ORIGINAL RESEARCH REPORT

Depletion of Lysyl Oxidase-Like 4 (LOXL4) Attenuates Colony Formation *in vitro* and Collagen Deposition *in vivo* Breast Cancer Model

Ni Luh Gede Yoni Komalasari[1,2*], I Gde Haryo Ganesha[1], I Gusti Nyoman Sri Wiryawan[1], Nahoko Tomonobu[2], Masakiyo Sakaguchi[2]

[1]Department of Histology, Faculty of Medicine, Udayana University, Bali, Indonesia.
[2]Department of Cell Biology, Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama University, Okayama, Japan.

Article Info

Article history:
Received 29-01-2024
Revised 17-04-2024
Accepted 08-05-2024
Published 01-07-2024

Keywords:
Good health
Lysyl oxidase
Extracellular matrix

*__Corresponding author__:
Ni Luh Gede Yoni Komalasari
yonikomalasari@unud.ac.id

ABSTRACT

Background: Lysyl oxidase (LOX) family proteins have recently become a topic in cancer progression. Our recent study found a high LOX-like 4 (LOXL4) expression in MDA-MB-231 cells. **Objective**: To reveal the impact of depleted LOXL4 in both *in vitro* and *in vivo* breast cancer models from a histological perspective. **Material and Method**: Endogenous LOXL4 was depleted using the CRISPR/Cas9 on MDA-MB-231 parental cells. Based on the LOXL4 protein expression, the clone was determined for the next experiment, thus generating MDA-MB-231 LOXL4 KO. Cell assay was conducted using colony formation assay (n=3) followed by crystal violet staining. The indicated cells were inoculated orthotopically to female BALB/c nude mice (n=5). At the end of the experiment, tumors were isolated, fixed, and prepared for Masson's Trichrome staining. **Result**: CRISPR/Cas9 completely depleted LOXL4 expression on clone number #2-22. Depletion of LOXL4 reduced the colony size formed by MDA-MB-231 cells. MDA-MB-231 LOXL4 KO #2-22 derived tumors showed depressed tumor volume compared to the parental group. Reduced collagen was also observed from the Masson's Trichrome staining ($p<0.001$). **Conclusion:** Depletion of LOXL4 downregulates the growth of MDA-MB-231 cells *in vitro* and collagen deposition *in vivo*.

How to cite:
Komalasari, N.L.G.Y., Ganesha, I.G.H, Wiryawan, I.G.N.S, et al. 2024. Depletion of Lysyl Oxidase-Like 4 (LOXL4) Attenuates Colony Formation *in vitro* and Collagen Deposition *in vivo* Breast Cancer Model. Majalah Biomorfologi-Biomorphology Journal, 34(2): 67-73.

Majalah Biomorfologi (Biomorphology Journal) p.ISSN:0215-8833, e.ISSN: 2716-0920
doi: 10.20473/mbiom.v34i2.2024.67-73

Highlights

1. Revealing LOX family members' mechanisms in promoting invasive breast cancer progression is essential for targeting specific molecules in invasive breast cancer.
2. Depletion of LOXL4 in invasive breast cancer shows attenuation of cell invasiveness *in vitro* and collagen deposition in tumor models *in vivo*.

BACKGROUND

Breast cancer is still the most prevalent cancer, with increasing rates along with age. According to the World Health Organization (WHO) data, 2.3 million women were diagnosed with breast cancer, resulting in 685.000 deaths (World Health Organization, 2024). In Asia, breast cancer is the most common cancer in women across most of the countries with high mortality-to-incidence (M/I) of 0.28 compared to the other parts of the world (Huang, et al., 2022; Lim, et al., 2022; Rajappa, et al., 2023). Breast cancer is still the leading cause of mortality related to cancer in the Southeast Asia region and the highest disability-adjusted life years (DALYs) among females (10.9 million). Cancer incidence increases along with life expectancy (Sharma, et al., 2024). Therefore, early detection and target therapy are still essential to be investigated to ease the burden of breast cancer.

In invasive cancer that develops from a solid tumor, breast cancer relies on microenvironment changes, including the extracellular matrix, blood vessels, and immune system (Brassart-Pasco, et al., 2020; Winkler, et al., 2020). Thus, searching for genes related to the extracellular matrix (ECM) for target therapy in breast cancer, we observed intriguing genes as members of the lysyl oxidase (LOX) family (Komalasari, et al., 2023). The lysyl oxidase (LOX) family comprises five members, including LOX, LOX-Like 1 (LOXL1), LOXL2, LOXL3, and LOXL4, which, in general, has been known to contribute to the maturation of collagen and elastin in the ECM (Grau-Bové, et al., 2015; Vallet & Ricard-Blum, 2019). To conduct the function, they depend on the presence of copper (Oldfield, et al., 2018). In recent studies, some LOX family members were observed also to have functions in cancer progression other than the ECM function. LOX is related to hypoxia and tumor invasion in bone (Reynaud, et al., 2017). LOXL2 was observed to induce tumor progression in esophageal squamous cell carcinoma (ESCC) and renal cell carcinoma (RCC) (Hong &Yu, 2019; Liu, et al., 2023). LOXL3 and LOXL4 were reported to promote the growth and invasiveness of melanoma, gastric cancer, and hepatocellular carcinoma (HCC) (Li, et al., 2019; Koorman, et al., 2022). Many studies have presented LOX family members in various cancers, but the mechanisms are still puzzling (Choi, et al., 2017; Shao, et al., 2019). There are some extracellular and intracellular mechanisms in which LOX family members promote tumor progression.

In our previous investigation, high expression of LOX-Like 4 (LOXL4) was found particularly in triple-negative breast cancer (TNBC) cells (Komalasari, et al., 2023). Additionally, low levels of LOXL1 and LOXL4 were observed in less aggressive MDA-MB-231 that were afflicted by ZEB1 mutation (Hirabayashi, et al., 2023). Some studies highlighted the importance of the LOX family members in various cancers. The inhibitions of LOX family members stalled the cancer progression (Chen, et al., 2020). However, which LOX family members are important, and the molecular mechanisms, especially in breast cancer progression, remain to be elucidated. Discovery of the specific molecule and the mechanisms related to breast cancer progression leads to a potential for target therapy, particularly for invasive breast cancer.

OBJECTIVE

In this study, we were interested in distinguishing the LOXL4 function through the depletion of LOXL4 in the TNBC cell line. First, to observe the impact of LOXL4 depletion on the cell function *in vitro*, and second, to use animal models to observe the effect of LOXL4 depletion from a histological perspective. It is essential to identify specific LOX family members that support solid tumor progression by establishing potential beneficial treatments to maintain the well-being of cancer patients.

MATERIAL AND METHOD

Cell lines

MDA-MB-231 cells (ATCC, Rockville, MD, USA), the TNBC cell line, were mainly used in this study. A subline derived from the MDA-MB-231 parental cell was generated using CRISPR/Cas9 methods as described before (Komalasari, et al., 2023). The clone number 22 from gRNA two was then selected based on the protein expression, which was further named MDA-MB-231 LOXL4 knockout (KO) #2-

22 for further experiments. Cells were maintained in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), cultivated mainly in a 10 cm dish, and incubated at 37ºC with 5% CO_2.

In vitro cell analysis

MDA-MB-231 parental and subline LOXL4 KO #2-22 were analyzed using colony formation assay (CFA) to observe the potential of cells to build a colony from a single cell without nudging the neighboring cells. 1 x 10^2 cells were planted in a 60 mm dish. Three dishes were prepared for each parental and LOXL4 KO #2-22 group (n=3) and then incubated for seven days. After colonies formed, 70% EtOH was used for fixation, followed by Crystal Violet staining. One colony was defined as more than or equal to 20 cancer cells (Hirabayashi, et al., 2023).

In vivo animal model and histology specimen

Ten female BALB/c nude mice were prepared and randomly separated into two groups, five mice per group. The mice in the first group were inoculated with $5x10^5$ cells of MDA-MB-231 parental into the right mammary fat pad. The mice in the second group were inoculated with MDA-MB-231 LOXL4 KO #2-22 using the same number of cells. Tumor volume was measured after the tumor formed. After 40 days of incubation, tumors were then isolated, fixed with 4% paraformaldehyde (PFA), and then preserved in a frozen tumor block. Histological specimens were obtained from the frozen slices and stained with Masson's Trichrome, Hematoxylin, and Eosin (H&E). Collagen from the Masson 's Trichrome staining was quantified using a Keyence BZ-X analyzer software to detect collagen fibers in the randomly selected slide.

Statistical analysis

Statistical tests were performed in Microsoft Excel for Mac version 16.81. Data were presented in mean and SD. Based on the Student t-test, $p<0.05$ was considered statistically significant.

RESULT

High LOXL4 expression in TNBC drove our attention to the importance of this gene in breast cancer progression. To investigate further this role, the LOXL4 gene was depleted from the MDA-MB-231 parental cells. The establishment of LOXL4 KO #2-22 has been shown in our previous study Komalasari, et al., (2023), the clone was determined based on the protein expression. To assert the concomitant of LOXL4 KO #2-22 in breast cancer cell proliferation, cells were equally spread in low numbers, and then the colony developed from each single cell was observed. After the designated observation time, the size of the colony number formed in the MDA-MB-231 parental group was 51±8.18, and the LOXL4 KO #2-22 group showed 35±3.6 (Figure 1). The number of colonies was significantly reduced ($p<0.05$).

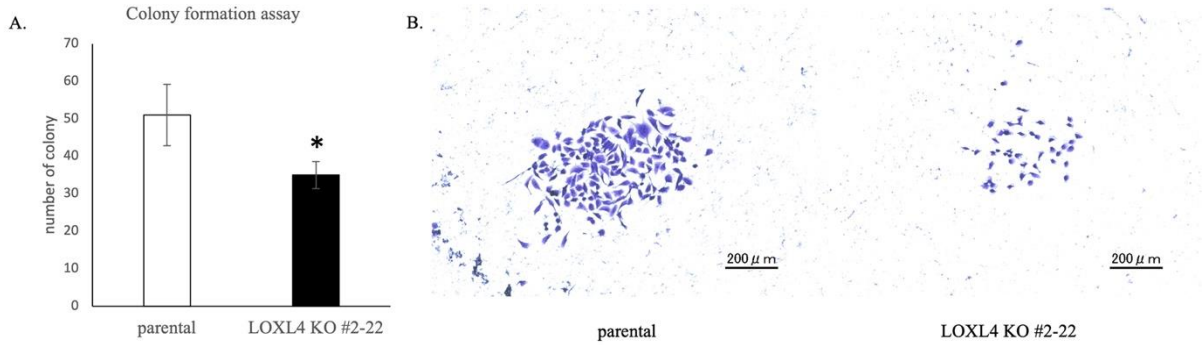

Figure 1. Depletion of LOXL4 affects the single-cell potential to form a colony. (A) Colony number count. (B) Representative of colony size from each group after seven days of incubation.

According to our previous study, the primary tumor growth in the LOXL4 KO group was stalled (Komalasari, et al., 2023). The primary tumor from the breast cancer model was then investigated histologically. The first staining showed a packed and dense configuration of tumor cells in the parental group with dense and regular stroma (Figure 2). LOX family is considered to exert function in the maturation of collagen. To assess the shared function as other LOX family members in the extracellular matrix, i.e., collagen configuration, tumor sections were stained with Masson's Trichrome. Quantification of Masson's Trichrome staining exhibited decreased collagen fibers in the MDA-MB-231 LOXL4 KO #2-22 (21.4±3.6) subline group compared to the parental group (29.5±2.3) with the $p<0.01$ (Figure 3).

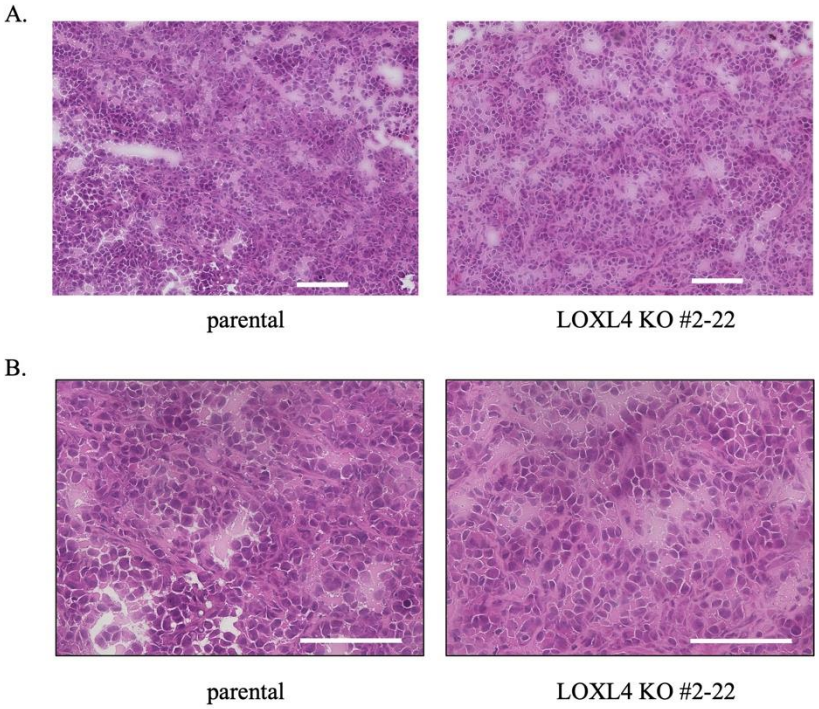

Figure 2. (A) The tumor section from the parental group shows densely packed cancer cells. (B) Larger magnification. The white scale indicates 100 µm.

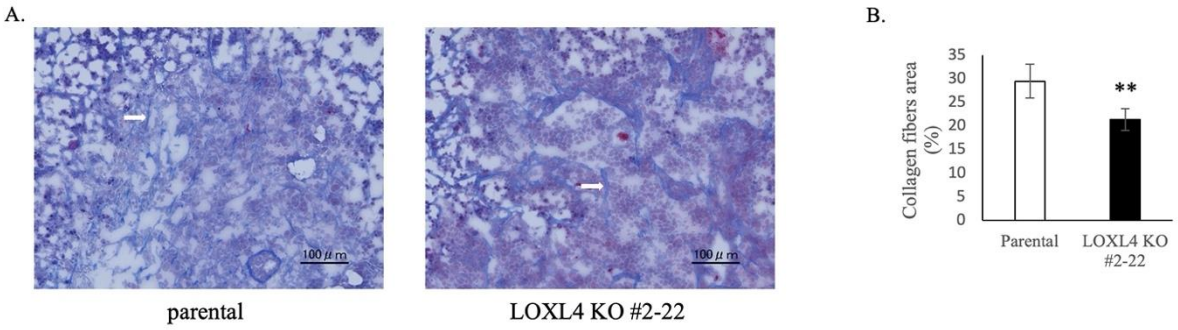

Figure 3. Collagen fibers with Masson's Trichrome staining from tumor section. (A) Representative of Masson's Trichrome staining. The white arrows indicate collagen fibers. (B) Quantification of collagen fibers ($p<0.01$). Scale: 100 µm.

DISCUSSION

In our first screening, we found a high expression of LOXL4 in TNBC, which was not observed in non-TNBC cell lines. To confirm this function, CFA was conducted to evaluate the impact of LOXL4

depletion. Depletion of LOXL4 attenuated the number and size of colonies in the LOXL4 KO #2-22 group, thus reducing cell-cell interaction. Yin, et al., (2020) also showed depressed proliferation of cancer cells on shRNA LOXL4 treatment. This result suggests that LOXL4, other than the ECM, exerts an atypical function in the cancer cell cycle. Additionally, low cell numbers reduce cell-cell interactions, thus preventing downstream mechanisms from mutually inducing cell proliferation. The capability of a single cell to form a colony is also used as a cancer cell stemness indicator (Rajendran & Jain, 2018). Ohta, et al., (2022) observed that slow-proliferated cancer cells have characteristics of stem cells.

After being inoculated orthotopically, the LOXL4 KO #2-22 group developed a smaller tumor size (Komalasari, et al., 2023). Staining of the LOXL4 KO #2-22 tumor showed less ECM with misshapen collagen fibers. LOX family members have been known to partake in ECM maturation which contributed to tumor progression (Maller, et al., 2021). LOXL4 was reported to be associated with collagen I, collagen IV, and liver fibrosis progression (Chen, et al., 2020; Tan, et al., 2021). Thus, LOXL4 supports cancer progression through a common function of LOX family members. Li, et al., (2019) introduce that exosomes in hepatocellular carcinoma (HCC) contain LOXL4 which drives invasion and metastasis. The stiff matrix also contributed to the release of exosomes (Wu, et al., 2023). Therefore, several mechanisms of LOXL4 to induce cancer progression are proposed.

Our previous investigation revealed the surface binding protein of LOXL4, annexin A2 (Komalasari, et al., 2023). This study showed that depletion of LOXL4 reduced collagen deposition, suggesting that secreted LOXL4 affects both neighboring cancer cells and their tumor microenvironment. The impact of LOXL4 in other aspects of TME, including matrix metalloproteinase (MMP) regulation, immune cells, and cancer-associated fibroblast, is also essential to be investigated. Based on current *in vitro* investigation, other intracellular functions of LOXL4 are our next concern. The stemness characteristic of LOXL4 KO #2-22 is an intriguing topic to be explored further.

Strength and limitations

This study supports the concept that LOXL4 has the potential for other functions in breast cancer progression; thus, it is highly expressed in highly invasive cancer cell lines. Our current study was limited to the histological approach of collagen in animal breast cancer models. Further studies are required to disentangle the function of the LOXL4 animal model related to the immune system, matrix metalloproteinase (MMP) regulation, and cancer-associated fibroblast.

CONCLUSION

Our *in vitro* study revealed that depletion of LOXL4 attenuates the ability of a single cell to form a colony. Furthermore, the depletion of LOXL4 decreased collagen from the histological examination in the *in vivo* breast cancer model. Based on these data, the depletion of LOXL4 attenuates the breast cancer model progression. This might be an insight to investigate LOXL4 as a potential target treatment in stalling breast cancer progression.

Acknowledgment

The authors would like to express gratitude to the Japanese Government for the scholarship and support during the study of NLGYK, to all members of the Cell Biology Department, Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama University, and members of the Histology Department, Faculty of Medicine, Udayana University for all their attention and support during the experiment, data collection, and manuscript preparation.

Conflict of Interest

All authors have no conflict of interest.

Ethic Consideration

The animal study was approved by Okayama University with approval number OKU-2020001 on 01-04-2020.

Funding Disclosure

This research was funded by JSPS KAKENHI grant no 20H03516 to M.S.

Author Contribution

NLGYK contributed to the conception and design, collecting analysis and interpretation of the data, drafting the article, and statistical analysis. IGHG drafted the article, performed critical revisions, and provided administrative support. IGNSW contributed to critical revision and administrative support. NT helped with data collection and interpretation and technical support. MS contributed to the conception, drafting of the article, critical revision, and obtaining funding. All authors agreed with the final approval of the article.

REFERENCES

Brassart-Pasco, S., Brézillon, S., Brassart, B., et al. 2020. Tumor microenvironment: Extracellular matrix alterations influence tumor progression. Frontiers in Oncology, 10. doi: 10.3389/fonc.2020.00397.

Chen, W., Yang, A., Jia, J., et al. 2020. Lysyl oxidase (LOX) family members: Rationale and their potential as therapeutic targets for liver fibrosis. Hepatology, 72(2): 729–741. doi: 10.1002/hep.31236.

Choi, S. K. Kim, H. S., Jin, T., et al. 2017. LOXL4 knockdown enhances tumor growth and lung metastasis through collagen-dependent extracellular matrix changes in triple-negative breast cancer. Oncotarget, 8(7): 11977–11989. doi: 10.18632/oncotarget.14450.

Grau-Bové, X., Ruiz-Trillo, I., Rodriguez-Pascual, F. 2015. Origin and evolution of lysyl oxidases. Scientific Reports, 5(1): 10568. doi: 10.1038/srep10568.

Hirabayashi, D., Yamamoto, K. I., Maruyama, A., et al. 2023. LOXL1 and LOXL4 are novel target genes of the Zn2+-bound form of ZEB1 and play a crucial role in the acceleration of invasive events in triple-negative breast cancer cells. Frontiers in Oncology, 13. doi: 10.3389/fonc.2023.1142886.

Hong, X., Yu, J. 2019. Silencing of lysyl oxidase-like 2 inhibits the migration, invasion and epithelial-to-mesenchymal transition of renal cell carcinoma cells through the Src/FAK signaling pathway. International Journal of Oncology. doi: 10.3892/ijo.2019.4726.

Huang, J., Ngai, C. H., Deng, Y., et al. 2022. Cancer incidence and mortality in Asian countries: A trend analysis. Cancer Control, 29: 107327482210959. doi: 10.1177/10732748221095955.

Komalasari, N. L. G. Y., Tomonobu, N., Kinoshita, R., et al. 2023. Lysyl oxidase-like 4 exerts an atypical role in breast cancer progression that is dependent on the enzymatic activity that targets the cell-surface annexin A2. Frontiers in Oncology, 13. doi: 10.3389/fonc.2023.1142907.

Koorman, T., Jansen, K. A., Khalil, A., et al. 2022. Spatial collagen stiffening promotes collective breast cancer cell invasion by reinforcing extracellular matrix alignment. Oncogene, 41(17): 2458–2469. doi: 10.1038/s41388-022-02258-1.

Li, R., Wang, Y., Zhang, X., et al. 2019. Exosome-mediated secretion of LOXL4 promotes hepatocellular carcinoma cell invasion and metastasis. Molecular Cancer, 18(1): 18. doi: 10.1186/s12943-019-0948-8.

Lim, Y. X., Lim, Z. L., Ho, P. J., et al. 2022. Breast cancer in Asia: Incidence, mortality, early detection, mammography programs, and risk-based screening initiatives. Cancers, 14(17): 4218. doi: 10.3390/cancers14174218.

Liu, Y., Liu, X., Chen, F., et al. 2023. LOXL2 promotes tumor proliferation and metastasis by FAK/Src signaling in esophageal squamous cell carcinoma. Electronic Journal of Biotechnology, 63: 18–28. doi: 10.1016/j.ejbt.2023.01.002.

Maller, O., Drain, A. P., Barrett, A. S., et al. 2021. Tumor-associated macrophages drive stromal cell-dependent collagen crosslinking and stiffening to promote breast cancer aggression. Nature Materials, 20(4): 548–559. doi: 10.1038/s41563-020-00849-5.

Microsoft Corporation. 2024. Microsoft Excel for Mac. Available at: https://learn.microsoft.com/en-us/officeupdates/update-history-office-for-mac.

Ohta, Y., Fuji, M., Takahashi, S., et al. 2022. Cell–The cell-matrix interface regulates dormancy in human colon cancer stem cells. Nature, 608(7924): 784–794. doi: 10.1038/s41586-022-05043-y.

Oldfield, R. N., Johnston, K. A., Limones, J., et al. 2018. Identification of Histidine 303 as the catalytic base of lysyl oxidase via site-directed mutagenesis. The Protein Journal, 37(1): 47–57. doi: 10.1007/s10930-017-9749-3.

Rajappa, S., Singh, M., Uehara, R., et al. 2023. Cancer incidence and mortality trends in Asia based on regions and human development index levels: An analyses from GLOBOCAN 2020. Current Medical Research and Opinion, 39(8): 1127–1137. doi: 10.1080/03007995.2023.2231761.

Rajendran, V., Jain, M. V. 2018. *In vitro* tumorigenic assay: Colony forming assay for cancer stem cells: 89–95. doi: 10.1007/978-1-4939-7401-6_8.

Reynaud, C., Ferreras, L., Di Mauro, P., et al. 2017. Lysyl oxidase is a strong determinant of tumor cell colonization in bone. Cancer Research, 77(2): 268–278. doi: 10.1158/0008-5472.CAN-15-2621.

Shao, J., Lu, J., Zhu, W., et al. 2019. Derepression of LOXL4 inhibits liver cancer growth by reactivating compromised p53. Cell Death & Differentiation, 26(11): 2237–2252. doi: 10.1038/s41418-019-0293-x.

Sharma, R., Abbastabar, H., Abdulah, D. M., et al. 2024. Temporal patterns of cancer burden in Asia, 1990–2019: A systematic examination for the Global Burden of Disease 2019 study. The Lancet Regional Health - Southeast Asia, 21: 100333. doi: 10.1016/j.lansea.2023.100333.

Tan, H., Wang, N., Zhang, C., et al. 2021. Lysyl oxidase-like 4 fosters an immunosuppressive microenvironment during hepatocarcinogenesis. Hepatology, 73(6): 2326–2341. doi: 10.1002/hep.31600.

Vallet, S. D., Ricard-Blum, S. 2019. Lysyl oxidases: From enzyme activity to extracellular matrix cross-links. Essays in Biochemistry. Edited by J. Adams, 63(3): 349–364. doi: 10.1042/EBC20180050.

Winkler, J., Abisoye-Ogunniyan, A., Metcalf, K. J., et al. 2020. Concepts of extracellular matrix remodelling in tumour progression and metastasis. Nature Communications, 11(1): 5120. doi: 10.1038/s41467-020-18794-x.

World Health Organization (2024) Breast cancer, WHO. Available at: https://www.who.int/news-room/fact-sheets/detail/breast-cancer.

Wu, B., Liu, D., Guan, L., et al. 2023. Stiff matrix induces exosome secretion to promote tumour growth. Nature Cell Biology, 25(3): 415–424. doi: 10.1038/s41556-023-01092-1.

Yin, H., Wang, Y., Wu, Y., et al. 2020. EZH2-mediated epigenetic silencing of miR-29/miR-30 targets LOXL4 and contributes to tumorigenesis, metastasis, and immune microenvironment remodeling in breast cancer. Theranostics, 10(19): 8494–8512. doi: 10.7150/thno.44849.