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Journal of Cellular Biochemistry 113:1569–1580 (2012)

Journal of Cellular Biochemistry

IL-4 Induces Proliferation in Prostate Cancer PC3 Cells Under Nutrient-Depletion Stress Through the Activation of the JNK-Pathway and Survivin Up-regulation

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ABSTRACT

Interleukin (IL)-4 plays a critical role in the regulation of immune responses and has been detected at high levels in the tumor microenvironment of cancer patients where it correlates with the grade of malignancy. The direct effect of IL-4 on cancer cells has been associated with increased cell survival; however, its role in cancer cell proliferation and related mechanisms is still unclear. Here it was shown that in a nutrient-depleted environment, IL-4 induces proliferation in prostate cancer PC3 cells. In these cells, under nutrient-depletion stress, IL-4 activates mitogen-activated protein kinases (MAPKs), including Erk, p38, and JNK. Using MAP-signaling-specific inhibitors, it was shown that IL-4-induced proliferation is mediated by JNK activation. In fact, JNK-inhibitor-V (JNKi-V) stunted IL-4-mediated cell proliferation. Furthermore, it was found that IL-4 induces survivin up-regulation in nutrient-depleted cancer cells. Using survivin-short-hairpin-RNAs (shRNAs), it was demonstrated that in this milieu survivin expression above a threshold limit is critical to the mechanism of IL-4-mediated proliferation. In addition, the significance of survivin up-regulation in a stressed environment was assessed in prostate cancer mouse xenografts. It was found that survivin knockdown decreases tumor progression in correlation with cancer cell proliferation. Furthermore, under nutrient depletion stress, IL -4 could induce proliferation in cancer cells from multiple origins: MDA-MB-231 (breast), A253 (head and neck), and SKOV-3 (ovarian). Overall, these findings suggest that in a tumor microenvironment under stress conditions, IL-4 triggers a simultaneous activation of the JNK-pathway and the up-regulation of survivin turning on a cancer proliferation mechanism. J. Cell. Biochem. 113: 1569–1580, 2012.

KEY WORDS: IL-4; JNK; SURVIVIN; PROSTATE CANCER; NUTRIENT-DEPLETION STRESS; PROLIFERATION

Cancer cell survival, proliferation, and metastasis are influenced by the cytokines and chemokines of the tumor microenvironment interacting with cells and regulating complex signaling pathways. Interleukin-4 (IL-4) is known as a T helper type 2 (T_H2) cytokine because it is produced by T_H2 cells, and it is primarily involved in promoting their differentiation and proliferation. However, IL-4 is also produced by other cells like natural killer T cells (NKT), mast cells, basophils, and eosinophils [Sokol et al., 2008; Yoshimoto et al., 2009; Wu et al., 2011]. Furthermore, increased IL-4 and IL-4R expression has been reported for several tumor cells including breast, ovarian, colon, lung, and thyroid.[Kawakami et al., 2002; Todaro et al., 2006; Todaro et al., 2007; Koller et al., 2010]. The direct effect of IL-4 in cancer cells is a controversial issue, and examples of both tumorigenic and anti-tumorigenic effects have been reported. Among anti-tumorigenic functions are the growth inhibition and induction of apoptosis [Gooch et al., 1998; Chang et al., 2000; Gooch et al., 2002; Yu et al., 2004]. However, more recent studies show instead that IL-4 can promote tumor formation by inhibiting apoptosis and enhancing proliferation [Prokopchuk et al., 2005; Todaro et al., 2006; Li et al.,

Conflict of interest: None.
Grant sponsor: National Institutes of Health; Grant number: CA093900 and P50 CA69568; Grant sponsor: Southwest Oncology Group; Grant number: CA32102; Grant sponsor: American Cancer Society Clinical Research Professorship.
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Received 29 November 2011; Accepted 1 December 2011 • DOI 10.1002/jcb.24025 • © 2011 Wiley Periodicals, Inc.
Published online 15 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

2008]. These conflicting results suggest that IL-4 function may vary, and a detailed analysis of the IL-4-induced signaling pathways that lead to tumor progression merits further investigation.

Survivin is a protein of particular importance to cytokine-induced signaling pathways that control the survival and proliferation of cancer cells. Survivin (encoded by BIRC5) is a member of the inhibitor of apoptosis (IAP) family of proteins that play an essential role in mitosis [Jeyaprakash et al., 2007]. Wild type p-53, commonly lost or mutated in many cancers, represses survivin levels both at the mRNA and protein level, while overexpression of tumor suppressor PTEN has also been shown to induce survivin down-regulation in a reaction reversed by re-expression of recombinant survivin [Mirza et al., 2002; Guha et al., 2009]. Furthermore, a conditional deletion of PTEN in mouse prostate resulted in increased survivin expression that preceded the epithelial dysplasia [Guha et al., 2009].

In the tumor microenvironment, individual cells in a tumor exist in various stages of proliferation, autophagy, and apoptosis and survivin has been shown to play different but important roles in all three areas [Altieri, 2008]. We have shown that CCL2, a cytokine that is highly expressed in the tumor microenvironment, protects prostate cancer PC3 cells from autophagic death by up-regulating survivin via the phosphatidylinositol 3-kinase/AKT-dependent pathway [Roca et al., 2008a]. Here we demonstrate that IL-4 promotes prostate cancer PC3 cell proliferation under nutrient-depletion stress and investigate the pathways and critical factors induced by IL-4 that mediate this response. The results presented here indicate that in a nutrient-depleted-stressed microenvironment, IL-4 activates the Jun N-terminal kinase (JNK) pathway and up-regulates survivin expression to induce proliferation in prostate cancer PC3 cells, a mechanism that could also function in other cancer types.

MATERIALS AND METHODS

CELL LINES

PC3-luciferase prostate cancer cells (designated PC3 for simplicity) were generated as described [Loberg et al., 2006]. MDA-MB-231 (breast cancer), A253 (head and neck cancer), and SKOV-3 (ovarian cancer) cell-lines were obtained from ATCC. All cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Invitrogen).

WESTERN BLOT ANALYSIS

Cells were grown to 80%-confluency in appropriate medium. Cells were synchronized by starvation in serum-free-RPMI for 16 h at 37°C. Cells were detached using 0.25 mM EDTA, then plated in six-well-culture plates at a density of 1.5×10^5 cells/ml and treated with IL-4 and (or) the inhibitors U0126 (MEK1/2 inhibitor), SB 220025 (p38-inhibitor), and JNK-inhibitor-V (JNKi-V); (EMD/Calbiochem) at the indicated concentrations. To analyze survivin expression during cell proliferation, cells were detached and plated in RPMI (0.5–1% FBS) supplemented with IL-4 (100 ng/ml, Symansis, 4004C). Protein lysates were collected at designated time-points and the blots performed as previously [Roca et al., 2008a].

GENERATION OF STABLE SHORT-HAIRPIN-RNA (shRNA) CELL LINES

Two independent shRNAs (shS-1 and shS-2; Cat. RHS4430-98520325 and RHS4430-99140887, respectively; Open Biosystems), as well as control Empty-Vector (EV) and Scrambled (Scr) sequences, were packaged into lentiviruses by the University of Michigan Vector Core. Cell-transfection was performed as previously described [Roca et al., 2008a]. Stable-transfected cell-lines were named: $PC3^{EV}$, $PC3^{Scr}$, $PC3^{sh\text{-}1}$, and $PC3^{sh2}$. From the whole population of $PC3^{sh\text{-}1}$, a sub-population sh1-7 ($PC3^{sh1\text{-}7}$) that showed the largest decrease in survivin expression was isolated and used in all experiments. $PC3^{sh2}$ represents the total population from shS-2-transfected PC3.

PRIMARY ANTIBODIES

All antibodies used in Western-analyses were obtained from Cell Signaling: Survivin (Cat. 2808), β-Actin mAb (Cat. 4967L), Phospho-Akt (Ser473) (Cat. 4058), Phospho-c-Raf (Ser338) (Cat. 9427), Phospho-Mek1/2 (Ser217/221) (Cat. 9154), Phospho-Erk1/2 (Ser217/221) (Cat. 9154), ERK1/2 (Ser217/221) (Cat. 4695), Phospho-p38 (Thr180/Tyr182) (Cat. 4631), p38 (Cat. 9212), Phospho-JNK (Thr183/Tyr185) (Cat. 4668), JNK (Cat. 4668), Phospho-ATF2 (Thr71) (Cat. 9221), Phospho-JUN (Ser63) (Cat. 9252), Phospho-p70S6K (Thr389) (Cat. 9234), LC3B (Cat. 3868).

WST-1 CELL VIABILITY ASSAY

Cell Proliferation Reagent WST-1 (Roche) was used to assess cell viability and chemosensitivity as described [Roca et al., 2008a]. To analyze cell proliferation in the presence of IL-4, synchronized-cells were plated in RPMI (0.5–1% FBS) and allowed to attach for 6 h. After attachment, cells were stimulated with IL-4 (100 ng/ml) and (or) treated with the inhibitors (U0126-MEK1/2 inhibitor), SB 220025 (p38-inhibitor) and JNKi-V; (EMD/Calbiochem) at the indicated concentrations.

IN VIVO BIOLUMINESCENT ANALYSIS OF PROSTATE CANCER GROWTH AND HISTOLOGY

Bioluminescent imaging of $PC3^{EV}$, $PC3^{Scr}$, $PC3^{sh1\text{-}7}$, and $PC3^{sh2}$ cells was done as previously described [Loberg et al., 2007]. Once individual mice reached critical tumor burden ($ROI > 1 \times 10^{10}$ photons per second), tumors were harvested from the left and right adrenal glands, fixed, paraffin-embedded, and 5-mm sections were placed on glass slides. Hematoxylin–eosin staining was performed according to the manufacturer's instructions (Sigma, Inc., St. Louis, MO). Identification of cell proliferation was accomplished by labeling with an anti-Ki-67 antibody (HPA001164, Sigma), and survivin staining was performed using anti-survivin antibody (Cell Signaling; Cat. 2808).

STATISTICAL ANALYSIS

Average values are presented as the means +/− SD. The data were analyzed using repeated measures mixed models of WST-1 ratio to baseline generated for each cell line separately with an unstructured

correlation matrix. Fixed covariates in the model included group, time, second order of time (to account for the shape of the growth), and each time covariate with group interaction. Pairwise comparisons using contrasts were generated to test the growth difference between groups. Additionally, the cross-sectional comparison at 120 h was made using an ANOVA model. Tumor growth measures were modeled to test the differences in tumor growth. All statistical models were performed using SAS 9.2 (SAS Institute, Cary, NC). Statistical significance was determined at a threshold of 0.05 unless otherwise stated. Bonferroni multiple-comparisons corrections were made to adjust for multiple-testing where appropriate.

RESULTS

IL-4 INDUCES PROSTATE CANCER CELL PROLIFERATION IN A NUTRIENT-DEPLETED ENVIRONMENT

Previous studies have suggested that IL-4 may have both stimulatory and inhibitory effects on the growth of malignant cells [Topp et al., 1995; Gooch et al., 1998; Chang et al., 2000; Gooch et al., 2002; Yu et al., 2004; Prokopchuk et al., 2005]. Here we investigated the effects of IL-4 on the proliferation of prostate cancer PC3 cells when subjected to nutrient-deprivation stress. To analyze this effect, PC3 cells were serum-starved for 16 h, plated in low serum (0.5% FBS), and stimulated with IL-4. Cells were trypsinized and counted at 24 h intervals (up to 72 h) using the trypan-blue exclusion assay. Figure 1A depicts the increase over time in the live cells counts of IL-4-treated samples relative to control (untreated) cells. A dose response in proliferation is also observed as an increase in live cells from 50 to 100 ng/ml of IL-4. In addition PC3 cell proliferation was assessed by performing the WST-1 assay at increasing time-points. As shown in Figure 1B, the IL-4-stimulated cells demonstrated a sustained increase in WST-1 values (up to 96–120 h) that corresponds to an increase in cell number as observed in Figure 1A. In contrast, the control cells showed modest proliferation at the expense of the initial nutrients and FBS (0.5%); however, as the cells became nutrient-depleted they were unable to proliferate further. To demonstrate that cells become nutrient-depleted under these culture conditions, protein samples were collected at different time-points and analyzed by immunoblotting using the LC3B antibody. Microtubule-associated protein LC3 is widely used to monitor autophagy [Mizushima and Yoshimori, 2007; Roca et al., 2008b]. Activation of autophagy involves the cleavage of LC3-I and its conjugation with phosphatidylethanolamine to form LC3-II, a process that is essential to autophagosome formation. As observed in Figure 1C at 24 h when the medium is fresh the LC3-I band is observed; however, at a later time (72 h and beyond) this band is almost undetected as a result of cleavage and conversion into LC3-II (compare lane 1 with 2–7), which serves as a good indication of higher autophagosome formation and activation of autophagy. Therefore, since autophagy is activated in response to nutrient scarcity, these findings suggest that these culture conditions generate a nutrient-depleted stressed environment where IL-4 is capable of inducing proliferation in the prostate cancer PC3 cells.

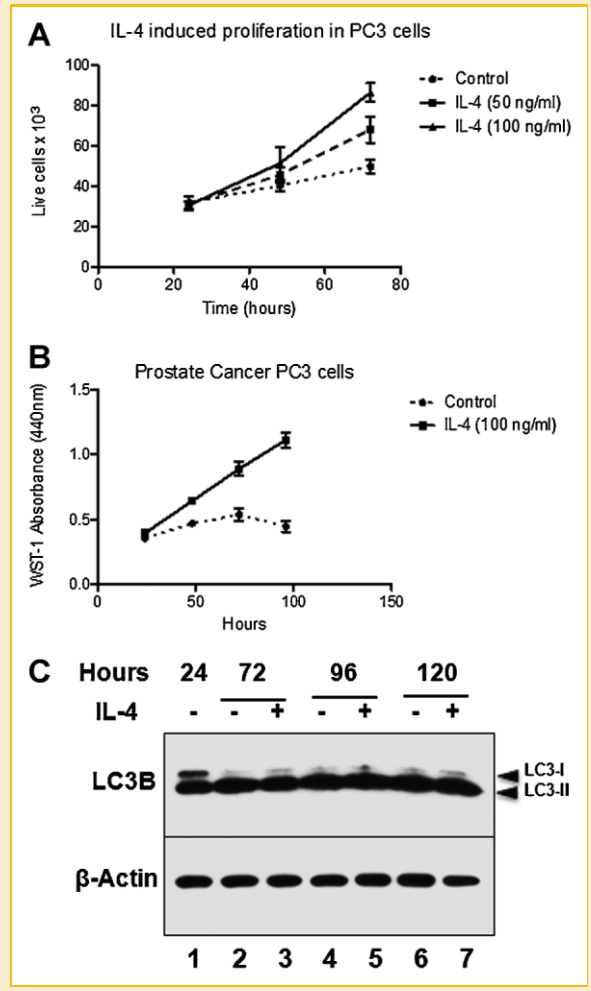

Fig. 1. IL-4 induces prostate cancer PC3 cell proliferation in a nutrient-depleted environment. A: To analyze the effect of IL-4 in prostate cancer proliferation, PC3 cells were serum-starved for 16 h, seeded in 24 well/plates (3×10^4 cells/well) in low serum (0.5% FBS), and stimulated with IL-4 at two different concentrations 50 and 100 ng/ml. Triplicate samples from control and IL-4-treated cells were trypsinized and counted at 24 h intervals (up to 72 h) using the trypan-blue exclusion assay and the automated cell counter (Countess, Invitrogen). The graph depicts the increase in the live cells counts over time in IL-4-treated and the control (untreated) cells. B: WST-1 cell proliferation assay. Cells were synchronized for 16 h by serum starvation and plated in 96 wells (3×10^3 cells/well) in low serum (0.5% FBS) with or without IL-4 (100ng/ml). The proliferation curves represent the WST-1 values (average absorbance (440 nm)) of $n = 10$ samples measured at 24 h increments up to 96 h. C: Autophagy activation in PC3 cells growing under nutrient-depleted conditions. Cells were synchronized as described above, seeded in 6 well/plates (2×10^5 cells/well) (0.5% FBS) and treated or not with IL-4 (100 ng/ml). Protein samples were collected at the indicated time-points and analyzed by Western Blot using the LC3B antibody. The immunoblot analysis depicts the autophagosome formation induced by nutrient-depletion, evidenced by the conversion of LC3-I protein into LC3-II from 24 h (low autophagosome formation, LC3-I is observed) to 72–120 h (high autophagosome formation, LC3-I almost undetectable and higher ratio LC3-II/LC3-I), which serves as a good indicator of autophagy activation.

JNK-PATHWAY ACTIVATION MEDIATES THE IL-4-INDUCED PROLIFERATION IN PROSTATE CANCER PC3 CELLS

The crucial role of mitogen-activated protein kinase (MAPK) signaling in the signal transduction of many mitogenic factors

and their up-regulation in human tumors has been abundantly documented [Lopez-Bergami et al., 2007; Wagner and Nebreda, 2009; Ferte et al., 2010]. To determine if MAP-kinases are involved in the mechanism of IL-4-induced PC3 proliferation, the activation of MAPK-pathways by IL-4 was investigated. The cells were plated in serum-free medium for 16 h, and following IL-4-stimulation, protein lysates were collected at increasing time-points as indicated in Figure 2A–C. The cells triggered a signaling-cascade with the activation of MAPK-pathways, including the extracellular signal-regulated kinase (ERK)1/2, p38, and JNK. As observed in Figure 2A–C, IL-4 induced phosphorylation of c-Raf, MEK1/2, ERK1/2, p38, and JNK, as well as downstream targets of p38 and JNK-signaling: The transcription factors ATF-2 and JUN, two members of the activator protein 1 (AP-1) family which are implicated as regulators of altered gene expression and proliferation in response to cytokines, growth factors and oncogenic transformations [Davis, 2000; Jochum et al., 2001; Shaulian and Karin, 2002].

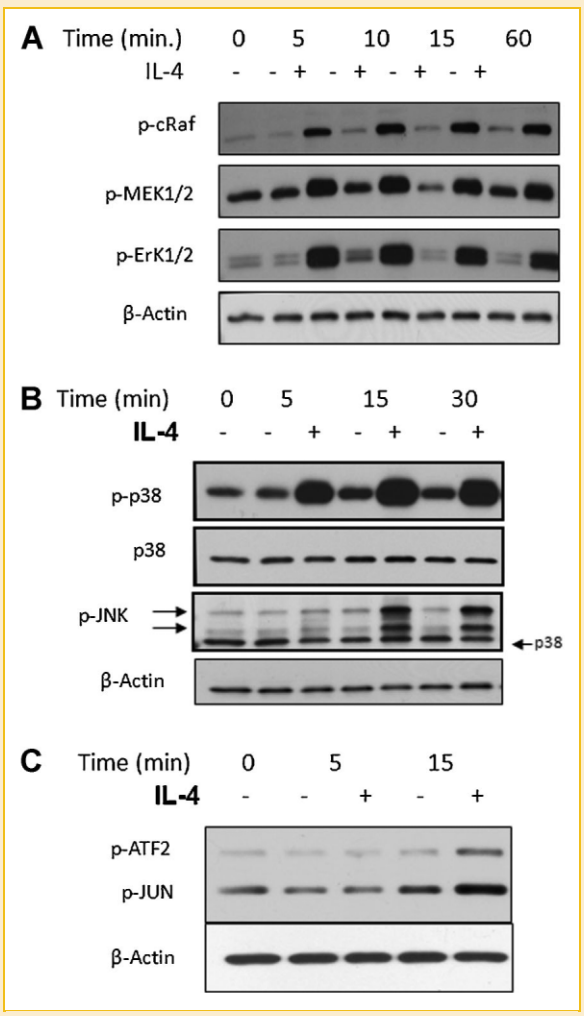

Fig. 2. IL-4 activates the ERK, p38, and JNK signaling pathways in cancer cells. Immunoblot analysis of prostate cancer PC3 cells demonstrates the activation of mitotic-activated protein kinase (MAPK) signaling pathways in response to stimulation with IL-4 (100 ng/ml). MAPK pathways include the activation of: A: Extracellular regulated kinases, ERK1/2, and (B) the stress-activated protein kinases, p38-MAPK and the JUN N-terminal kinase (JNK). The analysis also reveals the activation of downstream transcription-factors, JUN and ATF2 (C).

Next, using specific kinase-inhibitors for each signaling pathway, the role of MAP-kinases in the mechanism of IL-4-induced PC3 proliferation was assessed. The contribution of ERK1/2, p38, and JNK pathways was analyzed in independent experiments using the inhibitors U0126 (inhibits MEK1/2 upstream of ERK1/2), SB 220025 and (JNKi-V), respectively. First, even though MEK1/2-ERK1/2-inhibitor (U0126- 10 μM) and p38-inhibitor (SB 220025- 0.5 μM) demonstrated target-specific inhibition of phosphorylation (Fig. 3B,D), no effect on the cell proliferation induced by IL-4 was observed in a parallel assay (Fig. 3A,C). In contrast, the JNKi-V not only suppressed JNK phosphorylation (Fig. 3F), but also demonstrated a dose-dependent inhibition of the IL-4-mediated proliferation (blue lines, Fig. 3E) in this nutrient-depleted environment. This inhibitor further suppressed the basal proliferation observed in the control cells (red lines, Fig. 3E). Altogether these findings suggest that IL-4-induced activation of JNK is a function crucial to promoting prostate cancer PC3 cell proliferation.

IL-4 INDUCES SURVIVIN UP-REGULATION UNDER NUTRIENT-DEPLETION STRESS AS A CRITICAL FACTOR IN THE PC3 PROLIFERATION RESPONSE

The connection between cytokines and survivin has been established in different cancer cells; for example, it has been reported that different cytokines, like IL-2, IL-4, and GM-CSF, induce survivin up-regulation [Faderl et al., 2003; Decker et al., 2010]. Furthermore, survivin plays an essential role in mitosis and has been connected to cell proliferation networks [Altieri, 2008]. Recently, it was shown that CCL2 up-regulates survivin in nutrient-depleted PC3 cells [Roca et al., 2008a; Roca et al., 2009]. Therefore, it was hypothesized that IL-4 could also up-regulate survivin under nutrient-depletion stress as a critical mechanism to induce proliferation, and so the effect of IL-4 on the regulation of survivin was investigated. PC3 cells were serum starved for 16 h and plated in serum-free media for a total of 96 h to create a nutrient-depleted environment at later culture-times. Protein lysates were collected at different times and analyzed by immunoblotting. As shown in Figure 4A, survivin is up-regulated in nutrient-depleted cells in response to IL-4 compared to the untreated controls. In fact, the IL-4-induced survivin up-regulation becomes significant at later time-points (72 96 h; see lanes 7–10 Fig. 4A), when survivin levels drop as a result of nutrient depletion stress (after 48 h of culture).

Next, the impact of survivin up-regulation on the mechanism of IL-4-mediated proliferation was further investigated in prostate cancer cells through the generation of survivin-depleted cells using shRNAs. Two survivin-specific shRNAs (shS-1 and shS-2), as well as two corresponding controls: Empty vector (EV) and scrambled shRNA (Scr), were packaged into lentivirus and transfected into luciferase-expressing PC3 cells. Following selection, four stable-transfected cell lines were generated: $PC3^{EV}$ and $PC3^{Scr}$ corresponding to the control vectors, and $PC3^{sh1\text{-}7}$ and $PC3^{sh2}$ corresponding to the survivin-specific shRNAs: shS-1 and shS-2, respectively. These cells were further characterized in vitro to evaluate cell proliferation and the corresponding survivin levels. Both control and knockdown cells were plated in low serum (1% FBS), and the cell viability was

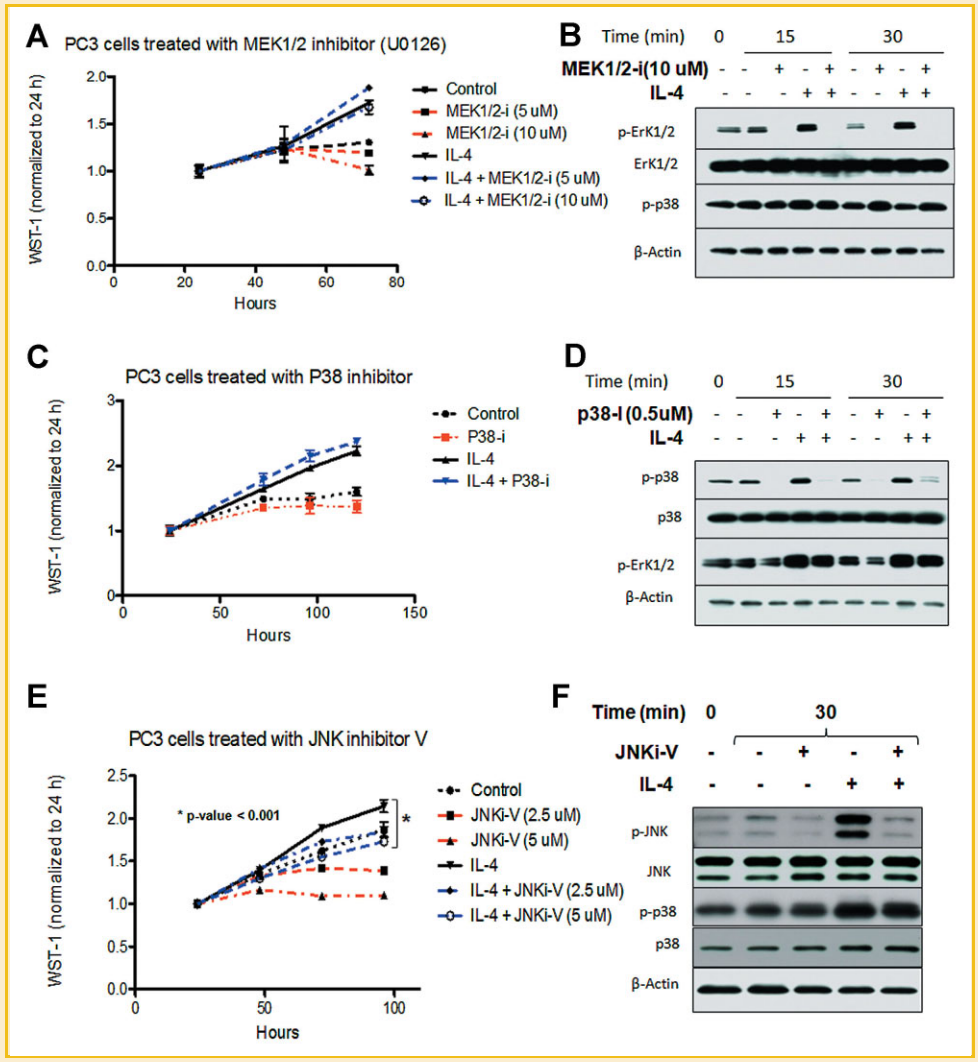

Fig. 3. JNK-pathway activation mediates the IL-4-induced proliferation in prostate cancer PC3 cells. IL-4-induced proliferation is JNK-pathway dependent and ERK and p38 independent. Specific kinase inhibitors were used to analyze the role of MAP-kinases in the IL-4-induced proliferation mechanism. Cell proliferation was assessed by WST-1 dye conversion at 24 h increments (as described in Fig. 1B; where the WST-1 values represent the average absorbance of n = 10 samples per condition) for IL-4 treated and untreated PC3 in the presence of the following inhibitors: (A) MEK1/2-i (U0126, inhibits MEK1/2 upstream of ERK1/2); (C) p38-i (SB 220025, inhibits p38 activation) and (E) JNKi-V (JNK-inhibitor-V, inhibits JNK activation). The inhibitors were used at the indicated concentrations. All WST-1 values were normalized to the values at 24 h. The specificity of the inhibitors was evaluated by Western blot in each case using phospho-specific antibodies as indicated in (B) (MEK1/2-i), (D) (p38-i), and (F) (JNKi-V).

measured using a WST-1 assay at 24 h intervals. As shown in Figure 4B, both knockdown and control lines demonstrated similar proliferation rates during the first 72 h. At this time, a parallel immunoblotting analysis revealed high levels of survivin in all cells, including the knockdown cells (Fig. 4C). However, after 72 h, $PC^{sh1\text{-}7}$, and $PC3^{sh2}$ showed a significant decrease in cell proliferation compared to controls (Fig. 4B). As seen in Figure 4C, at 144 h, survivin levels demonstrated a significant drop in knockdown cells ($PC3^{sh1\text{-}7}$ and $PC3^{sh2}$), which correlates with the nutrient exhaustion that occurs at a later times and a significant decrease in cell proliferation (Fig. 4B). Altogether, this analysis suggests that survivin-shRNAs could effectively induce knockdown only under conditions of limited nutrients. In fact the knockdown-shRNAs have a limited effect during conditions of abundant nutrients at the initial culture times (first 72 h), when survivin levels are high enough to sustain proliferation. However, when survivin drops below a critical threshold (after 72 h), as a result of nutrient-depletion and the effect of shRNAs, then the cell proliferation declines as observed in knockdown cells (Fig. 4B).

Following cell characterization, it was investigated how survivin-knockdown affects the IL-4-mediated proliferation in these cells. Three cell lines: PC3, $PC3^{Scr}$ (controls), and $PC3^{sh1\text{-}7}$ (knockdown) were serum starved and plated in 0.5% FBS to create a nutrient-depleted environment in these cultures and proliferation was assessed upon IL-4 stimulation. As shown in Figure 5A, IL-4-stimulated cells showed a major increase in proliferation relative to control (untreated) cells. However, the IL-4-mediated proliferation response was considerably lower in knockdown ($PC3^{sh1\text{-}7}$) when compared to controls. These findings suggest that the shRNA-mediated survivin knockdown reduces the proliferation-inducing

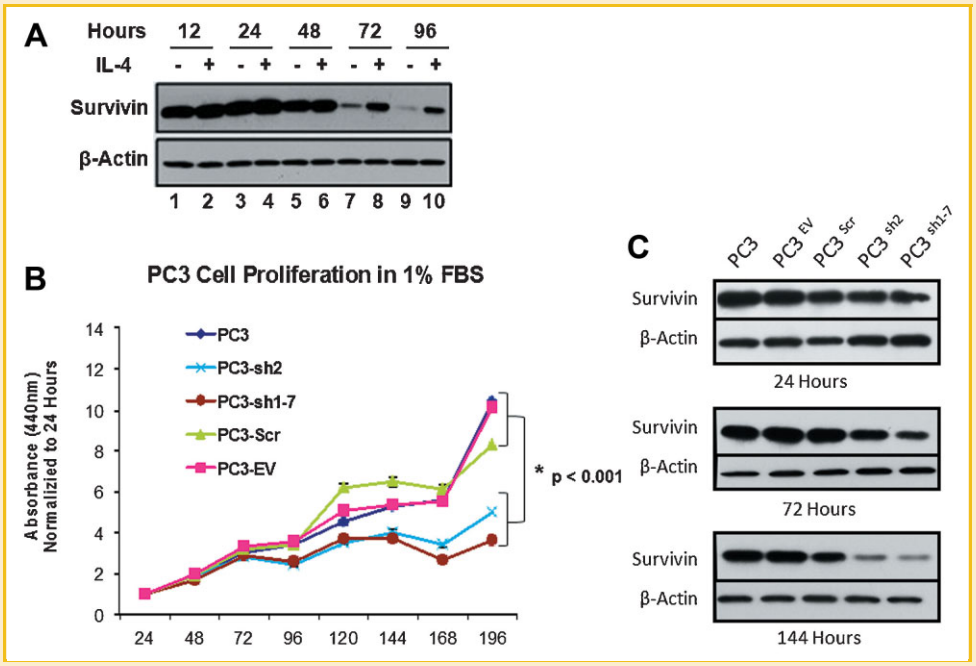

Fig. 4. IL-4 induces survivin up-regulation in nutrient-depleted PC3 cells. A: Immunodetection showing the time-course effect of IL-4 on survivin expression in nutrient-depleted prostate cancer cells. PC3 cells were serum starved for 16 h and cultured in serum-free media for a total of 96 h to create a nutrient-depleted environment. Protein lysates were collected at different time-points and analyzed by Western blot using survivin monoclonal antibody (Cell Signaling). The figure depicts the survivin up-regulation in nutrient-depleted PC3 in response to IL-4 stimulation compared to the untreated controls. Up-regulation of survivin by IL-4 was observed at 72 and 96 h (compare lanes 7 and 8 to 9 and 10). At these later times the nutrient scarcity is intensified and survivin levels significantly drop as a result of nutrient-depletion stress (after 48 h of culture, compare lanes 1–6 to 7 and 9). B–C: Progression of cell proliferation is dependent on survivin expression above a critical threshold level. Analysis of cell proliferation and survivin protein in PC3 cells expressing survivin-specific shRNA (sh1-7 and sh2) as compared to the controls (PC3, Scr (scrambled-shRNA) and EV (empty vector)). B: Cells were cultured in the presence of low serum (1% FBS) and cell proliferation was evaluated by measuring the WST-1 absorbances at increasing time points up to 196 h ($n = 10$ per cell-line at each time-point). C: Parallel Western analysis of survivin protein that correspond to 24, 72, and 144 h of the time course. Note that survivin protein levels significantly drop in the knockdown-cells ($PC3^{sh2}$ and $PC3^{sh1-7}$) from 72 to 144 h, when the conditions of nutrient scarcity are achieved and the differences in cell proliferation are accentuated compared to controls (PC3, $PC3^{Scr}$ and $PC3^{EV}$) (see Fig. 4B).

potential of IL-4 on prostate cancer cells. In a parallel assay, survivin levels were examined at two different time points: 48 and 96 h (Fig. 5B). The 96 h time-point corresponds to a more advanced nutrient-depletion stage in culture as compared with 48 h. As shown in Figure 5B survivin expression was higher in control cells (PC3 and $PC3^{Scr}$) as compared to $PC3^{sh1-7}$. Additionally, IL-4-stimulation induced a significant survivin up-regulation in the knockdown-cells (compare lanes 5 and 6 and 11 and 12). This increase was more striking at 96 h (lanes 7–12), when IL-4 was able to rescue the expression of survivin (compared to control $PC3^{Scr}$ and $PC3^{sh1-7}$ cells). The rescue of survivin correlates with the increasing slope in the proliferation curve from 96 to 120 h (Fig. 5A). Furthermore, the critical drop of survivin, observed in $PC3^{sh1-7}$ cells from 48 to 96 h (lanes 5 and 11, Fig. 5B), also correlates with the reduced proliferation when compared to control cells (PC3 and $PC3^{Scr}$).

To understand the mechanism of IL-4-induced survivin up-regulation, by which survivin expression is rescued in $PC3^{sh1-7}$ cells (Fig. 5B), the mRNAs were isolated from control and IL-4-treated cells and the relative survivin-mRNA expression was analyzed. As shown in Figure 5C no significant changes were observed in survivin-mRNA between control and IL-4-stimulated cells at two different times: 72 and 96 h. These results suggest that survivin up-regulation is not controlled by a transcriptional mechanism, but rather by differences in mRNA translation. Furthermore, in prostate cancer cells it has previously been shown that hyperactivation of mTORC1 and the downstream kinase p70S6K originate a differential survivin expression at the protein level through changes in mRNA-translation [Vaira et al., 2007; Roca et al., 2009]. In fact, as shown in Figure 5D, IL-4 induces a sustained activation of p70S6K (lanes 2 and 4), while the activated kinase is dramatically down-regulated in control cells by 96 h (lanes 2–3). Therefore, these findings suggest that IL-4 opposes the negative effect of survivin-shRNA by stimulating a sustained increase in the translated survivin. In total, these results are similar to previous reports showing that p70S6K activation mediates survivin protein up-regulation in prostate cancer cells by cytokines like CCL2 [Roca et al., 2008a; Roca et al., 2009] or IGF1 [Vaira et al., 2007].

Subsequently, the possible link between JNK-activation and survivin up-regulation in the IL-4-induced proliferation mechanism under nutrient-depletion stress was further assessed using $PC3^{sh1-7}$ cells. The experiment was performed as described in Figure 3E, and both control and IL-4-stimulated cells were treated with JNKi-V at 2.5 μM, a concentration known to affect cell proliferation. The cells were incubated for 72 and 96 h, and survivin expression was analyzed by immunoblotting at these time-points. As expected, survivin decreased at 96 h with the increase of nutrient scarcity

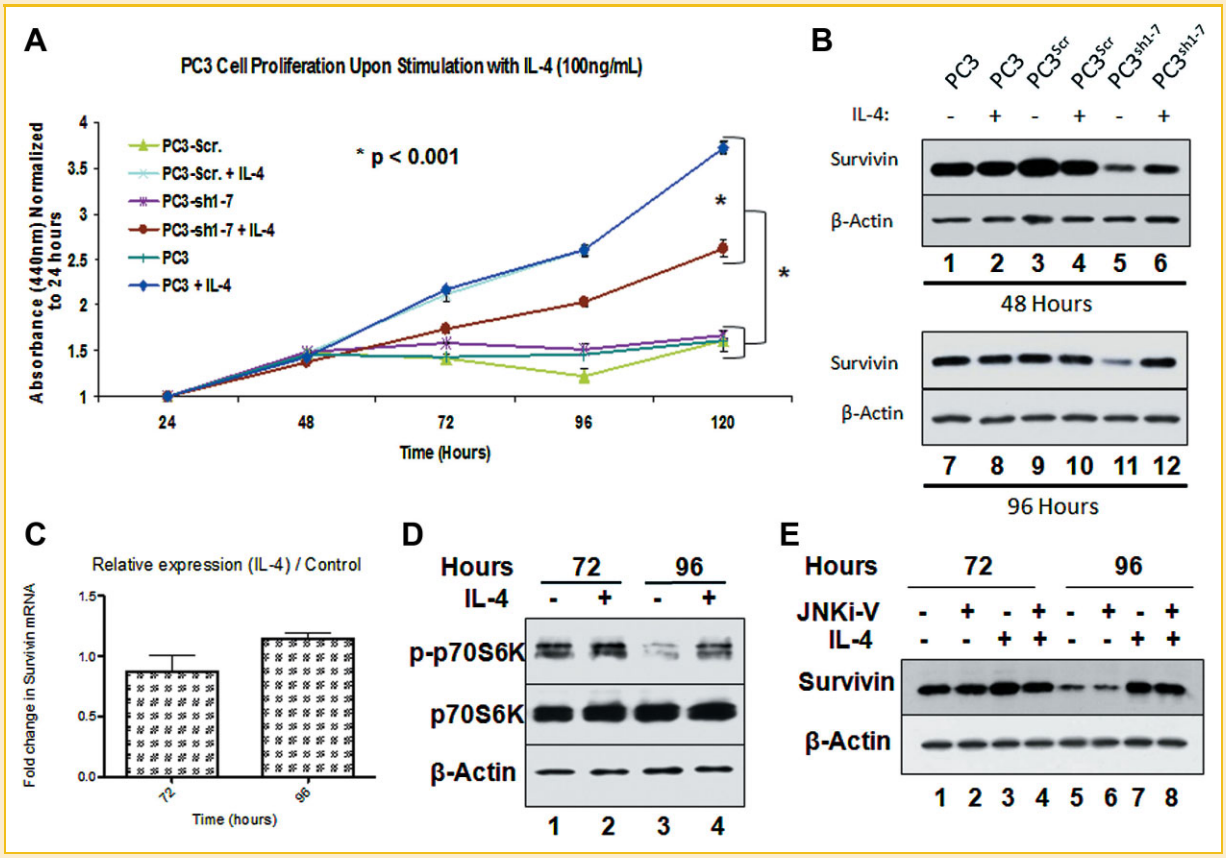

Fig. 5. IL-4 induces survivin up-regulation under nutrient-depletion stress as a critical factor in the PC3 proliferation response. IL-4-mediated cell proliferation was analyzed in survivin-depleted cells (PC3-sh1-7) and compared to controls (PC3 and PC3-Scr). A: Cell proliferation was assessed by measuring the WST-1 absorbance at increasing time points up to 120 h ($n = 10$). B: Parallel analysis by Western blot of survivin expression corresponding to 48 and 96 h of culture. C: Analysis of survivin mRNA in IL-4-treated $PC3^{sh1-7}$ compared to untreated cells. The total RNA was isolated from triplicate samples of IL-4-stimulated or control cells at 72 and 96 h of culture. The graph depicts the ratios of survivin mRNA in IL-4-treated relative to control samples after normalization to the β-Actin control-probe (survivin probe- Hs00977611_g1 and β-Actin-Hs99999903_m1; TaqMan Gene Expression Assays, Life Technologies). D: In a parallel experiment as described in (C), the protein was also collected and the activation of p70S6 kinase (a downstream target of mTOR Complex-1) was analyzed by immunoblotting using the phospho-specific antibody (phospho-p70S6K-Thr389). Note a sustained activation of p70S6 kinase in IL-4 stimulated cells (lanes 2 and 4), while a critical drop is observed at 96 h in control cells. E: Survivin expression is not affected by treatment with a JNK-inhibitor-V (JNKi-V). The experiment was performed as described in Figure 3E, and both control and IL-4-stimulated cells were treated with JNKi-V at 2.5 μM. The cells were incubated for 72 and 120 h, and survivin expression was analyzed by immunoblotting at these time points.

(lanes 5–6), and IL-4-stimulation induced survivin up-regulation in these cells (lanes 7–8); however, survivin expression was not affected by treatment with a JNK-inhibitor when used at a concentration that affects cell proliferation (Fig. 5E). Altogether these findings suggest that survivin up-regulation is independent of JNK-activation, and therefore, both survivin up-regulation and JNK-activation are two essential factors induced by IL-4 to sustain prostate cancer proliferation under nutrient-depletion stress.

SURVIVIN KNOCKDOWN DECREASES TUMOR PROGRESSION AND INCREASES MOUSE SURVIVAL IN CORRELATION WITH CANCER CELL PROLIFERATION UNDER NUTRIENT-DEPLETION STRESS

The significance of survivin up-regulation in a nutrient-depleted or stressed environment was further assessed in vivo. Control and survivin-knockdown cells were injected into the left ventricle of male SCID-mice (ICI model) [Arguello et al., 1988; Jenkins et al., 2003]. Mice were imaged weekly, and the total tumor burden was analyzed and calculated as regions of interest (ROI). Fifteen mice were injected per cell line, and survivin-knockdown cells, $PC3^{sh2}$ and $PC3^{sh1-7}$ were compared to the controls, $PC3^{EV}$ and $PC3^{Scr}$. Analysis of ROI-values revealed significant differences in tumor burden between controls and survivin-knockdown cells (Fig. 6A). This significant delay in tumor progression in the survivin-knockdown groups correlates with the differences observed in cell proliferation between these cells and the controls in a nutrient-depleted environment (Fig. 4B,C). Furthermore, as shown in Figure 6B, the Kaplan–Meier survival analysis also correlates with the tumor progression differences observed between the groups. In fact, mice injected with survivin-knockdown cancer cells showed a significant increase in survival when compared to control mice (average survival 76% for knockdown-injected mice compared to 36% for the control mice; P-value <0.05).

Once control mice reached critical tumor burden ($ROI > 1 \times 10^{10}$), tumors were dissected from adrenal glands for each group of mice. Collected samples were stained for hematoxylin and eosin (H&E), survivin, and Ki67, a known marker of cell proliferation [Whitfield

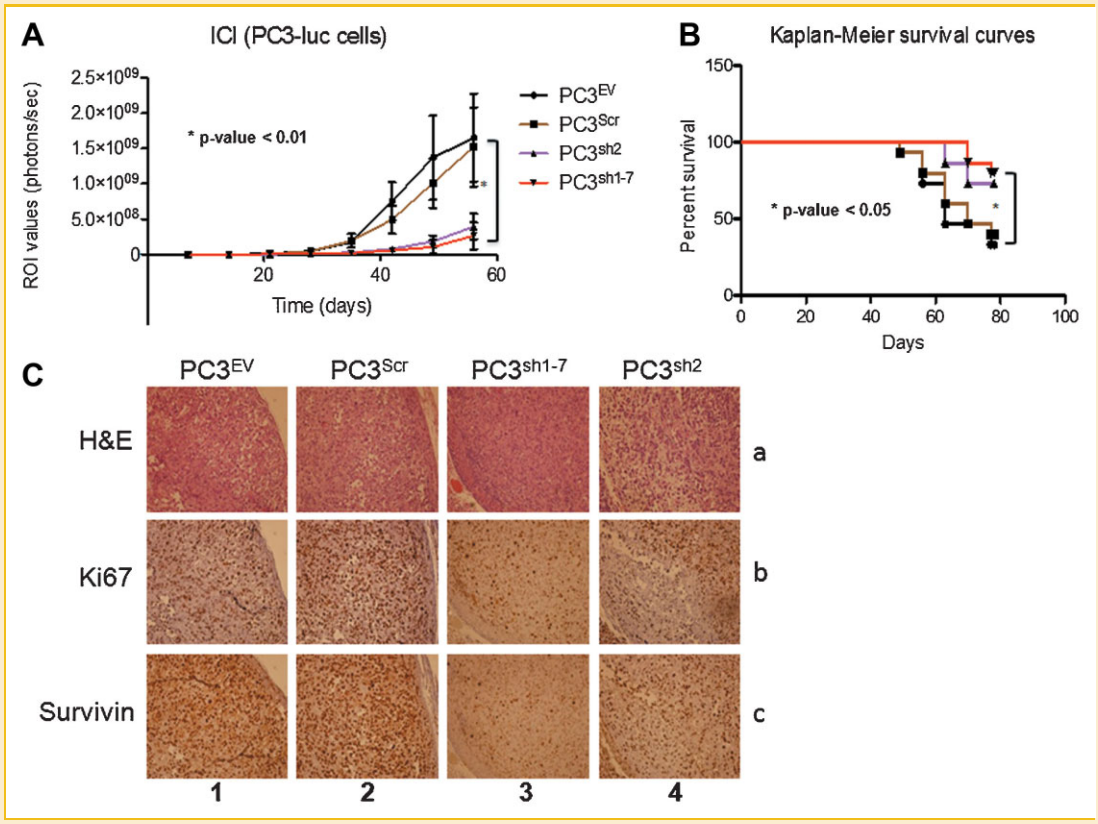

Fig. 6. Survivin knockdown decreases tumor progression and increases mouse survival in correlation with cancer cell proliferation under nutrient-depletion stress. In vivo analysis of tumor progression using the ICI model to compare survivin-knockdown and control cells. A: Bioluminescence analysis of tumor growth. The graph depicts the average tumor burden calculated from regions of interest (ROIs in photons/second) of all mice for every group (EV, Scr, sh1-7, and sh2). B: Kaplan–Meier cancer-survival curves showing mouse survival for every group. C: Immunohistochemical analysis of adrenal tumor samples from each group of mice stained for hematoxilin and eosin (H&E) (panels a1–a4), the proliferation marker, Ki67 (panels b1–b4), and survivin (panels c1–c4).

et al., 2002; Glinsky et al., 2005]. A representative staining is shown in Figure 6C. H&E staining revealed similar tumor morphology with high concentration of cancer cells in all groups (Fig. 6C; panels a1–a4). However, as expected, the control groups $PC3^{EV}$ and $PC3^{Scr}$ showed a significantly higher survivin staining compared to the knockdown (Fig. 6C; panels b1–b4). Furthermore, correlating to the in vitro data, the proliferation marker Ki67 revealed an increased staining in the controls compared to survivin-knockdown ($PC3^{sh1-7}$ and $PC3^{sh2}$) (Fig. 6C; panels c1–c4). Overall, these results indicate a direct correlation between the survivin levels and tumor-cell-proliferation, which also correlates with overall tumor progression and mouse-survival. Therefore, decreasing survivin levels in the cancer cells results in decreased cancer proliferation in the mouse microenvironment.

IL-4 INDUCES PROLIFERATION IN CANCER CELLS FROM DIFFERENT ORIGINS

As IL-4-induced cancer cell proliferation may have implications in the progression of other types of cancer, its effect was investigated in cancer cells from different origins: In breast cancer MDA-MB231, head and neck cancer A253, and ovarian cancer SKOV-3 cells. Using a similar approach as described for PC3, the effect of IL-4 on cell proliferation was assessed by performing a WST-1-assay at increasing time-points in low-serum conditions (0.25–0.5% FBS). As shown in Figure 7A, the IL-4-stimulated cells demonstrated a sustained increase in WST-1-values, while the control-cells showed modest proliferation up to the first 48 h of culture, the point when the cells encounter nutrient-scarcity and are unable to proliferate further. These results suggest that IL-4 has the potential to induce proliferation in environmentally stressed cancer cells of different origins similar as it does with PC3 cells.

Next, MDA-MB-231 cells were selected to investigate if JNK-pathway activation is essential to this proliferation mechanism. Similar to PC3, when MDA-MB-231 cells were treated with the JNKi-V, a dose-dependent inhibition of IL-4-mediated cell proliferation was achieved (Fig. 7B). These findings imply that IL-4-induced activation of JNK-signaling is crucial to promote cancer proliferation. Furthermore, survivin is also up-regulated by IL-4 in nutrient-depleted MDA-MB-231 cells (Fig. 7C), suggesting that both factors identified to be crucial in the mechanism of IL-4-induced proliferation in nutrient-depleted PC3, JNK-activation, and survivin up-regulation (Fig. 7D), could play a critical function in different cancer types. However, a complete elucidation in each case requires further investigation.

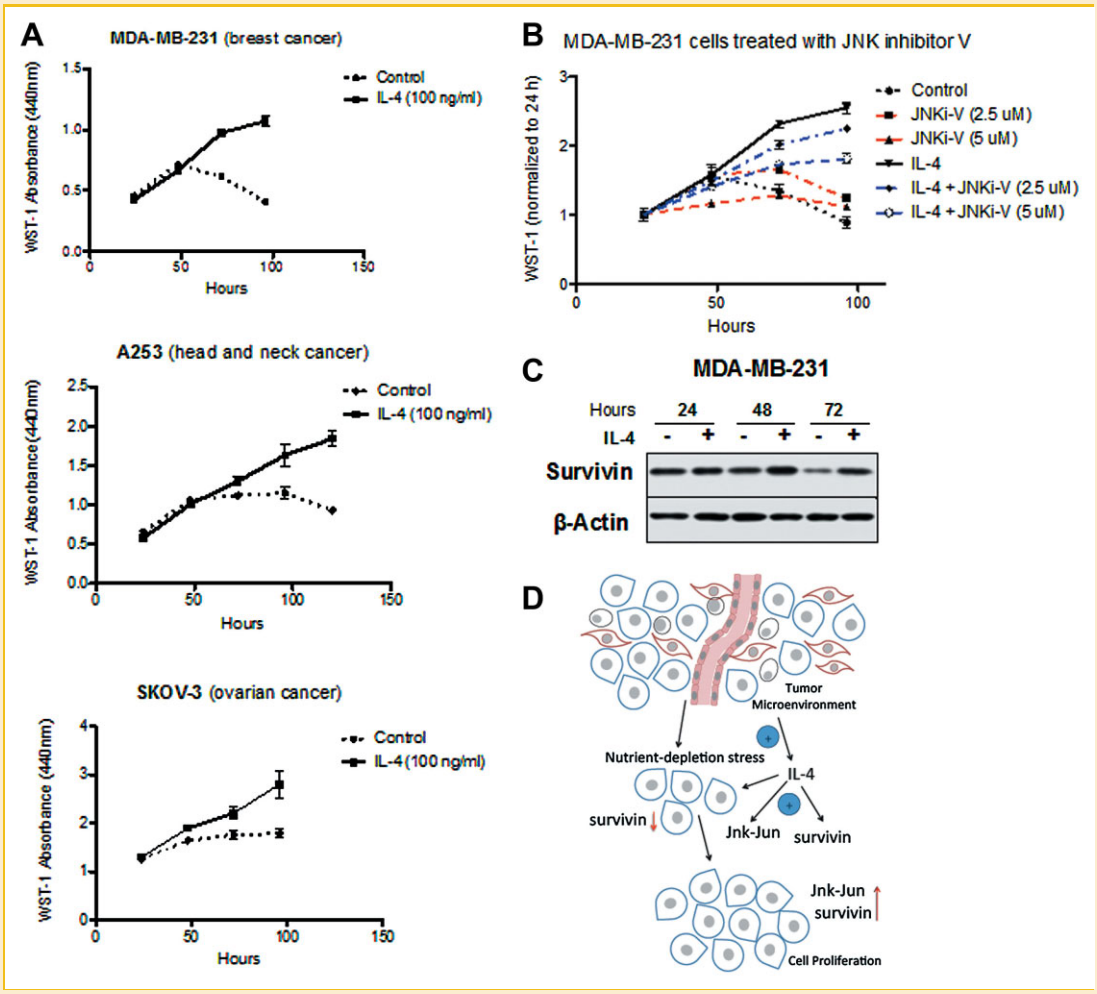

Fig. 7. IL-4 induces proliferation in cancer cells from different origins. A: The IL-4 effect on cell proliferation was investigated in the breast cancer MDA-MB231, head and neck cancer A253, and ovarian cancer SKOV-3 cells. Cells were synchronized for 16 h by serum starvation and then plated in low serum (0.25–0.5% FBS) with or without IL-4 (100ng/ml). The proliferation curves represent the WST-1 values (average absorbance (440 nm)) of n = 10 samples for each cell line measured at 24 h increments. B: The critical role of JNK-pathway activation was assessed in MDA-MB-231 cells by using the JNK inhibitor V (JNKi-V) as described in Figure 3E. C: IL-4 induces survivin up-regulation in serum-depleted MDA-MB-231 cells. Analysis by Western blot showing the time course effect of IL-4 on survivin expression in breast cancer MDA-MB-231 cells treated as described in (A). D: Proposed mechanism by which IL-4 induces cancer proliferation under nutrient-depletion stress. In the tumor microenvironment, cancer cells face a shortage of nutrients that results in the down-regulation of survivin and a consequent decrease in proliferation. However, in this challenging and stressed environment, IL-4 turns on a proliferation mechanism mediated by the simultaneous activation of the JNK-pathway and the up-regulation of survivin.

DISCUSSION

Several studies support the role of IL-4 as a contributor to tumor progression via its effect on the cells of the tumor microenvironment [Stremmel et al., 1999; Fukushi et al., 2000; Gordon, 2003; Gocheva et al., 2010]. For example, IL-4 induces the alternative activation of macrophages (M2-type) and contributes to the transition of macrophages into tumor-promoting that facilitate tumor growth, angiogenesis, and invasion [Mantovani et al., 2008]. Furthermore, increased levels of IL-4 receptor have been reported in a variety of human cancers [Kawakami et al., 2002; Koller et al., 2010], and IL-4 may actually promote tumorigenesis by a direct effect on the malignant cells [Conticello et al., 2004; Todaro et al., 2006; Li et al., 2008]. Aberrantly increased cell proliferation is a requisite of successful tumor progression and the ability to metastasize at distant sites. Although studies have found examples of IL-4 having both negative and positive effects on cell proliferation in general, studies with cancer cells have suggested that IL-4 promotes malignant cell proliferation, though the mechanism is still unclear [Myers et al., 1996; Prokopchuk et al., 2005]. The results presented here demonstrate that IL-4 is a potent inducer of prostate cancer PC3 cell proliferation when the cells are subjected to nutrient-depletion stress (Fig. 1). In fact the autophagy activation at 72 h strongly suggests that cells are subjected to nutrient-scarcity (Fig. 1C). In addition, critical factors in this mechanism have been elucidated in these prostate cancer cells. It was demonstrated that IL-4 activates three MAPK signaling pathways in these cells: ERK, p38, and JNK (Fig. 2). Using specific inhibitors that differentiate between each pathway, the role of each signaling in cell proliferation was further assessed. This approach allowed the identification of the

stress-activated kinase, JNK, as a major pathway that mediates the proliferation response induced by IL-4 in prostate cancer PC3 cells under a nutrient-depletion stress (Fig. 3). However, neither ERK nor p38 inhibition demonstrated a direct effect on cancer proliferation (Fig. 3A–D). Supporting the importance of JNK is the fact that a JNKi-V, which demonstrated specific inhibition of JNK phosphorylation (Fig. 3F), also showed suppression of IL-4-induced proliferation (Fig. 3E). The JNK-pathway is primarily activated by cytokines and exposure to environmental-stress [Davis, 2000]. Studies of JNK-signaling support the role of JNK in tumor development and progression. For example, a role for JNK in tumorigenesis has been reported in liver-cancer development, whereby p38α-deficiency increased proliferation resulting from sustained activation of the JNK-JUN-pathway [Hui et al., 2007]. In a recent report, it was demonstrated that a growth-promoting function of the death-receptor, CD95, is mediated by JNK-JUN-pathway [Chen et al., 2010]. In contrast to studies that demonstrate the pro-oncogenic role of JNK, the tumor-suppressor activity of JNK has been reported to be associated with its pro-apoptotic function [Kennedy and Davis, 2003]. Therefore, JNK may play a context-dependent role in tumorigenesis. Furthermore, the role of JNK in prostate cancer is of particular importance because the tumor-suppressor PTEN, that is frequently lost in this cancer, leads to Akt-activation and increased JNK-activity both in cell-lines and in clinical prostate-cancer samples [Vivanco et al., 2007]. As PTEN is frequently mutated in cancer, the JNK-mediated proliferation induced by IL-4 could be more accentuated in this particular context.

Next, it was further demonstrated that IL-4 induces survivin up-regulation in nutrient-depleted PC3 cells (Fig. 4A). Survivin is one of the nodal proteins differentially expressed in cancer and linked to multiple signaling pathways essential for tumor-progression and metastasis, including cell-division networks and cellular-stress responses [Altieri, 2008]. Survivin up-regulation by IL-4 has been reported in colon cancer stem cells [Di Stefano et al., 2010]. Under nutrient-depletion stress, the cell machinery forces the down-regulation of survivin, and therefore, it was hypothesized that up-regulation of survivin was crucial in the mechanism of IL-4-induced proliferation. By using survivin-shRNAs, it was demonstrated that the IL-4-induced prostate cancer cell proliferation was dependent on survivin levels. In fact, as demonstrated in Figure 5, IL-4-induced proliferation decreased substantially due to the shRNA-mediated survivin knockdown in PC3. It was further demonstrated that IL-4 induces a sustained activation of the p70S6-kinase (Fig. 5D), a downstream target of mTORC1, which have been demonstrated to enhance translation of survivin-transcripts [Vaira et al., 2007] that correlates with an increase in survivin protein (Fig. 5E, lines 5 and 7). Furthermore, by using JNKi-V, it was further determined that the IL-4-induced survivin up-regulation is independent of JNK-activation. Indeed, survivin levels were not affected by the inhibitor concentration that demonstrated a negative effect on cell proliferation (Fig. 5E, lines 7 and 8). These findings suggest that survivin expression above a threshold limit in a challenging nutrient-depleted environment is essential for cellular proliferation, and therefore, IL-4-mediates PC3 cell-proliferation through independent activation of JNK-signaling and up-regulation of survivin.

Further understanding of how survivin up-regulation in a nutrient-depleted environment contributes to cell proliferation came from in vivo experiments in the ICI-model of prostate cancer extravasation and metastasis using survivin-knockdown cells. In these cells, survivin-shRNAs induce knockdown under depleted nutrients; however, no differences in proliferation or survivin levels were observed in vitro when they grow in the presence abundant nutrients (Fig. 4B,C). When injected into mice, cancer cells in the bloodstream spread throughout the body and seed into various niches. This initial process of seeding and subsequent growth occurs in an environment that is hostile to the cancer cells and that contains a very limited supply of nutrients. Consequently, decreased survivin under this environmental stress, as found in our knockdowns, would obstruct this initial process of seeding and growth, essential for tumor progression. In fact, as shown in Figure 6A,B, a significant difference in tumor progression and survival was observed between mice injected with knockdown-cells compared to those injected with control. Furthermore, tumors isolated from survivin-knockdown cells demonstrated lower proliferation as evidenced by IHC-staining with antibody for the proliferation marker Ki67 in correlation with lower survivin staining (Fig. 6C).

Although the mechanism presented here is demonstrated in prostate cancer PC3 cells, it was shown that under nutrient-depletion stress, IL-4 could induce proliferation in cancer cells from multiple origins: Breast, head and neck, and ovarian cancer (Fig. 7A). Furthermore, the critical factors of this mechanism identified in PC3 could have a general implication in other cancer cells as suggested for breast cancer MDA-MB-231 (Fig. 7B,C).

Tumor metastases are characterized by high environmental-stress and shortage of nutrients. The results presented here suggest that survivin expression is up-regulated in this environment by IL-4, a cytokine highly expressed by the leukocyte infiltrate found in the tumor microenvironment [Gocheva et al., 2010; Gordon and Martinez, 2010]. In this context, the up-regulation of survivin above a necessary threshold limit is a pathological event, which combined with JNK-hyperactivation, will guarantee tumor growth even in the most adverse conditions (Fig. 7D). The goal to effectively target survivin could be difficult to achieve because according to the findings presented here, survivin levels and cell proliferation could be rescued by cytokines like IL-4 (Figs 4A and 5B,E). However, if the most critical factors that contribute to survivin expression and JNK-activation are identified in this milieu, a targeted-therapy against them may represent an effective approach to halt tumor proliferation (Fig. 7D). Alternatively, simultaneous targeting of JNK and survivin could be effective against metastatic tumors like prostate cancer, characterized by PTEN deletion and high survivin expression.

ACKNOWLEDGMENTS

The authors thank their colleague, Yee Seng Ng for his assistance in figure preparation. This work was supported by the National Institutes of Health [Grant numbers CA093900 and P50 CA69568 to KJP], the Southwest Oncology Group [Grant number CA32102 to KJP], and the American Cancer Society Clinical Research Professorship [KJP].

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