

Ph.D. Thesis

The interaction of lysyl oxidase with the hormone placental lactogen and their effect on mammary epithelial cell proliferation and migration

Noémi Polgár

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

Supervisors and consultants:

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

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

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Introduction

Breast cancer is the most common cancer among women, as it is diagnosed in 1×10^6 women in the world each year. The incidence of breast tumor development in women differs with age, mammary gland mass and exposure to endogenous and exogenous hormones. However, the function, molecular regulation, and interaction of genetic alterations in carcinogenesis, as well as those of tumor cell migration and invasion, and metastasis of breast carcinomas are not fully understood. As metastasis is a major challenge in cancer treatment, determination of molecular markers of metastatic potential and therefore understanding the process of metastatic tumor progression are important. Recently, comprehensive transcriptome profiling of normal breast, noninvasive breast tumor and invasive breast cancer cell lines identified lysyl oxidase (LOX) as a possible marker gene based on its differential expression. In addition, upregulated LOX expression and activity have been reported in breast cancer tissues and invasive/metastatic breast cancer cell lines as well. Lysyl oxidase has been primarily recognized as a matrix-crosslinking enzyme; hence regulators and interacting factors contributing to its novel role in breast cancer development and progression are yet to be discovered. Results of our previous yeast two-hybrid screens demonstrated the interaction of LOX with the pregnancy-specific hormone, placental lactogen (PL). PL is a member of the somatotropin/prolactin hormone family, expressed solely in the placental syncytiotrophoblasts under physiological conditions, that has a role in lactogenesis and the growth and development of the fetus. Similarly to growth hormone and prolactin, expression of PL has been associated with tumors of mammary origin. However, its role in tumorigenesis as well as the role of the LOX-PL interaction in such processes, remains to be elucidated.

Objectives

The goal of this work was to verify the novel LOX-PL interaction that likely promotes LOX's function during tumorigenesis, and to characterize this interaction and evaluate its biological significance. The following aims were proposed:

Specific Aim 1.

Determine the specificity of the interaction between LOX and PL represented by multiple clones in yeast two-hybrid screen using yeast direct studies.

Test the specificity of the interaction of the library proteins with the LOX bait proteins in yeast following the identification of LOX-interacting partners in yeast two-hybrid

screening by directly co-transforming various combinations of bait and target plasmids into yeast and assaying for reporter gene activation.

Specific Aim 2.

Confirm and characterize the LOX-PL interaction *in vitro*.

- a) Generate expression constructs for LOX and LOX fragments.
- b) Express and purify recombinant LOX proteins in quantities sufficient for further *in vitro* biochemical experiments.
- c) Perform *in vitro* binding analysis using pull-down and Far-Western assays.
- d) Perform solid phase binding assays to measure the equilibrium dissociation constant (K_d) of the protein interaction, and determine LOX-binding affinity for hGH and hPRL, two related members of the hormone family.

Specific Aim 3.

Investigate LOX-PL interactions *in vivo*.

- a) Test LOX and PL expression in breast cancer tissues by immunofluorescent staining and confirm their co-expression in mammary neoplasms that is also supported by data from the literature.
- b) Identify an appropriate *in vivo* model to test the native LOX-PL complex using co-immunoprecipitation.

Specific Aim 4.

Explore the effects of the identified LOX-PL protein interactions on the catalytic activity of LOX.

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

Specific Aim 5.

Investigate the biological significance of the identified protein interactions.

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

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

Materials and Methods

Over the course of this PhD study, I was validating and characterizing the protein interaction of lysyl oxidase and placental lactogen, identified in previous yeast two-hybrid screens. For the biochemical analysis, recombinant GST-LOX deletion constructs were cloned, expressed and purified from *E. coli* bacteria. Yeast direct interaction-studies, solid phase binding assays, pull-down assays verified the LOX-PL interaction *in vitro*, and the binding affinity was determined. *In vitro* enzyme activity assays were used to test the effect of PL-binding on the enzymatic activity of LOX. Expression of the proteins in breast tumor tissue sections was evaluated by co-immunostaining and confocal microscopy. Western blot analysis was used to evaluate expression of the proteins in breast cancer cell lines, and the interaction was tested *in vivo* by co-immunoprecipitation trials. The individual and combined effects of LOX and PL overexpression on cell behavior was assessed in immortalized normal mammary epithelial cells transduced by a lentiviral system to stably express LOX and/or PL. Changes in cell proliferation was studied in cell proliferation assays. Alterations of cell motility was assessed in these transduced cell lines first by staining the actin cytoskeleton to reveal its possible rearrangement, then by performing cell migration assays.

Results

In yeast direct interaction trials, pull-down and Far-Western assays we found that LOX indeed binds PL. Moreover, we discovered that in addition to LOX, another member of the enzyme family, LOXL2 also binds PL.

Recombinant, GST-tagged LOX deletion constructs were successfully cloned and expressed in *E. coli*. Following initial, partly successful attempts to purify these GST-LOX proteins in soluble form, we were able to purify them from the inclusion body fraction in quantities sufficient for further *in vitro* biochemical analysis.

In solid phase binding assays we were able to narrow down the LOX region (amino acids 169-348) that participates in PL-binding most likely in concert with LOX's CRL domain.

We demonstrated that LOX does not only bind PL, but has an affinity to two homologous members of the hormone family, growth hormone and prolactin as well, although LOX's binding affinity is approximately two orders of magnitude lower to these hormones than to its natural substrates.

In enzyme activity assays we determined that PL is not a substrate of LOX, as the enzyme did not show any activity toward the hormone.

Since LOX retains its enzymatic activity towards cadaverine as substrate in the presence of PL, we revealed that PL has neither an inhibitory, nor a stimulatory effect on LOX activity.

Co-expression LOX and PL was tested by co-immunostaining on a breast cancer tissue macroarray, as both LOX and PL expression in breast tumors have been previously reported. Upon demonstrating the co-expression of the proteins in tumor samples of mammary origin, we investigated their expression in breast cancer cell lines by Western blotting, and showed that the highly invasive and metastatic breast cancer cell lines Hs578T and MDA-MB231 did not only express LOX at elevated levels, but PL as well.

In co-immunoprecipitation studies using the PL and LOX co-expressing Hs578T breast cancer cells, we confirmed that the LOX-PL interaction can also occur *in vivo*.

In order to investigate the individual and combined effects of LOX and PL overexpression in mammary epithelium, we transduced immortalized normal mammary epithelial MCF-10A cells to stably express either or both of the proteins. As PL but not LOX has been demonstrated to induce cell proliferation, we tested cell proliferation of the stably transduced cell lines. We have found that overexpression of PL resulted in significantly elevated proliferation rates, and its co-expression with LOX further increased cell proliferation. Overexpression of LOX alone did not result in such effect.

Another cellular process we studied was cell migration, as overexpression of LOX reportedly leads to a more migratory phenotype in poorly invasive, nonmetastatic breast cancer cell lines. First, we stained the actin cytoskeleton using phalloidin to evaluate the possible actin rearrangement that would suggest changes towards a more motile phenotype. Interestingly, we observed the rearrangement of the actin cytoskeleton and the formation of numerous, long filopodia in all stably transduced cell lines. Therefore we decided to test the migratory abilities of these cell lines in cell migration assays. Our results reveal, that overexpression of LOX alone is not sufficient to induce a more migratory phenotype in normal breast epithelial cells, unlike in poorly invasive breast cancer cells. Similarly,

overexpression of PL alone does not induce changes in migratory behavior. The cell lines co-expressing both LOX and PL, however showed a significant increase in their cell motility.

Conclusions

In this study the interaction of the extracellular matrix enzyme, lysyl oxidase with the hormone placental lactogen was confirmed and characterized in *in vitro* assays. In addition to LOX, we demonstrated that another member of the enzyme family, LOXL2 is able to bind PL as well. Interestingly, these enzymes binding PL, LOX and LOXL2 have both been implicated in tumorigenic processes. Our results did not only support LOX binding to PL but also suggested binding to GH and PRL, two other members of the somatotropin/prolactin hormone family that are reportedly potent oncogenes as well.

In addition, *in vitro* amine oxidase activity assays showed that PL is neither a substrate nor an inhibitor of LOX. Therefore the exact mechanism and fashion of their interaction remains to be elucidated.

Using fluorescence-labeled immunostaining on a tissue macroarray, we detected LOX and PL expression in and around breast tumor cells. Subsequently, we tested protein expression of breast cancer cell lines, and found elevated PL expression in highly invasive cell lines, where LOX expression was also increased. Furthermore, we showed PL expression in poorly invasive cells at elevated and low levels, respectively. Since the highly invasive and metastatic breast cancer cell lines express both PL and LOX, we decided to study their individual and combined effects on cell behavior by overexpressing and co-expressing these proteins in immortalized normal breast epithelial cells. LOX plays a role in promoting cell migration, while PL was shown to induce cell proliferation, thus we tested these processes. Stably transduced MCF-10A normal mammary epithelial cells co-expressing PL and LOX had significantly increased proliferation rates compared to the parental and the PL-expressing cells, while LOX alone had no effect on proliferation. Therefore, the co-expression of LOX with PL appears to enhance the proliferation-inducing effect of PL. Co-expressing cells in addition showed a significantly higher migratory rate compared to cells expressing either or none of these proteins. Our results demonstrated that LOX, in addition to promoting tumor cell invasion through a H₂O₂-induced FAK/Src activation that we have described earlier, may further induce tumor cell migration in interaction with PL by activating independent signaling.

Publications of the author

Articles published

Ben Fogelgren, Noémi Polgár, Kornélia Molnárné Szauter, Zsuzsanna Újfaludi, Rozália Laczkó, Keith S. K. Fong, and Katalin Csiszar Cellular Fibronectin Binds to Lysyl Oxidase with High Affinity and Is Critical for Its Proteolytic Activation; *J. Biol. Chem.*, Jul 2005; 280: 24690 – 24697

Noemi Polgar, Ben Fogelgren, J. Michael Shipley, and Katalin Csiszar Lysyl Oxidase Interacts with Hormone Placental Lactogen and Synergistically Promotes Breast Epithelial Cell Proliferation and Migration; *J. Biol. Chem.*, Feb 2007; 282: 3262 - 32

Posters and presentations connected to the present research:

2005 96th Annual Meeting of the American Association for Cancer Research – poster

Ben Fogelgren, Noemi Polgar, Sheri F.T. Fong, Kornelia Molnarne Szauter, Zsuzsanna Ujfaludi, Keith S.K. Fong, Katalin Csiszar Cellular fibronectin binds to lysyl oxidase with high affinity and is critical for its proteolytic activation, *Proc Amer Assoc Cancer Res* 2005;46:[3765]

2005 96th Annual Meeting of the American Association for Cancer Research – poster

Sheri F. T. Fong, Ben Fogelgren, Kornelia Molnarne Szauter, Christopher Moon, Peter Hollosi, Noemi Polgar, Valerie Weaver, Dawn Kirschmann, Katalin Csiszar Lysyl oxidase (LOX) is expressed in the microenvironment of breast tumor cells and promotes epithelial plasticity, invasive properties and metastasis *Proc Amer Assoc Cancer Res* 2005;46:[3785]

2006 97th Annual Meeting of the American Association for Cancer Research – poster

Noemi Polgar, Ben Fogelgren, Katalin Csiszar The interaction of lysyl oxidase with the placental lactogen hormone and its potential role in breast cancer, *Proc Amer Assoc Cancer Res* 2006; 47:[3427]

N. Polgar, B. Fogelgren, J.M. Shipley and K. Csiszar

A lysyl oxidase interaction promotes epithelial cell migration • abstract for poster *Matrix Biology*, Volume 25, Supplement 1, November 2006, Page S92

Additional posters and presentations:

2003 Annual Meeting of the Hungarian Cardiologists' Association – presentations

Blazsó P, Sepp R, Polgár N, Rácz P, Pálincás A, Mogensen J, McKenna WJ, Csanády M, Forster T. Mutation analysis of the troponin I gene in hypertrophic cardiomyopathy
Cardiologica Hungarica 2003; 33: A73.

Polgár N, Sepp R, Rácz P, Blazsó P, Dongó Á, Jebelovszki É, Pálincás A, Csanády M, Forster T. Mutation analysis of the beta myosin heavy chain gene in dilated cardiomyopathy. *Cardiologica Hungarica* 2003; 33: A74

Rácz P, Sepp R, Blazsó P, Polgár N, Pálincás A, Mogensen J, McKenna WJ, Csanády M, Forster T. Mutation analysis of the troponin T gene in Hungarian hypertrophic cardiomyopathy patients. *Cardiologica Hungarica* 2003; 33: A72.