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Roles of Rho-associated kinase in lung cancer (Review)

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Abstract. Lung cancer is one of the most lethal forms of cancer known to man, affecting millions of individuals worldwide. Despite advancements being made in lung cancer treatments, the prognosis of patients with the disease remains poor, particularly among patients with late-stage lung cancer. The elucidation of the signaling pathways involved in lung cancer is a critical approach for the treatment of the disease. Over the past decades, accumulating evidence has revealed that Rho-associated kinase (ROCK) is overexpressed in lung cancer and is associated with tumor growth. The present review discusses recent findings of ROCK signaling in the pathogenesis of lung cancer that were conducted in pre-clinical studies. The significant role of ROCK in cancer cell apoptosis, proliferation, migration, invasion and angiogenesis is discussed. The present review also suggests the use of ROCK as a potential target for the development of lung cancer therapies, as ROCK inhibition can reduce multiple hallmarks of cancer, particularly by decreasing cancer cell migration, which is an initial step of metastasis.

Contents

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1. Introduction

Globally, lung cancer is the most prevalent type of cancer, affecting 2.09 million individuals and was responsible for 1.76 million associated deaths in 2018 (1). Smoking is the leading cause of lung cancer, as 86% of individuals with the disease have a history of smoking (2). Lung cancer can be divided into 2 major types, which are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for >85% of all lung cancer cases and can be classified into adenocarcinoma (50%), lung squamous cell carcinoma (SCC) (30-40%) and large cell carcinomas (10%) (3). NSCLC is often diagnosed at a late stage, resulting in poor therapeutic responses and high mortality rates (4,5). The 1-year survival rate for patients with lung cancer is 44%, and the 5-year survival rate is only 17% (6). The treatment options for lung cancer are surgery, radiotherapy, platinum-based chemotherapy and neoadjuvant chemotherapy. However, the efficacy of these treatments for some patients remains unsatisfactory, as lung cancer is a heterogeneous disease. Moreover, these treatments can have deleterious side-effects, while patients with metastatic tumors are vulnerable to developing a post-treatment resistance to such medications, rendering the treatment of lung cancer difficult (7). However, improvements in effectiveness and survival rates have been observed when histology-guided chemotherapy, maintenance therapy, or vascular endothelial growth factor (VEGF)-targeted therapy is combined with platinum doublet therapy, which is the standard therapy for unresectable and metastatic lung cancer (8,9). Nowadays, novel therapies targeting molecular aberrations or driver mutations have emerged as a therapy of choice due to the excellent effectiveness and lower side-effects, owing to the completely sequenced human genome allowing the identification of novel mutations that play a key role in lung carcinogenesis. An example of such a therapy, which has been tested and has yielded promising results, is tyrosine kinase therapy, that specifically targets the epidermal growth factor receptor (EGFR), a type of protein commonly altered in NSCLC (10). However, this novel treatment spectrum only includes a minority of patients that harbor the mutation (11). Therefore, targeting oncogenic pathways that play a central role in cancer is a sensible strategy, as it is likely to be effective. One such pathway that has recently become of interest

in cancer therapy is the Rho-associated kinase signaling pathway (ROCK).

ROCK plays an essential role in carcinogenesis, particularly in promoting cancer cell motility that causes metastasis. ROCK is an effector of the small GTPase Rho and has been studied in various malignancies, such as breast (12), skin (13), liver (14) and lung cancer (15). Studies on lung cancer usually use NSCLC cell lines or tissue biopsies from patients with NSCLC to assess changes in the proliferation, migration, and growth of cancer following the inhibition of knockdown of ROCK (16-21). These studies have found that ROCK is responsible for promoting lung cancer growth if upregulated or overexpressed, and this has led researchers to suggest that ROCK may be a novel target for the treatment of lung cancer.

In the present review, the role of ROCK in lung cancer is discussed and the published evidence from *in vitro* and *in vivo* studies that were performed to decode the function of ROCK in lung cancer is summarized.

2. Literature search

A literature search on ROCK and studies on lung cancer was conducted from January 1, 2020, to November 15, 2020 using Scopus, PubMed and Web of Science, with the following keywords: 'ROCK OR Rho' OR 'Rho-associated kinase' AND 'Lung cancer' OR 'NSCLC' OR 'Non-small cell lung cancer' OR 'SCLC' OR 'small-cell lung cancer'. Only original research articles on the roles of ROCK and ROCK inhibition in studies on lung cancer written in the English language were selected for reading. The full text of the articles concerned was retrieved following the screening of the titles and abstracts.

3. Overview of Rho-associated kinase signaling pathway

ROCK is a member of the protein kinase A/protein kinase G/protein kinase C (AGC) serine/threonine kinases family that plays an essential role in promoting cell motility by facilitating cytoskeleton contractility (22,23). As shown in Fig. 1, ROCK is activated by binding of the Rho GTPase (i.e., RhoA and RhoC) to its Rho-binding domain (RBD), which leads to the activation by phosphorylation of the myosin-binding subunit of the myosin light chain phosphatase (MYPT) (24), myosin light chain (MLC) (24) and LIM kinases (LIMK) (25,26). The activation of the MLC substrate leads to the activation of myosin II motor activity, leading to the crosslinking of myosin to filamentous actin (F-actin), enhancing actomyosin cytoskeleton contractility (27,28). On the other hand, the activation of MYPT inhibits MLC dephosphorylation, and the activation of LIMK phosphorylates cofilin, which renders it inactive and unable to polymerize F-actin (29). In brief, the phosphorylation of MLC and MYPT leads to increased levels of phospho-MLC, and thus promotes actomyosin contractility, which alters the migratory behavior of cells. Enhanced contractility resulting from ROCK activation also facilitates cancer cell proliferation and regulates cell adhesion (28,30-32).

There are 2 ROCK homologs, namely ROCK1 and ROCK2. Collectively they are referred to as ROCK. ROCK1 and ROCK2 consist of 1,354 and 1,388 amino acids, respectively, and both contain an N-terminally located kinase domain, a coiled-coil region followed by a Rho-binding domain (33). The homologs share ~65% similarity in their overall amino acid sequences, and the sequence similarity increases to 92% if only their kinase domains are compared (22). Although both ROCK proteins share the same function, which is to regulate cytoskeleton contractility and exert a redundant effect on MLC and MYPT phosphorylation (34), they have also been found to differ from each other in terms of tissue distribution and subcellular localization. ROCK1 is ubiquitously expressed in non-neuronal tissues such as the lungs, liver, thymus, stomach, spleen, kidneys, testes, placenta and embryo (22,28). ROCK1 has been found at the microtubule-organizing center, cytoplasm, plasma membrane, and cell-cell adhesion sites (19,35,36). Moreover, ROCK2 is abundant in the brain, muscle, placenta, lungs and heart (22,28). It has been found to be localized at the nuclei and pre-synapses, including active zones (31). Notably, different types of cancer seem to have various needs for the ROCK proteins, specifically expressing both ROCK1 and ROCK2, or either alone (37-40). According to Kümper *et al* (34), both ROCK isoforms are independently important to the cells since the cells that lack any of the ROCK isoforms are still capable of proliferating. However, the cells that lack both ROCK isoforms are unable to contract and become flattened out. Eventually, cell division and growth are attenuated. This evidence has demonstrated the essential role of ROCK in maintaining cell survival, and the heterogeneity of ROCK expression in different types of cancer has particularly suggested the need for the development of a ROCK isoform-specific cancer therapy.

ROCK signaling pathway and expression in lung cancer. ROCK activation is positively associated with tumor growth (13,41-43), and a growing number of studies continue to lend credence to the importance of the ROCK signaling pathway in lung cancer development. ROCK is one of the Rho pathway genes that is significantly upregulated in a number of KRAS mutant NSCLC cell lines (44,45), several NSCLC animal models (15,46), and tumor tissues derived from patients with NSCLC (37,47). Furthermore, ROCK is also upregulated in bronchial epithelial cell lines exposed to cigarette smoke (48) and even in the lungs of smokers (49). This high expression of ROCK in NSCLC cells and tissues indicates that ROCK plays an important role in the initiation of the development of NSCLC, as shown by ROCK inhibition and knockdown studies, which will be addressed below. The expression levels of the ROCK upstream effector, RhoA, and ROCK downstream substrates, MYL9 or MLC, have been found to be higher in late-stage (stage II and IV) compared with early-stage (stage I and II) lung cancer (50,51). ROCK1 and MLC expression levels have also been found to be higher in NSCLC with lymphatic metastasis than in NSCLC without lymphatic metastasis (19,37,50). According to Hu *et al* (37), ROCK1 expression has a significant positive association with tumor size and clinical stage, as measured in NSCLC tissues collected from the clinical setting. Moreover, a high ROCK1 expression has also been found to be associated with poor survival rates of patients with NSCLC. To support the findings of ROCK1 expression, Du *et al* (19) performed immunohistochemistry (IHC) on 10 NSCLC tissues with lymph node metastasis, which revealed a higher ROCK1 expression in 6 out of 10 tissues. However, IHC-screened

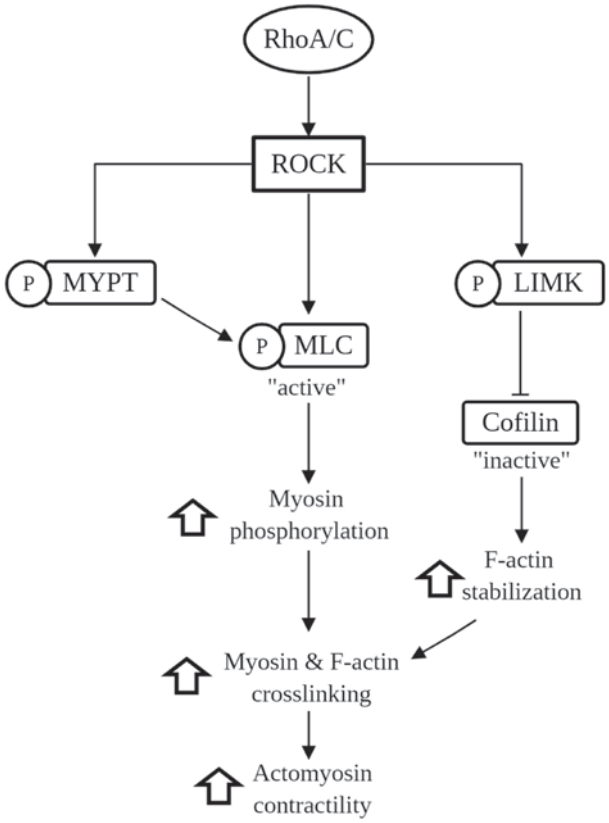

Figure 1. Overview of the ROCK signaling pathway. ROCK, Rho-associated kinase; MYPT, myosin light chain phosphatase; MLC, myosin light chain; LIMK, Lim kinase.

ROCK1 expression in NSCLC tissues without lymph node metastasis was only higher in 2 out of 10 tissues.

Factors potentiating the ROCK signaling pathway in lung cancer. The increased stiffness of the extracellular matrix (ECM) in a tumor is one of the factors recognized to activate Rho GTPases, as demonstrated in a 3D culture system with varying levels of tissue or substrate stiffness (12,52). The aim of this ROCK activation is to counterbalance the external force exerted on cells by increasing the contractility of the internal cytoskeletal structure (53). The increased stiffness of lung cancer is promoted by the increased production of cancer-associated fibroblasts and various ECM proteins, such as fibrillary collagen, fibronectin and tenascin C (54). Collagen, the main component of the ECM, has been shown to increase tensile strength in lung cancer and to interact with fibroblasts to activate ROCK (55). ROCK is activated by physical changes via the β-integrin receptor, a transmembrane protein (56), which phosphorylates focal adhesion kinase (FAK) (57). Activated FAK or p-FAK subsequently activate the downstream substrates, RhoGTPase and ROCK (58). Apart from increased tissue stiffness, tumor tissue also develops hypoxic regions with known partial oxygen pressure in lung cancer of only 16.6 mmHg (59) and it has been demonstrated that this condition can increase the expression of RhoA/ROCK in lung cancer (60). Cancer cells in hypoxic tumors are usually deprived of oxygen, which causes them to migrate to less hypoxic microenvironments through hypoxia-inducible factors (HIFs) and to later transactivate RhoA/ROCK. Since ROCK is a key protein that promotes cell motility, its upregulation in the hypoxic condition is crucial (61). During the process, cancer cells can escape the basement barrier and gain access to circulation through tumor vasculature by intravasation, which is the first step of metastasis (62). Collectively, this evidence indicates that both increased tissue stiffness and the hypoxic environment of a tumor may lead to the ROCK-mediated malignant transformation of lung cancer, indicating the important role of these biophysical properties in the promotion of carcinogenesis.

4. Roles of Rho/ROCK signaling pathway in modulating the behavior of lung cancer

Cancer cell apoptosis. The ROCK signaling pathway plays multiple roles in lung cancer carcinogenesis, including the suppression of apoptosis and conferring immortality to cancer cells (30). ROCK activation inhibits caspase-3, a crucial mediator of apoptosis, which eventually suppresses MYC-induced apoptosis (63,64). The reduction of caspase-3 has been suggested to inhibit the cell cycle, thereby providing a path for NSCLC to bypass senescence (65). The effect of ROCK on apoptosis via caspase-3 regulation was confirmed by Yang *et al* (66) who reported an increased level of active caspase-3 in a SCLC cell line following treatment with fasudil, a ROCK inhibitor. In addition, a previous study by Xin *et al* (67) reported that NSCLC cells treated with small interfering RNA (siRNA) against ROCK1 or ROCK1 knockdown resulted in apoptosis induced by the upregulation of the LATS2 and JNK signaling pathway, suggesting the functional role of ROCK in the regulation of lung cancer apoptosis. Another mechanism of the suppression of apoptosis is through the increment of phospho-signal transducer and activator of transcription 3 (p-STAT3) (64) and nuclear factor-κB (NF-κB) (68), which both are initially activated by RhoA (69). STAT3 upregulation can upregulate its downstream target responsible for the suppression pf apoptosis, such as c-MYC, cyclin D1 and survivin (70). However, NF-κB plays a critical role in desensitizing cells to apoptosis by suppressing reactive oxygen species (ROS) and antagonizing p53 (68). According to Gu *et al* (71), NF-κB expression was found to be highly expressed and associated with poor survival outcomes among patients with NSCLC, suggesting the essential role of NF-κB in driving carcinogenesis. In conclusion, the ROCK signaling pathway is capable of promoting the survival and growth of lung cancer cells by suppressing apoptosis and bypassing senescence through multiple mechanisms.

Cancer cell proliferation. The regulation of cell proliferation is another prerequisite for the development of lung cancer other than apoptosis (72). RhoA/ROCK has been shown to play an important role in promoting the proliferation of NSCLC in *in vitro* (20,73-75) and *in vivo* (34) studies. The majority of these studies used MTT or MTS tetrazolium assays to measure the level of cell proliferation. In ROCK-activated fibroblasts, Ras/MAPK increased the expression of multiple downstream signaling cascades, such as p27, cyclin D1 and cyclin A (76), which are known to promote cell cycle progression and cell proliferation (77). Notably, ROCK knockdown results in the downregulation of cyclin D1 and cyclin E in NSCLC cells (67),

suggesting the functional role of ROCK in promoting cell proliferation if overexpressed. In addition, other studies have also attempted to elucidate the role of ROCK in promoting cancer cell proliferation by using ROCK inhibitors or knockdown of ROCK. The studies by Liu *et al* (64) and Tang *et al* (78) demonstrated that the application of RhoA inhibitor and RhoE/rnd3 (RhoA competitor) was capable of inhibiting ROCK activation, thus reducing the proliferation of lung cancer cells. Of note, the study by Kümper *et al* (34) using NSCLC cells and animal models found that the depletion of both ROCKs led to a cell proliferation defect by affecting MLC and MYPT phosphorylation. However, the proliferation defect was not observed in NSCLC cells lacking either ROCK1 or ROCK2, demonstrating the functional role of both ROCK isoforms in cell proliferation. Thus, these studies indicated that ROCK activated multiple proteins that play a key role in promoting cell proliferation during lung cancer development.

Cancer cell migration. Since cell migration is a pivotal step in metastasis, it is important to identify molecular pathways that promote cancer cell motility or migration. Cancer cell migration is a dynamic process involving several biochemical and morphological changes. ROCK is the most well-known signaling pathway that promotes cancer cell migration as it can regulate cytoskeletal contractility (79). According to Hu *et al* (37), ROCK1 has been reported to enhance NSCLC cell migration by inhibiting phosphatase and tensin homolog (PTEN) activation, which then activates the phosphoinositide 3-kinase (PI3K)/AKT and FAK signaling pathways. This hierarchy of events promotes cell migration by increasing cytoskeletal contraction and by regulating cell-cell adhesion. ROCK further promotes the motility of NSCLC cells through the formation of lamellipodia at the edge of the cell surface by increasing pFAK colocalization with actin (37) and by cofilin inactivation (80), resulting in plasma membrane protrusion (81). Cancer cells employ 2 migration phenotypes that ROCK is capable of performing, the amoeboid and mesenchymal phenotype. ROCK is the most prominent signaling pathway that regulates amoeboid migration (82), characterized by losing of cell-cell and cell-ECM adhesion, eventually forming a bleb. The amoeboid type of migration uses a bleb-driven mechanism to pass through the holes in the surrounding 3D network of ECM filaments (82,83). In comparison, the mesenchymal mode of migration induced by the EMT process is characterized by the acquisition of mesenchymal characteristics which causes the loss of intact cell-cell contact and apical-basal polarity. EMT also mediates cytoskeleton contractility, which results in a change in cell shape from a cuboidal to a spindle-like shape that aids cell migration and has been found to be caused by TGF-β1 released by RhoC and ROCK activation in lung cancer (19,84-86). Therefore, ROCK is suggested as a worthy target to decrease cell migration since it plays an essential role in regulating the 2 main types of cell migration that aid metastasis.

Cancer cell invasion. Concurrently, cancer cells begin to invade their surroundings as they develop malignant phenotypes, particularly an enhanced cell motility. Invasion processes include extracellular matrix remodeling by matrix metalloproteinases (MMPs), which can degrade the basement membrane and stromal ECM, thereby providing a 'path' for cancer cells to invade (87). In this regard, ROCK has been found to play an essential role in the invasion of NSCLC cells by increasing the MMP-2 and MMP-9 expression (17), which are also known to promote angiogenesis and VEGF production (88). In addition, another family of MMPs, including MMP-10 and MMP-13, has been found to play a key role in the invasion of lung cancer by increasing the vascular permeability that aids in cancer cell intravasation into blood vessels (89). Notably, a high expression of MMPs has been reported in late-stage and metastatic lung cancer compared to early-stage and non-metastatic NSCLC (90). Their expression is also directly associated with the high potential of NSCLC invasion (91). This further supports to the role of ROCK and MMPs in promoting invasion, which will inevitably lead to metastasis.

Angiogenesis. RhoA/ROCK is responsible for the formation of a vascular structure or angiogenesis necessary for the growth of the tumor. The actin cytoskeleton regulated by ROCK plays a central role in the angiogenesis process involving endothelial cells (EC) proliferation, branching and sprouting (92). The study by Zhang *et al* (81) demonstrated that lung cancer cells that were conditioned with endothelial cells relied on RhoA/ROCK signaling to invade and metastasizes by angiogenesis. ROCK is also involved in the formation of a specific type of vascular pattern in NSCLC known as vasculogenic mimicry (VM), which preferably forms in the hypoxic environment of the tumor (93,94). VM is a supplement to endothelial cell-dependent angiogenesis induced by cancer cells (95). ROCK-induced VM formation is associated with the expression of the glycoprotein dimer known as Semaphorin 4D (Sema4D). Sema4D has been shown to be highly expressed in various types of solid tumors, including lung cancer (96). With respect to the pro-angiogenic factor, ROCK has also been reported to stimulate the secretion of VEGF, which is responsible for vascular formation from cancer cells and infiltrating immune cells. This regulation was reported by Zhu *et al* (17), who found a decreased VEGF expression in NSCLC cells treated with fasudil. They also reported decreased cell proliferation, migration, invasion and angiogenesis in cells with a low VEGF expression, suggesting the essential role of VEGF in lung cancer carcinogenesis. Similarly, another study by Zahra *et al* (97) reported decreased endothelial cell proliferation, migration and tube formation that induced angiogenesis following VEGF inhibition by RhoA knockdown. In summary, ROCK, Sema4D and VEGF are good targets for anti-angiogenic therapy as they play an essential role in angiogenesis. In particular, ROCK holds promise for use as a novel anticancer therapy, as it is capable of regulating a number of downstream signaling pathways and factors crucial in carcinogenesis, as shown in Fig. 2.

5. ROCK signaling pathway inhibition in lung cancer

Since the use of the first approved ROCK inhibitor (fasudil) for the treatment of cerebral vasospasms (98), immense efforts have been made to repurpose the therapeutic benefits of this agent for the treatment of cancer (81,99-101). Apart from fasudil, a wide range of compounds has also been tested against lung cancer, and as ROCK overexpression or upregulation is associated

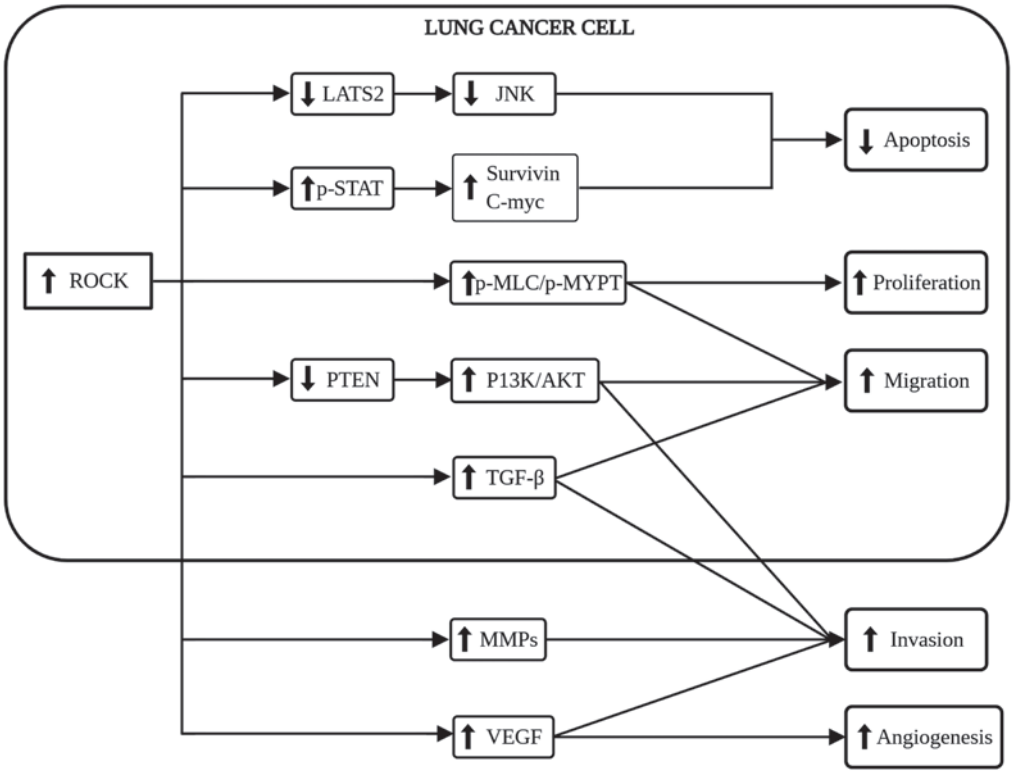

Figure 2. Diagram illustrating the signaling pathways and factors studied to be activated by ROCK in lung carcinogenesis. ROCK, Rho-associated kinase.

with an enhanced growth of lung cancer as discussed above, the current treatment modality is to inhibit or reduce ROCK expression. Researchers have been testing several inhibitors that may target ROCK upstream effectors or downstream substrates in lung cancer, such as RhoA inhibitor (64), RhoC inhibitor (85,102), ROCK pan-inhibitor (Y27632) (103,104) and LIMK inhibitor (BMS-5) (105). As these targets are intertwined in one signaling pathway, the inhibition of RhoA or LIMK, for example, also yields a preferable result, as with the inhibition of ROCK. Thus, the present review also includes studies on ROCK-associated substrate inhibitors instead of only studies on ROCK inhibition. Novel natural compounds have also been tested for their inhibitory action against ROCK in lung cancer, with promising results (46,80). Examples of the natural compounds studied are zerumbone derived from ginger (80), glabridin from licorice (106), plumbagin from chitrak (46), deguelin from cork bush (107), β-escin from horse chestnut (108), and XAP from Muruwa (102). In recent years, natural compounds have attracted the attention of researchers in developing novel cancer treatment, since they are believed to exert a less toxic effect on normal cells (109). β-escin (a RhoA/ROCK inhibitor) for instance, has been evaluated to not cause significant body weight loss or histologic cytotoxicity on normal mice after 34 weeks of treatment, which indicates that this natural compound is unable to jeopardize general health (108). As reviewed by Surien *et al* (110), a number of natural compounds have been proven to be beneficial in the treatment of lung cancer at pre-clinical studies. From the literature search, the present review identified 6 different studies that evaluated ROCK expression following treatment with natural compounds (46,80,102,106-108). Notably, these natural compounds have been documented to be capable of reducing RhoA, RhoC and ROCK expression, all of which promote cell proliferation, migration, invasion and angiogenesis.

The specificity of ROCK inhibition should also be considered, as some ROCK inhibitors, such as Y-27632 have additional off-target inhibitory activity against mitogen- and stress-activated protein kinase 1 (MSK1) (111). Therefore, a more potent and specific ROCK inhibitor should be developed to resolve these issues. Some researchers have begun to investigate this possibility by developing OXA-06, a potent ATP-competitive ROCK inhibitor that is structurally distinct from Y-27632, a ROCK1/2 inhibitor (15) and a Rho-kinase inhibitor (RKI) (112), both of which have been tested and proven to exhibit less *in vitro* off-target protein kinase inhibitory activity. OXA-06 also has the ability to block anchorage-independent cancer cell growth by causing cell cycle arrest in G0/G1 (15). siRNA and short hairpin RNA (shRNA) have also been used for the inhibition of ROCK activation in lung cancer studies. These short double-strand RNA molecules are a promising therapeutic approach that can be programmed to silence a specific target. Their use, either *in vitro* or *in vivo,* has allowed researchers to compare the effects of ROCK inhibition with those of their novel compounds and has enhanced our understanding of the underlying mechanisms that regulate this pathway. For example, siRNA targeting either ROCK1 or ROCK2 alone is capable of inhibiting ~90% of NSCLC anchorage-independent cells. Moreover, non-specific siRNA that targets both ROCKs effectively inhibits tumor growth as reflected by near-complete colony formation suppression. These findings demonstrate that siRNAs can specifically discriminate against ROCK homologs and give a clear image of their inhibition effects on

a specific ROCK homolog (15). Moreover, shRNA has been used to target RhoC (85) and ROCK1 (37) in lung cancer studies. These studies reported that the inhibition of RhoC and ROCK1 by shRNA significantly suppressed the EMT process and the migration of lung cancer, as with other ROCK inhibitors.

The use of microRNAs (miRNAs or miRs) for the treatment of lung cancer has also been documented. miRNAs are small, non-protein-coding RNA molecules that have been shown to be involved in carcinogenesis as either tumor suppressors or oncogenes (113). miRNAs are regarded as convenient biomarkers due to their better stability compared to mRNAs. As reviewed by Iqbal *et al* (114), several miRNAs have been identified to play a prominent role in lung cancer, and a few have been identified to target RhoA/ROCK. Throughout the literature, only 4 studies on miRNAs have been found that elucidate ROCK expression. These miRNAs have been reported to be downregulated in NSCLC tissue biopsies and their restoration *in vitro* has been reported to reduce the proliferation, migration and invasion of the A549, H1299 and SPC-A1 cell lines by decreasing ROCK expression (19,20,115,116). Furthermore, a previous meta-analysis and Kaplan-Meier data by Yang *et al* (117) and Wu *et al* (118) demonstrated that other miRNAs, such as miRNA-21, miRNA-155, miRNA-19b and miRNA-146a, can be used to predict recurrence, as a prognostic biomarker and to demonstrate association with the survival rate of patients with lung cancer.

Generally, studies on ROCK and lung cancer in literature can be divided into 2 major categories: Kinase-targeting ROCK inhibition (Table I) and non-kinase-targeting ROCK inhibition (Table II). Kinase-targeting ROCK inhibitors target upstream effectors, downstream substrates, or ROCK, whereas non-kinase-targeting ROCK inhibitors target signaling pathways other than ROCK, such as proteins, oncogenes and non-coding RNAs, but can still affect ROCK expression. The targets of the 24 ROCK inhibitors identified in the literature are presented in Fig. 3. Collectively, these inhibitors can inhibit MLC/LIMK phosphorylation, which activates cofilin and promotes F-actin depolymerization. As a result, crosslinking between F-actin and myosin is reduced, leading to a decrease in actomyosin contractility, which plays an essential role in the transformation of malignant phenotype in cancer cells (119). It was found that the majority of the kinase-targeting ROCK inhibition studies were conducted *in vitro* using NSCLC cell lines, while there is a lack of studies conducted *in vivo* or by using tissue biopsies of lung cancer patients. The most commonly used cell line is human A459 lung adenocarcinoma cells, followed by H1299 and H460 cells, which are also categorized as NSCLC.

Targeting proteins, oncogenes, or non-coding RNAs that coordinate Rho may also be a promising approach, as presented in non-kinase-targeting ROCK inhibition studies (Table II). Generally, these studies have reported that NSCLC tissue has a higher expression of DEK (73), keratin14 (KRT14) (120), chromodomain helicase DNA-binding protein 4 (CHD4) (74) and long non-coding RNA (lncRNA) NORAD (121); the depletion or disruption of these has been observed to reduce RhoA, ROCK and phospho-myosin expression in NSCLC cell lines. It has also been demonstrated that the depletion of these proto-oncogenes is capable of inhibiting NSCLC cell growth by promoting apoptosis and attenuating their proliferation, migration and invasion (73,74,120,121). In addition, a high DEK and CHD4 expression has been shown to be associated with poor survival rates among patients with NSCLC, as analyzed using Kaplan-Meier data, and to be positively associated with TNM staging, differentiation and nodal status (73,74). Therefore, it is suggested that DEK and CHD4 expression may be critical factors and potential biomarkers of NSCLC progression. Therefore, studies on DEK, CHD4, KRT14 and lncNORAD expression in NSCLC tissues provide insight into the possibility of these proteins to be translated into potential therapeutic targets for treating lung cancer.

Some studies have found inconsistent effectiveness in treating cancer either by targeting both ROCK isoforms, or only one of them. Furthermore, the characterization of the role of ROCK1/2 in lung cancer carcinogenesis is therefore essential. However, ROCK1 tends to be more significant than ROCK2 in lung cancer studies (34,37). ROCK1 is also the preferred isoform in most of the kinase-targeting ROCK inhibition studies, as shown in Fig. 3. It may be due to the predominant role of ROCK1 over ROCK2 in regulating cytoskeletal organization, which indicates isoform-specific regulation in lung cancer. ROCK1 has been regarded as a positive regulator of cell migration and invasion in several solid tumors, such as osteosarcoma (38), breast (12), pancreatic (122), gastric (123) and lung cancer (37,124,125). In addition, some ROCK inhibitors, such as Y-27632, which can target both ROCK isoforms, have been found to be more potent against ROCK1 than against ROCK2 (126). However, this should not be the reason to discount the role of ROCK2 in lung cancer growth. Further evidence is needed to demarcate the role of ROCK2 in lung cancer. Overall, the aim of these ROCK inhibition studies, either kinase-targeting or non-kinase-targeting ROCK inhibition, is to determine the effects of ROCK inhibition that elucidates the ROCK function in lung carcinogenesis. The majority of the studies included cell migration assays to investigate cellular motility changes following ROCK inhibition or knockdown, since it is a key mediator that regulates cytoskeletal rearrangement that affects cellular motility (37,104,116,125). Examples of the migration assays employed are Transwell, collagen invasion, tube formation and scratch wound healing assays. Their findings suggested that the ROCK signaling pathway should be considered as a potential therapeutic target for inhibiting lung cancer development, as ROCK action is diffuse and its activation can promote multiple hallmarks of cancer.

6. Other druggable targets of lung cancer

Identifying the genetic aberrations of lung cancer, such as KRAS and EGFR mutations is important in tailoring the appropriate therapy for the disease. NSCLC harboring KRAS mutations has been found to be more vulnerable to RhoA inhibitor-induced apoptosis compared to wild-type NSCLC (127). As KRAS mutations are common in lung cancer, occurring in 30% of adenocarcinoma and 5% of SCC cases, the inhibition if RhoA appears to be an excellent treatment option for NSCLC (45,128). Furthermore, the KRAS-mutant NSCLC cell line is also vulnerable to a combination of drug pairs that inhibit polo-like kinase 1 (PLK1), a synthetic lethal partner of Ras oncogene, and ROCK that exhibit marked apoptosis induction and colony form inhibition (45). Another common

Table I. Studies on kinase-targeting ROCK inhibition in lung cancer.

Direct ROCK inhibitor	Target	Type of lung cancer	Name of cell line	Effects	(Refs.)
Fasudil	- ROCK	- NSCLC	- 95D	- Decreases cell growth and metastasis	(16)
		- NSCLC	- A549	- Decreases cell proliferation - Decreases migration and invasion	(17)
		- NSCLC	- A549	- Decreases cell viability - Decreases cell migration - Anti-angiogenic	(81)
		- NSCLC	- H1299 - HCC827	- Anti-angiogenic	(94)
		- SCLC	- H446	- Decreases cancer cell growth in a dose-dependent manner - Prevents cell adhesion - Decreases cell proliferation - Decreases cell migration and invasion	(66)
		- SCLC	- H1339 - (Tissue biopsies) - (mouse model)	- Decreases cancer cell growth - Induces cell cycle arrest - Induces cell apoptosis	(18)
Y-27632	- ROCK	- NSCLC	- A549 - H1299 - PC-9 - HCC827	- Reduces lamellipodia formation - Decreases cell invasion	(103)
MYBH	- ROCK1	- NSCLC	- H460	- Inhibits actomyosin organization - Inhibits phosphorylation of LIMK and cofilin through ROCK1 - Decreases cell motility, invasion, and metastasis	(21)
Rho-kinase inhibitor (RKI) 18	- ROCK	- NSCLC	- A549 - H460	- Inhibits stress fiber formation, filopodia, and lamellipodia	(112)
Fasudil and BI-2536	- ROCK and PLK1	- NSCLC	- A549 - (mouse model)	- Induces cell apoptosis - Induces cell cycle arrest - Decreases colony number and size of the cell - Decreases tumor growth in KRAS mutant mouse model - Upregulates p53 signaling pathway	(45)
Y-27632 and afatinib	- ROCK and EGFR	- NSCLC	- A549 - H358 - H1650 - H1975	- Decreases cell growth - Decreases cell invasion	(104)
BMS-5	- LIMK	- NSCLC	- A549	- Inhibits microtubule assembly - Increases acetylated α-tubulin	(105)
Zerumbone	- ROCK1	- NSCLC	- A549	- Inhibits lamellipodia formation	(80)
Glabridin	- RhoA	- NSCLC	- A549	- Decreases the interaction of FAK and Src - Decreases MLC phosphorylation that can activate ROCK - Anti-angiogenic	(106)
Plumbagin	- FAK - ROCK1 - LIMK1/2	- NSCLC	- A549 - H1299 - (mouse model)	- Inhibits lamellipodia formation - Reduces cell invasion	(46)
Deguelin	- ROCK1	- NSCLC	- A549 - H460	- Decreases cell proliferation - Decreases cell migration and cell invasion - Inhibits filopodia and lamellipodia formation - Decreases tumor metastasis-related genes such as CD44, MMP2, and MMP9	(107)

Table I. Continued.

Direct ROCK inhibitor	Target	Type of lung cancer	Name of cell line	Effects	(Refs.)
B-Escin	- RhoA	- NSCLC	- H460 - (mouse model)	- Inhibits tobacco carcinogen-induced lung tumor formation by modulating RhoA/Rock signaling	(108)
XAP	- RhoC	- NSCLC	- A549	- Decreases cell proliferation - Decreases cell migration and invasion - Reduces CCR5 chemokine receptor expression	(102)
OXA-06	- ROCK	- NSCLC	- A549 - H1299 - H23 - H358 - H1703	- Induces cell cycle arrest - Decreases anchorage-independent growth and cell invasion	(15)
shRNA	- ROCK1 and PTEN	- NSCLC	- A549 - H1299 - H226 - SK-MES-1	- Decreases cell migration and invasion	(37)
	- RhoC	- NSCLC	- A549	- Decreases EMT induced by TGF-β1	(85)
siRNA	- ROCK	- NSCLC	- H1299	- Decreases cell growth with a near-complete suppression of colony formation	(15)
	- ROCK1	- NSCLC	- A549	- Decreases cell viability - Induces cell apoptosis - Decreases cell proliferation	(67)
MicroRNA-335-5p	- ROCK1	- NSCLC	- A549 - SPC-A1 - (Tissue biopsies)	- Decreases TGF-β1-induced EMT - Decreases cell migration and invasion	(19)
MicroRNA-186	- ROCK1	- NSCLC	- A549 - H1299 - H358 - H157 - (Tissue biopsies)	- Decreases proliferation - Decreases cell invasion and migration	(20)
MicroRNA-148a	- ROCK1	- NSCLC	- A549 - H1299 - (Tissue biopsies)	- Decreases EMT - Decreases cell invasion	(116)
MicroRNA-101	- ROCK2	- NSCLC	- A549 - NCI-460 - NCI-520 - NCI-H596 - (Tissue biopsies)	- Downregulation of miR-101 contributes to - EMT in cisplatin resistance-NSCCLC - Low miR-101 expression is associated a with poor survival time	(115)

gene aberration that has been studied as a drug target is EGFR mutations, which occur in 39% of adenocarcinoma and 58% of the SCC subtype of lung cancer (129). EGFR is a tyrosine kinase that plays an essential role in SCC pathogenesis by dimerizing its receptor (130). Therefore, inhibiting EGFR dimerization can inhibit the pathogenesis, and inhibiting RhoA by using lovastatin has been shown to yield the same effect as an EGFR inhibitor, as RhoA is necessary for EGFR localization and activation (131). Inhibiting EGFR activation can also reduce programmed death-ligand 1 (PD-L1) expression associated with the p-ERK1/2/p-c-Jun pathway (132). Reducing PD-1 and its interaction with the ligand is important in restoring the proliferation of T-cells and promoting the cytotoxic activity of immune cells to cancer cells (133). Importantly, an increased PD-L1 expression has been reported to be associated with EGFR mutations (134,135) and is currently the only approved biomarker for NSCLC immune checkpoint inhibitors (136).

Targeting proteins that regulate the ROCK signaling pathway has also been suggested to yield promising results. Osteopontin (OPN), a major non-collagenous bone matrix protein, has been found to be responsible for activating ROCK1, and to subsequently increase LIMK and cofilin phosphorylation in NSCLC cell lines, thereby promoting cancer cell migration and invasion (103). It is suggested that

Table II. Studies on non-kinase-targeting ROCK inhibition in lung cancer.

Indirect ROCK inhibitor	Target	Type of lung cancer	Name of cell line	Effects	(Refs.)
KRT14 lentivirus	- KRT14	- NSCLC	- A549 - H1975 - (mouse model)	KRT14 depletion reduces: - ROCK1 expression - Cell migration and invasion	(120)
DEK siRNA	- DEK	- NSCLC	- A549 - H1299 - (Tissue biopsies)	DEK depletion reduces: - RhoA expression - Cell proliferation - Cell migration	(73)
CHD4 siRNA	- CHD4	- NSCLC	- A549 - H1299 - (Tissue biopsies) - (mouse model)	CHD4 depletion reduces: - RhoA, ROCK, phosphor-myosin expression - Cell proliferation - Cell migration - Cell growth	(74)
lncNORAD siRNA	- lncNORAD	- NSCLC	- A549 - (mouse model)	lncNORAD depletion reduces: - RhoA/ROCK expression - Cell proliferation - Cell migration and invasion	(121)

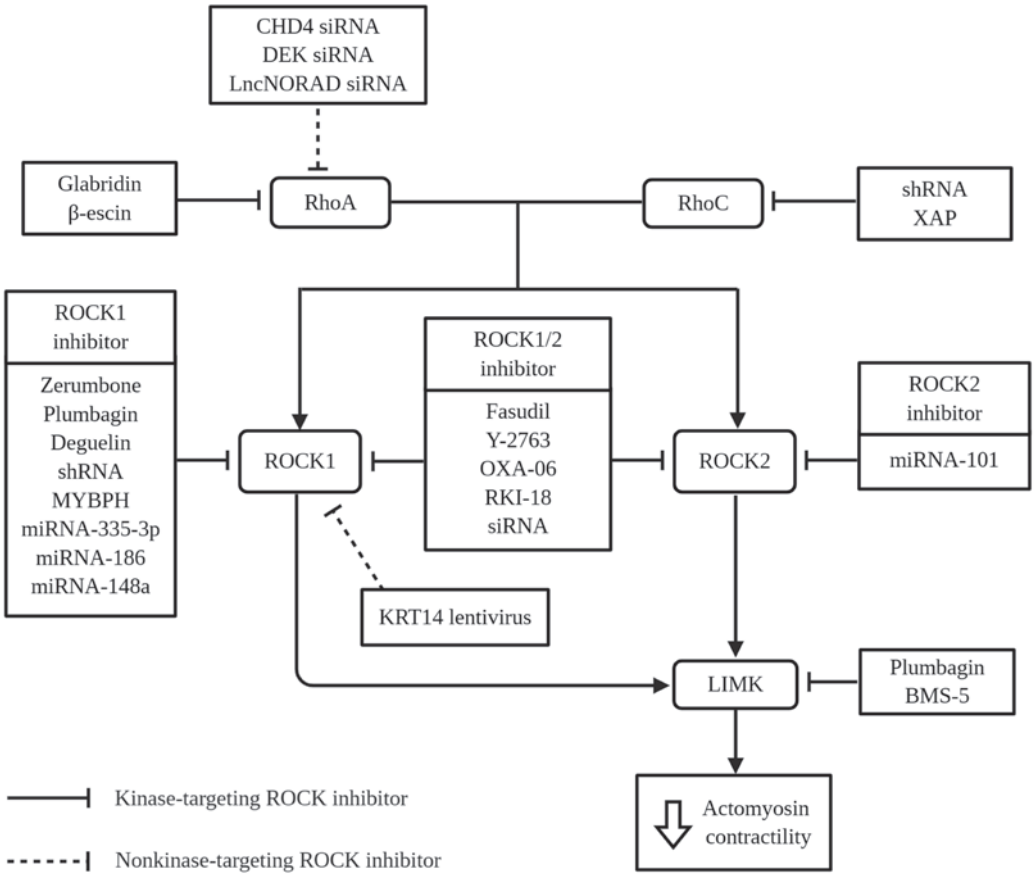

Figure 3. Targets of kinase-targeting ROCK inhibitors and non-kinase-targeting ROCK inhibitors. ROCK, Rho-associated kinase; LIMK, Lim kinase.

OPN is a worthy target as its inhibition can significantly reduce the tumor weight and volume of NSCLC as studied by Cho *et al* (137). In addition, OPN can reduce lamellipodia formation and actin polymerization via the ROCK signaling pathway following suppression with zerumbone and plumbagin, respectively (46,80). Since OPN plays an essential role in mediating tumor-stroma interaction and contributes to tumor growth and metastasis (138), targeting OPN may

hold promise for the prevention of lung cancer metastasis. In addition, caveolin-1 (CAV1), an ECM-associated oncogenic membrane protein that can activate the ROCK signaling pathway, may also be a potential druggable target. High levels of stromal CAV1 have been identified in various types of cancer, including lung cancer (139,140) and its interactions with Rho-GTPases have been demonstrated to promote metastasis through Src, Ras and Erk activation (141). This interaction promotes cell migration and invasion by regulating CAV1 tyrosine phosphorylation, which can lead to the regulation of focal adhesion dynamics (142).

7. Conclusion and future direction

The treatment of lung cancer has improved substantially over the years, involving various strategies and modalities, such as surgery, radiotherapy, chemotherapy, immunotherapy and molecular-targeted therapy (143). The search for a suitable target candidate to treat lung cancer is still ongoing. Herein, the ROCK signaling pathway is suggested as one of the potential targets that can be utilized for the treatment of lung cancer, since its inhibition has resulted in promising outcomes to reduce cancer cell proliferation, migration, and invasion in pre-clinical studies. Repurposing the use of already licensed drugs, such as fasudil for the treatment of lung cancer is a good start, as it provides a rapid translation of pre-clinical data into effective therapies for lung cancer patients. However, the use of fasudil is still associated with certain drawbacks, such as the off-target effect. On the other hand, other novel compounds may have insufficient efficacy apart from the concerns of side-effects and the selective binding of ROCK inhibitors, as the ROCK signaling pathway also plays an essential role in normal cell homeostasis. Nevertheless, the expression of ROCK in cancer is higher if compared to normal cells or tissues; thus, the use of ROCK inhibitors in cancerous and normal cells or tissues may yield different outcomes in term of expression following treatment. Specific pathway inhibition, limited to cancer cells, is preferable to prevent undesirable side-effects caused by current chemotherapeutic drugs. Obtaining a range of effective doses without causing adverse effects in patients is also another challenge that requires a comprehensive analysis of safety, efficacy and toxicity to be made. The majority of the ROCK and lung cancer studies were found to be performed *in vitro* only, while there is a lack of *in vivo* studies or the use of tissue biopsies from patients with lung cancer. Therefore, further research should also employ these more complex settings to fully elucidate the mechanisms of the ROCK signaling pathway in lung cancer.

To date, to the best of our knowledge, no single inhibitor targeting the ROCK signaling pathway has been approved for use in clinical trials against lung cancer. Nevertheless, defactinib, a drug that can inhibit FAK, a mechanosensor that can detect changes in the ECM and activate ROCK, has been tested in a clinical trial for the treatment of KRAS mutant NSCLC. However, defactinib has been shown to only yield modest and contrasting results from pre-clinical studies (144). This may be due to the insufficient efficacy and it being an unspecific target of FAK inhibitor. Therefore, it is suggested that the targeting of ROCK should be performed to yield a more profound impact in regulating cellular phenotypes, as discussed herein, thus treating lung cancer. A combination of agents in the treatment of lung cancer should also be considered in future research, as supported by better outcomes in pre-clinical studies targeting ROCK with EFGR or PLK1 (45,104). Moreover, an agent that can affect multiple oncogenic pathways and fine-tuning treatment strategies based on molecular aberrations can provide more effective treatment strategies, since cancer is highly adaptive and can acquire resistance rapidly (145,146). Targeting ROCK can also help to solve several issues, such as drug resistance seen in hypoxic tumors. According to Murakami *et al* (147), gefitinib was found to be ineffective in hypoxic EGFR mutation-positive NSCLC due to a vascular inadequacy that dampens the bioavailability of the drug in the target area. Therefore, targeting ROCK along with gefitinib can help in restoring the role of the blood vessel to deliver oxygen and increase the drug bioavailability. Taken together, the ROCK signaling pathway plays a critical role in the carcinogenesis of lung cancer, and is therefore suggested as a potential therapeutic target in the treatment of lung cancer. Further in-depth research is urgently required to enhance our understanding of this pathway, and further attempts should be made to elucidate the biological mechanisms between ROCK and lung cancer.

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Authors' contributions

MAZ and SFM contributed to the conceptualization, drafting and writing of the manuscript. SFM, EWC, NFR and GTS provided substantial contributions to the finalization, correction and critical reviewing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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