Introduction

Liver is a metabolic hub that plays a key role in driving the energy balance in the body (Nguyen et al., 2008; Rui, 2014). For all the metabolic processes to work in tandem with each other, gene expression is fine-tuned by an array of microRNAs (miRNAs) that function mainly by binding to the 3'UTR of their target transcripts, thereby suppressing their expression (Aryal et al., 2017; Dumortier et al., 2013; Rottiers & Näär, 2012). Target genes for miRNAs can be usually determined via sequence-based prediction algorithms or by experimental validation. Sequence-based prediction algorithms consider several factors into account such as seed pairing and sequence similarity among miRNA and target mRNAs, accessibility of mRNA AU content, GU wobble in seed match, folding energy as well as conservation (Garcia-Moreno & Carmona-Saez, 2020; Gomes et al., 2013; Yu et al., 2020a). However, binding of a particular miRNA to its potential target mRNA does not necessarily culminate in inhibition of gene expression downregulation(Garcia-Moreno & Carmona-Saez, 2020). TargetScan is one such algorithm that is based on seed matching and combs through mRNAs by looking for the presence of conserved 8mer, 7mer and 6mer sites of mRNAs that match each miRNA (Singh, 2017; Vyas et al., 2023; Yu et al., 2020). Additionally, predictions of mammalian miRNAs are also ranked based on the predicted efficacy of targeting using cumulative weighted context++ scores of the sites (Garcia-Moreno & Carmona-Saez, 2020). Context ++ scores are based on several factors such as 3'UTR target site abundance, predicted seed-pairing stability, 8mer sites, probability of conserved targeting and so on (Thomas et al., 2011).

Under conditions of liver damage, as in the case of NAFLD and NASH, profound re-programming of the hepatic gene expression takes place, that is orchestrated, in part, by altered miRNA expression profile in liver (Liu et al., 2018). Since the initial reports of altered miRNA expression profiles in NASH patients, miRNAs have been in the spotlight to decipher mechanisms by which gene expression is modulated in NAFLD/NASH by miRNAs. A study revealed 42 differentially expressed miRNAs in the steatotic vs. non-steatotic group of

individuals wherein; 8 miRNAs (hsa-miR-3663-5p, miRPlusI137*, miR-576-5p, miR-892a, miR-3924, miR-106b*, miR-103a-2* and miR-1282) that were most significantly upregulated had not been previously linked to NASH (Soronen et al., 2016). In another study, 5 miRNAs (upregulated: miR-146b-5p and downregulated: miR-139-5p, miR-30b-5p, miR-122-5p and miR-422a) were reported to be differentially expressed between healthy women and women with NASH (Latorre et al., 2017). Interestingly, subjecting cultured human hepatocytes with palmitate alone or in combination with high glucose altered expression levels of all the five miRNA species (Latorre et al., 2017). Further, in NASH patients, 46 differentially expressed miRNA species were reported and among these, miR-224, miR-34a, miR-200a, miR-146b and miR-222 were upregulated whereas, miR-617, miR-641, miR-198, miR-765 and miR-601 were downregulated (Cheung et al., 2008).

Among the key regulators of hepatic gene expression, miR-122 comprises of approximately 70% of the total miRNA population in adult liver (J. Hu et al., 2012)and studies have reported that miR-122 expression is sharply upregulated in both mouse and human liver during embryonic development, thus implying that miR-122 may play a crucial role in hepatocyte differentiation and liver development. (Laudadio et al., 2012) Further, in mice, four liver-enriched transcription factors such as hepatocyte nuclear factor (HNF) 1α, HNF-3β, HNF4α and CCAAT/enhancer-binding protein (C/EBPα) bind to miR-122 promoter and activate miR-122 expression (H. Xu et al., 2010). Moreover, miR-122 plays a key role in regulating hepatic lipid and cholesterol metabolism (Esau et al., 2006a; Wen & Friedman, 2012). A study revealed that miR-122 sequestration accounted for decreased cholesterol, LDL and HDL fractions both in liver and blood, and decreased hepatic lipid accumulation (Esau et al., 2006b). Further, miR-122 silencing in HFD-fed mice accounted for a decrement in hepatic steatosis with decreased cholesterol synthesis and enhanced fatty-acid oxidation (Y. Hu et al., 2022a). Another study had shown that intrahepatic miR-122 levels were significantly upregulated in HFD-induced NASH mice that

corroborated with the *in vitro* studies wherein free-fatty acid (FFA)-induced Huh7 cells recorded upregulated miR-122 levels (Escutia-Gutiérrez et al., 2021).

Previous studies from our laboratory had reported that diet and/or photoperiodic shifts induced circadian desynchrony in mice accounted for altered oscillations of circulating and intrahepatic miR-122 oscillations that corresponded with altered serum liver function markers, lipid profile and hepatic fatty manifestations (Devkar et al., 2023). These findings suggest that miR-122 plays a key role under conditions of circadian desynchrony induced metabolic changes in liver. Interestingly, a study had reported that miR-122 regulates hepatic Noct expression, thus providing an important link between hepatic metabolism and the circadian clock (Kojima et al., 2010). However, detailed studies in this regard are lacking. The previous two chapters explored the circadian basis of hepatic Noct under conditions of H and/or CD induced NASH pathology as well as investigating the possible melatonin-Noct interactions. This chapter delves into elucidating the epigenetic regulation of Noct under experimentally induced NASH pathology and employs computational algorithms and databases to explore the miRNA-mediated regulation of hepatic Noct in NASH pathology and the results are further assessed in experimental models.

Methodology

Computational studies

Potential epigenetic regulators of *Noct* were identified using TargetScan and miRNAs implicated in NAFLD/NASH were determined using Human MicroRNA Disease Database (HMDD). The two datasets were merged in Cytoscape 3.9.1 to decipher the miRNA control of *Noct* in NASH pathology. The most promising miRNA candidate was further scrutinized in TargetScan.

In vivo studies

C57BL/6J male mice were subjected to high-fat-high-fructose (H) diet alone or in combination with photoperiodic manipulations induced chronodisruption (CD) for 16 weeks.

Experimental Groups:

1. Control (C)
2. High-fat-high-fructose diet (H)
3. Chronodisruption (CD)
4. High-fat-high-fructose diet + Chronodisruption (HCD)

In vitro studies

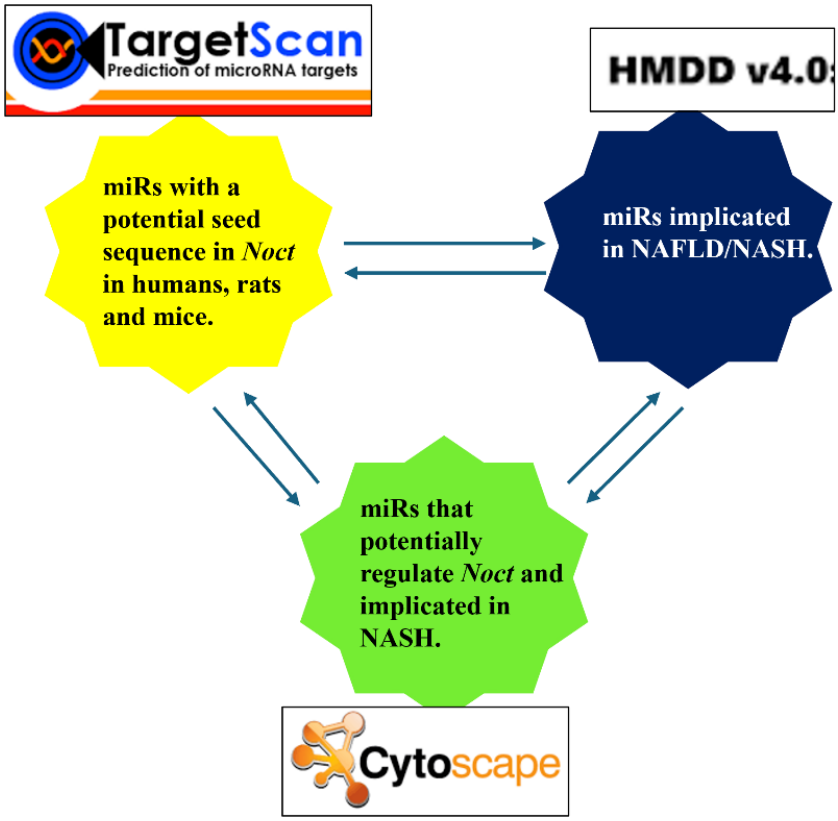
HepG2 cells were serum-synchronized and subjected to oleic acid (OA) alone or in combination with miR-122-specific inhibitor for 24 h. Cells were harvested at different time points for gene and protein expression studies.

Parameters:

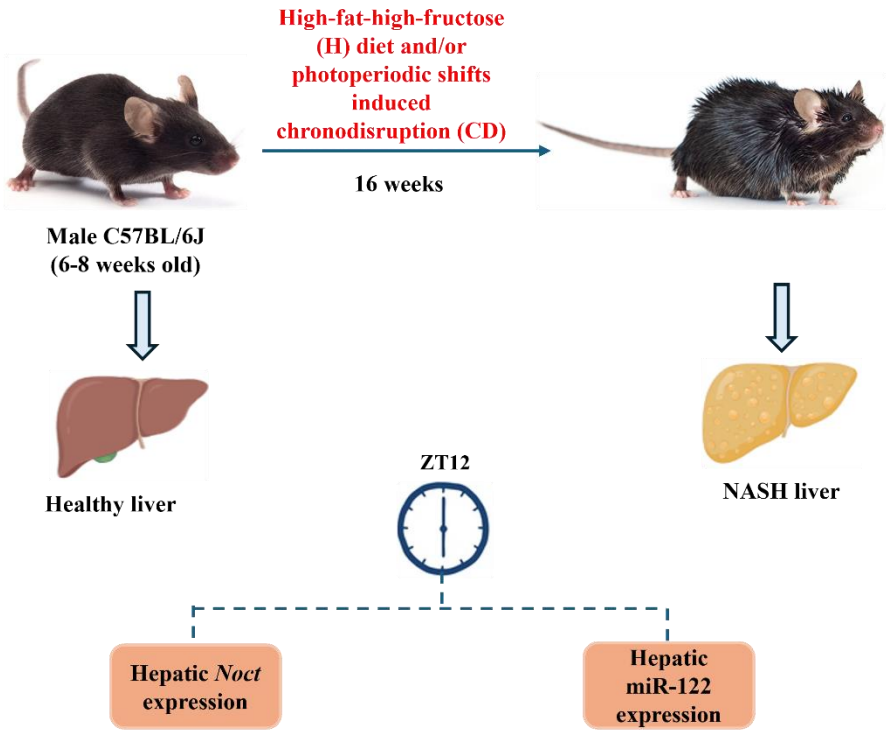
1. Cytotoxicity analysis to determine the optimum concentration of miR-122 inhibitor.
2. Mmu-miR-122 and hsa-miR-122 quantification by qPCR.
3. Gene expression studies by qPCR: *mmu-Noct* (at ZT12) and *hsa-NOCT* oscillations (at time points: 24 h, 28 h, 32 h, 36 h, 40 h, 44 h and 48 h).

4. Protein expression studies by Western Blotting: hsa-NOCT oscillations (at different time points: 24 h, 28 h, 32 h, 36 h, 40 h, 44 h and 48 h).
5. CircWave analysis to scrutinize Noct rhythmicity.

Computational studies

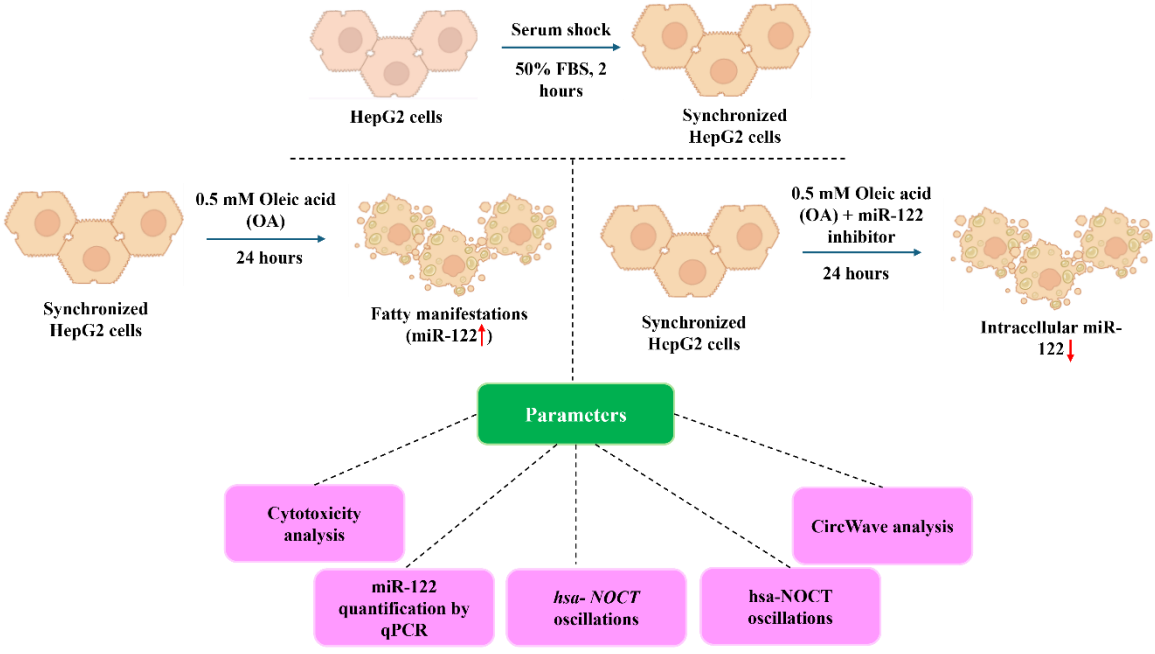


In vivo studies



In vitro studies

(Time-point based study)



Results

Algorithmic analysis identified all the potential miRNAs regulating *Noct* expression in humans, mice and rats.

Potential interactions for miRNA: *Noct* mRNA were studied using TargetScan V 7.0 wherein; seed regions were compared in the presence of conserved 8mer and 7mer and target site was predicted considering traits such as AU-rich regions near sites, co-express miRNA, 13- 16 residue pairing and position away from long UTR centre. In this study, we identified 1087, 240 and 89 miRNAs in humans [Fig. 3.1], mice [Fig. 3.2] and rats [Fig. 3.3] respectively that potentially bind to the *Noct* gene at the 3'UTR. These miRNAs were further assessed on the basis of context ++ scores and context ++ percentile scores.

Human MicroRNA Disease Database (HMDD) identified miRNAs implicated in NASH pathology.

To accurately identify all the miRNAs that have been reported to be implicated in NASH pathology, HMDD was employed that gave a list of 70 miRNAs [Fig. 3.4]. These miRNAs were further scrutinized in miRNet and subjected to enrichment analysis using default parameters of the online tool wherein; 40 miRNAs were functionally enriched [Fig. 3.5]. Among the most significantly enriched miRNAs ($p<0.05$) were miR-155, miR-122, miR-192 and miR-375.

Overlapping screening identified miR-122 as a potential epigenetic regulator of *Noct* expression in NASH.

An overlapping screening was performed between the above two datasets using statistical tools and default settings of the Cytoscape 3.9.1 software that identified 30 miRNAs that regulate *Noct* expression in NASH. These miRNAs are suggestive of the epigenetic regulation of *Noct* under the said pathology [Fig. 3.6]. miR-122 was one of the 30 miRNAs and based on the conserved seed-sequence of miR-122 across species [Fig. 3.7], its gene targets and their implications in liver disorders, miR-122 was chosen as the most potent epigenetic regulator of *Noct* in NASH.

MiR-122 and hepatic *Noct* expression inversely correlate in H and/or CD mice at ZT12.

MiR-122 was further subjected to scrutiny in the liver tissues of H and/or CD mice wherein; miR-122 and *Noct* expression were assessed by qPCR at ZT12. This specific timepoint was chosen since available literature and our findings have revealed that hepatic *Noct* expression peaks at the said time. Findings revealed that miR-122 inversely correlated with hepatic *Noct* expression. H group recorded ~4-fold increment in *Noct* mRNA that coincided with a very low intrahepatic expression of miR-122. In contrast, CD group recorded higher miR-122 expression and a lower-than control expression of hepatic *Noct.* HCD group recorded a moderate change [Fig. 3.8].

Intracellular inhibition of miR-122 in HepG2 cells

To further assess possible miR-122: Noct interactions, a synthetic inhibitor (NSC-5476) specific to miR-122 was employed to suppress miR-122 expression in HepG2 cells. This inhibitor specifically binds to primary and precursor miR-122 (pri-miR and pre-miR) transcripts, thereby inhibiting mature miR-122 synthesis. HepG2 cells were subjected to OA alone (0.5 mM), or in increasing concentrations of the inhibitor (0.05 to 10 μM) for 24 h to perform MTT assay. Cytotoxicity analysis identified 10 μM concentration of the inhibitor as a potent concentration [Fig. 3.9]. This concentration was further validated by treating HepG2 cells with OA alone or in combination with the inhibitor for 24 h. qPCR analysis revealed that OA-treated cells accounted for a significant upregulation ($p<0.01$) of intracellular miR-122 as compared to Control cells. However, OA along with the inhibitor recorded a significant downregulation ($p<0.05$) of miR-122 expression in HepG2 cells [Fig. 3.10].

MiR-122 inhibition in HepG2 cells upregulates *NOCT* expression in HepG2 cells.

Serum-synchronized HepG2 cells were treated with 0.5 mM OA alone or in combination with 10 μM miR-122 inhibition for 24 h and harvested at different time points for gene expression studies. HepG2 cells were treated with OA and NSC-5476 accounted for a 4-fold decrement in miR-122 levels as compared to OA-treated cells [Fig. 13 (A)]. miR-122 inhibition accounted for a significant increment in *NOCT* at 40h as compared to control cells ($p<0.001$) as well as OA-treated cells ($p<0.01$). Furthermore, non-significant increments were recorded at 44 h and 48 h [Fig. 3.11].

Intracellular inhibition of miR-122 in HepG2 cells disrupt *NOCT* rhythmicity.

Possible alterations in *NOCT* mRNA oscillations were assessed by CircWave analysis wherein; aberrant *NOCT* rhythms in HepG2 cells subjected to miR-122 inhibition were recorded [Fig 3.12 (a). Further, miR-122 inhibited HepG2 cells recorded a non-significant increment in the % relative amplitude when compared to OA-treated cells, albeit this increment was significant ($p<0.05$) when compared to Control cells [Fig.3.12 (b)]. The peak time of (CoG) of *NOCT* in OA + miR-122 inhibitor group shifted to ~40 h as compared to 36 h in control and ~32 h in OA-treated cells [Fig. 3.12 (c)]. Additionally, the cumulative *NOCT* expression recorded a non-significant increment in OA+ miR-122 inhibitor group as compared to OA group. However, this increment was significant ($p<0.01$) as compared to Control cells [3.12 (d)].

MiR-122 inhibition does not alter NOCT protein expression in HepG2 cells.

Serum synchronized HepG2 cells treated with OA alone or in combination with miR-122 inhibitor for 24 h were collected at different time points to assess NOCT protein expression. Inhibition of miR-122 accounted for a significant increment in NOCT protein expression at 28 h ($p<0.01$) and 32 h ($p<0.05$). The rhythmic expression of NOCT ranging from 40 h to 48 h in OA + inhibitor group was comparable to the OA treated group and significantly altered from the control group [Fig. 3.13 (a) and (b)].

MiR-122 inhibition alters NOCT protein oscillations without changes in NOCT expression.

CircWave analysis of NOCT protein oscillations revealed that significant perturbations in NOCT protein rhythmicity [Fig. 3.14 (a)]. MiR-122 inhibition in HepG2 cells accounted for a significant increment ($p<0.01$) in the % relative amplitude as compared to control cells though the amplitude was lower than that in OA-treated group [Fig. 3.14 (b)]. Further, the CoG shifted to ~36 h as compared to 48 h in control and ~38 h in OA-treated group [Fig. 3.14 (c)]. The cumulative NOCT expression recorded a non-significant increment in OA + Inhibitor group [Fig. 3.14 (d)].

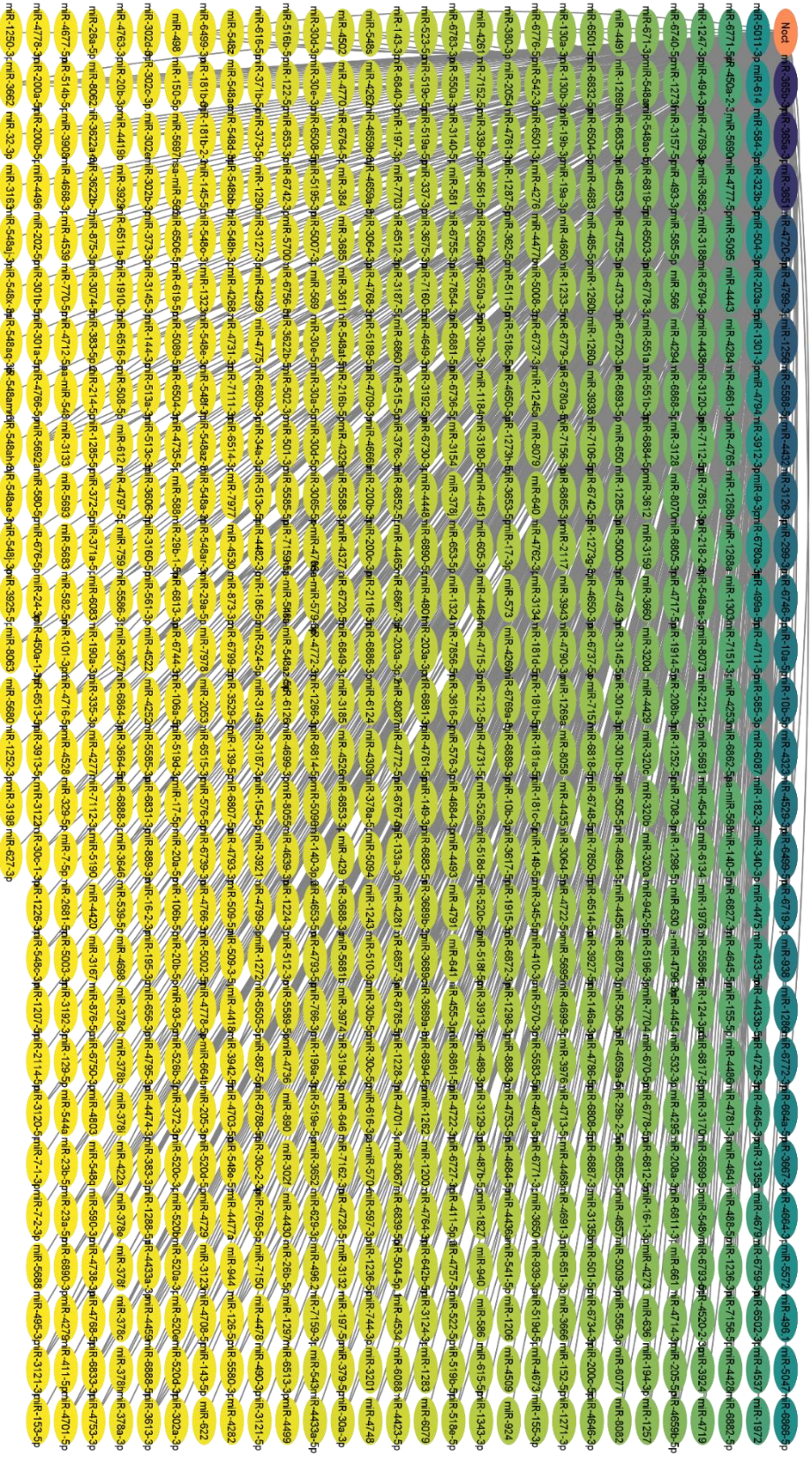


Figure 3.1. miRNAs regulating *NOCT* expression in humans.

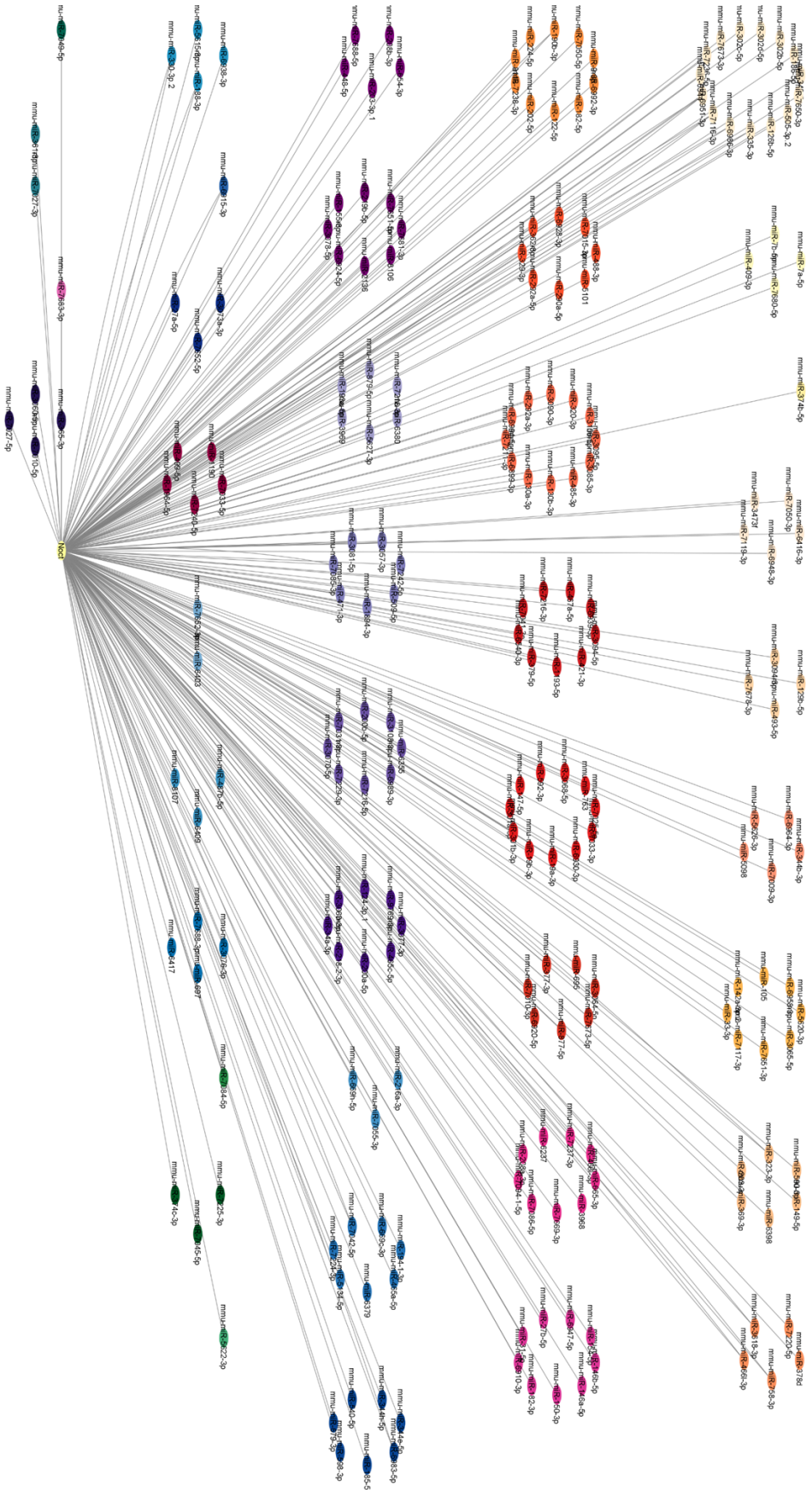


Figure 3.2. miRNAs regulating *Noct* expression in mice.

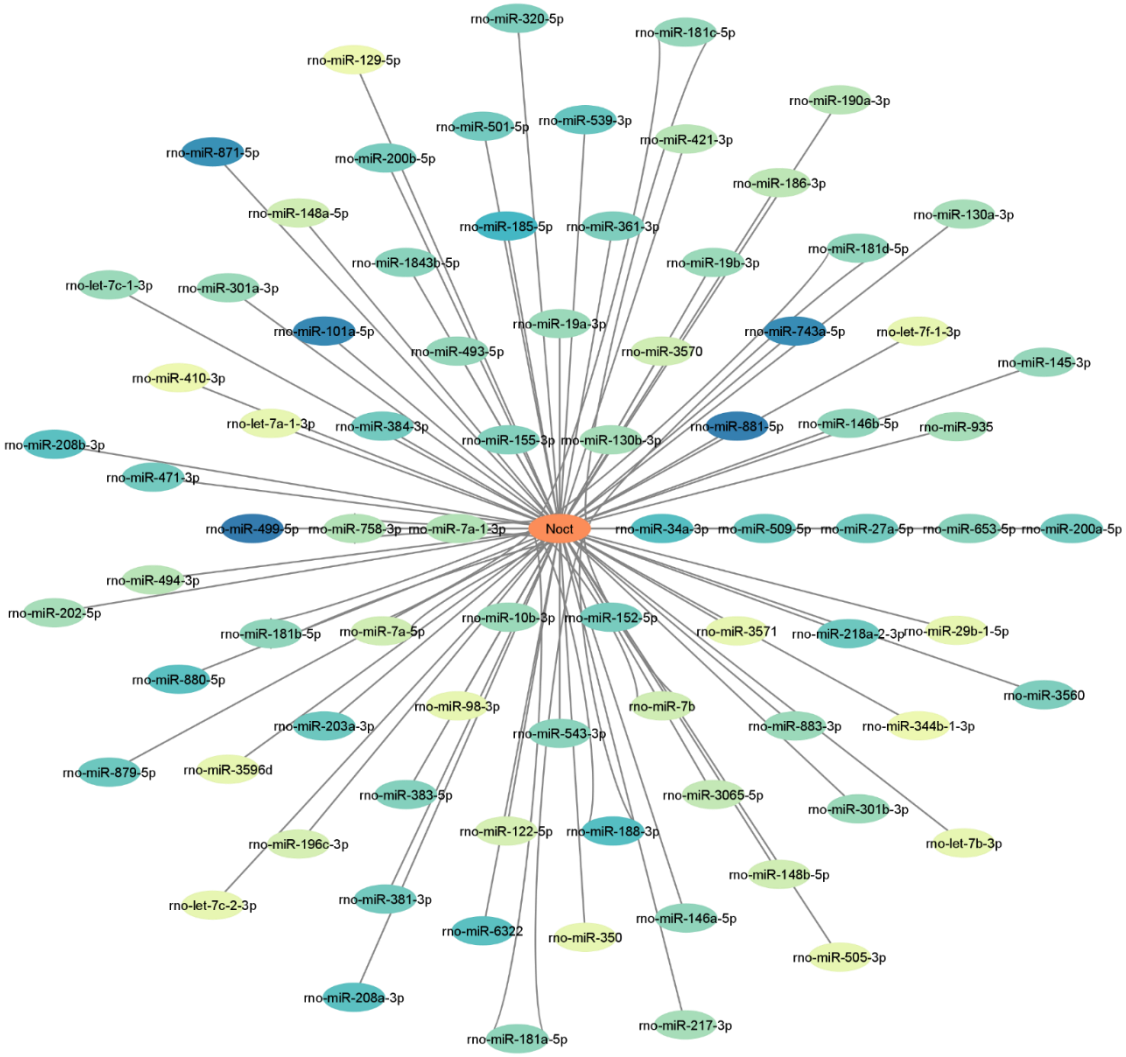


Figure 3.3. miRNAs regulating *Noct* expression in rats.

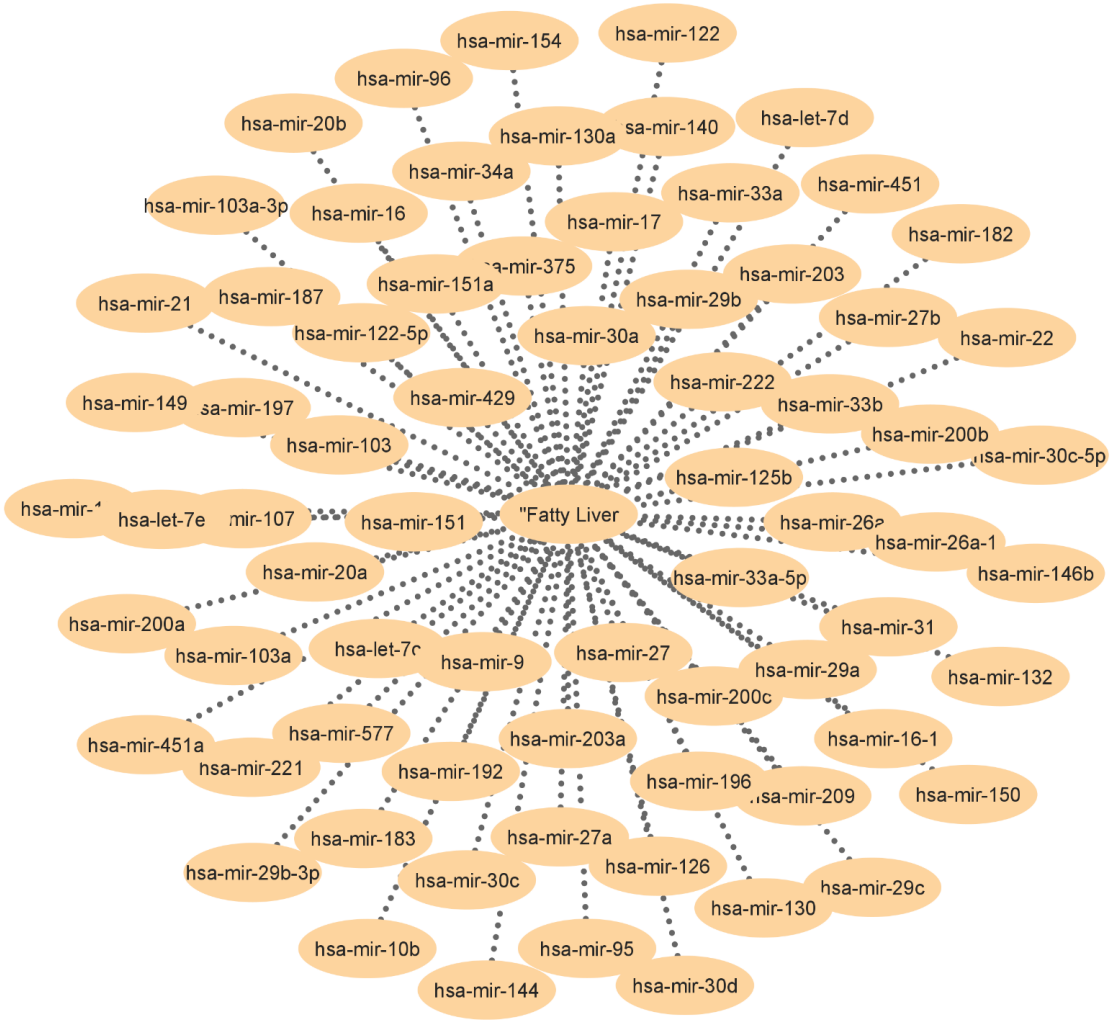


Figure 3.4. miRNAs implicated in NAFLD/NASH.

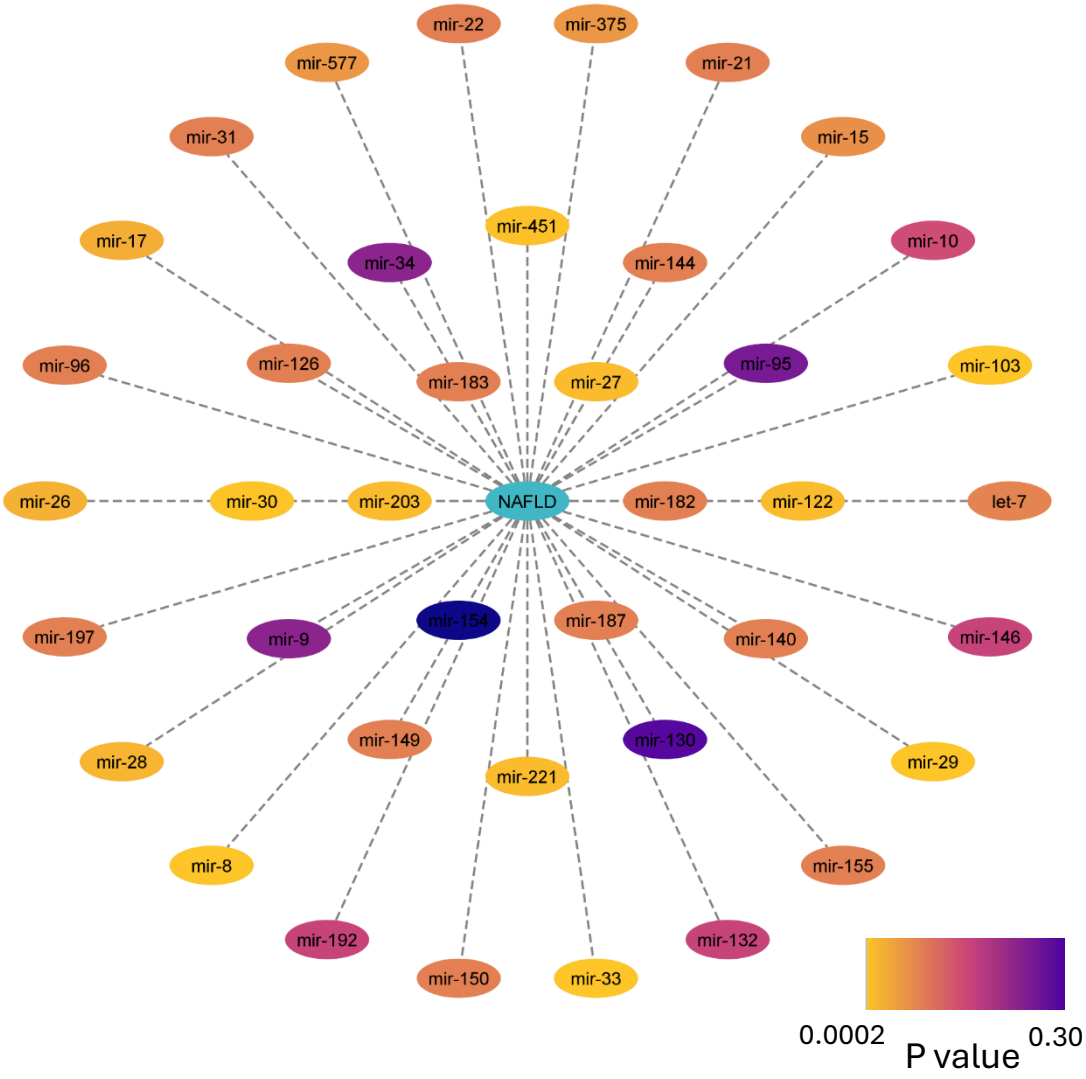


Figure 3.5. Functional enrichment in miRNet identified 40 miRs.

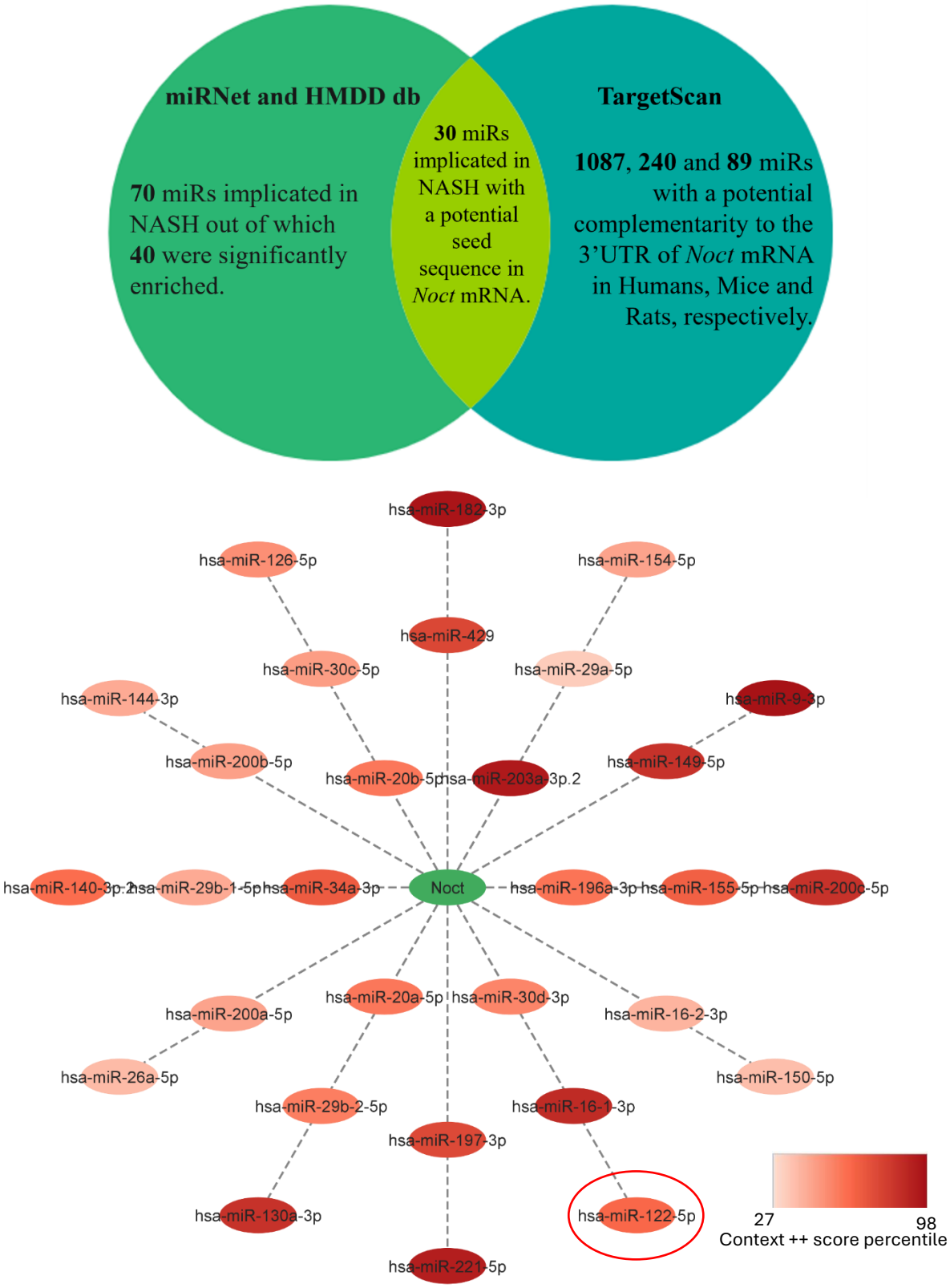


Figure 3.6. Overlapping analysis identified miR-122 as the most potent epigenetic regulator of *Noct* in NASH pathology.

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         .............................................................170................180...............190.............
Mouse    ----------------------------------GUU------UGCACUCCAG-UUUGAGCUUGUUGU-UCAUC---
  Rat    ----------------------------------GUU------UGCACUCCAG-UCCGAGUGUGUGCUGUCAUC---
Human    ----------------------------------GUU------UGCACUCCAA-UUUUGGCUUGUGUU-GUUUU--
Chimp    ----------------------------------AUU------UGCACUCCAA-UUUUGGCGUGUGUU-GUUUU--
Rhesus   ----------------------------------GUU------UGCACUCCAA-UUUUGGCUUGUAUU-GUUUU---
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Figure 3.7 miR-122 has a conserved seed sequence in Noct gene across species.

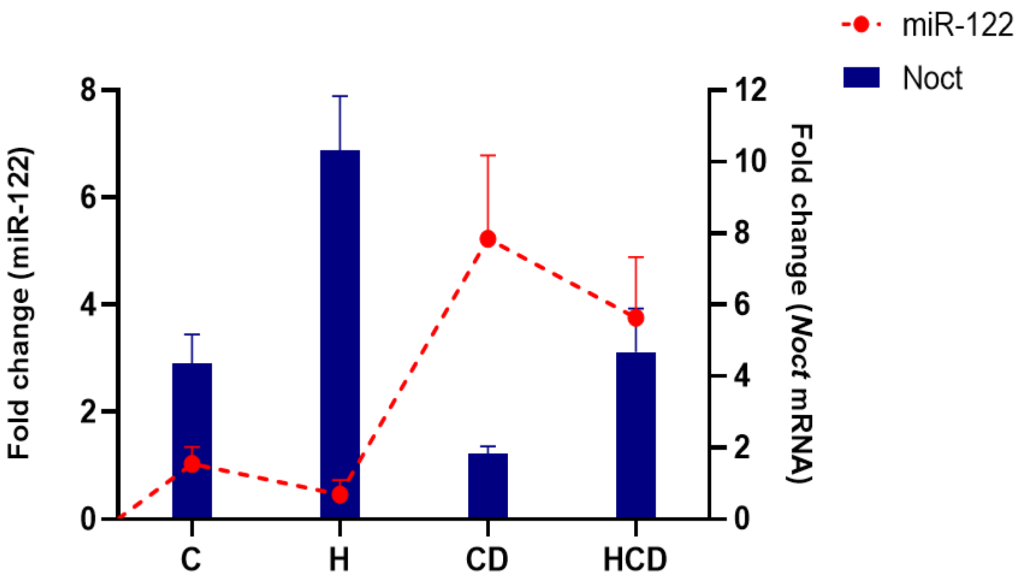

Figure 3.8. Hepatic *Noct* mRNA and miR-122 expression inversely correlate in H and/or CD mice at ZT12. Red dotted line indicates miR-122 levels, and the blue bars indicate *Noct* expression.

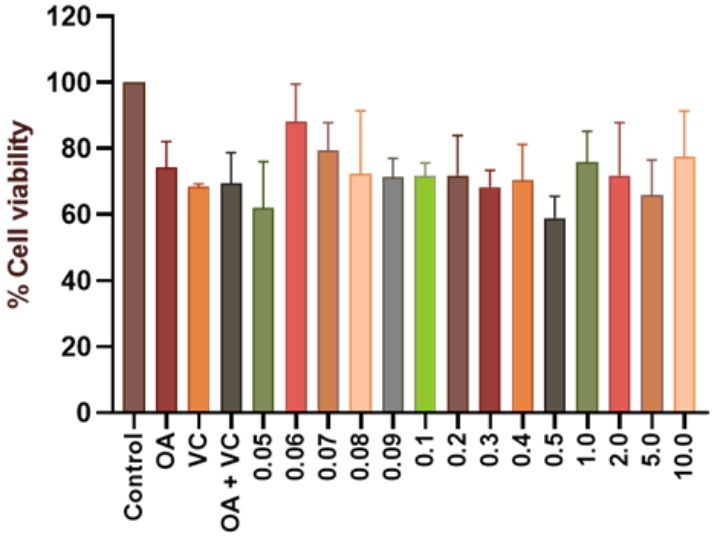

Figure 3.9. Dose standardization of miR-122 inhibitor: HepG2 cells were subjected to 0.5mM OA alone or in combination with increasing concentrations of miR-122 inhibitor (0.05µM-10µM) for 24h and subsequently, MTT assay was performed (n=4 for each treatment). From the results obtained, 10µM concentration of the inhibitor was selected for further experiments.

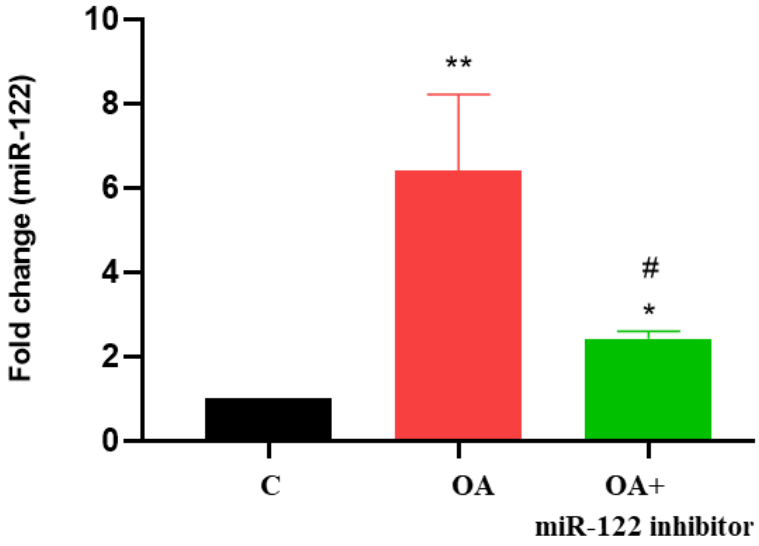

Figure 3.10. HepG2 cells were subjected to OA alone or in combination with 10µM miR-122 inhibitor for 24h and intracellular miR-122 levels were assessed by qPCR to validate the concentration of the inhibitor. Results are represented as mean ± SD. *p<0.05, **p<0.01 when treatments groups are compared to Control, #p<0.05 when OA+ inhibitor group is compared to OA group.

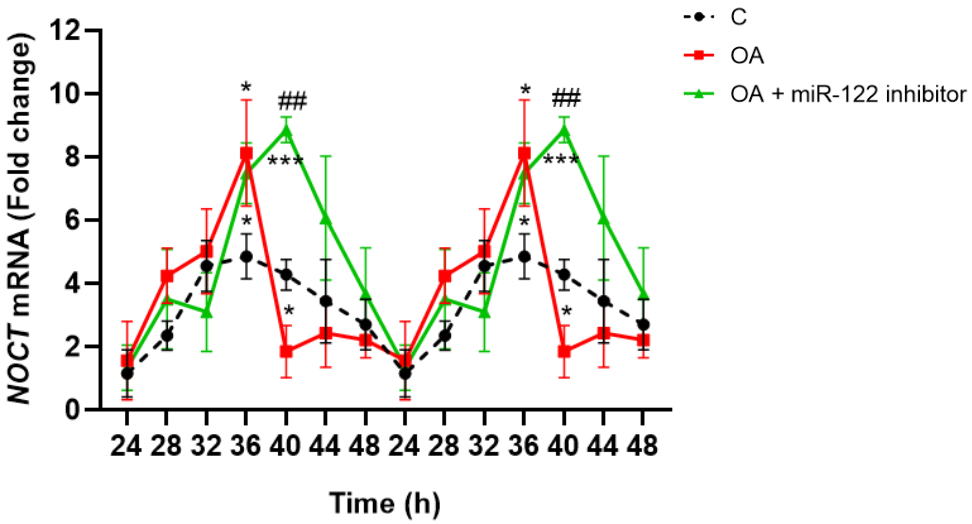

Figure 3.11. Serum-synchronized HepG2 cells were treated with 0.5 mM OA alone or in combination with 10 µM for 24 h and subsequently collected at different time points to assess *NOCT* mRNA oscillations. Results are expressed as mean ± SD (n=3 at each time point). *p<0.05, ***p<0.001 when treatment groups are compared with Control and ##p<0.01 when OA + inhibitor group is compared with OA group.

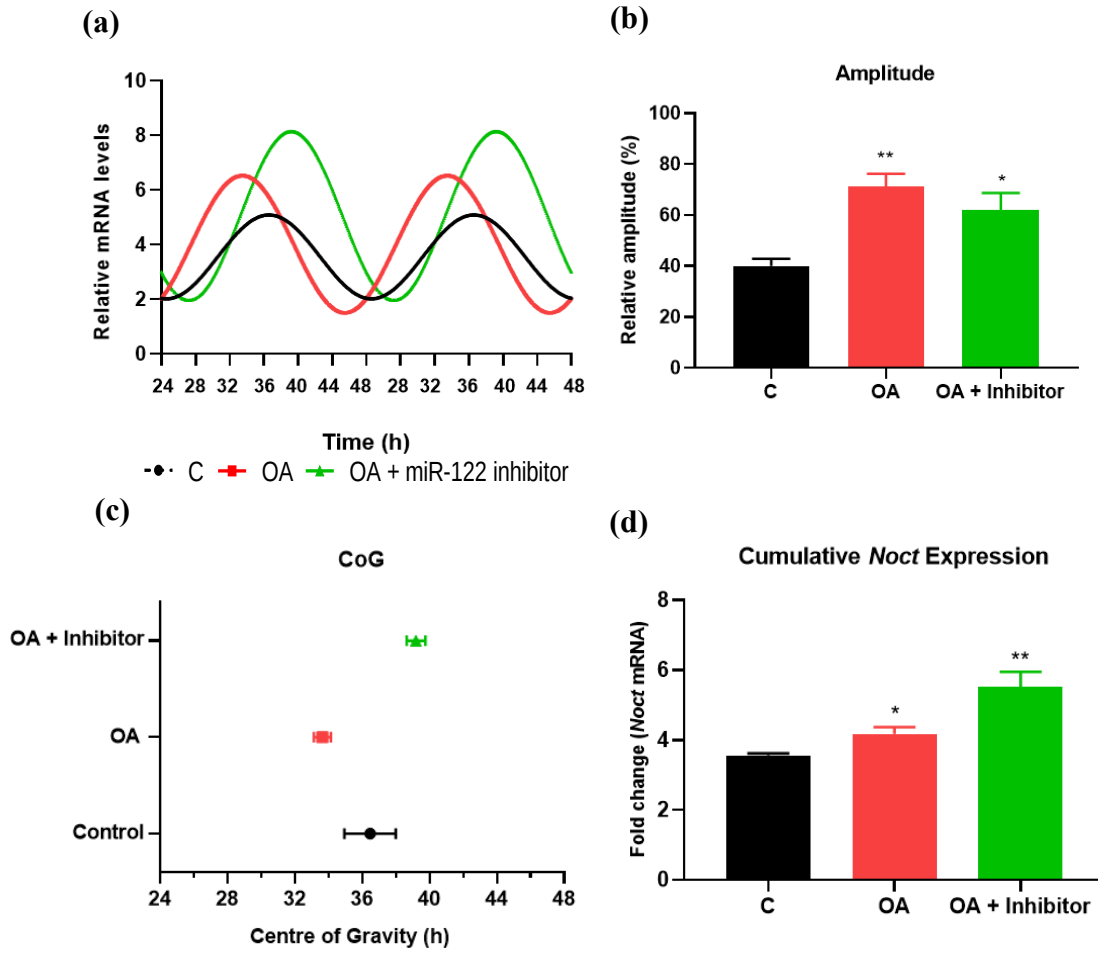

Figure 3.12. Alterations in *NOCT* mRNA rhythmicity analysed by CircWave analysis: (a) *NOCT* mRNA oscillations in the form of a sine-cosine wavefunction; (b) % relative amplitude; (c) peak time in the form of CoG and (d) Cumulative *NOCT* expression. Results are expressed as mean ± SD. *p<0.05, **p<0.01 when treatment groups are compared with control groups.

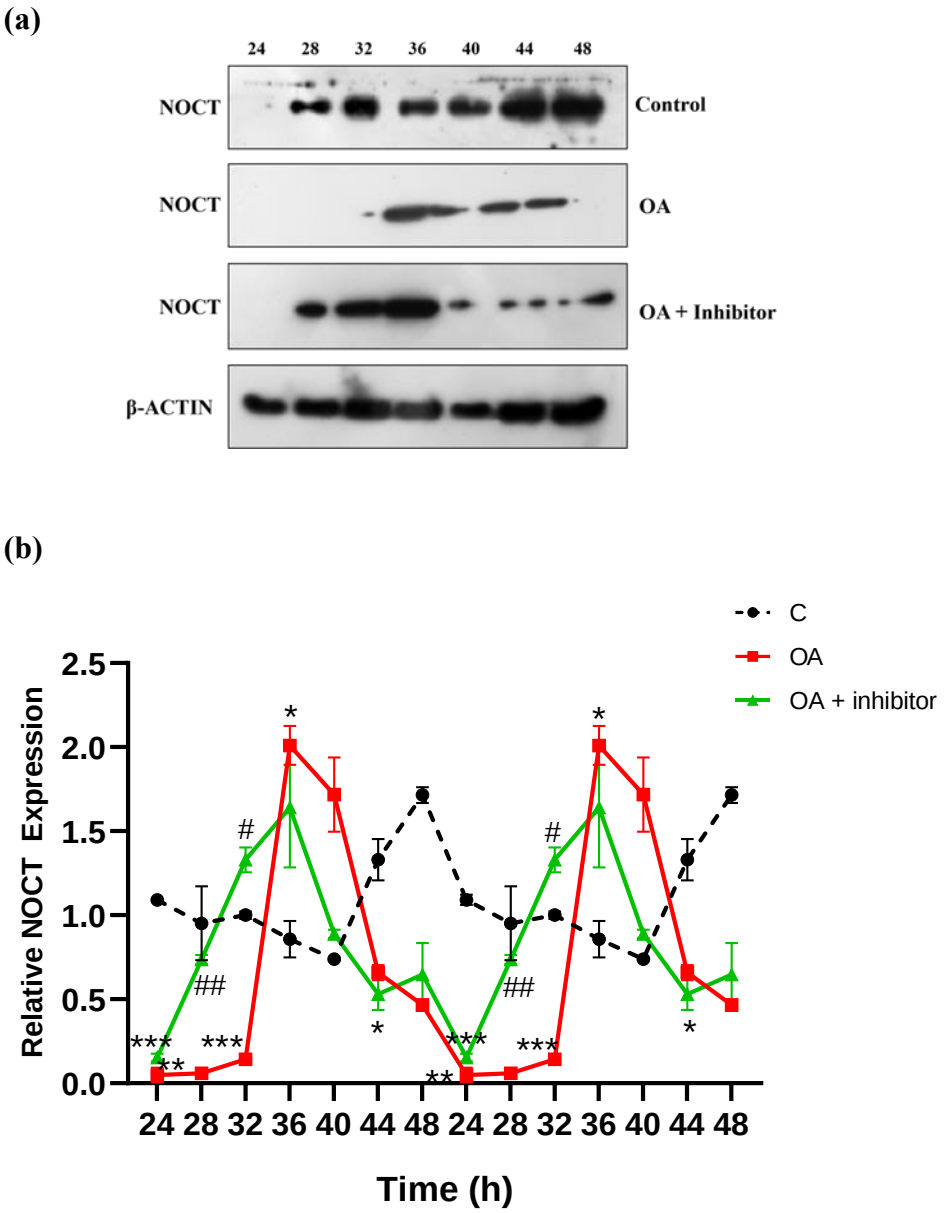

Figure 3.13. NOCT protein oscillation in serum-synchronized HepG2 cells treated with OA alone or in combination with miR-122 inhibitor for 24 h: (a) Immunoblots of NOCT at different time points and (b) NOCT oscillations. Results are expressed as mean ± SD (n=3 at each time point). *p<0.05, **p<0.01, ***p<0.001 when treatment groups are compared with Control and #p<0.05, ##p<0.01 when OA+ Inhibitor group is compared with OA group.

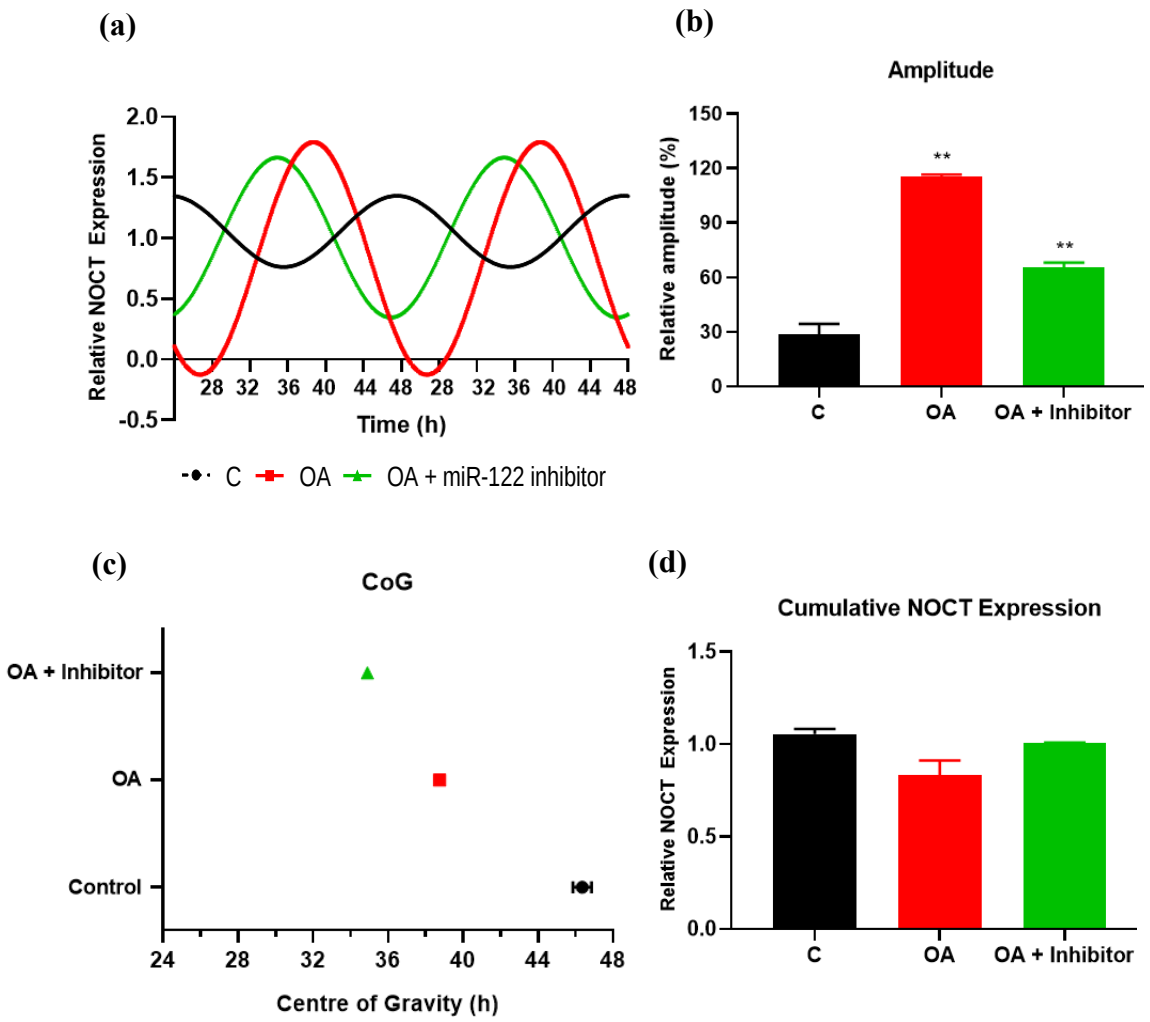

Figure 3.14. CircWave analysis of NOCT protein rhythmicity in OA alone in combination with miR-122-inhibitor-treated HepG2 cells: (a) NOCT oscillations in the form of a combinatory sine-cosine wavefunction; (b) % relative amplitude; (c) Peak time in the form of CoG and (d) Cumulative NOCT expression. Results are expressed as mean ± SD. **p<0.01 when treatment groups are compared with Control group.

Discussion

MiR-122 plays a key role in cholesterol and lipid metabolism pathways (J. Hu et al., 2012) in liver and its elevated expression is synonymous with the pathogenesis of NASH (Samy et al., 2024). MiR-122 inhibition alleviated lipid accumulation and inflammation by inhibition of TLR4/MyD88/NF- κBp65 signalling pathway in OA-treated L02 cells (Y. Hu et al., 2021). In another study, inhibition of miR-122-5p alleviated inflammation and oxidative stress in HFD-induced NASH by targeting FOXO3 (Y. Hu et al., 2022b). Further, intracellular miR-122 promoted hepatic lipogenesis by suppression of LKB/AMPK signalling pathway through its targeting of Sirt1 in HepG2 and Huh7 cells subjected to FFA overload (OA: PA= 2:1) (Long et al., 2019). Interestingly, miR-122 serves as a link between the circadian clock and metabolism since studies have reported that miR-122 locus is transcribed in a circadian manner and the orphan nuclear receptor, Rev-erb-α, drives the circadian transcription of miR-122 (Gatfield et al., 2009).

In the present study, computational analysis identified miR-122 as one of the important regulators of hepatic Noct expression in NASH. Additionally, miR-122 has a conserved seed sequence in *Noct* gene as inferred by our *in silico* analysis. Other studies had reported that the 3'-untranslated region (3'UTR) of *Noct* mRNA (conserved among mammals) consisted of one putative recognition site for miR-122 in mouse embryonic fibroblasts (MEFs). Overexpression of miR-122 in MEFs caused downregulation of *Noct* whereas; knock down of miR-122 in C57BL/6J mice had led to upregulation of Noct (Kojima et al., 2010). Along with these reports and our *in-silico* findings, we had hypothesized on miR-122- *Noct* interactions in NASH and the same was recorded in H, CD or HCD groups. This inverse correlation between miR-122 and *Noct* mRNA is suggestive of miR-122-mediated regulation of *Noct* under NASH.

In our study, miR-122 inhibition in HepG2 cells accounted for a significant upregulation of *NOCT* mRNA at 40 h and non-significant increments at 44 h

and 48 h, thus validating our *in vivo* findings. Findings of the study showcased herein imply that miR-122 plays a crucial role in maintaining the normal rhythmicity of *NOCT* gene expression. Inhibition of miR-122 not only alters the amplitude of *NOCT* but also shifts the peak time, delaying it significantly from 32 h (in OA group) and 36 h (in control group) to 40 h. Thus, miR-122 might be a circadian regulator of *NOCT* expression. Interestingly, miR-122 inhibition did not account for a significant increment when compared to OA-treated group. However, this increment was significant when compared to the Control group, indicating that miR-122 inhibition influences *NOCT* expression more dramatically in control cells, but less so when OA is already upregulating *NOCT.*

In hepatocellular carcinoma (HCC), miR-122 was found to regulate expression of genes such as *Cyclin G1* and *Adam10* but did not result in a significant suppression of their protein levels (Chun, 2022). Additionally, miR-122 has been demonstrated to repress *Wnt1* mRNA without fully inhibiting the translated protein in some cancer-related pathways (J. Xu et al., 2012), thus implying that the regulatory mechanisms of miR-122 may sometimes be limited to the transcriptional level and miR-122 can inhibit mRNA levels of its target genes without necessarily reducing the translated proteins. In our study, inhibition of miR-122 in HepG2 cells did not account for a significant upregulation of NOCT protein. Further, CircWave analysis revealed a significant increment in the % relative amplitude of NOCT protein oscillations as compared to control cells implying that inhibition of miR-122 causes dramatic fluctuations in NOCT rhythmicity, however, the amplitude was lower than that of the OA group. This indicates that while miR-122 inhibition has a significant effect, OA has a more pronounced impact on NOCT protein regulation, possibly due to its role in modulating lipid metabolism and energy balance. Additionally, miR-122 inhibition does not account for significant alterations in NOCT protein in HepG2 cells. Thus, miR-122 inhibition causes significant alterations in NOCT protein rhythmicity. However, the overall effect on NOCT protein expression is moderate when compared to the effects

of OA alone. Thus, our findings suggest that miR-122 is crucial for regulating NOCT protein rhythmicity that may consequently play a crucial role in metabolic or circadian regulation in hepatocytes.