# Tumor–stromal Interactions with Direct Cell Contacts Enhance Motility of Non-small Cell Lung Cancer Cells Through the Hedgehog Signaling Pathway

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**Abstract.** *The metastatic potential of non-small cell lung cancer (NSCLC) has been shown to be associated with interactions with the tumor microenvironment, which primarily comprises of cancer-associated fibroblasts (CAFs). Heterotypic cell–cell interactions occur via released signaling molecules and direct physical contact. To investigate the differential contribution of direct cell–cell contact and paracrine signaling factors to NSCLC metastasis, we performed two types of co-cultures: direct co-cultures of the NSCLC cell line H358 with primary cultures of CAFs from patients with resected NSCLC; and indirect co-cultures across a separable membrane. We showed that CAFs more potently induce epithelial-to-mesenchymal transition (EMT) in NSCLC H358 cells through direct contacts than through indirect interactions, as indicated by an elongated and disseminated appearance. Immunocytochemical experiments show that EMT accompanies the expression of mesenchymal cytoskeletal proteins, including vimentin. However, H358 cells proliferate more slowly in direct co-culture than in indirect co-culture. Real-time reverse transcription-polymerase chain reaction (RT-PCR) revealed that H358 cells in direct contact with CAFs up-regulate the expression of the pan-mesenchymal markers α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), transforming growth factor-β (TGFβ) signaling effector SMAD family number-3 (SMAD3), and hedgehog signaling effector GLI family zinc finger-1 (GLI1), compared with the indirect co-culture system. Furthermore, we found that the direct GLI1 transcription targets snail family zinc finger-1 (SNAI1) and SNAI2 are up-regulated, suggesting that the hedgehog signaling pathway is active in direct co-culture. A scratch wound assay showed that direct contact co-culture increases the motility of H358 cells. In conclusion, these findings provide evidence that paracrine factors and direct physical contact between NSCLC cells and CAFs might control the metastatic potential of NSCLC through the hedgehog signaling pathway.*

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Lung cancer is the leading cause of cancer-related death worldwide (1, 2), and 85% of all lung cancer cases are non-small cell lung cancer (NSCLC). NSCLC is typically characterized by a prominent desmoplastic stroma with abundant inflammation (3, 4). Desmoplastic stroma creates a tumor microenvironment conductive to tumorigenesis, angiogenesis, metastatic spread of tumor cells, and therapy resistance. Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment; they are among the predominant cell types in the tumor stroma and contribute to most of the phenotypes described earlier through remodeling the extracellular matrix (ECM) and influencing tumor and stromal cells, including immune and endothelial cells. However, the molecular and cellular mechanisms by which CAFs interact with tumor cells and other stromal cells remain ill-defined, especially for NSCLC.

CAFs are widely defined as all fibroblast cells within the tumor stroma. Therefore, CAFs are heterogeneous in origin and are derived from various progenitor cells, including fibroblasts, mesenchymal stem cells (MSC), and epithelial normal and transformed cells (5, 6). The process by which

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epithelial normal and transformed cells trans-differentiate into CAFs has been referred to as the epithelial-to-mesenchymal transition (EMT), which represents the loss of epithelial traits such as expression of cytokeratins, and the gain of mesenchymal traits, such as expression of vimentin, fibroblast-specific protein-1 (FSP1), and α-smooth muscle actin (α-SMA) (5, 7, 8). Phenotypically, CAFs resemble normal myofibroblasts (MFs) in that they express MF markers such as α-SMA, vimentin, or FSP1 and have an elongated spindle shape, but biologically, they are different in that MFs contain a contractile apparatus and provide a system for mechanotransduction to the surrounding ECM (9).

CAFs play a multifaceted role in tumorigenesis and metastasis thorough their interactions with carcinoma cells. The molecular mechanisms by which CAFs interact with carcinoma cells involves diffusible [*e.g.* hepatocyte growth factor (HGF) and chemokine (C-X-C motif) ligand 12 (CXCL12/SDF1)]) and insoluble factors (10). The paracrine factors of CAFs exert their action on carcinoma cells through the regulation of an interactive network of transcriptional repressors including snail family zinc finger 1 (SNAI1), SNAI2, zinc finger E-box binding homeobox 1 (ZEB1), ZEB2, transcription factor 3 (TCF3), forkhead box C2 (FOXC2), and twist basic helix-loop-helix transcription factor 1 (TWIST1) in carcinoma cells, leading to the activation of the transdifferentiation EMT program in carcinoma cells (11). However, a number of studies have shown that during tumorigenesis, cancer cells come into direct contact with the stroma, which has long been considered to be a microenvironment passive to the malignant transformation, leading to the stimulation of tumor-enhancing inflammation and angiogenesis (12). In support of this idea, recent studies have shown that fibroblasts and epithelial cells can form heterotypic cell–cell contacts through cadherin adhesion molecules (13, 14). However, the mechanisms by which direct heterotypic interactions between CAFs and carcinoma cells modulate tumorigenesis and the metastatic cascade *in vivo* remain unclear.

CAFs are a significant component of NSCLC tumors (15-19); however, the multifaceted mechanisms of CAFs underlying tumor progression have not been well-defined. We previously reported that NSCLC H358 cells grown in direct co-culture with primary cultures of CAFs exhibit greater motility through EMT induction (20). However, this direct co-culture system does not lend itself to the study of the relative contribution of soluble factors *vs.* direct physical contact underlying the multiple effects of CAFs on the behavior of NSCLC H358 cells in co-culture. In the current work, we have therefore established an indirect co-culture system in which cells can be co-cultured across a porous membrane, thus preventing intercellular contact while allowing paracrine factors to mediate the indirect intercellular interaction. We report that direct physical contact between these two cell types contributes to distinctive microenvironmental impacts on NSCLC progression.

## Materials and Methods

*Primary culture of fibroblasts.* CAFs and NFs were isolated from lung cancer specimens of patients who underwent surgical resection in 2012 at the Samsung Medical Center, Seoul, Korea, as described previously (20). Briefly, 30 min after surgery, fresh tissues (3 mm × 3 mm × 3 mm in size) were minced with surgical blades in 5 ml of Roswell Park Memorial Institute (RPMI)-1640 medium without serum to an approximate size of 40-60 μm in diameter, or 40-200 cells/clump. Tissue fragments were subjected to enzyme digestion in a solution containing a cocktail of collagenase I (450 U/ml; EMD, San Diego, CA, USA) and DNase I (60 U/ml; Roche, Mannheim, Germany) for 20 min and then seeded in a T25 culture flask in 5 ml of RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Fibroblasts were separated from epithelial cells by treatment with Dispase II (14 U/ml; Roche) and subsequent mechanical ablation with a pulled glass pipette tip. The culture medium was changed after 48 h to remove unattached cells and debris in suspension. Attached cells were cultured for 7-10 days to $1\times10^6$ cells. All fibroblasts were used after 3-7 passages following primary culture.

*Cell lines and co-culture conditions.* The human bronchioalveolar carcinoma H358 NSCLC cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and transfected with a lentivirus green fluorescence protein (GFP) construct. Stable GFP-expressing H358 (GFP-358) cells were selected and expanded in growth media (RPMI-1640 supplemented with 10% FBS). Two types of co-cultures were performed. Direct co-cultures are where two cell types are grown in physical contact. In brief, 10,000 CAFs were plated and grown for 24 h to approximately 80% confluency in growth media. The following day, GFP-H358 cells were seeded on top of the pre-incubated fibroblasts at a ratio of 4:1. To avoid the effects of reaching confluency or passage on the interpretation of data, co-cultures were maintained no longer than seven days. Indirect co-cultures are co-cultures where fibroblasts and cancer cells are grown separately using a membrane, but they are able to interact indirectly through paracrine factors. In brief, 10,000 CAFs were seeded in inserts with 8-μm pore size polycarbonate membrane in 24-well transwell plates (BD Biosciences; San Jose, CA, USA). The following day, 5,000 GFP-H358 cells in 500 μl of growth media were placed under the transwell inserts containing pre-incubated CAFs. Plates with direct and indirect co-cultures were cultured under normal culture conditions for seven days, after which inserts were removed and cancer cells were subjected to the downstream assays.

*Immunocytochemistry.* Fibroblasts were seeded on sterile glass coverslips and immunocytochemical staining was performed. Briefly, sterile coverslips were fixed with 4% paraformaldehyde for 10 min and were then permeabilized with 0.5% Triton-X 100 for 5 min. Cells were then blocked for 1 h with blocking solution [1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)], followed by incubation with primary antibody against vimentin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Subsequently, the cells were incubated with Alexa 594-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 60 min at room temperature. The cells were then washed with PBS, and the coverslips were mounted on a glass slide in 10% Mowiol 4-88, 1 μg/ml 4m,6-diamidine-2-phenylindole dihydrochloride (DAPI), and 25% glycerol in PBS. The cells were observed with a ZEISS FL Microscope Axiovert 200 (Zeiss, Oberkochen, Germany).

Table I. *Primers used in this study.*

| Gene | Name | Forward sequence (5' to 3') | Reverse sequence (5' to 3') |
|---|---|---|---|
| *SNAI1* | Snail family zinc finger 1 | CCTCCCTGTCAGATGAGGAC | CCAGGCTGAGGTATTCCTTG |
| *SNAI2* | Snail family zinc finger 2 | GGGGAGAAGCCTTTTTCTTG | TCCTCATGTTTGTGCAGGAG |
| *ZEB1* | Zinc finger E-box binding homeobox 1 | TTCAAACCCATAGTGGTTGCT | TGGGAGATACCAAACCAACTG |
| *ZEB2* | Zinc finger E-box binding homeobox 2 | GCGATGGTCATGCAGTCAG | CAGGTGGCAGGTCATTTTCTT |
| *SMAD2* | SMAD family number 2 | CGTCCATCTTGCCATTCACG | CTCAAGCTCATCTAATCGTCCT |
| *SMAD3* | SMAD family number 3 | CCATCTCCTACTACGAGCTGAA | CACTGCTGCATTCCTGTTGAC |
| *TWIST1* | Twist basic helix-loop-helix transcription factor 1 | GGAGTCCGCAGTCTTACGAG | TCTGGAGGACCTGGTAGAGG |
| *CDH1* | E-cadherin | TGCCCAGAAAATGAAAAAGG | GTGTATGTGGCAATGCGTTC |
| *CDH2* | N-cadherin | ACAGTGGCCACCTACAAAGG | CCGAGATGGGGTTGATAATG |
| *CK19* | Keratin-19 | CCCGCGACTACAGCCACTA | GCTCATGCGCAGAGCCT |
| *VIM* | Vimentin | GAGAACTTTGCCGTTGAAGC | GCTTCCTGTAGGTGGCAATC |
| *FAP* | Fibroblast activation protein | TCAACTGTGATGGCAAGAGCA | TAGGAAGTGGGTCATGTGGGT |
| *FSP* | Fibroblast-specific protein | GATGAGCAACTTGGACAGCAA | CTGGGCTGCTTATCTGGGAAG |
| *α-SMA* | α-smooth muscle actin | AGGGGGTGATGGTGGGAATG | GCCCATCAGGCAACTCGTAAC |
| *MKI67* | Antigen identified by monoclonal antibody KI-67 | AGTTTGCGTGGCCTGTACTAA | AGAAGAAGTGGTGCTTCGGAA |
| *GADD45A* | Growth arrest and DNA-damaging-inducible, alpha | GAGAGCAGAAGACCGAAAGGA | CAGTGATCGTGCGCTGACT |
| *CDKN1A* | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | TGTCCGTCAGAACCCATGC | AAAGTCGAAGTTCCATCGCTC |
| *CDKN1B* | Cyclin-dependent kinase inhibitor 1B (p27, Kip1) | AACGTGCGAGTGTCTAACGG | CCCTCTAGGGGTTTGTGATTCT |
| *BCL2L11* | BCL2-like 11 | TAAGTTCTGAGTGTGACCGAGA | GCTCTGTCTGTAGGGAGGTAGG |
| *SMO* | Smoothened, frizzled family receptor | ATCTCCACAGGAGAGACTGGTTCGG | AAAGTGGGGCCTTGGGAACATG |
| *PTCH1* | Patched 1 | TTCTCACAACCCTCGGAACCCA | CTGCAGCTCAATGACTTCCACCTTC |
| *GLI1* | GLI family zinc finger 1 | TGCCTTGTACCCTCCTCCCGAA | GCGATCTGTGATGGATGAGATTCCC |
| *HIP* | Hedgehog interacting protein | TCTGTCGAAACGGCTACTGC | CCTGGTCACTCTGCGGATGT |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | TGGACTCCACGACGTACTCAG | ACATGTTCCAATATGATTCCA |

*Cell sorting*. After 48 h of co-culture, cells expressing GFP were trypsinized and centrifuged at 300 ×*g* for 5 min. The cell pellet was suspended in 5 ml of PBS and cells were sorted by fluorescence-activated cell sorting (FACS) with FACSAriaIII (Becton Dickinson Biosciences, San Jose, CA, USA) analysis and segregated with a 488-nm optical filter. Sort gates were set by analyzing 50,000 cells from GFP-expressing and control cells. Cells were collected and centrifuged at 500 x*g* for 10 min and processed for analysis of gene expression.

*Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)*. Total RNA was extracted from the sorted co-cultured cells, followed by synthesis of first-strand cDNA using oligo-dT primers and M-MLV reverse transcriptase (Invitrogen). Real-time qPCR reactions were carried in triplicate in a final volume of 25 μl containing 1× SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA, USA), 10 ng of cDNA, and 20 pmol of each primer. Real-time qPCR was performed using an Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in each reaction. The PCR conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s, and then 60˚C for 1 min. To verify specific amplification, melting curve analysis was performed (55-95˚C, 0.5˚C/s). Relative expression quantification was performed by the ΔΔCT method. Genes and their primers are shown in Table I. Expression of each mRNA was normalized to that of GAPDH in the same sample.

*Cell proliferation analysis*. The fluorescence of cells in 96-well plates was quantified in triplicate using a Gloxmax plate reader (Promega, Fitchburg, WI, USA) with filter settings of excitation at 460 nm and emission at 550 nm with a bandwidth of 30 nm. Fluorescence intensity was calculated in relative fluorescence units (RFU). The non-specific signals of wells containing cell-free medium (blank value) were subtracted from the results to give the fluorescence signals of the GFP-expressing H358 cells.

*Co-culture wound healing assay.* Cells were allowed to grow to 80% confluence in a 24-well plate and were then wounded by making a single scratch in the monolayer with a pipette tip. The medium was then replaced to remove floating cells and debris, and cells were subsequently allowed to grow for three days to close the wound. Photographs of the same area of the wound were taken at 24 h post-wound using a phase-contrast microscope.

## Results

*Growth behavior of NSCLC H358 cells in co-culture*. Our previous study on co-culture of NSCLC H358 cells with CAFs demonstrated that heterotypic cell–cell interactions play an important role in metastasis (20). To further differentiate the effects of physical contact *vs*. soluble factors on tumor progression, we established co-cultures under two separate conditions (Figure 1A). Firstly, we conducted an indirect co-culture in which CAFs and NSCLC H358 cells were grown separated by a membrane through which interactions *via* soluble factors could still occur. While indirect transwell co-culture allows for exposure of H358 cells to the paracrine factors that CAFs secrete, it does not allow CAFs and H358s

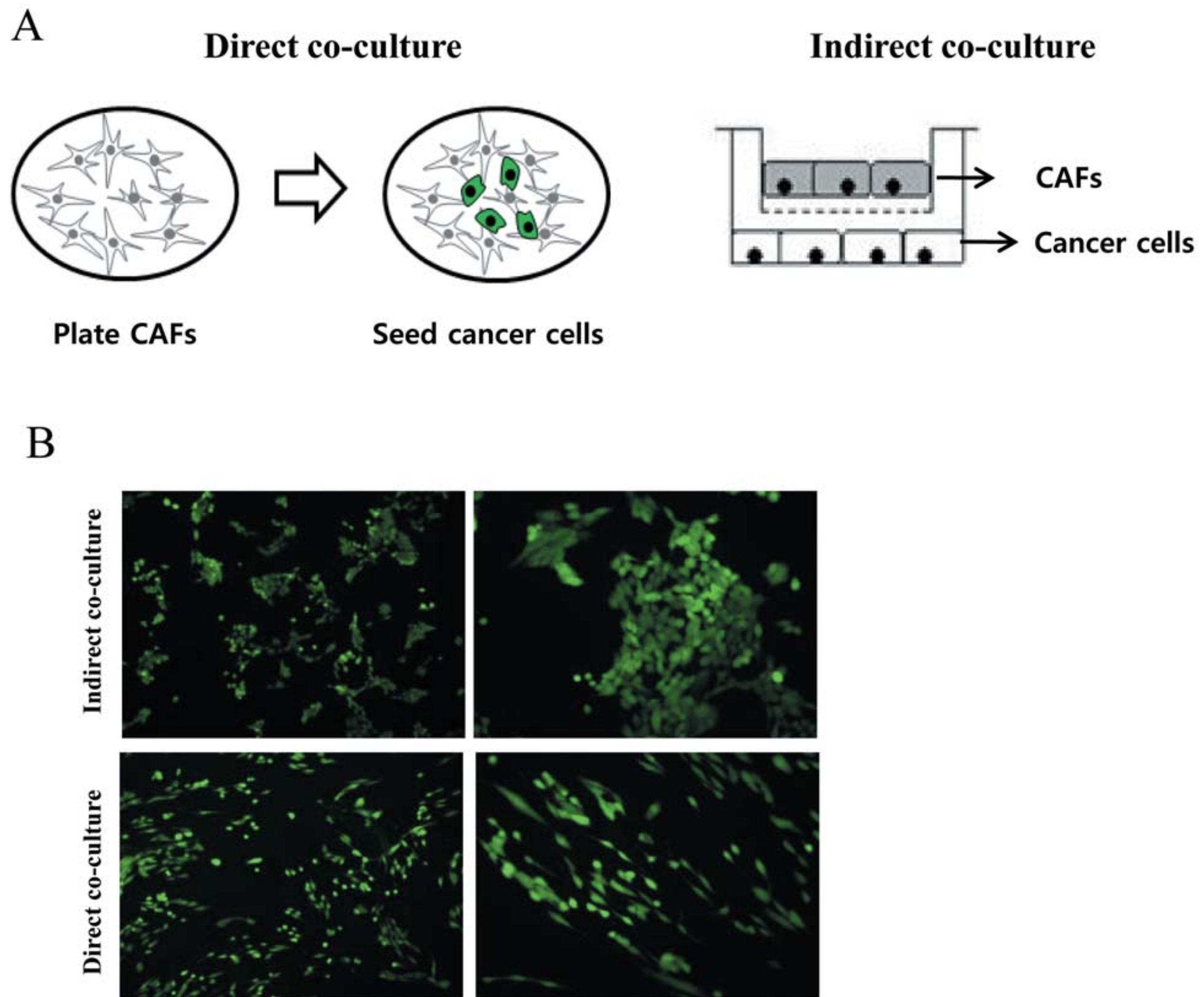


Figure 1. *Co-culture systems. A: Scheme of direct and indirect co-culture systems. Non-small cell lung cancer (NSCLC)-green fluorescence protein (GFP) cells were utilized in co-culture to delineate NSCLC cells from cancer-associated fibroblasts (CAFs). In indirect co-culture, CAFs were seeded on the insert layers of transwell plates with 8-μm pore size polycarbonate membranes in 24-well transwell plates, whereas cancer cells were grown in the bottoms of the wells. The cells could not pass through the membrane separating each population. By preventing direct cell-to-cell contact, this co-culture system can differentiate the effects of soluble factors on cell growth from the effects of cell-cell interactions. In direct co-culture, H358 cells and CAFs were grown in the same well, allowing the two cell types to physically contact. B: Phase-contrast micrograph (original magnification: ×1,000) showing the morphology of human NSCLC H358 cells grown in direct and indirect co-culture with CAFs.*

to directly contact each other. Hence, we conducted a direct co-culture where CAFs and H358s were grown in the same well, allowing for direct contact between the two cell populations. H358-GFP cells expressing green fluorescent protein were employed in co-cultures. While H358-GFP cells in indirect co-culture showed typical epithelial cobblestone morphology with homotypic cell adhesion and growth in clusters, H358-GFP cells grown in direct contact with CAFs displayed a loss of epithelial morphology and became elongated and non-polarized in morphology and dispersed in growth, which is reminiscent of the EMT phenotype (Figure 1B). Taken together, this observation suggests that CAFs influence the growth behavior of NSCLC H358-GFP cells differently depending on the mode of co-culture.

*Induction of EMT in human NSCLC H358 cells by CAFs.* To further examine the relative effects of physical contact *vs.* soluble factors on EMT induction in NSCLC H358 cells, we carried out experiments to observe the molecular and cellular features of EMT phenotypes under two separate co-culture

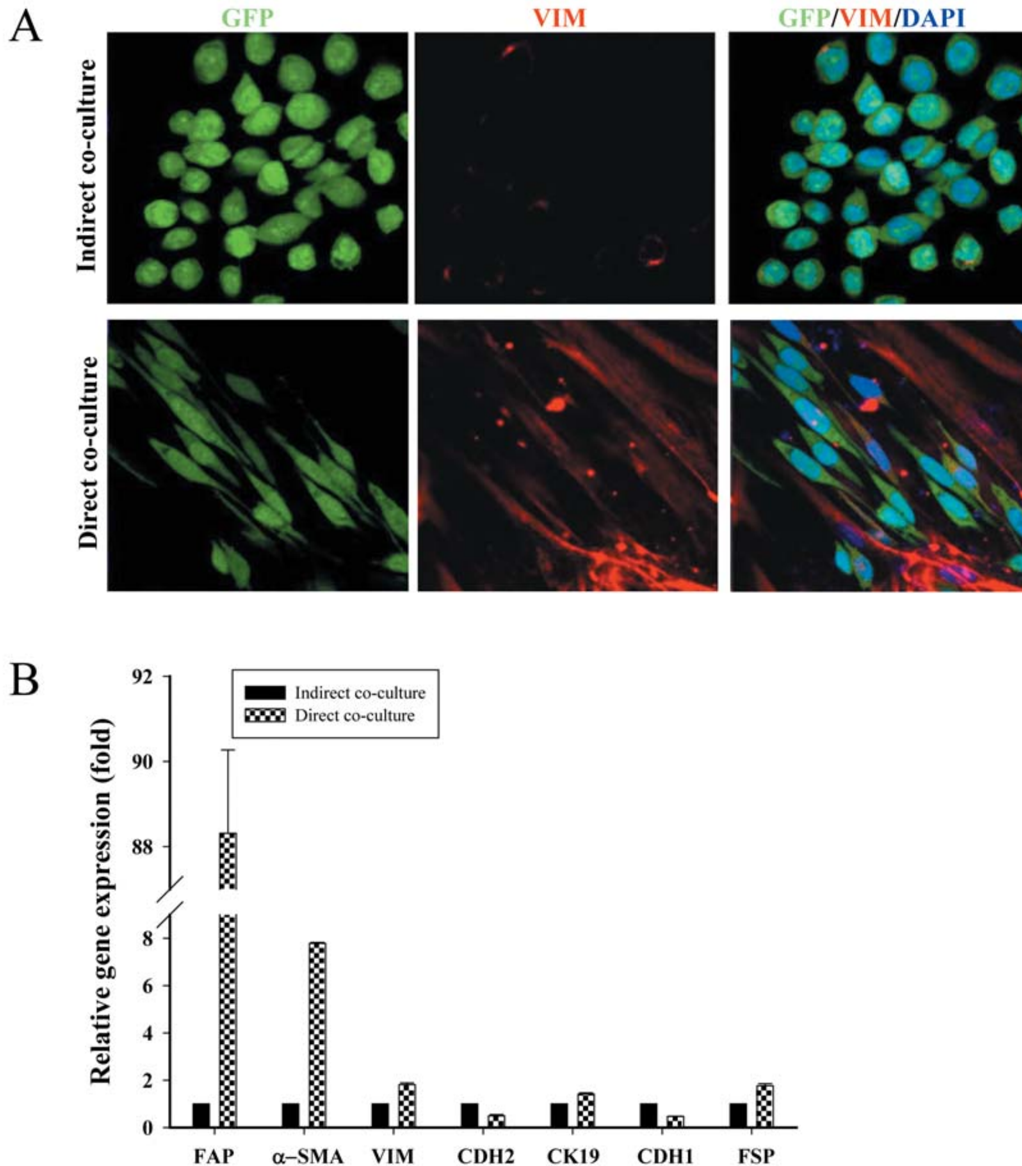


Figure 2. *Lung cancer-associated fibroblasts (CAFs) in direct co-culture induce epithelial-to-mesenchymal transition (EMT) phenotype. A: Representative images of immunofluorescence staining of non-small cell lung cancer (NSCLC) H358-green fluorescence protein (GFP) cells. Cells were stained with antibody targeting vimentin (Vim, green), while nuclei were counterstained blue with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI). Original magnification: ×1,500. B: Quantitative real time RT-PCR of human NSCLC H358 cells grown in direct and indirect co-culture with CAFs. Data shown are the mean±SEM from two independent experiments carried out in duplicate. The expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the same sample and is presented as the fold-change over that of indirect co-culture.*

conditions. Of note, EMT induction in H358-GFP cells was more pronounced in direct co-cultures than in indirect co-cultures, as evidenced by the more spindle-shaped morphology (Figure 2A). Immunofluorescence staining revealed that NSCLC H358 cells in direct co-culture up-regulate the expression of vimentin, which is one of the mesenchymal markers (Figure 2A). This finding is in good agreement with reports from others showing that the EMT phenotype accompanies the switch of intermediate filaments from cytokeratins to vimentins (8). Among the mesenchymal cytoskeletal markers, the expression of α-SMA was highly up-regulated in direct co-cultures (Figure 2B). *De novo* expression of α-SMA in the stroma stimulates the migration of stromal cells and cancer cells and contributes to alterations in cytoskeletal organization, which increases their contractile potential (21-23). Fibroblast activation protein (FAP)

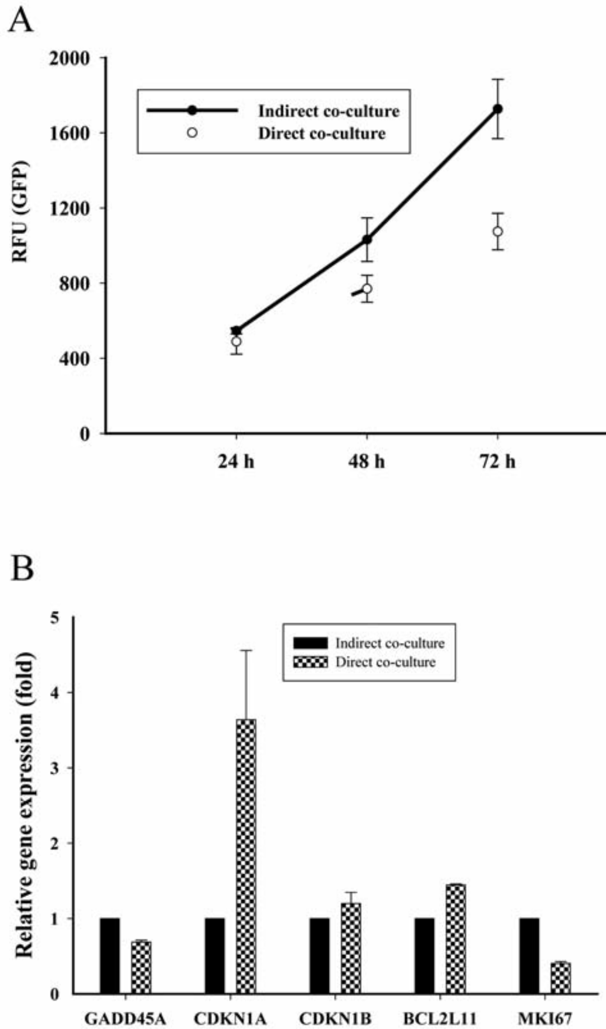


Figure 3. *Lung cancer-associated fibroblasts (CAFs) in direct co-culture retard cancer cell growth. A: Green fluorescence protein (GFP) fluorescence intensity of H358-GFP cells. CAFs stimulate the proliferation of H358 cells. Cell proliferation was measured by measuring the fluorescence intensity. Error bars represent SD (n=3). RFU, Relative fluorescence units. B: Quantitative real time RT-PCR of human NSCLC H358 cells grown in direct and indirect co-culture with CAFs. Data shown are the mean±SEM from three independent experiments carried out in duplicate. The expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the same sample and is presented as fold-change over that of indirect co-culture.*

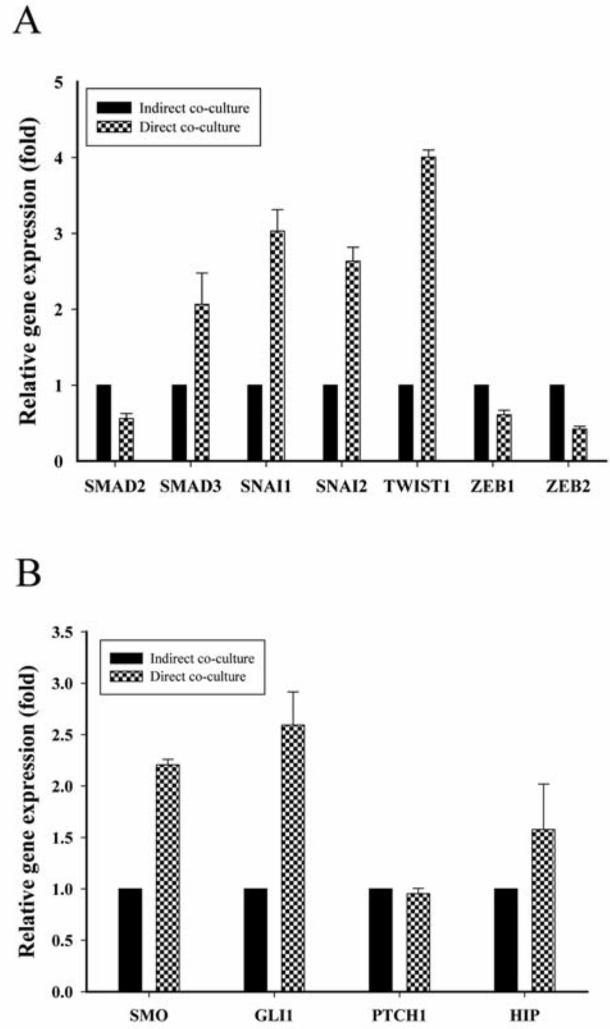


Figure 4. *Up-regulation of expression of the hedgehog signaling mediator GLI family zinc finger-1 (GLI1) and the transforming growth factor-β1 (TGFβ1) mediators SMADs in direct co-cultures. Quantitative real time RT-PCR of human NSCLC H358 cells for measuring TGFβ1 (A) and hedgehog signaling (B). Data shown are the mean±SEM from three independent experiments carried out in duplicate. The expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the same sample and is presented as fold-change over that of indirect co-culture.*

expression in direct co-culture was up-regulated by more than 80-fold compared with indirect co-culture (Figure 2B). FAP, a 95-kDa type II integral membrane glycoprotein belonging to the serine protease family, has both collagenase and dipeptidyl peptidase activities that contribute to the degradation of the ECM (24, 25). Cheas *et al*. showed that FAP is exclusively expressed by CAFs in over 90% of human epithelial carcinomas, including lung, ovarian, and breast cancers (26). However, the EMT cytoskeletal marker E-cadherin (CDH1) was down-regulated in direct co-culture compared with indirect co-culture, whereas the differentiated mesenchymal lineage marker vimentin was activated at a relatively lower level (Figure 2B). The finding that the transcriptional levels of vimentin did not agree with the immunocytochemistry trends suggests that post-transcriptional regulation is likely responsible for the discrepancy in the level of protein and

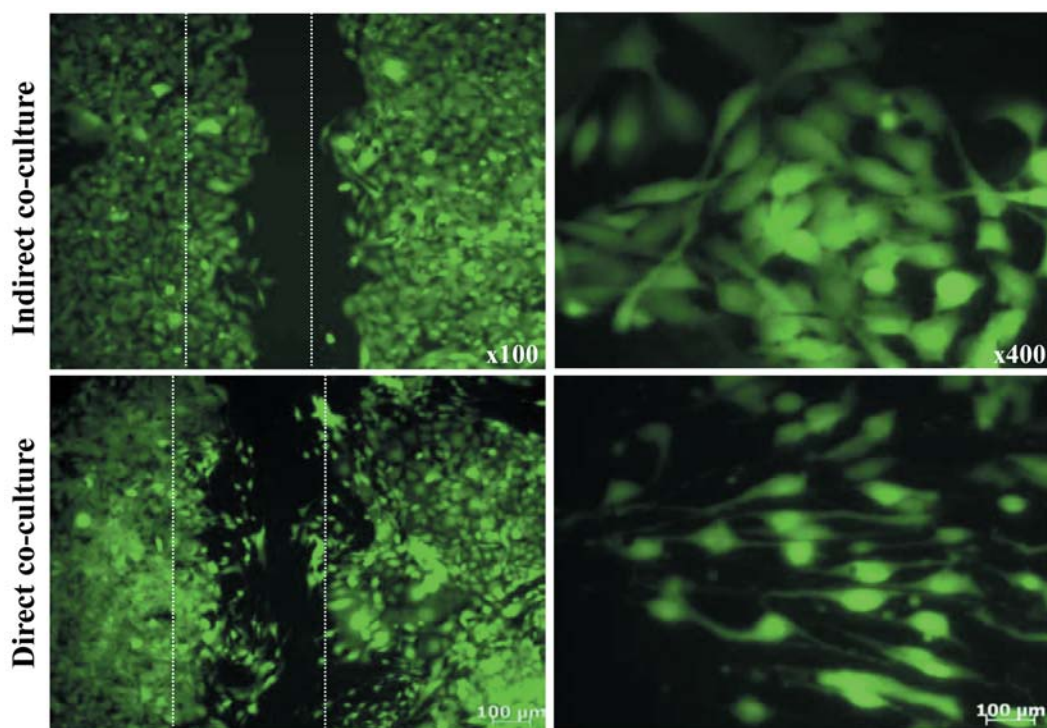


Figure 5. *The differential effect of lung CAFs on motility. Representative image of H358-GFP cells showing cell migration. In the wound healing assay, cells were scratched and allowed to grow for three days. Cells were observed using phase-contrast fluorescence microscopy to measure the wound width at 24 h post-wound. Original magnification: ×1,000.*

mRNA. Taken together, our findings suggest that CAFs in direct co-culture are more potent in inducing the mesenchymal markers including α-SMA and FAP, which confer motility on NSCLC H358 cells.

*Regulation of human NSCLC H358 cell proliferation by CAFs.* Several studies have shown that EMT-inducing signals attenuate proliferation, which may also compromise tumor growth (27-29). To examine the relative effects of physical contact *vs*. soluble factors on the proliferation of NSCLC H358 cells, we analyzed the fluorescence intensity of NSCLC H358 cells. The proliferation of H358-GFP cells in indirect co-culture was much faster than those in direct co-culture (Figure 3A). This result indicates that while the paracrine factors that CAFs secrete in co-culture enable H358-GFP cancer cells to proliferate, direct contact between CAFs and cancer cells inhibits cancer cell proliferation. Consistent with the results of the proliferation assay, among cell cycle-related proteins, cyclin-dependent kinase inhibitor CDKN1A ($p21^{Cip1}$) was specifically increased in the direct contact co-culture compared with indirect co-culture (Figure 3B). Taken together, these results suggest that CAFs in direct co-culture are more potent at inhibiting the proliferation of NSCLC H358 cells.

*Differential expression of the hedgehog signaling mediator GLI family zinc finger-1 (GLI1) and the transforming growth factor β1 (TGFβ1) signaling mediator SMADs under different co-cultures.* To further examine the molecular mechanisms underlying the observed differences in EMT induction and proliferation in direct and indirect co-cultures, we systematically studied the upstream signaling pathways and downstream mediators under the control of CAFs. Interestingly, the expression of the transcription factor SMAD3 was up-regulated in direct co-culture compared with indirect co-culture (Figure 4A). Recent studies have reported that SMAD3 promotes EMT through the transcriptional regulation of target genes, including $p21^{Cip1}$ and *α-SMA* (30, 31). This is consistent with our result showing that $p21^{Cip1}$ and *α-SMA* are up-regulated (Figure 2B and 3B). Of note, *SNAI1* and *SNAI2*, which impair cell cycle progress (28), were up-regulated in direct co-culture. Interestingly, the zinc finger transcription factor *GLI1*, which mediates hedgehog signaling (32), was highly up-regulated in direct-contact culture (Figure 4B). Studies of how GLI1 regulates cellular functions have shown that GLI1 is able to induce EMT, and that SNAI1 is a transcriptional target of GLI1 that mediates this cellular effect (32, 33). In light of the fact that hedgehog signaling influences EMT induction (33), our findings suggest that hedgehog signaling mediates paracrine and direct contact effects.

*CAFs stimulate motility.* To associate the differences in EMT induction and proliferation observed in direct and indirect co-cultures with cancer progression, we studied the motility of NSCLC cells in co-culture. To this end, H358-GFP cells with primary cultured fibroblasts were scratched and allowed to migrate to close the gap. Actively migrating H358-GFP cells were observed in direct co-culture with CAFs (Figure 5). H358 cells that are actively motile in direct co-cultures show more spindled morphology compared with indirect co-cultures. Taken together, this observation suggests that CAFs in direct contact co-culture affect the motility of cancer cells to a greater extent than in indirect co-culture through the induction of the EMT phenotype.

## Discussion

Cell–cell communication occurs *via* diffusible signaling molecules and intimate direct contact. To further differentiate the effects of physical contact *vs*. soluble factors on tumor progression, we established co-cultures under two separate conditions; direct and indirect co-cultures. Indirect co-culture using cell culture inserts with porous membrane has often been used to investigate how the diffusible factors that CAFs secrete affect the motility of cancer cells. On the other hand, the effects of cell–cell contact and juxtacrine signaling between cancer cells and CAFs have been investigated using direct co-culture, layering two cell types one on top of the other. In this study, comparisons of these two modes of co-culture led us to conclude that paracrine factors and heterocellular physical contact between CAFs and NSCLC H358 cells cooperate with each other, leading to enhanced motility. To the best of our knowledge, this is the first study to report the direct role of physical cell-to-cell contact in tumor progression in NSCLC.

It has been shown that TGFβ induces the EMT phenotype accompanied by suppression in proliferation (34). In light of these findings, our observation of the induction of EMT phenotype and the inhibition of proliferation invoked by direct cell-to-cell contact strongly suggests that a heterotypic direct interaction between NSCLC H358 cells and CAFs exerts its effect through the TGFβ signaling pathway. In support of this idea, we found that the EMT-inducing transcription activator SMAD3 was up-regulated in direct co-culture. Indeed, SMAD3 coordinates functions downstream of TGFβ-induced EMT through the transcriptional regulation of target genes, including $p21^{Cip1}$ and *α-SMA* (30, 31). TGFβ is very likely to be secreted both in indirect co-cultures and in direct co-cultures. Our observation that EMT is not induced in H358 cells in indirect co-culture, while proliferation is accelerated in comparison with direct co-culture, suggests that TGFβ creates crosstalk in signaling taking place through direct contact. Interestingly, GLI1 was significantly up-regulated in direct co-culture. Studies on GLI1 regulation of cellular functions have shown that this zinc finger transcription factor is able to induce EMT and that SNAI1 is a transcriptional target of GLI1 that mediates this cellular effect (32, 33). Taken together with these findings, our results prompted us to postulate that hedgehog signaling could be evoked by heterocellular contact and may create crosstalk in TGFβ signaling pathways, leading to enhanced cell motility. The mechanism of the interaction between these signals needs to be studied.

Our study demonstrates that CAFs and NSCLC H358 cells interact through heterotypic cell–cell adhesion. Indeed, several studies have shown that cadherin family proteins mediate heterotypic cell–cell adhesion between breast epithelial cells and fibroblasts (13, 14). Given these findings, we propose that cadherin proteins form heterotypic adhesions between CAFs and NSCLC H358 cells and bridge intracellular TGFβ signaling to hedgehog signaling. This idea also warrants further study.

In conclusion, our findings provide evidence that paracrine factors and direct physical contact between NSCLC cells and CAFs might cooperatively control the metastatic potential of NSCLC cells. Our study sheds light on the mechanisms by which lung CAFs might influence the differential metastatic potential of NSCLC cells, with cancer cells in contact with CAFs being more motile and cancer cells distant from CAFs being more proliferative. To the best of our knowledge, this is the first report that demonstrates the cellular and molecular mechanisms underlying the heterotypic contact between NSCLC cells and CAFS, which may be a potential target for therapeutic intervention.

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