

Functional Analysis of a Long Non-Coding RNA Associated with Lupus Protection

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ABSTRACT

A single-nucleotide polymorphism (SNP) in the complement receptor 2 (CR2) gene was previously identified in a large lupus association study to be associated with decreased risk of lupus and increased levels of a nearby long non-coding RNA (CR2 lncRNA). This project aims to further define the relationship between the CR2 lncRNA, CR1/CR2 expression, and mTOR, and possibly identify new mechanisms by which the CR2 lncRNA may confer protection against lupus and be utilized for future treatment studies. Knockdown of mTOR was performed on isolated primary B cells using previously developed siRNAs, and expression levels of mTOR and CR1 were assessed using qPCR. We also ran a standard curve qPCR protocol using known amounts of RNA template to assess our ability to accurately measure expression levels of CR2 and other lncRNAs of interest in anticipation for knocking down the CR2 lncRNA. Results showed successful knockdown of mTOR for one of the siRNAs at 1nM and 10nM concentrations, with knockdown efficiencies around 50%, in line with previous literature. The other two siRNAs showed no change in mTOR transcription. For CR1, no change was seen in expression levels for all knockdown samples. Additionally, while the CR2 standard curve showed satisfactory results, the assays for the CR2 lncRNA and other lncRNAs displayed high variability and poor efficiencies.

SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease that affects more than 1.5 million Americans, 90% of whom are women, with peak incidence between the ages of 15 and 45. As a chronic and lifelong disease, it has substantial morbidity and mortality, ranking among the top 20 leading causes of death in females between the ages of 5 and 64. In the last 50 years, only one drug has been developed for use in lupus, and more effective targeted therapies are desperately needed. One of the hallmarks of lupus is the production of autoantibodies to double-stranded DNA (dsDNA), which are key drivers in the pathogenesis of disease by virtue of their ability to form immune complexes that activate complement and cause organ damage that is cumulative over time. These antibodies increase prior to disease flare and are suppressed upon symptom resolution, suggesting that modifying their production will improve lupus outcomes.

BACKGROUND

The B Cell Complement Receptors

- Complement receptor 1 (CR1) and complement receptor 2 (CR2) on B cells play essential roles in the maintenance of tolerance to self-antigens (Figure 1). Without this regulation, autoantibodies can develop and may lead to SLE.
- Patients with SLE have been previously shown to have a 50% decrease in CR1 and CR2 protein levels

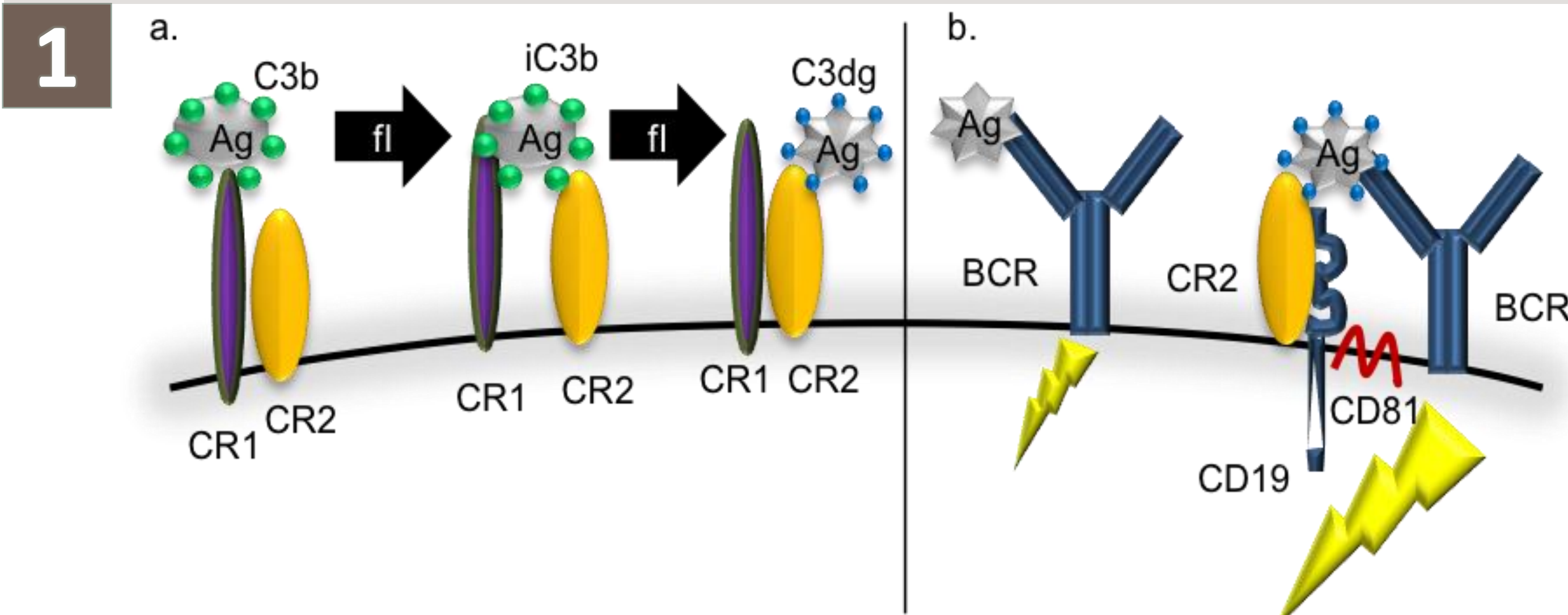


Figure 1: CR2 is found in two complexes on the B cell membrane: one containing CR1 (a) and a second containing CD19 and CD81 (b). CR1 binds C3b and acts as a cofactor for the factor I (fi)-mediated cleavage of C3b to iC3b and C3dg. C3dg is the specific ligand for CR2, which when coligated with the B cell receptor (BCR) lowers the threshold for B cell activation

CR2 lncRNA

- A single-nucleotide polymorphism (SNP) in the CR2 gene was identified in a large lupus association study to be associated with decreased risk of lupus and increased expression of CR1.
- The SNP is located within a putative B cell enhancer and is associated with increased levels of a nearby lncRNA (referred to as CR2 lncRNA)
- Previous work in our lab has also shown a positive correlation between CR1 expression and mTOR pathway activation in B cells, as well as increased mTOR pathway activation in the presence of the SNP

OBJECTIVES

We hypothesize that the CR2 lncRNA confers lupus protection by directly modulating the transcription of CR1 in B cells and subsequently promoting antigen-specific B cell tolerance via the mTOR pathway. To test this, we aimed to ultimately knockdown the CR2 lncRNA transcripts in primary B cells obtained from human subjects using small interfering RNA (siRNA), and subsequently measure the effects on CR1, CR2, and mTOR expression using RT-qPCR. Through this, we hope to identify new mechanisms by which the CR2 lncRNA may confer protection and be utilized for future lupus treatment studies.

METHODS

Optimizing Knockdown Procedure: mTOR Knockdown

Three different mTOR-siRNAs previously developed by Integrated DNA Technologies were independently introduced to SNP(-) primary B cells at three concentrations each (10 nM, 1 nM and 0.1 nM), along with a scrambled negative control siRNA that is absent in the human genome, using the Amaxa Nucleofector, an electroporation-based transfection method. RNA transcripts were harvested after a 24-hour incubation period, and expression of mTOR, CR1, and β -actin, an endogenous gene control, were assessed for each sample using qPCR. Relative expression levels of the knockdown samples and negative controls were evaluated using the comparative CT method.

Troubleshooting CR2 lncRNA Knockdown

We designed three CR2 lncRNA-siRNAs using the Integrated DNA Technologies' custom siRNA generator. The siRNAs were designed around a unique splice junction of the CR2 lncRNA as to avoid off-target effects on CR2 and other lncRNAs that are transcribed in the region (Figure 2). In order to confirm the absence of off-target effects, we needed to ensure that we could accurately measure expression levels of CR2 and other lncRNAs of interest via qPCR. To do this, we tested our qPCR assays for each transcript of interest using a standard curve qPCR protocol. Known amounts of RNA template obtained from primary B cells were tested in varying magnitudes, and qPCR efficiencies and Ct slopes were assessed for each assay.

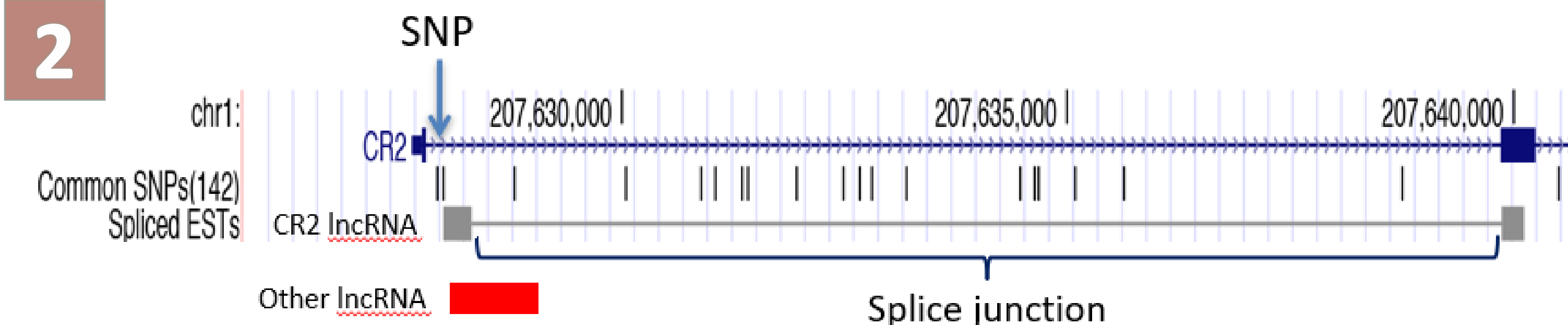


Figure 2: Gene map showing the location of the protective SNP and the regions coding for CR2, the CR2 lncRNA, and other lncRNA. The unique splice junction of the CR2 lncRNA, around which the siRNAs were designed, is labeled above. The splice junction is flanked by sequence coding for other lncRNAs on the left and sequence coding for CR2 exon 2 on the right.

RESULTS

mTOR Knockdown

mTOR siRNA 13.1 at 10 nM and 1 nM had knockdown efficiencies of 32% and 55%, respectively, in line with knockdown efficiencies of around 50% seen in transfected B cells from previous literature (28). All other samples either showed relatively no change in mTOR transcription or had increased levels of mTOR expression (Figure 3a). For CR1, no reduction was seen in expression levels for all samples (Figure 3b).

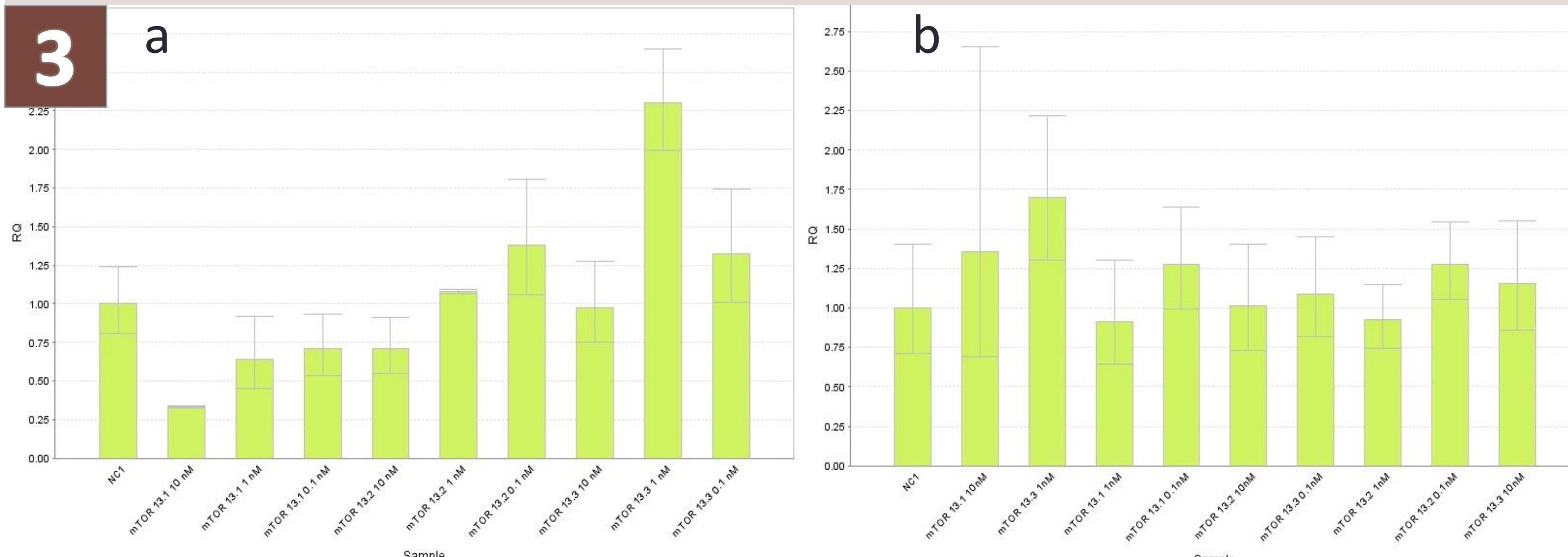


Figure 3: Relative expression levels of mTOR (left) and CR1 (right) following knockdown with three different mTOR siRNAs (13.1, 13.2, and 13.3) at three different concentrations (10 nM, 1 nM, and 0.1 nM) are shown above, as compared to a sample transfected with a negative control (labeled NC1).

CR2 and lncRNA Standard Curves

Only the CR2 assay showed satisfactory results with $R^2 > 0.99$ and slope of around -3.3 (the expected number of cycles between two dilutions). The assays for the CR2 lncRNA and a nearby lncRNA of interest had R^2 of 0.88 and 0.96, slopes of -4.3 and -3.5, and overall efficiencies of 70% and 93%, respectively.

LIMITATIONS

mTOR Knockdown

- Performed on only one subject that was negative for the SNP
- Primary B cells are notoriously difficult for achieving high transfection rates, limiting efficacy of siRNA knockdown methods

lncRNA Standard Curves

- Poor efficiencies were seen with the lncRNA standard curves
- Result may be due to a low copy number in the samples, particularly those at higher dilutions, resulting in stochastic error when measuring fold-changes.
- Other possible explanations include degraded assay primers, inadvertent introduction of RNases or DNases to samples, or primers needing to be redesigned entirely

CONCLUSIONS

mTOR Knockdown

- CR1 expression may not be dependent on mTOR activity
- More subjects and samples per subject are necessary to ensure the accuracy of the post-knockdown mTOR and CR1 expression levels and more thoroughly assess the efficacy of each mTOR siRNA

Path toward CR2 lncRNA Knockdown

- Further dissection into cause of poor efficiencies with lncRNA standard curves needs to be done
- Once lncRNAs can be accurately and reliably measured, we plan to test our CR2 lncRNA knockdown protocol on at least 5 individuals each with and without the protective SNP, and assess effects on expression of the CR2 lncRNA, mTOR, CR1/CR2, and other nearby lncRNAs
- If the CR2 lncRNA knockdown is successful, we will also perform RNA-sequencing on the samples to assess effects on global transcription
- If knockdown is unsuccessful or off-target effects are seen, we will pursue guided gapmers as an alternate method for inhibition

Looking Ahead

- Utilizing CRISPR-Cas9-mediated genome editing to generate double-strand DNA breaks upstream and downstream of the lncRNA exon to study effects on CR1 activity
- Further assessment of CR2 lncRNA tissue distribution and subcellular location
- Correlating CR2 lncRNA activity with lupus disease flares/remissions

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Conflicts of Interest

John Clinton Olivas and Susan Boackle have no conflicts of interest to report.

REFERENCES

- Kardava L, et al. "Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors." The Journal of clinical investigation 121.7 (2011): 2614-2624.
- Marquart HV, et al. "Complement receptor expression and activation of the complement cascade on B lymphocytes from patients with systemic lupus erythematosus (SLE)." Clin Exp Immunol. 101: 60-5
- Wu X, et al. "A role for the Cr2 gene in modifying autoantibody production in systemic lupus erythematosus." J. Immunol. 169: 1587-92
- Zhao J, et al. "Preferential association of a functional variant in complement receptor 2 with antibodies to double-stranded DNA." Ann Rheum Dis 75: 242-52