

Lysyl Oxidase, a Targetable Secreted Molecule Involved in Cancer Metastasis

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Abstract

Secondary metastatic cancer remains the single biggest cause of mortality and morbidity across most solid tumors. In breast cancer, 100% of deaths are attributed to metastasis. At present, there are no "cures" for secondary metastatic cancer of any form and there is an urgent unmet clinical need to improve the tools available in our arsenal against this disease, both in terms of treatment, but also prevention. Recently, we showed that hypoxic induction of the extracellular matrix modifying enzyme lysyl oxidase (LOX) correlates with metastatic dissemination to the bone in estrogen receptor negative breast cancer and is essential

for the formation of premetastatic osteolytic lesions. We showed that in models of breast cancer metastasis, targeting LOX, or its downstream effects, significantly inhibited premetastatic niche formation and the resulting metastatic burden, offering preclinical validation of this enzyme as a therapeutic target for metastatic breast cancer. Our work is the latest in an emerging body of work supporting the targeting of LOX and calls for greater efforts in developing therapeutics against this extracellular secreted factor in the prevention of cancer progression across multiple solid tumor types. *Cancer Res*; 76(2); 188–92. ©2016 AACR.

Cancer Metastasis and the Importance of the Cellular Microenvironment

The spread of primary tumors to nonadjacent unrelated organs has proven to be a complex and difficult problem for researchers to tackle. This is because metastasis is a systemic process involving not only primary tumor cells, but also contributions from a vast and diverse range of nonmalignant host cells at both primary and secondary sites over varying timescales.

Over the last two decades, it has become increasingly apparent in the field of cancer research that the genetic aberrations once seen as the primary drivers of cancer initiation and metastasis, can account for only a subset of the necessary steps required for progression. Additional contributions from the tumor microenvironment, dictated by both the tumor cells themselves, but more importantly by resident nonmalignant cells, provide an enormous repertoire of both soluble and insoluble cues, which facilitate metastasis. The dissemination of an isolated (and often surgically resectable) primary tumor to full-blown metastatic disease is a complex and reciprocal process, and it is currently unclear whether tumor cells or the microenvironment drives progression or they act equally. The deposition and posttranslational modification, as well as remodeling of the local extracellular matrix (ECM) has been shown to be critically important in metastasis both at primary and secondary sites. Similarly, the secretomes of cancer cells (and recruited host cells) have also been

shown to be key in controlling the recruitment and response of nonmalignant host cells within the primary tumor microenvironment generating both feed-forward and feed-back signaling loops.

The idea that a tumor can modulate not only the behavior of local cells and the resulting microenvironment at the primary site to facilitate its progression, but also at distant secondary sites prior to tumor cell arrival has gained acceptance in the last decade. It has been shown that secreted factors (secretomes) from cancer cells are capable of modulating the behavior of host cells resident in secondary organs in the absence of tumor cells. This leads to remodeling of local environments to create permissive niches (premetastatic niches) for tumor cells to subsequently colonize and form overt secondary metastases (1–3). The apparent systemic reprogramming of distant cells to generate premetastatic niches is thought to be one of the biggest contributors to the specific organotropism of certain tumors. The process of preparation has been shown to be mediated through elevated levels of circulating factors secreted from tumors, but also via tumor-secreted exosome-mediated cellular reprogramming. Both are currently under investigation to determine whether they offer potential prognostic or diagnostic markers of tumor progression and/or patient outcome. Similarly, they may also offer viable targets for therapeutic intervention to prevent or target secondary metastatic tumors.

Lysyl Oxidase and Its Role in Cancer Progression and Metastasis

Lysyl oxidase (LOX) is the prototypical member of a family of five secreted copper-dependent enzymes whose documented function is to oxidize primary amine substrates to reactive aldehydes. The best-characterized role is in the remodeling of the ECM through the oxidative deamination of peptidyl lysine residues in collagens and elastin to facilitate covalent cross-linking (4, 5). The action of LOX is important in establishing the structural integrity and stability of the ECM, thereby contributing to the tensile

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strength of many tissues, although intracellular functions of LOX and LOX family members have also been reported (6). In evolutionary terms, LOX proteins have been identified not only in animals, but also in many other eukaryotes, as well as in bacteria and archaea revealing a pre-metazoan origin for this gene family (7). The critical role of LOX in mammalian systems has been demonstrated in LOX^{-/-} mice, which die just before or soon after birth as a result of severe cardiovascular malformations, which have been attributed to defective elastogenesis (8).

LOX expression is typically induced under conditions of hypoxia as a result of the hypoxia response element (HRE) embedded within its promoter sequence. Hypoxia is a salient feature of almost all solid tumors and although evidence has been presented to identify a role for LOX in premalignant changes during breast cancer tumorigenesis (9), it is known that solid tumors are characterized by dysregulated growth (increased growth and decreased cell death), leading to significant oxygen deficiency (hypoxia). As such expression and activity of LOX in tumors is indicative of the transition from oxygen sufficiency to deficiency and has been associated with patient outcome and survival (10). To date, a functional role for LOX in cancer has been reported in breast, colorectal, prostate, gastric, and pancreatic cancer, head and neck squamous cell carcinoma (SCC), renal clear cell carcinoma, melanoma, oral and oropharyngeal SCC, as well as basal and squamous cell skin carcinoma. A comprehensive review of the role of LOX and LOXL family members in cancer can be found in refs. 6 and 11.

In our recent paper (12), we addressed the question of how the secretome of breast cancers may be responsible for conferring specific organotropism during metastasis. We retrospectively profiled a cohort of 344 lymph-node-negative primary breast tumor patients who received no adjuvant therapy (13). We noted that the hypoxic signature as defined by Chi and colleagues (14) was closely associated with poor survival and metastasis, specifically in estrogen receptor negative (ER⁻) breast cancer patients. Furthermore, we determined that this association pertained specifically to the occurrence of bone relapse and not other sites of relapse in ER⁻ patients but not ER⁺ patients. This was of particular interest because hormone receptor-positive tumors (including ER⁺) typically exhibit a higher tendency to develop bone metastasis and are associated with better survival outcomes. This is in comparison to hormone receptor-negative (including ER⁻) tumors, which show a tendency to primarily develop visceral metastases (15). Nonetheless, the data show that 17% of ER⁻ breast cancer patients show bone metastases and this accounts for approximately 7% of all bone relapse in breast cancer. These findings were subsequently validated in a second patient data set (16). To determine which hypoxia-regulated factors may be responsible in contributing to this apparent osteotropism, we profiled the hypoxic secretome from bone-tropic and parental MDA-MB-231 ER⁻ human breast cancer cells by quantitative mass spectrometry. We identified LOX as significantly associated with a hypoxic bone-tropic phenotype and further retrospective analysis of patient samples confirmed that LOX expression is associated with metastasis and bone relapse in ER⁻ patients, but not in ER⁺ patients. We subsequently demonstrated in *in vivo* models of breast cancer metastasis that high levels of LOX secreted from hypoxic tumors drives the generation of small focal premetastatic osteolytic lesions within the bones of tumor-bearing mice. The secretome-centric nature was further confirmed in tumor-free mice injected with hypoxic-conditioned media. Mechanistically,

we found that LOX activity modulates the normal bone homeostatic mechanisms, disrupting the delicate balance between osteoclasts (bone-degrading cells) and osteoblasts (bone-building cells). The presence of LOX not only decreases osteoblast proliferation, but is, more importantly, capable of driving *de novo* osteoclastogenesis *in vitro* and *in vivo*. This disruption of bone homeostasis leads to unbalanced coupling in favor of bone degradation and the formation of premetastatic osteolytic niches.

From a targeting perspective, we showed that silencing LOX expression at the primary tumor, or blocking the activity of tumor-secreted LOX with targeting antibodies was capable of abrogating the formation of focal premetastatic osteolytic lesions and reducing subsequent metastatic burden within the bones. Targeting the downstream activities of LOX, namely, the *de novo* generation of functionally active osteoclasts, through the administration of a powerful clinically approved third-generation amino-bisphosphonate, zoledronic acid, completely blocked the formation of focal premetastatic osteolytic lesions and almost completely eliminated the metastatic burden in metastasis models (Fig. 1).

Elucidating the early events associated with organotropism in solid tumor metastasis, including the key effectors of this process is an essential prerequisite for the development of effective targeted therapies. We provide a mechanistic link between the very early steps of bone metastasis of breast tumors and the expression of LOX at the primary tumor. However, further studies are required to confirm whether blocking LOX or its downstream actions may provide a target for preventive treatment for patients at a higher risk of bone metastasis, as well as a potential biomarker to identify these patients.

Therapeutic Potential and Clinical Implications for Future Therapies

When it comes to combatting cancer metastasis, target molecules tend to be organ-specific as a result of the complex interaction between multiple players operating in context-dependent roles. Rather than attempting to find global mediators associated with breast cancer metastasis, we instead focused on a subtype of breast cancer and a specific target organ of interest. LOX expression at the primary tumor offers the potential to predict the potential risk for bone-specific relapse in ER⁻ breast cancer patients as well as a potential avenue for therapeutic intervention. Along with other previously published work in the field (2, 6, 17–20) we have clearly highlighted the potential of LOX in solid tumor metastasis. Its implication in cell proliferation, invasion, and metastasis as well as in driving angiogenesis and malignant transformation has rapidly elevated it to a position as a viable target for therapeutic intervention within the solid tumor setting.

However, despite the success in many pre-clinical *in vivo* models, to date little progress has been made in the development of suitable small molecule inhibitors. The lack of a complete crystal structure for LOX currently precludes it from classical structure-driven fragment-based drug development and screening approaches and even the naturally occurring small-molecule inhibitor β -aminopropionitrile (BAPN) is nonspecific, showing affinity for multiple LOX 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 aid this endeavor. Importantly though, by elucidating the downstream mechanisms of LOX activity in facilitating breast cancer osteotropism we identify the potential to capitalize on

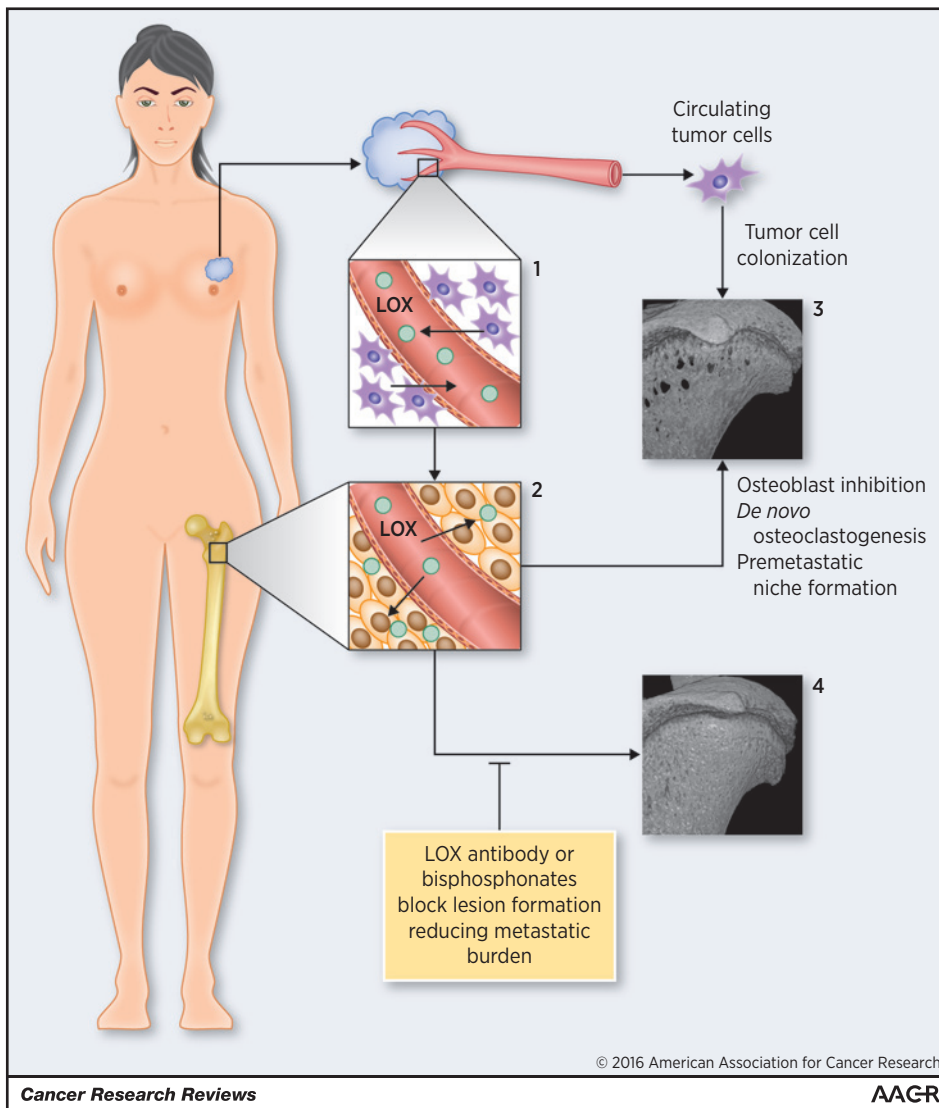


Figure 1.

The prometastatic effects of LOX secreted by hypoxic breast cancers. Tumor hypoxia drives the expression and secretion of LOX into the circulation (1), which travels to the bone (2) and acts to disrupt the osteoblast/osteoclast balance in favor of bone destruction, leading to the generation of focal premetastatic osteolytic lesions (3). These subsequently act to enhance metastatic colonization of circulating tumor cells. 4, treatment with anti-LOX antibodies or bisphosphonates abrogate premetastatic niche formation and reduce metastatic colonization.

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already approved inhibitors in a new setting. The use of bisphosphonates are currently approved for the treatment of breast cancer patients with clinically detectable bone metastases. However, we propose that LOX expression at the primary tumor may be used to identify a subset of ER⁻ breast cancer patients that will likely benefit from the preventative treatment with bisphosphonates to reduce the risk of bone relapse. Clinical trials of bisphosphonates as adjuvant therapy for breast cancer have had mixed results and highlights the need for more personalized approaches to selective administration. It would be of great interest to retrospectively evaluate primary tumor LOX expression in some of the larger randomized trials that have already been completed on bisphosphonates as adjuvant systemic therapy for breast cancer. Recently, a landmark report was published in *Lancet* from the Early Breast Cancer Trialists' Collaborative Group (21). It made the call for adoption of bisphosphonates as a standard of care for the adjuvant therapy of early-stage breast cancer in postmenopausal women where bone homeostasis is already disrupted (similar to that seen in

high LOX models). Their findings are based on the absolute reduction in the risk of breast cancer death, relatively mild and uncommon toxic effects, and the generic and relatively inexpensive availability of bisphosphonates. However, because bisphosphonate action is bone-centric, it seems unlikely that their use will prevent contralateral breast cancer, loco-regional disease, or metastases to non-osseous sites. Therefore, targeting LOX directly will likely have success over and above that of reducing metastatic bone disease, as well as offering a potential biomarker for response to bisphosphonate treatment.

Moving forward, it will be essential to continue to develop conditional and inducible knockout and knockin models of LOX expression to allow a greater degree of manipulation *in vivo*. This will allow us to rapidly increase our understanding of the multiple roles of LOX in both normal tissue homeostasis and diseases such as cancer and cancer metastasis. At present, LOX-neutralizing antibodies have proved to be the most effective, and specific approach showing good efficacy in many pre-clinical studies (2, 12, 17, 19, 20). However, current antibodies available are not

suitable for use in human patients and the translation to the clinic of targeting antibody approaches is often associated with a higher complexity of administration. That said, in the context of the systemic dissemination of high-LOX expressing primary breast cancer, the ability to readily target circulating LOX activity to prevent premetastatic niche generation at multiple sites negates the need to optimize tumor tissue penetrance.

Yet, despite the potential promise of targeting LOX in cancer, caution is needed, as evidenced by the lethality of complete LOX knockout in mouse models. *Lox*^{-/-} mice die at birth or soon after owing to impaired connective tissue formation, resulting in cardiovascular and diaphragm instability. This clearly demonstrates the importance of LOX-mediated biogenesis of fibrillar ECM during development (8, 22). It must also be noted that the nonspecific small molecule LOX inhibitor (BAPN), has previously been trialed as a topical treatment for hypertrophic fibrotic scarring and keloidal scars in humans, but despite promising efficacy these clinical trials were halted owing to non-tumorigenic toxicity. However, specific targeting of LOX for acute treatment of cancer and cancer metastasis in adults offers significant promise with minimal risk of long-term developmental defects. In support of this, we have observed no evidence of adverse developmental effects in our mouse models of cancer treated with anti-LOX therapies; however, it remains to be seen whether this would pertain to the patient setting. Nonetheless, 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 target. In addition to this, combination with site-specific delivery technologies (such as hypoxia-activated drugs) will make it possible to avoid the need for systemic inhibition approaches in treating cancer patients.

Beyond the scope of our recent study, is the question of whether the inhibition of LOX-mediated unbalanced coupling in the bone is capable of reverting the formation of already formed lesions. Currently, the focus of further on-going studies is the reversibility of premetastatic lesion generation. We believe that in the highly active microenvironment of the bone that the restoration of normal bone homeostatic mechanisms will allow the recovery of normal bone structure and removal of premetastatic sites. At present, the time scale is unknown but such a study has significant clinical implications. The ability to reverse tumor-induced changes in secondary tissues in an adjuvant setting after surgery could dramatically decrease the risk of relapse from circulating and/or already disseminated tumor cells.

In terms of targeting, an alternative approach to directly targeting the action of LOX would be indirectly through its expression and/or activity. As mentioned above, LOX possesses within its

promoter region an HRE, which was the basis of its identification in our study as a mediator of osteotropism. The clinical targeting of HREs, such as hypoxia-inducible factor, has shown relative promise in recent years (23, 24). However, it has been shown that LOX expression can be induced by cross-talk from other signaling pathways including TGF β (25–29). At present it is too early to determine whether the success of hypoxia-inducible factor inhibitors is in part attributed to depleting LOX expression. Another approach would also be to target the posttranslational activation of LOX, through targeting BMP-1, which is believed to be required to cleave the LOX propeptide after secretion releasing the mature active protein. However, due to the high expression of BMP-1 in some tissues, this may induce unwanted complications. A third, albeit indirect approach may be to target the catalytic activity of LOX by depleting its essential copper cofactor. The catalytic activity of LOX is copper-dependent with the carboxyterminus requiring the presence of a tightly bound active copper (II) ion (30). Although copper availability does not directly affect synthesis, it does markedly influence its functional activity (31, 32). Tetrathiomolybdate is a potent copper chelator and has demonstrated antiangiogenic, antifibrogenic, and anti-inflammatory actions in preclinical studies. The use of tetrathiomolybdate has been tested in several cancer settings, with limited or poor success, but recent reports from a small phase II clinical trial showed 62 of 75 advanced breast cancer patients show no detectable evidence of disease at 5 years. Although LOX has not been investigated in this setting, one would speculate that tetrathiomolybdate treatment leads to a significant downregulation in LOX activity, which may be contributing to efficacy of treatment. Currently, a larger phase III trial of tetrathiomolybdate for triple-negative breast cancer is being called for, but Wilson Therapeutics who hold the rights to tetrathiomolybdate have no immediate plans to test tetrathiomolybdate in cancer patients, nor sublicense the drug.

Altogether, our study has made major steps in increasing the current knowledge and understanding of the early stages of breast cancer metastasis to the bone and the importance of LOX mechanisms in facilitating this. Several challenges still lie ahead in studying cancer metastasis, yet we believe that in the not-too-distant future, targeting solid tumors with LOX inhibitors in the clinic will prove a powerful cancer treatment bringing hope to primary and metastatic cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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