In-silico Analysis and Experimental Validation of Osmoregulatory PRL Expression Models in *Oreochromis mossambicus*.

**Project Goal**

The goal of this project is to build a model of prolactin (PRL) osmoregulatory pathways by identifying transcription factors (TF), binding sites, and transcription factor modules (TFM) in PRL promoters in Mozambique tilapia (*Oreochromis mossambicus*), and analyzing associated osmoregulatory genes and pathways within *O. mossambicus* as well as other species.

We hypothesize that by performing a cross-species comparative genetic analysis based on *O. mossambicus* PRL\textsubscript{177} and PRL\textsubscript{188} promoters and functionally related genes using Genomatix Software Suite (GSS), we will be able to identify potential osmoregulatory elements and propose a mechanistic model of PRL regulation. This will include transcriptional and translational regulation of PRL, as well as how PRL may regulate other genes such as CLC-2C, AQP3 and NCC2. This model will be validated, in part, with experiments on Mozambique tilapia PRL cells and Human embryonic kidney (HEK) cell lines.

**Background and Significance**

Prolactin is involved in over 300 different biological processes, covering a range of categories including water and electrolyte balance, development, metabolism, behavior, reproduction, and immunoregulation (Bole-Feysot et al. 1998, Manzon 2001, Nicoll 1974, Nicoll and Bern 1972). The pleiotropic effects of PRL present significant challenges in attempts to characterize regulatory and signaling pathways, as PRL functions overlap with and are mediated by a number of other hormones. In addition, PRL may work both synergistically and in opposition to the same
hormone. Bossus et al. (2017) found that PRL and cortisol work together to upregulate several Claudin tight junction proteins. However, McCormick (2001) reported that several lines of evidence indicated cortisol worked to allow fish to adapt to seawater (SW), while PRL allowed fish to adapt to freshwater (FW). The cellular response to PRL may vary widely between species and even tissue types within the same organism. In some settings, PRL may cause an increase the quantity of specific cell types. For example, observations of increased ionocyte cell numbers during zebrafish PRL experiments have led researchers to conclude that PRL induces cells to proliferate and/or differentiate (Breves et al. 2014).

Experiments with mammals demonstrated that pituitary tissues secrete more PRL in hypo-osmotic media in-vitro, so the function of PRL with respect to osmoregulation may be well conserved (LaBella et al. 1975).

Our ability to extract PRL cells from Mozambique tilapia provide a unique opportunity for studying cells in-vivo and in-vitro, as Mozambique tilapia PRL cells are located in the rostral...
pars distalis (RPD) of the anterior pituitary, are spherical, and comprise tissue that is 95% homogenouss (Nishioka et al. 1988, Nishioka et al. 1993).

Osmoregulation is an essential adaptation allowing organisms to inhabit or migrate through environments with (sometimes sharply) fluctuating solute concentrations. One of the most challenging requirements in clinical medicine is maintaining salt and water balance in seriously ill patients (Guyton, 1995); plasma osmolality deviations of even 15% may lead to convulsions and coma in mammals (Berne et al., 1998). In humans, the sudden change of mineral and ion concentrations, usually from drinking large volumes of water in a short period of time, have led to coma and death in extreme cases (Gardner 2002, Garigan and Ristedt 1999, Yamashiro et al. 2013). In fish, maintaining an acceptable range of osmotic pressure may require a considerable investment of energy. Brill and co-workers (2001) reported O₂ consumption required for osmoregulation may account for up to 63% of the standard metabolic rate for a 100 g tilapia. Past experiments have demonstrated that PRL is upregulated in response to hypoosmotic environments and downregulated in response to hyperosmotic environments (Seale et al. 2012). However, the specific regulatory and osmosensitive elements have not been yet identified. This study seeks to further refine existing models of PRL regulation of expression and signal pathways. Radhakrishnan et al. (2012) published a PRL signaling pathway in humans based on PRL receptor interactions, depicted below:
Figure 2 – Prolactin signaling network in humans, from Radhakrishnan et al. 2012

The continued investigation of PRL promoters, transcription factors and other potential regulators such as miRNA and IncRNA associated with osmoregulation will provide insights into mechanisms of this important family of proteins and their connection with other protective homeostatic pathways. *O. mossambicus* is selected for in-silico analyses because direct study of the PRL cell in tilapia is currently the best available model for obtaining direct evidence of mechanistic osmoreception and osmoadaptation (Seale et al. 2006).

Results from these analyses will be of immediate value to planned and ongoing experiments by Dr. Seale at UH CTAHR, in collaboration with researchers in New York and Japan, aimed at
increasing our understanding of osmoregulation utilizing tilapia PRL cell models. This understanding has broad significance and is especially important in human osmoregulatory pathologies since these pathways are likely to be highly conserved across species (Seale et al. 2006). In addition to gaining insight related to mechanisms facilitating osmoregulation, we expect any discoveries made to be applicable to many of PRL’s other functions within *O. mossambicus* as well as other vertebrate species, including humans.

Two previously identified genes shown to play a role in osmoregulation, PRL\textsubscript{177} and PRL\textsubscript{188}, will be the initial focus of analysis. Related genes to be analyzed include CLC-2C, AQP3, and NCC2, which were recently proposed as PRL targets (2017). The primary analysis toolkit will be the web based Genomatix Software Suite (https://www.genomatix.de). Modules of this online toolkit will be utilized to build a model of PRL osmoregulation using multiple approaches, including searches for overrepresented transcription factor binding sites, co-regulatory patterns within species (including co-regulation and pathway links with CLC-2C, AQP3, and NCC2), and common framework elements in multiple sequence sets across species. The results of these analyses will include a report of putative transcription factors and associated genes responsible for osmoregulation, which will be used to build a model of osmoregulatory gene and signaling interactions. This in turn will be used as input for further laboratory experiments to validate predictions and elucidate osmoregulation pathways and mechanisms. The experimental results and updated model are the intended final result of the project.
Process/Methodology:

This hypothesis will be tested by addressing the following specific aims (SA):

**SA1: Conduct literature mining to identify the current knowledge about PRL regulation and create initial list of coregulated genes, transcription factors, and signaling molecules**

First, a review of existing literature will be conducted to obtain available knowledge related to PRL-mediated osmoregulation. Literature mining will be conducted primarily using Genomatix Literature Inspector. A written report summary with citations will be generated and submitted to the mentors for review.

**SA2: In-silico analysis of the PRL promoters with GSS**

Second, the information collected will then be applied to select species and sequences for comparative genomic analyses against PRL_{177} and PRL_{188}. Preliminary organisms for comparison include humans, mice, tilapia (*O. niloticus*) and zebrafish. The mentor will approve the final list of sequences to be analyzed. The analyses will then be performed in-silico using personal and UH Manoa computational resources (e.g. student laptop and Hamilton library PCs). The primary analysis toolkit will be the web-based Genomatix Software Suite (GSS - https://www.genomatix.de). Genomatix software modules will be used as follows:

- **Frame Worker**: Extracts common framework elements from regulatory gene sequences common to multiple species.
- **MatInspector**: Detects transcription factor binding site motifs in the target sequence.
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- **MatBase**: Contains the transcription factor knowledge base with weight matrix descriptions, gene-gene interactions, and literature citations.

- **ELDorado**: Identifies co-regulatory patterns across species, for example *O. mossambicus*, *O. niloticus*, *D. rerio*, *M. musculus* and *H. sapiens*.

In addition, other annotated genomes in ENSEMBL for species such as Cod and Medaka may be analyzed to provide insight into differences between and among euryhaline and stenohaline fish.

*SA2a: Identify regulatory elements (RE) - TF, TFM, ncRNA*

PRL\textsubscript{177} and PRL\textsubscript{188} promoter sequence searches within GSS will generate a list of potential TF binding sites. This list must be further processed and merged with results of promoter sequences from coregulated genes (for example GHR1, FSHB, CGB1) from the selected species to produce a list of the 20 most likely TF. NcRNA sequences obtained previously will be analyzed and potentially incorporated into the model.

*SA2b: Comparative genomics analysis to identify RE preserved by evolution that may play role in PRL expression and function*

Nucleotide sequences that are conserved across multiple euryhaline species may prove to be transcription binding site or other type of regulatory element that modulates the transcription or translation of PRL. Conserved sequences will be documented and investigated.

*SA2c: Analysis of RE unique to O. Mossambicus but have potential links to the osmoregulation pathways based on published literature*
PRL\textsubscript{177} and PRL\textsubscript{188} both respond to salinity change (but with different sensitivity).

Different patterns of regulation have been identified for PRL\textsubscript{177} versus PRL\textsubscript{188}. Also, one TF is unique to PRL\textsubscript{188}, so this will be analyzed to learn its function and whether it links to protein interaction networks in other organisms.

\textit{SA2c: Pathway links and co-regulatory patterns of the PRL}

TF, co-regulated genes, and signaling molecules will be identified and assembled to build the PRL regulatory and signaling pathway model. PRL\textsubscript{177} and PRL\textsubscript{188} have different patterns of phosphorylation, which will help in determining predicted and validated links to regulatory networks and pathways. RE in reference genomes will be aligned with available PRL\textsubscript{177} and PRL\textsubscript{188} upstream and downstream sequences obtained from previous experiments (Seale, unpublished data).

Other online resources will be utilized to cross-reference information, such as NCBI, ENSEMBL, USC genome browser, the genome and transcriptome database of Freshwater Hybrid Fish (http://rd.biocloud.org.cn/img/logo_hnu.jpg), SMART (smart.embl-heidelberg.de), Protein Data Bank, and UH Manoa Online Library. Mentors will be consulted to validate the feasibility and likelihood of proposed relationships discovered via GSS. Finally, the results of sequence analyses will be used to synthesize working model(s) of transcription binding sites, gene interactions and signaling pathways.

\textit{SA3: Synthesize regulatory and signaling pathway model(s)}

\textit{SA3a: Signal transduction (prolactin-producing and target cells)}
**Figure 3 – Signal transduction model for prolactin-producing cells and target cells**

A model of signal transduction will be diagrammed and describe how prolactin-producing cells are prompted to release prolactin and how receptor cells process prolactin as the signal.

**SA3b: Regulation PRL gene expression**

**Figure 4 – PRL related genes (produced in GeneMania Cytoscape)**

A gene association diagram will be included, with a description of how PRL upregulates or downregulates associated genes.

**SA3c: Resulting cellular response (membrane restructuring, differentiation, proliferation)**
A description of what types of changes cells may undergo, such as increased receptor or ion channel expression, depending on cell type, will be included in the model.

**SA4: Contribute to the regulatory/signaling pathway validation experiments**

*SA4a: Transcription factors (analyze available RNA sequence data or luciferase reporter assays)*

Transcription factors and pathways that are identified during the course of this research will be used as input for ongoing validation experiments being conducted by Dr. Stoytcheva. In addition, experimental results will be used as feedback into the model.

*SA4b: Evaluate whether IncRNA (XR_001224668.2) encodes for miRNA (analysis of the stem loop structure)*

Long non-coding RNA sequences obtained during previous experiments (Seale et al. unpublished data) will be analyzed and investigated to determine any potential role in translational regulation of PRL protein expression.

**SA5: Summarize results, submit Honors Thesis and present findings**

Final results will be submitted in the Honors Thesis and presented at the Fall 2018 Undergraduate Showcase.

**Timeline**

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| Task 3 – Model Synthesis |  |  |  |  |  |  |  |
| Task 4 – Model Validation |  |  |  |  |  |  |  |
| Task 5 – Summarize and Analyze Results; Prepare Final Thesis and Presentation |  |  |  |  |  |  |  |

**Applicant’s Role**

Under the supervision of Drs. Zoia Stoytcheva, I will be responsible to perform the bioinformatics analysis to discover regulatory elements involved in PRL-mediated osmoregulation and synthesize a model that can be tested in *Oreochromis mossambicus*. I have taken the requisite Biosafety and ethics training courses, relevant molecular biology, genetics, biochemistry and bioinformatics courses, and will participate in wet lab validation of regulatory and signaling pathways during Spring and Summer 2018. I will ensure that each objective is achieved, submit the final thesis and present the results at the Fall 2018 Undergraduate Showcase.
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