Early diagnosis and noninvasive detection of liver fibrosis and its heterogeneity remain as major unmet medical needs for stopping further disease progression toward severe clinical consequences. Here we report a collagen type I targeting protein-based contrast agent (ProCA32.collagen1) with strong collagen I affinity. ProCA32.collagen1 possesses high relaxivities per particle ($r_1$ and $r_2$) at both 1.4 and 7.0 T, which enables the robust detection of early-stage (Ishak stage 3 of 6) liver fibrosis and nonalcoholic steatohepatitis (Ishak stage 1 of 6 or 1A Mild) in animal models via dual contrast modes. ProCA32.collagen1 also demonstrates vasculature changes associated with intrahepatic angiogenesis and portal hypertension during late-stage fibrosis, and heterogeneity via serial molecular imaging. ProCA32.collagen1 mitigates metal toxicity due to lower dosage and strong resistance to transmetallation and unprecedented metal selectivity for Gd$^{3+}$ over physiological metal ions with strong translational potential in facilitating effective treatment to halt further chronic liver disease progression.
Nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are common causes of chronic liver disease (CLD), cirrhosis, and hepatocellular carcinoma (HCC), which are major causes of morbidity and mortality worldwide. CLD originates from a variety of causes such as viral hepatitis, metabolic dysfunction, as well as alcohol abuse and autoimmune disease, and almost all chronic liver injuries cause liver fibrosis. Nonalcoholic steatohepatitis (NASH) and hepatic fibrosis can develop in patients with any type of CLD, including alcoholic liver disease, hepatitis C, hepatitis B, NAFLD and autoimmune hepatitis. The Centers for Disease Control and Prevention (CDC) previously reported that 19.388 people died in the US from alcohol related liver diseases in 2014. Moreover, from 2000 to 2015, death rates for chronic liver disease and cirrhosis in the United States increased 31% among persons aged 45–64 years. Nonalcoholic fatty liver disease including NASH, is the primary cause of CLD in the United States, affecting an estimated 80–100 million Americans (30–40% of the population). NAFLD and ALD can further progress to cirrhosis or hepatocellular carcinoma. It has been projected that NASH occurs in 20% of patients with NAFLD (3–12% of the US population), and 30–40% of them with NASH will develop fibrosis.

Biopsy is the gold standard for diagnosis and staging of CLD as it relies on stage-dependent characteristic patterns of collagen expression. However, it has many limitations such as sampling errors, and high interobserver variability with 33–50% error rates even for diagnosis of advanced stages of liver fibrosis such as cirrhosis likely due to heterogeneity. Earlier studies have reported that imaging modalities can detect morphological characteristics of liver cirrhosis such as surface nodularity; however, in some cases, the cirrhotic liver can appear completely normal, and biopsy is required for confirmation of the diagnosis.

Tremendous effort has been devoted to the development of noninvasive imaging techniques associated with fibrosis and NASH, such as ultrasound; MR apparent diffusion coefficient (ADC); magnetic resonance elastography (MRE); T1, T2, relaxation in the rotating frame (T1p); magnetization transfer; and proton density fat fraction. However, these techniques cannot provide accurate detection of early stage liver fibrosis and NASH. A prerequisite for early detection and efficient treatment of liver fibrosis and NASH is a reliable noninvasive diagnostic method to accurately stage NASH and fibrosis progression and response to therapy. Magnetic resonance imaging (MRI) offers several unique advantages compared to other clinical imaging modalities with its deep tissue penetration, high spatial resolution, and coverage of the entire liver. MRI does not require the use of ionizing radiation, and it is well suited for detection and monitoring the progression and regression of NASH and fibrosis. However, MRI cannot unambiguously detect early stage patient liver fibrosis and NASH with regional heterogeneity due to major limitations such as lack of sensitive MRI contrast agents.

To differentiate invisible fibrotic cells from heterogeneous tissue background lacking a clear boundary requires novel MRI contrast agents and imaging methodology to have a strong specificity for both organ and diseased cells as well as improved relaxation properties. Iron oxide-based contrast agents such as ferumoxides create negative (dark) T2/T1* effects that reportedly yield images with signal voids and very limited accuracy. The majority of clinically approved Gd3+ MRI contrast agents have high r1 and r2 relaxivity values, but only r1 is strong enough to lead to a positive (bright) imaging. To date, two clinical agents Gd-BOPTA (MultiHance) and Gd-EOB-DTPA (Eovist, US; Primovist, Europe) exhibit liver distribution and are able to detect late-stage fibrosis (cirrhosis) in vivo using T1-weighted sequence at a short time point. However, there is an unmet medical need to quantify the severity or the stage of fibrosis when making decisions regarding diagnoses, and prognoses of CLDs.

The recent development of collagen-targeted contrast agents based on approved Gd-DTPA (Magnevist) appears to image liver fibrosis with improved sensitivity and specificity but these results depend on the animal models evaluated, while safety concerns about Magnevist have prevented the efforts to develop a safe MRI contrast agent capable of detecting early stage fibrosis, NASH and delineating disease heterogeneity. To address this, we reasoned that precision T1 and T2 imaging capability (r1 and r2) of stage-dependent characteristic expression patterns of collagen type I would lead to the detection of early stage of the disease and its heterogeneity associated with late-stage liver fibrosis.

In this study, we report the development of a protein MRI contrast agent (ProCA) based on rat α-parvalbumin expressed in Escherichia coli for molecular imaging of collagen I levels in vivo (ProCA32.collagen1) in three models of CLDs. ProCA32.collagen1 demonstrates high dual relaxivity values for r1 and r2 per Gd3+ at both 1.4 and 7.0 T, as well as a strong collagen-targeting capability. ProCA32.collagen1 enables the first robust detection of early and late-stage liver fibrosis and early stage NASH in addition to heterogeneous expression of collagen by multiple imaging techniques. Furthermore, it provides the first demonstration of possible vascular and architectural alterations caused by intrapapatic angiogenic and portal hypertension during late-stage fibrosis.

**Results**

**Design of ProCA32.collagen1.** Collagen-targeted protein MRI contrast agent, ProCA32.collagen1 was designed by engineering a collagen type I targeting peptide moiety to the C-terminal of protein contrast agent, ProCA32 (with two Gd3+ binding sites) (Fig. 1a). In the modeled structure, lysine residues are positioned as anchor points for polyethylene glycol (PEG) modification. A flexible hinge was used to maximize targeting capacity and relativity, while maintaining metal binding capability. PEGylation was used to improve relaxivities by tuning correlation time. It also increases stability and blood retention time. The designed ProCA32.collagen1 was bacterially expressed, purified, modified, and formulated (Supplementary Fig. 1). Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of the complex indicated the formation a 2:1 Gd-ProCA32.collagen1 complex.

Collagen type I binding affinity of ProCA32.collagen1 was determined using indirect enzyme-linked immunosorbent assay (ELISA) (Fig. 1b). The contrast agent exhibited high affinity to collagen type I with a dissociation constant of Kd = 1.4 ± 0.2 μM and a 1:1 binding model. ProCA32 without the collagen I targeting moiety did not show any specific binding to collagen I. The determined relaxivity values of r1 and r2 for ProCA32.collagen1 were 34.0 ± 0.12 mM−1 s−1 and 200 ± 0.16 mM−1 s−1 per Gd3+, respectively at 37 °C and 1.4 T, or 68.0 ± 0.25 mM−1 s−1 and 100.0 ± 0.32 mM−1 s−1 per particle, respectively (Table 1, Supplementary Fig. 2a, b, c). ProCA32.collagen1 exhibited the highest r1 (21.3 ± 0.5 mM−1 s−1 per Gd3+ or 42.6 ± 1 mM−1 s−1 per particle) and r2 (108.5 ± 12.2 mM−1 s−1 per Gd3+ or 217.0 ± 2.4 mM−1 s−1, per particle) compared to clinical contrast agents, at higher magnetic field strength of 7.0 T and 37 °C. ProCA32.collagen1’s r1 and r2 relaxivity values are 5–10 and 12–17 times greater than those of clinical contrast agents at 1.4 and 7.0 T, respectively. Therefore, this targeted contrast agent can be applied to both low- and high-magnetic field strengths with both T1- and T2-weighted molecular imaging. The water number q in the first coordination shell determined by luminescence resonance energy transfer (LRET) using Tb3+ luminescence life time decay, was 0.5 (Supplementary Fig. 2d and e).
We examined metal binding affinity and selectivity of ProCA32.collagen1. The Gd$^{3+}$ binding affinity of ProCA32.collagen1 was calculated to be $2.0 \pm 0.25 \times 10^{-22}$ M, which is comparable to the approved clinical contrast agents (Table 2 and Supplementary Fig. 3). Since competition of physiological metal ions such as Zn$^{2+}$ and Ca$^{2+}$ is considered to be one of the most important risk factors for Gd$^{3+}$ release in vivo, we then determined metal binding constants for these metal ions. As shown in Table 2, ProCA32.collagen1 exhibited 108–1016-fold greater metal selectivity (kinetic stability) for Gd$^{3+}$ over Ca$^{2+}$ and Zn$^{2+}$ compared with clinically approved contrast agents such as Dotarem.  

Transmetallation studies demonstrated that the relaxation rates of clinical contrast agents gradually decreased upon incubation with 2.5 mM zinc$^{2+}$ (Fig. 1c). In contrast, the relaxation rates of ProCA32.collagen1 remained unchanged suggesting that ProCA32.collagen1 resists transmetallation. Further, ProCA32.collagen1 remained intact without cleavage upon incubation with human serum at 37 °C for 13 days (Supplementary Fig. 4b). Pharmacokinetic properties of the contrast agent were also assessed (Fig. 1d). Moreover, ProCA32.collagen1 clinical chemistry test values were within the normal range, demonstrating normal function of organs including kidney and liver (Supplementary Fig. 5). No tissue damage or major Gd$^{3+}$ accumulation was observed (Supplementary Fig. 6).
Detection of early and late stages liver fibrosis and NASH. We next evaluated whether molecular MRI with ProCA32.collagen1 could unambiguously and quantitatively detect both early and late stage liver fibrosis and NASH in vivo in different mouse models, taking advantage of the high $r_1$ and $r_2$ values (Figs. 2 and 3, Supplementary Fig. 8). Figure 2a shows R1 map MRI collected pre, and 24 h after, intravenous (I.V.) injection of contrast agents (100 μL, 5 mM, 0.02 mmol/kg) of ProCA32.collagen1, ProCA32 (nontargeted), and Eovist in TAA-Alcohol-induced liver fibrosis mouse model. After 24 h post injection, ProCA32.collagen1 demonstrated an increase in R1 to 2.4 ($\Delta R1 \sim 0.78$ s$^{-1}$, an approximate 50% increase in the liver area) in early stage fibrotic liver. This change is nearly doubled with $\Delta R1$ enhancement to 1.40 s$^{-1}$ (a 78% increase) compared to preinjection for late-stage fibrotic liver. The R1 increase at 24 h post injection was highly specific, and gradually decreased after 48 h due to excretion. In contrast, the targeting agent did not result in a significant change in normal liver ($\Delta R1 < 0.2$ s$^{-1}$ and 10% increase in liver area) at 24 h time point. Consistent with the R1 map, T2 map results also exhibited the same pattern at 24 h for both early and late stages of fibrosis, with higher changes for the late-stage fibrotic liver (Fig. 2b, Supplementary Fig. 8a).

ProCA32.collagen1 is also capable of detecting early-stage NASH in nonalcoholic fatty liver in NASH diet-induced mouse model using both R1 and T2 maps (Fig. 3a, b, Supplementary Fig. 8c). At 24 h post-injection of ProCA32.collagen1, liver with NASH exhibited $\Delta R1$ of 1.02 s$^{-1}$ which is 4-fold higher than $\Delta R1$ of 0.25 s$^{-1}$ for normal liver (Fig. 3a). Consistently, $\Delta R2$ determined by T2 map is 11.1 s$^{-1}$ instead of 3.8 s$^{-1}$ of normal liver at the same time point (Fig. 3b, Supplementary Fig. 8c). In contrast, Eovist did not demonstrate significant enhancement in R1 map. In addition, ProCA32 without collagen binding capability did not result in any significant enhancement for R1 map changes at this time point (Fig. 2a).

Histological validation, and biodistribution. Detailed histological analysis and hydroxyproline content (Supplementary Fig. 16) confirmed early stage liver fibrosis (Ishak stage 3 of 6) and early stage NASH (Mild-1A or Ishak stage 1 of 6) based on the Ishak and NASH Clinical Research Network (NASH CRN) scoring systems with the help of a pathologist. Late-stage fibrosis (Ishak stage 5 of 6) was also confirmed by histology analysis for all animal models (Figs. 2f, 3f, g). Late-stage liver fibrosis (Ishak stage 5 of 6) induced by diethylnitrosamine (DEN) was also confirmed by histology analysis. Furthermore, ProCA32.collagen1 distribution in normal and fibrotic liver was well-correlated with the stage of the disease (Figs. 2d, e and 3d, e). During the late stage of liver fibrosis in both NASH diet and TAA/alcoholic models, αSMA levels (brown) were much higher compared to early-stage fibrosis, NASH and normal liver (Figs. 2f and 3f). Sirius red, and H&E staining of early stage NASH compared to normal liver demonstrate the presence of microvesicular and macrovesicular steatosis and collagen, as indications of the disease stage (Fig. 3g).

We have also reported the application of serial molecular imaging (SMI) over time of a targeted contrast agent to reveal additional differences between early and late stages of fibrosis and NASH. The serial change of R1 values can be seen in Fig. 4b, d, where all R1 values in both early stage of the disease and normal liver increased to 2.0–2.4 s$^{-1}$, 3 h post injection of ProCA32.collagen1. However, at 24 h post injection, R1 values of normal liver decreased but fibrotic and livers with NASH showed an increase to 2.4 and 2.8 s$^{-1}$, respectively. These values decreased for both livers as the contrast agent was washed out of the liver 48 h post injection. However, at 3 h post injection of ProCA32.collagen1, R1 of late-stage fibrotic livers for both animal models increased to >3.5–4.1 s$^{-1}$ with $\Delta R1 \geq 2.0$ s$^{-1}$ (Fig. 4a, c). The Increase of R1 at 3 h post-injection for late-stage fibrosis likely suggests the existence of intrahepatic angiogenesis. Histogram analysis further confirms the large difference between early-stage and late-stage liver fibrosis in TAA/Alcohol model at different time points (Supplementary Fig. 14). We have observed significantly increased vessel formation stained by CD31 with quantitative analysis supporting the vessel changes due to intrahepatic angiogenesis in late-stage fibrosis (Fig. 4g).

We further reported the existence of portal hypertension associated with late-stage fibrosis in mouse liver which may justify the slow washout of ProCA32.collagen1 from the liver (Fig. 4e, f, Fig. 6c, d).

Quantitative mapping of collagen heterogeneity. Detection of liver fibrosis heterogeneity can be regarded as one of the major limitations of liver biopsy and other imaging modalities. Collagen heterogeneity induced by DEN was observed with ProCA32.collagen1-enabled SMI (Fig. 5). The DEN-induced mouse model exhibited strong collagen heterogeneity mimicking patient cirrhosis. Figure 5a showed that the right area of the mouse (defined by A2: Area 2) liver has a collagen proportionate area (CPA) of 12% (Sirius red staining) while the left area (A1) has a CPA of 6.7%. All T1-, T2-weighted, and T1-inversion recovery images exhibited time-dependent liver enhancement at 3 and 24 h post injection of ProCA32.collagen1 with the maximum enhancement at 3 h post injection, specifically in A2, confirming the histology data (Fig. 5a, b). R1 maps and its corresponding histogram analysis further confirm collagen heterogeneity (Supplementary Fig. 15).

In order to demonstrate the time-dependent collagen heterogeneity detection by ProCA32.collagen1 and quantitatively assess the extent of enhancement, a color matrix was created (Fig. 5d, e). The first column shows the enhancement 3 h post injection compared to Prescan (called 3 h). The second column shows the
enhancement 24 h post injection compared to Prescan (called 24 h) and third and fourth columns represent the enhancement 24 h post injection compared to 3 h. If the voxels remained enhanced it was called Maintained and if the voxels had decrease in intensity, they were called Washout. The matrix shows a quantitative voxel analysis of MRI images by exhibiting the number of voxels and their percentage of increase in intensity at each time point post injection of ProCA32.collagen1 compared to preinjection which is an indication of the dynamic property and targeting capability of ProCA32.collagen1 based on collagen distribution differences in the liver. In this analysis, the short-T1 inversion recovery with long TE demonstrated the highest sensitivity in terms of number of voxels with ~100% increase in intensity for detecting collagen heterogeneity among other pulse sequences.

Disease staging with ProCA32.collagen1. In order to demonstrate different uptake and washout rates of ProCA32.collagen1 based on the stage of the disease and post injection time point, percentage of R1 and R2 enhancement curves were plotted based on R1 and R2 values from MR mapping. To assess the uptake, time point before injection was used as a reference to calculate an area under the curve (AUC) in the time window 0–48 h (AUC_0–48) for the entire liver in each MRI slice in NASH-diet model (Fig. 6a, b). To assess the washout, 24 h time point was selected, and the corresponding AUC was calculated (Fig. 6c, d). AUCs calculated from ΔR1 and ΔR2 were represented as boxplots for better demonstration of ProCA32.collagen1 ability to distinguish different stages of disease in NASH diet model (Fig. 6e). The difference in AUC_3–48 for normal vs. early, early vs. late and

Fig. 2 Early stage detection of liver fibrosis in TAA/Alcohol model. a R1 maps and ΔR1 values of normal (Ishak stage 0 of 6), early-stage (Ishak stage 3 of 6), and late-stage (Ishak stage 5 of 6) liver fibrosis before and 24 h after injection of ProCA32.collagen1, and ProCA32. b ΔR2 values derived from T2 maps demonstrating the highest enhancement for late-stage liver fibrosis at 24 h post injection. c ProCA32.collagen1 can distinguish early-stage liver fibrosis from normal and late-stage fibrotic liver with ΔR1 derived from a R1 map at 24 h time point. d Correlation between Gd^3+ concentration in liver with Ishak score at 24 h time point. e Percent injection dosage of ProCA32.collagen1 based on [Gd^3+] in fibrotic and normal livers at 24 h time point. f Sirius red, H&E and αSMA staining of early- and late-stage fibrotic liver compared to normal liver confirms the stage of liver fibrosis. scale bar, 100 µm, *P < 0.05, student’s t test, all data are represented as mean ± SD, n = 4 biologically independent animals in each group.
Several scatter-plots (Fig. 6f) showed that AUC_0–48 are positively correlated with CPA measured from histology (Supplementary Fig. 9b) showing the potential of ProCA32.collagen1 in staging NASH and liver fibrosis (Fig. 6f) as a noninvasive technique. The Pearson correlations between LogitCPA and AUC_0–48 were 0.88 (p value = 0.00016) and 0.89 (p value = 0.00013) for ΔR1 and ΔR2, respectively. ProCA32.collagen1 was also capable of distinguishing normal liver from early- and late-stage fibrosis in TAA/alcohol model (Supplementary Fig. 12) based on receiver operating characteristic (ROC) analysis.

Discussion

ProCA32.collagen1 exhibits higher relaxivity properties for both r1 and r2 compared to ProCA32 (Table 1). These increases of r1 and r2 are likely due to effect of PEG modification and targeting moiety on rotational correlation time (τR). ProCA32.collagen1 also exhibits the highest binding affinity to collagen type I (Kd of 1.4 μM) among all other MR imaging agents available3, due to the rational design of the targeting moiety. ProCA32.collagen1 also exhibits in vivo binding to collagen I in mouse tissues compared to ProCA32 without any binding (Supplementary Fig. 11). This strong binding affinity to collagen type I provides required...
**Fig. 4** Detection of portal hypertension during liver cirrhosis in TAA/alcohol- and NASH diet-induced cirrhosis. 

**a** R1 map and ΔR1 values of early-stage (Ishak stage 3 of 6), late-stage (Ishak stage 5 of 6), and normal liver (Ishak stage 0 of 6) before and 3 h after injection of ProCA32.collagen1 and Eovist (30 min) in TAA/alcohol model. **b** R1 changes of liver over different MRI time points after injection of ProCA32.collagen1, ProCA32, and Eovist in TAA/alcohol model (P < 0.05, paired student’s t test). **c** R1 map and ΔR1 values of NASH (Ishak 1 of 6), late-stage (Ishak stage 5 of 6), and normal liver (Ishak stage 0 of 6) before and 3 h after injection of ProCA32.collagen1 and Eovist (30 min) in NASH diet model. **d** R1 changes of liver over different time points after injection of ProCA32.collagen1 and Eovist in NASH diet model (P < 0.05, paired student’s t test). **e** Representative SEM images of sections from mice with late-stage liver fibrosis in TAA/alcohol model. Quantitation of number and size of fenestrations of liver sinusoids in mice with late-stage liver fibrosis measured by manually counting/measuring number and the diameters of fenestration in the SEM images (scale bar, 500 nm). **f** Velocity of portal vein blood flow as measured by Doppler ultrasound imaging shows high-portal hypertension detected at 3 h after injection of ProCA32.collagen1 in late-stage liver fibrosis in TAA/alcohol model. **g** Representative images of IHC stains of CD31 and quantitation of CD31 IHC stains of liver tissue in mice with late-stage liver fibrosis in TAA/alcohol model confirming intrahepatic angiogenesis. scale bar, 100 μm; *P < 0.05, **P < 0.001, unpaired two-tailed student’s t test; all data are represented as mean ± SD, n = 4 biologically independent animals in each group.
Fig. 5 Demonstration of time-dependent mapping of collagen heterogeneity in DEN model. a T1-, T2-weighted, and T1-inversion recovery images of DEN-induced diseased liver before and 3 and 24 h post injection of ProCA32.collagen1 demonstrating collagen heterogeneity of liver in Area 2 (A2). b Sirius red staining and CPA analysis confirmed that A2 has more collagen than A1 which correlates with MRI (scale bar, 100 µm). c T1-, T2-weighted, and T1-inversion recovery images all demonstrated a higher contrast to noise ratio (CNR) in A2 of liver 3 h after ProCA32.collagen1 injection compared to A1. Data are represented as mean ± SD, n = 4 independent images. d Percentage of relative enhancement and quantitative voxel analysis of T1-inversion recovery images demonstrating liver regions enhanced at 3 and 24 h after injection of ProCA32.collagen1 compared to pre-injection (color scale represents number of voxels). e Percentage of relative enhancement and quantitative voxel analysis of T2-weighted images of liver demonstrating regions enhanced at 3 and 24 h after injection of ProCA32.collagen1 compared to pre-injection (color scale represents number of voxels).
sensitivity to detect early stage secretion of collagen in the liver (1–20 nmol g\(^{-1}\))\(^3\). Our developed ProCA32.collagen1 has shown serum stability and no Gd\(^{3+}\) deposition in brain (Supplementary Fig. 6d) that are equivalent to the macrocyclic agents.

Early stage fibrosis and NASH can be reversed if it is detected early\(^3\).\(^4\),\(^9\). However, current techniques including FibroScan and MRE as well as MRI with current clinical contrast agents, failed to detect early stages of NASH and fibrosis\(^3\).\(^4\). We have shown that early-stage alcohol-induced liver fibrosis (Ishak stage 3 of 6) and early stage NASH (Mild-1A, zone 3, perisinusoidal or Ishak stage 1 of 6) can all be detected using both R1 and R2 maps (24 h post injection). The detected MRI signals for both early and late stages of the disease based on number of voxels that had ~100% increase in intensity.

ProCA32.collagen1 also has a high-\(r_2\) value that enables the application of several imaging methodologies which reduces the possibility of detecting artifacts. Because of high \(r_1\) and \(r_2\) short-T1 inversion recovery with long TE of 32.67 ms was used to increase the sensitivity and achieve a high contrast between normal areas of liver and fibrotic regions. Using this pulse sequence, in the first step, liver signal was suppressed by selecting appropriate inversion times and short liver T1 because of high \(r_1\) of contrast agent, then the second step suppression occurs with long TE because of short T2 of liver due to high-\(r_2\) value of ProCA32.collagen1 (Supplementary Fig. 10). The effect of short-T1 inversion recovery with long-TE methodology can also be observed in detecting collagen heterogeneity as it shows higher sensitivity compared to other imaging pulse sequences (Fig. 5d) based on number of voxels that had ~100% increase in intensity.

At 3 h post injection in late-stage fibrosis, ProCA32.collagen1 with SMI exhibits organ distribution and retention time for possible detection of vascular changes due to intrahepatic angiogenesis, and portal hypertension\(^4\), as well as collagen heterogeneity in liver. In addition, pharmacokinetic data (Table 3) suggest the long terminal half-life of ~10 h for ProCA32.collagen1 in blood and higher exposure time (AUC was significantly higher than ProCA32) with volume of distribution (\(V_d\)) of 1.77 L/kg (two-fold higher than that of ProCA32), which are the indications.
of targeting ability of contrast agent and its distribution and penetration in tissues.

The presence of portal hypertension can be observed in scanning electron microscopy (SEM) images and blood flow (Fig. 4e, f). Normal liver sinusoidal endothelial cells (SEC) have fenestrae. However, in late-stage fibrosis, SECs lose fenestrae when activated and express CD31 (Fig. 4e, g). Defenestration, as seen from SEM images, leads to portal hypertension in late-stage fibrosis, as the blood flow out of the liver is significantly reduced (Fig. 4f), therefore, it can be speculated that the increase of R1 and R2 at 3 h post injection of ProCA32.collagen1 and slow washout (Fig. 6c, d) is attributed to portal hypertension.

We have also shown that ProCA32.collagen1 at 3 h post injection, can demonstrate collagen distribution differences under MRI in three different animal models of NASH diet, TAA/alcohol and DEN (Fig. 7a, Supplementary Fig. 7). This information might be essential for understanding the molecular mechanism of fibrosis formation and determining effective treatment as well as image-guided biopsy. In addition, ProCA32.collagen1 is capable of monitoring liver fibrosis regression and treatment in cirrhotic mice generated from TAA/alcohol model (Supplementary Fig. 17). Our data suggest that ProCA32.collagen1 can distinguish pirfenidone-treated liver from normal liver. This capability can be an essential step prerequisite to human clinical trials, to monitor treatment efficacy. Taken together, ProCA32.collagen1 has been shown to have strong human translational potential and application because of its high binding affinity and specificity to collagen in human HCC tissues with cirrhosis (Fig. 7c) and significantly reduced dose because of high relaxivity and the greatest metal selectivity than all other clinically approved Gd^{3+}-based contrast agents. The contrast agent is expected to overcome the major clinical barriers in early diagnosis, noninvasive detection and staging of chronic liver diseases, and have strong translational potential for monitoring treatment efficacy.

Table 3 Summary of pharmacokinetic parameters in mouse for ProCA32.collagen1 and ProCA32

| Contrast Agent | ProCA32 | ProCA32.collagen1 |
|----------------|---------|------------------|
| t_{1/2} (h)   | 8.09    | 9.93             |
| V_c (L/kg)    | 0.20    | 1.53             |
| V_{ss} (L/kg) | 0.76    | 1.77             |
| CL (mL/min/kg)| 0.11    | 0.36             |
| MRT (h)       | 13.90   | 14.51            |
| AUC (ng h/mL blood) | 30773.00 | 140073.00 |

Methods

Protein expression, purification, and lysine PEGylation. In the contrast agent design of ProCA32.collagen1, collagen type I targeting peptide (GGGKKWHCY-TYPPIHYCVYG) for human type I collagen was linked to the C-terminal of ProCA32 by a flexible hinge and surface modification by PEGylation. ProCA32.
collagen1 was expressed in Escherichia coli BL21 (DE3) cell strain and was purified using established procedures.22 Purified ProCA32.collagen1 was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), ultraviolet-visible (UV-vis) spectrometry, and electrospray ionization mass spectrometry. Gd3+ was loaded to ProCA32.collagen1 at a 2:1 ratio. Other metals in ProCA32.collagen1 such as Ca2+ were removed by chelx-100 and the metal content in ProCA32.collagen1 was analyzed by ICP-OES. The protein was PEGylated with methoxy succinimidyl carboxymethyl ester (M-SCM-2000) reagent with molecular weight of 2 kDa (IenKen Technology) and further purified. PEGylated products were analyzed by SDS/PAGE with protein staining by Coomassie Brilliant Blue and PEG staining by I2. The protein absorbance was measured with UV–vis spectrometry by monitoring the Trp signal in the protein. The extinction coefficient of the protein was calculated based on the protein sequence and the final concentration was measured using Beer–Lambert law.

Mice experiments. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee (IACUC) of Georgia State University and the University of Georgia.

TAA/alcohol-induced liver fibrosis and cirrhosis. To induce early stage fibrosis, BALB/c mice (n = 4) were treated with a twice weekly injection of thioacetamide (TAA) (200 mg/kg) and 10% ethanol in their drinking water for 6 weeks. To induce cirrhosis, BALB/c mice (6–7-week-old, n = 4) were treated with twice-weekly i.p. injections of TAA (200 mg/kg) and 10% ethanol in their drinking water for 12 weeks. The body weights were recorded every week or every four days. At the end of the experiments, animals were sacrificed. Livers, other organs, and blood samples were collected. Liver weights were measured, and liver pictures were taken. Tissue sections were prepared and analyzed by IF, IHC, or H&E stains using commercially available antibodies as indicated. Serum samples were prepared from collected blood samples. The serum samples were analyzed through an external company, Comparative Clinical Pathology.

DEN-induced HCC. For all of our studies, we employed female and male mice with C57BL/6 genetic background. Animals were housed in a pathogen-free animal facility under a 12 h light/dark cycle and fed standard rodent chow and water ad libitum. In order to induce HCC, 12-day-old mice were treated with a single dose of DEN (Sigma-Aldrich # N7056) dissolved in saline at a dose of 25 mg/kg body weight by i.p. injection on day 12. Mice in one randomly preassigned group (n = 4) were killed 10 months after DEN administration for histological and biochemical analyses. Mice were randomly distributed in various groups having equal males and females. Immediately after euthanizing, livers were removed, weighed and lobes were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined microscopically.

NASH diet-induced NASH and cirrhosis. Two different groups of mice (n = 4 per group) on NASH diet were chosen for our studies in development of NASH and cirrhosis, in mice with NAFLD. To develop early stage NASH, WT group (n = 4) on NASH diet were chosen for our studies in development of NASH and cirrhosis, and the levels of serum markers in blood circulation were compared with normal mice (Supplementary Fig. 13). The serum levels of markers in blood circulation were compared with normal mice (Supplementary Fig. 13).

Metal-binding affinity studies. In order to investigate the Gd3+ binding affinity of ProCA32.collagen1, a buffer system was used in which Tb3+ binding affinity can be measured since Gd3+ is spectrophotometrically silent. Then the Gd3+–binding affinity was calculated using a competition assay.22,23 Determining Tb3+ binding affinity of ProCA32.collagen1 was based on the Tb3+-LRET experiment in which 30 µM ProCA32.collagen1 was prepared in 5 mM DTPA, 50 mM HEPES, and 150 mM NaCl at pH 7.2. The protein–Tb3+ LRET emission spectra were collected between 520 and 580 nm using an excitation wavelength of 280 nm. The free Tb3+ concentration ([Tb]free) in each titration point were calculated by Eq. (1). The dissociation constant between Tb3+ and ProCA32.collagen1 ([Kd(Tb,ProCA)]) was calculated by the Hill equation (Eq. (2)).

\[
\text{[Tb]}_{\text{free}} = \frac{K_{\text{Tb,DTPA}} \times [\text{Tb} - \text{DTPA}]}{[\text{DTPA}]_{\text{free}}} 
\]

\[
K_{\text{Tb,DTPA}} = \frac{[\text{Tb}]_{\text{free}}}{[\text{Tb} - \text{DTPA}]} \times \frac{[\text{Tb}]_{\text{free}}}{[\text{Tb} - \text{DTPA}]} \]

where [Tb]free is the free Tb3+ concentration calculated from the buffer system, Kd(Tb,DTPA) is the dissociation constant of Tb3+ to DTPA, and the dissociation constants of Tb3+ to DTPA were obtained from National Institute of Standards and Technology Standard Reference Database.44 [Tb–DTPA] is the concentration of Tb–DTPA complex that is formed during titrations, [DTPA]free is the free DTPA in the buffer, f is the fractional change of the LRET signal at each titration point, n is the Hill number, and Kd(Tb, ProCA) is the dissociation constant between Tb3+ and ProCA32.collagen1. A competition assay was performed to measure the binding affinity of ProCA32.collagen1 for Gd3+. The Tb3+ fluorescence spectra were recorded by tryptophan excitation at 280 nm and emission from 500 to 650 nm. In all, 10 µM of ProCA32.collagen1 was used and 20 µM Tb3+ was incubated with different concentrations of GdCl3 from 0 to 1000 µM at room temperature overnight. The Tb3+-FRET changes were measured by the emission at 545 nm. The apparent dissociation constants (Kd(app)) were calculated by fitting the plot of LRET peak intensities over different concentrations of Gd3+. (Eq. (3) and the dissociation constants of Gd3+ to ProCA32.collagen1 (Kd(Gd,ProCA)) were calculated by Eq. (4)).

\[
f = \frac{([\text{Tb}]_{\text{i}} + [\text{Gd}]_{\text{i}} + K_{\text{app}}) - \sqrt{([\text{Tb}]_{\text{i}} + [\text{Gd}]_{\text{i}} + K_{\text{app}})^2 - 4 \times ([\text{Tb}]_{\text{i}} \times [\text{Gd}]_{\text{i}})}}{2 \times ([\text{Tb}]_{\text{i}})} 
\]

\[
K_{\text{d,Gd,ProCA}} = \frac{K_{\text{d,app}} \times K_{\text{d,Tb,DTPA}} \times [\text{Gd}]_{\text{i}}}{[\text{Tb}]_{\text{i}}} 
\]

where f is the fractional change of the LRET signal, [Tb]i is the total Tb3+ concentration, [Gd]i is the total Gd3+ concentration at each titration point, and Kd(Gd,ProCA) is the dissociation constant between Gd3+ and ProCA32.collagen1 determined by Eq. (2).

The Kd(app) for ProCA32.collagen1 was calculated first and then this value was used to calculate the actual dissociation constant using the Eq. (4).

For determining the calcium binding affinity of ProCA32.collagen1, 10 µM of ProCA32.collagen1 was added to the calcium–EGTA buffer system containing 50 mM HEPES, 150 mM NaCl, and 5 mM EGTA at pH 7.2. The system was titrated with different concentrations of CaCl2, and the binding constant of the Ca-EGTA ([Ca-EGTA]) and free EGTA ([EGTA]free) were monitored. The tryptophan (Trp) fluorescence changes were monitored under the emission spectra between 300 and 390 nm as excited at 280 nm. The free calcium concentration at each titration point was calculated by Eq. (5).

\[
[\text{Ca}]_{\text{free}} = \frac{[\text{Ca} - \text{EGTA}]}{[\text{Tb}]_{\text{i}}} 
\]

where f is the fractional change of Trp fluorescence intensity, and [Ca]free is the free Ca2+ concentration in each titration point determined by Eq. (5).

The zinc-binding affinity of ProCA32.collagen1 was determined using a similar assay by competition titration using Fluoizin dye54.55 The fluorescence of 2 µM Fluozin-1 was excited at 495 nm and the emission spectra were collected between 500 and 600 nm in the presence of 2 µM Zn2+ and different concentrations of ProCA32.collagen1. The apparent dissociation constant (Kd(app)) was calculated by Eq. (7).

\[
f = \frac{[\text{Zn}]_{\text{i}} + [\text{ProCA}]_{\text{i}} + K_{\text{app}}}{[\text{Zn}]_{\text{i}} + [\text{ProCA}]_{\text{i}} + K_{\text{app}}} \cdot \frac{[\text{Fluozin}]_{\text{free}}}{[\text{Fluozin}]_{\text{free}}} 
\]

where [Zn]i, [ProCA]i, and [Fluozin]i are the free Zn2+, free ProCA, and free Fluozin concentrations, respectively, and [Fluozin]free is the total Fluozin-1 concentration. Measurement of water coordination number. To determine the number of coordination water molecules in the inner sphere of Gd3+–ProCA32.collagen1, the dissociation constant between Tb3+ and ProCA32.collagen1, which include western water d.t. (D120798) and fructose in drinking water (42 g/L) starting from week old for 6 months. Fructose in drinking water was autoclaved and changed twice a week. They were on western diet all the time. To develop cirrhosis, Liver-Specific Comparative Gene Identification-58 (CGI-58) knocked out (LivKO) mice were treated with the same exact procedure, and the levels of markers in blood circulation were compared with normal mice (Supplementary Fig. 13). The serum levels of markers in blood circulation were compared with normal mice (Supplementary Fig. 13).
**Serum and transplantation studies.** For serum stability experiments, human serum samples were incubated with the ProCA32.collagen1 antibody (1:2 ratio, polyclonal rabbit-anti-fibrotic and normal liver tissues using Anti-alpha smooth muscle Actin antibody (Catalog # ab8287) with concentration of 0.1-0.5 µg/mL. Heat mediated antigen retrieval was performed with citrate buffer pH = 6 before using the standard IHC staining protocol. An experienced pathologist was blinded to the groups of H&E staining. Liver sections were cut (4 μm) and mounted on slides (Probe On; Fisher Scientific). The sections were dried, deparaffinized, and hydrated with xylene and graded ethanol.

**Histology analysis.** For immunohistochemistry, paraffin-embedded liver sections were cut (4 μm) and mounted on slides (Probe On; Fisher Scientific). The sections were dried, deparaffinized, and hydrated with xylene and graded ethanol. Formalin-fixed samples were then stained with Sirius Red according to standard procedures. Sirius Red stained slides were analyzed by a pathologist, at Emory University School of Medicine, to score the amount of liver disease based on Ishak scoring system\(^1\). For H&E Staining, liver histological analysis was performed. The H&E-stained sections were examined using a light microscope (Olympus BX51, Tokyo, Japan) and digital images were captured using a camera (Hamamatsu, Japan). Immuno-fluorescence staining was performed on paraffin sections of mouse liver tissues using Anti-alpha smooth muscle Actin antibody (Catalog # ab8287) with concentration of 0.1-0.5 µg/mL. Heat mediated antigen retrieval was performed with citrate buffer pH = 6 before using the standard IHC staining protocol. An experienced pathologist was blinded to the groups of H&E staining to evaluate the organ toxicity of ProCA32.collagen1. For CD31 staining, liver sections were cut (4 μm) and mounted on slides (Probe On; Fisher Scientific). The sections were dried, deparaffinized, and hydrated with xylene and graded ethanol. Formalin-fixed samples were then stained with Sirius Red according to standard procedures. Sirius Red stained slides were analyzed by a pathologist, at Emory University School of Medicine, to score the amount of liver disease based on Ishak scoring system\(^1\). For H&E Staining, liver histological analysis was performed. The H&E-stained sections were examined using a light microscope (Olympus BX51, Tokyo, Japan) and digital images were captured using a camera (Hamamatsu, Japan).

**Immunofluorescence imaging.** Immunofluorescence staining was performed on fibrotic tissues collected from the mice livers post injection of ProCA32 and ProCA32.collagen1 at 24 h time point. Cryosections of fibrotic liver tissues were cut at a thickness of 5 μm. The tissues were fixed with 4% (vol/vol) formaldehyde for 10 min at room temperature and then washed three times with Tris-buffered saline with Tween 20 (TBST). Tissues were then blocked at room temperature for 1 h with 1% bovine serum albumin (BSA) in TBST and then incubated with two primary antibodies for 1.5 h. A ProCA32.collagen1 antibody rabbit antibody prepared by our lab was used as a primary antibody for the contrast agent at a 1:100 dilution. Another primary antibody for collagen type I, Anti-Collagen I antibody [COL11] (ab34710) was used with a 1:500 dilution. After washing three times with TBST, the slides were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen; catalog #A-110012) as a secondary antibody for collagen type 1 with a 1:200 dilution. Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen; catalog #A-11012) was used as a secondary antibody for ProCA32.collagen1 (1:200 dilution). The nuclei of the cells were stained by DAPI (blue, ThermoFisher scientific, catalog #62248, 1:1000 dilution).

**Determination of r1 and r2 and ΔR1 and ΔR2 analysis.** The ability of a contrast agent to change a relaxation rate is determined by either $\frac{\Delta r}{C_1}$ or $r_2$, where the subscript refers to either the longitudinal (1/T1) or the transverse (1/T2) relaxation time. Relaxivity is simply the change in relaxation rate after the introduction of the contrast agent. The relativity of enhancement vs time points. In this matrix, 12 bins were created, where the initial bin was the voxels with values of less than 10% change and the last bin included voxels with more than 100% change. Then the voxels within each bin were counted. As previously noted, this matrix represented positive values or changes for 3 and 24 h, maintained and negative washout values. The relaxivities of protein (r1 and r2) were determined using different concentrations of GdCl3 and protein (2:1) at 37 °C with 1.4 T Bruker Minispec NMR spectrometer. The relaxivities were calculated as $\frac{\Delta r}{C_1}$ or $r_2$, where the subscript refers to either the longitudinal (1/T1) or the transverse (1/T2) relaxation time. Relaxivity is simply the change in relaxation rate after the introduction of the contrast agent ($\Delta r_1/T_1$) normalized to the concentration of contrast agent or metal ion (M) as in Eq. (9).

$$r_1 \equiv \frac{\Delta (1/T_1) }{M}$$

In addition, the change in liver longitudinal relaxation rate ΔR1 defined as $R_1^{\text{post}} - R_1^{\text{pre}}$

**Enzyme-linked immunosorbent assay.** Collagen type 1 solution from rat tail (Sigma-Aldrich) was coated in 96-well plates at 4 °C overnight. After being blocked with 5% BSA, a series of different concentrations of ProCA32.collagen1 were incubated with the collagen type 1 solution. After incubation at 4 °C overnight, the...
unbounded ProCA32.collagen1 was washed away with 1× TBST. The homemade polyclonal rabbit-anti-ProCA32 antibody was applied with 1: 1000 dilution as the primary antibody against protein contrast agent. A stabilized goat-anti-rabbit HRP-conjugated antibody (Pierce) was used as the secondary antibody (1:200). After a robust wash with 1× TBST, the remaining secondary antibody in the 96 well plate was visualized using 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific). The absorbance intensity was detected by the FLUOstar OPTIMA plate reader at an absorbance wavelength of 450 nm.

Organ distribution analysis. Different organ tissue samples from mice were collected after euthanasia. The collected tissues (~0.2 g) were digested with 70% (wt/vol) ICP-grade HNO3 at 40 °C overnight. The HNO3 solution containing the digested tissues were collected the next day, filtered, and diluted with 2% (wt/vol) HNO3 to 1×, and then each sample containing different organ was analyzed by ICP-OES to measure the Gd3+ concentration at wavelength of 342.246 nm. YCl2 at 2 ppm was used as internal reference and different Gd3+ solutions with different concentrations ranging from 5 to 1000 ppb were used as reference.

AUC analysis. Percentage of R1 and R2 enhancement for each post-injection time point coming from R1 and R2 values in MR mapping was calculated as: % enhancement = (postinjection signal – preinjection baseline signal) / preinjection baseline signal × 100. First, the percentage of R1 and R2 enhancement curves were plotted and then the areas under the entire curve (AUC_0–48) were calculated. AUC values were calculated for each mouse from normal, early and late stage imaging intravenous injection of ProCA32.collagen1, and ProCA32 in mice (Fig. 6a, b). We also calculated the percentage of R1 and R2 enhancement curve in the time window 3–48 h, using the 3 h data point as a reference for each subject. Logit CPA is the log transformation of CPA values and can be calculated as LogitCPA = ln((CPA/100)/(1−CPA/100)) (Fig. 6f). Statistical significance was tested by unpaired two-tailed Student’s t test (Excel), with p < 0.05 as significant. ROC was calculated using SAS 9.4 (SAS Institute) and R 3.2.2 software.

CNR, ΔR1 and ΔR2 calculations. All CNR, R1, ΔR1, and ΔR2 values of all plots represent mean values across multiple MRI slices in each group. The total animal numbers in each group are denoted in each figure legend. Data were statistically analyzed by comparing different groups. The p values were calculated using unpaired two-tailed Student t test and have been denoted in figure legends. Statistical analysis was performed in GraphPad Prism 5 (GraphPad Software). The mouse group sizes were set to support statistically valid data and to minimize the use of the animal. Contrast to noise ratio (CNR) in the liver was calculated by choosing a region of interest (ROI) within the liver, estimating the mean intensity within ROI, and then subtracting it by the mean intensity of muscle. Then this value was divided by the standard deviation of a region outside the mouse torso.

Pharmacokinetic studies. Composite plasma concentration-time profiles following intravenous injection of ProCA32.collagen1, and ProCA32 in mice (n = 6) were analyzed using noncompartmental analysis tool of PhoenixWinNonlin software (Version 6.2). Gd3+ concentration in serum collected after injection of ProCA32.collagen1 at different time points was measured by ICP-OES at 342 nm up to 7 days. After injection, the Gd3+ concentration in serum decreased until it was below the detection limit of ICP-OES. The area under the concentration-time curve (AUC last and AUC inf) was calculated by linear trapezoidal rule. Values of clearance and volume of distribution were estimated values. The elimination rate constant value (kω) was obtained by linear regression of the log-linear terminal phase of the concentration–time profile using at least 3 nonzero declining concentrations in terminal phase with a correlation coefficient of >0.8. The terminal half-life value (t1/2) was calculated using the Eq. (11)

\[ t_{1/2} = \frac{0.693}{k_\omega} \]  

Measurement of hydroxypyroline content in liver tissues. In the Hydroxyproline Assay Kit (Sigma-Aldrich, St. Louis, MO), hydroxyproline concentration was determined by the reaction of oxidized hydroxyproline with 4-(dimethylamino) benzaldehyde (DMAB), which results in a colorimetric (560 nm) product, proportional to the hydroxyproline present. Hydroxyproline standards for colorimetric detection were prepared as follows: First, 10 µL of the 1 mg/mL hydroxyproline standard solution was diluted with 90 µL of water to prepare a 0.1 mg/mL standard solution. Then, 0, 2, 4, 6, 8, and 10 µL of the 0.1 mg/mL hydroxyproline standard solution was added into a 96-well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 µg/well standards. For sample preparation, 10 µg of liver tissue was homogenized in 100 µL of water and then transferred to a pressure-tight polypropylene vial with PTFE-lined cap. Then, 100 µL of concentrated hydrochloric acid (HCl, –12 M) was added, to hydrolyze at 120 °C for 3 h. Solutions were mixed and centrifuged at 10,000 × g for 3 min. About 10–50 µL of supernatant was transferred to a 96-well plate. All wells were evaporated to dryness under vacuum. The assay reagents were prepared as follows: Chloramine T/oxidation buffer mixture – 100 µL is required for each reaction well. For each well, 6 µL of Chloramine T concentrate was added to 94 µL of oxidation buffer and mixed well. Diluted DMAB reagent—100 µL is required for each reaction well. For each well, 50 µL of DMAB was added and concentrated to 50 µL of perchloric acid/isopropanol solution and mixed well. The Chloramne T/oxidation buffer mixture was added to each sample and standard well and incubated at room temperature for 5 min. Totally, 100 µL of the Diluted DMAB reagent was added to each sample and standard well and incubated for 90 min at 60 °C, and finally the absorbance was measured at 560 nm (A560).

Data availability
All data used for this paper are available from the authors upon request.

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Author contributions

M.Salarian, and J.I.Y. designed the research and experiments; R.C.T., M. Salarian, Y.Z., J.I.Y., and M. Sharma created animal models; M. Salarian, S.X., K.H., and M.N. analyzed the MRI data; A.B.F., L.Y., and Z.R.L. reviewed the histopathology and provided critical suggestions for animal models; S.K. provided the critical suggestions regarding MR imaging and data analysis; S.T. helped with the ELISA experiment and data analysis; M. Salarian and O.Y.I. purified the protein; P.M. provided the clinical contrast agent and suggestion for MR data analysis; Y.H., G.Q., and X.M. provided the suggestions and performed the statistical analysis; R.M. helped with the pharmacokinetic data analysis and M. Salarian. and J.I.Y. wrote the paper.

Additional information

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