A System For Investigating The Role of lncRNAs in ERa Regulated Transcription

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Introduction

Breast cancer is the most common cancer and one of the leading causes of cancer-related death. 1 in 8 women will develop some form of breast cancer in their lifetime. Current therapeutics are not always effective in treating the illness, with many patients experiencing a relapse within 5 or 10 years, and becoming resistant to chemotherapies. There is a pressing need to continue exploring the mechanisms that regulate breast cancer development and progression to develop new therapies.

Around 80% of all breast cancers are hormone receptor-positive (HR+), having either estrogen receptors (ER) or estrogen and progesterone receptors (PR). Both ERs and PRs are nuclear hormone receptors, meaning they bind to a ligand (estrogen or progesterone, respectively), then migrate to the cell nucleus to initiate gene expression. These receptors are responsible for initiating transcription, which is the process of making an RNA copy of a gene's DNA sequence. This copy, called mRNA, can then be translated into a protein. ERα is one isoform of the estrogen receptor and plays a key role in the development of HR+ breast cancer.

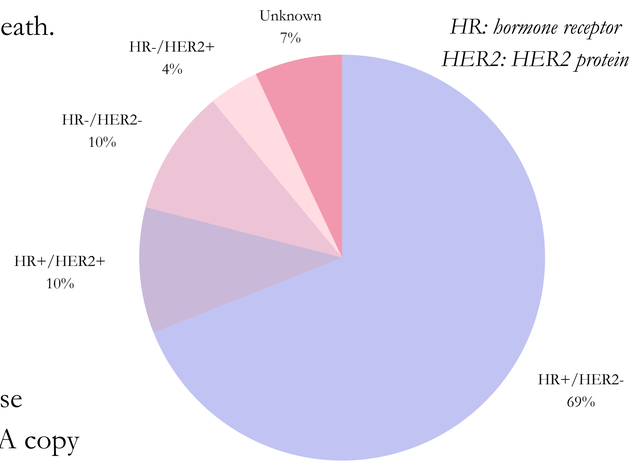

Figure 1. A diagram of the occurrence rate of the four breast cancer subtypes

In the classic model of transcription, transcription factors (TFs) interact with various cofactors to regulate gene expression. Almost all known transcription cofactors are proteins. However, emerging data strongly suggest a direct regulatory role for RNAs in gene regulation, potentially by acting as a scaffold for the assembly of transcription machinery as membrane-less compartments known as biomolecular condensates. In this project, we hypothesize that the direct interaction of transcription factors with specific RNAs regulates the transcription by changing the physical properties of the transcriptional condensates. Long non-coding RNAs (lncRNAs) are of particular interest due to their involvement in various cellular processes. They have been associated with the development, metastasis, and even drug resistance of various cancers. Given the poor outcomes of breast cancer treatment, developing alternative treatments to the classic chemo- and endocrine therapies is a clinically relevant question.

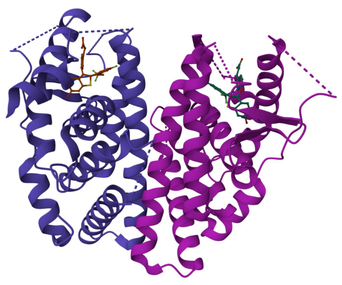

Figure 2. The ERα protein

To test this hypothesis, we performed UV-crosslinking and immunoprecipitation followed by next-generation sequencing (CLIP-seq). This assay identified several RNA candidates that interact with ERα and its cofactors. My project aimed to validate the functional relevance of this interaction by depleting the RNAs in the cells and measuring the impact of this transcript elimination in hormone-induced transcriptional activation of target genes. My goal this summer was to create a system we can use to knock down the RNAs that interact with ERα.

Methods and Results

Cell Culture

MCF7 breast cancer cells were cultured in DMEM media with 10% FBS in a humidified incubator with 5% CO2 at 37°C. To induce estrogen signaling, these cells were cultured for 72 hrs. in phenol-red free DMEM with 5% charcoal-stripped FBS and treated with 100nM 17β-estradiol (E2) for 1hr. Control samples were treated with ethyl alcohol, the solvent for 17β-estradiol.

UV Crosslinking and Immunoprecipitation

The CLIP-seq assay is used to identify the RNAs that interact with proteins of interest. MCF7 cells were treated with E2 or vehicle for 1 hr. The cells were washed twice with ice-cold PBS. The cells were then subjected to UV-crosslinking (254 nm at 400 mJ/cm2). The subsequent steps were performed using an established protocol. The sequencing data obtained from this experiment were analyzed using an in-house informatics pipeline to identify RNAs directly interacting with ERα.

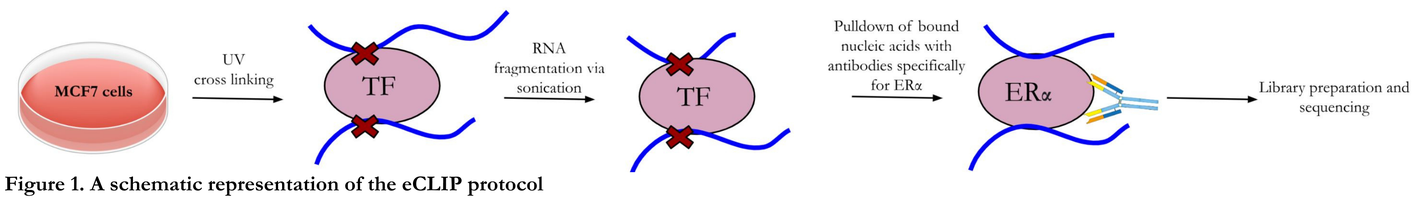

Figure 1. A schematic representation of the eCLIP protocol

Designing Guide RNAs and Cloning of gRNAs to a Lentivector Plasmid

To study the functional relevance of RNA candidates in estrogen-induced transcription, we decided to knock down the interacting RNAs and examine the transcription of target genes with quantitative real-time PCR. To this end, we employed a novel RNA-targeting CRISPR-Cas system (Cas13d). I designed guide RNA oligos *in silico* using an online gRNA design tool (https://cas13design.nygenome.org/). The guide RNA was then cloned into a lentiviral plasmid downstream of the U6 promoter to drive the expression of gRNA tethered to a direct repeat sequence. The plasmid with the correct inserts was confirmed using Sanger sequencing.

Figure 2. The sequencing result of the CRISPR plasmid used for cloning the gRNA oligos

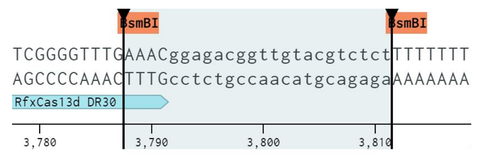

Figure 3. Original sequence with Enzyme Digestion Sites

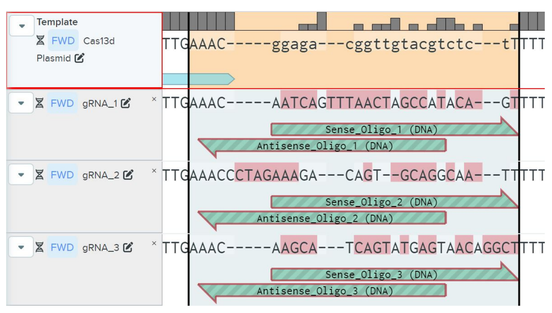

Figure 4. Alignment of Original Sequence and New Sequences with Inserts

Preparation of Lentivirus Particles

A lentivirus is part of a genus of retroviruses and is used in the lab setting to integrate genetic information into the genome of a host cell, allowing for the viral DNA to be passed on to progeny cells after cell division. This technique was used to deliver the gRNA to the host cells. The main components are a lentivector plasmid (made in the previous step) and a packaging plasmid. The first step was transfecting (a process by which foreign nucleic acids are delivered into a cell to modify the cell's genetic makeup) cells with these two plasmids using a lipid-mediated transfection (Lipofectamine 3000). 293T cells were used due to their efficient transfection rates. Over the course of 72 hours, lentiviral particles were collected from the cell media. These viral particles can now be used to infect cells and modify the genome.

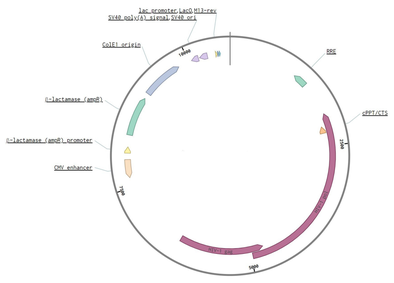

Figure 5. A sequencing map of the packaging plasmid used for the transvection

Conclusion & Further Directions

With the optimization of this protocol, I can directly examine the role of RNAs interacting with the transcription factors in hormone-induced transcription. I will accomplish this using the transduction of gRNA-containing plasmids against the RNA of choice. The transcriptional impact of the knockdown will be examined using quantitative real-time PCR of target genes or a luciferase reporter system. Once we identify more high-confidence RNA targets that act as cofactors for ERα, we will study the physiological relevance of these RNAs in normal estrogen-signaling programs and in breast cancer using animal models. New strategies and approaches are needed to promote breast cancer prevention, improve survival rates, and improve the health outcomes of racial/ethnic minorities, and though the diverse treatment outcomes are the result of an amalgamation of factors, one of these is likely the molecular interactions of transcription factor proteins and the noncoding genome.

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