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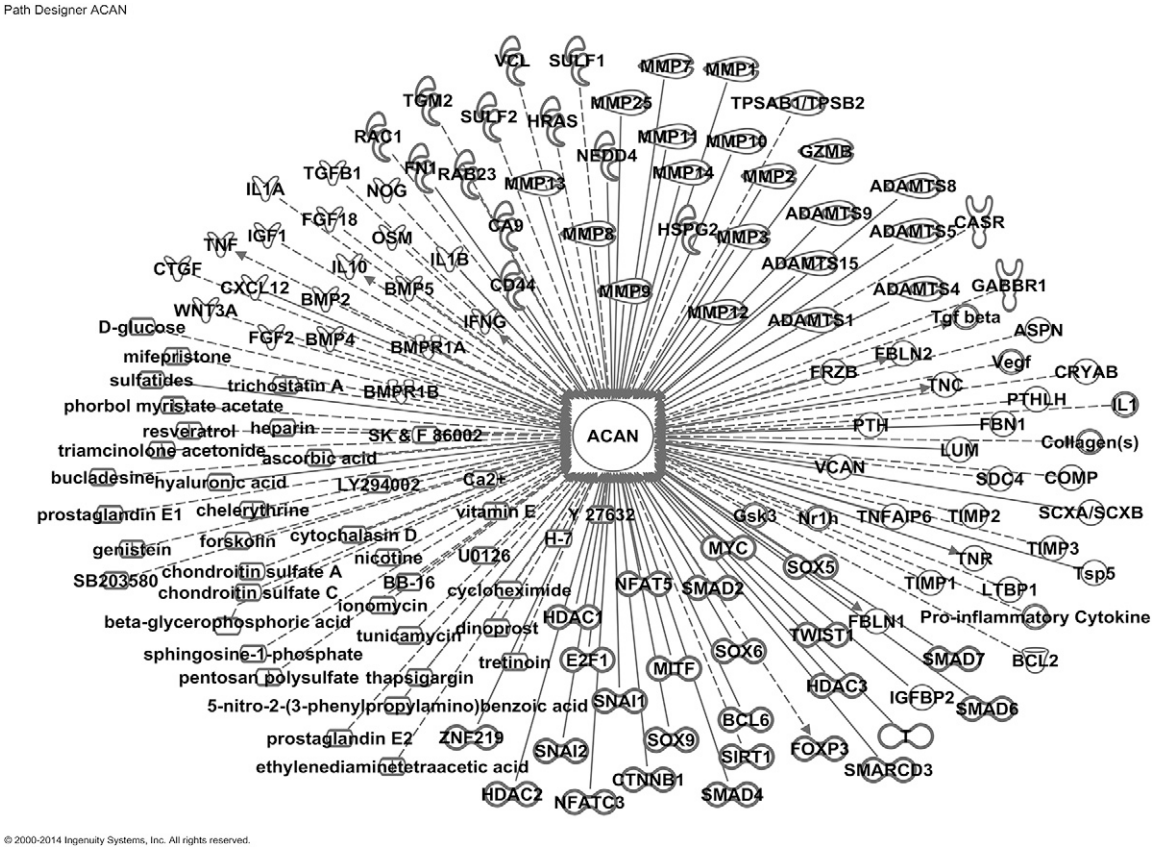

Fig. E-1

Representative diagram (generated by the IPA software) used to analyze ACAN, illustrating its possible interrelationships with genes for transcription regulators, cytokines and hormones, and the extracellular matrix of ligamentous tissues. RT-qPCR confirmed that ACAN was significantly upregulated in female compared with male study participants. Data were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

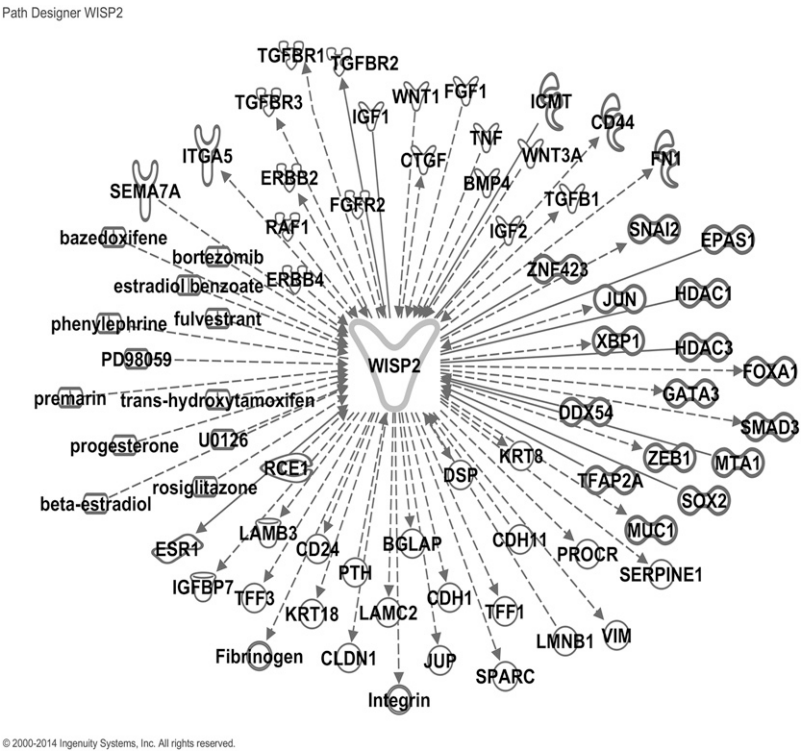

Fig. E-2

Representative diagram (generated by the IPA software) used to analyze WISP2, illustrating its possible interrelationships with genes for transcription regulators, cytokines and hormones (including estrogens and progesterone), and the extracellular matrix of ligamentous tissues. RT-qPCR confirmed that WISP2 was significantly downregulated in female compared with male study participants. Data were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

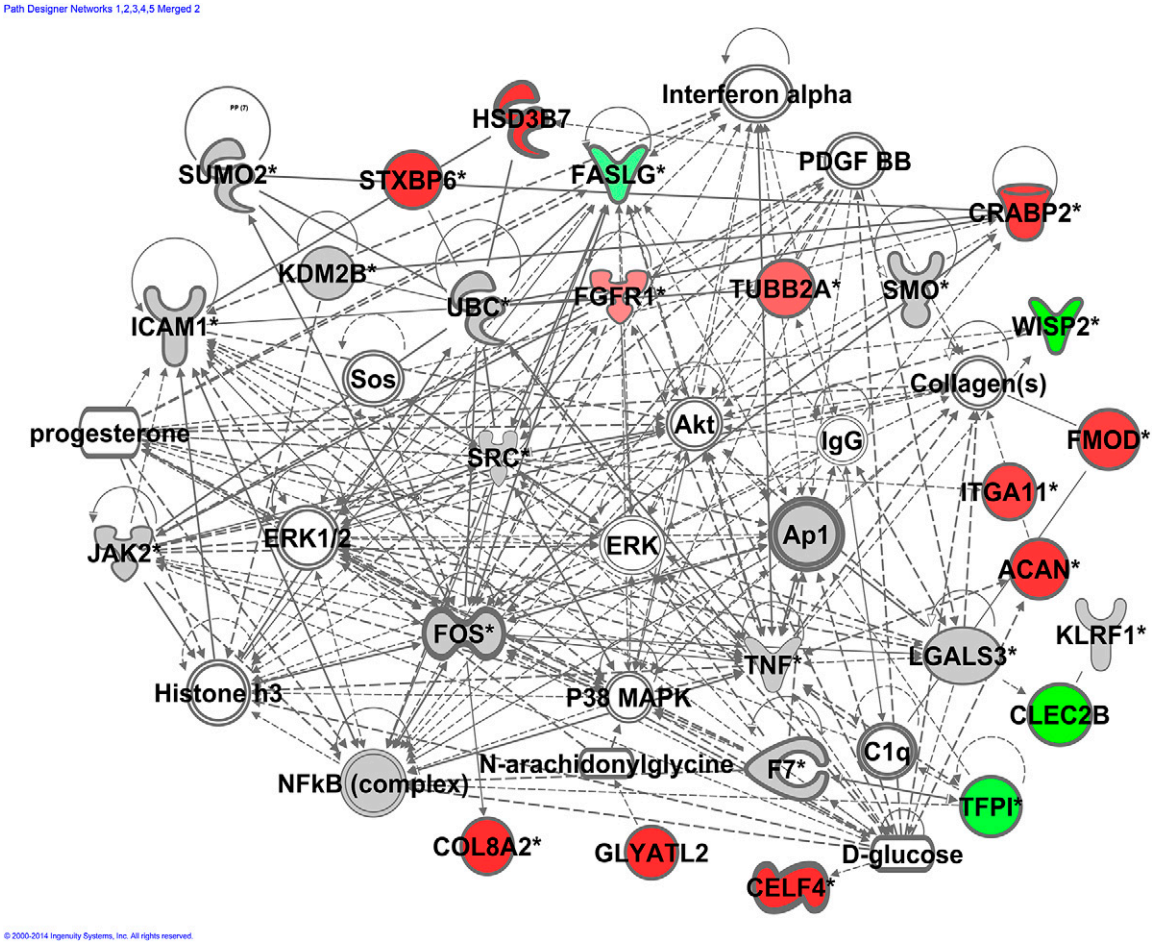

Fig. E-3

Representative diagram (generated by the IPA software) of four functional pathways that contain genes identified in the microarray analysis as significantly upregulated (red) or downregulated (green) in female compared with male study participants (see Table II for definitions of gene abbreviations). This diagram for genes that are not linked to the X or Y chromosome describes the functional pathways "gene expression," "organismal development and cell-to-cell-signaling and interaction," "developmental disorder," and "metabolic disease." Data were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

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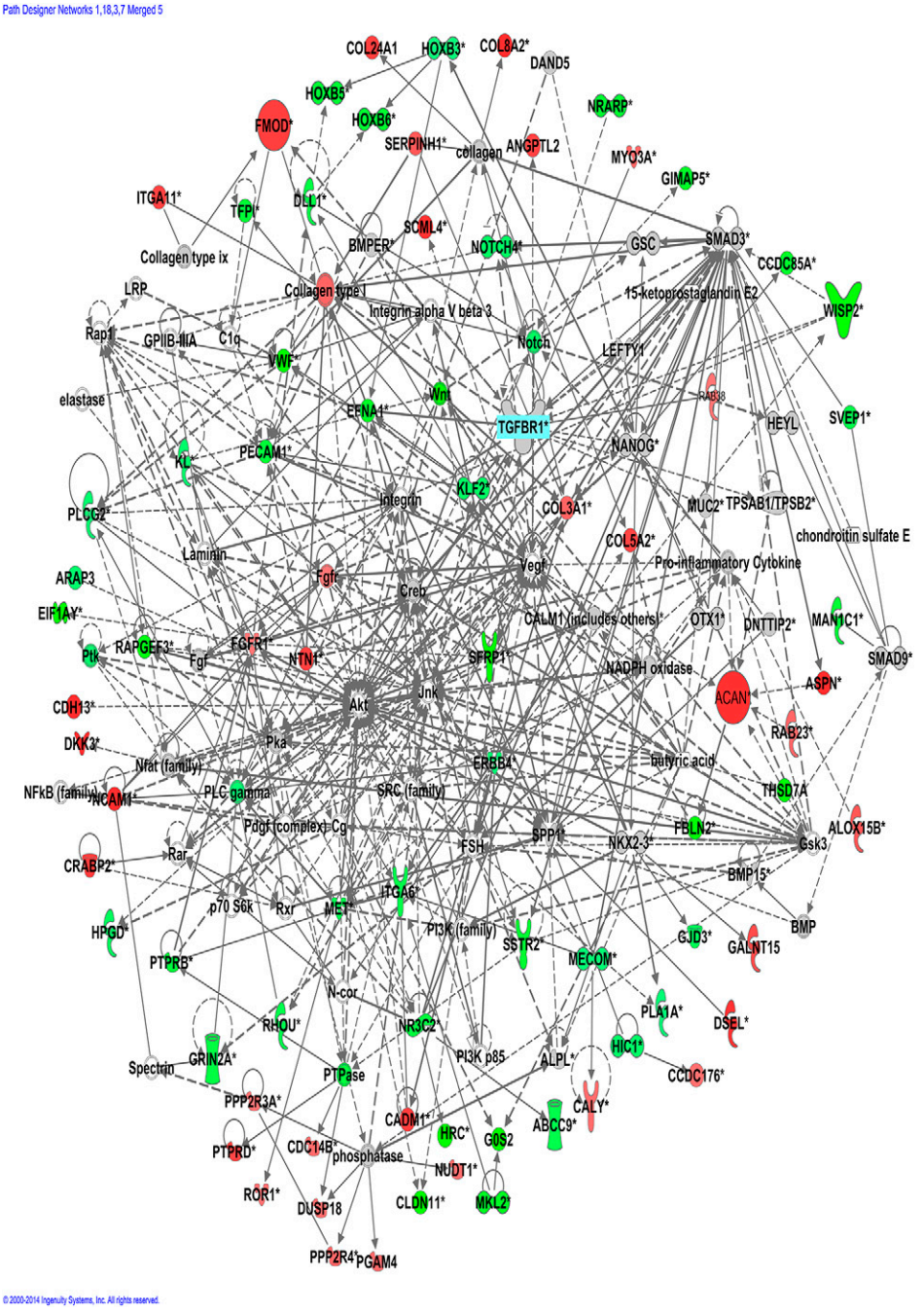

Fig. E-4

Representative diagram (generated by the IPA software) of several functional pathways that contain genes identified in the microarray analysis as significantly upregulated (red) or downregulated (green) in female compared with male study participants. These pathways for all genes found to be significantly upregulated and downregulated, including those that are linked to the X or Y chromosome, overlap those shown in Figure E-3 and also involve the larger functional pathways “connective tissue development and function” and “connective tissue disorders.” Data were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

Appendix E-1 Comments and Statistical Analysis of the Agilent Whole-Genome Array Data

The authors were very aware of numerous potential problems in controlling variables for gene-expression studies. Such variables in this study included the time from injury of the patients to ACL surgery, differences in BMI among the patients, the female menstrual cycle, and contact or noncontact injury type. Ordinarily, such variables would be expected to yield a poor correlation in intraclass statistical analyses of the microarray data for (i.e., analyses within the female subgroup and within the male subgroup). However, cluster analyses conducted by the Biomedical Genomics Core at Nationwide Children's Hospital in Columbus, Ohio, resulted in a highly significant p value (≤ 0.05) within each of these subgroups (see the Statistical Analysis of Agilent Whole Genome Array Data section below). Thus, there was a very high degree of confidence in the statements that all four female study participants yielded equivalent microarray data and that all three male participants did likewise. In other words, the variability in patient time to surgery, BMI, and other such possible factors had little or no effect on the statistical outcome, which demonstrated very high intraclass correlations.

This result was further confirmed by qPCR analysis, which included additional patients and variability. Here, the statistical analysis yielded the same significant results in the three genes that were selected on the basis of their relation to the extracellular matrix of ligaments. The qPCR analysis was also conducted to address the concern of false positives and to confirm the validity of the statistical significance of the microarray results. In this regard, the chosen false discovery rate (FDR) of 10 was considered highly restrictive and therefore limited the number of statistically significant genes to the eighteen that were reported. The choice of a low FDR of 10 thus reduced the possibility of false positives while still allowing eighteen genes to be identified as differentially expressed. An alternative choice of 15 for the FDR would have increased both the probability of false positives and the identification of statistically significant genes. Therefore, the ACAN, FMOD, and WISP2 genes reported were not false positives, as indicated by the use of the low FDR of 10 as well as the qPCR analysis confirmation.

The control of variables to achieve precise gene-expression studies also extended to surgical specimen handling and preparation. In the surgical suite, removal of ACL tissue from the knee of a patient was done with a sterile, sharp, and rather small arthroscopic knife. The knife was used one time only for specimen harvesting and was then discarded. The tissue excised was taken from the base of the tibial stump to the ruptured end of the ACL, with as much of the ACL stump retrieved as possible. Gene expression analysis of ACL tissue was done with an optimized methodology for preservation of RNA through use of RNAlater buffer[14]. The detailed protocol from the manufacturer of RNAlater was followed strictly, with no variation from that procedure for any of the ACL specimens. Freezing of the samples occurred twenty-four hours after ACL immersion in RNAlater (according to the manufacturer's protocol). Meticulous attention to methodology was necessary to prevent RNA degradation and to reduce sample variability that could result in increased standard error and loss of statistical significance. ■

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Nationwide Children's Hospital

The Research Institute
Biomedical Genomics Core
Peter White, PhD (Director)

Statistical Analysis of Agilent Whole Genome Array Data
for Robin Jacquet
December 19, 2011

This analysis is based on microarrays that were processed in the Biomedical Genomics Core at The Research Institute at Nationwide Children's Hospital, Columbus, Ohio. Sample labeling and hybridizations were performed by *David Newsom* and data analysis by *Peter White*. Filenames and samples were as follows:

FileName	Sample	Condition	Design	Replicate
US85103615_252800415479_S01_GE1_1010_Sep10_1_1.txt	F161	Female	1	1
US85103615_252800415479_S01_GE1_1010_Sep10_1_2.txt	F162	Female	1	2
US85103615_252800415479_S01_GE1_1010_Sep10_1_3.txt	F163	Female	1	3
US85103615_252800415479_S01_GE1_1010_Sep10_1_4.txt	F165	Female	1	4
US85103615_252800415479_S01_GE1_1010_Sep10_2_1.txt	M148	Male	2	1
US85103615_252800415479_S01_GE1_1010_Sep10_2_2.txt	M149	Male	2	2
US85103615_252800415479_S01_GE1_1010_Sep10_2_3.txt	M166	Male	2	3
US85103615_252800415479_S01_GE1_1010_Sep10_2_4.txt	M166	Male	2	4

All analysis was performed using analysis scripts that were developed in-house using the R software environment for statistical computing and graphics. These scripts call on several Bioconductor (http://www.bioconductor.org/) packages. Bioconductor is an open source and open development software project to provide tools for the analysis and comprehension of genomic data (Gentleman *et al.*, 2004).

Sample Processing

The concentration of the samples provided was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. RNA samples were analyzed by the FGC using an Agilent 2100 Bioanalyzer Lab-On-A-Chip Agilent 6000 Series II chip to determine the integrity of the samples. The RNA was of high quality and all samples passed our QC cutoff. Sample labeling and hybridization was performed according to the manufacturer's protocols. **Samples were hybridized to the SurePrint G3 Human GE 8x60K Microarray (AMADID 028004).**

Scanning and Image Analysis

Microarray slides were hybridized overnight, washed and then scanned with an Agilent G2505C Microarray Scanner. This high resolution scanner represents the very latest technology from Agilent for Arrays and features an industry-leading extended dynamic range of 10^6 (20-bits) for high sensitivity scanning without saturation, low-level detection resulting from optimized precision optics, broad dynamic range, and minimal spectral cross talk that enables detection of weak features. The information about each probe on the array was extracted from the image data using Agilent Feature Extraction 10.10 (FE). This data is stored in the FE ".txt" files. The raw intensity values from these files are imported into the mathematical software package "R", which is used for all data input, diagnostic plots, normalization and quality checking steps of the analysis process using scripts developed in-house by Peter White specifically for this analysis.

It is very important that you keep a copy of the TXT files as they are required for deposition of array data into repositories such as ArrayExpress or GEO (this has become a requirement for publication in most journals). Furthermore, from these files all of the subsequent analysis can be recreated.

Data Preprocessing

Preprocessing refers to the procedures used to convert raw probe intensities into useful gene expression measurements. For analysis of Agilent Expression data this process consists of

1. Reading in the FE extraction files and extracting the median intensity values for each probe on the array. These intensities are not background corrected (this has been shown to only introduce noise), but are corrected for any scanner offset that was added to the original raw values (see Analysis Log file for details).
2. The dataset is filtered to remove positive control elements and any elements that have been flagged as outliers.
3. Present (P), Marginal (M) or Absent (A) calls are made for each element on the array using in-house methodologies that take into account both the results of FE probe detection statistics and the intensities of the negative control elements on the array.
4. Data normalization is the process of removing unwanted non-biological variation that might exist between arrays in a microarray experiment. Sources of such variation might include scanner-setting differences, the quantities of mRNA hybridized, processing order and many other factors. Regardless of the normalization approach used, the underlying assumption of the normalization algorithm is that the number of genes changing expression between conditions is relatively small or that an equivalent number of genes increase and decrease in expression. The algorithm for normalization is depending upon whether the design of the experiment was a one-color or two-color approach:
    a. Agilent One-Color Analysis: The median green (Cy3) intensities are normalized between the arrays using the Quantile Normalization package in "R" (Bolstad *et al.*, 2003). Quantile normalization is a non-linear probe-level normalization that results in the same empirical distribution of intensities for each array This is a significantly more robust approach than simply normalizing to the median value of each array.

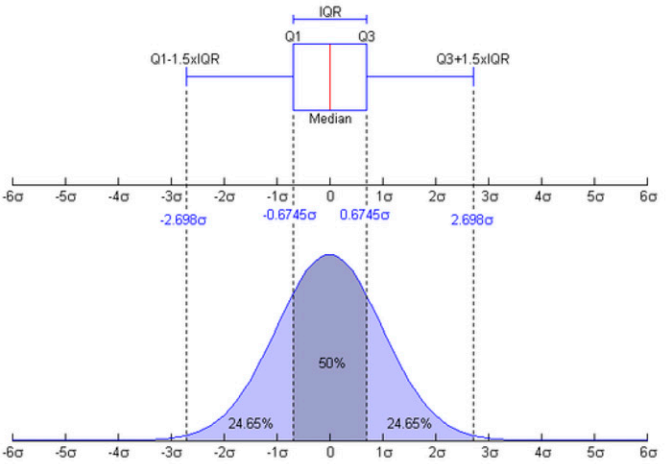
b. Agilent Two-Color Analysis: The median red (Cy5) and green (Cy3) intensities are used to calculate the ratio of expression for each element on the array, in terms of M (log2(Red/Green)) and A ((log2(Red) + log2(Green))/2)). The M values are then global loess normalized within each array using the LIMMA microarray processing package in "R" (Smyth, 2004). This intensity based normalization approach adjusts the red and green intensities relative to one another so that the red/green ratios are as far as possible an unbiased representation of relative expression.

Quality Control Diagnostics

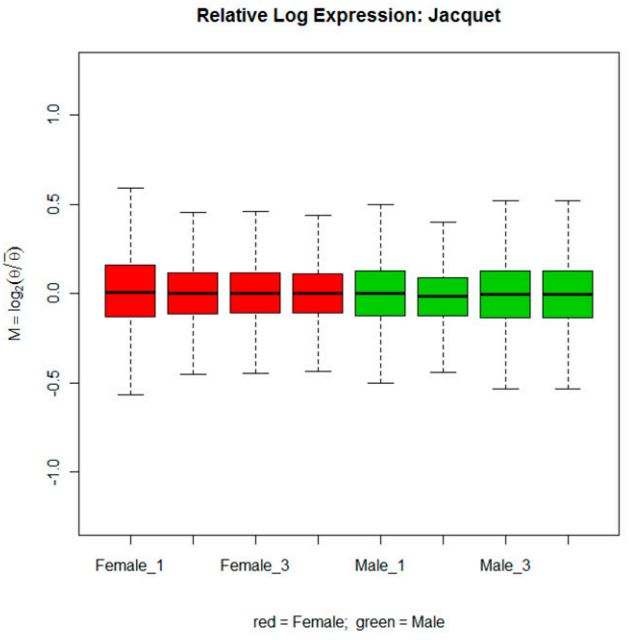
A frequently overlooked, yet critically important, additional step to data preprocessing is the ability to distinguish between good and poor quality arrays in a given data set. This is done by visualizing the data before and after the various preprocessing steps and through the generation of quality control (QC) metrics. In addition to the Agilent FE QC reports (see PDF files) we generate several QC plots that are designed to highlight outlying arrays or potential issues with samples.

Intensity Distribution Plots. One of the underlying assumptions for the normalization procedure is that the probe data all come from the same distribution, with the only differences being the location and scale. Basically, in the *Intensity Histogram* plots this means that we want the shape of the curves to be very similar, and we want the curves to be fairly close to each other.

Another useful visualization tool to display the distribution of the data is through the use of box plots. These plots are used to graphically depict numerical data using their five-number summaries: the sample minimum, lower quartile (Q1), median (Q2), upper quartile (Q3) and the sample maximum. Any observation that lies more than 1.5 x IQR lower than Q1 or higher than Q3 is considered an outlier, as indicated by the "whiskers". As such, they allow us to view the distribution of raw expression values and identify any arrays that are outliers. Furthermore, after normalization the distribution of probe intensities should be the same for all the arrays.



RLE Plots. Another quality assessment tool is Relative Log Expression (RLE) values. Specifically, these RLE values are computed for each probe set by comparing the expression value on each array against the median expression value for that probe set across all arrays. Assuming that most genes are not changing in expression across arrays means ideally most of these RLE values will be near 0. Boxplots of these values, for each array, provides a quality assessment tool. When examining this plot focus should be on the shape and position of each of the boxes. Typically arrays with poorer quality show up with boxes that are not centered about 0 and/or are more spread out. ***For this particular experiment, all array quality control metrics met or exceeded our quality control expectations.***



Statistical Analysis

Microarrays measure the expression of thousands of genes to identify changes in expression between different biological states. As such, methods are needed to determine the significance of these changes while accounting for the enormous number of genes tested. One significant challenge inherent to high-throughput analysis of large scale changes in gene expression is the development of statistical methods that will maximize both the sensitivity and specificity of detection of differentially expressed genes. Although several tools are available, we focus on two well validated and commonly utilized approaches to perform our statistical analysis:

Significance Analysis of Microarrays (SAM). SAM is a powerful tool for analyzing microarray gene expression data useful for identifying differentially expressed genes between two conditions (Tusher *et al.*, 2001). SAM calculates a test statistic for relative difference in gene expression based on permutation analysis of expression data and calculates a false discovery rate (FDR) using the q-value method presented in Storey (2003). In outline, SAM identifies statistically significant genes by carrying out gene specific t-tests and computes a statistic for each gene, which measures the strength of the relationship between gene expression and a response variable. This analysis uses non-parametric statistics, since the data may not follow a normal distribution. The response variable describes and groups the data based on experimental conditions. In this method, repeated permutations of the data are used to determine if the

expression of any gene is significantly related to the response. The use of permutation-based analysis accounts for correlations in genes and avoids parametric assumptions about the distribution of individual genes. For this experiment, SAM analysis was implemented in R using the Bioconductor Siggenes package. In a one-color experimental design a *two-class unpaired* analysis is typically performed for each experimental comparison, whereas in a two-color approach a *one-class* analysis is used. Typically, an FDR cutoff in the range of 10-20% is chosen to maximize sensitivity without significantly impacting accuracy. ***For the current study, a 10% FDR was used to generate the list of significantly differentially expressed genes. The q-values (FDR) for each gene are provided in the results table – the lower the value the more significant the result.***

Adjusted p-value. The moderated t-statistic (t) is computed for each probe and for each contrast in the experimental design. This has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, i.e., shrunk towards a common value (Smyth, 2004). This has the effect of borrowing information from the ensemble of genes to aid with inference about each individual gene. The p-value is obtained from the distribution of the moderated t-statistic. Finally these p-values are adjusted for multiple testing using Benjamini and Hochberg's (1995) step-up method for controlling the false discovery rate. This is the most popular method for p-value adjustment. If all genes with p-value below a threshold, say 0.05, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 5%. ***For the current study, the adjusted p-values were calculated using the Bioconductor limma package.***

Although we provide results using both methods, our recommendation is that one should initially focus on the gene list generated with the SAM FDR statistic, as this is often the most commonly reported. Furthermore, it can be considered to be more robust as it is a non-parametric approach and does not assume equal variance and/or independence of genes. Please note that prior to statistical analysis, all control probes were filtered out of the dataset.

Cluster Analysis

Hierarchical clustering was performed on the samples (arrays) using the "R" package "pvclust" (Suzuki and Shimodaira, 2006). This package calculates p-values for hierarchical clustering via multiscale bootstrap resampling. Hierarchical clustering is done for given data and p-values are computed for each of the clusters. It provides AU (approximately unbiased) p-values as well as BP (bootstrap probability) values computed via multiscale bootstrap resampling. The cluster dendrogram highlights AU p-values (printed in red) and BP values (printed in green), which are less accurate than AU values as p-values. One can consider that clusters (edges) with high AU values (e.g. 95%) are strongly supported by the data. Rectangles highlight those clusters with a highly significant p value (0.05), significant clusters within these highlighted clusters are not highlighted.

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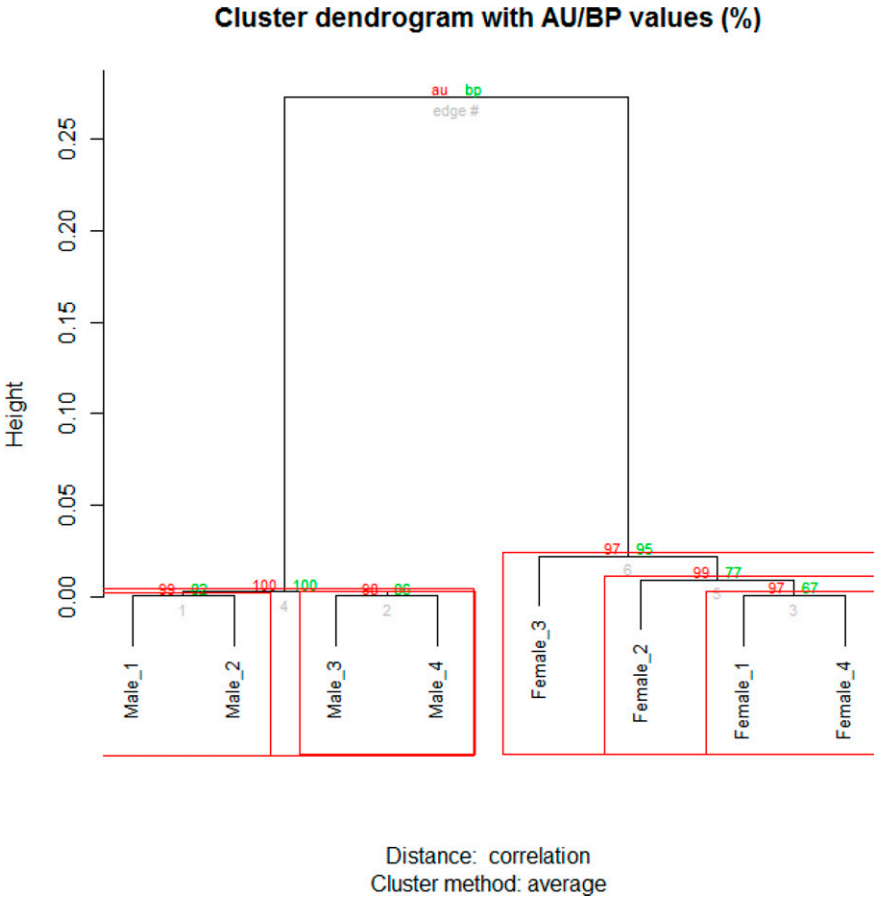

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