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

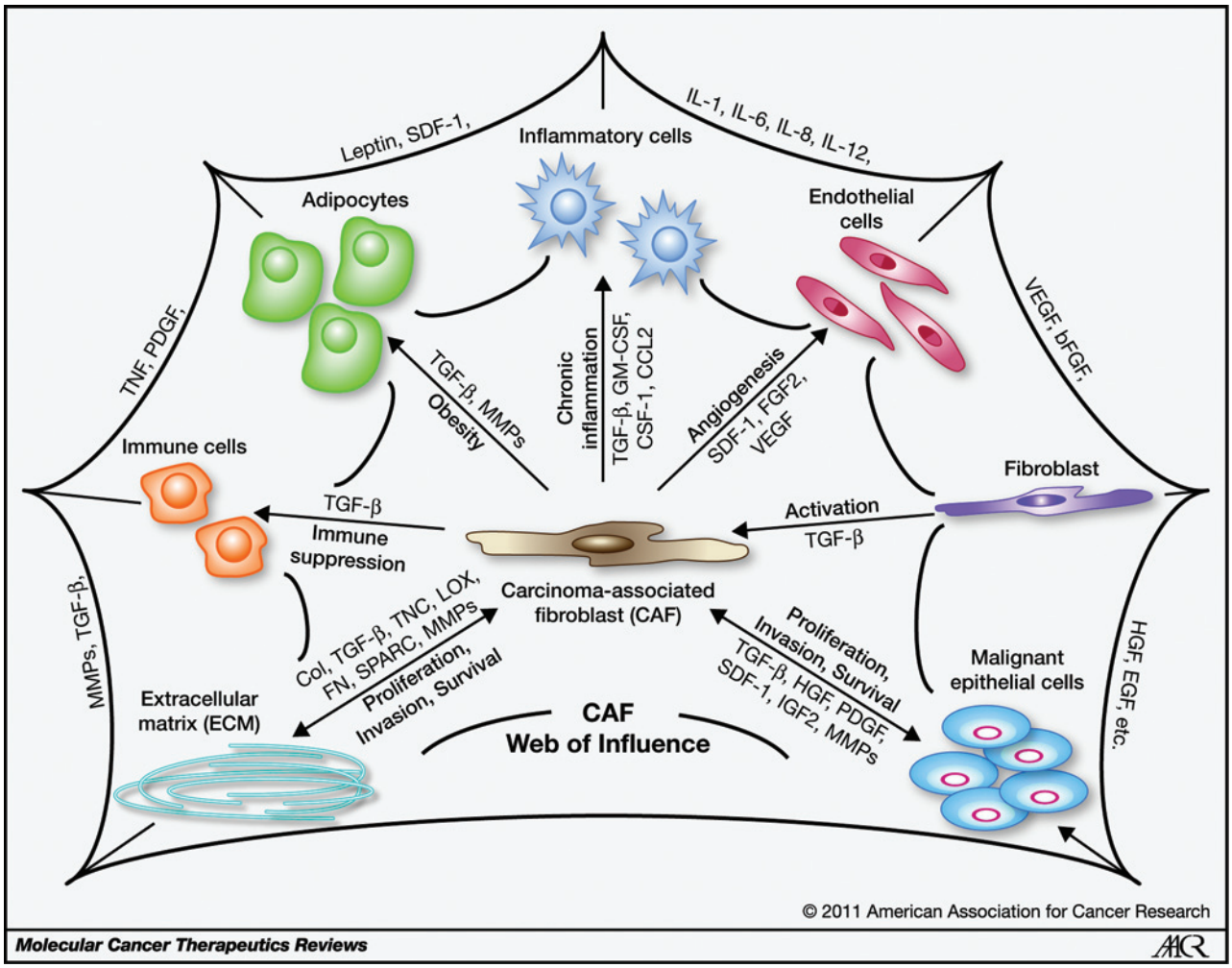

Figure 1. CAFs can promote tumorigenesis directly through multiple mechanisms, including increased angiogenesis, proliferation, invasion, and inhibition of tumor cell death. These effects are mediated through the expression and secretion of numerous growth factors, cytokines, proteases, and extracellular 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.

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)

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,

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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