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Genome Wide Analysis of Hepatic Gene Silencing in Mammalian Cell Variants

by

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HONORS THESIS

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I hereby recommend that this Honors Thesis be accepted as fulfilling this part of the undergraduate degree cited above:

Thesis Director ______ 5/3/12 Date

Honors Program Director ______ 3 May 2012 Date

Abstract

Using microarray data, a genome wide analysis of hepatic gene-silencing in four hepatoma variant cell lines was carried out. The purpose of this analysis was to identify candidate genes that may be able to restore liver function in non-functional cell variants, ultimately giving insight into the mechanisms behind global hepatic gene expression. Based on a selection scheme allowing only for genes activated or repressed at least 5 fold in 2 out of the 4 variant cell lines, 225 genes were found to be repressed while a total of 76 genes were found to be activated. Of the repressed genes identified, fourteen candidate genes were chosen based on known function as transcription factors or involvement in signal transduction pathways. One gene of particular interest, ONECUT1 (or HNF6), was analyzed for the capability to restore liver function in one of the cell variant lines. This was done by stable transfection of ONECUT1 into the H11-variant cell line, followed by qPCR to monitor liver gene reactivation. Measurement for liver reactivation was assessed by comparing gene expression values of known liver-specific genes (such as albumin) of parental hepatoma cells to those of variant cell lines. Data indicates that ONECUT1 is not able to directly restore liver function in the variant cell lines. However, the thorough analysis of hepatic gene silencing paves the way for genetic rescue experiments designed to identify genes involved with hepatic function and the genetic programs responsible for establishing and maintaining liver specific gene expression.

Introduction

The liver is responsible for a variety of functions essential to sustaining life amongst vertebrates.[1-8] The breadth of hepatic functions and their importance to maintaining homeostasis within the body is highlighted by the devastating effects that arise from liver failure. The liver plays a role in the digestive system by synthesizing and secreting bile salts (in bile) that solubilize dietary fat.[2] Along with its digestive functions, it also has excretory, degradative, endocrine, and clotting functions within the body.[1,3] Among these endocrine factors are secretion of IGF-1,which promotes growth in several tissues, and angiotensinogen (to form renin-angiotensinogen complex), which helps regulate blood pressure throughout the body.[4] It is involved with the excretory system by both excreting and biotransforming endogenous and foreign organic molecules, as well as destroying erythrocytes.[1] Production of clotting factors such as fibrinogen and prothrombin in the liver is essential to coagulation and hemostatic processes.[5,6]

Apart from these functions, the liver also plays an important role in several metabolic processes occurring throughout the body. Organic metabolic processes in the liver are highlighted by its involvement with maintaining blood glucose levels, a major energy source for many cells. The liver plays a central role in maintaining blood glucose levels by glycogenesis (uptake/storage), as well as glycogenolysis and gluconeogenesis, both of which are involved with the release of glucose.[7] Cholesterol, an important structural component of animal cell membranes responsible for providing fast alterations to permeability and fluidity, is also synthesized in the liver.[1]

Given the breadth of hepatic functions, it is understood why liver disease produces devastating and typically lethal effects. Combatting liver disease, however, poses significant obstacles. Hepatic fibrosis, or accumulation of extracellular matrix leading to scar in the liver, is developed from a wound healing in response to an injury.[8] This hepatic injury may be caused by a variety of effects, including viral, autoimmune, drug induced, or cholestatic and metabolic diseases. Regardless of cause, damage

and scarring to the liver resulting in hepatic fibrosis ultimately leads to cirrhosis. Cirrhosis, the end-stage consequence of fibrosis of the hepatic parenchyma, is characterized by nodule formation that can alter liver function, causing problems with one or several of the associated hepatic functions listed above.[8] Currently, there is no way to treat late stage fibrosis or cirrhosis without a transplant. For this reason, it is important to understand the basic underlying mechanisms by which genetic programs in the liver are established and maintained. In this approach, a tumor cell line model is used to provide insight into these questions.

The genetic programs responsible for establishing and maintaining liver specific gene expression have previously been identified; however, the mechanism by which these genes lead to the differentiated state remains unclear.[9,10] Regulatory circuitry within mammalian hepatocytes has been mapped and includes several master regulatory genes that encode proteins which bind to and activate liver-specific genes. These proteins include HNF1α, FOXA2, HNF4α, and ONECUT1, all of which seem to work together to activate hundreds of hepatic genes and also interact amongst each other in autoregulatory loops to activate expression of one another.[11, 12, 13] Other genetic programs involved with maintaining differentiated hepatocytes, however, have not been well established. The purpose of this study was to give insight into other genetic programs that may be responsible for leading to this differentiated state.

Identification of genes involved in hepatic phenotype was achieved by conducting a genome-wide analysis of several rat hepatoma cell lines. These included both a parental cell line (Fg14 - which was used as an indicator of functional liver) and four independent variant cell lines (M29, H11, M38, and HS2) that had been stringently selected against (<1 in 100,000 cells) to not possess liver function. Although these four variant cell lines are similar in that none of them possess liver function, no inference can be made about the mechanism by which each cell line has lost hepatic function. For this reason, it cannot be assumed that each cell line lost hepatic liver function via the same pathway. This is

further illustrated by previous experiments where stable transfection of certain genes (HNF4 or HNF1α) into variant cell lines resulted in a recovery of liver specific genes (e.g. $\alpha 1AT$) in certain variant cell lines (M38, H11), but not others (HS2, M29).[14,15,16]

From this genome wide analysis of parental and variant cell lines, 'candidate' genes that may ultimately be capable of restoring liver function in variant cell lines upon stable transfection were selected via a stringent selection protocol. This protocol for identification of candidate genes was based on two main criteria: First, the genes had to be activated or repressed at least 5 fold in 3/4 or 4/4 variant cell lines, and second, they must act as transcription factors or signal transduction factors within the cell. These can be labeled as candidate genes because given these two criteria, they have the greatest potential to restore liver function in variant cell lines upon transfection, thus giving insight into other genetic programs that may be involved with driving liver function. From here, a candidate gene of specific interest (ONECUT1/HNF6) was stably transfected back into a variant cell line (H11) and then analyzed for its capability to recover expression of liver specific genes in the variant cell line.

Methods and Materials

The cell lines were derived from a rat hepatoma tumor cell line H4IIEC3 from Mary Weiss's laboratory in the 1970's. The parental Fg14 cells are both an adenine phosphoribosyltransferase positive (ARPT+) and xanthine-guanine phosphoribosyltransferase positive (GPT+) cell line. This cell line was derived by stable transfection of *gpt* and *arpt* transgenes which are driven by human α1-antitrypsin (SERPINA1) gene promoter. The variant cell lines (M29, H11, M38, and HS2) had been derived from Fg14 cell lines by negative selection against ARPT and GPT transgene expression using 20 μg/ml 2,6-diaminopurine (DAP) and 30 μg/ml 6-thioxanthine, respectively. All cells were grown, expanded, and maintained in 1:1 Ham's F-12-Dulbecco's modified Eagles medium containing 5% fetal bovine serum and 5 μg/100 ml penicillin-streptomycin.

RNA was extracted using a Qiagen RNeasy Mini Kit with DNAseI as according to kit protocols. The cells were lysed with RLT buffer and spun through a Qiagen column shredder to homogenize, then collected with an RNEasy column. Samples were washed and digested with DNase I for 15 minutes at room temperature. RNA integrity was then measured by 28S and 18S rRNA after gel electrophoresis in 3-(N-morpholino)propanesulfonic acid (MOPS)with 1% agarose – 2.2 M formaldehyde gels. RNA purity was then determined by nano-drop spectrophotometry at 260 and 280 nm. Triplicate samples of RNA extracted from cultures of each cell line were then used to obtain microarray data.

Before gene expression levels could be analyzed by the Illumina microarray, the extracted RNA had to be converted to usable products for the bead assay. The quality of the RNA was tested using the Agilent 2100 Bioanalyzer, which was followed by the conversion of high quality RNA to single stranded cDNA using the Illumina Total Prep RNA Amplification Kit. This single stranded cDNA was then converted to double stranded cDNA via manufacturer protocol. The double stranded cDNA was used as a template for a 14 hour in vitro transcription reaction to produce cRNA. Quality of this cRNA was checked and followed by hybridization (16 hours) of 750 ng of each sample to RatRef-12 v1 Expression BeadChips. Following washing, staining, and scanning via manufacturer protocol, the BeadChips were analyzed using the GeneExpression Module (version 1.8.0) of the Illumina GenomeStudio software.

The Illumina microarray data was obtained from the W.M. Keck Center for Comparative and Functional Genomics in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. The initial process began by obtaining an average microarray reading for each gene in the parental cell line and the four variant cell lines. Fg14, H11, HS2, and M38 cell lines were run as triplicates to minimize false positives, while the M29 cell line only had one data point for each gene. Based on average data points, genes that were activated or repressed equal to or greater than fivefold (≥5 fold) in each variant cell line were extrapolated from the data set. Analyses of shared and unique genes (activated and repressed) to each variant cell line was then carried out.

Once this data was obtained, the genes that were repressed or activated 5 fold or more in 2 out of the 4 cell lines were screened for function using various literature and trusted internet sources. Coupled with this 2/4 criterion, candidate genes were then selected based on their involvement with signal transduction pathways or their ability to act as transcription factors in the body. After candidate genes had been selected, one of specific interest, ONECUT1 (HNF6) was reintroduced into one of the triplicates of the H11 cell line by stable transfection. After transfection, difference in ONECUT1 expression levels between the variant cell line (denoted H11) and the recently transfected cell line (H11-ONECUT1) was determined by using ΔΔCT values from a quantitative polymerase chain reaction (qPCR) with an Applied Biosystems StepOne Thermocycler using primer sets specific for ONECUT1. The same qPCR procedure was then used to determine if any global recovery of hepatic function had been achieved by priming for known genes that are established indicators of liver function. These included *Alb, α1at,* and *Pepck*.

Results

A genome wide analysis of parental (Fg14) and variant (H11, M29, HS2, and M38) hepatoma cell lines was carried out to provide insight into the genetic pathways responsible for driving mammalian liver function. To determine this, triplicate whole-genome experiments were ran using isolated RNA from parental and variant cell lines that had been reverse transcribed into single stranded cDNA, which was then used to produce double stranded cDNA and transcribed into cRNA, which could then labeled with biotin and hybridized to Illumina Beadchip Microarrays (see Materials and Methods).

To check variability between microarray triplicates of each cell line, analysis of ≥5 fold differences within the parental and variant cell line triplicates was carried out. My results showed very modest differences between the approximately 22,500 genes in each of the cell lines. The parental Fg14 cell line only showed 2 genes varying ≥5 fold, while two of the variant cell lines (M38 and H11) only

showed 5 genes and 0 genes (respectively) varying ≥5 fold amongst them. The HS2 cell line showed a total of 22 genes that varied ≥5 fold amongst the triplicates. The M29 cell line is not included because it did not include any replicates. (Figure 1).To minimize the possibility of false positives showing up in my results, two criteria were applied: 1) that genes must show a ≥5 fold difference between cell lines and 2) this difference must be shared in at least 2/4 variant cell lines.

Analysis of gene repression in cell variants

To analyze repression in cell variants, genes that were repressed at least 5 fold in cell variants were assessed. The number of genes repressed in each cell line ranged from 230-284, with M38, HS2, M29, and H11 having 232, 253, 284, and 230 genes repressed respectively (Figure 2A).Following identification of repressed genes in individual cell lines, comparisons of shared genes between variant cell lines was carried out (criteria #2). From this analysis, 268 genes were found to be shared between at least 2/4 cell lines, while 132 (49%) of these genes were shared between 4/4 cell lines (Figure 2B). There were 126 unique to each cell line, or only repressed in 1/4 cell lines. Many genes that are established indicators of liver function (Alb, Agt, Asl, Aldob, Ass, Pah, Fbb, Fgb, Hpx, Pah, Pck1, Tat, and Ttr) were amongst the genes repressed ≥5 fold, which was expected since the SerpinA1 promoter sequence was incorporated into the selection process.

Based on the 268 genes that were shared between at least 2/4 cells (fulfilling both criteria), candidate genes were selected based on their established or proposed function. These included genes that are known to act as transcription factors or those that are involved in signal transduction pathways. From the genes repressed in cell variants, 14 candidate genes were identified that have the best potential to provide insight into the genetic programs involved with liver function. These genes included Bhlhb2, Creb3l3, Creg, Dppa4, Dppa5, Gas2, Hhex, Igfbp1, Mdk, Onecut1, Rnfl25, Sec16b, Stra8, and Tcfap2b (Table 1).

Analysis of Activated Genes

It is also possible that the global genetic reprogramming seen in cell variants is the result of specific gene activation, which in turn may have downstream effects resulting in the repression of hepatic genes. Analysis of activated genes and identification of candidate genes was conducted using the same criteria as were applied to the repressed genes; genes had to be activated ≥5 fold in at least 2/4 cell lines and the genes had to be known or proposed to act as transcription factors or signal transduction factors.

The total number of genes activated ≥5 fold ranged from 51-113 in the four variant cell lines, which was lower than the number of genes repressed >5 fold (230-284)(Figure 3A). Among the 4 variant cell lines (M38 H11 HS2 M29), 113, 51, 98, and 100 genes were activated, respectively. The number of activated genes shared between cell lines was also lower. Only 2 genes were activated in 4/4 cell variant cell lines, while a total of 132 genes were shared among the four variant cell lines on the repressed side (Figure 3B).

Quantitative Polymerase Chain Reaction (qPCR)

Upon identification of candidate genes, a gene of specific interest, ONECUT1/HNF6, was stably transfected back into one of the variant cell lines (H11) and analyzed by ΔΔCT values using qPCR to see the effect of introducing ONECUT1 on the expression of genes indicative of liver function (eg. Alb, Pepck, etc.) Surprisingly, qPCR results of Fg14, H11, and H11-ONECUT1 with a ONECUT1 primer shows that ONECUT1 is still expressed significantly more (>10 fold) in the parental Fg14 cell line than the previously transfected H11-ONECUT1 cell line. This is derived from converting the cycle difference to an expression difference. Given the cycle difference, the repression value can be calculated by raising 2 to the power of the cycle difference (so a 3.3 cycle difference represents a 10 fold difference in expression). There was approximately a 6 cycle difference between H11-ONECUT1 and Fg14, representing a 64 fold difference in expression for ONECUT1.Results also showed that there is little to no difference (<2 fold) between the H11 cell line and the H11-ONECUT1 cell line (Figure 4).qPCR data with a PCK primer (PCK is an established indicator of liver

function) showed that recovery of PCK expression was modest (approximately 2 fold) between H11 and H11-ONECUT1. This is in comparison with Fg14, which shows a ≥ 100 fold expression difference in PCK expression when compared to H11 or H11-ONECUT1 (Figure 5).

Discussion

A total of 398 genes were repressed ≥5 fold in at least one of the variant cell lines, while 132 of these genes were repressed in all four variant cell lines. This indicates that: 1) gene silencing in each cell variant is extensive; and, 2) a high degree of overlap is present amongst cell variants. Despite a large degree of overlap, each cell line also has a distinct set of repressed and activated genes unique to that cell line, providing support that each variant cell line has lost hepatic function by a different mechanism. A total of 75 genes were activated in ≥ 5 fold in at least one of the variant cell lines, while only 2 of these genes were shared amongst all four of these cell lines. This shows that gene activation in cell variants is less extensive and that the overlap between cell lines is not nearly as profound. These results cannot give meaningful insight into the mechanism by which these variant cell lines lost hepatic function, as both the repressed and activated side may still be responsible for this differentiated state. In my study, thorough analysis of the repressed side was carried out, but that is not meant to downplay the potential importance of activated genes. Further genome-wide analysis of the activated genes and genetic rescue experiments designed for activated genes may provide meaningful insight into the mechanism by which these variant cell lines lost their hepatic function.

Analysis of variability within the cell lines showed that the parental Fg14 cell line and the H11 and M38 variant cell lines showed little variation between gene expression values throughout the genome, with only 0-5 genes showing ≥ 5 fold differences between the triplicates. The potential for false positives, however, was higher in both the HS2 and M29 cell lines. The HS2 cell line had a higher potential for false positives because of its much higher variability within the cell line, showing 22 genes with ≥ 5 fold

differences, while the M29 cell line had a higher potential for providing false positives because there was only one sample. To minimize the possibility of these false positives showing up in our final analysis, we used the criteria that genes must be repressed or activated in 2/4 cell lines. By employing this strategy, however, it is possible that we missed repressed or activated genes unique to each cell line that may have some involvement with driving them to their dedifferentiated state. For this reason, the list of genes presented cannot be considered completely inclusive.

A surprising result in our identification of repressed genes was that the transcription factors HNF1α and HNF4 did not meet our criteria for repressed genes. This is because HNF1α and HNF4 act as transcription factors driving expression of SERPINA1, the gene which is selected against in all variants and is significantly repressed in all the variant cell lines (55-95 fold). I believe, however, that this phenomenon could most likely be explained by the sensitivity limitations of the microarray data. Parental Fg14 microarray data for the transcription factors is around 400 units, and background noise from the microarray data is around 100 units, making a true fold repression not available by microarray. To test this, qPCR was used to determine relative expression values of both HNF1α and HNF4. The qPCR data showed a ~100 fold reduction in both of these genes, supporting the fact that low expression levels in the parent were the reason these genes did not meet our criteria. This indicates that genes expressed in low levels in the Fg14 cell line may not have been identified by our scheme, therefore increasing the potential pool of missed genes that may be involved with driving liver function.

Analysis by qPCR after stable transfection of ONECUT1 into the H11 cell variant showed that ONECUT1 expression differences between the variant cell line and the newly transfected cell line were modest (<2 fold) (Figure 4A). This is surprising because it is expected that ONECUT1 expression would increase upon transfection. The reason for this discrepancy is unclear. It is possible that the transfection process may not have worked as per protocol, and thus, expression levels are unaltered. It is also possible, however, that genetic or molecular mechanisms may be responsible for ONECUT1 not being expressed.

Further assessment needs to be completed to provide a more complete picture of why the gene is not being expressed after transfection. Since ONECUT1 is not being expressed at a higher level, the cell line (as is) is not an ideal model to test for the effect of reintroducing ONECUT1 on recovery of hepatic function, since there is modest difference between the H11 and the H11-ONECUT1 cell line at this point in time. Only one qPCR test for recovery of a hepatic indicator (PCK, Figure 5). is included in the results, but recovery of other hepatic genes (e.g., Alb, etc.) was not seen in additional tests.

In conclusion, gene silencing in each cell variant is extensive and highly overlapping. A distinct set of unique genes in each cell line, however, indicates that the molecular mechanisms by which these cell lines have lost hepatic function are varying. In my genome-wide analysis it is likely that some activated or repressed genes (potentially involved with hepatic function) were not included in my analysis based on limitations by microarray sensitivity and my specific criteria. Genetic rescue experiments, designed with both ONECUT1 and other candidate genes, are essential for identifying genes involved with hepatic function and the genetic programs responsible for establishing and maintaining liver specific gene expression.

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Table 1. List of candidate genes selected from genes that are repressed ≥ 5 fold in at least 2/4 cell lines. These genes were selected based on their established or proposed function, with emphasis on those known to act as signal transduction factors or transcription factors in the cell.

Fold Repression					
M38	**HS2**	**H11**	**M29**	**Gene Name**	**Description**
6.2	1.7	3.6	5.5	Bhlhb2	Basic helix-loop-helix domain containing, Class 2B
12.9	15.0	14.6	13.7	Creb3l3	cAMP responsive element binding protein 3-like 3
3.6	6.4	2.7	5.9	Creg	E1A-stimulated genes (predicted)
17.4	18.7	18.7	16.7	Dppa4	Developmental pluripotency associated 4 (LOC680293) (predicted)
12.2	11.9	11.9	10.1	Dppa5	Similar to DPPA5 developmental pluripotency associated 5 (RGD1564306) (predicted)
9.3	8.5	7.9	8.8	Gas2	Growth arrest-specific 2. (RGD1563167) (predicted)
5.1	6.0	2.8	5.8	Hhex	Hematopoietically-expressed homeobox protein
118.6	128.0	131.9	107.4	Igfbp1	Insulin like growth factor binding protein 1
8.3	9.6	0.1	7.4	Mdk	Midkine (neurite growth- promoting factor 2)
10.7	5.3	14.9	16.9	Onecut1	One cut homeobox 1
7.0	14.9	2.1	43.3	Rnf125	Ring Finger protein 125 (predicted)
9.4	10.3	10.6	5.3	Sec16b	SEC16 homolog B. (S. cerevisiae homolog)
19.1	19.7	19.7	19.0	Stra8	Stimulated by Retinoic Acid 8 (RGD1562852) (predicted)
3.5	9.5	1.0	21.0	Tcfap2b	Transcription factor AP-2 beta

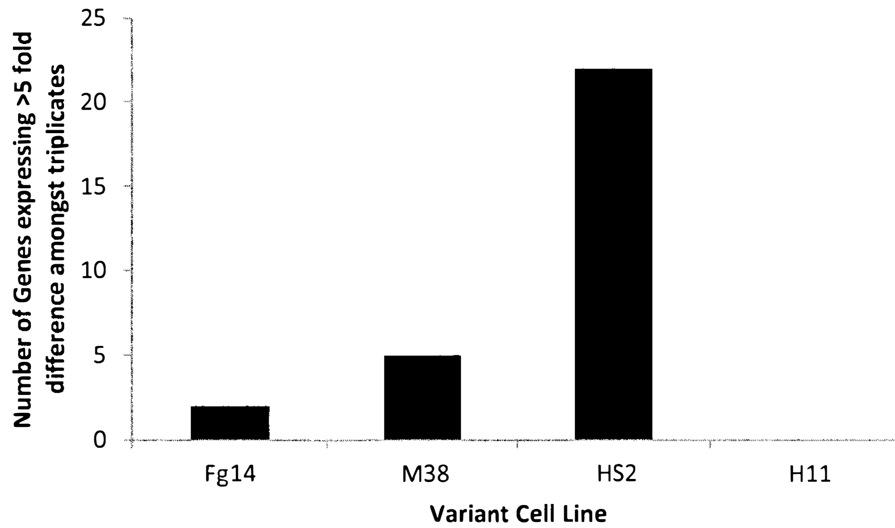


Figure 1. Variability amongst cell line triplicates in each cell line. Differences in expression values (≥ 5 fold) from Microarray data show the relative stability of each cell line. H11, Fg14, and M38 show only 0-5 genes repressed amongst the triplicates, while the HS2 cell line shows more. The M29 cell line is not included because it included only a single replicate.

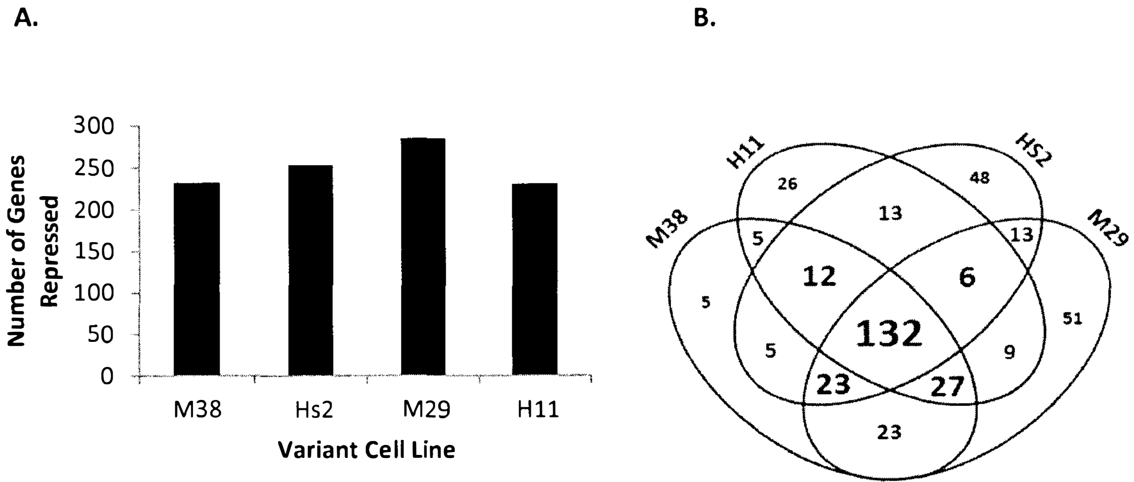


Figure 2. Number of total genes repressed ≥ 5 fold in each individual cell line (A) and the repressed genes that are shared between the four variant cell lines (B).

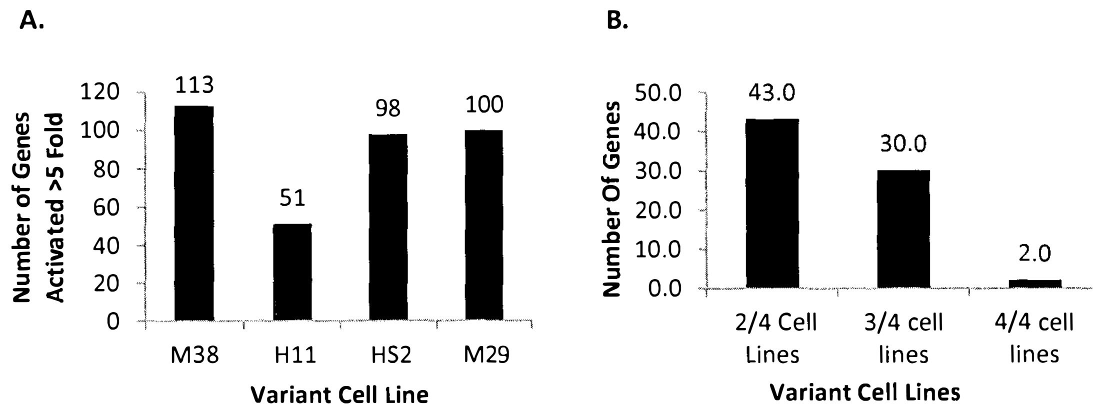
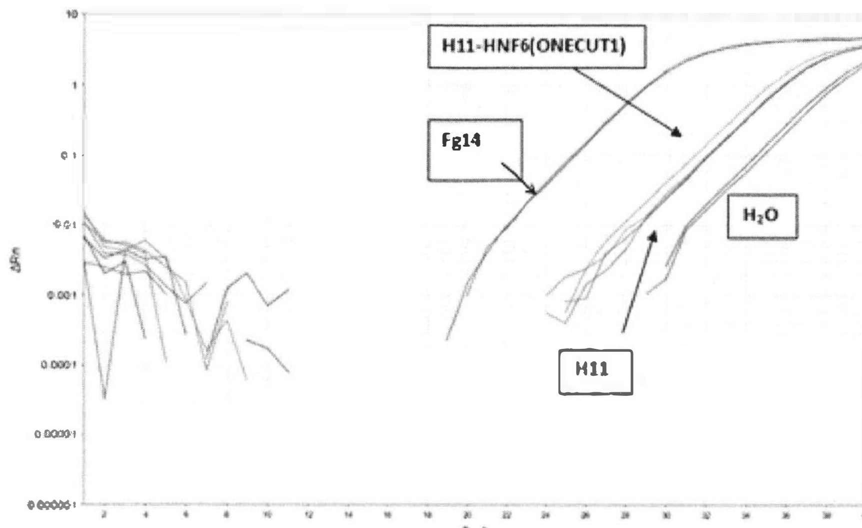


Figure 3. Activated genes in variant cell lines. The total number of genes activated in each cell line (A) and the number of activated genes shared amongst cell lines (B).

A.



B.

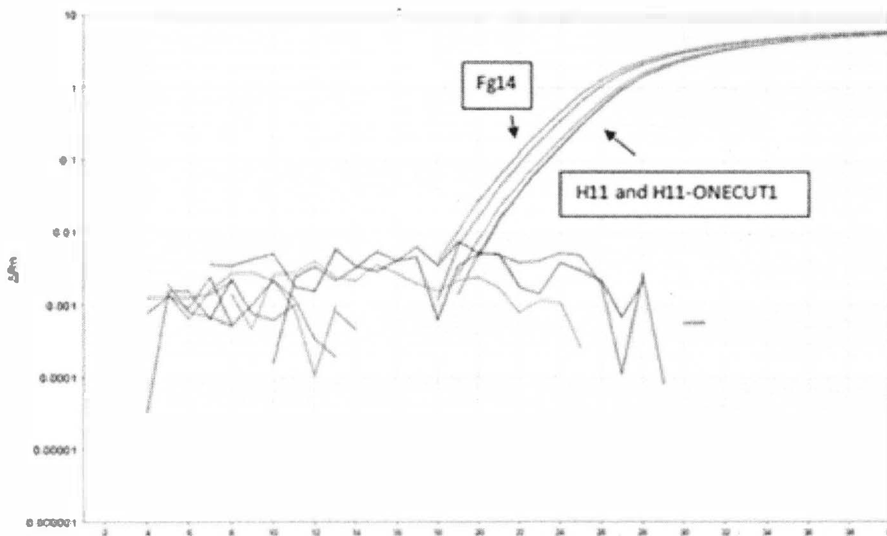


Figure 4. (A) shows qPCR data using ΔΔCT values for Fg14, H11, H11-HNF6, and H_2O with a rat primer for HNF6. Relative expression of ONECUT1 shows a ~5 cycle difference between Fg14 and the H11-HNF6 cell line even after stable transfection of HNF6 into H11. This 5 cycle difference corresponds to a 2^5 fold difference in expression. (B) shows the amplification plot of the three cell lines with a GAPDH control primer.

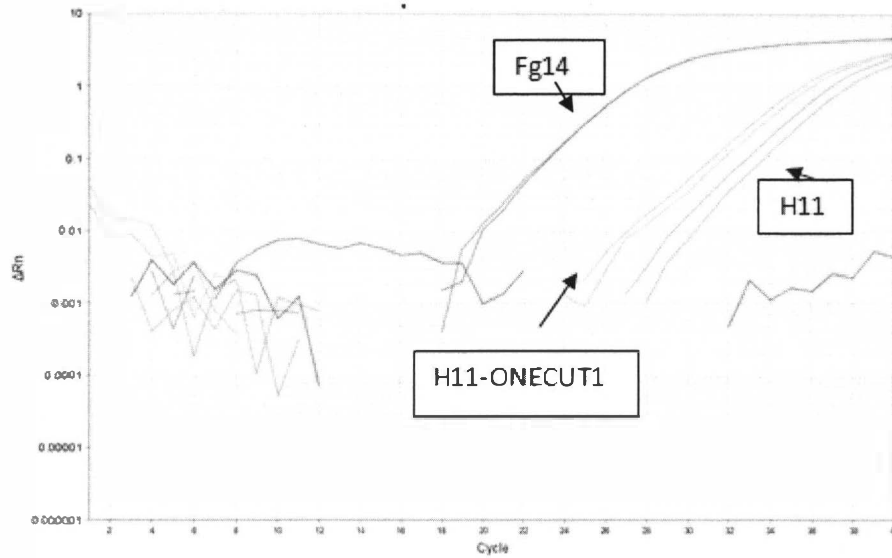


Figure 5. Amplification plot of parental, variant, and transfected cell lines with rPCK primer. Differences in expression of rPCK between Fg14, H11, and H11-ONECUT1 cell lines shows that parental Fg14 cells express rPCK at a much higher level than either H11 or H11-ONECUT1, as indicated by the 5-6 cycle difference. The expression differences between rPCK in H11 and H11-ONECUT1 are modest (≅ 2 fold.)

Eastern Illinois University

Certificate for awarding the degree of

Bachelor of Science with Honors

We, the undersigned, certify that

Jeffrey L. Kurkewich

has successfully completed the Honors Program in

Biological Sciences

Given at Charleston, Illinois, on this 27th day of April, Two thousand and Twleve.

Thesis Advisor

Honors Program Director