LYSYL OXIDASE INTERACTS WITH HORMONE PLACENTAL LACTOGEN AND SYNERGISTICALLY PROMOTES BREAST EPITHELIAL CELL PROLIFERATION AND MIGRATION

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Lysyl oxidase (LOX), an extracellular amine oxidase, catalyzes the crosslinking of collagen and elastin. LOX has been also shown to play an essential role in promoting invasive and metastatic potential of breast tumor cells. However, the LOX interacting factors in these processes are not known. In this study, we identified placental lactogen (PL), a member of the growth hormone/prolactin hormone family, as a LOX interacting partner using yeast two-hybrid screens. PL is normally only expressed in placental syncytiotrophoblasts, but PL genes are amplified and expressed in high percentage of invasive ductal breast carcinomas. We confirmed LOX-PL interactions using Far-Western and solid phase binding assays. In activity assays, PL was not a substrate or inhibitor of LOX. We further demonstrated that PL is expressed in breast tumor epithelial cells and detected LOX-PL interactions by coimmunoprecipitation in invasive breast cancer cells. In MCF-10A normal breast epithelial cells stably expressing LOX, PL or both, LOX had no effect on cell proliferation, PL alone increased proliferation by 49\%, while coexpression of LOX and PL led to a 121\% increase in cell proliferation. Unlike in tumor cells, LOX did not induce a more migratory phenotype in MCF-10A cells, nor did PL. However, their coexpression resulted in a 240\% increase in cell migration, suggesting that these interactions may be highly relevant to the transition of epithelial cells towards a migratory phenotype during the development and progression of breast carcinoma, and a significant role for LOX-PL interactions in epithelial cell behavior.

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that catalyzes the enzymatic step for generating lysine-derived covalent crosslinks that insolubilize collagen and elastin during the biosynthesis of the extracellular matrix (ECM) (1). Thus LOX plays an essential role in the development and maintenance of connective tissue in the dermis, cardiovascular, respiratory and skeletal systems (2). Recent studies also suggested the implication of LOX in cell motility (3, 4), transcriptional regulation (5), embryonic development (2, 6) and in pathological conditions including cancer. Increases in the expression and enzymatic activity of LOX has been demonstrated in highly invasive cutaneous and uveal melanoma (7), invasive and metastatic breast cancer cell lines (7, 8) and metastatic breast tumors (9). In addition, overexpression of the catalytically active LOX in non-invasive and poorly metastatic breast cancer cell lines induced an invasive cell phenotype (9) and increased the metastatic potential of LOX expressing cells in a mouse model for breast cancer (10).

Human lysyl oxidase is synthesized by diverse cell types as a 48 kD prepro-enzyme that is secreted into the extracellular space following glycosylation. In the ECM, the glycosylated proLOX is activated through the cleavage of the N-terminal propeptide by the C-terminal procollagen protease/bone morphogenic protein-1 (BMP-1), yielding a 30 kD active enzyme (2, 6, 11, 12). While
the proteolytic activation of LOX and its matrix cross-linking activity has been extensively studied, its significance in pathological conditions is less understood. Consequently, questions have arisen regarding the regulation of LOX activation, potential new substrates, and protein interactions in these processes. To elucidate the factors that might play a role in these molecular mechanisms, we focused on LOX-protein interactions and identified human placental lactogen as a potential interacting partner of LOX from yeast two-hybrid screens.

Human placental lactogen (PL) is a member of the growth hormone/prolactin peptide hormone family that includes growth hormone (GH), prolactin (PRL) and placental lactogen. PL is produced by the placental syncytiotrophoblasts as a single chain polypeptide hormone of 191 amino acids, that has been shown to form biologically active homodimers in vivo (13-15). In humans, PL is only expressed in the placenta during pregnancy and it replaces the downregulated pituitary PRL, as the placental growth hormone (GH-V) replaces the downregulated pituitary GH (16). Human placenta-derived PL stimulates mammary gland development, lactogenesis and the growth and metabolism of the fetus (17). Three PL genes have been identified within the growth hormone gene cluster on chromosome 17, PL-1 (also named PL-L or CS-L), PL-4 (PL-A or CS-A) and PL-3 (PL-B or CS-B) (18, 19). PL-3 and PL-4, but not PL-1, contribute to the mature hormone production (20), and the secreted protein products of these genes are identical (21).

PL has been implicated in breast cancer, although its significance remains unexplored. All three PL genes have been shown to be amplified in 22% of breast carcinomas (22). PL expression was demonstrated within tumors in 77% of the cases in this panel and another study reported PL expression in 82% of breast tumors (23). In addition, a trend was noted towards an increased incidence of lymph node metastases with amplification of the PL genes (22).

The two other members of this hormone family, GH and PRL, are potent oncogenes acting through their receptors, and PL exerts its effects via the same receptors. PL binds to the GH receptor (GHR) with lower affinity than GH (24) despite their significant (85%) amino acid sequence homology (25). PL has only 23 %, amino acid sequence homology to PRL (25), but binds to the PRL receptor (PRLR) with a higher affinity (24, 26). The PRL receptor mediates the growth and differentiating hormone effect of PRL in the breast. In breast cancer cells, stimulated PRLR activates signaling cascades associated with cytoskeletal alterations and enhances membrane ruffling, and cell motility, events that have been associated with the progression of mammary carcinoma in vivo (27). Epidemiologic evidence, indeed, supports a role for PRL in the pathogenesis (28), progression (29) and poor prognosis of breast carcinoma (30, 31). Although PL is expressed in a high percentage of breast carcinomas and can bind to PRLR, its expression and function in breast cancer cells has not been investigated.

In this study, we describe the expression of PL in breast cancer cell lines and report a yeast two-hybrid screen that identified PL as an interacting partner of LOX. In subsequent experiments we characterize LOX-PL binding affinity and binding site within LOX, and confirm this interaction in invasive breast tumor epithelial cells. To address the mechanism and significance of potential LOX-PL interactions, we analyze cell proliferation and migration using MCF-10A normal breast epithelial cell lines stably expressing LOX, PL or both. Our results support a proliferation inducing function for PL in normal epithelial cells, and furthermore, suggest a potential role for LOX-PL interactions in breast epithelial cell proliferation and migration.

**EXPERIMENTAL PROCEDURES**

*Antibodies and proteins used* - Polyclonal anti-PL antibody, polyclonal anti-GH antibody and monoclonal anti-PRL antibody were purchased from Novocastra Laboratories, Chemicon International and Upstate Biotechnologies, respectively. Monoclonal anti-glutathione S-transferase (GST) antibody was purchased from Upstate Biotechnology. Polyclonal anti-LOX antibody, which recognizes both the full-length and the processed forms of human LOX was previously characterized (32). Purified PL, GH and PRL proteins were purchased from the National Hormone and Peptide Program. Purified bovine aorta LOX (bLOX) was generously provided by Dr. Herbert Kagan, Boston University (33).
**Immunofluorescent microscopy** - Fixed human breast cancer tissues with corresponding normal tissues (AccuMax Array A712(I), ISU Abaxis Co.) were obtained. Tissue sections were deparaffinized by incubation at 65 °C for 20 min and immersion in xylene three times for 15 min each. Rehydration of the tissue sections was performed in decreasing concentration ethanol series. The sections were washed in PBS and in 0.1% Triton X-100 in PBS for 10 min each, and blocked in 5% normal goat serum in PBS for 30 min. The sections were then incubated with the primary antibodies in 5% normal goat serum in PBS for 2 h, washed in PBS for 15 min, blocked in 5% normal goat serum in PBS for 10 min, incubated with fluorescently labeled secondary antibodies for 30 min, and then incubated with To-PRO-3 nuclear stain (Molecular Probes) for 15 min. After mounting the samples with Vectorshield mounting medium, a Zeiss LSM Pascal confocal microscope was used for imaging.

**Cell lines and culture conditions** – Immortalized mammary epithelial MCF-10A cells and breast cancer cell lines Hs578T, MDA-MB-231, MCF-7 and T47D were kindly supplied by Dr. Dawn Kirschmann (Department of Anatomy and Cell Biology, University of Iowa) and were maintained as previously described (34, 35). Expression of PL and LOX in the tumor cell lines was evaluated by Western analysis of the cell lines’ conditioned cell medium (CCM). Briefly, at ~70% confluency, the cells were washed with PBS, and incubated in serum-free, phenol red-free medium for 48 h. The CCM was collected, and the protein concentration was measured using Bradford reagent (Biorad). To a volume of CCM containing 20 μg of proteins, 10 μl of Strataclean Resin (Stratagene) was added and mixed at RT for 10 min to bind the proteins (36). After centrifugation and the removal of the supernatant, the resin was resuspended in 10 μl of 2x Laemmli buffer, and the samples were boiled for 5 min before electrophoresis by SDS-PAGE.

**Yeast two-hybrid screen** - A yeast two-hybrid screen was performed according to Clontech’s System 3 protocol using a human placental cDNA library (Clontech), which contained over 3.5 x 10^6 independent clones generated from human placental mRNA with oligo-dT primers described previously (37). Primers were designed to introduce restriction sites 5’ and 3’ to sequence-verified human LOX cDNA in order to clone Clontech’s pGBK7T7 vector downstream and in frame with the GAL4 DNA-binding domain (GAL4-DB). Several LOX expression constructs were designed and cloned, including the full length proLOX (amino acids 1-417), the mature LOX (amino acids 169-417), the LOX propeptide (amino acids 1-168), the cytokine receptor-like (CRL) domain (amino acids 349-417), the proLOX without the CRL domain (amino acids 1-348) and the mature LOX without the CRL domain (amino acids 349-417). The finished constructs, pGBK7-LOX1_417, pGBK7-LOX169-417, pGBK7-LOX1_168, pGBK7-LOX349-417, pGBK7-LOX1_348, pGBK7-LOX169-348, were each verified by DNA sequencing. Similar cloning was done to create yeast two-hybrid bait constructs for human lysyl oxidase-like (LOXL) and lysyl oxidase-like 2 (LOXL2) genes, which resulted in the constructs pGBK7-LOXL26_574 (amino acids 26-574), pGBK7-LOXL-Nterm26_368 (amino acids 26-368), pGBK7-LOXL-Cterm338_574 (amino acids 338-574), pGBK7-LOXL2-Nterm1_547 (amino acids 1-547), and pGBK7-LOXL2-Cterm548_774 (amino acids 548-774).

The library was transformed into AH-109 yeast that contained pGBK7-LOX1_417 or pGBK7-LOX169-417, and clones containing interacting proteins were selected based on activation of the three reporter genes HIS3, ADE2, and lacZ. The library plasmids were isolated from the positive yeast clones and the cDNA inserts were sequenced. The interactions of the identified positive clones were further characterized by performing direct cotransformations into AH109 yeast of various combinations of LOX, LOXL, and LOXL2 deletion constructs with the positive clones and with parallel empty vector controls. These direct cotransformation assays also used selection based on HIS3 and ADE2 reporter gene expression.

**Bacterial expression of GST-LOX** - The plasmid pGEX-4T-1 (Amersham) was used to generate expression construct for the mature LOX containing an N-terminal glutathione S-transferase (GST) tag, as described previously (37). Briefly, the pGBK7-LOX169-417 plasmid was digested with EcoRI and Sall, and the LOX insert was ligated into the pGEX-4T-1 vector downstream and in frame with the GST tag. The pGEX-4T-1-LOX
expression plasmid was then transformed into the BL21 strain of E. coli (Stratagene), in parallel with the empty pGEX-4T-1 vector to use for GST-only negative controls. GST-LOX fusion protein expression was induced by adding 0.1 mM isopropyl beta-D-1-thio-galactopyranoside (IPTG) to growing cultures and shaking for an additional 2 hours at 37°C. Recombinant proteins were extracted from inclusion bodies using a solubilization buffer (8 M urea, 10 mM K$_2$HPO$_4$, 5 mM DTT, pH 8.2), filtered through a 0.45 µm syringe filter, and refolded with a rapid dilution into 10 mM K$_2$HPO$_4$ buffer (pH 8.2). GST-LOX fusion proteins were captured and purified using Glutathione Sepharose 4B (Amersham), and then eluted using the inclusion body solubilization buffer and refolded again. Protein concentrations were measured using the Bradford reagent (Biorad).

**Far-Western experiments** - Our protocol was based on the modified protocol for Far Westerns from the Molecular Cloning Manual – Protein-protein Interactions (38). Equal volumes of the purified GST-LOX$_{169-417}$ construct and GST only as a control were resolved by SDS-PAGE and blotted onto a pre-treated Immobilon-P membrane (Millipore Corp., Bedford, MA). To refold the blotted proteins, we incubated the membrane at room temperature (RT) with refolding buffer R6 (refolding buffer: 20 mM HEPES, pH7.7; 25 mM NaCl; 5 mM MgCl; 1 mM DTT with 6 M guanidinium hydrochloride added) for 1 h; then with refolding buffer R4 (containing 4 M guanidinium hydrochloride) and with refolding buffer R2 (with 2 M guanidinium hydrochloride) for 15 min each, and finally with refolding buffer R1 (containing 0.187 M guanidinium hydrochloride) for 30 min. The membrane was incubated in refolding buffer R1 overnight at 4°C with gentle shaking and then blocked with 5% Carnation non-fat dry milk in PBST (1x PBS with 0.1% Tween-20) for 1 h at RT. The membrane was then blocked with 3 µM reduced glutathione in 2.5% Carnation non-fat dry milk in PBST for 1 h at RT. The membrane was washed with PBST and incubated with 5 µg/ml of the target protein (PL) in PBST for 1 h at 4°C. After washing, the membrane was incubated with anti-PL primary antibody, then with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody for 1 h at RT. We used ECL reagents (GE Healthcare) for HRP detection.

**Solid phase binding assay** - To determine the binding affinity of LOX to PL, GH, and PRL, solid phase binding assays were performed as previously described (37, 39). Wells of high protein-binding EIA/RIA microplates (#3590; Corning) were coated with purified PL, GH and PRL proteins at 200 nM in PBS overnight at 4°C. After aspirating the liquid, the wells were blocked with 1% BSA in PBS for 3 hr at 37°C. After removing the blocking solution, wells were washed three times with 0.1% BSA in PBS. Purified soluble GST-LOX protein was added to the wells at various concentrations (0-5000 nM) in 10 mM K$_2$HPO$_4$ (pH 8.2) and incubated overnight at 4°C. The wells were then washed three times with PBST and bound ligand was reacted with anti-GST (Upstate Biotechnology) primary antibody and then detected with peroxidase-labeled secondary antibody (Amersham). Peroxidase activity was measured using the QuantaBlu Fluorogenic Peroxidase Substrate Kit (Pierce Biotechnology), with readings performed by a POLARStar Optima microplate reader (BMG Labtechnologies). All measurements were performed in triplicates. The dissociation constants were calculated using nonlinear regression analysis performed by Graphpad Prism4 statistical software (Graphpad, Inc.).

**Coimmunoprecipitation** - For immunoprecipitation experiments CCM from cultured Hs578T breast cancer cells was collected from cultures grown under serum free conditions for 2 days post-confluence, and the CCM was concentrated using Amicon Ultra tubes (Millipore). After preclearing the CCM by incubating it with 20 µl TrueBlot anti-rabbit IgG beads (eBioscience) for 30 min at 4°C, 3 ml of the sample was incubated with 3 µg of the immunoprecipitating antibody (anti-LOX or anti-PL) at 4°C overnight. An equal amount of purified rabbit IgG (Jackson Immunoresearch) was used as a negative control to test the specificity of the immunocomplex formation. The formed immunocomplexes were captured by addition of 50 µl of TrueBlot anti-rabbit IgG beads and incubation for 1 h at 4°C. The beads were then collected by centrifuging at 4000 rpm for 10 min at 4°C, and after taking aliquots of the flow-through for further analysis the beads were washed 3 times in PBST and resuspended in 2x Laemmli Buffer. The immunoprecipitated samples were analyzed by Western blotting.
LOX activity assays - The LOX enzyme activity was measured using the Amplex Red fluorescence assay (40) which was adapted for use in microplate format and described previously (37). The assay reaction mixture consisted of 50 mM NaBorate (pH 8.2), 1.2 M Urea, 50 µM Amplex Red (Molecular Probes, Inc.), 0.1 U/ml horse-radish peroxidase (HRP, Sigma), and 10 mM 1,5-diaminopentane substrate (Sigma). To measure if LOX activity is affected by binding to PL, 10 pmol of purified bLOX was incubated with an equal molar amount of purified PL for 1 hr at 4°C. To measure if PL is a substrate for LOX, we replaced the 1,5-diaminopentane in the reaction mix with various amounts of purified PL and measured the activity of 10 pmol of purified bovine LOX. The protein samples were added to the reaction mix in the presence or absence of 500 µM of the LOX inhibitor beta-aminopropionitrile (BAPN, Sigma), and the reactions were then incubated in a POLARStar Optima microplate reader at 37°C. The fluorescent product was excited at 560 nm and the emission was read at 590 nm. The LOX enzyme activity was calculated as the increase of fluorescent units over time (60 min) above the BAPN controls. All samples were assayed in triplicate to minimize experimental error. Statistical analysis was performed using Graphpad Prism4 statistical software.

Lentiviral constructs - PL and LOX were stably expressed in mammalian cells using the ViralPower™ Lentiviral Expression System (Invitrogen) according to the manufacturer’s protocol. Forward PCR primers were designed to contain the additional 5’CACC-3’ bases necessary for directional cloning on the 5′ end, while the reverse primers were designed to contain the native stop codon. The primers used to amplify the human PL sequence were: 5’ - CAC CAT GGC TCC AGG CTC - 3’ (forward) and 5’ - CTA GAA GCC ACA GCT GCC CT - 3’ (reverse). The primers designed to amplify LOX were: 5’- CAC CAT GCG CTT CGC CT - 3’ (forward) and 5’ - CTA ATA CGG TGA AAT TGT GCA GCC TG - 3’ (reverse). For the LOX template we used a previously-cloned expression vector pcDNA3.1-LOX, and for the PL-4 template, we used a positive clone isolated by the yeast two-hybrid screen. Both template plasmids were sequence verified. DNA fragments were amplified by PCR, in a total volume of 50 µl, which consisted of 1x PCR Buffer, 125 µM dNTP, 0.5 µM of each primer, 1 U of DeepVent polymerase (New England Biolabs, Inc.), and 200 ng of template DNA. The PCR cycling consisted of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and elongation for 1 min at 72°C, which was repeated 30 times. The first cycle was initiated with a denaturation step for 5 min at 95°C. The amplified fragments were then cloned into the pLenti6/V5 lentiviral expression vector using the pLenti6/V5 Directional TOPO Cloning Kit (Invitrogen), according to the manufacturer’s protocol.

Lentiviral transduction - HEK293FT human embryonic kidney epithelial cells (Invitrogen), used as hosts for the production of the designed lentiviruses, were maintained as recommended by the manufacturer. These cells were cotransfected with the pLenti6/V5-PL-4 or pLenti6/V5-LOX lentiviral DNA constructs and Viralpower Packaging Mix using Lipofectamine 2000 (Invitrogen) as a transfection reagent. Virus-containing supernatants were collected after 72 hours of transfection. MCF-10A cells were stably transduced with lentiviruses containing pLenti6/V5-PL-4, pLenti6/V5-LOX and as a positive control pLenti6/V5-GW/lacZ, according to the manufacturer’s recommendations (Invitrogen). The transduced cells were selected with 6 µg/ml Blasticidin (Invitrogen) and clonal selection was performed using Quixell™ Cell Selection and Automated Transfer System (Stoelting Co.). Expression of PL and LOX in the stably transduced MCF-10A cell lines was evaluated by Western analysis. Individual clones that were determined to have the highest levels of expression of PL and LOX were selected for further experiments.

Cell proliferation assay – Cells were seeded in two parallel 96-well plates in triplicates at 2000 cells per well. Cells were allowed to attach for 4 hours at 37°C, and then washed with 1x PBS to remove the unattached cells. The medium was replenished on one of the plates, and these cells were grown for 72 h at 37°C. The cells on the other plate were fixed in 95% ethanol, stained with methylene blue, washed, and lysed with 1% sarkosyl to solubilize the dye. After 72 h the same staining procedure was performed on the second plate. Colorimetric absorbance was measured at 620 nm using a POLARStar Optima microplate.
PL and LOX expression in breast tumors on tissue macroarrays using PL and LOX antibodies. All five tumor samples we analyzed were LOX positive, and of these, three stained for PL. In these tumors, both LOX and PL were detected within and surrounding tumor cells (Fig. 1A, B). To test if cultured breast tumor cell lines also express PL, total protein from preconfluent culture media was extracted and analyzed by immunoblotting. Figure 1C shows the secretion of PL by the invasive/metastatic cell lines Hs578T and MDA-MB-231, the non-invasive/poorly metastatic MCF-7 cell line, and a small amount by T47D cells. PL from cell culture media was detected as a 42 kD band, which is consistent with the molecular weight of a homodimer as previously reported (14). As a positive control for the immunoblots, cell medium protein was used from the PL-expressing choriocarcinoma cell line, JAR. On Western blots of media fractions of these highly malignant invasive cells, we also detected abundant amount of the processed 30 kD LOX (not shown).

Identification of placental lactogen as a LOX-interacting protein - To identify possible LOX-interacting proteins and novel mechanisms of LOX functions, we had previously performed a yeast two-hybrid screen. The expression of the lysyl oxidase gene family is high in placental tissues, and we anticipated that using a placental library would be optimal for discovering proteins with LOX-binding ability. Two independent screens of a human placental yeast two-hybrid library were performed with the full length 48 kD proLOX and the 30 kD mature LOX as baits. After transformation of pGBK7-LOX1-417 and pGBK7-LOX169-417 into yeast strain AH109, expression of the fusion proteins GAL4-BD-LOX1-417 and GAL4-BD-LOX169-417 were confirmed by Western blot (data not shown). Assays for expression of the reporter genes ADE2, HIS3, and lacZ showed no autonomous transcriptional activity by GAL4-BD-LOX1-417 or GAL4-BD-LOX169-417 alone. Subsequently, the placental cDNA library was transformed into the yeast strain containing pGBK7-LOX1-417 or pGBK7-LOX169-417, and the cells were plated to screen for expression of the 3 reporter genes. Yeast colonies that expressed all three reporter genes were isolated and the library insert cDNA was sequenced.
From the yeast two-hybrid screen with proLOX (LOX\textsubscript{1-417}), 7 of the 20 positive clones sequence-identified were PL cDNAs. From the screen with LOX (LOX\textsubscript{169-417}), 9 of the 39 positive clones sequence-identified were PL cDNAs. Most of the positive clones encoded the entire pro-PL protein of 217 amino acid residues, and all were identified as PL-4 transcript variant 1. The expression of PL-4 and PL-3, which result identical mature hormones, is initiated and upregulated in placental syncytiotrophoblasts during pregnancy, with the highest expression levels at term (19, 43). PL-4 gene’s transcript variant 1 that contains all five exons of the gene encodes isoform 1, the most abundant full-length isoform of the hormone (44).

To verify that the binding of PL was specific to LOX, and to further test which domain of LOX contains the PL binding site, several combinations of bait and target plasmids were cotransformed into yeast that were spotted on nutritional dropout plates to test reporter gene activation. Yeast were also cotransformed with combinations of LOX or PL proteins with empty plasmid vectors as negative controls. A non-interacting bait protein, nuclear lamin C, was also used as a negative control to show specificity of the PL interaction. Only the yeast cells that contained fragments of LOX or proLOX that interacted with PL were able to grow on the nutritional dropout plates (Fig. 2A,B). The bait constructs activating the reporter genes were proLOX (LOX\textsubscript{1-417}), the mature protein (LOX\textsubscript{169-417}), and deletion constructs LOX\textsubscript{1-348}, LOX\textsubscript{169-348}, LOX\textsubscript{169-417}, as illustrated in Figure 2. These data suggested that the interaction between LOX and PL was the result of specific binding, and that the possible PL-binding site of LOX is located between the amino acids 169-348. The LOX cytokine receptor-like (CRL) domain alone (amino acid residues 349-417), despite it’s amino acid sequence homology with the extracellular domain of the cytokine receptors, did not bind PL. Furthermore, the propeptide region of LOX (LOX\textsubscript{1-168}) alone did not interact with PL. While the presence of the propeptide domain in construct LOX\textsubscript{1-417} partially inhibited, in construct LOX\textsubscript{1-348} had no effect on the interaction with PL.

We further tested whether LOX-like proteins, LOXL and LOXL2, that have high sequence homology with LOX within this putative binding region, could also bind to PL using the yeast two-hybrid system. The full-length LOXL, N-terminal half LOXL, C-terminal half of LOXL, N-terminal half of LOXL2, and C-terminal half of LOXL2 were cloned into the pGBK7 bait vector and co-expressed with PL as described in methods. Expression of each bait protein was confirmed by Western blotting (data not shown). These bait vectors and the PL target plasmid were cotransformed into yeast that were spotted on plates to test reporter gene activation. Yeast were also cotransformed with empty vector and lamin C controls as described above. The only bait that was positive for activating the reporter genes was the C-terminal portion of LOXL2 (Fig. 2C,D). The homologous domain of LOXL failed to interact with PL enough to activate the reporter genes, suggesting that PL specifically binds to this putative binding site within LOX and LOXL2.

**In vitro assays confirm the binding of LOX and PL**

- For further *in vitro* experiments aimed to verify and characterize the interaction of LOX and PL, significant amounts of purified LOX protein was needed. In order to generate LOX, cDNA encoding the 30 kD active enzyme was cloned into the pGEX-4T-1 vector and was expressed in *E. coli* bacteria in a recombinant form containing a GST-tag on its N-terminus. The GST-LOX protein was purified from bacterial inclusion bodies using the binding affinity of the GST-tag to glutathione. The proteins were then refolded based on the method previously published by Jung et al. (45), and described for GST-LOX by Fogelgren et al. (37).

To verify the binding of PL to LOX, we performed Far-Western analysis. Equal volumes of purified GST-LOX fusion protein and GST alone as negative control were separated by SDS-PAGE, blotted onto a membrane, refolded, and incubated with purified PL. Using an antibody against PL, we detected the PL bound to purified PL to GST-LOX but not to GST (Fig. 3). We obtained the same result in three independent experiments. As a control, we stained parallel membranes with Coomassie Blue, which confirmed the presence of both GST and GST-LOX on the membrane.

To further confirm the interaction of LOX and PL and to determine an equilibrium dissociation constant (K\textsubscript{d}) for this binding, we performed solid phase binding assays. Since PL is a member of the GH/PRL hormone family and has sequence homology with both GH and PRL, we tested...
whether LOX could also bind to GH and PRL. Repeated experiments consistently demonstrated that GST-LOX bound to immobilized PL with the highest affinity (Kₐ 30-120 nM) (Fig. 4). Compared to its affinity to PL, GST-LOX exhibited a slightly lower binding affinity to GH (Kₐ 180-344 nM) and significantly less affinity to PRL. The Kₐ value for the GST-LOX–PRL interaction could not be established within the concentration range tested, and there was only minimal interaction of GST-LOX with the BSA control.

Coimmunoprecipitation demonstrates LOX-PL interactions in breast tumor cells - To confirm the in vitro yeast interaction data and demonstrate native LOX-PL interactions in breast tumor epithelial cells that express both LOX and PL, we performed coimmunoprecipitation using the conditioned cell medium of invasive Hs578T cells. In these experiments we found that LOX could be coimmunoprecipitated with PL using anti-PL antibody, confirming the interaction of these proteins in the cell medium (Fig. 5). We could not, however, immunoprecipitate the LOX-PL complex with the LOX-antibody. We hypothesize that binding of PL might have interfered with the LOX epitope and prevented the anti-LOX antibody from binding to LOX. The epitope used to generate the anti-LOX antibody resides within the N-terminal region of the mature LOX (amino acids 176-197), consistent with the putative PL-binding site within LOX (amino acids 169-348). The negative control purified rabbit immunoglobulin did not immunoprecipitate LOX, excluding any non-specific binding.

PL is neither an inhibitor nor a substrate of LOX enzyme activity - To determine if binding to PL inhibits LOX amine oxidase activity, we performed an activity assay with bLOX (purified from bovine aorta) that had been preincubated with an equal molar amount of PL. Purified bLOX, either preincubated with PL or without, was incubated with 1,5-diaminopentane substrate and the quantity of H₂O₂ generated during the oxidative reaction was measured using Amplex Red and horseradish peroxidase. To determine enzyme activity attributable to LOX, BAPN was added to parallel reactions to inhibit its activity, and parallel reactions were run without any substrate. LOX activity was calculated as the increase in fluorescence above BAPN-inhibited controls. The results showed no decrease in LOX activity when bound to PL, as compared with non-bound bLOX (Fig. 6A). Since it has been reported that LOX has the ability to oxidize lysyl residues of another secreted growth factor, basic fibroblast growth factor (bFGF) (46), we tested if LOX could oxidize lysyl residues of PL in vitro. We used a range of concentrations of PL substrate (0–1.8 µM) as described for bFGF (46). Purified bLOX was incubated with this range of PL and the quantity of H₂O₂ generated during the oxidative reaction was measured and calculated as described above. The results showed PL was not oxidized at statistically significant levels (Fig. 6B). Therefore, we concluded that in these in vitro assays, PL does not inhibit LOX's catalytic activity, nor is a substrate for LOX.

Generation and characterization of stably transduced cell lines – As the highly invasive/metastatic breast carcinoma cell lines Hs578T and MDA-MB-231 express both LOX and PL, we decided to evaluate the individual and combined effects of PL and LOX on cell proliferation and migration by overexpressing and coexpressing these proteins in normal breast epithelial cells. We used MCF-10A immortalized normal breast epithelial cells as the parental cell line and generated stably transduced cell lines. Lentiviral particles, containing cDNA for PL-4 transcript variant 1 (771 bp), and the full length LOX (1254 bp) were generated in HEK297FT cells and used to transduce MCF-10A cells. Stably transduced MCF-10A cells were selected using blasticidin and individual clones were isolated and characterized. To confirm expression of PL and LOX in these cell lines, proteins were extracted from the conditioned cell medium of preconfluent cultures and analyzed by Western blotting (Fig. 7). Two stably transduced clones showed elevated LOX (Fig. 7A), four elevated PL expression (Fig. 7B), and two clones coexpression of both proteins (Fig. 7C). The parental MCF-10A cells do not express detectable levels of PL or LOX at preconfluent stage (Fig. 6A-C). In the stable PL-expressing clones, PL was mostly present as a 21 kD monomer, but the 42 kD homodimer form we noted in the breast cancer cell lines was also present in small amounts (data not shown). In subsequent experiments, clones PL-1, LOX-1 and PL+LOX-1 were used with the parental MCF-10A cell line and data was confirmed with clones PL-3, LOX-3 and PL+LOX-5 in repeated trials.
Coexpression of LOX and PL increased cell proliferation – As PL has been shown to promote in vitro[^H] thymidine incorporation and mitogenesis in cultured fetal fibro- and myoblasts (47), and increase DNA synthesis in epithelial cells of human breast carcinoma (48), we tested if the expression of PL in stably transduced cell lines induced an increase in cell proliferation. The proliferation assay was based on the use of methylene blue to stain the cells attached to the microplate wells, and then measuring the absorbance of the solubilized dye. MCF-10A cells expressing PL alone had a significantly higher (49% increase, p=0.0001) proliferation rate as compared to the parental MCF-10A cells in proliferation assays (Fig. 8). LOX has not been reported to influence cell proliferation in any cell types. Indeed, LOX overexpression did not result in a significant increase in proliferation of the LOX transduced cell lines compared to control. We noted, however, that coexpression of LOX and PL resulted in a proliferation rate significantly higher than either the parental or the PL-expressing cell proliferation rate (121% increase, p<0.0001; 47% increase, p<0.0001, respectively). These results suggested that the coexpression of LOX with PL enhances the proliferation-inducing effects of PL.

Coexpression of PL and LOX induces cell migration - Since elevated LOX expression has been demonstrated to contribute to a migratory and invasive phenotype in breast cancer cells (9, 49), we tested if overexpression of LOX in the stably transduced normal breast epithelial cell lines also promoted migration, and tested the effect of LOX-PL coexpression on the migratory ability of these cells. First, we used phalloidin staining to visualize the actin cytoskeleton of our parental and transduced MCF-10A cells. Our results showed rearrangement of the actin cytoskeleton and the appearance of numerous, longer filopodia in the PL-LOX coexpressing cell lines compared to the parental MCF-10A cells (Fig. 9A). These changes of the actin cytoskeleton indicated changes towards a migratory phenotype in the stably transduced cells, which we subsequently quantified in cell migration assays.

In the cell migration assays, we tested the ability of our cell lines to migrate through a gelatin-coated polycarbonate filter. Breast epithelial cells individually expressing LOX or PL did not show significantly different levels of migration compared to the parental normal mammary epithelial cell line. In contrast, the LOX-PL coexpressing cell line had a strong migratory phenotype and significantly higher migratory rates than the parental or the LOX transduced cell lines (240% increase, p=0.0038; 93.5% increase, p=0.0052, respectively) (Fig. 9B). The migration assays were repeated in three independent trials with consistent results and similar data were obtained using the other overexpressing clones. These results suggested that LOX and PL coexpression, but neither LOX nor PL alone, was capable of inducing a strong migratory phenotype in normal breast epithelial cells. These findings were quite different from data we obtained with the non-invasive breast cancer cell lines (9) where the overexpression of LOX alone induced migratory and invasive phenotype.

**DISCUSSION**

Results of our study demonstrated the interaction of LOX with PL in in vitro assays and invasive Hs587T breast tumor cells and showed that these two proteins did not interact in an enzyme-substrate or enzyme-inhibitor fashion. PL is recognized as a member of the growth hormone family and has been reported in breast tumors, but its role in breast epithelial cells is unknown. We have detected PL expression in breast tumor cells and demonstrated that ectopic expression of PL promoted normal breast epithelial cell proliferation. Coexpression of LOX with PL significantly enhanced this cell proliferation promoting effect of PL, while LOX alone had no affect on cell proliferation, suggesting a functional relation between these proteins. It is important to note that LOX, while proved essential in promoting breast tumor cell migration, was not capable of enhancing migratory phenotype in normal epithelial cells. However, LOX-PL coexpression significantly increased migration of otherwise static normal epithelial cells, further supporting an in vivo role for potential LOX-PL interactions.

In yeast two hybrid screens, we found high representation of LOX-interacting PL cDNAs among the positive clones (23% and 35%). Considering that PL mRNA accounts for approximately 5% of the total placental mRNA at term (50), the high representation of LOX interacting PL clones was most likely not due to the high level
of PL cDNA in our placental library. This interaction was indeed verified to be specific in our direct interaction assays. In agreement with the observation that while both PL-4 and PL-3 are expressed during pregnancy, PL-4 expression increases approaching term, the cDNA clones we identified were all clones of PL-4 transcript variant 1. This variant encodes the most abundant PL-4 isoform 1 that contains all five exons of the gene.

As we aimed to narrow down the PL-binding site within LOX, our first candidate site for this interaction was the C-terminal LOX cytokine receptor-like (CRL) domain. The CRL domain is homologous to the ligand-binding extracellular domain of class I cytokine receptors (51, 52), and PRLR and GHR, the known rectors for PL, are also class 1 cytokine receptors.

However, the LOX-CRL domain (amino acids 349-417), did not bind PL in yeast direct interaction studies. Furthermore, the LOX deletion constructs lacking the CRL domain were able to bind PL both in yeast interaction and solid phase binding assays. Amino acid sequence analysis of the LOX-CRL domain revealed that of the two barrel-shaped modules of cytokine receptors, the LOX C-terminal region contains the conserved residues for the N-terminal barrel domain, but it lacks amino acid sequences corresponding to the C-terminal barrel module (6). It is possible that the truncated CRL domain generated by the yeast expression construct was not properly folded, or that this partial cytokine receptor domain within LOX is not sufficient for PL binding. The CRL domain may, however, participate in PL interactions, together with a more N-terminal binding site within LOX. In support of this hypothesis, the presence of the CRL domain in the GST-LOX construct significantly increased binding affinity to PL in solid phase assays (not shown) compared to the GST-LOX construct lacking the CRL-domain.

The PL-binding site within LOX proved to be a region between amino acids 169 and 384. Binding of PL within this region, however, did not appear to interfere with the active site of LOX including the Cu-binding site (amino acids 280-296) and the lysyl (314) and tyrosine (349) residues that form the LTQ cofactor.

LOX also demonstrated binding affinity not only for PL but the highly homologous GH in solid phase binding assays. As the LOX-GH interaction proved specific, LOX may bind human GH in vivo, but further studies are required to address the characteristics of this potentially important interaction.

We have demonstrated the expression of LOX in breast tumors and the highly invasive/metastatic breast cancer cell lines Hs578T and MDA-MB-231 (7, 9). PL has also been reported in high percentage of breast tumors (22, 23), but its expression and function in breast tumor cell lines had not been investigated. Therefore we asked the question whether PL is expressed in the same cell lines as LOX. Our immunoblot analysis detected PL expression in the invasive cell lines that expressed LOX, and we confirmed the interaction of LOX with PL in Hs578T cells by coimmunoprecipitation.

To begin to address the in vivo mechanism and significance of potential LOX-PL interactions, we selected two processes: cell proliferation and cell migration. Cell proliferation is induced by PL, but not LOX. Therefore, we tested if LOX would modify PL’s effect. Cell migration, in contrast, is promoted by LOX, but not PL, and this process was suitable to test if PL would modify LOX’s effect.

In agreement with reports on PL’s proliferation-promoting effect in normal fetal (47) and breast tumor cells (48), overexpression of PL in stably transduced normal breast epithelial cell lines significantly elevated proliferation rates. The exact mechanism of how PL induces cell proliferation is not known. It is known however, that PL can bind to the GH and PRL receptors and the presence of these receptors has been shown in normal and tumor breast epithelium (53, 54) and cell lines (55-57). Activation of GHR results in activation of the JAK-STAT signaling pathway (58) and proliferation in MCF-10A cells (59). However, the binding of PL to the GHR is at a low affinity (24) and human PL-induced signaling via the GHR has not been reported. The binding of PL to the PRL receptor, in contrast, is at a higher affinity (24) and PRLR activation by PL is more likely.

Activated PRLR stimulates proliferation in breast tumor cells through JAK2, and Src mediated FAK signaling (60). PL was also shown to stimulate tyrosine phosphorylation of JAK2 (61) and induce the JAK-STAT pathway in breast carcinoma cells (48). Therefore, the high prolifera-
tion rate of PL-overexpressing MCF-10A breast epithelial cells likely resulted from the activation of these pathways.

Though LOX can promote tyrosine phosphorylation of both FAK and Src in breast tumor cells, we did not note this activation to result in increased proliferation either in tumor (9), or normal breast epithelial cells. Therefore, it is likely that the enhanced cell proliferation in LOX-PL coexpressing cells was not due to separate effects, but resulted from the interactions of LOX with PL. However, it remains to be established how LOX-PL interactions may promote activation of a signaling pathway. LOX has been shown to alter the mitogenic potential of basic fibroblast growth factor (bFGF) through modification of lysyl residues within bFGF (46). In our experiments, however, PL was not a substrate for LOX and such modifications of PL by LOX seem unlikely.

Regarding cell migration, we anticipated that LOX might induce migration of normal breast epithelial cells as a strong FAK/Src mediated effect of LOX was noted in breast tumor epithelial cells (7, 9) and mouse mammary tumors (10). While normal MCF-10A cells can process and activate ectopic LOX (42), and overexpression of LOX in these cells induced some phenotypic changes and filopodia formation (42), LOX was not sufficient to induce a migratory phenotype. We therefore hypothesize that LOX’s effect may be moderated in normal cells, or that LOX may have different function in normal and tumor epithelial cells.

There are no reports regarding any effect of PL on cell migration, and our data did not show significant migration increase of the PL overexpressing normal epithelial cells. The stimulation of the PRL receptor, however, was reported to induce membrane ruffling, and motility of MCF-7, T47D and MDA231 cells (27). Consistent with these observations, we have also noted some rearrangement of the cytoskeleton in normal cells overexpressing PL, but cell migration was not altered.

Coexpression of LOX with PL, however, resulted in cytoskeletal changes, filopodia formation and doubled the migration rate of MCF-10A cells compared to those that overexpressed either LOX or PL alone. If both LOX and PL independently induced tyrosine phosphorylation and activation of FAK/Src, we would indeed observe an additive migratory effect in the LOX-PL clones. However, this may not be the case, as LOX or PL alone did not significantly increase cell motility. It is possible that FAK/Src activation through PL-activated PRLR initiated some cytoskeletal changes and primed cells towards a motile cell phenotype, but this was not sufficient to induce cell migration and that the additional tyrosine phosphorylation of FAK/Src due to LOX resulted in strong cell migration as a cumulative effect. It is also possible that, as in the case of cell proliferation, the interactions of LOX with PL enhanced signaling that was then sufficient to induce migration in normal epithelial cells. Further studies are needed to address the mechanistic details of how LOX binding to PL may promote highly migratory cell behavior.

LOX and PL are coexpressed in Hs578T and MDA-MB-231 cell lines and their interactions may contribute to the highly migratory/invasive phenotype of these cells. In addition to invasive breast epithelial cells, LOX and PL are coexpressed in the JAR choriocarcinoma cells that are also migratory/invasive. These results collectively demonstrate an important role for the LOX amine oxidase in epithelial cell behavior in novel interactions with a growth hormone family member, PL, and show that these interactions may be highly relevant to the transition of epithelial cells towards a migratory phenotype during the development and progression of breast carcinoma.

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REFERENCES


FOOTNOTES

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*The abbreviations used are: LOX, lysyl oxidase; ECM, extracellular matrix; BMP-1, bone morphogenic protein-1; PL, placental lactogen; GH, growth hormone; PRL, prolactin; GHR, growth hormone receptor; PRLR, prolactin receptor; PBS, phosphate-buffered saline; CCM, conditioned cell medium; CRL, cytokine receptor-like; GST, glutathione S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; RT, room temperature; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; DTT, dithiothreitol; PBST, PBS with Tween 20; BSA, bovine serum albumin; IgG, immunoglobulin G; HRP, horseradish peroxidase; BAPN, β-aminopropionitrile; PCR, polymerase chain reaction; HEK 293FT, human embryonic kidney 293FT

FIGURE LEGENDS

Fig. 1. LOX and PL immunostaining in breast tumor tissues, and Western blot analysis of PL protein secreted by breast cancer cell lines. A, LOX staining is shown in green, nuclear labeling is red. B, PL staining is in red, and nuclear labeling in green. Scale-bars = 20µm. C, On Western blots of the cell media high levels of PL-production is detected by invasive Hs578T, MDA-MB-231, and non-invasive MCF-7, and very low levels by non-invasive T47D cells. As positive control, JAR choriocarcinoma cell line was used. PL appears in a homodimer form of approximately 42 kD.

Fig. 2. Deletion constructs of LOX and their interactions with PL in yeast direct interaction studies. A,C: Diagrams of LOX, LOXL and LOXL2 constructs used in direct interaction experiments. Numbers represent the amino acids contained in the deletion constructs. Purple: pro-peptide; red: copper-binding site; yellow: scavenger receptor cysteine-rich (SRCR) domain; green: CRL, cytokine receptor-like domain; BMP: bone morphogenic protein cleavage site; LTQ: lysyl-tyrosine quinone cofactor; blue: PRD, proline-rich domain. B,D: Yeast expressing various combinations of lysyl oxidase bait proteins with PL, spotted in triplicate on nutritionally selective media. Negative controls included empty bait vector (pGBKT7) and empty target vector (pGADT7).

Fig. 3. Far-Western blot analysis confirms PL-LOX binding. Recombinant GST-LOX and GST as control were subjected to separation by SDS-PAGE, transformed onto a membrane, refolded in renaturation buffers, and incubated with purified PL. Bound PL was detected with immunoblot using anti-PL antibody (left image). PL bound to recombinant GST-LOX (55 kD) but not to GST (25kD). In a parallel
control experiment, GST and GST-LOX were separated by SDS-PAGE and transferred onto a membrane under the same conditions as before, and the membrane was then stained with Coomassie Blue. Staining of the membrane shows both GST and GST-LOX are present on the blot. The positions and apparent molecular weight of the Magic Mark XP and SeeBlue+2 are marked on the left side of the panels.

**Fig. 4.** Solid phase binding assay demonstrates GST-LOX binding to hPL. In microplate wells (coated with PL, GH, PRL, or negative control BSA) various concentrations of GST-LOX were incubated. Bound GST-LOX was detected with an anti-GST antibody in an enzyme-linked immunosorbent assay. All concentration points were measured in triplicates, error bars represent S.E.M., Kd values were calculated with Prism4 statistical software.

**Fig. 5.** Western blot of proteins immunoprecipitated from Hs578T cell medium demonstrates the *in vivo* binding of LOX to PL. *In vivo* interactions were tested by immunoprecipitation of PL and LOX using anti-PL and anti-LOX antibodies, with purified Rabbit IgG as the negative control. Immunoprecipitates were subjected to immunoblot analysis using the anti-LOX antibody as described in “Experimental Procedures”. LOX was coimmunoprecipitated from conditioned cell medium of Hs578T cells using the anti-PL antibody, but not with the negative control rabbit IgG. Positive controls are conditioned cell medium (CCM), and precleared CCM from Hs578T cells. IP: immunoprecipitating antibodies, WB: antibody used for Western blot

**Fig. 6.** LOX enzyme activity assays testing if PL inhibits LOX catalytic activity, or if PL is a substrate for LOX. A, To determine if binding to PL inhibited LOX activity, purified bovine LOX was incubated with PL at a molar ratio of 1:1 in the presence or absence of LOX inhibitor BAPN. No significant decrease in activity was measured. B, Lack of significant catalytic activity of LOX measured with PL as a substrate in a concentration range of 0-1.8 µM, in the presence or absence of BAPN at 60 min reaction time. As a control, 0 µM PL substrate was added to the reaction mix. Error bars are S.E.M.

**Fig. 7.** Western blot analysis of LOX, PL and their coexpression in stably transduced MCF-10A cells. MCF-10A cells were transduced with lentivirus containing PL and/or LOX expression constructs. Stably transduced single cells were selected and clones established. Protein production of LOX (A), PL (B), or both (C) was detected by immunoblot analysis. PL in these cell lines was mostly present as a monomer of ~21 kD. At pre-confluency, the parental cell line MCF-10A did not express PL or LOX.

**Fig. 8.** Proliferation assays demonstrate that PL expression promotes MCF-10A cell proliferation, and PL-LOX coexpression further enhances cell proliferation. 2,000 cells from each cell line were allowed to attach in the wells of two parallel microwell plates. At 0 h, the number of attached cells on one of the plates was determined by staining the cells with methylene-blue, and measuring the absorbance of the solubilized dye. The same measurement was performed on the second plate after 72 h of incubation. The proliferation rate was calculated as the difference of the absorbance at 72 h and 0 h. The PL-expressing cells showed increased proliferation (49%) compared to the parental MCF-10A cells. The PL-LOX coexpressing cells exhibited significantly increased proliferation rates compared to both the parental and PL-expressing cell lines (121% and 47%, respectively). The LOX-expressing cells do not show significant changes in their proliferation rate. *** marks P values ≤ 0.0001. These results were confirmed in repeated trials, error bars are S.E.M.

**Fig. 9.** PL and LOX coexpression in MCF-10A cells leads to increased cell migration. A, The actin cytoskeleton in MCF-10A and PL-LOX co-expressing cells was stained with phalloidin. Coexpression of PL and LOX resulted in actin cytoskeleton rearrangement and formation of numerous filopodia in PL-LOX co-expressing cells compared to parental MCF-10A cells. Scale-bars = 10 µm. B, Cell migration rates of the parental and the stably transduced cell lines were determined in a migration assay. 50,000
cells were seeded in triplicates on a gelatin-coated polycarbonate membrane, and were allowed to migrate for 4 h. The cells were then fixed, stained with haematoxilin-eosin, and counted. PL-LOX coexpression resulted in a significantly higher migratory rate (240% increase) compared to the parental MCF-10A cells. The expression of either LOX or PL alone did not increase cell migration. Errors bars are S.E.M. ** marks P values ≤ 0.005.
Figure 1

A

Anti-LOX  Control

B

Anti-PL  Control

C

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>42 kD</th>
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<tbody>
<tr>
<td>Hs578T</td>
<td></td>
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<tr>
<td>MDA-MB231</td>
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</tr>
<tr>
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<td>JAR</td>
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Figure 2

A

LOX1-417
LOX1-348
LOX1-168
LOX169-348
LOX169-417
LOX349-417

B

pGBKT7 only
Lamin C
LOX1-417
LOX1-168
LOX1-348
LOX169-348
LOX169-417
LOX349-417

C

LOXL2-574
LOXL-Nterm26-368
LOXL-Cterm338-574
LOXL2-Nterm1-547
LOXL2-Cterm548-774

D

LOXL2-574
LOXL-Nterm26-368
LOXL-Cterm338-574
LOXL2-Nterm1-547
LOXL2-Cterm548-774

PL (cl. 1)
Figure 3
Figure 5

IP: Rabbit IgG Anti-PL Anti-LOX CCM Preleared CCM

WB: LOX 30 kD
Figure 7

A

![Image](30 kD Anti-LOX)

B

![Image](20 kD Anti-hPL)

C

![Image](30 kD Anti-LOX)

![Image](20 kD Anti-hPL)
Figure 8
Figure 9

A

B

MCF-10A

PL+LOX-1

**

**

Migratory cells

MCF-10A  LOX  PL  PL+LOX
Lysyl oxidase interacts with hormone placental lactogen and synergistically promotes breast epithelial cell proliferation and migration
Noemi Polgar, Ben Fogelgren, J. Michael Shipley and Katalin Csiszar

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