Video Article

Development of an *In Vitro* Assay to Evaluate Contractile Function of Mesenchymal Cells that Underwent Epithelial-Mesenchymal Transition

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URL: https://www.jove.com/video/53974
DOI: doi:10.3791/53974

Keywords: Developmental Biology, Issue 112, Tissue fibrosis, transforming growth factor β1 (TGF-β1), human lung epithelial cells, epithelial mesenchymal transition (EMT), human lung fibroblasts, gel contraction assay, tumor necrosis factor (TNF)-α

Date Published: 6/10/2016

Citation: Mikami, Y., Matsuzaki, H., Takeshima, H., Makita, K., Yamauchi, Y., Nagase, T. Development of an *In Vitro* Assay to Evaluate Contractile Function of Mesenchymal Cells that Underwent Epithelial-Mesenchymal Transition. *J. Vis. Exp.* (112), e53974, doi:10.3791/53974 (2016).

Abstract

Fibrosis is often involved in the pathogenesis of various chronic progressive diseases such as interstitial pulmonary disease. Pathological hallmark is the formation of fibroblastic foci, which is associated with the disease severity. Mesenchymal cells consisting of the fibroblastic foci are proposed to be derived from several cell sources, including originally resident intrapulmonary fibroblasts and circulating fibrocytes from bone marrow. Recently, mesenchymal cells that underwent epithelial-mesenchymal transition (EMT) have been also supposed to contribute to the pathogenesis of fibrosis. In addition, EMT can be induced by transforming growth factor β, and EMT can be enhanced by pro-inflammatory cytokines like tumor necrosis factor α. The gel contraction assay is an ideal *in vitro* model for the evaluation of contractility, which is one of the characteristic functions of fibroblasts and contributes to wound repair and fibrosis. Here, the development of a gel contraction assay is demonstrated for evaluating contractile ability of mesenchymal cells that underwent EMT.

Video Link

The video component of this article can be found at https://www.jove.com/video/53974/

Introduction

Fibrosis is involved in the pathogenesis of various chronic progressive diseases, such as interstitial pulmonary disease, cardiac fibrosis, liver cirrhosis, terminal renal failure, systemic sclerosis, and autoimmune disease[1]. Among interstitial lung diseases, idiopathic pulmonary fibrosis (IPF) is a chronic progressive disease and shows poor prognosis. Pathological hallmark of IPF is the development of fibroblastic foci consisting of activated fibroblasts and myofibroblasts that are associated with the prognosis. The origins of such pulmonary fibroblasts are proposed to be derived from several mesenchymal cells, including originally resident pulmonary fibroblasts and circulating fibrocytes from bone marrow. Recently, epithelial-mesenchymal transition (EMT) has been proposed to be associated with the formation of mesenchymal cells[2], and to contribute to the pathogenesis of fibrotic disorders.

It is thought that EMT plays important roles in the process of fetal development, wound healing, and progression of cancer, including tumor invasion and metastasis[3]. Following the process of EMT, epithelial cells obtain the ability of mesenchymal cells by loss of epithelial markers, such as E-cadherin, and by expression of mesenchymal markers, such as vimentin, and α-smooth muscle actin (SMA)[4,5]. Previous studies showed the evidence that EMT process has been associated with the development of tissue fibrosis in the kidney[6] and lung[7]. Additionally, chronic inflammation promotes fibrotic disease[8]; furthermore, such inflammatory cytokines as Tumor necrosis factor superfamily member 14 (TNFSF14; LIGHT), tumor necrosis factor (TNF)-α, and interleukin-1β, have been shown to enhance EMT[9-12].

Collagen gel contraction assay, a collagen-based cell contraction assay in which fibroblasts are embedded in type I collagen gel three-dimensionally, is an ideal *in vitro* model for the evaluation of contractility. Contractility is one of the characteristic functions of fibroblasts and contributes to normal wound repair and fibrosis[13]. In this assay, it is thought that the attachment of fibroblasts to type I collagen through integrin-dependent mechanisms is supposed to produce mechanical tension under some conditions, and consequently lead to tissue contraction.

Here, the development of the gel contraction assay is reported to be adapted to evaluate the acquisition of contractile function in the cells that underwent EMT. This report demonstrates that this modified assay is suitable for evaluating contractility in mesenchymal cells that underwent EMT.

Protocol

1. Preparations and Culture of Lung Epithelial Cells

1. Culture A549 human lung epithelial cells (adherent cell line) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin.
2. Remove and discard the cell culture media from culture dish, and wash once with 5 - 10 ml of phosphate buffered saline (PBS). After washing, immediately aspirate the PBS.
3. Add 2 ml Trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%) and incubate at 37 °C and 5% CO_2 for 3 min.
4. Collect the detached cells in centrifuge tubes containing cell culture medium, centrifuge the tubes at RT for 4 min at 150 × g.
5. Resuspend the cells pellet in 2 ml of cell culture medium and remove a sample for cell counting. Count the number of the cells using a hemocytometer with Trypan blue stain to check cell viability.
6. Seed A549 cells on 10 cm polystyrene plates at a density of 0.5 - 1.0 × 10^6 cells/dish with 10 ml of medium for gel contraction assay. Seed the cells on 6 well polystyrene plates at a density of 0.5 - 1.0 × 10^5 cells/well with 2 ml of medium for EMT confirmation. Incubate the cells at 37 °C and 5% CO_2 for 24 hr.

2. EMT Procedure

1. Add 10 µl of TGF-β1 (5 µg/ml) and 10 µl of TNF-α (10 µg/ml) to the plate for gel contraction assay seeded in step 1.6. Add 2 µl of TGF-β1 (5 µg/ml) and 2 µl of TNF-α (10 µg/ml) to the plate for EMT confirmation seeded in step 1.6. Incubate at 37 °C and 5% CO_2 for 48 hr.

3. Confirmation of EMT Procedure by PCR and Western Blotting

1. Confirm change in morphology (from cobble stone-like to spindle shape) of treated cells using phase contrast microscopy.
   Note: Normal A549 cells have cobble stone-like and triangular shaped appearance that is a characteristic of epithelial cells, but after stimulation with TGF-β1 and TNF-α, the cells appear long and spindle shaped that is similar to mesenchymal cells[14,15].
2. Evaluate the expression of an epithelial marker, such as E-cadherin, and mesenchymal markers such as N-cadherin, vimentin, and α-smooth muscle actin using PCR or Western blotting.
   1. Extract total RNA from the cells using RNA extraction kit[16] and synthesize cDNA using reverse transcriptase[17] according to the manufacturer's protocol.
   2. Measure expression of mRNA levels using real time PCR system[18] and monomeric cyanine dye PCR kit according to the manufacturers' instructions. The specific primers for GAPDH (glyceraldehyde 3-phosphate dehydrogenase), ACTA2 (alpha-actin-2), CDH1 (cadherin-1; E-cadherin), and VIM (vimentin) are shown in **Table 1**.
   3. Lyse the cells using a lysis buffer (**Table 2**) solution containing 1% protease inhibitor cocktail. Measure all sample protein concentrations using a protein assay kit[19,20], and apply the same amounts of protein to a polyacrylamide gel.
      1. Perform SDS gel-electrophoresis and semi-dry transfer of the proteins to PVDF membrane[21]. Incubate the membrane with primary antibodies in blocking buffer for 1 - 2 hr. After incubation, wash twice the membrane with wash buffer and incubate the membrane 1 hr with second antibodies.
         Note: See the Materials/Equipment Table for antibodies and dilutions used in these studies. See **Table 2** for the components of blocking buffer.
      2. Wash the membrane with wash buffer twice. Take pictures of the membrane with the Western blotting detection kit[22] using a cold CCD camera[11,23].

4. Gel Contraction Assay for Evaluating EMT

1. Aspirate the conditioned media from the cell culture vessel, and wash well with 5 - 10 ml of PBS to remove dead cells. After washing, immediately aspirate the PBS.
2. Add 2 ml of 0.05% Trypsin/EDTA and incubate at 37 °C and 5% CO_2 for 3 min.
3. Collect the detached cells in centrifuge tubes containing DMEM supplemented with trypsin inhibitor (1 mg/ml), centrifuge the tubes at RT for 4 min at 150 ×g.
4. Mix type 1 collagen gel with distilled water, and 4× concentrated DMEM to adjust the volumes to achieve a collagen concentration of 1.75 mg/ml, and 1× DMEM concentration. Be sure to keep the gel medium on ice during this step.
   Note: To make 6 ml of gel medium, mix well 3.5 ml of type 1 collagen gel (3 mg/ml), 1.5 ml of 4× concentrated DMEM, and 1ml of distilled water.
5. Resuspend the cell pellet in 500 µl of PBS and remove a sample for cell counting. Count the number of the cells using a hemocytometer with trypan blue stain to check cell viability.
6. Add the gel medium to adjust the volumes to achieve a cell density of 3.0 × 10^5 cells/well (6.0 × 10^5 cells/ml) and gently but quickly mix it by pipetting without gelation.
7. Dispense 0.5 ml of the mixture into each well of a 24-well non-treated plate quickly and carefully to make neat cylindrical form. Be careful not to allow any air bubbles to contaminate the gels (**Figure 1A**).
   Note: The gel medium containing the cells is viscous and can easily gel and form crescent shape in the well.
8. Incubate the plate for 15 min in a cell incubator at 37 °C, 5% CO_2 and 95% humidity to gel completely.

9. Detach gels from the plate without breaking by moving a sterilized spatula in a manner to draw a circumference in one direction. Using a spatula, gently transfer the gels to 60 mm tissue culture dishes containing 5 ml of DMEM/1% FBS with or without TGF-β1 (5 ng/ml) and TNF-α (10 ng/ml).
10. Gently shake the dishes to ensure gels are floating on the medium. Incubate in a cell incubator at 37 °C, 5% CO_2 and 95% humidity.

5. Measurement of Gel Size

1. Measure the collagen gel size after 0, 24, 48, and 72 hr using an image analysis system.
    1. Turn on the gel documentation system (**Figure 2A**), and put dishes into the light shielding cabinet. Then take off the lid of dishes in the cabinet.
    2. Open the related gel analyzing software. Click the "image acquisition button" in the menu-bar to show the image of the gels in the cabinet. Then, click "acquire" in the menu bar to take pictures of the gels (**Figure 2B**).
    3. Click the "detection button" in the menu bar (**Figure 2C**) and adjust the measurement region (yellow circle in **Figure 2C**) by dragging the mouse. Then, click the "auto-detect button" in the menu bar (see button in **Figure 2D**). The software automatically detects the gels showing a heatmap (**Figure 2D**).
    4. Click "OK" to extract the outline of the gels by image processing and to calculate areas surrounded by the outline (**Figure 2E**). After the area is calculated, the calculated area is displayed in separate pop-up window.
    Note: If the gels overlap each other during imaging, move gels gently using a sterile pipette tip.

Representative Results

During EMT, epithelial cells lose epithelial markers, like E-cadherin, and gain the expression of mesenchymal markers, such as vimentin and α-smooth muscle actin[4,5]. Incubation of A549 human lung epithelial cells with TGF-β1 and TNF-α induces EMT. The appearance of normal A549 cells are cobble stone like shape and triangle shape that is a characteristic of epithelial cells (**Figure 3A**), but after stimulated with TGF-β1 and TNF-α, the appearance changed to long spindle shape that is similar to mesenchymal cells (**Figure 3B**).

The expression of epithelial and mesenchymal markers was evaluated to confirm that cells underwent EMT. Relative mRNA expression was calculated with ΔΔCt method. Individual data was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is a housekeeping gene. A549 cells treated with TGF-β1 and TNF-α for 48 hr had significantly reduced expression of *CDH1* and significantly increased expression of *VIM* and *ACTA2* (**Figure 4A**). The primers sequences used for q-PCR are shown in Table1. As shown in **Figure 4B**, stimulation with TGF-β1 and TNF-α attenuated E-cadherin expression, whereas the expression of vimentin, N-cadherin, and α-smooth muscle actin was induced.

The gel contraction assay was performed to evaluate the contractility of cells that underwent EMT. After inducing EMT with TGF-β1 and TNF-α, cells were cast into the collagen gel and incubated for 72 hr in media containing TGF-β1 and TNF-α, or PBS. As shown in **Figure 5A**, the gels containing cells treated with TGF-β1 and TNF-α were smaller than control gels containing cells treated with PBS. To quantify the changes in gel size, gels were analyzed using a gel analyzer 0, 24, 48, and 72 hr after treatment. After 72 hr, the sizes of gels containing cells treated with TGF-β1 and TNF-α were significantly reduced compared to the control gels (**Figure 5B**). We also confirmed that the gels containing cells treated with TGF-β1 alone were smaller than control gels but were not smaller than the gels treated with TGF-β1 and TNF-α (data not shown).

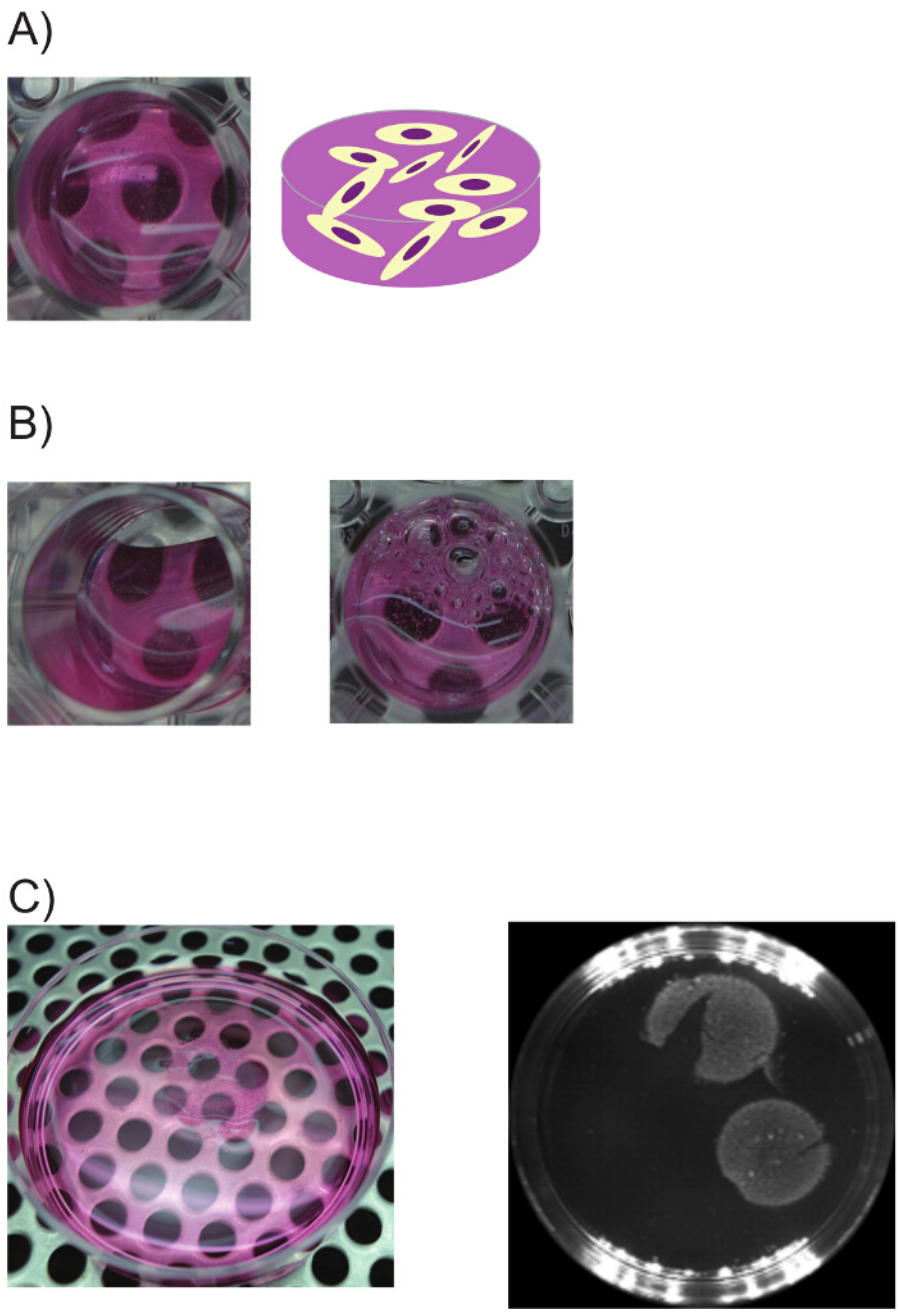

Figure 1. Supplementary Figure for Making Gels. These images show the gels cast into wells. The gel medium containing the cells is viscous and can easily gel and form crescent shape in the well. (**A**) The left image shows that the gels are casted correctly, and the right image shows the schema of "neat cylindrical form". (**B**)The left image shows that the gels deform, and the right image shows that air bubbles contaminate the gels. (**C**) The gels are soft, so easily damaged and usually deform like "Pac-Man". Please click here to view a larger version of this figure.

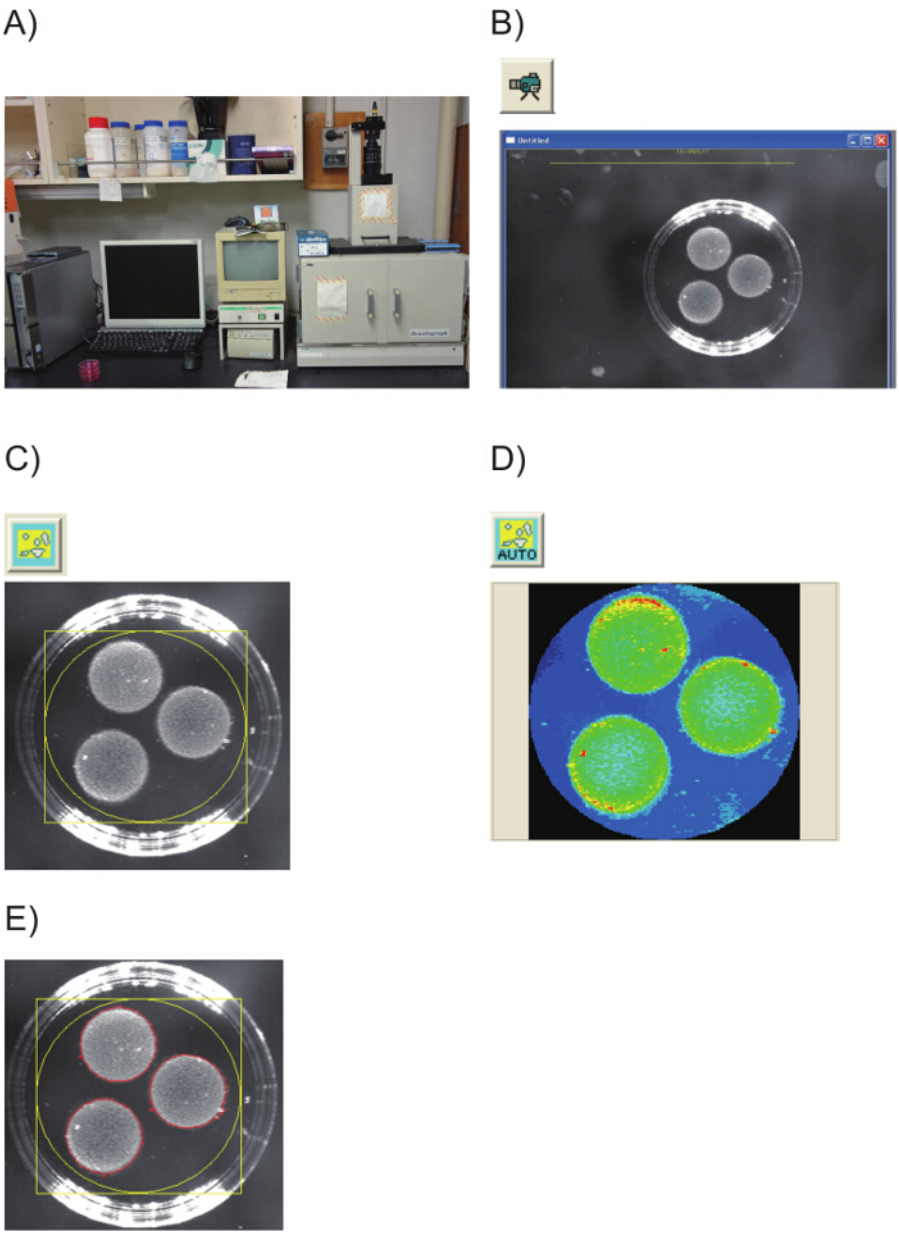

Figure 2. The Steps for Measuring the Gel Size. (**A**) The gel documentation system consists of a PC and a gel imaging system. (**B**) The "image acquisition button", and a picture of the gels. (**C**) The "detection button", and the window for adjusting the measurement region (yellow circle). (**D**) The "auto-detection button", and the software makes a heatmap of the gels from a picture to detect the outline of the gels. (**E**) The software extracts the outline of the gels by image processing, and calculates areas surrounded by the outline. Please click here to view a larger version of this figure.

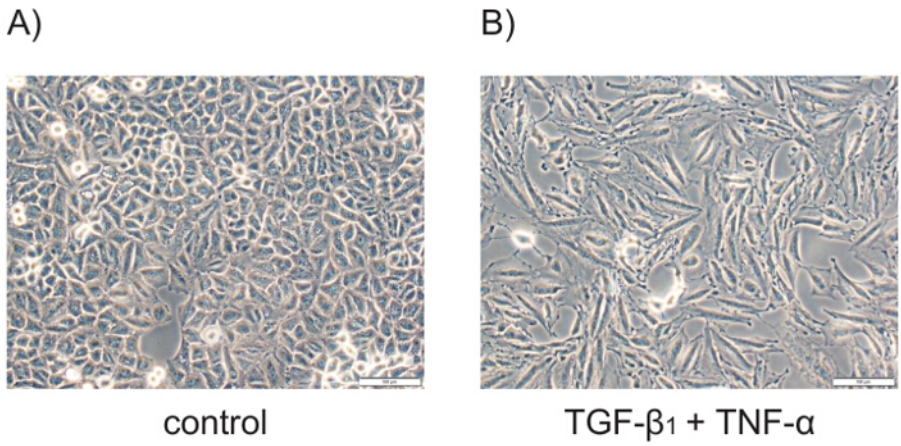

Figure 3. Morphologies of EMT-induced Cells. A549 human lung epithelia were plated at a density of 1.0 × 10^5 cells/well in a 6-well plate and incubated with or without 5 ng/ml TGF-β1 and 10 ng/ml TNF-α for 48 hr, followed by phase contrast microscopic imaging. (**A**) and (**B**) are images obtained under ×200. The scale bars in (**A**) and (**B**), 100 µm. Please click here to view a larger version of this figure.

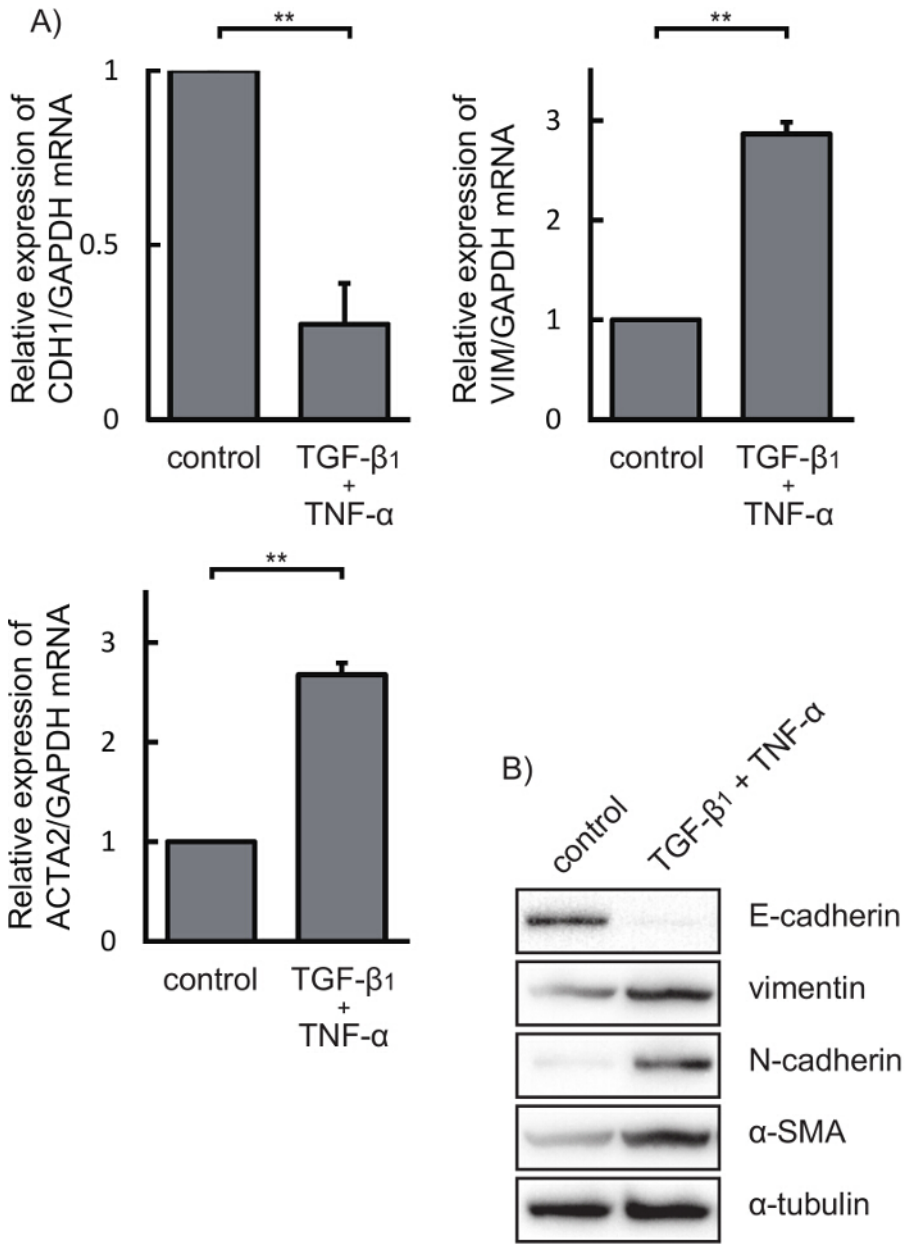

Figure 4. EMT Stimulation with TGF-β1 and TNF-α. (**A**) qRT-PCR analysis of EMT marker expression (*CDH1, VIM,* and *ACTA2*). (**B**) Western blot showing the expression of E-cadherin, N-cadherin, vimentin, and α smooth muscle actin proteins in A549 cells stimulated with or without TGF-β1 and TNF-α. α-tubulin was used as the internal control. A549 cells were cultured as in Figure 1. N = 3 independent experiments. ** p <0.01; error bars represent SEM. Please click here to view a larger version of this figure.

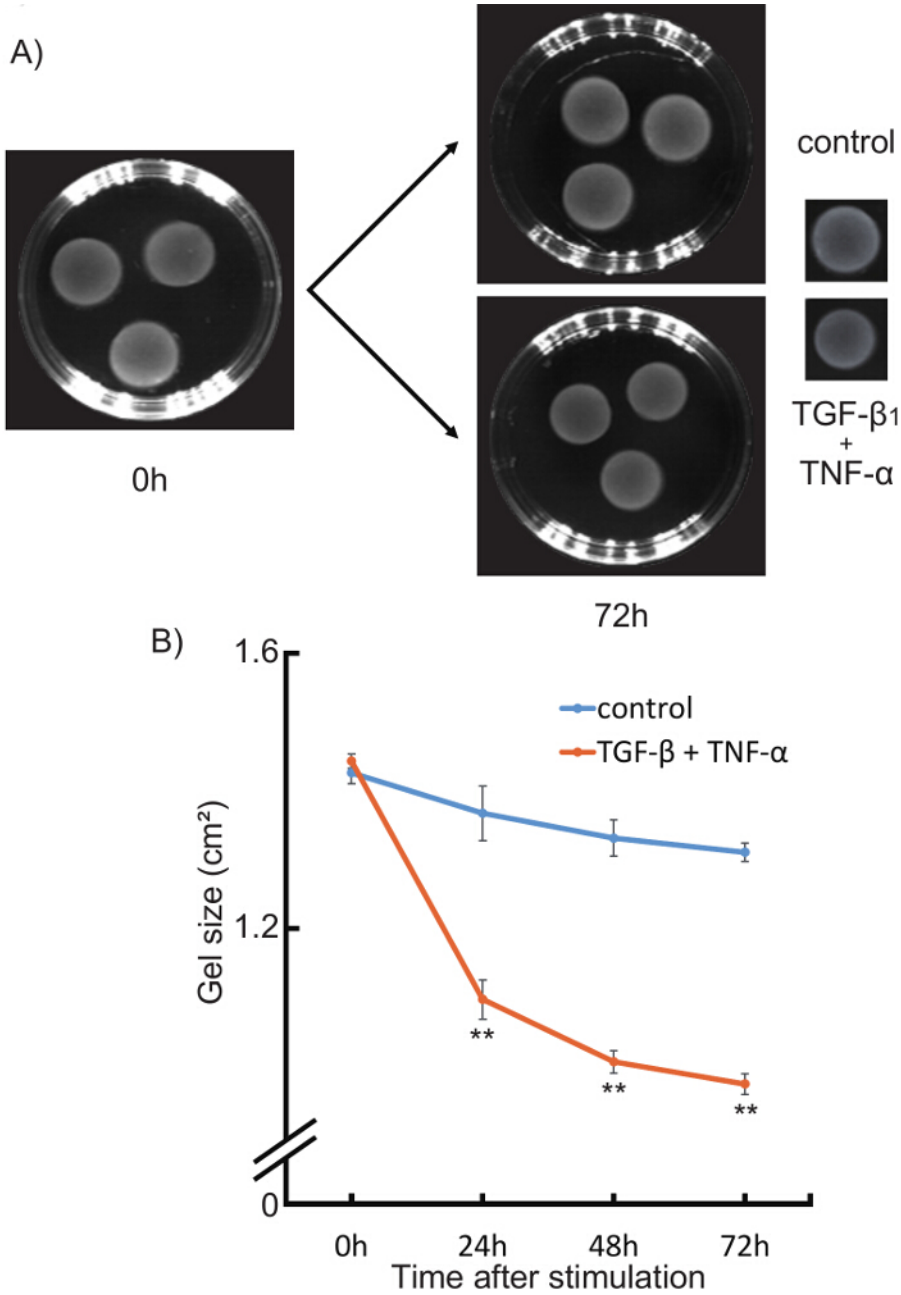

Figure 5. Cells Undergoing EMT Acquired Contractility. A549 cells were plated at a density of 1.0 x 10^6 cells/dish in 10 cm dishes and incubated with or without 5 ng/ml TGF-β1 and 10 ng/ml TNF-α for 48 hr. Cells were then cast into 500 µl of medium containing 1.75 mg/ml collagen gel at a density of 3.0 × 10^5 cells/well in a 24-well plate. After gel formation, they were added to medium with or without 5 ng/ml TGF-β1 and 10 ng/ml TNF-α in 60 mm dishes and incubated for 72 hr followed by imaging (**A**). Gel sizes were measured after 0, 24, 48, and 72 hr using an image analysis system. N = 9 independent experiments (**B**). ** p <0.01; error bars represent SEM. Please click here to view a larger version of this figure.

Gene Name	Forward 5'→3'	Reverse 5'→3'
ACTA2	GCACCCAGCACCATGAAGA	ACCGATCCAGACAGAGTATTT
GAPDH	GGTGAAGGTCGGAGTCAACGGA	GAGGGATCTCGCTCCTGGAAGA
VIM	GACAATGCGTCTCTGGCACGTCTT	TTCTTCTGCCTCCTGCAGGTTCTT
CDH1	CCCATCAGCTGCCCAGAAAATGAA	CTGTCACCTTCAGCCATCCTGTTT

Table 1. Primer Sequences. Primer sequences used for qRT-PCR. GAPDH was used as internal control.

Lysis Buffer: RIPA Buffer	
20 mM Tris-HCl pH 7.5	
150 mM NaCl	
1 mM EDTA	
1% Polyoxyethylene (9) octyiphenyl ether	
0.1% Na-deoxycholate	
0.1% SDS	
Blocking Buffer	
1% Blocking reagent in wash buffer	
Wash buffer: TBST buffer	
NaCl	45 g
1 M Tris pH 7.4	50 ml
Polyoxyethylene (20) Sorbitan monolaurate	2.5 ml
Distill Water	add to 5 L
Total	5 L

Table 2. The Components of Buffers Used. The components of lysis, blocking buffer and wash buffer.

Discussion

The protocol developed in this study comprises two steps. The first step is performed to induce EMT, while the second step is the gel contraction assay. Since it is important to confirm that cells underwent EMT, step 2 provides an excellent complement to the morphological and gene expression changes. Previous studies showed that EMT of A549 cells was induced by TGF-β1 only[24]; however, as we have reported previously[10], TNF-α treatment enhances EMT and the acquisition of mesenchymal cell markers. It is thought that the mechanisms of TGF-β-mediated EMT is smad-dependent[25]. TNFα enhances TGF-β1-induced EMT that leads to enhancement of gel contraction. It has been reported that the mechanisms of TNFα for enhancement of TGF-β1-induced EMT are not only phosphorylation of smad2 linker region, but also MAPK signaling regulation[26]. Therefore, the protocol developed in this study included stimulation by both TGF-β1 and TNF-α.

The embedding of cells that underwent EMT into the type I collagen gel, and floating on the medium, are central aspects of this assay (step 4). Because the gels are soft and easily damaged, and have different dimensions on each side, extreme caution is required when applying the gels to the medium and measuring their sizes. If it is difficult to carry out this step, there is an alternative method to measure the gel sizes in the well without moving the gels into 60 mm dish[27,28]. There are several common problems, the first problem is that the collagen gels easily gelate at RT, so the gels often deform as mentioned in step 4.7. Therefore, the plate must be gently shaken after casting the gels into wells to make sure they take on a neat cylindrical form. The second problem is that if any air bubbles enter the gels during gelation, then the size of the gels will be uneven. Be careful not to allow any air bubbles to contaminate the gels. There are two main differences between original method and this method. The first differences is that the final concentration of collagen gels of original method is 0.75 mg/ml but that of this method is 1.75 mg/ml in order to allow the gels to contract more than they do with the original method. The second differences is that the original method uses serum free medium for gel floating medium but this method uses 1% FBS in the gel floating medium in order to keep cell viability and maintain cell contractility.

The gel contraction assay originally developed for fibroblasts is an ideal *in vitro* model for evaluating the contractility of cells that contribute to the process of wound healing and fibrosis [13]. The attachment of fibroblasts to type 1 collagen is supposed to produce mechanical tensions that consequently leads to a reduction in the size of the collagen gels. Additionally, this assay has recently been used as an *in vitro* model for studying the contraction of airway cells in inflammatory diseases, including bronchial asthma and COPD[29-31].

The gel contraction assay is comparable to other assays, such as cell migration assays, in that it does not require specific devices and exhibits high quantitative reproducibility. However, there are few reports applying the gel contraction assay for evaluating EMT[32]. In this study, gels containing cells that underwent EMT significantly decreased in size from 24 to 72 hr after gelation, indicating cell contraction. There are however limitations to this method. The first is that in this assay, the size change of the gels show the sum of contractility of all cells in the gels. However, it is difficult to evaluate single cell contractility in the gels using this assay. The second is that A549 cells is a cancer cell line, not normal human epithelial cells. Therefore, it is difficult to extrapolate our results directly to the pathogenesis of pulmonary fibrosis. However, A549 cells are widely used to study the functions of airway epithelial cells, and several studies yielded evidence of EMT by using A549 cells as a model of pulmonary fibrosis [33,34].

In summary, the type I collagen gels containing mesenchymal cells that underwent EMT were reduced in size, indicating contraction of those cells. Thus, the gel contraction assay should be considered as an extended *in vitro* assay to evaluate acquisition of contractile function in the cells through EMT process.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgements

We thank Dr. Tadashi Koyama for technical help. This work was supported in part by JSPS KAKENHI Grant Numbers 23249045, 15K09211, 15K19172; a grant to the Respiratory Failure Research Group from the Ministry of Health, Labour and Welfare, Japan; a grant for research on allergic disease and immunology, Japan.

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