Identification of Two Distinct Carcinoma-Associated Fibroblast Subtypes with Differential Tumor-Promoting Abilities in Oral Squamous Cell Carcinoma

Daniela Elena Costea1, Allison Hills2, Amani H. Osman1, Johanna Thurlow5, Gabriela Kalna6, Xiaohong Huang4, Claudia Pena Murillo4, Himalaya Parajuli1, Salwa Suliman1,2, Keerthi K. Kulasekara7, Anne Chr. Johannessen1,3, and Max Partridge4

Abstract
Heterogeneity of carcinoma-associated fibroblasts (CAF) has long been recognized, but the functional significance remains poorly understood. Here, we report the distinction of two CAF subtypes in oral squamous cell carcinoma (OSCC) that have differential tumor-promoting capability, one with a transcriptome and secretome closer to normal fibroblasts (CAF-N) and the other with a more divergent expression pattern (CAF-D). Both subtypes supported higher tumor incidence in nonobese diabetic/severe combined immunodeficient (NOD/SCID) Il2g2(null) mice and deeper invasion of malignant keratinocytes than normal or dysplasia-associated fibroblasts, but CAF-N was more efficient than CAF-D in enhancing tumor incidence. CAF-N included more intrinsically motile fibroblasts maintained by high autocrine production of hyaluronan. Inhibiting CAF-N migration by blocking hyaluronan synthesis or chain elongation impaired invasion of adjacent OSCC cells, pinpointing fibroblast motility as an essential mechanism in this process. In contrast, CAF-D harbored fewer motile fibroblasts but synthesized higher TGF-β levels. TGF-β1 did not stimulate CAF-D migration but enhanced invasion and expression of epithelial–mesenchymal transition (EMT) markers in malignant keratinocytes. Inhibiting TGF-β1 in three-dimensional cultures containing CAF-D impaired keratinocyte invasion, suggesting TGF-β1–induced EMT mediates CAF-D–induced carcinoma cell invasion. TGF-β1–pretreated normal fibroblasts also induced invasive properties in transformed oral keratinocytes, indicating that TGF-β1–synthesizing fibroblasts, as well as hyaluronan-synthesizing fibroblasts, are critical for carcinoma invasion. Taken together, these results discern two subtypes of CAF that promote OSCC cell invasion via different mechanisms. Cancer Res; 73(13); 3888–901. ©2013 AACR.

Introduction
Induction of invasion is the key property differentiating malignant from benign lesions. Similar to other carcinomas, oral squamous cell carcinoma (OSCC) is a multistep process in which the altered epithelium undergoes malignant conversion over a period of time (1). As carcinoma evolve, changes in the epithelia result also in concomitant adaptations of the adjacent normal stroma and one of its main cell type, the fibroblast, and this was shown to be important for tumor progression (2, 3). There is now convincing evidence that dysplasia-associated fibroblasts (DAF) and carcinoma-associated fibroblasts (CAF) differ from those associated with normal epithelium (4, 5), and that these adaptations have functional consequences for tumor progression and invasion (6, 7). Initially, these carcinoma-promoting effects were attributed mainly to the subpopulation of myofibroblasts in the mixed CAF populations that had higher ability to secrete stimulative paracrine factors (3, 8, 9).

However, CAF were shown to show phenotypic and genotypic diversity (4, 10, 11), as well as complex changes in their secretory activity (12–14), but the functional significance of this heterogeneity has been poorly addressed so far. Only recently, the essential role of this heterogeneity and the cooperation between different subpopulations was shown for prostate tumorigenesis, with the differential TGF-β signaling in tumor stromal cells indicated to be pivotal (15). TGF-β1 essential role in carcinogenesis, either as tumor suppressor at early stages and/or as tumor promoter at late stages has been long recognized (16). One of the processes through which TGF-β1 participates to carcinogenesis is the epithelial–mesenchymal transition (EMT) phenomenon, via increased carcinoma...
cell motility, invasiveness, and ultimately metastasis, and this phenomenon was shown to occur in OSCC as well (17).

It was the aim of this study to investigate stromal heterogeneity in OSCC at both molecular and functional levels, and to identify the mechanisms by which different CAF subsets support oral carcinoma development and invasion. We show here a differential global gene expression profile for normal oral fibroblasts (NOF), DAF, and CAF derived from OSCC, but the novelty is the observation that unsupervised clustering could identify two subgroups of CAF. Detailed analysis of their migratory and secretory characteristics presented here indicates that the 2 subtypes of CAF are able to induce oral carcinoma cell invasion by different mechanisms.

Materials and Methods

Clinical samples, cell isolation, and characterization

OSCCs, lesions with histologic evidence of dysplasia, and contralateral normal oral biopsies were collected following ethical approval and written informed consent. CAF (n = 18), DAF (n = 6), and NOF (n = 17) were isolated and forwarded for morphologic examination, 2-dimensional (2D) cultures, 3-dimensional (3D) constructs in collagen type I biomatrices, gene microarray, quantitative reverse transcription PCR (qRT-PCR), immunohistochemistry (IHC), and flow cytometry analysis (7, 18). For flow cytometry, subconfluent cells (10^6) were suspended in 100 μL PBS with 2% FBS and 1% HEPES (all Sigma), and incubated with 20 μL of fluorochrome-conjugated antibody fluorescein isothiocyanate (FITC) mouse anti-human epithelial-specific antigen (ESA; Biomedica), phycoerythrin (PE) mouse anti-human CD31, CD45, CD146, CD140b (known as platelet-derived growth factor receptor B—PDGFRB), FITC, or PE mouse immunoglobulin G 1 (IgG1)x—iso type control (all BD Pharmingen) for 15 minutes and analyzed using a MoFlo cell sorter (Beckman Coulter). For IHC, fixed cells and tissues were stained with lineage-specific antibodies recognizing vimentin, αSMA (smooth muscle actin), CD31, pancytokeratin (panCK), S100A4 (FSP; all DAKO; 1:50), and fibroblast-activating protein (FAP; Affinity Bioreagents; 1:5), and visualized with EnVision kit (DAKO), as previously described (19). For scanning electron microscopy (SEM), 2 × 10^6 fibroblasts were fixed in 2% glutaraldehyde/0.1 mol/L phosphate buffer pH 7.2, for 2 hours at 4°C, mounted on grids and viewed using a Jeol JSM-7400 field emission-SEM. For transmission electron microscopy (TEM), cells were postfixed in 1% osmium tetroxide (Sigma) in PBS (30 minutes), dehydrated using graded ethanols, embedded in epoxy resin, ultrathin sectioned, double stained with uranyl acetate and lead citrate (Sigma). Specimens were examined using a TEM (JEOL 1230; Jeol Ltd.), and the micrographs processed using an Arcus II scanner (Agfa-Gevaert N.V.). For detailed characterization, see Supplementary Figs. S1 and S2.

Gene expression profiling

Cells (2 × 10^6) at passage 2 were seeded in 3D collagen type I (BD Biosciences) biomatrices in duplicates for 5 days. RNA was extracted using RNA Stat (Biogenesis Ltd.) for analysis with U133 plus 2 arrays (Affymetrix Inc.). CEL file data were normalized using Robust Multichip Average expression summary (RMAexpress; http://rmaexpress.bmbolstad.com/). CEL files for samples and normalized data matrix are available from http://www.ncbi.nlm.nih.gov/geo/ (GSE38517). Rank product analysis (20) was conducted to identify differentially expressed genes between NOF (n = 5), DAF (n = 4), and CAF (n = 7). Gene Ontology analysis was conducted for the differentially expressed genes using DAVID 6.7 (21). Data were analyzed using Spectral Clustering, an unsupervised algorithm (22), for all strains maintained in 3D biomatrices and 2D monolayers, or for 3D alone only.

Tumor xenografting in NOD/SCID IL2γr(null) mice

To assess tumor formation, 10^5 transformed, nontumorigenic oral dysplastic keratinocytes (DOK cell line; ref. 23) obtained from European Collection of Cell Cultures were suspended in 50 μL of growth factor–reduced matrigel (BD Biosciences), and inoculated alone (n = 6) or together with 10^5 fibroblasts of the strains NF5 (n = 6), CAF1 (n = 6), or CAF5 (n = 6) subcutaneously on the back of 12-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) IL2γr(null) mice (The Jackson Laboratory). Tumor incidence and development (volume) was assessed at every 3 days. All mice were sacrificed 45 days after inoculation and tissues were harvested for histologic assessment. The Norwegian Animal Research Authority approved all animal procedures.

Tissue engineering and evaluation of carcinoma cell invasion.

Fibroblasts at passage 3 to 7 (split ratio 1:4) were embedded in collagen type I biomatrix (BD Biosciences), and seeded on top with malignant or transformed oral keratinocytes in triplicate, as previously described (7). The majority of experiments were carried out using Ca1 malignant oral keratinocyte cell line (24) that showed minimal invasion when seeded onto NOF-populated biomatrices. Nontumorigenic DOK cells and additional stains of malignant oral keratinocytes UK1, H357, 5PT, CalH3 (25), and SCC25 (26) were used to validate the findings. For some experiments, NOF (n = 3) were pretreated with 10 ng/ml TGF-β1 (BD Biosciences) for 10 days, then used to construct 3D biomatrices. 3D constructs were harvested, formalin-fixed, and paraffin-embedded. To measure depth of invasion, 3-μm sections were stained for panCK (DAKO; 1:50). Subsequently, each section was divided into fifths. The central and the two outer fifths were excluded from measurements, depth of invasion being assessed in the remaining two fifths only. For this, a horizontal line was drawn (using the software Olympus DP.Soft 5.0) through the uppermost remnants of the collagen gel to visualize the basement membrane zone; depth of invasion was determined every 100 μm along this horizontal line as the vertical distance from this line to the limit of invading epithelial cells (19).

Secretory profile

Eighteen-hour serum-free conditioned media was collected from cells maintained in 2D and 3D culture at similar passages and analyzed for levels of growth factors, cytokines, matrix metalloproteinases (MMP), and hyaluronan by ELISA with Luminex beads (R&D Systems, Inc.), the Widescreen Human
Cancer Panel 2 (Novagen, Millipore), the Flukorine MAP TGF-β Multiplex Kit (R&D Systems, Inc.), and hyaluronan ELISA (Corgenix). The results are presented with values normalized for 10^6 cells; data represent the mean ± SD. Deposition in patient tissue and 3D constructs of hyaluronan was visualized by histochemical staining in deparaffinized, rehydrated tissue sections stained with a 5% acidic solution of Alcian blue (Sigma) for 45 minutes and then counterstained with nuclear fast red (Sigma).

Protein detection

Protein lysates were resolved by PAGE and the membranes were probed with antibodies recognizing Smad2, pMLC (both Cell Signaling Technology Inc.; 1:1,000), Smad2/3 (R&D Systems, Inc.; 1:1,000), Hyaluronan-mediated motility receptor (RHAMM) (gift from V. Assmann, Center for Experimental Medicine, Institute of Tumor Biology, University Hospital Hamburg, Germany; 1:400), and gyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam; 1:1,000) as previously described (7). For validation of microarray data, additional Western blot analysis and IHC of monolayers and tissue sections was conducted with antibodies recognizing ITGA6, ITGA5 (all Cell Signaling Technology Inc.), GAPDH, DUSP4 (both Abcam), MAFP5, COL15A1 (all Sigma), PMEPA1, CADPS (both Abnova), TAGLN (Leica), INTA11 (gift from Prof. D. Gullberg, Department of Biomedicine, University of Bergen, Norway), RAB27B (gift from M.C. Seabra, Imperial College London, UK), and CH3L1 (gift from S. Werner, Institute of Molecular Health Sciences, Zurich, Switzerland), all 1:1,000.

Quantitative reverse transcription PCR

Cells were lysed with RNA-Stat-60 (AMS Biotechnology Europe Ltd.), total RNA was extracted following the manufacturer’s instructions, and cDNA synthesis was conducted using High-Capacity cDNA Archive Kit system (Applied Biosystems). qRT-PCR was then conducted using inventoried TaqMan assays with exon-spanning probes detecting MAFP5, CADPS, ASPN, TAGLN, TAMI, MEST, EVI1, CDKN2, EDIL3, SRGN, VIM, S100A4, TWIST1, HMIG2, FAPA, PDGFRB, COL1A2, DDR, THY1, and ACTB. Comparative 2^(-ΔΔCt) method was used to quantify the relative mRNA expression.

Inhibition of hyaluronan

For some experiments, 0.3 mmol/L 4-methylumbelliferone (4-MU; Sigma) was added to collagen matrix and media for 24 hours to block elongation of hyaluronan chains and motility of cells. Specific inhibition of hyaluronan synthesis was achieved by hyaluronan synthase HAS2 short hairpin RNA (shRNA) lentiviral particles transduction of CAFs 1 and 3. Fibroblasts were seeded at 10^5 cells per well in 6-well plates and after 24 hours were infected with HAS2 shRNA or control shRNA lentiviral particles (Santa Cruz Biotechnology) at a multiplicity of infection (MOI) of 100 in presence of polybrene (5 μg/mL; Santa Cruz Biotechnology), then centrifuged at 32°C for 2 hours at 2,300 rpm. After 48 hours, puromycin (2 μg/mL; Santa Cruz Biotechnology) was added for selection of transduced cells, and after additional 5 days, the cells were split 1:4 in puromycin-containing medium.

TGF-β1 inhibition

A potent and specific inhibitor of TGF-β1 superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7, the small molecule SB431542 (Sigma), was used at a concentration of 10 μmol/L, and the antibody against TGF-β1 (R&D Systems, Inc.) or the isotype control were used at a concentration of 10 μg/mL. Inhibitors were added 30 minutes before TGF-β1 treatment in monolayer. In 3D constructs, these reagents were added each second day, with the change of medium.

Motility assays

Cell migration assays were conducted as previously described (27) with and without prior exposure of cells to 10 ng/mL TGF-β1 for 18 hours. For cell invasion assay, Transwell plates were coated with 6 μg/mL Matrigel (Invitrogen). In some experiments 0.3 mmol/L 4-MU (Sigma) was added to collagen matrix and media to block elongation of hyaluronan for 24 hours prior the assessment. For time-lapse microscopy, 10^6 cells were seeded on 1.7 mg/mL collagen (Nutacon) for 24 hours and then treated with CellTracker Green CMFDA (Molecular Probes, Invitrogen). One hour before imaging, medium was changed to 1% FBS/1% HEPES. Images were taken every 10 minutes for 18 hours with a Zeiss LSM 510 META confocal microscope to determine average track speed, straightness, length, and displacement using Open Source imaging, Fiji and Imaris software (MeasurementPro and ImarisTrack; Bitplane).

Statistical analysis

All data are presented as mean ± SD. Mann–Whitney U-test (SPSS version 18) was used for analysis of ELISA data, ANOVA with a post hoc Bonferroni test for RhoA/Rac G-LISA data, paired Student t test for the comparison of invasion scores, and independent Student t test for analysis of population doublings data. To examine the biologic significance of the genes over-expressed in CAFs, we examined whether they correlated with outcome using our independent microarray database of 71 head and neck squamous cell carcinoma (22), with disease-specific death as the primary endpoint. Cox univariate and multivariate analysis was conducted using the R environment (http://www.r-project.org) and Survival bioconductor package (http://www.bioconductor.org).

Results

CAFs show increased expression of TGF-β target genes

Rank product analysis showed that 335 genes were significantly upregulated and 347 were downregulated [false discovery rate (FDR) ≤ 0.01] when CAF and NOF were compared (Supplementary Table S1). These genes were linked to substrate adhesion, tissue remodeling, cell migration, secretion, growth regulation, and angiogenesis (Table 1). Gene Ontology analysis confirmed that the overexpressed genes clustered into many functional groups (Supplementary Table S1). Notably, of the top 100 genes (the unique gene symbols), 52 were TGF-β targets (Fig. 1A; ref. 28). In addition, many transcripts of factors that bind to, or modulate the bioactivity and cellular response to TGF-β1, including ASPN (29), BGN (30), DCN (31), PMEPA1 (32), and CTHRC1 (33) were upregulated when CAF were compared with NOF (Table 1). Other upregulated genes were
### Table 1. Summary of the top overexpressed and underexpressed genes by fibroblasts derived from normal human oral mucosa, dysplastic lesions, and OSCC

<table>
<thead>
<tr>
<th>Function</th>
<th>Overexpressed in CAF vs. NOF</th>
<th>Underexpressed in CAF vs. NOF</th>
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<tbody>
<tr>
<td>ECM-receptor interaction/adhesion</td>
<td>MFAP5, COL15A, CHI3L1, COL11A, ASPN, FN1, SULF1, GPC6, HMCN1, MEGF10, CDH13, MGP, ITGB1, ITGA6, ITGA11, COL10A1, PLOD2, HAS2, TM4SF1, DG2, PCDH7, CHRHC1, ITGAV, LGALS1, SDC2</td>
<td>MAP7, ATRNL1, GPC3, ROB2, EGFL6, SPON1, THBS4, SEMA4D, F11R, ITGA8, MATN2, PCDH10, DPT, CILP, ITGA6, CDH18, DCN, EMLIN2, FLRT3, CXCL12</td>
</tr>
<tr>
<td>Tissue remodeling</td>
<td>TAGLN, TIA1, KBTBD10, CDH13, BGN, MET, TM4SF1, TPM1, ACTC, CTHRC1, SDC2</td>
<td>MMP27, GPC3, CORIN, CTSC, CTSH, SOD3, MMP12, MMP10</td>
</tr>
<tr>
<td>Migratory machinery</td>
<td>COL15A, MEST, PITX1, OMD, ASPN, CFI, NFIB, EVI12A, HNCN1, CH1, BGN, ADCY2, FOXL2, DPT, ALDH1A1, CPXM, DPT, IGFBP3, PLXDC1, TMEM132B, SLC6A15, KCND2, MA8B12L2, PPAPDC1A</td>
<td>RAB3B</td>
</tr>
<tr>
<td>RhoGTPase/actin cytoskeleton associated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secretion/endothelial</td>
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<tr>
<td>Endothelial cell associated</td>
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<tr>
<td>Transcription</td>
<td></td>
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<tr>
<td>Growth regulation</td>
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<tr>
<td>Inflammatory/immune response</td>
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<td>Transcription factors</td>
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**NOTE:** The genes differentially expressed in CAF when compared with NOF (FDR < 0.01) were linked to substrate adhesion, tissue remodeling, cell migration, secretion, growth regulation, and angiogenesis. TGF-β1 targets are shown in italics. Fewer changes were found when DAF and NOF were compared (127 genes significantly upregulated and 75 downregulated, FDR rate of <0.01). The most overexpressed functional group in DAF when compared with NOF were transcription factors.
known modulators of cellular secretion, such as \textit{CADPS} (34), \textit{RAB27B} (35), and \textit{SRGN} (8-, 9-, and 6-fold, respectively; ref. 36). Fewer changes were found when DAF and NOF were compared, with 127 genes significantly upregulated and 75 downregulated (FDR ≤ 0.01). Interestingly, many of the upregulated genes in DAF were transcription factors (Table 1). The prognostic significance of the top-ranked 50 probe sets and TGF-β1 targets identified here by rank product analysis as being...
upregulated in CAF versus NOF was investigated on our independent microarray database of 71 head and neck SCC. Univariate analysis showed that 20 of these probe sets were significantly associated with reduced disease-free survival (P < 0.05). These genes included PMEP1 (P = 0.025), BGN (P = 0.0058), and CADPS (P < 0.001). Kaplan–Meier analysis showed the quartiles of cases with the lowest expression of these genes had the best outcome (Fig. 1B–D). Significant changes in gene expression were validated by qRT-PCR, IHC, and Western blot analysis with additional strains of fibroblasts to those profiled on Affymetrix arrays maintained in 3D culture (Supplementary Fig. S3).

Gene expression profile identifies two subgroups of CAF derived from OSCC

Spectral clustering showed a clear separation of fibroblasts grown in 2D cultures from those maintained in 3D biomatrices (Fig. 1E), and for the cells in 3D biomatrices, NOF and DAF grouped at different positions from CAF, reflecting their distinct gene expression signatures (Fig. 1E). Further analysis using hierarchical clustering (Fig. 1F), and various clustering arrangements of 3D cultures alone, showed that CAF 1–4 tightly grouped together, indicating that these strains were transcriptionally more similar to each other than the rest of the CAF strains (CAF 5–7). CAF 1–4 often clustered closely to NOF, and for these reasons were termed CAF-N; whereas the more transcriptionally divergent CAF 5–7 were termed CAF-D. Of note, the NOF strain (NF1) closest to CAF strains in some clustering arrangements was from a juvenile, and the DAF strain (DAF3) that also clustered closest to CAF strains was isolated from a patient who later developed OSCC. Gene Ontology analysis revealed that differentially expressed genes between CAF-N and CAF-D clustered in the functional groups of cell migration and cell surface signal transduction (Supplementary Table S1.11 and S1.12) Some of the genes upregulated in CAF-N versus CAF-D belonged to cell migration (e.g., TBX1, KIT, ITGA4, CXCL12, and NR2F1) and the angiogenesis functional group (Fig. 1G), whereas some of the genes upregulated in CAF-D versus CAF-N (e.g., PAX3, NR2P2, and EDNRB) were associated with mesenchymal/nerve crest development and ameboidal cell movement (Fig. 1H).

The two CAF subgroups provide differential support for tumor formation and invasion

DOK cells did not develop tumors when xenotransplanted subcutaneously in NOD/SCID IL2rg(null) mice. Tumor incidence increased from 0 (0 of 6), when DOK were inoculated alone, to 66.66% (8 of 12) by coinoculating DOK with CAF. A significantly higher tumor incidence was observed for DOK coinoculated with CAF-N (83.33%, 5 of 6) when compared with DOK coinoculated with CAF-D (50%, 3 of 6; Fig. 2A). In addition, DOK/CAF-N tumors developed after a shorter lag time (median of 7 days) than DOK/CAF-D tumors (median of 14 days; Fig. 2A), and showed invasion into the musculature layer (Fig. 2B), in contrast to the well-circumscribed DOK/CAF-D tumors (Fig. 2C). Quantification of the fibroblast support for carcinoma cell invasion using in vitro 3D constructs showed that CAF-N were significantly more effective in supporting deeper invasion of carcinoma cells than CAF-D (P = 0.005; Fig. 2D), with a mix of small islands and single carcinoma cells invading the 3D biomatrix (Fig. 2E). Nevertheless, both subtypes of CAF supported significantly deeper invasion of oral carcinoma cells when compared with NOF embedded in 3D biomatrix (Ca1 cell line; Fig. 2D and E). This was confirmed using other cell lines (DOK, SCC25, CalH3, and UK1; Supplementary Fig. S4). An intermediate pattern of invasion was observed when DAF were incorporated into the 3D biomatrix (Supplementary Fig. S4).

The two CAF subgroups show a differential secretory profile

To identify growth factors and multifunctional cytokines that could be linked to the key differences in CAF behavior, we investigated their secretory profile (Supplementary Fig. S5). Multiplex ELISA assay of conditioned medium from fibroblasts maintained in 3D biomatrices showed that CAF synthesized significantly more TGF-β1 (P < 0.001), interleukin (IL)-1α (P < 0.001), acidic fibroblast growth factor (aFGF; P = 0.008), TNF-α (P = 0.014), and keratinocyte growth factor (KGF) (P = 0.04) than NOF, whereas more VEGF (P = 0.003) and hepatocyte growth factor (HGF; P = 0.007) was secreted by NOF (Supplementary Fig. S5B). CAF-N secreted significantly more KGF (P = 0.019), HGF (P = 0.002), aFGF (P = 0.008), and MMP3 (P = 0.016) than CAF-D (Fig. 4A), whereas CAF-D secreted 9 times more TGF-β1 (P < 0.001; Fig. 3A) and more IL-1α (P = 0.006) than CAF-N (Fig. 3A).

TGF-β1 increases invasion and expression of EMT markers in OSCC cells, whereas TGF-β1 inhibition by SB431542 impairs CAF-D–induced invasion of OSCC cells in 3D constructs

Provided that CAF-D stimulated significantly deeper carcinoma cell invasion when compared with NOF, the observation that TGF-β1 was the most obvious change in their secretory profile determined us to assess the TGF-β1 effect on carcinoma cell migration and invasion. Transwell assays showed significant increase of oral carcinoma cell migration and invasion after treatment with TGF-β1 for all cell lines tested (P = 0.012 and 0.002, respectively), varying from 2- to 3-folds (Fig. 3B and C). Although the amplitude and kinetics of the response to TGF-β1 treatment varied between the cell lines investigated, qRT-PCR showed an increased expression of EMT-related markers after 5 hours of exogenous TGF-β1 exposure (Fig. 3D). IHC showed significant increase in vimentin expression as long as 5 days after TGF-β1 treatment (Fig. 3E–G). Significant decrease in cell invasion was observed when 3D biomatrices populated with CAF-D were seeded with DOK and various other carcinoma cell lines on top (n = 3), and 24 hours later treated with SB431542 (Fig. 3F–I). Significant decrease in invasion was also observed for Ca1 and 5PT cell lines occurring after treatment with the anti-TGF-β1 Ab, but this effect was not statistically significant (Fig. 3F–I). Inhibition of activation of TGF-β1 downstream pathways after treatment with inhibitors 30 minutes
before TGF-β1 stimulation could be shown with SB431542 but not anti-TGF-β1-Ab (Fig. 3J).

The two CAF subgroups have distinct motility phenotypes

To determine other critical differences between the 2 CAF subtypes that might be linked to their differential ability to support tumor development and invasion, we investigated fibroblast motility. Transwell migratory experiments showed that CAF-N contained a significantly higher percentage of intrinsically migratory fibroblasts than CAF-D or NOF (Fig. 4A). The presence of this subpopulation of migratory fibroblasts predominantly in CAF-N was also indicated by increased expression of pSmad2 when CAF-N and NOF were compared (Fig. 4B). The significantly higher levels of RhoA-GTPase and Rac1/2/3-GTPase in CAF-N when compared with NOF (P = 0.0001; Supplementary Fig. S6) reflected also the higher proportion of migratory fibroblasts in CAF-N when compared with NOF. Furthermore, time-lapse microscopy showed that CAF-N moved faster and for longer distances from the initial point (longer track displacement) than NOF (Fig. 4C and D).
CAF-N subpopulation of intrinsically motile fibroblasts is dependent on hyaluronan and is essential for supporting carcinoma cell invasion

Differential intrinsic migratory activity has been linked to inherent differences in the production of hyaluronan; therefore hyaluronan was next investigated (37). Significantly higher levels of hyaluronan were found when comparing all available CAF strains (n = 9) with NOF (n = 3) maintained in 3D biomatrices (P = 0.008; Supplemental Fig. S4A), and the levels of secreted hyaluronan were significantly higher for
CAF-N than CAF-D ($P = 0.002$; Fig. 5A). HAS2 was also upregulated 3-fold in CAF-N when compared with CAF-D (Supplementary Table S1A). Histological visualization of hyaluronan in the tumor stroma from which CAF-N strains were isolated (Supplementary Fig. S7D), and a fine, discontinuous deposition of hyaluronan around cords of invasive cells in 3D constructs with Ca1 cells onto CAF3 (Supplementary Fig. S7E and S7F). IHC and Western blot analysis revealed that hyaluronan receptors RHAMM (Fig. 5B and C) and CD44 (data not shown) were expressed by CAF. Blocking hyaluronan chain elongation by addition of 4-MU resulted in a significant decrease in the proportion of intrinsically migratory fibroblasts in CAF-N (Fig. 5D), identifying hyaluronan as a key factor for the maintenance of this CAF-N migratory subpopulation. SEM of control and 4-MU–treated CAF-N revealed a reduction in the number of lamellipodia and filopodia (Fig. 5E). The functional importance of hyaluronan–dependent subpopulation of intrinsically migratory CAF-N for oral carcinoma cell invasion was proven by a significant reduction in the depth of invasion of oral carcinoma cells when CAF-N pretreated with 4-MU were incorporated into 3D constructs and seeded on top with Ca1 cells ($P = 0.001$; Fig. 5F and G). 4-MU is described as a selective inhibitor of nonsulfated GlcUA-containing glycosaminoglycans, and thus of hyaluronan production, but other effects on related glycosaminoglycans cannot be excluded. For this reason, we also specifically targeted hyaluronan synthesis with HAS2 shRNA lentiviral particles. qPCR showed a $84.50\%$ reduction in HAS2 mRNA in HAS2 shRNA-treated CAF3 compared with control shRNA-treated CAF3 (Supplementary Fig. S8), and this downregulation induced a statistically significant inhibition of invasion of carcinoma cells in 3D constructs ($P = 0.0067$; Fig. 5H and I).

**TGF-β1 activation of NOFs is sufficient to promote invasion of transformed oral keratinocytes**

Upregulation of TGF-β1 target genes in CAF prompted us to establish whether this had any functional relevance for CAF motility and subsequent oral carcinoma cell invasion. Transwell assays revealed that after exposure to 10 ng/mL TGF-β1, a significantly higher proportion of migratory fibroblasts were present in CAF-N and NOF than in CAF-D (Fig. 6A), indicating that CAF-D contained only low numbers of fibroblasts that migrated in response to exogenous TGF-β1. This was also indicated by the increased expression of Smad2 in TGF-β1–treated CAF-N and NOF (Fig. 6B), whereas pSmad2 was almost undetectable in CAF-D either before or after exposure to exogenous TGF-β1 (data not shown). Broadly, similar percentages of naïve fibroblasts that migrated in response to exogenous TGF-β1 were observed when CAF-N and NOF were compared (Fig. 6A). These data show that CAF-N include, in addition to the subpopulation of intrinsically motile fibroblasts, another subset of naïve fibroblasts that are able to become motile after stimulation with exogenous TGF-β1, and that these subtypes of motile fibroblasts are largely absent in CAF-D (Fig. 6C). The functional importance for the TGF-β1–mediated activation of fibroblasts for carcinoma cell invasion was tested in 3D models by pretreating NOF with 10 ng/mL TGF-β1 for 10 days and embedding afterward these activated fibroblasts into a 3D biomatrix seeded on top with DOK cells. Nonactivated, matched fibroblasts supported only a minimal DOK cell invasion (Fig. 6D), whereas TGF-β1–activated fibroblasts supported significantly deeper invasion in 3D
biomatrices (Fig. 6E). The pattern of invasion induced by TGF-β1–activated NOF showed predominantly cords of cells and single cells invading vertically in the biomatrix (Fig. 6F), similar to the pattern of carcinoma cell invasion induced by CAF-N (Fig. 6G).

Discussion

Two distinct CAF subtypes were identified in this study by transcriptome and secretome analysis: CAF-N with a gene expression pattern and secretory profile closer to NOF, and CAF-D with a divergent transcriptome and secreting very high levels of TGF-β1. The CAF-N subtype included a high percentage of intrinsically motile CAFs, and the number of migratory cells could be increased in response to TGF-β1, whereas the CAF-D subtype included few motile cells and the motility phenotype was largely unchanged in response to TGF-β1.

Functional effects of the distinct CAF subtypes were elucidated by implanting transformed, nontumorigenic oral keratinocytes together with CAF into NOD/SCID IL2rg(null) mice. CAF-N significantly increased tumor incidence and shortened the lag time for tumor formation as compared with CAF-D. CAF-N also supported deeper oral carcinoma cell invasion than CAF-D in 3D models, with areas of noncohesive invasion as well as vertical proliferation of cords of carcinoma cells, a pattern of invasion previously correlated with poor prognosis (37). The observation that CAF-N supported the best tumor formation and invasion indicates the pivotal role of CAF migration for oral carcinoma cell invasion, in line with previous...
However, this was previously linked to the requirement for expression of the integrins A3 and A5. These integrins were not upregulated in the present study (Supplementary Table S1 and Supplementary Fig. S3), suggesting that alternative integrins, including A6, A10, and A11 that were upregulated in the present study (Supplementary Table S1 and Supplementary Fig. S3) and other reports (39, 40) may substitute for A3 and A5.

Figure 6. A, Transwell migration assay showing CAF distinct response to TGF-β1: CAF-N showed increase of the subpopulation of migratory cells after exposure to TGF-β1, whereas CAF-D were refractory to exposure to TGF-β1. B, Western blot analysis showing increased levels of pSmad2 after 1 hour TGF-β1 exposure of CAF-N and NOF. Skin fibroblasts (SF) were used as control for activation of TGF-β1 downstream pathway. C, the distribution of different cell phenotypes contributing to the heterogeneity of each fibroblast strain analyzed: (i) intrinsically motile cells; (ii) TGF-β1-responsive cells; and (iii) stationary/low-migratory and high-secretory cells. CAF-N and NOF contained significantly higher percentages of intrinsically and TGF-β1–induced motile cells when compared with CAF-D (P < 0.05). CAF-D contained a significantly high percentage of low-motile, highly secretory cells (P < 0.01). D and E, hematoxylin and eosin staining of representative 3D constructs with DOK cells seeded onto control NOF (D) or matched NOF pretreated with TGF-β1 (E), showing that TGF-β1–activated NOF supported a deeper invasion (magnification, ×200; scale bar, 200 mm). F, closer view (magnification, ×400; scale bar, 50 mm) showing invasion of DOK when seeded onto NOF pretreated with TGF-β1, with small islands, single cells, and cords of cells growing deep into the matrix (noncohesive pattern of invasion). G, closer view (magnification, ×400; scale bar, 50 mm) showing invasion of DOK when seeded onto CAF-N with a noncohesive, vertical pattern of invasion, similar to the invasion pattern seen when DOK were seeded onto NOF pretreated with TGF-β1.
heterogeneity of each fibroblast strain: (i) intrinsically motile cells; (ii) TGF-β1-responsive cells; and (iii) stationary/low-migratory cells (Fig. 6C). It has been previously shown that migratory cells are not active synthesizers (41), therefore it is most likely that the third subpopulation of stationary/low migratory fibroblasts is responsible for synthesis of various growth factors, cytokines, MMPs, and extracellular matrix (ECM) components including hyaluronan. As depicted in Fig. 6C, all fibroblast strains were heterogeneous, but presence of these subtypes in various proportions, and their cooperative effects was found to be essential for the functional differences observed between NOF, CAF-N, and CAF-D. CAF-N were most efficient at maximizing the potential contact with transformed keratinocytes due to the high proportion of intrinsically migratory fibroblasts that were able to create a network of tracks used by oral transformed cells for invasion, as previously suggested (38). The maintenance of this subpopulation and its effects on carcinoma cell invasion was dependent on hyaluronan secretion. The CAF-N strain that secreted the highest levels of hyaluronan (CAF3) supported the deepest invasion of malignant oral keratinocytes, pinpointing the notion that cooperation between different subpopulations of CAF (e.g., motile cells and hyaluronan-synthesizers) results in increased ability to promote carcinoma invasion. The broad similarity between CAF-N, which supported the highest rate of tumor formation and the deepest invasion, and NOF was unexpected but is consistent with the notion that naïve fibroblasts undergo adaptations in response to signals in the milieu (2).

The microarray analysis conducted in this study indicated the pivotal role of TGF-β1 in the stromal adaptations that occur as oral carcinoma evolves, as we found that approximately 50% (52 of 100) of the top genes upregulated in CAF compared with NOF were TGF-β1 targets. The importance of TGF-β1 for CAF activation was confirmed by demonstration of significant deeper invasion occurring when TGF-β1-pretreated NOF were embedded in 3D constructs and seeded on top with transformed oral keratinocytes, as compared with matched controls. Most likely, inflammatory cells that are present in vivo in the tumor stroma provide TGF-β1 that initiates this process (42), but over time, the change in the transcriptome, with increased expression of many TGF-β1 target genes, leads to emergence of the nonmotile (41), high TGF-β1–secreting fibroblast subtype (14). By correlating Transwell motility assay with secretome analysis, we found this subset of high TGF-β1 secretors to be present at significantly higher proportions in CAF-D strains. This finding might indicate that CAF-N and CAF-D are actually 2 different stages of CAF in the OSCC progression, with CAF-N representing an earlier stromal change than CAF-D. This is also suggested by the findings that CAF-N are closer to NOF and DAF in many aspects, and their impact on carcinoma cell invasion is more dramatic, as it might be necessary for inducing invasion at early, but not late stages of carcinogenesis. It has been previously shown that increased HGF synthesis suppresses TGF-β1 secretion, and that changes in the balance between HGF and TGF-β1 synthesis by fibroblasts might be decisive in pathogenesis of other diseases (43). Such switch might also occur with OSCC progression, as indicated by our results here. The high secretion of HGF might also maintain low the synthesis of TGF-β1 in NOF; it lowers progressively in CAF-N, and switches completely to low HGF and high TGF-β1 secretion in CAF-D (Fig. 3A). The mechanism responsible for this shift is currently unknown, as is the mechanism switching from TGF-β1–responsive to TGF-β1–refractory CAF. A possible explanation might be loss of TGFBR2 by CAF as previously suggested (44), or defects in Smad signaling as found for malignant keratinocytes (45).

Increased expression of genes that bind to, or ameliorate the response to TGF-β1, including BGN and ASPN (31, 46), or PAMEPA1 and CTHRC1 that interfere with phosphorylation of Smads 2/3 (32, 33, 47) may also maintain the distinct CAF subpopulations. Nevertheless, secretion of high levels of TGF-β1 not only induces NOF to evolve into CAF; it can also stimulate invasion of malignant keratinocytes directly through EMT (48). In line with this, our results suggest that CAF-D, being high TGF-β1 secretors, are able to stimulate EMT and oral cancer cell invasion via TGF-β1 (Fig. 3). However, this high TGF-β1–secreting CAF phenotype (CAF-D) did not support deep invasion in our experiments. Migration of keratinocytes deep into stroma probably requires additional mechanisms such as a subpopulation of highly motile fibroblasts, and a subpopulation of cells that also secrete hyaluronan, MMPs, or growth factors that support proliferation (KGF and HGF). In vivo, these other subpopulations of cells might be continuously renewed from bone marrow or adjacent normal connective tissue, but whether or not the fibroblast subtypes identified in this study share a common lineage is presently unknown. Highly motile fibroblasts are also known to be present in wounds (49), thus it will be instructive to tease out differences between wound-associated fibroblasts and CAF to establish whether they are broadly similar, or whether CAF have unique characteristics. Most likely, it is the persistence of the transcriptome changes in fibroblasts in the developing tumor milieu that drives the altered balance in the subpopulations identified in the present study. Our experiments indicate that such changes are of a dynamic nature, and the reduced life span of CAF (Supplementary Fig. S1) may indicate that in vivo, the fibroblasts with a changed transcriptome are eliminated and replaced continuously. Nevertheless, these adaptations support tumor formation and have prognostic significance, and we provide here for the first time evidence that CAF subtypes have functional relevance via different mechanisms to support tumor formation and invasion.

 Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.E. Costea, A.H. Osman, J. Thurlow, H. Parajuli, S. Suliman, A.C. Johannesen, M. Partridge
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.E. Costea, A.H. Osman, C.P. Murillo, H. Parajuli, S. Suliman, K.K. Keerthi, M. Partridge
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.E. Costea, A.H. Osman, J. Thurlow, G. Kalna, X. Huang, C.P. Murillo, H. Parajuli, S. Suliman, M. Partridge
Writing, review, and/or revision of the manuscript: D.E. Costea, A.C. Johannessen, M. Partridge

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.E. Costea, A. Hille, J. Thurlow, X. Huang, K.K. Keerthi, A.C. Johannessen, M. Partridge

Study supervision: D.E. Costea, A.C. Johannessen, M. Partridge

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Daniela Elena Costea, Allison Hills, Amani H. Osman, et al.


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