Type III and V collagens modulate the expression and assembly of EDA+ fibronectin in the extracellular matrix of defective Ehlers–Danlos syndrome fibroblasts

Nicoletta Zoppi *, Marco Ritelli, Marina Colombi

Division of Biology and Genetics, Department of Biomedical Sciences and Biotechnology, Medical Faculty, University of Brescia, 25123 Brescia, Italy

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Abstract

Background: Alternative splicing of EDA fibronectin (FN) region is a cell type- and development-regulated mechanism controlled by pathological processes, growth factors and extracellular matrix (ECM). Classic and vascular Ehlers–Danlos syndrome (cEDS and vEDS) are connective tissue disorders caused by COL5A1, COL5A2 and COL3A1 gene mutations, leading to an in vivo abnormal collagen fibrillogenesis and to an in vitro defective organisation in the ECM of type V (COLLV) and type III collagen (COLLIII). These defects induce the FN-ECM disarray and the decrease of COLLS and FN receptors, the αβ1 integrin and α5β1 integrins. Purified COLLV and COLLIII restore the COLL-FN-ECMs in both EDS cell strains.

Methods: Real-time PCR, immunofluorescence microscopy, and Western blotting were used to investigate the effects of COLLS on FN1 gene expression, EDA region alternative splicing, EDA+–FN-ECM assembly, α5β1 integrin and EDA+–FN-specific α9 integrin subunit organisation, α5β1 integrin and FAK co-regulation in EDS fibroblasts.

Results: COLLV-treated cEDS and COLLIII-treated vEDS fibroblasts up-regulate the FN1 gene expression, modulate the EDA+ mRNA maturation and increase the EDA+–FN levels, thus restoring a control-like FN-ECM, which elicits the EDA+–FN-specific α9β1 integrin organisation, recruits the α5β1 integrin and switches on the FAK binding and phosphorylation.

Conclusion: COLLS regulate the EDA+–FN-ECM organisation at transcriptional and post-transcriptional level and activate the α5β1–FAK complexes. COLLS also recruit the α9β1 integrin involved in the assembly of the EDA+–FN-ECM in EDS cells.

General significance: The knowledge of the COLLS-ECM role in FN isotype expression and in EDA+–FN-ECM-mediated signal transduction adds insights in the ECM remodelling mechanisms in EDS cells.

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1. Introduction

Ehlers–Danlos syndrome (EDS) refers to a heterogeneous group of rare autosomal dominant and recessive connective tissue disorders, characterised by joint hypermobility, hyperextensible, elastic and fragile skin with impaired wound healing. The involved genes, encoding type V, type I, and type III collagens (COLLS) or enzymes entering into the COLLS triple helices processing, differ between the six major EDS types [1,2]. Among them, the classical, or type I EDS (cEDS, or EDSI) and the vascular, or type IV EDS (vEDS, or EDSIV) are caused by mutations in COL5A1 or COL5A2 and in COL3A1 genes, respectively. Ultrastructural studies of in vivo cEDS skin biopsies showed a decrease of COLL fibrils density and the presence of large irregular COLL fibrils (cauliflower shape), whereas in vEDS biopsies a thinner dermis than control ones and a reduced number of COLL bundles with small diameter fibrils were reported [3]. The abnormal COLL fibrillogenesis might be due either to primary defect in COLL genes and to alteration of non-collagenous extracellular matrix (ECM) components, known to influence the COLL fibrils assembly. In vitro cultured skin fibroblasts derived from cEDS and vEDS patients do not organise fibronectin (FN) in the ECM [4–8], consequently to an altered COLL expression and deposition into the ECM, caused by COLL gene mutations [6,8]. In particular, the defective synthesis and organisation of type V COLL (COLLV) or type III COLL (COLLIII) are associated to a decreased organisation of the specific COLL receptor, the α2β1 integrin [6]; this event leads to the disorganisation of the FN-specific cell surface receptor, the α5β1 integrin, triggering an FN-ECM disarray [6].

The FN-ECM regulates a variety of biological processes, including cell adhesion, migration, proliferation, survival and gene expression, via signal transduction pathways which can be differentially activated by several ECM ligands interacting with specific integrin settings [9–12]. At least ten different FN specific integrins are reported; between them,
the α5β1 integrin is the main FN receptor in dermal skin fibroblasts, recognising the FN RGD binding site and regulating inside-out signalling for cell proliferation, survival and migration [9].

In EDS cells the α5β1 integrin is replaced by another FN receptor, the αvβ3 integrin [6,8], allowing cell survival in the absence of an organised COLL-FN-ECM [8]. Indeed, in these cells the αvβ3 integrin transduces signals through the FAK-independent paxillin. In this signalling the src family proteins are involved in the activation of paxillin, which maintains a pre-apoptotic cell behaviour: EDS cells proliferate in the absence of ECM, actin cytoskeleton and anti-apoptotic factors [8]. Purified exogenous COLLV and COLLIII restore the COLLS-ECM in cEDS and vEDS cells, respectively, through the recruitment of the α2β1 integrin and the αvβ3 substitution with the α5β1 integrin, finally supporting the FN-ECM re-organisation, through a yet unknown α5β1 integrin-mediated signal transduction pathway [6].

The defective FN-ECM assembly reported in different EDS fibroblasts' types has been associated to a decrease of EDA+ (or EDI or EIIIA) FN mRNA level [13], one of the alternative spliced regions of the FN1 gene [14–17]. FN alternative splicing, also acting at the EDB domain (or EIIId or EIIIib) and at the type III connecting segment (Illics), is regulated in a cell-type-, development- and age-dependent manner and in pathological processes. Skin fibroblasts preferentially synthesise the EDA+–FN mRNA [18–20]; the EDA−–FN enters in the cellular FN (cFN) isotype, organising in the fibrillar ECM [13]. Plasma FN (pFN) mainly contains the EDA isoform. In vivo, the EDA+–FN is poorly represented in the ECM of adult tissues and normal adult skin fibroblasts in vivo mainly produce EDA−–FN [21,22]. EDA+–FN is overexpressed in fibroblasts and epithelial cells during tissue remodelling and cell migration occurring in developing embryos [23–26], during wound healing [21,22,27], liver fibrosis, myofibroblast differentiation [28,29] and in some tumours [30,31]. Although in EDS patients the in vivo FN-ECM organisation was not investigated, the clinical defects and the impaired wound healing observed suggest a role of FN in the connective tissue haemostasis. Growth factors, cytokines, hormones and stress stimuli regulate FN alternative splicing either in vivo or in vitro [32–38]. Furthermore, different ECM proteins can control and modulate this process, i.e., laminin and type IV COLV [39].

Here we report the effect of purified human COLLIII and COLLV on the modulation of EDA+–FN mRNA synthesis and on the EDA−–FN organisation in ECM-defective EDS cells, leading to the αvβ3 and α5β1 integrin recruitment and signalling to FAK protein.

2. Materials and methods

2.1. Cell strains

Human control skin fibroblasts were established in our lab from three skin biopsies of age- and sex-matched healthy donors. cEDS and vEDS fibroblasts carried dominant missense mutations in the genes encoding for the α1 chains of COLLV (COLSA1, G1181C) and COLLIII (COL3A1, G769S), respectively [6,8]. Five cEDS cell strains (P1–P5) carrying different mutations in COLSA1 and COLSA2 genes [7 and unpublished results] and three vEDS cell strains (P1–P3) carrying different COL3A1 gene mutations [40] were also analysed in some experiments. The cells at the 8th in vitro passages were grown in Earle's Modified Eagle's Medium (MEM) (Invitrogen/Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technology), 100 IU/ml penicillin and 100 μg/ml streptomycin (complete MEM) at 37 °C in a 5% CO2 atmosphere. Alternatively, control and EDS cells were seeded in complete MEM supplemented with 1.0, 2.5, 5 and 10 μg/ml purified human COLV or COLLIII.

2.2. Antibodies and reagents

Mouse anti-αvβ3 (clone JB5), anti-αvβ3 (clone LM609) integrins monoclonal antibodies (mAbs) and purified human COLV and COLLIII were from Millipore, Chemicon (Billerica, MA). The goat anti-COLLV and anti-COLLIII polyclonal antibodies (Abs) were from LifeSpan BioSciences, Inc. (Seattle, WA). The anti-FAK and anti-integrin α9 Abs, the anti-phospho-tyrosine (anti-p-Tyr) (PY20) and the anti-GAPDH (clone 0411) mAbs were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-integrin α4 (EPIR35S5Y) Ab and the anti-α5 integrin subunit mAb (clone NKI-SAM1) were from Abcam (Cambridge, UK). Fluorescein isothiocyanate (FITC-) and rhodamine-conjugated goat anti-rabbit and goat anti-mouse secondary Abs were from Calbiochem-Novabiochem INTL. Horseradish-peroxidase (HRP-) conjugated anti-rabbit, anti-goat and anti-mouse IgGs, BSA, rhodamine-conjugated rabbit anti-goat IgGs, the affinity purified rabbit anti-human FN Ab (F3648) and anti-tenascin mAb (clone BC-24), recognising all of the human FNs and tenascins, respectively, were from Sigma Chemicals (St. Louis, MO). The anti-human EDA FN (clone DH1) mAb was from BIOHIT (Celbio, Italy). The anti-FN Ab and the anti-EDA FN mAb did not recognise the bovine FN (SM 1B to Fig. 1). The rabbit anti-u-PA Ab was from Technoclone GmbH (Alifax, Italy).

2.3. RNA extraction and retro-transcription reaction

Total RNA was isolated from 1.5 × 10^6 control and EDS fibroblasts grown for 48 h to confluence, treated or not with 5.0 μg/ml purified human COLLV and COLLIII, using TRIzol reagent according to the manufacturer's instructions (Invitrogen/Life Technologies, Carlsbad, CA). The RNA was recovered by precipitation with ethanol and dissolved in RNase free water. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer and quality was checked using the AGILENT Bioanalyzer 2100 lab-on-a-chip technology. Retro-transcription (RT) was performed using the Moloney murine leukemia virus reverse transcriptase (MMLV-RT) provided by Invitrogen. Total RNA (3 μg) from each cell strain was mixed with 2.2 μl of 0.2 ng/μl random hexamer, 10 μl of 5× buffer, 10 μl of 2 mM dNTPs, 1 μl of 1 mM DTT, 0.4 μl of 33 U/μl RNasin, 2 μl MMLV-RT (200 U/μl), in a final volume of 50 μl. The reaction mix was incubated at 37 °C for 2 h and the enzyme was heat-inactivated at 95 °C for 10 min. Samples were stored at −20 °C and cDNA was used for Real-time PCR.

2.4. Real-time PCR by TaqMan probes

The expression levels of EDA+– and EDA−–FN isoforms were analysed by means of an Applied Biosystem 7500 Real-time PCR system based on a 5′ nuclease assay (TaqMan). For the quantitative measurement, the comparative threshold cycle (ΔΔCt) method was used according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Porphobilinogen deaminase gene (PBGD) was used for normalisation of possible fluctuations in quantitative values of the target transcripts. PCR was carried out with TaqMan Universal PCR Master Mix (Applied Biosystem), which contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference and optimised buffer components. AmpErase UNG treatment was used to prevent the possible reamplification of carryover PCR products. Thermal cycling was initiated with incubation at 50 °C for 2 min and 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase, respectively. After this step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95 °C for 15 s for melting and 60 °C for 1 min for annealing and extension. 20 ng of cDNA, 7.5 pmol of each primer and 5 pmol of labeled probe were used in each Real-time PCR, in a final volume of 25 μl.

Primers and probes of the housekeeping gene PBGD and of the EDA+– and EDA−–FN mRNA isoforms were previously described [41]. In brief, the forward primer of EDA+–FN lies within the EDA region, whereas the forward primer of the EDA−–FN isoform does not include the EDA region. The reverse primers and probes are similar for EDA+– and EDA−–FN. The specificity and efficiency of both primer sets were...
already tested on plasmids containing the EDA\(^+\) or the EDA\(^-\) fragment [41].

The relative quantification of EDA\(^+\) and EDA\(^-\) mRNAs was determined by comparing the levels of COLLV- and COLLIII-treated cell lines and their untreated counterparts by the equation \(2^{-\Delta \Delta Cq}\). For the quantification of the levels of the two FN isoforms in untreated cell strains, control skin fibroblasts were used as calibrator. The reported values of relative quantification are the means ± SEM obtained analysing two different cell strains for each type (control fibroblasts C and C1, cEDS and cEDS P4 and vEDS and vEDS P3 cell strains), in two independent RT reactions and with each individual determination repeated in triplicate. Statistical analysis of these data was performed by two-way ANOVA and Bonferroni's post-hoc test carried out by means of GraphPad Prism 4 (GraphPad Software Inc., USA). p values < 0.05 were considered statistically significant.

2.5. Immunofluorescence microscopy analysis (IF)

The FN-ECM organisation was analysed by IF on \(1 \times 10^5\) control and EDS cells grown for 48 h on 24 x 24 mm glass coverslides in complete MEM supplemented or not with 1.0, 2.5, 5.0 and 10 \(\mu\)g/ml purified COLLV or COLLIII. The cells, washed in PBS and fixed in methanol for 40 min at \(-20°C\), were immunoreacted for 40 min at room temperature with anti-human FN Ab (1:100), recognising all of the FN isoforms in untreated cell strains, control skin fibroblasts used as calibrator. The reported values of relative quantification are the means ± SEM obtained analysing two different cell strains for each type (control fibroblasts C and C1, cEDS and cEDS P4 and vEDS and vEDS P3 cell strains), in two independent RT reactions and with each individual determination repeated in triplicate. Statistical analysis of these data was performed by two-way ANOVA and Bonferroni's post-hoc test carried out by means of GraphPad Prism 4 (GraphPad Software Inc., USA). p values < 0.05 were considered statistically significant.

2.6. Western blotting analysis

The levels of total and EDA\(^-\)FN, stored in the cytoplasmic compartment, secreted and organised into the ECM by control and EDS cells, were evaluated collecting the cell extract or deoxycholate (DOC)-soluble fraction (CE), the culture medium (CM) and the DOC-insoluble (DOC-IS) ECM of each untreated, COLLV- and COLLIII-treated cell strain, as previously reported [6]. In particular, \(3 \times 10^5\) control (C, C1 and C2), cEDS (cEDS, cEDS P4, cEDS P1) and vEDS (vEDS, vEDS P3, vEDS P1) cells were cultured for 48 h in complete MEM supplemented or not with 5 \(\mu\)g/ml COLLV or COLLIII. Media were collected in the presence of proteases inhibitors (25 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM aminobenzamidine). To collect the FN cytosplasmic DOC-soluble fraction, the cell layers were rinsed three times in cold PBS and treated for 10 min at 4°C with the DOC lysis buffer (0.5%M/10 mM Tris–HCl, pH 8.0, 1 mM phenylmethylsulfonylfluoride, 2 mM EDTA). The DOC-IS fractions were scraped into a mixture of 4% SDS, 20% glycerol, 25 mM Tris–HCl, pH 8.0 and 0.002% bromophenol blue. The quantitative evaluation of the protein concentrations was performed using the detergent compatible Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) and 20 \(\mu\)g of total proteins was separated by electrophoresis in 8% SDS-PAGE under non-reducing conditions. After nitrocellulose sheet transfer, the membranes were blocked overnight at 37°C with 3% non-fat milk (w/v) in 0.1 M Tris–HCl, pH 8.1 and immunoreacted for 2 h at 37°C with anti-FN Ab (1:1,000) and 1 \(\mu\)g/ml anti-EDA FN DH1 mAb diluted in 0.3% BSA, 0.01% NaN\(_3\) in PBS, washed 3 x 10 min in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl (TBS), 0.1% Tween 20 (v/v) (TBS-T) at room temperature and reacted for 2 h at 37°C with HRP-conjugated anti-rabbit and anti-mouse IgGs, respectively, diluted in 0.3% BSA/PBS. For each fraction (CE, CM and DOC-IS) a nitrocellulose filter was incubated with the anti-FN Ab or with the anti-EDA FN mAb, stripped, tested for the absence of residual signal, using the enhanced chemiluminescence (ECL) method (Pierce), and reprobed with an Ab directed against a housekeeping protein, i.e., GAPDH for CE, u-PA for CM and tenasin for DOC-IS fraction. Filters were immunoreacted for 2 h at room temperature with 1 \(\mu\)g/ml anti-GAPDH mAb, 1:1,000 anti-u-PA Ab and 2 \(\mu\)g/ml anti-tenasins mAb diluted in TBS-T. The experiments were performed twice.

To test the purity of the human COLLIII and COLLV proteins added to the culture cell media, a 6% SDS-PAGE was performed loading 10 \(\mu\)g of each protein and staining the gel by Coomassie brilliant blue; alternatively, after blotting, membranes were blocked with 3% non-fat milk and immunoreacted with 1:1,000 anti-COLLV and 1:500 anti-COLLIII Abs, as reported above. For FAK analysis, control, cEDS and vEDS cells, grown for 48 h in complete MEM supplemented or not with COLLV or COLLIII, were treated with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 10 mM NaF, 10 mM Na\(_3\)PO\(_4\), 1 mM Na\(_2\)VO\(_4\), 10 \(\mu\)g/ml leupeptin, 4 \(\mu\)g/ml pepstatin, 0.1% Igepal). Cell lysates were centrifuged at 10,000 rpm for 10 min at 4°C and quantified as reported above. The immunoprecipitation was performed treating 3 mg of proteins with the anti-FAK Ab at 4°C for 3 h, in the presence of 50 \(\mu\)l of protein A-sepharose beads (Pierce, Rockford, IL) diluted 1:1 in TBS. After washing, the bound proteins were recovered by boiling in 1% SDS containing β-mercaptoethanol. Immunoprecipitated FAK protein, following 8% SDS-PAGE and blotting on nitrocellulose sheet, was immunoreacted for 2 h at room temperature with 1:500 anti-FAK Ab and, after stripping, with 1 \(\mu\)g/ml anti-GAPDH mAb. The stripped filters were incubated with 0.4 \(\mu\)g/ml anti-p-Tyr mAb, or 1.0 \(\mu\)g/ml anti-c5¥1 mAb diluted in TBS-T. Alternatively, equal amounts of cell extracts were immunoprecipitated with the anti-c5¥1 mAb, immunoreacted, after the SDS-PAGE, with the anti-c®5¥1 mAb and, after stripping, with the anti-p-Tyr mAb and the anti-FAK Ab. As loading control, equal amounts of crude cell extracts were immunoreacted with the not-related anti-GAPDH mAb. The experiments were performed three times.

All of the detections were performed incubating 2 h at room temperature with HRP-conjugated anti-rabbit, anti-goat or anti-mouse secondary Abs diluted in TBS-T and using the ECL method. For each Ab, the same exposition and developing times were used in the different experiments. The semi-quantitative evaluation of the Integrated Optical Density (IOD) of the whole bands was performed using the Analytical Imaging Station (AIS) software (Imaging Research INC., St. Catherine, Ontario, Canada). The reported values are the means ± SEM of the ratios between the IOD of the sample and control protein band detected in the same lane, obtained in two independent experiments and calculated analysing three control (C, C1 and C2), three cEDS (cEDS, cEDS P4 and cEDS P1) and three vEDS (vEDS, vEDS P3 and vEDS P1) cell strains. Statistical analysis of these data was performed by two-way ANOVA and Bonferroni's post-hoc test carried out by means of GraphPad Prism 4 (GraphPad Software Inc., USA). p values < 0.05 were considered statistically significant.
3. Results

3.1. Purified COLIII and COLLV induce the organisation of an EDA⁺-FN-ECM in defective EDS fibroblasts

The FN-ECM organisation in EDS fibroblasts was analysed by IF on cells grown to confluence in complete MEM using either an anti-FN Ab, recognising all of the FN isotypes (total FN), or an anti-EDA FN mAb, recognising only the EDA⁺-FN isotype. Control fibroblasts organised an abundant EDA⁺-FN-ECM (Fig. 1A), comparable with that detected with the anti-total FN Ab, as shown in Supplementary material (SM) 1A to Fig. 1 and as previously reported [6,8]. On the contrary, cEDS and vEDS cells grown to confluence in complete MEM using either an anti-FN Ab, recognising only the EDA⁺-FN isotype. Control fibroblasts analysed by IF. COLLs-treated control cells the FN-ECM induced by the specific mAb, recognising only the EDA⁺-FN isotype. Control cells were grown in the presence of complete MEM using either an anti-FN Ab or the anti-EDA FN mAb. In the untreated control CE lower immunoreaction with the anti-EDA FN mAb showed that in EDS cells the EDA⁺-FN-ECM organisation was modulated only by the specific mAb they lack.

In the cEDS cells the COL5A1 gene mutation affects not only the COLLV-ECM organisation but also the assembly of COLIII in the extracellular environment; furthermore, both COLls are stored in the cytoplasm. The COL5A1 gene defect of vEDS fibroblasts prevents the COLIII-ECM organisation and leads to its intracellular storage, without affecting the COLLV deposition [6]. These results, confirmed by Western blotting analysis (data not shown), suggest a possible effect of purified human COLLV and COLIII in the rescue of EDS cells’ ECM disarray. A COLLV and COLIII dose–effect curve from 1.0 to 10.0 μg/ml was performed to establish the optimal protein concentrations inducing in COLLV-deficient cEDS and COLIII-deficient vEDS cells a fibrillar COLLV- and COLIII-ECM, respectively (SM 3 to Fig. 1). In the presence of 5 μg/ml of COLLV and COLIII cEDS and vEDS cells organised a fibrillar COLLV- and COLIII-ECM, respectively, covering the adhered cells (SM 3 to Fig. 1).

Therefore, control and EDS cells were treated with 5.0 μg/ml purified human COLLV and COLIII and the FN-ECM organisation was analysed by IF. COLLS-treated control fibroblasts organised the total FN in the ECM (SM 1A to Fig. 1), as previously reported [6]. Similarly, purified COLLV and COLIII induced in cEDS and vEDS cells, respectively, the assembly of a fibrillar FN-ECM (SM 1A to Fig. 1) [6]. The immunoreaction with the anti-EDA FN mAb showed that in EDS cells the FN-ECM induced by the specific COLl they lack contained EDA⁺-FN fibrils (Fig. 1B). In particular, cEDS cells and vEDS cells organised the EDA⁺-FN-ECM in a COLL dose-dependent manner; both EDS cells treated with 5 μg/ml COLLV and COLIII, showed, as well as untreated and COLls-treated control cells (Fig. 1), a fibrillar EDA⁺-FN array (Fig. 1B; SM 3 to Fig. 1), undetectable at lower COL concentrations (SM 3 to Fig. 1).

Similar results were obtained analysing other two control (C1, C2) (SM 4A to Fig. 1), five cEDS (cEDS P1–P5) (SM 4B to Fig. 1) and three vEDS (vEDS P1–P3) (SM 4C to Fig. 1) fibroblast strains, carrying different mutations in COL5A1/COL5A2 or COL3A1 mutations, respectively, grown in the absence and in the presence of 5 μg/ml COLLV and COLIII. These data suggest that the ECM disarray of total FN and EDA⁺-FN is a common feature of cEDS and vEDS skin fibroblasts, due to the COL5A1/COL5A2 and COL3A1 gene mutations. It is noticeable that the altered ECM array of untreated cEDS and vEDS cells and their rescue by COLLL and COLIII, respectively lack, are independent from the mutation type and position along the gene.

In order to investigate whether the ECM disarray observed in EDS cells could be rescued independently from the COL type added to the culture medium, purified COLLV and COLIII were grown for 48 h in complete MEM, in the absence (Untreated) (A) or in the presence of 5 μg/ml human purified COLLV (+COLLV) or COLIII (+COLLIII) (B) were immunoreacted with an anti-EDA FN mAb. Scale bar: 10 μm. Experiments were repeated three times. Representative images are shown.

Fig. 1. Purified COLLV and COLIII induce the assembly of an EDA⁺-FN-ECM in EDS deficient cells. Control, cEDS and vEDS fibroblasts, grown for 48 h in complete MEM, in the absence (Untreated) (A) or in the presence of 5 μg/ml human purified COLLV (+COLLV) or COLIII (+COLLIII) (B) were immunoreacted with an anti-EDA FN mAb. Scale bar: 10 μm. Experiments were repeated three times. Representative images are shown.
Fig. 2. Purified COLLV and COLLIII induce in cEDS and vEDS cells' cytoplasm, extracellular environment and ECM high levels of FN, incorporating the EDA⁺ isotype. A: Western blotting analysis was performed under non-reducing conditions with the anti-FN Ab, recognising all of the FN isotypes (Total FN) and the anti-EDA FN mAb, on the FN stored in the cell cytoplasm (CE), secreted in the culture medium (CM) or present in the ECM (DOC-IS fraction) of control, cEDS, and vEDS fibroblasts grown for 48 h in complete MEM in the absence (Untreated) or in the presence of 5 μg/ml purified COLLV (+COLLV) or COLLIII (+COLLIII). Each membrane, loaded with the CE fraction of untreated, COLLV-treated and COLLIII-treated cells, or with the CM, or with the DOC-IS samples, was immunoreacted with the anti-FN Ab or with the anti-EDA FN mAb, stripped and reprobed with the anti-GAPDH mAb for CE fractions, anti-u-PA Ab for CM fractions and with the anti-tenasin mAb for DOC-IS fractions. GAPDH, u-PA and tenascins were used as housekeeping proteins for normalisation. Tenascins are ECM proteins known to be organised in control and EDS cells' ECM with a similar array, which is not significantly modified by the presence of purified human COLLV and COLLIII (SM 1 to Fig. 2). Experiments were repeated two times. Representative images are shown. B: Quantitative evaluation by image analysis of the FN bands obtained analysing the CE, CM and the DOC-IS fractions. Equal amounts of proteins from control (C, C1 and C2), cEDS (cEDS, cEDS P4 and cEDS P1), and vEDS (vEDS, vEDS P3 and vEDS P1) fibroblast culture fractions were loaded and analysed (Fig. 2A and SM 2 to Fig. 2). The reported values are means ± SEM of the ratios between the Integrating Optical Density (IOD) of sample and the control protein specific for each fraction detected in the same lane, obtained in two independent experiments and calculated analysing three control (C, C1 and C2), cEDS (cEDS, cEDS P4 and cEDS P1) and vEDS (vEDS, vEDS P3 and vEDS P1) cell strains. Statistical analysis was performed using a two-way ANOVA and Bonferroni’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001). The statistically significant differences mentioned in the text are as follows: * compared to untreated control fibroblasts, # compared to untreated cEDS fibroblasts, ^ compared to untreated vEDS fibroblasts, in CM, CE and DOC-IS, respectively.
cell types was approximately 2 and 3-fold lower than in control fibroblasts, respectively (\( \leq p \leq 0.01 \)) (Fig. 2B,a). These data confirm the FN retention at cytoplasmic level observed by IF in EDS cells and the secretion in the culture media of low amounts of total FN, poorly assembled in the ECM (SM 1A to Fig. 1) [6]. The EDA\(^{-}\)-FN amounts measured in untreated control CM and DOC-IS fractions were similar to those evaluated in CE counterparts (Fig. 2A and SM 2 to Fig. 2). In the control CE and CM the EDA\(^{-}\)-FN isotype levels were significantly higher than those evaluated in cEDS and vEDS counterparts. In particular, in the cEDS and vEDS CE the EDA\(^{-}\)-FN levels were approximately 10-fold lower (\( \leq p \leq 0.001 \)) when compared with the EDA\(^{-}\)-FN amounts of the control. The EDS CM contained 3-fold less (\( \leq p < 0.001 \)) EDA\(^{-}\)-FN than the control CM (Fig. 2B,b). The DOC-IS fraction obtained from EDS cells contained levels of EDA\(^{-}\)-FN isotype, which were approximately 8-fold lower than in control cells (\( \leq p \leq 0.001 \)) (Fig. 2B,b). Altogether, these data suggest that EDS cells produce and store higher levels of total FN and lower levels of EDA\(^{-}\)-FN than control cells and secrete and organise in the ECM reduced amounts of EDA\(^{-}\)-FN isotype.

Treatment of control fibroblasts with purified COLLLIII enhanced the levels of total FN in the DOC-IS fraction (\( \leq p \leq 0.001 \)); on the contrary, COLLLIII had no effect (Fig. 2B,a). The levels of total FN in the CM and CE of the COLLLIII- and COLLLIII-treated control cells were comparable to those detected in the untreated ones (Fig. 2B,a). The EDA\(^{-}\)-FN level significantly increased in the CM (\( \leq p < 0.001 \)) and DOC-IS (\( \leq p < 0.005 \)) fraction of COLLLIII-treated control cells, compared to untreated counterparts (Fig. 2B,b). The COLLLIII treatment in all the fractions did not significantly modulate the EDA\(^{-}\)-FN levels (Fig. 2B,b). These data show that in control fibroblasts COLLLIII, but not COLLLIII treatment induces a higher FN-ECM deposition and higher EDA\(^{-}\)-FN levels in the CM and in the ECM. After COLLLV treatment, the levels of total FN detected in the CE of cEDS cells were not significantly different from those measured in the untreated counterpart, whereas in the CE of COLLLIII-treated vEDS cells the total FN levels were slightly higher compared with the untreated counterpart (\( \leq p < 0.05 \)) (Fig. 2B,a). The levels of total FN in the vEDS cells CE fraction were significantly induced when compared to untreated control cells (\( \leq p < 0.001 \)) (Fig. 2B,a). The EDA\(^{-}\)-FN isoform increased 27- and 22-fold in COLLLV-treated cEDS and COLLLIII-treated vEDS CE, respectively (\( \leq p < 0.001 \)) (Fig. 2B,b). Furthermore, the EDA\(^{-}\)-FN induced in both EDS cell types by the treatment with the specific COLL they lack reached significantly higher levels than those of treated and untreated control fibroblasts (\( \leq p < 0.001 \)) (Fig. 2B,b).

Increased amounts of total-FN were detectable in the COLLLV-treated CE and COLLLIII-treated vEDS CM fraction: in particular, approximately 3-fold (\( \leq p < 0.001 \)) and 2-fold increase (\( \leq p < 0.01 \)) were measured when compared to the untreated counterparts, respectively (Fig. 2B,a). Furthermore, in the DOC-IS fraction an approximately 4- and 6-fold increase (\( \leq p < 0.001 \)) and (\( \leq p < 0.001 \), respectively) was observed (Fig. 2B,a). In this fraction, the levels of total-FN after COLL treatment were significantly different from those measured in untreated control cells (\( \leq p < 0.001 \)) (Fig. 2B,a). COLL treatment induced a significant increase of EDA\(^{-}\)-FN levels either in the CM of the EDS cells (approximately 5-fold) (\( \leq p < 0.001 \)) and in the DOC-IS fraction (approximately 7-fold) (\( \leq p < 0.001 \)) (Fig. 2B,b).

These data, combined with the IF results, although limited to three strains for each cell type with a high variability (Fig. 2A and SM 2 to Fig. 2), show that in cEDS and vEDS cells the treatment with the specific COLL they lack might induce the synthesis, secretion and organisation of a FN-ECM, incorporating the EDA\(^{-}\)-FN isotype. In particular, COLLLIII and COLLLIII induce in the cell medium, in the cytoplasm and in the ECM higher EDA\(^{-}\)-FN isotype levels than those evaluated in untreated cells, confirming the EDA\(^{-}\)-FN-ECM restoring observed by IF analysis in these fibroblasts (Fig. 1; SM 4B, C to Fig. 1). The up-regulation of total-FN elicited by COLLLV in cEDS cells ECM was similar to the increase observed in COLLLV-treated control fibroblasts; on the contrary, the increase of EDA\(^{-}\)-FN in the ECM of COLLLV-treated cEDS cells was significantly higher than in control ECM counterpart (Fig. 2B). A similar increase of EDA\(^{-}\)-FN in the ECM was observed in COLLLIII-treated vEDS cells, but not in COLLLIII-treated control fibroblasts (Fig. 2B,b).

Similar effects were observed growing EDS cells in the presence of COLLLs in serum-free medium (data not shown), a condition known to induce apoptosis in these cells [8], indicating that the restored EDA\(^{-}\)-FN assembly into the ECM is independent from serum and should rescue from apoptosis. The high levels of EDA\(^{-}\)-FN stored in COLLLs-treated EDS cells cytoplasms and the increased amounts of protein in the CM and in the ECM suggest that purified COLLLs up-regulate not only the secretion of EDA\(^{-}\)-isotype and its assembly into the ECM but also its synthesis.

3.2. COLLLIII and COLLLV modulate the FN mRNA isoforms expression

In order to evaluate whether COLLLIII and COLLLV treatment, restoring in EDS cells their assembly into the ECM (SM 3 to Fig. 1), influences FN1 gene expression, we assessed in two different cEDS and vEDS cell strains, treated or not with 5.0 \( \mu \)g/ml purified COLLLs, inducing a control-like EDA\(^{-}\)-FN-ECM (SM 3 to Fig. 1), the levels of both EDA\(^{-}\) and EDA\(^{-}\)-FN mRNA isoforms by Real-time PCR. In two different control fibroblast strains either COLLLIII or COLLLV treatment was carried out.

Although this analysis was limited to two cell strains for each cell type and a high variability among these strains was observed, the relative quantification of EDA\(^{-}\) and EDA\(^{-}\)-FN expression showed in both EDS cell types amounts of both isoforms not significantly different from those detected in control fibroblasts (Fig. 3A).

The levels of the EDA\(^{-}\) and EDA\(^{-}\)-FN mRNAs in all COLLL-treated cell types were compared to those of their untreated counterparts (Fig. 3B, C, D). In control fibroblasts the treatment with COLLL did not modulate the levels of both FN mRNA isoforms, whereas COLLLIII significantly increased the levels of both isoforms by approximately 6-fold (\( \leq p < 0.001 \)) (Fig. 3B). This increase of FN mRNA levels, triggered by COLLLIII but not by COLLLV, is likely due to an improved gene transcription and/or to an enhanced mRNA stability.

COLLLV treatment induced in cEDS cell strains an approximately 11-fold (\( \leq p < 0.01 \)) increased level of the EDA\(^{-}\)-FN mRNA and an increase of approximately 34-fold (\( \leq p < 0.001 \)) of the EDA\(^{-}\)-FN mRNA (Fig. 3C). Therefore, in cEDS fibroblasts, COLLL treatment not only raises FN1 gene expression, but also significantly induces the specific maturation of the EDA\(^{-}\)-FN mRNA isoform (\( \leq p < 0.001 \)) (Fig. 3C).

Finally, in vEDS fibroblasts COLLLIII treatment increased by approximately 4-fold the levels of EDA\(^{-}\)-FN mRNA isoform (\( \leq p < 0.01 \)) and about 6-fold the EDA\(^{-}\)-FN mRNA levels (\( \leq p < 0.001 \)) (Fig. 3D). The EDA\(^{-}\)-FN mRNA levels induced by COLLLIII treatment were not significantly different from the increased EDA\(^{-}\)-FN mRNAs ones. Therefore, COLLLIII treatment up-regulates the FN1 gene expression either in control or vEDS fibroblasts, without significantly inducing the preferential maturation of the EDA\(^{-}\)-FN mRNA isoform.

These data, together with the results obtained at protein level, suggest that purified COLLLs modulate the expression of FN at different levels, i.e., transcriptional, post-transcriptional, translational and post-translational level, either in control or in EDS fibroblasts. In particular, COLLLV-treated control fibroblasts respond with an increased synthesis and organisation of total FN without modulating the mRNA levels; on the contrary, COLLLIII has no effect at protein level, even if the treatment induces higher mRNAs amounts of both isoforms. In both EDS cell types the treatment with the specific COLL they lack induces an up-regulation of both FN mRNA isoforms and higher EDA\(^{-}\)-FN amounts either in the intra- and extracellular compartment. Finally, only in COLLLV-treated cEDS cells a significant modulation of FN1 splicing at the EDA region takes place.
3.3. The EDA+ -FN-ECM induced by COLLs restores the α5β1 integrin-FAK signal transduction in EDS cells

The organisation of the αvβ3 and α5β1 integrin receptors was analysed by IF in control and EDS cells grown for 48 h in complete MEM in the absence and in the presence of COLLs. Untreated control fibroblasts organised the α5β1 integrin on the cell surface and only rare αvβ3 patches at the focal adhesion sites. EDS cells organised on the whole cell surfaces the αvβ3 integrin and rare α5β1 integrin patches in the focal contacts (Fig. 4A).

After COLL and COLLIII treatment, control fibroblasts did not modify their integrin setting (Fig. 4B). On the contrary, COLL-treated cEDS and COLLIII-treated vEDS fibroblasts organised α5β1 integrin patches on the whole cell surface, downloading the αvβ3 integrin patches (Fig. 4B). Similar results were also obtained in cEDS P1-P5 and vEDS P1-P3 cells grown in complete medium supplemented or not with COLLs and in the presence of COLLs-supplemented serum-free medium (data not shown), thus excluding that the integrin repatching observed after COLL treatment is serum-dependent.

A gene expression profile study, performed using a microarray assay on untreated, COLLV-treated cEDS and COLLIII-treated vEDS fibroblasts, showed that the levels of α5, β1, αv, β3 integrin subunits and FAK genes expression were not modulated by COLLV and COLLIII treatments (unpublished results). These results suggest that in EDS cells the rich EDA+ and EDA+ -FN-ECM induced by purified COLLs is associated with the restored organisation of the α5β1 integrin receptor; on the contrary, the αvβ3 integrin is preferentially recruited in the presence of the defective EDA+ -FN-ECM.

To investigate whether the EDA+ -FN-ECM induced by COLL treatment might influence the activation of a specific transduction pathway, through the recruitment of the α5β1 integrin receptor it binds, the expression and organisation of FAK were analysed in control and EDS cells, in the absence or in the presence of exogenous COLLs, by Western blotting of FAK immunoprecipitates and IF, performed using a specific anti-FAK Ab. Western blotting showed that the FAK protein, undetectable in EDS cells immunocomplexes before COLL treatment, was induced in COLLV and by COLLIII in vEDS at levels comparable with those detected either in untreated or in COLL-treated control cells' immunocomplexes (Fig. 5a). These results were confirmed by IF analysis: untreated and COLL-treated control cells organise FAK patches, preferentially localised in the focal adhesion sites (SM 1 to Fig. 5). EDS fibroblasts, without detectable FAK clusters before treatment, were induced by COLLs to organise FAK patches in the cell contact sites and on the whole cell surface (SM 1 to Fig. 5). These data suggest that COLLs, inducing the EDA+ -FN-ECM assembly through the α5β1 integrins, also promote FAK recruitment.

To investigate whether COLL treatment switches on an α5β1 integrin-FAK-mediated transduction signalling, the EDA+ -FN-ECM immunoprecipitates recovered from untreated, COLLV- and COLLIII-treated control and EDS cells, were analysed for their tyrosine phosphorylation and binding to α5β1 integrins using the anti-p-Tyr and the anti-α5β1 integrin mAbs. After COLL treatment, in EDS cells' extracts FAK co-immunoprecipitated with the α5β1 integrin and was tyrosine-phosphorylated, as in untreated and COLL-treated control fibroblasts (Fig. 5a). These results are suggestive of an α5β1 integrin-FAK signal transduction activation.

In order to evaluate whether the FAK recruitment and phosphorylation were associated to the α5β1 integrin activation, consequent to the EDA+ -FN-ECM assembly, the α5β1 integrins were immunoprecipitated from control and EDS fibroblasts with a specific anti-α5β1 integrin mAb and tested for tyrosine phosphorylation and binding to FAK, with the
anti-p-Tyr and the anti-FAK integrin Abs, respectively. In COLL-treated control fibroblasts increased amounts of α5β1 integrins immunoprecipitates were detected compared to their untreated counterparts. These integrins were tyrosine-phosphorylated and bound to the FAK protein (Fig. 5b). After COLL treatment, the α5β1 integrin immunoprecipitated from EDS cells at higher levels than those detected in untreated counterparts (Fig. 5b), thus confirming the data obtained by IF (Fig. 4). Furthermore, the α5β1 integrin co-immunoprecipitated with FAK and was tyrosine-phosphorylated to a level comparable to those detected in COLL-treated control fibroblasts (Fig. 5b). The FAK protein co-immunoprecipitated with the α5β1 integrin from the COLL-treated EDS cells was tyrosine-phosphorylated (data not shown), as reported in Fig. 5a. In COLL-treated EDS cells the α5β1 integrin phosphorylation levels were higher than those evaluated in untreated EDS cells (Fig. 5b), suggesting that the EDA+-FN-ECM binding to α5β1 integrin up-regulates the α5β1-mediated transduction signalling. The α5 integrin subunit co-distributed with the FAK protein in COLL-treated cEDS and COLLIII-treated vEDS cells, as shown by the double IF analysis and merging of the fluorescent signals (SM 2 to Fig. 5). The COLLIII treatment of cEDS or the COLL treatment of vEDS did not affect the organisation of the EDA+-FN-ECM (SM 5 to Fig. 1) and only induced few patches of the FAK protein (SM 3 to Fig. 5), suggesting that the EDA+-FN assembly improved the FAK recruitment in EDS fibroblasts. Furthermore, these results suggest that purified COLLV and COLLIII induce in cEDS and vEDS cells, respectively, the α5β1 integrin-FAK signal transduction activation. Still, the FAK binding to other integrin receptors, up-regulated by the COLL treatment, and its downstream activation are not excluded.

3.4. The EDA+-FN-ECM assembly induced by COLLs involves the α9β1 integrin recruitment on EDS cell membrane

The EDA+-FN-specific integrins α4β1, α4β7 and α9β1 receptors, organised by EDS fibroblasts induced to restore the EDA+-FN-ECM by COLL treatment, were investigated by IF analysis using Abs directed against the α4 and α9 integrin subunits, due to the non-availability of anti-human α4β1, α4β7 and α9β1 heterodimers Abs. Untreated EDS cells, COLLV-treated cEDS and COLLIII-treated vEDS fibroblasts did not express detectable levels of α4 integrin subunit, which was organised in untreated and COLLs-treated control cells either in patches on the cell membrane and in nuclear and cytoplasmic compartments (Fig. 6). These data suggest that in EDS cells COLLV and COLLIII treatment does not induce the recruitment of both α4β1 and α4β7 integrins. The α9 integrin subunit, undetectable in untreated and COLLs-treated control cells and in untreated EDS fibroblasts, was abundantly organised in COLLV-treated cEDS and COLLIII-treated vEDS fibroblasts. In particular, the α9 integrin subunit strips were preferentially localised at the focal adhesion sites (Fig. 6). These data, confirmed in all of the EDS cells strains analysed, suggest that the EDA+-FN-ECM restoring, induced in COLL-treated EDS fibroblasts, involves the organisation of the EDA+-FN specific α9β1 integrin.

4. Discussion

In this work we studied the effect of human purified COLLV and COLLIII on the EDA+-FN expression in the cytoplasm, in the cell medium and in the ECM of cEDS and vEDS skin fibroblasts, showing a
defective COLSs- and FN-ECM in the absence of treatment. The lack of an organised FN-ECM in cEDS and vEDS fibroblasts is a common downstream event induced by COLSA1/COLSA2 and COL3A1 genes mutations, responsible for the defective synthesis and assembly of COLV and COLIII fibrils, respectively [6,8, this work].

Co-cultivation with control fibroblasts, synthesising the EDA + -FN isotype, or supplementation with exogenous purified cFN, containing the EDA + isotype, restore the FN-ECM assembly in EDS cells, suggesting that the FN-ECM defect can be related to a reduction of the EDA + -FN isotype levels [4]. Here we report that in vitro grown cEDS and vEDS cells show high levels of total FN in the cytoplasm, low amounts of the protein in the extracellular compartment, and lack of an organised FN-ECM. In all of the EDS cell fractions, and particularly in the DOC-1584 cells, a significant reduction of the EDA + -FN isotype is observed.

We previously hypothesised that in EDS cells this EDA + -FN deficiency might be related to a different proportion of EDA region inclusion due to the FN pre-mRNA alternative splicing, giving rise to an altered ratio of EDA + /EDA − FN mRNAs, compared to control fibroblasts [13]. In the four EDS cell strains analysed in this work a reduced level of EDA + -FN mRNA isoform was not observed, compared to the two control fibroblasts studied and the amounts of the EDA + and EDA − FN mRNAs detected in all of the EDS cells were in the range measured in control fibroblasts. However, the low cytoplasmic amounts of EDA + -FN present in cEDS and vEDS cells suggest that the FN-ECM defect might be associated with a decrease of the EDA + -FN, independently from the EDA + and EDA − -FN mRNA levels.

As previously reported, we showed that COLIII and COLLV treatment restores in EDS cells the FN-ECM assembly [6]. In particular, COLII induce the organisation of a control-like FN-ECM incorporating the EDA + isotype and the storage in the cytoplasm of high levels of EDA + -FN. Until now, only type I and type IV collagen were reported to affect the FN1 gene expression in human epithelial cells; in particular, type IV collagen down-regulates the FN mRNA synthesis rate and the inclusion of the EDA region into the mature FN mRNA [39]. Here the treatment with purified COLLV and COLIII acts on FN1 gene expression at different levels and with some differences in the three cell types investigated. An up-regulation of the FN1 gene expression, which should be due to enhanced gene transcription and/or mRNA stability, and of EDA exon inclusion in the mature transcript in cEDS cells by COLLV is reported, as well as a stimulation of the FN1 gene expression triggered by COLIII in control and vEDS fibroblasts. In particular, the effect triggered by COLIII is observed either in control cells, secreting and assembling into the ECM high level of FN, or in vEDS cells depleted of FN, suggesting that the COLIII action on the FN1 gene expression is independent from the FN level available in the extracellular environment and from the presence of the mutation. Addition of COLV to the EDS cells results in an increase of FN mRNA levels in cEDS, but not in control fibroblasts, which a feedback mechanism could be activated, due to the presence of large amounts of secreted FN. COLV not only up-regulates the FN1 gene expression, but also induces a preferential EDA + -FN mRNA maturation in cEDS cells. The levels of EDA + -FN mRNA synthesised in COLLV-treated cEDS cells reaches higher levels
than those induced by COLLIII in vEDS control cells. Furthermore, the COLLIII treatment increases in vEDS cells either the EDA- and the EDA+ mRNA levels, without significantly modulating the specific maturation of the EDA+-FN mRNA isoform, which is in turn affected by COLLV in cEDS cells. These different behaviors result either in cEDS and vEDS cells in the rescue of the EDA+-FN-ECM defect, suggesting a COLLV and COLLIII regulation at different levels of the mechanisms involved in the FN-ECM assembly.

FN splicing is regulated through a network of different and cross-acting signalling pathways triggered by different extracellular stimuli and ultimately converging on trans-acting splicing regulatory factors which modulate their activity or cellular/nuclear localisation, changing their phosphorylation state [42]. Between these, signalling mechanisms involving the phosphatidylinositol 3-kinase and JNK activities which up-regulate and inhibit the EDA exon inclusion, respectively, are known [43–45]. Although the COLL-mediated signal transduction pathways leading to the modulation of FNI gene expression and splicing were not investigated in this work, the COLL effects here reported suggest that different factors, affecting gene expression, or mRNA half-life and/or splicing [46–49] should be involved. As previously reported, COLLSs organised in the EDS cells’ ECM bind to their specific receptor, the αvβ3 integrin [6], probably switching on signal transduction pathways, which might be mediated by FAK activation. These pathways should converge on specific transcription or splicing factors, modulating FNI gene expression and finally the EDA+-FN assembly in the ECM. In particular, COLLV might induce the EDA exon inclusion through the stimulation of SR proteins, or the inhibition of hnRNPs which favour the EDA exon skipping [48].

COLLs, restoring the FN-ECM assembly, rescue EDS cells from anoikis induced in these cells by the ECM deficiency when cultured in the absence of growth factors [8], indicating that FN-ECM induced by COLL treatment promotes the EDS cell survival. The EDA+-FN-ECMs induced in EDS cells by COLLSs should bind to the FN integrin receptors and differentially affect the transduction signalling for cell survival. In particular, EDS cells, recruiting the αvβ3 integrin, restore the αvβ3 integrin organisation after COLL treatment, associated to the EDA+-FN isoform assembly in the ECM. On the contrary, the EDA- isotype does not rescue the αvβ3 integrin, as shown by EDS cells treatment with pFN, which restores an EDA+-FN-ECM without modifying the αvβ3 integrin setting [8]. Therefore, FN-ECMs differentially assembling the two isotypes, depending on the COLLSs-αvβ3 integrin complexes availability, might influence FN integrin receptor organisation and signal transduction mechanisms in a different manner [8,50–54].

We previously reported that in EDS cells the αvβ3 integrin transduces survival and proliferation signals into the cytoplasm through paxillin, directly bound to the αvβ3 integrin receptor, in the absence of FAK activation [8]. Here we report that COLL and COLLIII treatment restores in EDS cells the αvβ3 integrin and the organisation of the FAK protein, which is tyrosine phosphorylated. These results suggest that in these cells the ECM containing COLLSs and EDA+-FN induces the αvβ3 integrin-FAK-mediated signal transduction. This pathway is not switched on by the ECM lacking the EDA+-FN induced by pFN treatment [8]. The FAK tyrosine phosphorylation observed in COLLSs-treated EDS cells should be explained by the possible recruitment of this kinase not only by the αvβ3 integrin, but also by the COLL-induced αvβ3 integrin [6] and by other FN integrins, including the EDA-specific receptors.
The authors have no conflict of interest to declare.

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[7] S. Vigilo, N. Zoppi, A. Sangalli, A. Galliani, S. Barlati, M. Mottes, M. Colombi, M. Valli, Resistant to anoikis defective EDS cells, whereas the detection of the αvβ3 integrin-mediated FN folding might improve the RGD presentation to the α5β1 receptor. However, functional data are needed to clarify the role of α5β1 integrin in the EDA–FN–ECM recovery in mutant EDS fibroblasts and its involvement in the up-regulation of FAK-mediated signal transduction.

Although normal adult skin fibroblasts in vivo mainly produce EDA–FN, it is known that in tissue remodelling, such as wound healing, the EDA–FN expression is up-regulated and promotes cell adhesion, proliferation and differentiation of fibroblasts into myofibroblasts [22,25,28]. COLLV treatment of in vitro wounded CEDS cells up-regulates the impaired EDS cells migration in wounded area [7]. The present work shows that COLLV treatment up-regulates the EDA exon inclusion, inducing the assembly of the EDA+–FN–ECM and suggests a similar mechanism as in vivo wound healing. As either EDA–FN or its specific receptors, i.e., the α5β1 integrin, are essential for an efficient skin wound healing and ECM organisation [21,52,53], the knowledge of the transduction signalling switched on by COLLV- and COLLV–ECM assembly and of the mechanisms regulating the expression of the different FN isoforms should add new insights in the research of therapeutic approaches for the defective wound healing in EDS patients.

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Declarazione di interesse

The authors have no conflict of interest to declare.