Mechanical compression induces VEGFA overexpression in breast cancer via DNMT3A-dependent miR-9 downregulation

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Tumor growth generates mechanical compression, which may trigger mechanotransduction in cancer and stromal cells and promote tumor progression. However, very little is known about how compression stimulates signal transduction and contributes to tumor progression. In the present study, we demonstrated that compression enhances a tumor progression phenotype using an in vitro compression model, and validated the results from the in vitro model with high- and low-compressed breast cancer tissues. Mechanical compression induced miR-9 downregulation by DNMT3A-dependent promoter methylation in the MDA-MB-231 and BT-474 breast cancer cell lines and in cancer-associated fibroblasts. The overexpression of miR-9 target genes (LAMC2, ITGA6, and EIF4E) was induced by miR-9 downregulation, which eventually enhanced vascular endothelial growth factors production. Demethylation and decompression could reverse compression-induced miR-9 downregulation and following overexpression of miR-9 target genes and VEGFA.

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The increase of cellularity in a restricted tissue space causes mechanical compression. Therefore, tumor growth generates mechanical compression, which may affect the signal transduction of cancer and stromal cells and promote tumor progression. This mechanotransduction may be a component of the cancer signaling axis. However, little is known about how mechanical compression contributes to tumor progression.

In a living organism, various mechanical stresses naturally occur and can modulate cellular signaling.1–4 Unlimited proliferation is a fundamental property of cancer.10 Therefore, tumor proliferation in a restricted tissue space increases mechanical compression, which may happen in almost all solid tumors. It was reported that increased mechanical compression acts in the interior and periphery of tumors.11 The solid stress by tumor proliferation is known to be a cause for the increase of compressive force in tumor tissue.12 In addition to tumor proliferation, an excessive deposition of extracellular matrix (ECM) contributes to solid stress by stiffening soft tissues.13 The degree of tissue stiffness is associated with breast cancer malignancy.14,15 Because uncontrolled proliferation and tissue stiffness are common features of cancer, it follows that compression-induced mechanotransduction may be important for tumor progression. It was demonstrated that mechanical compression can induce cancer cell migration by stimulating the formation of leader cells.16 Nonetheless, the signaling pathways downstream of mechanical stress are largely unknown in cancer.

Previously, we found that laminin-332 and integrin-α6β4 are overexpressed in the margin between the tumor and fibrotic stroma in breast invasive ductal carcinoma (IDC).17 The margin is the periphery where compression is induced by tumor growth; thus, laminin-332 and integrin-α6β4 overexpression may be induced by compression and may play an important role in breast cancer progression. Laminin-332 promotes tumorogenesis, tumor invasion, and survival.18,19 Integrin-α6β4 is also involved in cancer progression.20,21 Most importantly, the binding of laminin-332 to integrin-α6β4 contributes to angiogenic signaling. It was shown that integrin alpha6beta4 enhances EIF4E activity, leading to the increased production of vascular endothelial growth factors (VEGF) in breast cancer cells.22 Interestingly, LAMC2 (the gene encoding the gamma subunit of laminin-332), ITGA6 (the gene encoding integrin alpha6), ITGB4 (the gene encoding integrin beta4), and EIF4E have microRNA-9 (miR-9) binding sites in their 3'-UTRs (microRNA.org and TargetScan), and miR-9 is downregulated in advanced breast cancer.22,23 The connection between miR-9 and compression has not been reported. However, a mechanical stress is capable of altering miR expression.24 miR-18a is induced by a mechanical stress and leads to a reduction in phosphatase and tensin homolog (PTEN) expression.25 Taken together, it is plausible that compression can induce miR-9 downregulation, which would lead to the upregulation of miR-9 target genes.

In this study, we demonstrated that mechanical compression downregulates miR-9 expression in cancer-associated fibroblasts (CAFs), which are major component contributing to matrix stiffness, and breast cancer cells by using an agarose-scaffolded alginate bead culture for a static three-dimensional
(3D) compression model. In addition, we also showed that compression-induced miR-9 downregulation triggers and enhances a signaling axis for VEGFA.

Results

Mechanical compression downregulated miR-9 expression in breast cancer. miR-9 downregulation in advanced breast cancer may be associated with the mechanical compression (CF) generated by cancer cell proliferation. To confirm whether compression induces miR-9 downregulation in breast cancer, CAFs and breast cancer cell lines [MCF7, BT-474, MDA-MB-231, and SK-BR-3: representative cell lines for luminal A, luminal B, Claudin-low (triple negative), and Her2 amplification of human breast cancer, respectively26] were compressed with 0.5, 1, 2, 5, and 10 relative compression units (RCUs, 1 RCU = –0.773 kPa, the compression value of a native tumor microenvironment16) using an agarose-scaffolded alginate bead culture model. Agarose-scaffolded alginate bead culture was firstly developed for this study, since the previous compression models using agarose gel requires heating or chaotropic salts for agarose gel to endure the compression of native tumor microenvironment. Heat and chaotropic salts impair the integrity of RNA and protein.29,30 Alginate is one of the widely used biomaterials for cell culture31 and is easy to polymerize and depolymerize. However, alginate beads were not strong enough to endure the compression of native tumor microenvironment. To make alginate beads endurable to compression, they were embedded within an agarose gel (Figure 1a). The suitability of our model was evaluated as Supplementary Figure S1.

In our model, miR-9 expression was significantly decreased by compression in CAFs, MDA-MB-231, and BT-474 at all RCUs (Figures 2a and b). On the other hand, miR-9 expression was not changed or upregulated by compression in MCF7 and SK-BR-3 (Supplementary Figure S2A). To confirm compression-induced miR-9 downregulation, CAFs, MDA-MB-231, and BT-474 were compressed with 1 RCU for 24 h in the presence of a Cy5-conjugated miR-9 probe. As shown in Figure 2c, the fluorescence of miR-9 was diminished by compression in CAFs, MDA-MB-231, and BT-474 compared with the controls. miR-9 is derived from three precursors, mir-9-1, mir-9-2, and mir-9-3. Therefore, the expression of miR-9 precursors was further evaluated. Similar to miR-9 expression, mir-9-1, -2, and -3 were downregulated by compression in CAFs, MDA-MB-231, and BT-474 at all RCUs (Figures 2d and e).

Compression-induced miR-9 downregulation was associated with DNMT3A-dependent methylation. miR-9 downregulation in breast cancer is known to be associated with hypermethylation.22 Therefore, a compression-induced regression of miR-9 and its precursors was investigated in the absence or presence of the methylation inhibitor 5-azacytidine (5-aza). As shown in Figure 3a, the expression of miR-9 and its precursors in 5-aza-treated CAFs, MDA-MB-231, and BT-474 was higher than in untreated cells at the indicated RCUs. 5-aza treatment inhibited compression-induced miR-9 downregulation (i.e., increased miR-9 expression) at all RCUs.

Promoter hypermethylation can lead to gene repression. Thus, compression may be associated with the promoter hypermethylation of miR-9 precursors. To test this hypothesis, the region 2 kb upstream of each miR-9 precursor locus was analyzed to determine the putative promoter regions. Four, one, and two CpG islands were identified in the 2 kb region upstream of mir-9-1, mir-9-2, and mir-9-3, respectively (Supplementary Figure S3). For methylation-specific qPCR analysis, methylated and unmethylated DNA-specific primer sets were designed at the third CpG island of mir-9-1, the first CpG island of mir-9-2, and the second island of mir-9-3 since a largest number of CpG sites are located in the CpG islands. The relative methylation of the precursor miR-9 promoters was calculated by normalizing to unmethylated DNA using the ΔΔCt method. As shown in Figure 3b, the methylation of the miR-9-1 putative promoter was raised over 10-fold in CAFs, and 3–10-fold in MDA-MB-231 and BT-474 following compression. A similar, but less degree of methylation was observed in the mir-9-2 putative promoter region (3–8-fold increase). The methylation of the mir-9-3 putative promoter was also increased by compression in CAFs, MDA-MB-231, and BT-474. In BT-474, putative promoter regions of precursor miR-9 were methylated following compression, but to a lesser extent than in CAFs and MDA-MB-231.

De novo promoter methylation is catalyzed by the DNMT3 family of enzymes. Therefore, it was examined whether compression induces the overexpression of DNMT3A, 3B, and 3l. DNMT3A mRNA was upregulated by compression at all the RCUs in CAF, MDA-MB-231, and BT-474 (Figure 3c). DNMT3A protein was also overexpressed following compression (Figure 3d). However, unlike DNMT3A, DNMT3B mRNA was not elevated by compression, but rather downregulated at some RCUs (Supplementary Figure S4A). DNMT3L mRNA was not generally changed by compression at RCUs in CAF and MDA-MB-231, whereas was significantly decreased in BT-474 (Supplementary Figure S4B). The DNMT3B and 3L proteins showed very low expression and little difference between RCUs (Supplementary Figure S4C). Unlike CAF, MDA-MB-231 and BT-474, as shown in Supplementary Figure S5A, MCF7 and SK-BR-3 showed a significant downregulation of compression-induced DNMT3A mRNA expression. Similar to mRNA, the expression of DNMT3A protein was generally decreased in MCF7 by compression except for 1 RCU, whereas it was decreased in SK-BR-3 at the RCUs of 0.5, 5, and 10 (Supplementary Figure S5D).

To confirm whether the hypermethylation of the putative promoter regions was induced by DNMT3A, chromatin immunoprecipitation with anti-DNMT3A antibody was performed. As shown in Figure 3e, DNMT3A bindings to the putative promoter regions of miR-9 precursors were higher in compressed cells than uncompressed control cells. In the MDA-MB-231 stably overexpressing DNMT3A shRNA, compression did not induce the downregulation of precursors of miR-9 (Figure 3f).

Compression-induced miR-9 downregulation was reversed by decompression. To further validate the influence of compression on miR-9 expression, cells were
compressed for 24 h and then incubated for an additional 24 h without compression (i.e., decompression, or deCF). As shown in Figure 4a, miR-9 expression was upregulated by decompression in the CAF, MDA-MB-231, and BT-474, compared with the controls which were compressed for 24 h but without decompression. Similar to miR-9, the expression of each precursor miR-9 was also increased by the decompression in CAF, MDA-MB-231, and BT-474 compared with the controls. The red fluorescent miR-9 probe intensity, which had been weakened by compression, recovered by decompression (Figure 4b). Compression-induced miR-9 downregulation was induced by DNMT3A-mediated hypermethylation (Figures 3b-e). Therefore, restored miR-9 expression by decompression may be coupled with DNMT3A downregulation. As shown in Figures 4c and d, an upregulated expression of DNMT3A mRNA and protein by compression was downregulated by decompression.

Compression-induced miR-9 downregulation was associated with VEGF expression. Compression-induced miR-9 downregulation may contribute to angiogenesis, since miR-9 downregulation is observed in advanced breast cancer and tumor progression is dependent of angiogenesis. It was previously reported that the signaling axis consisting of laminin-332, integrin-α6β4, and EIF4E enhances the expression of VEGFs. Laminin-332 and integrin-α6β4 are also known to be overexpressed in the margin between tumor and fibrotic stoma, where compression is induced by tumor growth in breast IDC. LAMC2 encodes the gamma subunit...
of laminin-332, ITGA6 and ITGB4 encode the subunits of integrin-α6β4, and EIF4E encodes the EIF4E protein. Interestingly, LAMC2, ITGA6, ITGB4, and EIF4E have miR-9-binding sequences in their 3'-UTRs (microRNA.org and TargetScan). Accordingly, we hypothesized that compression-induced miR-9 downregulation would result in the upregulation of LAMC2, ITGA6, ITGB4, and EIF4E. To determine whether miR-9 binds to the 3'-UTRs of LAMC2, ITGA6, ITGB4, and EIF4E, wild and seed sequence deletion mutants of each 3'-UTRs were cloned into the pGL3 control vector (Supplementary Figure S6), and then miR-9 binding to the 3'-UTRs was measured by luciferase activity assay. The luciferase activities of wild-type 3'-UTRs of LAMC2, ITGA6, ITGB4, and EIF4E were all significantly decreased by miR-9 co-transfection compared with those of the mutant 3'-UTRs (Figure 5a).

Next, we determined whether miR-9 downregulation leads to the upregulation of LAMC2, ITGA6, ITGB4, EIF4E, VEGFA in CAF, MDA-MB-231, and BT-474. In the CAF, MDA-MB-231, and BT-474 transfected with anti-miR-9, the mRNA and protein expression of LAMC2, ITGA6, EIF4E, and VEGFA were significantly upregulated, whereas those of ITGB4 was not (Figures 5b and c).

Compression-induced expression of miR-9 target genes was suppressed by methylation inhibition and decompression. miR-9 downregulation induced the upregulation of LAMC2, ITGA6, EIF4E, and VEGFA expression (Figures 5b and c). Since compression-induced miR-9 downregulation was reversed by methylation inhibition (5-aza treatment) and decompression, the expression of LAMC2, ITGA6, EIF4E, and VEGFA may be also regulated by methylation inhibition and decompression. To confirm this assumption, we compressed CAF, MDA-MB-231, and BT-474 at the RCU of 1 for 24 h in the absence or presence of 5-aza, or further incubated for an additional 24 h without compression, and then analyzed mRNA expression. As shown in Figure 6a, a compression-induced upregulation of LAMC2, ITGA6, EIF4E, and VEGFA was decreased by 5-aza treatment and decompression in CAFs, MDA-MB-231, and BT-474. On the other hand, ITGB4 expression was generally not decreased by 5-aza treatment and decompression. It was decreased by 5-aza treatment only in CAF. Like mRNA expression, LAMC2, ITGA6, EIF4E, and VEGFA protein expression was increased by compression, but decreased by 5-aza treatment or decompression in CAFs, MDA-MB-231, and BT-474 (Figure 6b). ITGB4 protein expression was...
Figure 3 Compression-induced miR-9 downregulation via DNMT3A-dependent methylation. (a) Compression-induced miR-9 and its precursor expression of CAF, MDA-MB-231, and BT-474 in the presence or absence of 5-azacytidine (5-aza). Cell-alginate beads were pretreated with 10 μM 5-aza for 1h before compression. (b) Methylation-specific PCR analysis of putative promoters of pre-mir-9-1, pre-mir-9-2, and pre-mir-9-3. Methylated DNA was calculated by normalizing to unmethylated DNA using the ΔΔCt method. Compression-induced expression of (c) DNMT3A mRNA and (d) DNMT3A protein in CAF, MDA-MB-231, and BT-474. CAF1 was used as a representative for CAF. The intensity of western bands were quantified using Image J. Each band was normalized with GAPDH, and then presented as the relative intensity value of control versus compressed sample. (e) Chromatin immunoprecipitation (Chip) assay to examine DNMT3A binding to the promoter regions of miR-9 precursors. The CAF1 compressed at the RCU of 1 for 24 h were used for Chip assay, and then DNMT3A-bound DNA was analyzed using real-time PCR. Relative binding to DNMT3A was calculated using the ΔΔCt method. The Ct values of DNMT3A-bound DNA were normalized to those of input DNA. (f) DNMT3A-dependent downregulation of miR-9 precursors. The wild-type and DNMT3A shRNA overexpressing MDA-MB-231 were exposed to 1 RCU for 24 h to induce miR-9 downregulation. Data are represented as mean ± S.D. Statistical significance was determined using a control-versus-compressed sample t-test. *, **, and *** represent P-values of 0.01 to 0.05, 0.001–0.01, and <0.001, respectively.
largely unaffected by compression, decompression, or 5-aza treatment.

To confirm whether decreased expression of LAMC2, ITGA6, EIF4E, and VEGFA by 5-aza treatment or decompression was dependent on miR-9 expression, CAF was transfected with anti-miR-9, and then compressed in the presence or absence of 5-aza at the RCU of 1. For decompression, the compressed CAF was further incubated for 24 h without compression. As shown in Figures 6c and d, a decreased expression of LAMC2, ITGA6, EIF4E, and VEGFA by 5-aza treatment or decompression was reversed by anti-miR-9.

**miR-9 and its target gene expression are associated with the compressive state of the tissue.** To further validate the results of the 3D static compression model, we evaluated gene expression of low- and high-compressed breast cancer tissues from patients. Tissue stiffness or elasticity is significantly correlated with tumor cellularity, and increased cellularity by tumor expansion generally leads to high compressive state in tissue via solid stress. Therefore, low- and high-compressed tissues were classified using the mean compression values obtained by Shear-wave elastography (SWE, low-compression group, <100 kPa; high-compression group, >250 kPa; Figure 7a). miR-9 and its precursors were downregulated in high-compressed tissues compared with low-compressed tissues (Figures 7b-e). In contrast, DNMT3A expression was upregulated in high-compressed tissues compared with low-compressed tissues (Figure 7f). Among miR-9 target genes, LAMC2 expression was not associated with the compressed state of the tissues (Figure 7g). ITGA6 expression was increased in the high-compressed tissues compared with low-compressed tissues (Figure 7h). ITGB4 expression was not associated with the
compressive state of tissues (Figure 7i). EIF4E and VEGFA expression was increased in high-compressed tissues compared with low-compressed tissues (Figures 7j and k, respectively). The increased expression of ITGA6 and VEGFA in high-compressed tissues was similar to the increased expression observed in CAFs, MDA-MB-231, and BT-474. Therefore, the expression of ITGA6 and VEGFA was investigated by in situ proximity ligation assay (PLA) on tissue sections. As shown in Figure 71, a more amount of red-fluorescence was observed in high-compressed tissue than low-compressed one when performing double recognition for ITGA6 with ITGB4. Like ITGA6, a more amount of red-fluorescence was observed in high-compressed tissue than low-compressed one upon performing single recognition for VEGFA (Figure 7m).

Discussion
In our experimental model, mechanical compression induced miR-9 downregulation in CAFs, MDA-MB-231, and BT-474 (Figures 2a and b), whereas it did not induce miR-9 downregulation in MCF7 and SK-BR-3 (Supplementary Figure S2A). There may be two possible assumptions for this heterogeneous response of breast cancer cells to compression. One is...
cell-type specificity. For instance, stress-activated p53 induces a heterogeneous response in a cell-type-specific manner.\textsuperscript{33} Another is the different expression status of growth factors such as estrogen receptor (ER), progesterone receptor (PR), and Her2. All the receptors anchored in the cell membrane can function as mechanoreceptors.\textsuperscript{34} A ligand-independent function of ER has an essential role in osteocyte and osteoblast mechanotransduction.\textsuperscript{35} In CAFs, MDA-MB-231, and BT-474, compression-induced miR-9 downregulation contributed to VEGFA overexpression by inducing overexpression of LAMC2, ITGA6, EIF4E, and VEGFA by methylation inhibition and decompression in CAF. CAF1 was used as a representative for CAF. The cells were compressed with the RCU of 1 for 24 h in the absence or presence of 10 μM of 5-azacytidine. For decompression, the cells were cultured for additional 24 h without compression.

Data are represented as mean ± S.D. ** and *** represent P-values of 0.001–0.01 and < 0.001, respectively.

**Figure 6** Compression-induced expression of miR-9 target genes and VEGFA was suppressed by methylation inhibition and decompression. The effect of methylation inhibition and decompression on (a) mRNA expression and (b) protein expression of miR-9 target genes and VEGFA in CAF, MDA-MB-231, and BT-474. (c) miR-9 dependent downregulation of LAMC2, ITGA6, EIF4E, and VEGFA by methylation inhibition and decompression in CAF. CAF1 was used as a representative for CAF. The cells were compressed with the RCU of 1 for 24 h in the absence or presence of 10 μM of 5-azacytidine. For decompression, the cells were cultured for additional 24 h without compression.

DNMT3A is required for de novo methylation, but it is not needed for the maintenance of methylation.\textsuperscript{37,38} Hence, miR-9 reversal cannot be explained by decompression-induced DNMT3A downregulation. DNMT3A downregulation may be indirectly associated with miR-9 promoter demethylation. The balance in the dynamics of DNA methylation and demethylation is an epigenetic mechanism for gene expression.\textsuperscript{39,40} Therefore, decompression-induced DNMT3A downregulation can result in the imbalance between methylation and demethylation, which leads to demethylation of miR-9 promoter. With DNMT3A downregulation, decompression may activate demethylases such as the Ten-eleven translocation methylcytosine dioxygenase (TET) or the methylation-sensitive transcription factor to lead miR-9 upregulation such as Yin Yang 1 (YY1).\textsuperscript{41} Kangaspeska et al.\textsuperscript{42} reported transient cyclical methylation and demethylation of CpG dinucleotides in the estrogen (E2)-responsive pS2 gene. This may be an evidence of reversible methylation state.

The compression model with cell-agarose constructs has been developed in order to study the cells exposed to periodic...
compression and decompression such as osteoblasts and odontoblasts. The model should be suitable to investigate compression-induced mechanotransduction, but it has an obvious disadvantage in sampling. For extracting RNA and protein, agarose has to be depolymerized by heating or adding chaotropic salts, which adversely affect sample quality. 27,28 To figure out the problem, we encapsulated cells with alginate before embedding them into agarose. Alginate is one of the good biomaterials for 3D cell culture and easily depolymerized by EDTA.27 Unfortunately, since alginate bead was not strong enough to endure the compression of native tumor microenvironment, we scaffolded the cell-alginate beads with agarose. By using agarose-scaffolded cell-alginate bead constructs, we could recover cells easily after loading compression and then extract RNA and protein using common methods. Tse et al.16 reported that the compression more than 5.8 mm Hg (1 RCU) triggered apoptosis and only 40% of cells viable at 58 mm Hg (10 RCU). However, in our model, there was little difference in apoptosis between RCUs (Supplementary Figure S1D). This discrepancy may be caused by difference between 2D and 3D cell culture environment. We encapsulated cells with alginate for 3D cell culture, whereas Tse et al. cultured cells on the membrane of transwell chamber. 3D environment has two merits compared with 2D. 3D is more similar to in vivo situation than 2D, and 3D is more advantageous in ECM deposition. ECM is known as a transmitter of mechanical stress. 34,43 As shown in Supplementary Figure S1E, ECM deposition around cells was observed in alginate beads.

Here, we showed that compression induces miR-9 downregulation via DNMT3A-dependent promoter methylation, which leads to the upregulation of miR-9 target genes (LAMC2, ITGA6, and EIF4E) thus potentiating signal transduction for VEGFA expression in CAFs and breast cancer cells. We also showed that decompression can restore miR-9 expression and subsequent downregulation of miR-9 target genes. Therefore, compression-induced miR-9 downregulation may be an important mechanism for tumor angiogenesis.

Materials and Methods

Tissue acquisition. For CAF isolation, human breast tumor tissues were obtained from three IDC patients. Tissue stiffness is along with tumor growth-induced solid stress, 44 and the solid stress increases compression force. 12 Therefore, to study compression-dependent gene expression, low- and high-
Compressed breast tumor tissues were obtained from 20 breast cancer patient tissues after measuring compression by shear-wave elastography (SWE) using the Aixplorer US system (SuperSonic Imagine, Aix-en-Provence, France): 10 for low-compression below the mean value of 100 kPa and 10 for high-compression over the mean value of 200 kPa. All patients donating the tissues underwent surgery at Severance Hospital of the Yonsei University Health System, Seoul, South Korea. The protocol for the research was approved by the Severance Hospital Ethics Committee (IRB number 4-2008-0383). All participants signed consent forms and were informed of tissue use for comprehensive experiments of breast cancer.

Isolation of cancer-associated fibroblasts and cell cultures. CAFs were isolated as previously described. Briefly, tissue from early-stage IDC (stage 1) that was less than 10 mm in diameter was sliced and then digested overnight with a collagenase preparation (ISU ABXIS; Seoul, South Korea). Digested tissue was filtered through a 70 μm cell strainer (SPL Life Science; Pocheon-si, South Korea). Cells were separated by Ficol gradients, washed with PBS, resuspended in DMEM/F12 cell culture medium containing 20% (v/v) fetal bovine serum (FBS, 100 U/μl penicillin, and 100 μg/ml streptomycin (Gibco BRL; Grand Island, NY, USA) and cultured at 37 °C in a humidified incubator containing 5% CO₂.

Compression assay and sample preparation. Compression assays were performed as previously described. Briefly, for making the alginate beads containing cells, pellets of CAFs, MDA-MB-231, BT-474, MCF7, or SK-BR-3 were suspended in 0.5% alginate solution to yield 5 × 10⁶ cells/ml. Using a syringe with a stainless steel needle, the alginate solution was injected into 0.5% calcium chloride solution (50 μl in 1 ml) to yield a 2 mm diameter bead. The cell-alginate beads were washed with PBS twice, and then incubated for an additional 24 h without compression. For sample preparation, the cell-alginate beads were compressed for 24 h and then incubated with RCUs for 24 h. One RCU was 5.8 mmHg (~0.773 kPa), and the compression value of a native tumor microenvironment. An empty cube was placed in 4% agarose gel without compression (Supplementary Figure S1C). Alginate bead compression below the mean value of 100 kPa and high compression over 100 kPa were isolated as previously described. Briefly, tissue from early-stage IDC (stage 1) was obtained. Digestion was performed using a collagenase preparation (ISU ABXIS; Seoul, South Korea). The expression of c-Jun was examined. c-Jun is known to be upregulated by mechanical stress. To confirm whether compression was transferred to cells, Alginate bead compression (Supplementary Figure S1H). The deformation of agarose gel and compression (Supplementary Figure S1A, B). The deformation of agarose gel and compression (Supplementary Figure S1A, B). The deformation of agarose gel and compression (Supplementary Figure S1A, B). The deformation of agarose gel and compression (Supplementary Figure S1A, B).

Firefly luciferase reporter constructs and luciferase assays. The wild-type 3'-UTRs of human LAMC2, ITGA6, ITGB4, and EIF4E were amplified by PCR using CAF genomic DNA as template. Mutant 3'-UTRs of each gene with deletions of the miR-9 seed sequence (from 7 to 10 bp) were generated by an overlap extension PCR method. Wild-type and mutant 3'UTRs were inserted downstream of the firefly luciferase-coding gene at the XbaI site of the pGL3 control vector (Promega; Madison, WI, USA) and 500 ng of miR-9 or control miR plasmid were transfected into 293T cells in six-well plates using Lipofectamine LTX with Plus Reagent (Invitrogen). The cells were harvested 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega).

Fluorescent imaging of miR-9 in compressed cells. In the presence of the human C5s-conjugated miR-9-Sp probe (EMD Millipore; Billerica, MA, USA), the cell-alginate beads were compressed with 1 RCU for 24 h, or were incubated for additional 24 h without compression for the decoy treatment studies. For the control, the cell-alginate beads were cultured for 24 h without compression. The cells were isolated from the alginate beads by depolymerization, fixed with 4% paraformaldehyde, permeabilized with 0.1 M glycine, and mounted on a gelatin-coated glass slide. In situ fluorescence images were quantified by measuring red-colored area using ImageJ (Bethesda, MD, USA) (version 1.50i).

In situ proximity ligation assay (PLA). After deparaffinization and rehydration, the slides with tissue sections (4 μm) were washed twice with PBS and then blocked for 1 h at RT with 2% (w/v) BSA in PBS containing 0.1% (v/v) Triton X100. The slides were subsequently incubated at 4 °C overnight with anti-ITGA6 (1:100 dilution) (Abcam) and anti-ITGB4 (1:100) (Santa Cruz Biotechnology; Dallas, TX, USA) antibodies for double recognition or anti-VEGFA antibody (1:100) (Santa Cruz) for single recognition in a humidity chamber. Probe ligation was performed using Duolink (OLINK Biosciences; Uppsala, Sweden), as recommended by the manufacturer. Images were taken using a LSM710 confocal laser scanning microscope and ZEN software (Carl Zeiss; Oberkochen, Germany). Fluorescence images were quantified by measuring red-colored area using Image J (Versión 1.50i).

Treatment with anti-miR-9. Cells were cultured in six-well plates until 70% confluent, and then transfected with 0.1 μM of anti-miR-9 or negative control miR for...
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