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Activation of GPR30 inhibits the growth of prostate cancer cells through sustained activation of Erk1/2, c-jun/c-fos-dependent upregulation of p21, and induction of G_2 cell-cycle arrest

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G-protein-coupled receptor-30 (GPR30) shows estrogen-binding affinity and mediates non-genomic signaling of estrogen to regulate cell growth. We here showed for the first time, in contrast to the reported promoting action of GPR30 on the growth of breast and ovarian cancer cells, that activation of GPR30 by the receptor-specific, non-estrogenic ligand G-1 inhibited the growth of androgen-dependent and androgen-independent prostate cancer (PCa) cells *in vitro* and PC-3 xenografts *in vivo*. However, G-1 elicited no growth or histological changes in the prostates of intact mice and did not inhibit growth in quiescent BPH-1, an immortalized benign prostatic epithelial cell line. Treatment of PC-3 cells with G-1 induced cell-cycle arrest at the G_2 phase and reduced the expression of G_2-checkpoint regulators (cyclin-A2, cyclin-B1, cdc25c, and cdc2) and phosphorylation of their common transcriptional regulator NF-YA in PC-3 cells. With extensive use of siRNA-knockdown experiments and the MEK inhibitor PD98059 in this study, we dissected the mechanism underlying G-1-induced inhibition of PC-3 cell growth, which was mediated through GPR30, followed by sustained activation of Erk1/2 and a c-jun/c-fos-dependent upregulation of p21, resulting in the arrest of PC-3 growth at the G_2 phase. The discovery of this signaling pathway lays the foundation for future development of GPR30-based therapies for PCa.

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Estrogens can trigger rapid signaling responses through plasma membrane-mediated intracellular signaling independent of nuclear translocation.[1,2] Previous studies showed that ~5–10% of endogenous estrogen receptors [ERα/ERβ] and their splice variants present at the plasma membrane are responsible for these responses.[1–5] Recently, an orphan G-protein-coupled receptor (GPR30) with high-affinity and low-capacity binding to estrogens was localized to both plasma membrane and the endoplasmic reticulum. Upon binding to ligand, GPR30 induces rapid but transient activation of extracellular signal-regulated kinase-1/2 (Erk1/2) to stimulate proliferation in ER-negative breast cancer cells, and in endometrial and ovarian cancer cells.[6–10] By contrast, normal and malignant bladder urothelial cells respond to GPR30 activation with reduction in cell proliferation.[11] These findings suggest dual roles for GPR30 in the regulation of cell growth, depending on cell type. It has been suggested that GPR30 functions as a membranous estrogen receptor to elicit physiological estrogenic responses in the targeted cells,[10] but contradictory data have challenged this idea.[4,12] Thus, the biological significance of GPR30 remains unclear.

The identification of G-1 (1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone) as a GPR30-selective agonist[13] is a major advancement toward elucidating the biological significance of GPR30, especially in cells that express ERα and/or ERβ. Activation of

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Abbreviations: GPR30, G-protein-coupled receptor-30; G-1, 1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone; PCa, prostate cancer; cdc25c, cell division cycle-25 homolog-C; cdc2, cyclin-dependent kinase-1; NF-YA, nuclear transcription factor-Yα; MEK, mitogen-activated protein kinase kinase-1; Erk1/2, extracellular signal-regulated kinase-1/2; c-jun, v-jun avian sarcoma virus-17 oncogene homolog; c-fos, v-fos FBJ murine osteosarcoma viral oncogene homolog; p21, cyclin-dependent kinase inhibitor-1A; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; IC_{50}, half-maximal inhibitory concentration; DMSO, dimethyl sulfoxide; MPP dihydrochloride, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol; E2, 17β-estradiol; MSK1, nuclear mitogen- and stress-activated protein kinase-1; ATF1, activating transcription factor-1; Elk1, ETS-like gene-1

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GPR30 by G-1 was found to stimulate proliferation in BG-1 ovarian cancer cells and SKBR3 breast cancer cells through induction of c-fos expression.[9] In BG-1 cells, expression of cyclin-D1, cyclin-E, and cyclin-A1 was also found to be upregulated by G-1.[9] In SKBR3 cells, 17β-estradiol (E2)- or phytoestrogen-mediated stimulation of cell proliferation was shown to be mediated through GPR30, as these responses were abrogated by a GPR30-antisense oligonucleotide.[9,14] Similar findings were reported for ovarian, endometrial, and thyroid cancer cells in knockdown studies.[7–9] Collectively, these data show that G-1 is a useful investigative tool for studying GPR30-mediated signaling.

In this study, we showed the inhibitory role of GPR30 in the growth of prostate cancer (PCa) cells by investigating the effects of G-1 on an Erk1/2 kinase activation cascade that is causally linked to aberrant expression of early-response genes, upregulation of p21, and cell-cycle arrest at the G$_2$ phase in PCa cells.

Results

G-1-induced inhibition of cell growth *in vitro* and *in vivo*. To investigate the biological functions of GPR30 activation in PCa, we treated two androgen-deprivation-resistant cell lines, PC-3 (ERα- and ERβ-positive) and DU145 (predominantly ERβ-positive), for 4 days with 10^{-8}–10^{-5} M G-1. The treatment induced a dose-dependent inhibition of growth in both cell lines (Figure 1a, left panel). The half-maximal inhibitory concentration (IC$_{50}$) of G-1 for PC-3 cells was 1.02×10^{-6} M and for DU145 cells it was 3.21×10^{-6} M. Thus in all subsequent experiments we chose to use the more sensitive cell line (PC-3) and 1 μM of G-1 as the treatment regimen. The efficacy of G-1 to induce cell growth inhibition appeared to correlate with the level of GPR30 expression in these two cell lines (Figure 1a, right panel). Treatment with GPR30 siRNA significantly downregulated GPR30 mRNA expression (Figure 1b, left panel) and abrogated the G-1-induced growth inhibition in PC-3 cells (Figure 1b, right panel). Using a soft-agar assay,[15] G-1 was shown to inhibit anchorage-independent growth of PC-3 cells, which was blocked by the GPR30 siRNA (Figure 1c). These data support a direct link between GPR30 and G-1-induced growth inhibition in PC-3 cells.

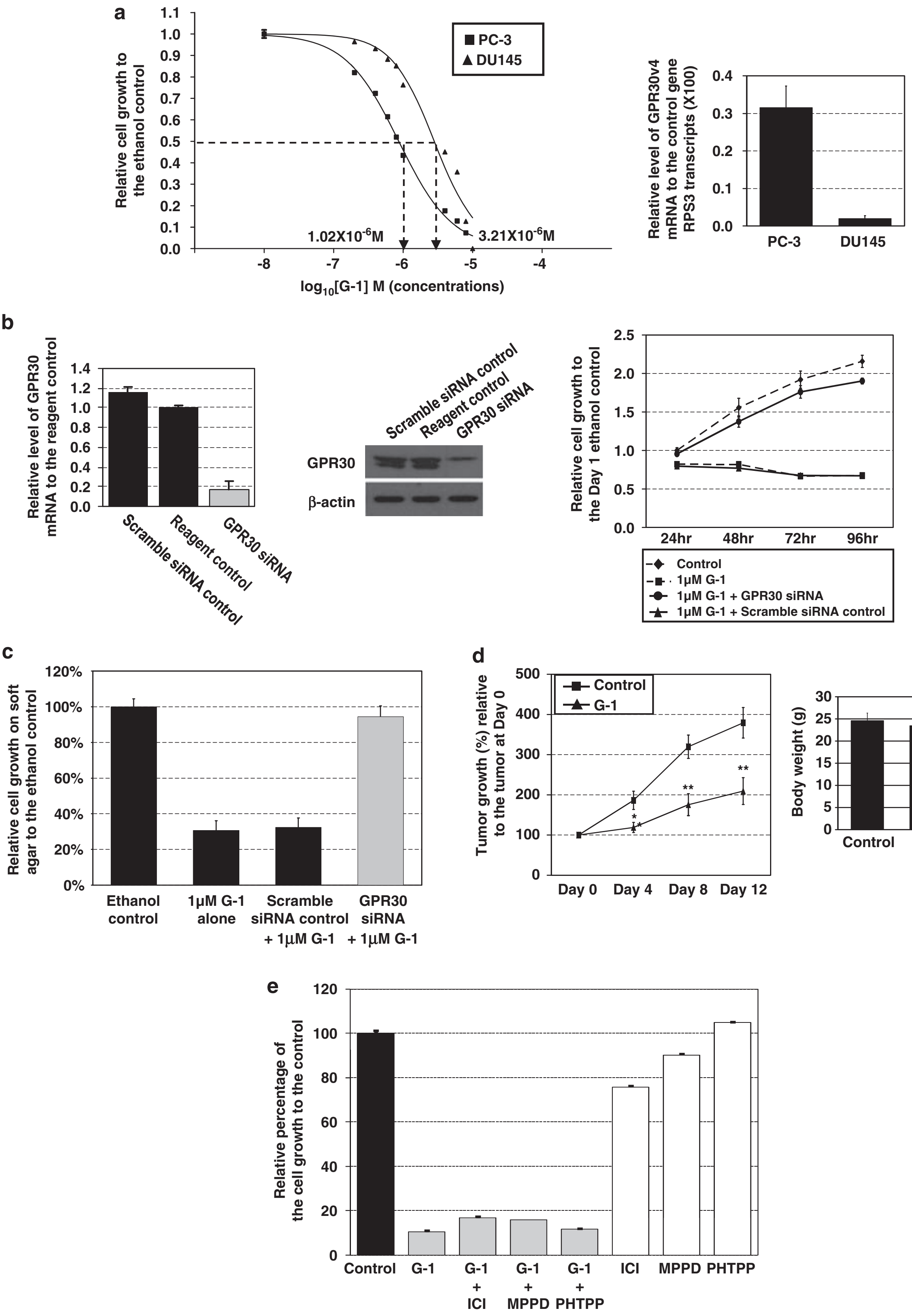
Similar to PC-3 and DU145 cells, androgen-dependent LNCaP cells also showed growth inhibition in response to G-1 treatment (Supplementary Figure S1). Regarding non-malignant prostatic epithelial cells, G-1 treatment only induced growth inhibition in actively proliferating BPH-1 cells grown in charcoal-stripped fetal bovine serum but not in growth-quiescent cells kept in serum-free medium (Supplementary Figure S2). The data suggest that non-malignant prostatic epithelial cells in a growth-quiescent state such as one found in the normal prostate[16,17] are not susceptible to G-1-induced growth inhibition.

To directly investigate the effect of G-1 on normal prostatic epithelial cells *in vivo*, we treated intact nude mice daily with G-1 at 4 mg/kg/day for 7 days. No significant difference was observed in total body weight, prostate weight, and histology of the G-1-treated mice when compared with that in vehicle-treated controls (Supplementary Figure S3). These findings indicate that normal prostatic epithelial cells *in vivo* are not responsive to G-1-treatment and G-1 has minimal toxicity in intact mice.

The PC-3 xenograft model was used to evaluate the effects of G-1 on PCa growth *in vivo*. Daily treatment with G-1 significantly suppressed the growth of the xenografts over the entire treatment period, as compared with vehicle treatment (Figure 1d). There was no difference between the body weights (without tumor) of the vehicle- and the G-1-treated mice, further supporting that G-1 was minimally toxic in nude mice (Figure 1d, right panel). Results of quantitative RT-PCR analysis of seven pairs of clinical PCa samples and their corresponding adjacent normal tissues showed a trend of slight reduction in GPR30 transcript levels in cancers ($P = 0.1288$), similar to DNA microarray data found in Oncomine (Supplementary Figure S4).

To rule out the involvement of ERα or ERβ in the G-1-induced growth inhibition, PC-3 cells were treated with G-1 in the absence or presence of an ER antagonist. Treatment of PC-3 with 1 μM G-1 alone induced a significant reduction in cell growth (Figure 1e). Co-treatment of PC-3 cells with G-1 and 1 μM of fulvestrant (an ERα and ERβ antagonist) or an ER subtype antagonist (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP dihydrochloride; an ERα antagonist) or 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; an ERβ antagonist)) was unable to reverse the G-1-induced growth inhibition, suggesting that the effect of G-1 is not mediated through one of these receptors. PC-3 cells were also treated with E2 (10^{-9}–10^{-6} M), and minimal or no inhibition of PC-3 cell growth was observed (data not shown), a finding similar to one of our previous reports.[18] E2 was also found to have no promoting or inhibitory effects on G-1-induced cell growth inhibition in PC-3 cells (Supplementary Figure S5).

Figure 1 G-1-induced inhibition of cell growth through G$_2$/M arrest and relative expression of GPR30 mRNA in PCa cells. (**a**) PC-3 (■) and DU145 (▲) cells were treated with 10^{-8}–10^{-5} M G-1 for 4 days and control cells were treated with ethanol. Cell growth relative to that of the control at day 4 was plotted against the concentrations of G-1 using sigmoid curve fitting, and IC$_{50}$ was determined. The R_2 of goodness of fit for the sigmoid curve was 0.9947 for PC-3 cells and 0.9762 for DU145 cells. The levels of expression of GPR30 mRNA in PC-3 and DU145 cells were quantified by real-time RT-PCR analysis. (**b**) siRNA specifically reduced GPR30 expression as determined by real-time RT-PCR analysis and Western blot analysis. PC-3 cells were treated with 1 μM G-1 for 4 days in the presence of GPR30 siRNA or scramble siRNA as a control. Two reagent controls with ethanol and with G-1 were included. The growth of the cells relative to that of the ethanol control at day 1 was determined. (**c**) G-1 inhibited anchorage-independent growth through GPR30-mediated signaling. Relative cell growth on soft agar to that of the ethanol-treated control cells for G-1-treated PC-3 cells with either GPR30 siRNA or scramble control siRNA are presented. (**d**) G-1 inhibited tumor growth of PC-3 xenografts *in vivo*. The percentage of tumor growth relative to the original size of the tumors at day 0 in the G-1-treated mice (▲) and control mice (■) and the body weights of mice after removal of xenografts are presented. $^{*}P < 0.05$; $^{**}P < 0.005$. (**e**). PC-3 cells were treated with 1 μM G-1 for 4 days in the presence of 1 μM ER antagonists (ICI, MPP dihydrochloride MPPD, and PHTPP). Cell growth relative to that of the ethanol control at day 4 is presented. The cell growth for the ethanol control was set as 1. Column, mean; bar, standard deviation


a
Relative cell growth to the ethanol control
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
■ PC-3
▲ DU145
1.02X10-6M
3.21X10-6M
-8
-7
-6
-5
-4
log10[G-1] M (concentrations)
Relative level of GPR30v4 mRNA to the control gene RPS3 transcripts (X100)
0.4
0.3
0.2
0.1
0.0
PC-3
DU145
b
Relative level of GPR30 mRNA to the reagent control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Scramble siRNA control
Reagent control
GPR30 siRNA
Scramble siRNA control
Reagent control
GPR30 siRNA
GPR30
β-actin
Relative cell growth to the Day 1 ethanol control
2.5
2.0
1.5
1.0
0.5
0.0
24hr
48hr
72hr
96hr
Control
1μM G-1
1μM G-1 + GPR30 siRNA
1μM G-1 + Scramble siRNA control
c
Relative cell growth on soft agar to the ethanol control
120%
100%
80%
60%
40%
20%
0%
Ethanol control
1μM G-1 alone
Scramble siRNA control + 1μM G-1
GPR30 siRNA + 1μM G-1
d
Tumor growth (%) relative to the tumor at Day 0
500
400
300
200
100
0
Control
G-1
*
**
**
Day 0
Day 4
Day 8
Day 12
Body weight (g)
30
25
20
15
10
5
0
Control
G-1
e
Relative percentage of the cell growth to the control
120
100
80
60
40
20
0
Control
G-1
G-1 + ICI
G-1 + MPPD
G-1 + PHTPP
ICI
MPPD
PHTPP

G-1 induced a G_2-phase cell-cycle arrest. Flow cytometric analysis (Figure 2a and Supplementary Figure S6) showed a significant increase in the number of cells in the G_2/M phase after treatment of PC-3 cells with G-1 for 1 day, as compared with that in ethanol-treated control cells. The increase lasted throughout the 4-day treatment period, indicating induction of a persistent cell-cycle arrest in the G_2/M stage of the cell cycle by G-1 in PC-3 cells. Prolonged G_2/M arrest by G-1 eventually triggered apoptosis, as the sub-G1 cell populations and Annexin-V-positive stained cells increased after treatment for 3–4 days (Supplementary Figures S6 and S7). Similar G_2/M arrest by G-1 was also observed in LNCaP cells (Supplementary Figure S8).

To determine whether the G-1-treated PC-3 cells were arrested at the G_2 or the mitotic phase, we examined changes in the expression of a panel of cell-cycle-specific markers over time (Figure 2b). The two mitotic phase markers, phosphorylated Bcl-2 and MPM-2, either were not expressed or showed no significant change in expression levels over time (Figure 2b). In contrast, expression levels of G_2-checkpoint regulators, including cyclin-B1, cyclin-dependent kinase-1 (cdc2), and its phosphorylated proteins, and cdc25c were significantly downregulated in PC-3 cells treated with G-1 over a 72-h period. In addition, the level of cyclin-A2, which functions in the transition from both G_1 to S and G_2 to the mitotic phase of the cell cycle, also decreased; however, expression of cyclin-E, which is involved in transition from G_1 to S, did not change. In agreement with changes in the protein level, expression of mRNAs of cyclin-B1, cdc2, cdc25C, and cyclin-A2, but not of cyclin-E, was also significantly downregulated (Figure 2c). Collectively, these data support the notion that G-1induces cell-cycle arrest at the G_2 phase in PC-3 cells. That nuclear factor-Y A-subunit (NF-YA) regulates the transcriptional expression of G_2-checkpoint regulators,[19,20] and G-1 reduced the phosphorylation of NF-YA, but did not change NF-YA protein level *per se*, in PC-3 cells (Figure 2d), further supports cell cycle arrest at the G-2 phase.

G-1 induced a sustained GPR30-dependent phosphorylation and nuclear accumulation of Erk1/2. Treatment of PC-3 cells with 1 μM G-1 over a 3-day period induced a sustained increase in the level of phosphorylated Erk1/2 but did not change the level of total Erk1/2 protein (Figure 3a, upper panels). The earliest time point at which Erk1/2 phosphorylation became noticeable is after 1 h of G-1 treatment (Figure 3a, lower panel). Such induced phosphorylation was also found in G-1-treated LNCaP cells (Supplementary Figure S9). The G-1-induced phosphorylation of Erk1/2 in PC-3 cells was then shown to be mediated by GPR30, as transfection of a GPR30-siRNA into these cells abrogated the G-1 action (Figure 3b).

Similar to a previous report,[21] in a temporal study, we observed a time-dependent increase in the nuclear localization of phosphorylated Erk1/2 in G-1-treated PC-3 cells that lasted for at least 24 h (Figure 3c). In contrast, G-1-induced nuclear accumulation of phosphorylated Erk1/2 in MCF-7 cells peaked at 1 h after treatment and rapidly declined to undetectable levels by 24 h (Figure 3c). Thus, PC-3 cells are capable of retaining phosphorylated Erk1/2 in their nuclei for a significantly longer duration than do MCF-7 cells.

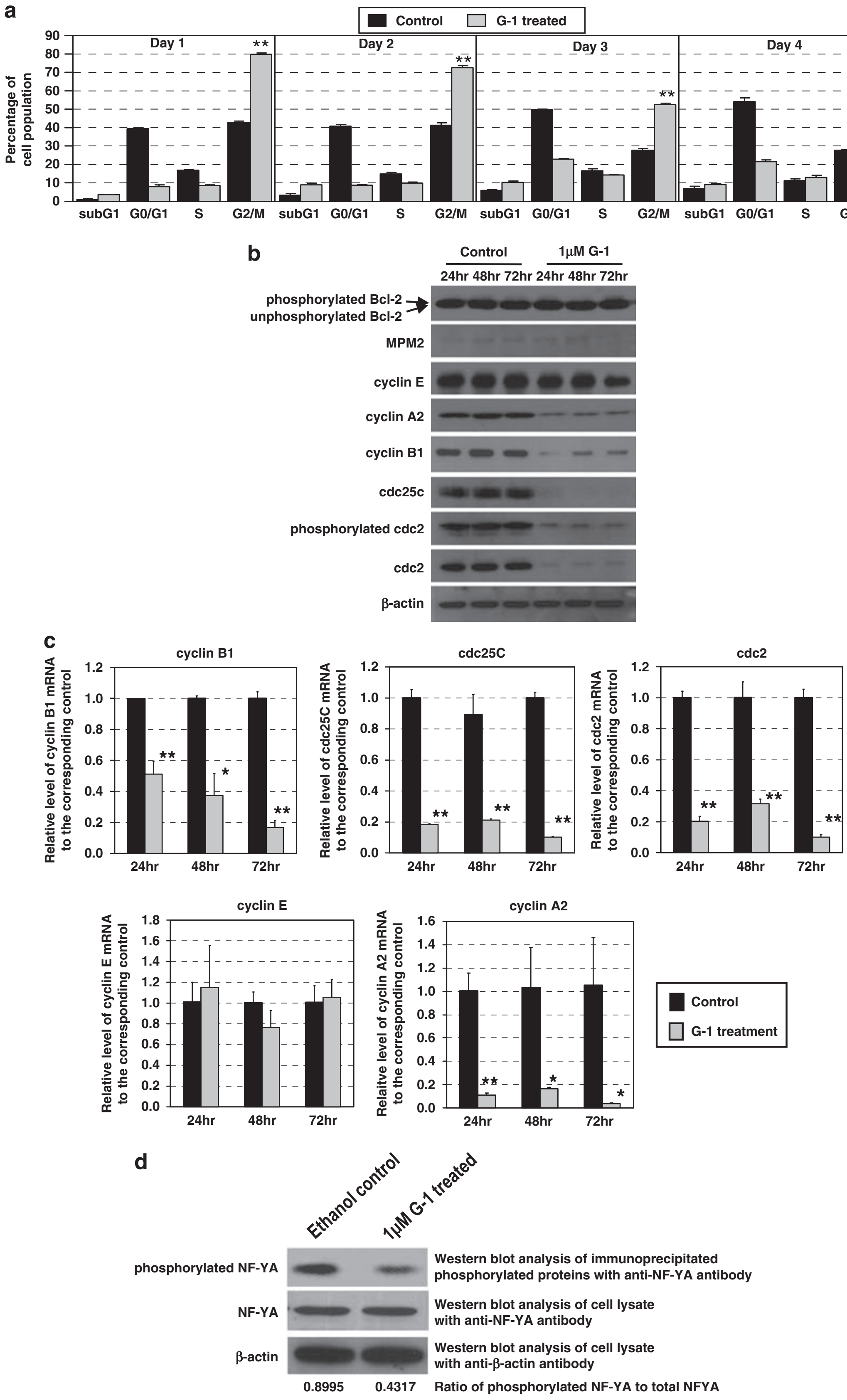
We next asked whether Erk1/2 activation, rather than phosphoinositide-3-kinase, is the principal downstream mediator of G-1 action in PC-3 cells. PC-3 cells were treated with the MEK inhibitor PD98059 or the phosphoinositide-3-kinases inhibitor LY294002. Only the former inhibitor blocked the G-1-induced inhibition of cell growth (Figure 3d), confirming our hypothesis that G-1's action in PC-3 cells is primarily mediated by Erk1/2 activation.

G-1 induced p21 expression to inhibit cell growth through GPR30-mediated signaling. Activation of GPR30 by G-1 markedly increased p21 expression at both the mRNA and protein levels in a dose- and time-dependent manner in PC-3 cells (Figure 4a). The overexpression was completely abolished by GPR30-siRNA but not by a scramble siRNA control (Figure 4b). G-1-induced p21 upregulation was also observed in LNCaP cells (Supplementary Figure S9). Importantly, an siRNA-mediated p21 knockdown in PC-3 cells (Figure 4d, fourth panel) effectively abrogated the growth-inhibitory action of G-1 on these cells (Figure 4c). In line with this abrogation, the knockdown also prevented the G-1-induced downregulation of cyclin B1, cdc25c, and cdc2 expression in the cells (Figure 4d). These findings establish G-1-induced p21 upregulation as a direct mediator of the growth-inhibitory response.

Obliteration of G-1-induced p21 upregulation by MEK inhibitor PD98059. Treatment with 1 μM G-1 for 48 h induced significant phosphorylation of Erk1/2 and upregulation of p21 expression in PC-3 cells. Pretreatment with 30 μM PD98059 obliterated the inductive effects of G-1, as this inhibitor completely abolished p21 upregulation and Erk1/2 phosphorylation in the cells (Figure 4e). These findings suggested that phosphorylation of Erk1/2 is essential for upregulation of p21 expression in G-1-treated PC-3 cells.

G-1 upregulated the expression and phosphorylation of two early-response genes and phosphorylation of their upstream regulators. Serum and growth factors are potent activators of Erk1/2 to stimulate the activator protein-1 (AP-1)

Figure 2 Effects of G-1 on the expression of cell-cycle regulators and markers, and NF-YA phosphorylation. (**a**) Percentages of PC-3 cells treated or not treated with 1 μM G-1 at different cell-cycle phases were determined by flow cytometry. (**b**) The lysates of PC-3 cells treated with 1 μM G-1 or ethanol were subjected to Western blot analysis to determine the protein level of phosphorylated bcl-2, MPM2, cyclin-E, cyclin-A2, cyclin-B1, cdc25C, phosphorylated cdc2, total cdc2, and β-actin. (**c**) Cells were subjected to real-time RT-PCR analysis to determine mRNA levels. $^*P<0.01$; $^{**}P<0.001$. (**d**) The cell lysates were immunoprecipitated with an antibody to phosphoserine/threonine/tyrosine residues. The levels of NF-YA protein in the immunoprecipitates were determined by Western blot analysis. The levels of β-actin protein were used as loading control for immunoprecipitation. The ratio of phosphorylated NF-YA to total NF-YA for ethanol and G-1 treated cells are presented


a
Control
G-1 treated
Percentage of cell population
0
10
20
30
40
50
60
70
80
90
Day 1
Day 2
Day 3
Day 4
**
subG1
G0/G1
S
G2/M
b
Control
1μM G-1
24hr 48hr 72hr 24hr 48hr 72hr
phosphorylated Bcl-2
unphosphorylated Bcl-2
MPM2
cyclin E
cyclin A2
cyclin B1
cdc25c
phosphorylated cdc2
cdc2
β-actin
c
cyclin B1
Relative level of cyclin B1 mRNA to the corresponding control
cdc25C
Relative level of cdc25C mRNA to the corresponding control
cdc2
Relative level of cdc2 mRNA to the corresponding control
0.0
0.2
0.4
0.6
0.8
1.0
1.2
24hr
48hr
72hr
**
*
cyclin E
Relative level of cyclin E mRNA to the corresponding control
1.4
1.6
1.8
cyclin A2
Relative level of cyclin A2 mRNA to the corresponding control
Control
G-1 treatment
d
Ethanol control
1μM G-1 treated
phosphorylated NF-YA
NF-YA
β-actin
Western blot analysis of immunoprecipitated phosphorylated proteins with anti-NF-YA antibody
Western blot analysis of cell lysate with anti-NF-YA antibody
Western blot analysis of cell lysate with anti-β-actin antibody
0.8995
0.4317
Ratio of phosphorylated NF-YA to total NFYA

complex, which is composed of two early-response gene proteins, c-jun and c-fos.[22] Treatment with 1 μM G-1 for 24 h induced c-jun and c-fos expression at the mRNA and protein level as compared with that in controls (Figure 5a). The expression of c-jun is regulated by a feed-forward mechanism.[23] Phosphorylation of c-jun activates its transcriptional ability, leading to induction of more c-jun expression.[24] We found that treatment with 1 μM G-1 for 24 h induced the phosphorylation of c-jun and c-fos, in which pretreatment with MEK inhibitor PD98059 before G-1 treatment significantly reduced the induction (Figure 5b). These findings supported that activation of MEK by G-1 induced the early expression of c-jun and c-fos in PC-3 cells.

As phosphorylation of MSK1 and ATF1 has been shown to be an upstream regulator of c-jun expression,[25] we treated PC-3 cells with 1 μM G-1 for 24 h and observed marked induction of these two proteins in their phosphorylated form (Figure 5c). Similarly, we found significant increase in phosphorylated Elk-1, an upstream regulator of c-fos, in G-1-treated PC-3 cells (Figure 5c). These findings indicate that activation of GPR30 by G-1 can act on early-response genes c-jun and c-fos through their respective upstream regulators.

Involvement of c-jun and c-fos in the G-1-induced upregulation of p21. G-1 treatment induced c-jun and c-fos expression along with upregulation of p21 and downregulation of cyclin-B1 in PC-3 cells, as described above. siRNA knockdown of either c-jun or c-fos alone (Figure 6a) blocked the G-1-induced cell growth inhibition (Figure 6b). In addition, double siRNA knockdown of c-jun and c-fos completely reversed the inhibitory effect of G-1 on cell growth (Figure 6b). Consistent with our hypothesis that induction of c-jun and c-fos is the downstream target of G-1-induced Erk1/2 activation in PC-3 cells, siRNA knockdown of c-jun or c-fos did not affect the phosphorylation of Erk1/2 by G-1 (Figure 6c). However, the knockdowns abolished the upregulation of p21 and downregulation of cyclin-B1 in these cells (Figure 6c), indicating that p21 and a G_2-checkpoint regulator (cyclin-B1) are downstream of these early-response genes. These findings indicate that upregulation of c-jun or c-fos is downstream from Erk1/2 activation but upstream from p21 upregulation.

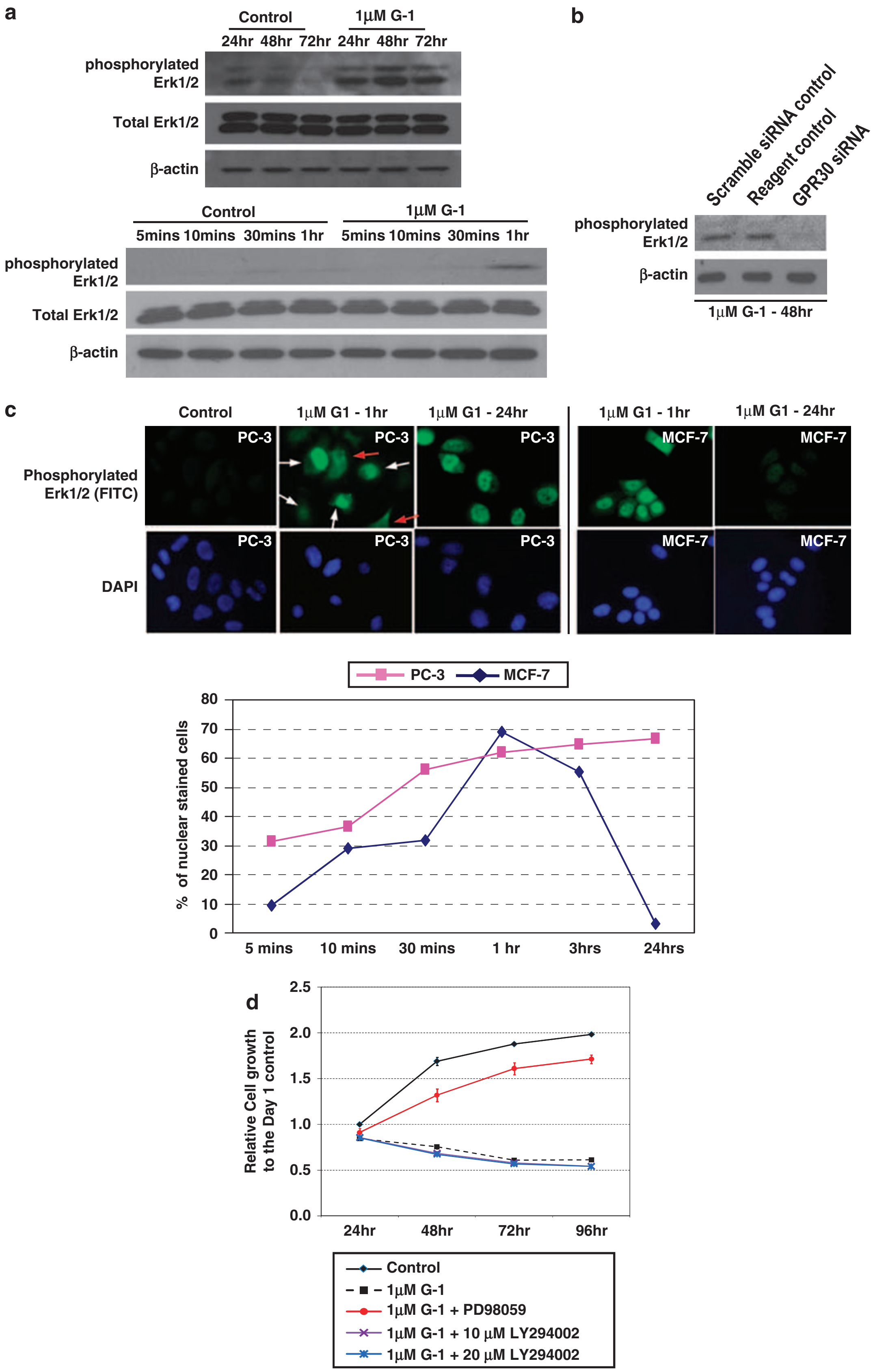
Discussion

Here we report that activation of GPR30 by the receptor-specific, non-estrogenic ligand G-1 leads to growth inhibition of PCa cells (PC-3, DU145, and LNCaP) in culture and as xenografts (PC-3) in nude mice. This finding is in stark contrast to the growth-promoting action of G-1 mediated by GPR30 activation in breast, endometrial, ovarian, and thyroid cancer cell lines.[7–9,14] Furthermore, we found G-1 to have little or no effects on mouse prostate (weight and histology) and on growth-quiescent immortalized benign prostatic epithelial cells in cultures. As depicted in Figure 7, we propose a model illustrating the steps involved in the G-1-induced, GPR30-mediated growth-inhibitory cascade in PCa cells, which were empirically validated by a comprehensive series of siRNA-knockdown experiments and the MEK inhibitor PD98059. In PC-3 cells, G-1, through GPR30 activation, caused prolonged activation and nuclear accumulation of Erk1/2. G-1 also activated the early-response genes c-jun and c-fos, leading to upregulation of p21, which in turn downregulated a number of key G_2-checkpoint regulators and ultimately led to cell-cycle arrest at the G_2 phase in PC-3 cells.

Previous studies by us[18] and others[26] showed that both ICI 182,780 (ICI) and E2 inhibited PC-3 cell growth in a modest manner. As PC-3 expresses ERα and ERβ, as well as GPR30, it is difficult to pinpoint which of the three receptors is responsible for mediating the growth-inhibitory action of the anti-estrogen/estrogen. However, E2 was shown to have a 15-fold higher affinity for ERα and ERβ than for GPR30.[13] Thus, it is thought that the action of ICI/E2 is mediated largely through transactivation of the ERs rather than GPR30 in PC-3 cells. To study the specific effect of GPR30 in ER-positive PC-3 cells, we used G-1, which is a selective ligand for GPR30, in this study. G-1 has little to no affinity for the two ERs,[13] suggesting that inhibition of PC-3 cell growth is attributable primarily to activation of GPR30. This premise was confirmed by siRNA knockdown of GPR30, which effectively blocked the inhibitory effects of G-1 on PC-3 cell growth. Furthermore, G-1 is unlikely to act through ERs, as blockade of ER's action by specific antagonists (ICI, MPP dihydrochloride, PHTPP) did not alter the growth-inhibitory effects of G-1 on PC-3 cells.

The mode of action of G-1 and its interaction with GPR30 in PCa cells have not been studied previously. In the ER-negative SKBR3 breast cancer cell line, GPR30 mediates the E2-induced rapid activation of Erk1/2 (<0.5 h), leading to cell growth[9] and cell migration.[27] In this study, G-1 has also been shown to induce Erk1/2 activation in PC-3 cells, but the biological consequence was a profound inhibition of cell growth. Both Erk1/2 activation and cell growth inhibition were dependent on GPR30, as siRNA knockdown of the receptor effectively blocked these responses. In addition, G-1-induced Erk1/2 activation and cell growth inhibition were both suppressed by the MEK inhibitor PD98059, indicating that the kinase is the common upstream regulator. In human umbilical vein smooth-muscle cells, G-1 exerted a similar biological action by inducing Erk1/2 activation and cell growth inhibition.[28]

Figure 3 G-1 induced rapid and sustained Erk1/2 phosphorylation with nuclear accumulation of phosphorylated Erk1/2. (**a**) PC-3 cells treated with 1 μM G-1 or ethanol for 3 days. Cells were lysed at early (5 min–1 h) and late (24–72 h) time points, and levels of total and phosphorylated Erk1/2 and β-actin protein were determined by Western blot analysis. (**b**) siRNA-treated PC-3 cells (GPR30 siRNA) and controls (scramble siRNA control and reagent control), all treated with 1 μM G-1, were lysed and levels of phosphorylated Erk1/2 and β-actin protein were determined by Western blot analysis. (**c**) PC-3 and MCF-7 cells treated with 1 μM G-1 from 5 min to 24 h. An antibody to phosphorylated Erk1/2 was used for immunofluorescence staining (green). The nuclei were stained with DAPI (blue). Representative micrographs of the 1 h- and 24 h-treated PC-3 and MCF7 cells, and ethanol-treated PC-3 cells are shown. The white arrows indicate cells with nuclear accumulation of phosphorylated Erk1/2; the red arrows indicate cells with diffuse cytoplasmic staining. Cells with and without nuclear staining were counted and the percentage of nuclear-stained cells from 5 min to 24 h were determined. (**d**) The growth of the cells treated with PD98059/LY294002 and G-1 relative to that of the ethanol control at day 1 was determined. G-1-treated cells without pretreatment with PD98059/LY294002 and the ethanol-treated cells were used as controls


a
Control
1μM G-1
24hr 48hr 72hr 24hr 48hr 72hr
phosphorylated Erk1/2
Total Erk1/2
β-actin
Control
1μM G-1
5mins 10mins 30mins 1hr 5mins 10mins 30mins 1hr
phosphorylated Erk1/2
Total Erk1/2
β-actin
b
Scramble siRNA control
Reagent control
GPR30 siRNA
phosphorylated Erk1/2
β-actin
1μM G-1 - 48hr
c
Control
1μM G1 - 1hr
1μM G1 - 24hr
1μM G1 - 1hr
1μM G1 - 24hr
Phosphorylated Erk1/2 (FITC)
DAPI
PC-3
MCF-7
% of nuclear stained cells
0
10
20
30
40
50
60
70
80
5 mins
10 mins
30 mins
1 hr
3hrs
24hrs
d
Relative Cell growth to the Day 1 control
0.0
0.5
1.0
1.5
2.0
2.5
24hr
48hr
72hr
96hr
Control
1μM G-1
1μM G-1 + PD98059
1μM G-1 + 10 μM LY294002
1μM G-1 + 20 μM LY294002

At this point we do not know why G-1 activation of GPR30 induces opposite effects on cell growth in different cell types, but these differences may be related to the duration of the nuclear occupancy of activated Erk1/2. Upon activation, the phosphorylated Erk1/2 translocates from the cytoplasm to the nucleus to phosphorylate their nuclear targets for transcriptional regulation.[21] It has been proposed that the signal duration of Erk1/2 activation could dictate a cell-fate decision,[29] with transient Erk1/2 activation leading to cell survival and proliferation and sustained activation with nuclear accumulation of activated Erk1/2 transmitting antiproliferative signals.[29–32] A previous study reported that the differences between the cell growth responses of osteoclasts/osteocytes and osteoblasts to estrogens depend on the kinetics of Erk1/2 activation and the duration of nuclear accumulation of phosphorylated Erk1/2.[31] In this study, we showed that the G-1-induced Erk1/2 activation in PC-3 cells was rapid but unusually prolonged ($\geqslant$24 h) as compared with that induced in MCF-7 cells, which peaked at 1 h and disappeared by 24 h. Whether the signal duration of Erk1/2 activation is central to the PCa cell growth response to G-1 requires future studies to clarify the role of GPR30 in cell-fate determination.

The cell-cycle regulator p21 was originally thought to inhibit cell-cycle progression in the G_1 phase, but recent evidence indicates that it is also involved in G_2/M arrest.[33,34] Upregulation of p21 has been shown to associate with nuclear translocation of G_2-checkpoint regulators.[35] An association between p21 induction and downregulation of cyclin-B and cdc25C has been linked to G_2/M arrest.[36,37] Here we report G-1-induced p21 upregulation and significant downregulation of several G_2-specific checkpoint proteins. One mechanism by which p21 upregulation suppresses multiple G_2-checkpoint regulators is by inhibiting the phosphorylation of the NF-YA, which binds to a common CCAAT motif in these proteins.[38,39] Indeed, we observed significant reduction in NF-YA phosphorylation after G-1 treatment. Finally, we found siRNA knockdown of p21 to effectively block the G-1-induced inhibition of cell growth and reverse the downregulation of G_2-checkpoint modulators. These findings provide credence for p21-mediation of the G-1-induced-cell cycle arrest at the G_2 phase in PC-3 cells.

We further investigated the mechanism underlying GPR30-mediated p21-induced growth inhibition in PC-3 cells. Kardassis *et al.*[40] showed that c-jun transactivated the p21 gene promoter. This finding prompted us to ascertain if the G-1-induced upregulation of p21 is caused by increased expression and/or activation of the early-response genes. We found that c-jun and c-fos were upregulated and activated in G-1-treated PC-3 cells. Furthermore, our study also showed that the transcriptional factors MSK1 and ATF1 that activate c-jun, as well as Elk-1 that activates c-fos,[25] were phosphorylated in G-1-treated cells. Knockdown of each of the early-response genes with small interfering RNA blocked p21 upregulation, cyclin-B downregulation, and growth inhibition induced by G-1 in PC-3 cells. Collectively, these data support the mediation of G-1induced p21 upregulation through activation of the early-response genes c-jun and c-fos in PC-3 cells.

Our data showed that GPR30 was expressed in variable but detectable levels in PCa tissues and cell lines, and G-1 induced cell growth inhibition in both androgen-responsive (e.g., LNCaP) and androgen-deprivation-resistant (e.g., PC-3) PCa cells *in vitro* and *in vivo*. The treatment was found to have minimal or no effects on normal prostatic epithelial cells in the mouse prostate. It also did not affect benign prostate epithelial cells (e.g., BPH-1) in a growth-quiescent state, which is the state most normal epithelial cells are in *in vivo*.[16,17] Therefore, this agent may have the potential to be used alone or in combination with androgen-deprivation therapies as a first-line treatment regimen for advanced PCa, local or metastatic. The treatment is likely to pose little or no harmful effects on normal prostatic tissues in patients. Additionally, it may add efficacy when used in conjunction with standard chemotherapies for metastatic PCa.

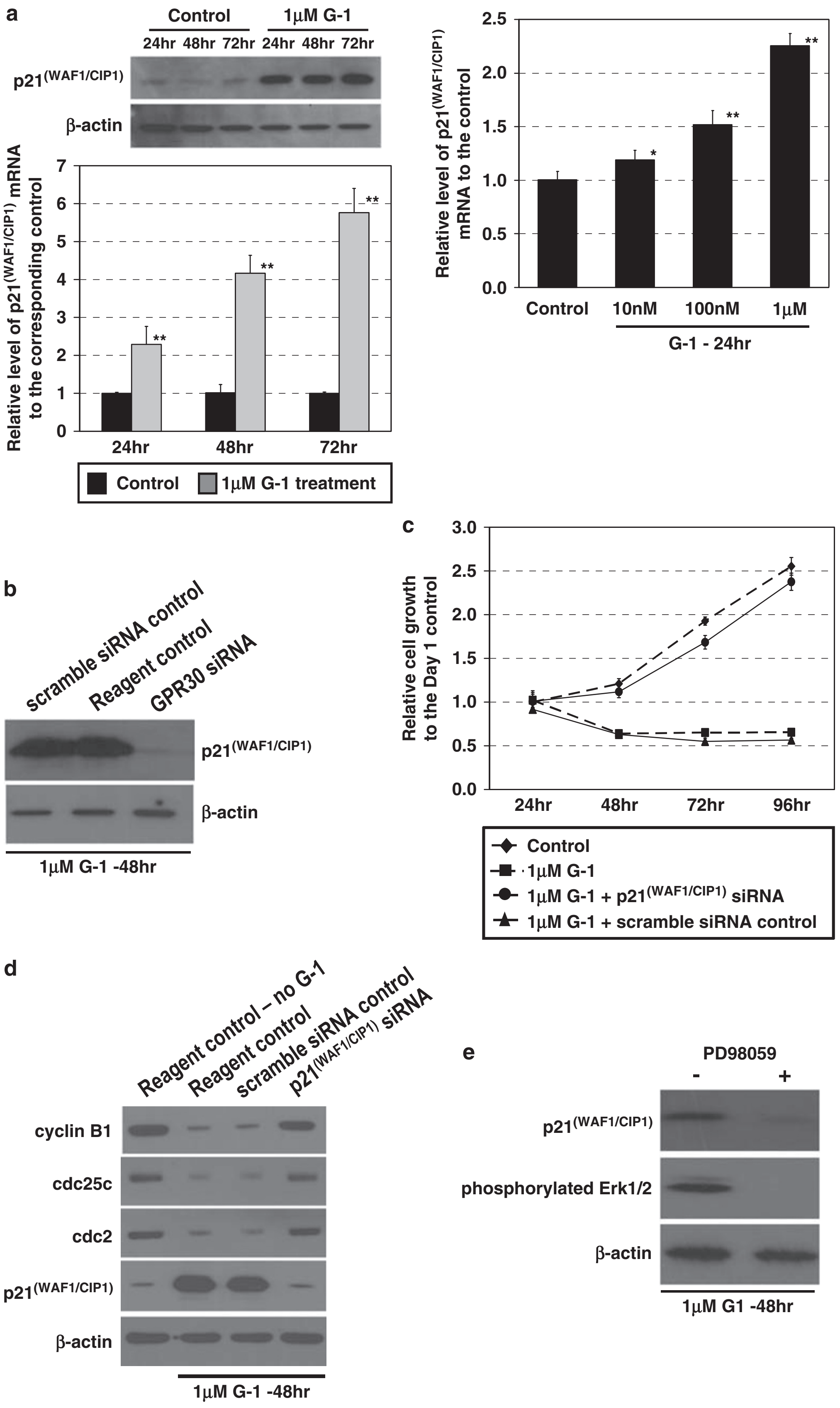
In conclusion, we showed that treatment of PCa cells with G-1 induced growth inhibition *in vitro* and *in vivo* through activation of GPR30 and cell-cycle arrest at the G_2 phase. We further provided evidence supporting a novel G-1/GPR30-signaling pathway that involves protracted activation of Erk1/2 that is linked to a c-jun- and c-fos-mediated increase in p21 expression. The discovery of this signaling pathway opens up new opportunities for the development of GPR30-based therapies for PCa by using G-1 or its derivatives.

Materials and Methods

Analysis by reverse transcription-PCR (RT-PCR). Total RNA samples were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random hexamer (Applied Biosystems, Foster City, CA, USA). Primer sequences are presented in Supplementary Table S1. PCR reactions with SYBR Green PCR Master-Mix (Applied Biosystems) were monitored in real-time with iCYCLER (Bio-Rad Laboratories, Hercules, CA, USA). Cycle thresholds (C_T) of the genes of interest were compared with those of ribosomal protein-3 (RPS3) to determine relative expression levels. Relative fold changes between the expression of the genes of interest in treated and control samples were determined by the equation: fold change $= 2^{-[\Delta\Delta C_T]}$, where $\Delta\Delta C_T = (C_{T\ \text{Gene of interest}} - C_{T\ \text{RPS3}})_{\text{Treated}} - (C_{T\ \text{Gene of interest}} - C_{T\ \text{RPS3}})_{\text{Control}}$.

Cell growth assay. The effects of G-1 (Cayman, Ann Arbor, MI, USA) treatment on PCa cell growth were determined by MTT (3-[4,5-dimethylhiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay; 1.5×10^3 cells per well were seeded in RPMI-1640 medium supplemented with 5% charcoal-stripped fetal bovine serum (CS-FBS) as day 0. After 24 h, the cells were treated with 1×10^{-8}, 2×10^{-7},

Figure 4 G-1 induced p21 mRNA and protein through GPR30 and downregulation of expression of G_2-checkpoint regulators for inhibition of cell growth by p21. (**a**) PC-3 cells were treated with 1 μM G-1 or ethanol. The levels of p21 protein and mRNA were determined by Western blotting and real-time RT-PCR analyses, respectively. Furthermore, the cells were treated with 10^{-8}–10^{-6} M G-1. The levels of p21 mRNA in cells were quantified by real-time RT-PCR analysis. *$P < 0.01$; **$P < 0.001$. (**b**) The siRNA-treated cells (GPR30siRNA) and controls (scramble siRNA control and reagent control), all treated with 1 μM G-1, were lysed and the levels of p21 and β-actin protein were determined by Western blot analysis. (**c**) The G-1-treated cells with p21 siRNA or the scramble siRNA control were subjected to Western blot analysis to determine levels of cyclin-B1, cdc25c, cdc2, p21, and β-actin protein. Two reagent controls (ethanol or 1 μM G-1) were included. (**d**) Effects of p21siRNA knockdown on the G-1-induced inhibition of cell growth are presented with the controls. Growths of the cells relative to that of the ethanol control at day 1 were determined. (**e**) G-1-induced expression of p21 was dependent on Erk1/2 activation. PC-3 cells were treated with 1 μM G-1 for 48 h in the presence or absence of 30 μM PD98059. The levels of p21 and β-actin protein were determined by Western blot analysis


a
Control
1μM G-1
24hr 48hr 72hr 24hr 48hr 72hr
p21(WAF1/CIP1)
β-actin
Relative level of p21(WAF1/CIP1) mRNA to the corresponding control
0 1 2 3 4 5 6 7
**
24hr
48hr
72hr
Control
1μM G-1 treatment
Relative level of p21(WAF1/CIP1) mRNA to the control
0.0 0.5 1.0 1.5 2.0 2.5
*
Control
10nM
100nM
1μM
G-1 - 24hr
b
scramble siRNA control
Reagent control
GPR30 siRNA
p21(WAF1/CIP1)
β-actin
1μM G-1 -48hr
c
Relative cell growth to the Day 1 control
0.0 0.5 1.0 1.5 2.0 2.5 3.0
24hr
48hr
72hr
96hr
Control
1μM G-1
1μM G-1 + p21(WAF1/CIP1) siRNA
1μM G-1 + scramble siRNA control
d
Reagent control – no G-1
Reagent control
scramble siRNA control
p21(WAF1/CIP1) siRNA
cyclin B1
cdc25c
cdc2
p21(WAF1/CIP1)
β-actin
1μM G-1 -48hr
e
PD98059
-
+
p21(WAF1/CIP1)
phosphorylated Erk1/2
β-actin
1μM G1 -48hr

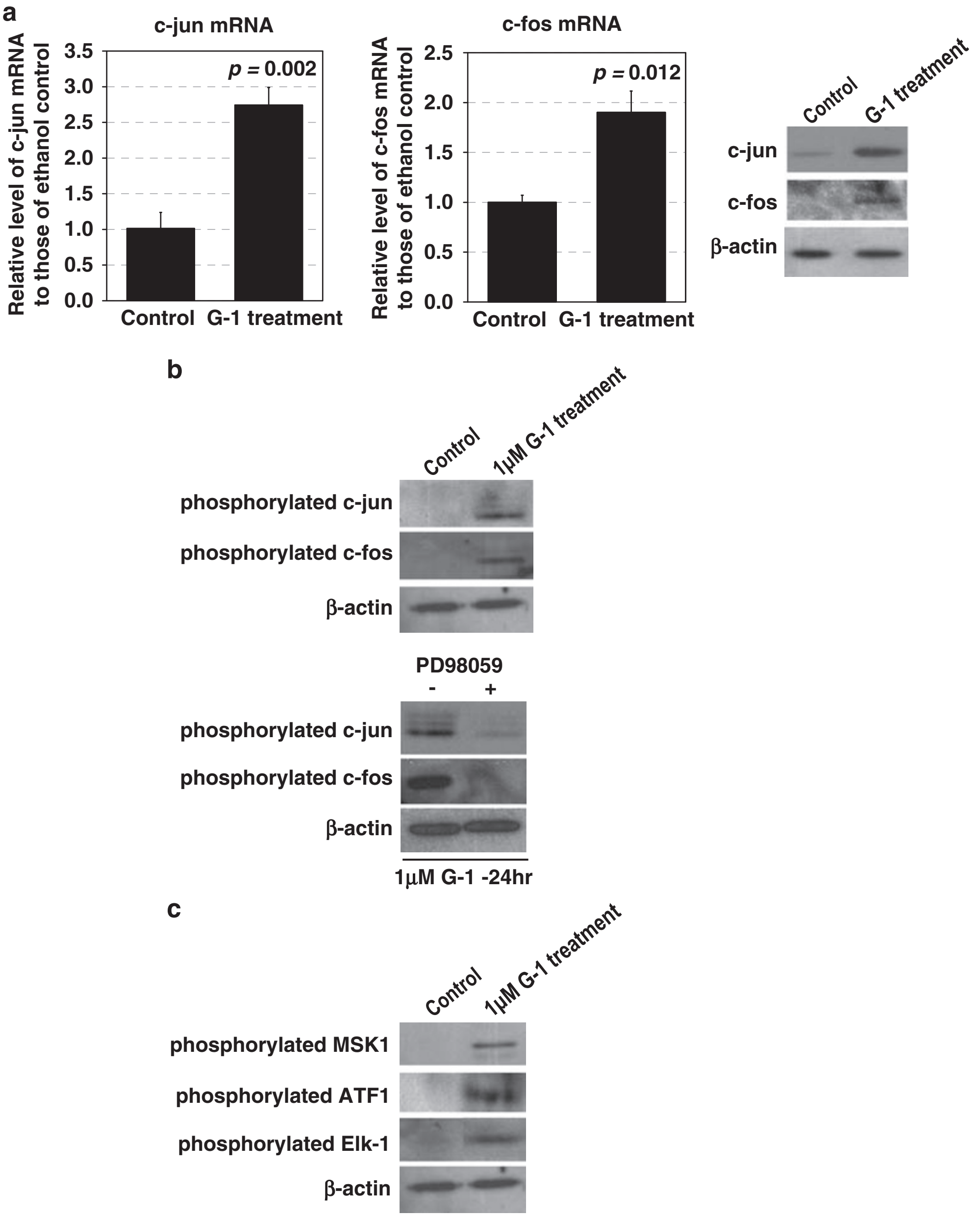


Figure 5 G-1 upregulated c-jun and c-fos expression and phosphorylation, and activated the upstream regulators of c-jun. PC-3 cells were treated with 1 μM G-1. (**a**) The levels of c-jun and c-fos mRNA and protein in G-1-treated cells, and ethanol-treated controls were determined by real-time RT-PCR and Western blot analyses, respectively. The levels of β-actin protein were used as the loading control. (**b**) PC-3 cells were treated with 1 μM G-1 in the presence and absence of 30 μM PD98059. The ethanol-treated cells were used as the control. The levels of phosphorylated c-jun and phosphorylated c-fos proteins in the cells were determined by Western blot analysis. (**c**) The levels of phosphorylated MSK1, ATF1, and Elk-1 in G-1- and ethanol-treated cells were determined by Western blot analysis

4×10^{-7}, 6×10^{-7}, 8×10^{-7}, 1×10^{-6}, 4×10^{-6}, 6×10^{-6}, 8×10^{-6}, and 1×10^{-5} M G-1 in 0.1% ethanol for 4 days in octuplicate; control cells were treated with drug vehicle (0.1% ethanol). Growth of the sample at various time points relative to that of the day 1 control was calculated by the formula $(OD_{Sample}-OD_{Blank})/(OD_{Day\ 1\ control}-OD_{Blank})$, and the relative growth of the day 1 control was set as 1. The concentration of G-1 (IC_{50}) that achieved 50% inhibition of cell growth was calculated from absorbance values on day 4.

In a set of experiments, PC-3 cells (American Type Culture Collection, Manassas, VA, USA) were treated with a nuclear receptor antagonist (1 μM) or kinase inhibitors (PD98059 or LY294002) either alone or with G-1. The antagonists used included ICI or fulvestrant (an ERα and ERβ antagonist; a gift from Zeneca Pharmaceuticals, Cheshire, UK), MPP dihydrochloride (an ERα antagonist; Tocris, Ellisville, MO, USA), and PHTPP (an ERβ antagonist, Tocris, Ellisville, MO, USA).

Flow cytometric analysis. PC-3 cells were cultured overnight in RPMI-1640 medium supplemented with 5% CS-FBS and then treated with 1 μM G-1 for 1–4 days. The treated cells were fixed and stained with propidium iodide. At least 20 000 stained cells were analyzed using FACSAria (Becton Dickinson-Biosciences, Franklin Lakes, NJ, USA).

Treatment of GPR30, p21 (p21), c-jun, or c-fos siRNAs. PC-3 cells (2×10^5) were cultured in 4 ml of RPMI-1640 medium supplemented with CS-FBS and 1 ml of siRNA-Lipofectamine-2000 mixture (40 nM siRNA for GPR30, p21, c-jun, or c-fos) and 10 μl Lipofectamine-2000 in Opti-MEM-I medium; Invitrogen, Carlsbad, CA, USA). The siRNA-treated cells were treated with 1 μM G-1 for another 2 days and then subjected to real-time RT-PCR and Western blot analysis. The predesigned and validated GPR30-, p21, c-jun, and c-fos-siRNAs were

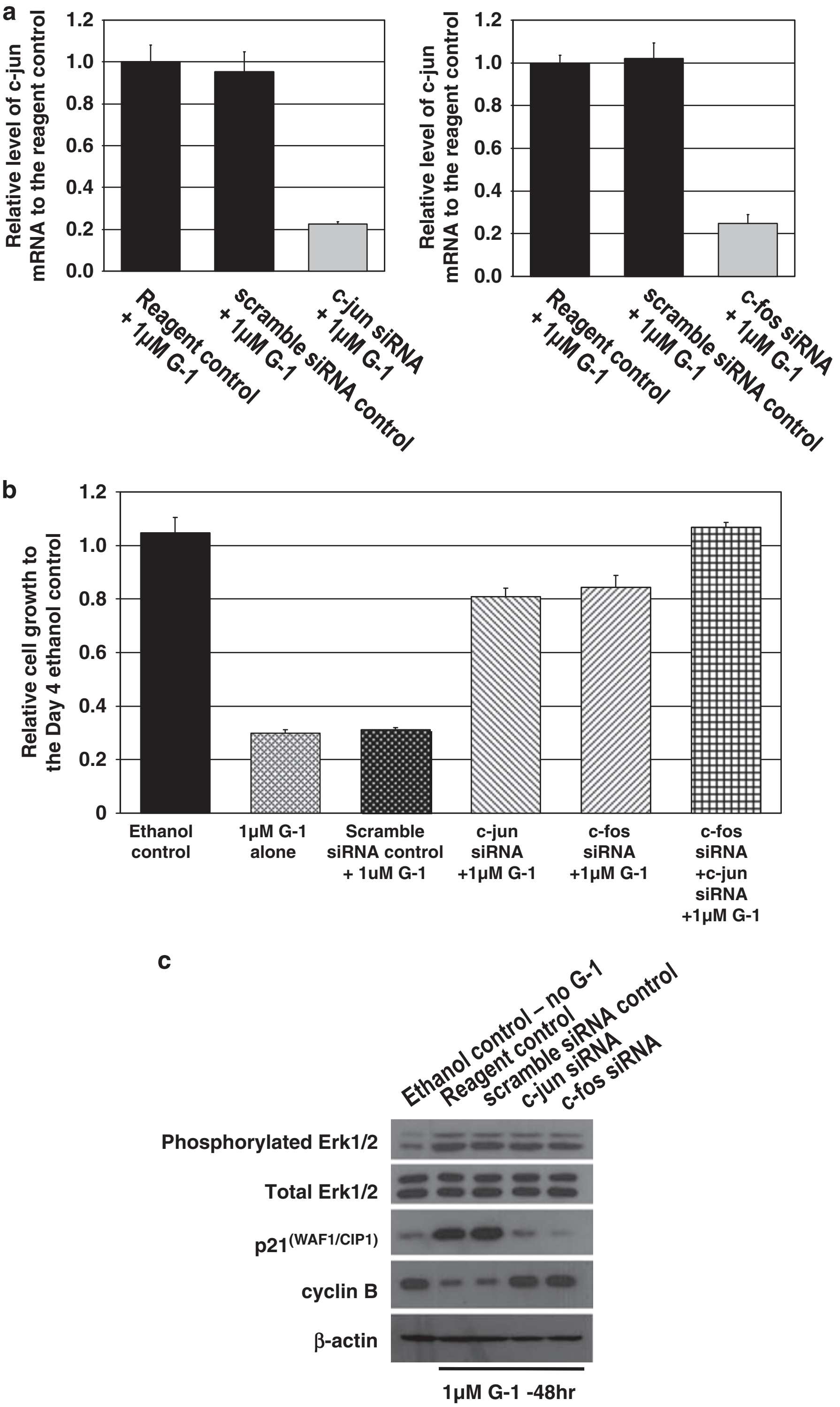


Figure 6 siRNA knockdown of c-jun and c-fos blocked the G-1-induced inhibition of cell growth, upregulation of p21, and downregulation of cyclin-B, but had no effect on G-1-induced phosphorylation of Erk1/2. (**a**) siRNA specifically reduced c-jun or c-fos expression as determined by real-time RT-PCR analysis. (**b**) PC-3 cells were treated with 1 μM G-1 for 4 days in the presence of c-jun siRNA, c-fos siRNA, or scramble siRNA as control. Two reagent controls with ethanol and with G-1 without any siRNA were included. The growth of cells relative to that of the ethanol control at day 1 was determined. (**c**) The G-1-treated cells with c-jun siRNA, c-fos siRNA, or scramble siRNA control were subjected to Western blot analysis to determine the levels of phosphorylated Erk1/2, p21, cyclin-B1, and β-actin protein. Two reagent controls (ethanol or 1 μM G-1) without any siRNA were included

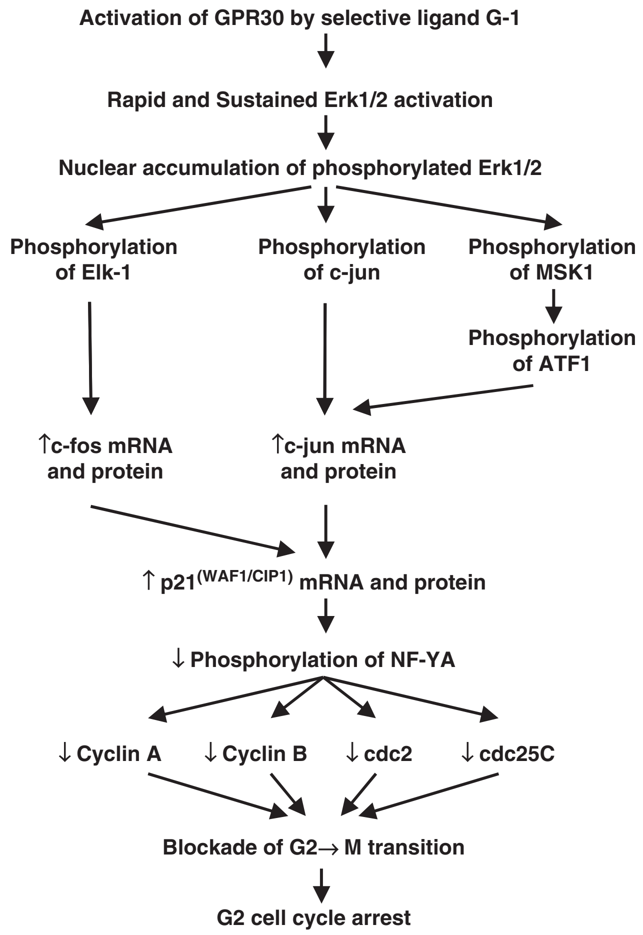

Activation of GPR30 by selective ligand G-1
Rapid and Sustained Erk1/2 activation
Nuclear accumulation of phosphorylated Erk1/2
Phosphorylation of Elk-1
Phosphorylation of c-jun
Phosphorylation of MSK1
Phosphorylation of ATF1
↑c-fos mRNA and protein
↑c-jun mRNA and protein
↑ p21(WAF1/CIP1) mRNA and protein
↓ Phosphorylation of NF-YA
↓ Cyclin A
↓ Cyclin B
↓ cdc2
↓ cdc25C
Blockade of G2→ M transition
G2 cell cycle arrest

Figure 7 The proposed GPR30-mediated signaling by G-1 in PCa cells for cell growth inhibition

obtained from Invitrogen. For controls, cells were treated with Lipofectamine-2000 in Opti-MEM-I medium without any siRNA as reagent control. Cells with scramble siRNA control (Invitrogen) and Lipofectamine-2000 in Opti-MEM-I medium also were used.

Immunoprecipitation. The lysates of G-1-treated PC-3 cells and controls in lysis-I buffer (Supplementary Table S2) with an equal amount of protein were incubated with an antibody to phosphoserine/threonine/tyrosine residues. Protein-A/G Plus-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to capture the complexes. The beads were heat-incubated with Laemmli sample buffer (Supplementary Table S2) and the supernatants were subjected to Western blot analysis.

Western blot analysis. After treatment, cells were lysed in lysis-II buffer (Supplementary Table S2). Five 20-μg protein lysates or immunoprecipitates were run onto 10–12.5% polyacrylamide gels with SDS and transferred onto polyvinylidene fluoride membranes. Primary antibodies (Supplementary Table S3) were incubated with the membranes and recognized with horseradish peroxidase-conjugated secondary antibodies. ECL-Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA) were used to visualize the complexes.

Immunofluorescence staining. PC-3 cells were cultured overnight in RPMI-1640 medium supplemented with CS-FBS and then treated with 1 μM G-1 for 5 min to 24 h. The cells were immunostained with an antibody to phosphorylated Erk1/2 recognized by FITC-conjugated secondary antibody. The nuclei were stained with DAPI. The stained cells were visualized by immunofluorescence microscopy.

Soft-agar growth assay. A 1-week 96-well soft-agar growth assay was performed as previously described.[15] Briefly, a mixture of 25 μl of 37°C pre-warmed 2 × RPMI-1640 medium with CS-FBS and 25 μl 56°C pre-warmed 1.2% Bacto Agar Select (BD Biosciences, San Jose, CA, USA) was plated onto each well of a 96-well microplate. The solidified agar layer was topped with a mixture of 10 μl of cell suspension (7×10^3 cells), 20 μl of 2 × of the culture medium, and 30 μl of 0.8% Bacto Agar Select in the presence of 11.25 pmole of siRNA targeting GPR30 or scrambled Sheath-negative RNAi control with Lipofectamine-2000 in Opti-MEM-I medium. A semisolid feeder layer containing 25 μl of 2 × of the culture medium and 25 μl of 1.2% Bacto Agar Select was added on the top of the solidified cell layer in each well. Each treatment was conducted in octuplicate. The cells were allowed to grow for 7 days and growth was determined by Invitrogen AlamarBlue cell viability assay in accordance with the manufacturer's protocol. Fresh medium (100 μl) was placed on top of the layer for 10 min and then removed to maintain the moisture of the layer. Absorbance at 570 and 600 nm in each well was measured with a Perkin Elmer Victor microplate reader. Relative cell growth on soft agar was calculated as the percentage difference in the reduction of AlamarBlue between the test and control cells, calculated as $[(O2 \times A1)-(O1 \times A2)]/[(O2 \times P1)-(O1 \times P2)] \times 100$, where O1 is the molar extinction coefficient (E) of oxidized AlamarBlue at 570 nm (i.e., 80586); O2 is the E of oxidized AlamarBlue at 600 nm (i.e., 117216); A1 and A2 are absorbance at 570 and 600 nm, respectively, of the well with GPR30 siRNA or control siRNA plus G-1-treated cells or G-1-treated cells; and P1 and P2 are absorbance at 570 and 600 nm, respectively, of the well with control ethanol-treated cells.

Xenograft experiments. PC-3 cells (1×10^6) in 100 μl of PBS were injected subcutaneously into the left and right sides of the flank of surgically castrated nude (*nu/nu*) athymic male mice (6 weeks of age, 24–29 g) (Taconic Laboratory, Hudson, NY, USA). Tumor volumes were expressed in mm^3: Volume (mm^3) = Long diameter (mm) × Short diameter $(mm)^2 \times 0.5236$. Mice with tumors of $\sim 150\ mm^3$ were treated daily with vehicle alone (2.5% DMSO, 5% ethanol) or G-1 in vehicle (4 mg/kg/day) administered by subcutaneous injection ($n = 5$). Tumors were measured every 4 days. At 12 days, mice were killed and weighed after xenografts were removed.

Conflict of interest

The authors declare no conflict of interest.

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