Pro-tumorigenic roles of fibroblast activation protein in cancer: back to the basics

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Introduction

Fibroblast activation protein (FAP) was first described in 1986 by Rettig, et al., who named it based on its expression by reactive fibroblasts, especially in cancer. Independently, Aoyama and Chen identified a serine protease on melanoma cells that they named seprase based on its enzymatic activity. By 1997, based on gene sequencing, it was demonstrated that FAP and seprase are the same molecule, and now FAP is the widely accepted nomenclature. FAP is a member of the dipeptidyl peptidase (DPP) family, and shares around 50% homology with DPPIV, its closest family member. Like other DPP enzymes, FAP has post-proline exopeptidase activity, but FAP is unique in also having gelatinase activity, which allows it to degrade denatured or MMP-cleaved collagen I.

Structurally, FAP consists of a 6 amino acid cytoplasmic tail, a single 20 amino acid transmembrane domain, and a 734 amino acid extracellular domain. This extracellular domain consists of an eight-bladed beta propeller, which acts as a substrate selectivity gate, and an alpha/beta hydrolase domain. FAP monomers are not active, but form active homodimers as well as heterodimers with DPPIV. Soluble FAP has also been detected in various contexts; this appears to be the dimerized extracellular domains alone. FAP is expressed during development, but only rarely in healthy adult tissues. However, it is highly upregulated—especially on fibroblasts—at sites of active tissue remodeling, including wound healing, fibrosis, and cancer. In the context of cancer, FAP has gained notoriety as a marker of cancer-associated fibroblasts (CAFs), which have a number of pro-tumorigenic functions. Moreover, FAP itself has been demonstrated to have pro-tumorigenic activity, both through enzymatic and non-enzymatic means. In this review, we cover recent advances in FAP expression profiling, molecular function, and targeted therapies in the context of cancer, and pose a number of major questions about FAP that remain to be answered.
Patterns of FAP expression in cancer

FAP expression is typically low to undetectable in most normal adult tissues, but is highly upregulated in a multitude of cancers, including almost all carcinomas. In tumors, various mesenchymal cells express FAP, including mesenchymal stem cells (MSCs), CAFs, sarcoma, and melanoma cells(15–18). FAP expression on epithelial tumor cells has also been reported, but the prevalence and significance of this remains to be established. One difficulty in interpreting reports of FAP expression comes from the fact that, while FAP-specific antibodies do exist, some antibodies on the market lack specificity. Therefore, studies reporting on FAP expression using only immune-based assays should be interpreted based on inclusion of appropriate controls. Below, we summarize recent findings about FAP expression in terms of both tissue and cell type, with a focus on potential prognostic value (Table 1).

FAP expression in normal tissues

Many human studies rely on using tumor-adjacent tissues for controls. Yet even when these tissues do not show histological evidence of tumor cells, the presence of tumor elsewhere in the same organ can have effects on distal tissue, and thus classifying these samples as “normal” is often misleading. In human tumor-adjacent tissues, FAP was detectable at the RNA level by RT-PCR in the context of esophageal squamous cell carcinoma (ESCC)(19), lung carcinoma(18), and glioma(17). At the protein level, FAP was found in pancreatic ductal adenocarcinoma (PDAC) adjacent tissue(20,21). However, data obtained from non-tumor-bearing subjects suggest that some of these instances of tumor-adjacent FAP expression are not reflective of FAP expression in healthy tissues. For example, protein-level analysis in the brain did not detect FAP expression in samples from non-tumor bearing patients(22). A more systemic approach to FAP expression profiling in mice with extra-chromosomal luciferase under the control of the FAP promoter suggests that low basal levels of FAP expression might be found in many tissues, including muscle, bone marrow, adipose, skin, and pancreas(23). In line with this, using mice expressing luciferase under the control of the endogenous FAP promoter our lab detected expression in healthy adult murine skin, bone, pancreas, and—to a lesser extent—kidney (Fig1). In the pancreas, single cell RNA sequencing revealed that FAP expression is specific to alpha-cells within normal islets(24). Though the cellular source is unknown, FAP can also be detected in the plasma of healthy donors(20,25). These data suggest that, while FAP up-regulation in tumors does provide a potential therapeutic window, its expression in healthy tissues may not be as restricted as previously thought, and must be taken into consideration when evaluating the potential side effects of targeting FAP.

FAP expression by various cell types

FAP expression by fibroblastic stromal cells is well established, but increasing evidence suggests that it may be expressed by additional cell types in the context of the tumor microenvironment (TME). Mature adipocytes undergo rapid de-differentiation when cultured in vitro, and this process was associated with induction of FAP expression(26). In human pancreas, FAP was expressed in glucagon+ alpha cells, often co-localizing with DPPIV expression(27). In glioma, using double immunofluorescence (IF) FAP was seen on
mesenchymal stromal cells, but also on astrocytes, neural stem cells, and scattered CD45+ cells(22). These CD45+ cells might be fibrocytes or possibly a macrophage subset, as in murine lung cancer, FAP was expressed by some M2 (CD206+) macrophages(28). This result is consistent with past reports of FAP on macrophages in human breast cancer(29). In reconstructed skin cultures consisting of layered collagen, fibroblasts, melanocytes, and keratinocytes, FAP expression could be induced in melanocytes but not in keratinocytes(30).

Whether epithelial tumor cells express FAP is an area of ongoing research. Using immunohistochemistry (IHC), apparent cytoplasmic FAP stain was observed in lung cancer(31); unfortunately, this study appears to have relied on an antibody that—while marketed as targeting FAP—was actually generated against a peptide fragment of fas-associated phosphatase(32); a protein with no relation to fibroblast activation protein. However, using different antibodies for IHC, FAP has been detected in lung(33), breast(34–36) and pancreatic tumor cells(20,37). Using flow cytometry, FAP was detected on a subset of pancreatic tumor cells which also up-regulate Thy-1 (CD90), perhaps indicative of their undergoing EMT(38). FAP expression has been reported in mesothelial cells in the context of ovarian(39) and gastric cancer(40). In line with these results, certain epithelial tumor cell lines can express FAP, however this expression is often very low-level and/or requires non-physiological levels of induction stimuli(41,42). Pearl, et. al. reported FAP expression on circulating ovarian tumor cells, using a cell isolation method which was based on the ability of circulating cells to migrate through collagen-rich matrix. They were able to detect low levels of FAP mRNA in this cell population, though the protein-level analysis was confounded by their simultaneous, single-color staining for both FAP and CD44. As such, they were able to demonstrate that a population of CD45- circulating cells was able to ingest collagen matrix and express either CD44 or FAP. It would be interesting to demonstrate conclusively if these cells are migrating epithelial tumor cells or, perhaps, are tumor-associated mesenchymal cells traveling with metastasizing cells(43).

**FAP expression and prognostic value in tumors**

While individual reports of FAP’s prognostic value vary from study to study, across a wide range of human cancers FAP is reported to correlate to higher tumor grade and worse overall survival. A recent meta-analysis reported that across a range of IHC-based studies (many of which are discussed individually below) in various tumor types, the most consistent results associated high FAP expression with increased lymph node metastasis and poor overall survival(44). In breast invasive ductal carcinoma (IDC), FAP associated to higher grade, and was reported to correspond with inflammatory-type stroma(34) and adipose-type stroma(36), though correlations between FAP expression and more classical subtypes (e.g. hormone receptor positivity) were not replicable between studies. FAP expression was higher in invasive lobular carcinoma (ILC) than in invasive carcinoma of no special type(35). Interestingly, FAP expression appeared to correspond to malignancy of breast phyllodes tumors, whose rare transition from benign to malignant has been difficult to predict(16). In colorectal cancer (CRC), high FAP expression associated with worse overall survival(45–47). FAP expression in primary CRC also correlated to grade, as well as the sarcomatoid phenotype, which itself putatively relates to high levels of epithelial to mesenchymal transition (EMT)(46). FAP+ primary tumors yielded more lymph node
metastasis, which themselves then expressed FAP(46). High FAP also corresponded to a shift in immune cell populations within the tumor: reduced CD3+ but increased CD11b+ cells(47). In gastric cancer, high FAP expression correlated to higher grade, lymph node and peritoneal invasion, and worse overall survival(40,48,49). In glioma, FAP expression associated to higher grade and the mesenchymal subtype(22). In intrahepatic cholangiocarcinoma (ICC), high FAP expression correlated to high CCL2 and STAT3 expression, along with reduced overall survival and increased probability of recurrance(50). In oral squamous cell carcinoma (OSCC) high FAP expression was reported to associate with higher tumor stage, lymph node metastasis, and reduced overall survival(51). In ovarian cancer, high FAP associated to platinum chemotherapeutic resistance and probability of recurrence(52) as well as higher stage, lymph node metastasis, and reduced survival(39). In pancreatic ductal adenocarcinoma (PDAC), high FAP expression correlated to higher grade/stage and reduced survival(21,37,38).

Conversely, in hepatocellular carcinoma (HCC), FAP expression did not significantly correlate to grade, stage, or survival(53). Tumor-associated FAP was not found to be a robust predictor of overall survival in ESCC unless used in conjunction with other stromal markers(54). Interestingly, another study in ESCC found that low FAP levels in plasma robustly associated with higher tumor stage and low plasma HDL(25). Levels of circulating FAP did not serve as a robust biomarker for CRC unless combined with other markers(55).

Collectively, these data indicate that, given the appropriate context and detection method, FAP may serve as a valuable prognostic marker across diverse human tumor types.

**Molecular biology of FAP**

While more groups are recognizing the usefulness of FAP as a marker for CAFs, the basic biology of FAP is understudied. Since FAP can and does play active pro-tumorigenic roles, understanding this basic biology might be crucial in exploiting FAP as a therapeutic target. Below we address recent advances in knowledge about the induction of FAP expression, its cellular localization, and what enzymatic and non-enzymatic roles it might play in cancer.

**Transcriptional regulation**

Multiple groups have demonstrated up-regulation of FAP expression driven by various tumor-derived factors (Fig 2). Fibroblasts and MSCs co-cultured directly with tumor cell lines show marked up-regulation of FAP(56,57), but no-contact co-culture with adenoma explants could also induce FAP expression by bone-marrow MSCs (BM-MSCs)(15), as could conditioned media from myeloma cells(57,58). Additionally, conditioned media from liver cancer cells enhanced FAP expression on umbilical cord MSCs(59) and hepatic stellate cells(60), the latter in a STAT3-dependent manner. Various factors may be implicated in tumor-mediated up-regulation of FAP. High doses of TNFα yielded increases in ERK1/2 phosphorylation along with modest increases in FAP expression in breast cancer cell lines(42), though similar doses did not yield significant up-regulation of FAP by BM-MSCs. BM-MSCs instead up-regulated FAP in response to IL-1β and TGFβ(61). Treatment of melanoma-bearing mice with an inhibitor of the A2BR adenosine receptor reduced the number of FAP+ cells in a transplant model of melanoma, while an A2BR agonist increased
FAP+ cells. These cells showed enhanced ERK1/2 phosphorylation and expression of FGF2 and CXCL12. Interestingly, hypoxia induced the expression of CD73, which produces extracellular adenosine, indicating that hypoxia in the TME might enhance signaling through A2BR and thus induce FAP expression(62). Treatment of a human fibroblast line with progranulin enhanced FAP expression, as did co-culture with progranulin-expressing colon cancer cells. When progranulin was silenced in the tumor cells, FAP was not up-regulated(63). Luo et. al. reported that stimulation of primary fibroblasts with estrogen caused phosphorylation of ERK1/2 and upregulation of both TGFβ and FAP(64). Jia et. al. investigated estrogen signaling in the context of prostate cancer and discovered opposing activity of the classic estrogen receptor alpha (ERα) and an alternate receptor, GPR30. They noted that CAFs expressed higher levels of GPR30 and lower ERα than normal fibroblasts, and using both receptor overexpression and silencing studies demonstrated that FAP expression is promoted by GPR30 but inhibited by ERα(65).

Malignant ascites-derived exosomes caused a modest increase in FAP expression by a mesothelial cell line, which was abrogated in the presence of a TGFβ inhibitor(66). Indeed, TGFβ seems to be at the center of most signaling pathways that have been implicated in FAP expression (Fig2). LPS signaling through TLR4 induced TGFβ expression by primary fibroblasts, which corresponded to high FAP expression(67). UV radiation induced FAP expression by up-regulating cathepsins which in turn induce TGFβ(30). hTERT induced both cathepsin D and EGR-1, resulting in increased FAP(68). This result has interesting implications for the use of cell lines in studying FAP expression, since any which have been immortalized by hTERT may show FAP up-regulation as an artifact of this process. The studies mentioned above demonstrate some link between FAP and various signaling pathways, though they do not demonstrate any direct action on the FAP promoter, meaning that the observed upregulation of FAP might be downstream of some master program of fibroblast activation. In contrast, promoter analysis has revealed direct roles for EGR-1(69) and SMAD3(70)—downstream of TGFβ signaling—in inducing FAP transcription. Promoter bioanalysis has also identified putative binding sites for AP-1, c/EBP and Ets proteins, and an E-box, though the functionality of these sequences is yet to be validated(70).

Less is known about what factors suppress FAP expression, either acutely or as part of basal regulation keeping FAP expression low in normal tissues (Fig2). As mentioned above, estrogen signaling through ERα specifically was observed to inhibit FAP expression, and ERα levels are higher in normal fibroblasts than in CAFs(65). Genistein—an isoflavone with multiple mechanisms of action on cells—was observed to reduce FAP expression on gastric cancer cell lines, through mechanisms unknown(71). Yet it is interesting to note that genistein is capable of acting as an agonist to various estrogen receptors(72,73). In the context of breast tumors, knockdown of PTEN induced FAP expression, indicating that PTEN is a FAP repressor. In the same study, miR-21 was demonstrated to be a negative regulator of PTEN, and thus a FAP inducer(16). Recently, miR-30a-5p was reported to target FAP mRNA directly, in OSCC cell lines(74). During their expression profiling of FAP in glioblastoma, Busek et. al. noted that the correlation between FAP mRNA levels (by RT-PCR) and protein levels (by ELISA) had an r value of only 0.32, and cases with detectable
RNA were negative for protein; these observations together may be indicative of post-transcriptional regulation of FAP(22).

One highly understudied question surrounding the regulation of FAP expression is what roles biomechanical stimuli might play. Ha et al. report that FAP expression was low to undetectable in fibroblasts cultured on 2D silicon surfaces, but reversibly up-regulated in culture on 3D silicon nanowire arrays. Levels of FAP were inversely proportional to wire length(75). What exact property of these arrays is responsible for the enhanced FAP expression has not been fully elucidated, though there are many intriguing possibilities. The 3D arrays induce formation of a larger number of filopodia, which is associated with higher adhesive force and expression of FAK(76). Arrays comprised of longer nanowires have lower spring constant than shorter wires, and this associates with reduced cell adhesion and expression of both FAK and alpha 2 integrin(77). Whether this—or some other—mechanosignaling pathway lies upstream of FAP expression is a very interesting question. Wäster, et. al. noted that extended in vitro culture of melanocytes caused both loss of FAP expression and acquisition of a senescent phenotype(30); our lab has had similar results with primary fibroblasts, which rapidly down-regulated FAP expression in monolayer culture on plastic (unpublished data). These results could indicate that FAP is highly sensitive to substratum mechanics.

**Cellular localization**

Active FAP resides in dimeric form on the plasma membrane(6). Wonganu and Berger identified a conserved region of the transmembrane domain that is necessary for dimerization. This sequence consists of 3 small, polar residues spaced four residues apart. If these residues are mutated, FAP monomers no longer associate, which also results in reduced enzymatic activity and intracellular accumulation of the mutant FAP, possibly indicating that FAP dimerization and cellular localization are linked(78). A rare human genetic mutation of FAP demonstrated that a single-nucleotide polymorphism in the beta-propeller domain also inhibited appropriate protein trafficking, resulting in an accumulation of mutant FAP in the endoplasmic reticulum. Mutant FAP was then degraded by the proteasome(79). Multiple groups have suggested that, once at the plasma membrane, FAP localizes to invadopodia(42,80,81). Knopf et. al. used a combination of mass spectrometry-based screening, co-immunoprecipitation (co-IP), and immunofluorescence (IF) to identify multiple FAP interaction partners, including caveolin-1 and stomatin which are known to localize to lipid rafts(81).

Secreted FAP can be found at varying levels in circulation, and appears to result from shedding of cell-surface FAP at invadopodia, but despite the fact that this has been known for some time, the exact mediators of FAP shedding are still unknown(80). This process might be very useful to understand, given that in more than one tumor type, levels of circulating FAP were inversely proportional to levels of tumor-associated FAP(20,25), suggesting that FAP activity within tumors might be at least partially regulated by cell-level retention. In support of this hypothesis, induction of FAP expression in fibroblasts did not always correlate to high levels of FAP shed into the supernatant(25). Up-regulation of FAP expression in response to various stimuli can be transient, and certain culture methods

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caused loss of FAP expression from previously expressing cells(26,30), indicating that FAP was undergoing turnover, though, again, the mechanisms controlling this phenomenon are unknown. Using western blotting, various groups observed protein bands which react with anti-FAP antibodies and yet have a smaller molecular mass than full length monomers; whether these are non-specific artifacts or indicate the presence of proteolytic fragments of FAP is unclear(19,78).

**FAP substrates**

FAP’s dual enzymatic activity gives it a range of putative substrates (Fig 3). Several reported substrates are shared with DPPIV, namely neuropeptides NPY, BNP, substance P, and PYY, which were all identified using an *in vitro* assay(82). Using *ex vivo* assays with addition of candidate substrates directly to FAP-containing plasma, Wong et. al. validated NPY as a physiological substrate of FAP(83). BNP, substance P, and PYY were far more efficiently cleaved by other proteases in the human plasma, indicating that physiologically, FAP is not likely responsible for cleavage of these factors(83). In the context of prostate cancer, FAP was able to cleave perlecan only after initial cleavage events by MMP7; this subsequent FAP activity generated unique fragments relative to MMP7 digestion alone(84). This result is similar to FAP activity on fibrillar collagens, which is dependent on initial cleavage by MMP collagenases(85,86). FAP cleavage of collagen I was shown to enhance macrophage adhesion *in vitro*, an effect which is independent of integrin binding but at least partially mediated by the macrophage scavenger receptor SR-A(87). Given that some tumor-associated macrophages express FAP, it is intriguing to speculate that FAP expression by macrophages might promote their recruitment to and/or retention by the TME. Taking a more global approach, Koczorowska, et. al. used a peptide library screen to identify a panel of putative FAP substrates, including ADAM15, IL-6, Serine Protease 23, Testican 1, and TGFβ1, though each of these candidates need to be validated as bona fide FAP substrates based on evidence of direct FAP activity on their proteolytic processing(88).

Three groups recently investigated FAP cleavage of FGF21, which—in primates—contains a canonical gly-pro DPP cleavage site at the c-terminus. Given that FGF21 plays important roles in responding to metabolic stress, it is possible that FAP-mediated inactivation of FGF21 could promote systemic programs of obesity and inflammation that accelerate cancer growth(89). *Ex vivo* assays of FAP activity on human or monkey plasma revealed that FAP is able to cleave and inactivate FGF21, though murine FGF21 contains a gly to glu substitution which renders it resistant to FAP activity(90–92). Additionally, administration of FAP inhibitor to monkeys resulted in an increase of intact FGF21 in circulation(91). FAP is also capable of cleaving the n-terminus of FGF21, an event required for FGF21 activation, but depletion studies in human plasma demonstrated that n-terminal cleavage can occur in the absence of FAP. Conversely, FAP is indispensable for the c-terminal, inactivating cleavage event(92). These results suggest that FAP might play a role in systemic metabolic regulation, which can greatly influence tumor risk and progression.

**Functional roles for FAP in cancer**

Taken together, recent published results demonstrate that FAP can directly enhance proliferation, migration, and invasion of cells by which it is expressed (Fig 3). Co-culture
with FAP⁺ cells can also promote proliferation, activation, and invasion of additional cell types including tumor, endothelial, and immune cells. One meta-analysis of TCGA gastric cancer data revealed that cases with high FAP expression also display enrichment of gene pathways for immune regulation, angiogenesis, cell migration, and cell differentiation and growth(49).

**Direct roles of FAP on fibroblasts**—Partial silencing of FAP in primary fibroblasts using siRNA resulted in modest reduction of both proliferation and production of the ECM molecules collagen I, fibronectin, and laminin(64). siFAP also inhibited UV-induced fibroblast invasion through basement membrane extract(30). Stable overexpression of FAP in a CAF line resulted in significant changes to the CAF secretome, including upregulation of proliferative, inflammatory, and ECM remodeling factors. Functionally, co-culture of these FAP⁺ CAFs with endothelial cells enhanced angiogenesis as measured by sprouting(88). This is consistent with prior results from FAP-null mice, that showed reduced vascularization in lung tumors(93).

**Direct roles of FAP on mesenchymal tumor cells**—Stable transfection of FAP in HT1080 fibrosarcoma cells resulted in increased adhesion to and migration on fibronectin and collagens I and IV; this could be reversed through antibody blocking of FAP, which itself resulted in internalization and loss of FAP from the cell surface. FAP⁺ cells expressed higher levels of integrin-signaling pathway components ILK, p-FAK, and Rac-1, and were also more sensitive to integrin blocking, displaying a greater loss of adhesion and migration than FAP⁻ HT1080 cells given the same treatment. Treatment with Src kinase II or PI3K inhibitors reduced adhesion and migration of only the FAP⁺ cells, indicating that FAP plays a role in cell-ECM interactions through classical integrin signaling pathways(94). Tansi, et. al. reported that, when injected with matrigel plugs into mice, FAP⁺ HT1080 cells formed smaller primary tumors than the parental line, but the FAP⁺ cells showed more evidence of invasion through the plugs and the mice displayed enhanced lung metastasis(95). Somewhat conversely, Baird et. al. reported that FAP⁺ HT1080 cells had enhanced primary tumor growth relative to the parental line(96). However, these two studies may not be directly comparable due to differences in the overexpression lines, mice, and injection techniques used, making it difficult to conclude which is the more physiologically relevant result. In the second study, FAP⁺ cells were more sensitive to doxorubicin (but not cisplatin), as measured by the MTT assay, but did not show up-regulation of classical markers of apoptosis or necrosis; instead, FAP⁺ cells treated with doxorubicin were found to have increased mitochondrial membrane permeability, lysosomal permeability, and oxidative stress(96). FAP⁺⁺ melanoma lines were more invasive in vitro, either TGFβ inhibition or direct silencing of FAP with shRNA reduced this invasiveness(97). In a separate study with FAP expressing melanoma cells in a zebrafish model, treatment with an anti-FAP antibody reduced invasive spread of tumor cells(30). Osteosarcoma lines with FAP knockdown showed reduced in vitro proliferation, migration, and adhesion to as well as invasion through matrigel(98).

**Direct roles of FAP on epithelial tumor cells**—In gastric cancer, tumor cell lines with more endogenous FAP expression proliferated faster, and siFAP treatment reduced this proliferation(71). Forced expression of FAP in MCF7 breast cancer cells, was able to
enhance proliferation, but reduced both adhesion and migration, though FAP expression in
the MDA-MB-231 line did not show comparable effects, suggesting that the impact of
overexpression may be context dependent(99). siFAP treated OSCC cells displayed reduced
colony formation, migration and invasion through matrigel(74). Similarly, shFAP treatment
of OSCC lines resulted in reduced proliferation and migration with enhanced adhesion to
fibronectin. This corresponded to enhanced primary tumor growth and experimental
metastasis in a xenograft model. In a separate study, shFAP-treated OSCC cells showed
decreased levels of cell-cycle promoters and markers of EMT with increased levels of
MMP2 and 9, all indicative of an overall shift in cellular phenotype. Silencing of FAP also
resulted in increased PTEN, associated with decreased PI3K, AKT, and ERK signaling.
Silencing of PTEN was able to reverse the shFAP-induced reduction of proliferation,
migration, and invasion, while transfection with constitutively active PTEN enhanced the
phenotypes, suggesting that a PTEN/FAP axis can mediate tumorigenic cell behaviors(51).

**Indirect effects of FAP on tumor cells**—Recombinant FAP added to the culture media
of ovarian cancer cells allowed enhanced survival in the presence of cisplatin, through
mechanisms unknown(52). Conditioned media (CM) from CAFs promoted the *in vitro*
proliferation and migration of gastric cancer cells, as well as survival of cancer cells when
treated with chemotherapeutic agents. All of these effects were partially abrogated when the
CAFs were treated with shFAP. Co-injection of cancer cells with FAP+ CAFs enhanced
tumor growth while reducing T-cell infiltration; again, treatment of CAFs with shFAP
reduced these tumor-promoting effects. shFAP CAFs allowed for improved T-cell function
and better overall survival of mice relative to FAP+ CAFs(48). Transwell co-culture of lung
cancer cells with FAP+ CAFs resulted in increased cancer cell proliferation, migration, and
invasion through matrigel. Treatment of the CAFs with an anti-FAP antibody that results in
FAP internalization had no effect on tumor cell proliferation, caused a slight reduction of
migration, and significantly reduced invasion. This would suggest that FAP expression by
CAFs can alter their secretome in such a way as to promote tumor cell invasion, but that
FAP might be dispensable in CAF promotion of migration or proliferation(100), a result
somewhat at odds with the prior study cited. This difference may be based on tumor context;
e.g. gastric vs. lung cancer, but highlights the importance of understanding mechanistic
pathways by which FAP expression on CAFs can alter tumor cell behavior. In direct co-
culture with pancreatic tumor cells, FAP+ 3T3 fibroblasts—relative to parental FAP− 3T3
cells—enhanced tumor cell invasion through matrigel and progression through cell cycle,
which was associated with increased phosphorylation of Rb in the tumor cells(37). Different
CAF lines subcultured from the same tumor can display variable endogenous levels of FAP;
in a transplant model of colon cancer, FAPhi CAFs—relative to FAPlo CAFs from the same
primary source—inhibited the efficacy of anti-PD1 therapy and altered the immune cell
profile in tumors, causing reduced numbers and functionality of T-cells but increased
myeloid-derived suppressor cells (MDSCs). FAPhi CAFs also expressed more of the
myeloid cell chemotactic factor CCL2, and inhibition of CCL2 abrogated their enhanced
tumor-promoting effects. Inhibition of FAP with the DPP inhibitor linagliptin also abrogated
these effects, but since linagliptin also inhibits DPPIV, we cannot conclude that FAP alone
was directly responsible(47).
**Indirect effects of FAP on immune cells**—Co-culture with BM-MSCs, which endogenously express FAP, promoted survival of myeloma cell lines treated with bortezomib, an effect that was reduced by siFAP. In the presence of FAP+ BM-MSCs, bortezomib-treated tumor cells had enhanced expression of β-catenin, inhibition of which resulted in tumor cell apoptosis. Treatment of tumor cells with various combinations of putative BM-MSC-derived soluble factors was unable to replicate the drug-resistance phenotype, which could indicate a role for cell-contact(57). Co-culture of T-cells with BM-MSCs suppressed CD4+ T-cell proliferation and promoted senescence. BM-MSCs from healthy donors caused a shift in T-cell phenotype towards Tregs, while BM-MSCs from multiple myeloma (MM) patients induced a Th17 phenotype along with increased phosphorylation of AKT. Treatment of the MM BM-MSCs with the DPP inhibitor PT100 was able to restore T-cell proliferation and cause a shift back to Treg phenotype along with reduced p-AKT, indicating that FAP expression by stromal cells can have a profound effect on the immune compartment of tumors(58).

**Non-enzymatic effects**

While the experiments above suggest that the presence of FAP can enhance various tumorigenic processes, it is not always clear whether this is based on FAP’s enzymatic activity (Fig 3). MCF-7 breast cancer cells transfected to express FAP displayed enhanced growth, adhesion, and migration, and this was true even if the FAP construct contained an S624A mutation that ablates enzymatic activity. Wild-type and mutant FAP caused comparable increases in p-PI3K, p-AKT, and MMP9 expression, suggesting that, at least in this context, FAP might have effects on intrinsic cell signaling independent of its enzymatic activity(101). In BM-MSC, shFAP caused reduced migration through activation of RhoA; but inhibition of FAP with two unique peptidase inhibitors did not replicate the RhoA activation and loss of migratory ability(61). As above, Yang et. al. found that FAP+ CAFs—relative to FAP− CAFS—express more CCL2, as well as CXCL2, CXCL12, and IL6. In co-injection experiments they saw that only FAP+ CAFs were able to enhance tumor growth relative to tumor cells alone, and that this associated with increased tumoral MDSCs. Transplants into CCR2-null mice resulted in none of these phenotypes, indicating that CCL2 is the primary mediator of these effects. Upstream, CCL2 was induced by p-STAT3, silencing of uPAR in the CAFs reduced both STAT3 phosphorylation and CCL2 secretion, and uPAR was able to co-IP with FAP. Yet inhibition of FAP with PT100 does not reduce levels of p-STAT3 or CCL2 secretion, which suggests that association of FAP with uPAR induced pro-tumorigenic effects in a non-enzymatic manner(50). Clues to FAP’s non-enzymatic roles in cancer may be discerned from discovering other proteins that it associates with. Knopf et. al. demonstrated that FAP interacts with DPPIV, erlin-2, adenosine deaminase (ADA), stomatin, prohibitin-2, thy-1, and caveolin-1. FAP association with ADA was dependent on DPPIV, but coIP experiments suggest direct binding of FAP to erlin-2, stomatin, and caveolin-1(81). As yet unknown is what the functional consequences of these associations might be.
FAP-targeted therapies

Since FAP is a useful marker of CAFs, many potential FAP-targeted therapies have the ultimate goal of depleting FAP+ cells. Promising preclinical approaches have included various FAP-targeting vaccines and immunotherapies(102–104). Although the anti-tumorigenic effects of deleting FAP+ cells was validated using transgenic mice with diphtheria toxin receptor in FAP+ cells, these studies also revealed that total ablation of FAP+ cells results in impaired hematopoiesis and development of cachexia(23), indicating that there is a window of efficacy in terms of stromal cell depletion but dosing must be designed to avoid these potential side effects. For targeting FAP at the molecular level, various small molecule inhibitors have been employed, but one difficulty in designing FAP-specific inhibitors is the close homology that the active site shares with other DPP family members as well as prolyl oligopeptidase (PREP).

FAP+ cell depletion

Various vaccination modalities have been employed to target FAP+ cells in preclinical models, including DNA vaccines(105–108), adenoviral vectors(109), peptide immunization(110), and whole-cell vaccines(111,112). In addition, various immunotoxins(113–115), antibodies(116,117), FAP-targeting liposomes(118–121), and FAP-directed chimeric antigen receptor T-cells(122–124) can also deplete FAP+ cells and thus provide therapeutic benefit. From this plethora of treatments, certain patterns emerge. Functionally, FAP+ cells play important roles in immunosuppression(111,113,115,123), especially of CD8+ T-cells(109,112), as well as promoting desmoplasia(105,111,124). In terms of therapeutic efficacy, FAP-directed treatments often combine effectively with chemotherapy(108,114), tumor antigen vaccines(109,115), or antibody treatments(116,117). This could be an important consideration, since prior single-agent human trials, such as of the anti-FAP antibody sibrotuzumab, have shown limited efficacy(125).

FAP-activatable prodrugs

Another avenue of FAP-mediated therapy is the use of prodrugs which can be activated by FAP cleavage in the TME, thus increasing the dose which can be administered without systemic toxicity(126,127). Additional safety can be built in by requiring iterative activation events, as with an emetine-based prodrug which requires dual self-cleavage and FAP cleavage events in order to become active(128). Heightened efficacy can be achieved by linking multiple drugs with a FAP-cleavable linker, as with a construct where doxycycline and light-activatable phthalocyanine inactivate each other until separated by FAP-mediated cleavage(129). More sophisticated delivery methods designed to increase bioavailability to tumors include loading chemotherapeutics into nanoparticle carriers; in this case FAP can be used to activate the drugs(130) or to help disassemble the carrier to mediate drug release and/or uptake(131,132). Similar concepts can be applied to imaging techniques for cancer detection by using FAP-cleavable reporter constructs(133,134).

Enzymatic inhibition

Some of the small-molecules most commonly used as FAP inhibitors in research are PT-100 (Val-boro-Pro, talabostat) and linagliptin, but these inhibitors can also act on DPPIV. Despite
promising preclinical studies, clinical trials of PT-100 have shown minimal efficacy, even in combination with chemotherapy(135–137), and the reasons for this disconnect are not fully known. More recently, in a murine transplant model of colon cancer, chemotherapeutic treatment with oxaliplatin increased CAF markers (FAP, vimentin) in tumor while reducing overall tumor volume and enhancing overall survival. Combination treatment with oxaliplatin and PT-100 reduced CAF markers as well as further reducing tumor growth and enhancing survival. Within tumors, the combined treatment promoted apoptosis, restricted accumulation of dendritic cells and macrophages, and reduced vascularization(138). The success of PT-100 in this specific model could be due to the fact that the chemotherapeutic appeared to first enhance the desmoplastic response thus expanding the tumor region that could be targeted by FAP inhibition. To extrapolate to human cases, FAP inhibition may be most useful for those patients with highly desmoplastic tumors.

Similarly, linagliptin was used in conjunction with anti-PD1 for treatment of murine gastric cancer transplant tumors. This combinatorial approach showed enhanced survival relative to either treatment alone, along with reduced tumor volume, reduced collagen accumulation, and an enhanced ratio of CD8+ T-cells to Tregs in both tumor and lymph node(48). A pseudopeptide inhibitor of both FAP and PREP was able to inhibit xenograft growth of lung and colon cancers, causing enhanced apoptosis, reduced vascularization and accumulation of thick collagen fibers within the tumors(139). While all these results are encouraging in terms of clinical benefit, since none of the inhibitors used are entirely specific to FAP, it cannot be determined whether inhibition of FAP is their sole mechanism of action. One approach to demonstrating the necessity of FAP activity to any phenotype is comparing a dual-specific inhibitor to a DPPIV specific inhibitor; using this approach in lung cancer demonstrated that inhibition of FAP reduced growth of murine lung and colon cancers(93).

Designing highly specific FAP inhibitors is not impossible, however, as a recent study by Jansen, et. al. examined around 60 structurally related small molecules for inhibition of FAP, PREP, DPPIV, DPP9, and DPP2 and discovered several structural components that can yield highly specific FAP inhibitors with low in vivo toxicity which may be good candidates for future preclinical studies(140). Since there is some indication that FAP and DPPIV may play opposing roles in some cancer contexts(71), it will be interesting to see if FAP-specific inhibitors are more efficacious than broader spectrum DPP inhibitors. In terms of delivery and integration into combinatorial therapies, conjugation of both a FAP inhibitor and a reporter fluorophore to a polymer carrier yielded tumor-specific fluorescent signal; this concept could be extrapolated to combined administration of FAP inhibitors with chemotherapeutics(141).

**Conclusions**

The majority of recent studies relating to FAP in cancer fall into two categories: those that observe up-regulation of FAP in response to some stimuli and use this as a surrogate marker for pro-tumorigenic stroma, and those that describe therapeutic methods of depleting tumor stroma, using FAP as the target. Many of these papers are scientifically fascinating and clinically promising, but the fact remains that our understanding of FAP’s basic biology is far outstripped by our use of it as a molecular marker. A better understanding of FAP would
allow for the design of even more physiologically relevant studies, which might in turn enhance the—thus far rather low—efficacy of FAP-targeted therapies in the clinic. Major consensus of recent results reveals that FAP expression is of prognostic value in multiple tumor types, and that FAP itself promotes pro-tumorigenic functions of various cell types, including the proliferation and motility of mesenchymal, tumor, and immune cells. In this review we have asked, at what levels might FAP be expressed in normal, healthy and/or tumor adjacent tissue? Answering this question would allow us to better delineate therapeutic windows of any FAP-targeted therapy and indicate the importance of tumor-targeted delivery of FAP-targeted therapies. We have also asked what cell types FAP is truly expressed on in vivo, and what role mechanosignaling plays in the regulation of FAP, understanding which would allow us to design better model systems for additional studies of FAP. Knowing what signaling pathways FAP interacts with, including which functional roles of FAP rely on enzymatic vs. non-enzymatic activity would expand our repertoire of FAP-related targets. These open questions create the possibility of much exciting research about FAP yet to come.

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Figure 1. FAP expression in healthy adult mouse
Genetically engineered mice with luciferase reporter knock-in at both FAP alleles were administered luciferin ten minutes before euthanasia and organs harvested for imaging. Results indicate that FAP is expressed in skin, bone, pancreas, and at very low levels in the kidney. (Data generated by Leslie Hopper and Michele Jacob)
Multiple environmental and soluble factors have been observed to alter FAP expression, though detailed mechanistic pathways for many of them are unknown. The best characterized is TGFβ activation of SMAD3, which binds directly to the FAP promoter. A2BR: adenosine 2B receptor. ADO: adenosine. EE: estrogens. EGR-1: early growth response protein 1. ERα: estrogen receptor alpha. ERK1/2: extracellular signal-related kinases 1 and 2. FAP: fibroblast activation protein. GPR30: g-protein coupled receptor 30. hTERT: human telomerase reverse transcriptase. IL-1β: interleukin 1 beta. LPS: lipopolysaccharide. miR: microRNA. PGRN: progranulin. PTEN: phosphatase and tensin homolog. SiNW: silicon nanowires. SMAD3: mothers against decapentaplegic homolog 3. STAT3: Signal transducer and activator of transcription 3. TGFβ: transforming growth factor beta. TGFβR: TGFβ receptor. TLR4: toll-like receptor 4. TNFα: tumor necrosis factor alpha. UV: ultraviolet radiation.
Figure 3. Selected mechanisms of action for FAP in the TME
Shown are some of the best-elucidated pathways by which FAP promotes tumor growth. FAP can be expressed by macrophages, tumor cells, and tumor-promoting mesenchymal stromal cells, and it exerts effects though both enzymatic and non-enzymatic means. CCL2: (MCP1) c-c motif chemokine ligand 2. Col I: type I collagen. FAK: focal adhesion kinase. FAP: fibroblast activation protein. hFGF-21: human fibroblast growth factor 21. MMPs: matrix metalloproteinases. NPY: neuropeptide Y. PI3K: phosphoinositide 3-kinase. PTEN: phosphatase and tensin homolog. RhoA: ras homolog gene family member A. SR-A: (MSR1) class A macrophage scavenger receptor. uPAR: urokinase plasminogen activator receptor.
### Table 1

### FAP expression profiling in various tumor types

Recent studies on patterns of FAP expression are summarized by tissue of cancer origin, cell type observed to express FAP, and methods of detection used. The relevant reagents, mostly antibodies, are reported with the same level of detail available in each study’s material and methods section.

| Cancer  | Cell                      | Method          | Reagents                                                                 | Reference |
|---------|---------------------------|-----------------|--------------------------------------------------------------------------|-----------|
| Bone    | Tumor                     | IHC             | Mouse antibody (Santa Cruz Biotechnology, Inc.)                          | 98        |
| Brain   | Astrocyte, stem cell, stroma, macrocyte | ELISA, WB, IF, qRT-PCR | DuoSet ELISA kit (R&D systems), rat monoclonal D8 and D28 (Vitatex), mouse monoclonal F19 | 22        |
| Breast  | Fibroblast                | IHC, qRT-PCR    | NR                                                                      | 16        |
| Breast  | Tumor, stroma             | IHC             | Rabbit polyclonal antibody ab53066 (Abcam)                              | 34        |
| Breast  | Tumor, stroma             | IHC             | Polyclonal antibody (Abcam)                                             | 36        |
| Breast  | Tumor, stroma             | IHC             | Polyclonal antibody (Abcam)                                             | 35        |
| Breast  | NR                        | qRT-PCR         |                                                                          | 99        |
| Colorectal | Stroma                   | IHC             | Unspecified antibody (Abcam)                                            | 47        |
| Esophageal | NR                       | WB, RT-PCR      | Rabbit polyclonal antibody NIN3 (GeneTex, Inc.)                          | 19        |
| Esophageal | Stroma                   | ELISA, IHC, IHC | DuoSet ELISA kit (R&D systems), sheep polyclonal antibody AF3715 (R&D) | 25        |
| Esophageal | Stroma                   | IHC             | Unspecified antibody (Abcam)                                            | 54        |
| Gastric | Stroma                    | IHC, IF         | Unspecified antibody (Boster Biological Technology)                      | 48        |
| Gastric | Mesothelia                | IHC             | Rabbit polyclonal antibody (Abcam)                                      | 40        |
| Liver   | Stroma                    | IHC             | Rabbit polyclonal antibody SAB2900181 (Sigma)                           | 59        |
| Liver   | Stroma                    | IHC             | Rat monoclonal D8 (Vitatex)                                             | 53        |
| Lung    | Tumor                     | IHC             | Rabbit polyclonal antibody (Assay Biotech)                              | 31        |
| Oral    | Tumor                     | IHC, IF         | Rabbit polyclonal antibody (LifeSpan BioSciences)                       | 51        |
| Ovarian | Stroma, tumor             | IHC             | Rabbit polyclonal antibody LS-A8023 (LifeSpan BioSciences)              | 52        |
| Ovarian | Tumor, mesothelia         | IHC, ISH        | Rabbit polyclonal antibody ab53066 (Abcam)                              | 39        |
| Pancreatic | Stroma, tumor            | ELISA, IHC, IF  | DuoSet ELISA kit (R&D systems), polyclonal antibody (LSBio), mouse monoclonal F19 | 20        |
| Pancreatic | Stroma, tumor            | IF              | Mouse monoclonal F11-24 (Santa Cruz Biotechnology, Inc.)                | 21        |
| Pancreatic | Stroma, tumor            | IHC             | Rabbit polyclonal antibody ab53066 (Abcam)                              | 37        |
| Pancreatic | Stroma, tumor            | IHC, IF         | Sheep polyclonal antibody AF3715 (R7D Systems), mouse monoclonal F19    | 38        |
| Parathyroid | NR                      | RT-PCR, qRT-PCR |                                                                          | 15        |
| Renal   | Stroma                    | IHC             | Rabbit polyclonal antibody ab53066 (Abcam)                              | 45        |
| Renal   | Stroma                    | IHC             | Rabbit polyclonal antibody ab53066 (Abcam)                              | 46        |

ELISA: enzyme linked immunoassays. IF: immunofluorescence. IHC: immunohistochemistry. ISH: in situ hybridization. NR: not reported. (q)RT-PCR: (quantitative) reverse transcriptase polymerase chain reaction. WB: western blot.