Chemokine receptor 4 targeted protein MRI contrast agent for early detection of liver metastases

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INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. Approximately 50% of UM patients will develop metastases (1). About 93% of UM metastases occur in the liver, which results in death in almost all cases due to the lack of effective treatments (2). Through histological analysis of postmortem patient samples, UM liver metastases can be classified into three stages based on size (i.e., diameter): stage 1 (≤50 μm in diameter), stage 2 (51 to 500 μm in diameter), or stage 3 (>500 μm in diameter) (3). Pathologically, UM hepatic metastases primarily have two growth patterns: infiltrative or nodular. The infiltrative pattern occurs when circulating metastatic UM cells lodge in the sinusoidal space and eventually replace the hepatic lobule. The nodular pattern metastases, however, originate in the perportal area. UM cells co-opt the portal vein, and when the tumor grows, it exhibits angiogenesis and effaces the adjacent hepatocytes (4).

There are major barriers facing clinicians in UM management, such as the lack of noninvasive and sensitive imaging methods for metastases, and the resistance of UM to traditional systemic chemotherapies (5, 6). Contrast-enhanced computed tomography (CT) is a widely used modality for screening of hepatic metastases (7); however, this method is not optimal for liver lesion characterization (8). 2-¹⁸F-fluoro-2-deoxy-D-glucose (¹⁸F-FDG) positron emission tomography/CT (PET/CT) not only can locate the “hotspot” for characterization of liver metastases but also has disadvantages due to the use of radiation dosimetry and the comparatively low specificity of the technology (9).

Magnetic resonance imaging (MRI) is the preferred clinical imaging modality for the assessment and characterization of liver malignancy because it does not use ionizing radiation and has high soft tissue penetration providing morphological, anatomical, and functional information. Dynamic-enhanced MRI, with liver-specific contrast agents, is widely used for liver lesion characterization, although its sensitivity and specificity are low for lesions less than 1 cm (10). In addition, MRI with the administration of clinically approved contrast agents can not differentiate the different growth patterns of UM metastases in the liver (11). Previous studies have demonstrated that molecular imaging of corresponding biomarker expression, such as HER2, improves detection sensitivity for cancers (12), but to date, diagnostic biomarkers for imaging UM liver metastases have not yet been established. Therefore, there is a pressing unmet medical need to develop MRI contrast agents for early detection and follow-up of liver metastases, especially for high-risk patients.

CXCR4 (chemokine receptor 4) plays a key role in cell migration and metastatic dissemination to several organs such as the liver, bone marrow, and lung, as these organs have intrinsically high concentrations of its natural ligand CXCL12 (Fig. 1A) (13–15). A CXCR4 antagonist, plerixafor (Mozobil, AMD3100), has been approved by the U.S. Food and Drug Administration (FDA) for stem cell mobilization to the peripheral blood for autologous transplantation (16). CXCR4 expression has been proposed as a prognostic factor and a potential therapeutic target. Elevated expression of CXCR4 has been reported in several UM cell line studies (17, 18). Blockage of CXCR4 gene expression by transfection with CXCR4 small interfering RNA (siRNA) has been found to inhibit invasive properties of UM cells exposed to soluble factors produced by human livers (14). On the basis of these data, we hypothesized that CXCR4 would be a potential biomarker with treatment implications for imaging UM metastases in the liver.

In this study, we confirmed and validated that CXCR4 is a diagnostic imaging biomarker by its elevated expression in liver metastases in
three different systems: ex vivo using samples of UM patients, in vitro UM cell lines, and in vivo mouse models. In addition, we have successfully designed a CXCR4-targeted, protein-based contrast agent, ProCA32.CXCR4, which can detect UM hepatic metastases as small as 0.1 mm$^3$. The detected liver micrometastases were further validated by histological analysis, which correlated with MRI results. Our results indicated that ProCA32.CXCR4 enables precision MRI capable of defining molecular signatures for identifying metastases.

### RESULTS

**CXCR4 is highly expressed in UM liver metastases**

To validate CXCR4 as a biomarker for imaging UM metastases, we determined CXCR4 expression in multiple systems, including six UM cell lines, UM patient–derived tissue, as well as a metastatic UM mouse model. Flow cytometry analyses of six UM cell lines revealed that CXCR4 is expressed across different UM cell lines. Among these, Mel290 and M20-09-196 cell lines exhibited more than 80% CXCR4 immunopositivity (Fig. 1B). Immunohistochemical (IHC) analysis of CXCR4 in UM patient liver tissue revealed that CXCR4 is highly expressed in liver metastases with both nodular and infiltrative growth patterns (Fig. 1C). We further observed elevated CXCR4 expression in primary ocular tumor and liver metastases in the metastatic UM mouse model generated by inoculation of M20-09-196 cells (Fig. 1, D and E), which have the BAP1 gene mutation that is often observed in aggressive UM liver metastases (19). In these M20-09-196 mice, the CXCR4 immunoreactive score (IRS) of UM metastases in the liver was significantly higher than in primary UM ($P < 0.05$, Fig. 1F). Together, these data indicated that CXCR4 expression is increased in UM metastases in the liver and may be a potential biomarker for diagnostic imaging of UM metastases.

**Design of the CXCR4-targeted protein contrast agent ProCA32.CXCR4 and in vitro validation of CXCR4 binding**

Figure 2A presents the design of ProCA32.CXCR4 and the interaction of ProCA32.CXCR4 with CXCR4. ProCA32.CXCR4 was generated by engineering a CXCR4-targeting moiety into a protein contrast agent, ProCA32, which incorporates two designed gadolinium ($\text{Gd}^{3+}$) binding sites (20). The viral chemokine analog viral macrophage inflammatory protein-II (vMIP-II) is encoded by the human herpes virus 8 and interacts with CXCR4. On the basis of the complex x-ray structure of CXCR4 and vMIP-II, we designed the CXCR4 targeting moiety, including key CXCR4 interaction residues from vMIP-II that reach into the binding pocket and interact with key residues D262, D97, S285, and E288 of CXCR4 in both chemokine recognition sites 1 and 2 (21). ProCA32.CXCR4 was bacterially expressed and purified following our previously reported protocol (20). ProCA32.CXCR4 is also composed of lysine or cysteine residues, which allow post-expression PEGylation (22). PEGylation was verified by Coomassie brilliant blue staining and iodine staining (fig. S1). The CXCR4-targeting capability of ProCA32.CXCR4 was verified and quantified by immunofluorescence staining and enzyme-linked immunosorbent assay (ELISA). We determined the dissociation constant ($K_d$) of ProCA32.CXCR4-CXCR4 interaction using indirect ELISA (Fig. 2B). Nontargeted ProCA32 was used as a negative control. The binding curve indicated a 1:1 binding stoichiometry, and the determined $K_d$ value was 1.10 ± 0.18 μM. The CXCR4 receptor number per Mel290 cell was 1.2 ± 0.1 × 10$^6$. Immunofluorescence

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**Fig. 1. CXCR4 expression is up-regulated in UM cell lines, hepatic metastases in UM patients, and metastatic UM mice.** (A) Tumor cells that express CXCR4 metastasize through CXCR4–CXCL12 interaction to specific organs that have intrinsically high concentrations of CXCL12 such as the lung, liver, and bone. (B) UM cell lines have elevated CXCR4 expression. Flow cytometry results measured elevated CXCR4 expression across different UM cell lines. Mel290 and M20-09-196 measured more than 80% of CXCR4 immunopositivity. Measurements of each cell line were done in triplicate. (C) CXCR4 IHC staining in liver tissue from metastatic UM patients ($n = 4$, IRS = 8.2 ± 1.3). The liver metastases displayed strong red intensity, denoting strong CXCR4 expression. (D and E) CXCR4 IHC staining of primary UM (D) and hepatic metastases (E) in metastatic UM mice. UM hepatic metastases have higher CXCR4 expression compared with primary UM, indicated by the red staining. (F) CXCR4 IRS of primary UM and metastases in the liver in metastatic UM mice. Hepatic UM metastases displayed stronger CXCR4 expression (IRS = 9.5 ± 0.8) than primary UM (IRS = 5.4 ± 0.3). $P \leq 0.05$. 

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staining of ProCA32.CXCR4 after incubating with the CXCR4-expressing cell line Mel290 confirmed that ProCA32.CXCR4 binds to CXCR4 through residue-residue and electrostatic interactions. ProCA32.CXCR4 has two Gd\(^{3+}\) binding sites. (B) CXCR4 targeting study of ProCA32. CXCR4 by ELISA. The dissociation constant of ProCA32.CXCR4 binding to CXCR4 was calculated as 1.10 ± 0.18 \(\mu\)M, measured by indirect ELISA. \(n = 3\). The nontargeted contrast agent ProCA32 did not exhibit CXCR4 targeting capability. (C) Fluorescence staining of Mel290 cells to study the CXCR4 binding effect of ProCA32.CXCR4. Blue fluorescence is nucleus staining with 4′,6-diamidino-2-phenylindole (DAPI), green color is fluorescein-labeled ProCA32.CXCR4, red color indicates CXCR4 staining, and composite is the combination of nucleus, CXCR4, and ProCA32.CXCR4 staining. ProCA32.CXCR4 exhibited good spatial colocalization with CXCR4; Pearson’s \(r\) is 0.82. (D) Working flow of ProCA32.CXCR4. ProCA32.CXCR4 was administered through tail vein injection and distributed with blood flow, and specific targeting to CXCR4 high expression metastatic UM (indicated by black cells) was shown over time.

**Fig. 2. ProCA32.CXCR4 binds to CXCR4.** (A) Model structure of ProCA32.CXCR4 interacting with CXCR4 [Protein Data Bank (PDB): 4RWS] through targeting moiety. ProCA32.CXCR4 was constructed by engineering the CXCR4 targeting moiety (red) to ProCA32 (blue) by a flexible linker (green). The targeting moiety of ProCA32.CXCR4 binds to CXCR4 through residue-residue and electrostatic interactions. ProCA32.CXCR4 has two Gd\(^{3+}\) binding sites. (B) CXCR4 targeting study of ProCA32. CXCR4 by ELISA. The dissociation constant of ProCA32.CXCR4 binding to CXCR4 was calculated as 1.10 ± 0.18 \(\mu\)M, measured by indirect ELISA. \(n = 3\). The nontargeted contrast agent ProCA32 did not exhibit CXCR4 targeting capability. (C) Fluorescence staining of Mel290 cells to study the CXCR4 binding effect of ProCA32.CXCR4. Blue fluorescence is nucleus staining with 4′,6-diamidino-2-phenylindole (DAPI), green color is fluorescein-labeled ProCA32.CXCR4, red color indicates CXCR4 staining, and composite is the combination of nucleus, CXCR4, and ProCA32.CXCR4 staining. ProCA32.CXCR4 exhibited good spatial colocalization with CXCR4; Pearson’s \(r\) is 0.82. (D) Working flow of ProCA32.CXCR4. ProCA32.CXCR4 was administered through tail vein injection and distributed with blood flow, and specific targeting to CXCR4 high expression metastatic UM (indicated by black cells) was shown over time.

**Improved \(r_1\) and \(r_2\) relaxivities of ProCA32.CXCR4**

The \(r_1\) and \(r_2\) values per Gd\(^{3+}\) for ProCA32.CXCR4 were 30.9 mM\(^{-1}\) s\(^{-1}\) and 43.2 mM\(^{-1}\) s\(^{-1}\), respectively, at 1.5 T (Fig. 3A and fig. S2A, relaxivity reported on the basis of “per Gd\(^{3+}\)” value). Both \(r_1\) and \(r_2\) relaxivity values were 8 to 10 times greater than the clinically approved Gd\(^{3+}\)-based contrast agents (GBCAs) (Fig. 3A and table S1). ProCA32. CXCR4 also exhibited good relaxivities at higher magnetic field of 7.0 T (fig. S2B). The \(r_1\) and \(r_2\) relaxivity values of non-PEGylated ProCA32.CXCR4 were 23.5 and 98.6 mM\(^{-1}\) s\(^{-1}\), respectively. The relaxivities of the non-PEGylated form of ProCA32.CXCR4, lysine-PEGylated ProCA32.CXCR4 (Lys-ProCA32.CXCR4), and cysteine-PEGylated ProCA32.CXCR4 (Cys-ProCA32.CXCR4) did not exhibit significant differences. The \(r_1\) and \(r_2\) relaxivities of ProCA32.CXCR4 were largely retained after PEGylation. Overall, ProCA32.CXCR4 exhibited improved \(r_1\) and \(r_2\) relaxivities when compared with clinical GBCA at both 1.5 and 7.0 T.

**ProCA32.CXCR4 has uniquely high metal selectivity against transmetalation and metal toxicity**

Gd\(^{3+}\)-related toxicity, such as the development of nephrogenic systemic fibrosis (NSF) in patients with chronic kidney disease and brain deposition of GBCA, is largely attributed to the kinetic and thermodynamic stability of GBCA (23). ProCA32.CXCR4 is stable up to 14 days when incubated with serum at 37°C (fig. S3A). The transmetalation
ProCA32.CXCR4 enables early detection of stage 2 nodular growth pattern UM metastases in the liver

We demonstrated the unique imaging capability of ProCA32.CXCR4 for detection of liver metastases, improving the current detection limit and enabling nodular pattern detection in metastatic M20-09-196 mice at 7.0 T. The early detection of UM metastases in the liver of M20-09-196 mice can be achieved using either Cys-ProCA32.CXCR4 or Lys-ProCA32.CXCR4. Liver micrometastases ranging from 0.01 to 0.08 mm³ were detected with spin echo acquisition and fast spin echo acquisition following tail vein injection of Cys-ProCA32.CXCR4 (0.025 mmol/kg) (Fig. 4A). Enhancement of UM metastases was not detected by MRI following administration of Eovist or Lys-ProCA32 without the targeting moiety (Fig. S5A). These results demonstrate the sensitivity and specificity of our system. These small liver lesions, detected by MRI with Cys-ProCA32.CXCR4, were further verified by detailed hematoxylin and eosin (H&E) staining analysis and found to be exclusively nodular growth pattern type (labeled by the yellow, blue, green, and red circles) (Fig. 4B). The interlesion distances and diameters of lesions on MRI correlated well with the corresponding measurements in H&E staining of tissue sections (\(y = 1.09x + 0.08\)) (Fig. 4C). A statistical analysis indicated that Cys-ProCA32.CXCR4 provides diagnostic validation for UM metastases in the liver. AUC = 0.84; \(P < 0.0001\). Three mice were used for the experiment. Analyses were based on 11 metastases found on MR images.

Fig. 3. Relaxivity (reported as “per Gd” value), transmetalation, and metal selectivity studies of ProCA32.CXCR4. (A) Relaxivity assessment of ProCA32.CXCR4 and GBCA with 60-MHz relaxometer; ProCA32.CXCR4 has 8 to 10 times higher \(r_1\) and \(r_2\) values than clinical GBCA. (B) Transmetalation study of ProCA32.CXCR4 and other GBCA in the presence of Zn\(^{2+}\). Thermodynamic index \([K] = 16.1\) (Fig. 3C and fig. S4C), which was 10\(^{11}\) to 10\(^{12}\) orders of magnitude higher than small chelator contrast agents. For other physiological metal ions such as Ca\(^{2+}\), ProCA32.CXCR4 also exhibited better metal selectivity for Gd\(^{3+}\) over Zn\(^{2+}\) \([\log (K_{Gd/Zn}) = 16.1]\) (Fig. 3C and fig. S4C). (C) Metal (Zn\(^{2+}\), Ca\(^{2+}\), Gd\(^{3+}\), and Tb\(^{3+}\)) binding affinity and metal selectivity values of ProCA32.CXCR4 in comparison with clinical contrast agents. N/A, not available; PEG, polyethylene glycol.

Fig. 4. MRI images of metastatic UM mice M20-09-196 and histological correlation. (A) \(T_1\)-weighted spin echo and \(T_2\)-weighted fast spin echo MR images of M20-09-196 before and 48 hours after Cys-ProCA32.CXCR4 injection. At 48 hours after injection, both \(T_1\) and \(T_2\)-weighted MR images revealed four lesions not observed before injection. The zoom-in view of the yellow rectangular region shows both gray and color scales. (B) H&E and IHC staining of M20-09-196 liver with UM metastases. H&E staining revealed four metastatic lesions, highlighted by different color circles, with similar locations as the metastases in MRI images. Higher-magnification images identified the growth pattern of metastases to be nodular pattern. S100 IHC labeling confirmed that the lesions were metastatic UM. CXCR4 immunohistological staining confirmed the CXCR4 expression on UM metastases. (C) The measurement of distances between metastases and the diameter of metastases in MRI images correlates with the H&E histological staining (\(y = 1.09x + 0.08\)). (D) Statistical analysis indicated that Cys-ProCA32.CXCR4 provides diagnostic validation for UM metastases in the liver. AUC = 0.84; \(P < 0.0001\). Three mice were used for the experiment. Analyses were based on 11 metastases found on MR images. a.u., arbitrary units.
Molecular MDCI and tumor permeability of ProCA32.CXCR4

We further evaluated and validated in vivo the molecular imaging capability of ProCA32.CXCR4 at 4.7 T by generating a liver-implanted UM murine model by inoculation of the Mel290 UM cell line. Molecular dynamic contrast imaging (MDCI) was performed to display implanted UM tumor in mouse liver by administration of Cys-ProCA32.CXCR4 via intravenous injection, followed by the acquisition of T1-weighted gradient echo MRI as a function of time. The nontargeted contrast agent Lys-ProCA32 was used as a control. The tumor regions exhibited different enhancement patterns between mice with Lys-ProCA32 and Cys-ProCA32.CXCR4 injection. In implanted Mel290 mice with Lys-ProCA32 injection, the tumor MRI signal intensity increased at 12 and 50 min after injection and decreased 3 hours after injection (Fig. 5A). However, the tumor MRI signal intensity in the Mel290 mice with Cys-ProCA32.CXCR4 injection gradually increased to the maximum at 22 hours after injection and then began to decrease due to excretion (Fig. 5A). The time plot of UM tumor signal-to-noise ratio (SNR) change followed by Cys-ProCA32.CXCR4 injection showed that UM tumor SNR increased more than 40% at 22 hours after injection when compared with before injection, whereas SNR of tumor region in the Mel290 mice with Lys-ProCA32 injection showed a mild increase (10%) immediately after injection (12 min) and then washed out at 3 hours after injection (Fig. 5B). On the other hand, MRI results of Mel290 mice with Cys-ProCA32.CXCR4 and Lys-ProCA32 injection exhibited similar patterns of SNR changes in the liver regions over time (Fig. 5C). The liver SNR of both mice with Cys-ProCA32.CXCR4 and Lys-ProCA32 injection increased drastically right after injection and up to 3 hours, with a percentage increase of SNR of approximately 45% at 3 hours after injection when compared with before injection. This enhancement of the liver region gradually decreased due to elimination. Cys-ProCA32.CXCR4 was observed to target and distribute across the tumor tissue in Mel290 mice. The MRI of tumor regions in Mel290 mice following Cys-ProCA32.CXCR4 injection revealed enhancement of the tumor rim immediately after injection and rapid penetration into the center (Fig. 5, A and D). The immunofluorescence staining of the administered Cys-ProCA32.CXCR4 into Mel290 murine tumor tissue exhibited intense and broadly distributed red immunofluorescence labeling (Fig. 5E). In contrast, red fluorescence staining was not observed with the tumor tissues of the Mel290 mice receiving the Lys-ProCA32 injection. Gd3+ content analysis using inductively coupled plasma optical emission spectrometry (ICP-OES) indicated that the tumor tissue of Mel290 mice receiving the Cys-ProCA32.CXCR4 injection exhibited significantly higher Gd3+ content than tumor tissue of Mel290 mice with the Lys-ProCA32 injection (Fig. 5F). These results further validated the CXCR4-targeting capability of ProCA32.CXCR4 in vivo with good tumor permeability.

Validation of the in vivo CXCR4 targeting capability of ProCA32.CXCR4 by receptor blocking experiment

We validated the in vivo CXCR4 targeting capability of Cys-ProCA32.CXCR4 by receptor blocking experiment. A subcutaneous UM murine model was developed to demonstrate that UM tumor signal intensity enhancement following Cys-ProCA32.CXCR4 administration could be blocked by first administering the CXCR4 blocking reagent (Fig. 6A). We specifically constructed a CXCR4 blocking reagent by fusing the CXCR4-targeting moiety (LGASWHRPDKPCGLYQKRPLP) of ProCA32.CXCR4 to the C terminus of glutathione S-transferase (GST) tag to ensure proper blocking. Injection of the nontargeted Lys-ProCA32 only resulted in initial SNR enhancement at 3 hours after administration due to blood pool distribution. This enhancement returned to baseline at 24 hours. In contrast, injection of Cys-ProCA32.CXCR4 resulted in maximum SNR enhancement at 24 hours after injection and returned to the baseline at 48 hours. Previous injection of CXCR4 receptor blocking reagent specifically eliminated the enhancement at 24 hours by Cys-ProCA32.CXCR4 but retained the 3-hour initial enhancement due to blood pool effect (Fig. 6, B and C, and fig. S6). These results support the view that ProCA32.CXCR4 is able to specifically bind to the CXCR4 receptor overexpressed on the tumors and enables molecular targeting MRI.

Toxicity study of ProCA32.CXCR4

A detailed pharmacokinetic study was carried out to study the bioavailability of Cys-ProCA32.CXCR4. The AUC0.72h (fig. S7A) of ProCA32.CXCR4 was 113.20 μg·h/ml. The clearance of ProCA32.CXCR4 was 0.31 ml/min per kilogram, slightly less than Eovist (0.4 ml/min per kilogram). ProCA32.CXCR4 had a half-time of 9.19 hours, with a mean residence time of 19.58 hours. The biodistribution study using ICP-OES showed very low amounts of Gd3+ in the brain (0.07% injection dosage (ID)/g tissue) at 5 days after injection of ProCA32.CXCR4 (fig. S7), with the liver displaying the highest concentration of Gd3+ (21.3% ID/g tissue). The biodistribution studies of Gd3+ demonstrated no potential Gd3+-dependent toxicity via brain deposition. Alkaline phosphatase (ALP) and Alanine transaminase (ALT) levels of the mice injected with ProCA32.CXCR4 were comparable with levels from control mice. Albumin, total bilirubin, bilirubin-conjugated, and bilirubin-unconjugated levels in mice injected with ProCA32.CXCR4 exhibited no substantial differences when compared with control mice (table S2). Detailed histological analyses of brain, liver, spleen, muscle, and kidney tissues showed no observable tissue damage (fig. S8). Thus, injection of ProCA32.CXCR4 did not indicate acute toxicity in the mouse study.

DISCUSSION

The liver is a common site for cancer metastases. UM almost exclusively metastasizes to the liver. The mechanism of the liver-specific metastases is not well understood. One of the hypotheses in the field postulates that tumor cells that overly express CXCR4 hijack the CXCR4/CXCL12 axis during the metastatic process and spread to the liver (13–15). This hypothesis is based on the findings that the liver microenvironment in UM is rich in multiple chemoattractants including CXCL12, the natural ligand of CXCR4 (24), and CXCR4 was found to be overexpressed on UM cells in several UM cell line studies (17, 25). CXCR4 is proposed to be a prognostic marker in multiple malignancies, including acute myelogenous leukemia, breast cancers, colorectal cancers, and cutaneous melanoma (17, 26–28). Thus, development of imaging agents for CXCR4 may be used as a diagnostic biomarker in cancer and potentially as a prognostic factor.

In this investigation, we validated the diagnostic value of CXCR4 as an imaging biomarker in UM by demonstrating elevated CXCR4 expression in three different biological systems: UM patient liver metastases, UM cell lines, and an in vivo UM murine model. Multiple attempts have been made toward the development of CXCR4 molecular imaging agents over the years using different imaging technologies including Single-photon emission computed tomography (SPECT), PET, and near-infrared imaging (29–31). MRI has the
advantage of being able to provide high spatial resolution imaging without ionizing radiation and depth limitation. Despite this advantage, the application of MRI in molecular imaging is very challenging due to the sensitivity of current contrast agents and the low concentration of biomedical receptors presented on the tumor cell surface (32). To overcome these challenges, we developed a CXCR4-targeted MRI contrast agent, ProCA32.CXCR4, which exhibits 8- to 10-fold increases in both \( r_1 \) and \( r_2 \) relaxivities over clinical GBCA and enables sensitive MRI detection of CXCR4. We generated a metastatic UM mouse model by inoculation of M20-09-196 melanoma cells to demonstrate the imaging capacity of ProCA32.CXCR4. MRI following ProCA32.CXCR4 administration is able to detect UM micrometastases (Fig. 4A and fig. S5B) as small as 0.1 mm\(^3\) in murine livers, which is a notable improvement in the detection limit of MRI for liver lesions (10). Several factors contributed to the robust detection of micrometastases at early stages. First, CXCR4 targeting enabled ProCA32.CXCR4 accumulation at metastasis sites. Second, the high relaxivities of ProCA32.CXCR4 substantially improved the sensitivity of MRI. ProCA32.CXCR4 has a secondary coordination shell and optimized rotational correlation time, which contributes
Comparison of UM tumor SNR change following administration of Cys-ProCA32.CXCR4 with and without previous administration of blocking reagent; subcutaneous UM tumors are represented by color heat map. Tumor from UM mice that received Cys-ProCA32.CXCR4 administration showed significant increase in MRI signal intensity after Cys-ProCA32.CXCR4 administration. This enhancement could be blocked by first administrating the CXCR4 receptor blocking reagent. As seen with the mice that received the blocking reagent and then the Cys-ProCA32.CXCR4 injection, the SNR of UM tumor substantially increased at 24 hours after administration. This enhancement was blocked by first administrating a blocking reagent. For the mice that received Cys-ProCA32.CXCR4 injection, the SNR of UM tumor was notably lower in comparison with the UM tumor SNR of the mice that received the blocking reagent and then the Cys-ProCA32.CXCR4 injection, the SNR of UM tumor was not significantly different from the mice that received Cys-ProCA32.CXCR4 administration. For the mice that received Cys-ProCA32.CXCR4 injection, the SNR of UM tumor was not significantly different from the mice that received Lys-ProCA32.CXCR4 injection.

The inertness of ProCA32.CXCR4 verified its strong stability against transmetalation. Moreover, the improved relaxivity of ProCA32.CXCR4 enabled excellent contrast enhancement in vivo with 75% reduction of Gd³⁺ brain deposition in patients and animals has raised concerns over the use of GBCAs (35–37). We have carefully considered these factors in the design of ProCA32.CXCR4 for translation into the clinic. The Gd³⁺ binding sites of ProCA32.CXCR4 were designed to balance Gd³⁺ binding for safety and water accessibility for relaxivities. ProCA32.CXCR4 has been shown to exhibit unprecedented Gd³⁺ kinetic and thermodynamic stability, with a log (K_Gd) of ProCA32.CXCR4 calculated at 21.89. Metal selectivity values of ProCA32.CXCR4 for Gd³⁺ over Zn²⁺ and Ca²⁺ are 10⁶ to 10¹² times greater than the clinically approved contrast agents Dotarem and ProHance. The inertness of ProCA32.CXCR4 in the presence of Zn²⁺ verified its strong stability against transmetalation. Moreover, the improved relaxivity of ProCA32.CXCR4 enabled excellent contrast enhancement in vivo with 75% reduction of Gd³⁺ brain deposition compared with other GBCAs. Acute toxicity and tissue/organ toxicity in the in vivo model were not observed. Collectively, ProCA32.CXCR4 has a safe profile, which includes strong Gd³⁺ binding affinity, unique metal selectivity, and inertness against transmetalation. In addition, no acute toxicity and/or tissue/organ toxicity was observed in the in vivo model. These results strongly support the safety of ProCA32.CXCR4 for diagnostic use due to the observed strong Gd³⁺ binding affinity, unique metal selectivity, and inertness against transmetalation.

We acknowledge potential limitations in the translatability of our system, as images with best tumor enhancement happened between 24 and 48 hours after injection. We are in the process of optimizing polyethylene glycol (PEG) modification of ProCA32.CXCR4 to tune the pharmacokinetic/pharmacodynamic (PK/PD) properties. We are aware that CXCR4 can be expressed on normal cells (i.e., immune cells). Further studies will be conducted to more extensively evaluate ProCA32.CXCR4 before it can be considered for clinical applications. The present research validates our hypothesis that CXCR4 may be a diagnostic imaging biomarker for liver metastases. These results were measured using UM patients' samples, UM cell lines, and animal models. In addition, we successfully designed a CXCR4-targeting protein-based contrast agent, ProCA32.CXCR4, for early detection of UM hepatic metastases. The detected liver micrometastases were validated by histological analyses and correlated with MRI results.
Collectively, our results indicate that this contrast agent can enable precision MRI capable of defining molecular signatures for identifying metastases and possibly for treatment stratification.

**MATERIALS AND METHODS**

**IHC analysis**

Metastatic liver tissue from UM patients was immunolabeled with anti-CXCR4 antibodies for IHC analyses. Briefly, liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. These blocks were sectioned at a thickness of 5 μm for the labeling. Paraffin-embedded sections were first deparaffinized and rehydrated following a mixture of one part of 30% hydrogen peroxide and nine parts of absolute methanol to quench endogenous peroxidase activity for 10 min. Samples were then washed three times using tris-buffered saline with Tween 20 (TBST), 5 min each wash. Antigen retrieval was achieved by boiling in target retrieval solution (Agilent Technologies) for 20 min. Slides were washed as before prior to blocking in a 5% bovine serum albumin (BSA; Thermo Fisher Scientific) in TBST for 2 h. Samples were incubated in a 1:300 dilution of the anti-CXCR4 primary antibody (Abcam, 12G5, ab189048) in TBST overnight at 4°C. IHC staining was performed with a red chromogen kit following the manufacturer’s guidelines. Counterstaining of the nucleus was performed with hematoxylin. A CXCR4-positive control (brain specimen) was processed with the same protocol. All cases of UM hepatic metastases exhibited high expression of CXCR4, indicated by the red-labeling intensity.

**Flow cytometry analysis**

Flow cytometry was performed to measure the percentage of CXCR4⁺ UM cells. Cultured human UM cells were dissociated with a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO), washed, and immunolabeled for 20 min at 4°C with an allophycocyanin (APC) mouse anti-human CD184 antibody (CXCR4 is also known as CD184, clone 12G5) (BD Biosciences, San Jose, CA). Data acquisition was performed by using a BD FACS Aria IIu cell sorter (BD Biosciences, San Jose, CA). FlowJo software (Tree Star, Ashland, OR) was used for data analysis.

**Molecular cloning, expression, purification, and PEGylation of ProCA32.CXCR4**

ProCA32.CXCR4 was constructed by engineering a CXCR4-targeting moiety (LGASWHRPDKFCILGYQKRPLP) to the C terminus of ProCA32; PEGylation was performed for surface modification. ProCA32.CXCR4 was expressed in BL21 (DE3) pLysS cell strain and purified following our established protocol (20). Two site-specific PEGylations, cysteine PEGylation and lysine PEGylation, were used for ProCA32.CXCR4 surface modification. For cysteine PEGylation, ProCA32.CXCR4 solution [concentration between 1 and 10 mg/ml, in 10 mM HEPES (pH 7.2)] was degassed by bubbling with nitrogen. Tris (2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich) solution was used to reduce disulfide bonds at room temperature for 20 min. Methoxy PEG maleimide (JenKem Technology) with a molecular weight of 2 kDa was reacted with reduced ProCA32.CXCR4 at a molar ratio of 1:1 overnight at 4°C. For lysine PEGylation, ProCA32/ProCA32.CXCR4 solution [concentration between 1 and 10 mg/ml, in 10 mM HEPES (pH 7.2)] was reacted with methoxy PEG succinimidyl carboxymethyl ester reagent (molecular weight of 2 kDa, JenKem Technology) at a molar ratio of 1:5 overnight at 4°C. Purification of the PEGylated protein sample was achieved by fast protein liquid chromatography. The PEGylation product was evaluated with Coomassie blue staining and iodine (I₂) staining (fig. S1).

**Determination of r₁ and r₂ relaxation values**

The relaxation times (T₁ and T₂) of ProCA32.CXCR4 were measured with 1.5 T Bruker minispec relaxometer and 7.0 T Bruker MRI scanner. We tested different concentrations of ProCA32.CXCR4 and GdCl₃ (1:2) prepared in a solution of 50 mM HEPES, 100 mM NaCl, with a pH of 7.2. Samples were incubated at 37°C for 1 h before measurement. T₁ and T₂ relaxation times of ProCA32.CXCR4 at 1.5 T were measured by a 1.5 T Bruker minispec relaxometer, and longitudinal (r₁) and transverse (r₂) relaxivities were calculated in Eq. 1. The slopes of curves were the r₁ and r₂ relaxivities (fig. S2A). Relaxivities of ProCA32.CXCR4 at 7.0 T were measured with a 7.0 T Bruker MRI scanner with saturation recovery and spin echo sequence (fig. S2B). Commercially available GBCAs (i.e., Dotarem, Magnevist, and Eovist) were prepared in the same buffer and measured using the same procedures.

\[
r_1 = \frac{1}{T_1} - \frac{1}{T_{buffer}}
\]

**Immunofluorescence staining**

Immunofluorescence staining of CXCR4 was performed on cultured Mel290 and M20-09-196 UM cells. Cultured cells were harvested upon reaching 50 to 70% confluency and fixed on cover slides with 3.7% formaldehyde solution at 4°C. Fixed cells were incubated with 5 μM fluorescein 5-carbamoylmethylthiopropionic acid N-hydroxysuccinimide ester-labeled ProCA32.CXCR4 (the control group) was incubated with fluorescein-labeled ProCA32 for 1 h at 37°C. Briefly, the slides were washed thoroughly with TBST buffer, and then the nucleus was labeled using 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) and imaged with a Zeiss microscope.

For the colocalization studies, cells were incubated with fluorescein-labeled ProCA32.CXCR4 followed by blocking with 5% BSA (prepared in TBST buffer) for 20 min at room temperature and overnight incubation with 0.1% dilution of anti-CXCR4 (Abcam, ab189048) at 4°C. UM cells were washed and incubated with a 0.1% dilution of a goat anti-mouse secondary antibody (Invitrogen, Alexa Fluor 555) for 60 min at room temperature. DAPI (Thermo Fisher Scientific) was applied for nuclear labeling before slides were covered and sealed. Colocalization analyses of red fluorescence (555-nm excitation) and green fluorescence (488-nm excitation) were done using Fiji’s plugin coloc2 (Fig. 2C).

Flash-frozen liver tissues of Mel290-inoculated mice were collected after injection of either ProCA32.CXCR4 or ProCA32 (control group) (Fig. 5E). Liver cryosections (4 μm) were thawed at room temperature for 20 min and rehydrated with TBST. Tissue sections were surrounded with a hydrophobic barrier using Dako pen (Agilent) and blocked with 5% BSA for 60 min at room temperature, followed by incubation with an anti-ProCA32.CXCR4 or anti-ProCA32 primary antibody (1:50 dilution) for 60 min at room temperature. After thoroughly washing with TBST, tissue slides were incubated with 0.1% dilution of goat anti-rabbit secondary antibody (Invitrogen, Alexa Fluo 555) for 60 min at room temperature. Nuclear labeling proceeded by using DAPI (Thermo Fisher); slides were covered and sealed.
Enzyme-linked immunosorbent assay
An indirect ELISA assay was used to quantify the CXCR4-targeting capability. Cell lysates of Mel290 cells in NaHCO₃ solution (pH 9.6) were incubated in 96-well plates overnight at 4°C. The 96-well plates were washed thoroughly in TBST buffer and blocked by 5% BSA solution (prepared in TBST) for 60 min at room temperature. Different concentrations of ProCA32.CXCR4, ranging from 0 to 5000 nM prepared in TBST, were added and incubated for 1 hour at room temperature. A 0.1% solution with an anti-ProCA32.CXCR4 antibody (in-house polyclonal rabbit antibody) was added for 60-min incubation at room temperature. After washing with TBST, 100 μl of 1-Step Ultra TMB-ELISA Substrate Solution (Thermo body (Pierce) for 45 min at room temperature. A 0.1% solution with an anti-ProCA32.CXCR4 antibody (in-house polyclonal rabbit antibody) was added for 60-min incubation at room temperature. As a secondary antibody, we used a stabilized goat anti-rabbit horseradish peroxidase–conjugated antibody (Pierce) for 45 min at room temperature. After washing with TBST, 100 μl of 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added into each well to visualize the color change. When a blue gradient color was observed, 100 μl of 1 M H₂SO₄ was added into each well to stop the reaction. The absorbance intensity at 450-nm wavelength was measured by a FLUOstar OPTIMA plate reader, and data were plotted using GraphPad Prism 5.

Metal binding studies
The Gd³⁺ binding affinity of ProCA32.CXCR4 was investigated by an Tb³⁺ competition assay in a chelator buffer system (20). QM1 fluorescence spectrophotometer (PTI) was used to collect fluorescence spectra at room temperature. A Tb³⁺ luminescence resonance energy transfer (LRET) experiment was used to determine the Tb³⁺ binding affinity of ProCA32.CXCR4. Tb³⁺ LRET emission spectra were recorded from 500 to 600 nm wavelength with tryptophan excitation at 280 nm (fig. S4A). The chelator buffer system consisted of 30 μM ProCA32.CXCR4, 5 mM diethylenetriamine pentaacetic acid (DTPA), 50 mM HEPES, and 150 mM NaCl (pH 7.2). DTPA is a strong chelator (K_d = 10⁻²¹ M, 25°C, National Institute of Standards and Technology). Upon titration of different concentrations of Tb³⁺ titrated into the solution, the “free” Tb³⁺ concentration can be calculated by

\[
[Tb^{3+}]_{\text{free}} = K_d(Tb,\text{DTPA}) \times \frac{[Tb - \text{DTPA}]}{[\text{DTPA}]_{\text{free}}} \tag{2}
\]

where [Tb-DTPA] is the Tb³⁺-DTPA complex concentration, and it is assumed that the “free” Tb³⁺ triggered the terbium–tryptophan LRET and caused fluorescence signal change. The Tb³⁺ binding affinity to ProCA32.CXCR4 was determined by

\[
f = \frac{[Tb^{3+}]_{\text{free}}^n}{K_d(Tb,\text{ProCA32.CXCR4})^n + [Tb^{3+}]_{\text{free}}^n} \tag{3}
\]

where f is the fractional LRET signal change and n is the Hill number.

Gd³⁺ binding affinity of ProCA32.CXCR4 was determined by competing with Tb³⁺-loaded ProCA32.CXCR4. ProCA32.CXCR4 (10 μM) and Tb³⁺ (20 μM) were prepared in 5 mM DTPA, 50 mM HEPES, and 150 mM NaCl at pH 7.2. Different concentrations of Gd³⁺, ranging from 0 to 200 μM, were added and incubated overnight. Gd³⁺ replacement of Tb³⁺ in the ProCA32.CXCR4 binding pockets resulted in a signal decrease in fluorescence spectra (fig. S4B), and an apparent K_d of Gd³⁺ competition was calculated by

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

where f is the fractional LRET signal change, [Tb]_T is the total Tb³⁺ concentration, [Gd]_T is the total Gd³⁺ concentration, and K_{app} is the apparent dissociation constant of Gd³⁺ in competition with Tb³⁺.

The dissociation constant of Gd³⁺ with ProCA32.CXCR4 was then calculated by

\[
K_{d_{Gd,ProCA32.CXCR4}} = K_{d_{app}} \times \frac{K_d(Tb,\text{ProCA32.CXCR4})}{K_d(Tb,\text{ProCA32.CXCR4}) + [Tb^{3+}]_T} \tag{5}
\]

where K_{d_{Tb,ProCA32.CXCR4}} is the dissociation constant of Tb³⁺ with ProCA32.CXCR4 and [Tb³⁺]_T is the total Tb³⁺ concentration.

The dissociation constant between ProCA32.CXCR4 and Ca²⁺ was determined in an EGTA buffer system. ProCA32.CXCR4 (10 μM) was prepared in EGTA buffer [5 mM EGTA, 50 mM HEPES, and 150 mM NaCl (pH 7.2)]. Free Ca²⁺ concentration was calculated by Tsien’s assay (38), using Eq. 6

\[
[Ca^{2+}]_{\text{free}} = \frac{[Ca^{2+}]_{\text{free}}^n}{K_d(Ca,\text{ProCA32.CXCR4})^n + [Ca^{2+}]_{\text{free}}^n} \tag{6}
\]

where f is the fractional fluorescence change, [Ca²⁺]_{free} is the free Ca²⁺ concentration, and n is the Hill number.

The dissociation constant between Zn²⁺ and ProCA32.CXCR4 was determined by a modified fluorescence competition assay, where 2 μM ZnCl₂ and FluoZin-1 (Thermo Fisher) were combined in a 1:1 ratio. Different concentrations of ProCA32.CXCR4 were titrated into the sample, and fluorescence emission spectra were recorded from 500 to 600 nm following excitation at 495-nm wavelength (fig. S4C). The apparent K_d of ProCA32.CXCR4 -Zn²⁺ competition was calculated by

\[
f = \frac{[Zn]_T + [\text{ProCA32.CXCR4}]_T + K_{app}}{[Zn]_T + [\text{ProCA32.CXCR4}]_T + K_{app}^2 - 4 \times [Zn]_T \times [\text{ProCA32.CXCR4}]_T \times (\text{2} + [Zn]_T)} \tag{7}
\]

Transmetalation studies
To characterize the resistance of the Gd³⁺-ProCA32.CXCR4 complex to transmetalation by endogenous ions such as Zn²⁺, a relaxometric transmetalation assay was performed using a previously reported test by Laurent and colleagues (39). Briefly, ProCA32.CXCR4 and other GBCA were mixed with the same concentration...
were inoculated in a volume of 20 µl of PBS. The needle was then reached just below the liver subcapsule. Two million Mel290 cells used to guide a 301/2-gauge needle into the liver until its point liver was exposed with a small retractor. A surgical microscope was placed at the right and left side of the back of NU/NU mice. After 6 weeks, subcutaneous tumors of 60 to 120 mm² in volume were formed.

MRI scan
M20-09-196 mice were scanned with a 7.0 T Agilent MRI scanner at University of Georgia. Mice were anesthetized by inhalation of isoflurane gas. The respiration rates of animals were monitored throughout the MRI scanning and controlled at 70 to 80 times per minute. T1- and T2-weighted images were collected by spin echo and fast spin echo sequence before and after one bolus injection of Lys-ProCA32.CXCR4 or Cys-ProCA32.CXCR4 (0.025 mmol/kg) at 3, 24, and 48 hours. Control mice were injected with one bolus at the same dosage of Lys-ProCA32 and imaged at the same time points with the same parameters. The parameters of spin echo sequence were as follows: repetition time (TR), 500 ms; echo time (TE), 4.89 ms; field of view (FOV), 3.5 cm × 3.5 cm by a matrix of 512 × 512; thickness, 1 mm with no gap. The parameters of fast spin echo sequence were as follows: TR/TE, 140 ms/11 ms; FOV, 4 cm × 4 cm by a matrix of 512 × 512.

Intrahepatic xenotransplantation Mel290 mice MR images were all collected on a 4.7-T small-bore Varian MRI scanner at Emory University. Mice were anesthetized following similar procedure, and T1-weighted images were collected before and after one bolus injection of Cys-ProCA32.CXCR4 or Lys-ProCA32 (0.025 mmol/kg) at 12 min, 50 min, and 3, 22, and 46 hours by gradient echo sequence. The parameters of gradient echo sequence were as follows: TR/TE, 560 ms/11 ms; FOV, 3.5 cm × 3.5 cm by a matrix of 512 × 512.

Subcutaneous Mel290 tumor mice MRI results were acquired with a 7.0 T Bruker MRI scanner at Yerkes National Primate Research Center. Mice were anesthetized following a similar procedure as detailed above, and T1-weighted images were collected before and after one bolus injection of Cys-ProCA32.CXCR4 or Lys-ProCA32 (0.025 mmol/kg) at 3, 24, and 48 hours. Blocking group mice received intravenous injections of CXCR4 blocking reagent (0.025 mmol/kg) 24 and 12 hours before the injection of Cys-ProCA32.CXCR4. The parameters of the rapid imaging with refocused echoes (RARE) sequence were as follows: TR/TE, 560 ms/11 ms; FOV, 3.5 cm × 3.5 cm by a matrix of 256 × 256. MRI data were processed and analyzed by Fiji and MRIcron.

Organ distribution analysis by ICP-OES
ICP-OES was used to analyze the Gd³⁺ distribution in different mouse organs after injection of ProCA32.CXCR4. Healthy CD-1 mice were injected with a bolus dosage of ProCA32.CXCR4 (0.025 mmol/kg). Animals were euthanized 46 hours after receiving an injection of ProCA32.CXCR4, and heart, liver, spleen, kidney, brain, and muscle tissues were subsequently collected and used for ICP-OES analysis. Tissues (0.1 to 0.5 g) were dissolved overnight in 1 ml of Nitric Acid 67-69%, Optima (Fisher Chemical). Undissolved particles were removed by filtration, and the supernatant was retained for Gd³⁺ content analysis by ICP-OES (fig. S7B).

Toxicity study
ProCA32.CXCR4 acute toxicity was tested by a bolus injection of 100 µl of 7 mM ProCA32.CXCR4 to 10-week-old healthy CD-1 mouse. ProCA32.CXCR4 solutions with two different PEGylation methods (Cys-ProCA32.CXCR4 and Lys-ProCA32.CXCR4) were tested. Each test group had three mice, and the control group was injected with
saline. Mice were observed every 8 hours after injection and then euthanized after 5 days. Terminal blood was collected by cardiac puncture, and serum was transferred immediately to microcentrifuge tube. Plasma was separated from blood cells by centrifugation at 14,000 rpm, 4°C for 10 min. Serum samples were used for basic blood biochemical tests and kidney function tests to measure ALT, ALP, and electrolyte levels (table S2). Tissues including heart, muscle, liver, spleen, kidney, lung, and brain were collected for analysis of gadolinium distribution using ICP-OES.

Pharmacokinetic study
Female CD-1 mice (8 to 10 weeks old) were used to determine the pharmacokinetic parameters of Cys-ProCA32.CXCR4. Cys-ProCA32.CXCR4 (100 μL, 0.025 mmol/kg) was administered through tail vein injection. Blood samples were collected at various time points using the saphenous vein up to 7 days using a sparse sampling design (three to six animals per time point). Immediately following blood sample collection, samples were stored on ice, serum was obtained through centrifugation, and Gd^{3+} concentration was determined using ICP-OES. Pharmacokinetic parameters were calculated using the noncompartamental analysis tool of Phoenix WinNonlin software.

Statistical analysis
SNR was calculated by the mean value across different slides of MRI results of the same subjects. Analyses of differences between the two groups were performed using two-tailed Student’s t test in GraphPad Prism 5 (GraphPad Software). The P values are denoted in figure legends, and differences were considered significant if P < 0.05. No estimation of sample size and blinding was performed for animal studies. Receiver operating characteristic (ROC) analyses were performed using R and SAS. AUC was reported to measure the performance of the contrast agent. Mice were randomly assigned to groups for the experiments.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/6/eaav7504/DC1

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Submitted 17 October 2018
Accepted 22 November 2019
Published 7 February 2020
10.1126/sciadv.aav7504