PH.D. DISSERTATION

Interaction of lysyl oxidase with the hormone placental lactogen and its effect on mammary epithelial cell proliferation and migration

Noémi Polgár

University of Szeged, Faculty of Natural Sciences, Department of Genetics and University of Hawaii,John A. Burns School of Medicine and Cardiovascular Research Center

Supervisors and consultants:

Mátyás Mink, PhD
University of Szeged, Faculty of Natural Sciences,
Department of Genetics

Katalin Csiszár, PhD
University of Hawaii,
John A. Burns School of Medicine and
Cardiovascular Research Center

Szeged, 2008

ACKNOWLEDGEMENTS

I would like to express my gratitude to all who helped me and gave me the possibility to complete this dissertation. First of all I would like to deeply acknowledge my supervisor Dr. Katalin Csiszár who made this research possible by giving me the opportunity to work in her laboratory at the Cardiovascular Research Center (CVRC), University of Hawaii, John A. Burns School of Medicine, and by taking me under her mentorship. I thank her for her support and helpful criticism throughout my work.

I am most grateful to have had the chance to work with Dr. Ben Fogelgren, post-doctoral fellow at CVRC, from whom I learnt the most and whose dedication and endless enthusiasm was indeed inspiring. I thank him for all his guidance, encouragement, and patience.

I would like to thank the members of Dr. Csiszár's laboratory for their assistance, and advice I received. Drs. Keith and Sheri Fong, Péter Hollósi, Matthias Jansen, Dr. Rozália Laczkó along with Dr. János Molnár and Dr. Kornélia Molnárné Szauter were very a helpful and lovely team to work with.

I would also like to acknowledge my supervisor Dr. Mátyás Mink at the Department of Genetics, University of Szeged for allowing me to join his team while I was writing my dissertation. I am grateful for his generosity and insight.

I would like to thank my immediate colleagues and friends Dr. Gyula Csanádi, Tünde Huszár, Anita Ordas and Péter Rácz for their friendship and encouragement all these years.

I owe a special note of gratitude to the Chairman of the Department of Genetics, University of Szeged, Dr. Péter Maróy and his staff for allowing me to become a member of their team.

I gratefully acknowledge my opponents who have given their time to read this manuscript for their detailed review, constructive criticism and advice.

Finally, this thesis would not have been written without the loving support of my parents and my sister. Thank you so much.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS USED

3D	three dimensional
A, Ala	alanine
aa	amino acid
AAS	α-aminoadipic-δ-semialdehide
ACP	aldol condensation product
AD	active domain
amp^r	ampicillin resistance
ATP	adenosine triphosphate
BAPN	β-aminopropionitrile
BD	binding domain
bFGF	basic fibroblast growth factor
bLOX	bovine lysyl oxidase
BMP-1	bone morphogenic protein-1
bp	base pair
BSA	bovine serum albumin
C, Cys	cysteine
cAMP	cyclic adenosine monophosphate
CCM	conditioned cell media
cDNA	complementary DNA
cFN	cellular fibronectin
cfu	colony-forming unit
cl	clone
cm	centimeter
CMV	cytomegalovirus
COL3A1	collagen III α1
coIP	co-immunoprecipitation
CRL	cytokine receptor-like
CSH	chorionic somatomammotropin
Cu	copper
D	Dalton
D, Asp	aspartic acid
DAO	diamine oxidase
DAPI	4',6-diamidino-2-phenylindole
DEAE	diethylaminoethyl
deLNL	dehydrolysinonorleucine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
E, Glu	glutamic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EtBr	ethidium bromide
EtOH	ethanol
F, Phe	phenylalanine
FAD	flavin adenine dinucleotide
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fmol	femtomole
FN	fibronectin
FSH	follicle-stimulating hormone
g	gram
g	acceleration due to gravity
G, Gly	glycine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GH	growth hormone
GHR	growth hormone receptor
GST	glutathione S-transferase
H, His	histidine
h-	human
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
HRP	horseradish peroxidase
I, Ile	isoleucine
IGF	Insulin-like growth factor
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-1-thio-β-D-galactopyranoside
IRF	interferon regulatory factor
K, Lys	lysine
kb	kilobase pair
K_d	equilibrium dissociation constant
K_i	inhibition constant
kD	kiloDalton
L, Leu	leucine
LB	Luria-Bertani
LiAc	lithium acetate
LOX	lysyl oxidase
LOXL	LOX-like

LOXL2	LOX-like 2
LOXL3	LOX-like 3
LOXL4	LOX-like 4
LS	lysozyme soluble
LTQ	lysyl-tyrosylquinone
M	molar (mole/liter)
mA	milliampere
MAO	monoamine oxidase
MCS	multiple cloning site
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
mTLD	mammalian tolloid
mTLL	mammalian tolloid-like
N, Asn	asparagine
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor-kappa B
ng	nanogram
nm	nanometer
nM	nanomolar
nmol	nanomole
OD	optical density
OHS	occipital horn syndrome
OMIM	Online Mendelian Inheritance in Man
oPL	ovine placental lactogen
ORF	open reading frame
	oxidase
P, Pro	proline
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PAO	polyamine oxidase
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween-20
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
pg	picogram
PGE2	prostaglandin E2
PL	placental lactogen
pM	picomolar
pmol	picomole
PRD	proline rich domain
PRL	prolactin
PRLR	prolactin receptor
proLOX	full-length LOX proenzyme
Q, Gln	glutamine
R, Arg	arginine
rDMLOXL-1	recombinant Drosophila LOXL-1
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
Rrg	*ras* recision gene
RT	room temperature
S, Ser	serine
SDS	sodium dodecyl sulfate
SE	standard error
SEM	standard error measurement
SPBA	solid phase binding assay
SRCR	scavenger receptor cysteine rich
SRS	Silver-Russell syndrome
SS	signal sequence
SSAO	semicarbizide-sensitive amine
Stat	signal transducer and activator of transcription
T, Thr	Threonine
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TGF-β	transforming growth factor-β
ThCS	thrombin cleavage site
TPQ	topaquinone
U	unit
UV	ultraviolet
V	volts
V, Val	valine
v/v	volume (of solute) per volume (of solvent)
VSMC	vascular smooth muscle cells
W, Trp	tryptophan
w/v	weight (of solute) per volume (of solvent)
x g	times gravitational force
Y, Tyr	tyrosine
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

INTRODUCTION

Breast cancer is the most common cancer among women, as it is diagnosed in $1x10^6$ women in the world each year [1]. The incidence of breast tumor development in women differs with age, mammary gland mass and exposure to endogenous and exogenous hormones [1]. However, the function, molecular regulation, and interaction of genetic alterations in carcinogenesis, as well as those of tumor cell migration and invasion, and metastasis of breast carcinomas are not fully understood. As metastasis is a major challenge in cancer treatment, determination of molecular markers of metastatic potential and therefore understanding the process of metastatic tumor progression are important. Recently, comprehensive transcriptome profiling of normal breast, noninvasive breast tumor and invasive breast cancer cell lines identified lysyl oxidase (LOX) as a possible marker gene based on its differential expression [2]. In addition, upregulated LOX expression and activity have been reported in breast cancer tissues and invasive/metastatic breast cancer cell lines as well [3, 4]. Lysyl oxidase has been primarily recognized as a matrix-crosslinking enzyme; hence regulators and interacting factors contributing to its novel role in breast cancer development and progression are yet to be discovered. The goal of this work was to identify novel LOX-interacting partners that likely promote its function during tumorigenesis, and to characterize such interactions and evaluate their biological significance.

Lysyl Oxidase and the LOX Enzyme Family

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that catalyzes the crosslinking of elastin and fibrillar collagens in the extracellular matrix (ECM) [5].

LOX was first discovered by Pinnell et al. [6] who demonstrated that the initial step of the covalent crosslinking of elastin and collagen, the formation of allysyl residues is mediated by an enzyme from bone extracts of embryonic chicks. This enzyme proved to be irreversibly inhibited by physiologically active levels of the lathyrogen β-aminoproprionitrile (BAPN), a compound that had been previously shown *in vivo* and in tissue culture to inhibit the cross-linking of collagen I and elastin by blocking the lysine-to-allysine conversion [7-9]. The enzyme was later given the non-systematic name lysyl oxidase [10].

The enzymes catalyzing the oxidation of primary amine substrates to reactive aldehydes are the amine oxidases. Members of the first class of amine oxidases, such as monoamine oxidases and polyamine oxidase, contain flavine adenine dinucleotide (FAD) as cofactor. The second class of amine oxidases, including LOX, diamine oxidase (DAO),

and monoamine metabolizing semicarbazide-sensitive amine oxidase (SSAO) contain a tightly bound Cu^{2+} ion and a tyrosine-derived cofactor such as topaquinone (TPQ) or lysyl-tyrosine quinone (LTQ) at their active site [5, 11, 12].

Lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) was initially considered as a single enzyme and its insolubility and tendency for aggregation did not facilitate the biochemical studies aimed to characterize the protein. Following the discovery that LOX can be solubilized in buffers with high urea content (4-6 M) and its activity can be recovered by the removal of urea [13] , the enzyme was purified from many tissues and organisms such as bovine aorta [14], piglet skin [15], embryonic chick cartilage [16], aorta [17] and human placenta [18]. The observation that LOX resolved upon DEAE chromatography into multiple isoforms [19] led to the hypothesis that there might be alternatively spliced or other forms of LOX or isoenzymes with similar properties.

So far LOX and four LOX-like (LOXL) proteins, (LOXL, LOXL2, LOXL3 and LOXL4) have been described as members of the LOX enzyme family. The lysyl oxidases act on peptidyl lysine residues as substrates in contrast to the other amine oxidases, which primarily act on small soluble polyamines [12]. The genes of these enzymes are located on different chromosomes. According to data at NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org) in the human genome, *LOX* localizes to chromosome 5q23.2 encoding 7 exons, *LOXL* localizes to chromosome 15q24-q25 with 7 exons, *LOXL2* is located on chromosome 8p21.3-p21.2 containing 14 exons as well as *LOXL3* on chromosome 2p13, while *LOXL4* was mapped to chromosome 10q24 encoding 15 exons. Based on their gene structure the LOX genes can be further divided into two subgroups **(Figure 1.)**. The *LOX* and *LOXL* genes share a similar exon structure in which five of the seven exons (exons 2-6) exhibit the highest level of homology as they are similar in size and encode amino acids of 76% sequence identity. Exons 1 and 7 of these genes, however, show significant differences. While exon 1 in LOX encodes a 5'-untranslated region, a signal peptide and a proenzyme region of the protein and the first amino acids of the mature enzyme, in LOXL it is larger and contains the sequences of a proline-rich region besides a signal and a propeptide sequence. Correspondingly, exon 7 of LOX varies from that of LOXL as it includes several differentially used polyadenylation signals in addition to five coding nucleotides, a stop codon and a 3'-untranslated region, thus resulting in a longer exon in the former [20]. Likewise, LOXL2, LOXL3 and LOXL4 show similarities in their gene structure. In addition to the 4 exons encoding the conserved LOX-domain that characterizes this enzyme family, they all differ from *LOX* and *LOXL* in their 5' exons that

are responsible for the coding of scavenger receptor cystein rich (SRCR) domains not present in either *LOX* or *LOXL* [11, 21].

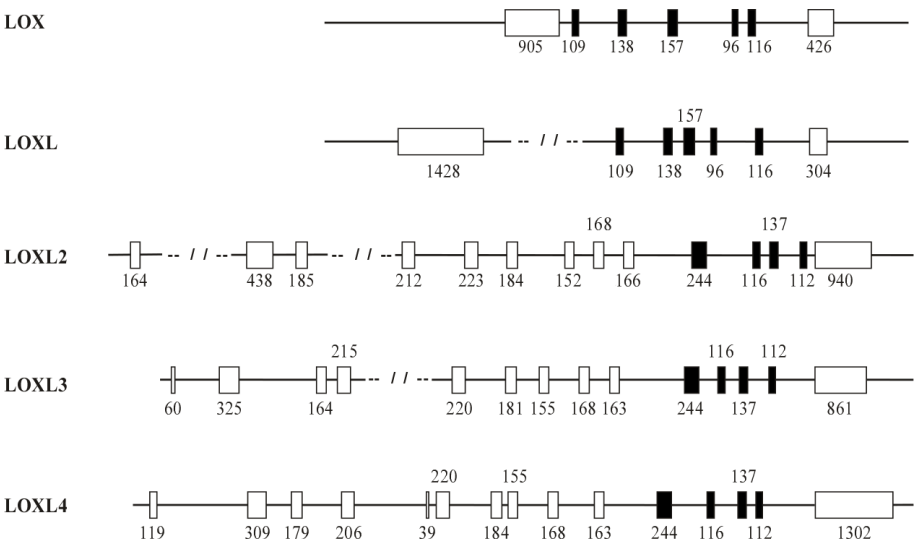

Figure 1. The gene structure of the lysyl oxidase gene family.
The numbers indicate the base pairs in each exon. The black boxes show the highly conserved exons coding the LOX domain.

Analysis of the corresponding amino acid sequences revealed that highly conserved residues define structural and functional domains within the carboxy-terminal region of the lysyl oxidases as demonstrated in **Figure 2.** The C-terminal region contains a copper-binding domain with four histidines that participate in binding of the copper (II) cofactor, a cytokine receptor-like (CRL) domain showing homology with the extracellular ligand-binding domain of the growth factor and cytokine receptor family, and the lysine and tyrosine residues that form the LTQ cofactor as well as ten cystein residues conserved among LOX isoenzymes [11, 21, 22]. Thus the catalytic region is very similar among the members of the LOX enzyme family. Furthermore, each member of the enzyme family contains a signal sequence at the amino terminus essential for their secretion into the extracellular space.

The N-terminal region of these proteins show amino acid sequence divergence as it is illustrated in **Figure 2.** LOX contains a propeptide region at its amino terminus (amino acids 1-168) that is proteolytically cleaved by procollagen-C proteinase activity [23]. The N-terminus of LOXL comprises a proline rich region with a proline content of 14,2% which makes it unique among the lysyl oxidases [24], while within LOXL2, LOXL3 and LOXL4 there are repeated scavenger-receptor cystein-rich (SRCR) sequence motifs [21, 22, 25-27] that can be found in several secreted and cell surface proteins and may be in-

volved in binding to other ECM and cell surface molecules [28]. The divergent N-terminal domains are thought to modulate protein-protein interactions, substrate specificity, activity, and the processing of the LOX protein in its physiological context [11].

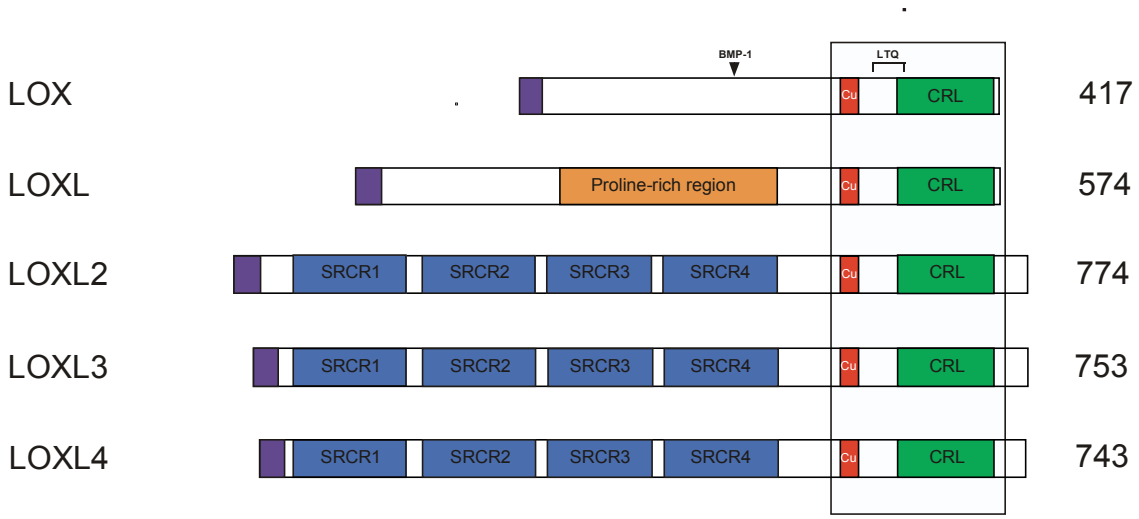

Figure 2. Schematic domain structure of the lysyl oxidases
Purple rectangle: signal peptide; BMP-1: site of proteolytic cleavage; Cu: Cu^{2+}-binding domain, CRL: Cytokine receptor-like domain, SRCR: scavenger receptor cysteine-rich motifs. The numbers indicate the number of amino acids.

Transcriptional and Post-transcriptional Regulation of LOX

Analysis of the human LOX gene revealed the presence of predicted transcriptional elements in the 5' flanking region such as AP-2, MalT, Sp-1 and glucocorticoid-receptor-binding sequences, as well as metal regulatory elements in the promoter and the first intron [29]. Enhancer sequences of the human *LOX* gene between -796 and -274, relative to the start codon and within the first intron [29], along with interferon regulatory factor (IRF-1)-binding sequences [30], suppressor elements, and transcription-modulating and serum deprivation-responsive elements for the murine LOX gene have been described [11, 31].

Both the LOX mRNA and protein were demonstrated to be present in various tissues under normal and pathological conditions. Therefore it is not surprising that a complex mechanism exists that coordinates the regulation of LOX expression and protein activity [11, 32]. The factors contributing to this regulation include metal ions [33, 34], growth factors and cytokines like transforming growth factor-β1 (TGF-β1) [35-38], basic fibroblast growth factor (bFGF) [39, 40], insulin-like growth factor (IGF) [41], and platelet-derived growth factor (PDGF) [42, 43], hormones like prostaglandin E2 (PGE2) [35,

37], follicle-stimulating hormone (FSH) [44], and testosterone [45], as well as signaling molecules like cAMP [46] and ras [47].

Biosynthesis and Proteolytic Processing of LOX

Lysyl oxidase is synthesized as a 46 kD prepro-enzyme with a 21-nucleotide signal sequence at its N-terminus [48, 49]. Sequential signal peptide cleavage, copper incorporation, and N-glycosylation within the propeptide region (amino acids 91-93) during its transport through the endoplasmic reticulum and the Golgi elements yield a 50 kD proenzyme [49, 50], which is then secreted into the extracellular space. Based on pulse-chase study results conducted in rat smooth muscle cell cultures, this 50 kD form is converted to a 32 kD non-glycosylated mature enzyme in the medium [49]. The enzymes responsible for the extracellular maturation of LOX are the procollagen C-proteinases bone morphogenic protein (BMP-1) and mammalian Tolloid (mTld) encoded by the *BMP-1* gene [51] as well as the genetically distinct mammalian Tolloid-like (mTLL)-1 and -2 proteinases [23, 52]. The proteolytic processing of LOX occurs at the Gly-Asp bond between residues 168 and 169 (numbered according to the human sequence) [53] yielding an 18 kD propeptide and a 32 kD active enzyme which retains the 10 cystein residues of proLOX, all of them in disulfide linkage [54]. We have identified fibronectin (FN) as a novel factor in the proteolytic activation of LOX [55]. Our results revealed the interaction of FN and LOX, suggesting an association between FN and proLOX as well. Furthermore, our data demonstrated a significant impairment in the proteolytic activation of LOX in embryonic fibroblast cultures derived from $FN^{-/-}$ mice that was not due to the absence of the processing enzyme BMP-1. Thus the presence of FN seems to be essential for the proteolytic activation of LOX supporting the hypothesis that the extracellular environment influences the activation rate of LOX.

Catalytic Activity of LOX

The reaction catalyzed by lysyl oxidase is a redox process that requires the presence of an electron donor and a final electron acceptor for the oxidation of the substrate and the restoration of the enzyme, respectively. LOX contains two cofactors essential for its catalytic activity, a tightly bound copper ion (Cu^{2+}) [19] and a lysyl tyrosylquinone (LTQ) cofactor [56] that is a unique characteristic of the LOX enzyme family.

The Copper (II) Ion

According to atomic absorption data, LOX contains 1 copper atom per 32 kD monomer. The removal of this copper results in the loss of enzyme activity [57, 58]. Active protein synthesis is required for the incorporation of ^{67}Cu into LOX which occurs prior to glycosylation and processing during protein trafficking in the Golgi elements [50]. Electron spin resonance studies have indicated that copper (II) is bound in a tetragonally distorted, octahedrally coordinated ligand field [57], similar to other divalent copper ion containing enzymes. The sequence W*X*WH*X*CH*X*HYHS in human corresponding to the copper-binding site contains four histidines that participate in copper binding with their nitrogen ligands [59]. These histidines are conserved in all members of the LOX family, suggesting that copper incorporates into the LOX-like proteins likely in a similar manner. The incorporated copper (II) is also important for the stabilization of the LTQ cofactor as it has been demonstrated for bovine LOX that exhibited a decrease in the LTQ content upon the removal of the enzyme-bound copper [60].

The Lysyl Tyrosylquinone (LTQ) Cofactor

Lysyl oxidase contains a covalently bound carbonyl cofactor in addition to copper. Raman and UV-visible spectroscopy revealed the quinone nature of the cofactor the structure of which is derived from the covalent crosslinking of the ε amino group of peptidyl lysine 314 with the side chain of tyrosine 394 [56, 61] as supported by mutagenesis studies. The formation of the LTQ cofactor presumed to be a self-processing reaction, that involves the autocatalytic hydroxylation and oxidation of Tyr394 (human LOX sequence) with the catalytic assistance of the copper atom at the nascent active site [62]. Indeed, studies of the recombinant form of *Drosophila* lysyl oxidase (rDMLOXL-1) revealed that the initially inactive rDMLOXL-1 that does not contain a carbonyl cofactor, following aerobic dialysis against copper shows catalytic activity and displays a characteristic electronic absorption spectrum of an organic cofactor present [63]. The developing cofactor is then attacked by the ε-amino group of Lys314, and the reoxidation of this quinol product yields the peptidyl LTQ [62]. This covalently linked lysyl component of the LTQ cofactor in LOX might play an important role in the preference of LOX for peptidyl lysine substrates [64].

Amine Oxidase Activity

Lysyl oxidase catalyzes the oxidative deamination of peptidyl lysine in elastin and collagen to δ-aminoadipic-β-semialdehyde or allysine during their posttranslational modification [65]. This reactive aldehyde can then spontaneously form covalent cross-links with neighboring amino groups or peptidyl aldehydes in the course of connective tissue biogenesis **(Figure 3.)**.

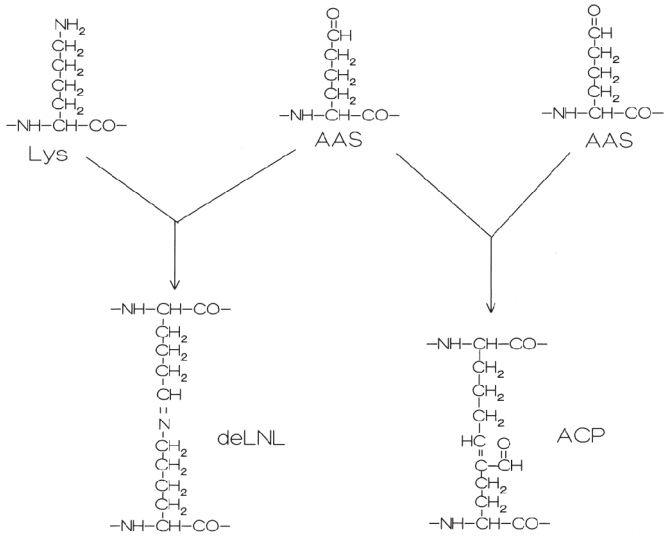

Figure 3. The LOX-catalyzed reaction.
Spontaneous development of crosslinkages from lysine (Lys) and peptidyl α-aminoadipic-δ-semialdehyde (AAS) yielding dehydrolysinonorleucine (deLNL) and aldol condensation product (ACP) [5].

The enzyme operates by a ping–pong mechanism that involves two half-reactions to complete the catalytic cycle. The mechanism can be summarized as demonstrated by the following equations [66].

(1) $LOX_{ox} + RCH_2NH_2 \rightarrow LOX_{red}(-NH_2) + RHC{=}O$

(2) $LOX_{red}(-NH_2) + O_2 + H_2O \rightarrow LOX_{ox} + H_2O_2 + NH_3$

The resulting cross-links are essential for the formation of collagen fibrils and insoluble elastin, the major components of the extracellular matrix. Recent studies have demonstrated that not only LOX but all the members of the enzyme family exhibit cross-linking activity, as it was suggested by the presence of a catalytic domain conserved throughout the protein family [11, 67-69].

Inhibition of LOX activity

Administration of β-aminoproprionitrile (BAPN) to growing animals is known to result in lathyrism, a disease characterized by an increased fragility of the connective tissues and an elevation of solubility of collagen from tissues due to decreased cross-linking. Thus BAPN is a potent lathyrogen capable of inhibiting crosslink formation *in vivo*. Pinnell and Martin were the first to demonstrate that micromolar concentrations of BAPN irreversibly inhibited lysyl oxidase activity *in vitro* [6] with an inhibition constant (K_i) of 3-5 μM [70]. BAPN binds to the active site of LOX and is enzymatically processed to a reactive intermedier, which is able to derivatize the enzyme [71]. BAPN was hence used to specifically inhibit the activity of lysyl oxidases and to identify additional irreversible inhibitors based on their structural similarities to BAPN, since LOX is a reasonable chemotherapeutic target for the control of fibrosis in pathological conditions. Such irreversible inhibitors are β-haloethylamines and β-nitroethylamine with inhibition constants similar to that of BAPN [72]. Certain para-substituted benzylamines [73] and 1,2-alkyldiamines [74] have also been found to be active site-directed inhibitors of lysyl oxidase. Furthermore, LOX can be inhibited by heparin [75], N-(5-aminopentyl)aziridine [76], and the non-competitive, reversible inhibitor trans-2-phenylcyclopropylamine [77].

Biological functions of LOX

Classical lysyl oxidase functions

Lysyl oxidase primarily targets collagen and elastin *in vivo* as the peptidyllysine products of amine oxidation are not broadly found among other mammalian proteins.

Collagens

Collagens are essential structural components of the extracellular matrix, and the most abundant proteins in the mammalian body. Collagens are important for a broad range of functions, including tissue scaffolding, cell adhesion, cell migration, cancer, angiogenesis, tissue morphogenesis and tissue repair. There have been at least 41 human collagen genes described that give rise to the numerous types of collagens conferring tensile strength to connective tissues. Collagens are characterized by the presence of at least one triple-helical collagenous domain with the repeating sequence motif $(Gly\text{-}X\text{-}Y)_n$, where X and Y positions are often occupied by proline and 4-hydroxyproline, respectively [78]. In addition to the collagenous domains, collagens contain noncollagenous structures as well and many of these have important functions distinct from those of the collagenous domains

[79, 80]. The collagen protein family can be divided into several subgroups based on their function and domain homology. One of these subgroups includes the so called fibril-forming collagens that are the principal source of tensile strength in mammalian tissues [81]. The precursors of the fibrillar collagens called procollagens following extensive post-translational modifications inside the cell and secretion into the extracellular space, undergo proteolytic conversion to collagen molecules. These collagen molecules then form fibrils that are stabilized by the formation of intra- and intermolecular crosslinks, a result lysyl oxidase activity [82].

Elastin

The elastic properties of many tissues capable of deforming in a repetitive and reversible manner are due to the presence of elastic fibers in the extracellular space. The most important component of these elastic fibers is elastin. Ultrastructural analysis have shown that the elastic fibers consist of two major elements: the laterally packed, thin ordered core component formed by elastin, and the fibrillar components called microfibrils serving as a scaffold during the incorporation of elastin [83, 84]. In the mammalian genome there is only a single elastin gene present, which is located on chromosome 7q11.1-21.1 [85]. Elastin is synthesized as a soluble tropoelastin precursor containing alternating hydrophobic domains rich in non-polar amino acids such as Gly, Val, Pro and Ala, and hydrophilic domains rich in Lys and Ala involved in cross-linking [86]. So far 11 human tropoelastin splice variants have been identified [87]. Tropoelastin undergoes very little post-translational modification such as the hydroxylation of Pro residues [88], and following secretion it is rapidly incorporated into the forming elastic fiber with no further proteolytic processing [89]. Tropoelastin is then subject to a process of ordered self-aggregation as a result of multiple specific interactions of hydrophobic domains, that concentrates and aligns tropoelastin molecules prior to crosslinking catalyzed by lysyl oxidase, the final step in the formation of the insoluble elastin polymer [83, 87].

In addition to the above-mentioned substrates of LOX, *in vitro* studies revealed that the enzyme is able to oxidize mono- and diamines [77, 90], synthetic elastin-like polypeptides [91], H1 histone [92] and the lysine residues of the secreted basic fibroblast growth factor (bFGF) [93] as well.

Novel functions of lysyl oxidase

In addition to its involvement in the cross-linking of collagens and elastin, LOX has been demonstrated to act as a tumor suppressor, to induce chemotaxis, and to have intracellular and moreover intranuclear activities [11, 66].

LOX's involvement in cell growth control was first implied in a study of sea urchin development and a report on *Xenopus* oocyte maturation. The enzyme specific activity was demonstrated to increase over six-fold during development, showing the greatest rise during gastrulation and larva formation in sea urchins. The treatment of the developing embryos with the specific inhibitor BAPN led to developmental arrest and the accumulation of non-crosslinked collagen molecules, suggesting a critical role for LOX in sea urchin gastrulation and morphogenesis [94].

Using a Xenopus model Di Donato et al. revealed the inhibitory effect of LOX on *ras*-dependent oocyte maturation when coinjected with oncogenic p21-Ha-*ras* into maturing oocytes. BAPN treatment of the injected oocytes successfully reversed this inhibition. This study also demonstrated that lysyl oxidase action depended on *de novo* protein synthesis and was therefore probably mediated by an unknown, newly synthesized protein that acted as an inhibitor of oocyte maturation [95]. Therefore this report suggested an intracellular role for lysyl oxidase.

LOX has also been implied in cellular senescence. Differential screening revealed several gene sequences, including a lysyl oxidase-like sequence WS9-14 , or LOXL2 that are overexpressed by both normal senescent human fibroblasts and fibroblasts derived from subjects with Werner's syndrome showing the characteristics of premature aging [25, 96]. Senescence in animals may depend on cellular senescence, cell death, modified proliferation of stem cells, interaction of different factors, as well as major qualitative and quantitative alterations in the components of extracellular matrix [97]. LOX and the members of the enzyme family might play an important role in the changes of the ECM associated with aging.

In addition, the 32kD purified LOX was described to have a strong chemoattractant effect on different cell lines such as vascular smooth muscle cells (VMSC) [98], human monocytes [99], fibroblasts [100], and breast cancer cell lines [3]. These experiments brought to light that the chemotactic response of the VSMCs depended on the H_2O_2 product of the LOX-catalyzed reaction, as cells were chemotactically responsive to the active enzyme, but not if the enzyme was inactivated or was administered in the presence of catalase [98]. Similarly, a hydrogen peroxide–based mechanism was found to mediate LOX's

migration-facilitating effect in breast cancer cells [4] as discussed in the *Lysyl oxidase in cancer* section.

The involvement of lysyl oxidase in modulation of cell phenotype was suggested upon the discovery of a *ras* recision gene (*rrg*) in mice. The *rrg* transcript was highly expressed in normal fibroblasts, significantly decreased in the tumorigenic derivatives of these cells, and reexpressed at high levels in the non-tumorigenic revertants [101]. Cloning and sequencing of mouse *rrg* cDNA revealed its 96% sequence homology with the rat LOX cDNA, while mouse rrg exhibits 89% homology with the human LOX at the amino acid level [102, 103]. Consistently, LOX mRNA expression was found to be markedly low in various malignantly transformed cell lines as well [104, 105]. Knock-down of LOX by its antisense cDNA caused transformation of rat fibroblasts, which showed anchorage-independent growth and were highly tumorigenic in nude mice [106]. Further investigation revealed that LOX expression was inhibited by an FGF autocrine pathway in the transformed tumorigenic cells, where administration of suramin, a general inhibitor of growth factor receptor binding resulted in a reversible tenfold increase in LOX expression [107]. The anti-oncogenic effect of LOX in *ras*-transformed fibroblasts was likely due to its ability to inhibit signaling pathways that lead to activation of NF-κB transcription factor, a mediator of *ras* activity [108].

Interestingly, several groups have reported that LOX mRNA expression and enzyme activity was upregulated in highly invasive rat prostatic carcinoma cell lines, human cutaneous and uveal melanoma [3], invasive and metastatic breast cancer cell lines [2, 3], and metastatic breast tumors [4]. Conversely, poorly invasive MCF-7 cells overexpressing LOX show an increase in migration, indicating an important role for the enzyme during the course of epithelial-mesenchymal transition [4]. The dual role of LOX in tumor suppression and promotion will be discussed in the *Lysyl oxidase in cancer* section.

Although information on functions of LOX mostly came from studies conducted with mesenchymal cell types, increasing evidence supports the necessity of a more adequate model to elucidate the novel roles of LOX in epithelial tissues as well. In this regard, it is important to refer to a recent study that showed expression, enzyme activity and the cytoplasmic localization of mature LOX in polarized epithelial MDCKII and MCF-10A cells [109]. These cell lines might serve as suitable model systems for addressing LOX's novel role in epithelia.

LOX enzyme function within the nucleus

Besides its cytoplasmic occurrence, nuclear localization of LOX has also been reported in various cell types. First, the enzyme was detected within the nuclei of cultured murine 3T3 fibroblasts and rat aortic smooth muscle cells, and evidence on its catalytic activity inside the nucleus of the latter was provided [110]. Then the fully processed LOX was demonstrated to translocate from the extracellular space into the nucleus of vascular smooth muscle cells in a specific manner, independent of the enzyme's catalytic activity, as intracellular uptake and distribution were unaffected by treatment with BAPN [111]. Additional data supported the presence of LOX within the nuclei of other cell types [66, 112], and another member of the enzyme family, LOXL3 was detected in mouse liver predominantly in the nuclei of hepatocytes [113]. Interestingly, nuclear localization was demonstrated for the *Drosophila melanogaster* lysyl oxidase (DmLOXL-1) as well in fruit fly embryos and adult salivary gland cells [114]. However, the mechanism by which LOX is transported into the nucleus is yet to be discovered. Evidence on LOX's ability to both act on histone H1 as a substrate [92], and to bind to histones H1 and H2 [115] *in vitro*, also to modify chromatin structure [113, 116] provides a possible explanation for the nuclear role of the enzyme, supporting this way the possibility of LOX functions different from its extracellular crosslinking activity.

In respect to its nuclear localization, it is of special interest that lysyl oxidase was reported to act as a specific inducer of transcription activity of the human collagen III α1 (COL3A1) promoter [117]. Experiments carried out in transfected monkey renal fibroblast (COS7) cells overexpressing the mature form of LOX showed a dramatic increase in COL3A1 promoter activity in a reporter gene assay when compared to the cells containing the vector without the LOX sequence. This inducer effect of LOX was abolished upon the administration of its specific inhibitor, BAPN. The Ku antigen heterodimer complex, which is involved in some of the main DNA repair and recombination processes [118, 119], was identified as a possible mediator of LOX's transcription-activator effect [117].

Lysyl oxidase in pathologic conditions

The expression and activity of lysyl oxidase have been reported to be altered in many human pathologies compared to normal conditions. The possibility of LOX's involvement in the pathogenesis of these diseases is supported by increasing evidence.

Pathologies associated with reduced LOX activity

The disorders characterized by decreased lysyl oxidase activity include lathyrism, nutritional copper deficiency, and recessively inherited human genetic maladies such as the X-linked Menkes disease (OMIM 309400) with its milder form, the occipital horn syndrome (OHS, OMIM 304150) [32, 120, 121].

Lathyrism occurs upon the ingestion of *Lathyrus odoratus* (sweet pea) containing β-(γ-glutamyl)aminoproprionitrile that is metabolized to β-aminopropionitrile (BAPN) a specific inhibitor of LOX activity. Administration of BAPN, leads to abnormal cross-linking of collagens and elastin, consequently lathyrism is characterized by kyphoscoliosis, bone deformities, weakening of the epiphyseal plates, tendons, ligament attachments, skin and cartilage, dislocation of joints, loss of teeth, hernias, and vascular rupture [122].

Copper is an essential cofactor of the lysyl oxidase isoenzymes, therefore decreased availability of copper in addition to affecting several copper-dependent enzymes, leads to diminished LOX enzyme activity and connective tissue defects as well as to manifestations closely resembling those of lathyrism [122]. Activity of lysyl oxidase has been demonstrated to change in response to variations in dietary copper levels [123-125]. Decreased lysyl oxidase activity due to copper deficiency leads to diminished crosslink-formation accounting for the connective tissue defects in growing animals such as aortic aneurysms, bone fragility and internal hemorrhages [126, 127].

Menkes' disease as well as OHS are due to mutations in the gene *ATP7A*, that encodes an ATP-dependent copper transporter [128, 129]. During normal copper metabolism, following ingestion, copper is absorbed in the small intestine, and stored in the liver. Biliary excretion via the gall bladder is the major route of copper elimination from the body and a small amount of Cu is found in urine [130]. Both OHS and Menkes disease are characterized by abnormal neural development, connective tissue abnormalities and death in infancy [131]. Manifestations in OHS include bladder diverticula with spontaneous ruptures, inguinal hernias, slight skin laxity and hyperextensibility, skeletal changes, such as occipital horn-like exostoses, and vascular tortuoisity. Manifestations in Menkes' disease also include bladder diverticula, hyperextensibility and laxity of skin, and skeletal abnormalities, and the disease is characterized by neurological degeneration, mental retardation, and generalized arterial disease with grossly abnormal elastic lamellae. The accumulation of Cu in intestinal cells, kidney and vascular endothelial cells in the blood brain barrier in Menkes' patients leads to Cu deficiency and defects in the activities of many Cu-dependent proteins [132], including lysyl oxidase. The reduced lysyl oxidase activity in these pa-

thologies has been demonstrated in skin samples and cultured fibroblasts [120, 133, 134]. Fibroblasts from patients with Menkes syndrome and OHS do not secrete LOX into their medium or contain any significant amounts of a copper-deficient, catalytically inactive lysyl oxidase protein in their cytoplasms suggesting that the synthesis of LOX protein itself is impaired [135, 136]

Increased LOX activity in fibrosis

Fibrotic diseases present major medical problems such as disfigurement, progressive disability and death. Pathological fibrosis can affect any organ or tissue, but it occurs most often in the lung, liver, kidney, and skin. Fibrosis by definition is the formation or development of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process, as opposed to formation of fibrous tissue as a normal constituent of an organ or tissue. Fibrotic diseases are thus characterized by the excessive production, deposition and contraction of the extracellular matrix components, especially insoluble collagen fibers [137]. An association between increased lysyl oxidase activity and organ fibrosis has been suggested in many experimental models including liver fibrosis [138-140], pulmonary fibrosis [141-143], kidney [144], and myocardial fibrosis [145]. Therefore LOX has become the object of pharmaceutical research searching for molecular targets for the treatment of fibrosis. Initially, the treatment of fibrosis with the specific LOX-inhibitor BAPN seemed plausible, but due to its unacceptable toxic effect it proved to be not feasible [146, 147].

Lysyl oxidase in cancer

Altered LOX expression and activity have been reported in various cancerous tissues and neoplastic cell lines, and subsequently the enzyme was identified as a potential modulator of tumorigenesis and tumor progression. Recent studies have demonstrated the upregulation of LOX expression in breast carcinomas, central nervous system cancers, head and neck sqamous cell carcinomas, lung adenocarcinomas, melanomas, osteosarcomas and prostate adenocarcinomas [3, 148-153]. Elevated LOX expression is associated with the early stromal reaction in breast cancer as well [154], similarly to another member of the enzyme family, LOXL that has been localized to the site of de novo fibrinogenesis in early stromal reaction of breast carcinoma [155]. LOXL and similarly, LOXL2 have been implicated in breast tumor progression as both enzymes are expressed in highly inva-

sive breast cancer cell lines, although LOXL2 appears to have a stronger association with the invasive/metastatic phenotype observed [3].

LOX's role in tumor progression in breast cancer was revealed when inhibition of the enzyme with either BAPN or antisense oligonucleotides to *LOX* mRNA resulted in the inhibition of *in vitro* invasion of highly invasive and metastatic breast cancer cell lines [3]. In addition, the poorly invasive MCF-7 breast tumor cells, when transfected with the murine *LOX* gene showed a 2-fold increase in invasiveness that was reversible by the administration of BAPN. Subsequently the involvement of a hydrogen peroxide-dependent mechanism was suggested to play a role in the regulation of cell migration in LOX-induced breast cancer invasion [4]. Erler et al. have demonstrated that the inhibition of LOX abolished lung and liver metastases in an *in vivo* mouse model for breast cancer [151]. Recent evidence also suggested LOX's implication in the process of epithelial-mesenchymal transition (EMT), a mechanism that facilitates cellular repositioning and redistribution during embryonic development, tissue reconstruction after injury, carcinogenesis, and tumor metastasis [109].

These reports are somewhat conflicting with the previous findings describing LOX as a tumor suppressor. The contradictory findings of LOX's role in tumor progression could be due to the different cell origins as LOX's tumor suppressor function was studied in cells of fibroblast and mesenchymal origin, while it showed tumor-promoting activities in carcinomas, tumors of ectodermal origin. In addition, many factors could influence the cellular response to LOX expression such as cell differentiation stage, genetic patterns, availability of LOX substrates, and cell-cell or cell-ECM interactions. Interestingly, not only LOX has such a complex role in tumor progression, as another member of the enzyme family, LOXL2 was demonstrated to be downregulated in ras-transformed fibroblasts [156], head and neck squamous cell carcinomas [157], and serous ovarian carcinomas [158], while its upregulation was noted in highly tumorigenic, metastatic mouse squamous and spindle cell carcinoma [159], and invasive breast cancer cell lines [3], as well as in pancreatic cancers [160] compared to normal controls. Fong et al. demonstrated that these differences in LOXL2 expression are likely due to the stage of tumor progression, since an increase in LOXL2 expression was observed in less-differentiated colon and esophageal tumors, while in well-differentiated colon tumors significant decrease in the expression of the enzyme was observed [161].

Future studies are therefore needed to address the mechanisms by which LOX and other members of the enzyme family may contribute to the tumorigenic processes.

During the course of our work we have identified human placental lactogen (hPL) as a LOX-interacting protein that might play a novel part in carcinogenesis, in concert with the enzyme. The following section gives a detailed introduction of the hormone hPL.

Human placental lactogen

In 1936 Ehrhardt was the first to describe lactogenic activity in the placenta [162]. Subsequent studies demonstrated the presence of a protein hormone in the placenta that had biological properties similar to that of human pituitary growth hormone and prolactin [163, 164]. This hormone was initially designated as human chorionic somatomammotropin (hCS) [165], and most databases use the nomenclature chorionic somatomammotropin hormone (CSH). However, as a similar abbreviation hCS had previously been assigned to human citrate synthetase [166], the terminology "human placental lactogen" (hPL) is more widespread in the scientific literature.

The somatotropin/prolactin gene family

Human placental lactogen (hPL) is a member of the somatotropin/prolactin gene family, an evolutionary related gene family which includes human growth hormone (hGH) and human prolactin (hPRL). The *hPRL* gene is located on chromosome 6 [167], while the growth hormone gene cluster, that includes three *hPL* and two *hGH* genes, localizes to chromosome 17q21-24 [166, 168, 169]. All five genes within the cluster have the same transcriptional orientation and their arrangement is as follows: 5'- *hGH-N* (N is for normal) – *hPL-1* (also known as hCS-L ; L for like) – *hPL-2* (also named hPL-4, hPL-A, hCS-A or CSH-1) – *hGH-V* (V is for variant) – and *hPL-3* (also referred to as hPL-B, hCS-B or CSH2) [170, 171]. The *hGH* and *hPL* genes share a common ancestral origin [172, 173] and probably have evolved by gene duplication. Alignment of the genes reveals an identical molecular architecture: the transcriptional units are split by four introns, and their open reading frames exhibit perfect codon colinearity [174]. 90-95% sequence homology seen in exons, introns, 5' and immediate 3' nontranscribed regions reflects the recent divergence of the genes. The regions downstream of the polyadenylation sites in the genes for hGH and its variant, but not for hPL, contain members of an Alu family repeat sequence [175].

Expression of the growth hormone and placental lactogen genes

While hGH-N is expressed in the pituitary gland, the other four genes have been found to be active in the placenta under normal physiological conditions. Although, expression of hPL has recently been discovered in the ovaries and testes as well [176, 177],

suggesting novel auto/paracrine functions for hPL in both the female and male reproductive system.

Alternative splicing generates several isoforms of each of the five hormones, leading to further diversity and potential for specialization. Differential splicing of the primary hGH-N transcript results in the generation of 22 kD and 20 kD isoforms with the former being the most abundant. The *hGH-V* gene has been demonstrated to be expressed as two alternatively spliced mRNAs in the placental syncytiotrophoblasts resulting in the expression of a cell-associated and a secreted protein product [178-180].

In a second intron 5'-splice donor site, characteristic to the other four genes, the *hPL-1* gene contains a G to A transition which destroys the normal donor site, that explains initial data on the absence of hPL-1-derived cDNA in a screening of a human placental cDNA library [181]. However, this particular family member is in fact actively transcribed in placental villi, while alternative splicing suggests that the majority of the transcripts would be unable to express a secreted protein [182, 183].

The *hPL-2* and *hPL-3* genes are expressed throughout pregnancy, in increasing amounts approaching term [184] as the term placenta contains up to 5 times more hPL mRNA than a comparable quantity of first trimester tissue [183]. The hPL-2 and hPL-3 mRNAs show a slight divergence in their nucleotide sequence, and their encoded prehormones differ in a single amino acid of the signal peptide region as at position -24 hPL-2 contains a proline, while hPL-3 contains an alanine. Nevertheless, the secreted protein products are identical [181].

The human placental lactogen protein

Human placental lactogen has been demonstrated to be a major protein component synthesized by the placental syncytiotrophoblasts as it represents about 5% of the total placental mRNA at term [174] and 2-4% of the total proteins [185]. The hormone can be detected by immunofluorescence 5-10 days following implantation in the syncytial layer [186, 187]. Three weeks post-fertilization, hPL can be detected in the maternal serum, and its levels rise throughout pregnancy [188]. The half-life of hPL in maternal serum is 10-30 min, which explains its rapid disappearance from maternal serum after delivery [189, 190].

Previous studies have revealed that the hormone is produced as a propeptide of 25 kD, that has a 26-amino acid signal sequence at its N-terminus [181]. Propeptide cleavage is associated only with a cell's membrane-bound ribosomal fraction [191, 192], and this pre-hPL is secreted post-translationally [193]. The mature human placental lactogen is a

single polypeptide hormone of approximately 22 kD consisting of 191 amino acids with two intramolecular disulfide bridges [194] that has been shown to participate in the formation of biologically active homodimers *in vivo* [195-197]. In accordance with their common genetic ancestry and high nucleotide sequence identity, hPL exhibits 85% and 25% amino acid sequence homology to hGH and hPRL, respectively [198] **(Figure 4)**.

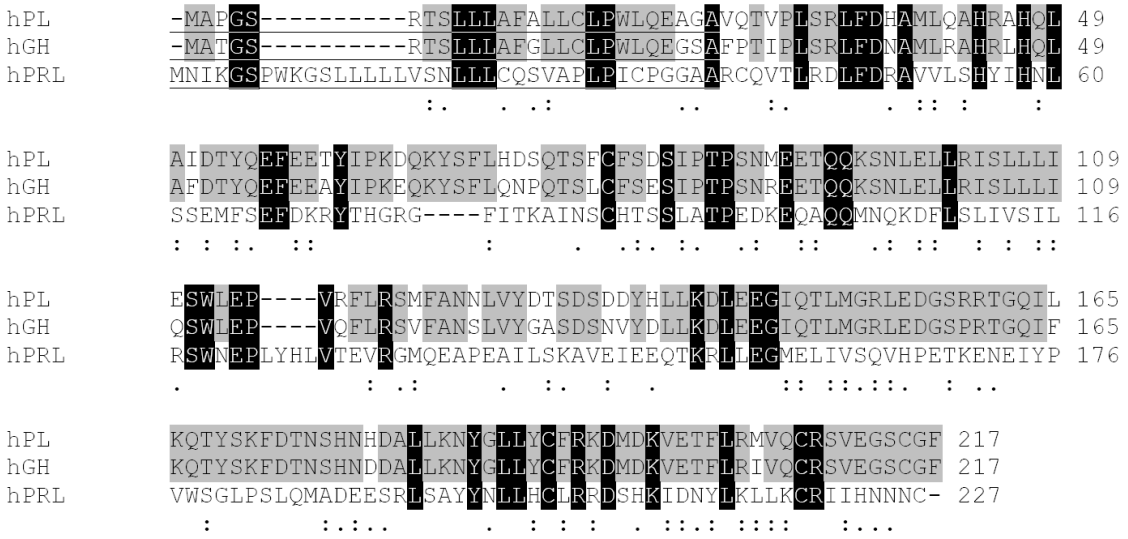

Figure 4. Comparison of the amino acid sequences of the somatotropin/prolactin hormone family demonstrates their homology.
The signal peptides are underlined; the residues conserved in all three proteins are white with black highlighting; amino acids conserved in hPL and hGH only are black with grey highlighting.; ":" marks conserved substitutions; "." marks semi-conserved substitutions.

Analysis of the 3D structure of the ovine PL reveals the long four-helix bundle structure, similar to that of hGH, with an up–up–down–down orientation of the four main helices as it is demonstrated on **Figure 5** [199]. Subtle differences in packing of the protein results in a slightly more elongated oPL molecule than hGH. In addition, oPL contains a 12-residue N-terminal extension. oPL and hGH also show a remarkable similarity of their receptor binding site as it is demonstrated by the molecular rendering of **Figure 5**.

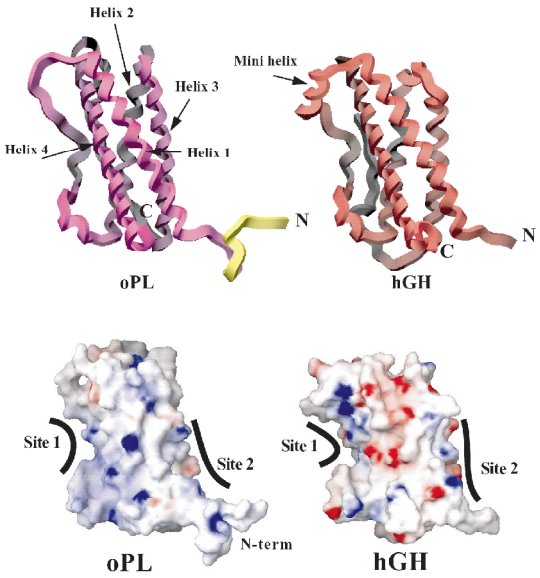

Figure 5. 3D structure comparison of ovine PL and human GH.
Helices 1–4 are labeled on the ribbon diagram of the oPL molecule. The N-terminal extension on oPL is shown in yellow. Site 1 and 2 are the receptor binding sites exhibiting a high degree of similarity. Adopted from Elkins et al. [199].

hPL in physiological and pathological processes

Based on its structural similarity to hGH, several studies focused on the possible growth-promoting effects of hPL. Recent investigations using isolated human fetal tissues demonstrate that hPL has a direct somatotropic and metabolic effect. Hill et al. found that hPL, but not hGH promotes amino acid transport, [^{3}H] thymidine incorporation, and mitogenesis in cultured fibroblasts and myoblasts of fetal origin, suggesting that hPL may contribute to the growth stimulus in human fetal connective tissues [200, 201]. In addition to its direct growth-promoting function, as it is suggested by substantial evidence, hPL likely influences fetal growth in an indirect manner by altering maternal metabolism as an insulin antagonist [189, 190]. Furthermore, hPL has been implicated in lipolysis as well. Human placental lactogen was shown to be lipolytic on adipose tissue from both pregnant and non-pregnant women as adipose tissue explants and adipocytes released glycerol and nonesterified fatty acids upon hPL stimulation [202]. hPL also induces glucose uptake, oxidation and incorporation into glycogen, glycerol and fatty acids in adipocytes [203, 204]. The current model of hPL's role in maternal metabolism is that the hormone increases glucose availability for the fetus by its insulin antagonist and lipolytic activity **(Figure 6)** [189].

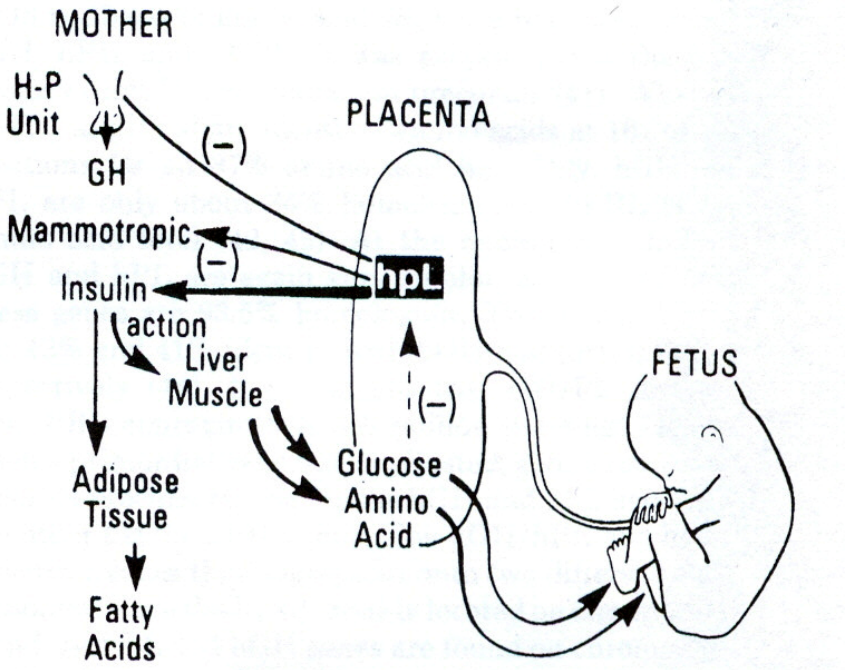

Figure 6. Physiological role of hPL during pregnancy.
hPL's role in maternal metabolism is to increase glucose availability for the fetus by its lipolytic and insulin antagonist activity. (-), inhibitory effect. Adopted from Walker et al. [189].

Placental lactogen was named after its *in vivo* lactogenic effect observed in rabbits [163], although this activity has not been demonstrated so far in humans. Nevertheless, hPL possesses a mammotropic activity, as it has been reported to stimulate DNA synthesis in tumorous breast epithelial cells *in vitro* [205], and in human breast tumors maintained in athymic mice [206]. The role of PL in human mammary gland may be to stimulate cell proliferation rather than milk secretion.

Deletion of the *hPL2* gene have been associated with the clinically and genetically heterogeneous Silver-Russell syndrome (SRS) [207], a congenital disorder characterized by intrauterine and postnatal growth retardation associated with dysmorphic features such as relative macrocephaly, a prominent forehead, a small triangular face, clinodactyly and asymmetry of head, limbs and trunk [208]. Data on hPL's role in fetal growth has been ambiguous as one group reported that hPL was not essential for normal pregnancy [209], while according to another group, the complete lack of hPL production resulted in severe growth retardation of an otherwise normal baby [210]. Recent investigation has revealed hypomethylation of the telomeric imprinting region on chromosome 11p15, another chromosomal region associated with SRS, that made the role of *hPL* deletion in the etiology of SRS unlikely [211].

Ectopic expression of PL has been reported in many cases of malignant transformations such as esophageal neoplasms [212], in cases of lung cancer [213], in epithelial ovarian cancer [214] and testicular germ cell tumors [215].

PL has also been implicated in breast cancer, although its significance remains unexplored. Ectopic expression of hPL by human breast tumors have been reported by Sheth et al. [216]. All three PL genes have been shown to be amplified in 22% of breast carcinomas [217] and PL expression was demonstrated within tumors in 77% of the cases in this panel, while another study reported PL expression in 82% of breast tumors [218]. In addition, a trend was noted toward an increased incidence of lymph node metastases with amplification of the PL genes [217].

Interestingly, the other two members of the protein family, GH and PRL have been reported as potent oncogenes, acting through their receptors. Similarly, hPL is able to bind to and dimerize the growth hormone receptor (GHR) and prolactin receptor (PRLR). PL binds to the GH receptor (GHR) with lower affinity than GH [219] despite their significant (85%) amino acid sequence homology, yet PL binds to the PRL receptor (PRLR) with a similar affinity as hGH [219]. 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* [220]. Epidemiologic evidence supports a role for PRL in the pathogenesis [221], progression [222], and poor prognosis of breast carcinoma [223, 224]. 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 thoroughly investigated.

SPECIFIC AIMS

Specific Aim 1. To determine the specificity of a LOX interaction with the yeast two-hybrid positive protein placental lactogen, represented by multiple clones using yeast direct interaction studies.

Test the specificity of the interaction of the library proteins with the LOX bait proteins in yeast by directly co-transforming various combinations of bait and target plasmids into yeast and assaying for reporter gene activation.

Specific Aim 2. To confirm and characterize the LOX-PL interaction *in vitro*.

a) Generate expression constructs for LOX and LOX fragments.

b) Express and purify recombinant LOX proteins in quantities sufficient for further *in vitro* biochemical experiments.

c) Perform *in vitro* binding analysis using pull-down and Far-Western assays.

d) Perform solid phase binding assays to measure the equilibrium disassociation constant (Kd) of the protein interaction, and determine LOX-binding affinity for hGH and hPRL, two related members of the hormone family.

Specific Aim 3. To investigate LOX-PL interactions *in vivo*.

a) Test LOX and PL expression in breast cancer tissues by immunofluorescent staining and confirm their co-expression in mammary neoplasms that is also supported by data from the literature.

b) Identify an appropriate *in vivo* model to test the native LOX-PL complex using co-immunoprecipitation.

Specific Aim 4. To explore the effects of the identified LOX-PL protein interactions on the catalytic activity of LOX.

Perform enzyme activity assays in order to clarify the nature of the protein interactions and determine whether it is an enzyme-substrate or enzyme-inhibitor/enhancer relation.

Specific Aim 5. To investigate the biological significance of the identified protein interactions.

a) Generate stable cell lines overexpressing either LOX or PL or both of the proteins for further phenotypic analysis.

b) Study the cellular phenotype including proliferative and migratory characteristics of the overexpressing cell lines in order to determine the biological role of the protein interaction.

Yeast Direct Interactions

Plasmid construction for yeast two-hybrid assays

A yeast two-hybrid screen was previously performed according to Clontech's System 3 protocol using a human placental cDNA library [225], which contained over $3.5x10^6$ independent clones generated from human placental mRNA with oligo-dT primers as described previously [55]. For the yeast direct interaction bait LOX constructs primers were designed to introduce EcoRI and BamHI restriction sites 5' and 3', respectively, to sequence-verified human LOX cDNA in order to clone the inserts into Clontech's pGBKT7 vector **(Figure 7)** into EcoRI and BamHI sites downstream and in frame with the GAL4 DNA-binding domain (GAL4-DB).

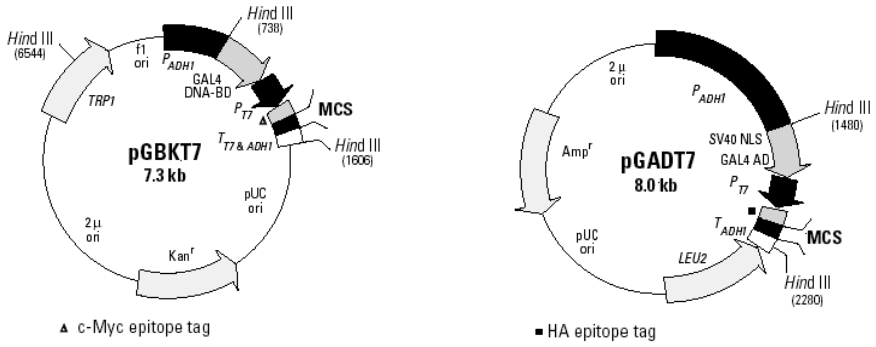

Figure 7. Vectors pGBKT7 and pGADT7.
Schematic diagram of the yeast expression vectors pGBKT7 and pGADT7 [225] used for the yeast two-hybrid screen and the direct interaction studies.

The LOX cDNA template used was a plasmid expression construct pcDNA3.1-LOX previously cloned in our lab to contain the complete LOX open reading frame (nt 1-1254). Several LOX expression constructs were designed and cloned, including the full-length pro-LOX (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 pro-LOX without the CRL domain (amino acids 1–348), and the mature LOX without the CRL domain (amino acids 349–417). The finished constructs, pGBKT7-$LOX_{1–417}$, pGBKT7-$LOX_{169–417}$, pGBKT7-$LOX_{1–168}$, pGBKT7-$LOX_{349–417}$, pGBKT7-$LOX_{1–348}$, and pGBKT7-$LOX_{169–348}$, 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

pGBKT7-LOXL$_{26–574}$ (amino acids 26–574), pGBKT7-LOXL-Nterm$_{26–368}$ (amino acids 26–368), pGBKT7-LOXL-Cterm$_{338–574}$ (amino acids 338–574), pGBKT7-LOXL2-Nterm$_{1–547}$ (amino acids 1–547), and pGBKT7-LOXL2-Cterm$_{548–774}$ (amino acids 548–774) were as shown in

Table 1.

Primer	Sequence	Restriction Site
pGBKT7-proLOX F	5'-ACA TGC CAT GGT GAT GCG CTT CGC CTG GAC CG-3'	*Eco*RI
pGBKT7-proLOX R	5'-CGC GGA TCC CTA ATA CGG TGA AAT TGT GCA GC-3'	*BamH*I
pGBKT7-LOX F	5'-CCG GAA TTC GAC GAC CCT TAC AAC CCC TA-3'	*Eco*RI
pGBKT7-LOX R	5'-CGC GGA TCC CTA ATA CGG TGA AAT TGT GCA GC-3'	*BamH*I
pGBKT7-LOX$_{1-168}$ F	5'-ACA TGC CAT GGT GAT GCG CTT CGC CTG GAC CG-3'	*Eco*RI
pGBKT7-LOX$_{1-168}$ R	5'-TAT GGA TCC TTA GCC CAC CAT GCC GTC CAC-3'	*BamH*I
pGBKT7-LOX$_{1-348}$ F	5'-ACA TGC CAT GGT GAT GCG CTT CGC CTG GAC CG-3'	*Eco*RI
pGBKT7-LOX$_{1-348}$ R	5'-GC GGA TCC CTA ACT CAA TCC CTG TGT GTG TG-3'	*BamH*I
pGBKT7-LOX$_{169-348}$ F	5'-CCG GAA TTC GAC GAC CCT TAC AAC CCC TA-3'	*Eco*RI
pGBKT7-LOX$_{169-348}$ R	5'-GC GGA TCC CTA ACT CAA TCC CTG TGT GTG TG-3'	*BamH*I
pGBKT7-LOX$_{349-417}$ F	5'-CCG GAA TTC CCT GGC TGT TAT GAT ACC TA-3'	*Eco*RI
pGBKT7-LOX$_{349-417}$ F	5'-CGC GGA TCC CTA ATA CGG TGA AAT TGT GCA GC-3'	*BamH*I

Table 1. The primers used to amplify the LOX fragments

After PCR amplification, the inserts were digested and ligated into the pGBKT7 vector, and sequence-verified prior to transformation into the AH109 yeast. The AH109 genome contains four GAL4-responsive reporter genes:ADE2, HIS3, *lac*Z, and MEL1. Following amplification and cloning into the pGADT7 vector the placental cDNA library obtained from Clontech was transformed into AH-109 yeast that contained pGBKT7-LOX$_{1-417}$ (the full length protein) or pGBKT7-LOX$_{169-417}$ (the mature enzyme), with an efficiency measured by dilutions of transformants on non-selective plates. Clones containing interacting

proteins were selected by growth on a drop-out minimal medium lacking leucine, tryptophan, histidine, and adenine and by activation of the lacZ gene. The positive clones were streaked multiple times on these selective plates to get single clones and to verify their growth under selective conditions. The library plasmids were isolated from the positive yeast clones and the cDNA inserts were sequenced using an ABI 310 capillary sequencer. The interactions of the identified positive clones were further characterized by performing direct co-transformations 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 co-transformation assays also used selection based on HIS3 and ADE2 reporter gene expression.

Plasmid Purifications from Yeast

To purify plasmids containing the positive clones from yeast, we used Quiagen Plasmid Mini Kit according to the manufacturer's protocol. Yeast colonies isolated as positives from the two-hybrid screen were inoculated in 3 ml of WL- dropout media and incubated at 30˚C overnight with shaking at 270 rpm. The next morning, 1.5 ml of the cultured cells were transferred to a microcentrifuge tube and centrifuged at 14,000 x g for 5 min to pellet the cells. The media supernatant was aspirated, and the cells were resuspended in 150 µl of Buffer P1 (Resuspension buffer: 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). To digest the yeast cell wall, 2 µl of Zymolase enzyme was added to the cell suspension and the sample was incubated at 37˚C for 60 min. 150 µl of Buffer P2 (Lysis Buffer: 200 mM NaOH; 1% SDS) was added, then 150 µl of Buffer P3 (Neutralization buffer: 3 M KAc, pH 5.5), and the sample was incubated on ice for 5 min. The sample was centrifuged at 14,000 x g for 20 min at 4˚C to spin down the yeast proteins. The supernatant containing the DNA was then transferred to a fresh microcentrifuge tube. The plasmid DNA was precipitated by adding 1/10th of the total volume of 3 M sodium acetate pH 5.2, and twice the total volume of ice-cold 100% EtOH. The sample was mixed well, and frozen at -80˚C for 60 min. The sample was centrifuged at 14,000 x g for 20 min at 4˚C to pellet the DNA, and then the supernatant was aspirated. The DNA pellet was washed once with 70% EtOH, and air-dried. Finally the plasmid DNA was dissolved in 20 µl of TE buffer (10 mM Tris, pH 8; 1 mM EDTA).

Yeast co-transformation

The yeast co-transformation was performed according to Clontech's protocol [225]. A 50 ml culture of AH109 yeast strain [225] was grown in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 10% (v/v) dextrose) overnight at 30°C with shaking, then was added to 250 ml of fresh YPD and incubated for 4 h until the OD_{600} was 0.5 ± 0.1. The cells were pooled by centrifugation at 1000 x g for 5 min at room temperature and were resuspended in 25 ml of H_2O. This suspension was centrifuged again at 1000 x g for 5 min at room temperature, and the cell pellet was resuspended in 1.5 ml of sterile 1x TE/LiAc solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5). To 0.1 ml of this competent cell suspension we added 0.1 μg of DNA-BD-bait plasmid (pGBKT7-LOX constructs), 0.1 μg AD-target plasmid (pGADT7-cl1, containing the full length PL sequence), 0.1 mg Herring testes carrier DNA and 0.6 ml of sterile PEG/LiAc solution (40% polyethylene glycol 4000, 1x TE/LiAc). After vortexing, the cells were incubated for 30 min at 30°C with shaking. Then 70 μl of DMSO was added and the cells were heat-shocked at 42°C for 15 min. After being chilled on ice for 2 min, the yeast centrifuged for 5 sec at 18000 x g, resuspended in 150 μl of 1x TE and plated on selective WL- dropout plates. Individual colonies of successfully co-transformed yeast were restreaked onto WL-master plates, and then restreaked onto AHWL- dropout plates. Subsequently, colonies of the co-transformants were suspended in the wells of a microplate in 100 μl 1x TE buffer. After measuring the OD at 595 nm, the concentrations were calculated and the samples were diluted to an identical final cell concentration. 5 μl of these yeast suspensions were spotted on AHWL- dropout plates in triplicates and incubated at 30˚C for 4 days. Differences in growth were the results of the different binding affinity of the interacting proteins, leading to different levels of reporter gene activation.

Bacterial protein expression

For *in vitro* and *in vivo* binding experiments we needed purified LOX protein, which is not available commercially. So we cloned the LOX gene fragments into the pGEX-4T-1 plasmid [226] **(Figure 8)** to generate bacterial expression constructs for LOX, proLOX, and various LOX cDNA fragments. The plasmid constructs were designed to produce LOX protein fragments with an N-terminal glutathione-S-transferase (GST) tag. The previously generated pGBKT7-LOX constructs used in the yeast direct interaction experiments were used as templates.

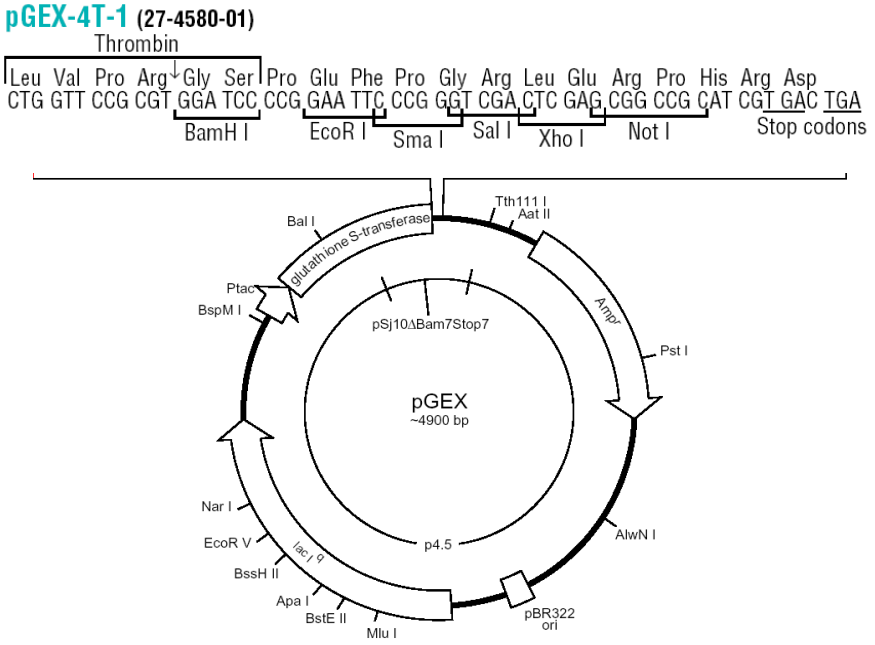

Figure 8. The pGEX-4T-1 bacterial expression vector.
Schematic diagram of the construct used for the bacterial expression of the LOX gene fragments

Restriction enzyme digestion

The pGBKT7-LOX plasmids (pGBKT7-proLOX, pGBKT7-LOX, pGBKT7-LOX_{1-168}, pGBKT7-LOX_{1-348}, pGBKT7-$LOX_{169-348}$, and pGBKT7-$LOX_{349-417}$), as well as the pGEX-4T-1 vector, were each restriction digested with EcoRI and SalI (New England Biolabs) enzymes. The double digestions were performed using EcoRI digestion buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl, 0.025% Triton X-100, pH 7.5) with 100 µg/ml BSA as recommended by the manufacturer. After incubation at 37˚C for 120 min, the digested DNA was analyzed by agarose gel electrophoresis to separate the digested fragments for following isolation of the inserts.

Agarose gel electrophoresis

The enzymatically digested samples were mixed with 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll), loaded onto an agarose gel (1,2% in 1x TAE with 0,5 µg/ml EtBr) and run at 100 V, 300 mA for 50 min. To determine the size of the separated DNA fragments, molecular weight markers (New England Biolabs) were run parallel with the samples. The gel was analyzed on a UV transilluminator with a Kodak EDAS 290 imaging system.

DNA purification from agarose

After separation of the samples on agarose gel, we isolated the digested fragments from the agarose using the Geneclean Spin Kit (Q-BIOgene). We melted the gel slices containing the plasmid DNA in 400 µl of Geneclean Spin Glassmilk in a Geneclean Spin Filter at 55°C for 5 min, while inverting a few times to prevent the settling of matrix. The liquid was centrifuged out of filter at 12 000 x g into a Catch Tube. We washed the filter with 500µl of Geneclean Spin New Wash solution, and then spun the wash out of the filter at 12000 x g for 30 sec. After drying the pellet by centrifuging for 2 min at 12 000 x g, and transferring the filter into a fresh Catch Tube, we eluted the DNA by resuspending the Glassmilk on the filter in 20µl of Geneclean Spin Elution Solution, and centrifuging for 30 sec at 12 000 x g.

Ligation

For the ligation of the insert LOX DNA fragments into the pGEX-4T-1 plasmid we made a ligation reaction using a T4 DNA Ligase [227], according to the manufacturer's protocol, in a total volume of 20 µl, which contained 1x Ligation Buffer, 60 fmol of the digested insert DNA, 20 fmol of the digested vector DNA and 0.1 U of T4 DNA Ligase. We also performed a ligation reaction with the vector only as a negative control. To calculate the appropriate amount of the insert and the plasmid DNA in a 3:1 molar ratio, we used the following formula:

$$y\ \mu g\ DNA \times \frac{pmol}{660pg} \times \frac{10^6\ pg}{1\mu g} \times \frac{1}{N} = z\ pmol\ DNA$$

N = the number of nucleotides and
660pg/pmol = the average molecular weight of a nucleotide pair
z = the amount needed in pmol; y = the mass of the DNA in µg

The ligation reaction was incubated for 75 min at room temperature.

Bacterial transformation

To select the correctly ligated constructs, we transformed XL-1 Blue *Escherichia coli* bacteria (Stratagene) with the ligation reaction mix. We used 50 µl of competent XL-1 Blue *Escherichia coli*, added 1.7 µl of β-mercaptoethanol and incubated for 10 min on ice. After adding 2 µl of the ligation reaction and incubating for 30 min on ice, we heat-shocked the bacteria at 42°C for 45 sec, then incubated the cells on ice for another 2 min.

Following this, the bacteria were incubated at 37°C for 1 h with 950 µl of LB media (1% (w/v) tryptone, 0,5 % (w/v) yeast extract, 1% (w/v) NaCl) to let the resistance gene be expressed. After shaking, we plated the bacteria on LB^{amp} plates on which only the bacteria transformed with an intact plasmid carrying the appropriate antibiotic resistance could grow. Positive clones were streaked consecutively until single colonies were obtained.

Plasmid preparation

Purification of plasmids from bacteria is similar to the method used in yeasts. For recovering the plasmid constructs from bacteria we used the Qiagen Plasmid Mini Kit according to the manufacturer's protocol as described above in the "Plasmid Purifications from Yeast" section. After the plasmids were purified from the *E. coli*, we sequenced our samples to verify that the fragments were inserted into the correct reading frame. Upon sequence verification, the plasmids pGEX-proLOX, pGEX-LOX, pGEX-$LOX_{1\text{-}168}$, pGEX-$LOX_{1\text{-}348}$, pGEX-$LOX_{169\text{-}348}$, and pGEX-$LOX_{349\text{-}417}$ were transformed into low protease BL21 *E. coli* strain.

Protein expression and extraction from soluble fractions

This protein expression/extraction method was based on the protocol from Amersham [226]. We inoculated the BL21 bacteria containing the pGEX-LOX plasmids in LB media containing 100 µg/ml ampicillin for overnight incubation at 37°C. The next morning we diluted the overnight culture 1:10 with fresh media and grew the bacteria at 37°C until the OD_{600} of the culture reached 0.6 (in about 2 h). We induced protein expression with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 2 h of induction, the bacteria were harvested from 1.5 ml of the culture by centrifugation at 18 000 x g for 2 min and resuspended in 200 µl of CellLytic BII solution (Sigma). Samples were then vortexed for 2 min. After centrifuging the samples at 4°C, 18 000 x g for 5 min, the supernatant was collected – this was termed the "soluble fraction". To get all of the soluble proteins, the resuspension of the pellets was repeated 3 more times: first in the presence of endonuclease (Sigma) and lysozyme solution, and twice with lysozyme only. Each suspension was incubated for 10 min at RT to allow optimal lysozyme activity. The final pellet of debris – the inclusion body fraction - was resuspended in 50 µl of 2x Laemmli sample buffer (0.125 M Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, and 0.1 M DTT) for analysis.

Protein expression from inclusion bodies

The protein expression in inclusion bodies was carried out using a modified protocol that described in Jung [228]. The BL21 *E. coli* containing pGEX-LOX plasmids were grown in 300 ml of LB medium containing 100 μg/ml ampicillin until the OD of culture was 0.6. For induction, 0.1 mM IPTG was added. After 4 h of induction the cells were harvested by centrifugation at 6000 x g for 20 min at 4°C and resuspended in 6 ml of chilled lysis buffer (50 mM Tris, pH 8.0; 1 mM EDTA; 100 mM NaCl; 1 mg/ml lysozyme). Triton X-100 (1%) and DNase was added (0.1 mg/ml) to lysates and suspension was mixed for 60 min at 4°C. Sonication was repeated 3 times at 70% efficiency with a Fisher Scientific Sonic Dismembrator Model 100 sonicator while the samples were on ice. The inclusion bodies were then pelleted by centrifugation at 6000 x g for 20 min at 4°C, washed with washing buffer (2 M urea; 10 mM K_2HPO_4, pH 8.2) and solubilized overnight at 4°C with solubilizing buffer (8 M urea; 10 mM K_2HPO_4, pH 8.2; 2 mM β-mercaptoethanol; 5 mM DTT).

Protein purification

The protein purification was based on Amersham protocol for GST-fusion protein purification [226]. Since the pGEX-LOX constructs contain an N-terminal GST tag, we used its affinity to glutathione by utilizing Glutathione Sepharose 4B beads [226], that facilitated the purification of the glutathione-S-transferase-fusion proteins. For the purification of the extracted soluble proteins we took 500 μl of the samples, added 50 μl of Glutathione Sepharose 4B beads previously washed with 1x PBS and incubated the samples at 4°C for 1 h with rotating. After capturing the proteins, the samples were centrifuged for 15 sec at 18 000 x g, and the beads were washed 3 times with 1x PBS. The washes and the flow-through were saved for further analysis.

For the purification of GST-fusion proteins from inclusion bodies, we used the same method after diluting the samples to reduce the urea concentration to 2 M by adding 10 mM K_2HPO_4, pH 7.0. This purification was carried out in a larger scale, so 1.5 ml of Glutathione Sepharose 4B resin was used for each inclusion body extraction. Elution of purified proteins from the Glutathione resin was performed using 1.5 ml of solubilization buffer as detailed above, overnight at 4°C.

Bradford assay

To measure the concentration of the proteins extracted or purified we performed a Bradford protein assay, commonly used to determine the total protein concentration of a sample. In a 96-well Costar 3599 microwell plate we mixed 160 µl of the dilutions of BSA, in a range of concentration 0-25 mg/ml, as a standard with 40 µl of Bradford reagent in triplicates. To measure the protein concentrations we mixed 40 µl of Bradford reagent with X µl of protein and (160-X) µl 1x PBS – X varies from 1 to 160 µl since the dilution of the protein sample was required for the resulting absorbance to fall within the linear range of the assay. We measured the absorbance at 600 nm using a BMG Polarstar microplate reader.

SDS-PAGE of the proteins

Equal amount of the proteins were dissolved in 2x Laemmli buffer and boiled for 5 minutes, then chilled on ice for 5 min. SDS-polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the samples was carried out using discontinuous polyacrylamide gels (5% stacking gel; 10% resolving gel) according to the method of Laemmli [229]. The gel were run on Hoeffer miniVE protein gel apparatus for 2 h at 300 V and were stained with Coomassie Brilliant Blue solution (0.25% Coomassie Blue in 45% methanol and 10% acetic acid) for 2 h, destained in destaining solution (35% ethanol and 10% acetic acid) 3 times for 30 min each and rehydrated in 2% glycerol. The gels were dried on Whatman paper at 85°C for 2 h using a Biorad Gel Dryer - Model 583 - with a Biorad Hydrotech Vacuum Pump.

Antibodies

For this study, we used an anti-LOX rabbit polyclonal antibody generated by Dr. Katalin Csiszar's lab and characterized previously [112]. We used this antibody for Western blots at a 1:400 dilution. For detection of GST fusion proteins, an anti-GST mouse monoclonal antibody was used [165 05-311, Upstate Biotechnology]. For detection of placental lactogen, an anti-PL rabbit polyclonal antibody was used [165 NCL-PLp, Novocastra Laboratories]. Anti-Src[pY418] and anti-GAPDH were purchased from Biosource Inc. (44-660G) and Abcam (ab22555), respectively. All commercial antibodies were used at their manufacturer recommended concentrations.

Western blot

Our protocol for Western blotting was based on the modified protocol of Towbin [230]. The purified protein samples were resolved by SDS-PAGE, as described above. The proteins were blotted onto a pre-treated Immobilion-P membrane (Millipore P-15552, Fisher) using a platinum Western Blot Transfer apparatus (Biorad) – 1 h at 2 mA/cm^2. After transfer, the membrane was blocked with 1x PBS; 0.1% Tween-20 (PBST); and 5% Carnation nonfat dry milk for overnight at 4°C. After rinsing the membrane with dH_2O 5 times, and washing once with PBST for 5 min, we incubated the membrane with the appropriate primary antibody in PBST for 1 h at room temperature. Each antibody's working concentration was based on the manufacturer's recommendation. The membrane was washed again as described above and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (Jackson Laboratory). A dilution of 1:50000 in 1x PBST was used for secondary antibodies. The membranes were washed as described above, and finally once more with 1x PBS for 3 min. We used ECL Plus reagent (GE Healthcare, Amersham Biosciences) [226] for chemiluminescent detection: ECL reagent #1 and #2 were mixed in a 1:40 ratio, and we incubated the blots in this mix for 5 min. The blots were then exposed to autoradiograph film for 1-15 min or overnight. The film was developed and fixed using standard solutions.

GST pull-down assay

Our protocol was based on the modified protocol for GST pull-downs from the Molecular Cloning Manual – Protein-protein Interactions [231]. After capturing equal amounts of the GST-LOX fusion proteins and GST only as a negative control by incubating the proteins with 50 µl Glutathione Sepharose 4B beads at 4˚C for 1h with rotating, we incubated the captured fusion proteins with the target protein hPL at 50 µg/ml for 1 h at RT. After this binding step, the beads were washed 3 times with 1 ml of 1x PBS, then 30 µl of 2x Laemmli sample buffer was added and the samples were analyzed either by SDS-PAGE or by Western blotting.

Far Western analysis

Our protocol was based on the modified protocol for Far Westerns from the Molecular Cloning Manual – Protein-protein Interactions [232]. Two microgram of each purified protein was resolved by SDS-PAGE as described above. The proteins were blotted

onto a pre-treated Immobilion-P membrane using a platinum Western Blot Transfer apparatus– 1 h at 2 mA/cm^2. To refold the proteins on the membrane, after blotting we washed the membrane with refolding buffer R6 (20 mM Hepes, pH7.7; 25 mM NaCl; 5 mM MgCl; 1 mM DTT, 6 M guanidinium hydrochloride) twice for 30 min each at RT; then with refolding buffer R4 (20 mM Hepes, pH 7.7; 25 mM NaCl; 5 mM MgCl; 1 mM DTT, 4 M guanidinium hydrochloride) once for 15 min at RT, then with refolding buffer R2 (20 mM Hepes, pH 7.7; 25 mM NaCl; 5 mM MgCl; 1 mM DTT, 2 M guanidinium hydrochloride) once for 15 min and finally with refolding buffer R1 (20 mM Hepes, pH 7.7; 25 mM NaCl; 5 mM MgCl; 1 mM DTT, 0.187 M guanidinium hydrochloride) twice for 15 min each. The membrane was incubated in refolding buffer R1 overnight at 4°C with gentle shaking and then was blocked with 5% Carnation non-fat dry milk in PBST 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 3 times in PBST for 10, 5 and 5 min; then was incubated with 5 μg/ml of the target protein (PL) in PBST for 1 h at 4°C. After washing again as above, the membrane was incubated with the appropriate primary antibody to the target protein. After washing with PBST 3 times for 10, 5 and 5 min, we incubated the membrane with the secondary antibody-horseradish peroxidase labeled conjugate for 1 h at RT. After the final washes – 3 times in PBST for 10, 5 and 5 min then in PBS for 3 min at RT - we used ECL Plus reagent for chemiluminescent detection as described above.

Solid Phase Binding Assay (SPBA)

This protocol was based on the protocol of A. Paul Mould [233]. The purified "receptor" protein was diluted to 10 μg/ml in sterile 1x PBS, and bound to the wells of an ELISA microplate (Costar 3950) by incubating 100 μl/well overnight at 4°C. The wells were then blocked with 200 μl of 1% BSA in each for 1h at 37°C, and then we washed the wells with PBST 3 times. We incubated the bound proteins with the purified "ligand" proteins in a range of concentrations (0.1-100 nM) for 1 h at RT. After removing the unbound "ligand", we washed the wells with PBST 3 times and incubated with the appropriate primary antibody against the ligand protein for 1 h at RT. The wells were washed for 3 times with PBST, and incubated with the secondary antibody-horseradish peroxidase (HRP) labeled conjugate for 1h at RT. After washing the wells 3 times with PBST, QuantaBlu Working Solution (from QuantaBlu Fluorogenic Peroxidase Substrate Kit, Pierce) was

added to detect the peroxidase activity of HRP. The solution was excited at 320 nm and the emitted fluorescence was measured at 405 nm using a BMG Polarstar microplate reader. Kd was calculated by Prism4 software based on these measurements.

Enzyme Activity Assay of bound LOX

This protocol was a modified version of Palamakumbura's activity assay protocol [234]. A 96-well microwell plate (Costar 3950) was coated overnight at 4°C with 100 μl of BSA as negative control or PL. Each sample was done in quadruplicates. The wells were blocked with 1% BSA for 3h at 37°C, washed with 0.1% BSA in 1x PBS and incubated with 100 μl of ligand: purified bovine aorta LOX, generously provided by Dr. Herbert Kagan, in various concentrations (0.1-100 nM) overnight at 4°C. We washed the wells 3 times with PBST, once with PBS and added the reaction mix to the wells: 1.2 M urea, 0.05 M sodium borate, pH 8.2, 40 μg of horseradish peroxidase, 0.25 mg sodium homovanillate (Amplex Red, Molecular Probes), 10 mM 1,5-diaminopentane. In the presence of horseradish peroxidase, hydrogen peroxide oxidizes homovanillate to give a fluorescent product with an excitation maximum at 564 nm and an emission maximum at 587 nm. The reactions were incubated at 37°C for 30 min, and the fluorescence was measured at 590 nm.

Immunofluorescent microscopy

Fixed human breast cancer tissues with corresponding normal tissues (AccuMax Array A712(II)) were obtained from ISU Abxis Co. 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 a 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 (anti-LOX 1:100, anti-hPL 1:200) in 5% normal goat serum in PBS for 2 h at room temperature, 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. The tissue sections were then either incubated with To-PRO-3 nuclear stain (Molecular Probes) for 15 min and mounted with Vectashield mounting medium (Vector Laboratories), or simply mounted with Vectashield mounting medium containing DAPI nuclear stain. 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 [235, 236]. Human embryonic kidney epithelial cells (HEK 293FT) were obtained from Invitrogen and maintained as recommended by the distributor [227].

Protein extraction from cultured cells

To evaluate expression of PL and LOX in the tumor cells, total protein from the cell lines' conditioned cell medium (CCM) was extracted and analyzed by Western blotting. Briefly, at ~70% confluence, the cells were washed with sterile 1x PBS and incubated in phenol red-free, serum-free medium for 48 h. The medium was then collected, and following removal of the cell debris by centrifugation, the protein concentration was measured using Bradford reagent (Bio-Rad). To a volume of CCM containing 20 µg of proteins, 10 µl of Strataclean Resin (Stratagene) was added and mixed at room temperature for 10 min to bind the proteins [237]. After pelleting the resin by centrifugation, the supernatant was removed, the resin was resuspended in 10 µl of 2x Laemmli buffer, and the samples were boiled for 5 min before electrophoresis by SDS-PAGE.

Co-immunoprecipitation

For immunoprecipitation experiments, conditioned cell medium (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 of 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 the 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 three times in PBST and resuspended in 2x Laemmli buffer. The immunoprecipitated samples were analyzed by Western blotting.

Generation of stably transduced cell lines

Lentiviral Constructs

PL and LOX were stably expressed in mammalian cells using the ViraPower™ Lentiviral Expression System (Invitrogen) [227] 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, whereas 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'-CTAGAAGCC 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 the previously cloned expression vector, pcDNA3.1-LOX, and for the PL-2 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 each primer, 1 unit of DeepVent polymerase (New England Biolabs, Inc.), and 200 ng of template DNA. The PCR cycling consisted of denaturation for 30 s at 95°C, annealing for 30 s 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 run on a 1,2% agarose gel and isolated from the agarose as described in the "Agarose gel electrophoresis" and "DNA purification from agarose" sections.

TOPO cloning

For the cloning of the blunt-end PCR product we used the TOPO Cloning Kit from Invitrogen. We mixed 0,5 µl PCR (~ 1 ng DNA) product with 1 µl Salt Solution, 3,5 µl sterile water, and 1 µl TOPO vector (pLenti6/V5), and incubated this mixture for 5 minutes at room temperature, then proceeded with transforming the constructs into XL-1 Blue supercompetent *E. coli* as described in the "Bacterial transformation" section. To verify the transformation efficiency, we used the pUC19 plasmid as a positive control. The transformants were selected on LB medium containing 100 µg/ml ampicillin and 50 µg/ml blasticidin (pLenti6/V5 constructs), and on LB medium only (pUC19). Six positive clones were selected from each transformation, and the plasmids were purified as described in the "Plasmid preparation" section. The positive clones were analyzed by both restriction enzyme digestion using BamHI and XhoI enzymes, and sequencing with the CMV forward and the appropriate reverse primers hybridizing within the insert.

Production of viral particles

HEK293FT human embryonic kidney epithelial cells [227], used as hosts for the production of the desired lentiviruses, were maintained as recommended by the manufacturer, in high glucose DMEM with 10% fetal bovine serum (FBS), 0,1 mM MEM Non-Essential Amino Acids (NEAA), 2 mM L-glutamine, 1% antibiotics/antimycotics with 500 µg/ml Geneticin (complete medium).

These cells were co-transfected at 90% confluency with the pLenti6/V5-PL-2 or pLenti6/V5-LOX lentiviral DNA constructs and Virapower packaging mix using Lipofectamine 2000 (Invitrogen) [227] as a transfection reagent.

For each transfection sample, we prepared DNA-Lipofectamine™ 2000 complexes. In a sterile 5 ml tube, we mixed 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti expression plasmid DNA (12 µg total) in 1.5 ml of Opti-MEM® I Medium without serum. In a separate sterile 5 ml tube, we diluted 36 µl Lipofectamine™ 2000 transfection reagent with 1.5 ml of Opti-MEM® I Medium without serum, incubated this mixture for 5 minutes at room temperature, and combined the diluted DNA with the diluted Lipofectamine 2000. To allow the DNA-Lipofectamine complexes to form, we incubated this mixture for 20 minutes at room temperature. During incubation we prepared the HEK 293FT cells by detaching them from the cell culture flask using trypsine-EDTA (0,25% trypsine, 1 mM EDTA), diluting them into the working concentration of $1{,}2x10^6$ cell/ml with Opti-MEM I medium with 10% FBS and incubating them in the CO_2 incubator at 37°C for 5 min. Subsequently, we added the DNA-Lipofectamine™ 2000 complexes to a T75 tissue culture flask containing 5 ml of Opti-MEM® I medium containing 10% serum, and gently mixed with 5 ml of the 293FT cell suspension ($6x10^6$ total cells). The cells were then incubated overnight at 37°C in a CO_2 incubator. The next day, the medium was replenished with complete culture medium containing sodium pyruvate (i.e. D-MEM with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate). Virus-containing supernatants were harvested 72 hours post transfection by removing medium to a 15 ml sterile conical tube. We pelleted cell debris by centrifuging at 3000 rpm for 15 minutes at +4°C and transferred the virus-containing supernatants into cryovials in 1 ml aliquots. Viral stocks were stored at -80°C.

Titering the viral stocks

To determine the titers of our lentiviral stocks, we chose to transduce our immortalized normal mammary epithelial MCF-10A cell line. The day before the transduction,

cells were plated in a 6-well plate such that they would reach 30-50% confluency at the time of transduction. On the day of transduction, the lentiviral stocks were thawed, and 10-fold serial dilutions were prepared in complete medium in a total volume of 1 ml, concentrations ranging from 10^{-2} to 10^{-6}. These dilutions were than added to one well of previously seeded cells each, in the presence of 6 μg/ml Polybrene. As a negative control, a mock-transduced well was included. Subsequently, the cells were incubated overnight at 37˚C in a CO_2 incubator. To select for transduced cell lines, the following day the medium was replenished with fresh complete medium containing 6 μg/ml Blasticidin. The medium was replaced with fresh complete medium containing antibiotic every 3 days. After 10 days of incubation there were no live cells left in the mock-transduced well, but discrete antibiotic resistant colonies appeared in some of the dilution wells. The medium was removed at this point, and the cells were washed twice with PBS. The cells were stained with crystal violet and the colonies were counted to determine the titer of the lentiviral stocks.

Viral transduction of MCF-10A cells

MCF-10A cells were seeded in 6-well plates at a density of 10^5 cells per well in complete medium and were incubated overnight at 37˚C in a CO_2 incubator. The following day the lentiviral stocks were diluted in complete medium to a MOI (multiplicity of infection) of 0.01 in a total volume of 1 ml. The medium containing the viruses was added to the cells in the presence of 6 μg/ml Polybrene. After incubating the cells overnight at 37˚C, the medium was replenished with fresh complete medium. The following day this was replaced with medium containing 6 μg/ml Blasticidin enabling us to select for stably transduced cells. The medium was replaced every 3-4 days until antibiotic resistant colonies could be identified. Clonal selection was performed using QuixellTM cell selection and automated transfer system (Stoelting Co.), the clonal cell lines were then maintained in complete medium containing 6 μg/ml Blasticidin. Expression of PL and LOX in the stably transduced MCF-10A cell lines was evaluated by Western analysis as decribed in the "Protein extraction from cultured cells" section. Individual clones that were determined to have the highest levels of expression of PL and LOX were selected for further experiments.

Immunofluorescent Staining of the Actin Cytoskeleton

Parental MCF-10A cells and stably transduced MCF-10A cells co-expressing PL and LOX were grown on coverslips to ~60% confluence. After fixing the cells in 4% formaldehyde in PBS for 10 min at room temperature, cells were treated with 0.1% Triton X-

100 in PBS and blocked with 0.1% BSA in PBS for 30 min at room temperature. The cells were stained with phalloidin-Alexa 488 conjugate (1:40 in 1% BSA) for 20 min and washed in 0.1% BSA in PBS. After mounting the samples with Vectashield mounting medium, a Zeiss LSM Pascal confocal microscope was used for imaging.

Cell Proliferation Assay

Our protocol for measuring proliferation rates was based on Olsson's photometric microassay [238]. Cells were seeded in two parallel 96-well plates in triplicates at 2000 cells/well, and were allowed to attach for 4 h 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 bound to the cells. 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 reader (BMG). Proliferation rates were calculated as the difference between the number of cells after 72 h and the initial number of seeded cells. Data were analyzed using Graphpad Prism4 software.

Cell Migration Assay

The *in vitro* cellular migration assay was based on the previously described membrane invasion culture system assay (41). MCF-10A cells and stably transduced MCF-10A cell lines expressing PL and/or LOX were seeded in serum-free medium at a density of 50,000 cells/well on the top of a gelatin-coated polycarbonate filter (10-μm pore size) suspended in a membrane invasion culture system chamber, whereas the chamber underneath the membrane contained complete medium. The cells were then incubated in a CO_2 incubator at 37°C for 4 h, fixed in methanol, and stained with hematoxilin-eosin, and the migratory cells were counted. The experiments were repeated three times, and data were analyzed using Prism4 software (Graphpad).

RESULTS

Yeast two-hybrid screening

In order to identify novel interacting partners of lysyl oxidase that contribute to the enzyme's non-classical functions, a yeast two-hybrid screen was performed in our laboratory. The expression of the lysyl oxidase gene family is high in placental tissues, and we assumed 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-kDa pro-LOX ($LOX_{1–417}$) and the 30-kDa mature LOX ($LOX_{169–417}$) as baits. Following the transformation of pGBKT7-$LOX_{1–417}$ and pGBKT7-$LOX_{169–417}$ into yeast strain AH109, expression of the fusion proteins GAL4-BD-$LOX_{1–417}$ and GAL4-BD-$LOX_{169–417}$ were confirmed by Western blot analysis (data not shown). Assays for expression of the reporter genes *ADE2*, *HIS3*, and *lacZ* showed no autonomous transcriptional activity induced by GAL4-BD-$LOX_{1–417}$ or GAL4-BD-$LOX_{169–417}$ alone. Subsequently, the placental cDNA library was transformed into the yeast strains containing either the pGBKT7-$LOX_{1–417}$ or the pGBKT7-$LOX_{169–417}$ construct, and the cells were plated onto selective plates to screen for expression of the three reporter genes. Yeast colonies that expressed all three reporter genes were then isolated, and upon plasmid extraction, the library insert cDNA was sequenced. From the yeast two-hybrid screen with pro-LOX ($LOX_{1–417}$), 7 of the 20 positive clones sequence-identified proved to be PL cDNAs. From the screen with the mature LOX ($LOX_{169–417}$), 9 of the 39 positive clones sequence-identified were PL cDNAs. Most of the positive clones encoded the entire pre-PL protein of 217 amino acid residues, and all were identified as PL-2 transcript variant 1 **(Figure 9)**. The expression of PL-2 and PL-3, which result in identical mature hormones, is initiated and up-regulated in placental syncytiotrophoblasts during pregnancy, with the highest expression levels at term [171, 188]. The PL-2 gene transcript variant 1 that contains all five exons of the gene encodes isoform 1, the most abundant full-length isoform of the hormone [183].

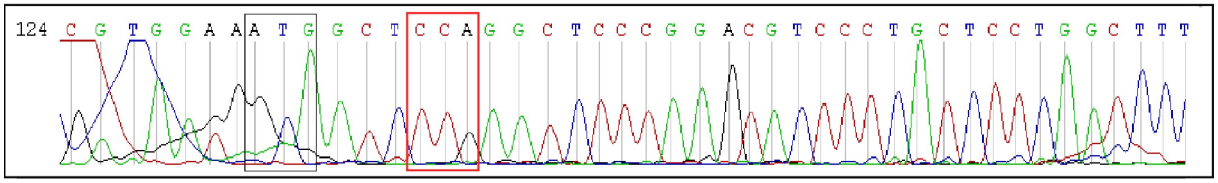

Figure 9. Representative DNA sequence of the yeast two-hybrid positive clones encoding PL-2 transcript variant 1.
The black box indicates the start codon, the red box shows the codon that differs in PL-2 (CCA) and PL-3 (GCA), thus enabling us to distinguish the two transcripts.

Verifying placental lactogen as a LOX-interacting protein

The yeast two-hybrid screens show a tendency towards false positive hits as a result of autonomous transcriptional activation by the bait protein or non-specific binding of library proteins. We have previously clarified that LOX had no autonomous transcription-activating effect, therefore, as the next step, it was necessary to confirm that the detected interaction was indeed specific between LOX and PL. To confirm the specificity of the PL-LOX binding as proposed in Specific Aim 1, and to further test which domain of LOX contains the PL binding site, several combinations of bait **(Figure 10)** and target plasmids were co-transformed into yeast that were spotted on nutritional dropout (Ade^-/His^-/Trp^-/Leu^-/X-Gal – AHWL^-/X-Gal) plates to test reporter gene activation and to maintain a selective pressure on the bait and library plasmids (TRP1, LEU2).

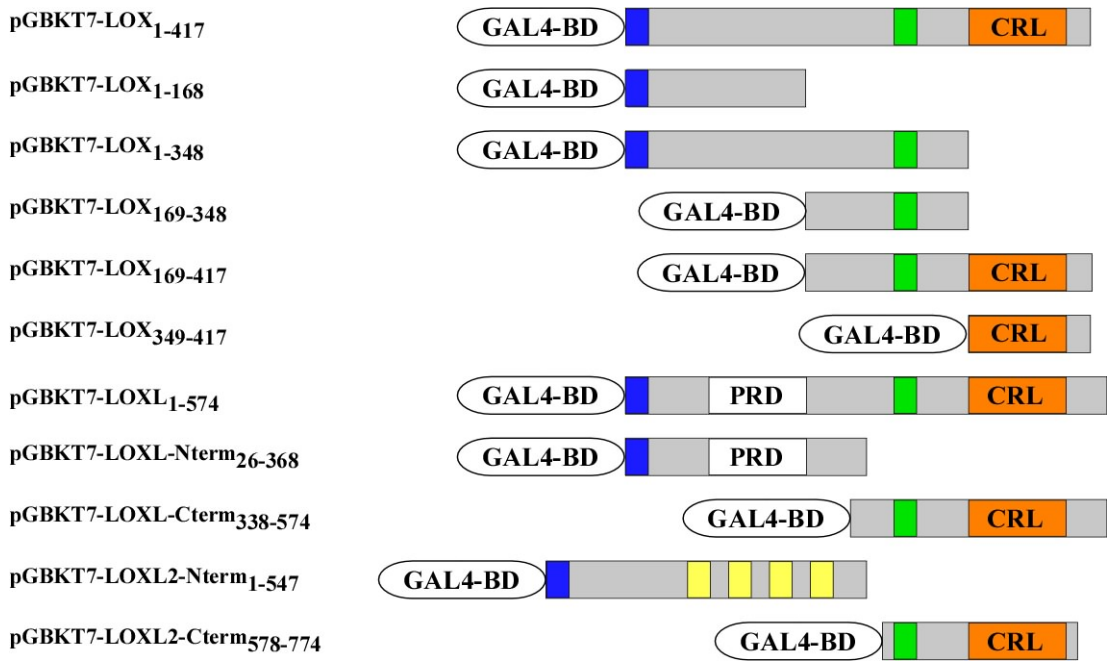

Figure 10. The LOX and LOX-like deletion constructs used as baits in yeast direct interaction trials. In addition to the original baits pGBKT7-LOX_{1-417} and pGBKT7-$LOX_{169-417}$ other constructs were included to narrow down the possible site of interaction and to test if homologous LOX-like proteins would bind the same target protein. GAL4-BD: the GAL4 binding domain; blue rectangle: signal peptide; green rectangle: Cu-binding site; CRL: cytokine receptor-like domain; PRD: proline-rich domain; yellow rectangles: SRCR (scavenger receptor cystein-rich) domain.

Yeast were also co-transformed with combinations of LOX or PL proteins with empty plasmid vectors as negative controls (**Figure 11** first lane and first row). A non-interacting bait protein, nuclear lamin C, was also used as a negative control to show specificity of the PL interaction (**Figure 11** second row). Only the yeast cells that contained fragments of LOX or proLOX (and in further experiments, fragments of LOXL and

LOXL2) that interacted with PL were able to grow on the nutritional dropout plates. **Figure 10** shows the schematic diagram of the LOX deletion constructs used in the direct interaction studies. The bait constructs showing interaction with PL were proLOX (LOX_{1-417}), the mature protein ($LOX_{169-417}$), and deletion constructs LOX_{1-348}, $LOX_{169-348}$, $LOX_{169-417}$. The yeast containing the proenzyme exhibited slower growth rate compared to that of the LOX_{1-348}, $LOX_{169-348}$, and $LOX_{169-417}$-containing cells. This phenomenon might be due to the different folding of the GAL4-BD-proLOX interfering with the site of the LOX-PL interaction. Furthermore, the propeptide region of LOX (LOX_{1-168}) alone did not interact with PL. While the presence of the propeptide domain in the construct LOX_{1-417} partially inhibited, in construct LOX_{1-348} had no effect on the interaction with PL. Initially we thought that the CRL domain would be a likely candidate containing the site of interaction, since it shows amino acid sequence homology with the extracellular domain of the cytokine receptors to which placental lactogen binds *in vivo*. However, the LOX cytokine receptor-like (CRL) domain alone (amino acid residues 349-417), did not bind PL **(Figure 11)** in our direct interaction tests.

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.

We further tested whether LOX-like proteins, LOXL and LOXL2, that share a 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 **(Figure 10)** were cloned into the pGBKT7 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 co-transformed into yeast that were then spotted on plates to test reporter gene activation. Yeast were also co-transformed with empty vector and lamin C controls as described above. The only bait positive for activating the reporter genes was the C-terminal portion of LOXL2 **(Figure 11)**. The homologous domain of LOXL did not interact with PL enough to activate the reporter genes, suggesting that PL specifically binds to a putative binding site within LOX and LOXL2.

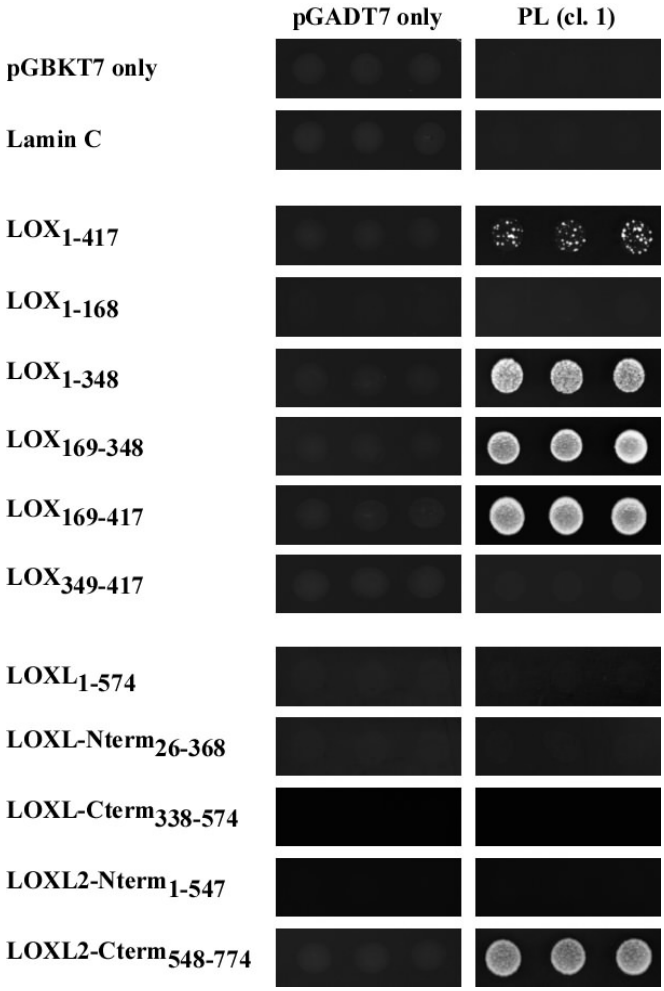

Figure 11. Yeast direct interaction studies
Each field represents yeast spotted in triplicates and grown on AHWL- dropout plates. Only yeast containing the combination of interacting protein fragments were able to grow under the nutritional selection. The bait constructs are indicated on the left, while the prey constructs are on the top of the figure. Negative controls were the empty vectors pGADT7 and pGBKT7 and the non-interacting Lamin-C protein.

Bacterial expression of recombinant lysyl oxidase

For further *in vitro* experiments aimed to verify and characterize the interaction of LOX and PL, as proposed in Specific Aim 2, significant amounts of purified LOX protein was needed. As purified LOX is not available commercially, we needed to express the protein in a bacterial protein expression system. We chose to express LOX as a glutathione-S-transferase (GST)-fused protein in *E. coli* bacteria. Expression of genes in *E. coli* offers a convenient system to produce large amounts of recombinant proteins that may otherwise be difficult to isolate from natural cells and tissues. In order to generate LOX, the LOX fragments used as bait in the yeast direct interaction studies were cloned into a pGEX-4T-1 vector generating GST-LOX constructs this way which were expressed in *E. coli* bacteria **(Figure 12)**.

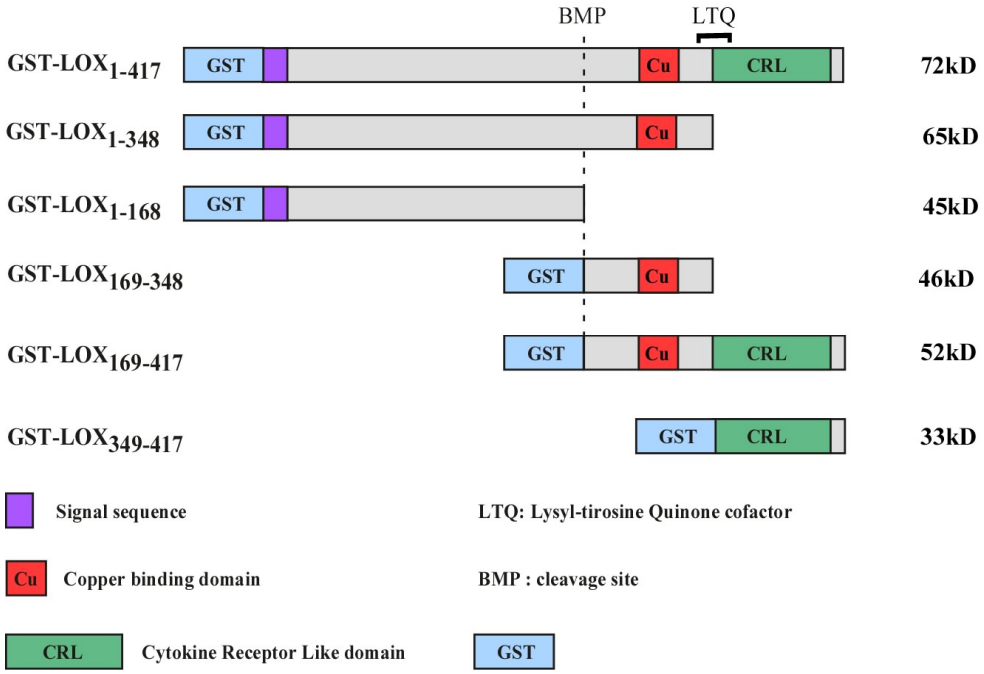

Figure 12. The GST-LOX constructs designed for bacterial protein expression.
The expected molecular weight of each fusion protein is indicated on the right.

The vector contained an ampicillin resistance gene (Amp^r), an IPTG-inducible Ptac promoter upstream of the GST coding sequences, and a multicloning site with a thrombin cut site between the GST and MCS sequences **(Figure 8)**. The thrombin site would allow proteolytic removal of the GST tag from the purified recombinant protein. Following EcoRI and SalI enzymatic digestion of all the pGBKT7-LOX constructs and the pGEX-4T-1 vector, the digested plasmids were separated by agarose gel electrophoresis, and isolated. The LOX fragments were then cloned into the pGEX-4T-1 plasmid in frame with the GST-tag and the thrombin cleavage site. Following transformation into XL-1 Blue supercompetent *E. coli*, the plasmids were isolated and the successful cloning was verified by restriction endonuclease digestion and agarose gel electrophoresis as well as by sequencing. The correct pGEX-LOX constructs and pGEX only were transformed into BL21 *E. coli* bacteria, a strain with low protease levels optimal for protein expression. The pGEX would express the GST only which can be used a negative control in our subsequent binding experiments. Following transformation the bacteria were incubated overnight in LB medium, diluted 1:10 and grown until the OD_{600} of the culture reached 0.6. The bacterial expression of the GST-fusion proteins was induced by adding IPTG to the culture at the log phase of growth. In our first effort we wished to induce the expression of the fusion proteins in their soluble form. Therefore we attempted to optimize the induction conditions by lowering the

temperature to 25°C, or using the lowest suggested concentrations of IPTG to decrease bacterial growth rate and inclusion body formation. We extracted the protein samples as different fractions: one soluble, three lysozyme soluble fractions and inclusion bodies. We analyzed our samples by SDS-PAGE and Western blotting to see whether the correct size fusion proteins were expressed. A representative Coomassie Blue-stained PAA gel is shown in **Figure 13**.

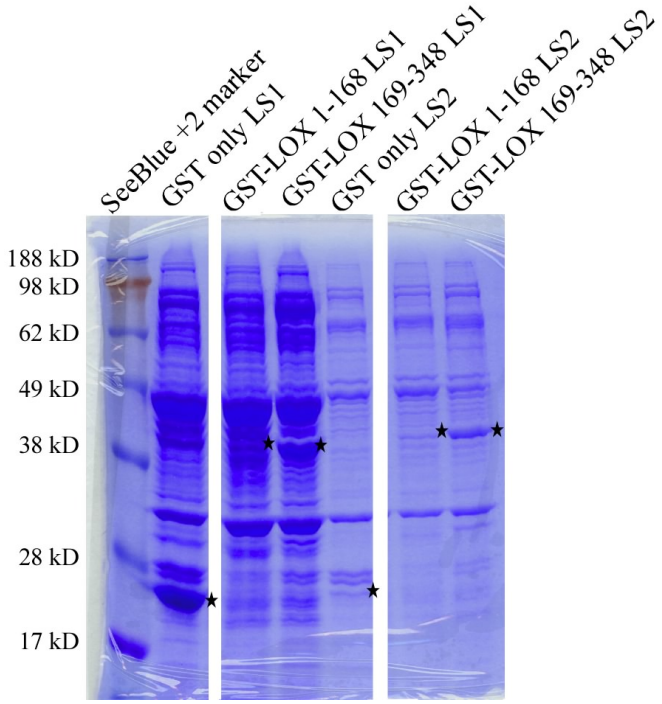

Figure 13. Representative SDS-PAGE gel of soluble bacterial proteins from E. coli expressing GST-LOX fusion proteins.
Stars indicate protein bands of correct molecular weight. The figure demonstrates the low levels of some of the recombinant proteins in these lysozyme-soluble (LS) fractions extracted in the presence of lysozyme.

We were able to express all GST-LOX constructs at the correct molecular weight except GST-LOX$_{1\text{-}417}$, containing the full-length protein. Furthermore, despite our attempts to optimize growth conditions to increase the solubility of the fusion proteins, only GST-LOX$_{169\text{-}417}$, GST-LOX$_{349\text{-}417}$, and GST-LOX$_{169\text{-}348}$ were present in the soluble or lysozyme soluble fractions in any significant amount. GST-LOX$_{1\text{-}348}$ and GST-LOX$_{1\text{-}168}$ were found almost entirely in the inclusion bodies. Moreover, the recombinant proteins found in soluble forms were present in the inclusion body fraction in much higher quantities than in the soluble fractions as it is shown on **(Figure 14)**, which demonstrates the relative distribution of GST-LOX$_{1\text{-}348}$ among the lysozyme soluble and inclusion body fractions with or without IPTG-induction.

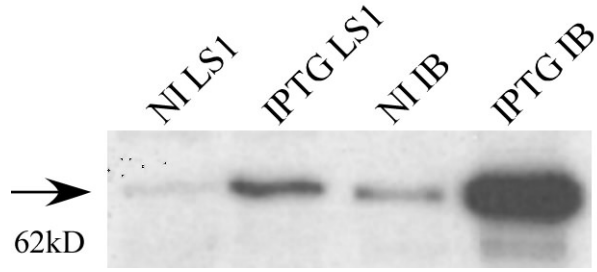

Figure 14. Western blot of the extracted GST-LOX$_{1-348}$, using an anti-GST antibody.
The figure illustrates the relative distribution of GST-LOX$_{1-348}$ between the lysozyme soluble and inclusion body-derived protein fractions. Interestingly, recombinant protein expression could be observed at a low level in the absence of the inducer IPTG. NI: non-induced, IPTG: IPTG-induced, LS1: lysozyme soluble fraction 1, IB: inclusion body fraction. The arrow shows the protein at correct molecular weight.

Due to the above mentioned limitations of soluble GST-LOX protein production and the insufficient quantities of the obtained fusion proteins, we decided to take another approach, and chose to extract the fusion proteins from inclusion bodies based on a similar protocol for His-tagged recombinant LOX [228]. This extraction method was carried out in a larger scale, with an increased IPTG-induction period of 4h. To extract the soluble proteins first, we used lysis buffer, lysozyme and sonication. The remaining inclusion bodies were then solubilized in the presence of 8M urea. As such a high urea concentration denaturates the proteins, and therefore inhibits the binding between the GST-tag and the glutathione beads, it was necessary to lower the urea concentration to enhance protein refolding and purification as well. Based on the method previously published by Jung et al. [228], we first diluted our protein samples by adding 10 mM K_2HPO_4, pH 7.0 lowering the concentration of urea this way, and we attempted to purify the proteins from solutions of equal volume containing 6 M, 4 M and 2 M urea. The GST-LOX proteins were then purified using the binding affinity of the GST-tag to glutathione. The 2 M urea concentration proved to be optimal for protein purification, as it allowed renaturation of the GST-LOX recombinants and resulted in sufficient amounts of purified protein **(Figure 15A and B)**. Using this method we were able to generate GST and GST-LOX proteins GST-LOX$_{349-417}$, GST-LOX$_{169-417}$, GST-LOX$_{1-168}$, and GST-LOX$_{169-348}$ from bacterial inclusion bodies **(Figure 15C)**.

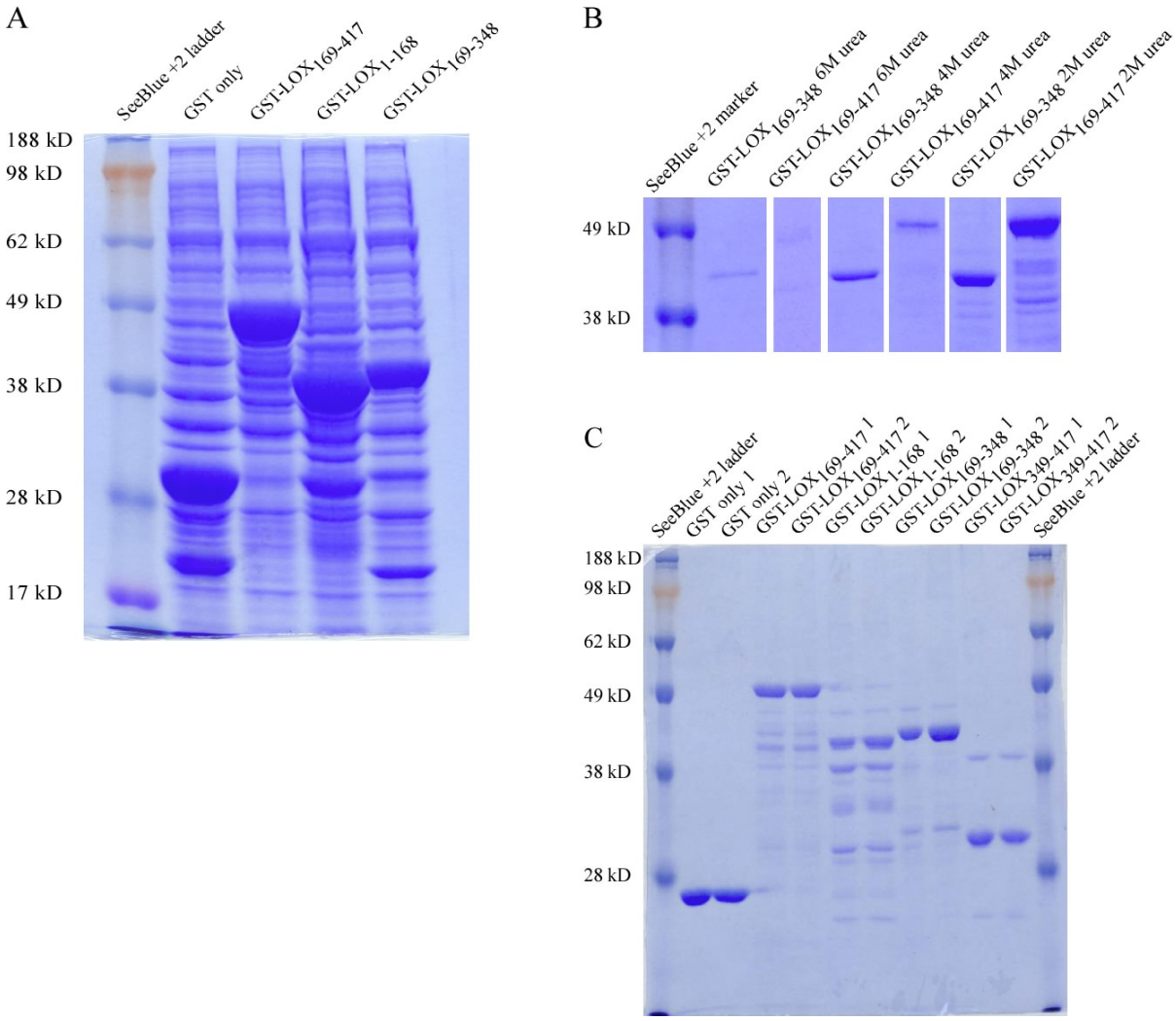

Figure 15. Protein extraction and purification steps.
A - Representative Coomassie Blue-stained protein gel of GST-LOX fusion proteins extracted from bacterial inclusion bodies. B – Purification efficiency could be increased by decreasing the urea concentration of the protein extracts. C – Purified GST-LOX protein constructs, paralel samples. GST-affinity based purification proved to be highly efficient as we were able to obtain milligram quantities of the recombinant LOX proteins.

In vitro assays confirm the binding of LOX and PL

Far-Western analysis

Having generated sufficient quantities of recombinant LOX protein fragments, we aimed to verify the interaction of PL with LOX, and performed different *in vitro* binding experiments. Far-Western blotting was initially used to screen protein expression libraries with a radiolabeled protein [239, 240], and is now widely applied for identification of protein-protein interactions such as receptor-ligand interactions. Far-Western blotting is similar to Western blotting. In Far-Western analysis, a "prey" protein (i.e. a GST-LOX protein) is separated by SDS-PAGE electrophoresis, transferred to a membrane and detected with a labeled or antibody-detectable "bait" protein, in our case hPL. During the blotting it is im-

portant to conserve or restore the native conformation of the studied proteins, as the denatured "prey" proteins might not be able to interact with the bait, or due to the altered denatured conformation, false-positive interactions might occur. Therefore, following the transfer of the proteins onto the membrane, we applied a renaturation step and incubated the membrane in decreasing concentration guanidinium-hydrochloride series. Briefly, equal volumes of purified GST-LOX$_{169-417}$ fusion protein and GST alone as negative control was separated by SDS-PAGE, transferred onto a membrane, refolded, and incubated with hPL as bait. Using an anti-hPL antibody, we detected the PL bound to GST-LOX fusion protein. No hPL was detected in interaction with the negative control GST **(Figure 16)**. We obtained the same result in three separate experiments. As a control, parallel membranes were stained with Coomassie Blue, to confirm the presence of both GST and GST-LOX on the membranes.

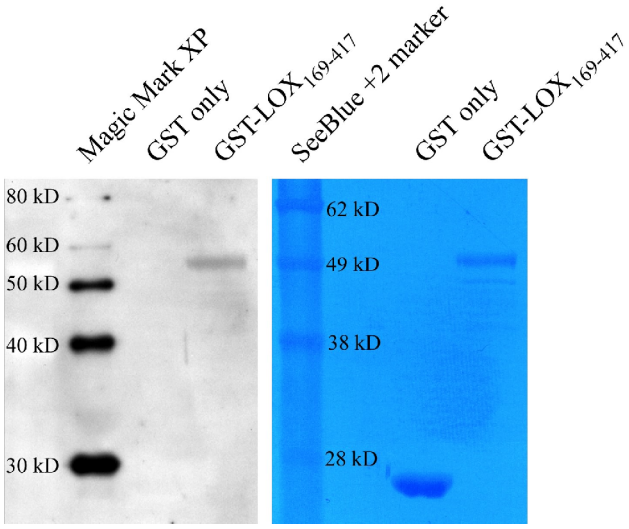

Figure 16. Far-Western analysis confirmed LOX-PL interaction.
Left panel - Far.Western blot with anti-hPL antibody. Right panel – Membrane stained with Coomassie Brilliant Blue for control.

Pull-down assay

We tested PL-LOX binding in GST pull-down assays. This method can be applied to confirm interactions between a probe and a known protein, where the probe protein contains a GST-tag that helps purification by affinity chromatography on glutathione beads. Therefore we captured equal amounts of GST-LOX$_{169-417}$, GST-LOX$_{349-417}$ and GST only as a negative control using Glutathione Sepharose 4B beads, and incubated the immobilized fusion proteins with purified hPL. Following removal of unbound proteins by washing the beads with 1x PBS, 2x Laemmli buffer was added, and equal amounts were resolved by SDS-PAGE. The bound hPL was detected using an anti-hPL antibody (NCL-

PLp). As a positive control for immunodetection 1 μg of hPL was also resolved parallel with the samples. As **Figure 17** demonstrates hPL was binding to GST-$LOX_{169-417}$, containing the mature enzyme, and to GST-$LOX_{349-417}$, containing the cytokine receptor-like domain of the protein, but not to GST alone.

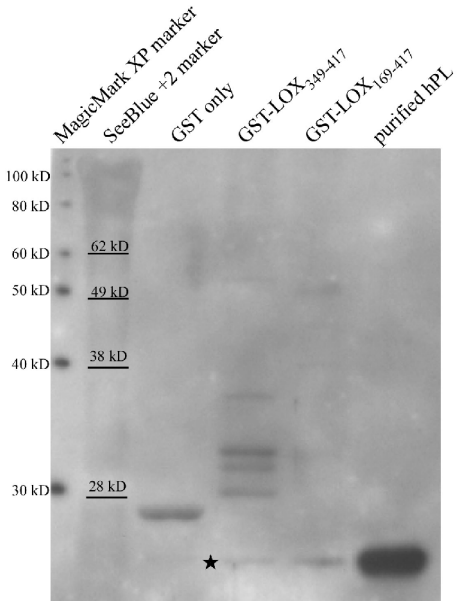

Figure 17. GST pull-down of human placental lactogen.
The star indicates the correct molecular weight hPL bands. This data reveals the binding of hPL to the CRL domain (GST-$LOX_{349-417}$) GST-fusion proteins but not to GST alone

However, this method had its limitations. We were not able to control the amount of GST proteins captured, as their binding efficiency to glutathione is different. In addition, we could not control the quantities of proteins loaded onto the lanes of the SDS-PAGE gels. Therefore we could only verify that hPL does bind to the mature enzyme and to the CRL domain, but we could not draw any conclusion regarding the possible differences in its binding affinity to the different LOX fragments.

Solid phase binding assays

To further confirm the interaction of LOX and PL and to determine an equilibrium dissociation constant (K_d) for this binding, we performed solid phase binding assays based on a previously described protocol [241]. The assay in principle is similar to radioreceptor assays developed to determine the binding affinity of a radiolabeled hormone to its receptor [242]. Equal amounts of the "receptor" protein were immobilized in the wells of an ELISA plate, and incubated with the "ligand" protein in a range of concentrations (0-2000nM or 0-5000nM). After removing the unbound "ligand", the bound protein was in-

cubated with the appropriate primary and a horseradish-peroxidase (HRP) conjugated secondary antibody. The bound ligand was detected by measuring the peroxidase activity of the HRP.

To narrow down the possible site of interaction within LOX, we chose to study the binding affinity of the different LOX deletion constructs to hPL. Repeated experiments showed that the construct containing the mature enzyme exhibits the strongest affinity to hPL. The deletion construct of the mature enzyme without the CRL domain showed slightly lower binding affinity, the construct containing the pro-region a significantly lower affinity, while the CRL domain alone the lowest binding affinity to hPL **(Figure 18)**. However, we were not able to determine the K_d during these tests in the given range of concentrations.

Since PL is a member of the GH/PRL hormone family and shares sequence homology with both GH and PRL, we investigated whether LOX could bind to GH and PRL as well. Replicate experiments showed that GST-LOX bound to immobilized PL with the highest affinity (K_d 30-120 nM) **(Figure 18)**. Compared to its affinity to PL, GST-LOX exhibited a slightly lower binding affinity to GH (K_d 180-344 nM) and significantly less affinity to PRL. The K_d 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.

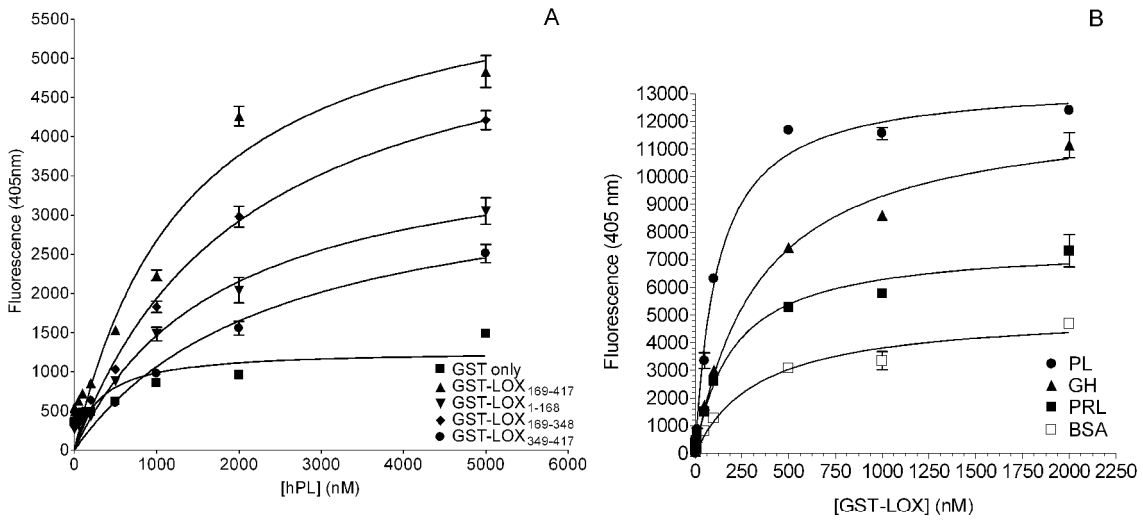

Figure 18. Solid phase binding assays determining binding affinities.
A: GST-LOX deletion constructs and GST as a negative control were immobilized in ELISA plates, hPL was added in a range of concentrations and detected by an anti-hPL antibody in an enzyme-linked immunosorbent assay. *B*: In microplate wells coated with PL, GH, PRL, or negative control BSA, various concentrations of $GST\text{-}LOX_{169\text{-}417}$ were incubated. Bound GST-LOX fusion protein was detected with an anti-GST antibody. Measurements were performed in triplicates in both experiments. *Error bars*, S.E.

Detection of PL and LOX expression in breast cancer tissues and cell lines

Our studies have previously demonstrated that the expression of LOX in the highly invasive and metastatic Hs578T and MDA-MB-231 breast cancer cell lines plays a role in their invasion and metastasis. In addition, we have shown that the active LOX induced a migratory/invasive behavior in the non-invasive and poorly metastatic breast cancer cell lines MCF-7 and T47D [3], and that in normal epithelial cells, overexpression of LOX triggered changes towards a mesenchymal, fibroblast-like phenotype [109]. PL expression, on the other hand, has not been previously investigated in these cell lines, even though the presence of the hormone has been detected in breast tumor samples at a high frequency [217]. First, we studied PL and LOX expression in breast tumor samples of tissue macroarrays using PL and LOX antibodies for immunofluorescent staining as proposed in Specific Aim 3. Briefly, the sections were deparaffinized, washed and incubated with the appropriate primary and secondary antibodies. The latter were conjugated with green (FITC) or red (Rhodamine) fluorescent dyes. Following mounting, confocal microscopy was used for capturing the immunofluorescent staining. Five of the six tumor samples we analyzed showed LOX expression, and three of these stained for PL as well. In these tumor samples, both LOX and PL were detected within and surrounding tumor cells of epithelial origin **(Figure 19)**.

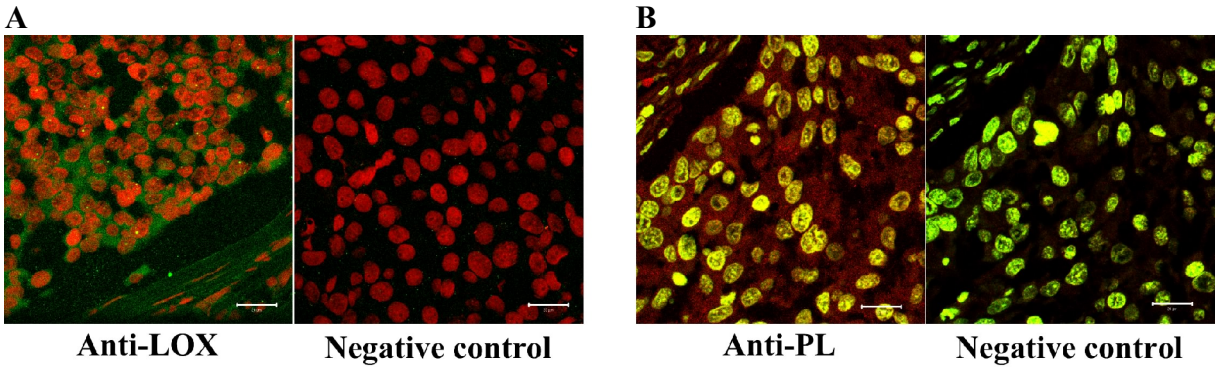

Figure 19. Immunofluorescent staining of breast cancer tissue samples.
A: LOX staining is green, nuclear staining by propidium iodide is red. *B:* PL staining is red, nuclear staining is green. Both anti-LOX and anti-PL stainings show the intra- and pericellular localization of the proteins. Secondary antibodies only were used as negative controls.

Subsequently, in a search for an appropriate *in vivo* model to study the LOX-PL protein interaction, we tested if cultured breast cancer cell lines also expressed PL by Western blot analysis using total protein extracts from preconfluent culture media of cells grown under serum-free conditions for 2 days. As **Figure 20** illustrates, PL is expressed in small quantities by T47D cells and in bigger quantities by the invasive and metastatic cell

lines Hs578T and MDA-MB-231, the non-invasive/poorly metastatic MCF-7 cells. PL was detected as an approximately 42 kD band from cell culture media. This is consistent with the molecular weight of a previously described homodimer form of the hormone [195]. Total protein extracts from the cell medium of the PL-expressing choriocarcinoma cell line, JAR were used as positive control for the immunodetection. In addition, elevated levels of LOX mRNA expression has been previously reported in the malignant and invasive Hs578T and MDA-MB-231 cell lines compared to the less invasive MCF7 and T47D cells [3].

Figure 20. Expression of placental lactogen in human breast cancer cell lines.
PL was detected by Western blotting. It appears in a homodimer form of approximately 42 kD.

Demonstration of the native LOX-PL interaction

Based on the above results, we chose the Hs578T cell line expressing both LOX and PL for our further experiments to demonstrate native LOX-PL interactions in a cellular environment. Using the conditioned cell medium of invasive Hs578T cells, we performed co-immunoprecipitations (coIPs). The method is mostly used to identify interacting proteins and complexes in cell lysates. In principle, the protein of interest believed to participate in an interaction is precipitated using the appropriate antibody, and the interacting partner captured can be identified. Once the protein-complex of interest is bound to the specific antibody it can be removed from the solution by an antibody-binding protein or a secondary antibody attached to some sort of a solid support such as agarose beads. Upon the removal of unbound proteins by consecutive washes, the binding of the target protein can be confirmed by immunoblotting. Immunoprecipitation is generally used in cell lysates as it requires relatively high protein concentrations. As both LOX and PL are secreted into and LOX is also processed in the extracellular space, we needed to use cell medium instead of cell lysates. For this reason it was necessary to modify the method so as to enable us to detect protein interactions in the cell medium. We decided to use a 10 kD-cutoff filter to concentrate our conditioned cell medium (CCM) samples by centrifugation increasing their protein concentration this way.

In these trials we found that, using the anti-PL antibody, LOX could be co-immunoprecipitated with PL, verifying the interaction of these proteins in the medium of the breast cancer cells **(Figure 21)**. However, we could not immunoprecipitate the LOX-PL complex using the LOX-antibody. We assume that interaction with PL might have interfered with the LOX epitope and hindered the binding of the anti-LOX antibody to the enzyme. The anti-LOX antibody was designed to recognize an epitope that resides within the N-terminal region of the mature LOX (amino acids 176-197), overlapping with the hypothetical PL-binding site within LOX (amino acids 169-348). We could exclude any aspecific interaction as the negative control purified rabbit immunoglobulin did not immunoprecipitate LOX.

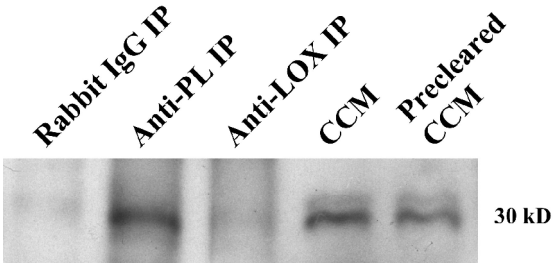

Figure 21. Co-immunoprecipitation demonstrates LOX-PL interaction in cell culture.
LOX immunoprecipitated with the anti-PL antibody, but not with the negative control rabbit IgG. However, the protein could not be captured by the positive control anti-LOX antibody, possibly because binding of PL interfered with LOX's epitope.

PL is neither a substrate nor a regulator of LOX

Following *in vitro* and *in vivo* verification of the LOX-PL interaction, we became interested in its nature, whether it is an enzyme-substrate or enzyme-regulator relation. To clarify if binding to PL inhibits LOX amine oxidase activity, we performed an enzyme activity assay with bLOX, purified from bovine aorta, that had been previously incubated with an equal or double molar amount of hPL. Bovine LOX, either alone or in the presence of PL, was incubated with 1,5-diaminopentane substrate and the H_2O_2 released during the oxidative reaction was measured using Amplex Red and horseradish peroxidase [243, 244].

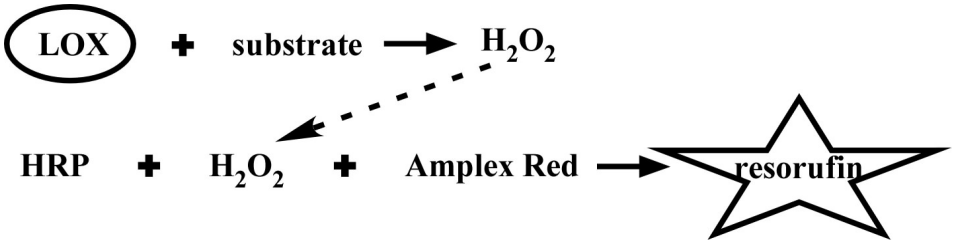

Figure 22. Reaction scheme of lysyl oxidase enzyme activity assay.
The hydrogen peroxide released upon oxidative deamination of a LOX substrate can be quantified as in its presence, HRP converts Amplex Red into fluorescent resorufin.

The LOX-inhibitor BAPN was used as negative control in parallel experiments to determine enzyme activity of LOX, and parallel reactions were tested in the absence of the substrate as well. LOX activity was calculated as the increase in fluorescence above controls under BAPN inhibition. The results did not show any decrease or increase in LOX activity when in its PL-bound state, as compared with non-bound bovine LOX **(Figure 23)**. The negative controls containing no substrate or the inhibitor BAPN show equally low levels of fluorescence, demonstrating that the fluorescence measured was due to the H_2O_2 release upon LOX's enzymatic activity. Thus we concluded that binding to PL does not alter LOX's catalytic activity, as it could be expected in an enzyme-regulator interaction.

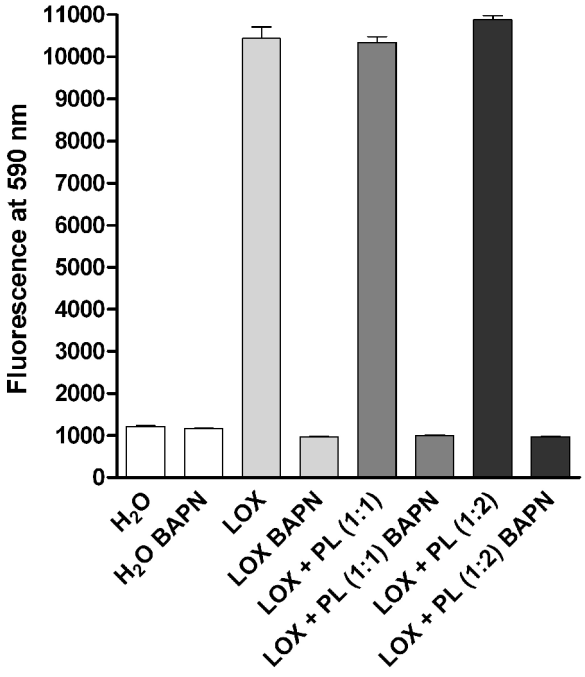

Figure 23. Lysyl oxidase enzyme activity in the presence of hPL.
No loss of enzyme activity was observed when LOX was bound to hPL compared to the activity levels of LOX alone. Negative controls contained either no substrate (only H_2O) or the specific inhibitor BAPN. Error bars represent Standard Error.

LOX has been reported to possess the ability to oxidize the lysyl residues of a secreted growth factor, basic fibroblast growth factor (bFGF) [93]. Hence we decided to test if LOX was able to oxidize lysyl residues of PL *in vitro*. Based on the experimental conditions previously described for bFGF [93], we used a range of concentrations of PL as substrate (0–1.8 μM). Purified bovine LOX was incubated with PL in the given range of concentrations and the quantity of H_2O_2 generated during the oxidative reaction was measured as described above. The results showed that PL was not oxidized at statistically significant

levels **(Figure 24)** as no significant difference could be observed in emitted fluorescence and therefore in H_2O_2 release between the samples containing PL as substrate or H_2O as a negative control. In addition, when compared to the previous activity assay in which the enzyme activity of PL-bound LOX was tested, it is clear that the fluorescence levels in this latter experiment are similar to those of the negative and BAPN-inhibited controls of the former. Based on this data, we concluded that in these *in vitro* assays, PL is not a substrate for LOX, completing Specific Aim 4.

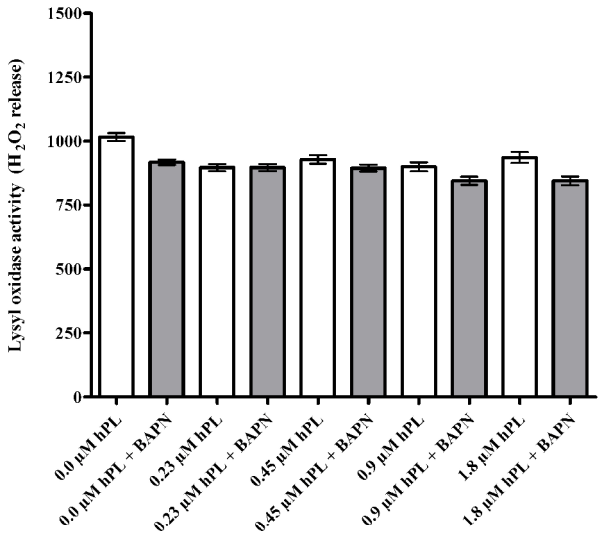

Figure 24. Activity of LOX against hPL as a substrate.
LOX did not show enzymatic activity against hPL, as fluorescence levels are comparable to those of the negative controls containing either no substrate or the inhibitor BAPN. Error bars represent Standard Error.

Investigation of the biological role for LOX-PL binding

Generation of cell lines stably expressing LOX and/or PL

The findings revealing co-expression of LOX and PL in highly invasive and metastatic breast cancer cell lines inspired us to study the biological significance of this protein interaction in a cellular model, as outlined in Specific Aim 5. We chose to investigate the individual and combined effects of LOX and PL on cell phenotype and behavior by testing the proliferative and migratory characteristics of normal breast epithelial cells overexpressing or co-expressing the proteins.

We used MCF-10A immortalized normal breast epithelial cells as the parental cell line and generated stably transduced cell lines. We elected to use a lentiviral system to introduce our genes of interest into the MCF-10A cells because the lentiviral infections have the advantage of high-efficiency in delivering a target gene into both dividing and non-dividing cells, and long-term stable expression of a transgene. Replication-incompetent

lentiviral particles, containing cDNA for PL-2 transcript variant 1 (771 bp) and the full length LOX (1254 bp) in a pLenti-based expression vector, were generated in HEK297FT cells. Following antibiotics-sensitivity tests, MCF-10A cells were infected with the lenti-viruses, and stably transduced cells were selected using 5 μg/ml blasticidin. Individual clones were isolated, expanded in culture and characterized. Since we only had a single antibiotics selection-system available, verifying the expression of the proteins and this way the integration of both PL and LOX sequences in co-transduced cells was necessary. To confirm expression of PL and LOX in these cell lines, proteins were extracted from the conditioned cell medium of preconfluent cultures grown under serum-free conditions for 2 days, and submitted to immunoblot analysis.

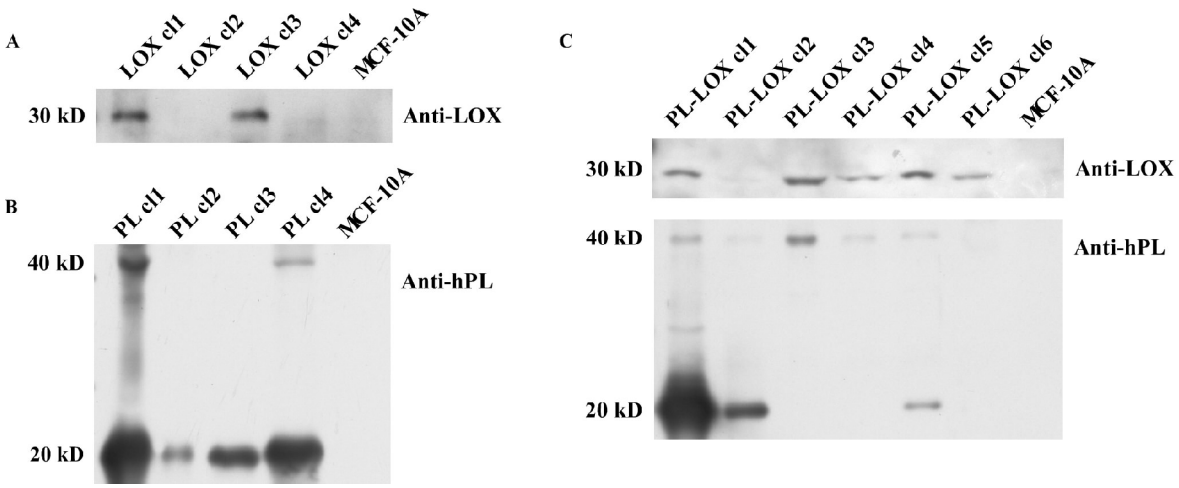

Figure 25. Generation of cell lines expressing either or both of LOX and hPL.
Western blotting was used to determine the expression of the proteins in the isolated stable clones.

As **Figure 25** demonstrates, two stably transduced clones showed elevated LOX **(Figure 25A)**, four elevated PL expression **(Figure 25B)**, and two clones co-expression of both proteins **(Figure 25C)**. The parental MCF-10A cells do not express PL under normal conditions and show no detectable levels of LOX at preconfluent stage. In the cell medium of the stable PL-expressing clones, the hormone was predominantly present as a 21 kD monomer, however, the 42 kD homodimer form we observed previously in breast cancer cells was also present in small amounts. In our further experiments, clones PL cl1, LOX cl1 and PL-LOX cl1 were studied with the parental MCF-10A cell line as control, and the observations were confirmed with clones PL cl3, LOX cl3 and PL-LOX cl5 in repeated tests.

Increased cell proliferation rates in cells co-expressing PL and LOX

PL has been reported to promote mitogenesis and *in vitro* [^{3}H]-thymidine incorporation in cultured fetal fibro- and myoblasts [200], and augment DNA synthesis in epithelial cells of human breast carcinoma [205]. Based on the above information we decided to test the effect of PL overexpression in stably transduced cell lines with particular interest on changes in proliferative attributes. Our proliferation assay was based on the use of methylene blue dye that stains the cells attached to the surface of microplate wells. Upon lysis of the cells the amount of the dye released was quantified by measuring the absorbance of the cell lysate. As a point of reference, cells were seeded in two parallel triplicates and following attachment and removal of the non-attached cells, one set of triplicates was stained with methylene blue and the "zero time point" absorbance was measured. The second set of triplicates were incubated for 72h, and stained with methylene blue. The rate of proliferation was calculated as the difference between the "zero time point" and the 72 h absorbance values. MCF-10A cells expressing PL alone had a significantly higher (49% increase, $p \leq 0.0001$) proliferation rate as compared to the parental MCF-10A cells in proliferation assays **(Figure 26)**.

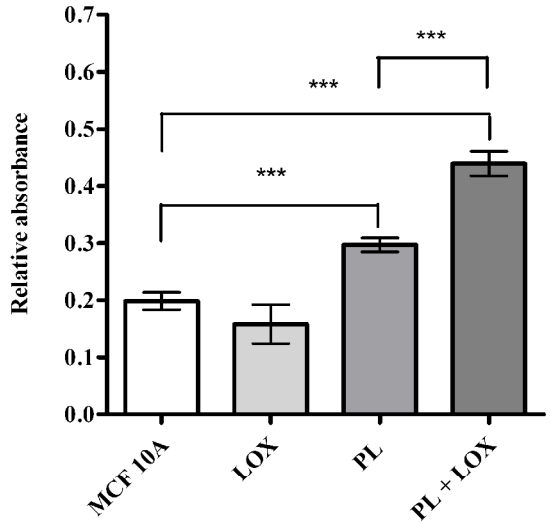

Figure 26. Proliferation assays revealed that PL expression induced MCF-10A cell proliferation, and PL-LOX co-expression further enhanced cell proliferation.
2,000 cells from each cell line were seeded in the wells of two parallel microwell plates. After 72 h of incubation, the measurement was performed on the second plate. Proliferation rate was calculated as the difference of the absorbance at 72 and 0 h. ***, $p \leq 0.0001$. P values were calculated using pairwise t-tests. These results were confirmed in repeated experiments. Error bars represent Standard Error.

LOX has not yet been reported to influence cell proliferation in any cell types. In fact, LOX overexpression did not lead to a significant increase in proliferation of the LOX

transduced cell lines compared to the control parental cell lines. We observed, however, that co-expression of LOX and PL resulted in a proliferation rate significantly higher than either the parental (121% increase, $p \leq 0.0001$) or the PL-expressing (47% increase, $p \leq 0.0001$) cell proliferation rate. These results suggested that the co-expression of LOX with PL enhances the proliferation-inducing effects of PL.

Co-expression of PL and LOX induces cell migration

Elevated LOX expression has been demonstrated to contribute to a migratory and invasive phenotype in breast cancer cell lines [4, 245]. Therefore, we tested if LOX over-expression in the stably transduced normal breast epithelial MCF-10A cells also resulted in migration, and investigated if LOX-PL co-expression had any effect on the migratory abilities of these cells. As a preliminary study, we chose to use phalloidin staining to visualize the actin cytoskeleton of our parental and transduced MCF-10A cells, as the possible rearrangement of the actin cytoskeleton would have enabled us to conclude if any changes toward a more migratory phenotype had occurred. Phalloidin is a toxin from Amanita phalloides, which binds actin, preventing its depolymerization, and therefore in a fluorophor-conjugated form it can be utilized to detect the cellular distribution of actin. The cells from each cell line were thus seeded on glass coverslips and grown until approximately 60% confluency. Upon fixation with formaldehyde, the cells were permealized, blocked in a BSA solution, and stained with fluorescence-conjugated phalloidin. High resolution images of the phalloidin-stained cytoskeleton were captured by confocal microscopy.

We have found the rearrangement of the actin cytoskeleton and the appearance of numerous, longer filopodia, in the PL and LOX expressing cell lines as well as in the PL-LOX co-expressing cell line compared to the parental MCF-10A cells **(Figure 27)** which had fewer and shorter filopodia.

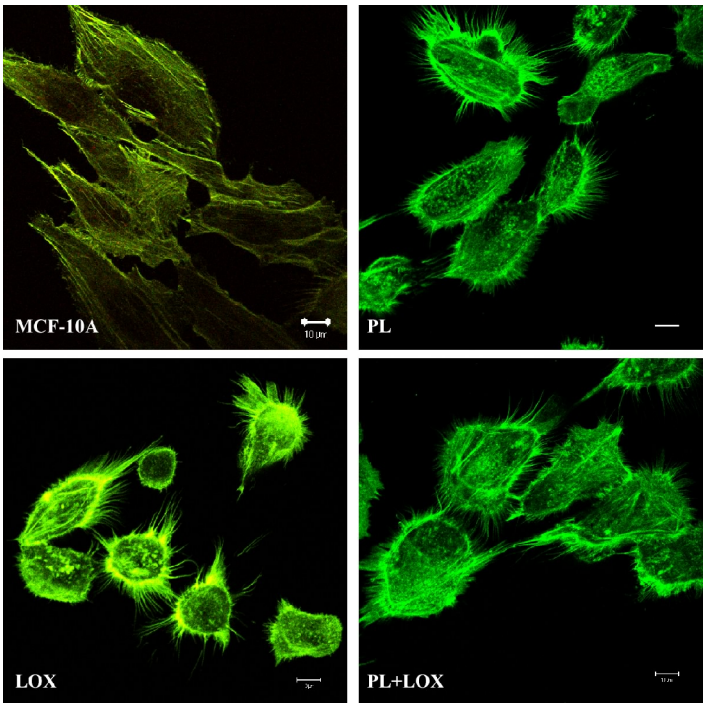

Figure 27. PL and LOX expression in MCF-10A cells leads to rearrangement of the actin cytoskeleton. The actin cytoskeleton in MCF-10A and stably transduced cells was stained with FITC-conjugated phalloidin. Expression and co-expression of PL and LOX resulted in actin cytoskeleton rearrangement and formation of numerous filopodia in transduced cells compared with parental MCF-10A. *Scale bars*, 10 μm.

These cytoplasmic projections are characteristic of a migrating cell. The above alterations of the actin cytoskeleton indicated changes towards a migratory phenotype in the stably transduced cells. Subsequently, migratory properties of the cells were evaluated and quantified in cell migration assays.

With the cell migration assays, we investigated the ability of our transduced and parental cell lines to migrate through a polycarbonate filter coated with gelatin. Breast epithelial cells individually expressing PL or LOX did not show significantly different levels of migration compared to the parental MCF-10A cells. In contrast, the LOX-PL co-expressing cell line exhibited a strong migratory phenotype and had significantly higher migratory rates than the parental (240% increase, p=0.0038) or the LOX overexpressing (93.5% increase, p=0.0052) cell lines **(Figure 28)**.

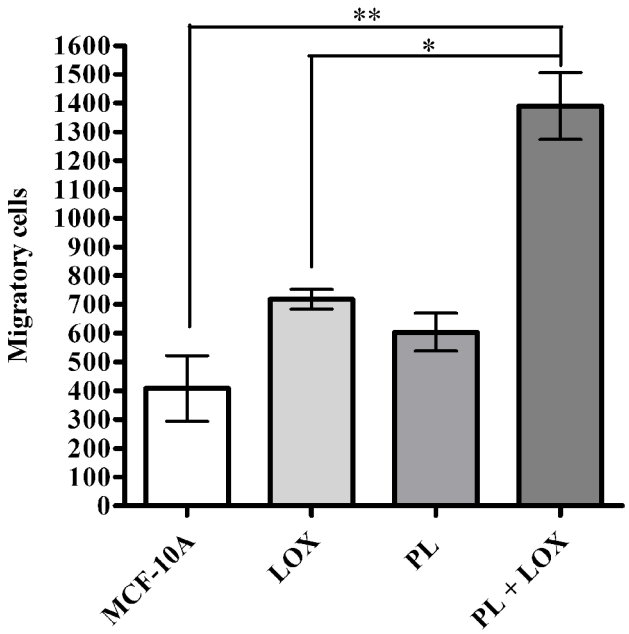

Figure 28. Migration assays used to determine migratory rates of parental and stably transduced cell lines.
50,000 cells were seeded on a gelatin-coated polycarbonate membrane in triplicates and were allowed to migrate for 4 h. The cells were then fixed, stained, and counted. PL-LOX co-expression led to a significantly higher migratory rate (240% increase) compared to the parental cell line MCF-10A. The expression of either PL or LOX alone did not significantly increase cell migration. Error bars represent Standard Error. **, $p \leq 0.005$. *, $p \leq 0{,}01$.

These results were confirmed in three independent experiments, and similar data were obtained using the other overexpressing and co-expressing clones. These results indicated that LOX and PL co-expression, was capable of inducing a strong migratory phenotype in normal breast epithelial cells, while the overexpression of either LOX or PL alone had no such consequence. These data revealed that, unlike in non-invasive breast cancer cells, overexpression of LOX in normal breast epithelial cells does not have a migration and invasion promoting effect.

DISCUSSION

In our previously performed yeast two-hybrid screens we have identified the pregnancy-specific hormone, placental lactogen as an interacting partner of lysyl oxidase. Although the yeast two-hybrid screening is a widely-used tool for identification of protein-protein interactions in a large scale, it holds the possibility of high percentage of false positives estimated by some to be up to 50% [246]. This can be due to many aspects of the screen as the overexpression and therefore the abundance of the studied proteins in yeast, the possibility of inaccurate posttranslational modifications of mammalian proteins in yeast, or expression of two proteins that would not be present in the same cell under physiological conditions can all result in false positive interactions. For this reason, it is necessary to confirm the yeast two-hybrid screen data by using other, more reliable methods as well.

In yeast two-hybrid screens, using the mature and the full-length enzyme as bait, we found a high representation of PL cDNAs among the LOX-binding positive clones (23% of clones for the mature and 35% for the full-length LOX form). Considering that PL mRNA accounts for only approximately 5% of the total placental mRNA produced at term [174], we could exclude that the high ratio of LOX-interacting PL clones present was due to the abundance of PL cDNA in our placental cDNA library.

Both PL isoforms hPL-2 and hPL-3 are expressed during pregnancy. In term placentas the hPL-2 transcripts have been found to be more abundant, possibly due to increased expression levels compared to that of hPL-3 [247]. Consistent with this observation the cDNA clones identified in our screen were all clones of PL-2 transcript variant 1. This variant encodes the most abundant PL-2 isoform that contains all five coding exons of the gene.

Supporting its specificity, the interaction was additionally confirmed in yeast direct interaction trials. We used these experiments not only to verify our yeast two-hybrid screen data but to define the possible PL-binding site within LOX. Initially, we hypothesized that LOX's C-terminal cytokine receptor-like domain would contain the site of interaction as it shows homology to the N-terminal extracellular domain of the cytokine receptor superfamily. The consensus sequence C-x9-C-x-W-x26-32-C-x10-13-C (C - cystein, W - tryptophan, xn - a defined number of any amino acid) conserved in human LOX and LOXL proteins is the consensus sequence of the extracellular ligand-binding domain for many receptors for cytokines as well as prolactin and growth hormone [20, 248]. As human placental lactogen binds the class 1 cytokine receptors for both prolactin and growth hormone

(PRLR and GHR, respectively) [219], the interaction of LOX's CRL domain with hPL seemed likely. However, the CRL domain itself did not exhibit any binding affinity to hPL in our yeast direct interaction studies. Cytokine receptors contain two barrel-shaped domains within their extracellular region, the CRL domain within LOX however lacks the C-terminal barrel module as it has been revealed by amino acid sequence analysis [11]. It seemed possible that this truncated cytokine receptor domain of LOX was not sufficient for binding PL.

Like the CRL domain, the propeptide region of LOX had no detectable binding affinity to PL either. It was interesting to observe that while the CRL region did not interfere with the protein interaction, the presence of the propeptide proved to have a partial inhibitory effect on LOX-PL binding, as yeast containing the full length LOX (i.e. the mature LOX with the propeptide, LOX_{1-417}) grew at a slower rate than yeast containing the mature enzyme lacking the propeptide ($LOX_{169-417}$), indicating lower levels of reporter gene expression and thus a weaker protein interaction. On the other hand the presence of the propeptide in assays using deletion construct LOX_{1-348} did not influence the LOX-PL binding to a measurable extent.

These discrepancies of data obtained in different assays might be the result of improper folding and/or posttranscriptional processing of the mammalian proteins in yeast, a known drawback of the experimental system. Based on the yeast direct interaction data, the PL-interacting domain of LOX appears to be located between amino acids 169 and 348. However, further confirmation of these findings was needed, and no final conclusion could be drawn concerning the site of interaction based solely on these data. Since this interacting fragment of LOX includes a region highly homologous among the LOX family members, the so called LOX domain, we also investigated the interaction of PL with fragments of LOXL and LOXL2. In the yeast direct interaction studies, only LOXL2 showed binding to PL, but not LOXL. Interestingly, the enzymes binding PL, LOX and LOXL2 but not LOXL have both been implicated in tumorigenic processes, as detailed in the Lysyl oxidase in cancer section. Nevertheless, because of the above discussed drawbacks of the method, verification of this data was needed.

Following these studies, we decided to establish the nature of the LOX-PL interaction in *in vitro* experiments. For these trials we needed significant amounts of the purified LOX. Although the purified form is not commercially available, the enzyme can be isolated from many different tissues such as piglet skin, human placenta or bovine aorta as it has been reported previously [14, 15, 18]. Since protein isolation from tissues does not

yield quantities sufficient for the proposed biochemical trials, we had to revise our strategy and find an alternate solution. We therefore chose to express LOX deletion constructs in *E. coli* fused with an N-terminal GST-tag that would enable rapid, affinity-based purification of the protein using solid carrier-bound glutathione. First, we attempted to isolate LOX fusion proteins in a soluble form These trials had their limitations, as we observed protein degradation that we were not able to eliminate and in addition, many of the LOX fusion constructs were present not in soluble form but in the inclusion body fractions. As successful production and purification of recombinant LOX proteins from *E. coli* expression systems had been reported, we decided to purify LOX from inclusion bodies based on methods described previously [69, 228]. We were able to generate significant amounts of GST-LOX following this protocol. To reconstitute the recombinant proteins we tried refolding them by dialysis against Cu2+-containing K_2HPO_4 buffer to ensure incorporation of the Cu^{2+}-cofactor that is essential for proper folding and catalytic activity. While the protocol we adapted reportedly yielded active recombinant LOX [69, 228], we were not able to detect any enzymatic activity of our recombinants. Still the expressed LOX proteins seemed to be in a conformation similar to that of native LOX because they showed binding affinity to LOX substrates.

Having generated sufficient amounts of recombinant LOX and its deletion constructs, we proceeded with biochemical studies intended to characterize the LOX-PL binding. Our data validated the interaction in *in vitro* assays. Furthermore, information on the PL-binding site of LOX in pull-down and solid phase binding assays was obtained. We demonstrated that the CRL domain did have a binding affinity for PL (see GST-$LOX_{349-417}$ in **Figures 17** and **18A**) that could not be detected in previous yeast direct interaction trials. In addition, solid phase binding data suggested that the CRL domain may participate in PL interactions, in concert with a more N-terminal binding site of LOX, since the presence of the CRL domain in the GST-LOX construct (GST-$LOX_{169-417}$) significantly increased binding affinity to PL compared with the GST-LOX construct lacking this domain (GST_$LOX_{169-348}$) (**Figure 18A**).

Our results also revealed that lysyl oxidase was able to bind not only hPL but the highly homologous hGH and the less homologous hPRL, additional members of the somatotropin/prolactin hormone family. Among their numerous biological roles, hGH and hPRL are required for normal mammary gland development [249]. Increasing evidence supports a role for both GH and PRL in breast cancer development as well [223, 250-252]. As no such interaction have been reported previously, further investigations are needed to reveal

the biological significance of these hormone-enzyme interactions in physiological as well as pathological processes such as breast neoplasm formation.

Our *in vitro* activity assays intended to characterize the LOX-PL binding and shed light on the characteristics of this protein interaction. Binding affinity of LOX to PL proved to be two orders of magnitude lower than its affinity to the tropoelastin and type I collagen substrates of the enzyme as determined by Fogelgren et al. [55]. We have clarified that despite LOX's ability to oxidize lysyl residues reported for the growth factor bFGF [93], the native enzyme does not oxidize any of the nine lysyl residues present in human placental lactogen as a substrate. Even though we conducted these trials using LOX isolated from bovine aorta, we hypothesize that human lysyl oxidase is acting in a similar manner, as bovine and human LOX share an overall amino acid sequence homology of approximately 89% with the catalytic domains showing the highest degree of homology and no substrate differences were noted between human and bovine LOX.

In addition, we have determined that binding to PL has neither an inhibitory nor a stimulatory effect on the enzyme activity towards cadaverine (1,5-diamino pentane) as substrate. Based on these results we assumed that the PL binding site is not overlapping with the enzyme's catalytic site.

As both LOX and PL expression has been detected in breast cancer tissues by others [4, 217], we chose to test a tissue macroarray to evaluate their possible co-expression in tumor samples of mammary origin. Of the six tumor samples tested three showed co-expression of our proteins of interest that supported the previous findings. We demonstrated the presence of both proteins in breast cancer tissues, and we became interested in evaluating this binding *in vivo* and investigating the possible role of the LOX-PL interaction in breast cancer. Despite its involvement in breast tumors, PL expression and function has not yet been characterized in tumor cell lines of mammary origin. Therefore, we tested PL-expression in breast cancer cell lines, and found that the highly invasive and metastatic cells did express PL in addition to LOX. Moreover, we were able to verify the interaction *in vivo* by co-immunoprecipitation, and we were confident that the interaction can indeed occur in tissues. These results gave rise to our hypothesis that the *in vivo* LOX-PL binding might contribute to tumorigenic processes. To test our theory we chose to investigate if the overexpression of either or both LOX and PL had any effect on immortalized normal breast epithelial cells as breast carcinomas originate from the epithelium of the mammary gland.

Placental lactogen reportedly induces cell proliferation, but such effect has not been attributed to LOX. Thus, we decided to investigate if PL had similar effect in normal breast epithelium and if LOX would alter hPL's such effect. Supporting previous data on hPL's cell proliferation promoting function [200, 201, 206, 253], we observed significantly elevated proliferation rates as a result of PL overexpression in stably transduced normal breast epithelial cell lines. The molecular mechanism of how PL triggers cell proliferation remains to be elucidated. It is likely, however that the hormone exerts its effect via growth hormone or prolactin receptors. GHR and PRLR are present in normal mammary epithelium and breast cancer cells [254-258], and PL has been demonstrated to bind to both receptors [219]. Activation of GHR results in signal transduction via the JAK/Stat pathway, predominantly through JAK2/Stat5 activation, and leads to cell proliferation in MCF-10A cells [251]. On the other hand, hPL signaling via the GHR has not been reported so far. It is possible that hPL primarily acts by binding not the growth hormone but the prolactin receptor, as it has a higher affinity for the latter [219]. Activation of the PRL receptor stimulates JAK2/Stat5 signaling as well as MAPK and Src/FAK kinase cascade that ultimately induce proliferation as it has been previously demonstrated in breast cancer cell lines T47D and MCF7 [259]. A similar signaling pathway has been demonstrated for mouse PL (mPL)-I which upon binding PRLR activated JAK2-mediated proliferation in Nb2 cells [260]. Furthermore, PL was shown to stimulate tyrosine phosphorylation of JAK2 and induce the JAK-STAT pathway in breast carcinoma cells [261]. We suggest that the high rate of proliferation observed in PL-overexpressing cells is due to activation of these signaling pathways. Although LOX has been reported to activate Src/FAK signaling in a hydrogen-peroxide mediated fashion in breast cancer cell lines [4], no proliferation-inducing effect have been observed so far. On the contrary, previous studies have shown that genetic inhibition of LOX increases cell proliferation [262]. In addition, LOX is capable of reducing the mitogenic potential of bFGF by oxidizing it [93]. In our experiments, however, PL did not prove to be a substrate of the enzyme, and proliferation was not negatively affected by LOX, since the LOX-expressing clones did not show any change in cell proliferation compared to parental cells, and the cell lines stably co-expressing PL and LOX demonstrated a significant increase in proliferation compared not only to parental but to PL-expressing cell lines as well. Therefore we hypothesize that this increase was not due to separate effects of the proteins but a result of their interaction. The exact mechanism by which LOX-PL interaction may promote such processes remains to be investigated.

We evaluated the effects of LOX and/or PL overexpression on another significant cellular process, cell migration. As LOX promotes migration in breast carcinoma cell lines [3, 4] and murine breast tumors [151] via FAK/Src signaling, we hypothesized that it would have similar effects in normal breast epithelial cells. It is important to note that MCF-10A cells express LOX in cell culture [109], therefore these cells are capable of processing and activating the ectopic LOX. On the other hand, PL has not been implicated in cell migration so far, therefore we became interested if PL would modify LOX's effect on the MCF-10A cells. Phenotypic changes were examined first by staining the actin cytoskeleton. We expected to find altered filament organization as a hallmark of altered cell motility. We observed the rearrangement of the actin cytoskeleton and the formation of numerous filopodia in all stably transduced cell lines, including those that expressed PL only, compared to the parental cell line. Filopodia are cytoplasmic projections that extend from the leading edge of migrating cells, and are characteristic of growth cones involved in axon development and fibroblasts participating in wound healing. These findings prompted us to further investigate the migratory abilities of these cell lines.

Migration assays revealed, that although MCF-10A cells are able to process ectopic LOX, and overexpression of the enzyme leads to formation of filopodia, LOX was not sufficient to induce a migratory phenotype in normal cells unlike in the poorly invasive, non-metastatic breast cancer cell line MCF7. We hypothesized that the effect of LOX may be moderated in normal MCF-10A cells or that LOX may have different function in normal breast epithelial and breast carcinoma cells. PL has not been implicated in cell migration so far, and our normal breast epithelial cells stably overexpressing PL did not exhibit any significant increase in their migratory abilities either. Previous findings demonstrated that activation of the prolactin receptor leads to membrane ruffling, cytoskeletal changes, and increased cell motility in breast cancer cell lines MCF7, MDA-MB231 and T47D cells [220]. In partial agreement with this study, we observed rearrangement of the actin cytoskeleton, although alterations in cell migration were not noted in our trials. The LOX and PL co-expressing cells on the other hand did show a significant increase in their motility compared to the PL or LOX overexpressing and the parental cell lines, in addition to cytoskeletal rearrangement and formation of numerous filopodia. Assuming that both LOX and PL acted through the FAK/Src signaling cascade and independently induced tyrosine phosphorylation and activation of FAK/Src, we would observe similar doubling of migratory rates in the co-expressing cell line. Yet neither LOX, nor PL alone induced a significant increase in cell migration, making the additivity of their effects unlikely. It is possible,

however, that FAK/Src activation by PL via PRLR led to cytoskeletal changes that primed cells toward a motile phenotype, although it was not sufficient to induce cell migration. Additional FAK/Src activation by LOX therefore possibly contributed to the emergence of a more migratory phenotype. Furthermore, it is also possible that LOX-PL interaction enhanced signaling to an extent where it was sufficient to induce cell motility in normal epithelial cells. The exact mechanism by which the LOX-PL interaction triggers highly migratory phenotype is to be evaluated in further studies.

Co-expression of LOX and PL occurs in Hs578T and MDA-MB231 breast cancer cell lines, where they likely contribute to their highly invasive and metastatic phenotype. LOX and PL are co-expressed in the choriocarcinoma cell line JAR as well, that is also migratory and invasive [263]. Furthermore, under physiological conditions, PL and LOX are both expressed by placental trophoblasts, and may possibly contribute to the trophoblast invasion. Similarities of trophoblast and cancer cells have been emphasized by recent research results. Although the placenta is a normal tissue, its trophoblastic cells have been described as pseudo-malignant cells because they exhibit high cell proliferation, lack of cell-contact inhibition, migratory and invasive properties as well as capacity to escape effectors of the immune system, especially during the first trimester of pregnancy. The emerging hypothesis is that trophoblastic and cancer cells use comparable mechanisms and similar molecular mechanisms in their proliferative, migratory and invasive processes. The PL-LOX interaction may possibly be a member of the molecular circuits shared by "pseudo-malignant" invasive trophoblasts and invasive breast cancer cells. Therefore, the comparison of signaling pathways in placental trophoblasts and tumor cells is essential to reveal the signaling cascades and key molecules implicated in the invasion and migration processes.

Our results collectively demonstrate an important role for the LOX amine oxidase in epithelial cell behavior in novel interactions with a somatotropin/prolactin hormone family member, placental lactogen, and show that these interactions may be highly relevant to the transition of epithelial cells toward a migratory phenotype during the development and progression of breast carcinoma.

SUMMARY

Introduction

Breast cancer is the most common cancer among women, as it is diagnosed in $1x10^6$ women in the world each year. The incidence of breast tumor development in women differs with age, mammary gland mass and exposure to endogenous and exogenous hormones. However, the function, molecular regulation, and interaction of genetic alterations in carcinogenesis, as well as those of tumor cell migration and invasion, and metastasis of breast carcinomas are not fully understood. As metastasis is a major challenge in cancer treatment, determination of molecular markers of metastatic potential and therefore understanding the process of metastatic tumor progression are important. Recently, comprehensive transcriptome profiling of normal breast, noninvasive breast tumor and invasive breast cancer cell lines identified lysyl oxidase (LOX) as a possible marker gene based on its differential expression. In addition, upregulated LOX expression and activity have been reported in breast cancer tissues and invasive/metastatic breast cancer cell lines as well.

Lysyl oxidase (LOX), a copper-dependent amine oxidase, contributes to the assembly and maintenance of the extracellular matrix (ECM) by initiating the formation of covalent cross-links in collagen and elastin. Besides its matrix-stabilizing function, LOX has recently been shown to play a role in cell motility, transcriptional regulation, embryonic development and several pathological conditions including breast cancer. Lysyl oxidase has been primarily recognized as a matrix-crosslinking enzyme; hence regulators and interacting factors contributing to its novel role in breast cancer development and progression are yet to be discovered. The goal of this work was to identify novel LOX-interacting partners that likely promote its function during tumorigenesis, and to characterize such interactions and evaluate their biological significance.

A previously performed yeast two-hybrid screen using a human placental cDNA library and both full-length and processed active form of LOX as baits identified placental lactogen (PL) as a possible interacting partner. PL, a member of the growth hormone (GH)-prolactin (PRL) hormone family, stimulates mammary gland development, lactogenesis and the growth and metabolism of the fetus. PL is expressed in the placental syncytiotrophoblasts under normal conditions, but its expression was shown in 77% of invasive ductal carcinomas, and the amplification of the PL genes has been reported in 22% of the cases. While PL was reported to promote epithelial cell proliferation in breast carcinomas, its role in breast tumors is not fully understood.

Specific aims

Upon the identification of a novel LOX-interacting partner, placental lactogen that likely promote LOX's function during tumorigenesis, the goal of this work was to characterize such interactions *in vitro* and *in vivo* and evaluate their biological significance.

Methods

E tanulmány során a korábbi élesztő két-hibrid vizsgálataink által kimutatott lizil oxidáz-placentális laktogén fehérjekölcsönhatást igazoltuk és jellemeztük. A biokémiai analízishez rekombináns GST-LOX deléciós konstruktumokat klónoztunk, majd a fúziós fehérjéket Escherichia coliban termeltettük és tisztítottuk. Az élesztő direkt kölcsönhatás-vizsgálatok, a szilárd fázisú kötési vizsgálatok és a pull-down kísérletek igazolták a LOX-PL kölcsönhatást *in vitro*, illetve a kötési affinitás is meghatározásra került. Enzim aktivitási mérésekkel teszteltük az interakció hatását a LOX katalitikus működésére. A fehérjék expresszióját immunfluoreszncenciával határoztuk meg egy konfokális mikroszkóp segítségével emlőrák eredetű szövetmetszetekben. Western blot analízissel teszteltük a fehérjék expresszióját emlőrák sejtvonalakban, az *in vivo* kölcsönhatást koimmunoprecipitációval vizsgáltuk. Immortalizált, normál emlő epitelium eredetű sejtvonalakat lentivirális vektorokkal transzdukáltunk, ezzel a LOX és/vagy a PL túltermelésére késztetve őket. Ezt követően a LOX és a PL túltermelésének egyedi és együttes, a sejtek viselkedésére gyakorolt hatását analizáltuk ezen sejtvonalakon. A proliferatív eltéréseket proliferációs tesztekkel, a motilitásbeli eltéréseket először az aktin citoszkeleton lehetséges átrendeződését feltáró falloidin festéssel, majd migrációs esszékkel tanulmányoztuk.

Results

Results from our direct interaction studies, pull-down assay, Far-Western analysis and solid phase binding assays supported LOX binding to PL and suggested binding to GH and PRL as well. In addition, *in vitro* amine oxidase activity assays showed that PL is neither a substrate nor an inhibitor of LOX. Since increased expression and enzyme activity of LOX have been reported in highly invasive and metastatic breast cancer cell lines as well as in metastatic breast tumors, and PL has been shown to be expressed in breast carcinomas, we tested their expression in tissue sections of mammary carcinomas and breast cancer cell lines. Using fluorescence-labeled immunostaining on a tissue macroarray, we detected LOX and PL expression in and around tumor cells. Subsequently, we tested protein expression of breast cancer cell lines, and found elevated PL expression in highly invasive

MDA-MB231 and Hs578T cell lines, where LOX expression was also increased. Furthermore, we showed PL expression in poorly invasive MCF-7 and T47D cells at elevated and low levels, respectively. Since the highly invasive and metastatic breast cancer cell lines express both PL and LOX, we decided to study their individual and combined effects on cell behavior by overexpressing and co-expressing these proteins in immortalized normal breast epithelial cells. LOX plays a role in promoting cell migration, while PL was shown to induce cell proliferation, thus we tested these processes. Stably transduced MCF-10A normal mammary epithelial cells co-expressing PL and LOX had significantly increased proliferation rates compared to the parental and the PL-expressing cells, while LOX alone had no effect on proliferation. Therefore, the co-expression of LOX with PL appears to enhance the proliferation-inducing effect of PL. Co-expressing cells in addition showed a significantly higher migratory rate compared to cells expressing either or none of these proteins. Our results demonstrated that LOX, in addition to promoting tumor cell invasion through a H_2O_2-induced FAK/Src activation that we have described earlier [3], may further induce tumor cell migration in interaction with PL by activating independent signaling.

ÖSSZEFOGLALÓ

Bevezetés

Az emlőrák a nők körében leggyakrabban előfordulő rákos megbetegedés, évente $1x10^6$ nőt diagnosztizálnak világszerte mellrákkal. Az emlődaganatok kialakulásának kockázata a nőkben az életkortól, az emlőmirigy tömegétől és endo- illetve exogén hormonhatásoktól függően eltérő lehet. Ugyanakkor a tumorigenezist kiváltó, genetikai változásokból eredő molekuláris mechanizmusok, kölcsönhatások és azok regulációja, illetve a tumorsejtek migrációjának, inváziójának valamint a karcinómák áttétképződésének pontos molekuláris folyamatai még nem tisztázottak. Mivel az áttétek keletkezése a rák gyógykezelésében komoly kihívást jelent, a metasztatikus képességet jelző molekuláris markerek azonosítása és az áttétes tumorképződés folyamatának megértése kiemelkedő fontosságú.

A közelmúltban egy normál, non-invazív emlő tumor és invazív emlőrák sejtvonalakon végzett, átfogó transzkriptóma-elemzés során a lizil oxidázt (LOX) eltérő expressziója alapján potenciális marker génként azonosították. Ezen felül a LOX emelkedett expresszióját és enzimaktivitását figyelték meg mellrák szövetekben és invazív/metasztatikus emlőrák sejtvonalakban. A lizil oxidáz (LOX), egy extracelluláris amin oxidáz, ami a kollagén és elasztin keresztkötésének első enzimatikus lépését katalizálja. A LOX tehát fontos szerepet tölt be az extracelluláris mátrix (ECM) kialakulásában, szerkezetének fenntartásában, ezáltal a normális kötőszöveti működésben, az embrionális fejlődésben és a sebgyógyulásban. A lizil oxidáz fő ismert funkciója az extracelluláris mátrix komponenseinek keresztkötése, így azon szabályozó és kölcsönható faktorok, melyek hozzájárulnak az enzim újonnan leírt, mellrákban betöltött funkciójához, még felderítésre várnak.

Korábbi élesztő két-hibrid szűréseink a lizil oxidáz és a terhesség-specifikus hormon, a placentális laktogén (PL) interakcióját mutatták ki. PL a szomatotropin-prolaktin hormoncsalád tagja, mely normál körülmények között a placenta szinciciotrofoblasztjaiban termelődik, és a laktogenezisben valamint a magzat növekedésében és fejlődésében játszik szerepet. A növekedési hormonhoz és a prolaktinhoz hasonlóan a placentális laktogén expresszióját is leírták már emlő eredetű tumorokban. Ugyanakkor a tumorképződésben betöltött szerepe, hasonlóan a LOX-PL kölcsönhatás szerepe ezekben a folyamatokban még tisztázásra vár.

Célkitűzés

A kutatás célja a lizil oxidáz, a fent említett patológiás folyamatokban potenciálisan közreműködő szubsztrátjainak és regulátorainak élesztő két-hibrid szűréssel történt azonosítása után, a fehérje-kölcsönhatások kísérletes igazolása, valamint ezen interakciók biokémiai jellemzése, biológiai szerepük elemzése normál és patológiás folyamatokban.

Módszerek

E tanulmány során a korábbi élesztő két-hibrid vizsgálataink által kimutatott lizil oxidáz-placentális laktogén fehérjekölcsönhatást igazoltuk és jellemeztük. A biokémiai analízishez rekombináns GST-LOX deléciós konstruktumokat klónoztunk, majd a fúziós fehérjéket Escherichia coliban termeltettük és tisztítottuk. Az élesztő direkt kölcsönhatásvizsgálatok, a szilárd fázisú kötési vizsgálatok és a pull-down kísérletek igazolták a LOX-PL kölcsönhatást *in vitro*, illetve a kötési affinitás is meghatározásra került. Enzim aktivitási mérésekkel teszteltük az interakció hatását a LOX katalitikus működésére. A fehérjék expresszióját immunfluoreszncenciával határoztuk meg egy konfokális mikroszkóp segítségével emlőrák eredetű szövetmetszetekben. Western blot analízissel teszteltük a fehérjék expresszióját emlőrák sejtvonalakban, az *in vivo* kölcsönhatást koimmunoprecipitációval vizsgáltuk. Immortalizált, normál emlő epitelium eredetű sejtvonalakat lentivirális vektorokkal transzdukáltunk, ezzel a LOX és/vagy a PL túltermelésére késztetve őket. Ezt követően a LOX és a PL túltermelésének egyedi és együttes, a sejtek viselkedésére gyakorolt hatását analizáltuk ezen sejtvonalakon. A proliferatív eltéréseket proliferációs tesztekkel, a motilitásbeli eltéréseket először az aktin citoszkeleton lehetséges átrendeződését feltáró falloidin festéssel, majd migrációs esszékkel tanulmányoztuk.

Eredmények

A LOX és a PL kölcsönhatását az élesztő két-hibrid szűrések után *in vitro* módszerekkel igazoltuk, és a LOX fehérjén belül a PL-kötő helyet a 169-348 aminosav rágióra szűkítettük le. Ugyanakkor valószínű, hogy a kötés stabilizálásában a LOX citokin receptorszerű (CRL), C terminális doménje is szerpet játszik. Emellett megállapítottuk a fehérjék kötődésének affinitását (K_d = 30-120nM), mely két nagyságrenddel bizonyult alacsonyabbnak az enzim szubsztrátjai felé mutatott affinitásánál. Az irodalmi adatokkal összhangban a két fehérje expresszióját igazoltuk mind mellrák szövetmintákban, mind emlőrák eredetű sejtvonalakban. Azt találtuk, hogy az invazív és erősen metasztatikus emlőrák sejtvonalak nemcsak a lizil oxidázt, hanem a placentális laktogént is emelkedett szinten

termelik. Az egyik ilyen rákos sejtvonalban végzett koimmunoprecipitációval bizonyítottuk *in vivo* interakciójukat is.

Mivel az erősen invazív és metasztaikus sejtvonalakban mindkét fehérje termelődik, úgy döntöttünk, megvizsgáljuk azok a sejtek viselkedésére gyakorolt egyedi és együttes túltermelésének hatását normál emlő epiteliális sejtekben.

MCF-10A normál emlő epiteliális sejteket lentivirális vektorokkal transzdukáltunk, hogy stabilan expresszálják a LOX enzimet és/vagy a Pl hormont. A LOX szerepet játszik a sejtvándorlás folyamataiban, míg a PL egyik funkciója a sejtosztódás indukciója; ezért tehát ezen sejtes folyamatok vizsgálatát végeztük. A PL-t és LOX-ot stabilan túltermelő normál emlő epiteliális MCF-10A sejtek emelkedett osztódási rátát mutattak a parentális és a csak PL-t termelő MCF-10A sejtvonalakhoz képest. A LOX túltermelés önmagában nem volt semmilyen detektálható hatással a sejtosztódási rátákra. A LOX koexpressziója a PL-nel tehát úgy tűnik, felerősíti a PL által indukált sejtosztódást.

A koexpresszáló sejtek ezen felül szignifikánsan magasabb migrációs szinteket is mutattak, mint a parentális vagy csak az egyik fehérjét termelő MCF-10A sejtek. Érdekes módon a LOX túltermelése a normál emlő epitheliális sejtekben önmagában nem volt elegendő egy jobban migráló fenotípus kialakításához, ellentétben a kevéssé invazív MCF-7 mellrák eredetű sejtvonalakban kifejtett ilyen irányú hatásával.

Eredményeink tehát demonstrálták, hogy a lizil oxidáz a tumorsejtek migrációját a már általunk korábban leírt, H_2O_2-indukált FAK/Src jelátviteli útvonal serkentése mellett, a placentális laktogénnel való kölcsönhatása révén, más független jelátviteli útvonalakon keresztül is elősegítheti.

REFERENCES

1. *Hormones and breast cancer.* Hum Reprod Update, 2004. **10**(4): p. 281-93.
2. Nagaraja, G.M., Othman, M., Fox, B.P., Alsaber, R., Pellegrino, C.M., Zeng, Y., Khanna, R., Tamburini, P., Swaroop, A., and Kandpal, R.P., *Gene expression signatures and biomarkers of noninvasive and invasive breast cancer cells: comprehensive profiles by representational difference analysis, microarrays and proteomics.* Oncogene, 2006. **25**(16): p. 2328-38.
3. Kirschmann, D.A., Seftor, E.A., Fong, S.F., Nieva, D.R., Sullivan, C.M., Edwards, E.M., Sommer, P., Csiszar, K., and Hendrix, M.J., *A molecular role for lysyl oxidase in breast cancer invasion.* Cancer Res, 2002. **62**(15): p. 4478-83.
4. Payne, S.L., Fogelgren, B., Hess, A.R., Seftor, E.A., Wiley, E.L., Fong, S.F., Csiszar, K., Hendrix, M.J., and Kirschmann, D.A., *Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism.* Cancer Res, 2005. **65**(24): p. 11429-36.
5. Kagan, H.M. and Trackman, P.C., *Properties and function of lysyl oxidase.* Am J Respir Cell Mol Biol, 1991. **5**(3): p. 206-10.
6. Pinnell, S.R. and Martin, G.R., *The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptide linkage to alpha-aminoadipic-delta-semialdehyde (allysine) by an extract from bone.* Proc Natl Acad Sci U S A, 1968. **61**(2): p. 708-16.
7. Mecham, R.P., Lange, G., Madaras, J., and Starcher, B., *Elastin synthesis by ligamentum nuchae fibroblasts: effects of culture conditions and extracellular matrix on elastin production.* J Cell Biol, 1981. **90**(2): p. 332-8.
8. Decker, J.L., Levene, C.I., and Gross, J., *Effect of beta-aminopropionitrile (lathyrus factor) on free hydroxyproline, proline and glycine in chick embryo.* Proc Soc Exp Biol Med, 1959. **101**(3): p. 472-4.
9. Miller, E.J., Martin, G.R., Mecca, C.E., and Piez, K.A., *The biosynthesis of elastin cross-links. The effect of copper deficiency and a lathyrogen.* J Biol Chem, 1965. **240**(9): p. 3623-7.
10. Martin, G.R., Pinnell, S. R., Siegel, R. C., and Goldstein, and R., E., *Chemistry and molecular biology of the intercellular matrix*, ed. E.A. Balazs. Vol. 1. 1970, New York: Academic Press. 405.
11. Csiszar, K., *Lysyl oxidases: a novel multifunctional amine oxidase family.* Prog Nucleic Acid Res Mol Biol, 2001. **70**: p. 1-32.
12. Floris, G. and Agro, A.F., *Amine Oxidases*. Encyclopedia of Biological Chemistry, ed. W.J. Lennarz and M.D. Lane. Vol. 1. 2004: Elsevier, Inc.
13. Narayanan, A.S., Siegel, R.C., and Martin, G.R., *Stability and purification of lysyl oxidase.* Arch Biochem Biophys, 1974. **162**(1): p. 231-7.
14. Kagan, H.M., Sullivan, K.A., Olsson, T.A., 3rd, and Cronlund, A.L., *Purification and properties of four species of lysyl oxidase from bovine aorta.* Biochem J, 1979. **177**(1): p. 203-14.
15. Shackleton, D.R. and Hulmes, D.J., *Purification of lysyl oxidase from piglet skin by selective interaction with Sephacryl S-200.* Biochem J, 1990. **266**(3): p. 917-9.
16. Siegel, R.C. and Fu, J.C., *Collagen cross-linking. Purification and substrate specificity of lysyl oxidase.* J Biol Chem, 1976. **251**(18): p. 5779-85.
17. Harris, E.D., Gonnerman, W.A., Savage, J.E., and O'Dell, B.L., *Connective tissue amine oxidase. II. Purification and partial characterization of lysyl oxidase from chick aorta.* Biochim Biophys Acta, 1974. **341**(2): p. 332-44.

18. Kuivaniemi, H., Savolainen, E.R., and Kivirikko, K.I., *Human placental lysyl oxidase. Purification, partial characterization, and preparation of two specific antisera to the enzyme.* J Biol Chem, 1984. **259**(11): p. 6996-7002.
19. Kagan, H.M., *Characterization and regulation of lysyl oxidase*. Biology of the Extracellular Matrix, ed. R.P. Mecham. 1986, Orlando: academic Press. 321-389.
20. Kim, Y., Boyd, C.D., and Csiszar, K., *A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase.* J Biol Chem, 1995. **270**(13): p. 7176-82.
21. Asuncion, L., Fogelgren, B., Fong, K.S., Fong, S.F., Kim, Y., and Csiszar, K., *A novel human lysyl oxidase-like gene (LOXL4) on chromosome 10q24 has an altered scavenger receptor cysteine rich domain.* Matrix Biol, 2001. **20**(7): p. 487-91.
22. Maki, J.M., Tikkanen, H., and Kivirikko, K.I., *Cloning and characterization of a fifth human lysyl oxidase isoenzyme: the third member of the lysyl oxidase-related subfamily with four scavenger receptor cysteine-rich domains.* Matrix Biol, 2001. **20**(7): p. 493-6.
23. Panchenko, M.V., Stetler-Stevenson, W.G., Trubetskoy, O.V., Gacheru, S.N., and Kagan, H.M., *Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C-proteinase.* J Biol Chem, 1996. **271**(12): p. 7113-9.
24. Kenyon, K., Modi, W.S., Contente, S., and Friedman, R.M., *A novel human cDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24-q25.* J Biol Chem, 1993. **268**(25): p. 18435-7.
25. Saito, H., Papaconstantinou, J., Sato, H., and Goldstein, S., *Regulation of a novel gene encoding a lysyl oxidase-related protein in cellular adhesion and senescence.* J Biol Chem, 1997. **272**(13): p. 8157-60.
26. Huang, Y., Dai, J., Tang, R., Zhao, W., Zhou, Z., Wang, W., Ying, K., Xie, Y., and Mao, Y., *Cloning and characterization of a human lysyl oxidase-like 3 gene (hLOXL3).* Matrix Biol, 2001. **20**(2): p. 153-7.
27. Maki, J.M. and Kivirikko, K.I., *Cloning and characterization of a fourth human lysyl oxidase isoenzyme.* Biochem J, 2001. **355**(Pt 2): p. 381-7.
28. Resnick, D., Pearson, A., and Krieger, M., *The SRCR superfamily: a family reminiscent of the Ig superfamily.* Trends Biochem Sci, 1994. **19**(1): p. 5-8.
29. Csiszar, K., Entersz, I., Trackman, P.C., Samid, D., and Boyd, C.D., *Functional analysis of the promoter and first intron of the human lysyl oxidase gene.* Mol Biol Rep, 1996. **23**(2): p. 97-108.
30. Tan, R.S., Taniguchi, T., and Harada, H., *Identification of the lysyl oxidase gene as target of the antioncogenic transcription factor, IRF-1, and its possible role in tumor suppression.* Cancer Res, 1996. **56**(10): p. 2417-21.
31. Gacheru, S.N., Thomas, K.M., Murray, S.A., Csiszar, K., Smith-Mungo, L.I., and Kagan, H.M., *Transcriptional and post-transcriptional control of lysyl oxidase expression in vascular smooth muscle cells: effects of TGF-beta 1 and serum deprivation.* J Cell Biochem, 1997. **65**(3): p. 395-407.
32. Smith-Mungo, L.I. and Kagan, H.M., *Lysyl oxidase: properties, regulation and multiple functions in biology.* Matrix Biol, 1998. **16**(7): p. 387-98.
33. Li, W., Chou, I.N., Boak, A., and Kagan, H.M., *Downregulation of lysyl oxidase in cadmium-resistant fibroblasts.* Am J Respir Cell Mol Biol, 1995. **13**(4): p. 418-25.
34. Zhao, Y., Gao, S., Chou, I.N., Toselli, P., Stone, P., and Li, W., *Inhibition of the expression of lysyl oxidase and its substrates in cadmium-resistant rat fetal lung fibroblasts.* Toxicol Sci, 2006. **90**(2): p. 478-89.

35. Boak, A.M., Roy, R., Berk, J., Taylor, L., Polgar, P., Goldstein, R.H., and Kagan, H.M., *Regulation of lysyl oxidase expression in lung fibroblasts by transforming growth factor-beta 1 and prostaglandin E2.* Am J Respir Cell Mol Biol, 1994. **11**(6): p. 751-5.
36. Feres-Filho, E.J., Choi, Y.J., Han, X., Takala, T.E., and Trackman, P.C., *Pre- and post-translational regulation of lysyl oxidase by transforming growth factor-beta 1 in osteoblastic MC3T3-E1 cells.* J Biol Chem, 1995. **270**(51): p. 30797-803.
37. Roy, R., Polgar, P., Wang, Y., Goldstein, R.H., Taylor, L., and Kagan, H.M., *Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-1 beta, and prostaglandin E.* J Cell Biochem, 1996. **62**(3): p. 411-7.
38. Shanley, C.J., Gharaee-Kermani, M., Sarkar, R., Welling, T.H., Kriegel, A., Ford, J.W., Stanley, J.C., and Phan, S.H., *Transforming growth factor-beta 1 increases lysyl oxidase enzyme activity and mRNA in rat aortic smooth muscle cells.* J Vasc Surg, 1997. **25**(3): p. 446-52.
39. Feres-Filho, E.J., Menassa, G.B., and Trackman, P.C., *Regulation of lysyl oxidase by basic fibroblast growth factor in osteoblastic MC3T3-E1 cells.* J Biol Chem, 1996. **271**(11): p. 6411-6.
40. Hong, H.H. and Trackman, P.C., *Cytokine regulation of gingival fibroblast lysyl oxidase, collagen, and elastin.* J Periodontol, 2002. **73**(2): p. 145-52.
41. Trackman, P.C., Graham, R.J., Bittner, H.K., Carnes, D.L., Gilles, J.A., and Graves, D.T., *Inflammation-associated lysyl oxidase protein expression in vivo, and modulation by FGF-2 plus IGF-1.* Histochem Cell Biol, 1998. **110**(1): p. 9-14.
42. Green, R.S., Lieb, M.E., Weintraub, A.S., Gacheru, S.N., Rosenfield, C.L., Shah, S., Kagan, H.M., and Taubman, M.B., *Identification of lysyl oxidase and other platelet-derived growth factor-inducible genes in vascular smooth muscle cells by differential screening.* Lab Invest, 1995. **73**(4): p. 476-82.
43. Smith-Mungo, L. and Kagan, H.M., *PKC-MEK-MAPK-dependent signal transduction pathway mediates the stimulation of lysyl oxidase expression by serum and PDGF in rat aortic smooth muscle cells.* J Cell Biochem, 2002. **85**(4): p. 775-84.
44. Harlow, C.R., Rae, M., Davidson, L., Trackman, P.C., and Hillier, S.G., *Lysyl oxidase gene expression and enzyme activity in the rat ovary: regulation by follicle-stimulating hormone, androgen, and transforming growth factor-beta superfamily members in vitro.* Endocrinology, 2003. **144**(1): p. 154-62.
45. Bronson, R.E., Calaman, S.D., Traish, A.M., and Kagan, H.M., *Stimulation of lysyl oxidase (EC 1.4.3.13) activity by testosterone and characterization of androgen receptors in cultured calf aorta smooth-muscle cells.* Biochem J, 1987. **244**(2): p. 317-23.
46. Ravid, K., Smith-Mungo, L.I., Zhao, Z., Thomas, K.M., and Kagan, H.M., *Upregulation of lysyl oxidase in vascular smooth muscle cells by cAMP: role for adenosine receptor activation.* J Cell Biochem, 1999. **75**(1): p. 177-85.
47. Hajnal, A., Klemenz, R., and Schafer, R., *Up-regulation of lysyl oxidase in spontaneous revertants of H-ras-transformed rat fibroblasts.* Cancer Res, 1993. **53**(19): p. 4670-5.
48. Hamalainen, E.R., Jones, T.A., Sheer, D., Taskinen, K., Pihlajaniemi, T., and Kivirikko, K.I., *Molecular cloning of human lysyl oxidase and assignment of the gene to chromosome 5q23.3-31.2.* Genomics, 1991. **11**(3): p. 508-16.
49. Trackman, P.C., Bedell-Hogan, D., Tang, J., and Kagan, H.M., *Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor.* J Biol Chem, 1992. **267**(12): p. 8666-71.

50. Kosonen, T., Uriu-Hare, J.Y., Clegg, M.S., Keen, C.L., and Rucker, R.B., *Incorporation of copper into lysyl oxidase.* Biochem J, 1997. **327 (Pt 1)**: p. 283-9.
51. Takahara, K., Lyons, G.E., and Greenspan, D.S., *Bone morphogenetic protein-1 and a mammalian tolloid homologue (mTld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues.* J Biol Chem, 1994. **269**(51): p. 32572-8.
52. Uzel, M.I., Scott, I.C., Babakhanlou-Chase, H., Palamakumbura, A.H., Pappano, W.N., Hong, H.H., Greenspan, D.S., and Trackman, P.C., *Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures.* J Biol Chem, 2001. **276**(25): p. 22537-43.
53. Cronshaw, A.D., Fothergill-Gilmore, L.A., and Hulmes, D.J., *The proteolytic processing site of the precursor of lysyl oxidase.* Biochem J, 1995. **306 (Pt 1)**: p. 279-84.
54. Williams, M.A. and Kagan, H.M., *Assessment of lysyl oxidase variants by urea gel electrophoresis: evidence against disulfide isomers as bases of the enzyme heterogeneity.* Anal Biochem, 1985. **149**(2): p. 430-7.
55. Fogelgren, B., Polgar, N., Szauter, K.M., Ujfaludi, Z., Laczko, R., Fong, K.S., and Csiszar, K., *Cellular fibronectin binds to lysyl oxidase with high affinity and is critical for its proteolytic activation.* J Biol Chem, 2005. **280**(26): p. 24690-7.
56. Wang, S.X., Mure, M., Medzihradszky, K.F., Burlingame, A.L., Brown, D.E., Dooley, D.M., Smith, A.J., Kagan, H.M., and Klinman, J.P., *A crosslinked cofactor in lysyl oxidase: redox function for amino acid side chains.* Science, 1996. **273**(5278): p. 1078-84.
57. Gacheru, S.N., Trackman, P.C., Shah, M.A., O'Gara, C.Y., Spacciapoli, P., Greenaway, F.T., and Kagan, H.M., *Structural and catalytic properties of copper in lysyl oxidase.* J Biol Chem, 1990. **265**(31): p. 19022-7.
58. Romero-Chapman, N., Lee, J., Tinker, D., Uriu-Hare, J.Y., Keen, C.L., and Rucker, R.R., *Purification, properties and influence of dietary copper on accumulation and functional activity of lysyl oxidase in rat skin.* Biochem J, 1991. **275 (Pt 3)**: p. 657-62.
59. Krebs, C.J. and Krawetz, S.A., *Lysyl oxidase copper-talon complex: a model.* Biochim Biophys Acta, 1993. **1202**(1): p. 7-12.
60. Tang, C. and Klinman, J.P., *The catalytic function of bovine lysyl oxidase in the absence of copper.* J Biol Chem, 2001. **276**(33): p. 30575-8.
61. Wang, S.X., Nakamura, N., Mure, M., Klinman, J.P., and Sanders-Loehr, J., *Characterization of the native lysine tyrosylquinone cofactor in lysyl oxidase by Raman spectroscopy.* J Biol Chem, 1997. **272**(46): p. 28841-4.
62. Dove, J.E. and Klinman, J.P., *Trihydroxyphenylalanine quinone (TPQ) from copper amine oxidases and lysyl tyrosylquinone (LTQ) from lysyl oxidase.* Adv Protein Chem, 2001. **58**: p. 141-74.
63. Bollinger, J.A., Brown, D.E., and Dooley, D.M., *The Formation of lysine tyrosylquinone (LTQ) is a self-processing reaction. Expression and characterization of a Drosophila lysyl oxidase.* Biochemistry, 2005. **44**(35): p. 11708-14.
64. Lucero, H.A. and Kagan, H.M., *Lysyl oxidase: an oxidative enzyme and effector of cell function.* Cell Mol Life Sci, 2006. **63**(19-20): p. 2304-16.
65. Siegel, R.C., *Lysyl oxidase.* Int Rev Connect Tissue Res, 1979. **8**: p. 73-118.
66. Kagan, H.M. and Li, W., *Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell.* J Cell Biochem, 2003. **88**(4): p. 660-72.

67. Vadasz, Z., Kessler, O., Akiri, G., Gengrinovitch, S., Kagan, H.M., Baruch, Y., Izhak, O.B., and Neufeld, G., *Abnormal deposition of collagen around hepatocytes in Wilson's disease is associated with hepatocyte specific expression of lysyl oxidase and lysyl oxidase like protein-2.* J Hepatol, 2005. **43**(3): p. 499-507.
68. Lee, J.E. and Kim, Y., *A tissue-specific variant of the human lysyl oxidase-like protein 3 (LOXL3) functions as an amine oxidase with substrate specificity.* J Biol Chem, 2006. **281**(49): p. 37282-90.
69. Kim, M.S., Kim, S.S., Jung, S.T., Park, J.Y., Yoo, H.W., Ko, J., Csiszar, K., Choi, S.Y., and Kim, Y., *Expression and purification of enzymatically active forms of the human lysyl oxidase-like protein 4.* J Biol Chem, 2003. **278**(52): p. 52071-4.
70. Narayanan, A.S., Siegel, R.C., and Martin, G.R., *On the inhibition of lysyl oxidase by -aminopropionitrile.* Biochem Biophys Res Commun, 1972. **46**(2): p. 745-51.
71. Tang, S.S., Trackman, P.C., and Kagan, H.M., *Reaction of aortic lysyl oxidase with beta-aminopropionitrile.* J Biol Chem, 1983. **258**(7): p. 4331-8.
72. Tang, S.S., Simpson, D.E., and Kagan, H.M., *Beta-substituted ethylamine derivatives as suicide inhibitors of lysyl oxidase.* J Biol Chem, 1984. **259**(2): p. 975-9.
73. Williamson, P.R. and Kagan, H.M., *Electronegativity of aromatic amines as a basis for the development of ground state inhibitors of lysyl oxidase.* J Biol Chem, 1987. **262**(30): p. 14520-4.
74. Gacheru, S.N., Trackman, P.C., Calaman, S.D., Greenaway, F.T., and Kagan, H.M., *Vicinal diamines as pyrroloquinoline quinone-directed irreversible inhibitors of lysyl oxidase.* J Biol Chem, 1989. **264**(22): p. 12963-9.
75. Gavriel, P. and Kagan, H.M., *Inhibition by heparin of the oxidation of lysine in collagen by lysyl oxidase.* Biochemistry, 1988. **27**(8): p. 2811-5.
76. Nagan, N., Callery, P.S., and Kagan, H.M., *Aminoalkylaziridines as substrates and inhibitors of lysyl oxidase: specific inactivation of the enzyme by N-(5-aminopentyl)aziridine.* Front Biosci, 1998. **3**: p. A23-6.
77. Shah, M.A., Trackman, P.C., Gallop, P.M., and Kagan, H.M., *Reaction of lysyl oxidase with trans-2-phenylcyclopropylamine.* J Biol Chem, 1993. **268**(16): p. 11580-5.
78. Kadler, K.E., Baldock, C., Bella, J., and Boot-Handford, R.P., *Collagens at a glance.* J Cell Sci, 2007. **120**(Pt 12): p. 1955-8.
79. Myllyharju, J. and Kivirikko, K.I., *Collagens and collagen-related diseases.* Ann Med, 2001. **33**(1): p. 7-21.
80. Ortega, N. and Werb, Z., *New functional roles for non-collagenous domains of basement membrane collagens.* J Cell Sci, 2002. **115**(Pt 22): p. 4201-14.
81. Kadler, K.E., Holmes, D.F., Trotter, J.A., and Chapman, J.A., *Collagen fibril formation.* Biochem J, 1996. **316 (Pt 1)**: p. 1-11.
82. Eyre, D.R., Paz, M.A., and Gallop, P.M., *Cross-linking in collagen and elastin.* Annu Rev Biochem, 1984. **53**: p. 717-48.
83. Kielty, C.M., Sherratt, M.J., and Shuttleworth, C.A., *Elastic fibres.* J Cell Sci, 2002. **115**(Pt 14): p. 2817-28.
84. Rosenbloom, J., Abrams, W.R., and Mecham, R., *Extracellular matrix 4: the elastic fiber.* Faseb J, 1993. **7**(13): p. 1208-18.
85. Fazio, M.J., Mattei, M.G., Passage, E., Chu, M.L., Black, D., Solomon, E., Davidson, J.M., and Uitto, J., *Human elastin gene: new evidence for localization to the long arm of chromosome 7.* Am J Hum Genet, 1991. **48**(4): p. 696-703.
86. Indik, Z., Yeh, H., Ornstein-Goldstein, N., Kucich, U., Abrams, W., Rosenbloom, J.C., and Rosenbloom, J., *Structure of the elastin gene and alternative splicing of*

elastin mRNA: implications for human disease. Am J Med Genet, 1989. **34**(1): p. 81-90.
87. Vrhovski, B. and Weiss, A.S., *Biochemistry of tropoelastin.* Eur J Biochem, 1998. **258**(1): p. 1-18.
88. Uitto, J., Hoffmann, H.P., and Prockop, D.J., *Synthesis of elastin and procallagen by cells from embryonic aorta. Differences in the role of hydroxyproline and the effects of proline analogs on the secretion of the two proteins.* Arch Biochem Biophys, 1976. **173**(1): p. 187-200.
89. Bressan, G.M. and Prockop, D.J., *Synthesis of elastin in aortas from chick embryos. Conversion of newly secreted elastin to cross-linked elastin without apparent proteolysis of the molecule.* Biochemistry, 1977. **16**(7): p. 1406-12.
90. Trackman, P.C. and Kagan, H.M., *Nonpeptidyl amine inhibitors are substrates of lysyl oxidase.* J Biol Chem, 1979. **254**(16): p. 7831-6.
91. Kagan, H.M., Tseng, L., Trackman, P.C., Okamoto, K., Rapaka, R.S., and Urry, D.W., *Repeat polypeptide models of elastin as substrates for lysyl oxidase.* J Biol Chem, 1980. **255**(8): p. 3656-9.
92. Kagan, H.M., Williams, M.A., Calaman, S.D., and Berkowitz, E.M., *Histone H1 is a substrate for lysyl oxidase and contains endogenous sodium borotritide-reducible residues.* Biochem Biophys Res Commun, 1983. **115**(1): p. 186-92.
93. Li, W., Nugent, M.A., Zhao, Y., Chau, A.N., Li, S.J., Chou, I.N., Liu, G., and Kagan, H.M., *Lysyl oxidase oxidizes basic fibroblast growth factor and inactivates its mitogenic potential.* J Cell Biochem, 2003. **88**(1): p. 152-64.
94. Butler, E., Hardin, J., and Benson, S., *The role of lysyl oxidase and collagen crosslinking during sea urchin development.* Exp Cell Res, 1987. **173**(1): p. 174-82.
95. Di Donato, A., Lacal, J.C., Di Duca, M., Giampuzzi, M., Ghiggeri, G., and Gusmano, R., *Micro-injection of recombinant lysyl oxidase blocks oncogenic p21-Ha-Ras and progesterone effects on Xenopus laevis oocyte maturation.* FEBS Lett, 1997. **419**(1): p. 63-8.
96. Jourdan-Le Saux, C., Tronecker, H., Bogic, L., Bryant-Greenwood, G.D., Boyd, C.D., and Csiszar, K., *The LOXL2 gene encodes a new lysyl oxidase-like protein and is expressed at high levels in reproductive tissues.* J Biol Chem, 1999. **274**(18): p. 12939-44.
97. Van Gansen, P. and Van Lerberghe, N., *Potential and limitations of cultivated fibroblasts in the study of senescence in animals. A review on the murine skin fibroblasts system.* Arch Gerontol Geriatr, 1988. **7**(1): p. 31-74.
98. Li, W., Liu, G., Chou, I.N., and Kagan, H.M., *Hydrogen peroxide-mediated, lysyl oxidase-dependent chemotaxis of vascular smooth muscle cells.* J Cell Biochem, 2000. **78**(4): p. 550-7.
99. Lazarus, H.M., Cruikshank, W.W., Narasimhan, N., Kagan, H.M., and Center, D.M., *Induction of human monocyte motility by lysyl oxidase.* Matrix Biol, 1995. **14**(9): p. 727-31.
100. Nelson, J.M., Diegelmann, R.F., and Cohen, I.K., *Effect of beta-aminopropionitrile and ascorbate on fibroblast migration.* Proc Soc Exp Biol Med, 1988. **188**(3): p. 346-52.
101. Contente, S., Kenyon, K., Rimoldi, D., and Friedman, R.M., *Expression of gene rrg is associated with reversion of NIH 3T3 transformed by LTR-c-H-ras.* Science, 1990. **249**(4970): p. 796-8.
102. Mariani, T.J., Trackman, P.C., Kagan, H.M., Eddy, R.L., Shows, T.B., Boyd, C.D., and Deak, S.B., *The complete derived amino acid sequence of human lysyl oxidase*

and assignment of the gene to chromosome 5 (extensive sequence homology with the murine ras recision gene). Matrix, 1992. **12**(3): p. 242-8.
103. Kenyon, K., Contente, S., Trackman, P.C., Tang, J., Kagan, H.M., and Friedman, R.M., *Lysyl oxidase and rrg messenger RNA.* Science, 1991. **253**(5021): p. 802.
104. Kuivaniemi, H., Korhonen, R.M., Vaheri, A., and Kivirikko, K.I., *Deficient production of lysyl oxidase in cultures of malignantly transformed human cells.* FEBS Lett, 1986. **195**(1-2): p. 261-4.
105. Hamalainen, E.R., Kemppainen, R., Kuivaniemi, H., Tromp, G., Vaheri, A., Pihlajaniemi, T., and Kivirikko, K.I., *Quantitative polymerase chain reaction of lysyl oxidase mRNA in malignantly transformed human cell lines demonstrates that their low lysyl oxidase activity is due to low quantities of its mRNA and low levels of transcription of the respective gene.* J Biol Chem, 1995. **270**(37): p. 21590-3.
106. Giampuzzi, M., Botti, G., Cilli, M., Gusmano, R., Borel, A., Sommer, P., and Di Donato, A., *Down-regulation of lysyl oxidase-induced tumorigenic transformation in NRK-49F cells characterized by constitutive activation of ras proto-oncogene.* J Biol Chem, 2001. **276**(31): p. 29226-32.
107. Palamakumbura, A.H., Sommer, P., and Trackman, P.C., *Autocrine growth factor regulation of lysyl oxidase expression in transformed fibroblasts.* J Biol Chem, 2003. **278**(33): p. 30781-7.
108. Jeay, S., Pianetti, S., Kagan, H.M., and Sonenshein, G.E., *Lysyl oxidase inhibits ras-mediated transformation by preventing activation of NF-kappa B.* Mol Cell Biol, 2003. **23**(7): p. 2251-63.
109. Jansen, M.K. and Csiszar, K., *Intracellular localization of the matrix enzyme lysyl oxidase in polarized epithelial cells.* Matrix Biol, 2007. **26**(2): p. 136-9.
110. Li, W., Nellaiappan, K., Strassmaier, T., Graham, L., Thomas, K.M., and Kagan, H.M., *Localization and activity of lysyl oxidase within nuclei of fibrogenic cells.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 12817-22.
111. Nellaiappan, K., Risitano, A., Liu, G., Nicklas, G., and Kagan, H.M., *Fully processed lysyl oxidase catalyst translocates from the extracellular space into nuclei of aortic smooth-muscle cells.* J Cell Biochem, 2000. **79**(4): p. 576-82.
112. Li, P.A., He, Q., Cao, T., Yong, G., Szauter, K.M., Fong, K.S., Karlsson, J., Keep, M.F., and Csiszar, K., *Up-regulation and altered distribution of lysyl oxidase in the central nervous system of mutant SOD1 transgenic mouse model of amyotrophic lateral sclerosis.* Brain Res Mol Brain Res, 2004. **120**(2): p. 115-22.
113. Molnar, J., Fong, K.S., He, Q.P., Hayashi, K., Kim, Y., Fong, S.F., Fogelgren, B., Szauter, K.M., Mink, M., and Csiszar, K., *Structural and functional diversity of lysyl oxidase and the LOX-like proteins.* Biochim Biophys Acta, 2003. **1647**(1-2): p. 220-4.
114. Molnar, J., Ujfaludi, Z., Fong, S.F., Bollinger, J.A., Waro, G., Fogelgren, B., Dooley, D.M., Mink, M., and Csiszar, K., *Drosophila lysyl oxidases Dmloxl-1 and Dmloxl-2 are differentially expressed and the active DmLOXL-1 influences gene expression and development.* J Biol Chem, 2005. **280**(24): p. 22977-85.
115. Giampuzzi, M., Oleggini, R., and Di Donato, A., *Demonstration of in vitro interaction between tumor suppressor lysyl oxidase and histones H1 and H2: definition of the regions involved.* Biochim Biophys Acta, 2003. **1647**(1-2): p. 245-51.
116. Mello, M.L., Contente, S., Vidal, B.C., Planding, W., and Schenck, U., *Modulation of ras transformation affecting chromatin supraorganization as assessed by image analysis.* Exp Cell Res, 1995. **220**(2): p. 374-82.
117. Giampuzzi, M., Botti, G., Di Duca, M., Arata, L., Ghiggeri, G., Gusmano, R., Ravazzolo, R., and Di Donato, A., *Lysyl oxidase activates the transcription activity*

of human collagene III promoter. Possible involvement of Ku antigen. J Biol Chem, 2000. **275**(46): p. 36341-9.
118. Jeggo, P., Singleton, B., Beamish, H., and Priestley, A., *Double strand break rejoining by the Ku-dependent mechanism of non-homologous end-joining.* C R Acad Sci III, 1999. **322**(2-3): p. 109-12.
119. Gu, Y., Jin, S., Gao, Y., Weaver, D.T., and Alt, F.W., *Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 8076-81.
120. Royce, P.M., Camakaris, J., and Danks, D.M., *Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome.* Biochem J, 1980. **192**(2): p. 579-86.
121. Kagan, H.M., *Characterization and regulation of LO*, in *Biology of the Extracellular Matrix*, R.P. Mecham, Editor. 1986, Academic Press: Orlando. p. 321-398.
122. Steinmann, B., Royce, P., and Superti-Furga, A., *The Ehlers-Danlos Syndrome. Connective Tissue and Its Heritable Disorders.* Molecular,
Genetic, and Medical Aspects., ed. S.B. Royce PM. 2002, New York: Wiley-Liss Inc. 651–685.
123. Harris, E.D., *Copper-induced activation of aortic lysyl oxidase in vivo.* Proc Natl Acad Sci U S A, 1976. **73**(2): p. 371-4.
124. Rucker, R.B., Romero-Chapman, N., Wong, T., Lee, J., Steinberg, F.M., McGee, C., Clegg, M.S., Reiser, K., Kosonen, T., Uriu-Hare, J.Y., Murphy, J., and Keen, C.L., *Modulation of lysyl oxidase by dietary copper in rats.* J Nutr, 1996. **126**(1): p. 51-60.
125. Rucker, R.B., Rucker, B.R., Mitchell, A.E., Cui, C.T., Clegg, M., Kosonen, T., Uriu-Adams, J.Y., Tchaparian, E.H., Fishman, M., and Keen, C.L., *Activation of chick tendon lysyl oxidase in response to dietary copper.* J Nutr, 1999. **129**(12): p. 2143-6.
126. Rucker, R.B., Riggins, R.S., Laughlin, R., Chan, M.M., Chen, M., and Tom, K., *Effects of nutritional copper deficiency on the biomechanical properties of bone and arterial elastin metabolism in the chick.* J Nutr, 1975. **105**(8): p. 1062-70.
127. Rayton, J.K. and Harris, E.D., *Induction of lysyl oxidase with copper. Properties of an in vitro system.* J Biol Chem, 1979. **254**(3): p. 621-6.
128. Vulpe, C., Levinson, B., Whitney, S., Packman, S., and Gitschier, J., *Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase.* Nat Genet, 1993. **3**(1): p. 7-13.
129. Das, S., Levinson, B., Vulpe, C., Whitney, S., Gitschier, J., and Packman, S., *Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse.* Am J Hum Genet, 1995. **56**(3): p. 570-6.
130. Pena, M.M., Lee, J., and Thiele, D.J., *A delicate balance: homeostatic control of copper uptake and distribution.* J Nutr, 1999. **129**(7): p. 1251-60.
131. Danks, D.M., Cartwright, E., Stevens, B.J., and Townley, R.R., *Menkes' kinky hair disease: further definition of the defect in copper transport.* Science, 1973. **179**(78): p. 1140-2.
132. Kaler, S.G., *Metabolic and molecular bases of Menkes disease and occipital horn syndrome.* Pediatr Dev Pathol, 1998. **1**(1): p. 85-98.
133. Byers, P.H., Siegel, R.C., Holbrook, K.A., Narayanan, A.S., Bornstein, P., and Hall, J.G., *X-linked cutis laxa: defective cross-link formation in collagen due to decreased lysyl oxidase activity.* N Engl J Med, 1980. **303**(2): p. 61-5.

134. Royce, P.M. and Steinmann, B., *Markedly reduced activity of lysyl oxidase in skin and aorta from a patient with Menkes' disease showing unusually severe connective tissue manifestations.* Pediatr Res, 1990. **28**(2): p. 137-41.
135. Kuivaniemi, H., Peltonen, L., and Kivirikko, K.I., *Type IX Ehlers-Danlos syndrome and Menkes syndrome: the decrease in lysyl oxidase activity is associated with a corresponding deficiency in the enzyme protein.* Am J Hum Genet, 1985. **37**(4): p. 798-808.
136. Kemppainen, R., Hamalainen, E.R., Kuivaniemi, H., Tromp, G., Pihlajaniemi, T., and Kivirikko, K.I., *Expression of mRNAs for lysyl oxidase and type III procollagen in cultured fibroblasts from patients with the Menkes and occipital horn syndromes as determined by quantitative polymerase chain reaction.* Arch Biochem Biophys, 1996. **328**(1): p. 101-6.
137. Leask, A. and Abraham, D.J., *TGF-beta signaling and the fibrotic response.* Faseb J, 2004. **18**(7): p. 816-27.
138. Siegel, R.C., Chen, K.H., Greenspan, J.S., and Aguiar, J.M., *Biochemical and immunochemical study of lysyl oxidase in experimental hepatic fibrosis in the rat.* Proc Natl Acad Sci U S A, 1978. **75**(6): p. 2945-9.
139. McPhie, J.L., *The activity of lysyl oxidase in experimental hepatic fibrosis.* Hepatogastroenterology, 1981. **28**(5): p. 240-1.
140. Carter, E.A., McCarron, M.J., Alpert, E., and Isselbacher, K.J., *Lysyl oxidase and collagenase in experimental acute and chronic liver injury.* Gastroenterology, 1982. **82**(3): p. 526-34.
141. Counts, D.F., Evans, J.N., Dipetrillo, T.A., Sterling, K.M., Jr., and Kelley, J., *Collagen lysyl oxidase activity in the lung increases during bleomycin-induced lung fibrosis.* J Pharmacol Exp Ther, 1981. **219**(3): p. 675-8.
142. Riley, D.J., Kerr, J.S., Berg, R.A., Ianni, B.D., Pietra, G.G., Edelman, N.H., and Prockop, D.J., *beta-Aminopropionitrile prevents bleomycin-induced pulmonary fibrosis in the hamster.* Am Rev Respir Dis, 1982. **125**(1): p. 67-73.
143. Tzortzaki, E.G., Tischfield, J.A., Sahota, A., Siafakas, N.M., Gordon, M.K., and Gerecke, D.R., *Expression of FACIT collagens XII and XIV during bleomycin-induced pulmonary fibrosis in mice.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **275**(2): p. 1073-80.
144. Di Donato, A., Ghiggeri, G.M., Di Duca, M., Jivotenko, E., Acinni, R., Campolo, J., Ginevri, F., and Gusmano, R., *Lysyl oxidase expression and collagen cross-linking during chronic adriamycin nephropathy.* Nephron, 1997. **76**(2): p. 192-200.
145. Tsuda, T., Gao, E., Evangelisti, L., Markova, D., Ma, X., and Chu, M.L., *Post-ischemic myocardial fibrosis occurs independent of hemodynamic changes.* Cardiovasc Res, 2003. **59**(4): p. 926-33.
146. Franklin, T.J., *Therapeutic approaches to organ fibrosis.* Int J Biochem Cell Biol, 1997. **29**(1): p. 79-89.
147. Kagan, H.M., *Intra- and extracellular enzymes of collagen biosynthesis as biological and chemical targets in the control of fibrosis.* Acta Trop, 2000. **77**(1): p. 147-52.
148. Kirschmann, D.A., Seftor, E.A., Nieva, D.R., Mariano, E.A., and Hendrix, M.J., *Differentially expressed genes associated with the metastatic phenotype in breast cancer.* Breast Cancer Res Treat, 1999. **55**(2): p. 127-36.
149. Perou, C.M., Jeffrey, S.S., van de Rijn, M., Rees, C.A., Eisen, M.B., Ross, D.T., Pergamenschikov, A., Williams, C.F., Zhu, S.X., Lee, J.C., Lashkari, D., Shalon, D., Brown, P.O., and Botstein, D., *Distinctive gene expression patterns in human*

mammary epithelial cells and breast cancers. Proc Natl Acad Sci U S A, 1999. **96**(16): p. 9212-7.
150. Ross, D.T., Scherf, U., Eisen, M.B., Perou, C.M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S.S., Van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J.C., Lashkari, D., Shalon, D., Myers, T.G., Weinstein, J.N., Botstein, D., and Brown, P.O., *Systematic variation in gene expression patterns in human cancer cell lines.* Nat Genet, 2000. **24**(3): p. 227-35.
151. Erler, J.T., Bennewith, K.L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q.T., Chi, J.T., Jeffrey, S.S., and Giaccia, A.J., *Lysyl oxidase is essential for hypoxia-induced metastasis.* Nature, 2006. **440**(7088): p. 1222-6.
152. Borczuk, A.C., Kim, H.K., Yegen, H.A., Friedman, R.A., and Powell, C.A., *Lung adenocarcinoma global profiling identifies type II transforming growth factor-beta receptor as a repressor of invasiveness.* Am J Respir Crit Care Med, 2005. **172**(6): p. 729-37.
153. Uzel, M.I., Shih, S.D., Gross, H., Kessler, E., Gerstenfeld, L.C., and Trackman, P.C., *Molecular events that contribute to lysyl oxidase enzyme activity and insoluble collagen accumulation in osteosarcoma cell clones.* J Bone Miner Res, 2000. **15**(6): p. 1189-97.
154. Peyrol, S., Raccurt, M., Gerard, F., Gleyzal, C., Grimaud, J.A., and Sommer, P., *Lysyl oxidase gene expression in the stromal reaction to in situ and invasive ductal breast carcinoma.* Am J Pathol, 1997. **150**(2): p. 497-507.
155. Decitre, M., Gleyzal, C., Raccurt, M., Peyrol, S., Aubert-Foucher, E., Csiszar, K., and Sommer, P., *Lysyl oxidase-like protein localizes to sites of de novo fibrinogenesis in fibrosis and in the early stromal reaction of ductal breast carcinomas.* Lab Invest, 1998. **78**(2): p. 143-51.
156. Zuber, J., Tchernitsa, O.I., Hinzmann, B., Schmitz, A.C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schafer, R., *A genome-wide survey of RAS transformation targets.* Nat Genet, 2000. **24**(2): p. 144-52.
157. Rost, T., Pyritz, V., Rathcke, I.O., Gorogh, T., Dunne, A.A., and Werner, J.A., *Reduction of LOX- and LOXL2-mRNA expression in head and neck squamous cell carcinomas.* Anticancer Res, 2003. **23**(2B): p. 1565-73.
158. Hough, C.D., Sherman-Baust, C.A., Pizer, E.S., Montz, F.J., Im, D.D., Rosenshein, N.B., Cho, K.R., Riggins, G.J., and Morin, P.J., *Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer.* Cancer Res, 2000. **60**(22): p. 6281-7.
159. Peinado, H., Del Carmen Iglesias-de la Cruz, M., Olmeda, D., Csiszar, K., Fong, K.S., Vega, S., Nieto, M.A., Cano, A., and Portillo, F., *A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression.* Embo J, 2005. **24**(19): p. 3446-58.
160. Gronborg, M., Kristiansen, T.Z., Iwahori, A., Chang, R., Reddy, R., Sato, N., Molina, H., Jensen, O.N., Hruban, R.H., Goggins, M.G., Maitra, A., and Pandey, A., *Biomarker discovery from pancreatic cancer secretome using a differential proteomic approach.* Mol Cell Proteomics, 2006. **5**(1): p. 157-71.
161. Fong, S.F., Dietzsch, E., Fong, K.S., Hollosi, P., Asuncion, L., He, Q., Parker, M.I., and Csiszar, K., *Lysyl oxidase-like 2 expression is increased in colon and esophageal tumors and associated with less differentiated colon tumors.* Genes Chromosomes Cancer, 2007. **46**(7): p. 644-55.
162. Li, C.H., Dixon, J.S., and Chung, D., *Primary structure of the human chorionic somatomammotropin (HCS) molecule.* Science, 1971. **173**(991): p. 56-8.

163. Josimovich, J.B. and Maclaren, J.A., *Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone.* Endocrinology, 1962. **71**: p. 209-20.
164. Kaplan, S.L. and Grumbach, M.M., *Studies Of A Human And Simian Placental Hormone With Growth Hormone-Like And Prolactin-Like Activities.* J Clin Endocrinol Metab, 1964. **24**: p. 80-100.
165. Li, C.H., Grumbach, M.M., Kaplan, S.L., Josimovich, J.B., Friesen, H., and Catt, K.J., *Human chorionic somato-mammotropin (HCS), proposed terminology for designation of a placental hormone.* Experientia, 1968. **24**(12): p. 1288.
166. Owerbach, D., Rutter, W.J., Martial, J.A., Baxter, J.D., and Shows, T.B., *Genes for growth hormone, chorionic somatommammotropin, and growth hormones-like gene on chromosome 17 in humans.* Science, 1980. **209**(4453): p. 289-92.
167. Owerbach, D., Rutter, W.J., Cooke, N.E., Martial, J.A., and Shows, T.B., *The prolactin gene is located on chromosome 6 in humans.* Science, 1981. **212**(4496): p. 815-6.
168. George, D.L., Phillips, J.A., 3rd, Francke, U., and Seeburg, P.H., *The genes for growth hormone and chorionic somatomammotropin are on the long arm of human chromosome 17 in region q21 to qter.* Hum Genet, 1981. **57**(2): p. 138-41.
169. Harper, M.E., Barrera-Saldana, H.A., and Saunders, G.F., *Chromosomal localization of the human placental lactogen-growth hormone gene cluster to 17q22-24.* Am J Hum Genet, 1982. **34**(2): p. 227-34.
170. Barsh, G.S., Seeburg, P.H., and Gelinas, R.E., *The human growth hormone gene family: structure and evolution of the chromosomal locus.* Nucleic Acids Res, 1983. **11**(12): p. 3939-58.
171. Barrera-Saldana, H.A., *Growth hormone and placental lactogen: biology, medicine and biotechnology.* Gene, 1998. **211**(1): p. 11-8.
172. Niall, H.D., Hogan, M.L., Sauer, R., Rosenblum, I.Y., and Greenwood, F.C., *Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene reduplication.* Proc Natl Acad Sci U S A, 1971. **68**(4): p. 866-70.
173. Bewley, T.A., Dixon, J.S., and Li, C.H., *Sequence comparison of human pituitary growth hormone, human chorionic somatomammotropin, and ovine pituitary growth and lactogenic hormones.* Int J Pept Protein Res, 1972. **4**(4): p. 281-7.
174. Barrera-Saldana, H.A., Robberson, D.L., and Saunders, G.F., *Transcriptional products of the human placental lactogen gene.* J Biol Chem, 1982. **257**(20): p. 12399-404.
175. Seeburg, P.H., *The human growth hormone gene family: nucleotide sequences show recent divergence and predict a new polypeptide hormone.* Dna, 1982. **1**(3): p. 239-49.
176. Schwarzler, P., Untergasser, G., Hermann, M., Dirnhofer, S., Abendstein, B., Madersbacher, S., and Berger, P., *Selective growth hormone/placental lactogen gene transcription and hormone production in pre- and postmenopausal human ovaries.* J Clin Endocrinol Metab, 1997. **82**(10): p. 3337-41.
177. Untergasser, G., Kranewitter, W., Schwarzler, P., Madersbacher, S., Dirnhofer, S., and Berger, P., *Organ-specific expression pattern of the human growth hormone/placental lactogen gene-cluster in the testis.* Mol Cell Endocrinol, 1997. **130**(1-2): p. 53-60.
178. Cooke, N.E., Ray, J., Emery, J.G., and Liebhaber, S.A., *Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins.* J Biol Chem, 1988. **263**(18): p. 9001-6.

179. Cooke, N.E., Ray, J., Watson, M.A., Estes, P.A., Kuo, B.A., and Liebhaber, S.A., *Human growth hormone gene and the highly homologous growth hormone variant gene display different splicing patterns.* J Clin Invest, 1988. **82**(1): p. 270-5.
180. Liebhaber, S.A., Urbanek, M., Ray, J., Tuan, R.S., and Cooke, N.E., *Characterization and histologic localization of human growth hormone-variant gene expression in the placenta.* J Clin Invest, 1989. **83**(6): p. 1985-91.
181. Barrera-Saldana, H.A., Seeburg, P.H., and Saunders, G.F., *Two structurally different genes produce the same secreted human placental lactogen hormone.* J Biol Chem, 1983. **258**(6): p. 3787-93.
182. Misra-Press, A., Cooke, N.E., and Liebhaber, S.A., *Complex alternative splicing partially inactivates the human chorionic somatomammotropin-like (hCS-L) gene.* J Biol Chem, 1994. **269**(37): p. 23220-9.
183. MacLeod, J.N., Lee, A.K., Liebhaber, S.A., and Cooke, N.E., *Developmental control and alternative splicing of the placentally expressed transcripts from the human growth hormone gene cluster.* J Biol Chem, 1992. **267**(20): p. 14219-26.
184. Lolis, D., Konstantinidis, K., Papevangelou, G., and Kaskarelis, D., *Comparative study of amniotic fluid and maternal blood serum human placental lactogen in normal and prolonged pregnancies.* Am J Obstet Gynecol, 1977. **128**(7): p. 724-6.
185. Friesen, H., Belanger, C., Guyda, H., Hwang, P., *Lactogenic Hormones*. 1972, London: Churchill Livingston, Ciba Foundation. 83-110.
186. Sciarra, J.J., Kaplan, S.L., and Grumbach, M.M., *Localization Of Anti-Human Growth Hormone Serum Within The Human Placenta: Evidence For A Human Chorionic 'Growth Hormone-Prolactin'.* Nature, 1963. **199**: p. 1005-6.
187. Beck, J.S., Gordon, R.L., Donald, D., and Melvin, J.M., *Characterisation of antisera to a growth-hormone-like placetal antigen (human placental lactogen): immunofluorescence studies with these sera on normal nd pathological syncytiotrophoblast.* J Pathol, 1969. **97**(3): p. 545-55.
188. Sciarra, J.J., Sherwood, L.M., Varma, A.A., and Lundberg, W.B., *Human placental lactogen (HPL) and placental weight.* Am J Obstet Gynecol, 1968. **101**(3): p. 413-6.
189. Walker, W.H., Fitzpatrick, S.L., Barrera-Saldana, H.A., Resendez-Perez, D., and Saunders, G.F., *The human placental lactogen genes: structure, function, evolution and transcriptional regulation.* Endocr Rev, 1991. **12**(4): p. 316-28.
190. Beck, P. and Daughaday, W.H., *Human placental lactogen: studies of its acute metabolic effects and disposition in normal man.* J Clin Invest, 1967. **46**(1): p. 103-10.
191. Szczesna, E. and Boime, I., *mRNA-dependent synthesis of authentic precursor to human placental lactogen: conversion to its mature hormone form in ascites cell-free extracts.* Proc Natl Acad Sci U S A, 1976. **73**(4): p. 1179-83.
192. Boime, I., Szczesna, E., and Smith, D., *Membrane-dependent cleavage of the human placental lactogen precursor to its native form in ascites cell-free extracts.* Eur J Biochem, 1977. **73**(2): p. 515-20.
193. Caulfield, M.P., Duong, L.T., and Rosenblatt, M., *Demonstration of post-translational secretion of human placental lactogen by a mammalian in vitro translation system.* J Biol Chem, 1986. **261**(24): p. 10953-6.
194. Li, C.H., *Recent knowledge of the chemistry of lactogenis hormones*. Lactogenic Hormones, ed. G.E.W. Wolstenholme, Knight, J. 1972, Edinburgh: Churchill Livingston. 22.
195. Schneider, A., Kowalski, K., and Sherwood, L., *Identification of "big" human placental lactogen in placenta and serum.* Endocrinology, 1975. **97**(6): p. 1364-1372.

196. Schneider, A.B., Kowalski, K., Buckman, G., and Sherwood, L.M., *Dimeric ("big") human placental lactogen. Immunological and biological activity.* Biochim Biophys Acta, 1977. **493**(1): p. 69-77.
197. Schneider, A.B., Kowalski, K., Russell, J., and Sherwood, L.M., *Identification of the interchain disulfide bonds of dimeric human placental lactogen.* J Biol Chem, 1979. **254**(10): p. 3782-7.
198. Peterson, F.C. and Brooks, C.L., *Different elements of mini-helix 1 are required for human growth hormone or prolactin action via the prolactin receptor.* Protein Eng Des Sel, 2004. **17**(5): p. 417-24.
199. Elkins, P.A., Christinger, H.W., Sandowski, Y., Sakal, E., Gertler, A., de Vos, A.M., and Kossiakoff, A.A., *Ternary complex between placental lactogen and the extracellular domain of the prolactin receptor.* Nat Struct Biol, 2000. **7**(9): p. 808-15.
200. Hill, D.J., Crace, C.J., and Milner, R.D., *Incorporation of [3H]thymidine by isolated fetal myoblasts and fibroblasts in response to human placental lactogen (HPL): possible mediation of HPL action by release of immunoreactive SM-C.* J Cell Physiol, 1985. **125**(2): p. 337-44.
201. Hill, D.J., Crace, C.J., Strain, A.J., and Milner, R.D., *Regulation of amino acid uptake and deoxyribonucleic acid synthesis in isolated human fetal fibroblasts and myoblasts: effect of human placental lactogen, somatomedin-C, multiplication-stimulating activity, and insulin.* J Clin Endocrinol Metab, 1986. **62**(4): p. 753-60.
202. Williams, C. and Coltart, T.M., *Adipose tissue metabolism in pregnancy: the lipolytic effect of human placental lactogen.* Br J Obstet Gynaecol, 1978. **85**(1): p. 43-6.
203. Friesen, H., *Purification Of A Placental Factor With Immunological And Chemical Similarity To Human Growth Hormone.* Endocrinology, 1965. **76**: p. 369-81.
204. Felber, J.P., Zaragoza, N., Benuzzi-Badoni, M., and Genazzani, A.R., *The double effect of human chorionic somatomammotropin (HCS) and pregnancy on lipogenesis and on lipolysis in the isolated rat epididymal fat pad and fat pad cells.* Horm Metab Res, 1972. **4**(4): p. 293-6.
205. Welsch, C.W. and McManus, M.J., *Stimulation of DNA synthesis by human placental lactogen or insulin in organ cultures of benign human breast tumors.* Cancer Res, 1977. **37**(7 Pt 1): p. 2257-61.
206. McManus, M.J., Dembroske, S.E., Pienkowski, M.M., Anderson, T.J., Mann, L.C., Schuster, J.S., Vollwiler, L.L., and Weisch, C.U., *Successful transplantation of human benign breast tumors into the athymic nude mouse and demonstration of enhanced DNA synthesis by human placental lactogen.* Cancer Res, 1978. **38**(8): p. 2343-9.
207. Eggermann, T., Eggermann, K., Mergenthaler, S., Kuner, R., Kaiser, P., Ranke, M.B., and Wollmann, H.A., *Paternally inherited deletion of CSH1 in a patient with Silver-Russell syndrome.* J Med Genet, 1998. **35**(9): p. 784-6.
208. Hitchins, M.P., Stanier, P., Preece, M.A., and Moore, G.E., *Silver-Russell syndrome: a dissection of the genetic aetiology and candidate chromosomal regions.* J Med Genet, 2001. **38**(12): p. 810-9.
209. Nielsen, P.V., Pedersen, H., and Kampmann, E.M., *Absence of human placental lactogen in an otherwise uneventful pregnancy.* Am J Obstet Gynecol, 1979. **135**(3): p. 322-6.
210. Rygaard, K., Revol, A., Esquivel-Escobedo, D., Beck, B.L., and Barrera-Saldana, H.A., *Absence of human placental lactogen and placental growth hormone (HGH-*

V) during pregnancy: PCR analysis of the deletion. Hum Genet, 1998. **102**(1): p. 87-92.
211. Eggermann, T., Schonherr, N., Eggermann, K., and Wollmann, H., *Hypomethylation in the 11p15 telomeric imprinting domain in a patient with Silver-Russell syndrome with a CSH1 deletion (17q24) renders a functional role of this alteration unlikely.* J Med Genet, 2007. **44**(4): p. e77.
212. Burg-Kurland, C.L., Purnell, D.M., Combs, J.W., Hillman, E.A., Harris, C.C., and Trump, B.F., *Immunocytochemical evaluation of human esophageal neoplasms and preneoplastic lesions for beta-chorionic gonadotropin, placental lactogen, alpha-fetoprotein, carcinoembryonic antigen, and nonspecific cross-reacting antigen.* Cancer Res, 1986. **46**(6): p. 2936-43.
213. Gropp, C., Havemann, K., and Scheuer, A., *Ectopic hormones in lung cancer patients at diagnosis and during therapy.* Cancer, 1980. **46**(2): p. 347-54.
214. Casper, S., van Nagell, J.R., Jr., Powell, D.F., Dubilier, L.D., Donaldson, E.S., Hanson, M.B., and Pavlik, E.J., *Immunohistochemical localization of tumor markers in epithelial ovarian cancer.* Am J Obstet Gynecol, 1984. **149**(2): p. 154-8.
215. Berger, P., Untergasser, G., Hermann, M., Hittmair, A., Madersbacher, S., and Dirnhofer, S., *The testis-specific expression pattern of the growth hormone/placental lactogen (GH/PL) gene cluster changes with malignancy.* Hum Pathol, 1999. **30**(10): p. 1201-6.
216. Sheth, N.A., Suraiya, J.N., Sheth, A.R., Ranadive, K.J., and Jussawalla, D.J., *Ectopic production of human placental lactogen by human breast tumors.* Cancer, 1977. **39**(4): p. 1693-9.
217. Latham, C., Zhang, A., Nalbanti, A., Maner, S., Zickert, P., Blegen, H., and Zetterberg, A., *Frequent co-amplification of two different regions on 17q in aneuploid breast carcinomas.* Cancer Genet Cytogenet, 2001. **127**(1): p. 16-23.
218. Horne, C.H., Reid, I.N., and Milne, G.D., *Prognostic significance of inappropriate production of pregnancy proteins by breast cancers.* Lancet, 1976. **2**(7980): p. 279-82.
219. Lowman, H.B., Cunningham, B.C., and Wells, J.A., *Mutational analysis and protein engineering of receptor-binding determinants in human placental lactogen.* J Biol Chem, 1991. **266**(17): p. 10982-8.
220. Maus, M.V., Reilly, S.C., and Clevenger, C.V., *Prolactin as a chemoattractant for human breast carcinoma.* Endocrinology, 1999. **140**(11): p. 5447-50.
221. Hankinson, S.E., Willett, W.C., Michaud, D.S., Manson, J.E., Colditz, G.A., Longcope, C., Rosner, B., and Speizer, F.E., *Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women.* J Natl Cancer Inst, 1999. **91**(7): p. 629-34.
222. Bhatavdekar, J.M., Patel, D.D., Shah, N.G., Vora, H.H., Suthar, T.P., Ghosh, N., Chikhlikar, P.R., and Trivedi, T.I., *Prolactin as a local growth promoter in patients with breast cancer: GCRI experience.* Eur J Surg Oncol, 2000. **26**(6): p. 540-7.
223. Clevenger, C.V., Chang, W.P., Ngo, W., Pasha, T.L., Montone, K.T., and Tomaszewski, J.E., *Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop.* Am J Pathol, 1995. **146**(3): p. 695-705.
224. Holtkamp, W., Nagel, G.A., Wander, H.E., Rauschecker, H.F., and von Heyden, D., *Hyperprolactinemia is an indicator of progressive disease and poor prognosis in advanced breast cancer.* Int J Cancer, 1984. **34**(3): p. 323-8.
225. Clontech Laboratories, I., *MATCHMAKER GAL4 Two-Hybrid System 3 & Libraries User Manual*. Third Edition ed. 1999. p. 19-20.

226. Amersham, *GST Gene Fusion System*. Third Edition ed. 2000: Amersham Pharmacia Biotech.
227. Invitrogen, L.T., *Growth and Maintenance of the 293FT Cell Line*. Version C ed. 2003.
228. Jung, S.T., Kim, M.S., Seo, J.Y., Kim, H.C., and Kim, Y., *Purification of enzymatically active human lysyl oxidase and lysyl oxidase-like protein from Escherichia coli inclusion bodies.* Protein Expr Purif, 2003. **31**(2): p. 240-6.
229. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(259): p. 680-5.
230. Towbin, H., Staehelin, T., and Gordon, J., *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
231. Einasson, M. and Orlinick, J.R., *Identification of protein-protein interactions with glutathione-S-tarnsferase fusion proteins*. Protein-protein Interactions, ed. E. Golemis. 2002, Cold Spring Harbor: Cold Spring Harbor Laboratory Press. 37-57.
232. Golemis, E., ed. *Protein-protein interactions. A molecular cloning manual.* 2002, Cold Spring Harbor Press: Cold Spring Harbor.
233. Streuli, C.H. and Grant, M.E., eds. *Extracellular matrix protocols*. Methods in Molecular Biology, ed. J.M. Walker. Vol. 139. 2000, Humana Press: Totowa.
234. Palamakumbura, A.H. and Trackman, P.C., *A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples.* Anal Biochem, 2002. **300**(2): p. 245-51.
235. Debnath, J., Muthuswamy, S.K., and Brugge, J.S., *Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures.* Methods, 2003. **30**(3): p. 256-68.
236. Kirschmann, D.A., Lininger, R.A., Gardner, L.M., Seftor, E.A., Odero, V.A., Ainsztein, A.M., Earnshaw, W.C., Wallrath, L.L., and Hendrix, M.J., *Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer.* Cancer Res, 2000. **60**(13): p. 3359-63.
237. Ziegler, J., Vogt, T., Miersch, O., and Strack, D., *Concentration of dilute protein solutions prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.* Anal Biochem, 1997. **250**(2): p. 257-60.
238. Olsson, N.O., Leclerc, A., Jeannin, J.F., and Martin, F., *A simple photometric microassay for the quantitative evaluation of macrophage-mediated cytotoxicity on adherent cancer cells.* Ann Immunol (Paris), 1982. **133D**(3): p. 245-54.
239. Blackwood, E.M. and Eisenman, R.N., *Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc.* Science, 1991. **251**(4998): p. 1211-7.
240. Kaelin, W.G., Jr., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S., Chittenden, T., Li, Y., Farnham, P.J., Blanar, M.A., and et al., *Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties.* Cell, 1992. **70**(2): p. 351-64.
241. Mould, P., *Solid phase assays for studying ECM protein-protein interactions*. Extracellular Matrix Protocols, ed. C.H. Streuli and M. Grant. 2000, Totowa, NJ: Humana Press. 295-299.
242. Lefkowitz, R.J., Roth, J., and Pastan, I., *Radioreceptor assay of adrenocorticotropic hormone: new approach to assay of polypeptide hormones in plasma.* Science, 1970. **170**(958): p. 633-5.
243. Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R.P., *A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen*

peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem, 1997. **253**(2): p. 162-8.
244. Zhou, M. and Panchuk-Voloshina, N., *A one-step fluorometric method for the continuous measurement of monoamine oxidase activity.* Anal Biochem, 1997. **253**(2): p. 169-74.
245. Payne, S.L., Hendrix, M.J., and Kirschmann, D.A., *Lysyl oxidase regulates actin filament formation through the p130(Cas)/Crk/DOCK180 signaling complex.* J Cell Biochem, 2006. **98**(4): p. 827-37.
246. Deane, C.M., Salwinski, L., Xenarios, I., and Eisenberg, D., *Protein interactions: two methods for assessment of the reliability of high throughput observations.* Mol Cell Proteomics, 2002. **1**(5): p. 349-56.
247. Martinez-Rodriguez, H.G., Guerra-Rodriguez, N.E., Iturbe-Cantu, M.A., Martinez-Torres, A., and Barrera-Saldana, H.A., *Expression of human placental lactogen and variant growth hormone genes in placentas.* Arch Med Res, 1997. **28**(4): p. 507-12.
248. Bazan, J.F., *Structural design and molecular evolution of a cytokine receptor superfamily.* Proc Natl Acad Sci U S A, 1990. **87**(18): p. 6934-8.
249. Kelly, P.A., Bachelot, A., Kedzia, C., Hennighausen, L., Ormandy, C.J., Kopchick, J.J., and Binart, N., *The role of prolactin and growth hormone in mammary gland development.* Mol Cell Endocrinol, 2002. **197**(1-2): p. 127-31.
250. Mukhina, S., Mertani, H.C., Guo, K., Lee, K.O., Gluckman, P.D., and Lobie, P.E., *Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone.* Proc Natl Acad Sci U S A, 2004. **101**(42): p. 15166-71.
251. Zhu, T., Starling-Emerald, B., Zhang, X., Lee, K.O., Gluckman, P.D., Mertani, H.C., and Lobie, P.E., *Oncogenic transformation of human mammary epithelial cells by autocrine human growth hormone.* Cancer Res, 2005. **65**(1): p. 317-24.
252. Perks, C.M., Keith, A.J., Goodhew, K.L., Savage, P.B., Winters, Z.E., and Holly, J.M., *Prolactin acts as a potent survival factor for human breast cancer cell lines.* Br J Cancer, 2004. **91**(2): p. 305-11.
253. Welsch, C.W., Dombroske, S.E., and McManus, M.J., *Effects of insulin, human placental lactogen and human growth hormone of DNA synthesis in organ cultures of benign human breast tumours.* Br J Cancer, 1978. **38**(2): p. 258-62.
254. Gebre-Medhin, M., Kindblom, L.G., Wennbo, H., Tornell, J., and Meis-Kindblom, J.M., *Growth hormone receptor is expressed in human breast cancer.* Am J Pathol, 2001. **158**(4): p. 1217-22.
255. Reynolds, C., Montone, K.T., Powell, C.M., Tomaszewski, J.E., and Clevenger, C.V., *Expression of prolactin and its receptor in human breast carcinoma.* Endocrinology, 1997. **138**(12): p. 5555-60.
256. Meng, J., Tsai-Morris, C.H., and Dufau, M.L., *Human prolactin receptor variants in breast cancer: low ratio of short forms to the long-form human prolactin receptor associated with mammary carcinoma.* Cancer Res, 2004. **64**(16): p. 5677-82.
257. Peirce, S.K., Chen, W.Y., and Chen, W.Y., *Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.* J Endocrinol, 2001. **171**(1): p. R1-4.
258. Decouvelaere, C., Peyrat, J.P., Bonneterre, J., Djiane, J., and Jammes, H., *Presence of the two growth hormone receptor messenger RNA isoforms in human breast cancer.* Cell Growth Differ, 1995. **6**(4): p. 477-83.
259. Acosta, J.J., Munoz, R.M., Gonzalez, L., Subtil-Rodriguez, A., Dominguez-Caceres, M.A., Garcia-Martinez, J.M., Calcabrini, A., Lazaro-Trueba, I., and Martin-Perez, J., *Src mediates prolactin-dependent proliferation of T47D and MCF7*

cells via the activation of focal adhesion kinase/Erk1/2 and phosphatidylinositol 3-kinase pathways. Mol Endocrinol, 2003. **17**(11): p. 2268-82.
260. Cohick, C.B., Dai, G., Xu, L., Deb, S., Kamei, T., Levan, G., Szpirer, C., Szpirer, J., Kwok, S.C., and Soares, M.J., *Placental lactogen-I variant utilizes the prolactin receptor signaling pathway.* Mol Cell Endocrinol, 1996. **116**(1): p. 49-58.
261. Takeda, T., Kurachi, H., Yamamoto, T., Homma, H., Morishige, K., Miyake, A., and Murata, Y., *Participation of JAK, STAT and unknown proteins in human placental lactogen-induced signaling: a unique signaling pathway different from prolactin and growth hormone.* J Endocrinol, 1997. **153**(1): p. R1-3.
262. Giampuzzi, M., Oleggini, R., Albanese, C., Pestell, R., and Di Donato, A., *beta-catenin signaling and regulation of cyclin D1 promoter in NRK-49F cells transformed by down-regulation of the tumor suppressor lysyl oxidase.* Biochim Biophys Acta, 2005. **1745**(3): p. 370-81.
263. Grummer, R., Hohn, H.P., Mareel, M.M., and Denker, H.W., *Adhesion and invasion of three human choriocarcinoma cell lines into human endometrium in a three-dimensional organ culture system.* Placenta, 1994. **15**(4): p. 411-29.