Biochimica et Biophysica Acta 1820 (2012) 1576–1587

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

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

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ARTICLE INFO

Article history:
Received 19 May 2011
Received in revised form 25 May 2012
Accepted 6 June 2012
Available online 15 June 2012

Keywords:
Fibronectin
EDA region
Alternative splicing
Collagen
Integrins
Ehlers–Danlos syndrome

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 $\alpha2\beta1$ and $\alpha5\beta1$ 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, $\alpha5\beta1$ integrin and EDA$^+$-FN-specific $\alpha9$ integrin subunit organisation, $\alpha5\beta1$ 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 $\alpha9\beta1$ integrin organisation, recruits the $\alpha5\beta1$ 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 $\alpha5\beta1$–FAK complexes. COLLs also recruit the $\alpha9\beta1$ 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.

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 $\alpha2\beta1$ integrin [6]; this event leads to the disorganisation of the FN-specific cell surface receptor, the $\alpha5\beta1$ 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,

Abbreviations: CE, cell extract; CM, culture medium; COLLIII, type III collagen; COLLV, type V collagen; COLLs, collagens; DOC-IS, deoxycholate-insoluble; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; FN1, fibronectin gene

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doi:10.1016/j.bbagen.2012.06.004

the α5β1 integrin is the main FN receptor in dermal skin fibroblasts, recognising the FN RGD binding site and regulating outside-in and 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 α2β1 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 EDII or EIIIB) and at the type III connecting segment (IIICS), 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^- isotype. *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 [20,30,31]. 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 COLL [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 FN-ECM defective EDS cells, leading to the α9β1 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 (*COL5A1*, G1181C) and COLLIII (*COL3A1*, G769S), respectively [6,8]. Five cEDS cell strains (P1–P5) carrying different mutations in *COL5A1* and *COL5A2* 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% CO_2 atmosphere. Alternatively, control and EDS cells were seeded in complete MEM supplemented with 1.0, 2.5, 5 and 10 μg/ml purified human COLLV or COLLIII.

2.2. Antibodies and reagents

Mouse anti-α5β1 (clone JBS5), anti-αvβ3 (clone LM609) integrins monoclonal antibodies (mAbs) and purified human COLLV 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 (EPR1355Y) 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 isopropyl alcohol, washed by 75% ethanol solution 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 were 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^-. 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 $2^{-(\Delta\Delta Ct)}$. 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 1×10^5 control and EDS cells grown for 48 h on 24×24 mm glass coverslides in complete MEM supplemented or not with 1.0, 2.5, 5.0 and 10 μg/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 isotypes, and with 1 μg/ml anti-EDA FN mAb diluted in 0.3% BSA, 0.01% NaN_3. To investigate the COLLV, COLLIII and tenascin organisation, the cells fixed in methanol were immunoreacted with 1:100 anti-COLLV and anti-COLLIII Abs or with 2 μg/ml anti-tenascin mAb, respectively, diluted in 1% BSA.

The α5β1, αvβ3 integrins, the α4 and α9 integrin subunits and the FAK distribution were analysed, in control and EDS fibroblasts grown in different culture conditions for 48 h, fixing the cells in 3% paraformaldehyde/60 mM sucrose for 6 min and permeabilising in 0.5% (v/v) Triton X-100 for 90 s. After 2×5 min washing in PBS/0.15 M glycine, the cells were reacted for 30 min at room temperature with 1 μg/ml anti-α5β1 and 4 μg/ml anti-αvβ3 integrin mAbs or with 1:50 anti-α4 mAb and anti-FAK Ab and with 1:25 anti-α9 Ab. A double IF analysis was performed using 1:100 anti-FAK Ab and 2.0 μg/ml anti-α5 integrin subunit mAb.

After 2×3 min washing in PBS, the cells were reacted with FITC- or rhodamine-conjugated anti-rabbit or rhodamine-conjugated anti-mouse or anti-goat IgGs (10 μg/ml in 1% BSA) for 45 min, washed 3×5 min in PBS and mounted on glass slides in 1:1 PBS/glycerol solution. The IF signals were acquired by a CCD black and white TV camera (SensiCam-PCO Computer Optics GmbH, Germany) mounted on a Zeiss fluorescence-Axiovert microscope and digitalised by Image Pro Plus program (Media Cybernetics, Silver Spring, MD). All of the experiments were repeated three times and were performed on the different control, cEDS and vEDS fibroblasts strains.

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, 3×10^5 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 μg/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 cytoplasmic 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% DOC in 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 D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA) and 20 μg 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 μg/ml anti-EDA FN DH1 mAb diluted in 0.3% BSA, 0.01% NaN_3 in PBS, washed 3×10 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 tenascins for DOC-IS fraction. Filters were immunoreacted for 2 h at room temperature with 1 μg/ml anti-GAPDH mAb, 1:1.000 anti-u-PA Ab and 2 μg/ml anti-tenascins 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 μg 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 1:1000 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 extracted 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 $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1KIU/ml aprotinin). 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 μl 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 μg/ml anti-GAPDH mAb. The stripped filters were incubated with 0.4 μg/ml anti-p-Tyr mAb, or 1.0 μg/ml anti-α5β1 mAb diluted in TBS-T. Alternatively, equal amounts of cell extracts were immunoprecipitated with the anti-α5β1 mAb, immunoreacted, after the SDS-PAGE, with the anti-α5β1 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 COLLIII 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 fibroblasts assembled rare EDA$^+$-FN fibrils in the ECM and showed a diffuse intracellular accumulation of protein (Fig. 1A). A similar organisation was detected using the anti-total FN Ab, as shown in SM 1A to Fig. 1 and as previously reported [6,8].

In the cEDS cells the *COL5A1* gene mutation affects not only the COLLV-ECM organisation but also the assembly of COLLIII in the extracellular environment; furthermore, both COLLs are stored in the cytoplasm. The *COL3A1* gene defect of vEDS fibroblasts prevents the COLLIII-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 COLLIII (SM 2 to Fig. 1) in the rescue of EDS cells' ECM disarray. A COLLV and COLLIII 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 COLLIII-deficient vEDS cells a fibrillar COLLV- and COLLIII-ECM, respectively (SM 3 to Fig. 1). In the presence of 5 μg/ml of COLLV and COLLIII cEDS and vEDS cells organised a fibrillar COLLV- and COLLIII-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 COLLIII 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 COLLIII 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 and vEDS cells organised the EDA$^+$-FN-ECM in a COLL dose-dependent manner; both EDS cells treated with 5 μg/ml COLLV and COLLIII, 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 COLL 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 COLLIII. 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 COLLV and COLLIII, they 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 COLL type added to the culture medium, five cEDS cells were grown in the presence of COLLIII and four vEDS fibroblasts of COLLV. These treatments rescued the organisation of a fibrillar FN matrix, without inducing the assembly of the EDA$^+$-FN isotype (SM 5 to Fig. 1), suggesting that in cEDS and vEDS cells the EDA$^+$-FN-ECM organisation is modulated only by the specific COLL they lack.

The evaluation of FN present in the cell extracts (CE), in the culture media (CM) and in the DOC-IS ECM fractions obtained from three control, cEDS and vEDS fibroblasts strains grown in the presence of complete MEM, supplemented or not with purified COLLV or COLLIII, was performed by SDS-PAGE, using either the anti-total FN Ab or the anti-EDA FN mAb. In the untreated control CE lower amounts of total FN were detected than those evaluated in CM and DOC-IS counterparts (Fig. 2A and SM 2 to Fig. 2). In the CE of both untreated EDS cells the amount of total FN was higher than in control fibroblasts, even if only cEDS cells showed a statistically significant difference of approximately 2-fold (Fig. 2B,a: $$ $p<0.01$). In the CM of EDS cells the total FN levels were approximately 2-fold lower than in control cells (***$p<0.001$ for both EDS cell types) (Fig. 2B,a). The total FN organised into the DOC-IS fraction of cEDS and vEDS

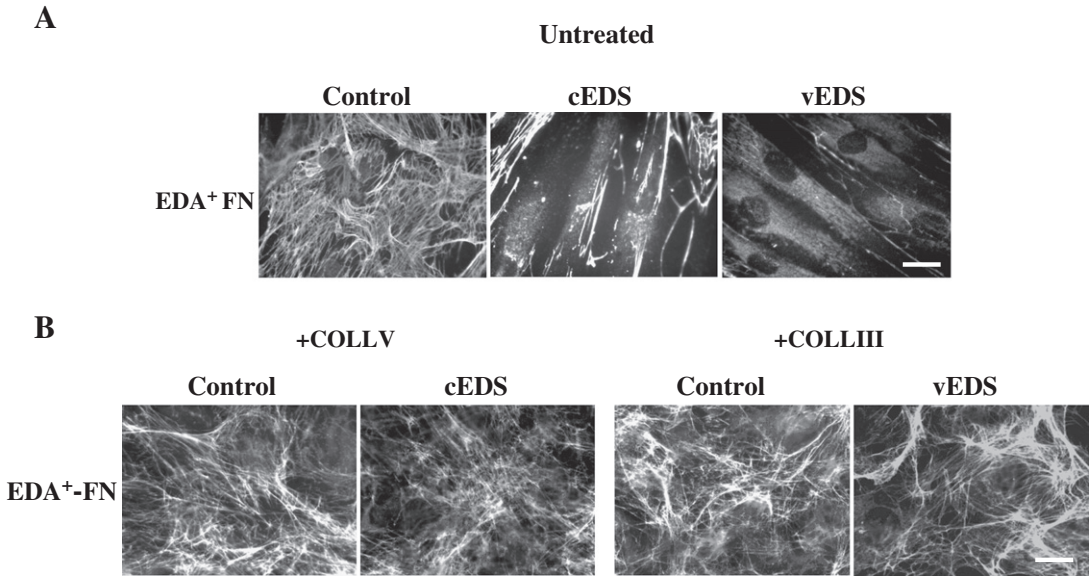

Fig. 1. Purified COLLV and COLLIII induce the assembly of an EDA$^+$-FN-ECM in EDS deficient cells. Control, cEDS and vEDS fibroblasts, grown for 48 h in complete MEM, in the absence (Untreated) (A) or in the presence of 5 μg/ml human purified COLLV (+COLLV) or COLLIII (+COLLIII) (B) were immunoreacted with an anti-EDA FN mAb. Scale bar: 10 μm. Experiments were repeated three times. Representative images are shown.

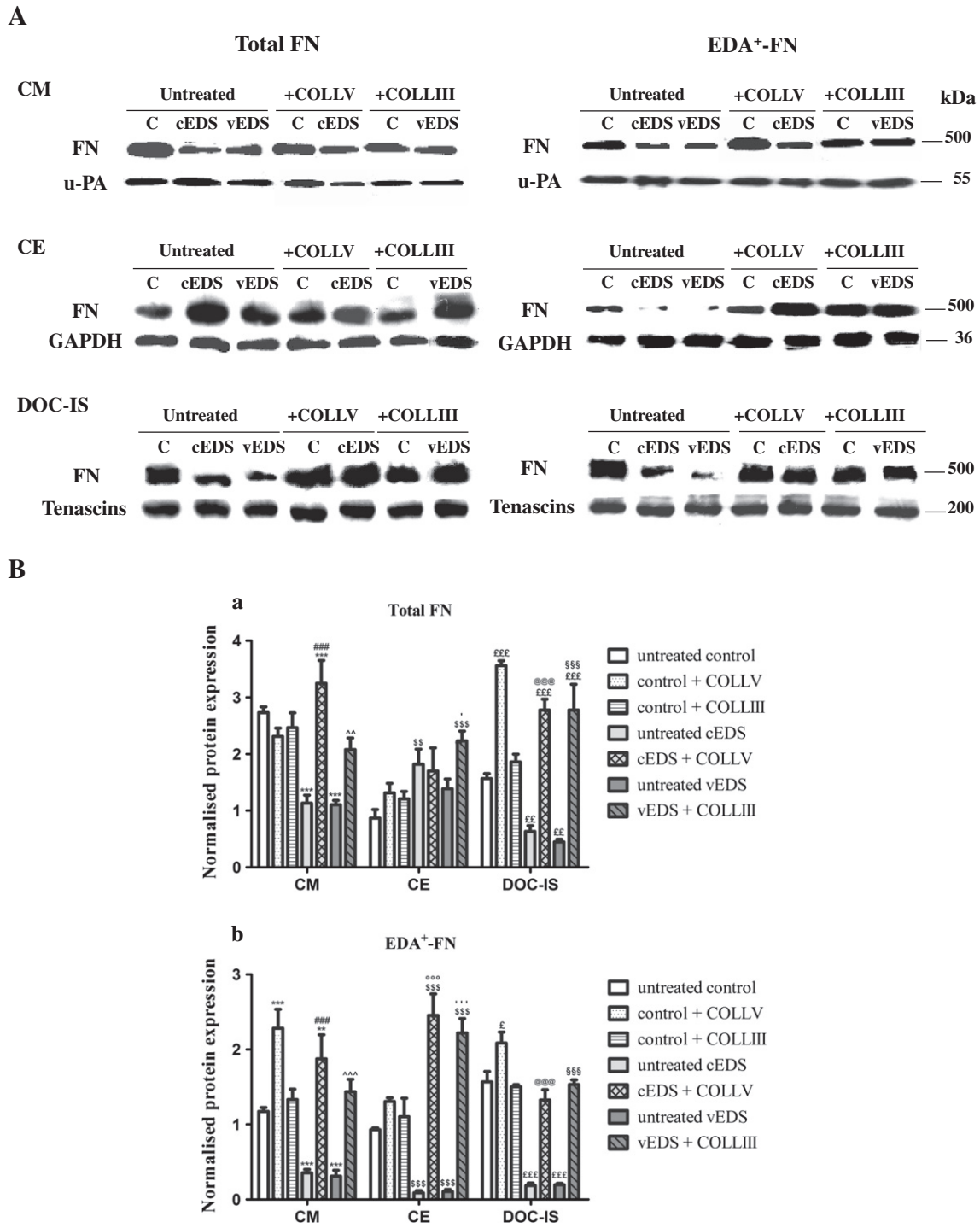

Fig. 2. Purified COLLV and COLLIII induce in cEDS and vEDS cells' cytoplasm, extracellular environment and ECM high levels of FN, incorporating the EDA$^+$ isotype. A: Western blotting analysis was performed under non-reducing conditions with the anti-FN Ab, recognising all of the FN isotypes (Total 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-tenascin 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: *, $, and £ compared to untreated control fibroblasts, #, º, and @ compared to untreated cEDS fibroblasts, ^, ', and § 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 (££ $p<0.01$) (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 ($$$ $p<0.001$) when compared with the EDA$^+$-FN amounts of the control. The EDS CM contained 3-fold less (***$p<0.001$) 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 (£££ $p<0.001$) (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 COLLV enhanced the levels of total FN in the DOC-IS fraction (£££ $p<0.001$); on the contrary, COLLIII had no effect (Fig. 2B,a). The levels of total FN in the CM and CE of the COLLV- and COLLIII-treated control cells were comparable to those detected in the untreated ones (Fig. 2B,a). The EDA$^+$-FN level significantly increased in the CM (***$p<0.001$) and DOC-IS (£ $p<0.05$) fraction of COLLV-treated control cells, compared to untreated counterparts (Fig. 2B,b). The COLLIII treatment in all the fractions did not significantly modulate the EDA$^+$-FN levels (Fig. 2B,b). These data show that in control fibroblasts COLLV, but not COLLIII treatment induces a higher FN-ECM deposition and higher EDA$^+$-FN levels in the CM and in the ECM.

After COLLV 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 COLLIII-treated vEDS cells the total FN levels were slightly higher compared with the untreated counterpart (' $p<0.05$) (Fig. 2B,a). The levels of total FN in the vEDS cells CE fraction were significantly induced when compared to untreated control cells ($$$ $p<0.001$) (Fig. 2Ba). The EDA$^+$-FN isotype increased 27- and 22-fold in COLLV-treated cEDS and COLLIII-treated vEDS CE, respectively (ººº and ''' $p<0.001$) (Fig. 2B,b). Furthermore, the EDA$^+$-FN induced in both EDS cell types by the treatment with the specific COLL they lack reached significantly higher levels than those of treated and untreated control fibroblasts ($$$ $p<0.001$) (Fig. 2B,b).

Increased amounts of total-FN were detectable in the COLLV-treated cEDS and COLLIII-treated vEDS CM fraction: in particular, approximately 3-fold (### $p<0.001$) and 2-fold increase (^^ $p<0.01$) 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 (@@@ and §§§ $p<0.001$, 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 (£££ $p<0.001$) (Fig. 2B,a). COLL treatment induced a significant increase of EDA$^+$-FN levels either in the CM of the EDS cells (approximately 5-fold) (### and ^^^ $p<0.001$) and in the DOC-IS fraction (approximately 7-fold) (@@@ and §§§ $p<0.001$) (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, COLLV and COLLIII 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 COLLV in cEDS cells ECM was similar to the increase observed in COLLV-treated control fibroblasts; on the contrary, the increase of EDA$^+$-FN in the ECM of COLLV-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 COLLIII-treated vEDS cells, but not in COLLIII-treated control fibroblasts (Fig. 2B,b).

Similar effects were observed growing EDS cells in the presence of COLLs 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 COLLs-treated EDS cells cytoplasms and the increased amounts of protein in the CM and in the ECM suggest that purified COLLs up-regulate not only the secretion of EDA$^+$ isotype and its assembly into the ECM but also its synthesis.

3.2. COLLIII and COLLV modulate the FN mRNA isoforms expression

In order to evaluate whether COLLIII and COLLV 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 μg/ml purified COLLs, 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 COLLIII or COLLV 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 COLLs-treated cell types were compared to those of their untreated counterparts (Fig. 3B, C, D). In control fibroblasts the treatment with COLLV did not modulate the levels of both FN mRNA isoforms, whereas COLLIII significantly increased the levels of both isoforms by approximately 6-fold (***$p<0.001$) (Fig. 3B). This increase of FN mRNA levels, triggered by COLLIII but not by COLLV, is likely due to an improved gene transcription and/or to an enhanced mRNA stability.

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

Finally, in vEDS fibroblasts COLLIII treatment increased by approximately 4-fold the levels of EDA$^-$-FN mRNA isoform (**$p<0.01$) and about 6-fold the EDA$^+$-FN mRNA levels (***$p<0.001$) (Fig. 3D). The EDA$^+$-FN mRNA levels induced by COLLIII treatment were not significantly different from the increased EDA$^-$-FN mRNAs ones. Therefore, COLLIII 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 COLLs 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, COLLV-treated control fibroblasts respond with an increased synthesis and organisation of total FN without modulating the mRNA levels; on the contrary, COLLIII 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 COLLV-treated cEDS cells a significant modulation of *FN1* splicing at the EDA region takes place.

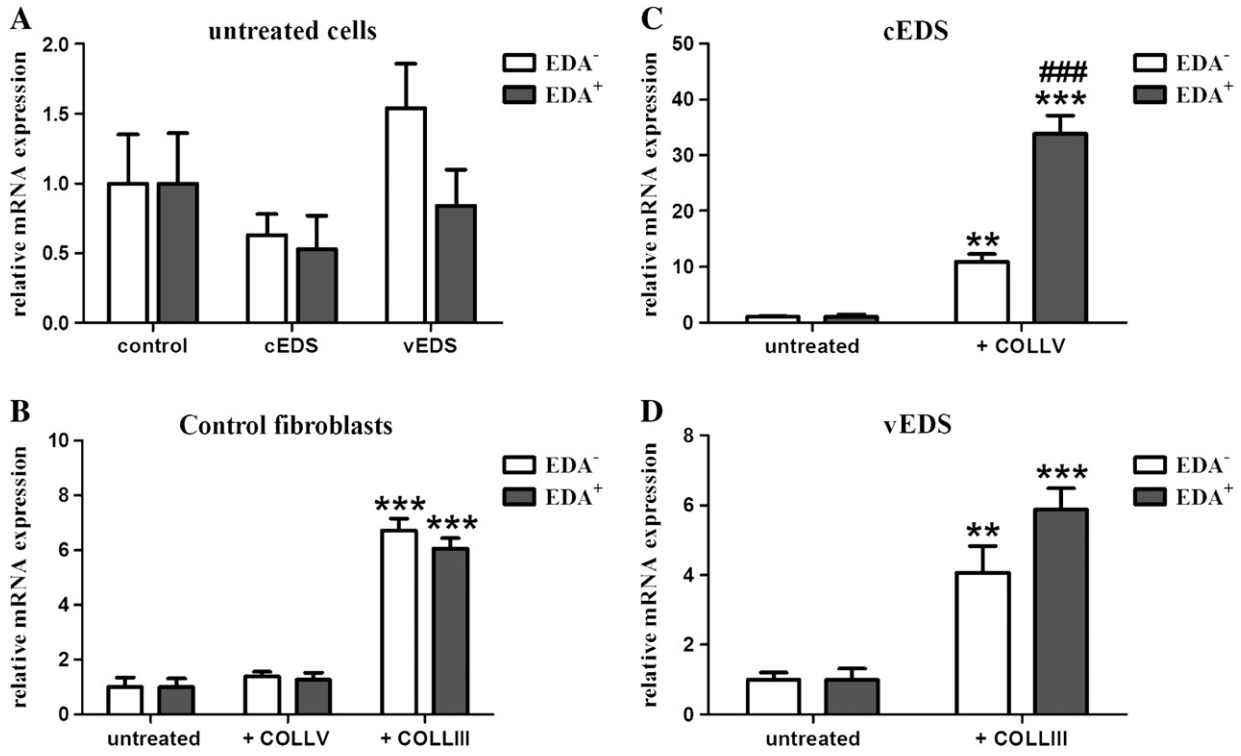

Fig. 3. Purified COLLV and COLLIII regulate the expression and the splicing of the *FN1* gene in EDS cells. The EDA$^+$ and EDA$^-$ FN mRNA isoforms were analysed by Real-time PCR using TaqMan probes and the ΔΔCt method in untreated control (C and C1), cEDS (cEDS and cEDS P4), and vEDS (vEDS and vEDS P3) fibroblasts (A), in COLLV- and COLLIII-treated control cells (B), in COLLV-treated cEDS (C), and in COLLIII-treated vEDS (D). A: The relative quantification of *FN1* gene expression was determined comparing the EDA$^+$ and EDA$^-$ mRNA levels of untreated cEDS and vEDS cell strains to those measured in untreated control fibroblasts. B, C, D: The relative quantification was obtained comparing the EDA$^+$ and EDA$^-$ mRNA levels of COLLV- and COLLIII-treated control cell strains, of COLLV-treated cEDS and COLLIII-treated vEDS cell strains with their untreated counterpart. The reported values are means + SEM obtained analysing two different cell strains for each type, in two independent RT reactions and with each individual determination repeated in triplicate. Statistical analysis was performed using a two-way ANOVA and Bonferroni's *post-hoc* test. **p<0.01, ***p<0.001 compared to untreated counterpart; ### p<0.001 EDA$^+$ *versus* EDA$^-$ mRNA levels in COLLV-treated cEDS fibroblasts.

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 COLLV and COLLIII treatment, control fibroblasts did not modify their integrin setting (Fig. 4B). On the contrary, COLLV-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 by COLLV in cEDS and by COLLIII in vEDS at levels comparable with those detected either in untreated or in COLL-treated control cells' immunoprecipitates (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 FAK 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

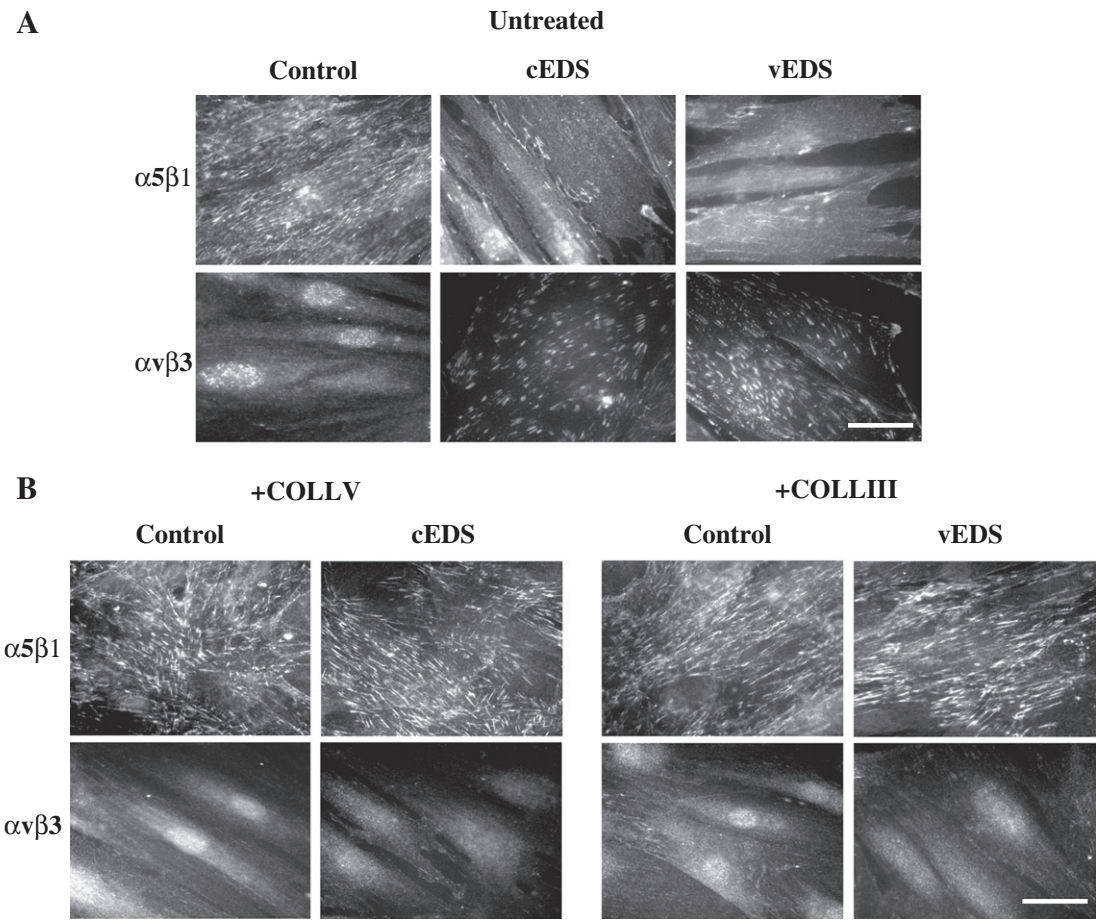

Fig. 4. Purified COLLV and COLLIII induce in αvβ3 integrin-expressing EDS fibroblasts, the αvβ3 to α5β1 receptor switch. Control and cEDS fibroblasts grown for 48 h in complete MEM, in the absence (Untreated) (A) and in the presence of 5 μg/ml purified COLLV (+COLLV) (B), and control and vEDS fibroblasts grown in the absence (Untreated) (A) and in the presence of 5 μg/ml purified COLLIII (+COLLIII) (B) were immunoreacted with the anti-α5β1 and anti-αvβ3 integrin mAbs. Scale bar: 10 μm. Experiment was repeated three times. Representative images are shown.

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 COLLV-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 COLLV 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 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

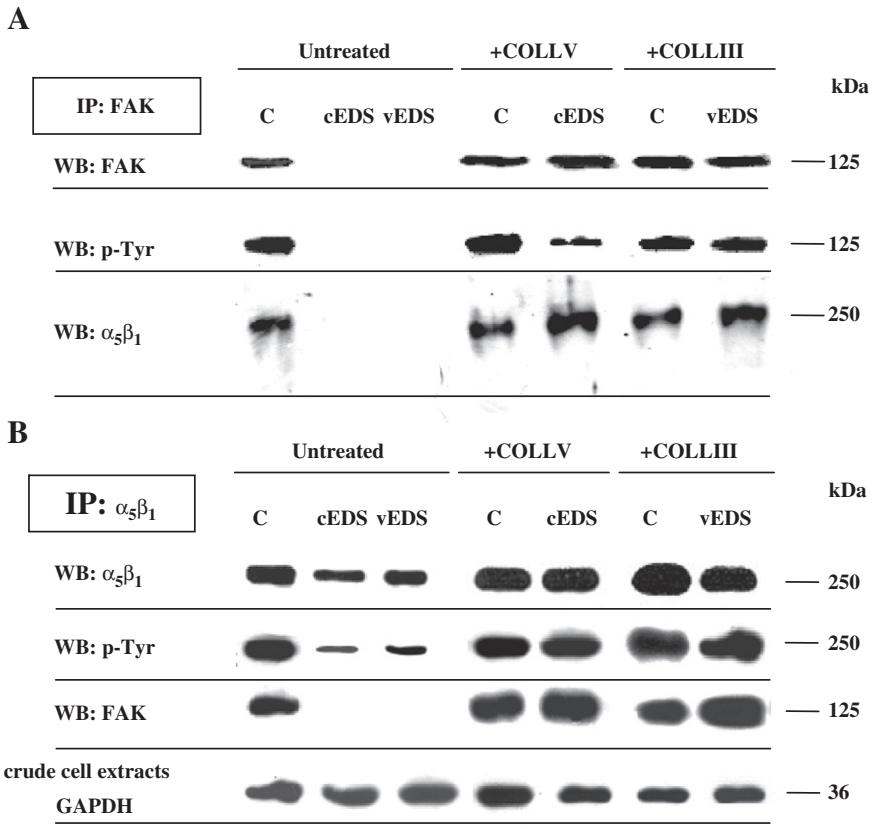

Fig. 5. Purified COLLV and COLLIII induce in FAK-deficient EDS fibroblasts the tyrosine phosphorylated FAK organisation. A): Western blotting of FAK immunoprecipitates from equal amounts of proteins obtained from control (C), cEDS and vEDS cells grown for 48 h in complete MEM (Untreated), from control (C) and cEDS cells grown in complete MEM supplemented with 5 μg/ml purified COLLV (+COLLV) and from control (C) and vEDS cells grown in complete MEM supplemented with 5 μg/ml purified COLLIII (+COLLIII), were immunoreacted with the anti-FAK Ab. After stripping, the blot was immunoreacted with the anti-α5β1 mAb, and subsequently with anti-p-Tyr and anti-GAPDH (not shown) mAbs, to test the activation of FAK and its co-immunoprecipitation with the canonic rescued FN α5β1 receptor. B): Alternatively, the immunoblotting of α5β1 immunoprecipitates was performed using the same Abs, to test the α5β1 integrin expression, phosphorylation and its co-immunoprecipitation with the FAK protein. After each immunoreaction, membranes were stripped and developed by using ECL solution in order to verify the absence of residual signals. Either in A) and B) GAPDH was undetectable when investigated in FAK and α5β1 immunoprecipitates. As loading control, equal amounts of crude cell lysates from untreated and COLLs-treated control and EDS cells were immunoreacted with the anti-GAPDH mAb. All of the experiments were performed three times. Representative images are shown.

defective COLLs- 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 *COL5A1*/*COL5A2* and *COL3A1* genes mutations, responsible for the defective synthesis and assembly of COLLV and COLLIII 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-IS one, 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 show that COLLIII and COLLV treatment restores in EDS cells the FN-ECM assembly [6]. In particular, COLLs 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 COLLIII 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 COLLIII in control and vEDS fibroblasts. In particular, the effect triggered by COLLIII 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 COLLIII 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 purified COLLV results in an increase of FN mRNA levels in cEDS, but not in control fibroblasts, where a feed-back mechanism could be activated, due to the presence of large amounts of secreted FN. COLLV 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

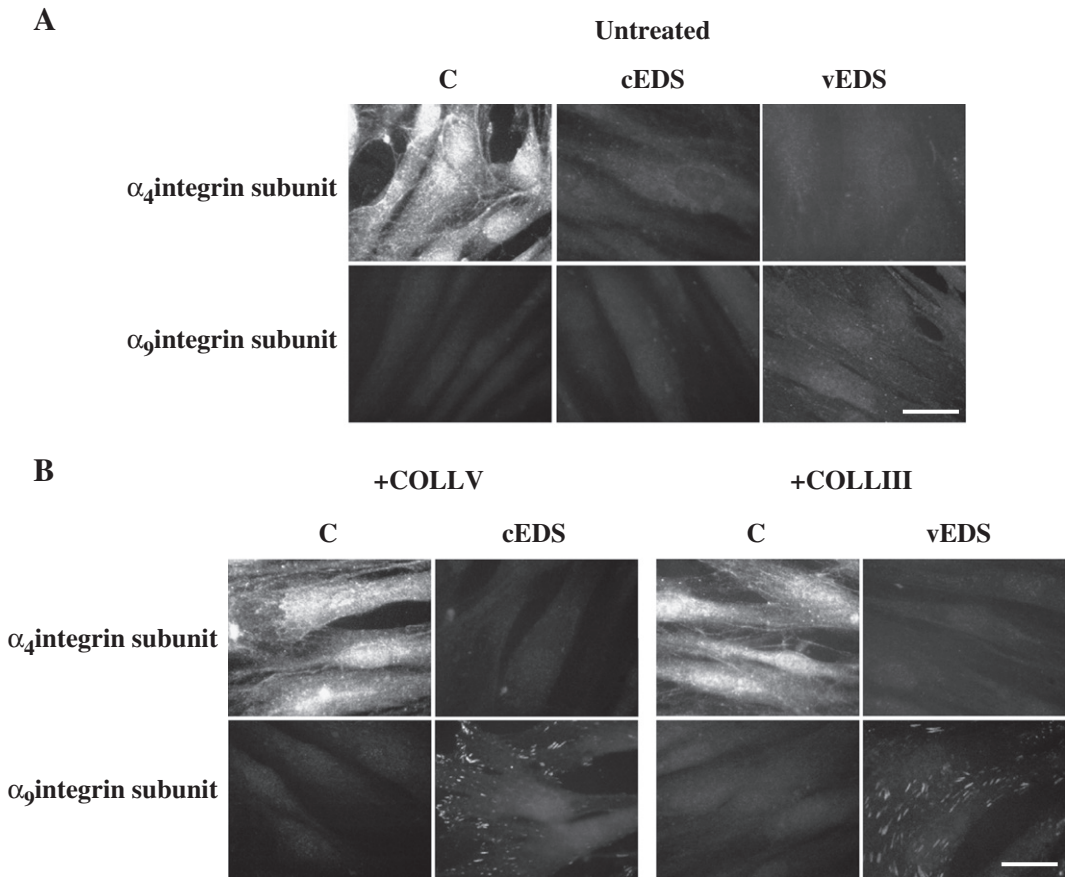

Fig. 6. In COLLV- and COLLIII-treated EDS fibroblasts the α9β1 integrin is involved in the restoring of the EDA$^+$-FN-ECM. To evaluate the involvement of EDA$^+$-FN-specific receptors α4β1, α4β7 and α9β1 integrins in EDA$^+$-FN-ECM assembly, control and cEDS fibroblasts grown for 48 h in complete MEM, in the absence (Untreated) (A) and in the presence of 5 μg/ml purified COLLV (+COLLV) (B), and control and vEDS fibroblasts grown in the absence (Untreated) (A) and in the presence of 5 μg/ml purified COLLIII (+COLLIII) (B) were immunoreacted with the anti-α4 integrin subunit mAb and with the anti-α9 integrin subunit Ab. Scale bar: 10 μm. Experiment was repeated three times. Representative images are shown.

than those induced by COLLIII in vEDS and 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 behaviours 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 *FN1* 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, COLLs organised in the EDS cells' ECM bind to their specific receptor, the α2β1 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 *FN1* 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 COLLs 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 α5β1 integrin organisation after COLL treatment, associated to the EDA$^+$-FN isotype assembly in the ECM. On the contrary, the EDA$^-$ isotype does not rescue the α5β1 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 COLLs–α2β1 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 COLLV and COLLIII treatment restores in EDS cells the α5β1 integrin and the organisation of the FAK protein, which is tyrosine phosphorylated. These results suggest that in these cells the ECM containing COLLs and EDA$^+$-FN induces the α5β1 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 COLLs-treated EDS cells should be explained by the possible recruitment of this kinase not only by the α5β1 integrin, but also by the COLL-induced α2β1 integrin [6] and by other FN integrins, including the EDA-specific receptors

[50,52–54]. The FAK protein recovered from COLLs-treated EDS cells was complexed and co-localised with high levels of phosphorylated α5β1 integrin. These data suggest that the ECM containing COLLs transduces through the α5β1–FAK axis and confirms that the modulation of cell responses to FN depends from the COLL-FN deposition into the ECM [55].

Since the EDA$^+$-FN-ECM assembly induced in EDS cells by exogenous COLLs leads to a marked phosphorylation of FAK and activation of the α5β1 integrin, which are not detected after FN treatment, restoring the EDA$^-$-FN-ECM [8], the α5β1 integrin might be more specific for the binding to EDA$^+$-FN than to FN lacking this region. The COLL-FN interaction or the inclusion of the EDA region could induce a conformational change of FN molecules, preferentially exposing the RGD domain to the α5β1 integrin receptor [51,25]. The EDA domain is recognised either in human and murine species, in normal or pathological conditions, by other integrins, *i.e.*, α4β1, α9β1 and α4β7 dimers [50,52–54]. The recruitment of these integrins by EDA$^+$-FN, which is organised in the EDS cells' ECM following COLL treatment, was investigated in order to clarify whether it plays a role in a more efficient EDA$^+$-FN-ECM assembly. The organisation of the α4β1 and α4β7 integrins was not induced by COLL treatments of deficient EDS cells, whereas the detection of the α9 integrin subunits in both EDS treated cells suggests that the α9β1 integrin is membrane-bound, involved in the EDA$^+$-FN-ECM binding and in a possible cross-talk with the RGD-specific α5β1 FN integrins, eliciting the FN-ECM deposition. COLLs, inducing the EDA$^+$-FN synthesis, might allow the correct protein presentation to the cells and the adequate FN assembly, up-regulating the EDA$^+$-FN binding to the EDA-specific α9β1 integrins. In turn, the α9β1 integrin-mediated FN folding might improve the RGD presentation to the α5β1 receptor. However, functional data are needed to clarify the role of α9β1 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 in *in vivo* wound healing. As either EDA$^+$-FN or its specific receptors, *i.e.*, the α9β1 integrin, are essential for an efficient skin wound healing and ECM organisation [21,52,29,53], the knowledge of the transduction signalling switched on by COLLV- and COLLIII-ECM assembly and of the mechanisms regulating the expression of the different FN isotypes should add new insights in the research of therapeutical approaches for the defective wound healing in EDS patients.

Supplementary related to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.06.004.

Declaration of interest

The authors have no conflict of interest to declare.

Acknowledgement

This work was funded by Ministero dell'Istruzione, dell'Università e della Ricerca, Fondo per gli Investimenti della Ricerca di Base 2009–2011 and Regione Lombardia (Network-Enabled Drug Design) 2011.

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