**Integrin linked kinase (ILK) expression and function in vascular smooth muscle cells**

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Vascular smooth muscle cell (SMC) migration and proliferation contribute to arterial wound repair and thickening of the intimal layer in atherosclerosis, restenosis and transplant vascular disease. These processes are influenced by cell adhesion to molecules present in the extracellular matrix, and regulated by the integrin family of cell-surface matrix receptors. An important signaling molecule acting downstream of integrin receptors is integrin-linked kinase (ILK), a serine/threonine kinase and scaffolding protein. ILK has been implicated in cancer cell growth and survival through modulation of downstream targets, notably Akt and glycogen synthase kinase-3β (GSK3β). Evidence also exists to establish ILK as a molecular adaptor protein linking integrins to the actin cytoskeleton and regulating actin polymerization, and this function may not necessarily depend upon the kinase activity of ILK. ILK has been implicated in anchorage-independent growth, cell cycle progression, epithelial-mesenchymal transition (EMT), invasion and migration. In addition, ILK has been shown to be involved in vascular development, tumor angiogenesis and cardiac hypertrophy. Despite the documented involvement of integrin signaling in vascular pathologies, the function of ILK has not been well characterized in the SMC response to vascular injury. This brief review summarizes and puts into context the current literature on ILK expression and function in the vascular smooth muscle cell.

A large body of research is dedicated to elucidating the mechanisms by which smooth muscle cells (SMCs) contribute to thickening of the arterial wall in pathologies such as atherosclerosis and restenosis. After arterial injury and during neointimal hyperplasia, SMCs undergo a phenotypic switch characterized by the transition from a quiescent to an active/synthetic phenotype, and they begin to synthesize an abundant extracellular matrix. In turn, interactions between cells and the matrix govern the process of neointimal thickening. Cell surface integrin receptors play important roles in signaling proliferative and migratory cellular responses during arterial wound repair. Integrin-linked kinase (ILK) is an important downstream mediator of integrin signaling, yet little is known of its function in the arterial response to injury.

Integrin-linked kinase (ILK) was originally identified as a serine/threonine kinase binding to the cytoplasmic domain of β1- and β3-integrin subunits. ILK functions to activate Akt and inhibit glycogen synthase kinase-3β (GSK3β), and has been implicated in cancer cell growth and survival through modulation of these downstream targets. Given its role in anchorage-independent growth, survival and cell cycle progression, epithelial-mesenchymal transition (EMT), and invasion and migration, it is often suggested that ILK be targeted for cancer treatment. ILK is also involved in vascular development and tumor angiogenesis. Concurrent studies in model organisms and cell cultures point to a role for ILK as a molecular scaffold linking integrins to the actin cytoskeleton and regulating actin polymerization. Furthermore, this scaffolding function may be independent of the kinase activity of ILK. In *C. elegans*, genetic ablation of pat-4/ilk (ILK homologue) leads to severe adhesion defects, muscle detachment and embryonic lethality. However, PAT-4/ILK does not phosphorylate GSK3β in *C. elegans*. Similarly, in *Drosophila melanogaster*, loss of function mutants for ILK resulted in severe embryonic muscle-attachment defects and detachment of F-actin from the cell membrane, and the muscle attachment defect was rescued by expressing a kinase-deficient ILK. Finally, tissue-specific conditional knockout of ILK in mouse chondrocytes results in defects in the skeleton, and inhibition of cell adhesion, spreading and cytoskeletal assembly in chondrocytes in culture. These deficiencies were not attributable to impaired Akt or GSK3β signaling. In fact, the importance of ILK kinase function appears to be cell type-dependent. Inhibition of ILK activity in transformed cells resulted in a decrease in Akt phosphorylation and apoptosis, but had no effect in non-transformed cell types including vascular SMCs, thus calling into question the importance of ILK as a kinase in non-cancerous cell types.

We have studied the function of ILK in vascular smooth muscle cell wound repair and found that ILK acted as a scaffolding protein at focal adhesion sites. In our experiments, immunostaining of cultured SMCs revealed co-localization of ILK and paxillin at focal adhesions, a finding which is consistent with a previous study. Several proteins such as PINCH1, parvins and paxillin interact directly with ILK to facilitate its localization to focal adhesions and coordinate actin organization and cell spreading. Overexpression

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of an ILK-binding-deficient PINCH protein in tracheal SMCs led to decreased recruitment of ILK and PINCH to focal adhesions, and decreased association between ILK, paxillin and vinculin.\textsuperscript{26}

We hypothesized that ILK acting as a scaffolding protein might regulate the SMC response to vascular injury. To study this, we examined ILK using in vitro models mimicking vascular injury. Silencing ILK expression with siRNA decreased cell adhesion to fibronectin, and accelerated cell proliferation and wound closure.\textsuperscript{21} However, silencing ILK in wounded SMCs did not attenuate the increase in Akt and GSK3\textsubscript{B} phosphorylation observed after wounding.\textsuperscript{21} Nonetheless, we observed rearrangement of focal adhesions and stress fibers in ILK-silenced SMCs, which may have contributed to the reduced adhesion to fibronectin and enhanced cell migration and proliferation. Thus it seems that the scaffolding role of ILK may be more important for focal adhesion dynamics and remodeling in SMCs than the kinase function of ILK. These results were also surprising because they imply that ILK functions to inhibit cell growth and motility, unlike several studies which have suggested that ILK signals to increase these processes.\textsuperscript{7,8,10}

To address in vivo arterial wound repair, we studied ILK expression after balloon catheter injury of the rat carotid artery. Following balloon injury, SMCs undergo a process of dedifferentiation which includes enhanced proliferation and migration from the media to the intima. We found that ILK protein expression was dramatically decreased in the media during the SMC proliferative and migratory responses.\textsuperscript{21} The rapid decrease in ILK protein expression is consistent with the effects of silencing ILK in cultured SMCs. We propose that the decrease in ILK following injury facilitates the rearrangement of focal adhesions, altering cell adhesion to facilitate SMC migration and proliferation. The decrease in ILK expression in SMCs following injury may be related to the transition of these cells to a de-differentiated state. A recent study has shown that increased ILK expression correlates with cell differentiation in the luminal layers of the epithelium in the esophagus, colon and intestines when compared to the basal layers.\textsuperscript{27} ILK was also prominent in more differentiated areas of malignant tumors. In our studies, we noted an increase in ILK expression in the layers of the intima closest to the vascular lumen. This was consistent with findings in another recent study reporting increased ILK protein expression in the intima of balloon-injured rat carotid arteries in vivo and in the developing intima of human saphenous veins cultured ex vivo.\textsuperscript{28} We suggest that ILK is upregulated here in coincidence with the re-establishment of SMC quiescence.

In addition to maintaining stable cell adhesion to matrix, in the quiescent differentiated SMC, ILK may function to mediate contraction and aid the cell in exerting force on surrounding extracellular matrix fibers. In SMCs, ILK is localized to myofilaments, and promotes cell contraction by directly phosphorylating myosin light chain (MLC) or myosin light chain phosphatase (MLCP).\textsuperscript{9,29,30} Alternatively, ILK may activate smooth-muscle contraction indirectly via phosphorylation and activation of MLCP inhibitors including CPI-17 and PHI-1.\textsuperscript{29} Consistent with a role for ILK in mediating contraction, stimulation of tracheal SMCs with acetylcholine recruits ILK and PINCH to the cell membrane, and overexpression of an ILK-binding-deficient mutant PINCH attenuated the localization of ILK at adhesion sites, and attenuated actin polymerization, the activation of the actin nucleation initiator N-WASP, and the development of tension.\textsuperscript{26} ILK has also been identified as a key regulator of cardiac myocyte contractility.\textsuperscript{31} Likewise, ILK is required in the skeletal muscle of zebrafish for integrin-matrix adhesion to maintain the stability of muscle fibres.\textsuperscript{32} Mice with a skeletal muscle-specific deletion of ILK develop muscular dystrophy and detachment of muscle cells from basement membranes.\textsuperscript{33} ILK mutants also showed displacement of several focal adhesion proteins and reorganization of the actin cytoskeleton.\textsuperscript{34}

Our results after silencing ILK expression differ somewhat from a previous study of ILK in vascular SMCs. Overexpression of wild-type ILK in SMCs increased cell migration in response to stromal derived factor-1 or angiotensin II, while overexpression of a kinase-dead mutant of ILK (E359K) suppressed SMC migration in Boyden chamber assays.\textsuperscript{35} In contrast to this study, we have shown the effects of inhibiting endogenous ILK by siRNA. ILK-induced quiescence of SMC may require tight regulation of intracellular ILK levels such that both its suppression and its upregulation promote cell motility. Taken together, these studies reveal that the functions of ILK are broader and more complex than originally thought. This molecule has the potential to function as an adapter protein regulating cytoskeletal assembly and signal transduction from focal adhesion sites, as a protein kinase activating several signaling axes, and as a regulator of the mitotic spindle.\textsuperscript{36,37} The breadth of ILK function in regulating cell-matrix interactions, cytoskeletal organization and cell signaling is of great importance to normal development and disease progression. Functional studies using both kinase-deficient ILK variants and ILK siRNA will allow researchers to specifically attribute cellular behaviors to the proposed functions of ILK, and to determine their relative importance in different cells and pathologies. Based on our studies using injury models mimicking cellular events in occlusive vascular disease, we propose that ILK functions to maintain SMCs in a stationary, contractile phenotype in the normal artery. Following arterial injury, decreased ILK expression facilitates the reorganization of focal adhesions and the actin cytoskeleton, allowing for more efficient SMC migration and proliferation to establish a thickened neointima.

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