

Interaction of tumor cells and Cancer-associated fibroblasts in radiation-induced senescence

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ABSTRACT

Background: Despite significant progress in tumor therapy, recurrences continue to pose a major challenge in clinical oral oncology. Recent studies suggest that senescence and intercellular signaling within the tumor microenvironment play a crucial role in post-radiation recurrence. As these mechanisms remain incompletely understood, this study examined the interactions between co-cultured tumor cells and cancer-associated fibroblasts (CAFs) under the influence of radiation.

Methods: A549 tumor cells and primary CAFs were cultured individually and in co-culture and then exposed to 16 Gray irradiation. Changes in cell morphology and secretome were measured, and medium transfer experiments assessed tumor cell migration. Six days post-irradiation, the proportions of apoptotic, necrotic, dead, and viable cells were assessed. Long-term cultures were used to document tumor cell repopulation.

Results: Post-irradiation fewer tumor cells underwent senescence in co-culture with CAFs compared to monoculture, without an increase in long-term tumor cell repopulation in the co-cultures. MCP-1 (CCL2) secretion was lower in co-culture, potentially contributing to the reduced senescence. IL-6 secretion was higher, consistent with its role in promoting tumor invasion. IL-8 expression was elevated in non-irradiated co-cultures; its reduction post-irradiation may explain the lower senescence in co-culture. CAFs produced OPG, potentially driving migration. TIMP-1 was consistently secreted across all conditions. CAF-conditioned medium promoted migration of A549 and FaDu cells.

Conclusion: This study demonstrates that CAFs modulate tumor cell senescence and enhance migration following irradiation. These findings highlight the role of CAFs in progression and radiation response. Targeting CAF-related mechanisms during radiotherapy may improve therapeutic outcomes across epithelial cancer types.

1. Background

One of the main topics of research in oncology is the treatment of epithelial tumors, which include malignancies such as lung, gastric,

colorectal and oral carcinomas (OSCC) [1]. These tumors are collectively responsible for a substantial global cancer burden, with rising incidence rates in several regions [2,3]. Despite the clinical relevance of these tumor types, therapeutic advances remain limited. For example,

Abbreviations: alpha-smooth muscle actin, α -SMA; cancer-associated fibroblasts, CAFs; C-chemokine ligand 2, CCL2; cluster of differentiation 31, CD31; cluster of differentiation 45, CD45; collagen type I, COL1A2; epithelial cell adhesion molecule, EPCAM; fibroblast activation protein, FAP; fibroblast-specific protein-1, FSP-1; Gray, Gy; head and neck squamous cell carcinoma, HNSCC; hemoglobin alpha chain, HBA1; interleukin 6, IL-6; interleukin 8, IL-8; monocyte chemoattractant protein-1, MCP-1; oral squamous cell carcinoma, OSCC; osteoprotegerin, OPG; phosphate-buffered saline, PBS; platelet-derived growth factor receptor A, PDGFRA; platelet-derived growth factor receptor B, PDGFRB; podoplanin, PDPN; protein tyrosine phosphatase, receptor type, C, PTPRC; quantitative polymerase chain reaction, qPCR; S100 calcium binding protein A4, S100A4; senescence-associated secretory phenotype, SASP; standard deviation, SD; TATA box binding protein, TBP; tissue inhibitor of metalloproteinase-1, TIMP-1; transferrin receptor, TFRC; vimentin, VIM.

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head and neck epithelial tumors have received relatively few new FDA-approved therapies in recent years compared to other cancers [4].

Therefore, efforts are being made to continuously improve surgical techniques, chemotherapy, and radiotherapy strategies [5]. The combination of these methods can significantly improve patients' chances of survival because recurrence remains a significant factor determining mortality with rates staying high at approximately 40 % over the past decades in certain tumor entities [6,7]. Consequently, the 5-year survival rate has also remained constant for a long time [7].

Adjuvant radiotherapy is commonly applied to eliminate residual tumor cells following surgical resection in epithelial cancers [8]. Advanced techniques such as 3D conformal or intensity-modulated radiotherapy are widely used in both definitive and adjuvant settings, ideally within six weeks after surgery [9]. For example, national guidelines in Belgium emphasize conventional fractionation (60–66 Gy over 6–6.5 weeks) and stress avoiding treatment interruptions to ensure efficacy in OSCC treatment [10]. More and more, relapse is also associated with tumor cells entering a dormant state called senescence [11,12]. As a result tumor cells not only lose their ability to divide but also undergo changes in their appearance and metabolism [13]. Senescence was previously considered irreversible and potentially desirable for cancer therapy [14]. However, recent research shows that re-proliferation can occur after a period of dormancy, leading to clinical recurrence [15,16]. Wang and colleagues demonstrated that patients whose tumors exhibited high levels of senescence-associated gene transcripts had reduced survival rates. [17].

Senescent cells modulate their microenvironment by producing a unique set of cytokines. This pattern of secretion is known as the senescence-associated secretory phenotype (SASP) [13]. It primarily consists of proinflammatory signaling molecules but can also vary depending on the context [18]. This secretory profile can activate key signaling pathways in surrounding tumor cells, promoting survival and proliferation. Some researchers consider SASP to be a protective mechanism that activates the immune response. This allows cells from the innate and adaptive immune systems to be directed towards cancer cells or precancerous lesions to eliminate them [19]. However, it also might have negative effects: Components of the SASP can promote cancer cell growth, invasion, metastasis, and tumor vascularization, further contributing to a tumor-supportive microenvironment. [20].

This tumor microenvironment is furthermore shaped by cancer-associated fibroblasts (CAFs). CAFs can originate from various precursor cells and have a long, spindle-shaped appearance [21]. They are characterized by ongoing activation and play a role not only as a basis and support but also by producing growth factors, chemokines, and cytokines that actively promote tumor cell growth, angiogenesis, and the invasion of immunosuppressive cells [22]. They are also involved in tumor invasion through different mechanisms among them the production of matrix metalloproteinases [23,24]. It is believed that interrupting the interaction and communication between CAFs and cancer cells could be a target for modern therapies [25].

However, the role of CAFs in radiation-induced senescence and their contribution to this process remain poorly understood. Importantly, within the tumor microenvironment, not only cancer cells but also CAFs are exposed to irradiation during radiotherapy. Yet, there is limited understanding of how CAFs themselves respond to radiation, including their activation status, secretory behavior, and potential to undergo senescence, or how radiation influences their communication with tumor cells and other components of the microenvironment. These interactions under irradiation remain poorly explored, representing a critical gap in our knowledge that is central to the focus of this study.

The aim of this study was to investigate the mutual effects between irradiated CAFs and tumor cells *in vitro*, with a particular focus on senescence. Important questions included whether the proportion of senescent tumor cells differs when CAFs are present compared to when they are cultured alone, whether there are differences in the proportion of necrotic, apoptotic, dead, or viable tumor cells, and ultimately,

whether changes in the secretion of cytokines may be responsible for these effects.

A better understanding of how senescence in tumor cells and CAFs influences cancer cell behavior and intercellular signaling could identify novel targets to disrupt tumor-promoting communication after radiotherapy.

2. Materials and methods

2.1. Cell isolation and cell culture of tumor cells and CAFs

The head and neck squamous cell carcinoma (HNSCC) cell line FaDu was obtained from a collaborating research group and its identity was verified via short tandem repeat (STR) analysis in 2016. H357 cells were purchased from Sigma-Aldrich (St. Louis, MO, USA), SCC-9 cells from the American Type Culture Collection (ATCC, CRL-1629™), and HNSCCUM-04 N cells were isolated in-house in our laboratory [26]. All tumor cells were maintained in DMEM/Ham's F12 medium (Sigma-Aldrich) supplemented with 5 % BCS (VWR Life Science, Radnor, PA, USA) and 2 % penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

The non-small cell lung carcinoma cell line A549, obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, was employed for the experiments due to its well-established propensity to undergo senescence after exposure to radiation. The decision to use A549 cells was based on the desire to eliminate additional variables introduced by primary tumor cells, which are inherently harder to standardize. Although head and neck carcinoma cell lines also exhibit radiation-induced senescence, as shown by Schötz et al., the proportion is only around 20 %, making them less suitable for our specific experimental setup [27]. A549 is a well-established cell line in senescence research [28–30]. After radiation, almost all cells become senescent [16,31]. Wiesmann and colleagues also showed that re-proliferation is possible. Therefore, this cell line is particularly suitable for studying the underlying mechanisms of senescence and radiation resistance [16].

Primary human CAFs were isolated from OSCC samples obtained during tumor resection from patients who underwent surgery at the Department of Oral and Maxillofacial Surgery, University Medical Center Mainz, Mainz, Germany. The tissue for the cultivation of CAFs was taken from the tumor tissue in such a way that the histological assessment of the tumor margins was not affected. A second tissue sample was taken from the same patient at the site of tumor resection from clinically healthy oral mucosa at the greatest possible distance from the tumor margin. This second tissue sample served as reference sample. It was used to isolate normal fibroblasts from the same patient and was utilized as control for the characterization of the CAFs via qPCR. This study was performed in agreement with the declaration of Helsinki on the use of human material for research. All patients provided signed informed consent prior to participating in the study. The Ethics Committee of the State Medical Association of Rhineland-Palatinate Ethics Committee approved this study (ethics vote Votum: 2022-16424_3; year of ethics vote: 2022). The fibroblasts were isolated from the tissue by out-grow from the sample avoiding enzymatic digestion. To prevent contamination by fungi, the samples were first swabbed in phosphate-buffered saline (PBS) with amphotericin before being fragmented. The sample was then divided into three to six fragments with a size of approximately 2–3 mm³ and placed in a 6-well plate. To improve the adhesion of the fragments to the well plate, they were incubated at 37 °C for seven minutes. Then they were submerged in culture medium (Dulbecco's modified Eagle medium with 10 % fetal bovine serum (Gibco®, Life Technologies™, Paisley, UK), 1 % penicillin-streptomycin-neomycin, 1 % L-glutamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)). When a sufficiently large number of cells was grown out of the tissue fragments, contamination with mycoplasma was ruled out. The primary fibroblasts were used for the experiments before reaching

the fourth passage.

For the co-culture experiments all cells were maintained in DMEM/Ham's F12 medium (Sigma-Aldrich) supplemented with 5 % BCS (VWR Life Science, Radnor, PA, USA) and 2 % penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Regular checks for mycoplasma contamination were carried out, and cells were incubated at 37 °C and 5 % CO₂.

2.2. Characterization of CAFs

In the first-place fibroblast growth was determined phenotypically. Their identity was then confirmed via qPCR by detecting the mRNA expression of typical fibroblast markers and by exclusion of the expression of markers of other cell types using a PrimePCR Custom 96-well Plate (Bio-Rad Laboratories Inc., Hercules, USA) with the following predesigned and validated PrimePCR™ Assays (see Table 1). To identify suitable reference genes, the PrimePCR Pathway Plate, Reference Genes H96, human (Bio-Rad Laboratories Inc., Hercules, USA), was used according to the manufacturer's instructions and TBP and TFRC were chosen.

The non-expression of EPCAM served to exclude the isolation of epithelial / tumor cells, the non-expression of CD31 to exclude endothelial cells, and the non-expression of CD45 to exclude lymphocytes. Fibroblasts furthermore exhibited expression of typical fibroblast markers such as vimentin, collagen type I, and S100A4. Furthermore, α -SMA, PDPN, PDGFRA, PDGFRB, and FAP were employed to verify isolated fibroblasts as CAFs based on their established use in identifying these cells [32,33]. Since fibroblasts, and especially CAFs, are very heterogeneous, it was decided to determine the identity of the cells using several markers [25,32,33].

2.3. Co-culture procedure and irradiation

Fig. 1 provides an overview of the experimental timeline: Beginning on the day before irradiation (Day -1) A549 cells were detached through trypsin digestion (Sigma-Aldrich, St. Louis, MO, USA), and treated with the CellTrace™ Cell Proliferation Kit (Thermo Fisher Scientific (Darmstadt, Hessen, Germany) following the manufacturer's instructions. With approximately 10⁶ cells/ml PBS, a final concentration of 1 μ M was applied for a 20-min incubation period. After deactivation and removal of the staining solution, cells were manually counted using a Neubauer counting chamber.

For monoculture experiments, 120,000 CAFs or 40,000 A549 cells were seeded into one 25 cm² cell culture flask. In co-culture experiments, 20,000 A549 cells were co-cultured with 80,000 CAFs 25 cm² cell culture flasks in direct contact. These cell ratios were selected based on preliminary validation experiments considering the approximate proliferation rates. Irradiation was administered after 24 h with a dose of 16 Gy using a Cs¹³⁷ gamma source (Day 0). This dosage is known to reliably induce senescence in a substantial proportion of cells without causing excessive cell death [16]. Additionally, this approach aligns with contemporary radiation therapy strategies that prioritize delivering higher dosages over fewer sessions to curb radiation resistance induction (FLASH radiotherapy) [34]. Cells were cultured until Day 6. Medium was changed on Day 2 and replaced with serum-free medium on Day 4. Cells were harvested on Day 6 post-irradiation for further analysis, encompassing flow cytometry, beta-galactosidase staining, and collection of the supernatant. Additional culture flasks of irradiated A549 cells and the co-culture were investigated every two days for 50 days to check whether repopulation occurred.

2.4. Senescence-associated beta-galactosidase staining

Senescent cells express increased amounts of lysosomal hydrolase, which reacts with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside to generate a perinuclear blue stain detectable by light microscopy. This staining is one of the standard methods for senescence detection [35].

The Senescence beta-Galactosidase Staining Kit (#9860 Cell Signaling Technology®, Danvers, MA, USA) was employed according to the manufacturer's instructions as previously described [16]. After medium removal, cells underwent PBS washing, fixation, and exposure to the staining solution at an exact pH of 6 overnight. Cells were covered with glycerin for long-term storage. To quantify the amount of senescence-associated β -galactosidase activity the integrated density (mean blue value $\times$ area) of the staining was assessed using ImageJ Fiji, allowing for integration of the intensity of the signal and the area occupied by it [36,37]. For this purpose, five representative images were acquired from each irradiated 25 cm² culture flask and the respective control flasks. A normalization to cell number was omitted, as the different cell lines showed substantial differences in both post-irradiation survival and cell size. To assess the relative increase in staining intensity, values from irradiated samples were normalized to their respective unirradiated controls, thereby providing a fold-change in senescence-associated β -galactosidase activity.

2.5. Flow cytometry

Flow cytometry was employed to measure the proportions of alive, dead, apoptotic, and necrotic cells within the tumor cell population. Within the living cell subset, senescent and non-senescent cell fractions were quantified. In co-cultures, the CAF-to-tumor cell ratio was also assessed. On Day 6, cells were harvested and washed twice with PBS. Subsequently, cells were suspended in Annexin V binding buffer. For apoptosis/necrosis staining, Annexin-FITC (apoptosis marker) and Sytox Blue (live/dead dye) were utilized. The previously mentioned CellTrace reagent (Red 660/20) was used to distinguish between A549 and CAFs. A high intensity of the CellTrace signal indicated arrested proliferation.

Analysis was carried out using a BD FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data interpretation was conducted using Cytobank (<https://community.cytobank.org/cytobank/login>). Cell identification began with forward scatter (FSC) and sideward scatter (SSC) analysis to exclude debris. To differentiate between A549 and CAFs within co-culture, the CellTrace signal (Red 660/20) was combined with FSC parameters, as only A549 cells were stained with the CellTracer. Apoptotic, necrotic, and live cells were distinguished based on FITC (Annexin-FITC) and Violet 450/50 (Sytox Blue) signals. Cells stained only with Annexin-FITC were classified as apoptotic, those stained only with Sytox Blue as necrotic, and cells positive for both dyes as dead. Viable cells were identified by non-staining with Sytox Blue.

Considering the anticipated cell cycle arrest observed in senescent cells, intracellular dye dilution of the CellTrace reagent was negligible in senescent cells. Accordingly, A549 cells exhibiting pronounced signals in this channel were classified as senescent. In contrast, the dominant population in untreated samples was designated non-senescent. For a detailed visualization of the gating strategy please refer to the supplementary file.

2.6. Cytokine array

To examine the secretion of key cytokines associated with senescence and potential alterations thereof, the Human Cytokine Array C5 (Ray-Biotech, Peachtree Corners, GA, USA) was employed, adhering to the manufacturer's guidelines. Table 2 shows the array map. Supernatants from irradiated and non-irradiated cells were collected on Day 6 after 48 h culture in serum-free medium, followed by centrifugation to remove cellular debris and deceased cells. The supernatant samples were stored at -80 °C until use. Deceased cells were not discarded but also included in the flow cytometry analysis. Membranes were blocked for 30 min and then incubated with supernatants overnight at 4 °C. Biotin-conjugated soluble antibodies and horseradish peroxidase-conjugated streptavidin facilitated detection.

Documentation and densitometric analysis were executed using the

Table 1
Marker genes used for identification and characterization of CAFs via qPCR.

Gene Symbol	Gene Name	Target Cell Type	Unique Assay ID	Amplicon Context Sequence
ACTA2	alpha smooth muscle actin (α -SMA)	CAFs, smooth muscle cells	qHsaCID0013300	GCGGCAGTGGCCATCTCATTTTCAAAGTCCAGAGCTACATAACACAGTTTCTCCTTGATGT CCCGGACAATCTCACGCTCAGCAGTAGTAACGAAGGAATAGCCACGC
COL1A2	collagen type I	fibroblasts	qHsaCED0003988	CTAGACAGAGATGAAGTGGGTCCTGTTTGTGTTTCATAATACAAAGGTGCTAATTAAT AGTATTTTCAGATACTTGAAGAATGTTGATGGTGCTAGAAGAATTGAGAAGAAATCTCTGT ATTGAGTTGTATCGTGTGGTGTATTTTTTAAAAAATTTGATTT
EPCAM	epithelial cell adhesion molecule	epithelial cells (tumor cells)	qHsaCID0023244	TGCACITTCAGAAGGAGATCACAACGCGTTATCAACTGGATCCAAAAATTTATCAGGATTTT TGTATGAGAATAATGTTATCACTATTGATCTGGTTCAAATTTCTTCTCAAAAACTCAGAA TGATGTGGACATAGCTGATGTGGCTTATTATTTGAAAAAGAT
FAP	fibroblast activation protein	CAFs	qHsaCID0018575	GCTTTAGGAAGTGGGTCACTGTGGGTGTATAAGTGGTTCGTGGACAGGCCGGATAAGCCGTG GTTCTGGTCAGAGTACCACATTGCCTGGAAATCCACTTGTGCAATTAAC
HBA1	CD31	endothelial cells	qHsaCED0020775	CAGAGCTTTAGCAATCTG CGTTAAGCTGGAGCCTCGGTGGCCATGCTTCTTGCCTCTGGGCTCCCCCAGCCCCCTCCTC CCCTTCTGCACCCGTACCCCGTGGTCTTTGAATAAAGTCTGAGTGGCGGCAAGGA
PDGFRA	platelet-derived growth factor receptor A, CD140A	CAFs	qHsaCID0007202	TTCCATCCGGCGTTCCTGGTCTTAGGCTGTCTTCTCAGGGCTGAGCCTAATCCTCTGCCAG CTTTCATTACCTCTATCCTTCCAAATGAAAATGAAAAGTTGTGCAGCTGAATTCATCCTTTTCTCTG
PDGFRB	platelet-derived growth factor receptor B, CD140B	CAFs	qHsaCID0013272	TGGTAGCTGAAGCCACGAGGTCCATGTAGCTTAGCAGTGGAGACTCGTTGATCAAAGTTGCTCGG CAGGTCTCTCAGGGGCGAGGGAACGTAGTTATCGTAAGGGGCCATGTAGTTGGAGGACTC GATGTCTGCATATTTGACG
PDPN	podoplanin	CAFs	qHsaCID0009013	GTGGCAACAAGTGTCAACAGTGTAAACAGGCATTGCGATCGAGGATCTGCCAACTTCAGAAAGCACAGTC CACGCGCAAGAACAAGTCCAAGCGCCACAGCCTCAAACGTGGCCACCAGTCACTCCACGGAGAAAGT GGATGGAGACACACAGACAACAGTTGA
PTPRC	CD45	lymphocytes	qHsaCED0038908	AAGGACGATGCTGTTTCTTAGGGACAGGCTGACTTCCAGATATGACCATGTATTTGTGGCTTAAAC TCTTGCAATTTGGCTTTGGCTTTCTGGACAC
S100A4	S100 calcium binding protein A4, fibroblast-specific protein-1 (FSP-1)	fibroblasts	qHsaCID0013749	CGATGCAGGACAGGAAGACACAGTACTCTTGAAGTCCACCTCGTTGTCCCTGTTGCTGTCCAAGT TGCTCATCAGCTTCTGGAAGCAGCTTCATCTGCTCTTTCCCAAGAAGCTGGGAGCTCCCGGT CAGCAGCTCCTTATGTTCTGACTTGTGAGCTTGAACCTGTACACCTCTTTGCC
TBP	TATA box binding protein	reference gene	qHsaCID0007122	AAATATGTATCCACAGTGAATCTTGGTTGTAAACTTGACCTAAAGACCAATTGCACCTTCGTGCCC GAAACGCCGAATATAATCCCAAGCGGTTTGCTGCGGTAATCATGAGGATAAGAGAGCCACGAA CCACGGCACTGATTTTCAGTTC
TFRC	transferrin receptor, CD71	reference gene	qHsaCID0022106	AGCATTCCCGAAATCTGTTGTTAGTCTGGAAGTAGCACGGAAGAAGTCTCCACGAGCAGAATACA GCCACTGTAAACTCAGGCCCATTTCTTTATGTCTGCTGTATTGGTTTCAGATCCCTCACAAATGA ATTGCAGGAGGAGATGCTTCAGAGAGAGGAAGCCGAAAACACCCCTGCAATCTTTCAGACAGGA
VIM	vimentin	fibroblasts	qHsaCID0012604	TGTTGACAAATGCGTCTCTGGCACGTCTTGACCTTGAACGCAAGTGGA

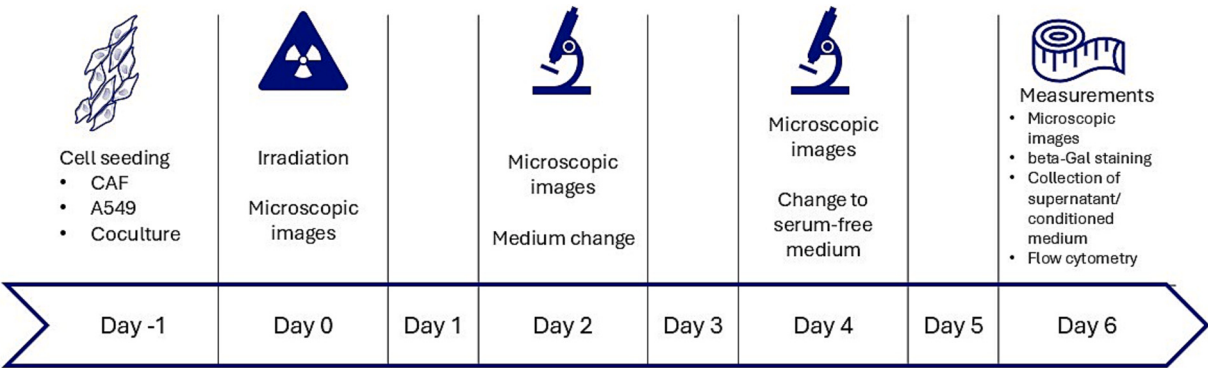


Fig. 1. Experimental Timeline Cells (CAF, A549, co-culture) were seeded on day –1 and cultured for six days following irradiation, with microscopic monitoring and medium changes every second day. Key measurements were conducted on day 6.

ChemiDoc XRS+ system (BioRad, Dreieich, Hessen, Germany) in conjunction with Image Lab software (BioRad, Dreieich, Hessen, Germany). Positive controls were used to standardize results across different membranes. The results were adjusted based on the occupied cell culture surface area in each respective culture, as determined using the software QuPath (Queen’s University, Belfast, Northern Ireland). Representative microscopic images were analyzed with the software by manually marking and outlining the proportion of the surface area covered by cells. If the entire surface was covered by cells, the adjustment factor was set to 1. However, if, for example, only 80 % of the surface was occupied, the values were multiplied by 1.25 to normalize the results. This adjustment ensured that the data accounted for variations in cell

coverage across different samples and provided a more accurate representation of the experimental outcomes.

2.7. ELISA

Guided by the results of the membrane-based cytokine array, interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) (alternative name: C-chemokine ligand 2 (CCL2)), osteoprotegerin (OPG) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were chosen for subsequent analysis via ELISA. For the selection of the cytokines their high relevance for cancer progression and their known influence on senescence were relevant but also for example the

Table 2
Human Cytokine Array C5.

	A	B	C	D	E	F	G	H	I	J	K
1	POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GM-CSF a/ b/g IL-8 (CXCL8)	GRO alpha (CXCL1)
2	I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN- gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
4	MIP-1 delta	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
5	OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8–1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
6	FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha	NAP-2 (CXCL7)	NT-3
8	NT-4	OPN (SPP1)	OPG (TNFRSF11)	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

extraordinary high signal for MCP-1 in the Cytokine Array. Procedures adhered closely to manufacturer's instructions, displaying minor differences between distinct kits (R&D Systems DuoSet ELISA kits: catalogue# DY206, DY208, DY279, DY805, DY970). Detection was performed using a plate reader (Multiskan Ascent®, Thermo Fisher Scientific GmbH, Rockford, USA). The raw data were analyzed using the MyAssays Data Analysis Tool (www.myassays.com, MyAssays Ltd., USA) with a four-parameter logistic (4-PL) curve fit, yielding a regression coefficient (R^2) of at least 0.9859. The results were adjusted according to the occupied cell culture surface area which was determined using the software QuPath.

2.8. Migration analysis

To investigate the influence of CAF-conditioned medium on the migration of tumor cells, we utilized the previously generated supernatants for stimulating scratch assays using A549 and FaDu cells. The conditioned media were obtained using CAF cultures from five different patients. The CAFs had been cultured for two days under serum-free conditions, resulting in conditioned media without FCS. Accordingly, control experiments were performed using medium without FCS to ensure consistent conditions. FaDu cells were used because they are among the most commonly employed cell lines in HNSCC research.

Cellular migratory capacity was assessed using culture inserts (ibidi® GmbH, Munich, Germany) as previously described [38]. The scratch assay chambers were placed into a 12-well plate. Twenty-four hours prior to treatment, 70 µl of a suspension containing 500,000 tumor cells / ml was seeded to each chamber. After 24 h of incubation, the inserts were removed, and the cells were treated with 1 ml CAF-conditioned medium. Since irradiation reduced the proliferation capacity of CAFs, we diluted the conditioned medium based on total cell count to improve comparability between the effects of the medium from irradiated and untreated CAFs. Tumor cells maintained in serum free medium served as control. The cell-free area in the monolayer was photographed at 8, 16, 24 and 48 h after adding the conditioned media. Gap closure was then objectively evaluated using the T-Scratch software (<https://www.cselab.ethz.ch/software/>, accessed on 21 September 2020) for a computer-assisted analysis.

2.9. Statistical analysis

Statistical significances for this exploratory study were assessed using a *t*-test, a one-way, or a two-way ANOVA respectively such as applicable, followed by post hoc Bonferroni corrections for pairwise comparisons. All analyses were conducted using GraphPad Prism 10 for Windows, Version 10.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as means ± SD, unless otherwise indicated. Statistical

significance levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Characteristics of CAFs

Tumor specimens were obtained from seven patients diagnosed with OSCC. All samples were collected during surgical resection, immediately preserved under standardized conditions, and subsequently processed according to established pathological protocols. Each specimen was classified according to the TNM staging system to ensure accurate stratification of tumor progression (See Table 3).

To identify the isolated cells as CAFs, and to perform a basic characterization, we analyzed the mRNA expression of key fibroblast and CAF markers using qPCR. Expression of all examined markers (see Fig. 2) was detectable in all cell samples; however, expression levels varied substantially between cells from different patients, as expected. Furthermore, cells of other cell types could be excluded in all samples by non-expression of CD45, CD31, and EPCAM. The following figure presents the mean relative expression of the marker genes, expressed as fold change relative to healthy fibroblasts derived from the same patient.

3.2. Radiation-induced cellular senescence in HNSCC and A549

To assess the contribution of radiation-induced cellular senescence to the response of head and neck tumor cells to irradiation, we initially investigated a panel of tumor cell lines (Fig. 3A). Fractionated irradiation was employed to closely mimic the clinically relevant radiotherapy regimen used in the treatment of head and neck cancers. Specifically, cells were irradiated with 2 Gy per fraction, five times per week,

Table 3

TNM classification of the seven CAF-containing tumor samples included in this study. The classification includes the primary tumor size and extent (T), the pathological lymph node status (pN), and the presence or absence of distant metastasis (M), according to the TNM staging system. Additional pathological parameters are reported: L (lymphatic invasion), V (vascular invasion), Pn (perineural invasion), and R (resection margin status).

CAF-Sample	TNM Classification
CAF_P1	pT2, pN0 (0/20), M0, L0, V0, Pn0 R0
CAF_P2	pT2, pN0 (0/44), M0, L0, V0, Pn0 R0
CAF_P3	pT4a, pN1, M0, L0, V0, Pn0 R0
CAF_P4	pT4a, pN2b (2/38), M0, L1, V0, Pn1 R0
CAF_P5	pT4a, pN3b (1/47), M0, L1, V0, Pn1 R0
CAF_P6	pT3, pN3b (1/42), M0, L0, V0, Pn0 R0
CAF_P7	pT3, pN3b (2/28), M0, L0, V0, Pn1 R0

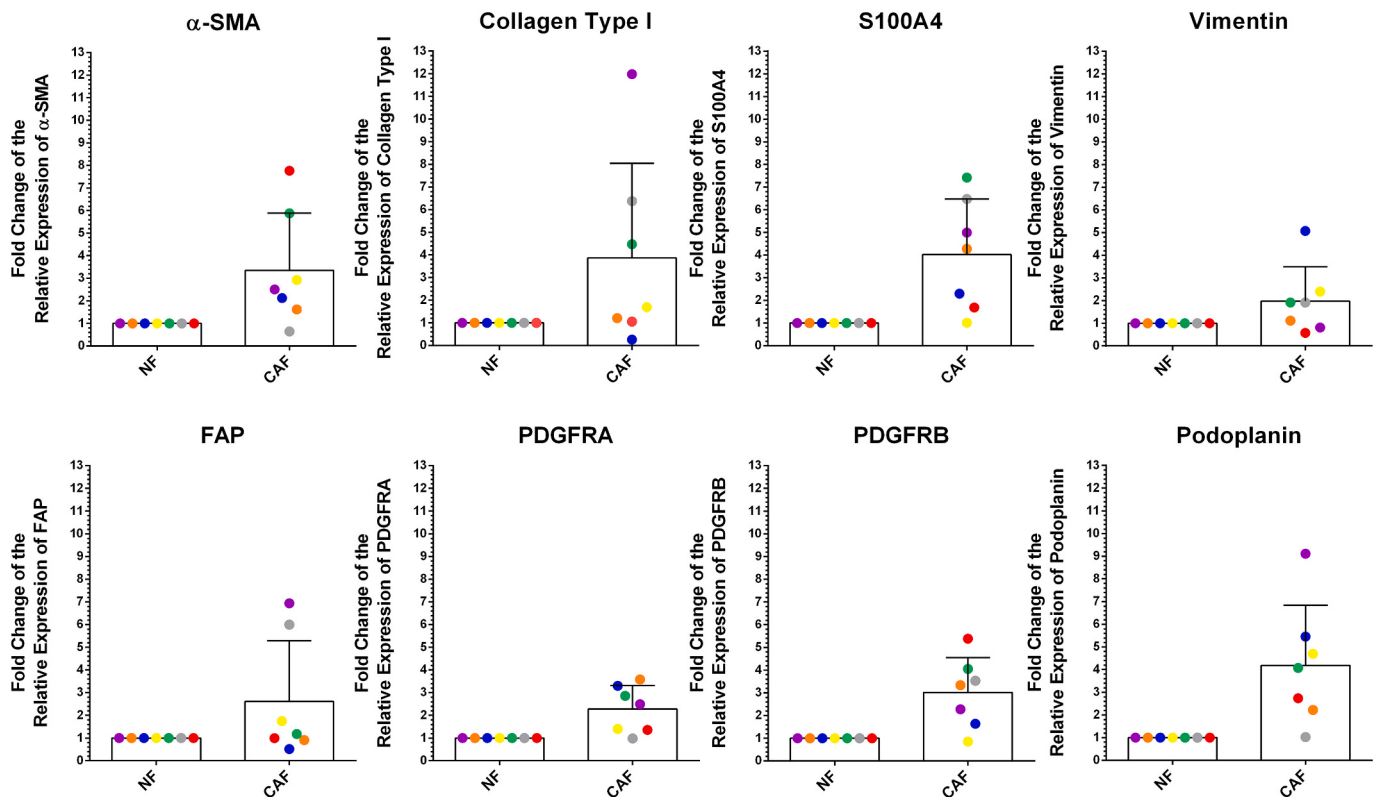


Fig. 2. Analysis of mRNA expression of key fibroblast and CAF marker, including α -SMA, Collagen Type I, S100A4, Vimentin, FAP, PDGFRA, PDGFRB, and Podoplanin in CAF samples relative to matched normal fibroblasts. The figure displays the mean fold change of the mRNA expression of the genes relative to matched normal fibroblasts and the standard deviation as well as the levels of individual mRNA expression of all primary CAFs from the respective samples, with each dot representing a single measurement and each color corresponding to a different patient. The data demonstrates that all investigated fibroblast markers were detectable across all cells, although the expression levels varied considerably between patients.

followed by a two-day break, over a total period of three weeks [8]. This schedule represented the maximum duration and dose that could be feasibly administered in vitro, as extending the treatment to the full six-week clinical protocol was not possible due to the increased radiation sensitivity of cells under in vitro conditions, which led to excessive cell death.

Our results demonstrated substantial inter-cell line variability in response to irradiation, consistent with the known heterogeneity of head and neck tumors (Fig. 3A). Both the extent of radiation-induced cell death and the induction of senescence varied among the different tumor cell lines. Notably, cells that survived fractionated irradiation frequently exhibited features of cellular senescence, as evidenced by increased senescence-associated β -galactosidase activity. These findings suggest that senescence may serve as a survival mechanism in response to radiation exposure in a subset of head and neck tumor cells.

The quantitative analysis of the senescence-associated β -galactosidase activity underlined this variability among the different tumor cell lines (Fig. 3B). A549 cells displayed a marked increase in senescence, with a fold-change of 16 relative to unirradiated controls, whereas the head and neck cancer cell lines FaDu, H357, SSC9, and HNSCCUM-04 N showed more moderate increases of 2, 3, 4, and 8, respectively. Manual cell counting alone did not fully capture these differences, as it fails to account for cell survival. To provide a more representative assessment, we quantified senescence using integrated density, which incorporates staining intensity and cell area and was normalized to unirradiated controls. This approach highlights that A549 cells exhibit the most robust senescence response overall, as nearly all cells survive and undergo senescence, while head and neck tumor cells show lower integrated density values. Furthermore, in the A549 model cell line, radiation-induced senescence was also effectively induced by a single 16 Gy dose, eliminating the need for a fractionated irradiation protocol.

Senescent features emerged within six days after irradiation and persisted up to day 18. These findings support A549 as a suitable model for studying radiation-induced senescence in vitro.

Therefore, we selected A549 cells as a model system, to facilitate a more detailed investigation of radiation-induced senescence in the context of interaction with CAFs. This cell line exhibited minimal radiation-induced cell death, while the vast majority of cells undergo senescence following irradiation (Fig. 3A last line). To optimize experimental conditions, we conducted additional preliminary experiments and determined that a single dose of 16 Gy induces a comparable extent of senescence as a fractionated regimen of 15×2 Gy (Fig. 3). Furthermore, we observed that senescence in A549 cells is fully established by day 6 post-irradiation and persists for several weeks (Fig. 3B). These observations were consistent with previous reports characterizing radiation-induced senescence in A549 cells [16].

Based on these preliminary findings, A549 cells were used for all subsequent experiments, with analyses performed on day 6 following irradiation.

3.3. Morphological changes and induction of senescence following irradiation

To illustrate the morphological changes following irradiation, as well as to demonstrate the behavior of A549 tumor cells and CAFs in co-culture, Fig. 4 presents representative microscopic images of the monolayer cell culture. The cells are shown at day 2, 4, and 6 post-irradiation with 16 Gy, alongside untreated controls. The last column features a senescence-associated beta-galactosidase staining to detect senescence (Fig. 4).

In monolayer culture, non-irradiated A549 cells exhibited an epithelial-like morphology with tightly packed, polygonal cells. They

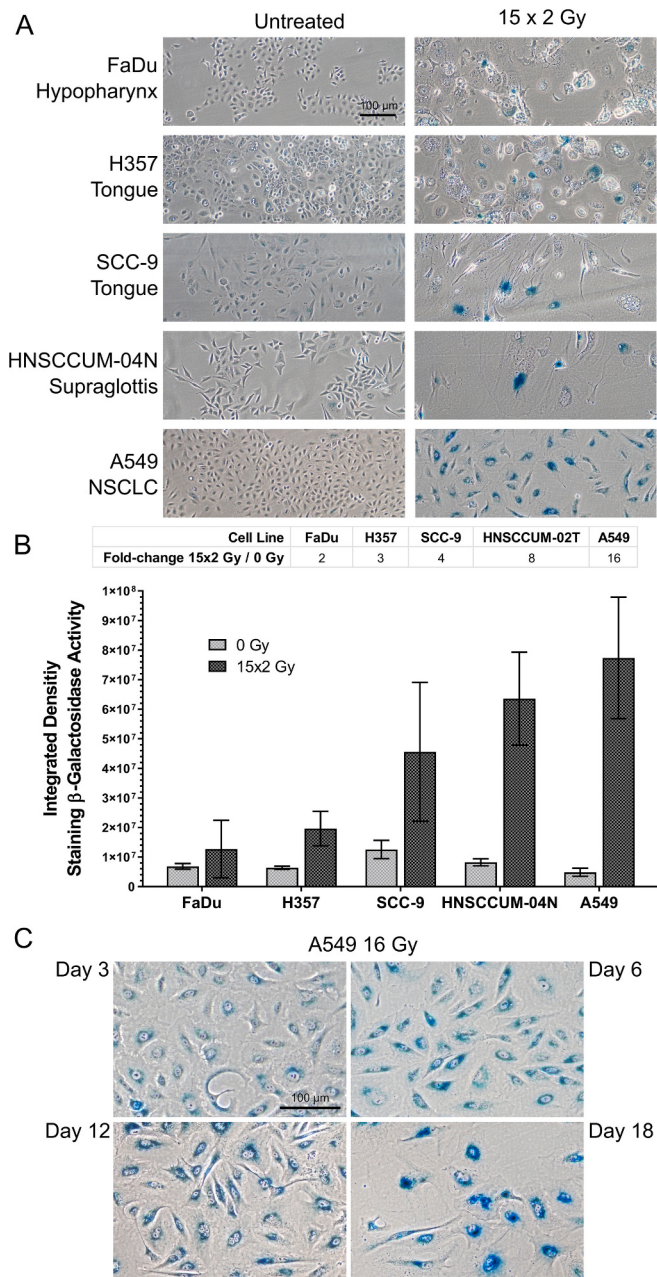


Fig. 3. Senescence-associated beta-galactosidase activity in HNSCC and A549 cells following irradiation. (A) Representative images of senescence-associated beta-galactosidase activity of FaDu, H357, SCC-9, HNSCCUM-04 N, and A549 cells following fractionated irradiation with 15 × 2 Gy, as well as in the non-irradiated control condition demonstrate that senescence is a prevalent phenomenon; however, its manifestation varies depending on the cell line. (B) Quantification of senescence-associated β -galactosidase activity following fractionated irradiation after exposure to 15 fractions of 2 Gy in comparison to untreated control cells is shown as integrated density values of the indicated cell lines using five representative regions of interest (ROI). Bar charts present mean $\pm$ SD. The table above the graph indicates the relative increase in staining intensity between irradiated and untreated samples as fold-change values. (C) In the A549 model cell line, radiation-induced senescence could also be effectively triggered by a single 16 Gy dose. Senescence became apparent within the first six days post-irradiation and persisted through to day 18. All microscopic images were acquired at 100 $\times$ magnification, and the scale shown in the first subfigure also applies to the remaining images within the panel.

displayed a relatively uniform shape with well-defined cell borders. Post-irradiation, A549 cells appeared enlarged with a pronounced nucleus, showing the typical fried-egg-morphology, which is a typical indicator of senescence. The senescence-associated beta-galactosidase staining in the last column further confirmed senescence, as evidenced by the prominent blue signal around the nucleus (Fig. 4A).

CAFs displayed a spindle-shaped morphology that changed only marginally following irradiation. Despite this, a clear positive signal in the senescence-associated beta-galactosidase staining indicated senescence in these cells as well. Even in the untreated culture, a slight

positive signal was present, likely due to the ubiquitous presence of this enzyme. However, the difference in staining intensity between treated and untreated cells was notable (Fig. 4B).

In the untreated co-culture, structures reminiscent of those seen in histological specimens from tissue sections were formed: tumor cell nests surrounded by CAFs. These structures did not form when the cells were irradiated. Furthermore, after irradiation, the distinct identification of cell types became difficult. Nonetheless, the senescence-associated beta-galactosidase staining indicated the induction of senescence in the irradiated co-culture as well (Fig. 4C).

3.4. Fraction of senescent cancer cells in mono-culture and in co-culture

The fraction of senescent tumor cells in the cell cultures was then further investigated using flow cytometry. Using gating, only the proportions of senescent cells among viable cells were recorded (Fig. 5). As expected, very few tumor cells were senescent in the untreated samples. This was observed in both mono-culture and co-culture with CAFs, with only 2.35 % (95 % CI: 1.08–3.63 %) and 1.8 % (95 % CI: 0.81–2.8 %) classified as senescent, respectively. Notably, after irradiation, the condition was particularly striking: While nearly all tumor cells in mono-culture became senescent (98.29 % (95 % CI: 97.42–99.17 %)), a significantly larger proportion of tumor cells in co-culture remained non-senescent (senescent cells in co-culture after irradiation: 84.24 % (95 % CI: 77.04–91.45 %)). The difference was statistically significant ($p < 0.001$).

3.5. Ratio of CAFs and tumor cells in co-culture

Furthermore, the flow cytometric analysis also allowed us to assess

the ratio of CAFs to A549 tumor cells within the co-cultures. It revealed that the ratio of CAFs to A549 tumor cells in the co-culture changed depending on whether the culture was irradiated (Fig. 6). The proportion of CAFs in the irradiated co-culture was significantly higher, at 68.96 % (SD: 8.47 %), compared to the untreated co-culture, with 23.63 % (SD: 10.81 %) ($p < 0.001$). Notably, the variability between individual patients, as indicated by the standard deviations (SD), was comparable in both conditions, suggesting a consistent response pattern among the seven samples.

3.6. Cellular viability and cell death in tumor cells

To quantify the impact of radiation, we measured the proportions of living, necrotic, apoptotic, and dead A549 tumor cells (Figs. 7 and 8) in untreated control cultures and following irradiation. Statistically significant differences were observed in the proportion of living and dead cells between irradiated and non-irradiated cultures. The proportion of viable cells decreased to ~68 % in monoculture and ~74 % in co-culture. Notably, there was no significant difference between co-

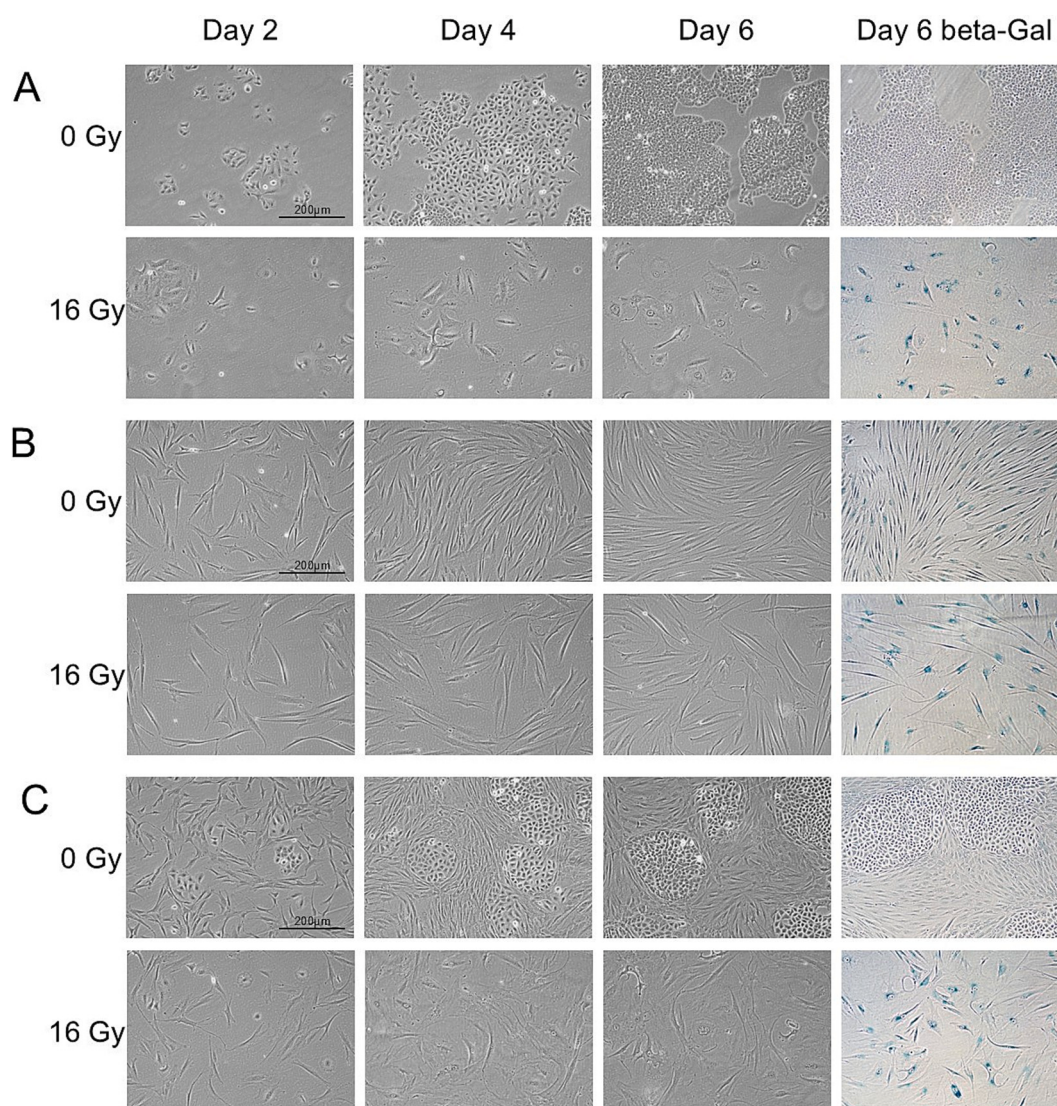


Fig. 4. Cell Morphology After 16 Gy γ -Irradiation. Representative images of A549 cells (A), CAFs (B), and their co-culture (C) are presented at day 2, 4, and 6 following 16 Gy γ -irradiation, alongside corresponding untreated samples. A549 cells exhibited a marked increase in cellular size following irradiation, while CAF morphology displayed minimal alterations. Cells in co-culture demonstrated characteristic formations: tumor cell nests were surrounded by CAFs. The right column depicts senescence-associated beta-galactosidase staining, which was evidently pronounced in irradiated cells across all types and culture conditions. The scale shown in the first subfigure also applies to the remaining images within the panel.

culture and mono-culture but we observed a trend towards a higher proportion of living cells ($p = 0,601$) and a lower proportion of dead cells ($p = 0,0575$) in co-culture in comparison to the mono-culture.

Only minimal differences were observed with respect to necrotic and apoptotic cells (Fig. 8). While an increase in the necrosis rate was observed in the irradiated tumor cells, particularly in the co-culture, strong fluctuations between individual cultures prevented a clearer picture from being drawn. A significant increase in apoptosis after irradiation was not observed in any of the cultures. Therefore, based on the available data, it is difficult to draw a definitive conclusion regarding the underlying cell death mechanism.

3.7. Long-term culture after irradiation

To assess whether the tumor cells, which survived irradiation, were able to repopulate four samples of tumor cell mono-culture and co-cultures with CAFs were cultured for up to 50 days (Fig. 9). The samples were monitored for signs of repopulation every two days. In all four mono-culture samples, cells were observed that no longer tested positive for senescence-associated beta-galactosidase activity and no longer exhibited the typical morphology of senescent A549 tumor cells. Repopulation was inferred based on these observations. This phenomenon was only observed in two of the four co-culture samples.

3.8. Cytokine secretion

To further decipher the influence of the CAFs on the tumor cells and assess the underlying mechanisms more systematically, an array was created for a preliminary evaluation of cytokine secretion (Fig. 10). In total, up to 80 cytokines could have been detected; however, most cytokines were below the detection threshold. Spots with a clear signal were quantified using densitometry. While a single experiment does not necessarily provide conclusive or generalizable results, the goal of demonstrating differences not only between irradiated and non-irradiated cells but also between culture conditions was achieved. Additionally, cytokines were identified that warranted further analysis

using more precise methods, namely ELISA. Since the proliferation of irradiated cells is restricted, but senescent cells occupy a larger surface area, the data obtained with the cytokine array was normalized based on the occupied area in the cell culture to make the groups comparable.

We identified IL-6 as relevant cytokine, because its levels behaved inversely between A549 tumor cells and CAFs. MCP-1 exhibited a particularly strong signal across all samples. IL-8, notably, showed higher levels in untreated co-cultures compared to mono-cultures, while TIMP-1 exhibited the opposite trend, with lower levels in co-culture. OPG was the only cytokine to show higher levels in irradiated compared to non-irradiated CAFs.

Based on the findings from the array, TIMP1, IL-6, IL-8, MCP-1, and OPG were selected for further quantitative investigation of secretion using ELISA. Fig. 11 presents the results of the ELISA analysis. When comparing irradiated and non-irradiated mono-cultures, only IL-8 and MCP-1 showed significantly higher secretion in A549 tumor cells. In contrast, CAFs exhibited no significant decreases or increases in secretion regardless of the irradiation status, indicating that the secretion of the investigated cytokines was maintained following irradiation. Comparing irradiated and non-irradiated co-culture, only MCP-1 displayed significant differences. Comparing non-irradiated cultures, IL-6 secretion was higher in co-culture compared to either tumor cells or CAFs alone, with this difference being significant only in comparison to A549 tumor cells. CAFs also exhibited a significantly higher secretion of IL-6 than A549. Following irradiation, the difference remained significant only between A549 and co-culture. IL-8 secretion showed no differences among non-irradiated groups but was significantly higher in irradiated A549 mono-cultures than in irradiated CAFs and co-culture. MCP-1 secretion was significantly elevated in co-culture compared to non-irradiated mono-cultures. However, after irradiation, MCP-1 secretion was significantly lower in co-culture and CAF mono-culture compared to the A549 mono-culture. No significant differences in TIMP-1 secretion were observed among non-irradiated cultures. However, irradiated CAFs and co-cultures secreted higher amounts of TIMP-1 compared to irradiated A549 cells. Both irradiated and untreated co-cultures and CAFs showed significantly higher OPG secretion than

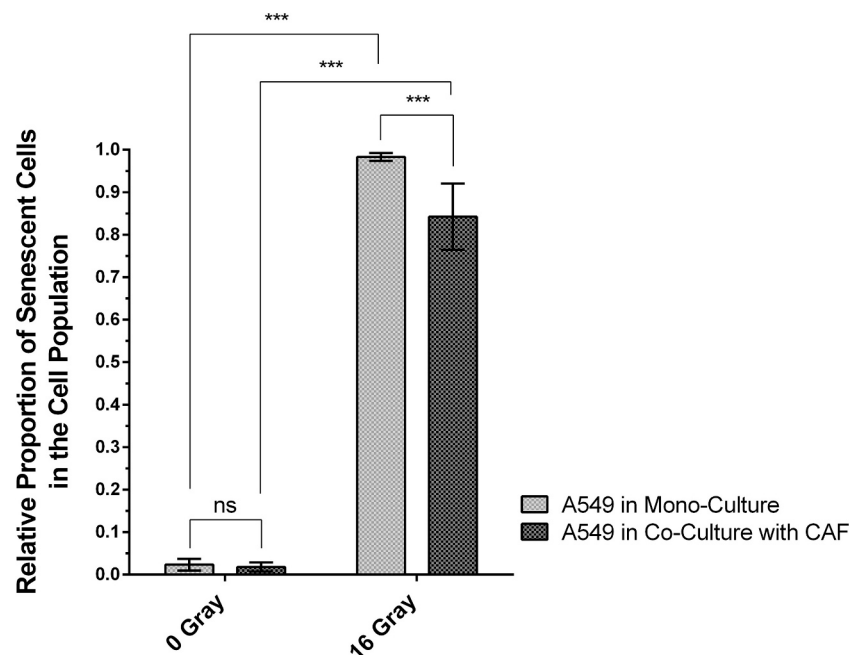


Fig. 5. Fraction of Senescent A549 Tumor Cells. This figure shows the relative proportion of senescent tumor cells under mono-culture and co-culture conditions, following irradiation in comparison to non-irradiated control samples. Without irradiation very few A549 tumor cells were senescent. While nearly all cells in mono-culture became senescent after irradiation, a significantly larger proportion of A549 cells in co-culture remained non-senescent. Shown are means $\pm$ SD. Ordinary one-way ANOVA was performed comparing between 0 Gy and 16 Gy and between mono-culture and co-culture, correction for multiple comparisons by Bonferroni, $n = 7$, (***) $p < 0.001$, ns = not significant).

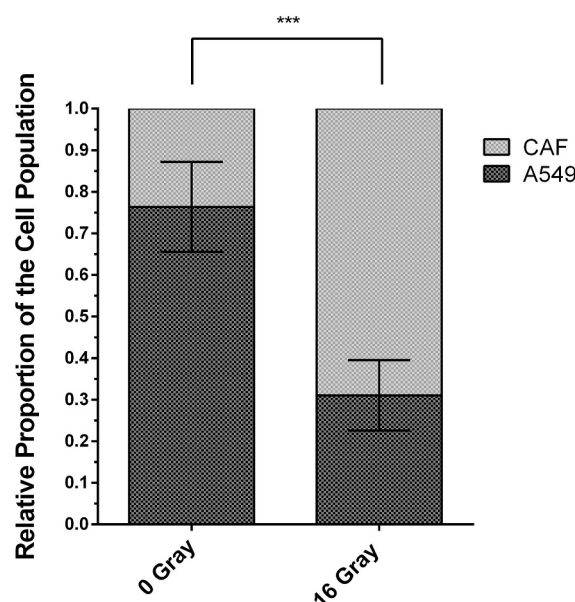


Fig. 6. Composition of Co-Cultures After Irradiation. This figure illustrates the relative abundance of A549 tumor cells and CAFs within co-cultures 6 days after irradiation. The proportion of CAFs in the irradiated co-culture was significantly higher compared to the untreated co-culture. Shown are means $\pm$ SD. An unpaired *t*-test was performed comparing between 0 Gy and 16 Gy, $n = 7$, (***) $p < 0.001$.

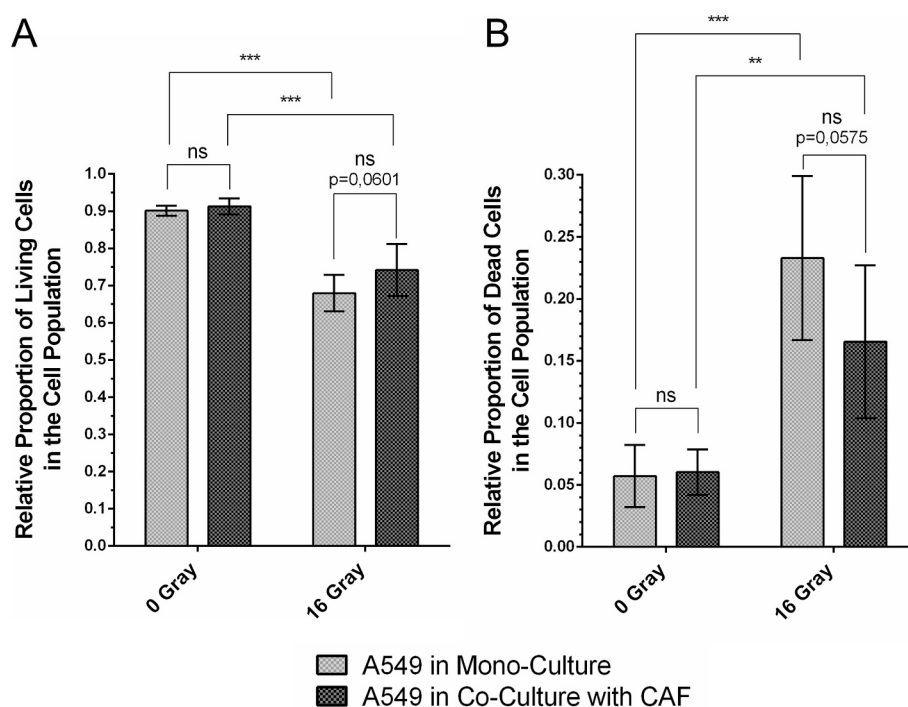


Fig. 7. Cell Viability Following Irradiation. Irradiation significantly reduced the viable tumor cell population ($p < 0.001$) in mono-culture as well as in co-culture with CAFs (A). The percentage of living cells in the cell population decreased to approximately 68 % and approximately 74 % in mono-culture and co-culture, respectively ($n = 7$). Accordingly, the proportion of dead cells was significantly increased after irradiation (B). The differences between mono-culture and co-culture narrowly missed statistical significance with a trend towards a higher proportion of living cells and a lower proportion of dead cells in co-culture. Shown are means $\pm$ SD. Ordinary one-way ANOVA was performed comparing between 0 Gy and 16 Gy and between mono-culture and co-culture, correction for multiple comparisons by Bonferroni, (** $p < 0.01$, *** $p < 0.001$, ns = not significant).

A549 mono-cultures.

3.9. Paracrine effects of CAF-conditioned media on tumor cell migration

The effects of conditioned media from irradiated and non-irradiated CAFs on the migration of A549 and FaDu cells were examined (Fig. 12). To account for differences in cell numbers, conditioned media were

adjusted according to the respective cell count prior to use. Migration was assessed by scratch assay and expressed as relative migration normalized to the control (medium without FCS).

Both A549 and FaDu cells showed enhanced migration when stimulated with CAF-conditioned media compared to the control. The increase in migration was more pronounced in cells treated with conditioned medium from irradiated CAFs than with medium from non-

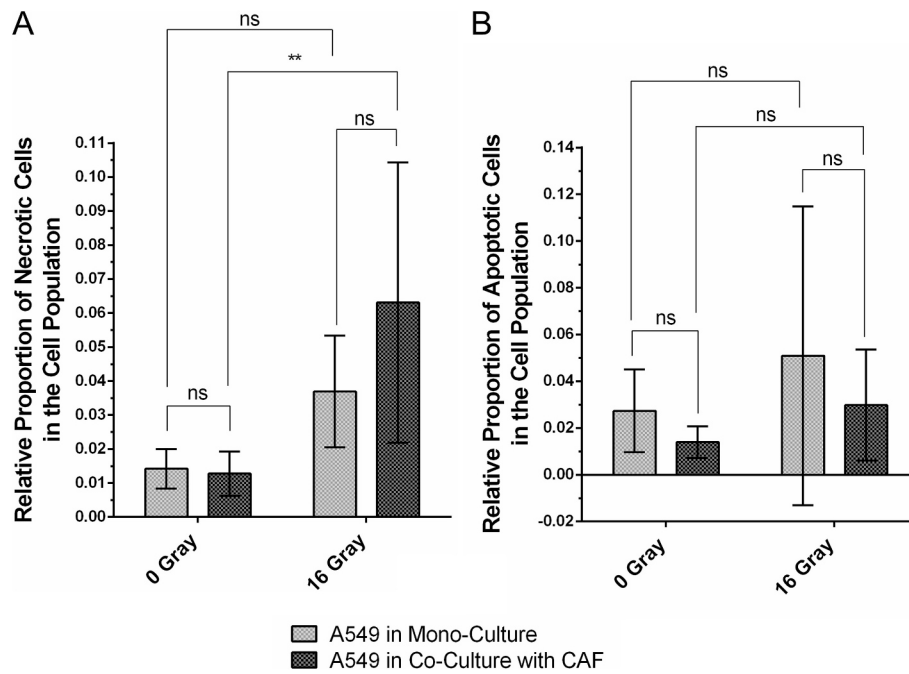


Fig. 8. Cell Death Following Irradiation. Irradiation led to an increase in the relative proportion of necrotic tumor cells (A) which was more pronounced in the co-culture. An increase in apoptotic cell death (B) could not be observed. Shown are means $\pm$ SD. Ordinary one-way ANOVA was performed comparing between 0 Gy and 16 Gy and between mono-culture and co-culture, correction for multiple comparisons by Bonferroni, $n = 7$, (** $p < 0.01$, ns = not significant).

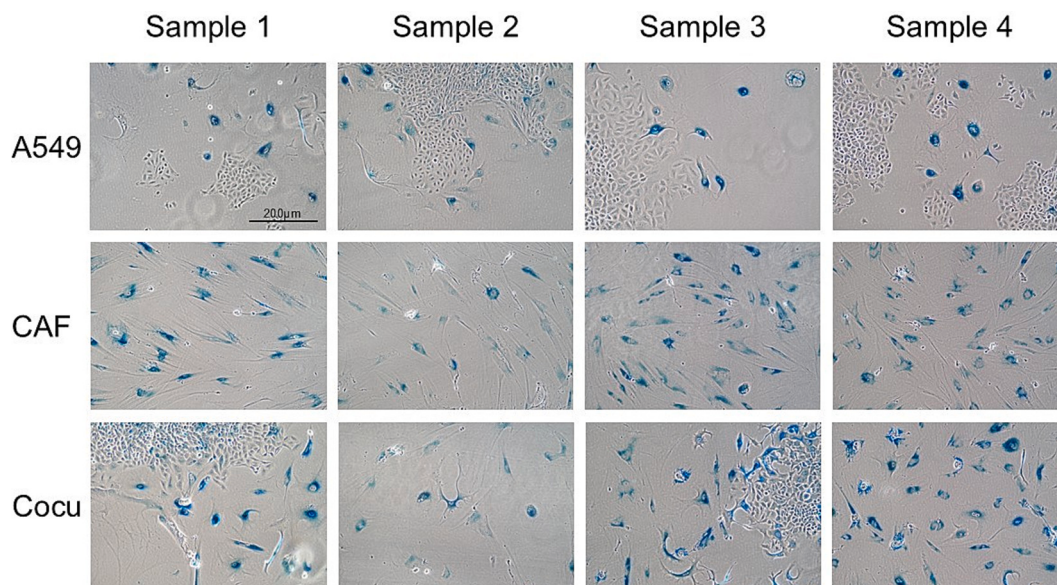


Fig. 9. Long-Term Culture Following Irradiation. Reproliferation was observed in all A549 tumor cell mono-culture samples (Samples 1–4), while only two out of four co-culture samples (Samples 1 and 3) exhibited this phenomenon after a maximum cultivation period of 50 days. The proliferating cells no longer tested positive for senescence-associated beta-galactosidase activity and did not display the characteristic morphology of senescent A549 tumor cells, suggesting a recovery of proliferative capacity. Representative images of the cells stained for senescence-associated beta-galactosidase activity are shown. The scale shown in the first subfigure also applies to the remaining images within the panel.

irradiated CAFs. For A549 cells, this difference was significant at 24 h ($p < 0.05$). In FaDu cells, an even stronger response was observed, with a significantly stronger migration at 16 h and 24 h of FaDu cells stimulated with conditioned medium from irradiated CAFs compared to medium of non-irradiated CAFs ($p < 0.001$).

4. Discussion

This study aimed to elucidate the effects of CAFs on the senescence and the behavior of A549 tumor cells following irradiation. We found

that the presence of CAFs can reduce induction of tumor cell senescence by irradiation, they might facilitate survival of tumor cells and promote tumor cell migration.

4.1. Senescence of tumor cells and CAFs

In our study, we could verify the A549 cell line as a well-established model to study senescence, as a high percentage of senescent A549 cells can be induced through irradiation [16,39]. We observed typical morphological changes of A549 cells following irradiation [40] and also

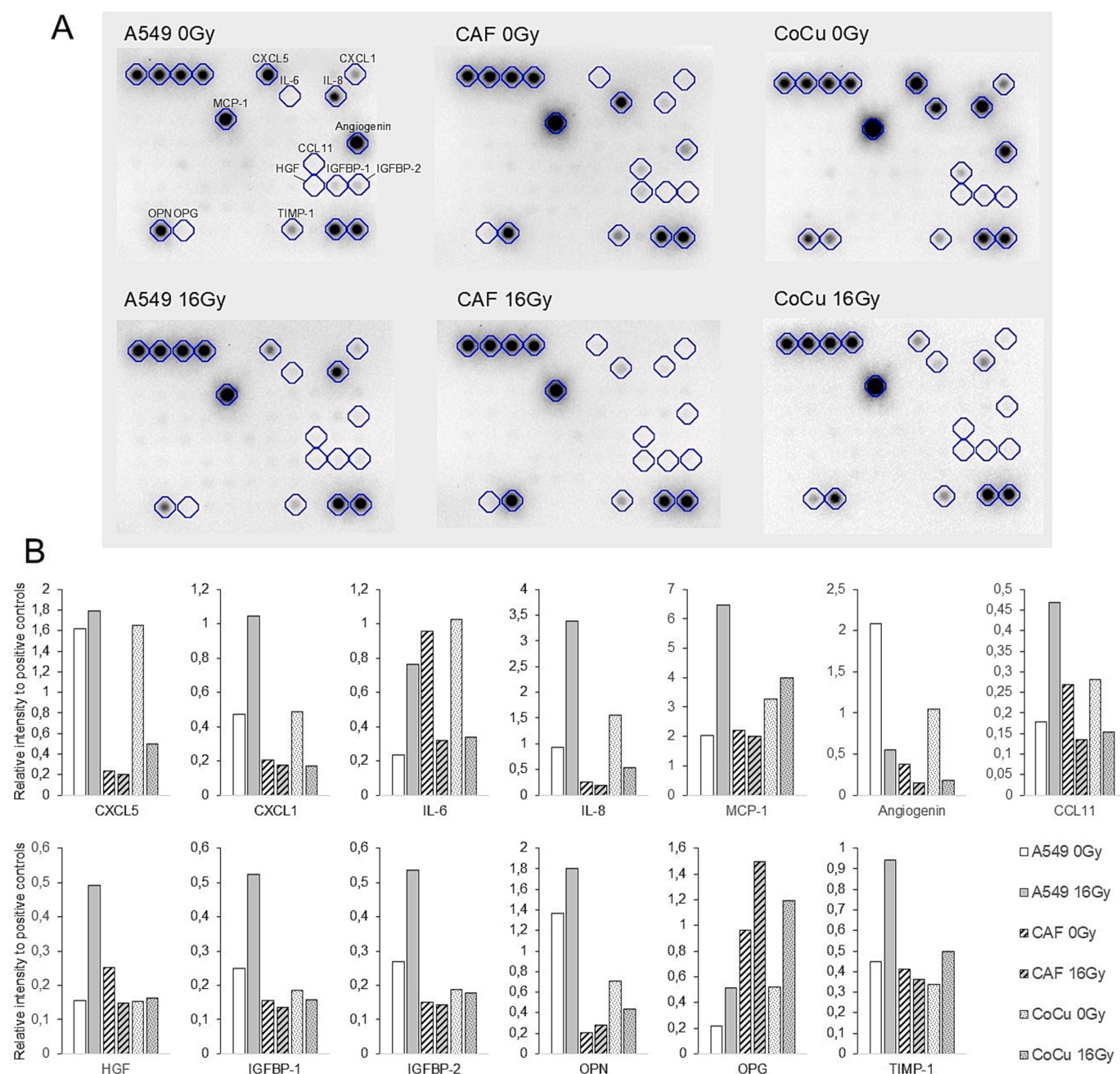
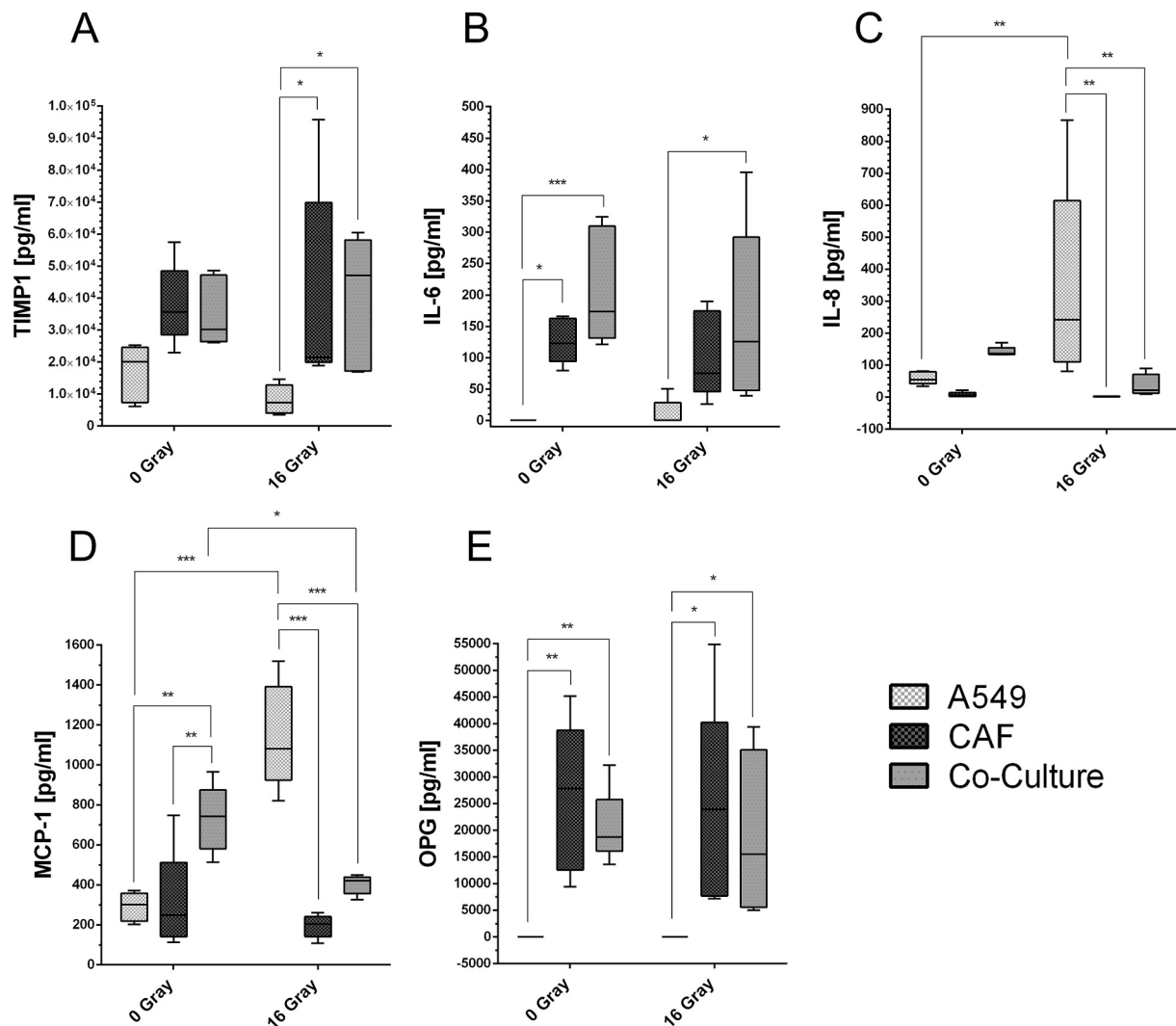


Fig. 10. Cytokine Secretion Profiles in A549 Tumor Cells, CAFs, and Co-Cultures After Irradiation. (A) Images of cytokine array membranes following antibody incubation and labeling show the secretion profile of 80 cytokines in A549 tumor cells, CAFs, and their co-culture after six days of culture with either 0 Gy or 16 Gy irradiation. (B) Signal intensity graphs display the secretion levels of key cytokines, normalized to the occupied cell culture surface area.

demonstrated altered lysosomal enzyme activity through senescence-associated beta-galactosidase staining – which are the most commonly used markers for senescence [41]. Our analysis furthermore demonstrated that radiation-induced senescence is a prominent phenomenon across different cell lines; however, A549 cells were characterized by particularly high post-irradiation survival and a very pronounced senescent response. The absence of dilution of the CellTracer dye clearly indicated a growth arrest following irradiation, or at least a significant slowing of tumor cell proliferation. Thus, our study model met the criteria for the investigation of senescence [42–44]. Whenever possible, senescence should be defined through multiple markers, and in this publication, we took care to consider various parameters: morphology, beta-galactosidase staining, and cell cycle arrest (via CellTracer) [40].

CAF showed a less pronounced morphological response to

irradiation. Affolter et al. also demonstrated that, compared to other cell types, CAFs exhibit a weaker response to radiation with high amounts of cell survival [45] and Liu et al. reported a clear dose-dependent restriction of their migration and proliferation [46]. Findings from studies on lung cancer suggest that even high doses of ionizing radiation fail to eliminate lung CAFs, instead pushing them into a senescent state [47]. CAFs are, nevertheless, a heterogeneous group of cells, and differences are to be expected [48]. CAF proliferation appeared to decline less sharply compared to A549 in our study, as indicated by the varying ratios of A549 cells and CAFs in co-culture. This suggests that while both cell types exhibited reduced proliferation, the more pronounced drop in A549 proliferation may be due to their initially higher proliferative capacity in the untreated state.



4.2. Co-culture of CAFs and tumor cells

After observing the two cell types individually, we aimed to investigate their direct interaction, initially in a non-irradiated co-culture: Morphologically, we were pleased to observe that the two-dimensional appearance in the monolayer closely resembled histological images of tumor tissue. In our experimental setup, tumor cell nests were surrounded by CAFs. Dissanayaka et al. describe various invasion types in their publication, including the so-called “pushing islands,” which show a notable resemblance to our setup [49]. Although a two-dimensional structure cannot fully replicate a three-dimensional *in vivo* environment, this aspect still supports the closeness of our experimental setup to the tissue environment *in vivo* [50]. Undoubtedly, three-dimensional *in-vitro* systems as well as *in-vivo* experiments can capture additional aspects of the complex situation of an irradiated multicellular tumor in the patient [51]. At the same time, these complex models involve a multitude of interacting factors, making it necessary to first investigate fundamental mechanistic relationships in a more simplified model system, as we have done in this pilot study. Future studies can then build on

this foundation by incorporating further aspects of this complexity. After irradiation, the cell nest structure failed to form. It is possible that the increased migratory capacity of A549 cells and reduced proliferation is responsible for that. It has been shown in melanomas that proliferation is particularly crucial for the formation of cell nests [52].

4.3. Senescence of tumor cells in co-culture

The next logical step was the detailed observation of the irradiated co-culture. CAFs represent a crucial element within the complex and varied landscape of the tumor microenvironment, influencing both tumor growth and the effectiveness of cancer treatments [48,53]. Given that CAFs are exposed to radiation alongside cancer cells during radiotherapy, it becomes essential to investigate how radiation affects their behavior and how such changes might influence the progression of tumors. Most current studies focus solely on the role of senescent fibroblasts in interaction with non-senescent cancer cells [54]. In our co-culture approach, both CAFs and tumor cells were irradiated together with direct physical contact. Although we did not specifically address

the question of which observed effects arise with or without direct physical contact, our contact-based model certainly provided a close approximation to the in-vivo situation.

Our investigations revealed that fewer A549 tumor cells underwent senescence in co-culture with CAFs than in monoculture. Since senescence is associated with a growth arrest, we hypothesized that fewer senescent cells would result in an increase in long-term tumor cell re-proliferation. [11,19]. However, what also became clear from our results is that although fewer cells became senescent in co-culture, re-proliferation was more prevalent in monoculture. One could argue that the non-senescent cells were already entering cell death, but this could be ruled out by specifically analyzing only living cells. It is therefore conceivable that senescent CAFs may potentially contribute to maintaining tumor cells in a senescent state in co-culture, thereby reducing their re-proliferation. Other studies examining the effect of senescent CAFs on non-senescent tumor cells have found that CAFs can promote proliferation [46,54]. Further research has shown that in colorectal cancer, certain levels of radiation-activated CAFs can actually aid in

cancer cell survival by stimulating the insulin-like growth factor 1 / insulin-like growth factor 1 receptor pathway [55]. As previously noted, senescent cells can reinforce their own senescence via autocrine mechanisms [20,56]. This mechanism might be more effective in monoculture – at least in the first days – and might ensure the long term survival of the tumor cells, which evidently can re-proliferate from their senescent state [16]. From these previous studies together with our results, it can be concluded that CAFs may initially protect tumor cells from or delay senescence induction, but in the long term they appear to stabilize the senescent state and thereby limit re-proliferation. In contrast, autocrine mechanisms in A549 monocultures may accelerate the onset of senescence, yet this senescence seems more prone to eventual re-proliferation. For the experiments within the scope of this study, we deliberately selected a cell line that undergoes pronounced radiation-induced senescence in monoculture. In subsequent studies, it will be interesting to investigate cell lines which share with CAFs a matched tissue origin and that display less prominent senescence in monoculture to determine whether co-culture with CAFs in these tumor

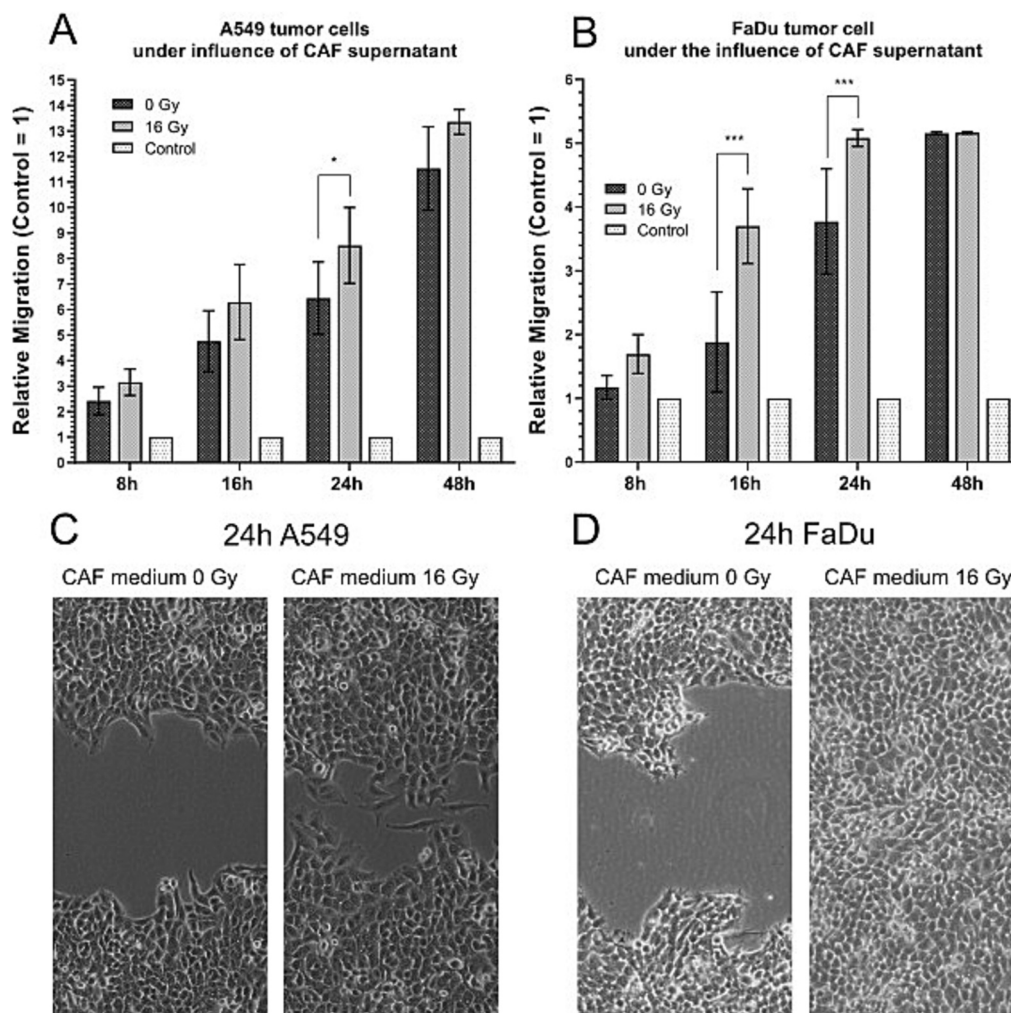


Fig. 12. Effects of CAF-conditioned media on A549 and FaDu tumor cell migration. (A, B) Migration of A549 lung carcinoma cells and FaDu HNSCC cells stimulated with conditioned media from non-irradiated and irradiated CAFs from 5 different patients, assessed by scratch assay and normalized to the control (medium without FCS, $n = 1$). A noticeable increase in migration was observed in both groups stimulated with CAF-medium compared to the control, indicating that CAF-conditioned media promoted tumor cell migration. At 24 h, A549 cells stimulated with medium from irradiated CAFs showed a significant increase in migration in comparison to those stimulated with medium from untreated CAFs ($p < 0.05$). In the FaDu cell line, this effect was even more pronounced, with a highly significant increase of migration in the tumor cells stimulated with medium from irradiated CAFs in comparison to medium from non-irradiated CAFs at 16 h and 24 h. (C, D) Representative microscopic images of the cell-free area at 24 h for A549 and FaDu cells, respectively. (left: stimulation with conditioned medium from non-irradiated CAFs, right: stimulation with conditioned medium from irradiated CAFs) Statistical analysis was performed using two-way ANOVA with Bonferroni correction across all time points comparing between irradiated and non-irradiated CAF-conditioned media for each respective time point, and significant differences, where present, were indicated in the figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cells similarly reduces senescence or, conversely, enhances it.

4.4. SASP as a potential driver of observed changes in senescence development and proliferation

To explain the observed cell behavior in our study, we hypothesized that the SASP could be responsible for the underlying mechanism. Several authors have already shown that the SASP of tumor cells can enhance the proliferation of premalignant cells [57–60]. However, the definition of SASP varies considerably among different authors, encompassing a broad spectrum of factors. This variability complicates the assessment of whether SASP is predominantly pro- or anti-tumorigenic [13]. Current therapy strategies aimed at inducing or blocking senescence may intervene at the wrong point in the process, because not the cancer cell is the target but the tme. CAFs and their signaling pathways could be the key [56,61,62]. Our study may be one of the first to uncover CAF-related mechanisms that prevent the onset of senescence in direct co-culture, which could represent a potential therapeutic target for more effective cancer treatment.

Our initial analysis using the cytokine array confirmed differences between cell types and treatment conditions. But CAFs are a heterogeneous group of cells, and radiation likely affects their metabolism in critical ways. This high variability might explain why the results of our array ($n = 1$) differed from the findings of the ELISA ($n = 5$). However, choosing primary CAFs that were used in our experiments immediately after isolation from the patients, we sought to establish an in vitro model that closely reflects the patient-specific situation also representing the inter-patient variability. Previous research by Desai et al. demonstrated that the secretion profile of cytokines and growth factors in various tumor cell lines, including A549 cells, changed post-irradiation [63]. Although the radiation dose in their experiments (2 Gy and 6 Gy) was lower than ours, both qualitative and quantitative differences were found, which were also dose-dependent [63]. In our ELISA analysis, however, only MCP-1 and IL-8 showed a significant difference between irradiated and non-irradiated cells. The reasons for this might lie in the specific cytokines we examined, the timing of analysis, or the radiation dose. It also must be noted that our results cannot specify which cells in the co-culture were responsible for the changes measured. Further research will be necessary to evaluate, for example whether CAFs produce these cytokines themselves or if they stimulate the neighboring tumor cells to do so and which mechanisms are involved.

We found that the IL-6 secretion was significantly higher in co-culture than in mono-culture. IL-8 was more strongly expressed in non-irradiated co-cultures compared to mono-cultures, however, this difference was not significant. Both IL-6 and IL-8 play important roles in tumor invasion, which aligns with the increased expression observed in the presence of CAFs. In breast cancer research, these cytokines have been used as prognostic serum markers, underscoring their significance [64–66]. For pancreatic cancer, Wang et al. suggested that IL-8 might mediate the pro-invasive effects of senescent CAFs [67]. In our experiments, the marked decrease in IL-8 secretion in the co-culture after irradiation could be attributed to changes in the ratio between CAFs and A549 cells. Interestingly, both IL-6 and IL-8 are thought to induce senescence [68,69]. In our co-culture model, this would suggest that post-irradiation, lower levels of IL-6 and IL-8 should be observed, which was at least somewhat true for IL-8. Taken together, the reduced levels of IL-6 and IL-8 in the co-culture after irradiation may explain the lower frequency of senescent tumor cells, as both cytokines promote senescence. At the same time, since IL-6 and IL-8 are linked to tumor invasion, their presence in the co-culture may contribute to the CAF-conditioned medium's ability to enhance migration, particularly after irradiation.

TIMP-1 was consistently secreted across all culture conditions. Despite the lower proportion of CAFs in non-irradiated co-cultures, no differences were observed between irradiated and non-irradiated conditions. Hellevik similarly found no alterations in TIMP secretion in irradiated lung CAFs [47]. Guccini et al. identified TIMP-1 as a

gatekeeper that limits the metastasis-promoting potential of senescent tumor cells. A loss of TIMP-1 reprograms the SASP to promote cell invasion and migration [70]. If CAFs indeed increase TIMP-1 secretion, this may suggest a protective role against tumor progression. In a publication by Pitiyage et al. TIMP levels correlated with the replicative lifespan of the cultures but not with the presence of senescent cells, as depleting senescent cells in oral submucous fibrosis fibroblast cultures did not reduce TIMP-1 or TIMP-2 secretion [71]. The influence on senescence seems minimal, but the stable presence of signaling molecules could be just as important, allowing intervention in both pre- and post-irradiation stages, as well as in senescent and non-senescent states. Thus, the stable TIMP-1 secretion across all conditions suggests that it does not directly contribute to the reduced senescence or repopulation observed in co-culture after irradiation. Its inhibitory role in invasion and migration could counteract, but not fully suppress, the pro-migratory effects of CAF-conditioned medium.

MCP-1 is a pro-inflammatory chemokine involved in the recruitment of monocytes and lymphocytes [72]. We focused on this cytokine because of its strong secretion in our initial array. Its elevated secretion following A549 irradiation suggested a role in self-inducing and reinforcing senescence. Previous in vitro experiments have shown that MCP-1 can induce senescence in normal human keratinocytes [73]. MCP-1 can promote senescence by increasing p53 and p21 protein levels via ROS or p38 MAPK signaling [74,75]. It is also involved in the migration and invasion of various cancer cell types, including breast, prostate, glioblastoma, ovarian, bladder, and chondrosarcoma [76–78]. Since CAFs secreted less MCP-1 than A549 cells, it is likely that tumor cells, particularly in their senescent state, drive the increased MCP-1 secretion, potentially leading to enhanced invasiveness. MCP-1 secretion was significantly lower in co-culture and CAF mono-culture compared to the A549 mono-culture, but this might only reflect the composition of the co-culture rather than an interaction between cell types. Overall, the lower MCP-1 levels in co-culture may help explain the reduced senescence after irradiation, while its role in promoting invasion suggests a link to the increased migration observed in response to CAF-conditioned medium.

As the name suggests, OPG is involved in bone remodeling but also plays roles in vascular biology and immunology. It can influence cell survival, proliferation, and migration [79–81]. Specifically, OPG can enhance endothelial cell survival and migration and has been associated with metastasis. Evidence suggests that OPG secreted by irradiation-induced senescent cancer cells promotes the migration of neighboring cancer cells and may serve as a therapeutic target to counteract the negative effects of senescent cancer cells generated by radiotherapy [82]. We found that only CAFs produced OPG, A549 cells did not, suggesting that it may drive the effects observed in our migration experiments with conditioned CAF medium.

Our study underlines the complexity of the interactions in the tumor microenvironment. A finely tuned interaction between these mechanisms cannot be ruled out, and contradictory effects are also possible. For example, IL-6 can have different effects in the tumor microenvironment depending on the context, and these effects may oppose each other [83]. It is likely that the SASP also exhibits similar opposing influences. In addition, the literature shows that CAFs and tumor cells can communicate not only through secreted SASP cytokines, but also via other mechanisms such as metabolites or exosomes [84,85]. To more precisely delineate the contributions of individual factors, genetic or pharmacological perturbations can be employed to guide targeted inhibition of signaling pathways and thus ultimately find ways to improve the radiotherapy outcome in patients.

4.5. Paracrine effects of the conditioned medium from CAFs on tumor cells

Given the observed cytokine changes, we next examined whether these translate into functional effects on tumor cell migration. The

results of these experiments complement our earlier findings, completing the overall picture presented in this work. It is now clear how essential it is to examine the migration capacity of tumor cells, given that many of the substances mentioned are known to promote metastasis and that this applies to multiple tumor types, including lung carcinoma and HNSCC.

The increased migration ability of A549 and FaDu cells mediated by CAF-conditioned medium is a significant finding confirming that this pro-migratory effect is not limited to lung carcinoma cells but also occurs in HNSCC cells. Even more important, however, is the observation that CAFs appear to have a stronger effect on migration after irradiation than without. This could represent a key mechanism, since we found repopulation more frequently in mono-cultures, but migration was more pronounced when A549 and FaDu cells were stimulated with conditioned medium indicating that both epithelial tumor types respond similarly to CAF-derived signals. It could be especially important since in OSCC, the presence of regional lymph node metastasis, especially with extracapsular spread, is the most important prognostic factor [86].

It is well-known that even non-irradiated CAFs influence both proliferation and migration. For example, Kim et al. demonstrated that in co-cultures of A549 cells and CAFs, there was a greater expression of α -SMA (alpha-smooth muscle actin) and increased migration, alongside reduced proliferation – especially compared to co-cultures with normal fibroblasts [61]. We were able to show that these effects could be induced by the conditioned medium in both A549 and FaDu cells. Coppe et al. supported these findings, suggesting that the collective factors secreted by senescent cells can affect the invasiveness of two breast cancer cell lines [18].

An in vitro study on the influence of senescent CAFs on oral carcinoma cells (Cal-27) found that co-cultured senescent CAFs significantly promoted Cal-27 cell proliferation, although this effect was reduced compared to non-irradiated CAFs. In terms of promoting Cal-27 cell invasion, senescent CAFs showed a positive but less pronounced effect compared to non-irradiated CAFs. Regarding migration, co-cultured senescent CAFs significantly promoted Cal-27 cell migration compared to single DMEM culturing, though the effect was not significantly different from that of non-irradiated CAFs [46]. Our results show that conditioned medium from irradiated CAFs enhanced migration of both A549 and FaDu tumor cells more prominently than medium from untreated CAFs could. A study from Suzuki et al. showed that fibroblast cell lines promoted HNSCC cell migration in coculture, which was further enhanced after irradiation. In our study, conditioned medium from primary CAFs likewise stimulated cancer cell migration, also with a stronger effect observed with medium from irradiated CAFs [87].

5. Conclusion

In conclusion, it is becoming increasingly evident that not only targeted cancer therapy is necessary but also targeted therapy against CAFs, since our study showed that CAFs modulate tumor cell senescence and enhance tumor cell migration following irradiation. A deeper understanding of the tumor microenvironment and the interplay of its individual components will improve our grasp of the mechanisms leading to treatment failure. Our study adds an important piece to this puzzle, helping to complete the overall picture.

CRediT authorship contribution statement

Johannes Raphael Kupka: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rita Gieringer:** Validation, Investigation. **Sebahat Kaya:** Validation, Investigation. **Victoria Langer:** Validation, Investigation. **Jürgen Brieger:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Nadine Wiesmann-Imilowski:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project

administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics approval and consent to participate

The Ethics Committee of the State Medical Association of Rhineland-Palatinate Ethics Committee approved this study (ethics vote Votum: 2022-16424_3; year of ethics vote: 2022)

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Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2025.112187>.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

- [1] T. Katoh, S. Kaneko, S. Takasawa, N. Nagata, H. Inatomi, K. Ikemura, et al., Human glutathione S-transferase P1 polymorphism and susceptibility to smoking related epithelial cancer; oral, lung, gastric, colorectal and urothelial cancer, *Pharmacogenetics* 9 (1999) 165–169.
- [2] J. Ferlay, M. Colombet, I. Soerjomataram, C. Mathers, D.M. Parkin, M. Piñeros, et al., Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods, *Int. J. Cancer* 144 (2019) 1941–1953, <https://doi.org/10.1002/ijc.31937>.
- [3] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global Cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA A Cancer J. Clin.* 71 (2021) 209–249, <https://doi.org/10.3322/caac.21660>.
- [4] E.C. Scott, A.C. Baines, Y. Gong, R. Moore, J.R. G.E. Pamuk, H. Saber, et al., Trends in the approval of cancer therapies by the FDA in the twenty-first century, *Nat. Rev. Drug Discov.* 22 (2023) 625–640, <https://doi.org/10.1038/s41573-023-00723-4>.
- [5] D. Alterio, G. Marvaso, A. Ferrari, S. Volpe, R. Orecchia, B.A. Jereczek-Fossa, Modern radiotherapy for head and neck cancer, *Semin. Oncol.* 46 (2019) 233–245, <https://doi.org/10.1053/j.seminoncol.2019.07.002>.
- [6] J.S. Cooper, T.F. Pajak, A.A. Forastiere, J. Jacobs, B.H. Campbell, S.B. Saxman, et al., Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck, *N. Engl. J. Med.* 350 (2004) 1937–1944, <https://doi.org/10.1056/NEJMoa032646>.
- [7] A. Capote-Moreno, P. Brabyn, M.F. Muñoz-Guerra, J. Sastre-Pérez, V. Escorial-Hernandez, F.J. Rodríguez-Campo, et al., Oral squamous cell carcinoma: epidemiological study and risk factor assessment based on a 39-year series, *Int. J.*

- Oral Maxillofac. Surg. 49 (2020) 1525–1534, <https://doi.org/10.1016/j.ijom.2020.03.009>.
- [8] J.-P. Machiels, C. René Leemans, W. Golusinski, C. Grau, L. Licitra, V. Gregoire, Squamous cell carcinoma of the oral cavity, larynx, oropharynx and hypopharynx: EHSN-ESMO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up†, *Ann. Oncol.* 31 (2020) 1462–1475, <https://doi.org/10.1016/j.annonc.2020.07.011>.
 - [9] H.F. Köhler, H. Mehanna, J.P. Shah, A. Sanabria, J. Fagan, M.A. Kuriakose, et al., Comparison of different guidelines for oral cancer, *Eur. Arch. Otorinolaringol.* 278 (2021) 2961–2973, <https://doi.org/10.1007/s00405-020-06423-8>.
 - [10] R. Leroy, C.D. Gendt, S. Stordeur, G. Silversmit, L. Verleye, V. Schillemans, et al., Quality Indicators for the Management of Head and Neck Squamous Cell Carcinoma: KCE = Federaal Kenniscentrum voor de Gezondheidszorg = Centre Fédéral d'Expertise Des Soins de Santé = Belgian Health Care Knowledge Centre, 2019.
 - [11] L. Wyld, I. Bellantuono, T. Tchkonja, J. Morgan, O. Turner, F. Foss, et al., Senescence and Cancer: a review of clinical implications of senescence and Senotherapies, *Cancers (Basel)* (2020), <https://doi.org/10.3390/cancers12082134>.
 - [12] K.-X. Song, J.-X. Wang, de Huang., Therapy-induced senescent tumor cells in cancer relapse, *J. Natl. Cancer Cent.* 3 (2023) 273–278, <https://doi.org/10.1016/j.jncc.2023.09.001>.
 - [13] J. Birch, J. Gil, Senescence and the SASP: many therapeutic avenues, *Genes Dev.* 34 (2020) 1565–1576, <https://doi.org/10.1101/gad.343129.120>.
 - [14] C.A. Schmitt, B. Wang, M. Demaria, Senescence and cancer - role and therapeutic opportunities, *Nat. Rev. Clin. Oncol.* 19 (2022) 619–636, <https://doi.org/10.1038/s41571-022-00668-4>.
 - [15] T. Saleh, L. Tyutyunyk-Massey, D.A. Gewirtz, Tumor cell escape from therapy-induced senescence as a model of disease recurrence after dormancy, *Cancer Res.* 79 (2019) 1044–1046, <https://doi.org/10.1158/0008-5472.CAN-18-3437>.
 - [16] N. Wiesmann, R. Gieringer, M. Viel, J. Eckrich, W. Tremel, J. Brieger, Zinc oxide nanoparticles can intervene in radiation-induced senescence and eradicate residual tumor cells, *Cancers (Basel)* (2021), <https://doi.org/10.3390/cancers13122989>.
 - [17] J. Wang, C.-C. Zhou, H.-C. Sun, Q. Li, J.-D. Hu, T. Jiang, S. Zhou, Identification of several senescence-associated genes signature in head and neck squamous cell carcinoma, *J. Clin. Lab. Anal.* 36 (2022) e24555, <https://doi.org/10.1002/jcla.24555>.
 - [18] J.-P. Coppé, C.K. Patil, F. Rodier, Y. Sun, D.P. Muñoz, J. Goldstein, et al., Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor, *PLoS Biol.* 6 (2008) 2853–2868, <https://doi.org/10.1371/journal.pbio.0060301>.
 - [19] T.-W. Kang, T. Yevsa, N. Woller, L. Hoenicke, T. Wuestefeld, D. Dauch, et al., Senescence surveillance of pre-malignant hepatocytes limits liver cancer development, *Nature* 479 (2011) 547–551, <https://doi.org/10.1038/nature10599>.
 - [20] S. Xiao, D. Qin, X. Hou, L. Tian, Y. Yu, R. Zhang, et al., Cellular senescence: a double-edged sword in cancer therapy, *Front. Oncol.* 13 (2023) 1189015, <https://doi.org/10.3389/fonc.2023.1189015>.
 - [21] O.D. Wever, P. Demetter, M. Mareel, M. Bracke, Stromal myofibroblasts are drivers of invasive cancer growth, *Int. J. Cancer* 123 (2008) 2229–2238, <https://doi.org/10.1002/ijc.23925>.
 - [22] B. Peltanova, M. Raudenska, M. Masarik, Effect of tumor microenvironment on pathogenesis of the head and neck squamous cell carcinoma: a systematic review, *Mol. Cancer* 18 (2019) 63, <https://doi.org/10.1186/s12943-019-0983-5>.
 - [23] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, *Nat. Rev. Cancer* 6 (2006) 392–401, <https://doi.org/10.1038/nrc1877>.
 - [24] A. Glentis, P. Oertle, P. Mariani, A. Chikina, F. El Marjoui, Y. Attieh, et al., Cancer-associated fibroblasts induce metalloprotease-independent cancer cell invasion of the basement membrane, *Nat. Commun.* 8 (2017) 924, <https://doi.org/10.1038/s41467-017-00985-8>.
 - [25] G. Biffi, D.A. Tuveson, Diversity and biology of Cancer-associated fibroblasts, *Physiol. Rev.* 101 (2021) 147–176, <https://doi.org/10.1152/physrev.00048.2019>.
 - [26] H.-J. Welkoborsky, R. Jacob, S.H. Riazimand, H.S. Bernauer, W.J. Mann, Molecular biologic characteristics of seven new cell lines of squamous cell carcinomas of the head and neck and comparison to fresh tumor tissue, *Oncology* 65 (2003) 60–71, <https://doi.org/10.1159/000071206>.
 - [27] U. Schoetz, D. Klein, J. Hess, S. Shnayden, S. Spoerl, M. Orth, et al., Early senescence and production of senescence-associated cytokines are major determinants of radioresistance in head-and-neck squamous cell carcinoma, *Cell Death Dis.* 12 (2021) 1162, <https://doi.org/10.1038/s41419-021-04454-5>.
 - [28] L. Yang, J. Fang, J. Chen, Tumor cell senescence response produces aggressive variants, *Cell Death Dis.* 3 (2017) 17049, <https://doi.org/10.1038/cddiscovery.2017.49>.
 - [29] I. Duran, J. Pombo, B. Sun, S. Gallage, H. Kudo, D. McHugh, et al., Detection of senescence using machine learning algorithms based on nuclear features, *Nat. Commun.* 15 (2024) 1041, <https://doi.org/10.1038/s41467-024-45421-w>.
 - [30] Jochems F, Thijssen B, Conti G de, Jansen R, Pogacar Z, Groot K, et al. The Cancer SenESCopedia: a delineation of cancer cell senescence. *Cell Rep.* 2021;36:109441. doi:<https://doi.org/10.1016/j.celrep.2021.109441>.
 - [31] X. He, A. Yang, D.G. McDonald, E.C. Riemer, K.N. Vanek, B.A. Schulte, G.Y. Wang, MIR-34a modulates ionizing radiation-induced senescence in lung cancer cells, *Oncotarget* 8 (2017) 69797–69807, <https://doi.org/10.18632/oncotarget.19267>.
 - [32] Z. Zhao, T. Li, Y. Yuan, Y. Zhu, What is new in cancer-associated fibroblast biomarkers? *Cell Commun. Signal* 21 (2023) 96, <https://doi.org/10.1186/s12964-023-01125-0>.
 - [33] D. Yang, J. Liu, H. Qian, Q. Zhuang, Cancer-associated fibroblasts: from basic science to anticancer therapy, *Exp. Mol. Med.* 55 (2023) 1322–1332, <https://doi.org/10.1038/s12276-023-01013-0>.
 - [34] N. Matuszak, W.M. Suchorska, P. Milecki, M. Kruszyna-Mochalska, A. Misiarz, J. Prac, J. Malicki, FLASH radiotherapy: an emerging approach in radiation therapy, *Rep. Pract. Oncol. Radiother.* 27 (2022) 344–351, <https://doi.org/10.5603/RPOR.a2022.0038>.
 - [35] M. Malavolta, R. Giacconi, F. Piacenza, S. Strizzi, M. Cardelli, G. Bigossi, et al., Simple detection of unstained live senescent cells with imaging flow cytometry, *Cells* (2022), <https://doi.org/10.3390/cells11162506>.
 - [36] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, et al., Fiji: an open-source platform for biological-image analysis, *Nat. Methods* 9 (2012) 676–682, <https://doi.org/10.1038/nmeth.2019>.
 - [37] A. Krzysztyniak, A. Gluchowska, G. Mosieniak, E. Sikora, Fiji-based tool for rapid and unbiased analysis of SA-β-gal activity in cultured cells, *Biomolecules* (2023), <https://doi.org/10.3390/biom13020362>.
 - [38] L.K. Müller-Heupt, N. Wiesmann, S. Schröder, Y. Korkmaz, N. Vierengel, J. Groß, et al., Extracts of Rheum palmatum and Aloe vera show beneficial properties for the synergistic improvement of Oral wound healing, *Pharmaceutics* (2022), <https://doi.org/10.3390/pharmaceutics14102060>.
 - [39] X. Chen, M. Yuan, T. Zhong, M. Wang, F. Wu, J. Lu, et al., LILRB2 inhibition enhances radiation sensitivity in non-small cell lung cancer by attenuating radiation-induced senescence, *Cancer Lett.* 593 (2024) 216930, <https://doi.org/10.1016/j.canlet.2024.216930>.
 - [40] E. González-Gualda, A.G. Baker, L. Fruk, D. Muñoz-Espín, A guide to assessing cellular senescence in vitro and in vivo, *FEBS J.* 288 (2021) 56–80, <https://doi.org/10.1111/febs.15570>.
 - [41] B.Y. Lee, J.A. Han, J.S. Im, A. Morrone, K. Johung, E.C. Goodwin, et al., Senescence-associated beta-galactosidase is lysosomal beta-galactosidase, *Aging Cell* 5 (2006) 187–195, <https://doi.org/10.1111/j.1474-9726.2006.00199.x>.
 - [42] N.E. Sharpless, C.J. Sherr, Forging a signature of in vivo senescence, *Nat. Rev. Cancer* 15 (2015) 397–408, <https://doi.org/10.1038/nrc3960>.
 - [43] A. Hernandez-Segura, J. Nehme, M. Demaria, Hallmarks of cellular senescence, *Trends Cell Biol.* 28 (2018) 436–453, <https://doi.org/10.1016/j.tcb.2018.02.001>.
 - [44] G.A. Romar, T.S. Kupper, S.J. Divito, Research techniques made simple: techniques to assess cell proliferation, *J. Invest. Dermatol.* 136 (2016) e1–e7, <https://doi.org/10.1016/j.jid.2015.11.020>.
 - [45] Affolter A, Schmidtmann I, Mann, Wolf, J., Brieger J. Cancer-associated fibroblasts do not respond to combined irradiation and kinase inhibitor treatment. *Oncol. Rep.* 2013;29:785–790. doi:<https://doi.org/10.3892/or.2012.2180>.
 - [46] Y. Liu, Y. Wu, M. Yang, J. Yang, R. Tong, W. Zhao, et al., Ionizing radiation-induced “zombie” carcinoma-associated fibroblasts with suppressed pro-radioreistance on OSCC cells, *Oral Dis.* 29 (2023) 563–573, <https://doi.org/10.1111/odi.13979>.
 - [47] T. Hellevik, I. Pettersen, V. Berg, J.O. Winberg, B.T. Moe, K. Bartnes, et al., Cancer-associated fibroblasts from human NSCLC survive ablative doses of radiation but their invasive capacity is reduced, *Radiat. Oncol.* 7 (2012) 59, <https://doi.org/10.1186/1748-717X-7-59>.
 - [48] G.J. Yoshida, Regulation of heterogeneous cancer-associated fibroblasts: the molecular pathology of activated signaling pathways, *J. Exp. Clin. Cancer Res.* 39 (2020) 112, <https://doi.org/10.1186/s13046-020-01611-0>.
 - [49] W.L. Dissanayaka, G. Pitiyage, P.V.R. Kumarasiri, Rubasinha Liyanage Pemith Ranura Liyanage, K.D. Dias, W.M. Tilakaratne, Clinical and histopathologic parameters in survival of Oral squamous cell carcinoma, *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* 113 (2012) 518–525, <https://doi.org/10.1016/j.oooo.2011.11.001>.
 - [50] S. Ballav, A.J. Deshmukh, S. Siddiqui, J. Aich, S. Basu, Two-dimensional and three-dimensional cell culture and their applications, *Cell Cult.—Adv. Technol. Appl. Med. Life Sci.* (2021), 30 pages 1–27.
 - [51] M.A.G. Barbosa, C.P.R. Xavier, R.F. Pereira, V. Petrikaitė, M.H. Vasconcelos, 3D cell culture models as Recapitulators of the tumor microenvironment for the screening of anti-Cancer drugs, *Cancers (Basel)* (2021), <https://doi.org/10.3390/cancers14010190>.
 - [52] P. Haridas, A.P. Browning, J.A. McGovern, D.L. Sean McElwain, M.J. Simpson, Three-dimensional experiments and individual based simulations show that cell proliferation drives melanoma nest formation in human skin tissue, *BMC Syst. Biol.* 12 (2018) 34, <https://doi.org/10.1186/s12918-018-0559-9>.
 - [53] W. Meng, Y. Wu, X. He, C. Liu, Q. Gao, L. Ge, et al., A systems biology approach identifies effective tumor-stroma common targets for oral squamous cell carcinoma, *Cancer Res.* 74 (2014) 2306–2315, <https://doi.org/10.1158/0008-5472.CAN-13-2275>.
 - [54] M. Takasugi, Y. Yoshida, N. Ohtani, Cellular senescence and the tumour microenvironment, *Mol. Oncol.* 16 (2022) 3333–3351, <https://doi.org/10.1002/1878-0261.13268>.
 - [55] J. Tommelein, E.D. Vlieghe, L. Verset, E. Melsens, J. Leenders, B. Descamps, et al., Radiotherapy-activated Cancer-associated fibroblasts promote tumor progression through paracrine IGF1R activation, *Cancer Res.* 78 (2018) 659–670, <https://doi.org/10.1158/0008-5472.CAN-17-0524>.
 - [56] D.V. Faget, Q. Ren, S.A. Stewart, Unmasking senescence: context-dependent effects of SASP in cancer, *Nat. Rev. Cancer* 19 (2019) 439–453, <https://doi.org/10.1038/s41568-019-0156-2>.
 - [57] C. Bavik, I. Coleman, J.P. Dean, B. Knudsen, S. Plymate, P.S. Nelson, The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms, *Cancer Res.* 66 (2006) 794–802, <https://doi.org/10.1158/0008-5472.CAN-05-1716>.

- [58] J.-P. Coppé, K. Kauser, J. Campisi, C.M. Beauséjour, Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence, *J. Biol. Chem.* 281 (2006) 29568–29574, <https://doi.org/10.1074/jbc.M603307200>.
- [59] J.W.M. Martens, A.M. Sieuwerts, J. Bolt-deVries, P.T. Bosma, S.J.J. Swiggers, J.G. M. Klijn, J.A. Foekens, Aging of stromal-derived human breast fibroblasts might contribute to breast cancer progression, *Thromb. Haemost.* 89 (2003) 393–404.
- [60] J.-P. Coppe, M. Boisen, C.H. Sun, B.J.F. Wong, M.K. Kang, N.-H. Park, et al., A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion, *Mol. Cancer Res.* 6 (2008) 1085–1098, <https://doi.org/10.1158/1541-7786.MCR-08-0062>.
- [61] S.-H. Kim, C. Choe, Y.-S. Shin, M.-J. Jeon, S.-J. Choi, J. Lee, et al., Human lung cancer-associated fibroblasts enhance motility of non-small cell lung cancer cells in co-culture, *Anticancer Res.* 33 (2013) 2001–2009.
- [62] Y.H. Kim, Y.W. Choi, J. Lee, E.Y. Soh, J.-H. Kim, T.J. Park, Senescent tumor cells lead the collective invasion in thyroid cancer, *Nat. Commun.* 8 (2017) 15208, <https://doi.org/10.1038/ncomms15208>.
- [63] S. Desai, A. Kumar, S. Laskar, B.N. Pandey, Cytokine profile of conditioned medium from human tumor cell lines after acute and fractionated doses of gamma radiation and its effect on survival of bystander tumor cells, *Cytokine* 61 (2013) 54–62, <https://doi.org/10.1016/j.cyto.2012.08.022>.
- [64] J. Milovanović, N. Todorović-Raković, M. Radulovic, Interleukin-6 and interleukin-8 serum levels in prognosis of hormone-dependent breast cancer, *Cytokine* 118 (2019) 93–98, <https://doi.org/10.1016/j.cyto.2018.02.019>.
- [65] H. Knüpf, R. Preiss, Significance of interleukin-6 (IL-6) in breast cancer (review), *Breast Cancer Res. Treat.* 102 (2007) 129–135, <https://doi.org/10.1007/s10549-006-9328-3>.
- [66] C. Yao, Y. Lin, C. Ye, J. Bi, Y. Zhu, S. Wang, Role of interleukin-8 in the progression of estrogen receptor-negative breast cancer, *Chin. Med. J.* 120 (2007) 1766–1772.
- [67] T. Wang, F. Notta, R. Navab, J. Joseph, E. Ibrahimov, J. Xu, et al., Senescent carcinoma-associated fibroblasts upregulate IL8 to enhance Prometastatic phenotypes, *Mol. Cancer Res.* 15 (2017) 3–14, <https://doi.org/10.1158/1541-7786.MCR-16-0192>.
- [68] P. Ortiz-Montero, A. Londoño-Vallejo, J.-P. Vernot, Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities in the MCF-7 breast cancer cell line, *Cell Commun. Signal* 15 (2017) 17, <https://doi.org/10.1186/s12964-017-0172-3>.
- [69] J.C. Acosta, A. Banito, T. Wuestefeld, A. Georgilis, P. Janich, J.P. Morton, et al., A complex secretory program orchestrated by the inflammasome controls paracrine senescence, *Nat. Cell Biol.* 15 (2013) 978–990, <https://doi.org/10.1038/ncb2784>.
- [70] I. Guccini, A. Revandkar, M. D'Ambrosio, M. Colucci, E. Pasquini, S. Mosole, et al., Senescence reprogramming by TIMP1 deficiency promotes prostate Cancer metastasis, *Cancer Cell* 39 (2021) 68–82.e9, <https://doi.org/10.1016/j.ccell.2020.10.012>.
- [71] G.N. Pitiyage, K.P. Lim, E. Gemenitzidis, M.-T. Teh, A. Waseem, S.S. Prime, et al., Increased secretion of tissue inhibitors of metalloproteinases 1 and 2 (TIMPs –1 and –2) in fibroblasts are early indicators of oral sub-mucous fibrosis and ageing, *J. Oral Pathol. Med.* 41 (2012) 454–462, <https://doi.org/10.1111/j.1600-0714.2012.01129.x>.
- [72] M. Baggiolini, B. Dewald, B. Moser, Human chemokines: an update, *Annu. Rev. Immunol.* 15 (1997) 675–705, <https://doi.org/10.1146/annurev.immunol.15.1.675>.
- [73] W.J. Lee, S.Y. Jo, M.H. Lee, C.H. Won, M.W. Lee, J.H. Choi, S.E. Chang, The effect of MCP-1/CCR2 on the proliferation and senescence of epidermal constituent cells in solar lentigo, *Int. J. Mol. Sci.* (2016), <https://doi.org/10.3390/ijms17060948>.
- [74] H.J. Jin, H.J. Lee, J. Heo, J. Lim, M. Kim, M.K. Kim, et al., Senescence-associated MCP-1 secretion is dependent on a decline in BMI1 in human mesenchymal stromal cells, *Antioxid. Redox Signal.* 24 (2016) 471–485, <https://doi.org/10.1089/ars.2015.6359>.
- [75] J.C. Acosta, A. O'Loughlin, A. Banito, S. Raguz, J. Gil, Control of senescence by CXCR2 and its ligands, *Cell Cycle* 7 (2008) 2956–2959, <https://doi.org/10.4161/cc.7.19.6780>.
- [76] M.-Y. Lien, A.-C. Chang, H.-C. Tsai, M.-H. Tsai, C.-H. Hua, S.-P. Cheng, et al., Monocyte chemoattractant protein 1 promotes VEGF-A expression in OSCC by activating ILK and MEK1/2 Signaling and downregulating miR-29c, *Front. Oncol.* 10 (2020) 592415, <https://doi.org/10.3389/fonc.2020.592415>.
- [77] Z. Ling, X. Yang, X. Chen, J. Xia, B. Cheng, X. Tao, CCL2 promotes cell migration by inducing epithelial-mesenchymal transition in oral squamous cell carcinoma, *J. Oral Pathol. Med.* 48 (2019) 477–482, <https://doi.org/10.1111/jop.12869>.
- [78] J.-F. Liu, P.-C. Chen, T.-M. Chang, C.-H. Hou, Monocyte chemoattractant Protein-1 promotes cancer cell migration via c-Raf/MAPK/AP-1 pathway and MMP-9 production in osteosarcoma, *J. Exp. Clin. Cancer Res.* 39 (2020) 254, <https://doi.org/10.1186/s13046-020-01756-y>.
- [79] W.C. Dougall, Molecular pathways: osteoclast-dependent and osteoclast-independent roles of the RANKL/RANK/OPG pathway in tumorigenesis and metastasis, *Clin. Cancer Res.* 18 (2012) 326–335, <https://doi.org/10.1158/1078-0432.CCR-10-2507>.
- [80] P.E. Reid, N.J. Brown, I. Holen, Breast cancer cells stimulate osteoprotegerin (OPG) production by endothelial cells through direct cell contact, *Mol. Cancer* 8 (2009) 49, <https://doi.org/10.1186/1476-4598-8-49>.
- [81] M. Weichhaus, P. Segaran, A. Renaud, D. Geerts, L. Connelly, Osteoprotegerin expression in triple-negative breast cancer cells promotes metastasis, *Cancer Med.* 3 (2014) 1112–1125, <https://doi.org/10.1002/cam4.277>.
- [82] H.J. Hwang, S.H. Jung, H.C. Lee, N.K. Han, I.H. Bae, M. Lee, et al., Identification of novel therapeutic targets in the secretome of ionizing radiation-induced senescent tumor cells, *Oncol. Rep.* 35 (2016) 841–850, <https://doi.org/10.3892/or.2015.4473>.
- [83] S.T. Orange, J. Leslie, M. Ross, D.A. Mann, H. Wackerhage, The exercise IL-6 enigma in cancer, *Trends Endocrinol. Metab.* 34 (2023) 749–763, <https://doi.org/10.1016/j.tem.2023.08.001>.
- [84] A. Mittal, M. Nenwani, I. Sarangi, A. Achreja, T.S. Lawrence, D. Nagrah, Radiotherapy-induced metabolic hallmarks in the tumor microenvironment, *Trends Cancer.* 8 (2022) 855–869, <https://doi.org/10.1016/j.trecan.2022.05.005>.
- [85] X. Chen, J. Liu, Q. Zhang, B. Liu, Y. Cheng, Y. Zhang, et al., Exosome-mediated transfer of miR-93-5p from cancer-associated fibroblasts confer radioresistance in colorectal cancer cells by downregulating FOXA1 and upregulating TGFβ3, *J. Exp. Clin. Cancer Res.* 39 (2020) 65, <https://doi.org/10.1186/s13046-019-1507-2>.
- [86] S. Wenzel, C. Sagowski, W. Kehrl, F.U. Metternich, The prognostic impact of metastatic pattern of lymph nodes in patients with oral and oropharyngeal squamous cell carcinomas, *Eur. Arch. Otorrinolaringol.* 261 (2004) 270–275, <https://doi.org/10.1007/s00405-003-0678-8>.
- [87] S. Suzuki, S. Toyoma, Y. Kawasaki, T. Yamada, Irradiated fibroblasts increase interleukin-6 expression and induce migration of head and neck squamous cell carcinoma, *PLoS One* 17 (2022) e0262549, <https://doi.org/10.1371/journal.pone.0262549>.