously reported protocol.[12] Briefly, tumors were minced with surgical scissors and further digested enzymatically in a collagenase/proteinase cocktail (0.1% collagenase and 0.1% dispase II; FUJIFILM Wako Pure Chemical, Osaka, Japan) for 60 minutes in a reciprocating water bath shaker at 37°C. The digested tissue was washed by phosphate-buffered saline (PBS) and fractionated according to the speed of sedimentation. Fast-settling fraction contained cell clumps, eventually forming floating CTOS in a low-attachment culture dish (AGC Techno Glass, Shizuoka, Japan). Slowly settling fraction contained fully digested single cells such as CAF and blood cells. After CAF attached to the plastic dish, floating cells and debris were removed and subsequent cell division of CAF further increased cell purity. We observed epithelial-like cancer cells occasionally attached to the plate but such cells were removed as a result of the much slower cell division compared to CAF (data not shown). Fetal lung-derived human fibroblasts (TIG-3) was purchased from JCRB Cell Bank (Osaka, Japan). The culture medium for CTOS was serum-free advanced DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with fibroblast growth factor-2 (10 ng/mL; ReproCell, Kanagawa, Japan), L-Alanyl-L-glutamine (2 mmol/L; FUJIFILM Wako Pure Chemical) and antibiotic cocktail (gentamicin, penicillin, streptomycin and amphotericin B; FUJIFILM Wako Pure Chemical). The culture medium for CAF and TIG-3 was advanced RPMI (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (GE Healthcare Hyclone, Chicago, IL, USA), L-alanyl-L-glutamine and antibiotic cocktail.

2.3 | Perturbation

Perturbation (functional alteration by external stimuli) was measured by comparing gene expression between control culture and particular stimulus. CAF were split into four or five dishes and used for the experiment. Culture condition of each stimulus was as follows: (i) hypoxia treatment: cells were incubated in hypoxic chamber (1% O_2; Astec, Fukuoka, Japan) for 6 hours; (ii) exposure to chemotherapy compound, 3.125 μg/mL cisplatin (Nichi-Iko Pharmaceutical, Toyama, Japan); (iii) exposure to 312.5 μg/mL fluorouracil (Towa Pharmaceutical, Osaka, Japan) for 3 days. Concentration of cisplatin or fluorouracil was determined based on IC_{50} in pilot experiments (data not shown) and only the cells surviving chemical exposure were harvested for analysis. (iv) Coculture with CTOS (fibroblasts and CTOS were separated by Millicell cell culture insert; Merck Millipore, Burlington, MA, USA) for 3 days. Culture insert (polycarbonate membrane) hindered carcinoma cells from contacting with fibroblasts directly, but allowed communication with each other through paracrine signals such as cytokines and extracellular vesicles.[13]

2.4 | Gene expression analysis

Total RNA was extracted from fibroblasts (CAF and TIG-3) with or without experimental stimulus (RNAiso plus; Takara Bio, Shiga, Japan). PrimeScript RT reagent kit with gDNA eraser (DNase) was used for reverse transcription (Takara Bio). PCR conditions and reagents are described elsewhere.[14] For data acquisition, we used a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the Taqman method (Thermo Fisher Scientific). Data were quantified according to the modified delta-cycle threshold (ΔC_T) method and the ΔC_T value was used for statistical analysis.[15] In figures, expression level is presented as $\log_2$ (relative expression) = 20 (arbitrary constant) − ΔC_T. We selected 18 genes that encode components for fibroblast activation, paracrine communications, extracellular matrix (ECM) remodeling, for monitoring dynamic behaviors during perturbation. Previous study indicated that the dynamics of biologically relevant markers could reflect the stepwise differentiation model of CAF.[16] Gene-specific Taqman probes were purchased from Thermo Fisher Scientific. Catalog number of each fluorescent probe is as follows: Eukaryotic 18S rRNA endogenous control (4352930E), *ACTA2* (Hs00426835_g1), *CXCL12* (Hs03676656_mH), *MMP2* (Hs01548727_m1), *S100A4* (Hs00243202_m1), *TGFB1* (Hs00998133_m1), *TNC* (Hs01115665_m1), *VEGFA*

(Hs00900055_m1), *VIM* (Hs00958111_m1), *FN1* (Hs01549976_m1), *HGF* (Hs00300159_m1), *IL6* (Hs00174131_m1), *LOX* (Hs00942483_m1), *MMP9* (Hs00957562_m1), *POSTN* (Hs01566750_m1), *TNF* (Hs01113624_g1), *CXCL8* (Hs00174103_m1), *PDGFA* (Hs00234994_m1), *PTGES* (Hs00610420_m1).

2.5 | Western blotting and fluorescent immunostaining

Cancer-associated fibroblasts under various perturbations were harvested and whole-cell proteins were dissolved with solubilizer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS; Thermo Fisher Scientific). The entangled genomic DNA was sheared by sonication for 30 minutes at 4°C. Procedures for western blotting have been described elsewhere.[14] Primary antibodies used are as follows: vascular endothelial growth factor (VEGF)-A from Protein Tech (#19003-1-AP, Rosemont, IL, USA) and GAPDH from Cell Signaling Technology (#5174, Danvers, MA, USA). Experiments were reproduced twice with different passage points. In the histological procedure, α-smooth muscle actin (α-SMA) and VEGF-A were detected using dual immunofluorescence staining. Paraffin-embedded sections (5-μm thick) underwent standard deparaffinization and rehydration steps. The sections were treated with antigen retrieval solution, pH 9, (Agilent Technologies, Santa Clara,

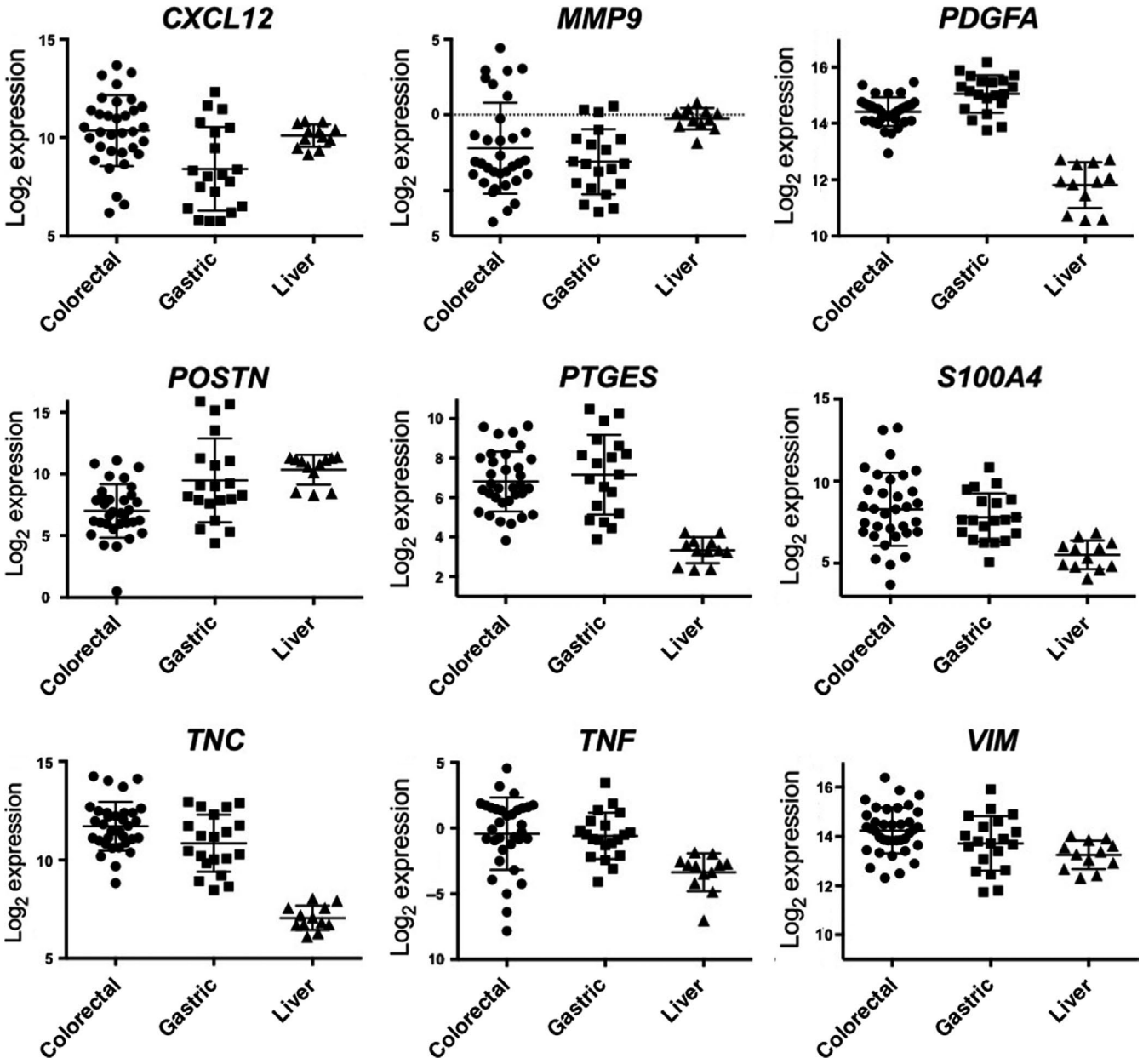

FIGURE 1 Cancer-associated fibroblasts show large variations of gene expression, partly explained by tissue of origin. Kruskal-Wallis multivariate analysis of variance detected the main effect by a difference in tissue of origin. Namely, inter-tissue variations exist for 11 genes (see Table 2). Nine representative genes are shown here. Scatterplots indicate $\log_2$ expression of designated genes. Error bars denote mean ± SD

TABLE 3 Fisher's least significant difference procedure by Friedman test (n = 13)

Dependent variable	Chi-squared	Significance
ACTA2	22.015	0.000*
CXCL8	27	0.000*
CXCL12	27.369	0.000*
FN1	20.077	0.000*
HGF	13.523	0.004*
IL6	27.185	0.000*
LOX	25.154	0.000*
MMP2	3.738	0.291
MMP9	5.585	0.134
PDGFA	0.969	0.809
POSTN	15.277	0.002*
PTGES	21.462	0.000*
S100A4	11.215	0.011
TGFB1	11.862	0.008*
TNC	19.8	0.000*
TNF	17.031	0.001*
VEGFA	32.354	0.000*
VIM	3.738	0.291

Comparison between hypoxia and control was not included due to insufficient samples (n = 5). Four levels were defined (the degree of freedom is three) as control, cisplatin, fluorouracil or coculture with cancer spheroids.
*The null hypothesis was rejected ($P < 0.01$, there exists main effect by perturbation).

CA, USA) for 1 minute at 105°C using an autoclave machine (TOMY Digital Biology, Tokyo, Japan). Subsequently, the sections were blocked with 5% (v/v) normal donkey serum in PBS with Triton X-100 (0.3%) for 60 minutes. The sections were incubated with goat α-SMA antibody (1:500; Novus Biologicals, Centennial, CO, USA) and rabbit VEGF-A antibody (1:500; Bioss Antibodies, Woburn, MA, USA) overnight at 4°C. The sections were then incubated with secondary antibodies (Alexa Fluor 488 antigoat IgG, 1:500; Abcam, Cambridge, UK, and Alexa Fluor 594 antirabbit IgG, 1:500; Cell Signaling Technology) for 2 hours at room temperature. Finally, the sections were counterstained and mounted with ProLong Gold Antifade with DAPI (Cell Signaling Technology). Microscopic digital images were taken using a fluorescent microscope (BZ-X710; Keyence Corp., Osaka, Japan).

2.6 | Statistical procedure

All statistical tests were carried out using SPSS software (IBM, Armonk, NY, USA). To detect the main effect by a difference in tissue of origin, Kruskal-Wallis multivariate analysis for variance (MANOVA) was used. Data of all perturbations were included, as the variance could be interpreted as clinically relevant change. The dataset of distinct perturbations was obtained from the same individual and was treated as paired samples. To detect the main effect by experimental stimulus, we adopted Fisher's least significant difference procedure for paired samples (Friedman test and two-sided Wilcoxon signed rank test as post-hoc analysis). For comparison of *VEGFA* in between TIG-3 and CAF, two-sided Mann-Whitney *U* test was used. *P* values <0.01 were considered significant. To estimate the dependency between tumor location and VEGF-A localization in CAF, we carried out chi-squared test ($P = 0.012$) but the conclusion was questionable as a result of a limited number of specimens.

3 | RESULTS

3.1 | Cancer-associated fibroblasts showed large variations of gene expression, partly explained by tissue of origin

To investigate the heterogeneity of CAF among colorectal, gastric and liver cancers, we measured mRNA expression of 18 marker genes and carried out Kruskal-Wallis MANOVA (Table 2). Main effect by tissue difference was considered significant for 11 genes: *CXCL12*, *FN1*, *HGF*, *MMP9*, *PDGFA*, *POSTN*, *PTGES*, *S100A4*, *TNC*, *TNF* and *VIM* (Table 2 and Figure 1). Standard deviation of $\log_2$ expression was especially large for *POSTN* (2.865: ± sevenfold difference in linear scale), *TNF* (2.582: ± sixfold) and *MMP9* (2.561: ± sixfold). Thus, variation of some marker genes was explained, in part, by the difference in tissue of origin.

3.2 | Perturbations in gene expression of CAF

Next, we estimated the perturbations by measuring marker gene expression. Friedman test detected main effects of experimental stimulus in 13 out of 18 marker genes (Table 3). As a post-hoc analysis, two-sided Wilcoxon signed rank test was carried out and 11 of 13 genes were significantly changed by the stimulus (Table 4). Cisplatin significantly affected gene expression of *ACTA2*, *CXCL8*, *CXCL12*, *LOX*, *PTGES* and *TGFB1* (Figure 2A). Fluorouracil affected gene expression of *ACTA2*, *CXCL12*, *HGF* and *VEGFA* (Figure 2B). CTOS affected gene expression of *ACTA2*, *CXCL8*, *FN1*, *IL6*, *LOX*, *POSTN* and *VEGFA* (Figure 2C). Overall, degree of expression change was small, compared to large variation among the same level of stimulus (Figure 2).

3.3 | Paracrine signal from CTOS did not induce *VEGFA* in fetal lung fibroblasts

Among 11 genes described in Figure 2, we particularly focused on *VEGFA*. We observed that hypoxia, fluorouracil exposure and coculture with CTOS remarkably upregulated *VEGFA* in CAF (Figure 3A). As the putative function of the coculture is a paracrine signal through membrane pores (see Methods), we investigated whether such a paracrine signal could induce *VEGFA* in normal fibroblasts. When TIG-3 was cocultured with CTOS from various cancers (Table 1), the change of *VEGFA* expression was not reproducible (Figure 3B). Basal expression of *VEGFA* tended to be higher in CAF, compared to TIG-3

TABLE 4 Post-hoc analysis by Wilcoxon signed rank test

Dependent variable	Comparison	N	*Z*	Significance
ACTA2	Cisplatin vs control	17	−2.675[nr]	0.007*
	5FU vs control	17	−3.479[nr]	0.001*
	CTOS vs control	13	−3.180[nr]	0.001*
CXCL8	Cisplatin vs control	17	−2.627[pr]	0.009*
	5FU vs control	17	−2.059[pr]	0.039
	CTOS vs control	13	−3.180[pr]	0.001*
CXCL12	Cisplatin vs control	17	−2.959[nr]	0.003*
	5FU vs control	17	−3.385[nr]	0.001*
	CTOS vs control	13	−1.572[nr]	0.116
FN1	Cisplatin vs control	17	−1.396[nr]	0.163
	5FU vs control	17	−0.828[nr]	0.407
	CTOS vs control	13	−2.621[nr]	0.009*
HGF	Cisplatin vs control	17	−2.107[pr]	0.035
	5FU vs control	17	−3.077[pr]	0.002*
	CTOS vs control	13	−1.782[pr]	0.075
IL6	Cisplatin vs control	17	−2.438[pr]	0.015
	5FU vs control	17	−2.249[pr]	0.025
	CTOS vs control	13	−3.180[pr]	0.001*
LOX	Cisplatin vs control	17	−3.006[pr]	0.003*
	5FU vs control	17	−0.118[nr]	0.906
	CTOS vs control	13	−2.970[nr]	0.003*
POSTN	Cisplatin vs control	17	−0.166[pr]	0.868
	5FU vs control	17	−0.592[nr]	0.554
	CTOS vs control	13	−3.040[nr]	0.002*
PTGES	Cisplatin vs control	17	−2.999[pr]	0.003*
	5FU vs control	17	−1.563[pr]	0.118
	CTOS vs control	13	−2.062[nr]	0.039
TGFB1	Cisplatin vs control	17	−2.912[pr]	0.004*
	5FU vs control	17	−0.828[nr]	0.407
	CTOS vs control	13	−2.201[pr]	0.028
TNC	Cisplatin vs control	17	−1.965[nr]	0.049
	5FU vs control	17	−2.343[nr]	0.019
	CTOS vs control	13	−1.083[pr]	0.279
TNF	Cisplatin vs control	17	−1.491[pr]	0.136
	5FU vs control	17	−0.544[pr]	0.586
	CTOS vs control	13	−2.314[pr]	0.019
VEGFA	Cisplatin vs control	17	−1.254[pr]	0.210
	5FU vs control	17	−3.621[pr]	0.000*
	CTOS vs control	13	−3.180[pr]	0.001*

5FU, fluorouracil; CTOS, cancer tissue-originated spheroid.
Comparison between hypoxia and control was not carried out due to insufficient samples (n = 5). nr, based on negative ranks; pr, based on positive ranks. Perturbation increases the expression when the test was based on positive ranks.
*The null hypothesis was rejected ($P < 0.01$, significant difference between perturbation and control).

($P = 0.046$ by Mann-Whitney *U* test). Higher expression of *VEGFA* in CAF was further augmented by coculture with CTOS (Figure 3B). Data indicated that CAF were competent to induce *VEGFA* in response to paracrine signal from CTOS, but fetal lung fibroblasts were not. In other words, the paracrine signal per se was not sufficient to induce *VEGFA*.

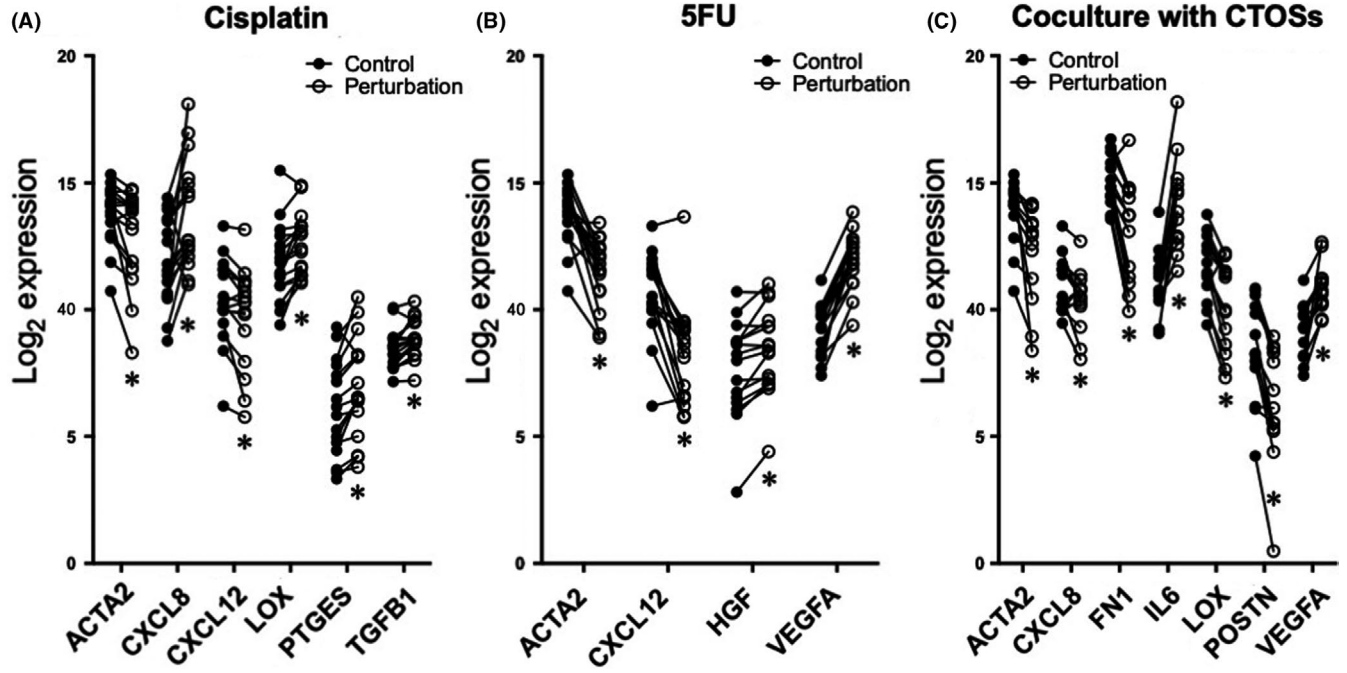

FIGURE 2 Perturbations in gene expression of cancer-associated fibroblasts. Friedman and two-sided Wilcoxon signed rank tests detected significant change in designated gene expression ($P < 0.01$, see Tables 3 and 4). Scatterplots indicate $\log_2$ expression. A, Expression change by cisplatin treatment (3.125 μg/mL for 3 d). B, Expression change by fluorouracil (5FU) treatment (312.5 μg/mL for 3 d). C, Expression change by coculture with cancer tissue-originated spheroids (CTOS) (for 3 d). Asterisk denotes significant difference between control and the experimental stimulus ($P < 0.01$, two-sided Wilcoxon signed rank test)

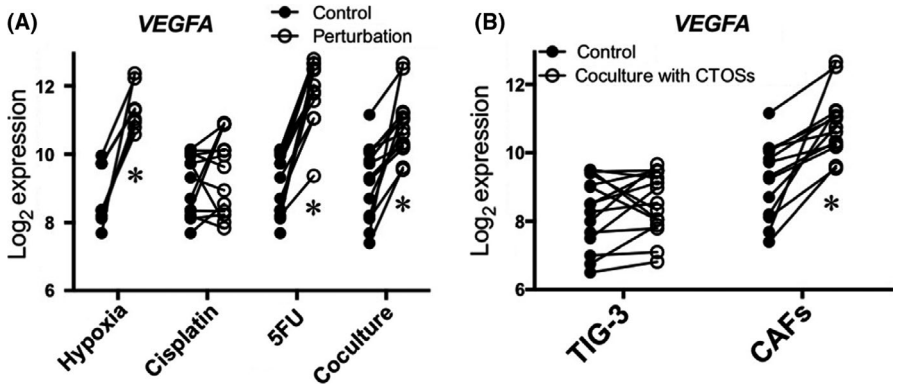

FIGURE 3 Vascular endothelial growth factor A (*VEGFA*), a gene for angiogenesis was induced by experimental stimulus in cancer-associated fibroblasts (CAF). Paracrine signal from cancer tissue-originated spheroids (CTOS) was not sufficient for the induction in human fetal lung fibroblasts. A, *VEGFA* was induced either by hypoxia (1% O_2 for 6 h), fluorouracil (5FU; 312.5 μg/mL for 3 d) or coculture with CTOS (for 3 d). B, When human fetal lung fibroblasts (TIG-3) were cocultured with CTOS, the induction of *VEGFA* was not reproducible. Basal expression of *VEGFA* tended to be higher in CAF (compare the filled circles between TIG-3 and CAF: $P = 0.046$ by Mann-Whitney *U* test) but the higher *VEGFA* in CAF was further augmented by coculture with CTOS. Asterisk denotes significant difference between control and the experimental stimulus ($P < 0.01$, two-sided Wilcoxon signed rank test)

3.4 | Vascular endothelial growth factor A protein was synthesized in CAF both in vitro and in vivo

Finally, we confirmed whether protein synthesis of VEGF-A was accompanied by mRNA induction. Consistent with mRNA induction, all the four perturbations increased VEGF-A synthesis in cultured CAF (Figure 4), whereas internal control GAPDH protein was largely unaffected (Figure 4). In paraffin-embedded tumor specimens, VEGF-A protein was colocalized with α-SMA (encoded by *ACTA2* gene, a surrogate marker of fibroblast activation) protein (Figure 5, ID16), suggesting that CAF synthesize VEGF-A in pathological conditions. However, syntheses of VEGF-A and α-SMA was not overlapping in some patients (Figure 5, ID13), presumably because other cell types (eg, smooth muscle cells surrounding venules) also synthesize α-SMA. We observed that some

α-SMA-negative cells also synthesized VEGF-A protein (Figure 5, ID11), indicating that CAF is not an exclusive cell type that induces angiogenesis in complex tumor microenvironments. When we compared the degree of colocalization among distinct tumor locations, some tendency existed in hepatocellular carcinomas, but not in liver metastasis (Table 5).

4 | DISCUSSION

Although stromal activation is considered critical for prognosis of patients, previous studies have analyzed snapshot data using microarray or immunohistochemistry of paraffin sections.[3–5] However, CAF in situ are known to be dynamic in terms of gene expression and function.[16,17] Stromal cells sense various environmental stressors and reorganize cellular and ECM compositions for tissue homeostasis.[18] In the present study, we measured 18 marker genes that encode components for fibroblast activation, paracrine communications, and ECM remodeling for monitoring dynamic functions.[16]

The significance of analyzing CAF from various types of cancer (Table 1) is twofold. One is to confirm the heterogeneity of fibroblasts among a distinct tissue of origin.[10] Indeed, several marker genes showed large variation in their expression level, which was explained, in part, by tissue of origin (Table 2 and Figure 1).

The other significance of analyzing the various CAF is to extrapolate the knowledge to distant stromal cells. When patients receive adjuvant chemotherapy (eg, cisplatin or fluorouracil), the systemic stressor also influences stromal cells in distant organs. We defined clinically relevant environmental stimuli and estimated perturbations of CAF in gene expression. Among such perturbations, coculture with CTOS is especially important, as cancer cells secrete various cytokines and extracellular vehicles.[13] In principle, the coculture experiments recapitulate the paracrine (but not the juxtaposed) interaction between cancer cells and CAF in tumors in situ. However, if the paracrine signal spreads through the vasculature, the systemic signal could influence stromal cells of distant organs. Such "remote control by primary tumor"

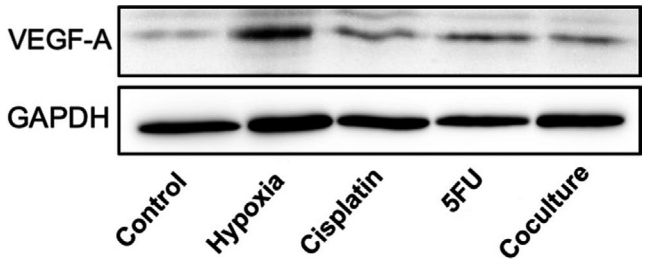

FIGURE 4 Protein synthesis of vascular endothelial growth factor A (VEGF-A) was induced by experimental stimulus in cancer-associated fibroblasts. 30 μg (for VEGF-A) or 10 μg (for GAPDH) of whole-cell protein lysate was loaded per lane. Western blotting against VEGF-A or GAPDH was carried out. GAPDH served as an internal control. 5FU, 5-fluorouracil

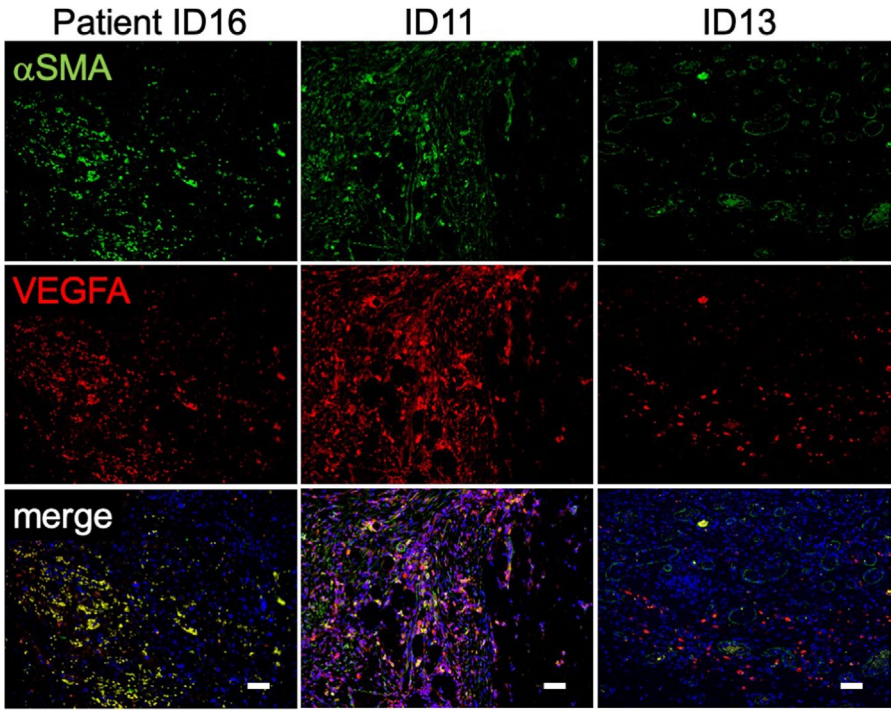

FIGURE 5 Vascular endothelial growth factor A (VEGF-A) protein was occasionally synthesized in cancer-associated fibroblasts in colorectal cancer specimens. Upper raw images: α-smooth muscle actin (α-SMA) protein is stained with green color in different tumor specimens. Middle raw images: VEGF-A protein is stained with red color in different tumor specimens. Lower raw images: Fluorescent signals of three different colors are merged. Cellular nuclei are stained with DAPI (blue color). Note that the immunoreactivities of α-SMA and VEGF-A are moderately overlapping in patient ID16 (liver metastasis of rectal cancer, left column images), whereas such colocalization is mild in ID11 (colon cancer, middle column images). In ID13 (rectal cancer, right column images), venule-like structures are stained with α-SMA antibody without colocalization with VEGF-A. Scale bars, 50 μm

TABLE 5 Degree of colocalization between α-smooth muscle actin and vascular endothelial growth factor A

Colocalization	−+	++	+++
Tumor location			
Gastric	3	1	0
Hepatocellular	0	0	3
Colorectal	3	1	0
Liver meta (colorectal)	3	2	0

Number of cases are depicted for each tumor location. Patient ID of each tumor specimen (Table 1) are as follows: gastric (4, 5, 6 and 7), hepatocellular (1, 2 and 17), colorectal (8, 9, 11 and 13) and liver metastasis (16, 19, 21, 23 and 27). −+, none or mildly colocalized; ++, moderately colocalized; +++, strongly colocalized.

has been intensively studied and the stromal cells are considered to reorganize the tissue microenvironments as premetastatic niches.[19,20]

In this respect, it is interesting that the coculture of CTOS remarkably induced *VEGFA* expression (Figure 3A). Data raised another question of whether this signal similarly affects fibroblasts in distant organs. To address this hypothesis, we carried out replacement of fetal lung fibroblasts with CAF. Opposite to our prediction, coculture of CTOS failed to induce *VEGFA* in the lung fibroblasts (Figure 3B). Tuveson and colleagues reported that pancreatic ductal adenocarcinoma cells induced CAF phenotype from pancreatic stellate cells.[17] The discrepancy could be due to the difference in choice of materials (ie, fetal lung fibroblasts vs pancreatic stellate cells) or to the difference in incubation time (3 vs 10 days). From our data, we presume that patients' CAF had acquired a type of "competence for *VEGFA* induction", which the lung fibroblasts do not have. CAF are known to resemble physiologically normal fibroblasts during tissue damage (termed as activated or primed) in terms of cytokine secretion and ECM remodeling.[7] However, the secretory phenotype of CAF is augmented and irreversibly switched, compared to primed fibroblasts.[7] Molecular identity for the paracrine signal competence remains unknown, but is an interesting theme for future study.

From the clinical perspective, it is intriguing that fluorouracil induced *VEGFA* in CAF (Figure 2B). Data suggest that fluorouracil potentially increases the risk of distant metastasis, although it is effective in decreasing tumor bulk. Indeed, in a mouse breast cancer model, fluorouracil enhances lung metastasis by activating infiltrating neutrophils.[21] In contrast, bevacizumab is a monoclonal antibody medication that inhibits VEGF-A, one of the most potent angiogenesis ligands. Standard use of bevacizumab in metastatic colorectal carcinoma is combined with fluorouracil. It is tempting to speculate that the combination of fluorouracil and bevacizumab could effectively produce synergy by targeting disseminated cancer cells and CAF in distant organs. Thus, the outcome of chemotherapy could lead to undesirable consequences unless we understand the interplay of the tumor microenvironment (ie, cancer cells, CAF, macrophages, neutrophils etc.).

Finally, collection of similar data is continuing and patient outcomes are being followed up. We noted that the perturbation was nearly consistent in coculture with CTOS, but not in cisplatin or fluorouracil exposure (ie, responder or non-responder existed for each gene: Figure 3). We carried out four-way MANOVA to detect interfactor interaction between experimental stimulus and three other factors (tissue of origin, histology, TMN stage, Table 1). However, we failed to detect interaction (data not shown), indicating that the "responder or non-responder" variations cannot be explained by known pathological factors. It is proposed that the drivers of cancer evolution are not genomic mutations, but intermittent changes of tissue microenvironments.[22] Apart from the malignancy of cancer cells per se, individual difference in tumor microenvironments could significantly influence disease prognosis.[1,2] Overall, prediction of behaviors of complex systems is difficult when more than three dynamic players interact with each other.[23,24] This could be the reason why most patients who receive genomic testing do not benefit from a genomic precision medicine strategy.[9] As alternatives, genome biologists are proposing "perturbation-based monitoring of cellular responses" for tackling the complexity inside cancer cells.[9,10] In the present study, we established perturbation-based monitoring of patients' CAF as a surrogate indicator for response of distant stromal cells against environmental stimuli. Combined with a follow-up study, we will identify critical marker genes to determine patient outcome.

5 | CONCLUSIONS

We established perturbation-based monitoring of patients' CAF. *VEGFA* was reproducibly induced by experimental stimuli. Paracrine signal from CTOS was sufficient to induce *VEGFA* in CAF, but not in fetal lung fibroblasts. Further data collection and individual patient follow up is ongoing to identify critical determinants for disease outcome.

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DISCLOSURE

Conflicts of Interest: Authors declare no conflicts of interest for this article.

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REFERENCES

1. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21:309–22.

2. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19:1423–37.
3. Erkan M, Hausmann S, Michalski CW, Fingerle AA, Dobritz M, Kleeff J, et al. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. Nat Rev Gastroenterol Hepatol. 2012;9:454–67.
4. Calon A, Lonardo E, Berenguer-Llergo A, Espinet E, Hernando-Momblona X, Iglesias M, et al. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. Nat Genet. 2015;47:320–9.
5. Moffitt RA, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, et al. Virtual microdissection identifies distinct tumor-and stroma-specific subtypes of pancreatic ductal adenocarcinoma. Nat Genet. 2015;47:1168–78.
6. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med. 2015;21:938–45.
7. Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer. 2016;16:582–98.
8. Rinn JL, Bondre C, Gladstone HB, Brown PO, Chang HY. Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS Genet. 2006;2:e119.
9. Letai A. Functional precision cancer medicine—moving beyond pure genomics. Nat Med. 2017;23:1028–35.
10. Eduati F, Doldan-Martelli V, Klinger B, Cokelaer T, Sieber A, Kogera F, et al. Drug resistance mechanisms in colorectal cancer dissected with cell type-specific dynamic logic models. Cancer Res. 2017;77:3364–75.
11. Wang R. Perturbation. In: Dubitzky W, Wolkenhauer O, Yokota H, Cho K.-H, (Eds.),. Encyclopedia of systems biology. New York: Springer, 2013; p. 1680–1.
12. Shibuya N, Inoue K, Tanaka G, Akimoto K, Kubota K. Augmented pentose phosphate pathway plays critical roles in colorectal carcinomas. Oncology. 2015;88:309–19.
13. Andaloussi SE, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov. 2013;12:347–57.
14. Tago K, Inoue K, Ouchi M, Miura Y, Kubota K. Receptor for advanced glycation endproducts signaling cascades are activated in pancreatic fibroblasts, but not in the INS1E insulinoma cell line: are mesenchymal cells major players in chronic inflammation? Islets. 2016;8:135–44.
15. Tanaka G, Inoue K, Shimizu T, Akimoto K, Kubota K. Dual pharmacological inhibition of glutathione and thioredoxin systems synergizes to kill colorectal carcinoma stem cells. Cancer Med. 2016;5:2544–57.
16. Busch S, Andersson D, Bom E, Walsh C, Ståhlberg A, Landberg G. Cellular organization and molecular differentiation model of breast cancer-associated fibroblasts. Mol Cancer. 2017;16:73.
17. Ohlund D, Handly-Santana A, Biffi G, Elyada E, Almeida AS, Ponz-Sarvise M, et al. Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. J Exp Med. 2017;214:579–96.
18. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341:738–46.
19. McAllister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. Nat Cell Biol. 2014;16:717–27.
20. Peinado H, Zhang H, Matei IR, Costa-Silva B, Hoshino A, Rodrigues G, et al. Pre-metastatic niches: organ-specific homes for metastases. Nat Rev Cancer. 2017;17:302–17.
21. Sasaki S, Baba T, Muranaka H, Tanabe Y, Takahashi C, Matsugo S, et al. Involvement of prokineticin 2-expressing neutrophil infiltration in 5-fluorouracil-induced aggravation of breast cancer metastasis to lung. Mol Cancer Ther. 2018;17:1515–25.
22. DeGregori J. Connecting cancer to its causes requires incorporation of effects on tissue microenvironments. Cancer Res. 2017;77:6065–8.
23. Mayr E. Cause and effect in biology. Science. 1961;134:1501–6.
24. Weisbuch G. Complex systems dynamics. Boca Raton: CRC Press; 2018.

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