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TGFβ1 induces apoptosis in invasive prostate cancer and bladder cancer cells via Akt-independent, p38 MAPK and JNK/SAPK-mediated activation of caspases

Ahmad Al-Azayzih [a,b], Fei Gao [a,b], Anna Goc [a,b], Payaningal R. Somanath [a,b,c,*]

[a] Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA, United States
[b] Charlie Norwood VA Medical Center, Augusta, GA, United States
[c] Department of Medicine and Vascular Biology Center, Georgia Health Sciences University, Augusta, GA, United States

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ABSTRACT

Recent findings indicate that advanced stage cancers shun the tumor suppressive actions of TGFβ and inexplicably utilize the cytokine as a tumor promoter. We investigated the effect of TGFβ1 on the survival and proliferation of invasive prostate (PC3) and bladder (T24) cancer cells. Our study indicated that TGFβ1 decreased cell viability and induced apoptosis in invasive human PC3 and T24 cells via activation of p38 MAPK-JNK-Caspase9/8/3 pathway. Surprisingly, no change in the phosphorylation of pro-survival Akt kinase was observed. We postulate that TGFβ1 pathway may be utilized for specifically targeting urological cancers without inflicting side effects on normal tissues.

1. Introduction

Transforming growth factor β (TGFβ) family of pro-fibrotic growth factors control physiological processes such as wound healing as well as clinical conditions such as tissue fibrosis by inducing fibroblast differentiation to myofibroblasts [1]. TGFβ plays fundamental role in many cell functions including cell migration, survival, proliferation, and differentiation [2,3]. The exact role of TGFβ in cancer is highly controversial. TGFβ is a known inhibitor of proliferation in epithelial and lymphoid cells [4], and hence considered to have a tumor suppressive role [5–13]. However, the exact role of TGFβ signaling in tumor development appears to be complex and is context-dependent. On one end, studies have shown that ablation of TGFβ signaling results in enhanced tumor growth [14–16]. On the other end, TGFβ has also been implicated in eliciting tumor promoting effects through its ability to induce epithelial-to-mesenchymal transition (EMT), immunosuppression and metastasis [17–21]. These controversies may also be due to the specific role of TGFβ in cancer cells vs. stromal fibroblasts that influence the tumor growth [22–24]. These discrepancies demand extensive research on TGFβ signaling in cancer with specific effects on the tumor and stromal cells.

Multiple studies have implicated the inter-dependent role of TGFβ-induced canonical (Smad-dependent) and non-canonical (Smad-independent) signaling pathways in both the tumor suppressive and tumor promoting activities [11]. The differential effects of TGFβ in multiple cell types in the regulation of various physiological and pathological processes are attributed mainly to a particular non-canonical pathway activated by this growth factor in a specific cell type. TGFβ stimuli can result in the activation of one or more of the pro-survival pathways such as PI3K/AKT/mTOR and RAF/MEK/ERK1/2 pathways, two major kinases often hyper-activated in cancer cells [25]. Alternatively, pro-apoptotic pathways such as P38 MAPK and JNK pathways are also involved in the regulation of tumor cell apoptosis [26,27].

In light of studies demonstrating both tumor suppressive and tumor promoting effects of TGFβ, we sought to determine the specific effects of TGFβ1, major TGFβ isoform on the survival, proliferation and foci formation of invasive prostate (PC3) and bladder (T24) cancer cell lines *in vitro* and characterize the underlying mechanisms regulating the process. We report that TGFβ1 inhibits invasive prostate (PC3) and bladder (T24) cancer cell proliferation, survival and foci formation, and induced apoptosis *in vitro* in a dose- and time-dependent manner via activation of p38 MAP kinase and JNK, subsequently leading to the cleavage of caspases 9, 8 and 3. We also demonstrate the Akt-independent effects of TGFβ on prostate and bladder cancer cell survival and apoptosis.

Abbreviations: TGFβ, transforming growth factor β; p38 MAPK, p38 mitogen activated protein kinase; JNK, c-jun N-terminal kinase; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix.

* Corresponding author. Address: Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, HM1200 Georgia Health Sciences University, Augusta, GA 30912, United States. Fax: +1 706 721 3994.
E-mail address: sshenoy@georgiahealth.edu (P.R. Somanath).

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2. Materials and methods

2.1. Reagents, cell lines and antibodies

Human PC3 and T24 cell lines were obtained from ATCC (Manassas, VA) and maintained in DMEM-High Glucose (Hyclone, Logan, UT) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 humidified atmosphere at 37 °C. Primary antibodies against p-AKT (S473), p-ERK1/2, p-P38-MAPK, p-SAPK/JNK, cleaved caspase 3, cleaved caspase 9 and cleaved caspase 8 were purchased from Cell Signaling (Boston, MA). Primary antibodies against β-actin were purchased from Sigma (St Louis, MO). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from BioRad (Hercules, CA). Recombinant human TGFβ1 was purchased from R&D systems (Minneapolis, MN).

2.2. Cell proliferation assay

Proliferation of PC3 and T24 cell lines was determined using the nonradioactive BrdU-based cell proliferation assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 and T24 cells were plated in 96-well flat bottom plates at a density of 5×10^3 cells per 100 μl, and allowed to grow for 24 h. Cells were then treated with (0.1, 0.5, 1, 2.5 and 5 ng/ml TGFβ1) for an additional 24 h in serum free conditions. Cells were then subjected to 5-bromo-2-deoxyuridine assay using the BrdU Labeling and Detection Kit III as done previously [28]. BrdU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690 nm on an ELISA plate reader.

2.3. Cell viability assay

Cell number was assessed indirectly by means of tetrazolium salt conversion into formazan crystals Briefly, PC3 and T24 cells were plated in 96-well plates at 5000 cells per well in the DMEM containing 10% FBS. 24 h after plating, the medium was replaced with the same medium containing (0.1, 0.5, 1, 2.5 and 5 ng/ml TGFβ1), After 24 h treatment, cells viability was measured using the Cell Proliferation Kit I (MTT) (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. The absorbance at 570 nm was measured using an ELISA reader and used to determine relative cell numbers in each well.

2.4. Apoptosis assay

Cytoplasmic histone-associated DNA fragments were quantified by using Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN) according to the manufacture's protocol. Briefly, PC3 and T24 cells were plated in 96-well plate at a density of either 10^4 cells/well. After 24 h, the cells were incubated in DMEM containing (0.1, 0.5, 1, 2.5 and 5 ng/ml TGFβ1) for 24 h. Control cells were incubated in DMEM alone. Cells were lysed and centrifuged at 200*g* for 10 min, and the collected supernatant was subjected to ELISA [29].The absorbance was measured at 405 nm (reference wavelength, 492 nm).

2.5. Western blot analysis

PC3 and T24 cell lines were cultured to reach a monolayer in DMEM in 6-well plates. The wells were treated with DMEM containing (0.1, 0.5, 1, 2.5 and 5 ng/ml of TGFβ1). Control cells were

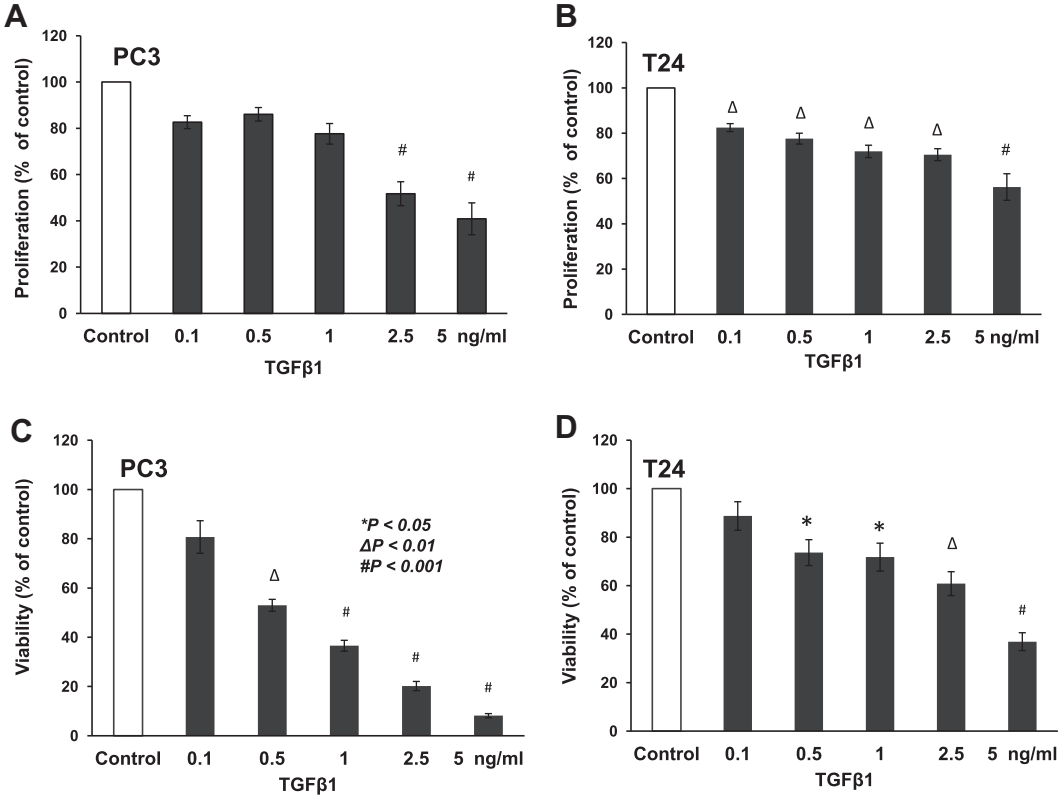

Fig. 1. TGFβ1 inhibited proliferation and viability of prostate (PC3) and bladder (T24) cancer cells. (A and B) Bar graphs showing a reduction in the proliferation (represented as absorbance normalized to control) in PC3 cells and T24 cells, respectively after 24 h treatment with TGFβ1 (0.1, 0.5, 1, 2.5, and 5 ng/ml) compared to control cells. (C and D) Bar graph showing a reduction in the MTT absorbance (represented as percentage change in the MTT absorbance compared to control) in PC3 cells and T24 cells, respectively after 24 h treatment with TGFβ1 compared to control cells.

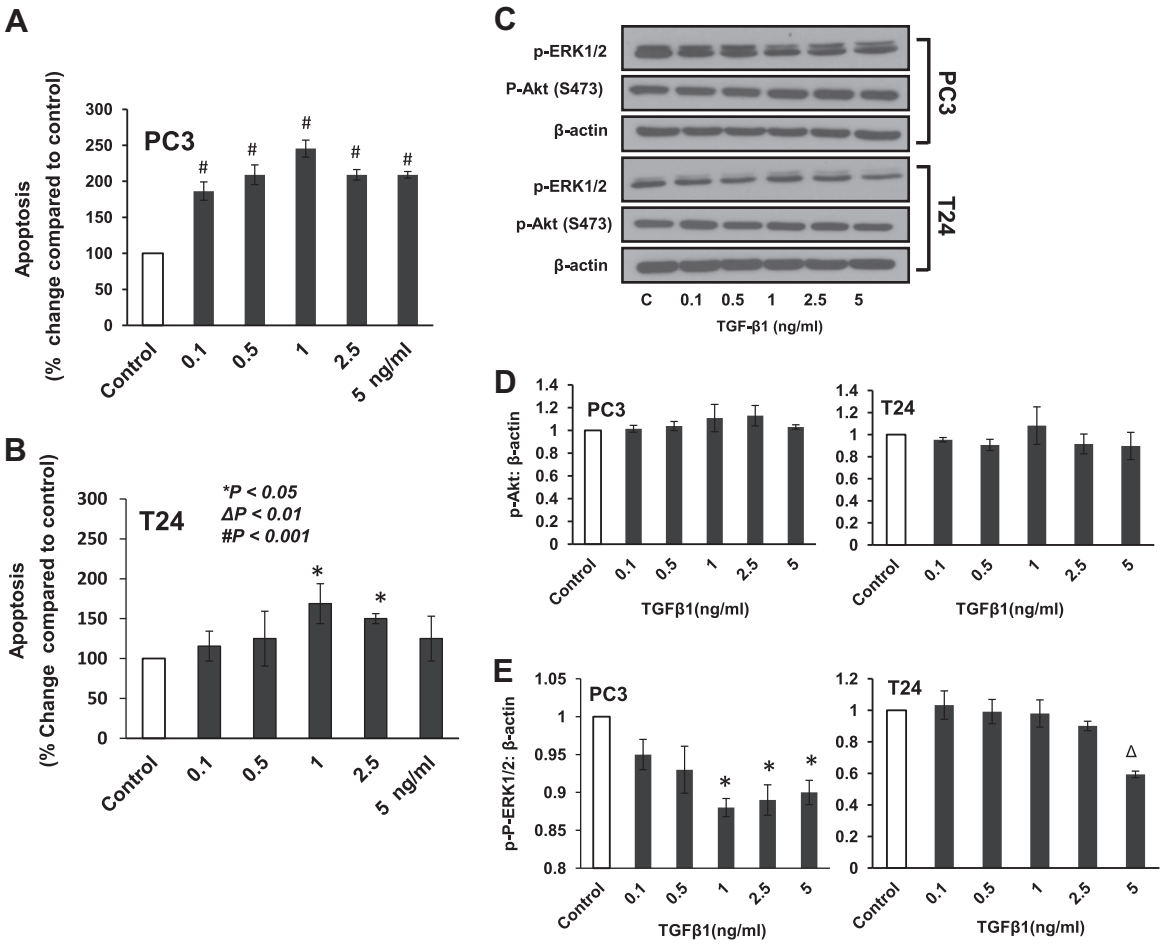

Fig. 2. TGFβ1 induced apoptosis of PC3 and T24 cells in an Akt-independent mechanism. (A and B) Bar graph showing apoptosis in PC3 and T24 cells treated with control (Serum free DMEM) and TGFβ1 (0.1, 0.5, 1, 2.5, 5 ng/ml) for 24 h. (C) Western blots of pAkt S473 and pERK1/2 expression in PC3 and T24 cells treated with control and TGFβ1 (0.1, 0.5, 1, 2.5, 5 ng/ml) for 24 h. (D and E) Band densitometry analysis of pAkt S473 and pERK1/2 expression in PC3 and T24 cells normalized to β-actin, respectively, treated with control and TGFβ1 (0.1, 0.5, 1, 2.5, 5 ng/ml) for 24 h.

grown in DMEM alone. Whole-cell lysates were prepared using lysis buffer [50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, and 1× complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. The protein concentration was measured by the D_L protein assay (Bio-Rad Laboratories, Hercules, CA). Western analyses were performed using the standard Laemmli's method as done previously [30].

2.6. Colony/foci formation assay

Colony formation assay was performed using the standard protocol [31]. In this approach, PC3 cells were cultured on 6-well plates until the monolayer was reached. After seven days of culture with TGFβ1 (0.1, 0.5, 1, 2.5 and 5 ng/ml) in serum containing medium, with changing the medium and growth factor every day, each of the wells was counted for the number of colonies and compared with the untreated controls. Plates were fixed using 2% paraformaldehyde, briefly stained with crystal violet, and counted visually or by using ImageJ software.

2.7. Statistical analysis

All data are presented as mean ± S.D of three to five independent experiments done at least in triplicates. A Student's two-tailed *t* test was used to determine significant differences between treatment and control values.

3. Results

3.1. TGFβ1 inhibits proliferation and viability of PC3 and T24 cells

We first determined the direct effect of TGFβ1 on prostate cancer (PC3) and bladder cancer (T24) cell proliferation and viability. Based on the results of a BrdU incorporation assay, our data revealed that treatment with TGFβ1 results in significant inhibition of PC3 cell proliferation by 50 to 60% for 2.5 and 5 ng/ml TGFβ1, respectively, compared control (Fig. 1A). TGFβ1 also inhibited proliferation of T24 cells by 18%, 23%, 28%, 30%, and 44% for doses of 0.1, 0.5, 1.0, 2.5 and 5 ng/ml of TGFβ1, respectively, compared to control (Fig. 1B). Our data from the cell viability assay (MTT assay) revealed that treatment with TGFβ1 resulted in significant inhibition of PC3 cell viability by 48%, 64%, 80%, and 90% for 0.5, 1.0, 2.5 and 5 ng/ml TGFβ1, respectively compared to control (Fig. 1C), and inhibition of T24 cell viability by 27%, 29%, 40%, and 63% for 0.5, 1.0, 2.5 and 5 ng/ml TGFβ1, respectively compared to control (Fig. 1D). Thus, our data indicate that TGFβ1 significantly inhibits PC3 and T24 cell proliferation *in vitro*.

3.2. TGFβ1 induces apoptosis of PC3 and T24 cells in an Akt-independent manner

We next determined the effect of TGFβ1 on apoptosis of prostate (PC3) and bladder (T24) cancer cells. Our study indicated that treatment with 0.1 ng/ml TGFβ1 resulted in significant increase in

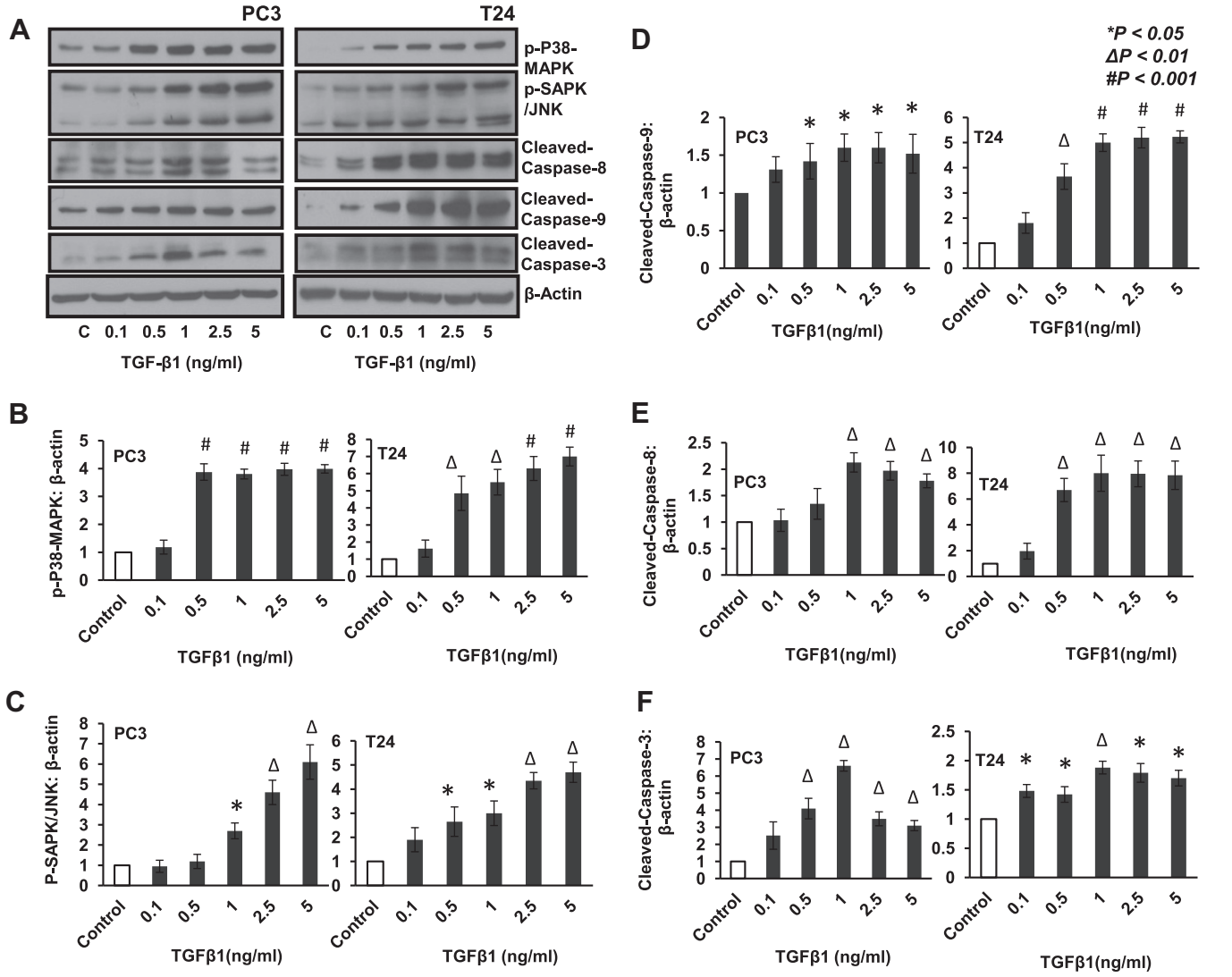

Fig. 3. TGFβ1 increased phosphorylation of p38-MAPK and SAPK/JNK and enhanced expression of cleaved caspases in PC3 and T24 cells. (A) Western blots showing increased phosphorylation of p38-MAPK and SAPK/JNK, as well as increased expression of cleaved caspase-9, cleaved caspase-8, and cleaved caspase-3 after 24 h treatment with TGFβ1 (0.1, 0.5, 1, 2.5, and 5 ng/ml) compared to control (DMEM) (B–F) Bar graphs showing band-densitometry analysis of phoshorylations of p38 MAPK, SAPK and expression of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3, respectively, with TGFβ1 treatment as mentioned above, normalized to β-actin.

PC3 cell apoptosis by 86% (Fig. 2A). This effect was further enhanced by treatment with 0.5 and 1.0 ng/ml TGF-β1 which exhibited more than a 2 and 2.5 fold increases in cell death. Treatment with 1.0 and 2.5 ng/ml of TGFβ1 resulted in significant increase in T24 cell apoptosis by 68 and 50%, respectively (Fig. 2B). Since Akt is the major cell survival kinase often deregulated in cancers, we sought to determine the effect of TGFβ1 on PC3 and T24 cells. Our data indicated that TGFβ1-induced apoptosis does not involve changes in the phosphorylation of Akt in either of the cancer cells (Fig. 2C and D). However, we observed a significant reduction in the phosphorylation of ERK1/2 in both PC3 and T24 cells, a kinase known to regulate cell proliferation (Fig. 2C and E). Our studies demonstrated that TGFβ1 induces invasive prostate and bladder cancer cell apoptosis *in vitro* in an Akt-independent manner and that impaired proliferation of PC3 and T24 cells in response to TGFβ1 may be due to reduced phosphorylation of ERK1/2.

3.3. TGFβ1 increases phosphorylation of p38 MAPK and SAPK/JNK pathways in the activation of caspases

We next sought to identify the signaling pathways responsible for the observed effects of TGF-β1 on the apoptosis and survival of invasive prostate and bladder cancer cells. Our analysis of other major pathways in these cells revealed that treatment with TGFβ1 results in significant increase in the phosphorylation of p38 MAPK and JNK (Fig. 3A–C) as well as in the increase in the expression of cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 in both PC3 and T24 cells, in a dose-dependent manner (Fig. 3A and D–F). Our data indicated that TGFβ1 induced invasive prostate and bladder cancer cell apoptosis *in vitro* involving p38 MAPK and JNK/SAPK-mediated activation of caspases 9, 8 and 3.

3.4. TGFβ1 inhibits colony/ foci formation by PC3 and T24 cells

Since the tumor growth mainly relies on the proliferation and apoptosis of cancer cell lines, we next determined the effect of TGFβ1 on the colony/foci formation of PC3 and T24 cells. Our data revealed that treatment with TGFβ1 significantly inhibited colony formation by PC3 cells by 40–60% for concentration 2.5 and 5 ng/ml of TGFβ1, respectively, compared control (Fig. 4A). TGFβ1 also inhibited colony formation ability of T24 cells by 40–45% for concentrations 2.5 and 5 ng/ml of TGFβ1, respectively, compared to control (Fig. 4B).

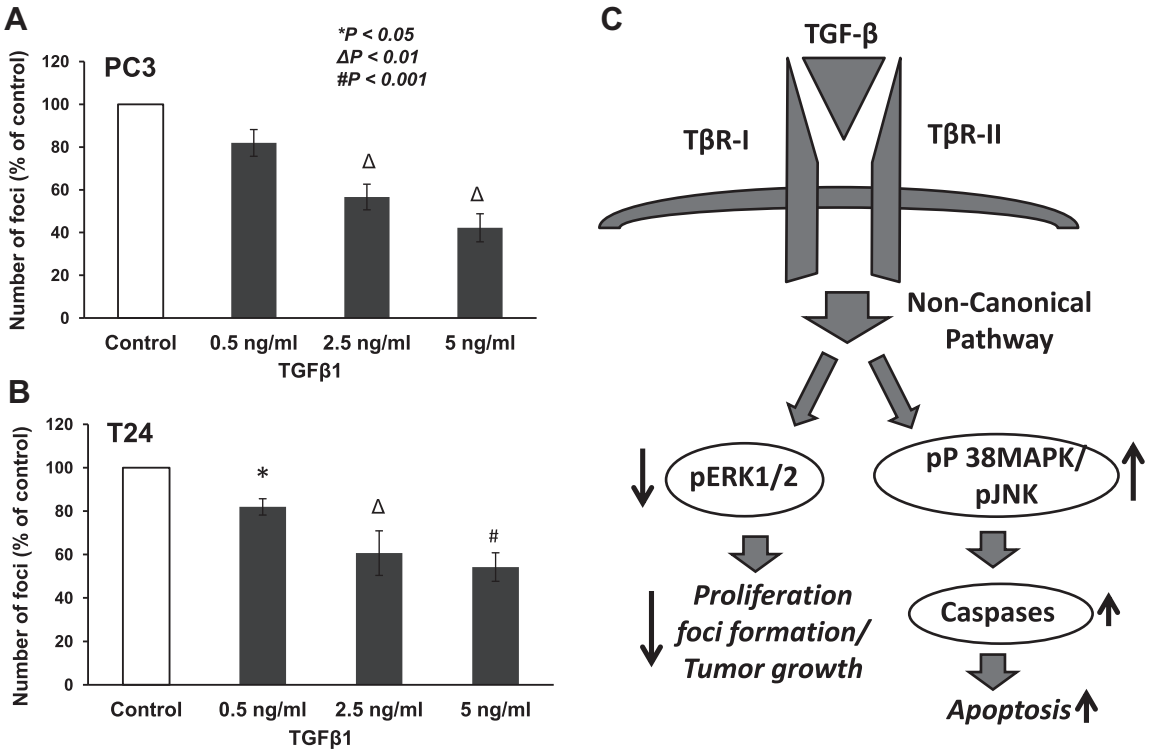

Fig. 4. TGFβ1 inhibited foci formation by PC3 and bladder cancer cells. (A) Bar graph showing foci formation by the PC3 cells treated with control (Serum free DMEM) and TGFβ1 (0.5, 2.5, 5 ng/ml) for 7 days. (B) Bar graph showing foci formation by the T24 cells treated with control (Serum free DMEM) and TGFβ1 (0.5, 2.5, 5 ng/ml) for 7 days. (C) Schematic representation of the working hypothesis.

4. Discussion

In the current study, we demonstrated that treatment with TGFβ1, a growth factor that elicits differential effects on various cell types resulted in enhanced apoptosis and reduced proliferation in invasive prostate (PC3) and bladder cancer (T24) cells *in vitro* in an Akt-independent, but ERK-, p38 MAPK- and JNK/SAPK-dependent activation of caspase-9, caspase-8 and caspase-3.

The role of TGFβ signaling in cancer is highly controversial. Recent publications reports both tumor suppressive [14–16] and tumor promoting effects of TGFβ [17–21]. These controversies, by itself speak about the complexity of TGFβ signaling in cancer and demands extensive research on the cell-specific effects of TGFβ on the tumor and stromal cells. Presence of multiple isoforms, differential effects on various cell types, source of cytokine in a specific tumor tissue, stage of tumor growth, varied effects in the presence and absence of other growth factors etc. may influence the total outcome [22–24]. Hence, individual analysis on the specific effects of TGFβ isoforms on tumor and stromal cells is essential to address the existing discrepancies on the role of TGFβ signaling in cancer. Our study demonstrated that even in invasive prostate and bladder cancer cells, TGFβ1 elicited a tumor suppressive role by inducing apoptosis as well as reducing viability and proliferation.

Akt and ERK are two major kinases that are often hyper-activated in most cancer cells and considered central to many signaling pathways [25,28,30]. While Akt is predominantly a cell survival kinase, it also controls other cell functions such as proliferation, migration and integrin activation [32–34]. On the other hand, proliferation and cell growth of cancer cells are more reliant on the activation of ERK kinases [35–37]. In contrast, p38-MAPK and JNK pathways are activated by environmental stress as well as by the growth factors and cytokines that induce apoptosis, and senescence [26,27,38,39]. We have shown previously that TGFβ1 activates Akt in normal fibroblasts and is necessary for the enhanced survival and expression of extracellular matrix (ECM) proteins by the fibroblasts [40]. However, although TGFβ1 treatment resulted in the apoptosis of both PC3 and T24 cells, surprisingly no significant changes in the phosphorylation of Akt was observed, thus indicating the involvement of an Akt-independent pathway in TGFβ1-induced apoptosis in PC3 and T24 cells. Even though we observed a modest, but significant reduction in the phosphorylation of ERK1/2 in PC3 and T24 cells with TGFβ1 treatment, our further analysis indicated that TGFβ1 is involved in the activation of stress activated kinases such as p38 MAPK and JNK/SAPK in PC3 and T24 cells. This, in turn, was associated with enhanced expression of cleaved caspases-8, -9 and -3, which are well known modulators of intrinsic and extrinsic apoptosis pathways [41]. Although physiological and clinical implications of these effects induced by TGFβ1 in prostate and bladder cancer cells is yet to be determined *in vivo*, our study provides supportive evidence that TGFβ1 plays a direct tumor suppressive role on invasive prostate and bladder cancer cells via inducing apoptosis and inhibiting cell proliferation.

Acknowledgments

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