REGULATION OF OXIDATIVE STRESS PATHWAY BY LNCRNA MALAT1 AS A LINCHPIN CONNECTING MALAT1 PLEIOTROPIC FUNCTIONS WITH MULTIPLE DISEASES

A Dissertation

by

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ABSTRACT

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long noncoding RNA and its overexpression is associated with poor prognosis of many types of cancers and its silencing has been reported to reverse the course of carcinogenesis *in vitro* and *in vivo* in laboratory animals. The MALAT1 is an evolutionarily conserved lncRNA with high level of expression in various tissues and cell types due to its unique stabilizing 3' triple helix structure. Its genome-wide involvement in RNA splicing and transcriptional regulation suggests that it has "utility type" of functionality in transcriptional regulation. However, its physiological functions are somewhat enigmatic as the MALALT1 null mice show no overt phenotype under normal laboratory conditions. Consistent with its pervasive involvement in gene regulation, results from recent studies indicate that MALAT1 has pleiotropic role in regulating physiological and pathophysiological functions.

In this study, we revealed a salient feature of MALAT1 is its novel function in regulating oxidative stress and immune/inflammatory responses which are important etiological factors for many diseases. In transcriptome analysis of MALAT1 null mice we found significant upregulation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) regulated antioxidant genes with significant reduction in reactive oxygen species (ROS). MALAT1 null mice exhibited sensitized insulin-signaling response and alleviated lipopolysaccharide-induced innate immune response. Furthermore, we established a mouse T2DM model, ob/ob model and investigated the role of MALAT1 in regulating the insulin signaling pathway and obesity. Consistent with our previous results, MALAT1 ablation alleviates the hyperglycemia, hyperinsulinemia, and insulin resistance in the obesity-induced T2DM in *ob/ob* mice, with significantly decreased fat deposition in liver

and visceral adipose tissue. In summary, we demonstrate that MALAT1 plays an important role in regulating oxidative stress-mediated diseases and has the potential as a therapeutic target for the treatment of diseases caused by excessive exposure to ROS.

DEDICATION

I would like to dedicate this thesis to my grandmother, who passed away June 11, 2013. She was an inspiration, a truly loving human being and unconditionally loved me. She was the one always encouraged me to pursue my own dream. I would also like to thank my mother and father, for their deeply love and strong support. I would also like to thank my grandfather, for the love and support he gave me.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Yanan Tian of the Department of Veterinary Physiology and Pharmacology, Dr. James J Cai and Dr. Jane Welsh of the Department of Veterinary Integrative Science, and Dr. Ian Tizard of the Department of Veterinary Pathobiology.

All work conducted for this dissertation was completed independently by Jingshu Chen. Conduction for the dissertation was completed by the student independently.

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1. INTRODUCTION

Recent genome-wide transcriptome analysis and Encyclopedia of DNA Elements (ENCODE) project revealed that only 2-3% of the mammalian genome is transcribed into protein-coding RNAs (mRNAs) (Consortium 2012, Djebali, Davis et al. 2012), and approximately 90% of the human genome is utilized for generating transcripts which do not have protein-coding potential. These transcripts are defined as noncoding RNAs (ncRNAs) (Consortium 2004, Wang and Chang 2011). These noncoding transcripts outstrip the number protein-coding transcripts, with approximately 60,000 to 12,000 annotated in human genome in comparison to 23,000 coding genes (Hangauer, Vaughn et al. 2013). NcRNAs are numerous in kinds and functions and their identification and classification are constantly evolving. For now, ncRNAs have been divided into two major groups based on size: small ncRNA (<200bp including rRNA, miRNA, snRNA, snoRNA, siRNA and piRNA), and long noncoding RNAs, a major category of transcripts emerging from the pervasive transcription, which are loosely defined as RNAs>200nt in length and majority of these transcripts have no apparent coding capacity. Recently these lncRNAs are further subcategorized according to their position relative to protein-coding mRNAs, as long intergenic ncRNAs (lincRNAs), intronic lncRNAs and antisense lncRNAs (Rinn and Chang 2012, Mattick and Rinn 2015). The information regarding these lncRNAs has been emerging piecemeal and several well characterized lncRNAs have been found to regulate various functions such as genomic imprinting (H19 and Air), X chromosome inactivation (Xist), stem cell differentiation (lincRNA-RoR), cancer metastasis (HOTAIR and MALAT1), and riborepressor (Gas5), among many others (Kino, Hurt et al. 2010, Tsai, Spitale et al. 2011, Lee and Bartolomei

2013, Flynn and Chang 2014). For the most part, functions of majority of these pervasively transcribed RNAs remain poorly understood. Much of the scientific community considered that vast majority of long ncRNAs (lncRNAs) are non-functional (Babak, Blencowe et al. 2005, van Bakel, Nislow et al. 2010, Palazzo and Lee 2015), while a statistic analysis showed striking correlation between the number of lncRNAs with organism complexity (Figure 1), suggesting a potential role of lnRNAs in driving evolution and complexity in animals (Amaral, Dinger et al. 2008, Mercer, Dinger et al. 2008, Wilusz, Sunwoo et al. 2009, Kapusta and Feschotte 2014). It has not been possible to systemically analyze the lncRNA until the recent advent of the massive parallel deep sequencing of nucleic acids, making it possible to illuminate the "dark matter" of the genome space allowing a glimpse of the transcriptome landscape.

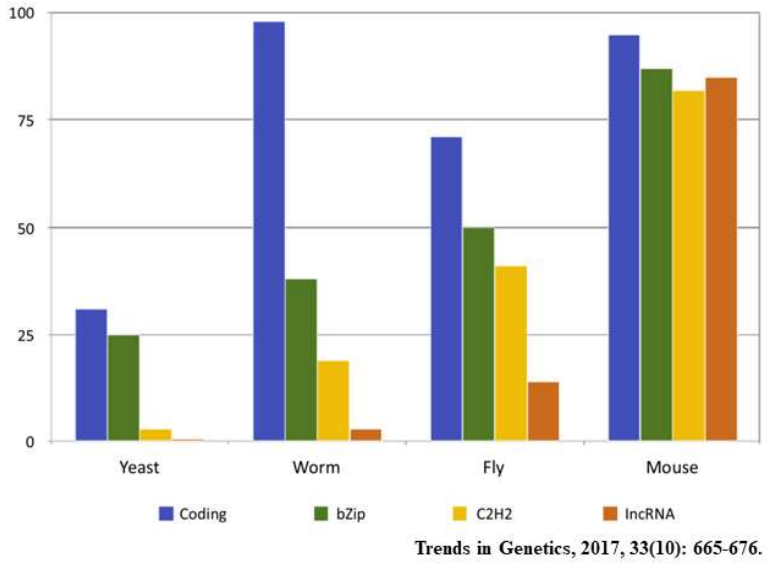

Figure 1. Comparative evolution of coding and long noncoding RNAs (lncRNAs). Numbers of coding genes (blue) and long noncoding genes (red) are shown relative to the numbers found in humans. While the numbers of coding genes do not correlate well with levels of organismal complexity, the exponential increase in the numbers of lncRNA genes does. Similarly, the numbers of C2H2 zinc-finger proteins (yellow) also show an exponential increase as opposed to the more linear increases in other types of transcription factors (bZip proteins shown in green).

To date, over 50,000 human lncRNAs have been identified (Iyer, Niknafs et al. 2015) and there have been many studies that showed these lncRNAs playing important roles in molecular functions including RNA processing, transcriptional and post-transcriptional regulation of gene expression and serving as precursors of small RNAs (Mercer, Dinger et al. 2009, Xie, Yuan et al. 2013). Many lncRNAs are "mRNA-like" possessing with a 5'cap and a polyadenylated (poly [A]) tail, while some of the lncRNAs with abundant expression are processed differently lacking these canonical structures (Wilusz 2016). It was also found that these lncRNAs possessed complex secondary and higher order structures, making them versatile for interaction with proteins and target genes. There is emerging evidence showing that many lncRNAs are involved in important biological processes, including cell proliferation (Ponting, Oliver et al. 2009), inflammation (Carpenter, Aiello et al. 2013, Atianand, Hu et al. 2016), genomic imprinting (Kanduri 2016), tumor progression and metastasis (Bhan, Soleimani et al. 2017). It has been hitherto difficult to associate the organisms complexity with the number of proteins coding genes and now as increasingly more lncRNA being discovered, striking correlation between the number of lncRNAs with organism complexity has been shown, suggesting a potential role of lnRNAs in driving the evolution (Amaral, Dinger et al. 2008, Mercer, Dinger et al. 2008, Wilusz, Sunwoo et al. 2009, Kapusta and Feschotte 2014).

The lncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) has been found as one of the most abundant (~3000 copies/cell) nuclear-retained lncRNAs in mammalian cells (Ji, Diederichs et al. 2003, Wilusz, Freier et al. 2008, Tripathi, Ellis et al. 2010). It is also known as nuclear-enriched abundant transcript 2 (NEAT2), HCN, LINC00047, NCRN00047 and PRO2853 (Hutchinson, Ensminger et al. 2007). MALAT1 was initially found to be associated with metastasis and poor patient survival in non-small cell lung cancer (Ji,

Diederichs et al. 2003). Unlike most other lnRNA which are expressed at low levels with a pattern of tissue specific expression, MALAT1 is highly conserved among mammalian species (Ji, Diederichs et al. 2003, Hutchinson, Ensminger et al. 2007, Bernard, Prasanth et al. 2010), and is ubiquitously expressed in almost all human tissues with the highest expression in the pancreas and lung (Ji, Diederichs et al. 2003). MALAT1 is one of the first identified lncRNAs to be associated with human diseases and has been extensively investigated (Gutschner, Hämmerle et al. 2013). Overexpression of MALAT1 has been found in various cancers, including lung cancers (Schmidt, Spieker et al. 2011), breast cancers (Praz, Jagannathan et al. 2004, Ellis, Ding et al. 2012), pancreatic cancers (Pang, Yang et al. 2015), myeloma (Amodio, Stamato et al. 2018), collectively suggesting that MALAT1 is a negative prognostic factor. Our previous studies show that the MALAT1 level is highly regulated during certain physiological processes, such as stress conditions induced by LPS, hyperglycemia and exposure to the xenobiotics (Chen, Ke et al. 2018). The up-regulation by the stress conditions suggests that transcriptional regulation is involved however, the mechanism involving the regulation has not been well understood. In this introduction section, we will discuss the latest finding on the roles of MALAT1 in regulating human metabolic diseases and underlying mechanisms of the actions. We will also discuss therapeutic potential of MALAT1 as the target for treating these diseases.

1.1 Structure and Biogenesis of MALAT1

MALAT1 gene is located in chromosome 11q13 in human, and in chromosome 19qA in mouse (Zhang, Arun et al. 2012, Wilusz 2016). It is ubiquitously expressed in every tissue, and the expression level is comparable or even higher than house-keeping protein coding genes such as □-actin and GAPDH (Zhang, Arun et al. 2012).

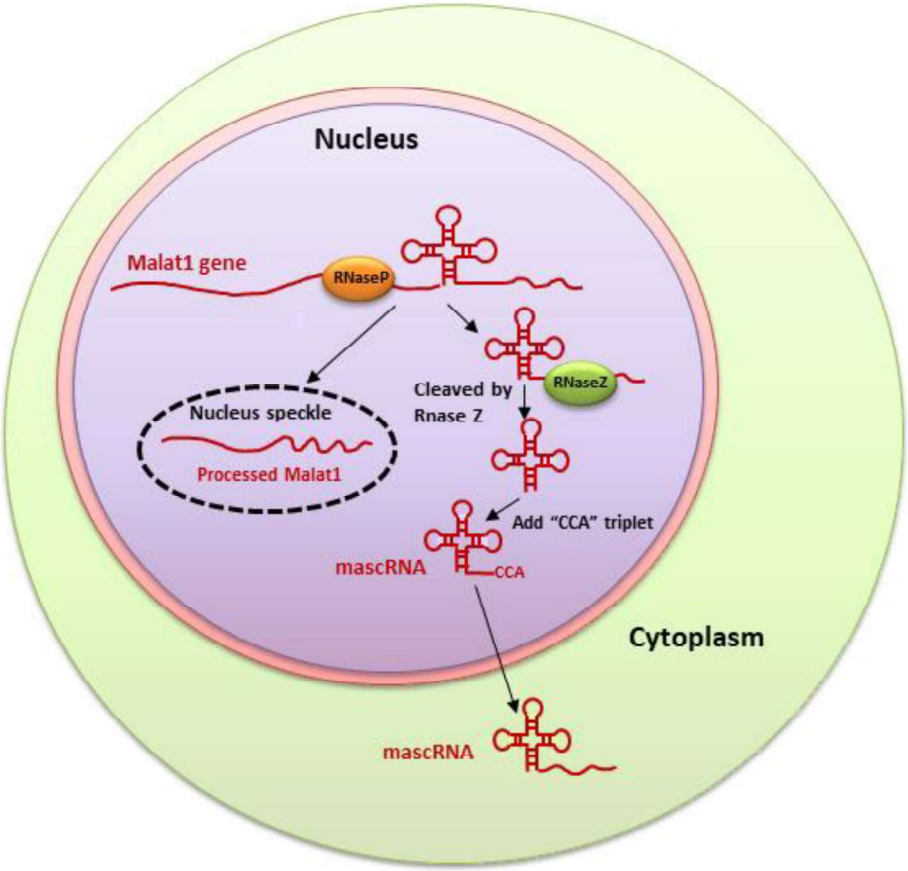

Figure 2. Biogenesis of MALAT1. Nascent MALAT1 transcript forms a tRNA-like structure at its 3' end, which can be recognized and processed by RNase P to generate stable MALAT1 with a U-A-U triple-helical structure at the 3' end, which can be recognized and binded by methyltransferase-like protein 16 (METTL16). The 3' end product was further cleaved by RNase Z to form mascRNA, which is ~60 nucleotides in length with unknown function.

MALAT1 expression is initiated from three different promoter regions. The major transcriptional start site is mapped to transcript FJ209305.1. The 3′-end processing of MALAT1 yields an additional short transcript (mascRNA). Unlike canonical mRNA processing by cleavage and polyadenylation machinery, MALAT1 transcript forms a tRNA like structure on its 3' end, which is recognized and cleaved by RNase P to generate a mature and stable transcript about 6.7 kb in length with a U-A.U triple-helical structure (. Denotes the Hoogsteen face and – denotes the Waston-Crick face) (Brown, Valenstein et al. 2012, Wilusz, JnBaptiste et al. 2012). The processed transcript was found to localize to nuclear speckles (Brown, Valenstein et al. 2012, Brown, Bulkley et al. 2014). The 3' end is further cleaved by RNase Z and form the mascRNA (MALAT1-associated small cytoplasmic RNA), which is around 60 nucleotides becomes localized in the cytoplasm with undefined functions (Wilusz, Freier et al. 2008, Sunwoo, Dinger et al. 2009, Brown, Valenstein et al. 2012) (Figure 2). The triple-helix structure at the 3' end of MALAT1 RNA is found to protect the 3' end from 3'-5' exonuclease (25). Brown et al. recently showed the detailed crystal structure of this triple-helix, and determine function of each nucleotide in maintaining the atypical triple-helix structure (Brown, Bulkley et al. 2014) and the stability of processed MALAT1 (Wilusz, JnBaptiste et al. 2012). The triple helix structure of MALAT1 was found to bind to methyltransferase-like protein 16 (METTL16), which is a putative RNA methyltransferase (Brown, Bulkley et al. 2014, Ruszkowska, Ruszkowski et al. 2018). With the triple helix structure in 3' end, MALAT1 was found to be generally stable in a variety of cells, including human B cells (Grote and Herrmann 2015), Hela cells (Tani, Mizutani et al. 2012), mouse 3T3 cells (Clark, Johnston et al. 2012), MEF cells (Bernard, Prasanth et al. 2010) and cancer cells (Tani, Nakamura et al. 2010). The degradation of the lncRNA is generally resemble that of the mRNA. Specifically, for MALAT1 however, its degradation pathway is not clear at

moment and because of its level is relevant to the prognosis of the cancers, how MALAT1 is degraded warrant further investigation. It was reported that Drosha-DGCR8 complex (a component of the Microprocessor) binds and controls the stability of MALAT1 by interacting with 5'end of MALAT1 transcript (Macias, Plass et al. 2012), and microRNA-9 (miR9) targeted AGO2-mediated regulation of MALAT1 for its degradation in the nucleus (Leucci, Patella et al. 2013).

1.2 The Role of MALAT1 Regulating Alternative pre-mRNA Splicing and Global Gene Expression

MALAT1 is a component of the splicing factor, as such perhaps it is not surprising that it is of high abundance and ubiquitously expressed in various cells and tissues. As one of the most abundant lncRNA in normal cells and tissues, MALAT1 has been investigated in multiple fields including its molecular function in nucleus. It was first published by Hutchinson et al. that MALAT1 co-localized with SC35 nuclear speckles using RNA fluorescent in situ hybridization (FISH) and protein immunofluorescent (IP) staining (Hutchinson, Ensminger et al. 2007). MALAT1 was then found to interact with the serine/arginine-rich family of splicing factors, and regulate alternative splicing of pre-mRNAs (Tripathi, Ellis et al. 2010). With the development of CHART-seq (Capture hybridization analysis of RNA targets) which is a technique used to identify the genomic binding sites of lncRNAs by purifying formaldehyde-cross-linked complexes using antisense oligonucleotides directed against specific lncRNAs, West et al. identified hundreds of MALAT1 binding sites which mostly enriched in the actively transcribed genes in human cells, indicating the function of MALAT1 in globally regulating RNA processing during gene transcription (West, Davis et al. 2014). It was also shown by Engreitz et al. using RNA antisense purification (RAP) method to show that MALAT1 interacted with associated U1

small nuclear RNA, a component of the spliceosome, and indirectly with nascent transcripts through proteins factors allowing MALAT1 to localize to chromatin sites containing actively transcribed genes (Engreitz, Sirokman et al. 2014). These two independent reports (Engreitz, Sirokman et al. 2014, West, Davis et al. 2014) showed the MALAT1 interacted actively with transcribed gene loci, suggesting the function of MALAT1 in globally regulating gene expression through regulating RNA processing.

1.3 Physiological and Pathological Function of MALAT1

In 2012, there were three MALAT1 knockout mouse models generated by different strategies in three independent laboratories, specifically Zhang et.al removed a 3kb genomic region encompassing promoter and 5' end region of MALAT1 (Zhang, Arun et al. 2012), Eissmann et al. knocked out the entire 7kb sequence of MALAT1 in mouse (Eißmann, Gutschner et al. 2012) and Nakagawa et al. disrupted MALA1 gene expression through inserting a transcriptional terminator (lacZ and the polyadenylation sequences) in the promoter region (Nakagawa, Ip et al. 2012) (Figure 3).

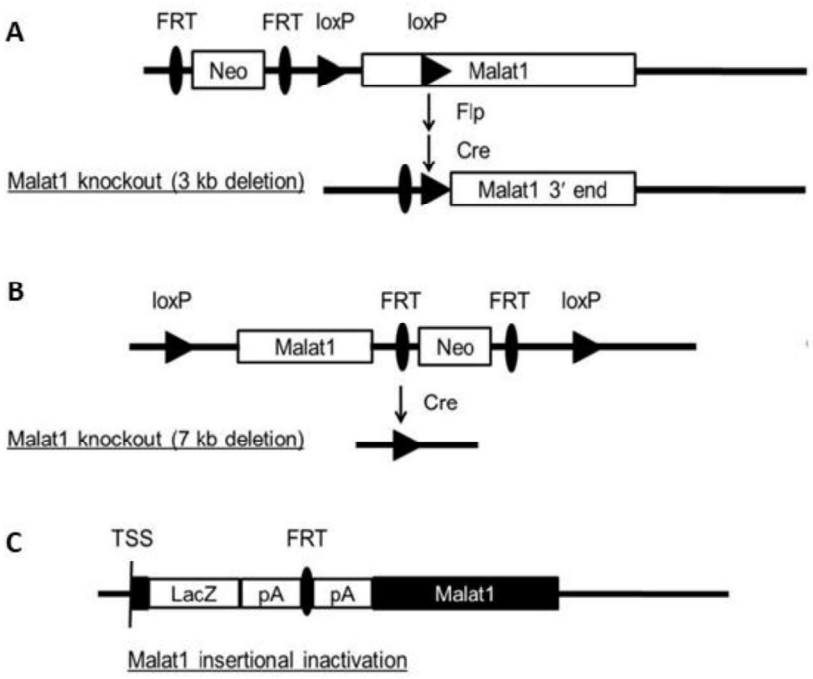

Figure 3. Different strategies used to generate MALAT1 knockout mice. (A) A 3 kb genomic region encompassing the 50 end of Malat1 and its promoter was deleted. (B) The full-length MALAT1, including 250 bp upstream of the transcriptional start site and 321 bp downstream of the 3 0 end of Malat1, was deleted. (C) The β-galactosidase gene (lacZ) with polyadenylation sequences (pA) was inserted 69 bp downstream of the transcriptional start site (TSS) of Malat1.

However, despite of the MALAT1 pervasively interacting with whole genome, MALAT1 null mouse models generated by these three independent laboratories showed no overt phenotype (Eißmann, Gutschner et al. 2012, Nakagawa, Ip et al. 2012, Zhang, Arun et al. 2012). Loss of MALAT1 in mouse did not affect global gene expression, nuclear speckles or alternative pre-mRNA splicing (Nakagawa, Ip et al. 2012). Collectively these findings lead us to consider MALAT1 may have a stress-dependent function *in vivo*. In our study, we found MALAT1 interacts with Nrf2 pathway and genetic ablation of MALAT1 leads to activation of Nrf2 pathway in the MALAT1 null cells (Chen, Ke et al. 2018) (Figure 4). Interestingly, the expression of NEAT1, which is located 30kb 5' side from MALAT1, is upregulated in MALAT1 null mice as shown in our results and others (Eißmann, Gutschner et al. 2012, Zhang, Arun et al. 2012), suggesting the possibility of additional factors compensate for the effect of MALAT1 ablation in mice.

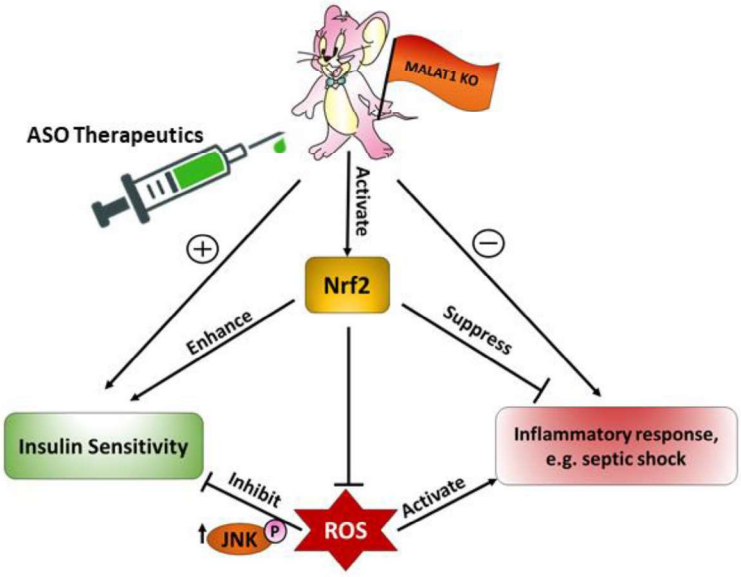

Figure 4. Proposed mechanism for MALAT1-regulated interactions between insulin signaling pathway and inflammatory response. MALAT1 ablation activates Nrf2 pathway, quenches ROS, and inhibits inflammatory responses leading to sensitized insulin signaling pathway. ASO, antisense oligonucleotides.

Increases in the prevalence of type 2 diabetes mellitus (T2DM) have become a global health crisis in recent years posing a serious threat to public health (Maruthur 2013). Diabetes mellitus is a clinical condition characterized by hyperglycemia either due to impaired insulin signaling pathway or decreased insulin secretion function of pancreatic islets. The T2DM is a metabolic disease with prolonged exposure to hyperglycemia which causes the oxidative stress which is a major underpinning etiological factor for various organ dysfunction and including brain, eye, heart, liver and kidney (Deshpande, Harris-Hayes et al. 2008, Hanefeld, Duetting et al. 2013). Recent studies including our new findings have shown that MALAT1 played a significant role in the various pathophysiological processes including oxidative stress, inflammation, glucose metabolism, insulin resistance, pancreatic islets dysfunction and diabetes progression (Chen, Ke et al. 2018, Lei, Chen et al. 2019). In this session, we will focus on the correlation between MALAT1 and diabetes-related complications and the underpinning mechanism. Upregulation of MALAT1 expression level has been found to be correlated with various of diabetes-related diseases in diabetes mouse model and T2DM patients. It was firstly published in 2015 that the expression of MALAT1 was upregulated in high-glucose treated endothelial cells as well as in kidney from streptozocin (STZ)-induced diabetic mouse, paralleled by upregulation of expression of genes involved in inflammation including serum amyloid antigen 3 (SAA3), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) (Puthanveetil, Chen et al. 2015). MALAT1 expression was also found to be significantly upregulated in the retinas of diabetic mice (*db/db* mice), in high-glucose retreated retinal endothelial cell line (RF/6A) and in the aqueous humor samples and fibrovascular membranes of diabetic patients (Yan, Tao et al. 2014). Based on these results, the same group further investigated the function of MALAT1 in regulating retinal function in diabetic rats, and found that silencing of MALAT1 using MALAT1

shRNA adenovirus decreased diabetes-induced apoptotic in RF/6A cells, reduced retinal inflammation and hyper-proliferation of retinal endothelial cells through p38 mitogen-activated protein-kinase (MAPK) signaling pathway, and ultimately alleviated retinal vessel impairment in diabetic condition in rats (Liu, Yao et al. 2014).

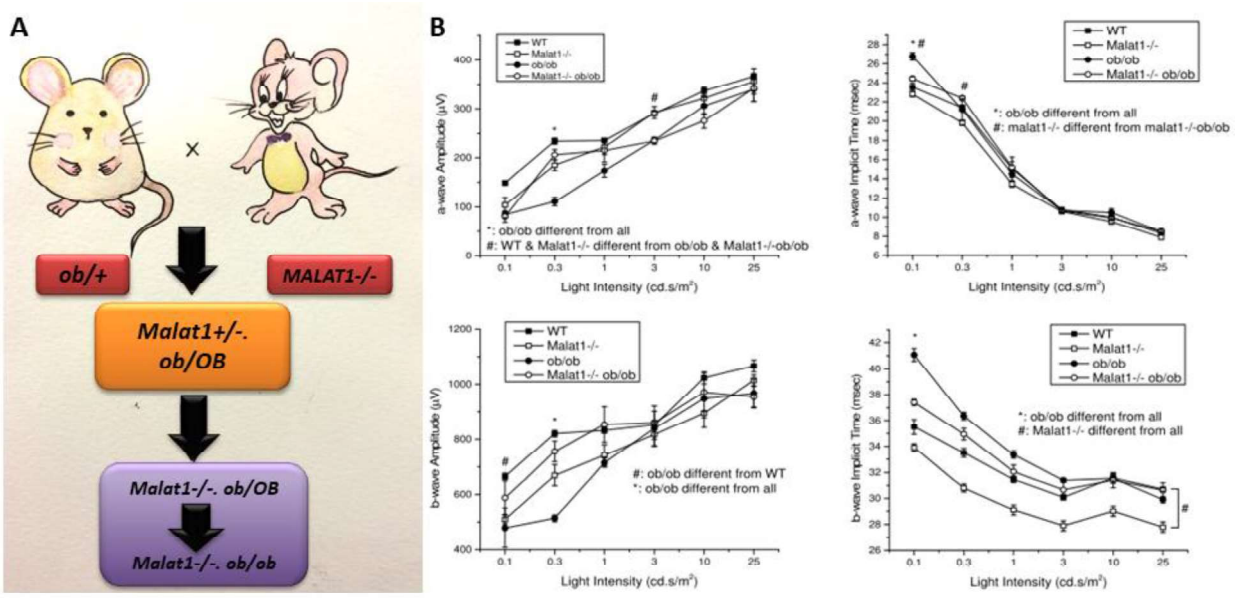

Figure 5. Ablation of MALAT1 ameliorates retinal function in *ob/ob* mice. (A) Generation of MALAT1 null mice in ob/ob background. (B) Electroretinogram (ERG) was recorded in anesthetized mice, including wild-type mice (WT), MALAT1 null mice (Malat1-/-), *ob/ob* diabetic mice (*ob/ob*) and MALAT1 ablated ob/ob mice (Malat1-/- *ob/ob*). The representative ERG-waves for different experimental groups are shown. Amplitudes of a- and b-were statistically analyzed.

Interestingly, our lab also found MALAT1 regulate the retinal function using ob/ob diabetes mouse model (Figure 5). We generated homozygous *MALAT1* $^{(-/-)}$*.ob/ob* mice by crossbreeding MALAT1 null mice with *ob/+* mice (Figure 5) and investigated the visual function by conducting visual electrophysiology experiments in the established mouse model. The amplitudes of a- and b-waves were found to be significantly reduced and the b-wave implicit time was significantly increased in the ob/ob mice, indicating that these obesity mice had worsen light responses and impaired visual function compared to wild type group. Ablation of MALAT1 in ob/ob mice alleviated the impaired visual function with improved amplitudes of a- and b- wave and significantly decreased b-wave implicit time, suggesting the MALAT1 ablation prevents electroretinogram (ERG) abnormality caused by obesity-induced diabetes in mice (unpublished data). The upregulation of MALAT1 expression level was also observed in the serum of T2DM patients (90 patients with 56 males and 34 females with diagnosed T2DM) compared to healthy individuals ($p<0.05$) (Liu, Zheng et al. 2019). Data from the clinical T2DM patient sample showed MALAT1 expression was positively correlation with HOMA-IR (homeostatic model assessment of insulin resistance) and resistin which is a adipocyte-derived hormone contributing to insulin resistance in the serum (Habib 2012, Liu, Zheng et al. 2019).

It has been shown by us and others that MALAT1 plays an important role in regulating insulin signaling pathway in mouse T2DM models (Yan, Chen et al. 2016, Chen, Ke et al. 2018), the human clinical evidence of the role of MALAT1 await to be discovered. Yan et al. showed that knock down of MALAT1 could decreased hepatic lipid accumulation by downregulating nuclear SREBP-1a protein level, and prevented insulin resistance both *in vivo* and *in vitro*. Using MALAT1 null mice model, we first showed ablation of MALAT1 sensitized insulin signaling pathway through decreasing ROS generation and improving antioxidant/detoxification signaling

pathway (Nrf2/ARE-driven pathway) (Figure 4). Furthermore, our newly finding showed MALAT1 ablation could alleviate obesity-induced hyperglycemia, hyperinsulinemia, and insulin resistance, with significantly decreased lipid accumulation in liver and visceral adipose tissue in *ob/ob* T2DM mouse model (unpublished data). These results shown by us and others provided strong evidence that MALAT1 could be an important therapeutic target for metabolic diseases.

MALAT1 was also found to regulate the function of pancreatic islets. It was reported that MALAT1 regulated the insulin secretion function through interaction with microRNA (miR)-17 and TXNIP protein (thioredoxin-interacting protein) in Min6 cells (a pancreatic β cell line) treated with cigarette smoke extract. Min6 cells treated with cigarette smoke extract showed increased MALAT1 expression and impaired insulin secretion function. Silencing of MALAT1 decreased the TXNIP level through increasing the expression of miR-17, alleviating the dysregulation of insulin secretion function in cigarette smoke extract treated Min6 cells. We have observed significantly enhanced insulin secretion function by performing glucose stimulated insulin secretion (GSIS) test using primary pancreatic islets isolated from MALAT1 null mice. With the recently rapid development of single-cell sequencing technique, it is one of the future directions to compare the cell subpopulation within the pancreas between MALAT1 null and wild type mice and investigate the molecular mechanisms regarding the transcriptome analysis (Carter, Bihannic et al. 2018, Zeisel, Hochgerner et al. 2018, Kalamakis, Brune et al. 2019, Mickelsen, Bolisetty et al. 2019).

1.4 Regulation of Epitranscriptomic Machinery by MALAT1

One interesting mechanistic connection between MALAT1 and global gene regulation warrant further investigation is the regulation of the methionine cycle by MALAT1 which may impact the homeostasis of the methyl donor and antioxidant capacity.

In mammalian methionine metabolism, S-adenosylmethionine (SAM) is produced, which occupies a central position in the metabolism of all cells as an essential methyl donor to maintain normal methylation of DNA, RNA, histones as well as synthesis of glutathione which is important for cellular redox system (Figure 6). It has been found that in human clinics and animal models, methionine metabolism is disrupted with greatly increases in the oxidative stress levels as indicated by the dysregulated GSH/GSSG level (Biolo, Antonione et al. 2007, Dong, Hu et al. 2013).

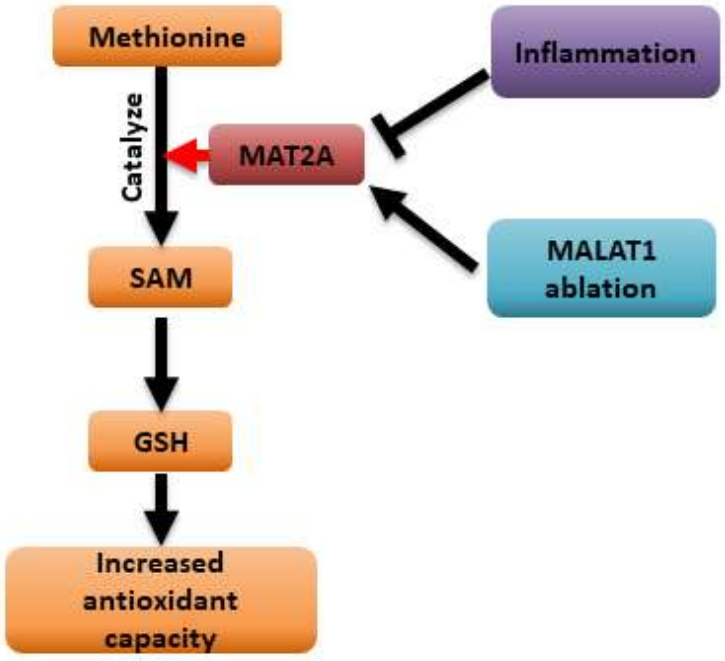

Figure 6. Schematic illustration of epitranscriptomic regulation of methionine cycle and putative role of MALAT1-RNA methylation regulating sepsis.

Methionine adenosyltransferase (MAT2A) is essential for the biosynthesis of SAM and the gene expression of MAT2A is regulated at transcriptional and post-transcriptional levels through m6A RNA methylation of the 3'UTR of MAT2A mRNA (Figs 4 and 5) (Pendleton, Chen et al. 2017, Shima, Matsumoto et al. 2017) . Post-transcriptionally, the mRNA of MAT2A stability is regulated by the m6A methylation mediated by RNA methylation writer METTL16 which methylates 3'UTR of MAT2A, thus controlling its level. Mechanistically, strong evidence from structural analysis indicates among the methylation sites of MAT2A, the hairpin 1(hp1) is the linchpin for regulating MAT2A expression (Doxtader, Wang et al. 2018) by regulating intron splicing(Pendleton, Chen et al. 2017) and mRNA decay(Shima, Matsumoto et al. 2017) of MAT2A, thus controlling methionine cycle (Figure 7). METTL16 forms a complex with MALAT1(Brown, Kinzig et al. 2016). The METTL16-binding site, which is the highly conserved triple helix, is present in every MALAT1 homolog (Stadler 2010, Zhang, Mao et al. 2017), thus it is highly likely MALAT1-associated proteins such as methyltransferases (METTLE 13/14 and16) may in turn modify the functions of MALAT1 thereby regulating gene expressions involving in wide range of the physiological processes.

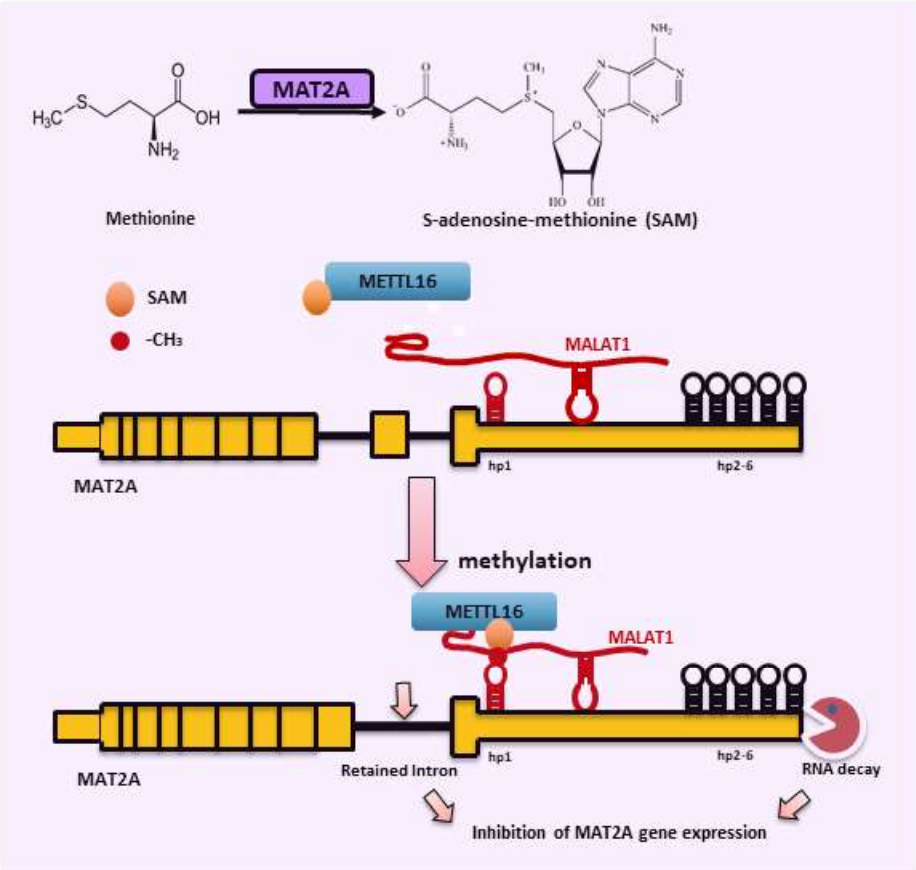

Figure 7. MALAT1 regulates MAT2A gene expression by interaction with METTL16 which methylates hairpin 1(hp1), a linchpin for controlling intron retention and/or mRNA decay of MAT2A.

1.5 Future Therapeutic Interventions Targeting MALAT1

MALAT1 over expression has been found to be associated with the progression of various types of cancers and oxidative stress-associated diseases. MALAT1 knockout mice are viable and develop normally, making MALAT1 an attractive target for treating these diseases through RNA silencing/knocking down approaches. For targeting MALAT1, one of the efficient way is utilizing the antisense oligo nucleotides (ASO) LNA GapmeRs, which are single-stranded oligonucleotides that consist of a DNA stretch flanked by LNA nucleotides to inhibit/knockdown MALAT1 *in vivo* (Au - Lin, Au - Hu et al. 2018). Previous researches have used modified antisense oligonucleotide base pairing with the targeted lncRNA in the nucleus induces degradation by an RNase H-dependent mechanism.

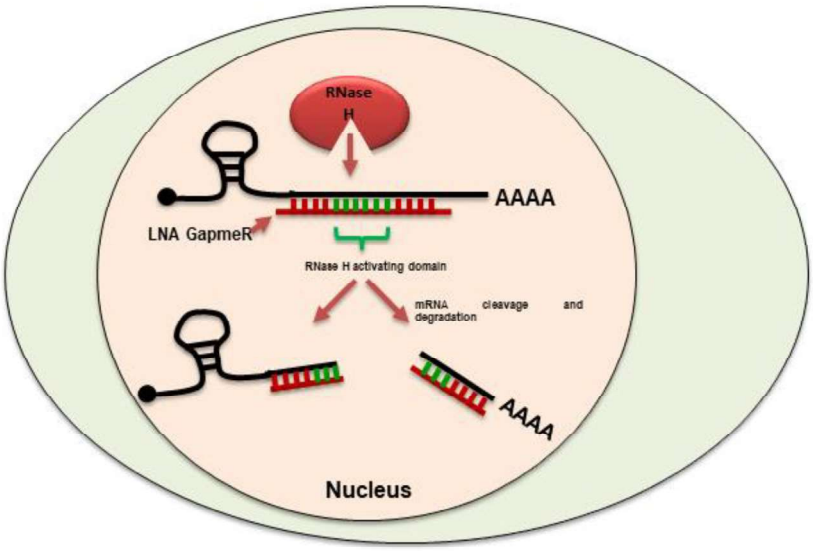

Figure 8. Structure of LNA GapmeRs and mechanism of action. LNA GapmeRs consist of two outer blocks of modified LNA nucleotides flanking an unmodified central stretch of bases. LNA GapmeRs bind to RNA transcripts by complementary base paring and are only able to bind in regions devoid of secondary structure. The unmodified central bases form an RNA/DNA duplex that is recognized and cleaved by RNase H through the RNA strand, resulting in degradation of the target RNA MALAT1.

Locked nucleic acid (LNA)–ONs are a new generation of antisense ONs. Unlike previous ON designs, the ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon nucleoside (Braasch and Corey 2001, Orum and Wengel 2001, Li, Xu et al. 2003, Vester and Wengel 2004). This modification provides ONs with an exceptionally high binding affinity for mRNA compared with conventional DNA ONs. Additionally, LNA–ONs are resistant to nucleases and therefore provide exceptional stability in plasma and cell culture medium (Figure 8) (Jepsen, Sørensen et al. 2004, Karkare and Bhatnagar 2006, GrÜnweiler and Hartmann 2007, Veedu and Wengel 2009). Many complex metabolic diseases such as T2DM, have proven to be resistant to therapeutic approaches based on single medicines. The mechanistic studies have revealed that MALAT1 interacts with Nrf2 (Chen, Ke et al. 2018), which has multiple natural and synthetic activators, suggesting a combinatory targeting strategy aiming at "MALAT1-Nrf2 axis" further broadening the therapeutic potential.

2. LONG NONCODING RNA MALAT1 REGULATES GENERATION OF REACTIVE OXYGEN SPECIES AND THE INSULIN RESPONSES IN MALE MICE*

2.1 Introduction

Insulin resistance occurs in the majority of patients with type 2 diabetes mellitus (T2DM) and the excessive exposure to the reactive oxygen species (ROS) induced by various stressors including inflammatory cytokines and hyperglycemia is an important mechanism in causing insulin resistance (Houstis, Rosen et al. 2006, Giacco and Brownlee 2010, Maruthur 2013). The NF-E2 p45-related factor 2 (Nrf2) is a cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factor, which plays a causative role in protecting cells from prooxidants and electrophiles by regulating antioxidant response element (ARE)–mediated expression of detoxifying and antioxidant enzymes (Itoh, Chiba et al. 1997). Suppression of Nrf2/ARE activity contributes to oxidative stress and mitochondrial dysfunction and has been found to be one of the factors in dozens of others which are involved in developing of insulin resistance (Cheng, Siow et al. 2011). Previous results showed that suppression of Nrf2 activity led to oxidative stress-induced insulin resistance and downregulated glucose utilization (Tan, Ichikawa et al. 2011).

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One molecular mechanism for insulin resistance is characterized by impairment of the insulin-induced activation of insulin receptor substrate (IRS)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway. One mechanism for ROS-induced insulin insensitivity is through activating stress-sensitive c-Jun N-terminal kinases (JNK) which, when activated by ROS, suppresses insulin signaling through inhibition of the major metabolic action of insulin including phosphorylation of IRS and Akt (Haruta, Uno et al. 2000, Greene, Sakaue et al. 2003, Gual, Le Marchand-Brustel et al. 2005, Hiratani, Haruta et al. 2005). It has been found that genetic deletion of JNK1 in mice resulted in improved insulin sensitivity in part through protection of pancreatic β-cells against oxidative stress, suggesting a central role for JNK in the development of T2DM (Hirosumi, Tuncman et al. 2002, Kaneto, Nakatani et al. 2006).

Glucose homeostasis is governed by many mechanisms involving complex interaction of a constellation of transcriptional and post-transcriptional regulatory factors (Thomsen, Ceroni et al. 2016). In addition to protein-coding genes, recent genomic studies have shown that mammalian genomes encode numerous noncoding RNAs (ncRNAs), which are emerging as important regulators for diverse cellular processes including lipid metabolism and carcinogenesis (Cabili, Trapnell et al. 2011). Noncoding RNAs can be broadly classified into small (18-200nt) and long noncoding RNAs (lncRNAs), which are longer than 200nt (Prasanth and Spector 2007). Although the physiological functions of most lncRNAs have not been well understood, some lncRNAs including MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) have been found to play critical roles in disease processes such as the carcinogenesis.

MALAT1 was initially reported to be a prognostic marker in stage I lung adenocarcinoma (Ji, Diederichs et al. 2003). Over expression of MALAT1 has now been found to correlate with poor prognosis of wide range of cancers (Gutschner, Hämmerle et al. 2013, Liu, Yao et al. 2014,

Ma, Wang et al. 2015, Puthanveetil, Chen et al. 2015). Interestingly, bioinformatic analysis of MALAT1 reveals its role in regulation of broad range of genes by regulating various aspects of transcription including pre-mRNA processing and transcription elongation (Tripathi, Song et al. 2012). Consistent with its pervasive involvement in gene regulation, MALAT1 has recently been reported to play a role in regulating lipid and glucose metabolism (Yan, Chen et al. 2016) and MALAT1 was found to stimulate production of inflammatory cytokines in the endothelial cells under treatment with high glucose (Puthanveetil, Chen et al. 2015). In the cultured endothelial cells, lentiviral expression of MALAT1 has been found to regulate Nrf2 expression (Zeng, Zhang et al. 2018).

Despite of the pervasive involvement of MALAT1 in gene regulation, ablation of MALAT1 gene in mice does not result in overt phenotype (Gutschner, Hämmerle et al. 2013). However, in transcriptome analysis we found significantly increased gene expressions of antioxidant/detoxification pathways including Nrf2/Kelch-like ECH-associated protein 1 (Keap1) in the MALAT1 null hepatocytes with significantly decreased ROS generation in hepatocytes and isolated pancreatic islets in response to the oxidative stimuli (Figure 9). The reduced ROS status in MALAT1 null mice is associated with enhanced insulin signaling capacity with improved insulin-signaling responses to fast-refeeding and glucose/insulin challenges indicating an increased insulin sensitivity. Our results suggest that MALAT1 may be utilized as a therapeutic target for the treatment of diabetes as well as other diseases caused by excessive exposure to ROS.

2.2 Materials and Methods

2.2.1 Reagents

Lipopolysaccharide (LPS), Fluorescent dye 2',7'-dichlorofluorescein (DCFH-DA) and Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) were purchased from Sigma. The primary antibodies used include those against phosphorylated c-Jun NH2 -terminal kinase (p-JNK), JNK, p-Akt (serine 473), Akt, and insulin receptor substrate 1 (IRS1) were purchased from Santa Cruz. Anti-insulin and anti-glucagon fluorescence staining antibody were purchased from eBiosience. Protein carbonyl content assay kit was purchased from Abcam. TNF-α was purchased from Roche. Fetal bovine serum, penicillin and streptomycin were purchased from Gibco.

2.2.2 Animals, drug treatment and metabolic parameters

MALAT1 null mice were from Texas A&M Institute for Genomic Medicine (TIGM) and randomly assigned to different treatments. Mice received a bolus injection of 1 unit/kg insulin or saline through a portal vein, and the liver tissues were harvested 5 min after the injection. Glucose tolerance tests were performed after mice were fasted overnight (16 hr) and injected intraperitoneally (i.p.) with glucose (in saline) at 2 g/kg. Insulin tolerance tests were performed by i.p. injecting insulin (0.75 unit/kg of body weight), and measuring blood glucose before (time = 0) and 15, 30, 60 and 120 min after injection. Blood glucose, insulin and glucagon were measured by using commercial assay kits (Nanajing Jiangcheng).

2.2.3 Isolation of mouse primary hepatocytes

The procedure for isolation of mouse primary hepatocytes was performed according to method described previously with modification (Gonçalves, Vigário et al. 2007). Briefly, mice were anesthetized (2.5% avertin, 0.4 mL/20g) and the livers was perfused through a needle

aligned along the inferior vena cava, with PBS buffer (pH 7.5) containing 0.5 mmol/L EGTA; followed by collagenase containing buffer, pH 7.5 which consists of 1×HBSS (Gibco) with 0.15 g/L collagenase type 2 (Worthington Biochemical Corporation). The collagenase-perfused liver was then dissected, suspended in DMEM (Gibco) without FBS, and filtered through cheesecloth and a 40 μm nylon membrane to remove connective tissue debris. Hepatocytes were subjected to centrifugation (65 g, 2 min at 4°C) and resuspended in DMEM with 10% FBS. Afterwards the hepatocytes were purified using density gradient centrifugation (50% Percoll solution, 65 g for 10 min at 4°C).

2.2.4 Immunohistochemistry

Immunohistochemical staining was performed in pancreatic tissues from MALAT1 null mice and wild type mice as described (Ouyang, Ke et al. 2010). Paraffin-embedded sections (4 mm thick) were deparaffinized, re-hydrated and microwave-heated for 15 min in 0.01 mol L^{-1} citric acid buffer (pH 6.0) for antigen retrieval. Then, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 15 min of blocking with normal serum (Invitrogen), the sections were incubated with antibodies (anti-insulin and anti-glucagon) overnight at 4 °C. Slides were washed three times with PBS, each for 5 min. After rinsing with PBS, the slides were immersed for 10 min in 3,3 0 -diaminobenzidine (Sigma) solution (0.4 mg ml^{-1}, with 0.003% hydrogen peroxide), and processes were monitored with microscope and reaction was terminated with distilled water, counterstained with haematoxylin, dehydrated, and coverslipped. The working concentration of all primary antibodies was 2 μg/ml.

2.2.5 ROS measurement

The level of oxidative stress was monitored by measuring the ROS (Swift and Sarvazyan 2000). The cultured cells treated for 12 h with high glucose, LPS and TNF-α were washed with

PBS and incubated with 5 μM solution of fluorescent probe (H2-DCFH-DA dissolved in culture medium without serum) at 37 °C for 60 min. The cultured cells were then washed three times with PBS and fluorescent signals were determined by (1) images analysis with an inverted fluorescence microscope (Olympus IX71), (2) signals were recorded and quantified with a fluorescence microplate reader at 488 nm, and (3) flow cytometry analysis using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with an excitation at 488 nm and an emission at 620 nm. Triplicate experiments were performed. For protein carbonylation assays, carbonyl levels were determined either by Western blotting with 10 μg per samples of total protein lysates or by ELISA using a commercial kit (Abcam) based on the manufacturer's instructions.

2.2.6 Western blot and immunoprecipitation

Western blot and immunopreciptation were performed as described (Tian, Ke et al. 1999). Mouse liver tissues were homogenized in the lysis buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EGTA, 2 mM Na_3VO_4 , 50 mM NaF, 20 m M $ZnCl_2$, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1×complete protease inhibitor mixture (Sigma)). After centrifugation (12,000×g in a microcentrifuge at 4 °C for 15 min), supernatant fractions were collected and incubated with antibodies and GammaBind Plus-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C on a rotary shaker. Proteins were analyzed by SDS-PAGE and then transferred to a nitrocellulose membrane. Individual proteins were detected with the specified antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Millipore).

2.2.7 Pancreatic islet cell isolation

Islets were isolated based on a published procedure (Szot, Koudria et al. 2007). Briefly, mice were killed by cervical dislocation. The pancreas was inflated with 3 mL of cold collagenase

solution (0.3 mg/mL) (Collagenase type 4, Worthington Biochemical Corporation) through the common bile duct with a 20 G needle, starting at the gall bladder. The pancreas was then removed from the body and placed in a siliconized vial containing 2 mL of 1 mg/mL collagenase solution and digested at 37 °C in a water bath for 12∼15 min. After three washes of the digested pancreas with centrifugation (97 × g at 4 °C for 1 min) to collect the tissues, islets were purified by density gradient centrifugation (cold polysucrose/sodium diatrizoate solution 1.1119g/ml, 560 × g at 4 °C for 15 min) and then dispersed in 96 well plates and cultured in RPMI (Gibco) with 10% FBS for further experiments.

2.2.8 Glucose stimulated insulin secretion assay (GSIS)

To determine the islet functions and to confirm the success of isolation of the functional islets, the insulin and glucagon levels of the cultured islets was determined. Briefly, a stable baseline insulin response was established at 5.6 mmol/l glucose, greater than a threefold response to 16.7 mmol/l glucose was considered as normal threshold. Supernatant insulin and glucagon levels were measured by a highly sensitive sandwich ELISA assay. For determination of the effects of PCB126 the islets were treated with PCB126 (1, 10 and 100 nM, 12 hr) and endocrine levels were determined using the ELISA.

2.2.9 Real time-PCR

Total RNA of primary hepatocytes, isolated pancreatic islets cells and the liver and pancreas was isolated using the Trizol (Qiagen) according to the manufacturer's protocol. RNA was eluted using 50 μl of RNase-free water and then stored at -80°C. Real-time (RT)-PCR was performed using iTaq Universal SYBR Green One-step Kit (Bio-Rad, Hercules, CA). The following primers were used (Table 1).

Table 1. Mouse primers related to oxidative stress pathways for real-time PCR

Gene Name	Forward Primer	Reverse Primer
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT
MALAT1	TGAAAAAGGAAATGAGGAGAAAAG	CTTCACAAAACCTCCCTTTACAAT
NQO1	CGACAACGGTCCTTTCCAGA	CCAGACGGTTTCCAGACGTT
SOD1	TGCAGGGAACCATCCACTTCG	AACATGCCTCTCTTCATCCGC
NRF2	TAGATGACCATGAGTCGCTTGC	GCCAAACTTGCTCCATGTCC
CAT	GGAGGCGGGAACCCAATAG	GTGTGCCATCTCGTCAGTGAA
C-JUN	GCAGAAAGTCATGAACCACG	AGTCCATCTTGTGTACCCTTG
KEAP1	ATGGCCACACTTTTCTGGAC	TCCTGTTGTCAGTGCTCAGG
GSTA1	CGCCACCAAATATGACCTCT	CCTGTTGCCCACAAGGTAGT

2.2.10 RNA pulldown assay

For RNA pulldown, a sets of pooled 13 biotinylated anti-MALAT1 oligonucleotides evenly spaced across the entire length of MALAT1 were synthesized (Engreitz, Sirokman et al. 2014). The nuclear proteins were extracted from mouse primary hepatocytes in freshly prepared polysome lysis buffer based on the published procedure (Tsai, Manor et al. 2010). Biotinylated RNA (10 pmol) was incubated with

200 μg per sample of nuclear lysate (1 h, room temperature) and then prewashed streptavidin beads (Promega) was added to the nuclear lysate and incubated for one hour at room temperature. The beads were washed with ice-cold RIP buffer (150 mM KCL, 25 mM Tris pH 7.4, 0.5 mM DTT, 0.5% NP40, 1 mM PMSF and protease inhibitor) five times and then boiled with 2x Laemmli loading buffer.

2.2.11 Fast-refeeding, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) on HFD treated MALAT1 ablated mice and wild type controls

We analyzed the effects of MALAT1 ablation on insulin sensitivity in vivo using GTT

and ITT assay. GTT was performed after mice are fasted overnight (16 hr) and i.p. injected with glucose (in saline) at 2 g/kg of body weight. ITT was performed by injecting insulin via i.p. (0.75 unit/kg of body weight) and measuring blood glucose before (time = 0) and 15, 30, 60 and 120 min after injection. Blood glucose and insulin were measured by ELISA.

2.2.12 Knockdown of MALAT1 using antisense oligonucleotides (ASO)

ASOs have been identified as an optimal method to degrade lncRNAs which localize in nucleus (Lennox and Behlke 2015, Lundin, Gissberg et al. 2015) in cells and animals. We synthesized a set of LNA GapmeR anti-MALAT1 oligonucleotides (Exiqon) (Frank-Kamenetsky, Grefhorst et al. 2008). The primary hepatocytes and isolated pancreatic islets were treated with ASO (10nM) for 24 hours and the ROS levels was measured after treatment.

2.3 Result

2.3.1 MALAT1 null mice exhibit attenuated ROS generation in responses to oxidative stress

MALAT1 null mice do not exhibit an overt phenotype. However, in a transcriptome analysis of the MALAT1 null hepatocytes, we found significantly increased expression of the genes involved in ROS detoxification including Nrf2/keap1-regulated pathways and decreased the expression of genes involved in the ROS generations (Figure 9) and the results were confirmed by qPCR (Figure 15). Exposure to the excessive level of ROS is known to cause insulin resistance and failure of the pancreatic β cells (Robertson, Harmon et al. 2003). ROS accumulation in the β cell mitochondrial inner membrane is a metabolic stressor and adversely affects mitochondrial structure and function leading to β cell failure (Ma, Zhao et al. 2012). We next measured ROS generation (Swift and Sarvazyan 2000) in fluorescence microscopy, flow cytometry and quantitative analysis of the fluorescence in hepatocytes as well as isolated islets using fluorescent

dye 2',7'-dichlorofluorescein (DCFH-DA) (Figure 10 A-F). We found that the common ROS-inducing stress factors such as lipopolysaccharide (LPS), TNF-α and high glucose levels all induced high levels of ROS in the wild type mice, and strikingly the induction of ROS was greatly diminished in the MALAT1-null hepatocytes and islet cells (Figure 10 A-F).

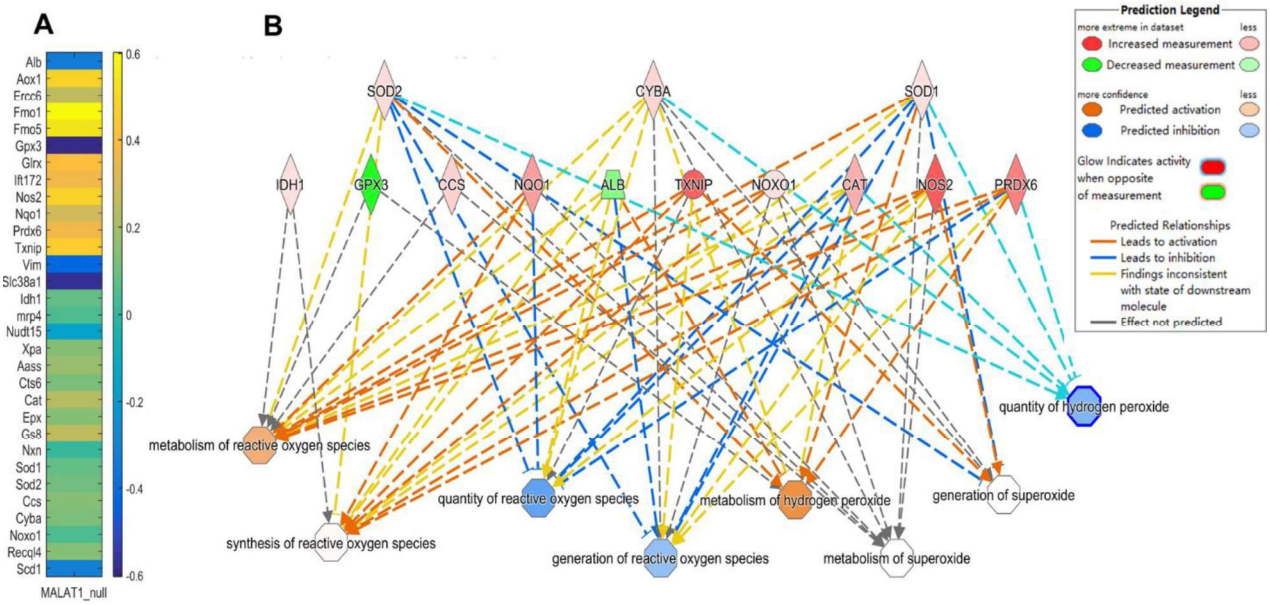

Figure 9. Ablation of MALAT1 suppresses ROS generation and enhances the ROS detoxification. A, Heat map illustration of differentially expressed genes related to ROS metabolism and detoxification in the MALAT1 ablated hepatocytes. B, MALAT1 ablation enhances the detoxification pathways of ROS and H2O2 and reduces the generation of ROS and H2O2 as illustrated by network diagram of ingenuity pathway analysis.

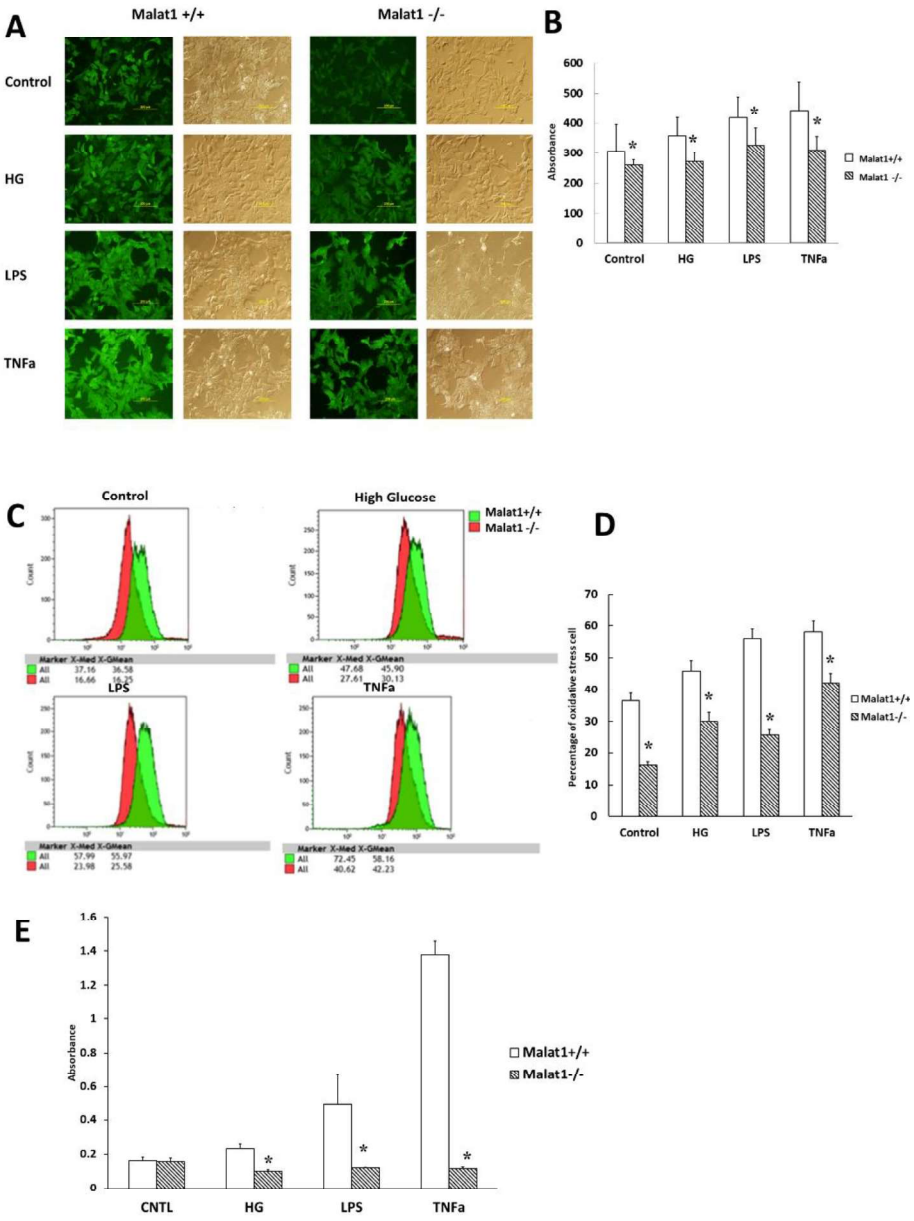

Figure 10. MALAT1 ablation inhibits ROS generation. (A) The ROS generation in mouse hepatocytes determined by immunofluorescence microscopy using DCFH-DA as a fluorescent dye. (B) ROS levels in MALAT1 null and wild type hepatocytes challenged with high glucose (HG), LPS and TNF-α for 12 h were determined by fluorescence quantification with the plate reader (BioTek). (C) and (D) Flow cytometric analysis of ROS generation in MALAT1 null and wild type hepatocytes. (E) ROS levels in isolated pancreatic islets from wild type and MALAT1 null mice challenged with high glucose, LPS and TNF-α determined by fluorescence quantification with the plate reader (BioTek). (F) Isolated mouse islets. The photos show the islets isolated from MALAT1 null mice and wild type mice, and then kept in cell culture medium. Scale bars represent 500 μm. Error bars show standard deviations. *, $P < 0.05$. Data are representative of at least three independent experiments.

2.3.2 Oxidative stress-induced protein carbonylation is suppressed in MALAT1 null mice

Protein carbonylation is a marker for cumulative oxidative stress. To determine the levels of oxidative stress in MALAT1 null mice we measured the protein carbonyl levels in the liver and pancreas. The basal protein carbonyl levels were significantly decreased in the MALAT1 ablated primary hepatocyte and pancreas compared to wild type mice which is consistent with the lower ROS levels in MALAT1 null mice (Figure 10 A-E). To induce oxidative stress, mice were i.p. injected with high glucose (2 g/kg, 3 times at 8, 16, and 24 h) and LPS (30mg/kg, 2h), and the liver and pancreas were collected for protein extraction. The high glucose and LPS treatments significantly induced protein carbonylation in the livers and pancreas of wild type mice, however, the inductions were significantly diminished in the MALAT1 null mice as determined by Western blotting (Figure 11A and B) and quantitated by ELISA (Figure 11C and D).

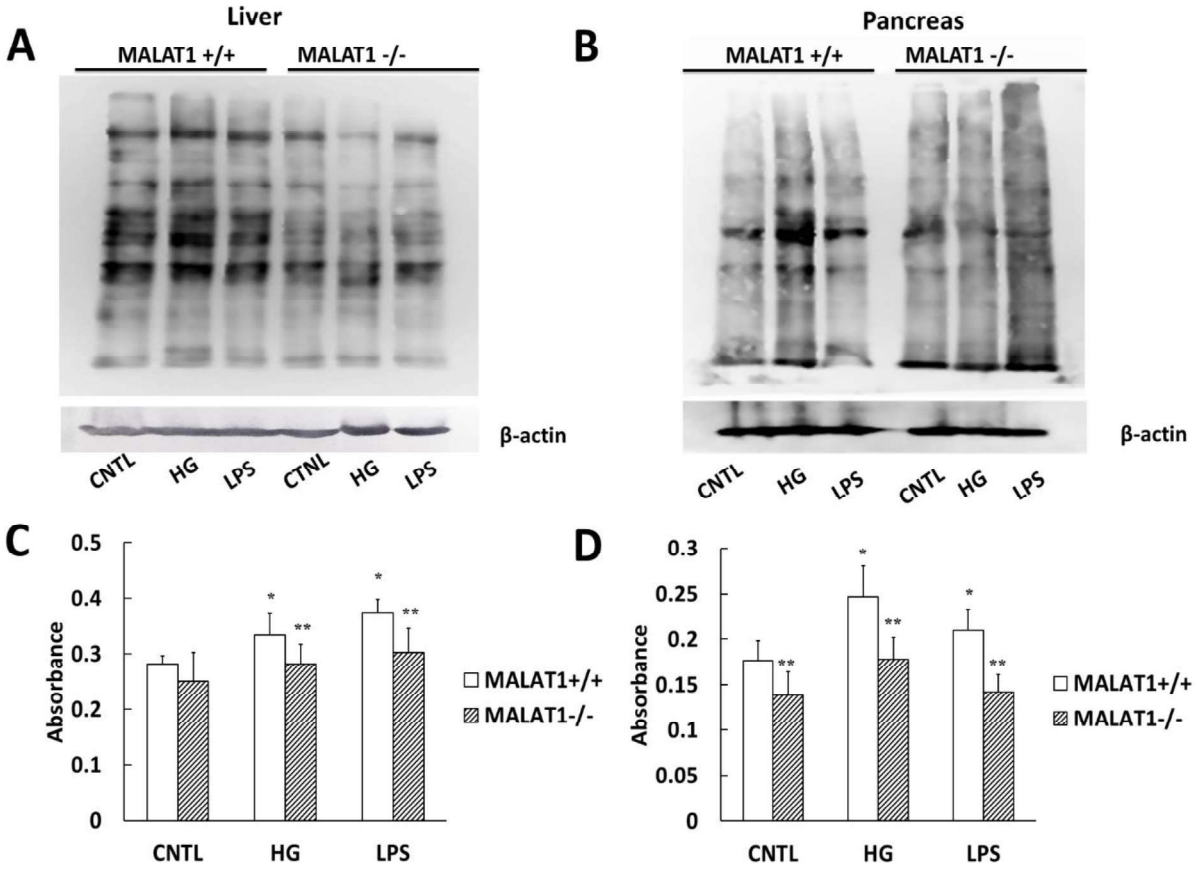

Figure 11. Measurement of total protein carbonylation in hepatocytes and pancreas of MALALT1 null and wild type mice. (A) and (B) Protein carbonylation determined by Western blotting. Equal amount of protein from primary hepatocytes (A) and pancreas (B) from the MALAT1 null and wild type control treated with high glucose (HG, 2 g/kg, 3 times in 24 h) or LPS (30 mg/kg, 2 h). (C) and (D) Quantification of protein carbonylation of hepatocytes and pancreas determined by ELISA (Absorbance was read at 375nm OD). Error bars show standard deviations. *, P <0.05; **, P <0.01. Data are representative of at least three independent experiments.

2.3.3 MALAT1 ablation inhibits c-Jun NH2 -terminal kinase activation and improves insulin sensitivity

Excessive exposure to ROS is known to cause insulin resistance leading to T2DM (Gao, Nong et al. 2010) and one pathological mechanism is through activation of the JNK pathway. We investigated the levels of expression and phosphorylation of the critical factors regulating insulin signaling pathway in the hepatocytes from MALAT1 null mice. As JNK was activated by oxidative stress, and the activation of JNK plays a central role in obesity-induced insulin resistance (Wellen and Hotamisligil 2003), we measured the expression of JNK and phospho-JNK in MALAT1 null hepatocytes under oxidative stress conditions. Levels of JNK and phospho-JNK induced by LPS and high glucose were higher in the wild type mice and these inductions were significantly inhibited in MALALT1 null hepatocytes (Figure 12A). The phosphorylation of IRS-1 and Akt are critical for the insulin responses. To analyze these insulin-induced responses, mice were fed with high fat diet for 12 weeks and were fasted overnight, and were administrated with either saline (control) or insulin, after which primary hepatocytes were isolated. Insulin-induced tyrosine phosphorylation of both the IRS-1 and Akt were significantly increased in MALAT1 null hepatocytes comparing with the wild type control (Figure 12B), suggesting heightened insulin sensitivity in MALAT1 null mice.

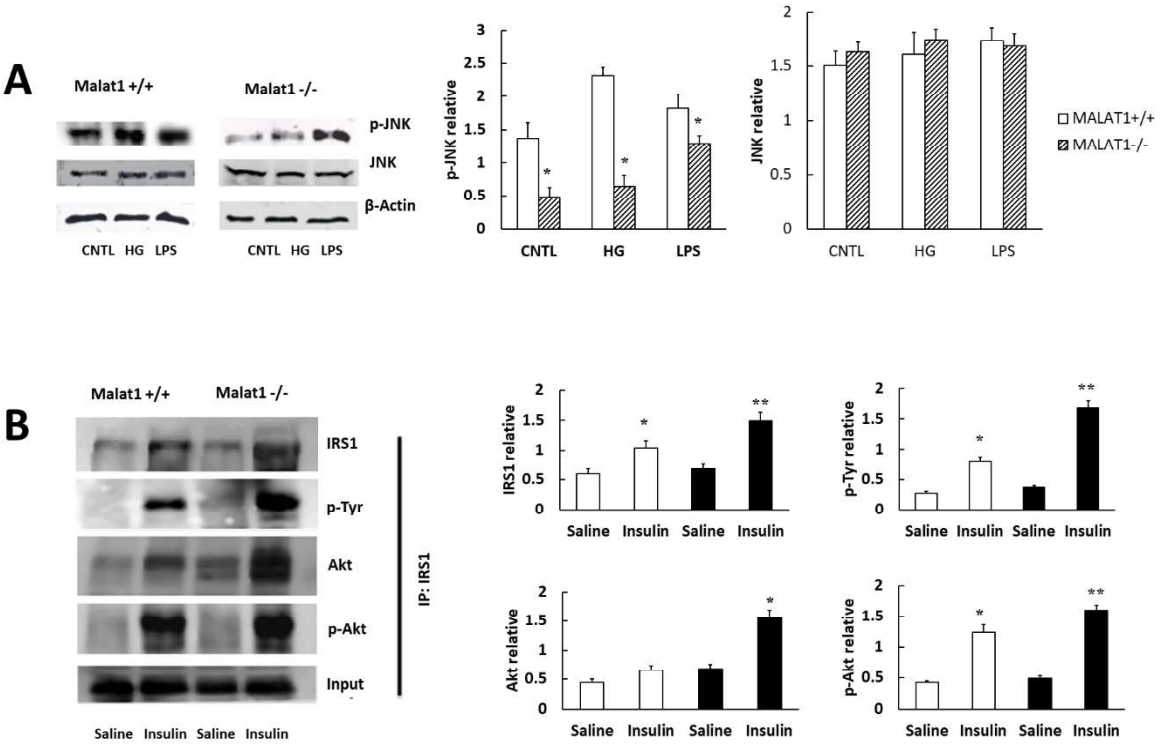

Figure 12. MALAT1 ablation inhibits JNK activation and enhances the insulin signaling capacity. (A) Western blotting analysis of the JNK phosphorylation as well as proteins levels of IRS1, p-Tyr of IRS1, Akt and p-Akt in primary hepatocytes from MALAT1 null and wild type mice. Hepatocytes were treated with high glucose (HG) and LPS. Quantifications (NIH ImageJ) of the Western blotting, right panel. (B) Mice fed on HFD (12 weeks) were injected with insulin (1 units/kg) through the portal vein and hepatocytes were harvested 5 min after insulin injection. Protein was extracted for Western blot analysis. Left: Protein extracted from liver was immunoprecipitated for IRS-1 and immunoblotted (IB) for phosphorylated proteins. Quantification are shown on the right panel. Data were presented as the ratios of the phosphorylated protein/un-phosphorylated protein. Error bars show standard deviations. *, P <0.05. Data are representative of at least three independent experiments.

2.3.4 MALAT1 null mice exhibit improved insulin-signaling responses

To investigate the role of MALAT1 in regulating the glucose homeostasis *in vivo*, we analyzed the parameters of glucose metabolism in the mice and found that the basal plasma levels of insulin and glucagon were lower in MALAT1 null mice compared to the wild type under the random fed conditions (data not shown), indicative of sensitized insulin signaling responses. Upon fast-refeed and glucose tolerance challenges (i.p. injection), MALAT1 null mice showed significantly lower blood glucose levels (Figure 13 A and B), suggesting an improved insulin response in these mice. Next, we performed insulin tolerance test (ITT) by i.p. injection of insulin to further investigate the insulin sensitivity of the MALAT1 null mice and observed that these mice showed sensitized responses to insulin (Figure 13 C). Under the fast-refeeding conditions, the blood glucose levels in MALAT1 null mice were lower comparing with the wild type control (Figure 13 A). Additionally, upon glucose challenge, the MALAT1 null mice responded with much higher insulin spike suggesting a highly efficient insulin response (Figure 13 D). Collectively, these results suggest that the insulin signaling pathway is highly sensitized in MALAT1-ablated mice (Figure 13 B-D).

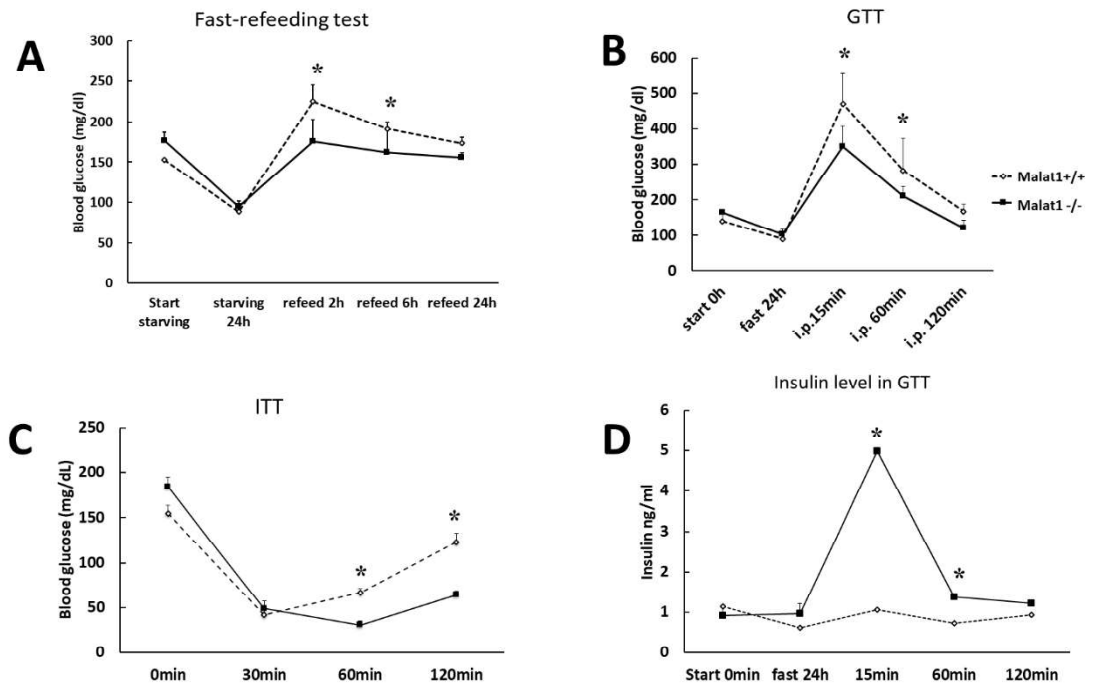

Figure 13. MALAT1 ablation improves insulin signaling responses in mice. (A) Blood glucose levels under fast-refeed challenge in MALAT1 null mice (N=30) and wild type mice (N=29). (B) Blood glucose levels of MALAT1 in glucose tolerance test (Data collected from 6 wild type and 6 MALAT1 null mice). (C) Blood glucose levels in insulin tolerance test (n=6). (D) Blood insulin levels in glucose tolerant test (n=6). *P < 0.01. Error bars show standard deviations. *, P <0.05; **, P <0.01.

2.3.5 MALAT1 ablation improves insulin secretion and increases pancreatic islet cellularity

MALAT1 ablation resulted in favorable hyperglycemic responses (Figure 13). We next investigated the pancreatic islet functions in an *in vitro* assay by isolating the islet and stimulating the insulin secretion in the culture islets. In MALAT1 deficient islets there was a significantly increased secretion of insulin in response to glucose challenge (Figure 14 A). Exposure to ROS-caused pancreatic β-cell dysfunctions which has been shown to ultimately lead to decreases in pancreatic β-cell mass through apoptosis (Robertson 2004). Significantly increased pancreatic islet cellularity was observed in MALAT1 ablated mice comparing with wild type control (Figure 14 B-D). The increases in the cellularity of the islet appear to be from both α and β cells based on the double immunohistological staining with antibodies against glucagon and insulin, respectively (Figure 14 C-G).

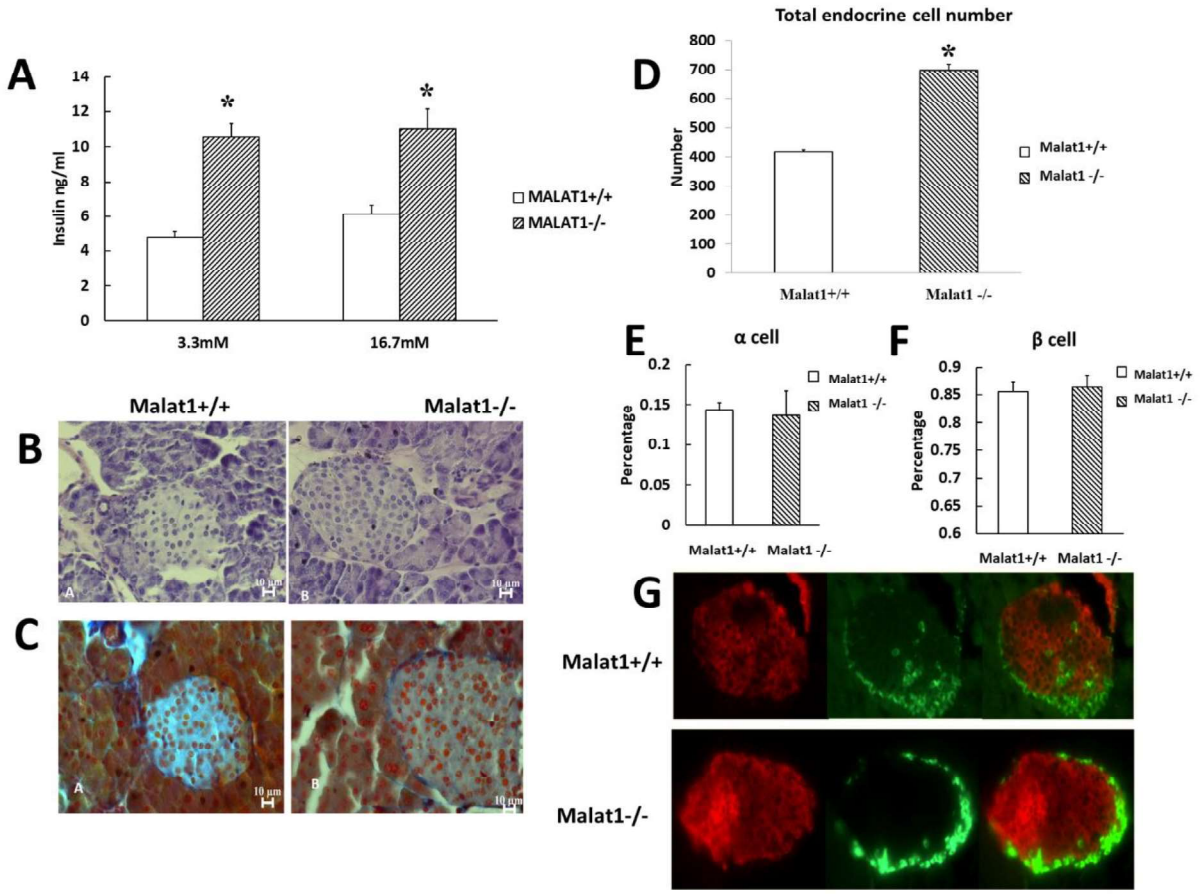

Figure 14. Pancreatic islets morphology and cellularity of MALAT1 null mice. (A) Glucose-stimulated insulin secretion (3.3 mM and 16.7 mM) was measured by ELISA (Rat/Mouse Insulin ELISA kit, Millipore) in isolated islets from MALAT1 null and wild type control mice. (B) Representative sections of pancreas from 6 week-old MALAT1 null and wild type mice using HE staining. (C) Sections of pancreas from 6 week-old MALAT1 null and wild type mice using Heidenhain's AZAN trichrome staining. (D) Quantification of total endocrine cell number per total pancreatic area. (E) and (F) Percentage of α and β cells in total endocrine cells per pancreatic area. (G) Representative sections of pancreas from 6-week-old MALAT1 null and wild type mice visualized by double immunofluorescence staining with anti-insulin (red) and anti-glucagon (green) antibodies. Error bars show standard deviations. *, P <0.05; **, P <0.01. Data are representative of at least three independent experiments.

2.3.6 MALAT1 interacts with Nrf2 and inhibits Nrf2/ARE-driven gene expression

We performed RNA-seq analysis and found that Nrf2 and Nrf2/ARE-driven genes such as Cat, and Nqo1 were increased in the MALAT1 null mice (Figure 15 A and B). To explore the role of MALAT1 in regulating Nrf2 and downstream ARE-driven pathways, we examined the Nrf2 expression in MALAT1 null and wild type primary hepatocytes collected from mice treated with high glucose (i.p. injected with 2g/kg glucose, 3 times, 8, 16 and 24 h) and LPS (i.p. injected 30mg/kg, 2h) and we found that the treated hepatocytes and pancreatic islets generated excessive ROS (Figure 10). Nrf2 and its regulated gene expressions were significantly increased in MALAT1 null hepatocytes in comparison with wild type groups under oxidative stress conditions. The basal level of Nrf2 was also upregulated in MALAT1 null hepatocytes (Figure 15 A) which is consistent with the results of transcriptome analysis suggesting that MALAT1 may play a role as a transrepressor of ARE-driven genes through interaction with Nrf2. To determine the mechanisms of MALAT1 in regulating Nrf2-driven gene expression, we performed lncRNA pulldown assay with biotinylated probe against MALAT1 (Hu, Feng et al. 2014) and found strong interaction between MALAT1 and Nrf2 (Figure 15 C).

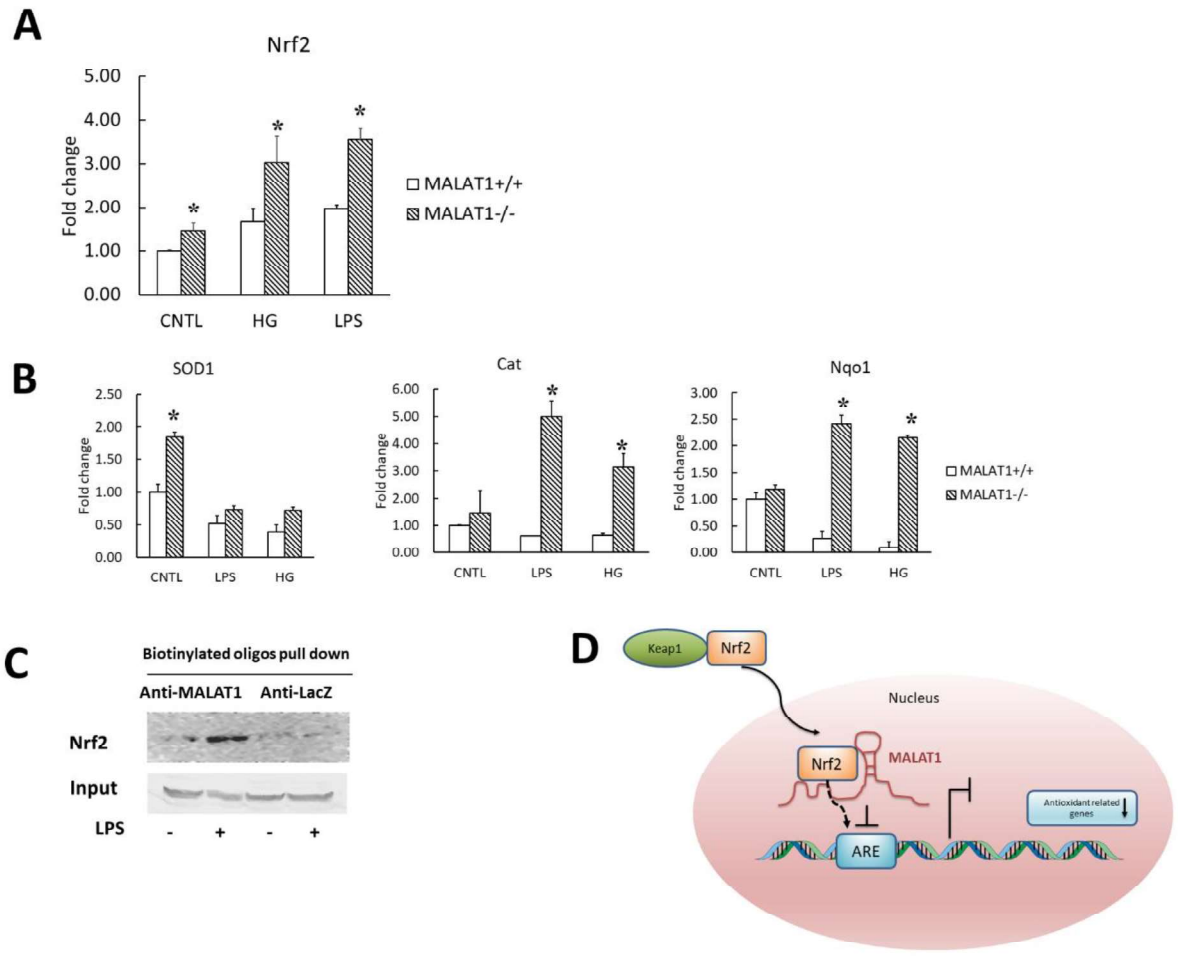

Figure 15. MALAT1 interacts with Nrf2 and inhibits the Nrf2/ARE-driven pathways. (A) Nrf2 expression in primary hepatocytes from MALAT1 ablated and wild type mice treated with high glucose (HG) and LPS. (B) Antioxidant genes expression in primary hepatocytes treated with HG and LPS. (C) Interaction between MALAT1 and Nrf2 in hepatocytes treated with LPS determined by the lncRNA pulldown assay. Western blot of Nrf2 expression pulled down by biotinylated MALAT1 oligos and LacZ oligos (negative control) from nuclear extracts from wild type mice treated with LPS. (D) Hypothesized mechanism for the interaction between MALAT1 and Nrf2 where MALAT1 acts as a negative modulator (riborepressor) inhibiting Nrf2-regulated transcription of antioxidant-related genes. Error bars show standard deviations. *, $P < 0.05$. Data are representative of at least three independent experiments.

2.3.7 MALAT1 ablation increases cell metabolic activity

Decreased insulin sensitivity of the liver accompanied by increased hepatic fat storage are two such major metabolic changes found in the diabetic patient. Mitochondria-intrinsic perturbations in obese, insulin-resistant patients with nonalcoholic steatohepatitis (NASH) include lower maximal respiration, increased mitochondrial uncoupling, and increased proton leak (Pinti, Fink et al. 2019). These findings are further strengthened by the observation of decreased ATP content and turnover in the T2D liver (Pinti, Fink et al. 2019).

Recent development of extracellular flux methods (Seahorse XF Analyzer, Agilent) allow us to monitor changes in oxygen concentration and pH to measure bioenergetic function in response to oxidative stress. Using the Seahorse XF Analyzer we have analyzed mitochondrial bioenergetics parameters and found greatly increased respiratory capacity in the MALAT1 null hepatocytes and resistant to high glucose challenges (Figure 16).

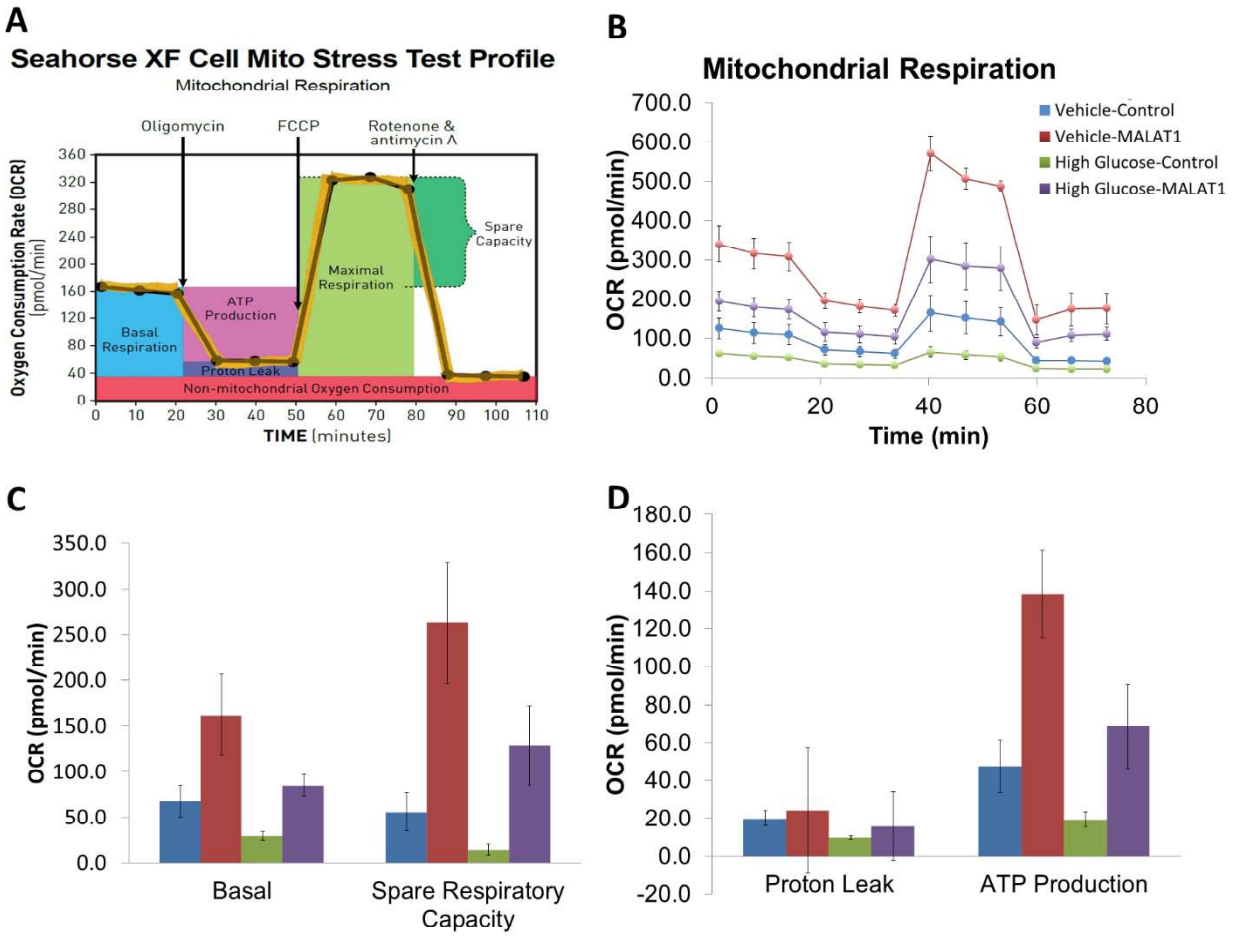

Figure 16. MALAT1 ablation regulates mitochondrial respiration capacity. (A) The schematic illustration of seahorse XF cell mito stress test. (B-C) Real-time measurement of oxygen consumption rates (OCR) in primary hepatocytes isolated from MALAT1 null and wild type mice. (D) ATP production level in isolated hepatocytes from MALAT1 null and wild type mice.

2.3.8 Deletion of the MALAT1 gene in genetic obese mice reverses the obesity-induced insulin resistant and sensitized insulin responses

Obesity induces oxidative stress/inflammation and predisposed animal to insulin insensitivity. *ob/ob* mouse model and HFD/STZ mouse model to explore the utility of MALAT1 as the therapeutic target. Leptin-deficient *ob/ob* mice are obese, insulin resistant with increased serum free fatty acid concentrations and have excessive internal ROS (Özcan, Yilmaz et al. 2006, Lindström 2007). To test the role of MALAT1 in *ob/ob* genetic background, we have generated the homozygous *MALAT1* $^{(-/-)}$*.ob/ob* mice. These mice were produced by firstly backcrossing MALAT1 null mice to C57BL/6J mice for ten generations. C57BL/6 *ob*/+ mice were purchased from the Jackson Laboratory. To generate *MALAT1* $^{(-/-)}$*.ob/ob*, *MALAT1*$^{(-/-)}$ were bred to *ob*/+ mice creating compound heterozygotes, *MALAT1*$^{(+/-)}$ *·ob/OB*. In the second cross, compound heterozygotes were bred to each other and *MALAT1*$^{(-/-)}$*·ob/OB* were identified and bred with each other to get *MALAT1*$^{(-/-)}$ *·ob/ob* mice. In parallel, *MALAT1*$^{(+/+)}$*·ob/OB* were bred to each other to generate *MALAT1*$^{(+/+)}$*·ob/ob ,* as well as *MALAT1*$^{(+/+)}$*·OB/OB* (Wild Type) mice. To generate the most reliable data, we will use littermate pairs for experiments. We found that that ablation of MALAT1 decreased the body weight initially (before week 6), even the weight gain eventually catch up due to the excessive food intake. Interestingly, the MALAT1 null ob/ob mice remained insulin sensitized as shown by GTT, ITT. MALAT1 ablation improved the hyperglycemia and hyperinsulinemia response in the obese mouse model (Figure 17).

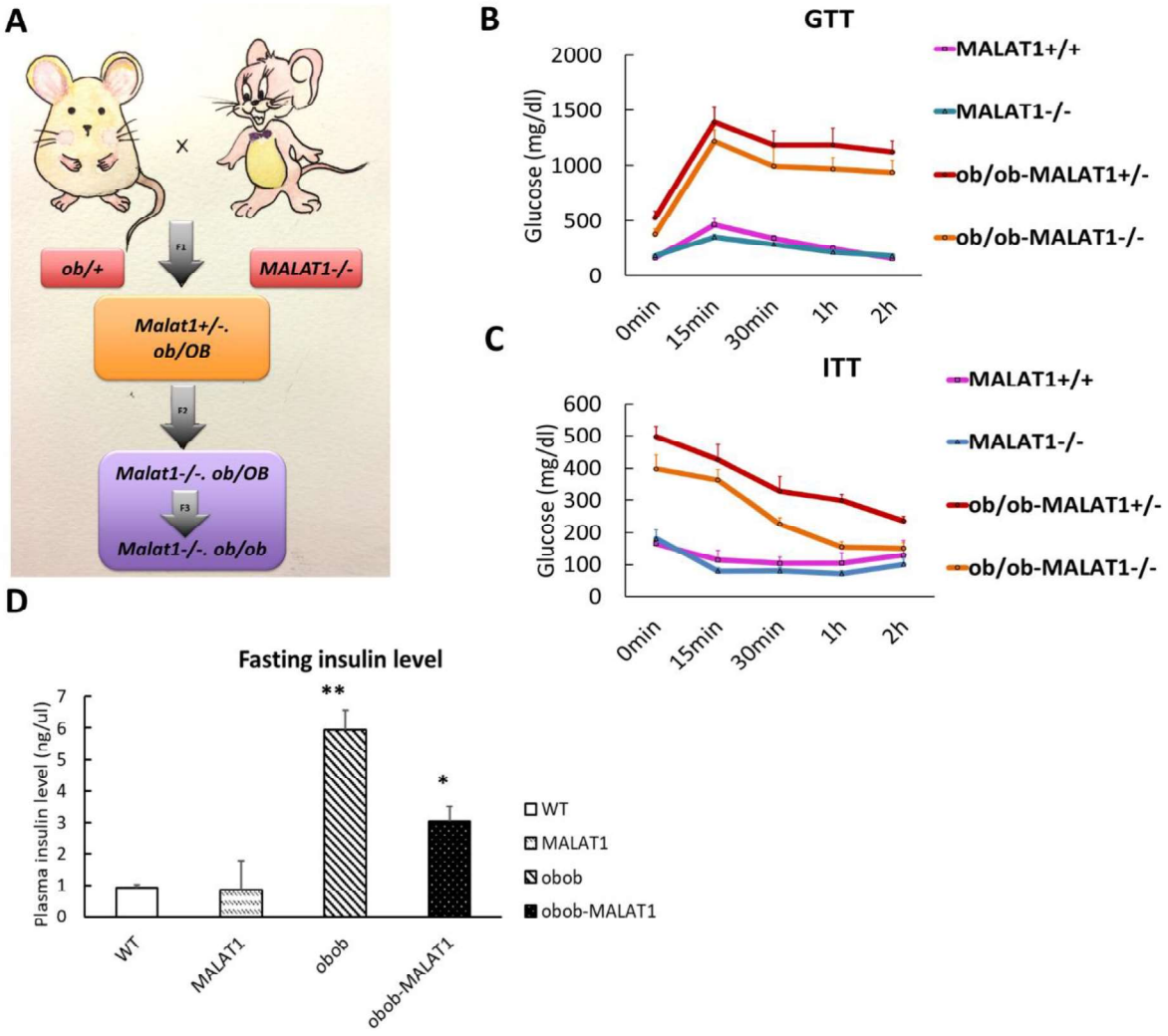

Figure 17. MALAT1 ablation alleviate obesity-induced hyperglycemia, hyperinsulinemia, and insulin resistance in mice. (A) Generation of MALAT1 null mice in ob/ob background. (B) (C) and (D), Glucose tolerance test (GTT), insulin tolerance test (ITT) and fasting insulin level in mice, respectively. *P < 0.05; **P < 0.01. Data are representative of at least three independent experiments.

2.3.9 Ablation of MALAT1 alleviates obesity-induced fatty liver syndrome

As *ob/ob* mice eats excessively due to the deletion of leptin receptor, we investigated that if MALAT1 ablation could alleviated the excessive food intake and we found that MALAT1 null *ob/ob* mice showed significantly lower body weight before 6 weeks old, however. Interestingly, the MALAT1 null *ob/ob* mice showed significantly alleviated the obesity-induced fatty liver syndrome as defined by histological analysis in mice (Figure 18). Collectively our result suggested MALAT1 deletion overcame the obesity-associated metabolic syndrome

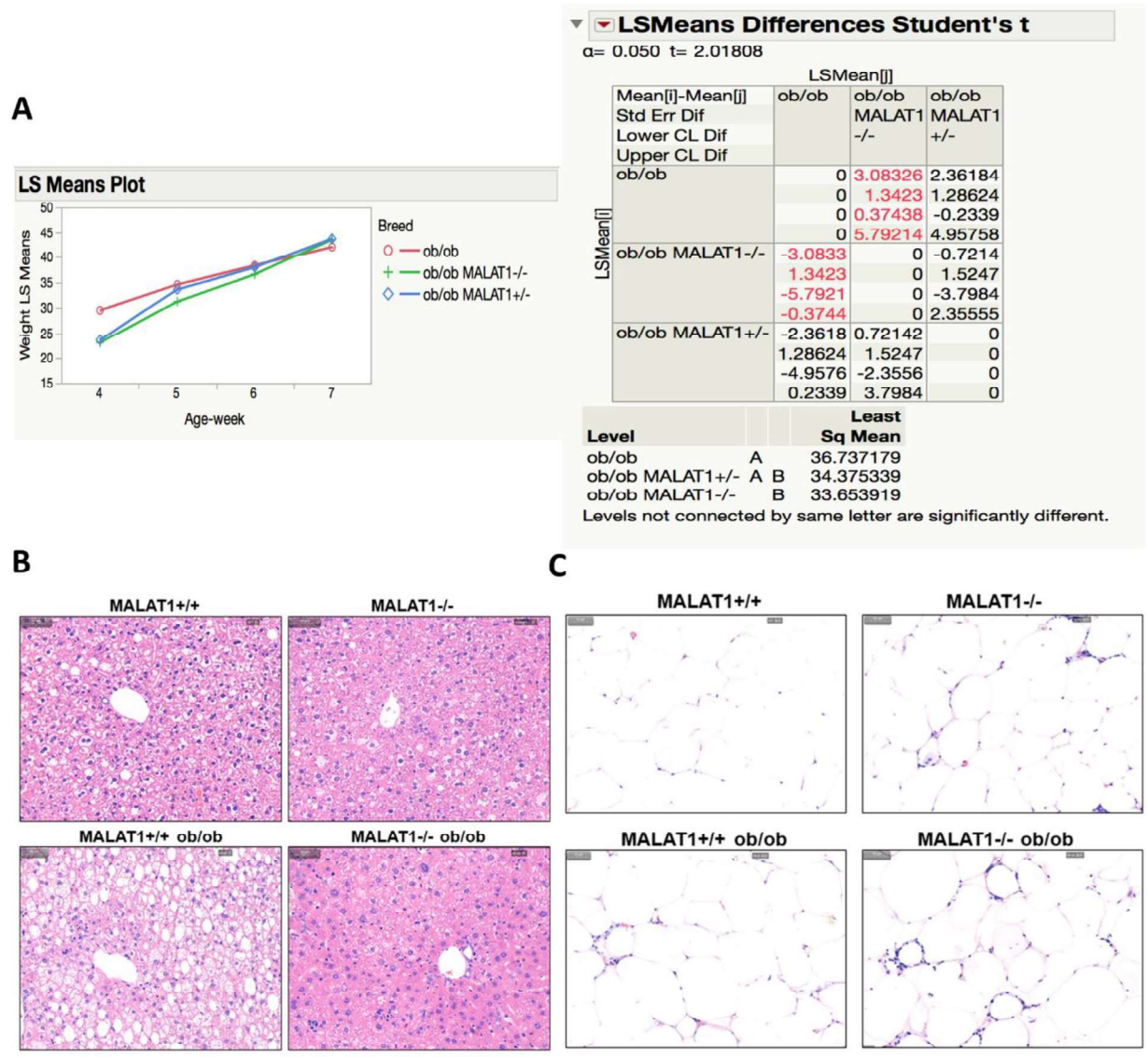

Figure 18. MALAT1 ablation alleviate obesity-induced fatty liver. (A) Bodyweight curve of ob/ob and MALAT1 null ob/ob mice. (B) Histological analysis of liver in mice. © Histological analysis of visceral adipose tissue in mice. Data are representative of at least three independent experiments.

2.3.10 Antisense silencing of MALAT1 reverse oxidative stress indicating therapeutic potential of the antisense approach for treating diseases

Many complex diseases have historically proven to be defiant to the best mono-therapeutic approaches. Obesity and its consequences, such as T2DM, have proven to be equally resistant to therapeutic approaches based on single medicines. Based on the results that MALAT1 null mice had significantly improved insulin responses (Figure 13) and reduced ROS-induced damage in liver and pancreas (Figure 10), we planned to investigate the therapeutic potentials by targeting MALAT1 *in vitro*. For targeting MALAT1, we used modified antisense oligonucleotide LNA GapmeRs, which are single-stranded oligonucleotides that consist of a DNA stretch flanked by LNA nucleotides. Base pairing with the targeted lncRNA in the nucleus induces degradation by an RNase H-dependent mechanism Locked nucleic acid (LNA)–ONs are a new generation of antisense ONs. Unlike previous ON designs, the ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon nucleoside (Braasch and Corey 2001, Orum and Wengel 2001, Li, Xu et al. 2003, Vester and Wengel 2004). This modification provides ONs with an exceptionally high binding affinity for mRNA compared with conventional DNA ONs. Additionally, LNA–ONs are resistant to nucleases and therefore provide exceptional stability in plasma and cell culture medium (Figure 19A) (Jepsen, Sørensen et al. 2004, Karkare and Bhatnagar 2006, GrÜnweiler and Hartmann 2007, Veedu and Wengel 2009).

ASOs has been identified as an optimal method to degrade lncRNAs which localize in nucleus (Lennox and Behlke 2015, Lundin, Gissberg et al. 2015) in cells and animals. We synthesized a set of LNA GapmeR anti-MALAT1 oligonucleotides (Frank-Kamenetsky, Grefhorst et al. 2008) and tested the sets of synthesized ASO individually in the pancreatic cells and hepatocytes to evaluate the inhibitory effects on the ROS levels and silencing effects on

MALAT1 level and then the selected ASO will be used in the T2DM mouse models. Our result showed that the ASO could knockdown MALAT1 with efficiency of 95% in isolated pancreatic islet cells (Figure 19). Interestingly, we found that knocking down of MALAT1 using ASO could significantly inhibit ROS generation (Figure 19). To explore the role of MALAT1-associated insulin resistance in mice, we plan to inject the T2DM mouse models with LNA GapmeR anti-MALAT1 oligonucleotides daily for 10 days via tail vein (2.5mg/Kg) (Michalik, You et al. 2014, Xing, Lin et al. 2014). MALAT1 expression will be measured in liver and pancreas using qPCR in the T2DM mouse model. GTT and ITT, as well as measurement of ROS, will be performed in the ASO-treated mice to investigate the therapeutic role of MALAT1 in regulating the insulin sensitivity in T2DM mice.

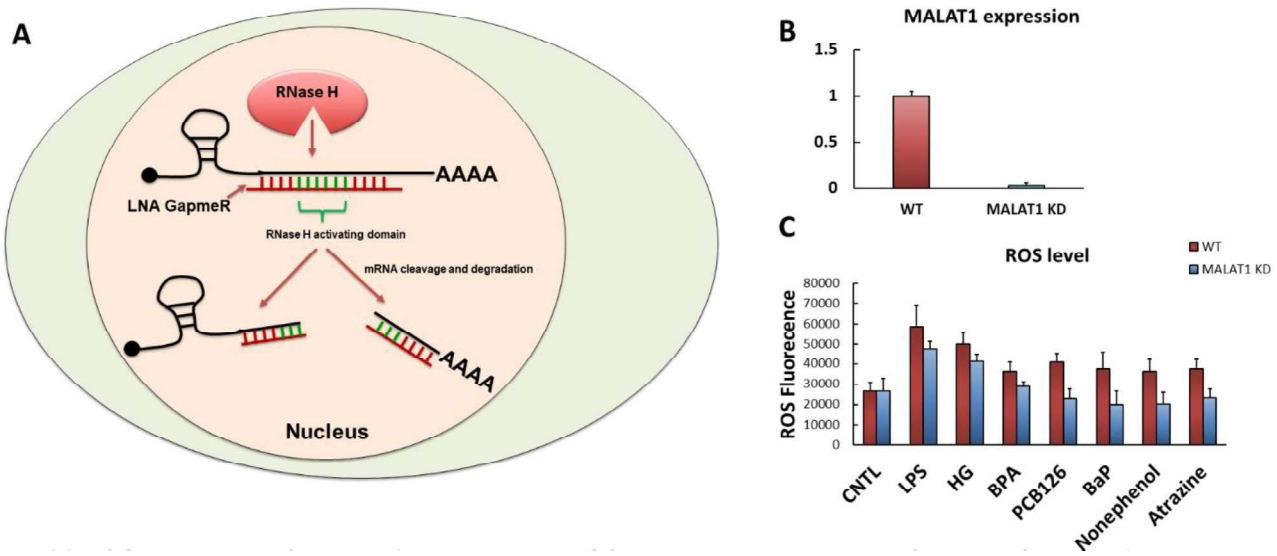

Figure 19. ASO silencing of MALAT1 suppresses ROS levels. A, Mechanism of action of MALAT1 silencing by LNA GapmeRs. LNA GapmeRs consist of two outer blocks of modified LNA nucleotides flanking an unmodified central stretch of bases. LNA GapmeRs bind to RNA transcripts by complementary base paring and are only able to bind in regions devoid of secondary structure. The unmodified central bases form an RNA/DNA duplex that is recognized and cleaved by RNase H through the RNA strand, resulting in degradation of the target RNA MALAT1. B, Effective silencing of MALAT1 by ASO in isolated pancreatic islet cells. C, Inhibition of oxidants induced-ROS through ASO silencing of MALAT1 in isolated pancreatic islets.

2.4 Discussion

MALAT1 is a lncRNA with hitherto reported gene regulatory functions mainly centering on its role in regulating transcription and RNA processing. Despite of its pervasive involvement in transcriptional regulation and RNA processing, MALAT1 null mice do not exhibit an overt phenotype. The lack of phenotype in MALAT1 null mice suggests a compensatory mechanism complementing the deficiency in these mice. Indeed, in the transcriptome analysis we found a related lncRNA NEAT1 was greatly increased (>10 fold) in the MALAT1 null hepatocytes (Figure 20) which is consistent with the results from another laboratory (West, Davis et al. 2014). We performed pathway analysis in MALAT1 null hepatocytes and found that the Nrf2-regulated antioxidant genes were activated comparing to the wild type. Consistent with the results of pathway analysis, we have found dramatic reduction of the ROS levels in MALAT1 null hepatocytes and pancreatic islets (Figure 10 and Figure 11) indicating these mice have higher antioxidant capacity and lower ROS milieu.

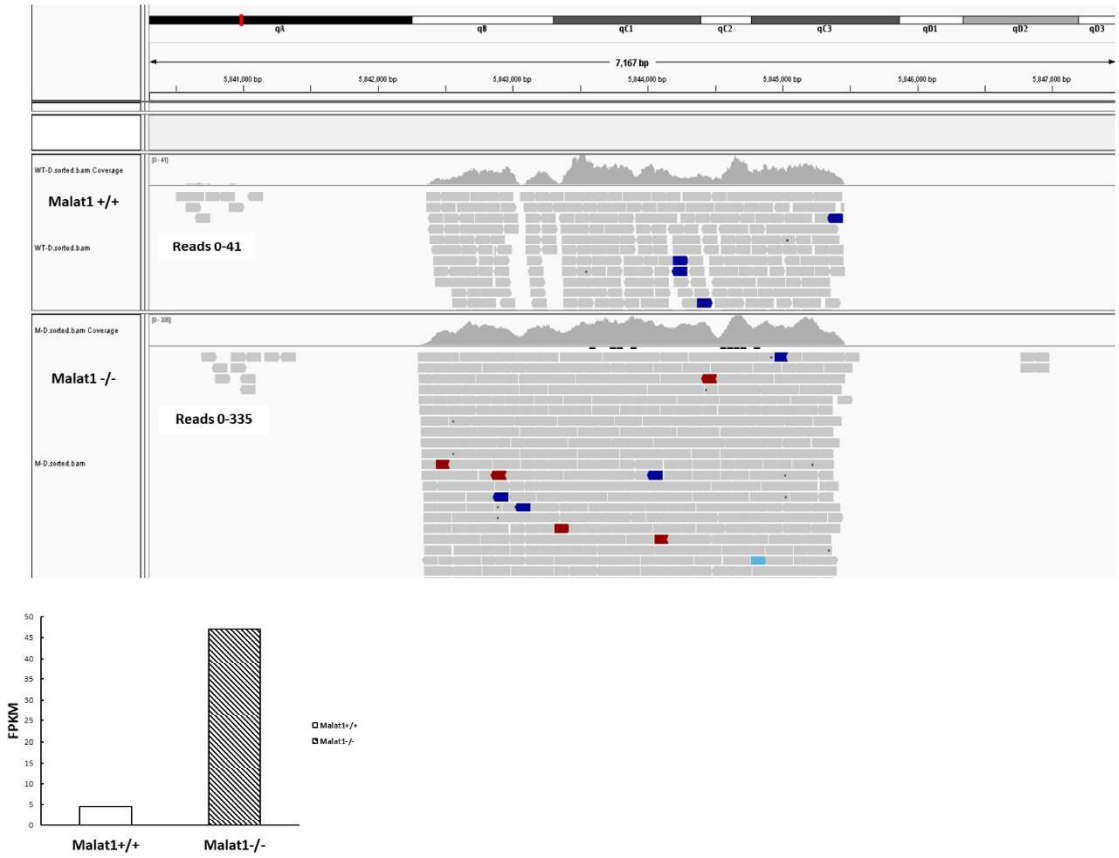

Figure 20. Ablation of MALAT1 upregulates the expression of NEAT1. IGV browser views for NEAT1 in both wild type (MALAT1+/+) and MALAT1 null hepatocytes.

Physiological levels of ROS are critical for cellular functions. However, exposure to ROS which exceeds the capacity of the antioxidant system results in the induction of oxidative stress, causing inflammation and tissue damage (Robertson, Harmon et al. 2003). Since pancreatic β cells express low level of antioxidant enzymes, it has been suggested to be a sensitive target for oxidative stress-mediated damage (Maechler, Jornot et al. 1999, Evans, Goldfine et al. 2002), which may lead to β cell apoptosis and decreased β cell mass (Marchetti, Bugliani et al. 2007). Reduced pancreatic functions and insensitivity of the insulin responses in tissues both contribute to the development of T2DM. The MALAT1 ablation reduced ROS levels suggests that these mice may have sensitized insulin responses and were protected against oxidative stresses. To investigate the role of MALAT1 in regulating ROS in both pancreas and liver, we determined the level of ROS generation in the hepatocytes and isolated pancreatic islets. Indeed, our results indicated that both basal ROS levels as wells as high glucose, LPS and TNF-α induced ROS levels were significantly lower in the MALAT1 ablated mice (Figure 10) with significantly decreases in the level of total protein carbonylation (Figure 11).

Exposure to excessive ROS has been shown to cause insulin resistance through activation of JNK which in turn leads to inhibition of the insulin-induced phosphorylation of IRS1 and Akt. We found in MALAT1 ablation suppressed JNK activity with concomitant activation of IRS-1 and insulin-induced phosphorylation of Akt (Figure 12). MALAT1 null mice exhibited favorable insulin-signaling response to hyperglycemic challenges including fast-refeeding, GTT and ITT (Figure 13), and significant increases in insulin secretion in response to glucose challenge in isolated MALAT1 null islets (Figure 14 A).

A pivotal factor in regulating the antioxidant responses is the Nrf2 which is translocated into the nucleus in response to ROS and binds to the ARE thereby transactivating ARE-driven

gene expressions. We searched the published data base (GSE55914) and found that MALAT1 interacted with the Nrf2/ARE-driven genes as well as Nrf2 transcriptional factor itself (Figure 21). Using lncRNA pulldown assay with the biotinylated MALAT1 oligos we have shown a strong interaction between MALAT1 and Nrf2 (Figure 15 C) suggesting it may function as a negative regulator for the Nrf2 transcriptional activity. Consistent with this hypothesis, we found Nrf2, as well as Nrf2-regulated antioxidant genes such as Nqo1, and Cat were upregulated in MALAT1 null hepatocytes (Figure 15 A and B).

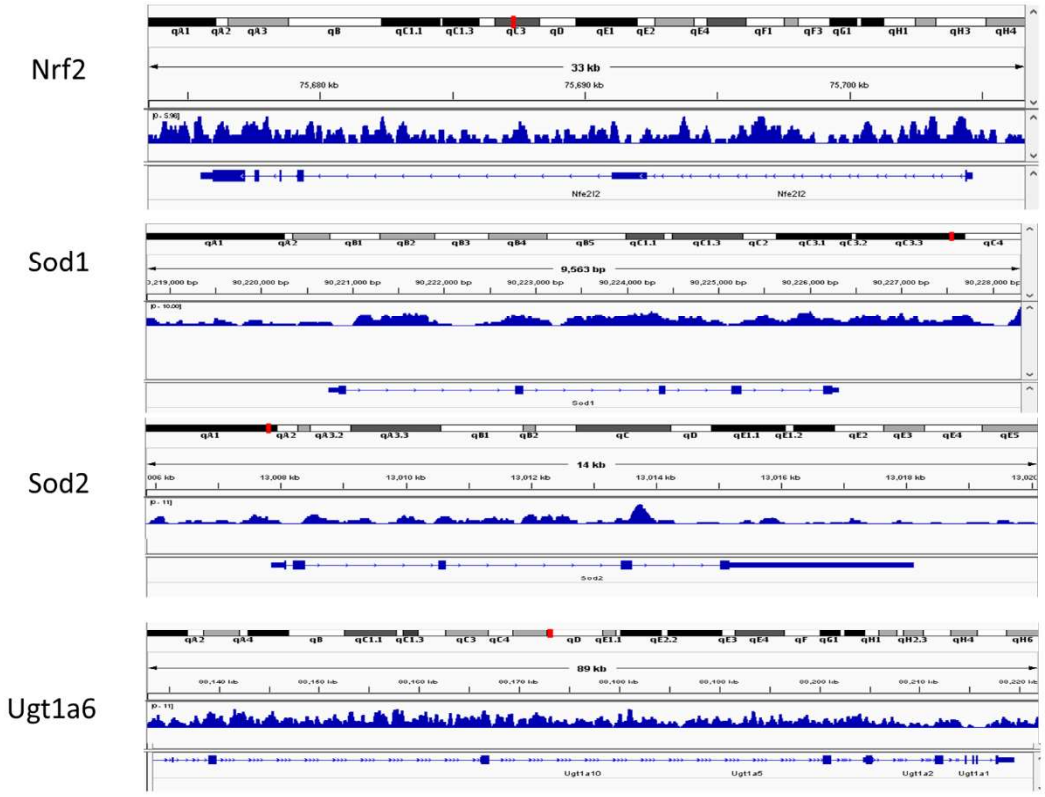

Figure 21. MALAT1 interacting with Nrf2/ARE-driven genes. IGV browser views for enrichment of MALAT1 on the genome of Nrf2, Sod1, Sod2 and Ugt1a6.

Two interconnected mechanisms may be involved in increased sensitization to insulin response in MALAT1 ablated mice: 1) MALAT1 ablation may de-repress the Nrf2/ARE-regulated antioxidant gene expressions as we have found MALAT1 interacted with Nrf2 in the nucleus where it functions as a transrepressor for Nrf2 (Figure 15) with concomitant decreases in the ROS levels (Figure 22); 2) MALAT1 null mice have improved insulin signal capacity via decreasing JNK phosphorylation and activating IRS1 and Akt pathways, both of which are due to the low internal ROS levels in the MALAT1 null mice (Figure 22). These results led us to hypothesize that MALAT1 function as a "riborepressor" (Kino, Hurt et al. 2010) in regulating the ROS metabolism through regulating Nrf2-mediated pathway. In this scenario, genetic deletion of MALAT1 gene causes de-repression of the expression of the genes involving in ROS detoxification as well as the genes in the insulin signaling pathways thereby improving the pancreatic β cell functions and sensitizing the insulin signaling responses.

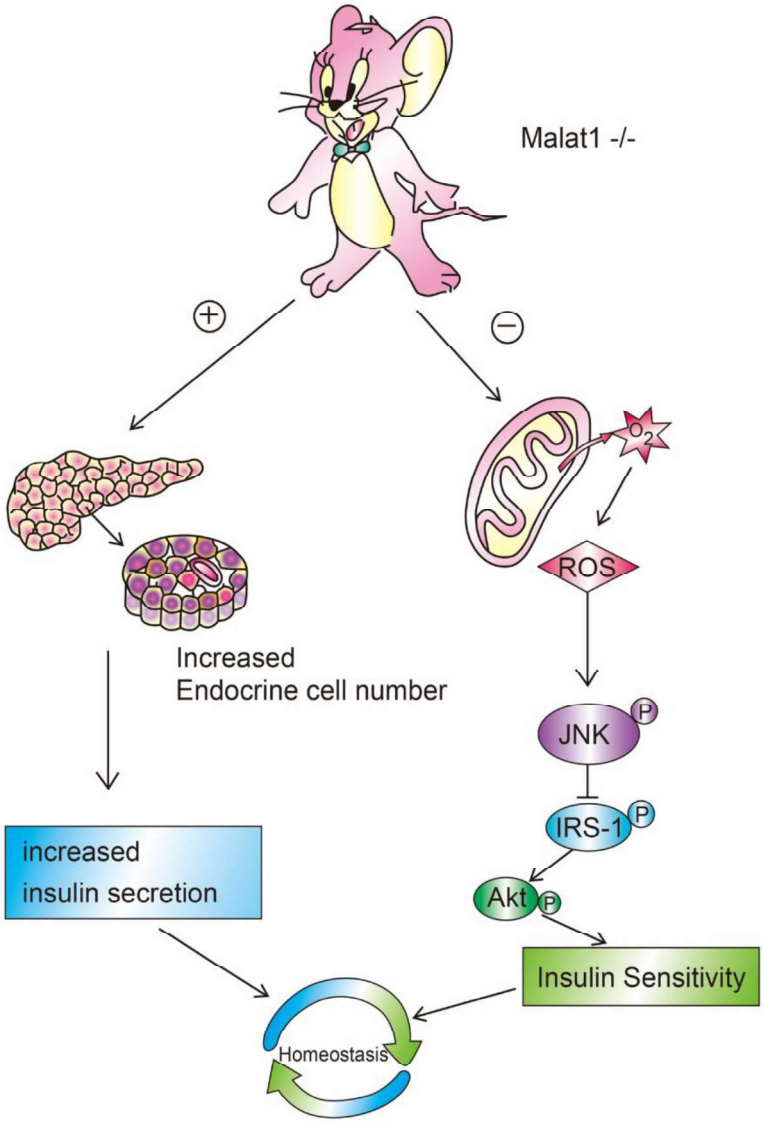

Figure 22. Proposed model illustrating how MALAT1 regulates insulin sensitivity and glucose homeostasis. Proposed mechanisms for the improved insulin signaling capacity in the MALAT1 ablated mice: 1) MALAT1 ablation improves insulin responses by upregulating the antioxidant gene expression thereby quenching ROS, and improving the insulin signaling capacity through inhibiting JNK activation and enhancing the functions of IRS1 and Akt; 2) MALAT1 ablation creates a low ROS internal environment which enhances pancreatic endocrine functions by increasing insulin signaling capacity.

Our results also suggest that MALAT1 is an important therapeutic target for not only the T2DM but also for wide range of diseases in which excessive production of ROS is an underpinning etiological factor.

3. A MURINE PANCREATIC ISLET CELL-BASED SCREENING FOR DIABETOGENIC ENVIRONMENTAL CHEMICALS*

3.1 Introduction

Exposure to certain environmental chemicals in human and animals has been found to cause cellular damage of the pancreatic β cells which will lead to the development of type 2 diabetes mellitus (T2DM). Although the mechanisms for the chemical-induced β cell damage were unclear and likely to be complex, one recurring finding is that these chemicals induce oxidative stress leading to the generation of excessive reactive oxygen species (ROS) which induce damage to the β cell. To identify potential diabetogenic environmental chemicals, we isolated pancreatic islet cells from C57BL/6 mice and cultured islet cells in 96-well cell culture plates; then, the islet cells were dosed with chemicals and the ROS generation was detected by 2',7'-dichlorofluorescein (DCFH-DA) fluorescent dye. Using this method, we found that bisphenol A (BPA), Benzo[a]pyrene (BaP), and polychlorinated biphenyls (PCBs), could induce high levels of ROS, suggesting that they may potentially induce damage in islet cells. This method should be useful for screening diabetogenic xenobiotics. In addition, the cultured islet cells may also be adapted for *in vitro* analysis of chemical-induced toxicity in pancreatic cells.

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*Reprinted with permission from "A Murine Pancreatic Islet Cell-based Screening for Diabetogenic Environmental Chemicals" by J., Chen, L., Zhong, J., Wu, S., Ke, B., Morpurgo, A., Golovko, N., Ouyang, Y., TianY., *J. Vis. Exp.* (136), e57327, doi:10.3791/57327 (2018). 152, 94-103, Copyright 2018 by JoVE Inc.

Increases in the prevalence of T2DM have become a global health crisis in recent years posing a serious threat to public health(Maruthur 2013). Many factors have been found to be causally linked to the development of T2DM, among which, recurring findings suggest that one common convergent point for these factors is the induction of oxidative stress which leads to the generation of excessive ROS(Houstis, Rosen et al. 2006, Ma, Zhao et al. 2012).

A wide spectrum of environmental chemicals including PCBs, dioxins, and BaP has been found to induce oxidative stress, which may impair the function of pancreatic β cells and lead to insulin resistance and T2DM(Valavanidis, Vlahogianni et al. 2006). Although the physiological level of ROS plays an important role in cellular functions, exposure to ROS that exceeds the capacity of the antioxidant system results in the damage to cells/tissues and leads to diseases(Robertson, Harmon et al. 2003). Pancreatic β cells express a low level of antioxidant enzyme, and thus are a sensitive target for the oxidative stress-mediated damage(Kaneto, Kajimoto et al. 1999, Maechler, Jornot et al. 1999). Chronic exposure to high levels of ROS has been shown to cause stress-induced pancreatic cell dysfunction(Robertson, Harmon et al. 2003) as well as insulin resistance in the liver and adipose tissue(Gao, Nong et al. 2010).

The overall goal of this project is to develop a cell-based assay to screen chemicals for their diabetogenic potentials based on their induction of ROS in pancreatic cells. The pancreas lacks metabolic detoxification and is a sensitive target for xenobiotic-induced damage (Kaneto, Kajimoto et al. 1999, Maechler, Jornot et al. 1999). Therefore, by directly measuring the ROS generated in the pancreatic cells, this assay should provide a direct approximation of the chemical-induced injury in the pancreas. To develop this method, we isolated mouse pancreatic islets, cultured the isolated islet under cell culture condition with chemicals, and utilized the chemical-induced ROS generation as the readout. This procedure is simple and effective in identifying

ROS-inducing chemicals in the isolated islet; it can be further developed for investigation of the mechanisms of toxicity that are specific to the pancreas *in vitro*.

3.2 Protocol

All animal experiments were executed in compliance with all relevant guidelines, regulations and regulatory agencies. The protocol being demonstrated was performed under the guidance and approval of the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M Institute for Genomic Medicine.

The protocol is outlined below as follows:

1. Solution Preparation

1.1. Dilute 10x Hank's balanced salt solution to 1x with double distilled H_2O, and store at 4 °C.

1.2. Prepare the isolation solution by adding HEPES (10 mM), $MgCl_2$ (1 mM), and glucose (5 mM). Adjust the pH to 7.4 and keep on ice.

1.3. Prepare the collagenase solution from collagenase Type 4.

Note: Specifically, collagenase Type 4 contains 160 U per mg of dry weight. 1 g of collagenase Type 4 is diluted in 5 mL double distilled H_2O with a concentration of 200 mg/mL. 2 mL of the diluted Collagenase is added to 30 mL ice cold isolation solution, and 6 mL of collagenase solution is transferred to a 50 mL tube for each mouse.

1.4. Prepare the wash solution by adding 1 mM $CaCl_2$ to the isolation solution.

1.5. Prepare the purification solution with cold polysucrose/sodium diatrizoate solution (1.1119 g/mL, 5 mL).

1.6. Prepare 500 mL of islet culture medium by adding L-glutamine (20 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and fetal bovine serum (FBS) (10%) into RPMI medium.

1.7. Prepare the DCFH-DA stock solution by dissolving the powder in distilled water at 200 μM and store at -20 °C.

Note: The ROS staining solution is freshly made by diluting DCFH-DA stock solution to the final concentration of 5 μM in RPMI. This reagent should be protected from light.

2. Surgical Preparation

2.1. Intraperitoneally inject (I.P.) a mouse with avertin (2.5% avertin, 0.4 mL/20 g). After the mouse is completely anesthetized and no longer responds to hind foot pinches, euthanize the mouse and move to the biological hood.

2.2. Place the euthanized mouse with the abdominal side facing up and spray the skin with 70% ethanol for sterilization.

2.3. Open the abdominal cavity with a 1 cm U-incision upon the upper abdomen and retract the skin in the rostral direction over the chest to expose the abdominal cavity.

3. Pancreas Perfusion and Removal

3.1. As illustrated in **Figure 23**, find the location of the ampulla. Use a pair of curved forceps to clamp the duodenum close to the position of sphincter of Oddi. Use another pair of curved forceps to clamp the duodenum wall to block the bile pathway in the duodenum.

Note: The ampulla has not been inserted in this step. This step is meant to block the bile pathway in the duodenum.

3.2. Reposition the mouse with the head proximal and the feet distal with regards to the researcher.

3.3. Find the common bile duct and slowly inject 3 mL of collagenase solution with a 30 G needle and 5 mL syringe. Make sure the common bile duct and the pancreas are inflated after the injection.

3.4. Remove the distended pancreas using the curved forceps and fine scissors to separate it from the descending colon, intestines, stomach, and spleen. Place the pancreas in a 50-mL tube containing 3 mL of collagenase solution and keep it on ice.

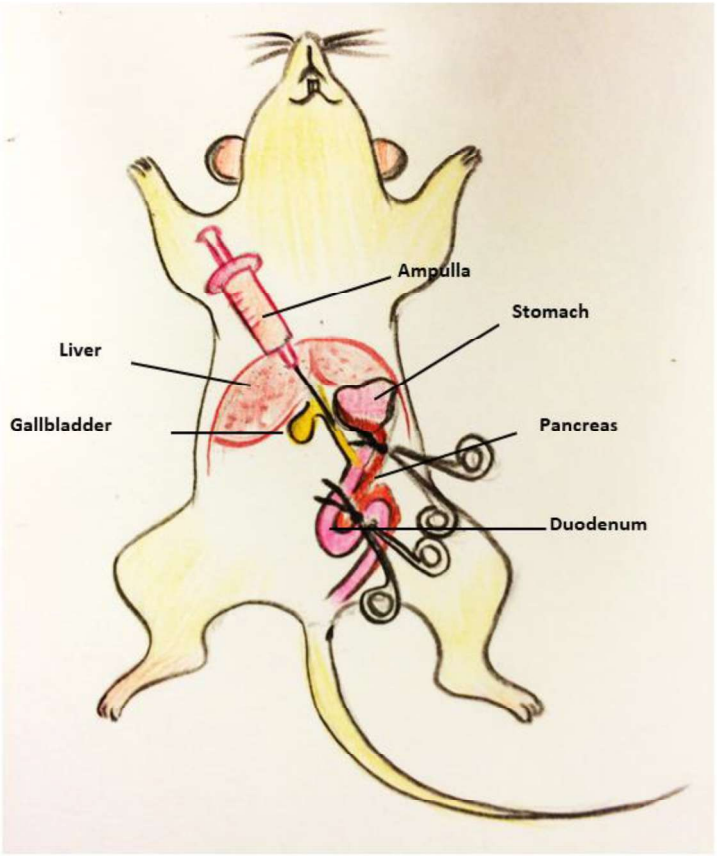

Figure 23. Procedure of mouse pancreas perfusion. The procedure consists of the following steps: (1) Find the ampulla and use two pairs of curved forceps to clamp the ampulla. (2) Insert the needle through the common bile. (3) Slowly inject 3 mL of collagenase solution stock in the 5 mL syringe. (4) Collect the distended pancreas starting from the duodenum.

4. Pancreas Digestion

4.1. Place each 50-mL tube into a 37 °C water-bath. Incubate for 15–20 min (time varies with the strain and age of the mice).

4.2. Vibrate the tube using a vortex.

4.3. Pool 4–5 digested pancreas in a large sterile sink strainer and use a scoop to make the pancreas fall apart thoroughly. Use a 23 G needle and 10 mL syringe containing wash solution to forcefully pipette the digested cells off the screen. Collect the wash solution in a 50-mL tube.

4.4. Centrifuge the tubes at 97 x g at 4 °C for 1 min and decant the supernatant.

4.5. Add 25 mL of wash solution to tubes and re-suspend the tissue by gentle vortexing. Spin down the tissues at 97 x g at 4 °C for 1 min and decant the supernatant. Repeat for 3 times. Be careful not to dislodge the pelleted tissue.

5. Purification of Islets

5.1. Add 10 mL of purification solution to the washed tissue pellet and gently resuspend the contents.

5.2. Gently overlay 5 mL of isolation solution on the purification solution. Be careful to keep a sharp liquid interface between the two solutions.

5.3. Centrifuge the tube at 560 x g at 4 °C for 15 min with slow acceleration and no brake. Insert the pipette into the interface and collect the islets layer into new 50 mL tubes.

5.4. Wash the islets as described in step 3.5.

6. Plating and Treatment of Islet Cells

6.1. Resuspend the islets in 10 mL of islets culture solution, gently mix well, and place 100 µL/well with a multichannel pipette to a 96-well plate. Place approximately 5 islets per 96 well.

6.2. Place the plate in the tissue culture incubator for 12 h.

6.3. Centrifuge (112 x g) the 96-well plate and remove the culture medium.

6.4. Dilute the environmental chemicals in the islets culture medium. Add 100 µL of each of the chemical diluents into separate wells of the plate. Resuspend the islets in each well and incubate for 12 h at 37 °C (the concentration varies for the different environmental chemicals).

7. ROS Staining and Measurement

7.1. After islets treatment, wash with 1x PBS (200 µL for each 96-well).

Note: The concentration of the chemicals is as follow: AFB1: 3 µM, Atrazine: 100 µM, BaP: 10 µM, BPA: 100 µM, PCB126: 10 µM, Nonylphenol, and BPA: 100 µM.

7.2. Use N-Acetyl Cysteine (NAC) as an antioxidant to quench ROS. Add 5 mM of NAC to the ROS-induced chemical diluents and incubate for 12 h at 37 °C.

7.3. Centrifuge the 96-well plate at 112 x g and incubate with 5 µM solution of fluorescent probe (H2-DCFH-DA dissolved in RPMI media) at 37 °C for 60 min.

7.4. Wash the treated islet cells 3 times with PBS and measure the fluorescence with a microplate reader at 488 nm.

Note: The accumulation of DCF in cells may be measured by an increase in fluorescence at 530 nm when the sample is excited at 488 nm.

7.5. Perform the glucose-stimulated insulin secretion (GSIS) assay(Efendić, Tatemoto et al. 1987) on ROS-inducing xenobiotic treated islets and untreated islets to measure the xenobiotic effects on the physiological function of the pancreatic islets.

3.3 Representative Results

A micrograph of the healthy Isolated islet is shown in Figure 24, in which islets have a round or oval = shape with relatively uniform size (although size uniformity can vary from strain to strain). We next investigated the pancreatic islet functions in an *in vitro* assay by isolating the

islet and stimulating the insulin secretion in the culture islets. Figure 25 shows our typical analysis of the GSIS assay from C57BL/6 mouse isolated islets induced by 3.3 mM and 16.7 mM glucose(Efendić, Tatemoto et al. 1987).

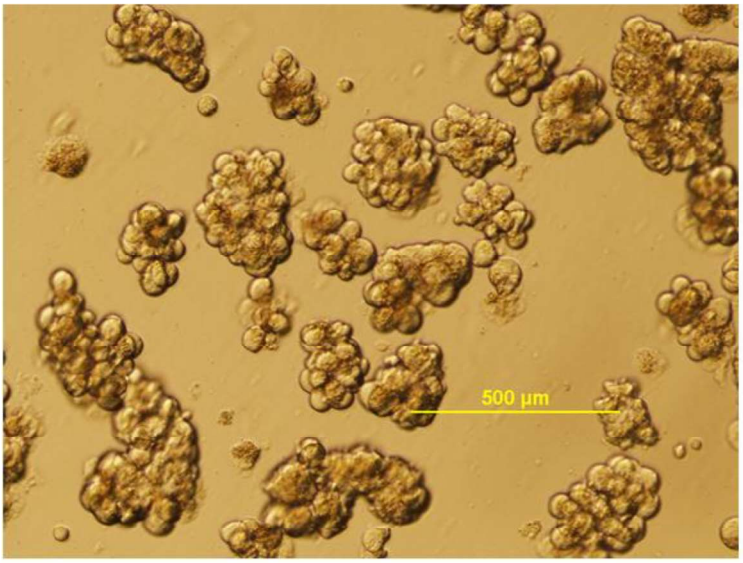

Figure 24. Isolated mouse islets. Isolated mouse pancreatic islets in culture medium are shown. Scale bar of 500 µm.

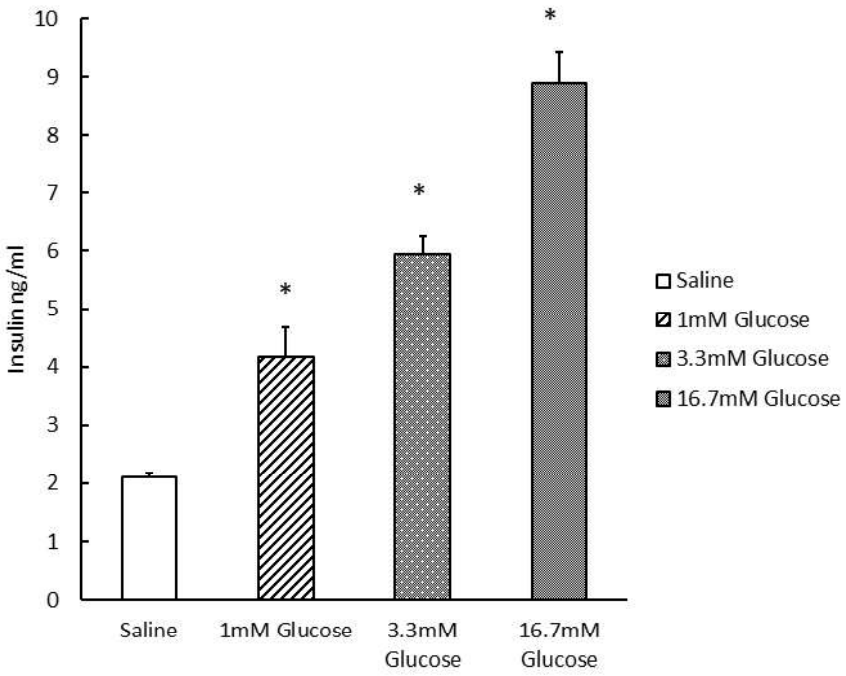

Figure 25. Glucose-stimulated insulin secretion of isolated pancreatic islets. Dose-dependent glucose-stimulated insulin secretion (1, 3.3, and 16.7 mM, treated for 15 min) was measured in isolated islets (average 5 islets per well), using a mouse insulin ELISA kit. Error bars show standard deviations. *$p < 0.05$.

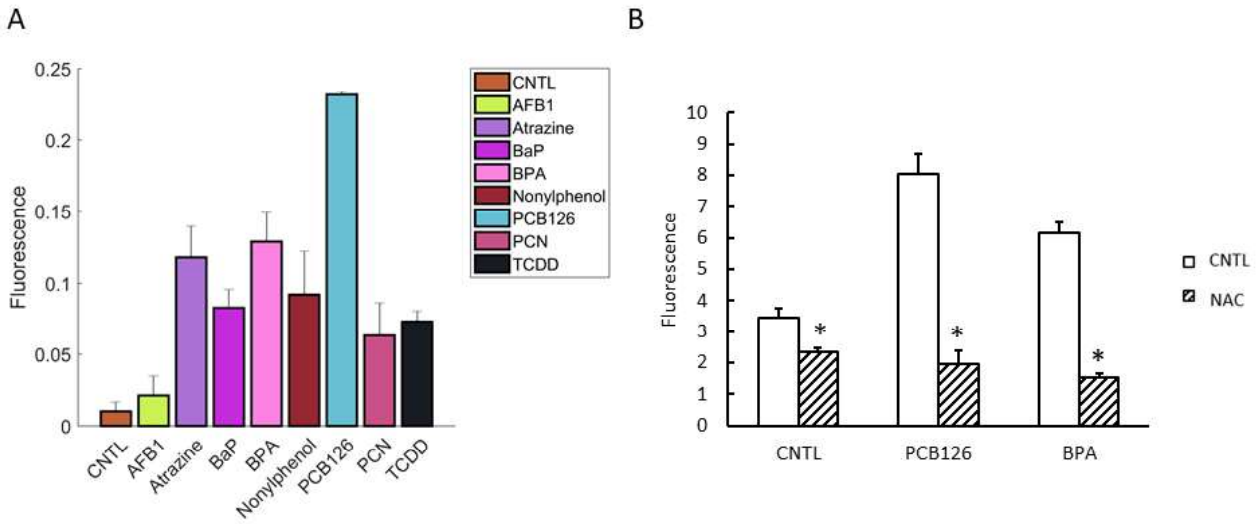

Figure 26. Induction of ROS by xenobiotics in isolated islets from C57BL/6 mice. (A) Isolated islets were treated for 12 h and stained with fluorescence dye 2',7'-dichlorofluorescein (DCFH-DA) for 1 h. The fluorescence was determined by a fluorescence plate reader. (B) The chemical-induced ROS were quenched by NAC (5 mM). (AFB1: 3 μM, Atrazine: 100 μM, BaP: 10 μM, BPA: 100 μM, PCB126: 10 μM, Nonylphenol, BPA: 100 μM, and NAC: 5 mM). Error bars show standard deviations. *$p < 0.05$.

Using the plate format screen method, we have identified several xenobiotics that caused significant induction of ROS in islet cells (Figure 26). We treated the isolated islets with different chemicals for 12 h and found that Atrazine, BPA, BaP, and PCB_{126} could cause significant induction of ROS in the isolated islets(Tian, Ke et al. 1999, Li and Wang 2005, Asahi, Kamo et al. 2010, Cui, Gu et al. 2017).

Although ROS is not equal to the diabetogenic induction, there is ample evidence indicating the important role of ROS in causing damage to the physiological functions of pancreatic β cells, which leads to T2DM(Gao, Nong et al. 2010). Therefore, the value of the work is to identify these ROS inducing chemical as the initial screen. Furthermore, the compounds identified can be further analyzed *in vitro* in the isolated pancreatic islet cell culture system described in the manuscript. We have shown the effects of ROS-inducing xenobiotics on glucose-stimulating insulin secretion of the isolated islets, which briefly provide diabetogenic data for these tested compounds. Pancreatic islets pre-treated with PCB126 and BPA showed a significant decrease in insulin secretion than the control group (Figure 27).

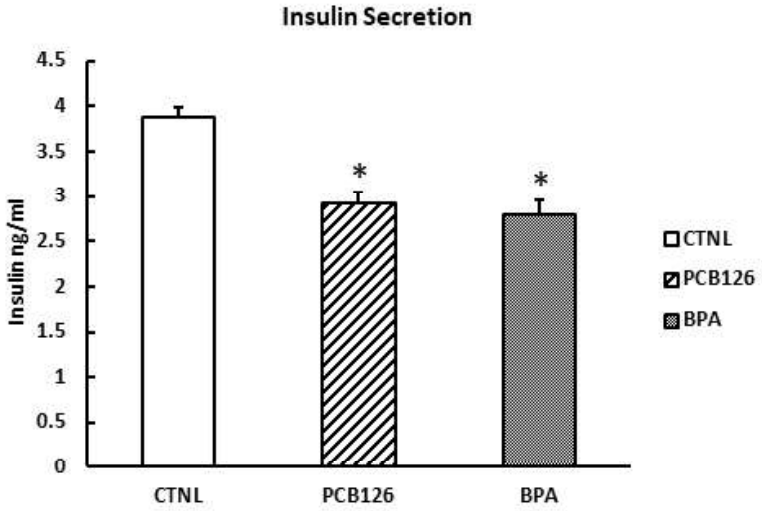

Figure 27. Xenobiotic effects on glucose-stimulated insulin secretion of isolated islets. Isolated islets were pre-treated with PCB126, and BPA (4 h) and kept in islet culture medium for 1 h. The supernatant of the islets was collected for measurement of insulin by a commercially available mouse insulin ELISA assay. Error bars show standard deviations. *$p < 0.05$.

3.4 Discussion

Accumulating evidence suggests that exposure to environmental chemicals plays an important role in the development of T2DM. Xenobiotics-induced ROS has been recognized as a potential etiological factor contributing to the development of T2DM. Humans are exposed to a wide range of xenobiotic chemicals and there is a great need for novel research techniques to effectively identify the pancreatic toxicants and to investigate the mechanism of toxicity specific to the pancreatic cells.

In this study, based on published procedures(Szot, Koudria et al. 2007), we have developed a protocol to isolate pancreatic islets from mouse and screen the cells with xenobiotic chemicals for oxidative stress-induced damage to the pancreatic cells. We also use this procedure to investigate the pancreatic specific toxic responses induced by the xenobiotic compounds. To obtain the pancreas from mouse, we prefer to use avertin over other anesthesia because it does not alter the blood glucose levels and does not affect the vasculature of the pancreas(Vaupel, McCoun et al. 1984, Kirstetter, Lagneau et al. 1997, Brown, Umino et al. 2005). We found that the density gradient centrifugation step is critical for isolation of the islet with high purity. Care should be taken to obtain the distinct layer between the polysucrose/sodium and isolation buffer phase to obtain a pure islets sample, devoid of debris and exocrine cells/tissues. The method can be used for *in vitro* analysis of environmental chemical effects on pancreatic islet physiology, such as the GSIS assay (as shown in Figure 25). The islets should be carefully dispersed with repeated pipetting into separate cells to ensure uniformed cell number in the 96-cell culture plate. The method described here combined the isolation of the islet and a rapid assay for ROS generation and therefore, is a simple and effective initial screen for potential pancreas-damaging chemicals.

We used insulin secretion in response to glucose challenge to confirm the identity of the isolated pancreatic islets. The procedure is highly adaptable for analysis of changes in parameters, including insulin secretion (Figure 25) and cAMP generation, in response to xenobiotic treatments(Neuman, Truchan et al. 2014).

4. ABLATION OF LNCRNA MALAT1 ACTIVATES THE ANTIOXIDANT PATHWAY AND ALLEVIATES INNATE INFLAMMATORY RESPONSE IN MICE

4.1 Introduction

Sepsis is a serious and expensive medical problem through out the world. In United states, the incidence of sepsis ranges from 400,000 to 750,000 cases annually, with a mortality rate around 30% in adults and 10% to 40% more in children and elderly people (Cohen 2002). Sepsis also could have long-term effects on quality of life in patients (Winters, Eberlein et al. 2010). However, there are no specific therapeutic interventions that are FDA-approved for clinically treating sepsis. The clinical treatment of sepsis is generally supportive in nature to eliminate the pathogen by administrating intervenous fluids, vasoactive fluids, vasoactive substances and oxygen with antibiotics (Wheeler and Bernard 1999, Gotts and Matthay 2016).

Sepsis is a pleiomorphic syndrome characterized by excessive inflammatory responses accompanying by systemic inflammatory response syndrome (SIRS) including high fever, hypotension, tachycardia and tachypnea, resulting in uncontrolled immune reaction after primary bacterial infection, which may lead to microcirculatory dysfunction, acute respiratory failure, tissue damage, multiple organ failure, and ultimately death (Oberholzer, Oberholzer et al. 2001). It is still unclear about the specific reason for uncontrolled inflammatory response and death happened in some septic patients (Angus, Linde-Zwirble et al. 2001). Based on the most clinical studies from 1990s, it was suggested that host's immune response to the pathogens during sepsis is complicated, involving the excessive innate immune response, immune suppression, and a failure to re-establish homeostasis in immune system (Hotchkiss, Monneret et al. 2013, Steinhagen, Schmidt et al. 2020) (Figure 28).

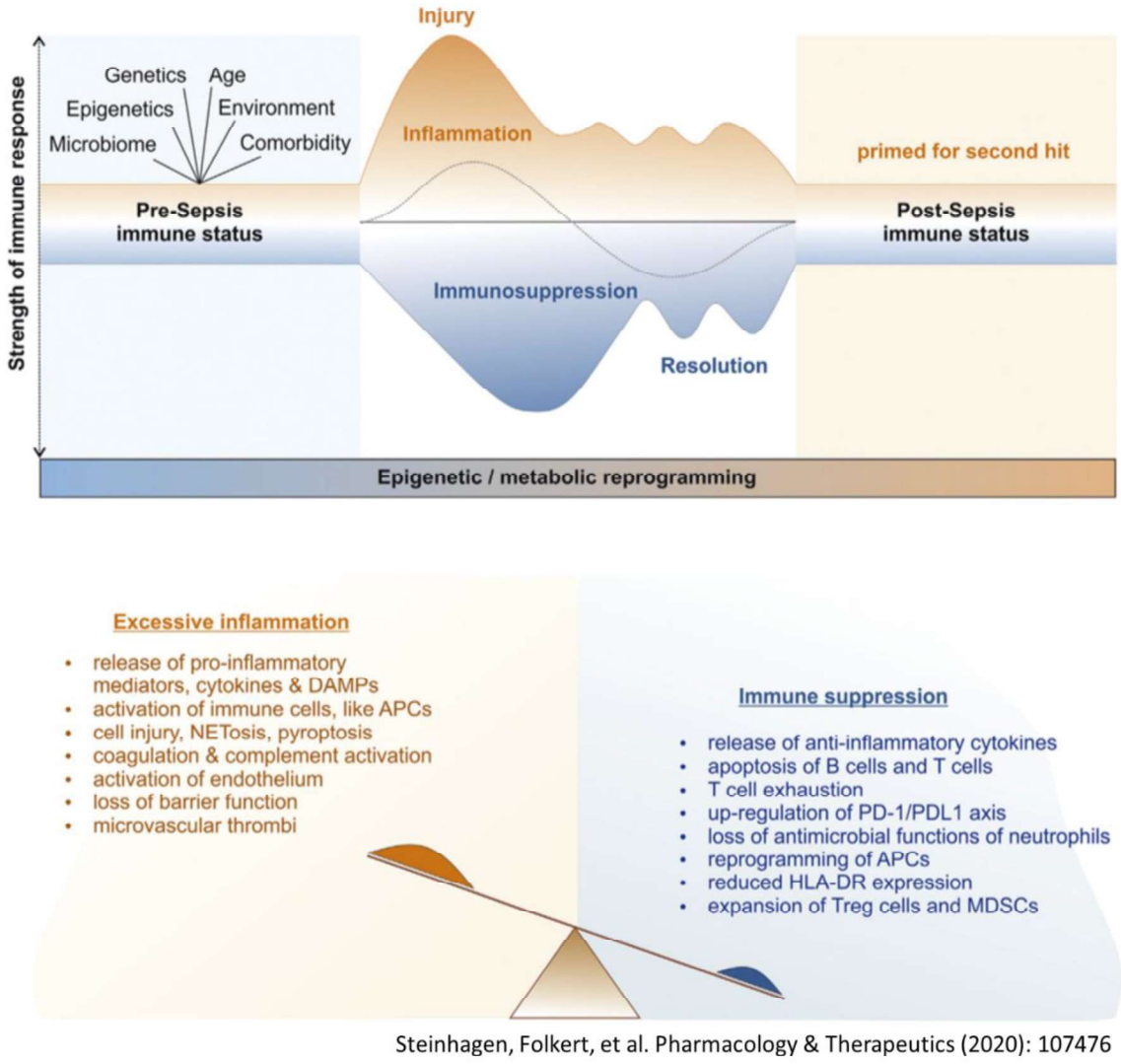

Figure 28. Illustration for the process of inflammatory and anti-inflammatory phases in sepsis.

Many factors can lead to SIRS, including the pathogen-associated molecular patterns (PAMP) such as lipoteichoic acid and bacterial lipopolysaccharide (LPS). Toll-like receptor 4 (TLR4), which leads to activation of inflammatory mediators, has been found to play an important role in sepsis pathophysiology. LPS, which is a main component of Gram-negative bacteria cell wall, is recognized by TLR4, which causes phagocytic cells to generate robustly a variety of pro-inflammatory cytokines. Recent clinical trials using a TLR4 antagonist (eritoran) involving 1800 septic patients were found to be ineffective. Currently we don't have adequate knowledge regarding the underlining mechanisms associated with the development of SIRS and sepsis in humans.

During sepsis, biochemical changes lead to the imbalance in the redox system, which will intensify SIRS and the downstream. It is possible that the complications developing in experimental sepsis, and perhaps in human sepsis, may be attenuated by therapeutic interventions that either reduce the level of pro-inflammatory mediators or restore degraded adaptive and innate immune responses. This review focuses on recent insights including our novel findings on regulations of some certain pathways and genes that may attenuate the loss of the redox balance in sepsis, thus reducing SIRS, multi-organ failure and lethality.

4.1.1 Interaction between pro-inflammatory and anti-inflammatory response

The host immune response to sepsis could be classified as pro-inflammatory responses and anti-inflammatory responses. The innate immune system acts as the first line of host immune through germ line-encoded pattern recognition receptors (PRR), such as toll-like receptors (TLRs), which recognize conserved motifs from pathogen-associated molecular patterns (PAMPs) such as microbial products (lipopolysaccharide, LPS), and induce the expression of hundreds of proteins involved in antimicrobial defense and adaptive immunity (Kawai and Akira 2008).

Leukocyte activation will in turn enhance the pro-inflammatory response. The anti-inflammatory response will be regulated by inhibition cytokine signals in innate immune system such as IL-10 and differentiation of immune cells (macrophage polarization).

4.1.2 Pattern recognition receptors

PPRs can be characterized by the cellular localization. Several classes of PRRs were discovered since mid- 1990s, including Nod-like receptors (NLRs) and RIG-I- like receptors (RLRs) (Vogl, Tenbrock et al. 2007, Lamkanfi 2011). Among these receptors, TLRs play an important role in immune response in sepsis. The intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain is critical for downstream signal transduction, leading to the transcriptional activation of inflammatory mediators (Kawai and Akira 2010). To date, thirteen mammalian TLRs have been discovered, in which ten of them are found in humans and TLR 11-13 are in mice. Some TLRs including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface, and some are expressed intracellularly including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10. The cellular localization of TLRs is important for ligand binding. TLRs are critical for the early initiation inflammatory response. It has been found that knock down of TLR function leads to a more enhanced susceptibility for infection, that MyD88-deficient mice showed vulnerable to a variety of infectious diseases (Kawai and Akira 2010). Accordingly, children deficient for MyD9929 or its direct downstream mediator IRAK-429 have frequent purulent infections (Ku, Von Bernuth et al. 2007, Von Bernuth, Picard et al. 2008). However, on the other hand, uncontrolled TLR activation and stimulation of downstream signals leads to inappropriate inflammation and tissue injury.

Several mechanisms are found to regulate of TLR signaling pathway in both positive and negative ways. Triggering receptor expressed by myeloid cells-1 (TREM-1) is a receptor that is

found on neutrophils and monocytes. It has been found that co-stimulation of TREM with TLRs leads to a synergistic increase in inflammatory response, while blocking of TREM led to attenuated cytokine secretion and improved survival in sepsis (Klesney-Tait, Keck et al. 2012, Arts, Joosten et al. 2013). These results provide the importance of balance in innate immune system, protecting host from infection and associated tissue damage.

TLR-mediated inflammatory response could be negative regulated by some transmembrane receptors including single immunoglobulin IL-1R-related molecule (SIGIRR and TIR8), IL-1 receptor-like 1 (ST2), and radioprotective 105 (RP105). Cells that deficient in either one of these receptors respond more avidly to TLR stimulation. Additionally, adaptor proteins involved in efficient signaling pathways of TLRs such as MyD88, TIRAP/Mal, TRIF, and TRAM) are also targets for negative regulation of TLR-mediated inflammation.

For instance, TAG (TRAM adaptor with Golgi dynamics domain) is a TRAM variant that competes with TRAM for TRIF binding, thereby inhibiting the TRIF-dependent pathway (Palsson-McDermott, Doyle et al. 2009). TAG is also found to mediate internalization of TLR4 to the endosomes for subsequent degradation (Carty, Goodbody et al. 2006). Inflammatory signaling pathway could also be shut down by ubiquitination, which is a process during which proteins are "tagged" with ubiquitin for proteasome-mediated degradation. SHP (small heterodimer partner) suppresses TLR signaling by regulating the ubiquitination of an essential downstream signaling molecule (TRAF6) (Yuk, Shin et al. 2011).

Epigenetic processes include chemical modifications of DNA and/or associated histones play an important role in regulating inflammation following PRR activation (Portela and Esteller 2010). Peripheral blood mononuclear cells isolated from sepsis patients show increased level of repressive histone modification at the promoter region of IL-1 and TNFa, which is regulated by

dimethylation of histone 3 lysine residue 9 (H3K9me2). Collectively results from previous research showed that early largely enhanced inflammatory response in sepsis may lead to regulation of epigenetic marks at promoter regions of pro-inflammatory genes in macrophages. Recently, noncoding RNA including micro-RNAs (miRNAs) and long noncoding RNAs were found to post-transcriptionally regulate inflammatory associated gene.

Several attempts have been made to find therapeutic treatment for sepsis. TLR4 was considered as an attractive target for treating sepsis as it played a causal role in cellular activation induced by several PAMPs (LPS) and DAMPs. However, one drug targeting TLR4, TLR4/MD2 antagonist eritoran didn't pass the phase III trial even though it had shown promising effects in phase II.

4.1.3 Cytokines regulation in immune response in sepsis

Cytokines played important roles in regulating immune response. The most investigated cytokines in sepsis are pro-inflammatory cytokines including TNFα, IL-1 and IL-6 (van der Poll and Opal 2008). TNFα was induced by LPS and secreted in large amount by activated macrophage, which were found extreme toxic to body and trigger septic shock during sepsis. IL-6 was also important in regulating sepsis, which has both pro-inflammaotory and anti-inflammatory properties. Other cytokines, including IL-8, IL-12, interferon (INF)-γ, and IL-10 were also involved in regulating immune response in sepsis. It was later found that IL-17, which mainly secreted by Th17 cells, is a key pro-inflammatory cytokine during sepsis by inducing the classical pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, providing crosstalk between the adaptive and innate immune system (Weaver, Hatton et al. 2007). Macrophage migration inhibitory factor (MIF) is another important cytokine, which is upregulated during

sepsis. Deficiency of MIF in mice was found to have defective effect on LPS challenge, which was later targeted for therapeutic treatment in sepsis (Yende, Angus et al. 2009).

4.1.4 Crosstalk between redox imbalance and inflammatory response in sepsis

During sepsis shock, biochemical change could lead to an imbalance redox state, during which excessive reactive oxygen species (ROS) could be generated from several potential sources, such as mitochondrial respiratory electron transport chain, xanthine oxidase activation, and respiratory burst (Macdonald, Galley et al. 2003). This redox imbalance will lead to oxidative stress often with decreased antioxidant capacity such as plasma GSH level, thioredoxins, selenium, together with inhibited mitochondria respiration rate with lower ATP production and increased lipid oxidation. ROS is a "weapon" of activated neutrophils and macrophages during inflammatory response, however, in the same time, which can cause chemical changes of proteins, DNA and lipids ultimately leading to membrane damage, alterations in membrane permeability, modification of protein structure and functional changes (Valko, Leibfritz et al. 2007).

Nuclear factor-erythroid 2–related factor 2 (Nrf2), as a basic leucine zipper redox-sensitive transcription factor, plays a pivotal role as a transactivator regulating a battery of antioxidant genes which collectively control intracellular ROS levels. Recent studies have found that Nrf2-regulated pathway plays an important role in regulating septic shock responses. When activated by oxidative stimuli, Nrf2 will translocate into nucleus from cytoplasm, resulting in enhanced activation of the ARE-driven antioxidant genes. Mice with disruption of Nrf2 have been shown to be highly sensitive to sepsis, with augmented lung inflammation (Thimmulappa, Lee et al. 2016). Nrf2 was found to suppress the acute inflammation and blunt cytokine storms in sepsis shock through inhibition of NF-□B activation by maintenance of redox balance. This indicates the importance of Nrf2-mediated maintenance of antioxidants.

4.1.5 Long noncoding RNA functions in regulating inflammatory response in innate immunity

Long non-coding RNAs (lncRNAs), which were once considered as part of "transcriptional noise", are emerging as important factors in regulating gene expressions involved in immune response. It has been increasingly recognized that lncRNAs have important regulatory functions in either activation or inhibition of innate immune response, and many of the immune regulatory functions are carried out by control of differentiation of innate immune-related cells (e.g. macrophage polarization). We will focus on the pleiotropic functions in the innate immunity and the underlying mechanism of these lncRNAs in either activating or restrain innate immune response at transcription, translation and post-transcriptional levels which are carried out through complex RNA-DNA, RNA-proteins and RNA-RNA interactions. In addition, we will also discuss the recent revelation on the post transcriptional modifications of RNA which is emerging as a new discipline (epitranscriptomics) and its role in regulating the inflammatory processes. Finally, we will provide insights into the important physiological role of lncRNA and the prospects of future therapeutic application by targeting these lncRNAs for treating inflammatory diseases.

The information regarding these lncRNAs has been emerging piecemeal and several well-characterized lncRNAs have been found to be involved in biological processes, such as genomic imprinting (H19), cancer metastasis (HOTAIR and MALAT1), riborepressor (Gas5), and stem cell differentiation (lincRNA-RoR), among many others (Mercer, Dinger et al. 2009, Kino, Hurt et al. 2010, Tsai, Spitale et al. 2011, Lee and Bartolomei 2013, Xie, Yuan et al. 2013, Flynn and Chang 2014). Surprisingly, lncRNAs discovered to date do not show a single/specific archetype, including interactions between nucleic acids (DNA and RNA) through base-pairing and through

interaction with proteins(Wang and Chang 2011). Thus, how lncRNAs function in cells has become an important question as extensive groundwork has been laid for investigation of their molecular functions. One example of these biological processes is the rapidly expanding body of work on lncRNAs regulating immune response (Robinson, Covarrubias et al. 2019). In this review, we will discuss the recent discoveries of lncRNAs involved in regulating innate immune response and macrophages diffenrentiation.

Innate immunity refers to nonspecific defense mechanisms that act immediately or within hours of an encountering the antigens in the body. Recently many lncRNAs have been reported to be transcriptionally induced or inhibited by inflammatory molecules and in turn regulate the immune response (Figure 29).

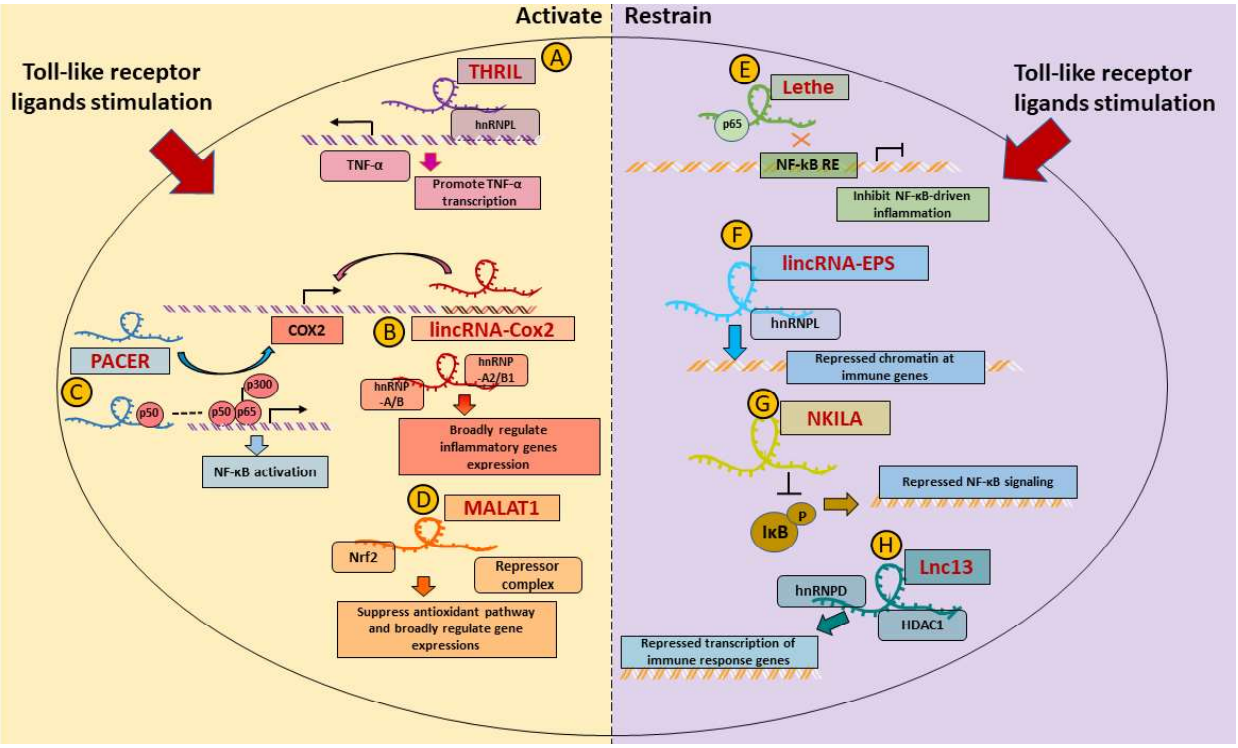

Figure 29. LncRNAs activate (A-D) or restrain (E-H)innate immune response. (A) THRIL was found as an antisense lncRNA to promote TNF-α transcription by forming an interactive complex with hnRNPL and binding to the promoter region of TNF-α. (B) LincRNA-COX2 defined as its location about 51 kb upstream of the protein-coding gene Cox2, was found to have globally effects on inflammatory gene expression via interacting with hnRNP-A2/B1 and hnRNP-A/B. (C) The PACER is another COX2-related nuclear antisense lncRNAs, which was found to facilitate the activation of NF-κB by sequestering the p50 away from the PTGS2 promoter. (D) MALAT1 has been found to play an important role in regulating antioxidant and detoxification process through interacting with nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) and its deletion led to activation of downstream antioxidant genes in the Nrf2-driven pathway and ablation of MALAT1 significantly attenuated pro-inflammatory response. MALAT1 was also reported to be associated with repressor complex (PRC2) and function as a ribo-repressor to pervasively regulate genes expression. (E) Lethe was found to negatively regulate NF-kB inflammatory signaling pathway by preventing RelA(p65) binding to NF-kB response elements. (F) LincRNA-EPS was found to be an important transcriptional brake that restrained the expression of genes encoding inflammatory by interacting with hnRNPL to alter nucleosome positioning and repress the transcription of immune response genes. (G) LncRNA NKILA was reported to be an essential lncRNA that regulated NF-κB signaling and repressed cancer-associated inflammation by binding to NF-κB/IκB forming a stable complex and directly mask phosphorylation motifs of IκB, thereby inhibiting IKK-regulated IκB and NF-κB activation. (H) LncRNA lnc13 was found to suppress the transcription of genes in inflammatory response through an RNA-protein complex by binding to hnRNPD and the histone deacetylase HDAC1, and suppressed the transcription of a set of immune response genes.

4.1.5.1 LincRNA-COX2

Among the lncRNAs identified during the process of inflammation, lincRNA Cox2, defined as its location about 51 kb upstream of the protein-coding gene Cox2, was found to be markedly induced more than 1,000-fold following the activation of Toll-like receptors TLR4, as well as TLR1, TLR2 and TLR8 (Guttman, Amit et al. 2009, Carpenter, Aiello et al. 2013). LincRNA-Cox2 is found to be transcribed depending on the TLR signaling adaptor protein Mdy88 and the transcription factor NF-κB. Ablation of lincRNA-Cox2 was found to cause more than 700 altered genes expression, which shows that lncRNAs can broadly regulate the expression of a large set of genes. Specifically, knockdown of lincRNA-Cox2 attenuated the immune response by suppressing expression of genes including TLR1, IL-6 and IL-23a in Pam3CSK4-stimulate macrophages. In addition, lincRNA-Cox2 was also found to regulate gene expressions in the absence of inflammatory stimulation. In resting macrophages, deletion of lincRNA-Cox2 increased the genes encoding chemokines (CCL5, CX3CL1), chemokine receptors (CCRI), as well as the interferon-stimulated genes (ISGs). LincRNA-Cox2 was found to control gene expression both in *cis* (where the lncRNA regulate the expression of neighboring genes) and in *trans* (where the lincRNA regulate genes on different chromosomes) (Elling, Robinson et al. 2018, Kopp and Mendell 2018). Transcriptomic studies showed that lincRNA-Cox2 functioned in *cis* as an enhancer RNA to regulate the expression of a neighboring gene prostaglandin-endoperoxide synthases (Ptgs2), which encodes a key enzyme in the prostaglandin biosynthesis pathway [17]. LincRNA-Cox2 was also found to broadly regulate the genes involved in inflammatory response *in trans (Hu, Gong et al. 2016, Covarrubias, Robinson et al. 2017, Xue, Zhang et al. 2019)*. One of the possible mode of action of lincRNA-cox2 in regulating immune response is through interaction with nuclear RNA-binding proteins, heterogeneous nuclear

ribboneoproteins hnRNP-A/B (Hnrnpab) and hnRNP-A2/B1 (Hnrnpa2b1) [16], which are involved in the process of splicing, RNA stabilization and transportation, transcription and translation of genes (Geuens, Bouhy et al. 2016). Additionally, ablation of Hnrnpab and hnRNP-A2/B1 in bone-marrow-derived macrophages was found to lead to dysregulation of genes involved in inflammation, which overlapped with the set of genes dysregulated by lincRNA-Cox2 ablation.

4.1.5.2 LncRNA THRIL

TNF-α and heterogeneous nuclear ribonucleoprotein L- (hnRNPL-) related immunetory LincRNA (THRIL) is another lncRNAs that functions through the mechanism of RNA-protein interaction with heterogeneous nuclear ribboneoproteins (hnRNP) (Li, Chao et al. 2014). It was first identified in an unbiased gene-expression screening in the human monocyte cell line THP1-defferentiated macrophages stimulated by Pam3CSK4. Depletion of THRIL in THP-1 cells was found to cause 300 genes differentially expressed in compared to the control THP-1 cells with sufficient level of THRIL. THRIL was found to be essential for TNF induction and knockdown of THRIL significantly suppressed of TNF-α and IL-6 secretion. Similar to lincRNA-Cox2, THRIL regulates the inflammation-related genes at resting state of macrophage. By screening using mass-spectrometry, THRIL was found to interact with RNA-binding protein hnRNPL and form a functional THRIL-hnRNPL interaction complex, which regulate transcription of TNF-α by directly binding to its promoter region. Clinical studies showed that THRIL expression positively correlated with acute respiratory distress syndrome (ARDS) risk, inflammatory response, severity of disease and also mortality in sepsis patients and could be used as a potential biological marker for diagnosis of sepsis (Fu, Yu et al. 2019).

4.1.5.3 PACER

The lncRNAs p50-associated COX-2 extragenic RNA (PACER) is another COX2-related nuclear antisense lncRNAs, which is transcribed from the upstream region of Cox2 gene (Krawczyk and Emerson 2014). The expression of PACER was induced by lipopolysaccharide (LPS), and positively regulated its neighboring gene PTGS2, that encoded COX-2 ('cycolooxygenase 2') (Krawczyk and Emerson 2014). Mechanistically, it has been found that chromatin-boundary/insulator factor CTCF created a permissive chromatin domain in the upstream promoter region of COX-2 and promoted PACER transcription. And PACER associated with the repressive subunit of NF-κB, p50, sequestered the p50 away from the PTGS2 promoter and facilitated the activation of NF-κB pathway with p65/p50 dimers. Recently PACER was also found to be induced by IL-1β in human chondrocyte (Pearson, Philp et al. 2016) and whether PACER physically interacts with COX2 and regulates IL-1β mediated inflammation is still unclear. It is also not clear if PACER acts *in trans* to regulate other NF-κB signaling pathway-mediated inflammatory genes.

4.1.5.4 MALAT1

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long noncoding RNA and has been found to play an important role in regulating diverse cellular process and diseases especially carcinogenesis and tumorigenesis (Ji, Diederichs et al. 2003). MALAT1 level was significantly upregulated after treatment of LPS and that inflammatory molecules such as TNF-α and IL-1β in mouse peritoneal macrophages, bone-marrow derived macrophages and human THP1 monocyte cell line (Zhao, Su et al. 2016). One recent study showed that MALAT1 played an important role in regulating antioxidant and detoxification

process through interacting with nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) and its deletion led to activation of downstream antioxidant genes in the Nrf2-driven pathway (Chen, Ke et al. 2018). In the current study of us we found MALAT1-Nrf2 interactive complex as a key regulator of LPS-induced immune response and ablation of MALAT1 significantly attenuated pro-inflammatory response in sepsis in mice. These results showed that MALAT1 had the potential to be a therapeutic target for treating inflammatory diseases including sepsis as well as other diseases caused by excessive generation of reactive oxygen species and imbalance redox state.

4.1.5.5 IL1β-eRNA and IL1β-RBT46

Through transcriptomic analysis of differentially expressed genes in human monocytes treated with LPS, one study showed that an eRNA (IL1β-eRNA) and a RBT (IL1β-RBT46) that adjacent to the IL-1β locus played important roles in regulating innate immune response (Ilott, Heward et al. 2014). Knockdown of these two lncRNAs was found to attenuate LPS-induced inflammatory genes transcription and suppress IL-1β and CXCL8 suppression.

4.1.5.6 CircRNAs

While most lncRNAs typically display “mRNA-like” features associated with linear sequence, including 5’ capping, polyadenylation (poly [A]) tail, and alternative splicing(Wilusz 2016), some non-codling RNA also be transcribed by back-splicing to form circular RNA (circRNAs), which are closed loops without 5’ capping containing multiple exons(Capel, Swain et al. 1993). Similar to lncRNAs, these circRNAs are found to be covalent in eukaryotic cells and frequently expressed in a tissue-specific manner. Interestingly, recent studies showed an emerging role of circRNAs in regulating genes encoding inflammatory molecules in innate immune response(Zhou, Fu et al. 2018). It is found that certain exogenous circRNAs could stimulate a

greater innate immune response than that stimulated by linear RNA with the same sequence (Chen, Kim et al. 2017). Interestingly, circRNAs generated by foreign introns induced the inflammatory response, while the same sequence of circRNA produced by endogenous introns was modified with m6A on the surface as "self" recognition to prevent immune activation (Chen, Kim et al. 2017, Chen, Chen et al. 2019). Another study showed that nearly 2,000 circRNAs were induced following TLR4 stimulation (Ng, Marinov et al. 2016, Ng, Marinov et al. 2017). Among those circRNAs, micircRasGEF1B was induced by LPS and its expression was dependent on NF-κB signaling pathway. Deletion of micrcRasGEF1B in LPS-treated macrophages was found to suppress the inflammatory adhesion molecule ICAM-1. The authors suggested that micircRasGEF1B was acting as a "sponge" in the cytoplasm that prevent miRNAs from targeting ICAM1 and in turn regulated the stability of ICAM1 mRNA. In addition to being present inside cells, circRNA has also been reported to show in extracellular fluids (Zhang, Yang et al. 2018), which has the potential to be developed as disease biomarkers (Li, Zheng et al. 2015).

4.1.5.7 Lethe

The lncRNAs Lethe was one of the first confirmed functional pseudogene lncRNA in the mammalian genome. It was found that lethe was largely induced in mouse embryonic fibroblasts after stimulation of pro-inflammatory cytokines TNF-α and IL-1β, and the anti-inflammatory agent, dexamethasone (Rapicavoli, Qu et al. 2013). The induction of lethe is dependent on NF-κB activation, and knockdown of lethe resulted in upregulation of NF-κB regulated genes, suggesting its negative regulatory role in inflammation. As expected, overexpression of lethe suppressed the activity of an NF-κB as lethe binded to the NF-κB subunit p65 (RelA) and prevent it from binding to the promoter of NF-κB-driven genes, thus suppressed the secretion of inflammatory cytokines, including IL-6, IL-8, and Nfkbia. Therefore, lethe played a role in

regulating immune response as a decoy receptor for NF-κB and suppressed inflammatory response. Overexpression of lethe was found to eliminate reactive oxygen species (ROS) generation in macrophages through modulation of NOX2 gene expression via NFκB signaling (Zgheib, Hodges et al. 2017). Additionally, lethe expression was associated with aging and it is highly likely that age-dependent decrease of lethe would be one important factor that increases NF-κB activity during aging (Adler, Sinha et al. 2007).

4.1.5.8 Lnc-IL7R

Lnc-IL7R is another newly reported immune-related lncRNAs and it overlaps with 3' untraslated region of the human interleukin-7 receptor α-submit gene (Cui, Xie et al. 2014). The expression level of lnc-IL7R was significantly upregulated in THP-1 cells after treated with LPS. The increased lnc-IL7R was found to diminish LPS-induced inflammatory response with a reduced secretion of E-selectin and VCAM-1. It was suggested that lnc-IL7R suppressed the transcription of E-selectin and VCAM-1 through regulating trimethylation of histone H3 at lysine 27 and the mechanism of lnc-IL7R in regulating IL-8 secretion needs to be further investigated in the future.

4.1.5.9 LincRNA-EPS

LincRNA-EPS was found to be an important transcriptional brake that restrained the expression of genes encoding inflammatory (Atianand, Hu et al. 2016). It is expressed in macrophages, dendritic cells and erythrocytes and its expressed was found to significantly decrease in cells following stimulation of microbial ligands including LPS. Ablation of lincRNA-EPS in mice does not show overt defects, however, these mice showed profound "hyper-activation" of immune response and increased susceptibility to LPS-induced inflammation *in vivo*. Consistently, gain-of-function and rescue studies further showed lincRNA-EPS as a potent

immune repressor. Genome-wide transcriptomic analysis of macrophage showed that lincRNA-EPS specifically repressed the expression of immune response genes. Mechanistically lincRNA-EPS localized in nucleus and associated with chromatin and interacted with hnRNPL (a member of a large family of heterogeneous ribonucleoproteins) to alter nucleosome positioning and repress the transcription of immune response genes. Recently another study showed that lincRNA-EPS expression was down-regulated in the monocytes from patients with active pulmonary uberculosisa in comparison to healthy groups, accompanied by activated JNK/MAPK signaling pathway leading to significant inhibited apoptosis and enhanced autophagy (Ke, Lu et al. 2020). Collectively, these results demonstrate that lincRNA-EPS plays a critical role in restraining immune response through acting directly on the chromatin to control chromatin accessibility and nucleosome positioning of the immune response genes.

4.1.5.10 LncRNA lnc13

Similar to LincRNA-EPS, lncRNA lnc13 was found to suppress the transcription of genes in inflammatory response (Castellanos-Rubio, Fernandez-Jimenez et al. 2016, Ray 2016). Lnc13 is expressed in macrophages and its expression is decreased after TLR4 activation. Lnc13 was reported to localize in the nucleus and functioned in an RNA-protein complex by binding to hnRNPD and the histone deacetylase HDAC1, and suppressed the transcription of a set of immune response genes including Myd88, Stat1, Stat3 and TNF. Upon inflammatory stimulation with TLR4 ligands, lnc13 expression level was reduced thereby resulting in increased expression of the repressed immune response genes. Additionally, lnc13 was found to harbor celiac disease associated single-nucleotide polymorphisms (SNPs) and a form of lnc13 transcribed *in vitro* containing alleles. Patients with celiac diseases were found to have lower expression of lnc13 and higher expression of lnc13-regulated immune response genes, suggesting the lnc13 plays a role

in maintaining immune homeostasis and dysregulation of lnc13 expression might be associated with the development of inflammation.

4.1.5.11 LncRNA NKILA

LncRNA NKILA was reported to be an essential lncRNA that regulated NF-κB signaling and repressed cancer-associated inflammation at the level of post-transcriptional control (Liu, Sun et al. 2015). NKILA is substantially induced in breast cancer cells after stimulated with IL-1β or TNFα. Similar to the function of lethe, knockdown of NKILA increased NF-κB signaling pathway activity and overexpression of NKILA attenuated immune response driven by NF-κB. Mechanistically NKILA was found to bind to NF-κB/IκB forming a stable complex and directly mask phosphorylation motifs of IκB, thereby inhibiting IKK-regulated IκB and NF-κB activation.

4.1.5.12 LncRNAs in regulating macrophage differentiation

Macrophages play critical roles in regulating both innate and adaptive immune system. These cells display remarkable phenotypic diversity that is largely dictated by their activation status. Under certain microenvironment, macrophages differentiate into two distinct functional phenotypes: pro-inflammatory macrophages (M1 macrophages) which are basically activated by IFN-γ and/or TLR ligands are generally associated with immunity to bacteria and intracellular pathogens, and alternatively activated anti-inflammatory macrophages (M2 macrophages) which are normally activated by IL-4/IL-13, IL-10, TGF-β or glucocorticoids and dominated by TH2 responses such as helminth immunity, asthma, and allergy. Macrophage polarization is closely associated with the pathophysiological states and it plays an important role in several diseases including infection, cancer and autoimmune diseases (Ruffell, Affara et al. 2012) (Patel, Rajasingh et al. 2017). The recent findings of molecular mechanism on macrophage plasticity and polarization provide insights for macrophage-centered diagnostic and therapeutic applications.

The regulatory mechanisms for the constellation of genes that control the differentiation and activation of macrophages are still not fully clear. Recently lncRNAs have been found to play emerging roles in regulating macrophage polarization. One study screened differentially expressed lncRNAs in polarized human monocyte-derived macrophages with treatment of IFN-γ+LPS or IL-4 (Hu, Goswami et al. 2019). It was shown that 9343 lncRNAs were deregulated in pro-inflammatory macrophages (treated with IFN-γ+LPS) and 4592 lncRNAs were deregulated in anti-inflammatory macrophages (treated with IL-4).

Among the deregulated lncRNAs, lncRNA TCONS_00019715 was found to show significantly higher expression in pro-inflammatory macrophages than that in anti-inflammatory macrophages. Consistently, expression of TCON_00019715 was decreased when proinflammatory macrophages convert to anti-inflammatory macrophages. Knockdown of TCONS_00019715 was found to diminish the expression of proinflammatory markers in macrophage and increase the expression of anti-inflammatory markers. Another example is lncRNA MALAT1, which we discussed previously about its function in regulating antioxidant/detoxification system (Chen, Ke et al. 2018) and innate immune response. One study showed that MALAT1 played an important role in regulating differential activation of macrophages and response to lung injury (Cui, Banerjee et al. 2019). Consistent with our findings, it was reported that MALAT1 ablation attenuated LPS-induced M1 macrophage activation, and on the contrary, enhanced IL-4-activated M2 macrophage activation. Mechanistically, deletion of MALAT1 caused decreased expression of Clec16a, and promoted IL-4 induced mitochondrial pyruvate carriers (MPCs) ad their mediation of glucose-derived oxidative phosphorylation (OxPhos). These findings suggested MALAT1 as an important regulator involved in pulmonary pathogeneses in association with aberrant macrophage differentiation. GAS5, previously

described as a “ribo repressor” and inhibit transcriptional activity of glucocorticoid receptor (Kino, Hurt et al. 2010), was also found to play a role in macrophage polarization (Ito, Asai et al. 2017). It was reported that reduction of GAS5 using nonsense-mediated RNA decay contributed to M2b macrophage differentiation. One possible mechanism was discovered that GAS5 expression level was decreased through interaction with miR-222 and the degradation of GAS5 caused M2b macrophage activation.

4.1.5.13 Regulation of epitranscriptomic modification in inflammation

Epigenetic studies have shown complex interactions between transcription factors involved in inflammation such as NF-κB, FOXP3 and STAT families, along with DNA methylation and covalent histone modifications, which play critical role in the regulation of inflammatory genes and the outcomes of these complex interactions are also modulated by physiological (Cunningham and Eghbali 2018) and dietary components (Das, Dickerson et al. 2018). In analogy to DNA and chromatin modifications, a diverse set of covalent modifications is detected on RNA nucleotides encoding the epitranscriptome, post-transcriptionally shaping gene expression through regulation of RNA stability, translation, and non-coding RNA functions. N6-methyladenosine (m^6A) is one of the most abundant RNA modifications, which present on 0.4%-0.6% of all cellular RNAs including mRNAs, lncRNAs, and circRNAs. Recently, m^6A modification has been reported to play emerging roles in regulating inflammatory response. One study showed that m^6A modification on circRNAs played important role in immune response (Chen, Chen et al. 2019). The unmodified foreign circRNAs from virus could activate antigen-specific T and B cell response by activating the RNA pattern recognition receptor (RIG-I) in presence of lysine-63-linked polyubiquitin chain and initiate MAVS filamentation, while the endogenous (self) circRNAs was marked by m^6A modification and abrogates the circRNA-

induced immunity. Mechanistically, YTHDF2, as one of the most common m6A reader proteins, was required for the "self" identification of m6A-marked circRNAs and in turn suppressed the immune stimulation by foreign circRNAs. Interestingly, this m^6A modification controlled circRNA immunity to distinguish self from foreign genes is strikingly similar to DNA modification-restriction systems which transforms nucleic acid chemical modification to organismal innate immunity.

Another example of epitranscriptomic modification of lncRNA involved in regulating inflammation is the interaction between MALAT1 and methionine cycle, which may impact the homeostasis of the methyl donor and antioxidant capacity. In mammalian methioninecycle, methionine adenosyltransferase (MAT2A) is essential for the biosynthesis of SAM and the gene expression of MAT2A is regulated at transcriptional and post-transcriptional levels through m^6A RNA methylation of the 3'UTR of MAT2A mRNA (Pendleton, Chen et al. 2017) (Shima, Matsumoto et al. 2017). It has been found that in human clinics and animal models, methionine metabolism is disrupted with greatly increases in the oxidative stress levels as indicated by the dysregulated GSH/GSSG level (Biolo, Antonione et al. 2007, Dong, Hu et al. 2013). Post-transcriptionally, the mRNA of MAT2A stability is regulated by the m6A methylation mediated by RNA methylation writer METTL16 which methylates 3'UTR of MAT2A, thus controlling its level. Mechanistically, strong evidence from structural analysis indicates among the methylation sites of MAT2A, the hairpin 1(hp1) is the linchpin for regulating MAT2A expression (Doxtader, Wang et al. 2018) by regulating intron splicing(Pendleton, Chen et al. 2017) and mRNA decay(Shima, Matsumoto et al. 2017) of MAT2A, thus controlling methionine cycle. METTL16 forms a complex with MALAT1(Brown, Kinzig et al. 2016). The METTL16-binding site, which is the highly conserved triple helix, is present in every MALAT1 homolog (Stadler 2010, Zhang,

Mao et al. 2017), thus it is highly likely MALAT1-associated proteins such as methyltransferases (METTLE 13/14 and16) may in turn modify the functions of MALAT1 thereby regulating gene expressions involving in wide range of the physiological processes.

4.1.6 A long noncoding RNA-MALAT1 mediates immune response in sepsis

Our recent studies showed that MALAT1 interacted with Nrf2/ARE as a negative co-regulator and its deletion led to activation of genes in the Nrf2/ARE pathway. Nrf2 is a pleiotropic transcriptional factor that regulates the cytoprotective genes required for resistance to oxidative stress, which induces reactive oxygen species and is the common etiological factor for many diseases. Indeed, we found MALAT1 ablation led to the activation of the insulin pathway and the mice become sensitized to the hyperglycemic challenge. In the current study we found MALAT1-Nrf2 interactive axis act as a key regulator for the LPS-induced inflammatory response. Oxidative stress, referred as a serious imbalance between ROS production and antioxidant defenses, has been implicated in the process of sepsis and has been causally associated for its severity. In this study, we define MALAT1 as an important regulator at transcriptome and epitranscriptome level that activate the inflammatory gene expression in hepatocytes and macrophages and in mice. Collectively, these results MALAT1 has the potential to be a therapeutic target for treating sepsis as well as other diseases caused by excessive generation of ROS.

4.2 Materials and Methods

4.2.1 Reagents

Lipopolysaccharide (LPS), Fluorescent dye 2',7'-dichlorofluorescein (DCFH-DA) and Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) were purchased from Sigma. The primary antibodies used include those against p65, p-p65, IκB, p-IκB, IKK, NLRP3,

IL-1β and casapase-1 were purchased from Abcam. TNF-α was purchased from Roche. Fetal bovine serum, penicillin and streptomycin were purchased from Gibco.

4.2.2 Isolation of mouse primary hepatocytes

The procedure for isolation of mouse primary hepatocytes was performed according to method described previously with modification (Gonçalves, Vigário et al. 2007). Briefly, mice were anesthetized (2.5% avertin, 0.4 mL/20g) and the livers was perfused through a needle aligned along the inferior vena cava, with PBS buffer (pH 7.5) containing 0.5 mmol/L EGTA; followed by collagenase containing buffer, pH 7.5 which consists of 1×HBSS (Gibco) with 0.15 g/L collagenase type 2 (Worthington Biochemical Corporation). The collagenase-perfused liver was then dissected, suspended in DMEM (Gibco) without FBS, and filtered through cheesecloth and a 40 μm nylon membrane to remove connective tissue debris. Hepatocytes were subjected to centrifugation (65 g, 2 min at 4°C) and resuspended in DMEM with 10% FBS. Afterwards the hepatocytes were purified using density gradient centrifugation (50% Percoll solution, 65 g for 10 min at 4°C).

4.2.3 ROS measurement

The level of oxidative stress was monitored by measuring the ROS (Swift and Sarvazyan 2000). The cultured cells treated for 12 h with high glucose, LPS and TNF-α were washed with PBS and incubated with 5 μM solution of fluorescent probe (H2-DCFH-DA dissolved in culture medium without serum) at 37 °C for 60 min. The cultured cells were then washed three times with PBS and fluorescent signals were determined by (1) images analysis with an inverted fluorescence microscope (Olympus IX71), (2) signals were recorded and quantified with a fluorescence microplate reader at 488 nm, and (3) flow cytometry analysis using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with an excitation at 488 nm and

an emission at 620 nm. Triplicate experiments were performed. For protein carbonylation assays, carbonyl levels were determined either by Western blotting with 10 μg per samples of total protein lysates or by ELISA using a commercial kit (Abcam) based on the manufacturer's instructions.

4.2.4 Western blot and immunoprecipitation

Western blot and immunopreciptation were performed as described (Tian, Ke et al. 1999). Mouse liver tissues were homogenized in the lysis buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EGTA, 2 mM Na_3VO_4 , 50 mM NaF, 20 m M $ZnCl_2$, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1×complete protease inhibitor mixture (Sigma)). After centrifugation (12,000×g in a microcentrifuge at 4 °C for 15 min), supernatant fractions were collected and incubated with antibodies and GammaBind Plus-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C on a rotary shaker. Proteins were analyzed by SDS-PAGE and then transferred to a nitrocellulose membrane. Individual proteins were detected with the specified antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Millipore).

4.2.5 Measurement of cytokines using multiplex microbead immunoassay

Supernatant of the LPS-treated hepatocytes will be collected and stored at -70°C until the analysis day. The cell will be pretreated with antioxidant reagent (including sulforaphane (40mM, 6h), N-Acetyl Cysteine (NAC) (5mM 12h) and methyl donor including (SAM 4mM, 6h, and Methionine 1mM, 6h). A multiplex biometric immunoassay, containing fluorescent dyed microspheres conjugated with a monoclonal antibody specific for a target protein, will be used for cytokine measurement according to the manufacturer's instructions (MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel- Immunology Multiplex Assay, Emdmillipore Inc., Darmstadt, Germany). Proinflammatory cytokines including IL-1α, IL-1β, IL-6, TNF-α, IL-

12 (p40), IL-12 (p70) will be measured. A range of 1.95–32,000 pg/ml recombindent cytokines will be used for establishment of standard curves and maximizing the sensitivity and the assay dynamic ranges. Cytokine levels will be determined using a multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). The analyte concentration was calculated using software provided by manufacturer (Bio-Plex Manager Software). The protein level of cytokines will also be confirmed using Mouse Cytokine Array C3 (RayBiotech, GA, USA).

4.2.6 M6A methylation RNA IP (immunoprecipitation)

The LPS-treated hepatocytes will be used for determination of the m6A methylation using anti-m6A antibody. As the adenine methylation residues are distributed in different segments of the MAT2A mRNA the antibody-precipitated RNA will be determined using different primer pairs bracketing different segments (3'UTR middle, 3'UTR end 5'UTR and coding N-terminus) of the RNA using RT-PCR as described in a previously published report by Shima et al. (Warda, Kretschmer et al. 2017).

4.2.7 Real time-PCR

Total RNA of primary hepatocytes, isolated peritoneal macrophages cells and the liver was isolated using the Trizol (Qiagen) according to the manufacturer's protocol. RNA was eluted using 50 µl of RNase-free water and then stored at -80°C. Real-time (RT)-PCR was performed using iTaq Universal SYBR Green One-step Kit (Bio-Rad, Hercules, CA). The following primers were used (Table 2).

Table 2. Mouse primers related to inflammation and RNA methylation for real-time PCR

Gene Name	Forward Primer	Reverse Primer
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT
MALAT1	TGAAAAAGGAAATGAGGAGAAAAG	CTTCACAAAACCTCCCTTTACAAT
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
TNF-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
NLRP3	TGGATGGGTTTGCTGGGAT	CTGCGTGTAGCGACTGTTGAG
Casespase1	TTGAAAGACAAGCCCAAGGTG	CTGGTGTTGAAGAGCAGAAAGC
METTL3	ATTGAGAGACTGTCCCCTGG	AGCTTTGTAAGGAAGTGCGT
METTL14	AGACGCCTTCATCTCTTTGG	AGCCTCTCGATTTCCTCTGT
METTL16	GACAAACCACCTGACTTCGCA	TCTGACTGCTTCGGGGTCTT
WTAP	GTTATGGCACGGGATGAGTT	ATCTCCTGCTCTTTGGTTGC
FTO	CTGAGGAAGGAGTGGCATG	TCTCCACCTAAGACTTGTGC
ALKBH5	ACAAGATTAGATGCACCGCG	TGTCCATTTCCAGGATCCGG
YTHDF1	CATTATGAGAAGCGCCAGGA	AGATGCAACAATCAACCCCG
YTHDF2	ACCAACTCTAGGGACACTCA	GGATAAGGAGATGCAACCGT
YTHDF3	TGCACATTATGAAAAGCGTCA	AGATGCGCTGATGAAAACCA
YTHDC1	TTCATAACATGGGACCACCG	TCATAGTCATGTACTCGTTTATCTC
MAT2A	GCTTCCACGAGGCGTTCAT	CATCAGGGTCTTGTTGAAGGTG
MAT2A-5’	GAAGCGATCCTCCCTCTGTG	TCAATGAACGCCTCGTGGAA
MAT2A-coding-N terminal	TCCACGAGGCGTTCATTGAG	TGCATCAAGGACAGCATCACT
MAT2A-stop codon	CCACTTTGGTAGGGACAGCTT	GGCCCTTTCCCTCAGAGCTT
MAT2A-3’ middle	GTCACAGGGCAGTACCTGAG	CCCTGGGAGGAGCTATTGTG
MAT2A-3’ end	GGGTTAGACCTACAGGGGGT	TTGCTTAGGGCAAGCAGTCA

4.3 Results

4.3.1 MALAT1 is upregulated in peritoneal macrophages exposed to microbial ligands (LPS)

Sepsis is a complex physiological response of an organism to harmful stimuli, such as pathogens and endotoxins, and is often caused severe oxidative stress which refers to a serious

imbalance between reactive oxygen species (ROS) production and antioxidant defenses in favor of the former, in a cell, tissue or organ. In our previous studies we have found that ROS generation is regulated by a long noncoding RNA (lncRNA) MALAT1, and genetic ablation of MALAT1 drastically reduced ROS generation and alleviated the oxidative stress induced by high level of glucose and other environmental chemicals including BPA and PCB126 in the primary hepatocytes. Previous research has shown that MALAT1 is highly expressed in immune related tissues and organs including bone marrow, lymph nodes, thymus and whole blood. Based on our and others studies, we hypothesize that MALAT1 should play a role in regulating immune response.

We firstly performed qRT-PCR to examine the kinetics of MALAT1 expression in peritoneal macrophages exposed to endotoxin LPS and TNFa which is a central mediator of sepsis. The expression of MALAT1 was significantly upregulated in response to both LPS and TNFa treatment in a time-dependent manner (Figure 30), which was correlated with the induction of pro-inflammatory genes including IL-1β, IL-6. Collectively, these results indicate that MALAT1 levels in peritoneal macrophages are dynamically regulated in response to endotoxin and inflammatory triggers.

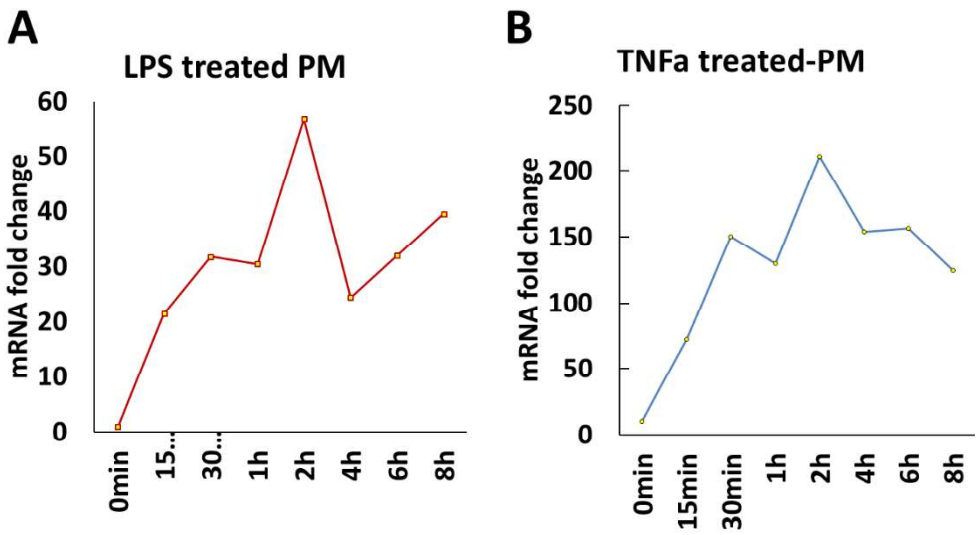

Figure 30. MALAT1 is upregulated in peritoneal macrophages exposed to microbial ligands. (A) and (B) MALAT1 expression increased in LPS-induced (A) and TNFa-induced (B) inflammation in peritoneal macrophage.

4.3.2 Ablation of MALAT1 leads to inhibited proinflammatory response and NF-kB signaling pathway

We have found that MALAT1 expression was significantly upregulated in peritoneal macrophages in response to LPS and TNFa, we next utilized MALAT1 null to test our hypothesis that MALAT1 would regulate immune response. We challenged the peritoneal macrophage isolated from MALAT1 null and wild type mice with LPS (1μg/ml, 2h, 4h, 6h, 8h) and test the immune response genes. The qRT-PCR results showed that pro-inflammatory genes including IL-1β, IL-6, TNFa, IL-12 (Figure 31A) and inflammasome complex including NLRP3 and Casepase-1 (Figure 33C) were significantly inhibited in MALAT1 ablated peritoneal macrophages.

Next, we measured macrophage-secreted cytokine levels using multiplex. Supernatant was collected in the cultured peritoneal macrophages after stimulation with LPS (1μg/ml, 2h, 4h, 6h, 8h). As expected, we found that the pro-inflammatory cytokines levels including IL-1b, IL-6, TNFa, and IL-12 were significantly inhibited in MALAT1 ablated peritoneal macrophage, of which IL-1b was the most inhibited cytokine (Figure 31B). Additionally, the cytokine antibody arrays were used to examine the levels of 42 cytokines related to immunity and inflammation in supernatant of peritoneal macrophages. As shown in Figure 32 A and B, MALAT1 ablation decreased proinflammatory cytokine levels compared with wild type group after LPS stimulation (1 μg/ml, 4h). We also found that levels of p65, and phosphorylation of p65 were significantly lower in the MALAT1 null peritoneal macrophages compared to wild type groups (Figure 32C). These results collectively indicated that MALAT1 ablation could inhibit inflammatory response in peritoneal macrophages.

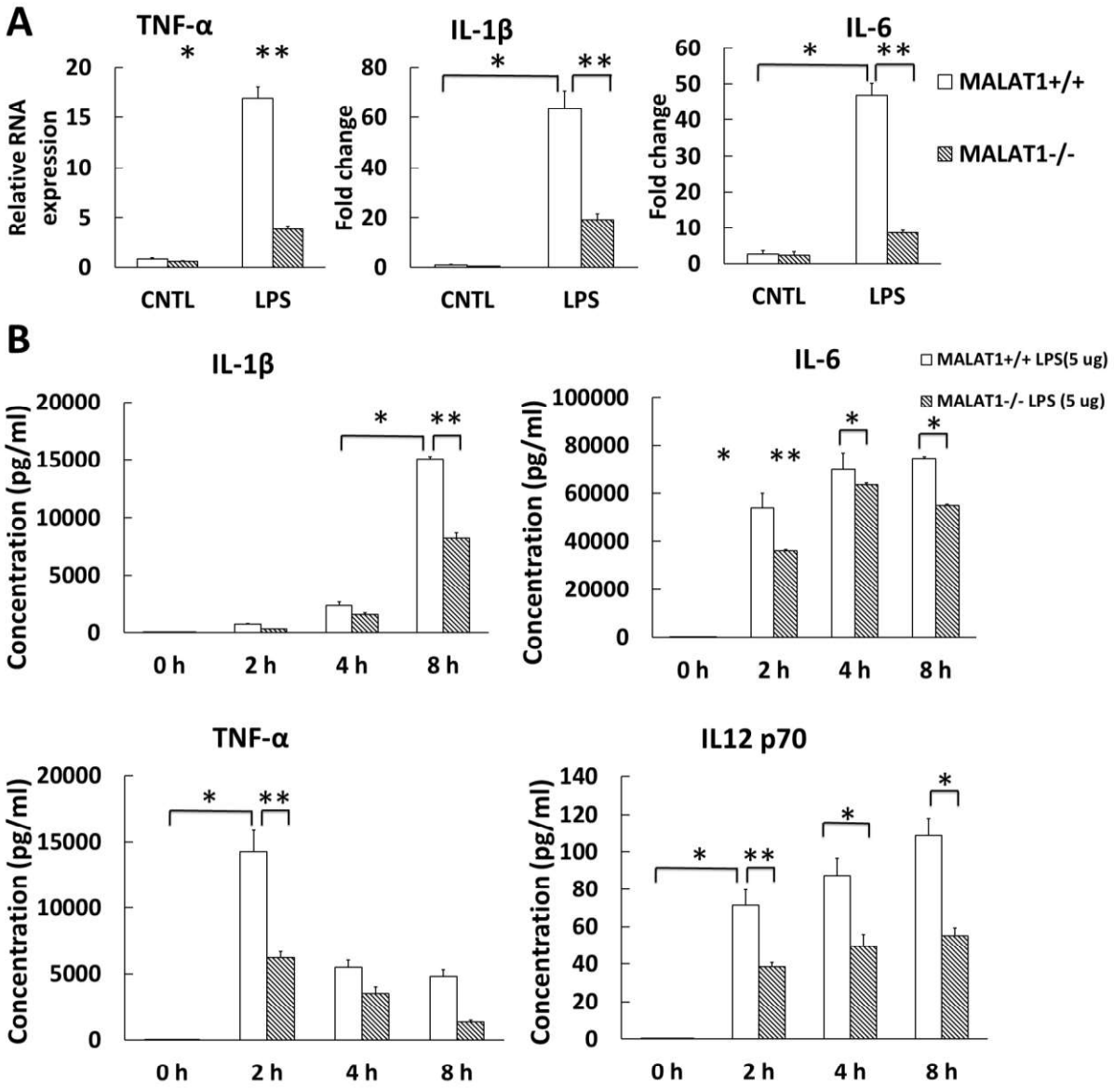

Figure 31. MALAT1 ablation decreases the levels of the inflammatory cytokines. (A) LPS induced-inflammatory related gene expression in wild type and MALAT1 ablated peritoneal expression determined by qRT-PCR. (B) inflammatory cytokine levels in the supernatant of peritoneal macrophage isolated from wild type and MALAT1 null mice treated with LPS (5μg).

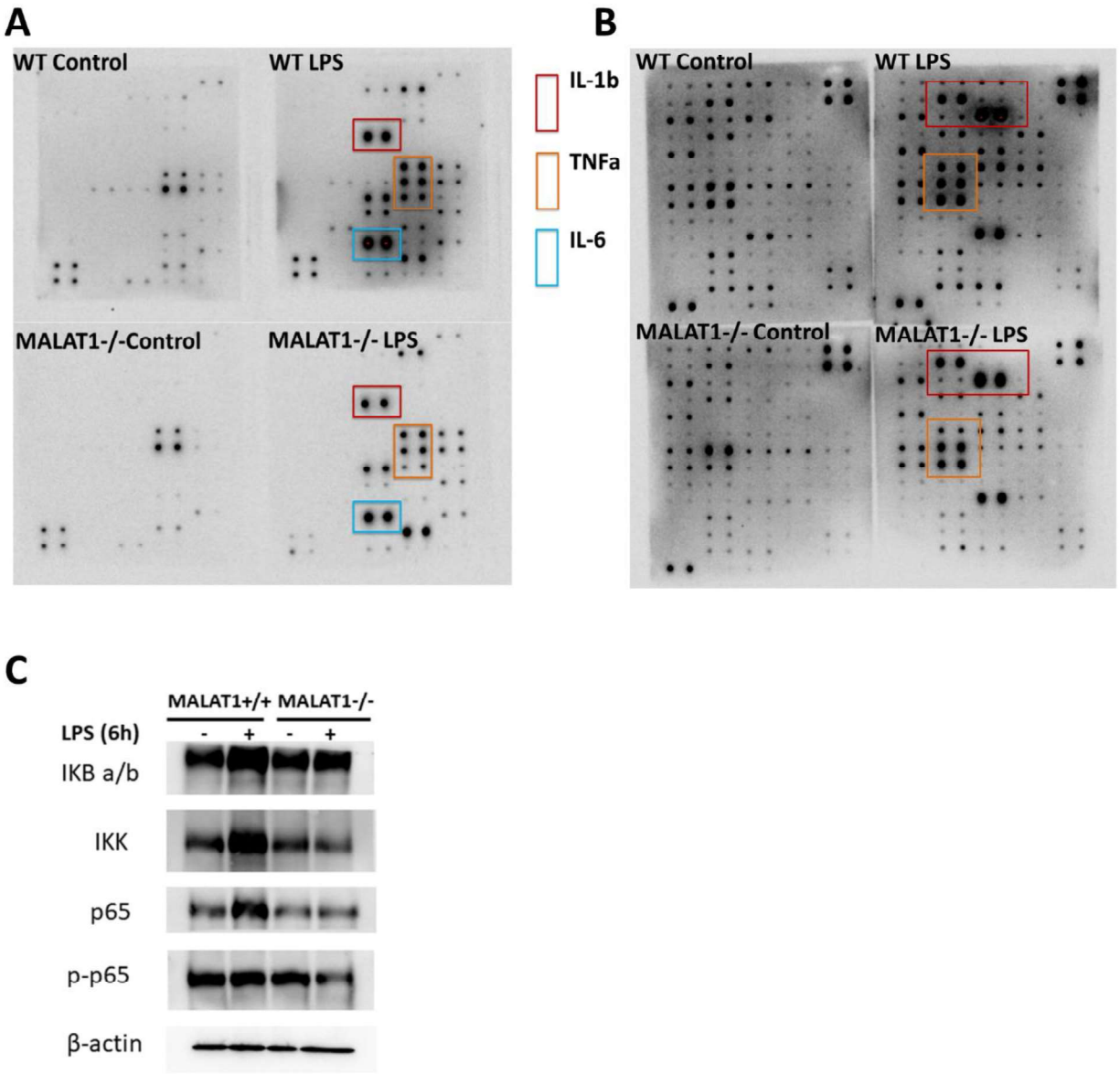

Figure 32. MALAT1 ablation suppresses NFkB signaling pathway activation. (A) and (B) cytokine levels from supernatant of peritoneal macrophage (A) and from blood serum of mice after 2 hours of LPS i.p. injection (B) were determined using a cytokine array. (C) MALAT1 ablation regulates NFkB signaling pathway.

4.3.3 MALAT1 ablation suppresses NLRP3 inflammasome activation by attenuating the LPS-induced oxidative stress

We have found that MALAT1 ablation could alleviate the inflammatory response in peritoneal macrophage challenged by LPS. Notably, IL-1b was the most inhibited cytokines compared to the other pro-inflammatory cytokines. Previous studies have shown that the maturation of IL-1b was predominately triggered by (NOD)-like receptor containing pyrin domain 3 (NLRP3) inflammasome, which is a multi-protein complex and could be activated by LPS, pathogen-associated molecular patterns(PAMS) and ROS. LPS and other bacterial infection have been found to cause sever oxidative stress with excessive generation of ROS. Our previous study has shown that Nrf2/ARE pathway was significantly activated in MALAT1 ablated hepatocytes. We next investigated the role of MALAT1 in regulating oxidative stress level and antioxidant capacity in mice, thus regulates the ROS-mediated NLRP3 inflammasome activation. We measured the ROS generation in the peritoneal macrophage, as well as in hepatocytes because liver played an important role in inflammatory response and detoxification process. As expected, we found that ROS generation was diminished in the MALAT1 ablated hepatocytes and peritoneal macrophages (Figure 33 A and B). With inhibited ROS generation, MALAT1 ablated hepatocytes were found to have significantly increased GSH/GSSG ratio and unregulated antioxidant genes expression. It was suggested that ROS could serve as a triggering factor to activate the NLRP3 inflammasomes, resulting in maturation of proinflammatory cytokines such as IL-1b and pathological processes (Abais, Xia et al. 2015). To investigate the possible function of MALAT1 regulating NLRP3 inflammasome pathway, peritoneal macrophage isolated from MALAT1 null and wild type mice were treated with LPS (5ug/ml, 4h). MALAT1 ablation significantly decreased the LPS-induced NLRP3 expression at both mRNA and protein level

(Figure 33 C and D). The sufficient NLRP3 protein level is critical for the formation and activation of NLRP3 inflammasome (Dowling and O'Neill 2012, Haneklaus, O'Neill et al. 2013). With significantly suppressed NLRP3 level, MALAT1 ablated macrophage showed decreased caspase-1cleavage and suppressed IL-1b secretion level (Figure 33 C and D). Collectively, these findings showed that MALAT1 ablation attenuated ROS generation thus suppressed NLRP3 inflammasome activation.

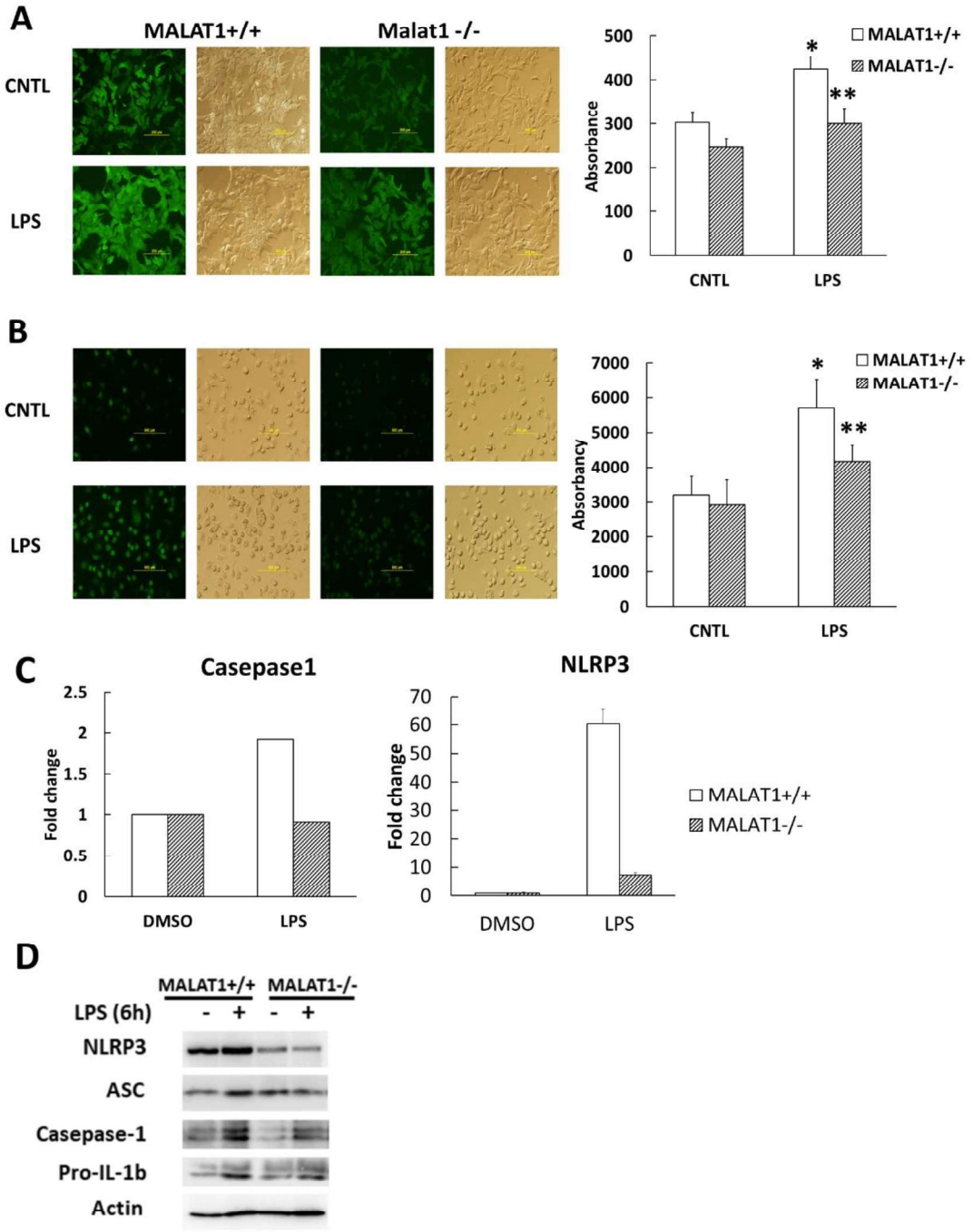

Figure 33. MALAT1 ablation suppresses NLRP3 inflammasome activation by attenuating the LPS-induced oxidative stress. A and B, The ROS generation in mouse hepatocytes (A) and peritoneal macrophage (B) determined by immunofluorescence microscopy using DCFH-DA. Antioxidant gene expressions in hepatocytes treated with oxidative stressors (LPS and High glucose (HG)). (C) NLRP3 inflammasome gene expression determined by qRT-PCR. (D) MALAT1 ablation regulates NLRP3 inflammasome signaling pathway determined by Western Blot. *, P <0.05.

4.3.4 MALAT1 ablation reulates cellular bioenergetics in sepsis

Sepsis is characterized by an initial hyperinflammation state accompanied with increased respiration and ATP production (Van den Bossche, O'Neill et al. 2017). During the process of inflammation, a hypometabolic state will eventually take in place with decreased mitochondrial respiration and ATP production. Recent development of extracellular flux methods (Seahorse XF Analyzer, Agilent) allows us to monitor changes in oxygen concentration and pH to measure bioenergetic function in response to oxidative stress. Using the Seahorse XF Analyzer we have analyzed mitochondrial bioenergetics parameters in primary hepatocytes and isolated peritoneal macrophages after treated with LPS, and found LPS treatment significantly decreased the respiratory capacity in hepatocytes, while increased the respiratory capacity in peritoneal macrophages, indicating the different physiological and pathological function of the hepatocytes and peritoneal macrophages (Figure 34 B-G). Interestingly, we found greatly increased respiratory capacity in the MALAT1 null hepatocytes and peritoneal macrophages, suggesting MALAT1 ablation increased the resistance to LPS-induced stress in cells (Figure 34 B-G). We also tested the ATP production in peritoneal macrophages and found that MALAT1 ablation significantly increased the ATP capacity (Figure 34 H). Collectively, these results indicated that MALAT1 ablation increased the mitochondrial respiratory capacity and bioenergetic metabolism, suggesting MALAT1 null mice could have a increased metabolic rate, The improved mitochondrial function and metabolism in MALAT null mice would provide more energy capacity during the process of sepsis.

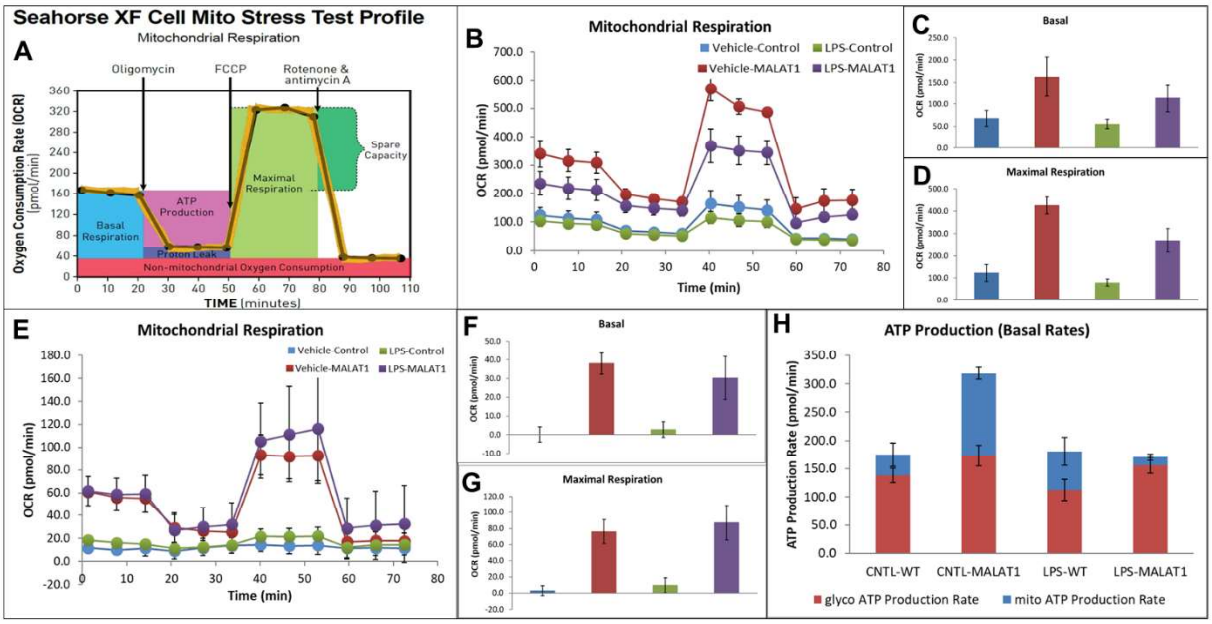

Figure 34. MALAT1 ablation regulates mitochondrial respiration capacity (A) The schematic illustration of seahorse XF cell mito stress test. (B-G) Real-time measurement of oxygen consumption rates (OCR) in primary hepatocytes and peritoneal macrophage isolated from MALAT1 null and wild type mice. (H) ATP production level in isolated peritoneal macrophage from MALAT1 null and wild type mice.

4.3.5 MALAT1 regulates GSH antioxidant pathway through m6A modification in mRNA

Sepsis is a disease with high lethality and morbidity (Hippensteel, Anderson et al. 2019, Yang, Langston et al. 2019) and is accompanied by profound metabolic disturbances of the methionine metabolism cycle(Malmezat, Breuillé et al. 2000, Semmler, Smulders et al. 2008, Erdem, Yerlikaya et al. 2012, Semmler, Prost et al. 2013). In mammalian methionine metabolism, S-adenosylmethionine (SAM) is produced, which occupies a central position in the metabolism of all cells as an essential methyl donor to maintain normal methylation of DNA, RNA, histones as well as glutathione which is important for cellular redox system. It has been found that in human clinics and animal sepsis models, methionine metabolism is disrupted with greatly increases in the oxidative stress levels as indicated by the dysregulated GSH/GSSG level (Biolo, Antonione et al. 2007). Methionine adenosyltransferase (MAT2A) is essential for the biosynthesis of SAM and the gene expression of MAT2A is regulated at transcriptional and post-transcriptional levels through m^6A RNA methylation of the 3'UTR of MAT2A mRNA (Pendleton, Chen et al. 2017, Shima, Matsumoto et al. 2017) . Despite of strong evidence for dysregulation of methionine and SAM in sepsis, currently there is no report about how RNA methylation, such as N^6-methyladenosine (m^6A) is regulated in sepsis**.** In this study, we found that RNA methylation writers METTL3, METTL14 and METTL16, readers Ythdc1, Ythdf1 and eraser FTO (Roundtree, Evans et al. 2017) have all been profoundly changed by LPS treatment (Figure 35. LPS-induced inflammation increases the total RNA global m6A/m with m6A machinery genes alternation. A, Global m6A machinery genes were changed in LPS-induced inflammation in peritoneal macrophage. B, Global m6A/m is transiently increased in peritoneal macrophage after treated with LPS (5μg). *P < 0.05; **P < 0.01. Data are representative of at least three independent experiments. A). Global m6A/m is transiently increased in peritoneal

macrophage after treated with LPS (Figure 35. LPS-induced inflammation increases the total RNA global m6A/m with m6A machinery genes alternation. A, Global m6A machinery genes were changed in LPS-induced inflammation in peritoneal macrophage. B, Global m6A/m is transiently increased in peritoneal macrophage after treated with LPS (5μg). *P < 0.05; **P < 0.01. Data are representative of at least three independent experiments. B). These results showed that RNA methylation was closely associated with the LPS-induced inflammation and reacted rapidly within 15 mins.

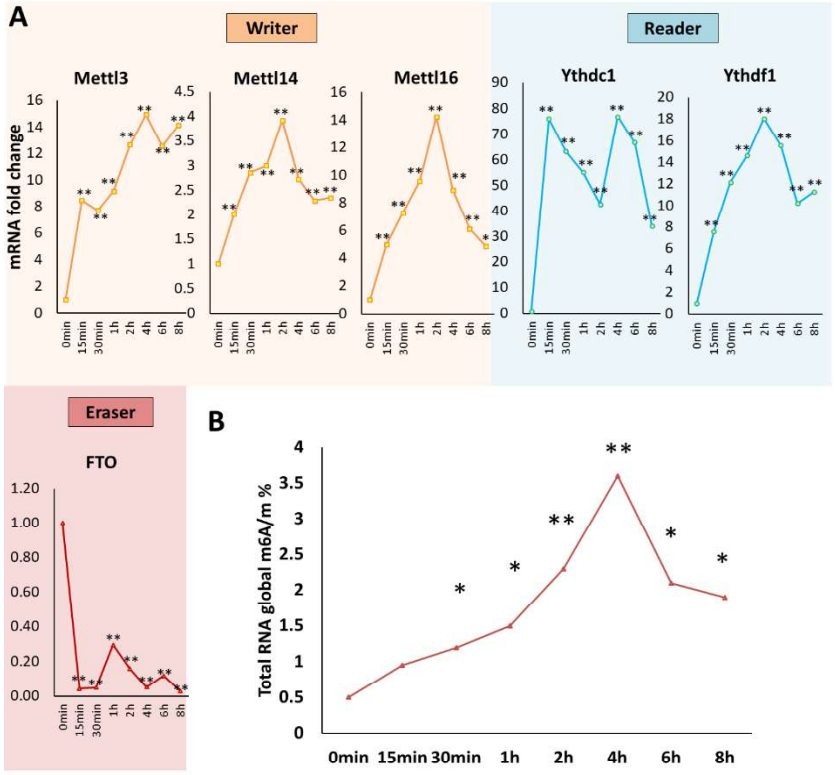

Figure 35. LPS-induced inflammation increases the total RNA global m6A/m with m6A machinery genes alternation. A, Global m6A machinery genes were changed in LPS-induced inflammation in peritoneal macrophage. B, Global m6A/m is transiently increased in peritoneal macrophage after treated with LPS (5μg). *P < 0.05; **P < 0.01. Data are representative of at least three independent experiments.

Using cultured peritoneal macrophage isolated from mice we found that methionine (1mM, 6h) and SAM (4mM, 6h) treatment decreased LPS-induced cytokine levels, and on the contrary the MAT2A competitive inhibitor cycloleucine (cLEU) (30mM, 6h) increased cytokine levels (Figure 36 A), further supporting the role of methionine cycle in regulating inflammatory response in sepsis. The GSH/GSSG ratio has also been increased by methionine/SAM suggesting an increased antioxidant capacity (Figure 36 B and C). Additionally, MALAT1 ablation significantly increased the GSH/GSSG ratio suggesting an improved redox state in the MALAT1 null peritoneal macrophages (Figure 36 B and C).

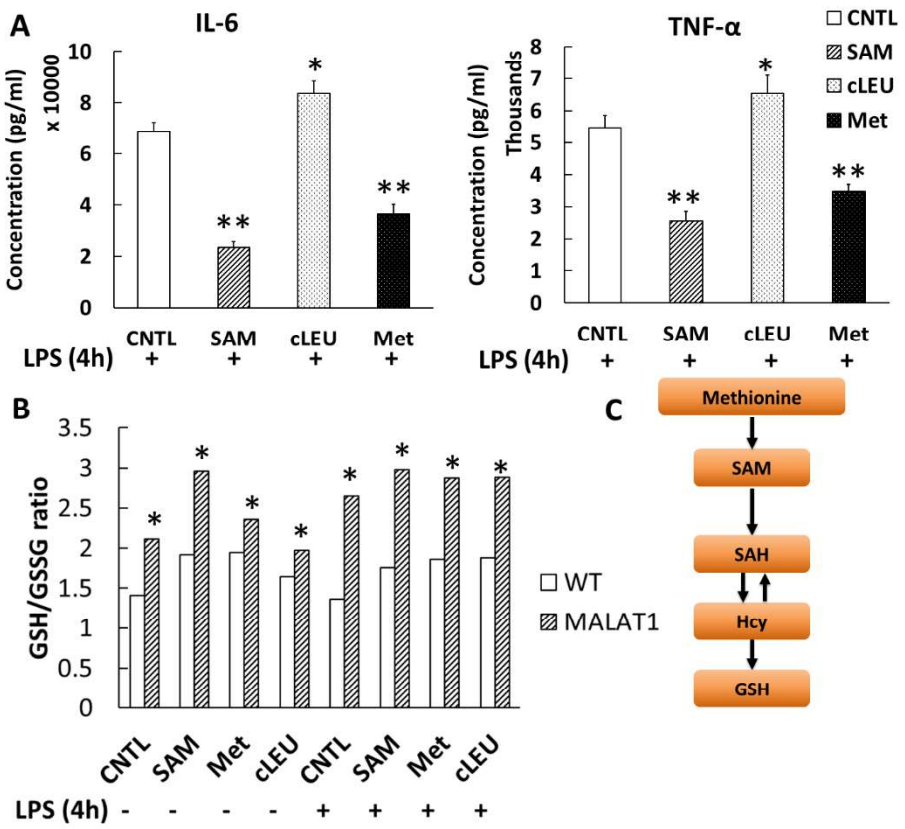

Figure 36. Treatment of methyl donor (SAM and Methionine)/SAM inhibitor cycloleucine regulate inflammatory cytokine secretion in peritoneal macrophage. A, Methyl donor treatment (SAM and Methionine (Met)) inhibit the pro-inflammatory cytokine level in peritoneal macrophage. SAM inhibitor cycloleucine (cLEU) exacerbate pro-inflammatory cytokines secretion in peritoneal macrophage. B, GSH/GSSG ratio in treatment of methyl donor (SAM and Met) and SAM inhibitor (cLEU) under LPS challenge in peritoneal macrophage. C, Methionine-SAM cycle. *P < 0.05; **P < 0.01.

Post-transcriptionally, the mRNA of MAT2A stability is regulated by the m^6A methylation mediated by RNA methylation writer METTL16 which methylates 3'UTR of MAT2A, thus controlling its level. Mechanistically, strong evidence from structural analysis indicates among the methylation sites of MAT2A, the hairpin 1(hp1) is the linchpin for regulating MAT2A expression(Doxtader, Wang et al. 2018) by regulating intron splicing(Pendleton, Chen et al. 2017) and mRNA decay (Shima, Matsumoto et al. 2017) of MAT2A, thus controlling methionine cycle (Figure 7 and Figure 37 A). METTL16 forms a complex with a lncRNA MALAT1(Brown, Kinzig et al. 2016) which is a pre-RNA splicing factor. We found that in genetically ablated MALAT1 mice, MAT2A gene expression is up-regulated (Figure 37 B) and LPS-induce ROS was significantly suppressed with increases in the levels of glutathione (Figure 33 A and B, Figure 36 B). M6A methylation pulldown assay results suggest MALAT1 ablation counteracted the LPS-induced methylation at hairpin 1(hp1 near the stop codon) (Figure 37 C) thus increasing MAT2A level (Figure 37 B). These exciting results suggested that MALAT1 regulates SAM biosynthesis by controlling MAT2A gene expression and MALAT1-regulated methionine metabolism pathway is a therapeutic target for sepsis.

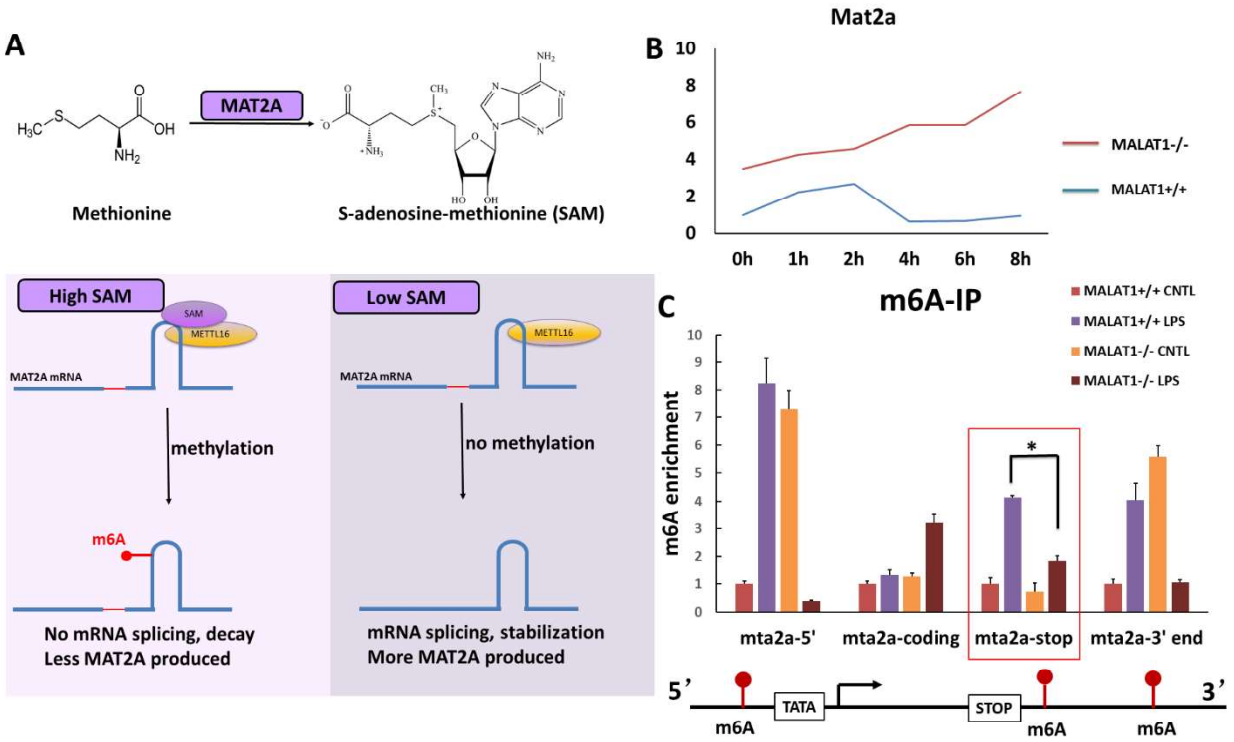

Figure 37. Metabolic regulation of epitranscriptome writer localization. (A) Under high SAM condition, METTL16 catalyzes methylation of an mRNA encoding the SAM biosynthetic enzyme MAT2A, limiting its splicing and reducing MAT2A mRNA stability. When SAM levels are low, METTL16 inefficiently methylates the MAT2A mRNA but for a stable interaction. This triggers splicing and mRNA stabilization that allows more MAT2A to be produced and for cellular SAM homeostasis to be maintained. (B) MAT2a gene expression was upregulated in MALAT1 null peritoneal macrophage in LPS-induced inflammation. (C) m6A methylation enrichment on MAT2A mRNA under treatment of LPS in wild type and MALAT1 ablated peritoneal macrophage.

4.5 Discussion

Inflammatory response during sepsis is remarkably versatile and complicated. Traditionally, the host immune response in the process of sepsis was considered to be excessive proinflammatory response leading to multi-organ failure, drastic drop in blood pressure and ultimately death. Recent recovered findings have shown that lncRNAs become active participants in regulating immune response in innate immune system. LncRNAs, with no apparent coding capacity, are found to play critical roles in regulating chromatin activity, RNA transcription, RNA splicing, and expression of protein-coding genes. It has been revealed that lncRNAs function *in cis* or *in trans* in activating or restraining inflammatory response by interacting with transcription factors and chromatin modifiers and regulates immune related gene expression and macrophage polarization. Although increasing cases have been reported about the lncRNAs expression are up/down regulated in cells treated with the inflammatory stimulators and modulate inflammatory response by regulating inflammatory signaling pathways including NF-kB and detoxification pathway.

In our previous studies, we found that MALAT1 interacted with Nrf2/ARE as a negative co-regulator and its deletion led to activation of genes in the Nrf2/ARE pathway. Nrf2 is a pleiotropic transcriptional factor that regulates the cytoprotective genes required for resistance to oxidative stress which induces ROS and is the common etiological factor for many diseases. Indeed, we found MALAT1 ablation led to the activation of the insulin pathway and the mice become sensitized to the hyperglycemic challenge. In the current study we found MALAT1-Nrf2 interactive axis act as a key regulator for the LPS-induced inflammatory response. It has been reported Nrf2-driven antioxidant system played pivotal role in the survival of sepsis as Nrf2 null mice showed significant decreased survival rate in the LPS-induced septic shock model. We

showed MALAT1 null mice were highly resistant to the septic shock with subsided "cytokine storm" (Figure 38). We performed a time-dependent LPS challenge in peritoneal macrophage isolated from MALAT1 null and wildtype mice and found the initial proinflammatory cytokines including TNF-α, IL1-β, IL-6 and IL-12 were significantly lower in the supernatant of the cultured peritoneal macrophages, as well as in the blood serum from the LPS-challenged mice. Consistently, the expression of proinflammatory related genes were significantly inhibited by MALAT1 ablation.

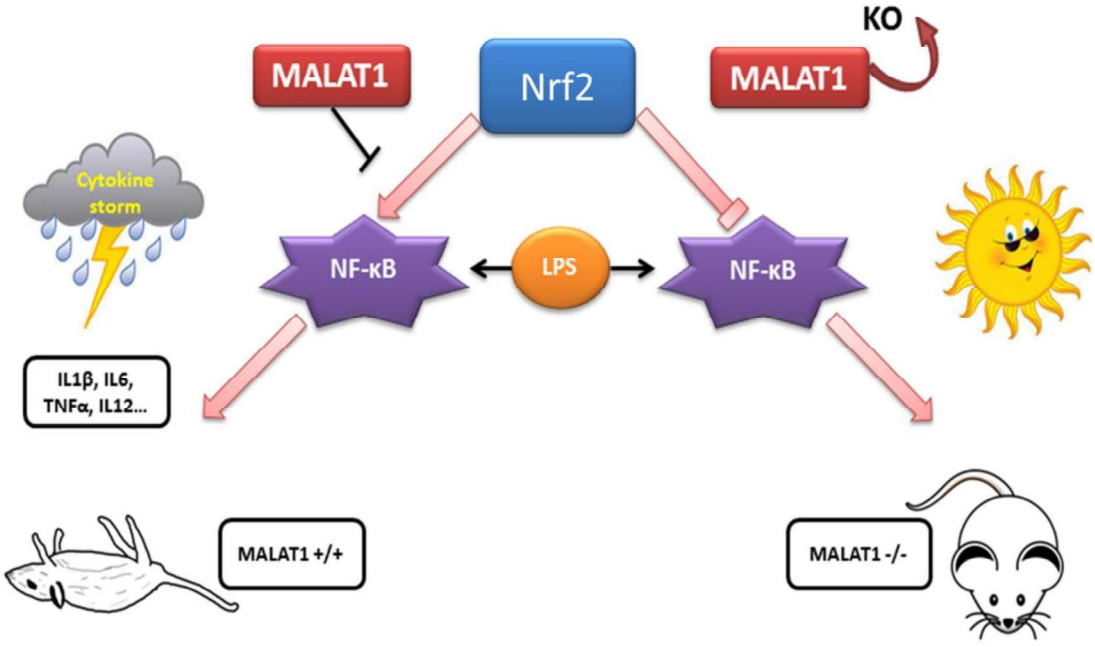

Figure 38. Schematic illustration of MALAT1-Nrf2 axis regulating inflammatory response in sepsis.

We have found that MALAT1 ablation could significantly attenuate ROS generation and oxidative stress in both peritoneal macrophage and hepatocytes, and increased the GSH/GSSG ration in mice. The ratio of GSH and GSSG has been considered as a useful estimation of the balance between oxidative reaction and endogenous antioxidant defense (Thimmulappa, Lee et

al. 2016). Cellular antioxidant defense including Nrf2 activation and GSH/GSSH pathway could counter inflammation by limiting ROS levels. The lower level of GSH has been found to augment inflammatory response in LPS. The cellular defense to oxidative stress and maintaining the balanced redox state is mutually supported by different pathways including Nrf2/ARE detoxification pathway and antioxidant products including GSH. We have reported that MALAT1 acted as a repressor by interacting with Nrf2 and suppress the transcriptional activity of Nrf2. Ablation of MALAT1 in hepatocytes was found to significantly activate the Nrf2/ARE detoxification pathway and increased Nrf2/ARE driven genes including GCLC which was one of the major determinants of GSH synthesis. The other major determinant of GSH synthesis was transsulfuration pathway converting methionine to cysteine, which was then converted to GSH via GSH synthetic pathway. In the transsulfuration pathway, methionine is sequentially converted to cysteine via several enzymatic steps, among which, the first and most important step is activation of methionine to S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyltransferase (MAT). SAM, as a major precursor of glutathione, and also as the principal biological methyl donor for methylation of DNA, RNA, chromatins, and proteins, becomes a convergent point by linking antioxidant pathway to methylation modification together. Recent studies have shown that the SAM synthesis is regulated by selective N6-adenosine methylation (m6A methylation) and m6A methyltransferase METTL16, which leads us to a brand-new area that whether MALAT1 is involved in the RNA methylation modifications in the LPS-induced inflammatory response. To investigate the effect of LPS-induced inflammation on the m6A RNA modification, we measured the total m6A content and key protein factors referring to “writer”, “eraser” and “reader” mRNA expression. The results showed that the total m6A RNA methylation level was dramatically increased by MALAT1 ablation, while the “writer” including

Mettle 3, Mettle 14, and Mettle 16 were significantly increased and the demethylase "eraser" FTO was significantly decreased in the MALAT1 null peritoneal macrophage (Figure 35). Additionally, we also observed the m6A RNA modification level was drastically increased in a time-dependent manner after LPS treatment which implicated that m6A modification could be a very important sensing mechanism regulating inflammatory response in mice (Figure 35).

Next, we investigated whether the m6A RNA methylation could affect the LPS-induced inflammatory response. Using cultured peritoneal macrophage isolated from mice we found that methionine and SAM treatment as methyl-donor decreases LPS induced cytokine levels and on the contrary the MAT2A competitive inhibitor cycloleucine (cLEU) increased cytokine levels (Figure 36) further supporting the role of methionine-SAM-regulated methylation in regulating inflammatory response in sepsis. These findings suggest that the total m6A level and the correlating protein factor including METTL3, METTL14, METTL16 and FTO play important roles in regulating inflammatory response.

Collectively, our research indicates MALAT1-Nrf2 interactive axis played an important role in regulating immune response during sepsis and MALAT1-regulated methionine metabolism pathway is a potential therapeutic target for treating inflammation diseases such as sepsis.

5. SUMMARY

Recent genomic studies have shown that mammalian genomes encode thousands of noncoding RNAs, which are emerging as key regulators of diverse cellular processes and pathogenesis (Cabili, Trapnell et al. 2011, Gutschner, Hämmerle et al. 2013, Ma, Wang et al. 2015, Sun, Hao et al. 2017). The mechanisms of lncRNAs in regulating these processes are under investigation and several modes of actions including lncRNA functions as signaling, decoy, guide and scaffold molecules have been proposed (Wang and Chang 2011). MALAT1 has been found to play critical roles in multiple cellular processes including modulating of chromatin structure and pervasively regulate gene expression. My dissertation mainly focused on two different function of MALAT1, one is regulating the insulin signaling pathway and T2DM, and the other is regulating the innate immune response. We found that the ROS generation and Nrf2 detoxification pathway could be the convergent point of mechanism for MALAT1 regulating T2DM and inflammatory response.

In Section two, our results reveal MALAT1, which is highly conserved across mammalian species and broadly involved in metastasis of various tumors (Gutschner, Hämmerle et al. 2013, Ma, Wang et al. 2015), plays a role in regulating the glucose homeostasis in mice. Interestingly, bioinformatic analysis of MALAT1 reveals its role in regulation of broad range of gene expression by controlling various aspects of transcription including pre-mRNA processing (Arun, Diermeier et al. 2016). MALAT1 interacts with transcriptional suppressor complex PRC2 through interacting with the EZH2 subunit (West, Davis et al. 2014) which is consistent with our results showing deletion or knocking down of MALAT1 de-represses gene expression including Nrf2 resulting in higher levels of antioxidant capacity(Chen, Ke et al. 2018).

W activity which is known to cause pancreatic damage (Piaggi, Novelli et al. 2007, Timme-Laragy, Sant et al. 2015, Shi, Jan et al. 2019) . This established cell-based assay could also be utilized for the future investigation in the environmental chemicals induced pancreatic islets cell dysfuncion using single-cell transcriptome analysis. The pancreatic islet consists of five known cell types (α, β, γ/PP, δ and ε cells). Very little is known about the xenobiotic detoxification pathways in these distinct types of cells. The recent rapid development of the single-cell RNA sequencing (scRNA-seq) technology, in particular, the droplet-based scRNA-seq makes it possible to measure the transcriptomic activities of these distinct cell population within pancreas (Carter, Bihannic et al. 2018, Zeisel, Hochgerner et al. 2018, Kalamakis, Brune et al. 2019, Mickelsen, Bolisetty et al. 2019). One of the future direction would be investigating the molecular mechanisms underlying environmental chemicals-induced toxicity in the distinct pancreatic cells using the bioinformatic pipelines and a machine learning workflow analyzing single-cell transcriptome data of the isolated pancreatic islet cells (Figure 39).

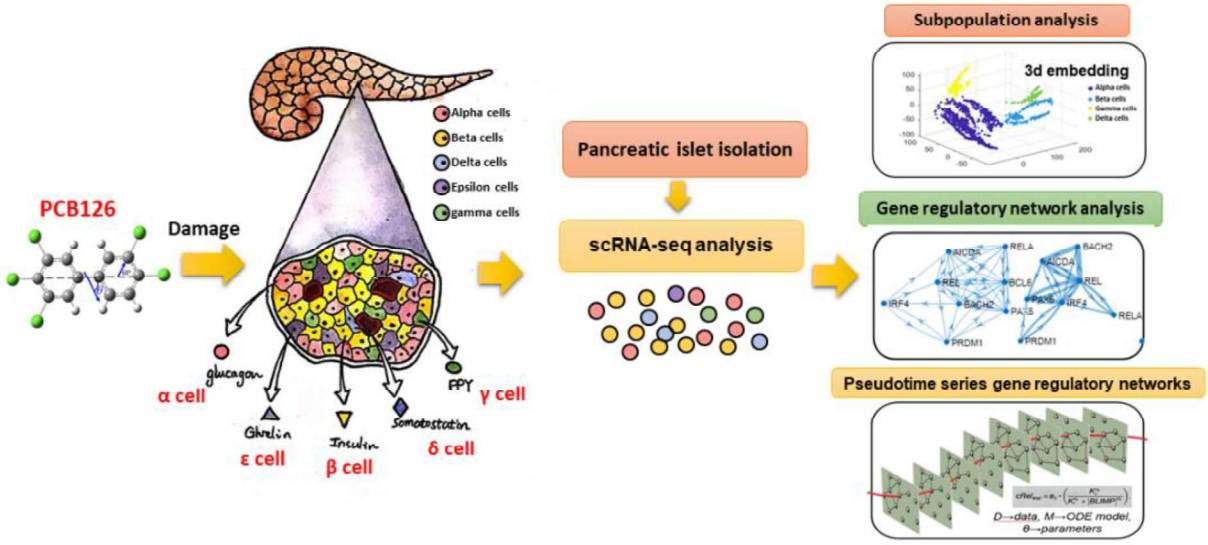

Figure 39. Workflow for obtaining and analyzing single-cell RNA-seq data from isolated pancreatic islets from mice treated with ROS-inducing chemicals such as PCB126.

In Section four we for the first time reported the function of MALAT1 in regulating innate immune response during sepsis in mouse. Sepsis is generally accepted as one major cause of death in the intensive care unit (ICU). Oxidative stress has been implicated in the process of sepsis and has been causally associated for its severity (Biesalski and McGregor 2007, Huet, Obata et al. 2007). The term ‘‘oxidative stress’ ’refers to a serious imbalance between reactive oxygen species (ROS) production and antioxidant defenses in favor of the former, in a cell, tissue or organ. We have reported that MALAT1-Nrf2 played an important role in regulating antioxidant/detoxification process in mice, and consistently, we found MALAT1 null mice are highly resistant to sepsis with significantly subsided “cytokine storm”.

Sepsis is a complex physiological response of an organism to harmful stimuli, such as pathogens and endotoxins, which requires the development of a sophisticated regulatory network to carry out functions as signal-specific and gene specific levels involving activation of antimicrobial defense, immune response and tissue repair. Epigenetic studies have shown complex interactions between transcription factors such as NF-κB, FOXP3 and STAT families, along with DNA methylation and covalent histone modifications, play critical role in the regulation of inflammatory genes and the outcomes of these complex interactions are also modulated by physiological(Cunningham and Eghbali 2018) and dietary components(Das, Dickerson et al. 2018). In analogy to DNA and chromatin modifications, a diverse set of covalent modifications is present on RNA nucleotides encoding the epitranscriptome, post-transcriptionally shaping gene expression through regulation of RNA stability, translation, and non-coding RNA functions. Currently there is no report about the how RNA methylation, such as N^6-methyladenosine (m^6A) which is the most abundant internal mRNA modification, is involved in inflammatory response in sepsis. In this section, we elucidated the role of m^6A and

its related enzymes in regulating the inflammatory response in sepsis by investigating the modes of action in regulating the gene expression of MAT2A. Using cultured peritoneal macrophage isolated from mice we found that methionine and SAM treatment decreases LPS-induced cytokine levels and on the contrary the MAT2A competitive inhibitor cycloleucine (cLEU) increased cytokine levels supporting the role of methionine-SAM-regulated methylation in regulating inflammatory response in sepsis. We have reported that MALAT1-ablated mice are highly resistance to hyperglycemia and LPS induced oxidative stress with significantly lower ROS level and increased antioxidant capacity. Additionally, genetic ablation of MALAT1 up-regulates MAT2A gene expression and drastically reduced ROS level in liver (Chen, Ke et al. 2018) and isolated peritoneal macrophage with increases in the levels of glutathione . Collectively these results suggested that MALAT1 regulated inflammatory response in sepsis through regulating methyl donor levels by epitranscriptomic mechanisms(Roundtree, Evans et al. 2017). Our novel finding of the role of MALAT1 in regulating methionine metabolism provides a potential therapeutic target for treatment of sepsis through RNA-based targeting approaches. As methionine metabolism can easily be manipulated by dietary strategies like supplementation commonly used in the clinics (Park, Kubicki et al. 2011, Dickerson, Deshpande et al. 2012) of Methionine/SAM (Bottiglieri 2002), such studies could lead to strategies of additive treatment of sepsis.

My Ph. D thesis have exclusively investigated the physiological role of MALAT1 in regulating glucose metabolism and inflammatory response in mice. Mechanistically, we and others have shown MALAT1 played an important role as a “ribo-repressor” in globally regulating gene expressions. We utilized aryl hydrocarbon receptor (AhR) signaling pathway as an example to investigate the mechanism of MALAT1 in regulating genes expression. AhR is a ligand-

activated transcription factor that regulates several immune processes involving both innate and adaptive immunity (Tian, Ke et al. 1999, Hao and Whitelaw 2013). Although the AhR was initially recognized as the receptor mediating the pathologic effects of dioxins and other xenobiotics, several dietary compounds and products of commensal flora activate AhR signaling at physiologically relevant doses and with significant potency. Thus, the AhR provides a molecular pathway through which xenobiotics as well as endobiotics modulate the immune response and, consequently, the development of immune-mediated disorders. Therefore, central to our understanding of dioxin-induced toxic responses and physiological functions of the AhR natural ligands is to elucidate the biology of the AhR and the role of AhR in regulating gene expression. The AhR has been shown to mediate most of the toxic effects induced by dioxin and related compounds. Nuclear factor kappa B (NF-κB) is a pleiotropic transcription factor that plays a pivotal role in a wide array of physiological and pathological responses including modulation of innate and adaptive immunity. In earlier studies, (Tian, Ke et al. 1999) as well as many other investigators have revealed a physical and functional interaction between AhR and NF-kB pathway (Kerkvliet 2009, Sekine, Mimura et al. 2009). Based on the interaction between AhR and NF-kB pathways, a "squelching" mechanism has been postulated in which AhR and NF-κB interact and compete for the co-regulator complex. The identity of the common and presumably limiting coregulatory factors remains unclear and likely candidates including nuclear co-regulators such as p300/CBP, NcoR/SMRT. We have found MALAT1 interacted with both AhR, NF-κB, NcoR, EZH2, as well as splicing facter SF2 (Figure 40) and may serve as a common transcriptional regulator which results in the mutual modulation between these pathways. Our studies and results from other have found that proinflammatory substances such as LPS induced MALAT1 expression. One mechanism is through the direct transcriptional activation of the

MALAT1 gene expression as the regulatory kB site has been identified in the regulatory regions of MALAT1 (Zhao, Su et al. 2016).

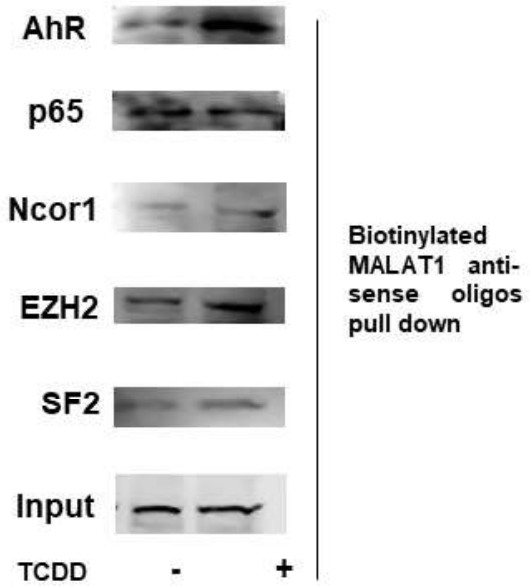

Figure 40 Association of MALAT1 with co-repressor NcoR1 as determined by RNA-Protein pull-down assay. A pooled biotinylated MALAT1 anti-sense oligos were used to precipitate native protein complexes from liver cell nuclear extract. The precipitates were subjected to Western blot analysis.

In order to understand the role of the MALAT1 in regulating the AhR pathway we performed transcriptome analysis of the TCDD-treated hepatocytes isolated from MALAT1 null mice as well as the wildtype control. We have found that the prototypic gene battery regulated by the AhR including *cyp1a1, cyp1b1, cyp1a2 and Nqo1* were significantly upregulated (average 3 fold) comparing with the wildtype control (Figure 41). Interestingly, a time course study of the cyp1a1 transcript indicated that the kinetics TCDD-induced transcription is different in the MALAT1-ablated hepatocytes suggesting the repressor functions of the lncRNA.

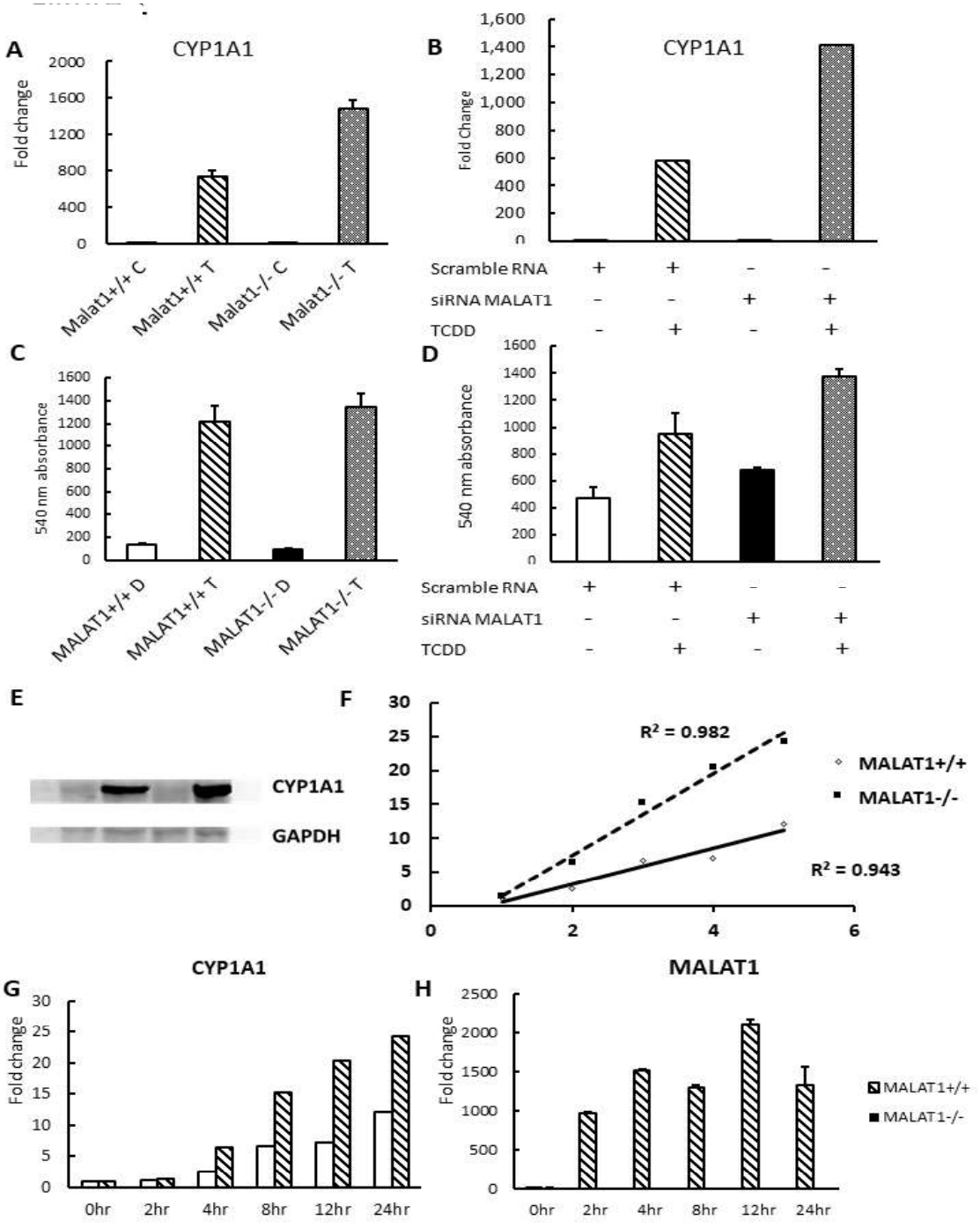

Figure 41 Regulation *cyp1a1* expression by MALAT1. (A) MALAT1 deficient augments the cyp1a1 expression. (B) Compatible expression level of EROD activity in malat1 mull in comparison with the wild type helpatocytes. (C) and (D) siRNA Malat1 knockdown malat1 potentiates *cyp1a1* expression and EROD activity. (E) CYP1A1 protein level in MALAT1 null hepatocytes and wild type controls. (F) and (G) MALAT1 deficiency alters *cyp1a1* transcription kinetics.

Chromatin Isolation by RNA Purification (ChIRP) and RNA-pull down assays results suggest malat1 is capable of associating with either AhR and the CYP1A1 gene (Figure 42) and/or nuclear repressors including NcoR1, SMRT as well as EZH2 a component in the polycomb repressive complex (Figure 40).

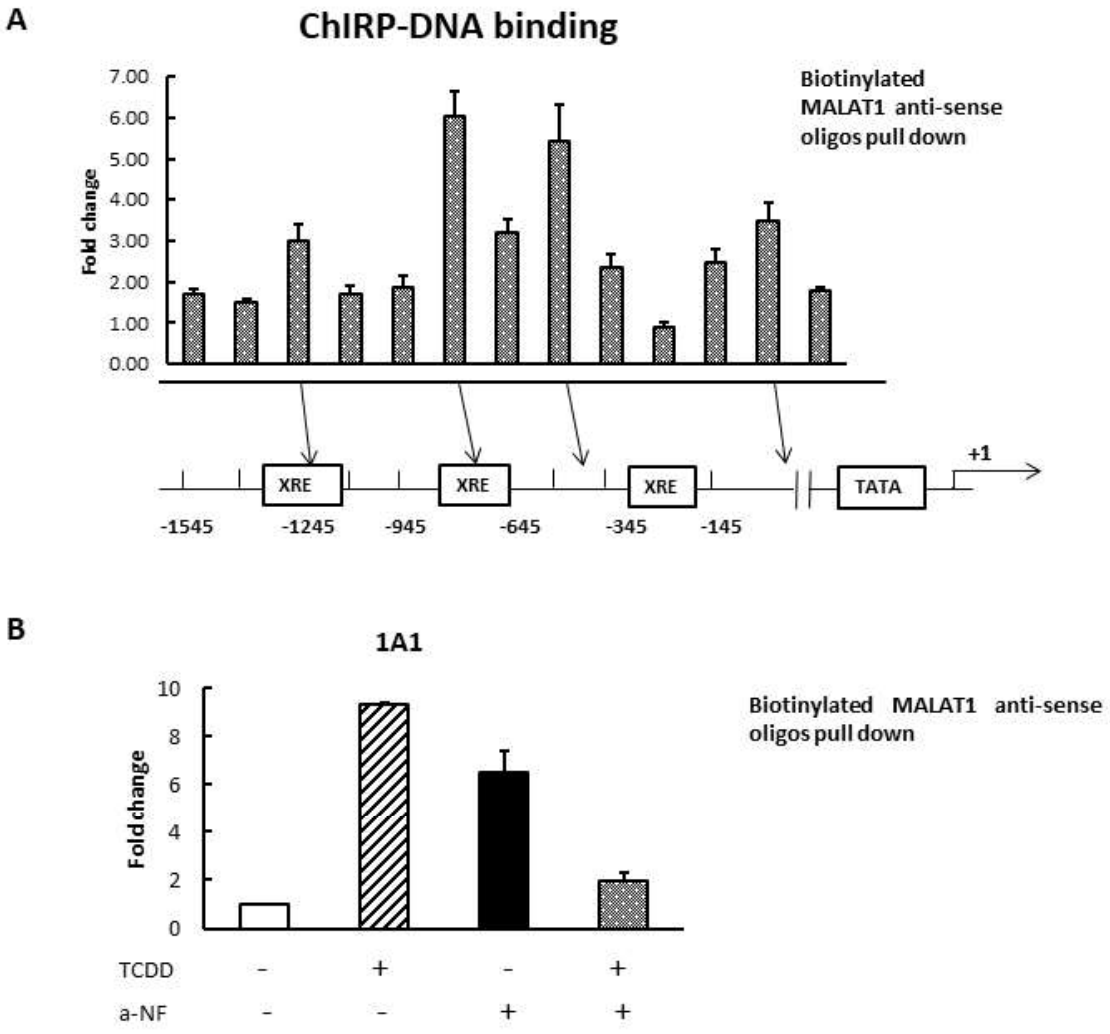

Figure 42. Chromatin isolation by RNA purification (ChIRP) using Malat1 biotinylated oligos. (A) and (B) ChIRP analysis of MALAT1 bindng sites in CYP1A1 gene.

Moreover, the results of transcriptomic analysis with RNA-seq suggest MALAT1 deficiency altered RNA processing mechanism leading to erroneous transcription including alternative splicing events (Figure 43).

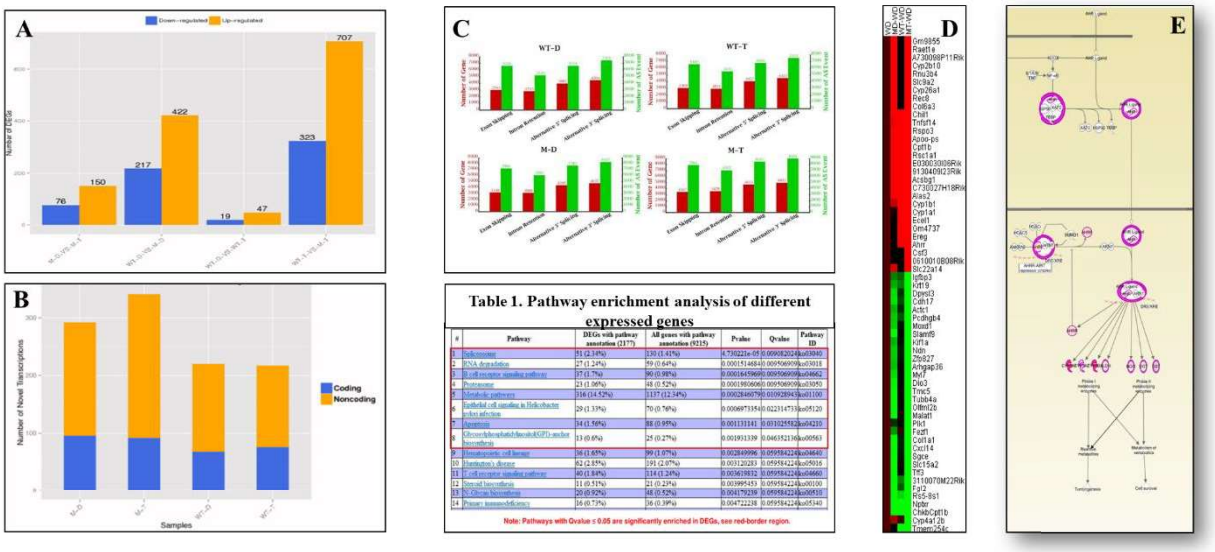

Figure 43. Bioinformatic analysis of AhR regulated transcription in MALAT1-/- and MALAT1 +/+ hepatocytes. (A) Differential gene expression in mutant and wild type hepatocytes. (B) Statistical analysis of novel transcripts in mutant and wild type hepatocytes. (C) Statistical analysis of alternative splicing events in malat1-/- and malat1 +/+ hepatocytes. (D) Heat map analysis of differential gene expression in mutant and wild type hepatocytes. (E) Ingenuity pathway analysis in AhR regulated gene expression. (Table 1) The enrichment analysis of the signaling pathway based on expressed genes of malat1-/- in comprison with wild type mice.

In summary, our results suggest MALAT1 functions as a scaffold giving rise to a platform gathering the actors of transcriptional activators and repressors thus playing a role as the "ribo-repressor". In MALAT1-ablated mice, the AhR pathway was significantly upregulated, imposing much more potent inhibitory effects on NF-κB pathway, resulting in significant repression of inflammatory cytokine levels (Figure 31). MALAT1 null mice showed alleviated oxidative stress, highly sensitized insulin response, and also suppressed pro-inflammatory response, suggesting MALAT1 as a potential target for treating ROS-mediated diseases.

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