Growth Hormone & IGF Research 20 (2010) 55–62

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Pomegranate extract induces apoptosis in human prostate cancer cells by modulation of the IGF–IGFBP axis

Satomi Koyama [a], Laura J. Cobb [a], Hemal H. Mehta [a], Navindra P. Seeram [b], David Heber [b], Allan J. Pantuck [c], Pinchas Cohen [a,*]

[a] Division of Pediatric Endocrinology, Mattel Children's Hospital, David Geffen School of Medicine, University of California, Los Angeles, USA
[b] Center for Human Nutrition, David Geffen School of Medicine, UCLA, USA
[c] Department of Urology, David Geffen School of Medicine, UCLA, USA

ARTICLE INFO

Article history:
Received 10 March 2008
Revised 7 September 2009
Accepted 8 September 2009
Available online 22 October 2009

Keywords:
Prostate cancer
Pomegranate
IGF-I
IGFBP-3

ABSTRACT

The IGF axis is critical for the regulation of apoptosis in many human cancer cell lines. Recently, potent anti-tumorigenic effects of pomegranate juice and extracts have been reported. Consequently, pomegranate has potential not only as a treatment but also as a preventative measure against certain types of cancer, including prostate. In this study, we investigated the relationship between pomegranate-induced apoptosis in human prostate cancer cells and the IGF/IGFBP system. Treatment of LAPC4 prostate cancer cells with 10 μg/ml POMx, a highly potent pomegranate extract prepared from skin and arils minus seeds and standardized to ellagitannin content (37% punicalagins by HPLC), resulted in inhibition of cell proliferation and induction of apoptosis. Interestingly, co-treatment with POMx and IGFBP-3 revealed synergistic stimulation of apoptosis and additive inhibition of cell growth. Western blot analysis revealed that treatment with POMx or POMx/IGFBP-3 combination resulted in increased JNK phosphorylation, and decreased Akt and mTOR activation, consistent with a growth inhibitory, pro-apoptotic function. We also investigated the relationship between IGF-1 and pomegranate-induced apoptosis in 22RV1 prostate cancer cells. Co-treatment with 100 ng/ml IGF-1 completely blocked apoptosis induction by POMx. In contrast, IGF-I failed to inhibit POMx-induced apoptosis in R^- cells, suggesting the importance of IGF-IR. POMx-treatment decreased *Igf1* mRNA expression in a dose-dependent manner indicating that its actions also involve tumor-specific suppression of IGF-1. These studies revealed novel interactions between the IGF system and pomegranate-induced apoptosis.

1. Introduction

Prostate cancer (CaP) is the leading cause of cancer in American men [1]. Treatment of recurrent CaP commonly involves hormone ablation therapy, however given sufficient time, tumors uniformly progress to a state of insensitivity to hormonal ablation. Once the disease is castrate-resistant and metastatic, the prognosis for the patient is poor. On the other hand, the protracted period of prostate carcinogenesis provides an ideal opportunity for chemoprevention, the administration of natural or synthetic agents to inhibit this process before the invasive phenotype is established [2–5]. This approach is ideally suited for CaP since it is typically diagnosed in men over 50 years of age, and therefore even a modest delay in disease onset or progression could significantly impact the quality of life of these patients [6]. In this regard, several naturally occurring antioxidants including lycopene, tea polyphenols and pomegranate as potential modulators of prostate carcinogenesis are currently being evaluated [7–9], and are demonstrating positive effects in CaP patients [6,10–13].

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and in beverage forms such as juice and wines [14]. Commercial pomegranate juice (PJ) shows potent antioxidant and anti-atherosclerotic properties attributed to its high content of polyphenols including ellagic acid (EA), gallotannins and anthocyanins (cyanidin, delphynidin and pelargonidin glycosides), and other flavonoids (quercetin, kaempferol and luteolin glycosides) [14–19]. The most abundant of these polyphenols is punicalagin, an ellagitannin implicated as the bioactive constituent responsible for >50% of the potent antioxidant activity of the pomegranate [14]. Punicalagin is abundant in the fruit husk and is extracted into PJ in significant quantities during processing, reaching levels of >2 g/L juice [14,18–20]. POMx is a highly potent pomegranate extract that was developed for use as a dietary ingredient, has received Generally Recognized as Safe status, is prepared from skin and arils minus seeds, and is standardized to ellagitannin content (37% punicalagins by HPLC) [21].

* Corresponding author. Tel.: +1 215 590 4504.
E-mail address: hassy@mednet.ucla.edu (P. Cohen).

1096-6374/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ghir.2009.09.003

The activity of insulin-like growth factor (IGF)-I and -II are regulated by a family of six high-affinity binding proteins. IGF-I is upregulated in a variety of cancers including prostate cancer, and functions as a potent mitogen and survival factor as well as being epidemiologically related to CaP incidence [22]. IGF binding protein (IGFBP)-3 is the most abundant of the IGFBPs in serum, where it forms a ternary complex with acid labile subunit and IGF [23]. In addition to its role in regulating IGF action, IGFBP-3 exerts many IGF-independent effects to inhibit cell proliferation and enhance apoptosis in many cell types, including prostate [24] and breast [25–27] cancers. As recent reports demonstrate that pomegranate juice and its extracts have potent anti-tumorigenic effects in prostate cancer, the purpose of this study was to evaluate the effects of pomegranate extracts on the IGF–IGFBP axis in prostate cancer cell lines.

2. Materials and methods

2.1. Reagents

Recombinant non-glycosylated IGFBP-3 was provided by Insmed (Glen Allen, VA). The mouse anti-β-actin was purchased from Sigma (St. Louis, MO). I-Block™ was purchased from Applied Biosystems (Foster City, CA). SDS–PAGE precast gels and blotting equipment were purchased from Bio-Rad (Hercules, CA). CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). Cell Death Detection ELISA^{PLUS} kit was purchased from Roche Applied Science (Germany). Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb, SAPK/JNK (56G8) Rabbit mAb, phospho-Akt (Thr308) antibody, phospho-Akt (Ser473) antibody, Akt antibody, phospho-mTOR (Ser2448) antibody, phospho-mTOR (Ser2481) antibody and mTOR antibody were purchased from Cell Signaling (Danvers, MA). IGF-I was provided by Tercica (Brisbane, CA).

2.2. Cell culture

The LAPC4 prostate cancer cell line was a generous gift from Charles Sawyers [28]. LAPC4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 nmol/L R1881 (Perkin–Elmer Life Sciences, Wellesley, MA). 22RV1 prostate carcinoma cell line (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. The R^- cell line were generously provided by Renato Baserga (Thomas Jefferson University, Philadelphia, PA); briefly, fibroblasts from an IGF-I receptor knock-out mouse was generated from 18-day embryos as described previously [29]. R^- cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. For individual experiments, cells were seeded at a final density of $1 \times 10^5/cm^2$ (24-well and six-well), or $2 \times 10^5/cm^2$ (96-well) in plates and grown to 80% or 50% confluence, respectively. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. All treatments were carried out as indicated in serum-free medium.

2.3. Cell proliferation assay

Cells were seeded on 96-well plates, and were allowed to attach overnight. Cells were incubated with 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 72 h in SF media. CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was performed according to

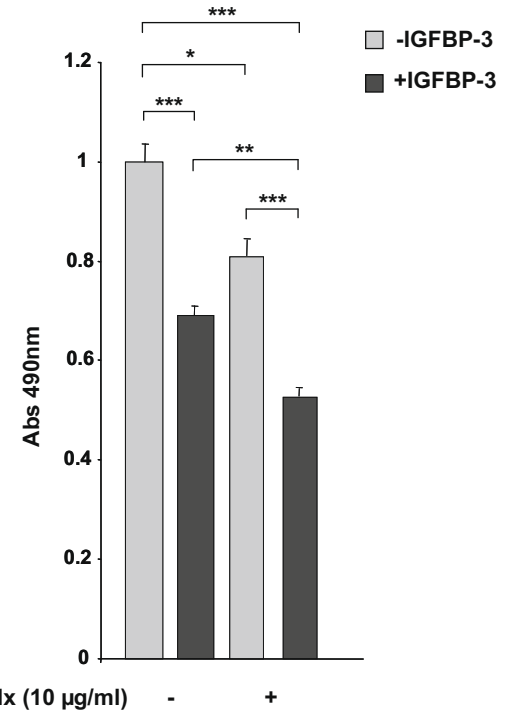

Fig. 1. IGFBP-3 and POMx additively inhibit the growth of LAPC4 cells. LAPC4 prostate cancer cells were incubated in the presence and absence of 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 72 h in SF media. Cell proliferation was assessed by enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), to a formazan product. $n = 3$; significance that mean is difference from untreated control: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

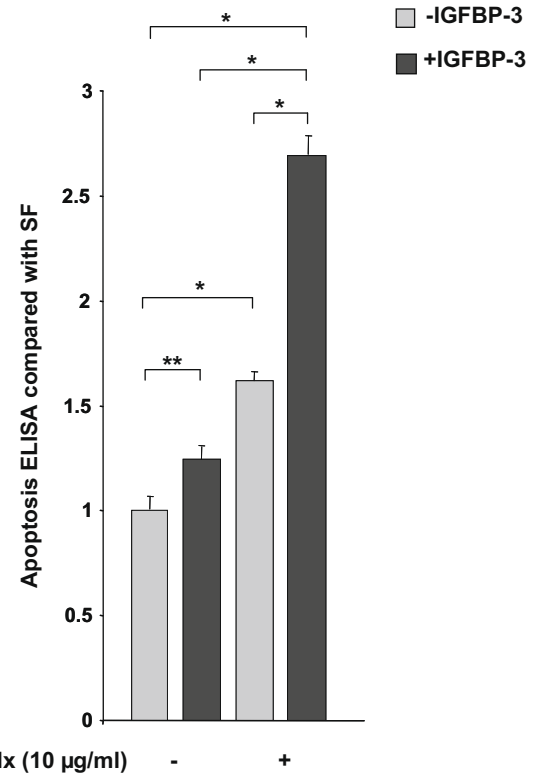

Fig. 2. Synergistic apoptosis induction by IGFBP-3 and POMx. LAPC4 cells were incubated in the presence and absence of 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 24 h in SF media. Apoptosis was assessed by ELISA for cytoplasmic histone-associated-DNA-fragments. $n = 3$; significance that mean is different from one (untreated control): $^*P < 0.05$, $^{**}P < 0.01$.

manufacturer's instructions. Mean ± SE values of the absorbance at 490 nm were plotted.

2.4. Analysis of apoptosis

Apoptosis was assessed in cells growing on 24 well plates using Cell Death Detection ELISA^{PLUS} for the determination of cytoplasmic histone-associated DNA-fragments (Roche Applied Science) following the manufacturer's instructions.

2.5. Immunoblotting

Cell lysates containing 50 μg protein were resolved by SDS–PAGE and transferred to PVDF membrane (Bio-Rad). Membranes were blocked on 0.2% I-Block™ in PBS containing 0.1% Tween 20 for 3 h at room temperature and then probed with the appropriate primary and secondary antibodies. Antibody–antigen complexes were visualized by Immobilon Western Chemilluminescence reagents (Millipore, Billerica, MA) and autoradiography.

2.6. mRNA extraction and reverse transcription polymerase chain reaction

Total RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed to cDNA (Bio-Rad Laboratories, Hercules, CA) as per manufacturer's instructions. PCR reactions were performed in 20 μl containing 1 μl cDNA and 5′ HotMasterMix (Eppendorf, Ontario, CA). Oligonucleotide primers (Invitrogen, Carlsbad, CA) were included in the reactions with sequences as follows: IGF-1: 5′TGG ATG CTC TTC AGT TCG TG 3′ (forward), 5'CCT GCA CTC CCT CTA CTT GC 3' (reverse); IGF-1: 5' GGA CCG GAG ACG CTC TGC GGG 3' (forward), 5' AGC TGA CTT GGC AGG CTT GAG 3' (reverse); β-actin: 5' CAC CTT CTA CAA TGA GCT GC 3' (forward), 5' AAG GTA GTT TCG TGG ATG CC 3' (reverse). Cycling conditions were as follows; denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min for 30 cycles.

2.7. Densitometrical and statistical analysis

Densitometric measurements of autoradiographs was performed by using computer-scanned densitometry. Experiments are means of triplicates, and each experiment was performed three times. Data are expressed as mean ± SEM. Statistical analysis was performed with by using an unpaired nonparametric Mann–Whitney test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. POMx and IGFBP-3 inhibit proliferation of LAPC4 prostate cancer cells and demonstrate additive effects

Recent reports demonstrate that pomegranate juice and its extracts have potent anti-tumorigenic effects. Since IGFBP-3 functions as a negative regulator of cell proliferation in cultured prostate cancer cells, we investigated the effects of co-incubation with POMx and IGFBP-3 on cell proliferation. LAPC4 human prostate cancer cells were treated with 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 72 h in SF media. POMx and IGFBP-3 independently inhibited cell proliferation by 20% and 30% (Fig. 1), respectively. However, co-treatment with both IGFBP-3 and POMx had an additive effect of growth inhibition, suggesting that co-treatment may have the potential for more potent inhibition of cancer growth.

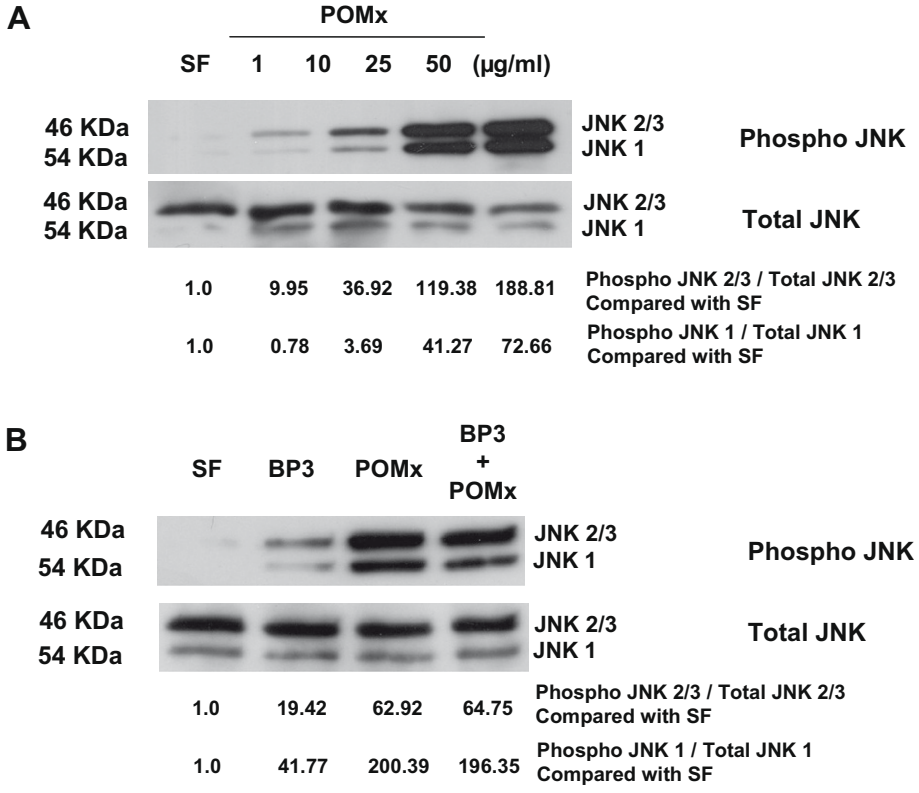

Fig. 3. Dose-dependent increase in JNK phosphorylation by POMx. (A) LAPC4 cells were incubated with increasing concentrations of POMx in SF media for 24 h. Levels of total and phospho-JNK were determined by immunoblotting, $n = 3$. (B) LAPC4 cells were treated in the presence or absence of 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 24 h in SF media. Levels of total and phospho-JNK were determined by immunoblotting, $n = 3$ (upper panel).

3.2. Synergistic apoptosis induction by IGFBP-3 and POMx

Pomegranate extracts and IGFBP-3 have been reported to enhance apoptosis in many cell lines [9,20,23–26,30–32]. Since co-treatment with IGFBP-3 and POMx additively inhibited the growth of prostate cancer cells, we evaluated the effects of co-treatment on apoptosis induction in LAPC4 cells. As previously demonstrated, treatment with 1 μg/ml IGFBP-3 for 24 h in SF media results in enhanced apoptosis induction by 25% in LAPC4 cells [33] (Fig. 2). Similarly, incubation of LAPC4 cells with 10 μg/ml POMx resulted in a 60% increase in apoptosis, as assessed by ELISA for cytoplasmic histone-associated DNA-fragments. Importantly, co-treatment of POMx and IGFBP-3 revealed synergistic induction of apoptosis (Fig. 2), an effect which was more pronounced than that seen for cell growth inhibition in Fig. 1.

3.3. POMx increases JNK phosphorylation in a dose-dependent manner in LAPC4 cells

It has been reported that pomegranate extracts reduce phosphorylation of a variety of different MAP kinases, including ERK1/2, JNK and p38, in human lung carcinoma A549 cells [34]. We investigated the influence of POMx and IGFBP-3 treatment on the key signaling pathways known to be important in cell proliferation and apoptosis in prostate cancer cells. LAPC4 cells were incubated with increasing concentrations of POMx in SF media for 24 h. Levels of total and phospho-JNK were determined by immunoblotting. POMx increased JNK phosphorylation in a dose-dependent manner (Fig. 3A). LAPC4 cells were also treated with 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 24 h in SF media. IGFBP-3 and POMx independently increase JNK phosphorylation. However, co-treatment resulted in no additional activation of JNK (Fig. 3B). This suggests that POMx and IGFBP-3 may activate JNK by the same mechanism, or that maximal phosphorylation is achieved after IGFBP-3-treatment.

3.4. Inhibition of Akt and mTOR phosphorylation by POMx and POMx/IGFBP-3 combination in LAPC4 cells

Recently, Khan et al. demonstrated reduced phosphorylation of Akt (Thr308) in A549 human lung carcinoma cells treated with pomegranate [34]. To further elucidate the mechanism of POMx

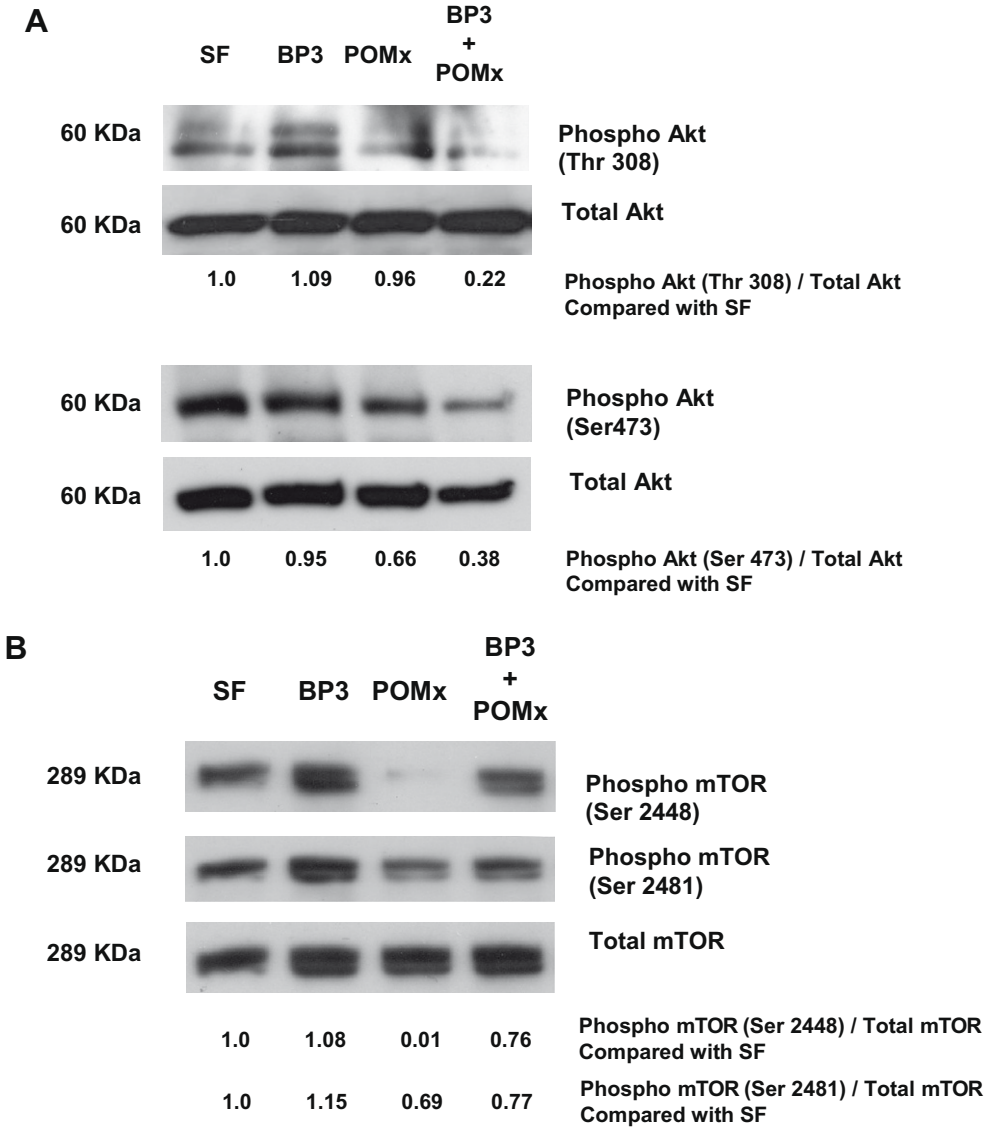

Fig. 4. Modulation of PI3-kinase/Akt/mTOR signaling by POMx. LAPC4 cells were treated in the presence or absence of 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 24 h in SF media. (A) Total and phospho-Akt (Thr308 and Ser473) were assessed by immunoblotting, $n = 3$. (B) Total and phospho-mTOR (Ser2448 and Ser2481) were assessed by immunoblotting, $n = 3$.

and IGFBP-3/POMx-induced apoptosis in LAPC4 cells, we assessed total and phospho-Akt and mTOR levels by immunoblotting. LAPC4 cells were incubated with 10 μg/ml POMx and/or 1 μg/ml IGFBP-3 for 24 h in SF media. Akt phosphorylation at position Thr308 and Ser473 is decreased by POMx (Fig. 4A) Interestingly, co-treatment with IGFBP-3 and POMx led to a synergistic decrease in Ser-473 phosphorylation, suggesting that this may be one of the mechanisms by which the synergistic induction of apoptosis occurs. mTOR is a downstream target of PI3-kinase/Akt signaling, and its regulation is frequently altered in tumorigenesis. PI3-kinase/Akt activity is also required for an autophosphorylation event at Ser-2481 of mTOR. POMx-treatment reduced mTOR phosphorylation at Ser2448, and slightly reduced at Ser2481. In contrast, IGFBP-3 slightly increased phosphorylation at both sites. Co-treatment with POMx and IGFBP-3 nullified the individual actions each, giving no overall change in mTOR phosphorylation at either serine residue. These data suggest that IGFBP-3 and POMx have differing effects on the PI3-kinase/Akt/mTOR signaling pathway, confirming the likelihood of distinct mechanisms of action for apoptosis induction.

3.5. IGF-I and POMx have opposing effects on apoptosis induction in prostate cancer cells

While IGFBP-3 is known to play an important inhibitory role in the regulation of many types of cancer, IGF-I is recognized to be a potent mitogen and survival factor. We therefore investigated the relationship between IGF-1 and pomegranate-induced apoptosis in prostate cancer cells. Apoptosis was assessed by ELISA for histone-DNA-fragments in 22RV1 cells incubated with 10 μg/ml POMx and/or 100 ng/ml IGF-I for 24 h in SF media. Treatment with POMx increases apoptosis by 20% in 22RV1 cells and 3-fold induction in R^- cells (Fig. 5). IGF-I reduced the apoptosis induced by serum withdrawal, however when added in combination with POMx, IGF-I completely antagonized the apoptosis induced by POMx in 22RV1 cells (Fig. 5A). In contrast, IGF-I failed to inhibit POMx-induced apoptosis in R^- (IGF-IR null MEFs) cells (Fig. 5B), suggesting the importance of the IGF-IR in antagonizing the effects of POMx.

3.6. IGF-I and POMx increase JNK phosphorylation in 22RV1 cells

Since POMx-treatment leads to a dose-dependent increase in JNK phosphorylation, we decided to investigate whether co-incubation with IGF-I could modulate this effect. 22RV1 cells were treated with 10 μg/ml POMx and/or 100 ng/ml IGF-I for 24 h in SF media, and total and phospho-JNK were assessed by immunoblotting. As demonstrated previously for LAPC4 cells (Fig. 3A), treatment with POMx increases JNK phosphorylation in a dose-dependent manner in 22RV1 cells (Fig. 6A). Interestingly, IGF-I treatment alone also caused a 1.5-fold increase in JNK phosphorylation (Fig. 6B). Co-treatment of 22RV1 cells with IGF-I and POMx synergistically enhance JNK phosphorylation (Fig. 6B), suggesting that the antagonistic actions of IGF-I on POMx-induced apoptosis are exerted via alternative pathways.

3.7. POMx decreases Igf1 mRNA expression in a dose-dependent manner

To elucidate additional mechanisms by which POMx modulate IGF-axis regulation of death and survival in prostate cancer cells, we investigated the effects of POMx-treatment on *Igf1* mRNA expression by RT-PCR in 22RV1 cells. POMx-treatment caused a dose-dependent reduction in *Igf1* mRNA levels (Fig. 7A). Treatment with either IGF-I or IGFBP-3 alone had no effect on *Igf1* levels (Fig. 7B) and neither was unable to antagonize the inhibitory effect of POMx alone. This suggests that inhibition of *Igf1* expression may

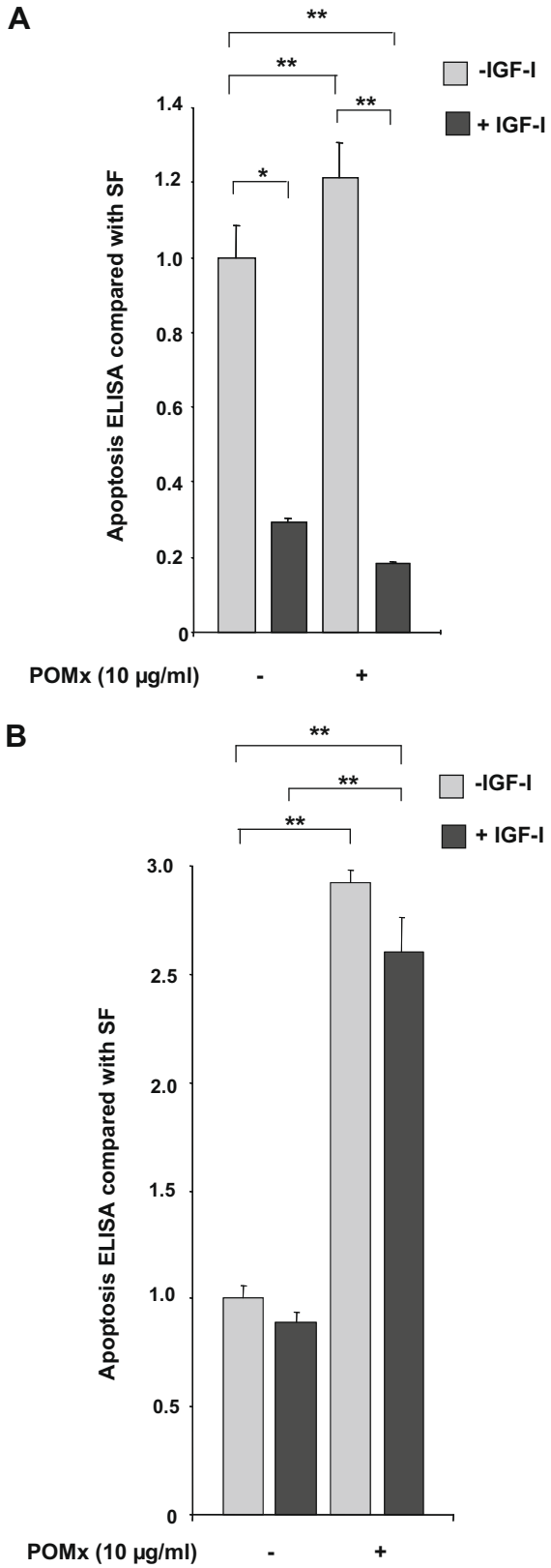

Fig. 5. IGF-I antagonizes POMx-induced apoptosis. (A) 22RV1 human prostate cancer cells were incubated in the presence or absence of 10 μg/ml POMx and/or 100 ng/ml IGF-I for 24 h in SF media. Apoptosis was assessed by ELISA for histone-associated DNA-fragments. $n = 3$; significance that mean is different from one (untreated control): $^*P < 0.05$, $^{**}P < 0.01$. (B) Apoptosis was assessed by ELISA for histone-associated-DNA-fragments in R^- cells (IGF-IR null MEFs) incubated in the presence and absence of 10 μg/ml POMx and/or 100 ng/ml IGF-I for 24 h in SF media. $n = 3$; significance that mean is different from one (untreated control): $^*P < 0.05$, $^{**}P < 0.01$.

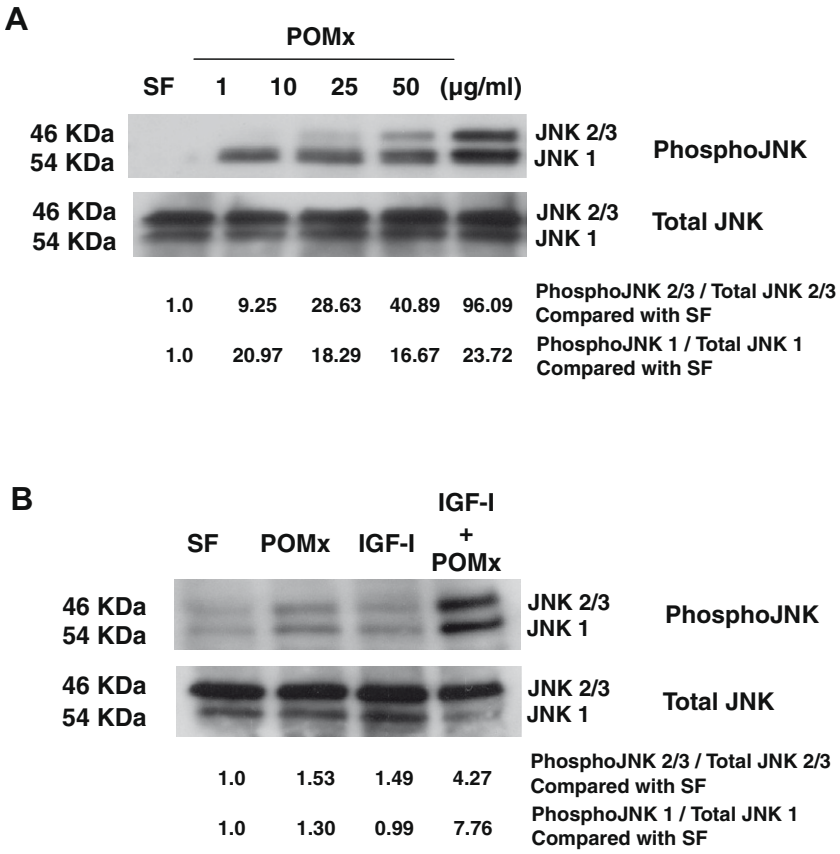

Fig. 6. Synergistic activation of JNK phosphorylation by IGF-I and POMx. (A) 22RV1 cells were incubated with increasing concentrations POMx in SF media for 24 h. Levels of total and phospho-JNK were determined by immunoblotting, blots are representative of three independent experiments. (B) 22RV1 cells were treated in the presence and absence of 10 μg/ml POMx and/or 100 ng/ml IGF-I for 24 h in SF media. Levels of total and phospho-JNK were determined by immunoblotting (upper panel). Blots are representative of three independent experiments.

be one of the mechanisms by which POMx causes reduced cell survival in prostate cancer.

4. Discussion

Multiple lines of evidence, including both *in vitro* and clinical studies, have demonstrated the centrality of the IGF system in cancers such as prostatic carcinoma, and point to IGFBP-3 as an anti-cancer molecule [35]. Furthermore, different pomegranate extracts have been shown to inhibit cell growth in a variety of human cancer cell lines. In 2005, Malik et al. demonstrated that pomegranate fruit extract (PFE) inhibits cell growth in PC3 cell lines in a dose-dependent manner. Recently, Kahn et al. also reported that PFE inhibits cell growth in A549 human lung carcinoma cells [34]. We have previously shown that punicalagin, ellagic acid (EA), total pomegranate tannin (TPT) and pomegranate juice (PJ) inhibit cell growth in RWPE-1 and 22RV1 prostate cancer cells, KB and CAL27 human oral cancer cells and HT-29, HCT116, SW480 and SW620 human colon cancer cells [20]. In addition, oral consumption of PFE inhibits the growth and progression of lung carcinoma in mice after chemically-induced tumor initiation [36]. In prostate cancer clinical studies, treatment with pomegranate juice was associated with significant prolongation of patients' PSA doubling time, and a decrease in cell proliferation and increase in apoptosis in prostate cancer cell lines grown in the presence of treated patients' serum (X). Here, we report that POMx and IGFBP-3 can additively reduce the proliferation of LAPC4 human prostate cancer cells. In addition to inhibiting cancer cell proliferation, we also previously reported that punicalagin, EA, TPT and PJ can induce apoptosis in HT-29 and

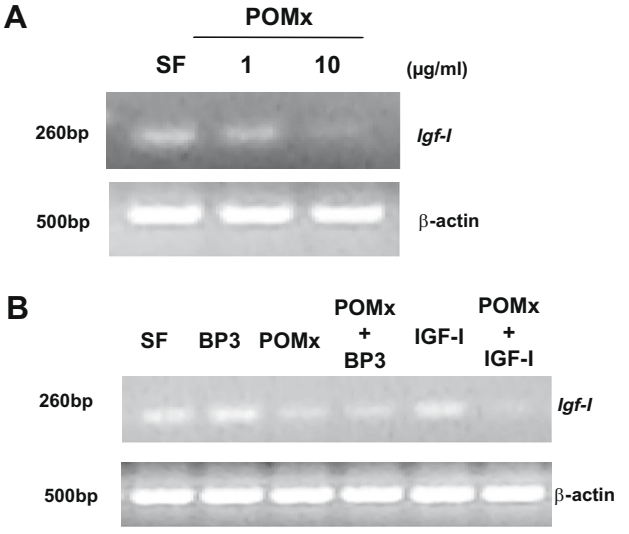

Fig. 7. POMx decreases *Igf1* mRNA expression in a dose-dependent manner. (A) 22RV1 cells were incubated with increasing concentrations POMx in SF media for 24 h. Total RNA was prepared using TRIzol Reagent. Levels of *Igf1* and β-actin mRNA were determined by RT-PCR. (B) 22RV1 cells were incubated with and without 1 μg/ml IGFBP-3, 100 ng/ml IGF-I and/or 10 μg/ml POMx for 24 h in SF media. Total RNA was prepared using TRIzol Reagent. Levels of *Igf1* and β-actin mRNA were determined by RT-PCR.

HCT116 human colon cancer cells [20]. Interestingly, although POMx and IGFBP-3 individually function as apoptotic factors, co-treatment leads to a synergistic induction of apoptosis in LAPC4 human prostate cancer cells. In addition to highlighting the potential of pomegranate and its extracts as possible therapeutics, these data also suggest that combination therapy of POMx with other therapeutics may have beneficial effects in the treatment of tumors. To analyze the synergistic mechanism of apoptosis induction by POMx and IGFBP-3, we investigated the signaling pathways known to be important in cell proliferation and apoptosis. Afaq et al. reported that PFE treatment in normal human epidermal keratinocytes (NHEK) prior to be UVB irradiation significantly inhibited UVB-mediated phosphorylation of ERK1/2, JNK1/2 and p38 in a time-dependent manner [37]. Khan et al. also reported reduced MAPK and PI3-kinase/Akt signaling after PFE treatment in A549 human lung carcinoma cells [34]. Here, we demonstrate reduced Akt/mTOR phosphorylation after POMx-treatment in LAPC4 human prostate cancer cell lines, but increased phosphorylation of JNK in both LAPC4 and 22RV1 human prostate cancer cell lines. This suggests that POMx has cell specific effects on JNK phosphorylation in different tumor models. We have previously demonstrated enhanced JNK phosphorylation in prostate cancer cells after treatment with IGFBP-3 [38]. Interestingly, however, co-incubation with POMx led to no further increase in JNK activation, suggesting that POMx and IGFBP-3 may activate JNK by the same mechanism, or that maximal phosphorylation is achieved after IGFBP-3-treatment. Surprisingly, IGF-I and POMx activated JNK synergistically. Since the two factors have opposing effects on cell proliferation and apoptosis, and as JNK phosphorylation can lead to both pro- and anti-apoptotic events, this would suggest that the activation of JNK by the different factors leads to the activation of distinct targets.

Intriguingly, POMx induced a 3-fold increase in apoptosis in R^- cells, compared with only a 20% induction in 22RV1 prostate cancer cells. This suggests that endogenous, in addition to exogenously added, IGF-I plays a key role in blocking POMx-induced apoptosis. Since POMx-treatment leads to a dose-dependent down-regulation of *Igf1* mRNA, this would suggest that some of the effects of POMx may involve suppression of endogenous tumor production of IGF-I. It is well established that manipulations of the IGF axis to promote apoptotic events in cancer may have beneficial therapeutic effects [39]. However, since POMx also induced apoptosis in MEF cells lacking the IGF-IR, it is unlikely that manipulation of *Igf1* levels is the only mechanism by which POMx induces apoptosis. Rather, it appears that pomegranate extracts act via multiple mechanisms, including enhancement of IGFBP-3 pro-apoptotic activity and through inhibition of the NFkB pathway [40]. It is possible that the IGFBP3-related activities of POMx are both IGF-dependent and -independent.

In conclusion, these studies reveal novel interactions between the IGF system and pomegranate-induced apoptosis, and suggest that pomegranate products modulate the tumor production and responsiveness to IGFs and the IGFBPs. As IGFBP-3 is currently being tested in humans as a treatment for prostate cancer and pomegranate supplements are becoming popular as adjuvant nutritional treatments for this disease, we propose that these agents may emerge as useful in the management of prostate cancer.

Acknowledgements

Supported in part by grants: 1R01CA100938, R01HD047013, P50CA92131, DOD idea development award (to PC) and a DOD fellowship award (to LJC).

References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, 2007, CA Cancer J. Clin. 57 (2007) 43–66.
[2] W.K. Hong, M.B. Sporn, Recent advances in chemoprevention of cancer, Science 278 (5340) (1997) 1073–1077.
[3] H.L. Parnes, I.M. Thompson, L.G. Ford, Prevention of hormone-related cancers: prostate cancer, J. Clin. Oncol. 23 (2005) 368–377.
[4] H. Mukhtar, N. Ahmad, Cancer chemoprevention: future holds in multiple agents, Toxicol. Appl. Pharmacol. 158 (1999) 207–210.
[5] Y.J. Surh, Cancer chemoprevention with dietary phytochemicals, Nat. Rev. Cancer 3 (2003) 768–780.
[6] M. Saleem, V.M. Adhami, I.A. Siddiqui, H. Mukhtar, Tea beverage in chemoprevention of prostate cancer: a mini-review, Nutr. Cancer 47 (2003) 13–23.
[7] M.L. Fraser, A.H. Lee, C.W. Binns, Lycopene and prostate cancer: emerging evidence, Expert Rev. Anticancer Ther. 5 (5) (2005) 847–854.
[8] I.A. Siddiqui, V.M. Adhami, M. Saleem, H. Mukhtar, Beneficial effects of tea and its polyphenols against prostate cancer, Mol. Nutr. Food Res. 50 (2006) 130–143.
[9] A. Malik, F. Afaq, S. Sarfaraz, V.M. Adhami, D.N. Syed, H. Mukhtar, Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer, Proc. Natl. Acad. Sci. USA 102 (2005) 14813–14818.
[10] P. Greenwald, Lifestyle and medical approaches to cancer prevention, Recent Results Cancer Res. 166 (2005) 1–15.
[11] E.A. Klein, I.M. Thompson, Update on chemoprevention of prostate cancer, Curr. Opin. Urol. 14 (2004) 143–149.
[12] H.L. Parnes, M.G. House, J. Kagan, D.J. Kausal, R. Lieberman, Prostate cancer chemoprevention agent development: the National Cancer Institute, Division of Cancer Prevention portfolio, J. Urol. 171 (2004) S68–S75.
[13] A.J. Pantuck, J. Leppert, N. Zomorodian, N. Seeram, W. Aronson, D. Seiler, H. Liker, H. Wang, R. Elashoff, D. Heber, M. Aviram, L. Ignarro, A. Belldegrun, Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer, Clin. Cancer Res. 12 (2006) 4018–4026.
[14] M.I. Gil, F.A. Tomas-Barberan, B. Hess-Pierce, D.M. Holcroft, A.A. Kader, Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing, J. Agric. Food Chem. 48 (2000) 4581–4589.
[15] M. Aviram, L. Dornfeld, M. Rosenblat, N. Volkova, R. Coleman, T. Hayek, D. Presser, B. Fuhrman, Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice, Am. J. Clin. Nutr. 71 (2000) 1062–1076.
[16] M. Kaplan, T. Hayek, A. Raz, R. Coleman, L. Dornfeld, J. Vaya, M. Aviram, Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis, J. Nutr. 131 (2001) 2082–2089.
[17] N.D. Kim, R. Mehta, W. Yu, I. Neeman, T. Livney, A. Amichay, D. Poirier, P. Nicholls, A. Kirby, W. Jiang, R. Mansel, C. Ramachandran, T. Rabi, B. Kaplan, E. Lansky, Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer, Breast Cancer Res. Treat. 71 (2002) 203–217.
[18] B. Cerda, J.J. Ceron, F.A. Tomas-Barberan, J.C. Espin, Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic, J. Agric. Food Chem. 51 (2003) 3493–3501.
[19] B. Cerda, R. Llorach, J.J. Ceron, J.C. Espin, F.A. Tomas-Barberan, Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice, Eur. J. Nutr. 42 (2003) 18–28.
[20] N.P. Seeram, L.S. Adams, S.M. Henning, Y. Niu, Y. Zhang, M.G. Nair, D. Heber, In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice, J. Nutr. Biochem. 16 (2005) 360–367.
[21] N.P. Seeram, R. Lee, M.L. Hardy, D. Heber, Rapid large-scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry, Sep. Purific. Technol. 41 (2005) 49–55.
[22] J.M. Chan, M.J. Stampfer, E. Giovannucci, P.H. Gann, J. Ma, P. Wilkinson, C.H. Hennekens, M. Pollak, Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study, Science 279 (5350) (1998) 563–566.
[23] S.M. Firth, R.C. Baxter, Cellular actions of the insulin-like growth factor binding proteins, Endocr. Rev. 23 (2002) 824–854.
[24] R. Rajah, B. Valentinis, P. Cohen, Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism, J. Biol. Chem. 272 (1997) 12181–12188.
[25] Z.P. Gill, C.M. Perks, P.V. Newcomb, J.M. Holly, Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner, J. Biol. Chem. 272 (1997) 25602–25607.
[26] H.S. Kim, A.R. Ingermann, J. Tsubaki, S.M. Twigg, G.E. Walker, Y. Oh, Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells, Cancer Res. 64 (2004) 2229–2237.
[27] Z.S. Gucev, Y. Oh, K.M. Kelley, R.G. Rosenfeld, Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor

beta2-induced growth inhibition in human breast cancer cells, Cancer Res. 56 (7) (1996) 1545–1550.
[28] N. Craft, Y. Shostak, M. Carey, C.L. Sawyers, A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase, Nat. Med. 5 (3) (1999) 280–285.
[29] C. Sell, M. Rubini, R. Rubin, J.P. Liu, A. Efstratiadis, R. Baserga, Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor, Proc. Natl. Acad. Sci. USA 90 (1993) 11217–11221.
[30] M. Albrecht, W. Jiang, J. Kumi-Diaka, E.P. Lansky, L.M. Gommersall, A. Patel, R.E. Mansel, I. Neeman, A.A. Geldof, M.J. Campbell, Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells, J. Med. Food 7 (3) (2004) 274–283.
[31] M. Larrosa, F.A. Tomas-Barberan, J.C. Espin, The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway, J. Nutr. Biochem. 17 (2006) 611–625.
[32] L.S. Adams, N.P. Seeram, B.B. Aggarwal, Y. Takada, D. Sand, D. Heber, Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells, J. Agric. Food Chem. 54 (2006) 980–985.
[33] L.J. Cobb, B. Liu, K.W. Lee, P. Cohen, Phosphorylation by DNA-dependent protein kinase is critical for apoptosis induction by insulin-like growth factor binding protein-3, Cancer Res. 66 (22) (2006) 10878–10884.
[34] N. Khan, N. Hadi, F. Afaq, D.N. Syed, M.H. Kweon, H. Mukhtar, Pomegranate fruit extract inhibits prosurvival pathways in human A549 lung carcinoma cells and tumor growth in athymic nude mice, Carcinogenesis 28 (2007) 163–173.
[35] O. Ali, P. Cohen, K.W. Lee, Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule, Horm. Metab. Res. 35 (2003) 726–733.
[36] N. Khan, F. Afaq, M.H. Kweon, K.M. Kim, H. Mukhtar, Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice, Cancer Res. 67 (2007) 3475–3482.
[37] F. Afaq, A. Malik, D. Syed, D. Maes, M.S. Matsui, H. Mukhtar, Pomegranate fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes paragraph sign, Photochem. Photobiol. 81 (2005) 38–45.
[38] K.W. Lee, L.J. Cobb, V. Paharkova-Vatchkova, B. Liu, J. Milbrandt, P. Cohen, Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3, Carcinogenesis 28 (2007) 1653–1658.
[39] R.P. Singh, R. Agarwal, Mechanisms of action of novel agents for prostate cancer chemoprevention, Endocr. Relat. Cancer 13 (2006) 751–778.
[40] M.B. Rettig, D. Heber, J. An, N.P. Seeram, J.Y. Rao, H. Liu, T. Klatte, A. Belldegrun, A. Moro, S.M. Henning, D. Mo, W.J. Aronson, A.J. Pantuck, Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-{kappa}B-dependent mechanism, Mol. Cancer Ther. 7 (2008) 2662–2671.