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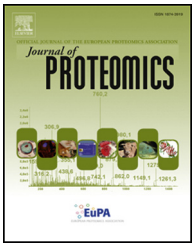

Regulation of PPAR-alpha pathway by Dicer revealed through proteomic analysis

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ABSTRACT

Dicer is a crucial RNase III enzyme in miRNA biogenesis pathway. Although numerous studies have been carried out to investigate the role of miRNAs and Dicer in the regulation of biological processes, few studies have examined proteomic alterations upon knockout of Dicer. We employed a Cre–loxP-based inducible knockout mouse system to investigate the proteome regulated by Dicer-dependent miRNAs. We utilized spiked liver lysates from metabolically labeled mice to quantify the subtle changes in the liver proteome upon deletion of Dicer. We identified 2137 proteins using high resolution tandem mass spectrometry analysis. The upregulated proteins included several enzymes involved in peroxisomal β-oxidation of fatty acids and a large majority of the upregulated proteins involved in lipid metabolism were known PPARα targets. MRM-based assays were carried out to confirm the upregulation of enzymes including peroxisomal bifunctional enzyme, phosphoenolpyruvate carboxykinase 1, cytochrome P450 3A13, cytochrome P450 3A41 and myristoylated alanine-rich protein kinase C substrate. Further, miRNA-124 which is predicted to regulate expression of peroxisomal bifunctional enzyme was confirmed to be downregulated in the Dicer knockout mice. Our study demonstrates the strength of coupling knockout mouse models and quantitative proteomic strategies to investigate functions of individual proteins *in vivo*.

Biological significance
Dicer dependent miRNA biogenesis is the major pathway for generation of mature miRNAs. We developed SILAC mouse-based proteomics screen to identify protein targets of Dicer-dependent

Abbreviations: PPAR, Peroxisome Proliferator-Activated Receptor; SILAC, stable isotope labeling by amino acids in cell culture; BRPLC, basic reversed phase liquid chromatography; FDR, false discovery rate; MRM, multiple reaction monitoring; Pbe, peroxisomal bifunctional enzyme; Pck1, phosphoenolpyruvate carboxykinase 1; Marcks, myristoylated alanine-rich protein kinase C substrate.

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miRNAs in liver of Dicer knockout mice. We spiked liver lysates of induced and uninduced Dicer knockout mice with liver lysate of SILAC labeled mice for identification of dysregulated proteome. We quantitated 1217 proteins of which 257 were upregulated in induced Dicer knockout mice. We observed enrichment of PPAR-α targets and proteins involved in lipid metabolism among upregulated proteins. We further carried out MRM-based validation of peroxisomal bifunctional enzyme, phosphoenolpyruvate carboxykinase 1, Cyp3A13, Cyp3A41 and myristoylated alanine-rich protein kinase C substrate. We further validated upregulation of peroxisomal bifunctional enzyme using Western blot analysis and downregulation of its predicted upstream miRNA, miR-124 using qRT-PCR. Our study demonstrates that upon ablation of Dicer, certain Dicer-dependent miRNAs are dysregulated which result in dysregulation of their target proteins such as proteins associated with lipid metabolism. Our study illustrates the use of SILAC strategy for quantitative proteomic investigations of animal model systems.

1. Introduction

MicroRNAs (miRNAs) are known to be one of the key mediators of post-transcriptional gene regulation [1]. Regulation of genes through miRNAs has been implicated in a variety of biological processes. MicroRNAs exert post-transcriptional regulation through binding to 3′ UTRs of target mRNAs and leading to halted translation or directing them for degradation [1]. Dicer is a ubiquitously expressed and evolutionarily conserved ribonuclease in the si/miRNA biogenesis pathway. It contains RNase III domains, whose role is to excise precursor miRNAs to generate mature miRNAs.

The role of Dicer has been investigated in several biological systems. Various *in vitro* and *in vivo* systems have been developed to explore the role of Dicer [2–6]. Dicer-deficient mouse embryonic stem cells exhibit defective proliferation, differentiation and centromeric silencing [7,8]. Depletion of Dicer is lethal at embryonic stage. Thus, inducible knockout systems were utilized to study the function of Dicer in specific cell types of adult mice including hepatocytes, CD8 cells and spermatogonia [9–12]. Deletion of Dicer results in dysregulation of enzymes involved in lipid metabolism in hepatocytes and small intestine [9,13]. Ablation of Dicer in hepatocytes results in the development of hepatic tumors in mice, indicating crucial regulatory role of Dicer and Dicer-dependent miRNAs [9]. In this study, we aimed to investigate the proteomic alterations that are induced by Dicer-dependent miRNAs in the liver.

We employed an inducible Cre–loxP knockout system for the deletion of Dicer. For quantitative profiling of the liver proteome, we used SILAC labeled mouse liver lysates for spiking. Previously, various *in vitro* labeling methods including DIGE, ^{18}O and iTRAQ labeling have been adopted for quantitative proteomic profiling of mouse systems which have various limitations [14–19]. Gel-based quantitative proteomics approaches are known for low reproducibility and separation due to the limited pI range. Back exchange of ^{18}O isotope, compression of iTRAQ ratios and probability of introducing manual errors in labeling are other limitations of *in vitro* quantitation approaches. Label free quantitation has been used as an alternative quantitative proteomic approach. However, it requires highly reproducible LC–MS conditions, which are often difficult to achieve, especially across multiple sample runs [20]. Therefore, *in vivo* labeling strategies such as ^{15}N labeling and stable isotope labeling by amino acids in cell culture (SILAC) are generally preferred for quantitative proteomics [21,22]. Some of the limitations of the ^{15}N labeling approach include challenges in achieving complete labeling and complexity of the quantitation data. As a result, SILAC has been the method of choice for *in vivo* labeling. Until recently, the use of SILAC was limited to cell lines, but with the development of $^{13}C_6$-lysine enriched chow, mice can also be labeled *in vivo*, extending the application of SILAC to animal systems [13,22,23]. This quantitation approach is relatively free of technical errors as proteins are labeled *in vivo*.

We developed a SILAC mouse-based quantitative proteomics assay to identify differentially expressed proteins upon depletion of Dicer in liver. We carried out high resolution mass spectrometry analysis and identified 2137 proteins. Of the 257 proteins that were upregulated in the liver of Dicer knockout mice, we observed enrichment of proteins involved in fatty acid metabolism. We further carried out MRM assays to validate candidate proteins, which include peroxisomal bifunctional enzyme, phosphoenolpyruvate carboxykinase 1, Cyp3A13, Cyp3A41 and myristoylated alanine-rich protein kinase C substrate. We also carried out Western blot analysis to validate upregulation of peroxisomal bifunctional enzyme. We further validated downregulation of miR-124 which is predicted to regulate expression of peroxisomal bifunctional enzyme using qRT-PCR assay. Our findings highlight crucial roles of Dicer and Dicer-dependent miRNAs in the regulation of proteins involved in lipid transport and metabolism in mouse liver. Our study also demonstrates the utility of SILAC mouse-based proteomics and MRM assays as robust mass spectrometry-based approaches for the development of *in vivo* quantitative proteomics strategies.

2. Materials and methods

2.1. Generation of inducible Dicer knockout mouse

We used Cre–loxP system to generate inducible knockout mice. ROSA26-CreERT2 mice and mice with floxed Dicer exons 21 and 22 were crossed. The progeny was responsive to tamoxifen, resulting in the deletion of floxed *Dicer* exons 21 and 22. ROSA26-CreERT2 mice without the treatment of tamoxifen were used as control. Mice were monitored daily for any obvious pathology. On day 8 post-induction, mice were starved for 3 h prior to euthanasia and necropsy.

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