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Driving role of head and neck cancer cell secretome on the invasion of stromal fibroblasts: Mechanistic insights by phosphoproteomics

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ABSTRACT

Background: Cancer-associated fibroblasts (CAFs) are major players in tumor-stroma communication, and participate in several cancer hallmarks to drive tumor progression and metastatic dissemination. This study investigates the driving effects of tumor-secreted factors on CAF biology, with the ultimate goal of identifying effective therapeutic targets/strategies for head and neck squamous cell carcinomas (HNSCC).

Methods: Functionally, conditioned media (CM) from different HNSCC-derived cell lines and normal keratinocytes (Kc) were tested on the growth and invasion of populations of primary CAFs and normal fibroblasts (NFs) using 3D invasion assays in collagen matrices. The changes in MMPs expression were evaluated by RT-qPCR and kinase enrichment was analyzed using mass spectrometry phosphoproteomics.

Results: Our results consistently demonstrate that HNSCC-secreted factors (but not Kc CM) specifically and robustly promoted pro-invasive properties in both CAFs and NFs, thereby reflecting the plasticity of fibroblast subtypes. Concomitantly, HNSCC-secreted factors massively increased metalloproteinases levels in CAFs and NFs. By contrast, HNSCC CM and Kc CM exhibited comparable growth-promoting effects on stromal fibroblasts. Mechanistically, phosphoproteomic analysis predominantly revealed phosphorylation changes in fibroblasts upon treatment with HNSCC CM, and various promising kinases were identified: MKK7, MKK4, ASK1, RAF1, BRAF, ARAF, COT, PDK1, RSK2 and AKT1. Interestingly, pharmacologic inhibition of RAF1/BRAF using sorafenib emerged as the most effective drug to block tumor-promoted fibroblast invasion without affecting fibroblast viability

Conclusions: Our findings demonstrate that HNSCC-secreted factors specifically fine tune the invasive potential of stromal fibroblasts, thereby generating tumor-driven pro-invasive niches, which in turn to ultimately facilitate cancer cell dissemination. Furthermore, the RAF/BRAF inhibitor sorafenib was identified as a promising

Abbreviations: CAFs, Cancer-associated fibroblasts; HNSCC, head and neck squamous cell carcinomas; CM, conditioned media; Kc, normal keratinocytes; NFs, normal fibroblasts; HPV, human papillomavirus; TME, tumor microenvironment; ECM, extracellular matrix; MMPs, matrix metalloproteinases; MS, mass spectrometry; KSEA, kinase-substrate enrichment analysis; EMT, epithelial to mesenchymal transition; FBS, fetal bovine serum; DTT, dithiothreitol; SPE, solid-phase-extraction; MSA, multistage activation.

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candidate to effectively target the onset of pro-invasive clusters of stromal fibroblasts in the HNSCC microenvironment.

1. Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1]. It represents more than 90 % of all head and neck cancers arising from the stratified epithelium of the upper aerodigestive tract, primarily located in the oral cavity, pharynx, and larynx. Tobacco and alcohol consumption and oncogenic viruses such as human papillomavirus (HPV) are recognized as major etiological factors [2]. Five-year overall survival for HNSCC is less than 50 % for advanced stage III and IV tumors, and patient mortality is mainly due to late diagnosis and failure of loco-regional control. Despite continuous advancements in treatment modalities, patients' life expectancy has barely increased over the last decades. At present, there are only few molecular-targeted therapies approved by the FDA for HNSCC treatment, i.e. cetuximab (anti-EGFR), nivolumab and pembrolizumab (anti-PD-1/PD-L1). Hence, increasing our understanding of HNSCC tumor biology is fundamental for the development of more effective personalized therapeutic strategies [3].

Mounting evidence has demonstrated that the tumor microenvironment (TME) is an integral and inseparable part of tumor development and progression [4,5], as it plays a key role in malignant transformation, cancer cell proliferation, invasion, and metastatic dissemination [6–8]. In addition to cancer cells, TME comprises a variety of stromal components, including cancer-associated fibroblasts (CAFs), endothelial cells, mesenchymal stem cells, immune cells from both innate and adaptive immune system, growth factors and cytokines, and the extracellular matrix (ECM) [7].

CAFs are the major component within the TME and participate in several hallmarks necessary for cancer progression, such as sustained growth, inflammation, angiogenesis, invasion, metastasis, and drug resistance [9,10]. It is nowadays well established that tumor cells can regulate stromal cells to release different paracrine factors that sustain cancer cell properties and facilitate tumor growth and spreading. In this respect, CAFs emerge as key players involved in enhancing tumor progression toward more aggressive phenotypes through several mechanisms, such as cell-cell contacts with cancerous or other stromal cells or by releasing a plethora of secreted factors, such as growth factors, chemokines, cytokines, or matrix metalloproteinases (MMPs) for ECM remodeling [11,12]. Accordingly, HNSCC specimens with a high density of CAFs have been associated with local recurrence and poor prognosis [13–16].

Paracrine communication between cancer cells and stromal cells by secreted proteins is of great importance during malignant transformation. Post-translational protein modifications play a key role in the conversion of these extracellular stimuli into gene expression changes in a rapid and reversible manner. In this sense, protein phosphorylation is one of the main post-translational modifications involved in signal transduction networks controlling enzymatic activity, protein stability and interactions, as well as cellular localization [17,18]. Consequently, protein phosphorylation plays a crucial role coordinating multitude of cellular processes such as transcriptional regulation, subcellular trafficking, cell proliferation and apoptosis, angiogenesis, invasion, migration, or inflammation among others [19]. As a result, protein phosphorylation is tightly regulated in the cellular environment and any perturbation affecting its regulatory mechanisms may lead to a diseased state. Altered protein phosphorylation has been associated to the oncogenesis of different types of cancer, including head and neck cancer, regulating various cellular functions involved in malignant transformation [2,20]. In addition, the role of protein phosphorylation in cell signaling is still poorly understood, especially, the molecules and pathways involved in the communication between cancer cells and fibroblasts during carcinogenesis. For all these reasons, phosphoproteome analysis emerges as a powerful and valuable tool for the study of the proteins and signaling pathways involved in the paracrine communication between cancer cells and stromal fibroblasts.

This work aimed to unravel key signaling networks involved in the crosstalk between HNSCC cells and CAFs, focusing on the impact of paracrine factors secreted by cancer cells on fibroblast biology. Functional characterization was combined with quantitative phosphoproteomics by mass spectrometry to further decipher mechanistic insights into the potential kinases and signaling networks involved. Our results demonstrate that HNSCC CM greatly enhanced the invasiveness of both CAFs and NFs, at least in part through the increase in MMP expression and the regulation of various signaling pathways and kinases such as MKK4, MMK7, Akt, RAF1 and BRAF. Furthermore, the impact of pharmacologic target inhibition on CAF invasion was evaluated to unveil the RAF/BRAF inhibitor sorafenib as a promising candidate to effectively target the onset of HNSCC-driven pro-invasive stromal fibroblasts disrupting the tumor-TME communication.

2. Methods

2.1. Cell culture

The HNSCC-derived cell line UT-SCC40 was kindly provided by Dr. R. Grenman (Department of Otolaryngology, University Central Hospital, Turku, Finland). FaDu and Detroit-562 cells were purchased to the ATCC. The HNSCC-derived cell line HCA-LSC1 was spontaneously generated in our laboratory from a primary laryngeal squamous carcinoma surgically resected at the Hospital Universitario Central de Asturias (HUCA, Asturias). Cancer-associated fibroblasts (CAFs) subpopulations, CAF2, CAF3 and CAF4, were obtained from minced tumor tissue of surgically resected HNSCCs at the HUCA. Normal fibroblasts (NFs) subpopulations, NF1 and NF2, were kindly provided by Dr. A. Gandarillas (IDIVAL, Santander). Primary normal keratinocytes (Kc) were obtained from the Tissue Engineering Unit (HUCA's Community Centre for Blood and Tissues). Cell lines were maintained in culture to a maximum of 10 passages, to avoid the accumulation of genetic changes, regularly tested for mycoplasma and authenticated by STR. HNSCC tissue samples from HUCA patients used in this study were provided by the Principado de Asturias BioBank (PT17/0015/0023) integrated into the Spanish National Biobanks Network. Samples were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees of the Hospital Universitario Central de Asturias and the Regional CEIm from Principado de Asturias (date of approval May 14th, 2019; approval number: 141/19, for the project PI19/00560).

Cells were grown in DMEM (Corning) supplemented with 10 % fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin (Biowest), 200 mg/mL streptomycin (Biowest), 2 mM L-glutamine (Corning), 20 mM HEPES (pH 7.3) (Biowest) and 100 mM MEM non-essential amino acids (Biowest).

2.2. Conditioned media production

The HNSCC-derived cell lines (FaDu, Detroit-562, UT-SCC40, and HCA-LSC1) and primary keratinocytes were grown until reach 80 % confluency, then washed with PBS, and media replaced with phenol red-free DMEM (Corning) without FBS for 72 h. Next, conditioned media (CM) were collected and filtered through a 0.45 μm pore filter (Sartorius) and frozen at −80 °C until use. Phenol red-free DMEM without FBS was used as control medium.

2.3. Drugs

PD153035 (EGFR inhibitor; Selleckchem), Sorafenib Tosylate (RAF1 and BRAF inhibitor; Selleckchem), SP600125 (MEKK4 and MEKK7 inhibitor; Selleckchem), GM6001 (MMPs inhibitor; Selleckchem) and GSK690693 (Akt inhibitor; Selleckchem) were prepared as 10 mM solutions in sterile DMSO, according to manufacturer's indications, aliquoted and stored at −80 °C until just before use, when they were brought to the desired final concentration. DMSO was used as the vehicle control condition.

2.4. Three-dimensional spheroid invasion assays

Fibroblasts (CAF2, CAF3, CAF4, NF1 and NF2) were suspended in DMEM plus 20 % Methyl cellulose (Sigma) at 80,000 cells/mL. Cell spheroids were subsequently obtained by serial pipetting of 25 μL into a non-adhesive bacterial Petri dish (2000 cells/spheroid) and incubated in an inverted position for 18 h. Next day, each cell spheroid was transferred to an individual well of a 96-well tissue culture plate and embedded into 110 μL of 2.3 mg/mL bovine collagen matrix (PureCol, Advanced Biomatrix). After 2 h of polymerization at 37 °C, each well was filled with 100 μL media (either control medium, HNSCC CM or Kc CM) with or without inhibitors (PD153035, sorafenib, SP600125, GM6001, GSK690693, or DMSO as vehicle control). 3D fibroblast spheroid invasion was monitored using a Zeiss Cell Observer Live Imaging microscope (Zeiss) coupled with a CO_2 and temperature-maintenance system. Time-lapse images were acquired every 60 min during 20 h using a Zeiss AxioCam MRc camera (Zeiss) [21]. The area of each individual spheroid was measured using Image J analysis program. The invasive area was determined by calculating the difference between the final and the initial area and data were represented relative to control condition.

2.5. Viability assays

Fibroblasts (NF1, NF2, CAF2, CAF3 and CAF4) were seeded into 96-well tissue culture plates at a density of 3000 cells *per* well. Next day, media were replaced with either control medium, HNSCC CM or Kc CM for 96 h. Treatment with the inhibitors (PD153035, sorafenib, SP600125, GM6001, and GSK690693) or DMSO (vehicle control) was for 24 h or 72 h. Cell viability was measured using a tetrazolium-based MTS test (CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega), reading the absorbance at 490 nm with a Synergy HT plate reader (BioTek). Data were represented as fold change and relative to control condition.

2.6. RNA extraction and mRNA analysis by real-time RT-PCR

Total RNA was extracted from CAF2 and NF1 using TRizol Reagent (MRC), and cDNA was synthesized with Superscript II RT-PCR System (Invitrogen) according to manufacturer's protocol. Gene expression was analyzed by real-time PCR following Power SYBR Green PCR Master Mix protocol (Applied Biosystems), using the StepOnePlus Real-Time PCR System (Applied Biosystems). The constitutively expressed RPL19 ribosomal coding gene was used as endogenous control. Reactions were run in triplicate and the relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data were expressed as fold-change, normalized to RPL19 mRNA levels and relative to control cells.

Reactions were carried out using the following primers: MMP1 (Fw: GACCAACAATTTCAGAGAG; Rv: TCAGTAGAATGGGAGAGT), MMP2 (Fw: CCCTGAGACCGCCATGTC; Rv: ATATTTGTTGCCCAGGAAAGTG), MMP8 (Fw: ACCAACACCTCCGCAAAT; Rv: GGATACATCAAGGCACCAG), MMP9 (Fw: TATGTACCGCTTCACTGAG; Rv: TTCAGGGCGAGGACCATA), MMP13 (Fw: TTCCTATCTACACCTACAC; Rv: TTCATCTCCTGGACCATA) and RPL19 (Fw: GCGGAAGGGTACAGCCAAT; Rv: GCAGCCGGCGCAAA).

2.7. Phosphoproteomic analysis by Mass Spec

Phosphoproteomic analysis was carried out as described in [22]. Three 15-cm-diameter dishes of CAF2 were seeded (5×10^6 cells *per* plate) for each experimental condition. Cells were treated for 15 min or 1 h with control medium, Kc CM, FaDu CM or HCA-LSC1 CM. Briefly, cells were washed twice with ice-cold PBS supplemented with phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF) and lysed with a denaturing buffer with phosphatase inhibitors (20 mM HEPES pH 8.0, 8 M urea, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM $Na_4P_2O_7$ and 1 mM ß-glycerol-phosphate). Cell lysates were further homogenized by sonication and insoluble material was removed by centrifugation at 16,000 g for 10 min at 4 °C. Protein concentration was calculated by Pierce BCA Protein Assay Kit (Thermo Fisher). For reduction and alkylation, 300 μg of protein were sequentially incubated with 4.1 mM dithiothreitol (DTT) and 8.3 mM iodoacetamide for 60 and 30 min respectively. For digestion, samples were diluted to 2 M urea with 20 nM HEPES pH 8.0 and incubated with immobilized TLCK-trypsin (20 TAME units/mg) (Thermo Fisher) for 16 h at 37 °C. Digestion was stopped by addition of TFA at a final concentration of 1 %.

The resultant peptide solutions were desalted by solid-phase-extraction (SPE) using Oasis HLB extraction cartridges (Waters UK Ltd.) according to manufacturer's instructions. Briefly, cartridges were activated with 1 mL of 100 % ACN and equilibrated with 1.5 mL of wash solution (1 % ACN, 0.1 % TFA in water). After the cartridges were loaded with peptide solution, they were washed with 1 mL of wash solution. Peptides were eluted with 0.5 mL of glycolic acid solution (1 M Glycolic acid in 50 % ACN and 5 % TFA). All the steps were done in a vacuum manifold, set at 5 mm Hg.

Next, phosphopeptide enrichment was performed. In short, eluates from Oasis cartridges were incubated during 30 min at room temperature with TiO_2 solution (50 % slurry; GL Sciences Inc.). TiO_2 beads were then packed by centrifugation in equilibrated C-18 spin columns (PepClean C-18 Spin Columns; Thermo Scientific). Beads were sequentially washed with 200 μL of glycolic acid solution and ammonium acetate solution (20 mM ammonium acetate pH 6.8 in 50 % ACN). For phosphopeptide elution, beads were incubated four times with 50 μL of 5 % NH_4OH for 1 min at room temperature and centrifuged and then, the four eluates were pooled. Samples were then dried using a SpeedVac and protein pellets were stored at −80 °C until the time of analysis.

Samples were solubilized and separated by nano-liquid chromatography as per the proteomic analyses, but a top 10 MSA (multistage activation) method was utilized for increased coverage for neutral loss phosphoproteomic data acquisition.

2.8. Mass Spec data analysis

Raw files were converted to peak lists (in the Mascot Generic Format) using Mascot Distiller (version 2.3.0) and searched using Mascot Server (version 2.3.01) against the SwissProt Uniprot database (2012–03–10) restricted to *Homo sapiens*. Mass windows for tolerance for MS scans were 10 ppm and 600 mmu for MS/MS. Fixed modification of carbamidomethylation of cysteine and variable modifications of oxidation of methionine and glutamine to pyroglutamate conversion were permitted. For phosphoproteomic data further variable modifications of phospho (S/T/Y) were included. Mascot result files were parsed using a Perl script that uses the Mascot Parser files provided by Matrix Science. The threshold for accepting peptides as being positively identified was set at an expectancy score of 0.05. Pescal was then used to automate the construction of the extracted ion chromatogram of all peptides identified in an experiment across all samples being compared, as described in [23]. Data were normalized by dividing each peptide intensity by the sum of all peptide intensities within a sample. Fold changes were calculated by averaging the normalized intensities of peptides within a sample group and dividing these by the intensities of the control group. Fold changes were then log transformed before calculation of

significance using an unpaired t-test.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD), unless otherwise stated, and compared using multiple t-test or one-way ANOVA with Holm-Sidaḱs post-hoc analysis for multiple comparisons. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software Inc). p values less than 0.05 were considered statistically significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$).

3. Results

3.1. Effect of HNSCC-derived factors on the invasion and proliferation of stromal fibroblasts

We functionally investigated the crosstalk between HNSCC cells and CAFs. For this purpose, conditioned media (CM) from different HNSCC-derived cell lines and normal keratinocytes (Kc) were obtained. These CMs were subsequently used to treat different populations of primary CAFs and NFs (see Methods for details) to explore the impact of tumor-secreted factors on fibroblast invasiveness.

Firstly, we studied the effect of CM from four different HNSCC cell lines on the ability of NF1 and CAF2 to invade into 3D collagen matrices. As shown in Fig. 1, CM from all four HNSCC cell lines tested (FaDu, HCA-LSC1, UT-SCC40 and Detroit-562) significantly increased to a similar extent the invasive capacity of both NF1 and CAF2, as compared to control cells. By contrast, CM from normal Kc had no stimulatory effect on fibroblast invasion.

These findings were further confirmed using different populations of primary CAFs (CAF3, CAF4) and NFs (NF2) (Fig. 2). Our results consistently showed that CM from both FaDu and HCA-LSC1 cells significantly enhanced the invasive potential of CAF3, CAF4 and NF2, whereas CM from Kc did not affect fibroblast invasion, except for a moderate increase observed in CAF4. These data clearly demonstrate that HNSCC-secreted factors exert a major influence on the surrounding stromal fibroblasts (both CAFs and NFs) by promoting pro-invasive properties.

Next, we evaluated the effects of HNSCC CM on the viability and growth of fibroblasts. Results showed that the presence of HNSCC CM

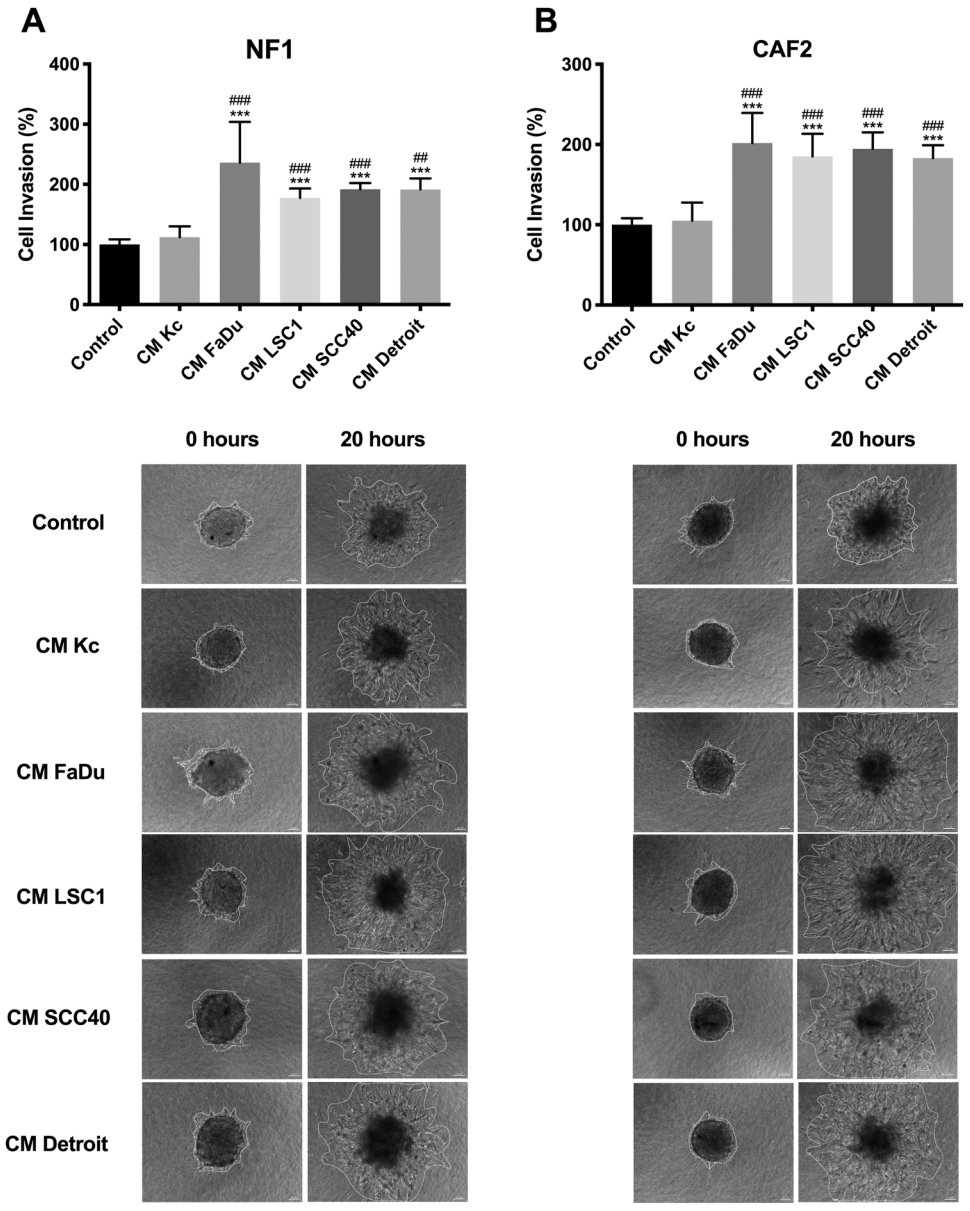

Fig. 1. Effect of HNSCC CM and normal Kc CM on fibroblast invasion. Bar chart showing the relative invasion of NF1 (A) and CAF2 (B) treated with CM from HNSCC-derived cell lines (HCA-LSC1, FaDu, UT-SCC40 and Detroit-562) or CM from normal Kc. Representative images from the 3D invasion assays of NF1 (A) and CAF2 (B) spheroids embedded into a collagen matrix for each condition shown in the bar chart. All data were normalized to control cells (untreated), and expressed as the mean ± SD of at least three independent experiments performed in quadruplicate. Statistical analysis and comparisons to control condition are labeled as "*" and comparisons to Kc CM condition are labeled as "#" (*** or ### $p < 0.001$ and ** or ## $p < 0.01$ by one-way ANOVA and Holm-Sidaḱs multiple comparisons test). Scale bar 100 μm.

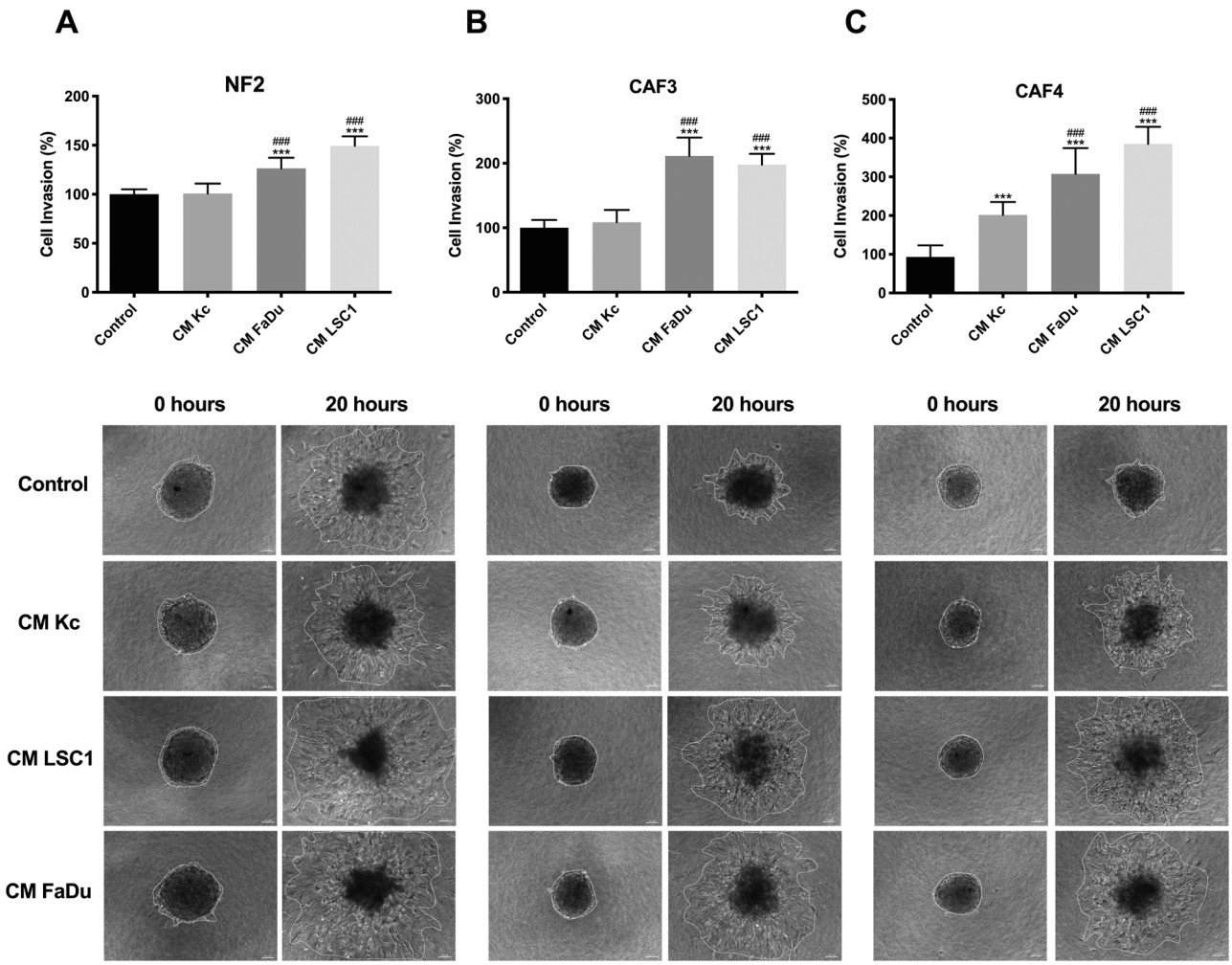

Fig. 2. Effect of HNSCC CM and Kc CM on the invasion of different populations of CAFs and NFs. Bar chart showing the relative invasion of NF2 (A), CAF3 (B) and CAF4 (C) treated with CM from HNSCC-derived cell lines (FaDu and HCA-LSC1) or CM from normal Kc. Representative images from the 3D invasion assays of NF2 (A), CAF3 (B) and CAF4 (C) spheroids embedded into a collagen matrix for each condition shown in the bar chart. All data were normalized to control (untreated) cells, and expressed as the mean ± SD of at least three independent experiments performed in quadruplicate. Statistical analysis and comparisons to control condition are labeled as "*" and comparisons to Kc CM condition are labeled as "#" (*** or ### $p < 0.001$ by one-way ANOVA and Holm-Sidaḱs multiple comparisons test). Scale bar 100 μm.

from FaDu, HCA-LSC1, UT-SCC40 and Detroit-562 cells as well as Kc CM significantly and consistently increased the proliferation of NF1, NF2, CAF2, CAF3 and CAF4 compared to control condition (i.e. culture media without FBS) (Fig. 3). Notably, there were no differences on the stimulatory effect on proliferation between HNSCC CM and Kc CM. These data indicate that CM from either HNSCC cells or normal Kc exhibit comparable growth-promoting effects on stromal fibroblasts.

Collectively, these results demonstrate that factors specifically secreted by HNSCC cells (but not normal Kc) fine tune the invasive potential of stromal fibroblasts, thereby leading to pro-invasive phenotypes, whereas secreted factors from HNSCC cells or Kc exhibited comparable stimulatory effects on fibroblast growth.

3.2. *HNSCC-derived factors widely induce the expression of metalloproteinases in stromal fibroblasts*

To characterize mechanistically the pro-invasive effects of HNSCC CM over fibroblast invasion, the expression levels of MMPs were analyzed by RT-qPCR in both NFs and CAFs treated with HNSCC CMs from FaDu and HCA-LSC1 cells. Thus, we selected for study MMP2 and MMP9, two well established regulators of HNSCC cell invasion [24,25], as well as the collagenases MMP1, MMP8 and MMP13, since our invasion experiments were performed into 3D collagen matrices.

We found that endogenous mRNA levels of all MMPs tested (MMP1, MMP2, MMP8, MMP9 and MMP13) were significantly and concordantly induced in both NF1 and CAF2 upon treatment with FaDu CM or HCA-LSC1 CM, as compared to both control media and Kc CM (Fig. 4). Noteworthy, even though normal Kc CM significantly increased MMP expression in both NFs and CAFs compared to control media, HNSCC CM led to a potent induction of all MMPs tested, in correlation with the observed differences in invasive potential.

These data indicate that HNSCC-secreted factors massively increase the endogenous levels of MMPs in CAFs and NFs, which could consequently contribute to ECM degradation and matrix remodeling by the surrounding stromal fibroblasts, generating tumor-driven pro-invasive niches to ultimately facilitate cancer cell dissemination.

3.3. *Identification of differential phosphorylation changes caused by HNSCC-secreted factors in CAFs by phosphoproteomics*

Quantitative phosphoproteomics by mass spectrometry (MS) was applied as a robust and valuable strategy for spatio-temporal monitoring of signaling network dynamics and detection of rapid phosphorylation changes caused by HNSCC-secreted factors in stromal fibroblasts, which

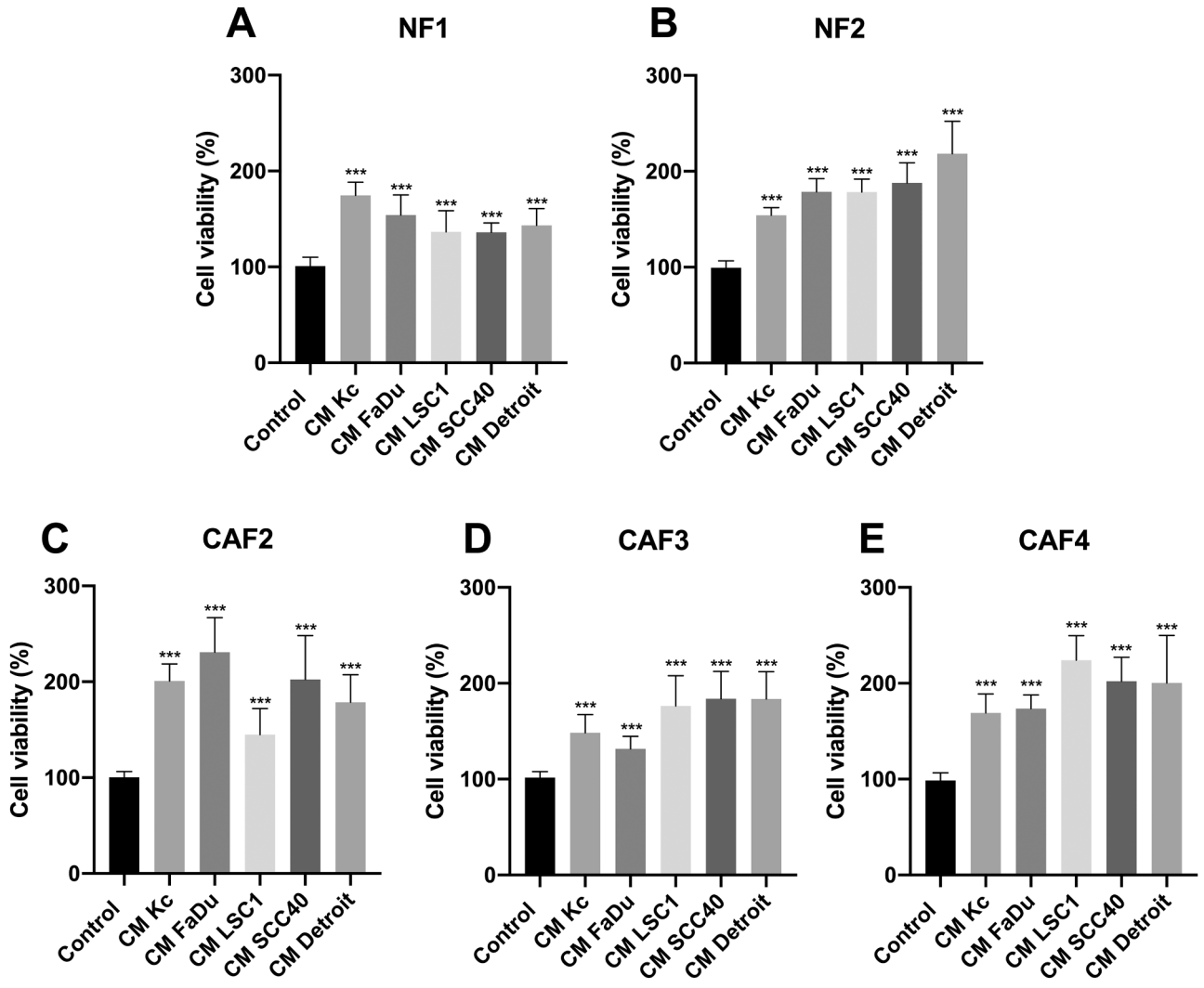

Fig. 3. Effect of HNSCC CM and normal Kc CM on the growth of stromal fibroblasts. NF1 (A) and NF2 (B), CAF2 (C), CAF3 (D) and CAF4 (E) were treated with CM from HNSCC-derived cell lines (FaDu, HCA-LSC1, UT-SCC40 and Detroit-562) or CM from normal Kc. Cell growth was estimated by tetrazolium-based MTS assay after 4 days of treatment. Data were normalized to the absorbance at day 0, and relative to control (untreated) cells. All data were expressed as the mean ± SD of at least three independent experiments performed in quadruplicate. ***$p < 0.001$ by one-way ANOVA and Holm-Sidaḱs multiple comparisons test.

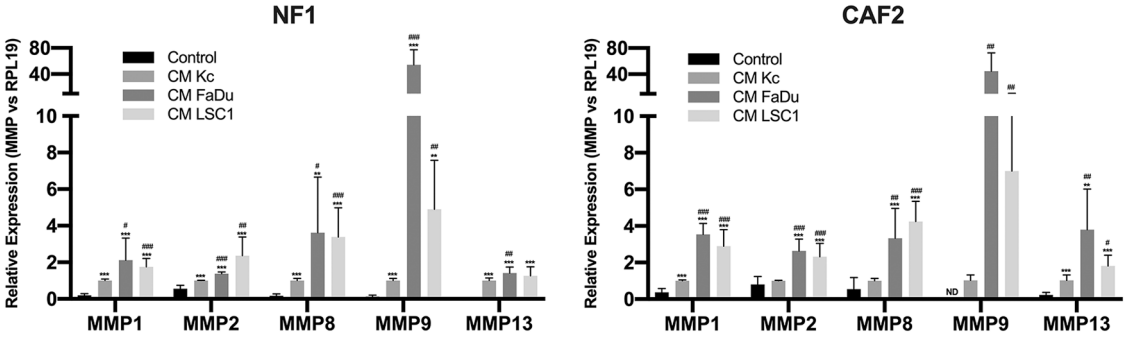

Fig. 4. Effect of HNSCC CM and normal Kc CM on MMP expression in fibroblasts. RT-qPCR analysis of MMP mRNA levels in NF1 and CAF2 treated with CM from HNSCC-derived cell lines (FaDu and HCA-LSC1) or CM from normal Kc. Data were normalized to RPL19 levels, and relative to CM Kc condition. All data were expressed as mean ± SD of at least three independent experiments performed in quadruplicate. Statistical analysis and comparisons to control condition are labeled as "* " and comparisons to Kc CM condition are labeled as "#" (ND = Not detected, * or # $p < 0.05$, ** or ## $p < 0.01$ and *** or ### $p < 0.001$ by multiple t-test corrected by Holm-Sidaḱs multiple comparisons test).

might be related to the pro-invasive behavior observed. Thus, MS-based phosphoproteomic analysis was performed to investigate rapid and specific changes in the phosphorylation status and kinase activation in CAFs upon treatment with HNSCC CM (from FaDu and HCA-LSC1 cells) or Kc CM. Comparisons of phosphorylation levels were conducted at two different time points (15 min and 1 h) using three different biological

replicates for each condition assayed.

As illustrated in Fig. 5A, increases in protein phosphorylation were predominantly and consistently detected at both 15 min and 1 h in CAFs treated with FaDu CM and HCA-LSC1 CM, compared to normal Kc CM. Furthermore, kinase-substrate enrichment analysis (KSEA) [26] was performed to link the differential patterns of phosphorylation sites identified in CAFs to the activation status of kinases known to catalyze such modifications (Fig. 5B). KSEA analysis revealed differential kinase activation changes in CAFs treated with FaDu CM and HCA-LSC1 CM compared to Kc CM, either up-regulated (in red) or down-regulated (in blue). Specifically, the activity of the following 11 kinases was found to concordantly increase in CAFs by treatment with both FaDu CM and HCA-LSC1 CM: MKK7, MKK4, MEKK6, ASK1, RAF1, COT, BRAF, ARAF, PDK1, RSK2 and AKT1 (Fig. 5B), which could potentially be involved in boosting tumor-driven pro-invasive effects on stromal fibroblasts.

3.4. Pharmacologic targeting of Kinases and MMPs effectively inhibit HNSCC-promoted invasiveness of stromal fibroblasts

Phosphoproteomic data provided valuable information on kinases and signaling pathways that could be contributing to enhance fibroblast invasiveness in the HNSCC TME. Based on these data, we selected several kinase inhibitors for the subset of kinases found to be differentially activated in fibroblasts: GSK690693 (AKT), SP600125 (MKK4 and MKK6) and sorafenib (RAF1 and B-RAF) as the best candidates to inhibit the pro-invasive phenotypes driven by HNSCC-secreted factors in the stromal fibroblasts. In addition, we also selected the inhibitor GM6001, a broad-spectrum MMP inhibitor, since we observed that HNSCC CM robustly and massively enhanced MMP expression in both CAFs and NFs. Since EGFR pathway activation is a major therapeutic target in HNSCC, the EGFR inhibitor PD153035 was also tested.

Firstly, dose response analyses were performed to evaluate the impact of the selected inhibitors on the viability of both NF1 and CAF2 and to determine the best drug concentration for fibroblast invasion assays, without having any cytotoxic effect on fibroblast viability. As shown in Supplementary Fig. S1, all the inhibitors tested (GSK690693, PD153035, SP600125, sorafenib and GM6001) increased fibroblast viability at low doses (0.1, 0.01 and 1 μM) in both CAF2 and NF1 at 24 h and 72 h, compared to vehicle condition (DMSO). However, some kinase inhibitors (PD153035, SP600125 and sorafenib) exhibited cytotoxicity at 10 μM (Supplementary Fig. S1). According to this, 1 μM was the selected dose to test the kinase and MMP inhibitors into the 3D invasion assays. Thus, we found that sorafenib and GM6001 significantly reduced fibroblast invasion by 50 % and 25 %, respectively, in both CAF2 and NF1 treated with HCA-LSC1 CM (Fig. 6). In addition, GSK690693 led to a much moderate reduction of fibroblast invasion (10–15 %), while SP600125 was only found to significantly reduce the invasion of NF1 (15 %).

These findings were further validated using three additional populations of primary CAFs (CAF3, CAF4) and NFs (NF2) (Fig. 7). Our results confirmed a robust and consistent reduction of fibroblast invasion by 50 % with 1 μM sorafenib, and also moderate but significant invasion reductions by GM6001 and GSK690693 (30 % and 15 %, respectively). Altogether, these results reinforce the prominent role of sorafenib, a RAF1 and BRAF inhibitor, as a highly promising drug to effectively counteract tumor-promoted pro-invasive effects of tumor stromal fibroblasts, while avoiding deleterious effects on normal fibroblast viability.

4. Discussion

Head and neck carcinomas, due to their epithelial origin, share several characteristics with epithelial cells, such as tight cell-cell junctions or the low capacity to ECM degradation, limiting their invasive potential [27,28]. Therefore, carcinomas must develop cooperative invasion strategies to be able to invade locally or even metastasize [29]. It is well-known that processes such as epithelial to mesenchymal transition (EMT) play a crucial role in tumor invasion, granting cancer cells the ability to invade the surrounding tissues [30,31]. However, there are examples of squamous cell carcinomas (SCC) that exhibit great invasive capabilities while retaining epithelial characteristics [27,32]. One possibility to this paradox could be a tight communication between tumor

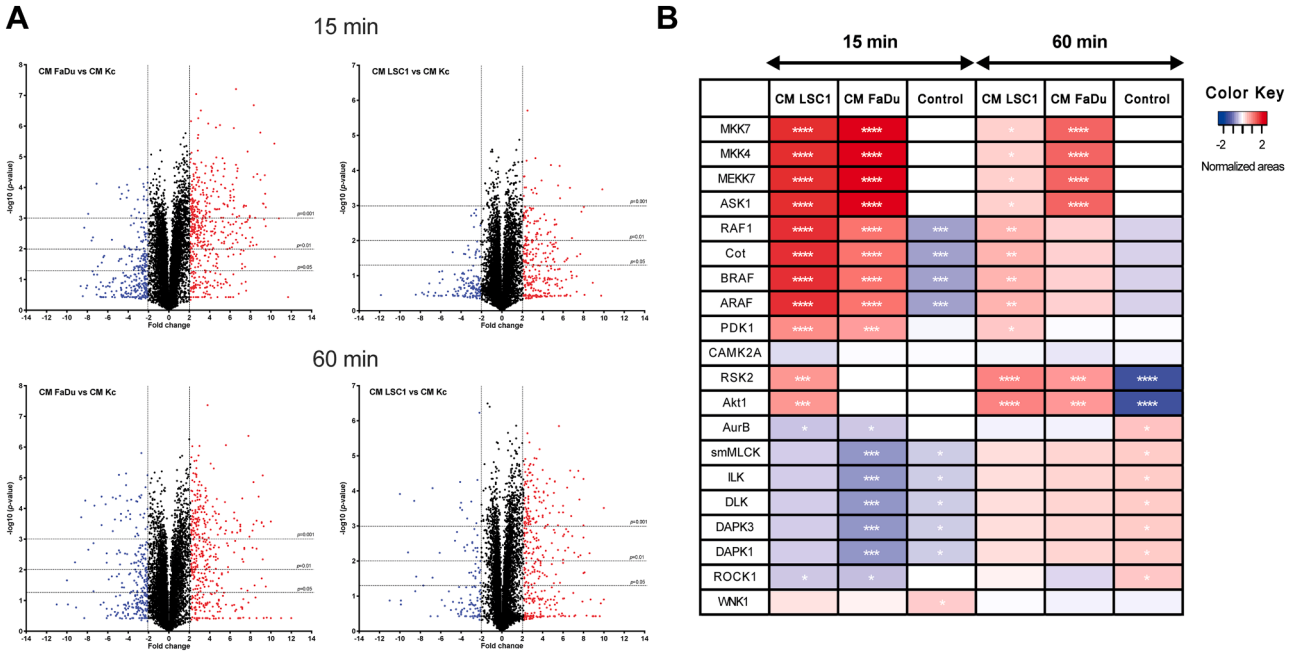

Fig. 5. Phosphoproteomic analysis by MS of differential changes in CAFs treated with HNSCC CM versus Kc CM. (A) Volcano plots showing the global phosphoproteomic changes, illustrating fold change (log base 2) and p-value (-log base 10) for CAFs treated with FaDu CM or HCA-LSC1 CM vs Kc CM for 15 min (upper panel) or 1 h (lower panel). Horizontal bars represent the significance $p = 0.05$, $p = 0.01$ and $p = 0.001$. Vertical bars represent the proteins with a fold change higher than 2 (in red) or below − 2 (in blue). (B) Heatmap represents the changes in the kinase-substrate enrichment analysis (KSEA). Three independent experiments were performed. Red indicates fold changes >0, and blue indicates fold changes < 0. *$p < 0.05$, * * $p < 0.01$ and * ** $p < 0.001$, * ** * $p < 0.0001$.

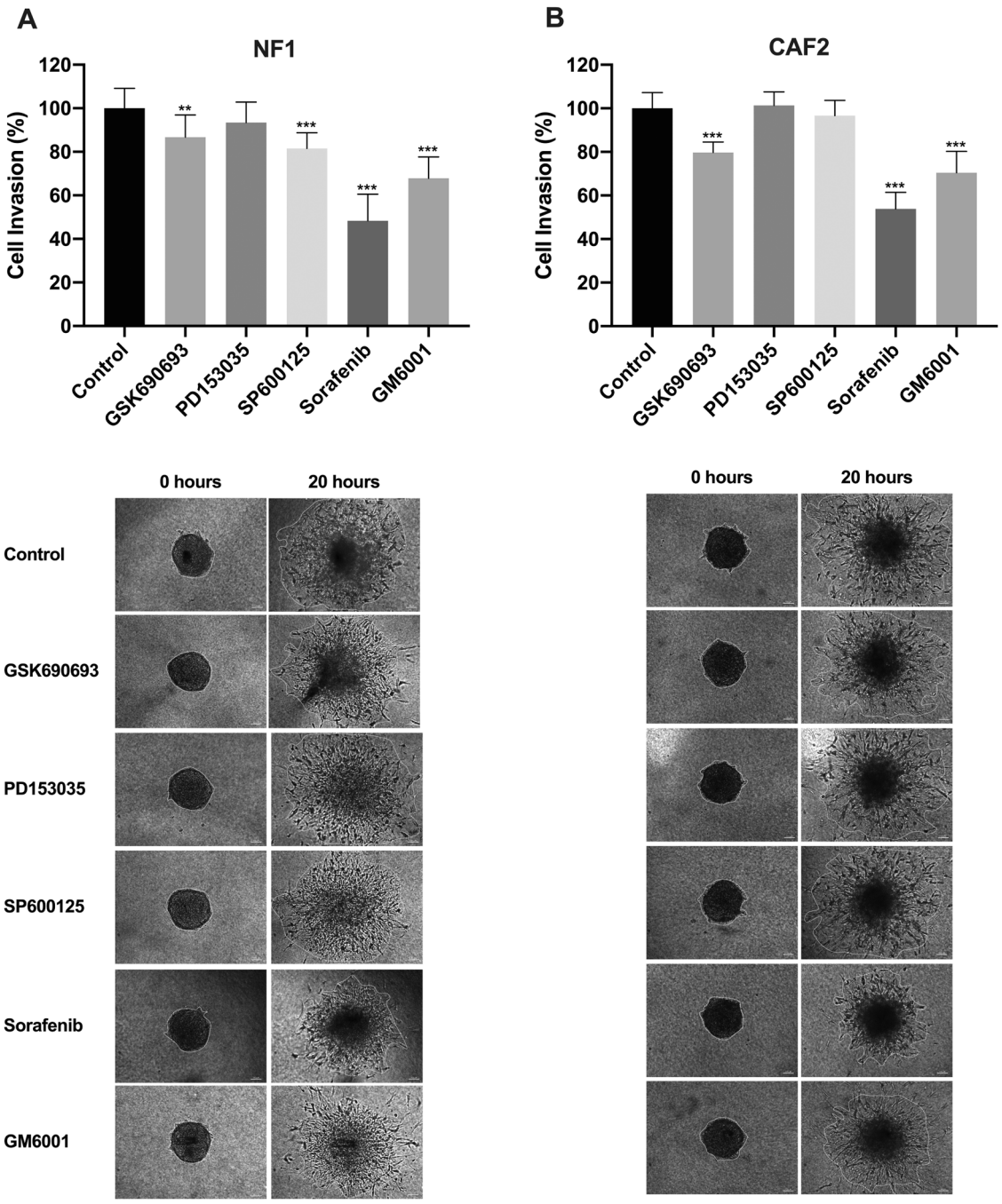

Fig. 6. Effect of kinase and MMP inhibition by GSK690693, PD153035, SP600125, sorafenib and GM6001 on HNSCC-driven fibroblast invasion. Bar chart showing the relative invasion of NF1 (A) and CAF2 (B) spheroids embedded into a 3D collagen matrix treated with HCA-LSC1 CM in the presence of GSK690693, PD153035, SP600125, sorafenib or GM6001 (1 μM) or vehicle (DMSO). Representative images of 3D fibroblast invasion for each condition assayed and plotted. All data were relative to vehicle-treated cells (control) and expressed as mean ± SD of at least three independent experiments performed in quadruplicate. * * $p < 0.01$ and * ** $p < 0.001$ by one-way ANOVA and Holm-Sidaḱs multiple comparisons test. Scale bar 100 μm.

cells and the surrounding non-malignant stromal cells to drive cooperative invasion [33,34].

Among the cells in the TME, CAFs are known for their ability to remodel ECM and creating tracks for cancer cells to migrate, making them the perfect partner for tumor invasion [35,36]. Then, CAFs might be responsible for the onset of pro-invasive TME, which favors dissemination of carcinoma cells from the primary tumor. In this context, several studies have revealed an important role of CAFs as `leading cells', creating paths for cancer cells to direct collective tumor invasion and ECM remodeling [36], which resemble the invading SCC cell clusters observed in human cancer samples [37]. Thus, epithelial cancer cells can take advantage of the mesenchymal characteristics of CAFs to develop cooperative invasion without undergoing EMT themselves. The Rho/ROCK signaling pathway, which regulates the actomyosin cytoskeleton and MMPs, has been identified as an important mediator of the pro-invasive role of fibroblasts in cancer [36]. Specifically, CAFs drive collective tumor invasion through an intercellular physical force mediated by a heterophilic adherence junction between N-cadherin on CAF membrane and E-cadherin on cancer cell membrane. This heterotypic adhesion junction between both cell types enables cell adhesion, migration and invasion. As a result, CAFs contribute to tumor invasion by pulling cancer cells away from the tumor, while cancer cells support

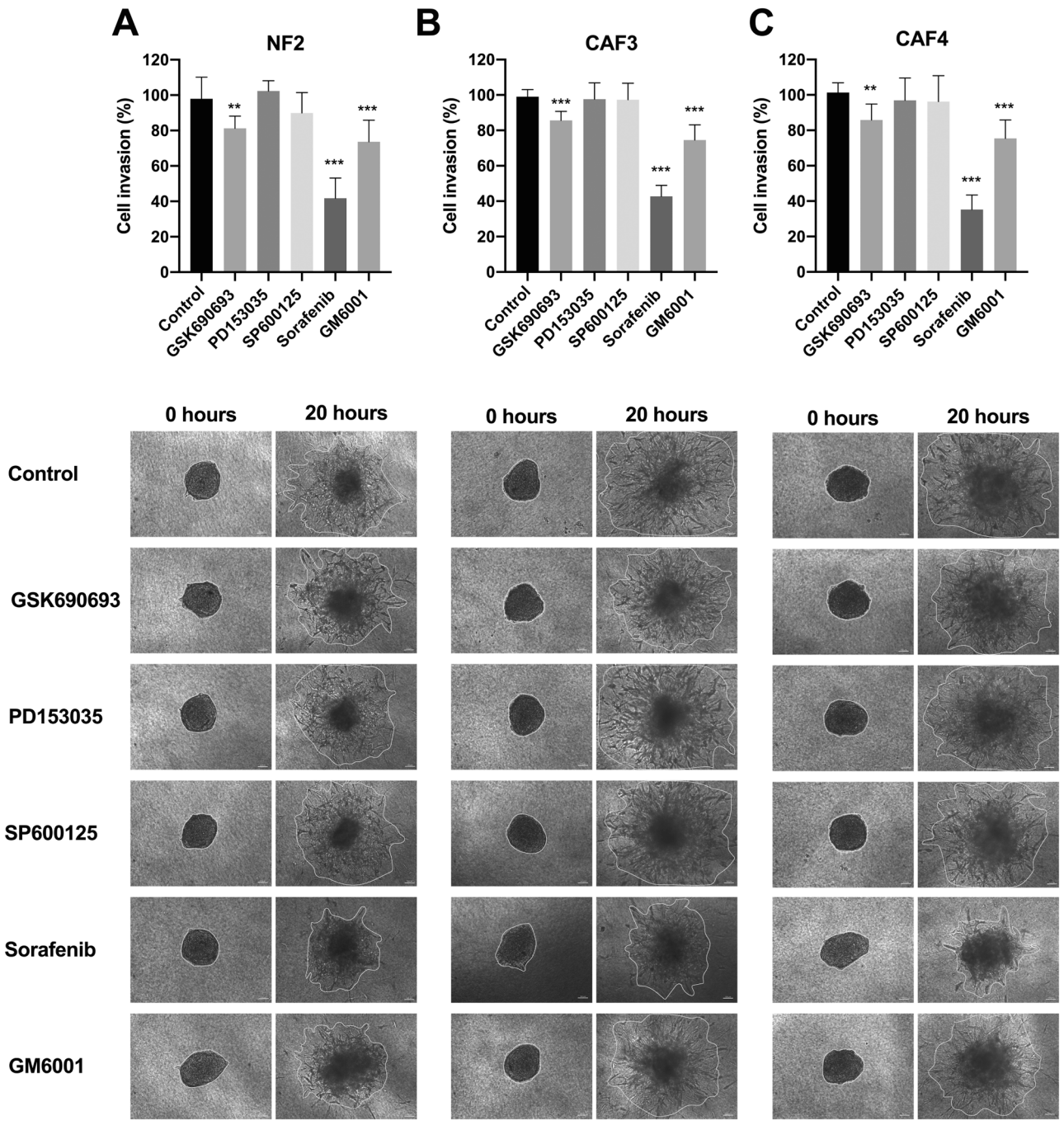

Fig. 7. Validation test of kinase and MMP inhibitors on the invasion of different fibroblast populations. Bar chart showing the relative invasion of NF2 (A), CAF3 (B) and CAF4 (C) spheroids embedded into a 3D collagen matrix treated with HCA-LSC1 CM in the presence of GSK690693, PD153035, SP600125, sorafenib or GM6001 (1 μM) or vehicle (DMSO). Representative images of 3D fibroblast invasion for each condition assayed and plotted. All data were relative to vehicle-treated cells (control) and expressed as mean ± SD of at least three independent experiments performed in quadruplicate. * * $p < 0.01$ and * ** $p < 0.001$ by one-way ANOVA and Holm-Sidaḱs multiple comparisons test. Scale bar 100 μm.

their own spread by polarizing CAF migration away from the tumor [29].

Paracrine signaling represents another important tumor-stroma communication mechanism through the secretion of soluble factors, such as growth factors or inflammatory cytokines, production of ECM proteins and MMPs, jointly generating chemotactic gradients to direct cell migration and invasion [38,39]. In particular, numerous studies have demonstrated the contribution of CAF-derived factors/molecules to HNSCC progression and spreading [40–42]. This study further and significantly extends these data providing evidence for the driving role of HNSCC-secreted factors on the invasiveness of stromal fibroblasts. Our results revealed that CM from different HNSCC cells greatly enhanced the invasiveness of both NFs and CAFs into 3D collagen matrices compared with control medium (non-supplemented). Moreover, CM from normal keratinocytes (Kc) was also included and tested in our experiments as an additional control that reproduces better the normal physiological conditions. However, it is noteworthy that Kc CM did not promote fibroblast invasion. These data evidence that HNSCC-secreted factors specifically increased the invasive potential of stromal fibroblasts in both CAFs and NFs. This was concomitantly

accompanied by induced expression of several MMPs. Furthermore, pharmacological blockade of MMP activity, using a broad-spectrum inhibitor of MMPs (GM6001), significantly reduced HNSCC-driven invasion in NFs and CAFs. Hence, these data demonstrate that paracrine signals from the tumors are able to educate the surrounding fibroblasts, making them more invasive, at least in part, through the involvement of MMPs. This supports other studies, where the communication between tumor cells and CAFs confers the latter a pro-invasive phenotype to degrade the ECM that will be used by tumor cells for their own dissemination [43–45]. Interestingly, our results demonstrate that HNSCC cells exert a comparable stimulatory effect on the invasive potential of CAFs and NFs. These findings reflect that stromal fibroblasts retain high plasticity and adaptability, being NFs equally responsive to HNSCC-driven paracrine signals than CAFs, despite their possible different origin and probably some irreversible epigenetic/activation changes.

Besides, we also found that HNSCC CM exerted a comparable growth-promoting effect to Kc CM in both NFs and CAFs. Therefore, our results show that secreted factors from HNSCC cells stimulate the invasive potential of stromal fibroblasts but do not have a significant effect on fibroblast growth and viability compared to Kc CM. Overall, these data indicate that under normal physiological conditions, fibroblast growth is controlled by normal Kc and seemingly, by cancer cells during HNSCC pathogenesis; however, cancer cells specifically exacerbate the invasive potential of stromal fibroblasts to support tumor invasion.

For all these reasons, the improvement of our comprehension of the tumor stroma components, especially CAFs, and their contribution to tumor progression, might be highly valuable to design novel therapeutic strategies aimed to disrupt the crosstalk between cancer cells and the TME components. Despite all the new knowledge generated in the last years on cancer biology, as well as therapeutic advancements, there has been little changes in the current therapeutic approaches to treat HNSCC patients and therefore, patients' survival has improved marginally in the last decades. In this scenario, the interest and publications destined to improve our understanding of CAF roles on HNSCC pathogenesis has been increasing. Hence, it is already well known that CAFs play an indispensable role in multiple processes related to malignant transformation, such as proliferation, migration, invasion, angiogenesis, inflammation, or drug resistance [10,46–48]. Therefore, the implementation of innovative therapeutic strategies that include the TME are needed to improve current HNSCC treatments. In spite a long-known recognition that CAFs are associated with poor prognosis in HNSCC [46,49,50], currently, no therapeutics targeting CAFs are clinically available for HNSCC patients.

In view of this background, our work also aimed to expand our comprehension of the functional changes that fibroblasts undergo when exposed to secreted factors from cancer cells. For this purpose, we performed a phosphoproteomic study to assess the rapid phosphorylation changes that fibroblast undergo in response to HNSCC-secreted factors and decipher key signaling transduction networks that could be involved in the pro-invasive phenotype of CAFs. Phosphoproteomic results show that HNSCC-secreted factors rapidly led to major protein phosphorylation changes in both NFs and CAFs, compared with factors derived from normal Kc. Overall, increased phosphorylation changes were predominantly detected in fibroblasts upon treatment with HNSCC CM, and various promising kinases were identified: MKK7, MKK4, ASK1, RAF1, BRAF, ARAF, COT, PDK1, RSK2 and AKT1. Several of these kinases have been reported to play important oncogenic roles, and to regulate tumor cell migration and invasion [51–54]. In this work, we studied the role of these kinases regulating the HNSCC-driven pro-invasive phenotype of stromal fibroblasts, which could therefore emerge as potential therapeutic targets in HNSCC. Interestingly, we found that pharmacologic inhibition of RAF1/BRAF using sorafenib was the most effective drug, blocking the invasion of both CAFs and NFs, at a dose that did not affect fibroblast viability. Sorafenib, a multi-kinase inhibitor with the highest affinity for Raf-1 and B-raf, has already been approved by the FDA for the treatment of metastatic renal cell carcinoma, hepatocellular carcinoma, and metastatic thyroid cancer [55]. In this work, we demonstrate that sorafenib inhibited fibroblast invasion, proving its potential as a promising drug to effectively target pro-invasive fibroblast clusters that lead HNSCC progression and spreading. Hence, sorafenib, in combination with conventional therapeutic regimens for HNSCC, focused on the proliferative abilities of cancer cells, could be a very valuable strategy to disrupt the stromal-cancer communication and the tumor-driven pro-invasive behavior displayed by stromal fibroblasts.

Noteworthy, this study demonstrates that both CAFs and NFs populations increased their invasion capabilities under the presence of CM from HNSCC cells. Besides, we observed that the enhanced invasiveness of fibroblasts is accompanied by an increase in MMPs expression and the activation of several signaling pathways. These results suggest that both CAFs and NFs displayed a common behavior under the presence of secreted factors from cancer cells. In a previous work we demonstrate that secreted factors from CAFs, but not from NFs, were able to sustain and robustly enhance stemness in HNSCC-derived cell lines, thereby increasing anchorage-independent growth, tumorsphere formation, and expression of various CSC markers [41]. Hence, we propose that CAFs, compared with NFs, undergo some permanent alterations, as epigenetic changes [9,56], that support cancer progression, such as stimulating cancer cell stemness [41]. However, CAFs are only able to display an exacerbated pro-invasive behavior when they are in close contact and exposed to tumor-derived factors, since invasion abilities of both fibroblasts' populations (NFs and CAFs) resulted equally enhanced by the presence of secreted factors from cancer cells. Therefore, it is important to understand the differences between CAFs and NFs, but it is also essential to comprehend the temporal regulations that tumor cells exert on fibroblasts within the TME [57].

Cancer cell invasion and metastatic dissemination are the main causes of relapses and recurrences, and ultimately of mortality in cancer patients. Despite the overwhelming consensus on the supportive role of CAFs during malignant transformation and tumor invasion, the molecular mechanisms underlying their driving role remain still unclear. Thus, a deeper comprehension of the key signaling networks involved in this process could be very beneficial for cancer diagnosis and therapy. In summary, we demonstrate that CM from cancer cells stimulate the invasiveness of both CAF and NF populations, at least in part through the increase in MMP expression and the regulation of signaling pathways that involve several kinases such as MKK4, MMK7, AKT, RAF1 and BRAF. The pharmacological inhibition of CAFs invasiveness revealed that sorafenib (a RAF and BRAF inhibitor) emerges as the best promising candidate (beyond the MMP inhibitor GM6001) to inhibit the exacerbated invasion abilities of CAFs driven by factors secreted from cancer cells.

5. Conclusions

This work unravels key signaling networks involved in the crosstalk between HNSCC cells and CAFs, demonstrating that HNSCC-secreted factors specifically fine tune the invasive potential of stromal fibroblasts to create pro-invasive environments that ultimately facilitate cancer cell dissemination. Specifically, our results show that HNSCC-derived factors enhanced the invasiveness of both CAFs and NFs, at least in part, through the increase in MMP expression and the regulation of various signaling pathways and kinases such as MKK4, MMK7, AKT, RAF1 and BRAF. Furthermore, the RAF/BRAF inhibitor sorafenib emerges as a promising candidate to inhibit the exacerbated invasion abilities of CAFs driven by factors secreted from cancer cells. Overall, our data elucidate a dynamic reciprocal signaling between HNSCC and fibroblasts, and unprecedentedly identify sorafenib as a promising adjuvant drug to include in the HNSCC current therapeutic strategies to effectively target the pro-invasive features of fibroblast in the TME.

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Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Samples were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees of the Hospital Universitario Central de Asturias and the Regional CEIm from Principado de Asturias (date of approval May 14th, 2019; approval number: 141/19, for the project PI19/00560). Patient informed consent was obtained for the human tissue samples used to isolate primary fibroblasts populations or to generate HNSCC-derived cell lines.

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

CRediT authorship contribution statement

Llara Prieto-Fernandez: Methodology, Investigation, Formal analysis and Visualization; **Maria de los Angeles Villaronga:** Methodology, Investigation, Formal analysis and Visualization; **Francisco Hermida-Prado:** Investigation; **Maruan Hijazi:** Investigation; **Irene Montoro-Jimenez:** Investigation; **Marta Pevida:** Investigation; **Sara Llames:** Investigation; **Juan Pablo Rodrigo:** Resources, Writing - Review & Editing; **Pedro Cutillas:** Formal analysis and Writing - Review & Editing; **Fernando Calvo:** Formal analysis and Writing - Review & Editing; **Juana Maria Garcia-Pedrero:** Supervision, Funding acquisition and Writing - Original Draft; **Saul Alvarez Teijeiro:** Conceptualization, Methodology, Investigation, Visualization, Formal analysis and Writing - Original Draft.

Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The mass spectrometry phosphoproteomics data produced in this study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035527 and 10.6019/PXD035527.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114176.

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