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Recombinant fusion protein of albumin-retinol binding protein inactivates stellate cells

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ABSTRACT

Quiescent pancreatic- (PSCs) and hepatic- (HSCs) stellate cells store vitamin A (retinol) in lipid droplets via retinol binding protein (RBP) receptor and, when activated by profibrogenic stimuli, they transform into myofibroblast-like cells which play a key role in the fibrogenesis. Despite extensive investigations, there is, however, currently no appropriate therapy available for tissue fibrosis. We previously showed that the expression of albumin, composed of three homologous domains (I–III), inhibits stellate cell activation, which requires its high-affinity fatty acid-binding sites asymmetrically distributed in domain I and III. To attain stellate cell-specific uptake, albumin (domain I/III) was coupled to RBP; RBP-albumin$^{domain\ III}$ (R-III) and albumin$^{domain\ I}$-RBP-albumin^{III} (I-R-III). To assess the biological activity of fusion proteins, cultured PSCs were used. Like wild type albumin, expression of R-III or I-R-III in PSCs after passage 2 (activated PSCs) induced phenotypic reversal from activated to fat-storing cells. On the other hand, R-III and I-R-III, but not albumin, secreted from transfected 293 cells were successfully internalized into and inactivated PSCs. FPLC-purified R-III was found to be internalized into PSCs via caveolae-mediated endocytosis, and its efficient cellular uptake was also observed in HSCs and podocytes among several cell lines tested. Moreover, tissue distribution of intravenously injected R-III was closely similar to that of RBP. Therefore, our data suggest that albumin-RBP fusion protein comprises of stellate cell inactivation-inducing moiety and targeting moiety, which may lead to the development of effective anti-fibrotic drug.

1. Introduction

Pathological features of hepatic and pancreatic fibrosis is the excessive production and deposition of extracellular matrix (ECM) components and there is overwhelming evidence that activated hepatic and pancreatic stellate cells are the major producers of the fibrotic neomatrix [1,2]. Studies showed that pancreatic stellate cells (PSCs) display similar cellular behavior to hepatic stellate cells (HSCs) [3,4]. PSCs, in their quiescent state, can be identified by their angular appearance and the presence of vitamin A-containing lipid droplets in their cytoplasm [5]. When activated by profibrogenic mediators, they transform into myofibroblast-like cells characterized by positive staining for α-smooth muscle actin (α-SMA), loss of vitamin A lipid droplets, and greatly increased synthesis of the ECM proteins, including type I collagen [6].

Retinoids (vitamin A and its metabolites) regulate multiple physiological activities, such as vision, reproduction, morphogenesis, cell proliferation and differentiation [7]. Vitamin A (retinol), acquired from the diet, circulates bound to retinol binding protein (RBP) in the bloodstream, and it is taken up initially by liver cells, transferred to HSCs via RBP receptor and stored as retinly ester in cytoplasmic lipid droplets [8–10]. Previous studies showed that intravenously injected RBP is taken up at relatively high levels by liver, especially HSCs [11–13].

Albumin is an abundant multifunctional plasma protein synthesized primarily by liver cells [14]. It comprises three homologous domains, each formed by two smaller subdomain, A and B [15]. Crystallographic analysis revealed asymmetric distribution of five high-affinity fatty acid binding sites in albumin (one in subdomain IB, one between IA and IIA, two in IIIA and one in IIIB) [16,17]. Our recent study showed that albumin is also expressed in stellate cells and directly involved in the formation of vitamin A-containing

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lipid droplets, thereby inactivating stellate cells [18,19]. In this study, we designed novel recombinant albumin-RBP fusion proteins and explored their biological activity. They were found to be internalized into stellate cells in a relatively selective manner and inactivated them.

2. Materials and methods

2.1. Materials

Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY). Male Sprague–Dawley rats and BALB/c mice of 6–8 weeks of age were purchased from Orient Charles River Korea, Seoul, Korea). All animals were maintained under temperature-, humidity-, and light-controlled conditions. Animal experiments were approved by the appropriate institutional committee and complied with the Guide for the Care and Use of Laboratory Animals.

2.2. Pancreatic stellate cells (PSCs) isolation and culture

Rat PSCs were isolated as described previously [6]. Briefly, the pancreas was finely minced, placed in a solution of Hank's buffered salt solution with 0.05% collagenase, 0.02% pronase and 0.1% DNase, and shaken for 20 min at 37 °C. After filtration through 150 μm mesh, cells were centrifuged on a 13.2% Nycodenz gradient at 1400*g* for 20 min. PSCs were collected from the band just above the interface of the Nycodenz solution and the aqueous layer, suspended in DMEM supplemented with 10% fetal bovine serum, and plated on non-coated plastic dishes. After reaching confluence in the primary culture, serial passages were obtained always applying a 1:3 split.

2.3. Construction of expression vector for albumin-RBP fusion proteins

Total RNA was isolated from rat liver tissue using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA using GeneAmp RNA PCR (Applied Biosystems, Foster city, CA). The entire open reading frame (ORF) of albumin or RBP was amplified by polymerase chain reaction (PCR) with the designed primers and inserted into a pBluescript vector. Expression plasmid encoding R-III was constructed as follows. The DNA fragments encoding RBP (1–585) and albumin (domain III: 1216–1827) were amplified from the pBluescript-RBP and pBluescript-albumin by PCR with a sense primer (5′ GCGGAATTCC ACCATGGAGT GGGTGTGGGC 3′ and 5′ GGGCTCGAGGAAGAACCTAAGAACTTG 3′) and an antisense primer (5′ CCCCTCGAGT CTGCTTTGAC AGTAACC 3′ and 5′ GGCTCTAGAT TAATGATGAT GATGATGATG GGCTAAGGCT TCTTTGCT 3′), respectively. The His-tag sequence was included in the antisense primer for albumin domain III. The PCR products were double digested with EcoRI/XhoI and XhoI/XbaI, respectively, and DNA fragments purified by agarose gel electrophoresis were ligated together. The resulting DNA fragment R-III was then inserted into the expression vector pcDNA3.1+ at EcoRI and XbaI site to yield pcDNA3.1-R-III. Expression plasmid encoding I-R-III was constructed as follows. The DNA fragments encoding albumin (domain I: 1–666) and RBP (55–585) were amplified with a sense primer (5′ GGGGTACCCC ACCATGAAGT GGGTAACCTT TC 3′ and 5′ GGGCAATTGG AGCGCGACTG CAGGGTG 3′) and an antisense primer (5′ CCCCAATTGC ATCCTCTGAC GGACAGC 3′ and 5′ CCCCTCGAGT CTGCTTTGAC AGTAACC 3′), respectively. The PCR products were double digested with KpnI/MFeI and MfeI/XhoI, respectively, ligated together, and cloned into KpnI/XhoI-cut pBluescript vector to yield pBluescript-I-R. The DNA fragment I-R was then ligated with the DNA fragment encoding albumin (domain III: 1216–1827) and inserted into pcDNA3.1+ vector at KpnI and XbaI site to yield pcDNA3.1-I-R-III. In pcDNA3.1-R-III and -I-R-III, the albumin-RBP coding region was located immediately upstream of 6-histidine tag coding sequence and stop codon in the same reading frame. The expression plasmids encoding mouse R-III were constructed in the same manner as rat R-III. Primers used for PCR are as follows: RBP (1–585) (sense) 5′ GCGGAATTCC ACCATGGAGT GGGTGTGGGC 3′ and (antisense) 5′ CCCCTCGAGC CTGCTTTGAC AGTAACC 3′; albumin (domain III: 1216–1827) (sense) 5′ GGGCTCGAGG AAGAGCCTAA GAACTTG 3′ and (antisense) 5′ GGCTCTAGAT TAATGATGAT GATGATGATG GGCTAAGGTG TCTTTGCA 3′. All constructs were then sequenced to confirm the albumin-RBP coding region by using an automatic sequencer.

2.4. Purification of (His)6-tagged R-III fusion protein

293 cells were stably transfected with the expression vector for mouse R-III and the high-expressing, clonal cell lines were selected by assessing levels of secreted R-III by Western blotting. For the purification of R-III, conditioned medium was prepared from transfected 293 cells grown in serum-free medium for 4 days, fractionated with ammonium sulfate (55%), and then subject to His Trap affinity column. The sample was further purified by Resource Q. Purified protein was dialyzed against deionized water, freeze-dried, and dissolved in saline solution. The purity of R-III is >95%, as determined by SDS–PAGE and protein staining.

2.5. Transfection

Activated PSCs (after passage 2) were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and subject to analysis after 24 h.

2.6. Western blot analysis

Cells were rinsed in ice-cold phosphate-buffered saline (PBS) twice and harvested by scraping in the lysis buffer [20]. Equivalent amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by immunoblot detection using the primary antibody. Primary antibodies were as follows: albumin (Santa Cruz, Santa Cruz, CA), His-tag (AB frontier, Seoul, Korea), α-SMA (Sigma, St. Louis, MO), α-tubulin (Cell signaling, Beverly, MA), and type I collagen (Calbiochem, San Diego, CA).

2.7. Immunofluorescence

PSCs were plated onto glass coverslips coated with gelatin. After the treatment, cells were fixed in paraformaldehyde and incubated with anti-albumin antibody (Santa Cruz #sc-58698) overnight at 4 °C in a moist chamber, followed by FITC-conjugated goat anti-mouse IgG (Santa Cruz #sc-2010). Cells were washed with PBS and mounted onto slides. Stained cells were visualized on a Zeiss AXIO Imager M1 microscopy.

2.8. Oil red O staining

PSCs were stained with Oil red O to visualize lipid droplets, essentially as described by Koopman et al. [21]. Oil red O were diluted in triethyl phosphate instead of isopropanol.

2.9. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Paired statistical analysis was done using *T*-tests. Comparisons were considered significant at $p < 0.05$ and p values were two tailed.

3. Results

3.1. Construction of recombinant albumin-RBP fusion proteins

It was previously reported that the expression of albumin inhibits stellate cell activation, which requires its high-affinity fatty acid-binding sites [18], and that RBP is internalized into stellate cells via receptor-mediated endocytosis [9,12,13]. For the specific targeting of albumin protein to stellate cells, we sought to design novel recombinant fusion proteins combining albumin (domain I/III, in which its high-affinity fatty acid binding sites are found [16,17]) and RBP; $RBP^{1-195a.a.}$-$albumin^{406-608a.a.(domain\ III)}$ (R-III) and $albumin^{1-222(domain\ I)}$-$RBP^{19-195}$-$albumin^{406-608(domain\ III)}$ (I-R-III) (Fig. 1A). Each protein part was connected to one another via restriction site linker, and polyhistidine tag was placed on C-terminus of the fusion proteins. We constructed expression plasmids for the fusion proteins (Fig. 1A) and assessed their biological activity in cultured pancreatic stellate cells (PSCs). Among the variants with different C-terminal ends of RBP, fusion proteins with $RBP^{-195a.a.}$ were found reproducibly expressed at relatively high levels (data not shown).

3.2. Expression of albumin-RBP fusion protein inactivates PSCs

PSCs were isolated from rat pancreas and activated by culture on uncoated plastic. PSC activation is directly correlated with the duration in *in vivo* culture and the expression of α-SMA, a marker for the activated PSC phenotype [22]. PSCs after passage 2 (PSCs-P2; activated PSCs) were transiently transfected with either empty vector or expression plasmids for His-tagged albumin, R-III or I-R-III, and cell lysates were analyzed by Western blot analysis. Anti-His tag antibody detected expression of albumin, R-III, and I-R-III at the expected size of ~66, 45 and 68 kDa, respectively (Fig. 1B). When grown in standard culture condition, control PSCs-P2 had an elongate fibroblastoid morphology and small, tiny dots could be observed in the perinuclear area (Fig. 2A). On the other hand, expression of R-III or I-R-III, like wild type albumin [18], led to the formation of autofluorescent lipid droplets and induced phenotypic reversal from the myofibroblast to the fat-storing cells with a more regular polygonal shape (Fig. 2B–D). Such a phenotypic change was accompanied with a decrease in levels of α-SMA and type I collagen (Fig. 1B), suggesting that expression of albumin domain III alone is sufficient to induce stellate cell inactivation. To investigate the cellular distribution of His-tagged fusion protein expressed in PSCs, we performed immunofluorescence using several commercially available anti-His tag antibodies but failed to detect positive staining for some unknown reason. It is interesting to note that albumin levels are increased not only in albumin-transfected, but also in R-III- or I-R-III-transfected cells (Figs. 1B and 2B–D). Such an increase in albumin levels was reproducibly observed in quiescent and inactivated PSCs [18,19], suggesting that albumin can be used as a reliable marker for the activation status of stellate cells.

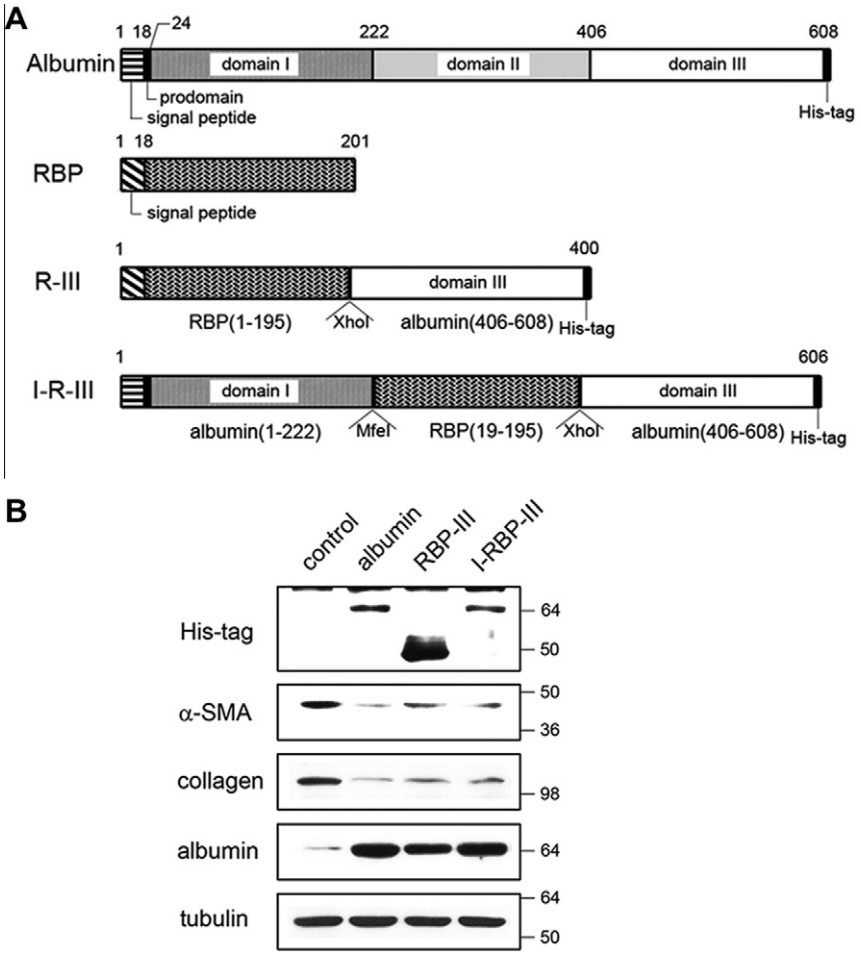

Fig. 1. Construction of recombinant albumin-RBP fusion proteins. (A) Schematic diagram of albumin-RBP fusion proteins, R-III and I-R-III, compared to albumin and RBP. Note that albumin, R-III and I-R-III protein products are histidine tagged at the C-terminal end. (B) PSCs after passage 2 were transiently transfected with either empty vector (control) or expression plasmids for His-tagged albumin, R-III or I-R-III, and cell lysates were analyzed by Western blotting. The Western blots are representative of three independent experiments from separate cell preparations. α-tubulin serves as loading control.

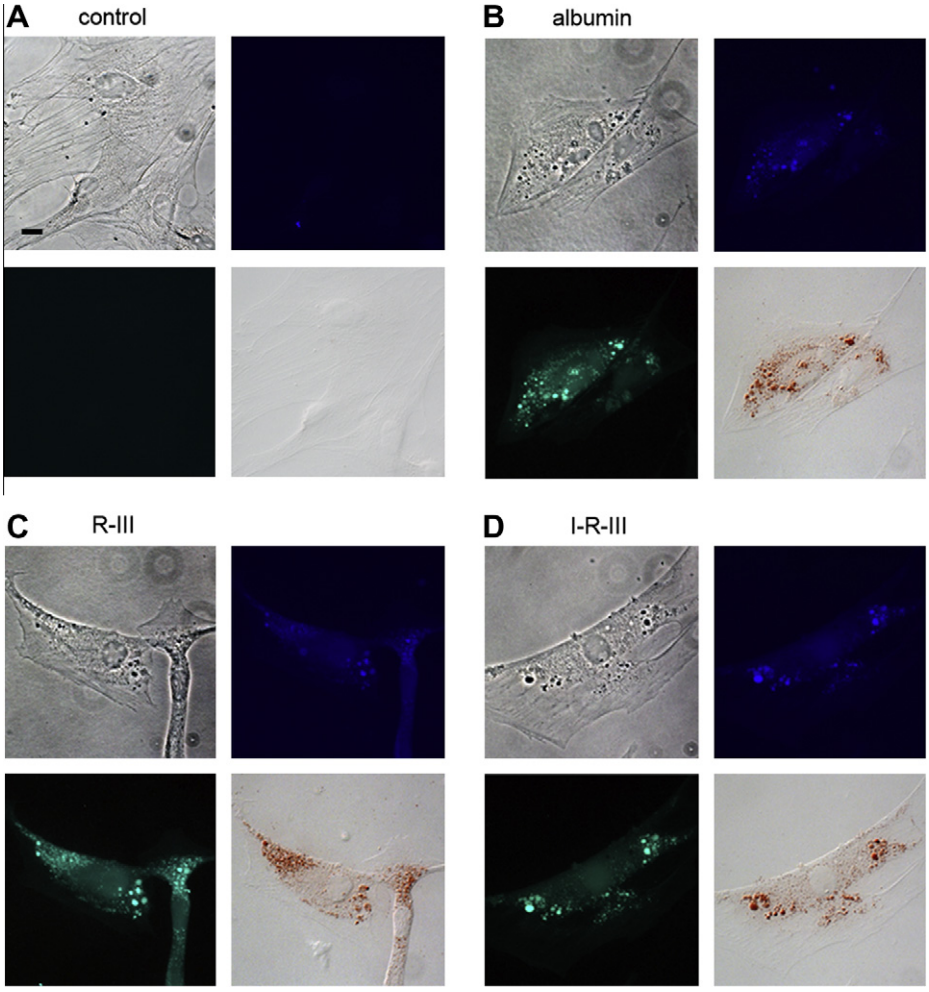

Fig. 2. Expression of albumin-RBP fusion protein in activated PSCs leads to reappearance of fat droplets. (A–D) PSCs after passage 2 were transiently transfected with either empty vector (A) or expression plasmids for albumin (B), R-III (C) or I-R-III (D) and examined for phenotypic changes. Phase contrast images (upper left), autofluorescence images (upper right), immunofluorescence images using anti-albumin antibody (lower left), and differential interference contrast images (DIC) of oil red O staining (lower right) are shown for the transient transfectants. Scale bar = 10 μm.

3.3. Albumin-RBP fusion proteins are internalized into PSCs

To determine whether RBP moiety facilitates cellular uptake of the fusion proteins, PSCs-P2 were incubated with conditioned medium taken from His-tagged albumin-, R-III- or I-R-III-stably transfected 293 cells for 24 h and cell lysates analyzed by Western blotting using anti-His tag antibody. Our previous study showed that intracellular albumin levels are low in activated stellate cells, regardless of the presence of large amounts of serum albumin in the medium [18]. Albumin produced and secreted from transfected 293 cells was not incorporated into PSCs, but R-III and I-R-III were successfully internalized (Fig. 3A). Moreover, cellular uptake of R-III and I-R-III induced lipid droplet formation (Fig. 3B). Such a phenotypic change was accompanied with a decrease in levels of α-SMA and type I collagen (Fig. 3A). Thus, this finding suggests that RBP moiety allows the fusion protein to cross the membrane of PSCs. We then investigated the mechanisms of internalization of the fusion protein. To this end, we purified R-III by FPLC to a purity of >95%. R-III was chosen since it was produced and secreted into the culture medium of transfected 293 cells at higher levels as compared with I-R-III (data not shown). PSCs-P2 were pretreated with inhibitor of clathrin-mediated (chlorpromazine) or caveolae-mediated endocytosis (filipin), and further incubated with R-III for 1 h. Western blot analysis of treated cell lysates revealed that cellular uptake of R-III is significantly inhibited by filipin but unaffected by chlorpromazine (Fig. 3C). This finding agrees with the previous report that RBP is taken up by liver cells by caveolae-mediated endocytosis [23].

3.4. RBP moiety mediates cell specific uptake of R-III

We then investigated the cell type specificity in the R-III uptake. For this experiment, different cell lines, including hepatic stellate cells (HSCs), Panc-1 pancreatic cells, HepG-2 hepatocellular cells, A549 lung epithelial cells, NIH3T3 cells, HT1080 fibrosarcoma cells, 293 embryonic kidney cells, HK2 renal proximal tubule cells, mouse mesangial cells (MMC) and podocytes, were incubated with R-III (0.15 μM) for 1 h and cell lysates analyzed by Western blotting. A significant level of cellular uptake of R-III was observed in HSCs and podocyte, and relatively little uptake also seen in NIH3T3, HK2 and MMC (Fig. 4A and B). Next, we examined *in vivo* tissue distribution of R-III. BALB/c mice were injected every day with R-III (3 or 10 μg) dissolved in 0.1 ml of saline solution through the tail vein for 7 days and liver lysates were analyzed by Western blotting. A distinct R-III protein band was seen in the injected mice and its density increased dose-dependently (Fig. 4C). When equivalent amounts of whole cell lysates obtained from different tissues were analyzed by Western blotting, we detected, in addition to a

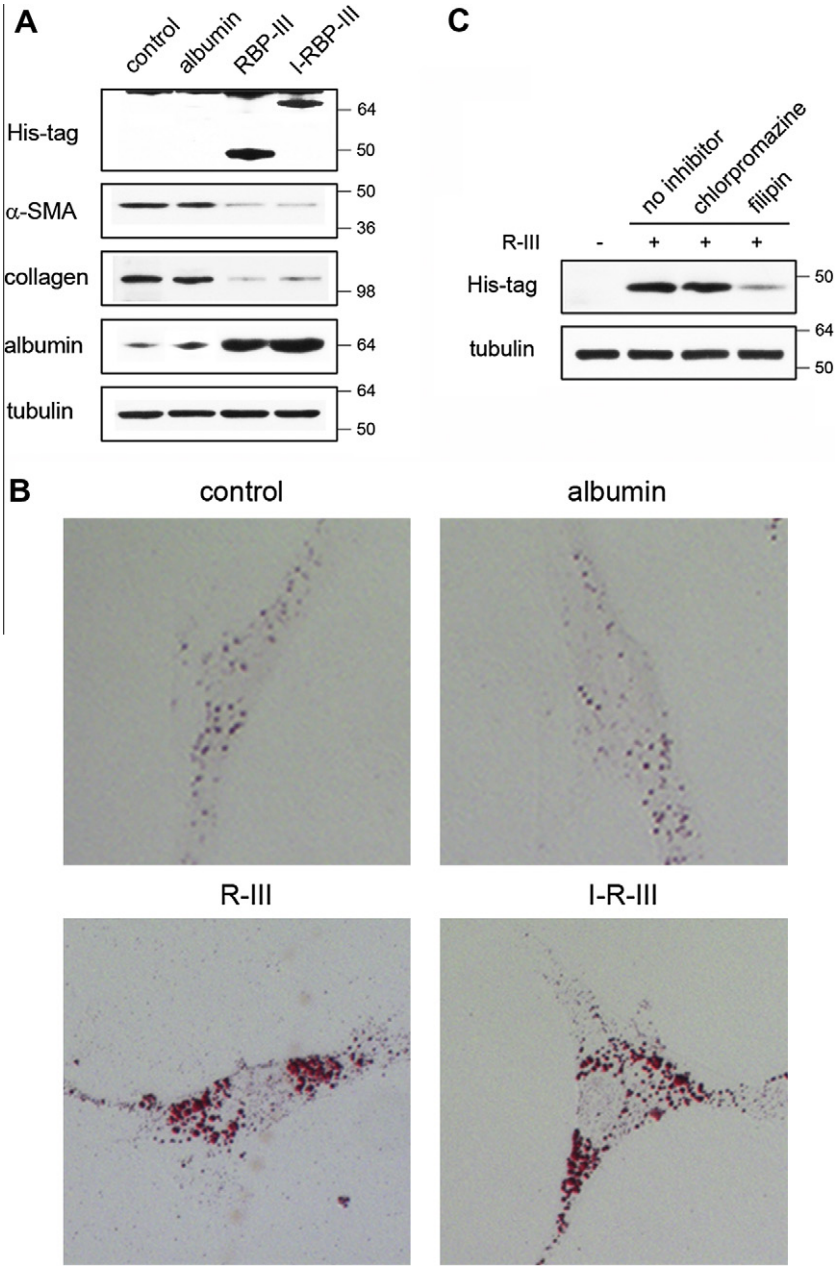

Fig. 3. Albumin-RBP fusion proteins are internalized into PSCs. (A and B) PSCs after passage 2 were incubated with the conditioned medium taken from His-tagged albumin-, R-III- or I-R-III-stably transfected 293 cells for 24 h and subjected to Western blotting (A) and oil red O staining (B). (C) PSCs after passage 2 were pretreated with chlorpromazine (10 μg/ml) or filipin (5 mg/ml) in normal culture medium for 30 min, then further incubated with purified R-III (0.15 μM) for 1 h. Cells were washed three times with PBS and cell lysates analyzed by Western blotting.

strong R-III signal in liver lysates, weak signal also in brain, lung, spleen, pancreas, kidney and intestine (Fig. 4D). This tissue distribution of R-III is closely similar to that of RBP [11]. Thus, our data suggest that R-III fusion protein comprises of stellate cell-inactivating moiety (albumin domain III) and -targeting moiety (RBP).

4. Discussion

Hepatic stellate cells (HSCs) play pivotal roles in both the regulation of retinoid homeostasis in the whole body and the development of liver fibrosis [24]. The retinoid-storing stellate cells are present in a number of tissues other than the liver, such as pancreas, kidney, spleen, intestine and lung [25]. They show the striking similarities in morphology and perivascular location, which suggests that activated stellate cells, may contribute to the myofibroblast cells seen in the fibrotic extrahepatic tissues. Studies have already shown that pancreatic stellate cells (PSCs) play a major role in pancreatic fibrosis [1] and have been undertaken to investigate the origin of renal myofibroblasts in chronic kidney disease [26].

Activated HSCs are considered an attractive target for antifibrotic therapy [27,28]. Treatments with aim of inactivating HSCs, downregulating proliferative, fibrogenic responses of HSCs, and promoting HSC apoptosis have been reported, but the efficacy of most treatment has not been proven in humans, due to the lack of cell specificity [29]. In this study, we designed novel recombinant albumin-RBP fusion proteins, R-III and I-R-III. Forced expression of fusion proteins in activated PSCs induces phenotypic

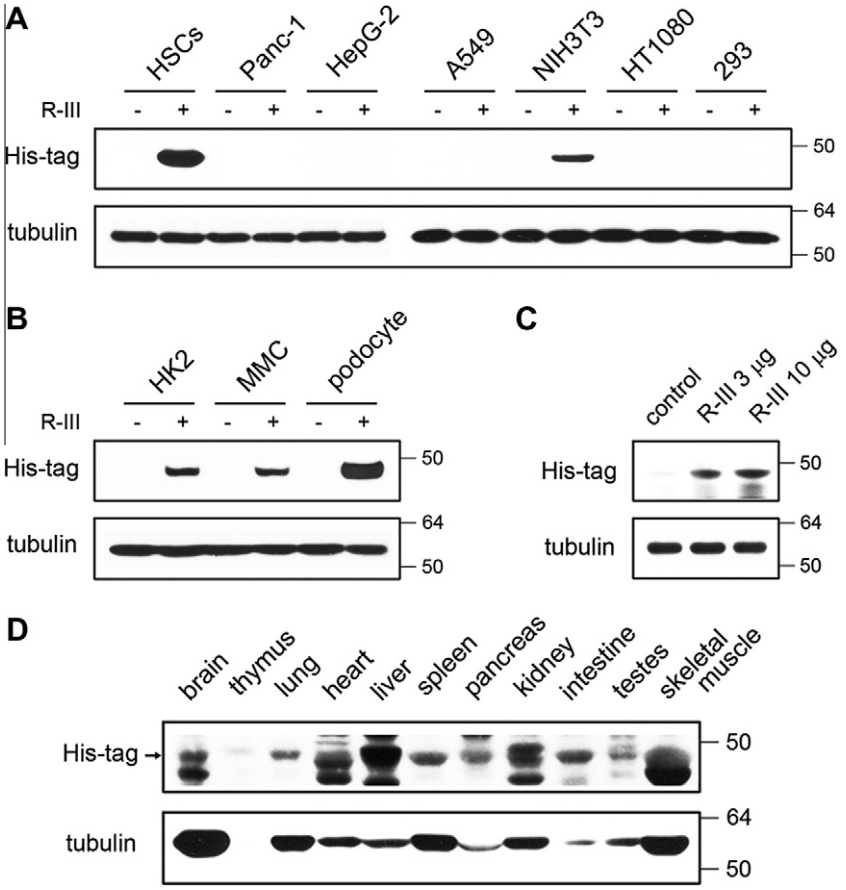

Fig. 4. RBP moiety mediates cell specific uptake of R-III. (A and B) After 1 h incubation with R-III (0.15 μM), different cell lines, including activated HSCs, Panc-1, HepG-2, A549, NIH3T3, HT1080, 293 cells (A), HK2, MMC and podocytes (B), were washed three times with PBS and cell lysates analyzed by Western blotting. (C) BALB/c mice were intravenously injected with R-III (3 or 10 μg) or saline alone (control) daily for 7 days and liver lysates were analyzed by Western blotting. (D) Equivalent amounts of whole cell lysates obtained from different tissues were analyzed for His-tagged R-III by Western blotting.

reversal from activated to fat-storing cells, and fusion proteins expressed on transfected 293 cells were successfully internalized into PSCs. Furthermore, tissue distribution of intravenously injected R-III is closely similar to that of RBP [11,12]. These findings suggest that albumin domain III, like full-length albumin, is also capable of inactivating stellate cells and that RBP moiety allows the fusion protein to cross the membrane of stellate cells. Further studies are required to determine the cellular distribution of intravenously injected R-III in tissues such as liver, pancreas and kidney, and to evaluate its anti-fibrotic potential using established animal models of fibrosis for each tissue.

Besides PSCs and HSCs, R-III was found to be internalized into podocytes, less efficiently in NIH3T3, HK2 and MMC. This finding is intriguing since the resident fibroblast is known to be a potential cellular source of myofibroblasts [2,30] and the role of each renal cell type in the development of renal fibrosis has been unraveling [26,30]. It is necessary to examine how R-III affects cellular behavior in these cell lines.

For the development of effective anti-fibrotic therapies, it is important to understand mechanisms for the activation of stellate cells, but it is not yet fully understood. Based upon previous reports, it is plausible to assume that the collapse of lipid droplets invariably observed during stellate cell activation may release profibrogenic molecules, possibly retinly ester and the resulting metabolites, and that albumin may retrieve them by inducing lipid droplet formation [18,31], thereby inactivating stellate cells. Involvement of albumin in the formation of cytoplasmic lipid droplets was demonstrated not only in stellate cells but also in 3T3-L1 adipocytes [31]. It is interesting to note that R-III- or I-R-III-mediated inactivation of PSCs is accompanied with an increase in albumin levels (Figs. 1B and 3A), supporting that albumin is important for acquisition of a quiescent phenotype in stellate cells [18]. Further study is needed to investigate the mechanism for the albumin-mediated inactivation of stellate cells.

In summary, we introduced novel recombinant albumin-RBP fusion protein and demonstrated that it may be a good candidate for stellate cell-targeted, anti-fibrotic therapy by inducing stellate cell inactivation.

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