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PROSPECTS

Paradoxical Roles for Lysyl Oxidases in Cancer—A Prospect

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Abstract Lysyl oxidase (LOX) is an extracellular matrix (ECM) enzyme that catalyzes the cross-linking of collagens or elastin in the extracellular compartment, thereby regulating the tensile strength of tissues. However, recent reports have demonstrated novel roles for LOX, including the ability to regulate gene transcription, motility/migration, and cell adhesion. These diverse functions have led researchers to hypothesize that LOX may have multiple roles affecting both extra- and intracellular cell function(s). Particularly noteworthy is aberrant LOX expression and activity that have been observed in various cancerous tissues and neoplastic cell lines. Both down and upregulation of LOX in tumor tissues and cancer cell lines have been described, suggesting a dual role for LOX as a tumor suppressor, as well as a metastasis promoter gene—creating a conundrum within the LOX research field. Here, we review the body of evidence on *LOX* gene expression, regulation, and function(s) in various cancer cell types and tissues, as well as stromal–tumor cell interactions. Lastly, we will examine putative mechanisms in which LOX facilitates breast cancer invasion and metastasis. Taken together, the literature demonstrates the increasingly important role(s) that LOX may play in regulating tumor progression and the necessity to elucidate its myriad mechanisms of action in order to identify potentially novel therapeutics. J. Cell. Biochem. 101: 1338–1354, 2007.

Key words: lysyl oxidase; cancer; tumor suppressor; metastasis promoter; epithelial-mesenchymal transition; extracellular matrix

Neoplastic transformation emerges as a result of genetic and epigenetic alterations in pathways that mediate cell growth, cell cycle arrest, and death. Genetic alterations can occur through mutational activation (e.g., oncogenes), mutational inactivation, and loss of heterozygosity (e.g., tumor suppressor genes), as well as epigenetically (e.g., methylation/demethylation of CpG dinucleotides) [Vogelstein and Kinzler, 2004]. Similar to tumorigenesis, metastatic progression also emerges as a result of genetic and epigenetic alterations in pathways that mediate cell invasion, survival outside of the primary tumor microenvironment, and colonization/growth at a distant organ site [Steeg, 2006]. However, the current hypothesis suggests that metastasis is a separate process utilizing the expression (metastasis promoting) or inactivation (metastasis suppressor) of genes distinct from those involved in tumorigenesis [Rinker-Schaeffer et al., 2006]. This paradigm

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suggests that metastasis-associated genes could be targeted therapeutically to stop cancer before colonization and formation of overt metastases—as the overwhelming majority of mortality in cancer patients is due to metastatic disease. With the advent of microarray technology, the transcriptosome for many cancer cell types has been identified and probed for genes that can be used as biomarkers for prognosis and/or targets for novel gene-specific therapeutic interventions. Thus, the identification of lysyl oxidase (LOX) as a potential modulator of tumorigenesis and/or metastatic tumor progression was revealed.

LOX (EC 1.4.3.13) is a copper amine oxidase and belongs to the emerging multigene family currently consisting of five members (LOX, LOXL, LOXL2, LOXL3, and LOXL4) with LOX being the most intensely studied to date [Smith-Mungo and Kagan, 1998; Csiszar, 2001; Kagan and Li, 2003; Molnar et al., 2003; Lucero and Kagan, 2006]. LOX is a copper-dependent amine oxidase, which was identified by Pinnell and Martin [1968] that initiates the covalent cross-linking of collagens or elastin in extracellular matrices. The formation of collagen or elastin cross-links leads to an increase in tensile strength and structural integrity, which is essential for normal connective tissue function, embryonic development, and adult tissue remodeling. Consequently, aberrant LOX expression or enzymatic activity leads to disease. Decreases in LOX expression and/or activity have been associated with such connective tissue disorders as cutis laxa, Menkes' syndrome, and spontaneous coronary artery dissection [Khakoo et al., 1997; Royce et al., 1980; Sibon et al., 2005]. Conversely, increases in LOX activity contribute to the development of fibrotic diseases such as atherosclerosis, scleroderma, and liver cirrhosis, and is implicated in senile plaque formation in Alzheimer's and non-Alzheimer's dementia [Kagan et al., 1981; Kagan, 1994; Chanoki et al., 1995; Gilad et al., 2005].

Although the extracellular matrix (ECM) maturation activity of LOX has long been thought to be its sole function, more recent evidence implicates the involvement of LOX in many critical biological functions other than collagen or elastin cross-linking. LOX has been shown to induce motility and migration in monocytes, vascular smooth muscle cells, and fibroblasts [Nelson et al., 1988; Lazarus et al., 1994; Li et al., 2000]. In addition, LOX expression and activity have been observed in the cytoplasm and nucleus [Wakasaki and Ooshima, 1990; Li et al., 1997; Nellaiappan et al., 2000; Kagan and Li, 2003; Lucero and Kagan, 2006; Jansen and Csiszar, 2007] and implicated in cell signaling and transcriptional gene regulation, as evidenced by utilization of histone H1 and H2 as substrates [Kagan et al., 1983; Giampuzzi et al., 2003a], altered chromatin condensation [Mello et al., 1995], activation of the collagen III α1 promoter through LOX-induced binding of Ku antigen [Giampuzzi et al., 2000], inactivation of the transcription factor NF-κB [Jeay et al., 2003], and regulation of cell adhesion through increased β-catenin and cyclin D1 expression [Giampuzzi et al., 2005]. Even more recent are the findings that LOX protein domains, other than the catalytic domain, can bind to proteins, as has been shown with binding to fibronectin [Fogelgren et al., 2005] and placental lactogen [Polgar et al., 2007] and that the cleaved 18 kDa LOX propeptide (LOX-PP) is also capable of regulating biological functions [Palamakumbura et al., 2004].

Since LOX protein structure and function are so complex and involve such vital biological processes as cell movement, signal transduction, and gene regulation, it is evident that aberrant regulation of LOX would lead to tumorigenesis and tumor progression. Indeed, loss of LOX expression and activity in a number of cancers and oncogene-transformed cell models has implicated LOX as a tumor suppressor gene. Likewise, LOX expression and activity in a number of cancers has also been observed and has implicated LOX as a metastasis promoting gene—creating a conundrum within the LOX research field with regard to LOX biological function(s) in cancer.

This review will summarize and examine the growing reports of aberrant LOX expression and activity (and where possible other LOX family members) in cancers, and assess the role of LOX in tumorigenesis and tumor progression with an emphasis on breast cancer metastatic progression.

LOX GENE ORGANIZATION AND PROTEIN STRUCTURE

The human *LOX* gene is located on chromosome 5 (5q23.3-31.2) and is comprised of seven

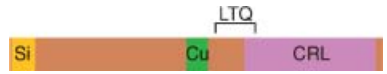
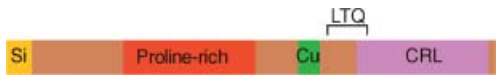
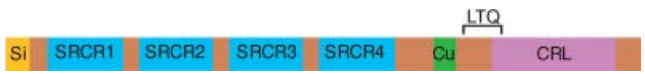
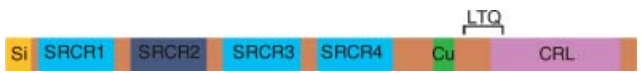
exons that encode a 417 amino acid protein [Hämäläinen et al., 1991, 1993; Mariani et al., 1992]. The entire gene spans across 15 kb of genomic DNA of which 5.5 kb is the 5′ UTR. The first exon encodes for the last 292 nucleotides of the 5′ UTR and approximately half of the coding sequence which contains the signal peptide, propeptide region, and 60 amino acids of the mature protein. In contrast, exon 7 only encodes the final two amino acids of the coding sequence, but contains a 3.8 kb 3′ UTR. The 3′ UTR contains both canonical and noncanonical polyadenylation sites that contribute to the observed size heterogeneity of mRNA transcripts [Boyd et al., 1995]. Differential usage of these alternative polyadenylation sites in the 3'UTR has been observed in adult and fetal membranes [Hämäläinen et al., 1991; Csiszar, 2001].

LOX is synthesized as a 48 kDa preproprotein (preproLOX) which includes a 21 amino acid signal sequence at the amino terminus [Trackman et al., 1992; Lucero and Kagan, 2006]. PreproLOX is N-glycosylated and secreted from the cell as a catalytically inactive 50 kDa proenzyme protein (proLOX). ProLOX is subsequently cleaved into its catalytically mature 32 kDa protein (LOX) and an 18 kDa LOX-PP by bone morphogenetic protein 1 (procollagen C-proteinase) and to a lesser extent by the tolloid proteinases mTLD, mTLL1, and mTLL-2 [Panchenko et al., 1996; Uzel et al., 2001]. The amino terminus of LOX contains the most unique sequence, whereas the carboxy terminus is highly conserved among LOX family members and is responsible for catalytic activity (Table I). The carboxy terminus contains a copper-binding site, lysyl tyrosyl quinine cofactor binding residues, catalytically active site, and a cytokine receptor and growth factor receptor-like domain [Csiszar, 2001; Lucero and Kagan, 2006]. Thus, the complexity of protein domains in LOX protein raises the possibility of multiple biological functions involving not only post-translational modifications of proteins via catalytic activity, but also protein–protein interactions that could potentially inactivate/activate signaling pathways involved in tumorigenesis or metastatic tumor progression.

LOX CATALYTIC ACTIVITY AND SUBSTRATE SPECIFICITY

The catalytic mechanism and substrate specificity of LOX have recently been reviewed in detail [Lucero and Kagan, 2006]. Briefly, LOX is a copper amine oxidase which oxidatively deaminates a substrate amine to an aldehyde product leaving the enzyme in a reduced state. Subsequently, molecular oxygen reduces the enzyme back to a catalytically active state and in the process generates ammonium and hydrogen peroxide. This process requires copper and lysyl tyrosyl quinone (LTQ) cofactors. The LTQ cofactor is covalently linked and plays a critical role in the mechanism of action of LOX as a transient electron. It is believed that copper is not directly involved in LOX catalytic activity; however, is thought to be essential for maintaining protein conformation and LTQ structural integrity. LOX utilizes at least three lysine residues in collagens and elastin as substrates for crosslinking; however, the amino acid sequences that surround these residues differ dramatically, suggesting that the specificity of LOX may be flexible [Lucero and Kagan, 2006]. Studies using lysine-containing oligopeptides demonstrated that LOX activity was sensitive to peptide length and to the specific positions of dicarboxylic amino acids near lysine residues in these peptides [Nagan and Kagan, 1994]. In addition, LOX activity is sensitive to electrostatic field effects between the enzyme and its protein substrate. For example, purified LOX readily oxidized peptidyl lysines in basic globular proteins with isoelectric points greater than pH 8; however, no oxidation was detected in neutral or acidic proteins with isoelectric points less than pH 8 [Kagan et al., 1984]. These results demonstrated that LOX substrate specificity was not limited to collagens and elastin. Subsequent studies have shown that LOX can utilize histones H1 and H2, as well as bFGF as substrates [Giampuzzi et al., 2003a; Li et al., 2003]. Vital to analyzing the role of LOX in biological processes is the necessity for a specific inhibitor of LOX catalytic activity. One such inhibitor that has extensively been used is β-aminopropionitrile [βAPN; Narayanan et al., 1972]. βAPN is an irreversible, competitive inhibitor of LOX activity which prevents catalytically active LOX from binding to target proteins. To date, the overwhelming majority of studies have only assessed LOX catalytic activity to determine its role in biological processes. With the growing realization that LOX can interact with alternative protein substrates by utilizing multiple domains, the

TABLE I. LOX Multigene Family: Genomic and Protein Organization

Gene	Chromosome (location and gene organization)	Protein domains	Protein coding sequence (length and size)	Catalytic activity (inhibitable by βAPN)	Reference
LOX	5q23.3-31.2, 7 exons spanning 15 Kb		417 amino acids, 50 kDa proenzyme, 32 kDa active enzyme	+	Hämäläinen et al., 1991; Mariani et al., 1992; Hämäläinen et al., 1993
LOXL	15q22, 7 exons spanning 25 Kb		574 amino acids, 63 kDa polypeptide	+	Kim et al., 1995; Szabó et al., 1997; Borel et al., 2001
LOXL2	8p21.2-21.3, 11 exons		774 amino acids, 87 kDa polypeptide	+ (not inhibitable by βAPN)	Murano et al., 1991; Saito et al., 1997; Jourdan-Le Saux et al., 1998; Jourdan-Le Saux et al., 1999; Vadasz et al., 2005
LOXL3	2p13.3, 14 exons spanning 21 Kb; splice variant *LOXL3-sv1* (10 exons, Δ exons 1,2,3,5)		753 amino acids, 80 kDa polypeptide; 392 amino acids, 44 kDa	+	Jourdan-Le Saux et al., 2001; Mäki and Kivirikko, 2001; Huang et al., 2001; Lee and Youngho, 2006
LOXL4	10q24, 14 exons		756 amino acids, 82 kDa polypeptide	+	Asuncion et al., 2001; Mäki et al., 2001; Kim et al., 2003

Si, signal peptide; Cu, copper binding domain; LTQ, LTQ cofactor binding site; CRL, cytokine receptor-like domain; SRCR, scavenger-receptor cysteine-rich domains.

use of βAPN and LOX-specific inhibitory RNAs or LOX domain mutants will be essential for future studies to determine the exact role of LOX in biological processes such as tumorigenesis and/or metastatic tumor progression.

LOX FUNCTION IN DEVELOPMENT

LOX initiates the covalent cross-linking of collagens and elastin in extracellular matrices. The formation of collagen or elastin cross-links by LOX leads to an increase in tensile strength and structural integrity which is essential for normal connective tissue function. As such the LOX catalytic domain is highly conserved across species and its activity has been observed in sea urchin [Butler et al., 1987], drosophila [Molnar et al., 2003; Molnar et al., 2005], zebrafish [Anderson et al., 2007], frogs [Geach and Dale, 2005], and mammals [Csiszar, 2001]. These studies demonstrated that LOX plays a pivotal role in embryogenesis and development. LOX expression is greatly increased in sea urchin embryos during gastrulation, and inhibition of LOX activity during 128 or 256 cell stages causes developmental arrest at the mesenchyme blastula stage [Butler et al., 1987]. Inhibition of LOX activity after the mesenchyme blastula stage had little effect on embryo development, suggesting that LOX plays a vital role in gastrulation and primary mesenchyme migration. Studies utilizing $LOX^{-/-}$ mice demonstrated that mice died at the end of gestation or as neonates due to structural cardiovascular and diaphragm instability from impaired connective tissue formation [Hornstra et al., 2003; Mäki et al., 2005]. It is interesting to note that the lethality of LOX depletion in embryonic mice could not be completely compensated for by other members of the LOX family even though they contain the conserved catalytic active domain, which may be accounted for by alternative functions, differential target proteins, and/or differential temporal expression during development for LOXL proteins [Hornstra et al., 2003]. In humans, LOX is highly expressed in early gestational amnion tissue (12–14 weeks) which corresponded to an increased collagen content and tensile strength observed in early versus late gestational age amniotic tissues [Casey and MacDonald, 1997; Hein et al., 2001]. The current paradigm for tumorigenesis and metastatic tumor progression is that developmental programs that mediate cell fate and structure in embryogenesis (such as epithelial to mesenchymal transition (EMT), Wnt, and Notch pathways) are inappropriately utilized, as may be the case with the re-expression of LOX in some cancers [Kelleher et al., 2006].

LOX IS A TUMOR SUPPRESSOR

Oncogene-transformed fibroblasts have been integral for determining the mechanism of LOX tumor suppressor gene function. Contente and colleagues were the first to isolate an mRNA (called the *ras* recision gene) that was downregulated in *ras*-transformed fibroblasts [Contente et al., 1990]. Persistent treatment of *ras*-transformed fibroblasts with IFNα/β yielded a revertant of the *ras*-transformed phenotype and a corresponding re-expression of the *ras* recision gene. It was later determined that the *ras* recision gene was in fact LOX [Kenyon et al., 1991]. Subsequently, Giampuzzi and colleagues demonstrated that the experimental downregulation of LOX in normal rat kidney fibroblasts (NRKF) led to increased cellular proliferation and anchorage-independent growth, loss of PDGF and IGF-1 regulation, and constitutive activation of *ras* [Giampuzzi et al., 2001]. A non-orthotopic injection of LOX knock out NRKF cells demonstrated increased tumorigenicity and metastasis. These investigators went on to demonstrate that the constitutive activation of *ras* (by downregulation of LOX) led to increased expression of β-catenin and cyclin D1 through a noncanonical *ras* signaling pathway [Giampuzzi et al., 2003b, 2005]. Moreover, re-expression of LOX in *ras*-transformed fibroblasts led to a decrease in the activation of NF-κB, a potent transcription factor capable of regulating cell growth and neoplastic transformation [Jeay et al., 2003]. The deactivation of NF-κB was not due to direct interaction with LOX, but by the inhibition of Akt/PI3K activation and membrane localization (a *ras* activated pathway). The most unanticipated results demonstrated that it was not LOX catalytic activity that mediated the suppression of neoplastic transformation signaling in fibroblasts, but it was the 18 kDa LOX-PP cleaved from proLOX by BMP-1 [Palamakumbura et al., 2004]. Although intracellular activity of the cleaved amino terminus of proLOX is a recent finding, it is not novel as the cleaved procollagen N-propeptide has also been shown to function

intracellularly to alter protein synthesis and phosphorylation, as well as cellular adhesion [Oganesian et al., 2006].

In addition to oncogene-transformed fibroblasts, a decrease in LOX activity has also been observed in fibrosarcoma, choriocarcinoma, and rhabdomyosarcoma cell lines compared to normal fibroblast cell lines, which was subsequently shown to be due to low quantities of LOX mRNA [Kuivaniemi et al., 1986; Hämäläinen et al., 1995]. With the introduction of microarray analysis, *LOX* has been shown to be modulated in various cancer cell lines and their corresponding tumor tissues. Thus, the majority of reports indicating alterations in LOX expression have been limited to mRNA transcript levels, which does not always correlate with catalytic activity [Uzel et al., 2000]. To date, a decrease in LOX mRNA and/or protein has been observed in basal and squamous cell, bronchogenic, colon, esophageal, gastric, head and neck squamous cell, pancreatic, and prostatic carcinomas, as well as melanoma (Table II). However, only two reports have definitively demonstrated a tumor suppressor role for LOX using in vitro/in vivo model systems. Bouez and colleagues demonstrated that downregulation of LOX (stable antisense expression) in keratinocytes induced their invasion into the dermis of an in vitro skin equivalent model [Bouez et al., 2006]. Interestingly, treatment of normal keratinocytes with βAPN did not induce invasion and suggests a role for LOX-PP in tumor suppression in this model. Alternatively, LOX protein may be capable of binding to novel target proteins outside of the catalytic domain to alter cell signaling involved in tumor progression. Kaneda and colleagues demonstrated that stable transfection of full-length LOX cDNA into an intestinal-type gastric cancer cell line decreased proliferation and anchorage-independent cell growth, as well as tumorigenesis in non-orthotopically injected nude mice [Kaneda et al., 2004]. Unfortunately, this study did not address a potential mechanism for the tumor suppressor activity. Taken together, these studies demonstrate that LOX is a potent tumor suppressor gene in fibroblasts, basal, and squamous cell and gastric carcinomas that occurs through inhibiting intracellular signaling pathways known to induce neoplastic transformation. It remains to be determined whether the tumor suppressor activity of LOX is as intimately involved in other cancers where downregulation of LOX mRNA has been observed.

Altered expression of other LOX family members has been identified as well (Table III). *LOXL* mRNA expression is downregulated in renal cell carcinomas cell lines in which the Von Hippel-Lindau (VHL) gene has been mutated, suggesting that loss of LOXL expression is associated with oncogenesis of type 2B VHL disease (mutations in the elongin-binding region) [Tsuchiya et al., 2005]. In addition, *LOXL* expression was upregulated in wildtype p53 reconstituted lung adenocarcinoma cell lines [Kannan et al., 2001]. Downregulation of *LOXL2* mRNA was observed in head and neck squamous cell carcinoma cell lines; however, more in-depth analyses are required to establish a tumor suppressor role for LOXL and LOXL2 in these cancers.

LOX IS A METASTASIS PROMOTER

In contrast, microarray technology has also demonstrated the upregulation of *LOX* mRNA in various cancer cell lines and their corresponding tumor tissues. To date, an increase in LOX mRNA and/or protein has been observed in breast, central nervous system cancer cell lines, head and neck squamous cell, prostatic, clear cell renal cell, and lung carcinomas, and in melanoma and osteosarcoma cell lines, compared to their normal or non-aggressive neoplastic counterparts (Table II). Statistically significant clinical correlations between *LOX* expression and tumor progression have been observed in breast [Erler et al., 2006], head and neck squamous cell [Erler et al., 2006], prostatic [Lapointe et al., 2004], and clear cell renal cell carcinomas [Stassar et al., 2001]. In these studies, the expression of high levels of LOX mRNA and/or protein was a poor prognostic factor and was associated with poorly differentiated, high grade tumors, increased recurrence rates, and decreased overall survival. The role of LOX in tumor progression has been most extensively studied in breast cancer using in vitro models of migration/invasion and in in vivo tumorigenesis and metastasis mouse models and will be further discussed in detail.

Upregulation of LOXL2 mRNA and/or protein has been reported in breast, esophageal, head and neck squamous cell, pancreatic, and prostatic carcinomas, melanoma, and

TABLE II. Tumor Tissues and/or Cell Lines in Which LOX Expression is Altered, Compared to Normal, or Non-Invasive Counterparts

Type of Cancer	Expression	Model System	Reference
Basal and squamous cell carcinoma	P, I; ↓	Tissues, cell lines	Bouez et al., 2006
Breast carcinoma	R, P, A, I, C; ↑	Tissues, cell lines	Kirschmann et al., 1999; Perou et al., 1999; Ross et al., 2000; Perou et al., 2000; Kirschmann et al., 2002; Nagaraja et al., 2005; Payne et al., 2005; Erler et al., 2006
Bronchogenic carcinoma	R, P; ↓	Tissues	Woznick et al., 2005
Central nervous system cancers	R; ↑	Cell lines	Ross et al., 2000
Choriocarcinoma	R, A; ↓	Cell lines	Kuivaniemi et al., 1986; Hämäläinen et al., 1995
Colon carcinoma	R; ↓	Cell lines	Csiszar et al., 2002; Mariadason et al., 2002; Kaneda et al., 2004
Esophageal carcinoma	R; ↓	Tissues	
Fibrosarcoma	R, A; ↓	Cell lines	Kuivaniemi et al., 1986; Hämäläinen et al., 1995
Gastric carcinoma	R, I; ↓	Tissues, cell lines	He et al., 2002; Kaneda et al., 2002; Kaneda et al., 2004
Head and neck squamous cell carcinoma	R; ↓	Tissues, cell lines	Rost et al., 2003
Head and neck squamous cell carcinoma	P, C; ↑	Tissues	Erler et al., 2006
Lung adenocarcinoma	R; ↑	Cell lines	Borczuk et al., 2005
Melanoma	R, A; ↓	Cell lines	Kuivaniemi et al., 1986
Melanoma	R; ↑	Cell lines	Kirschmann et al., 2002
Osteosarcoma	R, P, A; ↑	Cell lines	Fuchs et al., 2000; Uzel et al., 2000
Pancreatic cancer	R; ↓	Cell lines	Kaneda et al., 2004
Prostate adenocarcinoma	R, P; ↓	Tissues, cell lines	Ren et al., 1998
Prostate adenocarcinoma	R, C; ↑	Tissues, cell lines	Kirschmann et al., 2002; Lapointe et al., 2004
Clear cell renal cell carcinoma	R, C; ↑	Tissues, cell lines	Ross et al., 2000; Stassar et al., 2001; Young et al., 2001; Takahashi et al., 2001
Rhabdomyosarcoma	A; ↓	Cell lines	Kuivaniemi et al., 1986
Stromal reaction around:			
Basal cell carcinoma	P	Tissues	Bouez et al., 2006
Breast carcinoma	R, P	Tissues	Peyrol et al., 1997
Bronchioloalveolar carcinoma	P	Tissues	Peyrol et al., 2000
Lung adenocarcinoma	P	Tissues	Peyrol et al., 2000
Neuroendocrine carcinoma	P	Tissues	Peyrol et al., 2000
Small cell lung carcinoma	P	Tissues	Peyrol et al., 2000
Squamous cell carcinoma	P	Tissues	Trivedy et al., 1999; Bouez et al., 2006

R, RNA; P, protein; A, enzymatic activity; I, in vivo model validation; C, clinical validation; ↑, upregulation of LOX expression; ↓, downregulation of LOX expression.

TABLE III. Tumor Tissues and/or Cell Lines in Which LOXL, LOXL2, LOXL3, or LOXL4 Expression is Altered, Compared to Normal, or Non-Invasive Counterparts

Type of Cancer	Expression	Model System	Reference
LOX-PP:			
Her-2/neu-driven breast carcinoma	I	Cell lines	Palamakumbura et al., 2004
LOXL:			
Breast carcinoma	R, I; ↑	Cell lines	Montel et al., 2005
Lung adenocarcinoma (p53 reconstituted)	R; ↑	Cell lines	Tsuchiya et al., 2005
Renal cell carcinoma (VHL167 mutant)	R; ↓	Cell lines	Kannan et al., 2001
Stromal reaction around:			
Breast carcinoma	P	Tissues	Decitre et al., 1998
Bronchioloalveolar carcinoma	P	Tissues	Decitre et al., 1998
LOXL2:			
Breast carcinoma	R, P, C, I; ↑	Tissues	
		Cell lines	Perou et al., 2000; Kirschmann et al., 2002; Akiri et al., 2003; Nagaraja et al., 2005; Peinado et al., 2005
Esophageal carcinoma	R; ↑	Cell lines	Ban et al., 2005
Head and neck squamous cell carcinoma	R, C; ↑	Tissues	Chung et al., 2006
Head and neck squamous cell carcinoma	R; ↓	Cell lines	Rost et al., 2003
E1A immortalized kidney epithelial cells	R; ↑	Cell lines	Kiemer et al., 2001
Melanoma	R; ↑	Cell lines	Kirschmann et al., 2002; Peinado et al., 2005
Pancreatic cancer	P; ↑	Cell lines	Grønborg et al., 2006
Prostate adenocarcinoma	R; ↑	Cell lines	Kirschmann et al., 2002
LOXL3:			
Breast carcincoma	R; ↑	Cell lines	Peinado et al., 2005
Melanoma	R; ↑	Cell lines	Peinado et al., 2005
LOXL4:			
Head and neck squamous cell carcinoma	R; ↑	Cell lines	Holtmeier et al., 2003

R, RNA; P, protein; A, enzymatic activity; I, in vivo model validation; C, clinical validation; ↑, upregulation of LOX expression; ↓, downregulation of LOX expression.

E1A-immortalized kidney epithelial cell lines, compared to normal or poorly aggressive neoplastic counterparts (Table III). Chung and colleagues demonstrated that increased *LOXL2* expression (along with the expression of 74 other genes) was significantly associated with high-risk head and neck squamous cell carcinoma and could be used as a predictive biomarker for high-risk patients [Chung et al., 2006]. Akiri and colleagues demonstrated that stable expression of *LOXL2* in poorly invasive/non-metastatic MCF-7 breast cancer cells produced estrogen-dependent tumors in orthotopically injected nude mice with many fibrotic foci and cells that were capable of invading the tumor pseudocapsule and into surrounding blood vessels, nerves, and muscle tissue [Akiri et al., 2003]. Taken together, these reports demonstrate the potential of LOXL2 to promote metastatic tumor progression; however, more in-depth analyses are required to determine the mechanism in these cancers. In contrast, very few reports have demonstrated altered expression of *LOXL3* and *LOXL4* in cancers. At this time, very little is known about the biological function of these LOX family members in normal cell processes.

LOX FUNCTION(S) IN BREAST CANCER TUMOR PROGRESSION

Migration/Invasion/Metastasis

Our laboratory has demonstrated an increase in *LOX* expression in invasive breast cancer cells compared to poorly invasive cells and subsequently provided the first functional studies on the role of LOX in promoting tumor progression in breast cancer [Kirschmann et al., 1999, 2002]. Specifically, we demonstrated that the inhibition of LOX with βAPN or antisense oligonucleotides to *LOX* mRNA led to a significant inhibition of in vitro invasive potential in highly invasive breast cancer cell lines (MDA-MB-231, Hs578T). Conversely, exogenous expression of mature LOX in a poorly invasive cell line (MCF-7) led to a significant increase in invasion that was inhibited with βAPN. Subsequently, we demonstrated that LOX-facilitated breast cancer invasion involved the regulation of cell migration through a hydrogen peroxide-dependent mechanism, which is consistent with previous reports regarding motility and chemotactic responses induced by LOX in vascular smooth muscle cells (Fig. 1; Li et al., 2000; Payne et al., 2005). Specifically, exogenous expression of mature LOX (but not proLOX) facilitated the activation of Src and focal adhesion kinase (FAK; as measured by phosphorylation of key tyrosine residues in the kinase domains) in poorly invasive breast cancer cell lines. The activation of Src kinase was mediated by the production of LOX-generated hydrogen peroxide (through interaction with an unknown substrate) as treatment with catalase (which catalyzes the decomposition of hydrogen peroxide into molecular oxygen and water) markedly decreased Src phosphorylation to levels observed with βAPN inhibition of LOX activity. Activation of the FAK/Src signaling complex by exogenous expression of mature LOX, but not proLOX, promoted a migratory phenotype through the activation of the $p130^{Cas}$/Crk/DOCK180 signaling complex and subsequent activation of Rac1 and Cdc42, and inactivation of Rho [Payne et al., 2006]. Thus, mature LOX promoted a migratory phenotype through changes in actin filament polymerization in breast cancer cell lines.

In addition to our in vitro migration/invasion studies, Erler and colleagues demonstrated that inhibition of LOX by βAPN, blocking antibody, or LOX-specific inhibitory RNA resulted in an inhibition in the formation of lung and liver metastases, compared to controls, in an in vivo orthotopic mouse model of human breast cancer, as well as a tailvein lung colonization model [Erler et al., 2006]. Most importantly, no significant change in primary tumor growth upon LOX inhibition by βAPN, blocking antibody, or inhibitory RNA was observed. These results demonstrate that LOX is important for late-stage tumor progression to metastasis, but not for earlier stages involving tumor formation—the definition of a metastasis-promoting gene. In addition to promoting metastasis, hypoxia greatly increased LOX expression in MDA-MB-231 cells (an invasive/metastatic breast cancer cell line) and that high LOX expression levels co-localized to regions of hypoxia in MDA-MB-231-derived primary tumors [Erler et al., 2006]. Fluctuating oxygen levels, resulting from episodes of ischemia followed by reperfusion, is associated with promoting tumor progression and metastasis [Postovit et al., 2005].

Recently, Min and colleagues have demonstrated that stable expression of LOX-PP and to a lesser extent proLOX can reverse the *ras*- and Her-2/neu-transformed phenotype in

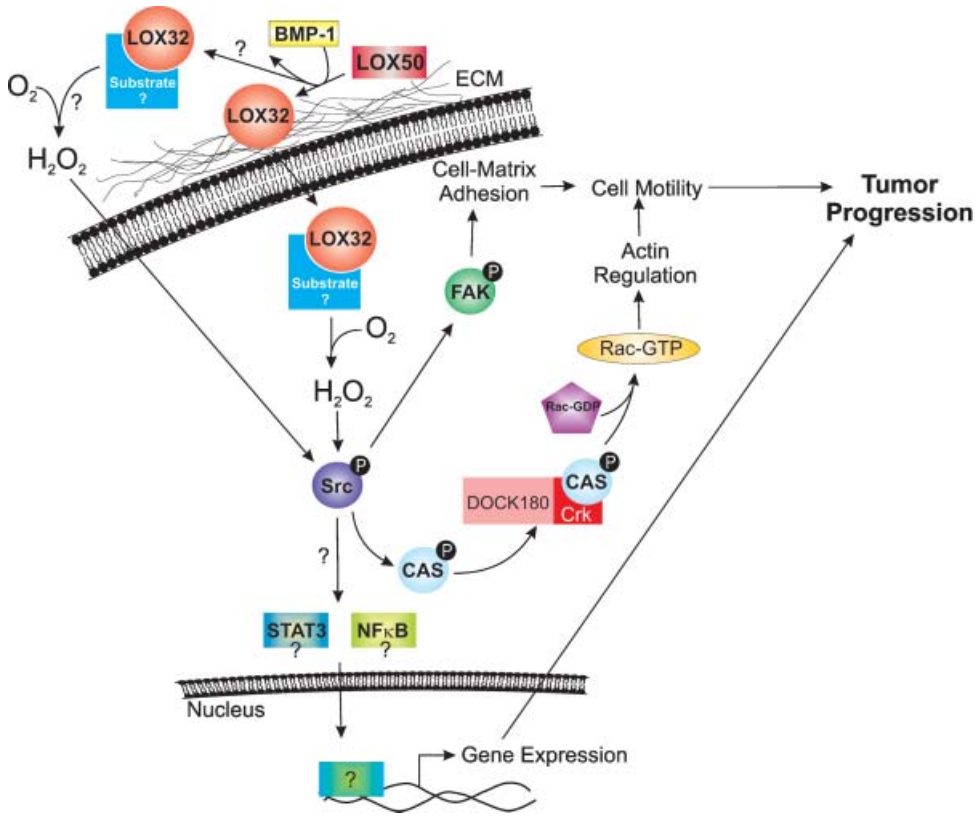


Fig. 1. Hypothetical model of LOX activity in promoting tumor progression in breast cancer. LOX is secreted as an inactive 50 kDa proenzyme into the ECM where it is cleaved by bone morphogenic protein-1 (BMP-1) to become the catalytically active 32 kD enzyme. Subsequently, active LOX can either translocate into the cell or remain in the ECM. Currently, it is not known where LOX target substrates localize. Subsequent catalytic interaction with substrate produces hydrogen peroxide and stimulates Src activation. Activated Src subsequently activates FAK (leading to changes in cell-matrix adhesion) and/or activates the $p130^{Cas}$/Crk/DOCK180 signaling pathway (facilitating actin filament formation). Activation of Src may also lead to activation of the transcription factors STAT3 and NFκB; however, this has not been validated in breast cancer cells. Together, stimulation of these pathways by LOX leads to cell motility and tumor progression in breast cancer.

mammary epithelial cells and suppress tumor formation in non-orthotopically injected nude mice [Min et al., 2007]. Currently, it is not known why LOX-PP in endogenously expressing LOX breast cancer cells (which requires LOX catalytic activity for invasion and thus catalytic cleavage of proLOX) does not prevent the metastatic promoting effects of catalytically active LOX. Nor is it known if LOX-PP can inhibit tumor progression in breast cancer cells that have not been transformed by the *ras* oncogene or genes working through *ras* kinases. Nonetheless, the concept of LOX-PP as a potential novel anti-metastatic therapeutic is exciting and merits further exploration. Taken together, these studies demonstrate that LOX is a potent metastasis-promoting gene in breast carcinomas that facilitates metastatic tumor progression by inducing cell motility and migration.

EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

Epithelial and mesenchymal cells represent distinct cell lineages and each have a unique gene expression profile that provides specific biological functions to each cell type [Lee et al., 2006; Thiery and Sleeman, 2006]. Epithelial cells are well differentiated, have an apical/baso-lateral polar morphology, demonstrate cell–cell adhesion and cell contact inhibition, and express E-cadherin and cytokeratins. In contrast, mesenchymal cells have a leading/trailing edge asymmetric morphology, demonstrate cell motility, have focal adhesions, express N-cadherin, vimentin, nuclear β-catenin, and transcription factors Snail, Slug, and Twist. Breast cancer cells are derived from an epithelial cell lineage and are hypothesized to use EMT to overcome cell–cell adhesion

constraints and acquire a migratory phenotype. Therefore, EMT is a fundamentally vital process for the dissemination and metastatic spread of cancer cells. Although the molecular mechanism by which breast cancer cells undergo EMT is not entirely clear, several potential pathways have emerged as putative mediators of EMT. The transcription factor Snail, which has been shown to be directly involved in developmental EMT, has been shown to be upregulated in breast cancers and is associated with lymph node metastasis and decreased relapse-free survival [Moody et al., 2005; Côme et al., 2006]. Recently, Peinado and colleagues demonstrated that the catalytic domain of LOX, LOXL, LOXL2, and LOXL3 interacted with Snail in the repressor SNAG domain and that Snail Lys98 and Lys137 were essential for LOX-Snail binding [Peinado et al., 2005]. Subsequently, they demonstrated that LOXL2 could partially repress E-cadherin promoter activity, but both Snail and LOXL2 were required for maximal repressive activity and induction of EMT in MDCK cells. Conversely, knock down of *LOXL2* expression by RNA interference in mouse squamous carcinomas induced a reversion in EMT characterized by phenotypic and genetic changes associated with epithelial cells. The authors indicate that proliferation of cells was not altered by inhibiting LOXL2 expression, further supporting the role of LOXL2 as a metastasis promoter. Unfortunately, the role of LOX and Snail in breast cancer cells or the requirement of LOXL2 catalytic activity for Snail activation was not evaluated in this study. Taken together, these studies suggest that LOX and LOXL family members play an integral role in EMT which is essential for metastatic tumor progression in some cancers.

Although not directly tested, there are suggestions of LOX mediating EMT as indicated by morphologic changes and as previously described in mediating mesenchyme migration during gastrulation [Butler et al., 1987; Giampuzzi et al., 2003b; Erler et al., 2006; Jansen and Csiszar, 2007]. In addition to direct interaction with Snail, a known inducer of EMT, LOX may be capable of inducing EMT through Src kinase activation. Src family kinases have the capacity to regulate fundamental cell processes including differentiation, cell shape, and migration [Parsons and Parsons, 2004]. Constitutively active Src can induce EMT and tumor progression contributing to metastasis [Boyer et al., 2002; Frame, 2002; Larue and Bellacosa, 2005; Galliher and Schiemann, 2006]. One potent downstream signaling molecule that is activated by Src and Rac1 is signal transducer and activator of transcription 3 (STAT3) [Silva, 2004; Clevenger, 2004]. Many studies in human tumor cells lines demonstrate an increase in STAT activation leading to a loss in cellular differentiation and increased survival [Silva, 2004; Diaz et al., 2006]. In addition to tumor cell lines, increases in Src, Rac1, and STAT3 activation and activity have been observed in breast tumors [Berclaz et al., 2001; Garcia et al., 2001]. As previously described, we have observed that LOX expression in poorly aggressive breast cancer cells induced Src and Rac1 activation. Therefore, it is enticing to speculate that LOX can activate STAT3 through Src or Rac1, potentiating a reversal in cellular differentiation and induction of an EMT; however, further experimentation is required to validate this hypothesis.

COPPER HOMEOSTASIS AND TUMOR PROGRESSION

Another piece of evidence (albeit indirect) suggesting that LOX is involved in breast cancer metastatic tumor progression comes from clinical trials in which patients with metastatic disease are treated with tetrathiomolybdate (TM), an anti-copper agent that complexes with copper and protein rendering copper unavailable for cellular uptake [Goodman et al., 2005]. When low copper levels were maintained for greater than 90 days, four of six patients maintained stable disease and one had regression of disease. The authors of this study concluded that TM (and thus copper deficiency) had a cytostatic, rather than cytotoxic effect, in bulky cancers leading to disease stabilization as opposed to reduction of tumor burden, suggesting that TM affected metastatic tumor progression. In Her2/neu transgenic mice, TM treatment impaired tumor growth by inhibiting vessel network formation and decreased activation of NF-κB [Pan et al., 2002]. Unfortunately, neither LOX activity nor the contribution of LOX inhibition to phenotypic alterations by TM treatment was assessed in in vivo mouse models of metastatic breast cancer. Rucker and colleagues discuss the role of dietary copper and LOX and indicate that LOX activity (crosslinking

capacity), but not mRNA or protein levels, is directly influenced by the amount of dietary copper intake [Rucker et al., 1998]. The requirement for LOX in vessel formation, the ability of LOX to affect NF-κB activation in *ras*-transformed fibroblasts, and the effect of copper homeostasis on LOX activity all point to a putative role for LOX in metastatic tumor progression.

STROMAL-TUMOR-ECM INTERACTIONS

Sommer and colleagues have identified LOX in the stromal reaction around breast, bronchopulmonary, and basal and squamous cell tumors [Peyrol et al., 1997; Trivedy et al., 1999; Peyrol et al., 2000]. In breast carcinoma, the highest levels of LOX expression were observed in myofibroblasts and myoepithelial cells surrounding in situ ductal tumors and in the fibrosis facing the invasion front of infiltrating tumors [Peyrol et al., 1997]. The authors of this study have suggested that the reactive stroma is a possible host defense mechanism to wall off the tumor. We would propose a different hypothesis in which the expression of LOX in the stroma increases collagen cross-linking and thus increases ECM stiffness leading to a loss of epithelial cell differentiation and tumor progression. Malignant transformation of neoplastic cells in the breast has been associated with changes in gland tension—characterized by increased compression forces, increased tension resistance forces, and an increased ECM stiffness [Paszek and Weaver, 2004]. Paszek and colleagues demonstrate that tumor tissues are stiffer than normal tissues and that matrix stiffness perturbs epithelial morphogenesis through elevated Rho-GTPase-dependent cytoskeletal tension, thus altering cell polarity, adherens junctions, and focal adhesions [Paszek et al., 2005]. Anderson and colleagues have theorized that harsh tumor microenvironments select for homogeneous tumor cells that have very aggressive traits, whereas mild tumor microenvironment conditions allow coexistence of heterogeneous tumor cells of varying metastatic aggressiveness [Anderson et al., 2006]. Indeed, our laboratory has observed that LOX expression appears to transition from the stroma in in situ ductal carcinoma to cytoplasmic tumor cell staining in high-grade invasive ductal carcinoma and metastases [Payne et al., 2005]. Based on these observations, it is enticing to speculate that a potential shift in LOX function has occurred with increasing metastatic potential caused by ECM-tumor cell interactions. However, further studies are required to validate these hypotheses.

CONCLUSION

In this review, we have analyzed the cancer literature and provided evidence to justify LOX as both a tumor suppressor and a metastasis promoter gene in cancer. This disparity could be due to cell origin, differentiation status, global genetic differences with regard to availability of LOX substrates, unbalanced expression/activity of LOX-PP and LOX, tumor-stromal cell interactions, ECM stiffness, and/or the intrinsic pleomorphic biological activities of LOX. The complex nature of LOX protein domain structure and biological functions precludes traditional microarray-based research to investigate tumor suppressor/metastatic promoting functions of LOX in human cancers. Future studies are needed to address activity, LOX-PP expression, and identification of alternative enzymatic and protein binding targets of LOX. Particularly noteworthy is the overwhelming evidence that LOX mediates metastatic progression in breast cancer. A clearer understanding of the mechanism(s) by which LOX contributes to tumor progression has the potential for novel anti-metastatic cancer therapeutics.

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