Biol 40031 (ID 48931) Professor Eckhardt
Clock lab: discovering alleles that modify human behavior Fall 2019

Lab session 1:10 to 5:00pm Thursdays
Room 813HN

OVERVIEW

An internal "biological clock" drives daily (circadian) rhythms of activity and rest, and central features of the "clock works" are conserved throughout the animal kingdom. A recent study revealed an allelic variant in a human clock gene (CRY1) that was both prevalent in the population and caused a serious sleep disorder (delayed sleep phase disorder = DSPD). Given the many pathways of human biology dependent on the clock, it is expected that variant alleles like this will affect not only sleep/wake cycles, but also may cause other pathologies, and ongoing studies are addressing this with respect to the CRY1 c.1657+3A>C allele.

In this course, you will use publicly-shared databases to identify additional alleles in clock genes (Cry1 and perhaps others) that both have significant representation in the human population and are likely to modify clock function. To make educated guesses about effects on clock function, you need to understand how these proteins work. You will be reading the published literature to help you develop your strategy (allele for study).

Once you have identified an allele of interest, you will give a PowerPoint™ presentation (with your lab partner) to the Young Laboratory at The Rockefeller University, explaining your reasoning. Professor Michael W. Young is one of the 2017 Nobel Laureates in Medicine, and your work is a collaboration with his laboratory to build on the findings described for CRY1 c1657+3A>C (Patke et al. 2017). After feedback from Young lab collaborators, you will modify pBluescript KS-hCry1 to generate the desired allele. This will then be subcloned into a lentiviral vector that the Young laboratory will "package" to test for function in a cell-based assay of clock function.

COURSE GOALS

This is a laboratory research course. You need to first investigate and understand the relevance of the research you are undertaking. You will then be fully responsible for understanding and carrying out the relevant methods required to achieve your research goals. Unlike earlier lab courses, you will not be given components in pre-determined amounts to carry out experiments. Rather, you will use available protocols (e.g. site-directed mutagenesis) to design your own experiments. When asked to do a restriction enzyme analysis of a plasmid, for example, you will determine the proper conditions to carry out the digestions and to analyze the products (making predictions based on plasmid DNA sequences). You will prepare buffers by calculating molarities, etc. In other words, this is like walking into any research laboratory and learning to be a productive member of the research team.

Specific goals:

1. Be able to describe the molecular components of the biological clock and how they come together to make the circadian pacemaker.

2. Identify the functional regions of the human Cry1 protein and make predictions of which amino acids are already known to (or are likely to) affect Cry1 function.
3. Be able to defend your ideas at a "laboratory meeting." Such meetings are the core of communication in a laboratory where multiple researchers are engaged in research on a variety of related, scientific questions.
4. Use DNA sequence databases to generate an annotated map of a human gene (in this case hCry1).
5. Use an annotated gene map to identify exon boundaries in a cDNA sequence.
6. Design primers for site-directed mutagenesis and for DNA sequencing
7. Devise methods to confirm plasmid structure prior to and after mutagenesis and/or after restriction-fragment subcloning.
8. Explain the components and output of the cell-based luciferase assay for clock function.

STUDENT ASSESSMENT

Course grade will be based 50% on laboratory work (work performed, problem-solving, experimental design and planning, laboratory notebook and notes), 25% on class participation (participating in class discussions of experiments and relevant research literature and lab meeting presentation), and 25% on the research paper. Each student will develop a research paper describing their work. This paper will consist of Introduction/Background/Significance, Research approach and goals, Materials and Methods, Experimental Results, Discussion.

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Office hours: by appointment

Assigned Research Literature:

Review of clock machinery

Dubowy, C. and Sehgal, A. Circadian Rhythms and Sleep in Drosophila melanogaster. Genetics 205: 1373-1397. 2017.

Crane, BR and Young, MW. Interactive features of proteins composing eukaryotic circadian clocks. Annu. Rev. Biochem. 83:191-219. 2014

hCry1

van der Horst et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630. 1999

Ukai-Tadenuma, M. et al. Delay in feedback repression by *Cryptochome 1* is required for circadian clock function. Cell 144: 268-281. 2011

Khan, S.K. et al. Identification of a novel cryptochrome differentiating domain required for feedback repression in circadian clock function. J. Biol Chem. 287:25917-25926. 2012

Patke, A. et al. Mutation of the human circadian clock gene *CRY1* in familial delayed sleep phase disorder. Cell 169: 203-215. 2017

The clock and human physiology

Masri, S. and Sassone-Corsi, P. The emerging link between cancer, metabolism, and circadian rhythms. Nat. Med. 24:1795-1803. 2018.

Cederroth, C.R. et al. Medicine in the fourth dimension. Cell Metabolism 30: 238-250. 2019.

Overview of lab projects:

a. **Establish Benchling account to download and analyze hCry1 gene and cloned cDNA**
b. **Use provided "raw" DNA sequence DNA to align sequence of recently-produced hCry1 mutants. Use results to predict change in hCry1 protein structure.**
c. **Confirm mutant cDNA plasmid structure by Restriction Enzyme analysis and DNA sequencing, respectively.**
d. **Isolate mutant cDNA inserts by gel extraction (insert will be given to Young lab to subclone and package as lentivirus)**
e. **Identify additional human allele(s) predicted to affect function of hCry1 protein (one allele/group)**
f. **Design and carry out mutagenesis of wt hCry1 cDNA plasmid (pBS-KS-hCry1)**
g. **Confirm mutant hCry1 cDNA plasmid structure and DNA sequence by restriction analysis and DNA sequencing, respectively.**
h. **Isolate mutant cDNA fragment to provide to Young lab for subcloning in lentiviral vector**

LAB SESSION 1 (AUG 29):

Reading prior to class: Reviews = Dubowy & Sehgal (Part 1 and intro to Part 2); Crane & Young

1. Student introductions (major; college year; prior research experience)
2. Safety instruction (iv-v, Bio 203 manual 2019)
3. Lab notebook instructions
4. Assign micropipets to each group (6 groups)
5. Prepare buffer for electrophoresis
6. Prepare growth media for bacteria
7. Start 1.5ml cultures of two mutants: A and Y
8. Establish Benchling account
9. Download DNA sequence for pBS-KS-hCry1 plasmid
10. Download DNA sequence for human Cry1 gene (https://www.ncbi.nlm.nih.gov/genome)
11. Annotate both cDNA and genomic DNA sequences (using Benchling tool)
12. Discuss review papers (as time permits)

LAB SESSION 2 (SEPT 12):

(Raena out of town)

Reading prior to class: hCry1 structure/function papers: van der Horst et al.; Ukai-Tadenuma, M. et al.;

1. Align sequences for alleles A and Y to pBS-KS-hCry1 plasmid sequence in Benchling.
2. Determine and discuss what these mutations should do to hCry1 structure.
3. Make mini-preps of A and Y mutant plasmids (Qiagen mini-prep kit)

4. Determine DNA concentration by Nano-Drop.
5. Discuss Review papers and van der Horst and Ukai-Tadenuma papers (hCry1 function in mammalian clock)

LAB SESSION 3 (SEPT 19):
(Laurel out of town)

1. Determine digestion conditions for single and double digests, using XbaI and MluI.
2. Make predictions for fragments expected; which order, in double-digest, would best guarantee XbaI/MluI fragment for subcloning?
3. Digest plasmids with XbaI, MluI, and XbaI/MluI
4. Run agarose gels (include MWM) on uncut plasmid and on digests
5. Record gel results
6. Make calculations for subcloning mutant inserts into lentiviral vector: molar ratios, CIP or not; ligation conditions.
7. Read and discuss Qiaquick Gel Extraction kit protocol.
8. Start cultures to make more mutant plasmid DNA, if needed.

LAB SESSION 4 (SEPT 26):
Reading before class: Khan, S.K. et al.; Patke et al.

1. Isolate inserts for subcloning:
    a. XbaI/MluI digests
    b. Run gels, cut out fragments
    c. Qiaquick gel extraction kit to isolate gel fragments
    d. Nanodrop to determine DNA yield
    e. If sufficient yield, will go to Young lab for subcloning in lentiviral vector
2. Discuss Khan and Patke papers

LAB SESSION 5 (OCT 3)
Reading before class: Quick-change Lightning Protocol

1. Explore database of human genomic DNA sequences: hCry1 alleles. https://gnomad.broadinstitute.org/gene/ENSG00000008405
2. Select candidate alleles to generate
3. Use online tool to design primers for mutagenesis
4. Start preparing PowerPoint presentations

LAB SESSION 6 (OCT 10)

1. Present PowerPoints to class; make revisions, as needed.
2. Discussion of Research paper each student will write for course.
3. Visit Rockefeller U. to present “allelic variant” plans to Young lab

LAB SESSION 7 (OCT 17)

1. Class-wide: Quick-change mutations
    a. Write out step-by-step protocol; generate table with amounts of each component needed

   b. Class-wide demonstration of PCR machine and programming
   c. Class-wide discussion of PCR "contamination" issues
2. Groups 1-3: Mutagenesis through digestion with DpnI (group one will use pWhitescript for control mutagenesis)
3. Groups 4-6:
   a. Make NZY+ broth
   b. Make Agar plates with ampicillin – need at least 22 plates

LAB SESSION 8 (OCT 24)

1. Groups 1-3: Transform competent bacteria with PCR products from mutagenesis experiment.
2. Groups 1-3: prepare additional growth media and amp plates, as needed (for groups 4-6)
3. Groups 5&6: Carry out mutagenesis experiment AND 4,5, 6 transform competent bacteria, if time (group 4 will be transforming with control pUC)

LAB SESSION 9 (OCT 31)

1. Groups 1-6: evaluate results of transformation. If promising, start growing 1.5ml cultures from 4 colonies of each mutant.
2. Groups 1 and 4, carry out mutagenesis. If time, transform competent cells, INCLUDING one sample of pWhitescript (to grow more of this for future experiments)

LAB SESSION 10 (NOV 7): *Preliminary draft of paper due at start of class!*

1. Groups 2,3,5,6
   a. streak plates with each (of 4) cultures and then make DNA from remainder of culture. Nanodrop for DNA concentration
   b. Select/design sequencing primers
2. Groups 1&4
   a. If haven't already, carry out PCR-mediated mutation and so on.
   b. If have already done mutation, transform and plate, or, if latter is already done, pick colonies to grow.

LAB SESSION 11 (NOV 14)

Read before class: Masri and Sassoni-Corsi

1. Groups 2,3,5,6 –
   a. receive sequencing results. Align with wt sequence in Benchling. If mutation accomplished, plan necessary sequencing to confirm no additional mutations.
   b. If did not achieve mutation, make plans for re-try
   c. Make plans for subcloning fragment (cut, gel isolate)
2. Groups 1 and 4 – continue, as needed (at the least, transformation of bacteria with mutagenized plasmid should be taking place this week)
4. Discuss Masri & Sassoni-Corsi review (this or next week, depending on stage of experiments)

LAB SESSION 12 (NOV 21)

1. Groups 1 and 4 – continue as needed. At the least, will have colonies to pick and grow.
2. All other groups – isolate DNA fragments or retry experiments
3. Continue discussion of Masri and Sassoni-Corsi review

LAB SESSION 13 (DEC 5)

Read before Dec 5 class: **Cederroth, C.R. et al.** review

Continue experiments, as required. Discuss review

LAB SESSION 14 (DEC 12):

Final Research Paper due by start of class, December 12

Goal is to have confirmed 6 new mutations and to have isolated each as an XbaI/MluI fragment for cloning in lentiviral vector.

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CUNY Policy on Sexual Misconduct Link:
http://www.cuny.edu/about/administration/offices/la/Policy-on-Sexual-Misconduct-12-1-14-with-links.pdf