Lysophosphatidic acid and sphingosine-1-phosphate promote morphogenesis and block invasion of prostate cancer cells in three-dimensional organotypic models

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Introduction

The mechanisms promoting local invasion and metastasis of castration-resistant, late stage prostate cancer (PrCa) are incompletely understood and poorly recapitulated by standard 2-dimensional (2-D) monolayer cell culture and invasion models (for example, transwell migration/Boyden chambers and scratch-wound assays). 2-D models fail to support the formation of multicellular structures and epithelial barriers such as the basement membrane (BM). In contrast, advanced model systems in three-dimensional (3-D) tumor microenvironment promote the formation of organotypic structures with relevant cell–cell and cell–matrix interactions (Brekhman and Neufeld, 2009), epithelial polarization and differentiation. In particular, models that utilize physiologically relevant ECM such as collagens or laminin (Matrigel) increasingly gain relevance (Reuter et al., 2009; Inman and Bissell, 2010; Ridky et al., 2010). 3-D cultures represent an opportunity to investigate dynamic morphogenetic processes like epithelial-to-mesenchymal transition (Chu et al., 2009), a central mechanism determining tumor cell motility, invasiveness and drug resistance (Mani et al., 2008; Kalluri and Weinberg, 2009). We have recently reported a miniaturized 3-D platform to analyze the morphology of PrCa cell lines in laminin-rich ECM (IrECM) (Harma et al., 2010). Malignant PC-3 and PC-3M cells initially show normal acinar differentiation, but later undergo spontaneous transformation into aggressive stellate structures.
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Results

Cell density and delipidated serum promote invasion in 3-D
The dynamics of the initial normal-like spheroid phase formed by PC-3 cells and their spontaneous transformation into invading structures were affected by external factors, including cell density. PC-3 cells were transferred into 3-D microwells at increasing cell densities. Sparser seeded cells (500–1000 cells/well) develop large, hollow spheroids lacking invasive processes for >12 days (Figure 1a, measured for 144 h). Onset of invasion is indicated by the loss of ‘roundness’, as measured by automated image analysis (ACCA). At high density (2500 cells/well), invasion commences preferentially in dense areas at earlier time points. Adding fresh medium to invasive 3-D cultures results in temporary retraction of invasive processes.

Next, medium was supplemented with increasing quantities of fetal bovine serum (FBS). In the absence of FBS, cells failed to form round spheroids, and invaded the lrECM from day 1 (Figure 1b). FBS concentrations >2.5% supported acinar morphogenesis and suppressed invasive behavior, indicating that differentiation-promoting factors were sufficiently supplied by 2.5% FBS. In contrast, cultures supplemented with charcoal-delipidated serum (CSS), removing the most lipophilic components including LPA and S1P (Lee et al., 1998a, 1998b), failed to develop spheroids even at 20% FBS (Figure 1c). This indicates repression of invasive properties by lipophilic factors.

The bioactive lipids LPA and S1P promote epithelial maturation and suppress invasive transformation
To identify the postulated lipophilic regulators, we tested a panel of steroids, growth factors and vitamins (Supplementary Figure S1). Re-constitution of androgens, estrogens, progesterone, glucocorticoids, vitamin A (retinoic acid) or D₃ (cholecalciferol) failed to support acinar differentiation. Next, we investigated lipids found at significant concentrations in serum, plasma and lymphatic fluids (prostaglandins, eicosanoids and thromboxanes). Only addition of LPA and S1P effectively suppressed invasion (Figure 2), maintaining differentiation, polarization and complete BM. LPA was less potent than S1P, with strong invasion-suppressive effects for 36 h and 42 h at 1.0 and 10 μM, respectively (Figure 2a). With S1P, effects lasted for >100 h (Figure 2b). In delipidated medium (Figure 2c, CSS), the BM started to disintegrate after 24 h and was completely disrupted after 72-96 h (compare also Figure 6). Both LPA and S1P potently repressed BM degradation and invasion. However, high concentrations of S1P, but not LPA, caused morphological defects and disorganization of the spheroids. Both LPA and S1P are thus potent regulators of acinar maturation, while S1P also affects cell growth and survival. LPA and S1P also potently blocked cell motility in monolayer settings, as shown by wound-healing assays (Supplementary Figure S2A).

LPAR1 mediates invasion-inhibiting signals, while LPAR2 and S1PR2 promote cell growth and proliferation
Next, we tested if siRNA knock down of the LPA and S1P receptors compromises the invasion-blocking effects of LPA and S1P (Figure 3a). The receptors LPAR1-3 and S1PR2–4 were silenced by RNA interference in monolayer culture for 72 h, and subsequently transferred into 3-D lrECM culture. The silencing effect of each siRNA was validated by RT–PCR. Knock down of LPAR1 in the presence of LPA or blocking of LPAR1 with the selective compound Ki-16425 (Figure 3b) caused rapid increase of invasiveness (loss of ‘roundness’ after 24 h, in contrast to
Figure 1 Factors supporting the invasive switch of acini formed by PC-3 cells in organotypic 3-D culture. (a) Increasing cell density promotes conversion of differentiated acini into invasive structures (phase-contrast images from live cell imaging; left panel). Disintegration of spheroids and invasive properties measured as loss of roundness by automated image analysis software ACCA (right panel; measured from days 4–7 of culture). (b) Addition of >2.5% FBS supports differentiation and suppresses invasive transformation of acini in 3-D. (c) FBS deprived of lipids by charcoal-stripping (CSS) fails to support differentiation even at 20%.
scrambled siRNA), but did not affect cell growth. In contrast, siLPAR2 resulted in proliferation-block. Similarly, in 2-D conditions, silencing of LPAR1 and 2 caused minor effects on cell proliferation (Supplementary Figure S2B). LPAR3 silencing did not result in a clear phenotype (not shown). Thus, LPAR1 appears as the key factor in LPA-mediated block of invasion, and both LPA and S1P can act via this receptor. Silencing of S1P receptors 2, 3 and 4 in 2-D and 3-D conditions caused growth inhibition (Supplementary Figure S2B), but did not affect invasion (Figure 3c; loss of ‘roundness’). The growth-promoting effect of S1PR2 was further validated with the specific antagonist JTE-013, causing severe growth retardation (not shown). As S1PR1-silencing was ineffective, we used the specific antagonist W146, which did not affect invasiveness (not shown).

Figure 2 Supplementary bioactive lipids LPA and S1P to CSS supports acinar differentiation and represses invasive transformation. (a) Concentrations of 1 and 10 μM LPA effectively repress invasive properties in lrECM for 36 and 42 h, respectively, maintaining the round phenotype. (b) S1P concentrations of 1 and 10 μM in CSS repress invasiveness for close to 100 h. (c) Immunofluorescent images of PC-3 spheroids stained for filamentous actin (F-actin) and laminin α-1 demonstrate disintegration of the actin cytoskeleton and first indication of the beginning disintegration of the BM after 24 h exposure to 10% lipid-deprived CSS versus complete medium. In CSS supplemented with 10 μM LPA or 1 μM S1P, both actin cytoskeleton and BM remain intact.

G-protein signalling cascades downstream of LPA and S1P receptors affect PC-3 spheroid formation and invasive switch
Gene set enrichment analysis (Figure 4a, Supplementary Table 1) (Mootha et al., 2003; Subramanian et al., 2005) of mRNA expression data, comparing PrCa cells in 2-D monolayer with early time points in 3-D culture (days 4–8), showed that a number of canonical pathways, gene sets and gene ontology categories were significantly enriched in all PrCa cell lines growing in 3-D (Harma et al., 2010), a characteristic of acinar morphogenesis. Most significantly induced were Rho-stimulatory Gα12/13 and Gαq signalling, whereas adenylate-cyclase activating Gαs signalling was reduced. Also adenylate-cyclase inhibitory Gαi signalling was induced, but failed false discovery rate filtering. A detailed analysis of genes within the Gα12/13 pathway (heatmap Figure 4b) indicated that the Rho-kinases ROCK1+2, G-protein Gα13 (GNA13), NF-κB p49/p100 (NF-κB2) and regulatory members of the NF-κB transcription factor complex (NF-κBIA and NF-κBIE) were significantly increased in 3-D compared with 2-D culture, peaking at days 4–8 when spheroids mature. As illustrated by Supplementary Figure S3, differential expression also occurred for many key components downstream of the Gα12/13 pathway. In invading cells (d13+15), expression of most Gα12/13 related transcripts is subsequently reduced. Silencing of GNA12 (Figure 4c) caused cell elongation in 2-D and severe growth defects in 3-D,
indicating the importance of G\(_{\alpha12/13}\) signalling in spheroid formation. Similar results were observed with siGNA13 (not shown), which was slightly less effective in 3-D. Furthermore, a detailed analysis of gene expression associated with LPAR1 functions (Figure 4d) indicates a peak of expression at days 4-8, followed by a decline in invasive cells (days 13-15) and failure of induction in CSS-treated spheroids.

**Blocking the \(G_{\alpha12/13}\) RhoA/ROCK pathway induces invasive properties in 3-D**

We executed a small-scale compound screen, based on inhibitors and activators targeting G-proteins and downstream signalling (Figure 5a, upper panel; Supplementary Table 2). Modulators of RhoA and Rho-kinases ROCK1/2 altered invasive properties in both 3-D and 2-D conditions (Supplementary Figure S4). The RhoA inhibitor CCG-1423 blocked invasion, but resulted in growth retardation, indicating a role for RhoA in proliferation. In cells treated with ROCK inhibitors Y-27632 and HA-1100, invadopodia formed within 24 h, similar to CSS treatment, silencing or biochemical blocking of LPAR1. Disrupting actin-cytoskeleton polymerization with cytochalasin-D caused substantial growth retardation, but the remaining structures showed pro-invasive structures. ROCK inhibitors effectively promoted motility in 2-D wound-healing/scratch-wound assays (Supplementary Figure S4A). These results indicate that LPA, LPAR1, \(G_{\alpha12/13}\), Rho proteins and downstream effectors are fundamental for the maturation of spheroids. Blocking these pathways promotes cell elongation, invadopodia formation and invasive/migratory properties.
Activating the Gαq/adenylate cyclase/cAMP pathway promotes invasion in 3-D

LPAR1 was reported to mediate downstream actions via Gαq and Gα12/13 proteins; together with Gα12/13 these counteract the stimulatory activity of Gαq signalling. Therefore, we tested modulators of Gαq downstream signalling pathways; activity of adenylate cyclase (AC) and phospholipase C, increased intracellular cAMP levels, protein kinase A (PKA) and the ERK1/2 kinases (not shown). The Gα12/13 inhibitors, pertussis toxin and NF023, or Gα12/13 effectors melittin and mastoparan, had only minor effects, possibly due to sequestration of peptides in matrigel. Treatment of spheroids with forskolin, a drug that activates AC and increases intracellular cAMP levels, resulted in a profound loss of acinar morphology and invasion (Figure 3a, lower panel), and an approximately 10-fold increase of intracellular cAMP levels (not shown). Accordingly, suppressors of cAMP accumulation (BPIPP and SQ22536) and PKA (KT-5720) effectively blocked invasion and promoted epithelial maturation. However, we detected only minor changes of intracellular cAMP levels in response to CSS, or in response to LPA and SIP, suggesting that this mechanism may only have a secondary role in downstream LPAR1 signalling. Possibly for this reason, modulators of cAMP/AC had only little effects on cell motility (Supplementary Figure S4B) or proliferation (Supplementary Figure S4C) in 2-D wound-healing assays, in contrast to RhoA/ROCK inhibitors.

Morphologic effects of compounds were analyzed by automated and computational image analysis (Figure 4 and Supplementary Figure S4). In Figure 4b, % roundness is used as representative measure for spheroid maturation, typically resulting in round structures, and usually strongly anti-correlated with measures for invasive structures. The box and whisker plots indicate deviation from the mean (control) as the result of lipid depletion (CSS), and inhibition of G-proteins or downstream effectors. Treatment with modulators of Gα12/13 did not cause measureable changes. ROCK inhibitors (HA-1100 and Y-26732) show strong invasion-promoting effects (= reduced roundness). In contrast, RhoA-inhibitor CCG-1423 blocks mainly proliferation and results in small, round structures. Promoting AC activity and increased cAMP accumulation favor invasiveness and reduce roundness (forskolin), while AC/PKA inhibitors increase maturation & roundness (BPIPP, KT5720 and SQ22536). The results of treatments on morphometric parameters are illustrated by segmented images (proprietary ACCA image analysis software), corresponding to the 3-D compound treatments (Supplementary Figure S5A). The heatmap (Supplementary Figure S5B, left panel) illustrates numerical changes of morphological image data for various treatments that affect invasive processes (roundness and area versus roughness, invasion-index, number, length or median). Supplementary Figure S5B, right panel, shows the Bonferroni-corrected P-values for these changes.

RhoA is counteracted by Rac and CDC42 proteins. Thus, we treated 3-D spheroids of PC-3 cells with Rac-inhibitors EHT-1864 (Shutes et al., 2007; Onesto et al., 2008) and NSC-23766 (Akbar et al., 2006; Devine et al., 2008), which blocked invasion effectively. This indicates a role of Rac proteins in formation of invasive structures, counteracted by RhoA in non-invasive and differentiating spheroids. We performed western blot analysis of PC-3 cells cultured in monolayer, compared with non-invasive early (d5), pre-invasive late acinus stage (d8) and invasive cells (d14) in 3-D. Furthermore, we analyzed invasive PC-3 aggregates in CSS versus acini, rescued with LPA (Figure 5d). RhoA activity, indicated by high levels of inactive p-RhoA Ser-188, is temporarily decreased in late-stage, pre-invasive acini (d8) and CSS treatment, but increased in cells rescued with LPA. Concomitantly, Rac1 is increased in both d14 invasive structures and CSS treatment, but reduced in LPA-rescued spheroids. Expression of ARHGAP1 (Rho-GTPase activating enzyme), which negatively regulates RhoA functions, is increased in d8 pre-invasive and d14 invasive structures, high in CSS-treated, but again repressed in LPA-rescued cultures. In contrast, ARHGEF11 (Rho guanine nucleotide exchange factor 11), which interacts with Gα12/13 proteins and stimulates RhoA functions, is low in monolayer and CSS treatment; but induced in early (d5) and late (d8) acini, as well as in LPA-rescued spheroids. Serum-response factor (SRF) expression corresponds most consistently with motility and invasiveness of PC-3 spheroids, and is highest in monolayer, invasive (d14) spheroids and CSS-treated cultures. The lack of changes of p-CREB1 may indicate the secondary nature of altered CAMP levels in response to LPA, as described above.

Figure 4: Evaluating the roles of G-protein signalling cascades and downstream pathways. (a) Gene Set Enrichment Analysis of mRNA gene expression data from PC-3 cells at early time points (day 4-8) of 3-D organotypic culture. Factors indicate relative enrichment of gene sets in 3-D compared with 2-D culture. Altered G-protein related signalling pathways highlighted in red. (b) Heatmap of mRNA gene expression data, illustrating relative changes for genes within the Gα12/13 pathway in PC-3 cells at various time points in 3-D culture, relative to monolayer. Multiple genes like ROCK1 are induced in well-differentiated spheroids (d4 and d8), but reduced after the invasive switch (d13 and d15). Inducible expression is abolished in 3-D culture supplemented with 10% CSS (24 and 72 h). (c) Silencing of Gα12 protein in 2-D monolayer conditions induces spindle-like morphology of PC-3 cells (upper panels). In contrast, silencing of Gα12 in 3-D condition causes growth inhibition. (d) Heatmap of LPA receptor-associated genes at various time points of 3-D culture and FBS versus CSS medium.
epithelial cell lines (EP156T and RWPE-1). CSS, the ROCK inhibitor Y-27632 and forskolin increased the otherwise limited invasive properties of malignant LNCaP cells. In contrast, treatment of non-transformed EP156T cells promoted the maturation of highly symmetrical spheroids, a hollow lumen, polarized epithelial layers, and an even more complete BM (Figure 6). Also, pre-malignant RWPE-1 and malignant DU145 spheroids responded to CSS, Y-27632 and forskolin, with the formation of invadopodia and degradation of the BM (Supplementary Figure S6). Our findings indicate that RhoA/ROCK and cAMP/AC/PKA pathways are differentially regulated in transformed versus non-transformed cell lines, resulting in opposing morphologic responses.

**LPAR1 is downregulated in metastatic PrCa**

We next analyzed the expression of LPA and S1P receptors in clinical mRNA expression sets (Figure 7). In both the expO and the MSKCC transcriptome datasets, expression of LPAR1 is reduced in PrCa versus normal tissues, and anti-correlates with clinical parameters associated with cancer progression and poor survival.

**Figure 6** Cell line-specific effects of ROCK inhibitors and increased cAMP levels on acinar morphogenesis versus formation of invasive structures. Spheroids formed by PC-3, LNCaP and EP156T cells in Matrigel were stained with Alexa488-phalloidin for filamentous F-actin, and an antibody against human laminin-α1 to highlight the BM. Inhibition of ROCK kinases (Y-27632) and enhanced AC/increased cAMP levels (forskolin) induce invasive features and disintegration of the BM in transformed PC-3 and LNCaP spheroids, but promote maturation of non-transformed EP156T cells.
outcome (Histological grade > 3; Gleason grade > 8; positive lymph node invasion). LPAR1 shows further decreased expression in metastatic samples compared with primary PrCa’s. In the MSKCC data, expression of LPAR1 is significantly reduced in 42% of primary and metastatic cancers (Supplementary Figure S7A; 98 of 218 tumors). The expression of other LPA and S1P receptors like S1PR3 was also altered in PrCa’s, but not as consistent as LPAR1. The heatmap (Supplementary Figure S7B) illustrates anti-correlation of LPAR1 with most other LPA/S1P receptors, except S1PR3. The high frequency of reduced LPAR1 expression and occasional deletion in PrCa’s is also demonstrated in Supplementary Figure S7C. Preliminary immune histochemical staining of formalin-fixed, paraffin-embedded PrCa tissues with an LPAR1 antibody showed a tendency towards reduced protein expression in Gleason Grade 4-5 and metastatic lesions (Supplementary Figure S8). This is currently further explored using larger panels of sections on tissue microarrays.

Discussion

LPA and LPAR1 act as potent negative regulators of cell motility and invasion by promoting epithelial maturation, while S1P receptors and LPA receptors 2-3 preferentially promote growth and survival (Daaka, 2002). LPA and LPAR1 thus stabilize the morphology of acini, and block or delay the re-organization of the actin cytoskeleton and invadopodia formation prior to invasion. Our findings are in contrast to other reports on PrCa (Hwang et al., 2006; Evelyn et al., 2007, 2010; Hao et al., 2007; Hasegawa et al., 2008) breast or ovarian cancer (Li et al., 2009a, b), but in line with findings on melanoma (Yamaguchi et al., 2003) and colon cancers (Shida et al., 2003, 2004), which similarly suggest a role of LPA, LPAR1 and downstream RhoA/Rac pathways in epithelial maturation. The Ga12/13 pathway with downstream effectors RhoA/ROCK, and Ga5 pathways with AC, cAMP, PKA and NF-κB are critical for the maturation of acini or spheroids. Ga12/13 is significantly induced in 3-D conditions, whereas Ga5 is repressed across all normal and cancer-derived cell lines, including PC-3, regardless of different morphological phenotypes (Harma et al., 2010) and invasive properties. Subsequently, the activity of G-proteins and downstream effector pathways are reversed during the invasive switch of PC-3 cells, coinciding with rearrangement of the actin cytoskeleton, increased expression of SRF, formation of invadopodia and degradation of the BM. SRF activity correlates with altered F-actin structures, collagen contraction and invasion in 3-D invasion assays (Morin et al., 2011), and integrates the expression of genes encoding structural and regulatory effectors of actin dynamics (Olson and Nordheim, 2010). Similarly, mechanisms downstream of Ga5 (AC, intracellular cAMP levels and PKA) affect cell morphology and invasiveness. In summary, LPA, LPAR1 and pathways downstream of Ga12/13, Ga5 and Ga1/0 promote multicellular differentiation of PrCa cells and repress...
the onset of invasiveness. These effects were most pronounced in PC-3 cells, but also observed across other transformed PrCa cell lines like LNCaP, DU145 and 22rV1. In contrast, inhibition of the same RhoA/ROCK and activation of cAMP-related pathways in non-transformed cells (EP156T) consistently enhanced epithelial differentiation. The complex tumor-specific nature of these cell biological effects, the activation of counteracting mechanisms, such as RhoA versus Rac1 in 3-D models, and the precise role of SRF and LIM kinases remain to be explored in more detail.

Most critical for understanding of these interconnected pathways related to LPA and S1P signalling is the use of appropriate model systems that properly address these mechanisms in multicellular differentiation, more similar to the (complex) clinical situation. Our finding that LPA and S1P promote differentiation has to take the tumor microenvironment, multicellular context, tissue specificity and cell transformation into account. None of the previous studies (in monolayer culture) reporting migration-promoting effects of LPA/SIP were based on organotypic, multicellular structures, and unable to track invasion of cell aggregates into the ECM. Additional studies rely on invasion models that monitor chemotactic motility of single cells, for example, Boyden chamber (Hope et al., 2009; Wang et al., 2010), or scratch/wound-healing assays (Fishman et al., 2001; Vogler et al., 2003; Wang et al., 2010). Our experimental settings focus instead on the plasticity of multicellular epithelial structures including the BM and dynamic turnover of cell-matrix contacts. In particular in PC-3 cells, this dynamic process resembles a spontaneous epithelial-to-mesenchymal transition, transforming metastable acini into mesenchymal-like, motile cell aggregates. Our results suggest a role of LPA and SIP in maturation of epithelia in general and prostate glandular cells in particular.

LPA + S1P are established as central regulators of many critical lipid-related pathways, like PPARγ (McIntyre et al., 2003; Zhang et al., 2004), and affected by phospholipases 2A in PrCa (Sved et al., 2004; Pino et al., 2010), thus linking LPA/S1P pathways to eicosanoid/arachidonic acid pathways and regulation of intracellular Ca$^{2+}$. LPA was also demonstrated to induce PC-3 migration, proliferation and survival via Erk1/2 and Akt (Gibbs et al., 2009) or MAPK p38 and ERK1/2 pathways (Hao et al., 2007)—again in 2-D monolayer conditions. These studies further suggested secretion of stimulatory chemokines, activation of STAT3 (Jeong et al., 2008; Seo et al., 2010; Snider et al., 2010a) and a key role for EGFR (Snider et al., 2010b), NFkappaB and RhoA (Hwang et al., 2006) in stimulating invasion. Similar associations were reported for SIP (Sekine et al., 2010). These observations are in opposition to our experimental findings, in which Gα12/13 and RhoA clearly have an important function in stabilization of acinar structures instead.

Additionally, other controversial aspects arise, that relate to the functions of LPA + S1P in clinical cancers. The role of autotaxin (ENPP2) and acyglycerol kinase, both of which produce LPA from lysophosphatidylcho-

line (Nouh et al., 2009; Zeng et al., 2009; Harper et al., 2010), or sphingosine kinases SHPK1 + 2, which generate SIP from sphingosine (Pchejetski et al., 2008, 2010; Brizuela et al., 2010), were suggested as valid anticancer targets (Liu et al., 2009a, 2009b), together with LPAR1. However, reduced expression of LPAR1 in a large portion of primary PrCa’s and distant metastases supports our observation that the LPA/LPAR1 axis actually promotes epithelial integrity and may also inhibit invasive properties in vivo. LPAR1 is unique among the LPA receptors in that it is also found in perinuclear localization and even in the nucleus (Gobeil et al., 2003; Waters et al., 2006). LPAR1 functions and intracellular transport/translocation are dependent on and regulated by RalA (Aziziyeh et al., 2009) and caveolin (Gobeil et al., 2003), related to both inflammatory and migratory mechanisms. Nuclear and perinuclear localization of LPAR1 is also regulated by cell density, Rho and ROCK inhibitors (Waters et al., 2006) and may correlate with activation of the Phospholipase A2, AKT and PI3K pathways, as well as integrin signalling. Within tumor tissues, LPAR1 may be employed to allow rapid, dynamic responses to a changing microenvironment. In those tumors that express significant levels of LPAR1, this pathway might contribute to the dynamics of epithelial plasticity and sense changing interstitial levels of sphingolipids and LPA, for example, in tumor cell intravasation, extravasation and transendothelial migration (Sequeira et al., 2008; van Golen et al., 2008). Invading and circulating tumor cells may encounter changing levels of LPA, SIP and related lipid signalling molecules in interstitial, lymphoid and vascular fluids, bone marrow and lymph nodes.

In summary, bioactive lipids like LPA + S1P have a decisive role in epithelial plasticity and the regulation of epithelial-to-mesenchymal transition, cell motility and dynamic adaptation to changes in the cellular microenvironment. On the basis of an organotypic 3-D cell culture models, our findings suggest that LPAR1 functions in PrCa are considerably more complex than previously thought, and deviate from generalized motility-promoting properties. Our report puts a cautionary note on the biological function of proposed anti-cancer targets like LPA/SIP receptors, associated enzymes involved in generation and turnover of LPA, and some of their downstream modulators. Such targets require stringent validation studies, utilizing a panel of diverse, biologically informative and relevant model systems.

Materials and methods

Cell lines and culture conditions

Cell lines were received from ATCC or originator laboratories. All lines were propagated in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% FBS, 1% penicillin/streptomycin and 1% l-glutamine. Immortalized prostate epithelial cells, EP156T and RWPE-1, were cultured in Keratinocyte Serum-Free Medium (Invitrogen, Carlsbad, CA, USA), supplemented with 50 mg/l bovine pituitary extract, 5 μg/l EGF and 2% FBS for 3-D conditions.
Serum delipidation by charcoal stripping
4 g of cell culture quality active charcoal (Sigma-Aldrich) was mixed with 40 mg of T70 dextran (GE Healthcare, Little Chalfont, UK), 4 ml of sterile 1× Tris–HCl, 4 ml of sterile 0.1× EDTA and sterile water to 400 ml. The slurry was stirred for 2–4 h at +4°C, centrifuged for 20 min at 5000 r.p.m., and the supernatant discarded. Activated dextran-charcoal was mixed with fresh FBS, and incubated twice for 30 min in a shaking water bath at +35°C. The charcoal was pelleted by centrifugation (20 min, 10 000 r.p.m., +4°C), supernatant collected, sterile filtered with 0.2μm suction filters (Thermo Fisher Scientific, Waltham, MA, USA), and stored at −20°C.

Miniaturized 3-D cell cultures and morphological image analysis with ACCA software
Miniaturized 3-D cultures were prepared as described previously (Harma et al., 2010). Density of spheroids across all experiments was controlled by seeding defined numbers of cells in each experiment (1000 cells/well for PC-3 and 1250 well for all other cell lines). For time-lapse experiments, 3-D cultures were imaged with the automated live-cell imager IncuCyte (Essen Bioscience, Ann Arbor, MI, USA). In endpoint assays, multicellular structures were stained with calcein-AM (Invitrogen), and apoptotic/dead cells highlighted by ethidium homodimer-2 (Invitrogen). 3-D confocal images were taken with a Zeiss Axiovert-200 M microscope with Yokogawa CSU22 spinning disc confocal unit, and a Zeiss Plan-Neofluar ×5 objective. Z-stacks were acquired with a step-size of 19 μm. Intensity projections were created with SlideBook, noise removed by normalization. Phase-contrast time-lapse and intensity projection images were analyzed with VTT’s proprietary ACCA software (version 1.95). ACCA segments individual cell structures based on morphological criteria, and assigns numerical values for features like area (size), roundness (shape), roughness (contour), and measures the number and length of invasive structures (median, index and maximum extension of ‘appendages’). Median cell density (cell Ratio) is quantified based on the ratio of cells to background signal. Raw numerical data were further statistically processed and visualized with R/Bioconductor.

Immunofluorescence labelling and imaging
Miniaturized 3-D cultures were fixed within microwells, using 2% paraformaldehyde, supplemented with 0.3% Triton X-100 (Sigma-Aldrich), 5 mM EGTA and 1 mM MgCl2 for 15–20 min at RT. Fixed cultures were washed 3x with PBS, and blocked for 1 h with 20% horse serum. Cultures were incubated overnight at 4°C, washed with PBS and incubated for 2 h at RT with secondary antibodies and Hoechst nuclear stain (1:5000). Anti-Laminin alpha-1 antibody (LAM-89) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) anti-Ki67 antibody from Abcam (Cambridge, UK) and Alexa-conjugated phalloidin from Invitrogen.

Immunoblotting
Protein samples were collected and lysed in WB-buffer (25 mM Hepes pH 7.5, 100 mM NaCl, 5 mM EDTA pH8.3, 0.5% Triton X-100, 20 mM β-glycerophosphate, 100 μM orthovanadate, 0.5 mM PMSF and 1 mM DTT). Protein concentration was measured by Bradford assay, and proteins separated by SDS-PAGE with precast PAGEr gels (Lonza, Basel, Switzerland), transferred on Protran nitrocellulose transfer membrane (GE Healthcare) and blotted with primary antibodies. Phospho-CREB1 (Ser133; no. 9198) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA), phospho-RhoA (p-Ser188; no. 9198) from ECM-Biosciences (Versailles, KY, USA), anti-Rac1 (ARCO3) from Cytoskeleton Inc. (Denver, CO, USA) and anti-ARHGEPF11 (HPA011026), anti-ARHGAPI (HPA004689) and anti-SRF (HPA00819) from Sigma-Aldrich.

RNA interference reverse transfection
All siRNA were purchased from Qiagen (Hilden, Germany). Cells were transfected with 81 nm siRNA for 48–72 h, using siLentFect (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in Opti-MEM medium (Invitrogen). After transfection, cells were embedded in Matrigel. Silencing efficacy of siRNAs was validated by quantitative RT-PCR (TaqMan), as described previously (Harma et al., 2010).

Drug treatments
All compounds were purchased from Tocris Bioscience (Bristol, UK) or Sigma-Aldrich, except for Ki16425 and JTE-013 (Cayman Chemical Company, Ann Arbor, MI, USA), W146 (Avanti Polar Lipids Inc., Alabaster, AL, USA) and CCG-1423 (Merck Chemicals Ltd., Darmstadt, Germany), and dissolved in appropriate vehicle (DMSO, Ethanol, or PBS/water). Drug treatments were performed in triplicates, with 2–4 different concentrations. Lipid-depleted CSS medium was used as a positive control to promote invasion. Multicellular structures were stained and imaged by spinning disk confocal microscopy, and analyzed by ACCA, as described above.

CellPlayer cell migration assays
Confluent cultures on 96-well ImageLock plates (Essen Bioscience) were scratched with WoundMaker (Essen Bioscience) and treated with compounds for a period of 48 h. Wound closure was monitored and quantified with IncuCyte live-cell imager (Essen Bioscience).

Bioinformatics: Clinical mRNA expression data analyses
Expression and correlation of LPA and S1P-related genes and pathways were analyzed in several large scale and clinical transcriptome studies: (1) The MSKCC data set containing >240 clinical PrCa samples and metastases (Grove-Kirkby, 2010; Taylor et al., 2010) (http://www.cbioportal.org/public-portal), (2) PrCa transcriptome data from expO (NCBI GEO dataset GSE2109); and (3) experimental expression data from our panel of PrCa cell lines, comparing 2-D and 3-D culture (GEO accession GSE19426). Normalized raw expression data were extracted from all data sets, median centered, and analyzed through an in-house HTML interface, using R/bioconductor-based algorithms. Hierarchical and K-Means clustering for S1P- and LPA-relevant genes and downstream pathways were extracted and plotted by TreeView/Cluster (Eisen et al., 1998).

Association of gene expression patterns with clinical annotation (grade, Gleason scores and TNM status) and patient survival were processed with R/Bioconductor. Gene Set Enrichment Analysis (MSigDb database) was performed across both clinical and in house datasets, to identify canonical pathways and gene ontology or GO categories enriched in clinical data and experimental settings.

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)