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ORIGINAL ARTICLE

The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation

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Recently, the orphan receptor G protein-coupled receptor 55 (GPR55) has been proposed as a potential cannabinoid receptor, although controversy remains on its physiological roles. Current evidence suggests a role for GPR55 as a receptor for the lysophospholipid lysophosphatidylinositol (LPI). In this study, we show that GPR55 is expressed in several prostate and ovarian cancer cell lines, both at the mRNA and at the protein level, and that it has a critical role in regulating proliferation and anchorage-independent growth. We further show that GPR55 mediates the effects of LPI in prostate and ovarian cancer cells. Indeed we demonstrate that LPI is able to induce calcium mobilization and activation of Akt and extracellular signal-regulated kinase (ERK)1/2 in these cells and that both pharmacological blockade of GPR55 and its downregulation using specific small interfering RNA strongly inhibits these processes. We further identify an autocrine loop by which LPI is synthesized by cytosolic phospholipase A2, pumped out of the cell by the ATP-binding cassette transporter ABCC1/MRP1, and is then able to initialize cascades downstream of GPR55. All together, these data demonstrate a role of LPI and its receptor GPR55 in cancer cells in activating an autocrine loop that regulates cell proliferation. These findings may have important implications for LPI as a novel cancer biomarker and for its receptor GPR55 as a potential therapeutic target.
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Keywords: G-protein-coupled receptor; lysophosphatidylinositol; GPR55; prostate cancer; ABC transporters; phospholipase A2

Introduction

Although the orphan G-protein coupled receptor (GPCR) G protein-coupled receptor 55 (GPR55) has been recently proposed as a cannabinoid receptor and evidence indicates that GPR55 is activated by certain cannabinoid ligands, the classification of GPR55 as a cannabinoid receptor remains a contentious issue (Ross, 2009). It is now well established that the lysophospholipid lysophosphatidylinositol (LPI) is able to bind and activate GPR55, triggering specific intracellular signaling cascades (Oka *et al*., 2007; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008; Henstridge *et al*., 2009; Kapur *et al*., 2009). Our previous work suggested that LPI is involved in cancer progression as it can be released in the medium of Ras-transformed FRT-Fibro fibroblasts and transformed thyroid cells and can function as an autocrine modulator of cell growth (Falasca and Corda, 1994; Falasca *et al*., 1995, 1998). Furthermore, we demonstrated that LPI stimulates cell proliferation in differentiated and k-ras-transformed thyroid cells and that increased levels of LPI, but not of other lysophospholipids, are present in thyroid as well as in other Ras-transformed cell lines (Falasca and Corda, 1994; Falasca *et al*., 1995, 1998). However, the mechanisms by which LPI is released and can signal inside the cells to stimulate proliferation were not known. It is noteworthy that levels of LPI and other lysophospholipids, such as lysophosphatidic acid, are increased in patients with ovarian cancer (Xiao *et al*., 2000, 2001; Sutphen *et al*., 2004), corroborating our hypothesis that LPI may function as an autocrine factor that regulates cancer cell growth. Despite this evidence, signaling mediated by LPI and its potential role in cancer were poorly investigated, mostly because no specific receptor for LPI was known. The recent discovery that LPI is able to activate some GPCRs, including GPR55 (Oka *et al*., 2007; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008; Henstridge *et al*., 2009; Kapur *et al*., 2009), GPR119 (Soga *et al*., 2005) and transient receptor potential channels (Monet *et al*., 2009), has fueled novel interest in the role of this lysophospholipid, in particular in cancer. Although it is known that GPR55 mRNA is mainly expressed in some regions of the central nervous system (Sawzdargo *et al*., 1999; Ryberg *et al*., 2007) and in the adrenal glands and gastrointestinal tract (Ryberg *et al*., 2007), very little is known about GPR55 expression in cancer cell lines or tissues. It has been recently reported that GPR55 is expressed and activated by LPI in IM-9 lymphoblastoid cells (Oka *et al*., 2010) and that LPI may have a role in migration, orientation and polarization of breast cancer cell lines MDA-MB-231 and MCF-7 overexpressing GPR55 (Ford *et al*., 2010). In addition, it has been

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recently found that LPI stimulates prostate cancer cell migration via transient receptor potential calcium channel activation (Monet *et al*., 2009). Taken together, these data suggest that GPR55 and LPI may indeed have a role in cancer.

In this study, we identified a novel role of the GPR55 receptor as a key component of an autocrine loop maintaining cancer cell proliferation. According to this, downregulation of GPR55 expression using small interfering RNA (siRNA) inhibits the basal phosphorylation state of Akt, cell proliferation and soft-agar colony formation. Moreover, we provide a strong evidence for the mechanism of action of LPI in cancer cells and strongly reinforce our original hypothesis that LPI has a specific role in cancer progression.

Results

GPR55 is expressed in prostate and ovarian cancer cell lines

To determine whether GPR55 is expressed in cancer cells, we first examined its expression in ovarian and prostate cancer cell lines. Western blot analysis using a commercial anti-GPR55 antibody revealed the presence of two bands in the range of predicted molecular weight for GPR55 (37 kDa) in the ovarian cancer cell line OVCAR3 and in the prostate cancer cell lines PC-3 and DU145 (Figure 1a). Expression of the receptor was also detected using a distinct commercially available antibody, showing GPR55 expression in all tested prostate cancer cell lines (PC-3, DU145, LNCaP) and the most abundant GPR55 expression in OVCAR3 and A2780 among ovarian cancer cell lines (Supplementary Figure 1a). Consistent with these data, reverse transcriptase–quantitative PCR (RT–qPCR) analysis revealed the expression of the GPR55 mRNA in some of these cell lines, being much higher in PC-3 and DU145 than in OVCAR3 (Figure 1b). As a positive control of expression, mRNA from human embryonic kidney 293 cells overexpressing GPR55 was loaded in the reaction (Figure 1b). To our knowledge this is the first demonstration of GPR55 expression in prostate and ovarian cancer cells.

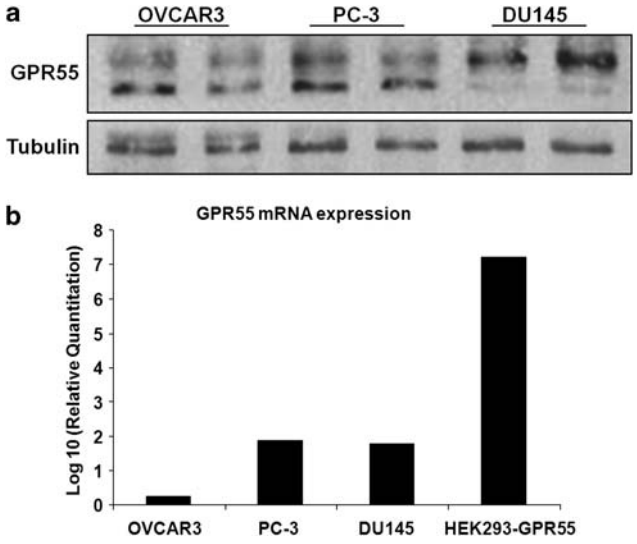

Figure 1 GPR55 is expressed in prostate and ovarian cancer cells. (**a**) Western blot of ovarian and prostate cancer cell lines with an antibody specific for GPR55 (Cayman Chemical). (**b**) GPR55 mRNA expression determined by RT–qPCR. The mRNA levels are expressed in log 10 scale after relative quantitation. As a positive control, mRNA from HEK293 cells overexpressing GPR55 was loaded in the reaction.

LPI regulates calcium mobilization, ERK and Akt activation in cancer cells

As already discussed above, several data have now demonstrated that the lysophospholipid LPI is able to activate GPR55. In an effort to characterize the specific role of GPR55 in cancer cells, we decided to investigate whether its ligand LPI was able to modulate intracellular functions in these cells. It was previously reported that LPI induces Ca^{2+}mobilization from intracellular stores in non-cancer cells in a mechanism involving GPR55 activation (Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008; Henstridge *et al*., 2009). Analysis of this process in cancer cells revealed that LPI was indeed able to stimulate a very rapid and transient increase in intracellular Ca^{2+} levels in PC-3 cells to the same extent as ATP (Figure 2a), indicating that prostate cancer cells are responsive to this lysophospholipid. In addition, we observed that LPI activated ERK in prostate and ovarian cancer cells (Figure 2b). Dose–response experiments revealed that 100 nM LPI was already able to stimulate ERK activation in OVCAR3, PC3 and DU145 (Supplementary Figure 1b), although a stronger activation was detected using 1 μM LPI in all the three cell lines tested (Figure 2b). Interestingly, LPI was also able to induce phosphorylation of protein kinase B/Akt at its residue Ser473 in all cell types (Figure 2b). Akt phosphorylation was rapid and transient in PC-3 and more sustained in DU145 (Figure 2b). These data indicate that LPI activates distinct signaling cascades in ovarian and prostate cancer cell lines.

LPI-dependent intracellular functions involve GPR55 activation

We then decided to investigate the potential involvement of GPR55 in mediating the LPI-dependent processes. We tested the effect of the antagonist cannabidiol (CBD) and of SR141716A (Rimonabant), a compound that has been shown to act as an agonist but also as an antagonist of GPR55 (Nevalainen and Irving, 2010; Sharir and Abood, 2010). Data revealed that treatment with CBD strongly blunted the LPI-mediated Ca^{2+}mobilization in PC-3 cells (Figure 3a) and inhibited activation of ERK in all three cancer cell lines tested (Figures 3b and c). Interestingly, a similar inhibition of all these processes was detected using SR141716A (Figures 3a, d and e), indicating that the compound clearly acts as a GPR55 antagonist in these experimental conditions. Similarly, Akt activation was blocked by

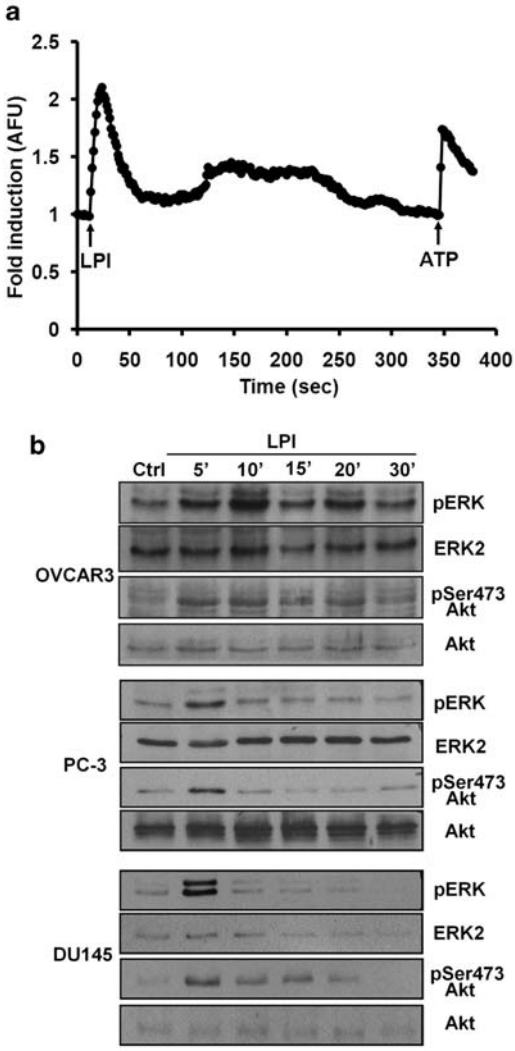

Figure 2 LPI induces mobilization of Ca^{2+} and kinases activation in cancer cells. (**a**) Starved PC-3 cells were loaded with 4 μM Fluo-4 and stimulated with 10 μM LPI and 1 mM ATP. Arrows indicate the time of treatment. Graph represents the temporal changes on fluorescence of a pool of at least 15 cells after normalizing as described in the Materials and methods section. The results are representative of three independent experiments. (**b**) Serum-starved cells were incubated in the presence or absence of 1 μM LPI at 37 °C for the indicated times. Akt and ERK activation was determined by analyzing their respective phosphorylation state by western blotting. Membranes were then stripped and re-probed with anti-Akt and anti-ERK antibodies.

CBD and SR141716A (Supplementary Figures 2a–d). These data indicate that pharmacological inhibition of GPR55 is able to block the LPI-dependent intracellular functions in prostate and ovarian cancer cells.

To confirm the role of GPR55 in these processes, we determined the effect of its downregulation using specific siRNA. Scrambled siRNA was used as a control in these experiments. The efficiency of the siRNA transfection was determined by RT–qPCR (Supplementary Figures 3a and b) and confirmed at the protein levels by western blot showing a reduction of both bands (Figure 4b). Data revealed that LPI was able to increase the levels of intracellular Ca^{2+} in PC-3 transfected with a scrambled siRNA (2.82 ± 0.24 arbitrary fluorescence units (AFU)) (Figure 4a). Downregulation of GPR55 expression strongly prevented the LPI-induced Ca^{2+} mobilization in PC-3 cells (1.51 ± 0.17 AFU) without affecting the ATP-induced Ca^{2+} release (Figure 4a). Furthermore, we observed that downregulation of GPR55 inhibited the LPI-dependent ERK activation in PC-3 and OVCAR3 (Figure 4b). Interestingly, downregulation of GPR55 strongly reduced the basal phosphorylation of Akt (Supplementary Figure 2e).

To further determine whether LPI specifically acts through GPR55 in prostate and ovarian cancer cells, we investigated the potential involvement of GPR119, another putative LPI receptor (Soga *et al*., 2005). First we determined the mRNA expression levels of this receptor in ovarian and prostate cancer cell lines. RT–qPCR revealed higher levels of GPR119 mRNA in PC-3 than in DU145 and OVCAR3 (Supplementary Figure 3c). Next, we determined the effect of GPR119 downregulation on LPI-dependent processes. The efficiency of the siRNA transfection was determined by RT–qPCR (Supplementary Figure 3d). Knockdown of GPR119 had no effect on ERK activation induced upon LPI stimulation in PC-3 and OVCAR3 cells (Figure 4c) and did not affect the LPI-dependent transient increase in intracellular Ca^{2+} levels induced by LPI in PC-3 cells (Figure 4d, siGPR119: 2.49 ± 0.40 AFU; siScrambled: 2.17 ± 0.51 AFU).

These data indicate that GPR55, but not GPR119, has a key role in mediating intracellular functions activated by LPI in prostate and ovarian cancer cells.

LPI activates distinct signaling pathways downstream of GPR55 in cancer cells

Rho and its downstream effector ROCK have been recently suggested to be upstream of LPI-dependent ERK phosphorylation (Oka *et al*., 2010). Therefore we decided to investigate the potential involvement of the Rho/ROCK pathway in the LPI-dependent signaling. Our data revealed that treatment of DU145 and OVCAR3 with the specific ROCK inhibitor Y-27632 strongly inhibited the LPI-dependent ERK phosphorylation (Figure 5a), confirming the involvement of Rho-ROCK downstream of GPR55 and upstream of ERK and in agreement with previous report (Oka *et al*. 2010). Interestingly, Y-27632 did not affect the LPI-dependent Ca^{2+} mobilization (Figure 5b), suggesting that LPI is able to increase the levels of intracellular Ca^{2+} in a mechanism involving GPR55 (Figure 4a), but independent of the Rho/ROCK pathway (Figure 5b). Taken together, these data indicate that the LPI-induced activation of GPR55 triggers parallel signaling pathways.

GPR55 regulates proliferation and growth in soft agar

We previously reported that LPI stimulates proliferation of differentiated and k-ras-transformed thyroid cells (Falasca and Corda, 1994; Falasca *et al*., 1995, 1998) and can be released by cancer cells (Falasca *et al*., 1998). To test the hypothesis that LPI may be released by

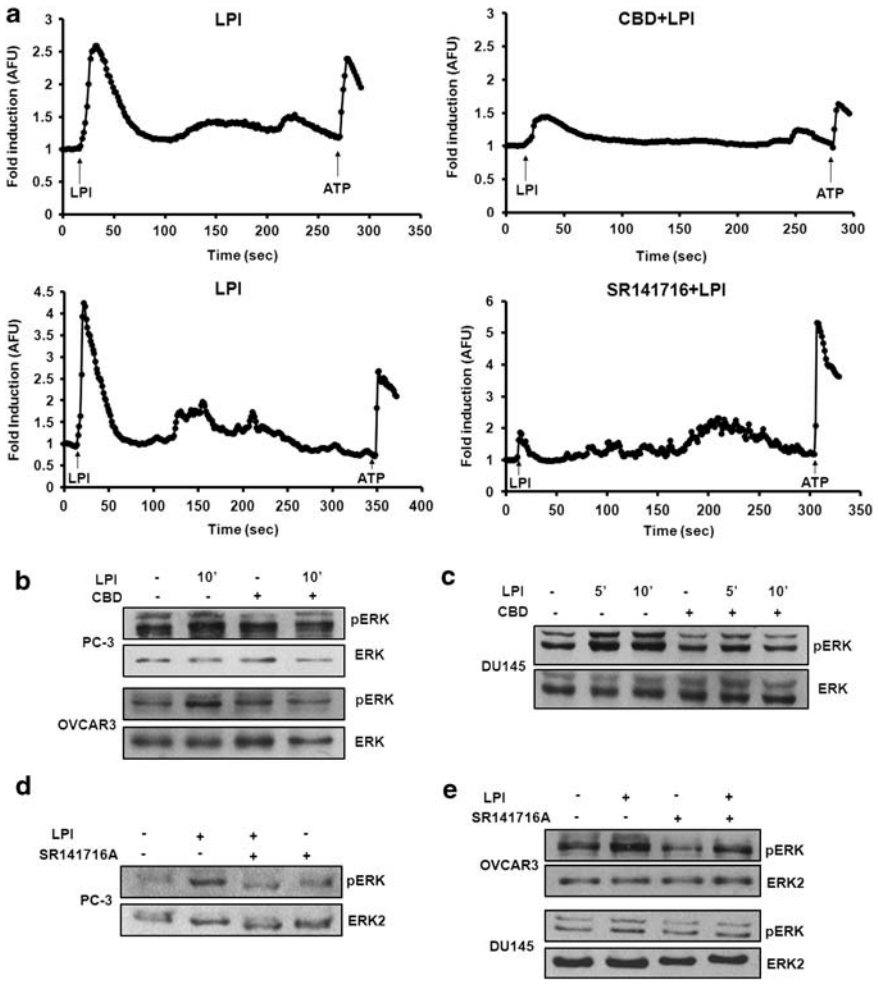

Figure 3 GPR55 regulates the mobilization of Ca^{2+} and activation of ERK induced by LPI. (**a**) Serum-starved cells were pre-incubated for 10 min with CBD (3 μM) or for 30 min with SR141716A (2 μM) before treatment with LPI (10 μM). (**b**, **c**) Cells were serum starved overnight and then pre-treated with CBD (3 μM) for 10 min before treatment with LPI (1 μM) for the indicated times. (**d**, **e**) Cells were serum starved overnight and then pre-treated with SR141716A (2 μM) for 30 min before treatment with LPI (1μM) for 5 min. Western blotting analysis of ERK activation is shown.

cancer cells in basal conditions and act as an autocrine factor through GPR55 activation, we first determined the effect of GPR55 inhibition on cell proliferation. Cell counting, at different days in the presence of serum, showed that downregulation of the receptor completely blocked proliferation of PC-3 and OVCAR3 cells (Figures 6a and b). A similar effect was observed in the presence of SR141716A (Figures 6c and d). Moreover, growth of PC-3 cells in soft agar was clearly inhibited by GPR55 downregulation (Figure 7). Notably, PC-3 only formed disorganized and more spread colonies in the absence of GPR55 compared with colonies formed by cells expressing a scrambled siRNA (Figure 7). These data indicate that GPR55 is critical for anchorage-dependent and anchorage-independent cell growth. Moreover, our data clearly indicate that GPR55 specifically regulates cell proliferation, as no induction of apoptosis was observed in cells transfected with the siRNA targeting GPR55, as assessed by caspase-3 activity and cell cycle profile analyses (Supplementary Figure 4).

Identification of a new autocrine loop regulating prostate cancer proliferation

To investigate the hypothesis that an autocrine loop by which cancer cells release LPI and promote their proliferation through GPR55 exists, we analyzed the potential involvement of the cytoplasmic isoform of phospholipase A2 (cPLA2) in generating the LPI that is eventually released. Downregulation of cPLA2 expression by siRNA reduced the number of PC-3 cells (Figure 8a) after 48 and 72 h compared with cells transfected with scrambled siRNA. cPLA2 mRNA levels after transfection were analyzed by RT–qPCR (Supplementary Figure 3e). Moreover, addition of exogenous LPI was able to rescue the effect of cPLA2 downregulation (Figure 8a), consistent with the hypothesis that cell proliferation is maintained by a released pool of LPI generated through cPLA2 activation. Based on a report demonstrating that the ATP-binding cassette transporter ABCC1 (multidrug resistance protein 1, MRP1) is involved in the export of sphingosine-1-phosphate from mast cells (Mitra *et al*., 2006), we

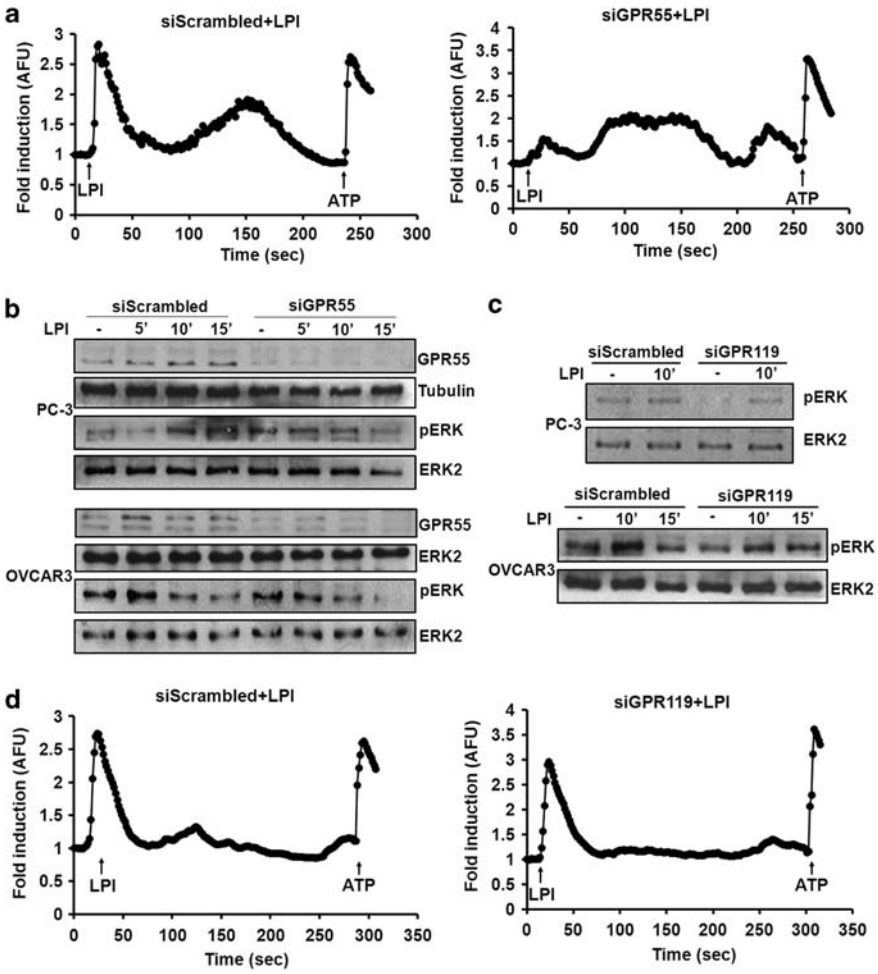

Figure 4 Downregulation of GPR55 inhibits the mobilization of Ca^{2+} and activation of ERK induced by LPI. (**a**) PC-3 cells were transfected with scrambled siRNA or siRNA targeting GPR55 and serum starved 24 h post-transfection. Intracellular Ca^{2+} increase was determined upon stimulation with LPI (10 μM), as described in Materials and methods. A pool of at least 15 cells was analyzed. Representative of three independent experiments. (**b**, **c**) Cells were transfected with scrambled siRNA or siRNA targeting GPR55 (**b**) or GPR119 (**c**), serum starved overnight and then stimulated with LPI (1 μM) at 37 °C for the indicated times. Western blotting analysis of ERK activation is shown. (**d**) Analysis of Ca^{2+} mobilization in PC-3 upon downregulation of GPR119.

decided to investigate its potential role in releasing LPI. The effect of ABCC1 downregulation on cell proliferation was comparable to the effect of cPLA2 knockdown, being able to inhibit proliferation (Figure 8b). The efficiency of the ABCC1 knockdown was also tested by RT–qPCR (Supplementary Figure 3e). As for cPLA2, addition of exogenous LPI was able to rescue the inhibitory effect of ABCC1 downregulation on cell proliferation of PC-3 cells (Figure 8b), supporting the hypothesis that ABCC1 mediates the extracellular release of cPLA2-dependent LPI. Taken together, these data indicate that cPLA2 and the transporter ABCC1 are involved in maintenance of a pool of LPI necessary to sustain prostate cancer cell proliferation.

GPR55 is the receptor involved in the LPI autocrine loop mediating proliferation of prostate cancer cells

Our data have shown that downregulation of GPR55 reduced cell proliferation in serum (Figure 6a) and this is consistent with data obtained in serum-free conditions (Figure 8c). Interestingly, exogenous LPI was not able to revert the effect of GPR55 downregulation (Figure 8c), consistent with its role as LPI receptor in these cells. To determine whether the released LPI also acts through GPR55 to stimulate proliferation, we generated a double knockdown combining siRNA targeting GPR55 and siRNA against cPLA2 or ABCC1. The efficiency of the siRNA was tested by RT–qPCR 48 h after transfection (Supplementary Figure 3f). The results showed a decreased number of cells compared with scrambled cells, which is not reverted by the addition of exogenous LPI (Figure 8d), as observed in the case of the single knockdown. These data indicate that LPI-induced rescue on proliferation occurs through GPR55 activation.

LPI is released in prostate cancer cells through ABCC1-mediated export

To definitely confirm the existence of an autocrine loop involving the intracellular synthesis of LPI by cPLA2

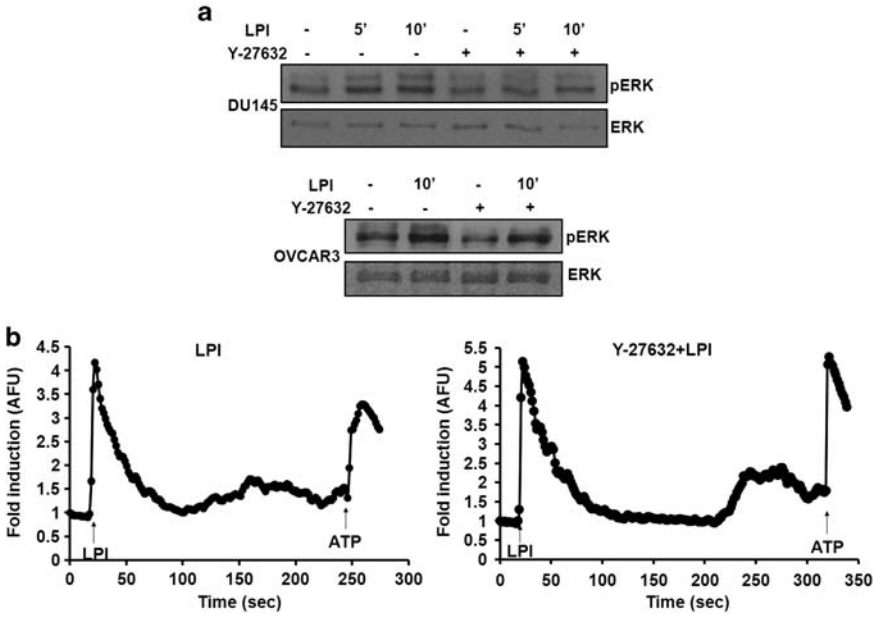

Figure 5 ERK phosphorylation downstream of GPR55 is dependent on Rho/ROCK activation. (**a**) Cells were serum starved overnight and then pre-treated with Y-27632 (20 μM) for 30 min before treatment with LPI (1 μM). ERK activation was determined by monitoring its phosphorylation in western blotting analysis. (**b**) Serum-starved cells were pre-incubated for 30 min with Y-27632 (20 μM) before treatment with LPI (10 μM) and analysis of intracellular Ca^{2+} increase.

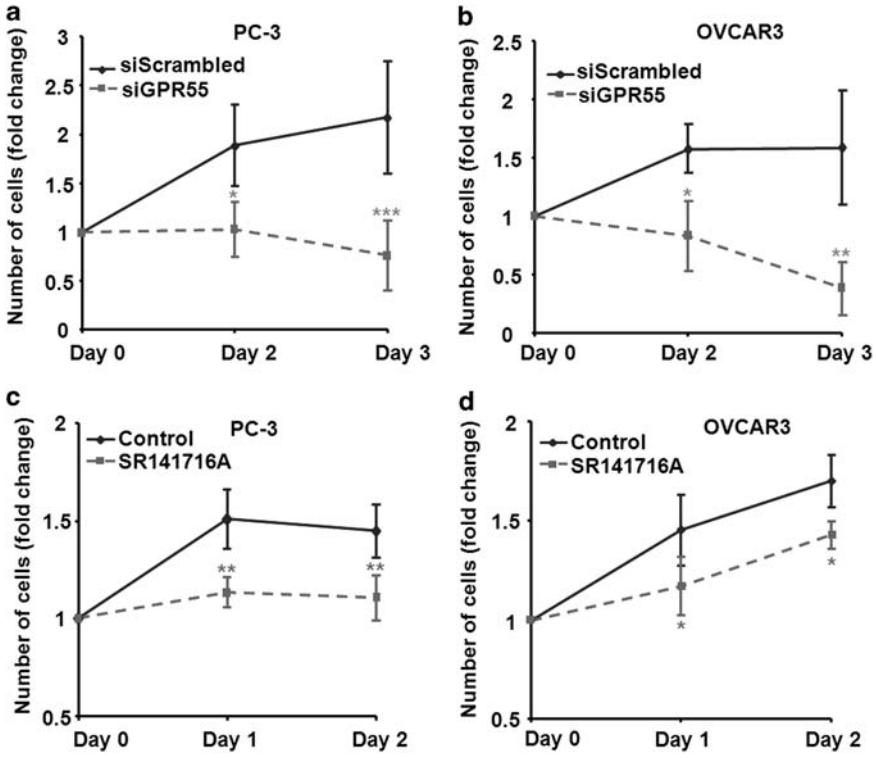

Figure 6 GPR55 regulates cell proliferation. Proliferation of PC-3 (**a**) and OVCAR3 (**b**) cells in serum was determined by counting the number of cells at days 2 and 3 after transfection with siRNA targeting GPR55. Data are mean ± s.d. of $n=4$ in PC-3 cells and $n=3$ in OVCAR3 cells, and they are expressed as the fold change compared with the number of cells on the siScrambled population at day 0; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The effect of SR141716A (2 μM) on PC-3 (**c**) and OVCAR3 (**d**) cells in serum-free conditions was determined in a similar manner. Data are mean ± s.d. of $n=4$ in PC-3 cells and $n=3$ in OVCAR3 cells, and they are expressed as the fold change compared with the number of cells on the control population at day 0; $^{*}P<0.05$, $^{**}P<0.01$. A full color version of this figure is available at the *Oncogene* journal online.

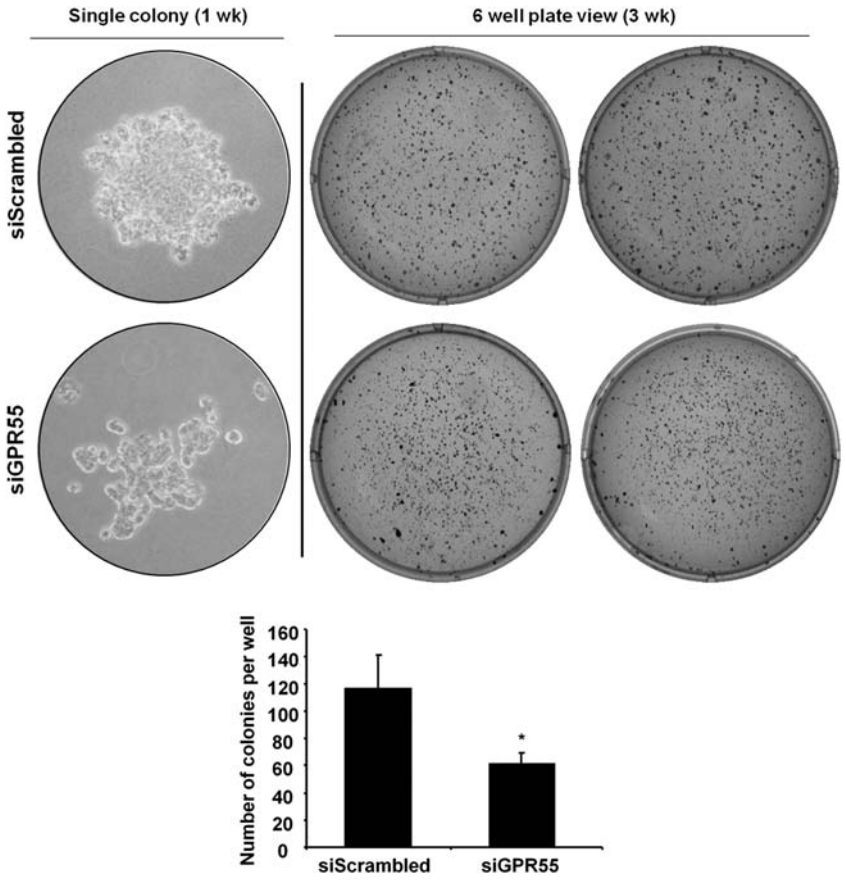

Figure 7 GPR55 regulates soft agar growth. Anchorage-independent growth of PC-3 cells was analyzed by soft agar colony formation assay as described in Materials and methods. Cells were transfected with a scrambled siRNA or a siRNA targeting GPR55. Representative images of total colonies after 3 weeks are shown together with images of a single colony for each condition after 1 week. Data are mean ± s.d. of the number of colonies from $n=3$ experiments performed in duplicate; $^*P<0.05$.

and the export in the extracellular medium by the transporter ABCC1, we first determined whether LPI could be released by PC-3 cells, using ^{3}H-labeling of the cells, lipid extraction of extracellular media and thin layer chromatography analysis of ^{3}H-LPI. Our data clearly showed the presence of LPI in the extracellular media of PC-3 cells, indicating that these cells are indeed able to synthesize and release LPI (Figure 8e). Moreover, downregulation of cPLA2 and ABCC1 significantly decreased the levels of ^{3}H-LPI in the culture media (Figure 8e), demonstrating the involvement of these two proteins in LPI synthesis and release.

Taken together, these data reveal the existence of an autocrine loop in prostate cancer cells by which LPI is synthesized intracellularly by cPLA2 and exported by the ABCC1 transporter in the extracellular media, where it can activate GPR55 and induce cell proliferation (Figure 9).

Discussion

In this paper, we reported the expression of the GPCR GPR55 in prostate and ovarian cancer cells and described a novel role for this receptor as a key regulator of cancer cell proliferation and anchorage-independent growth. Indeed downregulation of GPR55 expression using siRNA inhibits cell proliferation and soft agar colony formation. We further demonstrated that the lysophospholipid LPI is able to activate different intracellular signaling pathways in a mechanism requiring GPR55 activation. Moreover, we identified a novel autocrine loop involving the intracellular synthesis of LPI by the action of cPLA2 and its extracellular release, mediated by ABCC1, which is able to sustain prostate cancer cell proliferation. These data shed new light into the role of both GPR55 and LPI in cancer.

Several lines of evidence including our early work on k-ras-transformed cells have shown that LPI can act as a signaling molecule (Falasca and Corda, 1994; Falasca *et al*., 1995, 1998), but the lack of discovery of a specific receptor for LPI has been for long time a major impediment for the identification of the specific pathophysiological roles of this phospholipid (Oka *et al*., 2007; Ross, 2009). The identification of GPR55 as a LPI receptor has certainly disclosed novel avenues for this lipid mediator (Oka *et al*., 2007; Ross, 2009). Recent data have revealed an emerging role of LPI in

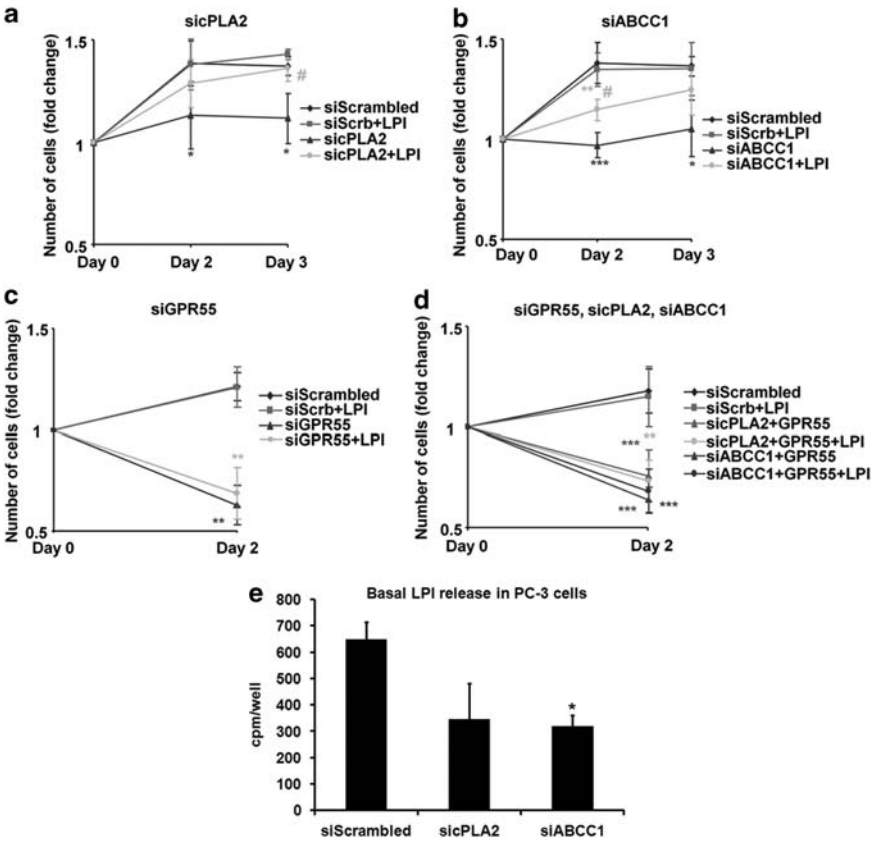

Figure 8 cPLA2 and ABCC1 mediate prostate cancer cell proliferation by maintaining the synthesis and release of a pool of LPI able to act through GPR55. Proliferation of PC-3 (**a**, **b**) cells in serum-free conditions was determined by counting the number of cells at days 2 and 3 after transfection with an siRNA scrambled and siRNA targeting cPLA2 or ABCC1 in the presence or absence of LPI (1 μM). Data are mean ± s.d. of $n=3$ experiments, and they are expressed as the fold change compared with the number of cells on the siScrambled population at day 0; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs siScrambled; $^{\#}P<0.05$ vs sicPLA2 or siABCC1. Proliferation of PC-3 (**c**, **d**) cells in serum-free conditions was determined by counting the number of cells at day 2 after transfection with a siRNA scrambled and siRNA targeting GPR55 (**c**) and the combination of GPR55 and cPLA2 or ABCC1 siRNA in the presence or absence of LPI (1 μM). Data are shown as mean ± s.d. of $n=3$ experiments (**c**) and $n=4$ (**d**), and they are expressed as the fold change compared with the number of cells on the siScrambled population at day 0; $^{*}P<0.05$, $^{**}P<0.01$ vs siScrambled. (**e**) LPI release was measured by TLC in media of PC-3 cells transfected with scrambled siRNA and siRNAs targeting cPLA2 or ABCC1 and labeled with ^{3}H-inositol for 48 h. The amount of ^{3}H-LPI in the medium was evaluated by liquid scintillation. Data are mean ± s.e.m. from $n=2$ (cPLA2) and $n=3$ (ABCC1). Data for ABCC1 are significant compared with scrambled siRNA ($P<0.05$). A full color version of this figure is available at the *Oncogene* journal online.

physiological context (Staton *et al*., 2008; Whyte *et al*., 2009). Specifically, it has been recently reported that GPR55, and its endogenous ligand LPI, has a role in bone physiology by regulating osteoclast number and function (Whyte *et al*., 2009). In addition, it has been shown that GPR55$^{-/-}$ mice are resistant to neuropathic and inflammatory pain (Staton *et al*., 2008). The recent demonstration that LPI can also activate other GPCRs, such as GPR119 (Soga *et al*., 2005), together with transient receptor potential channels (Monet *et al*., 2009), strongly suggests that this lipid may regulate several intracellular functions in different cellular contexts, indicating that still much needs to be understood about the role of this lipid in cell signaling. In this respect, it is worth mentioning that evidence suggests that LPI may have specific and distinct intracellular roles compared with other lysophopholipids. For instance, it has been reported that neither lysophosphatidic acid nor sphingosine-1-phosphate is able to stimulate GPR55 (Oka *et al*., 2007) despite the existence of structural similarities between the receptors for lysophosphatidic acid and sphingosine-1-phosphate and GPR55 (Kreitzer and Stella, 2009).

In this study, we demonstrated that LPI is able to activate distinct cellular signaling in prostate and ovarian cancer cells. These data are in agreement with our previous studies, suggesting that LPI is a bioactive molecule able to activate specific receptor-mediated signaling pathways. Specifically, we show that LPI activates Akt and increases intracellular Ca^{2+} levels in prostate and ovarian cancer cells. The effect on Akt is particularly significant considering the well-known role of this kinase in prostate cancer (Davies *et al*., 1999). More important, using different approaches, we demonstrated that the LPI-dependent cellular functions require activation of GPR55. First we show that inhibition of the receptor using the antagonist CBD strongly inhibits the LPI-dependent ERK activation and Ca^{2+} increase. Interestingly, a similar strong inhibition was detected in cells treated with SR141716A, indicating that, although

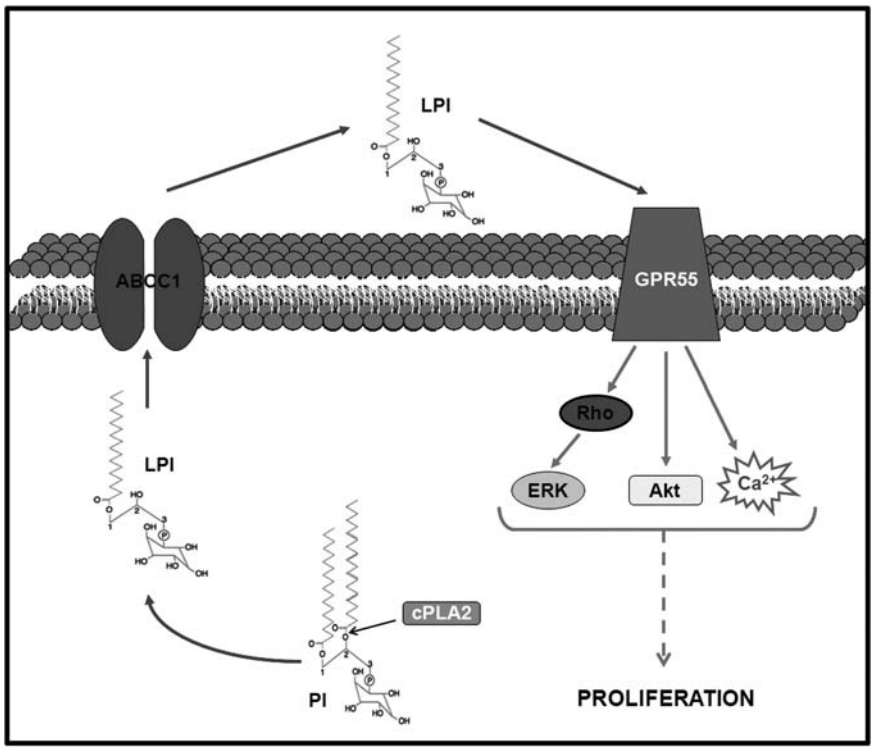

Figure 9 LPI and GPR55 define a new autocrine loop in cancer cells regulating cell proliferation. Once synthesized intracellularly by the action of cPLA2, LPI is released into the extracellular media by the transporter ABCC1. Once in the extracellular media, LPI can bind GPR55 and activate distinct intracellular signaling cascades, including ERK, Akt activation and Ca^{2+} mobilization, ultimately controlling cancer cell proliferation. The black arrow shows the action of cPLA2 on the sn-2 position of phosphatidylinositol to generate LPI. Blue arrows indicate mechanisms involved in LPI synthesis and release. Solid red arrows show effects induced by LPI through GPR55 activation and the dotted red arrow represents the signals leading to cell proliferation that remain to be established. A full color version of this figure is available at the *Oncogene* journal online.

this compound has been reported to possess both agonist (Henstridge *et al*., 2009; Kapur *et al*., 2009; Yin *et al*., 2009) and antagonist (Lauckner *et al*., 2008) effect on GPR55, it clearly acts as a receptor antagonist in our experimental models. Furthermore, we show that downregulation of GPR55 using specific siRNA is also able to blunt the LPI-dependent cellular functions, consistent with a previous report showing that the LPI-dependent activation of ERK in non-cancer cells was mediated by GPR55 (Oka *et al*., 2007). Our data indicate that GPR55 (but not GPR119, which has also been reported to be activated by LPI; Soga *et al*., 2005) has a key role in mediating the effect of LPI in prostate and ovarian cancer cells. Although we cannot rule out the possibility that LPI might be able to activate other GPCRs or transient receptor potential channels in prostate and ovarian cancer cells, our data indicating that downregulation of GPR55 completely blocks proliferation and that exogenous LPI is not able to rescue the inhibition on cell proliferation strongly suggest that GPR55 is a key receptor in mediating the LPI-dependent signaling leading to prostate cancer cell proliferation.

Moreover, we demonstrate that the LPI/GPR55 interaction is pivotal in maintenance of an autocrine loop, which sustains prostate cancer cell proliferation. Indeed we demonstrate that a pool of LPI, synthesized intracellularly by cPLA2, can be released in the extracellular media through the action of the transporter ABCC1 and can therefore activate GPR55 to promote cell proliferation (Figure 9). Consistent with this hypothesis, downregulation of either cPLA2 or ABCC1 reduces the levels of ^{3}H-LPI released in the extracellular media and inhibits prostate cancer cell proliferation. Addition of exogenous LPI is able to rescue the effect of ABCC1 or cPLA2 downregulation.

The involvement of cPLA2 and ABCC1 in cancer, and particularly in prostate cancer, has been previously reported and it has been shown that the expression or function of these proteins appears to be deregulated (Sullivan *et al*., 1998, 2000; Patel *et al*., 2008). Our study represents the first link between these molecules and GPR55/LPI axis in prostate cancer. The involvement of the ABCC1 transporter in this autocrine loop is of particular importance as the role of this transporter in cancer has been connected only to drug resistance so far (Hoffmann and Kroemer, 2004), whereas our data imply a direct role of ABCC1 in cancer cell proliferation by mediating the LPI export.

Our present work provides a strong evidence for the mechanism of action of LPI in cancer cells and supports our early hypothesis that LPI has a specific role in cancer progression (Falasca and Corda, 1994; Falasca *et al*., 1995, 1998). In addition, our data suggest that LPI may bind to its specific receptor on tumor cells in an autocrine or paracrine manner, thereby stimulating the growth and metastasis of prostate cancer.

In conclusion, our study identifies a novel role for the receptor GPR55 and LPI as a key regulator of cancer cell proliferation and anchorage-independent growth. We demonstrate that the phospholipid LPI can be synthesized and released by these cells in an autocrine loop involving cPLA2, ABCC1 and ultimately GPR55 activation, to induce and sustain cell proliferation. Therefore, these data show a new pathway representing a potential target for therapeutic intervention and identify LPI as a potential biomarker in prostate and ovarian cancer.

Materials and methods

Materials and cells
l-α-lysophosphatidylinositol sodium salt from soybean and Y-27632 dihydrochloride monohydrate were purchased from Sigma-Aldrich (St Louis, MO, USA); CBD from Tocris Bioscience (Ellisville, MO, USA); and ^{3}H myo-inositol from Perkin-Elmer (Waltham, MA, USA). Antibodies were from Cayman Chemicals (Ann Arbor, MI, USA) and Abcam plc (Cambridge, UK; anti-GPR55); Cell Signaling (Danvers, MA, USA, anti-phospho Ser473-Akt); Santa Cruz Biotechnology (Santa Cruz, CA, USA; anti-Akt, anti-phospho ERK1/2, anti-ERK2); and Sigma-Aldrich (anti-α-tubulin). Secondary antibodies were from Sigma (anti-rabbit), GE Healthcare (Amersham, UK; anti-mouse) and Dako (Glostrup, Denmark; anti-goat). The enhanced chemiluminescence detection system was from GE Healthcare. SR141716A (Rimonabant) was provided by the National Institute of Drug Abuse, Bethesda, MD, USA. PC-3 and DU145 were grown in RPMI (Invitrogen, Carlsbad, CA, USA); OVCAR3 cells were grown in DMEM (Invitrogen). Media were supplemented with 10% fetal bovine serum, sodium pyruvate, penicillin and streptomycin, and incubated in 5% CO_2 at 37 °C.

RNA interference transfections
PC-3 and OVCAR3 cells were transfected either with specific siRNAs for GPR55, cPLA2, ABCC1 (Pettus *et al*., 2004; Mitra *et al*., 2006; Oka *et al*., 2007), siGPR119 (purchased from ABgene Ltd, Epsom, UK) or with scrambled Silencer Negative Control siRNA (Ambion, Foster City, CA, USA) using Oligofectamine (Invitrogen) according to the manufacturer's instructions.

Gene expression
mRNA was extracted using the Qiagen RNeasy purification system (Qiagen, Chatsworth, CA, USA). cDNA was prepared using the Superscript II reverse transcriptase (Invitrogen) and analyzed using quantitative real-time PCR (Applied Biosystems 7500 Real-Time PCR System; Applied Biosystems, Carlsbad, CA, USA).

Calcium mobilization assay
Cells plated on optical chambers were serum starved overnight and incubated in HBSS medium containing 4 μM Fluo-4 AM (Molecular Probes, Eugene, OR, USA) for 50 min in the presence of 2 mM Ca^{2+}. After the incubation, chambers were washed and incubated in HBSS for 30 min. Cells were stimulated with LPI (10 μM) and ATP (1 mM) (Sigma) and the intensity of fluorescence was acquired every 1.3 s using a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Welwyn Garden City, UK). Data are expressed in AFU and are shown as fold increase over the basal fluorescence after subtracting the background. At least a pool of 15 cells was analyzed for each well.

Cell counting
PC-3 and OVCAR3 cells seeded in a 12-well plate were transfected and serum deprived overnight in the presence or absence of LPI (24 and 48 h). For experiments with SR141716A, cells were pre-treated for 30 min with the drug before LPI administration. In all cases, after the corresponding incubation time, cells were harvested, resuspended and manually counted.

Soft agar colony formation assay
Cells transfected with scrambled siRNA or siRNA targeting GPR55 for 24 h were trypsinized and counted; 30×10^3 cells were suspended in 0.3% molecular grade agarose (Bioline, London, UK), penicillin/streptomycin, 10% fetal bovine serum in RPMI 1640 medium and then layered on top of 1.5 ml of 0.6% low melting agarose, penicillin/streptomycin, 10% fetal bovine serum in RPMI 1640 in six-well culture plates and allowed to grow at 37 °C with 5% CO_2 for 3 weeks. Plates were stained with 0.005% crystal violet in methanol for 1 h and colonies were counted. Representative images of the colonies were taken with a Nikon Eclipse TE2000-S inverted microscope (Nikon, Kingston, UK).

Caspase-3 assay
Assay was performed in duplicates following the manufacturer's instructions (EnzChek Caspase-3 Assay Kit #2, Molecular Probes). Fluorescence was measured using a microplate fluorometer (FluoStar Optima, BMG LABTECH, Aylesbury, UK).

Cell cycle analysis
Cells were harvested, fixed in ice-cold 70% ethanol for at least 30 min and incubated with a solution containing RNAse (100 μg/ml, Sigma) and propidium iodide (final concentration 50 μg/ml; Sigma) for 15 min at room temperature. Cells were analyzed by flow cytometry collecting 25 000 events per sample using a BD FACSCanto II (BD Transduction Laboratories, Oxford, UK).

Metabolic labeling of cells and TLC analysis of LPI release
Cells grown in 12-well plates were transfected with the indicated siRNA. At 24 h after transfection, cells were labeled with [^{3}H]myo-inositol (5 μCi/750 μl) in Medium 199 (Sigma) for 48 h. Acid medium extraction and thin layer chromatography analysis were performed as reported (Falasca and Corda, 1994; Falasca *et al*., 1995). Total radioactivity of the ^{3}H-LPI-containing fractions was evaluated by liquid scintillation.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)