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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.

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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 [3-5] and has been linked to invasive, matrix-degrading behavior of malignant melanoma and breast cancer cells [6]. In animal models, over expression of FAP by the malignant cells also stimulates rapid growth of cancer [7, 8].

FAP is a member of the dipeptidyl peptidase clan and/or structural homologs (DASH) family of prolyl-specific peptidases [9-11]. The FAP protease is a dual function serine protease having both N-terminal prolyl-specific dipeptidase and endopeptidase activities [12-14]. The dipeptidase activity is presumably important for modifying chemokines [9, 11], 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 [15]. Recently our collaborator, Dr. Patrick McKee's laboratory identified low molecular weight proteolytic fragments of type I collagen that are produced by FAP cleavage [16]. 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) [17]. 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 [17]; 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.

Overall, although we enjoyed some success in parts of the project, several aspects of the project simply were not completed. We failed to use FAP-specific inhibitors designed by the partnering PI in an effort to test their effect on growth of tumors in an animal model of breast cancer. The biggest factor here was our under-estimation of the time it would take to synthesize sufficient quantities of FAP inhibitors to do the experiments. This means that several SOW objectives listed under AIM 1 were not accomplished because the inhibitors were not available.

We were also unsuccessful in developing and characterizing *in vitro* and *in vivo* models of micro emboli formation (SOW year 2, Aims 2 & 3, Kelly). We had several attempts at injecting GFP-expressing tumor cells into the tail veins of mice and then looking for microemboli in squashed preparations of lung tissue. We were not able to detect sufficient numbers of cells in this manner. We tried a different procedure for fixing the lungs after they were harvested. This involved inflating the lungs with agarose while perfusing the lungs with formalin. Although this preparation was much easier to detect cells and structures, we still failed to get enough fluorescently labeled tumor cells into the tail veins to detect more than an isolated cell or two in the lungs.

Despite these set-backs, progress was made in other areas of FAP tumor biology in breast cancer. This final period has been productive in terms of further investigating the role of FAP in angiogenesis. We have discovered that FAP may upregulate pro-angiogenic factors such as MMP-9, IL-8, and TGF-β while simultaneously down regulating anti-angiogenic factors such as thrombospondin. We present evidence suggesting that the EGR-1 transcription factor may be at the center of the pro-angiogenic response to FAP. Moreover, we have discovered that FAP and EGR-1 are both present in state of the art animal models of breast cancer.

Body

First year of the project:
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.

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 (Now published, Huang et al, 2011, Clin & Exp Metastasis, *28*:567-579).

Second year of the project:
For the first project period (above) we reported that plasmid cDNA constructs had been made in order to engineer MDA MB-231 cancer cells to express both FAP and GFP so that we could easily detect them after injecting them into the venous circulation of mice. Here we report that Avis E. Simms and Yan Huang have produced MDA MB-231 human breast cancer cells that over express both FAP and GFP together and MDA MB-231 control cells that do not express FAP but express high levels of GFP. Flow cytometry reveals that the FAP expression is high on 84% of these WTY-1 breast cancer cells but the GFP expression is seen in only about 25% of the cells. However, 66% of the GFP positive cells are also positive for FAP. We will continue sorting them using FACS technique to improve the percentage of cells expressing both GFP and FAP. At present, with the FAP^{high}/GFP and FAP^{Low}/GFP cells that we have in hand we will start the experiments outlined in the SOW under specific aims 2 & 3 indicating development of in vitro and in vivo models of microemboli formation. This will be accomplished under the one year no-cost extension period that we requested in July, 2010 and got approved in September, 2010.

Avis E. Simms, a graduate student in the lab, has produced data regarding fibrin degradation associated with cells expressing active FAP, mutant FAP, or very little FAP. These data specifically address of aspects of SOW specific aim 1. Initially Ms Simms evaluated the association of α2-antiplasmin (A2AP) with the cells by immunofluorescence microscopy. Ms Simms found no difference in the levels of A2AP associated with cells that don't express FAP (231), cells expressing active FAP (WTY6), or those expressing inactive mutant FAP (S5) (not shown). The fibrin degradation associated with 231, WTY-6, and S5 cells was determined using a commercially available ELISA kit for clinical measurement of the D-dimer product of fibrin breakdown, Asserachrom® D-Di (Diagnostica Stago, Asnieres-Sur-Seine, France). Equal numbers of cells were grown in 6 well plates and serum free medium. The cells received fibrinogen, thrombin and factor XIII (all wells). Some wells received A2AP alone, others received plasmin without A2AP. One group received all elements including plasmin and A2AP. The results show that there is both plasmin independent and plasmin dependent degradation of fibrin by these cells. Moreover, A2AP is very effective at suppressing plasmin-dependent fibrin degradation regardless of FAP expression by the cells (Fig. 3). Surprisingly, A2AP was also effective at plasmin independent fibrin degradation (Fig. 3, Fibrin, A2AP).
The observed plasmin-independent fibrin degradation suggested that other proteases produced by the cells might be mediating the fibrin degradation. Indeed, earlier we showed that expression of either wild type and active FAP or a catalytic mutant FAP caused elevated expression of matrix metalloproteinase-9 (MMP-9) (see first progress report, submitted manuscript in the Appendix). To determine if MMPs might be mediating the cleavage of fibrin, the cell-mediated fibrin cleavage was investigated in the presence or absence of the broad-spectrum MMP inhibitor BB-94. We found that MMP inhibition greatly decreased the levels of plasmin independent fibrin degradation (Fig. 4, Fibrin; Fibrin, A2AP). Not surprisingly, MMP inhibition does not inhibit the plasmin dependent fibrin degradation (Fig 4, Fibrin, Plasmin; Fibrin, Plasmin, A2AP).

The results are consistent with the idea that FAP expression on MDA MB-231 human breast cancer cells leads to an increase in MMP-9 release (see manuscript in the appendix of last year's progress report), and that this increased MMP-9 actually stimulates dissolution of fibrin and associated microemboli. If this is true then we would expect the opposite of the hypothesis driving specific aim 2 of the grant. Namely, that FAP should lead to increased incorporation of A2AP into fibrin and thereby stabilize fibrin coating of tumor cells. However, A2AP is still quite effective at blocking the plasmin dependent fibrin degradation and may have a lesser inhibitory effect on fibrin degradation mediated by MMPs. Therefore, the results are inconclusive as to

what the net result will be in vivo where there is abundant plasmin in the blood and extracellular fluids. We will test for fibrin accumulation and tumor platelet-cell aggregate formation in the presence or absence of A2AP for FAP expressing and non-expressing breast cancer cells. These experiments will be done in vitro under flow and in vivo as described in the grant proposal.

FAP suppresses DDPIV expression

FAP is closely related to DPPIV and the enzymatic functions of FAP and DPPIV overlap at least partially [18]. Because FAP expression elevated MMP-9 release and altered phosphotyrosine proteins (manuscript in Appendix of first year progress report) we investigated if there were also changes in surface expression DPPIV. The parental MDA MB-231 human breast cancer cells have very little FAP expression but reveal high levels of cell-surface DPPIV (Fig. 5A, Parental^{FAPLow}). On the other hand, these cells engineered to express FAP to high levels reveal very little surface expression of DPPIV (Fig. 5A, WTY-6$^{FAP\ High}$). Moreover, the FAP proteolytic activity is not required to suppress expression of DPPIV as it is reduced in parental MDA MB-231 cells expressing a mutant of FAP that is proteolytically inactive (Fig. 5A, S624A-5$^{FAP\ High}$). This FAP-mediated suppression of DPPIV expression is also observed at the mRNA level by RT-PCR (Fig 5B). These results suggest that FAP-mediated signaling causes a suppression of cell-surface DPPIV. This in turn may correlate with the already published aggressive behavior of FAP-expressing breast cancer cells [19].

In order to help link any observed alterations in microembolus formation to FAP and not to DPPIV, we sought to produce FAP suppressed cells from the WTY-6$^{FAP\ High}$ cells using shRNAs and lentiviral vectors. We purchased five FAP-specific cDNA clones encoding the FAP shRNA (TRCN0000006802, TRCN0000006803, TRCN0000006804, TRCN0000006805, and TRCN0000006806; which we abbreviate 802-806 respectively) from Open biosystems, packaging vector (pSPAX2), and the envelop vector (PCI-VSVG) from Addgene. We successfully grew the plasmid DNA for each of the clones, packaging, and envelop vector (Fig. 6A). Each of the clones was co-transfected with the packaging and envelope vectors into 293 cells to produce separate lentvirus progeny that could cause expression of each one of the five individual clones. After infection, the cells were selected using puromycin.. Subsequently, culture supernatants were harvested containing lentiviruses carrying the FAP-specific clones. These supernatants were used to infect WTY-6$^{FAP\ High}$ cells. After selection with puromycin, RT-PCR analysis of RNA isolated from infected WTY-6$^{FAP\ High}$ cells revealed that clones 802, 803, and 806 did not suppress FAP expression but clones 804 and 805 were effective at suppressing FAP at transcription level (Fig 6B, shows data for clone 804). To determine if cell surface expression of FAP was suppressed the cells were collected from the culture dish using EDTA treatment and gentle scraping. The cells were stained with F19 monoclonal antibody to FAP and subjected to flow cytometry. Consistent with the RT-PCR results, WTY-6$^{FAP\ High}$ cells infected with lentivirus encoding FAP-specific shRNA clones 802, 803 and 806 did not suppress cell surface expression of FAP (Fig. 7A-C) but clones 805 and 804 (Fig. 7D & E) did suppress surface expression of FAP relative to WTY-6$^{FAP\ High}$ cells (Fig. 7F). It was observed that MDA MB-231 cells that do not express FAP have high levels of DPPIV and that DPPIV is suppressed when these cells were engineered to express FAP (Fig. 5). We therefore investigated the possibility that suppression of FAP might result in an increase in DPPIV expression. Indeed, flow cytometry reveals that WTY-6$^{FAP\ High}$ cells have high levels of FAP expression (>82% cells positive) and low levels of DPPIV expression (0.38% positive cells) (Fig. 8A & C) while, WTY-6$^{FAP\ High}$ cells infected with lenti virus carrying clone 805 FAP-specific shRNA have suppressed FAP expression (7.9 % positive as compared to WTY-6$^{FAP\ High}$ cells where 91.5% cells are positive) but increased DPPIV expression (14.83 % positive cells versus the MDA MB-231 cells that are negative for FAP and have 47.21% cells positive for DPPIV) (Fig. 8B & D).

In this section we show that FAP expression suppresses DPPIV expression on breast cancer cells. Moreover, suppression of FAP leads to increased expression of DPPIV. This indicates that relative amounts of the two molecules on the cell surface may determine the overall phenotype of the cell. For example, we have shown that parental MDA MB-231 human breast cancer cells produce tumors that grow relatively slowly in immune compromised mice as compared to fast growing tumors formed by WTY-6$^{FAP\ High}$ cells and other populations of breast cancer cells that express FAP to high levels. Thus, DPPIV may be at one end of the spectrum promoting slower growth whereas FAP may be at the other end promoting rapid tumor growth. Given that FAP and DPPIV can form complexes it is not hard to envision such a system that is similar to the function of bcl-2 and bax proteins. These apoptotic proteins determine if the cell will survive or go into apoptosis by their relative amounts where bcl-2 like proteins favor survival and bax like proteins favor cell death.

FAP may suppress innate immunity.

The overall theme of this synergy grant is that FAP may promote survival of breast cancer cells in diverse microenvironments. One such environment is the blood stream where microembolus formation may be facilitated by FAP expression. Ability to evade the immune system may be important in several microenvironments that breast cancer cells encounter as they progress and metastasize. Micro-emboli themselves may promote tumor cell evasion of the immune system. Because of the homology of FAP to DPPIV and the fact that the substrate specificities of FAP and DPPIV share some overlap, we became interested in investigating a possible role for FAP in suppressing innate immunity by cleaving chemokines that attract NK cells. For the initial experiments, Ms Anna Mazur, a graduate student in the lab, used RT-PCR to evaluate the expression of a panel of chemokines known to be chemoattractive for NK cells.. She found that parental MDA MB-231 human breast cancer cells express IL-1β, IL-8, MCP1, and RANTES (Fig. 9, Parental). It was noted that IL-1β and IL-8 were expressed much more abundantly than the others (Fig 9, Parental). Similarly WTY-6$^{FAP\ High}$ cells also expressed IL-1β, IL-8, MCP1, and RANTES, but in addition they had detectable levels of IP-10 (Fig. 9, WTY-6$^{FAP\ High}$). Again the highest levels of expression were observed for IL-1β and IL-8 (Fig. 9, WTY-6$^{FAP\ High}$). The S624A$^{FAP\ High}$ cells that express high levels of an inactive FAP had high levels of IL-1β and IL-8 but the other chemokines were not detected (Fig. 9, S624A$^{FAP\ High}$).

We wondered if FAP might degrade chemokines? To begin to answer this question Ms Mazur partially purified FAP from detergent extracts of WTY-6$^{FAP\ High}$ using Wheat Germ Agglutinin (WGA) Agarose beads as we reported earlier [14]. This preparation is not FAP-specific in fact there many other proteins including DPPIV present in this preparation [14]. Nevertheless, the preparation does eliminate MMPs. We incubated the WGA Agarose beads containing our partially purified FAP with recombinant and homogeneous IL-8. We pelleted the WGA-Agarose and subjected the supernatant to analysis by Surface Enhanced Laser Desorption Time of Flight Mass Spectrometry (SELDI-TOF MS) as described by our collaborator, Dr. Larry Suva [20-22]. There is apparent increased degradation of IL-8 in WGA-Agarose beads containing active FAP from WTY-6$^{FAP\ High}$ cells (Fig. 10, WTY-6 & IL-8, blue trace). This as compared to no peaks in the bead control (Fig. 10, bead control, black trace) and just the intact peak of the IL-8 control (Fig. 10, IL-8, red trace). Low levels of degradation were observed with the extracts of MDA MB-231 parental cell mixed with IL-8 (Fig. 10, MDA MB-231 & IL-8, magenta trace). Surprisingly there is also somewhat elevated degradation of IL-8 from beads containing inactive FAP isolated from S624A$^{FAP\ High}$ cells (Fig. 10, S5 & IL-8, green trace). These results suggest that membrane extracts of cells overexpressing FAP can mediate degradation of IL-8. Future studies will be designed to confirm or deny that FAP is involved in this proteolytic degradation.

Final year of the project:
We included a submitted manuscript in the appendix of our first progress report submitted 9/30/2009. This article was published in 2011 and is included in the appendix of this report. A key experiment requested by the reviewers of the paper demanded that we develop a FAP specific protease activity assay. During this past project period we developed a FAP-specific protease activity assay that is both quantitative and relatively easy to perform as described below.

FAP protease activity assay was developed as follows. FAP was extracted from cells by using Triton X-100 using a procedure modified from that reported previously [14, 23]. Each cell type was grown to 90% confluence in three 75 cm^2 flasks. Media was removed; the cells washed three times with PBS, and then suspended using 1mM EDTA in PBS. Cells were pelleted by centrifugation at 10,000 X *g* for 5 minutes at 25^o C and the supernatant was removed. Cells were resuspended in 1 ml of extraction buffer (2.5%Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) and then the lysate was gently homogenized on ice in an 1.5 ml eppendorf tube with 30 strokes of the pestle. Care was taken to avoid excessive foaming. The homogenate was cleared by centrifugation for 5 min at 524 X g at 4^o C. The supernatant was transferred to a fresh tube and incubated with 100 µL of protein G magnetic Dynabeads (Invitrogen) that had been coated with approximately 5 µg F19 mAb to FAP for 30 min at 25^o C and washed according to the manufacturer's instructions. FAP collected on the protein G Dynabeads, was exposed to 100 µM of z-Gly-Pro-AMC (Bachem Bioscience Inc, King of Prussia, PA, USA) for 2 h at 37^o C in 400 µL of 50 mM Tris, 100 mM NaCl , 1 mM EDTA, pH 7.6 with shaking. To inhibit FAP, the FAP-coated protein G beads were first exposed to 10 µM of PT-100 or PT-630 in 390 µL of the above buffer for 30 min 25^o C in prior to addition of the 10 µL of 4 mM z-Gly-Pro-AMC to achieve a final concentration of 100 µM z-Gly-Pro-AMC. The beads were collected with the magnet and the fluorescence at 460 nm of the supernatants determined with excitation of 360 nM with the Tecan Safire microtiter plate reader. All samples were tested in duplicate. 7-Amino 4-Methyl Coumarin (AMC) (ACROS, New Jersey, USA) was used to develop a standard curve where the fluorescence emitted by free AMC at concentrations of 0.00125 µM, 0.0125 µM, 0.125 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM was determined by applying an excitation wavelength of 360 nm and measuring the fluorescence intensity at 460 nm (Tecan Safire fluorescent microplate reader using the Magellan software). This curve was used to relate fluorescence obtained from the unknown samples to µM free AMC.

To determine FAP activity in tumor tissues an extract was prepared by homogenizing 100 mg of tumor tissue in 500 µL 2.5%Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. The extract was cleared by centrifugation and the total protein determined using bicinchoninic acid assay (Pierce) where BSA was used to produce a standard curve. The F19-coated protein G dynabeads (50 µL) prepared as described above, were exposed to 5 mg of tumor extract protein to capture FAP. The FAP coated beads were then tested for FAP-activity using 100 µM z-Gly-Pro-AMC substrate as described above. Samples were tested in duplicate.

A FAP-specific protease activity assay was developed to confirm that FAP was inhibited in the tumors of the animals that were treated with talabostat and PT-630 but not in those treated with LAF-237. For the assay, FAP is immune precipitated from extracts of cells or tumor tissues and then exposed to the substrate z-Gly-Pro-AMC. FAP cleaves the peptide bond linking Pro to AMC and consequently the fluorescence emitted by AMC is increased. Extracts of MDA MB-231 cells that do not express FAP, revealed low activity in this assay (Fig. 11A, 231, black bars) and this activity was unchanged or showed a decrease when treated with PT-630 or talabostat (Fig. 11A, 231, white and gray bars). However, WTY-6 and WTY-1 transfectants of MDA MB-231 cells that express wild type and active FAP to high levels reveal high FAP activity in this

assay (Fig. 11A WTY-6 & WTY-1, black bars) that is inhibited by both PT-630 and talabostat (Fig. 11A WTY-6 & WTY-1, white and gray bars). In contrast, the mutant FAP reveals low levels of FAP activity similar to the FAP negative MDA MB-231 cells (Fig. 11A, S624A-5, black bars) that remains low when exposed to PT-630 and talabostat (Fig. 11A, S624A-5, white and gray bars). For comparison, intact z-Gly-Pro-AMC was used to determine back ground fluorescence (Fig. 11A, G-P-AMC, black, white, and gray bars).

Extracts of tumor tissues were prepared and subjected to the FAP-activity assay. In the case of WTY-6 tumors, Fresh frozen tissues were available from normal saline treated animals and PT-630 treated animals. The extracts from WTY-6 tumors of animals treated with normal saline had high FAP activity (Fig. 11B, saline) whereas the extracts of WTY-6 tumors from PT-630 animals had low FAP activity (Fig. 11B, PT-630). Similarly, extracts of WTY-1 tumors obtained from animals treated with LAF-237 that preferentially inhibits DPPIV and not FAP, had high FAP activity (Fig. 11C, LAF-237) but extracts of WTY-1 tumors with treated with PT-630 had low FAP activity (Fig. 11C, PT-630). Overall the results are consistent with the inhibitors acting as expected with LAF-237 producing relatively little inhibition of FAP proteolytic activity in the tumors but PT-630 causing significant inhibition of FAP activity.

These findings were incorporated into the text of our manuscript and it was accepted for publication (Huang et al, (2011) Clin. Exp. Metastasis. *28*:567-579. Included in the Appendix).

Role of FAP in tumor angiogenesis

Last year we reported that IL-8 and other chemokines are expressed by cells that also express FAP (Fig. 9 of this report) and evidence that FAP might cleave IL-8 (Fig. 10 of this report). We noted that the cleavage of IL-8 was detected using partially purified FAP that contained many other proteins and potentially other proteases and that more would need to be done to confirm or deny FAP cleavage of IL-8 (Figure 10 of this report). This year others have published that FAP does not cleave IL-8 as determined using purified FAP and IL-8 or IL-1β [24]. While we are still interested in whether or not this correct (because in fact the data are not shown and these are proteins expressed in insect cells that may differ from FAP and IL-8 made in human cells) our focus has begun to shift away from chemokines and the role of FAP in immunosuppression to angiogenesis. This is for a number of reasons, the first being that we were among the first to report that FAP expression correlates with rapid tumor growth and increased microvessel densities in a mouse model of human breast cancer [19]. This concept is supported by the fact that FAP was identified in a RT-PCR and gene expression profiling screens for serine proteases involved in angiogenesis [25, 26] and that inhibition of FAP protease activity can suppress angiogenesis in a mouse model of lung cancer [27]. More recently it was observed that FAP can cleave neuropeptide Y (NPY) [28]. NPY is a potent angiogenic factor that is known to be cleaved and activated by the related protease dipeptidyl peptidase IV [29]. The fact that FAP can also activate the pro-angigoenic (Y2 receptor binding) function of FAP is consistent with the role for FAP in angiogenesis. Moreover our recently published work indicates that FAP expression also causes an increase in MMP-9 [30] which is pro-angiogenic due to its ability to mobilize matrix-bound VEGF [31]. We wondered if FAP might promote angiogenesis through both its signaling functions and protease activities. A recent paper by Jonathan Cheng's group indicated that EGR-1 transcription factor can cause induction of FAP [32]. Interestingly, EGR-1 also has been implicated as an angiogenesis master switch because it causes increased expression of MMP-9, IL-8, PDGF-B, FGF and other pro-angiogenic factors [33-36]. We investigated the expression of EGR-1 in cell lines that differ in FAP expression.

FAP increases expression of EGR-1 Normal human skin fibroblasts derived from the skin of a reduction mammoplasty were purchased from ATCC (CRL-1947). These cells reveal high endogenous levels of FAP (Fig. 12A & B) and EGR-1 (Fig. 12C, Fibroblasts). Interestingly, forcing FAP expression in MDA MB-231 cells causes an increase in EGR-1 transcript (Fig. 12C, WTY-1 & WTY-6). The lack of EGR-1 in the FAP negative MDA MB-231 cells (Fig. 12C, 231) and increased EGR-1 expression in two different populations of these cells engineered to express FAP, suggests a positive-feedback loop where expression of FAP signals increased EGR-1 (Fig. 12C) and EGR-1 is known to drive FAP expression [32]. FAP expression alters cell signaling resulting in increased accumulation of MMP-9 that might contribute to the pro-angiogenic nature of FAP-expressing tumor cells (Fig. 13 & [30]). MMP-9 promotes angiogenesis through mobilization of matrix-bound VEGF [31]. Stable expression of EGR-1 could result in long term expression of EGR-1 response genes such as MMP-9, IL-8, VEGF and FGF that promote angiogenesis and rapid tumor growth.

FAP induction: FAP is not expressed in normal adult tissues but is highly expressed by activated fibroblasts. Inflammatory agents including TGF-β and IL-1β mediate increased FAP and EGR-1 expression as well as activation of fibroblasts [37-40]. The EGR-1 zinc finger transcription factor is at the center of the profibrotic response that is often seen in inflammation and response to TGF-β [41]. The FAP gene has an EGR-1 response element and FAP expression is increased by EGR-1 [32] which may explain increased FAP expression in response to TGF-β [38]. TGF-β also increases production of collagen through Runx-Smad2/3-Smad4 and ERK signaling [42, 43]. EGR-1 stimulates production of type I collagen by transcription of collagen IA gene [37]. EGR-1 also stimulates expression of a host of other genes that are involved in the stromal response to epithelial cancers including FAP [32], MMP-9 [33], and IL-8 [44] (Fig. 13).

FAP-Low MDA MB-231 cells and FAP-High WTY-6 and S624A-5 cells express IL-1β and IL-8 with slightly more robust expression of these cytokines in the FAP-High cells (Fig. 14A, see also progress report 10/2010, Figure 7). New preliminary findings suggest that suppression of FAP with shRNA-805 decreased expression of pro-angiogenic TGF-β and increased anti-angiogenic thrombospondin (Fig. 14B). Note that the suppression of FAP expression and the shRNAs specific to FAP are described in the progress report of 2010.

Our preliminary studies on late tumors from MMTV-PyMT mice (kindly provided by Drs. Jerry Ware and Sandy Gendler) identify FAP (Fig. 15) and EGR-1 (Fig. 16). FAP was concentrated from tumor extracts using WGA-Agarose beads as we have described [3, 14, 23]. FAP protein was identified on Western blots as a smear of bands running between 150 and 225 kDa that is consistent with active FAP (Fig 15A). High levels of FAP proteolytic activity were detected in the MMTV-PyMT tumor extracts using z-Gly-Pro-AMC substrate as we have done previously [30]. (Fig. 15B, Untreated). The protease activity was significantly decreased by Val-boroPro (PT-100), an inhibitor of FAP and related post prolyl peptidases (Fig. 15B, 10 μM PT-100). FAP is detected on fibroblasts (Fig. 15C, white arrows) and cells with very strong FAP staining were found in the stroma and intermixed with infiltrating inflammatory cells and tumor cells (Fig. 15C). FAP is not detected on the epithelial tumor cells (Fig 15D, black arrows).

EGR-1 positive fibroblast-like cells were found in the stroma by IHC of tissue sections (Fig. 16A & B, white arrows). EGR-1 is also found in epithelial tumor cells (Fig. 16B, black arrows). EGR-1 positive cells have black nuclei while the EGR-1 negative cell nuclei are blue due to the hemotoxylin stain (Fig. 16A & B). EGR-1 message was also detected in extracts of the MMTV-PyMT tumors by reverse-transcription PCR with EGR-1 specific primers (Fig. 16C). Blood vessels within the tumors are detected with CD34 antibody (Fig. 16D).

Key Research Accomplishments for the entire project period

Year 1.

- 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.

Year 2.

- Produced MDA MB-231 breast cancer cells expressing high levels of FAP and GFP
- Demonstrated plasmin dependent and plasmin independent fibrin degradation by FAP expressing cells
- Showed that even plasmin independent fibrin degradation is reduced by A2AP
- Demonstrated that MMPs mediate much of the plasmin independent fibrin degradation – This infers a role for FAP because we previously showed causes up-regulation MMP-9
- Produced lenti viruses for 5 different clones encoding shRNAs specific for FAP
- Two FAP-specific clones were successful in suppressing expression of FAP
- Demonstrated that FAP expression suppresses DPPIV expression
- Conversely, FAP suppression induces increased DPPIV expression – these findings support the notion that FAP stimulates cell signaling
- Identified several chemokines that attract innate immune cells and are expressed by MDA MB-231 cells and these cells engineered to express wild type or mutant FAPs.
- Found that partially purified FAP extracts can proteolytically degrade IL-8.
- Presented two abstracts at international meetings.

Year 3.

- Establishment of a FAP specific and fluorescence-based protease activity assay.
- Demonstration that the FAP activity is suppressed in tumors by FAP inhibitors given orally.
- Identification of high levels of FAP correlating with high levels of EGR-1 in fibroblasts and human breast cancer cells. This is in a limited number of samples.
- Development of the concept that FAP signaling through EGR-1 mediates both the pro-angiogenic and fibrotic functions of FAP.
- Initial finding that FAP upregulates IL-8, IL-1β, MMP-9, TGF-β and down regulates thrombospondin.
- Identified FAP protein and FAP protease activity in extracts of breast tumors excised from MMTV-PyMT mice.

- Identified FAP expression by stromal fibroblasts and not by tumor epithelial cells in histologic sections of MMTV-PyMT breast tumor tissue.
- Identified EGR-1 expression by RT-PCR and immunohistochemistry of breast tumor tissue.
- Showed microvessel staining of the MMTV-PyMT tumors.

Reportable Outcomes for the entire project period

Poster presentations (6 total)

1. T. Kelly, N. R. León, K. Kelly, B. Jones and N. Aziz "Tumor growth is slowed by an inhibitor of DASH proteases in a mouse model of human breast cancer" 3rd International Congress on Proteases and Dipeptidyl Peptidases, April 23-25, 2008, Antwerp, Belgium

2. Y. Huang, A. Mazur, A. E. Simms and T. Kelly "Fibroblast activation protein-α: A potential modulator of chemokines that regulate natural killer recruitment to breast tumors", Cell Adhesion and Stress Fibers: A symposium in honor of Keith Burridge, August 27-29, 2010, Chapel Hill, NC.

3. A. E. Simms and T. Kelly, "Alpha 2-Antiplasmin (α2AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin" Jackson Heart Study Scientific Conference. 9/23-24/2010. Jackson, MS. (Included in the appendix)

4. A. E. Simms, and T. Kelly (2011) "Alpha 2-Antiplasmin (α2-AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin" Arkansas Breast Challenge, 7/29-31/2011, Big Cedar Lodge, Ridgedale, MI (**Note** this was the same as the presentation Ms Simms delivered at the Jackson Heart Study that was reported in Progress report of October, 2010)

5. A. Mazur, Y. Huang, C. Washam, L. J. Suva, and T. Kelly (2011) Fibroblast Activation Protein-α: A potential modulator of biological functions in the tumor microenvironment. Arkansas Breast Challenge, 7/29-31/2011, Big Cedar Lodge, Ridgedale, MI

6. Y. Huang, A. Mazur, A. E. Simms, C. Washam, L. J. Suva, and T. Kelly (2011) "Fibroblast activation protein-α: A chemokine modulator regulating natural killer recruitment to tumors?" ERA of Hope, Congressionally directed medical research program, DoD breast cancer research program. 8/2-5/2011. Orlando, FL.

Papers over entire project (2 total):

1. Y. Huang, A. E. Simms, A. Mazur, S. Wang, N. R. León, B. Jones, N. Aziz, and **T. Kelly**. (2011) Fibroblast activation protein-α promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions. Clin. Exp. Metastasis. *28*:567-579. DOI: 10.1007/s10585-011-9392-x
2. **Kelly, T.**, Y. Huang, A.E. Simms, and A. Mazur, (2012) Fibroblast activation protein-α: A key modulator of the microenvironment in multiple pathologies. Intl. Rev. Cell & Mol. Biology, 297: *In press*.

Grants applied for (10) received (2) over entire project.

1) USAMRDC-BCRP Pre-doctoral fellowship BC093509 “Blood-borne tumor-host cell emboli in metastasis”, PI Avis Simms (Not Funded)

2) Arkansas Breast Cancer Research Program Predoctoral Fellowship “Blood-borne tumor-host cell emboli in breast cancer metastasis” PI Avis Simms (Inaugural recipient), (**Funded 7/1/2009)**

3) ARRA challenge grant NIH 1 RC1 CA145194-01 “Fibroblasts and platelets can cooperate with cancer stem cells to drive metastasis.” PIs J. Ware and T. Kelly (Not funded)

4) USAMRDC-BCRP Pre-doctoral fellowship BC100366 “Fibroblasts protect breast cancer cells from innate immunity”, PI Anna Mazur (Not Funded, Overall score 1.8, Excellent)

5) NIH, NCI, 1R21CA155057 Fibroblasts protect breast cancer stem cells from innate immunity, PI Thomas Kelly (Not Funded, Unscored).

6) USAMRDC-BCRP Idea grant application BC100281 “Matrix remodeling by cancer associated fibroblasts promotes breast tumor growth”, PI Thomas Kelly, (Not funded, Overall score 2.3, Good)

7) UAMS, Medical Research Endowment, Fibroblasts protect breast cancer stem cells from innate immunity, PI Thomas Kelly (**Funded)**

8) KG111394, Susan G. Komen for the Cure Post-baccalaureate training in disparities research, Proteolytic activity of fibroblast activation protein-alpha in the tumor microenvironment promotes outgrowth of triple negative breast cancer. PI Thomas Kelly, Co-PI Larry Suva (Not funded)

9) BC104059, USAMRC, CDMRP, BCRP, IDEX, Inhibition of fibroblast activation protein-alpha to increase immune-mediated destruction of breast cancer cells. PI Thomas Kelly (Not funded, Good, Good, Excellent)

10) 1R01CA168715-01, NIH, FAP expression by activated fibroblasts promotes tumor angiogenesis, PI Thomas Kelly (Not funded, Unscored)

Conclusion

Several key goals of the project were not completed due to technical difficulties. Nevertheless, this project has been successful in reporting the importance of non-catalytic functions of FAP, development of a FAP-specific protease activity assay, as well as discovery of a possible link of FAP and EGR-1 to the pro-angiogenesis function of FAP. This has resulted in preliminary findings indicating that FAP expression may lead to an increase in TGF-β expression and a suppression of thrombospondin expression. Together these findings support a role of FAP and EGR-1 in promoting angiogenesis in breast cancer. Finally, evidence is presented to show that the role of FAP and EGR-1 in breast cancer can be studied in the MMTV-PyMT mouse model of breast cancer.

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Total of 16 data figures (all but 1 are not yet published)

First Year Figures

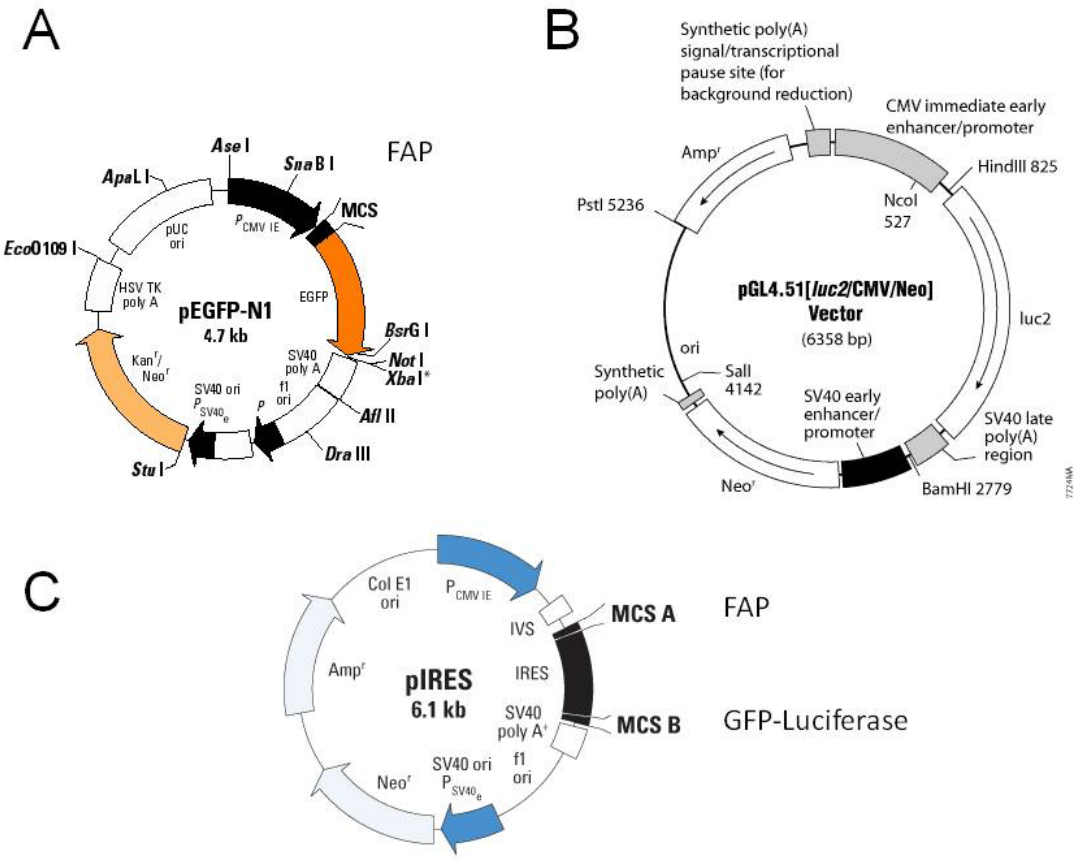


Figure 1. Plasmid vectors prepared to aid in identifying FAP tumor cells in microemboli. A) pEGFP has been produced with FAP cDNA in the multiple cloning site (MCS). B) pGL$.51 vector expresses luciferase and has been co-transfected with pEGFP into MDA MB-231 cells. C) The “pluc” vector is derived from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

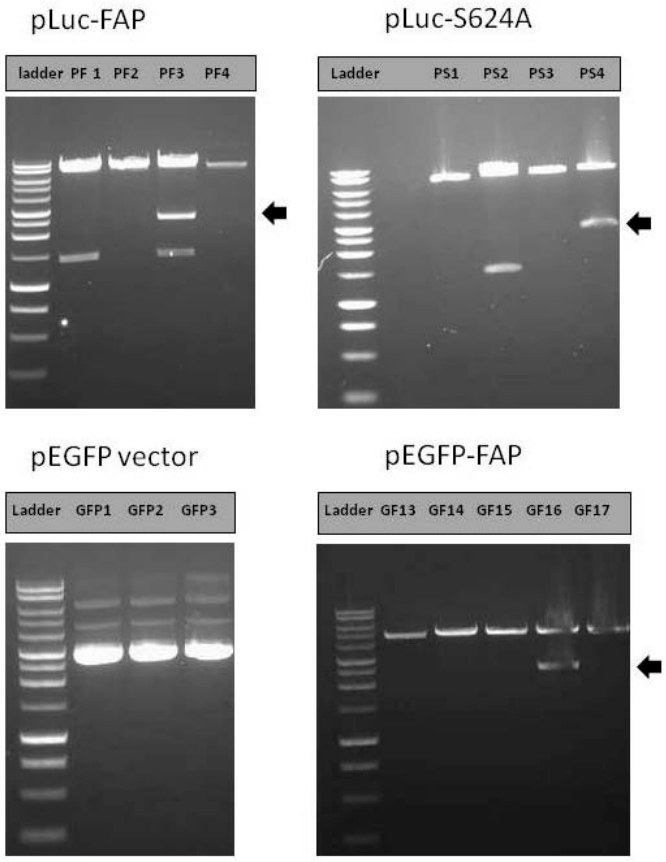
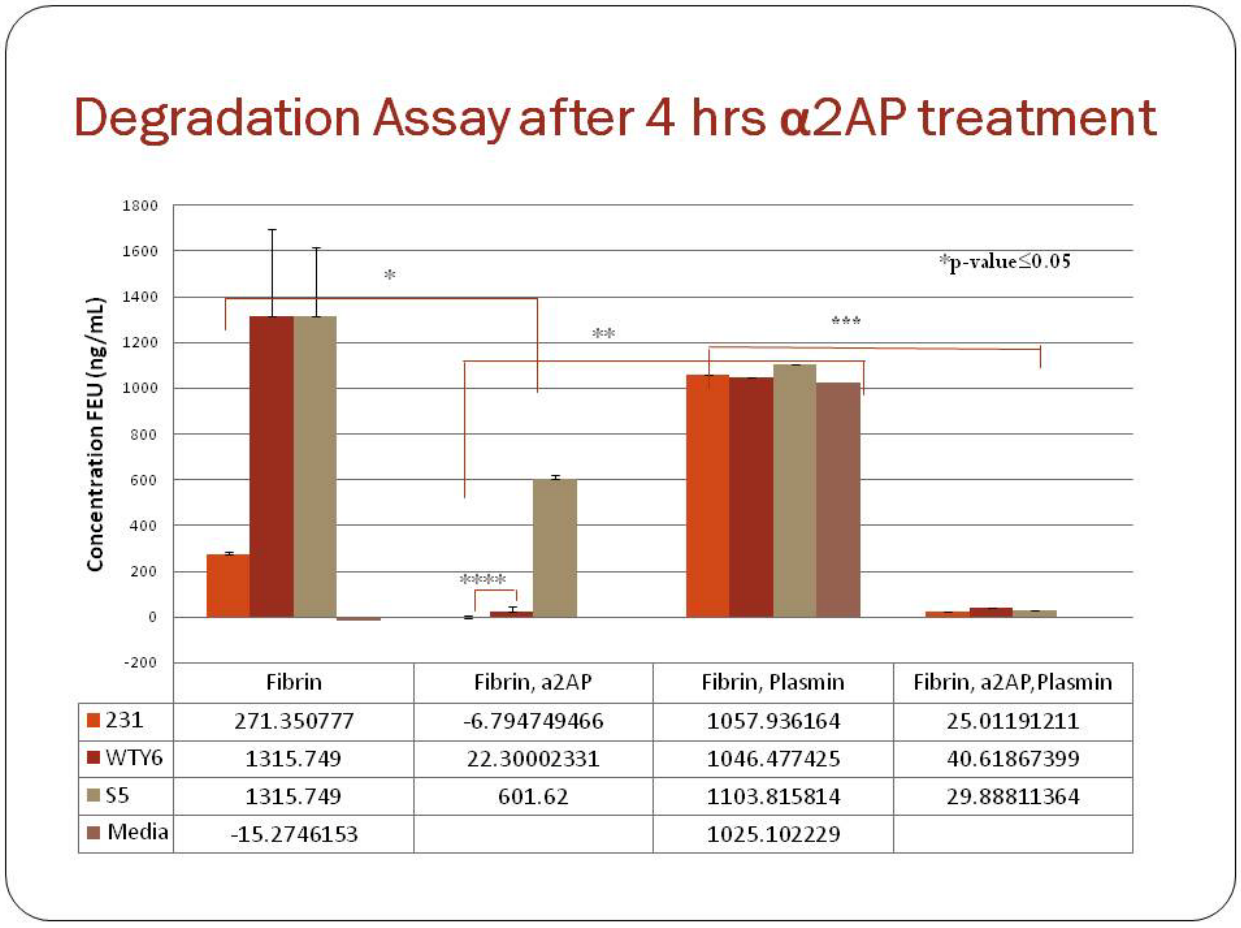


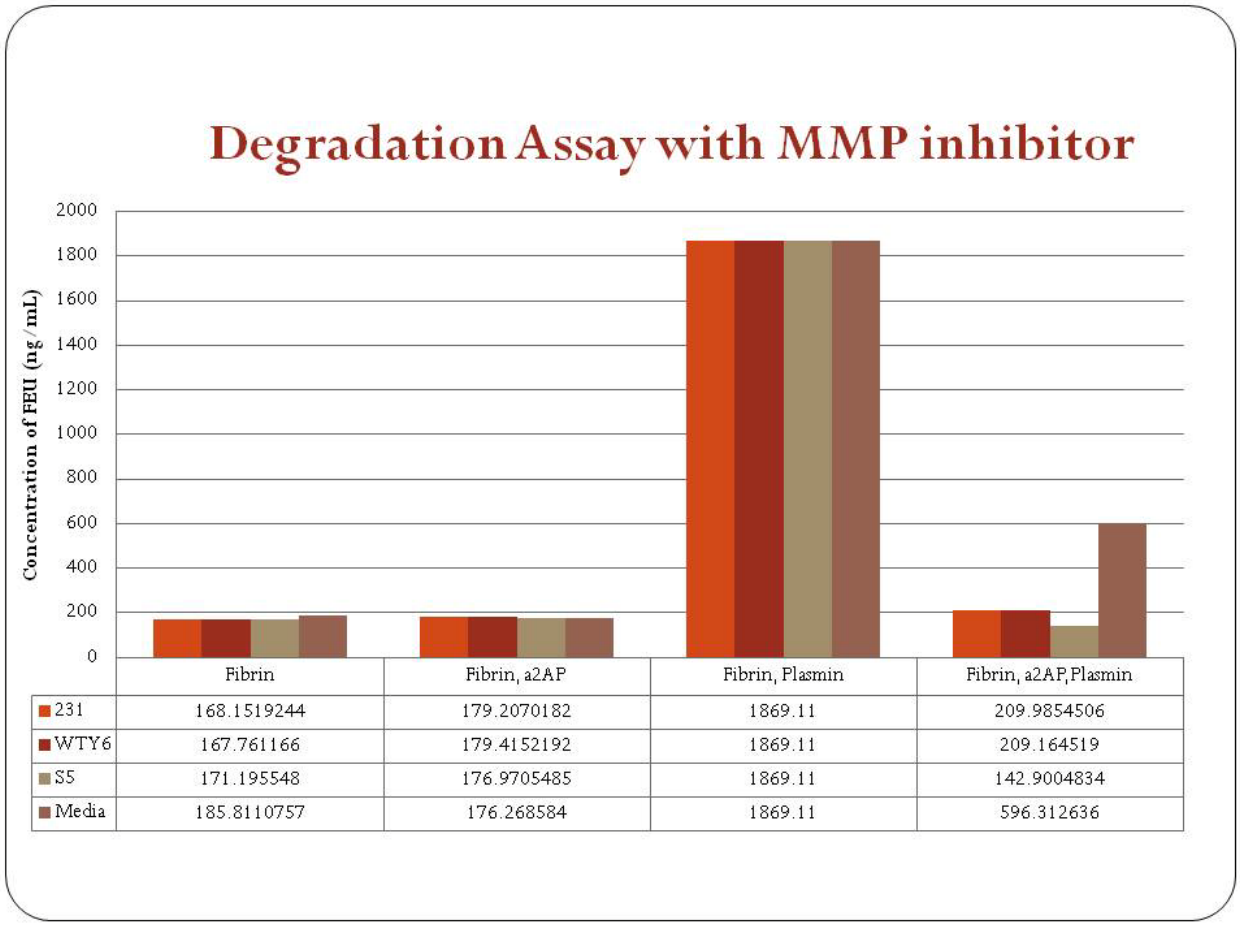
Figure 2. Plasmid vectors prepared to aid in identifying FAP tumor cells in microemboli. Top panels) Left panel shows pluc vector with the FAP insert (lane PF3, bold arrow). Right panel shows identification of the clone with cDNA encoding the S624A mutant FAP in pluc vector (lane PS4, bold arrow). **Bottom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

Second Year Figures



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	271.350777	-6.794749466	1057.936164	25.01191211
WTY6	1315.749	22.30002331	1046.477425	40.61867399
S5	1315.749	601.62	1103.815814	29.88811364
Media	-15.2746153		1025.102229	

Figure 3. After 4h treatment with α2-AP plasmin independent and dependent fibrin degradation was decreased. After 4h incubation in the absence of plasmin fibrin degradation was slightly elevated by 231 cells, greatly increased by WTY-6 and S5 cells and not detected in medium without cells (Fibrin group). When treated with A2AP but not plasmin the fibrin degradation was greatly diminished even in the absence of plasmin (Fibrin, A2AP group). When treated with plasmin and not with A2AP, fibrin degradation was high for all conditions (Fibrin, Plasmin Group) and was suppressed for all conditions by A2AP (Fibrin, A2AP, Plasmin, group).



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP, Plasmin
231	168.1519244	179.2070182	1869.11	209.9854506
WTY6	167.761166	179.4152192	1869.11	209.164519
S5	171.195548	176.9705485	1869.11	142.9004834
Media	185.8110757	176.268584	1869.11	596.312636

Figure 4. MMP inhibitors drastically decreased the plasmin independent fibrin degradation. Plasmin independent fibrin degradation greatly decreased when treated with BB94 (MMP inhibitor) regardless of whether or not A2AP was present (Fibrin group, Fibrin, A2AP group). Fibrin degradation still occurred in the presence of BB94 (1 µM) when treated with plasmin (Fibrin, Plasmin group). The plasmin dependent fibrin degradation was decreased when treated with α2-AP (Fibrin, A2AP, Plasmin).

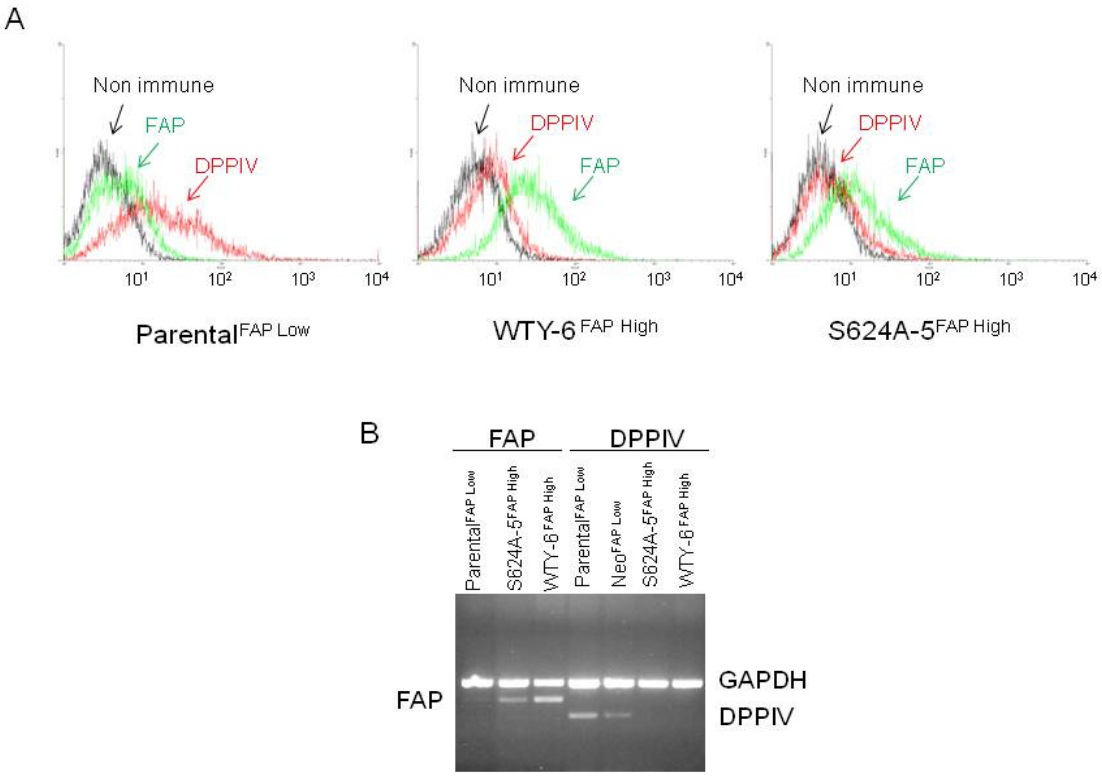


Figure 5. FAP expression suppresses DPPIV expression

A. Cell surface expression of DPPIV (red trace), FAP (green trace) and non immune IgG (black trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). **B.** FAP (left panel) or DPPIV (right panel) message signals were detected in cellular extracts by RT-PCR using specific primers. GAPDH serves as internal control.

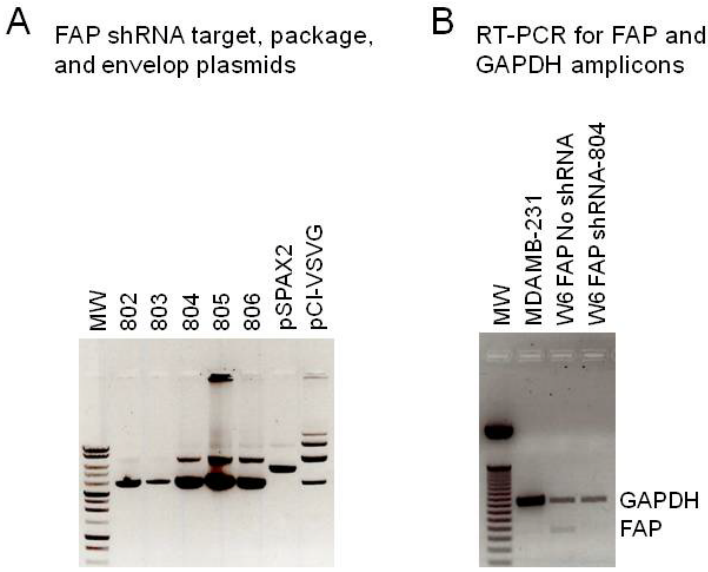


Figure 6. Supression of FAP expression by lenti viral vectors A. This gel shows the isolated DNA of the plasmids encoding FAP-specific shRNAs (802-806), the plasmid packaging vector DNA (pSPAX2) and the plasmid encoding the envelope DNA (pCI-VSVG) molecular weight standards are loaded in the left (MW). **B.** GADPH or FAP specific amplicons were detected by RT-PCR using primers specific for FAP and GAPDH. The MDA MB-231 cells do not express FAP and no FAP amplicon was detected (MDA MB-231). The WTY-6 cells express FAP to high levels and an amplicon was detected in uninfected cells (W6 FAP No shRNA) but not in cells infected with lenti viruses encoding FAP-specific shRNA-804 (W6 FAP shRNA-804).

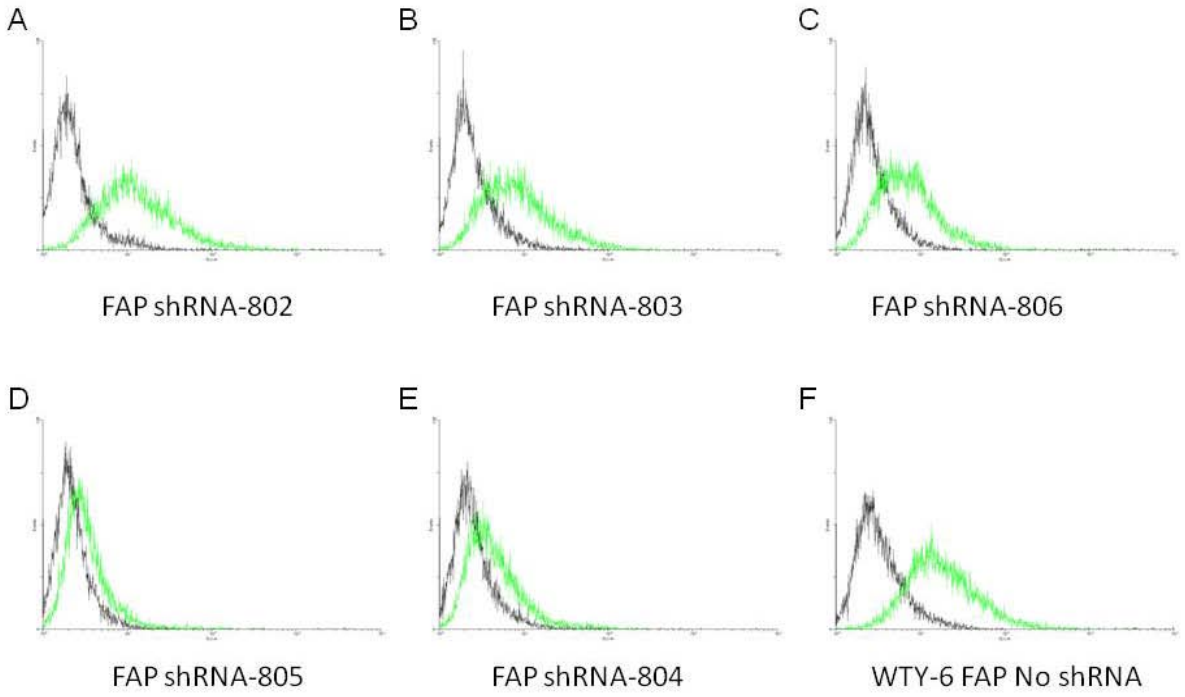


Figure 7. Suppression of FAP expression by lenti viral vectors containing clones 805 and 804 of FAP-targeted shRNA In histogram, the black trace is non immune IgG and the green trace is cell surface FAP expression **A-C.** WTY-6 cells were infected with lentiviruses encoding FAP-specific shRNAs 802 (A), 803 (B), and 806 (C). None of these were effective in reducing cells surface FAP expression. However WTY-6 cells infected with lentiviruses encoding FAP-specific shRNAs 805 (D) and 804 (E) showed suppression of FAP expression relative to the WTY-6 cells that were not infected with lentivirus (F).

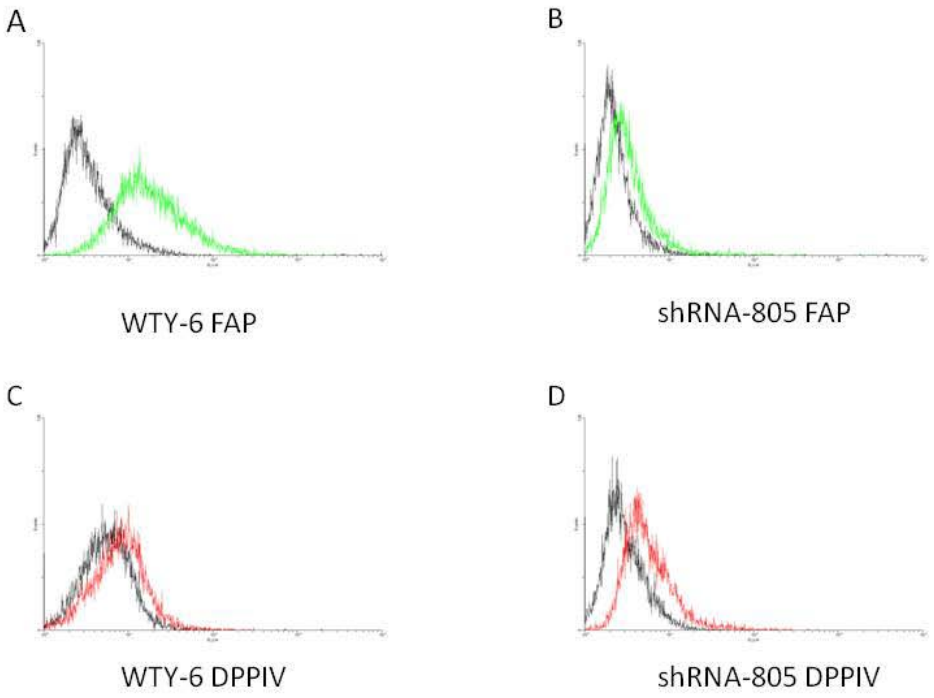


Figure 8. FAP suppression induces increased expression of DPPIV. A) FAP expression (green trace) is high on over 90% of the WTY-6$^{FAP\ High}$ cells. The black trace in all panels is the non-immune control. **B)** FAP expression (green trace) is suppressed in WTY-6$^{FAP\ High}$ cells by the shRNA from clone 805. **C)** DPPIV expression (red trace) on WTY-6$^{FAP\ High}$ cells is very low. **D)** DPPIV expression (red trace) is induced in cells where FAP is suppressed.

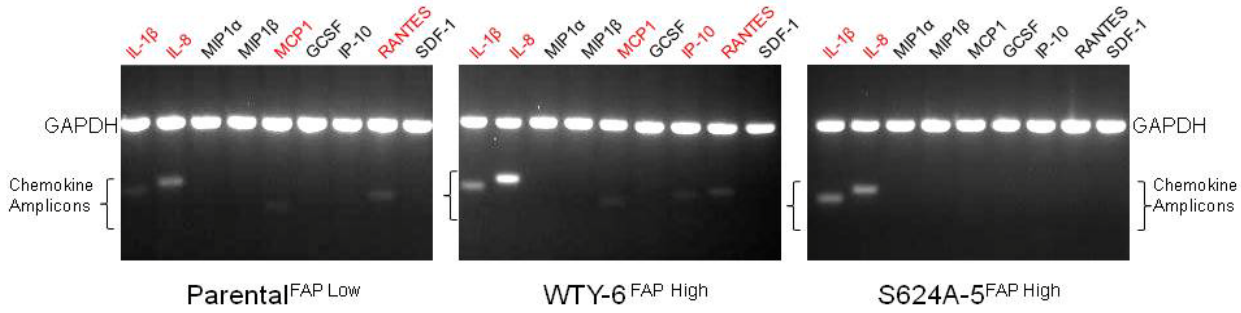


Figure 9. **IL-8 and other NK-cell attracting chemokines are expressed by the human breast cancer cells used in this study.** Chemokines were detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ cells using specific primers to produce amplicons between 180-220 bp. Reactions included primers for a 450bp GAPDH amplicon. Gel samples labeled red are positive for expression of the chemokine.

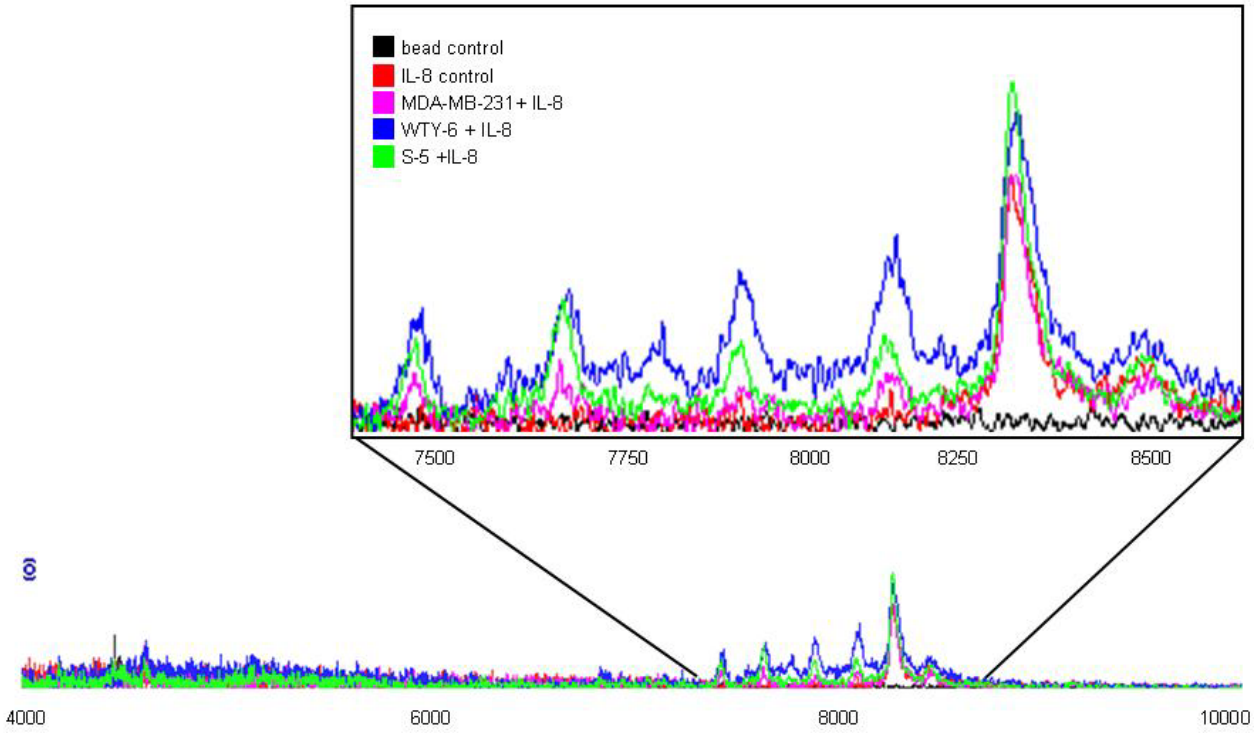


Figure 10. Increased proteolytic degradation of IL-8 is observed in cells that express FAP. Surface enhanced laser desorption time of flight mass spectrometry (SELDI) analysis of IL-8 proteolysis in the 4-10 kDa range. FAP from extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (Parental$^{FAP\ Low}$) levels or mutant (S624A-5$^{FAP\ High}$) FAP was bound to wheat germ agglutinin agarose beads, incubated with purified IL-8, and then the supernatant subjected to SELDI. Lower molecular weight peaks in high FAP (blue trace) and less extensively, mutant FAP samples (green trace) indicate proteolysis of IL-8. IL-8 break down is low in parental cells (magenta trace). Intact IL-8 occurs at 8.2 kDa (red trace) and no peaks are from the beads (black trace).

Third Year Figures

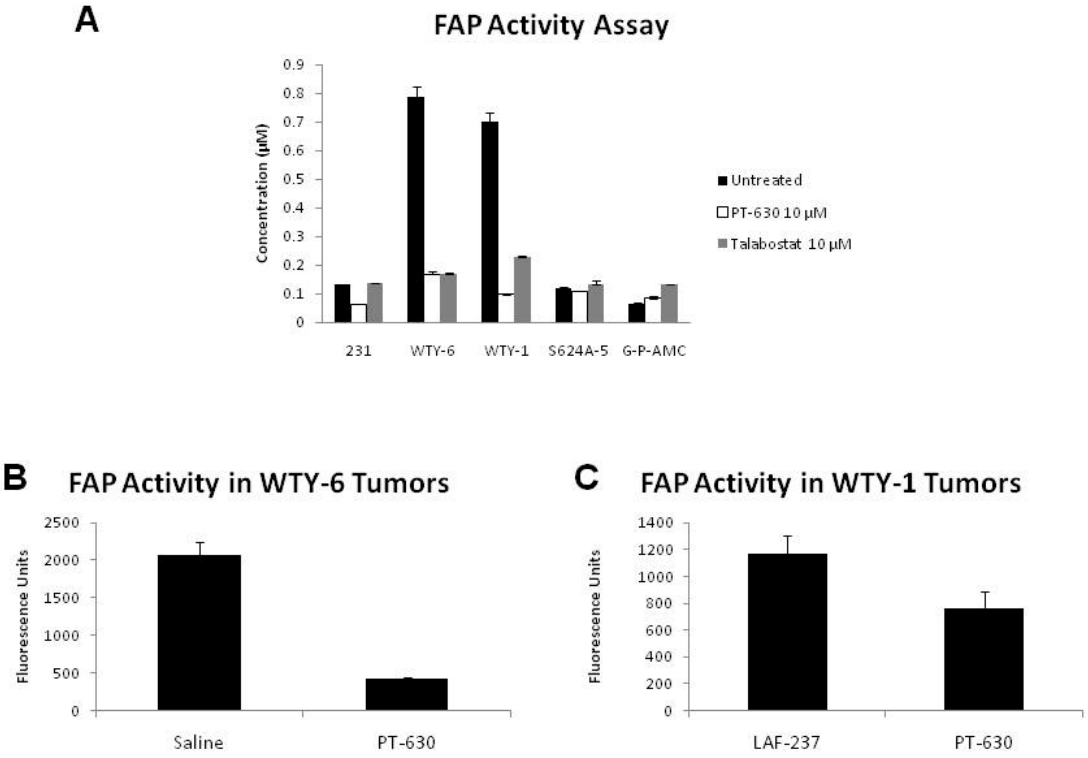


Figure 11. Effect of inhibitors on FAP activity. A. FAP activity was assessed by binding FAP in extracts of parental MDA-MB-231 (231) cells that do not express FAP; WTY-6 (WTY-6), WTY-1(WTY-1) cells that express wild type and active FAP, or S624A-5 cells that express a mutant FAP that is catalytically inactive (S624A-5) to F19 mAb immobilized on magnetic protein G beads. In addition, results are shown for substrate only (G-P-AMC). The FAP-bound beads were exposed to the FAP substrate z-Gly-Pro-AMC, the supernatant collected and the fluorescence emitted at 460 nm when excited by 360 nm was determined. A standard curve using free AMC allows plotting the concentration of free AMC on the y axis. Results are shown for FAP that is uninhibited (black bars) or for FAP inhibition by PT-630 (white bars) or PT-100 (gray bars). (From Huang et al, 2011, Appendix)

B & C. FAP activity was determined in extracts of tumor tissues derived from **B)** WTY-6 cells (WTY-6) exposed to normal saline (Saline), or PT-630 (PT-630), and **C)** extracts of tumor tissues derived from WTY-1 cells (WTY-1) and exposed to LAF-237 (LAF-237) or PT-630 as described in the animal experiments shown in figure 2. Results are given in fluorescence intensity determined at 460 nm wavelength with excitation wavelength of 360 nm.

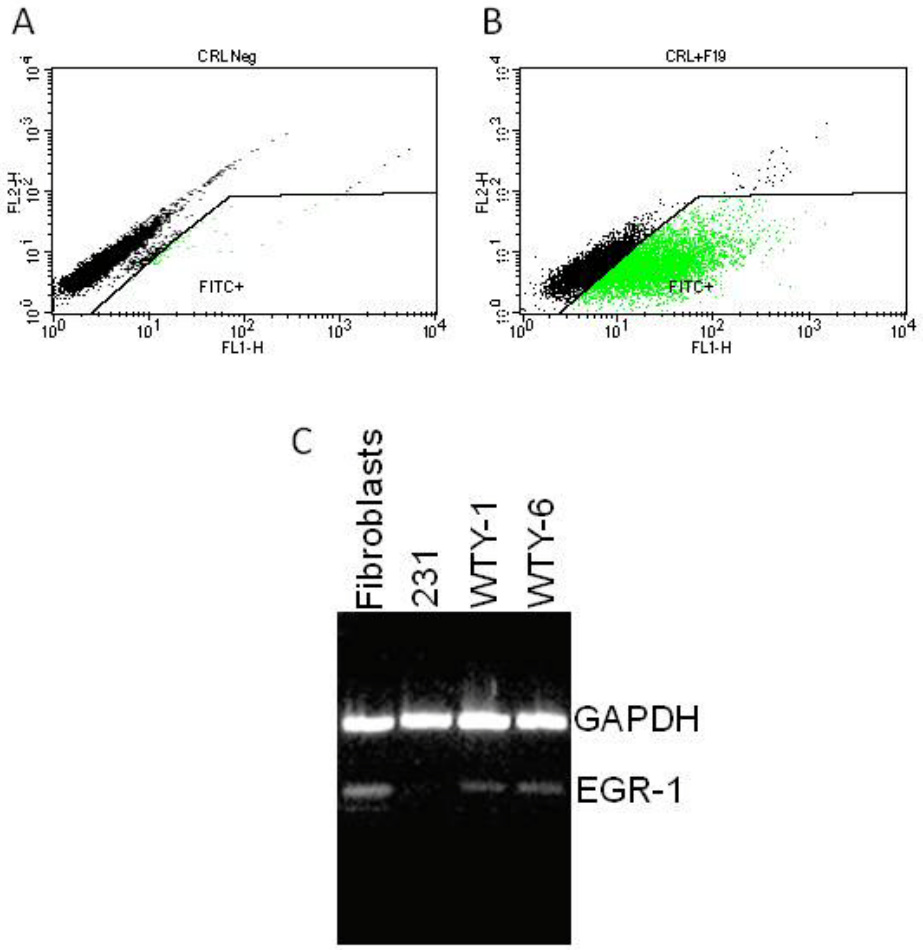


Figure 12. High FAP expression correlates with high EGR-1 expression. FACS analysis of human fibroblasts CRL-1947 with non-immune IgG **(A)** and F19 mAb to FAP **(B)** reveals about 50% fibroblasts expressing high levels of FAP. **(C)** Reverse transcription PCR showing robust expression of EGR-1 in fibroblasts and FAP-expressing cells

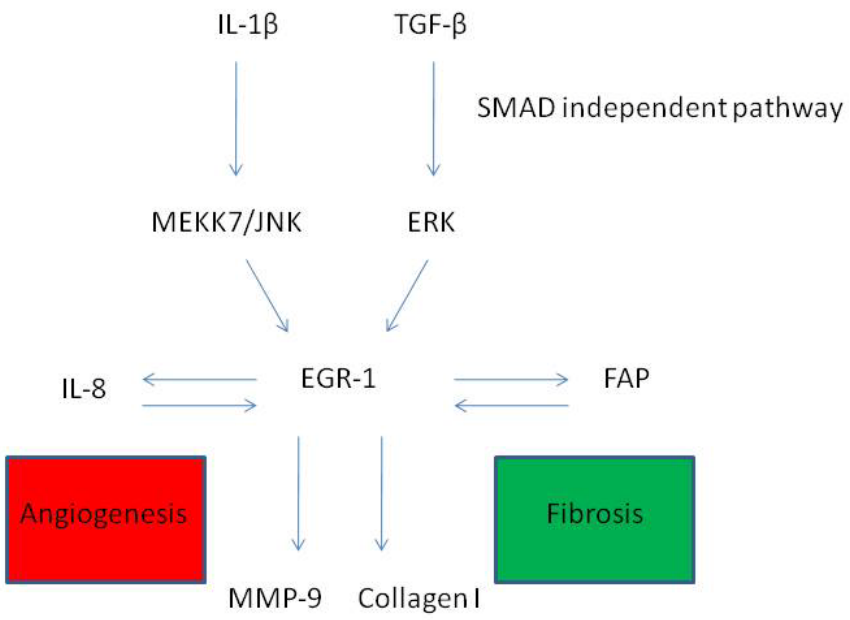


Figure 13. Schematic diagram of reported signaling that impacts fibroblast activation and FAP expression. Figure is based on references cited in the text and our results.

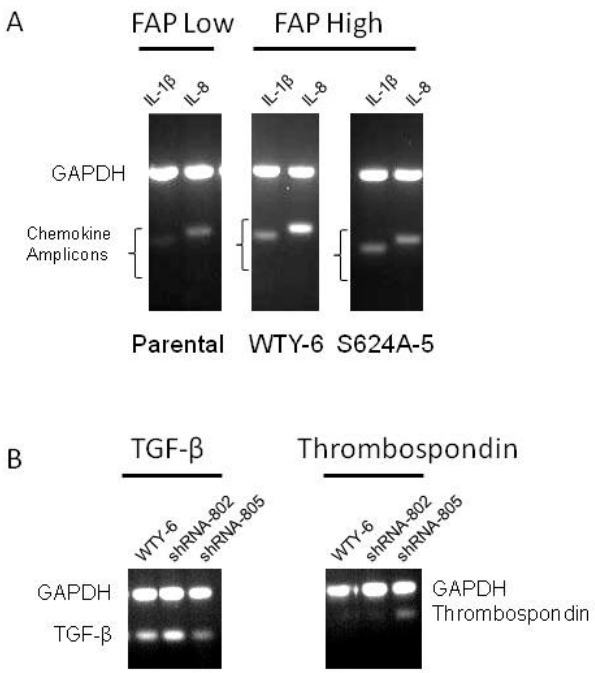


Figure 14. A) IL-1 and IL-8 are expressed by FAP low and FAP high cells. RT-PCR using primers specific for GAPDH, IL-1, & IL-8 in different cell lines. **B)** TGF-β and Thrombospondin expression in these lines. Note suppression of TGF-β and increased thrombospondin in cells where FAP is suppressed by shRNA-805.

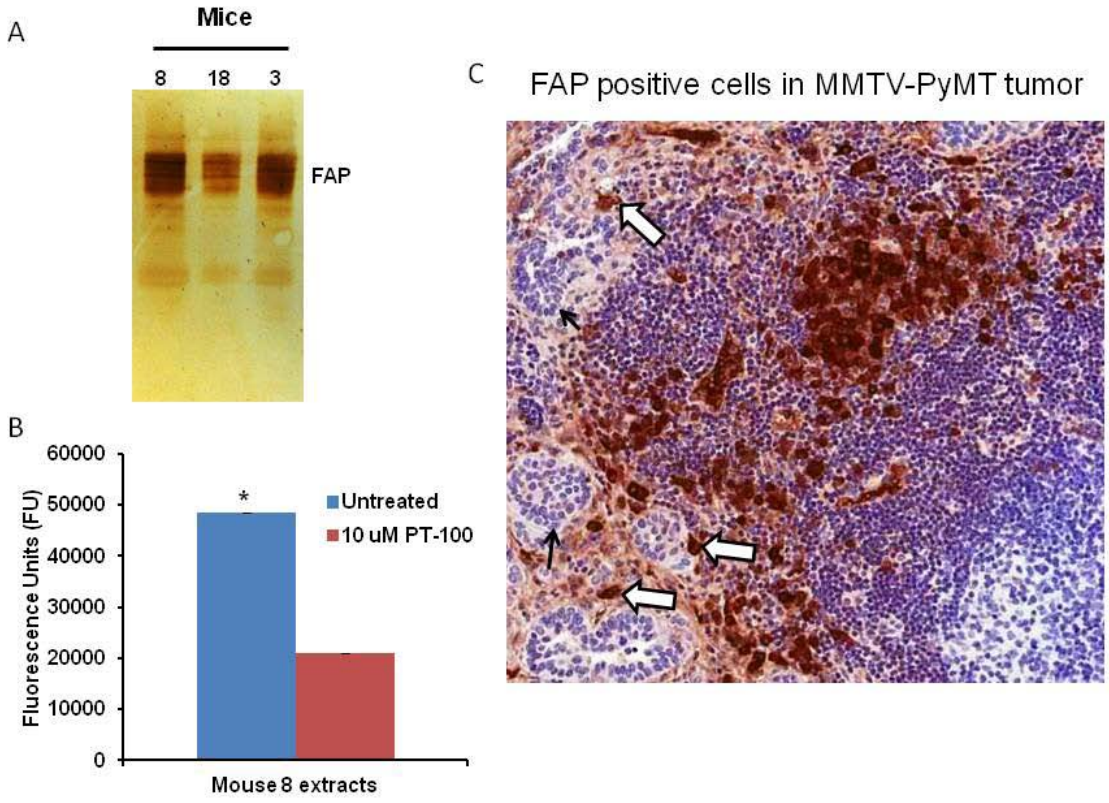


Figure 15. FAP and EGR-1 are expressed in tumor tissues of MMTV-PyMT mice. A) Western blot analysis of tumor tissue extracts from three different MMTV-PyMT mice with antibody to FAP showing immunoreactive bands between 150 and 225 kDa which identifies active FAP. **B)** High levels of cleavage of the FAP substrate, z-Gly-Pro-AMC (Blue bar) are found in tumor extracts that can be significantly decreased with the FAP inhibitor (Red bar) P≤0.05. **C)** Immunohistochemistry of breast tumors in MMTV-PyMT mice reveals intense FAP staining of stromal fibroblasts (black & white arrows) but epithelial tumor cells are negative (black arrows).

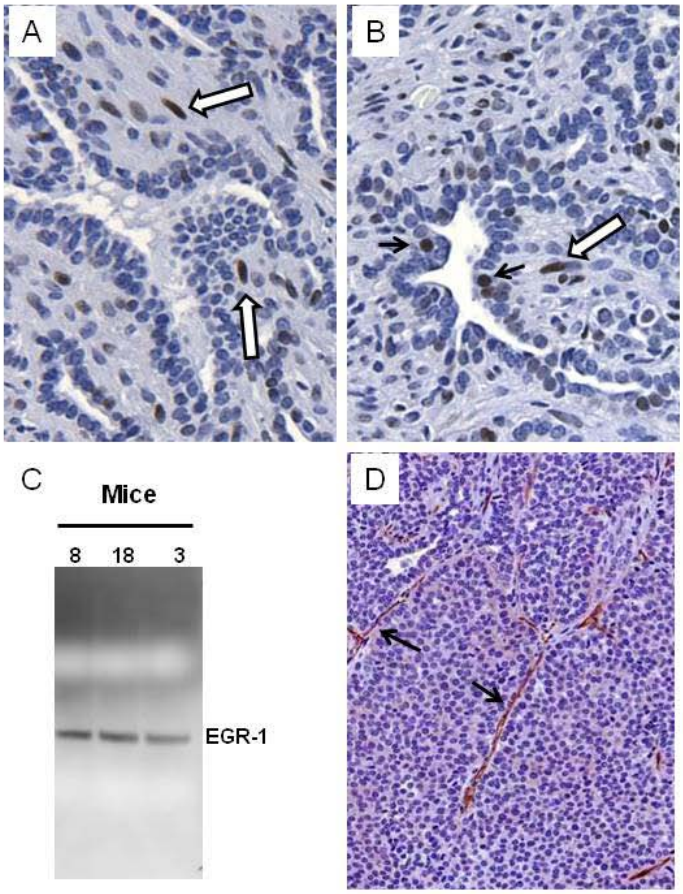


Figure 16. EGR-1 and CD34 expression in MMTV-PyMT tumors. A & B) Antibody to EGR-1 labels some fibroblasts (white arrows) and epithelial cells (black arrows) **C)** RT-PCR with primers to mouse EGR-1 reveals EGR-1 expression in tumor tissues from three different MMTV-PyMT mice. **D)** Blood vessels are apparent in sections of MMTV-PyMT breast tumors stained with CD34 antibody (arrows).

Appendix

Poster presentations (6 total)

1. T. Kelly, N. R. León, K. Kelly, B. Jones and N. Aziz "Tumor growth is slowed by an inhibitor of DASH proteases in a mouse model of human breast cancer" 3rd International Congress on Proteases and Dipeptidyl Peptidases, April 23-25, 2008, Antwerp, Belgium

2. Y. Huang, A. Mazur, A. E. Simms and T. Kelly "Fibroblast activation protein-α: A potential modulator of chemokines that regulate natural killer recruitment to breast tumors", Cell Adhesion and Stress Fibers: A symposium in honor of Keith Burridge, August 27-29, 2010, Chapel Hill, NC.

3. A. E. Simms and T. Kelly, "Alpha 2-Antiplasmin (α2AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin" Jackson Heart Study Scientific Conference. 9/23-24/2010. Jackson, MS. (Included in the appendix)

4. A. E. Simms, and **T. Kelly** (2011) "Alpha 2-Antiplasmin (α2-AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin" Arkansas Breast Challenge, 7/29-31/2011, Big Cedar Lodge, Ridgedale, MI (**Note** this was the same as the presentation Ms Simms delivered at the Jackson Heart Study that was reported in Progress report of October, 2010)

5. A. Mazur, Y. Huang, C. Washam, L. J. Suva, and **T. Kelly** (2011) Fibroblast Activation Protein-α: A potential modulator of biological functions in the tumor microenvironment. Arkansas Breast Challenge, 7/29-31/2011, Big Cedar Lodge, Ridgedale, MI

6. Y. Huang, A. Mazur, A. E. Simms, C. Washam, L. J. Suva, and **T. Kelly** (2011) "Fibroblast activation protein-α: A chemokine modulator regulating natural killer recruitment to tumors?" ERA of Hope, Congressionally directed medical research program, DoD breast cancer research program. 8/2-5/2011. Orlando, FL.

Papers (2 total):

1. Y. Huang, A. E. Simms, A. Mazur, S. Wang, N. R. León, B. Jones, N. Aziz, and **T. Kelly**. (2011) Fibroblast activation protein-α promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions. Clin. Exp. Metastasis. *28*:567-579. DOI: 10.1007/s10585-011-9392-x

2. Kelly, T., Y. Huang, A.E. Simms, and A. Mazur, (2012) Fibroblast activation protein-α: A key modulator of the microenvironment in multiple pathologies. Intl. Rev. Cell & Mol. Biology, 297: *In press*.

Tumor growth is slowed by an inhibitor of DASH proteases in a mouse model of human breast cancer

UAMS COLLEGE OF MEDICINE DEPARTMENT OF PATHOLOGY UNIVERSITY OF ARKANSAS FOR MEDICAL SCIENCES

Thomas Kelly[1], Noel R. León[1], Kevin Kelly[1], Barry Jones[2] and Nazneen Aziz[2]

[1]Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, AR and [2]Point Therapeutics, Inc., Boston, MA

point THERAPEUTICS

ABSTRACT

Dipeptidyl peptidases and structural homologs (DASH) are a family of 10 or more members that are implicated in the regulation of diverse biological processes. Fibroblast activation protein-α (FAP) in this family is a cell surface, post-prolyl serine protease that is over-expressed in human breast cancer but not expressed in normal adult tissues. Previously, we showed that expression of FAP by tumor cells increased tumor growth rates and microvessel densities of tumors in a mouse model of human breast cancer. Here we sought to determine if inhibition of the FAP protease has an anti-tumor effect. Derivatives of the human breast cancer cells MDA MB 231 engineered to over-express FAP (WTY-1 and WTY-6) or MDA MB 435 cells that express FAP endogenously were inoculated into the mammary fat pads of female SCID mice. The mice were treated with normal saline or inhibitors of the DPP proteases. Talabostat inhibits both extracellular and intracellular DPPs and via the inhibition of intracellular DPP8/9 invokes cytokine production and immune activation. In contrast, PT-630 does not readily permeate cells and appears to be restricted to the inhibition of extracellular DPPs (FAP and DPPIV) *in vivo*. Tumor take, growth rates, and wet weights were recorded. Talabostat slowed growth of tumors produced by all three FAP-α expressing cell lines; but PT-630 did not slow tumor growth. After 40 days the average wet weights of the WTY-1 tumors treated with talabostat were reduced compared to controls; however, this did not achieve statistical significance. WTY-1 tumors treated with talabostat had significantly lower microvessel density than untreated controls ($P<0.05$). Other DPP inhibitors did not significantly reduce microvessel density. The compounds were also tested *in vitro* for their ability to inhibit matrix degradation by the WTY-1 cells. Cells interacted for 48 hours with an artificial matrix of immobilized fluorescent fibronectin, which is a simple matrix as compared to natural matrix barriers encountered by cells *in vivo*. Talabostat and PT-630 were used at a concentration of 1 μM and fresh inhibitor was added after 24 hours. The films were fixed and evaluated microscopically. In four trials, 10 random microscopic fields were selected and manually scored for the presence or absence of visible matrix degradation. By this analysis all inhibitors reduced matrix degradation relative to the saline control. In another method, photomicrographs of five fields of each condition that exhibited degradation were taken. These were analyzed using the NIH ImageJ program for percent total area degraded. Treatment of cells with either inhibitor revealed reduced matrix degradation. While only talabostat slowed tumor growth, all DPP inhibitors inhibited matrix degradation.

QUESTION

Can inhibitors of DASH impede growth of human breast tumors in a mouse model?

EXPERIMENTAL DESIGN

Inoculate 1 x 10^6 FAP-expressing tumor cells in each of four mammary fat pads. Wait two days.

Begin dosing by oral gavage once daily with 1.3 mg/kg L-valine-L-boroproline called talabostat (extracellular & intracellular DASH), 13.3 mg/kg L-glutamyl L-boroproline called PT-630 (extracellular DASH); or 6.7 mg/kg 1-[[(3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidine called LAF237.

RESULTS

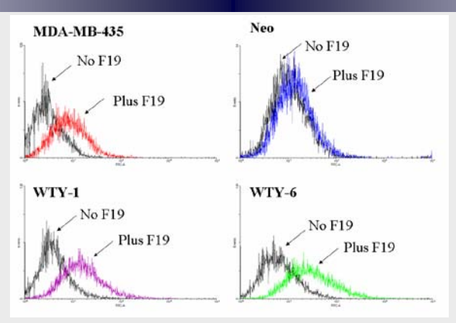


Figure 1. FAP expression by human breast cancer cells used in the study. FAP is expressed endogenously by MDA MB-435 human breast cancer cells (top left) and WTY-1 and WTY-6 cells that are MDA MB-231 human breast cancer cells engineered to express high levels of FAP (bottom left and right) but not by control transfected MDA MB-231 cells (top right).

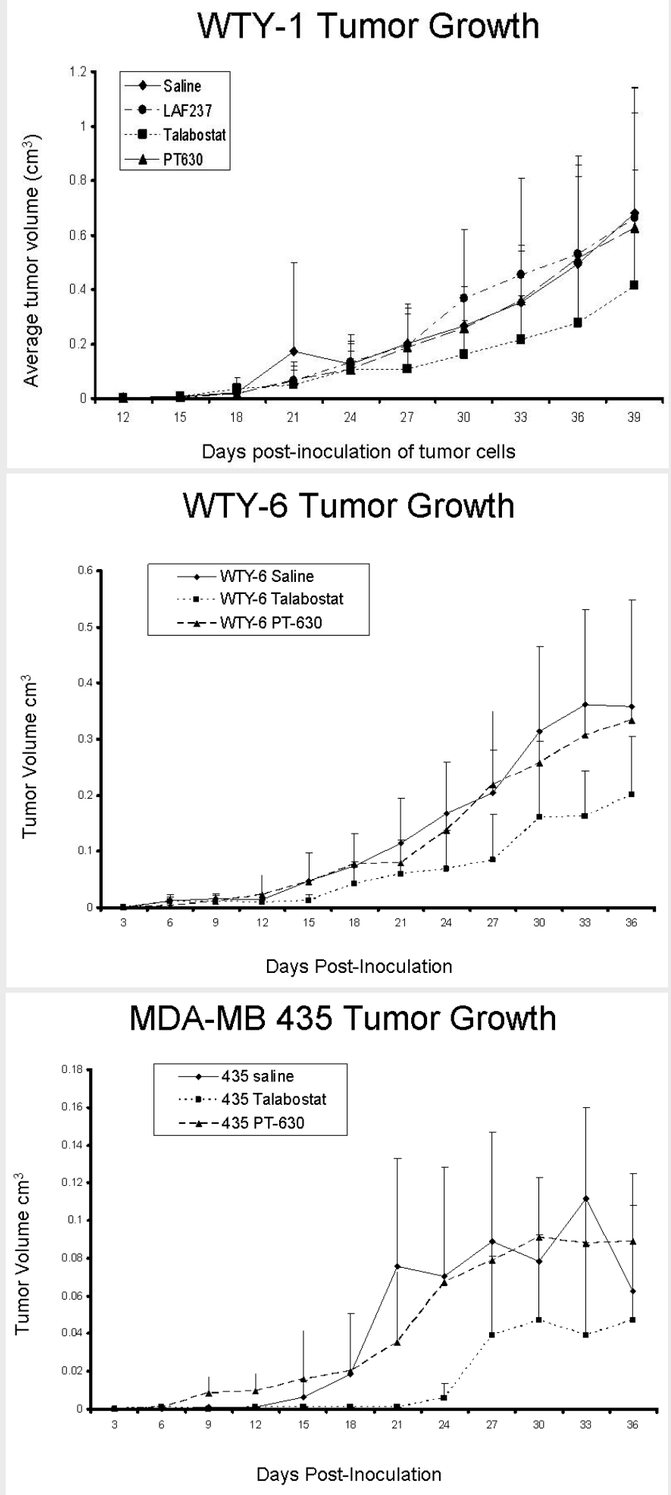


Figure 2. Inhibition of DASH proteases by talabostat slows growth of breast tumors in the mammary fat pads of SCID mice. Tumor volumes plotted over time of FAP-expressing human breast cancer cells in SCID mice treated with saline (♦), LAF237 (●), PT-630 (▲), or talabostat (■).

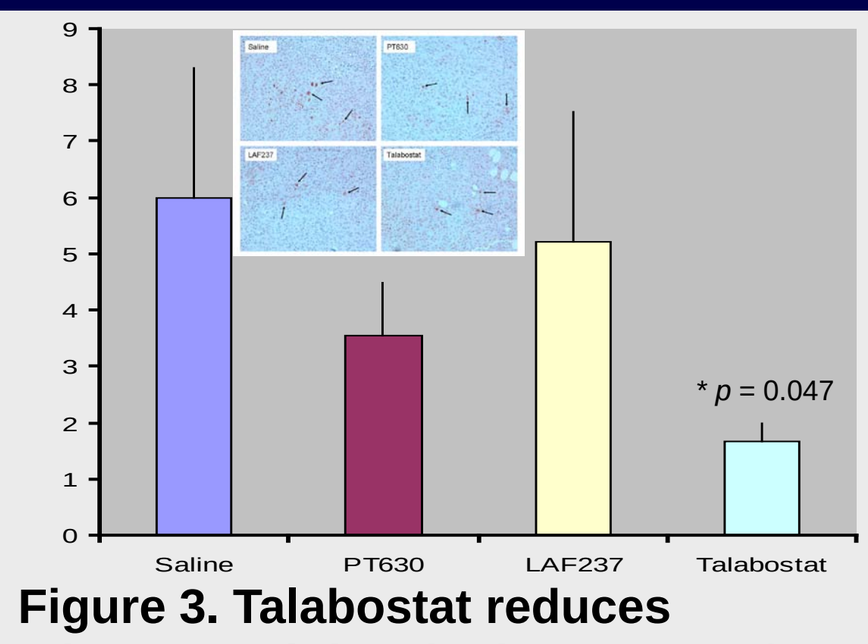


Figure 3. Talabostat reduces microvessel density in tumors of FAP-expressing breast cancer cells. Tumors of WTY-1 cells growing in animals treated with saline or DASH inhibitors were stained with antibody to CD34 (inset) and microvessels counted in 3 tumors from each group. Columns show the average vessel numbers and bars the standard deviations (graph).

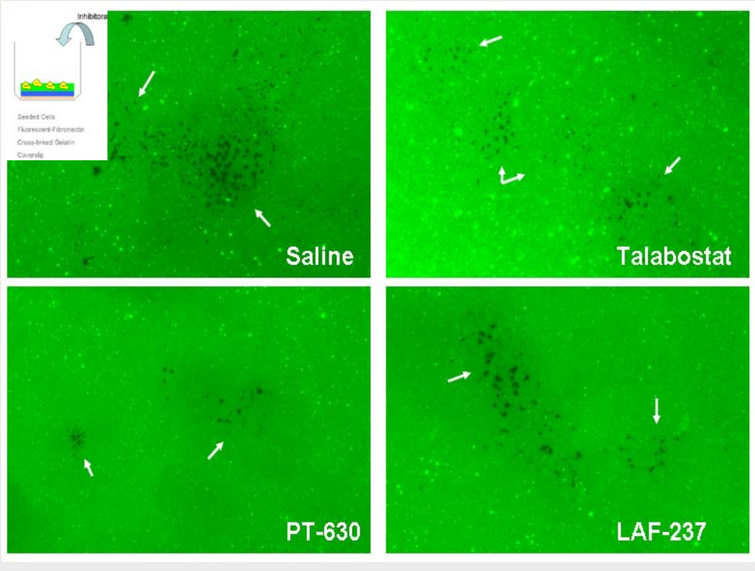


Figure 4. Inhibition of DASH proteases reduces matrix degradation by FAP-expressing breast cancer cells. WTY-1 cells were seeded onto glutaradehyde-crosslinked gelatin films that were fluorescent due to covalently-attached FITC-fibronectin (inset). Inhibitors were used at 1 μM and replenished after 24 h. After 48 h, matrix degradation appears as black holes in the matrix (arrows in photos).

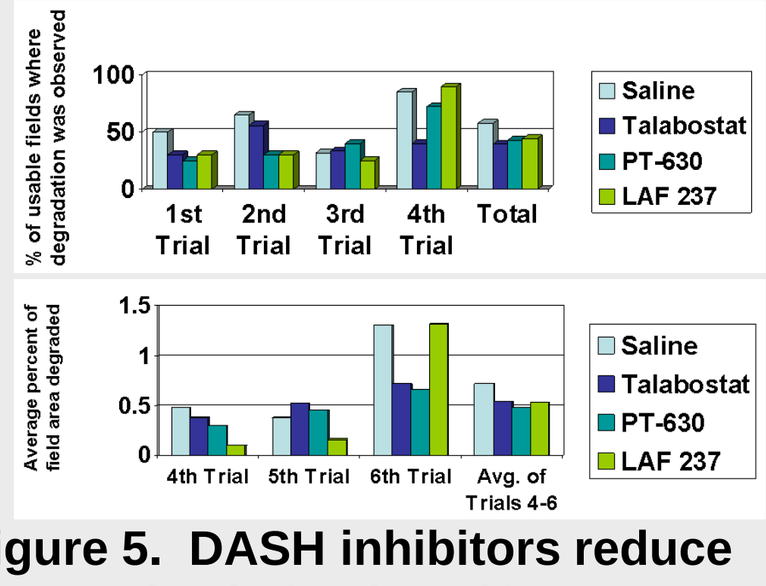


Figure 5. DASH inhibitors reduce aggressive behavior of breast cancer cells. Measuring the percentage of microscopic fields with matrix degradation (top panel) or percent of area degraded in fields with matrix-degradation as determined by the ImageJ program (bottom) reveals that all DASH inhibitors reduced matrix degradation. PT-630 and Talabostat showed the greatest inhibition.

CONCLUSIONS

1. Talabostat, but not other DASH inhibitors used, slowed the growth of FAP-expressing human breast tumors in a mouse model.

2. DASH inhibitors reduced matrix degradation caused by the breast cancer cells.

Fibroblast activation protein-α: A chemokine modulator regulating natural killer recruitment to tumors?

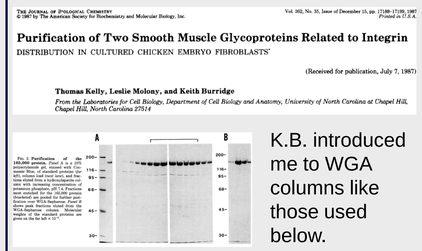
Yan Huang[1], Anna Mazur[1], Avis Simms[1], Charity Washam[2], Larry J.Suva[2], and Thomas Kelly[1]

[1]Department of Pathology, [2]Center for Orthopedic Research, Departments of Orthopedic Surgery and Physiology and Biophysics, Winthrop P. Rockefeller Cancer Institute, and University of Arkansas for Medical Sciences, Little Rock, AR

ABSTRACT

Dipeptidyl peptidases are a family of proteases that are implicated in the regulation of diverse biological processes. One member of the family, fibroblast activation protein-α (FAP), is a cell surface, post-prolyl serine protease that is over-expressed in human breast cancer but is not expressed in normal adult tissues. FAP has 50% amino acid sequence homology to another family member, dipeptidyl peptidase IV (DPPIV), and there is overlap in substrate specificities as both enzymes cleave NH_2-XAA-Pro peptides such as Gly-Pro-AFC. Importantly, DPPIV has been shown to cleave a number of chemokines including RANTES and SDF-1 that are known to be important in attracting innate immune cells such as natural killer (NK) cells. Proteolytic cleavage of RANTES and SDF-1 inactivates them and eliminates their attractive power for NK cells. Consistent with this, others have shown that inhibition of FAP by Val-boroPro, a broad spectrum inhibitor of post-prolyl proteases causes activation of immune responses of the host (Jones et al, 2003, Blood *102*:1641-8), perhaps through preventing the inactivation of chemokines. Previously, we showed that fast-growing tumors were produced by cells expressing proteolytically active FAP whereas parental MDA MB-231 do not express FAP and produce slow-growing tumors (Huang et al, 2004 Cancer Res. *64*:2712-2716). Here we investigate the tumor growth rates and differential expression of FAP, DPPIV, and chemokines in MDA MB-231 cells that do not express FAP and those engineered to express FAP to high levels. We extend our earlier findings by showing that cells expressing a catalytically inactive FAP produce rapidly growing tumors like cells expressing active FAP. We analyzed surface expression of FAP and DPPIV by flow cytometry to determine if DPPIV substitution for FAP results in the rapid growth of tumors produced by cells expressing a catalytically inactive FAP. We confirm that MDA MB-231 cells express little or no FAP and find that they express DPPIV to high levels. Conversely, these cells engineered to express wild type FAP or a catalytic mutant of FAP express FAP to high levels but reveal little DPPIV expression. These findings are confirmed by RT-PCR gene expression analysis. The results show that FAP expression correlates with rapid tumor growth and DPPIV expression correlates with slow tumor growth. Parental cells express DPPIV and the chemokines IL-1β, IL-8, MCP-1 and RANTES but little FAP; cells expressing proteolytically active FAP express these chemokines as well as IP-10 and little DPPIV; cells expressing inactive FAP express IL-1β and IL-8 only and little DPPIV. The findings are consistent with active FAP cleaving one or more of these chemokines and suppressing NK cell recruitment into the tumor thereby leading to rapid tumor growth. This work was supported by USAMRC, CDMRP, BCRP Synergistic Idea Award W91ZSQ7343N640 to TK and the Carl L Nelson Chair of Orthopaedic Creativity to LJS.

THIS STORY STARTED LONG AGO IN DR. KEITH BURRIDGE'S LAB



Purification of Two Smooth Muscle Glycoproteins Related to Integrin
DISTRIBUTION IN CULTURED CHICKEN EMBRYO FIBROBLASTS

Thomas Kelly, Leslie Molony, and Keith Burridge

K.B. introduced me to WGA columns like those used below.

Keith taught me so much about science and the wonder of discovery but while we worked hard….

….we also had a lot of fun!!

QUESTION

Does FAP expression suppress chemokines and recruitment of NK cells to tumors?

RESULTS

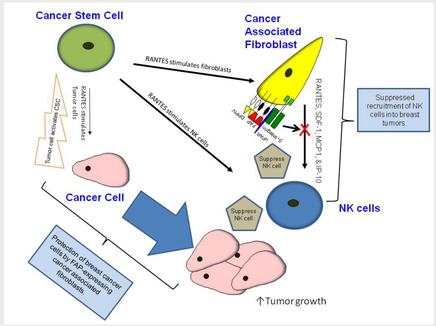


Figure 1. Cellular interactions in the tumor microenvironment. Cancer Associated Fibroblasts (CAFs, yellow) express FAP (red surface molecules) that may inactivate chemokines that attract NK cells and thereby suppress NK cell recruitment. The FAP activity of the CAFs may also decrease the tumor-killing function of NK cells.

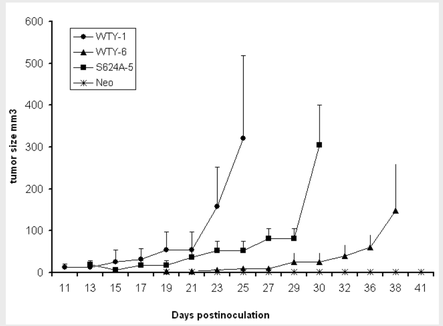


Figure 2. FAP promotes tumor growth. Growth of tumors of MDA MB-231 cells expressing wild type FAP (WTY-1 & WTY-6), inactive FAP (S624A-5) or no FAP (Neo). Growth curves for Neo, WTY-1 and WTY-6 were published previously (Huang et al, (2004) Cancer Research *64*:2712-2716. These human breast cancer cells were grown as tumors in the mammary fat pads of female SCID mice.

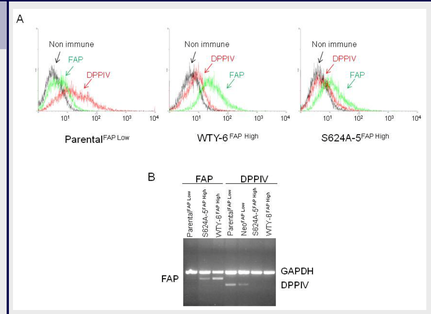


Figure 3. FAP expression suppresses DPPIV expression A. Cell surface expression of DPPIV (red trace), FAP (green trace) and non immune IgG (black trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). **B.** FAP (left panel) or DPPIV specific (Right panel were detected by RT-PCR using primers specific for FAP and for DPPIV as well as GAPDH.

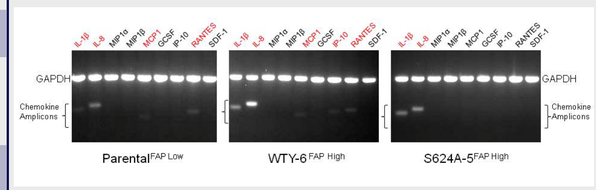


Figure 4. IL-8 and other NK-cell attracting chemokines are expressed by the human breast cancer cells used in this study. Chemokines were detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ using specific primers to produce amplicons between 180-220 bp. Reactions included primers for a 450bp GAPDH amplicon. Gel samples labeled red are positive for expression of the chemokine.

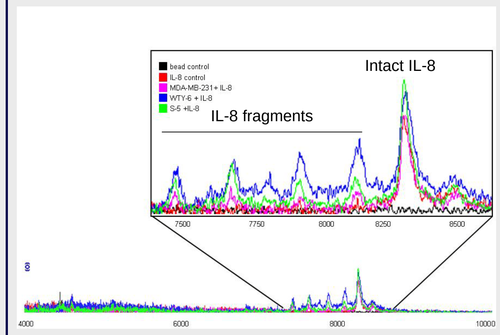


Figure 5. Increased proteolytic degradation of IL-8 is observed in cells that express FAP. Surface enhanced laser desorption time of flight mass spectrometry (SELDI) analysis of IL-8 proteolysis in the 4-10 kDa range. FAP from extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (Parental$^{FAP\ Low}$) levels or mutant (S624A-5$^{FAP\ High}$) FAP was bound to wheat germ agglutinin agarose beads, incubated with purified IL-8, and then the supernatant subjected to SELDI. Lower molecular weight peaks in high FAP (blue trace) and less extensively, mutant FAP samples (green trace) indicate proteolysis of IL-8. IL-8 break down is low in parental cells (magenta trace). Intact IL-8 occurs at 8.2 kDa (red trace) and no peaks are from the beads (black trace).

CONCLUSIONS

1. Expression of FAP coincides with suppression of DPPIV and is independent of FAP proteolytic activity.

2. FAP expression correlates with rapid tumor growth while DPPIV expression correlates with slow tumor growth in an animal model of human breast cancer.

3. FAP may facilitate enhanced cleavage of IL-8 and perhaps other chemokines that attract NK cells. FAP may cooperate with other proteases to degrade chemokines.

Alpha 2-Antiplasmin (α2AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin

Avis Simms[1],and Thomas Kelly[1]

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ABSTRACT

Fibrin, the major component of blood thrombi, increases the number of stable secondary tumor foci (metastases) arising from circulating tumor cells. Normally, fibrin is degraded by plasmin, but matrix metalloproteinases (MMPs) can also degrade fibrin. α2-Antiplasmin (α2-AP) is the primary inhibitor of plasmin and it stabilizes fibrin by protecting it from degradation by plasmin. α2-AP is a substrate of fibroblast activation protein -α (FAP) a serine protease found in elevated levels in human breast cancer. The FAP-cleaved version of α2-AP cross-links faster to fibrin during fibrin polymerization and maintains its inhibitory function towards plasmin. Our objective is to determine if FAP-cleaved or un-cleaved α2-AP will promote the metastasis of tumor cells by decreasing fibrin degradation around the cells. We hypothesize that FAP-cleaved α2-AP may prolong the life of fibrin by preventing degradation and promote encasement of FAP-expressing breast cancer cells in a fibrin sheath. To test this hypothesis, we used human breast cancer cells that do not express FAP (MDA- MB-231) or those engineered to express proteolytically active FAP (WTY-6) or a catalytically inactive mutant FAP (S5) as well as a mouse fibroblast line (NIH 3T3). First we used immunofluorescence microscopy to investigate how α2-AP interacts with cells. The cells were treated with α2-AP and then the cells were fixed, permeablized or not, and stained using an antibody to α2-AP. We find that α-AP binds to each cell type used regardless of FAP expression and/or activity. α2-AP attached to outer membrane of the cells and was not taken up by the cells. Next we examined proteolytic degradation of fibrin using a D-Dimer assay to determine if α2-AP inhibited fibrin degradation increased in the presence of the different cells. Each cell type was grown in serum free media and treated with fibrinogen, FXIIIa, and thrombin to produce fibrin. The experimental groups were treated with α2-AP and control groups were not. After an incubation period, the cells were either further treated with plasmin for 2 h or not. Media was collected and the D-Dimer Assay revealed that high levels of fibrin degradation occurred in the tumor cell media even in the absence of added plasmin. The broad spectrum inhibitor of MMPs (BB94) significantly reduced the plasmin-independent degradation of fibrin suggesting that MMPs mediate the plasmin-independent proteolysis of fibrin. Surprisingly, the addition of α2-AP decreases fibrin degradation caused by MMPs secreted from the tumor cells. The high levels of fibrin degradation caused by plasmin were also decreased when treated with α2-AP. The mechanism of α2-AP-mediated inhibition of fibrinolysis by MMPs is not clear but may be related to the crosslinking of α2-AP to fibrin. MMP cleavage sites on fibrin may be masked when fibrin is crosslinked by α2-AP or the crosslinking itself may make the fibrin bundles denser and prevent access by MMPs.

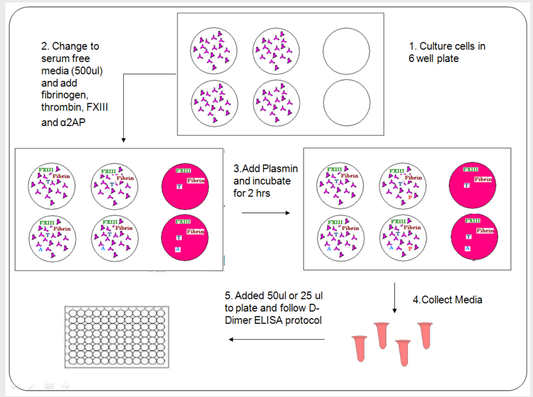
METHODS

α2-AP Interaction with Cells

Immunofluorescence microscopy was used

-Cells were cultured in
-They were treated with α2-AP and incubated for 1 h on ice or 5 h in 37ºC .
-Cell were then fixed with paraformaldehyde and then permabilized or not
-

METHODS cont.



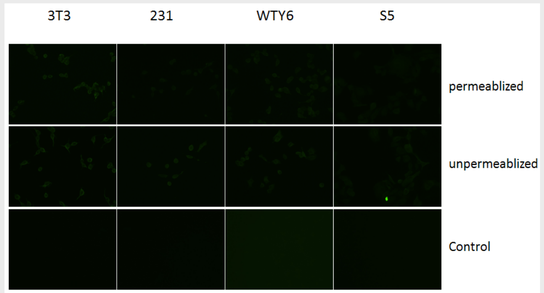
QUESTION

Can FAP-cleaved or un-cleaved α2-AP will promote the metastasis of tumor cells by decreasing fibrin degradation around the cells?

RESULTS

α2-AP treatment 1h on ice

A.



α2-AP treatment 5h 37^{0}C

B.

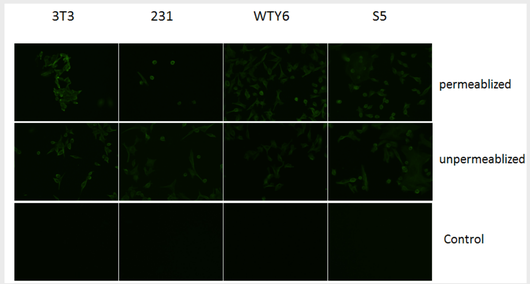
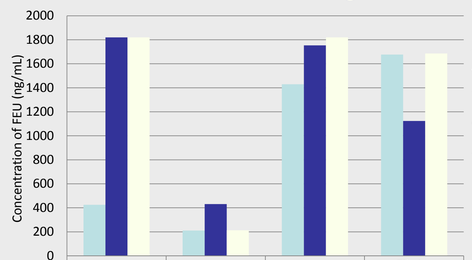
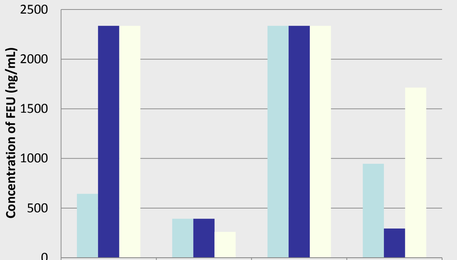


Figure 1. α2-AP attaches to the outer membrane of human breast cancer and mouse fibroblast cells. A. and B. α2-AP does interact with cancer and fibroblast cells by bind to the outer membrane of the cell. There is no visual difference in fluorescent staining of α2-AP in the permablized and unpermablized cells suggesting that α2-AP is not taken up by the cells (if so in small amounts). There is also no visual difference among the cell types. α2-AP binds to the cells whether FAP is present or not.

Concentration of Degraded Fibrin after 24 hrs α2-AP treatment



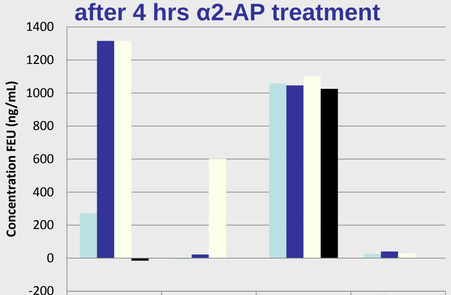
	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
Media	425.2115076	210.907952	1429.499335	1677.174938
231	1820.33	430.8356881	1753.334162	1123.360353
WTY6	1820.33	212.9716333	1820.33	1686.215093



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	643.1889886	392.1680946	2335.23	945.9183955
WTY6	2335.23	392.1680946	2335.23	294.1750612
S5	2335.23	260.4850052	2335.23	1713.511226

Figure 2. After 24h treatment with α2-AP plasmin independent and dependent degradation was decreased. After 24 h incubation in the absence of plasmin and in the presence of the proteolytically active FAP (WTY-6) or a catalytically inactive mutant FAP(S5) fibrin degradation greatly increased. When treated with α2-AP The degradation was inhibited. When plasmin was introduced we were able to see increased degradation in the FAP negative (231) and this degradation was decreased by α2-AP. (**A**.Concentration could not be calculated higher than 1820ng/mL so concentration may actually be higher. **B.** Concentrations could not be calculated higher than 2335ng/mL) (D-dimer are expressed in (FEU) Fibrinogen equivalent units)

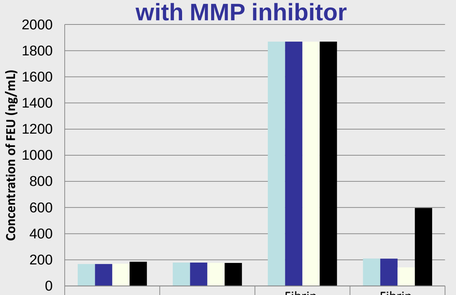
Concentration of Degraded Fibrin after 4 hrs α2-AP treatment



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	271.350777	-6.794749466	1057.936164	25.01191211
WTY6	1315.749	22.30002331	1046.477425	40.61867399
S5	1315.749	601.62	1103.815814	29.88811364
Media	-15.2746153		1025.102229	

Figure 3. After 4h treatment with α2-AP plasmin independent and dependent degradation was decreased. After 4 h incubation in the absence of plasmin and in the presence WTY-6 or S5 fibrin degradation greatly increased. When treated with α2-AP The degradation was decreased. When 231 was treated with plasmin degradation increased **(**Concentration could not be calculated higher than 1315.749ng/mL)

Concentration of Degraded Fibrin with MMP inhibitor



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	168.1519244	179.2070182	1869.11	209.9854506
WTY6	167.761166	179.4152192	1869.11	209.164519
S5	171.195548	176.9705485	1869.11	142.9004834
Media	185.8110757	176.268584	1869.11	596.312636

Figure 4. MMP inhibitors drastically decreased the plasmin independent fibrin degradation. Plasmin independent fibrin degradation greatly decrease when treated with BB94 (MMP inhibitor). Fibrin degradation still occurred in the presence of BB94 with treated with plasmin. The plasmin dependent was decreased when treated with α2-AP. **(**Concentrations could not be calculated higher than 1869.11ng/mL)

CONCLUSIONS

Fibroblast Activation Protein-α: A potential modulator of biological functions in the tumor microenvironment

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ABSTRACT

Prolyl peptidases are a family of serine proteases uniquely capable of cleaving peptides following proline residues (1) and are known for playing multiple roles in the regulation of diverse physiological processes. One member of the family, fibroblast activation protein-α (FAP), is a cell-surface serine protease that is expressed in wound healing, human breast cancer and other carcinomas, and some sarcomas (2). On the contrary FAP is not present in normal healthy adult tissues. α2-antiplasmin was the first natural substrate identified for FAP (3) but more recent work has found FAP capable of cleaving bioactive peptides including neuropeptide Y, B type natriuretic peptide, substance P and peptide YY (4). Due to its proteolytic function and specific pattern of expression, FAP is thought to be a regulator of biological processes within the tumor microenvironment that is required for tumor progression, vascularization and host's immune defense evasion. Here we investigate the potential of FAP to modulate functions within the tumor niche through induction of expression and proteolytic cleavage of certain cytokines. We show differential expression of FAP in the human breast cancer cell line MDA MB-231 and its transfectants engineered to express FAP to high levels. Further, we analyze effect of FAP on production of certain chemokines in those cell lines using RT-PCR. To investigate the potential of FAP to cleave various biological substrates we first established a fluorescence-release assay that allows us to monitor proteolytic activity of FAP that has been pulled down and bound to Wheat Germ Agglutinin (WGA) beads.
In our assay, synthetic, non-fluorescent substrate Gly-Pro-7-amino-4-methylcoumarin (Gly-Pro-AMC) is incubated with immobilized FAP and in turn subject to FAP-mediated proteolysis. Upon cleavage, the fluorescent molecule 7-amino-4-methylcoumarin (AMC) is released and level of fluorescence is assessed. This approach provides a quick and reliable system of enriching proteolytically active FAP from cellular extracts. To prove that the cleavage is performed by FAP-like proteases, we use small molecule inhibitor of FAP, PT-100 (Val-boroPro/Talabostat), shown to successfully suppress FAP dipetidyl peptidase activity in various enzymatic applications. Further we show that FAP possesses a potential to cleave chemokines. Recombinant IL-8 was incubated with cellular extracts containing no FAP, immobilized FAP, and immobilized but catalytically inactive mutant of FAP and then mixtures were analyzed by SELDI-TOF mass spectrometry. We found that IL-8 undergoes proteolytic digestion when incubated with active FAP, but remains intact when incubated with FAP-free cellular extracts. Surprisingly, we observed a low amount of IL-8 cleavage in preparations containing catalytically dead mutant of FAP, which is to be further investigated. In conclusion, evidence presented here suggests that FAP and/or FAP-like proteases are capable of cleaving of IL-8 that likely leads to its altered function. Therefore, these findings imply that FAP could be a key modulator of biological functions within tumor microenvironment hereby leading to modulation of tumor growth and invasion. This work is supported by grants from the DoD CDMRP-BCRP-BC074331 and University of Arkansas for Medical Sciences (UAMS) medical research endowment. Support also comes from the Department of Pathology at UAMS.

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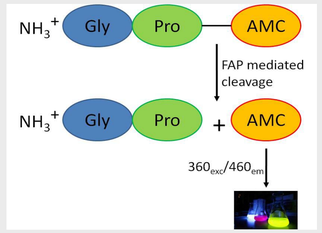
3. Lee KN *et al,* Antiplasmin-cleaving enzyme is a soluble form of fibroblast activation protein. *Blood* 107(4):1397-404, 2006.
4. Keane FN *et al,* Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are novel substrates of fibroblast activation protein-α. *FEBS J*, 2011, in press.

MATERIALS AND METHODS

▪ **Cell lines –** pcDNA 3.1 vector containing insert of wild type human FAP or insert of catalytically dead mutant of FAP was transfected into MDA MB-231 human breast adenocarcioma cell line to produce WTY-6 and S624A-5 cell lines that express wild type or mutant FAP to high levels (Fig. 2A).
▪ **Flow cytometry** – surface expression of FAP on parental human breast cancer MDA MB-231 and its transfectants engineered to either express active FAP (WTY-6$^{FAP\ High}$) or catalyticaly dead mutant of FAP (S624A-5$^{FAP\ High}$) was analyzed using F19 monoclonal antibody to FAP and FITC-conjugated secondary antibody in UAMS Flow cytometry core facility. Surface expression of DPPIV, dipeptidyl peptidase closely related to FAP, was also investigated (Fig.2A).
▪ **RT-PCR** – message RNA levels for various human cytokines in RNA cellular extracts of MDA MB-231, WTY-6 and S624A-5 cell lines were investigated using ThermoScript RT-PCR system (Invitrogen) (Fig. 2B).
▪ **Enrichment of FAP on Wheat Germ Agglutinin (WGA) beads** – total protein cellular extracts from MDA MB-231, WTY-6 and S624A-5 cell lines in 2.5% TX-100, 1xTBS, 5mM EDTA buffer were incubated overnight at 4°C with Wheat Germ Agglutinin beads (Vector Laboratories) to immobilize active FAP on the beads.



▪ **Fluorescence-release assay** – 100 µM Gly-Pro-AMC in 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.05% TX-100, 1% DMSO, pH 7.4 buffer was incubated for 2 hours at 37°C with FAP enriched from MDA MB-231, WTY-6 and S624A-5 total protein cellular extracts on WGA beads. The released fluorescence, proportional to the amount of released free AMC, was analyzed in a Synergy 2 fluorimeter (BioTek Instruments) (Fig.1, 3). For inhibition of dipeptidyl peptidase activity of FAP, 10 µM PT-100 was added for 30 minutes prior to incubation with Gly-Pro-AMC.

Figure 1. Scheme of the fluorescence –release assay. Gly-Pro-AMC is subject to incubation with FAP enriched on WGA beads. Due to dipepidyl peptidase activity of FAP, free AMC is released. Upon excitation at 360 nm and emisson at 460 nm, AMC emits fluorescence.

▪ **IL-8 cleavage** – human recombinant IL-8 (R&D Systems) diluted in 1x PBS to 10 mg/L was incubated with FAP enriched on WGA beads for 2 hours at 37°C. Then, reaction mixtures were analyzed by surface enhanced laser desorption time of flight mass spectrometry (SELDI-TOF) mass spectometry at UAMS Center for Orthopedic Research (Fig. 4).

RESULTS

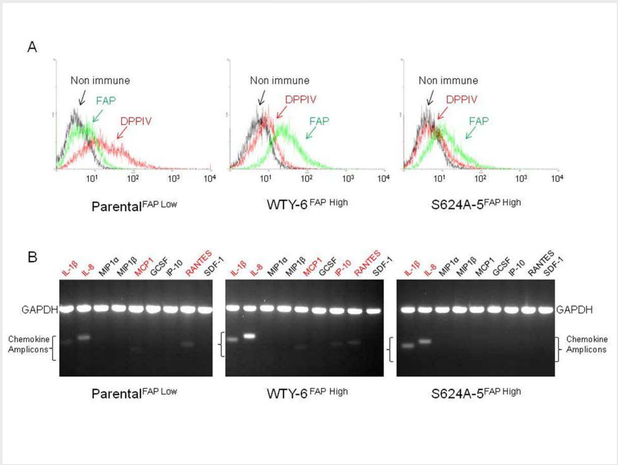


Figure 2. A. Cell surface expression of FAP (green trace) and DPPIV (red trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). Non immune IgG (black trace) serves as a control. **B.** Various chemokines detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ using specific primers to produce amplicons between 180-220 bp. GAPDH serves as an internal control. Samples labeled red are positive for expression of the chemokine.

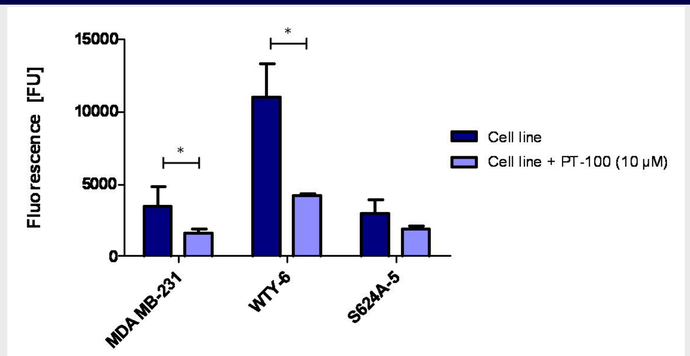


Figure 3. Fluorescence emitted in fluorescence-release assay. Gly-Pro-AMC was incubated with FAP enriched on WGA beads from cellular extracts of WTY-6$^{FAP\ High}$, MDA MB-231$^{FAP\ Low}$ and S624A-5$^{FAP\ High}$ cell lines. Upon FAP-mediated cleavage the molecule of AMC was released and fluorescence measured (Fig. 1). Amount of released fluorescence is proportional to activity of FAP enriched on WGA beads. Dark blue bars indicate preparations containing untreated cell line extracts. Light blue bars indicate preparations treated with inhibitor of dipeptidyl peptidase activity of FAP. Activity of FAP is not statistically different between MDA MB-231 and S624A-05 untreated preparations, while there is a statistical difference in activity of FAP between WTY-6 and both MDA MB-231 and S624A-5 untreated extracts (dark blue bars). In addition, PT-100 inhibits FAP activity in MDA MB-231 and WTY-6 preparations (horizontal bars). * indicates p-value < 0.05

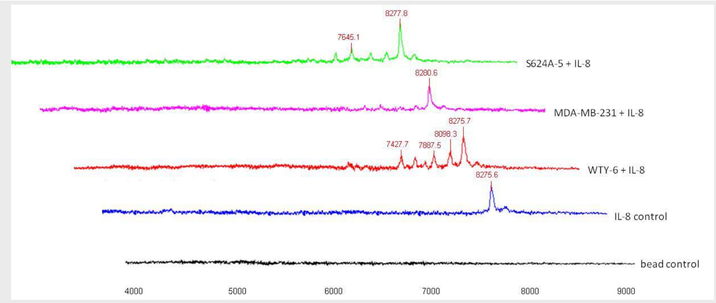


Figure 4. Proteolytic degradation of IL-8. Surface enhanced laser desorption time of flight mass spectrometry (SELDI-TOF) was employed to analyse the proteolysis of human recombinant IL-8 incubated with FAP enriched from protein extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (MDA MB-231$^{FAP\ Low}$) levels or cells expressing catalytically dead mutant of FAP (S624A-5$^{FAP\ High}$). Lower molecular weight peaks in high FAP preparation (red trace) and less extensively, in preparations containing mutant FAP (green trace), indicate proteolysis of IL-8. IL-8 break down is low in parental cells, that do not express FAP (magenta trace). Spectrum of intact IL-8 (8.2kDa) serves as a control (blue trace), as well as spectrum of empty WGA beads (black trace).

CONCLUSIONS

1. Our data suggest that FAP expression induced in MDA MB-231 human breast cancer cell line that does not endogenously express FAP (Fig. 2A) leads to change of phenotype of those cells possibly through altered expression of chemokines (Fig. 2B) that may play a role in tumor progression, angiogenesis and evasion of host's immunity.
2. Data obtained in fluorescence –release assay indicate that FAP enriched on WGA beads remains active and capable of proteolytic cleavage of its substrates. Treatment with inhibitor of dipeptidyl peptidase activity of FAP confirms this finding (Fig. 3).
3. Proteolytic degradation of human recombinant IL-8 observed in the presence of catalytically active FAP suggests that FAP might be a factor mediating proteolytic cleavage of IL-8. Less extensive IL-8 degradation in the presence of mutant FAP indicates that other proteases may contribute to this process.
4. Overall, our data presented here is consistent with finding that FAP is a potential candidate for a key regulator of various biological funtions within the tumor microenvironment.

Fibroblast activation protein-α: A potential modulator of chemokines that regulate natural killer recruitment to breast tumors

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[1]Department of Pathology, [2]Center for Orthopedic Research, Departments of Orthopedic Surgery and Physiology and Biophysics, Winthrop P. Rockefeller Cancer Institute, and University of Arkansas for Medical Sciences, Little Rock, AR

ABSTRACT

Dipeptidyl peptidases are a family of proteases that are implicated in the regulation of diverse biological processes. One member of the family, fibroblast activation protein-α (FAP), is a cell surface, post-prolyl serine protease that is over-expressed in human breast cancer but is not expressed in normal adult tissues. FAP has 50% amino acid sequence homology to another family member, dipeptidyl peptidase IV (DPPIV), and there is overlap in substrate specificities as both enzymes cleave NH_2-XAA-Pro peptides such as Gly-Pro-AFC. Notably, DPPIV has been shown to cleave a number of chemokines including RANTES and SDF-1 that are known to be important in attracting innate immune cells such as natural killer (NK) cells. Proteolytic cleavage of RANTES and SDF-1 inactivates them and eliminates their attractive power for NK cells. Consistent with this, others have shown that inhibition of FAP by Val-boroPro, a broad spectrum inhibitor of post-prolyl proteases causes activation of immune responses of the host (Jones et al, 2003, Blood *102*:1641-8), perhaps through preventing the inactivation of chemokines. Previously, we showed that fast-growing tumors were produced by cells expressing proteolytically active FAP whereas parental MDA MB-231 do not express FAP and produce slow-growing tumors (Huang et al, 2004 Cancer Res. *64*:2712-2716). Here we investigate the tumor growth rates and differential expression of FAP, DPPIV, and chemokines in MDA MB-231 cells that do not express FAP and those engineered to express FAP to high levels. We extend our earlier findings by showing that cells expressing a catalytically inactive FAP produce rapidly growing tumors like cells expressing active FAP (Huang et al., 2011, Clin. Exp. Metastasis, 28:567-579). We analyzed surface expression of FAP and DPPIV by flow cytometry to determine if DPPIV substitution for FAP results in the rapid growth of tumors produced by cells expressing a catalytically inactive FAP. We confirm that MDA MB-231 cells express little or no FAP and find that they express DPPIV to high levels. Conversely, these cells engineered to express wild type FAP or a catalytic mutant of FAP express FAP to high levels but reveal little DPPIV expression. These findings are confirmed by RT-PCR gene expression analysis. The results show that FAP expression correlates with rapid tumor growth and DPPIV expression correlates with slow tumor growth. Parental cells express DPPIV and the chemokines IL-1β, IL-8, MCP-1 and RANTES but little FAP; cells expressing proteolytically active FAP express these chemokines as well as IP-10 and little DPPIV; cells expressing inactive FAP express IL-1β and IL-8 only and little DPPIV. The findings are consistent with active FAP cleaving one or more of these chemokines and suppressing NK cell recruitment into the tumor thereby leading to rapid tumor growth.

QUESTION

Does FAP expression suppress chemokines that attract NK cells to tumors?

RESULTS AND METHODS

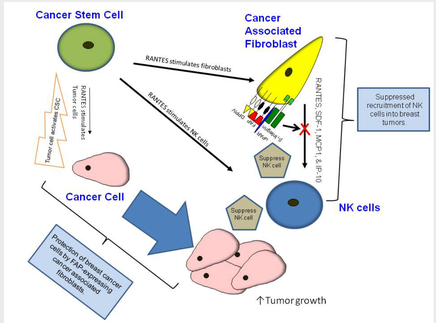

Figure 1. Cellular interactions in the tumor microenvironment. Cancer Associated Fibroblasts (CAFs, yellow) express FAP (red surface molecules) that may inactivate chemokines that attract NK cells and thereby suppress NK cell recruitment. The FAP activity of the CAFs may also decrease the tumor-killing function of NK cells.

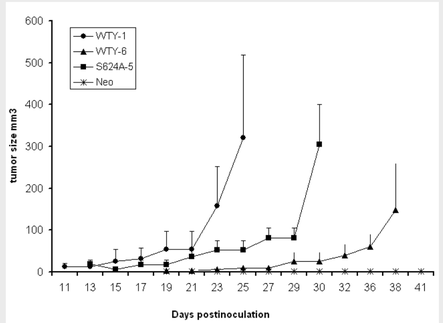

Figure 2. Proteolytically active and proteolytically inactive FAP promotes tumor growth. Growth of tumors of MDA MB-231 cells expressing wild type FAP (WTY-1 & WTY-6), inactive FAP (S624A-5) or no FAP (Neo). Growth curves for Neo, WTY-1 and WTY-6 were published previously (Huang et al, (2004) Cancer Research *64*:2712-2716.) and those for inactive FAP were recently published see Huang et al., (2011) Clin. & Exp. Metastasis *28*:567-579.) These human breast cancer cells were grown as tumors in the mammary fat pads of female SCID mice.

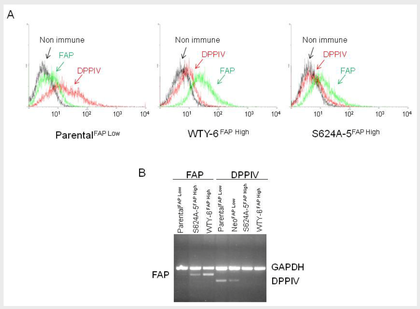

Figure 3. FAP expression suppresses DPPIV expression A. Cell surface expression of DPPIV (red trace), FAP (green trace) and non immune IgG (black trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). **B.** FAP (left panel) or DPPIV specific (Right panel were detected by RT-PCR using primers specific for FAP and for DPPIV as well as GAPDH.

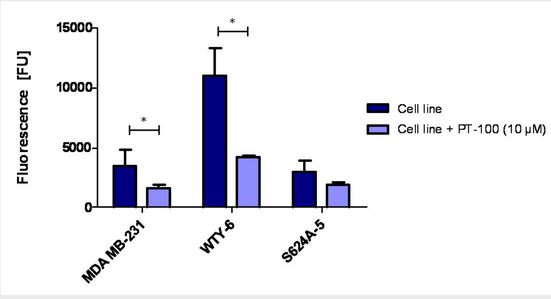

Figure 4. Fluorescence emitted in fluorescence-release assay. Gly-Pro-AMC was incubated with FAP enriched on WGA beads from cellular extracts of WTY-6$^{FAP\ High}$, MDA MB-231$^{FAP\ Low}$ and S624A-5$^{FAP\ High}$ cell lines and fluorescence of released AMC measured. Dark blue bars indicate untreated cell extracts. Light blue bars indicate extracts treated with the FAP inhibitor PT-100. * indicates *P*-value < 0.05

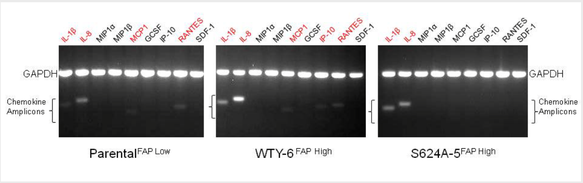

Figure 5. IL-8 and other NK-cell attracting chemokines are expressed by the human breast cancer cells used in this study. Chemokines were detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ using specific primers to produce amplicons between 180-220 bp. Reactions included primers for a 450bp GAPDH amplicon. Gel samples labeled red are positive for expression of the chemokine.

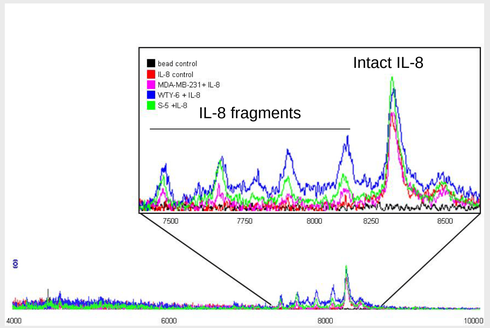

Figure 6. Increased proteolytic degradation of IL-8 is observed in cells that express FAP. Surface enhanced laser desorption time of flight mass spectrometry (SELDI) analysis of IL-8 proteolysis in the 4-10 kDa range. FAP from extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (Parental$^{FAP\ Low}$) levels or mutant (S624A-5$^{FAP\ High}$) FAP was bound to wheat germ agglutinin agarose beads, incubated with purified IL-8, and then the supernatant subjected to SELDI. Lower molecular weight peaks in high FAP (blue trace) and less extensively, mutant FAP samples (green trace) indicate proteolysis of IL-8. IL-8 break down is low in parental cells (magenta trace). Intact IL-8 occurs at 8.2 kDa (red trace) and no peaks are from the beads (black trace).

CONCLUSIONS

1. Expression of FAP coincides with suppression of DPPIV that is independent of FAP proteolytic activity.
2. FAP expression correlates with rapid tumor growth while DPPIV expression correlates with slow tumor growth in an animal model of human breast cancer.
3. FAP may facilitate enhanced cleavage of IL-8 and perhaps other chemokines that attract NK cells. FAP may cooperate with other proteases to degrade chemokines.

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RESEARCH PAPER

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

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Abstract Fibroblast activation protein-α (FAP) is a cell surface, serine protease of the post-prolyl peptidase family that is expressed in human breast cancer but not in normal tissues. Previously, we showed that FAP expression 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. Mammary fat pads of female SCID mice were inoculated with breast cancer cells that express FAP and the mice treated with normal saline or Val-boroPro (talabostat); Glu-boroPro (PT-630); or 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(*S*)-pyrrolidine (LAF-237) that inhibit prolyl peptidases. Other mice were injected with breast cancer cells expressing a catalytically inactive mutant of FAP and did not receive inhibitor treatment. PT-630 and LAF-237 did not slow growth of tumors produced by any of the three cell lines expressing FAP. Talabostat slightly decreased the growth rates of the FAP-expressing tumors but because PT-630 and LAF-237 did not, the growth retardation was likely not related to the inhibition of FAP or the related post-prolyl peptidase dipeptidyl peptidase IV. Breast cancer cells expressing a catalytically inactive mutant of FAP (FAP^{S624A}) also produced tumors that grew rapidly. In vitro studies revealed that cells expressing wild type FAP or FAP^{S624A} degrade extracellular matrix (ECM) more extensively, accumulate higher levels of matrix metalloproteinase-9 (MMP-9) in conditioned medium, are more invasive in type I collagen gels, and have altered signaling compared to control transfectants that do not express FAP and form slow growing tumors. We conclude that the proteolytic activity of FAP participates in matrix degradation, but other functions of the protein stimulate increased tumor growth.

Keywords Serine protease · Seprase · Dipeptidyl peptidase IV · Invadopodia · Metastasis

Abbreviations

ANOVA	Analysis of variance
GIP	Glucose dependent and insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorting
FAP	Fibroblast activation protein-α
FAP^{S624A}	MDA MB-231 cells expressing a catalytically inactive mutant of FAP
FITC	Fluorescien isothiocyanate
F19	Mouse monoclonal antibody directed against human FAP
LAF-237	(vildagliptin), 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(*S*)-pyrrolidine, an inhibitor of DPPIV and related proteases
MDA MB-231	A human breast adenocarcinoma cell line
MMPs	Matrix metalloproteinases
MMP-9	Matrix metalloproteinase-9
pcDNA3.1	A plasmid mammalian expression vector

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PT-100	Val-boroPro, an inhibitor of FAP and related proteases
PT-630	Glu-boroPro, an inhibitor of FAP and related proteases
RT-PCR	Reverse transcription-polymerase chain reaction
SCID	Severe combined immune deficient
WTY-1	Stable transfectant of MDA MB-231 engineered to express high levels of FAP
WTY-6	Stable transfectant of MDA MB-231 engineered to express high levels of FAP
4G10	Monoclonal antibody to phosphotyrosine
6-6B	Monoclonal antibody to MMP-9

Introduction

Fibroblast activation protein-α (FAP), also called seprase, is an integral membrane serine protease and member of the post-prolyl peptidase family [1, 2]. FAP is closely related to dipeptidyl peptidase IV (DPPIV) [3, 4] and exhibits a DPPIV-like fold, featuring alpha/beta-hydrolase and eight-bladed beta-propeller domains [5]. 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 [1, 6]. Like DPPIV, it cleaves NH2-X-Pro peptides [3]; but in addition, FAP possesses a unique endopeptidase activity and is able to degrade gelatin [3, 4]. Recently α2-antiplasmin was identified as the first natural substrate for FAP, and FAP-mediated cleavage of α2-antiplasmin results in more efficient cross linking of truncated α2-antiplasmin to fibrin while retaining the inhibitory action to plasmin [7]. Thus, FAP may have a role in regulating the dissolution of fibrin by plasmin.

Recent work has suggested many pathological functions for FAP [8]. FAP may have roles in osteoarthritis [9], pulmonary fibrosis [10], fibrosis in liver diseases such as hepatitis [11] and cirrhosis [12], and roles in numerous cancers, for example: multiple myeloma [13], pancreatic cancer [14], colon cancer [15], melanoma [16], ovarian carcinoma [17], and breast cancer [18]. FAP is expressed to high levels in human breast cancer but is not expressed in normal breast tissue [2, 18]. FAP protease activity is abnormally high in extracts of patient tumors indicating that increased expression leads to increased FAP protease activity in breast cancer [6]. The observation of increased expression of FAP generally in cancers of epithelial origin has led to the hypothesis that its proteolytic activity might be involved in tissue remodeling required for tumor progression and vascularization [19, 20].

Because of the conspicuous absence of FAP in normal adult tissues and its marked up-regulation in a variety of diseases, there is interest in the potential of small molecule inhibitors of the FAP protease to reduce pathogenesis. Moreover, DPPIV is a target for drug discovery in type 2 diabetes and hematopoietic stem cell engraftment [21]. Sitagliptin is a DPPIV inhibitor already approved for type 2 diabetes because it has insulinotropic effects caused by prolonging the half-life of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), both of which are substrates for DPPIV [21]. In this study we inhibited FAP using Val-boroPro (PT-100/talabostat), which inhibits FAP with a Ki of 6.2 nM and DPPIV with a Ki of 0.18 nM [22] and Glu-boroPro (PT-630), which inhibits DPPIV with a Ki of 3 nM and FAP with a Ki of 5 nM [22, 23]. Because DPPIV is expressed ubiquitously and constitutively in body organs [24], it was necessary to distinguish between the biological consequences of its inhibition from that of FAP. To do this, we also used 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(*S*)-pyrrolidine (LAF-237) which inhibits DPPIV with a Ki within a range of 17–51 nM [25, 26], but is a much less potent inhibitor of FAP with a Ki greater than 20 μM [27].

In this study, the role of the proteolytic activity of FAP in promoting tumorigenesis, tumor growth, extracellular matrix (ECM) degradation and invasiveness was investigated using small-molecule inhibitors of FAP and breast cancer cells engineered to express either active FAP or an inactive 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 also suggest that cell surface expression of FAP stimulates elevated production of factors such as MMP-9 that are required for ECM degradation and tissue invasion during tumor progression.

Materials and methods

MDA MB-231 human breast adenocarcinoma cells transfected with 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 [19].

Production of cDNA encoding S624A mutant

A cDNA for human FAP [19] 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 → 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′-TATCTTATCATGTCTGTAT

ACCGTCGACCTCTAGCT-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 performed by Alan Gies in the DNA sequencing core facility in the Department of Microbiology and 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 directed. The transfectants were selected with G418 (800 μg/ml) and sorted by fluorescence activated cell sorting based on staining with F19 antibody to FAP as described previously [19]. 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.

Gelatin zymography

To analyze FAP expression, extracts were prepared and subjected to gelatin zymography and western blotting as described previously [16, 19, 28]. Several steps were taken to enhance FAP-specific gelatinolytic activity while diminishing gelatinase activities of MMPs. First, FAP was partially purified from the extracts by pulling it down with WGA Agarose as described earlier [16, 19, 28]. Secondly, FAP gelatinolytic activity was preserved while that of MMPs was inhibited by including EDTA in the extraction and zymogram incubation buffers [16, 19, 28]. Finally, the FAP was eluted from the WGA beads in gel sample buffer containing *N*-acetyl glucosamine and dithiothreitol reducing reagent that inhibits MMPs but preserves FAP activity [16, 19, 28] and zymography was performed.

Tumor biology

2×10^6 tumor human mammary carcinoma cells were implanted into each of four mammary fat pads per female SCID mouse, and, starting on day 2 after tumor implantation, the mice were administered normal saline (placebo control) or protease inhibitors by gavage. Tumor growth was measured with calipers as previously described [19], and wet tumor weights were determined for tumors excised at the end of experiments. In the first animal experiment with S624A-5 cells there were seven animals per group, and in the second experiment, there were five animals per treatment group. For the inhibitor studies there were five 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 0.1 N HCl and PT-630 at a concentration of 28.1 mg/ml was supplied by Point Therapeutics (Boston, MA). Stock solutions were stored at −20°C in small aliquots (50–200 μl). Working solutions were prepared by diluting the 0.1 M acidified stock with sterile normal saline. Working solutions were made at concentrations that delivered 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, talabostat was not held at neutral pH for longer than 10 min before administration. Consequently, the working saline solutions were prepared with ice cold reagents in the animal facility, immediately prior to administration to mice. The inhibitor solutions (0.2 ml) were administered orally, once daily, via a blunt gavage needle fitted to a 1-ml syringe.

High titer F19 acites fluid

Five female Balb/c mice (6–8 weeks old) were housed for 1 week and injected IP 0.5 ml of pristane (Sigma) per mouse. One week later 5×10^6 F19 hybridoma cells were injected IP into each mouse. These hybridomas produce the F19 monoclonal antibody to FAP. When ascites fluid formed (approximately 3 weeks later) the fluid was harvested with a syringe. Ascites was collected at least twice per animal.

FAP protease activity assay

FAP was extracted from cells by using Triton X-100 using a procedure modified from that reported previously [16, 28]. Each cell type was grown to 90% confluence in three 75 cm^2 flasks. Media was removed; the cells washed three times with PBS, and then suspended using 1 mM EDTA in PBS. Cells were pelleted by centrifugation at $10{,}000 \times g$ for 5 min at 25°C and the supernatant was removed. Cells were resuspended in 1 ml of extraction buffer (2.5%Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5) and then the lysate was gently homogenized on ice in an 1.5 ml Eppendorf tube with 30 strokes of the pestle. Care was taken to avoid excessive foaming. The homogenate was cleared by centrifugation for 5 min at $524 \times g$ at 4°C. The supernatant was transferred to a fresh tube and incubated with 100 μl of protein G magnetic Dynabeads (Invitrogen) that had been coated with approximately 5 μg F19 mAb to FAP for 30 min at 25°C and washed according to the manufacturer's instructions. FAP collected on the protein G Dynabeads,

was exposed to 100 μM of z-Gly-Pro-AMC (Bachem Bioscience Inc, King of Prussia, PA, USA) for 2 h at 37°C in 400 μl of 50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.6 with shaking. To inhibit FAP, the FAP-coated protein G beads were first exposed to 10 μM of PT-100 or PT-630 in 390 μl of the above buffer for 30 min 25°C prior to addition of the 10 μl of 4 mM z-Gly-Pro-AMC to achieve a final concentration of 100 μM z-Gly-Pro-AMC. The beads were collected with the magnet and the fluorescence at 460 nm of the supernatants determined with excitation of 360 nM with the Tecan Safire microtiter plate reader. All samples were tested in duplicate. 7-Amino 4-Methyl Coumarin (AMC) (ACROS, New Jersey, USA) was used to develop a standard curve where the fluorescence emitted by free AMC at concentrations of 0.00125, 0.0125, 0.125, 1.25, 2.5, 5, 10 and 20 μM was determined by applying an excitation wavelength of 360 nm and measuring the fluorescence intensity at 460 nm (Tecan Safire fluorescent microplate reader using the Magellan software). This curve was used to relate fluorescence obtained from the unknown samples to μM free AMC.

To determine FAP activity in tumor tissues an extract was prepared by homogenizing 100 mg of tumor tissue in 500 μl 2.5%Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5. The extract was cleared by centrifugation and the total protein determined using bicinchoninic acid assay (Pierce) where BSA was used to produce a standard curve. The F19-coated protein G dynabeads (50 μl) prepared as described above, were exposed to 5 mg of tumor extract protein to capture FAP. The FAP coated beads were then tested for FAP-activity using 100 μM z-Gly-Pro-AMC substrate as described above. Samples were tested in duplicate.

Matrix degradation assay

Glutaraldehyde-crosslinked gelatin films with immobilized fluorescein isothiocyanate (FITC)-fibronectin were prepared on cover slips (18 mm circular glass) as described previously [29]. Cells were seeded onto the FITC-fibronectin matrices and grown in growth medium for 48 h at 37°C, 5% CO_2. Adherent cells were washed three times with sterile PBS and then fixed and prepared for fluorescence microscopy as described previously [29]. 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 UAMS. 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; [30]) were performed as described [31]).

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 [29], 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 [18, 32] and FAP promotes tumor growth and increased microvessel densities in a mouse model of breast cancer [19]. This study investigated the role of the proteolytic activity of FAP in promoting tumorigenesis, rapid tumor growth and invasive behavior of FAP-expressing tumor cells. Expression of a catalytically inactive FAP and inhibitors of post-prolyl peptidases were used to investigate the role of the FAP protease in determining 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, which do not normally express FAP, 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 (Fig. 1a) and FACS analysis of living cells with FAP-specific F19 monoclonal antibody (Fig. 1b). The protease activity of 170-kDa FAP was not detected in zymograms of extracts of S624A-5 cells that were enriched in FAP by wheat germ agglutinin chromatography (Fig. 1c, S624A-5). As expected, the protease activity was detected in extracts of transfectants expressing wild type FAP (Fig. 1c, WTY-1 and WTY-6,

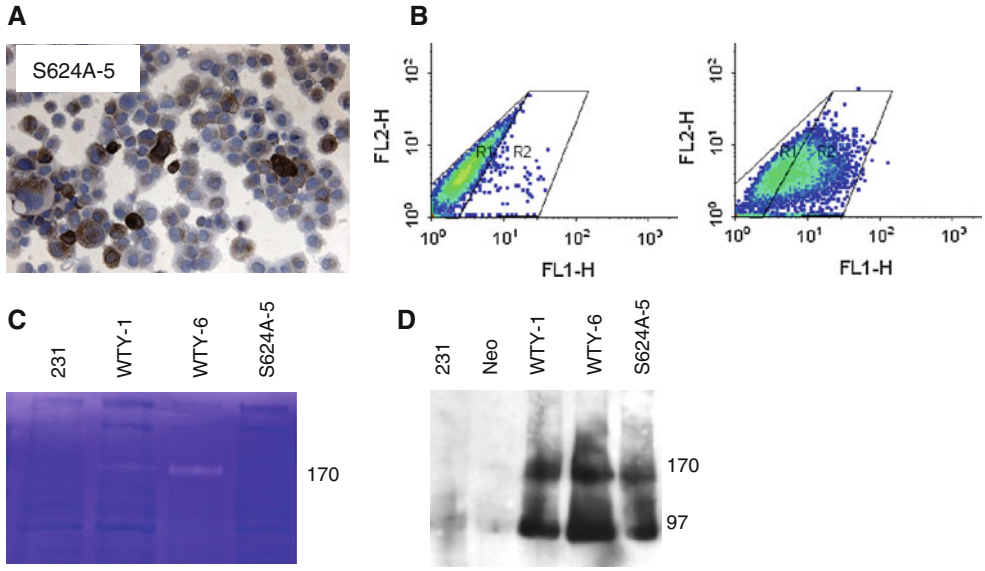

Fig. 1 Characterization of MDA MB 231 cells engineered to express FAP^{S624A}. **a** Photomicrograph of a cytospin of cells transfected with FAP^{S624A} catalytic mutant (called S624A-5) prior to flow sorting. Cells stained with F19 mAb to FAP appear *brown* due to the DAB reaction product while the blue hematoxylin detects all cells. **b** FACS showing density plots of cell surface FAP when cells expressing FAP^{S624A} were stained with non-immune IgG (*left panel*) or F19 mAb to FAP (*right panel*). The regions R1 and R2 cover identical areas in both *left* and *right panels* and serve to indicate the FAP negative cells (R1) and the FAP positive cells identified by F19 (R2). **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 and WTY-6). **d** The catalytic mutant can assemble into the 170-kDa dimer as detected by western blot in the cells expressing mutant FAP (*lane* S624A-5) and wild type FAP (*lanes* WTY-1 and WTY-6) but not in the parental MDA MB-231 cells (231) or these cells transfected with the empty vector (Neo)

see also [19]). 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 the reducing agents in gel-sample buffer used for optimal detection of FAP activity. MMPs are, however, released into the medium by these cells as described below. Importantly, western blot analysis using mAb F19 was performed on samples that were suspended in SDS sample buffer but not boiled to preserve the 170 kDa FAP dimer. This analysis revealed that the 170-kDa dimer of FAP was formed by the mutant FAP (Fig. 1d, 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. 1d, WTY-1 and WTY-6).

FAP expression promotes growth of tumors independent of its protease activity

In comparison to MDA MB-231 human mammary adenocacinoma cells that do not express FAP, MDA MB-231 cells engineered to express proteolytically active FAP (hereafter called WTY-1 and WTY-6) have greater tumorigenicity and form tumors that grow more rapidly in SCID mice [19]. In order to determine the role of the proteolytic activity of FAP in tumor growth in vivo, the effect of inhibitors of post prolyl peptidases on tumor growth was investigated in SCID mice implanted in mammary fat pads with human mammary tumor cell lines as previously described [19]. Starting on day 2 after tumor implantation in the mammary fat pads, mice were gavaged once daily with 1.3 mg/kg PT-100/talabostat, or 13.3 mg/kg PT-630; or 6.7 mg/kg LAF-237/vildagliptin. Tumors developed from inoculae of FAP-expressing human breast cancer cell lines WTY-1 (Fig. 2a) and WTY-6 (Fig. 2b), and from MDA MB-435 cells, which express FAP endogenously [33] (Fig. 2c). Of the post prolyl protease inhibitors tested, only PT-100/talabostat appeared to slow tumor growth (Fig. 2a–c), and this was especially pronounced in the case of the MDA MB-435 cells where production of measurable tumors was delayed by nearly 12 days relative to control (Fig. 2c). But even this reduction in tumor growth by PT-100/talabostat did not achieve statistical significance as compared to the rapidly growing MDA MB-435 tumors in saline-treated control mice. Also, weights of tumors excised on post-inoculation day 30–39 (depending on how fast the largest tumor in each treatment group reached 2 mm^3) indicated no statistically significant size differences between tumors grown in mice treated with

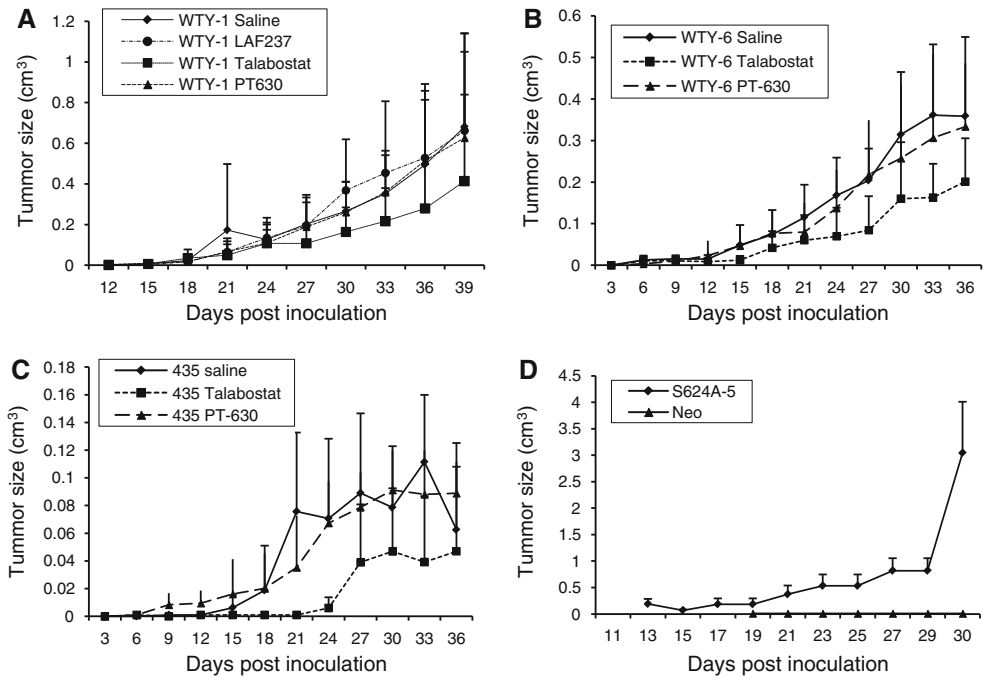

Fig. 2 Breast tumors expressing FAP grow rapidly even when FAP catalytic activity is inhibited. **a**–**c** Talabostat slows growth of breast tumors in the mammary fat pads of SCID mice. Tumor volumes plotted over time of FAP-expressing human breast cancer cells in SCID mice treated with saline (*filled diamond*), LAF-237 (*filled circle*), PT-630 (*filled triangle*), or talabostat (*filled square*). 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 (*filled diamond*) and Neo cells (*filled triangle*). In all cases tumor growth is recorded as volume (cm^3)

inhibitors versus saline ($P > 0.05$). However, the lowest average weights were consistently obtained for tumors treated with PT-100/talabostat.

The protease inhibitor results suggest that the tumorigenic effect of FAP is independent of its proteolytic activities. MDA MB-231 cells transfected with proteolytically inactive FAP^{S624A} were used to further investigate tumor growth stimulation by FAP in the absence of proteolytic activity, MDA MB-231 cells expressing FAP^{S624A} (42.6% positive cells) (S624A-5, Fig. 1b) and FAP-negative cells transfected with empty vector (Neo, Fig. 1) were injected subcutaneously into the mammary fat pads of female SCID mice as described above. The S624A-5 cells formed tumors that grew considerably faster than tumors of Neo transfectants that do not express FAP (Fig. 2d). The yield of tumors from S624A-5 cells expressing the proteolytically inactive FAP (26 tumors/28 injection sites; 93%) was greater than that observed for Neo control cells (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% [19]). Tumors of S624A-5 cells expressing FAP^{S624A} were considerably larger than those of cells lacking FAP (Neo) as judged by the average wet weight of the tumors excised on day 30 (Neo, 0.066 g $\pm$ 0.038; S624A, 0.8 g $\pm$ 0.41). The S624A-5 tumors were similar in size to those of cells expressing wild type FAP [19]. A second in vivo experiment was performed using a population of S624A-5 cells that was sorted by FACS to contain 41% FAP^{624A}-expressing cells. Implantation of these cells into the mammary fat pads of female SCID mice again resulted in rapidly growing tumors (not shown) with a high tumor yield (17 tumors/20 injection sites; 85%). The promotion of tumorigenesis and rapid tumor growth by the expression of proteolytically inactive FAP in mammary tumors in mice and the observation that small-molecule inhibitors of FAP protease activity did not significantly inhibit growth of tumors expressing proteolytically active FAP suggest that non-enzymatic functions of FAP may be responsible for increased tumor growth in vivo.

A FAP-specific protease activity assay was developed to confirm that FAP was inhibited in the tumors of the animals that were treated with talabostat and PT-630 but not in those treated with LAF-237. For the assay, FAP is immune precipitated from extracts of cells or tumor tissues and then exposed to the substrate z-Gly-Pro-AMC. FAP cleaves the peptide bond linking Pro to AMC and consequently the fluorescence emitted by AMC is increased. Extracts of MDA MB-231 cells that do not express FAP, revealed low activity in this assay (Fig. 3a, 231, black bars) and this activity was unchanged or showed a decrease when treated with PT-630 or talabostat (Fig. 3a, 231, white and gray bars). However, WTY-6 and WTY-1 transfectants of MDA

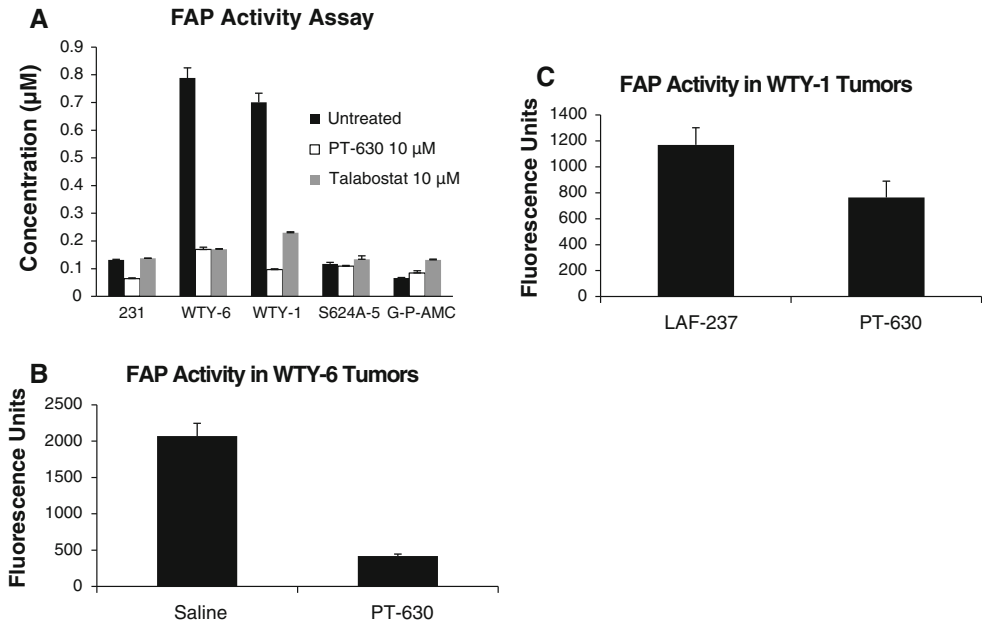

Fig. 3 Effect of inhibitors on FAP activity. **a**. FAP activity was assessed by binding FAP in extracts of parental MDA-MB-231 (231) cells that do not express FAP; WTY-6 (WTY-6), WTY-1(WTY-1) cells that express wild type and active FAP, or S624A-5 cells that express a mutant FAP that is catalytically inactive (S624A-5) to F19 mAb immobilized on magnetic protein G beads. In addition, results are shown for substrate only (G-P-AMC). The FAP-bound beads were exposed to the FAP substrate z-Gly-Pro-AMC, the supernatant collected and the fluorescence emitted at 460 nm when excited by 360 nm was determined. A standard curve using free AMC allows plotting the concentration of free AMC on the *y* axis. Results are shown for FAP that is uninhibited (*black bars*) or for FAP inhibition by PT-630 (*white bars*) or PT-100 (*gray bars*). **b**, **c** FAP activity was determined in extracts of tumor tissues derived from **b** WTY-6 cells (WTY-6) exposed to normal saline (Saline), or PT-630 (PT-630), and **c** extracts of tumor tissues derived from WTY-1 cells (WTY-1) and exposed to LAF-237 (LAF-237) or PT-630 as described in the animal experiments shown in Fig. 2. Results are given in fluorescence intensity determined at 460 nm wavelength with excitation wavelength of 360 nm

MB-231 cells that express wild type and active FAP to high levels reveal high FAP activity in this assay (Fig. 3a WTY-6 and WTY-1, black bars) that is inhibited by both PT-630 and talabostat (Fig. 3a WTY-6 and WTY-1, white and gray bars). In contrast, the mutant FAP reveals low levels of FAP activity similar to the FAP negative MDA MB-231 cells (Fig. 3a, S624A-5, black bars) that remains low when exposed to PT-630 and talabostat (Fig. 3a, S624A-5, white and gray bars). For comparison, intact z-Gly-Pro-AMC was used to determine back ground fluorescence (Fig. 3a, G-P-AMC, black, white, and gray bars).

Extracts of tumor tissues were prepared and subjected to the FAP-activity assay. In the case of WTY-6 tumors, Fresh frozen tissues were available from normal saline treated animals and PT-630 treated animals. The extracts from WTY-6 tumors of animals treated with normal saline had high FAP activity (Fig. 3b, saline) whereas the extracts of WTY-6 tumors from PT-630 animals had low FAP activity (Fig. 3b, PT-630). Similarly, extracts of WTY-1 tumors obtained from animals treated with LAF-237 that preferentially inhibits DPPIV and not FAP, had high FAP activity (Fig. 3c, LAF-237) but extracts of WTY-1 tumors treated with PT-630 had low FAP activity (Fig. 3c, PT-630). Overall the results are consistent with the inhibitors acting as expected with LAF-237 producing relatively little inhibition of FAP proteolytic activity in the tumors but PT-630 causing significant inhibition of FAP activity.

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 [16, 29]. These studies focused on the WTY-1 cells that over express proteolytically active FAP. Cells were administered 1 μM of the inhibitors and allowed to interact with the matrices for 48-h at 37°C. The inhibitors were replenished after 24 h. The reduction of matrix degradation was compared in treated and untreated cells. Matrix degradation remained high, even in the presence of FAP/DPPIV inhibitors (Fig. 4a). 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

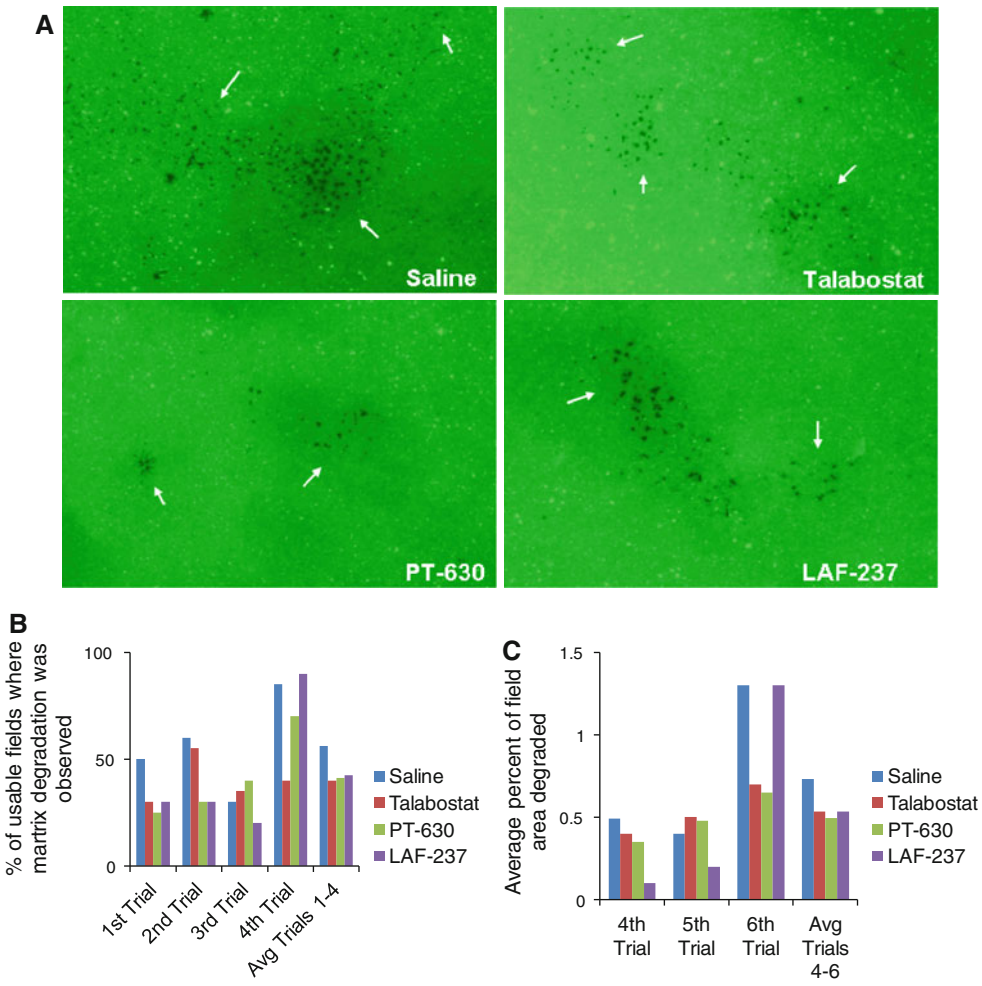

Fig. 4 Small-molecule inhibitors of FAP slightly reduce matrix degradation by cells expressing active FAP. **a** WTY-1 cells were seeded onto glutaradehyde-crosslinked gelatin films that were fluorescent due to covalently-attached FITC-fibronectin. Inhibitors were used at 1 μM and replenished after 24 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 inhibitors reduced matrix degradation. PT-630 and Talabostat showed the greatest inhibition

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 still remained relatively high (Fig. 4b). 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 occurred there was less matrix degradation where prolyl peptidases were inhibited relative to solvent control (Fig. 3c; $P < 0.05$, comparing average saline to average of inhibitors).

The extent of 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 and WTY-6) was also investigated. Cells expressing either mutant FAP^{S624A} or wild-type FAP degraded the matrix more extensively than control transfectants that do not express FAP (Fig. 5a, arrows). The matrix degradation was quantified in three random fields for each cell type using the Image J software and between 249 and 367 holes were detected (Fig. 5b). 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. 5b). Sixteen percent of the holes produced by cells expressing FAP^{S624A} had measurable degradation area (Fig. 5b). 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. 5b). 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. 5b).

Matrix metalloproteinases (MMPs) are major mediators of matrix degradation at invadopodia [16, 29]. Because

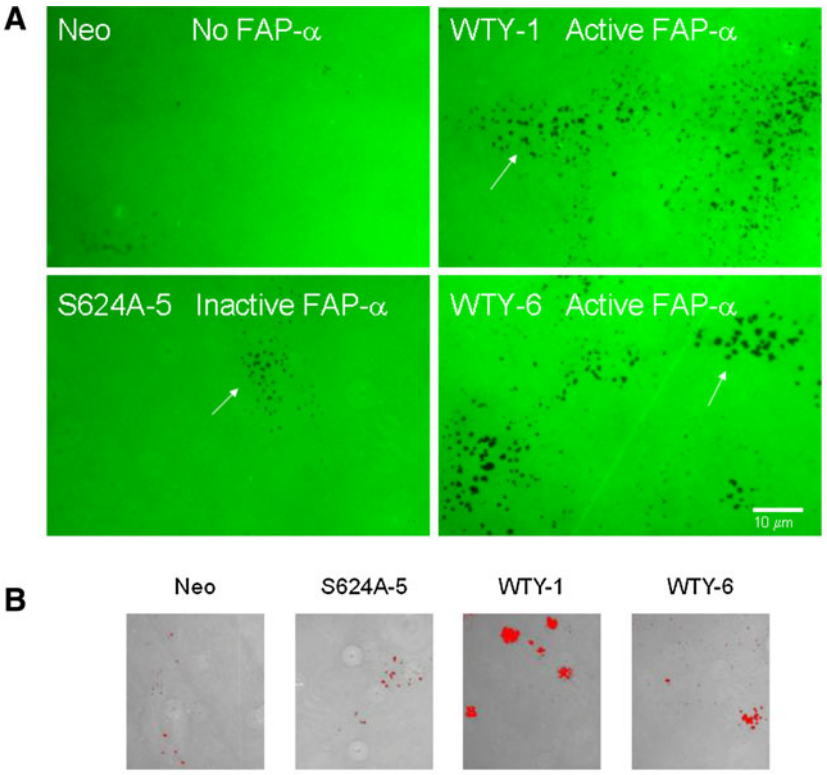

Fig. 5 Cells expressing a catalytic mutant of FAP maintain high levels of matrix degradation. **a** Matrix degradation (*arrows*) is barely detectable under the control transfectants after 48 h (Neo) but is visualized as *black areas* under FAP transfectants (WTY-1, WTY-6, and S624A). The FITC-fibronectin films were visualized with Zeiss Axioskop 2 *mot plus* microscope and 40× 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

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

prolyl peptidase inhibition produced a relatively modest decrease in matrix degradation, the possible effects of FAP expression on MMP accumulation in conditioned medium were investigated. MMPs in serum-free media were evaluated after equal numbers of each cell type had grown in an equal volume of medium for 24-h. Conditioned medium samples matched for the amount of total protein (30 μg) 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 previously [30, 31]. The major MMP detected by zymography in conditioned medium of these cells exhibited a molecular weight of 92-kDa, which corresponded to that of pro-MMP-9 (Fig. 6a, left panel). Moreover, the 92-kDa activity was elevated in conditioned medium from both FAP and FAP^{S624A}-expressing cell lines as compared to the control transfectants (Fig. 6a, 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. 6a, 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 [29]. For these experiments, type I collagen gels were used as described previously [29]. Briefly, a 1 ml gel of collagen is formed by mixing acid-soluble rat tail collagen with medium in the wells of a 24 well plate. Cells are seeded on top of the collagen gels, placed in an incubator, and then allowed to interact with the gel for 48 h. Cells that did not 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 [29]. The experiments were performed in triplicate. FAP^{S624A} and FAP-expressing cells invaded to a significantly higher degree than Neo cells that did not

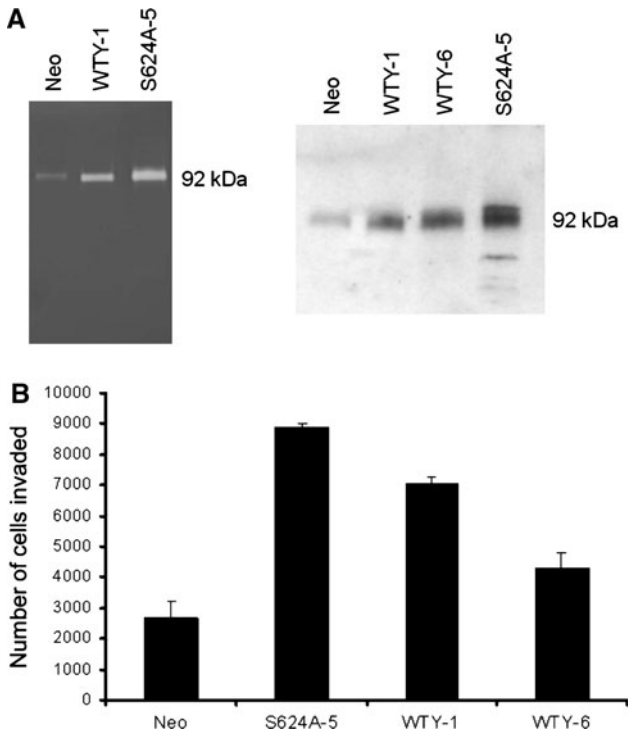

Fig. 6 Expression of either catalytically active or inactive FAP increases MMP-9 accumulation in media and invasive behavior of cells. **a** *Left panel* Zymogram of conditioned medium from control transfectant (Neo) and FAP overexpressing cells (WTY-1 and S624A-5). *Right panel* MMP-9-specific western 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

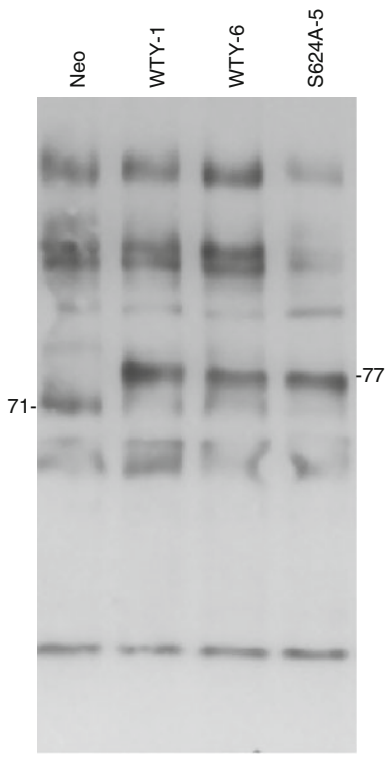

Fig. 7 Altered pattern of tyrosine phosphorylated proteins. Western blot of extracts of control transfectants (Neo) and FAP overexpressers (WTY-1; WTY-6; and S624A-5). The blot was probed with mAb 4G10 to phosphotyrosine

express FAP (Fig. 6b). 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 ECM proteins and invading collagen gels is consistent with the increased amounts of 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 invasive and tumorigenic phenotype of cells expressing active or inactive FAP suggested that FAP might alter cell signaling and prompted investigation of tyrosine phosphorylated proteins (YPPs) in FAP-expressing and non-expressing tumor cell lines. A 77-kDa YPP was identified in extracts of FAP^{S624A} and wild-type FAP-expressing cells was not detected in the control transfectants (Fig. 7). Conversely, the control transfectants expressed a 71-kDa YPP that was not detected in any of the FAP-expressing cell lines.

Discussion

This study demonstrates that the surface expression of human FAP increases tumorigenicity and tumor growth in vivo and invasiveness in vitro of human breast cancer cells and that the protease activity of FAP is not required for these functions. The 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 proteolytically inactive mutants of FAP are biologically active in tumor cells [34]. However, in a murine melanoma system, FAP acted as a tumor suppressor and expression of either wild type or inactive mutant FAP inhibited tumor growth [34]. The apparently contradicting findings of FAP-stimulation of growth in the human breast cancer model versus FAP-suppression in the murine melanoma suggest that the effect of FAP on tumor cell biology might depend on the type of tissue from which the tumor develops. The signaling molecules that are available for modulation by FAP will differ between different tumor cell types, resulting in the possibility that FAP expression will have different effect on tumor growth and phenotype in different cancers [20]. Additionally, there may be cellular contexts where the FAP protease activity is critical and dominates over non-

proteolytic signaling. For example, a human embryonic kidney cell line expressing wild type murine FAP formed rapidly growing tumors; but the same cell line expressing murine FAP^{S624A} formed slow growing tumors in a mouse xenograft model [35]. Moreover, catalytic function perturbing antibodies slowed the growth of these cells expressing active FAP [36]. Similarly inhibition of FAP proteolytic activity was effective at slowing tumor growth in mouse models of lung and colon cancers [23]. These findings suggest a role for the protease activity of FAP in tumor growth promotion in these models.

Several lines of evidence suggest that FAP activates cell signaling that changes cell behavior. Firstly, MMP-9 accumulation in conditioned medium is high for cells expressing FAP^{S624A} or wild-type FAP. The increase in MMP-9 levels of FAP-expressing cells is particularly relevant to tumor progression because it is a pro-angiogenic factor [37–40] and may be an important mediator of the increased angiogenesis previously observed in tumors expressing FAP [19]. This role is supported by earlier studies demonstrating that MMP-9 null mice exhibit a delay in angiogenesis in the growth plates of developing bone [37]. A subsequent study showed that MMP-9 promotes angiogenesis during carcinogenesis of pancreatic islets by releasing and mobilizing vascular endothelial growth factor (VEGF) [39], and MMP-9 also appeared to be associated with the pro-angiogenic switch in a mouse model of cervical cancer [41]. Moreover, MMP-9 is a potent mediator of the matrix degradation that facilitates tumor invasion and metastasis. Secondly, YPP expression appeared to be similar between cells that express FAP^{S624A} or FAP and different from that 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 ECM, contain four major YPPs identified as 150, 130, 81, and 77-kDa proteins [42]. YPPs of 150 and 71-kDa were identified as components of normal focal contacts [42]. In the present study, a 77-kDa YPP was readily detected in extracts of cells expressing active or inactive FAP without enriching for invadopodia, and the 71-kDa YPP was detected in extracts of control transfectants without enriching for focal adhesions.

The mechanisms by which FAP activates tumor cells are unknown, but the present 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 [20]). FAP has only six amino acids in its cytoplasmic domain [5, 36], and by itself, it does not appear capable of transmitting signals. However, integrins are known to signal and in turn increase the expression of several MMPs. For example, MMP-2 secretion can be up-regulated by signaling through the $\alpha v\beta_3$ integrin [43]. In addition, MMP-9 expression and invasion can be increased by FAK and Src activities, both of which can be activated by integrins [44]. In this context, it is particularly relevant that FAP can associate with β_1 integrins, including $\alpha_3\beta_1$ [45, 46]. Integrin $\alpha_3\beta_1$ is expressed by MDA MB-231 cells [47] and has been linked to elevated MMP-9 release by these cells [48]. Thus, the results suggest a mechanism in which FAP mediates its pro-growth and pro-invasive effects by associating with and activating integrins, thereby causing increased release of MMP-9. The extracellular MMP-9 can then act within the tumor microenvironment. Recent work has shown that FAP and MMP-9 can cooperate to degrade gelatin [49]. The integrin activation also coordinates with MMP-9 to promote tumor invasion and metastasis.

This study forced over-expression of FAP in the epithelial cancer cells and did not focus on the stromal cells. However, FAP is best known for its induced expression on stromal fibroblasts reacting to epithelial cancers [32, 50, 51]. Therefore, FAP expression on tumor cells themselves may have distinct effects on tumor growth as compared to the effects of the better known expression of FAP on stromal cells. In this regard, it is important to remember that there are several reports that FAP is expressed by epithelial cancer cells [52–54] including malignant breast cancer cells [18]. Moreover, others have shown that depletion and/or inhibition of FAP in the stromal compartment has a growth inhibitory effect on lung tumors [23]. Thus, it is likely that FAP expression in the tumor microenvironment is the critical feature that can stimulate growth. Moreover, the fact that the human breast cancer cells expressing the catalytic mutant of human FAP produced rapidly growing tumors while control transfectants did not suggests that in this model endogenous mouse FAP does not predominate because both tumors should elicit similar stromal responses. This may be a function of the xenograft model which produces tumors that generally have a significantly higher proportion of epithelial tumor cells and a lower proportion of stromal cells than are typically found in spontaneously formed tumors. In the case of the FAP/DPPIV inhibitors, these should have had similar inhibitory action against mouse FAP as against human FAP and thus mouse FAP was likely inhibited in the talabostat and PT-630 treated animals.

FAP is recognized as an excellent target for therapies directed against epithelial cancers because its expression is induced in the stromal cells of the tumor microenvironment but is very limited in normal adult tissues [55]. Current trials are utilizing FAP-specific antibodies in an effort to target toxic agents specifically to tumor tissue [55], 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 may not be effective in breast cancer; but there is insufficient data to predict the efficacy of such agents in other types of cancer. Inhibition of FAP protease activity may contribute to the antitumor effects of talabostat in mouse tumor models [23, 56]. More studies are required to clarify the roles of the protease activities and the intermolecular interactions of FAP in its biological functions.

Conclusions

We conclude that the proteolytic activity of FAP participates in matrix degradation, but other domains within the protein stimulate the production of biologically active factors, such as MMP-9, which enhance matrix remodeling and facilitate tumor growth.

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Conflict of interest The study was funded in part by Point Therapeutics who produced the talabostat and PT-630 compounds used in this research.

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Fibroblast activation protein-α: A key modulator of the microenvironment in multiple pathologies

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Running title: FAP builds tissue microenvironments

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Abstract

Fibroblast activation protein-α (FAP) is a serine protease that can provide target specificity to therapeutic agents because in adults its expression is restricted to pathologic sites, including cancer, fibrosis, arthritis, wounding or inflammation. It is not expressed in most normal tissues. The majority of FAP is expressed by activated fibroblasts responding to the pathologic situations. FAP is typically found as a type II transmembrane protein physically attached to cells and with the bulk of the protein, including the catalytic domain, exposed to the extracellular space and accessible to small molecules. In this chapter we review the structure, substrate specificities, signaling functions, and current design of FAP inhibitors. Evidence indicating the presence of FAP in multiple cancers, arthritis, fibrosis, keloids and other pathologies is described and indicates possible roles for FAP in facilitating cell invasion and growth. Separate sections are devoted to the role of FAP in coordinating the stromal response to cancer, including a role in angiogenesis and a potential role in modulation of the anti-tumor immune response. Finally studies attempting to demonstrate the clinical potential of FAP are discussed, as well as some novel applications employing FAP in therapy or diagnosis. Throughout the review, effort is made to highlight areas where information is lacking and to highlight important questions that require further investigation.

Key Words: seprase, dipeptidyl peptidases, metastasis, tumor microenvironment, breast cancer

Abbreviations

2SBPO	disulfonated benzo[*a*]phenoxazine
AMC	7-amino-4-methylcoumarin
α_2AP	α_2-antiplasmin
CAFs	cancer associated fibroblasts
DPPIV	dipeptidyl peptidase IV, also known as CD26
ECM	extracellular matrix
EDTA	ethylenediaminetetracetate
EGR-1	early growth response -1
EMT	epithelial to mesenchymal transition
ERK	extracellular signal-regulated kinase
F19	mouse monoclonal antibody to FAP
FAK	focal adhesion kinase
FAP	fibroblast activation protein-α
FAPᴛ-MT	1-methyl tryptophan bound to FAP β-propeller
GFAP	glial fibrillary acid protein
HASMC	human aortic smooth muscle cells
HSC	hepatic stellate cells
IHC	immunohistochemistry
IL-1β	interleukin-1β
IPF	idiopathic pulmonary fibrosis
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1-matrix metalloproteinase
NPY	neuropeptide-Y
OA	osteoarthritis
POP	prolyl oligopeptidase
PREP	prolyl endopeptidase

RA	rheumatoid arthritis
RT-PCR	reverse transcription-polymerase chain reaction
siRNA	small interfering RNA
αSMA	α-smooth muscle actin
TGF-β	transforming growth factor-β
TIMP-2	tissue inhibitor of metalloproteinase-2
TNF-α	tumor necrosis factor-α

1. Introduction

Fibroblast activation protein-α (FAP) was independently discovered in the mid 1980's and early 1990's by two groups pursuing fundamentally different questions. One group was investigating surface antigens to define activated fibroblasts. They produced a monoclonal antibody called F19 that strongly labeled the reactive stromal fibroblasts of epithelial tumors, cultured fibroblasts, fibroblasts in fetal mesenchymal tissues, and tumor cells of sarcomas (Garin-Chesa *et al.*, 1990; Rettig *et al.*, 1986; Rettig *et al.*, 1988). These investigators named the F19 antigen "fibroblast activation protein-α" (FAP) because of its strong expression on activated fibroblasts responding to cancers and in granulation tissue (Rettig *et al.*, 1993). A cDNA for the F19 antigen was subsequently cloned and homology to dipeptidyl peptidase IV (DPPIV) was recognized (Scanlan et al., 1994). The other group was investigating membrane bound proteases that might mediate the matrix-degrading functions of invadopodia, the membrane protrusions of invading tumor cells that degrade extracellular matrix (Chen, 1989; Mueller, 2008). This group identified a 170 kDa gelatinase activity on invasive melanoma cells that they named "seprase", for surface expressed protease (Aoyama and Chen, 1990; Monsky *et al.*, 1994). They produced monoclonal antibodies D8 and D43 to seprase and used these to clone a seprase cDNA (Goldstein *et al.*, 1997; Pineiro-Sanchez *et al.*, 1997). Molecular cloning revealed that seprase is identical to FAP and together with DPPIV belongs to a family of serine integral membrane proteases (Goldstein et al., 1997; Pineiro-Sanchez et al., 1997; Scanlan et al., 1994). As far as the names are concerned, both are partially correct. "FAP" implies that the

protein is only found on fibroblasts and this is not the case. Indeed early work investigating tumors by immunohistochemistry with F19 showed that neoplastic cells in malignancies of mesenchymal origin also express FAP (Rettig et al., 1993). Moreover, others have found FAP/seprase on epithelial tumor cells of breast, gastric, and colorectal cancers, as well as on melanocytes and melanoma cells (Aoyama and Chen, 1990; Iwasa *et al.*, 2005; Kelly *et al.*, 1998a; Monsky *et al.*, 1994; Mori *et al.*, 2004; Okada *et al.*, 2003). FAP was also identified in endothelial cells in an expression screen for serine proteases and by gene expression profiling (Aimes *et al.*, 2003; Ghilardi *et al.*, 2008). "Seprase" is not wholly accurate because a soluble form of FAP has been identified in sera (Lee et al., 2004; Lee et al., 2006) and immunohistochemistry reveals that there is a substantial intracellular pool in most cells that express FAP (Iwasa *et al.*, 2005; Kelly *et al.*, 1998a; Mori *et al.*, 2004; Okada *et al.*, 2003). Thus, FAP is not restricted to the cell surface. In any event, the name "fibroblast activation protein-α" and the symbol "FAP" predominate in the literature and are the official name and symbol listed in NCBI Gene and, consequently will be used throughout this review.

Almost since its discovery, it has been suggested that FAP could serve as a target for therapeutic agents, even though its precise biological functions remain largely unknown. FAP has potential as a therapeutic target because: 1) it is expressed at pathologic sites but not in normal adult tissues and could, therefore, lend exquisite target specificity to therapeutic agents; 2) it exists in a membrane-bound form that is physically attached to the plasma membrane of cells at the sites of pathology; and 3) the majority of the

protein including the serine protease domain is exposed on the surface of cells making it accessible to small molecules. In the past couple of years, major advances have been made in: i) identifying the natural substrate specificities of the FAP protease activity, ii) developing inhibitors that target the FAP protease, iii) defining the critical role of FAP in inflammation, angiogenesis, and suppression of the immune system by FAP expressing cells, iv) characterizing FAP function in producing extracellular matrices that permit cell movement, and v) developing novel therapies and techniques exploiting the protease activity of FAP. These will be the focus of the current review. In addition there are other earlier and excellent reviews on FAP and the prolyl peptidase family of proteases that are recommended to the reader (Chen and Kelly, 2003; Juillerat-Jeanneret and Gerber-Lemaire, 2009; Kelly, 2005; Lawandi *et al.*; O'Brien and O'Connor, 2008; Pure, 2009; Rosenblum and Kozarich, 2003; Wolf *et al.*, 2008).

2. Characterization of FAP

2.1 Classification, sequence and functional domain structure

FAP is a serine protease that cleaves the peptide bond between proline and other amino acids and this activity modifies various bioactive molecules (Goldstein et al., 1997; Pineiro-Sanchez et al., 1997; Scanlan et al., 1994). Other proteases with this activity include dipeptidyl peptidases 4, 6, 8, 9, prolyl endopeptidase (PREP, also called POP), proline amino peptidase, prolyl carboxy-peptidase, and attractin that with FAP comprise the family of post-prolyl peptidases (Chen *et al.*, 2003). FAP is most closely related to DPPIV. They share identical domain structure, and have 50% or 70% sequence identity in the entire sequence or the catalytic domain, respectively (Cheng et al., 2005; Pineiro-Sanchez et al., 1997; Scanlan et al., 1994). Most likely DPPIV and FAP are products of gene duplication (Irwin, 2002). Biochemically, FAP and DPPIV show similar dipeptidyl peptidase activity (N-terminal post-prolyl peptidase activity cleaving NH_2-Xaa-Pro peptide bonds). In addition FAP possesses a unique endopeptidase activity, which DPPIV lacks, enabling it to cleave gelatin, collagen type I, and α_2-antiplasmin (α_2AP) (Aoyama and Chen, 1990; Kelly, 1999; Lee *et al.*, 2004; Park *et al.*, 1999; Pineiro-Sanchez *et al.*, 1997).

Human FAP is a type II integral membrane protein with only six amino acids in the cytoplasm, a single membrane spanning domain of 18 amino acids, and a large extracellular domain of 736 amino acids encompassing the α/β hydrolase and 8 bladed β-propeller domains (Aertgeerts et al., 2005; Cheng et al., 2005). Within the catalytic domain, serine (S624), aspartate (D702) and histidine (H734) form an inverted catalytic triad, which defines FAP as a serine protease (Aertgeerts et al., 2005; Cheng et al.,

2005; Goldstein et al., 1997; Pineiro-Sanchez et al., 1997; Scanlan et al., 1994). Serine 624 mediates both the dipeptidyl peptidase and endopeptidase activities (Aoyama and Chen, 1990; Goldstein *et al.*, 1997; Pineiro-Sanchez *et al.*, 1997). When denatured, the 760 amino acid protein migrates as a 97 kDa protein on SDS-PAGE (Pineiro-Sanchez et al., 1997). However, FAP must assemble into a dimer of 170 kDa in order to be an active protease (Kelly, 1999; O'Brien and O'Connor, 2008; Pineiro-Sanchez *et al.*, 1997). Under non-denaturing conditions, FAP gelatinase activity can be readily detected at 170 kDa by zymography (Aoyama and Chen, 1990; Monsky *et al.*, 1994). FAP is also a glycoprotein (Rettig et al., 1988) and binds to the lectin, wheat germ agglutinin (Kelly, 1999; Kelly *et al.*, 1998a; Monsky *et al.*, 1994).

The FAP gene is highly conserved across species. Homologs of human FAP have been identified in mouse (Cheng *et al.*, 2002; Cheng et al., 2005; Niedermeyer *et al.*, 1998), xenopus (Brown *et al.*, 1996) and a variety of other species. Human and murine FAP genes show very similar genomic organization. The mouse FAP gene, located on chromosome 2, spans approximately 60 kB and contains 26 exons ranging in size from 46 bp to 195 bp (Niedermeyer et al., 1998). The human FAP, located on chromosome 2q23, spans approximately 73 kB and also has 26 exons. The FAP gene is organized similarly to the DPPIV gene. Human DPPIV maps to chromosome 2q24.3 and spans 70 kb and contains 26 exons ranging from 45 bp to 1.4 kb (Abbott *et al.*, 1994). Three different splice variants of FAP have been observed in mouse embryonic tissues, with all three predicted to encode proteins that include the complete catalytic domain and the membrane spanning domain, but with the splice variants lacking portions of the

membrane proximal extracellular domain (Niedermeyer *et al.*, 1997). In human melanoma cells an alternatively sliced FAP message has been described that encodes the carboxyl terminus of FAP including the entire catalytic triad (Goldstein and Chen, 2000). This would be a soluble enzyme, likely with functional roles distinct from full length FAP. Recent work characterizing the promoter revealed that the FAP gene has EGR1, HOXA4 and E2F1 transcription factor binding sites (Zhang *et al.*, 2010). Of these, EGR1 appears to be the most important transcription factor for driving FAP expression (Zhang *et al.*, 2010). This key discovery will allow for much greater understanding of how FAP is induced in cancer and in inflammation.

The crystal structure of human FAP reveals that the extracellular domain of FAP is composed of an eight-bladed β-propeller domain and αβ-hydrolase domain (Aertgeerts et al., 2005; Cheng et al., 2002; Cheng et al., 2005). The serine protease catalytic triad is located within the αβ-hydrolase subunit (amino acids 27-53 and 493-760) (Aertgeerts et al., 2005). The β-propeller domain of FAP (amino acids 54-492) also forms a flexible pore that is thought to be a structural feature confirming FAP substrate specificity similar to that shown for DPPIV (Aertgeerts *et al.*, 2005; Cheng *et al.*, 2002; Cheng *et al.*, 2005; Engel *et al.*, 2003; Gorrell *et al.*, 2001). In FAP and DPPIV, a second larger pore was noted to the side of the active site (Cheng *et al.*, 2005; Engel *et al.*, 2003). This was proposed as the means for substrates and cleavage products to exit (Engel et al., 2003). Additionally, the β-propeller domain might play a role in the binding of the enzyme to the components of extracellular matrix, as well as in forming complexes

between FAP and other transmembrane proteins (Kelly, 2005). Furthermore, the β-propeller contains a number of putative T-cell epitopes (Yi *et al.*, 2011).

Dimerization and glycosylation of FAP are necessary for its proteolytic activity. It has been demonstrated that glycosylated FAP exhibits both dipeptidyl peptidase and gelatinase activity *in vitro*, while the nonglycosylated isoform lacks enzymatic activity (Sun et al., 2002). The glycosylated 97 kDa monomer of FAP possesses the catalytic domain, but it is proteolytically inactive unless assembled into a dimer thereby forming the functional 170 kDa enzyme (Kelly, 1999; Pineiro-Sanchez et al., 1997). Furthermore, FAP can oligomerize with DPPIV into active complexes shown to be required for lung fibroblast migration on collagen (Ghersi *et al.*, 2002, 2003; Ghersi *et al.*, 2006; Rettig et al., 1993). Possibly, the two enzymes work together to elicit their functions. Further, FAP may also oligomerize with β1 integrins such α3β1 integrin (Artym *et al.*, 2002; Mueller *et al.*, 1999). This interaction with integrins may be critical for FAP-mediated cell signaling.

2.2. FAP substrates and inhibitors

A stated above, FAP possesses two lytic activities (Levy et al., 1999; Park et al., 1999), but is best known for the endopeptidase activity that distinguishes FAP from DPPIV. Gelatin is FAP's best described substrate and its gelatinase activity can be used to monitor its proteolytic activity (Kelly, 1999). For example, some effort has been made to study the endopeptidase activity with radiolabeled or fluorescent gelatin or type I

collagen substrates (in the presence of EDTA to inhibit metalloproteinase activities) (Kelly, 1999; Park *et al.*, 1999). The biological significance of FAP-cleavage of gelatin and type I collagen is not known. There is some suggestion that FAP may cooperate with MMPs to produce biologically active fragments of denatured collagen (Christiansen *et al.*, 2007; Huang *et al.*, 2011b). Given that zymography provides qualitative, but not quantitative analysis of endopeptidase/gelatinase activity, other synthetic substrates of FAP have been employed to study FAP enzymatic functions and their implications in cancer and other pathologies. The most common way to quantify FAP activity takes advantage of its dipeptidyl peptidase activity, using as substrates dipeptides in which the penultimate amino acid is proline linked to a fluorescent molecule of 7-amino-4-methylcoumarin (AMC), i.e., Gly-Pro-AMC (Huang *et al.*, 2011b; Park *et al.*, 1999; Santos *et al.*, 2009). Upon FAP mediated cleavage the free AMC is released and provides a measure of enzyme activity. The advantage of this approach is its ease, speed, and quantitative results. However, the potential to incorrectly assign the dipeptidyl peptidase activity of DPPIV or other post-prolyl peptidase to FAP is a potential drawback of the approach. Consequently, some other means to make the assay specific for FAP is required. For example, FAP-specificity can be achieved by measuring proteolytic activity of FAP that is purified from extracts using an antibody to FAP (Huang *et al.*, 2011b; Santos *et al.*, 2009).

The first identified physiologic substrate for the FAP endopeptidase was α_2AP, discovered by McKee's group while investigating a circulating antiplasmin-cleaving enzyme, later recognized as a soluble form of FAP (Lee et al., 2004; Lee et al., 2006).

Interestingly, FAP mediated cleavage of α_2AP results in a 13-fold increase in incorporation of cleaved α_2AP into fibrin, thereby promoting stabilization of fibrin by protecting it from plasmin degradation (Lee *et al.*, 2011b).

FAP endopeptidase activity was shown to cleave the SPRY2 protein (also called SPROUTY2), a natural inhibitor of receptor tyrosine kinase (Huang *et al.*, 2011a). Although SPRY2 is readily cleaved by FAP, it likely is not an *in vivo* substrate of FAP because it is localized inside the cells.

Recently, natural substrates for FAP dipeptidyl peptidase activity were identified by Gorrell's group (Keane *et al.*, 2011). Neuropeptide Y, B-type natriuretic peptide, substance P, and peptide YY were shown to be robust substrates for FAP and the first hormone substrates for FAP to be described. These molecules are also good substrates for DPPIV, however the half-life of substrates varied depending on the cleaving enzyme (DPPIV vs. FAP) (Keane *et al.*, 2011). The cleavage of peptide hormones by FAP implicates FAP in modifying tissue microenvironments.

There is considerable interest in identifying specific peptide substrates for FAP. This is based on the idea that once these sequences are known, specific peptides could be designed to inhibit FAP but not DPPIV or other prolyl peptidases (Edosada et al., 2006a; Edosada et al., 2006b; Lee et al., 2011b; Lee et al., 2009). Moreover,

knowledge of cleavable peptide sequences might be used to design pro-drugs that are specific for tumor cells and tumor microenvironment upon activation by FAP or fluorescent molecules that are unquenched by FAP activity.

Bachovchin and colleagues pioneered the design of inhibitors of post-prolyl peptidases. They took advantage of the well known NH_2-Xaa-Pro motif to synthesize boronic acid based inhibitors, such as Pro-boroPro, Ala-boroPro, and Val-boroPro (Connolly *et al.*, 2008; Flentke *et al.*, 1991; Kubota *et al.*, 1992; Poplawski *et al.*, 2011). These inhibitors are effective against most post-prolyl peptidases and, as such, have broad specificity.

Subsequent work has focused on developing inhibitors based on natural substrates of FAP. Studies focused on the FAP cleavage site within α_2AP (TSG**P-N**Q) indicated that FAP prefers Gly at P2 and Pro at P1 (Edosada *et al.*, 2006b). The identification of the FAP cleavage site in α_2AP inspired investigators to design highly specific inhibitors of FAP. Using the α_2AP FAP cleavage site as a model for FAP endopeptidase activity, it was shown that with Pro at P1 and Gly or D-amino acids at P2, FAP prefers small uncharged amino acids at P3 and tolerates most amino acids at P4 (Edosada *et al.*, 2006a). FAP has a preference for N-blocked peptide substrates, which are poor substrates for DPPIV. For example, FAP cleaves formyl-, benzyloxycarbonyl-, biotinyl-, and peptidyl-Gly-Pro substrates, which DPPIV cleaves poorly (Edosada *et al.*, 2006b). Accordingly, Acyl-Gly-boroPro has a markedly increased specificity for FAP versus other post prolyl peptidases (Edosada *et al.*, 2006b) and peptidyl chloromethyl ketones have been shown to inhibit FAP but not DPPIV (Edosada *et al.*, 2006a). McKee's group

also investigated FAP substrates based on the cleavage of α_2AP and found that glycine at P2 and proline at P1 are required (Lee *et al.*, 2009). This group also investigated amino acids further away from the cleavage site and found that α_2AP analogs with Arg at P7 had the highest affinity for FAP. They showed that a substrate analogue inhibitor, Phe-Arg-(8-amino-3,6-dioxaoctanoic acid)-Gly-[r]-fluoropyrrolidide, inhibited FAP with a K_i of 54 µM, but did not inhibit DPPIV even at 2 mM (Lee et al., 2009). This group recently developed acetyl-Arg-(8-amino-3,6-dioxaoctanoic acid)-D-Ala-L-boroPro that selectively inhibits FAP versus DPPIV, with the K_i for FAP (5.7 nM) approximately 1000 fold lower than that of DPPIV (6.1 µM) (Lee *et al.*, 2011b). Interestingly, this inhibitor also inhibits prolyl endopeptidase (PREP) with a K_i of 7.4 nM, which is comparable to that of FAP. Acetyl-Arg-(8-amino-3,6-dioxaoctanoic acid)-D-Ala-L-boroPro caused a dose-dependent decrease of FAP-mediated α_2AP cleavage, which ultimately shortened plasminogen activator-induced plasma clot lysis times (Lee et al., 2011b). Because there is no PREP in sera, it is likely that the suppressed cleavage of α_2AP by this compound is due to FAP inhibition. The shortened clot lysis times demonstrates a clear biological response that is regulated by FAP activity.

Other investigators used gelatin derived from type I collagen to identify FAP cleavage sites and found that PPGP and (D/E)-(R/K)-G-(E/D)-(T/S)-G-P are consensus sites for FAP (Aggarwal *et al.*, 2008). As observed with α_2AP, Gly at P2 and Pro at P1 are present in both consensus cleavage sites for gelatin derived from type I collagen (Aggarwal *et al.*, 2008).

Huang et al identified a consensus FAP cleavage site of SSG**P-V**A in SPRY2, consistent with earlier studies, confirming that Gly at P2 and Pro at P1 are necessary (Huang *et al.*, 2011a). This work predicts that FAP favors uncharged amino acid residues, regardless of their size, at the positions P1', P3, and P4 surrounding the cleavage site of the substrate (Huang *et al.*, 2011a).

2.3 Non-enzymatic activities of FAP There is growing appreciation that FAP activates cell signaling. Houghton and co-workers demonstrated that mouse melanoma cells engineered to express FAP or a catalytic mutant of FAP (in which Ser 624 is mutated to Ala) were less tumorigenic than the non-expressing parental cells (Ramirez-Montagut *et al.*, 2004). Further, the catalytic mutant cells were less tumorigenic than cells expressing wild-type FAP (Ramirez-Montagut *et al.*, 2004). This finding is in contrast to work by others that generally find FAP associated with increased invasive behavior and more aggressive tumor functions (Monsky *et al.*, 1994). Nevertheless, this study showed that FAP function could be mimicked by a catalytic mutant of FAP, and that biologically relevant cell signaling activated by FAP, was independent of its proteolytic activity.

During wound healing and tissue remodeling, as well as during tumor invasion, fibroblasts or tumor cells become motile. In these situations, motility and matrix formation activities may predominate. Houghton's group postulated that FAP expression may provide negative feedback to regulate otherwise uncontrolled

proliferation of activated melanocytes and fibroblasts during tissue remodeling, recognizing that an individual protein can have distinct functions in different cell types (Ramirez-Montagut *et al.*, 2004). Kelly's group observed the importance of the non-enzymatic functions of human FAP in a mouse xenograft model of human breast cancer. In this model FAP is associated with rapid tumor growth and increased angiogenesis (Huang *et al.*, 2004). In recent work, rapid tumor growth is associated with FAP expression and not with catalytic activity, as tumors expressing an S624A catalytic mutant of FAP grow rapidly (Huang *et al.*, 2011b). Moreover, cells expressing FAP invade more extensively into type I collagen gels than non-expressing parental cells and this is not dependent on the protease activity of FAP (Huang *et al.*, 2011b). In this model, there is evidence of FAP's effect on signaling, as cells that express wild type or mutant FAP also express elevated MMP-9 and have an altered pattern of proteins phosphorylated on tyrosine (Huang *et al.*, 2011b).

It is speculated that FAP activates cell signaling by forming complexes with other proteins. Indeed, as mentioned above, FAP can form complexes with DPPIV that are involved in adhesion and invasion into matrix (Ghersi *et al.*, 2002). The impulses needed to activate cell-signaling may also occur through complex formation involving FAP and other transmembrane proteins. Several studies indicate that FAP can complex with β_1 integrins as shown by co-immune precipitation of FAP with $\alpha_3\beta_1$ integrin (Mueller *et al.*, 1999). Moreover, FRET data suggests that FAP is in close proximity to urokinase plasminogen activator receptor (uPAR) and β_1 integrins (Artym *et al.*, 2002). Thus, it is possible that FAP alters signaling through integrins to effect cellular functions

as we suggested earlier (Kelly, 2005). Subsequent work has supported the idea that integrin signaling is involved in mediating FAP effects. For example, in a mouse model of lung cancer, tumors were produced on FAP-null or FAP-expressing backgrounds and lower levels of phosphorylation were observed in the FAP-expressing mice versus the FAP null mice, specifically decreased phosphorylation of FAK at Y397 and ERK (Santos *et al.*, 2009). There was also reduced expression of p21 (Santos *et al.*, 2009). The author's concluded that deletion of FAP increased p21 through increased FAK and ERK signaling (Santos *et al.*, 2009). FAK and ERK are well known downstream effectors of integrin signaling. In this system, FAP appears to suppress integrin mediated signaling.

3. Association of FAP with human disease. FAP has been linked to multiple disease states and to wound healing. Its enzymatic activities, along with its non-enzymatic activity, have been cited in the progression and even suppression of various diseases. This section will discuss and catalog FAP expression in disorders where FAP is postulated to play a role. Section 2.1 describes FAP in cancer and the reports are organized by disease site. Section 2.2 describes FAP in fibrosis of the liver, lung, and colon. Section 2.3 describes FAP in osteoarthritis and rheumatoid arthritis and section 2.4 describes several other pathologies where FAP expression is induced.

3.1. FAP in cancer

FAP is best known for its presence in stromal fibroblasts, found in over 90% of epithelial tumors. FAP is not expressed in healthy adult tissue other than a subset of pancreatic islet alpha cells that produce glucagon during tissue remodeling and in fetal

mesenchymal tissue during embryogenesis (Garin-Chesa et al., 1990; Rettig et al., 1986; Rettig et al., 1988). FAP is absent from the resting fibroblasts found in human sarcomas. It is detected in numerous epithelial cancers like colorectal, breast, ovarian, lung, bladder, and pancreas (Garin-Chesa *et al.*, 1990; Kelly, 1999; Kelly *et al.*, 1998a; Rettig *et al.*, 1988). Because FAP is mainly expressed on fibroblasts there is hope that improved understanding of the role of cancer associated fibroblasts will lead to novel interventions to improve outcomes in the many cancers where FAP is over-expressed. This section will focus on cataloging FAP expression and describing how it relates to available clinical information. Subsequent sections delve into the mechanistic role of fibroblasts and FAP in the tumor microenvironment (section 3) and the potential of a number of FAP-based interventions to improve outcomes (section 4).

Breast Cancer

FAP was identified in the reactive stroma of breast cancer using the F19 monoclonal antibody (Garin-Chesa *et al.*, 1990). It is clear that the F19 monoclonal antibody recognizes FAP on stromal cells. Later immunohistochemistry with FAP-specific anti-sera confirmed high FAP expression in breast cancer and aberrantly high FAP proteolytic activities in human breast cancer tissues, as judged by gelatinase activity (Kelly, 1999; Kelly *et al.*, 1998a) and dipeptidyl peptidase activity (Park *et al.*, 1999). The anti-sera also identified FAP expression in epithelial tumor cells in ductal carcinomas, a result that remains somewhat controversial (Kelly *et al.*, 1998a). The majority of the work assessing FAP in human tumors by immunohistochemistry argues that it is mainly expressed in the stroma by reactive fibroblasts. However, it is well

recognized that tumor cells themselves may express FAP at certain times and that FAP expression is observed in several breast cancer cell lines (Goodman *et al.*, 2003). Surprisingly, one study found that high FAP expression in the stroma of human breast cancers is associated with better outcomes (Ariga *et al.*, 2001). Elevated FAP in breast cancer is unquestioned, but the role of FAP in breast cancer is still not well defined.

Colon

FAP expression is more prominent in the early stages of colorectal cancer and in small colorectal tumor xenografts than in more advanced staged tumor tissues suggesting a greater role for FAP in early tumor development (Henry *et al.*, 2007). In colon, FAP is associated with an aggressive phenotype. High levels of stromal FAP correlate with rapid progression of disease and increased potential for development of metastatic cancer (Henry *et al.*, 2007). Iwaser et al. also reported a significant correlation between FAP expression and lymph node metastasis (Iwasa *et al.*, 2003). The poorer outcomes associated with FAP expression in colon cancer conflict with findings of a similar study in breast cancer where FAP is associated with better prognosis (Ariga *et al.*, 2001) and this apparent contradiction may be explained by the different roles of activated fibroblasts as discussed below.

Pancreas

90% of pancreatic adenocarinomas express FAP and it is found on tumor associated myofibroblasts adjacent to the tumor. Lower FAP expression was associated with increased fibrosis. Increased FAP expression was positively correlated with increased

number of tumor positive lymph nodes, higher risk of recurrence, and death of patients who did not receive preoperative therapy (Cohen *et al.*, 2008). The remodeling of ECM during tumor invasion involves some FAP-dependent alterations, which alter the structural design and composition of the ECM (Lee *et al.*, 2011a). Inhibiting FAP activity apparently reduces the organization of the matrix and invasive nature of pancreatic cancer. This inhibition is thought to disrupt FAP-directed re-organization of ECM by stromal cells (Lee *et al.*, 2011a).

Gastric carcinoma

Intestinal-type gastric cancer has more stromal cells and FAP expression than diffuse-type cancers (Okada *et al.*, 2003). Immunohistochemistry showed greater localization of FAP in gastric carcinoma cells compared to the minor expression in stromal cells and endothelial cells of microvessels in the tumor (Mori *et al.*, 2004). FAP expression appeared to identify different subtypes of cancer cells, being more prominent on moderately differentiated and diffuse type cells than on well-differentiated gastric carcinoma cells. Gastric carcinoma is a tumor type where FAP is also expressed by the epithelial cancer cells.

Brain

The abundance of FAP positive fibroblasts in malignant but not in benign tumors suggests a close correlation between the malignant potential of epithelial tumors and the FAP phenotype of the stromal components. Primary brain tumors are FAP negative, but carcinomas that metastasize to the brain induce FAP positive stromal cells

(Garin-Chesa *et al.*, 1990; Rettig *et al.*, 1986). In glioblastoma, the FAP gene is thought to be a target of the TWIST transcription factor that promotes invasion of mesenchymal-like cells that arise due to TWIST-driven epithelial to mesenchymal cell transition (Mikheeva *et al.*, 2010). FAP expression is upregulated in the most malignant of grade IV gilomas, compared to the lesser grades and correlates with mesenchymal differentiation (Mikheeva *et al.*, 2010). In astrocytic tumors, FAP and DPPIV enzymatic activities increase with tumor grade and are associated with neovascularization and necrosis (Stremenova *et al.*, 2007).

Ovaries

FAP is expressed in ovarian cancer but is not seen in benign tumors or those of low malignant potential (Garin-Chesa *et al.*, 1990; Rettig *et al.*, 1986). In ovarian cancer there is a significant association between the presence of reactive stromal markers, FAP, and α-smooth muscle actin (αSMA) and the occurrence of lymph node and omentum metastasis, as well as elevated lymphatic density (Schauer *et al.*, 2011). Moreover, FAP in peritoneal or pleural effusions from ovarian cancer patients was correlated with decreased survival (Zhang *et al.*, 2007). FAP can also be induced *in vitro* by adhesion of ovarian cancer cells to type I collagen (Kennedy *et al.*, 2009). FAP promotes proliferation, adhesion, increased invasion, and migration of ovarian cancer cells *in vitro* and also in an animal model (Kennedy *et al.*, 2009). Collagen matrices induce FAP expression in the tumor microenvironment presumably through binding β1 integrin. Supporting this, an antibody to β_1 integrin could substitute for the collagen gel and cause increased FAP expression invasiveness of ovarian tumor cells (Kennedy *et*

al., 2009). *In vitro*, TGF-β induces FAP expression that in turn promotes invasion of HO-8910PM ovarian carcinoma cells (Chen *et al.*, 2009).

Cervix

FAP was detected in microinvasive cervical carcinomas and all invasive carcinomas, but was not detected in atypical epithelial or cervical intraepithelial neoplasia (Jin *et al.*, 2003). As such, FAP could be an early marker of invasiveness in squamous lesions of the uterine cervix (Jin *et al.*, 2003). Cervical cancer is another type of cancer where FAP staining is reported in the epithelial tumor cells (Jin *et al.*, 2003).

Oral

FAP is upregulated in oral squamous cell carcinoma during fibroblast activation through a newly described mechanism called “nemosis” where activation occurs when fibroblasts cluster into dense cell aggregates (Rasanen *et al.*, 2009). Fibroblast gene expression differs among patients but FAP and αSMA are consistently seen in reactive fibroblasts of oral tumors. Therefore, FAP and αSMA can be prognostic markers of the stromal reaction in squamous cell carcinomas (Rasanen et al., 2009).

Myeloma

FAP is expressed by osteoclasts *in vitro*, along with osteogenic cells, adipocytes and vascular epithelial cells (Ge *et al.*, 2006). FAP is not expressed by myeloma plasma cells (Ge *et al.*, 2006). Inhibition of FAP expression by siRNA caused reduced myeloma plasma cell survival in co-cultures with osteoclasts; however, myeloma cell survival was not affected by FAP suppression when cultured by themselves (Ge *et al.*, 2006). Ge et

al. concluded that FAP is needed in the myeloma-bone marrow microenvironment (Ge et al., 2006). Further work by this group, using broad spectrum inhibitors of post-prolyl proteases (Val-boroPro and Glu-boroPro) showed *in vitro* that differentiation of osteoblasts and bone resorption by osteoclasts are not dependent upon post-prolyl peptidase activity (Pennisi et al., 2009). However, Pennisi et al. showed by RT-PCR arrays that osteoclasts up-regulate of 17 out of 85 adhesion molecules tested when co-cultured with myeloma cells. Treatment of the osteoclast-myeloma cell co-cultures with Val-boroPro down-regulated 18 of 85 genes tested and some of these are implicated to play a role in tumorigenesis and osteoclastogenesis (Pennisi *et al.*, 2009). Furthermore, in a severe combined immune deficient (SCID)-hu model of primary myeloma, Val-boroPro reduced osteoclast activity, bone resorption and tumor burden (Pennisi *et al.*, 2009).

Melanoma

The role of FAP in melanoma is controversial. There are moderate amounts of FAP positive fibroblasts in melanoma. Subsets of melanocytic cells show some FAP expression, but melanoma cells from metastatic melanomas are reported to be FAP negative (Huber *et al.*, 2003). Conversely, FAP is a marker for the invasive phenotype of melanoma cell lines (Aoyama and Chen, 1990). In these melanoma cell lines, FAP is found localized on the invadopodia of invasive melanoma cells (Pineiro-Sanchez et al., 1997). The presence of FAP on invadopodial membranes of melanoma cells suggests it could play a role in matrix proteolysis (Monsky *et al.*, 1994). Moreover, FAP can be

induced to localize to invadopodia by ligation of α1β6 integrin with laminin peptides (Nakahara *et al.*, 1996). In addition to degrading the matrix, FAP is associated with cell motility. Recently, it was shown that UV light can induce FAP expression in melanoma cells and in dermal fibroblasts and, thereby, promote melanoma invasion and metastasis (Waster *et al.*, 2011).

In summary, FAP is expressed in the tumor microenvironment of at least 10 different types of cancer. The widespread association of FAP expression with cancer suggests it has important functions in the disease. However, the nature of FAP's roles in cancer is still being determined.

3.2. FAP in fibrosis

Liver

FAP is not expressed by resting hepatic stellate cells (HSC). In chronic liver disease, however, HSC are activated and trans-differentiate into myofibroblasts that express FAP, αSMA and glial fibrillary acidic protein (GFAP) (Levy *et al.*, 2002). Activated HSC produce and degrade ECM (Levy et al., 1999; McCaughan *et al.*, 2000). Histology shows that the severity of fibrosis in chronic liver disease and hepatitis C infection correlates with increasing intensity of FAP immunostaining (Gorrell, 2005; Levy *et al.*, 2002). FAP expression is seen at the tissue remodeling interface in chronically injured liver. Moreover, FAP contributes to ECM changes in cirrhosis. FAP expression is localized to individual cells within the peri-sinusodial spaces rather than the ECM

structure (Gorrell, 2005). FAP overexpression in HSC causes increased cell adhesion, apoptosis, migration on ECM proteins, and invasion across transwells, with none of these functions requiring the enzymatic activity of FAP (Gorrell *et al.*, 2003).

Lung

Idiopathic pulmonary fibrosis (IPF) is categorized by aggressive fibrosis of the interstitium. FAP is expressed by fibroblasts in the fibrotic foci of the interstitium and is restricted to areas of active stroma where there is ongoing injury. IHC does not show FAP expression in normal lung tissue or in centriacinar emphysema tissue. FAP may contribute to maintaining a microenvironment with persistent activation of fibroblasts making it susceptible to epithelial injury (Acharya *et al.*, 2006).

Crohn's disease

A frequent complication of Crohn's disease of the colon is fibrosis and stricture formation that can require surgery. High levels of FAP expression has been noted in the fibrotic strictures of Crohn's that include the submucosa and muscle layers of the afflicted areas (Rovedatti *et al.*, 2011).

3.3 FAP in arthritis

Arthritis

Osteoarthritis (OA) patients show a significant elevation in FAP expression in their cartilage (Milner *et al.*, 2006), which suggests that FAP may play a supporting role in

cartilage degradation. FAP is expressed on chondrocytes in the cartilage tissue and initial degradation is observed around chondrocytes (Milner *et al.*, 2006). FAP expression is considerably higher in cartilage from OA patients compared to normal articular cartilage (Milner *et al.*, 2006).

In rheumatoid arthritis (RA) FAP is expressed by synovial fibroblasts (Bauer *et al.*, 2006). In RA, FAP is localized in the joint lining layer and correlates with accumulation of MMP-1 and MMP-13, an accumulation which exceeds that observed in the joints of OA patients (Bauer *et al.*, 2006). The level of FAP is much higher in RA tissue and correlates with the degree of synovial inflammation. The inhibitor L-Glutamyl-L-boroProline was used to inhibit FAP and DPPIV and resulted in increased invasion of cartilage by synovial fibroblasts in a mouse model of human rheumatoid arthritis (Ospelt *et al.*, 2010). The FAP and DPPIV inhibition also led to elevated SDF-1 (a DPPIV substrate) MMP-1, and MMP-13, which are downstream effectors of SDF-1 (Ospelt *et al.*, 2010). The authors concluded that FAP and DPPIV play a protective role in RA. We now know that FAP does not cleave SDF-1 (Keane *et al.*, 2011) and therefore the protection may be afforded primarily by DPPIV.

3.4 FAP in other pathologies

Heart

In human thin cap coronary atheromata, FAP is expressed by human aortic smooth muscle cells (HASMC) and not by macrophages (Brokopp *et al.*, 2011). However, FAP expression by HASMC is induced by TNF-α derived from macrophages, and levels of

FAP expression correlate with degree of macrophage infiltrate. FAP is linked to the plaque progression and fibrous cap thinning. Once FAP is expressed, it cleaves collagen in the fibrous caps of the human fibroatheromata. Cleavage of this type I collagen is inhibited by antibodies that neutralize FAP collagenolytic activity, indicating a role for FAP in degrading collagen within thin cap fibroatheromata (Brokopp *et al.*, 2011). Collagen breakdown by FAP is thought to render coronary fibroatheromata more prone to rupture.

Keloid

Keloids are fibroproliferative dermal tumors that develop as a result of dysregulated wound healing and are more frequently observed in black populations (Seifert *et al.*, 2008). Scar formation induces FAP expression during dermal incision (O'Brien and O'Connor, 2008). Keloid progression into surrounding healthy skin is aided by FAP and DPPIV expression in keloid fibroblasts, as shown using an irreversible inhibitor N-Gly-Pro diphenylphosphonate (Dienus *et al.*, 2010). Thus FAP and DPPIV promote the invasive growth of keloid fibroblasts (Dienus *et al.*, 2010). Interestingly, FAP expression is up-regulated 8 fold in the deeper part of the keloid, 7 fold in the superficial active part of the keloid and 4.8 fold in the active erythematous part of the keloid as compared to healthy skin (Seifert *et al.*, 2008).

4. Coordination of stromal responses in cancer

4.1 The activated fibroblasts

The stromal response to cancer is increasingly recognized as a critical element in the progression of cancer. In this regard, reactive stromal fibroblasts or cancer associated fibroblasts (CAFs) have generated much interest (De Wever *et al.*, 2008; Kalluri and Zeisberg, 2006; Orimo *et al.*, 2005; Orimo and Weinberg, 2006; Rasanen and Vaheri, 2010). Normally fibroblasts are relatively inactive in terms of protein synthesis and in histologic sections they appear as thin spindle shaped cells with heterochromatic nuclei that do not express FAP. However, fibroblasts become activated in response to epithelial cancers, wounding, and chronic inflammation. Activated fibroblasts synthesize and secrete proteins, are larger in size than inactivated fibroblasts, have enlarged euchromatic nuclei, and express FAP. Activated fibroblasts secrete proteins that promote invasion of cancer cells, activate endothelial cells and pericytes to promote angiogenesis, and regulate immune cell response to the tumor (Kalluri and Zeisberg, 2006; Rasanen and Vaheri, 2010).

The mechanism of fibroblast activation in cancer is not completely understood. Indeed even the origins of activated fibroblasts in tumors are incompletely described. Currently it is believed that the majority of CAFs arise from the activation of resident fibroblasts. However, activated fibroblasts can also originate from pericytes, vascular smooth muscle cells, bone marrow-derived mesenchymal cells, and from epithelial to mesenchymal cell transition (EMT) (Fig. 1). It is estimated that about 20%-25% of CAFs arise from mesenchymal stem cells in inflammation induced gastric cancer and in pancreatic cancer (Direkze *et al.*, 2004; Direkze *et al.*, 2006; Quante *et al.*, 2011). In adult tissues, differentiation from resident stromal fibroblasts into activated

myofibroblasts occurs through paracrine signaling with TGF-β generated by damaged or inflamed tissues (De Wever et al., 2008). Such TGF-β mediated activation of CAFs may occur in tumors. Both TGF-β and IL-1β induce differentiation of quiescent fibroblasts into activated myofibroblasts, but TGF-β is considered the predominate inducer (Chen *et al.*, 2009; Denys *et al.*, 2008). TGF-β is also a powerful factor inducing FAP expression in NIH3T3 fibroblasts (Chen *et al.*, 2009). The induction of fibroblasts and FAP by TGF-β is thought to convert fibroblasts to a proinvasive state (Denys et al., 2008). TGF-β has a key role in fibrosis, a pathologic condition characterized by activated fibroblasts and over-production of collagen. TGF-β causes a profibrotic response featuring non-SMAD3 mediated up-regulation of the early growth response -1 (EGR-1) transcription factor, increased type I collagen synthesis and myofibroblast differentiation (Bhattacharyya *et al.*, 2009; Chen *et al.*, 2006). Importantly, the FAP promoter has an EGR-1 binding site, and EGR-1 binding is important for FAP expression (Zhang *et al.*, 2010). Thus FAP may be induced in resident tissue fibroblasts that are activated by TGF-β mobilized from the matrix and/or by produced by the epithelial cancer cells (Fig. 2). Another possible mechanism for activation of fibroblasts and elevated FAP expression is epithelial to mesenchymal transition (EMT). In this regard it has been shown that in gliobastoma expression of the transcription factor TWIST1 causes an EMT that features elevated expression of FAP (Mikheeva *et al.*, 2010). TWIST is well known to produce EMT in breast cancer cells (Yang *et al.*, 2004). Thus it will be important to learn whether this entails induction of FAP expression as well.

In terms of the microenvironment in breast cancer, TGF-β apparently plays a critical role in supporting malignant growth through action on the stromal fibroblasts. TGF-β normally opposes growth and favors apoptosis of breast epithelial cells, but malignant cells may overcome negative TGF-β signals in a number of ways. These include crosstalk with other signaling pathways, such as pathways affected by estrogen receptor (Band and Laiho, 2011) and HER-2 (Wang, 2011). When epithelial cancer cells become resistant to growth arrest and apoptosis mediated by TGF-β, they continue to grow in the presence of TGF-β and the CAFs continue to be growth stimulated by TGF-β. This in turn leads to a stimulation of breast cancer cell invasion and metastasis to lung and to bone (Drabsch and Ten Dijke, 2011) in large part driven by stromal stimulation. It is not yet clear how FAP fits into the TGF-β-mediated activation of fibroblasts. A role for FAP in modification and organization of newly synthesized matrix seems likely. FAP may also be a key player in fibroblast motility that promotes fibroblast movement about the area where matrix is being created.

4.2 FAP effects on cell motility, adhesion and invasion

Expression of FAP on the cell surface has dramatic effects on the motility, matrix degradation, and invasive behavior of cells. Chen and co-workers pioneered identification of invadopodia, the membrane protrusions of invasive cells that contact and degrade extracellular matrix (Chen, 1989) [for reviews on invadopodia see (Chen and Kelly, 2003; Linder, 2007; Mueller, 2008)]. Chen's group went on to show that MMP-2, MT1-MMP and TIMP2 are localized to invadopodia (Chen and Wang, 1999;

Monsky *et al.*, 1993). Moreover, they found that FAP is localized to invadopodia and suggested that FAP is a potential marker of invasiveness (Chen *et al.*, 1994; Monsky *et al.*, 1994). Thus, invadopodia are sites where proteases are concentrated within the plasma membrane and these structures are capable of degrading multiple extracellular matrix components (Kelly et al., 1994). Chen and co-workers provided evidence that FAP-DPPIV complexes are responsible for the invasive phenotype of fibroblasts, endothelial cells, and ovarian cancer cells migrating into type I collagen gels (Ghersi et al., 2002; Ghersi et al., 2006; Kennedy *et al.*, 2009). Indeed, this work implies a two way communication between the collagenous matrix and the tumor cells as proposed some time ago (Bissell and Aggeler, 1987; Bissell *et al.*, 1982). FAP, through its ability to cleave type I collagen and gelatin, likely has an important role in the cell side of the communication that stimulates cell movement. FAP expression or lack of expression also alters the extracellular matrix, both in composition and organization (Lee *et al.*, 2011a; Santos *et al.*, 2009). FAP depletion caused dramatically increased accumulation of type I collagen in the stroma of lung tumors formed in FAP null animals (Santos *et al.*, 2009). This aberrant accumulation of type I collagen could impair cell motility, growth and tumor angiogenesis (Santos *et al.*, 2009). Recently it has been shown that FAP expression by fibroblasts results in alterations of organization and composition of the extracellular matrix that favors invasion (Lee *et al.*, 2011a). Furthermore, the protease activity of FAP increases the proteolytic degradation of matrix by FAP-expressing cells (Huang *et al.*, 2011b). The majority of the proteolytic degradation; however, is carried out by matrix metallproteinases (Kelly *et al.*, 1998b). In vitro, FAP positive fibroblasts produce a more organized matrix with more type I

collagen and fibronectin but less tenacin C than their FAP null counterparts (Lee *et al.*, 2011a). In addition, the matrix produced by the FAP positive cells promoted tumor cell motility and directed migration. But it is important to note that these effects were partly due to the protease activity of FAP, as they were attenuated by matrices produced by FAP positive cells in the presence of a FAP protease inhibitor (Lee *et al.*, 2011a). This finding suggests that FAP functions on activated fibroblasts to produce matrices with structures conducive for infiltration of new blood vessels and tumor cells. This activated matrix likely requires both FAP signaling and FAP protease activities for maximum effect.

4.3 FAP in angiogenesis

FAP has been consistently associated with angiogenesis. An early RT-PCR screen for serine proteases by Quigley's group identified FAP expression in human endothelial cells (Aimes *et al.*, 2003). This was subsequently confirmed by others using a gene expression profiling approach (Ghilardi *et al.*, 2008). FAP was found to be highly expressed in the endothelial cells and not on tumor cells cultured from human ovarian carcinoma (Ghilardi *et al.*, 2008). Kelly's group was first to identify increased microvessel densities associated with FAP expression using an animal model of human breast cancer (Huang *et al.*, 2004). They reported three fold higher microvessel densities in tumors formed by human breast cancer cells engineered to express FAP as compared to tumors formed by control cells with low FAP expression (Huang *et al.*, 2004). Consistent with these findings, Pure's group showed that genetic deletion or pharmacologic inhibition of FAP indirectly decreased tumor growth by reducing

microvessel density in animal models of lung cancer (Santos *et al.*, 2009). In this work, the FAP protease activity was important for the increased microvessel densities (Santos et al., 2009). However, in a xenograft model of human breast cancer, pharmacologic inhibition of FAP did not impede tumor growth, and expression of a catalytically inactive mutant FAP drove tumor growth (Huang *et al.*, 2011b) and elevated microvessel densities as efficiently as expression of a wild type and active FAP. Indeed, the tumors of MDA MB-231 breast cancer cells expressing the S624A mutant FAP have elevated microvessel density (151.8 vessels/mm^2 SD ±39.06, Huang and Kelly unpublished observations) that is statistically identical to the increased microvessel densities observed for tumors of these cells that express wild type and catalytically active FAP and significantly higher than that observed in tumors of control transfectants (Huang *et al.*, 2004). Thus, while the protease activity of FAP may compliment the pro-angiogenic effect, it is likely that expression of FAP on the cell surface alters cell signaling such that pro-angiogenic factors are elevated. For example, MMP-9 expression is elevated upon FAP expression in breast cancer cells and expression of FAP (Huang *et al.*, 2011b). The fact that FAP alters cell signaling has been shown by the apparent suppression of FAK and ERK phosphorylation in the presence of active FAP and the increased phosphorylation of FAK and ERK and expression of p21 in the absence of FAP or pharmacologic inhibition of the FAP protease (Santos et al., 2009). Clearly, FAP is participating in angiogenesis evoked by tumors but the precise nature of this response is not yet known. One way that the FAP protease may increase angiogenesis is through cleavage of neuropeptide Y (NPY). FAP dipeptidyl peptidase function cleaves neuropeptide Y to the NPY_{3-36} (Keane *et al.*, 2011). This cleavage stops signaling on

endothelial cells through the Y1 receptor but favors activation of the Y2 receptor which binds NPY_{3-36} (Zukowska *et al.*, 2003). The activated Y2 receptor stimulates proliferation of endothelial cells and thus can function as a vascular growth factor (Zukowska *et al.*, 2003).

Another way that FAP may encourage angiogenesis is through its role in assembling and arranging type I collagen matrices. As mentioned above, FAP-expressing fibroblasts promote assembly of a matrix that promotes tumor cell invasion (Lee *et al.*, 2011a). It is easy to envision such an invasion-stimulating matrix providing a pathway for endothelial cells to migrate and a substrate on which to form tubes that will become blood vessels. Consistent with this is the finding that lung tumors formed in FAP -/- animals exhibited aberrantly high collagen deposition that was poorly organized (Santos *et al.*, 2009). Such a poorly organized mass of collagen might inhibit migration. It is possible that a major function of FAP expressing CAFs is to produce a type I collagen rich matrix that promotes migration of fibroblasts and endothelial cells.

4.4 FAP in tumor immunity

Mounting evidence indicates that FAP-positive cells have a role in immune suppression, inhibiting anti-tumor responses by the immune system. Ablation of FAP positive cells has been shown to stimulate the anti-tumor activity of the immune system in animal models (Kraman *et al.*, 2010; Liao *et al.*, 2009). One study demonstrated that a DNA vaccine to FAP caused CD8+ T cells to kill FAP positive CAFs (Loeffler *et al.*, 2006). This vaccine led to decreased tumor growth, reduced levels of collagen in the tumors,

and increased sensitivity of tumor cells to chemotherapeutics (Loeffler *et al.*, 2006). Perhaps the reduced collagen allows better uptake of chemotherapeutics (Loeffler *et al.*, 2006). Destruction of the FAP positive cells could not only improve anti-tumor immune function but also increase efficacy of existing chemotherapeutics. Ablation of FAP-expressing cells in animals carrying Lewis Lung carcinoma cell tumors or pancreatic ductal carcinomas revealed that FAP-expressing cells had a non redundant immune suppressive function in the microenvironment (Kraman *et al.*, 2010). The FAP positive cells are primarily CAFs and only comprise about 2% of the tumor cells. Nevertheless, when FAP expressing cells were ablated there was rapid necrosis of cancer and stromal cells (Kraman et al., 2010). Similarly, in the 4T1 breast cancer cell-xenograft model of murine breast cancer, elimination of CAFs *in vivo* by a DNA vaccine targeted to FAP resulted in a shift of the immune microenvironment from a Th2 cytokine polarization to Th1 polarization. The Th2 polarization generally prevents tumor rejection and promotes tumor growth and is often observed in solid tumors. The Th1 cytokine polarization of the immune system is associated with cell mediated immunity and tumor rejection through the action cytotoxic T lymphocytes (Liao *et al.*, 2009). The microenvironment change resulting from DNA vaccine to FAP is characteristic of the Th1 response and features increased IL-2 and IL-7 proteins, decreased macrophages, myeloid-derived suppressers, T regulatory cells, and decreased tumor angiogenesis (Liao *et al.*, 2009). Reisfeld and co-workers showed that DNA vaccine to FAP improved the anti-tumor effects of doxorubicin by providing additional anti-tumor immune benefits and that a vaccine against FAP improved additional suppression of pro-angiogenic agents (Liao *et al.*, 2009).

Using a different approach, FAP was established as a tumor associated antigen. Du and co-workers chemically coupled 1-methyl-tryptophan to a portion of the β-propeller domain of murine FAP producing FAPт-MT (Yi *et al.*, 2011). The rationale for this approach is that the FAP portion would target uptake into antigen presenting cells within the tumor and in the process internalize 1-methyl-tryptophan, an inhibitor of the indolamine 2,3-dioxygenase in the antigen presenting cells (Yi *et al.*, 2011). It is believed that indolamine 2,3-dioxygenase contributes to tumor immune tolerance and thus inhibiting this enzyme might break tolerance and alleviate immune suppression in the tumor microenvironment. In fact, vaccination with FAPт-MT in Freund's incomplete adjuvant at several sites on the back of mice bearing tumors of 4T1 breast cancer cells was able to suppress tumor growth, increase survival, and elicit CD8+ T cell response (Yi *et al.*, 2011).

5. Clinical Potential of FAP.

The role of FAP and FAP-expressing cells in angiogenesis and immune suppression suggests strategies for targeting FAP as part of stroma-based therapeutic approaches to cancer. This section reviews what FAP-based treatments have been attempted clinically and describes some new approaches that are still in preclinical testing.

5.1 Small molecule inhibitors of FAP proteolytic activity

As mentioned above, FAP expression in stromal fibroblasts is associated with aggressive disease in colon cancer (Henry *et al.*, 2007). Therefore, inhibition of FAP proteolytic activity in colon cancer is logical for investigating the therapeutic potential of FAP inhibitors. A phase II clinical trial was conducted in patients with metastatic colon cancer using Val-boroPro to inhibit FAP proteolytic activity (Narra *et al.*, 2007). This study achieved significant but incomplete inhibition of FAP protease activity in the peripheral blood; unfortunately, there was minimal clinical activity in patients with previously treated colorectal cancer and these trials have been suspended (Narra *et al.*, 2007). Another phase II trial was performed in patients with non small cell lung cancer where Val-boroPro was given in conjunction with docetaxel. The hope was that Val-boroPro would stimulate the immune system and improve the docetaxel response. Eager et al. found no evidence that Val-boroPro increased the clinical activity of docetaxel (Eager *et al.*, 2009a). A similar phase II study was conducted for melanoma patients where Val-boroPro was given in conjunction with cisplatin. Again, no additional clinical response was observed with addition of Val-boroPro to the cisplatin regime (Eager *et al.*, 2009b). If FAP is important in promoting tumor growth, then why was no clinical response observed with inhibition of the FAP protease? There are several potential reasons: i) the Val-boroPro may have cyclized, as it is known to do, and become ineffective at the tumor site (Kelly *et al.*, 1993); ii) FAP mediated signaling independent of the protease activity drove tumor growth; or iii) the role of the FAP protease was compensated for by another enzyme. New and highly specific inhibitors of FAP are under development as described earlier (section 1.2) and these may prove more effective in eliciting clinical responses against tumors. Moreover, the pathways

and molecules that mediate FAP-signaling may prove valuable targets for therapeutic agents.

5.2 Antibodies to FAP

Early work investigated the distribution of ^{131}I-labeled F19, a mouse mAb to human FAP, in cancer patients. These studies demonstrated that mAb F19 to FAP could concentrate in colorectal tumor tissues (Welt *et al.*, 1994) and no toxicities were associated with intravenous administration of ^{131}I-labeled murine F19 mAb (Tanswell *et al.*, 2001; Welt *et al.*, 1994). The stromal targeting of this antibody was also confirmed in a mouse model where human skin implanted into immune compromised mice was used to support growth of human breast cancer cells (Tahtis *et al.*, 2003). Further work produced a humanized F19 (Schmidt *et al.*, 2001) and ultimately the production and use of sibrotuzumab, a humanized version of F19 antibody to FAP, in a dose escalation study (Scott *et al.*, 2003). This study, like those done before with F19, indicated that the sibrotuzumab is relatively non-toxic and that it is preferentially taken up in tumor tissues and not healthy organs (Scott *et al.*, 2003). Pharmacokinetic studies with this antibody showed that body weight affected the distribution of sibrotuzumab (Kloft *et al.*, 2004). Ultimately, unconjugated sibrotuzumab did not achieve a clinical response in a phase II trial of patients with metastatic colorectal cancer and the trial was discontinued (Hofheinz *et al.*, 2003). Apparently binding the unconjugated sibrotuzumab to tumor cells in the stroma did not cause enough antibody dependent cell cytolysis to register a clinical response. However, the fact that these antibodies reached the tumor tissues

suggests that attaching a cytotoxic agent might improve killing and elicit a clinical response. If antibodies are to be effective, the challenge will be to attach inactive pro- or caged forms of the cytotoxic agents that are specifically released upon binding to FAP. Such an approach would avoid off target cell killing because the FAP itself might release the pro-drug.

5.3 FAP release of prodrugs

The use of pro-drugs extends the overall strategy of targeting FAP expressing cells. Peptides that are specifically cleaved by FAP can be used to develop pro-drugs that take advantage of FAP expression at the pathology site to kill tumor cells (Lebeau *et al.*, 2009). Melittin is the main toxin of the European honeybee and is produced as pro-melittin, which contains a 22 amino acid N-terminal portion that is removed in the venom glands by a DPPIV-like enzyme to become the cell lytic mature melittin. Lebeau et al. reduced the size of the pro-domain and introduced a FAP-specific cleavage site. They showed *in vitro* that the modified pro-melittin selectively killed FAP expressing cells, but did not have appreciable activity against cells that didn't express FAP (Lebeau *et al.*, 2009). Moreover, antitumor activity of the FAP-cleavable pro-melittin was demonstrated in xenografts models of prostate cancer and breast cancer, where FAP-expressing reactive stroma occurs (Lebeau *et al.*, 2009). These studies show the promise of FAP-targeted pro-drugs. However the study relied on intra-tumoral injection for drug delivery which may not always be possible for anti-tumor drug delivery in patients.

Fluorescence release can be used to report protease activities. Lai et al developed Ac-GPGP-2SBPO and GPGP-2SBPO as reporters of FAP and DPPIV activities (Lai *et al.*, 2007). Molecular beacons based on the FAP cleavage sequence TSGP-NQEQK of α_2AP were used to demonstrate FAP activity in human and mouse models and might be useful for epithelial cancer detection and treatment (Lo *et al.*, 2009).

Another pro-drug strategy involves the use of a specific protease-targeted pro-domain that must be cleaved to expose an active protease inhibitor. This approach can preserve the inhibitory activity of the compound, so that it is only activated and reactive with the target protease. This is particularly relevant to Val-boroPro which rapidly cyclizes at physiologic pH, which attenuates its inhibitory activity 100 fold (Kelly *et al.*, 1993). Compounds designed for this approach are called “pro-soft”. The hope is not only to preserve activity, but also to reduce off-target and toxic side effects. The pro-soft compound cyclohexyl(glycine)-Pro-Val-boroPro (Chg-Pro-Val-boroPro) was used to show proof of principle (Poplawski *et al.*, 2011). Chg-Pro-Val-boroPro is specifically cleaved and activated by DPPIV, after which it exhibits long-lasting DPPIV inhibition with reduced toxicity in rats, as compared to Val-boroPro (Poplawski *et al.*, 2011). This strategy could be exploited to design a target portion of pro-soft Val-boroPro, Gly-boroPro or other dipeptide boronic acid derivative that makes it specific to FAP.

6. Conclusions and future directions

In the past few years much new information on aspects of FAP biology have begun to clarify its mechanisms of induction, substrate specificities of it proteolytic activities, activation of signaling pathways, and roles in cell migration and angiogenesis. The finding that FAP expressing cells are important suppressors of the anti-tumor immune response was an unexpected finding that holds much clinical promise. Studies have indicated that FAP is involved in a numerous different pathologies, including many cancers, rheumatoid arthritis, osteoarthritis, atherosclerosis, fibrosis and keloids. The striking occurrence of FAP in these pathologic states (but not in normal tissues) continues to suggest FAP is a good target for therapeutic agents and implies an important role for FAP in some or all of these clinical conditions. But, much work still remains to be done on the actual role of FAP in these various pathologic processes. FAP is associated with assembly of the extracellular matrix and, specifically, a matrix that permits cell migration. This cell migration could include movements of endothelial cells, fibroblasts, immune effecter cells, and invading tumor cells. The precise roles of FAP in modifying type I collagen and perhaps other proteins during assembly of the extracellular matrix is an area of great interest. Disruption of matrix assembly could impede both tumor invasion and tumor angiogenesis and, thereby, be manipulated to contain the tumor and inhibit its growth. This suggests new matrix-targeted therapies may be possible.

Two strategies for treating epithelial cancers, inhibition of FAP by small molecules and decorating FAP expressing cells with antibodies have little success to date in clinical trials. This might be due in part to the FAP signaling that compliments the protease

activity together with possible poor access of the antibody or low activity of the inhibitors tested to date. Most of the preclinical studies, and virtually all of the clinical studies, using small molecule inhibitors of FAP utilized Val-boroPro. This inhibitor is not specific to FAP and cyclizes at physiologic pH, which reduces its inhibitory activity by 100 fold. Therefore, other more specific FAP inhibitors may have a greater effect on tumor growth. Similarly, the antibody work has focused on F19 or its humanized form, sibrotuzumab. These antibodies were successful at concentrating in the tumors, but will require conjugation to some toxin or pro-toxin to kill cells and reduce the size of tumors.

Using FAP as a target for therapies that poison the CAFs and/or tumor cells may be the most efficacious use of FAP as a treatment modality. With a consensus sequence for the FAP protease activity in hand, design of a pro-melittin that requires proteolytic modification by FAP for toxic activity was accomplished. A study with this FAP-targeted pro-drug provided proof of principle that such a strategy could work to kill tumor cells and shrink tumors. However, it is not clear from this study if normal tissues would be spared were the FAP-targeted pro-drug given intravenously. Perhaps a future strategy can be devised in which a toxin that requires activation by the FAP protease is attached to FAP-specific antibodies to take full advantage of the target specificity provided by FAP to deliver and activate therapeutic agents. Moreover, imaging modalities that take advantage of FAP cleavage to activate fluorescent reporter molecules might be useful for monitoring FAP activity in living cells and monitoring tumor cell clearance.

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Figure Legends

Figure 1. Possible mechanisms of fibroblast activation in breast cancer. From top left: Epithelial cancer cells produce TGF-β that acts on resident fibroblasts causing increased expression of EGR-1. EGR-1 causes increased expression of FAP and type I collagen in CAFs. From top right, some of the CAFs are derived from EMT. EMT can be driven by the TWIST transcription factor and TWIST can also cause increased expression of FAP. Both mechanisms occur and emphasize the role of EGR-1 in the process.

Figure 2. Schematic diagram of reported signaling that impacts fibroblast activation and FAP expression. Figure is based on references cited in the text.

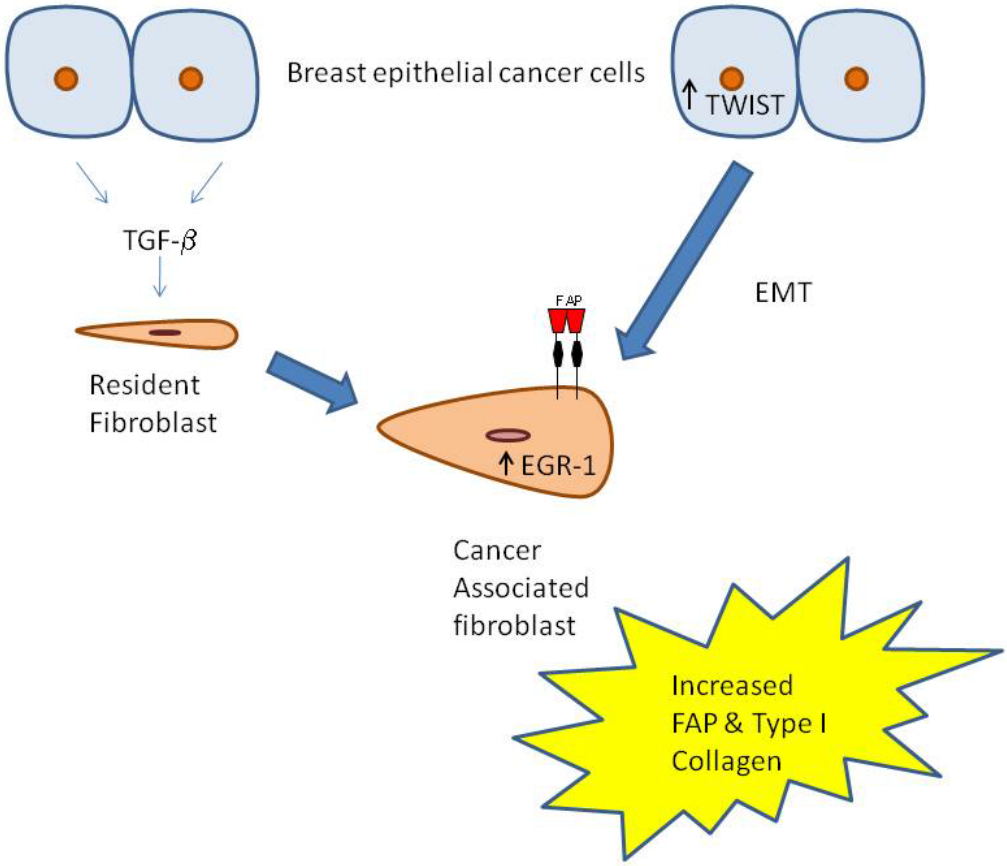
Breast epithelial cancer cells
TWIST
TGF-β
EMT
FAP
Resident
Fibroblast
EGR-1
Cancer
Associated
fibroblast
Increased
FAP & Type I
Collagen

Figure 1.

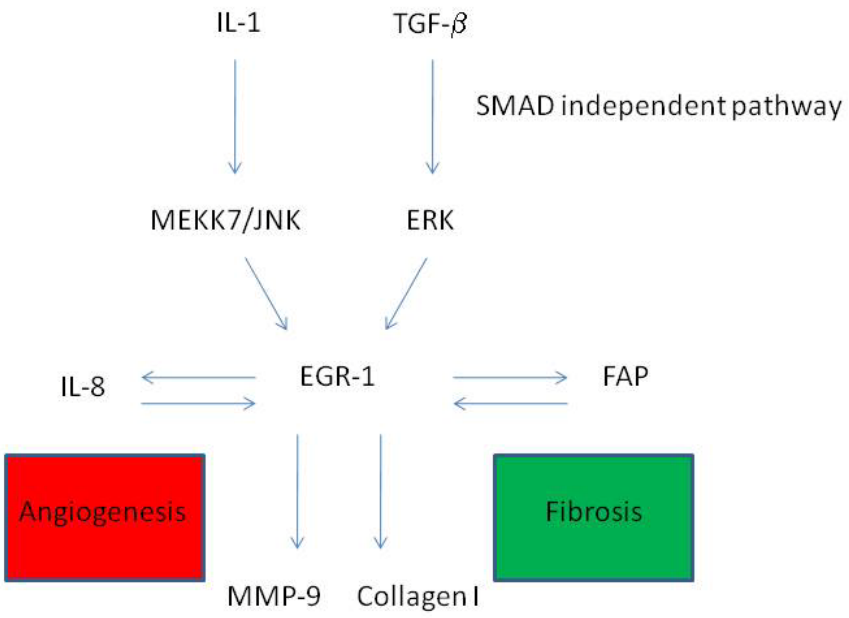
IL-1
TGF-β
SMAD independent pathway
MEKK7/JNK
ERK
IL-8
EGR-1
FAP
Angiogenesis
Fibrosis
MMP-9
Collagen I

Figure 2.