

IL-4 induces differentiation of human embryonic stem cells into fibrogenic fibroblast-like cells

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Background: Fibroblast heterogeneity is recognized, and fibroblasts from diseased tissues, including those of asthmatic subjects, have functional phenotypes that differ from normal tissue. However, progenitor-progeny relationships and the factors that control fibroblast differentiation are poorly defined.

Objective: We sought to determine whether IL-4 could alter the functional phenotype of fibroblasts during their differentiation from stem/progenitor cells.

Methods: Using a 3-dimensional collagen gel system, we obtained embryoid bodies derived from human embryonic stem cells and recovered spindle-shaped cells consistent with fibroblasts that had differentiated in the presence or absence of IL-4.

Results: IL-4-induced fibroblast-like cells were more active in contraction of collagen gels, migration, and production of fibronectin than control (without IL-4) cells. IL-4-induced cells demonstrated less expression of miR-155, which modulated contraction, migration, and fibronectin production. These differences persisted in culture without further addition of IL-4, suggesting the differentiated phenotype might be a permanent alteration.

Conclusion: The current study demonstrates that IL-4 induces differentiation of stem/precursor cells into fibroblast-like cells that demonstrate a more fibrogenic phenotype, which is due to reduced expression of miR-155. These findings provide a novel mechanism for the persistent abnormalities in IL-4-related diseases and a novel target to regulate tissue remodeling by fibroblasts. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: Embryonic stem cells, fibroblasts, IL-4, fibrosis, asthma, microRNA, miR-155, chemotaxis, collagen gel contraction, TGF- β

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Fibroblasts are ubiquitous mesenchymal cells that are believed to be responsible for the production and maintenance of extracellular matrix.^{1,2} They are also potent sources of growth factors and can modulate the proliferation of epithelial and endothelial cells. In addition, fibroblasts respond vigorously to a variety of mediators that are released at sites of injury.^{3,4} Thus fibroblasts are thought to mediate repair directly by their actions on matrix and indirectly by their actions on other structural cells. Moreover, fibroblast heterogeneity has been described in several fibrotic disorders.⁵⁻⁷ Similarly, a number of studies have demonstrated altered phenotypes of fibroblasts in airway diseases, including asthma⁸⁻¹⁰ and chronic obstructive pulmonary disease.¹¹⁻¹⁴ Although their heterogeneity is widely recognized, the source or sources of fibroblasts and the genesis of the various subpopulations characterized by distinct phenotypes remain undefined. Potential sources are thought to be expansion of locally resident fibroblasts, differentiation from a local stem/precursor population, and recruitment from circulating precursor populations.¹⁵⁻¹⁸ Importantly, the dynamic nature of the fibroblast population supports the concept that these cells can be targeted therapeutically.

Abbreviations used

α -SMA:	α Smooth muscle actin
bFGF:	Basic fibroblast growth factor
DMEM:	Dulbecco modified Eagle medium
EB:	Embryoid body
FITC:	Fluorescein isothiocyanate stain–green immunofluorescence
hESC:	Human embryonic stem cell
miRNA:	MicroRNA
PG:	Prostaglandin

We have established a reliable methodology that permits *in vitro* differentiation of fibroblast-like cells derived from human embryonic stem cells (hESCs). The current study was designed to determine whether a specific cytokine could induce an altered phenotype of fibroblast-like cells derived from stem cells in this system. In the current study we used IL-4, which is a profibrotic cytokine that is characteristically present in the airways in asthmatic subjects, and demonstrated that IL-4 induces differentiation of hESCs into fibroblast-like cells with a more fibrogenic phenotype and that this is due to reduced expression of the microRNA (miRNA) miR-155. This provides a novel target to regulate tissue remodeling by fibroblasts and a mechanism for asthma persistence despite anti-inflammatory therapy and long-term quiescence.

METHODS**Materials**

Native type I collagen (rat-tail tendon collagen) was extracted from rat-tail tendons by using a previously published method.¹⁹ Details relating to commercially available reagents are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

hESC culture and differentiation in 3-dimensional type I collagen gels

At the time of this study, the National Institutes of Health–approved and University of Nebraska Medical Center Embryonic Stem Cell Research Oversight and Institutional Review Board–approved hESC line H9.2 (WiCell Research Institute, Madison, Wis) was used. Undifferentiated hESCs were cultured on irradiated murine embryonic fibroblasts in 6-well plates with hESC culture medium containing 80% Dulbecco modified Eagle medium (DMEM)/F12, 20% KnockOut serum replacement, 1% nonessential amino acid, 1 mmol/L L-glutamine, 0.1 mmol/L 2-mercaptoethanol, and 4 ng/mL basic fibroblast growth factor (bFGF, Invitrogen, Carlsbad, Calif). Colonies were mechanically dissected with finely pulled glass micropipettes (1.0 mm OD; Clark Electromedical Instruments, Reading, United Kingdom) every 7 days and transferred to a freshly prepared mouse embryonic fibroblast layer.

To prepare embryoid bodies (EBs), hESCs were treated with 1 mg/mL collagenase, and cells were collected by means of centrifugation at 200g for 2 minutes. The pellet was resuspended in differentiation medium containing 90% DMEM/F12, 10% serum replacement, 1% nonessential amino acid, and 1 mmol/L L-glutamine without 2-mercaptoethanol and bFGF.²⁰ Cells were then placed in a bacteriological Petri dish (Sarstedt, Nümbrecht, Germany) and cultured for 4 to 5 days. Floating EBs were collected in a 50-mL polypropylene conical tube (Falcon; Beckton-Dickinson Labware, Franklin Lakes, NJ) and precipitated without centrifugation.

Collagen gels containing EBs were prepared as described previously.²¹ The cultured gels into which differentiated fibroblasts had migrated were dissolved with 1 mg/mL collagenase at 37°C in a 5% CO₂ atmosphere for 1 hour. The resulting cells were resuspended with DMEM containing 10% FBS (10% FBS-DMEM) and centrifuged at 200g for 5 minutes. The cells, including the EBs, were cultured in a 100-mm tissue-culture dish (Falcon) with 10% FBS-DMEM, 45 U/mL penicillin, 45 μ g/mL streptomycin, and

1 μ g/mL amphotericin B. When the fibroblasts, which grew as a monolayer, were near confluent, the cells were trypsinized and passaged in 10% FBS-DMEM. Contaminating EBs were lost with serial feeding.

Immunocytochemistry

Differentiated fibroblast-like cells were cultured until subconfluence in 8-chamber slides (Nunc, Inc, Naperville, Ill) in 10% FBS-DMEM and fixed in 4% paraformaldehyde for 30 minutes. Cells were washed in PBS before permeabilization and after each subsequent step. Blocking was performed with horse serum for 1 hour. Permeabilization was performed in 0.1% sodium citrate buffer with 0.1% Triton at 4°C for 5 minutes. Cells were incubated sequentially with monoclonal anti-vimentin (1:200 dilution), anti- α -smooth muscle actin (α -SMA; 1:50 dilution), anti-pancytokeratin (1:200), or anti-desmin (1:20) antibodies at 4°C overnight. Cells were then incubated with fluorescein isothiocyanate stain–green immunofluorescence (FITC)–conjugated anti-mouse IgG antibody and stained with propidium iodide after washing. Stained cells were visualized and photographed with a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan).

Collagen gel contraction assay

Collagen gel contraction assays were performed as described previously.²¹

Chemotaxis assay

Cell migration was assessed by using the Boyden blindwell chamber (Neuro Probe, Inc, Gaithersburg, Md), as previously described.²²

Proliferation assay

Cells were transferred to 12-well plates (2×10^4 cells/well) in 10% FBS-DMEM and cultured. Cell numbers from 3 separate wells were determined by using a Coulter electronic cell counter (Beckman Coulter, Inc, Fullerton, Calif).

Measurement of TGF- β 1, fibronectin, and prostaglandin E₂

Cells were cultured until subconfluent to evaluate mediator production. Cells were then treated with or without TGF- β 1 (100 pmol/L) in serum-free DMEM for 48 hours. Quantification of TGF- β 1 was performed by using an ELISA.²¹ Fibronectin was assayed with an inhibition ELISA that specifically detects human, but not bovine, fibronectin.²³ Prostaglandin (PG) E₂ production was determined by using an enzyme immunoassay (Cayman Chemical, Ann Arbor, Mich), according to the manufacturer's instructions.

Western blot analysis

α -SMA was quantified by means of Western blotting, as previously described.²⁴

miRNA preparation and microarray assay

miRNAs were extracted with the *mir*Vana miRNA Isolation Kit (Ambion; Applied Biosystems, Austin, Tex), according to the manufacturer's instructions. miRNA expression was profiled by using miRCURY LNA microRNA Array (version 11.0; Exiqon, Vedbaek, Denmark), according to the manufacturer's instructions. Raw data were analyzed with GenePix Pro 6.1 (Molecular Devices, Sunnyvale, Calif).

Real-time PCR

Real-time PCR for miR-155 quantification was performed with commercially available reagents. For details, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Transfection of miRNA mimic or inhibitor

miRNA mimic and inhibitor were transfected into cells with lipofectamine. For details, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Wound-closure assay

In vitro wound closure was performed and quantified as previously described.²⁵ The transfection was conducted on the second day of culture to explore the effect of miR-155 mimic or inhibitor on this assay.

Statistical analysis

Data are expressed as means $\pm$ SDs. All data were analyzed with GraphPad Prism 4 (GraphPad Software, Inc, San Diego, Calif). ANOVA was performed with the use of the nonparametric Kruskal-Wallis test. When applicable, the Mann-Whitney *U* test was used for comparisons between groups. For all comparisons, 3 different batches of control and IL-4-induced cells were evaluated, and significance was determined by using separate experiments performed on different occasions. A *P* value of less than .05 was considered significant.

RESULTS

IL-4-induced fibroblast-like cells demonstrate a fibrogenic phenotype

By using 3-dimensional type I collagen gel culture, fibroblast-like spindle-shaped cells were differentiated from hESCs. The presence of IL-4 during the differentiation did not visibly affect the outgrowth of fibroblast-like cells (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). When subsequently cultured in monolayers, fibroblast-like cells obtained both in the presence and absence of IL-4-induced cells demonstrated a characteristic spindle-shaped morphology (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Immunocytochemistry was performed with several cytoskeletal markers to evaluate the phenotypes of the fibroblast-like cells derived from hESCs. Both IL-4-induced and spontaneously differentiated (control) cells were entirely positive when stained for vimentin ([Fig 1, A and B](#)) but completely negative for cytokeratin ([Fig 1, C and D](#)) and desmin ([Fig 1, E and F](#)). Expression of α -SMA was less extensive in either cell type compared with expression of vimentin. However, IL-4-induced cells showed higher intensity of α -SMA staining compared with control cells ([Fig 1, G and H](#)). Expression of Thy-1 (CD90) was also less extensive compared with expression of vimentin, and no difference between control and IL-4-induced cells was observed (see [Fig E3, A and B](#), in this article's Online Repository at www.jacionline.org). Similarly, both control and IL-4-induced cells strongly expressed collagen type I (see [Fig E3, C and D](#)) but showed negative results on immunocytochemistry for CD34, CD45, and CD11b (see [Fig E3, E-J](#)).

Western blot analysis was performed to further confirm the difference of α -SMA expression between control and IL-4-induced fibroblast-like cells. Both control and IL-4-induced cells demonstrated significantly higher α -SMA expression than the undifferentiated hESCs and EBs ($P = .02$ each; [Fig 2](#)). IL-4-induced cells showed α -SMA expression to a greater degree compared with control cells (277% of control values, $P = .008$). After TGF- β 1 treatment, α -SMA expression increased in control fibroblast-like cells but not in IL-4-induced cells.

The expression of α -SMA is increased in fibroblasts present in areas of fibrosis.²⁶ This suggested that the increased expression of α -SMA in fibroblasts that had differentiated in the presence of IL-4 could be a feature of a distinct functional phenotype. To

further evaluate this possibility, we next performed several functional assays associated with repair and fibrosis, including contraction of 3-dimensional collagen gels, chemotaxis, wound closure, and proliferation. IL-4-induced cells were more active in contracting collagen gels and in chemotaxis than control cells (128% and 152% of control values, $P = .02$ and .009, respectively; [Fig 3, A and B](#)). Exogenous TGF- β 1 significantly stimulated contractile and chemotactic activities in control cells, although not in IL-4-induced cells. The wound-closure assay also indicated that IL-4-induced cells migrated significantly more in the absence of TGF- β 1 treatment ($P = .002$; [Fig 3, C](#), and see [Fig E4](#) in this article's Online Repository at www.jacionline.org). Control cells, but not IL-4-induced cells, clearly showed enhanced migration when treated with exogenous TGF- β 1. With regard to proliferation, IL-4-induced cells showed a 1.3-fold (day 3) and 1.2-fold (day 5) increase in cell numbers, but these differences did not differ significantly from control cells ([Fig 3, D](#)).

To further define the functional differences between control and IL-4-induced cells, we evaluated several secreted factors (ie, fibronectin, PGE₂, and TGF- β 1) that are believed to differ in cells from fibrotic compared with control tissues. IL-4-induced cells produced more fibronectin (260% of control values, $P = .002$; [Fig 4, A](#)) and, in contrast, less PGE₂ (35% of control values, $P = .002$; [Fig 4, B](#)). Control cells showed trends toward greater increases in both fibronectin and PGE₂ production after TGF- β 1 stimulation ($P = .13$ each), whereas there was no response to TGF- β 1 stimulation in IL-4-induced cells. IL-4-induced cells also demonstrated a trend toward a greater release of endogenous TGF- β 1, but this was not statistically significant ($P = .11$; [Fig 4, C](#)).

miR-155 modulates differentiated fibroblast-like cell phenotype

Because miRNAs are known to play a role in development and cell differentiation, we suspected that altered miRNA expression induced by IL-4 might contribute to the altered phenotype of the fibroblast-like cells derived from hESCs. Therefore we used microarray analysis to assess the miRNA profile of these cells using 2 different batches each of control and IL-4-induced cells. These 2 comparisons of miRNA profile indicated that miR-155 was underexpressed in IL-4-induced cells compared with control cells, and thus miR-155 was considered to be a candidate for differential modulation of the differentiated fibroblast-like cell phenotype (see [Fig E5](#) in this article's Online Repository at www.jacionline.org).

To further confirm the different expression of miR-155 in the 2 cell types, we next quantified miR-155 expression in undifferentiated hESCs, EBs, and differentiated fibroblast-like cells by using real-time PCR. Undifferentiated hESCs and EBs had essentially undetectable miR-155 ([Fig 5](#)). In contrast, miR-155 was readily detected in differentiated fibroblasts. Importantly, IL-4-induced cells expressed miR-155 levels that were 17-fold lower ($P < .002$) compared with levels seen in control cells. In addition, TGF- β 1 significantly reduced the expression of miR-155 in control cells but had no effect on the IL-4-induced cells.

To confirm a mechanistic role for miR-155 in the altered phenotype of IL-4-induced cells, we transfected control and IL-4-induced cells with miR-155 mimic or inhibitor. In control cells, which showed a high level of miR-155 expression,

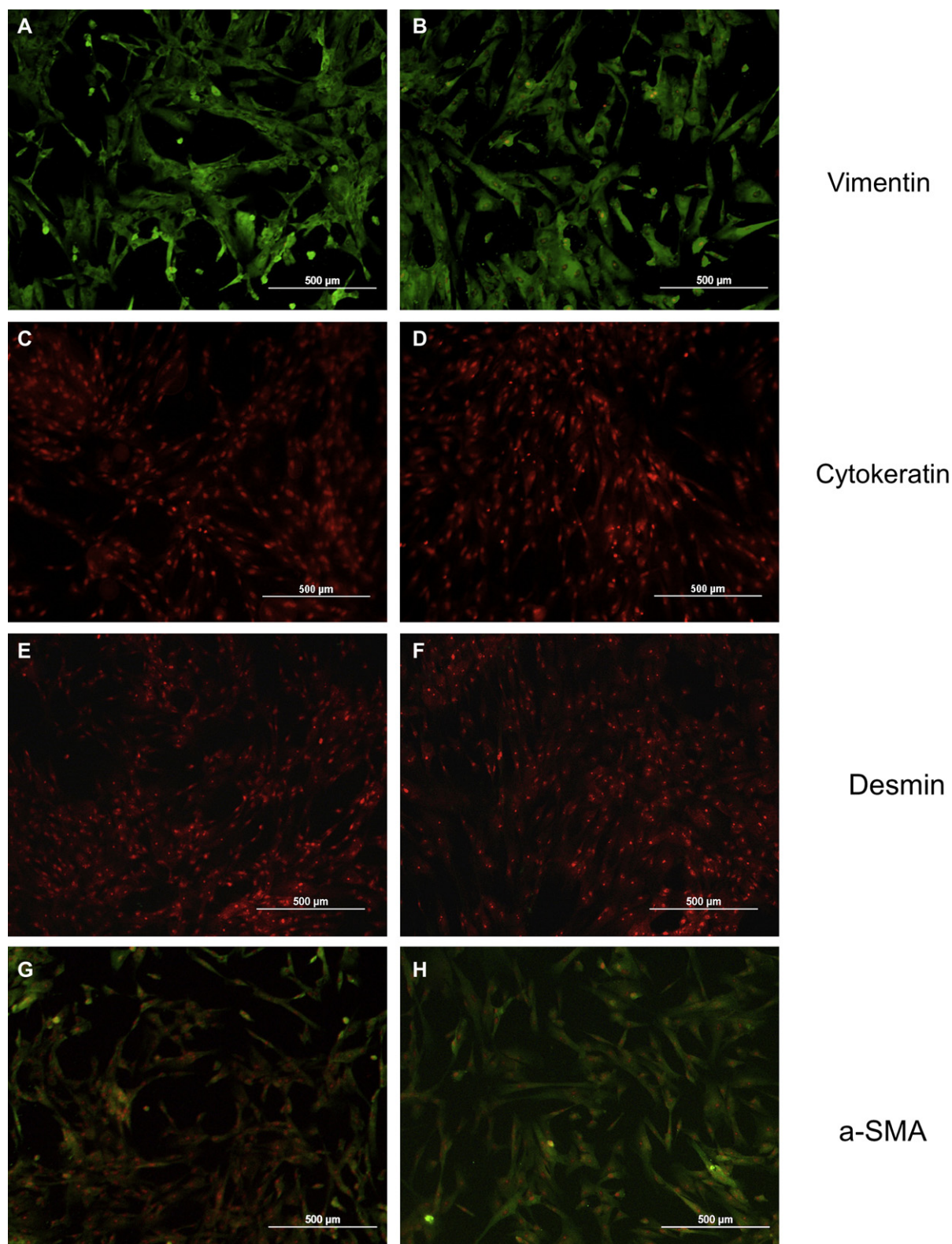


FIG 1. Representative immunocytochemistry of differentiated fibroblast-like cells. **A**, Vimentin in control cells. **B**, Vimentin in IL-4-induced cells. **C**, Cytokeratin in control cells. **D**, Cytokeratin in IL-4-induced cells. **E**, Desmin in control cells. **F**, Desmin in IL-4-induced cells. **G**, α -SMA in control cells. **H**, α -SMA in IL-4-induced cells.

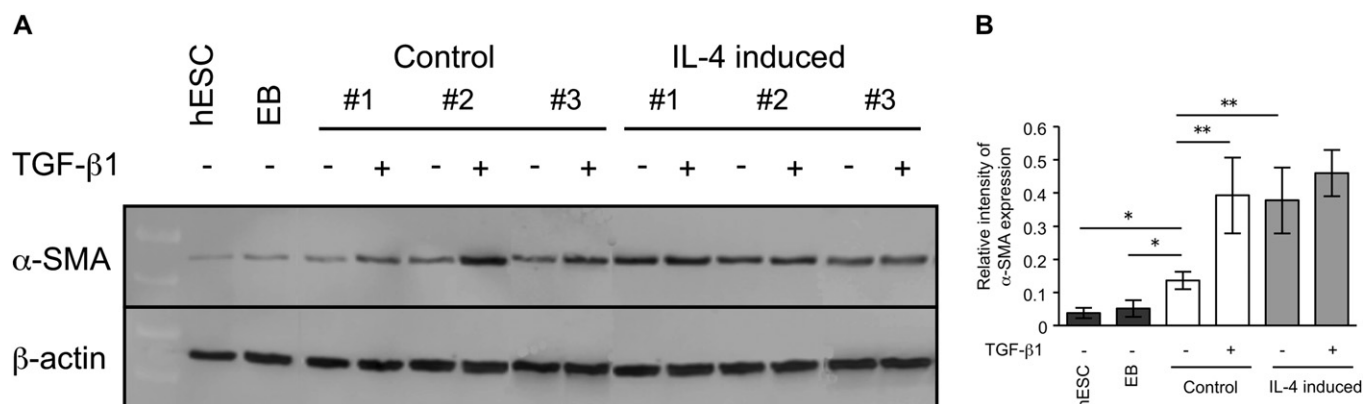


FIG 2. α-SMA expression in undifferentiated hESCs, EBs, and differentiated fibroblast-like cells. **A**, Representative Western blots for α-SMA. β-Actin expression is shown as a loading control. **B**, Densitometric analysis of α-SMA expression relative to the amount of β-actin. Three different batches of control and IL-4-induced cells (indicated by #1, #2, and #3 in Fig 2, A), each prepared from hESCs on separate occasions, were each evaluated 3 times. Values are presented as means ± SDs. * $P < .05$, ** $P < .01$.

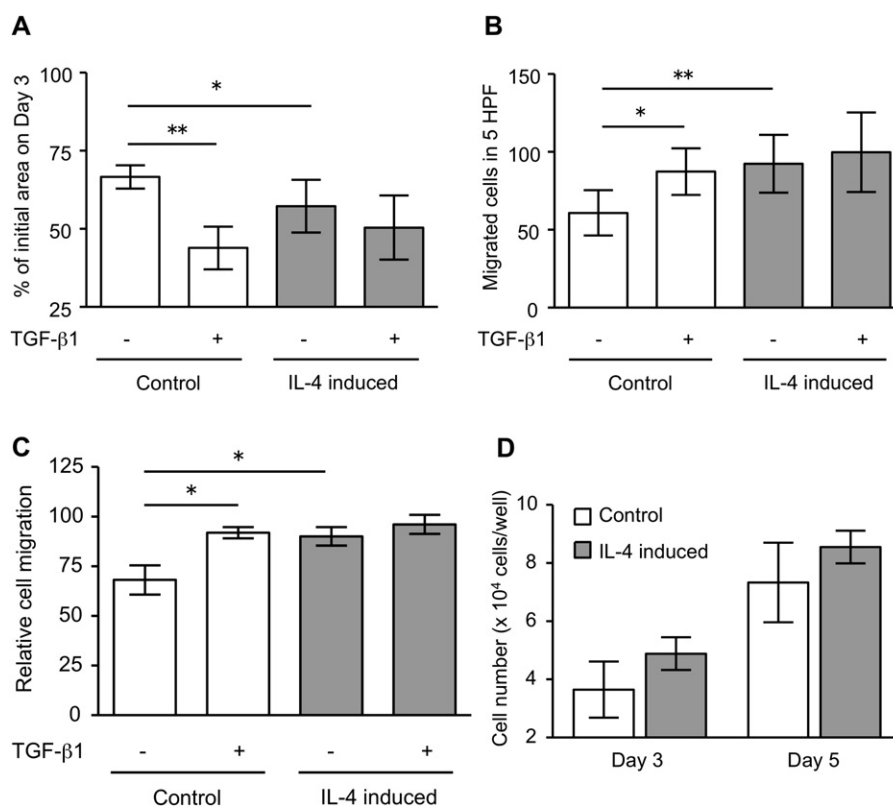


FIG 3. Physiological differences between control and IL-4-induced fibroblast-like cells derived from hESCs. **A**, Collagen gel contraction. The size of the gels was measured on day 3 and shown as the percentage of initial area. **B**, Chemotaxis toward fibronectin. Chemotaxis was assessed by counting the number of migrated cells in 5 high-power fields (HPF). **C**, Migration of fibroblast-like cells in the wound-closure assay. Cell migration is expressed as a percentage of the cell occupied area in the initial wound area. **D**, Cell proliferation. Cells were plated at a density of 2×10^4 cells/well and cultured. Cell numbers were determined on days 3 and 5 by using an electronic cell counter. For all experiments, 3 different batches of control and IL-4-induced cells were evaluated on 3 separate occasions, each performed in triplicate. Values are presented as means ± SDs. * $P < .05$, ** $P < .01$.

an miR-155 inhibitor enhanced collagen gel contraction ($P = .13$; Fig 6, A), chemotaxis ($P = .002$; Fig 6, B), wound closure ($P = .02$; Fig 6, C, and see Fig E6 in this article's Online Repository at www.jacionline.org), and fibronectin production ($P = .002$;

Fig 6, D). In contrast, an miR-155 mimic inhibited contraction ($P = .002$), chemotaxis ($P = .002$), wound closure ($P = .008$), and fibronectin production ($P = .002$) in IL-4-induced cells, which demonstrated low miR-155 expression. Neither cell type

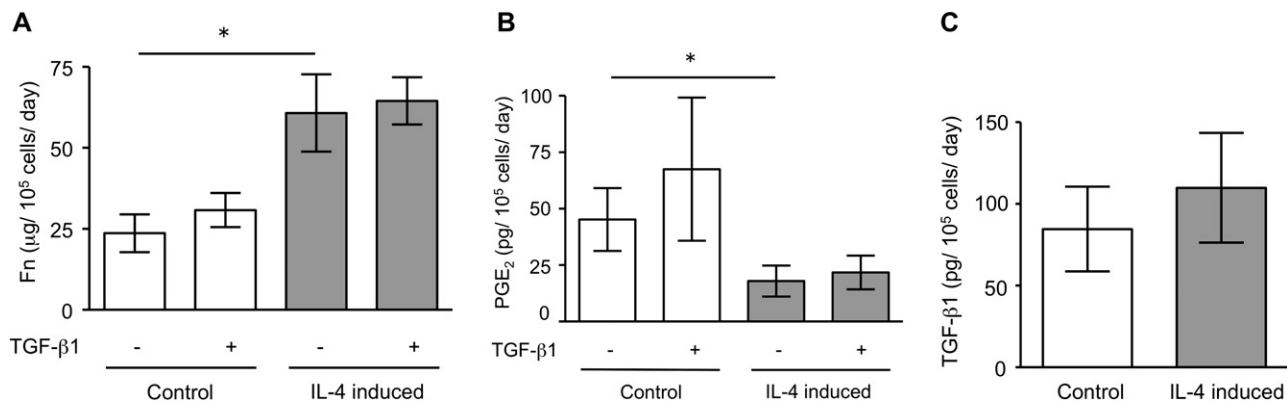


FIG 4. Production of biomarkers from control and IL-4-induced fibroblast-like cells derived from hESCs. **A**, Fibronectin production. **B**, PGE₂ production. **C**, TGF-β1 production. For the measurement of TGF-β1, only media without TGF-β1 were used. Three different batches of control and IL-4-induced cells were evaluated on 3 separate occasions, each performed in triplicate. Values are presented as means ± SDs. **P* < .01.

demonstrated significant changes in PGE₂ or TGF-β1 production or α-SMA expression in response to miR-155 (see Fig E7 in this article's Online Repository at www.jacionline.org). Also, miR-155 had no effect on the response to exogenous TGF-β1 in either type of differentiated fibroblast-like cell.

DISCUSSION

The present study demonstrates that in a 3-dimensional collagen gel culture system in which fibroblast-like cells differentiate from hESC-derived EBs, the presence of IL-4 during the differentiation process resulted in cells with an altered phenotype. Several functional and biochemical assays indicated that IL-4 induces differentiation of cells with a more fibrogenic phenotype compared with control cells, which are differentiated in collagen gels without cytokines. Furthermore, the expression of miRNA miR-155 is markedly reduced in cells that differentiated in the presence of IL-4. In support of a mechanistic role for reduced miR-155 expression in the altered functional phenotype, the introduction of an miR-155 mimic to IL-4-induced cells reversed several of the fibrogenic phenotypes toward the control phenotype. Conversely, an miR-155 inhibitor induced control cells to more closely resemble IL-4-induced cells. Taken together, these results suggest that IL-4 alters the differentiation of fibroblast-like cells from hESCs and that this results in reduced expression of miR-155, which, in turn, causes the cells to demonstrate features that resemble cells from fibrotic tissues.

Fibroblasts are heterogeneous mesenchymal cells that play important roles in the production and maintenance of extracellular matrix and regulate other structural cells.^{1,2,27} These cells are routinely cultured as adherent cells and demonstrate a characteristic spindle-shaped morphology. However, although fibroblast heterogeneity is clearly recognized, there are no specific markers to distinguish distinct subpopulations of fibroblasts. In the current study we demonstrated that the differentiated cells stained positively for vimentin, a nonspecific mesenchymal marker, and failed to stain both for cytokeratin, an epithelial marker, and desmin, a marker of muscle cells. Both control and IL-4-induced cells showed positive expression of α-SMA, which is often used as a marker of myofibroblasts. However, α-SMA fails to distinguish between myofibroblasts and smooth muscle cells.¹⁵ Although desmin is a marker of smooth muscle cells, it can be

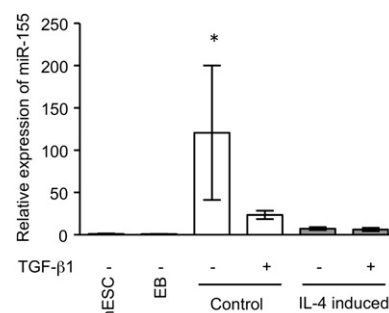


FIG 5. miR-155 expression in undifferentiated hESCs, EBs, and differentiated fibroblast-like cells. Cell layers of differentiated cells were extracted after 48 hours of treatment with or without TGF-β1 (100 pmol/L). miR-155 expression determined by means of real-time PCR is normalized to the amount of ribosomal RNA and expressed as $2^{-\Delta\Delta CT}$ values. **P* < .01 compared with other groups.

lost after smooth muscle cells are maintained in culture.²⁸ Thy-1 (CD90) is an outer-membrane leaflet glycoprotein that is expressed on subsets of neural cells, lymphocytes, and fibroblasts.²⁹ The current study showed positive staining for Thy-1 on both control and IL-4-induced fibroblast-like cells. Most normal lung fibroblasts show positive expression of Thy-1, whereas the majority of myofibroblasts from fibrotic sites are negative.³⁰ Similarly, fibroblasts are positive for collagen type I, as were both control and IL-4-induced cells. Fibrocytes, which are thought to be a circulating precursor of fibroblasts, are also positive for collagen I, CD34, CD45, and CD11b, although they are typically negative for α-SMA and Thy-1.³¹ Hence the markers used identify our cells as mesenchymal cells and indicate that they differ from both myofibroblasts and fibrocytes. However, the use of surface markers to identify subpopulations of mesenchymal cells is not fully developed, and we have opted to refer to the spindle-shaped differentiated cells as fibroblast-like cells.

We believe several other observations support our conservative approach to identifying our mesenchymal cells. First, in contrast to staining for vimentin, desmin, and keratin, the immunohistochemical staining of our fibroblast-like cells was not uniform for Thy-1, CD34, CD45, and CD11b, suggesting that the cell population that results from the differentiation process might be heterogeneous. Furthermore, *in vivo* it is likely that many

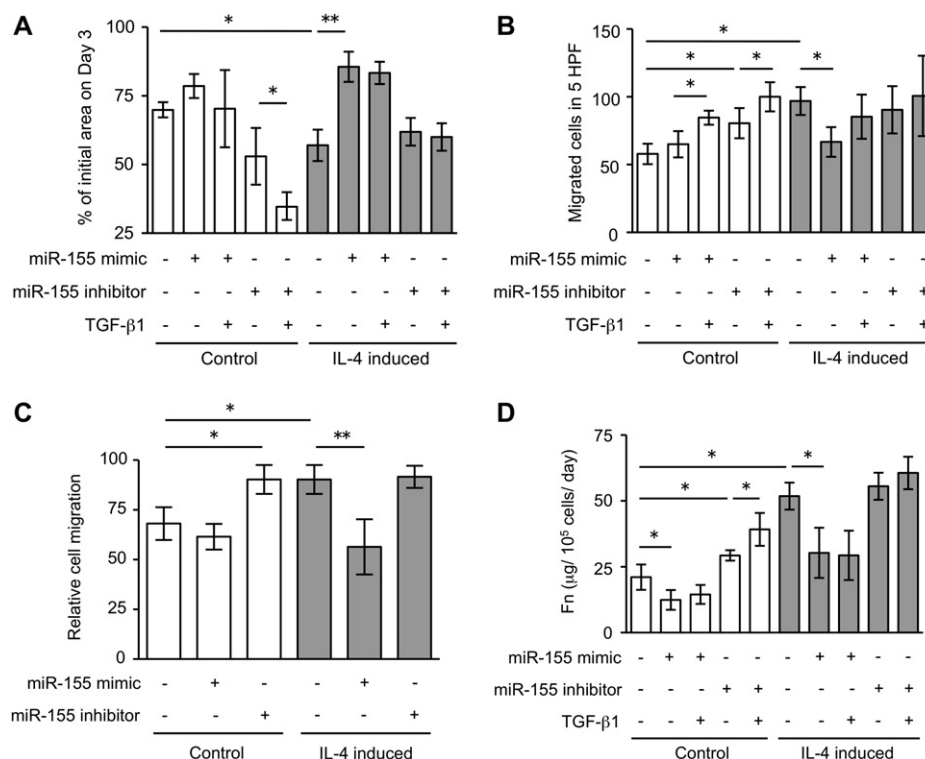


FIG 6. miR-155 regulates the differentiated fibroblast-like cell phenotype. **A**, Collagen gel contraction on day 3. **B**, Chemotaxis toward fibronectin. **C**, Cell migration determined by means of wound-closure assay. **D**, Fibronectin production. Three different batches of control and IL-4-induced cells were evaluated on 3 separate occasions in all experiments. Values are presented as means $\pm$ SDs. * $P < .05$, ** $P < .01$.

cytokines and growth factors contribute to the differentiation process, and thus it is unlikely that the differentiation of our cells exactly recapitulates *in vivo* differentiation. Nevertheless, the key finding reported in the current article is that by using the 3-dimensional culture system, we were able to demonstrate that the presence of the cytokine IL-4 can alter the final differentiated phenotype of the resulting fibroblast-like cells. We also have similar results with fibroblast-like cells from murine ESCs in the presence and absence of IL-4 (data not shown).

IL-4, a cytokine characteristically released by T_H2 cells, is known as a profibrotic cytokine. It can directly affect various functions of fibroblasts, including cytokine and adhesion molecule expression, extracellular matrix protein synthesis, and α -SMA expression.³²⁻³⁴ In a prior study we demonstrated that IL-4 also inhibits PGE₂ production. Because PGE₂ functions as an endogenous inhibitor of fibroblast repair functions, this is another mechanism by which IL-4 can augment fibroblast-mediated repair.³⁵ The current study extends these studies in an important and very distinct way: it shows that IL-4 might also result in differentiation of functionally distinct fibroblast populations from precursors. By using this mechanism, IL-4 could result in a persistent effect in contrast to the transient effects observed when IL-4 is added to differentiated fibroblast cultures.

The current study also demonstrates at least 1 mechanism by which IL-4 causes the fibrogenic phenotype. Specifically, IL-4-induced cells underexpress the miRNA miR-155. MiRNAs are a novel family of small, approximately 19- to 25-nucleotide, single-stranded noncoding RNAs. They are expressed in developmental stage-, tissue-, or cell type-specific manners or cause posttranscriptional gene repression by increasing mRNA

degradation or inhibiting translation.³⁶ This is achieved by direct binding between their 5' region and the 3' untranslated region of target mRNAs. miRNAs might also indirectly alter gene expression through epigenetic mechanisms.^{37,38} miRNAs have been associated with diverse biological processes, including development, cellular differentiation, and the pathogenesis of various diseases.

miR-155 represents as a typical multifunctional miRNA.³⁹ To date, evidence supports a role for miR-155 in hematopoiesis, inflammation, and immunity. Overexpression of miR-155 has been found to be associated with a variety of malignant tumors,⁴⁰ rheumatoid arthritis,⁴¹ cardiovascular diseases,^{42,43} and viral infections.⁴⁴⁻⁴⁶ A recent study demonstrated that mice deficient for miR-155 are immunodeficient and display increased lung airway remodeling.⁴⁷ Consistent with this, the current study shows that IL-4-induced cells, which expressed lower levels of miR-155, performed more robustly in *in vitro* models of tissue repair and remodeling, and importantly, an miR-155 mimic could, at least partially, modulate these functions toward control cells. Thus the current study also demonstrates that, in addition to its other suggested functions, miR-155 might be an important modulator of tissue repair and remodeling.

The current study does not define the specific targets of miR-155 in regulation of the fibroblast-like cell functions. Although in this study fibronectin production from the differentiated fibroblast-like cells was modulated by miR-155, no homology between miR-155 and the fibronectin gene was revealed by means of *in silico* assessment. A large number of predicted targets of miR-155 can be obtained from the online databases (991 from MicroCosm, 1985 from TargetScan, and 199

from PicTar), and many could interact either directly or indirectly with the functions assessed. However, not all *in silico* predicted targets have been found to be responsive to the predicted miRNA when tested experimentally.⁴⁸ In this context a limited number of target genes have been experimentally validated for direct binding to miR-155.³⁹ It has been reported that angiotensin II type 1 receptor is regulated by miR-155 in human lung fibroblasts and that TGF- β 1 treatment resulted in the decreased expression of miR-155 and the increased expression of angiotensin II type 1 receptor.⁴² Consistent with this, the current study demonstrated reduced expression of miR-155 by exogenous TGF- β 1 in control fibroblast-like cells. Because angiotensin II has been associated with fibrotic vascular remodeling, this study is also consistent with a profibrotic role for reduced miR-155 expression. In contrast, Pottier et al⁴⁹ have reported that miR-155 is upregulated in a murine model of lung fibrosis and that it increases human fibroblast motility and targets keratinocyte growth factor directly. Similarly, Kong et al⁵⁰ demonstrated increased expression of MiR-155 in response to TGF- β in a murine mammary epithelial cell line. To what degree these results depend on species, tissue of origin, and model systems is unknown. Thus additional investigation will be required to define the specific role of miR-155 in tissue fibrosis.

The current study also demonstrates that IL-4-induced fibroblast-like cells have diminished responses to TGF- β 1. TGF- β 1 is a widely recognized activator of fibroblasts, promoting myofibroblast development by inducing expression of α -SMA and extracellular matrix.^{51,52} TGF- β 1 is also known to stimulate fibroblast contraction and migration.^{53,54} In a prior study our group demonstrated that fibroblasts from patients with chronic obstructive pulmonary disease are also less responsive to TGF- β 1, which is due to the increased expression of Smad7, the inhibitory protein of the TGF- β -signaling pathway.¹³ However, we could not detect altered expression of Smad7 in control versus IL-4-induced fibroblast-like cells (data not shown). In the current study, however, the reduced response to exogenous TGF- β 1 in differentiated fibroblast-like cells was independent of miR-155. Thus the mechanism for the altered signaling among these 2 types of fibroblast-like cells remains undefined.

The current study evaluated differentiation of fibroblast-like cells from EBs derived from hESCs. When this occurred in the presence of the cytokine IL-4, cells with a more fibrotic phenotype resulted. There are no consensus criteria for distinct discrimination between fibroblasts and myofibroblasts. α -SMA is a well-known marker used to delineate myofibroblasts; however, some populations of fibroblasts are positive for α -SMA, and some myofibroblasts are negative. Because the current study showed that IL-4-induced cells had stronger α -SMA expression than control cells, they could have been described as myofibroblast-like cells. More importantly, these differences persisted in culture in the absence of IL-4, which is consistent with an altered differentiation, and this appears to be mediated, in part, by miR-155.

IL-4 is a mediator characteristically present in the airways in asthmatic subjects. In addition, fibroblasts cultured from the airways of asthmatic subjects have an altered, more fibrotic phenotype.⁸⁻¹⁰ The current study suggests that one mechanism for the accumulation of these functionally altered populations of cells is their differentiation in the presence of inflammatory cytokines. It is likely that many inflammatory cytokines in addition to IL-4 could alter the differentiation of fibroblasts. In

preliminary studies we evaluated several cytokines, such as TGF- β 1 and IL-13. All appeared to alter the differentiation of fibroblast-like cells in our system. Because the effects of IL-4 appeared to be relatively marked and consistent, we pursued IL-4 in detail. Thus our model system, which assessed only IL-4, is unlikely to recapitulate the entire milieu present in a disease such as asthma. Nevertheless, our study suggests that cytokine alteration of structural cell populations could play a role in asthma. Such a mechanism could account for the marked persistence of asthma despite anti-inflammatory therapy and long-term quiescence. We also believe our system has the potential to explore the effect of external factors on the cell development and differentiation.

We differentiated the cells from EBs but not ESCs directly. EBs, which form spontaneously from ESCs under appropriate culture conditions, have been used as precursor/stem cell sources for the differentiation of many cell types. By definition, ESCs are not present in the lung. However, there are clearly pluripotent precursors, as we and others have shown.⁵⁵⁻⁵⁸ A hierarchy of epithelial stem/precursor/progeny cells has been proposed,⁵⁹ and a similar hierarchy for mesenchymal cells seems likely. Whether the IL-4-induced differentiated cells characterized in the present report resemble a process that occurs in the lung or one that could affect a circulating source of mesenchymal cells, such as fibrocytes, remains to be determined.

In summary, the current study demonstrates that IL-4 induces differentiation of fibroblast-like cells that demonstrate a more fibrogenic phenotype, which was in part due to reduced expression of miR-155 compared with that seen in control cells. This demonstrates that distinct populations of fibroblast-like cells can originate if differentiation occurs in the presence of a cytokine and might represent a mechanism of disease development and maintenance.

We thank Lillian Richards for excellent secretarial support.

Key messages

- IL-4 induces the differentiation of fibroblasts with profibrotic features.
- The induction of miR-155 is one mechanism by which IL-4-induced differentiation results in the profibrotic phenotype.

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METHODS

Materials

Commercially available reagents were obtained as follows: recombinant human IL-4, TGF- β 1, monoclonal anti-TGF- β 1, and biotinylated anti-TGF- β 1 antibodies were from R&D Systems (Minneapolis, Minn); α -SMA antibody was from Abcam (Cambridge, Mass); monoclonal anti-pancytokeratin antibody, anti-vimentin antibody, anti-desman antibody, anti-mouse and anti-rabbit IgG FITC conjugate, anti-human fibronectin antibody, 3,3',5,5'-tetramethyl benzidine, propidium iodide, and 2-mercaptoethanol were from Sigma (St Louis, Mo); anti-Thy-1 antibody was from Santa Cruz Biotechnology (sc-9163; Santa Cruz, Calif); and CD11b, CD34, and CD45 antibodies were from BD PharMingen (San Diego, Calif). Anti-collagen type I was from Abcam. DMEM, FBS, penicillin-streptomycin, DMEM/F12 (1:1 mixture), KnockOut serum replacement, nonessential amino acids, L-glutamine, bFGF, collagenase type IV, 0.05% trypsin-EDTA, Trizol reagent, Opti-MEM, Lipofectamine 2000, horseradish peroxidase-conjugated streptavidin conjugate, and human fibronectin were from Invitrogen Life Technologies; Amphotericin B was from X-Gen Pharmaceuticals, Inc (Northport, NY); and Hsa-miRNA-155 mimic and inhibitor and meridian miRNA Mimic/Inhibitor Negative Control were from Dharmacon (Lafayette, Colo).

Preparation of collagen gels

Collagen gels were prepared as described previously.^{E1} Briefly, rat-tail tendon collagen, distilled water, and 4 $\times$ concentrated DMEM were combined so that the final mixture resulted in 0.75 mg/mL collagen, with a physiologic ionic strength of 1 $\times$ DMEM and a pH 7.4. EBs were then suspended in the neutralized collagen solution. Aliquots (1.0 mL/well) of the mixture of EBs in collagen were then cast into each well of a 12-well tissue-culture plate (Falcon) and allowed to gel. After gelation was completed, normally within 20 minutes at room temperature, basal media (1:1 mixture of differentiation media and DMEM/F12) with or without IL-4 (20 ng/mL) were added on the top of gels in a 12-well plate (1.0 mL/well). The basal medium was changed every 2 to 3 days, and EBs were cultured for 21 days in 3-dimensional type I collagen gels.

Immunocytochemistry

Differentiated fibroblast-like cells were cultured until subconfluence in 8-chamber slides (Nunc, Inc, Naperville, Ill) in 10% FBS-DMEM and fixed in 4% paraformaldehyde for 30 minutes. Cells were washed in PBS before permeabilization and after each subsequent step. Blocking was performed with horse serum for 1 hour. Permeabilization was performed in 0.1% sodium citrate buffer with 0.1% Triton at 4°C for 5 minutes. Cells were incubated sequentially with anti-Thy-1, anti-collagen type I, anti-CD34, anti-CD45, or anti-CD11b antibody at 4°C overnight. Cells were then incubated with FITC-conjugated anti-rabbit IgG or FITC-conjugated anti-rabbit IgG antibody and stained with propidium iodide after washing. Stained cells were visualized and photographed with a Nikon Eclipse TE300 microscope equipped with a DP71 digital camera (Olympus).

Collagen gel contraction assay

Collagen gel contraction assays were performed as described previously.^{E1} Fibroblasts were trypsinized and mixed with the neutralized collagen solution so that the final cell density in the collagen solution was 3 $\times$ 10⁵ cells/mL. Aliquots (0.5 mL/well) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon), and the mixture was allowed to gel. After gelation was completed, the gels were gently released from the 24-well tissue-culture plates and transferred into 60-mm tissue-culture dishes (3 gels in each dish), which contained 5 mL of freshly prepared serum-free DMEM with or without 100 pmol/L TGF- β 1. The gels were then incubated at 37°C in a 5% CO₂ atmosphere for 3 days. Gel contraction was quantified with an Optomax V image analyzer (Optomax, Burlington, Mass) daily. Data were expressed as a percentage of the initial gel size.

Chemotaxis assay

Cell migration was assessed by using the Boyden blindwell chamber (Neuron Probe, Inc, Gaithersburg, Md), as previously described.^{E2} Briefly,

26 μ L of serum-free DMEM containing human fibronectin (5 μ g/mL) was placed into the lower wells. Eight-micrometer-pore polycarbonate membranes (Neuron Probe, Inc) were used. Cells were trypsinized and suspended in serum-free DMEM at a density of 1 $\times$ 10⁶ cells/mL. Fifty microliters of cell suspension treated with TGF- β 1 was then added into each upper well. Cells were allowed to migrate at 37°C in a 5% CO₂ atmosphere for 12 hours. Cells that had not migrated were scraped off the upper surface of the membrane, and the membranes were air-dried. Cells were then stained with PROTOCOL (Fisher Scientific, Swedesboro, NJ) and mounted on a glass microscope slide. Chemotaxis was assessed by counting the number of cells in 5 high-power fields.

In vitro wound-closure assay

Before plating cells, the large end of a sterile pipette tip was placed in the central area of a 12-well tissue culture dish to prevent access of cells. After this, cells were gently seeded (10⁵ cells/well) in 10% FBS-DMEM and then cultured at 37°C until confluent. The wound was introduced in the central area of the confluent monolayer by gently removing the pipette tip. The cultures were then incubated in 1% FBS-DMEM to exclude an effect of proliferation with or without TGF- β 1 (100 pmol/L) for 48 hours, after which the cell layers were fixed and stained with PROTOCOL. Photomicrographs were taken with a Nikon Eclipse TE300 microscope equipped with an Olympus DP71 digital camera. Quantification of wound closure was performed by superimposing a rectangular grid on the digitized images and manually counting boxes that were free of cell nuclei, as previously described.^{E3} The transfection was conducted on the second day of culture, as described below, to explore the effect of miR-155 mimic or inhibitor on this assay.

Transfection of miRNA mimic or inhibitor

miRNA mimic and inhibitor were transfected into cells by using lipofectamine. Cells were plated into 6-well plates at a density of 1 $\times$ 10⁵ cells/well in 2 mL of 10% FBS-DMEM without antibiotics and amphotericin B. At 50% confluence, cells were transfected with miR-155 mimic or inhibitor. Lipofectamine 2000 (3.75 μ L) was mixed with 121.25 μ L of Opti-MEM medium and incubated for 5 minutes at room temperature to prepare the transfection solution. In a separate tube 20 μ mol/L miRNA mimic or inhibitor was mixed with 125 μ L of Opti-MEM. These 2 tubes were combined, gently mixed, and incubated for 20 minutes at room temperature. After incubation, Opti-MEM was added to obtain a final volume of 1 mL (final concentration of mimic or inhibitor, 50 nmol/L) for each well. Cells were washed with sterile PBS twice and incubated with transfection solution for 6 hours at 37°C. After 6 hours of transfection, cells were further cultured for 48 hours in 10% FBS-DMEM containing antibiotics and amphotericin B with or without TGF- β 1 (100 pmol/L).

Western blot analysis

α -SMA was quantified by means of Western blotting, as previously described.^{E4} Briefly, whole-cell lysates were prepared, and the total protein content of each was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif). After heating for 5 minutes at 95°C, 5 to 10 μ g of protein was loaded and separated by means of electrophoresis on 10% SDS-PAGE. After transfer of the proteins to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), the membrane was blocked with 5% milk in PBS and allowed to react with primary antibodies against α -SMA (1:200 dilution) at 4°C overnight. α -SMA proteins were subsequently detected and quantified with horseradish peroxidase-conjugated IgG (Rockland) with an enhanced chemiluminescence plus detection system (ECL plus) and Typhoon Scanner (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Real-time PCR

Total RNA was extracted with Trizol reagent. Real-time PCR for miR-155 quantification was conducted with the TaqMan MicroRNA Reverse Transcription Kit, TaqMan MicroRNA Assays, and the ABI Prism 7500 (Applied Biosystems), according to the manufacturer's instructions. As an internal control, the ribosomal RNA control kit (Applied Biosystems) was used.

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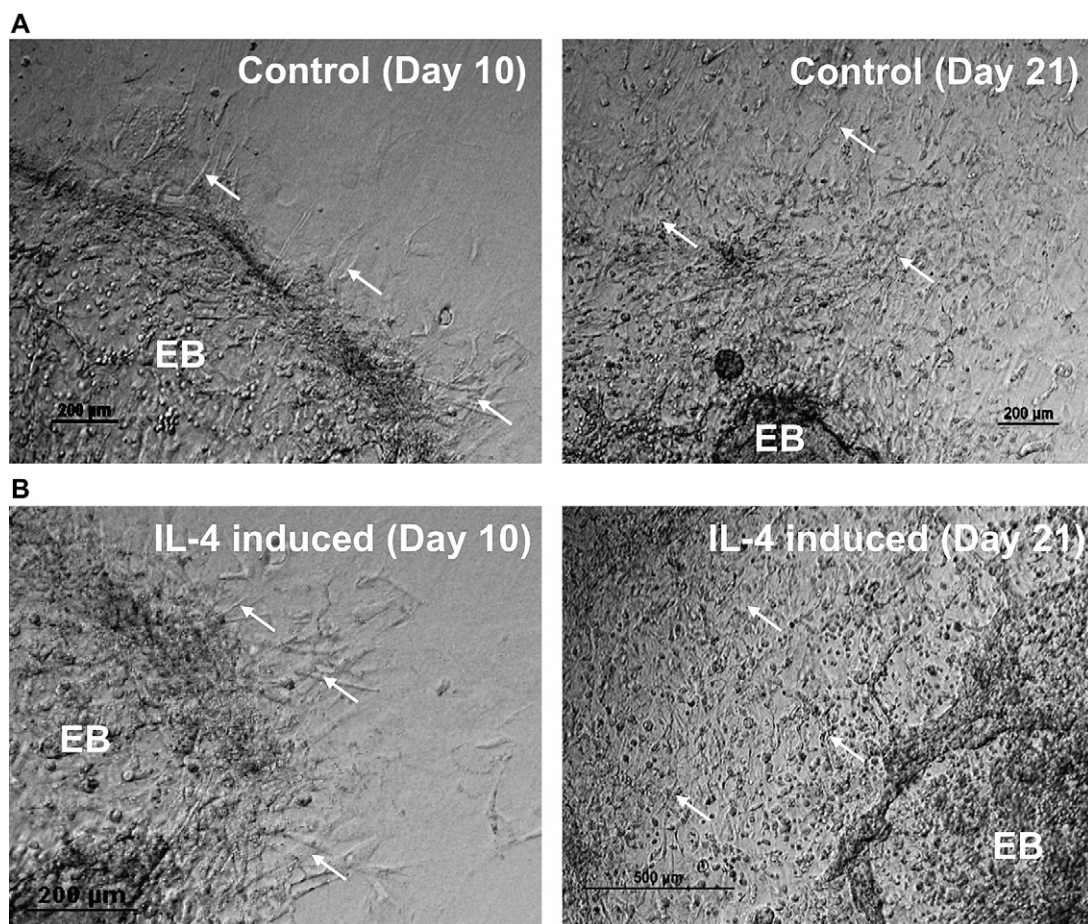


FIG E1. Morphology of EBs differentiating in 3-dimensional collagen gels. The differential EBs were cast into collagen gels and cultured in the absence (control; **A**) or presence (**B**) of IL-4. Spindle-shaped cells (*arrows*) start to appear surrounding EBs after 7 to 10 days of culture. Spindle-shaped cells increase in numbers and spread out around EBs by day 21. Images were taken on days 10 and 21.

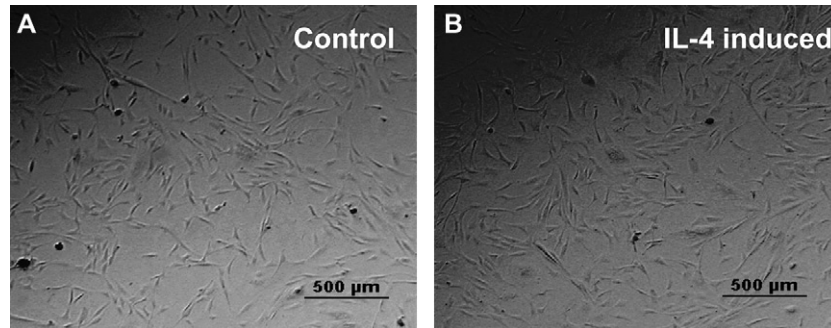


FIG E2. Morphology of differentiated fibroblast-like cells in monolayer culture. **A**, Control differentiated cells. **B**, IL-4-induced cells. The control and IL-4-induced cells released from collagen gels were cultured with 10% FBS-DMEM on monolayer. Images were taken at passage 4.

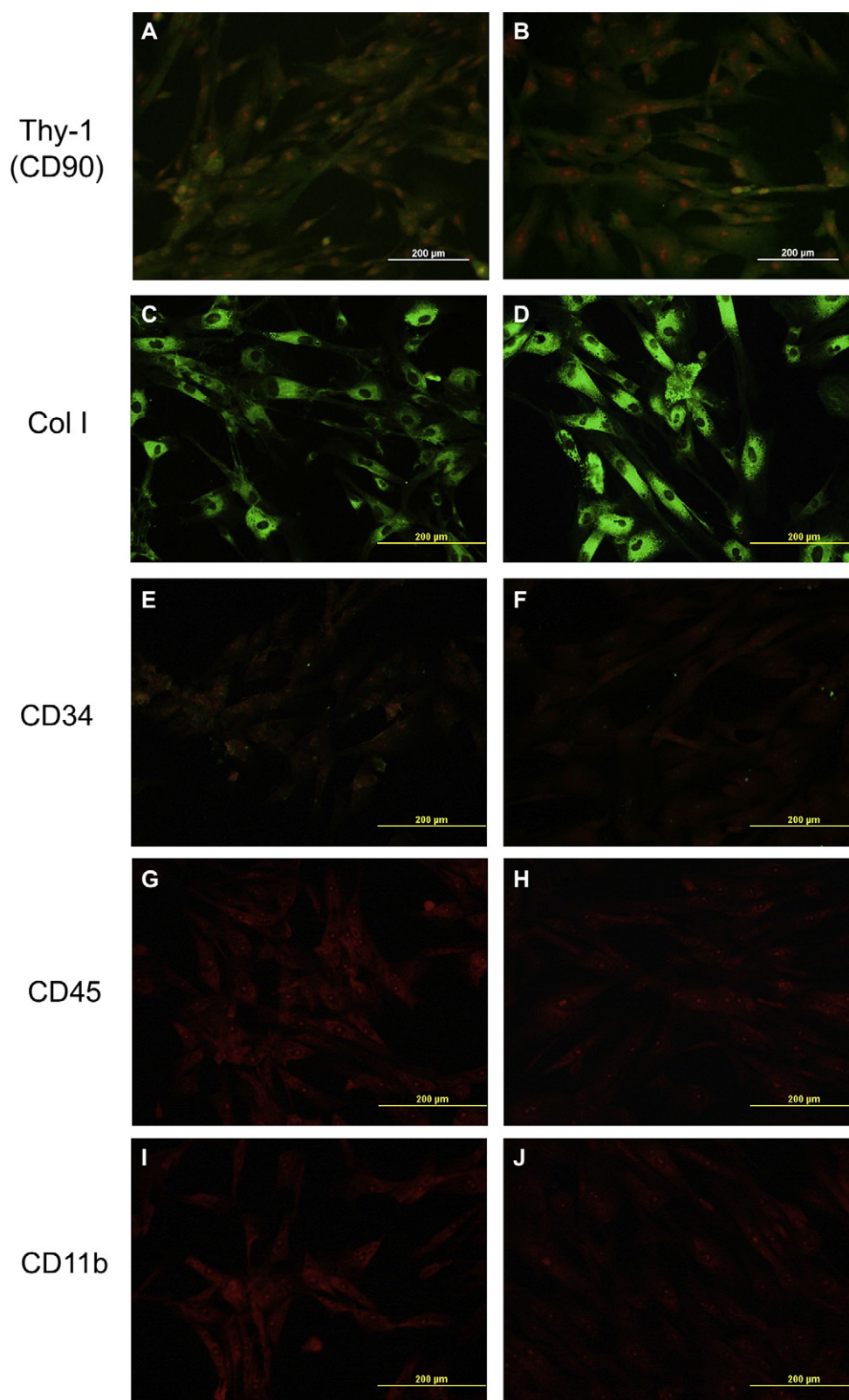


FIG E3. Representative immunocytochemistry for fibroblast-like cells. Collagen gels in which EBs had been cultured for 21 days with or without (control) IL-4 were dissolved by using collagenase and passaged into monolayer culture. Thy-1 (CD90): **A**, control cells; **B**, IL-4-induced cells. Collagen I (*Col I*): **C**, control cells; **D**, IL-4-induced cells. CD34: **E**, control cells; **F**, IL-4-induced cells. CD45: **G**, control cells; **H**, IL-4-induced cells. CD11b: **I**, control cells; **J**, IL-4-induced cells.

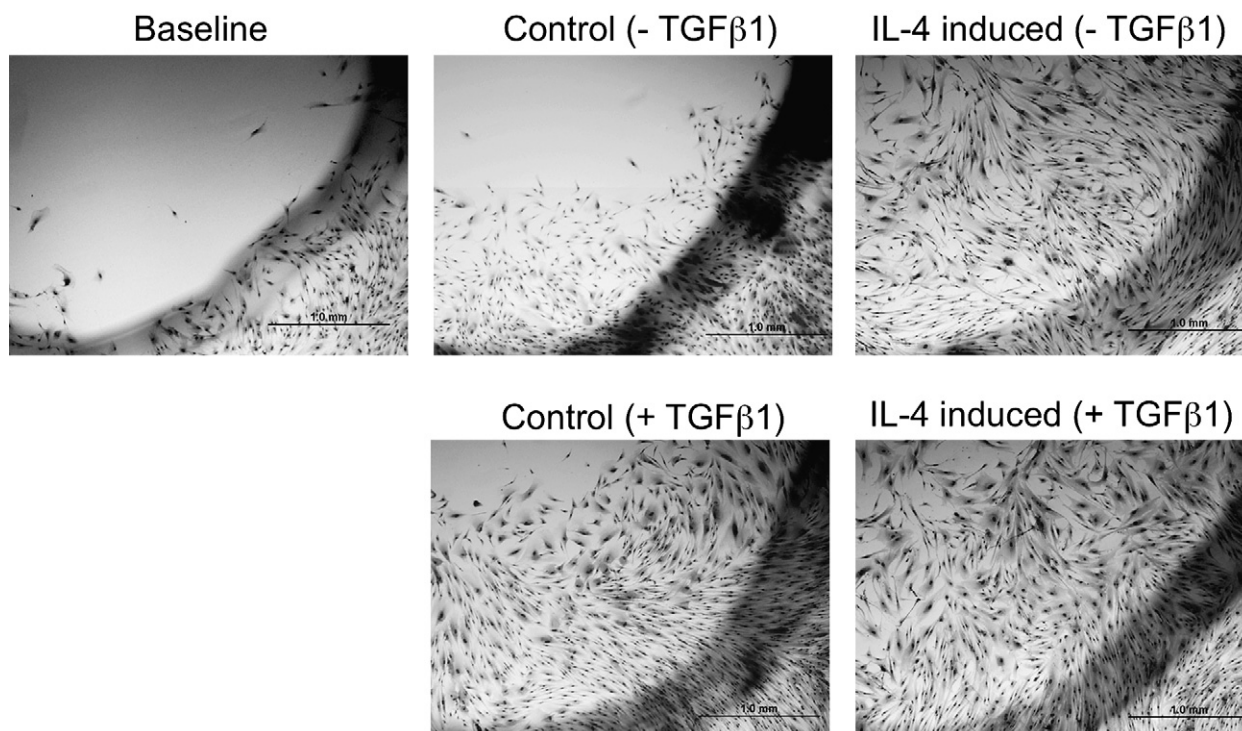


FIG E4. Migration of differentiated fibroblast-like cells in the *in vitro* wound-closure assay: representative photographs of *in vitro* wound-closure assay. Cells were seeded into a 12-well culture plate, and the wound-closure assay was performed in the presence or absence of TGF-β1 (100 pmol/L) for 48 hours, as described in the experimental procedures section. The *round solid line* shows an edge of the wound at the time of removal of the tip (baseline).

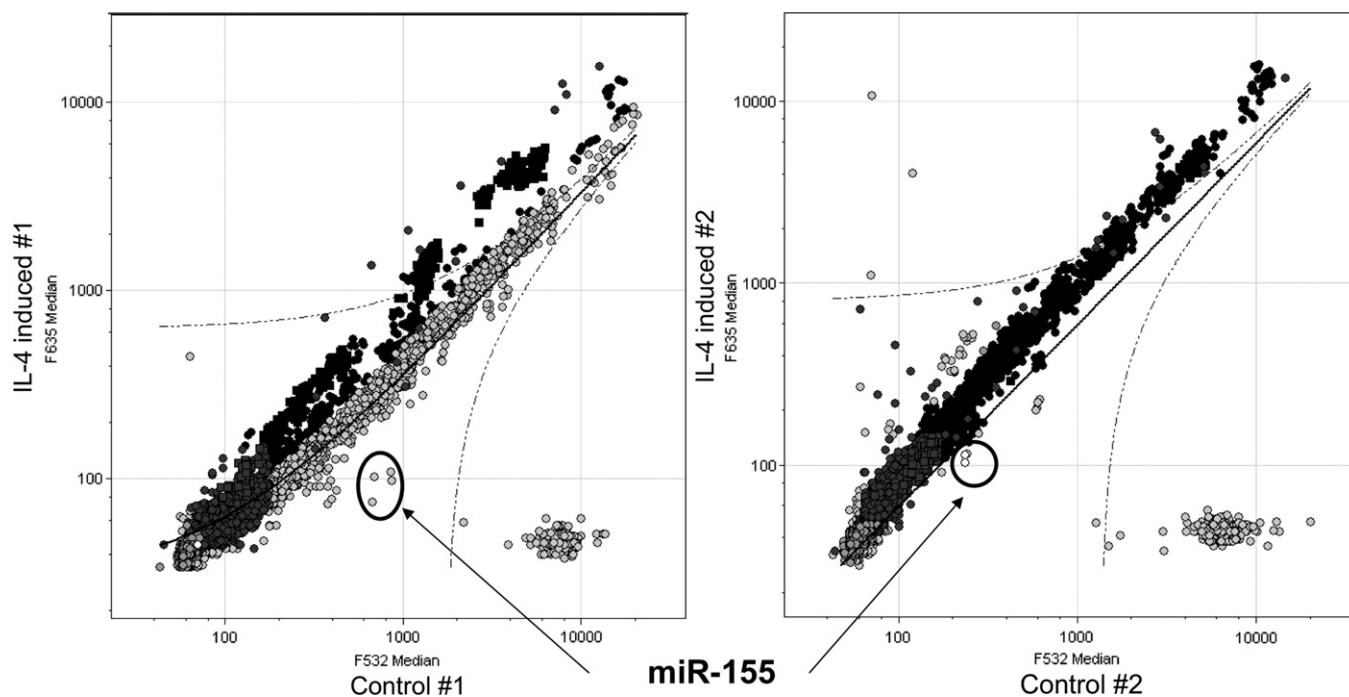


FIG E5. Microarray profiling of miRNAs in control and IL-4-induced fibroblast-like cells. The *vertical axis* represents relative expression in IL-4-induced cells, and the *horizontal axis* represents control cells. Two different batches of control and IL-4-induced cells were evaluated (#1 and #2), each in quadruplicate. miR-155 is shown to be reduced in all test spots of both comparisons in IL-4-induced cells compared with control cells.

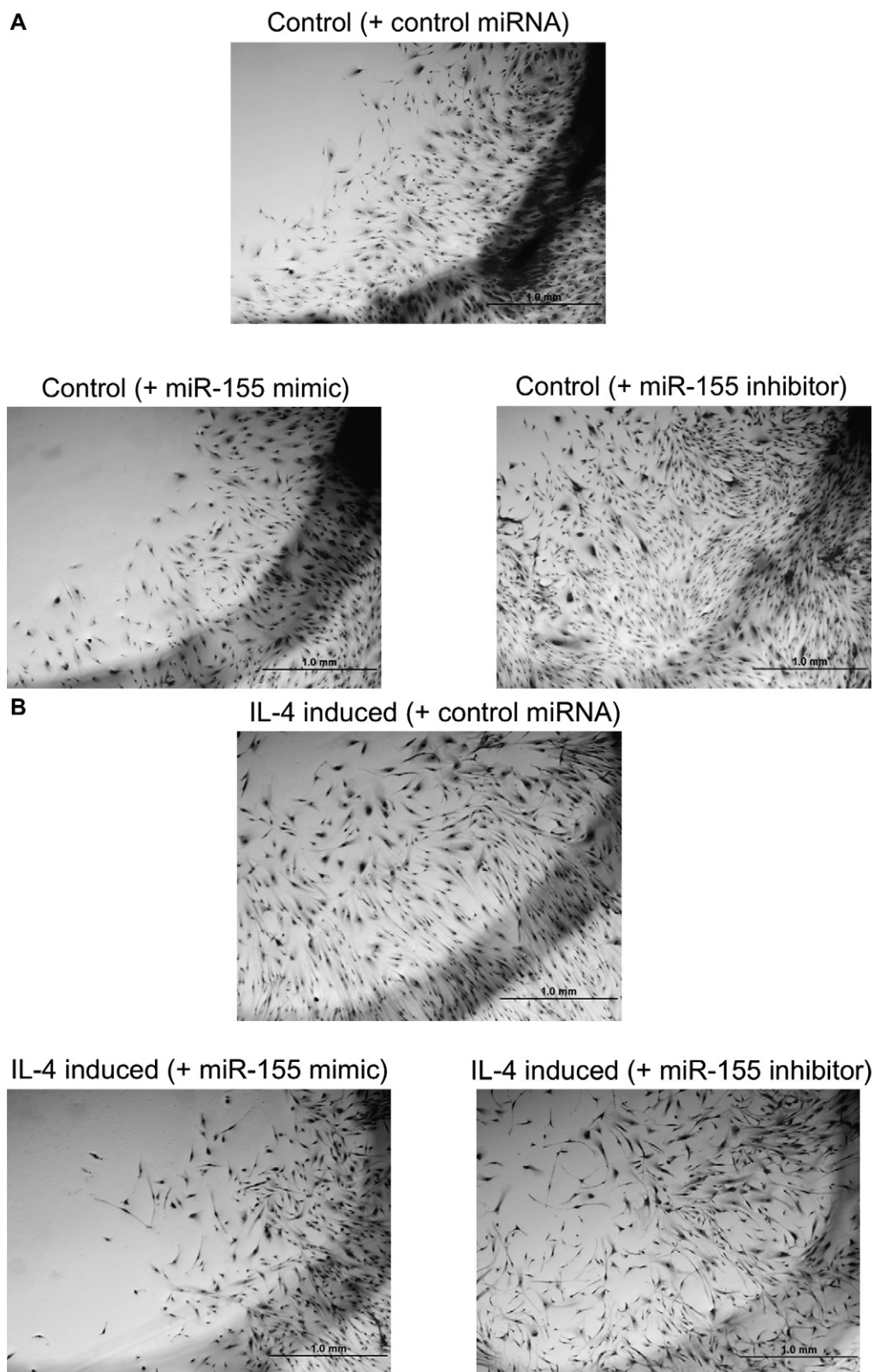


FIG E6. miR-155 regulates the motility of fibroblast-like cells. Representative photographs of the *in vitro* wound-closure assay. Cells were transfected with either miR-155 mimic, miR-155 inhibitor, or control miRNA. The wound-closure assay was then performed as described in the [Methods](#) section. The *round solid line* shows an edge of the wound at the time of removal of the tip.

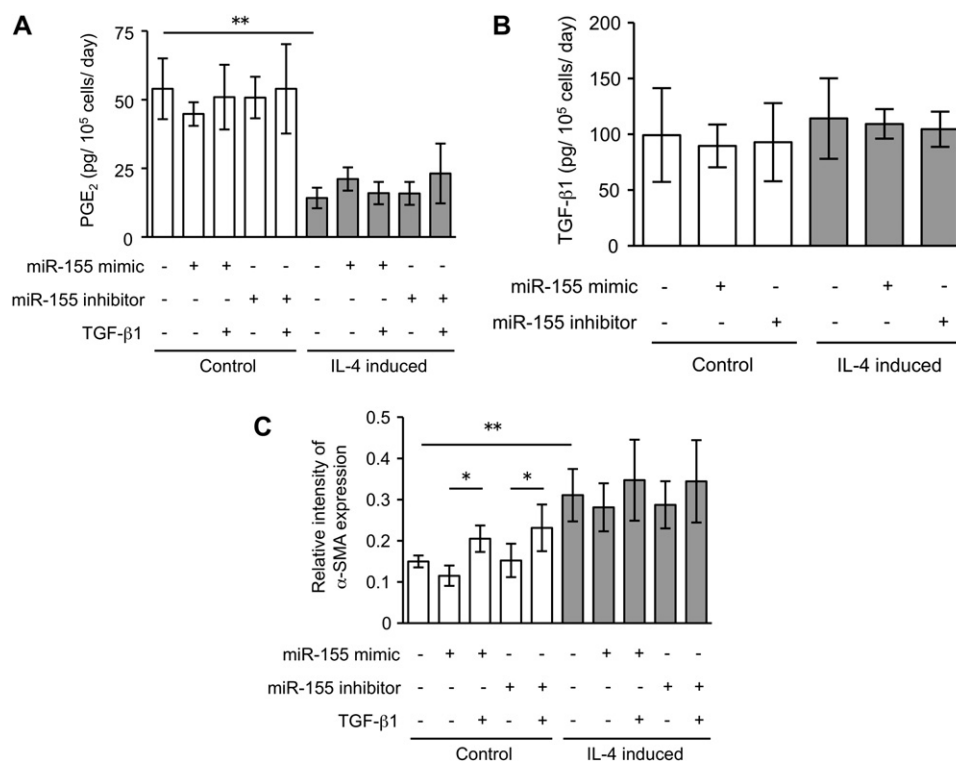


FIG E7. Effect of miR-155 on PGE₂ production, TGF-β1 production, and α-SMA expression in control and IL-4-induced fibroblast-like cells. **A**, PGE₂ production by means of enzyme immunoassay. **B**, TGF-β1 production by means of ELISA. **C**, Densitometric analysis of α-SMA expression expressed relative to the amount to β-actin by means of Western blot analysis. Cells were cultured in the presence or absence of TGF-β1 for 48 hours after the transfection of an miR-155 mimic, an inhibitor, or a control miRNA and then collected as either supernatants or cell layers. Three different batches of control and IL-4-induced cells were evaluated on 3 separate occasions. Values are presented as means ± SDs. **P* < .05, ***P* < .01.