THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 38, pp. 28057–28062, September 21, 2007

A Major Fraction of Fibronectin Present in the Extracellular Matrix of Tissues Is Plasma-derived*[S]

Received for publication, December 11, 2006, and in revised form, May 9, 2007 Published, JBC Papers in Press, July 19, 2007, DOI 10.1074/jbc.M611315200

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The origin of the fibronectin (FN) found in the extracellular matrix of tissues has not been defined experimentally. Previous studies suggest that there is contribution from both local tissue production and transfer from plasma, but the extent of this phenomenon has not been addressed. We have shown before that engineered mice constitutively expressing extra domain A-containing FN (EDA$^+$FN) have a significant decrease of FN levels in plasma and most tissues. We showed that hepatocytes modified to produce EDA$^+$FN have normal extracellular matrix-FN levels but secrete less soluble FN. When we performed a liver-specific EDA-exon deletion in these animals, FN levels were restored both in plasma and tissues. Therefore, an important fraction of tissue FN, approximately an equal amount of that produced by the tissue itself, is actually plasma-derived, suggesting that plasma is an important source of tissue FN. The present results have potential significance for understanding the contributions of plasma FN, and perhaps other plasma proteins, in the modulation of cellular activities and in the formation of the extracellular matrix of tissues.

Fibronectins (FN)[3] are a family of multifunctional glycoproteins known to play key roles in fundamental processes related to adhesive and migratory behavior of cells, such as embryogenesis, malignancy, homeostasis, wound healing, and maintenance of tissue integrity (1). FN generates protein diversity as a consequence of alternative processing of a single primary transcript at three different sites, the extra domain A (EDA), the extra domain B (EDB), and the type III homologies connecting segment (IIICS) (2–4). Two major forms of FN exist, plasma FN (pFN) and cellular FN. pFN is a soluble dimeric form that is secreted into the bloodstream by hepatocytes (5, 6) and found at 300 and 580 μg/ml in plasma of humans and mice, respectively (1, 7). pFN lacks both the EDA and EDB domains, whereas cellular FN is locally produced and deposited as insoluble fibrils in the extracellular matrix of tissues and contains these domains at variable proportions (1, 8, 9). Previous studies suggested that circulating pFN contributes to the extracellular matrix of tissues (10, 11) but the extent of the phenomenon has not been addressed.

The levels of FN in plasma are critical for hemostasis, tissue repair, and susceptibility to infections. Depletion of pFN (liver-specific knockout of FN) results in increased brain injury after transient focal cerebral ischemia (12), a delay in thrombus formation and decreased thrombus stability (13), decreased angiogenesis (14), and increased susceptibility to bacterial infections (15). Heterozygous null FN mice appear healthy and fertile (7) but show delayed thrombus growth in injured arterioles (16). Regrettably, the levels of FN present in the tissues of heterozygous null FN and in the pFN null mice have not been reported.

We have previously shown that knock-in mice having constitutive inclusion of the EDA exon of the FN gene (EDA$^{+/+}$ strain) had up to 70–80% reduction in the levels of plasma and tissue FN (17). Taking advantage of the "floxed" EDA exon present in those mice, we generated liver-specific EDA-null mice (EDA$^{+/+CRE}$) after crossing EDA$^{+/+}$ animals with a transgenic strain expressing the CRE recombinase only in hepatocytes (18). Consequently, hepatocytes of EDA$^{+/+CRE}$ mice, without the EDA exon, were able to produce and secrete pFN at normal levels.

We show here that the levels of pFN were restored in those mice and also that the levels of tissue FN were similar to those observed in EDA$^{WT/WT}$ animals. These results showed a major flow of pFN into the extracellular matrix of tissues and suggest the importance of the pFN as an essential source of FN for the tissues. The presented results might have potential significance for understanding the contributions of pFN, and perhaps other plasma proteins, to cellular activities and in the formation of the extracellular matrix of tissues.

EXPERIMENTAL PROCEDURES

Mice—The generation and genetic background of the mice devoid of regulated splicing at the EDA exon have been previously described (17). EDA$^{+/+}$ mice were mated with the transgenic strain Tg Alf pCRE mice, which have CRE recombinase under the control of the albumin promoter and enhancer (18). EDA$^{+/WT}$ mice having the CRE recombinase were mated in order to obtain EDA$^{+/+}$ mice with the CRE transgene (EDA$^{+/+CRE}$). This strain expresses the CRE recombinase exclusively in hepatocytes (18). The genotype of mice was determined by PCR from tail biopsies.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and supplemental Materials and Methods.
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[3] The abbreviations used are: FN, fibronectin; pFN, plasma FN; EDA, extra domain A; IIICS, type III homologies connecting segment; WT, wild type; RT-PCR, reverse transcription PCR.

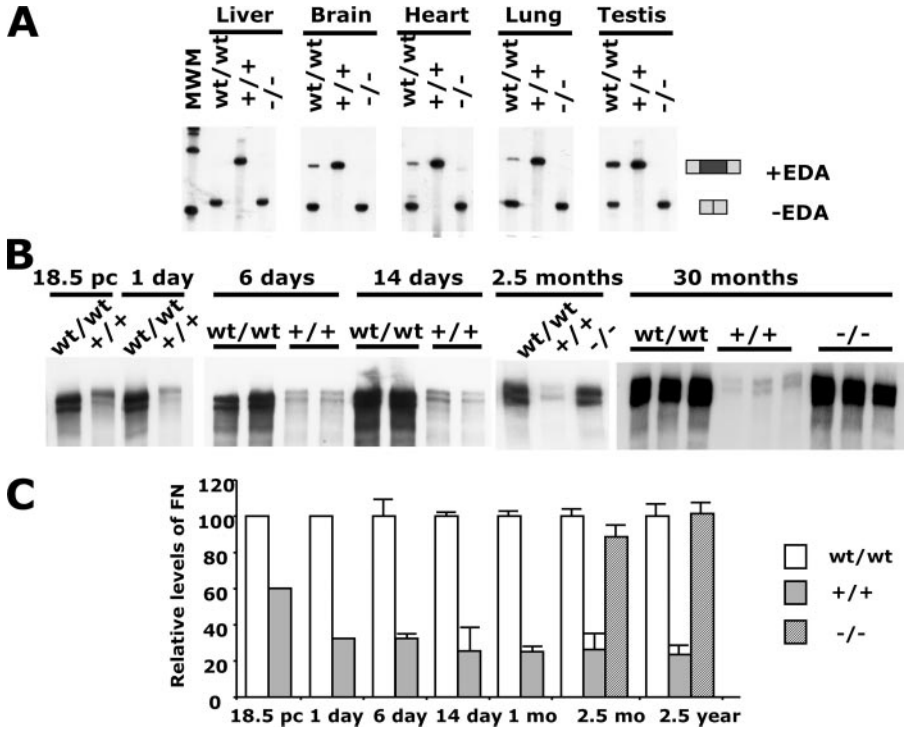

FIGURE 1. **EDA$^{+/+}$ embryos have a decrease in the levels of pFN.** *A*, radioactive RT-PCR analysis of the EDA exon of total RNA extracted from liver, brain, heart, lung, and testis from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ mice. The position of the EDA-containing (+*EDA*) and the EDA-lacking (−*EDA*) bands are indicated. *B*, Western blot analysis of pFN from 18.5-pc embryos, 1-, 6-, and 14-day-old babies, 2.5- and 30-month-old adult mice from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ animals. The samples corresponding to 18.5-pc and 1-day-old time points are pools of 4–6 animals/genotype. Note that the EDA^{+}FN migrates at a different size from the EDA^{-} FN by SDS-PAGE. *C*, the intensity of the signals in *panel B* was quantified with the help of the Quantity One software. The signal obtained in the EDA$^{WT/WT}$ samples was considered 100%. Data are presented as the mean ± S.D. of three independent animals/genotype.

RNA Preparation and Reverse Transcription (RT)-PCR Analysis—Total RNA was prepared from freshly extracted tissues and cells as described (19). The radioactive RT-PCR reactions were performed and quantified as previously described (20).

Protein Extracts and Western Blot Analysis—Mice were anesthetized with 2.5% Avertin (300 μl/20 g mouse), and organs were perfused with 25 ml of cold phosphate-buffered saline through the left ventricle of the heart. Organs were immediately dissected and snap-frozen in liquid nitrogen. Organs were homogenized, and protein content was determined by Bradford protein assay (Bio-Rad). Identical amounts of protein sample were run on a 6% SDS-PAGE and analyzed by Western blot with polyclonal rabbit anti-FN antibody (50 μg of protein extract, 1:1500; Sigma), anti-EDA 3E2 monoclonal antibody (100 μg of protein extract, 1:300; Sigma), or anti β-tubulin monoclonal antibody (20 μg of protein extract, E7, 1:3000; Developmental Studies Hybridoma Bank, University of Iowa) as described (17). Three animals per genotype were analyzed. To determine the efficiency of perfusion and elimination of plasma proteins in each of the organs analyzed, a Western blot analysis of 50 μg of protein extract was performed using an anti-mouse IgG antibody (1:2000; DAKO). Serial ECL expositions of the membranes were performed to determine the optimum linear range to quantify the signals. Films were scanned with the Versadoc (Bio-Rad) and quantified with the help of the Quantity One software package (Bio-Rad).

Bile was collected by holding the gallbladder with forceps. Two μl of each sample were mixed with protein loading buffer (0.125 Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromphenol blue) and boiled for 5 min. Bile FN was analyzed by Western blot as described above.

In Vivo Labeling of Hepatocytes—Hepatocytes were purified by the two-step collagenase perfusion method (21), using Liver Digest Medium (Invitrogen) as described by the manufacturers. Hepatocytes were plated in rat tail collagen for 1 h, washed, and then incubated for 24 h with Met-Cys-free medium supplemented with 300 μCi/ml of [^{35}S]Met/Cys (ProMix; Amersham Biosciences). The supernatant was collected, and a fraction was affinity-purified with gelatin-Sepharose as described (22, 23).

Southern Blot Analysis of Tissues—DNA was extracted from tissues, and 15 μg were digested with HindIII. The DNA was then run in an agarose gel and blotted onto Z-Probe membrane. A probe corresponding to the exon downstream to the EDA exon was used as described (17).

Immunohistochemistry of Tissue Sections—Organs were fixed in 4% formaldehyde and paraffin-embedded. 4-μm sections of each tissue were cut and incubated with affinity-purified polyclonal rabbit anti-FN antibody (1:200; Sigma). Then sections were incubated with biotinylated goat anti-rabbit IgG (5 μg/ml; VectaStain) followed by avidin-biotin-peroxidase mix (ABC Reagent; Vector Laboratories), 3,3′-diaminobenzidine peroxidase substrate (Vector Laboratories), and Gill's hematoxylin (Vector Laboratories). An AS LMD Leica microscope was used to visualize and photograph the sections.

RESULTS

Hepatocytes of EDA$^{+/+}$ Mice Have Normal Levels of Extracellular Matrix-FN but Do Not Secrete pFN—We have previously observed that mice having constitutive inclusion of the EDA exon of the FN gene (Fig. 1*A*) had a significant decrease of FN in plasma and in most tissues (17, 20). Further characterization of pFN levels (embryo, young, and adult mice) from EDA$^{+/+}$ mice showed very low amounts compared with EDA$^{WT/WT}$ and EDA$^{-/-}$ mice (Fig. 1, *B* and *C*). Embryos had 60% of the pFN levels in the control sample, whereas young and adult EDA$^{+/+}$ mice showed a higher decrease in pFN levels. The decrease in pFN was due neither to lower levels in mRNA in tissues of EDA$^{+/+}$ mice (17) nor to a reduced FN production by EDA$^{+/+}$ tissues, as FN secreted by EDA$^{+/+}$ embryonic fibroblasts or adult heart fibroblasts was similar to that pro-

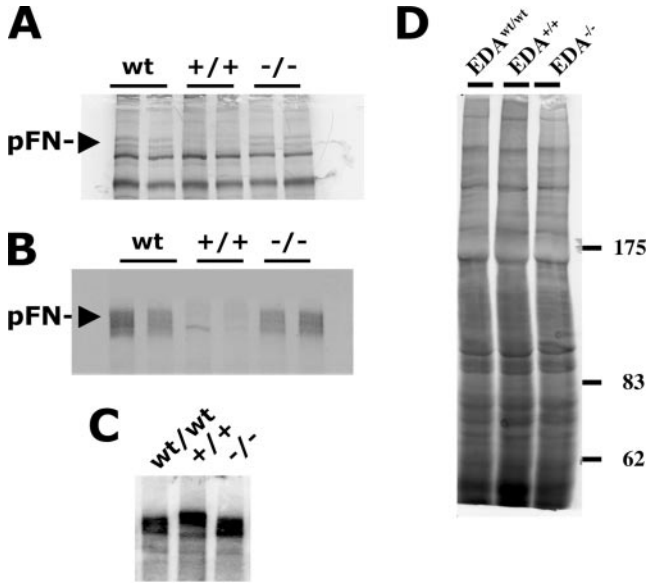

FIGURE 2. **Hepatocytes of EDA$^{+/+}$ mice do not secrete soluble EDA^{+} fibronectin.** *A*, hepatocytes from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ animals were isolated, plated, and metabolically labeled with [^{35}S]Met/Cys for 24 h. Identical aliquots of the conditioned medium were run in a 6% SDS-PAGE and autoradiographed. *B*, equal quantities of the samples prepared in *panel A* were affinity-purified with a gelatin-agarose resin, eluted, separated in an SDS-PAGE, and autoradiographed. *C*, total cell extracts prepared from equal number of hepatocytes from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ mice cultured in non-radioactive medium were collected after 24 h and analyzed by Western blot with a polyclonal anti-FN antibody. *D*, total cell extracts from the hepatocytes used in *panel A* were collected, and identical aliquots were run in a 6% SDS-PAGE and autoradiographed. Molecular weight markers are indicated.

duced by the EDA$^{WT/WT}$ fibroblasts, excluding a general defect (17).

The specific degradation of FN in the EDA$^{+/+}$ mice by proteases was ruled out by a series of experiments: 1) Metalloproteinase activity levels in plasma and tissues by gelatin zymography analysis were similar among EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ mice (supplemental Fig. S1, *A* and *B*); 2) [^{35}S]Met-labeled fragments of FN containing or not containing the EDA exon were not differentially degraded when incubated with EDA$^{WT/WT}$ or EDA$^{+/+}$ tissue extracts or plasma in the absence of protease inhibitors (supplemental Fig. S1, *C* and *D*); 3) no increase in FN degradation rate was observed after mixing protein extracts from EDA$^{+/+}$ or EDA$^{WT/WT}$ liver with those originating from different organs or plasma from EDA$^{+/+}$ mice in the absence of protease inhibitors (data not shown); 4) Western blot analysis of tissue extracts done with different sets of anti-FN polyclonal antibodies, run on 5–17% gradient gel, did not show any specific degradation products in EDA$^{+/+}$ mice (data not shown).

Because the hepatocytes are the source of pFN (5, 6), we performed metabolic labeling of hepatocyte primary cultures from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{-/-}$ livers followed by FN affinity purification of the conditioned medium to analyze pFN production. We observed a decreased secretion of pFN by the EDA$^{+/+}$ hepatocytes, suggesting that the reduced levels in pFN in the EDA$^{+/+}$ mice were the consequence of a defect in hepatocytes (Fig. 2, *A* and *B*). However, the FN amounts detected by Western blot in hepatocyte cell extracts were similar (Fig. 2*C*). Additionally, the decrease of pFN in EDA$^{+/+}$ embryos (Fig. 1*B*) but not in embryonic tissues (17) confirmed that the deficiency in FN secretion was limited only to EDA$^{+/+}$ hepatocytes. These results showed that EDA$^{+/+}$ hepatocytes were unable to secrete pFN in normal amounts. We hypothesized that the reduced levels of tissue FN could be due to the decreased supply of FN from plasma to tissues.

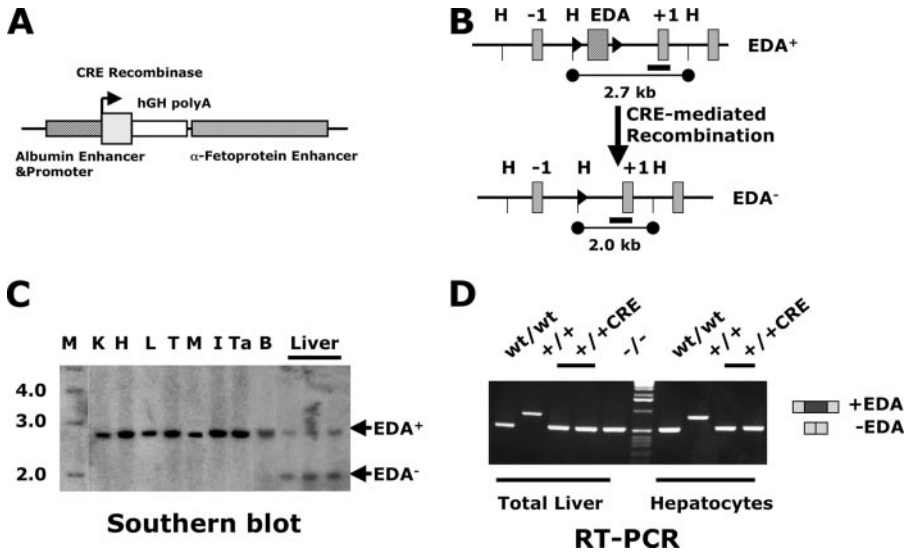

FIGURE 3. **Liver-specific deletion of the EDA exon.** *A*, scheme of the Alf P-Cre transgene. The CRE recombinase (*dotted box*) is expressed under the control of the mouse albumin enhancer and promoter (*dashed box*) and the mouse α-fetoprotein enhancer (*gray box*). Correct polyadenylation was directed by the hGH fragment (*empty box*). *B*, partial map of the EDA^{+} and EDA^{-} FN alleles. The EDA exon is indicated as a *dashed box* and the lox-P sites as *black triangles*. HindIII sites (*H*), the probe used in *panel C* (*black line below +1 exon*), the flanking exons (*−1* and *+1*), and the expected size of HindIII-digested fragments are as indicated. *C*, EDA$^{+/+}$ mice were crossed with a transgenic strain expressing the CRE recombinase in hepatocytes. Southern blot analysis of the different tissues from EDA$^{+/+CRE}$ mice was performed, and specific recombination was observed only in the liver. EDA^{+} and EDA^{-} bands are indicated. Liver DNA samples from three mice are shown. Tissues are as indicated: *K*, kidney; *H*, heart; *L*, lung; *T*, testis; *M*, skeletal muscle; *I*, intestine; *Ta*, tail; *B*, brain; *M*, molecular weight markers. *D*, RT-PCR analysis showing the inclusion/exclusion of the EDA exon in total RNA prepared from liver and purified hepatocytes from the different genotypes. The position of the EDA^{+} and EDA^{-} bands is indicated.

Generation of Liver-specific EDA Null Mice—To determine the extent of FN flow into tissues we restored the capacity of hepatocytes to produce pFN not containing the EDA exon by cross-breeding EDA$^{+/+}$ mice with a transgenic strain expressing the CRE recombinase only in hepatocytes (18). The aim was to perform a tissue-specific deletion of the EDA exon without modifying the EDA^{+} allele in other cell types and tissues (Fig. 3, *A* and *B*). Southern blot analysis of different tissues from the EDA$^{+/+}$ mice carrying the liver-specific CRE recombinase (EDA$^{+/+CRE}$ mice) showed CRE-mediated recombination only in the liver (Fig. 3*C*). The percentage

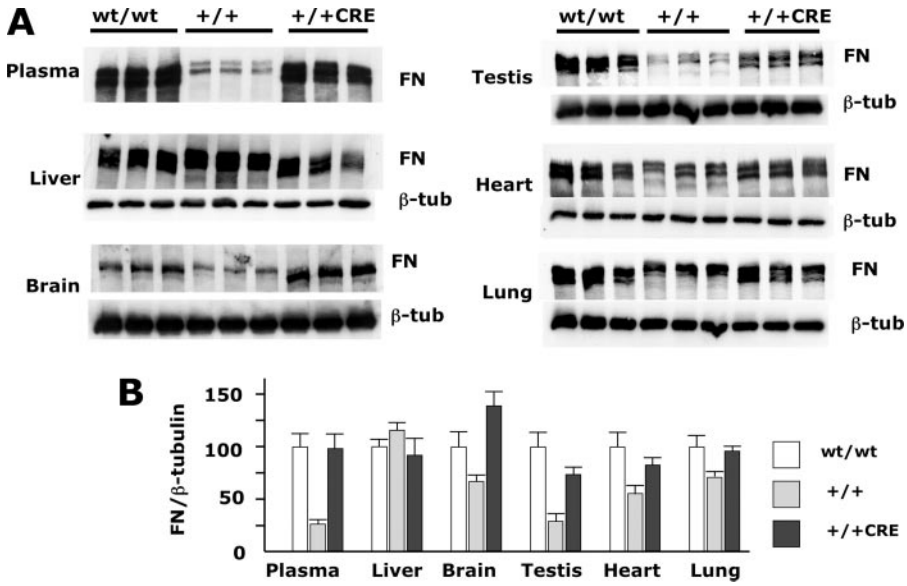

FIGURE 4. **Plasma and tissues from EDA$^{+/+CRE}$ mice have normal FN levels.** *A*, plasma and protein extracts were prepared from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{+/+CRE}$ mice (3 month-old), and total FN levels were detected by Western blot analysis. The protein load was controlled by detecting β-tubulin in the same extracts. *B*, the intensity of the signals in *panel A* was quantified with the help of the Quantity One software. The ratio between FN and β-tubulin signals was used for normalization, and results are shown in the bar graph. The ratio obtained in the EDA$^{WT/WT}$ samples was considered 100%. The mean ± S.D. of three independent experiments is shown.

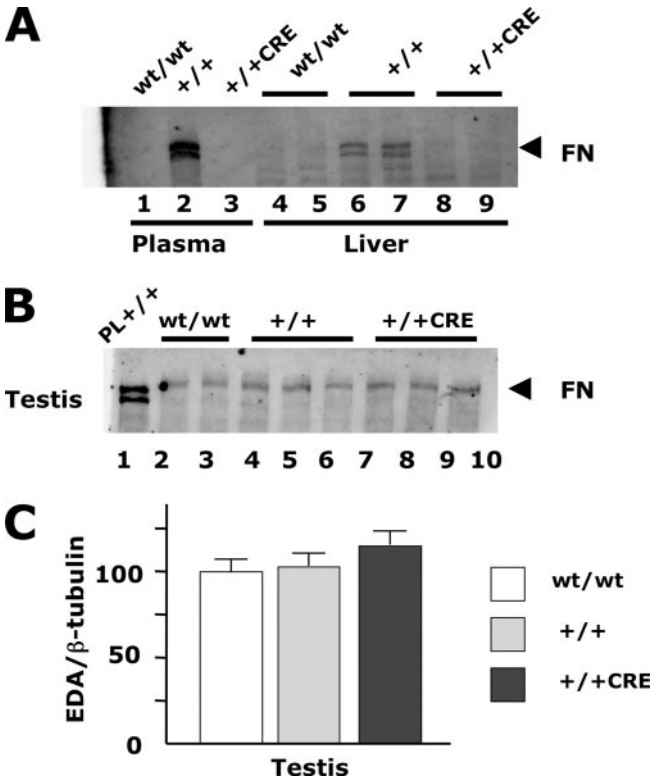

FIGURE 5. *A*, plasma from EDA$^{+/+CRE}$ mice has no EDA-containing FN. Plasma and liver protein extracts prepared from EDA$^{WT/WT}$, EDA$^{+/+}$, and EDA$^{+/+CRE}$ mice were analyzed for the presence of the EDA domain with the 3E2 anti-EDA monoclonal antibody. *B*, similar levels of EDA^{+}FN were seen in tissues from all three genotypes. The same protein extracts from testis used in Fig. 4 were analyzed by Western blot with the 3E2 anti-EDA monoclonal antibody. *C*, the ratio between EDA^{+}FN and β-tubulin signals was used for normalization, and results are shown in the bar graph. The ratio obtained in the EDA$^{WT/WT}$ samples was considered 100%. Data are presented as the mean ± S.D. of three independent animals/genotype.

of recombination in liver was ~60–70%. Because hepatocytes constitute 60% of the total number of cells present in the liver (24), one can consider that the percentage of recombination in hepatocytes was close to 100%. RT-PCR analysis confirmed the absence of the EDA exon in the FN mRNA both in liver and purified hepatocytes from EDA$^{+/+CRE}$ mice (Fig. 3*D*).

FN Levels Are Restored in EDA$^{+/+CRE}$ Mice—Tissues and plasma from EDA$^{+/+}$ mice expressing the hepatocyte-specific CRE recombinase were analyzed by Western blot with polyclonal anti-FN and anti-EDA-specific antibodies. As expected, pFN levels were completely restored in the EDA$^{+/+CRE}$ mice (Fig. 4*A*). Tissue extracts prepared from EDA$^{+/+CRE}$ mice contained amounts of FN similar to that found in EDA$^{WT/WT}$ mice (Fig. 4*A*). The amount of FN present in the extracellular matrix of tissues from EDA$^{+/+CRE}$ mice that was derived from plasma was ~60% in testis and ~40% in other tissues such as brain, heart, and lung (Fig. 4*B*). These results clearly indicate that an important proportion of the FN present in the extracellular matrix of adult tissues derives from plasma. Consequently, the amount of FN that is synthesized and deposited locally in tissues is much lower than believed.

We then used the monoclonal antibody 3E2 to specifically detect the EDA-containing FN isoform. As expected, no EDA^{+}FN was present in the plasma of EDA$^{WT/WT}$ mice. Additionally, we did not detect EDA^{+}FN in the plasma of EDA$^{+/+CRE}$ mice, indicating a high efficiency of CRE-mediated recombination in hepatocytes. The EDA^{+}FN isoform in liver was clearly visible only in the EDA$^{+/+}$ samples (Fig. 5*A*, *lanes 4–9*), confirming again the Southern blot and RT-PCR data (Fig. 3, *C* and *D*). The absence of EDA^{+}FN in the plasma of EDA$^{+/+CRE}$ mice also suggests that the FN flow is mainly from plasma to tissues and not vice versa (Fig. 5*A*, *lanes 1–3*). No differences were observed in the levels of EDA^{+}FN in testis (Fig. 5, *B* and *C*) and in other tissues (data not shown) between the EDA$^{WT/WT}$ and EDA$^{+/+CRE}$ samples, suggesting that the observed differences in tissue FN among the different genotypes corresponded to EDA^{-}FN incorporated from plasma into the extracellular matrix of tissues.

To ensure that the detected levels of FN in tissues were not due to non-complete perfusion of the organs, we performed Western blot analysis of the plasma globulins that remained in each tissue after perfusion and normalized the protein load with the β-tubulin signal. Supplemental Fig. S2 shows that the remaining globulin levels after tissue perfusion were ~10% of the levels seen in the non-perfused organs. Because most plasma proteins were washed out from the perfused organs, the

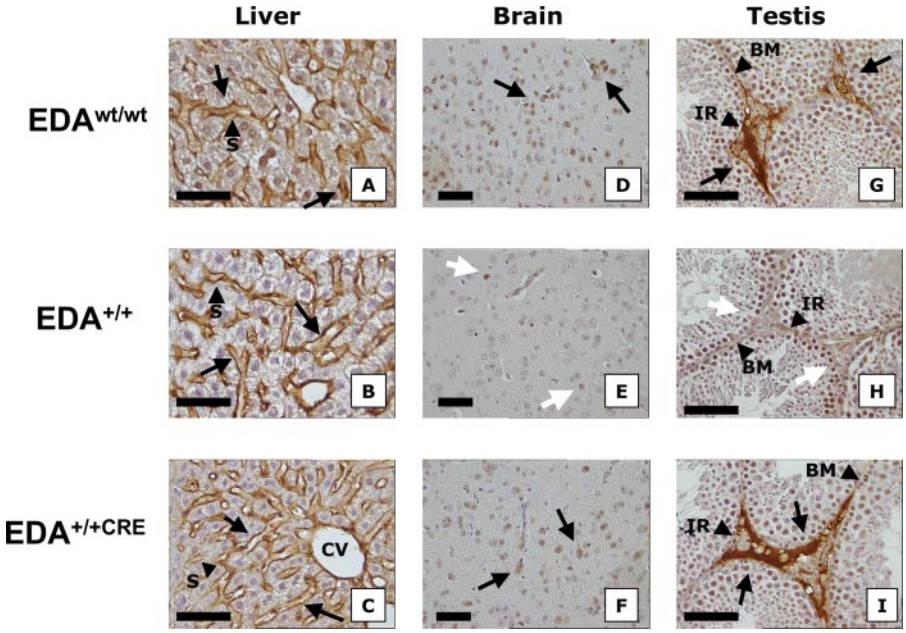

FIGURE 6. **Immunohistochemical analysis of liver, brain, and testis.** Tissue sections of liver (*A–C*), brain (*D–F*), and testis (*G–I*) from EDA$^{WT/WT}$ (*A*, *D*, and *G*), EDA$^{+/+}$ (*B*, *E*, and *H*), and EDA$^{+/+CRE}$ mice (*C*, *F*, and *I*) were stained with an affinity-purified polyclonal anti-FN antibody. The *black arrows* indicate FN in the extracellular matrix of EDA$^{WT/WT}$ and EDA$^{+/+CRE}$ from different tissues and EDA$^{+/+}$ liver. *White arrows* indicate the decrease in levels of FN from the same areas of brain and testis only in EDA$^{+/+}$ mice. *S*, sinusoids, *BM*, basement membrane, and *IR*, interstitial region, are indicated by *black triangles*. The *black bars* correspond to 50 μm.

differences of FN levels among the different strains were not due to residual contamination of pFN.

Immunostaining of Tissue Sections Confirmed the Decrease of FN Levels in EDA$^{+/+}$ Tissues and the Recovery in Tissues of EDA$^{+/+CRE}$ Mice—The above results were confirmed by immunohistochemical analysis of tissue sections. Similar levels of FN-specific signal were detected in the sinusoids of liver samples prepared from all three genotypes (Fig. 6, *A–C*, *black arrows*). In the brain sections of EDA$^{WT/WT}$ and EDA$^{+/+CRE}$ mice a stronger FN-specific signal, probably associated with the cell surface of glial cells (25–27), was observed when compared with the EDA$^{+/+}$ mice (Fig. 6*E*, *white arrows*). Similarly, in testis tissue sections, a stronger FN-specific signal was observed in the basement membrane of seminiferous tubules and interstitial regions of EDA$^{WT/WT}$ and EDA$^{+/+CRE}$ mice compared with EDA$^{+/+}$ samples (Fig. 6*H*, *white arrows*). However, we did not observe intracellular accumulation of FN in the tissue sections of EDA$^{+/+}$ mice by immunohistochemical analysis (Fig. 6, *B*, *E*, and *H*) or in primary culture of hepatocytes either by FN immunofluorescence (supplemental Fig. 3) or by metabolic labeling (Fig. 2*D*).

Gross histology of tissue samples from all three genotypes was similar, suggesting that the FN synthesized locally is sufficient to maintain the normal tissue architecture. These results indicate that there was a flow of FN from plasma to the extracellular matrix of tissues and plasma is an important source of tissue FN.

DISCUSSION

Our data demonstrate an important and novel role for plasma proteins, in particular that of fibronectin, in the formation of the extracellular matrix of tissues and, probably, in the modulation of cellular activities in tissues. The concept that there is FN flow from plasma into tissues or extracellular matrix of cells has been known for a long time. Addition or injection of soluble FN into the culture medium of cells or into the plasma of mice, respectively, resulted in the incorporation of FN into the extracellular matrix (10, 11, 28–30). However, the magnitude of the contribution of pFN to the extracellular matrix was not possible to address with either model. In the present report we have shown that in some tissues up to 60% of the fibronectin present in the extracellular matrix could be plasma-derived.

Sakai *et al.* (12) have recently shown that pFN supports neuronal survival and reduces brain injury following transient focal cerebral ischemia, suggesting the incorporation of pFN into the injured brain. Our results confirmed and extended their observations to non-injured tissues as we showed pFN incorporation into most normal organs, including brain. In fact, we are also demonstrating that this is a general mechanism that occurs in most normal tissues, and we suggest that other plasma proteins could also become incorporated into the extracellular matrix of tissues, modulating cellular activities.

Because FN is found both in blood and bile fluids, secretion of FN by hepatocytes seems not to be polarized as proposed for endothelial cells (31). Furthermore, we also observed a decrease in FN levels in the bile fluid of EDA$^{+/+}$ mice (data not shown), suggesting the absence of an EDA-dependent polarization of FN secretion in hepatocytes, as postulated for airway epithelial cells (32). The low levels of soluble FN in the plasma of EDA$^{+/+}$ mice and intermediate levels in EDA$^{+/WT}$ and EDA$^{+/-}$ mice (data not shown) point toward the existence of a mechanism, analogous to that observed for the secretion of the IIICS variants (33), that detects the EDA domain during the secretory pathway of pFN from hepatocytes and prevents the release of EDA^{+}FN into the bloodstream. The "defective" EDA^{+}/EDA^{+} pFN dimers might be formed but their transit through the pFN secretory pathway might be slower compared with EDA^{-}/EDA^{-} dimers. However, immunostaining of tissue sections, immunofluorescence of primary culture of hepatocytes, or metabolic labeling of hepatocytes did not reveal any intracellular accumulation of FN in the liver of EDA$^{+/+}$ mice, suggesting that the putative "misfolded" dimers are quickly degraded. We observed that the amount of pFN supplied to the extracellular matrix of tissues varied among the different organs analyzed, suggesting that there might be equilibrium between the amount of FN locally produced by the tissues and the availability of cellular receptors to incorporate FN from the plasma pool.

To conclude, the presented results might have potential significance for understanding the contributions of pFN, and perhaps other plasma proteins, to cellular activities and in the formation of the extracellular matrix of tissues, as we have shown that a major fraction of tissue FN is plasma-derived. Because plasma provides approximately an equal amount of FN as the tissue itself, we suggest that plasma should be considered an important source of FN for tissues. Furthermore, hepatocytes have normal levels of FN in the extracellular matrix but do not secrete soluble $EDA^{+}FN$, suggesting the existence of separate secretory pathways for soluble and fibrillar FN.

Acknowledgments—We thank Marcello Raspa and Günther Schütz for providing the Tg Alf pCRE mice, Meghan Walsh for critical reading of the manuscript and for comments regarding the use of English, and Giancarlo Lunazzi, Mauro Sturnega, and Stefano Artico for help in animal handling.

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SUPPLEMENTAL DATA

Supplementary Material and Methods

In vitro translation: cDNAs with or without the EDA exon were prepared from $EDA^{+/+}$ and $EDA^{-/-}$ mice by RT-PCR using the following primers: Forward 5' CGGGGTACCACCATGGGCACCATCACCCTGTATGCTGTCACT 3' and Reverse 5' CGCGGATCCTTATCAGAGTCCTGACACAATCACCGA 3' which produce a fragment of 803 bp and 533 bp, respectively. The PCR amplified products (EDA^{+} and EDA^{-}) were cloned in pBS-SKII vector. The *in vitro* translation was done by a single tube protein system (Novagen), based on a linked reaction in which transcription by a bacteriophage RNA polymerase is directly followed by translation with rabbit reticulocytes lysate. The translation products were analyzed by 10% SDS-PAGE followed by ON autoradiography. Fixed amount of the in vitro translated products were incubated in the absence of protease inhibitors with protein extracts (prepared in the absence of protease inhibitors) from tissues for 3h and 24h.

Gelatin Zymography: A total of 20 µg of tissue protein extract or 0.2 µl of plasma were diluted in 2X sample buffer (1:1) without adding any reducing agent. The samples were run on a 10% polyacrylamide gel polymerized in the presence of 0.2% gelatin. After electrophoresis, the gel was washed once for 15 minutes and then overnight in 100 ml wash buffer (2.5% Triton-X100, 50 mM Tris-HCl, pH 7.5 and 5 mM $CaCl_2$) to remove the sodium dodecyl sulfate (SDS). The gel was rinsed three times in water, followed by a 24 h incubation at 37ºC in incubation buffer (50 mM Tris-HCl, pH 7.5 and 5 mM $CaCl_2$) and was stained for 4 hours in Coomassie Brilliant Blue. The absence of coloration indicates the digestion of gelatin by metalloproteinases. In a parallel gel, the same extracts were run and stained with Coomasie Blue.

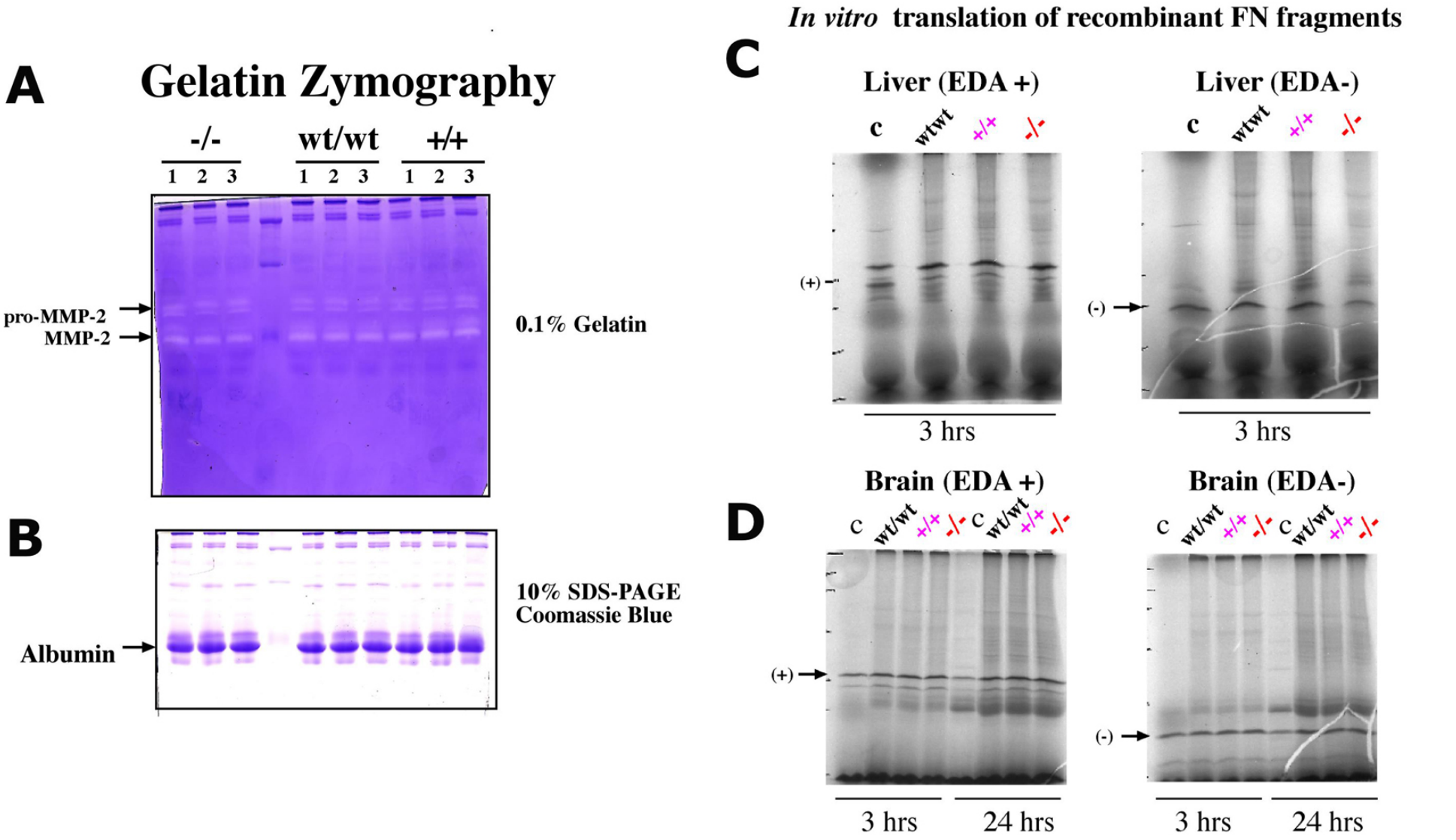
Immunofluorescence of primary cultures of hepatocytes: Hepatocytes were prepared as described in the Materials and Methods Section, plated onto rat-tail collagen coated cover slips and cultured for the indicated times. Cells were fixed and incubated with an affinity purified anti-FN rabbit antibody, then with a FITC labeled secondary antibody. Nuclei were stained with Hoetscht. Original magnification was 200x.

Legends to the Supplementary Figures.

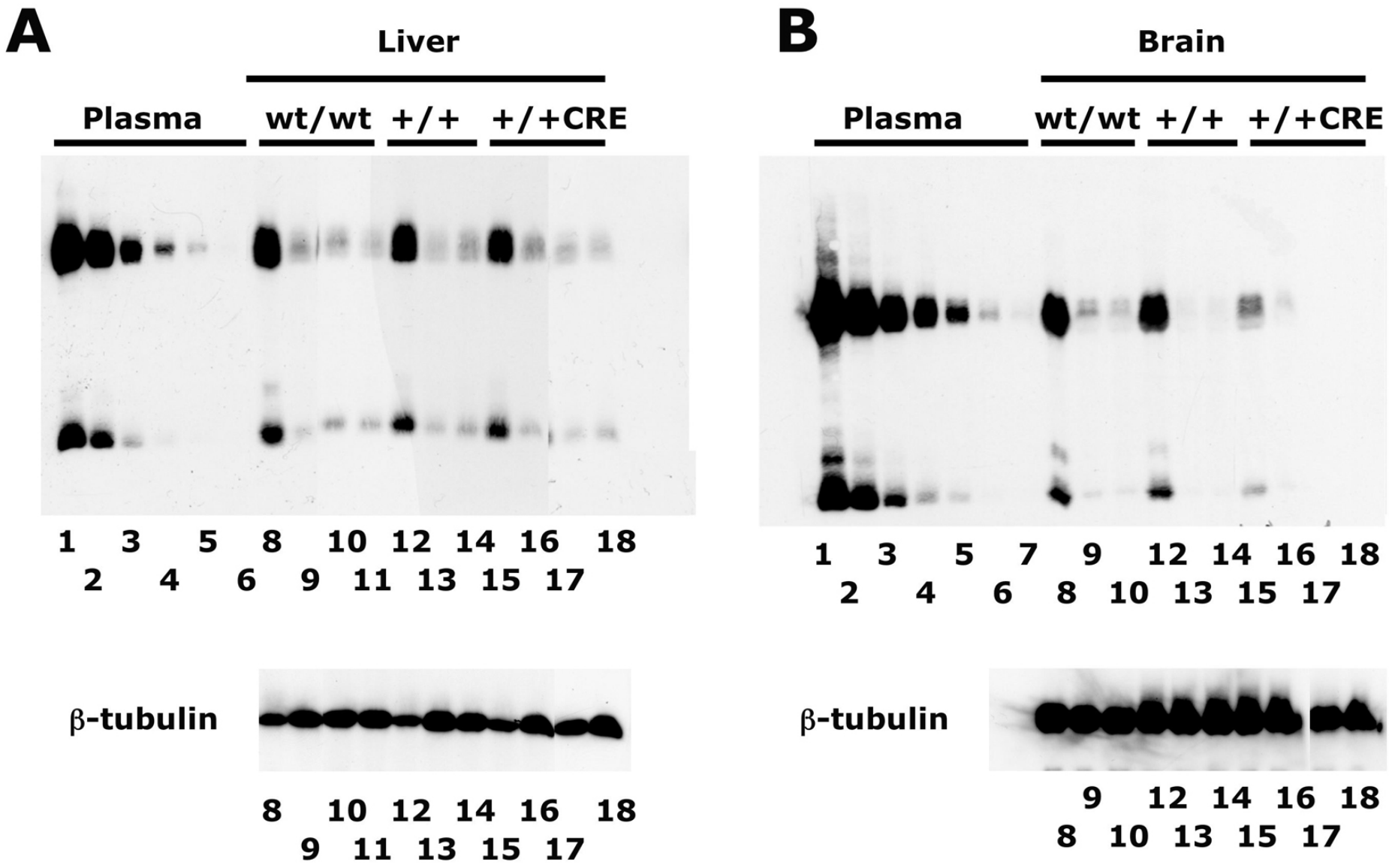
Supplementary Figure 1. A and B. MMPs activity was similar in tissues from all genotypes. Gelatin zymography analysis of plasma samples of $EDA^{wt/wt}$, $EDA^{+/+}$ and $EDA^{-/-}$ mice. The same amount of plasma was loaded in a gelatin-SDS gel, and the activity of metalloproteinases was determined. The same samples were loaded in a normal SDS-PAGE and stained with Coomassie Blue (Panel B) to normalize protein load. **C and D. EDA^{+} FN was not preferentially degraded.** FN fragments containing or not containing the EDA exon were in vitro translated in the presence of ^{35}S-Met/Cys and incubated for different time with tissue extracts in the absence of protein inhibitors.

Supplementary Figure 2. Perfusion of tissues showed almost no residual plasma proteins. Equal amounts of proteins samples (50 µg) were loaded, run on a 12% SDS-PAGE and analyzed by Western blot for the presence of plasma gamma globulins. The protein load was controlled by detecting β-tubulin in the same extracts. Lanes 1-7 correspond to serial dilutions of $EDA^{wt/wt}$ plasma (each lane is 1:2 of the previous one, starting from 0.05 µl to 0.00078 µl). Lanes 8, 12 and 15 correspond to non-perfused samples. Lanes 9-11, 13-14 and 16-18 correspond to perfused samples. The protein load was quantified by the detection of β-tubulin in the stripped membrane (lower panels).

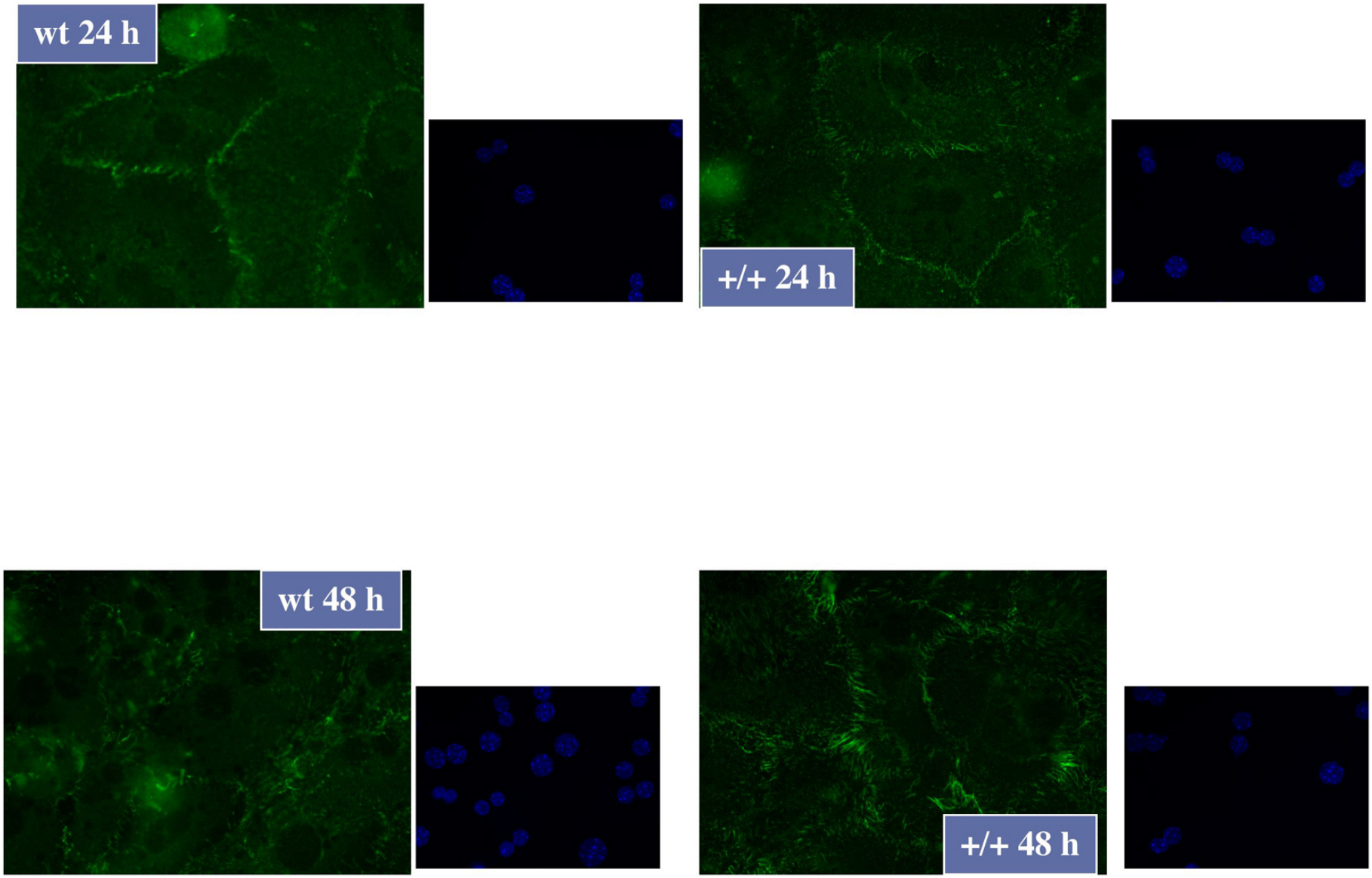
Supplementary Figure 3. No intracellular FN accumulation is seen in EDA+/+ hepatocytes. Hepatocytes were purified from $EDA^{wt/wt}$ and $EDA^{+/+}$ mice and plated onto rat tail collagen coated glass-slips for 24 or 48 h. Cells were fixed and stained with an affinity purified anti-FN polyclonal antibody. Nuclei of the same fields are shown (Hoetscht staining).



SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3

A Major Fraction of Fibronectin Present in the Extracellular Matrix of Tissues Is Plasma-derived

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J. Biol. Chem. 2007, 282:28057-28062.
doi: 10.1074/jbc.M611315200 originally published online July 19, 2007
