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Original Article

A landscape of patient-derived cancer-associated fibroblast signals in endometrial cancers

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Abstract: In conversation with endometrial tumor cells, the endometrial cancer-associated fibroblasts (CAFs) are the "partners in crime" of uterine neoplasm's highly heterogeneous tumor microenvironment (TME). We designed a laboratory-friendly method to culture endometrial CAFs on a patient-to-patient basis for studying the CAF-TME and CAF-tumor cell interaction(s). Here, we present a comprehensive characterization of endometrial CAFs derived from patients' tumor tissues (T) and tumor-adjacent normal tissues (N). We used more than 80 T and N from 53 consecutive consented patients with endometrial cancers at the Avera Cancer Institute. We derived TCAF and NCAF in a non-enzymatic feeder-layer culture and characterized their expression of markers by qRT-PCR, flow cytometry, immunocytochemistry, immunofluorescence, and Western blot. Although similar in the expression pattern of EpCAM-/CK18-/vimentin+ as in ovarian CAFs, endometrial NCAFs, and TCAFs characteristically presented dual morphology in culture. Endometrial CAFs were EpCAM-/CK18-/CD45-/CD31-/SMA+/TE-7+/PDGFRA+/CXCL12+/Meflin+/CD155+/CD90+ with patient-specific positivity for S100A4/FAP/PD-L1/CD44. Endometrial CAFs expressed mRNAs for signaling proteins of several pathways and receptor-ligands, including (1) cell cycle pathway, (2) TGF pathway, (3) FGF pathway, (4) Wnt-beta-catenin pathway, (5) HER pathway, (6) tyrosine kinase receptor ligands, and (7) steroid receptors. We tested the hypoxic response of CAFs to show that endometrial CAFs upregulate MMP1 in a HIF-1a-independent manner. In trying to delineate the relationship between expressions of CAF markers and T-cells in the tumor tissue, we observed that FAP-positive CAFs that are derived from CD4/CD8 positive tumor tissue expressed CXCL12 mRNA. The data indicate the role of the CXCL12-CXCR4 pathway of the CAF-rich stroma in the lymphocytic infiltration of the tumor. We demonstrate that endometrial CAFs can be cultured in an enzymatic-digestion-independent manner, and their signaling landscape can be mapped toward understanding CAF-TME dialogue. Our data will help unearth the functional relevance of endometrial CAFs in the context of clinical outcomes and designing CAF-inclusive therapy in the future.

Keywords: CAF markers, endometrial cancers, patient-specific CAF, primary culture

Introduction

Every tumor is a uniquely complex and vastly evolving anatomical conglomeration of assorted cells, neoplastic cells, and non-neoplastic cells. Prognostication is a built-in feature of all neoplastic changes. The success of neoplastic growth is dependent on its ecosystem, the tumor microenvironment (TME). A progressive tumor exists in and builds its favorable ecosystem within the TME. Evidence suggests that not only the tumor cells, but the TME contains an independent prognostic signature as a hallmark of tumor [1] by virtue of the roles of tumor endothelial cells, immune cells, and cancer-associated fibroblast (CAF) in the outcome of the disease [2]. Unlike T-cells, which support neoplastic growth by essentially ignoring tumor cells, CAFs

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assist and converse with tumor cells towards the progression of the disease and development of drug resistance.

The CAF component of TME is the host mesenchyme that immediately surrounds the tumor cells in several solid tumors and facilitates tumor growth and progression despite the therapy [3]. In patients with solid tumors, stromal CAFs play the perilous role of "turning the table" in favor of tumor cells towards the progression and metastasis of the disease leading to poor outcomes. Recently, we demonstrated the direct role of CAF in developing drug resistance in gynecological neoplasms like ovarian cancers [4]. In an evolving tumor, a rate-limiting conversion of CAFs from a protumor state to an anti-tumor state is called "stroma genesis or stromal-switch", which forms the concept of CAF as a target in cancer therapy [5]. This mechanism of reverse "tone" of conversation between CAFs and tumor cells will be essential to know to construct novel strategies of disease management based on targeting the reversal of the "stromal-switch" back to "normalization" (anti-tumor state) of the stroma [5].

In the world of CAF, ambiguity is a commonly used terminology to define the origin of CAFs, markers of CAFs, cell signaling in CAFs, CAF's function, as well as crosstalk(s) in real-time with every component within a tumor mass - all hallmarks of cancer, in situ and metastatic. In interrogating the current literature on CAF, two relevant characteristics of CAF are conclusively known [6]. First is the heterogeneity of the CAF origin, marker(s), subpopulation(s), and signaling, and the second is their undeniable role in determining the disease's outcome. Thus, one restricts us from targeting CAF to achieve a successful CAF-based therapeutic strategy. At the same time, the other prohibits us from denying the scope of the CAF-based therapeutic approach toward managing the disease [6].

Although CAF-based clinical trials are just beginning to gain momentum in a few solid tumors, including breast, lung, head-neck, oral, colorectal cancers, and pancreatic ductal adenocarcinomas [3, 7-10], clinical management of the disease based on targeting "stroma genesis" in endometrial cancers remains elusive at present. The paucity of data is predominantly due to the lack of experimental data to prove the origin, subpopulation, heterogeneity, CAF signaling, and the knowledge of definitive functions of CAF. While a review article interrogates the role of CAFs in endometrial cancers [11], far lesser experimental data on endometrial CAFs are available in the literature [6, 12-15]. Not surprising that in contrast to other solid tumors, there is limited knowledge about mechanistic markers for the CAF derived from patients with endometrial cancers [16-18].

Gynecological malignancies, including endometrial cancers, present a "crowded crosstalk between cancer cells and stromal microenvironment" [19]. TME of human endometrial cancers are highly heterogeneous [13], and the pillar population of endometrial stromal cells (after removing all the leukocytes with an anti-CD45) is represented by CD90+ fibroblasts, which in the presence of PDGF, can differentiate into SMA+ myofibroblasts [20]. As we interrogated the role of crosstalk between tumor cells and intra-tumoral CAFs in endometrial cancers, we began to appreciate the contribution of the CAF in the tumor ecosystem of endometrial neoplasm and hence on the management and outcome of the disease [6]. To mitigate the malevolent transformation of CAF and CAF-mediated support of tumor cells in favor of disease outcomes in endometrial cancers by targeting a CAF-based "stromal-switch", an in-depth characterization of endometrial CAF and their functional choreograph within endometrial tumor ecosystem is warranted.

The role of a fibroblastic response in epithelial, more specifically in endometrial cancers, directly indicated the clinical relevance of stromal CAFs in the context of tumor stromal ratio (TSR). Relatively recently, TSR was first described by Mesker et al. [21] as a prognostic factor in epithelial tumors, who studied the carcinoma-percentage as a derivative from the carcinoma-stromal ratio whose prognostic relevance has been reported in several epithelial cancers (See [22]). In endometrial cancers, TSR was reported as a continuous variable associated with worse OS in univariable Cox regression analysis. Low tumor stromal content in this study was associated with poor outcomes and other adverse prognostic indicators, although it was not found to be independently prognostic, suggesting the characteristic biology of tumor-stroma interactions in endometrial cancers [23]. Although in a more histo-

logically narrowed set of endometrioid endometrial carcinomas (a primary histological subtype in endometrial cancers), stromal signatures demonstrated that desmoplasia correlated positively with the DTF (a fibroblastic response of desmoid-type fibromatosis, DTF) expression signature [24], direct, in-depth characterization of patient-derived fibroblasts from the tumor and the tumor-adjacent normal tissue has not yet been reported.

To this end, an unmet need is an in-depth characterization of endometrial CAF, CAF's functional choreograph with endometrial tumor cells and other stromal cells within the uterine TME. Here we present a comprehensive characterization of endometrial CAFs derived and cultured from more than 80 tumors and tumor-adjacent normal tissues from 77 consecutive consented patients with endometrial cancers using a novel, non-enzymatic feeder-layer culture. Our characterization of CAF from the tumor (TCAF) and tumor-adjacent normal tissues (NCAF) included morphological evaluation, CAF marker expressions, mRNAs for CAF signaling, and differential hypoxic response in CAF markers.

Materials and methods

Tumor tissue collection at the time of surgery

All experimental protocols were approved by the Avera Institutional Review Board (IRB). The study reported here was conducted between October 2017-July 2022. Investigators had access to information that could identify individual participants during or after data collection. The written informed consent(s) (IRB Protocol Number: 2017.053-100399_ExVivo-001, Approval Date: October 4, 2017) was obtained from 53 adult patients (age: 18 years or older). The resected tumor (T) and tumor-adjacent normal (N) tissues were collected during surgery in designated collection media as per the guidelines and relevant regulations and provided by the pathologist, depending upon the availability of the tissue on a case-to-case basis. We included samples from consecutive consented patients with endometrial tumors at any stage/grade of the disease undergoing surgery with or without pre-treatment/history of any previous carcinoma. As tumors present intra-tumoral heterogeneity, the heterogeneity of the CAF population within the tumor sample is expected. We used the feeder layer from the entire tissue sample provided to us to set up the primary culture. Our CAFs from a single patient may have likely contained heterogeneous CAF subpopulations because of the patient's complex tumor microenvironment.

Cell lines and reagents

Human uterine fibroblasts (HUF; Primary Uterine Fibroblasts, Cat # PCS-460-010), HUVEC cells (cat # PCS-100-013), endometrial cells (RL-95-2 and AN3CA), MCF7 cells, and NCI-H441 cells were procured from ATCC and cultured according to the standard cell culture procedures per ATCC. Other cell lines for qRT-PCR were procured from ATCC. Antibodies for ICC were bought from Cell Marque, NOVUS, Abcam, Agilent-Dako, and Cell Signaling. All cells were used within 7-8 passages and tested negative for mycoplasma. The antibodies for WB were procured from Cell Signaling, USA.

Patient-derived primary culture of endometrial CAFs

The primary culture of CAFs (TCAF & NCAF) from the endometrial T and N samples was set up from the feeder layer. The initial seeding of cells was cultured in media containing DMEM/F-12 + Glutamax. The purity and the extent of epithelial cell contamination of the cultures were monitored by testing the expression of mRNA by qRT-PCR as well as protein expression by flow and ICC. A passage of the primary culture of CAF is qualified by (1) the negative expression of non-CAF markers, including epithelial cell markers CK 8, 18, 19, and EpCAM, leucocyte common antigen CD45, and endothelial cells marker, CD31, and (2) the positive expression of fibroblast/CAF markers, including SMAalpha, S100A4, CD90, FAP, CD155, TE-7, PDGFRA, and FGFR. Expression of stem cell marker, CD44, and immune checkpoint marker, PD-L1, were also monitored. The expression of fibroblast markers was monitored throughout each passage. The first 3 passages are used as early, followed by mid and late passages. Depending upon the viability and expression of markers, the late passage CAFs have been tested for senescence (beta-galactosidase assays). Ovarian CAFs are cultured, as mentioned earlier [4]. The late passages of CAFs at reduced growth rates were tested for beta-galactosidase positivity.

Expression of mRNA for CAF markers by qRT-PCR

The expressions of CAF markers, signaling proteins, and hypoxia signaling proteins were tested by qRT-PCR. RNA extraction and qRT-PCR were performed as mentioned elsewhere [25]. In short, RNA extraction was performed using the Qiagen RNEasy MiniKit and Qiashredder Kit according to the manufacturer's protocol. RNA was extracted from lysed cell pellets (Qiashredder system) and converted to cDNA using iScript Reverse Transcription Supermix. qRT-PCR was performed using the Roche LightCycler96 platform. Appropriate primers (Integrated DNA Technologies) for each gene of interest were mixed with Roche FastStart Essential DNA Green Master Mix and run in triplicates. FastStart Essential DNA Green Master (Roche) was used for product detection (Relative Ratio of the gene of interest to GAPDH) using the Roche LightCycler 96 Software version 1.1. The list of primers is presented in **Table 1**.

Expression of protein markers of CAF by flow cytometry

Flow cytometry was performed using SMA-FITC, FAP-PE, S100A4-PerCP, EpCAM-APC, CD31-FITC, CD155-PE, CD90 PE-Vio615, and PD-L1-APC. For flow cytometry, cells were trypsin released and rinsed in FACS Buffer (phenol red-free RPMI with 1% FBS). Cells were stained for 15 minutes with cell surface antibodies (CD31 Miltenyi, CD155 Miltenyi, CD90 Miltenyi, FAP R&D systems, PD-L1 Miltyeni) or corresponding isotype control antibodies (Miltenyi). Cells were fixed using the kit from Miltenyi for 30 minutes, followed by re-suspension in a permeabilization buffer from the same kit. Cells were stained for intracellular antibodies (SMA and S100A4, both from Novus biologicals) for 30 minutes. Stained cells were run on BD Accuri C6 flow cytometer and analyzed using FCS express (DeNovo software). TCAFs and NCAFs from all patients with established CAFs at every passage of the primary culture were tested for the expression of markers to confirm specificity. The list of antibody conjugates used in the study is presented elsewhere [4]. The expression of several CAF marker proteins was tested by Western blot, as mentioned earlier [4].

Subcellular localization of CAF markers by ICC

For ICC, CAFs were cultured on coverslips. Both NCAFs and TCAFs from each passage were stained for EpCAM, CK 8, 18, SMAalpha, S100A4, TE-7, and PD-L1 to confirm the specificity of the CAFs. ICC for protein was first validated and evaluated by a pathologist and then was run with corresponding positive and negative controls. Endometrial tumor cells (RL-95-2 and AN3CA) were used as the positive control for EpCAM and CK 8, 18 and as the negative control for SMAalpha, S100A4, and TE-7. HUF was the positive control for SMAalpha, S100A4, TE-7, and the negative control for EpCAM and CK 8, 18. HUVEC cells were used as the negative control for EpCAM, CK 8, 18, SMAalpha, S100A4, and TE-7 and as the positive control for CD31. NCI-H441 cells were used as the positive control for PD-L1, and MCF7 cells were used as the negative control for PD-L1. Hematoxylin was used as the counterstain. For ICC, pictures were taken at 20× and 40× dry-objectives of Olympus BX43 Microscope using cellSens 1.18 Life Science Imaging Software (Olympus Corporation). The list of antibodies used is presented elsewhere [4].

Results

Morphological features of endometrial CAFs

Both NCAFs and TCAFs are long, fusiform, or polygonal, with a wider middle part of the cell and protrusions at the ends. Majority of the cells bore an unclear outline. They typically spread out from a center point at the initial passages, while upon successive passages and trypsinization, that arrangement was lost, and cells tend to grow in a directional pattern when confluent in contrast to their growth in an irregular pattern in low confluency. Endometrial CAFs, both NCAFs and TCAFs (**Figure 1A**), bear characteristically dual morphologies, one of typical fibroblast-like shape with an unclear outline and the other with a more rounded well-knit shape and prominent borders (**Figure 1B**), as compared to uniformly shaped ovarian CAFs (**Figure 1C**). The dual morphology in CAFs was observed in 20-25% of the cases and their presence was observed independent of passage numbers and irrespective of histology, grade, and stage of the disease. In some cases, we observed characteristically multinuclear

Table 1. List of primers used for real-time quantitative PCR (Species: Human)

Gene	Sequence (5'-3')	TM (°C)	Amplicon (bp)
ACTA2/SMA-α2	F: CGTTACTACTGCTGAGCGTGA	60.14	137
	R: GCCCATCAGGCAACTCGTAA	60.39	
β-catenin/CTNNB1	F: AGCTTCCAGACACGCTATCAT	59.24	98
	R: CGGTACAACGAGCTGTTTCTAC	59.34	
BIRC5/SURVIVIN	F: CAAGGACCACCGCATCTCTAC	60.47	119
	R: AGTCTGGCTCGTTCTCAGTGG	61.76	
CCND1	F: TCTACACCGACAACTCCATCCG	61.78	133
	R: TCTGGCATTTTGGAGAGGAAGTG	60.81	
CCNE1	F: TGTGTCCTGGATGTTGACTGCC	62.77	123
	R: CTCTATGTCGCACCACTGATACC	60.55	
CD44	F: CATCACATGAAGGCTTGGAAGA	58.65	86
	R: TCCACCTGTGACATCATTCCT	58.73	
c-MYC	F: CGTCCTCGGATTCTCTGCTCT	61.35	195
	R: AAGGTGATCCAGACTCTGACCT	60.23	
CXCL12	F: ACACTCCAAACTGTGCCCTTC	60.75	139
	R: GTCCTTTTTGGCTGTTGTGCTT	60.42	
DAPLE/CCDC88C	F: CATCGAGCTGGAGCGGAAT	59.93	110
	R: TCAAGATCTGGCTGCTGAAGG	60.07	
EGF	F: TACTGCCTCCATGATGGTGTGTG	62.51	102
	R: GTCTCGGTACTGACATCGCTCC	62.45	
EPCAM	F: AGCGAGTGAGAACCTACTGGA	60.27	109
	R: CGCGTTGTGATCTCCTTCTGA	60.40	
Epiregulin (EREG)	F: ACAGGCAGTCCTCAGTACAA	58.28	114
	R: ATTGACACTTGAGCCACACG	58.77	
ERBB2	F: GGGAAACCTGGAACTCACCTA	59.02	96
	R: TTGTGAGCGATGAGCACGTAG	60.73	
ERBB3	F: CTATGAGGCGATACTTGGAACGG	60.86	144
	R: GCACAGTTCCAAAGACACCCGA	63.26	
ESR1	F: GCTTACTGACCAACCTGGCAGA	62.51	129
	R: GGATCTCTAGCCAGGCACATTC	60.55	
FAPα	F: GGAAGTGCCTGTTCCAGCAATG	62.29	154
	R: TGTCTGCCAGTCTTCCCTGAAG	61.93	
FGF2	F: GTGTGTGCTAACCGTTACCTG	59.20	140
	R: CAACTGGTGTATTTCCTTGACC	57.23	
FGFR1	F: AGGCTACAAGGTCCGTTATGC	60.13	101
	R: TGCCGTACTCATTCTCCACAA	59.38	
FGFR2	F: TTAAGCAGGAGCATCGCATTG	59.33	76
	R: GGGACCACACTTTCCATAATGAG	59.05	
FGFR3	F: CCTCGGGAGATGACGAAGAC	59.62	81
	R: CGGGCCGTGTCCAGTAAGG	62.66	
FGFR4	F: TGCAGAATCTCACCTTGATTACA	57.84	101
	R: GGGGTAACTGTGCCTATTCG	58.05	
GAPDH	F: GGCATGGACTGTGGTCATGAG	61.02	87
	R: TGCACCACCAACTGCTTAGC	61.17	
HGF	F: GAGAGTTGGGTTCTTACTGCACG	61.41	102
	R: CTCATCTCCTCTTCCGTGGACA	61.21	
HIF-1α	F: GAACGTCGAAAAGAAAAGTCTCG	58.57	124
	R: CCTTATCAAGATGCGAACTCACA	58.82	

HIF-2α/EPAS1	F: GTCTGAACGTCTCAAAGGGC	58.85	140
	R: AGCTCATAGAACACCTCCGTC	59.25	
ISLR/MEFLIN	F: AGAGGCTCCGCTTGACTAAGA	60.62	148
	R: ACAGTCGGCGATCTGGAAGC	62.56	
MMP1	F: GGCTTGAAGCTGCTTACGAAT	59.26	123
	R: AGGAGCTGTAGATGTCCTTGG	58.89	
MMP2	F: TACACCAAGAACTTCCGTCTGT	59.30	83
	R: CCAAGGTCAATGTCAGGAGAG	58.02	
MMP7	F: GGAGGAGATGCTCACTTCGAT	59.31	85
	R: CATGAGTTGCAGCATACAGGA	58.36	
MMP9	F: CGACGTCTTCCAGTACCGA	58.83	124
	R: CTGCAGGATGTCATAGGTCAC	58.17	
NRG1	F: GAGAACGCCCAAGTCAGCAA	60.88	101
	R: GATGGCTTGTCCCAGTGGTG	60.96	
PDGFRα	F: TTGAAGGCAGGCACATTTACA	58.41	119
	R: GCGACAAGGTATAATGGCAGAAT	59.18	
PD-L1/CD274	F: TGCCGACTACAAGCGAATTACTG	61.22	150
	R: CTGCTTGTCCAGATGACTTCGG	61.25	
PD-L2/PDCD1LG2	F: ACCCTGGAATGCAACTTTGAC	59.04	109
	R: AAGTGGCTCTTTCACGGTGTG	61.08	
PgR	F: TCAAGTTAGCCAAGAAGAGTTCC	58.36	115
	R: AATGTAGCTTGACCTCATCTCC	57.59	
S100A4	F: CAGAACTAAAGGAGCTGCTGACC	61.17	126
	R: CTTGGAAGTCCACCTCGTTGTC	61.12	
TGFβ1	F: TACCTGAACCCGTGTTGCTCTC	62.24	122
	R: GTTGCTGAGGTATCGCCAGGAA	62.63	
TGFβ2	F: GAGTGCCTGAACAACGGATTG	59.80	116
	R: CCATTCGCCTTCTGCTCTTGT	60.95	
TGFβR1	F: AGGACTGGCAGTAAGACATGA	58.46	158
	R: CCATTGCATAGATGTCAGCACG	60.03	
TGFβR2	F: TGTGATGTGAGATTTTCCACCTGT	60.45	124
	R: TGTTCTCGTCATTCTTTCTCCATAC	58.90	
VEGFA	F: TGCAGATTATGCGGATCAAACC	59.38	81
	R: TGCATTCACATTTGTTGTGCTGTAG	61.02	

cells within the NCAFs and TCAFs populations with varied nuclear numbers, ranging from 4 to 11 (**Figure 1D**, **1E**).

The multinuclear morphology in CAFs was observed in 10% of the cases, and their presence was observed independent of passage numbers and irrespective of histology, grade, and stage of the disease. We performed initial characterization of CAFs by immuno-fluorescence for CK 18-negativity, EpCAM-negativity, and vimentin-positivity as compared to epithelial tumor cell lines and ovarian CAFs. **Figure 2** presents CK 18-/EpCAM-/vimentin+/DAPI endometrial CAFs (**Figure 2A**) similar to ovarian CAFs (**Figure 2B**) and in contrast to CK 18+/EpCAM+/vimentin-/DAPI OVCAR3 and SUM149 epithelial tumor cell lines (**Figure 2C**, **2D**).

The immune marker expression was consistent through the passages, early through late. The late passages in different endometrial CAFs (**Figure 3A**) and NCAFs-TCAFs pairs (**Figure 3B**) were tested by the beta-galactosidase stain as compared to ovarian TCAFs and NCAFs (**Figure 3C**). Endometrial tumor cell lines, AN3CA, RL-95-2, and HUF cells in cultures were used as negative controls (**Figure 3A**, **3B**).

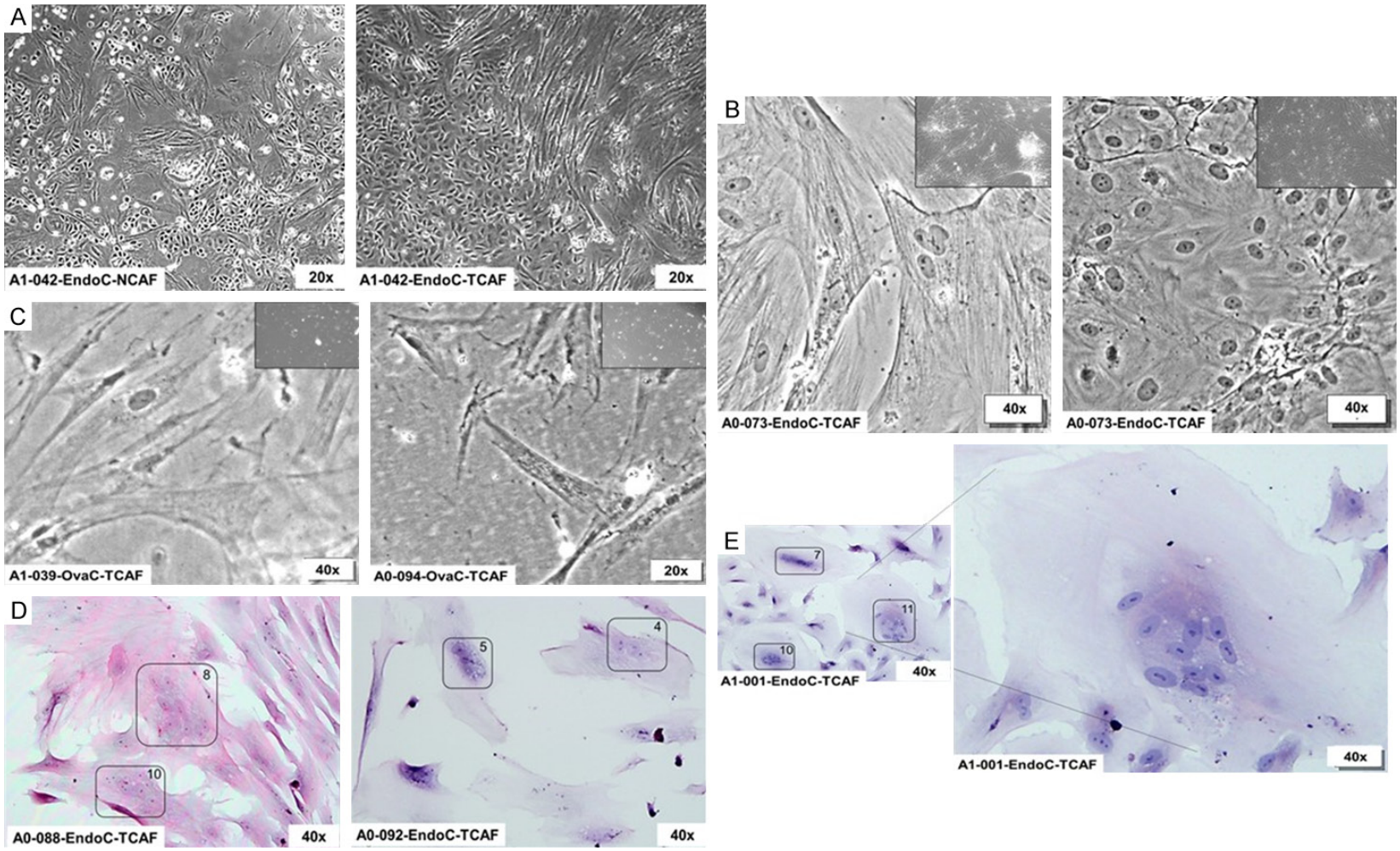


Figure 1. Characteristics morphological features of endometrial CAFs compared to ovarian CAFs: Dual morphology present within both NCAF and TCAF (A), in different passages of TCAFs from same patients (B) in contrast to ovarian CAFs (C), and presence of multinuclear cells in CAFs from samples from several patients (D, E). The numbers within the boxes indicate the number of nuclei of the particular multinucleated CAF.

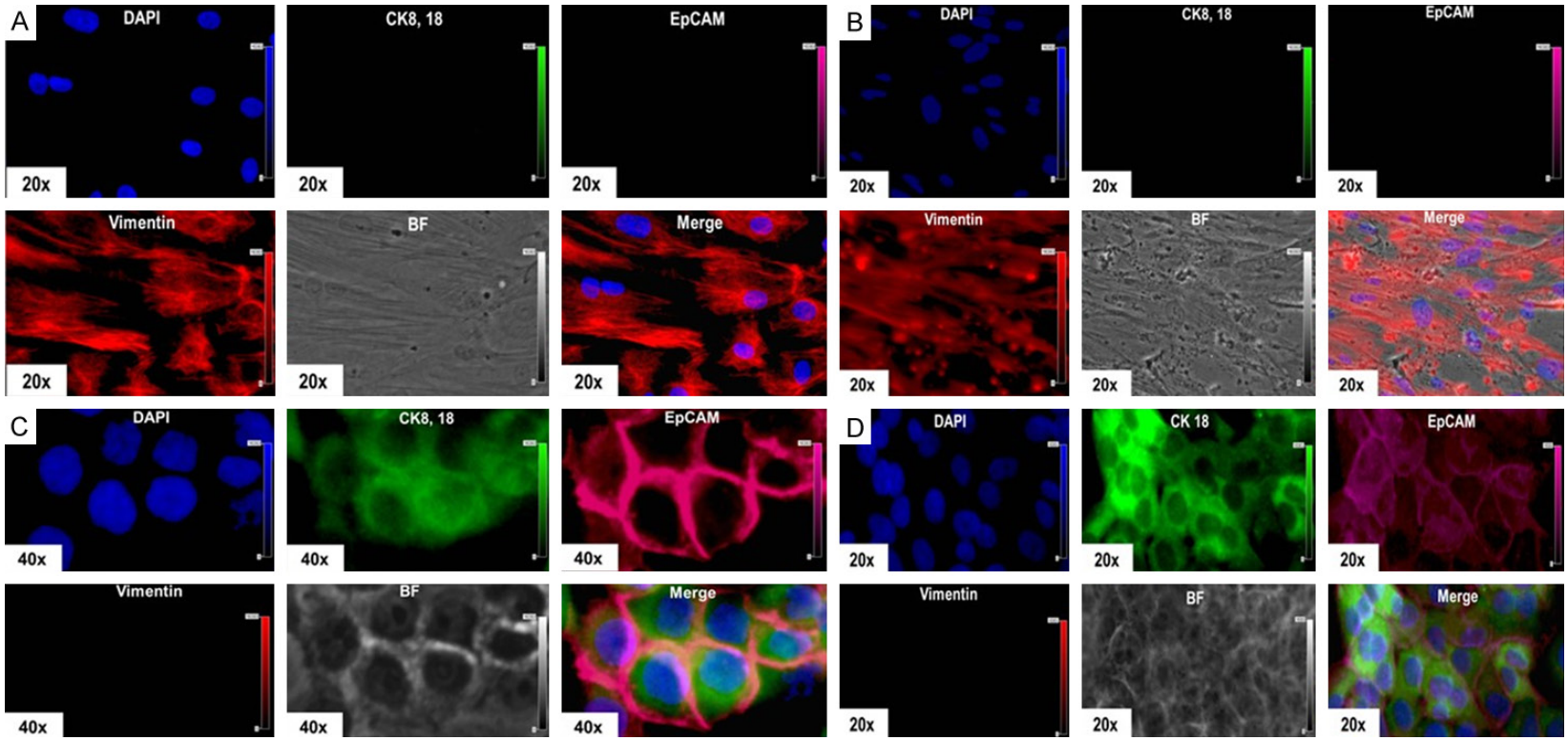


Figure 2. Immuno-fluorescent expression of CK 18, EpCAM, and vimentin in endometrial CAFs as compared to ovarian CAFs and epithelial tumor cell lines: Endometrial CAFs (A) were CK 18-/EpCAM-/Vimentin+/DAPI similar to ovarian CAFs (B) in contrast to CK 18+/EpCAM+/Vimentin-/DAPI of OVCAR3 ovarian (C) and SUM149 breast (D) cancer cell lines.

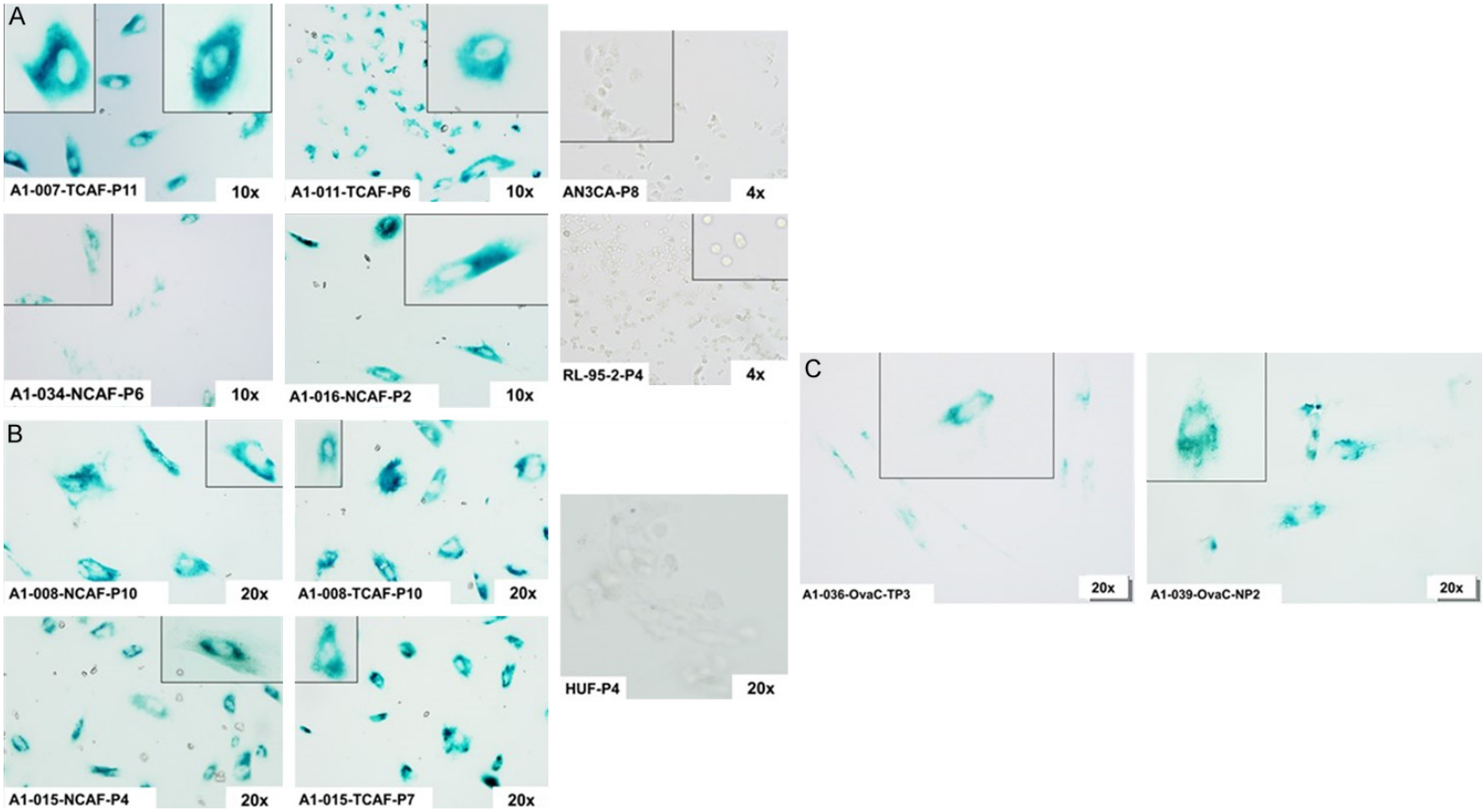


Figure 3. Beta-galactosidase stain of different endometrial CAFs of late passage compared to ovarian CAFs and in contrast to epithelial tumor cell lines: Endometrial CAFs stain positive in contrast to AN3CA and RL-95-2 endometrial cancer cell lines (A). Endometrial CAFs pairs (NCAF & TCAF) stain positive in contrast to human uterine fibroblasts, HUF (B), but similar to ovarian NCAFs and TCAFs (C).

Expression of mRNAs for markers of CAF in endometrial NCAFs and TCAFs

We tested the expression of EpCAM, FAPA, S100A4 & SMA (**Figure 4A**), CXCL12 & PDGFRA (**Figure 4B**), PD-L1, PD-L2 and Meflin (**Figure 4C**) in NCAFs and TCAFs derived from several endometrial tumors and tumor-adjacent normal tissues. We used HUF and ovarian CAFs as internal positive controls and several tumor cell lines as internal negative controls. The controls are color-coded. CAFs were negative for EpCAM, similar to HUF cells in contrast to RL-95-2 and MDA-MB-231 cell lines, while they were found to express mRNAs of FAPA, S100A4, SMAalpha, CXCL12, PDGFRA, PD-L1, PD-L2, and Meflin. While SMA, FAPA, CXCL12, PDGFRA, PD-L1, PD-L2, and Meflin were mostly absent in negative controls (epithelial tumor cells) but observed only in HUFs, the expression of S100A4 mRNA was found to be heterogeneous. Interestingly we observed Meflin only in all endometrial CAFs and not in ovarian CAFs. The expressions of PD-L1 and PD-L2 were found patient-specific.

Expression of CAF marker proteins in endometrial NCAFs and TCAFs by flow cytometry

We tested the expression of several CAF markers in patient-derived CAFs from several passages of culture using flow cytometry. **Figure 5** shows representative results from NCAF-TCAF pairs derived from two patients with endometrial cancers. Protein expression of SMA, FAP, CD155, PD-L1, EpCAM, CD31, and S100A4 in NCAF (**Figure 5A**), in TCAF (**Figure 5B**) and expression of SMA, FAP, CD155, CD90, EpCAM, CD31, S100A4 and PD-L1 in NCAF (**Figure 5C**) and TCAF (**Figure 5D**) showing a consistent pattern of expression as compared to mRNAs expression. Heatmap of % expression of CAF markers in NCAFs and TCAFs derived from 18 patients with endometrial cancers as compared to ovarian cancers are presented (**Figure 5E**, **5F**). Heatmap of passage-wise (early, mid, and late) expression of CAF markers by flow cytometry in early to mid-passages of NCAFs (green) and TCAFs (red) derived from 20 patients with endometrial (Peach) and ovarian (Teal) cancers. We presented the expressions conditionally formatted (All together) with a set of 5-rating icons with a rule of 3-color scale (light blue as the minimum, yellow as the mid-point, and dark blue as a maximum value), indicating that both NCAF and TCAF uniformly express SMA, CD155, and CD90, while EpCAM expression was absent. As observed before, the expressions of S100A4 and FAPA were found heterogeneous among both NCAFs and TCAFs as S100A4high/FAPAhigh, S100A4low/FAPAlow, S100A4high/FAPAlow, and S100A4-low/FAPAhigh CAFs. To understand the passage-wise distribution of expression of markers, we tested markers in NCAF and TCAF passages from 0-10 derived from one of our patient's T and N samples. Passage-wise Heatmap of expression (conditionally formatted with a set of 5-rating icons with gradient data) of CAF markers in the patient with endometrial cancer demonstrated a similar pattern of expression in different passages.

Expression of subcellular localization of CAF marker in endometrial NCAFs and TCAFs by ICC

We tested the ICC expression of positive, negative, and immune markers in endometrial CAFs and compared it with the expression in ovarian CAFs (**Figure 6**). The ICC stain from CAF from patients with endometrial cancer showed negative staining for EpCAM, CK 8, 18 along with PD-L1. A granular cytoplasmic TE-7 stain, as well as diffuse TE-7 stains (**Figure 6A**), were observed in endometrial CAFs in contrast to ovarian CAFs (**Figure 6B**). Heatmap of % expression of CAF markers by ICC of CAFs derived from patients with endometrial and ovarian cancers by semi-quantification demonstrate that endometrial CAFs bear the "triple negativity" feature of CAFs; negative for epithelial (EpCAM and CK 8,18), endothelial (CD31), and leucocyte common antigen markers (CD45) similar to the ovarian CAFs (**Figure 6C**). The expression of S100A4 and PD-L1 was found to be differential and patient-specific. Interestingly, endometrial CAFs derived from patients with the highest grade (3)/stage (III) expressed the lowest percentage of PD-L1 (0-10).

Expression of CAF marker proteins in endometrial NCAFs and TCAFs by western blot

Figure 7 shows the expressions of TGFbeta, S100A4, SMA, PD-L1, and CD44 in endometrial CAFs. SMA and TGF-B were found uniformly higher than HUF, while S100A4, CD44, and

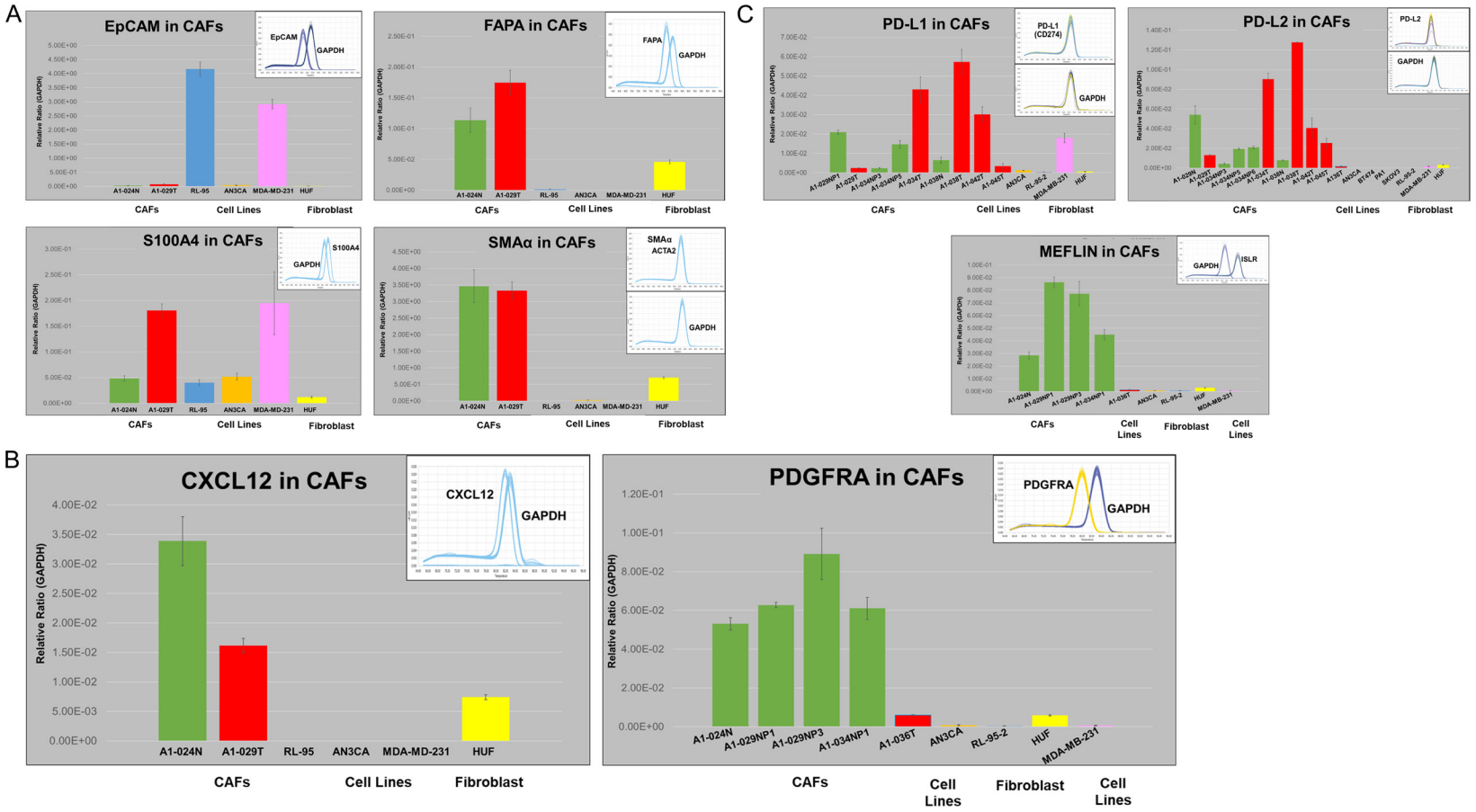
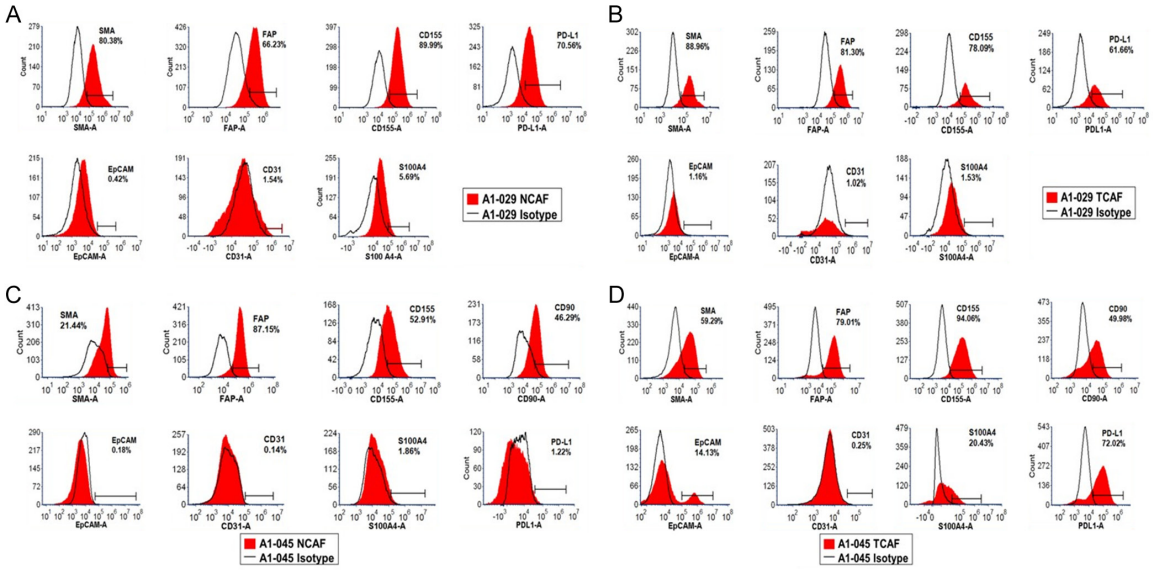


Figure 4. Expression of mRNAs for the markers of CAF in endometrial NCAFs and TCAFs: Expression of EpCAM, FAP, S100A4 & SMA (A), CXCL12 & PDGFRA (B), PD-L1, PD-L2 and Meflin (C) are presented. HUF and ovarian CAFs were used as internal positive controls, with several tumor cell lines as internal negative controls. The controls are color-coded. NCAFs and TCAFs were presented in green and red bars, respectively. Ovarian TCAFs were presented in red bars with a teal border.



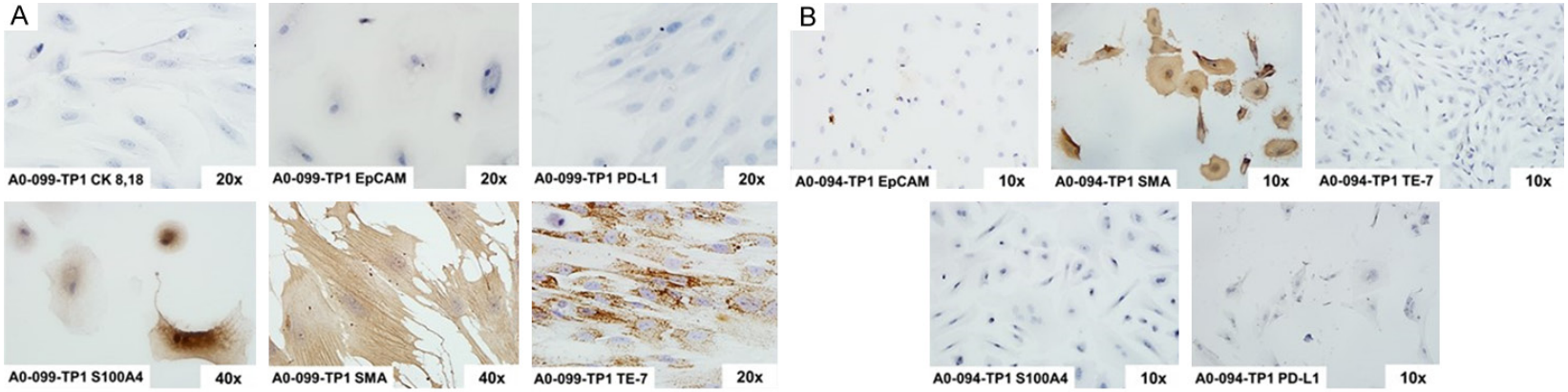
E

Patients: Histology of the disease	Grade	Stage	CAF Markers In Patient-Derived TCAFs & NCAFs: Endometrial & Ovarian Cancers											
			TCAF						NCAF					
			EpCAM	FAP	SMA	S100A4	CD155	CD90	EpCAM	FAP	SMA	S100A4	CD155	CD90
EEA	1	IA	0.11	67.55	63.33	38.82	99.5	99.61	ND	ND	ND	ND	ND	ND
Invasive EEA	1	IA	0.1	20.2	98.4	51.4	ND	ND	ND	ND	ND	ND	ND	ND
Invasive EEA	1	IA	4	45	96	54	ND	ND	1.5	40	71	14	ND	ND
Invasive EEA	3	IA	2.4	10.2	87.3	40.7	ND	ND	25.5	70.5	ND	ND	ND	ND
Invasive EEA	1	IA	0.1	34.3	97.2	40.2	ND	ND	ND	ND	ND	ND	ND	ND
EEA	3	IB	3.6	63.8	96.8	44.5	ND	ND	ND	ND	ND	ND	ND	ND
EEA	2	IA	4.1	42.6	42.6	20.4	ND	ND	0.7	24	74.3	23.5	ND	ND
EEA	2	IA	13.8	26.2	96.9	53.2	ND	ND	1.3	15.2	86.4	40.7	ND	ND
EEA	1	IA	4.7	73	27	59	ND	ND	1.9	64.5	87.9	17.8	ND	ND
Invasive HGS Adenocarcinoma	3	IIIC2	4.6	74.9	95.3	7.8	94.9	ND	5.5	91.5	93.3	53.4	98.5	35.1
HG mixed EAA, Clear cell & Serous 50%	3	IVB	17.6	2	78.8	79.1	94.9	67.2	ND	ND	ND	ND	ND	ND
EEA	2	IA	ND	80.5	78.3	22.6	94.4	72.1	4	81.9	60.2	76.7	98.1	71.6
EEA	1	IB	ND	34.8	ND	13.5	89.8	ND	1.9	94.3	83.2	0.6	99.2	71.7
EEA	1	IA	4	96.1	88.2	18	92.3	99.26	0	86.73	52	28	97.39	99.71
EEA	2	IA	5.5	32.6	10.3	3	96	33.6	2.1	84.6	47.3	3.1	92.94	97.51
EEA	2	I	0.2	92	47	8	98.8	99.73	0.6	88	65	3	97	99
Carcinosarcoma	3	IA	0	84.67	ND	15	97.74	99.89	0	42.2	ND	ND	93.99	98.38
EEA	1	NA	0.43	84.12	70.62	7.85	98.53	98.58	1.48	96.94	89.49	11.68	97.18	97.73
Adult Granulosa Cell Tumor	ND	IA	1	0	85	4.5	ND	ND	ND	ND	ND	ND	ND	ND
HGS Carcinoma	3	IIB	ND	ND	ND	ND	ND	ND	17.5	72.3	33.9	2.2	77.3	29.9

F

Patient	A1-008							
CAFs	TCAFs				NCAFs			
Passage	EpCAM	FAP	SMA	S100A4	EpCAM	FAP	SMA	S100A4
P0	0	40.7	96.7	ND	1.9	45	85.4	ND
P1	6.9	42.8	99.4	34.3	1.6	87.1	97.8	4.7
P2	4.5	27.3	95.2	59.3	0.7	53.7	98.7	36.6
P3	1.7	57.7	92.8	49.3	0.3	31.7	49.2	8
P4	12.4	9.2	94.8	75.9	1.7	25.4	83	8.5
P5	5	88	96	50	3	9	97.6	61.2
P6	10.8	77	99.1	60.1	2	23	50	2
P7	6.6	32.1	67.8	12.9	0.6	54	61	3.1
P8	20	32.3	45.7	27	1.8	29.5	41.4	1.5
P9	8.75	15	43	2	3	30	48	1.6

Figure 5. Expression of CAF markers by flow cytometry in endometrial NCAFs and TCAFs derived from patients with endometrial cancers: Expression of SMA, FAP, CD155, PD-L1, EpCAM, CD31, and S100A4 in NCAF (A), in TCAF (B), and expression of SMA, FAP, CD155, CD90, EpCAM, CD31, S100A4, and PD-L1 in (NCAF) (C) and TCAF (D) are presented. Heatmap of % expression of CAF markers in NCAFs and TCAFs derived from 18 representative patients with endometrial as compared to ovarian cancers are shown (E, F). Heatmap of passage-wise (early, mid, and late) expression of CAF markers by flow cytometry in early to mid-passages of NCAFs (green) and TCAFs (red) derived from 20 representative patients with endometrial (Peach) and ovarian (Teal) cancers. Expressions are conditionally formatted (all together) with a set of 5-rating icons with a rule of 3-color scale (light blue as minimum, yellow as a midpoint, and dark blue as a maximum value). Each column represents protein markers for CAFs. Each row represents a patient with histology, grade, and stage of the disease. NA: Not available; ND: Not detected; EEA: Endometrioid Endometrial Adenocarcinoma; HGS: High-Grade Serous (E). Passage-wise Heatmap of expression of CAF markers in one patient with endometrial cancer conditionally formatted with a set of 5-rating icons with gradient data bars with borders (Grey for EpCAM, Cyan blue for FAP, Orange for SMA, and Purple for S100A4). Each maker from TCAF (red) and NCAF (green) presented in each row is conditionally formatted row-wise. CAF markers are color-coded (F).



C

Organ Type		CAFs: Endometrial Cancers Patients									CAFs: Ovarian Cancers Patients			
Grade		1	2	1	1	3	3	1	1	3	X	1	X	1
Stage		IA	II	IA	IA	IIIC1	IV	IA	IA	IB	IIIC/IV	IIIC	IA	IVA
CAF Markers	*Ex Vivo* Series	% Expression by ICC												
		A0-080	A0-085	A0-087	A0-088	A0-089	A0-090	A0-095	A0-097	A0-099	AB91 (Biopsy)	A0-094	A1-036	A1-048
Positive Markers	SMA	100	90	100	100	100	95	100	60	90	ND	100	86	100
	TE-7	1	50	87	60	100	50	75	0	90	ND	0	ND	65
	S100A4	100	50	75	30	0	70	95	50	95	ND	0	3.2	0
Negative Markers	CK 8,18	0	0	0	0	0	0	1	0	0	ND	0	0	0
	EpCAM	0	0	0	0	0	0	1	0	0	ND	0	0	0
	CD45	0	0	0	0	0	0	0	0	0	ND	0	0	0
	CD31	0	0	0	0	0	0	0	0	0	ND	0	0	0
Immune Marker	PD-L1	0	1	50	30	10	1	0	0	0	ND	0	22	20

Figure 6. Subcellular expression of CAF markers in endometrial CAFs by ICC: Expression of CK 8, 18, EpCAM, PD-L1, S100A4, SMA, TE-7 in endometrial CAFs (A) as compared to expression of EpCAM, PD-L1, S100A4, SMA, TE-7 in ovarian CAFs (B) are presented. A Heatmap of % expression of CAF markers in CAFs derived from patients with endometrial cancers as compared to ovarian cancers is presented (C). Heatmap of % expression of CAF markers by ICC of CAFs derived from patients with endometrial (Peach) and ovarian (Teal) cancers by semi-quantification are presented. Expressions are conditionally formatted (all together) with a set of 5-rating icons with a rule of 3-color scale (light blue as the minimum, yellow as the midpoint, and dark blue as a maximum value). Each row represents protein markers (positive, negative, and immune), and each column represents a patient with the grade and stage of the disease. ND: Not available for detection; CAFs could not be derived from the biopsy sample. NA: Detection not performed due to the unavailability of CAFs.

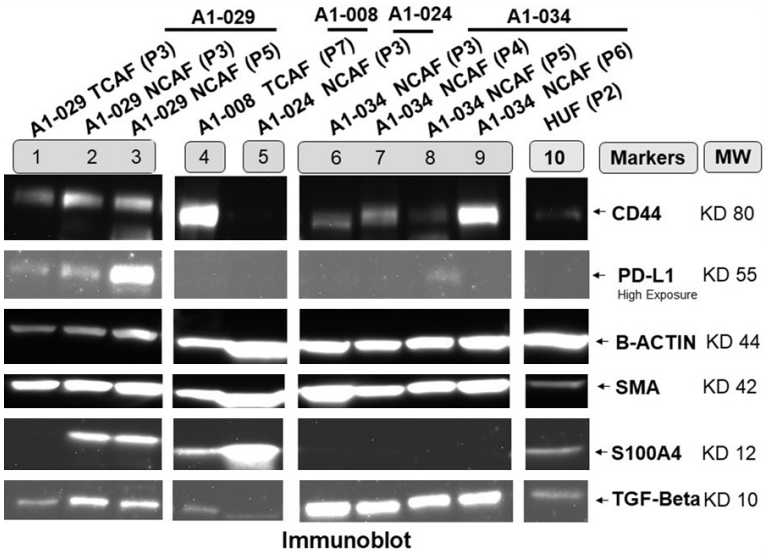


Figure 7. Western blot expression of CAF markers in endometrial NCAFs and TCAFs: Expression of TGFbeta, S100A4, SMA, PD-L1, and CD44 are presented. Beta-actin is used as the loading control. HUF is used as fibroblast control.

PD-L1 expression was differentially patient-specific.

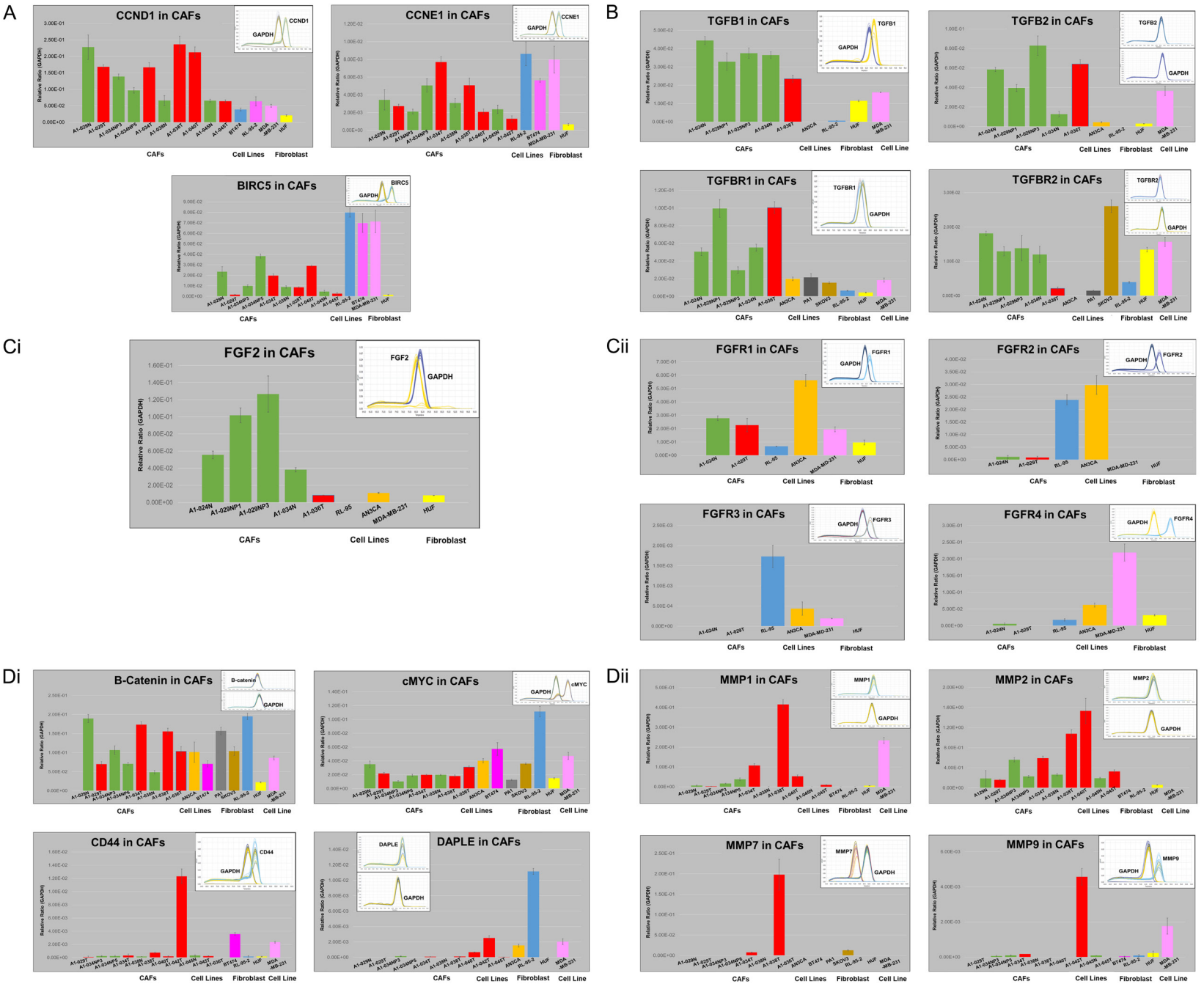
Expression of mRNAs for cell signaling proteins in endometrial NCAFs and TCAFs

To characterize the signaling landscape of the endometrial CAFs, we performed a comprehensive test on NCAFs and TCAFs for the expression of mRNAs for cell signaling (CCND1, CCNE1 & BIRC5) along with three relevant pathways in CAF signaling, including TGF (TGFbeta1, TGFbeta2, TGFBR1 & TGFBR2), FGF (FGF2, FGFR1, FGFR2, FGFR3, & FGFR4) and Wnt-beta-catenin (Beta-catenin, CD44, c-MYC, DAPLE, MMP1, MMP2, MMP7 & MMP9) pathways. We also tested endometrial NCAFs and TCAFs for the expression of receptors and ligands of HER (EGF, Epiregulin ERBB2, ERBB3 & NRG1), HGF, and steroid (ESR1 and PGR) pathways (**Figure 8A-H**).

Data showed that the mRNAs for CCND1 and CCNE1 were uniformly present in both TCAFs and NCAFs in contrast to patient-specific differential expression of the mRNA for the survivin protein. However, we did not observe any difference between TCAFs and NCAFs. Endometrial CAFs uniformly expressed mRNAs for TGFbeta1, TGFbeta2, TGFBR1 & TGFBR2 as compared to ovarian CAFs. In contrast, endometrial CAFs uniformly expressed FGF2 mRNAs, while ovarian CAFs had little expression for the same. Since we observed FGF2 mRNAs expression in endometrial CAFs, we also tested the expression of mRNAs for FGFR1, FGFR2, FGFR3, and FGFR4 to show that the CAFs differentially expressed FGFR1 mRNA, indicating that the FGF signaling in endometrial CAFs may be processed via FGF-FGFR1 axis. In the Wnt-beta-catenin pathway, we observed a uniformly expressed level of beta-catenin and its target protein c-MYC in endometrial NCAFs and TCAFs as compared to ovarian CAFs. In contrast, the expression of CD44 and DAPLE were found to be overall low (except one). Among the MMPs, uniformly low levels of MMP1 mRNA and moderate levels of MMP2 mRNAs were identified in contrast to MMP7 and MMP9, which were almost absent (except one). No mRNA for ERBB2 and ERBB3 was detected in the NCAFs and TCAFs in contrast to a uniform expression of NRG1. While low levels of epiregulin were detected in CAFs (except one), the expression of EGF and HGF were patient-specific among TCAFs and NCAFs. Similarly, while low levels of ESR1 were detected in CAFs (except one TCAF), the expression of PGR was patient-specific among TCAFs and NCAFs. Heatmap (patient-wise) of mRNA expression demonstrates a pattern of a characteristic of endometrial CAFs. Endometrial CAFs express mRNAs for cell cycle proteins along with survivin mRNAs and certain mRNAs of the TGF pathway, Wnt-beta-catenin, and FGF pathway. CAFs are devoid of mRNA for the ERBB family of proteins and ESR1 while expressing PGR, EGFs, HGF, and NRG1. No remarkable difference was observed between NCAFs and TCAFs.

Expression of mRNAs for hypoxic signaling proteins in endometrial TCAFs

We tested the hypoxic response in endometrial TCAFs to know how a hypoxic response in these non-transformed stromal cells characteristical-


A
CCND1 in CAFs
CCNE1 in CAFs
BIRC5 in CAFs
B
TGFB1 in CAFs
TGFB2 in CAFs
TGFBR1 in CAFs
TGFBR2 in CAFs
Ci
FGF2 in CAFs
Cii
FGFR1 in CAFs
FGFR2 in CAFs
FGFR3 in CAFs
FGFR4 in CAFs
Di
B-Catenin in CAFs
cMYC in CAFs
CD44 in CAFs
DAPLE in CAFs
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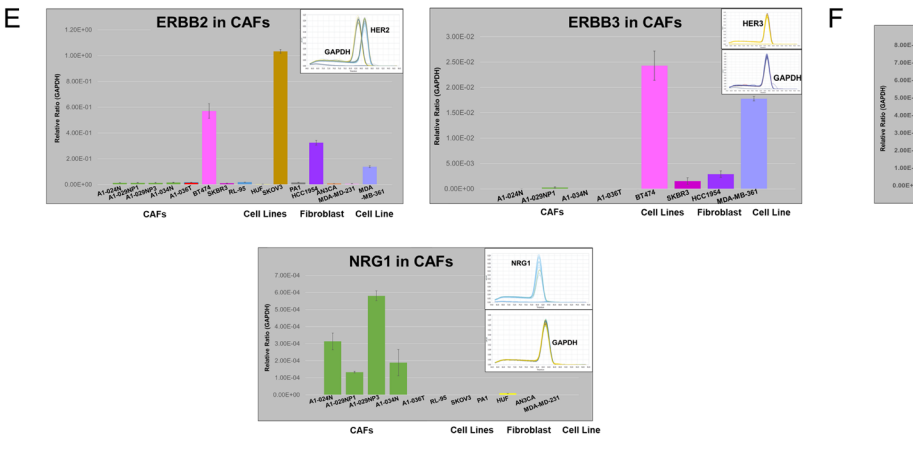
MMP9 in CAFs
GAPDH
CAFs
Cell Lines
Fibroblast



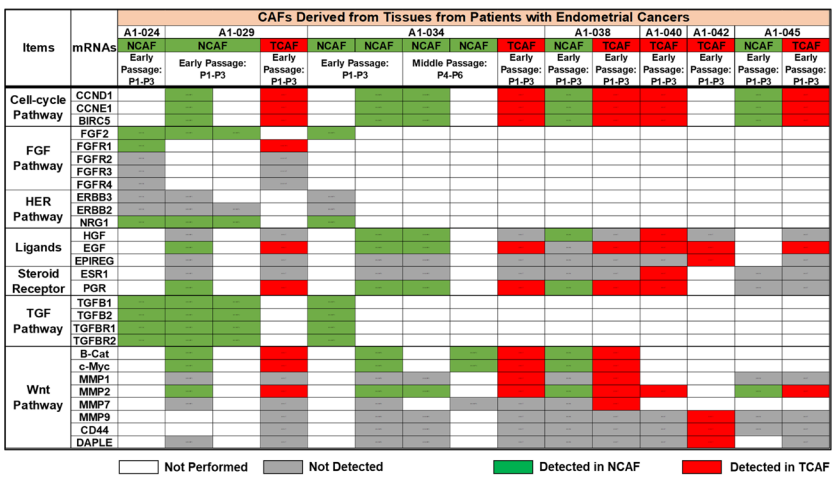


Figure 8. Expression of mRNAs for the signaling of CAF in endometrial NCAFs and TCAFs: Expression of signaling proteins of cell cycle pathway (CCND1, CCNE1 & BIRC5) (A), TGF pathway (TGFbeta1, TGFbeta2, TGFBR1 & TGFBR2) (B), FGF pathway, including FGF2 (Ci), FGFR1, FGFR2, FGFR3, & FGFR4 (Cii), Wnt-Beta-catenin pathway, including beta-catenin, CD44, c-MYC & DAPLE (Di), MMP1, MMP2, MMP7 & MMP9 (Dii), HER pathway including ERBB2, ERBB3 & NRG1 (E), ligands including EGF, HGF, & epiregulin (F), and steroid receptors, ESR1 and PGR (G) are presented. Green bars represented NCAF & Red bars represented TCAF. Cancer cell lines (represented in color bars) and human uterine fibroblasts (HUF, yellow bars) are used as controls. Inset shows the melting curves of the qRT-PCR reactions. Heatmap of mRNA expression is presented (H) patient-wise. Each column represents patients (Green for NCAF & Red for TCAF), and each row represents mRNA of the signaling proteins.

ly varies as compared to transformed epithelial tumor cells in endometrial cancers. Expression of hypoxia-induced mRNAs for HIF-1a, HIF-2a, VEGF, and MMP1 (**Figure 9A**) demonstrated no significant alterations in the mRNAs. Interestingly we observed an increase in MMP1 mRNA following hypoxia. A heatmap of ratios of hypoxic to the normoxic expression of signaling proteins of HIF-signaling, as well as a few growth factors, immune markers, steroid receptors, and the Wnt-beta-catenin pathway proteins (**Figure 9B**), clearly indicated that the increase in MMP1 in the hypoxic TCAFs was independent of HIF-1a/HIF2a transcription.

Relationship between expressions of CAF markers in tumor sample derived CAFs and presence of T-cells within the tumor at surgery

The expression of FAP mRNA and CXCL12 by qRT-PCR (**Figure 10A**; upper panel bar diagrams) and conditionally formatted expression of markers in the same CAFs by flow cytometry (**Figure 10A**; lower panel) showed EpCAM-/SMA+/FAPA+/CD155+/S100A4- TCAFs derived from the patients with endometrial cancer were positive for CXCL12, a receptor for CXCR4. A parallel IHC in the tumor sample from the same patient on day 0 (the day of the surgery) (**Figure 10B**) showed the presence of expression of CD3, CD4, and CD8 close to epithelial tumor cells.

Discussion

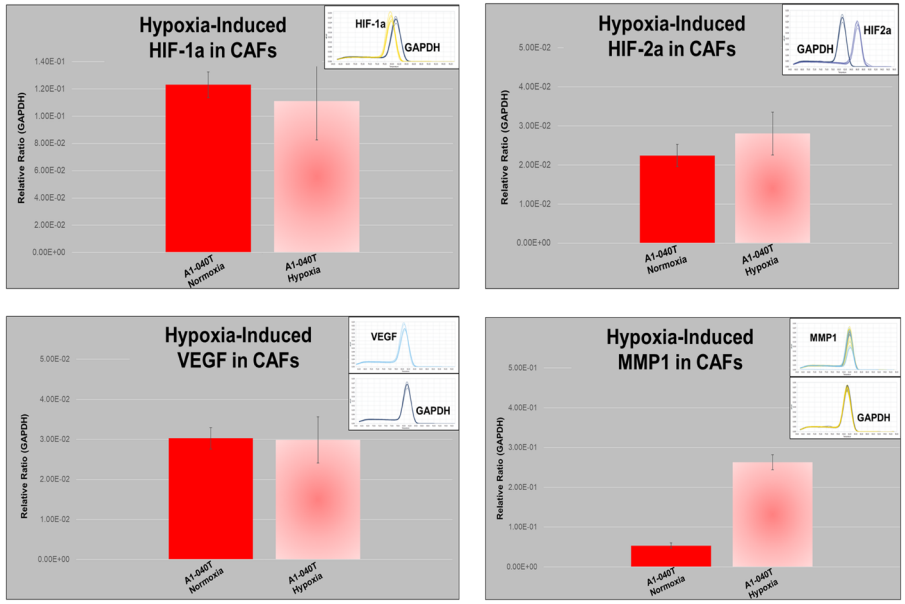
Tumor-TME is a state of cells where every cell is a citizen, yet tumor cells are aristocrats. A tumor presents a successful and evolving ecosystem structured around transformed tumor cells and non-transformed cells immediately to tumor cells, the tumor's microenvironment. Each component of TME has a bona fide role in the progression of a tumor as well as the development of treatment resistance in an organ-specific way, the two main determinants of the disease outcome. Hence, an unmet need remains to venture on the turf of CAFs in endometrial cancers. If the role of CAF is undeniable in shaping the two main determinants of the disease outcome, the evolving dialogue of CAF-tumor cells-rest of TME cells needs to be read. To solve the puzzle, we acquire knowledge about the signal-based function of CAF to establish a CAF-based stromal switch and to address the dynamic contribution of CAF in the progression of cancer [6]. Here we undertook an in-depth characterization of the landscape of endometrial CAF, which would be useful in delineating CAF's functional choreograph with endometrial tumor cells as well as other stromal cells within the endometrial cancer TME providing valuable insight for the future mechanistic study in organ-type specific CAF signaling. Our system of CAF culture is not artificial, as we derived CAFs from surgically resected tumors following pathological grossing in each case. As tumor samples presented diversity in their grossing pattern, histology, and stage/grade, we designed an exclusive way to set up the primary culture of CAFs from the feeder layer, which is distinct from the standard enzymatic digestion-based model.

We report unique morphological features of CAF in endometrial cancers. Since the data on the culture and features of endometrial CAFs are limited, we included a more frequently reported CAF from the closest gynecological cancer, ovarian cancer, as an internal comparator. Endometrial CAFs, both NCAF and TCAF, frequently exhibited dual morphology present in different passages from the same patients and presented a so far unreported multinuclear cell as compared to ovarian CAFs (**Figure 1**). In our study, the dual morphology was mutually exclusive to multinuclear status.

Considering the limited availability of patient-derived CAF data in endometrial cancers, and the extent of heterogeneity of CAF markers known so far in several organ-type cancers other than endometrial cancers, we devoted a significant part of our study to the characterization of endometrial CAFs and testing the expression (mRNA, & protein) of several CAF markers using multiple parallel techniques, including immunofluorescence, qRT-PCR, flow cytometry, ICC, and Western blot (**Figures 2-7**). Endometrial CAFs derived from any tumors, like primary fibroblasts, have rarely grown beyond 7-8 passages.

We used the triple-negative definition of Sahai et al., as "CAFs are defined as cells negative for epithelial, endothelial and leukocyte markers with an elongated morphology and no mutations" [26]. Indeed, endometrial CAFs were triple-negative; they did not express epithelial cell markers like CK 8, 18, and EpCAM, endothelial cell markers like CD31, and leucocyte common

A



B

Cell Signaling	mRNA for Proteins	A1-040T		
		GAPDH Ratio		Hypoxia/ Normoxia
		Normoxia	Hypoxia	Ratio
HIF signaling	**HIF-1A**	1.23E-01	1.11E-01	**0.903**
	HIF-2A	2.24E-02	2.80E-02	**1.25**
	VEGF	3.03E-02	2.98E-02	**0.986**
Ligands	**HGF**	5.32E-03	4.60E-04	**0.086**
	EGF	1.79E-03	8.04E-04	**0.448**
	EPIREG	7.06E-05	1.99E-04	**2.821**
Immune Markers	**PD-L1**	1.73E-02	1.51E-02	**0.872**
	PD-L2	4.72E-02	4.29E-02	**0.909**
Steroid Receptor	**ESR1**	7.43E-03	4.66E-03	**0.627**
	PGR	1.05E-02	7.14E-03	**0.681**
WP	**MMP1**	5.34E-02	2.63E-01	**4.924**
	MMP2	1.53E+00	9.57E-01	**0.624**
	DAPLE	6.73E-04	1.14E-04	**0.169**

Figure 9. Expression of hypoxic response in endometrial TCAFs derived from patients: Expression of hypoxia-induced HIF-1a, HIF-2a, VEGF, and MMP1 are presented (A). A heatmap of ratios of hypoxic to normoxic expression of signaling proteins of HIF-signaling, as well as a few growth factors, immune markers, steroid receptors, and Wnt-beta-catenin pathway proteins, are presented (B).

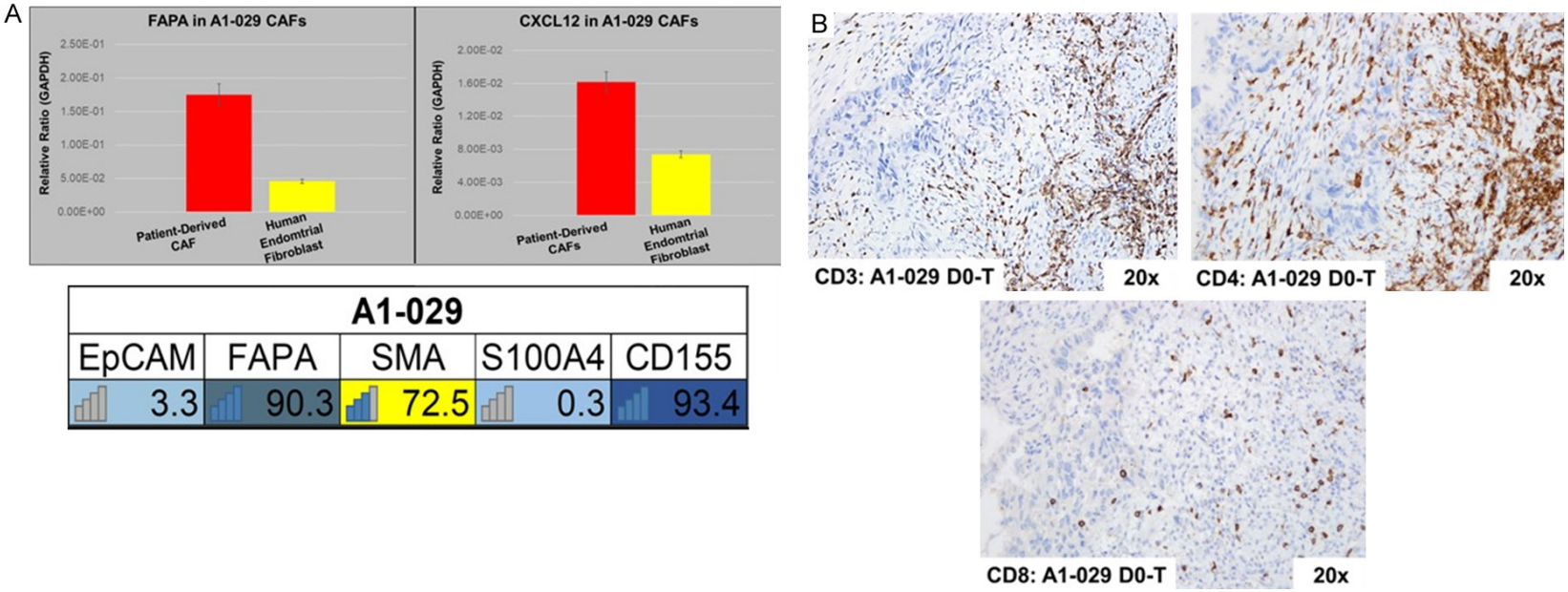


Figure 10. Relationship between expression of CAF markers in tumor-derived CAFs and T-cells in FFPE tumor sample: Expression of mRNA of FAP and CXCL12 by qRT-PCR (upper panel bar diagrams) and conditionally formatted percentage expression of markers in the same CAFs by flow cytometry (lower panel) are presented (A). HUF was used as the internal positive control. IHC expression of CD3, CD4, and CD8 in the tumor sample from the same patient on day 0 (the day of the surgery) is presented (B).

antigen, CD45 like, as we observed in ovarian CAFs [4]. Endometrial CAFs, both TCAFs and NCAFs uniformly expressed mesenchymal markers like vimentin, SMA, CD155, CD90, PDGFRA, Meflin, and TE-7, while expression of CD44, FAP, S100A4, PD-L1, and PD-L2 was patient-specific and passage specific. In fact, we observed a trend of inverse expression in FAP and S100A4 in some of the CAFs by flow cytometry. Five TCAFs with a high % expression of FAP (80-96%) had the lowest expression of S100A4 (less than 25%) expression, and five TCAFs with a high % expression of S100A4 (50-80%) had the lowest expression of FAP (less than 18%) expression (**Figure 5E**). The data may indicate the existence of a subtype of endometrial FAP-low/S100A4-high and FAP-high/S100A4-low CAFs, the functional study of which is beyond the scope of this work.

Acknowledging the fact that in a progressing solid tumor, CAFs signals are integrated to initiate (1) metabolic switch, (2) angiogenesis, (3) immunosuppression, (4) tumor invasion, (5) tumor proliferation, and (6) development of resistance to the treatment, we tested the expression of mRNAs pertaining to the landscape of several signaling pathways specific to endometrial cancers. We tested (**Figure 9**) the expression of signaling proteins of cell cycle pathway (CCND1, CCNE1 & BIRC5), TGF pathway (TGFbeta1, TGFbeta2, TGFBR1 & TGFBR2), FGF pathway (FGF2, FGFR1, FGFR2, FGFR3, & FGFR4), Wnt-Beta-catenin pathway (beta-catenin, CD44, c-MYC & DAPLE, MMP1, MMP2, MMP7 & MMP9), HER pathway (ERBB2, ERBB3 & NRG1 & ligands including EGF, HGF, & epiregulin), and steroid receptors (ESR1 and PGR) in 7 patient-derived NCAFs and TCAFs in different passages (wherever possible). The uniform expression of cell cycle proteins was in contrast to the absence of FGFR2, FGFR3, FGFR4, ERBB3, and ERBB2, whereas FGF2 and FGFR1 are more commonly expressed. The crosstalk between FGF2/FGFR1 signaling and estrogen was reported to lead to the migration and invasion of cancer cells, as reported by Santolla et al. [27], demonstrating a feedforward FGF2/FGFR1 paracrine activation coupling CAFs to cancer cells toward breast tumor progression. We observed NRG1 expression in every CAF tested. TME-derived (NRG1 in CAF supernatant) NRG1 has been demonstrated to promote antiandrogen resistance in prostate cancer [28]. To understand the specific role of NRG1 from CAF Berdiel-Acer et al. demonstrated that phosphorylation and activation of HER3 in luminal breast cancer cells occurs in a paracrine manner mediated by NRG1 expressed by CAFs [29]. CAFs increased the self-renewal of gastric cancer stem cells by secreting NRG1 via activation of NF-κB [30]. We observed patient-specific expression of HGF and EGF, while EPIREG and ESR1 were rare. HGF expression in CAFs was reported to be 10-fold higher than that in normal fibroblast, and HGF upregulated CD44 expression through the HGF/MET signaling resulting in the enhancement of metastasis in colorectal cancers [31]. In high-grade serous ovarian cancers, CAFs are reported to recruit cancer cells to form metastatic units in accelerating tumor metastasis by secreting EGF to maintain integrin alpha5 expression in ascetic cancer cells [32]. In contrast, the expression of PGR was more common among both NCAFs and TCAFs. Wnt-pathway's mechanistic involvement as a common culprit (*Un Colpevole Comune*) in endometrial tumor cells and endometrial cancer-associated fibroblast has been known [11]. As expected [33], we identified a prominent presence of both the TGF and the Wnt pathways mRNA in the endometrial CAFs. What the above summary of the expression heat map proves is the enormous degree of heterogeneity in endometrial CAFs. Recently, single-cell sequencing revealed the heterogeneity and intra-tumoral crosstalk in human endometrial cancers [13]. It raises the question of the existence of CAFs in different states exhibiting different expression patterns (pertaining to different signaling pathways), and more importantly, could these states be switched in favor of the management of the disease? In support of the above argument, TGF-B mediated autocrine and paracrine signaling has been reported to be the basis of CAF-mediated crosstalk of CAFs with cancer cells, which encouraged a CAF-inclusive targeted therapy. Cancer-derived TGF-B stimulated the expression of IL-6, C-X-C motif chemokine 12 (CXCL12), and VEGFA in CAFs, leading to metastasis in EC [34]. CAFs derived from EC tissues are known to promote the disease progression via the SDF-1/CXCR4 axis in a paracrine or autocrine manner [35]. Is it possible that CAFs bear patient-specific signatures in their expressions? If yes, the situation will be more complex. In that case, a patient-specific mRNA/pro-

tein expression in CAF should be taken into account in the context of personalized medicine and molecular diagnostics; *precision medicine might not be synonymous with tumor cell-based genomics.*

To the best of our knowledge, this study is the first to report the presence of CAF in the tumor-adjacent normal tissue in EC and establish NCAFs to evaluate the expression of mRNAs for the signaling proteins of different pathways in parallel to TCAFs. Our data indicate for the first time, the patients' samples generate NCAFs parallel to TCAFs, but once generated (in the patients), the overall expression pattern of mRNAs/proteins in NCAFs remains comparable to that in TCAFs in general. A wider perspective of this fact may provoke the fact that in patients with NCAFs, the residual tumor cells following surgery will be supported by the existence of these CAFs in the remaining post-surgery tumor-adjacent normal tissues. One straightforward way to test this hypothesis will be to categorize the patients into NCAFs-generating and no NCAFs-generating groups and follow their outcomes in the coming 3-5 years.

Since hypoxia is the fact of life of tumor cells in solid tumors, and the heterodimeric transcription factor, HIF plays an important role in CAF metabolic reprogramming in mediating the protumorigenic effect of CAFs [33], we tested the hypoxic response in TCAFs. No significant difference in the expression of both HIF-1a and HIF-2a, as well as their transcriptional readout, VEGF, was observed in hypoxic TCAFs as compared to normoxic conditions. Interestingly, all the normoxic TCAF samples exhibited significant levels of HIF-1a, HIF-2a, and VEGF. At present, we do not have any explanation for this observation. However, in its support, a study identified that stromal HIF-1a also affected cancer progression in a hypoxia-independent manner [36].

CAF forms a significant half of the desmoplastic reaction in any stroma-rich solid tumor. The prognostic value of desmoplastic reaction and lymphocytic infiltration in the management of breast cancers has been reported by Cardone et al. [37]. We demonstrated for the first time in endometrial cancers that the presence of CD3+, CD4+, and CD8+ cells (IHC from FFPE) in the tumor-bearing EpCAM-/SMA+/FAP-A+/CD155+/S100A4-/CXCL12+ CAFs indicating a probable role of CXCL12-CXCR4 pathway in the lymphocytic infiltration of the tumor [33]. It will be worth finding out if this event of correlation is associated with the pathological parameters and has any bearing on the disease outcome in the future.

The tumor-TME model has been mathematically framed as an evolutionary game by Wölfl et al. [38] to conceptualize and analyze biological interactions where tumor cells' fitness is not only influenced by their traits but also by the traits of cells of TME, and CAFs. The model implied that the progression of cancer is an interactive evolutionary competition between these different cell types. These interactions were explained through the Lotka-Volterra competition equations and their extensions term, the term "Deadlock game" and the term 'Leader game' in the context of the presence or absence of drug and/or cancer-associated fibroblasts. Kaznatcheev et al. [39] demonstrated that cancer-associated fibroblasts qualitatively switch the type of game being played by the in-vitro population from "Leader to Deadlock" in their non-small cell lung cancer model. In their system, they viewed an untreated tumor as similar to DMSO + CAF and thus designated it as the Leader game. Treating with Alectinib (Alectinib + CAF) or eliminating CAFs (through a stromal-directed therapy) moved the game into a Deadlock game.

In summary, our data proves that endometrial CAFs converse with the number of crosstalk that exist between tumor cells and CAF in endometrial cancers and confirm the importance of the intra-tumoral CAF-endometrial tumor cells ecosystem. Furthermore, it explains why cellular signals arising/involving CAFs should be folded into the strategy of disease management to counter the co-evolution of CAF-tumor cells during the process of metastatic progression of the disease and/or during the development of drug resistance following a clinical intervention. As we know more about the CAF landscape, the more we will pave the way to stratify the approach to "normalize" the stromal switch by targeting CAF in endometrial cancers.

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Written informed consents (IRB Protocol Number: 2017.053-100399_ExVivo001, Approval Date: October 4, 2017) were obtained for receiving resected tissue samples from 53 enrolled patients (age: 18 y or older) with endometrial cancers.

Disclosure of conflict of interest

None.

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References

[1] Pietras K and Ostman A. Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res 2010; 316: 1324-1331.
[2] Paulsson J and Micke P. Prognostic relevance of cancer-associated fibroblasts in human cancer. Semin Cancer Biol 2014; 25: 61-68.
[3] De P, Aske J and Dey N. Cancer-associated fibroblast functions as a road-block in cancer therapy. Cancers (Basel) 2021; 13: 5246.
[4] Sulaiman R, De P, Aske JC, Lin X, Dale A, Koirala N, Gaster K, Espaillat LR, Starks D and Dey N. Patient-derived primary cancer-associated fibroblasts mediate resistance to anti-angiogenic drug in ovarian cancers. Biomedicines 2023; 11: 112.
[5] Micke P and Ostman A. Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets 2005; 9: 1217-1233.
[6] Pradip D, Jennifer A and Nandini D. Cancer-associated fibroblasts in conversation with tumor cells in endometrial cancers: a partner in crime. Int J Mol Sci 2021; 22: 9121.
[7] De P, Aske J, Sulaiman R and Dey N. Bête noire of chemotherapy and targeted therapy: CAF-mediated resistance. Cancers (Basel) 2022; 14: 1519.
[8] Custódio M, Biddle A and Tavassoli M. Portrait of a CAF: the story of cancer-associated fibroblasts in head and neck cancer. Oral Oncol 2020; 110: 104972.
[9] Bienkowska KJ, Hanley CJ and Thomas GJ. Cancer-associated fibroblasts in oral cancer: a current perspective on function and potential for therapeutic targeting. Front Oral Health 2021; 2: 686337.
[10] Wu F, Yang J, Liu J, Wang Y, Mu J, Zeng Q, Deng S and Zhou H. Signaling pathways in cancer-associated fibroblasts and targeted therapy for cancer. Signal Transduct Target Ther 2021; 6: 218.
[11] De P, Aske JC, Dale A, Rojas Espaillat L, Starks D and Dey N. Addressing activation of WNT beta-catenin pathway in diverse landscape of endometrial carcinogenesis. Am J Transl Res 2021; 13: 12168-12180.
[12] Sun H, Chen L, Yan G, Wang R, Diao Z, Hu Y and Li C. HOXA10 suppresses p/CAF promoter activity via three consecutive TTAT units in human endometrial stromal cells. Biochem Biophys Res Commun 2009; 379: 16-21.
[13] Yu Z, Zhang J, Zhang Q, Wei S, Shi R, Zhao R, An L, Grose R, Feng D and Wang H. Single-cell sequencing reveals the heterogeneity and intratumoral crosstalk in human endometrial cancer. Cell Prolif 2022; 55: e13249.
[14] Li BL, Lu W, Qu JJ, Ye L, Du GQ and Wan XP. Loss of exosomal miR-148b from cancer-associated fibroblasts promotes endometrial cancer cell invasion and cancer metastasis. J Cell Physiol 2019; 234: 2943-2953.
[15] Fan JT, Zhou ZY, Luo YL, Luo Q, Chen SB, Zhao JC and Chen QR. Exosomal lncRNA NEAT1 from cancer-associated fibroblasts facilitates endometrial cancer progression via miR-26a/b-5p-mediated STAT3/YKL-40 signaling pathway. Neoplasia 2021; 23: 692-703.
[16] Voon YC, Omar IS, Wu MH, Said NABM and Chung I. Cancer-associated fibroblasts as cellular vehicles in endometrial cancer cell migration. Oncol Lett 2022; 23: 3.
[17] Subramaniam KS, Omar IS, Kwong SC, Mohamed Z, Woo YL, Mat Adenan NA and Chung I. Cancer-associated fibroblasts promote endometrial cancer growth via activation of interleukin-6/STAT-3/c-Myc pathway. Am J Cancer Res 2016; 6: 200-213.
[18] Aprelikova O, Palla J, Hibler B, Yu X, Greer YE, Yi M, Stephens R, Maxwell GL, Jazaeri A, Risinger JI, Rubin JS and Niederhuber J. Silencing of miR-148a in cancer-associated fibroblasts results in WNT10B-mediated stimulation of tumor cell motility. Oncogene 2013; 32: 3246-3253.
[19] De Nola R, Menga A, Castegna A, Loizzi V, Ranieri G, Cicinelli E and Cormio G. The crowded crosstalk between cancer cells and stromal microenvironment in gynecological malignancies: biological pathways and therapeutic implication. Int J Mol Sci 2019; 20: 2401.
[20] Chan RW, Schwab KE and Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod 2004; 70: 1738-1750.
[21] Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ and Tollenaar RA. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared

to lymph node status and tumor stage. Cell Oncol 2007; 29: 387-398.
[22] Rani P, Gupta AJ, Mehrol C, Singh M, Khurana N and Passey JC. Clinicopathological correlation of tumor-stroma ratio and inflammatory cell infiltrate with tumor grade and lymph node metastasis in squamous cell carcinoma of buccal mucosa and tongue in 41 cases with review of literature. J Cancer Res Ther 2020; 16: 445-451.
[23] Panayiotou H, Orsi NM, Thygesen HH, Wright AI, Winder M, Hutson R and Cummings M. The prognostic significance of tumour-stroma ratio in endometrial carcinoma. BMC Cancer 2015; 15: 955.
[24] Espinosa I, Catasus L, D' Angelo E, Mozos A, Pedrola N, Bértolo C, Ferrer I, Zannoni GF, West RB, van de Rijn M, Matias-Guiu X and Prat J. Stromal signatures in endometrioid endometrial carcinomas. Mod Pathol 2014; 27: 631-639.
[25] De P, Carlson JH, Jepperson T, Willis S, Leyland-Jones B and Dey N. RAC1 GTP-ase signals Wnt-beta-catenin pathway mediated integrin-directed metastasis-associated tumor cell phenotypes in triple negative breast cancers. Oncotarget 2017; 8: 3072-3103.
[26] Sahai E, Astsaturov I, Cukierman E, DeNardo DG, Egeblad M, Evans RM, Fearon D, Greten FR, Hingorani SR, Hunter T, Hynes RO, Jain RK, Janowitz T, Jorgensen C, Kimmelman AC, Kolonin MG, Maki RG, Powers RS, Puré E, Ramirez DC, Scherz-Shouval R, Sherman MH, Stewart S, Tlsty TD, Tuveson DA, Watt FM, Weaver V, Weeraratna AT and Werb Z. A framework for advancing our understanding of cancer-associated fibroblasts. Nat Rev Cancer 2020; 20: 174-186.
[27] Santolla MF, Vivacqua A, Lappano R, Rigiracciolo DC, Cirillo F, Galli GR, Talia M, Brunetti G, Miglietta AM, Belfiore A and Maggiolini M. GPER mediates a feedforward FGF2/FGFR1 paracrine activation coupling CAFs to cancer cells toward breast tumor progression. Cells 2019; 8: 223.
[28] Zhang Z, Karthaus WR, Lee YS, Gao VR, Wu C, Russo JW, Liu M, Mota JM, Abida W, Linton E, Lee E, Barnes SD, Chen HA, Mao N, Wongvipat J, Choi D, Chen X, Zhao H, Manova-Todorova K, de Stanchina E, Taplin ME, Balk SP, Rathkopf DE, Gopalan A, Carver BS, Mu P, Jiang X, Watson PA and Sawyers CL. Tumor microenvironment-derived NRG1 promotes antiandrogen resistance in prostate cancer. Cancer Cell 2020; 38: 279-296, e279.
[29] Berdiel-Acer M, Maia A, Hristova Z, Borgoni S, Vetter M, Burmester S, Becki C, Michels B, Abnaof K, Binenbaum I, Bethmann D, Chatziioannou A, Hasmann M, Thomssen C, Espinet E and Wiemann S. Stromal NRG1 in luminal breast cancer defines pro-fibrotic and migratory cancer-associated fibroblasts. Oncogene 2021; 40: 2651-2666.
[30] Han ME, Kim HJ, Shin DH, Hwang SH, Kang CD and Oh SO. Overexpression of NRG1 promotes progression of gastric cancer by regulating the self-renewal of cancer stem cells. J Gastroenterol 2015; 50: 645-656.
[31] Zhang R, Qi F, Shao S, Li G and Feng Y. Human colorectal cancer-derived carcinoma associated fibroblasts promote CD44-mediated adhesion of colorectal cancer cells to endothelial cells by secretion of HGF. Cancer Cell Int 2019; 19: 192.
[32] Gao Q, Yang Z, Xu S, Li X, Yang X, Jin P, Liu Y, Zhou X, Zhang T, Gong C, Wei X, Liu D, Sun C, Chen G, Hu J, Meng L, Zhou J, Sawada K, Fruscio R, Grunt TW, Wischhusen J, Vargas-Hernández VM, Pothuri B and Coleman RL. Heterotypic CAF-tumor spheroids promote early peritoneal metastatis of ovarian cancer. J Exp Med 2019; 216: 688-703.
[33] Fang Z, Meng Q, Xu J, Wang W, Zhang B, Liu J, Liang C, Hua J, Zhao Y, Yu X and Shi S. Signaling pathways in cancer-associated fibroblasts: recent advances and future perspectives. Cancer Commun (Lond) 2023; 43: 3-41.
[34] Subramaniam KS, Tham ST, Mohamed Z, Woo YL, Mat Adenan NA and Chung I. Cancer-associated fibroblasts promote proliferation of endometrial cancer cells. PLoS One 2013; 8: e68923.
[35] Teng F, Tian WY, Wang YM, Zhang YF, Guo F, Zhao J, Gao C and Xue FX. Cancer-associated fibroblasts promote the progression of endometrial cancer via the SDF-1/CXCR4 axis. J Hematol Oncol 2016; 9: 8.
[36] Rohwer N, Jumpertz S, Erdem M, Egners A, Warzecha KT, Fragoulis A, Kühl AA, Kramann R, Neuss S, Rudolph I, Endermann T, Zasada C, Apostolova I, Gerling M, Kempa S, Hughes R, Lewis CE, Brenner W, Malinowski MB, Stockmann M, Schomburg L, Faller W, Sansom OJ, Tacke F, Morkel M and Cramer T. Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis. Oncogene 2019; 38: 5670-5685.
[37] Cardone A, Tolino A, Zarcone R, Borruto Caracciolo G and Tartaglia E. Prognostic value of desmoplastic reaction and lymphocytic infiltration in the management of breast cancer. Panminerva Med 1997; 39: 174-177.
[38] Wölfl B, Te Rietmole H, Salvioli M, Kaznatcheev A, Thuijsman F, Brown JS, Burgering B and Staňková K. The contribution of evolutionary game theory to understanding and treating cancer. Dyn Games Appl 2022; 12: 313-342.
[39] Kaznatcheev A, Peacock J, Basanta D, Marusyk A and Scott JG. Fibroblasts and alectinib switch the evolutionary games played by non-small cell lung cancer. Nat Ecol Evol 2019; 3: 450-456.