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Perspective

Lactate and protein lactylation: the ugly duckling of energy as the sculpture artist of proteins

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Metabolites play important roles in numerous cell biology processes, such as cell proliferation, differentiation, stress response, and cell death [1]. Recently, lactate and lactate-derived lysine residue lactylation (Kla) have emerged as newly discovered epigenetic modifications that play critical roles in various physiological and pathological processes. In the history of lactate research, we can categorize the studies into three mile stones (Fig. S1 online). The first milestone event is the discovery of the "Warburg effect". In 1921, the German scientist Otto Warburg found that tumor cells could utilize glucose to generate a large amount of lactate even under aerobic conditions, leading to the re-examination of the role of lactate. Subsequent studies revealed that the "Warburg effect" is not limited to cancer cells, but also occurs in astrocytes, pluripotent stem cells (iPSC), and immune cells [2]. The second milestone event is the "Lactate shuttle theory" proposed by Brooks [3] in 1984. This theory explains how lactate is transported within the body and cells. McClelland et al. [4] found that lactate transport is dependent on monocarboxylate transporter (MCT), a family of 14 members of transmembrane proteins encoded by the *SLC16A* gene. The third milestone event is the discovery of protein lactylation. In 2019, the team led by professor Yingming Zhao [5] found that lactate-mediated lactylation of histone lysine residues, as a new epigenetic modification, could participate in the gene activation during M1 macrophage polarization. This breakthrough finding opens up a brand-new perspective on lactate research. Given the great potential roles of Kla, we would like to discuss the role and mechanisms of Kla in various physiological and pathological processes, the status of Kla on evolutionary process, and different methods for the identification of Kla sites and enzymes involved in protein Kla, including writers and erasers in different contexts.

The physiological and pathophysiological processes are not at the opposing sides but closely connected and able to transform into each other under certain conditions. Recently, a large amount of studies have suggested that lactate and Kla play critical roles in various physiological and pathophysiological processes in mammals. Fluctuations in lactate concentration can lead to changes in Kla in various physiological and pathophysiological processes which provide evidences for the substrate character of lactate on Kla. During the physiological processes, histone Kla sites, including H3K18la, H3K23la, and H4K12la have been demonstrated to play important roles in stem cell fate, embryo development, and neural development [6,7]. The mechanisms by which histone Kla regulates cell fate transition in physiological processes are associated with the chromatin state and gene expression (details are provided in the Supplementary materials online). Interestingly, these functional histone Kla sites differ from histone acetylation and crotonylation, indicating that histone Kla may regulate gene expression by cooperating with, rather than competing against, histone acetylation and crotonylation. However, the roles of non-histone protein Kla have not been studied in these physiological processes, which need further study. While during the pathophysiological processes such as tumor development, inflammation and immune process, and diseases development, the role of both histone Kla and non-histone protein Kla have been widely implicated. Histone Kla, including H3K18la and H3K59la, has been reported to regulate chromatin state and gene expression, and H3K18la has been regarded as a marker of various tumors [6,8]. The non-histone protein Kla, such as K28la of adenylate kinase 2, inhibits its enzyme

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activity, facilitating the proliferation and metastasis of HCC cells [9]. In summary, through involving in the chromatin status and gene expression, histone Kla has been found to play important roles both in physiological and pathophysiological processes. While the function of non-histone protein Kla is associated with enzyme activity, subcellular location or interactions with other proteins, which depends on the characteristics of the Kla proteins and may regulate the function of target protein differently (details are provided in the Supplementary materials online).

Considering the relationship between physiological and pathophysiological processes, we speculate that lactate and Kla may play important roles during the transformation between physiological and pathophysiological process. One cannot help but recall the "Yin-yang Taiji Fish Diagrams", one of the most famous philosophical symbols in China, which describes the balance and transformation of Yin and Yang in everything and fits well with the role of Kla in physiological and pathophysiological processes. The pathophysiological process could be regarded as the "Yin"side, while the physiological process could be viewed as the "Yang" side. The transformation and dependence of the pathophysiological and physiological process correspond to the transformation and dependence of "Yin" and "Yang". The role of Kla can be considered as the fish eyes in "Yin-yang Taiji Fish Diagram". Kla plays an important role in the transformation of pathophysiological (Yin) and physiological (Yang) processes (Fig. 1a). In this way, it could be easier to understand the role of lactate and Kla in pathophysiological (Yin) and physiological (Yang) processes by ancient wisdom. It would be interesting to explore the mechanisms by which lactate and Kla regulate the transformation of pathophysiological (Yin) and physiological (Yang) processes.

Kla was first identified in human HeLa cells and mouse bone marrow-derived macrophages (BMDMs) by Zhang et al. [5] in 2019. They identified 28 histone Kla sites and found that histone Kla participates in gene activation in the late phase of M1 macrophage polarization [5]. Their work revealed that Kla not only exists but also functions in mammalian cells, offering a new avenue for studying lactate and lactate-derived Kla. Numerous studies have demonstrated that Kla proteins, including histone and non-histone, exist in various species across the biological world on earth, including bacteria, protista, plantae, animalia and fungi [10] (Fig. 1b and Table S1 online). Based on the mountains of findings of Kla in different species, we deem that Kla may be conserved during the process of evolution on earth. This conservative property of Kla can be viewed at three levels. First, Kla sites and proteins have been discovered in multiple species, indicating their evolutionary conservation. Second, the generation of Kla is not limited to lower organisms such as *Escherichia coli* but is also observed in higher organisms such as humans, which implies an evolution conservation over the timeline of biological evolution on earth. Finally, pathway enrichment analysis has revealed that the functions of lactylated proteins are conserved in some cell biological processes, including RNA processing and metabolism, providing more evidences about the conservative status of Kla. In conclusion, lactate-derived Kla is a conserved epigenetic modification during the biological evolution process, and uncovering Kla sites and proteins in other species on earth would be of great value. There are also some exciting questions, such as whether Kla modification can communicate between different species, such as fungi and plant, and whether communication of Kla modification between different species can promote species evolution?

Currently, most research on protein lactylation proteomics utilizes liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, which combines liquid chromatography with mass spectrometry to separate and purify proteins in a mixture. Liquid chromatography separates proteins, while mass spectrometry can be used for qualitative, quantitative, and structural analysis of proteins with a certain purity. By combining these techniques, researchers can achieve the dual task of separation and identification of proteins in complex cells or tissues. Similar to other post-translational modifications (PTM) such as phosphorylation and acetylation, lactylation also has a characteristic ion with diagnostic significance. Wan et al. [11] found that a polypeptide carrying lactylated lysine forms chain imine ions after secondary fracture in the mass spectrometry collision chamber by synthesizing and studying the spectrum of the model lactated peptide segment. This ion forms secondary fragment-cyclic imine ions after deamination cyclization. The team analyzed the positive lactate peptide enriched in chemical modification and biological samples, and used the spectrum of non-modified synthetic peptide of nearly 100,000 human proteomes as a negative control to ensure the sensitivity and specificity of cyclic imine ions for lactylation. Based on this method, they showed that lactylation widely exists in human tissues and cells and revealed an important function of lactylation in the glycolytic enzyme Aldoa and Eno1 [11]. Although this method may identify Kla sites more precisely, the number of Kla sites and proteins identified is fewer compared to classical LC-MS/MS. The bioorthogonal chemical reporter strategy is also used to detect and identify Kla sites and proteins. Sun et al. [12] constructed an alkynyl-functionalized biorthogonal chemical reporter, YnLac. Using this reporter, they revealed many Kla sites on non-histone and demonstrated that lactylation of PARP1 could regulate its ADP-ribosylation activity. However, much less Kla proteins were detected using this method. Compared with experimental-dependent methods, computational-dependent methods for predicting Kla sites have great potential in Kla research. Two groups have developed such methods since the discovery of Kla. Jiang et al. [13] designed the first Kla site predictor named FSL-Kla. They claimed that FSL-Kla is not only a cutting-edge tool for profiling Kla sites but also provides candidates for further experiment. Lv et al. [14] proposed a method based on deep learning, called DeepKla, to accurately identify protein Kla sites. As an integrated deep learning framework, DeepKla consists of four closely connected subnetworks, including word embedding layer, convolutional neural network (CNN), bidirectional gated loop unit (BiGRU), and attention mechanism layer. Among them, the embedded layer uses the protein sequence as the only input to automatically extract sequence features, and BiGRU and attention mechanism are used to capture remote information and key location information from the protein sequence respectively. The researchers used the lactate data of rice and *Botrytis cinerea* as test data, and the results showed that this deep learning model has a strong predictive performance in identifying Kla sites. Recently, Lai et al. [15] also developed a new calculation model, automatic Kla (Auto-Kla). Depending on automatic machine learning, it can quickly and accurately predict the Kla sites in gastric cancer cells. They used the data of phosphorylation sites in host cells infected with SARS-CoV-2 and lysine cloning sites in HeLa cells to evaluate the performance of the PTM prediction ability of the machine learning (ML) model. The highlight of this machine model is that it is the first to use Auto ML method to predict Kla and PTM sites, which improves its generalization ability [15]. In comparison with these three methods, FSL-Kla is the first computational tool that can predict Kla sites. It utilizes a small dataset to summarize the physical and chemical properties, sequence features, and structural features of Kla. In the initial stage, it greatly improves the efficiency of tool utilization with a small number of data samples. The drawback is the inaccuracy caused by the insufficient dataset. Deep-Kla is a deep learning tool built for the lactylation sequencing data of a specific rice species, and its predictive ability for other species needs to be improved (the developers have made the source code of the tool available to facilitate its use by other researchers). Finally, Auto-Kla has made further improvements based on FSL-Kla and Deep-Kla. The

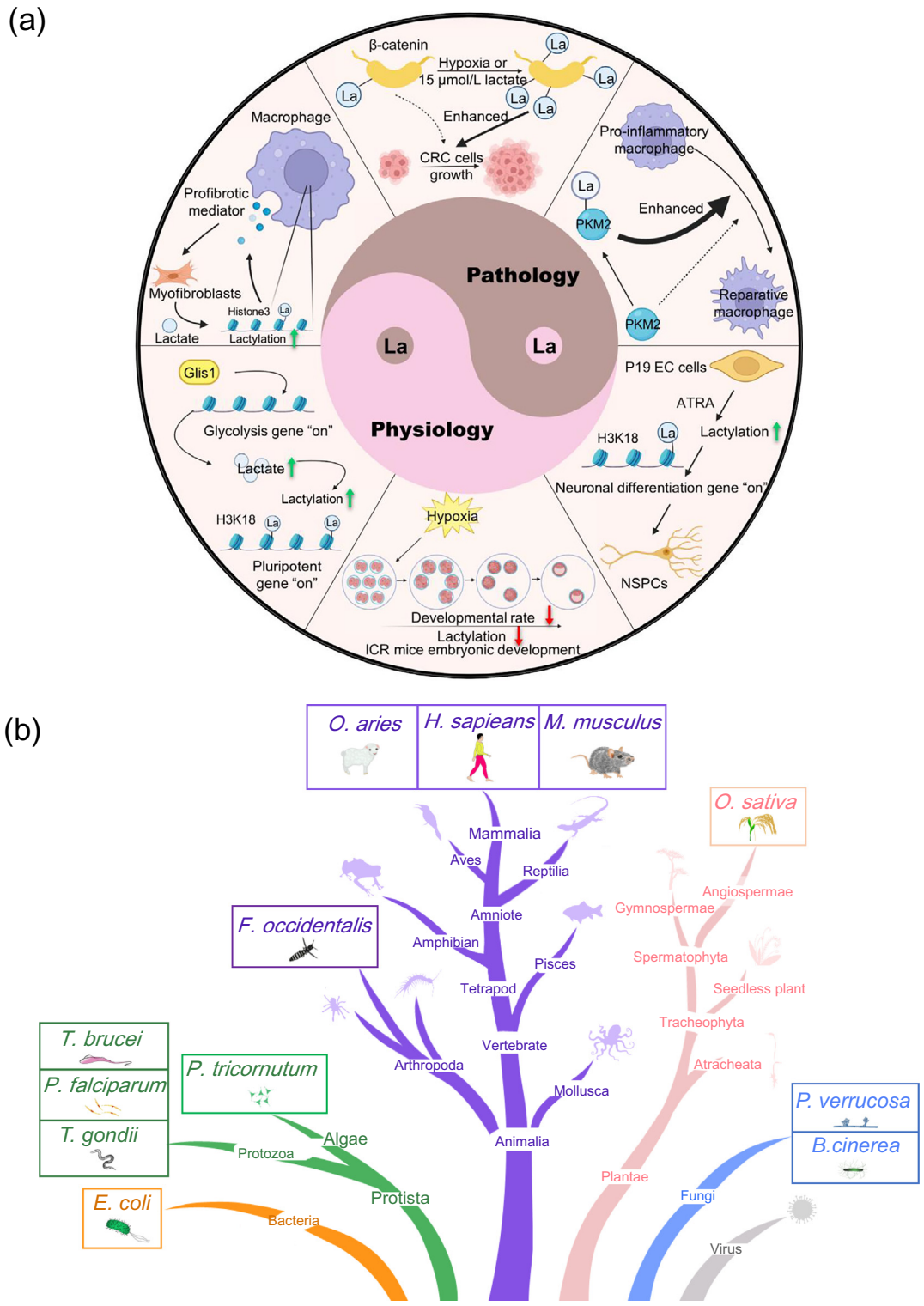

Fig. 1. The roles of lactate and lactylation during physiological and pathophysiological processes, as well as biological evolution process. (a) The summary of the roles of lactate and lactylation during many physiological and pathophysiological processes. The pathophysiological and physiological processes correspond to the "Yin" and "Yang" of "Yin-yang Taiji Fish Diagrams" respectively. Lactylation corresponds to the "fish eyes" of "Yin-yang Taiji Fish Diagrams" which represents the significance of lactylation during the transition between the pathophysiological and physiological processes. The figure here is created with BioRender.com. La: lactylation; CRC: colorectal cancer cells; PKM2: pyruvate kinase M2; P19 EC: P19 embryonic carcinoma cells; ATRA: all-trans-retinoicacid; NSPCs: neural stem and progenitor cells; H3K18: histone 3 lysine 18; ICR: Institute of Cancer Research. (b) The researches which have demonstrated the existence of lactylation in different species. It is indicated that lactylation exists in various species across the biological world on earth, including bacteria, protista, plantae, animalia, and fungi. Further, many studies have suggested that the function and pathway of lactylation protein is conservative in many species. *O. aries*: *Ovis aries*; *H. sapieans*: *Homo sapiens*; *M. musculus*: *Mus musculus*; *O. sativa*: *Oryza satival L*; *F. occidentails*: *Frankliniella occidentalis*; *T. brucei*: *Trypanosoma brucei*; *P. falciparum*: *Plasmodium falciparum*; *T.gondii*: *Toxoplasma gondii*; *P. tricornutum*: *Phaeodactylum tricornutum*; *E. coli*: *Escherichia coli*; *P. verrucosa*: *Phialophora verrucosa*; *B. cinerea*: *Botrytis cinerea*.

large number of open datasets greatly enhances the accuracy of Auto-Kla's predictions and allows for better adaptation to different species and protein post-translational modification sites. Currently, Auto-Kla may be the best computational tool for predicting lactylation sites. However, these predicting methods, including FSL-Kla, DeepKla, and Auto-Kla, rely on available Kla datasets, and further work is required for improve their accuracy in the prediction of Kla sites. Nevertheless, these prediction methods provide a valuable way to identify Kla sites and have a great potential for future study on Kla. In conclusion, classical LC-MS/MS is the main method to identify the sites and proteins of Kla, but downstream validation is required. The classical LC-MS/MS and those prediction methods would be the way to identify the sites and proteins of Kla in the future.

As the newest epigenetic modification, the enzymes, including writer (lactyltransferase) and eraser (delactylases), of Kla on histone and non-histone protein have gained great attentions since the discovery of Kla in 2019. P300, a histone acetyltransferase that has been known for many years, has recently been identified as a histone and non-histone protein lactyltransferase in various cell contexts cell contexts such as cancer cells, stem cells, and macrophages [6]. In the other hand, the eraser of histone delactylases have also been revealed. Moreno-Yruela et al. [16] showed that Class I histone deacetylases (HDAC1–3) are histone lysine delactylases. NAD^+-dependent deacylases Sirt2 and Sirt3 were also discovered as the histone delactylase in neuroblastoma cells [17] and as the non-histone delactylase in hepatocellular carcinoma, respectively [18]. However, due to the multi-function roles of P300 and HDAC family members, which can act as acetyltransferase and deacetylase in different cellular contexts, further research is needed to explore the existence of other lactyltransferase and delactylase.

Although the history of research on lactate-derived lactylation is very short, a large number of studies have confirmed the extensive and critical role of lactylation in a variety of physiological and pathological processes. Moreover, by summarizing the studies of Kla in different species, we proposed that Kla seems to be conserved during the process of species evolution on earth based on the numbers of Kla species, the span of Kla species and the overlapping biological functions of Kla. The function of Kla in physiological and pathological processes or species evolution process provides more considerations and views about the metabolism and epigenetic modification in the future. There are still many unresolved questions in the study of lactate and Kla, such as the identification of the enzymes of protein Kla, including the lactyltransferase and delactylases; the crosstalk between lactylation and other epigenetic modifications, and the identification of the Kla sites in others species. Understanding how different acylated modifications interact with each other and compete for the same sites could provide important insights into how cellular processes are regulated. Species-wide mapping of lactylation could provide new insights into the role of Kla in biological evolution and may lead to new breakthroughs in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary materials to this perspective can be found online at https://doi.org/10.1016/j.scib.2023.09.038.

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