EDA-containing cellular fibronectin induces fibroblast differentiation through binding to $\alpha_4\beta_7$ integrin receptor and MAPK/Erk 1/2-dependent signaling

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ABSTRACT   Fibroblast differentiation is an essential step during wound healing and fibrosis. Fibronectin (FN) is a major component of the extracellular matrix and occurs in two main forms: plasma and cellular FN. The latter includes the alternatively spliced domain A (EDA). Although EDA-containing cellular fibronectin (EDA-FN) is associated with fibroblast differentiation, how EDA-FN promotes differentiation is incompletely understood. In this study, we investigate the mechanism by which EDA-FN contributes to fibroblast differentiation with emphasis on the characterization of the EDA-FN receptor. We show that EDA-FN increases α-SMA expression (immunofluorescence), collagen deposition, cell contractility, and focal adhesion kinase (FAK) activation (immunoblotting); whereas plasma FN, a form lacking EDA, shows no effect. Primary lung fibroblasts constitutively express $\alpha_4\beta_7$ integrin receptor (FACS and RT-PCR). Blocking of $\alpha_4\beta_7$ reduces fibroblast adhesion to EDA-FN and inhibits α-SMA expression, collagen deposition, and FAK activation induced by EDA-FN. Using recombinant EDA-containing peptides, we demonstrate that the EDA segment is sufficient to induce fibroblast differentiation *via* binding to $\alpha_4\beta_7$. EDA-FN induces MAPK-Erk1/2 activation and inhibition of MEK1/2 attenuates EDA-FN-induced α-SMA expression. Our findings demonstrate that EDA-FN induces fibroblast differentiation by a mechanism that involves binding of EDA to $\alpha_4\beta_7$ integrin followed by activation of FAK and MAPK-associated signaling pathways.— Kohan, M., Muro, A. F., White, E. S., Berkman, N. EDA-containing cellular fibronectin induces fibroblast differentiation through binding to $\alpha_4\beta_7$ integrin receptor and MAPK/Erk 1/2-dependent signaling. *FASEB J.* 24, 4503–4512 (2010). www.fasebj.org

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FIBROBLASTS ARE RESPONSIBLE for the preservation of extracellular matrix (ECM) homeostasis by maintaining a balance between synthesis and degradation of structural proteins in all the organs of the body. These cells have the ability to alter their phenotype from noncontractile, quiescent fibroblasts, and intermediate "protomyofibroblasts" to "myofibroblasts", which are distinguished from their precursors by the expression of α-smooth muscle actin (α-SMA), their highly contractile capacity, and their ability to produce large amounts of ECM proteins (1, 2). The differentiation of fibroblasts into myofibroblasts requires the combined action of mechanical tension, TGF-β1 and EDA-containing fibronectin (FN) (1–3).

FN is a 440-kDa dimeric ECM glycoprotein that includes 3 different types of repeating segments, called type I, type II, and type III, the latter being the most abundant (4). The FN gene encodes 15 type III repeats, which are constitutively expressed, plus 2 that are alternatively spliced, the extra domain A (EDA) and B (EDB) (5). EDA and EDB are encoded by single exons, and their inclusion or exclusion from the FN mRNA is determined by a highly regulated alternative splicing mechanism (5). Two major forms of FN exist: plasma FN (pFN), a soluble dimeric form found in plasma secreted by hepatocytes, which lacks both EDA and EDB segments; and cellular FN (cFN), an insoluble cross-linked multimeric form found in ECM mainly secreted by activated fibroblasts as soluble dimers and contains variable proportions of EDA and/or EDB segments (5, 6).

Increased expression of the EDA-containing isoform of FN is found during embryogenesis, and once tissue development is completed its expression drops to negligible levels (7, 8). The FN isoform lacking both EDA and EDB domains is the most abundant form present in normal adult tissues (7–9). Under certain conditions such as wound healing or tissue fibrosis, where myofibroblasts are present in abundance, increased expression of EDA-containing cellular FN (EDA-FN) has been observed (10–12). Although EDA-FN is necessary for

myofibroblast differentiation (11, 12), the mechanism by which it contributes to this effect still remains unknown. Serini *et al.* (12) have proposed that EDA-FN might interact with an uncharacterized cell surface receptor on fibroblasts and possibly facilitate TGF-β1-driven signals.

Integrins are a family of heterodimeric transmembrane receptors that bind ECM components (13). It is well established that both plasma and cellular FNs bind several different integrins triggering intracellular signals, including early activation of focal adhesion kinase (FAK), which has been associated with fibroblast differentiation (14, 15). $\alpha_5\beta_1$ integrin, considered the "classic" FN receptor, binds to the RGD motif of FN and stimulates cell adhesion, migration, and proliferation (16, 17). Members of the α_4/α_9 subfamily of integrins include $\alpha_4\beta_1$, $\alpha_9\beta_1$, and $\alpha_4\beta_7$ (16, 18). $\alpha_4\beta_1$ and $\alpha_9\beta_1$ are expressed by fibroblasts (19, 20) and are known to bind the EDA segment of FN (21). In contrast, expression of $\alpha_4\beta_7$ integrin has not been reported in fibroblasts. In the present study, we show that lung fibroblasts constitutively express $\alpha_4\beta_7$ integrin and provide evidence that EDA-FN stimulates fibroblast differentiation *via* binding of the EDA segment to $\alpha_4\beta_7$ integrin followed by activation of downstream cell differentiation-linked signaling pathways.

MATERIALS AND METHODS

Antibodies and reagents

Anti-EDA-FN (3E2) and anti-α-smooth muscle actin (α-SMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-$\alpha_4\beta_7$ integrin (DATK32), FITC-conjugated anti-α_E integrin (2E7), and anti-EDA-FN (IST-9) were purchased from Abcam (Cambridge, UK). Anti-$\alpha_5\beta_1$ integrin (BMA5), anti-$\alpha_9\beta_1$ integrin (Y9A2), and irrelevant isotype-matched IgG antibodies were purchased from Chemicon (Billerica, MA, USA). LEAF purified anti-β_7 integrin (FIB27) was purchased from BioLegend (San Diego, CA, USA). Functional grade purified anti-β_1 integrin (HMb1-1) was purchased from eBioscience (San Diego, CA, USA). Anti-$\alpha_4\beta_1$ integrin (PS/2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-FAK (Tyr397), anti-FAK, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2), and anti-p44/42 MAPK (Erk1/2) (137F5) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Cy5-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit were purchased from Jackson Laboratories Inc (West Grove, PA, USA). EDA-FN was purchased from Sigma-Aldrich or isolated from media of cultured fibroblasts (11). pFN was purchased from Innovative Research (Novi, MI). MEK inhibitors U0126 and PD98059 were purchased from Cell Signaling Technology.

Fibroblast isolation and culture

C57Bl/6 mice were purchased from Harlan Laboratories Ltd (Jerusalem, Israel), and lung fibroblasts were extracted as described previously (22). The Hebrew University-Hadassah Med-

ical School Animal Ethics Committee approved all animal protocols. Human lung fibroblasts were obtained from lung biopsies of patients undergoing thoracic surgery, and cells were maintained in DME with 10% FCS, glutamine, penicillin, and streptomycin. Informed consent was obtained. Cells between passages 2 and 4 were used for all experiments (n=3–4).

Production of EDA-containing and EDA-lacking recombinant peptides

FN sequences containing or lacking the EDA segment were generated from full-length cFN and full-length pFN cDNAs, respectively. PCR amplifications were performed using the following primers: forward, 5′-ACCATCACTGTGTATGCTGT-CACT-3′; reverse, 5′-AAGTCCTGATACAACCACGGA-3′. These primers covered a sequence from base 4822 to base 5626 (numbers are as in the full-length cDNA sequence found at GeneBank) and generated two products: one of 804 bp containing the EDA segment flanked by the 11th and 12th type III repeats, and one of 534 bp lacking the EDA domain. PCR-amplified fragments were then cloned in the *Bam*HI site of the pProEX Htb expression vector (Invitrogen Corp, San Diego, CA, USA). Competent BL21 cells were transfected, and expression of the peptide was induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Cells were then lysed and disrupted by freezing/thawing and sonication. Protein extracts were loaded on a 12% SDS-PAGE gel. Bands corresponding to the EDA-containing and EDA-lacking peptides were detected by staining the gel with 1 M KCl, cut out from the gel and electroeluted in 50 mM carbonate-bicarbonate buffer, pH 9.6. Only the purified EDA-containing recombinant peptide showed positive immunological reactivity to the anti-EDA monoclonal antibody 3E2.

Immunofluorescence

Fibroblasts (10^4 cells/well) were cultured on coverslips placed in 24-well plates and treated with EDA-FN, pFN, EDA-containing peptides, or peptides lacking the EDA segment (10 μg/ml, 48 h, 37°C). Cells were then fixed in 75% methanol, permeabilized in 0.2% Triton 100×, blocked in 1% BSA, and incubated with anti-α-SMA. Binding was detected using Cy5-conjugated goat anti-mouse antibody. Nuclei were counterstained with propidium iodide (Sigma). Slides were examined using a Fluo-View FV 300 confocal laser scanning biological microscope (Olympus Corporation, Center Valley, PA, USA). Ten fields were examined per slide. Quantification was assessed using Image-Pro Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA) on images acquired at ×600 and expressed as integrated optical density (IOD) as described previously (22). For blocking experiments, cells were preincubated (10–20 μg/ml, 45 min, 37°C) with anti-$\alpha_4\beta_7$ integrin (DATK32), anti-$\alpha_5\beta_1$ integrin (BMA5), anti-β_7 integrin (FIB27), anti-β_1 integrin (HMb1–1), irrelevant isotype-matched IgG, MEK1/2 inhibitor U0126 (10 μM, 1 h, 37°C), MEK1 inhibitor PD98059 (20 μM, 1 h, 37°C) or anti-EDA (IST-9; 25 μg/ml, 45 min, 37°C).

To determine whether the $\alpha_4\beta_7$ integrin is expressed by fibroblasts *in vivo*, immunostaining was performed on 4–6 μm paraffined lung tissue sections from mice exposed to ovalbumin (22). After deparaffinization, slides were incubated with anti-$\alpha_4\beta_7$ integrin together with anti-α-SMA as a marker for myofibroblasts. Binding was detected using Cy5 anti-mouse or FITC anti-rat antibodies. Tissue slides were examined using a FluoView FV 300 confocal laser scanning biological microscope.

Collagen deposition by fibroblast monolayers

Fibroblasts (5×10^4 cells/well) were seeded in 24-well plates, and collagen deposition was determined using the Sircol colorimetric assay (Biocolor, Belfast, UK), as described previously (22), in the presence or absence of EDA-containing cFN, pFN, EDA-containing peptides, or peptides lacking the EDA segment (10 µg/ml). Collagen deposition was expressed as fold increase relative to deposition by untreated cells. For blocking experiments, cells were preincubated with anti-$\alpha_4\beta_7$ integrin (DATK32) (10 µg/ml, 45 min, 37°C) or anti-EDA (IST-9) (25 µg/ml, 45 min, 37°C).

Collagen gel contraction assay

Three-dimensional gel contraction was performed in collagen matrices as described previously (23). After 24 h at 37°C, gels were photographed and their size was determined using ImageJ (National Institutes of Health, Bethesda, MD, USA). Results were expressed as percentage of initial gel area.

ELISA for TGF-β1

ELISA for TGF-β1 was performed on cell culture medium using a commercial kit (R&D Systems, Minneapolis, MN, USA).

Fibroblast adhesion assay

Flat-bottom 96-well plates (BD Biosciences, San Jose, CA, USA) were coated with EDA-FN (20 µg/ml, 1 h, 37°C). Plates were then blocked with 1% BSA (30 min, 37°C). Fibroblasts (5×10^3 cells/well) were seeded into individual wells. Plated cells were centrifuged to ensure equal dispersion and were incubated for 20 min (37°C, 5% CO_2). Nonadherent cells were removed by centrifuging the plate upside-down for 5 min at 500 RPM. Adherent cells were fixed and stained with 0.5% crystal violet, 1% formaldehyde, 20% methanol followed by destaining with PBS. Relative adherence was determined by reading the plate at 550 nm in an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). For the indicated conditions, fibroblasts in suspension were preincubated with function-blocking antibodies (20 µg/ml, 30 min) on ice prior to plating.

RT-PCR for β7 integrin

Cellular RNA was isolated using TriReagent (Sigma). RT-PCR was performed using standard conditions (30 cycles) (Promega, Madison, WI, USA). cDNAs from murine lung fibroblasts and spleen-derived lymphoid cells were amplified using the following primers: forward, 5′-CCTACGACTCTGGGCTCTTG-3′; reverse, 5′-ACAGGTCAGCCTCAGAGCAT-3′.

cDNAs from human lung fibroblasts and blood-derived T lymphocytes were amplified using the following primers: forward, 5′-ACCGTGACCCTTGAACACTC-3′; reverse, 5′-TG-CAACTCCACAATCAGCTC-3′.

PCR products (194 bp for murine cells and 239 bp for human cells) were visualized by ethidium bromide-stained agarose gel electrophoresis. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as control. Some cells were exposed to 5 ng/ml of TGF-β1.

Flow cytometry

Cells were incubated with phycoerythrin-conjugated anti-$\alpha_4\beta_7$ integrin (DATK32); FITC-conjugated anti-α_E integrin (2E7) or irrelevant isotype-matched IgG for 30 min at room temperature. Fluorescence was analyzed using a FACStar analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). Data were processed with the CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blotting

Samples were prepared from cells grown in 6-well plates by addition of Nonidet P-40 lysis buffer supplemented with protease inhibitor cocktail (Pierce, Rockford, IL, USA), sodium vanadate (Sigma), and PMSF (Sigma). Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Lysates were then separated by 9% SDS-PAGE and samples were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% dry milk in TBS/Tween-20 (TBS-T) and incubated with anti-phospho-FAK, anti-FAK, anti-phospho-Erk1/2, and anti-Erk1/2 antibodies. Membranes were incubated with HRP-conjugated anti-rabbit IgG, and blots were developed with the SuperSignal West Pico Chemiluminiscent Substrate kit (Thermo Scientific, Rockford, IL, USA) and exposed to Fuji Medical X-Ray films (FujiFilm Corp., Tokyo, Japan). Image analysis was performed using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA).

Data analysis

The mean ± SE values are given for each group. One-way analysis of variance (ANOVA) followed by Bonferroni posttest was performed to compare groups. Values of $P < 0.05$ were considered statistically significant.

# RESULTS

EDA-FN induces fibroblast differentiation

We first compared the effects of EDA-FN and the non-EDA-containing form pFN on fibroblasts isolated from lungs of C57Bl/6 mice and evaluated different aspect of fibroblast differentiation, namely α-SMA expression, collagen production, collagen gel contractility, and TGF-β1 release.

Expression of α-SMA was assessed by quantitative immunofluorescence (22). EDA-FN (10 µg/ml, 48 h) increased α-SMA expression in comparison with untreated cells and pFN (**Fig. 1A**, $P<0.001$). pFN (10 µg/ml, 48 h) did not induce changes in α-SMA expression levels in comparison with untreated cells (Fig. 1A). Preincubation with anti-EDA antibody (IST-9) inhibited EDA-FN-induced α-SMA expression (Fig. 1A; $P<0.001$).

Fibroblast collagen deposition was measured by the Sircol colorimetric assay (22). EDA-FN (10 µg/ml, 48 h) increased fibroblast collagen deposition, whereas pFN (10 µg/ml, 48 h) showed minimal effect on collagen deposition (Fig. 1B; 28±2% for EDA-FN *vs.* 5±3% for pFN over untreated cells, $P<0.01$). Preincubation with anti-EDA antibody (IST-9) inhibited EDA-FN-induced collagen deposition (Fig. 1B; $P<0.01$).

Figure 1. EDA-FN induces markers of fibroblast differentiation. Primary lung fibroblasts were exposed to 10 μg/ml EDA-FN or non-EDA-containing pFN), and markers of fibroblast differentiation were evaluated as described in Materials and Methods. EDA-FN induced expression of α-SMA (A), ***$P < 0.001$ vs. untreated cells and pFN; collagen deposition (B), **$P < 0.01$ vs. pFN; cell contractility (C), *$P < 0.05$ vs. pFN, **$P < 0.01$ vs. untreated cells; and TGF-β1 release (D), **$P < 0.01$ vs. untreated cells and pFN. Preincubation with anti-EDA monoclonal antibody IST-9 inhibited the effects induced by EDA-FN. Experiments were performed 3 times; means ± SE are shown. Scale bars = 10 μm (A); 1 cm (C).
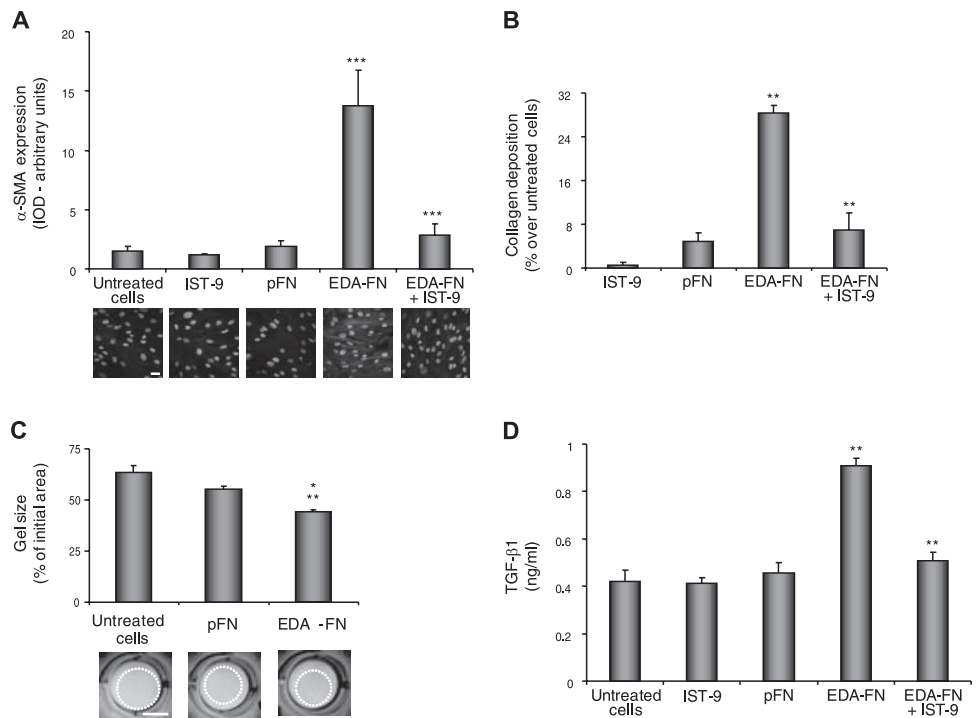
Fibroblast contractility was determined by the collagen gel contraction assay (23). After 24 h, untreated cells reduced the gel to 64% of its initial size at time 0 (Fig. 1C). EDA-FN (10 μg/ml, 24 h) reduced the size of the gel by an additional 20% in comparison with untreated cells (Fig. 1C, $P<0.01$), whereas pFN (10 μg/ml, 24 h) reduced the gel by only an additional 8% as compared to untreated cells.

TGF-β1 secretion into culture medium by lung fibroblasts was determined by ELISA. While EDA-FN increased TGF-β1 release by 2-fold in comparison with untreated cells (Fig. 1D, $P<0.01$), exposure to pFN did not affect TGF-β1 release. Pretreatment with anti-EDA antibody (IST-9) inhibited EDA-FN-induced TGF-β1 release (Fig. 1D, $P<0.01$).

$\alpha_4\beta_7$ integrin is constitutively expressed in lung fibroblasts and mediates fibroblast adhesion to EDA-FN

The effect exhibited by EDA-FN on fibroblast differentiation suggests the existence of a cell surface receptor that mediates this process. In an attempt to identify such a receptor, we first determined fibroblast adhesion to EDA-FN in the presence of blocking antibodies (20 μg/ml) for integrins $\alpha_4\beta_1$, $\alpha_9\beta_1$, and $\alpha_4\beta_7$. Blocking of integrin $\alpha_4\beta_7$ inhibited the adhesion of fibroblast to EDA-FN (**Fig. 2A**; $P<0.05$). In addition, blocking of $\alpha_4\beta_1$, an integrin receptor expressed by fibroblasts (19), which is known to bind the EDA segment (21), also abrogated cell adhesion to EDA-FN, whereas blocking of integrin $\alpha_9\beta_1$ showed no effect (Fig. 2A; $P<0.01$).

The α_4 subunit is known to be expressed on fibroblasts as part of the $\alpha_4\beta_1$ complex (15, 19). In contrast,

the β_7 subunit has not previously been identified on fibroblasts. We then evaluated the expression of both the β_7 subunit and the $\alpha_4\beta_7$ integrin complex in lung fibroblasts by RT-PCR and flow cytometry. Primary murine lung fibroblasts constitutively expressed β_7 integrin mRNA (Fig. 2B, lane 1). Addition of TGF-β1 (5 ng/ml, 48 h) did not increase β_7 integrin mRNA levels (Fig. 2B, lane 2). Primary human lung fibroblasts also expressed the β_7 integrin subunit (Fig. 2C). FACS analysis demonstrated expression of the $\alpha_4\beta_7$ complex in murine lung fibroblasts (mean fluorescence intensity − fold over control = 12.8±0.7) (Fig. 2D). We did not observe positive staining for the α_E subunit, which also forms a complex with the β_7 subunit (13) (data not shown). We also observed positive immunostaining for $\alpha_4\beta_7$ integrin in vivo in myofibroblasts from lung tissue of OVA-treated mice (Fig. 2E).

Blocking of $\alpha_4\beta_7$ integrin inhibits EDA-FN-induced myofibroblast differentiation

To investigate whether $\alpha_4\beta_7$ mediates EDA-FN-induced fibroblast differentiation, cells were preincubated with anti-$\alpha_4\beta_7$ blocking antibodies in the presence or absence of EDA-FN and α-SMA expression, collagen production and TGF-β1 release were determined. Blocking of $\alpha_4\beta_7$ inhibited EDA-FN-induced α-SMA expression ($P<0.001$; **Fig. 3A**). Preincubation with anti-$\alpha_5\beta_1$ blocking antibodies did not exert inhibitory effects on fibroblast differentiation. Fibroblasts were also preincubated with blocking antibodies against the β_7 and β_1 subunits. While pretreatment with anti-β_7 integrin antibodies completely inhibited the induction of α-SMA expression observed after exposure to 10
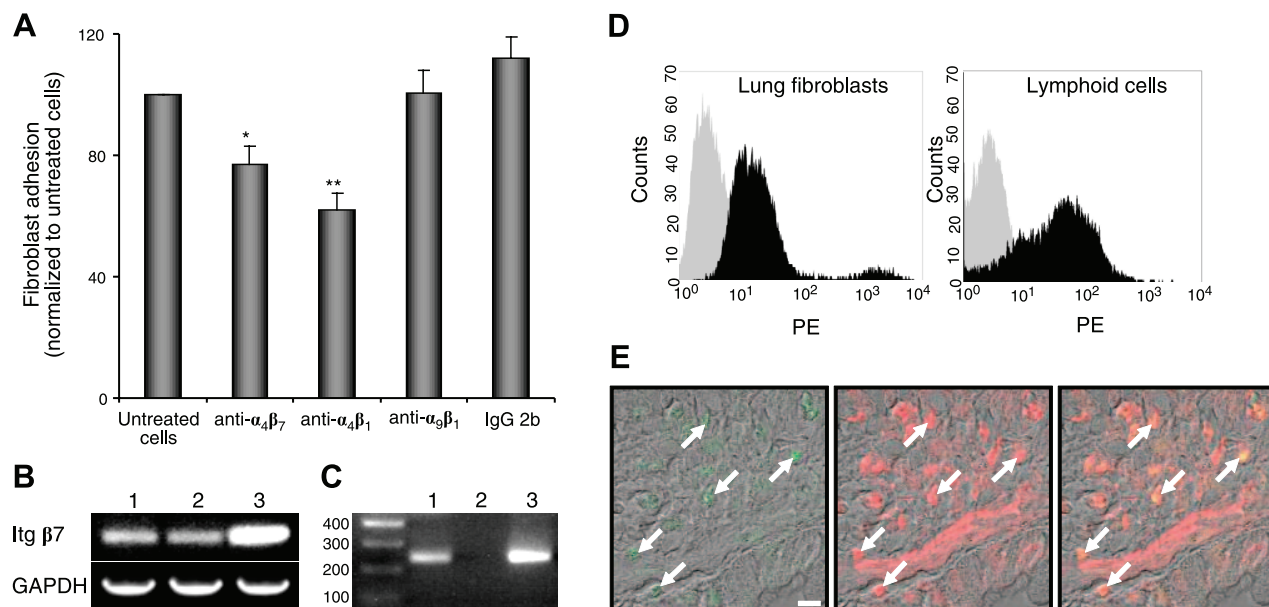
Figure 2. $\alpha_4\beta_7$ integrin is constitutively expressed in lung fibroblasts and mediates adhesion to EDA-FN. *A*) Lung fibroblasts were preincubated with blocking antibodies and seeded in 96-well plates previously coated with EDA-FN to determine cell adhesion. Blocking of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins inhibited fibroblast adhesion to EDA-FN. $*P < 0.05$, $**P<0.01$ *vs.* untreated cells). Experiments were performed 3 times; means $\pm$ SE are shown. *B*) mRNA expression of β_7 was evaluated by RT-PCR in murine primary lung fibroblasts (lane 1). Addition of 5 ng/ml TGF-β1 to lung fibroblasts did not alter β_7 mRNA expression (lane 2). Murine lymphoid cells (lane 3) were used as a positive control. *C*) mRNA expression of β_7 in human primary lung fibroblasts (lane 1) and in human T lymphocytes (lane 3, positive control). No band was observed in the absence of cDNA (lane 2, negative control). *D*) Staining for $\alpha_4\beta_7$ integrin (black histograms) and irrelevant IgG isotype control (gray histograms) was evaluated by flow cytometry on murine primary lung fibroblasts and lymphoid cells (positive control). *E*) Expression of $\alpha_4\beta_7$ integrin (green, left panel), α-SMA (red, middle panel), and colocalization (yellow, right panel) was evaluated in murine lung tissue by immunostaining (white arrows). Scale bar = 10 μm. Experiments were repeated 3 times; representative images are shown.

μg/ml EDA-FN (Fig. 3*A*; $P<0.001$); anti-β_1 integrin antibodies did not alter EDA-FN-induced α-SMA expression. The absence of effect with anti-β_1 antibodies rules out a role for β_1-containing integrins such as $\alpha_4\beta_1$ and $\alpha_9\beta_1$ on fibroblast differentiation. Pretreatment with anti $\alpha_4\beta_7$ integrin blocking antibodies also reduced EDA-FN-induced collagen production (Fig. 3*B*) and EDA-FN-induced TGF-β1 release (Fig. 3*C*) to levels similar to those observed for untreated cells (internal control) (31$\pm$5% over control without anti-$\alpha_4\beta_7$ *vs.* 7$\pm$4% over control with anti-$\alpha_4\beta_7$ for collagen production, $P<0.001$; 1.04$\pm$0.06 ng/ml without anti-$\alpha_4\beta_7$ *vs.* 0.63$\pm$0.05% ng/ml with anti-$\alpha_4\beta_7$ for TGF-β1 release, $P<0.001$).

The EDA segment is sufficient to induce a myofibroblast phenotype through binding to $\alpha_4\beta_7$ integrin

To determine whether the ability of EDA-FN to induce fibroblast differentiation is attributable to the EDA segment and—if so—due to direct binding of the EDA segment to $\alpha_4\beta_7$, we generated recombinant fragments of FN containing or lacking the EDA segment. Recombinant peptides (10 μg/ml) containing the EDA segment (EDA$^+$ peptides) increased α-SMA expression (**Fig. 4*A***; $P<0.001$ *vs.* untreated cells) and collagen production by 26 $\pm$ 2% (Fig. 4*B*; $P<0.01$ *vs.* untreated

cells). The effect observed for the EDA$^+$ peptides was similar to that observed for EDA-FN. Recombinant peptides lacking the EDA segment (EDA$^-$ peptides) were used as control and showed no effect.

To determine whether the effect of the EDA segment is *via* binding to $\alpha_4\beta_7$ integrin, we evaluated the expression of α-SMA after exposure to EDA$^+$ peptides in the presence or absence of anti $\alpha_4\beta_7$ blocking antibodies. We observed that preincubation with anti-$\alpha_4\beta_7$ antibodies completely abrogated the increased expression of α-SMA that was induced by 10 μg/ml EDA$^+$ peptides (Fig. 4*C*; $P<0.01$).

**EDA-FN enhances FAK activation *via* integrin $\alpha_4\beta_7$**

Binding of different sequences of FN to integrin receptors is known to activate FAK *via* phosphorylation of tyrosine residues (24, 25). The ratio between phosphorylated FAK and total FAK was determined by quantitative immunoblotting. EDA-FN (10 μg/ml) induced phosphorylation of the tyrosine 397 of FAK on lung fibroblasts in a time-dependent manner, with a maximal effect observed after 6 h of incubation (3.1-fold over untreated cells) (**Fig. 5*A***). We also compared FAK activation between EDA-FN and pFN and observed that cells exposed to 10 μg/ml EDA-FN significantly increased the phosphorylated FAK:FAK ratio in comparison to 10 μg/ml pFN (Fig. 5*B*; 1.04$\pm$0.08 for EDA-FN
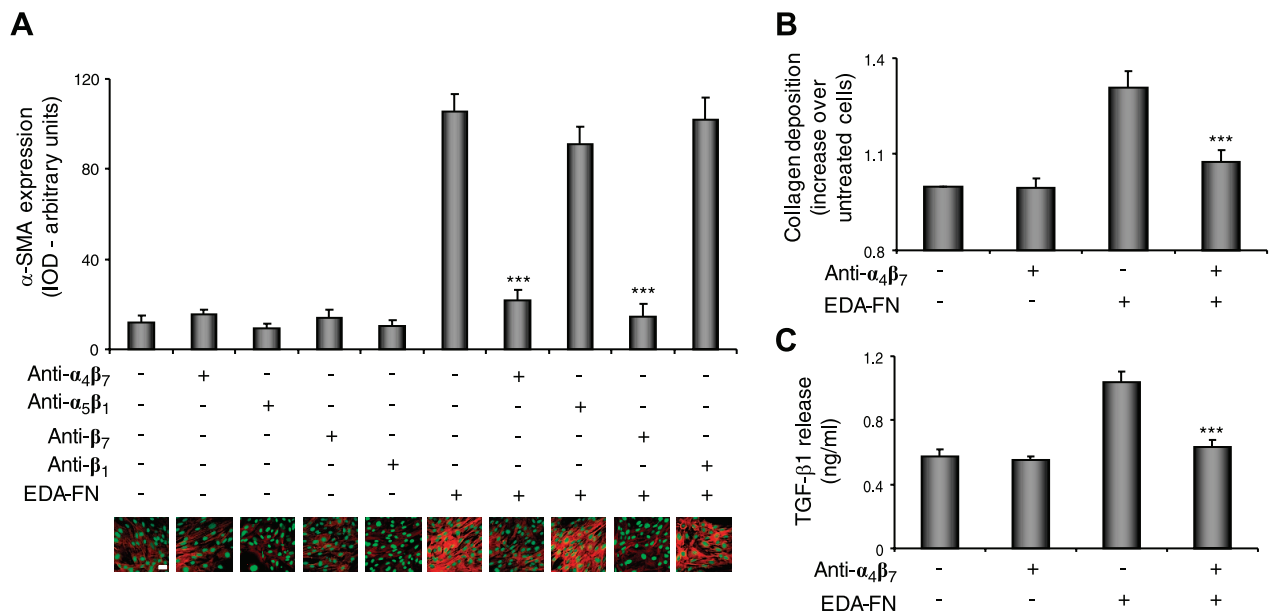
Figure 3. EDA-FN induces myofibroblast differentiation *via* $\alpha_4\beta_7$ integrin. Fibroblasts were exposed to 10 µg/ml EDA-FN in the presence of blocking antibodies for $\alpha_4\beta_7$, $\alpha_5\beta_1$, β_7, or β_1 integrins and expression of α-SMA was assessed by immunofluorescence, quantified and expressed as integrated optical density (IOD). Experiments were performed 4 times; representative images are shown. *A*) Preincubation with anti-$\alpha_4\beta_7$ or anti-β_7 blocking antibodies (45 min, 37°C) inhibited EDA-FN-induced α-SMA expression in fibroblasts. Scale bar = 20 µm. *B*, *C*) EDA-FN-induced collagen deposition, assessed by the Sircol assay (*B*) and EDA-FN-induced TGF-β1 release, determined by ELISA (*C*), were abrogated in fibroblasts preincubated with anti-$\alpha_4\beta_7$ blocking antibodies. ***$P < 0.001$.

vs. 0.59±0.07 for pFN; P<0.01). To determine whether the EDA segment can induce FAK activation, the phosphorylated FAK:FAK ratio was assessed in fibroblasts exposed to EDA$^+$ peptides or EDA$^-$ peptides. Exposure of cells to 10 µg/ml EDA$^+$ peptides for 3 h

increased phosphorylated FAK:FAK ratio in comparison with cells treated with 10 µg/ml EDA$^-$ peptides (Fig. 5*C*). No differences were detected between cells treated with EDA$^-$ peptides and untreated cells. To evaluate whether EDA-FN activates FAK *via* $\alpha_4\beta_7$ inte-
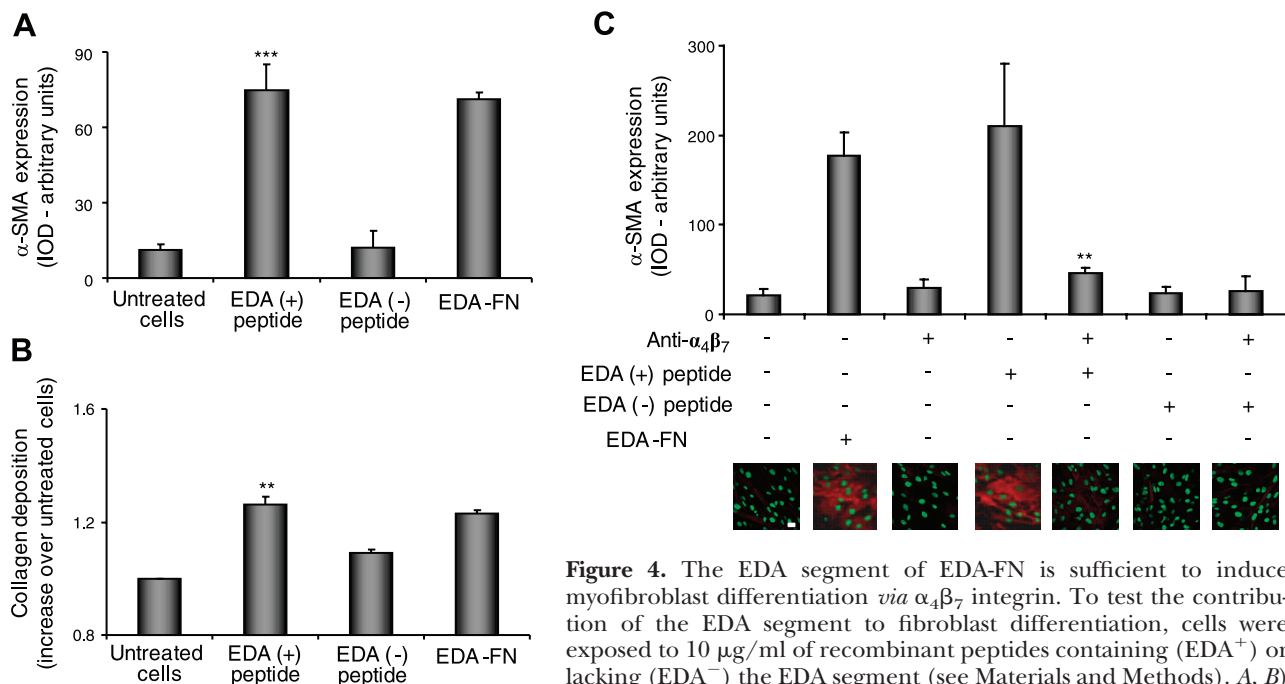
Figure 4. The EDA segment of EDA-FN is sufficient to induce myofibroblast differentiation *via* $\alpha_4\beta_7$ integrin. To test the contribution of the EDA segment to fibroblast differentiation, cells were exposed to 10 µg/ml of recombinant peptides containing (EDA$^+$) or lacking (EDA$^-$) the EDA segment (see Materials and Methods). *A*, *B*) EDA$^+$ peptides increased expression of α-SMA (*A*) and collagen deposition (*B*) in a manner similar to EDA-FN. No effect was observed after treatment with EDA$^-$ peptides used as control. *C*) Exposure to anti-$\alpha_4\beta_7$ integrin blocking antibodies abrogated the effect observed for EDA$^+$ peptides on α-SMA expression. Scale bar = 10 µm. **$P < 0.01$, ***$P < 0.001$. Experiments were performed 3 times; representative images are shown.
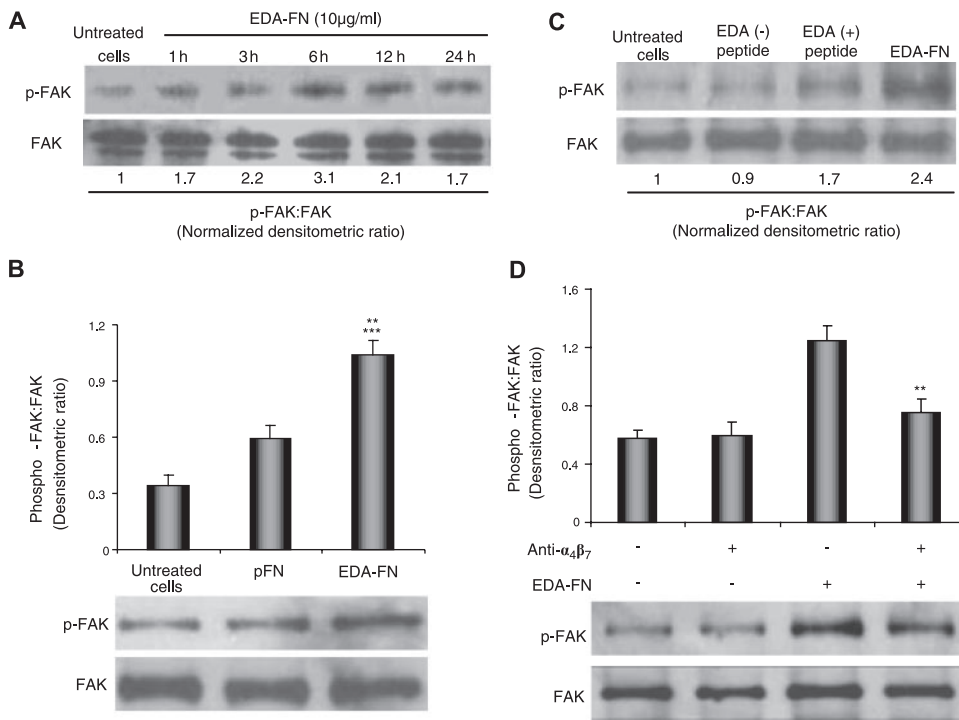
Figure 5. EDA-FN activates FAK *via* $\alpha_4\beta_7$ integrin receptor. Cell lysates obtained from fibroblasts exposed to EDA-FN, non EDA-containing pFN, EDA-containing peptides (EDA$^+$) or EDA-lacking peptides (EDA$^-$) were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-FAK and anti-FAK antibodies. Densitometric analysis calculated as ratio of phospho-FAK to total FAK was then assessed. *A*) Time course of FAK activation by EDA-FN showed a maximum effect after 3–6 h of incubation. *B*) Exposure to EDA-FN increased FAK activation in comparison with pFN. **P < 0.01 *vs.* pFN; ***P < 0.001 *vs.* untreated cells. *C*) Lysates from cells treated with EDA$^+$ peptides showed greater FAK activation than lysates treated with EDA$^-$ peptides. *D*) Blocking of $\alpha_4\beta_7$ integrin reduced EDA-FN-induced FAK activation. **P < 0.01. Blots shown are representative of 2 or 3 independent experiments from different lysates.

grin, cells were exposed to 10 μg/ml EDA-FN in the presence or absence of anti-$\alpha_4\beta_7$ integrin antibodies (preincubation for 45 min at 37°C). As shown in Fig. 5*D*, blocking of $\alpha_4\beta_7$ integrin decreased phosphorylated FAK:FAK ratio induced by EDA-FN to levels similar to untreated cells (0.75±0.09 in the presence of anti-$\alpha_4\beta_7$ integrin *vs.* 1.24±0.11 in the absence of anti-$\alpha_4\beta_7$ integrin; *P*<0.01).

EDA-FN-mediated fibroblast differentiation is MAP kinase Erk1/2 dependent

The intracellular signals involved in EDA-FN-mediated fibroblast differentiation are virtually unknown. We explored whether the p44/42 MAP kinase (ERK 1/2) signaling pathway, which is key in cell differentiation, proliferation and motility (26), contributes to fibroblast differentiation. Exposure to 10 μg/ml EDA-FN and EDA-containing peptides enhanced phosphorylation of p44/42 MAPK (Erk1/2) in lung fibroblasts in a time-dependent manner with maximal effect observed after 3 h of incubation (3.7-fold over untreated cells) (**Fig. 6*A***). Pretreatment (1 h, 37°C) with 10 μg/ml of MEK1/2 inhibitor U0126 or 20 μg/ml of MEK1 inhibitor PD98059 inhibited EDA-FN-induced α-SMA expression in fibroblasts (Fig. 6*B*; *P*<0.001 for U0126 and *P*<0.01 for PD98059 *vs.* EDA-FN). U0126 showed higher inhibitory capacity (96% inhibition) in comparison with PD98059 (62% inhibition).

DISCUSSION

Fibroblast differentiation to a myofibroblast phenotype is an essential step in the process of fibrogenesis (1, 2).

Although a link between EDA-FN and fibroblast differentiation has been known for more than 15 yr (10), the mechanism by which this interaction occurs has not been fully elucidated. In the present study, we show that $\alpha_4\beta_7$ integrin is constitutively expressed on lung fibroblasts and provide evidence that EDA-FN induces fibroblast differentiation through binding of the EDA segment to $\alpha_4\beta_7$ integrin receptor and activation of FAK and MAP kinase Erk1/2.

Previous studies have reported that EDA-FN is more potent than the EDA-lacking form pFN in promoting fibroblast adhesion, migration, and proliferation (27, 28). EDA-FN also induces the expression of α-SMA in lipocytes more efficiently than pFN, resulting in the differentiation of these cells into myofibroblast-like cells (10). In this study we show that EDA-FN, but not pFN, induces α-SMA expression, collagen production, gel contractility, and TGF-β1 release on primary lung fibroblasts. These effects are blocked by the anti-EDA monoclonal antibody (IST-9), thus adding further evidence for a role of the EDA segment of cFN on fibroblast differentiation.

Although ours and several other studies suggest the presence of a specific cell surface receptor that binds EDA to mediate the effect of EDA-FN on fibroblast differentiation (2, 10, 12, 29), no such receptor has been identified to date. In this study we demonstrate that $\alpha_4\beta_7$ integrin is constitutively expressed in murine and human lung fibroblasts and that cell adhesion to EDA-FN is partially mediated by this receptor. Expression of the $\alpha_4\beta_7$ integrin has previously been reported in human and murine eosinophils, basophils, NK cells, CD4$^+$ T, B lymphocytes and endothelial cells (30–32). This integrin plays a role in cell adhesion, rolling, differentiation, and survival of eosinophils and lympho-
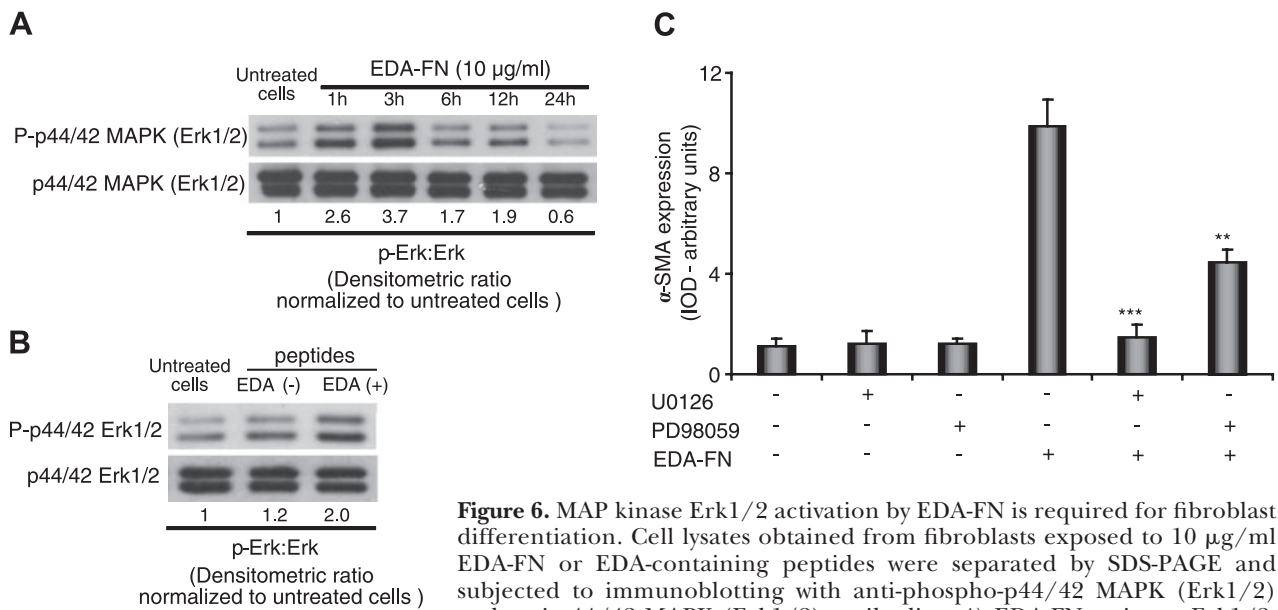
Figure 6. MAP kinase Erk1/2 activation by EDA-FN is required for fibroblast differentiation. Cell lysates obtained from fibroblasts exposed to 10 µg/ml EDA-FN or EDA-containing peptides were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-p44/42 MAPK (Erk1/2) and anti-p44/42 MAPK (Erk1/2) antibodies. *A*) EDA-FN activates Erk1/2, showing maximum effect after 3 h of incubation. *B*) Lysates from cells treated with EDA$^+$ peptides showed greater Erk1/2 activation than lysates treated with EDA$^-$ peptides. Blots shown are representative of 2 experiments from different lysates. *C*) Fibroblasts were preincubated (1 h, 37°C) with MEK inhibitors U0126 (10 µg/ml) and PD98059 (20 µg/ml) and then exposed to 10 µg/ml EDA-FN for 48 h. Cell expression of α-SMA in response to EDA-FN was totally inhibited by U0126 and partially inhibited by PD98059, as assessed by immunofluorescence. **$P < 0.01$; ***$P < 0.001$. Experiments were performed 3 times; data are presented as means ± SE.

cytes (30–34). β_7-deficient mice have impaired recruitment of lymphocytes, mast cell progenitors and eosinophils to the gastrointestinal tract (35). We did not observe the expression of the α_E subunit, which has also been shown to form complexes with β_7 (13, 30). Cytokines and growth factors are likely to regulate the expression of $\alpha_4\beta_7$. While tumor necrosis factor α and interleukin (IL)-1 β increase β_7 expression in endothelial cells (32), IL-5 and platelet-activating factor (PAF) do not affect $\alpha_4\beta_7$ expression on eosinophils (34, 35). Addition of TGF-β1 increases the expression of α_4 subunit on lung fibroblasts (15); however, we found that it does not alter the expression of the β_7 subunit in lung fibroblasts.

We found that EDA-FN induces lung fibroblast differentiation *via* $\alpha_4\beta_7$ integrin, since blocking of this integrin attenuates this effect. Furthermore, using β_7 blocking antibodies, we demonstrated that the β_7 subunit is essential for the stimulatory effect of EDA-FN. $\alpha_4\beta_7$ integrin binding sites have been previously identified in minimal sequences that share the dipeptide DV present on the CS-1 segment of the IIICS region (36, 37) and in the KLDAPT sequence present on the III5 repeat (38) of all forms of FN. A previous study has reported that EDA-FN increases eosinophil survival through $\alpha_4\beta_7$ integrin and these authors hypothesize that this effect is mediated by the $\alpha_4\beta_7$ integrin binding site on the CS-1 peptide (33). Our findings clearly demonstrate that fibroblast differentiation is not mediated *via* the $\alpha_4\beta_7$ binding site present on the IIICS or the III5 regions as pFN does not induce differentiation. In the present study, we provide evidence for the existence of another binding site for $\alpha_4\beta_7$ located on

the EDA-segment; however, the amino acid sequence that binds $\alpha_4\beta_7$ integrin has not yet been identified. It has been shown that the EDGIHEL sequence included in the EDA segment binds $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins (21). The EDA domain includes DV segments (ref. 39, GenBank accession number AF 095690) and it is possible that $\alpha_4\beta_7$ binds to EDA *via* DV-containing sequences. Further studies are necessary to address this issue.

It has been proposed that the EDA segment might not induce fibroblast differentiation directly, but rather its insertion into the FN molecule brings about a conformational change thereby increasing the accessibility of the RGD binding site to $\alpha_5\beta_1$ (27) or exposing other integrin binding sites present on FN. Our study demonstrates that this is unlikely to be the case for fibroblast differentiation. This is supported by three lines of evidence: EDA-FN-induced α-SMA expression is not affected by the addition of blocking antibodies against $\alpha_5\beta_1$ and β_1 integrins; recombinant EDA-containing peptides are sufficient to induce fibroblast differentiation; and the addition of anti-$\alpha_4\beta_7$ blocking antibodies inhibits recombinant EDA-containing peptides-induced α-SMA expression. Thus, taken together, these findings strongly suggest that the EDA segment itself binds to $\alpha_4\beta_7$ integrin and thereby induces a myofibroblast phenotype. Our results are consistent with previous studies showing that antibodies against EDA (clone IST-9) inhibit fibroblast differentiation and that addition of a recombinant EDA segment promotes 3T3 cell adhesion (10, 12, 29). Jarnagin *et al.* (10) found that recombinant EDA-containing peptides increased α-SMA expression in lipocytes; while Serini *et*

al. (12) showed inhibitory effects for recombinant EDA fragments on α-SMA expression in skin fibroblasts. These discrepancies may be due to the different structure of the peptides used.

The intracellular signal transduction pathways triggered following the binding of EDA-FN to integrin receptors have yet not been elucidated. Binding of different isoforms of FN to integrins activates focal adhesion kinase (FAK) (24), an early mediator of integrin-linked intracellular signals (25). FAK activation has been previously associated with fibroblast differentiation (14, 15). We show in this study that the EDA segment triggers FAK activation following binding to $\alpha_4\beta_7$ integrin. Interestingly, Thannickal *et al.* (15) found that induction of myofibroblast differentiation *via* TGF-β1 requires integrin-dependent FAK activation and they suggest that this effect may be through induction of integrin receptors and/or enhanced FN expression in response to TGF-β1. Mitogen-activated protein kinase (MAPK) Erk1/2 is involved in cell differentiation (26). We found that EDA-FN and EDA-containing peptides activate MAPK Erk1/2 in primary fibroblasts. Blocking of MAPK Erk1/2 by biochemical inhibitors of MAPK kinase MEK1 and MEK2 abrogated EDA-FN-induced α-SMA expression. Activation of Erk2 by EDA-FN has also been observed in CHO cells but, in contrast to our findings, in this study, Erk2 activation was observed in the absence of FAK (28). Our results, therefore, demonstrate that activation of MAPK Erk1/2 is essential for fibroblast differentiation and is mediated by the EDA segment of FN.

In summary, we demonstrate that EDA-FN contributes to fibroblast differentiation through binding of the EDA segment to $\alpha_4\beta_7$ integrin and activation of the intracellular signal transduction proteins FAK and MAP kinase Erk1/2. Our study advances present understanding of the mechanism by which EDA-FN mediates fibroblast differentiation and fibrogenesis. How EDA-FN interacts with TGF-β1 and/or mechanical tension to induce fibroblast differentiation requires further investigation. FJ

REFERENCES

1. Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R. A. (2002) Myofibroblasts and mechano-regulation of connective tissue remodeling. *Nat. Rev. Mol. Cell. Biol.* **3,** 349–363
2. Gabbiani, G. (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J. Pathol.* **200,** 500–503
3. Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., and Gabbiani, G. (2007) The myofibroblast: one function, multiple origins. *Am. J. Pathol.* **170,** 1807–1816
4. Hynes, R. O. (1990) In *Fibronectins* (1st Ed.) (Rich, A., Editor), Springer-Verlag, New York, New York, USA
5. Kornblihtt, A. R., Pesce, C. G., Alonso, C. R., Cramer, P., Srebrow, A., Werbajh, S., and Muro, A. F. (1996) The fibronectin gene as a model for splicing and transcription studies. *FASEB J.* **10,** 248–257
6. Muro, A. F., Chauhan, A. K., Gajovic, S., Iaconcig, A., Porro, F., Stanta, G., and Baralle, F. E. (2003) Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. *J. Cell Biol.* **162,** 149–160
7. Chauhan, A. K., Iaconcig, A., Baralle, F. E., and Muro, A. F. (2004) Alternative splicing of fibronectin: a mouse model demonstrates the identity of in vitro and in vivo systems and the processing autonomy of regulated exons in adult mice. *Gene* **324,** 55–63
8. Oyama, F., Murata, Y., Suganuma, N., Kimura, T., Titani, K., and Sekiguchi, K. (1989) Patterns of alternative splicing of fibronectin pre-mRNA in human adult and fetal tissues. *Biochemistry* **28,** 1428–1434
9. Pagani, F., Zagato, L., Vergani, C., Casari, G., Sidoli, A., and Baralle, F. E. (1991) Tissue-specific splicing pattern of fibronectin messenger RNA precursor during development and aging in rat. *J. Cell Biol.* **113,** 1223–1229
10. Jarnagin, W. R., Rockey, D. C., Koteliansky, V. E., Wang, S. S., and Bissell, D. M. (1994) Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J. Cell Biol.* **127,** 2037–2048
11. Muro, A. F., Moretti, F. A., Moore, B. B., Yan, M., Atrasz, R. G., Wilke, C. A., Flaherty, K. R., Martinez, F. J., Tsui, J. L., Sheppard, D., Baralle, F. E., Toews, G. B., and White, E. S. (2008) An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *Am. J. Respir. Crit. Care. Med.* **177,** 638–645
12. Serini, G., Bochaton-Piallat, M. L., Ropraz, P., Geinoz, A., Borsi, L., Zardi, L., and Gabbiani, G. (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by TGF-β1. *J. Cell Biol.* **142,** 872–881
13. Takada Y, Ye X, and Simon S. (2007) The integrins. *Genome Biol.* **8,** 215
14. Fernandes, D. J., Bonacci, J. V., and Stewart, A. G. (2006) Extracellular matrix, integrins and mesenchymal cell function in the airways. *Curr. Drug Targets* **7,** 567–577
15. Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., Horowitz, J. C., Day, R. M., and Thomas, P. E. (2003) Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J. Biol. Chem.* **278,** 12384–12389
16. Leiss, M., Beckmann, K., Giros, A., Costell, M., and Fassler, R. (2008) The role of integrin binding sites in FN matrix assembly in vivo. *Curr. Opin. Cell Biol.* **20,** 502–507
17. Akiyama, S. K., Yamada, S. S., Chen, W. T., and Yamada, K. M. (1989) Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* **109,** 863–875
18. White, E. S., Baralle, F. E., and Muro, A. F. (2008) New insights into form and function of fibronectin splice variants. *J. Pathol.* **216,** 1–14
19. White, E. S., Thannickal, V. J., Carskadon, S. L., Dickie, E. G., Livant, D. L., Markwart, S., Toews, G. B., and Arenberg, D. A. (2003) Integrin $\alpha_4\beta_1$ regulates migration across basement membranes by lung fibroblasts. *Am. J. Respir. Crit. Care Med.* **168,** 436–442
20. Kanayama, M., Kurotaki, D., Morimoto, J., Asano, T., Matsui, Y., Nakayama, Y., Saito, Y., Ito, K., Kimura, C., Iwasaki, N., Suzuki, K., Harada, T., Li, H. M., Uehara, J., Miyazaki, T., Minami, A., Kon, S., and Uede, T. (2009) Alpha9 integrin and its ligands constitute critical joint microenvironments for development of autoimmune arthritis. *J. Immunol.* **182,** 8015–8025
21. Liao, Y. F., Gotwals, P. J., Koteliansky, V. E., Sheppard, D., and Van De Water, L. (2002) The EIIIA segment of fibronectin is a ligand for integrins alpha 9beta 1 and alpha 4beta 1 providing a novel mechanism for regulating cell adhesion by alternative splicing. *J. Biol. Chem.* **277,** 14467–14474
22. Kohan, M., Breuer, R., and Berkman, N. (2009) Osteopontin induces airway remodeling and lung fibroblast activation in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* **41,** 290–296
23. Puxeddu, I., Bader, R., Piliponsky, A. M., Reich, R., Levi-Schaffer, F., and and Berkman, N. (2006) The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. *J. Allergy Clin. Immunol.* **117,** 103–110

24. Garcia, A. J., and Boettiger, D. (1999) Integrin-fibronectin interactions at the cell-material interface: initial integrin binding and signaling. *Biomaterials* **20,** 2427–2433

25. Guan, J. L., and Shalloway, D. (1992) Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* **358,** 690–692

26. Zhang, Y., and Dong, C. (2007) Regulatory mechanisms of mitogen-activated kinase signaling. *Cell. Mol. Life Sci.* **64,** 2771–2789

27. Manabe, R., Ohe, N., Maeda, T., Fukuda, T., and Sekiguchi, K. (1997) Modulation of cell-adhesive activity of fibronectin by the alternatively spliced EDA segment. *J. Cell Biol.* **139,** 295–307

28. Manabe, R., Oh-e, N., and Sekiguchi, K. (1999) Alternatively spliced EDA segment regulates fibronectin-dependent cell cycle progression and mitogenic signal transduction. *J. Biol. Chem.* **274,** 5919–5924

29. Xia, P., and Culp, L. A. (1995) Adhesion activity in fibronectin's alternatively spliced domain EDa (EIIIA): complementary to plasma fibronectin functions. *Exp. Cell Res.* **217,** 517–527

30. Shaw, S., and Brenner, M. (1995) The β_7 integrin in mucosal homing and retention. *Semin. Immunol.* **7,** 335–342

31. Lundahl, J., Sehmi, R., Hayes, L., Howie, K., and Denburg, J. A. (2000) Selective upregulation of a functional beta7 integrin on differentiating eosinophils. *Allergy* **55,** 865–872

32. Brezinschek, R. I., Brezinschek, H. P., Lazarovits, A. I., Lipsky, P. E., and Oppenheimer-Marks, N. (1996) Expression of the beta 7 integrin by human endothelial cells. *Am. J. Pathol.* **149,** 1651–1660

33. Meerschaert, J., Vrtis, R. F., Shikama, Y., Sedgwick, J. B., Busse, W. W., and Mosher, D. F. (1999) Engagement of alpha4beta7 integrins by monoclonal antibodies or ligands enhances survival of human eosinophils in vitro. *J. Immunol.* **163,** 6217–6227

34. Walsh, G. M., Symon, F. A., Lazarovils, A. L., and Wardlaw, A. J. (1996) Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. *Immunology* **89,** 112–119

35. Brandt, E. B., Zimmermann, N., Muntel, E. E., Yamada, Y., Pope, S. M., Mishra, A., Hogan, S. P., and Rothenberg, M. E. (2006) The alpha4beta7-integrin is dynamically expressed on murine eosinophils and involved in eosinophil trafficking to the intestine. *Clin. Exp. Allergy* **36,** 543–553

36. Rüegg, C., Postigo, A. A., Sikorski, E. E., Butcher, E. C., Pytela, R., and Erle, D. J. (1992) Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol.* **117,** 179–189

37. Hynes, R. O. (1987) Integrins: a family of cell surface receptors. *Cell* **48,** 549–554

38. Moyano, J. V., Carnemolla, B., Domínguez-Jiménez, C., García-Gila, M., Albar, J. P., Sánchez-Aparicio, P., Leprini, A., Querzé G, Zardi, L., and Garcia-Pardo, A. (1997) Fibronectin type III5 repeat contains a novel cell adhesion sequence, KLDAPT,. which. binds. activated. alpha4beta1. and. alpha4beta7. integrins. *J. Biol. Chem.* **272,** 24832–24836

39. Muro, A. F., Iaconcig, A., and Baralle, F. E. (1998) Regulation of the fibronectin EDA exon alternative splicing. Cooperative role of the exonic enhancer element and the 5′ splicing site. *FEBS Lett.* **437,** 137–141