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Reduced Type I Collagen Utilization: A Pathogenic Mechanism in COL5A1 Haplo-Insufficient Ehlers–Danlos Syndrome

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Abstract To examine mechanisms by which reduced type V collagen causes weakened connective tissues in the Ehlers–Danlos syndrome (EDS), we examined matrix deposition and collagen fibril morphology in long-term dermal fibroblast cultures. EDS cells with COL5A1 haplo-insufficiency deposited less than one-half of hydroxyproline as collagen compared to control fibroblasts, though total collagen synthesis rates are near-normal because type V collagen represents a small fraction of collagen synthesized. Cells from patients with osteogenesis imperfecta (OI) and haplo-insufficiency for proα1(I) chains of type I collagen also incorporated about one-half the collagen as controls, but this amount was proportional to their reduced rates of total collagen synthesis. Collagen fibril diameter was inversely proportional to type V/type I collagen ratios (EDS > control > OI). However, a reduction of type V collagen, in the EDS derived cells, was associated with the assembly of significantly fewer fibrils compared to control and OI cells. These data indicate that in cell culture, the quantity of collagen fibrils deposited in matrix is highly sensitive to reduction in type V collagen, far out of proportion to type V collagen's contribution to collagen mass. J. Cell. Biochem. 92: 113–124, 2004. © 2004 Wiley-Liss, Inc.

Key words: Ehlers–Danlos; collagen; type V; extracellular matrix

Type V collagen is a member of the fibrillar subclass of collagens, which have in common a triple helical domain composed of an uninterrupted series of Gly-X-Y triplets. Type V collagen is a quantitatively minor component in type I collagen-rich fibrils. In contrast to other fibrillar collagens, a portion of the type V collagen amino-terminal propeptide is retained after the collagen molecule is incorporated into fibrils [Linsenmayer et al., 1993; Niyibizi and Eyre, 1993, 1994; Moradi-Ameli et al., 1994]. This non-collagenous domain projects out through the gap region between adjacent type I collagen molecules with major portions present on the fibril surface [Marchant et al., 1996]. These surface domains have been proposed to limit lateral growth of the fibril by steric hindrance and charge interactions; this may be the mechanism underlying the observed negative correlation between collagen fibril diameter and tissue type V collagen content [Adachi and Hayashi, 1986; Fichard et al., 1995].

Heterozygosity for mutations in COL5A1 or COL5A2, which code for the proα1(V) and proα2(V) chains of type V collagen, respectively, is the underlying cause of up to one-half of all cases of the *classic* form of Ehlers–Danlos syndrome (EDS types I/II) [Sokolov et al., 1991; Wordsworth et al., 1991; Burrows et al., 1996, 1997; Nicholls et al., 1996; Toriello et al., 1996; Wenstrup et al., 1996; De Paepe et al., 1997; Michalickova et al., 1998;

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Richards et al., 1998; Bouma et al., 2001] which is characterized by joint laxity, fragile, hyperextensible skin, widened, atrophic scars, and other manifestations of connective tissue weakness [Beighton, 1992; Steinmann et al., 1993; Beighton et al., 1998]. Functional loss of one COL5A1 allele is the most commonly reported molecular mechanism in *classic* EDS, involving about 30% of cases [Schwarze et al., 2000; Wenstrup et al., 2000]. The consequent reduction in type V collagen synthesis is consistent with previous reports of larger than normal collagen fibrils in dermis of EDS patients [Vogel et al., 1979], but the larger diameter fibrils are themselves inconsistent with the reduced mechanical strength of connective tissue observed in EDS since larger diameter collagen fibrils correlate with increased rather than decreased structural properties (stiffness and maximum force) and material properties (modulus and maximum stress) [Parry and Craig, 1988]. This suggests that type V collagen has another function(s) in connective tissue biogenesis that is highly sensitive to quantitative differences in type V collagen content. To address this hypothesis, we examined matrix deposition and collagen fibril morphology in long-term cultures of mutant fibroblast cell strains that would be expected to have up to fourfold differences in the ratio of type V to type I collagen gene expression.

MATERIALS AND METHODS

Cell Strains

Cultured fibroblasts from two individuals with *classic* EDS and two individuals with osteogenesis imperfecta (OI) type I were used for these investigations. EDS8 and EDS53 were heterozygous for mutations in COL5A1 that have been previously published [Wenstrup et al., 2000]. EDS8 has a deletion of a GA dinucleotide at position 3957–3958 or 3959–3960 in the cDNA sequence of COL5A1 that leads to a frameshift and a premature stop codon. EDS53 has a C to T transition that results in a premature stop codon at position 2603 in the cDNA sequence. Both COL5A1 mutations result in undetectable levels of mRNA from the mutant allele, presumably due to nonsense mediated decay. OI2 and OI4 are fibroblasts from two patients with OI type I that are heterozygous for mutations in COL1A1 that result in haplo-insufficiency for proα1(I) chains of type I collagen. OI2 is patient F4 and OI4 is patient F6 in the report by Willing et al. [1994].

Human dermal fibroblasts were cultured in complete medium Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 100 U penicillin, 100 μg/ml streptomycin at 37°C, 95% humidity, and 5% CO_2. For biochemical analysis of collagen deposition and synthesis, 11,500–25,000 cells/cm^2 were plated in complete media plus 0.2 M ascorbate-2-P and grown for 3–28 days. Cultures were re-fed with fresh media twice weekly.

Measurement of Total Collagen Deposited in the Cell Layer

To measure accumulation of the collagenous matrix deposited by the cells, the cell layers from quadruplicate dishes were collected on days 7, 10, 14, and 21, washed with PBS, then suspended either in 6 N HCl for hydrolysis, or in lysis buffer (1 mM $MgCl_2$, 0.2% NP-40) for assay of protein. Colorimetric analysis of the hydroxyproline content of the cell layer was performed after acid hydrolysis [Berg, 1982]. The protein content of each sample was quantified by the method of Lowry et al. [1951] using bovine serum albumin as a standard.

Analysis of Collagen Synthesis

To measure short-term synthesis and secretion of type I collagen, media from cells incubated with 50 μCi 2,3,4,5-[^{3}H] proline (New England Nuclear) for 16 h was harvested with inhibitors (25 mM EDTA, 6.6 mM NEM, 0.1 mM PMSF) and the procollagen was precipitated with 30% ethanol using calf skin collagen as a carrier. The procollagen was digested with pepsin and the resultant collagen chains were separated by SDS–polyacrylamide gel electrophoresis in 5% bis/acrylamide 2 M urea gels under non-reducing conditions as previously described [Bonadio et al., 1985]. Relative amounts of secreted type I collagen were determined by calculating ratios of α1(III) to α1(I) chains after scanning densitometry of autoradiofluorograms.

Collagen biosynthesis as a proportion of total protein synthesized was performed by a modified method of Peterkofsky and Diegelmann [1971]. Media and cells were incubated for 2 h with 20 μCi 2,3,4,5-^{3}[H] proline and then were harvested separately into inhibitors. The cell layer was sonicated to release intracellular proteins. Extensive dialysis was used to remove

unincorporated ^{3}[H]-proline. Equal portions of the dialyzed solution were incubated in a bovine serum albumin carrier solution with or without 10 U of high purity bacterial collagenase (Sigma Chemical Co., St. Louis, MO) for 4 h at 37°C. Proteins were precipitated with a solution of trichloroacetic acid and tannic acid and relative amounts of protein synthesized were obtained by scintillation counting the acid soluble fraction. The relative rate of collagen synthesis was determined correcting for the high amount of proline in collagen.

Morphologic Analysis

Transmission electron microscopy. Cells were grown in 4-well chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL) or in 24-well transwell culture dishes (Costar, Corning, Inc., Corning, NY) for 21 days. Cell cultures were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4 with 8.0 mM $CaCl_2$ for 2 h at 4°C, and processed as previously described [Birk and Trelstad, 1984; Birk et al., 1997]. Briefly, the cultures were post-fixed with 1% osmium tetroxide and enbloc stained with 2% uranyl acetate/50% ethanol. After dehydration in an ethanol series followed by propylene oxide, the tendons were infiltrated and embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenylsuccinic anhydride, and DMP-30 (Electron Microscope Sciences, Fort Washington, PA). Thick sections (1 μm) were cut and stained with methylene blue–azur blue for examination and selection of comparable stratified regions for analysis. Thin sections were then prepared using a Reichert UCT ultramicrotome and a diamond knife. Staining was with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid pH 3.2. Sections were examined and photographed at 75 kV using a Hitachi 7000 transmission electron microscope.

Fibril analyses. The different cell strains were examined in four independent experiments. In each experiment, 1–3 different cultures were analyzed. At least five different regions were selected from thick sections where the cells were multilayered and comparable. Micrographs from non-overlapping regions were taken at 31,680×. The microscope was calibrated using a line grating. For measurements of fibril diameter, micrographs were randomly chosen in a masked manner from the different groups, digitized and diameters were measured using a RM Biometrics-Bioquant Image Analysis System (Memphis, TN). For measurements of fibril number, negative representatives of the different experimental groups were chosen. Three 10 mm lines, corresponding to 1.6 μm, were drawn perpendicular to the cell layer across the calibrated micrographs. The fibrils intersected by the line were counted and the mean determined for each sample. The fibril area fraction was determined in randomly selected micrographs using the Bioquant Image Analysis System. An area was blocked and the fibril area was determined after definition of a threshold value. The area occupied by fibrils, area fraction, was determined by dividing the fibril area by the total area.

Statistics. In the analyses of fibril diameters and fibril counts the field-to-field variability and correlation among the measurements from the same field (always 3 counts per field and 4–93 fibril diameter measures per field) were accounted for in the analyses. This was accomplished fitting linear mixed effect models to fibril diameters and to the square root transformed counts. For the fibril diameters, about 4% of the data was excluded from the model as influential outliers. Residuals were visually examined to check for departures from normality. In the analysis of area densities we used the Kruskal–Wallis test for the overall difference and Wilcoxon two-sample test for the pairwise differences. All analyses were performed using SAS 8.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Type V collagen represents only about 2–4% of collagen in dermis as opposed to 85–90% for type I collagen [Hong et al., 1979; Weber et al., 1984; Smith et al., 1986]. However, these collagens interact within heterotypic fibrils [Birk et al., 1988]. To determine the role of type V/I collagen interactions in the regulation of dermal collagen fibrillogenesis, mutant fibroblast cell strains were examined that would be expected to have up to fourfold differences in the ratios of type V collagen to type I collagen (Table I). Fibroblasts from individuals with OI type I with COL1A1 haplo-insufficiency synthesize type I collagen at approximately 50% or control rates, consistent with previous observations that reduced availability of proα1(I) chains encoded by COL1A1 limits type I collagen synthesis [Barsh et al., 1982; Rowe et al.,

TABLE I. Phenotype and Genotype of Human Cell Lines

Cell strain	Mutation	Clinical phenotype	Collagen I:V, relative ratio	Reference
EDS8	COL5A1+/del 3958–3959	*Classic* EDS[a]	2	Wenstrup et al. [2000]
EDS53	COL5A1+/C2603T (Ter)	*Classic* EDS[a]	2	Wenstrup et al. [2000]
OI2	COL1A1+/C2089T (Ter)	OI[b] type I	0.5	Willing et al. [1994]
OI4	COL1A1+/C3421T (Ter)	OI[b] type I	0.5	Willing et al. [1994]
Co81	—	Normal control	—	—
Co99	—	Normal control	—	—

[a]Ehlers–Danlos syndrome.
[b]Osteogenesis imperfecta.

1985]. Assuming that availability of proα1(V) chains is also rate limiting in type V collagen synthesis, then EDS cells with COL5A1 haplo-insufficiency would be expected to synthesize collagens with one-fourth of the type V: type I ratio of OI type I cells and one-half that of control cells.

Cell lines were chosen specifically for haplo-insufficiency mutations to avoid dominant negative effects from structurally abnormal collagen chains. These effects could include capture of normal proα chains in trimers containing one or more mutant chains or disruption of fibrillar architecture by structurally abnormal trimers. There also appeared to be no effect of the mutations on total protein synthesis or on other parameters of cell health (data not shown).

Fibroblasts cultured from EDS cells with demonstrated COL5A1 haplo-insufficiency (EDS8, EDS53), OI cells with COL1A1 haplo-insufficiency (OI2), and control cells (CO99) were plated in long-term cultures and total collagen accumulation was measured in the cell layers of long-term cultures supplemented with ascorbate. After 7 days, very little collagen was detected. A substantial portion of what collagen that was measured was likely to be procollagen in the intracellular secretory pathway rather than extracellular fibrillar collagen (Fig. 1). After 14 days of culture, there was a substantial difference in cell layer associated hydroxyproline between control cells and either EDS or OI cells. These differences almost certainly represent extracellular collagen, since the amount of intracellular procollagen would be expected to remain constant between 7 and 14 days and to be an increasingly minute fraction of measured hydroxyproline in the cell layer of long-term fibroblast cultures. The differences between EDS and control cultures after 21 days were even more pronounced: mean amounts of hydro-

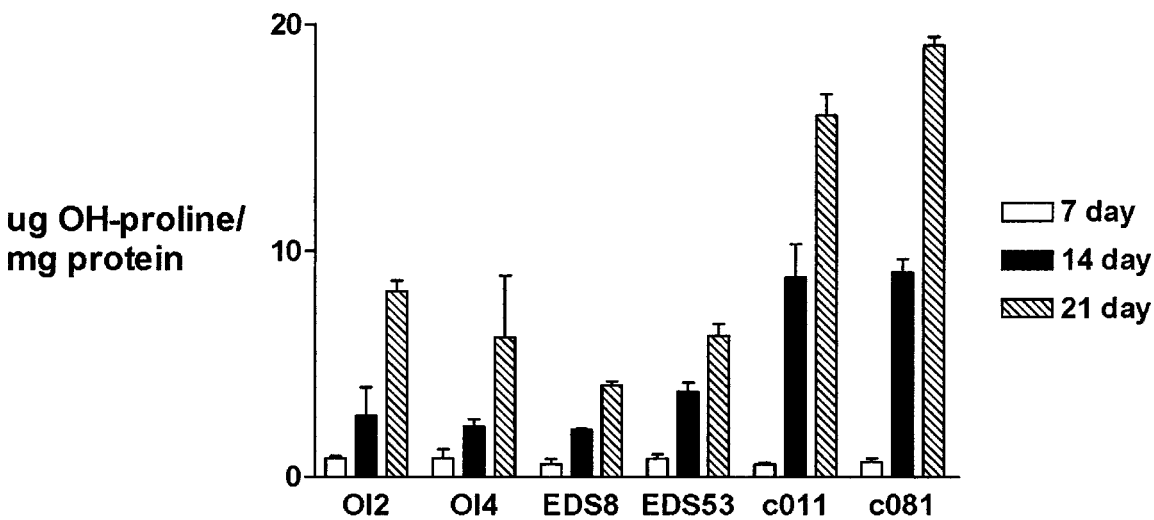

Fig. 1. COL5A1 haplo-insufficiency caused reduction total collagen deposition. Type V collagen deposition as determined by spectrophotometric measurement of hydroxyproline from cell layers of cells from patients with osteogenesis imperfecta (OI) and haplo-insufficiency for proα1(I) chains (OI2, OI4), patients with EDS and haplo-insufficiency for proα1(V) chains (EDS8, EDS53) and control cells (c011, c081).

xyproline deposited in the cell layer of OI and EDS cells were 46 and 30%, respectively of the mean values for two control cell lines (CO99, CO81).

In contrast to OI cells, in which the low rate of hydroxyproline deposition correlated with a proportionate decrease in the rate of collagen synthesis, EDS cells synthesized type I collagen—the major component of dermal collagen—at rates comparable to control cells, when calculated as the ratio of type I to type III collagen secreted into fibroblast media after overnight cultures (Fig. 2). The finding that the type I:type III ratio in COL1A1 haplo-insufficient cells is actually less than half of control cells has been previously observed, and is thought to be related to a compensatory increase in type III collagen in cultured cells [Rowe et al., 1985]. Short-term collagen synthesis, measured as a percentage of total protein production, was approximately equivalent in EDS and control cells, both of which had substantially higher rates than OI cells (Fig. 3). The near normal values of total collagen synthesis in EDS cells, in contradistinction to OI cells, is reflective of the fact that the expected 50% reduction in type V collagen synthesis in EDS cells represents a minute fraction of total collagen synthesized. Thus, there is major discordance between collagen synthesis and collagen utilization in EDS cells with COL5A1 haplo-insufficiency.

Fibroblasts cultured from COL5A1 haplo-insufficient cells (EDS8, EDS53), COL1A1 haplo-insufficient cells (OI2), and control cells (CO99) were plated in transwell culture dishes and examined by transmission electron microscopy. Fibrils from all cell lines synthesized fibrils with characteristic 67 nm periodicity (Fig. 4). Cells with COL1A1 haplo-insufficiency assembled significantly smaller diameter collagen fibrils than the normal control cells (Fig. 5). The mean diameters of fibrils from COL1A1 haplo-insufficient cultures versus control cell cultures was 24 ± 5 nm versus 34.6 ± 7 nm ($P < 0.0001$). The mean values of fibrils from the two COL5A1 haplo-insufficient cell cultures were consistently larger than from controls (EDS8: 38.6 ± 9 nm; EDS53: 36.4 ± 9 nm), however, the differences were significantly different only for the EDS8 cultures ($P = 0.02$ and 0.1, respectively). In both EDS8 and EDS53 cultures, the diameter distributions were broader than the Co99 control cultures and a larger population of fibrils was observed consistently.

Ultrastructural analyses indicated that fewer fibrils were being assembled/deposited in the EDS cultures. This was examined further by determining the number of collagen fibrils assembled in the different cultures. Fibril number in long-term fibroblast cultures was measured by counting collagen fibrils per unit length from electron micrographs. EDS cell cultures assembled significantly fewer collagen fibrils than normal control cultures (Fig. 6A). The mean number of fibrils per 10 m were: 95 (95% CI: 86, 103), 77 (95% CI: 70, 84), 45 (95% CI: 38, 53), 79 (95% CI: 61, 101) for CO99, EDS8, EDS53, and OI2, respectively. The decreased

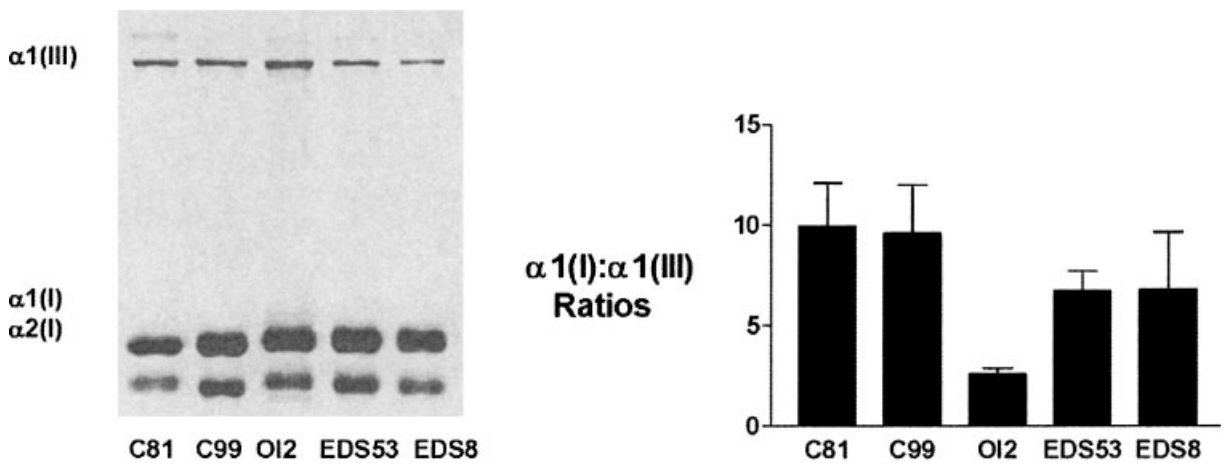

Fig. 2. Secreted ratios of type I:type III collagens. **Left**: Autoradiofluorogram of ^{3}[H]-proline labeled α chains of types I and III collagen harvested from fibroblast culture medium from OI (OI2, OI4), EDS (EDS8, EDS53), and control (C99) cells. **Right**: Ratios after densitometric scanning of gels.

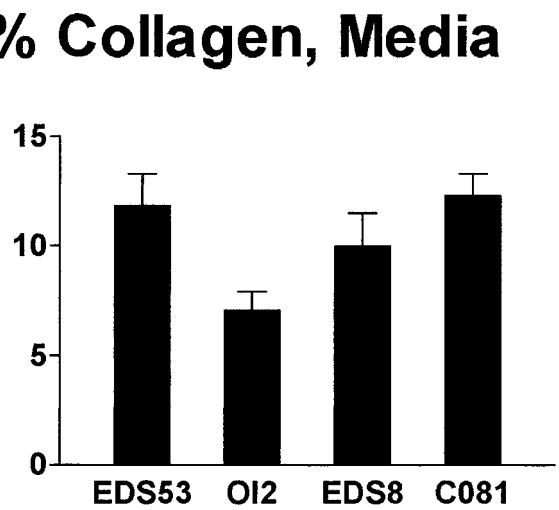

Fig. 3. Short-term synthesis of collagens. Collagen synthesis as a percentage of total protein synthesized during overnight labeling of proteins with ^{3}[H]-proline.

number of fibrils seen in the EDS cultures relative to the control cultures were significant (EDS53 $P < 0.0001$, EDS8 $P = 0.002$). There was no significant difference in the number of fibrils assembled by normal control or OI cultures. However, the fibrils assembled by the OI cells had significantly smaller diameters than either control or EDS fibrils (Fig. 5).

To further examine the relationship between fibril diameter and the number of fibrils deposited in the different cultures, the fibril area fraction was determined. In control cultures, fibrils occupied 44% of the extracellular space. This was reduced to 26 and 36% in the EDS53 and EDS8 cultures, respectively. This is a significant reduction ($P < 0.0001$ and 0.002, respectively). The COL1A1 haplo-insufficient cultures also had a significant reduction in fibril area fraction versus normal control cultures (Fig. 6).

DISCUSSION

Collagen fibrils formed by cultured dermal fibroblasts that synthesize different type V: type I collagen ratios exhibit differences in diameter consistent with previously reported notions of type V collagen's role as a negative regulator of collagen fibril diameter [Birk et al., 1990; Fichard et al., 1995; Marchant et al., 1996]. The observation that cells haplo-insufficient for COL1A1—with consequently elevated type V:type I ratios—synthesize smaller diameter fibrils than control cells also supports earlier in vitro fibrillogenesis experiments that showed an inverse relationship between type V:type I ratios and fibril diameter over a wide range of relative concentrations of the two collagen types [Birk et al., 1990]. However, reduction of proα1(V) chains also had quantitative effect on collagen fibril assembly/deposition. The 50% reduction of proα1(V) chains encoded by COL5A1 in the EDS strains caused a proportionate decrease in the number of collagen fibrils formed in the pericellular space adjacent to dermal fibroblasts. Total collagen deposited into the cell layer was reflected by a reduced number of fibrils adjacent to cells. This decrease was more than an order of magnitude greater than what would be predicted from simple loss of one-half of type V collagen's contribution to total collagen mass. The percentage reduction in total collagen deposition in long-term cultures of OI or EDS cells compared to control was accurately predicted by factoring the relative pericellular fibril number times the mean fibrillar cross sectional area.

These findings support a nucleating function for type V collagen in type I collagen-rich fibrils. The fibril nucleation function is consistent with prior observations by Birk et al. [1988] that type V triple helical epitopes were buried within collagen fibrils, and may therefore may represent the earliest deposited molecules within a fibril. However, the retained amino-terminal propeptide projecting at right angles to the main axis of the fibril [Linsenmayer et al., 1993] may alter the kinetics of fibril formation (reviewed by Birk et al. [1997]). Under conditions in which type V collagen is not limiting, new fibril formation may be favored over lateral expansion of existing fibrils at a given site within crypts formed by fibroblast membranes (reviewed by Kadler et al. [1996]). This model is supported by our observation that collagen fibril number adjacent to COL5A1 haplo-insufficient cells is reduced (Fig. 6). Also, *COL5A1* is a more ancient gene than *COL1A1* [Takahara et al., 1995; Tillet et al., 1996; Exposito et al., 2000] and fibril initiation may be a residual function of COL5A1, at least in some tissues.

We propose that control of collagen fibril formation is complex, and that in addition to transcriptional control of constituent molecules, post-translational control is exerted at the level of fibril nucleation; this process appears likely to be affected by the availability of type V collagen molecules. An additional

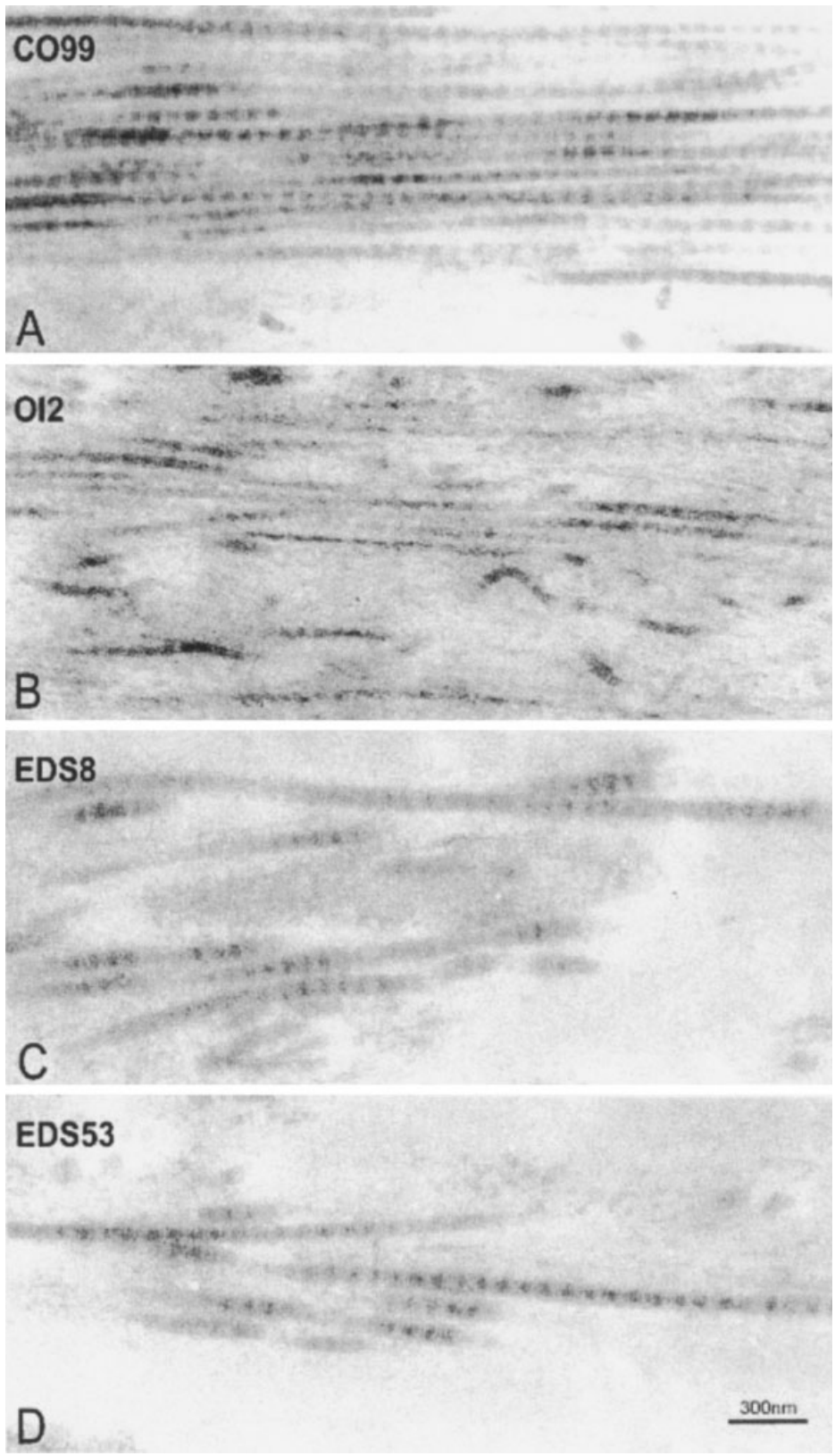

Fig. 4. Type I/V collagen ratios alter the structure of the deposited extracellular matrix. Normal dermal fibroblasts (CO99), fibroblasts haplo-insufficient in col1a1 (OI2), and fibroblasts haplo-insufficient in col5a1 (EDS8 and EDS53) all deposited normal, 67 nm striated collagen fibrils in long-term cultures. The cultures with an increased collagen type I/V ratio (EDS8 and EDS53) had larger diameters and fewer fibrils than the control cultures (CO99). In contrast, the cultures with decreased type I/V ratios (OI2) had a matrix similar to the control cultures, but with smaller diameter fibrils. Bar = 300 nm.

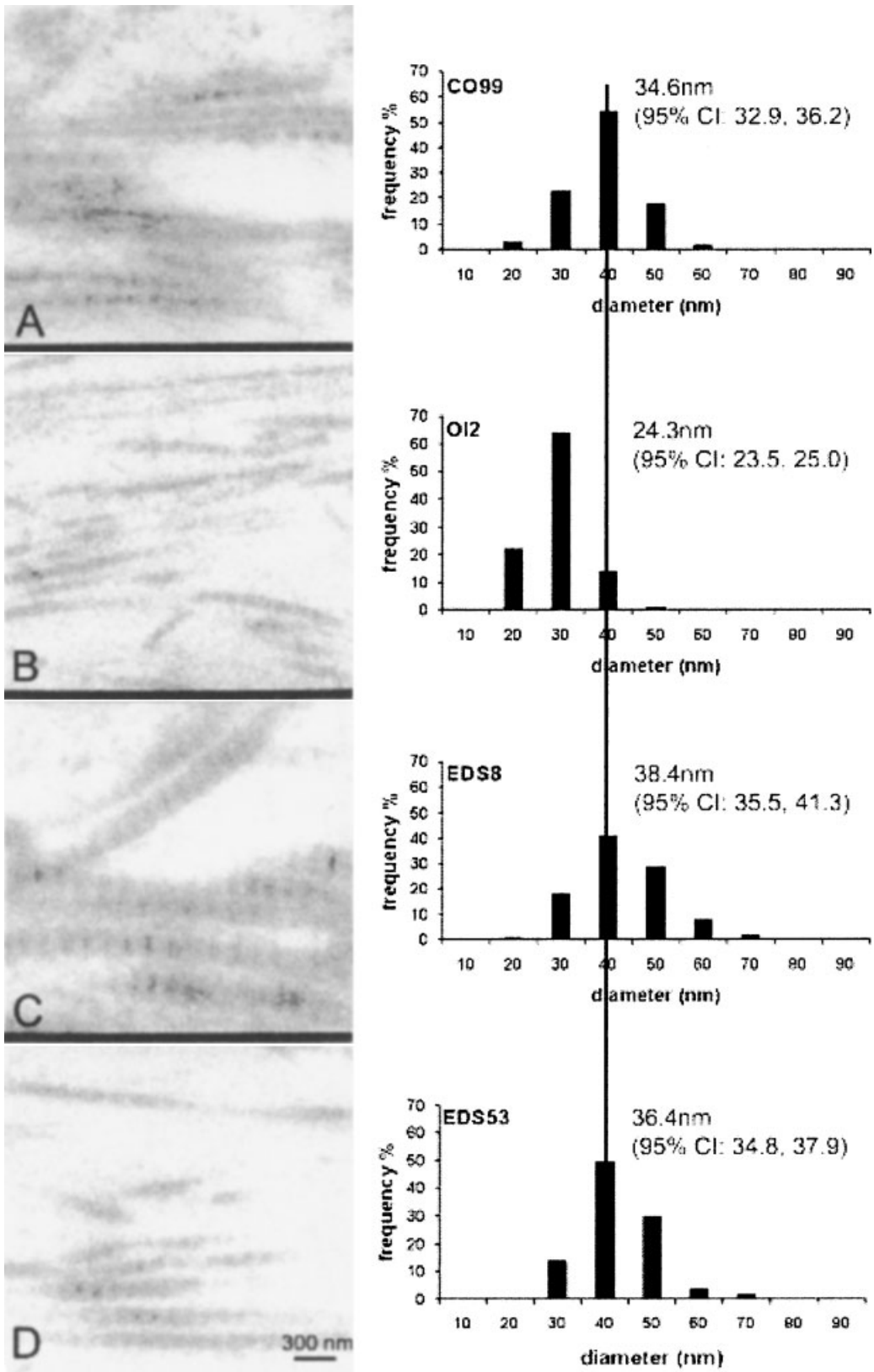
A
B
C
D
300 nm
CO99
34.6nm
(95% CI: 32.9, 36.2)
OI2
24.3nm
(95% CI: 23.5, 25.0)
EDS8
38.4nm
(95% CI: 35.5, 41.3)
EDS53
36.4nm
(95% CI: 34.8, 37.9)
frequency %
diameter (nm)

Fig. 5.

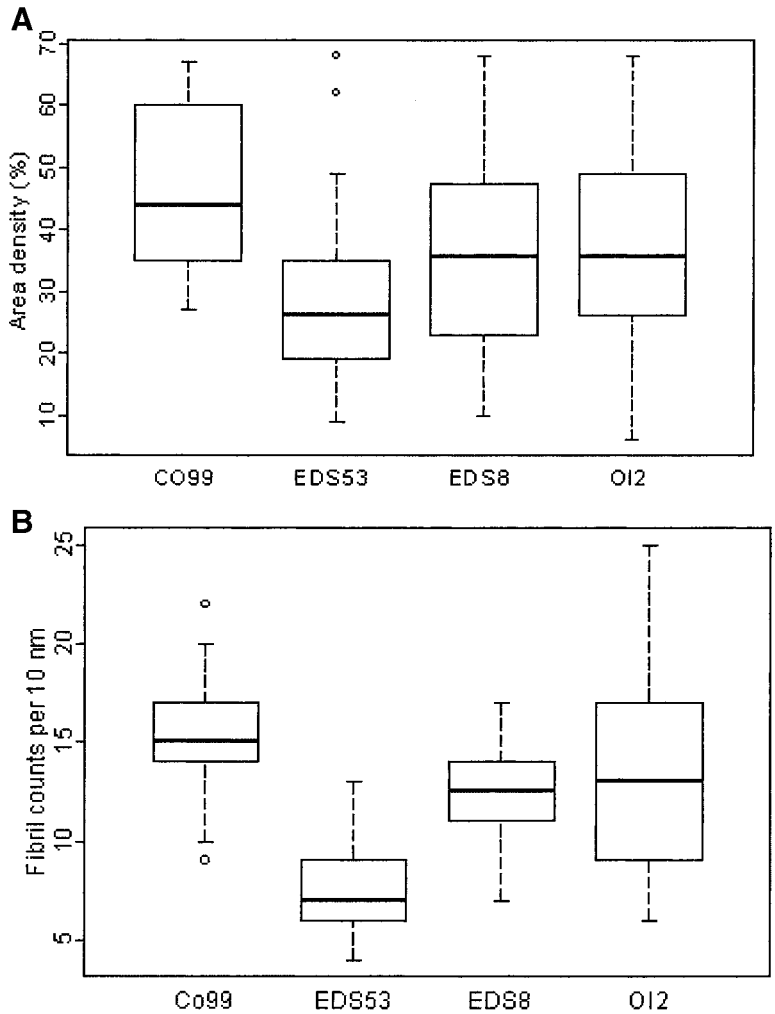

Fig. 6. Type V collagen regulates assembly/deposition of collagen fibrils. Box plots of (**A**) fibril number and (**B**) fibril area fraction. The box shows the inter-quartile range including the middle 50% of the data between 25th and 75th percentiles (**lower** and **upper boundaries** of the box, respectively). The **middle line** shows the median. **Lower** and **upper hatch lines** represent lowest and highest data within 1.5 times the inter-quartile range from the boundaries. For the fibril numbers, the whole inter-quartile ranges of EDS53 and EDS8 are below the inter-quartile range of CO99 consistent with significantly lower fibril counts in EDS53 and EDS8 as compared to CO99. For the fibril area fractions, the downward shift in inter-quartile ranges is less pronounced, but both medians of EDS53 and EDS8 are lower than 25th percentile (first quartile) of CO99.

layer of post-translational control may also be exerted by a mechanism that is sensitive to stoichiometric ratios of multiple collagenous and noncollagenous fibril components, by an unknown mechanism. For example, complete deficiency of small leucine rich repeat proteoglycans (SLRPs) with collagen binding properties, including decorin (DCN), biglycan (BGN), fibromodulin (FM), and lumican (LM), are associated with abnormal fibril size and morphology in mouse dermis [Danielson et al., 1997; Chakravarti, 1998; Ameye et al., 2002; Corsi et al., 2002; Jepsen et al., 2002] (see for review Ameye and Young [2002]). A skin fragility phenotype was reported for DCN deficient animals; DCN, LM, and FM deficient mice show

Fig. 5. Type V collagen content is inversely related to fibril diameter. Histograms of fibril diameter distributions (**right column**) and representative electron micrograph. **A**: Normal dermal fibroblasts (CO99), (**B**) fibroblasts haplo-insufficient in col1a1 (OI2), (**C**) fibroblasts haplo-insufficient in col5a1 (EDS8), and (**D**) (EDS53). The cultures with decreased type V collagen (C, D) have significantly larger mean diameters and a distinct population of larger fibrils.

abnormal collagen fibril morphology by electron microscopy, possibly as a consequence of abnormal lateral fusion or growth of collagen fibrils [Danielson et al., 1997; Svensson et al., 1999; Jepsen et al., 2002]. A reduction of type I collagen deposition was also observed in cultured dermal fibroblasts from tenascin X (TNX) deficient mice, which had normal type I collagen synthesis but reduced collagen deposition in cell culture [Mao et al., 2002]. TNX deficiency in humans is a rare cause of a relatively mild EDS phenotype that is associated with abnormal collagen fibril morphology, but lacks the characteristic wound healing pathology seen in *classic* EDS [Schalkwijk et al., 2001]. The mechanism by which TNX deficiency causes a reduction in collagen deposition is unknown.

Because complete deficiency of SLRPs and TNX results in only mild dermal phenotypes in mice, these molecules may have only a secondarily affect the kinetics of collagen fibrillogenesis, and rather may modify chemical or physical characteristics of fibrils, with some functional overlap. This is in contradistinction to type V collagen. Our data suggest a central role for this collagen in the initiation of fibril formation and therefore matrix assembly in dermal cells. The decreased fibril number/density is reflected in the structural/functional alternations seen in EDS. This central role is supported by data indicating that a complete deficiency of proα1(V) chains in mice is embryonic lethal and in the hemizygous state causes a dermal phenotype similar to the EDS, *classic* type (R.J. Wenstrup and D.E. Birk, unpublished results).

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