Subtypes of oral carcinoma-associated fibroblasts

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 Thurlow³, Gabriella Kalna⁴, Xiaohong Huang², Claudia Pena Murillo², Himalaya Parajuli¹, Salwa Suliman¹,⁵, Kulasekara K Keerthi¹, Anne Chr. Johannessen¹,⁶, and Max Partridge².

¹Section for Pathology, The Gade Institute, University of Bergen, Bergen, Norway.
²Head and Neck Unit, Guy’s and St. Thomas’ Hospitals NHS Foundation Trust, London, UK.
³Center for Stem Cell Biology, Department of Biomedical Science, University of Sheffield, Sheffield, UK.
⁴Beatson Institute, Glasgow, UK.
⁵Institute of Clinical Dentistry, University of Bergen, Bergen, Norway.
⁶Department of Pathology, Haukeland University Hospital, Bergen, Norway.

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Address for correspondence: Daniela Elena Costea, Section for Pathology, The Gade Institute, Haukeland University Hospital, 5021, Bergen, Norway. Tel: +47 5597 2564; Fax: +47 5597 3158. E-mail: daniela.costea@gades.uib.no

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**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 NOD/SCID Il2γ2(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 (HA). Inhibiting CAF-N migration by blocking HA 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-β1 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 3D 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 HA-synthesizing fibroblasts, are critical for carcinoma invasion. Taken together, these results discern two subtypes of CAF that promote OSCC cell invasion via different mechanisms.
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Introduction

Induction of invasion is the key property differentiating malignant from benign lesions. Similar to other carcinomas, oral squamous cell carcinoma (OSCC) is a multi-step 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 sub-population of myofibroblasts in the mixed CAF populations that had higher ability to secrete stimulative paracrine factors (3, 8, 9).

However, CAF were demonstrated 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 demonstrated 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 phenomenon (EMT), 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 identify the mechanisms by which different CAF subsets support oral
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carcinoma development and invasion. We show here a differential global gene expression profile for normal oral fibroblasts (NF), 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 two subtypes of CAF are able to induce oral carcinoma cell invasion by different mechanisms.


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Materials and Methods

Clinical samples, cell isolation and characterization

OSCCs, lesions with histological evidence of dysplasia and contra lateral normal oral biopsies were collected following ethical approval and written informed consent. CAF (n=18), DAF (n=6), and NF (n=17) were isolated and forwarded for morphological examination, 2D cultures, 3D constructs in collagen type I biomatrices, gene microarray, qRT-PCR, immunohistochemistry (IHC) and flow cytometry analysis (7, 18). For flow cytometry, subconfluent cells (10^5) were suspended in 100μl PBS with 2% FBS and 1% HEPES (all Sigma, St. Louis, MO), and incubated with 20μl of fluorochrome-conjugated antibody FITC mouse anti-human ESA (epithelial specific antigen, Biomedica, Foster City, CA), PE mouse anti-human CD31, CD45, CD146, CD140b (known as platelet-derived growth factor receptor B - PDGFRB), FITC or PE mouse IgG1 κ - isotype control (all BD Pharmingen, San Jose, CA) for 15 min and analyzed using a MoFlo cell sorter (Beckman Coulter, Brea, CA). For IHC, fixed cells and tissues were stained with lineage specific antibodies recognizing vimentin, aSMA (smooth muscle actin), CD31, pancytokeratin (panCK), S100A4 (FSP) (all DAKO, Golstrup, DK; 1: 50), and FAP (fibroblast activating protein, Affinity Bioreagents, Rockford, IL; 1:5), and visualized with EnVision kit (DAKO), as previously described (19). For scanning electron microscopy (SEM) 2x10^4 fibroblasts were fixed in 2% glutaraldehyde/0.1 M phosphate buffer pH = 7.2, for 2 hours at 4°C, mounted on grids and viewed using a Jeol JSM-7400 field emission-scanning electron microscope. For transmission electron microscopy (TEM), cells were post-fixed in 1% osmium tetroxide (Sigma) in PBS (30 min), dehydrated using graded ethanol, embedded in epoxy resin, ultra-thin sectioned, double stained with uranyl acetate and lead citrate (Sigma). Specimens were examined using a transmission electron microscope (JEOL 1230, Jeol Ltd, Tokyo, Japan), and the micrographs processed using an Arcus II scanner (Agfa-Gevaert N.V, Mortsel, Belgium). For detailed characterization, see supplementary Figs. S1-S2.

Gene expression profiling
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Cells (2x10^6) at passage 2 were seeded in 3D collagen type I (BD Biosciences, San Jose, CA) biomatrices in duplicates for 5 days. RNA was extracted using RNA Stat (Biogenesis Ltd, Pool, UK) for analysis with U133 plus 2 arrays (Affymetrix Inc., Santa Clara, CA). CEL file data was 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 (RP) analysis (20) was performed to identify differentially expressed genes between NF (n=5), DAF (n=4), and CAF (n=7). Gene ontology analysis was performed for the differentially expressed genes using DAVID 6.7 (21). Data was 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 IL2rγ(null) mice**

To assess tumor formation, 10^3 transformed, non-tumorigenic oral dysplasic keratinocytes (DOK cell line) (23) obtained from European Collection of Cell Cultures (Salisbury, Wiltshire, UK) were suspended in 50μl 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 weeks old NOD/SCID IL2rγ(null) mice (Jackson laboratories, US). Tumor incidence and development (volume) was assessed at every 3 days. All mice were sacrificed 45 days after inoculation and tissues were harvested for histological assessment. The Norwegian Animal Research Authority approved all animal procedures.

**Tissue engineering and evaluation of carcinoma cell invasion.**

Fibroblasts at passage 3-7 (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 performed using Ca1 malignant oral keratinocyte cell line (24) that showed minimal invasion when seeded onto NF-populated biomatrices. Non-tumorigenic 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, NF (n=3) were pretreated with 10ng/ml TGF-β1 (BD Biosciences) for 10 days, then
Subtypes of oral carcinoma-associated fibroblasts used to construct 3D biomatrices. 3D constructs were harvested, formalin fixed and paraffin embedded. To measure depth of invasion, 3µm sections were stained for pancytokeratin (DAKO; 1:50). Subsequently, each section was divided into 5ths. The central and the two outer 5ths were excluded from measurements, depth of invasion being assessed in the remaining two 5ths 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 100µm 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 (MMPs), and hyaluronan (HA) by ELISA with Luminex beads (R&D Systems, Inc, Minneapolis, MN), the Widescreen Human Cancer Panel 2 (Novagen, Millipore, US), the Flurokine MAP TGF-β multiplex kit (R&D Systems), and HA ELISA (Corgenix, Broomfiel, CO). The results are presented with values normalized for 10^6 cells; data represent the mean +/- SD.

Deposition in patient tissue and 3D constructs of HA was visualized by histochemical staining in deparaffinised, rehydrated tissue sections stained with a 5% acidic solution of alcian blue (Sigma) for 45 min 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 Signalling Technology Inc., Beverly, MA; 1:1 000), Smad2/3 (R&D Systems; 1:1000), RHAMM (gift from V Assmann; 1:400), and GAPDH (Abcam, Cambridge, UK; 1: 1 000) as previously described (7). For validation of microarray data, additional western blot and IHC of monolayers and tissue sections was performed with antibodies recognizing ITGA6, ITGA5 (all Cell Signalling Technology Inc., Beverly, MA), GAPDH, DUSP4 (both Abcam, Cambridge, UK), MFAP5, COL15A1 (all Sigma), PMEPA1, CADPS (both Abnova,
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Aachen, Germany), TAGLN (Leica, Espoo, Finland), INTA11 (gift from prof. D. Gulberg), RAB27B, gift from MC Seabra) and CHI3L1 (gift from S. Werner), all 1:1000.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cells were lysed with RNA-Stat-60 (AMS Biotechnology Europe Ltd, UK), total RNA was extracted following the manufacturer's instructions, and cDNA synthesis was performed using High-Capacity cDNA Archive Kit system (Applied Biosystems, US). qRT-PCR was then performed using inventoried Taqman assays with exon-spanning probes detecting MFAP5, CADPS, ASPN, TAGLN, TIAMI, MEST, EVII, CDKNIC, EDIL3, SRGN, VIM, S100A4, TWST1, HMGA2, FAPA, PDGFRB, COL1A2, DDR, THY1, and ACTB. Comparative $2^{-\Delta\Delta C_t}$ method was used to quantify the relative mRNA expression.

Inhibition of HA

For some experiments, 0.3 mM 4-methylumbelliferone (4-MU, Sigma) was added to collagen matrix and media for 24 hours in order to block elongation of HA chains and motility of cells. Specific inhibition of HA synthesis was achieved by hyaluronan synthase HAS2 shRNA lentiviral particles transduction of CAFs 1 and 3. Fibroblasts were seeded at $10^5$ cells/well in 6 well plates and after 24 hours were infected with HAS2 shRNA or control shRNA lentiviral particles (Santa Cruz) at a multiplicity of infection (MOI) of 100 in presence of Polybrene (5µg/ml, Santa Cruz), then centrifuged at 32°C for 2 hours at 2300rpm. After 48 hours Puromycin (2µg/ml, Santa Cruz) 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 10µmol/L, and the antibody against TGF-β1 (R&D Systems) or the isotype control were used at a concentration of 10 µg/ml. Inhibitors were added 30 min before TGF-β1 treatment in monolayer. In 3D constructs these reagents were added each second day, with the change of medium.
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Motility assays

Cell migration assays were performed as previously described (27) with and without prior exposure of cells to 10 ng/ml TGF-β1 for 18 hours. For cell invasion assay, transwells were coated with 6 µg/µl matrigel (InVitrogen, US). In some experiments 0.3 mM 4-MU (Sigma) was added to collagen matrix and media to block elongation of HA for 24 hours prior the assessment. For time-lapse microscopy, 10^5 cells were seeded on 1.7 mg/ml collagen (Nutacon, Leimuiden, Netherlands) for 24 hours and then treated with CellTracker™ Green CMFDA (Molecular probes, In Vitrogen). 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).

Statistics

All data are presented as means ± SD. Mann–Whitney U test (SPSS version 18) was used for analysis of ELISA data, ANOVA with a posthoc Bonferroni test for RhoA/Rac G-LISA data, paired Student’s t-test for the comparison of invasion scores, and independent Student’s t-test for analysis of population doublings data. To examine the biological 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 performed using the R environment (http://www.r-project.org) and Survival bioconductor package (http://www.bioconductor.org).
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Results

CAF show increased expression of TGF-β target genes.

Rank product analysis demonstrated that 335 genes were significantly up-regulated and 347 were down-regulated (FD rate of ≤0.01) when CAF and NF were compared (Supplementary Table S1). These genes were linked to substrate adhesion, tissue remodelling, cell migration, secretion, growth regulation and angiogenesis (Table 1). Gene ontology analysis confirmed that the over expressed genes clustered into many functional groups (Supplementary Table S1). Notably, of the top 100 genes (the unique gene symbols), 52 were TGF-β targets (28) (Fig. 1A). 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 up-regulated when CAF were compared to NF (Table 1). Other up-regulated genes were known modulators of cellular secretion, like CADPS (34), RAB27B (35), SRGN (36) (8, 9, and 6-fold respectively). Fewer changes were found when DAF and NF were compared, with 127 genes significantly up-regulated and 75 down-regulated (FD rate of ≤0.01). Interestingly, many of the up-regulated 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 RP analysis as being up-regulated in CAF versus NF 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 PMEPA1 (P=0.025), BGN (P=0.0058), and CADPS (P<0.001). Kaplan-Meier analysis showed the quartile 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, immunohistochemistry and western 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, NF and DAF grouped at different positions from CAF, reflecting their distinct gene expression signatures (Fig. 1E).
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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 NF, and for these reasons were termed CAF-N; whilst the more transcriptionally divergent CAF 5-7 were termed CAF-D. Of note, the NF 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 S1.11-12) Some of the genes up-regulated in CAF-N versus CAF-D belonged to cell migration (e.g. TBX1, KIT, ITGA4, CXCL12, NR2F1) and the angiogenesis functional group (Fig. 1G), while some of the genes up-regulated in CAF-D versus CAF-N (e.g. PAX3, NRP2, EDNRB) were associated with mesenchymal/neural crest development and amoeboidal 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 (0/6), when DOK were inoculated alone, to 66.66% (8/12) by co-inoculating DOK with CAF. A significantly higher tumor incidence was observed for DOK co-inoculated with CAF-N (83.33%, 5/6) when compared with DOK co-inoculated with CAF-D (50%, 3/6) (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 to NF embedded in
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3D biomatrix (Ca1 cell line, Fig. 2D,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), IL-1α (P<0.001), aFGF (P=0.008), TNFα (P=0.014) and KGF (P=0.04) than NF, whereas more VEGF (P=0.003) and HGF (P=0.007) was secreted by NF (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, while 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 to NF, 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 p=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 5h of exogenous TGF-β1 exposure (Fig. 3D). Immunohistochemistry 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
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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 min prior to TGF-β1 stimulation could be demonstrated 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 two 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 NF (Fig 4A). The presence of this subpopulation of migratory fibroblasts predominantly in CAF-N was also indicated by increased expression of pMLC and pSmad2 when CAF-N and NF were compared (Fig. 4B). The significantly higher levels of RhoA-GTPase and Rac1/2/3-GTPase in CAF-N when compared with NF (P= 0.0001, Supplementary Fig. S6) reflected also the higher proportion of migratory fibroblasts in CAF-N when compared to NF. Furthermore, time lapse microscopy showed that CAF-N moved faster and for longer distances from the initial point (longer track displacement) than NF (Fig. 4C-D).

CAF-N subpopulation of intrinsically motile fibroblasts is dependent on HA and is essential for supporting carcinoma cell invasion.

Differential intrinsic migratory activity has been linked to inherent differences in the production of HA; therefor HA was next investigated (37). Significantly higher levels of HA were found when comparing all available CAF strains (n=9) with NF (n=3) maintained in 3D biomatrices (P=0.008, Supplemental Fig. S4A), and the levels of secreted HA were significantly higher for CAF-N than CAF-D (P= 0.002, Fig. 5A). HAS2 was also up-regulated 3 fold in CAF-N when compared to CAF-D (Supplementary Table S1.4). Histochemical visualization of HA by alcian blue staining showed abundant HA in the tumor stroma from which CAF-N strains were isolated (Supplementary Fig. S7D), and a fine, discontinuous deposition of HA around cords of invasive cells in 3D constructs.
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with Ca1 cells onto CAF3 (Supplementary Fig. S7E, F). Immunohistochemistry and western analysis revealed that HA receptors RHAMM (Fig. 5B-C) and CD44 (data not shown) were expressed by CAF. Blocking HA 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 HA 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 HA-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-G). 4-MU is described as a selective inhibitor of non-sulphated GlcUA-containing glycosaminoglycans, and thus of HA production, but other effects on related glycosaminoglycans cannot be excluded. For this reason, we also specifically targeted HA 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-I).

TGF-β1 activation of NFs is sufficient to promote invasion of transformed oral keratinocytes.

Up-regulation 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 10ng/ml TGF-β1, a significantly higher proportion of migratory fibroblasts were present in CAF-N and NF 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 pMLC and pSmad2 in TGF-β1-treated CAF-N and NF (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 NF were compared (Fig. 6A). These data show that CAF-N include, in addition to the subpopulation of
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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-migratory activation of fibroblasts for carcinoma cell invasion was tested in 3D models by pre-treating NF with 10ng/ml TGF-β1 for 10 days and embedding afterwards these activated fibroblasts into a 3D biomatrix seeded on top with DOK cells. Non-activated, matched fibroblasts supported only a minimal DOK cell invasion (Fig. 6D), while TGF-β1–activated fibroblasts supported significantly deeper invasion in 3D biomatrices (Fig. 6E). The pattern of invasion induced by TGF-β1–activated NF 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).
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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 NF, 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, non-tumorigenic oral keratinocytes together with CAF into NOD/SCID IL2rγ(null) mice. CAF-N significantly increased tumor incidence and shortened the lag time for tumor formation as compared to CAF-D. CAF-N also supported deeper oral carcinoma cell invasion than CAF-D in 3D models, with areas of non-cohesive 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 publications (38). However, this was previously linked to the requirement for expression of the integrins A3 and A5. These integrins were not up-regulated in the present study (Supplementary Table 1 and Fig. S3), suggesting that alternative integrins, including A6, A10 and A11 that were up-regulated in the present study (Supplementary Table 1 and Fig. S3) and other reports (39, 40) may substitute for A3 and A5.

Analysis of data from migration assays identified at least three different phenotypes of fibroblasts contributing to the heterogeneity of each fibroblast strain: 1) intrinsically motile cells; 2) TGF-β1-responsive cells; and 3) stationary / low-migratory cells (Fig. 6C). It has been previously demonstrated 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
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growth factors, cytokines, MMPs and ECM components including HA. As depicted in Fig. 6C, all
fibroblast strains were heterogenous, but presence of these subtypes in various proportions, and
their co-operative effects was found to be essential for the functional differences observed between
NF, 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 HA secretion. The CAF-N strain that secreted the highest levels of HA (CAF3)
supported the deepest invasion of malignant oral keratinocytes, pinpointing the notion that co-
operation between different subpopulations of CAF (e.g. motile cells and HA-synthesizers) results
in increased ability to promote carcinoma invasion. The broad similarity between CAF-N, that
supported the highest rate of tumor formation and the deepest invasion, and NF was unexpected, but
is consistent with the notion that naïve fibroblasts undergo adaptations in response to signals in the
milieu (2).

The microarray analysis performed in this study indicated the pivotal role of TGF-β in the stromal
adaptations that occur as oral carcinoma evolves, since we found that approximately 50% (52 out of
100) of the top genes up-regulated in CAF compared to NF were TGF-β targets. The importance of
TGF-β1 for CAF activation was confirmed by demonstration of significant deeper invasion
occurring when TGF-β1-pretreated NF 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 non-motile (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 two different stages of CAF in the OSCC progression,
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with CAF-N representing an earlier stromal change than CAF-D. This is also suggested by the findings that CAF-N are closer to NF 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 the low the synthesis of TGF-β1 in NF; 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. 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 PMEPA1 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 NF 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 secrete also HA, 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
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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.
**Figure legends**

**Fig 1** A). Heat map showing differentially expressed transcripts of TGF-β target genes between NF, and CAF. Expression values are log2, mean centered (red - higher expression, green - lower expression). B). Kaplan-Meier analysis of 71 head and neck SCC cases for PMEPA1 (probe set 222450_at). The black line shows the quartile with the lowest PMEPA1 expression (0-0.25; 4 events /18 cases); the red line the other quartiles (0.25-1; 23 events/ 53 cases). C). Kaplan-Meier analysis of 71 HNSCC cases for BGN (201261_x_at). The black line shows the quartile with lowest BGN expression (0-0.25; 1 events /18 cases); the red line the other quartiles (0.25-1; 26 events/ 53 cases). D). Kaplan-Meier analysis of 71 head and neck SCC cases for CADPS (1568603_at). The black line shows the quartiles with lowest CADPS expression (0-0.75; 15 events/ 54 cases); the red line shows the quartile with highest CADPS expression (0.75-1; 12 events /17 cases). E). Spectral clustering showing distinct grouping of fibroblasts grown in 2D and 3D, and for those maintained in 3D separate grouping of NF, DAF and CAF. CAF clustered into two subgroups: one with a transcriptome closer to NF (CAF 1-4, termed CAF-N), the other with a more divergent expression profile (CAF 5-7, termed CAF-D). F). Hierarchical clustering showing the tight grouping of the more homogenous group of CAF-N (closest to DAF and NF) when compared to the more heterogenous and divergent group of CAF-D. G). Heat map showing differentially expressed probe sets between CAF-N and CAF-D of genes enriching GO BP 5, GO:0016477~cell migration. Expression values are log2, mean centered (red - higher expression, green - lower expression). H). Heat map showing differentially expressed probe sets between CAF-D and CAF-N of genes enriching GO:0014032~neural crest cell development and GO BP 5, GO:0016477~cell migration. Expression values are log2, mean centered (red - higher expression, green - lower expression).

**Fig 2.** A). Graph showing incidence and lag time of tumors formed by DOK cells co-inoculated subcutaneously with CAF-N (CAF1) or CAF-D (CAF5) in NOD/SCID/Iγ2(null) mice. B). Microphotograph showing the tumor front of invasion into the muscular layer of a tumor developed after co-inoculation of DOK and CAF1; hematoxylin-eosin (H&E) staining. C) Microphotograph
Subtypes of oral carcinoma-associated fibroblasts
showing a well demarcated tumor developed after co-injection of DOK and CAF5 (H&E staining, 200x magnification, scale bar 200μm). D) Graph showing that the depth of invasion of malignant oral keratinocytes (Ca1) was significantly higher when CAF-Ns as opposed to CAF-D and NF, were embedded into the biomatrix. Significant differences (P=0.001) are marked by a star. E). Upper panel shows representative H&E stained sections of 3D constructs with malignant oral keratinocytes (Ca1) seeded onto CAF-N, CAF-D, or NF. The lower panel shows pancytokeratin (panCK) staining of serial sections. 200x magnification. Scale bar 200μm.

Fig. 3. A). Graph showing significant differences in levels of secretion of various growth factors, cytokines and MMPs determined for NF, CAF-N, and CAF-D maintained in 3D biomatrices. CAF-N secreted higher levels of KGF and HGF than CAF-D, whereas more TGF-β1 was secreted by CAF-D, P<0.05. B). Graph showing cell migration in a transwell assay for OSCC-derived cell lines and DOK cells. Cells treated with 1ng/ml TGF-β1 for 18 h migrated significantly more than the control group. C). Graph showing cell invasion in a matrigel transwell assay for the same cell lines. TGF-β1 treated cells invaded significantly more than the control group. D). Graph showing the expression of mRNA for various EMT markers in Ca1 cells treated with TGF-β1 relative to control cells. E). Expression of vimentin as detected by immunohistochemistry (IHC) in Ca1 cells. F). Expression of vimentin as detected by IHC in TGF-β1 treated Ca1 cells. G). Graph showing that the number of vimentin positive cells after TGF-β1 treatment was significantly higher than in control cells. Statistical significant differences between the groups P <0.05 are highlighted by a star. H) Representative H&E stained sections of 3D constructs with malignant oral keratinocytes (CaLH3) seeded onto CAF-D and 24 hours later treated either with SB431542 or anti-TGF-β1 monoclonal Ab for 10 days (200x magnification, scale bar 200μm). I) Graph showing quantification of the depth of invasion of malignant keratinocytes in 3D constructs with and without addition of TGF-β1 inhibitors. J). Westernblot for pSMAD2 and SMAD2/3 showing inhibition of TGF-β1 downstream pathway in Ca1 cells treated with with SB431542 but not when treated with anti-TGF-β1 monoclonal Ab.
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Fig. 4 A). Transwell migration assay showing various fibroblast strains with distinct migratory characteristics: CAF-N with the highest proportion of intrinsically migratory cells and the relatively stationary CAF-D. B). Western analysis showing increased levels of pMLC and pSmad2 in CAF-N when compared to NF, reflecting the presence of a higher proportion of intrinsically migratory fibroblast in CAF-N. C). Track speed and D). track displacement of CAF-N and NF quantified by time-lapse microscopy over 18 hours: CAF-N migrated at higher speed and showed greater displacement (the shortest path between the start and the end points of the total cell movement track) than NF. Statistical significant differences between groups, P<0.05 are highlighted by a star.

Fig. 5 A). Graph showing that CAF secreted significantly more HA than NF (P=0.008) and that CAF-N synthesized significantly higher levels than CAF-D (P=0.002). B). IHC and C). western analysis confirmed that CAF and malignant oral keratinocytes expressed HA- receptor RHAMM. D). Graph showing that significantly fewer CAF-N migrated through transwells after exposure to 4-MU that prevents elongation of HA chains, P<0.001. E). CAF-N morphology as depicted by SEM showing larger, flattened cells with fewer filopodia after exposure to 4MU when compared to untreated controls. Upper panel 1500x magnification, scale bar 10μm; lower panel 5000x magnification, scale bar 1μm. F). Pancytokeratin immunohistochemistry showing reduced invasion of Ca1 carcinoma cells after CAF-N were exposed to 4-MU, confirmed by G). Measurement of the depth of invasion. 200x magnification. Scale bar 200μm. H). Representative H&E stained sections of 3D constructs with malignant oral keratinocytes (Ca1 and CaLH3) seeded onto ctrl-shRNA and HAS2shRNA treated CAF3 (200x magnification, scale bar 200μm). I) Graph showing quantification of the depth of invasion of malignant keratinocytes in 3D constructs with ctrl-shRNA and HAS2shRNA treated CAF3.
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Fig. 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 while CAF-D were refractory to exposure to TGF-β1. B). Western analysis showing increased levels of pSmad2 after 1h TGF-β1 exposure of CAF-N and NF. Skin fibroblasts (SF) were used as control for activation of TGF-β1 downstream pathway. C). Graph showing the distribution of different cell phenotypes contributing to the heterogeneity of each fibroblast strain analysed: (1) intrinsically motile cells; (2) TGF-β1-responsive cells; and (3) stationary / low-migratory and high secretory cells. CAF-N and NF contained significantly higher percentages of intrinsically and TGF-β1-induced motile cells when compared to CAF-D (P=0.05). CAF-D contained a significantly high percentage of low-motile, highly secretory cells (P<0.01). D). H&E staining of representative 3D constructs with DOK cells seeded onto control NF or E). matched NF pre-treated with TGF-β1, showing that TGF-β1 activated NF supported a deeper invasion. 200x magnification, scale bar 200μm. E). Closer view (400x magnification, scale bar 50μm) showing invasion of DOK when seeded onto NF pre-treated with TGF-β1, with small islands, single cells and cords of cells growing deep into the matrix (non-cohesive pattern of invasion). F). Closer view (400x magnification, scale bar 50μm) showing invasion of DOK when seeded onto CAF-N with a non-cohesive, vertical pattern of invasion, similar to the invasion pattern seen when DOK were seeded onto NF pre-treated with TGF-β1.

Legend to Table

Table 1. Summary of the top over expressed and under expressed genes by fibroblasts derived from normal human oral mucosa, dysplastic lesions and OSCC. The genes differentially expressed in CAF when compared to NF (FD rate of ≤0.01) were linked to substrate adhesion, tissue remodelling, cell migration, secretion, growth regulation and angiogenesis. TGF-β1 targets are shown in italics. Fewer changes were found when DAF and NF were compared (127 genes
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significantly up-regulated and 75 down-regulated, FD rate of $\leq 0.01$). The most over expressed functional group in DAF when compared to NF were transcription factors.
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Acknowledgments

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Author Contributions

The study was designed and supervised by MP, ACJ and DEC. MP, DEC, AH, HP, SS, AHO, and KKK, initiated the fibroblast strains, performed the tissue culture, animal experiments, migration assays, immunohistochemistry, western and growth factor analysis. DEC performed and analysed the time-lapse microscopy. Microarray analysis and qRT-PCR was performed and analysed by JT, GK, CPM, XH and MP. All authors approved the final manuscript.
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References


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### Top 30 genes overexpressed in CAF v NF

<table>
<thead>
<tr>
<th>Function</th>
<th>Over expressed in CAF v NF</th>
<th>Under expressed in CAF v NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeleton associated RhoGTPase/actin</td>
<td>Map7, ATRNL1, GPC3, ROBO2, EGFL6, SPON1, THBS4, SEMA4D, F11R, ITGA8, MATN2, PCDH10, DPT, CILP, ITGA9, CDH18, DCN, EMILIN2, FLRT3, CXLCL12,</td>
<td></td>
</tr>
<tr>
<td>Inflammatory/Immune transcription factors</td>
<td>CADPS, SRGN, TPMS, DAB2, ENPEP, ASB5, IL24,</td>
<td></td>
</tr>
<tr>
<td>Endothelial associated</td>
<td>KCTD10,</td>
<td></td>
</tr>
<tr>
<td>Tissue remodeling</td>
<td>CTSB, SPATN, SPC4,</td>
<td></td>
</tr>
<tr>
<td>Migratory machinery</td>
<td>TAGLN, TIM1, KBTBD10, CDH13, BGN, MET, TMSF1,</td>
<td></td>
</tr>
<tr>
<td>RhoGTPase/actin cytoskeleton associated</td>
<td>WASF2, DAA1M, C1orf11, PP1c, GARNL4, PLD5M,</td>
<td></td>
</tr>
<tr>
<td>Secretion/endocytosis</td>
<td>CAMPS, SRGN, RAB27B, SCG2, TACST2, SGNE1, INHBA,</td>
<td></td>
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<tr>
<td>Endothelial cell associated</td>
<td>COL154A, EDIL3, THBD, DCBL2, PEAR1, BGN, NOTCH3, GDF5</td>
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<tr>
<td>Transcription</td>
<td>TSHZ1, GATA6, NOTCH3, EBF, SPEN, INHBA, FOXL2,</td>
<td></td>
</tr>
<tr>
<td>Growth regulation</td>
<td>SRGN, IGBP5, ERK1, IGBP4, IGBP6, INHBA, GDF5,</td>
<td></td>
</tr>
<tr>
<td>Inflammatory/Immune response</td>
<td>NPT2, SCG2, ENPEP, ASB5, IL24,</td>
<td></td>
</tr>
<tr>
<td>Transcription factors</td>
<td>TSHZ1, GATA6, NOTCH3, EBF, SPEN, INHBA, FOXL2,</td>
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### Top 30 TGFβ targets overexpressed in CAF v NF

<table>
<thead>
<tr>
<th>Function</th>
<th>Over expressed in CAF v NF</th>
<th>Under expressed in CAF v NF</th>
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<tbody>
<tr>
<td>Cytoskeleton associated RhoGTPase/actin</td>
<td>MAP7, ATRNL1, GPC3, ROBO2, EGFL6, SPON1, THBS4, SEMA4D, F11R, ITGA8, MATN2, PCDH10, DPT, CILP, ITGA9, CDH18, DCN, EMILIN2, FLRT3, CXLCL12,</td>
<td></td>
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<tr>
<td>Inflammatory/Immune transcription factors</td>
<td>CADPS, SRGN, TPMS, DAB2, ENPEP, ASB5, IL24,</td>
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<tr>
<td>Endothelial associated</td>
<td>KCTD10,</td>
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<tr>
<td>Tissue remodeling</td>
<td>CTSB, SPATN, SPC4,</td>
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<tr>
<td>Migratory machinery</td>
<td>TAGLN, TIM1, KBTBD10, CDH13, BGN, MET, TMSF1,</td>
<td></td>
</tr>
<tr>
<td>RhoGTPase/actin cytoskeleton associated</td>
<td>WASF2, DAA1M, C1orf11, PP1c, GARNL4, PLD5M,</td>
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<tr>
<td>Secretion/endocytosis</td>
<td>CAMPS, SRGN, RAB27B, SCG2, TACST2, SGNE1, INHBA,</td>
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<tr>
<td>Endothelial cell associated</td>
<td>COL154A, EDIL3, THBD, DCBL2, PEAR1, BGN, NOTCH3, GDF5</td>
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<tr>
<td>Transcription</td>
<td>TSHZ1, GATA6, NOTCH3, EBF, SPEN, INHBA, FOXL2,</td>
<td></td>
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<tr>
<td>Growth regulation</td>
<td>SRGN, IGBP5, ERK1, IGBP4, IGBP6, INHBA, GDF5,</td>
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<tr>
<td>Inflammatory/Immune response</td>
<td>NPT2, SCG2, ENPEP, ASB5, IL24,</td>
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<tr>
<td>Transcription factors</td>
<td>TSHZ1, GATA6, NOTCH3, EBF, SPEN, INHBA, FOXL2,</td>
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### Top 30 genes overexpressed in DAF v NF

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<th>Under expressed in DAF v NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extra cellular matrix-receptor interaction/ adhesion</td>
<td>BGN, CADPS, SRGN, TPM1,</td>
<td></td>
</tr>
<tr>
<td>Tissue remodeling</td>
<td>GDF5</td>
<td></td>
</tr>
<tr>
<td>Migratory machinery</td>
<td>INHBA,</td>
<td></td>
</tr>
<tr>
<td>RhoGTPase/actin cytoskeleton associated</td>
<td>INHBA,</td>
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</tr>
<tr>
<td>Secretion/endocytosis</td>
<td>INHBA,</td>
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<tr>
<td>Endothelial cell associated</td>
<td>INHBA,</td>
<td></td>
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<tr>
<td>Transcription</td>
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</tr>
<tr>
<td>Transcription factors</td>
<td>INHBA,</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

A. Disease-specific survival

B. PMEPA1

C. BGN

D. CADPS

Follow-up (months) Follow-up (months) Follow-up (months)

E. Divergent transcriptome (CAF-D)

F. Homogeneous, close to normal transcriptome (CAF-N)

G. Close to normal transcriptome (CAF-N)

H. Transcriptome comparison
Fig. 2

A. 

B. 

C. 

D. 

E. 

F.
Fig. 3

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I. 

J. 

K. 

L.
Fig. 4

A.

B.

C.

D.
Fig. 5

A. Secretion of hyaluronan

B. RHAMM

C. CAF-N and NF GAPDH

D. CAF-N and NF

E. CAF1-M, CAF1+4MU, CAF3-M, CAF3+4MU

F. Ca1/CAF1 and Ca1/4MU-treated CAF1

G. Depth of invasion (μm)

H. Ca1 and Ca1H3

I. CAF3 ctrl-shRNA and CAF3 HAS2 shRNA

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Fig. 6

A. 

B. 

C. 

D. DOK/NF16

E. DOK/TGF-β1-pretreated NF16

F. TGF-β1-pretreated NF16

G. DOK/CAF1
Identification of two distinct carcinoma-associated fibroblast subtypes with differential tumor-promoting abilities in oral squamous cell carcinoma

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

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