Lysyl oxidase in colorectal cancer

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Colonial cancer (CRC), encompassing both colon and rectal cancer, is the third most prevalent form of cancer worldwide and the fourth-leading cause of cancer-related mortality, leading to ~600,000 deaths annually (22). Of note, CRC is most prevalent in developed countries, where cases account for over 60% of the total 1.2 million worldwide diagnoses. The primary treatment for CRC typically involves surgical resection, which remains the gold standard of care for patients. However, greater than 50% of CRC-specific deaths occur as a result of metastatic dissemination, with advanced-stage CRC patients exhibiting a median survival of only 6–9 mo from diagnosis (63). Thus the early identification of patients harboring metastatic tumors will allow the rapid deployment of adjuvant therapies to help improve their survival. Therefore, understanding the molecular mechanisms behind the spread of CRC is key to stratifying patients for emerging antimetastatic therapies.

Lysyl oxidase (LOX) is a secreted copper-dependent amine oxidase whose primary function is to catalyze the covalent cross-linking of collagens and elastin in the extracellular matrix (ECM). This occurs through the oxidative deamination of peptidyl lysine residues in ECM components (33, 64). The action of LOX is important in establishing the structural integrity and stability of the ECM, thereby contributing to the tensile strength of many tissues. LOX is the classical member of a larger family of proteins consisting of five currently identified paralogs: LOX, LOX-like 1 (LOXL1), LOX-like 2 (LOXL2), LOX-like 3 (LOXL3), and LOX-like 4 (LOXL4). Each of these members shares a high degree of sequence homology within their carboxy terminal region, leading to conservation of including a conserved catalytic domain, copper-binding motif, a lysyl-tyrosyl-quinone (LTQ) cofactor, and cytokine receptor-like (CRL) domain (Fig. 1). The amino terminal regions are more divergent and are thought to be important in protein-protein interactions. The presence of prodomains in LOX and LOXL1 enables their secretion as inactive proenzymes, which are then activated extracellularly. LOXL2, LOXL3, and LOXL4 contain no prosequences but instead contain four scavenger receptor cysteine-rich domains that are thought to be important for function. Although all of the family members have been implicated in various stages of cancer progression (55), and despite their high sequence homology, little is known about the precise molecular functions of LOXL1, LOXL3, and LOXL4. The well-studied member of the family remains LOX, although the recent explosion in interest in LOXL2 is rapidly expanding our knowledge base. The matrix remodeling function of LOX has been most extensively studied, and similar function of the other family members is presumed on the basis of conserved sequence homology of the catalytic domain.

To date, there is little evidence to suggest a functional redundancy between the family members during normal development; this is exemplified in the LOX knockout mouse (29, 49), which shows perinatal lethality due to aortic aneurysms and diaphragm rupture (29, 49, 50). Although the expression levels of the other LOX family members were not measured, remaining LOX family enzymatic activity was detected, suggesting that their expression levels were not affected (49, 50). The critical nature of LOX was shown in further studies where remaining LOX family enzymatic activity was detected, suggesting that their expression levels were not affected (49, 50). The end result is aortic aneurysms and diaphragm rupture (29, 49, 50).

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levels have been observed in preterm lamb lungs compared with full-term (8). Together this data highlights the importance of LOX in the assembly, tensile strength, and mechanical stability of collagen fibrils and for the assembly and reversible deformation of elastin. This function is critical in organs containing fibrillar collagen and/or elastic fibers such as skin, lung, cartilage, the cardiovascular system, and the fibrous lamina propria in the small intestine and stomach. In contrast to LOX, the LOXL1 knockout mouse is viable yet does not deposit normal elastic fibers in the uterine tract postpartum and develops pelvic organ prolapse, enlarged air spaces of the lung, loose skin, and vascular abnormalities with concomitant tro- poelastin accumulation (46). Distinct from LOX, LOXL1 localizes specifically to sites of elastogenesis and interacts with fibrulin-5, playing a critical role in elastin homeostasis (46).

To date, knockout mice for the other LOXL family members have yet to be successfully established. Thus far, it appears that LOX alone is critically important in development but that LOXL1 and likely the other family members are vital for the maintenance of normal tissue homeostasis. This is further reinforced by many publications in which abnormal LOX expression or activity is associated with a wide spectrum of human pathological states including scleroderma (11), Menkes disease, Ehler-Danlos syndrome (61), occipital horn syndrome (10, 38), lung and liver fibrosis (14), and liver cirrhosis (31), as well as in many cancers (reviewed in Ref. 7). Despite a strong role in ECM cross-linking, it has also been reported that LOX may act on other substrates. For example, it has been reported that LOX is capable of oxidizing the basic fibroblast growth factor (bFGF) (45), platelet-derived growth factor receptor β (PDGFRβ) (48), and histones H1 and H2 (21) in vitro. However, the implications of these LOX-mediated modifications are not yet fully understood.

**LOX Synthesis and Secretion**

The human LOX gene is located on chromosome 5q23.3–31.2 (25). LOX is initially synthesized as an inactive 50-kDa proenzyme of 417 amino acids, of which the first 21 amino acids represent the signal peptide (25) (Fig. 1). During biosynthesis, the propeptide region is subject to N-linked (65) and O-linked glycosylation (68). Glycosylation of the propeptide has been shown to be essential for LOX enzymatic activity, but it is not required for secretion of the proenzyme (23). Once secreted into the ECM, the proenzyme is proteolytically cleaved by procollagen C-proteinase (15). Cleavage releases a 18-kDa propeptide domain and the 32-kDa active mature enzyme (32). Although LOX is classically described as an ECM protein, immunohistochemical profiling has suggested that it may also be localized within the cytoplasm (69) and the nucleus (44) of some cell types. The precise role of LOX in these locations is still under intense investigation.

**LOX in Cancer**

**LOX as a tumor suppressor.** The LOX gene was originally identified as a tumor suppressor on the basis of in vitro experiments demonstrating that its reexpression was capable of reverting H-Ras-mediated transformation of NIH 3T3 fibroblasts (13). However, both the upregulation and downregulation of LOX at the mRNA level have since been reported in cancer, with CRC being no exception. Nonetheless, data continues to emerge implicating a critical role for LOX in the progression of numerous solid tumors, including breast (9, 17, 18, 54), head and neck (42), brain (41, 58), uveal melanoma (1), lung cancer (47), oral and oropharyngeal squamous cell carcinoma (2), as well as CRC, which will form the topic of this short review. Despite being classed as a secreted protein, LOX has been shown to have both intracellular and extracellular functionalities. These seemingly opposing reports suggest that LOX possesses complex and paradoxical roles and may act to function as both a tumor suppressor and metastasis promoter depending on its localization.

There are several reports that support a role for LOX as a tumor suppressor, whereby decreased LOX expression at both the mRNA and protein level has been observed in tumor tissue compared with matched normal tissue and in vitro cell lines, and this downregulation is thought to be controlled through promoter methylation (35, 60). In the studies, the silencing of LOX is associated with a more aggressive phenotype and decreased patient survival, supporting a putative function as a metastasis promoter. However, compelling evidence from the field has indicated that the tumor suppressor activity of LOX lies not within the active enzyme itself, but within the propeptide domain following extracellular cleavage from the mature enzyme (51–53, 72). It has been shown that the LOX propep-
tide achieves its tumor suppressor effects by reentering the cell following cleavage (24), although the precise mechanism by which this is achieved remains unknown. Indeed, the propeptide contains a putative nuclear localization sequence (68), which suggests that it may be actively directed to the nucleus once released from the proenzyme, and it has been shown to be capable of both repressing the oncogenic bcl-2 gene in breast cancer (71) and inhibiting FGF-2 signaling in prostate cancer (53). It has also been shown to possess an ability to inhibit signaling pathways that lead to the activation of nuclear factor-κB (NF-κB) in prostate and lung cancer (51, 52, 62), suggesting that there are likely multiple mechanisms by which it may exert tumor suppressor activity.

**LOX as a metastasis promoter.** In contrast to the above, it has been reported by many groups that the levels of LOX mRNA and protein are increased in invasive or metastatic breast cancer and melanoma cell lines, and invasion of these lines can be prevented in vitro by the addition of β-aminopropionitrile, a nonspecific small molecule inhibitor of LOX activity (18, 40), or immunologically by using LOX targeting antibodies (4, 6, 17, 18). These studies would suggest that in these situations LOX may be functioning as a metastasis promoter rather than tumor suppressor. More recently, LOX has been clinically validated as a prognostic biomarker for breast carcinoma (2), whereby high expression is correlated with poor disease-free and overall survival. This data therefore further supports a role for LOX in promoting cancer progression rather than attenuating it.

Voloshenyuk and colleagues have shown that LOX expression is induced by TGF-β (67) and TNF-α (66) in cardiac fibroblasts, and data from Kagan and Li (32) has shown that IFN-γ can also induce LOX expression. Since all of the aforementioned are generally implicated in tumor development and progression, it would seem contradictory that LOX would be acting in a tumor-suppressor role in these situations, unless one assumed its potency was insufficient to override such strong contextual drivers. Furthermore, LOX expression is induced under hypoxic (low oxygen) conditions through hypoxia-inducible factor-1 (HIF-1) transcription factor binding to a functional hypoxia-responsive element in the promoter region (18). Hypoxia is a well-known driving force of cancer progression, including CRC; thus again it is unlikely that LOX functions as a tumor suppressor in these conditions.

There are a number of studies that have reported no effect of LOX on primary cancer cell proliferation, including models such as breast cancer (18) and uveal melanoma (1). However, an equal number of studies have shown that LOX expression is capable of increasing proliferation of certain cell types, for example osteoblasts (24) and breast cancer (34) as well as CRC (discussed shortly). Coupled with reports that the LOX propeptide will inhibit cell proliferation in models of prostate (53) and breast cancer (3, 51), data suggests that the precise location of LOX, its activity, its processing, and more importantly the context of its expression are all important in determining its function. It has thus become necessary to reconcile the contrasting roles of the LOX propeptide and the mature enzymatically active LOX in terms of cancer progression, and much work is being done to address this. Given the body of evidence, one would speculate that it is entirely possible that the tumor suppressor properties of the LOX propeptide act to control the poinvusive properties of the LOX enzyme, and that the tipping of this balance during cancer progression could be an important metastatic trigger (Fig. 2). At present, however, it is too early to tell and further studies are currently being undertaken by several groups to evaluate this. An exciting notion from a therapeutic perspective would be the simultaneous administration of both the recombinant LOX propeptide and inhibitors of LOX enzymatic activity in cancer treatment. Nonetheless, a more detailed understanding of LOX-associated signaling will certainly be required before this can be realized.

**LOX in CRC.** It has only been within recent years that the role of LOX in the progression of CRC has really begun to be investigated. Initial reports showed decreased LOX mRNA expression levels in CRC patients with nonmetastatic disease (16), supporting early reports of LOX having a tumor suppressive role. However, this sample set was composed almost exclusively of samples from patients with early-stage disease, a stage that later studies have shown to exhibit LOX protein levels roughly equivalent to normal tissue (6). A more recent study has shown that LOX mRNA expression is associated with a diffuse cytoplasmic expression of CEA (carcinoembryonic antigen) in patients. The authors conclude that, since CEA is often correlated with an increased invasive potential of the tumor (39), in this case LOX may be acting to promote tumor progression. In support of this, our and other groups have supported a role for LOX in promoting CRC progression (4, 6, 56). In all of these studies, LOX mRNA and protein expression was shown to be increased during CRC disease progression and metastatic dissemination. Hence there is a large cohort of emerging data that supports a role for LOX in promoting tumor progression, especially in the context of CRC rather than in a tumor-suppressive capacity as was first proposed. It should be noted, however, that in these later papers detection of the LOX protein typically focused on the LOX enzyme itself and the authors did not profile the presence and or localization of the propeptide in these studies. We will now briefly cover the broader areas of the seemingly paradoxical nature of LOX in CRC.

**LOX as a tumor suppressor in CRC.** LOX was first proposed to be a tumor suppressor in CRC following a paper in 2002 by Csiszar and colleagues (16). Their work built on a previous report that had constructed a chromosome imbalance map based on over 300 cases of colon cancer and had revealed that ~15% of chromosome 5q14–5q31 is lost. This was of interest, since a higher percentage of the loss was typically confined to 5q21–31, a region that harbors the APC and MCC genes, but also the LOX gene (5q23). This prompted the authors to test the mutational status and expression level of the LOX gene in a cohort of patients with CRC. Analysis of this locus and the flanking loci in matched tumor and blood DNA samples from a panel of CRC patients demonstrated that 38% (16/42) of informative samples were affected by loss of heterozygosity (LOH) or allelic imbalance. Furthermore, 75% (6/8) of these tumor samples were shown to have significantly reduced LOX mRNA levels, and a similar reduction in LOX mRNA levels was detected in a panel of matched normal colon and colon tumor samples. Tumor samples demonstrating LOH by restriction fragment length polymorphism were also subjected to mutational analysis, including RT-PCR, exonic deletion detection by PCR, cDNA and genomic DNA sequencing, and were
found to have a spectrum of alterations and mutations directly affecting the LOX gene. Thus the authors were able to conclude that loss or reduction of LOX function during tumor development was a direct consequence of somatic mutations and must be critically associated with CRC pathogenesis. However, the authors were unable to evaluate metastatic tumors or metastases, with the majority of samples analyzed coming from patients with Dukes stage A–C nonmetastatic disease (stage A, 9%; stage B, 73%; stage C, 14%; stage D, 4%). Despite the implication of LOX in multiple cancers including CRC, there are only sparse reports of point mutations, deletions, or epigenetic alterations in the LOX gene, suggesting that tumor associated changes in expression may be due to a combination of the more frequent loss of heterozygosity, transcriptional (i.e., hypoxic induction) and translational regulation, which act as the main drivers behind LOX-mediated cancer/CRC progression. This is confirmed by the lack of LOX mutations identified in colon cancer patient samples published on the TCGA website (http://www.cbioportal.org/public-portal).

LOX as a promoter of cancer cell proliferation in CRC. Several years later, we showed that LOX expression is significantly elevated in tumor tissue compared with normal colon tissue in an archive of 559 colorectal adenocarcinoma patient samples, with the greatest increases being observed in metastatic tissue (6). We then went on to show that elevating the expression of LOX in the SW480 CRC cell line, which normally expresses low levels of LOX and is tumorigenic but not metastatic in models of CRC, led to significantly increased rates of cellular proliferation in 3D collagen type I cultures (6). Similarly, silencing of LOX in the matched SW620 metastatic line led to decreases in the rate of proliferation under the same conditions. The context here is important since collagen I is one of the primary substrates of LOX. LOX-mediated effects on proliferation did not pertain to cells plated onto tissue

Fig. 2. The tumor suppressor properties of the LOX propeptide are thought to be mediated through its nuclear localization and act in opposition to the prometastatic action of the mature enzyme and as such may act to control the proinvasive properties of the LOX enzyme. Thus the tipping of this balance during cancer progression could be an important metastatic trigger. ECM, extracellular matrix. BMP-1, bone morphogenic protein-1; PRO, propeptide.
LOX in CRC

LOX drives invasion and metastasis of CRC. As mentioned earlier, work by Kim and colleagues (39), using real-time PCR analysis of matched tumor and normal tissue specimens from 104 colorectal adenocarcinoma patients (28 patients with AJCC stage I, 37 with stage II, 33 with stage III and 6 with stage IV disease), found an upregulation of LOX mRNA in patients with a diffuse cytoplasmic expression pattern of CEA. However, this upregulation did not statistically correlate with tumor location, stage, growth type, or differentiation status, but its association with CEA expression led the authors to conclude that LOX upregulation may be associated with increased invasiveness and metastatic potential in colorectal cancer. Following this, in 2011, work from our laboratory also showed that silencing LOX in human CRC lines inhibits metastasis whereas LOX overexpression in human CRC lines promotes metastasis (6). At this point, data was also beginning to emerge on the potential mechanisms by which LOX may be exerting these tumor-promoting effects in CRC. These are discussed below and outlined in Fig. 3.

Mechanisms of Action: Stimulation of Proliferation and Invasion

That LOX is involved in tumor cell invasion potentiates it as a strong candidate for preventing tumor spread. Studies into the underlying mechanisms through which LOX may act to drive invasion have shown that LOX expression leads to associated increases in SRC kinase and focal adhesion kinase (FAK) activation, and the associated downstream signaling cascades (7). Indeed, evidence from our group suggests that both SRC and FAK are activated through LOX-mediated increased collagen cross-linking, resulting in increased matrix stiffness, which is sensed by mechanosensitive transmembrane integrins that then stimulate downstream activation of SRC and FAK (4, 6). These results are consistent with previous reports in breast cancer (43). We showed that this activation of SRC and FAK resulted in increased tumor cell proliferation and invasion and was associated with the formation of distant metastases (4, 6). Moreover, use of small molecule inhibitors of FAK or SRC was able to abrogate these LOX-driven effects on in vitro invasion (4), and inhibitors of SRC were able to abrogate effects on proliferation, tumor growth, and metastasis in vivo (6). The use of integrin-blocking antibodies successfully prevented LOX-mediated activation of SRC, confirming this mechanism of action (6). LOX-driven effects could be blocked directly by use of a function-blocking LOX antibody or through expression of a mutant version of LOX lacking enzymatic activity because of a point mutation in the LTQ domain (K320A), confirming a role for its enzymatic function (4). Our

Fig. 3. LOX regulates multiple signaling networks in colorectal cancer, both directly and indirectly, affecting signaling, transcription, and translation. Its action leads to changes in cellular proliferation, adhesion, and motility. FAK, focal adhesion kinase; AKT-PI3K, protein kinase B-phosphatidylinositol-3-kinase; HIF1α, hypoxia inducible factor 1α.

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data further suggests the potential to use primary tumor LOX expression as a useful diagnostic marker in indicating which CRC patients may be responsive to SRC kinase inhibitors such as dasatanib.

Pez et al. (56) proposed an alternative mechanism whereby LOX-mediated H₂O₂ production, a by-product of enzymatic activity, results in activation of the PI3K (phosphoinositide 3-kinase)-Akt signaling pathway, which leads to subsequent upregulation of HIF-1α protein synthesis and thus further LOX production creating a positive regulation loop and leading to enhanced CRC proliferation. Indeed, H₂O₂ production by LOX has also been implicated in the activation of SRC and FAK in breast cancer models (54). It would thus seem as though LOX drives CRC progression through activation of multiple pathways through multiple mechanisms, biochemical and biomechanical. A schematic of proposed LOX action is shown in Fig. 3.

Mechanisms of LOX Action: Stimulation of Angiogenesis

Angiogenesis is a rate-limiting factor for primary and metastatic tumor growth, and thus critically required for tumor progression (19, 37). Indeed, Hanahan and Weinberg (27, 28) have ascribed it as one of their “hallmarks of cancer”, and as such the process of angiogenesis is the subject of extensive study in the context of tumorigenesis and metastasis (26). The vascular endothelial growth factor (VEGF) signaling pathway plays a pivotal role in promoting angiogenesis, both under normal conditions and also during the pathogenesis of cancer. As such, it has become a major target for pharmaceutical intervention in the tumor setting (30, 36).

Studies from the LOX knockout mouse have shown that correct LOX expression is critical for the development of the cardiovascular system during embryogenesis (29). There have, however, been few studies on the effect of LOX expression on tumor-driven angiogenesis. The first study into the potential link between LOX and angiogenesis was published by our group earlier this year (5). In this paper, we provided compelling evidence of a novel link between LOX expression and VEGF secretion in vitro, in vivo, and in patients. We demonstrated that regulation of VEGF expression at both the mRNA and protein level occurs through PDGFRβ-mediated activation of Akt. Using clinically relevant inhibitors of angiogenesis (bevacizumab and sunitinib) or inhibitors of PDGFRβ- or Akt-mediated signaling, we were able to abrogate LOX-mediated increases in VEGF mRNA and protein expression and LOX-mediated stimulation of endothelial cells in vitro and in vivo. Finally, we showed that our proposed mechanism (Fig. 3) pertains to breast cancer models as well, suggesting a high degree of commonality between solid tumor types.

LOXLs in CRC

The evaluation of the other paralogs of the LOX family has received much less attention than LOX itself in CRC. Fong et al. (20) analyzed LOXL2 expression in colon (and esophageal) tumors and investigated methylation as a regulator of LOXL2 expression. Immunohistochemistry demonstrated intracellular localization of LOXL2 in normal colonic enteroendocrine cells, but not in mitotically active cells. Analysis of 52 colon adenocarcinomas revealed presence of LOXL2 expression in 83% of the samples and showed that there was a significant association between LOXL2 expression and less-differentiated colon carcinomas. The authors also determined that the methylation status of the 1,150 bp 5’ CpG island may contribute to the regulation of the gene. Loss of heterozygosity studies further suggested the loss of LOXL2 was unlikely to play a major role in colon tumors and that increased LOXL2 expression may contribute to colon cancer progression.

In 2009, Kim et al. (39) attempted to address the expression of the LOX family genes in colorectal adenocarcinomas, by performing real-time PCR analysis of matched tumor/normal tissue specimens from the previously mentioned patient database. The authors found that the expression of the LOX-like family genes was not statistically associated with tumor location, stage, growth type, or differentiation status. However, they did find, consistent with the previously mentioned findings above, that as well as LOX there was an upregulation at the mRNA level of LOXL2 and LOXL4 and that this was significantly correlated with the absence of lymphovascular invasion. They postulated that the oxygen tension in and around tumors may be an important regulator for the differential expression of LOXL2 and LOXL4 and important in the progression of CRC. Although these studies require further investigation, it would seem likely that LOXL2 and possibly also LOXL4 may play a role in colon cancer progression.

Therapeutic Targeting and Concluding Thoughts

The importance of LOX in solid tumors in general and in CRC is beyond doubt. Its implication in cell proliferation, invasion, and metastasis, driving angiogenesis and malignant transformation, has elevated it to a position as a viable target for therapeutic intervention. Indeed, cancer cells expressing high levels of LOX protein have an increased propensity to proliferate, invade, and metastasize in multiple solid tumor models, and there is compelling evidence from several laboratories to suggest that, in CRC, targeting LOX not only inhibits cancer cell invasion and metastasis but also reduces tumor angiogenesis since LOX regulates multiple signaling networks (Fig. 3). However, development of suitable drugs has to date been limited. The lack of a complete crystal structure for LOX or indeed any of the family of proteins currently precludes classical structure-driven fragment-based drug development and screening methodologies, and even the small-molecule inhibitors (e.g., β-aminoproprionitrile) currently available are nonspecific, showing affinity for multiple family members as well as other amine oxidases. Although continuing efforts to screen for novel inhibitors are underway, the elucidation of crystal structures would greatly increase our understanding of this family of proteins in terms of their function and regulation. Similarly, the development of robust readouts, which at present are limited, to accurately measure LOX and LOXL enzymatic activity is needed to fine-tune mechanistic studies into the action of family members. Although the LOX knockout mouse currently exists, the establishment of conditional and inducible knockout and knockin models will allow a greater degree of manipulation of LOX and LOXL family member expression in vivo, which would rapidly increase our understanding of these proteins, not only in normal development, but in disease in general, including cancer.

LOX-neutralizing antibodies have proved effective and specific but are accompanied by a higher complexity of adminis-
tration and preclude intracellular targeting of the family, which may be both advantageous and disadvantageous depending on context. Of course, alternative approaches to targeting the family directly include downregulation at the transcriptional and/or translational level and/or targeting posttranslational processing and activation. For example, forced expression of GATA3 reduces metastasis through decreased LOX expression (12), and targeting of HIF-1 prevents LOX-driven metastasis (70) in animal models. However, current knowledge and understanding of LOX mechanisms of action in different cellular compartments is not yet great enough to know which strategies would be most effective, and there is a critical need for network biology-driven studies to understand the context of when and also why LOX is important. The extracellular function of LOX, together with its relatively low expression levels in normal tissues and the dramatic abrogation of cancer progression when it is inhibited, make LOX a highly attractive drug target in CRC. Despite the challenges that lie ahead, the development of inhibitors to target LOX brings hope that effective therapies against primary and metastatic colorectal cancers will be available in the not-too-distant future.

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AUTHOR CONTRIBUTIONS

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