Role for p27Kip1 in Vascular Smooth Muscle Cell Migration

Ji Sun, PhD; Steven O. Marx, MD; Hong-Jun Chen, MD; Michael Poon, MD; Andrew R. Marks, MD; LeRoy E. Rabbani, MD

Background—Rapamycin is a potent inhibitor of smooth muscle cell (SMC) proliferation and migration. Rapamycin-mediated inhibition of SMC proliferation is associated with upregulation of the cyclin-dependent kinase inhibitor p27Kip1. Previously, we showed that mixed embryonic fibroblasts obtained from p27Kip1/−/− mice were relatively rapamycin-resistant, suggesting that p27Kip1 plays an integral role in modulating the antiproliferative effects of rapamycin. We hypothesized that the antimigratory effect of rapamycin may also be mediated by p27Kip1.

Methods and Results—Rapamycin (1 to 10 nmol/L) inhibited basic fibroblast growth factor–induced migration of wild-type (WT) but not p27Kip1/−/− SMCs in a dose-dependent manner (P<0.05) in a modified Boyden chamber. The effects of rapamycin on aortic SMC explant migration were also studied with WT, p27(+/−), and p27(−/−) mice. Rapamycin 4 mg·kg−1·d−1 IP for 5 days inhibited SMC migration by 90% in the WT and p27Kip1(+/−) (P<0.05) but not p27Kip1(−/−) animals.

Conclusions—Lack of p27Kip1 reduces rapamycin-mediated inhibition of SMC migration. These novel findings suggest a role for p27Kip1 in the signaling pathway(s) that regulates SMC migration. (Circulation. 2001;103:2967-2972.)

Key Words: muscle, smooth ■ cells ■ inhibitors ■ rapamycin

Vascular smooth muscle cell (SMC) migration is believed to play a major role in the pathogenesis of many vascular diseases, including atherosclerosis and restenosis after PTCA and coronary stenting.1 Previously, we and others demonstrated that rapamycin, a macrolide antibiotic, inhibited SMC proliferation in vitro and in vivo by blocking cell-cycle progression at the G1/S transition.2–4 The inhibition of cellular proliferation was associated with a marked reduction in cyclin-dependent kinase (CDK) activity and in retinoblastoma protein phosphorylation.2–4 Pretreatment of rat and human SMCs with rapamycin (2 nmol/L) for 48 hours inhibited platelet-derived growth factor (PDGF)-induced migration in a modified Boyden chamber. Acute rapamycin treatment (6 hours) of rat and human SMCs, however, had no effect on migration, suggesting that longer exposure to rapamycin is essential for its antimigratory actions.5 The finding that rapamycin possessed both antiproliferative and antimigratory properties led us to propose that rapamycin may have important applications in the treatment of such disorders as accelerated arteriopathy that occurs in transplanted hearts and restenosis after PTCA and placement of coronary stents.2,5,6 Recently, implantation of rapamycin (sirolimus)-coated stents in de novo lesions was shown to be safe and effective in inhibiting neointimal formation at 4 months of follow-up.7

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Downregulation of the CDK inhibitor (CDKI) p27Kip1 by mitogens is blocked by rapamycin.8 In p27Kip1/−/− mice, relative rapamycin resistance was demonstrated in mixed embryonic fibroblasts and splenic T lymphocytes.9 In rapamycin-resistant myogenic cells, constitutively low levels of p27Kip1 were observed, which did not increase with serum withdrawal and rapamycin.9 Rapamycin (1 nmol/L) inhibited p70S6k phosphorylation and activity in both p27Kip1/−/− mixed embryonic fibroblasts and rapamycin-resistant myogenic cells.9 Although rapamycin blocks p70S6k in both p27Kip1/−/− cells and rapamycin-resistant myogenic cells, constitutively low levels or absence of p27Kip1 inhibits the antiproliferative properties of rapamycin. Decreased levels of p27Kip1 in the vessel wall have been associated with increased neointimal response after PTCA.10 Overexpression of p27Kip1 in SMCs inactivated cdk2 and cdk4 activity, adenoviral gene transfer of p27Kip1 after femoral artery balloon angioplasty significantly inhibited intimal cell proliferation.11 Similar inhibition of neointima formation has also been reported for adenovirus-mediated overexpression of p21cip1 in the rat carotid artery after angioplasty.12 In this study, we demonstrate that p27Kip1 plays a critical role in the mediation of the antimigratory properties of rapamycin.

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Methods

Reagents
DMEM and trypsin were from Gibco, recombinant basic fibroblast growth factor (bFGF) was from Biosource International, paclitaxel was from Sigma, and the mouse monoclonal p27Kip1 antibody was from Santa Cruz. Rapamycin was a gift from Dr Suren Sehgal (Wyeth-Ayerst Laboratories).

Expression of C3 Exoenzyme
The expression of GST-C3 exoenzyme (cDNA was a gift from Dr Judy Meinkoth, University of Pennsylvania) was prepared as previously described.13,14

Cell Culture
The aortic SMCs (≤5 passages) were obtained from the explant migration experiments described below and were subcultured in DMEM containing 20% FBS at 37°C in a humidified 95% air-5% CO2 atmosphere. Identification of SMCs was performed with α-actin immunofluorescence.

Fluorescence-Activated Cell Sorter Analysis
Wild-type (WT) [p27Kip1 (+/+)], heterozygous [p27Kip1 (+/−)], and homozygous [p27Kip1 (−/−)] SMCs were placed in DMEM+0.5% FBS and various concentrations of rapamycin for 48 hours to achieve quiescence. The cells were washed, harvested, and labeled with propidium iodide solution–RNase for 1 hour. The cells were analyzed on a fluorescence-activated cell sorter (FACS), with a minimum of 15,000 cells counted as previously described.6

Assessment of Cell Viability
Trypan blue exclusion assays were performed at the conclusion of all experiments and demonstrated >95% viability. The effects of rapamycin (0.1 to 100 nmol/L, 48-hour incubation) on SMC viability were also determined by a cytotoxicity assay (Promega CytoTox 96).

SMC Adhesion Assay
SMCs were treated with rapamycin or vehicle for 48 hours. SMCs (5×10^5/mL in DMEM with 0.2% BSA) were loaded onto plates precoated with laminin or fibronectin. After 3 hours, the media containing nonadherent cells were removed, and cell numbers were determined in triplicate with a Coulter Counter.

SMC Migration Assay
Migration was measured by use of a 48-well modified Boyden chamber housing a polycarbonate filter with 8-μm pores as described.8 Rapamycin or FK506 was added to the growth medium for 48 hours, or C3 exoenzyme was added for 16 hours. The lower chambers were filled with either 50 ng/mL of bFGF or 0.2% BSA (negative control) in DMEM. An equal number of cells (2×10^5/mL) in 50 µL were loaded into the top chamber of each well. After 6 hours, nonmigrating cells were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with Giemsa stain. The number of SMCs on the lower chamber housing a polycarbonate filter with 8-μm pores was counted as previously described.6

Aortic SMC Explant Migration
The p27Kip1 (+/+) and p27Kip1 (−/−) knockout mice were kindly provided by Dr Andrew Koff.15 WT controls were obtained from 2 sources: C57BL/6 mice and p27Kip1 (+/+) littermates were purchased from Jackson Laboratory (Bar Harbor, Me). No differences in sensitivity to rapamycin were detected between the 2 groups. All mice used in these experiments were genotyped by Southern hybridization as previously described13 and immunoblot analysis of p27Kip1. The mice received 1 of 3 treatment protocols of rapamycin: 9 mg·kg⁻¹·d⁻¹ IP for 7 days, 4 mg·kg⁻¹·d⁻¹ IP for 5 days, or 2 mg·kg⁻¹·d⁻¹ IP for 2 days. The control group was treated with vehicle alone (0.2% sodium carboxymethyl cellulose, polysorbate 0.25%; Sigma). At the conclusion of the protocol, the mice were euthanized with 100 mg/kg of pentobarbital, the aortas were excised, and the adventitia and surrounding connective tissue were removed. The aortas were opened by a longitudinal cut, and the intimas and a thin portion of the subjacent media were removed. The descending aorta was weighed and trimmed to normalize the weight to 10 mg. The aortas were cut into 4 pieces (~2×2 mm), and each piece was placed into a separate well of a 6-well plate containing DMEM+20% FBS. The number of cells that explanted from each piece was counted daily and the total number of cells/animal added. The results in Figure 4 are presented as the mean percentage (±SD) of inhibition of migration compared with control for ≥4 animals from each group.

Immunoblots
Lysates from SMCs growing in log phase or treated with rapamycin (100 nmol/L for 48 hours) were prepared as previously described.9

Statistics
Data are presented as the mean±SD of the independent experiments. Statistical significance was determined by 1-way ANOVA and Fisher’s protected least significant difference test. A paired t test (StatView 4.01) was used to analyze all data. A value of P<0.05 was considered statistically significant.

Results
The inhibitory effects of rapamycin on the migration of SMCs isolated from WT and p27Kip1 (−/−) mice were determined. In WT SMCs, rapamycin treatment for 48 hours demonstrated a significant inhibitory effect on bFGF-induced migration (Figure 1A, open bars). The inhibition was concentration dependent between 1 and 100 nmol/L, with an IC50 of ~2 nmol/L. In contrast, no significant inhibition of migration by rapamycin (1 to 10 nmol/L) was observed in p27Kip1 (−/−) SMCs (Figure 1B, open bars). At higher concentrations (100 nmol/L), an ~35% inhibition was observed; the IC50 in p27Kip1 (−/−) cells was ~200 nmol/L, representing an ~100-fold increased IC50 compared with WT SMCs. Addition of rapamycin to either the upper or lower chamber immediately before incubation had no effect on SMC migration (data not shown). FK506, an agent that binds to the same cytosolic receptor (FKBP12) as rapamycin, had no effect on SMC migration (Figure 1A and 1B, solid bars). The anti migratory effect of rapamycin was competitively inhibited by a molar excess of FK506 (Figure 1C and 1D). These data indicate that rapamycin inhibits SMC migration by binding to FKBP12.

Treatment of WT SMCs with rapamycin (100 nmol/L for 48 hours) caused an increase in p27Kip1 protein levels (Figure 1A, inset); in contrast, no p27Kip1 was detected in p27Kip1 (−/−) SMCs (Figure 1B, inset). Although rapamycin inhibits SMC proliferation,2 these findings cannot be explained by differences in proliferation, because equal numbers of cells were loaded into the Boyden chamber, and the total number of cells in the upper and lower chambers after the 6-hour incubation was equal in the untreated and treated WT and p27Kip1 (−/−) SMCs. This is to be expected, because the cell cycle requires ~24 hours in the murine SMCs and rapamycin inhibits cells in late G1. In addition, we counted the number of cells in the upper chamber after 6 hours of incubation without bFGF in the lower chamber. Rapamycin

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had no significant effect on the total number of cells in the upper chamber [WT and p27Kip1(−/−)] (P=NS) (Figure 1E).

No differences in cell viability were noted between untreated and rapamycin-treated SMCs obtained from WT and p27Kip1(−/−) animals as measured by trypan blue exclusion. To further assess cellular viability, we determined that rapamycin (0.1 to 100 nmol/L) did not increase LDH (a marker for cell viability) release in either WT, p27Kip1(−/−) and control. Inset, Immunoblot demonstrating increased p27Kip1 levels between untreated and rapamycin-treated (100 nmol/L) was measured in p27(+/+) and p27(+/−) vascular SMCs as a measure of cytotoxicity. No significant differences were observed between untreated and rapamycin-treated cells, indicating that the antimigratory properties of rapamycin are not caused by cytotoxicity. Similar results were obtained with 0.1 and 10 nmol/L rapamycin.

Because migration is dependent on the adhesion of the SMCs to the Boyden chamber membrane, we performed adhesion assays with fibronectin- and laminin-coated plates. SMCs obtained from p27Kip1(−/−) animals demonstrated no differences in adhesion compared with SMCS obtained from WT animals on both fibronectin- and laminin-coated plates. Furthermore, rapamycin treatment (100 nmol/L) for 48 hours had no significant effect on the total number of cells in the upper chamber [WT and p27Kip1(−/−)] (P=NS) (Figure 1E).

Because differences in migration may be dependent on rapamycin-induced alterations in the distribution of cells in the cell cycle, we synchronized the vascular SMCs by serum starvation for 48 hours before loading into the modified Boyden chamber. FACS analysis of the 3 cell types (Figure 2A) indicated that varying concentrations of rapamycin in addition to serum starvation did not alter the distribution of cells in G0, S, and G2/M phases. Under these conditions, rapamycin treatment for 48 hours demonstrated a significantly inhibited bFGF-induced migration in p27Kip1(+/+) and p27Kip1(+/−) cells (Figure 2B). The inhibition of migration was concentration dependent between 1 and 100 nmol/L of rapamycin, with an IC50 of ≈2 nmol/L. In contrast, the IC50 for rapamycin in p27Kip1(−/−) cells was ≈200 nmol/L, representing an ≈100-fold increased IC50 compared with WT SMCS (Figure 2B). Synchronization of SMCS in G0 by serum starvation (Figure 2A) did not alter the antimigratory properties of rapamycin (Figure 2B).

Rapamycin is Not Cytotoxic to Vascular SMCS

<table>
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<th>Rapamycin</th>
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<th>p27Kip1(+/+)</th>
<th>p27Kip1(+/−)</th>
<th>p27Kip1(−/−)</th>
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<tbody>
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<td>1.00</td>
<td>1.30</td>
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</tr>
<tr>
<td>Control</td>
<td>1.00</td>
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Values are %±SD. SMC LDH release after incubation (48 hours) with rapamycin (100 nmol/L) was measured in p27(+/+), p27(+/−), and p27(−/−) vascular SMCS as a measure of cytotoxicity. No significant differences were observed between untreated and rapamycin-treated cells, indicating that the antimigratory properties of rapamycin are not caused by cytotoxicity. Similar results were obtained with 0.1 and 10 nmol/L rapamycin.

Figure 2. Low concentrations of rapamycin (Rapa) inhibit migration in WT but not in p27Kip1(−/−) SMCs. A, Migration of quiescent SMCs isolated from WT mice was determined in modified Boyden chamber after Rapa and FK506 treatment. Rapamycin (open bars: 1, 10, and 100 nmol/L) significantly inhibited bFGF-induced migration in p27Kip1(+/+) and p27Kip1(+/−) SMCs. A, FACS analysis of the 3 cell types (Figure 2A) indicated that varying concentrations of rapamycin in addition to serum starvation did not alter the distribution of cells in G0, S, and G2/M phases. Under these conditions, rapamycin treatment for 48 hours demonstrated a significantly inhibited bFGF-induced migration in p27Kip1(+/+) and p27Kip1(+/−) cells (Figure 2B). The inhibition of migration was concentration dependent between 1 and 100 nmol/L of rapamycin, with an IC50 of ≈2 nmol/L. In contrast, the IC50 for rapamycin in p27Kip1(−/−) cells was ≈200 nmol/L, representing an ≈100-fold increased IC50 compared with WT SMCS (Figure 2B). Synchronization of SMCS in G0 by serum starvation (Figure 2A) did not alter the antimigratory properties of rapamycin (Figure 2B).

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that p27Kip1 (SMC antimigratory actions of rapamycin. To demonstrate dependent and p27Kip1-independent pathways mediate the inhibition were observed in WT, p27Kip1 (equivalent levels of rapamycin-mediated inhibition of migration were determined to number of untreated WT cells. No significant differences were noted between treated and untreated cells.

To assess the in vivo effects of rapamycin on SMC migration in the p27Kip1(−/−) animals, we examined the ability of SMCs to migrate out from aortic explants and establish cell cultures. Rapamycin was not added to the culture medium after the aortas were explanted. Explant migration of aortic SMCs was performed with p27Kip1(+/+) littersmates, p27Kip1(+/-), or p27Kip1(−/−) mice. SMCs from p27Kip1(+/+) p27Kip1(+/-), and p27Kip1(−/−) mice migrated out of the aortic explant by day 2. In animals treated with rapamycin 4 mg · kg⁻¹ · d⁻¹ IP for 5 days, ~85% inhibition of migration compared with untreated animals was observed in the p27Kip1(+/-) and p27Kip1(−/−) groups (P<0.05). In contrast, no rapamycin-mediated inhibition of migration was observed in the p27Kip1(−/−) group (P<0.05, Figure 4A), indicating that p27Kip1 plays a critical role in the rapamycin-mediated inhibition of SMC migration. At higher doses, equivalent levels of rapamycin-mediated inhibition of migration were observed in WT, p27Kip1(+/-), and p27Kip1(−/−) cells (Figure 4B). At lower doses (2 mg · kg⁻¹ · d⁻¹ for 2 days), no rapamycin-mediated inhibition of migration was observed (data not shown). This assay, while primarily assessing SMC migration, may also reflect proliferation, especially after several days of culture. These results are consistent with the findings obtained in the modified Boyden chamber for p27Kip1(−/−) cells and suggest that both p27Kip1-dependent and p27Kip1-independent pathways mediate the SMC antimigratory actions of rapamycin. To demonstrate that p27Kip1(−/−) SMC migration could be inhibited by agents that act independently of the p27Kip1 pathway, we treated WT and p27Kip1(−/−) mice with taxol 20 mg · kg⁻¹ · d⁻¹ IP for 7 days. No differences in taxol-mediated inhibition were observed in the 2 groups (Figure 4C).

Recent data suggest that the Ras/RhoA mitogenic pathway regulates the degradation of p27Kip1, C3 exoenzyme, which did not affect cell adhesion in either WT or p27Kip1(−/−) SMCs (Figure 3).

ADP-ribosylates and inactivates RhoA, inhibited PDGF-induced p27Kip1 degradation. These findings suggest that activation of RhoA by mitogens is necessary for degradation of p27Kip1. In addition, thrombin-induced vascular SMC DNA synthesis and migration were inhibited by C3 exoenzyme. We sought to determine whether this inhibition of migration compared with untreated animals was observed in the p27Kip1(−/−) and p27Kip1(−/−) groups (P<0.05). In contrast, no rapamycin-mediated inhibition of migration was observed in the p27Kip1(−/−) group (P<0.05, Figure 4A), indicating that p27Kip1 plays a critical role in the rapamycin-mediated inhibition of SMC migration. At higher doses, equivalent levels of rapamycin-mediated inhibition of migration were observed in WT, p27Kip1(+/-), and p27Kip1(−/−) cells (Figure 4B). At lower doses (2 mg · kg⁻¹ · d⁻¹ for 2 days), no rapamycin-mediated inhibition of migration was observed (data not shown). This assay, while primarily assessing SMC migration, may also reflect proliferation, especially after several days of culture. These results are consistent with the findings obtained in the modified Boyden chamber for p27Kip1(−/−) cells and suggest that both p27Kip1-dependent and p27Kip1-independent pathways mediate the SMC antimigratory actions of rapamycin. To demonstrate that p27Kip1(−/−) SMC migration could be inhibited by agents that act independently of the p27Kip1 pathway, we treated WT and p27Kip1(−/−) mice with taxol 20 mg · kg⁻¹ · d⁻¹ IP for 7 days. No differences in taxol-mediated inhibition were observed in the 2 groups (Figure 4C).

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Migration in both WT and p27 Kip1 (-/-) mice. Migration of SMCs isolated from WT mice (open bars) and p27 Kip1 (-/-) (solid bars) was determined in modified Boyden chamber after C3 exoenzyme (2 and 20 µg/mL) treatment for 16 hours. SMCs derived from p27 Kip1 (-/-) mice demonstrated 25% relative migratory resistance to C3 exoenzyme. *P<0.05 vs control.

Discussion

Rapamycin has been shown to inhibit rat, porcine, and human SMC migration. In addition, rapamycin reduces intimal thickening by 50% after PTCA in a porcine model. The rapamycin antiestenotic effect is characterized by an inhibition of the SMC response to coronary injury with a concomitant decrease in retinoblastoma protein phosphorylation as well as an increase in p27Kip1 levels, thereby resulting in cell-cycle arrest.

The data indicate that the CDK1 p27Kip1 plays a critical role in mediating the antiproliferative and antimigratory properties of rapamycin. The lack of p27Kip1 in the null mice was associated with an ~100-fold increase in the IC50 for migration compared with WT SMCs in Boyden chamber assays (Figures 1A, 1B, and 2B). Similarly, when aortic explants of mice treated with rapamycin 4 mg · kg⁻¹ · d⁻¹ for 5 days were used, p27Kip1(-/-) mice demonstrated significantly less inhibition of SMC migration than WT animals. At higher concentrations of rapamycin (9 mg · kg⁻¹ · d⁻¹ IP for 7 days), no significant difference was observed between p27Kip1(-/-) and WT animals. These findings suggest that at higher doses and duration of treatment, p27Kip1-independent actions of rapamycin can inhibit migration. Both p27Kip1(-/-) and WT animals demonstrated no inhibition of migration after lower doses of rapamycin (2 mg · kg⁻¹ · d⁻¹ for 2 days). This is consistent with previous reports in rats demonstrating that low doses of rapamycin (1.5 mg · kg⁻¹ for 14 days) had no effect on alloimmune injury and only a 45% reduction in intimal hyperplasia after mechanical injury. In contrast, higher concentrations of rapamycin (6 mg · kg⁻¹ · d⁻¹ for 7 days followed by 3 mg · kg⁻¹ for 7 days) diminished intimal thickening by 98%. Mice treated with rapamycin 6 mg · kg⁻¹ · d⁻¹ for 14 days showed no evidence of renal, cardiac, or liver damage, and marrow cellularity was normal.

We have previously shown that splenic T lymphocytes derived from p27Kip1(-/-) mice required a 15- to 30-fold higher concentration of rapamycin than do WT cells for a comparable level of growth inhibition. The inhibition of migration in both WT and p27Kip1(-/-) SMCs was inhibited by excess FK506, implying that the antimigratory properties of rapamycin are mediated by FKBP12. Similarly, we have previously demonstrated that the antiproliferative properties of rapamycin are mediated through FKBP12 binding. Ap-
rapamycin in WT and p27\(^{Kip1}\)(−/−) cells.\(^9\) These findings suggest that in a subset of SMCs, rapamycin inhibits migration via a p70\(^{S6K}\) and p27\(^{Kip1}\)-independent pathway that has yet to be identified (Figure 6).

p27\(^{Kip1}\) levels are regulated in part by the Ras/RhoA mitogenic pathway (Figure 6). Overexpression of a dominant negative Ras or RhoA inhibits PDGF-induced degradation of p27\(^{Kip1}\). C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, inhibits PDGF-induced p27\(^{Kip1}\) degradation\(^17,24\) and inhibits thrombin-mediated vascular SMC proliferation and migration.\(^14\) Because loss of p27\(^{Kip1}\) reduced C3 exoenzyme (20 \(\mu\)g/mL) inhibition by 25%, the Rho pathway mediates migration through both p27\(^{Kip1}\)-dependent and independent pathways. Higher concentrations of C3 exoenzyme led to decreased cell viability. In Swiss 3T3 fibroblasts, it has been shown that Rho can be activated by extracellular ligands and that Rho activation can lead to the assembly of contractile actin-myosin filaments and focal adhesion complexes.\(^25\) These observations suggest 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 Rho\(^7,24\) and of mTOR\(^26\) are both associated with increased levels of the CDK1 p27\(^{Kip1}\) (Figure 6).

The function of p27\(^{Kip1}\) is clinically relevant because of the connections that have been made between the downregulation and enhanced degradation of p27\(^{Kip1}\) in colorectal, stomach, breast, and small-cell lung cancers.\(^2\) Our findings suggest that agents that increase p27\(^{Kip1}\) levels in vivo may have both an antiproliferative and antimigratory effect. Rapamycin-mediated inhibition of SMC migration appears to depend on the presence of p27\(^{Kip1}\). This intriguing finding implicates p27\(^{Kip1}\) in the signaling pathway(s) that regulates both SMC proliferation and migration. Pharmacological and/or recombinant technologies aimed at increasing p27\(^{Kip1}\) may have dramatic effects on the amelioration of restenosis after angioplasty/stent placement and accelerated arteriopathy after cardiac transplantation.

Acknowledgments

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References

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