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(54) Title: P27 PREVENTS CELLULAR MIGRATION

(57) Abstract: This invention provides methods of preventing cellular migration and of treating cardiovascular diseases and tumor metastasis by increasing the intracelllular concentration of cyclin-dependent kinase inhibitor p27 or C3 exoenzyme or decreasing the intracellular concentration of Rho-kinase, and methods of identifying chemical compounds for use in such treatments.

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P27 PREVENTS CELLULAR MIGRATION

This application claims priority of U.S. Serial No. 10/172,027, filed June 14, 2002, which is a continuation-in-part of U.S. Serial No. 09/766,944, filed January 22, 2001, the contents of which are incorporated herein by reference.

The invention disclosed herein was made with Government support under grant numbers RO1HL56180, RO1A139794, and RO3TW00949 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Background Of The Invention

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Vascular smooth muscle cell (SMC) migration is believed to play a major role in the pathogenesis of many vascular diseases, such as atherosclerosis and restenosis after both percutaneous transluminal angioplasty (PTCA) and coronary stenting (Schwartz, 1997). In normal blood vessels, the majority of SMC

reside in the media or middle coat of the vessel, where they are quiescent and possess a "contractile" phenotype, characterized by the abundance of actin- and myosin-containing filaments. In disease states, SMCs migrate from the media to the intima or inner coat of the blood vessel. The process of SMC migration in pathological states involves the synthesis of extracellular matrix, protease enzymes, growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), and cytokines that further contribute to proliferation and migration (Clowes and Schwartz, 1985; Ferns et al., 1991; Grotendorst et al., 1981; Ihnatowycz et al., 1981; Jawien et al., 1992). Fibroblast growth factor-2 (FGF-2) appears to modulate SMC migration by changing extracellular matrix (ECM)-β1 integrin interactions (Pickering et al., 1997). FGF-2 augments SMC surface expression of α2β1, α3β1 and αvβ1 integrins, thereby resulting in enhanced cellular motility through disassembly of the α-actin stress fiber network (Pickering et al., 1997).

Rapamycin, a macrolide antibiotic, inhibits SMC proliferation both *in vitro and in vivo* by blocking cell cycle progression at the transition between the first gap (G1) and DNA synthesis (S) phases (Cao et al., 1995; Gallo et al., 1999; Gregory et al., 1993; Marx et al., 1995). The inhibition of cellular proliferation is associated with a marked reduction in cell cycle dependent kinase activity and in retinoblastoma protein phosphorylation *in vitro* (Marx et al., 1995) and *in vivo* (Gallo et al., 1999). Down-regulation of the cyclin-dependent kinase inhibitor

(CDKI) $p27^{kipl}$ by mitogens is blocked by rapamycin (Kato et al., 1994; Nourse et al., 1994). Pre-treatment of rat and human SMC with rapamycin (2 nM) for 48 hours inhibited PDGF-induced SMC migration in a modified Boyden chamber. However, acute rapamycin treatment (6 hours) of rat and human SMC had no effect on migration, suggesting that longer exposure to rapamycin is essential for its anti-migratory actions. In support of these findings, acute 6 hour treatment with rapamycin (1-100 nM), wortmannin and LY294002 of both SMC and Swiss 3T3 cells failed to inhibit PDGF-induced chemotaxis (Higaki et al., 1996). The findings that rapamycin possesses both anti-proliferative and anti-migratory SMC properties led to the suggestion that rapamycin may have important applications in the treatment of disorders such as accelerated arteriopathy that occurs in transplanted hearts and restenosis after percutaneous transluminal angioplasty and placement of coronary stents (Marx et al., 1995; Marx and Marks, 1999; Poon et al., 1996). Rapamycin significantly inhibited neointimal proliferation in a porcine angioplasty model (Gallo et al., 1999) and reversed chronic graft vascular disease in a rodent heart allograft model (Poston et al., 1999). Recent clinical studies have implicated the importance of rapamycin in treating stent restenosis (Sousa et al., 2000).

In $p27^{kipl}$ (-/-) knockout mice, relative rapamycin resistance was demonstrated, and in rapamycin resistant myogenic cells, constitutively low levels of $p27^{kipl}$ were observed, which were not increased with serum withdrawal and rapamycin (Luo et al., 1996).

These findings suggested that the ability to block $p27^{kip1}$ down-regulation contributes to the growth inhibitory effects of rapamycin. Transfection of the cyclin-dependent kinase inhibitor $p21^{cip1}$ was shown to inhibit the spreading and attachment of SMC to extracellular matrices and migration in a modified Boyden chamber assay. These findings suggested that $p21^{cip1}$ is probably an adhesion inhibitor, as it prevented the assembly of actin filaments and the translocation of adhesion molecules (Fukui et al., 1997).

The present application discloses that rapamycin has potent inhibitory effects on SMC migration in wild type and p27 (+/-) mice, but not in p27 (-/-) knockout mice, indicating that the cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$ plays a critical role in rapamycin's anti-migratory properties and in the signaling pathway(s) that regulates SMC migration.

Summary Of The Invention

The present invention is directed to a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby preventing migration of the cell.

The invention is also directed to a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration of C3 exoenzyme, thereby preventing migration of the cell.

The invention provides a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby preventing migration of the cell.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby alleviating the subject's cardiovascular disease.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular concentration of C3

exoenzyme, thereby alleviating the subject's cardiovascular disease.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby alleviating the subject's cardiovascular disease.

The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby inhibiting tumor metastasis.

The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration of C3 exoenzyme, thereby inhibiting tumor metastasis.

The invention provides method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby inhibiting tumor metastasis.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of cyclin-

dependent kinase inhibitor p27 is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration of p27, and detecting an increase in the intracellular concentration of p27 in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of cyclin-dependent kinase inhibitor p27 is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration of p27;

(b) determining if the intracellular concentration of p27 is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of p27 is increased in the presence of each compound included in the plurality of chemical compounds, so as to

thereby identify any compound included therein as a compound which inhibits cellular migration.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of C3 exoenzyme is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration of C3 exoenzyme, and detecting an increase in the intracellular concentration of C3 exoenzyme in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of C3 exoenzyme is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration of C3 exoenzyme;

(b) determining if the intracellular concentration of C3 exoenzyme is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of C3 exoenzyme is increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the chemical compound under conditions suitable for decreasing the intracellular concentration of Rho-kinase, and detecting a decrease in the intracellular concentration of Rho-kinase in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for decreasing the intracellular concentration of Rho-kinase;

(b) determining if the intracellular concentration of Rho-kinase is decreased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of Rho-kinase is decreased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration of cyclin-dependent kinase inhibitor p27 and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration of C3 exoenzyme and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any

of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to decrease the intracellular concentration of Rho-kinase and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

The invention provides a pharmaceutical composition comprising an amount of a chemical compound identified using any of the methods described herein effective to inhibit cellular migration and a pharmaceutically acceptable carrier.

The invention provides a method for preparing a pharmaceutical composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical compound identified by any of the methods described herein or a novel structural and functional analog or homolog thereof.

The invention provides a method for making a composition of matter which inhibits cellular migration which comprises identifying a chemical compound using any of the methods described herein, and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

The invention provides a method of treating a subject with a cardiovascular disease which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by

any of the methods described herein, or a novel structural and functional analog or homolog thereof.

The invention provides a method of inhibiting tumor metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by any of the methods described herein, or a novel structural and functional analog or homolog thereof.

Brief Description Of The Figures

Figure 1A-1D. Rapamycin potently inhibits migration in smooth muscle cells from wild type, but not p27 (-/-) knockout mice.

(A) Migration of SMCs isolated from wild type mice was determined in the modified Boyden chamber following rapamycin and FK506 treatment. Rapamycin (open bars; 1, 10 and 100 nM) significantly inhibited SMC migration, whereas FK506 demonstrated no effect (blackened bars). * $p < 0.05$ as compared to control. The inset shows an immunoblot demonstrating increased $p27^{kipi}$ levels after rapamycin (100 nM for 48 hours) treatment (lane 2) as compared to untreated proliferating SMC (lane 1).

(B) Migration of SMCs isolated from p27(-/-) knockout mice was determined in the modified Boyden chamber following rapamycin and FK506 treatment. Only at high concentrations did rapamycin (open bars; 100 and 1000 nM) significantly inhibit SMC migration, whereas FK506 demonstrated no effect (blackened bars). * $p < 0.05$ as compared to control. The inset shows an immunoblot demonstrating the absence of $p27^{kipi}$.

(C and D) FK506 competes with rapamycin for binding to FKBP12 and inhibits the effects of rapamycin on wild type (C) and p27 (-/-) (D) SMC migration.

Figure 2A-2B. Lack of effect of rapamycin on murine SMC adhesion.

Wild type (open bars) and p27(-/-) (blackened bars) SMC were incubated with rapamycin for 48 hours before plating onto either fibronectin (A) or laminin (B) coated plates for 3 hours. The number of adhering cells was determined with a Coulter counter in triplicate and normalized to the number of untreated wild type cells. No significant differences were noted between treated and untreated cells.

Figure 3A-3C. *In vivo* administration of rapamycin potently inhibits explant migration of SMC from wild type but not p27(-/-) knockout animals.

(A) p27 (+/+), p27 (+/-) and p27 (-/-) mice were injected with rapamycin (4 mg/kg/day) for 5 days. The aortas were explanted, and migration of SMC was quantified and is presented as the rapamycin-mediated inhibition of migration as a % of control. Rapamycin significantly inhibited migration in both p27 (+/+) and p27 (+/-) SMC; rapamycin had no effect on p27 (-/-) SMC explant migration

(B) p27 (+/+), p27 (+/-) and p27 (-/-) mice were injected with rapamycin (9 mg/kg/day) for 7 days. Rapamycin inhibited migration in p27 (+/+), p27 (+/-) and p27 (-/-) SMC explants.

(C) p27 (+/+) and p27 (-/-) mice were injected with taxol (20 mg/kg/day) for 7 days. Taxol inhibited migration in p27 (+/+) and p27 (-/-) SMC.

Figure 4. Impaired migration-inhibitory response to C3 exoenzyme in SMC derived from p27 (-/-) knockout mice.

Migration of SMC isolated from wild type mice (open bars) and p27 (-/-) mice (blackened bars) was determined in the modified Boyden chamber following C3 exoenzyme (2 and 20 µg/ml) treatment for 16 hours. SMC derived from p27 (-/-) mice demonstrated a 25% relative migratory resistance to C3 exoenzyme.
* $p < 0.05$ as compared to control.

Figure 5. Rapamycin and C3 exoenzyme inhibit SMC migration through $p27^{kipl}$-dependent and -independent pathways.

Rapamycin (Rapa)-FKBP12 inhibits target-of-rapamycin (TOR)-mediated activation/phosphorylation of protein translation modulators 4E-BP1 (a translation initiation factor) and p70 S6 kinase (S6 is a ribosomal protein) (Marx and Marks, 1999) and prevents mitogen-induced down-regulation of $p27^{kipl}$ through an unknown mechanism (dashed lines). Rapamycin inhibits SMC migration through $p27^{kipl}$-dependent and -independent mechanisms. C3 exoenzyme, which specifically ADP ribosylates and inhibits RhoA, inhibits SMC migration through $p27^{kipl}$-dependent and -independent (cytoskeleton changes) pathways.

Detailed Description Of The Invention

The present invention is directed to a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby preventing migration of the cell. In one embodiment, the concentration of cyclin-dependent kinase inhibitor p27 is increased by increasing the concentration and/or activity of C3 exoenzyme.

The invention is also directed to a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration and/or activity of C3 exoenzyme, thereby preventing migration of the cell. In one embodiment, the compound is C3 exoenzyme.

The invention provides a method of preventing migration of a cell in a subject which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby preventing migration of the cell.

In one embodiment of any of the methods described herein, the cell is a smooth muscle cell. In one embodiment, the cell is a tumor cell.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which

increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby alleviating the subject's cardiovascular disease. In one embodiment, the concentration of cyclin-dependent kinase inhibitor p27 is increased by increasing the concentration and/or activity of C3 exoenzyme.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular concentration and/or activity of C3 exoenzyme, thereby alleviating the subject's cardiovascular disease. In one embodiment, the compound is C3 exoenzyme.

The invention provides a method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby alleviating the subject's cardiovascular disease.

In one embodiment of any of the methods described herein, the cardiovascular disease is atherosclerosis. In one embodiment, the cardiovascular disease is arteriopathy after heart transplantation. In one embodiment, the cardiovascular disease is restenosis after angioplasty or vascular stent placement. In different embodiments, the stent placement is in a coronary vessel, a peripheral vessel, or a cerebral vessel. In one embodiment, the blood vessel is an artery.

The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby inhibiting tumor metastasis. In one embodiment, the concentration of cyclin-dependent kinase inhibitor p27 is increased by increasing the concentration and/or activity of C3 exoenzyme.

The invention provides a method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration and/or activity of C3 exoenzyme, thereby inhibiting tumor metastasis. In one embodiment, the compound is C3 exoenzyme.

The invention provides method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby inhibiting tumor metastasis.

In different embodiments of the methods described herein, the compound increases the endogenous amount of cyclin-dependent kinase inhibitor p27. In different embodiments, the compound decreases the endogenous amount of Rho-kinase.

Chimeric molecules in which the active site of C3 exoenzyme or other agents is fused to regions of toxins that are rapidly taken up into cells can be

generated to enhance the uptake of C3 exoenzyme or the agent into cells. Similarly, viral agents can be used to enhance entry of C3 or other agents into cells. Other ways of enhancing entry of C3 or an agent into a cell include, but are not limited to, combining C3 or the agent with any of the following: a peptide added with C3 exoenzyme or the agent, a leader sequence comprised of an amino acid sequence (e.g., 9 arginines or 9 lysines or combinations thereof) fused to C3 exoenzyme or to the agent, or a TAT sequence based upon HIV-1 viral sequence.

In different embodiments of the methods described herein, the method does not comprise administration of a gene or gene therapy.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of cyclin-dependent kinase inhibitor p27 is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration of p27, and detecting an increase in the intracellular concentration of p27 in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit

cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of cyclin-dependent kinase inhibitor p27 is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration of p27;

(b) determining if the intracellular concentration of p27 is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of p27 is increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

In different embodiments of the methods described herein, cyclin-dependent kinase inhibitor p27 is detected using immunoblots. P27 is a regulator of cell cycle progression. Increased levels of p27 are associated with cell cycle arrest, which can be assessed by cell proliferation assays, phosphorylation status of the retinoblastoma protein (pRb) and activity assays of various cell cycle dependent kinases such as cdk2 or cdk4.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration and/or activity of C3 exoenzyme is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration and/or activity of C3 exoenzyme, and detecting an increase in the intracellular concentration and/or activity of C3 exoenzyme in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration and/or activity of C3 exoenzyme is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration and/or activity of C3 exoenzyme;

(b) determining if the intracellular concentration and/or activity of C3 exoenzyme is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration and/or activity of C3 exoenzyme is increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

In different embodiments of the methods described herein, C3 exoenzyme activity is detected by measuring p27, since C3 exoenzyme increases p27 levels. P27 can be assessed using Western blots, by cell proliferation assays, phosphorylation status of the retinoblastoma protein (pRb) and activity assays of various cell cycle dependent kinases such as cdk2 or cdk4. C3 exoenzyme ADP-ribosylates Rho, which inhibits Rho activity. Inhibition of Rho leads to increased p27 levels in smooth muscle. In different embodiments, C3 levels are measured by measuring Rho-kinase. The amount of C3 could also be quantified using an anti-C3 antibody.

The invention provides a method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the chemical compound under conditions suitable for decreasing the intracellular concentration of Rho-kinase, and detecting a decrease in the intracellular concentration of Rho-kinase in the presence of the chemical compound so as to

thereby identify the chemical compound as a compound which inhibits cellular migration.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for decreasing the intracellular concentration of Rho-kinase;

(b) determining if the intracellular concentration of Rho-kinase is decreased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of Rho-kinase is decreased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

In one embodiment of any of the methods described herein, the compound is not previously known to inhibit cellular migration.

In different embodiments of the methods described herein, the cells are smooth muscle cells or tumor cells. In one embodiment, the cells are vertebrate cells. In a further embodiment, the vertebrate cells are mammalian cells. In a still further embodiment, the mammalian cells are human cells.

Rho-kinase can be assayed using well known methods (e.g. Sander et al. 1999, Alblas et al. 2001, Beqaj et al. 2002). For example, in one assay (Beqaj et al. 2002) based on the capability of GST-rhotekin to bind to GTP-Rho (Ren et al. 1999), cells are lysed with Rho-binding lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ with 10 micrograms/ml leupeptin, 10 micrograms/ml aprotinin, and 1mM PMSF). Lysates are cleared by centrifugation, and active RhoA precipitated with 20 micrograms of GST-tagged fusion protein (residues 7-89 of mouse rhotekin Rho binding domain). The precipitates are washed in washing buffer (50 mM Tris, pH7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM PMSF, 10 micrograms/ml aprotinin and 10 micrograms/ml leupeptin), and the bound proteins are eluted and resolved in 14% SDS-PAGE, followed by transfer to nitrocellulose and blotting using a rabbit polyclonal RhoA antibody. Active RhoA is retained on the GST rhotekin fusion protein and can be quantified. Other assays (Sander et al. 1999, Alblas et al. 2001) involve use of Western blots and anti-RhoA monclonal antibody (Santa Cruz Biotechnology).

The invention provides a chemical compound identified by any of the methods described herein.

This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration of cyclin-dependent kinase inhibitor p27 and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration and/or activity of C3 exoenzyme and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. This invention provides a pharmaceutical composition comprising (a) an amount of a chemical compound identified using any of the methods described herein, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to decrease the intracellular concentration of Rho-kinase and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

The invention provides a pharmaceutical composition comprising an amount of a chemical compound identified using any of the methods described herein

effective to inhibit cellular migration and a pharmaceutically acceptable carrier.

The invention provides a method for preparing a pharmaceutical composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical compound identified by any of the methods described herein or a novel structural and functional analog or homolog thereof.

The invention provides a method for making a composition of matter which inhibits cellular migration which comprises identifying a chemical compound using any of the methods described herein, and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

The invention provides a method of treating a subject with a cardiovascular disease which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by any of the methods described herein, or a novel structural and functional analog or homolog thereof. In different embodiments, the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement. In one embodiment, the cardiovascular disease is restenosis after vascular stent placement. In different embodiments, the stent placement is in a coronary vessel, a peripheral vessel, or a cerebral vessel. In one embodiment, the blood vessel is an artery.

The invention provides a method of inhibiting tumor metastasis in a subject which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by any of the methods described herein, or a novel structural and functional analog or homolog thereof.

The invention provides a use of a chemical compound identified by any of the methods described herein for the preparation of a pharmaceutical composition for treating an abnormality, wherein the abnormality is alleviated by inhibiting cellular migration. In different embodiments, the abnormality is a cardiovascular disease or a tumor metastasis. In different embodiments, the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

In the subject invention, a "pharmaceutically effective amount" is any amount of a compound which, when administered to a subject suffering from a disease against which the compound is effective, causes reduction, remission, or regression of the disease. Furthermore, as used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

This invention provides homologs, analogs, isomers, isoforms, or isozymes of any of the compounds or agents described herein. A structural and functional analog of a chemical compound has a structure similar to that of the compound but differing from it in respect to a certain component or components. A structural and functional homolog of a chemical compound is one of a series of compounds each of which is formed from the one before it by the addition of a constant element. The term "analog" is broader than and encompasses the term "homolog". Isomers are chemical compounds that have the same molecular formula but different molecular structures or different arrangement of atoms is space. The isomers may be structural isomers, positional isomers, stereoisomers, optical isomers, or cis-trans isomers. The invention also provides for keto-enol tautomers. Isoforms are multiple forms of a protein whose amino acid sequences differ slightly but whose general activity is identical. Isozymes (isoenzymes) are multiple forms of an enzyme that catalyze the same reaction but differ from each other in properties such as substrate affinity or maximum rate of enzyme-substrate reaction.

This invention provides prodrugs or metabolites of any of the compounds or agents described herein. In general, prodrugs will be functional derivatives of compounds which are readily convertible *in vivo* into the required compound. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in Design of Prodrugs, ed. H. Bundgaard, Elsevier, 1985.

Metabolites include active species produced upon introduction of compounds into the biological milieu.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Materials And Methods

Reagents: Dulbecco Modified Eagle Medium (DMEM) and trypsin were obtained from GIBCO (Grand Island, NY), recombinant bFGF was obtained from Biosource International (Camarillo, CA), and paclitaxel was obtained from Sigma (St. Louis, MO). Rapamycin was a gift from Dr. Suren Sehgal (Wyeth-Ayerst Laboratories, Princeton, NJ).

Expression of C3 exoenzyme: C3 exoenzyme was prepared as previously described (Dillon and Feig, 1995). The Glutathione S Transferase (GST)-C3 exoenzyme cDNA (gift of Dr. Judy Meinkoth, University of Pennsylvania) was transformed into competent BL21. Protein expression was induced with 200 μM isopropylthiogalactoside (IPTG) at 32°C for 3 hours. Lysates were prepared and incubated with GST-sepharose beads for 1 hour at 4°C. The beads were washed and incubated overnight at 4°C with 3 units/ml thrombin (for cleavage of the C3 exoenzyme from the GST fusion protein), which was removed by incubating the supernatant with antithrombin-sepharose beads for 1 hour at 4°C. The supernatant was concentrated with a Centricon-10 (Amicon Inc, Beverly, Mass). Protein concentration was determined by Bradford assay and the supernatant was aliquoted and frozen in liquid nitrogen. The samples were run on SDS-PAGE and stained with Coomassie to confirm correct expression of the GST fusion protein and cleavage/purification of C3 exoenzyme before use (Seasholtz et al., 1999).

Cell Culture: The murine aortic SMCs were obtained from the explant migration experiments described below, and were subcultured in DMEM containing 20% fetal bovine serum (FBS) at 37°C in a humidified 95% air-5% CO_2 atmosphere (Kobayashi et al., 1993). The growth medium was changed every other day until 80% confluence was reached. The cells used for experiments were from passages #3-6. Verification of SMC phenotype was determined by positive fluorescent staining for α-actin and negative staining for Factor VIII antigen. Cell viability was 95% or greater as determined by trypan blue exclusion at the conclusion of each experiment.

SMC Adhesion Assay: The adhesion assay was performed as previously described (Wang et al., 1997). Murine SMCs were treated with rapamycin or vehicle for 48 hours. SMCs (5 X 10^5/ml in DMEM supplemented with 0.2% bovine serum albumin (BSA)) were loaded onto 12-well plates pre-coated with laminin or fibronectin. After 3 hours, the media containing nonadherent cells were removed, and cell numbers were determined by triplicate counts using a Coulter Counter (Model Z1, Coulter Electronics, Beds, England).

SMC migration assay: Migration was measured using a 48 well modified Boyden chamber housing a polycarbonate filter with 8 μm pores as described previously (Bornfeldt et al., 1994; Poon et al., 1996). Each membrane was coated with 0.1 mg/ml of collagen in 0.2 M acetic acid for 24 hours before each assay. For each assay, 50 ng/ml of bFGF in DMEM

was loaded in quadruplicate wells in the bottom chamber. BSA (0.2% in DMEM without bFGF) was used as a negative control. Rapamycin, FK506 or C3 exoenzyme was directly added to the growth medium for either 48 hours (rapamycin and FK506) or 16 hours (C3 exoenzyme) before the cells were trypsinized, and counted with a hemacytometer. An equal number of cells (2 X 10^5/ml) in 50 µl was loaded to the top chamber of each well. After 6 hours, non-migrating cells were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with Giemsa stain (Fisher Scientific, NY). The number of SMC on the lower surface of the filter was determined by counting four high power (X200) fields of constant area per well. Values are expressed as the percentage of cells migrating in response to bFGF after subtraction of the negative control (DMEM + BSA). Experiments were performed at least twice using quadruplicate wells.

Aortic SMC explant migration: Wild type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). The p27(+/-) and p27 (-/-) knockout mice were kindly provided by Dr. Andrew Koff of Memorial Sloan-Kettering Cancer Institute (Kiyokawa et al., 1996). The mice received one of three different treatment protocols (9mg/kg/day for 7 days, 4 mg/kg/day for 5 days, or 2 mg/kg/day for 2 days) of rapamycin via intraperitoneal (IP) injection. The control group was treated with vehicle alone (0.2% sodium CMC, polysorbate 0.25%; Sigma, St. Louis, MO). At the conclusion of the treatment protocol, the mice were euthanized with 100 mg/kg of pentobarbital, the

aortas excised and the adventitia and surrounding connective tissue were removed. The aortas were then opened by a longitudinal cut and the intima, as well as a thin portion of the subjacent media, were removed. The media were divided into 2 mm X 2 mm pieces and placed in 6 well tissue culture plates (35mm, 22.6mm diameter, Costar, Cambridge, MA) containing DMEM with 20% FBS. The culture media was changed every other day. The migration of SMC out of the explant was observed under the microscope daily following explant. The total number of cells explanted was determined for each animal's explants on a daily basis. The results in Figure 5 are presented as the mean percentage (± SD) of inhibition of migration (by rapamycin or taxol) as compared to control (untreated) for at least 4 animals from each group. The SMC phenotype was confirmed as previously described (Spector et al., 1997).

Immunoblots: Immunoblots were prepared using procedures previously described in Luo et al. (1996). SMC growing in log phase or treated with rapamycin (100 nM for 48 hours) were washed twice with ice cold phosphate buffered saline (PBS) and lysates prepared using a modified RIPA buffer as previously described (Poon et al., 1996). Lysates were clarified by centrifugation for 20 minutes at 14,000 rpm at 4°C. Protein concentrations were determined by Bradford assay with BSA as a standard (Bradford, 1976). Protein extracts (30 µg) were size-fractionated on SDS-12% polyacrylamide gels and transferred to nitrocellulose. Filters were blocked with PBS-0.1% Tween 20 and 5% dry milk for 1 hour at room

temperature, followed by incubation with a mouse monoclonal $p27^{kip1}$ antibody (F8 antibody, Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 2 hours. Filters were washed with PBS-0.1% Tween 20 and then incubated with a secondary antibody conjugated to peroxidase for 1 hour. Filters were washed with PBS-0.1% Tween 20; signals were detected using chemiluminescence detection system (ECL) followed by exposure to Kodak XAR film.

Statistics: Data are presented as the mean ± standard deviation (SD) of the independent experiments. Statistical significance was determined by one way analysis of variance (ANOVA) and Fisher's PLSD test (StatView 4.01; Brain Power, Inc., Calabasas, CA). A paired t test (StatView 4.01) was used to analyze all data. A p value of < 0.05 was considered statistically significant.

Results

The inhibitory effects of rapamycin on the migration of SMCs isolated from wild type and p27 (-/-) knockout mice were determined. In wild type murine SMC, rapamycin treatment for 48 hours demonstrated a significant inhibitory effect on bFGF-induced SMC migration (Figure 1A, open bars). The inhibition was concentration dependent between 1 nM and 100 nM, with an IC_{50} of ~2 nM. In contrast, no significant inhibition of migration by rapamycin (1 nM to 10 nM) was observed in the p27 (-/-) SMC (Figure 1B, open bars). At higher concentrations (100 nM), an approximately 35% inhibition was observed; the IC_{50} in

p27 (-/-) cells was ~200 nM, representing a 100 fold increased IC_{50} as compared to wild type SMC. Addition of rapamycin to either the upper or lower chambers immediately prior to incubation had no effect on SMC migration. FK506, an agent that binds to the same cytosolic receptor (FKBP12) as rapamycin, had no effect on murine SMC migration (Figure 1A and 1B, blackened bars). The inhibition of migration of wild type murine SMC by rapamycin (10 nM) was competitively inhibited by a 100-fold molar excess of FK506 (Figure 1C). The rapamycin-induced inhibition of migration (100 nM) in the p27 (-/-) SMC was also competitively inhibited by a 20 fold molar excess of FK506 (Figure 1D). These data indicate that the inhibition of migration was mediated through rapamycin's binding to FKBP12. Treatment of wild type murine SMC with rapamycin (100 nM for 48 hours) caused a significant increase in $p27^{kip1}$ protein levels (Figure 1A, inset); in contrast, no $p27^{kip1}$ was detected in p27 (-/-) SMC (Figure 1B, inset). Although rapamycin inhibits SMC proliferation, the differences in migration do not reflect proliferation as equal numbers of cells were loaded into the Boyden chamber. To confirm this, the numbers of cells in the upper and lower chambers after the 6 hour incubation were equal in the untreated and treated wild type and p27 (-/-) SMC. In addition, no differences in cell viability were noted between untreated and rapamycin treated SMC obtained from wild type and p27 (-/-) animals. No morphologic differences were observed between untreated and rapamycin (100 nM for 48 hours) treated SMC isolated from wild type mice and p27 (-/-) mice.

Since migration is dependent upon the adhesion of the SMC to the Boyden chamber membrane, adhesion assays were performed using fibronectin and laminin-coated plates. SMC obtained from p27 (-/-) animals demonstrated no differences in adhesion as compared to SMC obtained from wild type animals on both fibronectin and laminin coated plates. Furthermore, rapamycin treatment (100 nM for 48 hours) did not affect cell adhesion in either wild type or p27 (-/-) SMC (Figure 2).

To assess the *in vivo* effects of rapamycin on SMC migration in the p27 (-/-) animals, the ability of SMC to migrate out of the murine aortic explants and establish cell cultures was examined. Rapamycin was not added to the culture medium after the aortas were explanted. Explant migration of aortic SMC was performed using wild type C57BL/6, p27 (+/-), or p27 (-/-) mice. SMC from wild type, p27 (+/-) and p27 (-/-) migrated out of the aortic explant by day #2. In animals treated with rapamycin (4 mg/kg/day for 5 days), ~85% inhibition of migration as compared to untreated animals was observed in the wild type and p27(+/-) groups ($p<0.05$). In contrast, no rapamycin-mediated inhibition of migration was observed in p27 (-/-) group ($p< 0.05$, Figure 3A), indicating that $p27^{kip1}$ plays a critical role in the rapamycin-mediated inhibition of SMC migration. At higher doses (9 mg/kg/day for 7 days), equivalent levels of rapamycin-mediated inhibition of migration were observed in wild type, p27 (+/-) and p27 (-/-) cells (Figure 3B). At lower doses (2 mg/kg/day for 2

days), no rapamycin-mediated inhibition of migration was observed. These results are consistent with the findings obtained in the modified Boyden chamber for p27 (-/-) cells and suggests the presence of both $p27^{kipl}$-dependent and $p27^{kipl}$-independent pathways mediating rapamycin's SMC anti-migratory actions. In order to demonstrate that agents that did not perturb the $p27^{kipl}$ pathway could inhibit migration in p27(-/-) animals, wild type and p27 (-/-) animals were treated with taxol (20 mg/kg/day for 7 days) (Sollott et al., 1995). No differences in taxol-mediated inhibition were observed in the two groups (Figure 3C).

Recent data suggests that the Ras/RhoA mitogenic pathway regulates the destruction of $p27^{kipl}$. C3 exoenzyme, which adenosine diphosphate (ADP)-ribosylates and inactivates RhoA, inhibited PDGF-induced $p27^{kipl}$ degradation. These findings suggest that activation of RhoA by mitogens is necessary for degradation of $p27^{kipl}$ (Weber et al., 1997). In addition, thrombin-induced vascular SMC DNA synthesis and migration were inhibited by C3 exoenzyme (Seasholtz et al., 1999). We sought to determine whether this inhibition of migration was mediated, in part, by regulating $p27^{kipl}$ levels. SMC from wild type and p27 (-/-) animals were exposed to either 2 µg/ml or 20 µg/ml C3 exoenzyme for 16 hours, trypsinized and loaded into the upper chamber of the Boyden chamber. C3 exoenzyme significantly inhibited bFGF-mediated SMC migration in wild type cells (Figure 4, open bars). SMC from p27 (-/-) animals demonstrated a 25% relative resistance to C3 exoenzyme (Figure 4,

blackened bars). SMC that were acutely exposed to C3 exoenzyme demonstrated no inhibition of migration. These results implicate $p27^{kip1}$ as a regulator, in part, of both rapamycin and C3 exoenzyme-mediated inhibition of SMC migration.

Discussion

Rapamycin has been shown previously to inhibit rat, porcine, and human SMC migration (Poon et al., 1996). In addition, rapamycin reduces intimal thickening by 50% after coronary angioplasty in the porcine model (Gallo et al., 1999). The rapamycin anti-restenotic effect is characterized by an inhibition of the SMC response to coronary injury with a concomitant decrease in retinoblastoma protein (pRb) phosphorylation as well as an increase in $p27^{kip1}$ levels, thereby resulting in cell-cycle arrest (Gallo et al., 1999; Marx et al., 1995). The cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$ inhibits the regulatory activities of cyclin/CDK complexes including cyclinE/CDK2 by directly binding to them and, in turn, blocking the phosphorylation of retinoblastoma protein (pRb) (Kato et al., 1994; Nourse et al., 1994). Thus, $p27^{Kip1}$ is a regulator of cell proliferation; reduction of $p27^{kip1}$ protein levels during the late G_1 phase is required for cyclin/CDK complex activation and cell cycle progression in certain cell lines. The CDKI $p27^{kip1}$ is present at high levels in quiescent cells and upon mitogenic stimulation is downregulated (Kato et al., 1994; Nourse et al., 1994). Down-regulation of $p27^{kip1}$ by

mitogens can be blocked by the immunosuppressant rapamycin (Nourse et al., 1994).

The function of $p27^{Kip1}$ is clinically relevant because of the connections that have been made between the down-regulation and enhanced degradation of $p27^{Kip1}$ in colorectal, stomach, breast, and small-cell lung cancers (Steeg and Abrams, 1997). Furthermore, the regulation of the CDKI $p27^{kip1}$ plays a critical role in the regulation of SMC proliferation *in vivo*. Decreased levels of $p27^{kip1}$ in the vessel wall has been associated with increased neointimal response after percutaneous transluminal angioplasty (PTCA) (Braun-Dullaeus and al., 1997; Tanner et al., 1998). Angiotensin II stimulation of quiescent vascular SMC in which $p27^{kip1}$ levels are high results in SMC hypertrophy but induces SMC hyperplasia when levels of $p27^{kip1}$ are low as occurs in the presence of mitogens (Braun-Dullaeus et al., 1999). The findings disclosed in the present application suggest that agents that increase $p27^{kip1}$ levels *in vivo* may have both an anti-proliferative and anti-migratory effect.

Although the regulation of $p27^{kip1}$ can occur at the mRNA level (Hengst and Reed, 1996), most studies have supported the concept that $p27^{kip1}$ is regulated post-transcriptionally and involves ubiquin (Ub)-proteasome dependent degradation (Pagano et al., 1995). Targeting of $p27^{kip1}$ for ubiquitin is believed to involve phosphorylation of $p27^{kip1}$ by cyclin E-cdk2 complex (Sheaff et al., 1997; Vlach et al., 1997). Recently, a ubiquin-proteasome independent pathway has been described that involves proteolytic

processing that rapidly clips off the cyclin-binding domain. This ubiquitin independent processing is ATP-dependent and sensitive to proteasome-specific and chymotrypsin inhibitors (Shirane et al., 1999).

In addition, $p27^{kip1}$ levels have been shown to be regulated by the Ras/RhoA mitogenic pathway. Overexpression of a dominant negative Ras or RhoA inhibited the platelet derived growth factor (PDGF) induced degradation of $p27^{kip1}$. C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, inhibited PDGF-induced $p27^{kip1}$ degradation (Hirai et al., 1997; Weber et al., 1997) and inhibited thrombin-mediated vascular SMC proliferation and migration (Seasholtz et al., 1999). In Swiss 3T3 fibroblasts, it has been shown that Rho can be activated by extracellular ligands (lysophosphatidic acid) and that Rho activation can lead to the assembly of contractile actin-myosin filaments and focal adhesion complexes (Hall, 1998). Rac, a member of the Rho subfamily, has been shown to induce actin-rich surface protrusions (filopodia); Rac can activate Rho (although in fibroblasts this is interaction is weak and delayed) (Hall, 1998). Generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by PI 3-kinase activity is essential for receptor-mediated activation by Rac in mammalian cells and a PI3 kinase homolog, TOR2 (target of rapamycin 2) controls Rho1p activation in Saccharomyces cerevisiae (Hall, 1998; Schmidt et al., 1997). These observations suggests that the Rho GTPase family is one of the key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton.

Rapamycin has not been shown to interact with the Rho GTPase family, although it is interesting that inhibition of both Rho (Hirai et al., 1997; Weber et al., 1997) and mTOR (Brown et al., 1994; Nourse et al., 1994; Sabatini et al., 1994) are both associated with increased levels of the CDKI, $p27^{kip1}$.

The extracellular matrix (ECM) plays an essential role in the regulation of cell proliferation. Human capillary endothelial cells that were prevented from spreading (either mechanically or pharmacologically with cytochalasin or actomyosin) exhibited normal activation of mitogen-activated kinases, but failed to progress through G1 phase (Huang et al., 1998). This shape dependent block in the cell cycle was correlated with a failure to down-regulate $p27^{kip1}$, up-regulate cyclin D1 and phosphorylate pRb (Huang et al., 1998). Therefore, the accumulation of $p27^{kip1}$ in cells prevented from spreading suggests that $p27^{kip1}$ could play a role in the shape-dependent cell cycle arrest produced by cell rounding. Signaling pathway components that could be responsible for transducing the accumulation of $p27^{kip1}$ include Rho, which is involved in integrin-mediated changes in the cytoskeleton tension and shape, and the integrin-linked kinase, which has been shown to reduce the inhibitory actions of $p27^{kip1}$ and to promote anchorage-independent growth (Chrzanowska-Wodnicka and Burridge, 1996; Hotchin and Hall, 1995; Huang et al., 1998; Radeva et al., 1997).

The p21 CDKI (Cip1) has been shown to inhibit SMC migration *in vitro* (Fukui et al., 1997; Witzenbichler

et al., 1999). The spreading and attachment of the $p21^{Cip1}$ transfected rabbit aortic SMC to extracellular matrices (ECM) were inhibited compared to that of control vector-transfected cells. Cip1 transfected SMC maintained a round conformation on fibronectin. Moreover, $p21^{Cip1}$ transfected SMC demonstrated significantly reduced PDGF-BB mediated migration in a modified Boyden chamber (with fibronectin coated membranes). Therefore, $p21^{cip1}$ probably acts as an adhesion inhibitor, since it prevents the assembly of actin filaments and the translocation of adhesion molecules (Fukui et al., 1997). Interestingly, our study indicates that induction of $p27^{kip1}$ with rapamycin did not affect adhesion to collagen of either wild type or p27 (-/-) cells.

The homeobox transcription factor Gax is expressed in quiescent vascular SMC and is down-regulated during SMC proliferation and vascular injury (Witzenbichler et al., 1999). Gax up-regulates $p21^{cip1}$ and inhibits vascular SMC proliferation and migration (Witzenbichler et al., 1999). $p21^{cip1}$ mediates the growth inhibitory actions of Gax; overexpression of Gax does not have anti-proliferative or anti-migratory effects in cells derived from p21 (-/-) mice (Smith et al., 1997; Witzenbichler et al., 1999). Gax was unable to inhibit the migration of fibroblasts which lacked $p21^{cip1}$ (Witzenbichler et al., 1999). Transfection of a Gax cDNA inhibited PDGF-, bFGF-, and hepatocyte growth factor-induced vascular SMC migration (Witzenbichler et al., 1999). Cell cycle arrest by either p16 or p21 is essential for Gax-induced inhibition of migration. Interestingly,

overexpression of Gax cDNA, which increases $p21^{cipl}$, had no effect on the adhesion of cells to collagen and vitronectin coated plates. Therefore, in contrast to the fibronection adhesion defect shown in cells transfected with $p21^{cipl}$, cells transfected with Gax cDNA demonstrated no collagen/vitronectin adhesion defect. However, the studies reported conflicting information regarding the effects of overexpression of $p21^{cipl}$ on SMC migration; $p21^{cipl}$ transfection of rabbit vascular SMC inhibited migration in a fibronectin coated Boyden chamber (Fukui et al., 1997), whereas $p21^{cipl}$ transfection in rat vascular SMC had no effect in a collagen/vitronectin Boyden chamber (Witzenbichler et al., 1999).

In conclusion, rapamysin and C3 exoenzyme inhibit smooth muscle cell migration through $p27^{kipl}$-dependent and independent pathways (Figure 5). This intriguing finding implicates $p27^{kipl}$ in the signaling pathway(s) that regulate both SMC proliferation and migration. Technologies (e.g., pharmacologic, recombinant and/or gene therapy) aimed at increasing $p27^{kipl}$ are expected to have dramatic effects on the amelioration of restenosis after angioplasty or stent placement, or on accelerated arteriopathy after cardiac transplantation, as well as in cancer therapy where cellular migration is a key element in tumor metastasis.

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PCT International Publication No. WO 99/03508, published January 28, 1999.

PCT International Publication No. WO 99/65939, published December 23, 1999.

What is claimed is:

1. A method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby preventing migration of the cell.

2. A method of preventing migration of a cell in a subject which comprises administering to the subject a compound which increases intracellular concentration of C3 exoenzyme, thereby preventing migration of the cell.

3. A method of preventing migration of a cell in a subject which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby preventing migration of the cell.

4. The method of claim 1, 2 or 3, wherein the cell is a smooth muscle cell or a tumor cell.

5. A method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby alleviating the subject's cardiovascular disease.

6. A method of treating a subject's cardiovascular disease, which comprises administering to the

subject a compound which increases intracellular concentration of C3 exoenzyme, thereby alleviating the subject's cardiovascular disease.

7. A method of treating a subject's cardiovascular disease, which comprises administering to the subject a compound which decreases intracellular concentration of Rho-kinase, thereby alleviating the subject's cardiovascular disease.

8. The method of claim 5, 6 or 7, wherein the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

9. A method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration of cyclin-dependent kinase inhibitor p27, thereby inhibiting tumor metastasis.

10. A method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which increases intracellular concentration of C3 exoenzyme, thereby inhibiting tumor metastasis.

11. A method of inhibiting tumor metastasis in a subject, which comprises administering to the subject a compound which decreases intracellular

concentration of Rho-kinase, thereby inhibiting tumor metastasis.

12. The method of claim 2, 6 or 10, wherein the compound is C3 exoenzyme.

13. The method of claim 1, 5 or 9, wherein the concentration of cyclin-dependent kinase inhibitor p27 is increased by increasing the concentration of C3 exoenzyme.

14. A method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of cyclin-dependent kinase inhibitor p27 is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration of p27, and detecting an increase in the intracellular concentration of p27 in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

15. A method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of cyclin-dependent kinase inhibitor p27 is

increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration of p27;

(b) determining if the intracellular concentration of p27 is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of p27 is increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

16. A method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of C3 exoenzyme is increased, or contacting an extract from said cells, with the chemical compound under conditions suitable for increasing the intracellular concentration of C3 exoenzyme, and detecting an increase in the intracellular concentration of C3 exoenzyme in the presence of the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

17. A method of screening a plurality of chemical compounds not known to inhibit cellular

migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of C3 exoenzyme is increased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for increasing the intracellular concentration of C3 exoenzyme;

(b) determining if the intracellular concentration of C3 exoenzyme is increased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of C3 exoenzyme is increased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

18. A method of identifying a chemical compound that inhibits cellular migration, which comprises contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the chemical compound under conditions suitable for decreasing the intracellular concentration of Rho-kinase, and detecting a decrease in the intracellular concentration of Rho-kinase in the presence of

the chemical compound so as to thereby identify the chemical compound as a compound which inhibits cellular migration.

19. A method of screening a plurality of chemical compounds not known to inhibit cellular migration to identify a chemical compound which inhibits cellular migration, which comprises:

(a) contacting cells whose migration is inhibited when intracellular concentration of Rho-kinase is decreased, or contacting an extract from said cells, with the plurality of chemical compounds under conditions suitable for decreasing the intracellular concentration of Rho-kinase;

(b) determining if the intracellular concentration of Rho-kinase is decreased in the presence of the plurality of chemical compounds; and if so

(c) separately determining if the intracellular concentration of Rho-kinase is decreased in the presence of each compound included in the plurality of chemical compounds, so as to thereby identify any compound included therein as a compound which inhibits cellular migration.

20. The method of claim 14, 16, or 18, wherein the compound is not previously known to inhibit cellular migration.

21. The method of any of claims 14-19, wherein the cells are smooth muscle cells or tumor cells.

22. The method of any of claims 14-19, wherein the cells are vertebrate cells.

23. The method of claim 22, wherein the vertebrate cells are mammalian cells.

24. The method of claim 23, wherein the mammalian cells are human cells.

25. A pharmaceutical composition comprising (a) an amount of a chemical compound identified using the method of claim 14 or 15, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration of cyclin-dependent kinase inhibitor p27 and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

26. A pharmaceutical composition comprising (a) an amount of a chemical compound identified using the method of claim 16 or 17, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to increase the intracellular concentration of C3 exoenzyme and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

27. A pharmaceutical composition comprising (a) an amount of a chemical compound identified using the method of claim 18 or 19, or a novel structural and functional homolog or analog thereof, capable of passing through a cell membrane and effective to decrease the intracellular concentration of Rho-kinase and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

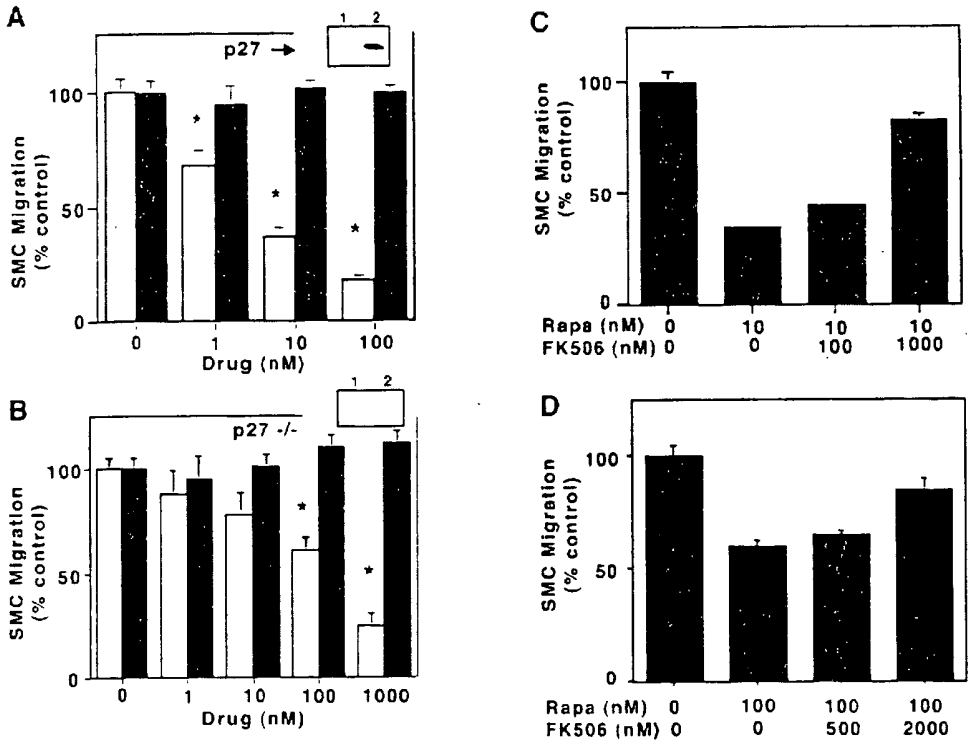
28. A method for preparing a pharmaceutical composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical compound identified by the method of any of claims 14-19 or a novel structural and functional analog or homolog thereof.

29. A method of treating a subject with a cardiovascular disease which comprises administering to the subject a therapeutically effective amount of a chemical compound identified by the method of any of claims 14-19, or a novel structural and functional analog or homolog thereof.

30. The method of claim 29, wherein the cardiovascular disease is atherosclerosis, arteriopathy after heart transplantation, or restenosis after angioplasty or coronary stent placement.

31. A method of inhibiting tumor metastasis in a subject which comprises administering to the

subject a therapeutically effective amount of a chemical compound identified by the method of any of claims 14-19, or a novel structural and functional analog or homolog thereof.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p27
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50
0
0 1 10 100
Drug (nM)
B
p27 -/-
1 2
SMC Migration (% control)
100
50
0
0 1 10 100 1000
Drug (nM)
C
SMC Migration (% control)
100
50
0
Rapa (nM) 0 10 10 10
FK506 (nM) 0 0 100 1000
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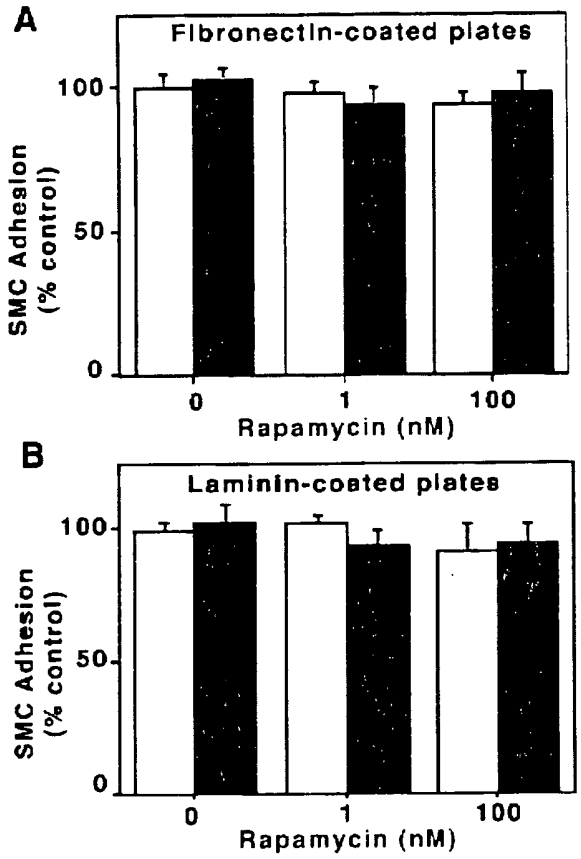
Figure 1A-1D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100
Rapamycin (nM)
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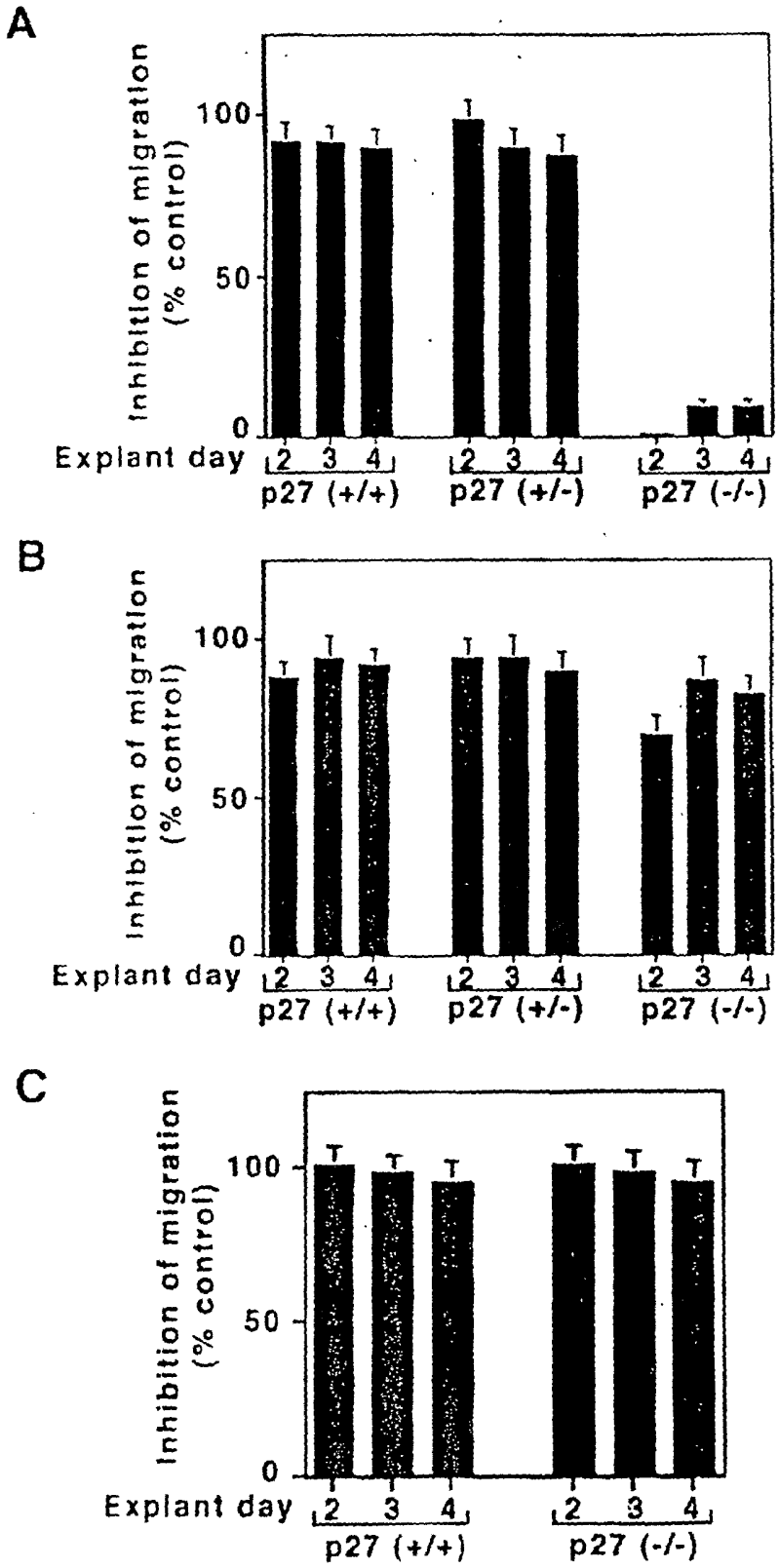
SMC Adhesion (% control)
100
50
0
0
1
100
Rapamycin (nM)

Figure 2A-2B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Inhibition of migration (% control)
100
50
0
Explant day
2 3 4
p27 (+/+)
p27 (+/-)
p27 (-/-)
B
Inhibition of migration (% control)
100
50
0
Explant day
2 3 4
p27 (+/+)
p27 (+/-)
p27 (-/-)
C
Inhibition of migration (% control)
100
50
0
Explant day
2 3 4
p27 (+/+)
p27 (-/-)

Figure 3A-3C

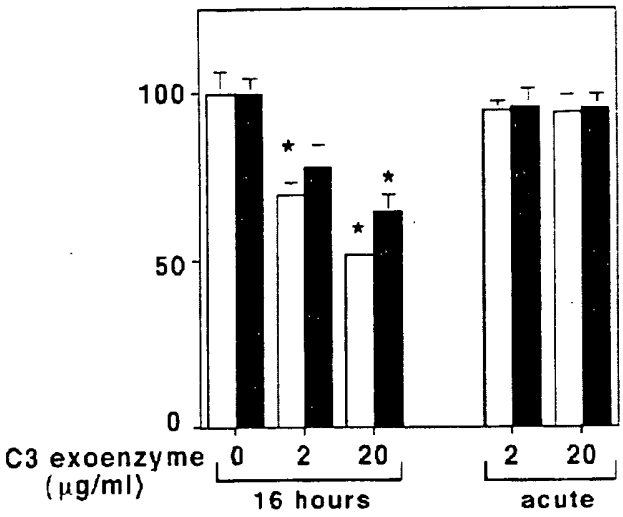
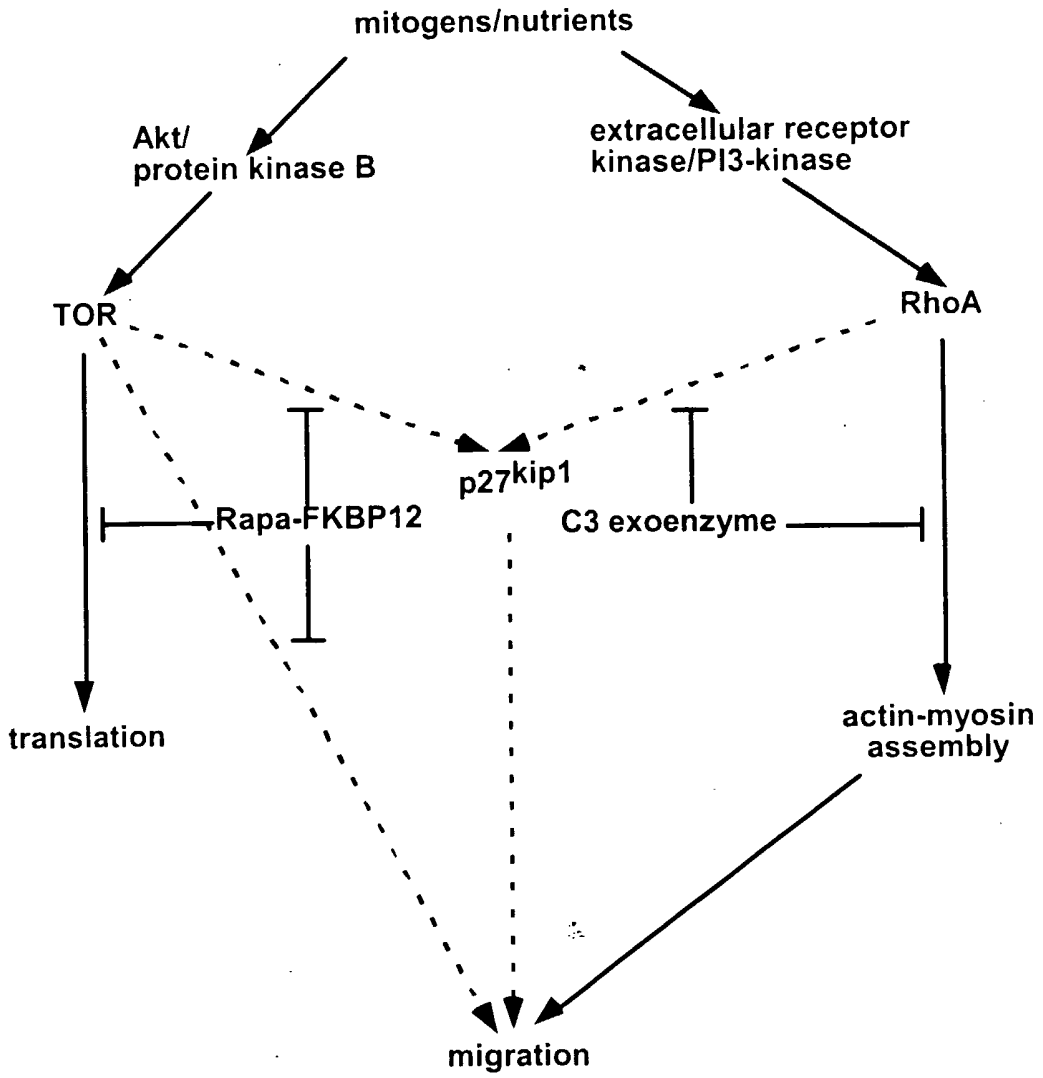


Figure 4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Akt/
protein kinase B
extracellular receptor
kinase/PI3-kinase
TOR
RhoA
p27kip1
Rapa-FKBP12
C3 exoenzyme
translation
actin-myosin
assembly
migration

Figure 5