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Research Article

LXR Inhibits Proliferation of Human Breast Cancer Cells through the PI3K-Akt Pathway

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Abstract. The oxysterol receptors, LXRs, have recently been shown to reduce cell and tumour growth in various model systems. Activation of LXRs could therefore provide a novel approach for treatment of cancers. Here we show that LXRβ is the main executor of the antiproliferative effect in human breast cancer cells. LXR inhibits the activation of growth factor-induced triggering of the PI3K-Akt pathway. Phosphorylation of several protein kinases in this pathway, including Akt and the PI3K itself, is reduced upon activation of LXR. Both mRNA and protein expression levels of the PTEN and PHLPPL protein phosphatases were induced by LXR and the amount of the second messenger PIP3 reduced—a pivotal activator signalling molecule in the PI3K. This suggest that the intracellular signalling cascade mediating proliferative cues from growth factors is the responsible mechanisms underlying the antiproliferative effects of LXR in human breast cancer cells. This provides novel and in-depth insights of how LXR works in cancer cells where the LXRs control the activity of intracellular signalling cascades that regulate proliferation.

Keywords: LXR; proliferation; breast cancer; PI3K/AKt

1. Introduction

The oxysterol receptors (liver X receptors; LXRs) encoded by the genes *Nhr1h3* (LXRα) and *Nhr1h2* (LXRβ) are members of the nuclear receptor (NR) family and established ligand-regulated transcription factors involved in many metabolic processes including lipid, cholesterol, and carbohydrate metabolism [1]. Considerable evidence has since emerged identifying both LXRs as anti-inflammatory transcription factors and physiological regulators of innate and adaptive immune responses [2]. New insight into LXR signalling suggests an important role in cell proliferation and tumour development and current reports suggest that LXRs are potential targets for both prevention and treatment of several cancers [1, 3]. We have shown that activation of LXRs significantly reduced proliferation in human breast cancer cells lines [4]. LXRs suppressed expression of ERα, Skp2, cyclin A2, and cyclin D1 and increased the expression of p53 and maintained the retinoblastoma (Rb) protein in a hypophosphorylated active form. Further we observed that genome-wide transcriptional responses in human breast cancer cell lines

indicated common responsive gene sets. Upregulated LXR-target genes annotated to metabolic pathways whereas downregulated LXR-target genes include genes annotated to cell proliferation-related processes [5]. Hierarchical clustering of breast cancer patients based on the expression profiles of the commonly downregulated LXR ligand-responsive genes showed a strong association of these genes with patient survival. Moreover, we showed that LXRs inhibit proliferation of human colorectal cancer cell lines and LXR-deficient mice had increased proliferation in the colonic epithelial, while activation of LXRs reduced epithelial proliferation [6].

LXRs ability to induce efflux of cholesterol from cells also contributes to their antiproliferative effects. For instance, lymphocytes are dependent upon excess of intracellular cholesterol for the synthesis of cellular membranes during the proliferative events of clonal expansion. Activation of LXRs decreased intracellular levels of cholesterol via ABCA1/G1 cholesterol transporters thereby decreasing proliferation of lymphocytes [7]. In line with this, LXR knockout mice showed splenomegaly and an increased number of splenic lymphocytes compared to control mice. It was also shown that LXRs promoted tumour cell death in glioblastomas, the most common cancer malignancy of the brain [8]. Activation of LXRs led to increased expression of ABCA1 and IDOL, triggering degradation of the LDL-receptor, consequently decreasing cholesterol levels and thereby reducing growth and survival of the tumour. However, the hitherto strongest indication that LXRs are therapeutic targets for cancers was recently published showing that activation of LXRβ suppressed melanoma invasion, angiogenesis, tumour progression, and metastasis [9]. LXRβ activation displayed melanoma-suppressive cooperatively with the frontline regimens dacarbazine, B-Raf inhibition, and CTLA-4 antibody and robustly inhibited melanomas that had acquired resistance to B-Raf inhibition or dacarbazine. Here we unveil new insights of LXRs that intercepts growth hormone-induced intracellular signalling cascades in human breast cancer cell lines.

PI3Ks kinases generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P(3)). Activation of receptor tyrosine kinase, including the receptor for IGF1, results in PIP3/PIP2 production. Akt interacts with these phospholipids and becomes activated whereby Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth. During the last decade, it has been shown that PI3K-Akt signalling pathway components are frequently altered in human cancers and particularly breast cancer cell growth and progression is often dependent on a highly active PI3K pathway [10]. As much as 70% of breast cancers are associated with a hyperactive PI3K-Akt pathway [11, 12]. The components of the PI3K-Akt signalling pathway represent attractive drug targets for breast cancers [13]. Several protein phosphatases including PTEN, PHLPP (PHLPP1), and PHLPPL (PHLPP2) dephosphorylate PIP3 and Akt, and in this way act as a negative regulators of the PI3K-Akt pathway. These phosphatases are thus regarded as tumour suppressors. Mutations in PTEN have been found in many types of cancers [14, 15]. PHLPP and PHLPPL directly dephosphorylate Akt, thereby reducing its activity [16, 17] and recent observations link genetic deletion of the PHLPPs to progression of cancer [18] displaying the importance of these factors in the PI3K-Akt pathway in progression of cancers. To investigate the role of LXRs in this pathway we have taken advantage of GW3965, a commonly used selective LXR synthetic agonist [19]. Our data points towards an important role of PI3K-Akt pathway in the antiproliferative effect of LXR signalling.

2. Materials and Methods

2.1. Cell culture and reagents. The human breast adenocarcinoma cell line (MCF7) and the hepatocellular carcinoma cell line (HUH7) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Cat.no:11330-032, Life Technologies) while the SW480 human colorectal adenocarcinoma (epithelial like) cell line was cultured in DMEM/F-12 (1:1) (Cat.no:11039-021, Life Technologies). The human estrogen receptor- (ER-) negative breast cancer cell line MDA-MB-231 was cultured in RPMI 1640 medium (Cat.no: BE12-167F). Heat inactivated foetal bovine serum (FBS, Life Technologies) was added at 1-10% as described in the figure legends. All cell culture media were supplemented with 100 IU/ml penicillin and 100 g/ml streptomycin (Life Technologies) and cells kept in 5% CO2 humidified atmosphere at 37 ˚C. LY294002 (PI3K-Akt/mTOR pathway inhibitor) and IGF-1 (Insulin-like growth factor-1) were purchased from Sigma Aldrich. PP242, Rapamycin (mTORC inhibitor), and MK-2206 (specific Akt inhibitor) were purchased from Selleckchem. The GW3965 was a kind gift from Tim Wilson at GSK.

2.2. RNA interference. 5×10^5 or 25×10^5 MCF7 cells were plated in 24- or 6-well plates, respectively, for gene expression profiling and viability assays. Cells were grown to 30-50% confluence and transfected with 10 nM LXRβ (Sigma Aldrich) or control Luc (Dharmacon/Thermo Fisher Scientific) small interfering RNA (siRNA) oligos using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Medium was replaced 48 h after transfection and cells were treated with vehicle (DMSO) or 5 μM GW3965 LXR agonist for 16 h before harvest. MCF7 and MDA-MB-231 cells were transduced with lentiviral vectors

encoding shLXRα Sigma, MISSION pLKO.1-puro shRNA TRCN0000022237), shLXRβ Sigma, MISSION pLKO.1-puro TRCN0000022214, or non-target shRNA Sigma, MISSION pLKO.1-puro non-target shRNA Control Plasmid). LXRα and LXRβ evels were assessed by qPCR.

2.3. Viability assay. 25×10^5cells were plated in 6-well plates and treated with 5 μM GW3965 in 1% serum for 48 h. The absolute number of viable cells (proliferation) and per cent viable cells of total cell population (viability) were analysed by trypan blue (Life Technologies, Carlsbad, CA) exclusion method according to manufacturer's protocol. Viability is measured by the number of live cells versus dead cells (dead cells are coloured by the trypan blue). The absolute number of viable cells (not trypan blue marked) is then a measure of how many cells there are in each well and that represents a quantitative measure of proliferation.

2.4. Quantitative PCR. Total RNA was isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA). Five hundred nanograms total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). qPCR reactions were performed using the SYBR Green technology with the Fast SYBR mastermix (Applied Biosystems) and amplified using the Mx3005P instrument (Agilent/Stratagene). Primers were designed using the Primer Express software; primer sequences are available on request. We calculated relative changes by the comparative C_T method with 18S as the reference gene.

2.5. Western blot analysis. Proteins for whole cell lysate were extracted in PBS with 1% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA containing protease inhibitor (complete mini ETDA free, Roche), and phosphatase inhibitor (PhosSTOP, Roche). Cell lysates were centrifuged at 4°C for 10 min at 13000 rpm. Some samples were subfractionated using NE-PER (Nuclear and Cytoplasmic Extraction Reagents) (Thermoscientific) as described in the figures. 50 μg proteins were denatured by boiling for 5 min and separated with SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane using iBlot transfer machine (Invitrogen). Membranes were incubated overnight at 4°C with primary antibodies against P-GSK-3α/(ser21/9)/β(SER21/9), total GSK-3α, P-PDK1(ser241), total PDK1, P-PI3K p85(Tyr459)/p55(Tyr199), total PI3K-p85, P-mTOR(ser2481), P-mTOR(ser2448), total mTOR, P-Akt(ser473), total Akt(pan), Akt2(D6G4), P-Rictor(Thr1135), P-4E-BP1(The70), P-4E-BP1(The37/46), total 4E-BP1, P-p70 S6 Kinase(Thr389), total p70S6k, P-MDM2(Ser166), P-Bad(Ser112), P-Bad(Ser136), P-FoxO1(Thr24)/FoxO3a(Thr32) Pten (all from Cell Signalling), total PHLPPL, total PHLPP and total Rictor (all from Bethyl laboratory), Akt1(Sigma Aldrich), PI3 kinase, p85(Millipore), 14-3-3ζ(C-16), Histon 1 (Santa Cruz), GAPDH (Ambion), and LXRα/β [20]. Secondary antibodies are IRDye 680D Goat (Polyclonal) Rabbit (red) and IRDye 800D Goat (Polyclonal) (all from LI-COR Biosciences). The Odyssey Infrared Imaging System (LI-COR) and the Image J software were used for protein detection and quantification of Western blots. The density of the phosphoprotein of the gene of interest was related to the density of the total protein. The average of this ratio within each group was used to compare the difference between the treatments.

2.6. PI3K assays. For direct functional assessment of PI3K activity class IA PI3K was isolated from MCF7 cells by immunoprecipitation using the PI3K-p85 regulatory subunit to isolate the PI3K complex. The complex' ability to convert a standard PIP2 to PIP3 in a kinase reaction was assessed by measuring the generated PIP3 using ELISA according to the manufacture's protocol. Briefly, 5×10^6 cells were washed three times with buffer A (137 mM NaCl, 20 mM Tris HCl pH7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM Na-orthovanadate) and lysed in 1 ml of buffer B (buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich) and 1% nonyl phenoxypolyethoxylethanol (NP40) (Calbiochem)) for 20 min at 4°C. Cell lysates were centrifuged at 13,000 rpm for 10 min to remove insoluble material and supernatants stored at -80°C. Frozen lysates containing 750 μg protein were thawed on ice and the PI3K complex was immunoprecipitated by incubation with 5 μl anti-PI3K p85 (Millipore) for 1 h at 4°C on a rotating wheel followed by addition of 60 μl of a 50% Protein A agarose slurry coupled to magnetic beads in PBS for 2 h. Using a magnetic stand the immunoprecipitated complex was collected. Pellets were washed three times in buffer C (buffer A plus 1% NP40), three times in washing buffer D (0.1 M Tris-HCl, pH 7.4, 5 mM LiCl, and 0.1 mM Na orthovanadate) and twice with buffer E (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM Na orthovanadate). Pellets were resuspended in 30 μl kinase reaction buffer (KBZ; supplemented with 5 mM DTT and 50 □M ATP) and incubated for 3 h at 37°C with 40 pmol PI(4,5)P2 substrate (Echelon Biosciences, Salt Lake City, UT, USA). The reaction was terminated with EDTA at a final concentration of 5 mM and the reaction mixture centrifuged at 13,000 rpm at 4°C. Supernatants were transferred to a microtiter plate for a competitive ELISA (Echelon Biosciences K-1000) to quantify the PIP3 generated in the kinase reaction. Duplicates of 60 μl of

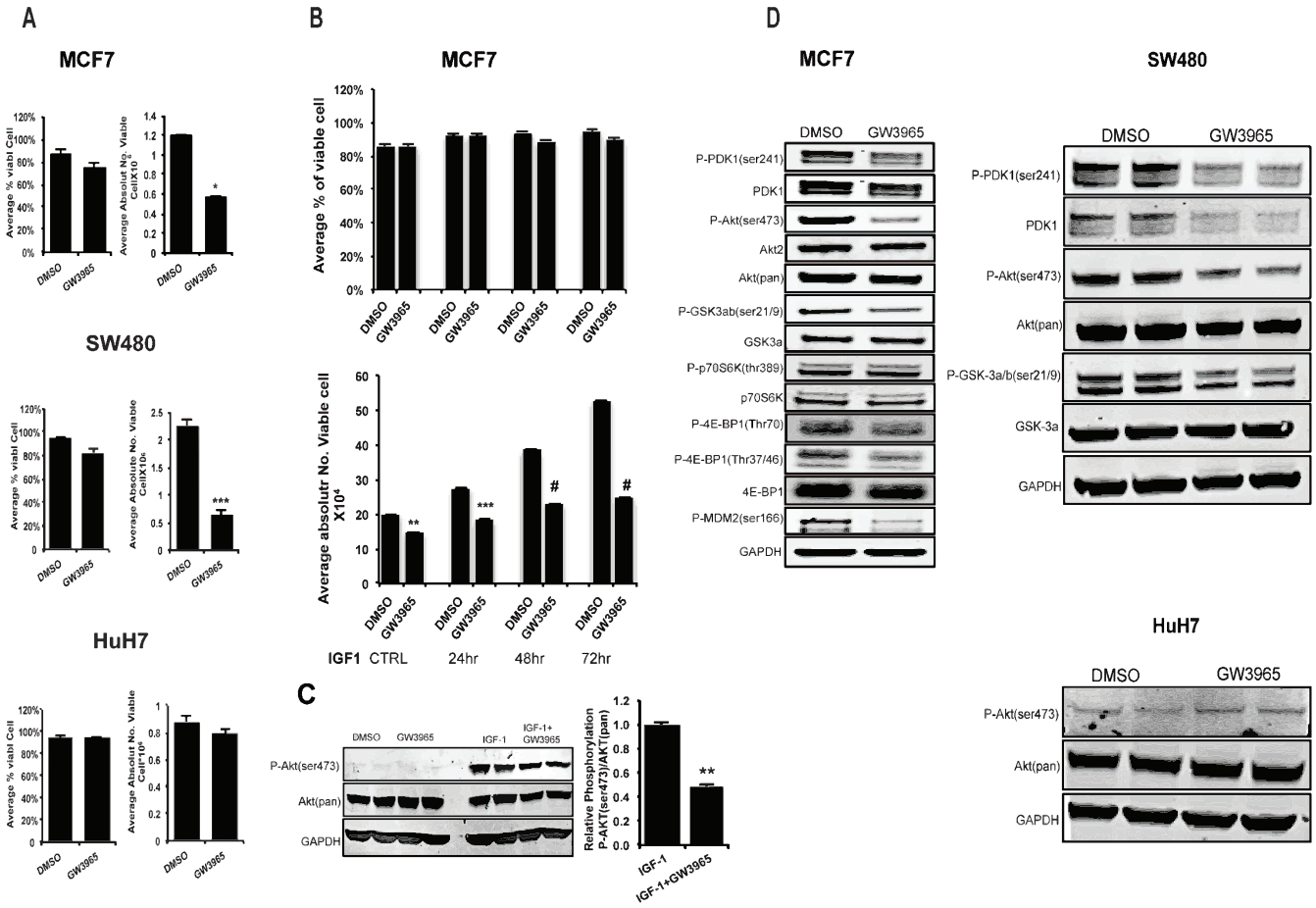

Figure 1: **LXR activation inhibits proliferation and reduces Akt phosphorylation. (A)** MCF7, SW480, and HUH7 cells were treated with vehicle (DMSO) or 5 μM GW3965 for 48 h. Cells were harvested, a viability test was performed, and the absolute number of viable cells was counted. MCF7 cells were cultured in 1% serum medium and then treated with DMSO and 5 μM GW3965 for 16 h. Cells were harvested either for the viability assay test to count absolute number of viable cells after activating the cells with IGF-1 (50ng/ml) in different time points **(B)** or for immunoblotting after activating them with (50ng/ml) IGF-1 (20 min) **(C)**. **(D)** MCF7, SW480, and HUH7 cells were cultured with 1% serum in growth medium and treated with 5 μM GW3965 for 16 h. Cells were given IGF-1 (50ng/ml) for 20 min, whole cell extract isolated and Western immunoblotting performed with various antibodies as described in the figure. GAPDH was used as an internal control demonstrating equal protein loading. The data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and # $P < 0.0001$ versus vehicle by Student's t-test.

the supernatants were incubated with 60 μl of anti-PIP3 antibody for 1 h at room temperature. The reaction mixture was then transferred to a microtiter plate coated with PIP3 and incubated for 1 h in the dark. After three washes with Tris-buffered saline (TBS) plus 0.05% Tween 20 (Sigma), 100 μl of horseradish peroxidase- (HRP-) conjugated anti-PIP3 (secondary detector) was added to each well and incubated for 1 h at room temperature in the dark. Following three additional washes with TBS plus 0.05% Tween 20, 100 μl of tetramethyl benzidine (TMB) substrate (Echelon Biosciences) was added and the reaction was terminated after 20 min with 50 μl 1N H_2SO4. Absorbance of the samples was measured using a TECAN plate reader at 450 nm and the PIP3 was quantified by comparison to a PIP3 standard curve run in parallel with the experimental samples.

2.7. Statistical analysis. Statistical analysis was performed using Student's t-test. Data are presented as mean ± SEM. Experiments were performed at least three times. Results were considered to be statistically significant at $P \leq 0.05$.

3. Results

We have previously reported a strong antiproliferative effect of LXR in human breast and colon cancer cell lines. Figure 1A shows that activation of LXR by the specific LXR agonist GW3965 did not alter cell viability (the number of dead trypan blue labelled cells) but robustly suppressed proliferation (we use the absolute number of cells counted as a comparative and quantitative measure of proliferation

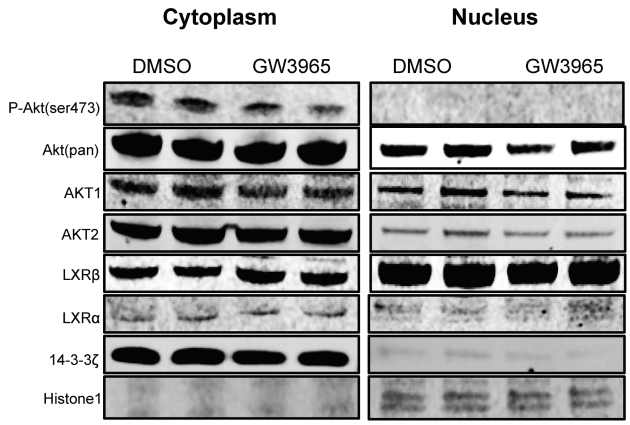

Figure 2: **Akt levels are reduced in the nucleus upon activation of LXR.** MCF7 cells were treated with vehicle or 5 μM GW3965 for 16 h. Cells were activated with IGF-1 (50ng/ml) for 20 min, cytoplasmic or nuclear cell extract isolated and Western immunoblotting performed with various antibodies as described in the figure. 14-3-3ζ and histone 1 antibodies were used as control for fractionation of cytoplasm and nuclease, respectively.

between treatments) in the human MCF7 breast cancer cell line and the human SW480 colon cancer cell line. No LXR effect was observed in the human hepatocellular cell line HuH7, indicating that the antiproliferative properties of LXR signalling are cell/tissue specific. We have further shown that the antiproliferative effect of LXR was mediated via several molecular pathways including altered phosphorylation status and transcriptional regulation of the important cell cycle regulators retinoblastoma protein (Rb) and p53 [4–6]. To continue in-depth analyses along these lines we investigated additional LXR mediated changes on factors involved in intracellular pathways controlling cell cycle and proliferation. We triggered growth-signalling pathways by treating cells with insulin-like growth factor 1 (IGF1). IGF1 induces cell growth in a time-dependent manner and this induction is strongly repressed by addition of the GW3965 LXR agonist (Figure 1B). The intracellular phosphorylation events precede the end point effect on proliferation; thus we use shorter incubation times to investigate phosphorylation versus proliferation. We also detected a strong induction of phosphorylation on intracellular pathways using IGF1 (Figure 1C). Further, we observed a LXR-mediated reduction of Akt(ser473) phosphorylation, and downstream targets of Akt, in both the MCF7 and SW480 cell lines, but not in the non-proliferative-responsive cell line (HuH7) (Figure 1D). This indicates that LXRs alter the phosphorylation status of Akt, however, in a cell specific manner.

Subcellular compartmentalisation is known to play a significant role in Akt-signalling and nuclear Akt has been shown to control cell cycle processes [21]. We separated a cytoplasmic and a nuclear fraction from MCF7 cells as indicated by the expression of the cytoplasm-specific protein 14-3-3ζ and nuclear-specific histone 1 protein (Figure 2). We observed expression of both LXRα and LXRβ in both compartments; however, LXRβ was highly enriched in the nucleus. Phosphorylation of Akt(ser473) was reduced in cytoplasm by addition of GW3965. The expression of phosphorylated Akt was too low in the nucleus to be detected. We identified expression of Akt1 and Akt2 in both the cytoplasm and in the nucleus using specific Akt1 and Akt2 antibodies as well as a "panAkt" antibody (recognising both Akt1 and Akt2). Activation of LXR reduced the protein expression level of panAkt, Akt2, and Akt1 in the nuclear fraction. Taken together, this indicated that LXR suppressed phosphorylation of Akt(ser473) as well as reduced Akt levels in the nucleus.

We investigated the PI3K-Akt pathway in more detail where the ability of the LXR to inhibit proliferation in MCF7 cells was monitored in the presence of specific inhibitors of PI3K (LY-294002), Akt (MK-2206), and mTORC1/2 (PP242 and rapamycin). Both concentration and time curves were performed to establish the optimal conditions in MCF7 cells for these inhibitors (Supplementary Figure 1). None of the inhibitors affected cell viability (data not shown). The phosphorylation status of the PI3K-Akt signalling pathway using the specific inhibitors had a strong effect on the PI3K-Akt signalling pathway as expected. LY-294002 reduced phosphorylation of many components in the entire pathway (Figure 3A). Rapamycin reduced phosphorylation of p70S6K and Rictor (Figure 3B). The Akt inhibitor MK-2206 reduced phosphorylation of Akt itself as well as phosphorylation of p70S6K and Rictor (Figure 3B). The mTORC1/2 inhibitor PP242 reduced phosphorylation of Rictor, Akt, and mTOR itself (Figure 3C).

In the presence of inhibitors the effect of the GW3965 agonist was abolished. We did not observe any effect on phosphorylation status when the PI3K was inhibited with LY294002. No effect was observed on Akt or downstream of Akt when the Akt-specific MK-2206 was used. When the PI3K pathway was inhibited downstream of Akt with rapamycin, GW3965 still decreased phosphorylation of Akt(ser473). The expression levels of total protein were not altered by addition of GW3965 or any of the inhibitors. These results suggested that LXRs targeted the PI3K-Akt pathway upstream, rather than downstream of Akt in the intracellular signalling cascade of PI3K-Akt. None of the inhibitors interfered with ability of LXRs to induce expression of the known cholesterol transporter gene ABCG1 (Figure 3D). However, the inhibitors reduced and often abolished the effect of GW3965 on the PTEN and PHLPP expression

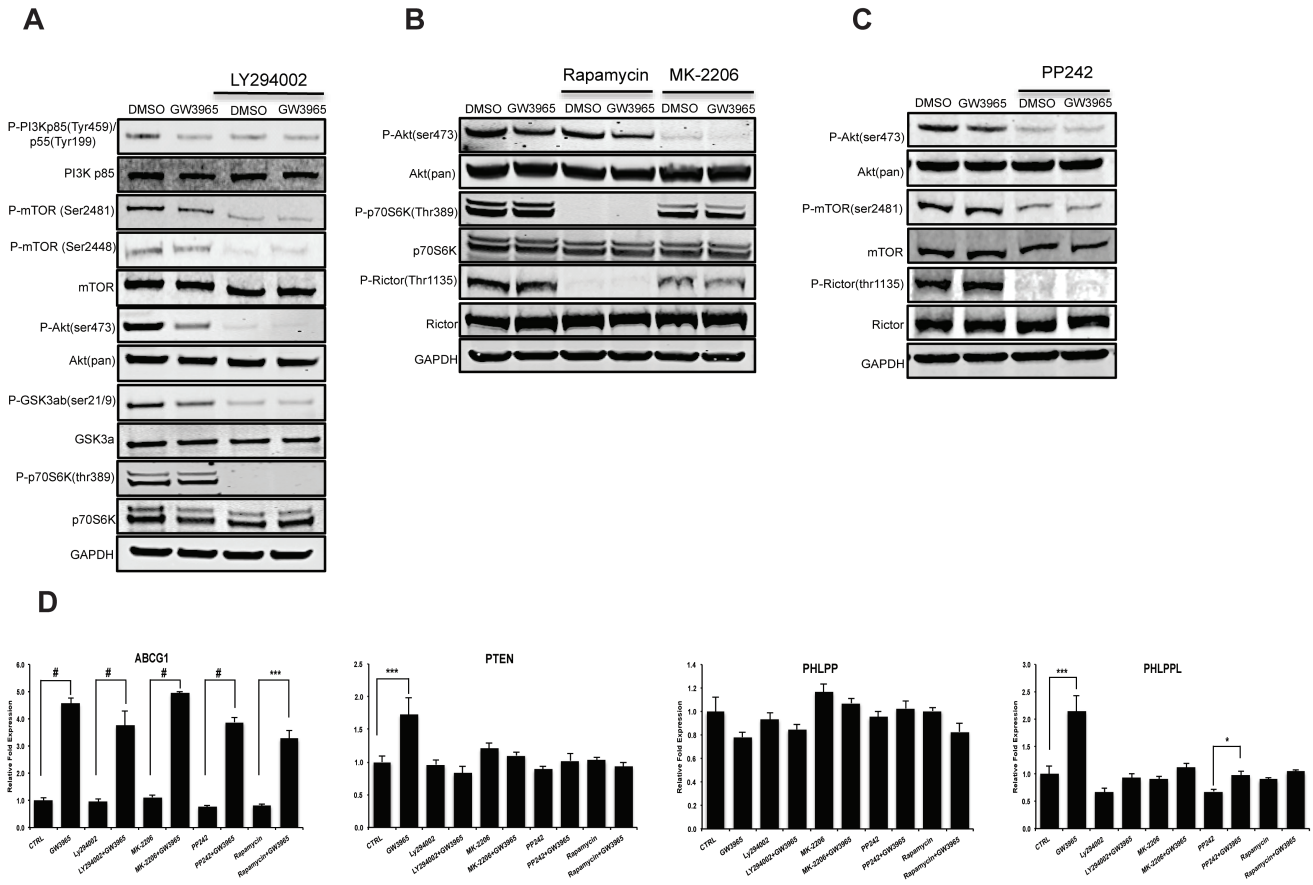

Figure 3: **Inhibition of PI3K or Akt abolished the effect of LXR on intracellular protein phosphorylation.** MCF7 cells were treated with DMSO and 5 μM GW3965 for 16 h and then cells treated with different inhibitors of PI3K/AKT pathway 30 μM LY-294002 for 30 min, 10 nM Rapamycin for 2 h, 500 nM MK2206 for 2 h, and 1 μM PP242 for 30 min. Cells were harvested to analyze either western immunoblotting by whole cell lysate extraction using various antibodies as described in the figure **(A-C)** or real time PCR by measuring relative mRNA expression levels for various regulatory gens that control PI3K/AKT pathway (PTEN, PHLPP, and PHLPPL) as described in the figure **(D)**. ABCG1 was used as a key LXR target gen. GAPDH was used as an internal control demonstrating equal protein loading. The data are presented as mean ± SEM. $*P < 0.05$, $***P < 0.001$, and # $P < 0.0001$ versus vehicle by Student's *t*-test.

further indicating that LXRs cross talk with the PI3K-AKT pathway.

We observed that activation of LXRs mediated transcriptional control of key players in the intracellular signalling cascade at the level of PI3K. Firstly, expression of the well-known LXR target gene, ABCA1, was significantly induced by GW3965 indicating a robust response of LXR (Figure 4A). mRNA expression of positive mediators of PI3K (Akt1) was reduced by LXRs while expression of phosphatases (PTEN and PHLPPL), whose roles are to reduce the effect of the PI3K cascade, was induced. We also observed reduced expression of the PHLPP phosphatase by GW3965. Similar effects at the protein level, where GW3965 induced protein expression of PTEN, PHLPPL (no difference was observed for PHLPP), further supported the effect on mRNA (Figure 4B). The change in mRNA levels differs from Akt1 and Akt2 where only the former is responsive to the GW3965 treatment. This is not reflected at the protein level which could be explained by the antibody that might have different affinity to Akt1 versus Akt2. Moreover, we observed that GW3965 reduced the phosphorylation status of the p85 catalytic subunit of the PI3K (Figure 4C) where a reduction in phosphorylation is associated to a reduced activity of the PI3K. This was observed in both the MCF7 and SW480 breast and colon cancer cell lines. Our results suggested that LXRs reduce the activity of the PI3K as well as induce the expression of phosphatases (PTEN) that consequently should lead to less amount of PIP3. Thus, we asked if treatment with GW3965 did reduce intracellular concentration of PIP3. MCF7 cells were treated with GW3965 and the catalytic subunit (p85) immunoprecipitated and used in a functional ELISA based assay to measure its efficiency to convert PIP2 to PIP3. Addition of GW3965 significantly reduced

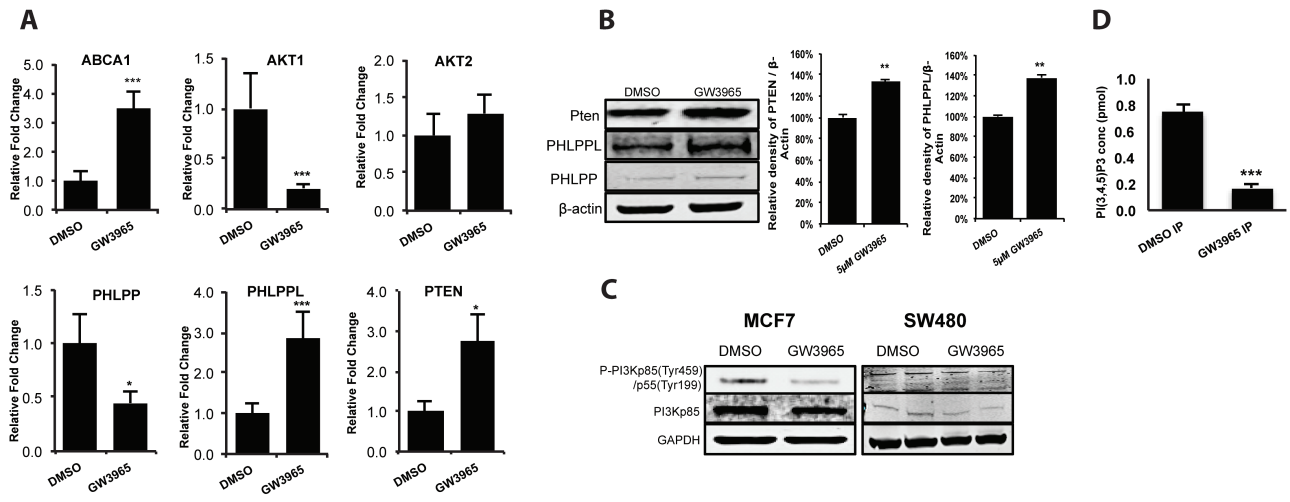

Figure 4: **Activation of LXRs regulates expression of kinases, phosphatases and reduces PI3K signalling. (A)** MCF7 cells were treated with DMSO or 5 μM GW3965 for 16 h. Relative mRNA expression levels of the key LXR target gene (ABCA1) and genes involved in the control of AKT signalling pathway (AKT1, AKT2, PHLPP, PHLPPL, and PTEN) were quantified by qPCR. **(B)** Cell lysates from MCF7 cells were isolated and protein expression analysed by Western blotting for phosphatase (PTEN, PHLPP, and PHLPPL). β-actin was used as loading control. Relative density of the protein's band was quantified as described in material and methods. **(C)** MCF7 and SW480 cells were cultured with 1% serum in growth medium and treated with DMSO or 5 μM GW3965 for 16 h. Cells were activated with IGF-1 (50ng/ml) for 20 min, whole cell extract isolated and Western immunoblotting performed with various antibodies targeting PI3K. GAPDH was used as an internal control demonstrating equal protein loading. **(D)** Inhibition of class IA phosphoinositide 3-kinase (PI3K) activity with 5 μM GW3965 for 16 h measured as phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generated from immunoprecipitated PI3K in a kinase assay using a competitive ELISA method. The data are presented as mean ± SEM. *P< 0.05, **P< 0.01, and ***P< 0.001 versus vehicle by Student's t-test.

the level of PIP3 (Figure 4D). Addition of GW3965 did not change the amount of p85 in the whole cell lysate or the immunoprecipitated fraction (Supplementary Figure 2). This confirmed that the effect of LXR takes place at the beginning of the PI3K-Akt pathway affecting both the activity of PI3K itself and phosphatases that reduce the amount of PIP3 and dephosphorylate Akt.

We next determined if the antiproliferative effect was mediated by LXRα, LXRβ, or both and took advantage of small hairpin RNA (shRNA) and small interfering RNA (siRNA) techniques to knock down expression of LXRs. Expression levels of both LXRα and LXRβ were robustly knocked down as analysed by Western immunoblotting (Figure 5A). Addition of GW3965 inhibited phosphorylation of Akt(ser473) in the presence of shControl and shLXRα, but not in the presence of LXRβ in the MDA-MB-231 cell line (Figure 5B). The importance of LXRβ in phosphorylation of Akt was further established and quantified in the MCF7 cell lines (Figure 5B and C). Proliferation assays in MCF7 cells confirmed that the antiproliferative effect of LXR was abolished when the expression of LXRβ was robustly knocked down (Figure 5D) indicating that LXRβ is the main LXR isoform inhibiting proliferation in human breast cancer cell lines.

4. Discussion

Accumulating evidence indicates that activation of the LXRs inhibits proliferation and tumour growth in various models. We have previously shown that proliferation of several human breast and colon cancer cell lines is significantly inhibited by administration of an LXR agonist [4–6]. Here we continue to investigate the antiproliferative effects of LXRs in depth at the molecular level; particularly with respect to inhibition of growth factor-induced activation of intracellular signalling cascades. Using IGF1 to activate pro-proliferative signalling cascades we showed that activation of LXRs by GW3965 significantly reduced the activity of the PI3K-Akt pathway.

Here we show that LXRs via GW3965 activation reduce proliferation of both breast (MCF7) and colon (SW480) cancer cell lines, but no effects were observed in a hepatocellular cell line (HuH7). In both the MCF7 and SW480 cell lines the antiproliferative effects were associated to reduced activity of the PI3K pathways while this was not observed in Huh7. Specifically the phosphorylation of the p85 subunit of the PI3K and Akt(ser473) was reduced, thereby inactivating these protein kinases. We found that expression levels of protein phosphatases that dephosphorylate PIP3

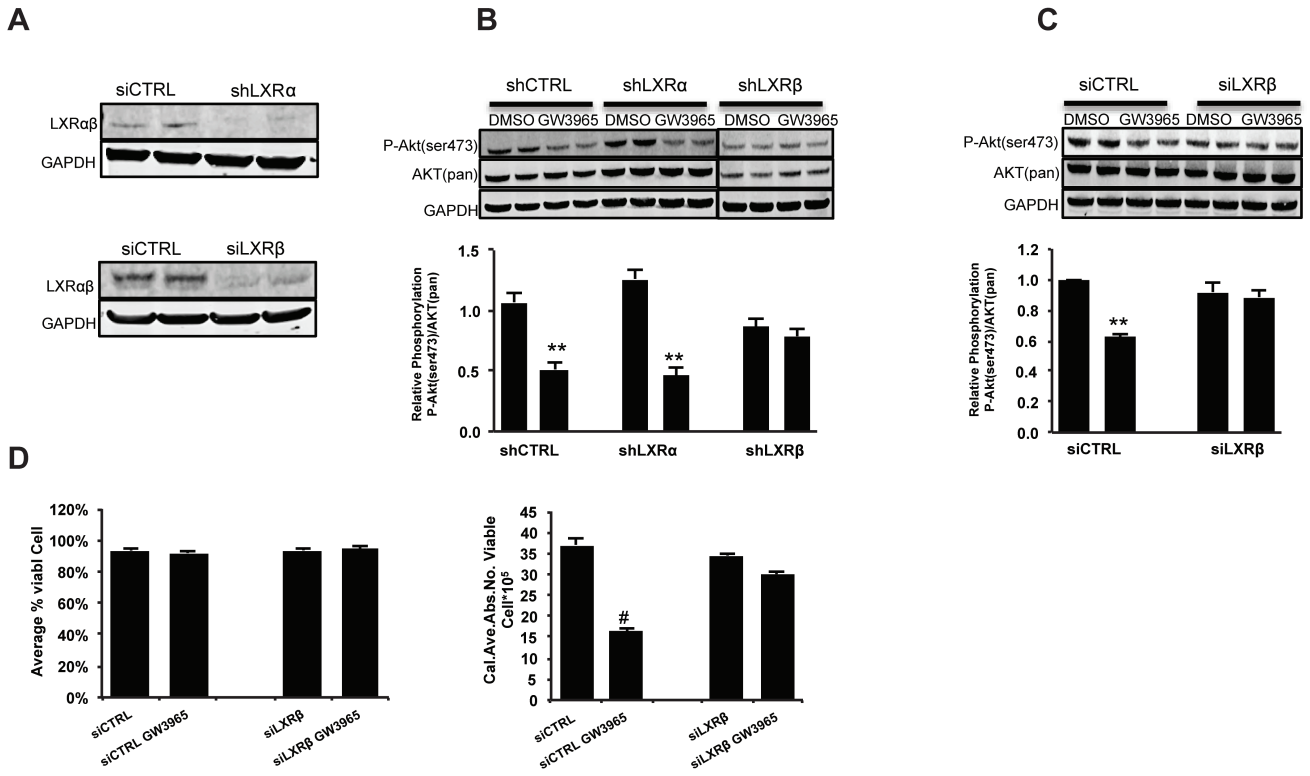

Figure 5: **LXRβ mediates the antiproliferative effect of LXR. (A)** The efficacy of silencing LXRs was analyzed under the condition mentioned in (B and C). **(B)** MDA-MB-231 cells were transduced with LXRα and LXRβ shRNA lentivirus and **(C)** MCF7 cells were transfected with 10 nM siLXRβ, for 48 h, and treated with DMSO or 5 μM GW3965 for 16 h. Cells were given IGF-1 (50ng/ml) for 20 min, whole cell extract isolated and Western immunoblotting performed. GAPDH was used as an internal control demonstrating equal protein loading. The relative level of phosphorylation was quantified as described in material and methods. **(D)** MCF7 cells were treated with 5 μM GW3965 for 48 h, cells were harvested, a viability test was performed, and the absolute number of viable cells was counted. The data are presented as mean $\pm$ SEM. $^{**}P < 0.01$ and $\#\, P < 0.0001$ versus vehicle by Student's t-test.

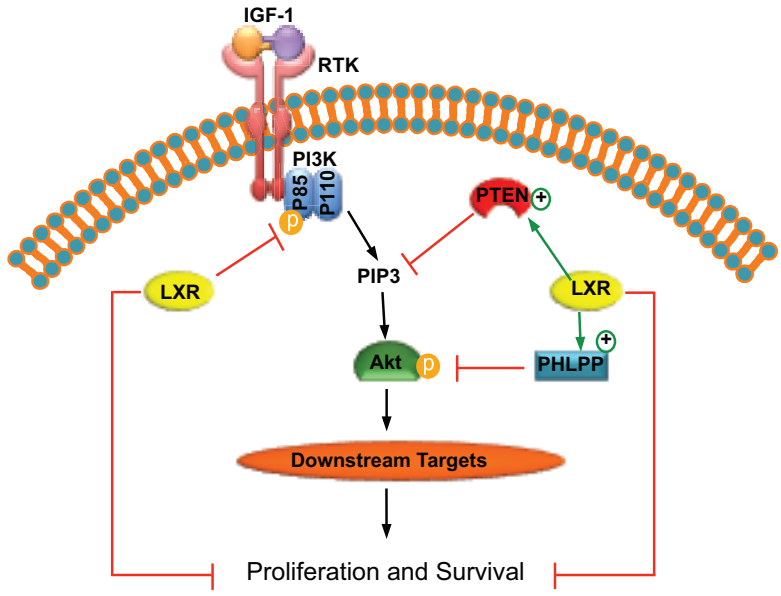

Figure 6: **Schematic presentation of the LXR-effects on the PI3/Akt intracellular signalling pathway.**

to PIP2 (PTEN) and that dephosphorylate Akt (PHLPPL) were induced. Thus, the specific molecular mechanisms responsible for the LXR-mediated antiproliferative effect includes 1) induction of processes that inhibit degradation of PIP3 and 2) suppression of processes that generate PIP3 (depicted in Figure 6). The second messenger PIP3 is vital in mediating proliferative signals from growth factors/growth factor receptors and reducing the level of PIP3 is a powerful mean to inhibit cell growth.

Intriguingly, addition of GW3965 intercepts Akt activation via metabolic pathways that consequently also led to reduced proliferation of prostate cancer cells. Activation of LXRs decreased cholesterol lipid rafts in the cellular plasma membrane in human LNCap prostate cancers cells by induced cholesterol efflux via the ABCG1 cholesterol transporter [22]. The disruption of the lipid rafts led to decreased phosphorylation of Akt and inhibition of Akt survival signalling. Interestingly, the observations made here, as well as findings in previous work in breast and colon cancer cell lines, did not indicate LXR-mediated increase of apoptosis [4–6]. However, LXR-induced apoptosis was reported as the underlying effect of the antiproliferative effects of LXR in other types of cancer including glioblastoma [8] and prostate [22, 23]. Taken together, this suggests that multiple mechanisms exist whereby LXRs execute their anti-proliferative effects.

We observed that GW3965-activation of LXRs significantly reduced the survival of human breast cancer cell lines and inactivated intracellular growth factors pathways. We propose that in breast cancer cells the inactivation of the growth receptor and PI3K-Akt intracellular signalling cascades plays an important role in the antiproliferative effects of LXRs. Hence, modulators of LXR activity could potentially be a strategy for molecular therapy of certain cancers including breast cancer. Data here further suggest that LXRβ is the most potent inhibitor of cell proliferation; thus targeted approaches could be narrowed down to LXRβ-specific modulators. The crystal structure of LXRs revealed a highly flexibility ligand binding pocket, allowing compounds of highly different structure to bind [24]. This further suggests the potential to develop specific small molecule modulators targeting LXRs, giving promise that there are still unexplored opportunities in drug discovery for LXRs.

Conflict of Interest

The authors declare no conflict of interest.

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