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The third type III module of human fibronectin mediates cell adhesion and migration

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Fibronectin (FN) is a major extracellular matrix protein involved in various biological events. This study demonstrated that the third FN type III repeat (FnIII3) and several fragments containing the repeat promote cell spreading and migration of human dermal fibroblasts (HDFs), whereas the fourth repeat (FnIII4) did not. A variety of cell types also spread on FnIII3 in a cell-type-specific manner, but not on FnIII4. Immunofluorescence assays revealed that FnIII3 induced the organization of focal contacts and stress fibres in HDFs. Cyclic [RGDFV] peptides with a D-Phe residue, which are selective inhibitors of cell adhesion to vitronectin, inhibited HDF spreading on FnIII3 equally with GRGDS, indicating little involvement of αV-integrins in FnIII3 spreading. An anti-β1 integrin mAb inhibited cell spreading on FnIII3 and FN. To our knowledge, this is the first demonstration that a novel domain of FnIII3 functions in cell spreading and migration through an interaction with unresolved β1 integrin(s) in an RGD-dependent manner.

Keywords: Cell adhesion/fibronectin/type III3 repeat/migration.

Abbreviations: FN, fibronectin; FnIIIa-b, His-tagged fusion protein of an FN fragment comprising type III repeats a-b; HDFs, human dermal fibroblasts; IPTG, isopropyl-β-D(−)-thiogalactopyranoside; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; mAb, monoclonal antibody.

Many types of cells can attach, spread, and migrate on plastic coated with extracellular matrix (ECM) proteins such as fibronectin (FN), vitronectin (Vn), laminin (Ln) and collagen *in vitro*. These interactions occur via a specific cell-surface receptor called integrin (*1*). All known integrins consist of one α and one β subunit, each of which can determine ligand specificity (*1*). In mammals, 18 α and 8 β subunits have been identified that combine to produce 24 integrins. Six type III FN repeats, including the fifth (III5) (*2*), III9 (*3*), III10 (*4*), EDA (*5*), III14 (*6*) and IIICS (*7*), are recognized by their respective integrin.

FN plays a key role in many biological processes, including cell adhesion, migration, proliferation and differentiation (*8*). Asaga *et al.* (*9*) previously showed the involvement of FN in collagen gel contraction and developed a monoclonal antibody (mAb A3A5) that binds to FN and inhibits contraction. Subsequently, we mapped its epitope within FNIII4-5 (*10*). In the present study, we aimed to reveal the biological significance of the epitope regions by testing their effects on cell adhesion and migration. FN cDNA fragments containing three consecutive or single FN type III repeats were expressed as 10× His-tagged proteins, affinity-purified to homogeneity with metal chelating columns, and further examined *in vitro* for their adhesive properties with a variety of cell types, including human dermal fibroblasts (HDFs). We describe the presence of a novel FN type III3 repeat that promotes cell spreading when used as a substrate. To elucidate the integrin(s) involved in the spreading, we tested the effect of an anti-β1 integrin mAb and several RGD-peptides on cell spreading. In addition, we conducted cell migration assays and found that FNIII3 has the potential to promote HDF migration, similar to FN.

Experimental procedure

Cell culture

HDFs were obtained from Kurabo Industries (Osaka, Japan). Human G361 melanoma cells, human HepG2 hepatocarcinoma cells, and murine Swiss 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Murine MC3T3-E1 pre-osteoblast cells and B16 melanoma cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Cells were maintained in dishes containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) or Minimum Essential medium alpha (α-MEM, Wako, Japan), supplemented with 10% (w/v) fetal bovine serum (Hyclone, Logan, UT, USA), 10 mM sodium bicarbonate, 20 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2--ethanesulfonic acid], 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C and 5% CO_2.

Recombinant FN fragments

To clone the cDNA fragments of the human FN type III repeats into an expression vector, the corresponding regions were amplified by RT–PCR. Briefly, first-stand cDNA was prepared from Trizol-treated HDFs, followed by RT with an oligo dT12-15 primer (Invitrogen, Carlsbad, CA, USA). Target cDNA fragments were amplified with LA Taq DNA polymerase (Takara, Shiga, Japan) and a set of *Nde1* site-flanked FN primers (Table 1). PCR-amplified FN cDNAs were electrophoretically purified in agarose gels, digested with *Nde I* and then ligated into *Nde I*-digested pET-16b plasmid vectors encoding an N-terminal 10× His-tag (Novagen, Madison, WI, USA). Correct insertion and the absence of PCR-generated errors in the constructs were verified by DNA sequencing.

Table 1. Primer used for amplification.

Clones	PCR product[a]	Primer Sequence (5′-3′)[b]
FnIII2-4	S708-T995	2f:TTATTATTCATATGTCTCCTCTTGTGGCCACTTC 4r:TTATTATTCATATGGGTT GTCTGTTGAGCAGTCAGAG
FnIII3-5	A810-L1085	3f:TTATTATTCATATGGCGCCTGATGCCCCTC 5r:TTATTATTCATATGCAGTGTGGTAAAGACTCCAGTGGC
FnIII4-6	T906-P1173	4f:TTATTATTCATATGACAGTGCCCTCTCCCAGG 6r:TTATTATTCATATGTGTCACCACTTTGTTTACAATTGG
FnIII5-7	K996-P1265	5f:TTATTATTCATATGAAACTGGATGCTCCCACTAACC 7r:TTATTATTCATATGTGGGATGATGGTATCAGAGATAGG
FnIII3	A810-D905	5f:TTATTATTCATATGAAACTGGATGCTCCCACTAACC 3r:TTATTATTCATATGATCTGAGCGTGGGGTG
FnIII4	T906-T995	4f:TTATTATTCATATGACAGTGCCCTCTCCCAGG 2r:TTATTATTCATATGTGTTGTTTGTGAAGTA

[a]The numbers indicate the first and last amino acid residues, numbered according to the sequence of mature FN (GenBank:AB191261). All primers contained an Nde1 restriction site (underlined). [b]Upper- and lower-case letters indicate forward and reverse primers, respectively.

The resulting constructs were named pET/FNIII2-4, pET/FNIII3-5, pET/FNIII4-6 and pET/FNIII5-7. To clone FNIII3 and FNIII4, we performed an inverse polymerase chain reaction (IPCR) as previously described (*11*). Briefly, IPCR for cloning FNIII3 was performed with PrimeSTAR HD DNA polymerase (Takara) using pET/FNIII3-5 as a DNA template and 3r and 5f as oligos (Table 1). The resulting products contained both III3 and III5 in the pET-16b vector and were partially digested with *NdeI*, circularized by ligation and transfected into *Escherichia coli* DH5α cells. The transformant containing FNIII3 was identified with a colony-PCR method using the 3r and 3f oligos (Table 1) and was named pET/FNIII3. pET/FNIII2-4 was used with the 2r and 4f oligos (Table 1) to clone FNIII4 by IPCR. We named this recombinant pET/FNIII4.

Bacterial expression and purification of FN fusion proteins

The cloned DNAs bearing His-tagged FN type III repeats were expressed in BL21(DE3) *E. coli* cells as instructed by the manufacturer (Novagen). In brief, protein expression was induced by adding 1 mM IPTG, and the expressed protein was purified on a HiTrap chelating column (GE Healthcare, Uppsala, Sweden), extensively dialysed against phosphate-buffered saline (PBS) using an Oscillatory microdialysis system (Daiichi Pure Chemicals, Tokyo, Japan), and recovered for use in cell adhesion assays. Protein concentration was quantified using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA). FN fusion proteins were separated by 12.5% (w/v) SDS–PAGE and visualized with dye staining. Standard proteins were purchased from New England Biolabs (NEB, Beverly, MA, USA) or Daiichi Pure Chemicals.

Cell spreading assay

Cell adhesiveness of the purified FN fusion proteins was determined by cell spreading assays as previously described (*12*). Proteins were coated on 96-well tissue culture plates (No. 3072, Beckton Dickinson, San Jose, CA, USA) at a concentration of 0.3–10 μg/ml. Human plasma FN or Fn-120kDa (*13*) was used as a reference. Cultured cells were detached, suspended in Eagle's minimum essential medium (MEM, Sigma, St Louis, MO, USA), and pre-incubated at 37°C for 10 min with or without mAb, before plating. After incubation for 80 min to 2 h at 37°C, the plates were washed with PBS, fixed with 4% (w/v) paraformaldehyde (PFA). The percentage of cells adapting a well-spread morphology was estimated by counting cells in five random fields under phase contrast microscopy and used to evaluate spreading efficiency. The effect of synthetic peptides on cell spreading was similarly evaluated in the presence of each peptide. The following were purchased: normal rat non-immune IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Fn-120kDa, Telios, San Diego, CA, USA; Vn, synthetic peptides of GRGDS and GRGES, Iwaki Glass (Tokyo, Japan); cyclo[RGDFV] peptide (D-Phe residue underlined), Biomol International, Plymouth Meeting, PA, USA; and EHS-Ln, Upstate, Lake Placid, NY, USA. Rat mAb13 directed against human integrin β1 (*14*) and human plasma FN were kindly provided by Dr K.M. Yamada, NIH, Bethesda, MD, USA.

Cell migration assays

Cell migration assays for the FN fusion proteins were performed by using Oris Universal Cell Migration Assembly kit as instructed by the manufacturer (Platypus Technologies, WI, USA). Briefly, proteins were coated on Oris-compatible 96-well plastic plates at 10 μg/ml, blocked with heat-denatured BSA, washed with PBS and dried. Oris stoppers were inserted into each well before adding the cell. In each well, 5×10^4 HDF cells suspended in DMEM were added and incubated to allow cell attachment for 3 h at 37°C in 5% CO_2 before starting the assay. The stoppers were then removed to allow cell migration to a central detection zone for 12 to 24 h at 37°C in 5% CO_2 in an incubator. Thereafter, cells were fixed with 4% PFA and stained with 0.25% (w/v) toluidine blue (*15*). The migrated cells in the plate equipped with a black detection mask were observed with a microscopy at low magnification (40×) or photographed using transmitted light.

Fibroblast growth arrest

Cell growth of HDFs was arrested by treating with mitomycin-C (MMC) (Sigma). MMC is an antibiotic with anti-proliferative properties. HDFs were suspended in regular culture medium, added to each well in 96-well culture plate (No.3072, Beckton Dickinson) at 5×10^3 cells/well, and allowed to adhere for 3 h at 37°C in 5% CO_2 before treating with the reagents. Cells were treated with different concentration of MMC at 0.62 to 40 μg/ml for 2 h. At the end of treatment, the MMC-untreated cells were fixed with PFA and used later in comparison as a reference. Meanwhile, HDF cells were fed with fresh medium and incubated further for 3 days, washed with PBS, fixed with PFA, stained with toluidine blue and solubilized into 0.5% SDS in PBS. The absorbance at 640 nm (A_{640}) was determined on a Spectrophotometer (Model V-520, JASCO, Tokyo, Japan). The absorbance was found to be a linear function of number of cells. In cell migration assay for growth-arrested cells, we treated HDFs with MMC at 10 μg/ml for 2 h at 37°C in 5% CO_2, detached to add cells to each well in 96-well plate (No. 3072) that was pre-coated with Fn and Fn fusion proteins. As the control, MMC-untreated cells were included for comparison. We incubated cells for 14 h in the cell migration assay as described above and evaluated the cell migration-promoting activity for each protein with or without MMC.

Immunofluorescence microscopy

HDFs were plated onto EZView 24-well culture dishes (glass bottom, Asahi Techno Glass, Tokyo, Japan) and subjected to immunofluorescence analysis for vinculin. The dishes were coated with 5 μg/ml fusion protein or cellular FN (Fibrogenex, Morton Grove, IL, USA), treated with heat-denatured BSA and washed with PBS. HDFs were plated onto the dishes and incubated for 4 h at 37°C in 5% CO_2. After washing with PBS, the cells were fixed with 4% (w/v) PFA, permeabilized with 0.25% (v/v) Triton X-100, treated with BSA, incubated with anti-vinculin mAb (clone VIN-11-5; Sigma) as the primary antibody, and treated with biotinylated anti-mouse IgG as the secondary antibody, followed by Alexa Fluor 488-conjugated streptavidin (Molecular Probes, Eugene, OR, USA). F-actin was visualized with the binding of Alexa Fluor

594-phalloidin (Molecular Probes). Nuclei were stained with Hoechst 33342 (Molecular Probes). Images were obtained using a fluorescence microscope (Nikon, Tokyo, Japan).

Results and discussion

The results of our previous study of fibroblast-mediated collagen gel contraction in 3D matrices suggested that FNIII4-5 may be involved in the FN matrix assembly of 3D matrices (*10*). This possibility was recently demonstrated in 2D cell cultures, by showing the inhibitory effect of the repeats on FN matrix assembly (*16*). However, it remains unclear whether the type III repeats have an effect on cell adhesion in various types of cells. To address this question, we used molecular biological techniques to prepare His-tagged FN fusion proteins that spanned the protein from III2 to III7. These fusion proteins were designated as clones FnIII3, FnIII4, FnIII2-4, FnIII3-5, FnIII4-6 and FnIII5-7 (Fig. 1A), and the size of each was in agreement with the length of the sequence deduced from the original fragment (Fig. 1B).

We first tested cell spreading activity using clones FnIII2-4, FnIII3-5, FnIII4-6 and FnIII5-7. As a reference, we included Fn-120kDa in the assay (Fig. 1C). On Fn-120kDa, HDFs spread in a dose-dependent manner, with maximal spreading at the Fn-120kDa concentration of 1.0 μg/ml, as previously shown (*13*). Among the four clones tested, clone FnIII3-5 stimulated the highest dose-dependent spreading activity. FnIII2-4 also stimulated dose-dependent spreading, but it was less active than FnIII3-5. In contrast, clones FnIII4-6 and FnIII5-7 displayed substantially reduced activities ($\geqslant$95% decrease). These results suggest that the potential for the promotion of cell spreading may be located in the III3 repeat, but not in the III4 repeat.

Subsequently, we separately prepared each repeat. As shown in Fig. 1C, FnIII3 exhibited substantially higher activities, whereas FnIII4 had greatly reduced activity. This finding indicates that FNIII3 has potential activity for promoting cell spreading of HDFs. The amounts of protein needed to promote 50% spreading activity were 1.0 μg/ml (8.3 nM) for Fn-120kDa, 1.7 μg/ml (106 nM) for FnIII3, 2.8 μg/ml (76 nM) for FnIII3-5 and 6.2 μg/ml (162 nM) for FnIII2-4 (Table 2). The molar ratios with respect to FnIII3 were 1.5 for FnIII2-4 and 0.7 for FnIII3-5, indicating that the potential FNIII3 activity was retained similarly between the two clones. It is unclear why the activity with the III3 repeat was lower than that with clone FnIII2-4. We speculate that the tertiary structure of the FNIII3 repeat may be different in clones FnIII2-4 and FnIII3-5 compared with clone FNIII3 after immobilization on plastic. Previously, Erickson showed the elastic properties of the FN type III repeat (*17*). If this is also true of our clones, the FNIII3 in FnIII2-4 would have transitioned reversibly into the unfolded state owing to its labile properties, resulting in a conformation unsuitable for promoting cell spreading. Importantly, these data do not rule out the possibility that either FnIII4 or FnIII2 might have suppressed FnIII3-dependent cell spreading when the repeats were aligned directly in this order.

The adhesive activity of FnIII3 was markedly lower than that of Fn-120kDa; specifically, 10-fold weaker on a molar basis. To determine whether FnIII3 is responsible for only a small fraction of the Fn-120kDa activity, we compared the rate of cell spreading on FnIII3 with the rates on well-known cell adhesive proteins such as Vn (Fig. 1D). At 20 min, cell spreading on FnIII3 was about 10%, which was much less than that on Fn-120kDa, Vn, or Ln. At 40 min, there was 40% cell spreading on FnIII3 and no spreading on FnIII3-5, whereas Fn-120kDa and Vn had promoted nearly complete cell spreading. At 60 min, cell spreading on FnIII3-5 finally became evident. At 80 min, FnIII3 had promoted nearly 80% spreading, although FnIII3-5 had promoted only 40%. FnIII4 had no activity during the assay. Collectively, these results clearly show that there was a time lag for the promotion of cell spreading by FnIII3 and FnIII3-5, suggesting that a different process may govern these spreading events.

Does the finding of a novel adhesion site of FnIII3 explain the actual mechanism of inhibition of collagen gel contraction by mAb A3A5? We still do not know about it precisely. The A3A5-binding site resides on FnIII4-5 (*10*). Although we never know whether FnIII4-5 promotes cell spreading, but it may not be active because the repeats lacks FnIII3 from the active repeat of FnIII3-5 in cell spreading. However, FnIII4-5 binds to Fn and inhibits the Fn matrix assembly (*16*). If Fn matrix formation is critical for collagen gel contraction, FnIII3-5 could be involved in the assembly as shown for FnIII4-5 unless FnIII3 is harmful for the assembly. Therefore, it may be worth to examine whether FnIII3-5 or FnIII3 can bind Fn and inhibit Fn matrix assembly. Alternatively, if we take the indirect model of inhibition by A3A5, this effect might be secondary one, that is, A3A5 may bind to FnIII4-5 so as to inhibit the interaction of FnIII3 to cellular component(s) including integrins by a steric hindrance. Anyhow, further mechanistic study to reveal the molecule(s) interacting with FnIII3 at cell surfaces is needed.

The role of the III3 repeat in the regulation of cytoskeletal organization was evaluated by examining its effect on the distribution of actin microfilaments (Fig. 2). HDFs were cultured in serum-free medium on glass plates that had been pre-coated with FN, FnIII3-5, FnIII3, or FnIII4. The plates were blocked with BSA, fixed, and stained for F-actin using Alexa Fluor 594-phalloidin (red, Fig. 2A, D, G, J and M).

Focal contacts are known to be sites of integrin engagement with various ECM proteins together with intracellular proteins, including paxillin, vinculin and tailin (*18*). Cells cultured on FN, FnIII3-5, and FnIII3 displayed thick microfilament bundles (Fig. 2A, D and G), in contrast to the unorganized pattern characteristic of cells on FnIII4 (Fig. 2J) and BSA (Fig. 2M). The merged images of the cells that spread on FN (C), FnIII3-5 (F), or FnIII3 (I) showed colocalization of vinculin with actin. These findings

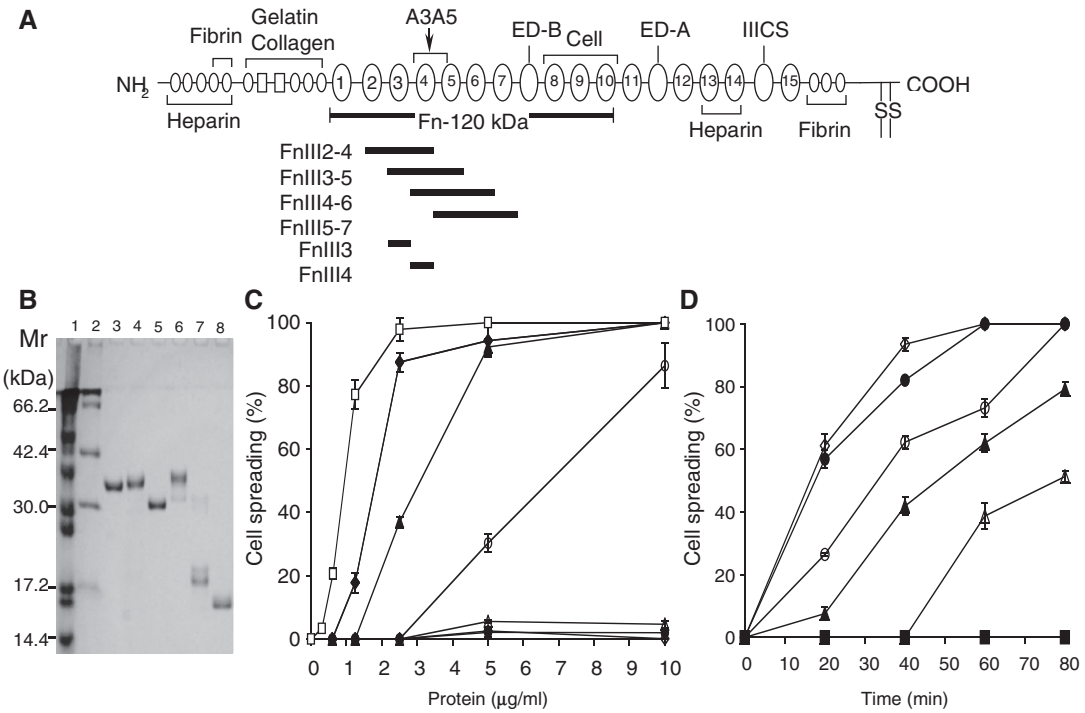

Fig. 1 Adhesive activity of various fibronectin fusion proteins. (A) Schematic of the FN monomer and its expression constructs. The three types of repeating homology units are shown, and the FN type III repeats are numbered. The locations of recombinant FN fusion proteins and Fn-120 kDa, which were used in the cell adhesion assay, are shown below in bold lines. The ligands listed around each domain are bound by that domain. ED-A, ED-B and ED-C, alternatively spliced domain; A3A5, the binding site for a mAb that inhibits collagen gel contraction (*9*). (B) Affinity purification of recombinant FN fusion proteins. His-tagged FN fusion proteins were bacterially expressed, purified and analysed electrophoretically. Lane 1, pre-stained protein standard (New England Biolabs); lane 2, standard proteins (Daiichi); lanes 3–8: affinity purified FN fusion proteins expressing III2-4 repeats (FnIII2-4, lane 3), III3–5 repeats (FnIII3-5, lane 4), III4-6 repeats (FnIII4-6, lane 5), III5-7 repeats (FnIII5-7, lane 6), the III3 repeat (FnIII3, lane 7), and the III4 repeat (FnIII4, lane 8). Proteins were stained with Coomassie blue. Mr represents the molecular weight determined by standard proteins (kDa). The bands of lower molecular mass in lanes 4 and 6 may be degradation products due to *E. coli* proteases. Judging from the sizes of the bands, proteolytic cleavages might have occurred within the C-terminal, non-His tagged, carrier sequence in the vector. (C) Cell spreading of human dermal fibroblasts (HDFs) on various substrates. Fn 120-kDa (open square), FnIII2-4 (open circle), FnIII3-5 (filled triangle), FnIII4-6 (open triangle), FnIII5-7 (filled circle), FnIII3 (filled diamond) and FnIII4 (open diamond) at various concentrations, ranging from 0.3 to 10.0 μg/ml, were coated onto plastic as substrates. HDFs were plated, incubated for 2 h, fixed and scored for spreading. Each point is the average percentage (±SD) of cells that spread in five random fields. (D) Time course of HDF spreading on fusion proteins. Substrates were prepared by coating 96-well clusters with 2 μg/ml Fn 120-kDa (open diamond), 2 μg/ml Vn (filled circle), 10 μg/ml Ln, (open circle) and 5 μg/ml of FnIII3-5 (open triangle), FnIII3 (filled triangle) and FnIII4 (filled square). At the indicated times, cells were fixed and scored for spreading. Each point is the average percentage (±SD) of cells that spread in five random fields.

Table 2. Activities of His-tag fusion proteins.

Protein	Number of aa residues	MW(kDa)[a]	Protein concentration needed to promote 50% cell spreading[b] (μg/ml)	(nM)	Molar ratio
FnIII2-4	332	38.2	6.2	162	1.5
FnIII3-5	320	36.8	2.8	76	0.7
FnIII4-6	312	35.9	No spreading		
FnIII5-7	314	36.1	No spreading		
FnIII3	140	16.1	1.7	106	1
FnIII4	134	15.8	No spreading		
Fn-120kDa		120	1	8.3	0.1

[a]An average MW of 115 Da for a single amino acid was used to calculate the MW of each protein. [b]Cell spreading activity was assayed for each proteins, at concentrations up to 60 μg/ml.

suggest that FnIII3 or FnIII3-5 may organize actin at focal contacts, presumably via integrin(s).

Integrin receptors participate in a number of biological processes, including cell adhesion, cell migration and FN matrix assembly (*8*). In the present study, we evaluated the activity of FN fragments for cell migration using an Oris Universal Migration Assembly Kit (Fig. 3A–C). We tested cell migration activity using FnIII2-4, FnIII3-5, FnIII4-6, FnIII5-7, FnIII3 and III4. As a reference, we included human cellular Fn in the assay (Fig. 3D). As demonstrated in Fig. 3D, Fn stimulated cell migration more efficiently

compared with a non-specific protein BSA as a control. When migration is examined over time, cell migration on Fn in detection zone became evident within 4 h and was mostly linear over 24 h. Among the six clones tested, clones FnIII2-4, FnIII3-5 and III3 stimulated cell migration and followed the same kinetics of migration as shown for Fn. In contrast, three clones, FnIII4-6, FnIII5-7 and III4, displayed substantially reduced activity (Fig. 3D). These results suggest that the potential for the promotion of cell migration may be located in the III3 repeat, but not in the III4 repeat. This conclusion coincides with results on cell spreading analysis for all proteins tested (Fig. 1), suggesting that cell adhesion is the key step for controlling cell migration. Exceptionally, FnIII2-4 was less active than FnIII3-5 at the beginning of cell spreading, but showed similar potential for cell migration each other, implying that initial step of cell to ECM interaction is not always rate-limiting in cell migration, but may be affected differently depending upon the properties of the ECM. The possible intrinsic difference between FnIII2-4 and FnIII3-5 should be clarified in future.

Cell adhesion is a prerequisite for molecular interaction between the cell and the ECM substrate. Cell movement on the substrate involves a number of individual steps, such as adhesion, protrusion, de-adhesion and contraction. During migration on substrate, cells assemble adhesion machinery that is composed of integrins and other intracellular components at focal contact in the leading edge and disassemble it at the trailing edge (*19*). Active remodeling of the ECM by proteolysis and secretion may promote such adhesion dynamics. Cell contraction is also needed to move the body of the cell and may promote the process of disassembly (*20*). To generate motility forces, complexes that interplay between the actin cytoskeleton and adhesion sites organized with ECM proteins are also needed (*19*, *20*). In addition, signals for controlling cell migration are transduced to intracellular proteins by matrix-bound integrin receptors (*18*–*20*). Considering these findings, we propose that

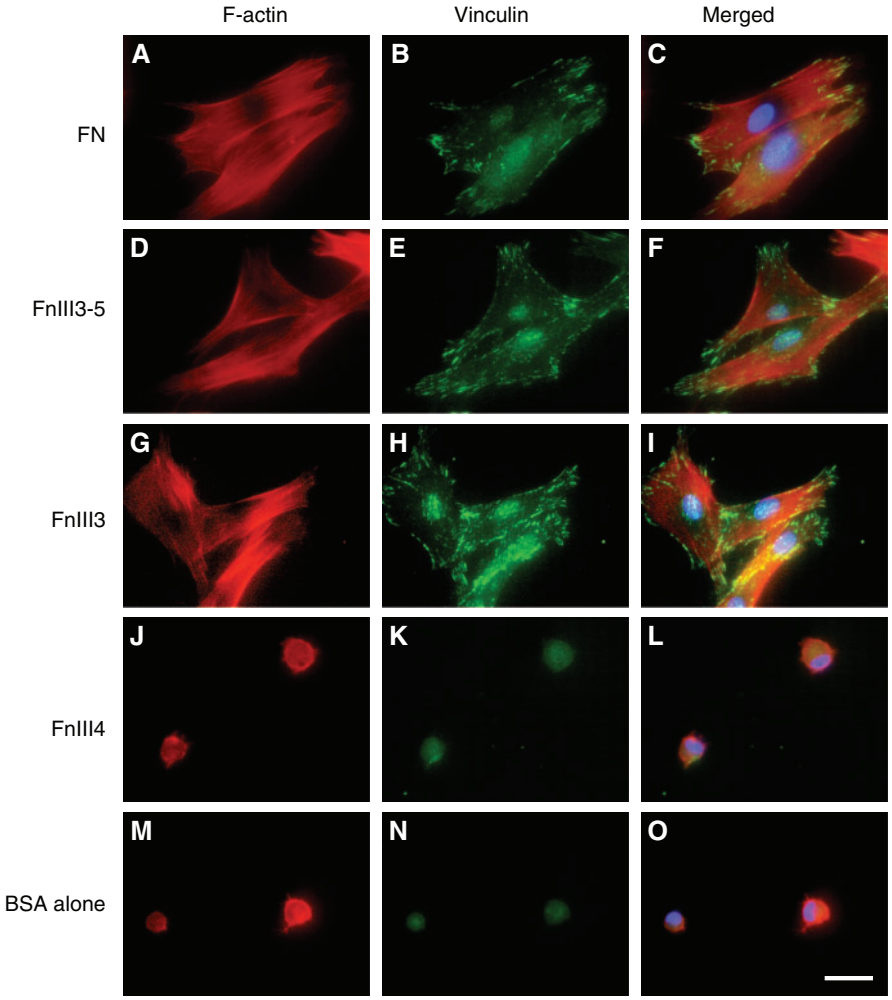

Fig. 2 Organization of focal contacts and stress fibers. Human dermal fibroblasts were incubated on glass plates coated with human cellular FN (A–C), FnIII3-5 (D– F), FnIII3 (G– I) or FnIII4 (J–L) for 4 h and stained for actin with Alexa Fluor 594-phalloidin (red; A, D, G, J and M) or immunologically stained for vinculin to localize the focal contacts with streptavidin-conjugated Alexa Fluor 488 (green; B, E, H, K and N). Overlapping actin and vinculin signals appeared yellow. Cell spreading on plates coated with FnIII4 or BSA alone (M–O) was negligible. The nucleus was stained with Hoechst 33342 (blue). Nuclear images were overlaid with digital Alexa Fluor 594 and Alexa Fluor 488 images (C, F, I, L and O). Bar, 10 μm.

integrin(s) are involved in cell migration mediated by FnIII3, as was previously shown for whole FN molecules (*21*).

ECM is known to involve in many biological events, such as cell adhesion, migration, proliferation and differentiation (*1*). To evaluate the role of cell proliferation on cell migration during the assay, we arrested cell proliferation by treating the cell with an anti-proliferative antibiotic, mitomycin-C (MMC). To determine the lowest effective dose for arresting, we treated HDF cells at different concentration of MMC for 2 h, detached, harvested and incubated in normal culture medium for 3 days. At the beginning of the assay, control cells were fixed and used later in comparison. We stained fixed cells with toluidine blue to quantify cell numbers by measuring absorbance at 640 nm. Figure 4A shows the results. We observed a dose-dependent growth inhibition by MMC in the range of 0.62–40 μg/ml of MMC. The lowest effective dose of MMC was turned to be 10 μg/ml. At the concentration of 20–40 μg/ml, MMC severely damaged the cell. In the absence or presence of MMC at 10 μg/ml, we cultured HDFs for 2 h and used them for cell migration assay. Figure 4B shows the results. The ratio of cell number of MMC-treated to MMC-untreated was 0.91 for Fn; 0.91, FnIII2-4; 0.92, FnIII3-5; 0.89, FnIII4-6; 0.97, FnIII5-7, 0.90, FnIII3, 0.89, FnIII4 and 0.92, BSA, respectively. These results indicate <10% of migrated cells were proliferative and growth suppression has only negligible effect on cell migration. Therefore, we suggest that the results shown in Fig. 3D mostly reflect the difference of potentials for cell migration in each protein tested.

We examined the effect of rat anti-human integrin β1 antibody (mAb13) (*14*) on cell spreading to identify the integrin receptors involved in FnIII3-mediated cell spreading (Fig. 5A). In the absence of antibody, HDFs fully spread on all proteins tested. In the presence of mAb13, spreading was severely inhibited on FN, FnIII3-5, FnIII3, and Ln, but not on Vn. This effect was not seen with rat control IgG, indicating the specificity of the antibody in the assay. These results are consistent with the recognition of a mAb13 shared by FN and Ln (*14*). Thus, cell spreading on FnIII3-5 and FnIII3 may be mediated by an integrin consisting of β1 and one or more unresolved α subunits.

To obtain more information on the unresolved α subunit(s), we examined the cellular specificity of spreading on FnIII3 using the human cell lines, such as HDF, G361 and HepG2, and the murine MC3T3-E1, Swiss 3T3 and B16 cell lines. The adhesiveness of each cell line was tested with FN, FnIII3 and FnIII4 as 2D substrates (Fig. 5B). FnIII4 showed no adhesion with any of the cell lines tested. The HepG2 and B16 cells did not spread on FN or FnIII3. G361 and Swiss 3T3 cells showed intermediate activity on FnIII3, but negligible activity on FN, suggesting the presence of cryptic activity in FnIII3. HDFs and MC3T3-E1 cells showed the highest spreading activity on FN and FnIII3. These results suggest the involvement of a unique type of β1 integrin in cell spreading on FnIII3.

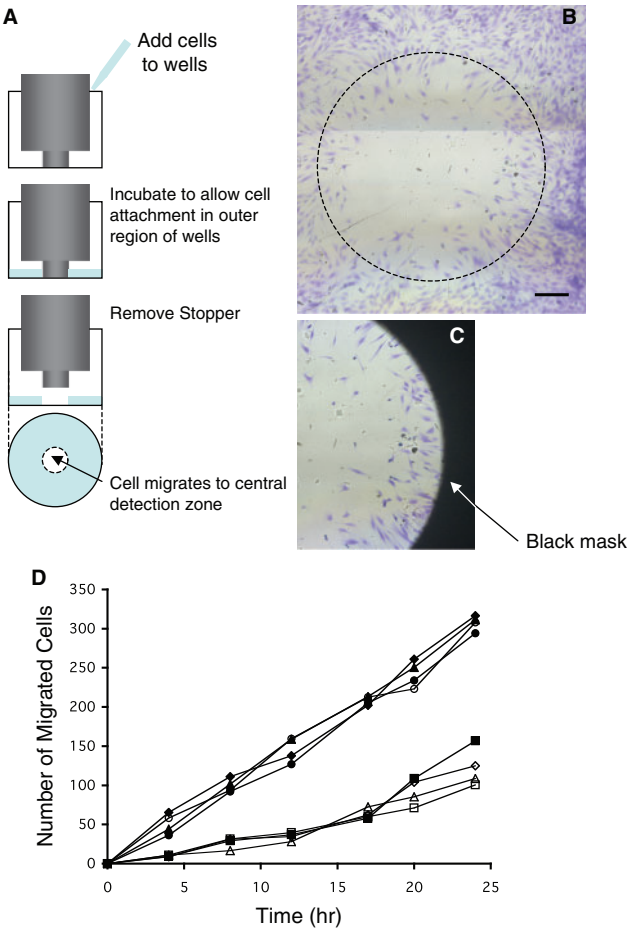

Fig. 3 Cell migration of human dermal fibroblasts on FN fragments. Cell migration on various substrates was assayed with an Oris migration assembly kit as instructed (Platypus Technologies). (A) HDF cells were added to the wells inserted with stoppers and incubated to allow cell attachment at 37°C for 3 h. Then, stoppers were removed to allow the cell migration to central detection zone. Cells were fixed, dye-stained and photographed at low magnification (40×). (B) A photograph of the cells migrated on human cellular FN coated at 10 μg/ml for 20 h. Detection zone is encircled by the black dotted line. Bar, 500 μm. (C) Migrated cells shown in (B) were photographed through the well equipped with a black mask (#) at its bottom. (D) Kinetics of HDF cell migration. The Oris 96-well plates were coated with various substrates, such as human Fn (open circle), FnIII2-4 (filled triangle), FnIII3-5 (filled circle), FnIII4-6 (open triangle), FnIII5-7 (filled square), FnIII4 (open diamond) and FnIII3 (filled diamond) at 10 μg/ml, blocked with BSA, and used for the assay. Control wells were only treated with BSA (open square). At time points indicated, cells appeared within the detection zone were counted through microscopy. At the last time point of 24 h, cells were fixed, stained and counted their numbers in detection zone. Each time point represents the mean of three determinations.

To our knowledge, FN interacts with α4β1, α5β1, α8β1, αVβ1, αIIbβ3, αVβ3, αVβ6, α4β7 and α9β7 integrins (*1*, *5*). Flow cytometry studies have revealed that HDFs express α3β1, α4β1, α5β1, αVβ1, αVβ3 and αVβ5 integrins (*22*). The cell adhesion function of these six integrins is differentially affected by RGD peptides; the first two integrins, α3β1 and α4β1, are classified as non-RGD integrins and are insensitive to the RGD-peptide, whereas the other four are RGD-integrins and sensitive to the peptide (*1*). A previous study with immobilized integrins showed that the inhibitory activity of the cyclic RGD-peptide

cyclo[RGDFV], which contains a D-amino acid residue (underlined), was about 10-fold that of GRGDS for αVβ3 and was equal to that of GRGDS for α5β1 (*23*). Aumailley *et al.* (*24*) reported that cyclo[RGDFV] was more effective than GRGDS for inhibiting cell spreading mediated by Vn in melanoma A375, mammary epithelia HBL and fibrosarcoma HT1080 cells. Three αV integrins (αVβ1, αVβ3 and αVβ5) participate in the recognition of Vn (*1*).

To learn more about the involvement of these integrins in cell spreading on FnIII3, we examined the effects of GRGDS and cyclo[RGDFV] on the spreading of HDFs. The peptide GRGES was also included as an inactive control peptide (Fig. 6). The inhibition profiles for GRGDS and cyclo[RGDFV] were clearly similar to each other, suggesting that the involvement of αVβ1, probably αVβ3 and αVβ5 in FnIII3-dependent cell spreading of HDFs could be excluded. As shown in Fig. 5A, spreading of HDFs on FnIII3 is likely to be mediated by β1 integrins. Taken together, this information leads us to speculate that one of two β1 integrins (α4β1, or α5β1) may be a candidate integrin for FnIII3 in HDFs. However, the involvement of α4β1 is unlikely for the following two reasons. First, α4β1 and α9β1 recognize the LDV sequence and related sequences (*5–7*), but FnIII3 does not contain any of those sequences. Second, although B16 cells preferentially spread on substrates containing the LDV sequence (*6*, *7*), we observed no B16 cell spreading on FnIII3 (Fig. 5B). Therefore, we speculate that α5β1 interacts with FnIII3 in HDFs. Taking into account the complexity of ligand recognition for α5β1, it is possible that it may bind FnIII3. The FN interaction with α5β1 is not restricted to the RGD sequence in FNIII10, as this integrin binds to repeats III9 and III10 as well as to type I(1-9) and type II(1-2) repeats (*25*). Furthermore, α5β1 recognizes another site

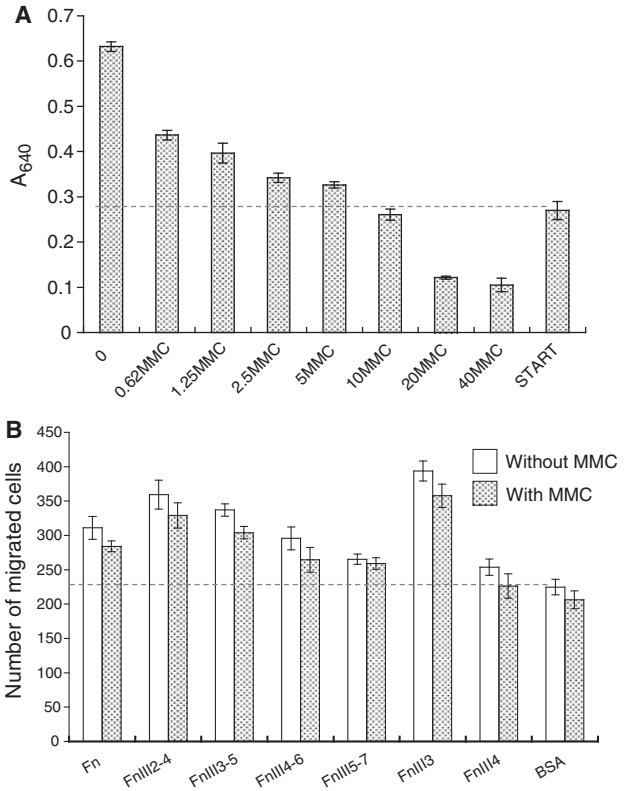

Fig. 4 Effect of mitomycin-C on cell migration. (A) Determination of the lowest effective dose of mitomycin-C (MMC) needed to inhibit cell proliferation. HDF cells were suspended in regular medium, plated onto 96-well culture plate (No. 3072, Beckton Dickinson), and allowed to adhere for 3 h at 37°C. Cells were treated with different concentration of MMC at 0.62 (0.62MMC) to 40 μg/ml (40MMC) as indicated for 2 h at 37°C. At the beginning of the assay, MMC-untreated cells were fixed with PFA and used as a control (START). Meanwhile, HDF cells were fed with fresh medium and incubated further for three days, washed with PBS, fixed with PFA, dye-stained and solubilized into 0.5% SDS in PBS. A_{640} was determined on a Spectrophotometer. Each time point represents the mean of three determinations. The dotted line shows the level of non-proliferated cells. (B) Cell migration of growth-arrested cells. HDF cells were treated with MMC at 10 μg/ml for 2 h at 37°C, harvested by centrifugation and suspended in fresh DME to use for cell migration assay as described in Experimental Procedure. We incubated cells for 16 h in the assay. Each time point represents the mean of three determinations. The dotted line shows the level of a non-specific protein, BSA without treatment by MMC.

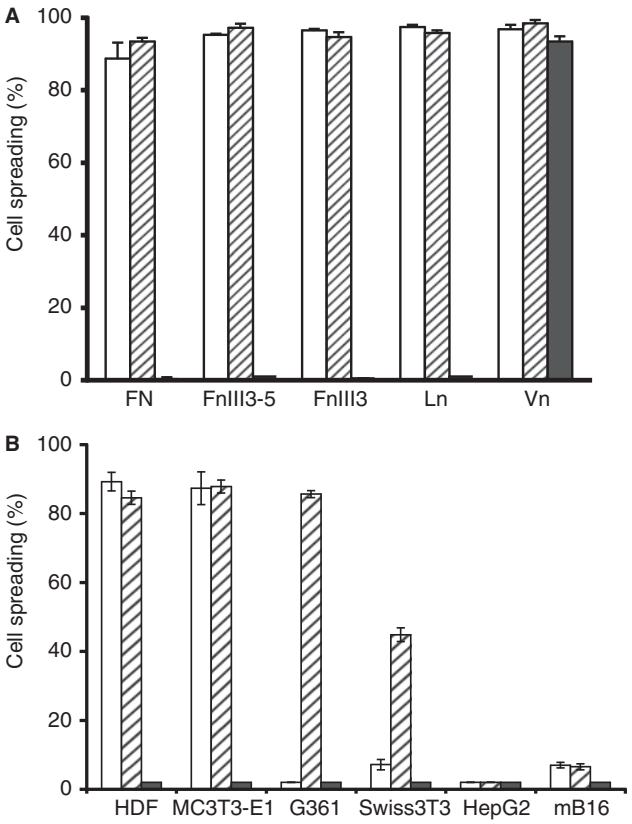

Fig. 5 Analysis of cell adhesion mediated by FN III3. (A) Effect of anti-β1 integrin mAb on human dermal fibroblast (HDF) spreading. Substrates were prepared by coating plates with a 10 μg/ml concentration of plasma FN, FnIII3-5, FnIII3 and Ln, or 5 μg/ml of Vn. HDFs were added alone (white bars), or in the presence of non-immune control IgG (10 μg/ml, crosshatched bars) or anti-β1 mAb13 (5 μg/ml, black bars). Each point is the average percentage (±SD) of cells that spread in five random fields. (B) Cell type-specific cell spreading on FnIII3. The 96-well plastic plates were coated with 5 μg/ml of plasma FN (white bars), FnIII3 (crosshatched bars) or FnIII4 (black bars) as substrates. Different types of cells as indicated were plated onto the substrates and cultured for 2 h, to examine their adhesive properties. Each point is the average percentage (±SD) of cells that spread in five random fields.

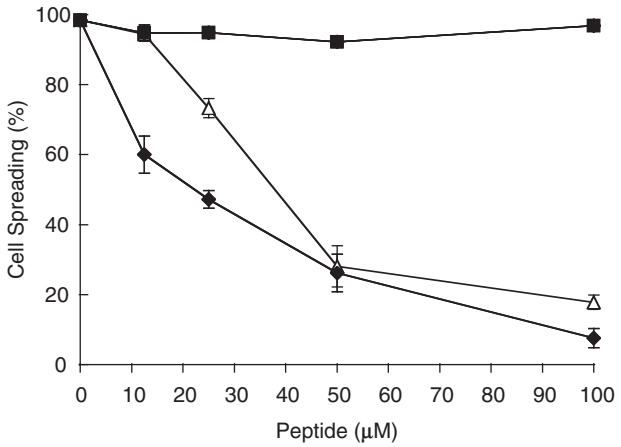

Fig. 6 Effect of RGD-peptides on fibronectin III3-dependent human dermal fibroblast spreading. Cell spreading of human dermal fibroblasts on FnIII3 substrates was examined in the presence of GRGDS (filled diamond), cyclo[RGDFV] (open triangle) and GRGES (filled square). Underlined letter is the D-Phe residue. The concentration of FnIII3 was 5 μg/ml. Each point is the average percentage (±SD) of cells that spread in five random fields.

in addition to RGD that synergizes with RGD for high-affinity FN recognition (*3*, *12*). Our RT-PCR analysis revealed that HepG2 cells express negligible levels of α5 mRNA (data not shown), and HepG2 cells were never observed to spread on FN or FNIII3 (Fig. 5B). Alternatively, if we assume that α5β1 integrin is a functionally dominant receptor needed for spreading on Fn in G361 and Swiss 3T3 cells, those cells might have to spread also on the FN. However, we found that G361 and Swiss 3T3 cells spread on FNIII3, but not intact FN (Fig. 5B). Therefore, this result suggests that receptors other than α5β1 integrin could be involved in the cell spreading on FNIII3. It is also conceivable that secondary receptor(s), such as those described for α3Aβ1 (*26*), may be involved in cell adhesion to FnIII3. More importantly, our studies do not rule out the involvement of other integrins in the interaction with FnIII3.

In summary, we demonstrated for the first time that FnIII3 promotes cell spreading of both HDFs and mouse pre-osteoblast cells and mediates cryptic cell spreading of human melanoma cells and mouse Swiss 3T3 cells. We also revealed the inhibitory effect of an anti-human integrin β1 antibody and RGD peptides on FnIII3-mediated cell spreading of HDFs. The results also provide evidence that FnIII3 promotes cell migration, presumably via RGD-dependent integrins. Consequently, this study raises the interesting possibility that FnIII3 may contain a novel sequence(s) recognized by β1 integrin(s) combined with a specific, but unresolved, RGD-dependent α-subunit(s). Further studies on FnIII3-mediated cell spreading should allow the determination of key sequences important for cell adhesion as well as the identification of their binding receptor integrins.

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Conflict of interest
None declared.

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