

JunD Is Required for Proliferation of Prostate Cancer Cells and Plays a Role in Transforming Growth Factor- β (TGF- β)-induced Inhibition of Cell Proliferation*

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TGF- β inhibits proliferation of prostate epithelial cells. However, prostate cancer cells in advanced stages become resistant to inhibitory effects of TGF- β . The intracellular signaling mechanisms involved in differential effects of TGF- β during different stages are largely unknown. Using cell line models, we have shown that TGF- β inhibits proliferation in normal (RWPE-1) and prostate cancer (DU145) cells but does not have any effect on proliferation of prostate cancer (PC3) cells. We have investigated the role of Jun family proteins (c-Jun, JunB, and JunD) in TGF- β effects on cell proliferation. Jun family members were expressed at different levels and responded differentially to TGF- β treatment. TGF- β effects on JunD protein levels, but not mRNA levels, correlated with its effects on cell proliferation. TGF- β induced significant reduction in JunD protein in RWPE-1 and DU145 cells but not in PC3 cells. Selective knockdown of JunD expression using siRNA in DU145 and PC3 cells resulted in significant reduction in cell proliferation, and forced overexpression of JunD increased the proliferation rate. On the other hand, knockdown of c-Jun or JunB had little, if any, effect on cell proliferation; overexpression of c-Jun and JunB decreased the proliferation rate in DU145 cells. Further studies showed that down-regulation of JunD in response to TGF- β treatment is mediated via the proteasomal degradation pathway. In conclusion, we show that specific Jun family members exert differential effects on proliferation in prostate cancer cells in response to TGF- β , and inhibition of cell proliferation by TGF- β requires degradation of JunD protein.

TGF- β is a secreted cytokine that acts as a major anti-proliferative factor in the initial stages of prostate cancer, whereas in the advanced stages of prostate cancer, it acquires pro-oncogenic and pro-metastatic properties (1–3). The TGF- β cytokine exists in three major isoforms: TGF- β 1, TGF- β 2, and

TGF- β 3. TGF- β ligands bind to a heterodimeric receptor complex consisting of two serine-threonine kinase receptors, designated TGF- β type I and type II receptors, and leads to activation of several intracellular pathways (4–7). Concomitant with the switch of TGF- β from growth-inhibitory to growth-promoting signal, expression of TGF- β ligands and receptors is known to be altered in prostate cancer relative to normal prostate cells and is further altered in more aggressive androgen-refractory prostate cancer cells (8, 9). Expression of TGF- β and its family members is also associated with poor prognosis (10–12). It has also been shown that loss of TGF- β type II receptor expression correlates with increasing tumor aggressiveness in prostate cancer (8). However, a significant fraction of prostate cancers become TGF- β -resistant without mutation, deletion, or down-regulation of TGF- β receptors or Smads or other downstream signaling molecules.

Previous studies have shown different effects of TGF- β 1 on proliferation of different prostate cancer cell lines; TGF- β inhibits proliferation of DU145 cells but has no effect on proliferation of PC3 cells in the presence of functional TGF- β receptors and Smad signaling (13–16), indicating differences in signaling mechanisms in two cell lines downstream of receptor-dependent Smad activation that are responsible for differential effects of TGF- β on cell proliferation. TGF- β is a pleiotropic cytokine whose signaling outcome is known to depend on the combination of available contributing factors and active pathways in each target tissue. Previous reports have shown that other intracellular proteins influence TGF- β effects (17–19). It has been well established that extensive interactions exist between the TGF- β signaling pathway and other major signaling pathways, including Wnt, Notch, Hedgehog, JNK, MAPK, and AKT/PI3K (20–24). It is also becoming apparent that TGF- β signaling intersects with several transcription factors and regulators, such as GLI, SOX4, Tieg3/Klf11, Id, and AP-1 proteins (25–29). Many studies have implicated AP-1 proteins in TGF- β signaling (30–32). The AP-1 family consists of dimeric protein complexes composed of different Jun proteins (c-Jun, JunB, and JunD) and four Fos proteins (c-Fos, FosB, Fra1, and Fra2). These proteins form Jun-Jun homodimers and Jun-Fos heterodimers and bind to the 12-O-tetradecanoylphorbol-13-acetate response element, T_GACTCA palindromic sequence, in the promoters of target genes (33, 34). AP-1 proteins have been shown to be involved in cell proliferation, inflammation, differentiation, apoptosis, wound healing, and carcinogenesis (35–38). Among the AP-1 proteins, there is growing evidence that Jun proteins play a major role in the

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This paper is dedicated to the memory of Dr. Natalya Klueva, who was involved in the initiation of these studies but passed away in 2010 after a courageous fight against breast cancer.

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control of cell proliferation and cell death by regulating the expression of cell cycle regulators (39–42). In prostate cancer, expression of AP-1 proteins has recently been associated with disease recurrence and more aggressive clinical outcome (43, 44).

Within the last few years, several studies have suggested the involvement of Jun proteins in prostate cancer growth, survival, and metastasis. For example, c-Jun has been shown to enhance androgen-dependent cell proliferation and inhibition of apoptosis in LNCaP cells (43, 45), but it mediates the action of a metastasis suppressor gene, *KAI1*, in PC3 and DU145 cells (46). JunD, along with Fra1 and Fra2, has also been reported to be essential in prostate cancer proliferation and confers protection against radiation-induced cell death (47). In a recent report, JunB was shown to play an important role in maintaining cell senescence that blocks malignant prostate cell transformations (48) and has been shown to be a potent activator of *KAI1* (49). Jun proteins by themselves or in combination with members of the Fos proteins have also been implicated in the actions of androgens (50, 51), atmospheric pollutants (52), growth factors (53), phytochemicals (54–56), peroxides (57), isothiocyanates (58), glycoproteins (59), and, most recently, proteasome inhibitors (60). AP-1 proteins form multiple homo- and heterodimers, and the composition of these dimers may dictate expression of specific genes involved in specific biological responses. However, the specific roles of individual AP-1 family members in the development and progression of prostate cancer are still largely unknown. Few reports have shown the effects, if any, of TGF- β on AP-1 in prostate cancer (61–63).

The present study was carried out to determine specific roles of Jun family members in TGF- β effects on proliferation in prostate cancer cells. Our results indicate that JunD is essential for proliferation of prostate epithelial cells, and the inhibitory effects of TGF- β on cell proliferation are dependent on degradation of JunD protein in these cells.

Results

Effects of TGF- β 1 on Proliferation of Prostate Cell Lines—We have previously shown that TGF- β 1 exerts differential effects on proliferation of different prostate cancer cell lines (15, 64). To confirm these studies, we first determined the effects of TGF- β 1 on proliferation of prostate cell lines representing specific stages of prostate cancer progression. Cells were plated overnight (1×10^4 cells), serum-starved for 24 h, and then treated with TGF- β 1 (1 and 10 ng/ml) for 18 h. Fig. 1 shows the effects of TGF- β 1 on cell proliferation. As measured by [3 H]thymidine incorporation, TGF- β 1 caused a significant dose-dependent inhibition of cell proliferation in RWPE1 and DU145 cells but not in PC3 and LNCaP cells. Treatment with TGF- β 1 resulted in 30% (1 ng/ml) ($p < 0.05$) and 41% (10 ng/ml) ($p < 0.05$) inhibition in RWPE1 cells and 24% (1 ng/ml) and 38% (10 ng/ml) ($p < 0.05$) inhibition of [3 H]thymidine incorporation in DU145 cells. LNCaP cells, which do not express TGF- β receptor II, served as negative control (Fig. 1). Next, we treated DU145 and PC3 cells with TGF- β 1 (5 ng/ml) to determine the stage of the cell cycle where TGF- β 1 exerted its inhibitory effects. TGF- β 1 treatment led to an elevated num-

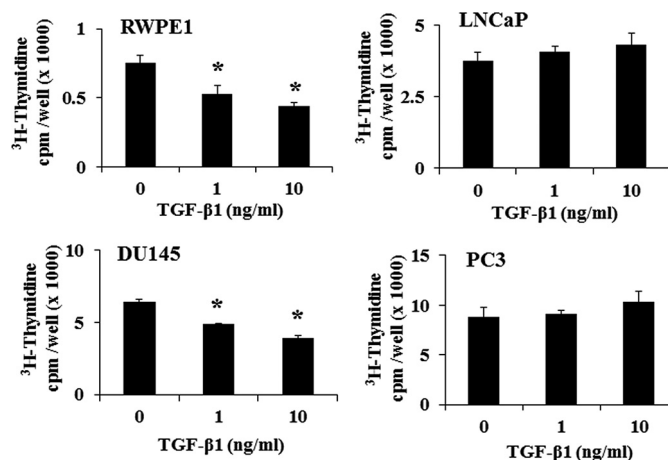


FIGURE 1. Effects of TGF- β 1 on cell proliferation in different prostate cell lines. RWPE1, LNCaP, DU145, and PC-3 cells were treated with different doses of TGF- β 1 (5 ng/ml) for 18 h, and [3 H]thymidine incorporation into DNA was determined during a 4-h period. Each bar represents mean \pm S.D. (error bars) from a representative experiment. *, significantly different from appropriate controls ($p < 0.05$).

ber of cells in the G₁ phase with a concomitant decrease in the number of cells in S phase in DU145 cells (Table 1). Similar treatment in PC3 cells did not cause any changes in cell numbers in different stages of the cell cycle.

Expression of Jun Family Members and Their Regulation by TGF- β 1 in Prostate Cancer Cells—To establish a prostate cancer model system in which to observe any correlation of Jun expression with prostate cancer progression, we first analyzed expression of Jun family members in four prostate cell lines using semiquantitative RT-PCR. Using gene-specific primers to amplify mRNA encoding each member of this protein family, all members of the Jun family were detectable in all four prostate cell lines (Fig. 2A). To examine the presence of Jun proteins in these prostate cell lines, the total cell lysate proteins were analyzed using Western blotting analysis (Fig. 2B). All Jun proteins were differentially expressed in all prostate cell lines. Lower levels of c-Jun and JunD proteins were detected in RWPE1 and LNCaP cells compared with DU145 and PC3 cells. JunB levels, on the other hand, were higher in RWPE1 and PC3 cells.

To investigate a possible role of Jun family members in TGF- β effects on cell proliferation, we determined the effects of TGF- β 1 on expression of Jun family members in DU145 and PC3 cells. DU145 and PC3 cells (1×10^4) were plated overnight and treated with TGF- β 1 (5 ng/ml) for different times. As shown in Fig. 3A, TGF- β 1 had only a minor effect, if any, on the mRNA levels of all Jun family members. However, at the protein level, TGF- β 1 exerted significant effects on the levels of JunD, c-Jun, and JunB in DU145 and/or PC3 cells in a time-dependent manner (Fig. 3, B and C). JunB was significantly up-regulated in both DU145 and PC3 cells after treatment with TGF- β 1 for 2 h (DU145, 4.9 ± 0.56 -fold; PC3, 2.8 ± 0.40 -fold) ($p < 0.05$) and 8 h (DU145, 4.0 ± 0.96 -fold; PC3, 2.5 ± 0.39 -fold) ($p < 0.05$) (Fig. 3C). Interestingly, TGF- β 1 significantly up-regulated c-Jun in PC3 cells starting at 2 h (2.4 ± 0.24 -fold; $p < 0.05$), which stayed elevated for 24 h (1.8 ± 0.46 -fold, $p < 0.05$) but did not have any effect on c-Jun protein levels in DU145 cells.

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TABLE 1

Cell cycle phase distributions of DU145 and PC3 cells after treatment with TGF- β 1 (5 ng/ml)

Cell lines	Treatment	G ₁	S	G ₂ /M
DU145	Control	65.5 \pm 1.5	16.1 \pm 1.5	19.6 \pm 1.8
	TGF- β 1	73.7 \pm 0.2 ^a	11.9 \pm 1.9 ^a	17.0 \pm 2.7
PC3	Control	45.4 \pm 1.3	18.3 \pm 2.1	36.3 \pm 0.8
	TGF- β 1	46.3 \pm 2.0	20.6 \pm 5.4	33.1 \pm 3.4

^a $p < 0.05$, significantly different from appropriate controls.

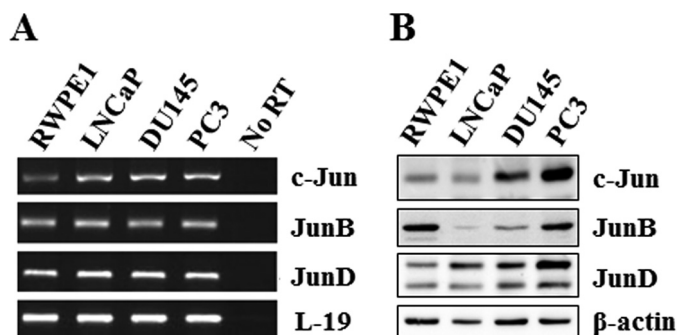


FIGURE 2. Basal expression of Jun family members in prostate cell lines. Cells were cultured under normal conditions for 24 h and lysed, and total RNA and total proteins were prepared. *A*, total RNAs were analyzed for expression of mRNA for individual Jun family members by RT-PCR. *B*, Jun family member protein levels were determined by specific Western blotting analysis. L-19 and β -actin were used as controls in RT-PCR and Western blotting analysis, respectively.

TGF- β 1 caused a significant down-regulation of JunD protein in DU145 cells starting at 8 h (0.6 \pm 0.18-fold, $p < 0.05$) but not in PC3 cells. TGF- β induced an increase in the levels of c-Jun and JunB and caused a significant decrease in the levels of JunD in RWPE-1 cells similar to those seen in DU145 cells, but not in PC3 cells (data not shown). These results suggested that down-regulation of JunD protein levels and/or up-regulation of c-Jun and JunB protein levels in DU145 cells may play a role in TGF- β 1 effects on inhibition of cell proliferation.

Role of Jun Proteins in Proliferation of Prostate Cancer Cells—To determine the possible role of individual Jun proteins in proliferation of prostate cancer cells and whether they play a role in the effects of TGF- β on cell proliferation, we used specific siRNAs to transiently knock down individual Jun proteins in DU145 and PC3 cells (Fig. 4A). Control siRNAs were also transfected to serve as negative control. Expression of Jun proteins was determined by Western blotting analysis, which confirmed a marked down-regulation of the corresponding Jun proteins in comparison with the cells transfected with the control siRNA (Fig. 4A). We analyzed proliferation of DU145 and PC3 cells after knockdown of individual Jun proteins (Fig. 4A). Transfection of JunD siRNA into DU145 or PC3 cells caused a significant reduction in proliferation of both DU145 (82% inhibition, $p < 0.05$) and PC3 (71% inhibition, $p < 0.05$) cells. On the other hand, knockdown of either c-Jun or JunB had no significant effect on cell proliferation in both cell lines. These results suggested that JunD is required for proliferation of both DU145 and PC3 cells.

To determine whether TGF- β 1 can further inhibit cell proliferation after siRNA-mediated repression of JunD, DU145 and PC3 cells were treated with TGF- β 1 (5 ng/ml) for 48 h after JunD siRNA transfection (Fig. 4B). As expected, after transfection

with control siRNA, we observed a significant decrease ($p < 0.05$) in proliferation of DU145 cells and no effect in PC3 cells after treatment with TGF- β 1. In both cell lines, transfection with JunD siRNA resulted in significant inhibition of proliferation, and TGF- β 1 treatment did not cause further decrease in cell proliferation. These results suggest that TGF- β 1 effects on proliferation of DU145 cells may be due to its effects on down-regulation of JunD protein in these cells.

Knockdown of JunD and TGF- β Treatment Exert Similar Effects on Cell Cycle Arrest in the G₁ Phase—To demonstrate that TGF- β effects on cell cycle arrest are mediated via down-regulation of JunD, we compared the effects of JunD knockdown with TGF- β 1 treatment on cell cycle machinery in DU145 cells by FACS analysis. Knockdown of endogenous JunD in DU145 cells resulted in an accumulation of cells in G₁ fraction and a corresponding reduction in cells in S and G₂/M phases as compared with the siControl (Fig. 5A). These effects are similar to those presented for TGF- β 1 treatment shown in Table 1.

We also determined the levels of several proteins that play a role in cell cycle regulation in DU145 cells after knockdown of endogenous JunD or after treatment with TGF- β 1 for 24 h. We have previously shown that TGF- β 1 induces a decrease in c-Myc and Id1 proteins and an increase in p21 in DU145 cells (15) as a part of its inhibitory effects on cell proliferation.

As shown in Fig. 5B, treatment with TGF- β 1 for 24 h or knockdown of endogenous JunD exerted identical effects on several cell cycle-associated proteins. There was a significant decrease in the levels of c-Myc, Ki-67, and Id-1 proteins, whereas there was a significant increase in the levels of p21. Both treatments did not affect the levels of p27 in DU145 cells. Interestingly, there was no significant decrease in the levels of cyclin D1, and there was a slight increase in cells treated with TGF- β 1.

JunD Knockdown Does Not Affect Cell Viability—To determine whether or not the knockdown of JunD results in decreased cell viability, we used a TUNEL assay to assess apoptosis in JunD siRNA transfected in DU145 cells (Fig. 5, C1 and C2). We observed very few (~1–2%) apoptotic positive cells in both siControl and JunD siRNA-transfected cells. In addition, we also determined the integrity of total nuclear DNA by DAPI staining in both treatments (Fig. 5, C3 and C4). Again, there was no significant differences in the nuclear DNA between the treatments, indicating that knockdown of JunD has no effect on viability of DU145 cells.

TGF- β 1 Does Not Affect JunD Phosphorylation or the SAPK/JNK Pathway—To determine whether TGF- β 1 induces phosphorylation of JunD in DU145 cells prior to its degradation, DU145 cells were treated with TGF- β 1 (5 ng/ml) at different time points and analyzed for the levels of phospho-JunD and total JunD by Western blotting analysis. As shown in Fig. 6A, TGF- β 1 did not induce phosphorylation of JunD. In fact, TGF- β 1 degraded basal phospho-JunD, mirroring total JunD degradation. We also examined whether TGF- β 1 activates the SAPK/JNK pathway. As shown in Fig. 6B, TGF- β 1 did not induce phosphorylation of SAPK/JNKs.

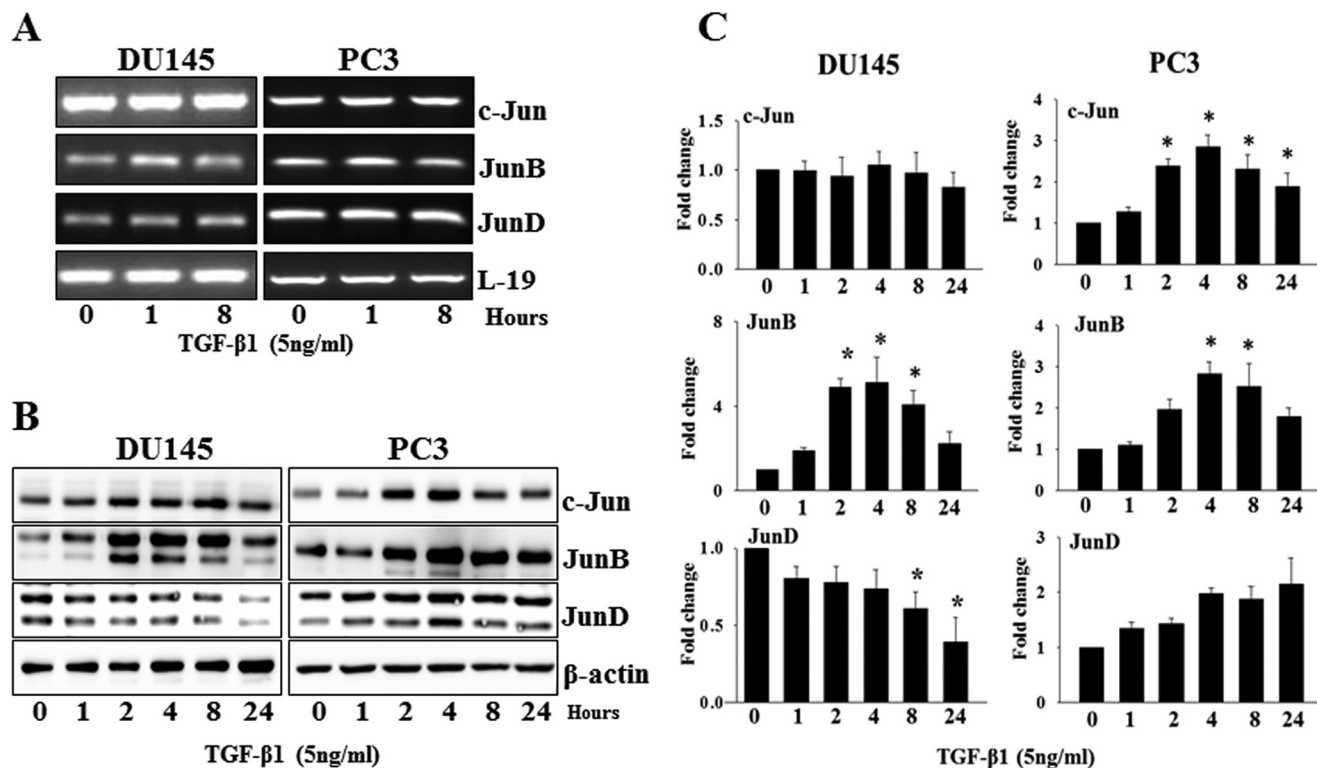


FIGURE 3. **Effects of TGF- β 1 on expression of Jun family members in DU145 and PC3 cells.** A, levels of mRNA of c-Jun, JunB, and JunD in DU145 and PC3 cell after treatment with TGF- β 1 (5 ng/ml) as determined by RT-PCR. B, the protein levels of c-Jun, JunB, and JunD from whole cell lysates of DU145 and PC3 cells after treatment with TGF- β 1 (5 ng/ml) at different time points as determined by Western blotting analysis. C, quantitative analysis of relative levels of c-Jun, JunB, and JunD proteins in DU145 and PC3 cells after treatment with TGF- β 1. Normalization was performed relative to the signal obtained with β -actin. Each bar represents mean \pm S.E. (error bars) ($n = 3$). *, significantly different from untreated controls ($p < 0.05$).

Generation of Stable DU145 Cell Lines Overexpressing Jun Proteins and Effects of TGF- β 1 on Their Cell Proliferation—To confirm the role of JunD in proliferation of prostate cancer cells, pcDNA3.1 constructs carrying c-Jun, JunB, or JunD were stably transfected into DU145 cells, and stable transfectants of DU145 overexpressing c-Jun, JunB, or JunD cells were generated. Empty vector (pcDNA3.1) was also transfected into DU145 cells to serve as a vector control. Multiple cell lines were selected for each transfection, and their Jun protein levels were analyzed by Western blotting analyses (Fig. 7A). Three lines overexpressing each Jun protein were selected for cell proliferation assays (Fig. 7B). Cells were plated at an initial density of 1×10^5 and counted manually after 4 days. As shown in Fig. 7B, DU145 cell lines overexpressing either c-Jun or JunB protein exhibited similar or decreased proliferation compared with the controls. In contrast, all cell lines overexpressing JunD exhibited a significant increase in cell proliferation rate (2.7 ± 0.07 -fold (D1), 3.4 ± 0.34 -fold (D5), 4.1 ± 0.07 -fold (D6); $p < 0.05$). To determine the effects of TGF- β 1 on the proliferation of DU145-overexpressing Jun proteins, cells were plated overnight at an initial density of 1×10^5 cells/well and were treated the next day with TGF- β 1 (5 ng/ml). After 4 days, cells were counted, and the data were analyzed. As shown in Fig. 7C, TGF- β 1 significantly inhibited proliferation of DU145 cells transfected with the empty vector (45% inhibition, $p < 0.05$). It also caused an inhibition of proliferation of DU145 cells overexpressing JunB and c-Jun proteins, which was not statistically significant. Surprisingly, TGF- β 1 also caused a significant inhi-

biton of proliferation in DU145 cells overexpressing JunD protein compared with the untreated cells (40% inhibition, $p < 0.05$).

JunD Promotes Colony Formation in DU145 Cells—To determine differential effects of Jun proteins on colony formation, DU145 cells overexpressing Jun proteins and carrying empty vector were cultured in soft agar and allowed to form colonies. Each of the DU145 sublines was able to form colonies; however, DU145 cells that overexpressed JunD showed greater (2.7 -fold) ability to grow in soft agar (30.3 ± 3.2 clones, $p < 0.001$ clones) in comparison with the vector-only DU145 cells (11.0 ± 0.8 clones) or the JunB (15.2 ± 1.6 clones)- and c-Jun (10.2 ± 0.5 clones)-transfected DU145 cells (Fig. 8).

Down-regulation of JunD by TGF- β 1 Is Dose-dependent and Is Mediated through TGF- β Receptors and via Ubiquitination and Proteasome Degradation—The effect of TGF- β 1 on down-regulation of JunD in DU145 cells was already significant at 1 ng/ml TGF- β 1 as shown in (Fig. 9A). No significant additional effect can be observed with higher TGF- β 1 dosages. Preincubation with inhibitors of TGF- β RI (SB31542) and RII (LY2157299) blocked TGF- β 1 induced reduction in JunD levels in DU145 cells, even potentiating JunD levels (SB31542, 1.3 ± 0.13 -fold, $p < 0.05$; LY2157299, 1.6 ± 0.26 -fold, $p < 0.05$), indicating that JunD degradation was mediated through the classical TGF- β and Smad3 signaling mediated by TGF- β receptors (Fig. 9B).

To determine whether down-regulation of JunD protein in DU145 cells in response to TGF- β treatment involves protea-

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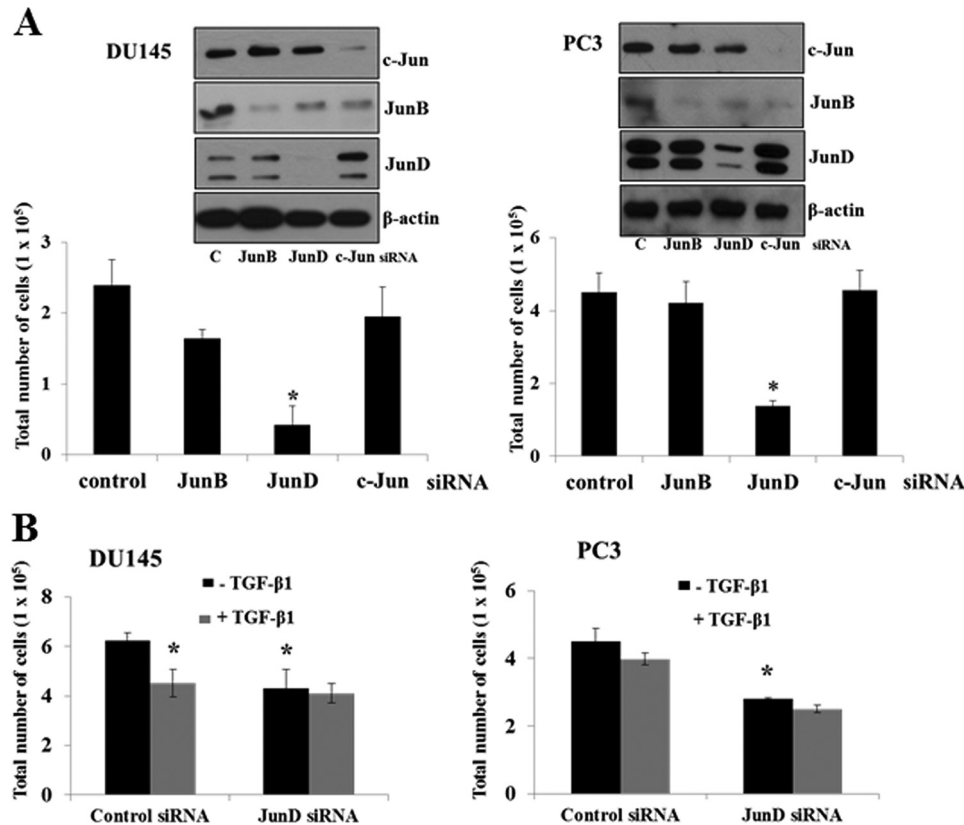


FIGURE 4. Role of individual Jun family members in proliferation of DU145 and PC3 cells. DU145 and PC3 cells were transfected with either control or specific Jun protein siRNA to knock down expression of individual family members. *A*, cell proliferation in DU145 and PC3 cells after transfection with control (siControl-A), or JunB, JunD, or c-Jun siRNA. Each bar represents mean \pm S.E. (error bars) ($n = 3$). *, significantly different when compared with appropriate controls ($p < 0.05$). Levels of Jun proteins after transfection with control siRNA and specific Jun siRNAs were determined by Western blotting analysis (inset). *B*, cell proliferation in DU145 and PC3 cells after transfection with control or JunD siRNA and treatment with TGF- β 1 (5 ng/ml) for 4 days.

somal degradation, we tested the effect of proteasome inhibitor, MG132, on the levels of JunD protein following TGF- β 1 treatment (Fig. 10A). After a 2-h pretreatment with MG132 (25 μ M), DU145 cells were treated with TGF- β 1 (5 ng/ml) for 8 h. JunD levels in DU145 cells, as expected, declined after 8 h (0.70 \pm 0.14-fold, $p < 0.05$) of TGF- β 1 treatment. MG132 itself raised the JunD basal level in DU145 cells (1.74 \pm 0.33-fold, $p < 0.05$). However, TGF- β 1 treatment failed to cause a reduction in the levels of JunD in the presence of MG132, suggesting that TGF- β 1 down-regulates JunD levels via proteasomal degradation. Experiments described earlier showed that TGF- β 1 inhibits proliferation in DU145 cells overexpressing JunD (Fig. 7C), suggesting that TGF- β 1 treatment may result in proteasomal degradation of JunD in these cells as well. To confirm this notion, DU145 cells overexpressing JunD (D6) were plated overnight (4×10^5), pretreated with MG132 for 2 h, and then treated with TGF- β 1 for 8 h. TGF- β 1 was able to down-regulate JunD protein in D6 cells (0.41 \pm 0.16-fold, $p < 0.05$), and MG132 pretreatment inhibited these effects of TGF- β 1 (Fig. 10B).

To examine the effects of TGF- β on ubiquitination of proteins, total cell lysates from DU145 cells were treated with and without TGF- β 1 in the presence and absence of MG132 and analyzed by immunoblotting with anti-ubiquitin antibody (Fig. 10C). The majority of ubiquitinated protein was observed in cells treated with MG132, indicating that all proteins conjugated with ubiquitin were prevented from being degraded by

the proteasome. To examine the ubiquitination of JunD, total cell lysates from the same experiment were subjected to immunoprecipitation using JunD antibody, and the resulting precipitates were analyzed by Western blotting analysis with anti-ubiquitin antibody (Fig. 10D). The majority of polyubiquitinated JunD was detected in DU145 samples treated with TGF- β 1 in the presence of MG132 compared with those without TGF- β 1 treatment. These results confirmed that down-regulation of JunD by TGF- β is achieved through ubiquitination followed by proteasomal degradation.

Discussion

In this study, we demonstrate for the first time that JunD plays an essential role in the proliferation of prostate epithelial cancer cells. We also show that inhibitory effects of TGF- β on the proliferation of prostate epithelial cells depend on proteasomal degradation of JunD, and failure of JunD degradation may induce resistance to inhibitory effects of TGF- β in advanced stages of prostate cancer.

The AP-1 family proteins have been studied for many years, and their involvement in cell proliferation, differentiation, differentiated functions, and apoptosis has been documented extensively (40, 65–67). It has also been suggested by several studies that expression of AP-1 proteins is associated with a more aggressive clinical outcome in prostate cancer (47, 68, 69). Most of these studies have, however, focused on the expression and/or function of activated AP-1 complex containing c-Jun

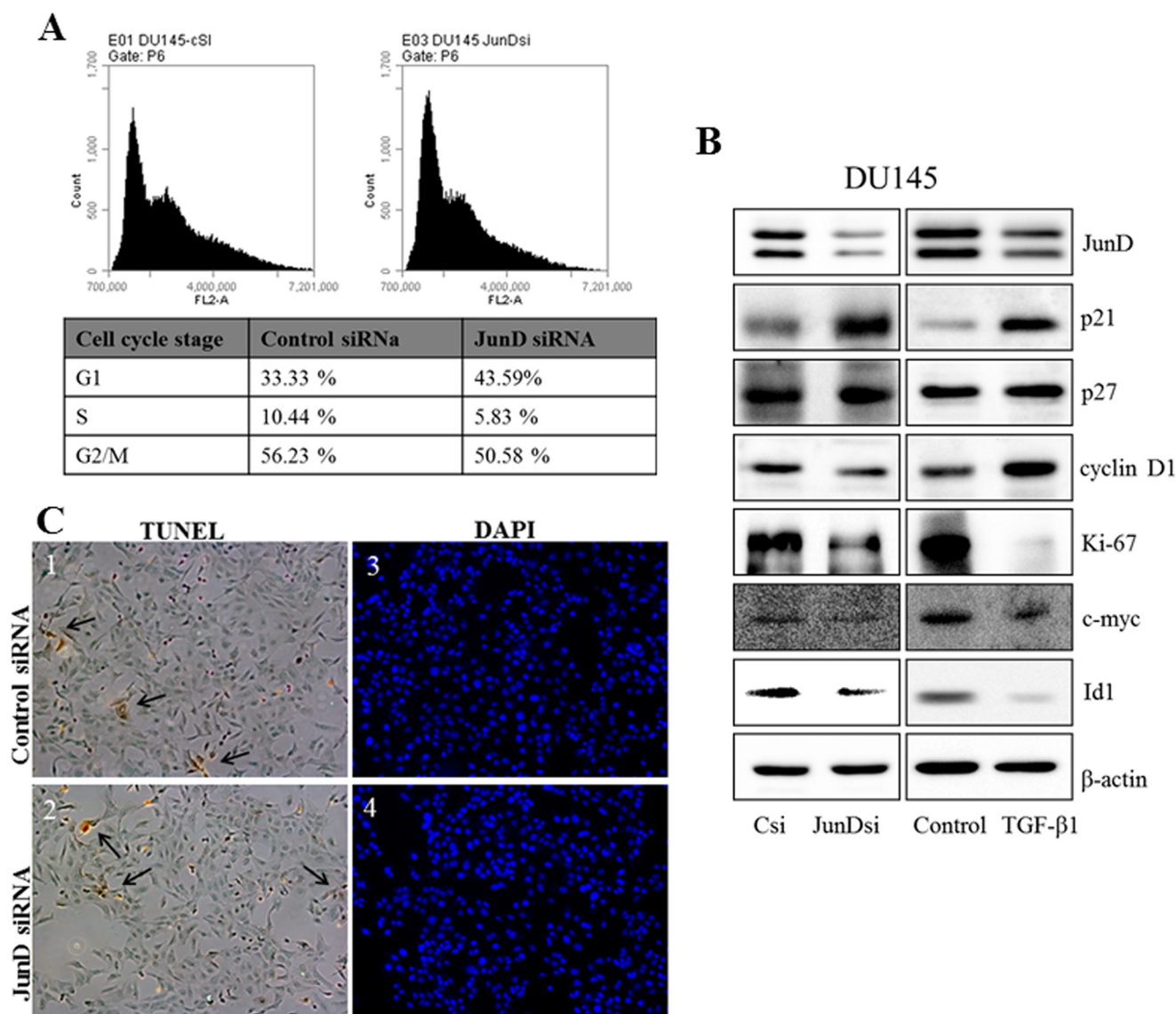


FIGURE 5. Mechanism of inhibition of proliferation in DU145 cells by JunD. *A*, DU145 cells were transfected with either control (siControl-A) or JunD siRNA. After 72 h, cells were fixed with 70% ethanol and stained with propidium iodide (50 μ g/ml). The DNA contents of the cells were measured by flow cytometry, and the percentage distribution of the cells in the G₁, S, and G₂/M phases was determined. *B*, DU145 cells were either transfected with JunD siRNA for 72 h with appropriate controls or treated with TGF- β 1 (5 ng/ml) for 24 h. A decrease in JunD levels in the JunD knockdown is compared with the decrease in the levels of JunD after treatment with TGF- β 1. Protein levels for p21, p27, cyclin D1, Ki-67, c-myc, and Id-1 were determined in total cell lysates by Western blotting. *C*, assessment of apoptosis in DU145 cells transfected with JunD siRNA (*bottom*) and siControl-A (*top*). DU145 cells were transfected with JunD siRNA and control siRNA for 72 h and then subjected to the TUNEL assay and DAPI staining. The TUNEL apoptosis assay (*C1* and *C2*) was carried out to assess apoptotic cells. The *brown stain* indicates apoptotic cells, whereas methyl green was used as a nuclear counter stain. DAPI staining (*C3* and *C4*) was carried out to reveal any condensation and formation of dense bodies characteristic of apoptosis.

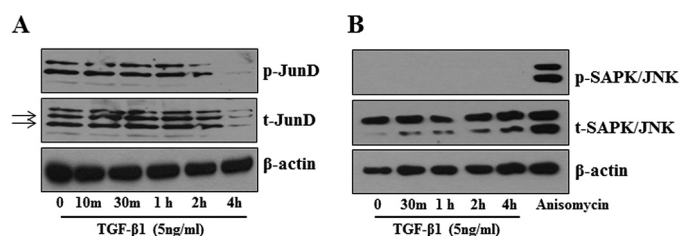


FIGURE 6. TGF- β 1 did not induce JunD or SAPK/JNK phosphorylation in DU145 cells. *A*, DU145 cells were treated with TGF- β 1 (5 ng/ml) for 0 min, 10 min, 30 min, 1 h, 2 h, and 4 h before cell lysis. Protein levels of phosphorylated (*p*-) and total (*t*-) JunD and β -actin were determined using Western blotting analysis. *B*, DU145 cells were treated the same way with TGF- β 1 and then analyzed by Western blotting analysis for phosphorylated and total JNK and β -actin. Anisomycin (250 ng/ml) was included as a positive control for JNK activation.

and c-Fos (70). Consequently, the specific functions of individual AP-1 family members and various homo- and heterodimers in the regulation of specific cellular processes remain largely unknown. Therefore, in the current study, we focused on elucidating the comparative roles of Jun family of proteins in prostate cancer cell proliferation. Our results showed that all Jun family members are constitutively expressed in various prostate cell lines at the mRNA level but exhibited differences in the levels of Jun proteins in various cell lines, indicating differential regulation of proteins in different cell lines. The specific knockdown of individual Jun family members by specific siRNA showed that JunD plays an essential role in proliferation of both DU145 and PC3 cells, whereas c-Jun and JunB knockdown had

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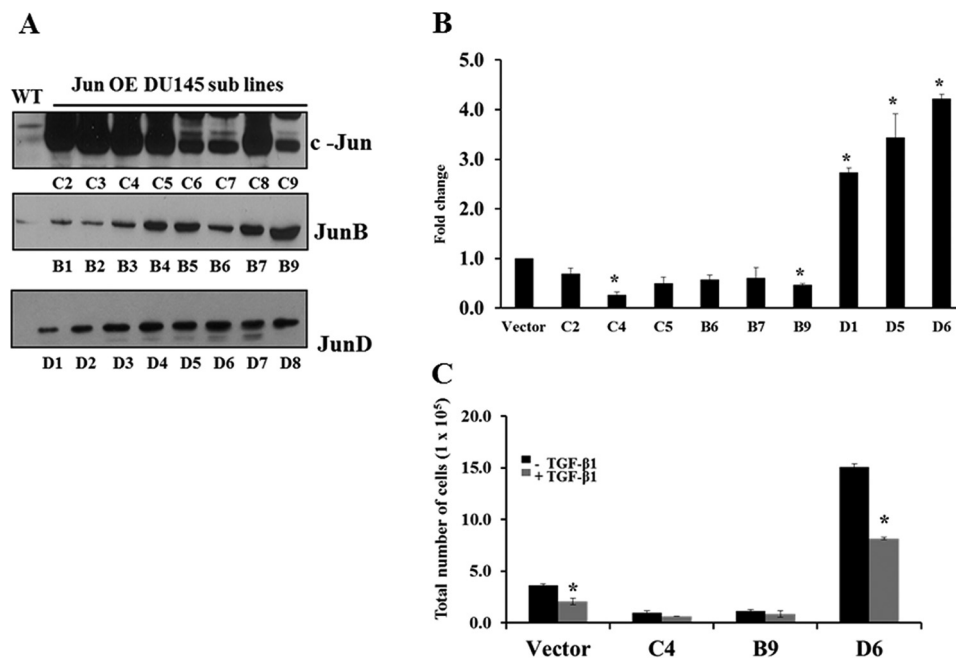


FIGURE 7. **Effects of overexpression of individual Jun proteins on cell proliferation in DU145 cells.** *A*, Western blotting analyses showing levels of c-Jun, JunB, and JunD proteins in DU145 subline cells after transfection with control (WT) or individual Jun plasmids. *B*, fold change in the cell proliferation rate of selected DU145 lines overexpressing c-Jun (C2, C4, and C5), JunB (B6, B7, and B9), and JunD (D1, D5, and D6). DU145 cells containing the empty vector were used as controls. *C*, representative stable DU145 cell lines expressing each Jun protein were treated with TGF- β 1 (5 ng/ml) for 4 days, and total cell numbers were counted. Each bar represents mean \pm S.E. (error bars) ($n = 3$). *, significantly different when compared with appropriate controls ($p < 0.05$).

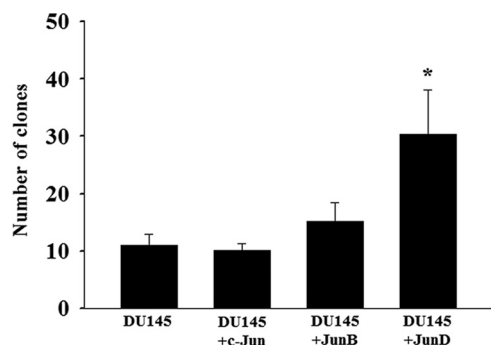


FIGURE 8. **Stable overexpression of JunD in DU145 enhances the anchorage-independent growth of prostate cancer cell colonies on soft agar.** DU145 overexpressing c-Jun, JunB, or JunD and DU145 cells containing empty vector, pcDNA3.1, were plated in 0.4% soft agar in complete growth medium for 21 days, and cell colonies were counted. Each bar represents mean \pm S.E. (error bars) from three independent wells.

minimal effects on proliferation in these cell lines. The essential role of JunD in proliferation of prostate cancer cells suggests that an AP-1 complex containing a Jun-Jun homodimer or Jun-Fos heterodimer containing JunD is responsible for positive regulation of proliferation of these cells. Because c-Jun and JunB knockdown did not influence cell proliferation, it is logical to assume that they do not form dimers with JunD to induce cell proliferation. Therefore, a dimer containing JunD-JunD or Jun-Fos may be responsible for these effects. Additional studies are needed to identify the JunD dimer partner that is required for induction of cell proliferation in prostate cancer cells. The essential role of JunD was also confirmed by our experiments where we generated DU145 cell lines overexpressing individual Jun family members. Whereas JunD overexpression resulted in a significant increase in the proliferation rate, overexpression of c-Jun or JunB resulted in a reduced proliferation rate. These

studies were further supported by increased colony formation in DU145 cells overexpressing JunD compared with DU145 cells overexpressing c-Jun or JunB. These results suggest that individual Jun family members exert distinct and even opposite effects on proliferation of prostate cancer cells. Our results are very similar to recently reported findings indicating that the inhibition of JunD results in induction of several molecules, such as *GADD* genes and JNKs, ultimately leading to prostate tumor cell death and inhibition of tumor development (71). These results are also supported by recent studies demonstrating that hydrogen peroxide-mediated proliferation is due to binding of Fra-1/JunD and phospho-c-Jun to the HARP promoter sites (57). JunD was also reported to be involved in migration and invasion of prostate cancer cells by inducing the expression of metalloproteinase-1-mediated by the Wnt5a signaling pathway (72). Interestingly, this action of JunD on prostate cancer cell migration was reported to be opposite to the effect of JunB and c-Jun (73). JunD plays important regulatory roles in both androgen-dependent and androgen-independent prostate cancer cells by functioning as a co-activator for the androgen receptor to mediate androgen-induced oxidative stress in LNCaP cells (51, 74) or by interacting with the NF κ B pathway to induce IL-6, an important mediator of metastatic, hormone-refractory prostate cancer (75). On the other hand, Church *et al.* (50) reported that cell growth inhibition in LNCaP cells after treatment with androgens resulted in a concomitant increase in JunD expression. This suggests that AP-1 regulation of cell proliferation depends on the cellular "context" and on the combination of available contributing factors and active pathways in each target tissue.

TGF- β is a multiple-function protein that acts as a tumor suppressor in normal epithelial cells and in early stage cancer

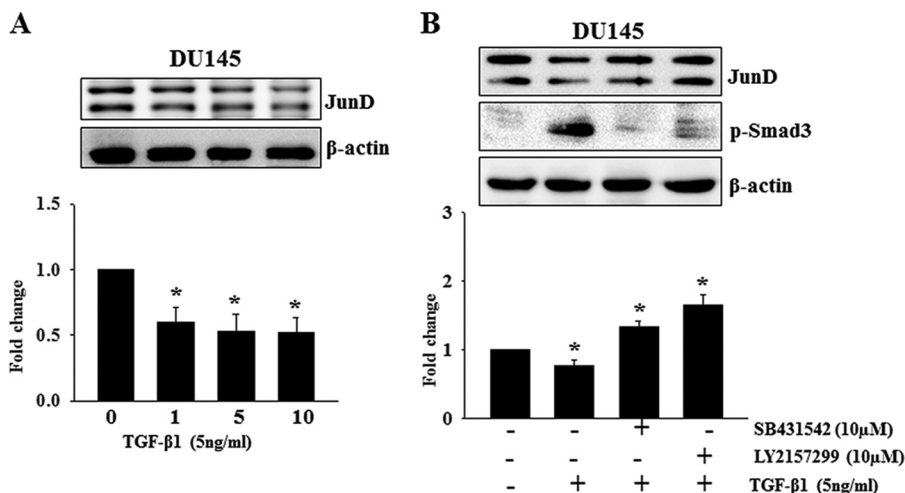


FIGURE 9. **Down-regulation of JunD by TGF- β 1 is dose-dependent and is mediated through TGF- β receptors.** *A*, effects of different doses of TGF- β 1 (0, 1, 5, and 10 ng/ml) on JunD levels in DU145 cells treated for 8 h. *B*, effects of TGF- β 1 (5 ng/ml) on JunD levels in DU145 cells in the presence and absence of inhibitors of TGF- β RI (SB431542; 5 μ M) and/or TGF- β RII (LY2157299; 10 μ M). Quantitative differences in JunD levels after different treatments are presented in the bar graphs. Each bar represents mean \pm S.E. (error bars) from three independent experiments. *, significantly different when compared with controls ($p < 0.05$).

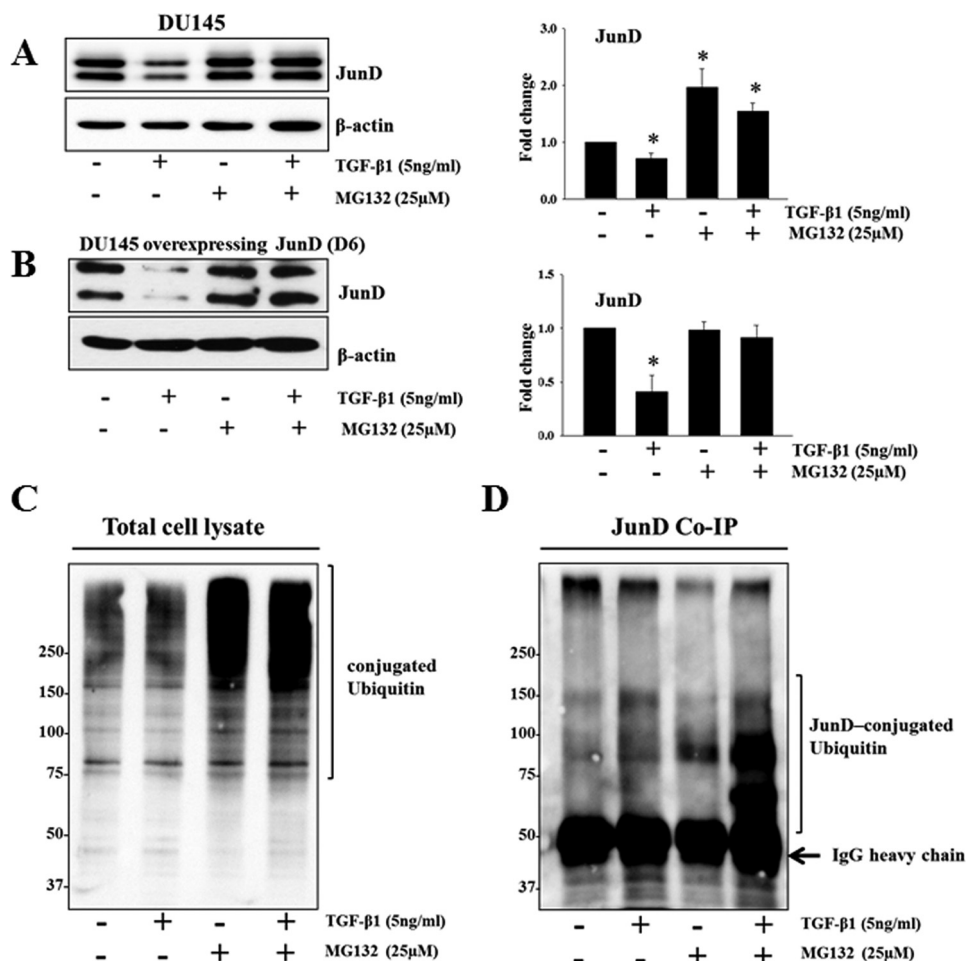


FIGURE 10. **TGF- β 1 induces ubiquitination and proteasomal degradation of JunD.** Western blotting analyses of JunD and β -actin in DU145 (*A*) and DU145 cells overexpressing JunD (*B*). Cells were pretreated with MG132 (25 μ M) for 2 h and then treated with TGF- β 1 (5 ng/ml) for 8 h. Quantitative analysis of JunD in DU145 and DU145 cells overexpressing JunD after treatment with TGF- β 1 in the presence or absence of MG132 is shown in bar graphs. Each bar represents mean \pm S.E. (error bars) ($n = 3$). *, significantly different when compared with controls ($p < 0.05$). *C*, equal amounts of total cell protein from DU145 cells treated with and without TGF- β 1 for 8 h in the presence or absence of MG132 (25 μ M, 2 h pretreatment) were subjected to SDS-PAGE. All ubiquitinated proteins were detected by Western blotting with anti-ubiquitin antibody. *D*, total cell lysates from different treatments were immunoprecipitated (Co-IP) using anti-JunD antibody, and the immunoprecipitates were resolved on an SDS-PAGE and immunoblotted with anti-ubiquitin antibody.

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cells; cells in later stages of cancer become resistant to its growth-inhibitory effects (13, 76). In advanced stages of the disease, TGF- β acts as a tumor promotor by virtue of its effects on epithelial to mesenchymal transition, cell migration and invasion, angiogenesis, and metastasis (77, 78). Our previous studies and present results show that TGF- β inhibits proliferation in RWPE-1 and DU145 cells, whereas PC3 cells are resistant to these growth-inhibitory effects (15). On the other hand, TGF- β induces cell migration and invasion in PC3 cells but does not affect migration and invasion in DU145 cells (16, 29, 64). Because both DU145 and PC3 cells express functional TGF- β receptors and Smad signaling (15, 29) but exhibit differential responses to TGF- β treatment, we have exploited these cell lines to understand differences in the signaling mechanism that may act either downstream or parallel to Smad signaling and are responsible for their differential responses to TGF- β . Our results show that TGF- β induces a reduction in JunD protein levels in DU145 cells but not in PC3 cells. Because JunD is required for cell proliferation, these results suggest that reduction of JunD levels in DU145 cells in response to TGF- β may lead to reduction in cell proliferation in these cells. On the other hand, the lack of TGF- β effects on PC3 cell proliferation may be due to their resistance to TGF- β -induced reduction of JunD levels. We previously showed that TGF- β caused a down-regulation of Id1, a transcriptional regulator, in DU145 cells but not in PC3 cells (15). Because Id1 knockdown by siRNA in both DU145 and PC3 cells resulted in decreased proliferation in both cell lines, we concluded that inhibitory effects of TGF- β required down-regulation of Id1 in prostate cancer cells (15). Our current study shows that knockdown of JunD affected the expression of several cell cycle regulatory proteins and also caused a reduction in the expression of Id1. These results indicate that JunD may regulate the expression of Id1 which, in turn, may be required for cell proliferation. Our previous studies and our present results identify at least one distinct signaling cascade, downstream of Smad2/3 activation, which may be required for inhibitory effects of TGF- β on cell proliferation and which may be altered in the cells that become resistant to growth-inhibitory effects of this cytokine. Treatment with TGF- β had no effect on *JunD* mRNA levels in both DU145 and PC3 cells but caused a significant decrease in JunD protein levels in DU145 cells. Furthermore, the effects of TGF- β did not involve phosphorylation of JunD or the activation of the JNK/SAPK pathway, suggesting that reduced intracellular protein levels are primarily responsible for the inhibitory effects of TGF- β on cell proliferation. Interestingly, TGF- β treatment also resulted in down-regulation of JunD protein and inhibition of cell proliferation in DU145 cell lines overexpressing JunD, suggesting post-transcriptional regulation of JunD expression. The reduction in JunD protein levels was inhibited in the presence of proteasomal inhibitor, which also resulted in an increase in the levels of ubiquitinated JunD protein in TGF- β -treated cells, suggesting that TGF- β -induced reduction in JunD levels is due to ubiquitination followed by proteasomal degradation of the protein. The ubiquitin-proteasome pathway for targeted degradation of proteins plays critical roles in a variety of biological processes, such as cell cycle progression, signal transduction, transcriptional regulation, receptor down-regu-

lation, and endocytosis (79, 80). The ubiquitin-proteasome pathway tightly regulates TGF- β family signaling (81–84). In this pathway, E3 ubiquitin ligases play a crucial role in the recognition and degradation of target proteins by the 26S proteasomes (85). Smad degradation regulates TGF- β family signaling (86, 87). Our laboratory has previously shown that TGF- β -induced degradation of Ski protein, a co-repressor of Smad2/3, mediated by the proteasomal pathway is required for TGF- β -induced biological responses in prostate cancer cells (16). The identity of specific E3 ligase(s) and other components involved in the degradation of JunD protein in response to TGF- β is currently not known, and it is plausible to assume that alterations in these components may result in lack of responsiveness to growth-inhibitory effects of TGF- β in advanced stages of prostate cancers.

On the basis of the results of the present study, we conclude that JunD plays a critical role in the proliferation of prostate cancer cells and may provide a significant therapeutic target for the treatment of prostate cancers. Our studies also show that c-Jun and JunB play a minimal role in induction of cell proliferation in prostate cancer. In addition, proteasomal degradation of JunD in response to TGF- β treatment is a prerequisite for growth-inhibitory effects of this cytokine, and alterations in the proteasomal pathway leading to lack of TGF- β effects on degradation of JunD may be partially responsible for resistance to growth-inhibitory effects of TGF- β in advanced stages of cancer. These phenomena may underlie many cases of prostate cancer with an otherwise intact TGF- β signaling mechanism and may represent an event earlier than the loss of TGF- β receptors in prostate cancer progression.

Experimental Procedures

Chemicals and Reagents—Recombinant human TGF- β 1 was purchased from PeproTech (Rocky Hill, NJ). Proteasome/calpain inhibitor, MG132, was acquired from Calbiochem. Inhibitors of TGF- β RI (SB431542) and RII (LY2157299) were purchased from Tocris Biosciences (Ellisville, MO) and Xcess Biosciences Inc. (San Diego, CA), respectively. Anisomycin was purchased from Calbiochem.

Human Jun cDNAs (c-Jun, JunB, and JunD) subcloned into the pcDNA3.1(–) Mycstop expression vector along with the empty vector were gifts from Dr. Curt Pfarr (Texas Tech University, El Paso, TX). Antibodies and siRNAs for Jun proteins and antibodies against p21, p27, cyclin D1, c-Myc, and Id1 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The antibodies against ubiquitin, phospho-Smad3, phospho-JunD, phospho-SAPK/JNK, and total SAPK/JNK were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The antibody against Ki-67 was purchased from Calbiochem. Anti- β -actin (clone AC-15) antibody was purchased from Sigma-Aldrich. Goat anti-rabbit IgG HRP was purchased from Life Technologies, Inc. Anti-mouse IgG HRP was obtained from GE Healthcare.

Cell Lines and Cell Culture Conditions—Human prostate cell lines (RWPE1, LNCaP, DU145, and PC3) were obtained from American Type Cell Culture Collection (Manassas, VA). All cells were cultured in the recommended growth media at 37 °C

with 5% CO₂ and 100% humidity as described previously (29, 64).

Cell Proliferation Assays—Cell growth assays were performed using three techniques: thymidine incorporation, flow cytometry, and manual cell counting. Each assay was performed at least three times. For thymidine incorporation, cells were seeded in a 24-well plate at a density of 4×10^4 cells/well and maintained with 5% fetal bovine serum (FBS) overnight. Cells were serum-starved for 24 h and treated with different doses of TGF- β 1 (0, 1, and 10 ng/ml) in the presence of 1% FBS for 18 h. Cells were then pulse-labeled for 4 h with 1 μ Ci/ml [³H]thymidine (GE Healthcare), and the radioactivity incorporated into DNA was determined by liquid scintillation counting as described previously (64). Flow cytometry was used to determine the cell cycle distribution of cells. Cells were plated at an initial density of 1×10^6 cells/well overnight and then serum-starved for 24 h. After treatment with TGF- β 1 (5 ng/ml) for 16 h, cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and fixed with cold 70% ethanol for 1 h on ice. Cells were spun down and were incubated with propidium iodide (50 μ g/ml) (Molecular Probes, Inc., Eugene, OR) and RNase A (Bio-Rad) at room temperature for 30 min before they were analyzed by flow cytometry. Cell cycle phase distribution was determined from 1×10^4 cells using a BD Accuri Cytometer (Ann Arbor, MI), following the manufacturer's instructions. For manual cell counting, cells were seeded at a density of 1×10^5 cells/well overnight in a 6-well plate and, if needed, treated the next day with TGF- β 1 (5 ng/ml) with 1% FBS at specific time points. Cells were then trypsinized and counted using a hemocytometer.

RNA Isolation, cDNA Synthesis, and RT-PCR—Cells were seeded at a density of 5×10^5 /well into a 6-well plate overnight in the presence of 5% FBS. For DU145, PC3, and LNCaP cells, their culture media were changed to 1% FBS after 24 h, whereas for RWPE1 cells, culture medium was changed to EpiLife[®] medium. The cells were then treated with TGF- β 1 (5 ng/ml) for 0, 1, and 8 h. Total RNA was isolated from the cells using TRIzol (Life Technologies) as described previously (88). 2 μ g of total RNA were reverse-transcribed, and the resulting cDNAs were diluted 4-fold with RNase-free water. 4 μ l of the diluted cDNA were added to separate RT-PCR mixtures following established procedures. All gene-specific primers were designed with Beacon-Designer version 5.0 as described previously (29). The following primers were used: c-Jun forward, 5'-TGGAAACGACCTTCTATGACGA-3'; c-Jun reverse, 5'-GTTGCTGGACTGGATTATCAGG-3'; JunB forward, 5'-TACCACGACGACTCATACACA-3'; JunB reverse, 5'-CGCTTTGAGACTCCGGTAGG-3'; JunD forward, 5'-CAAACCTGCCTTTCCTTTAC-3'; JunD reverse, 5'-GGCGAACCAAGGATACAAA-3'; L19 forward, 5'-GAAATCGCCAATGCCAACTC-3'; L19 reverse 5'-TCTTAGACCTGCGAGCCTCA-3'. The PCR products were visualized on 1% agarose gels stained with ethidium bromide (Amresco, Solon, OH). The relative intensities of specific PCR bands were determined by ImageJ version 1.48 (National Institutes of Health).

Western Blotting Analysis—Cells from different experiments were washed twice with ice-cold PBS and were lysed in cell lysis buffer (Cell Signaling Technology) containing 20 mM Tris-HCl

(pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 \times protease inhibitor mixture (Calbiochem). Protein concentrations were determined by the Lowry HS assay using the Bio-Rad DC protein assay kit according to the instructions provided by the manufacturer. Cell lysates were mixed with Laemmli's buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β -mercaptoethanol, and 10% glycerol), and individual samples (35 μ g of protein) were subjected to SDS-PAGE in 10% gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h at room temperature in 1 \times PBS (136 mM NaCl, 2.6 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and 5% fat-free skim milk. The blots were then incubated overnight at 4 $^{\circ}$ C with appropriate dilutions of specific primary antibodies (1:2000 dilution for anti-JunD; 1:1000 dilution for anti-c-Jun, anti-JunB, anti-p21, anti-p27, anti-cyclin D1, anti-ubiquitin, anti-phospho-JunD, anti-phospho-SAPK/JNK, anti-total SAPK/JNK, and anti-phospho-Smad3; 1:500 dilution for anti-c-Myc and anti-Ki-67; 1:10,000 dilution for anti- β -actin). After washing, blots were incubated with appropriate immunoglobulin coupled to horseradish peroxidase (dilution 1:20,000) for 1 h. The blots were developed in Millipore Luminata Forte (EMD Millipore, Billerica, MA) for 5 min and exposed to an x-ray film and visualized either by autoradiography or by the Syngene PXI 6 imaging system (Syngene, Frederick, MD). Western blots for β -actin were carried out in parallel as loading controls. The relative intensities of specific protein bands were determined by ImageJ version 1.48 (National Institutes of Health).

Immunoprecipitation—DU145 cells were pretreated with MG132 (25 μ M) for 2 h and then treated with TGF- β 1 (5 ng/ml) for 8 h. Cells were lysed in cell lysis buffer (Cell Signaling Technology). Total cell lysates containing 500 μ g of proteins were used for immunoprecipitation using procedures described previously (16). The resulting supernatants were incubated with 2 μ g of anti-JunD antibody overnight at 4 $^{\circ}$ C. Immunocomplexes were collected by centrifugation after incubation with protein A/G-Sepharose beads (Santa Cruz Biotechnology) and were analyzed by Western blotting analysis with anti-ubiquitin antibody.

Transfection with Specific Jun Protein and Control siRNAs—Cells were seeded at a density of 1.5×10^5 cells in 6-well plates in 2 ml of antibiotic-free normal growth medium supplemented with 5% FBS and incubated overnight at 37 $^{\circ}$ C. siRNAs (60 nM) for the Jun proteins (c-Jun, JunD, JunB) or control siRNA were transfected in DU145 and PC3 cells using transfection reagent (Santa Cruz Biotechnology) following the manufacturer's recommendations. 48–72 h after transfection, cells were treated with TGF- β 1 and/or subjected to different functional analyses.

Generation of DU145 Sublines Overexpressing Jun Proteins—DU145 cells (1.5×10^5) were grown in a serum-free medium in a 6-well plate and then transfected with the empty vector, pcDNA3.1 Mycstop, or with pcDNA3.1 JunB Mycstop, pcDNA3.1 Mycstop JunD, and pcDNA3.1 c-Jun Mycstop using FuGENE[®] HD transfection reagent (Promega, Madison, WI) following the manufacturer's instructions. For each gene transfection, after 48 h, cells were trypsinized and replated to dilutions of 1:2, 1:5, 1:10, 1:20, 1:40, and 1:80 in a 6-well plate, supplemented with 5% FBS and G418 (800 μ g/ml) (Calbi-

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ochem). Cells were continuously fed with fresh medium containing G418 every 2–3 days. About 1 week post-transfection, single G418-resistant colonies were picked using sterile cloning discs (Scienceware, Wayne, NJ) and grown to propagate. Total proteins from the transfected lines were extracted, and Western blotting analysis was performed to determine overexpression of the specific Jun proteins. The effects of these transfections on cellular proliferation were determined by cell counting.

TUNEL Assay and DAPI Staining—DU145 cells were seeded onto coverslips at a density of 1.0×10^5 cells in 6-well plates in 2 ml of antibiotic-free normal growth medium supplemented with 5% FBS. Transfection of control and JunD siRNA was performed as described above. Cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 20 min at room temperature and then washed three times with PBS. Fixed cells were evaluated for apoptosis using two independent methods; a TUNEL assay was used to detect DNA fragmentation, which is a hallmark of apoptosis in mammalian cells, and DAPI staining was used to visualize intact total nuclear DNA. For the TUNEL assay, apoptotic cells were detected using the proTUNEL-IHC DNA fragmentation assay kit (GeneTex, Irvine, CA) following the manufacturer's recommendation. For nuclear visualization, fixed cells were stained with DAPI (3 μ g/ml; Roche Applied Science) following standard fluorescence staining protocols. Coverslips were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and then images were captured using $\times 10$ magnification with an Axiovision camera of a Carl Zeiss 200M inverted fluorescence microscope (Carl Zeiss, Thornwood, NY).

Soft Agar Colony Formation—A soft agar colony formation assay was performed on DU145 cells overexpressing Jun proteins in 6-well plates. Each well contained 2 ml of 0.6% agar in complete medium as the bottom layer, 1 ml of 0.4% agar in complete medium and 3000 cells as the feeder layer, and 1 ml of complete medium as the top layer. Cultures were maintained under standard culture conditions. The number of colonies was determined with an inverted phase microscope (Carl Zeiss 200M) at $\times 100$ magnification. A group of ≥ 2 cells was counted as a colony. The data presented are means \pm S.E. of 12 wells (total number of colonies from 4 random fields/well) from two independent experiments at an optimum time of 21 days after cell plating. The experiment was repeated twice, with each experiment using a different Jun-overexpressing DU145 cell line.

Statistical Analysis—All experiments were performed at least three times using different cell preparations. Data from representative experiments are shown in the figures. The significance of the differences among treatments was determined using one-way analysis of variance and Duncan's multiple pairwise comparison tests using the statistical package from Sigma-Plot version 11.0 for Windows.

Author Contributions—A. C. M. and B. T. V. performed and analyzed the experiments and contributed the preparation of the manuscript. S. A. K. conceived and coordinated the study and helped in analysis of the data and preparation of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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