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14. ABSTRACT
Our overarching hypothesis is that FAP functions with other proteases in an extracellular communication network to digest certain proteins, thereby exposing signals stored in peptide regions that enable breast cancer cells to thrive in diverse microenvironments. FAP likely has important functions in two parts of the metastatic cascade: 1) FAP and proteases such as MMP-1 and MMP-9 cooperate to produce fragments of ECM proteins during adjacent tissue remodeling and these derivative peptides promote fibroblast growth, ECM deposition and angiogenesis; 2) cancer cell membrane FAP cleaves precursive A2AP to generate the more effective derivative for protecting and stabilizing fibrin within ECM margins of the expanding neoplastic cell mass as well as fibrin within cancer cell/fibrin/platelet emboli that lead to hematogenous metastasis. We believe that peptides that target and inhibit FAP on FAP-expressing cells can be produced by taking advantage of the substrate/active-site binding specificity of FAP.

This progress report documents the initial experiments and preparation of cell lines needed to complete the aims of the project. Substantial progress has been made and has resulted in production of 1 manuscript, 1 abstract presented at an international meeting, and three grant proposals one of which was funded and another still pending.

15. SUBJECT TERMS
Seprase, extracellular matrix, microembolism, tumor biology, angiogenesis

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Introduction

Fibroblast **a**ctivation **p**rotein-α (FAP), a prolyl-specific serine proteinase, is a constituent membrane protein of activated fibroblasts that synthesize some of the major components of the ECM. FAP is recognized on embryonic mesenchymal tissue and on fibroblasts during wound healing, but it is not expressed by normal, quiescent fibroblasts or benign tumor fibroblasts. FAP, however, is clearly over expressed by stroma within epithelial-derived cancers such as breast (1-3); however, its precise role is unknown. Although best known on activated fibroblasts, FAP is also expressed on epithelial cancer cells (2,4-6) and has been linked to invasive, matrix-degrading behavior of malignant melanoma and breast cancer cells (7,8). In animal models, over expression of FAP by the malignant cells also stimulates rapid growth of cancer (9,10).

FAP is a member of the dipeptidyl peptidase clan and/or structural homologs (DASH) family of prolyl-specific peptidases (11-13). The FAP protease is a dual function serine protease having both N-terminal prolyl-specific dipeptidase and endopeptidase activities (14-16). The dipeptidase activity is presumably important for modifying chemokines (11,13), but the role of FAP's endopeptidase activity (gelatinase) that degrades denatured collagen remains unclear; the latter activity is not possessed by dipeptidyl peptidase IV (17). Recently our collaborator, Dr. Patrick McKee's laboratory identified low molecular weight proteolytic fragments of type I collagen that are produced by FAP cleavage (18). His group also purified an enzyme in trace amounts in human plasma, which turned out to be a soluble form (sFAP) of membrane-bound FAP (mFAP) (19). Except for lacking the first 26 residues of its N-terminus that comprise the intramembrane and cytosolic segments, the soluble FAP's primary structure and proteolytic properties appear identical to naturally-occurring FAP. McKee's group made the unique discovery that sFAP cleaves precursive Met-alpha2-antiplasmin (methionine as the N-terminus: Met-A2AP) to yield a 12-residue N-terminally shortened derivative, Asn-alpha2-antiplasmin (19); both A2AP forms circulate in plasma and are responsible for >95% of the inhibition of plasmin activity towards fibrin, the latter also a recognized component of ECM as well as platelet/malignant cell/fibrin microemboli. Importantly, the FAP-cleaved A2AP maintains antiplasim activity and incorporates more readily into fibrin than its uncleaved counterpart. We reasoned that FAP on breast cancer cells might promote metastasis by increasing the formation of microemboli.

This synergy award is designed to determine if FAP proteolytic activity promotes formation of microemboli, tumor growth, and degradation of the extracellular matrix. This first period has been productive in terms of preparing the cells needed to pursue these questions and for working out the conditions for applying inhibitors while evaluating FAP-mediated matrix degradation. Kelly's part of the project involves determining microembolus formation in vivo and in vitro as well as aggressive matrix behavior in vitro.

Body

For in vivo experiments investigating blood borne tumor cells, it is imperative that fluorescently labeled tumor cells are made. We engineered FAP-GFP constructs and FAP-pluc plasmid constructs and obtained a luciferase expressing vector (see figure 1 for vector maps). The purpose of the GFP is two-fold. First it eases sorting of the the transfected cells by FACS because it eliminates the need for antibody incubations. Secondly, it enables us to easily distinguish the tumor cells from the host cells. Moreover, luciferase allows detection of tumor metastases in living animals using the IVIS camera.

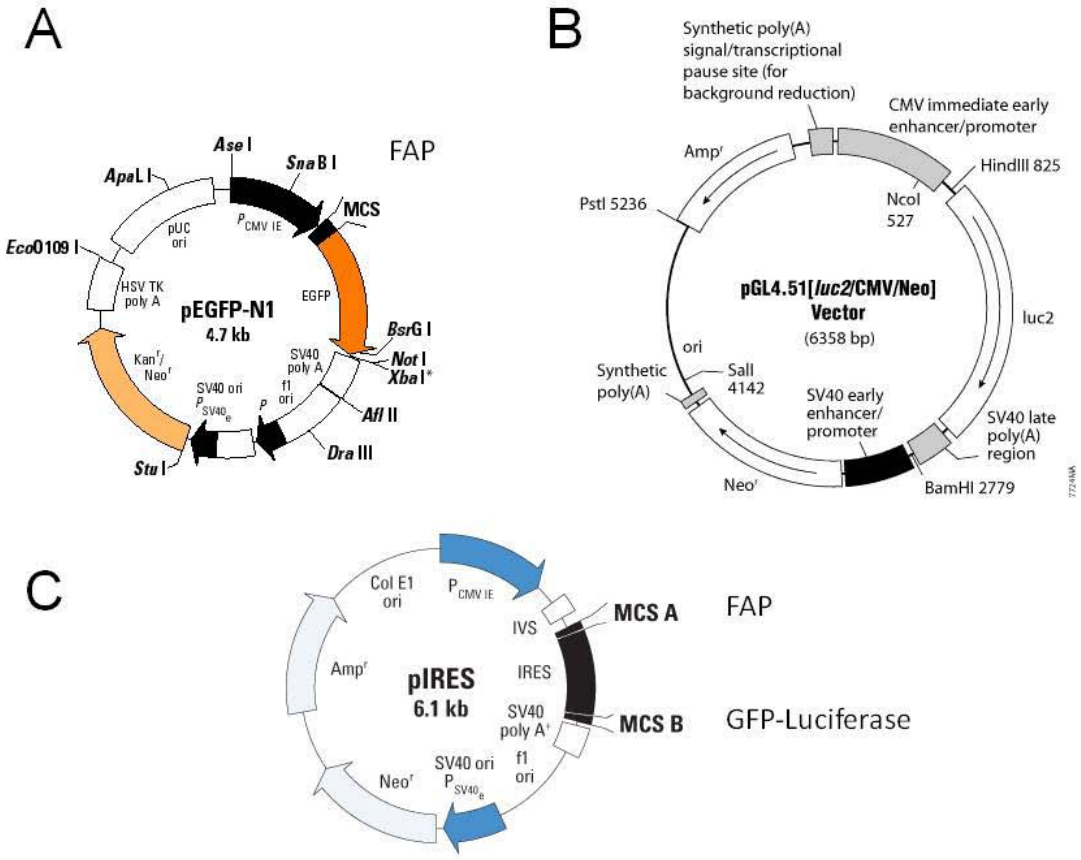


Figure 1. Plasmid vectors prepared to aid in identifying FAP tumor cells in microemboli. A) pEGFP has been produced with FAP cDNA in the multiple cloning site (MCS). B) pGL$.51 vector expresses luciferase and has been co-transfected with pEGFP into MDA MB-231 cells. C) The "pluc" vector is derived from pIRES and has been made to simultaneously express FAP, And a GFP-luciferase fusion protein.

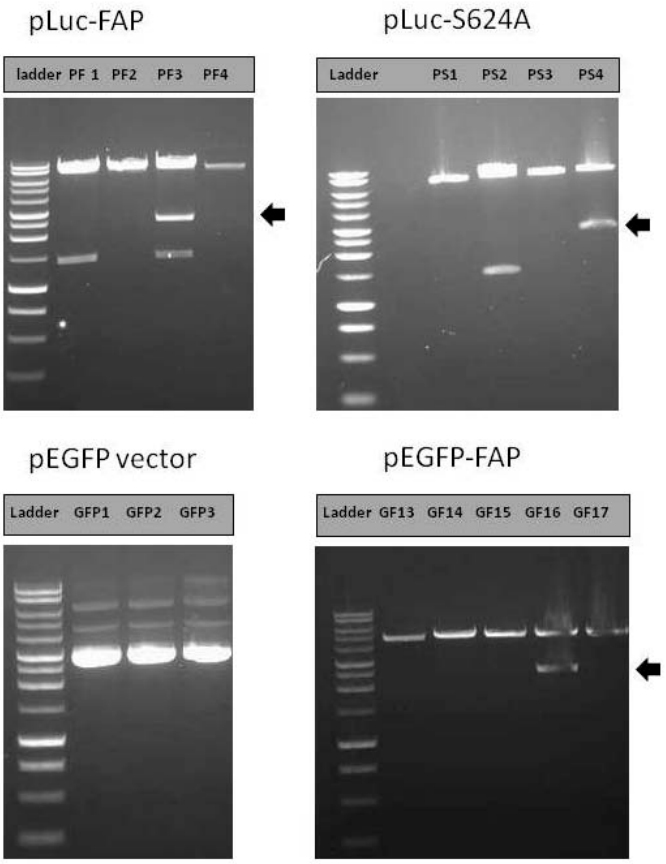
Two vector types were prepared. Ideally we will use the "pluc" vector which is based on the pIRES vector and simultaneously expresses FAP, GFP and luciferase (Fig. 1C). However, we have found that there are sometimes problems with the pluc vector. For one, it is difficult to efficiently transfect cells because of its size. Occasionally, it will alter the invasive phenotype of the cell regardless of FAP expression. Therefore, we also produced FAP-GFP constructs (Fig. 1A) to co-transfect with the luciferase expressing vector pGL4.51 (Fig. 1B). Figure 2 shows gels indicating successful isolation of bacteria colonies expressing FAP-GFP, FAP-pluc and pluc encoding a catalytically inactive mutant FAP where the catalytic serine has been changed to an alanine (S624A). (Needed for Specific aims (1 & 2).

Restriction and/or sequence analysis indicates that we were successful in introducing the FAP cDNA into the pluc vector. Similarly, FAP cDNA has also been inserted into the EGFP plasmid (Figure 2). These cells will be used for experiments to determine emboli formation (Specific aim 2).

We transfected FAP-expressing breast cancer cells with either: FAP-pluc or pluc only. We selected the cells shortly after transfection based on GFP expression and are performing

multiple sorts to achieve populations where a high percentage of cells express FAP or mutant FAP, GFP, and lucifierase. We also transfected GFP into MDA MB-435 breast cancer cells that express FAP endogenously and are working towards cloning populations of these cells.

Another area of progress has been our preparations for the work with the FAP-specific inhibitors that will be sent to us by the Co-PI Dr. McKee (Specific Aim 1 & 3). To prepare we have been testing the existing FAP inhibitor, PT-100 or talabostat for ability to inhibit matrix degradation. Our initial studies indicate that FAP inhibitors modestly suppress matrix degradation (see attached manuscript). We have begun investigating the effect of inhibiting both FAP (using PT-100 or PT-630) and MMPs (using BB-94) on matrix degradation by aggressive breast cancer cells. We have completed initial experiments with FAP inhibitors and FAP-expressing cells growing on fluorescent extracellular matrices and are now analyzing these results.



We have begun experiments with alpha2 AP and fibrinogen to learn how to set this up properly (Specific Aim 2 & 3).

We prepared a manuscript for publication on FAP inhibition and growth of tumors of breast cancer cells expressing mutant FAP (appendix).

Figure 2. Plasmid vectors prepared to aid in identifying FAP tumor cells in microemboli. Top panels) Left panel shows pluc vector with the FAP insert (lane PF3, bold arrow). Right panel shows identification of the clone with cDNA encoding the S624A mutant FAP in pluc vector (lane PS4, bold arrow). **Bottom panels)** Left panel shows pEGFP vector. Right panel shows a pEGFP vector with the mutant FAP insert (lane GF16, bold arrow).

Key research accomplishments:

- Produced pluc and EGFP plasmid vectors that express FAP and obtained a luciferase vector.
- Transfected MDA MB-231 with EGFP and pluc vectors
- FACS on MDA MB-435 and MDA MB-436 to grow FAP high and FAP low populations
- Did sorts for WTY-1 and WTY-6 to produce clones of cells expressing high levels of FAP
- Performed initial experiments to determine baseline matrix degradation with FAP-expressing cells
- Began trials with FAP inhibitors on the matrix degradation of FAP-expressing cells.
- Set up a protocol that will allow us to observe fibrinolysis. This will be used to determine if FAP cleavage of A2AP stabilizes fibrin and promotes fibrin accumulation.

Reportable outcomes

Manuscript:
"Fibroblast activation protein-α (FAP) promotes tumor growth and invasion through protease activities and non-enzymatic functions**"** Yan Huang, Sophie Wang, Noel R. León, Barry Jones, Nazneen Aziz and Thomas Kelly Submitted

Abstract/Presentation:
"Tumor growth is slowed by an inhibitor of DASH proteases in a mouse model of human breast cancer" Thomas Kelly, Noel R. León, Kevin Kelly, Barry Jones and Nazneen Aziz 3rd International Congress on Proteases and Dipeptidyl Peptidases, April 23-25, 2008, Antwerp, Belgium

Funding applied for:
Two predoctoral fellowships
1) USAMRDC-BCRP Pre-doctoral fellowship BC093509 "Blood-borne tumor-host cell emboli in metastasis", PI Avis Simms **Decision September, 2009**
2) Arkansas Breast Cancer research Fellowship "Blood-borne tumor-host cell emboli in breast cancer metastasis" PI Avis Simms (Inaugural recipient), **Funded 7/1/2009**

ARRA challenge grant NIH 1 RC1 CA145194-01 "Fibroblasts and platelets can cooperate with cancer stem cells to drive metastasis." PIs Jerry Ware and Thomas Kelly (Not funded)

Ongoing development of cell lines
FAP-GFP-luc cells in production that is originally derived from MDA MB-231 human breast cancer cells. FAP-pluc cells in production that is originally derived from MDA MB-231 human BrCa cells

Research opportunities Avis Simms passed her qualifying and is now a candidate for a PhD in Interdisciplinary Biomedical Sciences.

Conclusions
In summary we have made significant and important progress on the SOW for aims 1-3 by positioning ourselves to complete the experiments that will directly test the possibility that FAP promotes formation of microemboli and that these facilitate metastasis of breast cancer.

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Appendix

Manuscript: Fibroblast activation protein-α promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions*

Fibroblast activation protein-α promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions*

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Running Title: Tumor growth is promoted by catalytically-inactive FAP

Key Words: serine protease; seprase; dipeptidyl peptidase IV; invadopodia; angiogenesis, metastasis

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Abstract

Fibroblast activation protein-α (FAP) is a cell surface, post-prolyl serine protease that is expressed in human breast cancer but not in normal adult tissues. Previously we showed that FAP expression by tumor cells increased tumor growth rates in a mouse model of human breast cancer. Here the role of the proteolytic activities of FAP in promoting tumor growth, matrix degradation and invasion was investigated. Breast cancer cells expressing active FAP were injected into the mammary fat pads of female SCID mice. The mice were treated with normal saline or inhibitors of prolyl peptidases Gly-boroPro (talabostat, PT-100); Glu-boroPro (PT-630); or 1-[[(3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidine (LAF-237, vildagliptin). Both boroPro compounds are effective against FAP at nanomolar concentrations; however, micromolar LAF-237 is required to inhibit FAP. All three compounds inhibit the closely related dipeptidyl peptidase IV (DPPIV) at nanomolar concentrations. PT-630 and LAF-237 did not slow growth of tumors produced by any of the three cell lines expressing active FAP. Talabostat slightly decreased the growth rates of the FAP-overexpressing tumors but because PT-630 and LAF-237 did not, the growth retardation was likely not related to the inhibition of FAP or DPPIV. Moreover, mice inoculated with breast cancer cells expressing a catalytically inactive mutant of FAP (FAP^{S624A}) also produced rapidly growing tumors. *In vitro* experiments revealed that all three inhibitors reduced matrix degradation by cells expressing active FAP relative to solvent control indicating a role for the FAP proteolytic activity in matrix degradation. However, even with prolyl peptidase inhibition, matrix degradation by FAP-expressing cells remained relatively high. Moreover, matrix degradation by FAP^{S624A} cells was higher than that of cells that

did not express FAP and comparable to those that expressed active FAP. Matrix metalloproteinase-9 (MMP-9) accumulated to higher levels in medium conditioned by MDA MB-231 cells expressing inactive FAP^{S624A} or active FAP than it did in medium conditioned by control transfectants that did not express FAP. The MMP-9 is biologically active because cells expressing FAP^{S624A} or FAP invade into type I collagen gels more efficiently than control transfectants that lack FAP and accumulate low levels of MMP-9. Interestingly, FAP^{S624A} or FAP expression alters cell signaling because extracts of these cells contain a 77 kDa tyrosine-phosphorylated protein (YPP) that is not detected in the extracts of control transfectants. We conclude that the proteolytic activity of FAP increases matrix degradation, but FAP primarily drives rapid tumor growth independent of its protease activity by stimulating tumor cells to increase production of biologically active factors such as MMP-9.

Introduction

Fibroblast activation protein-α (FAP), also called seprase, is an integral membrane serine protease (1-4) that is a member of the SC clan of post-prolyl peptidases and is closely related to dipeptidyl peptidase IV (DPPIV) (5-7). FAP exhibits a DPPIV-like fold, featuring an alpha/beta-hydrolase domain and an eight-bladed beta-propeller domain (8). FAP has both exopeptidase and endopeptidase activities. Like the closely related DPPIV, it cleaves NH3-X-Pro peptides (6) but FAP is uniquely able to degrade gelatin (6,7). α2-antiplasmin has been identified as a natural substrate of FAP and cleavage of α2-antiplasmin by FAP results in more efficient cross linking of α2-antiplasmin to fibrin while retaining the inhibitory action of α2-antiplasmin to plasmin (9). Thus FAP may have a role in regulating the dissolution of fibrin by plasmin.

Recent work has suggested many biological functions for FAP (10). FAP may have roles in osteoarthritis (11,12), pulmonary fibrosis (13), fibrosis in liver diseases such as hepatitis (14,15) and cirrhosis (16), as well as a role in numerous cancers including multiple myeloma (17,18), pancreatic cancer (19), colon cancer (20), melanoma (2,21,22), ovarian carcinoma (23,24), squamous cell carcinoma of the esophagus (25), and breast cancer (26,27) to name a few. Because of the conspicuous absence of FAP in normal adult tissues and its marked up-regulation in these pathologic states, there has been interest in the potential of small molecule inhibitors of the FAP protease. Moreover, the closely related DPPIV has already been the target of therapeutic agents. Indeed, several DPPIV inhibitors have been approved for type 2 diabetes because they increase the levels of insulin by prolonging the half life of glucagon like peptide-1 (GLP-

1) and glucose dependent insulinotropic polypeptide (GIP) that are substrates for DPPIV. NVP LAF-237 or vildagliptin is one of the DPPIV inhibitors approved for type 2 diabetes (28). In this study we inhibited FAP using Val- boroPro (also called PT-100 or talabostat) which inhibits FAP with a Ki of 6.2 nM and DPPIV with a Ki of 0.18 nM (29) and Glu boroPro (also called PT-630) which inhibits DPPIV with a Ki of 1.8 nM and FAP in the nM range (29). To distinguish between the biological consequences of inhibition of FAP and DPPIV, we also used LAF-237 which inhibits DPPIV with a Ki of 17 nM (28) or 51 nM (30) but is a much less potent inhibitor of FAP with Ki of over 20 μM (31,32).

FAP is expressed to high levels in tumors of human breast cancer but is not expressed in normal breast tissue (4,26). FAP is a serine protease that is anchored to the plasma membrane by an N-terminal signal sequence that is not cleaved (5,33-35). FAP is synthesized as a 97 kDa protein that is proteolytically inactive and requires assembly into a dimer of 170 kDa to become an active protease (35-37). FAP protease activity is abnormally high in extracts of patient tumors indicating that increased expression leads to increased FAP protease activity in breast cancer (36).

In this study, the role of the proteolytic activity of FAP in promoting tumor growth and angiogenesis was investigated using small molecule inhibitors of FAP and breast cancer cells engineered to express a catalytic mutant of FAP. Our findings indicate that the protease activity of FAP is not critical for its growth promoting and invasive functions in tumors of human breast cancer cells. The results suggest that cell surface expression of FAP stimulates elevated production of factors such as MMP-9.

Materials and Methods

MDA MB-231 human breast adenocarcinoma cells transfected with empty pcDNA 3.1 (Neo) or this vector containing the insert for wild type FAP (WTY-1 and WTY-6) were produced and maintained as described earlier (38).

Production of cDNA encoding S624A mutant A cDNA for human FAP (38) in the pcDNA3.1 vector was subjected to oligonucleotide-directed mutagenesis (TransformerTM site-directed mutagenesis kit; Clontech, Palo Alto, CA) to yield constructs having a single point mutation (T $\rightarrow$ G) that changed the codon for serine 624 (TCC) to a codon for alanine (GCC). The mutagenic primer was 5'-CATATGGGGCTGG**G**CCTATGGAGGATAC-3' (mutant base in bold) and the selection primer eliminated a BSTZ174 site and was 5'-TATCTTATCATGTCTGTATACCGTCGACCTCTAGCT-3'. Sequence analysis of the entire insert was performed to confirm that the cDNA was identical to wild-type FAP, except for the point mutation. The analysis was done by Alan Gies in the DNA sequencing core facility in the Department of Microbiology & Immunology at the University of Arkansas for Medical Sciences (UAMS).

Transfection of cells The cDNA encoding S624A mutant was transfected into the human breast cancer cell line MDA MB-231 using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as recommended by the supplier. The transfectants were selected with G418 (800 μg/ml) and sorted by Fluorescence activated cell sorting based on immunofluorescence staining with F19 antibody to FAP by Ashley Whitlow using a

FACSCaliber instrument (Becton Dickinson) using FACScan, FACStar with turbo sort in the flow cytometry core in the Department of Microbiology, UAMS. The flow cytometry procedures and cytospin procedures were described previously (38,39). Once, sorted the cells were maintained in growth medium with G418 (400 μg/ml). The transfectants were used to form tumors in animals when the population of FAP-positive cells comprised at least 40% of the cells. To analyze FAP expression, extracts were prepared and subjected to gelatin zymography and western blotting as described previously (38).

Tumor Biology Implantation of (2×10^6) tumor cells into each of 4 mammary fat pads per female SCID mouse, monitoring tumor growth with calipers was performed as previously described (38). In the first animal experiment there were 7 animals per group. In the second experiment there were 5 animals per group.

Preparation of talabostat, PT-630 and LAF-237. A 0.1-M stock solution prepared by dissolving talabostat at a concentration of 30.6 mg/ml in 0.1N HCl and PT-630 at a concentration of 28.1 mg/ml was supplied by Point Therapeutics. Stock solutions were stored at -20^oC in small aliquots (50-200 μl). Working solutions are prepared by diluting the 0.1M acidified stock solution into sterile normal saline. Working solutions were made at concentrations that will deliver 10 μg/mouse talabostat; 200 μg/mouse PT-630, and 100 μg/mouse LAF-237 in 0.2 ml. To avoid excessive production of the cyclic form of the compounds, it is essential that talabostat was not held at neutral pH for longer than 10 minutes before administration. Consequently, the working saline solutions were

prepared with ice cold reagents in the animal facility, immediately prior to administration to mice.

Talabostat and PT-630 administration. 0.2 ml of talabostat or PT-630 solution was administered orally via a blunt gavage needle fitted to a 1-ml syringe once daily.

Matrix degradation assay Glutaraldehyde-crosslinked gelatin films with immobilized FITC-fibronectin were prepared on cover slips (18 mm circular glass) as described previously (40). Cells were seeded onto the FITC-fibronectin matrices and grown in growth medium for 48 hours at 37^{o} C, 5% CO_2. Adherent cells were washed three times with sterile PBS and then fixed and prepared for fluorescence microscopy as described previously (40,41). Coverslips were mounted in 80 % glycerol, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and a trace amount of *p*-phenylenediamine (Sigma, St. Louis, MO). Cells were observed and images captured using a Zeiss Axioskop 2 mot plus microscope and the Zeiss AxioCam MRc digital camera in the digital microscopy core laboratory at University of Arkansas for Medical Sciences. The digital images were analyzed using the NIH Image J program.

MMP-9 To investigate MMP-9 accumulation in conditioned medium from the cells, gelatin zymography and western blots using the 6-6B monoclonal antibody to MMP-9 (Oncogene Sciences; (42)) were performed as described (43,44)).

YPPs To investigate proteins phosphorylated on tyrosines, western blots of cell extracts were probed with monoclonal antibody 4G10 to phosphotyrosine (Upstate Cell

Signaling, Lake Placid, NY). Protein was determined by the bicinchoninic acid assay (Pierce).

Invasion assay Type I collagen gels (0.3 mg/ml) were prepared and used for invasion assays as described previously (40) except that invading cells were directly counted using a Coulter Z1 counter (Coulter Corporation, Miami, FL).

Results

Elevated expression of FAP is associated with human breast cancer (26,45) and FAP promotes tumor growth and increased microvessel densities in a mouse model of human breast cancer (38). This study investigates the role of the proteolytic activity of FAP in promoting the rapid tumor growth and aggressive behavior of FAP-expressing tumor cells. Expression of a catalytically inactive FAP and inhibitors of the SC clan of post-prolyl peptidases were used to investigate the role of the FAP protease on tumor biology. To produce a proteolytically-inactive form of FAP, the catalytic serine at position 624 was mutated by site-directed mutagenesis to alanine. MDA MB-231 cells were transfected with the mutant cDNA in the pcDNA3.1 expression vector, selected with G418, enriched by fluorescence activated cell sorting for cells positive for FAP, and termed S624A-5. The S624A-5 cells are not a clonal population, but a mixed cell population that stably expresses FAP^{S624A}. There were 42% cells positive for surface expression of FAP^{S624A} as judged by immunohistochemistry and FACS with F19 monoclonal antibody to FAP on living cells (Fig. 1; FACS top right panel). The 170 kDa FAP activity was not detected in zymograms of extracts of S624A-5 cells that were enriched using wheat germ agglutinin chromatography and loaded with equal protein relative to the other extracts (Fig. 1, bottom left, S624A-5). However, the protease activity was detected in extracts of transfectants expressing wild type FAP (Fig. 1, bottom left, WTY-1 & WTY-6, see also (38)). Note that MMPs were not detected by zymography in these cell extracts due to the enrichment of FAP by binding to wheat germ agglutinin, the presence of EDTA in the extraction buffer, and reducing agents in the gel sample buffer used for optimal identification of FAP activity. MMPs are released

into the medium by these cells as shown below. Importantly, western blot analysis with mAb F19 revealed that the 170 kDa dimer of FAP was formed by the mutant FAP (Fig. 1, bottom right, S624A-5). Thus, the failure to detect protease activity in the S624A-5 extracts is due to the loss of the catalytic serine and not to a defect in folding of the 97 kDa monomer and subsequent degradation. Comparable levels of the 97 kDa monomer were also detected in extracts of cells expressing active and mutant FAP (Fig. 1, bottom right).

FAP expression promotes growth of tumors independent of its protease activity

The growth of tumors of breast cancer cells expressing proteolytically active FAP was investigated to determine if small-molecule inhibition of the post prolyl peptidases including FAP could perturb tumor growth. Mice were given 1.3 mg/kg L-valine-L-boroproline called PT-100 or talabostat, 13.3 mg/kg L-glutamyl L-boroproline called PT-630; or 6.7 mg/kg 1-[[(3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidine called LAF237 by oral gavage once daily. Tumors formed of FAP-expressing human breast cancer cells, including WTY-1 (Fig. 2A) and WTY-6 (Fig. 2B) which are MDA MB-231 cells that engineered to overexpress wild type FAP and MDA MB-435 cells that express FAP endogenously (Fig. 2C). Of these molecules only PT-100 or talabostat appeared to slow tumor growth (Fig. 2A-C) and this appeared especially pronounced in the case of the MDA MB-435 cells where production of tumors large enough to measure was delayed by nearly twelve days relative to control (Fig. 2C). But even this reduction in tumor growth by PT-100 did not achieve statistical significance as compared to the rapidly growing tumors in saline treated animals with active FAP. Moreover, when

animals were sacrificed and tumors weighed, there was no statistically significant difference in size in tumors grown in the presence of normal saline versus those exposed to the inhibitors ($P > 0.05$). However, the lowest average weights were consistently obtained for tumors treated with PT-100. So although PT-100 slowed tumor growth, the tumors still grew rapidly relative to tumors that did not express FAP.

The inhibitor work suggested that FAP expression was sufficient to stimulate tumor growth independent of its protease activities. To further investigate the growth stimulation of FAP in the absence of proteolytic activity, transfectants of MDA MB-231 cells that do not express FAP (Neo) and those that express membrane-bound and inactive FAP^{S624A} (S624A-5, Fig. 1) were injected subcutaneously into the mammary fat pads of female SCID mice as previously described (38). The S624A-5 cells formed rapidly-growing tumors that grew considerably faster than tumors of Neo transfectants that do not express FAP (Fig. 2D) and at rates similar to tumors of cells expressing proteolytically-active FAP (38). The yield of tumors from cells expressing the inactive FAP (S624A-5, 26 tumors/28 injection sites; 93%) was greater than that observed for Neo control (19 tumors/28 sites; 68%) and comparable to that observed with WTY-1 and WTY-6 cells expressing active FAP (23 tumors/28 sites; 82% and 28 tumors/28 sites respectively; 100% (38)). Tumors of cells expressing FAP^{S624A} were considerably larger than those of cells lacking FAP (Neo) as judged by the average wet weight of the excised tumors (Neo, 0.066 g ± 0.038; S624A, 0.8 g ± 0.41) and similar to those of tumors of cells expressing wild type FAP (38). A second animal experiment was performed using S624A-5 cells that were sorted to 41% positive cells. Implantation of

these cells into the mammary fat pads of female SCID mice again resulted in rapidly growing tumors (Fig. 2D). The second experiment also had a high tumor yield (17 tumors/20 injection sites; 85%). The fact that tumors grew rapidly with catalytically inactive FAP and small-molecule inhibition of FAP proteolytic activity did not appear to dramatically suppress tumor growth suggested that non-enzymatic functions of FAP may responsible for the increased tumor growth.

FAP promotes matrix degradation and invasion by tumor cells independent of its protease activity The proteolytic degradation of fluorescent fibronectin matrices by cells expressing proteolytically active FAP was investigated in the presence or absence of FAP inhibitors. In this assay, the cells adhere to the matrix surface and fluorescence-negative black spots are observed where the matrix has been degraded (2,40). These studies focused on the WTY-1 cells that over express proteolytically active FAP. Cells were inhibited with 1 μM of the inhibitors and allowed to interact with the matrices for 48 hours. The inhibitors were replenished after 24 hours. Inhibition of FAP/DPPIV reduced matrix degradation as compared to uninhibited cells. However, matrix degradation remained high, even in the presence of FAP/DPPIV inhibitors (Fig 3A). Two different analyses were used to quantify the matrix degradation. In the first, the fluorescent matrices were examined by randomly choosing five fields of the matrices and scoring these for presence or absence of proteolytic degradation. The combined results of four trials revealed that all prolyl peptidase inhibitors decreased matrix degradation relative to solvent control but that the matrix degradation remained relatively high (Fig. 3B). Next, the matrices with obvious degradation were identified

and analyzed for extent of matrix degradation. Three separate fields were analyzed for each conditioned. The percentage of matrix field area that was degraded was determined using the NIH ImageJ program. This analysis revealed that in areas where matrix proteolysis was detected there was less matrix degradation where prolyl peptidases were inhibited relative to solvent control (Fig. 3C).

The proteolytic degradation of fluorescent fibronectin films by cells that do not express FAP (Neo), cells that express FAP^{S624A}, and cells expressing wild type and active FAP (WTY-1 & WTY-6) was also investigated. Cells expressing either mutant FAP^{S624A} or wild-type FAP degraded the matrix more extensively than control transfectants that did not express FAP (Fig. 4A, arrows). The matrix degradation was quantified in three random fields for each cell type using the Image J program from NIH and between 249 and 367 holes were detected (Fig. 4B). The majority of the matrix degradation holes produced by the different cell types were too small to register a percent area degraded. This threshold proved useful for quantifying the relative levels of extensive matrix degradation. Only 4% of the holes produced by the cells that did not express FAP were large enough to record a percent area degraded (Fig. 4B). Sixteen percent of the holes produced by cells expressing FAP^{S624A} had measurable degradation area (Fig. 4B). This was four-fold greater than Neo cells that do not express FAP and similar to the percentage observed with cells expressing active FAP. In the FAP-expressing cells, WTY-1 and WTY-6, 16-22% of the holes had measurable areas (Fig. 4B). FAP^{S624A} and FAP-expressing cells also had a higher percentage of the total matrix area degraded in three fields. The differences in overall matrix degradation by the different cell types

were significant (ANOVA, with $P = 0.0003$), and each type of FAP-expressing cell showed greater matrix degradation than the Neo control as shown by T Tests (Fig. 4B).

MMPs are major mediators of matrix degradation at invadopodia (2,40,46) and because prolyl peptidase inhibition produced a relatively modest decrease in matrix degradation we investigated the possible effects of FAP expression on matrix metalloproteinase (MMP) accumulation in conditioned medium. MMPs in serum-free media were evaluated after equal numbers of each cell type grew in an equal volume of medium for 24 h. Thirty micrograms of protein were suspended in sample buffer devoid of reducing reagent and used for gelatin zymography or Western blotting with mAb 6-6B to MMP-9 as described earlier (42,43). The major MMP detected by zymography in conditioned medium of these cells was at 92 kDa corresponding to pro-MMP-9 (Fig. 5A, left panel). Moreover, the 92 kDa activity was elevated in conditioned medium from FAP and FAP^{S624A}-expressing cells as compared to the control transfectants (Fig. 5A, left panel). Western blotting confirmed that MMP-9 release was increased 2-4 fold in conditioned medium from FAP expressing cells relative to the control transfectants (Fig. 5A, right panel). Results shown are representative of three separate determinations.

The invasion potential of the different cell types was investigated to determine if the positive correlation previously established between matrix degradation by functional invadopodia and invasiveness existed in this system (40). For these experiments we used type I collagen gels as described previously (40). Cells that didn't invade into the gels remained on top and were removed by limited collagenase digestion and trypsin

treatment. The collagen gels, with embedded invading cells, were digested by extensive collagenase digestion and the cells were counted (40). FAP^{S624A} and FAP-expressing cells invaded to a significantly higher degree than Neo cells that did not express FAP (Fig. 5B). The experiments were performed in triplicate. A statistically significant increase in invasion was observed for all FAP-expressing cells as compared to Neo controls (T-test; S624A $P = 0.00004$; WTY-1 $P = 0.000001$; and WTY-6 $P = 0.009$). The fact that cells expressing the catalytic mutant of FAP are active in degrading the matrix and invading is consistent with increased MMP-9 functioning to degrade matrix and promote invasion by the FAP^{S624A} and FAP-expressing cells.

The increased accumulation of MMP-9 and the more aggressive phenotype of cells expressing active or inactive FAP suggested that FAP might alter cell signaling. Tyrosine phosphorylated proteins (YPPs) were investigated in regard to molecular mechanisms of growth stimulation and invasion of FAP expressing cells. A 77 kDa YPP was identified in extracts of FAP^{S624A} and FAP-expressing cells that is not detected in the control transfectants (Fig. 7). Conversely, the control transfectants express a 71 kDa YPP that is not detected in any of the FAP-expressing cells. These molecular weights correspond well to the invadopodial specific 77 kDa YPP and the 71 kDa YPP of normal focal contacts identified earlier by Mueller and co-workers (47).

Discussion

This work demonstrates that surface expression of human FAP is sufficient for enhanced tumor growth and invasion and that the protease activity of FAP is not required for these functions. These findings imply that surface expression of FAP stimulates cell signaling independent of its protease activity. Our findings are consistent with those of others showing that both wild-type and catalytic mutants of FAP are biologically active (48). However, in a murine melanoma system, FAP acts as a tumor suppressor and expression of either wild type or mutant FAP inhibits tumor growth (48). The apparently contradicting findings of FAP-stimulation of growth in our human breast cancer model versus FAP-suppression in the murine melanoma model is likely due to the cellular context in which the FAP is expressed. Signaling molecules that are available for activation by FAP expression may determine the type of signal (stimulatory or inhibitory) that is transmitted (5). Additionally, there may be contexts where the FAP protease activity is critical. For example, human embryonal kidney cells expressing wild type murine FAP form rapidly growing tumors but these cells expressing murine FAP^{S624A} form slow growing tumors in an animal model (49). These findings suggest an important role for the protease activity in growth promotion in this model system. One potential drawback of work with catalytic mutants of FAP in animal models is the presence of wild type and active mouse FAP. Small molecule inhibitors of prolyl peptidases overcome this problem because they inhibit all exposed prolyl eptidases whether they are in the stroma or on the tumor cells themselves.

Several lines of evidence suggest that FAP activates cell signaling that changes cell behavior. First, MMP-9 accumulation in conditioned medium is high for cells expressing FAP^{S624A} or FAP. The increase in MMP-9 levels of FAP-expressing cells is particularly important because it is a pro-angiogenic factor (50-53) and thus may be an important mediator of the increased angiogenesis observed in tumors of cells expressing FAP. For example, early studies revealed that MMP-9 null mice have a delay in angiogenesis in the growth plates of developing bone (50). Subsequent work showed that MMP-9 promotes angiogenesis during carcinogenesis of pancreatic islets by releasing and mobilizing vascular endothelial growth factor (VEGF) (52). MMP-9 is also associated with the pro-angiogenic switch in a mouse model of cervical cancer (54). Moreover, MMP-9 is a potent mediator of the matrix degradation that facilitates tumor invasion and metastasis. Second, changes in YPPs are similar between cells that express FAP^{S624A} or FAP and different from those in cells that lack FAP. Interestingly, others have shown that extracts of membrane preparations enriched for invadopodia or podosomes--the membrane protrusions of invasive cells that contact and degrade extracellular matrix--contain four major YPPs at 150 kDa, 130 kDa, 81 kDa, and 77 kDa (47). These workers also identified YPPs at 150 kDa and 71 kDa as components of normal focal contacts (47). Here, the 77 kDa YPP identified in extracts of cells expressing active or inactive FAP was identified without enriching for invadopodia and the 71 kDa YPP was identified in control transfectants without enriching for focal adhesions.

The mechanisms of FAP activation of tumor cells are unknown but the findings are consistent with the idea that FAP mediates its effects by forming complexes with

signaling molecules at the cell surface (for a review see (5)). FAP has only six amino acids in its cytoplasmic domain and these do not appear capable of transmitting signals. However, integrins are known to signal increases in expression of several MMPs. For example, MMP-2 secretion can be up-regulated by signaling through $\alpha v\beta_3$ integrin (55). In addition, MMP-9 expression and invasion can be increased by FAK and src activities, both of which can be activated by integrins (56). Particularly relevant is the fact that FAP can associate with β_1 integrins, including $\alpha_3\beta_1$ (57,58). Integrin $\alpha_3\beta_1$ is expressed by MDA MB-231 cells (59) and has been linked to elevated MMP-9 release by these cells (60). Thus, the results are consistent with a mechanism where FAP mediates its pro-growth and pro-angiogenic effects by associating with and activating integrins, thereby causing increased release of MMP-9. The MMP-9 then acts within the tumor microenvironment. Recent work has shown that FAP and MMP-9 can cooperate to degrade gelatin (61). The integrin activation also coordinates with MMP-9 to promote tumor invasion and metastasis.

FAP is recognized as an excellent target for therapies directed against epithelial cancers because its expression is induced in the tumor microenvironment but is very limited in normal adult tissues (62). Current trials utilize antibodies to FAP in an effort to provide target specificity for toxic agents (62,63) and such studies continue to have promise. However, the serine protease activity is another obvious target for anti-FAP therapeutics. The results of this study suggest that targeting FAP with agents designed to inhibit the FAP protease activity will not be effective. However, antibodies that inhibit the protease activity of FAP were effective in reducing the growth of HT-29 colorectal

carcinoma cells in an animal model (64). Moreover, talabostat had anti-tumor effects by countering immune suppression apparently caused by FAP protease activity (65,66). Nevertheless consistent with the findings of this study, a phase II trial using Val boroPro failed to produce a clinical effect in colorectal cancer patients with metastatic disease (67). More studies are required to clarify the roles of the protease activity and complex formation by FAP in its biological functions. While, inhibition of FAP protease activity may prove clinically important in some settings another intriguing possibility is to attempt to use the proteolytic activity of FAP against tumor cells. Progress is already being reported on this front by a couple of groups including a focus on photodynamic therapy based on FAP to target tumor cell killing (68,69) and prodrug toxin that is activated by FAP to kill stromal cells of tumors (70).

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Figure Legends

Figure 1. Characterization of MDA MB 231 cells engineered to express FAP^{S624A}.

A. Photomicrograph of a cytospin of cells transfected with FAP^{S624A} catalytic mutant (called S624A-5). Cells stained with F19 mAb to FAP appear brown due to the DAB reaction product while the blue hematoxylin detects all cells. **B.** FACS showing cell surface FAP when cells expressing FAP^{S624A} were stained with F19 mAb to FAP (blue trace) but not when stained with non-specific IgG (black trace). **C.** Gelatin zymogram showing a lack of gelatinolytic activity in catalytic mutant lane (S624A-5) but there is gelatinase activity in cells expressing wild type FAP (lanes WTY-1 & WTY-6). **D.** The catalytic mutant can assemble into the 170 kDa dimer as detected by Western blot (S624A-5).

Figure 2. Breast tumors expressing FAP grow rapidly even when FAP catalytic activity is inhibited.

A-C. Inhibition of DASH proteases by talabostat slows growth of breast tumors in the mammary fat pads of SCID mice. Tumor volumes plotted over time of FAP-expressing human breast cancer cells in SCID mice treated with saline (♦), LAF237 (●), PT-630 (▲), or talabostat (■). The tumors are formed from the following cells: A) WTY-1; B) WTY-6; and C) MDA MB-435. **D.** Growth of tumors from S624A-5 cells (♦) and Neo cells (▲). In all cases tumor growth is recorded as volume (cm^3).

Figure 3. Small-molecule inhibitors of FAP slightly reduce matrix degradation by cells expressing active FAP.

A. WTY-1 cells were seeded onto glutaradehyde-crosslinked gelatin films that were fluorescent due to covalently-attached FITC-fibronectin. Inhibitors were used at 1 μM and replenished after 24 h. After 48 h, matrix degradation appears as black holes in the matrix.

B & C. Measuring the percentage of microscopic fields with matrix degradation (B) or percent of area degraded in fields with matrix-degradation as determined by the ImageJ program (C) reveals that all DASH inhibitors reduced matrix degradation. PT-630 and Talabostat showed the greatest inhibition.

Figure 4. Cells expressing a catalytic mutant of FAP maintain high levels of matrix degradation.

A. Matrix degradation (arrows) is barely detectable under the control transfectants after 48 h (Neo) but is visualized as black areas under FAP transfectants (WTY-1, WTY-6, & S624A). The FITC-fibronectin films were visualized with Zeiss Axioskop 2 *mot plus* microscope and 40 X objective.

B. Spots indicating matrix degradation were captured by the image J program in three random fields of matrices degraded by each cell type (examples for each cell type in the upper panels. The spots were counted and assessed for percentage of the entire field that was degraded and the table in the lower panel reports these data.

Figure 5. Expression of either catalytically active or inactive FAP increases MMP-9 accumulation in media and invasive behavior of cells.

A. Left Panel: Zymogram of conditioned medium from control transfectant (Neo) and FAP overexpressing cells (WTY-1 & S624A-5).

Right Panel: MMP-9-specific western blot of conditioned medium from control transfectants (Neo) and FAP overexpressers (WTY-1, WTY-6, S624A-5).

B. FAP expression increases invasiveness of breast cancer cells. The number of cells invading into type I collagen gels was determined as described previously. S624A-5, WTY-1, and WTY-6 all invade significantly more than Neo with P values given in the text.

Figure 6. Altered pattern of tyrosine phosphorylated proteins Western blot of extracts of control transfectants (Neo) and FAP overexpressers (WTY-1; WTY-6; and S624A-5). The blot was probed with mAb 4G10 to phosphotyrosine.

Figure 1.

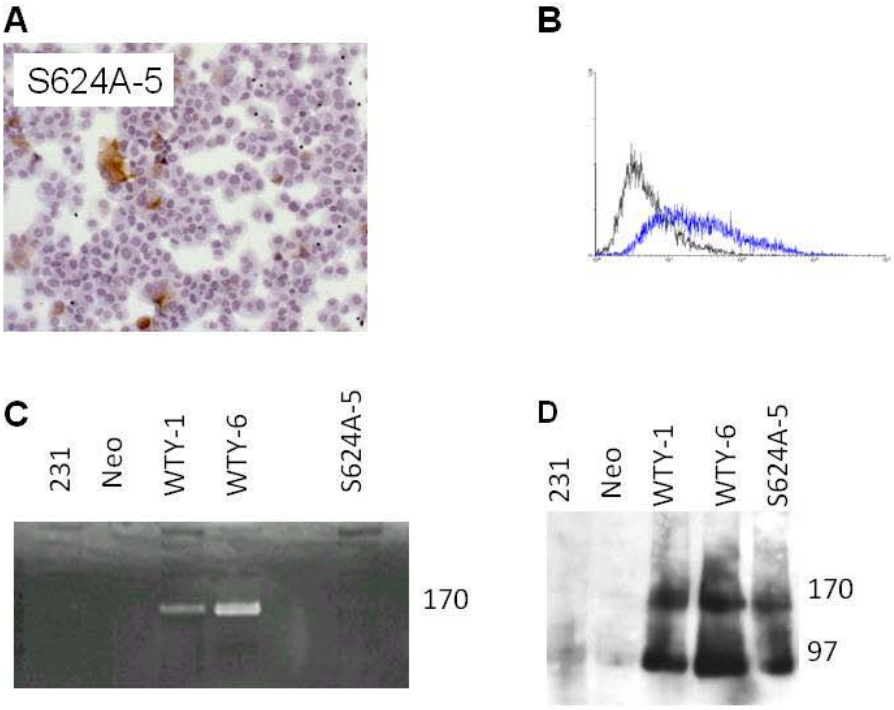

A
S624A-5
B
C
231
Neo
WTY-1
WTY-6
S624A-5
170
D
231
Neo
WTY-1
WTY-6
S624A-5
170
97

Figure 2.

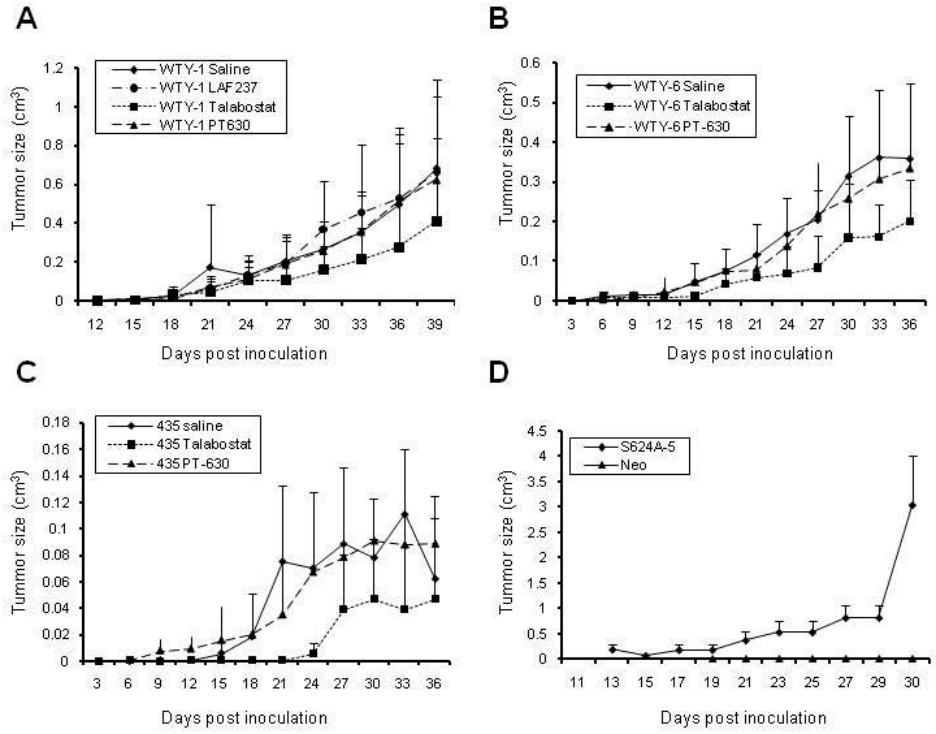

A
WTY-1 Saline
WTY-1 LAF237
WTY-1 Talabostat
WTY-1 PT630
Tummor size (cm3)
Days post inoculation
B
WTY-6 Saline
WTY-6 Talabostat
WTY-6 PT-630
Tummor size (cm3)
Days post inoculation
C
435 saline
435 Talabostat
435 PT-630
Tummor size (cm3)
Days post inoculation
D
S624A-5
Neo
Tummor size (cm3)
Days post inoculation

Figure 3.

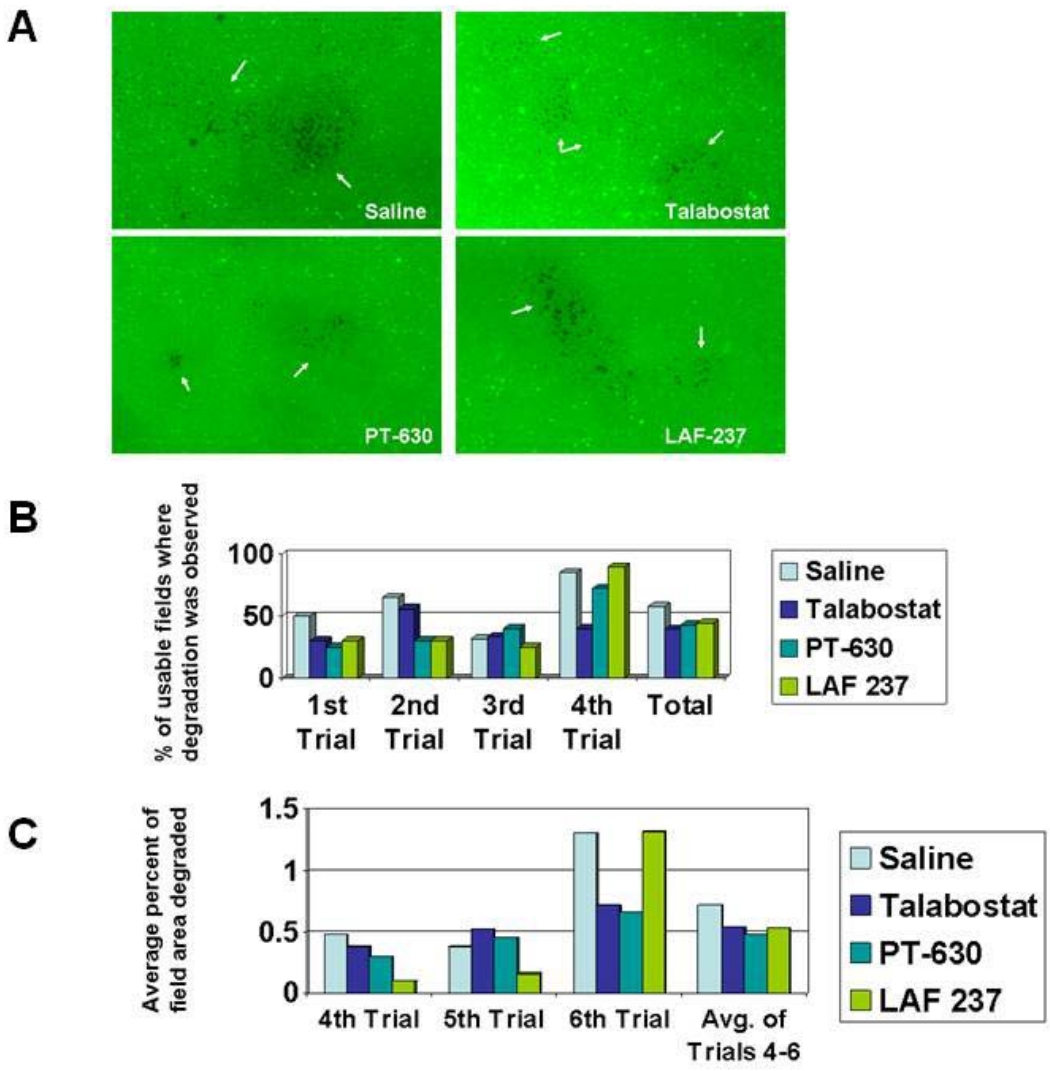
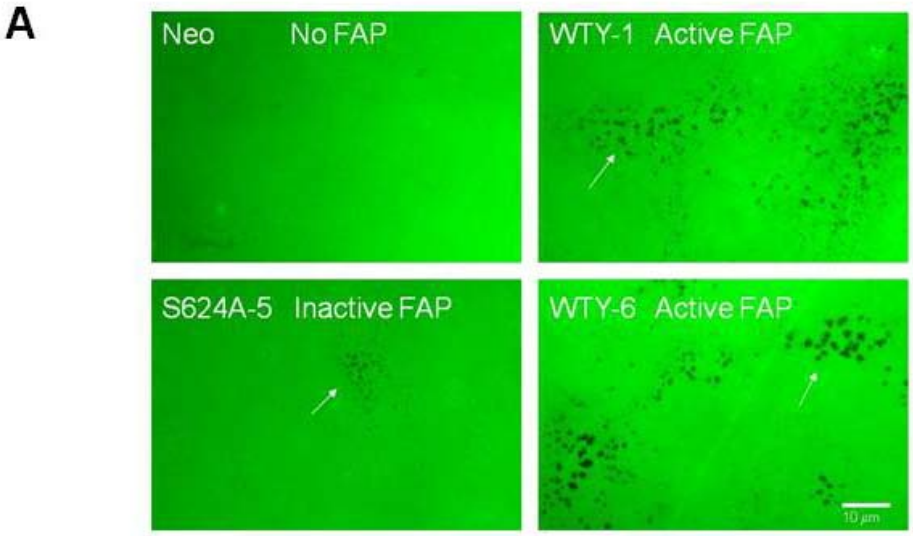

A
Saline
Talabostat
PT-630
LAF-237
B
% of usable fields where degradation was observed
100
50
0
1st Trial
2nd Trial
3rd Trial
4th Trial
Total
Saline
Talabostat
PT-630
LAF 237
C
Average percent of field area degraded
1.5
1
0.5
0
4th Trial
5th Trial
6th Trial
Avg. of Trials 4-6
Saline
Talabostat
PT-630
LAF 237

Figure 4.



Cells	Spots counted	% Spots with measurable area	% Area degraded matrix	*P* value for area degraded versus Neo
Neo	251	4	0.010	
S624A-5	272	16	0.047	0.031
WTY1	367	22	0.257	0.0017
WTY6	249	16	0.068	0.00026

Figure 5.

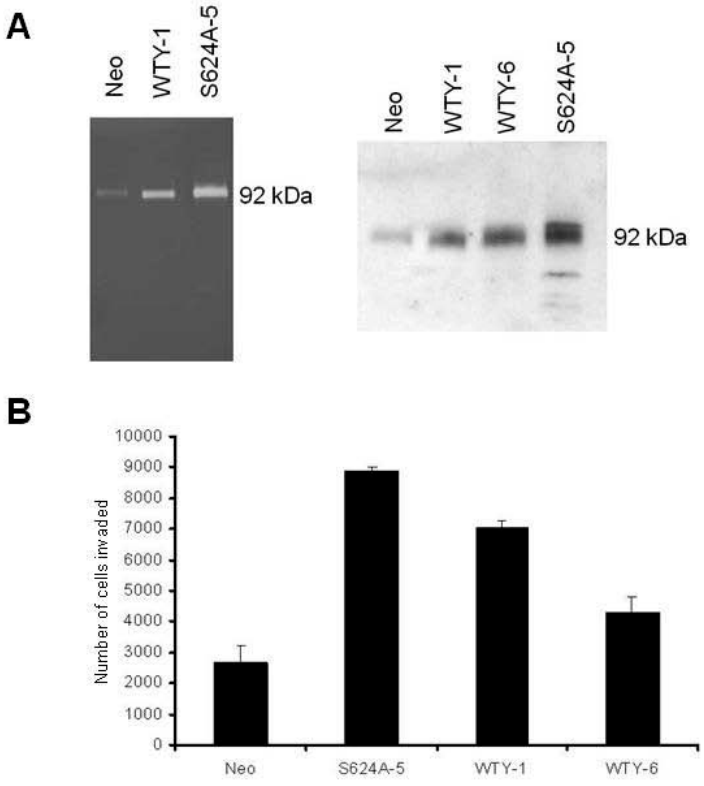

A
Neo
WTY-1
S624A-5
92 kDa
Neo
WTY-1
WTY-6
S624A-5
92 kDa
B
Number of cells invaded
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Neo
S624A-5
WTY-1
WTY-6

Figure 6.