Published in final edited form as:

Bioconjug Chem. 2012 August 15; 23(8): 1704–1711. doi:10.1021/bc300278r.

An Activatable Near Infrared Fluorescent Probe for *In Vivo* Imaging of Fibroblast Activation Protein-alpha

Jinbo Li^{1,2}, Kai Chen¹, Hongguang Liu¹, Kai Cheng¹, Meng Yang¹, Jiping Zhang³, Jonathan D. Cheng³, Yan Zhang^{2,*}, and Zhen Cheng^{1,*}

¹Molecular Imaging Program at Stanford (MIPS), Department of Radiology, and Bio-X Program, Canary Center at Stanford for Cancer Early Detection, Stanford University, Stanford, California, 94305-5344

²School of Chemistry and Chemical Engineering, Institute of Chemical Biology and Drug Innovation, Nanjing University, Nanjing 210093, China

³Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

Abstract

Fibroblast activation protein-alpha (FAP α) is a cell surface glycoprotein which is selectively expressed by tumor-associated fibroblasts in malignant tumors but rarely on normal tissues. FAP α has also been reported to promote tumor growth and invasion and therefore has been of increasing interest as a promising target for designing tumor-targeted drugs and imaging agents. Although medicinal study on FAP α inhibitors has led to the discovery of many FAP α -targeting inhibitors including a drug candidate in a phase II clinical trial, the development of imaging probes to monitor the expression and activity of FAP α *in vivo* has largely lagged behind. Herein we report an activatable near infrared (NIR) fluorescent probe (ANP_{FAP}) for *in vivo* optical imaging of FAP α . The ANP_{FAP} consists of a NIR dye (Cy5.5) and a quencher dye (QSY21) which are linked together by a short peptide sequence (KGPGNQC) specific for FAP α cleavage. Because of the efficient fluorescence resonance energy transfer (FRET) between Cy5.5 and QSY21 in ANP_{FAP}, high contrast on the NIR fluorescence signal can be achieved after the cleavage of the peptide sequence by FAP α both *in vitro* and *in vivo*. *In vitro* assay on ANP_{FAP} indicated the specificity of the probe to FAP α . The *in vivo* optical imaging using ANP_{FAP} showed fast tumor uptake as well as high tumor to background contrast on U87MG tumor models with FAP α expression, while much lower signal and tumor contrast were observed in the C6 tumor without FAP α expression, demonstrating the *in vivo* targeting specificity of the ANP_{FAP}. *Ex vivo* imaging also demonstrated ANP_{FAP} had high tumor uptake at 4 h post injection. Collectively, these results indicated that ANP_{FAP} could serve as a useful NIR optical probe for early detection of FAP α expressing tumors.

Keywords

FAP α ; NIRF; glioma; activatable probe; optical imaging

INTRODUCTION

Fibroblast activation protein- α (FAP α) is a cell surface glycoprotein and a member of the serine protease family.¹ It has been found to be selectively produced by tumor-associated

* Author to whom correspondence should be addressed: Zhen Cheng, Ph.D., Molecular Imaging Program at Stanford, Department of Radiology and Bio-X Program, Canary Center at Stanford for Cancer Early Detection, 1201 Welch Road, Lucas Expansion, P095 Stanford University, Stanford, CA 94305-5484, 650-723-7866 (V), 650-736-7925(Fax), zcheng@stanford.edu, or, Yan Zhang, Ph.D., School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China, njuzy@nju.edu.cn.

fibroblasts² and expressed in over 90% of malignant breast, colorectal, skin, and pancreatic tumors.³ In contrast, most normal adult tissues do not have detectable FAP protein expression.² FAP α is also known as a biomarker of the cancer-associated fibroblasts (CAFs) in the stroma, which plays an important role in affecting the proliferation, invasion and metastasis of cancer cells.^{4–7} Besides, recent investigations have revealed that FAP α -expressing cells provide a non-redundant, immune-suppressive component of the tumor microenvironment.⁸ Consequently, FAP α has been increasingly accepted and pursued as a promising tumor target for designing novel pharmaceuticals.⁹ These efforts have led to discovery of many small molecular FAP α inhibitors including Val-boroPro (Talabostat) which has been tested in a phase II clinical trial for treatment of patients with metastatic colorectal cancer.¹⁰ Considering the important role of FAP α in tumor biology and cancer therapy, development of molecular probes for *in vivo* FAP α detection and quantification is thus of great importance. These probes could serve as non-invasive imaging tools for early detection of cancer, stratification of cancer patients for FAP α targeted therapy, monitoring treatment response, and even for future theranostic applications.

In vivo optical imaging using near infrared (NIR) fluorescent probes has been of great interests because of the relatively deep tissue penetration ability of NIR dyes as well as low tissue autofluorescence.^{11–13} Activatable NIR probes that can generate a strong NIR fluorescence signal upon enzyme activation could further enhance the tumor to background signal ratio. Therefore this type of probes has been considered as promising smart probes for early detection of small tumor lesions.¹⁴ Many activatable NIR probes based on fluorescence resonance energy transfer (FRET) and targeting proteases including matrix metalloproteinases (MMPs),^{15–19} cysteine proteases^{20–22} and caspase^{23, 24} have been successfully developed and widely explored in biomedical research. However, to the best of our knowledge, activatable NIR probes for FAP α *in vivo* imaging have not been reported despite of the great potential of FAP α in tumor theranostics.

The difficulty in the construction of activatable NIR probes specific for FAP α lies in the fact that FAP α shares the same peptide substrates for other post-prolyl peptidases that are capable of cleaving the Pro-Xxx amino acid bond.²⁵ The most closely related prolyl peptidase family member of FAP α is the dipeptidyl peptidase-IV (DPPIV). The important substrate-binding domain and the key substrate-binding residues in FAP α and DPPIV are in very similar positions.²⁶ However, systematic structural and kinetic analysis of the substrate specificity of FAP α ²⁷ and peptide substrate profiling revealed that FAP α possesses endopeptidase activities.²⁸ Then peptide substrates such as Ac-GPGP-2SBPO²⁹ and Pyro-TSGPNQEYK(BHQ)⁹ were found to be able to discriminate FAP α from DPPIV. Therefore it is possible for us to develop novel activatable NIR probes which can be selectively activated by FAP α and consequently be used as *in vivo* optical imaging probes for FAP α .

Here we report our work on the development of the novel activatable NIR probe for FAP α (ANP_{FAP}) and the successful application of the probe for *in vivo* imaging of tumor with FAP α expression. The FRET pair used in the probe is Cy5.5 and QSY21, which are linked by the peptide substrate (KGPGPNQC) specific for FAP α (Scheme 1). *In vitro* assay on the fluorescence activation by FAP α , DPPIV and MMP-2 proteins indicated the specificity of the probe for FAP α . *In vivo* imaging using the probe on two tumor models with and without FAP α expression showed high contrast from the FAP α -positive tumor model and much lower contrast from FAP α -negative tumor model. *Ex vivo* imaging further revealed the tissue distribution of the activated probe, which may provide important information on further modification and optimization of the probe for *in vivo* imaging.

MATERIALS AND METHODS

General

All N-Fmoc-protected amino acids and rink amide MBHA resin were purchased from CS Bio Company (Menlo Park, CA). Cy5.5-mono-maleimide was purchased from GE health care (Piscataway, NJ). QSY21-NHS was purchased from Invitrogen (Carlsbad, CA). All other standard chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Recombinant FAP α and DPPIV were obtained from R&D systems (Minneapolis, MN), recombinant MMP-2 was purchased from EMD Millipore (Billerica, MA). The C6 and U87MG cells were obtained from American Type Tissue Culture Collection (Manassas, VA). Female athymic nude mice were purchased from Charles River Laboratories (Boston, MA).

The peptide Lys-Gly-Pro-Gly-Pro-Asn-Gln-Cys was synthesized on an automatic peptide synthesizer (CS Bio) using Fmoc-protected amino acids and rink amide MBHA resin. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Dionex Ultimate 3000 HPLC system (Sunnyvale, CA) for analysis and preparation of peptides and bioconjugates. Both semi-preparative (Vydac, Hesperia, CA. 218TP510-C18, 10 mm $\times$ 250 mm) and analytical (Dionex, Sunnyvale, CA. Acclaim120 C18, 4.6 mm $\times$ 250 mm) RP-HPLC columns were used. The mobile phase was solvent A, 0.1% trifluoroacetic acid (TFA)/H₂O, and solvent B, 0.1% TFA/acetonitrile. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Framingham, MA) was performed by Stanford Protein and Nucleic Acid Biotechnology Facility to verify the preparation of compounds. Absorption and emission spectra of ANP_{FAP} were measured on an Agilent UV-visible ChemStation (Agilent Technologies, Wilmington, DE) and an Inifite M1000 spectrofluorometer (Tecan, Austria), respectively. *In vivo* and *ex vivo* imaging were performed with an IVIS200 small animal imaging system (Caliper, Hopkinton, MA).

Synthesis of ANP_{FAP}

The crude peptide Lys-Gly-Pro-Gly-Pro-Asn-Gln-Cys on the resin was first acetylated by anhydrous acetic anhydride with hydroxybenzotriazole (HOBt)/N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF). Ac-Lys-Gly-Pro-Gly-Pro-Asn-Gln-Cys was then cleaved from the resin by addition of a 92.5/2.5/2.5/2.5 (v/v) mixture of TFA/triisopropylsilane/ethanedithiol/water for 2 h at room temperature (RT) and then precipitated by cold anhydrous diethyl ether. The resulted peptide was dried and further purified by a preparative RP-HPLC. The flow rate was 4 mL/min, with the mobile phase starting from 95% solvent A and 5% solvent B (0–3 min) to 35% solvent A and 65% solvent B at 33 min. Fractions containing the product were collected and lyophilized. The target product was characterized by MALDI-TOF-MS and ready for use in the next step.

The purified peptide (450 μ g) was then conjugated with Cy5.5-mono-maleimide (300 μ g) in 300 μ L of phosphate buffered saline (PBS, 0.1 M, pH=7.4) with a molar ratio of 2:1 for 2 h in dark at RT. The Cy5.5 labeled peptide was purified by RP-HPLC using the same flow rate and gradient as that for the un-modified peptide listed above. The product was characterized by MALDI-TOF-MS and then used for next step. Further conjugation with QSY21-NHS ester in dimethyl sulfoxide (DMSO) and 2% DIPEA (1.5 equivalents of QSY21-NHS ester per equivalent of peptide) for 2 h resulted the final product, ANP_{FAP}. The probe was also purified by HPLC and lyophilized using the same conditions. ANP_{FAP} was characterized by MALDI-TOF-MS and purity was confirmed by analytical RP-HPLC using the same gradient as the preparative runs.

***In vitro* assay of ANP_{FAP} activation by FAP α**

ANP_{FAP} was dissolved in water as a stock solution with a concentration of 1 mM. For enzymatic assays, ANP_{FAP} was mixed with FAP α , DPPIV or MMP-2 and diluted with PBS to a final concentration of 10 μ M. Different concentrations of FAP α (0.25 μ g/mL, 0.5 μ g/mL, 1 μ g/mL) were used for dose dependent study and 1 μ g/mL of DPPIV and MMP-2 were used to demonstrate the specificity of ANP_{FAP}. All the assays were performed in triplicate on a 96-well plate and the emission spectra of ANP_{FAP} were recorded at 37 °C every 10 minutes on spectrofluorometer for 1 h. The excitation wavelength was set at 675 nm and the emission spectra were recorded from 680 nm to 800 nm. After 1 h incubation, samples were imaged immediately by the IVIS200 imaging system for phantom study.

Cell culture and animal models

C6 and U87MG cells were cultured in DMEM medium (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA). Cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. C6 and U87MG tumor models were developed by subcutaneous injection of 3×10^6 and 5×10^6 cells, respectively, into the right shoulder of female athymic nude mice. Tumor-bearing mice were subjected to *in vivo* imaging studies when tumor volume reached 200–300 mm³. All animal studies were performed according to the protocol approved by the Stanford University Administrative Panels on Laboratory Animal Care (APLAC).

Western blot analysis

The expression of FAP α in C6 and U87MG tumor tissues was analyzed by western blot analysis with anti-FAP α monoclonal antibody. Tumors were collected right after the end of *in vivo* experiment and quickly frozen for preservation. Before protein extraction, tumors were weighted, homogenized and lysed with M-PER mammalian protein extraction reagent (Thermo Fisher, Waltham, MA). After centrifugation, clear cell lysates were transferred to new tubes. The protein concentrations were determined by using Bradford reagent (Bio-Rad, Hercules, CA). The cell lysates (50 μ g) were loaded onto NuPAGE 4–12% Bis-Tris Gel gel (Invitrogen, Carlsbad, CA) and transferred to Immun-blot PVDF membranes (Invitrogen, Carlsbad, CA). Anti-FAP monoclonal antibody was used at a 1:100 dilution. Anti-rabbit HRP conjugated secondary antibody was purchased from Thermo Fisher (Waltham, MA) and used at a 1:5000 dilution. Actin antibody (Abcam, Cambridge, MA) was used at a 1:3000 to detect expression of actin as a control for protein loading. All immunoblots were analyzed by using the ECL detection system (Thermo Fisher, Waltham, MA).

***In vivo* and ex vivo imaging**

For *in vivo* imaging, C6 and U87MG tumor-bearing mice (n=5 for each group) were injected intravenously *via* tail vein with 1 nmol of ANP_{FAP} in 150 μ L of PBS. NIRF imaging was then performed with the IVIS200 small animal imaging system. To acquire fluorescence of ANP_{FAP} *in vivo*, Cy5.5 filter (excitation 615 nm – 665 nm; emission 695 – 770 nm) and Cy5.5 background filter (580 nm – 610 nm) were used. Identical illumination settings (lamp voltage, filters, f/stop, field of views, binning) were used for acquiring all images. Fluorescence images were taken at 0.5, 1, 2, 3 and 4 h post injection (p.i.) using a 2 second exposure time (f/stop=1, binning=4). Images were then analyzed using Living Image 3.2 software (Caliper, Hopkinton, MA). The Cy5.5 background filter image was subtracted from the Cy5.5 filter image and the fluorescence emission was normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr). For comparison of tumor contrast, mean fluorescence intensities of the tumor area (T) at the right foreleg of the animal and of the area (N) at the right flank (normal tissue) were calculated by the region-of-interest (ROI)

function of Living Image software integrated with Igor (Wavemetrics, Lake Oswego, OR). Dividing T by N yielded the contrast between tumor tissue and normal tissue.

For *ex vivo* imaging, mice were sacrificed 4 h p.i. Tumor and other major tissues were dissected, rinsed with PBS, dried with tissue paper, and then put on black paper. The *ex vivo* images were acquired immediately using IVIS200 system with the same illumination setting as the *in vivo* imaging. Images were also processed with the same method as described above.

Statistical analysis

All data are shown as mean $\pm$ SD (standard deviation). Difference between groups was assessed by Student's t test. Statistical significance was considered if *P* values are less than 0.05.

RESULTS

Design and Synthesis of the FAP α -specific Probe

To design an optical probe suitable for *in vivo* imaging, the NIR dye Cy5.5 with maximum emission wavelength at 695 nm was selected to label the peptide substrate. Since the emission of Cy5.5 was known to be efficiently quenched by the dye QSY21³⁰, QSY21 was chosen as the FRET pair with Cy5.5 to label the peptide substrate. The selection of the peptide sequence (GPGPNQ) was based on the reported specific FAP α substrate sequences which could be cleaved by FAP α but not DPPIV.^{9, 28, 29} For efficient and site specific labeling the peptide substrate with Cy5.5 and QSY21, a lysine residue with a free amine group on the side chain and a cysteine with a thiol group were inserted to the N and C terminal of the peptide, respectively. More specifically, Conjugation of the two dyes with the peptide substrate was easily realized through the reaction of the cysteine thiol group with the Cy5.5 maleimide and the lysine amine with QSY21 NHS ester in ~26% yield and >95% purity (Scheme 1). Successful conjugation of Cy5.5 and further modification with QSY21 were indicated by the peak with *m/z* at 1880.6 (calculated as 1879.6) and 2544.6 (calculated as 2544.9) on the MALDI-TOF-MS spectrum of the purified product, respectively. The UV spectra of Cy5.5, QSY21 and ANP_{FAP} were shown in Figure 1A. There were two significant absorption bands centered at 620 nm and 676 nm respectively for ANP_{FAP}. In addition, the strong fluorescent emission at ~695 nm of the substrate labeled with Cy5.5 alone was efficiently quenched by QSY21 as shown in the fluorescence spectrum of ANP_{FAP} (Figure 1B).

Sensitivity and Specificity of ANP_{FAP} to FAP α

Sensitivity of ANP_{FAP} to FAP α was firstly examined *in vitro*. Upon incubation with FAP α at 37 °C, fluorescence emission ANP_{FAP} showed significant increase on the intensity of the corresponding to that of Cy5.5. It indicated that the probe could be efficiently cleaved by FAP α to release the Cy5.5 labeled residue and split the FRET pair. Fluorescent spectra of ANP_{FAP} incubated with FAP α at different time points were collected as shown in Figure 2A. It showed that the activation of ANP_{FAP} was fast in kinetics, and an increase of 12 fold in fluorescence emission intensity at 695 nm could be achieved within 1 h. As a contrast, ANP_{FAP} which was not incubated with any enzyme showed no increase in fluorescence emission intensity. Besides, ANP_{FAP} incubated with the same amount of post-prolyl peptidase DPPIV and MMP-2 did not show significant activation on fluorescent emission either (Figure 2B). These results clearly suggested that the activation of ANP_{FAP} was caused by the specific recognition and cleavage of the peptide substrate by FAP α .

Dose-dependent activation of the ANP_{FAP} was also observed when the probe was incubated with different concentrations of FAP α . Fluorescent spectra of ANP_{FAP} incubated with different amounts of FAP α at 37 °C for 1 h were recorded as shown in Figure 2C. The increase of the fluorescence intensity at 695nm was significant upon titration of FAP α concentration from 0.25 μ g/mL to 1 μ g/mL (Figure 2D). Phantom study on the ANP_{FAP} incubated with FAP α at concentrations of 0, 0.25, 0.5 and 1 μ g/mL also showed increased brightness of the probe (Figure 2D). The brightness of ANP_{FAP} under optical imaging corresponded well with the amount of FAP α presented within the target.

In Vivo Imaging

After successful validating the specificity and sensitivity of ANP_{FAP} *in vitro*, its potential for *in vivo* imaging was further investigated. For this purpose, U87MG tumor model was developed because of the high expression of FAP α in U87MG tumor. To evaluate the specificity of ANP_{FAP} *in vivo*, another tumor model C6, which is not supposed to express FAP α , was also established. Expression of FAP α in these two tumor models was measured, and it was found that FAP α was highly expressed in U87MG tumors, while almost no detectable expression in C6 tumor (Figure 3).

Based on the expression of FAP α in these tumors, C6 and U87MG were used as a negative and positive tumor model, respectively, for evaluation of the FAP α imaging ability of ANP_{FAP} *in vivo*. ANP_{FAP} was first injected into C6 and U87MG tumor-bearing mice through tail vein. *In vivo* imaging was recorded over 4 hrs. Representative *in vivo* whole-body images of mice at different time points (0.5, 1, 2, 3, and 4 h) were shown in Figure 4A. Strong fluorescent signals in FAP α positive tumor U87MG were observed, while much lower fluorescent signals were detected in FAP α negative tumor C6 in all the corresponding time points ($P < 0.05$). Early activation of ANP_{FAP} in U87MG tumor was observed at 0.5 h, and the fluorescent signals increased from 0.5 h to 4 h p.i.. Quantification analysis of fluorescent signals in tumors was calculated by drawing ROI, and the results were shown in Figure 4B. The fluorescence intensities of U87MG tumor increased since 0.5 h and reached maximum at 2 h p.i. and then remained unchanged. While fluorescence intensities of C6 tumor did not alter over the same time period. The fluorescence ratios between tumor and normal tissue were also calculated and shown in Figure 4C. The tumor to normal tissue ratios of U87MG increased fast from 0.5 h (2.218 ± 0.403) to 4 h (4.630 ± 0.728), suggesting accumulation of ANP_{FAP} in tumors. In contrast, tumor to normal tissue ratios of C6 remained constant at all time points, and these ratios were significantly lower than the corresponding ratios obtained from U87MG tumor model, highlighting the *in vivo* targeting specificity of the ANP_{FAP}.

Ex Vivo Imaging

To further confirm the *in vivo* imaging results and specific activation and accumulation of ANP_{FAP} in tumors, *ex vivo* imaging of tumors and other normal organs was performed after *in vivo* imaging finished. Representative *ex vivo* images of tumors and normal organs were shown in Figure 5A. Similarly as the *in vivo* imaging (Figure 4A), U87MG tumor was much brighter than C6 tumor ($P < 0.05$), which demonstrated successful activation of ANP_{FAP} by FAP α *in vivo*. Quantitative analysis of the fluorescent signals confirmed that activated ANP_{FAP} mainly located in U87MG tumor, but not in FAP α negative tumor C6 (Figure 5B). Strong signals from stomachs of both U87MG and C6 tumor bearing mice were also observed, which were likely from the autofluorescence of remained mouse chow. Lastly, it was notice that the other major organs gave comparable signals (Figure 5B). The tumor-to-normal tissue signal ratios were also calculated (Figure 5C), significant difference of the ratios between U87MG and C6 was clearly viewed ($P < 0.05$). All these findings were

consistent with the *in vivo* imaging results, demonstrating that ANP_{FAP} can be efficiently applied for *in vivo* imaging of FAP α .

DISCUSSION

Enzymes play important roles in many biological and physiological processes, and sometimes they can serve as valid biomarkers for various diseases. Thus, imaging enzymes help to investigate enzyme function and even diagnose diseases. Unique catalytic activities of enzymes enable design of smart optical probes that can be turned on upon meeting with the enzyme target. The self-quenching probe, Ac-GPGP-2SBPO, demonstrates specificity to FAP α , and it could be activated in FAP α transfected cells²⁹. The dual functional probe, Pyro-TSGPNQEQK(BHQ), developed by Zheng et.al can image FAP α and produce cytotoxicity simultaneously, by combination of pyropheophorbide α acid (Pyro) as a photosensitizer as well as a fluorophore whose fluorescence can be quenched by BHQ.⁹ The specificity of these probes to FAP α indicates the importance of the peptide sequence used as a linker in the activatable probes.

Different from previous studies, a new specific peptide substrate was selected by us according to the reported substrates specific to FAP α .^{9, 28, 29} Unlike Ac-GPGP-2SBPO,²⁹ which utilizes the strategy of self-quenching of the dye, ANP_{FAP} employs a quencher (QSY21) to make a NIR dye (Cy5.5) silent (Figure 1B), and the quenching efficiency was found to be 94%. In the enzymatic assay, ANP_{FAP} shows good sensitivity and selectivity toward FAP α (Figure 2). Approximately 12 fold fluorescence recovery of ANP_{FAP} can be achieved after incubation with 1 Mg/mL of FAP α for 1 h (Figure 2B). Furthermore for testing the specificity of ANP_{FAP}, DPPIV, which also belongs to the serine protease family and has 50% amino acid similarity to FAP α , was selected. Coupled expression of DPPIV and FAP α has been found in transformed astrocytic cells.³¹ The difference of these two enzymes mainly lies in their catalytic activities. FAP α is a strict endopeptidase while DPPIV is a strict exopeptidase.^{25–28} Based on the high similarity between DPPIV and FAP α , DPPIV was chosen as a specificity control for investigating the specificity of ANP_{FAP}. When incubating ANP_{FAP} with 1 Mg/mL of DPPIV, no activation of ANP_{FAP} can be observed (Figure 2C). Incubating ANP_{FAP} with another enzyme, MMP-2, also give similar results. Titration studies show nonlinear dose dependent activation of ANP_{FAP} toward FAP α (Figure 2D). One possible reason is that fluorescence recovery of ANP_{FAP} has reached maximum at 1 h incubation with higher concentration of FAP α such as 1 μ g/mL (Figure 2A), while that fluorescence intensities of the probe incubating with smaller amounts of FAP α for 1 h may not fully recovered. Collectively, these *in vitro* assays demonstrate that ANP_{FAP} is highly specific and sufficient to be used for further *in vivo* evaluation.

Recently, FAP α was found to be expressed in U87MG cells, making U87MG tumor model a good candidate for FAP α *in vivo* imaging.³¹ C6 tumor model which is FAP α negative was also generated as a control. Western blot results confirm that FAP α is indeed expressed in U87MG tumor but not in C6 tumor (Figure 3). Then the ability of ANP_{FAP} for *in vivo* imaging was evaluated in these mice models through intravenous tail vein injection of the probe. Despite limited delivery efficiency of Pyro-TSGPNQEQK(BHQ) after systematic administration reported before⁹, ANP_{FAP} shows efficient accumulation in U87MG tumor but not in C6 tumor (Figure 4A). By quantitative analysis of fluorescence intensities in U87MG tumor region, it can be seen that the signal increases from 0.5 h to 2 h and reaches maximum at 2 h (Figure 4B), indicating fast activation of the probe *in vivo* and 2 h is a good imaging time for future applications of ANP_{FAP}. For C6 tumor, only a slight increase of the fluorescence intensities is observed from 0.5 h to 1 h, which might be due to influx of activated probes from blood into tumor through enhanced permeability and retention effect.

Nevertheless, difference of fluorescence intensities between C6 tumor and U87MG tumor is significant ($P < 0.05$). It is worthy to note that since the probe is not actively targeted, it may have different levels of accumulations in two tumor models, which may make some contributions to the different fluorescence signals in tumors. Moreover, different from the absolute fluorescence intensities, tumor to normal tissue ratio of U87MG mice keeps increasing from 0.5 h to 4 h (Figure 4C), suggesting reduction of background signals all the time. *Ex vivo* imaging also shows significant difference of tumor-to-normal tissue ratios between C6 tumor and U87MG tumor (Figure 5C), which is consistent with the *in vivo* results. All these results demonstrate the high specificity of ANP_{FAP} and its high potential for serving as a NIR probe to image FAP α *in vivo*.

Interestingly, signals from the mouse body were also observed. Though autofluorescence was subtracted using the appropriate function of IVIS system, some signals could still be caused by autofluorescence of mice. An alternative imaging system, Maestro, may help further improve the imaging quality by using spectral unmixing function. Another possible reason is that FAP α can also be secreted from tumors, and soluble forms of FAP α have been found in bovine serum.³² Therefore, the soluble FAP α expressed in the blood or other tissues could cleave the probe to generate background signal. The *ex vivo* imaging study also confirms that besides the high uptake of ANP_{FAP} in U87MG tumor, background signals can be seen in liver, stomach, intestine and kidney (Figure 5B). Specific activation of probes in tumor is really hard to achieve and represents a common hurdle for enzyme imaging using activatable probes in this field, our finding here is really not a surprise. Take an example, for imaging MMPs, relatively high uptake of MMPs activatable probes in organs beside tumor has also been observed.^{18, 19} Regardless of the background signal, the encouraging thing is that we still can clearly visualize target tumor. To further optimize the probe and overcome the production of the non-specific background signal, the activatable probes could be encapsulated in polymers for protection during the circulation while be released when reaching tumors.

It should be pointed out that some of the limitations of optical imaging techniques can also hinder the use of ANP_{FAP} *in vivo*. For example, it is very hard to get quantitative information about FAP α , and the optical signals sometimes are misleading as they can be easily affected by many other factors such as depth of the probe presented *in vivo*, absorption and refraction of tissues, etc. To solve these problems, other imaging modalities such as positron emission tomography (PET) and magnetic resonance imaging (MRI) can be combined with fluorescence imaging. For example, MRI/NIRF activatable imaging of MMP-2 has been realized by loading Cy5.5 labeled MMP substrates onto iron oxide nanoparticles.³³ It is thus possible to use iron oxide nanoparticles to quench fluorescence of Cy5.5 labeled peptide substrate of FAP α and to realize both MR/NIRF imaging. An alternative strategy is to directly link ANP_{FAP} with iron oxide nanoparticles, and then dual modality imaging (NIRF/MRI) can be performed to image FAP α more efficiently. One example is pre-quenched MT1-MMP probe was anchored to magnetic nanocrystals for molecular imaging of invasive cancer cells.³⁴ It is expected that this approach could be adapted for improving the imaging efficiency of ANP_{FAP}. Lastly, it is worthy to note that FRET based activatable probe itself could provide a target-dependent photoacoustic signal.³⁵ With the photoacoustic signal, FAP α expressing tumors could be visualized more easily and clearly. Therefore, combining fluorescence imaging with other imaging modalities, ANP_{FAP} may serve as a more efficient probe for better understanding the distribution and function of FAP α *in vivo*.

CONCLUSION

A FAP α specific NIRF probe ANP_{FAP}, which is composed by a NIR dye Cy5.5, peptide substrate of FAP α , and a quencher QSY21, was designed and successfully synthesized. Fluorescent signals of ANP_{FAP} can be recovered in the presence of FAP α , while not by DPPIV or MMP-2, suggesting the high specificity of ANP_{FAP} toward FAP α . *In vivo* imaging studies further demonstrate that ANP_{FAP} can be activated rapidly in FAP α positive U87MG tumor but not in the negative C6 tumor. Overall, our study provide a new type of FAP α specific probe that can be used to image FAP α *in vivo* and to better understand the roles of FAP α *in vitro* and *in vivo*.

Acknowledgments

This work was supported in part by National Cancer Institute (NCI) 5R01 CA119053 (ZC) and the National Basic Research Program of China (No. 2011CB935800). J. L. was supported by a fellowship from the Chinese Scholarship Council (CSC).

ABBREVIATIONS

FAPα	Fibroblast activation protein-alpha
NIR	near infrared
FRET	fluorescence resonance energy transfer
p.i	post injection
HPLC	high-performance liquid chromatography
ROI	regions of interest

References

1. Rettig WJ, Garin-Chesa P, Beresford HR, Oettgen HF, Melamed MR, Old LJ. Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. *Proc Natl Acad Sci U S A*. 1988; 85:3110–4. [PubMed: 2896356]
2. Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, Old LJ, Rettig WJ. Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc Natl Acad Sci U S A*. 1994; 91:5657–61. [PubMed: 7911242]
3. Garin-Chesa P, Old LJ, Rettig WJ. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci U S A*. 1990; 87:7235–9. [PubMed: 2402505]
4. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006; 6:392–401. [PubMed: 16572188]
5. Cheng JD, Dunbrack RL Jr, Valianou M, Rogatko A, Alpaugh RK, Weiner LM. Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal model. *Cancer Res*. 2002; 62:4767–72. [PubMed: 12183436]
6. Huang Y, Simms AE, Mazur A, Wang S, Leon NR, Jones B, Aziz N, Kelly T. Fibroblast activation protein-alpha promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions. *Clin Exp Metastasis*. 2011; 28:567–79. [PubMed: 21604185]
7. Lee HO, Mullins SR, Franco-Barraza J, Valianou M, Cukierman E, Cheng JD. FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells. *BMC Cancer*. 2011; 11:245. [PubMed: 21668992]
8. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, Gopinathan A, Tuveson DA, Fearon DT. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*. 2010; 330:827–30. [PubMed: 21051638]

9. Lo PC, Chen J, Stefflova K, Warren MS, Navab R, Bandarchi B, Mullins S, Tsao M, Cheng JD, Zheng G. Photodynamic molecular beacon triggered by fibroblast activation protein on cancer-associated fibroblasts for diagnosis and treatment of epithelial cancers. *J Med Chem.* 2009; 52:358–68. [PubMed: 19093877]
10. Narra K, Mullins SR, Lee HO, Strzemkowski-Brun B, Magalong K, Christiansen VJ, McKee PA, Egleston B, Cohen SJ, Weiner LM, Meropol NJ, Cheng JD. Phase II trial of single agent Val-boroPro (Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer. *Cancer Biol Ther.* 2007; 6:1691–9. [PubMed: 18032930]
11. Petrovsky A, Schellenberger E, Josephson L, Weissleder R, Bogdanov A Jr. Near-infrared fluorescent imaging of tumor apoptosis. *Cancer Res.* 2003; 63:1936–42. [PubMed: 12702586]
12. Cheng Z, Levi J, Xiong Z, Gheysens O, Keren S, Chen X, Gambhir SS. Near-infrared fluorescent deoxyglucose analogue for tumor optical imaging in cell culture and living mice. *Bioconjug Chem.* 2006; 17:662–9. [PubMed: 16704203]
13. Miao Z, Ren G, Liu H, Jiang L, Cheng Z. Cy5.5-labeled Affibody molecule for near-infrared fluorescent optical imaging of epidermal growth factor receptor positive tumors. *J Biomed Opt.* 2010; 15:036007. [PubMed: 20615009]
14. Weissleder R, Tung CH, Mahmood U, Bogdanov A Jr. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol.* 1999; 17:375–8. [PubMed: 10207887]
15. Pham W, Choi Y, Weissleder R, Tung CH. Developing a peptide-based near-infrared molecular probe for protease sensing. *Bioconjug Chem.* 2004; 15:1403–7. [PubMed: 15546208]
16. Chen J, Tung CH, Allport JR, Chen S, Weissleder R, Huang PL. Near-infrared fluorescent imaging of matrix metalloproteinase activity after myocardial infarction. *Circulation.* 2005; 111:1800–5. [PubMed: 15809374]
17. Yoon SM, Myung SJ, Ye BD, Kim IW, Lee NG, Ryu YM, Park K, Kim K, Kwon IC, Park YS, Park CS, Moon DH, Kim do H, Do MY, Byeon JS, Yang SK, Kim JH. Near-infrared fluorescence imaging using a protease-specific probe for the detection of colon tumors. *Gut Liver.* 2010; 4:488–97. [PubMed: 21253297]
18. Zhu L, Xie J, Swierczewska M, Zhang F, Quan Q, Ma Y, Fang X, Kim K, Lee S, Chen X. Real-time video imaging of protease expression in vivo. *Theranostics.* 2011; 1:18–27. [PubMed: 21461134]
19. Zhu L, Zhang F, Ma Y, Liu G, Kim K, Fang X, Lee S, Chen X. In vivo optical imaging of membrane-type matrix metalloproteinase (MT-MMP) activity. *Mol Pharm.* 2011; 8:2331–8. [PubMed: 22014151]
20. Jaffer FA, Kim DE, Quinti L, Tung CH, Aikawa E, Pande AN, Kohler RH, Shi GP, Libby P, Weissleder R. Optical visualization of cathepsin K activity in atherosclerosis with a novel, protease-activatable fluorescence sensor. *Circulation.* 2007; 115:2292–8. [PubMed: 17420353]
21. Kim DE, Kim JY, Schellingerhout D, Shon SM, Jeong SW, Kim EJ, Kim WK. Molecular imaging of cathepsin B proteolytic enzyme activity reflects the inflammatory component of atherosclerotic pathology and can quantitatively demonstrate the antiatherosclerotic therapeutic effects of atorvastatin and glucosamine. *Mol Imaging.* 2009; 8:291–301. [PubMed: 19796606]
22. Abd-Elgalil WR, Cruz-Monserrate Z, Logsdon CD, Tung CH. Molecular imaging of Cathepsin E-positive tumors in mice using a novel protease-activatable fluorescent probe. *Mol Biosyst.* 2011; 7:3207–13. [PubMed: 21935563]
23. Bullok K, Piwnica-Worms D. Synthesis and characterization of a small, membrane-permeant, caspase-activatable far-red fluorescent peptide for imaging apoptosis. *J Med Chem.* 2005; 48:5404–7. [PubMed: 16107137]
24. Barnett EM, Zhang X, Maxwell D, Chang Q, Piwnica-Worms D. Single-cell imaging of retinal ganglion cell apoptosis with a cell-penetrating, activatable peptide probe in an in vivo glaucoma model. *Proc Natl Acad Sci U S A.* 2009; 106:9391–6. [PubMed: 19458250]
25. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem.* 1999; 274:36505–12. [PubMed: 10593948]

26. Edosada CY, Quan C, Wiesmann C, Tran T, Sutherlin D, Reynolds M, Elliott JM, Raab H, Fairbrother W, Wolf BB. Selective inhibition of fibroblast activation protein protease based on dipeptide substrate specificity. *J Biol Chem*. 2006; 281:7437–44. [PubMed: 16410248]
27. Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, Zhang Y, Kraus ML, Salakian S, Sridhar V, Wijnands R, Tennant MG. Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha. *J Biol Chem*. 2005; 280:19441–4. [PubMed: 15809306]
28. Edosada CY, Quan C, Tran T, Pham V, Wiesmann C, Fairbrother W, Wolf BB. Peptide substrate profiling defines fibroblast activation protein as an endopeptidase of strict Gly(2)-Pro(1)-cleaving specificity. *FEBS Lett*. 2006; 580:1581–6. [PubMed: 16480718]
29. Lai KS, Ho NH, Cheng JD, Tung CH. Selective fluorescence probes for dipeptidyl peptidase activity-fibroblast activation protein and dipeptidyl peptidase IV. *Bioconjug Chem*. 2007; 18:1246–50. [PubMed: 17489551]
30. Kong Y, Yao H, Ren H, Subbian S, Cirillo SL, Sacchettini JC, Rao J, Cirillo JD. Imaging tuberculosis with endogenous beta-lactamase reporter enzyme fluorescence in live mice. *Proc Natl Acad Sci U S A*. 2010; 107:12239–44. [PubMed: 20566877]
31. Balaziová E, Busek P, Stremenova J, Sromová L, Krepela E, Lizcova L, Sedo A. Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-alpha in transformed astrocytic cells. *Mol Cell Biochem*. 2011; 354:283–9. [PubMed: 21526345]
32. Collins PJ, McMahon G, O'Brien P, O'Connor B. Purification, identification and characterisation of seprase from bovine serum. *Int J Biochem Cell Biol*. 2004; 36:2320–33. [PubMed: 15313476]
33. Cha EJ, Jang ES, Sun IC, Lee IJ, Ko JH, Kim YI, Kwon IC, Kim K, Ahn CH. Development of MRI/NIRF 'activatable' multimodal imaging probe based on iron oxide nanoparticles. *J Control Release*. 2011; 155:152–8. [PubMed: 21801769]
34. Park J, Yang J, Lim EK, Kim E, Choi J, Ryu JK, Kim NH, Suh JS, Yook JI, Huh YM, Haam S. Anchored proteinase-targetable optomagnetic nanoprobe for molecular imaging of invasive cancer cells. *Angew Chem Int Ed Engl*. 2012; 51:945–8. [PubMed: 22162331]
35. Levi J, Kothapalli SR, Ma TJ, Hartman K, Khuri-Yakub BT, Gambhir SS. Design, synthesis, and imaging of an activatable photoacoustic probe. *J Am Chem Soc*. 2010; 132:11264–9. [PubMed: 20698693]

Figure 1.

A) Absorption spectrum of QSY21, Cy5.5 and ANP_{FAP}. B) Fluorescence emission spectrum of Cy5.5 labeled substrate and ANP_{FAP}.

Figure 2.

A) Time dependent fluorescence spectrum of 10 μM ANP_{FAP} with 1 $\mu\text{g/mL}$ FAP α at 37 $^{\circ}$ without FAP α , with 1 $\mu\text{g/mL}$ C. B) Time dependent fluorescence change of 10 μM ANP_{FAP} FAP α , 1 $\mu\text{g/mL}$ DPPIV or 1 $\mu\text{g/mL}$ MMP-2. C) FAP α concentration dependent fluorescence spectrum of 10 μM ANP_{FAP} with different concentrations of FAP α after a 1 h incubation at 37 $^{\circ}\text{C}$. D) Fluorescence increase ratio of ANP_{FAP} in the presence of various concentrations of FAP α after a 1 h incubation. Insert: phantom study of activated ANP_{FAP}.

Figure 3.
Western blot analysis of tumor tissue lysates.

Figure 4.

A) *In vivo* fluorescent imaging of subcutaneous C6 and U87MG tumor-bearing mice at 0.5, 1, 2, 3 and 4 h post tail vein injection of ANP_{FAP} (1 nmol). B) Fluorescence ROI analysis of tumor and muscle in mice bearing C6 and U87MG tumor from 0.5 to 4 h p.i. C) ROI analysis of tumor-to-normal tissue ratios of ANP_{FAP} in mice bearing C6 and U87MG tumor from 0.5 h to 4 h. Data are shown as Mean $\pm$ SD (n = 5 per group), * $P < 0.05$.

Figure 5.

A) *Ex vivo* imaging of tumor and normal tissue of C6 and U87MG mice after mice were sacrificed at 4 h post injection. The numeric label for each organ is as follow: 1, blood; 2, heart; 3, lung; 4, liver; 5, spleen; 6, pancreas; 7, stomach; 8, intestine; 9, kidney; 10, skin; 11, muscle; 12, bone; 13, tumor; 14, brain. B) ROI analysis of fluorescent signals from tumors and normal tissues. C) Tumor to normal tissue ratio of fluorescence intensities based on ROI analysis. Data are shown as Mean $\pm$ SD (n = 5 per group), * P < 0.05.

Scheme 1.
Chemical strategy for synthesis of ANP_{FAP}.