Arousal of cancer-associated stromal fibroblasts
Palladin-activated fibroblasts promote tumor invasion

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Cancer-associated fibroblasts (CAF), comprised of activated fibroblasts or myofibroblasts, are found in stroma surrounding solid tumors; these myofibroblasts promote invasion and metastasis of cancer cells. Activation of stromal fibroblasts into myofibroblasts is induced by expression of cytoskeleton protein, palladin, at early stages in tumorigenesis and increases with neoplastic progression. Expression of palladin in fibroblasts is triggered by paracrine signaling from adjacent k-ras-expressing epithelial cells. Three-dimensional co-cultures of palladin-expressing fibroblasts and pancreatic cancer cells reveals that the activated fibroblasts lead the invasion by creating tunnels through the extracellular matrix through which the cancer cells follow. Invasive tunneling occurs as a result of the development of invadopodia-like cellular protrusions in the palladin-activated fibroblasts and the addition of a wounding/inflammatory trigger. Abrogation of palladin reduces the invasive capacity of these cells. CAF also play a role in cancer resistance and immuno-privilege, making the targeting of activators of these cells of interest for oncologists.

Myofibroblasts, also referred to as cancer-associated fibroblasts (CAF), have smooth muscle cell-like contractile properties and positive α-smooth muscle actin (α-SMA) staining.1

The mechanism by which myofibroblasts enhance tumorigenesis and metastases is complex and may involve the enhanced secretion of soluble growth factors, increased contractility and mechanostimulation of the cancer cells, and physical remodeling of the extracellular matrix to create metastasis-promoting channels.2-8 Myofibroblasts can have a critical influence on immune surveillance, as well as chemo and radio-resistance to tumors.8-11 Moreover, hypoxic conditions caused by the exuberant growth of CAF surrounding cancer may also contribute chemotherapy resistance through increased hydrostatic pressure and compression/loss of local vasculature.7,12 Recent breakthroughs shed light on the timing and mechanism of this important step in tumorigenesis: fibroblast activation in cancer.

Stromal Fibroblasts are Activated Early in Tumorigenesis

Stromal fibroblast activation occurs early prior to cancer development. In human pancreatic cancer and mouse models of pancreatic cancer, CAFs are present surrounding the high-grade dysplastic lesions in the pancreas and even to a lesser extent in the low-grade dysplastic lesions.13,14 Similar findings in hepatocellular carcinoma and oral squamous cell carcinoma and their dysplastic precursor lesions have been found.15,16 These data suggest that cancer is not necessary for the transformation of CAF, but rather myofibroblast activation...
occurs earlier in the process of neoplastic progression when dysplasia is present.

**The Mechanism of Fibroblast Activation Implicates Two Factors: Palladin Expression and Inflammation**

Palladin appears to play a key role in fibroblast transformation in some cancers, including pancreatic cancer and breast cancer. Palladin is an embryonic protein that plays a key role in cellular migration. It is a cytoskeletal protein that acts as a scaffold and serves to crosslink the components of stress fibers, actin bundles, Z discs, focal adhesions and other subcellular structures. Palladin is upregulated in the leading edge of wounds and in the cancer-associated fibroblasts of metastatic cancers. Interestingly, palladin has also been detected in expression screens for invasion-specific genes in pancreatic and breast cancer.

Palladin is upregulated in the CAF of pancreatic cancer early during tumorigenesis—it is overexpressed in the stromal fibroblasts immediately surrounding low and high-grade dysplasia. The expression of palladin closely correlates with the expression of α-SMA in these pre-cancerous lesions. Our recent studies to unravel the role of palladin in fibroblast activation in cancer reveal that simply co-culturing a normal human fibroblast next to a pancreatic cancer cell is sufficient to impart myofibroblast properties to the fibroblast; this process occurs in a palladin-dependent fashion (Fig. 1). Because myofibroblasts can be detected early in tumorigenesis, we tested whether the initiating event in pancreatic ductal adenocarcinoma, e.g., a k-ras mutation in an epithelial cell, was sufficient to cause transformation of an adjacent resting fibroblast into a myofibroblast. Transwell experiments involving normal fibroblasts co-cultured with normal epithelial cell expressing wild-type or mutated k-ras were performed: activated k-ras (wild-type or mutated) paracrine signaling is sufficient to induce the adjacent, but not-touching, quiescent fibroblasts to become myofibroblasts. Abrogation of palladin, using siRNA, causes loss of the myofibroblast phenotype, including loss of common myofibroblast markers such as α-SMA, and loss of myofibroblast function, such as migration and invasion.

Curiously, palladin-expressing fibroblasts appear to be primed but not activated. The palladin-expressing fibroblasts have the phenotype of a myofibroblast: an elongated shape and expression of α-SMA and vimentin; however, an inflammatory or wounding signal is required for the myofibroblasts to become migratory and invasive. In absence of the inflammatory signal, the palladin-expressing myofibroblasts remain dormant, with diminished capability for migration or invasion. This finding might be one reason for the underlying inactivity of some indolent tumors, if inflammation is absent the fibroblast-led invasion cannot occur. In summary, while a palladin-expressing fibroblast is primed, expressing all of the proteins one would expect in a myofibroblast, it does not yet act as a leading partner for cancer cell invasion without the inflammatory signal. In the clinical setting, such inflammation could be driven from environmental factors such as smoking, infections or inflammatory cytokines associated with obesity.

**Stromal-Assisted Cancer Invasion and Metastases**

Myofibroblasts can produce tracks within the extracellular matrix, which in effect, create tunnels for the carcinoma cells to follow. More recently, we have identified the mechanism of how this fibroblast-led cancer invasion occurs. Upregulation of palladin causes the fibroblast to develop a fusiform/mesenchymal shape with apparent invadopodia—feet that contain proteolytic enzymes (Fig. 2). To identify the contents of the “feet” of palladin-activated myofibroblasts, we ensnared the myofibroblasts in the act of invasion in a sieve that was large enough to let the feet through, but too small for a whole cell to pass through. Proteomic analysis performed on the ensnared and isolated “feet” revealed the overexpression of proteolytic enzymes such as metalloproteinases and cathepsin, invadopodia proteins and proteins associated with poor prognosis in cancer. Functional studies demonstrated that, in the setting of an inflammatory or wounding signal, the palladin-activated fibroblasts can both rip and destroy the extracellular matrix literally creating tunnels through which the cancer cells follow. Fibroblasts without palladin expression have markedly diminished capacity to create tunnels and cancer cells do not follow them (Fig. 3). Remarkably, once the activated myofibroblasts escort the cancer cells through tunnels in the organ of origin, labeling studies have shown that the cancer cell and myofibroblasts invade together through blood vessels.
vessels and implant in metastatic sites.\textsuperscript{24,25} In support of these studies, we found palladin-expressing fibroblasts adjacent to cancer cells in lymph node and liver metastases.\textsuperscript{14}

**Metastasis Can Occur before Cancer Formation**

Elegant studies by Rhim et al., using lineage tracing in a pancreatic cancer engineered mouse model, revealed that mutant ductal cells undergo epithelial mesenchymal transformation (EMT), invade into the blood stream, and lodge into metastatic sites such as the liver prior to histologic evidence of cancer.\textsuperscript{26} The invasion of these mutant epithelial cells occurs in 2.7\% of all PanIN 2 (low-grade dysplasia) and 6.8\% of PanIN 3 (high-grade dysplasia) lesions, but never in the setting of PanIN 1 (hyperplasia). Inflammation is required for the dissemination of PanIN 2 and 3 cells to occur. Not surprisingly, COX-2, an inflammatory mediator, is increasingly overexpressed between PanIN lesions and malignant pancreatic tissues.\textsuperscript{27} In the studies by Rhim, if dexamethasone was added as an anti-inflammatory agent, the dissemination of mutated circulated pancreatic epithelial cells was abolished. Even more amazing was the loss of PanIN lesions and associated myofibroblasts within the pancreatic parenchyma in the setting of dexamethasone taken on a daily basis: the pancreata return to a normal appearance, while the control mice proceed to get pancreatic adenocarcinoma. Taken as a whole, this work implicates the invasion of mutated cells earlier than originally thought in cancer and would help explain the very lethal nature of some cancers, such as pancreatic, even when the tumors are quite small. The early activation of fibroblasts into tunneling myofibroblasts by k-ras mutated epithelial cells fits in mechanistically with the model of earlier invasion of epithelial cells prior to cancer formation. Abolition of inflammation reverses the invasion process.

**Targeting the Stromal Fibroblasts**

Because of the interdependent behavior of cancer cells and stromal fibroblasts, the latter have become a target of interest for oncologists.\textsuperscript{28} Pancreatic cancer cells have increased resistance to gemcitabine, in part due to direct activation of the Hedgehog pathway resulting from cross-talk between myofibroblasts and adjacent cancer cells.\textsuperscript{29,30} Recent chemotherapy using a Hedgehog inhibitor results in a significant loss of the tumor-associated fibroblasts in pancreatic cancer and prolonged survival in mouse models.\textsuperscript{7} However, in the latter trial the mice relapse when the myofibroblasts repopulate. This finding, combined with the negative outcome of a recent human phase III clinical trial testing the efficacy of chemotherapy and hedgehog

![Figure 2](image-url)

**Figure 2.** Normal human fibroblasts transfected with an empty vector (EV) remained boxy in appearance and had no effect on collagen or matrigel when exposed to wounding media (A and B). In contrast, fibroblasts transfected with wild-type palladin (WT) became elongated with mesenchymal features (C), caused destruction of the collagen matrix (D) with apparent clumping of the collagen edges. Additionally, palladin-expressing fibroblasts created tunnels in matrigel (stained red) when exposed to wounding media in 3D invasion cultures (E). Fibroblasts, stained white in (F), became quite elongated when tunneling. Tunnel is delineated by yellow arrowheads. Scale bars indicate 20 \(\mu\)m.
pathway inhibition, suggests that compensatory pathways may exist if only one pathway in the targeting of CAF is abrogated. This is particularly of issue because there are usually myofibroblasts remaining at the surgically resected pancreatic cancer margins and the sources for tumor stromal fibroblasts may be derived from both local and potentially non-local sources.31

Other methods of directly targeting the CAF have included use of monoclonal antibodies, drugs, and vaccines. A novel monoclonal antibody targeting fibroblast activation protein (FAP), a cell surface protease of activated tumor fibroblasts, has been shown to induce long-lasting inhibition of tumor growth and complete regression in xenograft models of lung, pancreas, and head and neck cancers.32 Vaccination against stromal fibroblasts targeting FAP has also shown some promise in mouse models.33 While no current therapy targets palladin expression...
in fibroblasts, we have performed preliminary studies suggesting that reagents that target anti-SMA and regulate hepatic stellate cell activation, (such as PPARγ agonists and metformin) are useful when used in combination to block palladin expression in myofibroblasts and human CAF. These combined palladin-targeting therapies concomitantly block α-SMA expression and the myofibroblast phenotype (unpublished data). Further investigation using small molecular and high-throughput drug screening is required to identify which drugs are most effective in blocking palladin and whether these drugs are effective in the early and the late stages of cancer in mouse models.

Therapies to decrease the inflammatory component of carcinogenesis have included use of aspirin, COX2 inhibitors such as celecoxib, NFκB inhibitors such as curcumin and PPARγ agonists such as troglitazone. Some of these anti-inflammatory drugs have been used in human phase II trials with mixed success, where many of the patients had later stage tumors. With our current knowledge of the role of inflammation in driving forth the early dissemination of myofibroblast-aided cancer cells, it is possible that the inflammation needs to be treated earlier in the neoplastic progression—before the cancer cells have escaped. In keeping with this concept, the effective use of anti-inflammatory drugs has been reported in chemoprevention trials.

Cross-talk between fibroblasts and cancer cells is essential to invasion and

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**Figure 4.** Stromal fibroblasts in normal pancreas are quiescent and without palladin staining. K-ras activation in ductal cells leads to paracrine signaling that is sufficient to induce palladin-associated myofibroblast transformation of the adjacent fibroblasts. This event occurs early in tumorigenesis, when epithelial cells are dysplastic, and increases with neoplastic progression. In the setting of a wounding signal, the palladin-activated fibroblasts develop cellular protrusions (feet) that express invadopodia proteins, proteases, and enhance the capacity for invasion. The palladin-activated fibroblasts create tunnels through the matrix, assisting the escape of cancer cells into the neo-vasculature. The activated fibroblasts appear to accompany the cancer cells to their metastatic niche in breast and pancreatic cancer models.
potentially chemotherapeutic agents that disrupt this process could be effective. Inflammatory cytokines and signaling molecules including TNF-α, IL-6, IL-1α/β, NFκB and TGF-β play key roles in paracrine signaling between tumor cells and fibroblasts, as outlined in an excellent review by Bhomick and Moses.45-49 Therapeutics designed to modulate these molecules are described elsewhere; in general most of these therapies are relatively new and thus trials of some of these agents are just being undertaken in humans.40,50

Figure 4 summarizes steps and mechanism of myofibroblast activation and the partnership these cells play in cancer invasion. Although difficult, targeting the stromal fibroblasts remains an attractive strategy in the fight of aggressive cancers because of the interdependence of the CAF and the cancer cells. In addition, it may be valuable to determine when during the neoplastic process targeting the CAF is most effective: in the initiating stages of tumorigenesis or whether the strategy can be effective in an established, even metastatic cancer.

Summary

The arousal of the stroma is a key and transformative event in the invasive stages of tumorigenesis in many solid tumors. Stromal fibroblasts can be transformed through paracrine signaling of adjacent k-ras overexpressing epithelial cells. The fibroblast then undergoes phenotypic change into a myofibroblast that is mediated through palladin, a cytoskeletal protein essential in cell motility. However, this change is insufficient to cause fibroblast-assisted cancer cell invasion and migration. For the latter events to occur, an additional wounding or inflammatory signal is required. The presence of these three events (overexpression of k-ras, palladin-expression in the fibroblasts and inflammatory signal) instigates the dynamic relationship between the stroma and the mutated epithelial cell. These three events are sufficient for the activated myofibroblast to tunnel through the extra-cellular matrix and provide avenues for the dysplastic and cancerous epithelial cells to follow. Abrogation of palladin or the inflammatory signal is sufficient to shut down the process.13 Future studies will help elucidate the role of epithelial-mesenchymal transition to enhance the migration of cancer cells through the fibroblast-created tunnels and the potential for chemotherapeutic targeting of the initiating events in cancer invasion.

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