THE ROLE OF p21 ACTIVATED KINASE 1 IN CELL SPREADING AND COLLAGEN REMODELING

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Dentistry

University of Toronto

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Abstract

Background: Cell adhesion and spreading on matrix substrates are fundamental processes contributing to periodontal wound healing. p21-activated kinases (PAKs) are involved in regulating cell spreading, the mechanism for which remains undefined. Objective: Examine the role of PAK-1 in collagen remodeling by cell extension formation. Methods: NIH-3T3 mouse fibroblasts were treated with control or PAK-1 siRNA. The number and length of cell extensions were measured in cells cultured on floating collagen matrices. Activated β1 integrins and focal adhesions per cell were measured. Further, cell contraction and collagen remodeling were examined. Finally, cell contractile activity was estimated. Results: PAK-1 knockdown cells exhibited fewer and shorter cell membrane extensions, reduced cell area, and reduced activated β1 integrin staining and focal adhesions per cell. PAK-1 knock-down cells showed reduced contraction and collagen fiber alignment. Conclusions: PAK-1 regulates cell adhesion to collagen by promoting the generation of cell extensions, which enhance tractional remodeling of collagen.
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Chapter 1 – Literature Review

I. Extracellular Matrix

Structure and Function

The extracellular matrix (ECM) comprises the major structural components of connective tissues [1], which provide support and attachment for organs, ligaments, fascia and other specialized components of organs and tissues [2]. The ECM enables attachment and orientation of layers of cells at the basement membrane. In the interstitium of connective tissues, the ECM provides a substrate for cell migration and for remodeling to optimize matrix structure and function. Some ECM molecules are ligands for cell adhesion, mechanosensing and signaling through cell surface receptors. Arising from these processes of adhesion, mechanosensing and signaling, the ECM is now recognized to be essential for cell differentiation, proliferation, survival, polarity, and migration [1-4]. In addition to the biochemical traits of ECM proteins that endow them with specific functional and structural elements, the mechanical properties of the ECM are thought to regulate many aspects of cellular behavior [2].

Mammalian genomes encode hundreds of ECM proteins, including collagens, laminins, fibronectins, proteoglycans and glycosaminoglycans [1, 2, 4, 5]. Variations in the number, organization, composition and distribution of ECM proteins contribute to the distinctive and tissue-specific structure and function of connective tissues [2]. The most abundant component of the ECM is comprised of the large family of collagens [3, 5-7], which together endow the ECM with impressive structural and information-processing properties.
i. Collagens

Collagens are a family of at least 29 known proteins [3]. Type I collagen makes up approximately 80% of the global extracellular matrix in mammals [6] although other collagens such as type II collagen are abundant in cartilage, while type III collagen exhibits increased expression in development and wound healing [3, 6, 8, 9]. All collagens share a common structural motif of helically-arranged fibrils that are formed from three protein subunits. The primary function of fibrillar collagens is to provide structural support for connective tissues but collagens also interact with other ECM proteins such as fibronectin to regulate cell metabolism [3].

Interstitial collagens consist of three α chains, each of which is composed of approximately 1000 amino acid residues and with a characteristic repeating Gly-X-Y motif of triplets in which the amino acids X and Y are commonly proline and hydroxyproline respectively [10]. Arising from the high amino acid content, the tight molecular packing provided by the small amino acid glycine and the consistent feature of the tri-peptide unit repeats, each α chain assumes a tightly wound left-handed helical conformation; this is the 95 kDa pro-collagen α chain subunit. At the next level of structural organization, the three left-handed α chains intertwine with one another to form a right-handed collagen fibril, which then assemble and align in a very precise fashion with other collagen fibrils to form the collagen fiber [10-12].

The triple-helical conformation of collagen and its arrangement into a tight, triple helix endows interstitial collagens with remarkable resistance to
most proteinases [10]. In vertebrates, intracellular lysosomal hydrolases known as cathepsins and extracellular interstitial collagenases (which are members of the matrix metalloproteinase or MMP family) can cleave triple-helical collagens to enable degradation and remodeling of the ECM in health, development and disease [10, 11]. Notably, deregulation of collagen degradation leads to imbalances of matrix homeostasis [7, 13]. These imbalances can manifest as net loss of normal matrix structure, tissue overgrowth, or fibrosis in a wide variety of connective tissue lesions that include osteoarthritis, gingival hyperplasia and heart failure [13].

ii Elastic Fibers

Elasticity and tensile strength are important physical features that are necessary for the normal function of vertebrate tissues such as skin, blood vessels and lungs [1]. Elastic fibers are produced by fibroblasts and smooth muscle cells within the ECM and endow connective tissues with the requisite resilience for tissue recoil after transient stretch [1, 14]. The main component of elastic fibers is elastin, a highly hydrophobic protein about 750 amino acids in length that is rich in proline and glycine residues [1]. The elastin protein is composed largely of two types of short segments that alternate along the polypeptide chain: hydrophobic segments, which are responsible for the elastic properties of the molecule; and alanine- and lysine-enriched α-helical segments, which form cross-links between adjacent molecules [1].

Notably, elastic fibers do not consist solely of elastin. The elastin core is coated with a sheath of microfibrils, which appear before elastin is expressed in
developing tissues and which may provide a scaffolding function to guide elastin deposition in the tissues [1]. Mutations in the coding sequences of elastin or fibrillin genes can lead to narrowing of the aorta or other arteries, and is associated with rapid proliferation of smooth muscle cells in the arterial wall. Mutations of these genes are manifest in disorders such as Cutis laxa, Williams syndrome and Marfan’s syndrome [1, 15-17].

iii Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are molecules consisting of unbranched polysaccharide chains that exhibit repeating disaccharide units [1, 18]. One of the two sugars in the repeating disaccharide is always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), which in most cases is sulfated [1, 18]. The second sugar is usually an uronic acid (glucuronic or iduronic acid) [1]. GAGs are negatively charged and typically comprise less than 10% by weight of most soft connective tissues. GAGs form porous hydrated gels; their net negative charge attracts cations, especially Na+, which are osmotically active [1]. This feature of GAGs mediates absorption of large amounts of water into the matrix, thereby increasing swelling and turgor pressure. These features in turn enable the extracellular matrix to withstand high amplitude compressive forces [1].

Proteoglycans are composed of GAG chains covalently linked to a core protein [1]. Many different forms of proteoglycans are present in the ECM of connective tissues [19]. The major biological function of proteoglycans derives from the physicochemical characteristics of the GAG component of the
molecule, which through hydration and swelling enables these tissues to withstand large compressive forces [1, 19]. Proteoglycans exhibit very large ranges of size. For example, aggrecan is a very large molecule (~2500 kDa) whereas decorin is much smaller (~90-140 kDa) [1]. Aggrecan is the most abundant proteoglycan in cartilage and, as described above, enables cartilage to tolerate high amplitude compressive forces [1, 19]. Decorin on the other hand, is more widely distributed in many connective tissues, including the periodontal ligament, and contributes to many matrix functions such as regulating collagen fibril formation and in modifying the signaling activity of transforming growth factor-beta [1, 19].

iv Adhesive Glycoproteins
Many ECM proteins are glycated (e.g. collagen, fibronectin) and the glycated amino acid residues play crucial roles in cell recognition, adhesion, migration and proliferation [20]. Glycoproteins contain oligosaccharide chains (glycans) covalently attached to polypeptide side-chains [21]. The major adhesive glycoproteins of the ECM include fibronectin, tenascin, laminin and nidogen [20]. Adhesive glycoproteins can mediate specific molecular interactions in the matrix such as binding to cells through transmembrane receptors (e.g. integrins) or to other matrix macromolecules, or both [22].

Function of Extracellular Matrices in Development, Health and Disease
The ECM is not an inert structure that surrounds cells and only occupies space. On the contrary, the ECM provides several specific functions that play crucial roles in the regulation of cell function and metabolism [23]. In certain
connective tissues, such as tendons, cartilage, and bone, the ECM exhibits a prominent mechanical support function [23, 24]. In tendons for example, the highly specialized organization of fibrous ECM proteins enables these tissues to resist stretch-induced deformation, whereas in cartilage and bone, the ECM resists both stretching and compressive forces [23, 25].

During embryogenesis, the ECM influences cell proliferation, differentiation, and migration [23]. In particular, migration is important during early stages of morphogenesis, when cells from the primitive streak migrate to reach their final destination and there, differentiate [23]. Adhesion to the ECM also influences the capacity of cells to form tissues or migrate within extracellular matrix boundaries [23]. This latter feature is particularly relevant in the pathogenesis of certain diseases such as cancer and psoriasis [23]. Indeed in cancer, the ability of transformed cells to invade an organ and to form metastases depends upon their ability to migrate in the vasculature, cross the blood vessel wall and invade the ECM of the target organ [23].

The ECM is centrally involved in many fundamental processes that are required for the normal function of organisms such as glomerular filtration and blood clotting [23]. Glomerular filtration is mediated by the basal laminae of the endothelium and by specialized structures synthesized by glomerular cells, which together form a porous network that not only prevents leakage of blood cells and plasma proteins, but also allows filtration of low molecular weight molecules [23]. Blood clotting is dependent upon the interaction of platelets with connective tissue proteins [23]. Indeed, the cascade of events that
mediates blood clot formation is induced by initial damage to blood vessels, exposure of the underlying ECM, and interaction of platelets with ECM components to promote hemostasis [23].

Finally, it is notable that the ECM binds many different growth factors and hormones, a process which serves to concentrate and focus signaling molecules in the vicinity of cells that are in close proximity to these chemical signals. Cellular responses to these signals can then be directly modulated by cell-ECM interactions [23].

**Overview of Matrix Remodeling**

The dynamic remodeling of the extracellular matrix is essential for normal development, wound healing and preservation of organ and tissue homeostasis [26]. Life-threatening pathological conditions can arise when ECM remodeling is excessive or poorly controlled [26]. Following synthesis, ECM components are secreted into the interstitial space that surrounds and supports cells and, as described above, provide the main structural scaffolding for tissues and organs [26]. The extracellular matrix also plays a role in protecting cells against mechanical forces by buffering tissues subjected to deforming stresses [26].

As described above, the ECM is composed of a large and complex array of biochemically and structurally diverse components [27]. An effective strategy for enabling matrix remodeling is by the timely and focal removal of one or more of its components [27]. This process of matrix degradation is necessary for tissue remodeling in a large array of metazoans including, for example, insect and amphibian metamorphosis and mammary gland involution [27, 28].
All proteins inside or outside cells are subject to degradation and modification [27]. One of the most important families of proteins that mediate ECM remodeling is the metalloproteinases. Two main families of the metalloproteinases include matrix metalloproteinases (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Both of these families of proteins are specialized for ECM degradation [27] although it is now recognized that they contribute to the processing of many other molecules, including those involved in cell signaling [29]. Serine proteinases, which include plasmin and cathepsin G, are active at neutral pH and can degrade ECM proteins in the extracellular space [27]. In contrast, cysteine, aspartate, and threonine proteinases are predominantly active at acidic pH and mainly digest proteins in vacuolar compartments [27, 30]. Notably, the cysteine proteases cathepsins B and L can be secreted into the extracellular space and there perform limited digestion of the ECM because their catalytic properties are not optimized for the pH of this environment [27, 31].

A second important strategy for enabling ECM remodeling is by altering the abundance and organization of ECM components that comprise protein networks [27]. For example interstitial collagens (e.g. type I) are subject to a myriad of post-translational modifications, which include covalent and non-covalent cross-linking [27]. Both lysyl oxidases and lysyl hydroxylases can affect the extent of intermolecular cross-linking in collagens and elastin [27]. In this context, enhanced collagen cross-linking that is mediated by high levels of
lysyl oxidase activity can increase the tensile strength of collagen fibers and markedly stiffen the ECM to modify various cell behaviors [27, 32, 33].

**Collagen Remodeling**

i. **Collagen synthesis**

The biosynthesis of collagens is initiated by activation of collagen gene transcription and, through a complex series of subsequent multi-step processes, leads ultimately to the secretion of collagen molecules and the extracellular aggregation of collagen heterotrimers into large fibrils [34]. The regulation of the transcriptional activities of collagens depends largely on the cell type (e.g. fibroblasts are prominent collagen-expressing cells), but synthesis is also controlled spatio-temporally by signaling networks that include various growth factors and cytokines [34].

Most collagen genes exhibit a complex organization of exons and introns in which as few as 3 and as many as 117 exons are transcribed. The mRNAs of fibrillar collagens are encoded by more than 50 exons [34]. Alternative mRNA splicing has been reported for many collagen types. In addition to splicing, the pre-mRNA undergoes capping at the 5’ end and polyadenylation at the 3’ end [34]. The mature mRNAs of fibrillar collagens are transported to the cytoplasm and translated on the rough endoplasmic reticulum [34], which is particularly prominent in fibroblasts. Ribosome-bound mRNAs are translated into procollagen molecules, which extend into the lumen of the rough endoplasmic reticulum by targeting processes that involve a signal recognition domain [34].
After removal of the signal peptide (by the signal peptidase), procollagen molecules undergo multiple post-translational modifications, which include hydroxylation of proline and lysine residues (catalyzed by procoll 3-hydroxylase, procoll 4-hydroxylase, and lysyl hydroxylase, respectively [34]). The presence of 4-hydroxyproline (an imino acid) is essential for the formation of intra-chain hydrogen bonds (which contribute to the thermal stability of the triple helical structure), the integrity of the collagen monomer and ultimately to the formation of the collagen fibril [34]. Hydroxylysine residues can form stable inter-molecular cross-links between collagen molecules in fibrils and they are sites for the attachment of carbohydrates [34].

Prior to the formation of triple helices, the C-terminal domains of the three α-chains are aligned, which initiates the formation of the triple helix. The alignment process progresses from the C-terminus to the N-terminus of collagen molecules [34]. The globular structure of C-terminal pro-peptides is stabilized by intra-chain disulphide bonds; an N-linked carbohydrate group is added by the oligo-saccharyl transferase complex [34]. After processing and pro-collagen assembly, triple-helical collagen molecules are packaged in the Golgi complex into secretory vesicles and released into the extracellular space for fibril assembly [34]. The C-terminal pro-peptides and the N-terminal pro-peptides are cleaved by two specific proteases, the pro-collagen N-proteinase and the pro-collagen C-proteinase [34]. Extracellular processing and fiber assembly then takes place depending on the collagen type [34].

Fibril-forming collagens spontaneously aggregate after processing of
pro-collagens into ordered fibrillar structures [34]. The nascent fibrils are oriented in distinct and function-specific fashions in different types of connective tissues [34]. The molecular arrangement of collagen into fibrils is further stabilized by the formation of covalent crosslinks, which contributes to the mechanical resilience of collagen fibrils and the formation of a stable collagen network [34].

ii Remodeling by Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are multi-domain, zinc-dependent endopeptidases that are important in connective tissue remodeling during development, homeostasis and wound healing [35]. Dysregulation of MMP expression and function have been implicated in a variety of diseases including arthritis, glomerulonephritis, periodontitis, tissue ulceration in diabetes, and tumor cell invasion and metastasis [34-39]. A number of MMP family members exhibit triple-helicase and collagenolytic activity, which are critical processes for ECM turnover [35]. The cleavage sites of collagen hydrolysis have been determined for several MMPs and occur in the same general region of fibril-forming collagen molecules [35]. Detailed studies of collagenolytic activity have shown that in general, three critical processes are involved: (a) the enzyme binds to collagen efficiently, (b) the enzyme can unwind the triple helix, and (c) the enzyme can cleave the individual strands of the triple helix [35, 40].

It has long been recognized that mammalian collagenases initially cleave interstitial (types I–III) collagens into 1/4- and 3/4-length fragments [35]. The sites within the collagen molecule of these initial cleavages have been
identified [35] and are thought to be a slightly more thermally unstable regions of the molecule, which enables initial catalytic attack of the molecule by collagenases. Several investigations have defined which MMP domains in collagenases are required for generating collagenolytic activity [35]. For the MMP family, the catalytic domain can unwind and cleave a triple-helical structure, while the C-terminal hemopexin-like domain appears to be responsible for the proper recognition and binding of collagenase to collagen molecules [35]. It is also possible that so-called “exosites” within the catalytic and/or C-terminal hemopexin-like domains may regulate the ability of some MMPs to cleave collagen [35].

### iii Remodeling by Phagocytosis

As discussed above, collagens and other ECM constituents in most connective tissues are subject to continuous remodeling and turnover, a phenomenon that occurs in both physiological and pathological conditions. Collagen degradation can be mediated in the extracellular space by MMPs and by a separate system in the vacuolar system of fibroblasts that includes lysosomal hydrolases [41]. Collagen degradation by the phagocytic pathway involves the participation of several proteolytic enzymes including members of the following proteinase families: matrix metalloproteinases (e.g. collagenase, gelatinase and stromelysin) on the cell surface, cysteine proteinases (e.g. cathepsin B and L) in lysosomes and serine proteinases (e.g. plasmin and plasminogen activator) on the cell surface [41].

Convincing evidence [36] indicates a pivotal role for matrix
metalloproteinases in certain types of matrix remodeling, particularly in those tissues in which there is rapid destruction of the ECM as is seen in inflammatory sites [41]. In contrast, in steady state conditions, such as physiological turnover of soft connective tissues, collagen degradation is more likely to take place within lysosomes after phagocytosis of collagen fibrils [41]. This process involves the following steps: (i) recognition of the fibril by membrane-bound receptors (integrins), (ii) segregation of the fibril, (iii) partial digestion of the fibril and/or its surrounding non-collagenous proteins by matrix metalloproteinases, and finally (iv) lysosomal digestion by cysteine proteinases, such as cathepsin B and/or L [41]. Modulation of this pathway is carried out under the influence of growth factors and cytokines, including transforming growth factor β and interleukin 1. While phagocytosis of collagen occurs in many healthy tissues, it is also manifest in several pathological conditions including certain tumours, Hurler syndrome, epidermolysis bullosa, and arthritis [41, 42].

Under physiological conditions that are characterized by an equilibrium between synthesis and degradation, the intracellular pathway is a particularly important route for collagen digestion [41, 43, 44]. When this equilibrium is disturbed, pathological consequences to the matrix may occur. Notably, extracellular (primarily collagenase-mediated) degradation occurs when, in a relatively short time interval, large amounts of collagen need to be degraded (e.g. inflammation) although phagocytosis of collagen is observed also in the rapid turnover of matrix that occurs in involution of the uterus [41].
II. **Cell Adhesion to Matrix**

A. **Transmembrane Collagen Receptors**

i. **Integrins**

Integrins are a major family of heterodimeric cell adhesion receptors for ECM proteins, other integrins and a wide variety of glycoproteins [45]. Different combinations of α and β integrin sub-units collectively enable the formation of at least 28 integrin pairings, many of which exhibit marked ECM binding specificity. Collagen-binding integrins contribute to physiological function and disease in a wide variety of tissues and disorders including inflammation, wound healing, fibrosis, and tumor angiogenesis [46]. Integrins are composed of non-covalently associated α and β subunits [46]. Four members of the β1 integrin subfamily primarily function as collagen receptors: α1β1, α2β1, α10β1, and α11β1 [46]. The most widely distributed collagen receptors are the α1β1 (a collagen monomer receptor, predominantly expressed in mesenchymal cells) and the α2β1 (a fibrillar collagen receptor, primarily expressed in epithelial cells, platelets and fibroblasts) [46].

Integrin activation is subject to complex allosteric control. Different integrin conformations are related to low- and high-affinity binding states for extracellular matrix ligands [2, 47]. Inactive integrins exhibit low binding affinity to matrix molecules and are generally thought to be in a compact, closed conformation in which the ligand-binding, extracellular headpiece is bent toward the leg region [46]. Activation is believed to convert the receptors to a fully extended form in which the headpiece faces away from the cell membrane,
toward extracellular ligands, which enhances ligand binding and subsequent signaling [46].

ii Discoidin Domain Receptors (DDRs)

The discoidin domain receptors (DDRs) are receptor tyrosine kinases that recognize fibrillar collagens as their ligands [48]. The DDR family comprises two distinct members, DDR1 and DDR2, which were initially discovered in the early 1990s and are characterized as receptor tyrosine kinases, based on the presence of a kinase domain [49-55]. Subsequently, collagens were identified as ligands for DDRs, thus establishing the unique characteristic of these receptors among other members of the receptor tyrosine kinase superfamily [48, 56]. Upon collagen binding, DDRs undergo tyrosine auto-phosphorylation with slow but distinctive activation kinetics, which elicit signaling programs that regulate a variety of cell-collagen interactions in both normal and pathological conditions [48]. Despite our limited understanding of the mechanisms by which DDRs mediate their multiple biological effects, DDRs are rapidly emerging as novel therapeutic targets for control of fibrosis and the dysregulated cell migration seen in cancer [48].

iii Glycoprotein VI (GPV1)

The GPVI ectodomain consists of two immunoglobulin-like domains followed by a mucin-like stalk region; the short cytoplasmic tail of GPVI contains binding motifs for calmodulin and SH3 domains [46, 57]. The functional receptor is a stable complex between GPVI and the Fc receptor γ chain (Fc-Rγ), which contains, in its cytoplasmic region, an immunoreceptor tyrosine-based
activation motif [46]. The complex is stabilized through an arginine-aspartate salt bridge between the transmembrane domains of GPVI and Fc-Rγ [46]. The crystal structure of the collagen-binding immunoglobulin domain pair of human GPVI shows that the immunoglobulin domains are oriented in a bent conformation with an inter-domain angle of 90° [46, 58]. This conformation is thought to be particularly important for the regulation of platelet activation [58].

GPVI may specifically recognize fibrillar collagen as it can interact with the appropriately folded quaternary structure of assembled collagen fibers but cannot bind acid-solubilized collagen [46, 57, 59]. GPVI is generally believed to play a central role in activating platelet signals because ablation of its function results in loss of collagen-induced platelet activation [46, 60, 61]. GPVI along with α2β1 are thought to engage collagen fibers to mediate firm vascular adhesion and platelet activation, thereby suggesting a synergistic role of GPVI with other platelet collagen receptors [46].

iv Leukocyte-associated Ig-like receptor-1 (LAIR-1)

LAIR-1 belongs to the inhibitory immunoglobulin superfamily and is structurally related to several family members whose genes are located in the leukocyte receptor complex on human chromosome 19 [46, 62]. The LAIR subfamily consists of two members, LAIR-1 and LAIR-2. LAIR-2 exhibits a similar amino acid sequence as the extracellular domain of LAIR-1 but lacks the transmembrane and cytoplasmic domains [46]. Similar to other inhibitory immunoglobulin receptors, LAIR-1 contributes to the regulation of the immune system by delivering inhibitory signals [46]. The discovery that collagens are
functional ligands for LAIR-1 revealed a novel role for collagen in the regulation of immune function [46, 63]. LAIR-1 binds to several different collagen types, including fibrillar collagens I–III, collagen IV, and certain transmembrane collagens [46, 63, 64]. LAIR-1 is expressed by almost all cells of the immune system [46]. Its inhibitory functions have been described in a variety of cellular systems [46, 62]. The negative signals delivered by LAIR-1 inhibit signals relayed by immunoreceptor tyrosine-based activation motif-bearing receptors [46]. Examples of these signals include the inhibition of target cell lysis by natural killer cells, the inhibition of the cytotoxic activity of effector T cells, and the down-regulation of B cell function [46]. LAIR-1–collagen interactions may be exploited by tumor cells that are known to up-regulate collagen expression. This type of interaction could dampen anti-tumor responses directed against the tumor by various effector cells [46, 62].

**Cell Adhesion Molecule Interactions**

The ability of cells to adhere to one another as well as to ECM proteins through specific molecular interactions suggests a critical role for cell adhesion in a wide array of biological processes that include tissue homeostasis, immunological responses, embryogenesis, maintenance of vascular and endothelial integrity and neuronal tissue development [65-75]. The loss of appropriate adhesive interactions or excessive stimulation of adhesion is associated with various diseases such as pemphigus vulgaris and Leukocyte Adhesion Deficiency 1 and 2 [65]. The ability to characterize cell adhesion molecules at the molecular level has enabled classification of molecules into
several discrete groups that include integrins, cadherins, members of the immunoglobulin superfamily, and selectins [65, 76]. These proteins can all participate in: 1) homotypic adhesion events, such as recruitment of platelets to the site of thrombus formation and in establishing the epithelial layer of the intestinal lining; 2) heterotypic adhesion events such as the binding of a neutrophil or lymphocyte to an endothelial cell layer; and 3) adhesion of cells to ECM proteins, as is seen in the adhesion of cells to the basement membrane along the vascular wall [65]. In addition to the observation that cells of the same or different embryological origin can adhere to each other and mediate homotypic or heterotypic adhesion, adhesion molecules themselves may interact in a homotypic (e.g. E cadherin to E cadherin) or heterotypic (e.g. Integrin to ICAM-1) fashion [65].

Dynamics of Cell Adhesions and Role in Matrix Remodeling

Cell adhesions mediate important bidirectional interactions between cells and the ECM [77]. Adhesions provide an interactive interface between the extracellular chemical and physical environment of the cell with the cytoskeleton and signaling machinery [77]. The formation of adhesions and the regulation of their dynamics are crucial for embryogenesis, immune cell function, and wound repair. But they also contribute to pathological states such as cancer invasion and metastasis, as well as various immune disorders [77-83].

Adhesive interactions can occur with remarkable temporal and spatial precision [77]. These interactions not only link cells together into functional
tissues and organs, but they also convey to adhering cells, positional
information concerning their cellular and extracellular environments [77]. This
information can, in turn, affect many facets of cell metabolism [77]. In addition
to responding and attaching to the matrix, cell adhesions can actively remodel
the ECM, driving reciprocal, bidirectional interactions between the cell and its
surrounding matrix [77].

The integrin family of matrix protein receptors plays a central role in the
formation, maturation, and function of a variety of cell adhesions [77]. Over the
past several decades, surveys of the molecular constituents of cell–matrix
adhesions, particularly integrin-mediated adhesions, indicate that they are
composed of multiple molecules, which together participate in both the
physical/structural and sensing/signaling activities of these adhesion structures
[77]. Based on their localization, interactions with other adhesion components,
or involvement in the regulation of the organization and function of these sites,
the entire collection of molecules associated with integrin adhesions has been
termed the integrin “adhesome” [84].

Currently the adhesome network includes ~180 components, a
catalogue that is based on immunolocalization studies, binding assays, RNA
interference and yeast two-hybrid analyses [85]. What is particularly impressive
is the large number of direct interactions (>700) reported among these various
components, which can be divided into “scaffolding interactions” (direct binding
between specific components) and “regulatory interactions” (involving specific
modification of one component by another component; e.g., phosphorylation,
GTPase activation) [77]. Adhesive constituents assemble themselves into different types of cell adhesion structures that vary in molecular complexity and can change over time after cell adhesion [77]. The complexity of protein interactions in cell adhesions contributes to the crucial roles played by these organelles in cell migration, proliferation, and determination of cell fate [77].

III. Cell Spreading

A. Biological importance of spreading in development, health and disease

Cell adhesion and spreading on matrix substrates are fundamental processes that are intimately involved in development, tissue remodeling, angiogenesis and wound healing. Cell spreading is frequently characterized by the formation of cell protrusions or extensions, examples of which include filopodia and lamellipodia [86-89]. The elucidation of the biology of cell membrane extensions is crucial to our understanding of normal cell function and of pathological conditions such as cell invasion, tumor growth and metastasis in cancer [90-92].

Cell Structures involved in Cell Spreading and Migration

i. Filopodia and Lamellipodia

The coordinated polymerization and elongation of actin filaments that extend peripherally towards the plasma membranes provides the propulsive force for several processes such as cell spreading, migration, morphogenesis, endocytosis and phagocytosis [93]. During these processes, actin filaments are
organized into three-dimensional arrays that undergo constant adjustment to fulfill varying metabolic and structural requirements of the cell [93]. The smallest protrusive structures at the leading edge of a motile cell are designated as filopodia, spike-like protrusions whose formation is mediated by the small GTPase, Cdc42. Broader, fan-shaped cell extensions designated as lamellipodia, are dependent on the small GTPase, Rac for their formation [93-96]. A lamellipodium is a relatively broad protrusion (1–2 µm diameter in cultured cells) that is filled with a branched network of actin filaments [93]. By contrast, filopodia are relatively thinner (0.2–0.5 µm diameter) spike-like structures that are filled with tightly packed, parallel bundles of actin filaments [93]. For both filopodia and lamellipodia, the fast-growing barbed ends of actin filaments are oriented towards the plasma membrane [93]. The elongation of these filaments by the addition of actin monomers on to the actin barbed ends pushes the leading edge of the cell forward and thus promotes cell migration [97, 98]. Filopodia are often found embedded in, or protrude from, the lamellipodial actin network [99-101]. In addition to cell migration, filopodia and lamellipodia are involved in several other cellular processes including wound healing, adhesion to the extracellular matrix, chemotaxis, neuronal growth-cone path-finding and embryonic development [102, 103].

**ii. Invadopodia**

The spread of cancer cells to distant sites in the body is a major cause of cancer mortality [104]. A growing body of evidence links specialized subcellular structures designated as invadopodia (or invasive foot processes) to
cancer invasion and metastasis [104]. Invadopodia are subcellular structures that are found prominently in invasive cells compared with non-invasive cancer cells and are involved in pivotal processes of cancer invasion, including the degradation of the ECM [105].

Invadopodia are organelles that protrude from the ventral side of the plasma membrane and form a complex pattern of invaginations that are in direct contact with the ECM [104]. Small, branched formations (~100 nm thick) from invadopodia penetrate into the surrounding matrix, which facilitates ECM degradation [106, 107]. The extracellular surfaces of invadopodia are enriched with integrins and proteases whereas the cytoplasmic face of invadopodia are comprised of signaling molecules such as Src, Cdc42, and ADP-ribosylation factors (which are involved in cytoskeletal assembly and organization) and molecules that facilitate membrane trafficking [104]. Invadopodia are not required for maintenance of cell viability in normal cells, suggesting that they may be potential targets for novel cancer therapeutics, since their deletion may be expected to have fewer side effects than many current chemotherapeutic or radiotherapy-based approaches that are not cancer cell-selective [104].

**Regulatory Systems in Cell Spreading**

Most cultured cells respond to plating on surfaces coated with ECM proteins by initial adherence and then spreading to acquire a flattened morphology [108]. This process of cell adhesion and spreading is mediated in part by integrins and involves complex dynamic rearrangements of the actin cytoskeleton [108]. These dynamic processes appear to be coordinated in space and time by
intracellular signaling pathways involving a very large range of molecules including tyrosine kinases, protein kinase C, arachidonic acid metabolites and, in some cases, intracellular calcium levels [108-113]. How specific signaling pathways are integrated to regulate the reorganization of cytoskeleton in spreading cells is only partly understood [108].

**p-21 Activated Kinases (PAKs) and Cell Spreading**

Cells spread by generating extensions that contact the underlying surface, form adhesions, and then exert tension to induce outward movement [108]. This process is reminiscent of the extensions and adhesions that are induced by the small GTPases Rac and Cdc42 [108]. These small G-proteins are closely related members of the Ras superfamily of GTPases, which, like other Ras family members, act as guanine nucleotide-regulated switches [108]. As described above, Cdc42 mediates formation of long, thin, actin-dependent extensions called filopodia, whereas Rac mediates formation of curtain-like extensions called lamellipodia and ruffles [94, 96]. Rac and Cdc42 interact with a number of effector proteins [108]. Some of the best characterized of these effectors are the p21-activated kinases (PAKs) [108]. Both Rac and Cdc42 in the GTP-bound state interact specifically with PAKs and strongly stimulate PAK kinase activity [114-116]. Rac and Cdc42 mutants that do not bind and activate PAK-1 can, under specific circumstances, induce lamellipodia and filopodia, respectively [117, 118]. Notably, constitutively active PAK-1 mutants can induce lamellipodia and actin cytoskeletal rearrangements independent of
Cdc42 or Rac [119]. Currently, a complete definition of how PAK-1 mediates cytoskeletal remodeling and influences cell spreading is elusive [108].

IV. p-21 Activated Kinases (PAKs)

A. Roles in Cell Metabolism and Signaling

Mammalian PAKs have been implicated in a large number of cellular activities including the regulation of MAP kinase signaling pathways (JNK, p38, ERK), apoptosis and the cell cycle, oxidant generation in phagocytic leukocytes and cytoskeletal dynamics [120]. Due to their involvement in many important biological signaling pathways and processes, abnormalities of PAK family members are likely to contribute to various disease states if PAK function is perturbed [120]. Examples of PAK-related dysfunction include inflammatory diseases that involve enhanced leukocyte influx and activation, cancers that exhibit high levels of metastasis, defects in neuronal formation or degeneration, autoimmune disorders, developmental abnormalities, and cancers in which defective control of apoptosis plays an etiologic role [120].

Major Isoforms

The p21-activated kinases (PAKs) comprise a family of serine/threonine kinases that are central to signal transduction and cellular regulation [114, 121-125]. In humans, six PAK isoforms have been identified; they are classified into two subfamilies based on domain architecture and regulatory mechanisms [114, 121-125]. Group I PAKs (PAK-1–3) are activated upon binding the Rho GTPases, Cdc42 and Rac1, whereas the activation of group II PAKs (PAK4–6)
are independent of GTPases [125]. For this literature review, I will focus on Group I PAKs, which contain the major PAK isoforms, PAK-1-3.

i Group I PAKs

Group I PAKs, which comprise PAK-1-3, are implicated in a wide range of cellular processes including cell proliferation, apoptosis, migration and adhesion to the extracellular matrix [126-128]. These three serine/threonine kinases are structurally very similar but differ with respect to tissue distribution: PAK-1 is expressed in brain, connective tissue cells, muscle, and spleen; PAK2 is ubiquitously expressed; and PAK3 expression is specific to neurons[128].

The Group I PAKs share a number of defining structural characteristics (Fig 1). They exist as an inactive homodimer; maintenance of inactivation is mediated by interactions between the auto-inhibitory domain (AID) and the kinase domain of PAK monomers [129, 130]. A p21-binding domain (PBD) is found in the N-terminal region of each protein and exhibits at least 88% amino acid identity amongst PAKs 1, 2, 3 [124]. The kinase domain, at the C-terminus, exhibits at least 93% amino acid identity among these three kinases [124]. PAKs 1,2,3 also have an acidic region and a variable number of proline-rich regions throughout the N-terminus [124]. In addition, each of the Group I PAKs has an auto-inhibitory domain (AID) which overlaps with the PBD [124]. Upon binding to Cdc42 or Rac, the dimeric structure relaxes allowing for phosphorylation by phosphoinositide-dependent kinase 1 (PDK1), AKT, and other PAK molecules [130]. Two phosphorylation sites, ser144 and thr423 (In
PAK-1), are particularly important for PAK activity [121, 130]. Group I PAKs can also be activated by non-GTPase-dependent mechanisms [121, 130, 131], and they can serve as scaffolds for SH3 domain-containing proteins [121, 130, 132]. Group II PAKs also bind Rac and Cdc42, but they lack an AID, exist as active monomers, and have not been reported to have a scaffolding function [124, 130].

![Diagram of PAK structures](image)

Fig. 1. Structural comparison of the Groups I and II PAKs. Although the defining structural features, the PBD and the kinase domain are highly conserved among all six PAKs, the degree of conservation is greater within the two subgroups. Percent amino acid identities for both domains are indicated, relative to PAK-1 for Group I and relative to PAK4 for Group II. Although the Group II PAKs may be regulated intramolecularly, they do not possess an identifiable AID. All six PAKs have proline-rich regions. In the case of Group I PAKs, interactions with SH3 domain containing proteins, such as Nck and Pix have been demonstrated. For the Group II PAKs, no such binding partners
have yet been identified (diagrams are not drawn to scale). This figure is derived from Reference [130].

**Structure**

PAK family kinases contain a highly conserved kinase domain (aa 255–529, aa 235–509, aa 254–528, in PAK-1, 2, 3 respectively) and an N-terminal regulatory domain[120]. Distinguishing features of the regulatory domain include three conserved proline-rich motifs (Fig. 2), a 95% homologous p21-binding domain, and a stretch of highly acidic amino acids preceding the kinase domain[120].

![Fig. 2. Structure of p21\textsuperscript{Rac/Cdc42}-activated kinase 1 (PAK-1). The PAK N-terminal regulatory domain consists of three proline-rich domains (Pro), a GTPase (Rac/Cdc42) binding domain (PBD) and an acidic region (ED). Certain mutations in the PBD domain (for example at positions 83/86) abolish GTPase binding and result in partial activation of PAK. Mutation of the conserved threonine 423 to glutamic acid in the C-terminal serine/threonine protein kinase domain renders PAK-1 constitutively active. This figure is derived from Reference [121].](image)

The p21-binding domain (PBD) is comprised of approximately ninety amino acids located between the second and third poly-proline motif[120]. Rac and Cdc42 minimally bind to the CRIB (Cdc42 and Rac interactive binding) domain (aa 75-90 in PAK-1) [121]. The association of the GTPase with PAK at the regulatory N-terminus induces a conformational change which `opens' the
kinase domain (Fig. 2), leading to auto-phosphorylation and catalysis of substrates [120]. Activated PAK is auto-phosphorylated on seven sites, which have been mapped to S21, S57, S144, S149, S199, S204 and T423 [133]. Mutations in the PBD domain of PAK-1 (e.g. H83,86L) abolish GTPase binding and induce moderate constitutive kinase activity, while replacement of threonine 423 with glutamic acid in the conserved kinase domain increases kinase activity substantially [134].

The crystal structure of PAK-1 in an auto-inhibited conformation has been determined at 2.3-Å resolution [135]. PAK-1 exists as a homodimer in solution and in cells [135, 136], with the protein in a trans-inhibited conformation where the N-terminal regulatory domain of one PAK-1 molecule binds and inhibits the C-terminal catalytic domain of the other [121]. The dimerization interface overlaps the PBD/CRIB and inhibitory switch domains [121]. The inhibitory switch consists of a bundle of three α helices with a short N-terminal β hairpin [121].

**Mechanism of Activation**

Structural data [135, 137-140] as well as genetic and biochemical studies [141-149] support a model in which GTPase binding disrupts dimerization and leads to a series of conformational changes that destabilize the folded structure of the inhibitory switch domain, inducing its dissociation from the catalytic domain, and that rearrange the potential kinase active site into a catalytically competent state (Fig. 2) [121].

Phosphorylation at Thr 423 in the activation loop of the PAK-1 catalytic
domain is important both for maintaining relief from auto-inhibition and for full catalytic function [144, 147, 148]. Although Thr423 will auto-phosphorylate when PAK-1 is activated in solution, there is little evidence that this readily occurs. Instead, phosphorylation of this site by an exogenous kinase, such as PDK1, may be required for effective PAK activation in vivo [150]. Exogenous phosphorylation and/or auto-phosphorylation of PAK at additional site(s) also contribute to kinase activation and/or maintenance of kinase activity, including Ser144 (PAK-1) [143, 144, 146, 148]. Interestingly, Ser144 auto-phosphorylation is not required for PAK activation by sphingosine, in contrast to activation by GTPases [143]. In combination with the observation that PAK-1 activation by sphingosine was more sensitive to PDK1-mediated phosphorylation and activation than the GTPase-activated enzyme[150], these data suggest structural differences in the conformational changes of PAK evoked by lipids versus those induced by GTPase binding[121].

**PAK-1 as a regulator of Cell Spreading, Cell Extension Formation and Cellular Motility**

The PAK family of kinases evidently plays a significant role in regulating a wide range of biological functions as suggested by the plethora of interacting proteins and phosphorylation substrates for PAKs1-3, and because of the many signals that regulate PAK activity [121]. PAKs are involved in the regulation of cytoskeletal dynamics, cell motility, neurogenesis, angiogenesis, cancer metastasis and several other cellular functions [121]. For the purposes of this thesis, I will focus on the involvement of PAK in cytoskeletal dynamics, cell spreading and cell extension formation [133, 151, 152].
The best characterized effectors of Rac and Cdc42 are the PAKs. In their GTP-bound state, both Rac and Cdc42 interact specifically with PAKs and increase the kinase activity of PAK [114, 115, 153]. Mutants of Rac and Cdc42 that do not bind and activate PAK-1 can still induce lamellipodia and filopodia formation [117, 118]. Notably, activated PAK-1 mutants themselves induce lamellipodia and cytoskeletal rearrangements [119]. Thus, the role of PAK-1 in mediating small GTPase signals, and other regulatory pathways in cytoskeletal dynamics has not been fully elucidated [108].

One of the first indications that PAK-1 might be involved in cytoskeletal regulation via Rac and Cdc42 came from localization studies, which showed that PAK-1 redistributed from the cytosol into cortical actin structures after stimulation of cells by PDGF, insulin or in vitro wounding of cell monolayers [154]. These cortical actin structures included lamellae at the leading edge of polarized cells, circular dorsal ruffles, and in some cases, peripheral membrane ruffles [121, 154]. Moreover, PAK-1 localizes to focal adhesions [133, 151, 152]. Constitutively active PAK-1 can induce filopodia formation, accumulation of large polarized membrane ruffles and retraction of the trailing edge of cells [119]. Certain PAK-1 mutants cause loss of actin stress fibers and increased focal adhesion turnover [119]. Consistent with these observations, PAK-1 activity is required for the formation of actin micro-spikes and the loss of focal adhesions and stress fibers promoted by active Rac and Cdc42. These data were obtained with the use of an auto-inhibitory domain fragment from PAK-1 that inhibits endogenous PAK activity [149, 151].
PAK-1 is recruited to the leading edge and lamellae of motile cells, including polarized fibroblasts [154] and human leukocytes [155]. Expression of either constitutively active or inhibitory PAK-1 mutants markedly alters cell motility as seen in NIH-3T3 cells expressing active versions of PAK-1. These cells exhibited broad, leading edge lamellipodia and were more motile than their wild type counterparts. In contrast, cells transfected with kinase inactive PAK-1 exhibited randomly formed, multiple lamellipodia, which reduced the ability of these cells to move in a directed manner. The inhibition of motility was accompanied by effects on the dynamics and stability of focal adhesions. Notably, expression of active PAK-1 enhanced overall cell contractility associated with increased phosphorylation of myosin II regulatory light chain [121].
Chapter 2

Statement of the problem

Cell adhesion and spreading on matrix substrates are fundamental processes that are required for mammalian development, tissue remodeling and wound healing. Cell spreading is characterized by the formation of cell extensions, which include the formation of filopodia and lamellipodia. An improved definition of the biology of cell extension formation is crucial for our understanding of physiological processes in tissue homeostasis and in pathological conditions including cell invasion in cancer. The formation of extensions in cells spreading on extracellular matrix proteins like collagen is regulated by complex pathways that regulate cell adhesion and matrix signaling. These pathways involve transmembrane adhesion receptors that bind fibrillar collagen (e.g. β1 integrin), small GTPases (e.g. Rac and Cdc42), and a large group of actin-binding proteins, which mediate remodeling and dynamic rearrangements of the actin cytoskeleton. Currently, the specific signaling pathways by which the actin cytoskeleton is regulated in cell protrusion formation are not well-understood and the role of matrix stiffness on cell spreading is not defined. The p-21 activated kinases (PAKs) are important effectors of Rac and Cdc42 GTPase activity and play an important role in regulating a wide range of biological functions such as the regulation of cytoskeletal dynamics, cell spreading, cell extension formation and possibly mechanosensing of matrix stiffness. Presently the mechanisms by which PAKs regulate cell extension formation on collagen of varying stiffness are not well-defined.
Hypothesis

PAK-1 influences collagen remodeling by regulating cell extension formation.

Objectives

1. Treat NIH 3T3 fibroblasts with control and knockdown PAK 1 and evaluate their phenotype when spreading on collagen.

2. Examine cell extension formation on collagen gels.

3. Investigate the role of PAK 1 in cell adhesion, contraction and collagen remodelling.
Materials and Methods

Antibodies and Reagents

Rabbit polyclonal antibody to PAK-1 was purchased from Millipore. Rabbit monoclonal antibody to PAK-1 was obtained from Abcam. Rabbit monoclonal antibody to phospho (ps144) PAK-1 was obtained from Epitomics. Rabbit polyclonal antibodies to phospho-PAK-1 (Thr423)/PAK2 (Thr402) to phospho-myosin light chain 2 (Ser19) were obtained from Cell Signaling. Mouse monoclonal antibody to β-actin (clone AC-15) and rabbit HRP (horseradish peroxidase)-conjugated goat IgG was purchased from Sigma–Aldrich. IRDye 800CW goat anti-rabbit IgG (H + L) and IRDye 680RD goat anti-mouse antibody was obtained from Mandel (Toronto, ON). A rat anti-mouse monoclonal antibody against activated β1-integrin (9EG7) and a mouse monoclonal antibody (KMI6) against β1-integrin were obtained from BD Biosciences. Anti- rabbit HRP-conjugated goat IgG was purchased from GE Healthcare. Fluorescein isothiocyanate (FITC)- conjugated goat anti-rabbit IgG (H+L) was obtained from Jackson Immuno-Research Laboratories. FITC-conjugated anti-rat IgG was obtained from Biolegend. FITC-conjugated collagen type I was obtained from Anaspec Euorgenec Group. Alexa Fluor 488 phalloidin was obtained from Invitrogen.

Cell Culture

NIH 3T3 mouse fibroblasts were plated on either tissue culture plastic, collagen-coated plasma treated glass (concentration=1 mg/ml) or collagen gels supported by nylon mesh grids. Cells were plated in DMEM (Dulbeco’s
modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum) and 10% antibiotics (146 units/ml penicillin G, 50 µg/ml gentamicin and 0.25 µg/ml amphotericin). Cells were maintained in a humidified incubator with 95% air and 5% CO₂, and were passaged with 0.05% trypsin with 0.53mM EDTA (Gibco, Burlington, ON). For cells plated on collagen gels supported by nylon grids, very low densities of cells were used to avoid the inclusion of more than one cell in a single grid space. Cells adhered rapidly to collagen gels and spread within 4 hours.

**Transfection**

Cells were trypsinized, plated on 35mm tissue culture dishes and grown overnight in antibiotic-free medium before transfection with ON-TARGETplus Mouse PAK-1 siRNA (Thermo Scientific) or an irrelevant control siRNA using DharmaFECT® 1 transfection reagent, according to the manufacturer’s protocol (Thermo Scientific).

**Preparation of nylon meshes and collagen gels**

Floating collagen gels supported by rigid supports were constructed as described [156]. Nylon mesh sheets with square openings (200 µm wide) were obtained from Dynamic Aqua-Supply (Surrey, BC). Mesh was cut into 2 cm x 2 cm pieces that fit into 35 mm non-tissue culture plastic dishes. Collagen gels were prepared from pepsin-treated, bovine dermal type I collagen (6.0 mg/ml; ~97% type I collagen; Advanced BioMatrix, San Diego, CA). Prior to experiments, collagen solutions were neutralized with 0.1M NaOH to pH=7.4 and diluted to a final concentration of 1 mg/ml collagen. Glass dishes were
covered with stretched Parafilm to make a smooth, hydrophobic surface.

Collagen solutions (~70 µl) were poured on the prepared hydrophobic surface. The nylon mesh was then placed on to the collagen, which filled the nylon grids with collagen solution. The amount of collagen solution incubated with each gel was adjusted depending on the area of each nylon mesh that was created.

Samples were incubated at 37°C in 5% CO₂ until collagen polymerization was complete (>90 minutes). Polymerized, collagen-coated nylon meshes were gently detached from hydrophobic surface by addition of 1X PBS and the floating collagen-coated meshes were inverted and immersed in cell culture medium.

**Morphology and imaging**

Cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 0.2% Triton X-100 for 10 minutes. Cell morphology was visualized by FITC phalloidin staining (10⁻⁶ M) and imaging with an inverted microscope (Nikon Eclipse). Morphometric analyses were performed with ImageJ (version 1.44) software. The number of extensions per cell as well as the mean and sum of the length of cell extensions were measured. The length of cell extensions was measured from the cell centroid to the tip of each cell extension. The sum of the lengths of cell extensions was computed by adding the length of all cell extensions for each cell.

**Immunoblotting and Immunostaining**

Cells were lysed and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Protein concentrations of cell lysates were
determined using the BCA™ protein assay (Pierce). Equal amounts of protein were loaded in individual lanes. Nitrocellulose membranes were probed for the indicated antibody, followed by incubation with mouse or rabbit HRP-conjugated secondary antibodies. ECL (enhanced chemiluminescence) detection was performed according to the manufacturer’s instructions (GE Healthcare). In some experiments radiographic films were exposed for standardized lengths of time using conventional methods. In other analyses, an imaging system (Licor, Mandel, Mississauga, ON) was used along with IRDye 800CW goat anti-rabbit IgG (H + L) and IRDye 680RD goat anti-mouse secondary antibody to visualize bound proteins according to the manufacturer’s instructions (LiCor) and developed using the LiCor computerized detection system. In experiments to examine cell-surface expression of β1-integrin, cells were detached quickly (<20 s) from dishes with versene, fixed, immunostained with KMI6 (anti-β1-integrin) antibody, counter-stained with FITC-conjugated anti-rat IgG and analyzed by flow cytometry.

**Flow Cytometry**

Cells were seeded onto plasma-treated, collagen-coated (1 mg/mL type I rat tail) cell culture plastic for the indicated lengths of time. To measure β1 integrin activation, cells were immunostained with the neo-epitope antibody, 9EG7 [157], which recognizes activated β1 integrins. Prior to immunostaining, cells were quickly (~20 sec) harvested in ice cold versene and fixed in ice-cold versene containing 1% paraformaldehyde, a procedure that preserves the integrin activation state of attached fibroblasts [158, 159]. Bound 9EG7 was
stained with FITC-conjugated anti-rat IgG2a antibody and the fluorescence of single cells was analyzed by flow cytometry (Altra, Beckman-Coulter). To measure total β1 integrin surface expression, cells that were not fixed or permeabilized were immunostained with KMI6 antibody and fluorescence was measured using flow cytometry.

**Total internal reflection fluorescence (TIRF) microscopy**

Analysis of immunostaining for PAK-1 and β1 integrin was performed on the ventral surfaces of attached cells (collagen-coated glass) by TIRF as described [158, 159].

**Gel Contraction Assays**

Type 1 rat tail collagen (1.36 mg/mL) was added to a solution of DMEM, 0.24M NaHCO₃, FBS, 10X antibiotic, and 0.1N NaOH. Cells were added to the solution to produce a final concentration of 150,000 cells/mL. Aliquots of the gel-cell solution (0.2 mL) were pipetted onto the center of each well of a 24 well non-tissue culture plate, ensuring that no gel contacted the side of the well. The cell-gel solution was allowed to polymerize for 5 minutes at room temperature before adding 1 mL of growth medium to each well. We used a floating gel contraction assay to measure collagen remodeling by cells [160]. After polymerization, gels containing cells were released from the base of the dish before incubation at 37°C. Measurements were made of the collagen gel diameters with a dissecting microscope and an inter-ocular grid at time 0 hrs and every 24 hours for a total of 72 hours.

To study collagen contraction mediated by cells, an attached gel
contraction assay was used [160]. After collagen polymerization, gels containing cells were incubated at 37°C for 3 days before being released from the base of the dish with a pipette. Measurements of gel diameter were made at 0 minutes and every 30 minutes thereafter until contraction stopped (4 hours). Data obtained from three separate experiments were plotted to analyze the change in collagen gel diameter over time.

**Collagen remodeling**

Collagen was applied to plasma-treated, glass-bottom micro-well dishes (35 mm dishes with 14 mm micro-well inserts, MatTek) and polymerized at pH=7.4 buffer. The collagen consisted of a 50:50 mixture of DQ-labeled type I collagen prepared from bovine skin (1 mg/mL, InVitrogen). Cells (10,000 per well) were incubated for 12 or 24 h at 37°C. After incubation the samples were rinsed twice with PBS, fixed in 3.7% formaldehyde, blocked with 1% BSA and stained for 30 min at 37°C with rhodamine phalloidin (Invitrogen). At least three separate fields for each sample were imaged by confocal microscopy (Leica) and the images were quantified to estimate the remodeling activity of the different cells with Image J. Remodeling was quantified from the fluorescence intensities of FITC-labeled collagen in fixed area, regions of interest in each sample. Fluorescence was normalized for samples of collagen gels without cells.

**Confocal Microscopy**

Collagen fibers were imaged using confocal reflectance microscopy with an inverted confocal laser scanning microscope (Leica TCP) with a 20 X dry or 40
X oil-immersion objective lens. Cells immunostained with phospho-myosin light chain 2 (Ser19) antibody were visualized with 40X oil immersion objective lens using the FITC channel.

**Analysis of local collagen fiber alignment**

As described earlier [156] Fast Fourier Transform (FFT) was used to extract the orientation of collagen fibers from acquired images using confocal reflectance microscopy. FFT produces a spectrum image in the frequency domain of the original intensity image in the spatial domain. Notably, the resulting image contains frequencies orthogonal to those in the original image. The FFT function of ImageJ (version 1.44) software was used to generate the frequency content of the gray-scale images. To quantify the directionality of the original image, the pixel intensities in the resulting FFT image were summed along a straight line from the centre to the edge of the image at different angles. The summation process was performed using Oval Profile, an ImageJ plug-in (http://rsbweb.nih.gov/ij/plugins/oval-profile.html). The resulting plot was the sum of pixel intensities between 0-180°. A perfect random image would result in constant pixel intensity at different angles. However, orientation of collagen fibers in a specific direction would result in higher pixel intensities at a corresponding angle. An alignment index was quantified by calculation of area under the intensity curve within ±10° of the peak.
Results

Cell Characterization

We examined the role of PAK-1 in cell extension formation in cultured fibroblasts. NIH3T3 fibroblasts that constitutively expressed PAK-1 were transfected with irrelevant siRNA or with PAK-1 siRNA. The efficacy of the knockdown was examined in parallel experiments in which cell lysates were immunoblotted for total PAK-1 expression (Fig. 1A). Cultured cells were immunostained for PAK-1 and viewed by TIRF microscopy (Fig. 1B). PAK-1 was readily detected in NIH3T3 wild-type cells but PAK-1 levels were strongly reduced in cells transfected with PAK-1 siRNA. Immunoblots probed for β-actin (used as a loading control) showed equivalent protein loading in individual lanes but there was reduced PAK-1 in cells with PAK-1 knockdown.

**PAK-1 phosphorylation is enhanced in adherent cells**

S-144 and T-423 in PAK-1 are phosphorylated when PAK-1 is activated [136, 161]. We examined whether cell adhesion and spreading on substrates affected PAK-1 phosphorylation. NIH3T3 mouse fibroblasts were grown overnight to 50% confluence, trypsinized and were re-plated or maintained in suspension for 60 minutes. Cells were lysed and immunoblotted for phospho-PAK-1 S144 or phospho-PAK-1 T423. Densitometry showed a 4.2-fold increase (n=3, p<0.005) of phospho-PAK-1 S144 and a 2.6 fold increase (n=3, p<0.005) of phospho-PAK-1 T423 in adherent cells compared with suspended cells (Fig. 2), indicating that PAK-1 is activated by substrate adhesion and spreading.
Involvement of PAK-1 in formation of cell extensions

We examined the effect of PAK-1 knockdown on the formation of cell extensions with a cell culture system that employs collagen gels of low stiffness [156] (Fig. 3). Nylon mesh sheets with square openings (200 µm wide) were coated with 1 mg/ml type I rat-tail collagen, allowed to polymerize and then placed in culture dishes containing medium. Very low plating densities were used (~200 cells total per well) to reduce the likelihood of the formation of intercellular adhesions. Cells were allowed to spread and, after 4 hours, cells were fixed, permeabilized, stained with FITC-phalloidin and imaged with an inverted fluorescence microscope (Fig. 4A). An identical experiment was performed except this time cells were allowed to spread on plasma-treated collagen-coated glass. This approach was used to assess whether cells behaved similarly when allowed to spread on collagen gels of low stiffness. Representative images (although not analyzed quantitatively) suggest similar morphologies in both gel systems (Fig. 4A).

Morphometric analysis of FITC-phalloidin-stained cells spread on floating collagen matrices showed that PAK-1 knockdown cells exhibited smaller projected cell area (p<0.005) and fewer cell extensions (p<0.005) of shorter length (p<0.005) than control cells (Figs. 4B-D).

**PAK-1 knockdown diminishes activated β1 integrins in focal adhesions**

We examined whether reduced PAK-1 expression affects the activation and recruitment of β1 integrins to focal adhesions. NIH 3T3 mouse fibroblasts transfected with control or PAK-1 siRNA were cultured for 4 hours on collagen-
coated dishes (5 µg/cm² type I rat tail collagen). Cells were stained with 9EG7, a neo-epitope antibody that binds to activated β1 integrins [157] prior to analysis by TIRF microscopy. This imaging method restricts the z-axis focal plane to ~100 nm of the cell-substrate interface [162]. Analysis of 9EG7 staining showed that PAK-1 knockdown cells contained fewer adhesions with activated β1 integrins than control cells (Fig. 5A). Analysis of focal adhesions stained with 9EG7 and imaged by TIRF microscopy showed that the number of activated β1 integrin-containing focal adhesions was 2.1-fold (p<0.05) greater in PAK-1 control cells than PAK-1 knockdown cells (Fig. 5B). The length of 9EG7-stained focal adhesions and focal adhesion area were not significantly different (p=0.707, p=0.417 respectively) between PAK-1 knockdown and control cells (Fig. 5C, D).

**PAK-1 and β1 integrin expression**

We examined the effect of PAK-1 knockdown on cell surface β1 integrin staining. NIH 3T3 mouse fibroblasts treated with control siRNA or PAK-1 siRNA were cultured for 4 hours on collagen-coated dishes (5 µg/cm² type I rat tail collagen) and cells were rapidly (<25 seconds) detached and fixed to preserve integrin activation state [158]. Detaching cells from floating collagen matrices was not possible for this experiment and hence, cells were cultured on collagen coated dishes as opposed to floating collagen matrices. Total cell surface β1 integrin expression was estimated by immunostaining (KMI6 antibody) and flow cytometry. Total cell surface β1 integrin staining was not significantly different (p=0.955) between control and PAK-1 knockdown cells (Fig. 6A). We also
measured activated β1 integrin staining on the whole cell surface. In cells treated identically as described in the aforementioned experiment, analysis of total 9EG7 staining showed that total cell surface activated β1 integrins was 1.25 fold (p<0.05) larger in control cells than PAKI knock-down cells (Fig. 6B).

**PAK-1 regulates collagen remodeling and contraction**

We examined the role of PAK-1 in remodeling and contraction of collagen in gels in two related assays. First, collagen remodeling was measured with a floating gel assay [160], which examines the ability of cells in compliant gels to extrude fluid from the gels and reorganize collagen fibrils through migratory activity. For this assay, cells were incubated in collagen gels during polymerization, the gels were floated on growth medium and gel contraction was measured over 3 days. Floating collagen gel contraction was 2.2-fold (p<0.01) faster in control cells than PAK-1 knock-down cells (Fig. 7A).

Collagen gel contraction that is attributable to cell-mediated contractile behavior was measured with an anchored gel contraction assay [10]. Cells were incubated in collagen gels, which were attached to the bottom of the wells in 12-well plates. After 3 days of attachment, cells build up tension in the gels. After release of the gels from the base of the dish, gel contraction was measured over several hours. Anchored collagen gel contraction was 2.6-fold (p<0.01) greater in control than PAK-1 knockdown cells (Fig. 7B).

Collagen remodeling was examined at the single cell level by examining regions of collagen clearance (peri-cellular collagen degradation) by cells plated on FITC-labeled type I collagen. We were unable to develop a working
protocol for studying peri-cellular collagen degradation in collagen gels of low stiffness. Hence, FITC-labelled collagen plates were used as a substitute to study this phenomenon. As indicated earlier, it appears that cell morphology appears to be similar for both culture systems and consequently suggest that study of peri-cellular collagen degradation on plates was a reasonable approach for the purposes of this investigation. After incubation for 12-24 hours, cells were counter-stained with rhodamine phalloidin and two color imaging was obtained by confocal microscopy (Fig. 7C). Representative images were quantified to estimate collagen remodeling from the fluorescence intensities of FITC-labeled collagen in fixed size, regions of interest in each sample. Background fluorescence of samples of collagen gels without cells was subtracted from test images. PAK-1 knockdown cells exhibited less collagen clearance per unit area than control cells (p<0.05; Fig. 7D). Further, the normalized collagen clearance (calculated by dividing total cell area by the area of collagen clearance used to estimate collagen clearance per cell) was less in PAK-1 knockdown cells than controls (p<0.0005; Fig. 7E).

**PAK-1 affects phosphorylation of non-muscle myosin IIA**

Non-muscle myosin II is an actin-dependent motor protein that plays a central role in cell migration, polarity formation and cytokinesis [163]. Myosin II contractile activity is associated with increased phosphorylation of the regulatory light chain [164]. Mono-phosphorylation of myosin light chain at Ser19 is accompanied by actin-activated Mg\(^{2+}\)-ATPase activity and increased stability of myosin II filaments [165], which leads to increased contractility. We
examined the effect of reduced PAK-1 expression on phosphorylation of non-muscle myosin regulatory light chain. NIH 3T3 mouse fibroblasts treated with control or PAK-1 siRNA were cultured for 4 hours on collagen-coated dishes (5 µg/cm² type I rat tail collagen) and on floating collagen gels. Cells that were immunostained with antibody to phospho-myosin light chain 2 (Ser19) were visualized with a 40X oil immersion objective lens. Staining for phospho-Ser19 in cell extensions was lower in PAK-1 knockdown cells than control cells (Fig. 8).

**PAK-1 affects collagen fiber alignment**

We examined how PAK-1 expression affects collagen fiber alignment in floating collagen gels supported by grids. Very low plating densities were used (~200 cells per well) and cells were allowed to spread. After 4 hours, collagen alignment was assessed by reflectance confocal microscopy (Fig. 9A). Fast Fourier Transform analysis showed reduced collagen fiber alignment in PAK-1 knockdown cell compared with control cells (1.09 vs 1.83, p <0.005; Fig. 9B).
Discussion

Our principal finding is that PAK-1 is involved in cell spreading and contributes to collagen remodeling and fibrillar reorganization, in part by regulating the formation of cell extensions. PAK-1 expression levels were associated with cell adhesion to collagen since we found reduced β1 integrin activation and focal adhesion formation in cells with PAK-1 knockdown. Further, knockdown of PAK-1 was associated with diminished contractility, as shown by inhibition of the phosphorylation of the non-muscle myosin regulatory light chain in cell extensions and attenuated contraction of attached collagen gels. Finally, PAK-1 expression affected collagen remodeling as shown by reduced contraction of floating collagen gels, decreased pericellular collagen clearance and diminished collagen fiber alignment in PAK-1 knockdown cells.

Cell adhesion and spreading

The phosphorylation of Ser-144 and Thr-423 of PAK-1 are reliable indicators of the activation status of PAK-1 [121]. We found enhanced phosphorylation of these residues in spreading cells compared with suspended cells, implicating PAK-1 activation in cell adhesion. Since adhesion is one of the requirements for subsequent spreading, PAK-1 may play an important role in this phenomenon.

Many cultured cells adhere and then spread out to acquire a flattened morphology when plated on surfaces coated with matrix proteins [108]. These processes of adhesion and spreading are mediated in part by integrins and involve complex, dynamic rearrangements of the actin cytoskeleton [108].
When we examined cells with PAK-1 knockdown, there was reduced activation and recruitment of β1 integrins in focal adhesions compared with controls. This observation suggests that PAK-1 is important for early events of cell adhesion to collagen. Notably, while attempts were made to study focal adhesions in floating collagen gels, one of the limitations of TIRF microscopy is that it does not readily allow the study of cells cultured on floating collagen matrices. Hence, collagen-coated glass-bottom dishes were used to study focal adhesions for this investigation. As pointed out earlier, cell morphology appeared to be similar for cells on collagen-coated dishes and floating collagen matrices. Accordingly, we suggest that examining focal adhesion formation would be informative when examined on collagen-coated dishes.

**Contractility**

Contractility is an important determinant of cell extension formation and spreading on matrix substrates [166]. In an anchored collagen gel contraction assay [10] we found that PAK-1 knockdown cells exhibited decreased gel contraction and hence, diminished cellular contractility. We extended this analysis by measuring the phosphorylation status of non-muscle myosin IIA regulatory light chain in cell extensions, a post-translational modification which is associated with actin-activated Mg\(^{2+}\)-ATPase activity and enhanced stability of myosin II filaments [165]. Since staining intensity in extensions of cells cultured on low stiffness collagen was reduced in PAK-1 knockdown cells, we consider that PAK-1 plays a role in cell-mediated matrix contraction.
Collagen remodeling

We explored the role of PAK-1 in \( \beta_1 \) integrin-mediated remodeling and reorganization of collagen, processes which are necessary for connective tissue homeostasis [46]. Data from floating collagen gel assays, which measure the remodeling of collagen fibrils as a result of through migratory and reorganizational activities of cells [167], indicated that collagen gel contraction was diminished by PAK-1 knockdown. We also considered that collagen remodeling through pericellular collagen degradation may be affected by PAK-1. Accordingly, in cells plated on FITC-labeled type I collagen, we found that PAK-1 knockdown cells exhibited reduced collagen degradation and clearance, indicating that PAK-1 is involved in the functional interactions of \( \beta_1 \) integrin-containing adhesions with fibrillar collagen that regulate collagen remodeling.

We also examined this process in a related assay by measuring collagen fiber alignment and found that with PAK-1 knockdown exhibited reduced fiber alignment. Evidently PAK-1 plays an important role in collagen remodeling.

Role of PAK-1 in cell extension formation

The coordinated assembly of actin monomers on the barbed ends of actin filaments in subcortical sites generates forces that act on cell membranes to enable cell protrusions in several processes including cell spreading, migration, morphogenesis, endocytosis and phagocytosis [93]. Cells spread by extending processes that contact the underlying substrate, form adhesions, and then exert contractile forces that help to create outward movement of the cell extension [108]. This process is coordinated in part by the small GTP-binding
proteins, Rac and Cdc42 [108]. The role of PAK-1 in influencing cell spreading is presently unclear [108]. We examined the effect of PAK-1 knockdown on cell extension formation on to collagen gels with low stiffness. We discovered that PAK-1 knockdown cells exhibited smaller surface areas and with fewer numbers of extensions that were reduced length. These findings, coupled with the data above on the role of PAK-1 in cell adhesion and contraction, suggest that PAK-1 is involved in the formation of cell extensions on collagen substrates. We consider that PAK-1 is a downstream mediator of small GTPases that directly influence cell adhesion and spreading on collagen.
Future Directions and Conclusions

Our data provide new insights into the role of PAK-1 in cell spreading and how PAK-1 may contribute to collagen remodeling and fibrillar reorganization, in part by regulating the formation of cell extensions. This finding may be particularly important for mammalian development, tissue remodeling, angiogenesis and wound healing. Our experimental approach primarily used PAK-1 knockdown of cells spreading on and remodeling collagen. In this context it would be helpful to study the effect of PAK-1 over-expression on these aforementioned processes, and which could provide insight on the role of PAK-1 in periodontal wound healing. Accelerated wound healing is of specific interest following periodontal surgical procedures, ranging from conventional flap approaches to more advanced mucogingival correction. As post-surgical morbidity following periodontal surgical intervention is often a major deterrent for patients requiring periodontal surgery, manipulation of PAK-1 function could be an approach for accelerating wound healing and decreasing post-operative sequelae associated with periodontal surgery. Conceivably, this could include the fabrication of periodontal dressings infused with molecules that affect PAK-1 function to hasten wound healing.

Although we investigated the role of PAK-1 in cell spreading and collagen remodeling, it would be of great interest to investigate the rate of cell migration on collagen by utilizing a cell migration assay [168-170]. Another point of investigation would be to study the functional co-operation of PAK1 and
its known binding partners (such as filamin A) in cell spreading and ECM reorganization.
Figure Legends

Figure 1. Cell Characterization. A) Immunoblot of NIH3T3 mouse fibroblasts demonstrating PAK-1 in control (C) and siRNA knockdown (KD) cells. B) Immunostaining of cells treated with PAK-1 siRNA show reduced staining compared with controls.

Figure 2. PAK-1 phosphorylation in adherent cells. NIH 3T3 mouse fibroblasts expressing PAK-1 were spread on collagen or maintained in suspension for 60 minutes. Cells were lysed and immunoblotted for phospho PAK-1 S144 or phospho PAK-1 T423 (antibodies from Cell Signaling). These residues are phosphorylated when PAK-1 is activated. β-actin was used as a loading control. Adherent cells showed a 4.2-fold increase of phospho PAK-1 S144 and a 2.6-fold increase of phospho PAK-1 T423 compared with suspended cells.

Figure 3. Grid-supported collagen gels. Nylon mesh sheets with square openings (200 µm wide) were cut into 2 cm x 2 cm pieces that fit into 35 mm non-tissue culture plastic dishes. Collagen gels were prepared from pepsin-treated, bovine dermal type I collagen. Prior to experiments, collagen solutions were neutralized with 0.1M NaOH to pH=7.4 and diluted to a final concentration of 1 mg/ml collagen. Glass dishes were covered with stretched Parafilm to make a smooth, hydrophobic surface. Collagen solutions (~70 µl) were poured on the prepared hydrophobic surface. The nylon mesh was placed on to the collagen, which filled the nylon grids with collagen solution. The amount of collagen solution poured on each gel was adjusted depending on the area of
each nylon mesh that was created. Samples were incubated at 37°C in 5% CO₂ until collagen polymerization was complete (>90 minutes). Polymerized, collagen-coated nylon meshes were gently detached from the hydrophobic surface by addition of 1X PBS and the floating collagen-coated meshes were inverted and immersed in cell culture medium. Very low plating densities (200 cells per well) were used to seed the dishes and cells allowed to spread. After the desired length of the experiment, the cells were fixed, permeabilized, and stained for imaging.

**Figure 4. PAK-1 involvement in formation of cell extensions.** A) Effect of PAK-1 knockdown on formation of cell extensions evaluated with a cell culture system with low collagen stiffness using grid-supported collagen gels described in Fig. 3 legend. Cells were allowed to spread and, after 4 hours, cells were fixed, permeabilized, stained with FITC-phalloidin and imaged with an inverted fluorescence microscope. B) Representative images of FITC-Phalloidin staining indicate that PAK-1 under-expressing cells appear to have smaller cell areas (***p<0.005) and C) cell membrane extensions that are fewer in number (***-p<0.0005) D) and smaller in length (***-p<0.0005)

**Figure 5. PAK-1 expression and activated β1 integrins in focal adhesions.** A) NIH 3T3 mouse fibroblasts were treated with control or knockdown siRNA for PAK-1 and cultured for 4 hours on collagen-coated dishes (5ug/cm² type I rat tail collagen). Cells were stained with 9EG7 antibody prior to analysis by TIRF microscopy. Representative images of 9EG7 staining indicate that PAK-1 knockdown cells exhibited fewer adhesions with activated β1 integrins
compared with controls. Data (mean±standard errors of mean) are indicated in histograms. B) Number of adhesions containing activated β1 integrins. There were more adhesions with activated β1 integrins in control than PAK-1 knockdown cells (*-p<0.05). C) Focal adhesion length data show no difference between cell types (p=0.707). D) Focal adhesion area show no difference between cell types (p=0.417). Metamorph was used to quantify focal adhesions. (n=20 cells analyzed for each cell type).

**Figure 6. β1 integrin surface expression and activation.** A) Cells were cultured on type I collagen-coated dishes for 4 hours. Total β1 integrin surface expression was measured by immunostaining non-fixed and non-permeablozed cells with KMI6 antibody, followed by flow cytometry. Analysis of staining indicated that expression of total cell surface β1 integrins was not significantly different (4.9 vs 4.8 fluorescent units) between control and PAK-1 knockdown cells. B) Total activated β1 integrin on cell surface was measured by immunostaining with 9EG7 antibody and analysis by flow cytometry of non-fixed and non-permeablozed cells. Analysis indicated that in cells plated on type I collagen-coated dishes for 4 hours, cells with PAK-1 knockdown exhibited 1.25 fold (*-p<0.05) reduced total activated β1 integrins than controls.

**Figure 7. Collagen remodeling and contraction.** A) Effect of PAK-1 on collagen remodeling in floating gels. Cells were incubated in collagen gels during polymerization and gels were floated in growth media. Gel diameters were measured over 3 days. Data are mean±standard errors of mean of gel diameters. Collagen gel contraction was 2.22 fold (*-p<0.01) faster in control
cells than cells with PAK-1 knockdown. B) Collagen gel contraction attributable to contractile behavior was measured with an anchored gel contraction assay. Cells were incubated in collagen gels that were then attached to the bottom of wells. After 3 days of attachment, cells build up tension and are then released from the base of the dish. Gel diameters were measured over several hours. Anchored collagen gel contraction was 2.55-fold (*p<0.01) faster in control than PAK-1 knock-down cells. C) Collagen clearance by cells plated on FITC-labeled type I collagen. After incubation for 12-24 hours, cells were stained with rhodamine phalloidin and imaged by confocal microscopy. D) Image analysis of control and PAK-1 knockdown cells show mean±standard error of the mean of collagen clearance per mean cell area. More clearance in control than PAK-1 knockdown cells (***p<0.0005). E) Data of normalized collagen clearance. Calculated by dividing total cell area by area of collagen clearance: collagen clearance per cell. Data show reduced collagen clearance in PAK-1 knockdown cells compared with vehicle controls (***p<0.0005).

Figure 8. PAK-1 expression and phosphorylation of non-muscle myosin

IIA regulatory light chain in cell extensions. NIH 3T3 mouse fibroblasts treated with vehicle (control) or with PAK-1 knockdown were cultured for 4 hours on collagen-coated dishes (5 μg/cm² type I rat tail collagen) and on floating collagen gels. Cells were immunostained with antibody to phospho- Ser 19 myosin light chain 2 and imaged with confocal microscopy (40X oil immersion objective lens). Staining intensity in cell extensions (indicated by the red circles) was lower in cells with PAK-1 knockdown than controls.
Figure 9. PAK-1 and collagen fiber alignment. A) In grid-supported collagen gels, cells were allowed to spread and after 4 hours, cells were fixed, permeablized, and imaged using reflectance confocal microscopy B) Images analyzed by Fast Fourier Transform indicate that PAK-1 knockdown cells show reduced collagen fiber alignment compared with controls (1.09 vs 1.83, ***-p <0.0005).
Figure 1

A

C  KD

~68kDa  PAK-1

~36kDa  Beta Actin

B

C  KD
Figure 2

Suspended     Attached

pPAK-1 S144

Beta Actin

Suspended     Attached

pPAK-1 T423

Beta Actin
This Model design:

✓ Enables in-plane mechanosening by adherent cells
✓ Obviates mechanical interference from the rigid underlying foundation of tissue culture plastic
✓ Controls the distance of cells from physical boundaries.
Figure 4

A

C

KD

Collagen Coated Glass

B

Floating Collagen Matrices

C

D

Mean Cell Extension Number (units)

***
Figure 5

A

B

C

KD

D
Figure 6

A

Fluorescence Channel #: KM16 Staining

B

Fluorescence Channel #: 9EG7 Staining Collagen
Figure 7

A

Percentage change in Diameter of Gel (%)

Time (hr)

B

Percentage change in Diameter of Gel (%)

Time (hr)
Collagen Clearance per mean cell area

C

KD

Rhodamine Phalloidin

FITC Collagen

D

E

Colagen Clearance per mean cell area

C

KD

***
Figure 8
Figure 9

A

No Cells

C

KD
References


