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PRE-CLINICAL RESEARCH

Telomerase Inhibition by Everolimus Suppresses Smooth Muscle Cell Proliferation and Neointima Formation Through Epigenetic Gene Silencing

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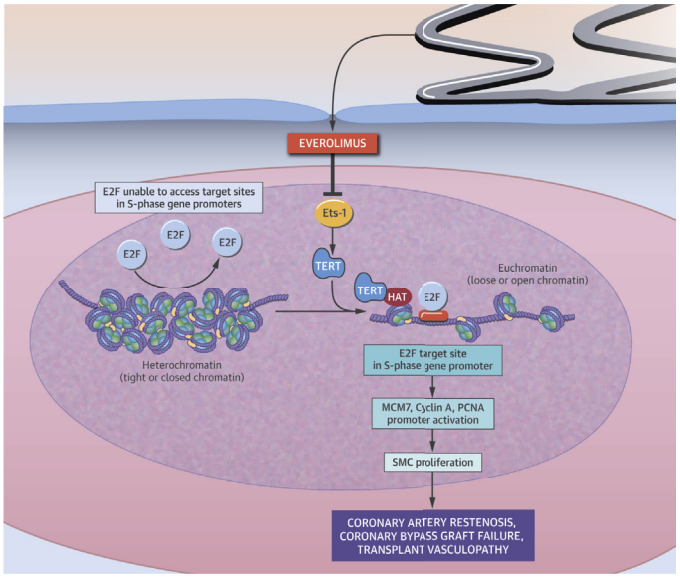
VISUAL ABSTRACT



Aono, J. et al. J Am Coll Cardiol Basic Trans Sci. 2016; 1(1-2):49-60.

HIGHLIGHTS

- The proliferative capacity of smooth muscle cells (SMC) during neointima formation is prevented by everolimus-coated drug-eluting stents.
- Everolimus failed to inhibit neointima formation by in mice overexpressing telomerase reverse transcriptase (TERT).
- Everolimus reduced TERT-dependent SMC proliferation through inhibition of Ets-1-dependent promoter activation.
- The inhibition of TERT-dependent SMC proliferation by everolimus occurred as a result of a G1→S-phase arrest, rather than telomerase shortening.
- Chromatin immunoprecipitation assays demonstrated that TERT induced E2F binding to S-phase gene promoters and supported histone acetylation.
- These studies identify a novel mitogenic pathway in SMC that depends on the epigenetic activation of S-phase gene promoters by TERT.

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SUMMARY

Proliferation of smooth muscle cells (SMCs) during neointima formation is prevented by drug-eluting stents. The replicative capacity of mammalian cells is enhanced by telomerase expression; however, the contribution of telomerase to the proliferative response underlying neointima formation and its potential role as a pharmacological target are unknown. The present study investigated the mechanisms underlying the mitogenic function of telomerase, and tested the hypothesis that everolimus, which is commonly used on drug-eluting stents, suppresses SMC proliferation by targeting telomerase. Inhibition of neointima formation by everolimus was lost in mice overexpressing telomerase reverse transcriptase (TERT), indicating that repression of telomerase confers the anti-proliferative efficacy of everolimus. Everolimus reduced TERT expression in SMC through an Ets-1-dependent inhibition of promoter activation. The inhibition of TERT-dependent SMC proliferation by everolimus occurred in the absence of telomere shortening but rather as a result of a G1→S-phase arrest. Although everolimus failed to inhibit phosphorylation of the retinoblastoma protein as the gatekeeper of S-phase entry, it potently repressed downstream target genes. Chromatin immunoprecipitation assays demonstrated that TERT induced E2F binding to S-phase gene promoters and supported histone acetylation. These effects were sensitive to inhibition by everolimus. These results characterize telomerase as a previously unrecognized target for the antiproliferative activity of everolimus, and further identify a novel mitogenic pathway in SMC that depends on the epigenetic activation of S-phase gene promoters by TERT. (J Am Coll Cardiol Basic Trans Sci 2016;1:49–60)

In addition to endothelial dysfunction and inflammation, proliferation of vascular smooth muscle cell (SMC) constitutes an essential component for atherosclerosis formation and neointimal remodeling (1). Once luminal obstruction in the course of atherosclerotic remodeling occurs, revascularization procedures represent primary treatment strategies (2). However, the clinical success of these procedures is limited by post-angioplasty restenosis, coronary artery bypass graft failure, and transplant vasculopathy. Considering the critical role of SMC proliferation in the pathophysiology of these treatment failures, the implantation of drug-eluting stents has become standard-of-care to prevent restenosis after angioplasty (3). Among the widely employed mammalian target of rapamycin (mTOR) inhibitors, recent clinical evidence has indicated that particularly second-generation, everolimus-eluting stents provide improved revascularization outcomes (4,5). Although everolimus potently inhibits neointima formation (6) and SMC proliferation (7), the detailed molecular mechanisms by which mTOR inhibitors elicit their antiproliferative efficacy and prevent cell cycle progression in SMC remain controversial. Early studies indicated that mTOR inhibitors induce G1 cell cycle arrest by targeting the retinoblastoma protein (RB), the key gatekeeper of the cell cycle, which represses S-phase gene expression induced by the transcription factor E2F (8). However, more recent evidence in SMC that are deficient for the RB protein have indicated that mTOR inhibitors may repress cell cycle progression independently of RB protein phosphorylation (9). Considering this controversy, the precise mechanisms by which commonly implanted everolimus-eluting stents prevent in-stent restenosis remain elusive.

By maintaining the stability of telomeres, repetitive deoxyribonucleic acid (DNA)-protein complexes that protect the ends of chromosomes, telomerase is rate limiting for cell proliferation and tissue renewal (10). Overexpression of the catalytic subunit telomerase reverse transcriptase (TERT) confers a virtually unlimited replicative capacity (11). In contrast, most somatic cells are thought to repress TERT, resulting in telomere shortening and limited replicative potential (12). However, accumulating evidence revealed that TERT is inducible in response to various environmental cues (13), which enhance proliferative responses during tissue renewal (10). Similarly as in other somatic tissues, we (14–16) and others (17) have previously reported that TERT expression is induced in proliferating SMC and in response to neointima and atherosclerosis formation. However, whether TERT expression in the vascular wall causes neointima formation and the transcriptional mechanisms by which TERT induces aberrant SMC proliferation remain unknown. Moreover, the possibility that TERT constitutes an alternative molecular target for the RB-independent antiproliferative efficacy of mTOR inhibitors on drug-eluting stents has previously not been investigated. Here, we demonstrate that TERT

ABBREVIATIONS AND ACRONYMS

ChIP = chromatin immunoprecipitation

MCM7 = minichromosome maintenance protein 7

mTOR = mammalian target of rapamycin

PCNA = proliferating cell nuclear antigen

RB = retinoblastoma protein

SMC = vascular smooth muscle cells

TBP = TATA binding protein

TERT = telomerase reverse transcriptase

TERTtg = telomerase reverse transcriptase-overexpressing transgenic

WT = wild type

expression bypasses RB phosphorylation as the common cell cycle checkpoint to directly activate an S-phase gene expression program through epigenetic modification. Consistent with this mitogenic activity of TERT, we further characterize TERT-dependent downstream histone acetylation of S-phase genes as a previously unrecognized mechanism underlying the inhibition of SMC proliferation by everolimus.

METHODS

CELL CULTURE. Primary human coronary artery vascular smooth muscle cells were purchased from Lonza (Allendale, New Jersey). Primary mouse vascular smooth muscle cells were isolated from 8- to 12-week-old littermate wild-type (WT) and telomerase reverse transcriptase–overexpressing transgenic (TERTtg) mice (18) as previously described (15). The SMC were treated with everolimus as indicated.

ENDOTHELIAL DENUDATION INJURY. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Guidewire-induced endothelial denudation injuries were performed on the femoral arteries of 8- to 12-week-old male littermate WT and TERTtg mice (15). Immediately after injury, the perivascular area of the femoral artery was treated with vehicle or 100 μg of everolimus. After 28 days, femoral arteries were removed and stained with an elastic Verhoeff-van Gieson staining kit.

For a more detailed methods summary, please see the Supplemental Appendix.

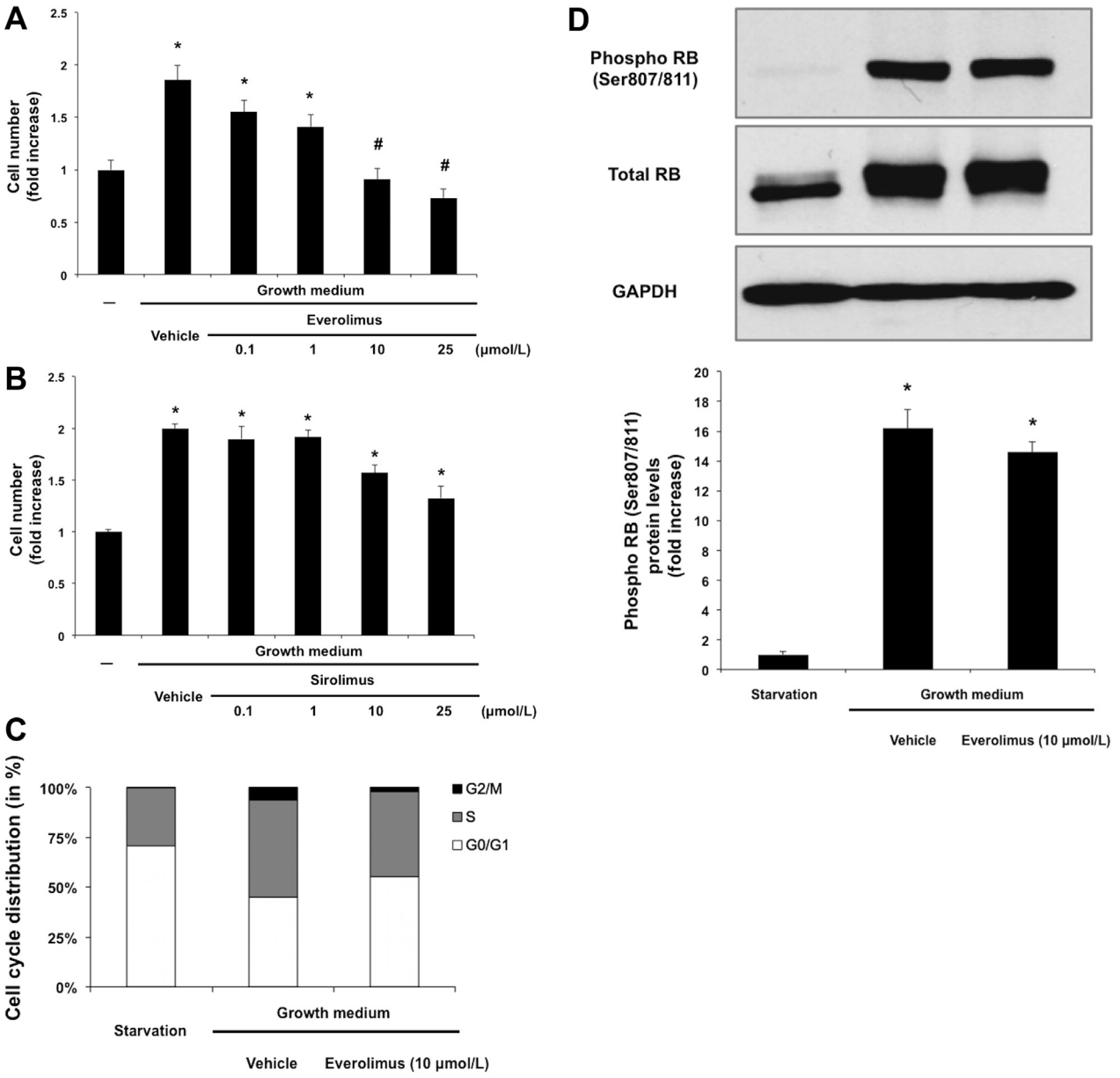
RESULTS

EVEROLIMUS INHIBITS SMC PROLIFERATION AND CELL CYCLE PROGRESSION. Due to the well-established antimitotic activity of mTOR inhibitors, sirolimus (8) and everolimus (7) constitute primary agents used on first- and second-generation drug-eluting stents, respectively. Therefore, we first evaluated the efficacy of everolimus and sirolimus to inhibit SMC proliferation in vitro. Consistent with a previous study (7), everolimus dose-dependently inhibited SMC proliferation (**Figure 1A**). Compared with everolimus, the previously reported antiproliferative efficacy of sirolimus (8) was limited to higher equimolar doses (**Figure 1B**). To further determine the mechanism by which everolimus prevents SMC proliferation, we next examined cell cycle distribution. As depicted in **Figure 1C**, everolimus inhibited mitogen-induced G1→S phase progression. On the basis of this finding, we investigated whether everolimus suppressed phosphorylation of the RB protein as the rate-limiting step for G1→S-phase progression. RB phosphorylation releases the transcription factor E2F, which in turn activates target genes required for DNA synthesis (19). However, surprisingly, everolimus inhibited neither site-specific ser807/811 nor total phosphorylation of the RB protein (**Figure 1D**), although it caused potent inhibition of G1→S-phase progression. These results indicate that everolimus inhibits cell cycle progression through a mechanism independent of RB phosphorylation.

EVEROLIMUS INHIBITS SMC PROLIFERATION THROUGH TRANSCRIPTIONAL REPRESSION OF TELOMERASE. Whereas RB phosphorylation promotes cell proliferation through release of the S-phase transcription factor E2F, telomerase overexpression endows cells with an unlimited replicative potential by overcoming the limitations of senescence (10,11). Although this mitogenic activity of telomerase was long thought to result from its ability to maintain telomeres, recent evidence indicates that TERT enhances proliferation through mechanisms independent of telomere elongation (20,21). Considering this evidence and our previous observation that telomerase is required for SMC proliferation (14,15), we hypothesized that the antimitotic activity of everolimus may depend on the repression of TERT. To address this question, we evaluated the antiproliferative efficacy of everolimus in SMC isolated from mice overexpressing a murine TERT transgene (18). As depicted in **Figure 2A**, expression of the murine TERT transgene induced SMC proliferation even in the absence of mitogenic stimulation. Furthermore, TERT expression prevented the inhibition of mitogen-induced SMC proliferation by everolimus, indicating that TERT constitutes an important target for the growth arrest induced by everolimus. On the basis of these findings, we next investigated whether TERT expression is inhibited by everolimus in SMC. Western blotting (**Figure 2B**) and immunohistochemistry (**Figure 2C**) revealed that everolimus dose-dependently inhibited TERT protein expression. Consistent with a primarily transcriptional regulation of TERT in SMC (14,15), everolimus repressed mitogen-induced TERT messenger ribonucleic acid expression (**Figure 2D**) and promoter activity (**Figure 2E**). Collectively, these experiments confirm that everolimus inhibits SMC proliferation through transcriptional repression of mitogen-induced TERT expression.

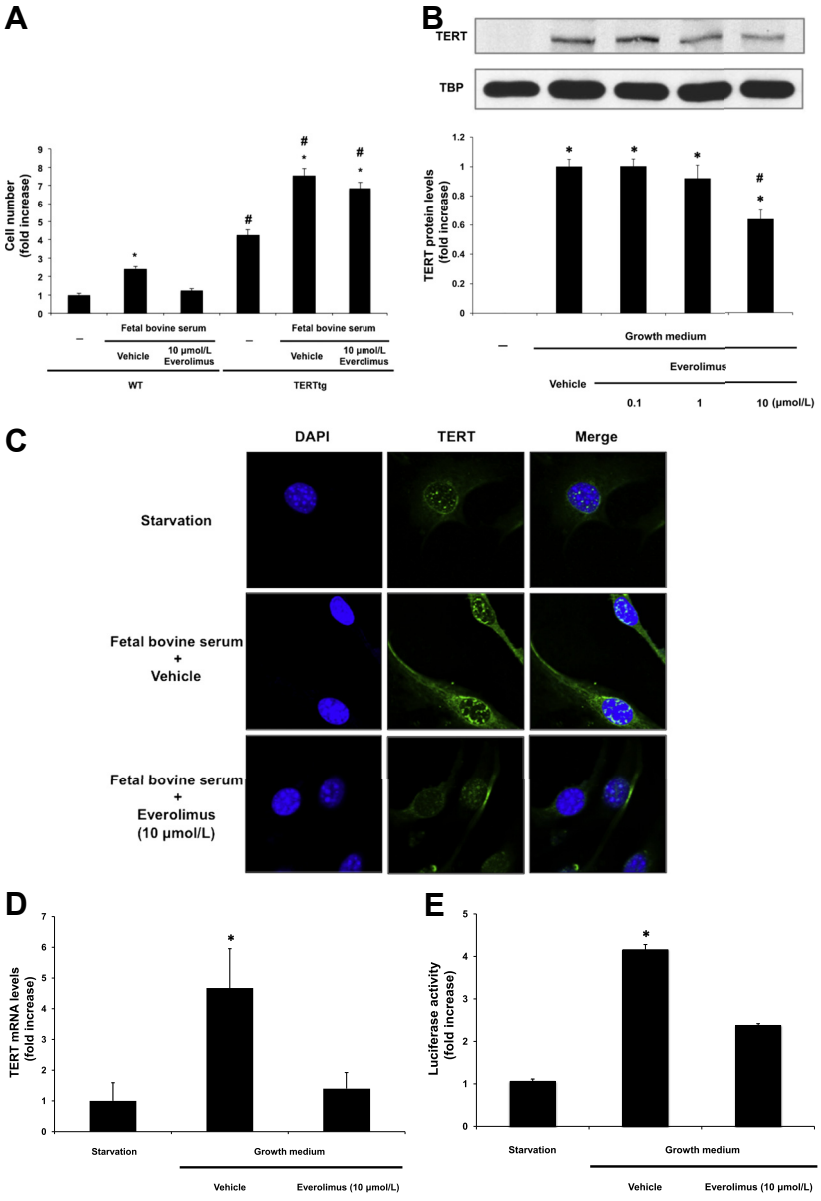
EVEROLIMUS REPRESSES Ets-1–DEPENDENT TRANSACTIVATION OF THE TERT PROMOTER. Having characterized TERT as a transcriptional target for everolimus, we next investigated the mechanisms

FIGURE 1 Everolimus Inhibits SMC Proliferation and Cell Cycle Progression



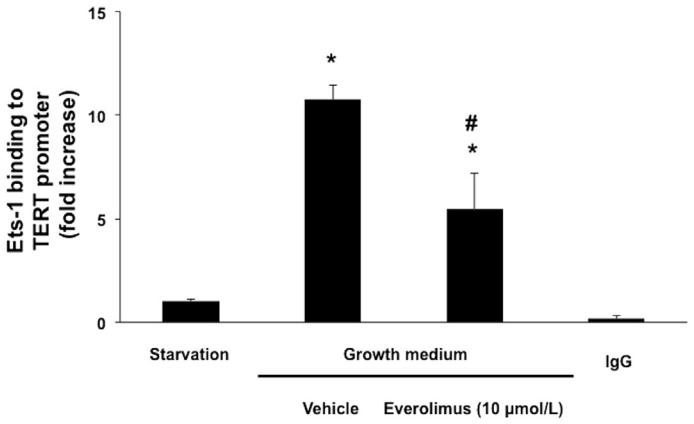
(A and B) Primary human coronary artery vascular smooth muscle cells (hSMC) were serum-deprived (−) and treated with vehicle or the indicated doses of everolimus **(A)** or sirolimus **(B)** in growth medium. Cell counts are expressed as mean ± SEM (n = 12 samples/group) relative to quiescent hSMC. **(A)** Significance levels versus quiescent cells (−): growth medium + vehicle, *p < 0.001; growth medium + 0.1 μmol/l everolimus, *p = 0.007; growth medium + 1 μmol/l everolimus, *p = 0.009. Significance levels versus growth medium + vehicle: growth medium + 10 μmol/l everolimus, #p < 0.001; growth medium + 25 μmol/l everolimus, #p < 0.001. **(B)** Significance levels versus quiescent cells (−): growth medium + vehicle, *p < 0.001; growth medium + 0.1 μmol/l sirolimus, *p = 0.010; growth medium + 1 μmol/l sirolimus, *p = 0.008; growth medium + 10 μmol/l sirolimus, *p = 0.008; growth medium + 25 μmol/l sirolimus, *p = 0.009. **(C)** Deoxyribonucleic acid histograms showing cell cycle distribution of hSMC after treatment with 10 μmol/l everolimus. **(D)** Western blotting for phosphorylated (phospho) retinoblastoma protein (RB), total RB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cell lysates isolated from hSMC. The autoradiograms shown represent 3 independently performed experiments using different cell preparations. Values are normalized to GAPDH and expressed as mean ± SEM (n = 6 samples/group) relative to quiescent hSMC (*p < 0.001 versus starvation).

FIGURE 2 Everolimus Inhibits SMC Proliferation Through Transcriptional Repression of Telomerase



(A) Cell proliferation assay in primary murine vascular smooth muscle cells (mSMC) isolated from wild-type (WT) or telomerase reverse transcriptase–overexpressing transgenic (TERTtg) mice. Cells were serum-deprived (−) and treated with vehicle or 10 μmol/l everolimus in 20% fetal bovine serum (FBS). Values are expressed as the mean ± SEM (n = 12 samples/group) relative to quiescent WT mSMC. Significant levels versus quiescent cells (−): FBS + vehicle (WT), *p = 0.008; FBS + vehicle (TERTtg), *p < 0.001; FBS + 10 μmol/l everolimus (TERTtg); *p < 0.001. Significance levels versus WT: quiescent cells, #p < 0.001; FBS + vehicle, #p < 0.001; FBS + 10 μmol/l everolimus, #p < 0.001. **(B)** Western blotting for TERT expression in nuclear extracts isolated from hSMC treated with everolimus. Densitometric data are expressed as TERT normalized to TATA binding protein (TBP) and presented as mean ± SEM fold-increase over vehicle from 3 independently performed experiments (n = 9 samples/group). Significance levels versus quiescent cells (−): growth medium + vehicle, *p < 0.001; growth medium + 0.1 μmol/l everolimus, *p < 0.001; growth medium + 1 μmol/l everolimus, *p < 0.001; growth medium + 10 μmol/l everolimus, *p < 0.001. Significance level versus growth medium + vehicle: growth medium + 10 μmol/l everolimus, #p = 0.022. **(C)** Immunostaining for TERT expression **(green)** in WT mSMC treated with 10 μmol/l everolimus. 4′-6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Images are representative of 3 independently performed experiments. **(D)** TERT mRNA expression in hSMC treated with 10 μmol/l everolimus analyzed by real-time PCR. Values are normalized to the housekeeping gene TBP and expressed as mean ± SEM (n = 12 samples/group) relative to quiescent hSMC (*p = 0.047 versus quiescent cells). **(E)** hSMC were transfected with a reporter construct driven by the full length TERT promoter. Following transfection, cells were treated with everolimus and analyzed for luciferase activity. Values are mean ± SEM (n = 12 samples/group) relative to luciferase activity of quiescent hSMC (*p < 0.001 versus quiescent cells).

FIGURE 3 Everolimus Represses Ets-1-Dependent Transactivation of the TERT Promoter



hSMC were treated with 10 μmol/l everolimus and stimulated with growth medium. Chromatin was harvested for ChIP assays using an antibody raised against Ets-1. Nonimmune IgG was used as a negative control. The graph depicts quantification of immunoprecipitated chromatin by real-time PCR using primer pairs that cover the Ets-1 site in the proximal TERT promoter. Cycle threshold (Ct) values were normalized to Ct values of input samples and are expressed as mean ± SEM (n = 12 samples/group). Significance levels versus quiescent cells (starvation): growth medium + vehicle, *p = 0.007; growth medium + 10 μmol/l everolimus, *p = 0.009. Significance levels versus growth medium + vehicle, #p = 0.041.

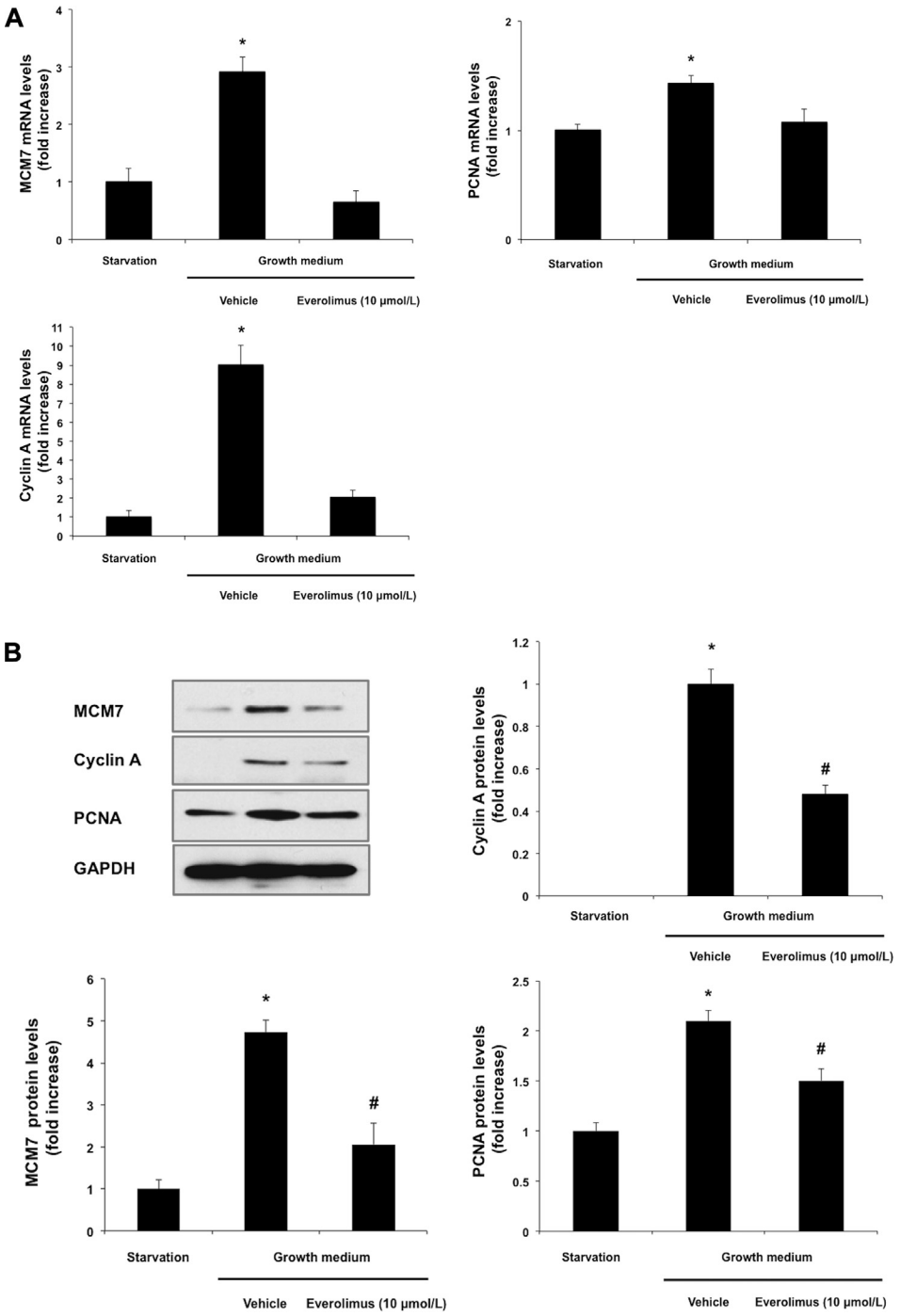
underlying everolimus-induced repression of the TERT promoter. Inducible TERT transcription during mitogenic responses of SMC is mediated through transactivation of 2 adjacent Ets-1 consensus motifs located at -23 and -18 within the TERT promoter (15). To analyze whether everolimus inhibits recruitment of Ets-1 binding to the endogenous TERT promoter, we employed chromatin immunoprecipitation (ChIP) assays. PCR amplification using primer pairs that cover the Ets-1 site at -23 in the TERT promoter demonstrated that everolimus suppressed Ets-1 binding to the TERT promoter (**Figure 3**). These data confirm that everolimus attenuates TERT transcription by inhibiting Ets-1 recruitment to the TERT promoter.

EVEROLIMUS SILENCES E2F TARGET GENE EXPRESSION WITHOUT INDUCING TELOMERE ATTRITION OR SENESCENCE. The observation that everolimus inhibits SMC proliferation through transcriptional repression of TERT raised the question of how TERT supports the mitogenic response. Furthermore, our data showing that everolimus induces G1 cell cycle arrest without inhibiting RB phosphorylation indicate that TERT signaling may feed directly into the S phase through a previously unknown mechanism, which is inhibited by everolimus. To address these questions, we assessed whether the antiproliferative effect and the repression of TERT by everolimus are associated with telomere attrition. Quantification of telomere lengths revealed no telomere attrition in SMC treated with everolimus (Supplemental Figures 1A and 1B). Consistent with these data, everolimus did not induce senescence-associated β-galactosidase activity in everolimus-treated SMC (Supplemental Figure 1C). These results indicate that the antiproliferative activity of everolimus is mediated through TERT repression; however, this inhibition of SMC proliferation by everolimus does not require telomere attrition.

Having confirmed a telomere-length independent mechanism, we next addressed the mechanisms by which everolimus-mediated TERT repression induced cell cycle arrest. As discussed earlier, the primary mechanism underlying cell cycle progression involves phosphorylation of the RB protein, release of the E2F transcription factor, and subsequent S-phase gene transcription of E2F target genes (19). Because everolimus inhibited mitogen-induced G1→S-phase progression independently of RB phosphorylation, we first investigated whether everolimus attenuates the expression of downstream E2F target genes, which are required for S-phase entry. As shown in **Figures 4A and 4B**, messenger ribonucleic acid and protein expression levels of the *bona fide* E2F target genes minichromosome maintenance protein (MCM)-7, Cyclin A, and proliferating cell nuclear antigen (PCNA) were silenced in response to everolimus treatment, although RB phosphorylation was not affected by the drug. Similarly, everolimus did not inhibit E2F protein expression (Supplemental Figure 2). These data suggest that the mechanism underlying everolimus-induced G1 arrest is mediated by TERT repression, bypasses RB phosphorylation, and acts directly on the transcription of E2F target genes causing altered S-phase gene expression.

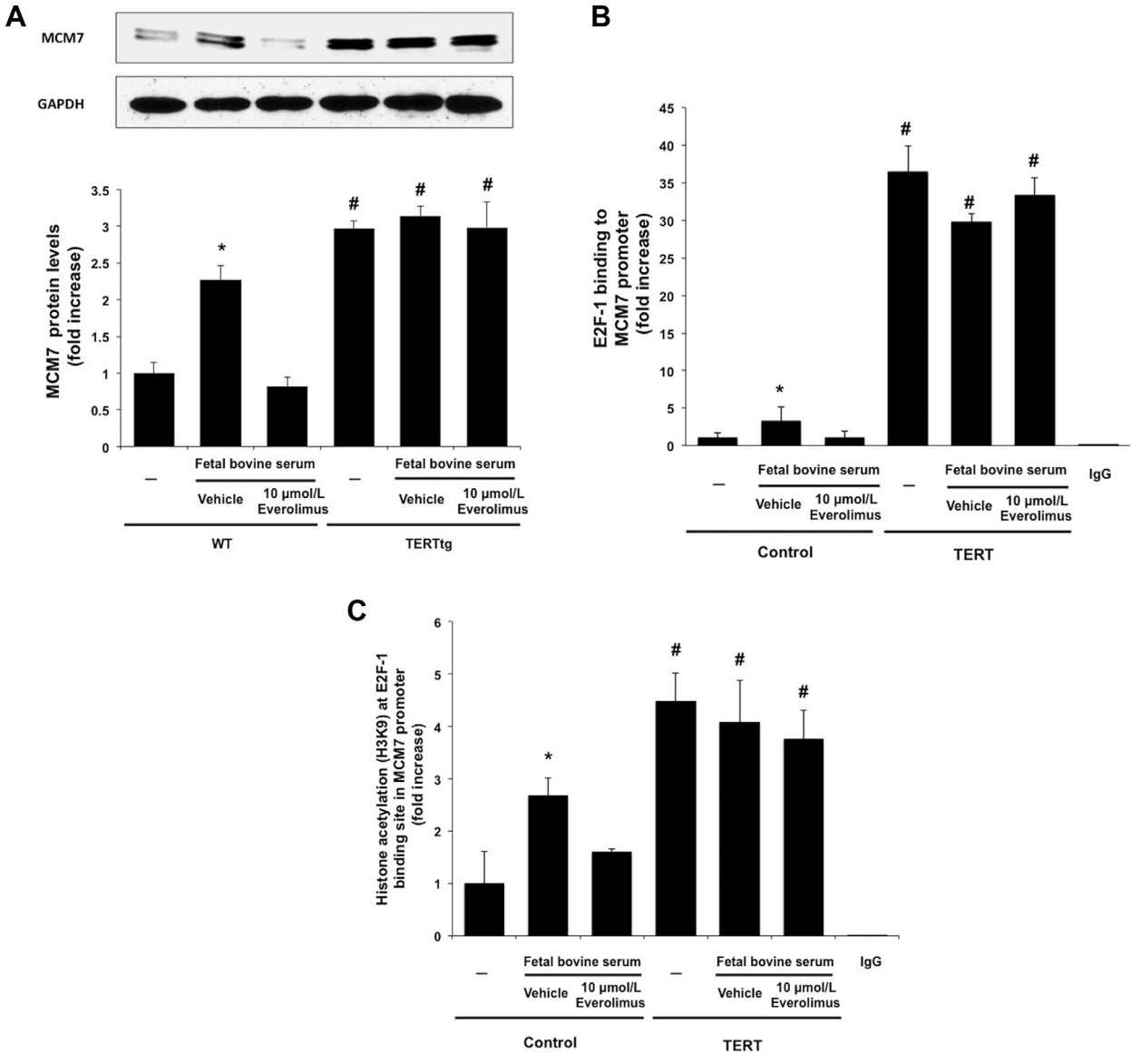
EVEROLIMUS SILENCES E2F TARGET GENES THROUGH TERT-DEPENDENT EPIGENETIC HISTONE MODIFICATION. TERT has previously been described to induce gene expression through direct chromatin activation at target gene promoters and interaction with transcription factors (22). To investigate whether TERT activates E2F-dependent transcription, we next employed again SMC isolated from TERTtg mice. As depicted in **Figure 5A**, constitutive TERT expression induced the primary E2F target gene MCM7. Although everolimus potently inhibited mitogen-induced MCM7 expression, this efficacy was completely lost in SMC overexpressing TERT. Similar data were obtained for the E2F target genes PCNA and Cyclin A (data not shown). Using ChIP experiments, we

FIGURE 4 Everolimus Silences E2F Target Gene Expression



hSMC were treated with 10 μmol/l everolimus and stimulated with growth medium. **(A)** Minichromosome maintenance protein 7 (MCM7), Cyclin A, and proliferating cell nuclear antigen (PCNA) mRNA expression levels were analyzed by real-time PCR and normalized to transcript levels of the housekeeping gene TBP. Data are presented as mean ± SEM (n = 9 samples/group) relative to quiescent hSMC. Significance levels versus quiescent cells: MCM7, *p < 0.001; Cyclin A, *p < 0.001; PCNA, *p = 0.008. **(B)** Western blotting for MCM7, Cyclin A, and PCNA expression in hSMC treated with everolimus. Densitometric quantification from 3 independent experiments was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as mean fold increase ± SEM (n = 6 samples/group) relative to quiescent hSMC. Significance levels versus quiescent cells: MCM7, *p < 0.001; Cyclin A, *p < 0.001; PCNA, *p = 0.006. Significance levels versus growth medium + vehicle: MCM7, #p < 0.001; Cyclin A, #p < 0.001; PCNA, #p = 0.032.

FIGURE 5 Everolimus Silences E2F Target Genes Through TERT-Dependent Epigenetic Histone Modification



(A) Western blotting for MCM7 in WT and TERTtg mSMC treated with 10 μmol/l everolimus in 20% fetal bovine serum (FBS). Densitometric quantification from 3 independent experiments was normalized to GAPDH and expressed as mean fold increase ± SEM (n = 6 samples/group) relative to quiescent WT mSMC. Significance levels versus quiescent cells: *p = 0.041. Significance levels versus WT: quiescent cells, #p = 0.018; FBS + vehicle, #p = 0.046; FBS + 10 μmol/l everolimus, #p = 0.012. **B** and **C**, HEK293 cells were transfected with a TERT expression vector (TERT) or empty vector (control). Cells were serum-deprived (−) and treated with vehicle or 10 μmol/l everolimus in 20% FBS. Chromatin was harvested for chromatin immunoprecipitation assays using antibodies raised against: **(B)** E2F-1 (significance levels versus quiescent cells: *p = 0.045. Significance levels versus control: quiescent cells, #p < 0.001; FBS + vehicle, #p < 0.001; FBS + 10 μmol/l everolimus, #p < 0.001) or **(C)** acetyl-histone H3 (Lys9) (significance levels versus quiescent cells: *p = 0.048. Significance levels versus control: quiescent cells, #p = 0.034; FBS + vehicle, #p = 0.043; FBS + 10 μmol/l everolimus, #p = 0.038). Nonimmune IgG was used as a negative control value. Quantification of immunoprecipitated chromatin was performed by real-time PCR using primer pairs that cover the E2F-1 site in the proximal MCM7 promoter. Ct values were normalized to Ct values of input samples and are expressed as mean ± SEM (n = 12 samples/group) relative to quiescent cells transfected with empty vector. Abbreviations as in Figures 1 and 3.

further confirmed that ectopic TERT expression (Supplemental Figure 3) increases E2F occupancy of the MCM7 promoter and abolishes the inhibition of E2F recruitment induced by everolimus (**Figure 5B**). Considering that TERT has been suggested to activate chromatin allowing subsequent transcription factor binding (22), we hypothesized that TERT facilitates E2F binding through chromatin modification rather than by increasing E2F availability. In support of this hypothesis, TERT overexpression did not increase E2F levels (Supplemental Figure 4). Moreover, ectopic E2F expression using adenoviral transduction did not reverse the inhibition of SMC proliferation by everolimus, indicating that E2F does not constitute a primary target for everolimus and that, even under conditions of abundant E2F availability, everolimus inhibits SMC proliferation (Supplemental Figure 5). These data could be reconciled through a mechanistic model in which everolimus represses TERT-mediated chromatin activation, thereby restricting subsequent E2F binding to the target promoter. Consistent with this model, TERT expression induced histone acetylation at the E2F target site and reversed the inhibition of histone acetylation observed in response to everolimus (**Figure 5C**).

TERT OVEREXPRESSION ABOLISHES THE ANTIPROLIFERATIVE EFFECT OF EVEROLIMUS ON NEOINTIMA FORMATION. Finally, to provide in vivo confirmation that the antiproliferative effect of everolimus may be attributed to the repression of TERT, we induced endothelial denudation injuries in WT and TERTtg mice. As depicted in **Figures 6A and 6B**, endovascular injury resulted in concentric neointima formation in WT mice treated with vehicle. As expected, everolimus treatment significantly attenuated neointima formation. Consistent with a key role of TERT in inducing SMC proliferation in vitro, neointima formation was increased in TERTtg mice. However, the inhibition of neointima formation in response to everolimus was abolished in TERTtg mice. These findings establish a key role of TERT in neointima formation and characterize TERT as previously unrecognized primary target for the antiproliferative efficacy of everolimus.

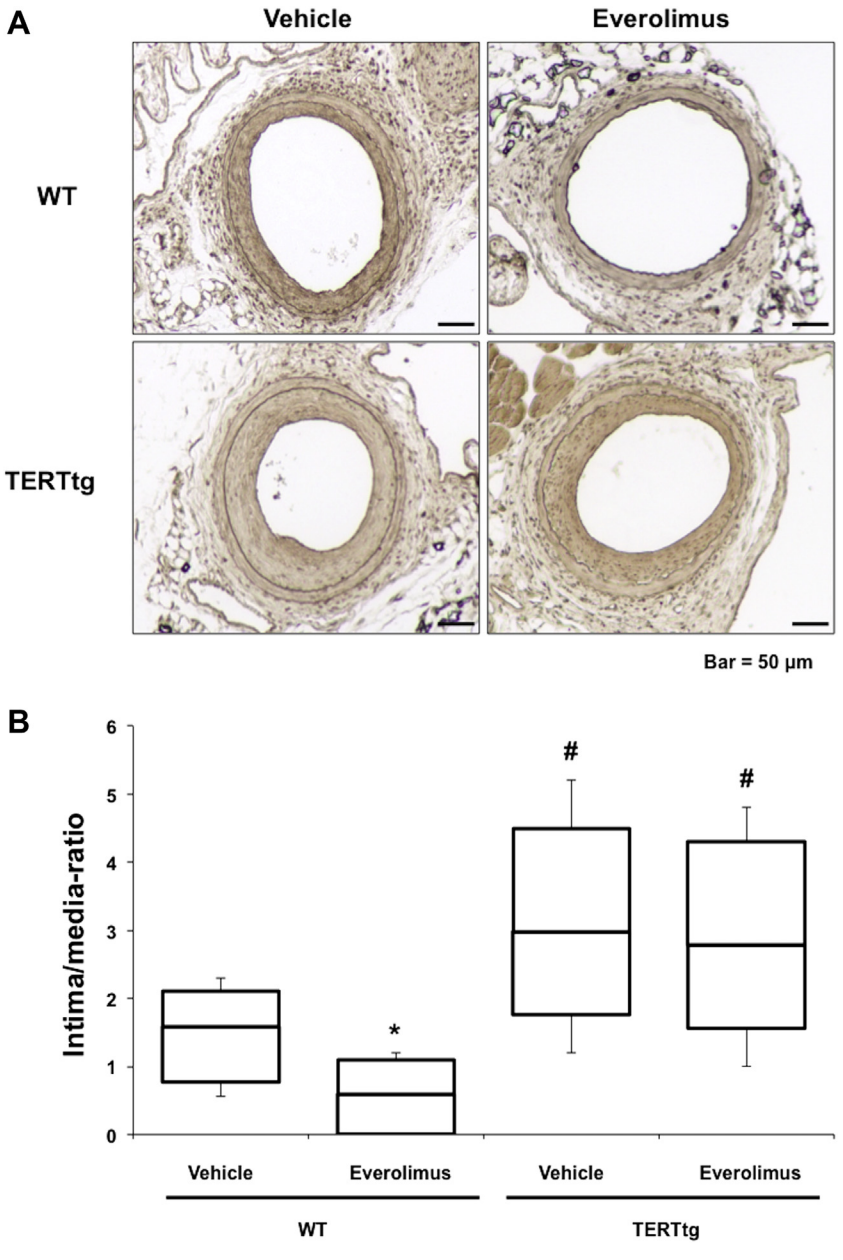
DISCUSSION

Everolimus-eluting stents constitute the most widely utilized stent platform to prevent restenosis after coronary angioplasty (4,5). Although more than 800,000 stents are implanted annually in the United States, the molecular mechanisms by which mTOR inhibitors prevent restenosis remain controversial. In cultured rat aortic SMC, sirolimus and everolimus prevent mitogen-induced phosphorylation of RB as a key step for this transition (7–9). However, using primary human coronary artery SMC isolated from in-stent restenotic lesions, Rosner et al. (9) have previously provided compelling evidence that sirolimus inhibits the progression of the cell cycle independently of RB. Therefore, the lack of an overt reduction of RB phosphorylation by everolimus at concentrations that inhibit cell cycle progression in our experiments using human coronary artery SMC is consistent with an RB-independent inhibition of G1→S phase progression by everolimus. Importantly, these discrepant findings point to distinct proliferative mechanisms between primary human coronary SMC and rat aortic SMC, and suggest that human coronary SMC, in particular those isolated from in-stent restenotic lesions, may be able to escape common checkpoints and maintain alternative mechanisms that signal toward S-phase and mitogenic gene expression.

It currently remains unknown whether the increased expression of TERT during neointima formation contributes to the proliferative response (14,15,17). Using a transgenic model of TERT overexpression, our data establish that constitutive TERT expression accelerates neointima formation. We further show that the well-established inhibition of neointima formation by everolimus is lost with constitutive TERT expression. Therefore, these data provide the first evidence to suggest a fundamental role of TERT expression during neointima formation and characterize TERT as a key molecular target for antiproliferative efficacy of everolimus. It has previously been reported that inhibition of telomerase activity using oligonucleotides inhibits SMC proliferation in vitro (17). Using small interfering ribonucleic acid and TERT overexpression approaches (14,15), our own research has further confirmed that TERT is necessary and sufficient for SMC proliferation in vitro. However, the detailed molecular mechanisms by which TERT induces SMC proliferation have previously not been addressed and remain unknown.

Our data illustrate repression of mitogen-induced TERT promoter activity as the key mechanism by which everolimus inhibits TERT expression. Although certain cancer and stem cells constitutively express TERT, quiescent somatic cells express low levels of TERT resulting in telomere shortening and impaired self-renewal (12). However, recent studies revealed that TERT is primarily regulated at the transcriptional level and inducible in response to mitogenic stimulation (13). This inducible TERT expression has been observed in multiple somatic tissues and demonstrated to enhance cell function

FIGURE 6 TERT Overexpression Prevents the Antiproliferative Effect of Everolimus on Neointima Formation



(A) Endothelial denudation injury was performed in femoral arteries of WT (n = 16) and TERTtg (n = 16) mice treated with vehicle or everolimus. Tissues were harvested 28 days after injury, and elastic Verhoeff-van Gieson staining was performed to visualize the internal and external elastic lamina. **(B)** Intima to media ratio was calculated as the intimal area divided by medial area. Data are presented as median and quartiles (significance level versus vehicle: *p = 0.046. Significance level versus WT: vehicle, #p = 0.048; Everolimus; #p = 0.034). Magnification × 100; scale bar = 50 μm. Abbreviations as in Figure 2.

during the proliferative response of tissue renewal (10). Similarly as in other somatic tissues, we (14,15) and others (17) have previously reported that TERT expression is induced in response to growth factor secretion during injury of the vasculature. TERT promoter activation during mitogenic stimulation of SMC is mediated by 2 adjacent Ets-1 motifs at -23 and -18 (15), and Ets-1 expression is induced during neointimal SMC proliferation (23). Consistent with our recent finding of Ets-1-dependent TERT

promoter transactivation during SMC proliferation (15), ChIP experiments confirmed Ets-1 binding to these consensus sites. Moreover, our data revealed that everolimus reduces Ets-1 binding to the endogenous TERT promoter in SMC. mTOR inhibitors have previously been demonstrated to attenuate Ets-1 protein expression in SMC (24), suggesting that the repression of TERT transcription by everolimus may be mediated at least in part through an inhibition of Ets-1 expression.

Everolimus-induced repression of TERT and the ensuing inhibition of SMC proliferation do not involve mechanisms that are dependent on telomere shortening or cellular senescence. In support of this notion, previous work has confirmed that TERT induces cell proliferation independently of its reverse transcriptase activity and ability to elongate telomeres (25,26). We further find that everolimus treatment of SMC induces cell cycle arrest in the G1 phase and is associated with altered downstream E2F-dependent S-phase gene expression. Conversely, TERT expression in SMC isolated from mice overexpressing a TERT transgene induces E2F-dependent transcription of MCM7, PCNA, and Cyclin A in SMC and reverses the repression of these *bona fide* S-phase genes by everolimus. Consistent with these data, ectopic TERT expression drives stem cells into S phase and induces the activity of E2F in epithelial cells (27,28). Our data extend this prior research and indicate that TERT enhances E2F recruitment to the chromatin of its target genes, whose protein products are required for DNA synthesis during S phase.

The molecular mechanisms that link TERT to gene transcription and increase E2F binding at S-phase gene promoters in our experiments remain elusive. However, recent analyses have documented that TERT induces differential gene expression and activates specific transcriptional programs independently of its catalytic activity (20,21). The broader changes in gene expression affected by TERT raised the possibility that TERT activates transcription through epigenetic mechanisms. Indeed, we observed that TERT expression induces histone acetylation at E2F motifs, which represents a reasonable mechanism for the increased E2F binding. Furthermore, ectopic E2F expression failed to reverse the inhibition of cell proliferation by everolimus, which could be explained by the acquisition of a silenced chromatin state at E2F sites in cells treated with everolimus and subsequent depletion of TERT. The hypothesis of an epigenetic mechanism is supported not only by our data demonstrating that TERT overexpression induces histone acetylation at E2F target sites, but also by previous work, in which TERT has been suggested to activate the chromatin at Wnt-dependent promoters in stem cells (22). Interestingly, TERT itself appears to be recruited to chromatin at selective transcription factor binding sites, where it associates with chromatin-modifying proteins (22). Alternatively, TERT may increase E2F binding through protein stabilization at the chromatin, which has recently been reported for myc-dependent gene transcription (29). Therefore, characterization of the specific chromatin signature at TERT-activated transcription sites will be necessary in future studies to determine the detailed mechanisms by which TERT modulates gene transcription.

CONCLUSIONS

We identified TERT as a previously unrecognized activator of an S-phase gene expression program. In response to mitogenic stimulation, TERT facilitates E2F transcription factor binding by supporting histone acetylation at its target promoters leading to SMC proliferation. We further establish that everolimus transcriptionally repressed TERT, which subsequently silences downstream E2F target gene expression. Collectively, these studies illustrate a novel mitogenic mechanism underlying SMC proliferation and identify TERT repression as a principal mechanism for the antiproliferative efficacy of everolimus.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Increased expression of the telomerase component TERT in response to vascular injury may serve a mitogenic function and accelerate aberrant neointima formation. In pre-clinical models, this inducible TERT expression constitutes an important target for the well-established antiproliferative efficacy of everolimus.

TRANSLATIONAL OUTLOOK: Future studies are warranted to investigate whether everolimus-eluting stent systems repress TERT after implantation in human coronary arteries and whether it is feasible to target TERT selectively in SMC while preserving re-endothelialization.

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APPENDIX For a supplemental methods section as well as figures, please see the supplemental appendix.