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Matrix Assembly Induction and Cell Migration and Invasion Inhibition by a 13-Amino Acid Fibronectin Peptide*

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Fibronectin (FN) is an extracellular matrix (ECM) protein involved in tumor growth and metastasis. Five human FN cDNA segments encoding for FN fragments, all starting with the II1 repeat and ending with different C-terminal extensions, have been stably expressed in chick embryo fibroblasts (CEF). These FN cDNAs induce the formation of an organized ECM in CEF as long as they retain a sequence coding for a 13-amino acid stretch (FN13), with collagen binding activity, localized between type II2 and I7 repeats. An FN13 synthetic peptide induces in control CEF the assembly of an FN-ECM comparable with that observed in CEF-expressing FN fragments. The activity of FN13 is specific for its amino acid sequence, although the cysteine present in the 6th position can be substituted with a polar serine without affecting the induction of a fibrillar FN-ECM. A less fibrillar matrix is induced by FN13-modified peptides in which the cysteine is methylated or substituted by a non-polar alanine. FN13 induces the assembly of an FN-ECM also in Rous sarcoma virus-transformed CEF lacking the ECM and in hepatoma (SK-Hep1) and fibrosarcoma (HT-1080) human cell lines. FN13 also promotes the adhesion of CEF and Rous sarcoma virus-CEF at levels comparable with those obtained with purified intact FN. Finally, FN13 inhibits the migratory and invasive properties of tumorigenic cells, whereas intact FN favors their migration. All FN13-modified peptides show similar effects, although with reduced efficiency. None of these activities is supported by a scrambled peptide. These data suggest a possible role of FN13 in tumor growth and metastasis inhibition and its possible use as anti-tumorigenic agent.

Fibronectin (FN)[1] is an adhesive heterodimeric glycoprotein present in the extracellular matrix (ECM) of connective tissues in disulfide cross-linked insoluble fibrils and in the blood in dimeric soluble form. FN contains three types of homologous repeats (I–III), organized in functional domains, connected by flexible, protease-sensitive segments, which allow the binding of the molecule to ECM components (fibrin, heparin, collagen, FN, and integrin receptors) (1–3). Through these multiple interactions, FN provides a scaffold for ECM assembly and takes part in different physiological and pathological processes (4–7).

One of the earliest observations concerning FN was that *in vitro* transformed and tumor-derived cells often fail to deposit a matrix, whereas the normal counterparts do have a matrix (8, 9). Because addition of FN to tumor-derived cultured cells improves cell adhesion and induces ECM and cytoskeleton organization, supporting the normal cell morphology, FN has been associated with the normal cell phenotypes (6, 10, 11). The inability of the transformed cells to deposit the ECM has been related to the proteolytic fragmentation of FN generated by the enhanced levels of proteases released by tumors and transformed cells (12–14), as well as to the down-regulation of the expression of integrin receptors binding to FN and supporting ECM assembly (7). Along these lines are also observations that an excessive ECM, containing collagens and FN, *i.e.* desmoplasia, is often deposited in stroma surrounding invasive carcinomas (15). It has been proposed that this stromal response temporarily antagonizes tumor growth and invasion (14, 16). Thus, the absence of an ECM of FN is associated with the transformed phenotype, whereas the presence of the ECM restricts cell invasion and migration in many tumor cells. The transition from assembly to non-assembly of the ECM may therefore be an important stage in cancer progression.

The assembly of FN into fibrils *in vitro* and *in vivo* requires multiple binding sites within FN including an N-terminal region consisting of the first five type I repeats (17–21), the RGD cell-binding site (22–23), the synergistic cell adhesive regions (24–25), the I_{12} repeat involved in the stabilization of FN fibrils (26), and a site located at or near the junction of type I_9 and type III_1 repeats (27–28). In addition, an FN-FN-binding site has been identified in a 14-kDa FNfg containing the first two type III repeats (29). A 76-amino acid FN peptide, including this site, corresponding to a C-portion of the type-III_1 repeat, has been shown to convert FN into a polymeric fibrillar form called superfibronectin (sFN) (29). sFN resembles the matrix fibrils produced by cultured fibroblasts, is highly adhesive, can inhibit cell spreading and migration *in vitro*, prevents tumor formation in nude mice injected with human tumorigenic cells, and has a strong antimetastatic activity (30). Recently, the

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[1] The abbreviations used are: FN, fibronectin; Ab, polyclonal antibody; CEF, chick embryo fibroblasts; COL-domain, collagen-binding domain; COLI, type I collagen; COL-site, collagen-binding site; ECM, extracellular matrix; FNfgs, fibronectin fragments; FN13, 13-amino acid collagen-binding fibronectin peptide; hFN, human fibronectin; IF, immunofluorescence microscopy; QISH, quantitative *in situ* hybridization; RCASBPFN, retroviral fibronectin constructs; RSV, Rous sarcoma virus; sFN, superfibronectin; mAb, monoclonal antibody; RT, reverse transcriptase; FBS, fetal bovine serum; MEM, minimum Eagle's medium; PBS, phosphate-buffered saline; IOD, integrated optical density; sFN, superfibronectin.

76-amino acid peptide assembling FN *in vitro* has been reported to suppress tumor growth, angiogenesis, and metastasis in mice even in the absence of FN (31).

The study of proteolytic FNfgs has disclosed cryptic biological activities not shared by the native protein (32–36). In particular, the FN collagen binding domain (COL-domain), in addition to binding with collagens and gelatin (3, 37–40), has been shown to possess a number of other biological activities including the expression of collagenase activity (35, 41–42), the promotion of odontoblast differentiation (43), and the substratum-dependent stimulation of fibroblast migration (44).

In this work we expressed five human recombinant FNfgs, spanning from the II_1 to the III_2 repeat of the molecule and encompassing the COL-domain in CEF, and we studied their ability to induce the FN-ECM organization. All FNfgs but one, which lacked the sequence coding for a 13-amino acid stretch with collagen binding activity, induced the organization of the FN-ECM either in control or in RSV-CEF. Therefore, we restricted the ECM organization capability of FNfgs to this 13-amino acid sequence. We demonstrate that the synthetic peptide corresponding to this sequence (FN13) induces the assembly of FN in cultured cells, enhances cell adhesion, and inhibits Matrigel matrix invasion of RSV-CEF and human tumor cell lines.

MATERIALS AND METHODS

Antibodies and Synthetic Peptides—In this work we used an anti-human FN rabbit polyclonal antibody (Ab) (Sigma) and three anti-FN mAbs: the f33 anti-human FN (hFN), recognizing an epitope located in the C terminus of the 120–140-kDa catheptic FNfg located downstream of the COL-domain (45); the f25 mAb, recognizing an epitope located at the N terminus of the same fragment, either in human or, improved with efficiency, in chick FN[2]; and the f29 anti-hFN mAb, recognizing an epitope in the 14-kDa hFN catheptic fragment flanking the COL-domain (46). Anti-chick FN and anti-chick COLI Abs were kindly provided by A. Colombatti (Aviano, Italy).

Five 13-amino acid synthetic peptides (Primm, Milan) were used: FN13 (AHEEICTTNEGVM), containing 13 amino acids of hFN COL-site and differing from this binding site as regards the absence of an N-terminal A (47); FN13Ser and FN13Ala, differing from FN13 regarding substitution of the cysteine in the 6th position from the N terminus with a serine, or an alanine, respectively; FN13Mod, identical to the FN13 peptide sequence with a methylated cysteine and FN13 scrambled sequence peptide (ScrFN13) (ITCETNEGEVAMH). Mass spectrometry was performed by the manufacturer for all peptides, with a 95% purity assessed by high pressure liquid chromatography.

Cloning System—The cloning system consisted of two vectors: the miniplasmid Cla12NCO and the retroviral vector RCASBP (48). Cla12NCO is an adaptor plasmid for RCASBP in which it is possible to clone DNA in a polylinker flanked by *Cla*I restriction sites. After *Cla*I restriction, the cloned DNA can be inserted in the unique RCASBP *Cla*I site. RCASBP contains the 5′- and 3′-long terminal repeats and the complete coding sequence for Gag, Pol, and Env proteins. The expression of the DNA inserted in this site is under the control of the long terminal repeat promoters and is translated from the start site present in the *Cla*I polylinker; after transfection and integration in CEF, RCASBP genes are expressed at high levels and direct the production of non-transforming retroviruses that infect all cells in culture.

FN cDNA Constructs—The cDNA coding for the human FN type II_1-III_2 repeats (II_1-III_2 fragment, 1.47 kb) (Fig. 1) was obtained by pFH134 plasmid *Hin*dIII and *Pvu*II restriction (49) and dimerized with T4 ligase. A palindromic self-annealing *Sac*I linker (5′GTGTG-GAGCTCCACAC3′) was ligated to the *Pvu*II blunt ends; the modified II_1-III_2 cDNA was digested with *Hin*dIII and *Sac*I and inserted in the *Sac*I- and *Hin*dIII-linearized/Cla12NCO plasmid. A 95-bp cDNA fragment coding for the hFN secretion signal peptide was obtained by PCR on pgHF3.7 plasmid (50) with the primers 5′dACATGCTTAGGGGTC-CGG3′ and 5′rCCTCTTGCTCTTCGAGGC3′ and Vent *Taq* DNA polymerase (New England Biolabs, Beverly, MA), and inserted in-frame upstream of the II_1-III_2 cDNA in the filled-in Cla12NCO polylinker *Eco*RI site. From this recombinant plasmid, 4 FN cDNA fragments, maintaining a common 5′ end and lacking progressive portions at the 3′ end, were generated by restriction digestion (Fig. 1) as follows: the II_1-I_9 (0.9 kb), lacking type III_1 and III_2 cDNA; the II_1-I_8 (0.7 kb), lacking the I_9 cDNA; the II_1-II_2 (0.5 kb), lacking I_8, and a portion of I_7 (114 bp from the 3′ end) cDNA. The II_1-II_2 cDNA contains 30 bp of I_7 5′ end and maintains the COL-site sequence (AAHEEICTTNEGVM) spanning from the type II_2 to the adjacent type I repeat (47). The last cDNA fragment, the II_1-II_2del (461 bp), derives from the II_1-II_2 construct without the 39 bp at the 3′ end encoding for the COL-site without the first alanine (47).

Recombinant Cla12NCO plasmids were transformed in KH802 *Escherichia coli* strain (51); after amplification, they were purified by Endo Free Plasmid Maxi and QIAprep Spin Miniprep kits from Qiagen (Diagen, GmbH, Germany); the FN cDNA inserts were excised by *Cla*I digestion and ligated in the *Cla*I-linearized RCASBP. After transformation in *E. coli*, the recombinant retroviral vectors, carrying the FN cDNA inserts in the correct orientation, were selected by restriction mapping and PCR analysis. Five recombinant RCASBPs containing the different FN cDNAs were generated (RCASBPFN constructs) with FN cDNAs inserted in-frame, as ascertained by sequence analysis.

Cell Cultures—Primary cultures of CEF were prepared as described previously (52). Secondary CEF were grown in Hanks' MEM with 5% (v/v) newborn calf serum (Invitrogen), 10% (v/v) tryptose phosphate broth, 100 μg/ml streptomycin, and 100 IU/ml ampicillin. Normal human adult skin fibroblasts were prepared in our laboratory from control donor skin biopsy. Human fibrosarcoma (HT-1080), hepatoma (SK-Hep1), cervix epithelioid carcinoma (HeLa), and rhabdomyosarcoma tumor-derived cell lines were from the ATCC. Human cells were grown in Earle's MEM, 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. For SK-Hep1 cells the medium was supplemented with 1% (v/v) sodium pyruvate and non-essential amino acids.

CEF Transfection and Infection—Secondary cultures of CEF were transfected using the calcium phosphate method (53) with empty RCASBP expression vector or with RCASBPFN constructs using the CellPhect transfection kit (Amersham Biosciences). In particular, 15 μg of retroviral vectors were co-precipitated with calcium phosphate for 30 min and added to monolayers of CEF. After 4 h of incubation at 37 °C, the media were removed. The cells were washed twice with PBS, and complete medium was added. The cells were maintained at 37 °C in a 5% CO_2 incubator.

Secondary CEF were seeded on round coverslips (1.5 cm inner diameter in a 24-well plate (Corning Costar Corp., Cambridge, MA)) (40,000 cells/well) in Hanks' MEM supplemented as above and infected with 30 *focus*-forming units of PA1 SR-RSV, subgroup D mutant (52). After 24 h of incubation at 35 °C, the medium was changed, and after 24 h, the cultures were treated with purified FN and FN13.

RT-PCR—The expression of human FN cDNAs, transfected in CEF in the RCASBP vector, was analyzed by RT-PCR on total RNAs and purified from control and transfected CEF at the 4th *in vitro* passage after transfection, with Trizol reagent (Invitrogen). One μg of total RNA was reverse-transcribed by standard procedures; PCR was carried out using the primers 5′dGCGGTAGCTGGACGTGC3′, corresponding to 17 nucleotides of Cla12NCO linker, and 5′rCCTCTTGCTCTTCGAGGC3′, corresponding to 18 nucleotides of FN secretion signal sequence, present in all constructs, using a standard protocol. The product of the reaction was a 133-bp DNA fragment in all transfectants integrating and expressing the RCASBP constructs. The endogenous FN mRNA expressed by control and by transfected CEF was evaluated by RT-PCR performed with a set of primers amplifying a 419-bp segment of chick FN cDNA encoding for a fragment that spans from the III_8 to the III_{10} repeat absent in hFNfgs (54).

Quantitative in Situ Hybridization (QISH)—The expression of human FN cDNAs in CEF transfected with the different RCASBP constructs was analyzed at different *in vitro* passages by *in situ* hybridization with a 461-bp FN cDNA probe (*i.e.* the II_1-II_2del fragment) labeled by nick translation (Invitrogen) in the presence of unlabeled dCTP, dGTP, and dTTP (6 μM each) and of an excess of [α-^{33}P]dATP (2000 Ci/mmol) (PerkinElmer Life Sciences), according to the supplier's instructions. *In situ* hybridization was performed as reported previously (55) on CEF cultured on microscope slides, and the hybridization grains were quantitatively evaluated with the Magiscan Image Analysis System (Joyce Loebl, Gateshead, UK).

FN-ECM Analysis by Immunofluorescence Microscopy (IF)—8×10^4 CEF not transfected and transfected with the different RCASBPFN constructs were seeded on 22 × 22-mm glass coverslips and grown in Hanks' MEM, 10% tryptose phosphate broth, and 5% newborn calf serum at 37 °C in a 5% CO_2 incubator. After 48 h, the cells were washed in PBS, methanol-fixed twice for 20 min at 4 °C, air-dried, and immu-

[2] S. Barlati and A. Vaheri, unpublished results.

noreacted with anti-chick FN and anti-chick-COLI Ab and with the f25 mAb. Anti-FN and anti-COLI Ab, 1:100 in 0.3% bovine serum albumin, 0.01% NaN_3 (diluting buffer), and f25 mAb, 1:10 in diluting buffer, were reacted for 30 min at room temperature. After washing 3 times for 3 min in PBS, the cells were reacted with rhodamine- or fluorescein-conjugated anti-rabbit or anti-mouse IgG (1:100 in diluting buffer) for 30 min at room temperature, washed 3 times for 5 min in PBS, mounted on glass slides in 1:1 PBS/glycerol solution, and photographed with a Leitz fluorescence microscope.

The assembly of the FN-ECM was also analyzed by IF in non-transfected CEF cultured in the presence of purified hFN (New York Blood Center Inc.) or of FN13, FN13Mod, FN13Ser, FN13Ala, and ScrFN13 synthetic peptides. Increasing concentrations of peptides were added to the medium (from 5 to 80 μg/ml) for 48 h, and the cells were immunoreacted either with anti-hFN f25 mAb or with the anti-chick COLI Ab.

The FN-ECM IF analysis was also performed on secondary CEF, before and after RSV infection, and on human skin fibroblasts, hepatoma (SK-Hep1), and fibrosarcoma (HT-1080) tumor-derived cell lines, grown in the absence and in the presence of conditioned media recovered from transfected CEF expressing the different FNfgs, or of 5–10 μg/ml hFN, or of 40 μg/ml FN13. After 48 h treatment, the cells were immunoreacted with f25 (CEF) and f33 (human cells) FN mAbs as reported above.

Quantitative evaluation of the fluorescence (associated with the ECM organized by control, transfected, and peptide-treated CEF and by SK-Hep1 and HT-1080 tumor cells) was performed as follows. The fluorescent images, with the same spatial resolution and comparable light intensity, were captured by a CCD black and white TV camera mounted on a Zeiss Axiovert 10S/H fluorescence microscope. By using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD), a task list file was developed that could be executed in semi-automatic mode on different images. Each image was processed by applying Sharpen and Rank nonlinear filters to optimize the image contrast and to remove the background noise. A binary image, visualized as a red overlay, corresponding to the fluorescence signal, was obtained by setting two thresholds in the fluorescence peak corresponding to the gray tones of the image visualized, with the lighter tones at the highest value of the peak and the darker ones below the slice background. The sequence of options in the task list was repeated on four images captured on each slide; the area without cells was subtracted from the field area containing the fluorescence signal, and the integrated optical density (IOD), related to the field area containing the signal, was measured and normalized to the cell number present in each image.

Cell Adhesion Assay—The effect of FN13 on CEF and CEF-RSV adhesion was tested by seeding 4×10^4 cells in 24-well plates (Corning Costar, Italy) coated with 0.5–10 μg/ml FN13 or with 10 μg/ml hFN. The adhesion assay was also performed on human control skin fibroblasts, HT-1080 and SK-Hep1 tumor cell lines in the presence of 10 μg/ml hFN or FN13. Coating of hFN and FN13 diluted in PBS was performed for 2 h at 37 °C followed by blocking with 0.5% bovine serum albumin for 1 h at room temperature. After medium removal, the cells were allowed to adhere for 1 h at 37 °C in a humidified 5% CO_2 incubator. Cell adhesion was quantified by staining the cells with crystal violet in 20% methanol, rinsing with water, and counting the cells with an optic microscope. The reported values are means ± S.D. of three independent measurements.

Cell Migration Assay—The effect of FN13, FN13Mod, FN13Ser, FN13Ala, and ScrFN13 peptides on cell migration was studied using the Transwell 8-μm filter (Corning Costar, Cambridge, MA) migration assay. 5×10^4 control and RSV-CEF, human control skin fibroblasts, HT-1080 and SK-Hep1 cells, resuspended in serum-free culture medium, were plated onto the upper chamber and allowed to migrate for 6 h through the polycarbonate filter into the lower chamber. The bottom wells were filled with culture medium supplemented with or without 10 μg/ml hFN and the different FN13 peptides. Migrating cells collected in the bottom chamber were counted, and their number is reported as the average of three independent experiments.

Cell Invasion Assay—Transwell 8-μm filters were coated with 150 μg/filter Matrigel basement membrane matrix (BD Biosciences) diluted in cold PBS. 2×10^5 human control fibroblasts, HT-1080, and SK-Hep1 tumor cells, suspended in Dulbecco's modified Eagle's medium containing 10% heat-decomplemented FBS supplemented or not with 40 μg/ml FN13, FN13Mod, FN13Ser, FN13Ala, ScrFN13 peptides or purified hFN, were added to the upper Transwell chamber. Conditioned medium from human control fibroblasts, grown in the absence of FBS, was placed in the lower chamber. The assays were carried out at 37 °C in 5% CO_2 atmosphere for 48 h; the non-invasive cells were removed with a cotton swab, and the filters were fixed in methanol and stained with 5% (v/v) Giemsa. All cells adhering to the filters were counted. Each assay was performed in triplicate.

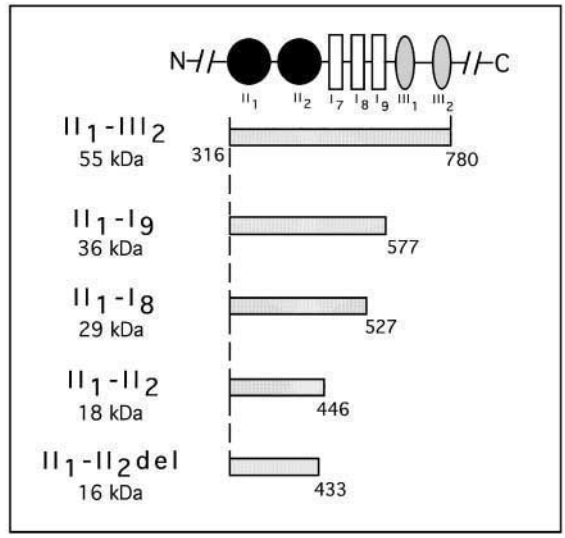


FIG. 1. **FNfgs coded by the cloned hFN cDNAs segments aligned with a portion of the FN monomer showing its structural organization.** All FNfgs start at amino acid 316 and end at the amino acid indicated *below* each fragment.

RESULTS

Construction and Expression of Recombinant FN cDNAs—Five cDNAs encoding for human FNfgs with a common N terminus and different C termini (Fig. 1) were inserted in the retroviral vector RCASBP and transfected in secondary CEF. The transfected cells integrated and expressed the recombinant FN cDNAs, as shown by RT-PCR performed on RNAs purified at the 4th *in vitro* cell passage (Fig. 2*A*). The level of endogenous FN mRNA was similar in control and transfected CEF, as shown by RT-PCR performed with a set of primers amplifying an FN mRNA region between type III_8 and III_{10} repeats absent in the hFN cDNAs (not shown) (54).

By using the RCASBP vector and the CEF as a cloning system, selection of the transfectants was not necessary because the cells expressing the retroviral RNA also produced recombinant viruses that infected the non-transfected cells present in the cultures. In order to obtain the different CEF populations homogeneously expressing the recombinant FN cDNAs, the transfectants were analyzed at increasing *in vitro* passages by QISH, using the II_1-II_2del radiolabeled FN cDNA as a probe. Starting from the 4th *in vitro* passage, QISH showed in all transfectant populations the presence of a given percentage of CEF expressing high levels of FN mRNA hybridizing with the II_1-II_2del probe (about 400 pixels per cell) and a low hybridization signal (less than 50 pixels per cell) in non-transfected CEF, corresponding to the basal endogenous FN mRNA level detected in these cells with the human probe (Fig. 2*B*). The number of CEF expressing high levels of hFN mRNAs increased following the *in vitro* passages and reached 100% between the 8th and the 10th culture passage.

Extracellular Assembly of FN and COLI in Transfected CEF—Cultures of CEF, CEF transfected with the empty RCASBP and with the different RCASBPFN constructs at the 10th *in vitro* passage, were analyzed by IF using the f25 mAb (Fig. 3*A*) and the anti-COLI Ab (Fig. 3*B*). In control CEF, as well as in CEF transfected with RCASBP empty vector, f25 mAb detected extracellular FN not organized in a fibrillar ECM

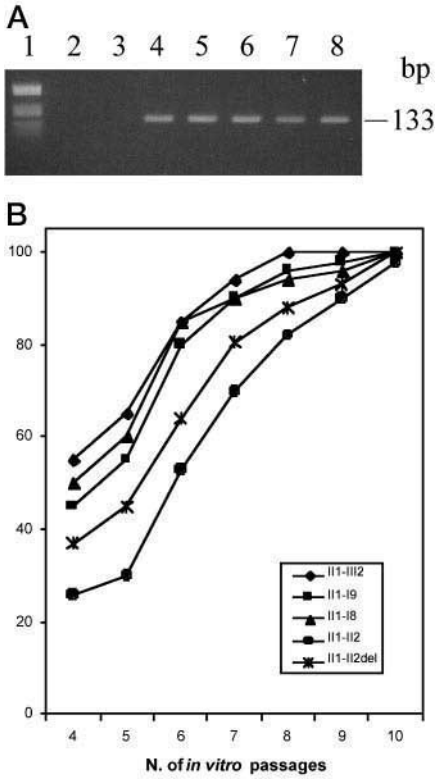


FIG. 2. **FN recombinant cDNAs are expressed by transfected CEF.** The expression of FN mRNA fragments was detected by RT-PCR using primers specifically recognizing these inserts (*A*) and by QISH (*B*). *A*, DNA markers (*lane 1*); control CEF (*lane 2*); CEF transfected with RCASBP empty vector (*lane 3*), with RCASBP-FNII$_1$-III$_2$ (*lane 4*), with RCASBP-FNII$_1$-I$_9$ (*lane 5*), with RCASBP-FNII$_1$-I$_8$ (*lane 6*), with RCASBP-FNII$_1$-II$_2$ (*lane 7*), and with RCASBP-FNII$_1$-II$_2$del (*lane 8*) recombinant vectors. *B*, percentage of transfected cells expressing the different hFN mRNA fragments at increasing *in vitro* passages as detected by QISH performed with the II$_1$-II$_2$del radiolabeled fragment.

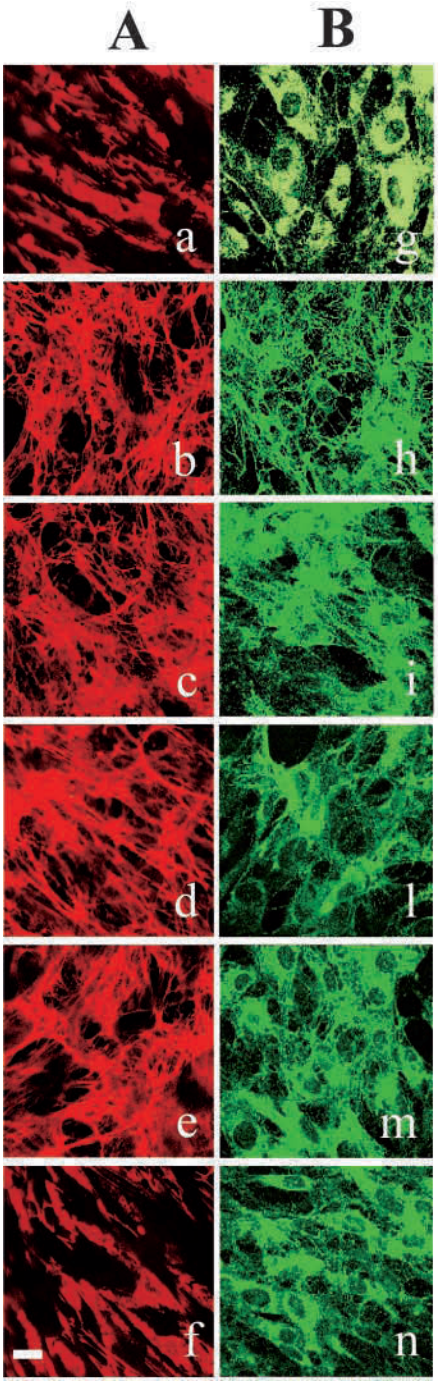


FIG. 3. **Transfected CEF expressing hFNfgs organize the FN-ECM.** IF on RCASBP (*a* and *g*) and on RCASBPFNs transfected CEF (*all other panels*) using the f25 anti-FN mAb (*A*) and the anti-COLI Ab (*B*). The transfectants, at the 10th *in vitro* passage, contain and express the II$_1$-III$_2$ (*b* and *h*), the II$_1$-I$_9$ (*c* and *i*), the II$_1$-I$_8$ (*d* and *l*), the II$_1$-II$_2$ (*e* and *m*), and the II$_1$-II$_2$del (*f* and *n*) cDNAs. *Scale bar*, 5–10 μm.

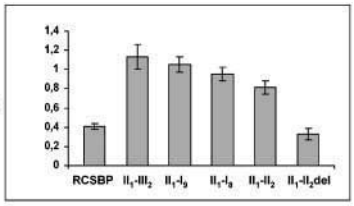
(Fig. 3*A*, *a*); on the contrary, in CEF transfected with the different cDNA fragments, a well organized and fibrillar FN-ECM was detected (Fig. 3*A*, *b–e*), the only exception being the FNII$_1$-II$_2$del (Fig. 3*A*, *f*). FNII$_1$-II$_2$del-transfected cells expressed and organized an FN-ECM only when they were maintained for 1 week in culture; at this time the matrix was still absent in control CEF (not shown). Quantitative evaluation by image analysis of the fluorescent FN signals shown in Fig. 4 (*a* and *b*) shows that the highest level of the IF signal was present in CEF transfected with the FNII$_1$-III$_3$ cDNA, overlaid by an FN-ECM with a 2.7-fold average increase in fluorescence compared with CEF transfected with the empty RCASBP vector. The fluorescent signal was slightly decreased (from 2.6- to 1.9-fold) in CEF transfected with the shorter FN cDNAs. On the contrary, a slight reduction (0.8-fold increase), compared with CEF transfected with the RCASBP vector, was measured in cells expressing the FNII$_1$-II$_2$del. An FN organization very similar to that reported above was obtained by detecting FN with a polyclonal anti-chick FN Ab. Similar FN-ECM structures were also obtained by adding to culture medium of control CEF 10 μg/ml purified hFN or by growing control CEF for 2 days in the presence of the conditioned media from the five transfectants (not shown). These results indicate that the transfected CEF express FN polypeptides capable of inducing the assembly of endogenous FN in an organized ECM, as well as intact FN. The expression of the human FNfgs could only be verified in cells transfected with the largest FN cDNA containing an epitope recognized by the f29 anti-hFN mAb (45), but not in the other transfectants expressing FNfgs, for which antibodies have never been isolated, due to the evolutionary conservation of this sequence (not shown).

Comparable results were also obtained following IF analysis of COLI (Fig. 3*B*, *g–n*). Whereas control CEF mainly showed COLI at a cytoplasmic level, transfected CEF showed the organization of this protein into the ECM. In the FNII$_1$-II$_2$del transfectant COLI was mainly detectable as an intracellular signal. Quantitative evaluation of the COLI organized by the transfectants, performed by image analysis on the immunore-

a

Sample	IOD	FI
RCASBP	0.41 ± 0.03	1.0
II_1-III_2	1.13 ± 0.13	2.7
II_1-I_9	1.05 ± 0.08	2.6
II_1-I_8	0.95 ± 0.07	2.3
II_1-II_2	0.81 ± 0.07	1.9
II_1-II_2del	0.33 ± 0.06	0.8

b



c

Sample	IOD	FI
RCASBP	0.21 ± 0.02	1.0
II_1-III_2	0.39 ± 0.01	1.8
II_1-I_9	0.36 ± 0.02	1.7
II_1-I_8	0.34 ± 0.01	1.6
II_1-II_2	0.37 ± 0.01	1.7
II_1-II_2del	0.35 ± 0.03	1.6

d

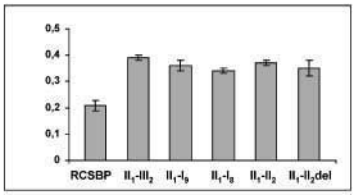


FIG. 4. **Quantitative image analysis of the FN-ECM (*a* and *b*) and COLI-ECM (*c* and *d*) organized by CEF transfected with hFN cDNAs.** The quantitative evaluations were performed in triplicate and are reported as IOD; each evaluated field contained on average 23 ± 1 cells. *FI*, fold increase.

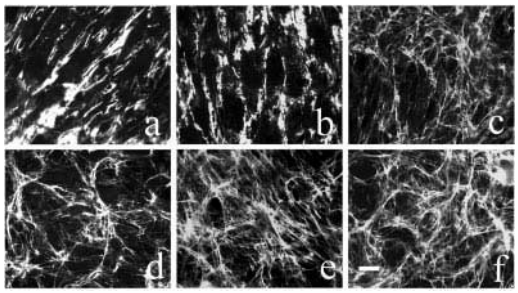


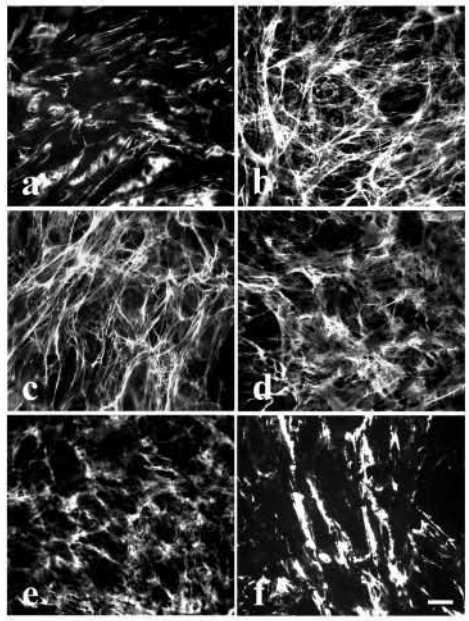
FIG. 5. **Effect of increasing concentrations of FN13 on the FN-ECM assembled by control CEF, as detected by IF with the f25 anti-hFN mAb.** *a*, untreated CEF; *b–f*, CEF treated with 5, 10, 20, 40, and 80 μg/ml FN13. *Scale bar*, 8 μm.

acted cells and shown in Fig. 4 (*c* and *d*), shows that irrespective of the degree of COLI organization, all transfectants expressed about a double amount of the protein compared with control CEF.

Identification of an FN Peptide Inducing FN and COLI Organization—The FNfgs expressed by CEF, the only exception being the $FNII_1$-II_2del fragment, contain the main COL-site of the molecule (47), the AHEEICTTNEGVM peptide absent in the $FNII_1$-II_2del fragment. This 13-amino acid peptide (FN13) was synthesized, and its effect on the assembly of FN was evaluated. Increasing concentrations of FN13 were added to the culture medium of control CEF, and the organization of FN into the ECM was analyzed by IF with the f25 anti-FN mAb. As shown in Fig. 5, FN13 starting from 5–10 μg/ml was capable of inducing the assembly of a fibrillar FN-ECM. At increasing peptide concentrations a thicker and closer FN-ECM network was organized; in particular, at 40 μg/ml, FN13 induced in control CEF a matrix comparable with that detected in transfected CEF (Fig. 3, *b–e*). This peptide concentration was therefore used in the following experiments.

Because FN13 contained a cysteine in the 6th position, to ascertain whether its possible effect on FN assembly could be due to the SH-reactive group, FN13 activity was analyzed in comparison with that exhibited by the following: a modified peptide carrying the SH residue methylated (FN13Mod); an FN13 peptide carrying a serine (FN13Ser) in place of the cysteine; an FN13 with a hydrophobic alanine (FN13Ala) instead of cysteine. Finally, the activity of these 4 peptides was compared with that of an FN13 scrambled-sequence peptide (ScrFN13) containing a cysteine in the 3rd position.

A



B

a

Sample	IOD	FI
CEF-peptide	0.10 ± 0.03	1.0
CEF+FN13	0.64 ± 0.07	6.2
CEF+FN13Ala	0.43 ± 0.07	4.2
CEF+FN13Ser	0.47 ± 0.07	4.5
CEF+FN13Mod	0.47 ± 0.04	4.5
CEF+ScrFN13	0.09 ± 0.02	0.9

b

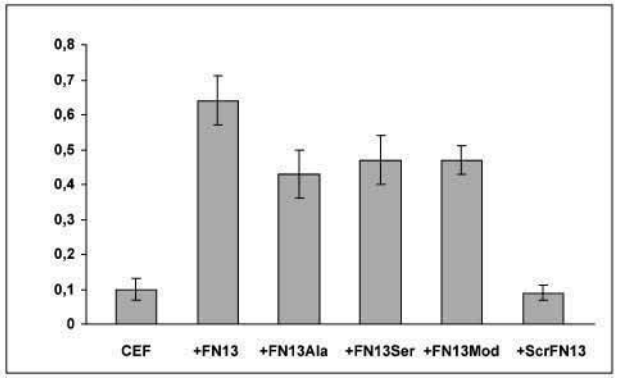


FIG. 6. **Effect of FN13 and of FN13-derived peptides on the organization of FN of cultured CEF.** *A*, IF was performed with the f25 mAb on control CEF (*a*) and on CEF treated with 40 μg/ml FN13 (*b*), FN13Ser (*c*), FN13Mod (*d*), FN13Ala (*e*), and ScrFN13 (*f*). *Scale bar*, 8 μm. *B*, quantitative image analysis of the FN ECMs (*a*) are reported as IOD. The quantitative evaluation of the FN-ECM was performed in triplicate; each evaluated field contained on average 23 ± 1 cells (*b*). *FI*, fold increase.

At a concentration of 40 μg/ml, FN13, FN13Ser, and FN13Mod induced the organization in cultured CEF, analyzed by IF with the f25 mAb, of a fibrillar network of FN overlaying the cells (Fig. 6*A*, *b–d*), which was absent in untreated cells. FN13Ala induced the formation of an abundant ECM that was less fibrillar (Fig. 6*A*, *e*) than that detected after treatment with the other FN13 peptides. The ScrFN13 peptide did not induce either enhancement or organization of FN into the ECM (Fig. 6*A*, *f*). Fig. 6*B* (*a* and *b*) shows the quantitative evaluation, performed by image analysis of the fluorescent FN signals, detected by IF with f25 mAb, in CEF in basal conditions and after treatment with the different peptides. The highest level of labeled FN was de-

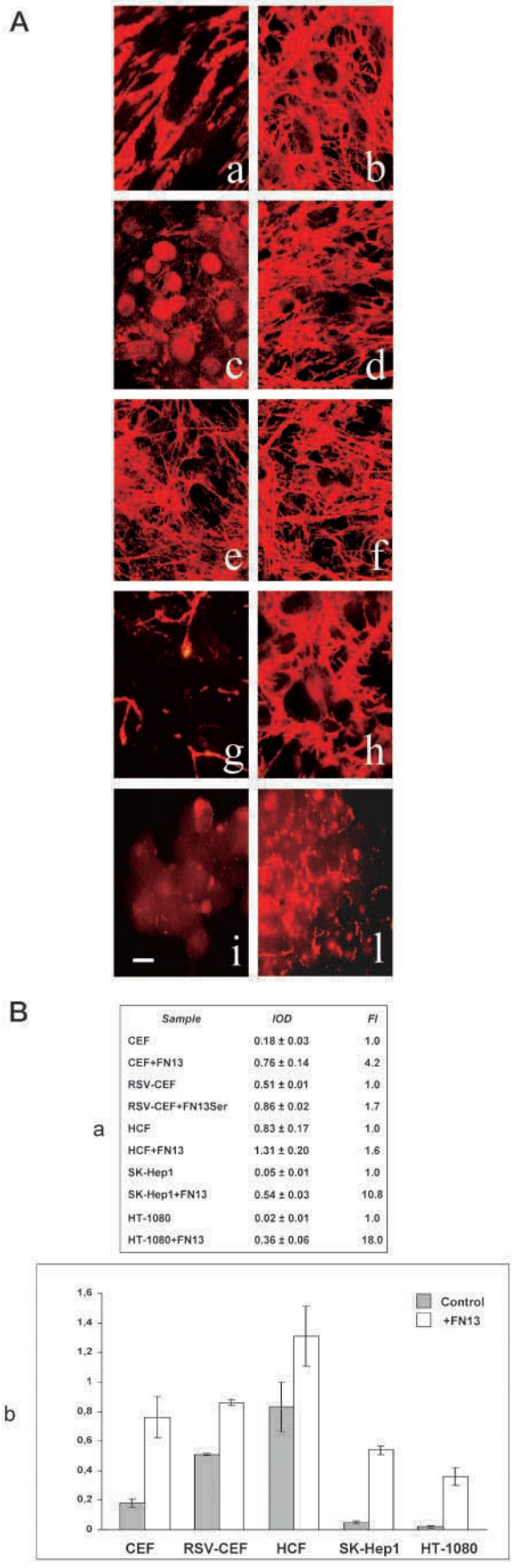


FIG. 7. **FN13 organizes the FN-ECM in avian and human normal and malignant cells.** *A*, IF of FN organized by control (*a* and *b*) and RSV-CEF (*c* and *d*), by human control fibroblasts (*e* and *f*), by SK-Hep1 (*g* and *h*), and by HT-1080 (*i* and *l*) malignant cells, before (*a*, *c*, *e*, *g*, and *i*) and after treatment with 40 μg/ml FN13 (*b*, *d*, *f*, *h*, and *l*). The avian cells were immunoreacted with the f25 mAb, whereas the human cells were reacted with f33 mAb. *Scale bar*, 8 μm. *B*, quantitative evaluation of the FN-ECM performed by image analysis is indicated as IOD. The number of cells for each evaluated field is as follows: 23 ± 1 CEF, 33 ± 6 ± RSV-CEF, 14 ± 1 SK-Hep1, and 27 ± 2 HT-1080. *FI*, fold increase.

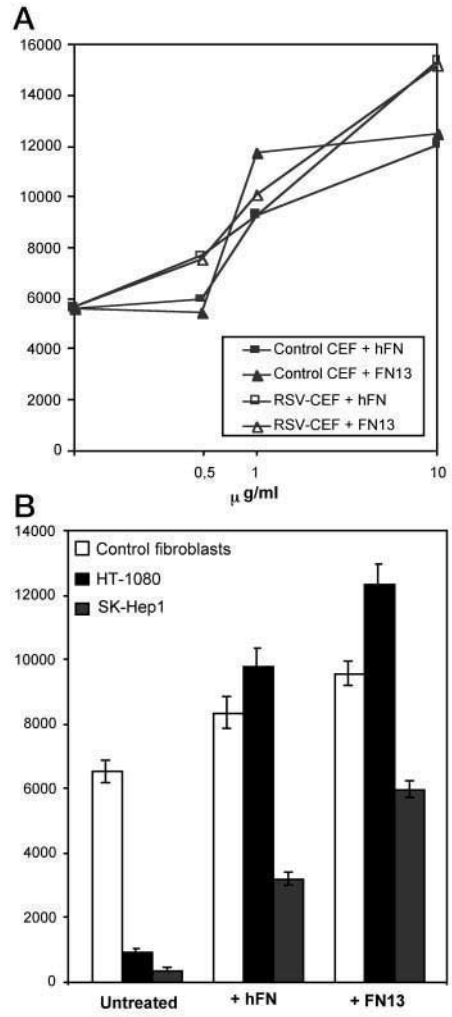


FIG. 8. **FN13 and hFN favor the cell adhesion of CEF, RSV-CEF (*A*), human control fibroblasts, HT-1080, and SK-Hep1 tumor cells (*B*).** *A*, increasing concentrations of FN13 and hFN (from 0.5 to 10 μg/ml) were coated on the plates before cell adhesion. *B*, 10 μg/ml FN13 or hFN was used for coating.

tected in CEF treated with FN13, showing about a 6-fold increase in FN compared with the control cells. The other peptides all induced a 4-fold increase in FN in CEF, whereas ScrFN13 had no effect on the organization of FN. Similar results were obtained when 10 μg/ml peptides were added to cultured CEF, although the signals were lower than those shown in Fig. 6*A* (not shown). These results indicate that induction of the FN-ECM in CEF is due to the FN13 sequence, although the presence of a reactive group (SH– or OH–) in the 6th position is associated with the formation of a more fibrillar FN-ECM.

IF analysis of COLI-ECM in CEF treated with the different peptides showed that they were not capable of inducing a fibrillar matrix, although they did induce the organization of COLI into a structure comparable with that observed in the

a

	Untreated	ScrFN13	FN13	FN13Mod	FN13Ser	FN13Ala	hFN
CEF	216±18	269±41	1365±109	644±70	490±37	586±43	1441±96
Control Fibroblasts	422±39	445±50	1299±74	777±52	583±21	552±39	873±84
RSV-CEF	3500±221	3660±304	1078±87	1092±147	1400±121	1079±58	4200±251
SK-Hep1	3224±299	3256±183	1195±147	1314±154	1656±122	1294±85	3995±229
HT-1080	2678±248	2565±166	689±49	1122±84	1526±113	831±73	3041±304

b

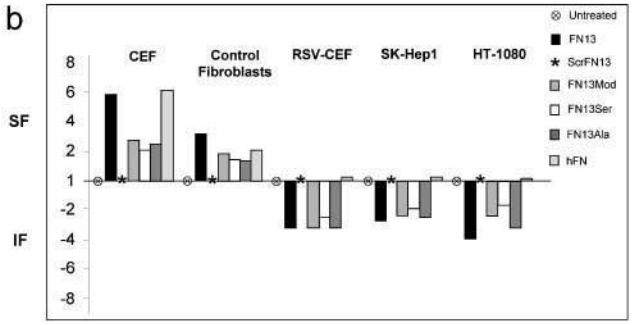


FIG. 9. **FN13 inhibits the migration of malignant cells.** Effect of FN13 and of hFN on the migration in a Transwell chamber of control and RSV-CEF, of human control fibroblasts, and of SK-Hep1 and HT-1080 tumor-derived cell lines. The values reported in *a* are the average ± S.D. of the measurements performed in three independent experiments. *b*, the enhancement of cell migration is indicated as stimulation factor (*SF*) and its inhibition as inhibition factor (*IF*), compared with the untreated cells corresponding to 1.

a

	Untreated	ScrFN13	FN13	FN13Mod	FN13Ser	FN13Ala	hFN
Control Fibroblasts	1892±18	1959±32	4261±582	4710±210	2882±43	3723±23	4957±132
SK-Hep1	7011±472	7361±384	1590±166	4964±578	1780±465	3932±8	9333±429
HT-1080	6111±275	6148±344	1183±16	4654±896	1310±682	3483±347	8506±318

b

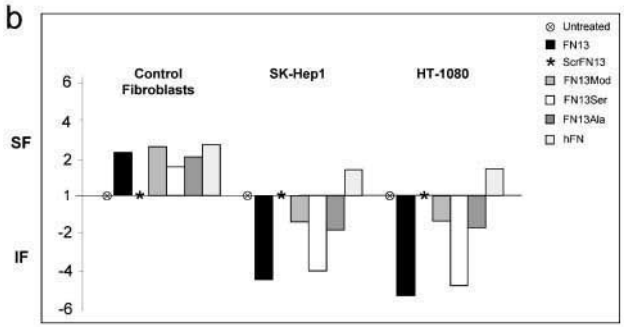


FIG. 10. **FN13 inhibits the invasion of malignant cells.** Invasion assay on Matrigel matrix performed with human control fibroblasts, HT-1080 and SK-Hep1 tumor-derived cells, in the absence and in the presence of FN13 peptides and of hFN. The values reported in *a* are the average ± S.D. of the measurements performed in three independent experiments. *b*, the enhancement of cell invasion is indicated as stimulation factor (*SF*) and its inhibition as inhibition factor (*IF*), compared with the untreated cells corresponding to 1.

transfected CEF (Fig. 3*B*, *i–m*). The ScrFN13 peptide had no effect on COLI organization (not shown).

FN13 Induces FN-ECM Assembly in RSV-CEF and in Human Tumor Cells—The effect of FN13 on FN-ECM organization was studied in RSV-CEF and in human tumor-derived cell lines. Cultured CEF were massively infected with the mutant PA1 of the SR-RSV strain, and, after the appearance of transformation *foci* they were grown in the absence and in the presence of FN13 and analyzed by IF with the f25 mAb. Fig. 7*A*, *c*, shows that RSV-CEF are round-shaped and not overlaid by the ECM of FN before treatment with FN13, whereas in the presence of the peptide a well organized fibrillar ECM of FN overlaying the cells can be observed (Fig. 7*A*, *d*).

A human hepatoma- (SK-Hep1) and a human fibrosarcoma-derived cell line (HT-1080), unable to organize the FN-ECM (Fig. 7*A*, *g* and *i*) after treatment with FN13, deposited FN in the extracellular environment. Whereas in SK-Hep1 cells FN was organized in a compact extracellular structure (Fig. 6*A*, *h*), differing from that observed in human control fibroblasts (Fig. 7*A*, *e* and *f*), in HT-1080 it was deposited in thick extracellular aggregates and lacked a fibrillar appearance (Fig. 7*A*, *l*). Quantitative evaluation of FN overlaying the cells, which was performed by image analysis, showed an increase in fluorescence after FN13 cell treatment (Fig. 7*B*). The enhancement of FN assembled in the ECM was highest in SK-Hep1 and HT-1080 tumor cells. Similar results were obtained when treating normal and malignant cells with 10 μg/ml purified hFN. This indicates that FN13 is able, to a varying extent, to promote the formation of an FN network surrounding transformed and tumor-derived cells.

Effect of FN13 on Cell Adhesion—In order to ascertain whether the ECM induced by FN13 in control and RSV-CEF influenced their adhesive properties, we performed an adhesion assay on these cells with and without the peptide. Increasing amounts of FN13 were coated on plastic; hFN was coated as a positive control. Fig. 8*A* shows that the number of adherent control and RSV-CEF increases with hFN and FN13 concentration, indicating that FN13 can support the adhesion of these cells as well as purified FN.

Human control fibroblasts and tumor-derived cell lines were also tested for their adhesive properties in the absence and in the presence of 10 μg/ml hFN and FN13. As shown in Fig. 8*B*, control fibroblasts adhere to the substrate more efficiently than HT-1080 and SK-Hep1 cells. In the presence of hFN and FN13, the adhesiveness of control fibroblasts was slightly enhanced (1.3- and 1.5-fold, respectively). The adhesiveness of SK-Hep1 and HT-1080 tumor cells increased several times (10.4- and 8.4-fold, respectively) in the presence of hFN, and 13- and 16-fold, respectively, in the presence of FN13. Therefore, FN13 effectively favors the adhesion of human tumor cells not organizing an FN-ECM in basal conditions. The effect of FN13 on the adhesion of these cells is comparable with that produced by purified intact hFN.

Effect of FN13 on Cell Migration and Invasion—We studied the effect of FN13 on the migration of control CEF, RSV-CEF, human control fibroblasts, HT-1080, and SK-Hep1 tumor-derived cells in Transwell chambers. Fig. 9 shows that, in the absence of any treatment, control CEF and human control fibroblasts migrated less efficiently than RSV-CEF and tumor cells. FN13 and hFN stimulated the migration of CEF and human control fibroblasts about 6- and 2–3-fold, respectively. On the contrary, the ScrFN13 peptide did not have any effect on the migration of either type of control cell. FN13 had the opposite effect on the migration of RSV-CEF and human tumor cells compared with control cell strains. Indeed, the peptide inhibited the migration of RSV-CEF, SK-Hep1, and HT-1080 cells 2–3-fold (Fig. 9). The ScrFN13 peptide did not show any effect on the migration of these cells, whereas hFN slightly enhanced their migration. In order to elucidate the different effects on cell migration produced by FN13 and hFN, FN13Ser, supporting fibrillar FN-ECM assembly, and FN13Mod and FN13Ala, inducing the aggregation of FN in a

non-fibrillar meshwork, were also tested. These peptides induced the migration of control cells, although to a lower extent than FN13 and hFN, and inhibited the migration of transformed and tumor cells.

Human control fibroblasts and tumor cells were also tested for their ability to invade a Matrigel basement membrane matrix. In this system, FN13, all FN13-derived peptides, and hFN, but not ScrFN13, slightly enhanced the invasive properties of control cells by 1.5–2.6-fold (Fig. 10). FN13 reduced by 4- and 5-fold the invasive capability of SK-Hep1 and HT-1080 tumor cell lines, respectively; the inhibitory activity of FN13Mod, FN13Ser, and FN13Ala was lower than that of FN13 both on SK-Hep1 (by 1.4-, 3.9-, and 1.8-fold, respectively) and on HT-1080 (by 1.3-, 4.7-, and 1.7-fold, respectively) (Fig. 10). ScrFN13 had no effect on the tumor cell invasion, whereas hFN slightly enhanced the invasiveness of these cells by 1.3- and 1.4-fold, respectively. Therefore, FN13 but not hFN, shows an anti-invasive effect on *in vitro* transformed avian cells and on human tumor-derived cells, whereas it favors the invasion of control avian and human cells. FN13 also showed an anti-invasive effect in HeLa (9.0-fold reduction) and in rhabdomyosarcoma (2.0-fold reduction) tumor cell lines (not shown). All FN13-modified peptides, but not ScrFN13, inhibited the invasive properties of transformed avian cells and human tumor-derived cells. In particular, FN13Ser, carrying a reactive –OH on the 6th amino acid, inhibited cell invasion to the highest extent, suggesting that the anti-migratory activity of FN13 peptide is associated not only with its sequence but also with a reactive group on the amino acid in the 6th position. Taken altogether, these data indicate that FN13 and FN13-related peptides have an anti-invasive potential on tumor and transformed cells which is not associated with the intact FN molecule.

DISCUSSION

The adhesive ECM protein FN, in synergy with its integrin receptors, plays an important role in several stages of embryo and tumor development. Embryonic and tumor cells are less adhesive than the normal adult counterpart and deposit less ECM. A diminished adhesion to the ECM may contribute to the migratory properties of embryo and tumor cells in the surrounding tissues (7).

FN is a multidomain protein containing binding sites for the cell (*i.e.* integrin receptors), for other ECM components, and for FN itself; through these interactions FN participates in many physiological and pathological processes (3, 7). The proteolysis of purified FN with several enzymes, as well as the construction and expression of recombinant FNfgs, has provided evidence that FNfgs can perform activities that are not present in the intact molecule (32–36).

In this work we investigated the involvement of FN COL-domain (37–39) and its adjacent regions in ECM formation by using a set of FN cDNA deletion segments that were stably expressed in CEF after cloning in a retroviral vector. All FN cDNAs started with the sequence encoding the first type II repeat of the molecule and encompassed downstream regions of different lengths. The data reported here show that recombinant hFN cDNA, retaining the sequence coding for the 13-amino acid stretch, falling in the boundary between the end of type II_2 and the beginning of type I_7 repeat, and corresponding almost completely to the major COL-site of FN (39, 47), was capable of inducing in CEF the assembly of an organized ECM of FN and of COLI. Only the largest FNfg, spanning to the end of type III_2 repeat, retains the 76-amino acid C-terminal sequence acting as an FN-FN binding region (29). However, the other three FN cDNAs also lacking this sequence induced the assembly of the FN-ECM. The absence in hFN cDNA of the sequence, coding for the last 13 amino acids, was associated with a strong reduction of the FN-ECM, indicating that this sequence probably plays an important role in the organization of the FN-ECM. The hFN cDNAs expressed in CEF also to different extents supported the organization of COLI into the ECM. This indicates that COLI is assembled in the ECM of CEF even in the absence of the major COL-site, in agreement with previous reports showing that the two type II FN repeats, upstream of the COL-site, share a 50% homology with the three type III repeats of type IV collagenase, which notoriously binds to COLs (56). Furthermore, it is noticeable that a gelatin binding activity has also been disclosed in a 21-kDa FNfg containing the I_8-I_9 repeats (39). Therefore, FN also contains multiple COL-sites, three of which are clustered between the type II_1 and the type I_9 repeats. This might explain the differential deposition of COLs into the ECM observed in the transfected CEF expressing FNfgs containing one or more COL-sites.

The 13-amino acid peptide FN13, which corresponds almost exactly to the main FN COL-site, was synthesized and analyzed to determine its effect on the assembly of FN and of COLI in cultured cells. FN13 is able to direct the organization in cultured CEF of an FN-ECM comparable with that observed in transfected CEF. FN13 also induces in CEF the formation of a COLI-ECM in structures lacking a clear-cut fibrillar organization. The linking activity of FN13 is partly due to the presence of a central cysteine possibly involved in disulfide bonds with other cysteines distributed in FN type I and II repeats (3) and on pro-α1(I) and pro-α2(I) collagen chains (57). When the cysteine is methylated (FN13Mod) or substituted with a non-polar amino acid such as alanine (FN13Ala), FN and COLI are still organized by CEF in compact and less fibrillar aggregates than those induced by FN13. Fibrillar FN organization into the ECM is also induced by another peptide carrying a reactive –OH group on the 6th amino acid (FN13Ser). These findings indicate that a reactive amino acid in the 6th position of FN13 is important for the formation of a fibrillar FN- and COLI-ECM and that the presence of a cysteine, which might be involved in the formation of disulfide bonds, is not required for the assembly of a fibrillar FN- and COLI-ECM. The sequence of FN13, therefore, is sufficient to induce the aggregation of an organized ECM, whereas a reactive amino acid in the 6th position favors the organization of fibrillar structures. The role of FN13 sequence in FN assembly is emphasized by the observation that the ScrFN13 peptide, which contains a cysteine in the 3rd position, does not induce FN and COLI organization into the ECM.

The 13-amino acid FN13 sequence is fully conserved in six different species from *Homo sapiens* to zebrafish (ExPASy BLAST2 Interface), indicating that this amino acid stretch must be maintained intact in order to allow the accomplishment of very important and common functions in all Vertebrata. This sequence is very ancient since it has also been found, entirely or for internal portions, with a 47–96% homology in all living organisms. The conservation of this sequence, mainly found in FN molecules, also explains the lack of immunogenicity of the COL-domain of FN (3).

The induction of the FN-ECM by recombinant FNfgs and FN13 has also been studied in RSV-CEF and in human tumor-derived cell lines. Transformed and tumor cells do not assemble the FN-ECM (4, 6, 8, 9, 23) and, due either to FN degradation by tumor proteases or to integrin receptors modulation (12–14, 16, 58), this phenotypic trait has been associated with tumor growth, invasion, and metastasis (30, 31). We observed that all conditioned media from transfected CEF, with the only excep-

tion of that expressing the II_1-II_2del FNfg, were able, with comparable efficiency, to induce the organization of FN-ECM even over the *foci* of RSV-transformed cells, which are normally deprived of FN-ECM. A similar FN-ECM network overlaying the transformation *foci* was observed in RSV-CEF grown in the presence of FN13 even with a low serum concentration. Following these treatments, the morphology of transformed CEF shifted from round-shaped to fibroblast-like, indicating that the FN-ECM induced in these cells temporarily restored a "normal" cell phenotype. SK-Hep1 and HT-1080 human tumor cell lines, which do not organize FN, assembled an FN-ECM in the presence of FN13. These results show that FN13 is sufficient to induce FN-ECM formation in ECM–transformed and tumor cells. Through its ability to induce an organized FN-ECM, the FN13 peptide also influenced the adhesive properties of control and transformed CEF. The stimulation of adhesiveness was much higher for transformed RSV-CEF, compared with control CEF. Indeed, FN13 treatment enhanced the adhesion of RSV-CEF to a level comparable with that obtained after hFN treatment. These data show that FN13 induces an FN-ECM supporting cell adhesion in a manner comparable with that retained by a hFN-induced matrix and suggest a similar organization for these structures in the different cell types.

FN13 also acted on the migration of control, transformed, and tumor cells in a Transwell chamber. In this assay, FN13 enhanced the migration of control avian and human fibroblasts as well as hFN. On the contrary, FN13 inhibited the migration of RSV-CEF and of tumor cells, whereas hFN slightly stimulated the migration of these tumorigenic cells.

The anti-migratory property of FN13 was also confirmed in a Matrigel matrix invasion assay. In this case too, hFN and FN13 both favored the migration of control fibroblasts, whereas FN13 strongly reduced the migration of SK-Hep1 and HT-1080 cells, unlike hFN, which enhanced the Matrigel invasion of these tumor cells as well. Comparable effects on the migratory and invasive properties of control, transformed, and tumor cells were obtained with FN13-related peptides, but not with ScrFN13, indicating that the FN13 sequence plays a crucial role in these biological functions, although, to different extents, the residue present on the 6th amino acid affects the efficiency of cell invasion and migration. Because HeLa (cervix epithelioid carcinoma) and rhabdomyosarcoma tumor cell lines, following FN13 treatment, also showed a drastic reduction in their migratory activity in the Matrigel matrix, FN13 seems capable of inhibiting the invasion both of ectoderm- and of mesenchyme-derived tumor cells.

Similar results were obtained when treating tumor cells of different embryonic origin with sFN (29) and with the III_1 FNfg, which is also defined *anastellin* and organizes FN in sFN (31). Either sFN or *anastellin* blocked tumor cell spreading and migration *in vitro* through the organization of FN. Although FN13 organizes FN, as well as COLI in cell cultures, its effect on the adhesion and migration of control, transformed, and tumor cells is comparable with that of *anastellin*. The different effect of purified hFN and of FN13 or *anastellin* on tumor cell migration can be explained by assuming that the matrix organized by these short FN peptides has a different structure, if compared with the physiological FN-ECM. Because *anastellin* and sFN have been reported to exert in mice strong tumor formation-prevention, together with antimetastatic and anti-angiogenetic activity (30, 31), we can hypothesize that FN13, which induces FN-ECM organization and shows an anti-migratory effect on tumor but not on normal cells and low immunogenic activity, will have similar biological activities *in vivo*. Treatment of mice with FN13 is in progress in order to evaluate its toxicity, its turn-over after intravenous and intraperitoneal injection, and its role in tumor growth and metastasis *in vivo*.

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