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Trailblazing LIM kinases take the lead in collective tumor cell invasion

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The spread of tumor cells from primary sites often occurs as associated cell collectives. In this form of invasion, the contribution of cells leading the way may differ from those that follow. By implication, proteins that regulate the actin cytoskeleton, a major driver of cell motility, may have different roles depending on whether they are in leading or following cells. The LIM kinases 1 and 2 (LIMK) phosphorylate and inactivate the filamentous actin severing function of cofilin proteins. Using siRNA or pharmacological inhibitors, LIMK was found to be required in leading cells of collectively invading tumor cells, or in cancer-associated stromal fibroblasts, for effective extracellular matrix degradation that facilitates three-dimensional invasion. The decreased extracellular matrix degrading activities were associated with an inability to form the stable filamentous actin structures necessary to make matrix-degrading protrusive structures. However, LIMK was not required for cell motility or for path-following in associated collectives. These findings show that leading and following cells in collective invasion have different properties and indicate that targeting the activities in leading cells is sufficient to significantly inhibit tumor cell invasiveness.

Key words: actin, LIMK, motility, invasion, metastasis, Rho, cofilin

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The metastatic spread of tumor cells from primary to distal sites mounts the most significant challenge to the well-being of cancer patients.[1] As a result, there is intense interest in understanding the underlying molecular mechanisms that drive the local invasion, dissemination and ultimately metastasis of cancer cells. In particular, a major objective sought by academic and industrial researchers is the identification of potentially druggable targets that if inhibited would limit cancer spread without affecting normal cell and tissue functions. The actin cytoskeleton is the motor that powers cell motility, and numerous proteins contribute to the dynamic flux of cytoskeletal structures that facilitate movement.[2] Therefore, proteins that regulate the actin cytoskeleton are attractive as targets for inhibiting cancer spread. Given that relatively straightforward enzymatic assays and focussed chemical libraries have been developed to help kinase inhibitor discovery, we decided that attractive candidate targets for potential anti-metastatic agents were the LIM kinase 1 and 2 (LIMK).

LIM kinases act as network hubs in signaling pathways that communicate from Rho GTPase proteins to the regulation of the actin cytoskeleton.[3] Their primary vocation appears to be the phosphorylation and inactivation of the filamentous-actin (F-actin) severing cofilin family proteins,[4] although there is some evidence that LIMK may also have cofilin-independent functions.[5] When unphosphorylated, cofilin proteins sever F-actin within aged regions in which ATP hydrolysis to ADP has induced a conformational change. As a result, activation of LIMK through the phosphorylation of a Threonine residue within the activation loop by upstream kinases including ROCK, PAK and MRCK leads to increased F-actin stability,[3] while LIMK inhibition would be expected to have the opposite effect and decrease F-actin stability.

In order to examine the contribution of LIMK to tumor cell invasiveness, we decided to adopt a two-pronged approach using siRNA-mediated knockdown and a potent, selective and non-cytotoxic

LIMK inhibitor that had been developed by Bristol-Myers Squibb.[6] Using three-dimensional invasion assays we found that LIMK inhibition by either method resulted in significantly decreased invasion.[7] Interestingly, although many reports in the literature have implicated a specific role for either LIMK1 or LIMK2 in various processes,[8-11] we found that the selective knockdown of either protein alone had only small effects that were additive when both were targeted or inhibited simultaneously. Similar results were reported for individual versus combined knockdown of LIMK1 and LIMK2 in a zebrafish xenograft model of pancreatic cancer metastasis.[12] Given the role of LIMK in regulating F-actin and the significant effects on decreasing three-dimensional invasion, we were surprised that both siRNA-mediated knockdown and LIMK inhibition did not affect cell motility using a modified scratch would assay or when intrinsic motility on fibroblast-derived matrix was measured. The discrepancies between the effects of LIMK inhibition on three-dimensional invasion through matrix protein and two-dimensional motility suggested that the most likely explanation was an effect on the ability of cells to create a path through the mechanically-resistant protein environment through which they could travel.

Although the importance of studying cell invasion through three-dimensional environments has become widely accepted as being more relevant to the in vivo situation than simple two-dimensional motility models,[13] it is often the behavior of highly-invasive tumor cells that invade as individuals that is examined. However, the ability of tumor cells to escape from the primary tumor mass and locally invade their surroundings as individuals is actually rare and somewhat unusual. Instead, epithelial cancer cells often invade collectively in strands, sheets and clusters without necessarily losing their cell-cell adhesions.[14] The cell collective may remain in contact with the tumor, or may break free and move away from the primary site. In this form of invasion, the leading cells create paths of reduced physical resistance through a combination of protease activity and mechanical force to remodel the extracellular matrix.[15] As a result, cells may have different roles in the invading collective; for example path-generating cells would require extracellular matrix remodeling activities versus path-following cells which would only require the ability to move through these paths. Upon reflection, this observation makes a great deal of sense, the acquisition of all the properties required for a tumor cell to break away from the primary mass and become independently invasive is actually relatively improbable. As long as the occasional tumor cell is able to be a trailblazer, a proportionally greater number of cells will probably have acquired the smaller subset of properties that enable them to be path-followers. An additional possibility is that non-tumor stromal cells may be selected for, or re-programmed through the secretion by tumor cells of paracrine factors such as TGFβ,[16] to provide the path-generating activity. Again, this would enable the more probably acquired properties required for path-following to be manifested in collective tumor cell invasion.

As mentioned above, many of the invasive cell lines commonly used in three-dimensional assays invade as individuals. Although the MDA MB 231 breast cancer cell line is often described as being mesenchymal-like,[17] it retains sufficient epithelial characteristics that it invades three-dimensional matrigel as collective strands.[18] In order to delineate the contribution of LIM kinases to three-dimensional collective invasion, we created two pools of cells stably expressing either membrane-targeted red or green fluorescent proteins (RFP or GFP) by drug selection followed by enrichment for fluorescence intensity by live cell sorting. When the two colored pools were mixed, the overall invasion was comparable to the original parental cell line, and image analysis revealed that there were equal representations of RFP or GFP expressing cells at the lead position of each strand. One of the most challenging aspects of these experiments was the confocal imaging and three-dimensional reconstructions required to identify each strand's leading cell. However, the effort was rewarded as this system allowed us to query whether specific proteins were necessary in leading cells. Knocking down LIM kinases in one color cell line while transfecting the other color cell line with control siRNA revealed that LIMK was required for path-generation, since the corresponding color cell was under-represented in the leading position. However, the identity of the cell immediately after the leading cell was equally split between the LIMK-targeted and the control siRNA-transfected colors, indicating that path-following abilities were unaffected by LIMK inhibition. These results were again consistent with the conclusion that LIMK activity was required for matrix remodeling that facilitates path-generation but not for cell motility that enables cells to follow paths.

Using a variety of two-dimensional and three-dimensional models, we found that knocking down or inhibiting LIMK reduced matrix degradation and the secretion of active matrix metalloprotease activity. These effects were associated with an inability to form protrusive matrix-degrading invadapodia structures, which previous research has shown to be dependent on F-actin stabilization.[19-21] Not only did LIMK inhibition result in an overall decrease in F-actin staining intensity, but fluorescence recovery after photobleaching (FRAP) experiments revealed that F-actin stability was significantly reduced. As a result, we concluded that at least one way that LIMK inhibition impaired the ability of cells to lead collective invasion was through reduced F-actin stability, which directly impacted upon the ability of cells to degrade matrix. In support of this conclusion, direct knockdown of matrix metalloprotease 9 also reduced the ability of cells to be path-generating leading cells. However, it cannot be excluded that an additional factor might be a reduction in the delivery of matrix metalloproteases from the Golgi to the plasma membrane or extracellular surroundings, given that a role for cofilin in cargo sorting at the trans-Golgi network has been reported.[22]

We also examined whether inhibiting LIMK would affect the ability of cells to physically remodel the extracellular matrix by measuring collagen contraction. One possibility is that the decreased F-actin stability resulting from LIMK inhibition would lower internal

actomyosin contractile force and cellular tension, leading to a reduction in external pulling force on matrix protein fibrils. We did indeed observe that knocking down or inhibiting LIMK reduced collagen contraction by cancer-associated fibroblasts (CAFs).[23] Given that inhibiting LIMK also reduced matrix degradation, we also wondered whether proteolysis actually facilitated contraction. When MT1-MMP, which is the major matrix metalloprotease in CAFs,[7] was knocked down there was a significant inhibition in collagen contraction. Therefore, we concluded one way LIMK activity contributes to matrix remodeling is to facilitate the proteolysis of cross-linked matrix proteins that would otherwise be too rigid for contraction to occur. It remains a possibility that an additional contribution of LIMK activity is to promote F-actin stability which contributes to actomyosin contractile force generation. There currently is heated debate about whether the extent of matrix protein cross-linking in three-dimensional model systems is an accurate reflection of the in vivo situation,[13,24] with implications for the interpretation of studies in which the motility modes of individual tumor cells has been examined. However, it seems likely that the dogmatic division of three-dimensional motility modes into two distinct categories as either requiring matrix degradation or force-mediated remodeling[25] may be somewhat artificial since force-mediated remodeling actually appears to be dependent on some level of matrix degradation. Further research will reveal the extent of the interdependence of these two matrix remodeling activities.

An interesting and important concept that has emerged from recent studies is the remarkable plasticity of cancer cell motility modes.[26] Although some cells seem to prefer to force their way through three-dimensional matrix while others tend to use proteolysis to generate their paths, tumor cells will adopt the most favorable mode when meeting an impasse. Similarly, cells that are capable of invading as individual cells may switch to collective invasion if circumstances dictate. We observed that the individual three-dimensional matrix invasion by BE colon carcinoma cells[27] converted to collective invasion when LIMK was inhibited (**Fig. 1**). This observation suggests that collective invasion may indeed be a reflection of relatively less effective invasive abilities than individual cell invasion. An additional possibility is that LIMK inhibition promotes the restoration of cell-cell adhesions that favor collective over individual invasion.

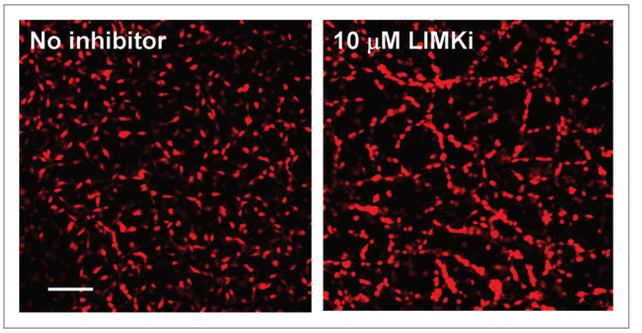

Figure 1. LIMK inhibition converts the invasion mode of BE colon carcinoma cells from individual to collective. Optical slices of three dimensional matrigel invasion assays of propidium iodide stained BE colon carcinoma cells revealed that their typical individual invasion mode (left) was converted to invasion of collective strands by LIMK inhibitor treatment (right). These findings suggest that individual tumor cell invasion has higher demands for the activities that contribute to invasiveness (e.g., remodeling of the extracellular matrix, reduced cell-cell contacts) than collective invasion, in which tumor cells may remain in contact with each other and matrix remodeling need only be performed by leading cells. Scale bar = 100 μm.

The major challenge for the future will be providing the "proof of principle" evidence in clinical studies which demonstrates that specifically targeting the processes that contribute to invasion and metastasis translates into patient benefit. Given the tremendous plasticity and adaptability of tumor cells when encountering changes in their environment, this may prove to be an unachievable goal. However, since it has been suggested that metastasis plays a significant role in the mortality of 90% of cancer sufferers,[1] even incremental successes could have substantial impacts upon the quality of life and survival of numerous cancer sufferers. Time will tell whether the effects of inhibiting LIMK on cancer cell invasion that have been reported by us and others will lead to eventual cancer therapies, but the potential for positive clinical outcomes is a strong motivator for continued research.

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