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Insights into the Role of Fibroblasts in Non-small Cell Lung Cancer Cell Lines Progression as a Potential Therapeutic Target

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ABSTRACT

Objectives: Fibroblasts are one of the most abundant cells in the tumor microenvironment, often playing a dual role in the cancer progression. Normal fibroblasts can have tumor suppressive properties at the initial stages of cancer, but upon stimulation by cancer cells they acquire tumor promoting qualities by transitioning into Cancer associated fibroblasts. The present study aimed to investigate the cross-talk that interplays between fibroblasts and cancer cells and its impact on cancer progression, metastasis and apoptosis reflected by the expression of epidermal growth factor receptor (EGFR) and its ligands, fibroblast growth factor-3 (FGF-3) and its receptor, angiogenic markers, vascular endothelial growth factor (VEGF) and matrix metalloproteinases, and various caspases. **Methods:** Fibroblast and cancer cell co-culture was done directly and indirectly through conditioned media. ELISA technique was used to assess the concentration of the studied biochemical markers. **Results:** Our results revealed an upregulation in the aforementioned parameters in A549 cell lines upon co-culture with fibroblasts. Our study also revealed a temporal aspect, as their expression increased overtime. Our study also revealed that the markers' overexpression was more pronounced in direct co-culture method, implicating the significance of direct cell-cell interaction**. Conclusion:** In conclusion, our study provides insight on the complex nature of the interaction between fibroblasts and cancer cells highlighting the need for further studies.

***Keywords**: Fibroblasts, cancer, co-culture, EGFR, FGF-3.*

INTRODUCTION

Fibroblasts are recognized as one of the most prevalent cell types in the tumor microenvironment (TME) [1,2]. In normal tissues, fibroblasts serve essential roles in maintaining homeostasis and facilitating wound healing by secreting various factors involved in the formation of the ECM, as well as other growth factors and cytokines crucial for tissue repair [3]. Normal fibroblasts possess a range of suppressive functions

against cancer initiation and metastasis, including direct cell-cell contact, paracrine signaling via soluble factors, and maintenance of ECM integrity. However, the loss of these inhibitory mechanisms signifies a natural phase in the development of cancer. Cancer cells induce the transition of normal fibroblasts into cancer-associated fibroblasts (CAFs), which subsequently initiate a cascade of pro-tumorigenic signals while disrupting the architecture of normal tissue, thereby creating an optimal niche for extensive cancer cell growth [1]. CAF-secreted factors include fibroblast growth factor (FGF), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), amphiregulin, epiregulin [4], a collection of matrix metalloproteinases (MMPs), and more [56]. Through inducing cancer cell proliferation, enhancing pro-tumor immune responses, modifying ECM, influencing drug resistance in tumor cells, and fostering angiogenesis, CAFs are essential in facilitating tumor progression and metastasis [7].

The cross-talk interaction between cancer cells and the TME amplifies neovascularization through angiogenic factors like MMPs and VEGF [8]. VEGF serves as the primary mediator of angiogenesis [9], which is one of the hallmarks of cancer [10]. On the other hand, MMPs participate by degrading the ECM, to create a pathway for endothelial cells migration which is an essential requirement for angiogenesis [11]. VEGF overexpression is exhibited in the majority of cancers, including NSCLC [12]. It has been reported that cytokines secreted by fibroblasts play a major role in the overexpression of VEGF [13]. It has also been reported that FGF initiates the Hedgehog signaling cascade, which regulates VEGF signal transduction [14]. Furthermore, the cooperative interaction between EGFR and FGFR has been shown to promote tumor growth. FGFR4 induces the expression of ErbB family ligands, resulting in the activation of EGFR [15]. EGFR belongs to the ErbB family of receptors, including ErbB1/EGFR/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 [16]. Numerous ligands, including amphiregulin, betacellulin, EGF, heparin-binding EGF-like growth factor, TGF-α, epiregulin, epigen, and Neuregulins (NRGs), can activate these receptors [17]. Currently, EGFR tyrosine kinase inhibitors (EGFR-TKIs) have demonstrated efficacy in anticancer therapy; however, the clinical efficacy of EGFR-TKIs can be altered by CAF-derived survival signaling to cancer cells [18,19]. CAF-secreted EGF-containing fibulin-like ECM protein-1 (EFEMP1) is known to promote tumor sphere formation, anchorage-independent growth, and cancer stemness maintenance in head and neck squamous cell carcinoma (HNSCC) [20]. Furthermore, the CAF-mediated EGFR signaling pathway plays a role in tumor invasion and metastasis. Collective invasion of squamous cell carcinoma (SCC) cells can be driven by matrix-dependent mechano-sensitization to EGFR signaling [21]. FGFs transmit signals by binding to FGF receptors (FGFRs), which play an integral role in various diseases [22]. FGFRs are receptor tyrosine kinases (RTKs) consisting of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain [23]. Upon FGF binding, FGFRs activate downstream signaling cascades, such as the PI3K/AKT and Ras/MAPK pathways, leading to an increased cell proliferation and resistance to apoptosis [242526]. TGF-β is a representative inducer of fibroblast activation, these TGF-β-activated fibroblasts overexpressed a CAF marker gene, alpha-smooth muscle actin, which is correlated with the overexpression of FGF [27]

In this study we aimed to investigate the role of fibroblasts on cancer progression, metastasis, angiogenesis, and apoptosis reflected by the expression of biochemical markers essential for such processes. We also aimed to compare between the direct and indirect co-culture methods to understand whether fibroblasts affect cancer cells directly or through secreted factors.

MATERIALS AND METHODS

Cells and Reagents

NSCLC cell lines A549 and normal human skin fibroblasts (HSF) were purchased from the American Type Culture Collection (ATCC) and maintained at the National Cancer Institute (NCI), Cairo, Egypt. Roswell Park Memorial Institute (RPMI-1640) medium, DMEM (Dulbecco's Modified Eagle's medium), fetal bovine serum (FBS), penicillin /streptomycin, and trypsin-EDTA were purchased from Sigma Aldrich Chemical Co., St. Louis, Mo, U.S.A.

Cell Culture

HSF and the A549 cell lines were maintained in DMEM supplemented with 10% FBS and 1% penicillin /streptomycin.

Preparation of conditioned media

NSCLC cell lines A549 cells were incubated in DMEM and RPMI 1640 respectively supplemented with 1% FBS for 24 h. After 24 h of incubation with tumor cells, the medium was recovered, centrifuged at 300 x g for 20 min to discard cell debris, and used to culture fibroblasts for 24 h, to obtain their activated forms, superimposable to native CAFs [28,29]. The activated form of fibroblasts was used to obtain AF CM. Activated fibroblasts were seeded in T75 culture flasks in 25 mL DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. The cells were incubated at 37 °C in a humidified incubator with 5% CO2 and allowed to grow until they reached 70–80% confluence to condition the media. Media was collected and centrifuged twice at 700 x g for 3 min., filtered, to remove dead cells/cellular debris. Supernatants (NF CM) were collected and stored at - 70°C to be used for the incubation of NSCLC cell lines [30].

Co-cultures of fibroblasts and NSCLC cell lines

For direct co-culture, A549 cells were seeded in 96-well plates with HSF cells in a ratio of 3:1 in RPMI medium supplemented with 10% FBS for 24 , 48, and 96 h [31]. As for the indirect co-culture, A549 cells were seeded in 96-well plates in AF CM for 24 , 48, and 96 h.

Enzyme-Linked Immunosorbent Assay (ELISA)

Human EGFR, Amphiregulin , Epiregulin, FGF-3, FGFR-3, caspases 3,7 ,8 ,9, VEGF, MMP2, MMP9 were analyzed using ELISA Kits (Catalog # ab100505), (Catalog # ELH-AR), (Catalog # ab213775), (Catalog # E2696Hu), (Catalog # MBS8291351), (Catalog # SEA626Hu), (Catalog # CSB-EL004552H), (Catalog # MBS452285), (Catalog # ab119508), (Catalog # MBS355343), (Catalog # MBS9135926) and (Catalog # MBS175780) in accordance with the manufacturer's instructions respectively. These parameters were measured for A549 monocultures, A549 co-cultured with HSF and A549 co-cultured with AF CM after 24, 48, and 96 h of co-culture.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation. To compare the expression of biochemical markers between monocultures and different co-culture conditions at 24 h, one way ANOVA was used followed by pairwise comparisons using Bonferroni correction. To evaluate changes in biomarker expression overtime, repeated measures ANOVA was employed. At each time point biomarker expression was compared between direct and indirect co-culture using two-sided unpaired t-test. For all tests, a two-tailed p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data visualizations were performed using "ggpubr" package (R package version 0.6.0).

RESULTS

Comparing mono- vs. co-culture on the expression of the studied parameters

Upon evaluating the expression of biochemical parameters, it was observed that the expression of the majority of such parameters was significantly increased in A549 cells upon co-culture for 24 h. For instance, the concentration of amphiregulin in A549 monocultures was 1,112.16±17.76, but it's concentration significantly increased to levels amounting to 30% increase in direct co-cultures, and 19% increase in indirect co-cultures (Table 1). The expression of epiregulin, FGF3, FGFR3, MMP 9, VEGF and the various caspases measured followed the same pattern (**Table 1**). Our results also revealed that the expression of the biochemical parameters was significantly higher in A549 cell line in direct co-culture than in indirect co-culture with AF CM 9 (**Figure 1 - 3**). Although MMP2 expression in A549 cells in direct co-cultures was higher than in monocultures, it showed no statistical significance ($p > 0.05$) (**Figure 3**).

Biochemical changes under different culture and co-culture conditions at various time points.

Our results revealed a significant time-dependent increase in the expression of the studied biochemical markers (**Table 2**). After direct co-culture with HSF for 24h, the concentration of amphiregulin was 1,449.61±8.88, but its concentration increased by 33% and 46.5% after 48h and 96h, respectively. The expression of other parameters, Epiregulin, FGF3, FGFR3, EGFR, VEGF, MMP9 and various caspases, also followed the same pattern; significantly increasing over time in both direct and in-direct co-cultures (**Table 2**). Our results also revealed that over time the expression of the studied biochemical parameters was significantly more pronounced in direct co-cultures compared to indirect co-cultures (**Figures 4-6**). MMP 2 levels, however, were significantly higher in co-cultures with AF CM at 96 h (**Figure 6**).

Table 1. Comparing cellular signaling events expression in A549 monocultures and after 24 h of co-culture.

Characteristic	Mono-culture	AF CM	HSF	p-value[1]
Amphiregulin	1,112.16±17.76	1,328.25±40.04	1,449.61±8.88	**<0.001**
Epiregulin	124.67±0.86	131.75±0.21	138.83±2.15	**<0.001**
FGFR3	1,790.32±9.22	2,352.74±18.44	2,472.59±9.22	**<0.001**
FGF3	792.81±10.14	817.15±14.20	898.29±6.09	**<0.001**
EGFR	1,104.35±9.29	1,104.35±0.00	1,139.18±2.32	**<0.001**
VEGF	408.02±2.58	411.89±3.87	429.94±1.29	**<0.001**
Caspase3	0.38±0.02	0.75±0.01	0.99±0.02	**<0.001**
Caspase7	112.55±8.98	458.12±4.49	494.03±4.49	**<0.001**
Caspase8	0.43±0.01	0.66±0.01	0.71±0.02	**<0.001**
Caspase9	3.08±0.30	4.49±0.10	5.49±0.10	**<0.001**
MMP2	573.16±32.37	562.37±64.74	605.53±43.16	0.6
MMP9	962.30±35.30	1,068.21±23.54	1,138.82±23.54	**<0.001**

Data were presented as mean±SD of 3 independent experiments.
Statistical significance is calculated using [1] One-way ANOVA (p<0.05 is considered significantly different)
FGFR3; fibroblast growth factor receptor-3: FGF3; fibroblast growth factor: EGFR; epidermal growth factor receptor: VEGF; vascular endothelial growth factor: MMP2; matrix metalloproteinase 2: MMP9; matrix metalloproteinase 9; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

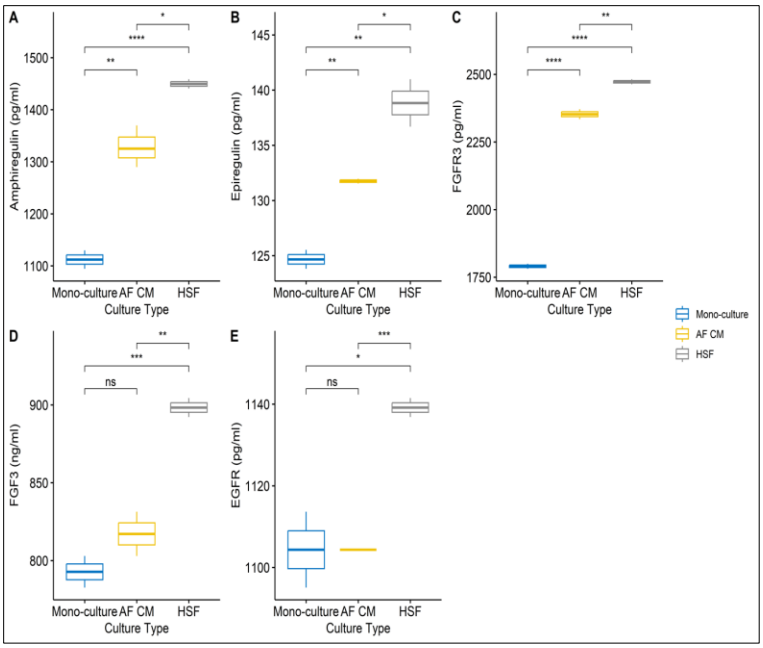

Figure 1. Differential expression levels of growth factors (amphiregulin, epiregulin, and FGF-3) and growth factor receptors (FGFR-3 and EGFR) in mono-culture and co-culture conditions. Data are presented as mean ± SD of 3 independent experiments. Statistical comparisons among groups were carried out using t-tests with Bonferroni correction. ns denotes nonsignificant, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

FGFR3; fibroblast growth factor receptor-3: FGF3; fibroblast growth factor: EGFR; epidermal growth factor receptor; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

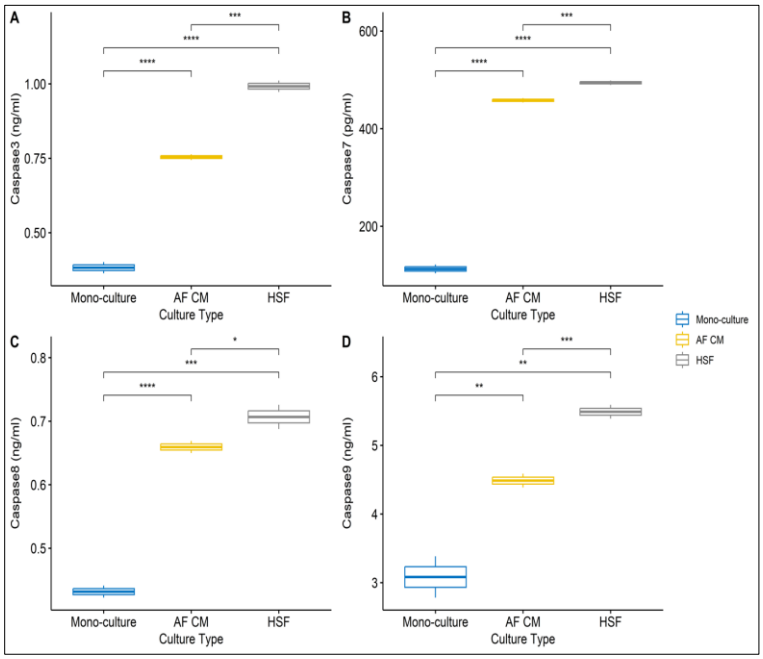

Figure 2. Differential expression levels of caspases in A549 cancer cell line mono-culture and under different culture and co-culture conditions. Data are presented as mean±SD of 3 independent experiments. Statistical comparisons among groups were carried out using t-tests with Bonferroni correction. ns denotes nonsignificant, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

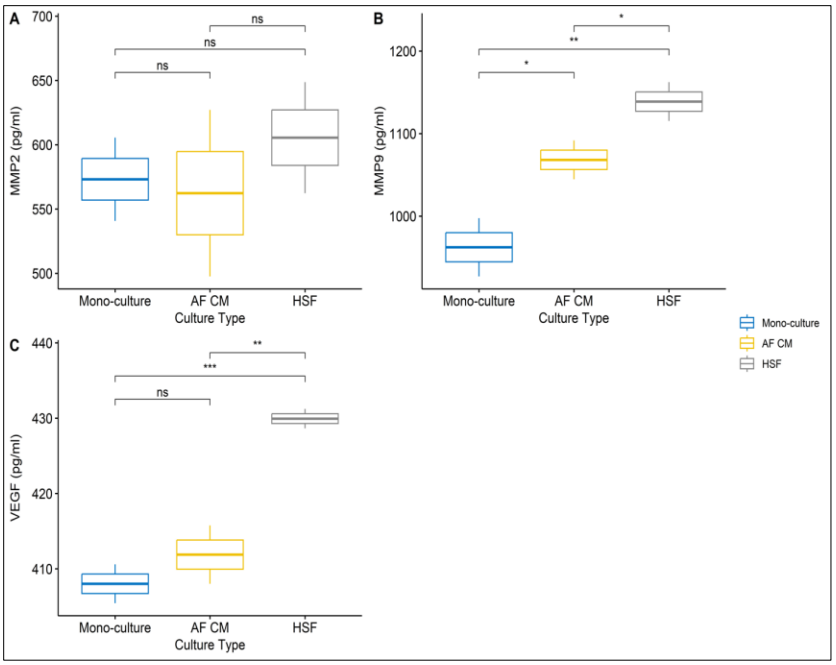

Figure 3. Differential expression of metalloproteinases (MMPS) and vascular endothelial growth factor (VEGF) in A549 mono-culture and different culture conditions. Data are presented as mean ±SD of 3 independent experiments. Statistical comparisons among groups were carried out using t-tests with Bonferroni correction. ns denotes nonsignificant, ** indicates p < 0.01, *** indicates p < 0.001, and **** indicates p < 0.0001.

MMP2; matrix metalloproteinase 2: MMP9; matrix metalloproteinase 9; VEGF: vascular endothelial growth factor; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

Table 2. Effect of coculture on the expression of different parameters over time

	AF CM				HSF			
Characteristic	**24 h**	**48 h**	**96 h**	**p-value[1]**	**24 h**	**48 h**	**96 h**	**p-value[1]**
Amphiregulin	1,328.25±40.04	1,578.38±22.20	1,782.62±22.20	**<0.001**	1,449.61±8.88	1,933.59±22.20	2,124.51±53.28	**<0.001**
Epiregulin	131.75±0.21	143.13±1.72	157.93±0.21	**<0.001**	138.83±2.15	156.43±0.86	174.67±0.21	**<0.001**
FGFR3	2,352.74±18.44	2,518.69±18.44	2,693.87±27.66	**<0.001**	2,472.59±9.22	2,675.43±9.22	2,850.61±18.44	**<0.001**
FGF3	817.15±14.20	894.23±6.09	940.89±16.23	**<0.001**	898.29±6.09	912.49±4.06	1,032.17±2.03	**<0.001**
EGFR	1,104.35±0.001	1,146.14±4.64	1,187.93±4.64	**<0.001**	1,139.18±2.32	1,141.50±4.64	1,234.36±4.64	**<0.001**
VEGF	411.89±3.87	438.97±2.58	436.39±2.58	**<0.001**	429.94±1.29	422.20±1.29	473.80±14.19	**<0.001**
Caspase3	0.75±0.01	0.85±0.01	1.07±0.02	**<0.001**	0.99±0.02	0.93±0.03	1.21±0.03	**<0.001**
Caspase7	458.12±4.49	628.66±22.44	660.08±8.98	**<0.001**	494.03±4.49	624.18±8.98	704.96±8.98	**<0.001**
Caspase8	0.66±0.01	0.74±0.02	0.87±0.01	**<0.001**	0.71±0.02	0.79±0.01	0.92±0.01	**<0.001**
Caspase9	4.49±0.10	8.50±0.10	9.60±0.20	**<0.001**	5.49±0.10	9.20±0.20	10.30±0.10	**<0.001**
MMP2	562.37±64.74	573.16±32.37	864.50±21.58	**<0.001**	605.53±43.16	605.53±21.58	551.58±32.37	0.06
MMP9	1,068.21±23.54	1,138.82±47.07	1,256.50±23.54	**0.001**	1,138.82±23.54	1,185.89±23.54	1,303.57±47.07	**<0.001**

Data were presented as mean±SD of 3 independent experiments.

Statistical significance is calculated using [1] Repeated Measures ANOVA (p<0.05 is considered significantly different)

FGFR3; fibroblast growth factor receptor-3: FGF3; fibroblast growth factor: EGFR; epidermal growth factor receptor: VEGF; vascular endothelial growth factor: MMP2; matrix metalloproteinase 2: MMP9; matrix metalloproteinase 9; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

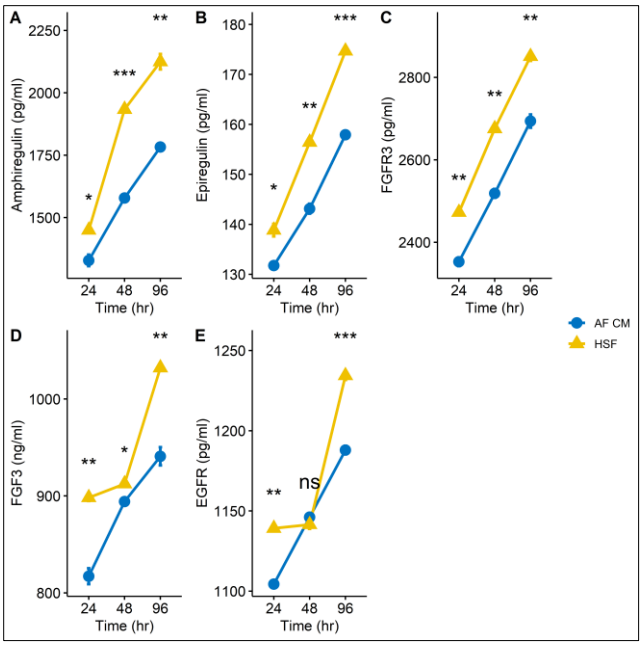

Figure 4. Expression Patterns of Growth Factors and Growth Factor Ligands Over Time in Co-cultures. Data is presented as mean ±SD of 6 replicates. p-values calculated using two-sided unpaired t-test; 'ns' indicates a nonsignificant difference, '*' indicates $p < 0.05$, '**' indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

FGFR3; fibroblast growth factor receptor-3: FGF3; fibroblast growth factor: EGFR; epidermal growth factor receptor; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

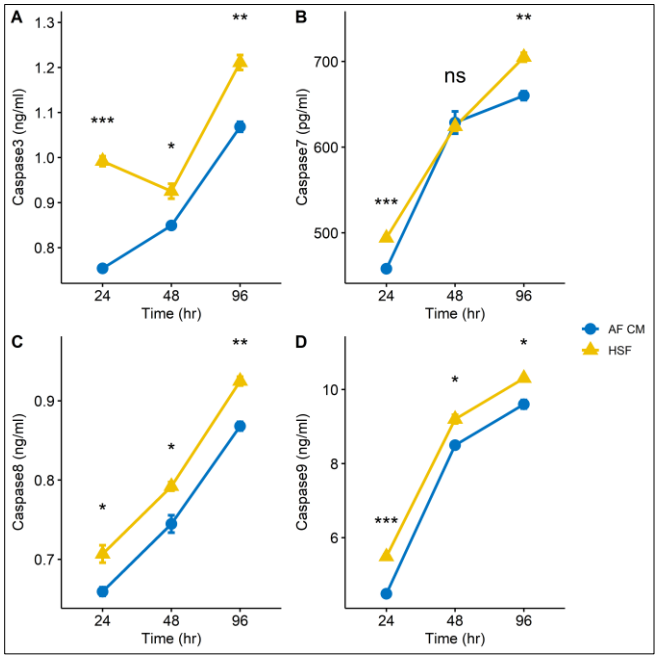

Figure 5. Expression Patterns of Caspases Over Time in Co-cultures. Data is presented as mean ±SD of 6 replicates. p-values calculated using two-sided unpaired t-test; 'ns' indicates a nonsignificant difference, '*' indicates $p < 0.05$, '**' indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

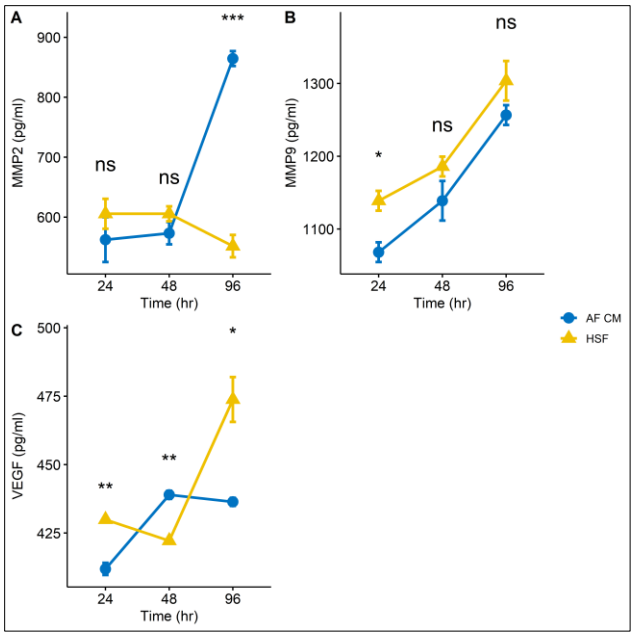

Figure 6. Expression Patterns of Metalloproteinases and VEGF Over Time in Cocultures. Data is presented as mean ±SD of 6 replicates. p-values calculated using two-sided unpaired t-test; 'ns' indicates a nonsignificant difference, '*' indicates $p < 0.05$, '**' indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

MMP2; matrix metalloproteinase 2: MMP9; matrix metalloproteinase 9; VEGF: vascular endothelial growth factor; AF CM; activated fibroblast conditioned media; HSF: human skin fibroblasts.

DISCUSSION

Fibroblasts are recognized as one of the most prevalent cell types in the tumor microenvironment (TME) [1,2]. Normal fibroblasts possess a range of suppressive functions against cancer initiation and metastasis. However, the loss of these inhibitory qualities signifies a natural phase in the development of cancer. Tumor cells induce a transition of normal fibroblasts into cancer-associated fibroblasts (CAFs), which subsequently initiate a cascade of pro-tumorigenic signals while disrupting the architecture of normal tissue, thereby creating an optimal niche for extensive cancer cell growth [1]. Fibroblasts are essential for preserving the homeostasis of surrounding epithelial cells, operating indirectly through paracrine pathways via growth factors [32] or directly through cell-cell interactions [33]. In this study we aimed to investigate the role of fibroblasts on NSCLC cells proliferation, angiogenesis, and metastasis through evaluating the expression of essential signaling pathways such as EGFR, FGF3/FGFR3 and the expression of VEGF and selected MMPs 2 and 9. We also sought to compare the effects of direct co-culture versus conditioned media, investigating whether fibroblasts interacted with NSCLC cells directly or indirectly.

Aiming to investigate the effect of fibroblasts on proliferation, angiogenesis, ECM remodeling and metastasis of cancer cells. NSCLC cell lines were co-cultured with fibroblasts for 24h, and quantitative analysis was performed to evaluate the expression of EGFR and its ligands; amphiregulin and epiregulin, FGF3 and its receptor FGFR3 which play a crucial role in cancer cell proliferation and overall survival. Our findings showed an upregulation in the expression of EGFR and its ligands and the overexpression of FGF3 and FGFR3 upon co-culture with fibroblasts which indicate an activation of cell survival mechanisms. It has also been suggested that co-overexpression of FGF3 and Epidermal growth factor receptor (EGFR) plays a pivotal role in the pathogenesis of NSCLC [34]. EGFR overexpression is linked to a more aggressive cancer phenotype, a poor clinical prognosis, and development of chemoresistance. Six mammalian ligands that bind to EGFR have been characterized, including epidermal growth factor (EGF), transforming growth factor-α(TGFα), amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin [35]. It has been reported that EGFR is constantly stimulated because of the continuous production of EGFR ligands in the TME [3637]. EGFR hyperactivation leads to an increase in drug efflux, DNA damage repair, and apoptosis inhibition [38]. Furthermore, excessive mitogenic signaling through the

FGF/FGFR axis may induce carcinogenic effects by promoting cancer progression and increasing the angiogenic potential, which can lead to metastatic tumor phenotypes. Dysregulated FGF/FGFR signaling is associated with aggressive cancer phenotypes, enhanced chemotherapy resistance and poor clinical outcomes [39]. Moreover, FGFs might trigger FGFRs activation and downstream signaling cascades, such as the Ras/MAPK and PI3K/AKT signaling pathways [40] leading to an increased cell proliferation and resistance to apoptosis[24-26]. In support of our findings, Magan et al. [41] showed that upon co-culture with CAFs, head and neck cancer cells showed an increase in EGFR expression. Hong et al [42] also identified that FGFR4 overexpression secretes EGFR ligands such as amphiregulin with consequent activation of EGFR. This result was also revealed in in vivo study and the cooperative interaction between EGFR and FGFR4 led to the promotion of cancer growth. In addition, FGFR4 overexpression was shown to reduce cetuximab-induced cytotoxicity and the combination of FGFR4 inhibitor (BLU9931) and cetuximab showed profound antitumor effect compared to cetuximab. Furthermore, when Fujita et al. (2009) [43] aimed to study tumor-stromal interactions in an in vitro coculture model between human pancreatic ductal adenocarcinoma and fibroblasts, they observed that co-culture conditions increased FGF-7 secretion and α-SMA expression, characterized by fibroblast activation and decreased epithelial marker E-cadherin in tumor cells.

In the present study, we aimed to evaluate the expression of VEGF and MMPs 2 and 9 which play a major role in regulating the process of angiogenesis. Our findings showed that VEGF and MMPs 2 and 9 were upregulated in NSCLC cells co-cultured with fibroblasts for 24 h. Overexpression of VEGF has been found in most human tumors, including NSCLC, and is associated with increased tumor recurrence, metastasis, and death [44]. VEGF is the main mediator of angiogenesis. In addition, VEGF contributes to cancer growth and metastasis [45]. On the other hand, MMPs is a family of structural-related zinc-dependent endopeptidase which generally does its actions by degrading macromolecules of the extracellular matrix and has around 28 members in the family, all comprise different types of actions. MMPs have a dual role in tumor growth and metastasis processes. They promote tumor growth by degrading matrix barriers and by enhancing angiogenesis [11]. It has been reported that the presence of CAFs is essential for angiogenesis [46]. Other reports have shown that gene expressions related to tumor angiogenesis and ECM degradation are enhanced when NSCLC tumor cells are cocultured with fibroblasts [47]. Wang L et al. (2017) [48] also found that IL-6 present in CAF-conditioned media (CM) and normal fibroblasts-CM induces overexpression of EMT-related genes and proteins, including vimentin, MMP2, MMP9, and VEGF, supporting our results. Strengthening our claim, when Liu et al. (2016) [49] employed a three-dimensional (3D) cell co-culture collagen gel model, containing human lung adenocarcinoma cells (HCC), human lung fibroblast cells (MRC-5), and macrophages, MMP-1 and VEGF were secreted at higher levels in mixed cell groups rather than mono-culture groups.

To investigate the effect of fibroblasts co-culture on the apoptosis of NSCLC cells , we evaluated the expression of various caspases. Members of the caspase family of proteases play essential roles in the initiation and execution of apoptosis. These caspases are divided into two groups: the initiator caspases (caspase-2, -8, -9 and -10), which are the first to be activated in response to a signal, and the executioner caspases (caspase-3, -6, and -7) that carry out the demolition phase of apoptosis [50]. Data regarding the effect of fibroblasts on apoptosis in the literature are contradictory, for instance, ….et al, demonstrated that when colon cancer cells were co-cultured with various types of fibroblasts and change in the apoptotic rate was witnessed depending on the type of fibroblast [51].

In the current study, co-culture was conducted directly and indirectly though conditioned media to investigate whether the interaction between fibroblasts and cancer cells requires the close proximity of both cell types or the majority of the interactions occur via the secretion of growth factors. Interestingly, we observed that direct co-culture was more effective in upregulating the expression of these biomarkers compared to conditioned media, indicating the importance of direct cell-cell contact in mediating the effects of fibroblasts on NSCLC cells. Similarly, Dhungel et al. (2023) demonstrated that specifically upon direct contact with fibroblasts, cancer cells undergo profound reprogramming and develop a partial EMT phenotype in which EMT-inducing growth factors, as well as ECM remodeling proteins, are highly upregulated [52]. On the other hand, Saad et al (2000) et al observed that co-culture of breast cancer cells and bone marrow fibroblasts resulted in augmentation of the levels of the matrix metalloproteases MMP-1 and MMP-2 in culture supernatants. The authors stated that soluble factors produced by bone marrow fibroblasts were responsible for the increase in MMP-1 levels, however, maximal MMP-2 production was dependent on direct contract between the breast cancer cells and the bone marrow fibroblasts [53]. Taken together, these findings highlight the complexity of the interactions that occur within the TME, indicating that some interactions depend on direct contact between cells while others simply depend on secreted factors.

Furthermore, our study revealed a temporal aspect to the effects of co-culture, with the expression of biomarkers increasing over time with prolonged co-culture. Despite this temporal increase, direct co-culture remained the most effective method for inducing

biomarker expression. This suggests that the effects of fibroblasts on NSCLC cells are sustained over time and are dependent on continuous interaction between the two cell types.

Similarly, Salvatore et al. (2015) [54] evaluated the interactions of fibroblasts and osteosarcoma cells in a transwell co-culture system over 24 h, 48 h, 72 h, and 96 h. the authors analyzed the contributions of these populations to the TME during cancer progression, as measured by multiple markers, focusing on those involved in cancer cell invasion, inflammatory responses, and angiogenesis: *TNF alpha*, *IL-6*, *MMP-1*, *MMP-9*, and *VEGF*. It was observed that the gene expression levels of the mentioned markers exhibited similar trends to our findings, reaching the highest level at 72 h and 96 h. Moreover, Liu et al (2022) [55] performed co-culture experiments with tumor cells and fibroblasts embedded in 3D collagen I matrices. The authors investigate the impact of fibroblasts on the migratory behavior of neighboring tumor cells and on the evolution of the surrounding ECM, their results indicate time-dependent evolution of the fibroblast-mediated microenvironment toward a state that facilitates tumor migration.

These discrepancies highlight the complexity of tumor-stroma interactions and the context-dependent nature of fibroblast function in cancer. Factors such as the specific tumor microenvironment, cancer cell type, and experimental conditions may contribute to the divergent results observed across studies.

There are major limitations in this study that could be addressed in future research. The study model used only one cell line from one cancer type, the authors believe that the results may have been enforced by several cell lines.

CONCLUSION

In conclusion, our study provides valuable insights into the role of fibroblasts in NSCLC progression and identifies potential therapeutic targets for intervention. By elucidating the signaling pathways involved in tumor-stroma interactions, we contribute to the growing body of knowledge aimed at developing targeted therapies for cancer. Future research should further investigate the mechanisms underlying fibroblast-mediated tumor progression and explore novel strategies to disrupt this interaction for therapeutic benefit.

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Conflict of interest

The author declares that there isn't any conflict of interest regarding the publication of this paper.

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