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Connective Tissue Growth Factor with a Novel Fibronectin Binding Site Promotes Cell Adhesion and Migration During Rat Oval Cell Activation

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Abstract

Oval cell activation, as part of the regenerative process after liver injury, involves considerable cell-matrix interaction. The matricellular protein, connective tissue growth factor (CTGF), has been shown to be critical for oval cell activation during liver regeneration following N-2-acetylaminofluorene/partial hepatectomy. To understand the mode of action of CTGF during this process, N-terminal CTGF was used as bait to screen a yeast two-hybrid complementary DNA library specific for regenerating livers with massive oval cell presence. Fibronectin (FN), a prominent component of hepatic extracellular matrix (ECM), was found to specifically bind to a new site on CTGF. In addition to module IV, this study showed that module I of CTGF was sufficient for binding to FN in both solid-phase *in vitro* binding assays and immunoprecipitation. Immunofluorescent staining revealed a dynamic ECM remodeling characterized by an FN-concentrated provisional matrix during oval cell–aided liver regeneration. Abundant CTGF protein was colocalized with FN in the provisional matrix. When expressed as recombinant proteins and immobilized on plastic surfaces, modules I and IV of CTGF were selectively adhesive to thymus cell antigen 1–positive ($Thy1^+$) oval cells, stellate cells, and sinusoidal endothelial cells but not to hepatocytes. The adhesion of these two modules on $Thy1^+$ oval cells required heparan sulfate proteoglycan and integrin $\alpha_5\beta_1$. Recombinant CTGF promoted an integrin $\alpha_5\beta_1$–dependent migration but not proliferation on Thy13 oval cells. *Conclusion:* Modules I and IV enabled the linkage of CTGF to FN and activated hepatic cells. Through these bindings, CTGF on the FN-concentrated provisional matrix promoted cell adhesion and migration, thereby facilitating oval cell activation.

The mammalian liver possesses robust regenerative capability with a two-tier system. For example, 70% partial hepatectomy (PHx) causes compensatory hyperplasia of the remnant liver by the replication of mature residual cells, including hepatocytes, stellate cells, and

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Potential conflict of interest: Bryon E. Petersen is an inventor of a patent or patents related to this technology and may benefit from royalties paid to the University of Florida related to its commercialization. Dr. Petersen owns stock in, is a consultant for, and holds intellectual rights with RegenMed.

sinusoidal endothelial cells (SECs).[1] However, when hepatocyte proliferation is inhibited with a low dose of carcinogenic compound N-2-acetylaminofluorene (2-AAF), 70% PHx activates a unique cell population termed *oval cells*, which sustain the restoration of normal hepatic mass and function. Oval cells are characterized by expressing phenotypic markers such as thymus cell antigen 1 (Thy1) and α-fetoprotein.[2,3] A large expansion of oval cells takes place at first in the portal triad areas, followed by infiltration into the parenchyma toward the central veins, where they differentiate into hepatocytes.[2] Although waves of hepatic cell proliferation, migration, and differentiation around the periportal regions have been uniformly observed, the extracellular matrix (ECM) remodeling during oval cell–aided liver regeneration has not been fully characterized.

Connective tissue growth factor (CTGF), also known as CCN2, is a secreted matricellular protein that belongs to the CCN family named after CTGF, cyr61, and NOV. It can act on various types of cells and exert cellular functions including growth, migration, differentiation, angiogenesis, apoptosis, and ECM protein production.[4] This protein comprises four mosaic conserved modules. Module I is homologous to the N-terminal cysteine-rich regions of the six classic insulin-like growth factor-binding proteins and has a low-affinity binding to insulin growth factor. So far, the relationship between CTGF and the insulin-like growth factor-binding protein superfamily is still controversial,[5] and the physiological function of this module on CTGF is unknown. Module IV has been found to be adhesive to several types of cells and to bind to different integrin subtypes, sometimes in a heparan sulfate proteoglycan (HSPG)– dependent manner.[6,7] Module IV has been shown to directly bind to fibronectin (FN).[8] CTGF knockout in mouse embryo fibroblasts causes a defect in cell adhesion to FN.[9,10] Accumulating evidence has shown that CTGF is involved in modulating cell-matrix interaction by contextually binding to ECM and cell surface proteins.

CTGF is normally expressed at a very low level in liver. However, CTGF up-regulation has been found in diverse repair processes after chronic or acute liver injuries.[11] Previously, we found that CTGF induction was critical for oval cell activation during liver regeneration after 2-AAF/ PHx.[12] To further understand the mechanism governing CTGF action during this process, we used the yeast two-hybrid approach and identified FN as a CTGF binding protein. A region from amino acid 21 to 107 of CTGF that mainly contains module I (abbreviated to module I in this study) was found to be a novel FN binding site. This present study showed that, like module IV, module I was not only an FN binding site but also a cell adhesion motif that selectively bound to hepatic cells. The binding between CTGF and FN was biochemically characterized. The biological function of CTGF interaction with FN in the regenerative process was investigated.

Materials and Methods

Animals Treated with 2-AAF/PHx

Male Fisher 344 rats (age: 6–8 weeks, weight: 130–150 g) were purchased from Charles River Laboratories. The procedure for induction of oval cell activation by 2-AAF/PHx in rats was performed as previously published.[12] All animal protocols were approved by the University of Florida Animal Care and Usage Committee and were conducted following their guidelines.

Yeast Two-Hybrid Complementary DNA (cDNA) Library Screening

A cDNA library was constructed with livers with maximal oval cell activation (day 8 after 2-AAF/PHx) according to the manufacturer's instructions with a HybriZAP-2.1 XR library construction kit (Stratagene, La Jolla, CA). The cDNA fragment that encodes mainly

module I was amplified and cloned into pXDGATcy86 bait vector with P1 primer 5′-GTC-GACGCCTGCCACCGGCCAGGCCT-3′ and P2 primer 5′-GCGGCCGCTTAACACGGACCCAC-CGAAGACACA-3′. The resulting construct was transformed into the HF7C strain and mated with the Y187 strain, which was pretransformed with the cDNA library. Detailed information about the bait vector and the procedure for the library screening has been described by Ding et al.[13] Candidates that grew on a synthetic drop-out minimal medium lacking leucine, tryptophan, and histidine but with 5 mM 3-amino-1,2,4-triazol were analyzed by sequencing with primer 5′-AGGGATGTTTAATACCACTAC-3′, and their identities were revealed by a Blast search in the Gene-bank database.

The cDNAs of CTGF and its mutants were cloned into the bait vector with a similar strategy, except with the following primer sets for DNA amplification: P1 (see above) and P3 5′-GCGGCCGCCTTACGC-CATGTCTCCATACATCTTCCTGT-3′ for CTGF, P1 and P4 5′-GCGGCCGCTTACCTCTAGGT-CAGCTTCACAGGG-3′ for I-III, and P3 and P5 5′-GTCGACACCCTGTGAAGCTGACCTAGAGG-AAAACA-3′ for IV. The amplified products were verified by DNA sequencing.

Expression and Purification of 3xFLAG Epitope Tagged CTGF and Module I

Primer sets P6 5′-CTCGGATCCGACCATGCTCGCCTCCGTCG-3′ and P7 5′-TAGTCGACCACGGACCCACCGAAGA-CACA-3′ for module I and P6 and P8 5′-TAGTCGAC-CGCCATGTCTCCATACATCTTCCT-3′ for CTGF were used to amplify the corresponding DNA fragments with signal peptide. The 3xFLAG epitope that was added to the C-terminus of recombinant proteins was from pME Flag vector (a gift from Dr. Naohiro Terada). Stable cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) with 1 mg/mL G418. A conditioned culture medium containing recombinant proteins was harvested after a 2-day incubation of stable transfectants in serum-free medium.

The purification of the recombinant proteins was performed with M2-conjugated agarose bead and eluted by 3xFLAG peptides according to the manufacturer's protocol (Sigma, St. Louis, MO). Purified proteins were concentrated with centricon (Millipore, Billerica, MA), and the purity was verified by silver staining (Invitrogen, Carlsbad, CA).

Immunoprecipitation Assay and Western Analysis

To detect the binding of CTGF and module I to FN, conditioned media with or without recombinant proteins were incubated with 11.4 nM FN, trishydroxymethyl-aminomethane-buffered saline (TBS), a protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and M2-conjugated agarose (Sigma). The immunoprecipitates were washed in TBS with 0.1% Nonidet P40, boiled, and separated on 5% sodium dodecyl sulfate/ polyacrylamide gel before being transferred to a polyvinylidene fluoride membrane. Anti-FN and horseradish peroxidase– conjugated anti-rabbit immunoglobulin G (IgG; Amersham Biosciences, Piscataway, NJ) were used as primary and secondary antibodies. The detection was performed with the ECL Plus kit (Amersham Biosciences).

To monitor FN protein expression during the regeneration process, total cell lysates were isolated according to Kim et al.[14] The same conditions for Western analysis mentioned previously were used. Loading quantitation was normalized with mouse anti–β-actin antibody (Abcam, Cambridge, MA).

Immunoprecipitation to detect integrin $\alpha_5\beta_1$ on primary Thy1$^+$ oval cells was conducted as described by Gao and Brigstock.[7] Anti-integrin α_5 (Chemicon, Temecula, CA) and protein A/G– conjugated beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to pull

down immunocomplexes. Immunoblotting was the same as mentioned previously, except that anti-integrin β_1 (Chemicon) was used as the primary antibody.

Solid-Phase Binding Assay

Microtiter plates were coated with FN (10 μg/mL), blocked in TBS (with 0.05% Tween 20) with 1% bovine serum albumin (BSA), and incubated with recombinant proteins at the desired concentrations. Amounts of bound recombinant proteins were detected with horseradish peroxidase– conjugated M2 antibody and tetramethylbenzidine as the substrate (R&D Systems, Minneapolis, MN). The resulting optical density was determined by a microplate reader at 450 nm.

Immunofluorescent Staining and Laser Scanning Confocal Microscopy

All immunostaining was performed on cells or frozen liver sections with standard staining protocols. Integrin $\alpha_5\beta_1$ staining was carried out on methanol-fixed primary Thy1$^+$ oval cells with JBS5 antibody (Chemicon). FN (R&D Systems) and CTGF (Santa Cruz) were stained on acetone-fixed frozen liver sections. Fc receptor block (Innovex Biosciences) was used before normal serum incubation in CTGF staining. Liver sections from animals perfused with 4% paraformal-dehyde in phosphate-buffered saline were stained with Thy1 (Phaminogen) and Desmin (Dako) antibodies. Secondary antibodies were Alexa Fluor 488–conjugated or Alexa Fluor 594–conjugated donkey anti-rabbit, anti-goat, and anti-mouse antibodies (Molecular Probes, Carlsbad, CA). All preparations were imaged with a Leica laser scanning confocal microscope at equivalent laser settings.

Primary Hepatic Cell isolation and Culture

To prepare primary cultured cells, livers were perfused with a two-step protocol as previously described.[12] Thy1$^+$ oval cells, SECs, and hepatocytes were isolated from livers harvested at day 7 post 2-AAF/PHx and cultured according to existing protocols.[3,15,16] To eliminate the contamination by oval cells that were predominantly present in regenerating livers, stellate cells were isolated from normal rats and cultured according to a published method.[17] The purity of each cell type was more than 85% on the basis of the characterization of phenotype by staining with cell type–specific markers. Thy1$^+$ oval cells were cultured in Iscove's medium containing 10% FBS and 0.1% insulin.

Expression of Maltose Binding Protein (MBP) Fused I and IV from Escherichia coli

The cDNAs of modules I and IV were removed from the bait vector and in-frame fused to the 3′ terminus of MBP. Fusion proteins were induced in the *E. coli* DE3 strain with 0.3 mM isopropyl-beta-D-thiogalactopyranoside. Protein purification was performed with amylase beads (Biolabs, Ipswich, MA) with column buffer (20 mM trishydroxym-ethylaminomethane-HCl, 200 mM NaCl, and 1 mM ethylene diamine tetraacetic acid, pH 7.4) and eluted with 10 mM maltose.

Adhesion Assay

Proteins were diluted in TBS to the desired concentration, coated on 96-well, flat-bottomed plates (Coastar, Corning, NY), and blocked with TBS containing 1% BSA. Adherent cells were stained by CyQUANT NF dye reagent (Invitrogen), and the fluorescence intensity of each sample was measured with a fluorescence microplate reader (Tecan, Durham, NC) with excitation at ~492 nm and emission detection at ~530 nm.

Transwell Migration Assay

Thy1$^+$ oval cells (1×10^5) were inoculated in the FN-precoated, upper chamber of 8.0-μm-pore transwells in 24-well companion plates (Corning Inc., Lowell, MA). Cells were

incubated in the presence or absence of 200 ng/mL CTGF in the lower chamber for 6 hours. The transwell membranes were fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with 0.1% Coomassie blue in 10% methanol and 10% acetic acid. The upper surface of the transwells was rubbed with a cotton-tipped applicator. Cells on the undersurface of the transwells were counted in 10 random fields at 40× magnification.

Cell Proliferation Assay

Thy1$^+$ oval cells were serum-starved for 24 hours. Cells (2×10^4) in Iscove's medium and 0.2% FBS were inoculated onto an FN-precoated 96-well plate for 8 hours. Then, the cells were grown in CTGF or FBS with the desired concentration for 2 days. [^{3}H]thymidine (0.1 μCi) was added to the cells, and they were incubated for an additional 18 hours. DNA was collected on glass filters with a vacuum filtration cell harvester. Tritium incorporation was assessed by liquid scintillation counting.

Statistical Analysis

The values reported in this study represent the average ± standard deviation (SD) of the measurements from at least 3 independent experiments. SD and statistical significance were determined by the Student *t* test with the statistical software included in Microsoft Excel (Microsoft, Redmond, WA). *P* values of < 0.05 were considered to be statistically significant.

Results

Module I Was Another FN Binding Site on CTGF

N-terminal CTGF that mainly consisted of module I was used as bait to screen a rat cDNA library specific for oval cell activation. One clone was identified as rat fibronectin 1 (Genebank accession number NM_019143). This clone encodes a truncated FN fragment from amino acid 2236 to the C-terminus including the last three FN type 1 repeats (Fig. 1A). Our yeast two-hybrid analyses showed that module IV, module I, and a truncated mutant without module IV were able to bind to the FN fragment (Fig. 1B). The module I binding to FN was specific because it did not interact with an unrelated plant protein, Pi-d2 binding protein 1 (PIB1, Os08g01900 in Rice Functional Genomic Expression Database), which encodes a ubiq-uitin-protein ligase (X. Ding and W.-Y. Song, unpublished work, 2007). The module I interaction with FN was further confirmed by solid-phase binding assay and immunoprecipitation (Fig. 1C and 1D), even though fewer FN proteins appeared to bind to module I protein in comparison with that of CTGF. Taken together, these data indicated that module I of CTGF was another FN binding site, although its affinity was lower than that of intact CTGF.

FN and CTGF Were Colocalized on the Provisional Matrix Around Periportal Regions During Oval Cell Activation

Previously, we showed that FN was up-regulated at the RNA level during oval cell activation with a pattern similar to that of transforming growth factor β, CTGF, and procollagen type I.[12] Here, immunofluorescent staining and Western analysis were performed to test if FN up-regulation was associated with the provisional matrix during oval cell activation. In normal liver, FN was confined to the portal areas, the central veins, and the hepatic capsule, as shown by immunostaining (Fig. 2A). In response to 2-AAF/PHx injury, we observed that FN deposition changed in the interstitial space around periportal regions, where the oval cell activation took place (Fig. 2B-F). With the infiltration of proliferating hepatic cells toward the parenchyma region, FN-concentrated areas appeared as early as day 3, then enlarged gradually to a peak around days 7-11, and disappeared

completely by day 22. Western analysis also showed that FN protein was increased until day 11 during oval cell activation (Fig. 2G). More direct evidence about the relationship between the FN-rich matrix and oval cell activation was obtained by a comparison of microarchitectures around periportal regions between normal and regenerating livers. The periportal regions of normal liver were characterized by thin FN fibrils and well-organized hepatic plates, which were composed of hepatocytes with large cytoplasm (Fig. 3A). In contrast, the periportal regions of the regenerating liver were packed with a large number of cells with scanty cytoplasm. These represented heterogeneous populations of activated hepatic cells that were infiltrating the hepatic plates and migrating toward central veins. Among the cells was the provisional matrix with uneven distribution of granular FN, which formed not only a scaffold but also a continuous network for cell migration (Fig. 3B). $Thy1^+$ oval cells that are barely present in a normal liver are known to be a hallmark of the regenerating liver after 2-AAF/PHx.[3] Stellate cell activation occurs simultaneously to regulate the oval cell response by secreting growth factors, cytokines, and ECM components.[2] Using cell type–specific markers for staining, we found that both $Thy1^+$ oval cells and $desmin^+$ stellate cells were surrounded by the FN-rich provisional matrix (Fig. 3C,D). The results provide direct evidence for an association between the FN-concentrated provisional matrix and activated hepatic cells.

To verify the binding between FN and CTGF during oval cell activation, an antibody against the N-terminal region of CTGF was used to stain sections of normal liver and a regenerating liver harvested at day 7 after 2-AAF/ PHx. In normal liver, CTGF was present only around portal veins (Fig. 4A-C), whereas a high level of CTGF was observed in the regenerating liver. CTGF was colo-calized with FN in many extracellular areas, as shown in Fig. 4D-F. IgG control staining was performed and confirmed antibody staining specificity (data not shown). These results indicated that CTGF was bound to FN fibrils in the provisional ECM around periportal zones in regenerating livers.

Modules I and IV Were Selectively Adhesive to Hepatic Cells

In view of the emerging role of CTGF and FN interaction in adhesion responses, we investigated the affinity of the two FN binding modules (I and IV) to several major primary hepatic cell types. Maltose binding protein fused module IV (MBP-IV) has been shown to be adhesive to stellate cells[6] and was used as a positive control. Two kinds of recombinant module I proteins were generated. One was I-3xFLAG generated from Chinese hamster ovary cells, and the other one was maltose binding protein fused module I (MBP-I) produced from *E. coli*. As shown in Fig. 5, MBP-IV and I-3xFLAG displayed high adhesivity to stellate cells, $Thy1^+$ oval cells, and SECs but not to hepatocytes in comparison to the BSA-negative control. The possibility that the 3xFLAG epitope of module I was responsible for adhesion activity was ruled out because it had no adhesivity to those cells. In addition, MBP-I showed much less adhesivity than I-3xFLAG. This could be attributed to misfoldings or to incorrect modifications during bacterial expression such that the configuration required for adhesion was missing. Taken together, these results indicated that bioactive modules I and IV were selectively adhesive to stellate cells, $Thy1^+$ oval cells, and SECs, all of which participated in oval cell activation.

Adhesion of Modules I and IV to $Thy1^+$ Oval Cells Required Heparan Proteoglycans and Integrin $\alpha_5\beta_1$

Integrin and HSPGs have been found to be able to serve as coreceptors for CTGF during cell adhesion.[6] We found that integrin $\alpha_5\beta_1$, the FN primary receptor, was expressed on the cell surface of $Thy1^+$ oval cells as detected by immunofluorescent confocal microscopy analysis using JBS5 antibody (Fig. 6A,B). This result was confirmed by immunoprecipitation with antibodies against integrin subtypes α_5 and β_1 (Fig. 6C). JBS5 antibody has been widely

used to block integrin $\alpha 5\beta 1$–mediated cell adhesion in human cells. We found that this antibody blocked Thy1$^+$ oval cell attachment to FN (Fig. 6D), which indicated that it could be used on rat cells. The involvement of integrin $\alpha_5\beta_1$ and HSPGs in Thy1$^+$ oval cell attachment to immobilized I-3xFLAG and MBP-IV proteins was determined. FN was used as a positive control. We found that pretreatment of cells with JBS5 antibody and heparin greatly inhibited cell adhesion compared with control IgG. Heparinase I, which removed the polysaccharide modification on HSPG(s), also significantly inhibited cell attachment (Fig. 6D). These results indicated that the adhesion of modules I and IV to Thy1$^+$oval cells required HSPGs and integrin $\alpha_5\beta_1$.

CTGF Promoted the Migration, but Not Proliferation, of Thy1$^+$ Oval Cells on FN

The motogenic and mitogenic activities of CTGF were assessed with primary Thy1$^+$ oval cells on FN-coated tissue culture plates. CTGF promoted a nearly 2.5-fold increase of cell migration in comparison to the control (Fig. 7A). Pretreatment with JBS5 antibody blocked CTGF-induced cell migration, and this indicated an integrin $\alpha_5\beta_1$– dependent motogenic activity on CTGF. However, CTGF did not show any proliferative effect in a ^{3}H-thymidine incorporation assay (Fig. 7B). These results indicated that the activities of CTGF *in vitro* were primarily to promote the adhesion and migration rather than proliferation of Thy1$^+$ oval cells.

Discussion

CTGF up-regulation has been found to be important for oval cell activation during liver regeneration triggered by 2-AAF/PHx.[12] As an extension of our *in vivo* observation, the current study focused on the mode of action of CTGF by characterizing its interaction with FN, a candidate interactor identified from a yeast two-hybrid cDNA library screening. FN has been considered an organizer of the remodeling process because of its rapid induction during various chronic and acute liver injuries.[14,18] We found that an FN-concentrated provisional matrix was associated with oval cell activation. CTGF was colocalized with FN in the provisional matrix. Biochemical characterization showed that modules I and IV of CTGF bound to FN. Moreover, the two FN binding modules selectively mediated hepatic cell adhesion. Their adhesion to Thy1$^+$ oval cells required HSPGs and integrin $\alpha_5\beta_1$. CTGF primarily promoted Thy1$^+$ oval cell migration but not proliferation *in vitro*. These observations suggested that CTGF acted as an adhesive molecule to promote cell adhesion and migration in the provisional matrix of regenerating livers after 2-AAF/PHx.

A region from amino acid 21 to 107 of CTGF that is mainly module I was sufficient for binding FN (Fig. 1). In contrast, a shorter human module I (aa27-101) was unable to bind to FN in a yeast two-hybrid analysis by Hoshijima.[8] Obviously, the different lengths of module I used for the yeast two-hybrid analyses made a difference. It seems that the extra amino acids in the long module I (aa21-107) are critical for FN binding. These amino acids may be directly involved in the FN interaction by serving as part of the binding site. Alternatively, they may be involved in the formation of the tertiary conformation critical for module I and FN binding. Understanding the exact interaction between module I and FN may rely on resolving the CTGF crystal structure in the future. In addition, module I alone had much weaker FN binding than the intact CTGF protein did (Fig. 1C,D). The synergistic effect of the two FN binding sites on intact CTGF (modules I and IV) accounts for the high affinity of this protein for FN.

The interaction between ECM and cells is essential for normal homeostasis and hepatic repair. Hepatic ECM that separates parenchyma from sinusoids in the space of Disse is characterized by a basement membranelike matrix with a low density and is critical for maintaining the differentiated functions of resident hepatic cells.[19] During oval cell–aided

liver regeneration, the composition of the matrix must change to suit the need for cell activation, movement, and differentiation. A rapid reorganization of selected ECM components is one of the key events required for the regenerative process. The present study showed that FN and CTGF were highly deposited in the provisional matrix that was associated with oval cell activation (Figs. 2–4). It is conceivable that high FN and CTGF proteins may modify the adhesivity of the provisional matrix, allowing certain hepatic cells to migrate in and locate to sites where they are needed.

CTGF binding to FN may have profound effects regulating oval cell response. First, CTGF may selectively induce the adhesion of such cell types as $Thy1^+$ oval cells, stellate cells, and SECs, but not hepatocytes (Fig. 5). Second, CTGF may differentially control the growth of certain hepatic cells. For instance, CTGF promotes the growth of stellate cells[20] but not $Thy1^+$ oval cells (Fig. 7B). Third, CTGF may be involved in revascularization during the regenerative process because it has been found to bind to vascular endothelial growth factor[21] and modules I and IV were found to be adhesive to SECs in our study. Finally, CTGF binding to FN may also be a way of storage. Small bioactive isoforms have been found in body fluids,[22] and those isoforms may be released from the provisional matrix after CTGF proteolysis.

One key feature in oval cell–aided liver regeneration is the orchestrated balance of communication between cell proliferation, adhesion, migration, and matrix deposition. A rapid turnover of the FN-rich provisional matrix must take place during differentiation to restore the normal hepatic architecture. Otherwise, uncontrolled deposition of a high-density matrix with concentrated FN and CTGF could compromise hepatocyte function, leading to liver fibrosis.[23] Overexpression of CTGF has been considered a hallmark for fibrosis.[11] Therefore, studies about the molecular mechanism governing the action of CTGF in cell and matrix interaction during tissue remodeling will not only have an impact on the basic knowledge about CTGF function but also help to develop therapeutic strategies for the treatment of tissue fibrosis.

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Abbreviations

2-AAF	N-2-acetylaminofluorene
AD	activation domain
BD	binding domain
BSA	bovine serum albumin
cDNA	complementary DNA
CTGF	connective tissue growth factor
CV	central vein
ECM	extracellular matrix
FBS	fetal bovine serum
FN	fibronectin

HSPG	heparan sulfate proteoglycan
IgG	immunoglobulin G
MBP	maltose binding protein
MBP-I	maltose binding protein fused module I
MBP-IV	maltose binding protein fused module IV
PHx	partial hepatectomy
PT	portal triad
SD	standard deviation
SEC	sinusoidal endothelial cell
TBS	trishydroxymethylaminomethane-buffered saline
Thy1	thymus cell antigen 1

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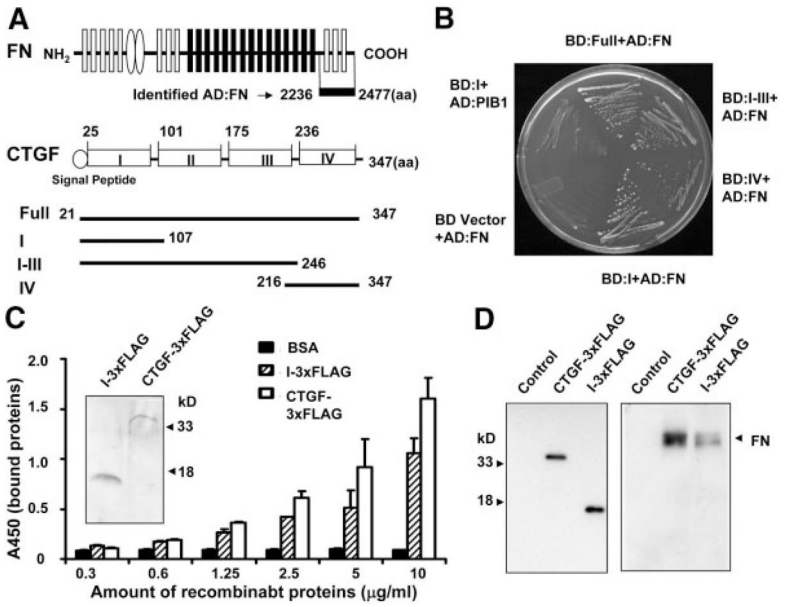

Fig. 1.

Module I of CTGF is another FN binding site. (A) Schematic representations of FN and CTGF proteins as well as constructs used for yeast two-hybrid analyses. The GAL4 activation domain (AD) fused FN fragment obtained from library screening (indicated as AD:FN), GAL4 DNA binding domain (BD) fused CTGF, and mutants (BD:Full, BD:I, BD:I-III, and BD:IV) are shown. Numbers label the amino acid position of the indicated proteins. Type I, II, and III repeats on FN are labeled as open rectangles, open ovals, and dark rectangles, respectively. Putative positions of the modules on rat CTGF are marked on the basis of a similar classification on the human ortholog.[4] (B) Yeast two-hybrid analyses of cotransformants for the binding of CTGF or its mutants to AD:FN on selection media lacking histidine, tryptophan, and leucine. (C) *In vitro* binding of recombinant proteins to FN in solid-phase assays. The insert shows a silver staining gel with purified CTGF-3xFLAG (~40 kDa) and I-3xFLAG (12 kDa). (D) FN was specifically pulled down by recombinant CTGF-3xFLAG and I-3xFLAG in immunoprecipitation. Equal amounts of recombinant proteins in conditioned media were input and detected by M2 antibody against epitope in the left panel. Conditioned media from cells transfected with an empty vector were used as controls. Immunocomplexes were pulled down by M2 antibody– conjugated agarose and detected by FN antibody in Western analysis (right panel).

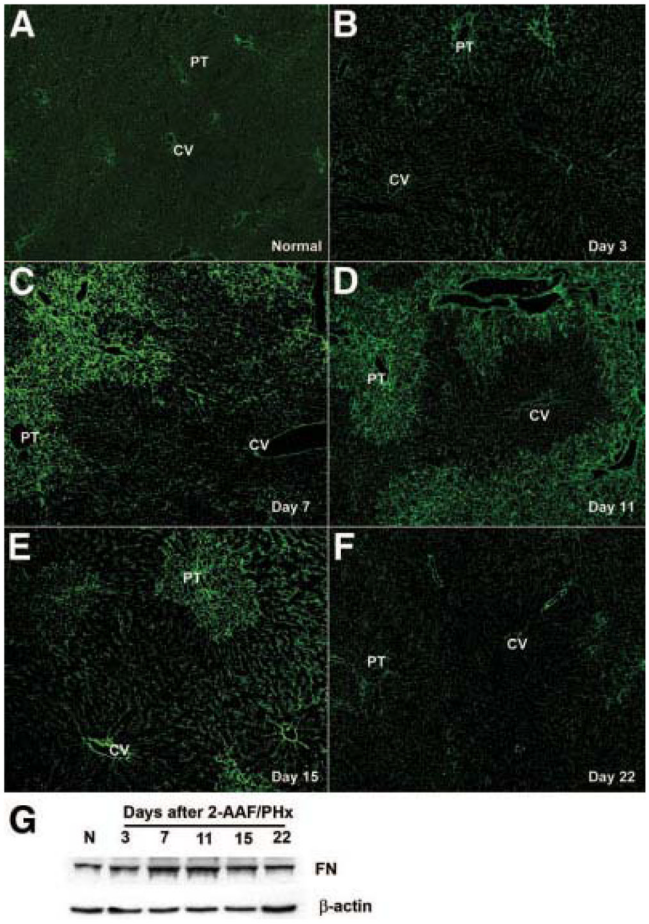

Fig. 2.

Increased FN deposition in a provisional matrix around portal areas was associated with oval cell activation. (A-F) Immunofluorescent staining of FN in a normal liver and regenerating livers at days 3, 7, 11, 15, and 22 after 2-AAF/PHx. All images were taken at 100× magnification. The central vein (CV) and portal triad (PT) are indicated. The time points for regenerating livers are defined as days after PHx surgery of 2-AAF implanted livers. (G) FN protein expression in total liver homogenate by Western analysis. N indicates normal liver.

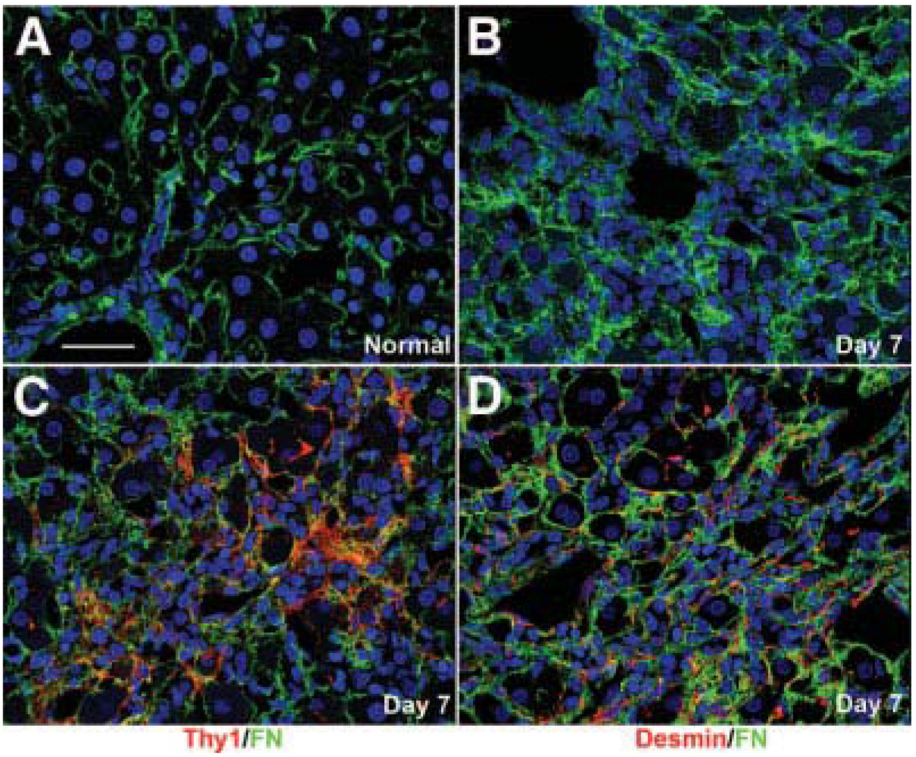

Fig. 3.

The FN-concentrated provisional matrix formed a continuous network that surrounded hepatic cells including Thy1$^+$ oval cells and desmin$^+$ stellate cells in the analysis of immunofluorescent confocal microscopy. (A,B) Staining with FN on liver sections from normal and regenerating livers, respectively. (C) Staining with FN in green and Thy1 in red on the regenerating liver section. (D) Staining with FN in green and desmin in red on the regenerating liver section. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole. Bar: 50 μm.

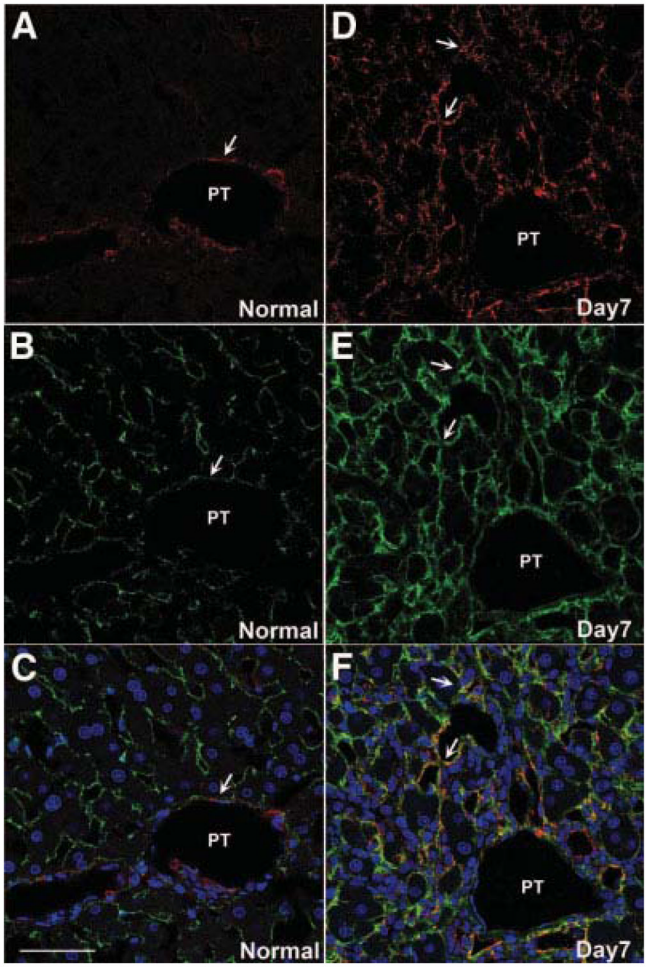

Fig. 4.

Colocalization between CTGF and FN in the provisional matrix around periportal areas in liver sections with massive oval cell activation (day 7 after 2-AAF/PHx). (A-C) Normal liver and (D-F) regenerating liver were stained with CTGF in red and FN in green. Colocalized areas, as indicated by white arrows, were yellow in (C,F) merged pictures. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Pictures were analyzed by immunofluorescent confocal microscopy. PT indicates portal triad. Bar: 50 μm.

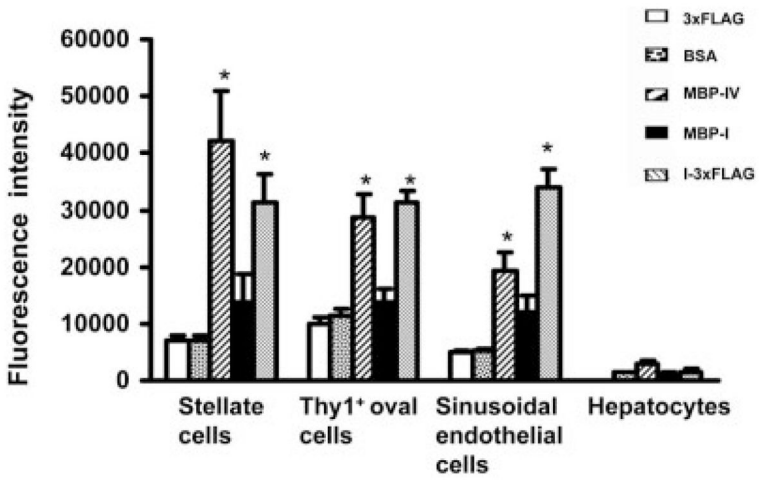

Fig. 5.

Modules I and IV were selectively adhesive to hepatic cells. Adhesion assays were performed in microplates that were precoated with 8 μg/mL 3xFLAG peptide, 2 μg/mL BSA, and I-3x Flag as well as 8 μg/mL MBP-IV and MBP-I. Adherent cells were stained by CyQUANT NF dye, and the fluorescence intensity of each sample was measured with excitation at ~492 nm and emission detection at ~530 nm. Each value is the average ± SD in triplicate. *$P < 0.05$ versus BSA in each group.

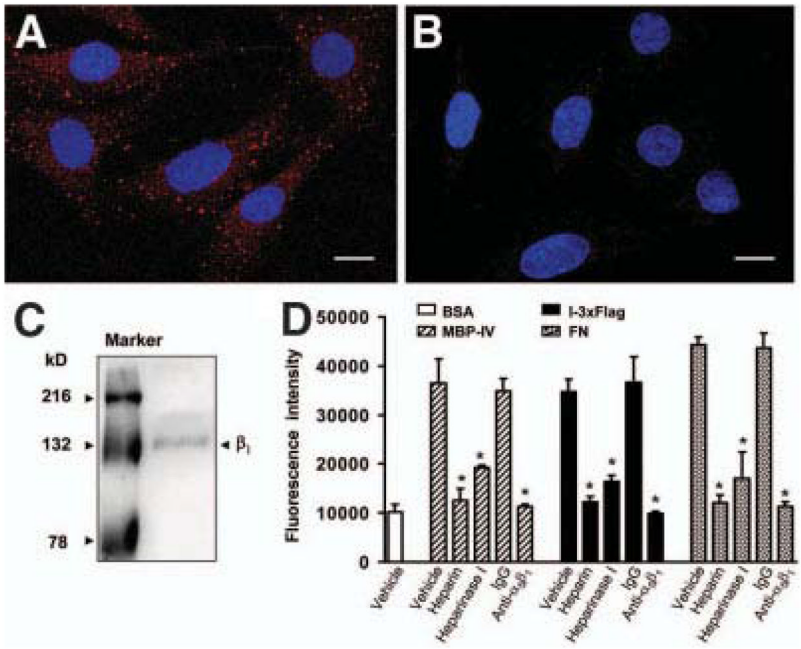

Fig. 6.

The adhesion of modules I and IV to Thy1$^+$ oval cells required integrin $\alpha_5\beta_1$ and HSPGs. (A) The expression of integrin $\alpha_5\beta_1$ on a cell surface stained with JBS5 antibody. (B) The negative control stained with normal mouse IgG. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Images were analyzed by confocal microscopy. Bar: 10 μm. (C) The association of integrin subunit α_5 with β_1 in immunoprecipitation. (D) The requirement of integrin $\alpha_5\beta_1$ and HSPGs on module I–mediated and module IV–mediated Thy1$^+$oval cell adhesion. Micro-plates were coated with 2 μg/mL BSA, 2 μg/mL I-3xFLAG, 2 μg/mL FN, and 8 μg/mL MBP-IV. Cells were pretreated at 37°C for 30 minutes with vehicle buffer, 2 μg/mL heparin, 2 units/mL heparinase I, 25 μg/mL JBS5, and 25 μg/mL normal mouse IgG before cell adhesion assay. Each value is the average ± SD in triplicate. *$P < 0.05$ versus vehicle in each group.

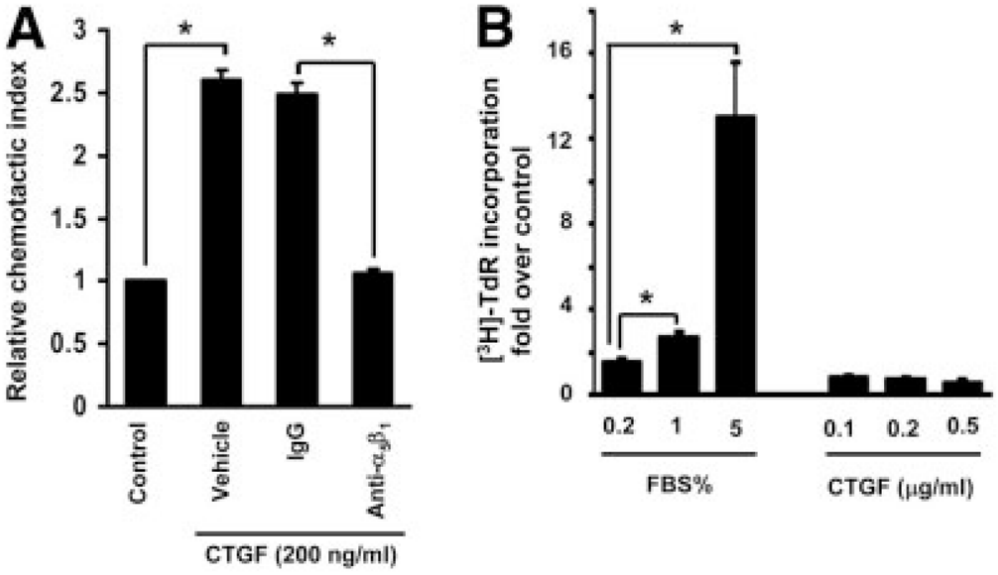

Fig. 7.

CTGF promoted the migration but not proliferation of Thy1$^+$ oval cells. (A) A transwell migration assay was performed in the presence or absence of 200 ng/mL CTGF in the lower chambers. Cells were pretreated with vehicle buffer, JBS5 (25 μg/mL), or mouse IgG (25 μg/mL) for 30 minutes at 37°C before being inoculated into the upper chamber of transwells. Cells on the undersurface of the transwells were counted in 10 random fields at 40× magnification. The control was without CTGF. Each value is the average ± SD in triplicate. Data were normalized with respect to the control. *$P < 0.05$. (B) CTGF alone had minimal activity on Thy1$^+$ oval cell proliferation in a ^{3}H thymidine incorporation assay. The control was cultured only in Iscove's medium with 0.2% FBS. Data were normalized for each independent experiment with respect to controls. *$P < 0.05$.