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NF-κB-mediated transactivation of telomerase prevents intimal smooth muscle cell from replicative senescence during vascular repair

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Abstract

OBJECTIVE—Functional telomerase is essential to the replicative longevity of vascular cells. To gain insights into mechanisms by which intimal hyperplasia interferes with the repair process, expression and function of the telomerase catalytic subunit (TERT) were investigated following vascular injury.

METHODS AND RESULTS—We found that TERT was *de novo* activated in intima of the injured arteries, involving activation of the nuclear factor κB (NF-κB) pathway. Stimulation of the isolated intimal smooth muscle cell (SMC) by basic fibroblast growth factor or tumor necrosis factor α resulted in increased TERT activity. This depends on the activation of c-Myc signaling since mutation of the E-box in the promoter or over-expression of MAD1, a c-Myc competitor, abrogated the transcriptional activity. Inhibition of NF-κB in both intimal SMC and in the injured artery attenuated TERT transcriptional activity through reduction of c-Myc expression. Pharmacological blockade of TERT led to SMC senescence. Finally, depletion of telomerase function in mice resulted in severe intimal SMC senescence following vascular injury.

CONCLUSIONS—These results support a model whereby vascular injury induces *de novo* expression of TERT in intimal SMC via activation of NF-κB and up-regulation of c-Myc. The resumed TERT activity is critical for intimal hyperplasia.

Keywords

telomerase; nuclear factor-κB; smooth muscle cell; intimal hyperplasia

Introduction

Telomerase is a complex of ribonucleoproteins containing two core components, a catalytic telomerase reverse transcriptase (TERT) and a telomerase RNA component (Terc). Activation

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of TERT is implicated in the synthesis of new telomeric DNA repeats, thereby overcoming telomeric DNA attrition from the ends of the cell's chromosomes during each round of division[1, 2]. Although TERT activity is low or absent in the majority of human adult somatic cells[3], many lines of evidence suggest that TERT can be reactivated in some organs such as liver and spleen with self-renewal capacity [4-6]. Moreover, TERT activity has also been found in inflamed lungs[7], injured liver[8] and hypertensive blood vessels[9], indicative of a potential role for TERT in the process of tissue repair and remodeling. More recent studies indicate that TERT is involved in vascular smooth muscle cells (SMC) proliferation both *in vitro*[10] and *in vivo*[11, 12]. However, the regulation of TERT and its function in the artery remain elusive.

Vascular SMC can be activated from quiescent into proliferative status in atherogenesis and in response to vessel injury. Proliferation of SMC is modulated by proinflammatory cytokines and growth factors expressed in atherosclerosis and restenotic lesions through activation of nuclear factor κB (NF-κB) signal transduction pathway[13-17]. We have recently shown that NF-κB plays a crucial role in intimal hyperplasia[18]. Given the fact that TERT expression in many tissues could be activated by mitogenic stimuli, we assessed the TERT expression and regulation by NF-κB activation in vascular cells using a rat model of carotid artery injury and the functional relevance of telomerase using arterial ligation model in *Terc*$^{-/-}$ mice.

Materials and Methods

An extended version of the Materials and Methods section can be found in the supplemental materials.

Rat model of angioplastic injury and inhibition of NF-κB

All procedures were approved by the regional ethical committee for animal research at Karolinska Institute. Male Sprague-Dawley rats (300-350g) were subjected to angioplastic injury to the left common carotid artery under general anesthesia by intraperitoneal injection of pentobarbital (2 mg/kg) plus Hypnorm® (50 mg/kg, Janssen Pharmaceutica, Belgium) as previously described[19]. Carotid arteries were transduced with 50 μL adenovirus encoding dominant-negative IKKβ (dnIKKβ) or *E coli* β-Galactosidase (β-Gal) at concentration 4×10^{10} pfu/mL and incubated for 40 minutes after the injury. The animals were sacrificed by overdosing pentobarbital two weeks after the injury and common carotid arteries were excised.

Carotid artery ligation model in *Terc*$^{-/-}$ mice

G4 m*Terc*$^{-/-}$ mice were used in this study[20]. Briefly, Mice were anesthetized with intraperitoneal injections of Hypnorm/Dormicum (Roche, Basel Schweiz) solution, the right common carotid artery and its bifurcation were exposed after mid cervical incision, and is subsequently subject to ligation. All surgery and post surgery treatment was carried out on heating pads. Three weeks after surgery the mice were sacrificed, the carotid arteries were dissected out for different purposes. Five cryosections between 200 to 1000 μm of ligated arteries were subjected to hematoxylin and eosin (H&E) staining and analysed for intimal area using Leica Qwin image analysis software (Leica Cambridge, UK).

Statistics

Statistical analyses were performed using the 2-tailed Student's *t* test or Mann-Whitney test, for experiments comparing 2 groups, and ANOVA with Tukey's Multiple Comparison post test, for 3 or more group experiments and values of $p<0.05$ were considered statistically significant.

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Results

Activation of TERT expression is via NF-κB in the injured artery

Using a rat carotid artery injury model, expression of TERT was examined in normal and injured vessels. Immunostaining analysis showed that TERT protein was highly induced in the injured vessels at day 14, and predominantly localized in intimal lesion (60% of intimal cells vs. 20% of medial cells, Fig 1A, top left panel, d and top right panel, $p<0.05$). Yet it was not detected in normal vessels, (Fig 1A, top left panel, b). To confirm the immunostaining findings, TERT transcript levels were analyzed by RT-PCR. Consistent with the results of TERT protein, levels of TERT mRNA were increased about 30-fold in the injured artery at day 14 when compared with normal artery (Fig 1A, lower panel, $p<0.05$). Intriguingly, immunostaining for NF-κB p65 displayed the similar expression as TERT in the intima (Fig 1A, c). Due to the pivotal role that NF-κB serves in vascular remodeling, we assessed the possibility that activation of NF-κB signal transduction pathway is involved in regulation of TERT expression. Utilizing adenovirus encoding dnIKKβ, the carotid artery was transduced at the time of balloon injury. This resulted in inhibition of the injury-induced NF-κB activation as described previously[18, 21], as well as marked suppression of TERT expression at both transcriptional and protein levels relative to that of β-Gal transduced injured vessels at day 14 (Fig 1B).

Induction of TERT activity in isolated vascular SMC by bFGF or TNFα

To directly assess TERT activity in SMC, isolated intimal and medial SMC were stimulated with bFGF or TNFα *in vitro*. As shown in Fig 2A, by TRAP analysis, a low level of basal TERT activity was detected in medial SMC, but higher level was observed in intimal SMC. Nevertheless, TERT activity was further enhanced by both TNFα and bFGF in medial SMC and to even greater levels in intimal SMC.

E-box in TERT promoter is crucial for bFGF and TNFα in transcriptional regulation of TERT in intimal SMC

To clarify molecular mechanisms for the transactivation of the TERT gene upon vascular injury, intimal SMC were transiently transfected with a 1.4 kb (encompassing -1353 to +9 of the transcriptional start site) TERT promoter-reporter or a 181 bp short proximal promoter-reporter [22, 23]. Our data showed that the long TERT promoter conferred minor transcriptional activity in intimal SMC upon either bFGF or TNFα stimulation (Fig 2B, left panel). In contrast, the short proximal TERT promoter was strongly activated by serum, bFGF or TNFα, consistent with a previous report (Fig 2B, right panel)[22]. These data indicate that the short proximal TERT promoter, so called core promoter, confers the essential activity for the upregulation of TERT expression by bFGF or TNFα, therefore, was further investigated.

Previous studies show that an E-box element (located at -165 within the proximal promoter region), which functions as the binding motif of transcription factor c-Myc, is conserved in both murine and human TERT gene promoter, and vital to transcriptional activation of the gene [23]. To determine the functional role of E-box in the bFGF and TNFα- induced activation of TERT, we examined activity of TERT core promoter in the condition with intact or mutated E-box. As shown in Fig 2C, TNFα and bFGF caused 6- and 9-fold increase of the promoter activity with the intact E-box, respectively in intimal SMC. Abrogation of the E-box by mutation, however, resulted in marked decrease in the promoter activity to both TNFα and bFGF. Similarly, enforced expression of MAD1, a competitor of MAX that forms a heterodimer with c-Myc for the subsequent binding to E-box [24], could also attenuate the promoter activity. These data suggest that bFGF and TNFα induced c-Myc activation and subsequent binding to the E-box in the TERT promoter contribute to the transactivation of TERT in the intimal SMC. We also examined the involvement of NF-κB in transcriptional regulation of TERT in intimal SMC by overexpressing NF-κB inhibitor, dnIKKβ. Interestingly,

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despite no consensus NF-κB response element in TERT proximal promoter region, dnIKKβ markedly diminished both bFGF and TNFα-induced promoter activity in intimal SMC (Fig 2C).

NF-κB regulates c-Myc expression and subsequent binding to the E-box in the TERT promoter in intimal SMC

Given the functional importance of c-Myc in TERT expression, the hypothesis that NF-κB regulates c-Myc and consequently the TERT expression in the vascular repair process was tested. Our *in vivo* data showed that c-Myc mRNA levels increased 3-fold in the carotid artery at day 14 after injury, but was reduced to nearly pre-injury levels in the vessels transfected with dnIKKβ (Fig 3A). Consistently, dnIKKβ infection also resulted in suppression of c-Myc and TERT expression *in vitro* in intimal SMC subjected to bFGF stimulation. (Fig 3B, lane 6 versus lane 4; lane 12 versus lane 10)

Analysis of c-Myc by western blotting validated that blockade of NF-κB by dnIKKβ led to reduction of both cytoplasmic and nuclear c-Myc protein (Fig 4A, lane 4 versus lane 2 or 3). Subsequently, the binding of c-Myc onto two different E-box sequences in TERT gene promoter was assessed by ChiP assay in the chromatin context of intimal cells exposed to bFGF stimulation. E-box 1 designates the sequence -114 to +108 in the proximal region of rat TERT gene promoter, while E-box 2 sequence is retrieved from -690 to -468 in the distal region corresponding to the E-box included in the long promoter (Fig 4B). We showed that increased in vivo c-Myc binding to E-box 1 was detected in the intimal cells exposed to bFGF for 2h (Fig 4B, c-Myc antibody precipitated panel, namely C-Myc ab, lanes 2 and 3), while it remained undetectable in untouched (lane 1 under c-Myc ab panel) and dnIKKβ infected cells (lane 4 under c-Myc ab panel), indicating that dnIKKβ treatment abrogates c-Myc binding to TERT promoter. Similar results were also observed for the binding of c-Myc to E-box 2 (Fig 4B bottom middle c-Myc ab panel).

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Both inhibition of TERT and Terc knockout results in replicative senescence of intimal SMC

To assess the relevance of TERT reactivation to SMC proliferation, intimal SMC were treated with bFGF in conjunction with different doses of TERT inhibitor BIBR1532. Supplemental figure IA shows that BIBR1532 at 1 μmol/L significantly impeded bFGF-induced intimal cell proliferation at 3, 7 and 14 days and at 5 μmol/L fully inhibited the cell proliferation at all time points. However, 10 μmol/L of this inhibitor revealed cell toxicity characterized by massive cell death at day 3 (data not shown). Morphologic changes of the cells exposed to BIBR1532 were also evaluated microscopically. As shown in supplemental figure IB, at day 7, BIBR1532 (1 μmol/L)-treated intimal cells became flat and enlarged in morphology, a characteristic of cellular senescence, and apparently growth arrested when compared to bFGF stimulated intimal SMC. At day 14, BIBR1532-treated intimal cells displayed signs of apoptosis as indicated by membrane blebbing.

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To further assess *in vivo* function of TERT during vascular repair process, we utilized a telomerase deficient mouse model by knocking down the RNA template of telomerase (*Terc*$^{-/-}$ mice). As early generations of these mice, up to G3, display relatively normal phenotype, compared to later generations[25] we chose G4 *Terc*$^{-/-}$ mice, and ligated common carotid artery, after 3 weeks, mice were sacrificed and arteries removed for analysis. Fig 5A and B show that *Terc*$^{-/-}$ mice displayed a severe cellular senescence restricted to the intimal area as detected by SA-βGal staining, compared to wild type mice. Intimal lesion was determined from 200 μm to 1000 μm from ligation site, and no clear distinction in the lesion size was observed up to 800 μm between the two groups of mice (data not shown). However a tendency of intimal reduction, albeit not statistically significant incomparison with the wild type mice, was noticed at 1000 μm level in *Terc*$^{-/-}$ mice (Fig 5C).

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Discussion

The present study reports for the first time two interesting findings. First, vascular injury, through activation of NF-κB, induces transcriptional upregulation of TERT selectively in intimal SMC, and c-Myc has been identified as NF-κB modulated transcription factor directing TERT transactivation upon vascular injury. Secondly, depletion of Terc in mice results in severe intimal SMC senescence following vascular injury. Our study supports the published work[11] that TERT was reactivated following vascular injury, and this also further extend our knowledge on NF-κB signaling modulating TERT in other cells types[26-28]. Our findings establish activation of TERT in intimal SMC by the injury induced NF-κB signaling as a basic mechanism underlying regulation of intimal hyperplasia.

Molecular mechanism that regulates intimal hyperplasia remains elusive. We demonstrated here that a cell type specific activation of TERT expression in intimal SMC undertakes in the process of intimal hyperplasia, and is tightly associated with activation of NF–κB signaling. To understand the regulation of TERT expression in SMC following vascular injury, we established an in vitro model in which TERT activity can be easily assessed in medial and intimal SMC exposed to TNFα or bFGF. Previous studies demonstrate that angioplastic injury to the artery provokes a pronounced inflammatory response, including induction and activation of the mitogen bFGF as well as proinflammatory cytokines such as TNFα, implicated in modulating SMC migration, growth and apoptosis [29-32]. In addition, both TNFα and bFGF are able to directly activate NF-κB [16, 32]. Initial analysis of TERT activity reveals that although both intimal and medial SMC possess a very low level of basal TERT activity, this can be substantially enhanced, in particular in the intimal SMC, when exposed to TNFα or bFGF, suggesting that TERT in SMC could be regulated by bFGF and TNFα produced by activated SMC and other inflammatory cells in the injured vessel. This view also supports a recent study that bFGF induces TERT expression in rat lung fibroblasts[33]. However, bFGF and TNFα may regulate TERT through different mechanisms. bFGF strongly activates the TERT promoter and induces the gene expression in intimal SMC, indicating that it regulates TERT primarily via activation of gene transcription. Conversely, TNFα is unable to induce TERT transcription in SMC (data not shown), although this cytokine could induce TERT activity and to some extent activate the TERT promoter. In view of recent findings that TNFα can rapidly activate TERT translocation from the cytoplasm to the nucleus[26], we postulate that TNFα regulates TERT activity via an alternative mechanism probably acting on posttranslational events.

Regulation of TERT activity can be achieved at various levels, including transcriptional[27] and post-transcriptional[26, 33-35]. A recent study[10] has shown that an increased TERT activity was due to phosphorylation of TERT in the proliferative vascular SMC, the present data, however, show that TERT in the injured vessel is modulated primarily via transcription regulation and involves NF-κB activation. Immunostaining analysis shows that both TERT and activated NF-κB signal were localized in the intima, and TERT expression was diminished when inhibiting NF-κB by dnIKKβ. These findings indicate that NF-κB activation serves as a critical regulatory mechanism contributing to *de novo* activation of TERT transcription. Previous studies have clarified that TERT transcription could be directed by several transcription factors, including c-Myc, NF-κB and STAT3 [22, 23, 36-39], suggesting that TERT expression may be regulated by different factors in different cellular context[40]. To gain further insight into the mechanism by which NF-κB modulate TERT expression in SMC, we assessed the activity of the TERT gene promoter in intimal SMC upon bFGF and TNFα stimulation. Study on TERT gene promoter has identified that activation of an E-box element in the proximal core promoter by Myc/Max dimer is essential for transcriptional activation in immortalized and cancer cells[22 24, 41], and also required for the TERT transcription in normal epithelial and fibroblast cells[22, 42, 43]. Results of the present promoter assay indicate that the transcription activity of TERT to a great extent depends on c-Myc function and E-box in the TERT core promoter region since mutation

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of the E-box or overexpression of MAD1 abolishes the promoter activity. Intriguingly, blockade of NF-κB also substantially attenuates the activity of bFGF and TNFα and since no NF-κB element exists in the tested TERT core promoter, these data imply an indirect rather than a direct role for NF-κB in the regulation of TERT gene transcription.

In this situation, activation of c-Myc has also been established as a central mechanism. We showed that c-Myc is induced in the injured vessels in an NF-κB dependent manner. Furthermore, overexpression of dnIKKβ in cultured intimal SMC blocks the cytokine-induced rapid c-Myc expression, and TERT expression. ChiP assay demonstrates NF-κB-mediated direct binding of c-Myc onto the TERT promoter in the chromatin context of living cells exposed to bFGF treatment. Taken together, the established correlation between activation of NF-κB and up-regulation of c-Myc and transcriptional activation of TERT gene provides an indirect role of NF-κB through c-Myc in the regulation of TERT gene expression following vascular injury.

Activation of telomerase is believed to be critical for cell proliferation and immortalization; finally, we explored the functional role of TERT reactivation in intimal hyperplasia in two ways. First, pharmacological inhibition of TERT in intimal SMC restrains the cell growth suggesting a critical role for TERT in supporting intimal SMC proliferation. Second, mimicing vascular injury by performing carotid artery ligation of Terc deficient mice induced dramatic cellular senescence in the intima of *Terc*$^{-/-}$ mice in comparison to wild type mice, affirming that reactivating SMC TERT upon injury is required for intimal development. These data may provide new understanding and solution for restenosis.

In conclusion, the present data demonstrate that expression of TERT can be *de novo* activated in vascular SMC in response to injury and that this is mediated by the activation of the NF-κB signal transduction pathway. In view of the critical role of NF-κB in the regulation of intimal hyperplasia and TERT activity, advances in the understanding of the regulation of TERT activity may open new avenues for therapeutic intervention of intimal hyperplasia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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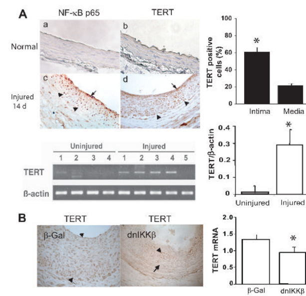

Figure 1. Activation of TERT expression in the injured artery is NF-κB dependent

A: *Top panel left*: Immunohistochemistry for TERT and NF-κB p65 in the rat carotid artery. The arrowheads indicate internal elastic lamina, and the arrows indicate cells showing representative positive signals for TERT and p65, respectively (brown color). (Original magnification: X400). *Lower panel left*: RT-PCR analysis of TERT and β-actin from uninjured and injured carotid arteries. **B: Left panel:** Immunohistochemistry for TERT in artery transduced with adenovirus carrying either *E coli* β -Galactosidase (β-Gal) or dominant-negative mutant of IκB kinase β (dnIKKβ) at day 14, the arrow heads indicate TERT positive cells and the arrows indicate internal elastic lamina. **Right panel:** qRT-PCR analysis of TERT from the injured carotid arteries transduced with β-Gal or dnIKKβ at day 14, respectively (n=5 to 7). TERT transcripts normalized to Hprt, data are presented as mean ± SEM *$p<0.05$.

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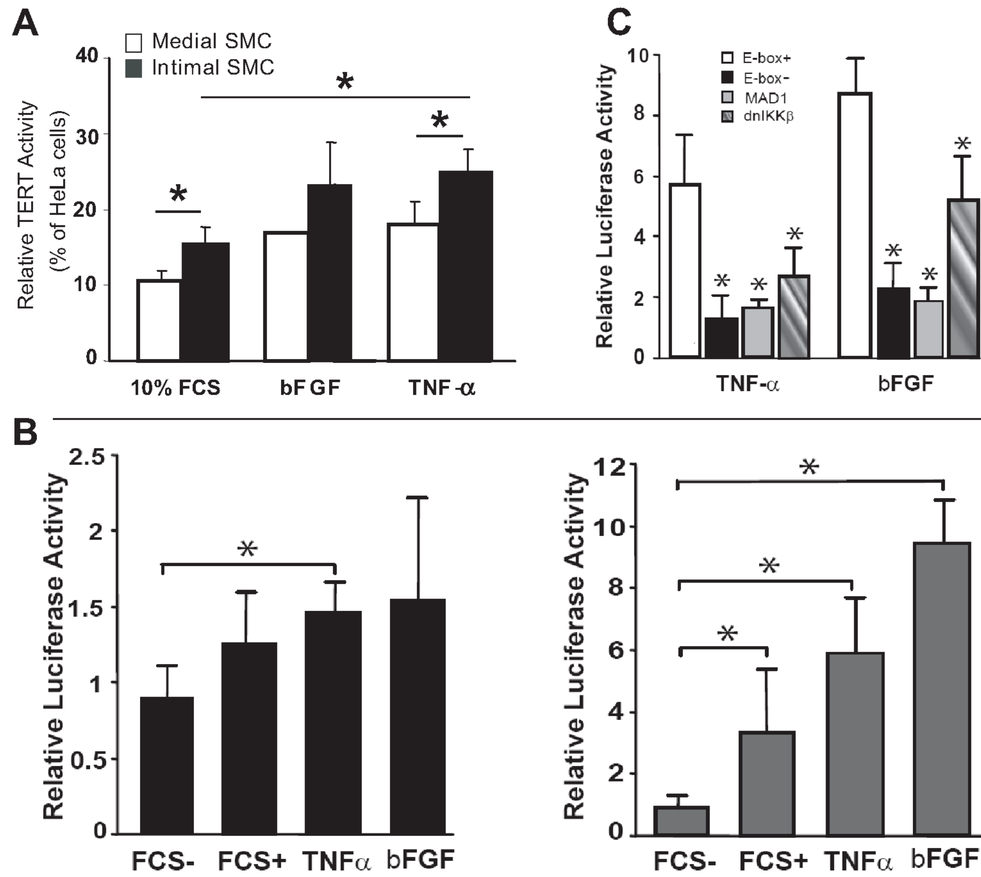

Figure 2. Enzymatic and transcriptional activity of TERT in medial and intimal SMC

A: Induction of TERT activity in medial and intimal SMC by bFGF and TNFα. The TERT activity was analyzed by TRAP assay (see Materials and Methods). Results are expressed as relative activity to HeLa cells as a positive control. *$p<0.05$ **B:** Modulation of TERT promoter activity by bFGF and TNFα Intimal SMC were transfected with the long (1.4 kbp, *left panel*) or the proximal (181 bp, *right panel*) TERT promoter. Luciferase activity was determined in the cells 18 h after treatment with 0.5% FCS (FCS-), 10% FCS (FCS+), bFGF or TNFα, respectively. *$p<0.05$ vs FCS-. **C:** E-box signaling for the transcriptional activity of TERT. Intimal SMC were transfected with the proximal TERT promoter bearing mutated E-box (E-box-) or with intact E-box (E-box+) plus co-transfection with pEF-MAD1 (MAD1) or, Adv-dnIKKβ in intimal SMC in response to TNFα or bFGF. Luciferase activity was analyzed 18 h after being treated with bFGF or TNFα. P*< 0.05 vs E-box+. Data in (B) and (C) are corrected for transfection efficiency using Renilla luciferase, and the levels of luciferase activity are expressed in relative to the unstimulated cells transfected with empty pcDNA3. Data are mean ± SEM from three independent experiments.

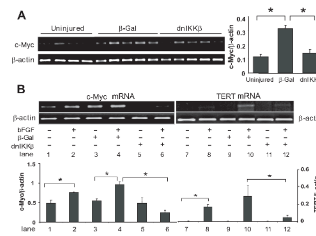

Figure 3. Regulation of c-Myc and TERT expression by NF-κB

A: RT-PCR analysis of c-Myc and β-actin mRNA from uninjured or injured arteries transduced with β-Gal or dnIKKβ at day 14, respectively. **Right panel:** Quantification of c-Myc transcripts normalized to β-actin. Data are presented as mean ± SEM, n= 4-5 rats *$p<0.05$. **B: upper panel:** RT-PCR products corresponding to c-Myc, TERT and β-actin (as an internal control) from intimal SMC stimulated with or without bFGF for 2 h or 24 h, respectively, prior to infection with or without β-Gal or dnIKKβ for 1 h. **Lower panel:** Quantification of c-Myc and TERT transcripts normalized to β-actin. Data are presented as mean ± SEM from three independent experiments, *$p<0.05$.

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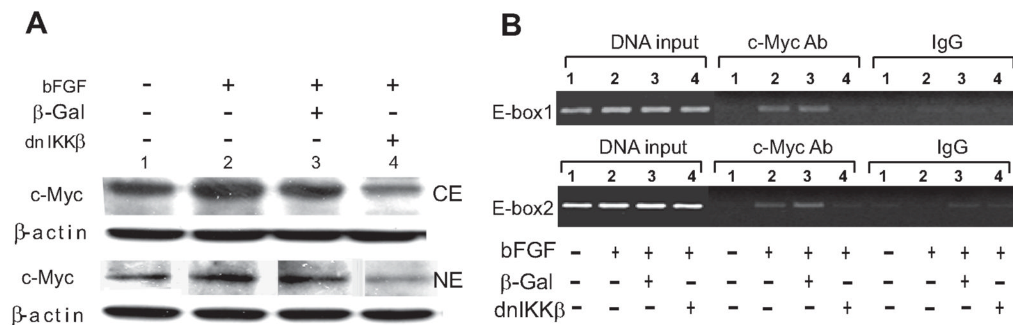

Figure 4. Regulation of c-Myc expression and its binding to E-box by NF-κB

A: Western blotting of c-Myc in intimal SMC stimulated with bFGF for 12 h in the presence of β-Gal or dnIKKβ (see Material and Methods). Cytoplasmic and nuclear extracts (CE and NE) were immunoblotted with c-Myc antibody, reprobed with β-actin antibody for loading control. A representative from three independent experiments is shown. **B:** Chromatin immunoprecipitation assay: Intimal SMC were exposed to bFGF stimulation or not for 2 h in the presence of β-Gal or dnIKKβ (lane 1: control; 2: bFGF stimulation; 3: β-Gal infection and bFGF stimulation; 4: dnIKKβ infection and bFGF stimulation). Chromatin extracts were immunoprecipitated with specific antibody to c-Myc or an IgG control. Panels of DNA INPUT, c-Myc ab and IgG correspond to the control genomic DNA input, c-Myc antibody or IgG pull-down respectively. The detection of captured TERT promoter was performed by PCR as described in Materials and methods.

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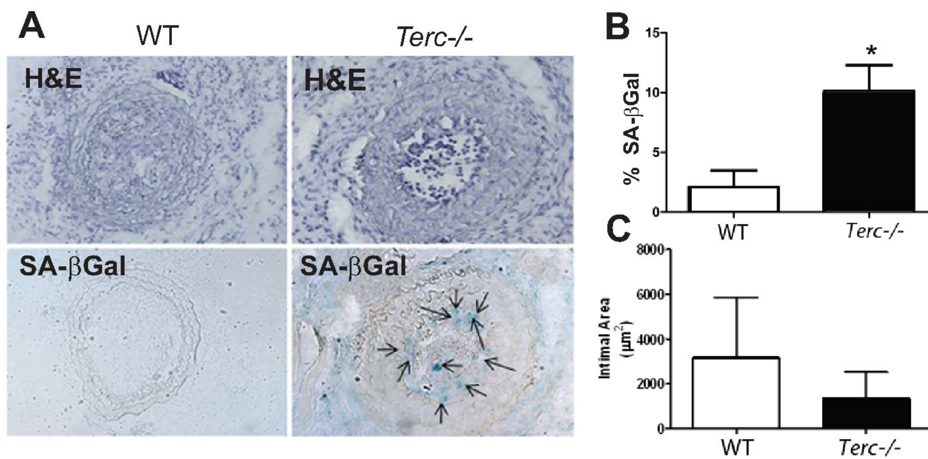

Figure 5. Terc deficiency in mice induces intimal cellular senescence in the ligation induced artery injury model

A: H&E (top panel) and SA-βgal stainings (bottom panel) for carotid arteries after ligation for 3 weeks in *Terc*$^{-/-}$ mice and wild type mice. The arrows indicated SA-βgal positive staining cells. **B:** quantificaiton of senescence visible (positive area of SA-βgal stainings) in the carotid arteries of *Terc*$^{-/-}$ mice (n=8) and wild type mice (n=6). Data are expressed as mean±SEM, *p<0.05. **C**: quantificaiton of intimal area for carotid arteries at 1000 μm from the ligation from *Terc*$^{-/-}$ (n=8) and wild type mice (n=6).