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PINX1 and TERT Are Required for TNF- α -Induced Airway Smooth Muscle Chemokine Gene Expression

Karl Deacon and Alan J. Knox

Airway smooth muscle (ASM) cells contribute to asthmatic lung pathology with chemokine hypersecretion and increased ASM cell mass. With little recent progress in the development of asthma therapies, a greater understanding of lung inflammation mechanisms has become a priority. Chemokine gene expression in ASM cells is dependent upon NF- κ B transcription factor activity. The telomerase/shelterin complex maintains chromosomal telomere ends during cell division. Telomerase is a possible cofactor for NF- κ B activity, but its role in NF- κ B activity in airway tissue inflammation is not known. In this study, we sought to address two key questions: whether telomerase is involved in inflammation in ASM cells, and whether components of the shelterin complex are also required for an inflammatory response in ASM cells. Telomerase inhibitors and telomerase small interfering RNA (siRNA) reduced TNF- α -induced chemokine expression in ASM cells. Telomerase siRNA and inhibitors reduced NF- κ B activity. An siRNA screen of shelterin components identified a requirement for PIN2/TERF1 interacting-telomerase inhibitor 1 (PINX1) in chemokine gene expression. High-level PINX1 overexpression reduced NF- κ B reporter activity, but low-level expression amplified NF- κ B activity. Coimmunoprecipitation studies showed association of PINX1 and p65. Overexpression of the N terminus (2–252 aa) of PINX1, but not the C-terminal telomerase-inhibitor domain (253–328 aa), amplified TNF- α -induced NF- κ B activity. GST pull-downs demonstrated that the N terminus of PINX1 bound more p65 than the C-terminal telomerase-inhibitor domain; these observations were confirmed in whole cells with N-terminal and C-terminal PINX1 immunoprecipitation. We conclude that telomerase and PINX1 are required for chemokine expression in ASM cells and represent significant new targets for future anti-inflammatory therapies for lung diseases, such as asthma. *The Journal of Immunology*, 2018, 200: 000–000.

Inflammation plays a central role in the pathology of asthma. In the last 15 y, asthma therapeutics have seen little improvement over the efficacy of combined β -adrenergic receptor agonist and inhaled corticosteroid treatment of mild and moderate asthma. Patients with severe asthma can receive “add-on therapies” in the form of leukotriene receptor antagonists, theophylline, omalizumab (anti-IgG-E), or mepolizumab (anti-IL-5), but these therapies can require long-term administration and are (relative to corticosteroid therapies) very expensive. For these reasons, there is a continuing focus on discovering novel approaches to anti-inflammatory therapeutics. The airways in asthma have a markedly thickened airway smooth muscle (ASM) cell layer that secretes a wide range of proinflammatory cytokines and mediators. The NF- κ B transcription factor has a

central role in inflammation. The active nuclear NF- κ B transcription factor is a heterodimer of members of the Rel family of proteins (c-Rel, Rel-A, Rel-B, p52/NF κ B2, and p50/NF κ B1). c-Rel, Rel-A (p65), and Rel-B proteins are sequestered as inactive monomers in the cytoplasm by members of the inhibitor of κ B family of proteins (I κ B α , I κ B β , I κ B ϵ). The canonical pathway of NF- κ B activation is dependent upon proinflammatory amplifiers, such as TNF- α , IL-1 β , innate immune receptors, the TLRs (TLR3, TLR4), or acquired immunity TCRs and BCRs inducing the activity of the I κ B protein kinases, IKK α and IKK β . IKK kinase activity causes phosphorylation of I κ B, leading to I κ B ubiquitination and proteasomal degradation that result in Rel protein accumulation in the nucleus to form the active NF- κ B transcription factor (1).

The telomerase holoenzyme comprises the telomere-end reverse transcriptase (TERT) and the telomerase RNA component (2, 3). Telomerase was identified as the enzyme primarily responsible for maintaining the telomere ends of somatic cell chromosomes, preventing gene fusions and DNA damage. During normal development, telomerase expression is suppressed, and somatic cells have a set limit of telomere length; as cells divide, telomeres shorten and eventually take part in inducing replicative senescence and cell death. Unlimited cell growth in cancer has been linked to increased telomerase expression in vitro and in patient tumor tissue (4). Telomerase associates with proteins that form the shelterin complex: TPP1, TERF2, POT1, TIN2, RAP1, and TERF1 (5). Binding of the shelterin complex to telomere ends prevents DNA damage-sensing apparatus from recognizing the open telomere ends as dsDNA breaks, potentially leading to inappropriate programmed cell death (6). PIN2/TERF1-interacting telomerase inhibitor 1 (PINX1) is an inhibitor of telomerase activity that is also necessary for TERT/shelterin component binding at

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K.D. conceived and coordinated the study; designed, performed, and analyzed the experiments shown in all of the figures; and wrote the manuscript. K.D. and A.J.K. reviewed the results and edited and approved the final version of the manuscript.

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Abbreviations used in this article: ASM, airway smooth muscle; Co-IP, coimmunoprecipitation; JIP1, JNK inhibitory protein 1; PINX1, PIN2/TERF1-interacting telomerase inhibitor 1; QPCR, quantitative PCR; siRNA, small interfering RNA; TERT, telomere-end reverse transcriptase.

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Table I. The telomerase inhibitors MST-312 and BIBR1532 reduce TNF- α -induced chemokine secretion from ASM cells

Chemokine	Inhibitor	Inhibition of Maximal (%)	$\pm$ SEM of Inhibition	Statistical Significance
CCL2	MST312	64	2.3	***
CCL2	BIBR1532	18	2.2	**
CCL5	MST312	97	0.68	***
CCL5	BIBR1532	55	5.0	***
CCL11	MST312	96	0.285	***
CCL11	BIBR1532	21	5.4	**
CXCL10	MST312	82	4.5	***
CXCL10	BIBR1532	74	1.93	***

ASM cells were treated with MST312 (5×10^{-6} M), BIBR1532 (5×10^{-6} M) or DMSO vehicle control for 60 min prior to addition of TNF- α (1 ng/ml) for 24 h. ELISA for CCL5, CCL2, CXCL10, and CCL11 was carried out on culture supernatants (normalized to cell counts) followed by a calculation of the percentage inhibition of maximal chemokine secretion by each inhibitor. All measurements represent the mean $\pm$ SEM of three independent experiments. Assay data were analyzed with a two-tailed paired *t* test.

p* = 0.01–0.05, *p* = 0.001–0.01, ****p* < 0.001.

telomere and nontelomere sites within chromosomes (7–9). PINX1 has been identified as a tumor suppressor with decreased expression (haploinsufficient) in a number of cancers (10). It has been hypothesized that the combination of increased TERT expression and loss of PINX1 (normally functioning as a TERT inhibitor) supports tumor growth. The current understanding is that PINX1 is a key component of TERT/telomerase homeostasis through its ability to bind TERT and inhibit TERT activity.

Recent studies have shown that TERT is required for the activity of the transcription factors with roles in chemokine expression and inflammation: Myc (11), TCF/LEF (12), and, particularly, NF- κ B (13). Asthma is characterized by persistent airway inflammation. Our group and other investigators have demonstrated that the asthmatic lung contains an increased amount of ASM cells and that ASM cells are a rich source of cytokines. Our aim was to determine whether telomerase is involved in regulating chemokine expression in ASM cells and to identify components of the telomerase/shelterin complex that play a role in the expression of chemokine genes by effecting the activation of NF- κ B in ASM cells. We found that telomerase was required for NF- κ B activity and chemokine gene expression in ASM cells and that PINX1 small interfering RNA (siRNA) decreased TNF- α -induced chemokine gene expression. Furthermore, PINX1 associated with NF- κ B and functioned as a positive regulator of NF- κ B activity in ASM cells. These studies give a novel insight into the roles of telomerase and PINX1 in regulating NF- κ B activity and chemokine expression in ASM cells, providing the first evidence that telomerase and PINX1 play a key role in inflammatory mechanisms in airway cells.

Materials and Methods

Reagents

MST312 was purchased from Sigma-Aldrich, BIBR1532 was purchased from Tocris Biosciences, and CCL2, CCL5, CCL11, CXCL8, and CXCL10 DuoSet ELISA kits and recombinant human TNF- α were purchased from R&D Systems. TERT (SC-7212), PINX1 (SC-292115), p65 (Rel-A; SC-372) and GAPDH (SC20357) Abs were purchased from Santa Cruz Biotechnology (Dallas, TX). κ B α (4814) and κ B α -S32P (5209) Abs were purchased from Cell Signaling Technology.

Plasmids

p6 κ B.TK.LUC was a kind gift from Prof. R. Newton (University of Calgary, Calgary, AB, Canada) (14). pRLSV40 was purchased from Promega. pGEX-FLAG-PINX1-N (2–252) and pGEX-FLAG-PINX1 (253–328) were a kind gift from Prof. C. Counter (15). pCDNAiii-c-FLAG was a gift from Prof. S. Smale (16). pCMVSPORT6-PINX1 (IMAGE clone 3914396) was obtained from Source Biosciences (Nottingham, U.K.) and subcloned into pCDNAiii at EcoRI/XhoI. pCDNAiii-FLAG-PINX1 (2–252) and pCDNAiii-FLAG-PINX1 (253–328) expression constructs were created by EcoRI/XhoI subcloning of FLAG-PINX1 from pGEX-FLAG-PINX1-N (2–252) and pGEX-FLAG-PINX1 (253–328). pCMV5-hTERT; pBABE-hTERT was a gift from Prof. B. Weinberg (17). cDNA was excised with EcoRI and SalI and subcloned into pCMV5 at the equivalent sites.

Human ASM cell culture

Human ASM cells were explant cultured from human tracheas obtained post mortem, grown in DMEM supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS (Life Technologies) at 37°C with 5% CO₂ and 100% humidity, and used at passage six (18). ASM cells were characterized by immunocytochemical analysis for positive α -smooth muscle actin expression and negative cytokeratin-18, as described (19). ASM cells were 95% pure. Ethical approval for this study was obtained from the North Nottinghamshire Research Ethics Committee.

Table II. The telomerase inhibitors MST-312 and BIBR1532 reduce TNF- α -induced chemokine mRNA accumulation in ASM cells

Chemokine	Inhibitor	Inhibition of Maximal (%)	$\pm$ SEM of Inhibition	Statistical Significance
CCL2	MST312	96	0.125	***
CCL2	BIBR1532	14	1.2	*
CCL5	MST312	98	1.1	***
CCL5	BIBR1532	13	0.2	*
CCL11	MST312	98.5	2.3	***
CCL11	BIBR1532	30	1.15	**
CXCL10	MST312	96.5	1.3	***
CXCL10	BIBR1532	50	2.6	**

ASM cells were treated with MST312 (5×10^{-6} M), BIBR1532 (5×10^{-6} M), or DMSO vehicle control for 60 min prior to the addition of TNF- α (1 ng/ml) for 24 h. Quantitative real time PCR was performed for CCL5, CCL2, CXCL10, and CCL11 after total RNA extraction and first-strand DNA synthesis followed by a calculation of the percentage inhibition of maximal chemokine RNA accumulation by each inhibitor. All measurements represent the mean $\pm$ SEM of three independent experiments. Assay data were analyzed with a two-tailed paired *t* test.

p* = 0.01–0.05, *p* = 0.001–0.01, ****p* < 0.001.

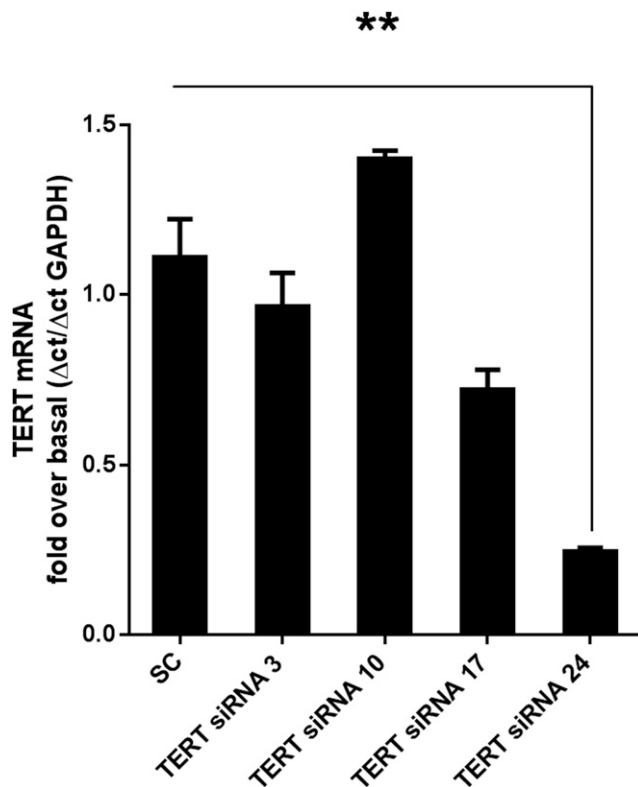


FIGURE 1. siRNA knockdown of TERT in ASM cells. ASM cells were transfected with 4 nM a scrambled control (SC) or TERT-specific siRNA (TERT siRNA 3, 10, 17, or 24) for 48 h prior to RNA extraction and analysis of TERT mRNA levels by QPCR against first-strand cDNA. $^{**}p = 0.001-0.01$.

ELISA

Cell lines were plated on 24-well plates and grown to 100% confluence, and the media were replaced with DMEM, with L-glutamine only, for 24 h. ELISA was performed, according to the manufacturer's protocol. All assay points were performed in triplicate on 24-well plates in a final media volume of 500 μ l. ELISA data were normalized to subsequent cell counts to give pg/ml for a standard 1×10^4 cells.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

RNA extraction, first-strand cDNA, synthesis and quantitative real-time PCR were performed as described previously (20). QPCR was performed with the following primer sets: GAPDH forward:

5'-CGGAGTCAACGGATTTGGTT-3' and GAPDH reverse: 5'-GCTC-CTGGAAGATGGTGA-3'; CCL2 forward: 5'-GCTCAGCCAGATGCAAT-3' and CCL2 reverse: 5'-GCTTGTCAGGTGGTCCATG-3'; CCL11 forward: 5'-GCCAGTTCTGTCCCAACC-3' and CCL11 reverse: 5'-GGAGTTG-GAGATTTTGTG-3'; CCL5 forward: 5'-GCTCCAACCCAGCAGTCG-3' and CCL5 reverse: 5'-GCCTCCCAAGCTAGGAC-3'; CXCL10 forward: 5'-GCCAATTTTGTCCAC-3' and CXCL10 reverse: 5'-GGCAGCCTCTGT-GTGGTCCAT-3'; PINX1 forward: 5'-CGATGCCAGTCCCTCCAC-3' and PINX1 reverse: 5'-GGCCTTAGGCTGGAGGTAAC-3'; and TERT forward: 5'-GCGTGCGCAGCTACCTGCCC-3' and TERT reverse: 5'-GCCTTCGGGG-TCCACTAGCG-3'.

All gene-specific quantifications were calculated as Δ ct (target ct – housekeeping ct) relative to control or untreated cell experiment control to give a final Δ ct (test)/ Δ ct (basal). All ct calculations were performed using Stratagene MxPro 3.2 software (Agilent).

siRNA knockdown of TERT, TERF1, and PINX1

Scrambled control siRNA (4 nM; All-Stars; QIAGEN) or TERT, TERF1, or PINX1 siRNA (4 nM; ON-TARGETplus siRNA; GE Dharmacon) was incubated for 10 min with 3 μ l of HiPerFect Transfection Reagent (QIAGEN) in 100 μ l of serum-free DMEM. siRNA complexes were incubated with 100 μ l of ASM cells (in complete media) at 6×10^5 cells per milliliter per well in a 24-well plate for 3 h. Complete media were added to a total volume of 600 μ l, and the plates were incubated at 37°C with 5% CO₂ and 100% humidity for 48 h. siRNA-transfected cells were serum starved for the last 24 h and then treated with the relevant agonist prior to RNA isolation or conditioned media harvesting.

NF- κ B-LUC reporter gene assays

Five hundred microliters of ASM cells were plated on 24-well plates, at 2×10^4 cells per milliliter, in DMEM supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS (Life Technologies) at 37°C with 5% CO₂ and 100% humidity. ASM cells were grown overnight, and the media were replaced with 400 μ l of DMEM with 4 mM L-glutamine (serum and antibiotic free) for 6 h. A total of 0.5 μ g of p6 κ B.TK.LUC was incubated with 0.001 μ g of pRLSV40 and 0.5, 1.0, or 2.0 μ g of pCMV5-WT-hTERTwt, pCDNAiii-PINX1, pCDNAiii-FLAG-PINX1 (2–252), or pCDNAiii-FLAG-PINX1 (253–328) for 18 h with FuGENE 6 (Promega). Transfected ASM cells were induced with 10 ng/ml of TNF- α or vehicle control for 6 h, washed once with PBS and lysed in 100 μ l of passive lysis buffer. Relative luciferase activity (firefly luciferase [LUC]/Renilla luciferase [REN]) was assayed with the Dual-Luciferase assay kit (Promega), according to the manufacturer's protocol. Luciferase assays were carried out on a Berthold MicroLumat Plus LB 96V luminometer (Jencons). For high-level protein coexpression with luciferase reporter constructs, the above transfections were repeated with FuGENE HD (Promega) at 80% cell confluence for up to 72 h prior to the reporter gene assays.

Coimmunoprecipitation assays

ASM cells were grown to confluence in 10-cm tissue culture dishes. Cells in two 10 cm tissue culture dishes were treated with TNF- α (1 ng/ml) for 30 min, placed on ice, washed with PBS, and lysed in a total of 600 μ l of

Table III. Telomerase siRNA reduces TNF- α -induced chemokine mRNA accumulation in and protein secretion from ASM cells

Chemokine	siRNA	Inhibition of Maximal (%)	$\pm$ SEM of Inhibition	Statistical Significance
CCL2 mRNA	TERT	82.4	0.2	***
CCL2 protein	TERT	9.4	0.23	*
CCL5 mRNA	TERT	96	1.3	***
CCL5 protein	TERT	21	1.24	*
CCL11 mRNA	TERT	77.3	3.1	**
CCL11 protein	TERT	7.6	0.51	*
CXCL10 mRNA	TERT	96.7	1.2	***
CXCL10 protein	TERT	32.5	0.52	**

ASM cells were transfected with TERT siRNA or scrambled control siRNA for 48 h prior to the addition of TNF- α (1 ng/ml) for 24 h. QPCR for CCL5, CCL2, CXCL10, and CCL11 mRNA was carried out on first-strand cDNA synthesized from total RNA followed by a calculation of the percentage inhibition by TERT siRNA of maximal chemokine mRNA accumulation, compared with maximal chemokine mRNA accumulation in ASM cells transfected with scrambled control siRNA. ASM cells were transfected with TERT siRNA or scrambled control siRNA for 48 h prior to the addition of TNF- α (1 ng/ml) for 24 h followed by ELISA for CCL5, CCL2, CXCL10, and CCL11 on culture supernatants (normalized to cell counts) and a calculation of the percentage inhibition of maximal chemokine secretion by TERT siRNA compared with scrambled control siRNA. All measurements represent the mean $\pm$ SEM of three independent experiments. Assay data were analyzed with a two-tailed paired *t* test.

* $p = 0.01-0.05$, ** $p = 0.001-0.01$, *** $p < 0.001$.

RIPA buffer with protease inhibitors. Lysates were incubated on ice for 20 min, vortexed for 10 s, and cleared by centrifugation at $14,000 \times g$ at 4°C for 20 min. Cleared lysates were incubated with $1 \mu\text{g}$ of control IgG or $1 \mu\text{g}$ of target Ab for 60 min on ice. Coimmunoprecipitation (Co-IP) assays were incubated on a bottle roller at 4°C with $70 \mu\text{l}$ of a 20% slurry of 50:50 protein A Sepharose/protein G Sepharose (GE Life Sciences) for 18 h at 4°C . Co-IP assays were washed with $500 \mu\text{l}$ of $3\times$ RIPA buffer and resuspended in $40 \mu\text{l}$ of $1\times$ SDS-PAGE loading buffer. Co-IP assays were analyzed by standard SDS-PAGE/Western blot. Primary blotting Abs were detected with L chain-specific goat anti-rabbit or rabbit anti-mouse HRP-linked secondary Abs at a 1:100,000 dilution (Jackson ImmunoResearch).

GST fusion protein production and GST pull-down assays

pGEX6t1 (GE Life Sciences), pGEX-FLAG-PINX1-N (2-252) (PINX1-C protein product), and pGEX-FLAG-PINX1 (253-328) (PINX1-N protein product) were transfected into BL21 *Escherichia coli*. Clones were grown overnight in 10-ml cultures of Luria broth ($100 \mu\text{g}/\text{ml}$ ampicillin), inoculated to 500 ml of Magic Media (Thermo Fisher), and grown at 37°C with shaking at 200 rpm for 6 h, followed by incubation at 30°C for 18 h without ampicillin. GST or GST-PINX1 was recovered with Glutathione Sepharose 4B (GE Healthcare Life Sciences), as follows. Bacterial pellets were recovered by centrifugation at $9000 \times g$, resuspended in 50 ml of GEX lysis buffer (20 mM Tris-HCl [pH 7.5], 1 M NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1% Triton X-100, 1% *N*-lauryl-sarcosine, 0.5 mM PMSF, $1\times$ protease inhibitor mixture [Sigma-Aldrich], 0.5 mM DTT), and frozen at -20°C for 24 h. Frozen pellets were rapidly defrosted in water at 37°C and cooled on ice prior to sonication with a probe sonicator set at 100% power for five 30-s intervals. Sonicated lysates were cleared by centrifugation at $10,000 \times g$ for 30 min at 4°C . Cleared lysates were incubated with 1 ml of 30% w/v Glutathione Sepharose 4B (previously washed in a $10\times$ volume of GEX lysis buffer) for 60 min on a bottle roller at 4°C . Loaded Glutathione Sepharose 4B beads were washed four times in 50 ml of GEX lysis buffer and resuspended in 1 ml of GEX lysis buffer. Protein-loaded beads were quantified by Coomassie Brilliant Blue-stained SDS-PAGE and compared to a Coomassie Brilliant Blue-stained SDS-PAGE standard curve constructed with BSA. ASM cells were grown to confluence in two 10 cm tissue culture dishes, washed in PBS, lysed in $600 \mu\text{l}$ of RIPA buffer, vortexed for 10 s, and incubated on ice for 20 min prior to centrifugation at $14,000 \times g$ for 20 min at 4°C . Cleared supernatants were rolled at 4°C with $20 \mu\text{g}$ of protein for GST control, PINX1-C, or PINX1-N for 1 h. Beads were washed four times with $600 \mu\text{l}$ of RIPA buffer, resuspended in $40 \mu\text{l}$ of $1\times$ SDS-PAGE loading buffer, and analyzed by SDS-PAGE/Western blot.

Nuclear and cytoplasmic protein separation

Nuclear and cytoplasmic proteins were prepared from ASM cells using a Nuclear Extract kit (Active Motif).

Protein quantitation and sample equilibration

All protein extracts were quantified by BCA assay (Thermo Fisher) with four replicates per extract. All protein loadings were equilibrated prior to SDS-PAGE. Co-IP assays were performed on identical lysates split for test (specific IgG) and control (preimmune IgG) immunoprecipitation. GST pull-down assays were performed on identical protein extracts that were split prior to incubation with protein-loaded beads.

SDS-PAGE Western blot analysis

Western blot analyses were performed as detailed previously (21).

Statistical analysis of data

Assay data from three separate experiments are presented as the SEM, and comparative data were analyzed with a two-tailed paired *t* test using Prism (GraphPad, San Diego, CA) (**p* = 0.01–0.05, ***p* = 0.001–0.01, ****p* < 0.001).

Results

Telomerase inhibitors reduce TNF- α -induced chemokine secretion and mRNA accumulation

ASM cells were treated with the telomerase inhibitors MST312 or BIBR1532 for 24 h prior to a 24-h induction with TNF- α . ELISA analysis of culture supernatants demonstrated that MST312 suppressed the secretion of CCL5, CCL2, CXCL10, and CCL11 (Table I). We chose these as asthma-relevant chemokines that all

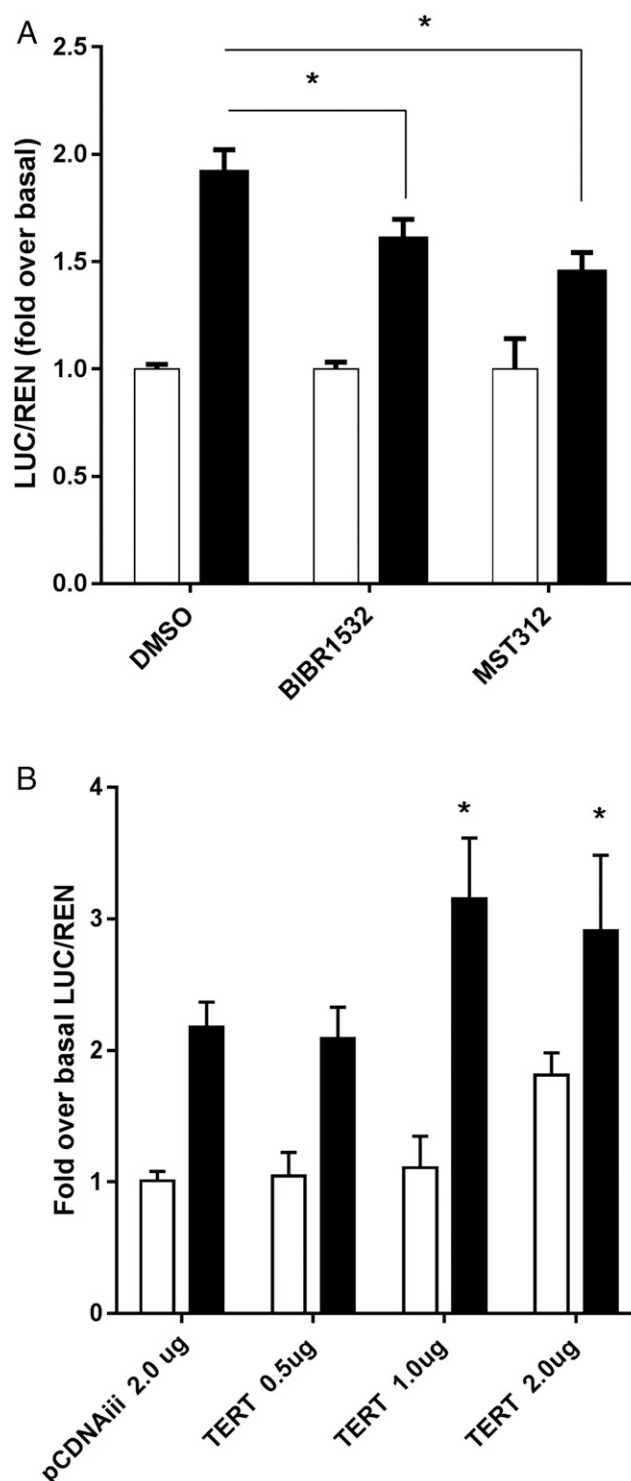


FIGURE 2. TERT inhibition reduces NF- κ B luciferase reporter gene activity, and TERT overexpression increases NF- κ B luciferase reporter gene activity. **(A)** ASM cells were transfected with $0.5 \mu\text{g}$ of p κ B.TK.LUC and $0.001 \mu\text{g}$ of pRLSV40. Transfected cells were treated with vehicle control (DMSO), MST312 (5×10^{-6} M), or BIBR1532 (1×10^{-6} M) for 30 min prior to induction with TNF- α (1 ng/ml) for 6 h (black bars) or no induction (white bars), followed by a Dual-Luciferase assay. **(B)** ASM cells were transfected with $0.5 \mu\text{g}$ of p κ B.TK.LUC, $0.001 \mu\text{g}$ of pRLSV40, and 0.5, 1.0, or 2.0 μg of pCMV5-TERT-wt (TERT) or 2.0 μg of pCDNAiii and then induced with TNF- α (1 ng/ml) for 6 h (black bars) or not (white bars), followed by a Dual-Luciferase assay. All data are mean $\pm$ SEM of six independent experiments. **p* = 0.01–0.05.

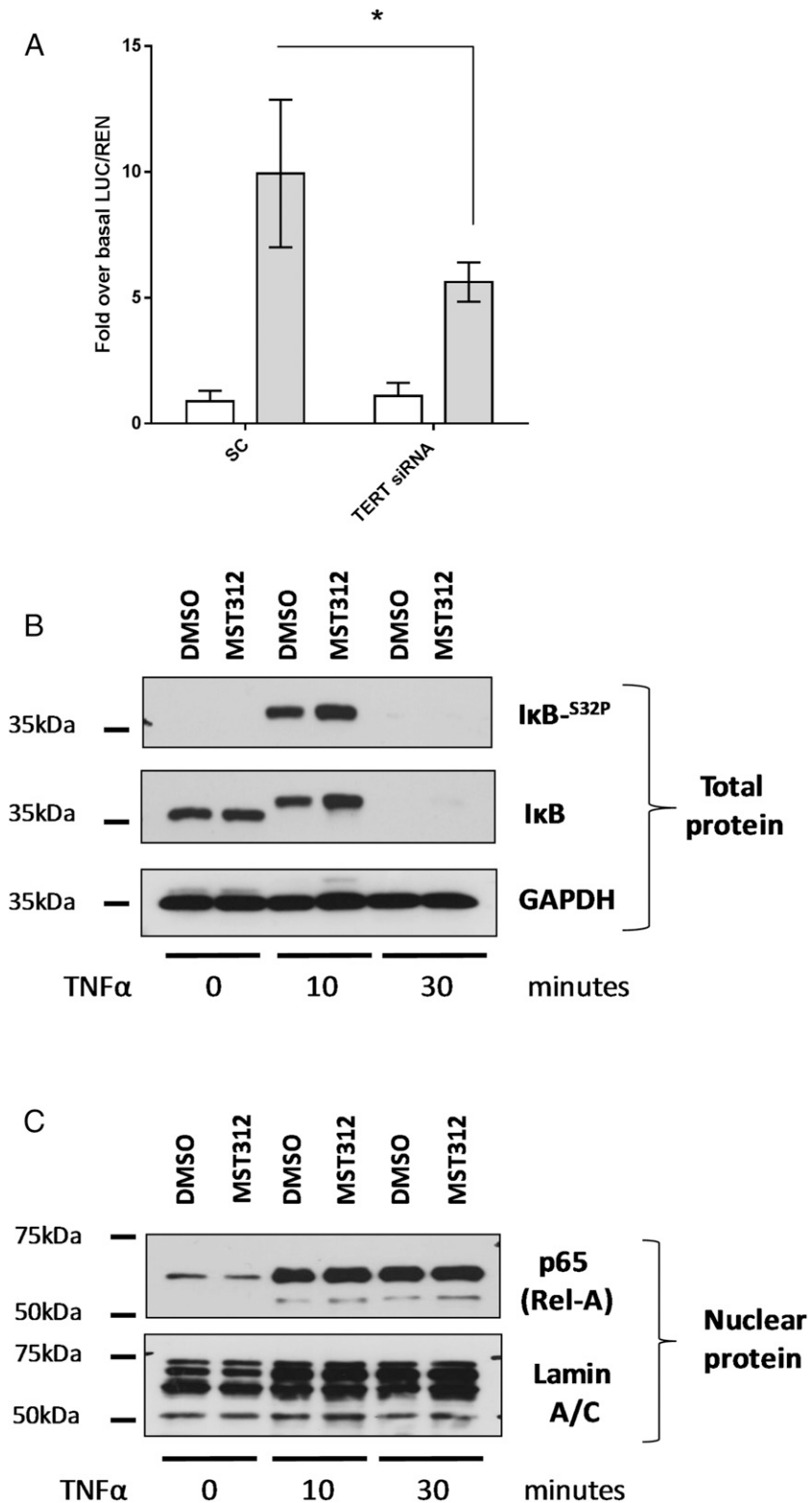
have NF- κ B response elements in their promoters. Quantitative PCR (QPCR) analysis of chemokine mRNA in ASM cells showed that MST312 suppressed mRNA accumulation in response to TNF- α (Table II). ELISA analysis of culture supernatants from cells treated with BIBR1532 showed a reduction in TNF- α -induced chemokine secretion (Table I). BIBR1532 was not as effective as MST312 in reducing chemokine secretion; this was mirrored by a smaller effect of BIBR1532 on TNF- α -induced

chemokine mRNA accumulation compared with MST312 (Table II).

TERT siRNA reduces TNF- α -induced chemokine mRNA accumulation

Because the two pharmacological telomerase inhibitors reduced chemokine mRNA expression with differing efficacy, we sought to confirm, using molecular approaches, that telomerase was required

FIGURE 3. TERT siRNA reduces NF- κ B reporter gene activity. The TERT inhibitor MST312 does not affect I κ B α phosphorylation or p65 (Rel-A) nuclear translocation in ASM cells. **(A)** ASM cells were transfected with scrambled control (SC) or TERT siRNA for 48 h prior to transfection with 0.5 μ g of p6 κ B.TK.LUC and 0.001 μ g of pRLSV40. Transfected cells were treated with TNF- α (1.0 ng/ml) for 6 h (gray bars) or were not treated (white bars), prior to a Dual-Luciferase assay. **(B)** ASM cells were treated with MST312 (5×10^{-6} M) or vehicle control (DMSO) for 60 min prior to induction with TNF- α (1.0 ng/ml) for up to 30 min. Total protein extracts were analyzed by Western blot for I κ B α phosphorylation (I κ B α -S32P) and total I κ B α with a control blot for GAPDH. **(C)** ASM cells were treated with MST312 (5×10^{-6} M) or vehicle control (DMSO) for 60 min prior to treatment with TNF- α (1.0 ng/ml) for up to 30 min. Nuclear extracts were blotted and probed for p65 (Rel-A) and then re probed for Lamin A/C. All transfection and luciferase measurements represent the mean $\pm$ SEM of eight independent experiments. Western blots are representative of three independent experiments. * $p = 0.01$ – 0.05 .



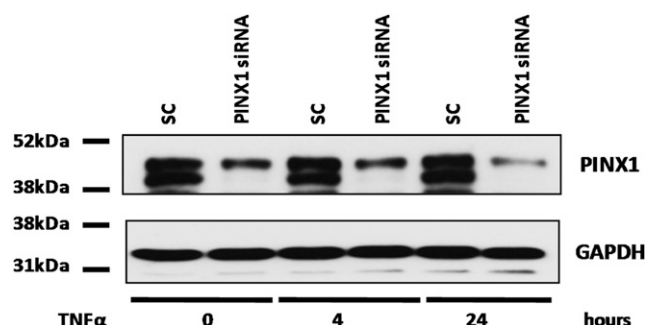


FIGURE 4. PINX1 siRNA reduces PINX1 expression in ASM cells. To assess PINX1 protein ablation with siRNA, ASM cells were transfected with a scrambled control siRNA (SC) or PINX1 siRNA (4 nM) for 48 h. Transfected cells were serum starved for 24 h and then induced with TNF- α (1.0 ng/ml) for 4 or 24 h. PINX1 protein was quantified by Western blot.

for TNF- α -induced chemokine gene expression. Four TERT siRNAs were tested for their ability to reduce TERT mRNA accumulation. We selected TERT siRNA-24 for further study, because it caused the greatest reduction in TERT mRNA accumulation (70% reduction) (Fig. 1). TERT siRNA reduced CCL5, CCL2, CXCL10, and CCL11 mRNA accumulation in response to TNF- α (Table III). TERT siRNA also reduced CCL5, CCL2, CXCL10, and CCL11 protein secretion from ASM cells (Table III). TERT siRNA does not cause further reductions in the level of chemokine protein secretion from ASM cells if observations are taken out from 24 to 48 or 72 h post-TNF- α addition (data not shown). We find that telomerase inhibition and TERT siRNA will cause a marked reduction in TNF- α -induced chemokine mRNA accumulation that is not always reflected in the reduction of chemokine protein secretion (Tables I–III). We have not directly investigated the disconnect between mRNA accumulation and protein secretion levels within this study, but chemokine mRNA (specifically CCL2, CCL5, and CXCL10) have documented translation-control mechanisms that could account for the difference between total mRNA and the quantity of chemokine protein secreted from ASM cells (22–25).

Telomerase inhibitors reduce TNF- α -induced (p6xkB.TK.LUC) NF- κ B reporter gene activity

With Gosh et al. (13) reporting a role for TERT in NF- κ B activity and our observations of reduced chemokine expression when

TERT expression was reduced or TERT activity was inhibited, we sought to determine whether TERT has a role in NF- κ B activity in ASM cells. Consistent with this hypothesis, we found that TNF- α -induced NF- κ B-LUC reporter activity was reduced by pretreatment with MST312 and BIBR1532 (Fig. 2A).

TERT overexpression increases NF- κ B-LUC activity

To confirm that TERT plays a role in NF- κ B activity in ASM cells, we cotransfected pCMV-TERT with the (p6xkB.TK.LUC) NF- κ B reporter. TNF- α -induced NF- κ B reporter activity was increased by coexpression with TERT (Fig. 2B).

TERT siRNA reduces TNF- α -induced NF- κ B-LUC activity, but the TERT inhibitor MST312 does not affect I κ B α phosphorylation or p65 (Rel-A) nuclear translocation

Because TERT inhibitors reduced TNF- α -induced chemokine gene expression, we performed experiments to determine whether TERT plays a role in NF- κ B activation. TERT siRNA significantly reduced NF- κ B-LUC activity (Fig. 3A). To determine whether TERT's role in TNF- α signal transduction was due to changes in I κ B α phosphorylation/degradation and subsequent p65 (Rel-A) nuclear translocation changing transcription factor activity, we treated ASM cells with MST312 and then induced them with TNF- α for 30 min. Western blot analysis of total protein extracts showed that I κ B α phosphorylation and subsequent I κ B α degradation were not affected by TERT inhibition (Fig. 3B). After the same treatment of ASM cells, nuclear extracts were Western blotted for p65 (Rel-A) nuclear accumulation. MST312 had no effect on the nuclear translocation of p65 (Rel-A) (Fig. 3C).

PINX1 siRNA, but not TERF1 siRNA, reduces TNF- α -induced chemokine mRNA accumulation and protein secretion

Previous studies have established the role of TERT in NF- κ B activity and chemokine gene expression in cancer cells; however, the shelterin complex and PINX1 have not been examined in this context. We have used a brief siRNA screen for a central component of shelterin, TERF1, and the TERT inhibitor PINX1. siRNA collections were tested for mRNA knockdown of PINX1 and TERF1, with two siRNAs selected for each target. Ninety percent knockdown of PINX1 protein was achieved (Fig. 4). Between 60 and 80% TERF1 mRNA knockdown was achieved for TERF1; however, despite knockdown of TERF expression, there was no effect on TNF- α -induced chemokine mRNA accumulation in ASM cells (data not shown). PINX1 siRNA decreased

Table IV. PINX1 siRNA reduces TNF- α -induced chemokine mRNA accumulation and chemokine protein secretion in ASM cells

Chemokine (mRNA or Protein)	siRNA	Inhibition of Maximal (%)	$\pm$ SEM of Inhibition	Statistical Significance
CCL2 mRNA	PINX1	89	0.82	***
CCL2 protein	PINX1	17	1.7	*
CCL5 mRNA	PINX1	42	2.1	**
CCL5 protein	PINX1	25	0.3	*
CCL11 mRNA	PINX1	46	0.23	**
CCL11 protein	PINX1	51	3.5	***
CXCL10 mRNA	PINX1	40	8	*
CXCL10 protein	PINX1	49	1.35	**

ASM cells were transfected with PINX1 siRNA or scrambled control siRNA for 48 h prior to the addition of TNF- α (1 ng/ml) for 24 h. QPCR for CCL5, CCL2, CXCL10, and CCL11 mRNA was carried out on first-strand cDNA synthesized from total RNA, followed by a calculation of the percentage inhibition by PINX1 siRNA of maximal chemokine mRNA accumulation, compared with maximal chemokine mRNA accumulation in ASM cells transfected with scrambled control siRNA. ASM cells were transfected with PINX1 siRNA or scrambled control siRNA for 48 h prior to the addition of TNF- α (1 ng/ml) for 24 h followed by ELISA for CCL5, CCL2, CXCL10, and CCL11 on culture supernatants (normalized to cell counts) and a calculation of the percentage inhibition of maximal chemokine secretion by PINX1 siRNA compared with scrambled control siRNA. Assay data were analyzed with a two-tailed paired *t* test. All measurements represent the mean $\pm$ SEM of three independent experiments.

p* = 0.01–0.05, *p* = 0.001–0.01, ****p* < 0.001.

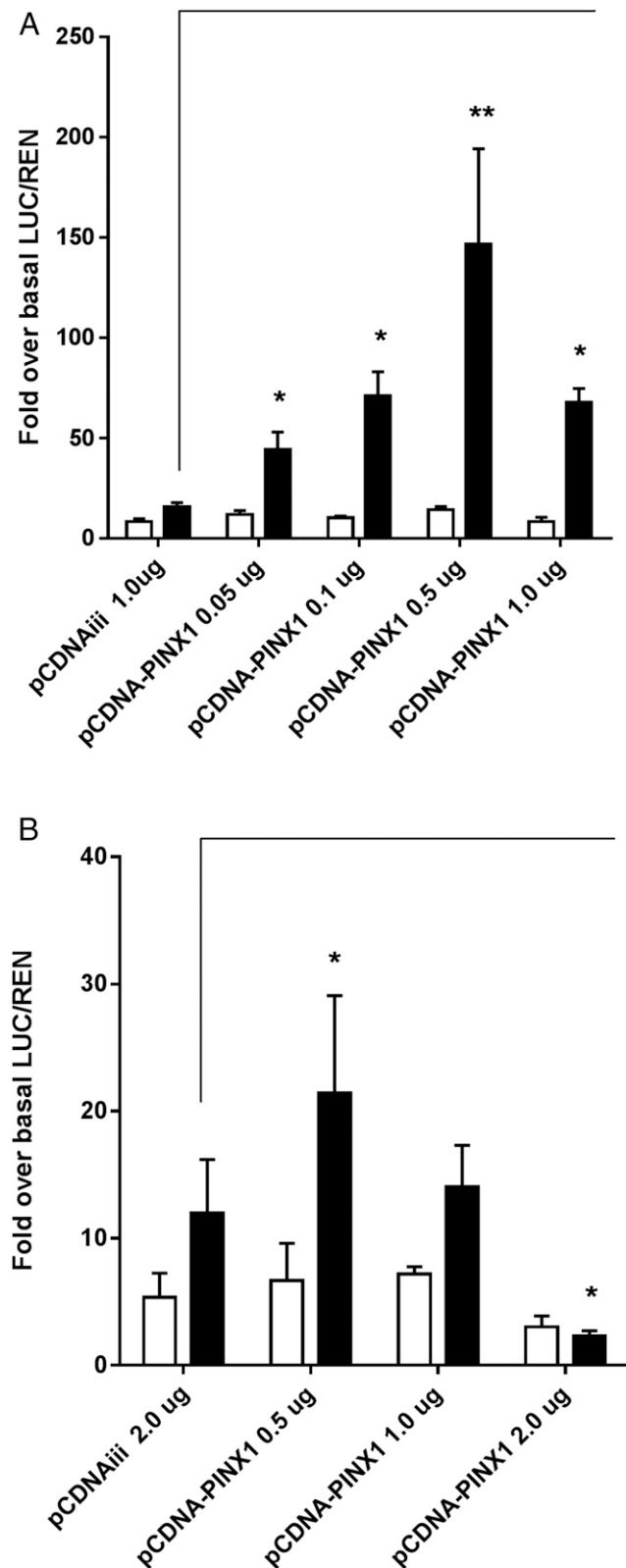


FIGURE 5. PINX1 overexpression increases NF- κ B-LUC reporter gene activity at low levels of transfected DNA but suppresses NF- κ B-LUC reporter gene activity at high levels of transfected DNA. **(A)** ASM cells were transfected with pCDNA-PINX1 (0.05–1.0 μ g) or pCDNAiIII and p6 κ B.TK.LUC with FuGENE 6 for 24 h. Transfected cells were treated with TNF- α (1.0 ng/ml) for 6 h (black bar) or were not treated (white bars), followed by a Dual-Luciferase assay. **(B)** ASM cells were transfected with pCDNA-PINX1 (0.5–2.0 μ g) or control pCDNAiIII and p6 κ B.TK.LUC with pRLSV40 using FuGENE HD for 72 h. (Figure legend continues)

TNF- α -induced mRNA accumulation of CCL5, CCL2, CXCL10, and CCL11 (Table IV). PINX1 siRNA also decreased chemokine secretion from ASM cells (Table IV).

PINX1 overexpression causes biphasic changes in NF- κ B reporter activity

Using siRNA, we have established that PINX1 plays a role in TNF- α -induced chemokine expression in ASM cells. Previous studies have shown that the chemokine genes require NF- κ B activity at their promoters to induce mRNA synthesis and that the PINX1-interacting protein TERT is involved in regulating NF- κ B activity. Cotransfection of 0.5 μ g of PINX1 expression construct with the NF- κ B-LUC reporter increased TNF- α -induced reporter activity to 100-fold over a reporter-only transfection (Fig. 5A). However, 1.0 or 2.0 μ g of PINX1 expression construct reduced or abolished NF- κ B activity, respectively (Fig. 5B). Fig. 5A contains data from transfections with FuGENE 6 with cells plated at 2×10^4 cells per milliliter (giving 20% confluence at the time of the transfection); under these conditions, DNA is only transfected for 18 h. Therefore, data in Fig. 5A are from a reporter gene system with a short period of transfection in a very small number of cells. We find that reporter gene systems of this type do not express much protein (very difficult to monitor by Western blot), but the signal/noise ratio for reporter gene induction is excellent, and the variability is low (see SEM bars in Fig. 5A). We have gone further with chromatin immunoprecipitation experiments to quantify active transcription factor binding to native promoter chromatin and found a very good agreement for the timing and relative fold over basal levels for activity for native transcription factors and versus transcription factor-specific reporter genes. Fig. 5B contains data from a transfection with FuGENE HD that was conducted with the same amounts of DNA as the FuGENE 6 experiment (Fig. 5A) but with many more cells and over a 72-h period rather than an 18-h period. The longer period of transfection, combined with more cells and FuGENE HD (less toxic to ASM cells than FuGENE 6), leads to higher relative levels of DNA transfection and protein expression. Higher levels of DNA lead to very high reporter signals (mean of 20,000 light units for an uninduced reporter) but greater error (high SEM) and a very poor signal/noise ratio. We have used the latter technique to allow the level of PINX1 expression to build up in ASM cells to determine whether high levels of expression would lead to suppression of NF- κ B activity. They do, but there is a comparative loss in the signal/noise ratio (>100 -fold [FuGENE 6] versus 15-fold [FuGENE HD] for 0.5 μ g of reporter gene DNA).

With a known role for PINX1 in TERT inhibition and TERT established as an NF- κ B activator, high-level PINX1 expression and consequent reduced NF- κ B activity are easily explicable; however, lower levels of PINX1 produced very large increases in relative NF- κ B activity, a novel and unexplained observation.

PINX1 siRNA decrease TNF- α -induced NF- κ B reporter activity without affecting I κ B α phosphorylation, I κ B α degradation, or p65 (Rel-A) nuclear translocation

To confirm that native PINX1 is functionally coupled to NF- κ B activity, we have shown that PINX1 siRNA reduced TNF- α -induced NF- κ B reporter activity (Fig. 6A); however, PINX1 knockdown

Transfected cells were treated with TNF- α for 6 h (black bars) or were not treated (white bars), followed by a Dual-Luciferase assay. All data represent the mean $\pm$ SEM of eight independent experiments. * p = 0.01–0.05, ** p = 0.001–0.01.

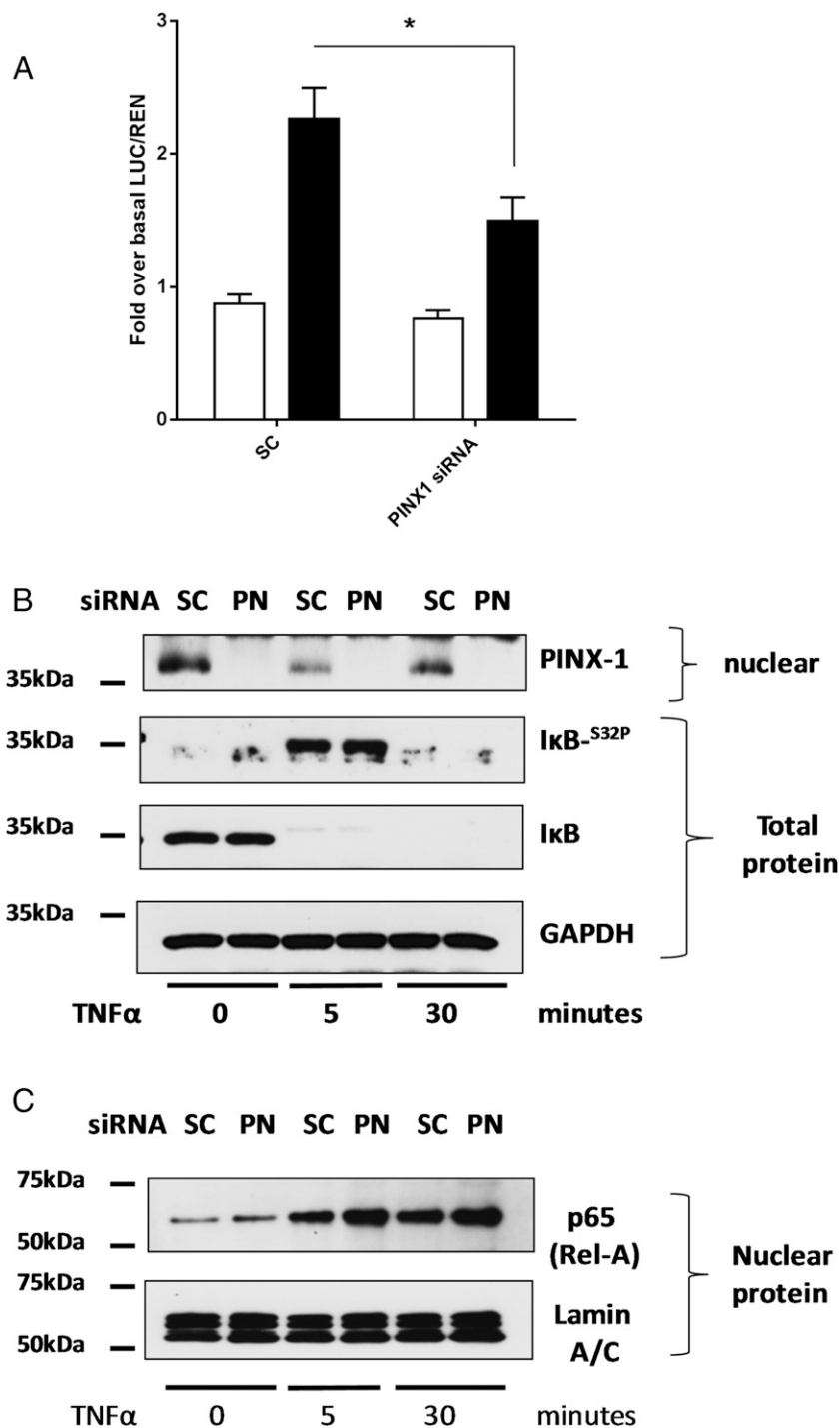


FIGURE 6. PINX1 siRNA reduces NF-κB reporter gene activity. PINX1 siRNA does not affect IκB phosphorylation or p65 (Rel-A) nuclear translocation in ASM cells. **(A)** ASM cells were transfected with scrambled control siRNA (SC) or PINX1 siRNA for 48 h, transfected with 0.5 μg of p6κB.TK.LUC and 0.001 μg of pRLSV40, and treated with TNF-α (1 ng/ml) for 6 h (black bars) or were not treated (white bars), followed by a Dual-Luciferase assay. **(B)** ASM cells were transfected with SC or PINX1 siRNA (PN) for 48 h prior to treatment with TNF-α (1.0 ng/ml) for up to 30 min. Total protein extracts were analyzed by Western blot for PINX1 and IκBα phosphorylation (IκBα-S^{32P}), reprobed for total IκBα, and then reprobed for GAPDH. **(C)** ASM cells were transfected with SC or PN for 48 h prior to treatment with TNF-α (1.0 ng/ml) for up to 30 min. Nuclear extracts were blotted and probed for p65 (Rel-A) and then reprobed for Lamin A/C. All luciferase assay measurements represent the mean ± SEM of eight independent experiments. Western blots are representative of three independent experiments. **p* = 0.01–0.05.

does not affect IκBα phosphorylation or degradation (Fig. 6B). PINX1 knockdown marginally increases the nuclear translocation of p65 (Rel-A) (<1-fold) (Fig. 6C), removal of PINX1 decreases NF-κB reporter gene activity and NF-κB-dependent chemokine gene expression, and the slight increase in p65 nuclear translocation induced by PINX1 removal does not appear to be sufficient to counteract the overall suppression of NF-κB activity by PINX1 ablation. Therefore, PINX1 does not affect signal transduction between TNF-α and the subsequent translocation of p65 (Rel-A) to the nucleus.

Native PINX1 and p65 (Rel-A) associate in whole cells and in vitro

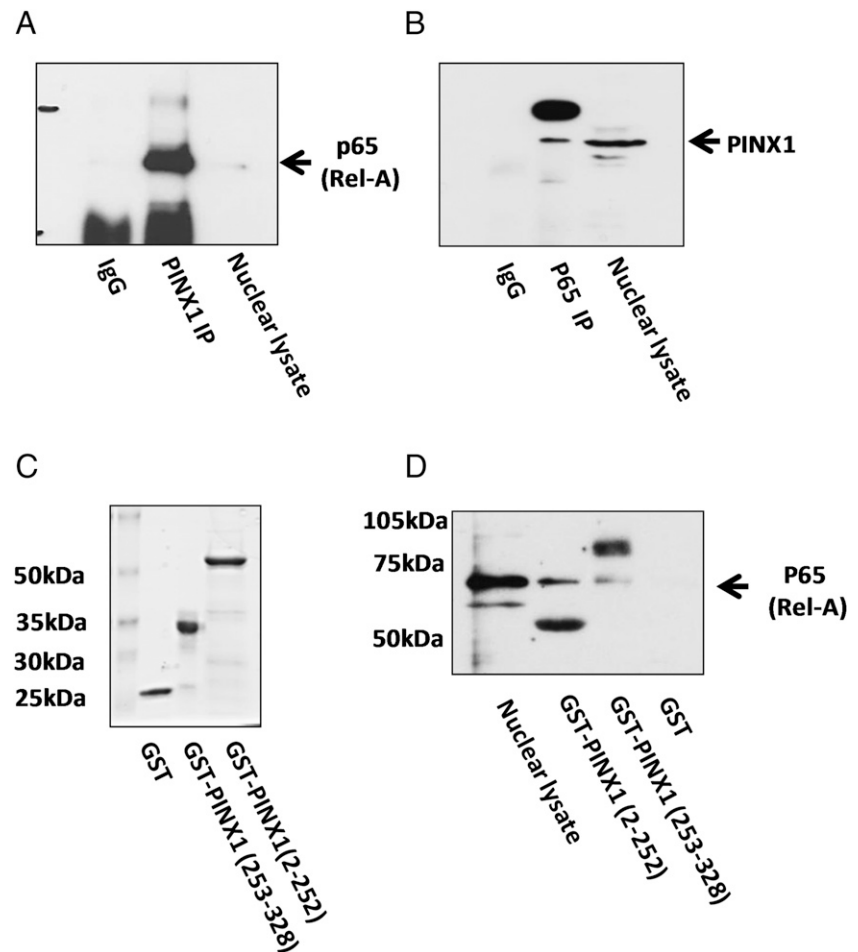
Immunoprecipitation of PINX1, followed by Western blot for p65 (Rel-A), showed that the two proteins associated with each other

(Fig. 7A). To confirm the protein–protein association, we immunoprecipitated p65 (Rel-A) and showed that PINX1 was recovered (Fig. 7B). PINX1 and p65 (Rel-A) associations were confirmed by GST pull-down assays, using equivalent quantities of GST, GST-PINX1 (2–252), or GST-PINX1 (253–328) (Fig. 7C), with the GST-PINX1 N-terminal amino acids (2–252) showing greater affinity for p65 (Rel-A) than the GST-PINX1 C-terminal TERT inhibitory domain aa (253–328) in ASM cell lysates (Fig. 7D).

The PINX1 N terminus and C terminus have a different ability to activate and interact with NF-κB

Expression of a FLAG-tagged N-terminal construct (2–252) of PINX1 amplified the NF-κB–LUC reporter gene response to TNF-α, whereas the FLAG-tagged C-terminal TERT inhibitor domain

FIGURE 7. Native p65 Rel-A coimmunoprecipitates with PINX1. GST-FLAG-PINX1 (2–252) (N-terminal) has a greater binding capacity for p65 (Rel-A) than GST-FLAG-PINX1 (253–328) (C-terminal). **(A)** ASM cells were treated with TNF- α (1 ng/ml for 30 min), followed by PINX1 immunoprecipitation (IP) with a parallel IgG control from total cell lysates. IPs and nuclear lysates were analyzed by Western blot for p65 (Rel-A). **(B)** ASM cells were treated with TNF- α (1 ng/ml for 30 min), followed by p65 (Rel-A) IP, with a parallel IgG IP control from total cell lysates. IPs and nuclear lysates were analyzed by Western blot for PINX1. **(C)** Ten micrograms of GST, GST-PINX1 (253–328), or GST-PINX1 (2–252) was loaded onto glutathione beads and analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. **(D)** ASM cells were treated with TNF- α (1 ng/ml for 30 min), followed by GST pull-down from total cell lysates with 10 μ g of GST, GST-PINX1 (253–328), or GST-PINX1 (2–252). GST pull-downs were analyzed by Western blot for p65 (Rel-A), with an ASM cell nuclear cell lysate positive control. Each panel is representative of three individual experiments.



(253–328) did not affect NF- κ B activity (Fig. 8A). An anti-FLAG Western blot of transfected ASM cells confirmed that both pCDNA-FLAG-PINX1 constructs express their relevant sections of PINX1 (data not shown). Anti-FLAG immunoprecipitation of pCDNAii-FLAG-PINX1 (2–252) and pCDNAii-FLAG-PINX1 (253–328) transfected to ASM cells were probed with anti-p65 (Rel-A) anti-sera, and the resulting Western blot demonstrated that the N terminus of PINX1 preferentially binds p65 (Fig. 8B). Repeat transfections and anti-FLAG immunoprecipitation showed that TERT bound equally to the PINX1 N terminus (2–252) and C terminus (253–328) (Fig. 8C).

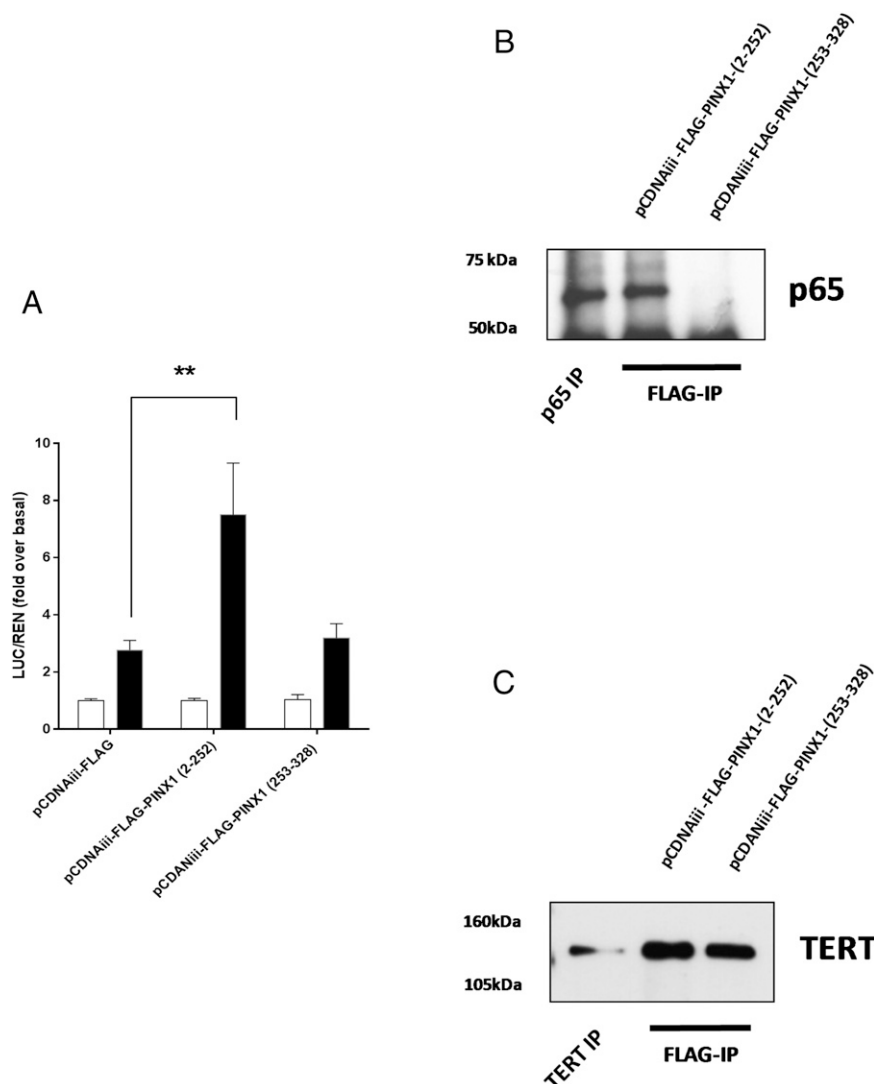
Discussion

There are a number of novel findings in this study that are of general biological relevance. First, we found that telomerase was essential for TNF- α -induced chemokine expression. Second, overexpression of the TERT subunit of the telomerase holoenzyme and siRNA knockdown of TERT changed NF- κ B activity in response to TNF- α . Third, PINX1 knockdown with siRNA decreased NF- κ B activity and chemokine mRNA accumulation and secretion in response to TNF- α . Fourth, overexpression of PINX1 inhibited NF- κ B activity at very high levels of expression; however, at low levels, it produces a very large increase in NF- κ B activity. Finally, we showed that PINX1 associated with p65 to activate NF- κ B via the N terminus (2–252 aa) of PINX1. This novel series of observations establishes a central role for TERT and PINX1 in ASM cells, which have well-established roles in airway inflammation and asthma biology. We have identified TERT and PINX1 as key components of the ASM cell response to TNF- α and conclude that telomerase and PINX1 will have as yet unexplored roles in asthma pathology.

Lung mesenchymal cells produce a large range of chemokines in response to inflammatory stimuli. In this study we show that normal human somatic cells with a high capacity for cytokine output require TERT for chemokine expression and subsequent secretion. A few studies have indirectly probed the role of telomerase or TERT in lung diseases. In chronic obstructive pulmonary disease, absolute telomere length was reduced (26), but the study did not address the activity of telomerase in lung tissue or the role of TERT in inflammation. Airway fibroblasts from patients with interstitial lung disease and idiopathic pulmonary fibrosis have altered telomerase activity (27, 28), but this study did not assess the effect of TERT inhibition on idiopathic pulmonary fibrosis physiology. A study of circulating leukocytes from asthma patients demonstrated that telomere length was reduced, as well as that airway smooth muscle cells had TERT expression, but the level of telomerase activity and its consequences for inflammation in the asthmatic airway were not investigated (29). In a separate study of memory T cells derived from asthma patients and control subjects, there was increased telomerase activity in response to house dust mite allergen, with a far greater increase in activity in the asthma patient group (30). Despite these studies indicating that telomerase activity may change in inflammatory lung diseases, there has not been any assessment of the role of TERT activity in structural airway cells. In this study we demonstrate that telomerase inhibition reduced inflammatory chemokine expression in a key cellular player in lung inflammation.

Prior to this study, a positive role for PINX1 in inducing inflammation has not been explored. Li et al. (31) have demonstrated that PINX1 siRNA induces MMP-2 expression in clear cell renal cell carcinoma and that PINX1 siRNA increases p65/Rel-A

FIGURE 8. Cotransfection of pCDNAiii-FLAG-PINX1 (2–252) increased the p65κB.TK.LUC (NF-κB reporter) response to TNF-α, whereas cotransfection of pCDNAiii-FLAG-PINX1 (252–328) did not. FLAG-PINX1 (2–252) had a greater capacity to bind native p65 (Rel-A) than FLAG-PINX1 (252–328) in ASM cells, and TERT bound PINX1 N terminus and PINX1 C terminus. **(A)** ASM cells were cotransfected with the p65κB.TK.LUC reporter with either pCDNAiii-FLAG-PINX1 (2–252), pCDNAiii-FLAG-PINX1 (252–328), or pCDNAiii-FLAG control. Transfected cells were treated with TNF-α (1 ng/ml) for 6 h (black bars) or were not treated (white bars), followed by a Dual-Luciferase assay. **(B)** ASM cells were transfected with pCDNAiii-FLAG-PINX1 (2–252) or pCDNAiii-FLAG-PINX1 (252–328) and treated with TNF-α (1.0 ng/ml) for 30 min. Anti-FLAG immunoprecipitation (IP) and a control p65 (Rel-A) IP were Western blotted and probed with a p65 (Rel-A) Ab. **(C)** ASM cells were transfected with pCDNAiii-FLAG-PINX1 (2–252) or pCDNAiii-FLAG-PINX1 (252–328) and treated with TNF-α (1.0 ng/ml) for 30 min. Anti-FLAG IP and a control TERT IP were Western blotted and probed with a TERT Ab. All luciferase assay measurements represent the mean ± SEM of eight independent experiments. Each Western blot figure is representative of three individual experiments. ***p* = 0.001–0.01.



expression and p65 entry into the nucleus. However, they did not monitor NF-κB activity or the role of PINX1 in cytokine-induced inflammatory responses in the carcinoma tissue/cells studied, because all of the studies were carried out in an uninduced “ground state” (31). PINX1 has previously been characterized as a negative regulator or inhibitor of TERT activity (8). We expected PINX1 depletion by siRNA to increase chemokine expression (by virtue of PINX1 loss deregulating suppression of TERT activity) or not affect TNF-α-induced chemokine expression; however, loss of PINX1 produced a statistically significant decrease in ASM cells’ ability to synthesize chemokine mRNA and secrete chemokine proteins.

Because PINX1 depletion affected chemokine gene expression, we assessed the role of PINX1 in NF-κB activity. At low levels of DNA cotransfection, PINX1 produced marked increases in NF-κB reporter gene activity (some 100-fold over the NF-κB reporter alone). At higher levels of PINX1 cotransfection and with a greater period of time to express the PINX1 protein, PINX1 coexpression led to eventual suppression of NF-κB activity. We hypothesize that PINX1 is capable of binding TERT and NF-κB and that the introduction of small quantities of PINX1 protein helps to form TERT/PINX1/NF-κB complexes that increase the transcription of genes with NF-κB binding sites at their promoters. If the interaction is optimal at a 1:1:1 ratio, then increasing PINX1 protein levels will eventually prevent TERT and NF-κB from

interacting (if the amount of TERT and NF-κB is constant) by titrating them apart, leading to an inhibition of NF-κB activity and, thus, explaining our observations in Fig. 5. The biphasic relationship between PINX1 and NF-κB is reflective of the relationship first characterized between JNK and JNK inhibitory protein 1 (JIP1). High levels of JIP1 were effective inhibitors of JNK activity (32), but further studies established that the inhibitory effect was due to disruption of a signaling complex normally required for JNK activation (33). At “normal” somatic cell levels of expression, JIP1 binds MLK3, MKK7 (the MLK3 substrate), and JNK (the MKK7 substrate), creating a signal-transduction pathway on a protein scaffold. PINX1 is known to organize the binding of TERT to chromatin associated with telomeres during cell division. PINX1 also functions to bind and stabilize TERT at centrosomes during cell division. To test the hypothesis that PINX1 is required to bind TERT and maintain the interaction with p65 (Rel-A), we tested the interaction between PINX1 and p65 (Rel-A). We found that native PINX1 bound to p65 (Rel-A) in vitro from ASM cell extracts and in whole cells. Initial investigation of the role of PINX1 in TERT inhibition established that TERT binds to the C-terminal TERT-inhibitory domain (aa 253–328) and the remaining N terminus of PINX1. Scaffold proteins have separable interactions with components of the same signal-transduction pathway. We demonstrated that TERT is required for NF-κB activity and bound to the N-terminus and C terminus of

PINX1 (Fig. 8C). In contrast, p65 (Rel-A) preferentially bound the N terminus of PINX1. Further overexpression of the N terminus of PINX1 amplified TNF- α -induced NF- κ B activity, whereas that of the C terminus did not. PINX1 bound TERT and p65 (Rel-A), but only the N terminus was functionally coupled to TNF- α -induced NF- κ B activity. Therefore, PINX1 has the characteristics of a scaffold for TERT and NF- κ B required for chemokine gene expression.

We have consistently found that GST-PINX1 (253–328)-loaded beads will pull down native p65 (Rel-A) from ASM cell lysates (although to a lesser extent than the equivalent amount of protein for GST-PINX1 [2–252]-loaded beads) when immunoprecipitation of a FLAG-tagged PINX1 (253–328) does not coimmunoprecipitate p65. There are two possible reasons why this is the case. First, the relative ratio of GST-fusion protein to “in-cell” expressed FLAG-tagged PINX1 is very high (more GST fusion protein than FLAG-tagged protein per immunoprecipitation per lane of a gel for p65 Western blot), leading to a more sensitive detection of a slight (potentially nonspecific) interaction between GST-PINX1 (253–328) and p65 (Rel-A). Second, FLAG-tagged PINX1 proteins are expressed “in cell”; therefore, despite their epitope tag, they will have a closer resemblance to native PINX1 than that expressed in GST fusion in a prokaryotic system. For this reason, an association between FLAG-tagged PINX1 (2–252) and p65 (Rel-A), and not FLAG-tagged PINX1 (253–328), would more closely reflect the native interaction between PINX1 and p65 (Rel-A). To confirm the second observation, we looked at the ability of FLAG-tagged PINX1 (2–252) and FLAG-tagged PINX1 (253–328) to influence the activity of NF- κ B when NF- κ B activity was induced by TNF- α . With only FLAG-tagged PINX1 (2–252) capable of amplifying TNF- α -induced NF- κ B activity, there is a stronger argument for a specific interaction between PINX1 (2–252) and p65 (Rel-A) than between PINX1 (253–328) and p65 (Rel-A).

With TERT and PINX1 overexpression capable of driving increased NF- κ B activity in response to TNF- α and siRNA ablation of TERT and PINX1 reducing chemokine mRNA, we have come to the initial conclusion that both TERT and PINX1 are required for NF- κ B activity at the promoters of the chemokines in this study. TERT siRNA causes a far greater reduction in chemokine mRNA accumulation than siRNA directed against PINX1 (Tables III, IV). This is also reflected in the greater reduction of TNF- α -induced NF- κ B activity with TERT siRNA (55% reduction) than with PINX1 siRNA (42% reduction). The reasons for the differences between the efficacy of TERT and PINX1 siRNA are not clear from the current experimental evidence; however, the very low level of TERT expression compared with PINX1 in ASM cells (TERT can only be detected with 100 μ g of nuclear extract in a single Western blot lane, whereas PINX1 can be detected in 10 μ g of total ASM cellular protein extract) may mean that there is no pool or reserve of TERT protein post-siRNA ablation, leading to a more effective loss of TERT function after siRNA treatment. PINX1 siRNA may not remove sufficient PINX1 protein to have an effect comparable to that of TERT siRNA.

To summarize, we have established that telomerase is required in ASM cells for TNF- α -induced NF- κ B activity and chemokine gene expression. We also established that PINX1, originally identified as a telomerase inhibitor, is required for TNF- α -induced NF- κ B activity and chemokine gene expression and that the level of PINX1 protein expression is critical for NF- κ B activity. Furthermore, we demonstrated that TERT and PINX1 interact with p65/Rel-A and that the position of those interactions is separable for p65/Rel-A, leading to the conclusion that PINX1 functions as a scaffold protein for TERT and NF- κ B in ASM cells. With limited recent success in identifying effective novel asthma therapies,

novel targets are required. Telomerase has not been studied in the context of asthma pathology, but there has been drug development centered on TERT activity in cancer pathology. To this end, TERT is a novel and “druggable” target for future asthma therapeutics. Prior to this study, PINX1 has never been considered as a target for inflammation or, specifically, asthma therapy. Our work suggests that it is a potential novel future target for anti-inflammatory asthma therapy development.

Disclosures

The authors have no financial conflicts of interest.

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