

# Cancer Microenvironment-Stimulated Mesenchymal Stem Cells in an Indirect Co-Culture System Influence Cancer Cell Growth and Apoptosis

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**Keywords:** Mesenchymal stem cells, Conditioned media, Cancer-associated fibroblasts, Tumor microenvironment, Lung cancer, Extracellular matrix

## Abstract

Mesenchymal stem cells (MSCs) migrate to injured tissues, aiding tissue repair, remodeling, and wound healing. As tumors are often considered to have traits of “injured tissues,” MSCs are recruited to tumor microenvironments where they can have pro- and antitumorigenic influence.

This study assesses whether human mesenchymal stem cells (hMSCs) of shared ancestry exhibit similar tumorigenic properties. Bone marrow-derived (hBM-MSCs) and umbilical cord-derived (hUC-MSCs) MSCs embedded in collagen are cultured in conditioned media from lung

adenocarcinoma (A549) cells to mimic the extracellular matrix and soluble cues of the cancer microenvironment. Cell viability, proliferation, and immunofluorescence analyses evaluate MSC behavior under these conditions. Further, A549 cells are exposed to conditioned media from cancer-stimulated MSCs to simulate indirect co-culture, and their response is assessed through viability, immunofluorescence, and flow cytometry.

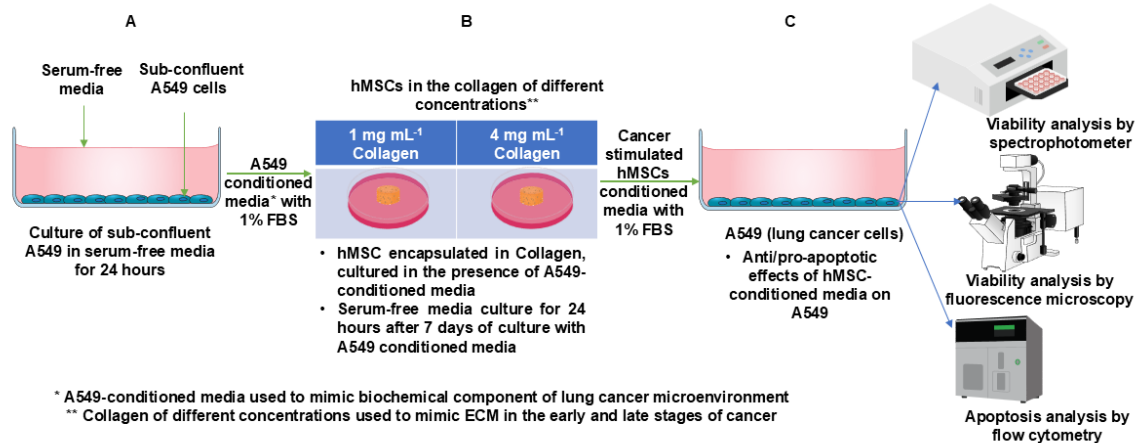
Results show increased viability and proliferation of hBM-MSCs, morphological changes, and elevated alpha-smooth muscle actin expression, suggesting a transition toward cancer-associated fibroblasts. In contrast, hUC-MSCs display reduced viability and no morphological alterations. Conditioned media from cancer-exposed hUC-MSCs induce apoptosis in A549 cells, whereas hBM-MSCs support A549 growth. These findings demonstrate that, despite their common origin, hUC-MSCs and hBM-MSCs exhibit opposing responses to tumor cues and influence lung cancer cell behavior differently.

## **1. Introduction**

As one of the most common malignancies, lung cancer is the principal factor contributing to mortality from cancer.<sup>[1]</sup> Furthermore, the general five-year survival rate for lung cancer is only 25%, and the disease still claims a substantial number of deaths despite advancements in surgery, chemotherapy, and radiation therapy. As reported by Siegel et al., lung cancer will claim the lives of more than 340 individuals per day, surpassing the combined deaths from pancreatic, breast, colorectal, and prostate cancers by 2.5 times.<sup>[2]</sup> Therefore, there is an urgent need for new therapies to treat this fatal disease. It is evident from extensive research studies that conceptualizing cancer as a disease solely focused on tumor cells is not a valid approach to identifying therapeutic targets. The extracellular matrix and stromal cell populations that comprise tumor stromal tissue are very different from the quiescent counterparts in homeostatic tissues and contribute to tumor establishment.<sup>[3]</sup> Mesenchymal stem cells (MSCs) have attracted considerable attention because of their potential therapeutic applications and unique properties, including their ability to undergo clonal expansion, multi-lineage differentiation, tissue repair/regeneration, and homing on sites of injury or inflammation.<sup>[4]</sup> Owing to these characteristics, MSCs have been proposed as a therapeutic intervention for various fibroproliferative illnesses, including cancer and fibrosis. To date, 48 clinical trials assessing MSC-based therapies for treating or alleviating cancer conditions have been registered on

*ClinicalTrials.gov*. The global database contained 21 completed studies.<sup>[5]</sup> However, it has not been thoroughly studied whether the mechanical and biochemical microenvironments in these pathological situations cause MSCs to adopt a pro-fibrotic myofibroblast fate.<sup>[6]</sup> The effect of mesenchymal stem cells on various cancer types has been extensively studied.<sup>[7–10]</sup> However, these findings are debatable, and no reliable conclusion has yet been reached. According to prior research, different outcomes can be attained based on the type of tumor, MSC origin, MSC dosage, and animal model used.<sup>[11,12]</sup> Researchers have recently studied the interactions between MSCs from different sources and cancers of various origins.<sup>[13,14]</sup> However, most studies have focused on the interaction between MSCs and cancer cells without considering the extracellular matrix (ECM), a crucial cancerous tissue component. The ECM is dynamic during cancer progression. Therefore, the state of the ECM should be well understood when deciding the treatment regime of cancer tissue. Tripartite communication between MSCs, cancer cells, and ECM has been poorly explored. The prime element of the extracellular matrix (ECM) is collagen type I, which also serves as a scaffold for the tumor microenvironment. Therefore, the most significant ECM-related element in creating the biochemical processes that form a supporting niche is the quantity of collagen present in the host tissue.<sup>[15]</sup> Variations in collagen concentrations may be significant for the survival and expansion of tumors. It has also been shown that other cells, like fibroblasts and mesenchymal stem cells, respond to the surrounding extracellular matrix's mechanical characteristics through cellular mechanosensing.<sup>[16,17]</sup> A study by Kuczek et al. showed that a cancerous tissue's collagen density regulates tumor-infiltrating T cells' activity.<sup>[18]</sup> It remains speculative whether the density of collagen, the most abundant component of the tumor ECM, can modulate the tumor-responsive properties of MSC and thereby support cancer cell survival. Thus, there is a gap in research regarding the effect of mesenchymal stem cells cultured in relevant cancer microenvironments on cancer cell survival. It has been demonstrated that MSCs traveling from the yolk sac to the placenta during the fetal developmental phase and returning to the bone marrow (BM) through the umbilical cord (UC) become entrapped in the Wharton's jelly.<sup>[19]</sup> Ultimately, homed hBM-MSCs and the hUC-MSCs share ancestry.

In this study, we employed a 3D-matrix-based hydrogel model to investigate the impact of two hMSCs of the same developmental origin on A549 cells (**Figure 1**).



**Figure 1. A schematic of the experimental workflow. (A)** A549 cells were cultured to obtain conditioned medium. **(B)** hMSCs were encapsulated in collagen and exposed to A549-conditioned media (supplemented with 1% FBS) for 7 days, followed by culture in serum-free media for 24 hours to obtain cancer-stimulated hMSC-conditioned media. **(C)** A549 cells were then treated with cancer-stimulated hMSC-conditioned media to assess effects on cell viability and apoptosis.

Specifically, we investigated the viability of A549 cells in response to conditioned media (CM) derived from the culture of cancer-stimulated hMSC at varying collagen concentrations, representing different stages of cancer. The use of collagen gel rather than Matrigel was appropriate for our model for various reasons. Type I collagen is a well-defined, single-component extracellular matrix protein, making it easier to control and reproduce experimental conditions. Although Matrigel contains a complex mixture of growth factors and ECM proteins, its batch-to-batch variability can cause inconsistent experimental results. Collagen gels allow tuning of mechanical properties like stiffness by varying concentration, which is important for studying cell–matrix interactions in 3D. Matrigel has limited tunability and degrades faster, making it less ideal for long-term studies. <sup>[20]</sup>

The Young's modulus of non-cancerous lung tissue ranges from 1 to 5 kPa.[21] In our study, 1 mg mL<sup>-1</sup> collagen (10 kPa) mimicked the initial cancer stage, while 4 mg mL<sup>-1</sup> collagen (40 kPa) mimicked progressive cancer ECM.[7,18,21] These studies were conducted with a 3D culture of hMSC in a cancer microenvironment where conditioned media of A549 mimicked a biochemical component of the microenvironment, and the biomechanical part was mimicked by collagen of

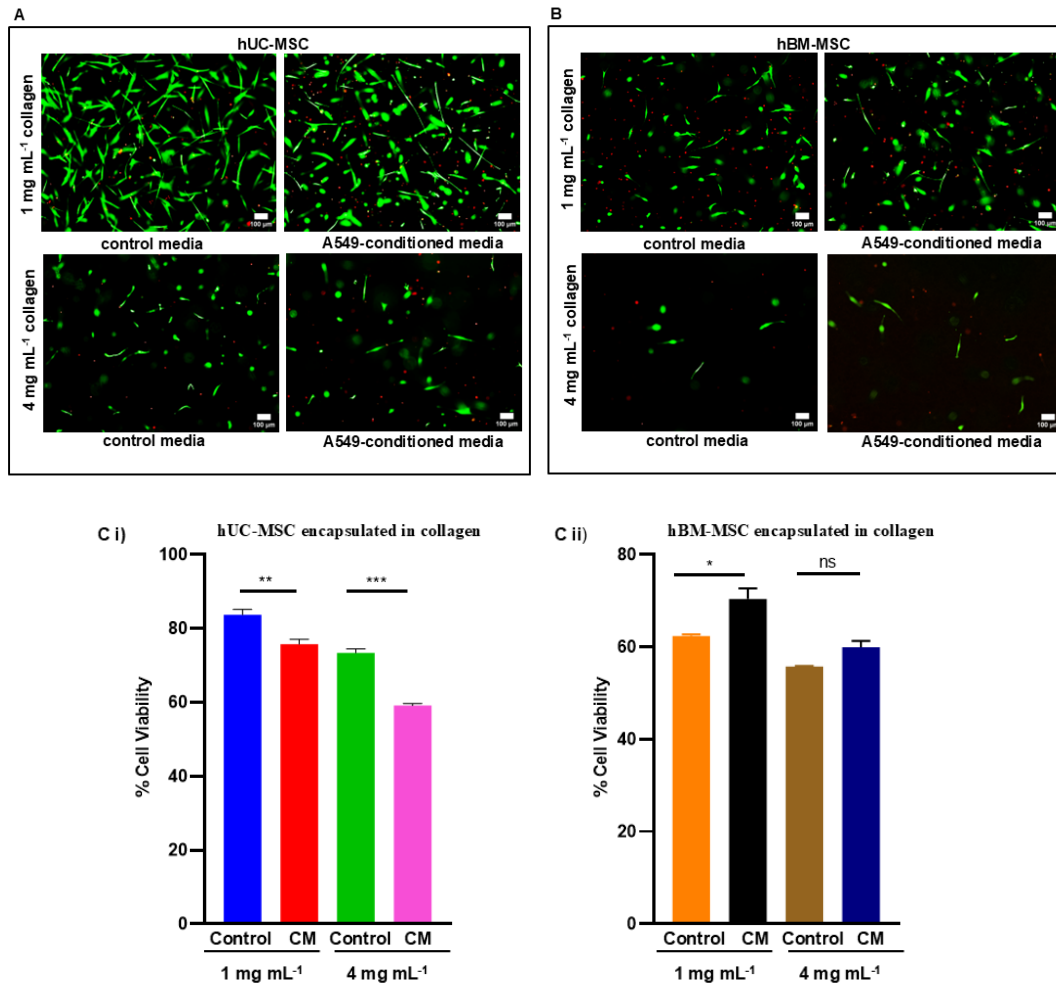
two different concentrations. After confirming hMSCs' viability, proliferation, and transition to cancer-associated fibroblasts (CAF) in the lung cancer microenvironment using a live/dead assay, a tetrazolium salt assay (WST) assay, and immunofluorescence, respectively, we investigated the effects of cancer-stimulated hMSCs' conditioned media on lung cancer cells. Furthermore, using a live/dead staining, WST assay, Hoechst nuclear staining, and a flow cytometer, we assessed the viability, alterations in nucleus morphology, and the apoptosis trends in A549 cells, respectively, after exposure to cancer-stimulated hMSCs CM.

We demonstrated that hMSCs of exact developmental origin have different effects on the viability of A549 cells. We anticipate using this in vitro model to facilitate early-phase hMSC-based therapeutic development in preclinical studies to evaluate the role of hMSCs from different tissue sources in multiple types of cancers more efficiently at a relatively low cost and in a high-throughput manner.

## 2. Results

### 2.1. Lung cancer-like microenvironment influences the viability and proliferation of hMSCs

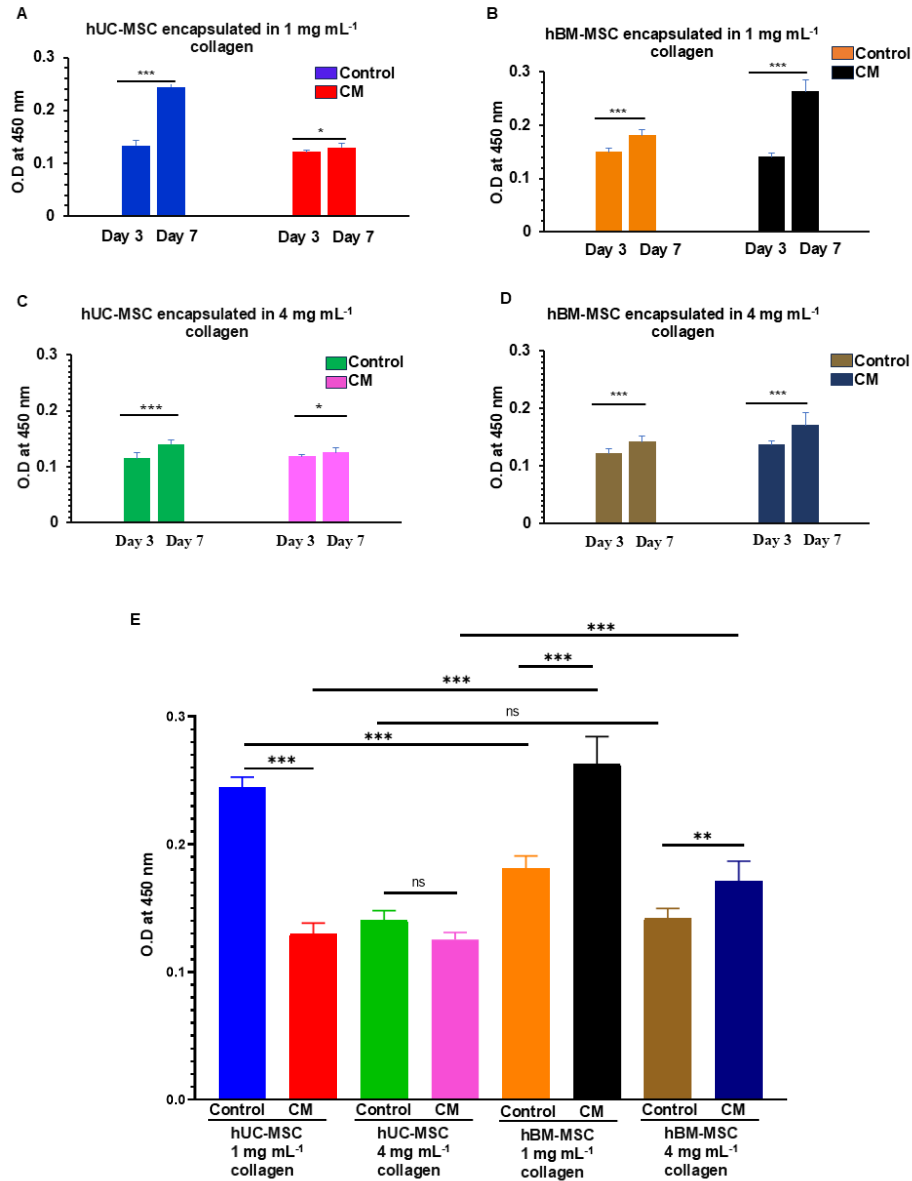
To investigate the effect of A549 conditioned media on the viability of 3D cultured hMSCs in collagen gels of two different concentrations, we first evaluated the viability of hUC-MSCs and hBM-MSCs after seven days of culture. Calcein-AM staining produced green fluorescence, indicating live cells. Non-viable cells were identified by red fluorescence obtained from EHD labeling (**Figure 2A-B**). hUC-MSCs encapsulated in 1 mg mL<sup>-1</sup> collagen gels cultured in conditioned media were less viable (84.96 %) than in control media (95.73 %,  $p = 0.0083$ ). Also, hUC-MSCs encapsulated in 4 mg mL<sup>-1</sup> collagen gels cultured in conditioned media were less viable (59.09 %) than in control media (73.37 %,  $p = 0.009$ ) (**Figure 2Ci**). In contrast, hBM-MSCs showed the opposite trend, where hBM-MSCs encapsulated in 1 mg mL<sup>-1</sup> collagen gels cultured in conditioned media (80.18 %) were more viable than in control media (71.4 %;  $p = 0.014$ ) (**Figure 2Cii**). The hBMSCs encapsulated in 4 mg mL<sup>-1</sup> collagen gels did not show significant differences in percent cell viability when compared between the control and conditioned media groups (**Figure 2**).



**Figure 2. Cell viability assay of collagen-encapsulated hMSCs cultured with control and A549-conditioned media. A) hUC-MSCs encapsulated in 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> collagen gel. B) hBM-MSCs encapsulated in 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> collagen gel. Live cells (green) were stained with Calcein-AM, and dead cells (red) were stained with Ethidium homodimer. Scale bar = 100  $\mu$ m. C) Quantification of live/dead cells on day 7. One-way ANOVA with Tukey test was performed to assess statistical significance. Data are presented as mean  $\pm$  standard deviation (SD). Error bars represent SD. (\* $p$  < 0.05 ; \*\* $p$  < 0.001, \*\*\* $p$  < 0.001).  $n$  = 6 images. CM: Conditioned media**

Having confirmed the viability of hBM-MSCs and hUC-MSCs, we next quantified the cell proliferation rate in each condition based on metabolic activity using the WST-8 colorimetric assay. Over one week, hMSCs encapsulated in collagen gradually increased proliferation within the control and conditioned media groups (**Figure 3A-D**). However, we observed different effects of the conditioned and control media on the proliferation rate of hUC-MSCs and hBM-MSCs in the 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> collagen gels. In the hUC-MSC group, cells in the

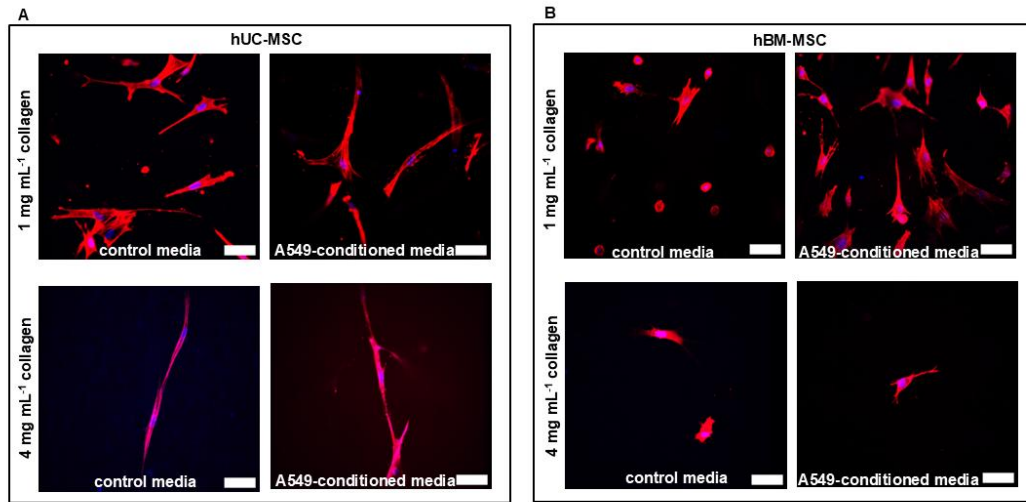
1 conditioned media showed a lower proliferation rate over time compared to the higher  
2 proliferation rate in control media, with the effect being more evident in 1 mg mL<sup>-1</sup> collagen gel  
3 than in the 4 mg mL<sup>-1</sup> collagen gel (**Figure 3A and C**). However, hBM-MSCs showed higher  
4 proliferation in conditioned media than in control media, with a more pronounced effect in the 1  
5 mg mL<sup>-1</sup> collagen gel compared to the 4 mg mL<sup>-1</sup> collagen gel (**Figure 3B and D**). In addition,  
6 hUC-MSCs showed higher proliferation in control media than hBM-MSCs, ~~with a more~~  
7 ~~significant difference in the 1 mg mL<sup>-1</sup> collagen gel than in the 4 mg mL<sup>-1</sup> collagen gel~~ a  
8 statistically significant (p<0.0001) difference was observed in the 1 mg mL<sup>-1</sup> collagen gel, while  
9 the difference in the 4 mg mL<sup>-1</sup> condition was not significant. In the case of conditioned media,  
10 the opposite effect was observed, with a higher proliferation rate for hBM-MSCs (p<0.0001)  
11 than hUC-MSCs in both 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> collagen gels (**Figure 3E**). Also, it was  
12 observed that when both hUC-MSCs and hBM-MSCs were cultured in the same concentrations  
13 of collagen (either 1 mg mL<sup>-1</sup> or 4 mg mL<sup>-1</sup>) with A549 conditioned media, hBM-MSCs had a  
14 higher proliferation rate. Additionally, on comparison between hBM-MSCs encapsulated in 4  
15 mg mL<sup>-1</sup> and hUC-MSCs encapsulated in 1 mg mL<sup>-1</sup> and vice-versa, hBM-MSCs still had a  
16 higher rate of proliferation (**Figure S1**)



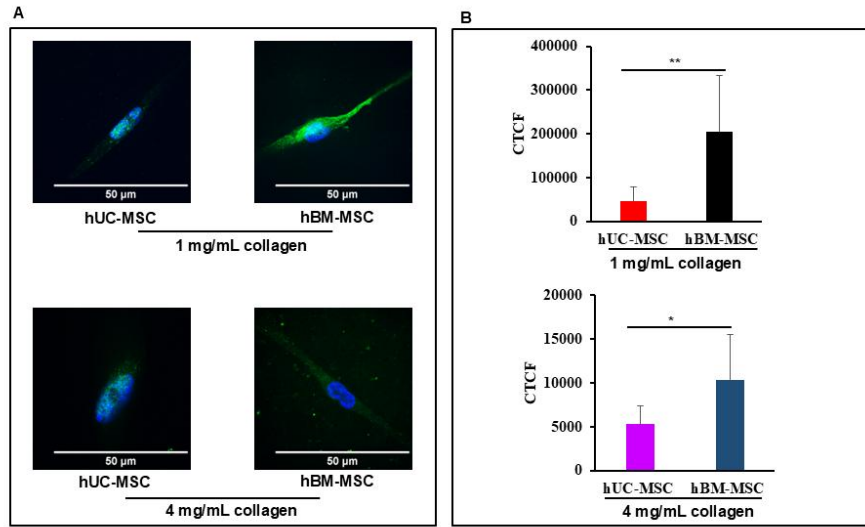
**Figure 3. Proliferation analysis of hMSCs in lung cancer-like microenvironment using WST-8 assay on days 3 and 7 in control and A549-conditioned media for A) 3D culture of hUC-MSC in 1 mg mL<sup>-1</sup> collagen. B) 3D culture of hBM-MSC in 1 mg mL<sup>-1</sup> collagen. C) 3D culture of hUC-MSC in 4 mg mL<sup>-1</sup> collagen. D) 3D culture of hBM-MSC in 4 mg mL<sup>-1</sup> collagen. E) On day 7, cell proliferation was compared between hUC-MSCs and hBM-MSCs embedded in the collagen of 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> concentrations. One-way ANOVA with Tukey test was used to test the statistical significance. Data are presented as mean  $\pm$  standard deviation (SD). Error bars represent SD. (\* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ )  $n = 6$  experiments. O.D., Optical density; CM, Conditioned media.**

1  
2       2.2.   *Lung cancer-like microenvironment influences hMSCs differently in*  
3               *terms of morphology and transition to cancer-associated fibroblasts*  
4               *form*

5 Mechanical forces linked to filamentous actin (F-actin) are crucial for the growth and  
6 differentiation of stem cells. We assessed the expression of F-actin to investigate the  
7 morphological changes in hMSC in the cancer microenvironment.  $\alpha$ -SMA expression was  
8 analyzed to confirm the differentiation of hMSC into cancer-associated fibroblasts (CAF) in the  
9 lung cancer microenvironment. hUC-MSCs did not display any significant change in  
10 morphology when comparing cells cultured with the control and those cultured with conditioned  
11 media, independent of the concentration of the collagen gels (**Figure 4A** and **Figure S2**).  
12 However, hBM-MSCs cultured with control media displayed a round morphology, independent  
13 of the concentration of collagen gels (**Figure 4B, left**). On the other hand, the cells cultured in  
14 conditioned media spread more and possessed organized stress fibers than those cultured in  
15 control media (**Figure 4B, right** and **Figure S2**). An inverse relationship was observed between  
16 cell spreading and circularity, with more spread cells exhibiting lower circularity values,  
17 indicating a transition from rounded to elongated morphology.



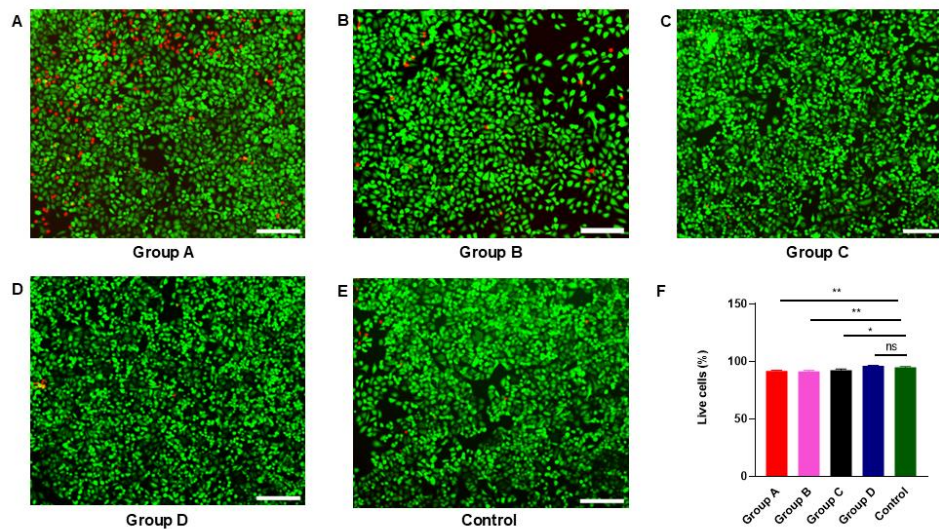
**Figure 4. Immunofluorescence assay for F-actin (red) and Hoechst (blue) to determine the morphology of hMSCs in lung cancer-like microenvironment at day 3 in control and A549-conditioned media. A) Morphology of hUC-MSCs encapsulated in collagen of two different concentrations (1 and 4 mg mL<sup>-1</sup>) B) Morphology of hBM-MSCs encapsulated in collagen of two different concentrations (1 and 4 mg mL<sup>-1</sup>). Scale bar = 200  $\mu$ m. Higher magnification images are provided in Supplementary Figure S2 for better visualization.**



**Figure 5. Immunofluorescence of CAF marker ( $\alpha$ -SMA) in hMSCs cultured with A549-conditioned media encapsulated in 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup> collagen gels. A)  $\alpha$ -SMA (green) and nuclei (blue). B) Fluorescence quantification of  $\alpha$ -SMA using CTCF (Corrected total cell fluorescence). Scale bar = 50  $\mu$ m; The student's t-test was used to test the statistical significance. Data are presented as mean  $\pm$  standard deviation (SD). Error bars represent SD. (\*p < 0.05 and \*\*p < 0.001) n = 30 cells. A representative single-cell image is presented; multiple similar fields are shown in Supplementary Fig. S3 to demonstrate consistency.**

### 2.3. Exposure to conditioned media of cancer-stimulated hMSCs influences the viability of A549 cells variably, depending on the type of hMSCs

To investigate the reciprocal effect of cancer-stimulated hMSCs on the survival of cancer cells, the viability of A549 cells was analyzed after exposure to conditioned media of cancer-stimulated hMSCs for 24 hours. Qualitative analysis was done using Calcein-AM and EHD staining. Green fluorescence indicates live cells, while red fluorescence represents dead cells. Group A (91.7% live cells; 8.2 % dead cells; p = 0.003) and Group B (91.1% live cells; 8.8 % dead cells; p = 0.001) cell viability was less compared to the control group (94% live cells; 5.2% dead cells). (Figure 6 A, B, and E).

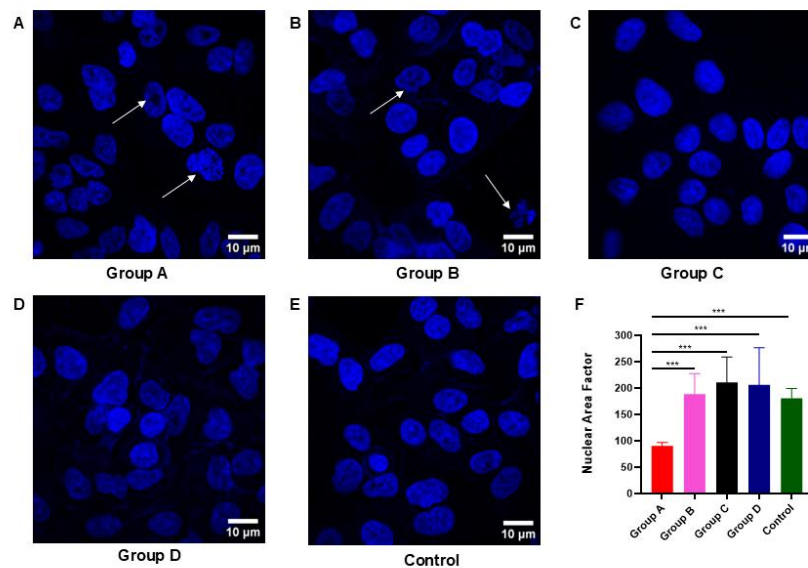


**Figure 6. Cytotoxic effects of the cancer-stimulated hMSC conditioned media on A549 cells after 24 hours of treatment. Epifluorescence microscopy images of A549 cells stained with Calcein-AM (live cells, green fluorescence) and ethidium homodimer-1 (dead cells, red fluorescence) for Live and Dead assay. A) Group A: A549 cells cultured with conditioned media of hUC-MSCs cultured in a 1 mg mL<sup>-1</sup> collagen gel. B) Group B: A549 cells cultured with conditioned media of hUC-MSCs cultured in a 4 mg mL<sup>-1</sup> collagen gel. C) Group C: A549 cells cultured with conditioned media of hBM-MSCs cultured in a 1 mg mL<sup>-1</sup> collagen gel. D) Group D: A549 cells cultured with conditioned media of hBM-MSCs cultured in a 4 mg mL<sup>-1</sup> collagen gel. E) Control: A549 cells cultured with control media. F) Percentage quantification of live cells. One-way ANOVA with Tukey test was used to test the statistical significance. Scale bar = 200  $\mu$ m, Data are shown as the mean  $\pm$  SD. Error bars represent the standard deviation of the mean. (\* $p \leq 0.05$  and \*\* $p < 0.001$ ). n = 3 images per group. SD., standard deviation. For details of group names, refer to Table 1**

Quantitative analysis was performed using a CCK-8 counting kit. After 24 h of incubation of A549 cells with conditioned media of cancer-stimulated hMSCs, the cells treated with conditioned media of cancer-stimulated hUC-MSCs, i.e, Groups A (65.4 % cell viability;  $p = 0.015$ ) and B (94.28 % cell viability), showed a decline in viability compared to the control group (100% cell viability). However, cells incubated with conditioned media of cancer-stimulated hBM-MSCs, i.e, Groups C (150 % cell viability;  $p = 0.0006$ ) and D (149.9 % cell viability;  $p = 0.0006$ ), showed higher viability than the control group (100% cell viability) (Figure S 1).

## 2.4. Cancer-stimulated hMSC conditioned media alter the nuclear morphology of A549 cells

After 24 h of treatment, the nuclear morphology of control and cancer-stimulated hMSC-conditioned media-treated A549 cells was analyzed. Untreated control cells had a typical blue appearance and no discernible morphological alterations. Nevertheless, hUC-MSC-conditioned media-treated cells showed signs of nuclear alterations in some cells, indicating chromatin condensation, nuclear shrinkage, and nuclear fragmentation (**Figure 7A and B**). Meanwhile, hBM-MSC CM-treated cells showed no nuclear alterations but resembled a typical blue appearance (**Figure 7C and D**). Nuclear Area Factor (NAF) was evaluated as a preliminary indicator of apoptosis. NAF is inversely proportional to the extent of apoptosis. It was observed that Group A had the least value of NAF among all the experimental groups, though the difference was not significant when compared with the control group (**Figure 7B**). To ascertain the scope and duration of the apoptotic process, NAF calculation should be paired with other tests since apoptosis is a multi-step, intricate process.



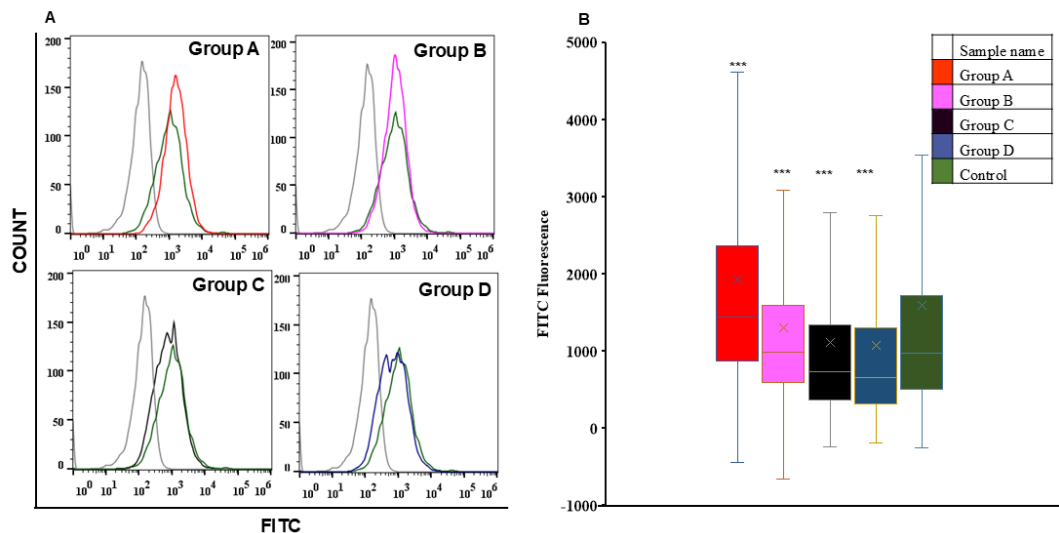
**Figure 7. Morphological analysis of the Apoptotic nucleus of A549 cells was identified with Hoechst 33258 staining. A) Group A: A549 cells cultured with conditioned media of hUC-MSCs cultured in a 1 mg mL<sup>-1</sup> collagen gel. B) Group B: A549 cells cultured with conditioned media of hUC-MSCs cultured in a 4 mg mL<sup>-1</sup> collagen gel. C) Group C: A549 cells cultured with conditioned media of hBM-MSCs cultured in a 1 mg mL<sup>-1</sup> collagen gel. D) Group D: A549 cells cultured with conditioned media of hBM-MSCs cultured in a 4 mg mL<sup>-1</sup> collagen gel. E) Control: A549 cells cultured with control media. Control cells exhibit normal morphology, and treated cells showing**

condensed chromatin and fragmented nuclei are indicated with white arrows. F) Quantitative comparison of Nuclear Area Factor (NAF). The mean NAF values (Area/circularity) derived from fluorescent nuclear imaging are displayed in a bar graph. Increased nuclear condensation and irregularity, which are frequently linked to apoptosis, are indicated by a decreased NAF. Scale bar = 10  $\mu$ m. Each group's data is displayed as mean  $\pm$  SD. n = 12 images. Statistical significance was assessed using one-way ANOVA with Tukey's post hoc test. \*\*\*p < 0.0001. For details of group names, refer to Table 1

## 2.5. Apoptotic and anti-apoptotic effects of cancer-stimulated hMSC-conditioned media on A549 Cells

To investigate the apoptotic and anti-apoptotic role of cancer-stimulated hMSC-conditioned media, we performed a flow cytometric apoptosis analysis of A549 cells. The cells were incubated with cancer-stimulated hMSC-conditioned and control media for 24 h. Afterward, cells were stained and subjected to flow cytometric analysis to measure apoptosis. A suitable gating technique was implemented to exclude dead cells, debris, and doublets.

hUC-MSCs conditioned media increased the ratio of apoptotic cells in A549 cells, whereas hBM-MSCs conditioned media showed a decreased proportion of apoptotic cells compared to control media (**Figure 8A-B**).



**Figure 8.** Apoptotic effect of the cancer-stimulated hMSC-conditioned media on A549 cells evaluated using flow cytometry analysis of Annexin V- FITC stained cells. A) The histogram compares the distribution of annexin V-negative cells and positive cells. The grey color peak shows unstained cells. B) The box plot shows the distribution of FITC fluorescence throughout the sample

analyzed. Whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, whereas center lines display the medians, and box bounds reveal the 25th and 75th percentiles. The Student's t-test was used to test the statistical significance.  $n = 10,000$  events. The control and experimental groups demonstrated a significant difference ( $***p < 0.0001$ ). For details of group names, refer to Table 1

### 3. Discussion

This study conclusively established an in vitro culture model for simulating the tumorigenic response of hMSCs in a lung cancer microenvironment. We found that soluble factors from lung cancer cells are critical for hMSC survival in the tumor microenvironment. hBM-MSCs showed an increase, while hUC-MSCs showed a decrease in survival rate in the presence of soluble factors from lung adenocarcinoma cells (**Figure 2** and **Figure 3**), with the effect being more evident in early-stage, which might be similar to the lung adenocarcinoma microenvironment (1 mg mL<sup>-1</sup> collagen gel) compared to late-stage model of the lung cancer (4 mg mL<sup>-1</sup> collagen gel). Also, it was observed that hBM-MSCs have a higher rate of proliferation than hUC-MSCs, independent of the concentration of collagen ECM in which the cells were encapsulated (**Figure S2**). This highlights the major contribution of the biochemical component of lung cancer microenvironment (mimicked by A549-conditioned media in this study) over the biomechanical (mimicked by collagen of two different concentrations in this study) in making the environment conducive for hBM-MSCs and inimical for hUC-MSCs survival.

In this study, hUC-MSCs displayed a more dispersed phenotype, whereas BM-MSCs preserved a spherical shape when cultured with control media. These variations most likely represent the inherent characteristics of MSCs derived from various tissue types. Originating from a more primitive and perinatal tissue source, hUC-MSCs exhibit more proliferative capacity and improved 3D environment adaptation. Higher expression of integrins and cytoskeletal regulatory proteins, which promote contact with and adaptation to 3D surroundings, may be the cause of their improved spreading.[22] On the other hand, BM-MSCs are said to have a tendency to maintain a rounder shape in less rigid matrices, as well as decreased proliferation and migratory activity. These cells are generated from a more quiescent adult niche.<sup>[7]</sup>

There is growing evidence that hMSCs differentiate into CAFs upon exposure to soluble substances released by cancerous cells.[7,23,24] We found that hBM-MSCs retained a small and

compact shape when cultured in a non-cancerous 3D microenvironment. In contrast, when cultured in the cancer 3D microenvironment, they showed spreading morphology, expressed stress fibers, and higher expression of  $\alpha$ -SMA marker, characteristic of cancer-associated fibroblasts (**Figure 4** and **Figure 5**). These results implied that hBM-MSCs survived with soluble factors from lung adenocarcinoma cells and differentiated into CAFs. Our findings corroborate the results of Ishihara et.al, who reported a transition from round to spread morphology of hBM-MSC when cultured with cancer cells' conditioned media.<sup>[7]</sup> At the same time, hUC-MSCs did not demonstrate any characteristic changes in morphology and lower expression of  $\alpha$ -SMA markers compared to hBM-MSCs when cultured in a cancer microenvironment, implying that they do not transform into cancer-associated fibroblasts. One of the study's shortcomings is that the CAF phenotype of cells was only evaluated using  $\alpha$ -SMA. Although more markers would offer a more thorough definition, additional marker-based validation was not possible at this time due to logistical and resource limitations. To support and corroborate these findings, future research will try to use a larger panel of markers.

Furthermore, conditioned media of cancer-stimulated hBM-MSCs increased lung adenocarcinoma cell growth. However, conditioned media derived from hUC-MSCs cultured under the same conditions suppressed lung adenocarcinoma cell growth and elevated cell death. Calcein AM and EHD staining showed that A549 cells treated with conditioned media of cancer-stimulated hUC-MSCs cultured in 1 mg mL<sup>-1</sup> collagen gel showed maximum percent of dead cells stained with ethidium homodimers (**Figure 6A**). In the context of this kind of assay, which includes fluorescent probes such as calcein AM (acetoxymethyl ester of calcein), esterase in apoptotic cells can cleave the ester group of the dye, leading to the accumulation of fluorescent calcein within apoptotic cells. This phenomenon complicates the interpretation of the results of this assay, which relies on esterase activity because both viable and apoptotic cells exhibit some degree of esterase activity. Apoptotic cells exhibit varying levels of metabolic activity, depending on the stage of apoptosis and the specific metabolic pathways involved. Also, as a result of WST-8 assay-based quantitative analysis, only A549 cells treated with conditioned media of cancer-stimulated hUC-MSCs cultured in 1 mg mL<sup>-1</sup> collagen gel (group A) have shown a significant decrease in percent cell viability in comparison to the control group. Groups C and D have shown a substantial increase in percent viability compared to the control group, supporting the hypothesis that cancer-stimulated hBM-MSCs have induced proliferation in A549

cells in both the early and late stages of lung adenocarcinoma model (**Figure S 1**). Hoechst 33342 staining revealed striking alterations in the A549 cells' nuclear morphology treated with CM from cancer-simulated hUC-MSCs, confirming apoptosis (**Figure 7**). To understand the reason behind viability trends and nuclear alteration in A549 cells exposed to cancer-stimulated hMSCs, flow cytometric analysis was performed, which showed that the apoptosis ratio of lung cancer cells treated with cancer-stimulated hUC-MSC-conditioned media was considerably higher than that of the control group, indicating the pro-apoptotic role of cancer-simulated hUC-MSCs in the cancer microenvironment. At the same time, the apoptosis ratio of lung cancer cells treated with hBM-MSC-conditioned media was considerably lower than that in the control group, indicating the anti-apoptotic role of cancer-simulated hBM-MSCs in the cancer microenvironment (**Figure 8A-B**).

A recent study by Pang et al. showed that apoptosis of MSCs is necessary for the therapeutic effects exerted by infusion into the lungs.<sup>[14]</sup> Our research reported a decline in the survival and proliferation rate of hUC-MSCs in a lung cancer microenvironment (**Figure 2C i** and **Figure 3E**). Conditioned media collected from the same microenvironment of hUC-MSCs resulted in the apoptosis-based mortality of A549 cells (**Figure 8A-B**), suggesting that hUC-MSCs might undergo apoptosis in the lung cancer microenvironment and potentially release apoptosis-inducing biomolecules with therapeutic relevance. These findings point to a possible negative feedback interaction between hUC-MSCs and lung adenocarcinoma cells in this in vitro tumor model. ~~thereby establishing that hUC MSCs undergo apoptosis in the lung cancer microenvironment, releasing apoptosis-inducing biomolecules in the conditioned media necessary for the therapeutic effects of hMSC therapy. The results showed a negative feedback loop between hUC-MSCs and lung adenocarcinoma cells in the lung tumor microenvironment.~~

In the case of hBM-MSCs, we have reported an increase in the survival and proliferation rate of hBM-MSCs in a lung cancer microenvironment (**Figure 2C ii** and **Figure 3E**). Conditioned media collected from the same microenvironment of hBM-MSCs resulted in the anti-apoptosis-based growth of A549 cells (**Figure 8A-B**); therefore, a positive feedback loop between hBM-MSCs and lung adenocarcinoma cells in the lung tumor microenvironment.

In a study by Subramanian et al., it was reported that Wharton's jelly stem cells showed different behavior than bone marrow mesenchymal stem cells after being subjected to conditioned media

of two different cancer cell lines, ovarian (TOV-112D) and breast (MDA-MB-231) carcinoma. [22] According to our findings, the only difference in the culturing conditions of the final A549 cells was the culture medium derived from cancer-stimulated hBM-MSCs or cancer-stimulated hUC-MSCs cultured in the 3D hydrogel. We can speculate that the different outcomes, either proliferation of A549 cells or killing of A549 cells, are caused by different biochemical molecules in the conditioned media of cancer-stimulated hBM-MSCs and hUC-MSCs. Conditioned media can include cargo-carrying membrane-wrapped vesicles such as exosomes, microvesicles, and free agents, such as growth factors, cytokines, and even microRNAs.[25] Exosomes have a decreased chance of immunological rejection after in vivo allogeneic delivery, are more robust and resilient than cells, and may offer an alternate treatment for many illnesses.[26]

As per previous reports, lung cancer during gestation is rare, with fewer than 70 cases reported in recent years, and placental or fetal invasion is a rare impediment in pregnant women with cancer.[27,28] This gives us some insights into our reported results, where hUC-MSC conditioned media resulted in declining lung cancer cell survival. Thus, hUC-MSCs might play a role in preventing placental invasion by lung cancer in pregnant women.

Per previous reports, circulating MSCs from bone marrow, adipose tissue, or tumor stroma cells can differentiate into CAFs [29]. Our study also concluded that hBM-MSCs were converted into CAF (**Figure 5**) and favored lung adenocarcinoma growth by preventing apoptosis induction in cancer cells (**Figure 8A-B**).

Conditioned media of hUC-MSCs cultured in a 1 mg mL<sup>-1</sup> collagen gel, stimulated with biochemical cancer component (A549 CM), showed significant apoptotic effects on lung cancer cells. In contrast, this effect became less critical when hUC-MSCs were cultured in 4 mg mL<sup>-1</sup> collagen gel (**Figure 8**). This proves the considerable role of the ECM in hindering the diffusion of soluble components to the target site because of the dense collagen network during the later stages of cancer progression. According to early research, stiffness can enhance fibroblast activation, influence cell behavior, and generate a protumorigenic positive feedback loop.[30] Comparable observations were observed in our study, where the pro-tumorigenic effect of hBM-MSCs was maintained, and the anti-tumorigenic effect of hUC-MSCs was decreased when cultured in ECM with higher stiffness owing to the high concentration of collagen (4 mg mL<sup>-1</sup>).

1 Conditioned media of hBM-MSCs cultured in 1 mg mL<sup>-1</sup> collagen gel, stimulated with a  
2 biochemical cancer component (A549 CM), showed significant anti-apoptotic effects on lung  
3 cancer cells, further maintaining the effect when hBM-MSCs were cultured in 4 mg mL<sup>-1</sup>  
4 collagen gel.

5 hMSCs have been reported to be paradoxical in cancer biology [31]. Given their shared ancestry,  
6 the nature and characteristics of hUC-MSCs and hBM-MSCs may be similar. However, as  
7 reported in our study, some noteworthy distinctions between them are likely caused by the  
8 microenvironments of their new sites. Though the current study shows that conditioned media  
9 from cancer-stimulated hMSCs have anti- or pro-tumor effects on lung cancer cells, the exact  
10 profile of the media was not explored, which is a valid limitation of the current study and will be  
11 studied in the future.

12 Our results suggest that the state of the ECM in cancer tissue concerning the density of collagen,  
13 which is the most prevalent element of ECM and a tissue source of MSCs being used, is critical  
14 for understanding MSCs' role as therapeutic agents in cancer biology.

#### 15 **4. Conclusion**

16 This work studied the tumorigenic properties of two different hMSCs in a lung cancer-like  
17 microenvironment. Biochemical and biomechanical components of the lung cancer  
18 microenvironment were mimicked using lung cancer-derived conditioned media and collagen  
19 hydrogel, respectively. Our in vitro results showed an overall negative feedback loop between  
20 lung cancer cells and hUC-MSCs and a positive feedback loop between lung cancer cells and  
21 hBM-MSCs. Specifically, hUC-MSCs have shown antitumor response by inducing apoptosis in  
22 lung cancer cells. However, hBM-MSCs have shown protumor response by inducing anti-  
23 apoptosis in lung cancer cells.

24 In future studies, confirming the cytokine profile of cancer-stimulated hUC-MSCs and hBM-  
25 MSC-conditioned media will be crucial. Additionally, identifying potential targets by analyzing  
26 the gene expression profiles of cancer cells treated with cancer-stimulated hUC-MSC  
27 conditioned media will help establish a solid foundation for the clinical application of hUC-  
28 MSCs. Furthermore, there is great potential to achieve synergistic effects and improve treatment  
29 results by combining the hUC-MSC-conditioned media approach with complementary

therapeutic modalities, including immunotherapy, radiotherapy, or chemotherapy. In addition, the anti-apoptotic role of cancer-stimulated hBM-MSC-conditioned media should be explored for its lung tissue regenerative potential.

## 5. Materials and Methods

### 5.1. Cell Culture

A549 cells (RIKEN BRC, RCB0098) were cultured in DMEM HG (Sigma-Aldrich, Darmstadt, Germany), complemented with 10% fetal bovine serum (FBS) (EU origin, Biowest, Nuallie, France) and 1% antibiotics. A549 cells were cultured for less than 15 passages. hBM-MSCs (PromoCell, C-12974) were expanded in mesenchymal stem cell basal media (Lonza, Walkersville, MD, USA, PT-323) supplemented with MSCGM SingleQuots (Lonza, PT-4105). hUC-MSCs (PromoCell, C-12971) were expanded in mesenchymal stem cell growth media 2 (PromoCell, Heidelberg, Germany, C-28009) supplemented with a supplement mix. hMSC cells cultured for five passages were used. Cells were maintained at 37°C and 5% CO<sub>2</sub> with 95% humidity. The culture media was replaced every two to three days, and the cells were passaged at 85–90% confluence. For the 2D culture of all cells, a commercial collagen-coated 100 mm dish was used.

### 5.2. hMSC culture in 3D Collagen hydrogel

Collagen gel neutralization and cell embedding in the neutralized gel were performed as described elsewhere.[32] Briefly, both hUC-MSCs and hBM-MSCs were trypsinized to obtain cell suspensions. The acidic stock solutions of collagen gel were diluted from the commercial 3 mg mL<sup>-1</sup> solution (Cellmatrix Type I-A, Nitta Gelatin Inc., Japan) and 5 mg mL<sup>-1</sup> (R&D Systems, Minneapolis, USA) to obtain 1 mg mL<sup>-1</sup> and 4 mg mL<sup>-1</sup>, respectively, with the use of phenol-red containing 10X media and buffer provided with a collagen gel culturing kit (Nitta Gelatin Inc., Osaka, Japan) and brought to pH 7.4. A cell-loaded hydrogel with a concentration of  $0.1 \times 10^6$  cells mL<sup>-1</sup> was prepared by mixing the cell suspension with neutralized collagen gel. One well of a six-well plate contained 400 µL of the cell–hydrogel mixture. The well plates were incubated at 37°C for 30 min to 1 h for complete gel polymerization, followed by adding A549 conditioned and control media (DMEM HG) with 1% FBS to the respective wells. 3 mL of the respective

media was added to each well. During the seven days of culture, the media were changed every three days.

### 5.3. *Preparation of Conditioned Media*

~~A549 cells were grown in a 100 mm dish to 85–90% confluency in DMEM HG media supplemented with 10% FBS and 1% penicillin-streptomycin.~~ A549 cells were cultured in 100 mm dishes to 85–90% confluency in DMEM-HG with 10% FBS and 1% penicillin-streptomycin. Sub-confluent cells were washed with prewarmed 1X PBS and cultured in 12 mL of DMEM HG media (nonserum, 1% penicillin-streptomycin) for another 24 h. The media were collected and filtered through a 0.22-mm pore PES filter (Thermo Scientific) and preserved at –80°C until further use.

~~For hMSC cells, encapsulated in collagen were cultured with 3 mL of A549 CM supplemented with 1% FBS and 1% penicillin-streptomycin per well of 6 well plate till day 6, gels were washed with 1X PBS three times and cultured in 3 mL of DMEM HG media (nonserum, 1% penicillin-streptomycin) per well of 6 well plate for 24 h.~~ For hMSCs, cells encapsulated in collagen were cultured with 3 mL of A549 CM (1% FBS and 1% penicillin-streptomycin) per well of a 6-well plate until day 6. The concentration of FBS is the same in both control and conditioned media, i.e, 1 % FBS. The serum concentration was kept low enough to support cell viability while avoiding suppression or dilution of cancer cells' secreted cytokine effects.<sup>[33]</sup> Gels were then washed three times with 1X PBS and further cultured for 24 h in 3 mL of serum-free DMEM-HG containing 1% penicillin-streptomycin per well. Collected filter-sterile conditioned media were stored at –80°C for further studies.

### 5.4. *Calcein AM/Ethidium Homodimer Staining*

Live/dead staining was performed to understand the effect of A549 CM and control media on the viability of collagen-encapsulated hMSC using a LIVE/DEAD<sup>TM</sup> Viability kit (Invitrogen, Life Technologies Corporation, Eugene, Oregon, USA) according to the manufacturer's instructions. The significant advantage of this live/dead staining method is that fluorescence microscopy can clearly distinguish the green and red fluorescence of calcein and ethidium homodimer (EHD), respectively. To stain target cells, the cells embedded in collagen gels were first washed with 1X PBS, followed by incubation with calcein acetoxymethyl (AM) (2 µM) and EHD (4 µM) for 30

min, protected from light. Fluorescently stained samples were observed using an Axiovert 200 Zeiss microscope (Oberkochen, Germany) with a mercury arc lamp. Viability (%) was calculated using ImageJ using the following equation, where MI stands for mean intensity :

$$\text{Viability (\%)} = (\text{MI}_{\text{(green)}} / (\text{MI}_{\text{(green)}} + \text{MI}_{\text{(red)}})) \times 100$$

### 5.5. *WST-8 assay of 3D hMSC*

The proliferation of hMSCs in collagen gels was analyzed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. ~~Cells encapsulated in collagen gels were cultured in 96-well plates (50 µL gel/well), incubated with 100 µL A549 CM (supplemented with 1% FBS and 1% penicillin-streptomycin), and control medium complemented with 1% FBS and 1% penicillin-streptomycin at 37°C and 5% CO<sub>2</sub> atmosphere.~~ Cells encapsulated in collagen gels (50 µL per well) were cultured in 96-well plates. They were incubated with 100 µL of A549 conditioned medium (CM) or control medium, both supplemented with 1% FBS and 1% penicillin-streptomycin, at 37 °C in a 5% CO<sub>2</sub> atmosphere.

The gels were washed with prewarmed 1X PBS and incubated in 100 µL DMEM HG containing 15 µL of CCK-8 stock solution, covering 50 µL of gel for one hour. Absorbance at 450 nm was measured using a microplate photometer (Thermoscientific, China). The experiments were performed in triplicate.

### 5.6. *Immunofluorescence staining*

Confocal microscopy investigated the morphology and differentiation fate of cultured hMSCs encapsulated in the 3D collagen hydrogel. Immunofluorescence staining was used to determine the expression of filamentous actin (F-actin) and alpha-smooth muscle actin (α-SMA) using the standard protocol described elsewhere.[33] Briefly, 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) was used to fix the 3D cultures for 20 min, and permeabilization was done using 0.5% Triton X-100 (Sigma) treatment for 10 min. Succeeded by 30 min of incubation with 3% bovine serum albumin (Nacalai tesque) at room temperature. Next, the cells were incubated with rabbit anti-human primary antibody of α-SMA at a dilution of 1: 150 (ab5694, Abcam) overnight at 4°C. Following further washing, the samples were incubated with Alexa Fluor 568 phalloidin

(Invitrogen) at a dilution of 1:250 for one hour in the dark. Hoechst 33342 (Invitrogen) was used for nuclear staining at a dilution of 1:1000 for 10 min in the dark. Fluorescence images were obtained under an IX-81 microscope (Olympus, Tokyo, Japan) equipped with a UPlanSApo (20x) and UMPlanFLN (60x) lens and an Andor CCD camera (SONA 4BV6U, UK) using the Metamorph software (Molecular Devices, Sunnyvale, CA). Images were processed and analyzed using the ImageJ software.

### 5.7. Viability assay of A549 cells

Ten thousand cells per well were seeded in 96-well plates and incubated with 100  $\mu$ L of DMEM HG complete media at 37°C in a 5% CO<sub>2</sub> environment for 24 h. After 1X PBS washing, cells were treated with 100  $\mu$ L of either control media (DMEM-HG supplemented with 1% FBS) or conditioned media from cancer-stimulated MSCs (containing 1% FBS and 1% penicillin-streptomycin). The concentration of FBS is the same in both control and conditioned media, i.e, 1 % FBS. The serum concentration was kept low enough to support cell viability while avoiding suppression or dilution of hMSC-secreted cytokine effects.<sup>[34]</sup> ~~Afterward, cells were washed with 1X PBS, and the complete media was replaced with 100  $\mu$ L of control (DMEM HG with 1% FBS) and conditioned media derived from cancer-stimulated hMSC cultured in different concentrations of collagen.~~ After another 24-h culture, the viability of cells was analyzed using Calcein-AM EHD staining and cell counting kit-8. Live cells (%) and dead cells (%) were calculated by quantifying the images via ImageJ using the following equation, where MI stands for mean intensity:

$$\text{Live cells (\%)} = (\text{MI}_{\text{green}} / (\text{MI}_{\text{green}} + \text{MI}_{\text{red}})) \times 100$$

$$\text{Dead cells (\%)} = (\text{MI}_{\text{red}} / (\text{MI}_{\text{green}} + \text{MI}_{\text{red}})) \times 100$$

For qualitative viability analysis, A549 cells were first washed with 1X PBS, followed by incubation with calcein-AM (2  $\mu$ M) and EHD (4  $\mu$ M) for 30 min in the dark. Fluorescently stained samples were observed under an Axiovert 200 Zeiss microscope. The viability of A549 cells was also analyzed using the Cell Counting Kit-8. After a 1X PBS wash, the cells were incubated in 100  $\mu$ L DMEM containing 10  $\mu$ L CCK-8 stock solution for one hour at 37°C. Absorbance at 450 nm was spectrophotometrically evaluated using a Multiskan FC photometer microplate reader (Thermoscientific, China). A549 cells cultured in DMEM HG media with 1%

FBS were used as a control, and media without cells were used as a blank. The following formula was used to calculate the percent viability:

$$\text{Percentage cell viability} = (A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%,$$

where A represents absorbance at 450 nm. Depending on the source of hMSC-conditioned media, there were four experimental and one control group (Table 1).

**Table 1. Study groups and the source of conditioned media used. A549 cells are divided into four experimental and one control group based on the source of cancer-stimulated hMSC-conditioned media to which they are exposed. NA = Not applicable.**

| Groups  | Treated cells<br>(exposed to cancer-stimulated hMSC-conditioned media) | Cell source of cancer-stimulated hMSC-conditioned media | Collagen concentration used for hMSC culture |
|---------|------------------------------------------------------------------------|---------------------------------------------------------|----------------------------------------------|
| Group A | A549                                                                   | hUC-MSC                                                 | 1 mg mL <sup>-1</sup>                        |
| Group B | A549                                                                   | hUC-MSC                                                 | 4 mg mL <sup>-1</sup>                        |
| Group C | A549                                                                   | hBM-MSC                                                 | 1 mg mL <sup>-1</sup>                        |
| Group D | A549                                                                   | hBM-MSC                                                 | 4 mg mL <sup>-1</sup>                        |
| Control | A549<br>Treated with control media<br>(DMEM HG with 1% FBS)            | NA                                                      | NA                                           |

### 5.8. Hoechst 33342 staining

The Hoechst 33342 DNA staining technique was used to assess nuclear condensation or fragmentation, which is one of the characteristics of apoptosis. The experimental setup, including cell density, media volume, and media composition, was used as mentioned in section 5.7 of the Materials and Methods. After treating A549 cells for 24 h with the stimulated hMSC-conditioned and control media, they were washed with 1X PBS and stained, as mentioned in the previous

section on immunofluorescence staining. After staining, the cell was rinsed again with 1X PBS and examined under an IX-81 microscope (Olympus, Tokyo, Japan). In order to detect abnormal nuclear phenotype, microscopic images were evaluated for nuclear area fractions. For this, using Image J software, raw images were first converted to an 8-bit greyscale format. The following sequence was followed: Image → Type → 8-bit → Image → Adjust → Threshold → Process → Binary → Analyze → Set Measurements → Analyze Particles.

Further, via the analyze particle option, the area and circularity of nuclei were obtained, and then the following equation was used:

$$\text{NAF} = \text{Nuclear Area} / \text{circularity of nucleus}$$

### 5.9. *Flow cytometric analysis of Apoptosis*

Apoptosis was determined using an Annexin V-FITC apoptosis detection kit (Nacalai Tesque, Inc.) following the manufacturer's specifications. A549 cells were seeded at a density of  $50 \times 10^4$  cells per well in a 24-well plate, cultured with 700  $\mu\text{L}$  of DMEM HG complete media per well at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  environment for 24 h. The next day, 700  $\mu\text{L}$  of cancer-stimulated hMSC-conditioned media supplemented with 1% FBS and 700  $\mu\text{L}$  of the control media (DMEM HG with 1% FBS) were added to the respective wells after removing the DMEM HG media and washing with 1X PBS. Spent culture media were recovered along with A549 cells exposed to the desired cancer-stimulated hMSC-conditioned and control media for 24 h. For positive control of Annexin-V-FITC, cells were treated with 700  $\mu\text{L}$  of 1  $\mu\text{M}$  staurosporine for 4 hours before harvesting the cells for flow cytometry. Cells were suspended in an Annexin-V buffer with Annexin-V-FITC and propidium iodide (PI) for 15 min in the dark. Unstained cells were used as a negative control for Annexin-V-FITC. Next, 10,000 stained cells were analyzed by flow cytometry using a Cell Sorter SH800 (Sony Corp., Tokyo, Japan). The data were processed and analyzed using FlowJo software.

### 5.10. *Statistical analysis*

Statistical analyses were performed using GraphPad Prism. Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis were performed based on data normality and the number of comparisons. The findings are reported as mean (standard deviation; SD), and a statistically significant value is specified as \* $p < 0.05$ , \*\* $p < 0.001$ , and \*\*\* $p < 0.0001$ .

## Supporting Information

Supporting Information is available from the Wiley Online Library or the author.

## Declaration of competing interest

The authors declare no competing interests

## Authors' contributions

Conception and design of the study: Pragati Sharma, Jun Nakanishi, Subha Narayan Rath

Experiments, analysis, and interpretation of data: Pragati Sharma, Jun Nakanishi

Writing, reviewing, and revising the manuscript: Pragati Sharma, Jun Nakanishi, Subha Narayan Rath

## Data availability

The data supporting the findings of this study are available in this manuscript and the supplementary materials.

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