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RNA sequencing analysis reveals the GnRH induced citrullinome

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Key words: histone modification, peptidylarginine deiminase, gene regulation, RNA-sequencing

Secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus is critical for the secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH binds to its receptors on the plasma membrane of anterior pituitary gonadotropes and stimulates the release of these hormones. LH is crucial for regulating the function of the testes in men and the ovaries in women. In women, LH is important in carrying out functions in women's menstrual cycles and stimulates the release of progesterone if pregnancy occurs.[1] FSH is also important for reproduction, with its main roles also responsible for the maintenance of the menstrual cycle, release of estrogen and progesterone, as well as the growth of ovarian follicles.[2] Given the essential role of gonadotropin production to fertility, major effort has gone into defining the mechanisms initiated by GnRH to regulate gonadotropin gene expression at the promoter/transcription factor level. Evidence suggests that PADs are both expressed and regulated in female reproductive tissues. With this evidence, RNA-sequencing was performed to identify key genes critical in maintaining gonadotrope function and are associated with PAD activity.

PADS

Peptidylarginine deiminases (PADs) are a family of calcium-dependent enzymes. PADs are involved in converting positively charged arginine residues on key proteins to neutrally charged citrulline within histone tail residues, a process known as citrullination which controls the expression of genes and modification on histones.

[1] Luteinising Hormone. January 7, 2015. Accessed May 6, 2016. http://www.yourhormones.info/hormones/luteinising_hormone.aspx/.
[2] Goldberg, Joanna, and Lydia Kraus. Follicle-Stimulating Hormone (FSH) Test. January 12, 2016. Accessed May 6, 2016. http://www.healthline.com/health/fsh#Overview1.

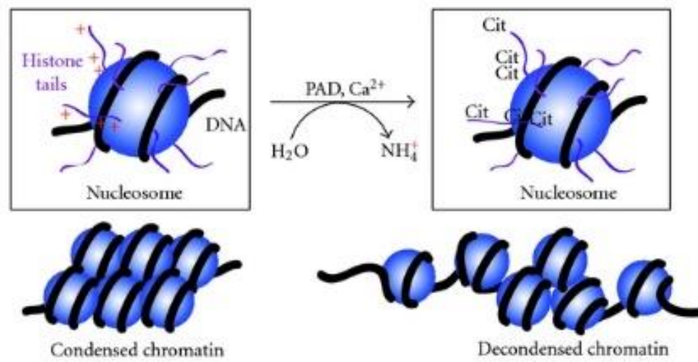
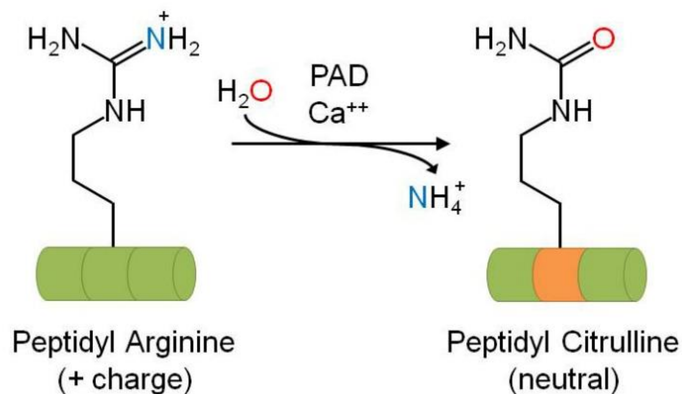


Fig. 1

Currently, five different PADs exist, including PAD1, 2, 3, 4, and 6. PADs exist within mammalian vertebrate genomes and each are involved particular roles within the immune and reproductive system and skin[3]. Evidence also shows that citrullination by PADs is important in regulating gene expression in female tissues. Currently, little is known about PAD expression in the anterior pituitary gland. This is of interest because estrous cycle dependent hormonal changes are needed to stimulate gonadotrope cells to produce processes essential for ovulation and female fertility.[4]



[3] Wang, Shu, and Yanming Wang. "Peptidylarginine Deiminases in Citrullination, Gene Regulation, Health and Pathogenesis." Biochimica Et Biophysica Acta 1829, no. 10 (July 2013): 1126-135. doi:10.1016/j.bbagrm.2013.07.003.

[4] Khan, Shaila A., Brian S. Edwards, Paul R. Thompson, Brian D. Cherrignton, and Amy M. Navratil. "GnRH Stimulates Peptidylarginine Deiminase Catalyzed Histone Citrullination in Gonadotrope Cells." Unpublished. Accessed May 1, 2016.

Fig. 2

The Role of PAD2

PAD2 is the most widely expressed of all the PADs and is expressed throughout several tissues. These tissues include secretory glands, brain, uterus, spleen, pancreas, and the skeletal muscle.[5] Overall, PAD2 associated citrullination seems to have limited activity within the nervous tissue. PAD2 mediated citrullination has other significant roles within the body, including citrullination of CXCL8 in suppression of inflammatory response, IKK-gamma in macrophages and citrullination of vimentin, resulting in the breakdown of filament network breakdown. Even more though, PAD2 appears to target histones for citrullination, suggesting that PAD2 plays a role within gene regulation.[6]

PAD2 and Gonadotropes

To begin to define the role of PADs in gonadotropes, mRNA expression levels of PAD1-4 from LβT2—cells derived from gonadotropes--cells were first examined. Past analysis found that PAD2 mRNA was significantly elevated compared to other forms of PADs by 600 fold higher. In order to confirm the results, it was also found that PAD2 protein is higher than other PADs in LβT2 cells by western blot analysis, consistent with results the earlier mRNA data. Overall, it was found that PAD2 mRNA and proteins levels are higher than other PADs in LβT2 cells and shows PAD expression in a gonadotrope derived cell line[7].

PAD2 and Citrullination

It is known that histone citrullination is a known target of PAD activation and that GnRHa stimulates citrullination of arginine residues on histone H3 tails. However, what is in

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[5] Wang, Shu, et al.
[6] Horibata, Sachi, Scott A. Coonrod, and Brian D. Cherrington. "Role for Peptidylarginine Deiminase Enzymes in Disease and Female Reproduction." Journal of Reproduction and Development 58, no. 3 (2012): 274-82. NCBI.
[7] Khan, Shaihla, et al.

question is if PAD2 expression increases citrullinated histones in gonadotropes. A time course experiment was performed in which LβT2 cells were treated with either a control with no treatment or a GnRHa treatment for 0, 30, 60, and 180 minutes and then examined with a western blot. It was detected that histone H3 arginine residues 2, 8, and 17 were citrullinated. Compared to the control, a statistically significant difference was found between GnRHa treated cells and the control at 30 minutes, therefore confirming that GnRHa stimulation of LβT2 cells results in PAD2 catalyzed histone citrullination.[8]

PAD Inhibitior

Biphenyl-benzimidazole-Cl-amidine (BB-ClA) is a known inhibitor of PAD2. Past research used BB-ClA to confirm the role of PADs in citrullinating histones in gonadotropes. With only the GnRH treatment at 30 minutes, LβT2 cells increased the level of citrullinated histone H3 arginine residues 2, 8, and 17, consistent with the previous results. However, treating the LβT2 with the BB-ClA treatment decreased GnRHa induced citrullination of histone H3 arginine residues 2, 8, and 17. Thus, GnRHa induced histone citrullination is significantly inhibited when PAD activity is blocked, as found from statistical significance between the two different treatments.[9]

RNA-Sequencing and Gene Analysis

RNA Sequencing (RNA-Seq) is a successful method in identifying key genes and to characterize the transcriptome of a population of cells with next-generation sequencing. RNA-seq was performed in order to identify the genes affected by GnRH versus the GnRH and BB-ClA treatments.

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[8] Khan, Shaihla et al.
[9] Khan, Shaihla et al.

In general, several methods can be used to create an RNA-seq library, but there are common steps that all RNA-seq methods utilize. First, the RNA must be purified. Most of the RNA within a cell are ribosomal RNA (rRNA) and should be removed before the creation of the library of interest. Messenger RNA (mRNA) isolation was used for the sequencing of the data sets discussed here. This involves targeting the polyadenylated tails to separate non-coding RNA from mRNA. Afterwards, the mRNA is fragmented into smaller pieces so sequencing may occur. Once the mRNA is split, reverse transcription and second-strand cDNA synthesis occurs. Here, an enzyme then is able to reverse transcribe mRNA into cDNA strands to create the library. Afterwards, the library created is ready for amplification and can be used for sequencing. In this last step, one is able to match the cDNA to a reference gene to see which genes were up and down-regulated as well as the amplification of each gene.[10]

In order to pick which genes to analyze, the genes must first have a threshold of at least one in their fragments per kilobase million (FPKM) values. FPKM is simply a unit of measurement and not a method in which to select genes. This unit normalizes the data by dividing by the total length of all the exons—part of a gene that will become a part of the final RNA produced after introns have been removed by RNA-splicing--and allows the comparison between genes within different samples. FPKM takes the rate of the counts per base and divides it by the total number of reads sequenced. In order to find the FPKM, first the counts (random variable X_i) and the effective length, which is the number of possible start sites a gene could have generated for a fragment of that particular length. This is calculated as:

$$l_i^* = l_i - \mu_{FLD} + 1$$

Where μ_{FLD} is the mean of the fragment length distribution from the reading.

[10] "Getting Started with RNA-Seq." New England BioLabs. Accessed May 6, 2016. https://www.neb.com/tools-and-resources/usage-guidelines/getting-started-with-rna-seq.

The interpretation is that if one were to sequence this pool of RNA again, the expectation is to see $FPKM_i$ fragments for each thousand bases in the feature for every $N/10^6$ fragments sequenced. Mathematically, this is described as:

$$\text{FPKM}_i = \frac{X_i}{\left(\frac{l_i^*}{10^3}\right)\left(\frac{N}{10^6}\right)} = \frac{X_i}{l_i^* * N} * 10^6$$

If the sample is able to meet the threshold for the FPKM values, then analysis of the genes may continue.[11]

Mean of Ratios and Ratio of Means

Two different methods were used to summarize and make inferences for the paired data: mean of the ratio and ratio of the means. Instead of looking at the typical incremental changes that t-tests would provide, these two methods were used to examine the relative change occurring within the different hormone and inhibitor treatments. By using these statistics, one is able to take into account not only the starting values of the control means, but also the amount of change between the control and the treatments to the genes. These two different methods can provide some interesting insight: the ratio of the means examines the relative change in population as a whole, while the ratio of the mean is primarily concerned with the average, relative change in the individual unit.

Mathematically, both the mean of ratios and the ratio of the means are a result of a weighted average, however it is the weighting of these averages that differ. Where R_i is the ratio between the control and treatment for each group, the equation is the following:

$$\bar{R} = \frac{1}{n}\sum R_i$$

[11] Pimental, Harold. "What the FPKM? A Review of RNA-Seq Expression Units." May 8, 2014. Accessed May 5, 2016. https://haroldpimentel.wordpress.com/2014/05/08/what-the-fpkm-a-review-rna-seq-expression-units/.

Here, it is seen that the weighting of the data values is considered equal. Using this, we are provided with the average amount of relative change between the genes.

On the other hand, in the ratio of the means, the weighting is determined by the denominator term, or the values within in the control group. Overall, the ratio of means is simply the mean of ratios, but the weighting differs from gene to gene instead of being applied equally. The representation for the ratio of the means is a follows:

$$\frac{T_y/n}{T_x/n} = \frac{\bar{y}}{\bar{x}}.$$

This method will provide the overall relative change within the population of genes under the different treatments.

Overall, the MoR and the RoM can be important indicators of information within the system; however one cannot which method is more important to discuss or verify as the correct one. It is of interest to describe the system both in terms of how the entire population changes and the average amount of change from gene to gene within the system. Both, as seen, will provide results that may prove interesting biologically between the different treatments.

For each of the four different treatments, the MoR and the RoM were performed using the original values in the data set. The results for each of the treatments are as following:

Treatment	**MoR (Standard Error)**	**RoM (Standard Error)**
GnRH v GnRH + BBCIA	0.99 (0.011)	1.12 (0.005)
BBCIA v GnRH + BBCLA	2.51 (0.194)	1.0 (0.03)
UNT v GnRH	3.12 (0.266)	1.0 (0.029)
UNT v BBCIA	1.1 (0.011)	0.995 (0.003)

Fig. 3

Between the different treatments, the MoR and the RoM produced extremely different answers for some treatments. While the RoM remained mostly constant around 1, both the untreated vs

GnRH data and the BBCLA vs GnRH + BBCLA produced extremely largest MoR compared to the rest of the treatments.

From the original data, meaningful subsets were created chosen based on their potential biological significance and their potential for interesting changes within the RoM and MoR to occur. The RoM and Mor methods were performed to see if information was either lost or gained by focusing on only separate aspects of the data. The first subset included only fold changes greater than the absolute value of 2. In this case, only the genes who were the most up-regulated or down-regulated within the different treatments were considered. The next subset contains only the starting control means greater than 5. This was chosen to only consider genes that were already significant producers rather than focusing on genes whose original production was miniscule. Finally, the last subset contains both fold changes greater than 2 and control means greater than 5 in order to find not only those genes with the largest expressions and impact, but also those in which the largest changes were observed. This way, the genes who were most affected by the treatments and are the largest contributors in gene expression can be found.

The data set of most interest is the control of GnRH hormones versus the treatment with both hormone GnRH and the BBCLA inhibitor. As indicated earlier, the original MoR was 0.99 (Std error=0.011) and the RoM 1.9 (Std Error=0.12). The results are as follows for the MoR and the RoM foe each subset:

GnRH v GnRh + BBCIA	**MoR (Std Error)**	**RoM (Std Error)**
Fold >2	0.65 (0.07)	1.9 (0.12)
Control Mean>5	0.98 (0.0016)	1.06 (0.0033)
Fold >2 & Control Mean >5	1.12 (0.278)	1.05 (0.323)

Fig. 4

As expected, some differences were found between the original data and the various subsets, the most substantial one being the subset consisting of only fold changes greater than 2.

Here, the MoR, or the average amount of changes between the genes, is lower than the original, while the RoM, or overall change, also decreased, though not as severely as its counterpart. Another interesting change between the original and a subset was the group consisting of both the fold changes greater than 2 and the control means greater than 5. There, the MoR increased by approximately 13 and the RoM decreased by 6. The subset containing control means greater than 5 did not provide any meaningful difference worth mentioning.

Also worth examining are any large differences that occurred between MoR and the RoM within each subset, indicating how the different methods can provide very different results and how care should be provided in deciding the best method to summarize data. Still focusing on the GnRH vs GnRH and BBClA treatment group, even within the original data set, a large difference between the MoR and the RoM was found. Each subset produced a similar story. The subset of control means greater than 5 also produced a MoR greater than the RoM, while the subsets of fold changes greater than 2 and the subset containing both large fold changes and control means both produced an RoM greater than the MoR. Overall, there was a large difference within each of the answers for RoM and the MoR.

Within each of these subsets, a list of genes were generated in order to find the genes that have the most potential and considered important within the context of reproduction, PADs, and the release of the hormones FSH and LH. The final list of genes chosen were from the GnRH versus GnRH and BB-ClA treatment. A discussion on what these genes are and their role in the process will ensue.

Biologically, there is not a known reason why these large changes occurred within the data and produced different RoM and MoR. But, because these large differences were observed not only between the original and each of the subsets, one can conclude that each of the different

subsets is providing different information and that some of this information is being gained or lost. Future discussion might revolve around why these significant changes occurred and what value they may carry biologically within the system.

Another Method for Gene Analysis

While the RoM, MoR, and subsets might provide some interesting insight on the system and cells as a whole, other methods exist in which to analyze the gene data and discover important genes in the process. This particular method focuses more on incremental change rather than relative change and provides a process to find statistical significance within the gene changes.

First, a t-test difference in means is performed for each gene. Then the genes which have a p-value less than a pre-specified significance level are considered for further analysis. From the p-values of each test, a q-value is formed. For the *i*-th test with test statistic t, this can be read as:

$$q\text{-value}(t_i) = \text{pFDR}(T \geq t_i)$$

where the pFDR with rejection region Γ and prior probability π_0 that the null hypothesis is true for any of the hypothesis tests is:

$$pFDR(\Gamma) = \frac{\pi_0 \Pr(T \in \Gamma \mid H = 0)}{\Pr(T \in \Gamma)} = \Pr(H = 0 / T \in \Gamma).$$[12]

Since several thousand t-tests are being performed during the process of analysis, it is necessary to make adjustments in order to account for the false positives that arises during the analysis. Q-values are designed to handle this by adjusting the p-values and to help establish the false positive rate. Using the p-values, a distribution is created. If there are few changes within the experiment, one would expect to see a distribution similar to the following figure:

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[12] Kimes, Patrick. "Understanding Q-values as a More Intuitive Alternative to P-values." April 10, 2099. Accessed May 7, 2016. http://pages.pomona.edu/~jsh04747/student theses/kimes_final.pdf.

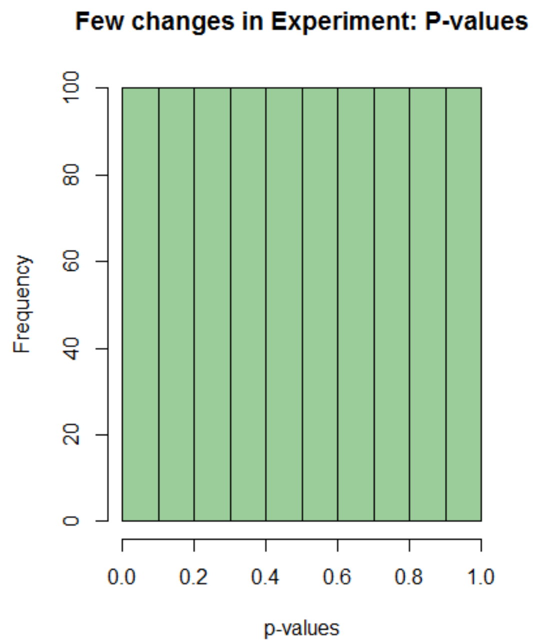

Fig. 5

While more significant changes within an experiment will appear to look more like the following:

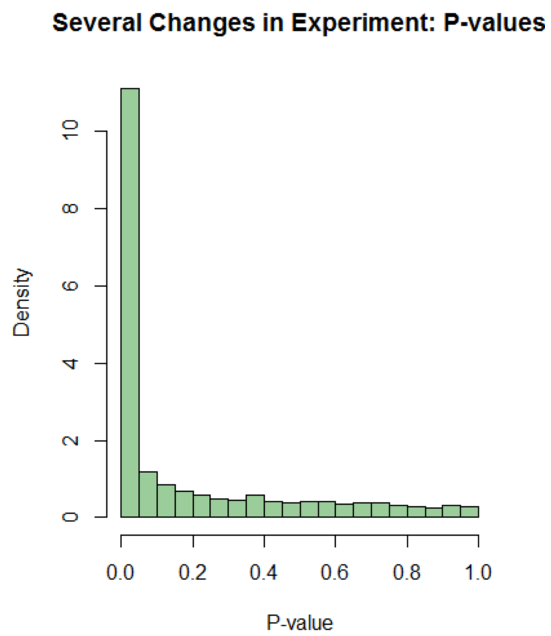

Fig. 6

When using the p-value approach, the height in which the p-value distribution begins to flatten is incorporated into the adjusted p-values. In order to have consideration, a gene must then have a q-value of at a pre-specified level or the level in which the p-value distribution flattens, which should reduce the number of false reads.[13]

Once genes are found with significant differences between their control and treatment means, have a q-value less than the specified level, then one more step is taken into account. The remaining genes are then sorted by fold change and the largest changes in genes who were both up-regulated and down-regulated are selected for further analysis in their role.

Key Genes

Known Genes

There are a few known genes important for their role with GnRH and female reproduction.

GnRHR

This particular gene encodes the receptor for the gonadotropin releasing hormone and is expressed on the surface of pituitary gonadotrope cells. Upon binding with GnRH, GnRHR associates with G-proteins which activate a phosphatidylinositol-calcium second messenger system. Upon the activation of this receptor, the luteinizing hormone (LH) and follicle stimulating hormone (FSH) are secreted.

LHB

LHB encodes the beta subunit of the luteinizing hormone and is a member of the glycoprotein hormone beta chain family. It acts as a protein coding gene. LHB is associated with

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[13] "P-values, False Discovery Rate (FDR) and Q-values." Nonlinear Dynamics. Accessed May 5, 2016. http://www.nonlinear.com/support/progenesis/comet/faq/v2.0/pq-values.aspx.

several diseases including leydig cell hypoplasia due to lhb deficiency and hypogonadotropic hypogonadism 23 with or without anosmia.

FSHB

FSHB is also a protein coding gene. This gene encodes the beta subunit of the follicle-stimulating hormone. FSHB is associated with several diseases including hypogonadotropic hypogonadism 24 without anosmia and infertility.

Genes Found During Analysis

Upon RNA-sequencing, a list of significant genes was generated from the GnRH treatment versus the GnRH with BB-ClA treatment. Several of the genes found are RNA modifying genes associated with gonadotrope function. However, only two genes will be discussed here.

Snora65

Snora65 is a small nucleolar RNA (snoRNA). This particular gene functions as a guide RNA and is noncoding RNA. Overall, snoRNA plays an essential role as guide RNA in the post-transcriptional synthesis of 2′-O-methylated nucleotides and pseudouridines in rRNAs, small nuclear RNAs (snRNAs) and possibly other cellular RNAs.[14] While snora65 was found and is a part of the snoRNA family associated with guiding chemical modifications of other RNA, additional details are unknown about this gene.

Rpph1

Another significant gene found was Rpph1, an endoribonuclease that cleaves transfer RNA (tRNA) precursor molecules and is associated with ribonuclease P (RNase P). RNASE P is

[14] Kiss, Tamas. "Small Nucleolar RNAs: An Abundant Group of Noncoding RNAs with Diverse Cellular Functions." Cell 109, no. 2 (April 19, 2002): 145-48. Accessed May 12, 2016. doi:10.1016/S0092-8674(02)00718-3.

an endoribonuclease that cleaves tRNA precursor molecules and is responsible for other RNA precursors.[15]

Conclusion

Evidence suggests that PADs are expressed and regulated in female reproductive tissues, especially PAD2. Knowing the importance of GnRH to regulate expression of LH, it was hypothesized that GnRH stimulates PAD catalyzed citrullination to regulate LHβ expression. It was found that GnRH rapidly stimulates PAD catalyzed citrullination of histones and this activity is inhibited by BB-ClA. Additionally, studies indicate that GnRH induced histone citrullination regulates LHβ expression adding to our understanding of GnRH stimulated histone modifications and the function of PADs in female reproduction. Future work would include demonstrating that PADs are expressed in gondaotrope cells not only *in vitro*, but also *in vivo*. Further gene analysis after RNA-sequencing is necessary for identification of gonadotrope genes regulated by PAD catalyzed histone citrullination. While genes were found, further study is necessary to find the true scope of these genes and identification. Future studies on significant gonadotrope genes and understanding of PAD expression will provide insight on female reproduction and novel approaches to fertility of regulation.

[15] James, Brown W., Elizabeth S. Haas, Donald G. Gilbert, and Norman R. Pace. "The Ribonuclease P Database." Nucleic Acids Research: Oxford Journals 22, no. 17 (1994): 3660-662. Accessed May 12, 2016. doi:10.1093/nar/22.17.3660.

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Horibata, Sachi, Scott A. Coonrod, and Brian D. Cherrington. "Role for Peptidylarginine Deiminase Enzymes in Disease and Female Reproduction." *Journal of Reproduction and Development* 58, no. 3 (2012): 274-82. NCBI.

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Luteinising Hormone. January 7, 2015. Accessed May 6, 2016. http://www.yourhormones.info/hormones/luteinising_hormone.aspx/.

"P-values, False Discovery Rate (FDR) and Q-values." Nonlinear Dynamics. Accessed May 5, 2016. http://www.nonlinear.com/support/progenesis/comet/faq/v2.0/pq-values.aspx.

Pimental, Harold. "What the FPKM? A Review of RNA-Seq Expression Units." May 8, 2014. Accessed May 5, 2016. https://haroldpimentel.wordpress.com/2014/05/08/what-the-fpkm-a-review-rna-seq-expression-units/.

Kiss, Tamas. "Small Nucleolar RNAs: An Abundant Group of Noncoding RNAs with Diverse Cellular Functions." *Cell* 109, no. 2 (April 19, 2002): 145-48. Accessed May 12, 2016. doi:10.1016/S0092-8674(02)00718-3.

Wang, Shu, and Yanming Wang. "Peptidylarginine Deiminases in Citrullination, Gene Regulation, Health and Pathogenesis." *Biochimica Et Biophysica Acta* 1829, no. 10 (July 2013): 1126-135. doi:10.1016/j.bbagrm.2013.07.003.

Appendix A

An analysis of the MoR and RoM was performed. The results of that data is included here:

BBCIA v GnRH + BBCLA	**MoR (Std Error)**	**RoM (Std Error)**
Fold >2	5.52 (0.5)	1.56 (0.106)
Control Mean>5	1.13 (0.02)	1.12 (0.2)
Fold >2 & Control Mean >5	1.47 (0.072)	1.52 (0.108)

UNT v GnRH	**MoR (Std Error)**	**RoM (Std Error)**
Fold >2	6.9 (0.88)	1.49 (0.11)
Control Mean>5	1.13 (0.017)	1.12 (0.017)
Fold >2 & Control Mean >5	0.72 (0.07)	0.8 (0.11)

UNT v BBCIA	**MoR (Std Error)**	**RoM (Std Error)**
Fold >2	1.91 (0.06)	0.99 (0.115)
Control Mean>5	1.01 (0.001)	0.99 (0.002)
Fold >5 & Control Mean >5	0.77 (0.21)	0.7 (0.19)

Appendix B

Lists of key genes were generated for each group of treatments as well as each of their subsets. Here, only the top 20 fold changes both directions is provided. Each is listed as follows:

BB-ClA versus GnRH+BB-ClA

Fold Changes >2	*Control Mean>5*	*Fold Changes >2 and Control Mean > 5*
Rd3	Vgf	B3gnt6
Actc1	Gm13889	Kctd14
Gm11714	Btg2	Tsku
Prss56	Tnfrsf12a	Rpp25
Myod1	Junb	Pramef6
Cckbr	Ier3	Gm13083
Egr1	Rgs2	Klhl14

Plau	Zyx	Gpr39
Tmbim1	Zfp36	Lmo3
Egr4	Spp1	Gper1
Tnfsf18	Klf6	Nudt7
Fosl1	Gadd45a	Rab19
Sult1e1	Srxn1	Fam69b
Egr3	Tubb6	Prrt3
Sh2d1b1	Rhob	Dhrs3
Sprr1a	Cdkn1a	Tfap4
Traf1	Arc	Phyhip
Pou2f3	Slc2a1	Zfp108
Plaur	Timp1	Gm6446
Krt17	Zswim4	Dnajc28
Serpinb2	Fhl3	Akap5
Nat1	Akap5	Fhl3
Gm5269	Dnajc28	Zswim4
Pik3c2g	Gm6446	Timp1
Gm12185	Zfp108	Slc2a1
Gm13078	Phyhip	Arc
Btnl5-ps	Tfap4	Cdkn1a
Insl3	Dhrs3	Rhob
Hoxa7	Prrt3	Tubb6
Gm13225	Fam69b	Srxn1
Gsx2	Rab19	Gadd45a
Lrrtm1	Nudt7	Klf6
Olfr531	Gper1	Spp1
Gja1	Lmo3	Zfp36
Gm7358	Gpr39	Zyx
Pdgfrb	Klhl14	Rgs2
Slc27a5	Gm13083	Ier3
Gal3st3	Pramef6	Junb
Zkscan16	Rpp25	Tnfrsf12a
Hoxa6	Tsku	Btg2
Tbx1	Kctd14	Gm13889
B3gnt6	B3gnt6	Vgf

GnRH versus GnRH+BB-ClA

Fold Changes >2	*Control Mean>5*	*Fold Changes >2 and Control Mean > 5*
Gm362	Snora65	Snora65
Gm11639	Gm23238	Gm23238
Prr33	Rmrp	Rmrp
Kcnk2	Rpph1	Rpph1
Vcan	Boll	Boll
Dcdc2c	Gm24451	Gm24451
Gm13857	Gm27421	Gm27421
Oscar	Gm26244	Gm26244
H2-DMb2	Gm16710	Gm16710
Lypd6	Rn7sk	Rn7sk
Tyrp1	Yam1	Snora34
Snora65	Gm25945	Gm11966
Cysrt1	Gm13581	Gm23027
Atp6v0d2	Dzank1	Dgcr8
Casq2	Gpr4	Gm27640
Kcne2	Calcb	Snora64
Gm8079	Gm14175	
Sepp1	Cryab	
Gm15638	Gm14897	
Lrrtm1	Gm14970	
Zic3	Tmed3	
Gm16540	Tmem185b	
Gm8244	Rbm8a	
Arhgap8	Polr3g	
Cel	Rpl22-ps1	
Fam124b	Slc7a14	
Gm28047	Snora17	
Prss38	Cmip	
Slc5a11	Atp6v0e	
Cidec	Cdca4	
Pcdh19	Mis12	
N4bp2os	Rer1	
Gata3	Fam214b	
Rab25	Zfp800	
Pdia2	Gm9727	
Ky	Mtmr11	

Pax8	Snora34	
Gm3543	Gm11966	
Slc18b1	Gm23027	
ANKRD23	Dgcr8	
Gm28693	Gm27640	
Gm21973	Snora64	

Untreated versus BB-ClA

Fold Changes >2	*Control Mean>5*	*Fold Changes >2 and Control Mean > 5*
Gm14440	Snora17	Snora17
Clec4a2	Gm25791	Gm25791
Hotairm1	Gm22442	Gm22442
Gm11413	Gm12039	Gm12039
Padi6	Dio1	Dio1
Gm6946	Gm24616	Gm24616
Mia	Gm13685	Gm13685
Gm14925	Gm26130	Gm26130
Glt1d1	Gm4482	Gm4482
Oasl1	Bloc1s2-ps	Bloc1s2-ps
Slc25a45	Tmem218	Tmem218
Gm26083	Anapc16	Anapc16
Cpa2	Pithd1	Pithd1
Ddit4l	Gm15421	Gm15421
Klk1b24	Slc37a2	Slc37a2
Muc2	Hist1h4j	Dgcr8
Phldb2	Gm3788	Gm23458
Theg	Pomp	Mt2
Mylk3	Gm18699	
Lif	Per2	
Gm14216	Smg9	
Aldh8a1	Slc30a1	
Gm20422	Ccdc73	
Acox2	Nsun3	
Wdr17	C8g	
Ctxn2	Gm25835	
Col26a1	Nlrp1b	
Arpp21	Gm12065	

Gm13695	Ddit3	
Xpnpep2	Gm27640	
Sema3b	Speer4a	
Mmp11	Gm12728	
Qrich2	Slc18a1	
Gm2862	Speer4e	
Gm12185	Gm21671	
Prokr2	Trib3	
Gm13150	Mt1	
Tmeff2	Gm14897	
Il1rn	Gm20479	
N4bp2os	Dgcr8	
Gm13225	Gm23458	
Fmo2	Mt2	

Untreated versus GnRH

Fold Changes >2	*Control Mean>5*	*Fold Changes >2 and Control Mean > 5*
Snora61	Gpr39	Gpr39
Btnl5-ps	B3gnt6	B3gnt6
Gm684	Gm13083	Gm13083
Gm10699	Kctd14	Kctd14
Gm13078	Lmo3	Lmo3
ANKRD23	Pramef6	Pramef6
Nat2	Klhl14	Klhl14
Btnl7-ps	Tsku	Tsku
Pabpc4l	Gper1	Gper1
Fyb	Zfp239	Zfp239
Gja1	Phyhip	Phyhip
Gpr113	Tfap4	Tfap4
Btnl4	Rab19	Rab19
Pdia2	Akap5	Akap5
Npbwr1	Gm6446	Gm6446
Gpr39	Fam69b	Fam69b
Gm11426	Gm996	Gm996
Zkscan16	Mrgpre	Mrgpre
B3gnt6	Dhrs3	Dhrs3
Btnl6	Thap3	Thap3

Gm13083	Zfp454	Zfp454
Traf1	Hmga1-rs1	Hmga1-rs1
Plau	Hmga1	Hmga1
Krt8	Gadd45a	Gadd45a
Pou2f3	Arc	Arc
Egr1	Timp1	Timp1
Tmbim1	Klf6	Klf6
Slc6a4	Cdkn1a	Cdkn1a
Cckbr	Slc2a1	Slc2a1
Crtam	Rhob	Rhob
Gm14474	Zfp36	Zfp36
Arhgap40	Tnfrsf12a	Tnfrsf12a
Inhba	Tubb6	Tubb6
Egr4	Spp1	Spp1
Sh2d1b1	Junb	Junb
Ifi202b	Srxn1	Srxn1
Sprr1a	Rgs2	Rgs2
Egr3	Ier3	Ier3
Serpinb2	Zyx	Zyx
Tnfsf18	Btg2	Btg2
Il1rn	Gm13889	Gm13889
Plaur	Vgf	Vgf