Review

Molecular Cancer Therapeutics

Rationale Behind Targeting Fibroblast Activation Protein–Expressing Carcinoma-Associated Fibroblasts as a Novel Chemotherapeutic Strategy

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Abstract

The tumor microenvironment has emerged as a novel chemotherapeutic strategy in the treatment of cancer. This is most clearly exemplified by the antiangiogenesis class of compounds. Therapeutic strategies that target fibroblasts within the tumor stroma offer another treatment option. However, despite promising data obtained in preclinical models, such strategies have not been widely used in the clinical setting, largely due to a lack of effective treatments that specifically target this population of cells. The identification of fibroblast activation protein α (FAP) as a target selectively expressed on fibroblasts within the tumor stroma or on carcinoma-associated fibroblasts led to intensive efforts to exploit this novel cellular target for clinical benefit. FAP is a membrane-bound serine protease of the prolyl oligopeptidase family with unique post-prolyl endopeptidase activity. Until recently, the majority of FAP-based therapeutic approaches focused on the development of small-molecule inhibitors of enzymatic activity. Evidence suggests, however, that FAP's pathophysiological role in carcinogenesis may be highly contextual, depending on both the exact nature of the tumor microenvironment present and the cancer type in question to determine its tumor-promoting or tumor-suppressing phenotype. As an alternative strategy, we are taking advantage of FAP's restricted expression and unique substrate preferences to develop a FAP-activated prodrug to target the activation of a cytotoxic compound within the tumor stroma. Of note, this strategy would be effective independently of FAP's role in tumor progression because its therapeutic benefit would rely on FAP's localization and activity within the tumor microenvironment rather than strictly on inhibition of its function. *Mol Cancer Ther*; *11(2)*; *257–66*. *©2012 AACR.*

Introduction

There is an increasing awareness of the necessity to understand a tumor within the context of its surroundings, i.e., the tumor microenvironment. Investigations that take into consideration the complex network of interactions and regulatory signals that exist between the stroma and tumor itself have become essential for the full elucidation of both oncogenesis and tumor progression. The stroma associated with a tumor commonly contributes a significant portion of the mass of many malignancies, and it can account for >90% of the tumor mass in carcinomas characterized by a desmoplastic reaction, such as breast, colon, and pancreatic carcinomas (1). It is well documented that the tumor is dependent on the reactive stroma for survival and growth signals, as well as the nutritional support required for maintenance of the primary mass. Additionally, the ability of the stroma to not only contribute to but also potentially drive the progression of cancerous cells into a highly aggressive and metastatic phenotype has only recently begun to be truly appreciated (2, 3), even though the first observations linking nonmalignant cells of the tumor microenvironment to tumorigenesis were made more than a century ago.

The stroma has been shown to undergo morphological alterations; recruit reactive fibroblasts, macrophages, and lymphocytes; increase secretion of growth factors and proteases; induce angiogenesis; and produce an altered extracellular matrix (ECM) when associated with a transformed epithelium (4). The tumor and its microenvironment exist in a dynamic and interconnected network of reciprocal interactions that can influence such varied processes as proliferation, migration, invasion, survival, and angiogenesis, to name a few. These effects are mediated through both paracrine and autocrine stimulation by a variety of growth factors and cytokines, including transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), VEGF, platelet-derived growth factor (PDGF), and interleukins [IL (4)]. These growth factors can be liberated from the ECM through the action of proteases, such as the matrix metalloproteinases (MMP), in addition to being secreted from cancer cells

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and activated fibroblasts. The presence of these growth factors, together with the remodeling of the ECM and induction of neovascularization, leads to a tumor microenvironment that is conducive to the growth, progression, and eventual metastasis of the tumor and has been termed a "reactive" stroma. The induction of a desmoplastic or reactive stroma is associated with a poor prognosis in multiple carcinomas, including breast, pancreatic, and colorectal cancers (5–7).

Fibroblasts in particular have been shown to consistently undergo several changes in both morphology and expression profiles when present in the tumor microenvironment (8). Indeed, the presence of activated fibroblasts that have acquired a myofibroblast-like phenotype within the tumor microenvironment serves as a primary indicator of reactive stroma formation (4). Evidence suggests that these activated fibroblasts, also known as carcinoma-associated fibroblasts (CAF), are central to regulating the dynamic and reciprocal interactions that occur among the malignant epithelial cells, the ECM, and the numerous noncancerous cells that are frequently found within this tumor milieu, including endothelial, adipose, inflammatory, and immune cells (Fig. 1; ref. 9).

CAFs have been implicated in nearly all stages of oncogenesis, from initiation through progression to metastasis, and have been shown to enhance epithelial cell growth, tumorigenicity, angiogenesis, and the metastatic potential

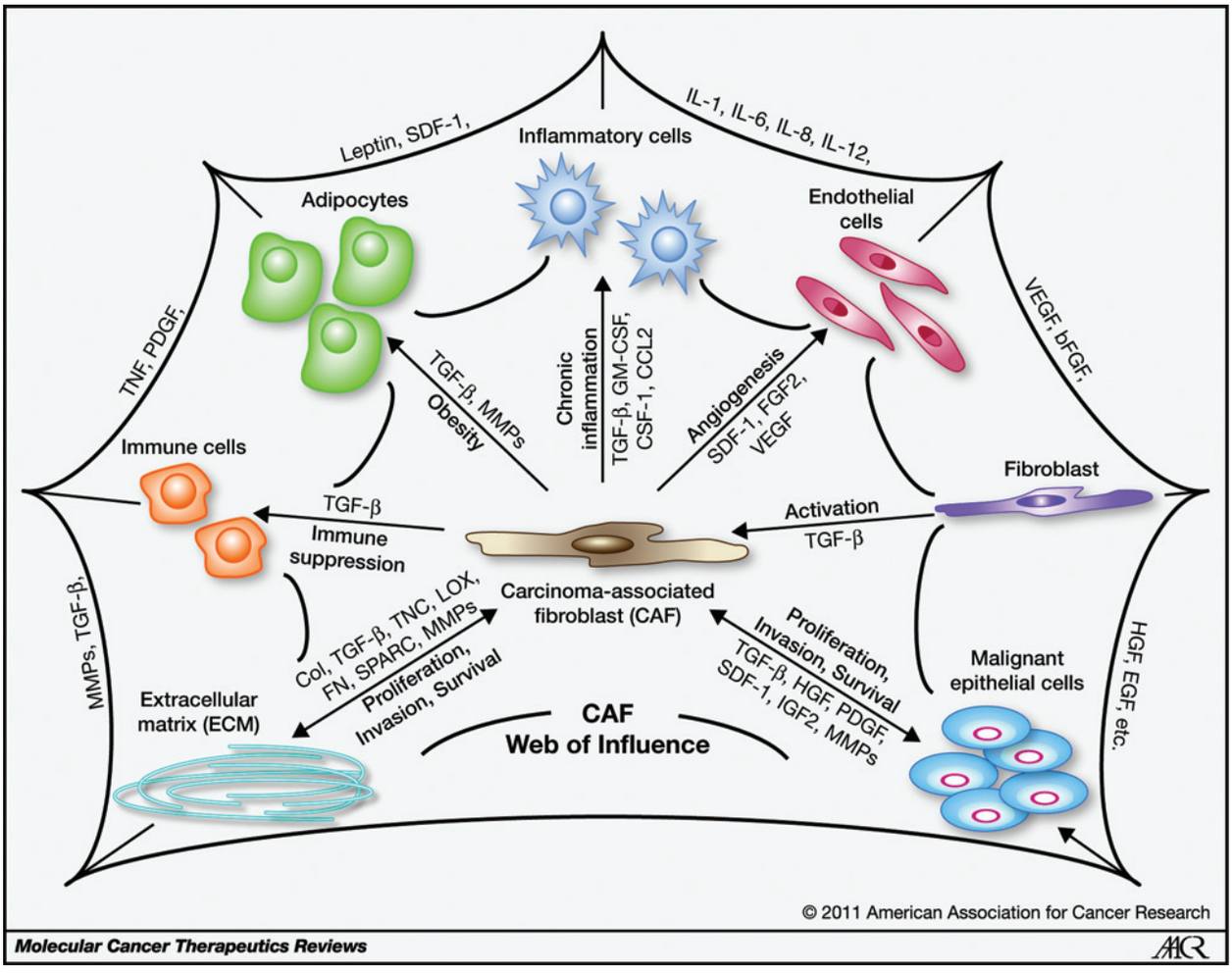

Figure 1. CAFs can promote tumorigenesis directly through multiple mechanisms, including increased angiogenesis, proliferation, invasion, and inhibition of tumor cell death. These effects are mediated through the expression and secretion of numerous growth factors, cytokines, proteases, and extracellular matrix proteins, such as SDF-1, FGF2, VEGF, TGF-β, HGF, tenascin-c, LOX, and the MMPs. CAFs can additionally influence tumorigenesis indirectly through effects on a multitude of other cell types, including adipocytes and inflammatory and immune cells. Furthermore, paracrine signals (examples listed around the perimeter of the web) derived from these accessory cells feed back to promote tumor growth. Ac, acetyl; AFC, 7-amino-4-(trifluoromethyl) coumarin; bFGF, basic fibroblast growth factor; CCL2, chemokine (C-C motif) ligand 2; Col, collagen; DPP-II (IV, 6, 7, 8, 9, 10), dipeptidyl peptidase-II (IV, 6, 7, 8, 9, 10); FN, fibronectin; GM-CSF, granulocyte macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IGF2, insulin-like growth factor 2; LOX, lysyl oxidase; SDF-1, stromal cell-derived factor 1; SFRP-1, secreted frizzled-related protein 1; SPARC, secreted protein, acidic and rich in cysteine; TNC, tenascin-c.

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of transformed cells compared with their normal fibroblast counterparts (2, 9). Knockout of the TGF-β type II receptor (TGFβR2) using the fibroblast-specific protein 1 promoter ($Tgfbr2^{fspKO}$) resulted in a loss of TGF-β responsiveness in stromal fibroblasts and led to the development of prostatic intraepithelial neoplasia, a precursor lesion of prostate cancer, in mice (10). CAFs grown with initiated but nontumorigenic human prostatic epithelium in male athymic mice resulted in tumors 500 times larger than controls grown with normal fibroblasts (11). Comparable studies involving the coimplantation of CAFs with a variety of neoplastic cells, including breast, ovary, and pancreas, into immunodeficient mice showed similar increases in tumorigenicity (12–14). Bone marrow–derived mesenchymal stem cells, which are known to localize to malignant tissues where they have the ability to differentiate into CAFs or myofibroblast-like cells, have been shown to enhance the metastatic spread of breast cancer cells up to 7-fold (15). These results clearly suggest a role for CAFs in tumor initiation, progression, and malignancy.

Fibroblast Activation Protein and the Post-Prolyl Peptidase Family

A key characteristic of CAFs is the expression of fibroblast activation protein α [FAP (16, 17)], which was originally identified as an inducible antigen expressed in reactive stroma (16, 18). Subsequently, it was independently identified as a gelatinase expressed by aggressive melanoma cell lines and given the name seprase [for surface expressed protease (19)]. Subsequent cloning revealed that FAP and seprase are the same cell-surface serine protease (17).

FAP is a type II integral membrane serine protease of the prolyl oligopeptidase family (also known as the S9 family), and it is further classified into the dipeptidyl peptidase (DPP) subfamily (S9B), of which dipeptidyl peptidase IV (DPPIV/CD26) is the prototypical member. Enzymes in this class are distinguished by their ability to cleave the Pro-Xaa peptide bond (where Xaa represents any amino acid), and they have been shown to play a role in cancer by modifying bioactive signaling peptides through this enzymatic activity (20). FAP, like all enzymatically active members of the subfamily, is a dipeptidase characterized by its ability to cleave after a proline residue (Table 1; ref. 21). The crystal structure of FAP has confirmed that the enzyme exists as a homodimer and that dimerization is necessary for enzymatic function (22). There is also evidence that FAP can additionally form heterodimers with DPPIV that are localized to invadopodia of migrating fibroblasts (23, 24). Normal, healthy adult tissues have no detectable FAP expression outside areas of tissue remodeling or wound healing; however, FAP-positive cells are observed during embryogenesis in areas of chronic inflammation, arthritis, and fibrosis, as well as in soft tissue and bone sarcomas (23, 25). Additionally, expression of FAP has been detected on mesenchymal stem cells derived from human bone marrow (26, 27).

A soluble form of FAP has been found in both bovine serum (28) and human plasma (29). Currently, the functional significance of this soluble form of FAP, as well as the role of the full-length membrane-bound form, is poorly understood. Even the mechanism leading to FAP's presence in the plasma is not known. Whether FAP's presence in the plasma is the result of shedding from the membrane surface or the biosynthesis of an alternatively spliced isoform is not clear at this point. Despite our poor understanding of how FAP enters the circulation, its presence there raises the possibility of using serum levels of FAP as a biomarker for cancer prognosis. Sequencing has shown that this extracellular, soluble form of FAP found in human plasma is highly homologous to antiplasmin-cleaving enzyme (APCE), which has been shown to cleave α_2-antiplasmin into a form that cross-links to fibrin more efficiently, resulting in greater plasmin inhibition (29). The suggested cleavage site within α_2-antiplasmin is not conserved evolutionarily, which implies that this is probably not the primary function for which FAP originally diverged from DPPIV during a duplication event (30). Neuropeptide Y (NPY), B-type natriuretic peptide (BNP), substance P, and peptide YY (PYY) were

Table 1. Characteristics of known post-prolyl peptidases

Prolyl peptidase	Enzymatic activity	Cellular localization	References
DPPIV	Dipeptidase	Membrane	(25, 36–38)
FAP	Dipeptidase/endopeptidase	Membrane	(25, 36, 38)
DPP6	Inactive	Membrane (K_v^+ channel)	(25, 37)
DPP8	Dipeptidase	Cytoplasm	(25, 37, 38)
DPP9	Dipeptidase	Cytoplasm	(25, 37, 38)
DPP10	Inactive	Membrane (K_v^+ channel)	(25, 37)
AAP	Acylpeptide hydrolase	Cytoplasm	(38, 39)
POP	Prolyl oligopeptidase	Cytoplasm	(38)
DPPII (DPP7)	Dipeptidase	Intracellular vesicles	(37, 38)
PCP	Prolyl carboxypeptidase	Lysosome	(38)

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recently identified as N-terminal dipeptide substrates for FAP *in vitro*, and further investigation into the physiological relevance of these substrates should prove interesting (31).

FAP appears to be conserved among chordates, with especially high homology in many mammals, including primates, rodents, dogs, and ungulates; however, homologs have also been found in zebrafish and 2 amphibian species of the *Xenopus* genus. Both the FAP and DPPIV genes are located on the 2q23 locus. This proximity, coupled with their high degree of homology (48% overall amino acid sequence identity), suggests a common ancestry, and it is believed that FAP evolved from DPPIV via a gene duplication event (30).

The FAP homolog found in the mouse genome [herein termed murine FAP (mFAP)] is expressed on the surface of reactive stromal fibroblasts, and it shares an 89% sequence identity, including the catalytic triad, with the human enzyme (32). FAP expression is observed during mouse embryogenesis in primitive mesenchymal cells in areas undergoing active tissue remodeling (33); however, $FAP^{-/-}$ mice are viable and manifest no apparent developmental defects (34). This lack of phenotype is likely the result of compensation by other proteases. It is also possible, however, that defects in these FAP-null mice may only manifest under the appropriate stressed or pathogenic conditions. Like its human counterpart, mFAP expression is not observed in normal adult murine tissues outside areas of tissue remodeling, such as wound healing (34). Of interest, FAP-null mice have displayed a decreased tumorigenicity, at least in the context of endogenous K-ras^{G12D}-driven lung cancer and syngeneic CT26 colon tumors (35).

In addition to FAP and DPPIV, the prolyl oligopeptidase family includes DPP6, DPP8, DPP9, DPP10, prolyl oligopeptidase [POP, also known as prolyl endopeptidase (PEP)], and acylaminoacyl peptidase [AAP, also known as acylpeptide hydrolase (APH); Table 1; refs. 25, 36–39]. Prolyl carboxypeptidase (PCP) and DPPII (also known as DPP7) of the S28 family are structurally related proteases with similar enzymatic activity that are localized to lysosomes and intracellular vesicles, respectively (Table 1; refs. 25, 36–39). The substrate preferences for many of these post-prolyl peptidases are not entirely known, but similar to DPPIV, most have dipeptidase activity (Table 1). AAP is enzymatically distinct in that it cleaves intracellular *N*-acylated amino acids from the NH_2-terminus of peptides, resulting in a single free *N*-acetyl amino acid as part of the protein catabolism pathway (Table 1; ref. 39). POP is a cytoplasmic protease whose oligopeptidase activity allows it to cleave after internal proline residues in short (<30 aa) peptide sequences (Table 1). This is in contrast to most members of the family, which are limited to exopeptidase activity. DPP6 and DPP10 are inactive due to an amino acid substitution in the catalytic triad, but they were recently found to be critical components of voltage-gated potassium (K_v^+) channels (Table 1; ref. 37). Despite FAP's high homology to DPPIV and the fact that FAP and DPPIV are the only 2 enzymatically active members of the family that are synthesized as integral membrane proteins with extracellular domains, there are distinct differences in their enzymatic properties (Table 1; refs. 25, 40). Unique to FAP among the DPPIV family is its collagen type I-restricted gelatinase activity (41, 42), which classifies it as both an endopeptidase and an exopeptidase.

FAP Expression in the Tumor Microenvironment

In contrast to DPPIV, FAP is not expressed in normal, healthy adult tissues outside of granulation tissue during times of wound repair (40). However, studies showed that in the disease state, FAP expression was detected on the surface of fibroblasts in the stroma surrounding >90% of the epithelial cancers examined, including malignant breast, colorectal, skin, and pancreatic cancers, as well as in some soft tissue and bone sarcomas (16, 18). In a small study, FAP expression was also detected in the stroma of all 7 human prostate cancer specimens examined (43). FAP expression has also been observed on the surface of fibroblasts or pericytes in areas of tumor angiogenesis (23, 35, 44).

To date, FAP expression has been most extensively characterized in breast tissue. In 14 samples analyzed, strong (12/14) to moderate (2/14) expression of FAP was observed in the stroma of human breast carcinomas but not in malignant epithelial cells or adjacent normal tissue (16). Furthermore, minimal or no expression was observed in samples obtained from fibrocystic disease (10/10) or fibroadenomas (2/2) in the same study. In another study, Ariga and colleagues (45) analyzed tissue samples from 112 Japanese women diagnosed with invasive ductal carcinoma of the breast, and they confirmed that FAP expression is exclusively localized to the stroma adjacent to FAP-negative tumor cells but is not present in the stroma of normal tissues. The semiquantitation of FAP levels in these samples showed strong expression in 61 of 112 patients and low levels of expression in the remaining 51 samples. Longer overall and disease-free survival rates were associated with increased FAP expression in that study, and a multivariate analysis showed FAP expression levels to be an independent prognostic factor (45).

In contrast, in a study examining FAP expression in patients with colon cancer, elevated levels were associated with aggressive disease as well as an increased risk of recurrence and metastasis (46). This observation led to multiple phase I and II trials to evaluate FAP as a therapeutic target in the treatment of colorectal cancer (47–49). Additionally, FAP expression has been associated with an overall poorer prognosis in multiple other cancer types, including pancreatic (50), hepatocellular (51), colon (52), ovarian (53), and gastrointestinal carcinomas (54). The mechanisms underlying these seemingly contradictory observations regarding FAP's role in tumorigenesis are still unknown, but they may be related to differences in the tumor microenvironment among different tumor types,

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including variations in the ECM, as well as the immune and inflammatory cell infiltrates present.

It is a well-known phenomenon that fibroblasts and other stromal cells of murine origin constitute the stroma surrounding tumorigenic human cell line xenografts in immunodeficient mice (32). Both murine fibroblasts in the tumor microenvironment and mouse embryonic fibroblasts grown *in vitro* (33) were found to express mFAP transcripts. Similar to human FAP expression patterns, mFAP has not been detected in normal adult mouse tissues. Using a polyclonal antibody produced within their laboratory, Cheng and colleagues (32) showed abundant mFAP expression in the stroma surrounding human HT-29 xenografts. Data from our laboratory, obtained with the same antibody, support these observations and show that murine stromal cells invade human tumor xenografts to various degrees depending on the xenograft being used and that a subset of these invading cells expresses mFAP (W.N. Brennen and S.R. Denmeade, unpublished data).

Role of FAP in the Biology of Cancer

Currently, not a lot is known about the regulation of FAP expression, and further investigations are necessary to fully elucidate the mechanisms underlying FAP's dichotomous role in tumorigenesis. Zhang and colleagues (55) characterized the minimal FAP promoter and showed that early growth response 1 (EGR1) is an important regulator of FAP transcription. Of note, the EGR1 transcription factor itself has also been shown to have contradictory roles in tumorigenesis depending on the tumor type. Furthermore, treatment with TGF-β, 12-*O*-tetradeconyl-phorbol-13-acetate (TPA), and retinoids is known to induce the upregulation of FAP expression on fibroblasts *in vitro*, whereas stress induced by serum starvation has no effect (56). Of interest, retinoids have been shown to have both chemopreventive and chemotherapeutic benefits in multiple cancer types (57). TGF-β is known to act as either a tumor promoter or suppressor, depending on the tumor type and stage of the disease. TGF-β is a potent inducer of the reactive phenotype in fibroblasts, and its regulation of FAP may underlie the context-dependent promotion or suppression of tumor growth that has been observed clinically.

Although the physiologic substrates of FAP have yet to be fully determined, investigators are beginning to elucidate a role for FAP in cancer biology. It has been proposed that FAP plays a role in matrix digestion and invasion through its gelatinase activity (58). The cleavage product generated from NPY in the presence of FAP has been shown to be proangiogenic, which may explain the correlation observed between FAP expression and increased microvessel density in tumors (31, 35, 59).

Using a variety of *in vivo* models, researchers have directly implicated FAP in tumor promotion by showing increases in tumor incidence, growth, and microvessel density (32, 35, 59, 60). Cheng and colleagues (32) reported an increase in both tumor incidence (2- to 4-fold) and growth (10- to 40-fold) in mFAP-transfected HEK293 human embryonic kidney cells grown as xenografts compared with mock-transfected controls. Administration of polyclonal rabbit antisera that was shown to inhibit FAP enzymatic activity significantly attenuated the growth of HT-29 human colorectal xenografts (32). In another study, Huang and colleagues (59) generated FAP-expressing human breast cancer cells (MDA-MB-231) that formed tumors with increased growth rates and a 3-fold higher microvessel density compared with vector controls when implanted into the mammary fat pads of murine hosts. Of interest, both FAP-positive cells and vector controls grew at the same rate *in vitro*, suggesting that FAP's effect on tumor growth is mediated through the tumor microenvironment *in vivo*. Combined with data showing an upregulation of FAP transcription in endothelial cells undergoing capillary morphogenesis and reorganization (61), this suggests that this tumor-promoting effect may be due in part to making the tumor microenvironment more conducive to angiogenesis. Most convincingly, using both syngeneic colon and endogenous K-ras^{G12D}–driven lung models of murine cancer in which they recapitulated the physiologic stromal-restricted expression of FAP, Santos and colleagues (35) showed that both pharmacologic inhibition and genetic deletion of FAP resulted in decreased tumor proliferation and altered stromatogenesis.

More recently, Kraman and colleagues (27) implicated FAP-expressing cells in immunosuppression, and selective elimination of this population of cells using transgenic mice expressing the diphtheria toxin receptor under the control of the FAP promoter restored host immunological control of tumor growth. A significant proportion of the FAP-expressing cells identified in this study, which are likely responsible for this immunomodulatory capability, share known markers (CD45−/CD34+/Sca-1+) associated with multipotent MSCs. MSCs are known to be immune-privileged due to a lack of antigenic stimulatory molecules, including major histocompatability complex class II antigens and costimulatory molecules, in addition to promoting an immunosuppressive and anti-inflammatory local environment (62). Circulating bone marrow–derived MSCs have been shown to express FAP by multiple groups, including our own (S. Chen and J.T. Isaacs, unpublished data) and are known to traffic to tumor sites at frequencies comparable to those observed in previous studies (26, 27). Of importance, FAP activity itself was not shown to mediate this immunosuppressive activity, because the LL2 carcinoma cells themselves were shown to express FAP. This indicates that inhibition of FAP activity alone by pharmacological agents will not restore host immunological defenses.

In contrast, other studies showed that expression of FAP decreased tumorigenicity in mouse models of melanoma (63), and it was associated with longer survival in patients with invasive ductal carcinoma of the breast (45). These conflicting observations suggest that the physiologic response to FAP may depend not only on the *in vivo*

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tumor microenvironment but also on the exact context of the expression within different microenvironments.

Potential for Therapeutic Exploitation of FAP by a Novel Prodrug

The unique enzymatic activity and highly restricted expression of FAP in the reactive stroma associated with >90% of epithelial cancers examined thus far make it a very attractive candidate for tumor-specific therapies. A number of potential therapeutic strategies can be envisioned, including inhibition of enzymatic function by a small molecule or antibody, and immunotherapies that deliver radioisotopes, drugs, or toxins to the tumor stroma. These approaches are currently being developed by several pharmaceutical companies, and promising progress along these lines is being made. Significantly, targeting of FAP-expressing cells in tumor-bearing mice using an oral DNA vaccine resulted in a considerable increase in intratumoral drug uptake, likely due to a concomitant decrease in the amount of type I collagen present (64).

Two phase I studies have been done to evaluate the biodistribution of a ^{131}I-labeled mFAP monoclonal antibody (mAb F19) in presurgical patients with hepatic metastases from colorectal cancer and in soft-tissue sarcoma. These studies showed selective accumulation of the F19 mAb in tumors, with minimal localization to any other normal tissue, indicating selective FAP expression within the tumor microenvironment (47). In another study, Scott and colleagues (47) used a humanized version of F19 mAb sibrotuzumab, which is under development by Boehringer Ingelheim Pharma KG, and tested its safety and distribution in 26 patients with colorectal and non–small cell lung cancer (NSCLC). They observed no objective tumor response, but they did see selective uptake in tumors 24 to 48 hours after infusion, with no significant normal organ uptake (47). In a phase II study, Hofheinz and colleagues (48) treated 25 patients with colorectal cancer with unconjugated sibrotuzumab. Although the therapy was found to be safe and well tolerated, no responses were observed and the trial was stopped. Studies to evaluate the effects of radioisotope- or toxin-labeled antibodies are currently under development.

Another FAP-targeted therapeutic strategy would be to inhibit its enzymatic function. A number of studies have characterized the dipeptide substrate requirements for FAP, leading to the development of small-molecule inhibitors that selectively inhibit FAP over other prolyl peptidases by companies such as Boehringer Ingelheim (65), Point Therapeutics (35), and Genentech (21). For example, Genentech developed a boronic acid–based inhibitor (Ac-Gly-prolineboronic acid) with a K_i of 23 nm and a reasonable (9-fold) to significant (5,400-fold) selectivity against all other members of the prolyl peptidase family (21). Point Therapeutics also developed a boronic acid–based inhibitor, Val-prolineboronic acid (PT-100, or talabostat), that is being evaluated in a variety of cancer types (66). Talabostat selectively inhibits both FAP and DPPIV, and it stimulates the production of immunomodulatory cytokines and chemokines through a mechanism that is not completely understood (65, 66). In phase II clinical trials, talabostat has been tested alone or in combination as therapy for metastatic colorectal cancer (49), NSCLC (67), stage IV melanoma (68), and chronic lymphocytic leukemia (66). Although one complete response (in NSCLC) and a few partial responses were reported in these studies, no clinical benefit could be attributed to talabostat or combination therapy over the single-agent, standard-of-care arms in the studies. However, no dose-limiting toxicities were observed in these trials, and the most commonly reported adverse event linked to talabostat was peripheral edema, likely resulting from stimulation of IL-6 or other immunomodulatory effects (66). On the basis of these trial results, investigators initiated 2 phase III trials in which talabostat was administered to patients with late-stage NSCLC in combination with either docetaxel or pemetrexed. However, these trials were halted at the interim evaluation. As reported by Kennedy (69) in the *Wall Street Journal*, these trials were terminated early because neither the primary nor the secondary goals were being met, and the patient group in the docetaxel-combination study appeared to have a lower survival rate than the group in the placebo arm. Additional therapies involving immunoliposomes that target FAP to deliver TNF are also in preclinical development (70).

FAP inhibition may not inhibit the growth of all tumor types and may even promote tumor growth, based on current conflicting data regarding FAP's role within the tumor microenvironment. Furthermore, FAP activity may not be as critical as the role of the FAP-expressing cell itself; consequently, merely inhibiting its enzymatic activity alone may not be as beneficial as eliminating the cell type in question altogether. A more viable strategy, which would circumvent this uncertainty regarding FAP's role in tumorigenesis, would be to take advantage of the protein's restricted tumor expression and unique enzymatic activity to selectively target FAP-activated prodrugs designed to deliver very potent cytotoxic agents to the tumor microenvironment (Fig. 2). This strategy entails the systemic administration of an inactive prodrug composed of a cytotoxin coupled to a peptide carrier containing a FAP cleavage site. This peptide carrier inactivates the prodrug by preventing it from crossing the cell membrane and consequently from reaching its intracellular target. The prodrug circulates throughout the body in this nontoxic, inactive form until it is proteolytically activated by FAP, which is present on CAFs localized to the tumor microenvironment. The cytotoxin itself has no inherent specificity; therefore, once it becomes activated, it nonspecifically targets any cell in close proximity to the region of activation, including fibroblasts, tumor cells, and endothelial cells. This propensity to kill neighboring cells that do not express the target once the prodrug has been activated is a phenomenon known as the bystander effect, and it can lead to a greater antitumor effect. This strategy should allow increased delivery of the drug specifically to

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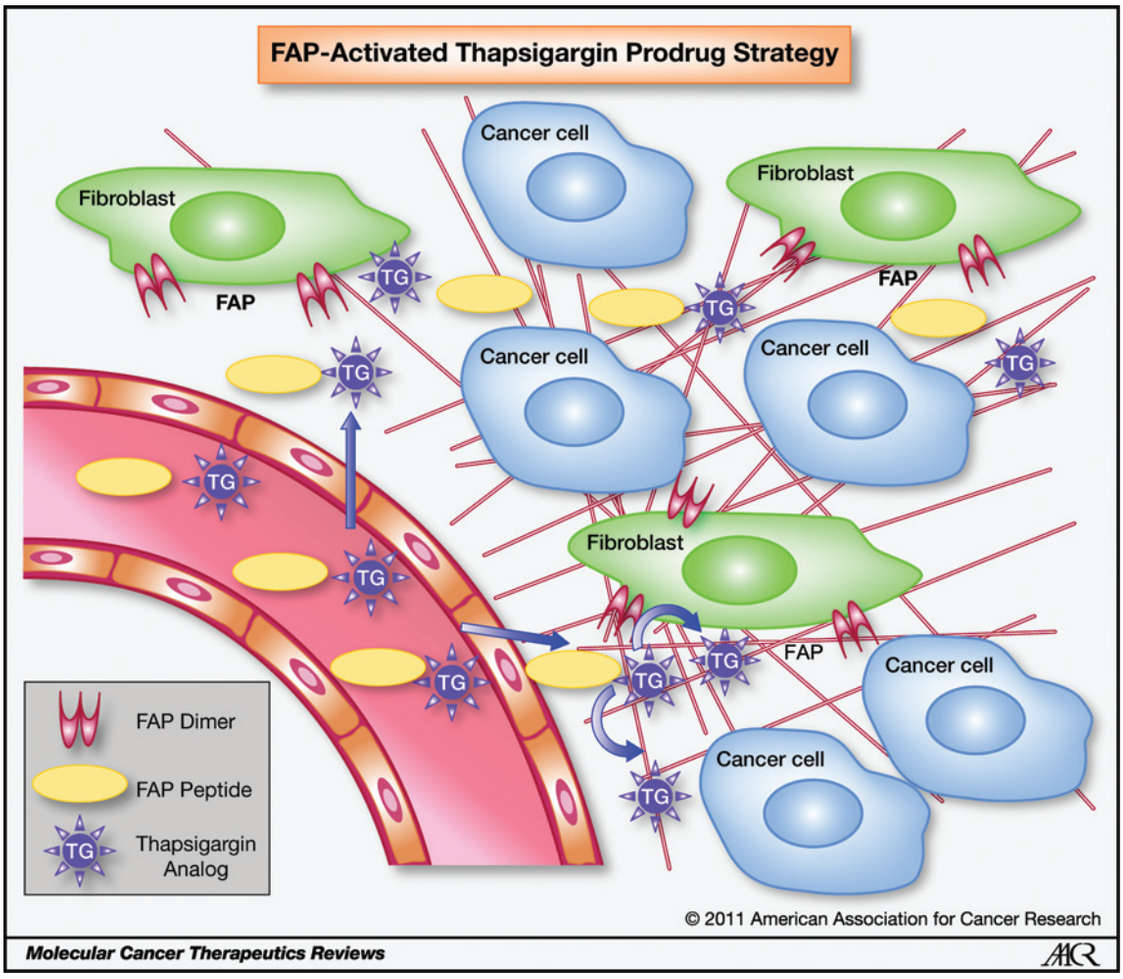

Figure 2. Schematic of the FAP-activated prodrug strategy. A FAP-activated prodrug is administered systemically and circulates throughout the body in an inactive form. The inactive prodrug diffuses into the tumor microenvironment where it encounters CAFs expressing FAP, which selectively activate the cytotoxin by cleaving off the inactivating peptide containing the FAP recognition sequence. Once activated, the thapsigargin analog used in this example directly kills the FAP-expressing cells, and it also targets neighboring cancer and endothelial cells through a bystander effect. In theory, any cytotoxic agent can be used as the warhead in this strategy, provided that its structure allows it to be conjugated to a protease-accessible peptide substrate. TG, thapsigargin.

the site of the tumor while minimizing the systemic toxicity associated with traditional chemotherapeutic modalities.

In this regard, several studies have focused on the peptide substrate requirements for FAP cleavage and specificity. These studies were primarily limited to analysis of dipeptide substrates. Aertgeerts and colleagues (22), the group responsible for determining the crystal structure, confirmed that FAP is an endopeptidase that is capable of cleaving N-terminal amine-blocked dipeptides that are resistant to cleavage by DPPIV. An analysis of known DPPIV dipeptide fluorescent substrates by Park and colleagues (71) revealed a preference for Ala-Pro-AFC (Km = 460 μM) over Lys-Pro-AFC (Km = 900 μM) or Gly-Pro-AFC (Km = 1,150 μM). Using zymography techniques, they also showed that FAP is able to cleave gelatin and human collagen I, but not human fibronectin, laminin, or collagen IV (71). To more clearly define the preferred substrates to use in the generation of an inhibitor, Edosada and colleagues (72) undertook a more extensive evaluation of dipeptide substrates. In this study, P2-Pro and Acetyl-P2-Pro dipeptide libraries were generated in which P2 was various amino acids, and hydrolysis resulted in the release of a fluorescent leaving group. FAP preferred Ile, Pro, or Arg in the P2 position of the first library, and it exclusively cleaved Ac-Gly-Pro in the acetylated library. This is distinct from DPPIV, which showed broad specificity (P2-Pro) and minimal reactivity (Ac-P2-Pro) in the 2 libraries, respectively. Lee and colleagues (73) profiled the extended substrate specificity of FAP using the sequence surrounding the APCE site found in α_2-antiplasmin. They used this sequence as a base for amino acid substitution at each position and did a comparative analysis of the cleavage kinetics that resulted from the substitution. Their analysis confirmed the Gly-Pro preference in the first 2 positions on the

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amino-terminal side of the scissile bond and revealed a preference for the positively charged amino acid Arg in the P6 or P7 position (73). In another study, our group profiled the substrate specificity of FAP by analyzing cleavage sites identified from the digestion of gelatin derived from human collagen I by FAP, and we determined a consensus sequence, (D/E)-(R/K)-G-(E/D)-(T/S)-G-P (41).

On the basis of these substrate-specificity data, we designed a FAP-activated protoxin generated from bee venom, and the results showed an antitumor effect against both breast and prostate cancer xenografts (74). These proof-of-principle experiments provided the initial validation for FAP as a viable target for prodrug activation in the tumor microenvironment. More recently, Huang and colleagues (75) generated a doxorubicin-based prodrug by conjugating the drug to the FAP-selective N-terminal benzyloxycarbonyl (z)-blocked dipeptide, z-Gly-Pro. Treatment of 4T1 breast cancer xenografts with this FAP-activated doxorubicin prodrug showed an antitumor efficacy *in vivo* similar to that observed with the parent compound, with a minimal effect on body weight. Significantly lower concentrations of doxorubicin were detected in nontarget tissues, including the heart, of prodrug-treated animals; however, as much as 20% of the total administered dose was converted to the active form of the drug, likely due to nonspecific activation of the prodrug (75).

Although this represents a considerable improvement in the toxicity profile over doxorubicin, there is certainly room for improvement in the targeting sequence itself. One could potentially eliminate much of this nonspecific activation by designing prodrugs with extended substrate sequences in order to enhance the selectivity of prodrug activation for FAP over nontarget proteases. With this in mind, our group generated and characterized a series of thapsigargin-based, FAP-activated prodrugs with extended substrate cleavage sites based on a previously described gelatin cleavage map (41) in both *in vitro* and *in vivo* models. These FAP-activated prodrugs showed a significant inhibition of tumor growth in both MCF-7 (breast) and LNCaP (prostate) xenograft models of cancer, with <0.1% of the total administered dose converted to the active form in mouse plasma (W.N. Brennen and S.R. Denmeade, unpublished results).

Conclusions

FAP is a post-prolyl protease with distinct substrate requirements, and its expression is restricted to the surface of reactive fibroblasts localized to the tumor microenvironment in normal, healthy adults. At present, these 2 characteristics make FAP uniquely suited for therapeutic strategies aimed at targeting CAFs. The evidence suggests that elimination of the FAP-expressing population would lead to an antitumor effect through multiple mechanisms. Such mechanisms include decreases in blood vessel density to deprive tumor cells of required nutrients and oxygen, reductions in the amount of collagen to allow for greater drug uptake, disruption of MSC-mediated immunosuppression to promote natural defense mechanisms and tumor rejection, and elimination of the CAFs themselves, which have been shown to promote growth, survival, and invasion of their malignant epithelial neighbors through numerous signaling pathways.

Together, the uncertainty regarding FAP's role in tumorigenesis and the realization that elimination of the FAP-expressing population of cells as a whole may be more beneficial than just inhibiting its enzymatic activity serve to promote the use and strength of the prodrug therapeutic strategy described herein. This approach seeks to exploit the restricted expression and localized enzymatic activity of FAP in order to activate a cytotoxic prodrug within the tumor microenvironment to trigger death in the targeted FAP-expressing population and neighboring cells through a bystander effect. Therefore, this strategy would be effective independently of FAP's function. Furthermore, a FAP-activated prodrug would have enhanced therapeutic efficacy compared with simple inhibition strategies because it would eliminate entire populations of cells with known tumor-promoting capabilities, including CAFs and MSCs.

The tumor microenvironment and CAFs, in particular, represent a reservoir of potential chemotherapeutic targets that remain largely untapped despite an ever-growing body of preclinical data indicating that they play a significant role in all stages of carcinogenesis, from initiation to metastasis. Strategies that attempt to exploit cellular targets within the tumor stroma offer several potential advantages over traditional approaches. These include providing a more genetically stable target that is not only less heterogeneous than its malignant epithelial counterparts but also less likely to acquire resistance to a cytotoxic agent. Additionally, many solid tumor malignancies share common alterations in their tumor microenvironment, and consequently, approaches that target these alterations may provide pan-tumor treatment options. These qualities make CAFs and other stromal cells potentially more amenable to therapeutic intervention. Unfortunately, this is still a relatively underappreciated treatment strategy, largely due to a lack of evidence suggesting that it is a practical option in the clinical setting. However, the surge in both pharmaceutical and academic efforts to develop FAP as a therapeutic target offers a promising outlook for negating that statement in the not-so-distant future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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