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PTTG/securin activates expression of *p53* and modulates its function

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Abstract

Background: Pituitary tumor transforming gene (*PTTG*) is a novel oncogene that is expressed abundantly in most tumors. Overexpression of *PTTG* induces cellular transformation and promotes tumor formation in nude mice. *PTTG* has been implicated in various cellular processes including sister chromatid separation during cell division as well as induction of apoptosis through *p53*-dependent and *p53*-independent mechanisms. The relationship between *PTTG* and *p53* remains unclear, however.

Results: Here we report the effects of overexpression of *PTTG* on the expression and function of *p53*. Our results indicate that overexpression of *PTTG* regulates the expression of the *p53* gene at both the transcriptional and translational levels and that this ability of *PTTG* to activate the expression of *p53* gene is dependent upon the *p53* status of the cell. Deletion analysis of the *p53* gene promoter revealed that only a small region of the *p53* gene promoter is required for its activation by *PTTG* and further indicated that the activation of *p53* gene by *PTTG* is an indirect effect that is mediated through the regulation of the expression of *c-myc*, which then interacts with the *p53* gene promoter. Our results also indicate that overexpression of *PTTG* stimulates expression of the Bax gene, one of the known downstream targets of *p53*, and induces apoptosis in a human embryonic kidney cell line (HEK293). This stimulation of *bax* expression by *PTTG* is indirect and is mediated through modulation of *p53* gene expression.

Conclusions: Overexpression of *PTTG* activates the expression of *p53* and modulates its function, with this action of *PTTG* being mediated through the regulation of *c-myc* expression. *PTTG* also up-regulates the activity of the *bax* promoter and increases the expression of *bax* through modulation of *p53* expression.

Background

The pituitary tumor transforming gene (*PTTG*), a securin, was cloned initially from a rat pituitary tumor [1]. Subsequently, we and others cloned the human *PTTG* gene [2,3] and characterized its function. *PTTG* protein is expressed at higher than normal levels in several tumors, including those of the pituitary [4], thyroid [5], colon [6], ovary [7], testis [7] and breast [8], as well as in hematopoietic neoplasms [9]. In some tumors, including those of the thyroid [10], pituitary [11], esophagus [12] and colorectum [6], high levels of expression of *PTTG* correlate with tumor invasiveness and, more recently, *PTTG* has been identified as a key "signature gene", with high expression predicting metastasis in multiple tumor types [13]. Overexpression of *PTTG* enhances cell proliferation, induces cellular transformation, and promotes tumor formation in nude

mice [2,3]. The involvement of *PTTG* in several cellular functions, such as mitosis [14,15], cell cycle progression [16], DNA repair [17] and secretion and expression of basic fibroblast growth factor (bFGF) [18] and vascular endothelial growth factor (VEGF) [19] has been reported.

Structural homology suggested that PTTG may be a mammalian securin and this has been confirmed by its involvement in regulating sister chromatid separation during mitosis [16]. The involvement of *PTTG* in cell signaling via the MAP kinase cascade [20] and its interaction with the *c-myc* promoter suggest its involvement in cellular transformation [21].

There is now considerable evidence supporting the concept that *PTTG* can regulate apoptosis in both a *p53*-dependent and a *p53*-independent manner [22]. In their studies, Yu *et al*. [22] have shown that overexpression of *PTTG* results in the induction of apoptosis of cells that are *p53* deficient (osteosarcoma MG-63 cells), as well as cells that express functional *p53* (breast tumor MCF-7 cells), with overexpression of both *PTTG* and *p53* resulting in an increase in apoptosis of MCF-7 cells but not MG-63 cells. However, overexpression of E6, which targets *p53* for degradation, eliminated *p53* in MCF-7 cells but did not inhibit apoptosis on overexpression of *PTTG*. Analysis of the molecular basis for the interaction between *PTTG* and *p53* by these investigators [22] showed that overexpression of *PTTG* in MCF-7 cells resulted in an increase in translocation of *p53* protein to the nucleus. Subsequently, Bernal *et al*. [23] using phage-display screening identified an interaction of the *p53* and *PTTG* proteins *in vitro* and *in vivo*. These investigators found that this interaction of *p53* with *PTTG* inhibited the transcriptional activity of *p53* and its ability to induce cell death in MCF-7 cells. These investigators did not find activation of *p53* expression on overexpression of *PTTG* in the lung tumor cell line, H1299. In these cells, overexpression of both *PTTG* and *p53* resulted in down regulation of *p53*-induced apoptosis together with down regulation of the *p53*-induced expression of downstream signaling genes, including *Bax*, SFN, and CDKN1A suggesting that, in these cells, *PTTG* inhibits the function of *p53* rather than its expression.

Our previous analysis of the *PTTG* promoter revealed tumor-specific activation of the promoter in various cell lines and indicated that Sp1 and NF-Y binding sites within the *PTTG* promoter are involved in its activation [24]. Zhou *et al*. [25] recently confirmed that Sp1 and NF-Y are involved in the expression of *PTTG* and that these binding sites play an important role in the regulation of the expression of *PTTG* by *p53*. We therefore undertook an analysis of the mechanisms by which *PTTG* regulates *p53* expression and function. Here, we report that *PTTG* regulates *p53* expression and function by modulating the activity of the *p53* promoter indirectly through regulation of the expression of *c-myc*, which binds directly to the *p53* promoter sequence.

Results

PTTG *upregulates expression of* p53 *in HEK293 and MCF-7 cells*

To determine the relationship between *PTTG* and *p53*, we first determined the effect of overexpression of *PTTG* on *p53* expression. For this purpose, we used three different cell lines. Two of these HEK293, which is a human embryonic kidney cell line, and MCF-7, which is a human breast cancer cell line, express wild-type *p53*, and one, PC3, which is a human prostate cancer cell line expresses a mutant form of *p53*. As shown in Figure 1A, western blot analysis of the lysates prepared from HEK293 and MCF-7 cells that had been transiently transfected with pcDNA-*PTTG* revealed a significant increase in expression of both *PTTG* and *p53* proteins compared to cells transfected with vector only. Consistent with the data reported by Rokhlin *et al*. [26], we did not detect *p53* protein on Western blotting of lysates prepared from PC-3 cells that had been transfected with pcDNA3.1-*PTTG* or pcDNA3.1 vector only. To confirm that overexpression of *PTTG* activates expression of *p53*, we performed double immunohistochemical analysis of HEK293 cells transfected with pcDNA3.1-*PTTG*. As shown in Figure 1B, the cells that exhibited staining of the *PTTG* protein and showed high levels of expression of *PTTG* also exhibited intense staining of the *p53* protein. Staining of *PTTG* and *p53* was either absent or very weak in HEK293 cells that had been transfected with pcDNA3.1 vector alone. These results suggested that *PTTG* upregulates the expression of *p53*.

The up-regulation of expression of *p53* by *PTTG* could be attributed to an alteration in the stability of the mRNA, an alteration in the efficiency of translation, or altered gene transcription. To determine whether the change in expression of *p53* is due to an alteration in the transcription of the *p53* gene, we analyzed the *p53* gene promoter activity and its regulation by *PTTG*. As shown in Fig. 2, transient transfection of cells with pcDNA3.1-*PTTG* resulted in a 3-fold higher level of *p53* promoter activity in MCF7 cells, a 2.3-fold higher level in PC3 cells, and a 1.6-fold higher level in HEK293 cells as compared to the levels of promoter activity in the same cell lines on transfection with pCDNA3.1vector only. Taken together, these results clearly demonstrate that up-regulation of *p53* expression is, at least in part, due to up-regulation of transcription of the *p53* gene by the *PTTG* protein.

Up-regulation of p53 *promoter activity by* PTTG *is mediated by regulation of expression of the* c-myc *gene*

To map the region of the *p53* promoter sequence that is required for activation by *PTTG*, we performed 5' and 3'-

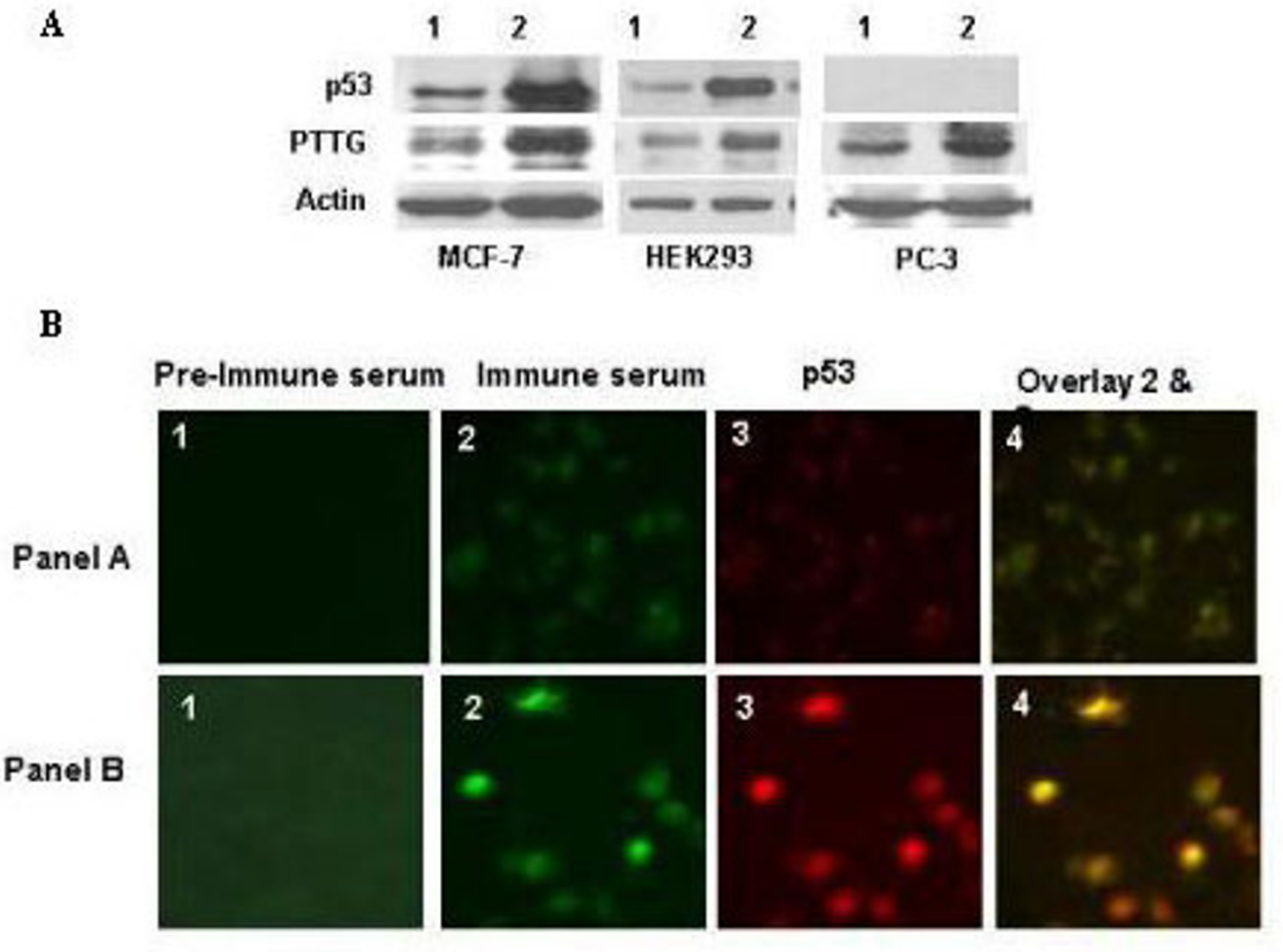

Figure 1
Expression of *PTTG* and *p53* proteins in MCF7, HEK293 and PC3 cells transfected with either pcDNA 3.1 (Lane 1) or pcDNA3.1-*PTTG* cDNA (lane 2). **A**: Expression of *PTTG* and *p53* proteins was analyzed by western blot analysis using a *PTTG*- or *p53*-specific antibody as described in Methods. A β-Actin antibody was used as a control to determine the variation in protein concentration and loading. **B**: Double immunocytochemical analysis of HEK293 cells for the co-expression of *PTTG* and *p53* proteins. HEK293 cells were transiently transfected with *PTTG* cDNA. After 48 hours of transfection, the cells were subjected to immunofluorescence analysis using *PTTG*-specific antiserum and a p53 monoclonal antibody. **Panel A:** Cells transfected with pcDNA3.1 vector. **Panel B:** Cells transfected with pcDNA3.1-*PTTG* cDNA. 1, pre-immune serum; 2, *PTTG* antiserum; 3, p53-specific monoclonal antibody, and 4, overlay of 2 and 3 showing co-expression of *PTTG* and p53 proteins.

deletion analysis of the *p53* gene promoter sequence and identified a small segment of 84 base pairs (from nucleotide -172 to -89) located near the transcription start site of *p53* gene that is responsible for activation (Fig. 3). This region has been demonstrated previously to be important for *p53* promoter activity [27]. DNA foot printing and gel shift assays using the 84 bp *p53* promoter sequence and purified *PTTG* recombinant protein did not indicate direct binding of the *PTTG* protein to the *p53* promoter sequence (data not shown), suggesting that the *PTTG* protein does not bind the *p53* promoter sequence directly.

Sequence analysis of the 84 bp activation region of the *p53* promoter revealed binding sites for the nuclear factors, NFκB and *c-myc*/*max* heterodimers. The importance of these transcription factors in the regulation of *p53* promoter activity has been demonstrated previously [28]. Pei [22] had demonstrated binding of *PTTG* to the *c-myc* pro-

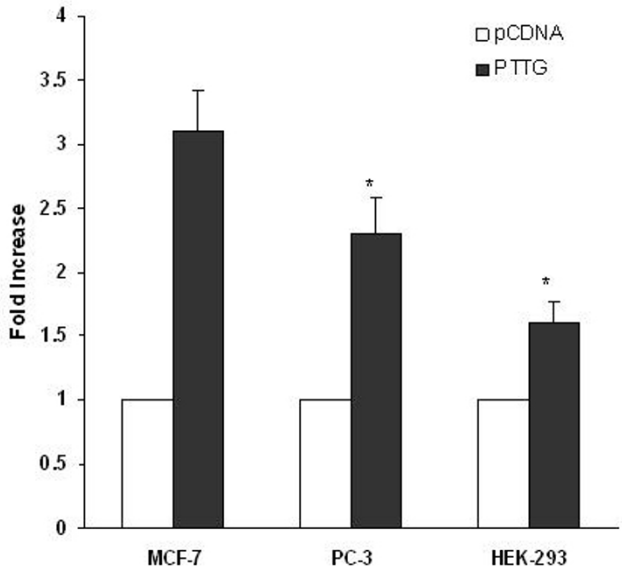

Figure 2
Overexpression of *PTTG* activates *p53* gene promoter and expression. MCF7, PC3 and HEK293 cells were co-transfected with the p53 gene promoter and *PTTG* expression vectors. The fold increases in luciferase activity on transfection of cells with *PTTG* expression vector (solid bars) compared to cells transfected with vector only (open bars) is shown. Transfections were performed in duplicate and the results are expressed as mean ± S.E.M of four independent experiments (*, $p < 0.05$ by Student's *t*-test).

moter sequence near its transcription start site, and stimulation of *c-myc* transcription by *PTTG*. As *PTTG* does not exhibit direct binding to the *p53* promoter sequence, we hypothesized that *PTTG* may modulate *p53* promoter activity indirectly through its interaction with *c-myc/max*. To test our hypothesis, we investigated the role of *c-myc* in the regulation of *p53* promoter activity. We first confirmed the effect of overexpression of *PTTG* on *c-myc* expression in HEK293 cells (data not shown). We then determined the effects of *PTTG* on binding of *c-myc* to the *p53* promoter sequence by creating a mutation within the *c-myc*-binding sequence, CATGTG, that is known to abrogate binding of *c-myc/max* to the *p53* promoter sequence and result in a loss of *c-myc* effects [28]. As shown in Fig. 4, mutation of the *c-myc/max* CATGTG binding sequence to CTTGGA resulted in a complete loss of the effects of *PTTG* on *p53* promoter activity in both HEK293 and PC-3 cell lines, suggesting that *PTTG* regulates *p53* promoter activity by modulating the expression of the *c-myc* gene, which in turn binds to the *c-myc/max* sequence of the *p53* promoter.

To further characterize the effect of *PTTG* on the interaction of *c-myc*/max with the p53 promoter sequence, we performed gel shift analysis using a γ-[^{32}P]-labeled probe derived from the *p53* gene promoter sequence (from nucleotide -172 to -89) containing either the wild-type (CATCTG) or mutated (CTTGGA) *c-myc/max* binding sequence. As shown in Fig. 5, gel mobility shift assays of nuclear extracts prepared from HEK293 cells transfected with pcDNA3.1-*PTTG* indicated an enhanced binding of the *c-myc* protein to the *c-myc/max* sequence (Fig. 5A and 5B, Lane 2) compared to that observed in extracts prepared from HEK293 cells transfected with pcDNA3.1 vector only (Fig. 5A and 5B, Lane 1). This binding of *c-myc* to the *c-myc/max* sequence was specific, since the inclusion of a 20-fold molar excess of unlabeled specific sequence in the reaction mixture resulted in almost complete abrogation of this binding (Fig. 5A, Lane 3), and pre-incubation of the nuclear extract with a *c-myc*-specific antibody directed against the N-terminal region of the *c-myc* protein resulted in a supershift (Fig. 5B, Lane 3). Furthermore, on use of the mutated *c-myc* sequence (*p53*/-172/-89-Δ-*c-myc*) as the probe in the gel shift assays, neither binding of *c-myc* (Fig. 5B, Lane 5) nor supershift of the binding complex (Fig. 5B, Lane 6) was observed. It is known that the *c-myc* protein is highly unstable and has only a short half-life but that it can be stabilized by the binding of its partner, *max*, at its C-terminal [29]. This *c-myc/max* heterodimer binds to the *c-myc/max* target sequence on the *p53* promoter and mediates various effects [30]. To confirm whether *c-myc* binds to the *c-myc/max* sequence alone or in association with *max*, we used antibodies directed against the C-terminal of the *c-myc* protein in the supershift assays. Inclusion of these antibodies negated the supershift of the DNA-protein complex (Fig. 5B, Lane 4), indicating that in these circumstances the C-terminal of *c-myc* is not accessible and is probably associated with *max*.

To further validate the role of *c-myc* in the induction of *p53* promoter activity on *PTTG* overexpression, we utilized a dominant-negative chimeric protein (myc DN), a *myc-max* fusion containing the DNA-binding domain of *max* fused to a transactivation-deficient *c-myc* [31]. It has been shown previously that when myc DN is overexpressed, it can bind to E boxes with high affinity, but that it cannot induce transcription and antagonizes *c-myc* function in a dominant-negative way [31]. As shown in Fig. 6, co-transfection of Myc DN with the *p53*-promoter construct resulted in a complete loss of activation of the *p53* promoter (Fig. 6A) and the induction of its expression by *PTTG* (Fig. 6B). These effects of Myc DN appear to be specific, since no changes in *p53* promoter activity or its expression were observed in HEK293 cells when co-transfected with Myc DN and the pcDNA3.1 vector (Fig. 6A and 6B). In addition, expression of Myc DN in HEK293 cells did not block the expression of the *PTTG* protein

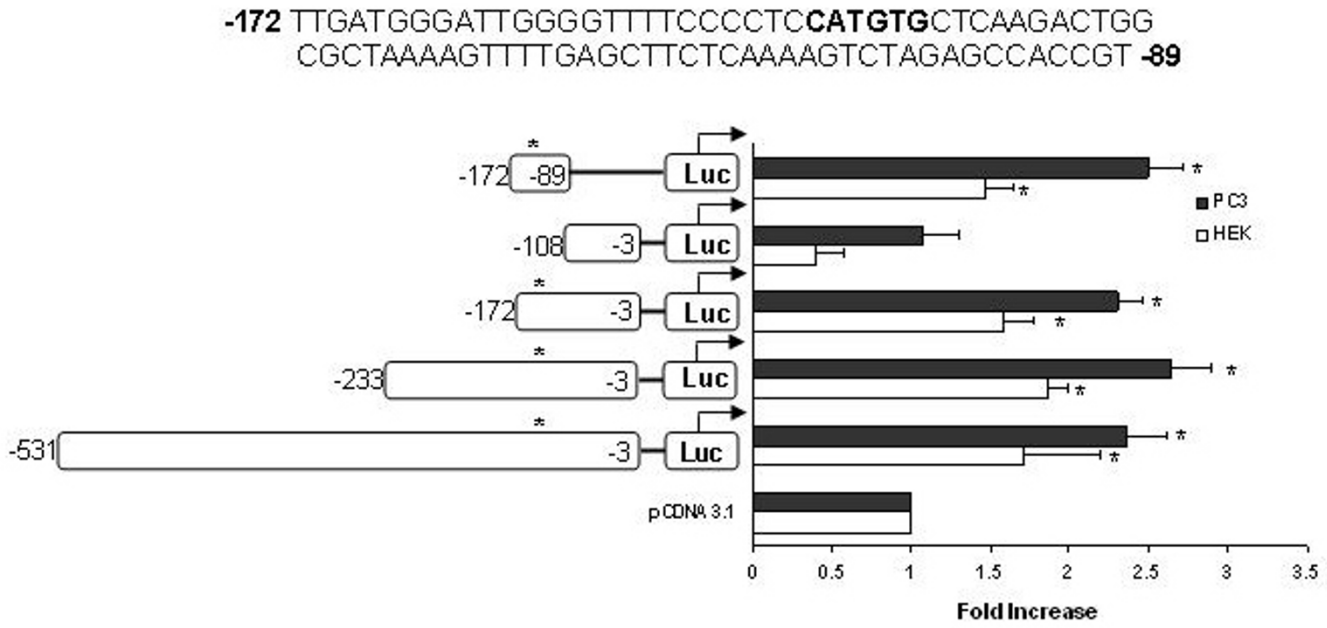

Figure 3
Mapping of the *PTTG* interacting region in the *p53* promoter sequence. HEK293 cells (open bars) and PC3 cells (solid bars) were co-transfected with various 5' and 3' deleted constructs of *p53* promoter and pcDNA3.1-*PTTG*. The sequence of the *p53* promoter construct (p53/-172/-89) containing the *c-myc*/*max* binding sequence is shown. The '*' indicates the position of *c-myc*/*max* binding sequence. Transfections were performed in duplicate and the results are expressed as mean ± S.E.M of four independent experiments (*, $p < 0.05$ by Student's *t*-test). Results are represented as the fold increase compared to control (pcDNA3.1)-transfected cells.

(Fig. 6B), suggesting that *PTTG* protein up-regulates the transcription and expression of the *p53* gene and this action of *PTTG* is achieved through modulation of *c-myc* expression and function.

PTTG *activates* bax *gene expression by inducing* p53 *expression*

To assess whether overexpression of *PTTG* modulates the transactivation properties of *p53*, we determined the effect of *PTTG* on *bax* promoter activity, as *bax* is a known to be a proapototic molecule that is induced by *p53*. The *bax* gene is a pro-apoptotic member of the *bcl-2* gene family [32] and its regulation by *p53* is well documented [33]. Co-transfection of MCF7 and HEK293 cells with pcDNA3.1-*PTTG* and the *bax* gene promoter showed a dose-dependent stimulation of the b*ax* promoter activity in both HEK293 and MCF-7 cell lines (Fig. 7A). Western blot analysis of lysates of HEK293 and MCF-7 cell lines transfected with pcDNA3.1-*PTTG* revealed enhancement of the levels of *bax* protein compared with the levels in cells transfected with pCDNA3.1 vector only (Fig. 7B). These results suggest that the *PTTG* protein up-regulates *bax* gene transcription and expression. It remains unknown if this effect of *PTTG* is direct or mediated through *p53* gene regulation; however, transfection of PC-3 cells, which do not express functional *p53*, with pcDNA3.1-*PTTG* did not result in an increase in the expression of *bax* (Fig. 7B). Taken together, these results suggest a relationship between *PTTG* and *p53* in the regulation of *p53* downstream signaling pathways by *PTTG*.

Overexpression of PTTG *induces apoptosis*

Finally, we determined the effect of *PTTG* overexpression on apoptosis. Apoptotic cells were not detectable on TUNEL staining of HEK293 cells that had been transiently transfected with the pcDNA 3.1 vector alone (Fig. 8A). In contrast, apoptotic cells were apparent on transient transfection of the HEK293 cells with either *PTTG* or *p53* cDNA (Fig. 8A). Furthermore, flow cytometric analysis of HEK293 transfected with *PTTG* or *p53* cDNA indicated that a greater number of cells in G1 phase in both *PTTG*-transfected (12%) and *p53*-transfected (22.6%) cells than in pcDNA 3.1-transfected cells (Fig. 8B), indicating that

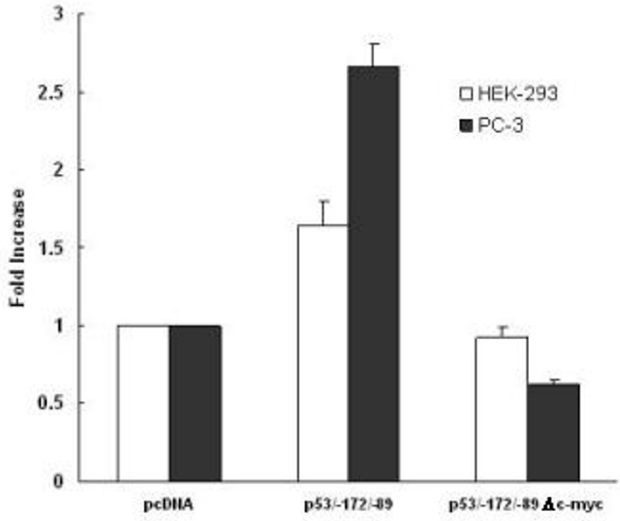

Figure 4
Activation of the *p53* promoter by *PTTG* is mediated through the *c-myc*/*max* sequence. HEK293 (open bars) and PC3 (solid bars) were co-transfected with the pcDNA3.1-*PTTG* and p53/-172/-89 (wild-type *myc*/*max* binding sequence) or the p53/-172/-89-Δ-*c-myc* (mutated *myc*/*max* binding sequence) promoter construct. Transfections were performed in duplicate and the results are expressed as mean ± S.E.M of four independent experiments (*, $p < 0.05$ by Student's *t*-test).

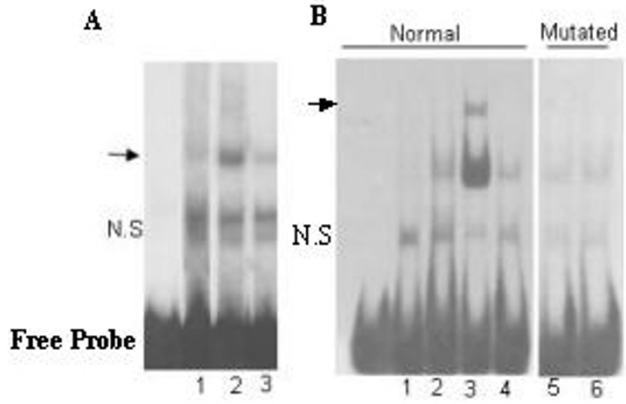

Figure 5
Electrophoretic mobility shift assays show the binding of *c-myc* protein to the *c-myc*/*max* sequence. **A**: Nuclear extract prepared from HEK293 cells transfected either with pcDNA3.1 (lane 1) or pcDNA3.1-*PTTG* (lane 2) and [^{32}P]-labeled *p53* gene promoter sequence carrying a normal *c-myc*/*max* binding site. Addition of a 20-fold molar excess of specific unlabeled DNA resulted in almost complete disappearance of the DNA-protein complex (lane 3). An arrow indicates the specific DNA-protein complex. **B**: Nuclear extracts prepared from HEK293 cells transfected either with pcDNA3.1 (lane 1) or pcDNA3.1-*PTTG* (lane 2) and [^{32}P]-labeled p53 promoter sequence carrying a normal *c-myc*/*max* binding site. Addition of antibody directed against the N-terminal of *c-myc* resulted in a supershift (lane 3, indicated by an arrow) whereas no supershift was obtained when the [^{32}P]-labeled-D-172/-89 *c-myc* sequence was used as a probe (lane 5). Addition of antibodies directed against the C-terminal of *c-myc* did not result in supershift (lanes 4 & 6) when either of the probes was used in the binding reaction indicating that C-terminal of *c-myc* is not assessable. N.S indicates non-specific complex.

PTTG and *p53* cDNA induce cell cycle arrest at the G1 phase.

Discussion

The present study was focused on determining the mechanism(s) by which *PTTG* regulates the expression of *p53*. For this purpose, we used three different cell lines: HEK293 and MCF7, which express wild-type *p53*; and PC-3, which expresses a mutant form of *p53* that is degraded rapidly. Overexpression of *PTTG* led to activation of *p53* expression in the HEK293 and MCF-7 cell lines, but not the control PC-3 cells (Fig. 1A). Immunohistochemical analysis of the HEK293 cells that had been transfected with pcDNA3.1-*PTTG* confirmed that overexpression of *PTTG* protein resulted in high levels of expression of the *p53* protein (Fig. 1B). The activation of the expression of the *p53* protein by *PTTG* in MCF-7 cells is consistent with the findings of Yu *et al*. [22] who reported an increase in expression of *p53* and its translocation into nucleus on overexpression of *PTTG* in these cells. In their studies, Yu *et al*. [22] did not find simultaneous accumulation of *p53* and mdm2 in the nucleus, indicating that *PTTG* may not use the ARF mechanism to induce *p53* expression. It is possible that *PTTG* may induce *p53* nuclear accumulation by inhibiting mdm2 expression through other mechanisms [34]. It also is possible that enhancement of the expression of *p53* may lead to the activation of other pathways, which could alter the expression of other genes or the interaction of *p53* with other proteins [35]. For example, enhanced *p53* may activate *bax*, an antagonist of Bcl-2 [36], or enhance the synthesis of insulin-like growth factor-I (IGF-I) receptor and one of its binding proteins, IGF-BP3 [37].

The mechanisms by which *PTTG* up-regulates the expression of *p53* may include enhancement of the stability of mRNA, an improvement in translation efficiency, or enhanced transcription of the gene. Our studies suggest that the enhanced expression of the *p53* gene is due, at least in part, to an increase in *p53* promoter activity. It has

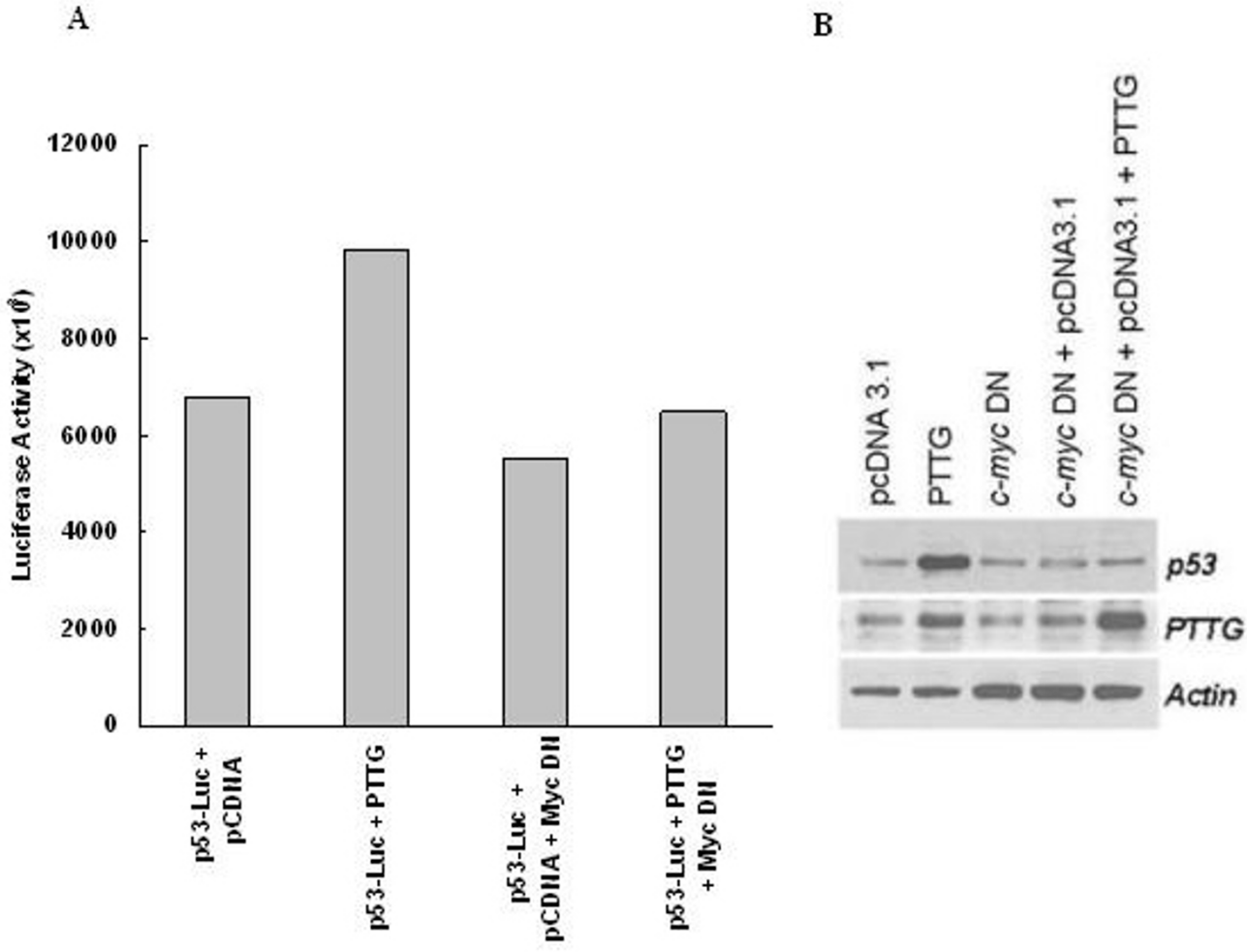

Figure 6

c-myc is essential for activation of p53 expression by *PTTG*. **A**: Co-transfection of HEK293 cells with the *c-myc* dominant-negative (Myc DN) construct, *p53* gene promoter and *PTTG* cDNA. Transfections were performed in duplicate and results are expressed as mean of two independent experiments. **B:** Western blot analysis of HEK293 cells transfected with either pcDNA 3.1, the *c-myc* dominant-negative (Myc DN) and/or *PTTG* cDNA. Expression was detected using specific antibodies. β-Actin antibody was used as a control to determine the variation in protein concentration and loading.

been shown that the *PTTG* possesses transactivating [38] and DNA binding properties [21]. We therefore expected that *PTTG* might regulate *p53* promoter activity by virtue of its direct binding to the *p53* gene promoter sequence; however, our DNA foot printing and gel shift assays showed no direct binding of *PTTG* protein to the *p53* promoter sequence, suggesting that *PTTG* modulates *p53* promoter activity through an indirect mechanism. Subsequent 5' and 3' deletion analysis of the *p53* gene promoter identified the sequence between nucleotides -172 to -89, which contains a *c-myc*/*max* binding sequence, as being responsive to *PTTG* activation. In previous studies, Pei [21] demonstrated binding of *PTTG* protein to the *c-myc* gene promoter sequence near its transcription start site and its activation by *PTTG*. Therefore, it is possible that the enhancement of expression of the *p53* gene by *PTTG* is a result of enhancement of the expression of *c-myc*. The resulting *c-myc* would then interact with its partner, *max*, to form a heterodimer that binds to the *p53* promoter and regulates its transcription. Binding of the *c-myc* protein to this sequence was confirmed by gel mobility shift and super shift assays, which revealed specific bind-

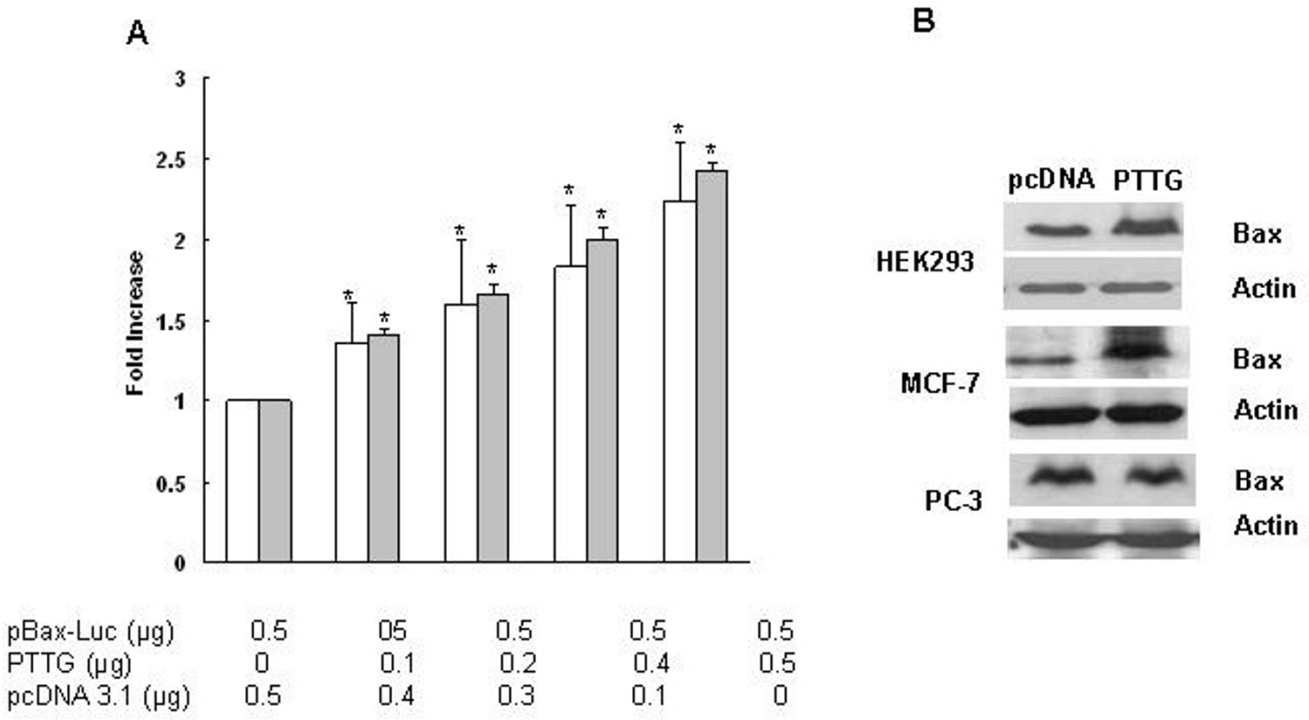

Figure 7
Overexpression of *PTTG* activates the expression of *bax* by upregulating *p53* expression. **A:** MCF7 cells (open bars) and HEK293 cells (solid bars) were co-transfected with the *bax* promoter and increasing amounts of the pCDNA3.1-*PTTG* expression vector. Transfections were performed in duplicate and the results are expressed as the mean ± S.E.M of four independent experiments (*, $p < 0.05$ by Student's *t*-test). Results are represented as the fold increase compared to control. **B**: Western blot analysis of HEK293, MCF7 and PC3 cells transfected with *PTTG* for the expression of *bax* after 48 hours of transfection. β-Actin antibody was used as a control to assess any variation in protein concentration and loading.

ing of the *c-myc* protein to this sequence. Site-directed mutagenesis of the *c-myc/max* sequence resulted in a complete loss of binding of the *c-myc* protein and *p53* promoter activation by *PTTG*. The importance of *c-myc* in regulating expression of the *p53* gene by *PTTG* is further supported by the results generated using a *c-myc* dominant-negative construct that abolished the induction of *p53* gene expression by *PTTG*. These results demonstrate clearly that *PTTG* up-regulates the expression and transcription of the *p53* gene by modulating the expression of the *c-myc* gene and its binding to the *c-myc/max* sequence on the *p53* promoter.

PTTG is an oncogene and has been shown to induce cellular transformation *in vitro* and promote tumor formation in nude mice [2]. In an attempt to define the mechanism by which *PTTG* contributes to tumorigenesis, Bernal *et al.* [23] used phage display screening and determined that the *p53* protein interacts with the *PTTG* protein both *in vitro* and *in vivo* leading to inhibition of the transcriptional activity of *p53* and its ability to induce cell death. In contrast to our results, these investigators did not find that *PTTG* altered the expression of *p53*. In their studies, these investigators showed a very low or marginal increase in the levels of *PTTG* protein on transfection with *PTTG* cDNA. Therefore, the discrepancy between Bernal's findings and ours may be attributable to the requirement for a threshold amount of *PTTG* protein for induction of *p53* promoter activity. Zhou *et al.* [25] showed suppression of *p53* expression in cells treated with the DNA-damaging drugs doxorubicin and bleomycin. This drug-induced suppression of *PTTG* was shown to be dependent on the presence of functional *p53*. In their studies, these investigators [25] showed direct suppression of *PTTG* expression by *p53* through its interaction with the NF-Y transcription factor binding sequence of the *PTTG* promoter, suggesting that the *PTTG* gene is a target of *p53* and may play a role in the *p53*-mediated cellular response to DNA damage.

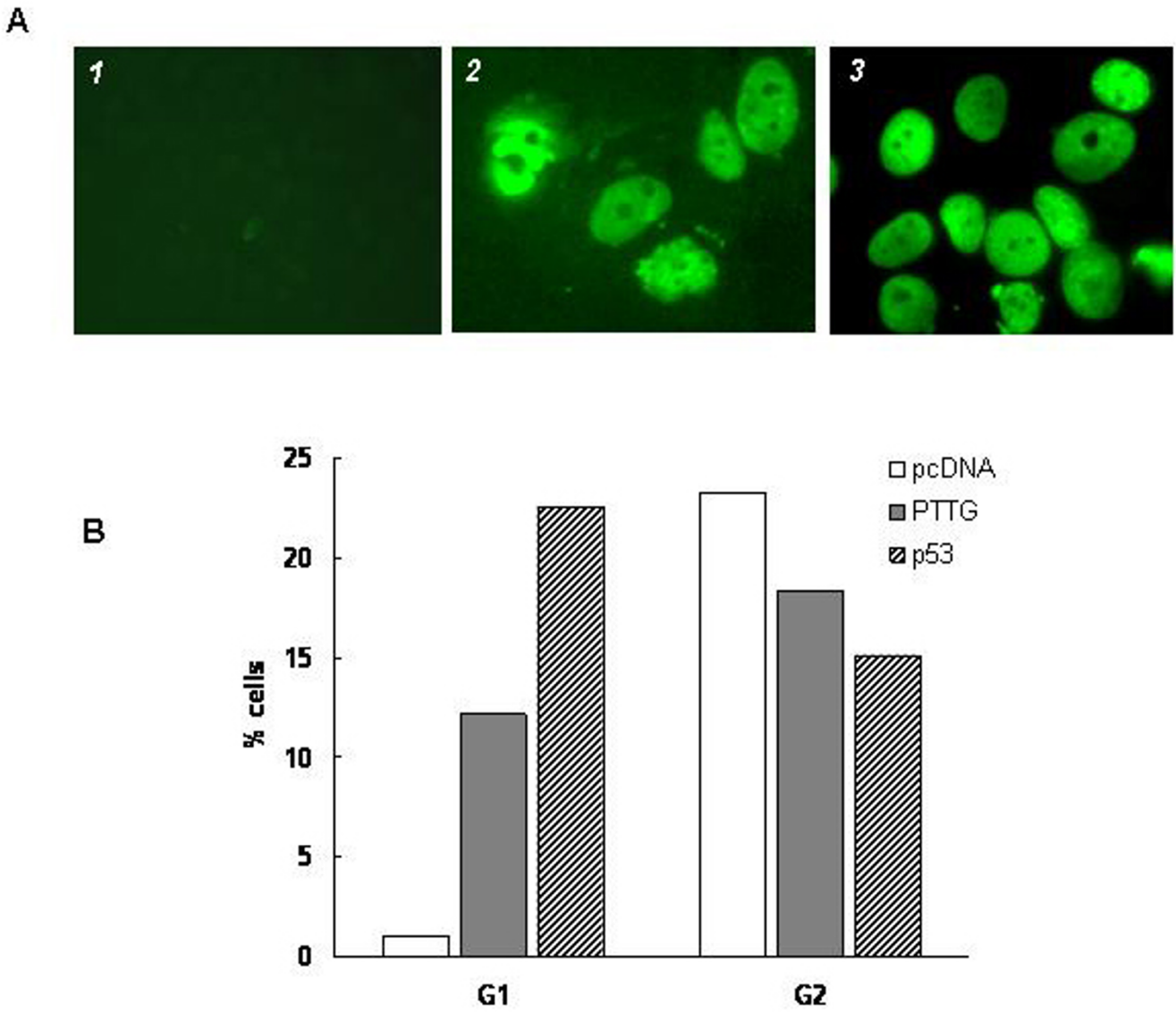

Figure 8
PTTG overexpression induces apoptosis. **A**: HEK293 cells were transfected with pcDNA 3.1 vector (1), *PTTG* cDNA (2), or p53 cDNA (3) for 48 hours before the TUNEL assay. **B**: Transfected HEK293 cells also were analyzed for apoptosis by flow cytometry. Plots show an accumulation of G1 cells upon transfection with *PTTG* and both an induction of sub-G1 and loss of G2 cells upon *p53* transfection. Data are representative of two independent experiments, performed in triplicate.

Our results show that overexpression of *PTTG* activates *p53* expression, and that this action of *PTTG* is achieved through the regulation of the expression of *c-myc*, which in turn regulates the expression of the *p53* gene, by its direct binding to the *c-myc/max* sequence of the *p53* promoter. The importance of *c-myc* in the induction of the expression of *p53* by *PTTG* is further revealed by our studies using a *c-myc* dominant-negative construct (Fig 6A and 6B). These results are consistent with those of Kirch *et al*. [28] and Levine [39] who also documented the importance of *c-myc* for *p53* expression and activation. Furthermore, *c-myc* has been reported to promote apoptosis by destabilizing mitochondrial integrity in cooperation with proapoptotic members of the BCL-2 family including *bax* [40] and it is considered as an important transcription factor [41]. Thus, its role in the induction of *p53* expression seems to be of physiologic importance in the control of genomic stability in cells.

Table 1: Sequence of different primers used in PCR amplification Construct

Construct	Primer Sequence
p53/-233/-3	Sense 5'-ATGAGCTCGGATTACTTGCCCTTACT-3' Antisense 5'-TACTCGAGAATCCAGGGAAGCGTGTC-3'
p53/-172/-3	Sense 5'-ATGAGCTCTTGATGGGATTGGGG TTTTC-3' Antisense 5'-TACTCGAGAATCCAGGGAAGCGTGTC-3'
p53/-108/-3	Sense 5'-ATGAGCTCCAAAAGTCTAGAGCCACCGT-3' Antisense 5'-TACTCGAGAATCCAGGGAAGCGTGTC-3'
p53/-172/-89	Sense 5'-ATGAGCTCTTGATGGGATTGGGGTT TTC-3' Antisense 5'-TACTCGAGACGGTGGCTCTAGACTTT-3'
p53 cDNA	Sense 5'-TAGGATCCGACACGCTTCCCTGGATT-3 Antisense 5'-TAGAATTCACACCTATTGCAAGCAAG-3'

Restriction enzymes SacI and XhoI sequences are underlined.

To assess the significance of the activation of *p53* by *PTTG* with respect to the function of *p53* in apoptosis and activation of downstream signaling genes, we analyzed apoptosis using TUNEL staining as well as the activation of the *bax* promoter. The *bax* gene encodes a pro-apoptotic member of *BCL-2* gene family [32] and its regulation by *p53* is well documented [33]. Our results indicate that overexpression of *PTTG* induces *bax* promoter activity (Fig. 7A). *PTTG* overexpression also induces apoptosis in HEK293 cells (Fig. 8). These results are in agreement with the earlier reports of *PTTG* overexpression inducing apoptosis by *p53*-dependent and independent mechanisms [22]. It would seem that when both of these apoptotic pathways fail, *PTTG* can support the survival of aneuploid cells, thereby supporting tumor growth.

Conclusions

Our studies reveal that the *PTTG* protein can up-regulate expression of *p53* in cells dependent on their *p53* status. This stimulatory effect of *PTTG* is indirect and is mediated through *c-myc*. *PTTG* also up-regulates the activity of the *bax* promoter and increases the expression of *bax* through modulation of *p53* expression.

Methods

Construction of reporter plasmids

We cloned the human *p53* gene promoter from nucleotides -531 to -3 (*p53*/-531/-3) into the promoterless and enhancerless basic pGL3 vector (Promega, Madison, Wisconsin). Briefly, the *p53* gene promoter sequence (from nucleotide -531 to -3) was amplified using human genomic DNA (Promega, Madison, Wisconsin) as a template and selected sense/antisense primers (sense; 5'-TAGAGCTCGGGAGAAAACGTTACGGT-3' ; antisense; 5'-TACTCGAGAATCCAGGGA AGCGT GTC-3') in PCR. The 5' and 3' deleted constructs of the *p53* promoter were generated by amplifying the desired sequence using *p53*/-531/-3 cDNA as a template and specific primers (Table 1) followed by their subcloning into pGL3 vector. The deleted constructs were designated as *p53*/-233/-3, *p53*/-172/-3, *p53*/-108/-3 and *p53*/-172/-89. The primers were designed to contain sequences for SacI and XhoI restriction enzymes (underlined) for cloning purpose. We generated a pcDNA3.1-*PTTG* plasmid by subcloning the full-length *PTTG* cDNA amplified from the human testis into pCDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA) as described previously [2]. We generated pcDNA3.1-*p53* plasmid by subcloning the full length human *p53* cDNA into pCDNA3.1. *c-myc* dominant-negative expression construct pBabe-cyc/max-64 was a generous gift from Dr. Hartmut Land, University of Rochester.

Cell culture and transfection

We purchased HEK293, PC-3 and MCF-7 cell lines from American Type Culture Collection (ATCC). All cells were cultured under conditions recommended by the supplier. For transient transfections we seeded the cells in six-well tissue culture plates 24 hours prior to transfection. Cells were transfected with appropriate plasmid DNA (1 μg/well) using Fugene-6 as a transfection reagent (Boehringer Mannheim) as described previously [2]. We used pRenila-Luc (100 ng) (Promega, Madison, Wisconsin) as an internal control. After 48 hours of transfection, the cells were harvested and assayed for luciferase and renilla luciferase activities using dual-luciferase reporter system (Promega) and quantified using a Zylux Femtometer FB12 luminometer.

Western blot analysis

The cells were harvested in lysis buffer (50 mM Tris-HCl, 8.3; 100 mM NaCl; 0.1% Triton X-100; 1 mM PMSF; 1 μg/ml leupeptin; 1μg/ml pepstatin and 1μg/ml aprotinin) and denatured in loading SDS sample buffer by heating at 95°C for 3 minutes. Fifty μg of proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to Hybond nitrocellulose membrane (Amer-

sham Biosciences). The membranes were blocked in 5% non-fat milk for 1 hour at room temperature, followed by incubation in primary antibody for 1 hour. The antibodies used were anti-*PTTG* (1:1,500 dilution) [42], anti-*p53* (1:2,000 dilution, Zymed Laboratories, San Francisco, California), anti-*bax* (1:500 dilution, Sigma, St. Louis, Missouri), and actin (1:5,000 dilution, Sigma). Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 was used to dilute the antibodies and wash the membranes. Proteins were visualized using the ECL system (Amersham Biosciences) according to the supplier's instructions.

Site-directed mutagenesis

Site-directed mutagenesis of the putative *c-myc/max* binding sequence of the human *p53* gene promoter carrying a 3-nucleotides change in *p53*/-172/-89 construct was carried out using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) using *p53*/-172/-89 construct as a template and selected primers (sense 5'-TCCCCTCCCTTGGACTCAAGACTGGCGCTA-3' ; antisense 5'-TAGCGCCAGTCT TGAGTCCAAGGGAGGGGA-3') in PCR. Changing the sequence CATCTG in the wild-type sequence to CTTGGA created the mutation in the *c-myc/max* binding sequence. The construct was sequenced to confirm the mutation and was designated *p53*/-172/-89-Δ-*c-myc*.

Preparation of nuclear extracts and DNA footprinting analysis

Nuclear extracts from HEK293 cells transfected with pcDNA3.1 or pcDNA3.1-*PTTG* were prepared according to Panek *et al*. [43]. Briefly the cells were collected in ice-cold phosphate-buffered saline (PBS) and pelleted at 3,700 rpm for 10 minutes. The packed cell volume (PCV) of the cells was measured and then the cells were resuspended (3 volumes of PCV) in hypotonic buffer (1 M HEPES, pH 7.9, 1 M $MgCl_2$ and 1 M KCl) and incubated for 10 minutes on ice. The resuspended cells were then transferred to a glass homogenizer and homogenized. The lysis of the cells was confirmed by Trypan blue dye exclusion. After homogenization, the nuclei were sedimented by centrifugation at 4,600 rpm for 15 minutes and then resuspended (3 volumes of the sedimented nuclei) in low-salt buffer (1 M HEPES, pH 7.9, 0.15 M $MgCl_2$, 0.5 M EDTA, 20 mM KCl and 25% glycerol) and mixed gently, followed by addition of high-salt buffer (1 M HEPES, pH 7.9, 0.15 M $MgCl_2$, 0.5 M EDTA, 1 M KCl and 25% glycerol). The high-salt buffer was added drop wise to the mixture with gentle mixing for 30 minutes. The mixture was centrifuged at 5,000 rpm for 15 minutes. The supernatant containing the nuclear extract was dialyzed against 100 volumes of dialysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) for 1 hour. The mixture was centrifuged for 20 minutes at 5,000 rpm to pellet any precipitated proteins. and the supernatant containing the nuclear extract was analyzed to determine the protein content and stored at -70°C.

The 84 base pair (-172 to -89) fragment of human *p53* promoter was amplified using [γ-^{32}P] end-labeled sense or antisense primer (see Table 1 for primer sequences) and the *p53*/-172/-89 construct as a template in PCR as described above. This [^{32}P]-labeled DNA fragment was purified on a 2% agarose gel. DNA footprinting analysis was carried out in a 25 μL reaction volume containing 200 mM HEPES, pH 7.9, [^{32}P]-labeled DNA fragment (40,000 cpm) and 50 ng/μl purified *PTTG* recombinant protein [42]. The binding reaction was allowed to proceed for 10 minutes on ice. The reaction was terminated by addition of 50 μL of stop solution A (10 mM $MgCl_2$, 5 mM $CaCl_2$ and 6.5 ng/μL of yeast transfer RNA) followed by digestion of DNA with 0.2 unit of DNase I (Promega Biotech, WI) for 1 minute at room temperature. The DNA digestion reaction was terminated by addition of stop solution B (20 mM EDTA, pH 8.0, 1.0% SDS and 0.2 M NaCl). DNA was extracted with phenol/chloroform, and separated by electrophoresis through 8% acrylamide/7 M urea gels. The gel was dried and subjected to autoradiography.

Electrophoretic mobility shift assay (EMSA)

The [^{32}P]-labeled DNA probe (84 bp) containing either the wild-type or mutated *c-myc/max* binding sequence was purified from agarose gel and used in gel shift assays. Briefly, 4 μg of nuclear extract was incubated with the γ-[^{32}P] labeled probe (25,000 cpm) in EMSA buffer (50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 250 mM NaCl; 2.5 mM EDTA; 2.5 mM DTT and 20% glycerol) and 250 ng/ml poly (dI-dC) in a 25 μl reaction volume for 30 minutes at room temperature. For super shift assays, the nuclear extract was incubated with *PTTG* antiserum (1:1,500 diluted), N-terminal anti-*c-myc* rabbit polyclonal antibody (0.5 μg, Santa Cruz Biotechnology, Santa Cruz, CA) or C-terminal anti-*c-myc* monoclonal antibody (0.5 μg, Zymed Laboratories, San Francisco, CA) for 30 minutes at room temperature prior to the addition of labeled probe. DNA-protein complexes were separated on 4% polyacrylamide gels and analyzed by autoradiography.

Double immunostaining of cells for PTTG *and* p53 *proteins*

HEK293 cells were grown on polylysine-coated chamber slides (Nunc International Corp., Naperville, IL), for 24 hours and then transfected with pcDNA3.1 or pcDNA3.1-*PTTG* cDNA as described above. After 48 hours of transfection, the cells were fixed with 4% freshly prepared paraformaldehyde for 8 minutes and then treated with 0.1% Nonidet P-40 for 5 minutes. Cells were treated with 5% normal goat serum for 60 minutes to block nonspecific binding followed by incubation with preimmune serum

(1:1,500 dilution) plus *p53* antibody (1:100 dilution) or *PTTG* antiserum (1:1,500 dilution) [42] plus *p53* antibody (1:100 dilution) for 60 minutes at room temperature. After several rinses with PBS buffer, the cells were incubated with Alexa Flour® 594 goat anti-mouse and Alexa Flour® 488 goat anti-rabbit secondary antibodies (1:500 dilution, Molecular Probes, Eugene, OR) for 45 minutes. Cells were rinsed with PBS and analyzed using a fluorescent microscope (Olympus X-70).

Apoptosis assay and flow cytometric analysis

Apoptosis analysis of the cells was carried out using TUNEL assay kit (Roche, Indianapolis, IN). HEK293 cells were transiently transfected with pcDNA 3.1-*PTTG* or *p53* plasmid as described above. After 48 hours of transfection, the cells were subjected to TUNEL assay following the supplier's instructions. Briefly, the cells were washed with PBS, air-dried and fixed in 4% paraformaldehyde pH (7.4) for 1 hour at room temperature followed by incubation in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice. The cells were then washed twice with PBS and air-dried. Fifty µl of TUNEL reaction mixture was added to each sample and incubated in the dark in a humidified chamber for 60 minutes at 37°C. The cells were washed three times with PBS and examined under Olympus X-70 fluorescence microscope. For flow cytometric analysis cells were transfected with pcDNA3.1-*PTTG* or pcDNA3.1-*p53* cDNA for 24 hours and analyzed as described by Zhou et al, [25].

List of abbreviations

PTTG, pituitary tumor transforming gene; Myc DN, *c-myc* dominant-negative expression construct; EMSA, electrophoretic mobility shift assay.

Authors' contributions

TH carried out most of the experimental work; SSK carried out the immunohistochemistry experiments and helped in evaluation of the data and manuscript preparation. Both authors read and approved the final version of the manuscript.

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