## SUPPLEMENTAL MATERIALS AND METHODS

### Synthesis of HYNIC-Conjugated HK Peptide

Sodium succinimidyl 6-(2-(2-sulfonatobenzaldehyde) hydrazono)nicotinate (HYNIC-NHS) was prepared according to the literature method (*1*). A solution of 2 μmol of Fmoc-HK was mixed with 6 μmol of HYNIC-NHS in 500 μL of a mixture containing *N*,*N*-dimethylformamide (DMF) and water (1:1 = v:v). The pH was adjusted to 8.5~9 using 0.1 N NaOH. After stirring for 2 h at room temperature, Fmoc-HK-HYNIC was isolated by semi-preparative HPLC. Fmoc-HK-HYNIC was obtained in 52% yield with >95% purity. After removal of the Fmoc group with 20% piperidine in DMF, the final product, HK-HYNIC (HHK), was generated. Analytical HPLC (Rt = 13.59 min) and mass spectrometry (MALDI-TOF-MS: m/z, 2585.12 for $[MH]^+$ ($C_{109}H_{179}N_{37}O_{34}S$, calculated molecular weight 2583.88) analyses confirmed the identity of the product.

### Flow Cytometry

The expression status of integrin $\alpha_v\beta_6$ in BxPC-3 and HEK293 cells was tested by fluorescence-activated cell sorting (FACS) analysis. Briefly, the cells were harvested and suspended in PBS supplemented with 1% bovine serum albumin (BSA). The cells were incubated with the mouse anti-human integrin $\alpha_v\beta_6$ antibody (clone E7P6; 1:150; Chemicon, Millipore, Billerica, USA) for 1 h at 4°C. After washing with cold PBS, the cells were incubated with the FITC-conjugated goat anti-mouse secondary antibody (1:200; Jackson Immuno-Research Laboratories, West Grove, USA) for 30

min at 4°C. The cells were washed, re-suspended in PBS, and then analyzed using a flow cytometer (Becton Dickinson, Germany).

**Cell Binding Assay**

The in vitro integrin $\alpha_v\beta_6$ binding affinity and specificity of the HK peptide were assessed via a cellular displacement assay using $^{99m}$Tc-HHK as the integrin $\alpha_v\beta_6$-specific radioligand. The experiments were performed on BxPC-3 cells using the same procedure as described in the *MATERIALS AND METHODS* of the main text. The best-fit 50% inhibitory concentration ($IC_{50}$) values were calculated by fitting the data with nonlinear regression using GraphPad Prism 4.0 (GraphPad Software). Experiments were repeated twice with quadruple samples.

**Serum Stability and Metabolism**

$^{99m}$Tc-HHK was incubated in human serum for 0, 1, 2, 4, 8, 12, and 24 h at 37°C to test the in vitro serum stability. After passing through a 0.22 μm Millipore filter, the samples were analyzed by radio-HPLC. For metabolism studies, female BALB/c mice (n = 3) were injected with $^{99m}$Tc-HHK at a dose of 74 MBq in 0.1 mL PBS via tail vein. At 0.5 and 1 h time points, the blood and urine samples were collected. The mice were then sacrificed, and the kidneys were removed and homogenized. The samples (serum, urine, and homogenized kidney) were centrifuged at 8000 rpm for 15 min. The supernatant was collected, filtered through a 0.22 μm Millipore filter, and then analyzed by radio-HPLC.

**Immunofluorescence Staining**

To validate the imaging results, immunofluorescence staining studies were performed to test the integrin $\alpha_v\beta_6$ expression in tumor tissues. Frozen BxPC-3 and HEK293 slices (5 μm thickness) from the tumor-bearing mice were fixed with ice-cold acetone, rinsed with PBS, and blocked with 10% FBS for 30 min at room temperature. The slices were incubated with the rat anti-mouse CD31 (1:100; BD Biosciences, San Jose, USA) and mouse anti-human integrin $\alpha_v\beta_6$ (clone E7P6; 1:100; Chemicon, Millipore, Billerica, USA) antibodies, after which visualization was performed with Cy3-conjugated goat anti-rat and FITC-conjugated goat anti-mouse secondary antibodies (1:200; Jackson Immuno-Research Laboratories, West Grove, USA) under a Leica TCS-NT confocal microscope (Wetzler, Heidelberg, Germany).

**REFERENCES**

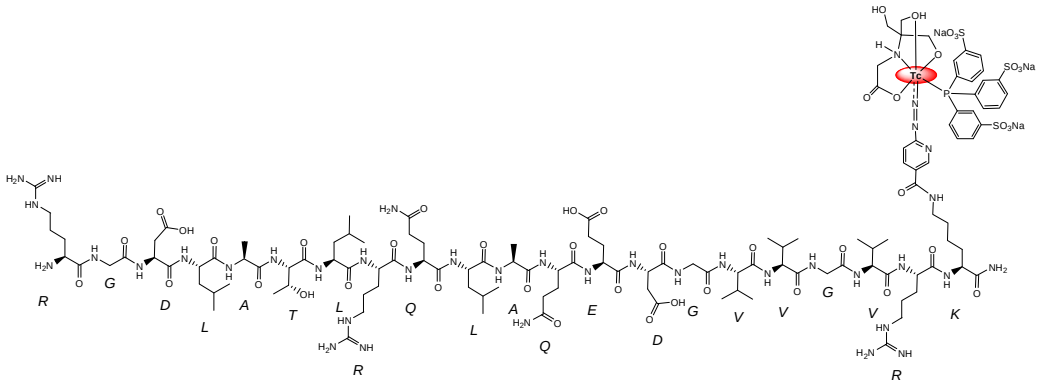
1. Harris TD, Sworin M, Williams N, et al. Synthesis of stable hydrazones of a hydrazinonicotinyl-modified peptide for the preparation of $^{99m}$Tc-labeled radiopharmaceuticals. *Bioconjug Chem.* 1999;10(5):808-814.

**SUPPLEMENTAL TABLE 1. Characterizations of $^{99m}$Tc-HHK and other integrin $\alpha_v\beta_6$-targeting radiotracers.**

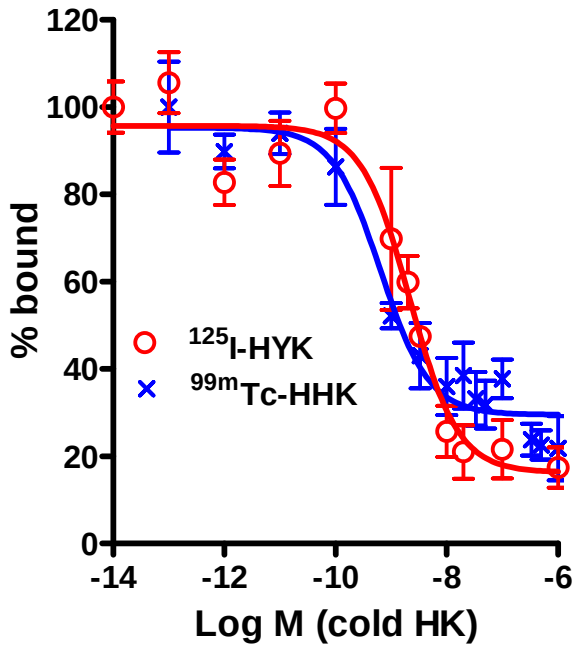
| Radiotracer | Targeting peptide | Labeling yield (%) | RCP | Synthesis time | Serum stability (%) | Ref.* |
|---|---|---|---|---|---|---|
| $^{99m}$Tc-HHK | RGDLATLRQLAQE DGVVGVRK | 94.8±1.1 | >98% | 30 min | 94.4±1.2 (24 h) | |
| [$^{18}$F]FBA-A20FM DV2 | NAVPNLRGDLQVL AQKVART | 3.6 | >98% | 130 min | NM | [7] |
| [$^{18}$F]FBA-PEG$_{28}$-A20FMDV2 | NAVPNLRGDLQVL AQKVART | 6.4±2.0 | >97% | NM | NM | [8] |
| [$^{18}$F]FBA-(PEG$_{28}$)$_2$-A20FMDV2 | NAVPNLRGDLQVL AQKVART | 5.4±2.5 | >97% | NM | NM | [8] |
| $^{111}$In-DTPA-A20F MDV2 | NAVPNLRGDLQVL AQKVART | NM | 99% | >30 min | ~50 (4 h) | [23] |
| $^{125}$I/$^{131}$I-HBP-1 | SPRGDLAVLGHKY | NM | NM | NM | NM | [22] |
| $^{64}$Cu-DOTA-R$_0$1 | GCILNMRTDLGTL LFRCRRDSDCPGA CICRGNGYCG | >80% | >95% | >60 min | ~80 (24 h) | [9] |
| $^{64}$Cu-DOTA-S$_0$2 | GCRSLARTDLDHL RGRCSSDSDCLAE CICLENGFCG | >80% | >95% | >60 min | >95 (24 h) | [9] |
| $^{18}$F-fluorobenzoate -R$_0$1 | GCILNMRTDLGTL LFRCRRDSDCPGA CICRGNGYCG | 7±1 | 93% | >105 min | 87 (2 h) | [10] |
| $^{18}$F-fluorobenzoate -S$_0$2 | GCRSLARTDLDHL RGRCSSDSDCLAE CICLENGFCG | 6±1 | >99% | >105 min | 94 (2 h) | [10] |

**Note:** RCP: radiochemical purity; NM: not mentioned.

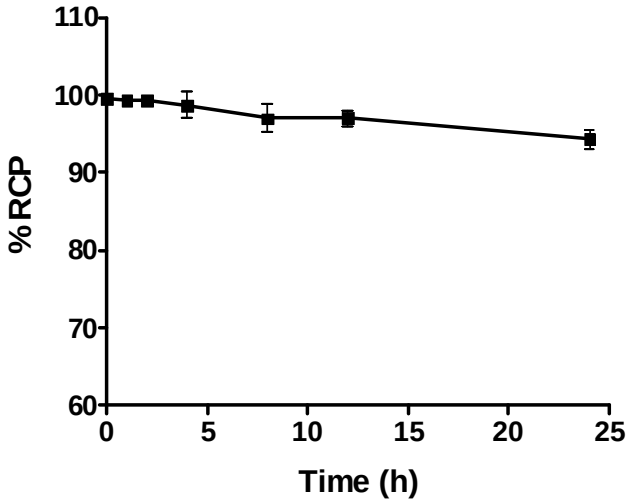
*Ref. number corresponds to references list in the main text.



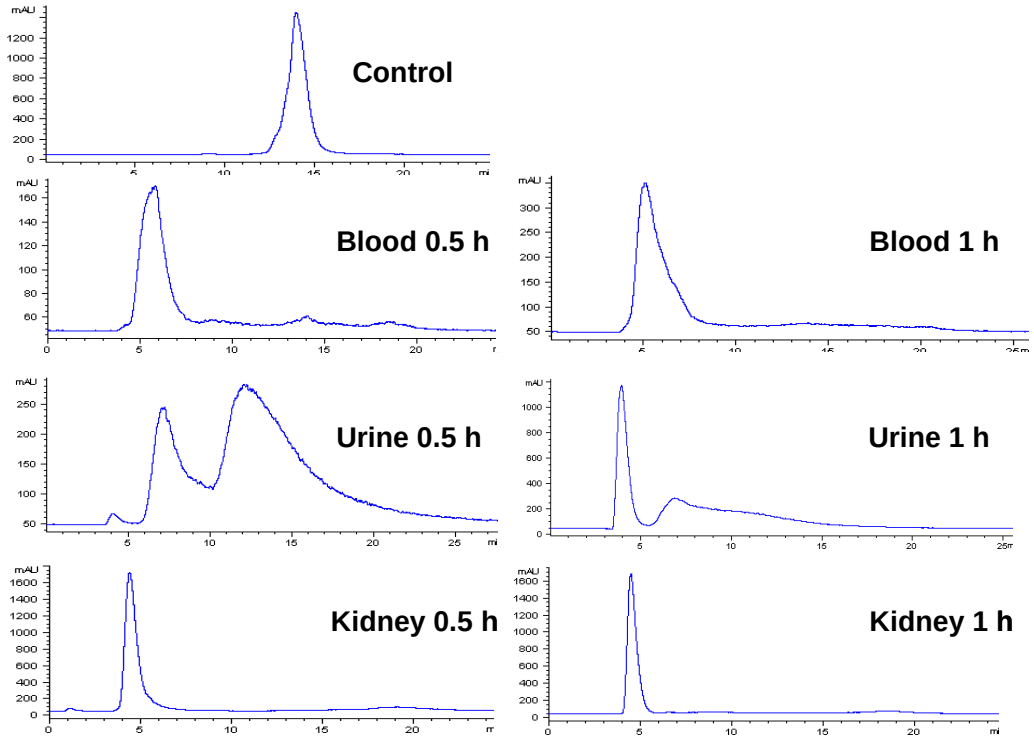
**Supplemental Figure 1.** Chemical structure of $^{99m}$Tc-HYNIC(tricine)(TPPTS)-HHK ($^{99m}$Tc-HHK).



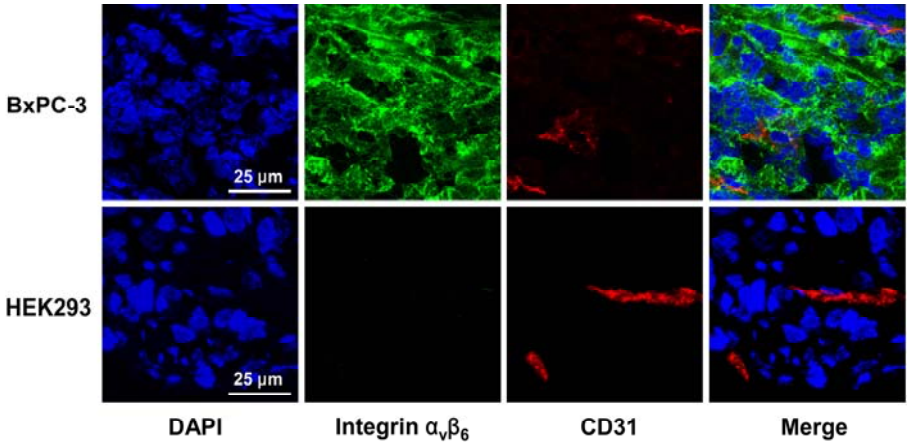

**Supplemental Figure 2.** Inhibition of $^{99m}$Tc-HHK or $^{125}$I-HYK binding to integrin $\alpha_v\beta_6$ on BxPC-3 cells by cold HK peptide (n = 4, means ± SD).




**Supplemental Figure 3.** Solution stability data for $^{99m}$Tc-HHK in serum.




**Supplemental Figure 4.** Typical radio-HPLC chromatogram and metabolic stability of $^{99m}$Tc-HHK in mouse blood, urine, and kidney after 0.5 and 1 h injection.




**Supplemental Figure 5.** Immunofluorescence staining of human integrin $\alpha_v\beta_6$ and mouse CD31 in BxPC-3 and HEK293 tissues.