REVIEW

Functional importance of lysyl oxidase family propeptide regions

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Abstract The lysyl oxidase family of proteins is primarily known for its critical role in catalyzing extracellular oxidative deamination of hydroxylysine and lysine residues in collagens, and lysine residues in elastin required for connective tissue structure and function. Lysyl oxidases have additional important biological functions in health and disease. While the enzyme domains are highly conserved, the propeptide regions are less uniform, and have biological activity, some of which are independent of their respective enzymes. This review summarizes what has been published regarding the functions of the propeptide regions of this family of proteins in the context of extracellular matrix biosynthesis, fibrosis and cancer biology. Although much has been learned, there is a need for greater attention to structure/function relationships and mechanisms to more fully understand these multifunctional proteins.

Keywords Lysyl oxidases · Propeptides · Extracellular matrix · Fibrosis · Cancer

Lysyl oxidases are classically understood to catalyze the final enzyme reaction required for biosynthetic cross-linking of collagens and elastin (Kagan and Trackman 1991). This family of enzymes is made up of five members: lysyl oxidase (LOX) and lysyl oxidase like-1 through 4 (LOXL1 – LOXL4). As reviewed previously by us and others, all five members have the active enzyme domain at the C-terminal

region that includes conserved copper binding residues and a conserved tyrosine and lysine residue that become the lysyl tyrosyl quinone (LTQ) cofactor required for enzyme activity (Finney et al. 2014, Moon et al. 2014, Trackman 2016a, 2016b). The propeptide regions begin at the respective predicted signal peptide cleavage sites at residue 21 for LOX, and residue 25 for LOXL1 – LOXL4, and extend to the beginning of the respective propeptide cleavage site. For LOX (Cronshaw et al. 1995) and LOXL1 (Borel et al. 2001) the propeptide cleavage sites processed by procollagen C-proteinases corresponds to residue 169 in human LOX, and likely at residue 338 in human LOXL1, while the catalytic domains in LOXL2 – LOXL4 begin around residue 535 (Trackman 2016a, 2016b). While processing is required for activation of LOX and LOXL1 enzymes (Trackman et al. 1992; Borel et al. 2001), it appears that LOXL2 – LOXL4 are active enzymes in both processed and non-processed molecular species (Rodriguez et al. 2010).

The 147 amino acid propeptide region of LOX and the 313 amino acid region of LOXL1 are each largely unique. Comparison of LOX and LOXL1 propeptide region sequences revealed a short region of 28 amino acids which are 71% identical, downstream of, but near, the signal peptide cleavage site (Fig. 1). By contrast, the propeptide regions of LOXL2 – LOXL4 of about 520 amino acids each have four conserved cysteine-rich scavenger receptor domains of approximately 100 amino acids each in length. LOXL2 – LOXL4 is considered a subgroup within the five member LOX family, while LOX and LOXL1 make up another subgroup. The current review will summarize published information regarding the biological functions of the propeptide regions of the LOX family. This is still an incomplete story, and more needs to be learned regarding the binding partners and biological roles that these domains play in biology. Evidence for the notion that propeptide regions of LOX family proteins

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et al. 2016a). Mutations in fibulin-4 result in cutis laxa accompanied by cardiovascular and bone abnormalities in humans. The E57K mutation in fibulin-4 that leads to cutis laxa in particular has reduced binding to recombinant LOX-PP (Sasaki et al. 2016a), strongly implicating impaired pro-LOX processing and activation as a contributing mechanism to the observed phenotype. Interestingly, this impaired binding is dependent upon post-translational glycosylation of LOX-PP since K57E fibulin-4 and wild type fibulin-4 were found to bind equally well to LOX-PP expressed in bacteria compared to glycosylated LOX-PP expressed in HEK293 cells (Sasaki et al. 2016a). Fibulin-4 knockout mice, and fibulin-4 knockin mice harboring equivalent mutations found in human cutis laxa, each experience poor elastin and collagen cross-linking, cardiovascular and skeletal abnormalities (Igoucheva et al. 2015; Papke et al. 2015). The fibulin-4 knockout study showed that pro-LOX processing and release of LOX-PP was unaffected in MEFs, but that LOX activity was low (Papke et al. 2015). Data suggest that mature LOX was either not optimally active in the absence of fibulin-4, or that mature LOX half-life is shorter in fibulin-4 knockout mice. Functional cooperation of fibulin-5 with LOXL1 in elastin cross-linking occurs in the context of pelvic organ prolapse (Liu et al. 2004; Liu et al. 2006; Zhao and Zhou 2012), but more detailed binding interaction networks or structure/function studies that include LOXL1 have not yet been reported to our knowledge.

Interestingly, a study focused on bone, osteoblasts and collagen structure demonstrated that newborn fibulin-4 homozygous knockout mice have abnormal collagen characterized by a low degree of mature collagen cross-links, increased collagen fibril diameter typical of LOX deficiency, and low amounts of mature LOX (Sasaki et al. 2016b). Fibulin-4 supplementation to cultures of fibulin-4 knockout calvaria osteoblasts restored mature LOX levels to normal, while LOX mRNA levels were not altered (Sasaki et al. 2016a). Data are consistent with the notion that fibulin-4 direct or indirect interactions with LOX facilitate proteolytic processing of pro-LOX, and with studies summarized above in the elastin field. A limitation of the bone study is that 50 kDa pro-LOX processing was not directly observed in the presence or absence of fibulin-4 possibly due to issues related to antibody specificity. Fibulin-4, in addition was shown to bind to fibrillin-1, a major component of microfibrils (El-Hallous et al. 2007), and fibronectin (Trask et al. 2000) which adds another layer to extracellular matrix complexes that must work together in a coordinated fashion to build functional extracellular matrices.

Fibronectin is a scaffolding protein and contains binding sites for a variety of cell surface and extracellular matrix proteins, and participates in extracellular matrix biosynthesis and remodeling. Mature and propeptide regions of pro-LOX expressed in *E. coli* as GST-fusion proteins were assayed for interaction with cellular fibronectin. The mature region of

LOX bound to fibronectin while the propeptide region did not (Fogelgren et al. 2005). Note that the mature region of LOX expressed in *E. coli* is typically enzymatically inactive. However, immunofluorescent staining for LOX and fibronectin supported that they co-localize in human cells and tissues, and the authors further reported that fibronectin was not a substrate for active purified LOX enzyme isolated from bovine aorta. These findings suggest that active LOX binds to fibronectin, but does not oxidize fibronectin. Finally, forms of LOX found in conditioned medium from fibronectin knock-out MEFs were compared to LOX produced by heterozygous fibronectin MEFs. Fibronectin null cells produced only pro-LOX, while heterozygous cells produced primarily mature LOX and detectable levels of pro-LOX (Fogelgren et al. 2005). Thus, taken together data support that fibronectin binds to pro-LOX via the mature LOX enzyme domain to facilitate biosynthetic processing of pro-LOX.

Studies summarized above suggest that different domains of LOX bind to different extracellular matrix proteins: fibronectin (mature enzyme domain) and elastin (propeptide domain) (Fogelgren et al. 2005; Thomassin et al. 2005). Such interactions facilitate proenzyme processing. Interestingly, BMP-1, which processes pro-LOX and several additional extracellular matrix precursor proteins, also binds to fibronectin. Pull down studies showed that the non-protease domains of BMP-1 mediate binding to fibronectin, while BMP-1 bound fibronectin at two or more sites. Binding of BMP-1 to fibronectin increased its processing of type 1 procollagen, perlecan and chordin (Huang et al. 2009). Pro-LOX processing was not studied in this series of experiments, but is presumed to be enhanced by fibronectin, consistent with (Fogelgren et al. 2005).

Periostin is a matricellular fibronectin-binding extracellular matrix protein which contains four central FAS1 domains flanked by an N-terminal EMI domain and a C-terminal domain. Co-immunoprecipitation studies revealed that the active enzyme domain of BMP-1 binds to the central FAS1 domains of periostin. Transfection of C3H10T1/2 cells with periostin promoted fibronectin and BMP-1 deposition, and collagen cross-linking measured by ELISA (Maruhashi et al. 2010). The overall concept emerges that periostin increases BMP-1 binding to fibronectin leading to optimal fibronectin-bound pro-LOX processing and activation. The finding that periostin knockout mice exhibit abnormal collagen structure in connective tissues is consistent with this model (Norris et al. 2007; Shimazaki et al. 2008).

A scheme is provided in Fig. 2 which summarizes some of the extracellular interactions of LOX and LOXL1 that play roles in extracellular enzyme targeting and activation. Higher resolution of coordinated temporal and molecular interactions in relevant tissues between pro-LOX, BMP-1, fibronectin and periostin, collagens, fibulins, microfibrillar proteins and elastin remain to be understood. It is clear that

Fig. 2 Scheme showing interactions between lysyl oxidase (a) or lysyl oxidase like-1 (b) with other extracellular matrix proteins summarized in the text. Blue arrows indicate demonstrated binding- or functional interactions. The scissors in (b) indicate that it is likely that more than one BMP-1 cleavage of LOXL1 could occur, and that the precise BMP-1 processing sites are not fully defined for LOXL1 at this time.

activation of LOX and probably LOXL1 in vivo consists of multiple coordinated interactions of these and possibly other proteins, some of which involve binding with the respective propeptide regions of pro-LOX and pro-LOXL1.

LOX-PP and tumor suppression The nearly simultaneous publication of the first cDNA sequence of rat LOX and the discovery of a novel mouse *ras* *recision* gene that turned out be *Lox* (Contente et al. 1990; Kenyon et al. 1991) was consistent with earlier studies that showed relatively low LOX enzyme activity from several cancer cell lines compared to corresponding non-tumorigenic cell lines (Kuivaniemi et al. 1986). The assumption at this point was that lysyl oxidase enzyme activity acts a tumor suppressor. However, inhibition of LOX activity with the classic inhibitor β -aminopropionitrile unexpectedly failed to re-transform phenotypic revertant clones of the *ras*-transformed fibroblasts (Palamakumbura et al. 2004). By contrast, gene knockdown experiments caused the phenotypic revertants to become tumorigenic (Contente et al. 1990). These findings suggested that the tumor suppressor property of LOX is independent of its enzyme activity, but dependent on its expression. Indeed LOX-PP, and not active LOX enzyme, inhibits hallmarks of the transformed phenotype of *ras*-transformed fibroblasts including *ras*-dependent signaling and growth in soft agar, and xenograft growth in mice (Palamakumbura et al. 2004).

Mutation or under-expression of tumor suppressors promote cancer development. An early indication that LOX-PP is a tumor suppressor in humans first came from a study in gastric cancer in which a polymorphism in the LOX gene causing a single amino acid change in the LOX-PP sequence (rs1800449, Arg158Gln) appeared to be associated with a high incidence of cancer (Kaneda et al. 2004). A subsequent epidemiology study that directly assessed for an effect of the

rs1800449, Arg158Gln LOX-PP polymorphism supported that LOX-PP is a tumor suppressor in an African American population of triple-negative human breast cancer patients (Min et al. 2009). Additional data demonstrated the loss of activity of the polymorphic LOX-PP variant in assays of murine NF639 breast cancer cells of cell proliferation, expression of EMT markers, Matrigel outgrowth, and xenograft growth in nude mice (Min et al. 2009). Independent epidemiology studies in humans have further corroborated the relationship between the rs1800449 LOX-PP polymorphism and poor outcomes in a variety of cancers, two of which revealed high expression of mature LOX enzyme protein correlated with a poor outcome (Ren et al. 2011; Yoon et al. 2011; Liu et al. 2012; Shi et al. 2012; Wang et al. 2012; Wu et al. 2012; Bu et al. 2014; Friesenhengst et al. 2014; Han et al. 2014; Gao et al. 2015; Wang et al. 2016; Fraga et al. 2017). In vitro data, however, indicate that the polymorphism does not alter secretion or processing of pro-LOX (Min et al. 2009). As far as is known now, only LOX-PP which is largely unique in sequence compared to other LOX family propeptide regions, is a tumor growth inhibitor/tumor suppressor.

To our knowledge, no direct evidence exists that propeptides from the other four isoforms are tumor suppressors. However, a publication by Sebban et al. identified alternatively spliced variants of LOXL4 that disrupt the sequence of one of four cysteine rich scavenger receptor (SRCR) domains in its propeptide region. These variants increased the invasive phenotype of ovarian cancer cells (Sebban et al. 2009; Sebban et al. 2013). Overexpression of the splice variants in tumor cells increased the invasive phenotype measured by Matrigel invasion assays, MMP2 expression and activity assays, cell morphology, cell attachment assays, and measurement of metastases in an ovarian orthotopic cancer model in mice. Interestingly, full length non-alternatively spliced

LOXL4 did not increase the invasive phenotype of cancer cell lines, but rather appeared to inhibit modestly some assays. Cells transfected with alternatively spliced forms of LOXL4 did not appear to produce the predicted protein, suggesting that the effects of these splice variants may occur at the RNA level. The authors concluded that the alternatively spliced LOXL4 RNA in some way stimulates the invasive character of tumor cells, while full length LOXL4 may have tumor suppressor function (Sebban et al. 2013). Another study of LOXL4 in bladder cancer identified mutations in exon 8 that encodes one of the SRCR domains in two of 8 bladder cancer cell lines, and in 7 out of 30 bladder cancer tumors, while surrounding normal tissue had no LOXL4 mutations. The possibility was raised that LOXL4 mutations in SRCR domains (which are located in the propeptide region of LOXL4) could compromise a tumor suppressor role of LOXL4 (Wu et al. 2007). The same study also showed that LOXL4 is silenced by promoter methylation in a variety of bladder cancer cell lines, and that pharmacologic demethylation with 5-aza-2'-deoxycytidine stimulates LOXL4 expression and inhibition of ERK activation, again suggesting tumor inhibitory properties of full length LOXL4 (Wu et al. 2007). Understanding the functional mechanism of the spliced out or mutated SRCR sequences in LOXL4 to suppress tumor development could be of considerable significance.

LOXL2 has been implicated as a tumor or metastasis promoting protein in a variety of cancers, with most studies showing dependence of tumor promotion dependent on enzyme activity (Wong et al. 2014; Ezzoukhry et al. 2016; Le Calve et al. 2016; Chang et al. 2017; Peng et al. 2017). A few studies have shown biological activity of LOXL2 independent of enzyme activity, raising the possibility that the proregions of LOXL2 could have function. For example, LOXL2 mutated to cause disruption of the active site of the enzyme still promoted EMT in a model of breast cancer (Cuevas et al. 2014; Cuevas et al. 2017) and still inhibited keratinocyte differentiation (Lugassy et al. 2012). Another study has shown that

enzymatically inactive LOXL2 may collaborate with active LOXL2 in the formation and subsequent stabilization of capillaries in the process of angiogenesis (Bignon et al. 2011). Structure/function studies to clarify the mechanisms of these activities have not yet been published to our knowledge. Interestingly, Lopez-Jimenez et al. (Lopez-Jimenez et al. 2017) have recently reported that full length LOXL2 is active against low molecular weight non-peptidyl substrate 1,5-diaminopentane, but is not active against type IV collagen. Activity against type IV collagen required proteolytic processing that removes the two N-terminal SRCR domains in the propeptide region. Moreover, LOXL2 enzyme molecules appear to become covalently bound to its type IV collagen substrate, dependent on active enzyme. Thus, the first two SRCR domains function to effectively inhibit productive LOXL2 oxidation of type IV collagen, but not 1,5-diaminopentane. Data imply that the proregion of LOXL2 serves to provide for interactions with type IV collagen and a processing protease in a protein complex required for subsequent proteolytic processing and collagen IV oxidation by properly located LOXL2 enzyme (Lopez-Jimenez et al. 2017).

LOX-PP mechanism Several molecular targets of LOX-PP mediate its inhibitory effects on tumor promoting signaling pathways and have been identified in breast cancer and prostate cancer cell lines, summarized in Fig. 3. Targets in prostate cancer cell lines include attenuating FGF-2 signaling mediated by FGFR1 and AKT activation (Palamakumbura et al. 2009; Vora et al. 2010b), and inhibition of DNA repair pathways mediated by binding to MRE11-containing DNA repair foci after nuclear localization (Bais et al. 2015). In breast cancer cells, targets are fibronectin-stimulated C130 CAS, FAK signaling and ERK1/2 activation in haptotaxis assays (Zhao et al. 2009), inhibition of β -catenin by targeting the receptor-type protein tyrosine phosphatase kappa (Sanchez-Morgan et al. 2011), inhibition of Ras-signaling by direct targeting of Hsp70 and Raf leading to reduced Erk1/2 activation (Sato et al. 2011), inhibition of CIN-85-mediated invasion (Sato

Fig. 3 Summary of known interactions and targets of LOX-PP, and physiologic substrates for LOX enzyme covered in the text. Note that intracellular pro-LOX and LOX have been described, and enzymatically inactive intracellular pro-LOX in principle could have similar functions as intracellular LOX-PP. Intracellular substrates for active LOX in a physiologic context have not been identified, to our knowledge

et al. 2013), and inhibition of proliferation and stimulation of apoptosis in vivo (Bais et al. 2012). Study of the phenotype of a spontaneous mutation in the mouse *NNA1* transcription factor gene revealed ataxia caused by overexpression of LOX in Purkinje cells in the brain that was linked to LOX-PP and not LOX enzyme (Li et al. 2010). Elevated LOX expression resulted in reduced Purkinje cell growth. LOX-PP was shown to inhibit the RelA subunit of NF- κ B from entering the nucleus, resulting in deficient mRNA and protein levels of MAP1B and MAP2 which are microtubule binding proteins required for Purkinje cell growth. Thus, in normal mice, normal *NNA1* represses production of LOX. If LOX is over-expressed, LOX-PP then inhibits RelA nuclear localization leading to deficient MAP1B and MAP2 expression, deficient Purkinje cell production, contributing to ataxia. As noted above, LOX-PP targets many aspects of RAS signaling (Sato et al. 2011), and RAS can drive NF- κ B activation (Jeay et al. 2003). By contrast, LOX enzyme oxidizes collagens and elastin and PDGFR all of which, when in excess, have been suggested to contribute to aspects of tumor growth or metastasis (Erler et al. 2006; Eliades et al. 2011).

Structure/function studies of LOX-PP performed so far have identified residues 26–100 to contain the RAF and HSP70 interaction residues (Sato et al. 2011), and residues 111–116 as binding sites for competitive inhibition of CIN85 interaction with c-CBL and the invasive character of breast cancer cell lines (Sato et al. 2013). The CIN85 protein is an adaptor protein upregulated in breast cancer and which is important for ubiquitination activity of a complex that degrades the extracellular matrix and facilitates tumor cell migration (Sato et al. 2013). Interestingly, the RAF and CIN85 binding sites are independent of each other since mutation of the CIN85 binding site of LOX-PP has no effect on RAF-LOX-PP binding (Sato et al. 2013). RAF is a well-established binding partner and effector of RAS function in the context of cancer [see (Samatar and Poulikakos 2014) for a review], and as noted LOX-PP inhibits a variety of RAS-dependent signaling pathways (Trackman 2016a, 2016b). Although there is now ample evidence that the rs1800449 polymorphism attenuates the ability of LOX-PP to suppress tumor development, no binding partner for the specific amino acid region around LOX-PP residue 158 of human LOX-PP has been identified. This suggests that LOX-PP has additional undiscovered functionally relevant targets that mediate its tumor suppressor activity.

LOX-PP is generated extracellularly by proteolytic processing by procollagen C-proteinases (Trackman et al. 1992; Uzel et al. 2001). Molecular binding partners and targets of LOX-PP identified so far in the context of cancer are intracellular, except possibly cell surface FGFR1. Therefore, the pathways of LOX-PP cell uptake were investigated in a variety of cell lines to assess for possible avenues to enhance uptake and therefore potentially block cancer cell growth and metastatic

potential. Data demonstrated that macropinocytosis is employed by most cell lines, while clathrin-dependent pathways serve as secondary uptake pathways in some cell lines (Ozdener et al. 2016). Ongoing work is focusing on modifying the structure of LOX-PP to enhance its uptake by cancer cells and increase its effectiveness, and to establish which targets are the most important in mediating the tumor inhibitor properties of LOX-PP.

The findings that pro-LOX is enzymatically inactive (Trackman et al. 1992), while LOX-PP has tumor inhibitory and tumor suppressor properties (Palamakumbura et al. 2004), and the active enzyme can stimulate metastasis (Erler et al. 2006), raise the question whether proteolytic processing could be a regulatory aspect of tumor suppression vs tumor promoting functions of LOX gene products. For example, if the LOX-PP domain is active as a tumor growth inhibitor while attached to the still inactive LOX enzyme domain, then in principle the efficiency of pro-LOX processing could regulate the balance between tumor suppression and promotion of metastasis. It is of interest that pro-LOX has been found in the nucleus of MC3T3-E1 osteoblasts (Guo et al. 2007), suggesting that pro-LOX, like LOX-PP (Ozdener et al. 2016) could be taken up by some cells and target intracellular tumor promoting signaling proteins.

It has become clear that propeptides of all LOX isoforms are likely to participate in extracellular protein-protein interactions that facilitate correct localization and functions of these enzymes. In the case of LOX and LOXL1 that require proteolytic processing for activation, evidence for extracellular hierarchies and regulatory control mechanisms to control enzyme activation and collagen and elastin crosslinking are apparent. In spite of this impressive progress, the binding partners and functions of propeptide regions of LOXL2 – LOXL4 are just beginning to emerge. The potential biological roles played by alternative splicing of LOX isoforms is intriguing, and still requires creative analyses. Finally, the mechanism by which the rs1800449 LOX-PP polymorphism causes loss of tumor suppressor function will be of great interest, and has potential in the context of development of novel therapeutic approaches.

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