RNAi Mediated Knock Down of Prolyl Hydroxylase II in Liver Tissue Enhances Lactate Clearance in Mice

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RNAi Mediated Knock Down of Prolyl Hydroxylase II in Liver Tissue Enhances Lactate

Clearance in Mice

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Abstract

Patients with respiratory chain disorders can present with lactic acidosis (Jain et al., 2016). At present, mitochondrial diseases caused by mutant mtDNAs remain largely untreatable (Ogasawara, Nakada, & Hayashi, 2010). A proposed mechanism for reducing lactic acidosis in these patients is to target the expression of HIF prolyl hydroxylases (PHDs). In the presence of oxygen, hypoxia inducible factor α (HIFα) becomes prolyl hydroxylated by PHDs (Epstein et al., 2001), which results in ubiquitination and proteasomal degradation. However, under hypoxic conditions, where oxygen is not readily available, PHD activity is decreased. The result is HIFα stabilization and translocation to the nucleus, upon which HIFα activates multiple genes that promote glycolysis over oxidative metabolism (Balligand, Feron, & Dessy, 2009). In the human genome, PHD2 is encoded by *Egln1*. Here we designed an siRNAs that lead to the depletion of PHD2, by reducing *Egln1* expression. *Egln1* expression was reduced *in vitro* and *in vivo*. On day 7 and day 14 after a single dose of 20 mg/kg with an siRNA targeting *Egln1* in c57/BL6 mice demonstrated enhanced lactate clearance when submitted to a lactate challenge, demonstrating the potential of PHD2 reduction via siRNA-mediated knockdown to clear excess lactate production associated with mitochondrial disease.

Dedication

I dedicate this achievement to my family for without their love and support this would not have been possible. My parents Alan and Penny, for instilling in me the belief to follow my passions. My darling wife Kari, who always encourages me to do my best when I feel my worst. Her championing of my spirit has made this all possible and I would be lost without her. To my daughter Sophie and my son Henry. Although your journey's have just begun, you remind me every day to get lost in the wonder that is science. Seeing you learn how to experience the world from a blank slate has reinforced the magic of the biological world in my mind. Finding this magic in the daily experience is nothing short of breath taking.   To my friends that have helped my deal with the stress of this project, and my co-workers who are probably tired of hearing about it yet let me vent anyways. Finally, to patients. To the patient that is suffering with very little hope I dedicate this small effort as a stepping stone towards greater advancement of my skills and knowledge with a firm desire way to expand my ability to help improve your situation.

Acknowledgments

Science is not an individual pursuit. Like all good work this project was built on the shoulders of researchers who had come before me and in collaboration with researchers who helped me in the present. I would like to acknowledge the amazing researchers at Alnylam that have helped me develop this project. William Querbes who first toyed with this target before I joined Alnylam, Don Foster who completed some initial screening data. The entire group of chemists that synthesize oligonucleotides at Alnylam, they are industry leading. Fred Tremblay who helped develop the therapeutic hypothesis and mentored my work. Dhaval Oza, who spent hours in the lab alongside me helping to advance data collection. Rohan Degaonkar, for repeatedly answering my *in vitro* questions. Elane Fishilevich, who reminded me that I could write this, one small piece at a time, and for providing many edits. I look forward to this being the first of many collaborations

Lastly, to Amy Chan-Daniels. My advisor and colleague for the last 3, almost 4 years. Watching Amy work in the high paced world of biotech where delivering results in hyper speed is not the exception, but the reality, has been influential on my career. Speed is of the upmost importance in a situation where people need an innovative medicine for unmet medical need. The way Amy balances the demand to move science forward quickly, with a scientist code and ethics to generate quality data, is exactly why she is a great mentor. I am fortunate to have worked with her.

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Chapter I

Introduction

There are many pathways of metabolism that can be utilized to create cellular

energy. The respiratory chain utilizes four macromolecular complexes to ultimately drive

ATP synthesis. Glycolysis is a method of respiration that utilizes glucose metabolism. In

the absence of oxygen, gluconeogenesis is the preferred pathway of metabolism.

Regulation of these pathways is controlled by hypoxia inducible factor α (HIFα). HIFα

becomes prolyl hydroxylated by HIF prolyl hydroxylases (PHDs), which results in

ubiquitination and proteasomal degradation. However, under hypoxic conditions, where

oxygen is not readily available, PHD activity is decreased. In instances of mitochondrial

disease, perturbation of normal metabolism can cause the buildup of lactic acid. Under

normal hypoxic conditions the lactic acid is utilized by the Cori cycle. In instances of

mitochondrial disease where normal oxygen is present, siRNA targeting PHD's may help

drive a hypoxic response, clearing the pathogenic build-up of lactate through up-

regulation of gluconeogenesis.

Mitochondria

Mitochondria are central to energy production in eukaryotic cells. Interestingly,

mitochondria's key feature, the oxygen consuming respiratory chain, is significantly

more efficient than an independent eukaryotic cell (Rich, 2003). Common consensus tells

us that the eukaryotic cells were invaded more than a billion years ago by a bacterium

containing this respiratory chain. After millions of years, this genetic information has

transferred to the nucleus, rendering these once independent bacteria as manifest

mitochondrial organelles in eukaryotic cells (Rich, 2003). This organelle has been

extensively studied; Peter Mitchell won a Nobel Prize in 1978 for his chemiosmotic

proposal, *i.e.,* the coupling of biological electron transfer to ATP synthesis. Mitchell

demonstrated that protons moving across the impermeable inner mitochondrial

membrane are linked to electron transfer and the creation of energy potential (Mitchell &

Moyle, 1967).

The respiratory chain

The respiratory chain (RC) is a pivotal process in the creation of cellular energy

which is utilized across the mitochondrial membrane. As reviewed by (Vafai & Mootha,

2012) (Koopman , Willems , & Smeitink 2012; Rich, 2003) the RC is defined as four

macromolecular complexes that catalyze electron transfer from reducing equivalents,

which are derived from the intermediary metabolism to molecular oxygen (Vafai &

Mootha, 2012). Complex I facilitates electron transfer from nicotinamide adenine

dinucleotide (NADH), and Complex II facilitates electron transfer from flavin adenine

dinucleotide (FADH$_2$). Electrons are the passed on to the mobile electron carrier

coenzyme Q. Other biological mechanisms can also transfer electrons to Coenzyme Q,

including *de novo* pyrimidine biosynthesis, amino acid oxidation, choline oxidation, and

glycolysis. Complex III is an adaptor that receives two electrons from reduced coenzyme

Q and funnels individual electrons to cytochrome *c*. Complex IV ends the RC by

accepting electrons from cytochrome *c* and using them to fully reduce oxygen to water

(Vafai & Mootha, 2012). The energy released by electron transport is used at complexes

I, III, and IV to expel protons from the mitochondrial matrix, thus establishing a proton

gradient (Koopman et al., 2012). This energy is subsequently used by complex V to drive ATP synthesis, which ultimately result in the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate. Enzymes that maintain cellular function can utilize ATP hydrolysis to release energy that is stored in ATP (Rich, 2003).

Comprehension of the proteins involved in the complex processes of the mitochondria, as well as how these proteins relate to human disease is essential. Mitochondrial proteome analysis (Pagliarini et al., 2008) demonstrates approximately 1000 genes encoding mitochondria. At present, over 150 gene mutations encoded by mitochondrial genomes have been identified as disease causing (Jain et al., 2016). These mutations are characterized, however the extensive heterogeneity of phenotypes presented in disease states renders direct correlation of mutations to phenotype difficult. For example, patients with respiratory chain disorders can present with a range of symptoms that includes blindness, deafness, gray or white matter brain disease, cardiomyopathy, skeletal muscle myopathy, anemia, ataxia, liver disease and kidney disease (Jain et al., 2016). Despite our knowledge of the genetic perturbations associated with disease, mitochondrial diseases caused by mutant mtDNA's remain largely untreatable (Ogasawara et al., 2010). Little is known of the actual epidemiological burden of mitochondrial disease. Individual mutations are considered rare, however, mitochondrial disease as a whole can present in 1 out of 4300 births (Gorman et al., 2015).

Glycolysis

In addition to the RC, glucose metabolism through the glycolysis pathway is a method of respiration that the cell can utilize to produce ATP. This pathway's mechanism was elucidated by the 1940s, and is comprised of ten steps, each catalyzed by a specific enzyme, including hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase, triosephosphate isomerase (TPI), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase, and pyruvate kinase (PK) (Otto Meyerhof, 1945; O. Meyerhof & Junowicz-Kocholaty, 1943; Otto Meyerhof & Oesper, 1947).   This pathway results in the production of 2 ATP and pyruvate. Pyruvate is then utilized in mitochondria via the TriCarboxylic Acid (TCA) cycle. The TCA cycle, also known as the Krebs cycle (Krebs, 1940), utilizes pyruvate to produce Acetyl-CoA, which is fully oxidized, resulting in the production of an additional 36 ATP molecules. One glucose molecule utilizing glycolysis and the TCA cycle is able to generate 38 total ATPs during oxidation. This glucose metabolism requires oxygen and is thus known as aerobic respiration.

Gluconeogenesis

Metabolism can still occur even in the absence of oxygen. Lack of oxygen drives pyruvate to be converted to lactate. This takes place in the muscle. The lactate is secreted by the muscle cells and enters the blood. This circulating lactate is taken up by the liver by monocarboxylate transporters (MCTs), specifically MCT2. MCT1 fulfils the role of actively secreting lactic acid from skeletal muscle. MCT2 (*Slc16a7*) has a high affinity for lactate and is directly involved in the liver uptake (Halestrap & Meredith, 2004). Once the lactate has entered the liver, it is utilized in the Cori cycle(Waterhouse & Keilson,

1969). During this part of the Cori cycle which takes place in the liver, lactate is converted into pyruvate by lactate dehydrogenase A. (LDHA). How this pyruvate is then ultimately converted into glucose depends on the metabolic conditions. Under aerobic conditions, pyruvate is converted to acetyl COA, a component of the TCA by pyruvate dehydrogenase (PHD). This enzyme is regulated by PDH Kinase 1 (PDK1). PDK1 is induced by hypoxia inducible factor (HIF) and negatively regulates the activity of PDH by phosphorylation(Kikuchi, Minamishima, & Nakayama, 2014). The above metabolism is down regulated under hypoxic conditions. Under hypoxia, gluconeogenesis converts the pyruvate to glucose that is then secreted back into the blood. Summarized, pyruvate is imported into the mitochondria converted to oxaloacetate (OAA) by pyruvate carboxylase (PC). OAA is converted to malate by mitochondrial malate dehydrogenase 2 (MDH2). Malate is then exported form the mitochondria and converted back to OAA by cytoplasmic malate dehydrogenase 1 (MDH1). This cytoplasmic OAA is then converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (ATP). The final stage of gluconeogenesis involves the dephosphorylation G6p and the excretion of the resulting glucose via the glucose transporter GLUT2 into the blood stream(Halestrap & Meredith, 2004; Karim, Adams, & Lalor, 2012; Kikuchi et al., 2014; Suhara et al., 2015). In instances of mitochondrial mutations, ineffective or nonfunctioning aspects of the metabolic pathways involved in aerobic respiration can subsequently lead to lactic acidemia (chronic buildup of lactic acid), a mitochondrial disease caused by mutant mitochondrial DNA (Larsson & Clayton, 1995). It is possible that this chronic lactic acidosis is itself pathogenic in terms of the progression of mitochondrial disease phenotypes (Merante et al., 1993; Stacpoole, Barnes, Hurbanis, Cannon, & Kerr, 1997;

Thompson Legault et al.). In healthy anaerobic metabolism that is unrelated to

mitochondrial dysfunction, but rather due to the specific cells' need to produce energy in

low oxygen environments, pathways can activate to clear any lactate that is produced. In

cells with mitochondrial dysfunction, it is well established that chronic over-production

of lactate transpires (Wallace, 1999). When mutations to proteins in the RC occur,

pyruvate is inadequately utilized at the level of re-oxidation of reduced cofactors. The

glycolytic pathway is accelerated to produce ATP without mitochondrial respiration.

Thus, the inhibition of accelerated lactic production, or enhanced lactate clearance, is a

conceivable treatment strategy in patients with mitochondrial disease (Ogasawara et al.,

2010).

Hypoxia inducible factor

Organisms in non-disease states have utilized various molecular mechanism to

compensate with for changing oxygen states that result in lactate production. In the

presence of oxygen, hypoxia inducible factor α (HIFα) becomes prolyl hydroxylated by

HIF prolyl hydroxylases (PHDs) (Epstein et al., 2001), which results in ubiquitination

and proteasomal degradation. However, under hypoxic conditions, where oxygen is not

readily available, PHD activity is decreased. With this decreased enzymatic activity, the

degradation of HIFα is perturbed. The result is HIFα stabilization and translocation to the

nucleus, upon which HIFα activates multiple genes that promote glycolysis over

oxidative metabolism (Balligand et al., 2009). One such gene is Glut2. This Glucose

transporter is up regulated in hypoxic states and increased cell membrane Glut2

expression increases glucose transport, which is necessary from the metabolic changes

hypoxia induces and has been directly correlated with HIF1α expression (Fujino et al.,

2016). Similarly, Pdk1 expression has been correlated with inhibition of PHD2 as an

indication of Cori cycle activation (Suhara et al., 2015; Zhang, Behrooz, & Ismail-Beigi,

1999). Studies have demonstrated that this response, presumably mediated through PHD

inactivation, may provide a protective effect in settings of mitochondrial dysregulation

and increased lactate (Jain et al., 2016).

Therapeutic hypothesis

Inactivation of PHD2 by reducing *Egln1* mRNA expression represents a potential

therapeutic approach in the treatment of mitochondrial disease. Of key interest would be

to determine the impact of PHD2 reduction on lactate clearance, and its resulting

implications under disease conditions. It has been shown in PHD2 knockout (KO) mice

that inactivation of PHD2 results in a decrease in blood lactate levels (Suhara et al.,

2015). Suhara et al. observed decreased blood lactate levels and enhanced lactate

clearance in these PHD2 KO mice at rest and after exercise. Activation of the Cori cycle

increased gluconeogenesis from lactate. This utilization of lactate correlated with a

drastic improvement in survival under lactic acidosis conditions in the liver specific

PHD2 KO mice as compared to that observed in naïve animals. Moreover, there is

evidence that in addition to liver specific knockdown (KD) of PHD2, muscle specific

reduction of PHD2 may also lower lactate levels (Mason et al., 2004). Under non-

disease conditions, hypoxic pathways should remain inactivated when regular

metabolism is favored. However, in mitochondrial disease states that result in lactic

acidosis, up-regulation of pathways designed to manage excess lactate may be beneficial.

In a state of mitochondrial dysfunction, activation of hypoxia pathways may help clear

excessive lactate levels and alleviate damage caused by lactic acidosis. My goal is to

build upon the findings of the highlighted studies and demonstrate the potential of PHD2 reduction via siRNA-mediated knockdown of *Egln1* to clear excess lactate production associated with mitochondrial disease.

RNAi as a therapeutic tool

The ability to selectively suppress disease-causing genes via RNAi has emerged as a novel and highly promising therapeutic approach for the treatment of genetic, metabolic, infectious and malignant diseases (Sehgal, Vaishnaw, & Fitzgerald, 2013; Vaishnaw et al., 2010). RNA interference is a natural biological process by which a short interfering RNA (siRNA) that is double stranded can direct sequence-specific degradation of messenger RNA (mRNA), leading to reduction of the synthesis of the corresponding protein (Fire et al., 1998). This mechanism can be utilized to target the expression of genes associated with disease. This process as reviewed in (Dana et al., 2017) can be summarized as follows: processing and cleavage of double-stranded RNA into siRNA with a 2 nucleotide overhang on the 3' end of each strand occurs via a processing enzyme called Dicer (Bernstein, Caudy, Hammond, & Hannon, 2001; Kim et al., 2004; Nishina et al., 2008). These siRNAs then associate with a multiprotein complex known as RISC (RNA induced silencing complex) (Chen et al., 2008; Hammond, Bernstein, Beach, & Hannon, 2000). Upon RISC incorporation, the two strands of the siRNA become separated, thus allowing the antisense strand to bind the complementary sequence in the target mRNA. The sense strand is degraded after strand separation. After pairing with the antisense strand of the siRNA, the target mRNA is precisely cleaved by the argonaute-2 (Ago2) endonuclease within the RISC/siRNA enzyme complex, thereby preventing synthesis of the corresponding target protein (Liu

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et al., 2004; Meister et al., 2004; Zamore, Tuschl, Sharp, & Bartel, 2000). siRNA targeting a specific gene can be conjugated with β-D-galactose or N-acetylglactosamine (GalNAc) allowing for subcutaneous injections of siRNA oligonucleotides and selective delivery to liver cells utilizing the hepatic asialoglycoprotein receptor (ASGPR) affinity for GalNAc (Zimmermann et al., 2017). Herein, I describe the application of RNAi technology to explore the relationship between HIFα stabilization, and the regulation of lactate clearance, through targeted knockdown of hepatic *Egln1* gene expression.

Chapter II

Materials and Methods

siRNAs were designed based on the target *Egln1*. Once created, an *in vitro*
evaluation was conducted looking at knockdown efficacy in both human and mouse liver
cells via transfection and RT-qPCR. Once a suitable candidate was chosen, an *in vivo*
dose response and time course was conducted to establish a suitable dose and elucidate
duration of *Egln1* knockdown. A lactate challenge was optimized as a means for
increasing circulating lactate levels in naïve mice. Ultimately, the lactate challenge was
performed on mice that had received a dose of our siRNA.

siRNA Design

Egln1 siRNAs were designed using the reference nucleotide sequence
NM_022051.2 (National Center for Biotechnology Information Reference System). To
achieve specificity the 19-mer candidate sequences were subjected to a homology search
against the RefSeq mRNA database. Mismatches in the seed region, non-seed region, and
at the cleavage site were determined relative to all other transcripts in the data base.
Single-stranded RNA's were synthesized at Alnylam Pharmaceuticals on controlled pore
glass using standard phosphoroamidites obtained from Proligo Biochemie. Ion exchange
HPLC was used for deprotection and purification of the crude oligonucleotides. Yield
and concentration were determined by UV absorption at 260 nm. siRNA duplexes were
generated by annealing equimolar amounts of complementary strands in a water bath at
90ºC for 3 min followed by cooling at room temperature for several hours (Yasuda et al.,
2014).

In vitro evaluation of siRNAs targeting *Egln1*

Once the candidates were synthesized, they were evaluated for their ability to reduce the target gene expression in both human and mouse liver cells, cell lines Hep3B (Sigma Cat # 86062703) and Mouse Hepatocytes (Bioreclamation IVT, Lot #URX) respectively. This *in vitro* assay for target knock down started on Day 1 with the transfection using; Gibco by Lifetech 1X Opti- MEM media with reduced serum (Cat #31985-062), Invitrogen Lipofectamine RNAi max (Cat #13778-150 , 150 mLs), 384 Biocoat Corning plate precoated with collagen (Cat # 354666), Integra multichannel pipettor, Viaflo384, Benchtop Centrifuge, 37C incubator, Gibco by lifetech 1X Williams media(- L-glutamine, -phenol red) (Cat # A12176-01, 500 ml). The Williams media was prepared ahead by adding 50 ml of Fetal bovine serum. On Day 2 the cells were lysed. mRNA was isolated from the cell lysate, with the aid of Dynabeads (Lifetech dynabeads oligodt25 Cat # 10902D).

RNA isolation and RT-qPCR evaluation of target mRNA

Tissue isolation was performed using RNeasy Mini Kit (Qiagen Cat # 74106). Once RNA was isolated it was quantified on a Nano Drop and diluted in DI water to a concentration of 20 ng/µL. 20 ng of RNA was added to Applied Biosystems high capacity cDNA reverse transcription kit. qPCR to analyze the mRNA expression of the target gene *Egln1* was performed utilizing the resulting cDNA. Gene expression was evaluated utilizing TaqMan probes and primers. *Egln1* (assay ID Mm00459770_m1), *Egln2* (assay ID #Mm00519067_m1), *Glut2* (assay ID Mm00446229_m1), *Pdk1* (assay ID Mm00554300_m1), *Gapdh* (assay ID Mm99999915_g1) and TaqMan universal PCR

master mix (cat#4304437). On a Roche Lightcycler 480. Relative transcript levels were calculated using ddCT method, with *Gapdh* transcript as a reference.

<center>IC$_{50}$</center>

The IC$_{50}$ experiment followed the above described transfection assay and was performed in mouse hepatocytes. This time the siRNA was transfected 4 times in replicate, with a starting concentration was 10 nM going down 6-fold to generate 8 points. Plot generated utilizing XL Fit software.

<center>*In vivo* dose response and time-course of siRNA response</center>

c57/Bl6 mice were dosed subcutaneously with 0.3, 1, 2, and10 mg/kg of siRNA diluted in 1X PBS, in groups of three, with one group receiving PBS. Ten days after the dose, the animals were sacrificed, and their livers collected and flash frozen. Tissue was ground and RNA isolated, and RT-qPCR was performed to evaluated expression of the target gene utilizing previously described methods.

<center>*In vivo* time course</center>

Once a dose of 10 mg/kg was selected from the dose response data, a time course experiment was performed. c57/Bl6 mice were dosed subcutaneously with 10 mg/ml of our candidate in groups of three with one group receiving PBS. One group received an additional dose at day fourteen. Animals were then sacrificed on Days 3, 7, 10, 14, 21, and 28. At each time point, 3 PBS treated animals were also sacrificed. The liver was flash frozen. RNA was isolated the levels of *Egln1* were evaluated by RT-qPCR.

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Lactate challenge

To evaluate an increase in lactose levels *in vivo*, we performed lactose challenge experiments. Lactate challenge experiments were developed and described by (E, Lu, Selfridge, Burns, & Swerdlow, 2013; Summermatter, Santos, Pérez-Schindler, & Handschin, 2013). Briefly summarized as follows: a solution of 200 mg/ml sodium lactate with a pH of 7.41, (Sigma USP grade, Cat 71718) dissolved in sterile phosphate buffered saline was administered at 2 g/kg by intraperitoneal injection, following AF-005 Alnylam Animal Facility protocol. Blood samples were obtained by tail nick/transection section following at intervals of 10 mins and analyzed for lactate levels using a lactometer (Nova Biomedical). The lactometer requires 0.7 µL of blood to analyze a sample, which eliminates the risk of exceeding recommended daily blood volume collection limits.

Efficacy in the lactate challenge

Based on in-house optimization Lactate challenge was performed under the following conditions. C57/Bl6 mice were dosed subcutaneously with the siRNA1 at 10 mg/kg and 20 mg/kg with an N=5. A control group received PBS. Lactate challenge was performed 7 days and 14 days post dose. Lactate readings were recorded every 10 mins for the first hour and then once at two hours.   2-way Anova, Bonferroni's multiple comparisons test was performed.

Chapter III

Results

To target *Egln1*, which encodes PHD2 protein, siRNAs were designed and

synthesized as described in the methods. An *in vitro* screen in primary mouse hepatocytes

(**Table 1**) identified at least three siRNAs that lowered *Egln1* mRNA by 50% or more.

Table 1. in vitro screen of Egln1 siRNAs. *Mouse Primary hepatocytes were transfected with siRNA1-18 at 10nM and 0.1nM. Table shows percent of mRNA remaining and standard deviation at 24 hours post-transfection measured by qPCR N=4 replicate transfections.*

Cell Line	Primary Mouse Hepatocytes			
Condition	10nM Avg	10nM SD	0.1nM Avg	0.1nM SD
siRNA1	2.04	0.68	18.73	6.30
siRNA2	26.55	2.79	51.83	12.67
siRNA3	2.73	1.00	40.98	3.89
siRNA4	4.45	0.65	41.43	6.87
siRNA5	4.28	2.08	50.10	7.53
siRNA6	25.13	1.57	57.67	8.93
siRNA7	4.04	1.35	77.25	10.01
siRNA8	89.54	11.82	95.25	12.62
siRNA9	5.43	1.65	59.90	17.40
siRNA10	10.59	1.44	88.12	9.96
siRNA11	105.56	9.98	97.81	17.54
siRNA12	31.20	6.48	64.33	11.05
siRNA13	37.52	10.09	98.79	14.48
siRNA14	10.36	1.78	89.76	15.72
siRNA15	3.95	2.69	40.70	9.67
siRNA16	4.60	2.18	99.75	10.43
siRNA17	7.01	1.06	95.74	20.23
siRNA18	8.91	1.38	102.19	24.98

After transfection at 10 nM and 0.2 nM in human liver cells (Hep3B), siRNA1, 2, and 3 lowered the *Egln1* mRNA levels in a dose-dependent manner. All three siRNAs reduced *Egln1* mRNA by greater than 60% at 10 nM and greater than 40% at 0.1 nM **(Figure 1A)**. Similarly to results in Hep3B cells, all three siRNAs showed dose-dependent knockdown in primary hepatocytes **(Figure 1B)**. siRNA1 and 2 demonstrated greater than 90% reduction in *Egln1* mRNA levels. siRNA3 reduced EGLN1 mRNA by 75%. At the lower dose of 0.1nM, siRNA1 was the superior siRNA and demonstrated greater than 80% reduction of the target mRNA. SCR siRNA, not sequence specific to target *Egln1*, did not reduce *Egln1* mRNA levels (data not shown).

To confirm its potency, the half maximal inhibitory concentration (IC_{50}) was approximated using serial dilutions of the siRNA1 and calculated to be 0.0249 nM. Subsequently, siRNA1 was selected as the lead candidate and utilized in further experiments.

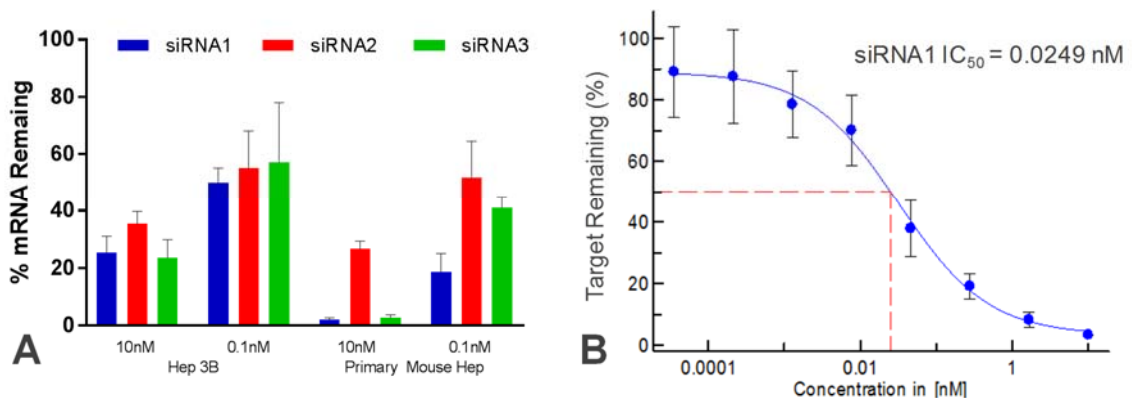Figure 1. siRNA *In vitro* Screen identifies potent siRNAs targeting *Egln1*

A. Hep3B, human liver cells and mouse primary hepatocytes were transfected with siRNA1, 2, and 3 at 10 nM and 0.1 nM. Plot shows percent of mRNA remaining (+/- standard deviation) at 24 hrs post transfection as measured by qPCR. N = 4 replicate transfections. B. Dose response curve for siRNA1 at 6 dilutions that ranged from 100 nM

to 10^{-4} nM. The half maximal inhibitory concentration (IC$_{50}$) for siRNA1 was calculated via XL Fit software and was approximated as 0.0249 mM.

Following on from *in vitro* efficacy, it was important to determine an appropriate dose for siRNA1 *in vivo*. A dose response experiment that introduced GalNAc-conjugated siRNA subcutaneously was conducted with a day 10 evaluation of *Egln1* mRNA reduction in mouse liver. Lowering of *Egln1* mRNA expression was observed in all treatment groups. siRNA1 demonstrated 40%, 70%, 80%, and 90% knockdown of the target mRNA at 0.3, 1, 3, and 10 mg/ml respectively. At 10 mg/ml, siRNA1 showed 90% knockdown of *Egln1* in mice; this dose was selected for the subsequent *in vivo* experiments.
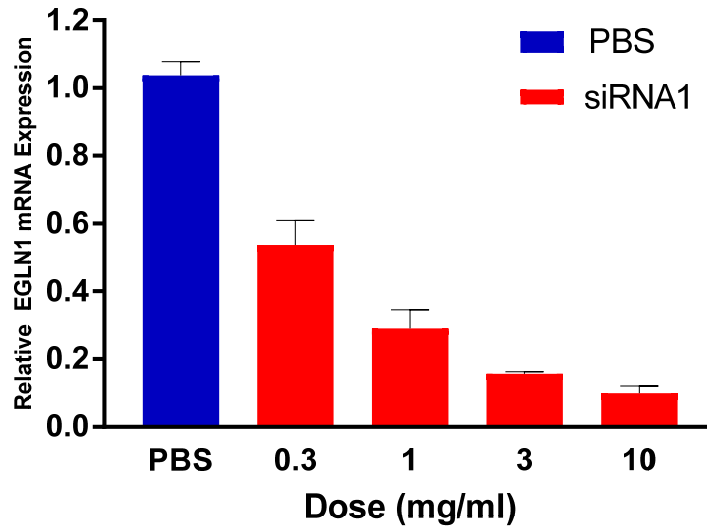

Figure 2. Dose Response for *Egln1* siRNA1 in mice

C57/BL6 mice (n=3) were dosed with siRNA subcutaneously at various dose concentrations; 0.3, 1, 3, 10 mg/ml with an additional group (n=3) dosed with PBS as a control. At Day 10 liver was harvested and Egln1 mRNA expression was determined

relative to the PBS control group. Levels of Egln1transcript in mouse livers (ΔΔC^T), relative to PBS-injected control, are plotted on the Y-axis (+/- SEM).

A time course experiment was conducted look at the ability of siRNA1 to knock down *Egln1* mRNA up to 28 days after a single injection **(Figure 3A)**. An additional group was dosed twice in one month (Day 0 and Day 14), to determine whether there would be an additive effect on *Egln1* silencing. *Egln1* mRNA was reduced maximally by 80% at Day 3, following a single dose administration, which was maintained throughout the 28 day time course. There was no additive effect observed on *Elgn1* mRNA reduction following administration of two doses on Days 0 and 14 (see group 28(2) in **Figure 3A**).
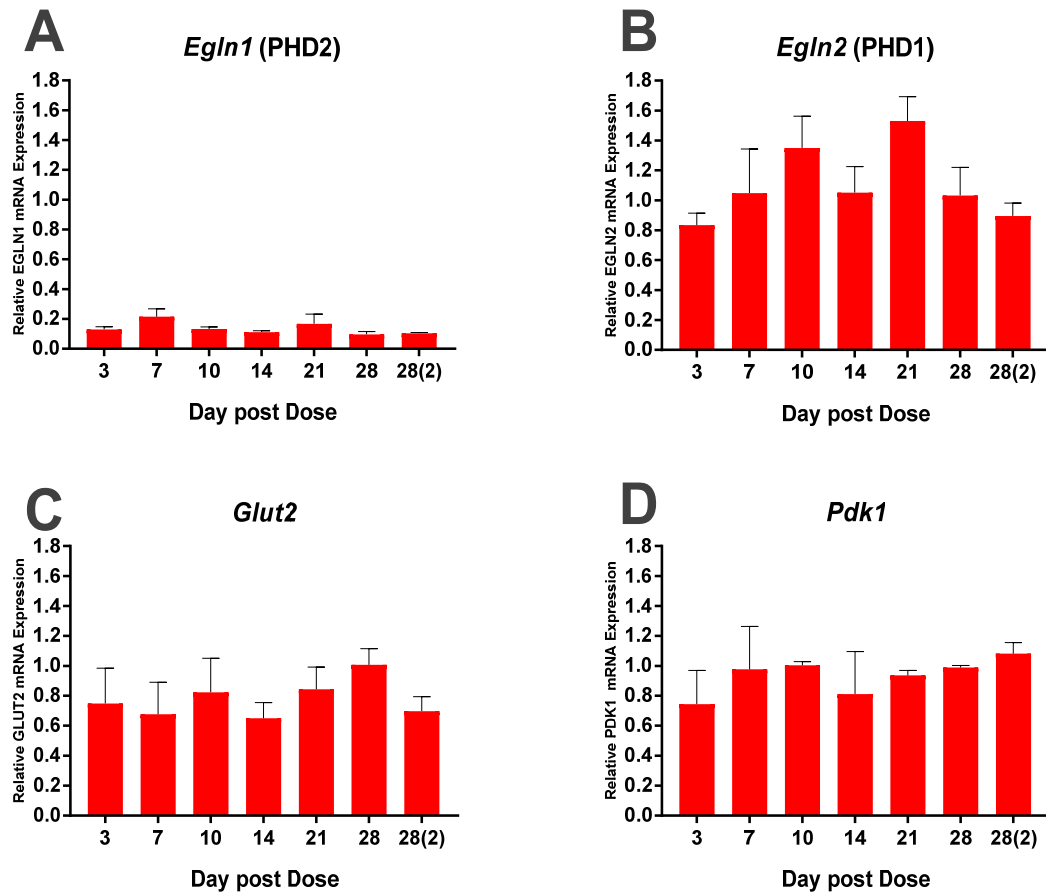
Figure 3. siRNA1 Efficacy *In vivo* Time Course

Relative expression of (A) Egln1, (B) Egln2, which encodes PHD2, (C) Glut2, and (D) Pdk1 transcripts in C57/BL6 mice (N=3 per time-point) that were dosed with Egln1 siRNA1 at 10 mg/kg subcutaneously. Livers were harvested at days 3, 7, 10, 14, 21, 28. Mice were also dosed with PBS (N=3) per time-point. In addition, one group, 28(2), received an additional dose of siRNA1 at 10 mg/kg subcutaneously at day 14, and then liver was harvested at day 28.

In addition to monitoring *Egln1* silencing, we also measured messenger levels of

Egln2, *Glut2*, and *Pdk1*. *Egln2* mRNA (not a direct target of siRNA1) was minimally

reduced at day 3. An increase in of *Egln2* mRNA was observed through the time course,

peaking at day 21 with an 80% increase, suggesting upregulation as a result of *Egln1*

reduction **(Figure 3B)**. *Glut2* was slightly reduced by 10-30% throughout the time

course, peaking at day 14. Although *Glut2* levels returned to baseline at day 28 following a single dose, a 20% reduction was observed in the group that received two doses **(Figure 3C)**. A slight reduction of *Pdk1* was observed at day 3 but remained at baseline levels throughout the time course **(Figure 3D)**. Although no enhanced reduction was observed on the collective group of targets after two doses, the changes in *Glut2* and *Egln2* suggested that there was a modest impact on the hypoxia pathway by day 14, which rapidly subsides thereafter. Consequently, days 7 and 14 post-single dose of siRNA1 were selected as the timepoints to evaluate the ability of *Egln1* silencing to enhance increased blood lactate clearance.
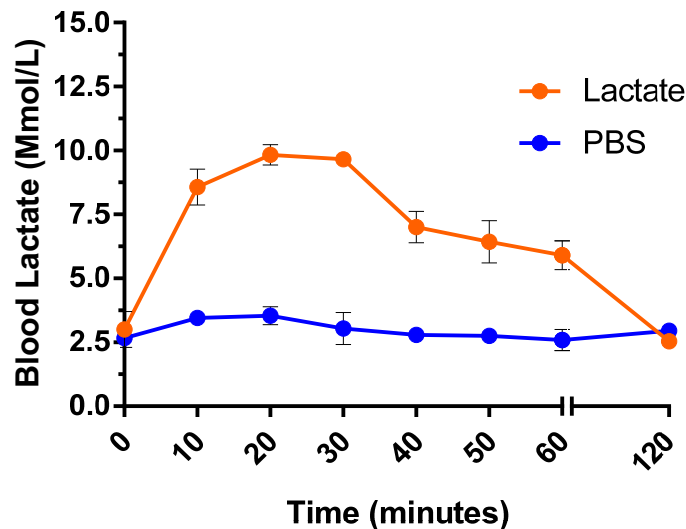

Figure 4. Lactate Challenge Baseline

Blood lactate levels (mM/L) measured with a lactometer every 10 mins for the first hour and then again at 2 hours after sodium lactate administration. C57/BL6 mice were split into two groups (N=3) and either 200 mg/mL sodium lactate with a pH of 7.41 dissolved in sterile PBS or PBS was administered at 2 g/kg by intraperitoneal injection.

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To establish a test system where we could safely increase blood lactate, we performed a blood lactate baseline experiment (**Figure 4**). After 10 minutes, mice dosed with lactate demonstrated an increase from baseline levels of blood lactate of about 2.5 mM/L to 8 mM/L. Mouse blood lactate remained elevated through 60 minutes, returning to baseline by 120 minutes post-lactate administration.
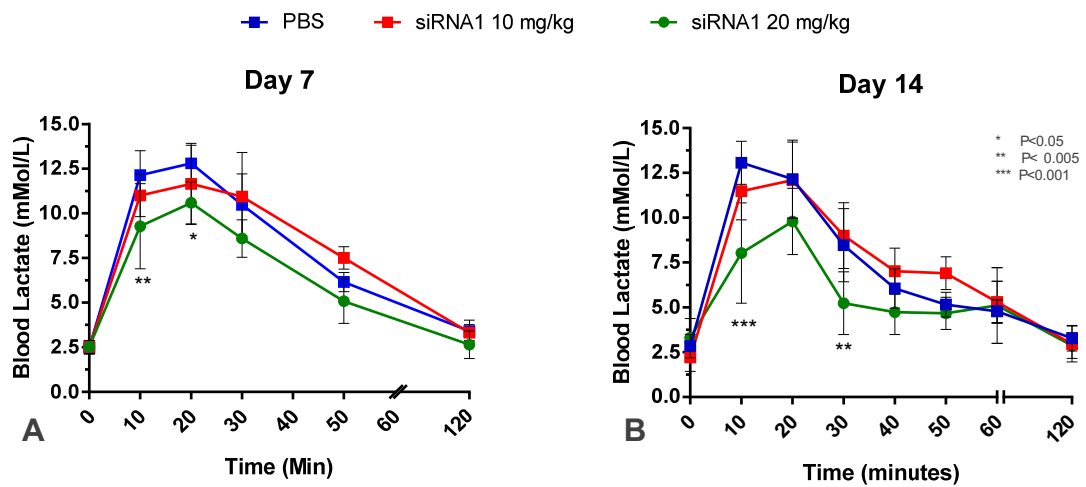
Figure 5. Single Dose Time Course Lactate Challenge

Mouse blood lactate levels after single subcutaneous administration of PBS, Egln1 siRNA1 at 10 or 20 mg/kg. c57/BL6 mice were subjected to a lactate challenge at Day 7 (A) and Day 14 (B) post dose (N=5) 2way ANOVA with Bonferroni's multiple comparisons test.

Having demonstrated the ability to safely increase blood lactate with lactate administration, we proceeded to design the lactate challenge experiment **(Figure 5)**. Due to the dose dependent performance of *Egln1* siRNA1, and the limited ability to impact hypoxia pathway genes observed in our time-course study, we decided to evaluate an additional high dose of 20 mg/kg in the lactate challenge experiment. At 10 mg/kg *Egln1*

siRNA1 did not demonstrate any significant reduction of peak lactate levels when compared to the PBS control. However, mice dosed with *Egln1* siRNA1 at 20 mg/kg demonstrated lower levels of peak lactate at Day 7 and Day 14 **(Figure 5)**. Furthermore, lactate-challenged mice demonstrated faster clearance of lactate by 30 mins, at the Day 14 timepoint when compared to PBS controls.

Chapter IV

Discussion

Under non-disease states, hypoxic pathways should remain inactivated when regular metabolism is favored. However, in mitochondrial disease states that result in lactic acidosis, up-regulation of pathways designed to manage excess lactate may be beneficial. In a state of mitochondrial dysfunction, activation of hypoxia pathways may help clear excessive lactate levels and alleviate damage caused by lactic acidosis. RNA interference (RNAi) is a naturally occurring biological process by which small interfering RNA (siRNA) can direct sequence-specific degradation of mRNA, leading to inhibition of synthesis of the corresponding protein (Elbashir et al., 2001; Fire et al., 1998). The ability to selectively suppress disease-causing genes via RNAi has emerged as a novel and highly promising therapeutic approach for the treatment of genetic, metabolic, infectious and malignant diseases. To date, mutations in over 150 genes, encoded by mitochondrial genomes, have been identified as disease causing (Jain et al., 2016). Patients with respiratory chain disorders can present with blindness, deafness, gray or white matter brain disease, cardiomyopathy, skeletal muscle myopathy, anemia, ataxia, liver disease and kidney disease(Jain et al., 2016). At present, mitochondrial diseases caused by mutant mtDNA's remain largely untreatable (Ogasawara et al., 2010). Due to the large number of mutations associated with this disease state, targeting each mutation with an siRNA would be unlikely to result in broad improvements in disease state. However, by enhancing the clearance of the lactic acid, a single siRNA has the ability to reduce the lactic acidosis state and potentially reduce symptoms associated with this pathology.

In the present study we took the approach of developing an siRNA to lower *Egln1* and then utilized this siRNA to explore the ability of *Egln1* reduction to enhance lactate clearance. Our initial *in vitro* screen **(Table 1)** demonstrated the power of the power of siRNA technology and its ability to knock down our target, *Egln1*. All 18 of the siRNAs tested showed some efficacy in primary mouse hepatocytes. To further distinguish the best siRNA to use in mouse experiments we then compared the silencing efficacy in Hep3b cells. By also exploring efficacy in a human cell line we were able to generate data with an siRNA whose potential would not just be limited to murine experiments. The subsequent *in vitro* screen identified a highly potent and specific siRNA against *Egln1* mRNA, designated as siRNA1. siRNA1 ablated *Egln1* mRNA almost completely in primary mouse hepatocytes **(Figure 1)**. While knockdown in cell lines such as primary mouse hepatocytes may give good indication on in vivo activity, not all siRNAs that are potent *in vitro* may translate to the mouse. We therefore set about to demonstrate *in vivo* silencing. *In vivo* evaluation of this siRNA1 demonstrated robust liver *Egln1* mRNA reduction of 80% by day 10 following a single dose of 10 mg/kg **(Figure 2)**. This level of mRNA reduction replicates other liver-targeted gene silencing that utilized GalNAc conjugated siRNAs (Butler et al., 2016). The follow-up time course evaluation demonstrated that onset of silencing was rapid (by day 3) with prolonged duration of action (28 days) **(Figure 3A)**.

In mouse models of mitochondrial disease, hypoxia has been shown to increase life span (Jain et al., 2016). HIF transcriptional activity drives the cellular respiration away from conventional mitochondrial oxidative energy metabolism. One alternative pathway of metabolism is utilization of lactate in the Cori Cycle. With the rapid

inhibition of *Egln1*, we hoped to stimulate this metabolic response and use it to drive the clearance of excess lactate. The activation of the Cori cycle has previously been observed in liver-specific PHD2 KO mice, suggesting a direct link between *Egln1* reduction and activation of this metabolism (Suhara et al., 2015).

Changes in the markers for Cori cycle activation (increases in *Glut2)* and hypoxia pathway activation (increase in *Pdk1)* were not observed during our time course experiment **(Figure 3)**. This would suggest that despite substantial knockdown of *Egln1,* the activation of hypoxic genes may require near-complete ablation of *Egln1* (>80%). Interestingly, we observed an increase in *Egln2* expression in correlation with *Egln1* silencing. It could be that this *Egln2* expression dampened the transcriptional activity of *Glut2* and *Pdk1*.

Although transcriptional changes were not observed in our target pathways during the time-course evaluation, the functional readout of enhanced lactate clearance was observed during the lactate challenge experiments. Upon more robust *Egln1* reduction, with and increased dose of siRNA1, enhanced lactate clearance was observed at days 7 and 14 post-dose **(Figure 4)**. Additional experiments would have to been performed to evaluate *Glut1* and *Pdk1* transcriptional activity in animals that received the higher dose. At this time, we don't have direct evidence that the higher dose of *Egln1* siRNA1 is sufficient to upregulate these genes. To gain a better understanding of this mechanism, it would be important to include additional genes in the analysis. One could focus on the enzymes responsible for driving key elements of the Cori cycle. When gluconeogenis from lactate occurs in the liver, it follows a path of lactate to pyruvate to glucose. To determine if lactate import and conversion to pyruvate is accelerated after treatment with

siRNA1, it would be important to examine the activation of monocarboxylate transporters and lactate dehydrogenase A (LDHA). Monocarboxylate transporter 2 (*Slc16a7*) has the highest affinity with lactate(Halestrap & Meredith, 2004) and is expected to be up-regulated with increasing transport of lactate into the liver. We would also expect to see up-regulation of LDHA in livers after treatment with high dose of siRNA1, indicating a higher conversion of lactate to pyruvate.

Following on from the observed enhanced clearance of lactate in the lactate challenge **(Figure 4)**, it would be important to take *Egln1* siRNA1 or an siRNA with similar or greater potency into mouse models of mitochondrial disease. Disruption of NDUFS4 gene, with codes for NADH:ubiquinone oxidoreductatse subunit S4 would be an interesting model to evaluate the ability of *Egln1* siRNA to enhance lactate clearance and rescue the disease phenotype. *Ndufs4* mice mimic the complex I deficiency, which has is the most frequent cause of leigh syndrome, a pediatric mitochondrial disease(Kruse et al., 2008). This model demonstrates increased levels of lactate that can be lowered after exposure to hypoxic conditions(Jain et al., 2016). The hypoxic conditions are created by limiting the percentage of oxygen in the mouse environment rather than driving hypoxic gene expression molecularly. The hypoxic conditions seemed to reduce the levels of lactate resulting in an increased lifespan. This improvement in lifespan linked to lactate reduction creates a potential opportunity to demonstrate therapeutic benefit with an *Egln1* siRNA treatment. While not performed in a disease model, the results of the current lactate challenge highlight an opportunity to apply siRNA-mediated transcript silencing under conditions of increased lactate levels, such as in mitochondrial disease states. The dose-dependent enhancement of lactate clearance (**Figure 5B**) and

the data from the PHD2KO mice suggest that the level of mRNA knock-down may be the most challenging hurdle to achieving activation of the hypoxic response. The sensitivity of the induction of hypoxic genes is not well understood. Previous data demonstrating activation of genes in mouse model utilized reduction of oxygen in the air to stimulate a response (Jain et al., 2016). There may be other mechanisms in place that regulate the hypoxic pathway, which may inhibit our ability to effectively induce hypoxic genes. Aerobic respiration is the more efficient system, and thus biologically it makes sense to skew any metabolism toward these aerobic pathways. Forcing a hypoxic response, especially in the presence of normal oxygen levels may only be possible with complete ablation of *Egln1* in the liver.

The poly mutational nature of mitochondrial disease and the variation in tissue manifestation suggests that it remains a very difficult target for historically used pharmaceutical interventions. The current treatments focus on vitamin cofactors (co-enzyme Q, α-lipoic acid, riboflavin, or L-carnitine (Parikh et al., 2009). However, to date, there remains no clinically effective treatment. While the reduction of lactate does not "fix" the causal mutation or improve mitochondrial function, it may prove a novel mechanism to prevent further system damage. The development of *Egln1/*PHD2-based treatment will need to consider the effects of driving gluconeogenesis through the Cori cycle long term, and whether the reduction of lactate would have only a transient effect on improving a patient's symptoms due to the rapid nature of the normal hypoxic response. Furthermore, it would be important to study how the reduction of lactate and increase of anaerobic metabolism would impact functional muscle. While type I muscle, dense in mitochondria, would likely experience the greatest impact in a state of

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mitochondrial dysfunction, it is not clear what the impact of reducing circulating lactate would have on the remodeling of muscle tissue. While many questions remain on the long-term viability of *Elgn1* siRNA treatment hypothesis, the ability to implement an RNAi-mediated approach to improve lactate clearance has broad implications on treatment of patients with mitochondrial disease.

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