Rho Kinase Proteins—Pleiotropic Modulators of Cell Survival and Apoptosis

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Abstract. Rho kinase (ROCK) proteins are Rho-GTPase activated serine/threonine kinases that function as modulators of actin-myosin cytoskeletal dynamics via regulation of Lin11, Isl-1 & Mec-3 domain (LIM) kinase, myosin light chain (MLC), and MLC phosphatase. A strong correlation between cytoskeletal rearrangements and tumor cell invasion, metastasis, and deregulated microenvironment interaction has been reported in the literature, and the utilization of pharmacological inhibitors of ROCK signaling for the treatment of cancer is actively being pursued by a number of pharmaceutical companies. Indeed, in many preclinical models ROCK inhibitors have shown remarkable efficacy in reducing tumor growth and metastasis. Interestingly, ROCK signaling has been shown to be either pro-apoptotic or pro-survival in a cell type and context dependent manner, though the molecular mechanisms controlling ROCK-mediated cell fate decisions are unknown. This review summarizes the many pleiotropic roles of ROCK signaling in survival and apoptosis, and suggests that controlled modulation of ROCK activity in tumor cells has the potential to significantly affect tumor survival and patient outcome.

ROCK Proteins

Rho associated protein kinases (ROCK, also known as Rho kinase) belong to a family of serine/threonine kinases modulated by interactions with Rho GTPases to serve as key regulators of actin cytoskeletal dynamics, and therefore control cell migration and motility (1). Specifically, ROCK proteins promote the formation of stress fibers and focal adhesions (Figure 1), but have also been implicated in diverse processes such as cell junction integrity and cell cycle control (2). ROCK activity is responsible for stabilization of actin microfilaments as well as promoting cellular contraction and cell substratum contact. ROCK stimulates actin polymerization via an inhibitory phosphorylation of the actin severing LIM kinase (Figure 2). ROCK promotes cellular contraction and attachment via an activating phosphorylation of myosin light chain (MLC) to increase myosin ATPase activity, and an inhibitory phosphorylation of MLC phosphatase leading to increased activation of MLC (Figure 3). Additionally, numerous other downstream targets of ROCK proteins have been identified including, but not limited to, intermediate filaments, ezrin/radixin/moesin (ERM) family proteins, collapsing response mediator protein 2 (CRMP2), calponin and adducin.

Two paralogs of ROCK have been identified in mammals (ROCK1 and ROCK2). These proteins were originally isolated as RhoA-GTP interacting proteins, and share 65% overall identity and 92% identity in their kinase domains (1). ROCK1 and ROCK2 are widely expressed from C. elegans to mammals and demonstrate both overlapping and unique tissue expression patterns and signaling functions within the cell. ROCK1 and ROCK2 knockout mice show distinct phenotypes, suggesting these proteins perform, at least to some degree, non-overlapping roles during development. ROCK1 knockout mice exhibit failure of eyelid and ventral body wall closure resulting in lethality soon after birth (3), while ROCK2 knockout mice exhibit embryonic lethality due to intrauterine growth retardation and placental dysfunction (4). The generation of heterozygote ROCK1 and ROCK2 mice leads to viable, fertile litters with no obvious phenotypic abnormalities, however a detailed examination of
ROCK1(+/–) mice revealed reduced neointima formation following carotid artery ligation correlating with decreased vascular smooth muscle cell proliferation and survival, decreased levels of proinflammatory adhesion molecule expression, and decreased leukocyte infiltration (5). Moreover, ROCK1(+/–) mice exhibit increased resistance to perivascular fibrosis, accompanied by decreased expression of tissue growth factor-beta, connective tissue growth factor and type III collagen (6). ROCK2(+/–), but not ROCK1(+/–), mice demonstrate no obvious cardiac phenotype, however they display decreased platelet endothelial cell adhesion molecule staining of endothelial cells in the lung, suggesting that ROCK2 plays a strong role in capillary development (7).

Deregulation of Rho/ROCK signaling has been reported across diverse tumors types. For instance, Rho-signaling proteins are elevated in, and contribute to the metastatic behavior of a variety of tumors (8-12). Several preclinical and clinical studies have utilized inhibitors of Rho/ROCK signaling for anticancer therapeutics in prostate, lung, melanoma, glioblastoma and many other tumor types with remarkable success (13-17). Many of the positive outcomes claimed from targeting Rho/ROCK signaling have been attributed to a reduction in invasion/metastatic potential of the cancer cells; however a wealth of findings have demonstrated that ROCK proteins are key modulators of cell survival and apoptosis, suggesting that cell viability may also be affected by ROCK inhibition.

An Overview of Apoptosis

Apoptosis is a controlled form of cell death that involves cell shrinkage, membrane blebbing, cellular disintegration, chromosome condensation, and subsequent removal of the apoptotic fragments by phagocytosis (1). This process is initiated by activation of caspase cystein proteases which cleave a large number of downstream protein targets to induce orderly morphological and biochemical changes within the cell, involving reorganization of actin microfilaments, microtubules, and intermediate filaments. The initial stages of apoptosis involve partial detachment of the cell from the extracellular matrix (ECM) due to caspase-mediated cleavage of focal adhesion kinase (FAK) as well as other structural proteins linking actin to focal adhesions (18). Following focal adhesion disassembly at the cell periphery, extensive cellular retraction occurs due to a loss of stress fibers and reorganization of actin microfilaments to form short fibers that bundle together and increase the tensile strength of the cell. As a consequence of retraction, cells undergoing apoptosis round up and reassemble new focal adhesion complexes ventral to the retracted cell body. Moreover, the formation of dynamic membrane protrusions...
called blebs is driven by modulation of actin-myosin activity, creating hydrodynamic forces during contraction to induce collapse at points of structural weakness within the cell (19, 20). Occurring concomitant with this process is caspase-8 mediated activation of deoxyribonuclease, which catalyzes internucleosomal DNA cleavage (21). Finally, apoptotic bodies of varying size and composition are produced in an actin/myosin dependent manner and are phagocytized by nearby cells and scavenging immune cells, to be ultimately degraded by lysosomal enzymes (22).

Apoptosis can be activated in the cell by two major processes: the intrinsic and extrinsic apoptotic pathways. The extrinsic apoptotic pathway responds to secreted death ligands (such as apoptosis stimulating fragment [Fas] ligand, tumor necrosis factor [TNF] alpha and tumor necrosis factor alpha related apoptosis inducing ligand [TRAIL]) that bind specifically to transmembrane death receptors (such as TNF-R, Fas and TRAIL-R) in the target cell, initiating a signal for apoptosis. Death ligand activation of these receptors induces the formation of a death-inducing signaling complex (DISC) composed of the death ligand, a trimeric death receptor and death domain containing adaptor proteins which trigger cleavage of caspases into their active form (23). This process leads to further rounds of caspase cleavage and activation, resulting in cellular apoptosis.

The intrinsic apoptotic pathway is initiated as a p53 induced cascade in response to DNA damage, defective cell...
cycle progression, or other severe cell stresses. This pathway is regulated by the fine balance of B-cell CLL/lymphoma 2 (Bcl2) family proteins within the cell (24). The Bcl2 proteins are apoptotic regulatory proteins that modulate mitochondrial membrane permeability, with some members being pro-apoptotic and others anti-apoptotic. Under normal conditions, the anti-apoptotic Bcl2 proteins (such as Bcl2, Bcl-xl, BclW, bifunctional Bcl2 family protein 1 [Bfl1], myeloid leukemia cell differentiation protein 1 [Mcl1], Bcl2 related protein A1 [A1], and Bcl2 homologue of ovary [Boo]) maintain mitochondrial integrity by counteracting the activation and function of pro-apoptotic Bcl2 family members (such as Bcl2 associated X protein [Bax], Bcl2 homologous antagonist killer [Bak], BclX5, Bcl2 associated death promoter [Bad], BH3 interacting domain death agonist [Bid], Bcl2 interacting killer [Bik], and hara-kiri [Hrk]) whose role is to induce mitochondrial damage. When pro-apoptotic Bcl2 proteins are activated, cytochrome-c is released from the mitochondria where it binds with apoptotic protease activating factor 1 (Apaf-1), forming the apoptosome. The activation of initiator caspases by the apoptosome begins a cascade of cleavage events ultimately leading to cellular apoptosis.

Role of ROCK Proteins in Apoptosis/Survival

Both disassembly and excessive crosslinking of the actin microfilament cytoskeletal architecture has been extensively demonstrated to induce apoptosis in numerous cell types (25-28) through modulation of signaling components such as Bcl2 activation (29), death receptor activation (30, 31), caspase activation (32), and p53 signaling (33). Moreover, an intimate association exists between cytoskeletal dynamics, the extracellular microenvironment, cell-to-cell adhesions and cell-to-substratum adhesions, where alterations in any of these components could be detrimental to the survival of the cell (34). ROCK protein signaling reportedly acts in either a pro- or anti-apoptotic fashion depending on cell type, cell context and microenvironment. For instance, ROCK proteins are essential for multiple aspects of both the intrinsic and extrinsic apoptotic processes, including regulation of cytoskeletal-mediated cell contraction and membrane blebbing, nuclear membrane disintegration, modulation of Bcl2-family member and caspase expression/activation and phagocytosis of the fragmented apoptotic bodies (discussed extensively below, Figure 4). In contrast, ROCK signaling exhibited pro-survival roles in a number of experimental studies (Figure 5) (14, 15, 35-40). Though a wealth of data exists to suggest both pro- and anti-survival roles for ROCK proteins, the molecular mechanisms that modulate these pleitropic roles are largely unknown.

ROCK Protein Regulation of Apoptosis

Essential roles of ROCK proteins in apoptosis. ROCK proteins are direct targets of caspase activity, whereby caspase 2 and 3 cleavage of ROCK proteins occurs in early apoptosis, thus removing the ROCK autoinhibitory C-terminal domain. This results in constitutive kinase activity of ROCK and its subsequent regulation of actin-myosin cellular contraction (41-47). Of note, ROCK cleavage also occurs early in apoptosis in a caspase-independent manner during endothelial release of microparticles and during invasion of colorectal cancer cells (48, 49). Granzyme-B has been shown to directly cleave ROCK2 in a caspase-independent manner, leading to cytotoxic lymphocyte granule-induced apoptosis (46). Whether via a caspase dependent or independent route, ROCK cleavage is an
essential step for apoptosis given that pharmacological inhibition of its kinase activity effectively abrogates apoptosis in a number of cell types.

In blebbing cells, caspase-cleaved ROCK-mediated phosphorylation of MLC is increased, thereby inducing contraction of cortical actin within the cell (50, 19, 44, 45). Indeed, transfection of cells with either a truncated (constitutively activated) ROCK1 gene or overexpression of a wild type ROCK2 gene is sufficient to induce MLC-mediated membrane blebbing independently of apoptotic stimuli (44, 51). Studies using cytoskeletal or ROCK inhibitors have identified multiple stages in apoptotic blebbing. For instance, caspase independent blebbing (zeiosis) occurs immediately after cytochrome c release from the mitochondria into the cytoplasm, whereby surface swellings at the active edges of cells form small blebs that dynamically extend and retract (52). This early phase of apoptotic blebbing, which occurs at the point where adherent cells begin to retract away from their neighbors and partially detach from the substratum (53), is critically dependent on ROCK/MLC cytoskeletal signaling (52). Late phase blebbing leads to morphologically distinct blebs that are relatively stable, fewer in number than those seen during early blebbing, exhibit an absence of visible organelles, and contain a distinct layer of endoplasmic reticulum which envelopes chromatin. Formation of these late blebs is efficiently blocked with Latrunculin A (an actin microfilament inhibitor), Blebbistatin (an inhibitor of myosin II), or Nocodazole (a microtubule inhibitor); however pharmacological inhibition of ROCK proteins only partially prevents the formation of late blebs (52).

In hypertrophic cardiomyocytes, Rho/ROCK signaling is necessary for apoptotic DNA fragmentation via activation of p53 and Bax (54). Conversely, inhibition of RhoA or ROCK protein signaling in hypertic stellate cells increases DNA fragmentation and condensation of nuclear chromatin (55). These limited data suggest that a more thorough examination is necessary before any consensus can be made regarding the role of ROCK proteins in apoptotic DNA fragmentation. Apoptotic nuclear disintegration, an actin microfilament-dependent and microtubule-independent process, requires ROCK modulation of the actin-myosin contractile force coupled with a ROCK-independent caspase-mediated degradation of nuclear lamin proteins (56). In addition to regulating nuclear disintegration, ROCK signaling is necessary for Golgi organelle fragmentation in apoptotic adrenal medulla pheochromocytoma cells (57). In this model, overexpression of constitutively active ROCK proteins induces Golgi fragmentation even in the absence of apoptotic stimuli. Moreover, ROCK proteins regulate protein traffic to and from cellular organelles during the apoptotic cascade. For instance, when myeloid leukemia cells become apoptotic, activated extracellular signal regulated kinase (ERK) is unable to translocate into the nuclei. Pharmacological inhibition of ROCK signaling is not only capable of rescuing these cells from apoptosis, but successfully restores the nuclear translocation of activated ERK (58). Furthermore, in apoptotic myeloid leukemia and fibroblast cells, caspase-independent ROCK signaling leads to nuclear exclusion of C1/C2 heterogenous nuclear ribonucleoproteins (hnRNPs), which play important roles in the packaging of nascent transcripts, alternative splicing and translational regulation (59). ROCK-mediated control of protein localization is well documented, as modulation of actin polymerization by ROCK has been shown to regulate nuclear localization of serum response factor (SRF) and sex determining region Y-box 9 (Sox9) during non-apoptotic conditions (60, 61). ROCK control of subcellular protein localization could potentially be a commonplace mechanism by which rapidly changing cytoskeletal dynamics during apoptosis alters cellular function.

Apoptotic body formation is driven by actin-myosin contraction initiated by caspase-mediated activating cleavage of ROCK1. In fibroblast and B-lymphoma cells this process is prevented by pharmacological inhibition or small interfering RNA (siRNA) knockdown of ROCK1, but not by inhibition of ROCK2 (57, 62). Moreover, ROCK activation is necessary for efficient phagocytosis of fragmented apoptotic bodies, and has been demonstrated to control the expression of N-acetylglucosamine (GlcNAc), a carbohydrate that serves as a major phagocytic marker (57, 63).

**ROCK control of extrinsic apoptosis.** The extrinsic apoptotic receptor Fas is linked to the actin cytoskeleton via an interaction with ezrin, radixin and moesin (ERM) proteins, whose function is to connect transmembrane proteins to the cytoskeleton (64, 65). The disruption of actin cytoskeleton dynamics or down-regulation of either ezrin or moesin inhibits extrinsic apoptotic signaling by blocking Fas aggregation and redistribution of Fas into lipid rafts, and by preventing association of flavin adenine dinucleotide (Fad) associated protein with death domain proteins (FADD) with its procaspases (31, 64, 66). These data suggest that ligand-mediated activation of the extrinsic apoptotic pathway initiates a cytoskeleton driven clustering of the activated death receptor with its downstream death domain proteins and their associated caspases. This process is dependent on ROCK signaling as pharmacological inhibition or siRNA downregulation of ROCK proteins blocks clustering of FAS proteins to lipid rafts, inhibits ROCK-mediated phosphorylation of ezrin and disrupts procaspase 8 and 10 association with FAS and FADD (64, 66-68). A similar ROCK-driven cytoskeletal regulation has been demonstrated for extrinsic apoptotic induction following ligand driven Fas receptor clustering (69, 70). In addition to modulating death receptor activity, ROCK signaling controls the expression...
levels of several extrinsic apoptotic regulators. Pharmacological inhibition of ROCK signaling results in a decrease in Fas, FasL and TRAIL expression during androgen induced apoptotic regression of prostate cancer cells and following cisplatin cytotoxicity in neuroblastoma cells (71-73). In contrast, ROCK inhibition reportedly enhances FasL expression in melanoma tumors (35).

**ROCK control of intrinsic apoptosis.** ROCK proteins perform a key role in cell cycle inhibition and impinge on the p53-driven intrinsic apoptotic cascade at multiple points from initial activation to output. However, the ROCK-mediated regulation of cell cycle and intrinsic apoptotic regulators seems to function in a cell type and context specific manner as conflicting results have been reported throughout the literature. Moreover, unlike that seen for ROCK regulation of the extrinsic apoptotic pathway, few consistent mechanisms have been proposed as to how ROCK proteins control intrinsic apoptotic regulation. ROCK inhibition has been shown to increase phosphorylation of p53 in neuronal cells, suggesting that ROCK signaling promotes murine double minute oncogene 2 (Mdm2)-mediated ubiquitination and degradation of p53 (74, 75). In contrast, fasudil treatment following nephropathy leads to decreased p53 expression, suggesting the opposite (76). No direct physical association has been reported in the literature between ROCK and p53, indicating that ROCK mediated regulation of p53 levels is likely modulated through indirect signaling crosstalk. For instance, ROCK activity has been shown to regulate phosphoinositol-3-kinase (PI3K)/Akt transforming (AKT) signaling (a negative regulator of p53 stability) through ROCK-dependent assembly of focal adhesions (77). A large proteomic screen demonstrated that ROCK2 physically associates and is activated by the serine/threonine kinase Polo-like kinase (Plk1) (78, 79), an important regulator of mitotic events such as centrosome maturation, mitotic spindle, mitochondrial matrix, sister chromatid cohesion, and cytokinesis. This interaction could modulate p53 status given that Plk1 is a strong inhibitor of p53 function through a direct physical interaction between the two proteins (80). Moreover, Plk1 induces an inhibitory phosphorylation on the Sumo E3 ligase topoisomerase I-binding protein (Topors) leading to inhibition of p53 sumoylation and its subsequent ubiquitination and degradation (81). Another possibility that deserves further study involves ROCK/LIMK mediated regulation of tubulin-dynein motor protein transport into the nucleus. p53 has been shown to localize to cellular microtubules, and transport of p53 into the nucleus following DNA damage is tubulin-dynein motor protein dependent (82-84). ROCK activity has repeatedly been demonstrated to control microtubule activity in a number of systems ranging from cell protrusions to tubulin-dynein vesicular trafficking (85-87), but whether p53 nuclear localization is regulated via this ROCK/LIMK/motor protein process has yet to be determined.

In a number of studies, ROCK signaling reportedly controlled Bcl-2 family member gene expression in favor of apoptosis (54, 73, 88-90) and modulates activation of multiple caspases (54, 64, 67, 77, 88, 91). ROCK modulation of Bcl2 expression may occur via the PI3K mediated pathway (discussed below) or through c-jun N-terminal kinase (JNK) activation. The JNK pathway is primarily activated by cytokines or exposure to various environmental stresses and plays an important role in regulating stress-induced apoptosis by triggering cytochrome c release from the mitochondria through modulation of Bcl2 and Bcl-xl activity (92). It has been demonstrated that ROCK1 directly phosphorylates JNK-interacting protein (JIP)-3, a scaffolding protein responsible for recruitment and activation of JNK protein, leading to subsequent triggering of apoptosis (93). This process can be prevented by sequestration of ROCK1 into stress granules, thus blocking ROCK1 interaction with JIP3 and protecting cells from apoptosis (94).

**ROCK Protein Regulation of Cell Survival**

control of cell survival by Rock proteins. Inhibition of ROCK promotes survival following balloon surgery and stent implantation of the carotid artery (95, 96), in autologous vein grafts (97), in pulmonary hypertension (98-100), following renal damage (101-106), in vaculogenic erectile dysfunction (107-110) and in diabetic retinal microvasculopathy (111). While it is highly likely that the effects of ROCK inhibition on numerous disease models are multifactorial, few mechanisms have been proposed to explain such observations. The PI3K/Akt pathway plays a central role in promoting cell survival by regulation of the activity and expression of Bcl2 family members, forkhead box 0 (FoxO) transcription factors, and p53 stability (112). PI3K activation is countered by phosphatase and tensin homolog (PTEN), a phosphatase that dephosphorylates proteins and phosphoinositide substrates (113). Activation of ROCK proteins by caspase cleavage or oncogene overexpression induces a direct phosphorylation of PTEN by ROCK, leading to the increased phosphatase activity and enhanced protein stability of PTEN. Activated PTEN then directly counters the pro-survival PI3K/AKT pathway, suggesting that ROCK activation blocks cell survival (41, 114-116). In addition, the PI3K/Akt pathway promotes the nitric oxide-mediated survival of endothelial cells by stimulating the expression of endothelial nitric oxide synthase (eNOS), the enzyme that converts the amino acid arginine to nitric oxide (117). Thus, ROCK-mediated activation of PTEN leads to a subsequent decrease in nitric oxide (NO) production and reduced cell survival of endothelial cells (118, 119), however ROCK’s regulation of NO-driven survival is reportedly
PI3K/Akt independent in some cells and involves activation of the PKC pathway (120-122).

ROCK signaling regulates chemotherapy resistance in several tumor cell types, and thus affects overall tumor resilience and survival. For instance, in multiple myeloma cells, ROCK-mediated attachment to the extracellular matrix is an essential component of cell adhesion-mediated drug resistance, a process whereby integrin interactions lead to upregulation of anti-apoptotic Bcl2 family members and overexpression of multidrug resistant genes (123). Similarly, inhibition of ROCK activity leads to enhancement of cisplatin-induced cytotoxicity in lung carcinoma cells through a focal adhesion kinase-independent mechanism (124). Conversely, following cisplatin injury to a panel of cultured neuroblastoma cells, pharmacological inhibition of ROCK activity resulted in increased cell survival, rapid acquisition of a chemoresistant phenotype and enhanced in vivo tumor survival. The increased chemoresistance in ROCK-inhibited neuroblastoma cells was attributed primarily to enhanced DNA damage repair, with observable alterations in the expression of multidrug resistance genes, p53, p21, Bcl2 family members and death receptors and their ligands (73).

**ROCK Protein Regulation of Proliferation**

*Control of cell cycle progression by ROCK proteins.* siRNA or pharmacological inhibition of ROCK blocks the G1/S transition in a number of cell types. Indeed, ROCK signaling promotes cell cycle progression into the S phase through a diverse array of downstream targets including upregulation of cyclin A/D1/D3 and cyclin dependent kinase (CDK) 2/4/6, nuclear translocation of CDK2 and cyclin E, and downregulation of the cell cycle inhibitors cyclin dependent kinase 4 inhibitor B (CDKN4B), CDKN2A, CDKN2C, CDKN2D (p21), CDKN1A, and CDKN1B (125-130) ROCK utilizes multiple downstream signaling cascades to modulate proliferation where it activates Ras/MAPK to regulate cyclin D and p21 expression, and, alternatively, LIM Kinase 2 to regulate cyclin A expression (128). Moreover, ROCK increases the expression of the F-box protein s-phase kinase-associated protein 2 (Skp2) which is required for the degradation of the cell cycle inhibitor p27(Kip1) (126, 128). Inhibition of ROCK signaling leads to cell cycle arrest in the G1 phase, decreased JNK, extracellular signal-regulated kinase (ERK), Ephrin-related tyrosine kinase (ELK), early growth response protein 1 (Egr1), and globin transcription factor (GATA) transcription factor activation, decreased c-FBJ murine osteosarcoma viral oncogene homolog (c-fos), jun proto-oncogene (c-jun), FasL, and Bcl2 expression, and increased Bax expression (15, 35, 39, 96, 131-134). Alternatively, a handful of papers suggest ROCK activity is capable of blocking cell cycle progression under certain conditions. For instance, during phorbol ester-induced apoptosis in prostate cancer cells, increased expression of the cell cycle inhibitor p21 is dependent on ROCK-mediated regulation of cytoskeleton dynamics (135). Additionally, pharmacological inhibition of ROCK activity in human Wharton’s jelly stem cells leads to downregulation of the pro-apoptotic Bax gene and the cell cycle regulators p21 and p53, as well as upregulation of the anti-apoptotic Bcl2 gene (89, 90), suggesting ROCK can inhibit cell cycle progression under certain conditions.

In addition to the requirement of early growth factor-mediated progression through the cell cycle, microenvironment-dependent changes in cell shape and cytoskeleton regulation modulate the G1/S transition whereby the major mitogenic-responsive pathways such as Ras, Rho, and PI3K are regulated by integrin mediated cell adhesion to the ECM (136). Disrupted integrin signaling is responsible for the change from anchorage dependent to anchorage independent cell growth in tumor cells, demonstrating a strong linkage between the extracellular microenvironment, cell adhesion, cellular morphology, and cell survival. Fibronectin/integrin interactions have been shown to stimulate cell proliferation in a ROCK-dependent manner by suppression of p21 and stimulation of cyclin D1 mRNA expression levels (130, 137, 138). Moreover, the degree of cell spreading on the ECM is a potent modulator of cell proliferation (139), and density dependent growth control is regulated by cell-to-cell adhesions via cadherin-mediated activation of p21 and p27 (140-143) and reduction in the strength and stability of cell-ECM contacts (144). Interestingly, cells that are restricted from spreading, such as fully confluent monolayers, exhibit a shape-dependent failure to increase the expression of cyclin D1, down-regulate p27 and phosphorylate retinoblastoma protein in late G1 (145, 146) and low ROCK activity (147). Cell spreading and mechanical stretch (mimicking non-confluent cell density) has been shown to activate RhoA and ROCK in smooth muscle cells, resulting in membrane association of RhoA, leading to ROCK-dependent hyperphosphorylation of Rb and enhanced proliferation (147-149). The loss of cadherin-mediated cell-to-cell contacts as seen in subconfluent cultures leads to the formation of a signaling complex composed of ROCK, novel protein kinase C (nPKC), and sarcoma proto-oncogene (Src) family kinases (SFKs), resulting in protein kinase D-dependent activation of the pro-proliferation nuclear factor kappa-B (NFkappaB) protein (37). These findings suggest that the extracellular microenvironment, particularly the effect of cell density, may affect the outcome of ROCK signaling in the control of cell fate. Therefore, simple differences in cell plating density may explain the numerous conflicting observations regarding the role of ROCK proteins in cell survival.
Implications for Cancer Therapy

Despite the obvious complexity and the ever growing number of publications linking ROCK as well as cytoskeletal regulation in the cellular decision between life and death, no sufficient comprehensive mechanism has been established which comes close to explaining the fundamental intricacies governing the pleiotropic roles of the ROCK proteins in cell survival. Despite this shortcoming, targeting of ROCK signaling in animal models of tumor progression has manifested outstanding results in many cases particularly with regard to tumor cell invasion and metastasis, suggesting that manipulation of this pathway could hold the key to pushing cancer cells just over the edge so that patients might gain an upper hand not afforded by chemotherapy or radiation alone. Perhaps the greatest challenge to researchers and clinicians is the dissection of these conflicting signaling roles, thereby learning which tumor types and what physiological conditions are appropriate for the proper manipulation of ROCK signaling.

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