

# Homocysteine Modifies Structural and Functional Properties of Fibronectin and Interferes with the Fibronectin–Fibrillin-1 Interaction

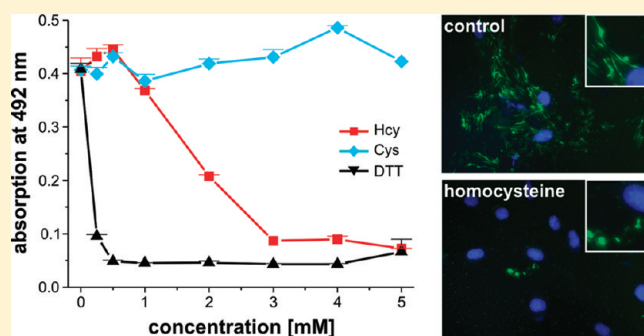
Dirk Hubmacher,<sup>†,‡</sup> Laetitia Sabatier,<sup>†</sup> Douglas S. Annis,<sup>‡</sup> Deane F. Mosher,<sup>‡</sup> and Dieter P. Reinhardt<sup>\*,†,§</sup>

<sup>†</sup>Department of Anatomy and Cell Biology, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

<sup>‡</sup>Departments of Biomolecular Chemistry and Medicine, University of Wisconsin, Madison, Wisconsin 53706, United States

<sup>§</sup>Faculty of Dentistry, Division of Biomedical Sciences, McGill University, Montreal, Quebec, Canada

**ABSTRACT:** Homocystinuria is a genetic disorder resulting in elevated levels of homocysteine in plasma and tissues. Some of the skeletal and ocular symptoms such as long bone overgrowth, scoliosis, and ectopia lentis overlap with symptoms seen in Marfan syndrome. Marfan syndrome is caused by mutations in the extracellular matrix protein fibrillin-1. We previously showed that fibrillin-1 is a target for homocysteine and that the deposition of homocysteinylation fibrillin-1 in the extracellular matrix is compromised. Since the assembly of fibrillin-1 is critically dependent on fibronectin, we analyzed the consequences of fibronectin homocysteinylation and its interaction with fibrillin-1. Cellular fibronectin and proteolytic fragments were homocysteinylation and tested in various interaction assays with recombinant fibrillin-1 and heparin. Fibronectin homocysteinylation consistently compromised the fibronectin–fibrillin-1 interaction, while the interaction with heparin was not affected. Fibronectin homocysteinylation, but not cysteinylation, reduced the fibronectin dimers to monomers as shown by Western blotting. ELISA analyses of homocysteinylation fibrinectin with three monoclonal antibodies demonstrated structural changes in the disulfide-containing FNI domains FNI<sub>2</sub>, FNI<sub>4</sub>, and FNI<sub>9</sub>. Using fluorescently labeled fibronectin, we studied the consequence of fibronectin homocysteinylation on assembly in cell culture. Modified fibronectin showed deficiencies in denovo matrix incorporation and initial assembly. In conclusion, we define here characteristic structural changes of fibronectin upon homocysteinylation that translate into functional deficiencies in the fibronectin–fibrillin-1 interaction and in fibronectin assembly. Since fibronectin is a major organizer of various extracellular protein networks, these structural and functional alterations may contribute to the pathogenesis of homocystinuria and Marfan syndrome.



Homocystinuria (MIM #236200) is a rare genetic disorder caused by mutations in the cystathionine- $\beta$ -synthase, which lead to an elevated concentration of the sulfhydryl-containing amino acid homocysteine in plasma and tissues. Cystathionine- $\beta$ -synthase mutations block the conversion from homocysteine to cystathionine in the transsulfuration pathway.<sup>1,2</sup> Some characteristic features of homocystinuria overlap with those seen in Marfan syndrome (MIM #154700) caused by mutations in the extracellular protein fibrillin-1.<sup>3,4</sup> These common symptoms include long bone overgrowth, scoliosis, kyphosis, ectopia lentis, and occasionally arachnodactyly.<sup>5</sup> However, both disorders differ markedly in other symptoms, such as thromboembolism and mental retardation found exclusively in homocystinuria versus aortic aneurysm and dissection found only in Marfan syndrome. Inter- and intrafamilial variability is a common feature of Marfan syndrome, suggesting that other gene products, metabolic compounds, or environmental factors may play a modifying role in the individual etiopathology. Homocysteine was described as a potential modifier in Marfan syndrome by correlating the severity of aortic aneurysms in patients with elevated homocysteine levels.<sup>6–8</sup>

The sulfhydryl group of homocysteine can reduce disulfide bonds in proteins due to its higher acid dissociation constant

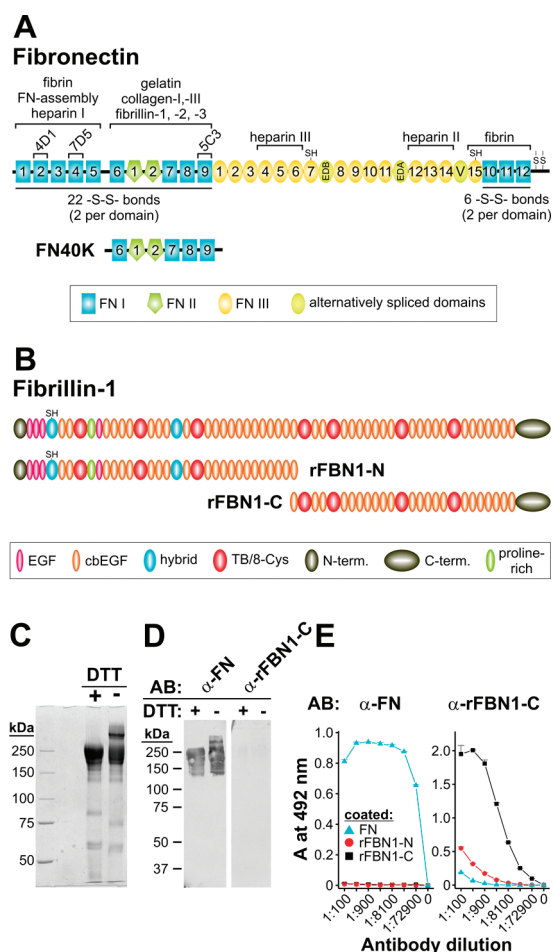
( $pK_a$ ) for the thiol group ( $pK_a \sim 10$ ) compared to that for cysteine ( $pK_a \sim 8.3$ ).<sup>9</sup> This renders homocysteine a reactive nucleophile, stronger than cysteine and able to form mixed disulfides or induce reorganization of disulfide bond patterns in proteins including fibronectin and fibrillin-1.<sup>10–13</sup> Altered disulfide bonds in turn influence the structural integrity, stability, and/or the function of target proteins.<sup>9</sup> As a consequence of the chemical properties of homocysteine, mixed homocysteine–cysteine disulfide bonds are more stable than pure cysteine–cysteine disulfide bonds. Therefore, protein-bound homocysteine can hardly be reduced by other cellular thiols, such as cysteine or glutathione.<sup>9</sup>

Fibronectin exists in two forms: as a cellular fibrous form secreted and assembled by mesenchymal cells and as soluble plasma fibronectin, synthesized by hepatocytes. Fibronectin is a modular protein composed of type I, II, and III repeats (FNI, FNII, and FNIII) (Figure 1). FNI and FNII domains are each stabilized by two intermolecular disulfide bonds, while FNIII domains lack disulfide bonds.<sup>14</sup> Cellular fibronectin is secreted as

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**Figure 1.** Reagents used in this study. (A) Domain organization of human fibronectin. The distribution of the disulfide bonds is indicated below the fibronectin model, and the binding sites for relevant proteins and monoclonal antibodies 4D1, 7DS, and 5C3 are indicated on top. The FN40K fragment is a proteolytic fragment comprising the entire gelatin binding region. FN, fibronectin domain; EDA, EDB, and V are alternatively spliced domains; -SH, free cysteine. (B) Domain structure of full-length fibrillin-1 and the recombinant N-terminal (rFBN1-N) and C-terminal (rFBN1-C) halves are indicated. EGF, epidermal growth factor-like domain; cbEGF, calcium-binding EGF domain; hybrid, hybrid domain; TB/8-Cys, transforming growth factor- $\beta$  binding protein like/8-cysteine domain; N-term., unique N-terminal domain; C-term., unique C-terminal domain; proline-rich, proline-rich domain; -SH, free cysteine. (C) Analysis of purified fibronectin by SDS-PAGE. Samples (5  $\mu$ g/lane) were separated on a 6% polyacrylamide gel in the presence of 20 mM DTT (+) or under nonreducing conditions (-). (D) Western blot analysis of purified fibronectin confirming the identity and integrity of the purified protein using the monoclonal antibody "clone 15" against human fibronectin ( $\alpha$ -FN, left) and the polyclonal anti-rFBN1-C antiserum ( $\alpha$ -rFBN1-C, right) raised against human fibrillin-1 (both 1:1000 dilution). No residual fibrillin-1 was detected in the fibronectin preparation. The positions of globular marker proteins are indicated in kDa. (E) ELISA analysis with soluble "clone 15" antibody ( $\alpha$ -FN, right) and anti-rFBN1-C antiserum ( $\alpha$ -rFBN1-C, left) against immobilized fibronectin (FN), recombinant N-terminal half (rFBN1-N) and C-terminal half (rFBN1-C) of fibrillin-1. The antibody dilution on the X-axis is plotted against the color absorbance at 492 nm on the Y-axis. In subsequent assays, the fibronectin "clone 15" antibody was used 1:500–1:1000 diluted and the fibrillin-1 anti-rFBN1-C antiserum was used 1:1000 diluted. AB, antibody.

a disulfide-bonded dimer with a molecular mass of 230–270 kDa for the monomer and is assembled into a fibrillar network mediated by interactions with cell surface RGD-dependent integrins.<sup>15</sup> The dimer structure is stabilized by a pair of disulfide bonds in the C-terminus of each subunit. This dimer linkage is critical for the assembly and multimerization of fibronectin whereas monomers lacking the relevant cysteine do not form fibrils.<sup>16</sup> Another important region for assembly is located in the first five N-terminal FNI domains, which are indispensable for fibronectin assembly.<sup>16–19</sup> This portion of the molecule is directly followed by a multiprotein interaction site located between FNI<sub>6</sub> and FNI<sub>9</sub>, which provides binding sites for collagen/gelatin and all three fibrillins.<sup>20–23</sup> The assembly of a number of matrix proteins depends on the presence of fibronectin rendering it a "master organizer" of the extracellular matrix. These proteins include fibrillin-1, collagen type I and III, thrombospondin-1, fibulin-1, and LTBP-1.<sup>23–30</sup> It has been demonstrated that homocysteine incorporates into plasma fibronectin and inhibits its binding to fibrin without altering binding to gelatin/collagen.<sup>10</sup> Homocysteinylated regions in fibronectin were localized to the N- and C-terminus but not to the gelatin binding region.<sup>10</sup>

The three fibrillins are characterized by a typical modular organization primarily including calcium-binding epidermal growth factor like domains, transforming growth factor- $\beta$  binding/8 cysteine domains and hybrid domains.<sup>31</sup> Of all matrix proteins, fibrillins contain with 12–13% the most cysteine residues and almost all of them are engaged in intradomain disulfide bonds (Table 1). This exceptionally high cysteine content makes the fibrillins a prime target for the modification with homocysteine in the extracellular matrix. We and others provided evidence that fibrillin-1 is a target for homocysteine modification, resulting in an enhanced susceptibility for various proteases, in reduced self-interaction, heparin binding, and matrix deposition.<sup>11–13</sup> We previously demonstrated that the C-terminal halves of fibrillin-1, -2, and -3 interact with the gelatin binding site in the N-terminal region of fibronectin and that disruption of the fibronectin network compromised fibrillin-1 network formation.<sup>23,32</sup>

In the current study we analyze the effect of homocysteine on fibronectin and show as a consequence that the interaction of fibronectin with fibrillin-1 is compromised but not the interaction with heparin. We further demonstrate that homocysteine, but not cysteine, is able to dose-dependently reduce the dimeric form of fibronectin to the monomeric form and selectively modifies epitopes for disulfide-dependent binding of monoclonal antifibronectin antibodies. Homocysteinylated fibronectin compromises denovo matrix deposition and initial assembly. These consequences of homocysteine on fibronectin could represent contributing factors in the development of clinical symptoms in homocystinuria and Marfan syndrome.

## MATERIALS AND METHODS

**Primary Cells and Cell Lines.** Human dermal fibroblasts were derived from foreskin explants obtained from circumcisions. Informed consent was obtained from the parents prior to the surgery, and the procedure was approved by the local ethics committee (PED-06-054). The generation of the transformed human dermal fibroblast line MSU1.1 and the fibronectin-null mouse embryonic fibroblasts were described previously.<sup>33–35</sup> The human embryonic kidney cell line 293 (HEK 293) was purchased from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Wisent), including penicillin, streptomycin, and glutamine in the

**Table 1. Cysteine Content of Selected Extracellular Matrix Proteins<sup>a</sup>**

protein	accession no.	kDa	# AA	# Cys	% Cys
fibronectin	NP_997647.2	272	2477	63	2.5
fibrillin-1	NP_000129.3	312	2871	361	12.6
fibrillin-2	NP_001990	315	2912	363	12.5
fibrillin-3	NP_115823.3	300	2809	356	12.7
ADAMTSL 2	NP_001138792.1	105	951	62	6.5
ADAMTSL 6	NP_079093.2	112	1018	60	5.9
ADAMTS 10	NP_112219.2	121	1103	66	6.0
collagen I	NP_000079.2	139	1464	18	1.2
collagen III	NP_000081.1	139	1466	22	1.5
collagen VIII	NP_001841.2	73	744	2	0.3
collagen XVI	NP_001847.3	158	1604	32	2.0
collagen VI	NP_001839.2	109	1028	20	1.9
decorin	NP_001911.1	40	359	6	1.7
Delta-like 1	NP_003827.3	41	383	38	9.9
elastin	NP_000492.2	63	724	2	0.3
emilin-1	NP_008977.1	107	1016	23	2.3
emilin-2	NP_114437.2	116	1053	22	2.1
fibulin-1	NP_006477.2	77	703	72	10.2
fibulin-2	NP_001158507.1	132	1231	107	8.7
fibulin-3	NP_004096.2	55	493	40	8.1
fibulin-4	NP_058634.3	49	443	42	9.5
fibulin-5	NP_006320.2	50	448	41	9.2
fibulin-6	NP_114141.2	613	5635	180	3.2
fibulin-7	NP_694946.2	47	439	30	6.8
laminin a3b	NP_937762.1	367	3333	162	4.9
lysyl oxidase	NP_002308.2	47	417	12	2.9
lysyl oxidase-like 1	NP_005567.2	63	574	12	2.1
LTBP 1	NP_996826.2	187	1721	148	8.6
LTBP 2	NP_000419.1	195	1821	160	8.8
LTBP 3	NP_001123616.1	139	1303	126	9.7
LTBP 4	NP_001036009.1	173	1624	160	9.9
MAGP 1	NP_002394	21	183	13	7.1
MAGP 2	NP_003471.1	20	173	8	4.6
Notch-1	NP_060087.3	273	2555	246	9.6
tenascin C	NP_002151.2	241	2201	99	4.5
TGF- $\beta$ 1	NP_000651.3	44	390	12	3.1
Thrombospondin-1	NP_003237.2	129	1170	70	6.0
versican	NP_004376.2	373	3396	35	1.0

<sup>a</sup>The protein sequences were derived from the NCBI Web page and the accession numbers are indicated. The sequence analysis was performed with the ProtParam algorithm (<http://ca.expasy.org/tools/protparam.html>). If different splice variants exist, only the longest isoform was included in the analysis. ADAMTSL, A disintegrin and metalloproteinase with thrombospondin motifs; LTBP, latent transforming growth factor- $\beta$  binding protein; MAGP, microfibrillar-associated glycoprotein; TGF- $\beta$ , transforming growth factor- $\beta$ .

presence or absence of 10% fetal calf serum at 37 °C in a humidified incubator under a 5% CO<sub>2</sub> atmosphere.

**Recombinant Fibrillin-1 and Fibronectin Fragments and Antibodies.** Cloning and production of the N- and C-terminal fibrillin-1 fragments (rFBN1-N and rFBN1-C, respectively) are described elsewhere.<sup>36,37</sup> In short, the recombinant fragments were expressed in HEK 293 cells with an N-terminal BM40 signal peptide and a C-terminal hexa-histidine tag. The proteins were

purified from culture supernatants using a Ni-NTA resin (GE Healthcare) and an Äktapurifier 10 (GE Healthcare). The monoclonal antibody against fibronectin (mouse ascites, “clone 15”) was purchased from Sigma. The polyclonal rabbit antibody against rFBN1-C (anti-rFBN1-C) was extensively characterized previously.<sup>38,39</sup> Notably, no cross-reactivity for fibronectin and rFBN1-C could be detected for both antibodies (Figure 1D,E). Secondary goat-antimouse and goat-antirabbit antibodies coupled to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories. The generation of a 40 kDa, gelatin-binding fragment from human plasma fibronectin followed an established procedure described previously.<sup>40</sup> Monoclonal fibronectin antibodies 4D1, 7D5, and 5C3 were generated against the recombinant human FN70K fragment, comprising the fibrin/heparin- and gelatin-binding region, and the epitopes were mapped as described.<sup>41</sup>

**Purification of Fibronectin.** Cellular fibronectin was purified from 2.5 L of serum-free conditioned medium derived from fibroblasts using a 50 mL gelatin sepharose column (GE Healthcare) according to the manufacturer’s instructions using an Äktapurifier 10 chromatography system. Fibronectin was eluted with 50 mM sodium acetate, pH 5.0 including 1 M sodium bromide. Fractions were analyzed by SDS-PAGE, pooled, and dialyzed against 50 mM Tris, pH 7.4, 150 mM NaCl (TBS) including 1 mM EDTA. After determination of the protein concentration with the bichinonic acid (BCA) assay kit (Fisher Thermo Scientific), the final purity was assessed by SDS-PAGE and Western blotting. Aliquots were stored at –80 °C. The purification of plasma fibronectin was described previously.<sup>42</sup>

**Homocysteine Preparation and Homocysteinylation of Fibronectin and rFBN1-C.** Homocysteine was prepared according to the method described by Duerre with minor modifications.<sup>11,12,43</sup> Briefly, free homocysteine was generated by reacting cyclic homocysteine thiolactone (Sigma) with sodium hydroxide, and the mixture was neutralized with a 1 M potassium phosphate solution. Aliquots were snap-frozen in liquid nitrogen for one-time use only. The concentration of free sulfhydryl groups was determined using Ellman’s reagent.<sup>44</sup> Cysteine was prepared by dissolving cysteine hydrochloride in the same buffer as used for the homocysteine preparation, and the concentration was determined with Ellman’s reagent. Proteins were incubated with different homocysteine concentrations for 24 h at 37 °C. A second aliquot was added after 10–12 h to account for the limited half-life of free homocysteine and cysteine in solution due to oxidation. The proteins were then dialyzed against TBS including 2 mM CaCl<sub>2</sub>, the protein quality was assessed by SDS-PAGE, and the concentration was determined using BCA. If the protein was coated and used as the immobilized ligand, homocysteine and cysteine were not dialyzed out, and the protein concentration was calculated using the initial concentration adjusted for the volume of homocysteine or cysteine added. In this case, residual homocysteine and cysteine were washed out after coating (see below). For the cell-based fibronectin assembly assay, fluorescently labeled fibronectin was incubated for 16 h at 37 °C with homocysteine, cysteine, or DTT, and a second aliquot was added after 4 h. The proteins were used for the assay without dialyzing out the reducing agents.

**Solid-Phase Binding and ELISA Assays.** Solid-phase binding assays were performed as described previously in detail.<sup>12,37,45</sup> In brief, 1  $\mu$ g/well (100  $\mu$ L/well) of either human cellular full-length fibronectin or the proteolytic fragment FN40K was immobilized in TBS overnight at 4 °C in 96-well plates (Maxisorp; Nalge Nunc



International) and blocked with 5% (w/v) nonfat milk in TBS. All washing steps were performed with TBS including 2 mM  $\text{CaCl}_2$  and 0.05% Tween 20 three times for 5 min. Serial dilutions (starting at 100  $\mu\text{g/mL}$ , 1:2 dilution steps) of the fibrillin-1 C-terminal fragment, rFBN1-C, in TBS including 2 mM  $\text{CaCl}_2$  and 5% (w/v) nonfat milk (binding buffer) were incubated for 2 h at 22 °C with the immobilized proteins. To detect the bound ligands, the wells were incubated for 90 min with 1:1000 diluted anti-rFBN1-C in binding buffer, followed by a 90 min incubation with horseradish peroxidase—conjugated goat antirabbit antibody (1:800 dilution in binding buffer) and the color reaction. For the data analysis, only the values for the highest concentration of soluble ligand (100  $\mu\text{g/mL}$ ) were used.

For the enzyme-linked immunosorption assays (ELISA), 0.5  $\mu\text{g/well}$  (100  $\mu\text{L/well}$ ) of protein was coated on each well, and after blocking with 5% (w/v) nonfat milk in TBS, the antibodies were added in serial dilutions (1:3; initial dilutions of 1:100) in the blocking buffer for 2 h at room temperature. All the subsequent steps were performed as described above for the solid-phase binding assays.

**Heparin Binding.** A 1 mL heparin column (HiTrap Heparin HP, GE Healthcare) was equilibrated in 20 mM Tris, 50 mM NaCl, 2 mM  $\text{CaCl}_2$ , pH 7.4. 50  $\mu\text{g}$  of modified or nonmodified fibronectin was injected at a flow rate of 0.1 mL/min. Bound protein was eluted with a linear gradient of 50–900 mM NaCl at a flow rate of 0.5 mL/min. Peak fractions were analyzed by 4–20% SDS-PAGE under reducing or nonreducing conditions followed by silver-staining.

**Western Blot Analysis.** Conditioned medium from human dermal fibroblasts was incubated with different concentrations of homocysteine and cysteine at 37 °C for 24 h as described above. The medium was then dialyzed against TBS using Slide-A-Lyzer Mini dialysis units (Thermo-Fisher Scientific) to remove any residual free homocysteine and cysteine. Controls using the Ellman's reaction verified complete dialysis of both amino acids. 32  $\mu\text{L}$  of sample was mixed with 8  $\mu\text{L}$  of 5-fold concentrated SDS-PAGE sample buffer with (reducing) or without (nonreducing) 20 mM DTT and incubated for 5 min at 95 °C. The proteins were separated by 6% SDS-PAGE and transferred onto a 0.45  $\mu\text{M}$  nitrocellulose membrane (BioRad) for 1.5 h at 400 mA in 10 mM borate buffer. Nonspecific binding sites were blocked for 1 h in TBS including 5% (w/v) nonfat milk. The blot was incubated with the first antibody (1:500 diluted in 5% BSA in TBS) overnight at 4 °C. After washing  $3 \times 10$  min with TBS including 0.02% Tween 20, the blot was incubated with the secondary goat-antimouse antibody coupled to horseradish peroxidase (1:800 diluted in TBS) for 2 h at room temperature. Color reaction was performed with TBS, including 0.5 mg/mL 4-chloro-1-naphthol, 17% (v/v) methanol, and 0.02% (v/v)  $\text{H}_2\text{O}_2$ . The net pixel intensities of the bands was measured using the ImageJ software.<sup>46</sup>

**FITC Labeling of Fibronectin and Cell-Based Fibronectin Assembly Assay.** Plasma fibronectin was labeled with fluorescein isothiocyanate isomer I (FITC) (Sigma) according to a procedure published previously.<sup>18</sup> FN-FITC was incubated with homocysteine, cysteine, or DTT as described above and seeded together with 10 000 fibronectin-null mouse embryonic fibroblasts per well in 8-well chamber slides (BD Biosciences) in DMEM without fetal calf serum, including 0.2% bovine serum albumin, fraction V (Thermo-Fisher Scientific).<sup>47</sup> The final concentration of FN in the medium was 45  $\mu\text{g/mL}$  (~200 nM), and the final concentrations of the modifying agents were 48 and 300  $\mu\text{M}$  for samples modified with 1 and 5 mM, respectively.

However, the half-life of DTT at pH 7.5 and 20 °C is 10 h and cysteine becomes quickly oxidized at neutral pH values (Sigma). Cells were fixed after 6 h with 4% paraformaldehyde, mounted with Vectashield (Vector) and examined with an Axioskop 2 microscope (Zeiss), equipped with an Axiocam camera. Images were recorded with the AxioVision software version 3.1.2.1 (Zeiss).

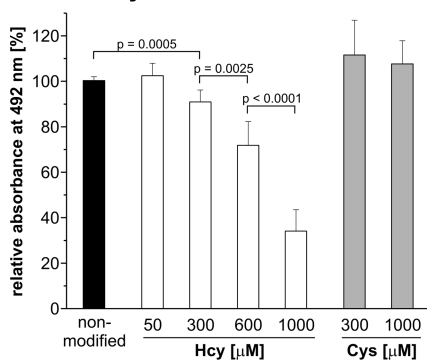
## RESULTS

**Cysteine Content of Fibronectin and Fibrillin and Characterization of the Purified Cellular Fibronectin.** Figure 1A,B illustrates the domain organization of human fibronectin and fibrillin-1, including the respective proteolytic (fibronectin) and recombinant (fibrillin) fragments used in this study. Fibronectin contains 2.5% cysteines, whereas the fibrillins are the most cysteine-rich proteins in the extracellular matrix with a cysteine content of 12.6%, 12.5%, and 12.7% for fibrillin-1, -2, and -3, respectively (Table 1). In fibronectin, the disulfide-bond containing domains (FNI, FNII) are localized in the N-terminal third and the C-terminal region of the molecule, whereas in fibrillins the disulfide bonds are distributed equally over the entire molecule. The C-terminus of fibronectin harbors two cysteine residues involved in the dimerization and self-assembly of fibronectin.<sup>18,40</sup> In addition, there are two nonpaired cysteines with unknown function in the FNIII<sub>7</sub> and FNIII<sub>15</sub> modules that are inaccessible to titration in the folded modules, suggesting no surface exposure of these two nonpaired cysteines (Figure 1A).<sup>48,49</sup>

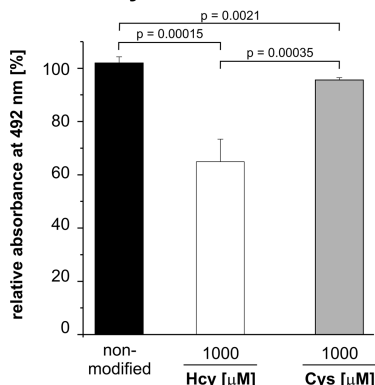
The cellular fibronectin used in our assays was purified from conditioned medium collected from primary human fibroblasts using gelatin chromatography. Figure 1C shows a sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified fibronectin. Under reducing conditions, the major band present at approximately 240–250 kDa correlates with the size range reported for the fibronectin monomer, depending on its specific splice variant (230–270 kDa). Under nonreducing conditions, the typical fibronectin dimer was detectable albeit the ratio of fibronectin dimer to monomer varied from preparation to preparation. No larger fibronectin aggregates have been observed. Variable dimer-to-monomer ratios likely was caused by the purification procedure possibly in combination with some degradation caused by copurified metalloproteinases despite the presence of EDTA as inhibitor. Fibronectin analyzed directly in cell culture medium was almost entirely dimeric (see Figure 4). The interactions of fibronectin with either fibrillin-1 or with heparin are not dependent on the dimerization state of fibronectin, as similar binding properties of fibronectin monomers and dimers were observed with fibrillin-1 (data not shown) and with heparin (Figure 3). Dimer linkage, however, is important for fibronectin assembly.<sup>16</sup>

The monoclonal antibody “clone 15” against fibronectin reacted with monomers and dimers under both, reducing and nonreducing conditions (Figure 1D, left), and did not show any cross-reactivity with fibrillin-1 fragments spanning the entire molecule (Figure 1E, left). A polyclonal antiserum against human fibrillin-1 (anti-rFBN1-C) did not detect residual fibrillin-1 in the fibronectin preparations as demonstrated by Western blotting (Figure 1D, right) or by ELISA (Figure 1E, right). Anti-rFBN1-C showed only some cross-reactivity with the N-terminal half of fibrillin-1 at dilutions lower than 1:900 (Figure 1E, right). Therefore, anti-rFBN1-C was used at a 1:1000 dilution in relevant subsequent experiments.

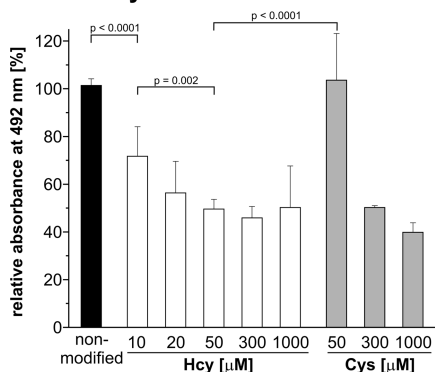
### A: Homocysteine modified fibronectin



### B: Homocysteine modified FN40K

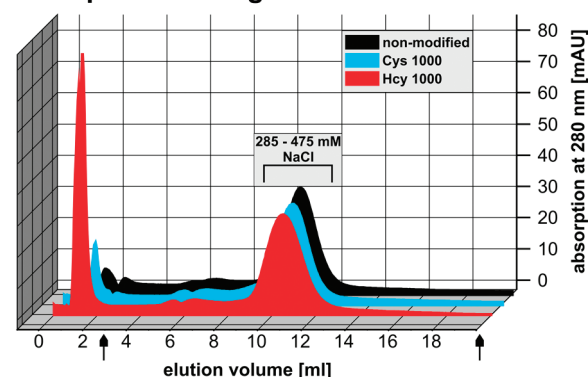


### C: Homocysteine modified fibrillin-1

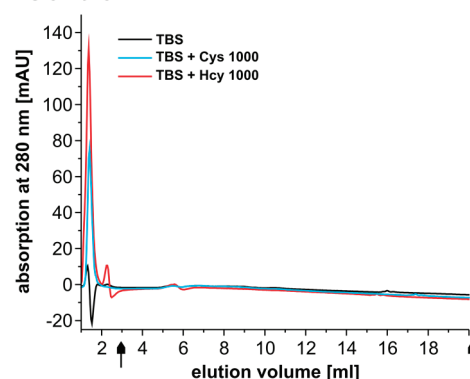


**Figure 2.** Consequences of homocysteinylation on the fibronectin–fibrillin-1 interaction. Fibronectin, fibronectin derived fragment FN40K, and fibrillin-1 C-terminal half (rFBN1-C) were treated for 24 h with homocysteine (Hcy) and cysteine (Cys) at concentrations indicated. Interactions were analyzed in solid phase assays between immobilized untreated and homocysteinylation fibronectin and soluble untreated rFBN1-C (A), immobilized untreated and homocysteinylation FN40K and soluble untreated rFBN1-C (B), and immobilized untreated fibronectin and soluble untreated or homocysteinylation rFBN1-C (C). Immobilized ligands were coated at 10  $\mu\text{g}/\text{mL}$ , and soluble ligands were used at 100  $\mu\text{g}/\text{mL}$ . Serial dilutions of the soluble ligands showed typical binding curves with similar results (data not shown). Nonmodified controls were set to 100%. The interaction between fibronectin or the FN40K fragment with rFBN1-C requires immobilized fibronectin ligands which likely stabilizes a permissive conformation.<sup>23</sup> P-values derived from a two-sided Student's *t* test for significant inhibition of the interaction are indicated. Sample size ranges from  $n = 4$ –8, except for the modified rFBN1-C at 300  $\mu\text{M}$  homocysteine and cysteine in (C) ( $n = 2$ ).

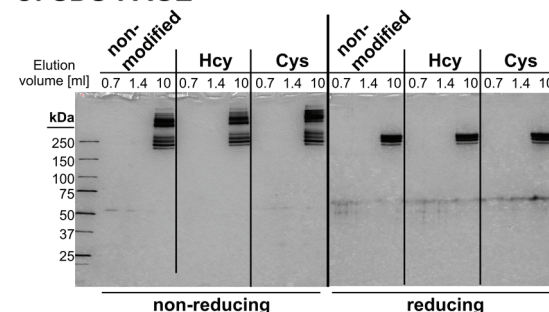
### A: Heparin Binding



### B: Control



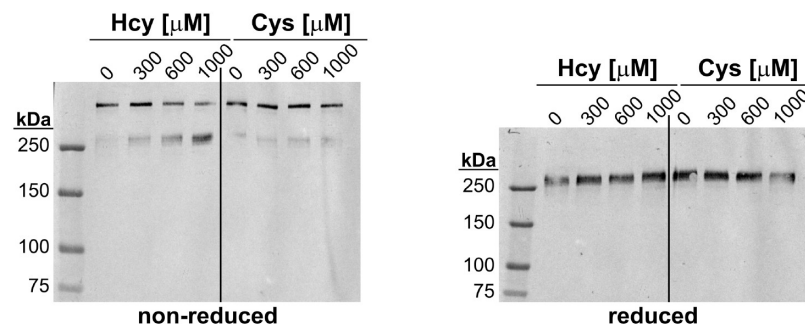
### C: SDS-PAGE



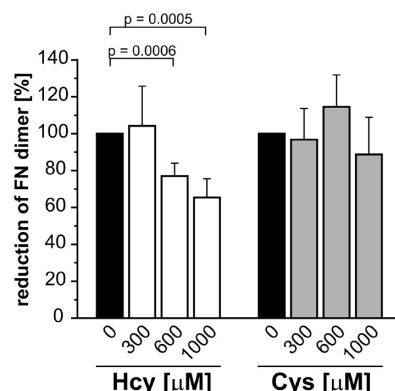
**Figure 3.** Binding of fibronectin to heparin is not affected by homocysteine. (A) Elution profiles of fibronectin (50  $\mu\text{g}$ ) bound to a 1 mL heparin column. Fibronectin modified by homocysteine (Hcy) or cysteine (Cys) as well as nonmodified fibronectin eluted between 285 and 475 mM sodium chloride. The start and end of the linear 50–900 mM NaCl gradient is indicated with an arrow. (B) Control heparin chromatographies of buffer alone (TBS), buffer with 1000  $\mu\text{M}$  cysteine (TBS + Cys 1000), and buffer with 1000  $\mu\text{M}$  homocysteine (TBS + Hcy 1000) confirm that the peaks at about 1 mL (flow through) are caused by the injection of homocysteine or cysteine. The start and end of the linear 50–900 mM NaCl gradient is indicated with an arrow. (C) SDS-PAGE analysis under nonreducing and reducing conditions of representative fractions from (A) on a 4–20% polyacrylamide gel followed by silver staining. Nonmodified and modified fibronectin was detected at an elution volume between 10 and 12 mL but not in the flow through. The positions of globular marker proteins are indicated in kDa. The bands in some lanes at  $\sim 50$ –60 kDa represent typical artifacts of silver-stained gels due to keratin contamination.

**Effect of Homocysteine on the Fibronectin–Fibrillin-1 Interaction.** Evidence is accumulating that fibronectin is a master

# A: Fibronectin dimer reduction by homocysteine



# B: Quantification



**Figure 4.** Homocysteine but not cysteine reduces fibronectin dimers in conditioned cell-culture medium. (A) Western blot analysis of conditioned medium from primary human dermal fibroblasts using the monoclonal fibronectin “clone 15” antibody (1:500 diluted). The conditioned medium was incubated for 24 h with homocysteine (Hcy) or cysteine (Cys) in concentrations as indicated, dialyzed and separated on a 6% polyacrylamide gel either without DTT (left panel) or after subsequent treatment with 20 mM DTT (right panel) for complete reduction. The positions of globular marker proteins are indicated in kDa. (B) Quantification of the fibronectin dimer reduction shown in (A) by quantification of the net pixel intensities. The reduction with 600 and 1000  $\mu$ M homocysteine is significant ( $p < 0.0006$ ; two-sided Student’s  $t$  test;  $n = 4$ ).

organizer for several extracellular matrix proteins, including collagen type I and III, thrombospondin-1, and fibulin-1.<sup>24–29</sup> In addition, we and others recently showed that the formation of the fibrillin-1 network is dependent on a preexisting fibronectin network and that fibronectin can interact with all three fibrillins through its gelatin-binding region.<sup>23,32</sup> To test whether homocysteinylation of fibronectin affects the fibronectin–fibrillin-1 interaction, we employed solid phase binding assays with immobilized homocysteinylation fibronectin and soluble nonmodified recombinant fibrillin-1 C-terminus (rFBN1-C) (Figure 2A). We observed statistically significant dose-dependent reduction of the fibronectin–fibrillin-1 interaction with homocysteine concentrations  $\geq 300 \mu$ M while cysteine as a control had no effect. Additional control experiments demonstrated that binding of homocysteinylation, cysteinylation, and nonmodified fibronectin to immobilized gelatin showed no difference, as reported previously by others (data not shown).<sup>10</sup> Homocysteinylation of the small proteolytic FN40K fibronectin fragment comprising only the gelatin binding region (see Figure 1A) resulted in a similar significant reduction of the interaction with fibrillin-1 compared to the full-length fibronectin (Figure 2B). Cysteinylation of the FN40K fragment also reduced this interaction significantly, but much less than homocysteinylation. The relative reduction in binding to rFBN1-C was not as strong with the homocysteinylation FN40K fragment ( $34.1 \pm 9.4\%$ ) compared to

the homocysteinylation full-length fibronectin ( $65.0 \pm 8.4\%$ ). In an inverse experiment, we tested the effect of homocysteine modification of rFBN1-C on its interaction with immobilized nonmodified fibronectin (Figure 2C). The interaction decreased dose-dependently after homocysteine modification. In contrast to the modification of fibronectin, relatively low concentrations of homocysteine, reaching a plateau at  $50 \mu$ M, were sufficient to account for the inhibitory effect, while cysteine concentrations of  $\leq 50 \mu$ M did not inhibit this interaction. At higher homocysteine and cysteine concentrations (300–1000  $\mu$ M), however, similar inhibition levels were observed. In summary, homocysteinylation of either fibronectin or rFBN1-C affected the fibronectin–fibrillin-1 interaction although higher homocysteine concentrations were required for fibronectin than for rFBN1-C.

**Homocysteinylation of Fibronectin Does Not Affect Heparin Binding.** Fibronectin interacts with heparin through several regions (see Figure 1A). Only the N-terminal HepI binding site contains disulfide bonds in FNI domains that are potentially modifiable by homocysteine.<sup>50</sup> Therefore, we compared the interaction of nonmodified and modified fibronectin by heparin affinity chromatography (Figure 3). Fibronectin was injected in the soluble phase, and bound protein was eluted with a linear sodium chloride gradient. Fibronectin bound quantitatively to the heparin column, and the interaction and affinity with heparin was not affected by either homocysteinylation or cysteinylation

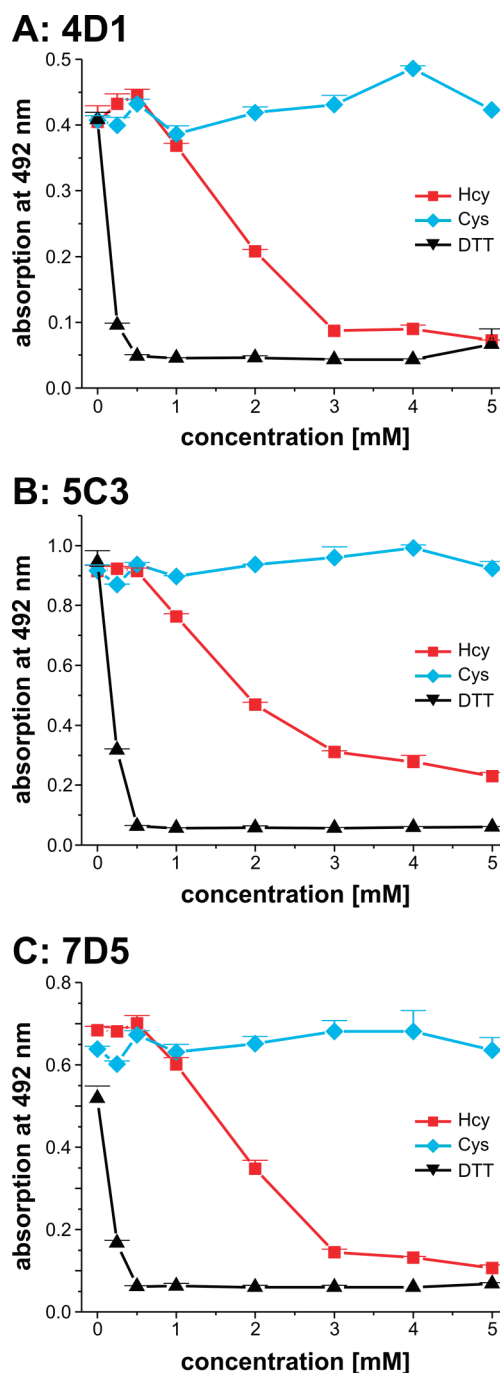


(Figure 3A). The sharp peak at 1 mL was caused by the injection of homocysteine or cysteine attributable to the absorption of disulfide-linked homocysteine or cysteine at 280 nm (Figure 3B).<sup>51</sup> Both the monomeric and the dimeric form of fibronectin interacted with heparin and eluted with ~285–475 mM sodium chloride (Figure 3A,C).

**Homocysteine Disrupts Dimerization of Fibronectin.** Primary human dermal fibroblasts secrete fibronectin into the cell culture medium predominantly in a dimeric form. Treatment of conditioned cell culture medium with increasing homocysteine concentrations (0–1000  $\mu$ M) resulted in a dose-dependent reduction of the fibronectin dimers to monomers, whereas cysteine did not cause dimer reduction at comparable concentrations (Figure 4A, left). As a control, the identical homocysteine and cysteine-treated conditioned media samples were subsequently treated with DTT for complete reduction and as loading control (Figure 4A, right). To quantify the effect of homocysteine on the reduction of the fibronectin dimer, we determined pixel intensities of the dimeric fibronectin bands on Western blots at various concentrations of homocysteine or cysteine (Figure 4B). This analysis corroborated the strong and statistically significant reduction of dimeric to monomeric fibronectin in the presence of 600 and 1000  $\mu$ M homocysteine to  $77.1 \pm 7.0\%$  and  $65.4 \pm 10.1\%$ , respectively, compared to nontreated fibronectin. No significant fibronectin dimer reduction was observed after incubation with cysteine.

**Sensitivity of Individual FNI Domains in Fibronectin to Homocysteine.** Monoclonal antibodies with cognate binding epitopes in the disulfide-containing domains FNI<sub>2</sub>, FNI<sub>4</sub>, and FNI<sub>9</sub> (see Figure 1A) were used to test structural alterations in these domains upon homocysteinylation.<sup>41</sup> First, we tested whether the respective epitopes in fibronectin are stabilized by disulfide bonds (Figure 5A–C, black symbols). Reduction of disulfide bonds in fibronectin by increasing concentrations of DTT dose-dependently reduced the binding of all three antibodies reaching maximum loss of the epitopes at 0.5 mM DTT. Next, we analyzed the consequences of homocysteine or cysteine treatment on the reactivity of the monoclonal antibodies with their epitopes (Figure 5A–C, red and blue symbols). All three epitopes were sensitive to the reduction by homocysteine. Approximately a 6-fold higher concentration of homocysteine (~3 mM) was required for complete loss of the antibody epitopes compared to the treatment with DTT. The reduction in epitope recognition was specific for homocysteine, since cysteine did not exert any effect in similar concentrations. These results demonstrate that homocysteine modifies the FNI<sub>2</sub>, FNI<sub>4</sub>, and FNI<sub>9</sub> domains resulting in sufficient structural changes to abolish binding to the respective antibodies. The concentrations of homocysteine disrupting the antibody epitopes are above the concentration range observed in severe forms of homocystinurias (up to 0.5 mM in patient serum). However, it should be noted that in the described experiments we attempted to compensate for the extended time (years) of a constant presence of elevated homocysteine levels in human patients using higher concentrations of homocysteine over feasible incubation periods (24 h).

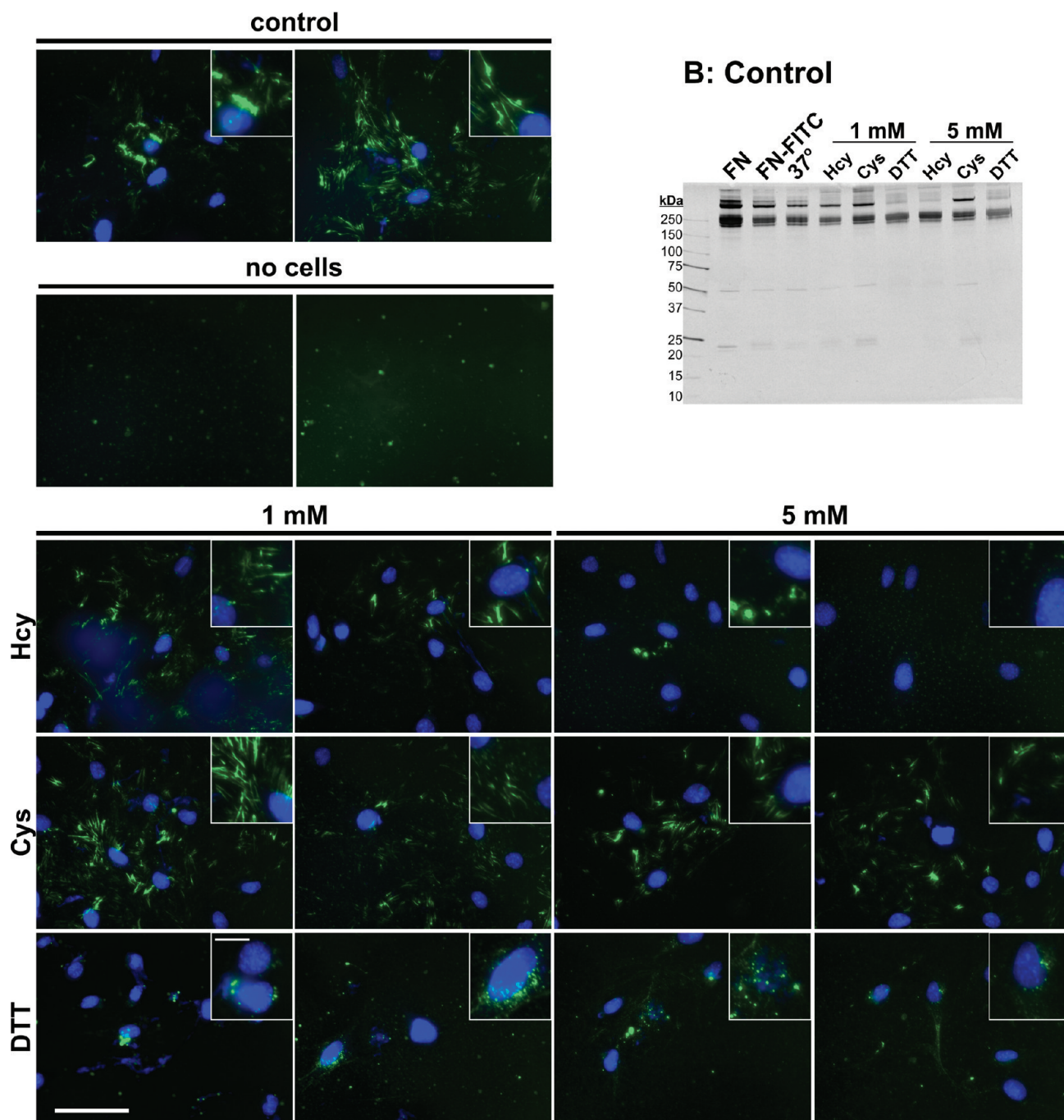
**Homocysteinylation Modifies De Novo Assembly of Fibronectin on Cells.** To analyze fibronectin deposition and assembly mechanisms in dependence of its homocysteinylation status, we employed an established procedure using fluorescently labeled fibronectin exogenously added to cells.<sup>47</sup> FITC-labeled fibronectin was treated with increasing concentrations of homocysteine and its controls and added to fibronectin-null cells in



**Figure 5.** Fibronectin epitopes for monoclonal antibodies are sensitive to homocysteinylation. Fibronectin was treated with homocysteine (Hcy), cysteine (Cys), and DTT at the concentrations indicated on the X-axes and coated on ELISA plates (0.5  $\mu$ g/well). Binding of monoclonal antifibronectin antibodies 4D1 (A), 5C3 (B), and 7D5 (C) was tested at antibody dilutions of 1:1,000. Error bars represent duplicate values.

serum free medium (Figure 6A). Homocysteine reduced (1 mM Hcy) or abolished (5 mM Hcy) fibronectin deposition and initial network formation on cells. The fibronectin assembly with cysteine-treated FITC fibronectin did not differ qualitatively and quantitatively from the untreated control. DTT-treated controls always showed complete loss of fibronectin deposition and network formation. Controls demonstrate that the modified fibronectin was not degraded and showed the typical reduction

# A: FITC-Fibronectin assembly



**Figure 6.** Effect of homocysteine on fibronectin assembly. (A) 10  $\mu$ g of FITC-labeled fibronectin in 10  $\mu$ L was incubated with homocysteine (Hcy), cysteine (Cys), and DTT at concentrations indicated for 16 h and added to human dermal fibroblasts for 6 h at the time of seeding. The signal for cell associated fibronectin (green) was visualized after fixation with paraformaldehyde, and cell nuclei were counterstained with DAPI (blue). Two representative pictures are shown for each condition. The insets in higher magnification show assembled fibronectin fibers. As controls, untreated fibronectin FITC was used in the presence (control) or absence (no cells) of cells. The scale bars represent 100  $\mu$ m for overview images and 25  $\mu$ m for all insets. The final concentration of FN in the medium was 45  $\mu$ g/mL, and the final concentration of the modifying agents was 48 and 300  $\mu$ M, respectively. (B) Control of homocysteinylation of FITC-labeled fibronectin used in (A). Similar quantities (2  $\mu$ g) of fibronectin before (FN) and after (FN-FITC) the FITC labeling reaction, FN-FITC incubated at 37  $^{\circ}$ C for 16 h (37 $^{\circ}$ ), and the modified FITC-FN samples as indicated were loaded on a 4–20% SDS-PAGE and silver-stained. Note that there is no degradation occurring during the time scale of the experiment and that the modification reaction with Hcy, Cys, and DTT was successful (selective reduction of the fibronectin dimer only for Hcy and DTT).

of the fibronectin dimer after homocysteinylation (Figure 6B). These results demonstrate that fibronectin homocysteinylation

negatively regulate initial fibronectin deposition and early network formation.



## DISCUSSION

In the present study, we analyzed the effect of homocysteine modification on the interaction of fibronectin with fibrillin-1 and on biochemical properties of fibronectin. Fibronectin is indispensable for fibrillin-1 assembly, and fibrillin-1 is mechanistically involved in the connective tissue phenotypes observed in patients with severe homocystinuria. Thus, results from this study add important information to previous studies and reveal potential biochemical mechanisms involved in the pathology of this group of genetic disorders. The major findings are (i) modification of fibronectin and fibrillin-1 resulted in a homocysteine-specific impairment of the interaction of fibronectin with fibrillin-1, but not with heparin; (ii) homocysteinylation of fibronectin reduces dimers to monomers; (iii) epitopes for monoclonal fibronectin antibodies in the FNI<sub>2</sub>, FNI<sub>4</sub>, and FNI<sub>9</sub> domains are specifically inactivated by homocysteinylation; (iv) fibronectin homocysteinylation disrupts initial deposition and assembly of fibronectin.

Fibrillin-1 primarily interacts with fibronectin through binding of a C-terminal fibrillin-1 region with the fibronectin gelatin-binding region.<sup>23</sup> In this study we show that homocysteinylation of both fibronectin and the C-terminal fibrillin-1 fragment rFBN1-C negatively affects the interaction between these proteins. It was previously shown that rFBN1-C only interacts with fibronectin in a disulfide-bonded multimeric form but not as monomers.<sup>23,52</sup> Furthermore, the disulfide-bonded rFBN1-C multimers are susceptible to homocysteine reduction resulting in monomers.<sup>12</sup> Therefore, one reason for the compromised binding of homocysteinylation of rFBN1-C to fibronectin could be due to an altered multimeric state of rFBN1-C. Generally, higher homocysteine concentrations were required for fibronectin to result in a similar inhibition of the interaction. Homocysteinylation of the proteolytic fibronectin fragment FN40K, comprising the gelatin-binding region, also compromised the interaction with rFBN1-C, demonstrating that this region in fibronectin is susceptible to homocysteine modification. We recently demonstrated that the interaction between fibronectin and rFBN1-C can be inhibited by gelatin.<sup>23</sup> On the other hand, it was shown by others that homocysteinylation of plasma fibronectin had no adverse effect on the interaction with gelatin.<sup>10</sup> Since the interaction between fibronectin and rFBN1-C is sensitive to homocysteinylation, but not the interaction with gelatin, the data indicate that rFBN1-C interacts differently with the fibronectin gelatin-binding region compared to gelatin. Fibrillin-1 potentially requires different residues to bind to fibronectin or the interaction is more dependent on the structural integrity of the region mediated via disulfide bonds compared to gelatin binding.

One of the three heparin binding sites in fibronectin at the N-terminus (FNI<sub>1-5</sub>) correlates with domains that are susceptible to homocysteine modification, suggesting that heparin interaction may be affected by this modification<sup>10</sup> (Figure 1A). However, our study shows that binding of fibronectin to heparin is not altered by the modification with either homocysteine or cysteine. The data demonstrate either that the structural changes introduced by homocysteine in these region are not sufficient to affect heparin binding or that subregions of domains are involved in heparin binding that are not susceptible to homocysteine modification. Since monomers and dimers of fibronectin both bound to the heparin affinity column, the dimer reduction observed after homocysteine modification had no effect on heparin binding properties. This is in line with a study by Kozaki and co-workers using a recombinant fibronectin isoform without the

dimerization domain which displayed normal heparin binding properties.<sup>53</sup>

The integrities of binding epitopes for three monoclonal antibodies against the FNI<sub>2</sub> and FNI<sub>4</sub> domains in the N-terminal heparin-binding region and FNI<sub>9</sub> domain in the gelatin-binding region of fibronectin were compromised by the modification with homocysteine but not with cysteine. This indicates direct binding of homocysteine to these domains and blocking antibody binding by interfering with disulfide bonds. Indeed, reduction of disulfide bonds by DTT in excess entirely blocked the ability of the antibodies to bind. Alternatively, the inhibitory mechanisms could potentially involve long-range structural effects and reshuffling of disulfide bonds. In a previous study, it was shown that homocysteine, but not cysteine, bound to fibronectin.<sup>10</sup> This correlates with our observations that high concentrations of cysteine had no effect on the antibody epitopes. In the same study the authors localized the regions of the molecule which homocysteine reacted with to the N-terminus and to C-terminal domains close to the fibrin binding site.<sup>10</sup> None of these domains are detected by the monoclonal antibodies used in our study, which bind to domains in and close to the gelatin binding region. Therefore, long-range effects from these homocysteinylation domains may be transferred to the gelatin binding region. Alternatively, it is possible that not all homocysteine binding areas were detected in the previous study.

For cellular fibronectin, we show here a dose-dependent and homocysteine-specific reduction of the dimeric to the monomeric form in conditioned medium of primary human dermal fibroblasts. In experiments not shown to clarify whether dimeric or monomeric fibronectin interacts with fibrillin-1, we found that gel-filtrated pools of fibronectin dimers and monomers both bound to fibrillin-1. In addition, the C-terminus of fibrillin-1 interacts with subfragments of fibronectin lacking the dimerization domain strengthening the fact that monomeric fibronectin interacts with fibrillin-1.<sup>23</sup> Whether or not fibronectin monomerization affects other protein interactions remains to be established. The target of homocysteine for the effect on the dimerization is likely the two cysteine residues in the fibronectin C-terminus (Figure 1A). Fibronectin, lacking these cysteine residues, was not able to assemble into multimeric fibronectin fibrils.<sup>16,53</sup> These authors demonstrated a combined mechanism of monomerization and reduced binding to  $\alpha_5\beta_1$  integrin contributing to the loss of fibronectin assembly. In our study, we observed reduced fibronectin deposition and initial assembly of homocysteinylation of fibronectin. Monomerization of the fibronectin dimer might be an important contributing factor. In addition, homocysteinylation of assembly epitopes at the N-terminus might also play a role. In our previous study, we did not observe altered formation of the fibronectin network in human dermal fibroblasts when homocysteine was added to serum-containing cell culture medium, while direct fibronectin homocysteinylation in the present study clearly leads to assembly defects.<sup>12</sup> It is likely that the serum in the culture medium quenches the effect of free homocysteine as it was shown that serum albumin is a target for homocysteine.<sup>54</sup> This type of protection was not present in the present experimental setup and would be diminished in the extracellular space.

In summary, the current study analyzes the effect of homocysteine on structural and functional properties of fibronectin. Fibronectin is a master organizer of extracellular matrices including fibrillin-containing microfibrils, which in turn organize elastogenesis. We found that the fibronectin–fibrillin interaction is

impaired when fibrillin or fibronectin is homocysteinylated and that homocysteinylated fibronectin is impaired in its ability to assemble. We suggest that these consequences of homocysteinylation contribute importantly to derailed fibrillin functions. For example, fibrillins and microfibrils are known to be involved in the storage and activation of TGF- $\beta$  as well as bone morphogenetic proteins, and recent studies implicate fibrillin-1 in bone metabolism.<sup>55,56</sup> Altered activation of TGF- $\beta$  and BMPs through homocysteinylated fibrillin and fibronectin could potentially contribute to the skeletal phenotype in homocystinuria (long bone overgrowth and scoliosis) as well as to changes in the bone mineral content observed in milder forms of homocysteinemias.<sup>57,58</sup> With the current study we completed a series of experiments showing the potential influence of homocysteine modification on the fibronectin–fibrillin–elastic fiber axes in the extracellular matrix.<sup>11,12</sup> Homocysteinylation of each of these proteins is a potential contributor to the overall connective tissue pathogenesis observed in patients with homocystinuria and could represent modifying mechanisms in Marfan syndrome and related disorders.

## AUTHOR INFORMATION

### Corresponding Author

\*Phone: +1 514 398 4243. Fax: +1 514-398-5375. E-mail: dieter.reinhardt@mcgill.ca.

### Present Addresses

<sup>†</sup>Lerner Research Institute, Cleveland Clinic, Cleveland, OH.

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## ABBREVIATIONS

Cys, cysteine; DTT, dithiothreitol; FBN1, fibrillin-1; FITC, fluorescein isothiocyanate; FN, fibronectin; Hcy, homocysteine; LTBP, latent transforming growth factor- $\beta$  binding protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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