Lysyl Oxidase Pro-peptide Inhibits Prostate Cancer Cell Growth by Mechanisms that Target FGF-2-Cell Binding and Signaling

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Abstract

Enhanced RAS signaling and decreased androgen dependence of prostate cancer cells accompany poor clinical outcomes. Elevated autocrine FGF-2 signaling promotes prostate cancer cell growth and survival. Expression of lysyl oxidase (LOX) inhibits RAS transforming activity. LOX is secreted as 50 kDa pro-lysyl oxidase protein and then undergoes extracellular proteolytic processing to form ~30 kDa lysyl oxidase enzyme and ~18 kDa pro-peptide (LOX-PP). We have previously shown that LOX-PP inhibits breast cancer cell transformation and tumor formation, but mechanisms of action of LOX-PP have not been fully elucidated. Here we report that LOX expression is reduced in prostate cancer cell lines and that recombinant LOX-PP protein inhibits serum-stimulated DNA synthesis and MEK/ERK and PI3K/AKT pathways in DU 145 and PC-3 androgen-independent cell lines. In DU 145 cells, treatment with a pharmacologic FGF-receptor inhibitor or a neutralizing anti-FGFR1 antibody mimicked LOX-PP inhibition of serum-stimulated DNA synthesis. FGF-2-stimulated DNA synthesis, ERK1/2, AKT, and FRS2α activation were found all to be inhibited by LOX-PP in DU 145 cells. LOX-PP reduced specific binding of FGF-2 to DU 145 cells, suggesting that LOX-PP targets FGF signaling at the receptor. Interestingly, PC-3 cells did not respond to FGF-2, consistent with previous reports. We conclude that LOX-PP inhibits proliferation of DU 145 cells by interfering with FGFR(s) binding and signaling, and that LOX-PP has other mechanisms of action in PC-3 cells.

Introduction

Prostate cancer is a leading cause of cancer-related deaths in men (Samid *et al.*, 1993). Prostate cancer initially requires androgen for growth and responds to hormone ablation strategies (castration and/or anti-androgen). Disease progresses to a state of reduced hormone dependence for which there is no effective treatment (Weber and Gioeli, 2004).

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RAS signaling is activated in advanced prostate cancer (Erlich *et al.*, 2006). Activation of mitogen activated protein (MAP) kinases via RAS correlates positively with prostate cancer progression and drives androgen independence (Gioeli *et al.*, 1999). A RAS antagonist, farnesylthiosalicylate, suppresses growth of prostate cancer in vivo (McPherson *et al.*, 2004). Activation of RAS signaling is sufficient for progression of androgen dependent LNCaP and CWR22 cells towards androgen independence (Weber and Gioeli, 2004). RAS signaling is highly active in androgen independent DU 145 and PC-3 cell lines (Gioeli *et al.*, 1999) and overexpressed Her-2/neu plays a major role in growth by elevating RAS activity (Kominsky *et al.*, 2000). Activating RAS mutations are rare in prostate cancer (Erlich *et al.*, 2006), suggesting that RAS activation predominantly occurs through growth factor receptor activation (Culig *et al.*, 1994; Planz *et al.*, 2001).

Fibroblast growth factors (FGFs) play an important role in growth and maintenance of normal prostate tissues (Ropiquet *et al.*, 2000). FGFs are produced by stromal cells and contribute to paracrine stimulation of epithelial growth (Giri *et al.*, 1999). In particular, FGF-2 has a major role in prostate epithelial cell proliferation (Ropiquet *et al.*, 1999). FGF-2 antisense studies in prostate cancer cell lines show that FGF-2 is required for cell survival and proliferation (Shain, 2004). Effects of FGFs are mediated by binding to high-affinity cell surface receptors (Forsten-Williams *et al.*, 2005; Johnson and Williams, 1993; Natke *et al.*, 1999; Nugent and Edelman, 1992; Powers *et al.*, 2000). Binding of FGF-2 to its receptors (FGFR1-4) is enhanced by cell surface heparan sulfate proteoglycans and leads to FGFRs autophosphorylation and activation (Johnson and Williams, 1993; Nugent and Iozzo, 2000). Ultimately, activation of FGFRs leads to signal transduction through multiple pathways downstream of activated RAS including ERK MAP kinases, the AKT/ phosphoinositol 3-kinase (PI3K) pathway, and by Fibroblast Receptor Substrate-2α (FRS2α), an FGF pathway-specific mediator (Eswarakumar *et al.*, 2005; Kwabi-Addo *et al.*, 2004; Mohammadi *et al.*, 1991; Schlessinger, 2004; Weber and Gioeli, 2004). Androgen independent DU 145 and PC-3 cell lines express higher amounts of FGFR1 compared to androgen independent LNCaP cells (Nakamoto *et al.*, 1992). Unlike DU 145 cells, however, PC-3 cells are both unresponsive to exogenous FGF-2 and express higher levels of c-MYC (Jones *et al.*, 1997; Nakamoto *et al.*, 1992).

Lysyl oxidase (LOX) enzyme catalyzes the final enzymatic step required for collagen and elastin cross-linking (Kagan and Li, 2003; Kagan and Trackman, 1991). LOX is synthesized as a 50 kDa glycosylated pro-enzyme (Pro-LOX), and then secreted where it undergoes extracellular proteolytic processing by procollagen C-proteinases to functional ~30 kDa enzyme and an ~18 kDa pro-peptide (LOX-PP) (Kagan and Li, 2003; Trackman *et al.*, 1992; Uzel *et al.*, 2001). Generation of LOX enzyme and LOX-PP occurs as a consequence of extracellular post-translational biosynthetic proteolytic processing of secreted Pro-LOX (Trackman *et al.*, 1992).

Expression of the LOX gene was found to inhibit RAS transforming activity and was hence named the "ras recision" gene (rrg) (Contente *et al.*, 1990; Kenyon *et al.*, 1991). Reduced LOX levels were observed in many cancers and cancer-derived cell lines (Contente *et al.*, 1990; Hajnal *et al.*, 1993; Hamalainen *et al.*, 1995; Krzyzosiak *et al.*, 1992; Kuivaniemi *et al.*, 1986). Furthermore, LOX expression is reduced in primary and metastatic prostate

cancers (Ren *et al.*, 1998). We have reported that LOX-PP, and not LOX enzyme, inhibits RAS-dependent transformation of NIH 3T3 cells (Jeay *et al.*, 2003; Palamakumbura *et al.*, 2004). LOX-PP is a potent inhibitor of the transformed phenotype of breast cancer cells (Min *et al.*, 2007). Moreover, LOX-PP expression attenuates growth of breast cancer cells implanted into mice (Min *et al.*, 2007). By contrast, active LOX enzyme promotes invasion by some tumor cells (Kirschmann *et al.*, 2002; Payne *et al.*, 2005) suggesting that LOX-PP may function to control negative effects of LOX enzyme with respect to cancer development.

We report here that LOX-PP inhibits growth responses to serum and to FGF-2 of androgen independent DU 145 prostate cancer cells. A mechanism of action of LOX-PP is shown here for the first time: LOX-PP inhibits FGF-2 binding to DU 145 cells, thereby interrupting autocrine and/or paracrine pathways. Data obtained with PC-3 cells indicates that LOX-PP similarly inhibits serum-stimulated growth responses, but by mechanisms that differ from those in DU 145 cells.

Results

Prostate cancer cells express lower levels of LOX than normal prostate cells

We evaluated <u>LOX</u> expression levels in six different human prostate cancer cell lines and in a control prostate epithelial cell line (PWR-1E). Cells were grown (Bonaccorsi *et al.*, 2004), total RNA isolated, and <u>LOX</u> expression determined by qPCR. All cancer cell lines express less than 10% of <u>LOX</u> mRNA levels compared to PWR-1E prostate epithelial cells (Figure 1A). To evaluate relative LOX protein expression, conditioned media samples were subjected to Western blotting. High levels of ~50 kDa pro-LOX protein (Trackman *et al.*, 1992) were detected in conditioned medium from PWR-1E cells (Figure 1B). In addition, ~18 kDa and ~35kDa protein bands were observed in media from PWR-1E cells (Figure 1B) that correspond, respectively, to glycosylated and non-glycosylated forms of LOX-PP previously seen in conditioned media samples (Guo *et al.*, 2007). No LOX-PP immunoreactive bands were seen in conditioned media samples from any of the six prostate cancer cell lines tested, consistent with findings at the RNA level seen in Figure 1A.

rLOX-PP inhibits growth of prostate cancer cells

We next evaluated the ability of rLOX-PP to inhibit serum-stimulated DNA synthesis of DU 145 and PC-3 cells. rLOX-PP inhibits serum-stimulated thymidine incorporation in both cell lines in a dose-dependent manner compared to controls (Figure 2).

Effects of rLOX-PP on cell cycle regulatory proteins expression was next measured. Serum-stimulated cyclin D1, CDK4, CDK6 and PCNA expression were all decreased by rLOX-PP in a dose-dependent manner (Figure 3). That rLOX-PP inhibits serum-stimulated expression of these proteins further supports the notion that rLOX-PP interferes with proliferative responses in prostate cancer cells.

rLOX-PP inhibits ERK1/2 and AKT signaling

Both DU 145 and PC-3 cells are androgen independent and express high amounts RAS protein. As an index of RAS activity, we determined levels of serum-stimulated phosphorylation of ERK and AKT in the presence and absence of LOX-PP. Data in Figures 4 and 5 demonstrate that serum-stimulated ERK1/2 and AKT phosphorylation is inhibited by rLOX-PP in a dose-dependent manner. These data support the notion that rLOX-PP inhibits growth factor-mediated signaling pathways in these cells.

Serum-induced proliferation of prostate cancer cells and FGF-2 signaling

In prostate cancer, wild type RAS is activated by autocrine and paracrine growth factor regulation (Weber and Gioeli, 2004) often by FGF-2 dependent pathways (Gioeli, 2005; Giri *et al.*, 2001; Ozen *et al.*, 2001). We, therefore, investigated effects of FGF-2 on DNA synthesis of DU 145 and PC-3 cells. FGF-2 increases the DNA synthesis of DU 145 cells and is dose-dependent (Figure 6A). However, PC-3 cells did not significantly respond to exogenous FGF-2, consistent with previous reports (Jones *et al.*, 1997; Nakamoto *et al.*, 1992) (data not shown). Hence DU 145 cells were employed to further investigate effects of rLOX-PP on FGF-2-stimulated DNA synthesis.

We next investigated whether LOX-PP inhibits serum-stimulated proliferation by interfering with FGF-2-dependent signaling in DU 145 cells. PD173074 is an ATP competitive reversible inhibitor of FGFR kinase that attenuates FGFR1-mediated signaling. DU 145 cells cultured in media containing 0.1% FBS were treated with 0 - 200 nM PD173074 for 18 hours. These concentrations of PD173074 are known to specifically inhibit FGF-2 induced proliferation (Bansal *et al.*, 2003). Cells were then stimulated with 10% serum for 6 hours and [^{3}H]thymidine incorporation was determined. As shown in Figure 6B, 100 nM and 200 nM PD173074 inhibit serum-induced DNA synthesis of DU 145 cells, and inhibition is dose-dependent.

To confirm that serum-induced proliferation of DU 145 cells depends mostly upon FGF receptor mediated signaling, neutralizing anti-FGFR1 monoclonal antibody (clone VBS1) was used to block FGFR1 activity. This antibody inhibits FGF-2 induced DNA synthesis in coronary venular endothelial cells by blocking FGF-2 binding to its receptor (Blanckaert *et al.*, 2002). DU 145 cells cultured in 0.1% serum were treated with 0 or 10 μg/ml anti-FGFR1 antibody or control IgG for 18 hours. Cells were then stimulated with 10% serum for 6 hours and [^{3}H]thymidine incorporation was determined. Anti-FGFR1 antibody inhibits serum-induced DNA synthesis (Figure 6C). Blocking of the anti-FGFR antibody by pre-treatment with rFGFR1 diminished its ability to inhibit serum-induced DNA synthesis, confirming the specificity of the anti-FGFR antibody employed (Figure 6C). Separate experiments show that neutralizing FGFR1 antibody fully blocks FGF-2-stimulated DNA synthesis in DU 145 cells, as expected (Figure S1).

rLOX-PP inhibits FGF-2 stimulation of DNA synthesis in DU 145 cells

Effects of rLOX-PP on FGF-2-dependent DNA synthesis of DU 145 cells were investigated. Cells grown in serum-free medium containing 0.1% BSA for 24 hours were treated with increasing concentrations of rLOX-PP, and cells were then treated with FGF-2 followed by

addition of [³H]thymidine in the absence or continuous presence of rLOX-PP for 6 hours. [³H]Thymidine incorporation was then determined. rLOX-PP inhibits FGF-2-stimulated DNA synthesis of DU 145 cells in a dose-dependent manner to levels near non-stimulated control cells (Figure 7). Taken together, results suggest that rLOX-PP inhibits FGF-2 induced proliferative responses in DU 145 cells.

rLOX-PP inhibits FGF-2 induced signaling

We next asked whether rLOX-PP can interfere with FGF-2 induced activation of ERK1/2 and AKT. Cells in serum-free media containing 0.1% BSA were treated with rLOX-PP (0 or 10 μg/ml), and then stimulated with 0 or 10 ng/ml FGF-2 for 5 or 15 minutes. Cell layer proteins were harvested and analyzed by Western blotting. As shown in Figure 8, rLOX-PP strongly inhibits FGF-2 induced ERK1/2 and AKT phosphorylation.

To further confirm that rLOX-PP interferes with FGF-2 induced signaling, effects of rLOX-PP on activating phosphorylations of Fibroblast Growth Factor Receptor Substrate-2α (FRS2α) were determined. FRS2α is an FGF pathway-specific signaling intermediate (Eswarakumar *et al.*, 2005). DU 145 cells grown in serum-free media containing 0.1% BSA were treated with rLOX-PP (0 or 10 μg/ml) followed by 0 or 10 ng/ml FGF-2 for 2 minutes, when FRS2α phosphorylation is maximum. Cell layer proteins were then harvested and analyzed by Western blotting. As shown in Figure 9, rLOX-PP potently inhibits FGF-2 induced FRS2α phosphorylation.

rLOX-PP inhibits binding of FGF-2 to DU 145 cells

Binding of FGF-2 to heparan sulfate proteoglycans facilitates its binding to receptors (Chu *et al.*, 2004; Chua *et al.*, 2004). LOX-PP is rich in arginine and is cationic with a calculated pI of 12.5. Due to its highly basic nature, LOX-PP could potentially interfere with FGF-2 binding to heparan sulfate proteoglycans and/or to FGFRs, and thereby inhibit FGF-2 dependent signaling. Hence, the specific saturation binding curve of FGF-2 to DU 145 cells was determined by competitive binding assays under equilibrium conditions. Cells cultured in medium containing 0.1% BSA for 24 hours were treated with ^{125}I-FGF-2 (0 - 600 pM) for 2 hours at 4°C. Bound ^{125}I-FGF-2 was extracted and quantified using a gamma counter. Nonspecific binding was determined in the presence of excess non labeled FGF-2 (230 nM). FGF-2 binding to DU 145 cells reached saturation at concentrations of 350 pM of total FGF-2 (data not shown), a biologically active concentration that corresponds to 6 ng/ml FGF-2 as seen in Figure 7. This concentration of FGF-2 was then used to determine whether rLOX-PP can inhibit FGF-2 binding to DU 145 cells. Cells were treated with ^{125}I-FGF-2 and rLOX-PP (0 - 10 μg/ml) for 15 minutes, and FGF-2 binding was determined. Data in Figure 10 demonstrates that rLOX-PP inhibits specific binding of FGF-2 in a dose-dependent manner. An IC_{50} of 6.8 μg/ml was obtained from the same data. This value is consistent with concentrations of LOX-PP required for inhibition of serum- and FGF-2-stimulated activities. Taken together, data show that rLOX-PP inhibition of FGF-2 induced signaling and biological activity in DU 145 cells occurs at the level of inhibition of FGF-2 cell binding in DU 145 cells.

Discussion

Prostate cancers typically begin as androgen sensitive lesions but develop into androgen insensitive lesions with progression to advanced stages (Erlich *et al.*, 2006). Increases in autocrine and paracrine growth factor loops correlate with prostate cancer progression and with androgen independence (Culig *et al.*, 1994; Weber and Gioeli, 2004). Increased expression of growth factors including FGF-2, Epidermal Growth Factor (EGF), Transforming Growth Factor-α, (TGF-α), Keratonocyte Growth Factor (KGF), and Insulin Like Growth Factor-1 (IGF-1) and increased expression of their respective cognate receptors are responsible for driving progression to androgen independence (Culig *et al.*, 1994; Planz *et al.*, 2001). This understanding predicts that a variety of signaling events may drive the transformed phenotype of prostate cancer cells. Interactions between different signaling pathways are likely to change as prostate cancer progresses from androgen dependent to androgen independent.

FGF-2, however, is expressed during all stages of human prostate cancer and plays a critical role in prostate cancer progression (Giri *et al.*, 1999; Kwabi-Addo *et al.*, 2004). Hemi- or homozygous inactivation of FGF-2 alleles in mice with transgenic adenocarcinoma of the prostate (TRAMP) by crossing with FGF-2 null mice, have increased survival by inhibiting progression to a poorly differentiated tumor cell phenotype and to metastatic disease (Polnaszek *et al.*, 2003). Higher levels of FGF-2 are expressed by androgen independent DU 145 and PC-3 cell lines compared to androgen dependent LNCaP cells (Cronauer *et al.*, 1997). Hence, it has been suggested that FGF-2 is a major growth factor responsible for increased proliferation and metastasis in prostate cancer cells by paracrine and autocrine pathways.

FGF-2 signaling is an attractive therapeutic target for cancer therapy. Suramin, a compound that can inhibit FGF signaling, was reported to enhance anti-tumor effect of doxorubicin on human prostate cancer (Zhang *et al.*, 2001). Moreover, inhibition of export of FGF-2 by Anvirzel, a novel treatment for cancer, contributes to its anti-tumor activity (Smith *et al.*, 2001). FGF-2 activation of MEK/ERK signaling plays a critical role in cancer cell migration (Hatziapostolou *et al.*, 2006; Wesley *et al.*, 2005). In endothelial cells, FGF-2 increases activation of integrin $\alpha_v\beta_3$ to facilitate cell migration via the ERK pathway (Kwabi-Addo *et al.*, 2004). Moreover, FGF-2 and PDGF synergistically promote tumor angiogenesis and pulmonary metastasis (Nissen *et al.*, 2007). Thus, FGF-2/FGFR signaling can play an important role in invasion and migration of cancer cells. Several different approaches have been taken to inhibit FGFR mediated signaling, such as developing antibodies to target receptors (He *et al.*, 2003) or using small molecule inhibitors that target the catalytic kinase domain (Mohammadi *et al.*, 1997).

Although LOX-PP reduces cancer cell transformation and inhibits RAS-dependent signaling pathways (Jeay *et al.*, 2003; Min *et al.*, 2007; Palamakumbura *et al.*, 2004; Wu *et al.*, 2007), mechanisms by which this occurs have remained largely unknown. Here we show for the first time that LOX-PP inhibits FGF-2-induced signaling in DU 145 prostate cancer cells. Data further indicate that one mechanism by which LOX-PP inhibits cell proliferation is to interfere with binding of FGF-2 to its receptor(s) (Figure 11). Findings obtained in PC-3

prostate cancer cells show that LOX-PP effectively inhibits proliferative responses to serum, but this inhibition occurs by a mechanism that appears not to include inhibition of FGF-2 signaling. LOX-PP has recently been shown to interfere with integrin-mediated haptotaxis and FAK activation that is driven by activated EGF-receptor (HER2) initiated signaling in MDA-MB-231 (human) and NF639 (mouse) breast cancer cell lines (Zhao *et al.*, 2009). Taken together, data support the notion that LOX-PP acts by more than one mechanism to inhibit growth responses of cancer cells. We suggest that the effectiveness of LOX-PP as a tumor suppressor is related to its ability to work by multiple mechanisms of action, rather than by only one mechanism.

Data indicate that LOX expression is low in several prostate cancer cells compared to a normal prostate epithelial cell line (Figure 1). We have previously shown that an FGF-2 autocrine pathway decreases expression of LOX in RAS-transformed NIH 3T3 fibroblasts (Palamakumbura *et al.*, 2003). Thus, increased expression of FGF-2 in DU 145 cells could contribute to low expression of LOX itself, by these and perhaps other, cancer cell lines. It is notable that LOX levels were reported to be diminished in primary human prostate cancer tissues and that LOX expression decreases as a function of prostate cancer progression (Ren *et al.*, 1998). Taken together with data presented here, we suggest that high levels of FGF-2 in prostate cancer tissues help to both down-regulate LOX expression, and therefore LOX-PP levels, and promote tumor cell proliferation (Figure 11).

There are some conflicting reports regarding whether LOX is a tumor suppressor or a tumor promoter. Increased expression of LOX under hypoxic conditions correlates with increased invasiveness of breast cancer tumors (Erler *et al.*, 2009; Erler and Weaver, 2009). β-Aminopropionitrile, a specific irreversible inhibitor for LOX enzyme activity, reduces breast cancer metastasis significantly in mice, suggesting that LOX enzyme, and not LOX-PP, is responsible for increased metastasis (Erler *et al.*, 2006; Erler and Giaccia, 2006; Kirschmann *et al.*, 2002; Payne *et al.*, 2005).

Based on Western blotting data alone, it has recently been suggested that Pro-LOX, and not LOX-PP, is a tumor suppressor (Contente *et al.*, 2009). The present study, and previous work, show directly that LOX-PP is active as an inhibitor of prostate, breast, lung, and pancreatic tumor cell growth and RAS-dependent signaling (Min *et al.*, 2007; Wu *et al.*, 2007). Moreover, LOX-PP expression inhibits breast cancer cell growth in xenografts in vivo (Min *et al.*, 2007). LOX-PP itself, therefore, is clearly active as a tumor suppressor. Data do support that ectopic Pro-LOX expression (that contains the LOX-PP domain) inhibits signaling pathways that contribute to cell transformation (Min *et al.*, 2007; Wu *et al.*, 2007). Processing of Pro-LOX to LOX-PP was, however, not inhibited in these reports, and it is not currently possible to definitively conclude which molecular form of LOX-PP is more active as a tumor suppressor. We suspect, however, that Pro-LOX that appears to be enzymatically inactive (Trackman *et al.*, 1992) and free LOX-PP are both active tumor suppressors. This notion raises the possibility that regulation of extracellular proteolytic processing of Pro-LOX and turnover of LOX proteins may contribute to the respective balance between the tumor suppressor function of Pro-LOX and LOX-PP, and promotion of invasion by active LOX enzyme.

Materials and Methods

Materials

DU 145 and PC-3 and PWR-1E cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Anti-phospho ERK1/2, anti-ERK1/2, anti-phospho AKT (S473), anti-AKT, Anti-β-actin anti-cyclin D1, anti-PCNA, anti-CDK4, anti-CDK6, anti-phospho-FRS2α(Tyr196), anti-phospho-FRS2α(Tyr436) and anti-rabbit secondary antibody were purchased from Cell Signaling Technology (Danvers, MA). Anti-FGFR1 monoclonal antibody (clone VBS1) was purchased from Millipore (Billerica, MA). Sterile 7.5% bovine serum albumin (BSA) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St Louis, MO). Human recombinant FGF-2 was purchased from Peprotech (Rocky Hill,NJ). Cell culture media (Dulbecco's Modified Eagle's Medium (DMEM), F12K, RPMI-1640, or Keratinocytes Serum Free Medium and reagents (penicillin and streptomycin) were purchased from Invitrogen (Carlbad, CA) and ATCC. [^{3}H]thymidine and ^{125}I-Bolton-Hunter reagent were obtained from DuPont NEN (Boston, MA). PD173074, FGF/VEGF receptor tyrosine kinase inhibitor was purchased from Calbiochem (San Diego, CA). Recombinant FGFR1β(IIIc)/Fc Chimera was purchased from R & D Systems (Minneapolis, MN).

Cell culture

Cells were grown in 100 mm cell culture plates as follows, DU 145 in DMEM, PC-3 in F12K, LNCap, CWR22 and 22RV1 in RPMI 1640, and PWR-1E in Keratinocytes Serum Free Medium. All growth media contained 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Cells in logarithmic growth phase were dissociated with trypsin/EDTA, and plated at a desired density. After cells became 75% confluent, they were serum starved for 24 hours and then treated as described in each experiment.

RNA isolation and real time PCR

Total RNA was isolated from PWR-1E, LNCaP, CWR22, 22RV1, DU 145 and PC-3 cell cultures grown in complete media, and purified using RNeasy mini-RNA purification kits (Qiagen). RNA samples (1 μg each) were added to 30 μl of reverse transcription (RT) reactions using random primers and Applied Biosystems Reverse Transcription kit. Thermal cycler conditions for RT were 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min. Two μl of each RT reaction were used per 25 μl of real time PCR (qPCR) reactions. TaqMan probes (Applied Biosystems) for lysyl oxidase (Cat. # Hs00942480_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat. # Hs99999905_m1) were used in qPCR analyses performed in an Applied Biosystems GeneAmp Prism 7500 System. Data were analyzed using the $2^{-\Delta\Delta ct}$ method (Huggett *et al.*, 2005) and mRNA levels were normalized to GAPDH mRNA.

Expression and purification of LOX-PP

Recombinant LOX-PP (rLOX-PP) was expressed and purified to homogeneity from TREX 293 cells as described (Hurtado *et al.*, 2008).

DNA synthesis assay

Cells grown in 12-well plates were serum-starved for 24 hours and then treated according to each experimental condition. Proliferation was induced with serum (10%) or FGF-2 (10 ng/ml) followed by incubation with 2 μCi/ml [^{3}H]thymidine for 6- (for DU 145 cells) or 16- (PC-3 cells) hours. Experiments were terminated by washing cells with ice cold PBS, followed by 10% TCA. DNA was extracted with 0.2 M NaOH, 0.1% SDS (Bonaccorsi *et al.*, 2004). Incorporated radioactivity was measured by liquid scintillation counting.

Western blotting

Proteins were extracted from cell layers with sample buffer (0.1 M Tris-HCl, 4% SDS, 10% glycerol, 5% β-mercaptoethanol). In selected experiments, 24 hour-conditioned serum-free media samples were collected and concentrated using Amicon Ultra concentrators (Millipore). Protein concentrations were determined using Nano Orange assay kits (Molecular Probes). Equal amounts of each sample were then subjected to 10% SDS PAGE and Western blotting as described for each experiment. Signals were quantitated using a digital densitometry system (Versadoc, BioRad).

^{125}I-FGF-2 binding studies

Preparation of ^{125}I-FGF-2 with ^{125}I-Bolton-Hunter reagent was conducted as previously published (Nugent and Edelman, 1992; Richardson *et al.*, 1999). Equilibrium binding was measured in cultures at 4°C in binding buffer (DMEM, low glucose, with 25 mM HEPES, pH 7.2 and 0.1% BSA) (Fannon and Nugent, 1996; Nugent and Edelman, 1992). Cultures were incubated for 2 hours at 4°C with ^{125}I-FGF-2 and rinsed with binding buffer. Bound FGF-2 was released by washing with 1N NaOH and quantified using a Cobra II auto-gamma 5005 counter (Packard Instruments, Meridian, CT). To determine effects of LOX-PP on FGF-2 binding, DU 145 cells were cultured in media containing 0.1% BSA for 24 hours. Cells were then treated with rLOX-PP (0 - 10 μg/ml) for 15 minutes, followed by incubation with ^{125}I-FGF-2 (350 pM) for 2 hours at 4°C. Bound FGF-2 was then released and quantified. Non specific binding was determined in the presence of excess of non-labeled FGF-2 (230 nM).

Statistics

Statistical tests were performed using the Students *t*-test assuming equal variances or one-way ANOVA with Tukey HSD Post Hoc analysis using SPSS software, as indicated in Figure Legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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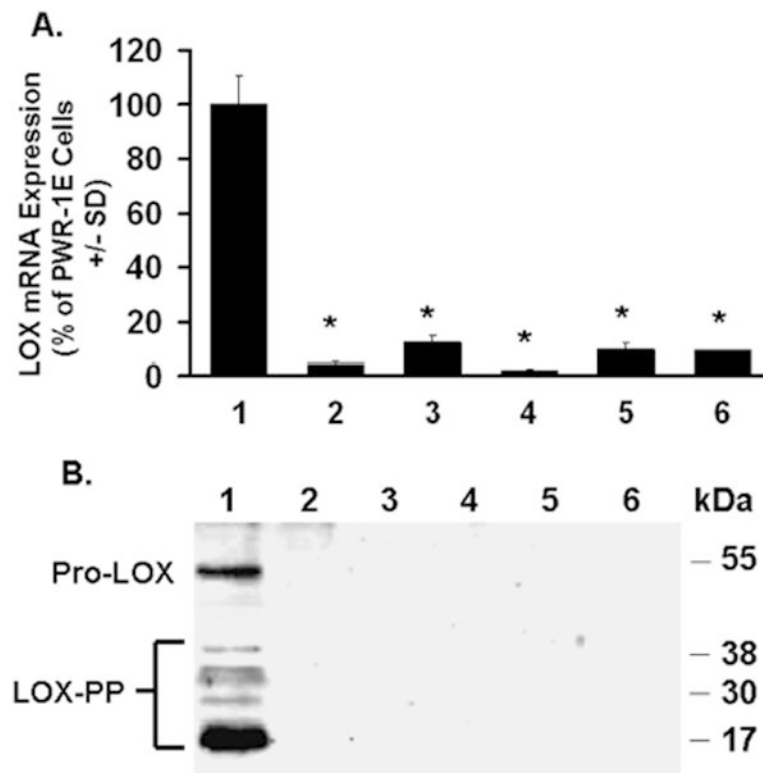
Figure 1. Real time qPCR analysis and Western analyses show reduced LOX expression in prostate cancer cells

Cells were cultured, and RNA and media proteins were isolated as described in "Materials and Methods". (A) Total RNA samples (1 μg) were reverse transcribed and cDNA was subjected to qPCR using Taqman probes (assays On Demand, Applied Biosystems). LOX mRNA levels were calculated relative to GAPDH levels, and RNA isolated from normal prostate epithelial cells (PWR-1E) cells was used as the normal control RNA. Data are expressed as fold change relative to PWR-1E RNA. (*, $p < 0.05$ as determined by ANOVA with Tukey HSD Post Hoc.)

(B) Conditioned serum-free media (24 hour) from confluent cultures of prostate cell lines were collected, concentrated, and protein concentrations determined. Equal amounts of proteins were subjected to SDS PAGE and Western blotting with anti-LOX-PP antibody. Data are from one representative experiment performed twice. Lanes 1-6, PWR-1E, LNCaP, 22RV1, PC-3, DU 145, CWR22 respectively
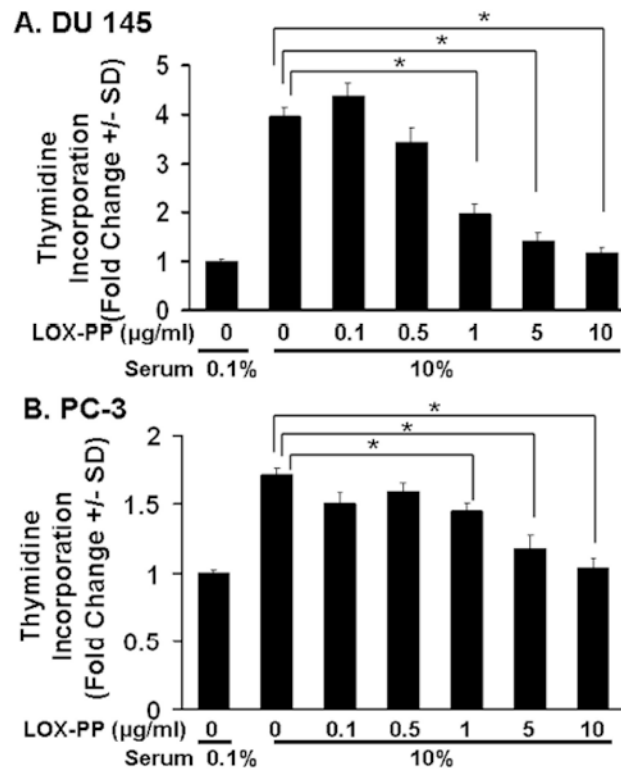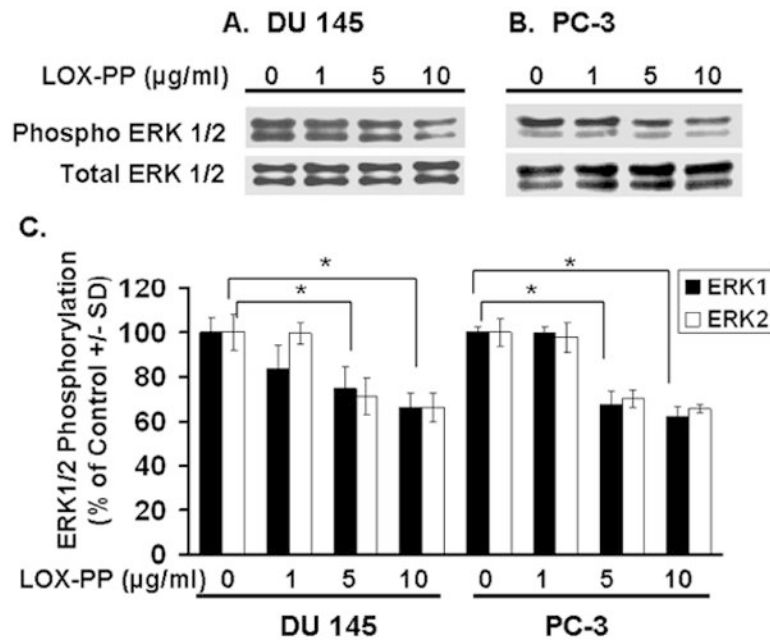
Figure 2. LOX-PP inhibits serum-stimulated DNA synthesis by (A) DU 145 and (B) PC-3 cells
Cells cultured in media containing 0.1% FBS for 24 hours, were then treated with rLOX-PP (0 - 10 μg/ml) for an additional 24 hours. Cells were stimulated with 10% serum and [³H]thymidine for the final 6- (DU 145) or 16 hours (PC-3) of the final 24 hour culture period, and rLOX-PP was continuously present at the indicated concentrations. [³H]thymidine incorporation was determined after TCA precipitation. Values are expressed as fold change in thymidine incorporation relative to non-stimulated control samples lacking rLOX-PP, and represent averages of 3 independent cultures grown at the same time. Data shown are from one representative experiment performed three times with similar results. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc.)
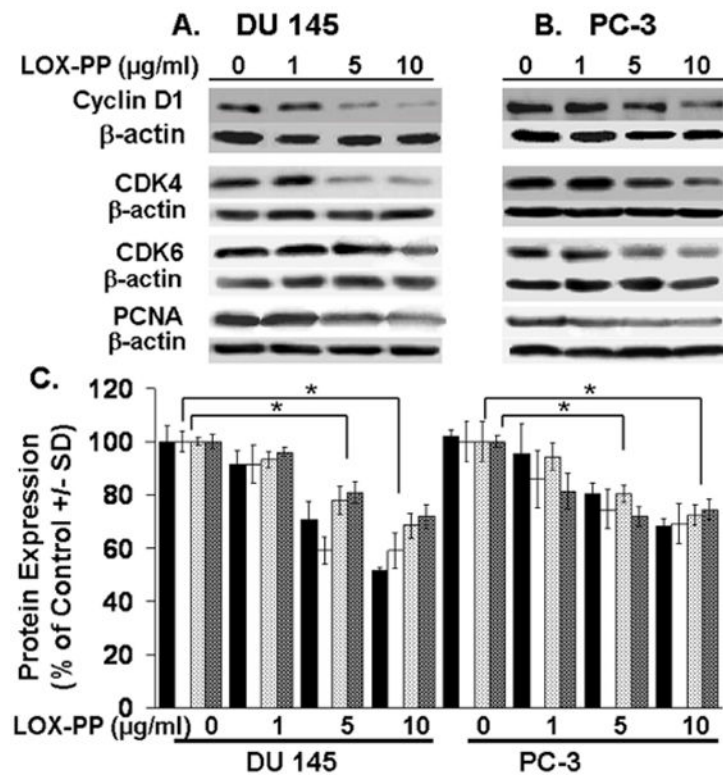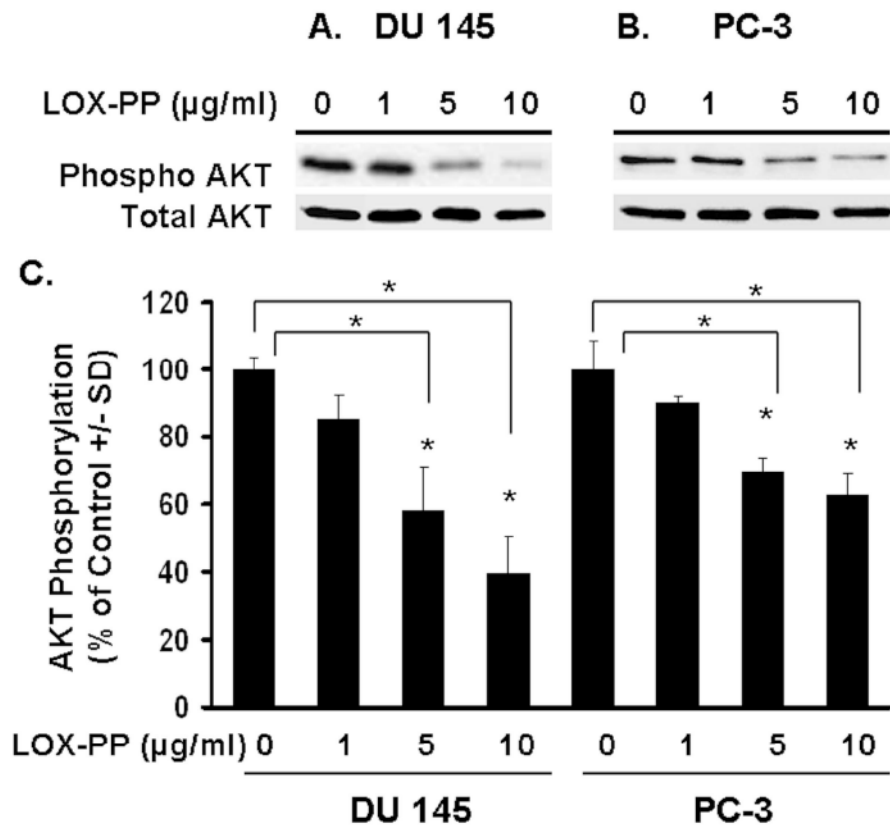
Figure 3. LOX-PP inhibits serum stimulated cyclin D1, CDK4, CDK6, and PCNA expression in DU 145 and PC-3 cells

Cells cultured in media containing 0.1% FBS for 24 hours were treated with 0, 1, 5 or 10 μg/ml rLOX-PP. After 8 hours, cells were stimulated with 10% FBS for 16 hours, followed by extraction of total cell layer proteins. Equal amounts of proteins from DU 145 (A) and PC-3 (B) were subjected to 10% SDS PAGE and Western blotting using anti-cyclin D1, anti-CDK4, anti-CDK6, and anti-PCNA antibody and anti-β-actin antibody for normalization. Results shown are from a representative experiment performed twice with similar results. (C). Densitometric analysis of cyclin D1 (■), CDK4 (□), CDK6 (▦), and PCNA (▨) protein expression normalized to β-actin expression. Values are expressed as percent normalized expression relative to control samples lacking rLOX-PP that were stimulated with 10% FBS (as 100%), and data are combined from 2 independent experiments. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc., n=6).

Figure 4. LOX-PP inhibits serum-stimulated ERK1/2 phosphorylation in DU 145 and PC-3 cells
Cells cultured in media containing 0.1% FBS for 24 hours were treated with rLOX-PP (0, 1, 5 or 10 μg/ml). After 24 hours, cells were stimulated with 10% FBS for 15 minutes, followed by extraction of total cell layer proteins. Equal amounts of proteins from DU 145 (A) and PC-3 (B) were subjected to 10% SDS and Western blotting using anti-phospho ERK1/2 antibody and anti-total ERK1/2 antibody for normalization. Results shown are from a representative experiment; (C), densitometric analysis of ERK1/2 phosphorylation normalized to total ERK1/2 expression. Values are expressed as percent normalized ERK1/2 phosphorylation relative to control cultures lacking rLOX-PP that were stimulated with 10% FBS (as 100%), and data are combined from 3 independent experiments each performed in triplicate. (*, $p < 0.05$ as determined by ANOVA with Tukey HSD Post Hoc.)

Figure 5. LOX-PP inhibits serum-stimulated AKT phosphorylation in DU 145 and PC-3 cells
Cells cultured in media containing 0.1% FBS for 24 hours were treated with rLOX-PP (0, 1, 5 or 10 μg/ml). After 24 hours, cells were stimulated with 10% FBS for 15 minutes, followed by extraction of total cell layer proteins. Equal amounts of proteins from DU 145 (A) and PC-3 cells (B) were subjected to 10% SDS PAGE and Western blotting using anti-phospho AKT antibody and anti-total AKT antibody for normalization. Results in (A) and (B) shown are each from one representative experiment performed three times with similar results; (C), densitometric analysis of AKT phosphorylation normalized to total AKT expression. Values in (C) are expressed as percent normalized AKT phosphorylation relative to control cultures lacking rLOX-PP that were stimulated with 10% FBS (as 100%), and data combined from 3 independent experiments each performed in triplicate. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc.)
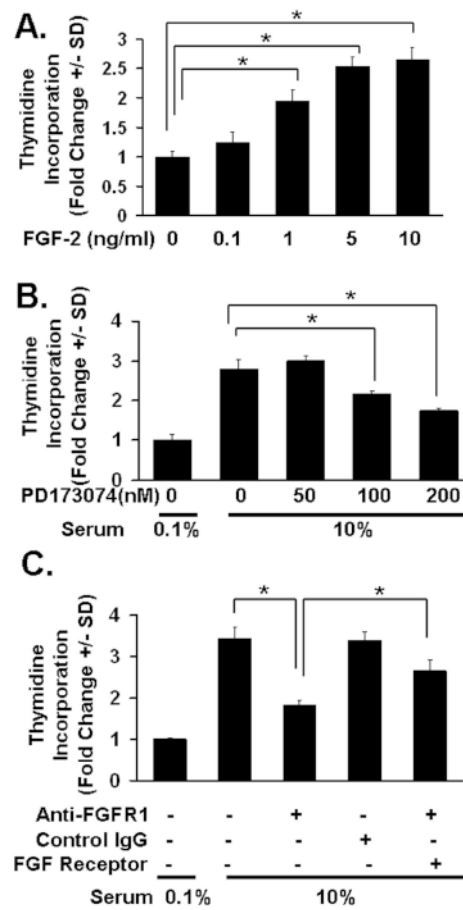
Figure 6. FGF-2 stimulates DNA synthesis of DU 145 cells and serum-induced DNA synthesis depends substantially upon FGF receptor signaling
(A) Cells grown in medium containing 0.1% BSA for 24 hours were treated with 0, 0.1, 1, 5 or 10 ng/ml FGF-2 followed by addition of [^{3}H]thymidine for 6 hours. After TCA precipitation, [^{3}H]thymidine incorporation was determined. Values are expressed as fold change in thymidine incorporation relative to cultures that were not stimulated with FGF-2 and are averages of 3 independent cultures grown at the same time. Data shown are from one representative experiment performed three times with similar results. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc). (B) Cells cultured in media containing 0.1% FBS for 24 hours were treated with 0, 50, 100 and 200 nM PD173074 (FGFR inhibitor) for 18 hours. Cells were then stimulated with 10% serum for 6 hours in the presence of [^{3}H]thymidine. After TCA precipitation, [^{3}H]thymidine incorporation was determined. Values are expressed as fold change in thymidine incorporation relative to cultures which were not stimulated and lacked PD173074, and are averages of 3 independent cultures grown at the same time. Data shown are from one representative experiment performed three times with similar results. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc). (C) Cells cultured in media containing 0.1% FBS for 24 hours were treated with 10 μg/ml anti-FGFR1 antibody or anti-FGFR1 antibody pre-incubated for 30 min with either recombinant FGFR1β(IIIc)/Fc Chimera, or control IgG, for 18 hours. Cells were then stimulated with 10% serum for 6 hours in the presence of
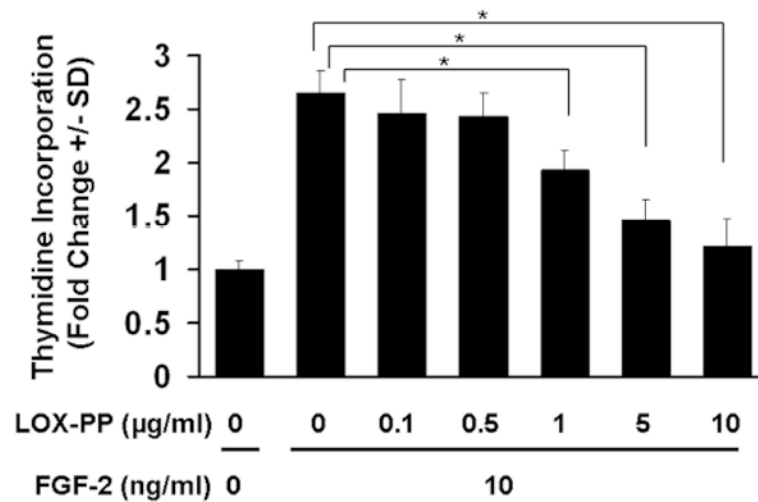
[^{3}H]thymidine. After TCA precipitation, [^{3}H]thymidine incorporation was determined. Values are expressed as fold change in thymidine incorporation relative to cultures which were not stimulated with serum and are averages of 3 independent cultures grown at the same time. Data shown are from one representative experiment performed twice with similar results. (*, p<0.05 as determined by students *t*-test).
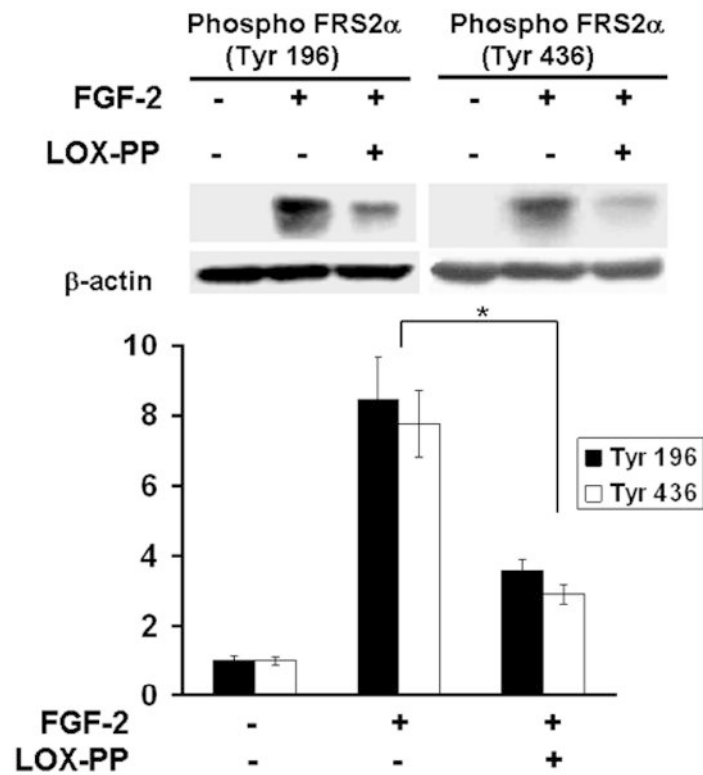
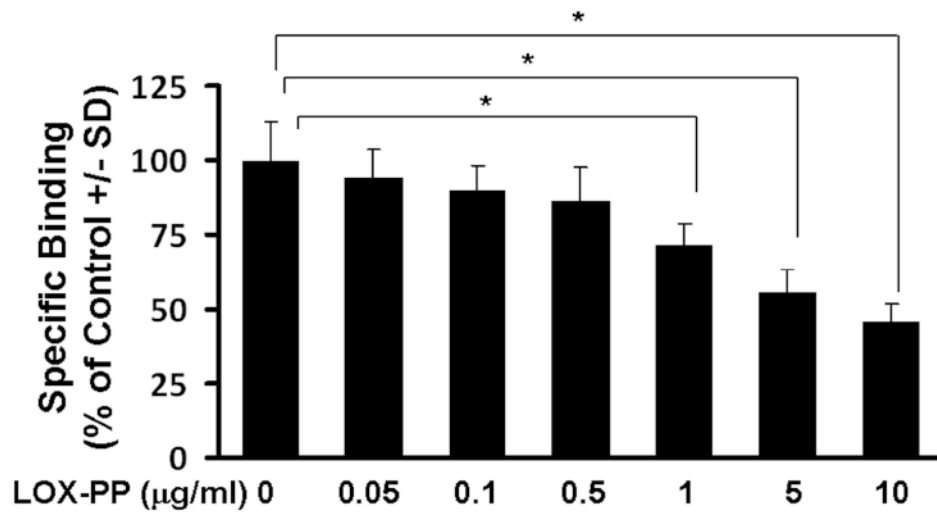**Figure 7. LOX-PP inhibits FGF-2 stimulated DNA synthesis in DU 145 cells**
Cells grown in medium containing 0.1% BSA for 24 hours were treated with 0, 0.1, 0.5, 1, 5 or 10 μg/ml rLOX-PP. After 18 hours, cells were stimulated with FGF-2 (10 ng/ml) followed by addition of [³H]thymidine for 6 hours. After TCA precipitation, [³H]thymidine incorporation was determined. Values are expressed as fold change in thymidine incorporation relative to un-stimulated control cultures lacking rLOX-PP, and are averages of 3 independent cultures grown at the same time. Data shown are from one representative experiment each, performed three times with similar results. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc.)
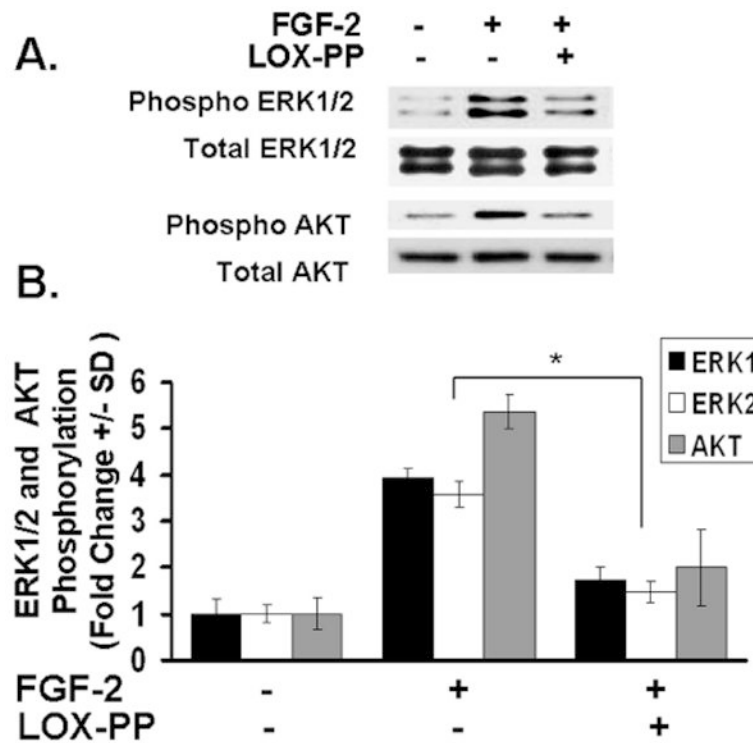
Figure 8. LOX-PP inhibits FGF-2 stimulated ERK and AKT phosphorylation in DU 145 cells
Cells grown in medium containing 0.1% BSA for 24 hours were treated with 0 or 10 μg/ml rLOX-PP. After 24 hours, cells were treated with 10 ng/ml FGF-2 for 5 minutes (for ERK1/2) or 15 minutes (for AKT), followed by extraction of total cell layer proteins. (A) Equal amounts of proteins from DU 145 were subjected to Western blotting using anti-phospho ERK1/2 or anti-phospho AKT antibody and anti-total ERK1/2 or anti-total AKT antibody for normalization. Results shown are from a representative experiment performed three times with similar results (B) Densitometric analysis of ERK1/2 and AKT phosphorylation normalized to total ERK1/2 and total AKT expression respectively. Values are expressed as fold change in normalized ERK1/2 or AKT phosphorylation relative to un-stimulated control cultures lacking rLOX-PP. Data shown are from 3 independent experiments combined each performed in triplicates. (*, $p<0.05$ as determined by student *t*-test).

Figure 9. LOX-PP inhibits FGF-2 stimulated FRS2α phosphorylation in DU-145 cells
Cells grown in medium containing 0.1% BSA for 24 hours were treated with 0 or 10 μg/ml rLOX-PP. After 24 hours, cells were treated with 10 ng/ml FGF-2 for 2 minutes followed by extraction of total cell layer proteins. (A) Equal amounts of proteins from DU 145 were subjected to 10% SDS PAGE and Western blotting using anti-phospho FRS2α (Tyr196) or anti-phospho FRS2α (Tyr436) antibody and anti-β-actin antibody for normalization. Results shown are from a representative experiment performed three times with similar results. (B) Densitometric analysis of FRS2α normalized to β-actin expression. Values are expressed as fold change in normalized FRS2α phosphorylation relative to un-stimulated control cultures lacking rLOX-PP. Data shown are from 3 independent experiments combined, each performed in triplicate. (*, p<0.05 as determined by student *t*-test).

Figure 10. LOX-PP inhibits binding of FGF-2 to DU 145 cells
Cells grown in medium containing 0.1% BSA for 24 hours were treated with LOX-PP (0, 0.05, 0.1, 0.5, 1, 5 or 10 μg/ml) for 15 minutes, followed by incubation with labeled 6 ng/ml FGF-2 for 2 hours at 4 °C. Bound FGF-2 was released by washing with 1M NaOH and quantified using a gamma counter. Non specific binding was determined in the presence of excess non-labeled FGF-2 (230 nM) and subtracted from the original values. Values are expressed relative to the specific binding of cultures which were not treated with rLOX-PP (as 100%), and are averages of 3 independent cultures grown at the same time. Data shown are from 3 independent experiments combined each performed in triplicates. (*, p <0.05 as determined by ANOVA with Tukey HSD Post Hoc).
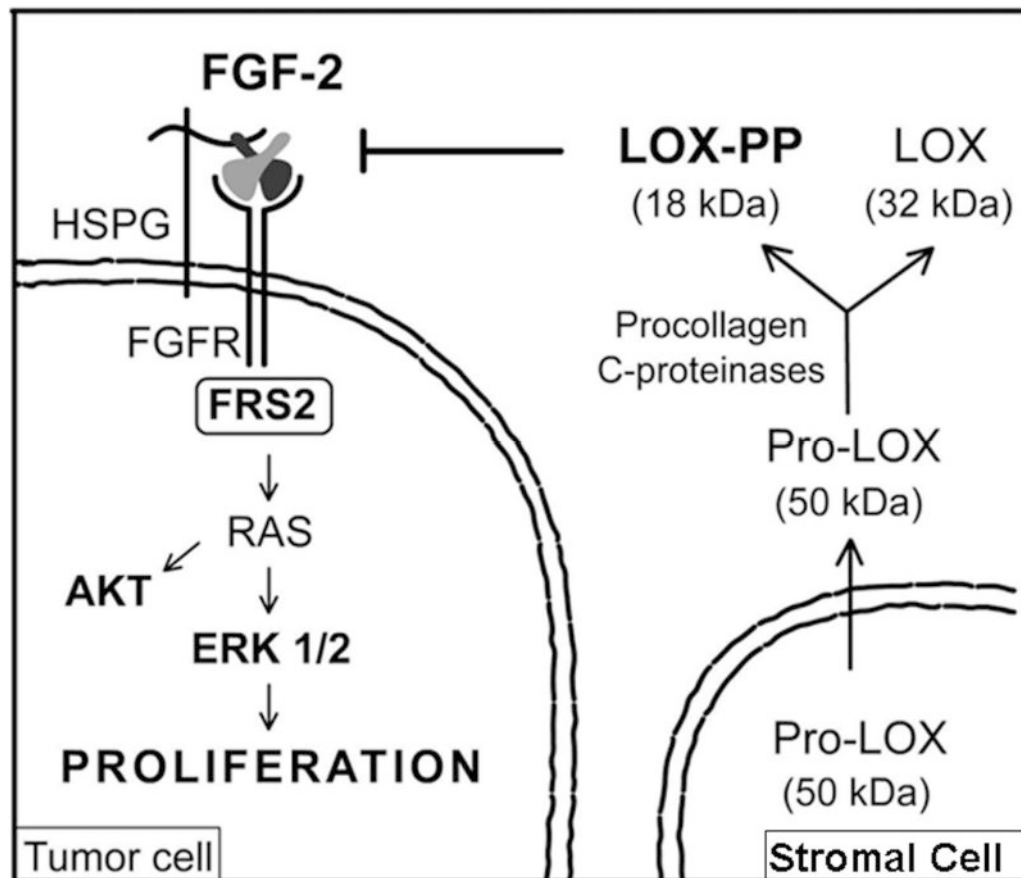
Figure 11. Proposed model for inhibition of FGF-2 signaling and proliferation by LOX-PP in DU 145 cells

FGF-2 autocrine and paracrine signaling is elevated in prostate cancer, and hence FGF-2 signaling is seen to be abnormally high in prostate cancer cells. FGF-2 uniquely phosphorylates and activates FRS2α, followed by RAS, AKT and ERK MAP kinase-dependent signaling resulting in an elevated proliferative response and tumor growth. Pro-LOX is synthesized and secreted as a 50 kDa pro-enzyme from stromal cells or from other non-transformed epithelial cells, or from tumor cells under hypoxic conditions, and levels of Pro-LOX production in prostate cancer epithelium may decrease as a function of prostate cancer progression (Ren *et al.*, 1998). Therefore, as prostate cancer progresses, levels of LOX-PP may decline. LOX-PP, either as free LOX-PP after procollagen C-proteinases processing, and possibly as intact 50 kDa LOX proenzyme, when expressed, inhibits FGF-2 binding and signaling, and FGF-2 stimulated proliferation of tumor cells.