Functions of leptin in tuberculosis and diabetes: multi-omics studies across species

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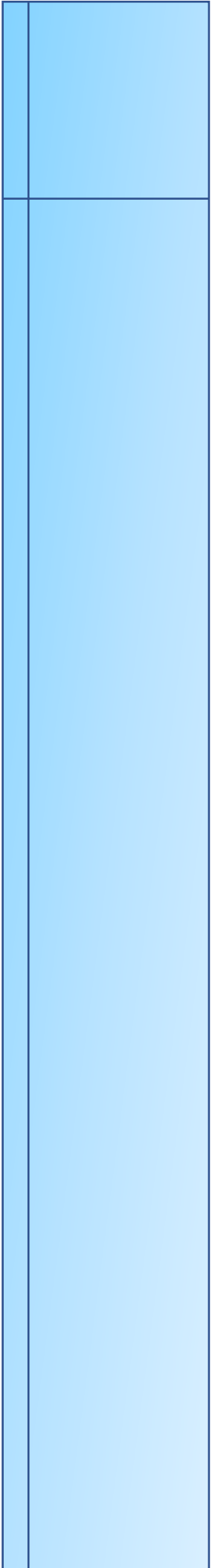

Summary

Chapter 1 provides a general background of metabolic wasting syndrome (also called cachexia), functions of the leptin gene, and the main omics technologies and an outline of this thesis. We particularly focus on two diseases resulting in cachexia at a later stage: tuberculosis (TB) and diabetes mellitus (DM). TB remains a big threat to global public health and one of the most important means to control the disease is to develop new efficient, reproducible and cost-effective diagnostic methods. Here we show that omics-based approaches are promising for biomarker discovery of TB as well as for prediction of disease therapy outcomes. DM is a chronic metabolic disease caused by defects in insulin signaling. Leptin has been extensively studied in DM because of its crucial role in control of body weight, energy homeostasis and metabolism. Leptin has also been implicated to play a role in the progression of tuberculosis. We illustrate that zebrafish is a versatile animal model to study the progression and the pathogenic mechanisms of TB and DM and functions of leptin in wasting syndrome. Furthermore, we compare the strengths and weaknesses of the most common used analytical tools: mass spectrometry and nuclear magnetic resonance spectroscopy. We also summarize the currently most common short-reading RNAseq methods and the future possibilities for the extensive use of long-reading sequencing methods.

Tuberculosis is a highly infectious and potentially fatal disease accompanied by wasting symptoms, which cause severe metabolic changes in infected people. Measurements of metabolites in human TB patients have shown to be a promising method for diagnostic purposes. In **Chapter 2**, we have compared the effects of mycobacterial infection on the level of metabolites in blood of humans and mice and whole zebrafish larvae using one highly standardized mass spectrometry pipeline, ensuring technical comparability of the results. Quantification of a range of circulating small amines show that the levels of the majority of these compounds were significantly decreased in all three groups of infected organisms. Our study identifies 10 common biomarkers for tuberculosis disease in humans, mice and zebrafish comprising: methionine, asparagine, cysteine, threonine, serine, tryptophan, leucine, citrulline, ethanolamine and phenylalanine. Furthermore, we use the zebrafish model to further investigate the metabolic changes after infection using NMR analyses based on a large number of specimens. The NMR analysis of zebrafish larvae confirms the MS data and also identifies new markers for infection including identifying general effects on metabolism such as a change of glucose levels. Our results show across-species conservation of metabolic reprogramming processes during TB infection and disease in humans, mice and zebrafish. Apparently, the mechanisms underlying these processes are independent of environmental, developmental and vertebrate evolutionary factors. The zebrafish larval model is highly suited to further investigate the mechanism of metabolic reprogramming and the connection with wasting syndrome due to infection by mycobacteria.

Summary

Leptin is a hormone which functions in the regulation of energy homeostasis via suppression of appetite. In zebrafish, there are two paralogous genes encoding leptin, called *lepa* and *lepb*. In a gene expression study, we found that the *lepb* gene was significantly downregulated under the state of insulin-resistance in zebrafish larvae, suggesting that the *lepb* plays a role in glucose homeostasis. In **Chapter 3**, we generate *lepb*-deficient zebrafish by using the CRISPR/CAS9 gene editing approach. This study aims to investigate whether the disruption of the *lepb* gene would result in the development of type 2 diabetes mellitus (T2DM) and diabetic complications in adult zebrafish. To address this question, we examined the body weight and length, blood glucose levels, and the body fat distribution in 1.5 years old *lepb*$^{-/-}$ adult zebrafish and compared them to age-matched wild type controls. Furthermore, we examine the renal histopathologic changes of these zebrafish by performing hematoxylin and eosin (HE) or periodic-acid schiff (PAS) staining, and transmission electron microscopy (TEM) methods. We observe that *lepb*$^{-/-}$ adult zebrafish have an increase in body weight, length and visceral fat accumulation, compared to age-matched control zebrafish. In addition, *lepb*$^{-/-}$ zebrafish have significantly higher blood glucose levels compared to control zebrafish. These data collectively indicate that *lepb*$^{-/-}$ adult zebrafish display the features of T2DM. Furthermore, we show that *lepb*$^{-/-}$ adult zebrafish have glomerular hypertrophy and thickening of the glomerular basement membrane, compared to control zebrafish, suggesting that *lepb*$^{-/-}$ adult zebrafish develop early signs of diabetic nephropathy. In conclusion, our results demonstrate that *lepb* regulates glucose homeostasis and adiposity in zebrafish, and suggest that *lepb*$^{-/-}$ mutant zebrafish are a promising model to investigate the role of leptin in the development of T2DM and are an attractive model to perform mechanistic and therapeutic research in T2DM and its complications.

Leptin plays a critical role in the regulation of metabolic homeostasis. However, the molecular mechanism and cross talks between leptin and metabolic pathways leading to metabolic homeostasis across different species are not clear. In **Chapter 4,** we have compared the metabolic changes resulting from leptin deficiency in blood of adult *ob/ob* mice and extracted and intact zebrafish larvae using MS, solution-state NMR and high-resolution magic-angle spinning NMR (HR-MAS NMR) spectrometry. In addition, we have compared the transcriptomic changes resulting from leptin deficiency in *ob/ob* mice heads, a published dataset for *ob/ob* mice liver and *lepb* mutant zebrafish larvae using deep RNA sequencing (RNA-seq). Thirteen metabolites were identified as common biomarkers discriminating *ob/ob* mice and *lepb*$^{-/-}$ zebrafish larvae from their respective wild type controls: alanine, citrulline, ethanolamine, glutamine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, putrescine, serine and threonine. Moreover, we also observe that glucose and lipid levels are increased in *lepb*$^{-/-}$ zebrafish larvae compared to the *lepb*$^{+/+}$ group. RNAseq show that many genes involved in proteolysis and arachidonic acid metabolism are dysregulated in *ob/ob* mice heads and *lepb* mutant zebrafish larvae compared to their wild type controls, respectively. Leptin deficiency in adult mice and larval zebrafish leads to highly similar metabolic alterations in amino acid levels. These metabolic changes show the same key features as

observed during progression of tuberculosis in human patients, rodents and zebrafish larvae. Moreover, by studying the transcriptome, we found highly similar changes in gene regulation related to proteolysis and arachidonic acid pathways in these two test systems. These results show a remarkable similarity of the effects of leptin knockdown on the metabolomes and transcriptomes of adult mice and zebrafish larvae that might be related to wasting syndrome. Apparently, the metabolic control by leptin is similar in adult and embryonic stages in mammals and fish, respectively.

Leptin plays an evolutionary conserved role in regulating glucose homeostasis and system metabolism as well as cellular and systemic inflammatory responses. In accordance, the leptin gene plays a role in many diseases such as cancer, tuberculosis and diabetes. In **Chapter 5**, we investigate the metabolism of leptin mutants in the absence and presence of mycobacterial infection in mice and zebrafish larvae. Metabolites in the blood of *ob/ob* mice and entire *lepb* mutant zebrafish larvae are studied using mass spectrometry and HR-MAS NMR spectroscopy, respectively. Our results show that leptin mutations and mycobacterial infection lead to a similar metabolic syndrome, characterized by the decrease of 11 amine metabolites. In both species, this metabolic syndrome is not aggravated when the leptin mutant was infected by mycobacteria. Therefore, we conclude that leptin and mycobacterial infection are both impacting metabolism non-synergistically. Subsequent transcriptome studies in zebrafish larvae showe that mycobacteria induced a very distinct transcriptome signature in the leptin mutant compared to the wild type sibling control. Apparently, different transcriptomic responses can lead to the same metabolic end states. Therefore, we conclude that leptin and mycobacterial infection control metabolism in different ways despite share metabolic features.

In **Chapter 6**, we discuss the major findings described in the previous chapters of this thesis and present perspectives for future studies. We demonstrate that zebrafish are an excellent animal model for studies of metabolic diseases including tuberculosis and diabetes due to various particular advantages. The effects of *lep* or *lepr* mutation on obesity in zebrafish as reported by different research groups are controversial. We postulate that differences in breeding schemes and compensatory genetic mechanisms might be responsible for the reported inconsistencies. Subsequently, we discuss that the metabolic changes in leptin mutants and infectious disease reported in this thesis are highly conserved in several species and different developmental stages considering the following points. Firstly, metabolic changes resulting from mycobacterial infection is highly similar in human, mice and zebrafish larvae. Secondly, metabolic and transcriptomic changes resulting from leptin mutation is very similar in mice and zebrafish larvae. Thirdly, metabolic changes resulting from mycobacterial infection and leptin mutation are highly similar in mice and zebrafish larvae. Fourthly, metabolic changes are shown to be highly similar based on different sample preparation and metabolomics technologies: mass spectrometry and nuclear magnetic resonance spectroscopy. Furthermore, we discuss the role of leptin in tuberculosis and

the potential utility of leptin deficient zebrafish for gestational diabetes mellitus studies and ongoing work that is currently performed. Finally, we provide perspectives of three additional technologies for future studies of new research questions resulting from this study. (1) Potential applications of a method called positional isotopomer nuclear magnetic resonance tracer analysis (PINTA) and magnetic resonance microimaging (μMRI) are proposed for further studies in zebrafish. (2) A vertebrate automated screening technology (VAST) bioimager platform will be applied for high throughput measurements of muscle wasting during development in mycobacteria-infected wild type and leptin mutant zebrafish larvae. (3) A sensitive targeted analysis HPLC-MS/MS platform can be used for studies of fatty acids, especially derivatives from arachidonic acid signaling pathway. By using these advanced platforms, we aim to provide new insights into the mechanisms of metabolic changes underlying tuberculosis and diabetes.