Analyzing gene *Insig2* and *Slc51β* in relation to the off-target analysis of an N-Acetylgalactosamine-siRNA using nanopore sequencing

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1. Abstract

Primary sclerosing cholangitis (PSC) is a chronic liver disease. An siRNA against a specific tight junction protein could influence the progression of PSC in the future. The aim of this thesis was to investigate potential off-target effects of this siRNA. For this purpose, the gene *Insig2* was selected as a possible off-target because significant downregulation was observed in previous experiments with a specific target tight junction protein knockout mouse model. The selection of gene *Insig2* is therefore based on previous experimental findings. The expression of *Insig2* under siRNA treatment was analyzed using a mouse model with two different conditions (untreated compared to the treated group). The samples were analyzed using direct RNA nanopore sequencing. Unfortunately, not enough read counts for the specific tight junction protein were measured, nevertheless there were high read counts for *Insig2,* and it was significantly downregulated in the treated group compared to the untreated group. The siRNA could influence the mRNA of *Insig2*, but because there were no read counts for the specific tight junction protein, the significance of the data is not assured. Further optimization in preparing the nanopore sequencing in the future is necessary.

Keywords: siRNA, *Insig2*, nanopore sequencing

2. Introduction

PSC is a chronic inflammatory disease of the bile ducts, which can be caused by many different conditions. Unfortunately, a liver transplantation is currently the only effective treatment to improve a patient's life expectancy [3].
Off-target analysis was performed to detect unintended effects of siRNA on non-target genes [1]. The focus was placed on *Insig2*, as this gene is involved in the regulation of cholesterol levels, bile acid production, and lipid metabolism by controlling cellular signaling in the endoplasmic reticulum [4].

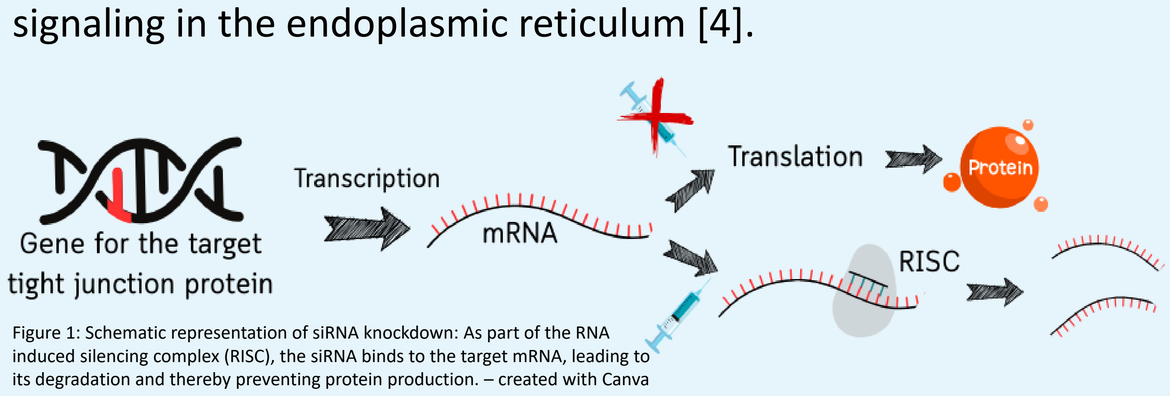


Figure 1: Schematic representation of siRNA knockdown: As part of the RNA induced silencing complex (RISC), the siRNA binds to the target mRNA, leading to its degradation and thereby preventing protein production. – created with Canva

In Switzerland, approximately 0.01% of the population is affected by PSC [2]. The goal of the project group is to develop a specific treatment option with the mechanism of RNA interference for PSC. A schematic representation of the therapeutic approach is visualized in Figure 1. To improve patient's quality of life and minimize the risk of liver transplants in the future. Therefore, analyzing the potential side effects with the off-target analysis is essential to ensure the safety of the treatment.

3. Aim and leading question

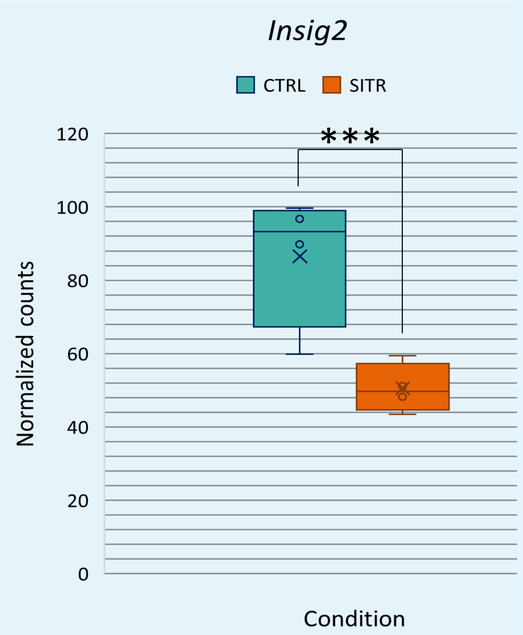
Evaluate the effects of the target protein specific N-Acetylgalactosamine (GalNAc)-siRNA application in an in vivo mouse model on the expression of the *Insig2* gene.

Which effects does the treatment with the specific GalNAc-siRNA have on the up- or down-regulation of the *Insig2* gene?

4. Methods and material

A mouse experiment with male wild-type mice was conducted. Five were treated with a subcutaneous injection of 10 mg/kg specific siRNA, the other four were not treated. After two days they were harvested. The whole liver was harvested, and a small part of it was snap-frozen in liquid nitrogen. RNA was extracted from the liver tissue. The extraction was performed using a RNeasy Qiagen Kit. Afterward, the quality and quantity were assessed using the NanoDrop spectrophotometer and the Agilent 2100 Bioanalyzer. Then the RNA was sent to the Institute for infectious disease (IFIK) to perform the library preparation followed by the nanopore sequencing.

5. Results



The following boxplot in Figure 2 provides an overview to visualize the distribution of the reads per group for the *Insig2* gene. On the Y-axis, the normalized read counts from the nanopore sequencing and on the X-axis, the two experimental conditions, first the untreated control group (CTRL) and second the treated group with siRNA (SITR), are shown.

Figure 2: Normalized counts for the nanopore sequencing results. Statistical significance was evaluated with two-way t-test. ns=non significant; *p-value <0.05; **p-value <0.01; ***p-value <0.005 – created with Excel

6. Discussion and outlook

Transcript detection of the target protein was unsuccessful, meaning that siRNA knockdown could not be confirmed. Technical limitations, such as an overloaded flow cell caused by multiplexing, could be the cause for the absence of read counts for the target transcript of the specific tight junction protein. *Insig2* was significantly downregulated in the siRNA-treated mouse group, the biological significance of this change remains unclear, and further testing is needed.

Further off-target analyses are planned for upcoming mouse experiments to rule out non-specific effects. To further increase sensitivity, cDNA amplification and alternative methods such as Illumina sequencing could be tested. In addition, assays are planned to precisely determine the possible effects of *Insig2* expression on liver function.

List of References

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[2] Primär sklerosierende Cholangitis (PSC): Chronisch entzündete Gallenwege. (2025). USZ Universitäts Spital Zürich. https://www.usz.ch/krankheit/psc/
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Figures

Figure 1 Loenders, M. (2025) Schematic representation of siRNA knockdown: As part of the RISC complex, the siRNA binds to the target mRNA, leading to its degradation and thereby preventing protein production. medi.
Figure 2 Loenders, M. (2025) Normalized counts for the nanopore naopore sequencing results. medi.