Class 12: RNASeq Analysis

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Background

Today we will analyze some RNASeq data from Himes et al. on the effects of a common steroid (Dexamethasone) on airway smooth muscle cells (ASM cells).

Our starting point is the "counts" data and "metadata" that contain the count values for each gene in their different experiments (i.e. cell lines with or without the drug).

Data import

```
# Complete the missing code
counts <- read.csv("airway_scaledcounts.csv", row.names=1)
metadata <- read.csv("airway_metadata.csv")
```

Let's have a peek at these objects:

```
head(counts)
```

```
                SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003        723        486        904        445       1170
ENSG00000000005          0          0          0          0          0
ENSG00000000419        467        523        616        371        582
ENSG00000000457        347        258        364        237        318
ENSG00000000460         96         81         73         66        118
ENSG00000000938          0          0          1          0          2
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1097        806        604
ENSG00000000005          0          0          0
ENSG00000000419        781        417        509
ENSG00000000457        447        330        324
ENSG00000000460         94        102         74
ENSG00000000938          0          0          0
```

```r
head(metadata)
```

```
          id     dex celltype     geo_id
1 SRR1039508 control   N61311 GSM1275862
2 SRR1039509 treated   N61311 GSM1275863
3 SRR1039512 control  N052611 GSM1275866
4 SRR1039513 treated  N052611 GSM1275867
5 SRR1039516 control  N080611 GSM1275870
6 SRR1039517 treated  N080611 GSM1275871
```

Q1.How many genes are in this dataset?

```r
nrow(counts)
```

```
[1] 38694
```

Q. How many different experiments (columns in counts or rows in metadata) are there?

```r
ncol(counts)
```

```
[1] 8
```

```r
nrow(metadata)
```

```
[1] 8
```

Q2. How many 'control' cell lines do we have?

```r
table(metadata$dex == "control")
```

```
FALSE  TRUE 
    4     4 
```

There are 4 control cell lines.

Toy differential gene expression

To start our analysis let's calculate the mean counts for all genes in the "control" experiments.

Step 1: Extract all "control" columns from the `counts` object. Step 2: Calculate the mean for all rows (i.e. genes) of these "control" columns. Steps 3-4: Do the same for "treated" Step 5: Compare there `control.mean` and `treated.mean` values.

```r
control.inds <- metadata$dex == "control"
control.counts <- counts[ , control.inds]
control.mean <- rowMeans(control.counts)
head(control.mean)
```

```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460 
         900.75            0.00          520.50          339.75           97.25 
ENSG00000000938 
           0.75 
```

Q3. How would you make the above code in either approach more robust? Is there a function that could help here?

Use `rowMeans()` instead of `rowSums(control.counts) / ncol(control.counts)`

Q4. Follow the same procedure for the treated samples (i.e. calculate the mean per gene across drug treated samples and assign to a labeled vector called treated.mean)

```r
treated.inds <- metadata$dex == "treated"
treated.counts <- counts[ , treated.inds]
treated.mean <- rowMeans(treated.counts)
head(treated.mean)
```

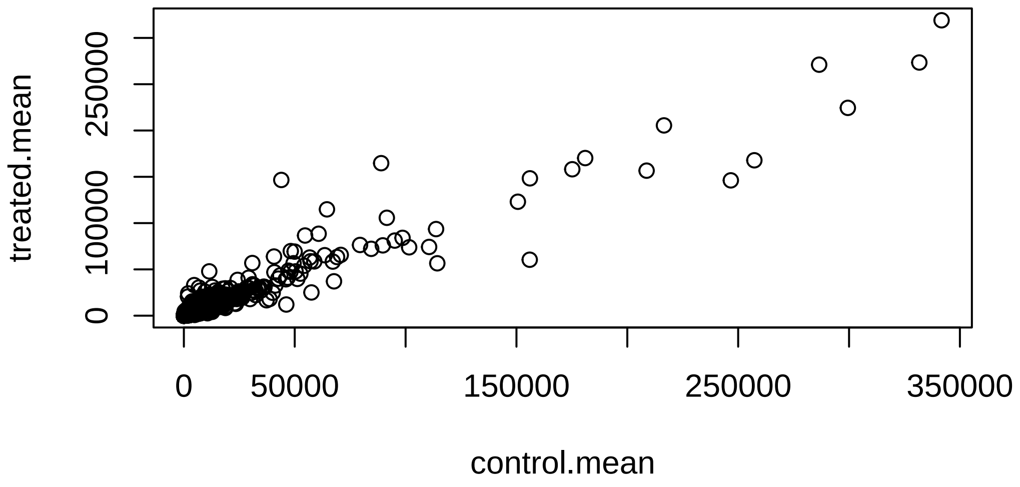
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         658.00            0.00          546.00          316.50           78.75
ENSG00000000938
           0.00
```

```
meancounts <- data.frame(control.mean, treated.mean)
colSums(meancounts)
```

```
control.mean treated.mean
    23005324     22196524
```

> Q5 (a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples. Your plot should look something like the following.

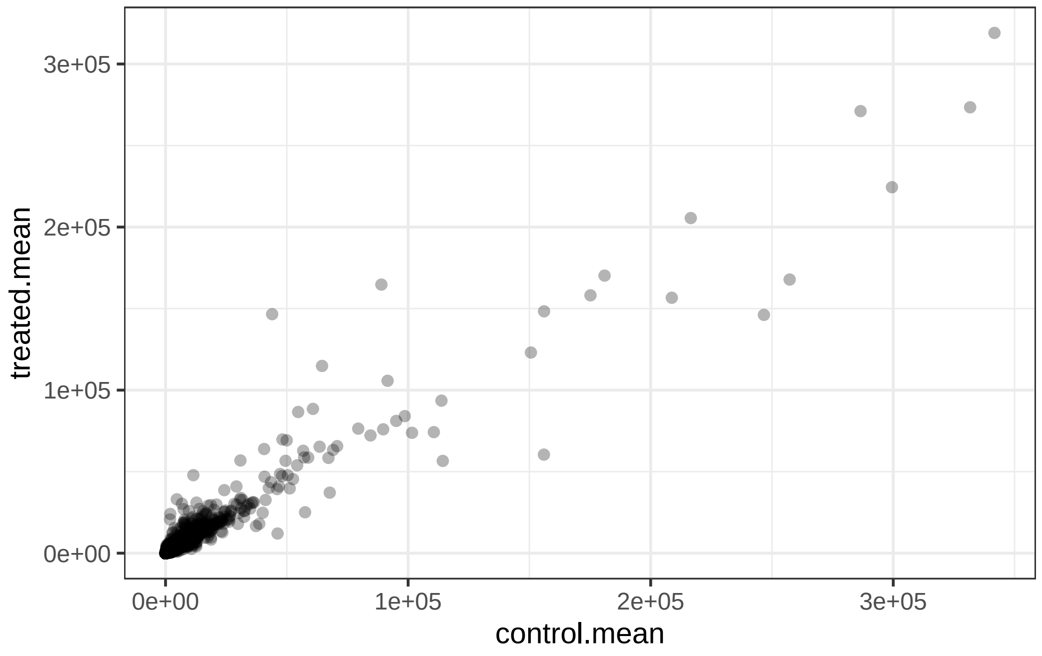
```
plot(meancounts)
```



> Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

We can ggplot and geom_point to generate the plot.

```r
library(ggplot2)
ggplot(meancounts,aes(control.mean, treated.mean)) +
  geom_point(alpha = 0.3) +
  theme_bw()
```



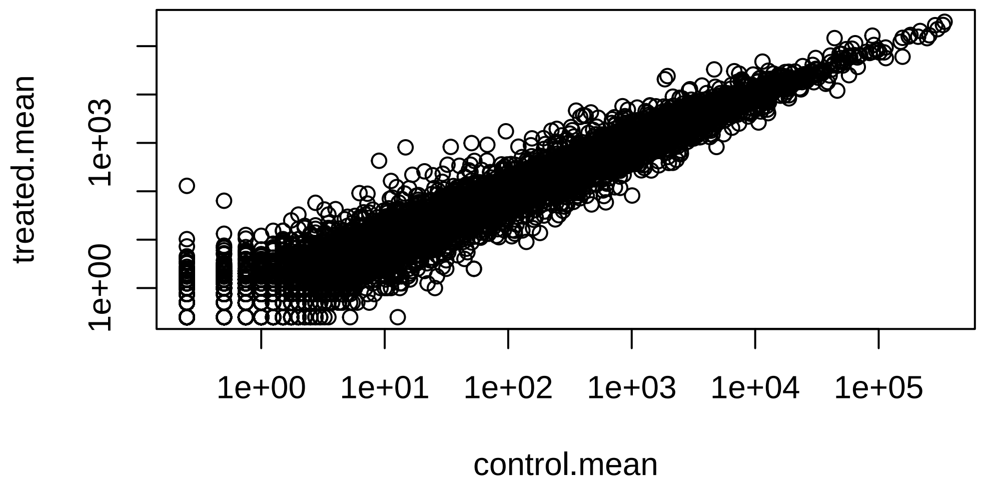
> Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

We can use `log` to do this.

```r
plot(meancounts, log="xy")
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 x values <= 0 omitted
from logarithmic plot
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 y values <= 0 omitted
from logarithmic plot
```



We often talk metrics like "log2 fold change"

```
# treated/control
log2(10/10)
```

```
[1] 0
```

```
log2(10/20)
```

```
[1] -1
```

```
log2(20/10)
```

```
[1] 1
```

```
log2(10/40)
```

```
[1] -2
```

```
log2(40/10)
```

```
[1] 2
```

Let's calculate the log2 change for our treated over control mean counts.

```
meancounts$log2fc <- log2(meancounts$treated.mean / meancounts$control.mean)
head(meancounts)
```

```
                control.mean treated.mean      log2fc
ENSG00000000003       900.75       658.00 -0.45303916
ENSG00000000005         0.00         0.00         NaN
ENSG00000000419       520.50       546.00  0.06900279
ENSG00000000457       339.75       316.50 -0.10226805
ENSG00000000460        97.25        78.75 -0.30441833
ENSG00000000938         0.75         0.00        -Inf
```

A common "rule of thumb" is a log2 fold change cutoff of +2 (upregulated genes) and -2 (downregulated genes).

Number of upregulated genes: Number of downregulated genes:

```
sum(meancounts$log2fc >= +2, na.rm=T)
```

```
[1] 1910
```

```
sum(meancounts$log2fc <= -2, na.rm=T)
```

```
[1] 2330
```

```
zero.vals <- which(meancounts[,1:2]==0, arr.ind=TRUE)

to.rm <- unique(zero.vals[,1])
mycounts <- meancounts[-to.rm,]
head(mycounts)
```

```
                control.mean treated.mean      log2fc
ENSG00000000003       900.75       658.00 -0.45303916
ENSG00000000419       520.50       546.00  0.06900279
ENSG00000000457       339.75       316.50 -0.10226805
ENSG00000000460        97.25        78.75 -0.30441833
ENSG00000000971      5219.00      6687.50  0.35769358
ENSG00000001036      2327.00      1785.75 -0.38194109
```

> Q7. What is the purpose of the arr.ind argument in the which() function call above? Why would we then take the first column of the output and need to call the unique() function?

The purpose of the arr.ind argument will tell us which genes (rows) and samples (columns) have zero counts. We are going to ignore any genes that have zero counts in any sample so we just focus on the row answer. That way we can avoid issues with weird answers like -inf and NaN.

> Q8. Using the up.ind vector above can you determine how many up regulated genes we have at the greater than 2 fc level? Q9. Using the down.ind vector above can you determine how many down regulated genes we have at the greater than 2 fc level?

```r
up.ind <- mycounts$log2fc > 2
sum(up.ind)
```

```
[1] 250
```

```r
down.ind <- mycounts$log2fc < (-2)
sum(down.ind)
```

```
[1] 367
```

> Q10. Do you trust these results? Why or why not?

While this provides some interesting information, a key limitation is we do not actually know how significant are the differences (missing p-values and other statisitcal analysis) between the means of the control and treated groups.Means are not a robust measure of center, and are susceptible to outliers. This can lead us to make incorrect conclusions.Fold change can be large (e.g. »two-fold up- or down-regulation) without being statistically significant (e.g. based on p-values).

```r
library(DESeq2)
```

For DESeq analysis we need three things: - count values (`countData`) - metadata telling us about the columns in `countData` (`colData`) - design of the experiment (i.e. what do you want to compare)

Our first function from DEseq2 will setup the input required for analysis by storing all these 3 things together.

```r
dds <- DESeqDataSetFromMatrix(countData = counts,
                              colData = metadata,
                              design = ~dex)
```

```
converting counts to integer mode
```

```
Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
design formula are characters, converting to factors
```

The main function in DESeq2 that runs the analysis is called `DESeq()`

```r
dds <- DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```r
res <- results(dds)
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195      -0.350703  0.168242 -2.084514 0.0371134
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160       0.206107  0.101042  2.039828 0.0413675
ENSG00000000457 322.664844       0.024527  0.145134  0.168996 0.8658000
ENSG00000000460  87.682625      -0.147143  0.256995 -0.572550 0.5669497
ENSG00000000938   0.319167      -1.732289  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163017
ENSG00000000005        NA
ENSG00000000419  0.175937
ENSG00000000457  0.961682
ENSG00000000460  0.815805
ENSG00000000938        NA
```

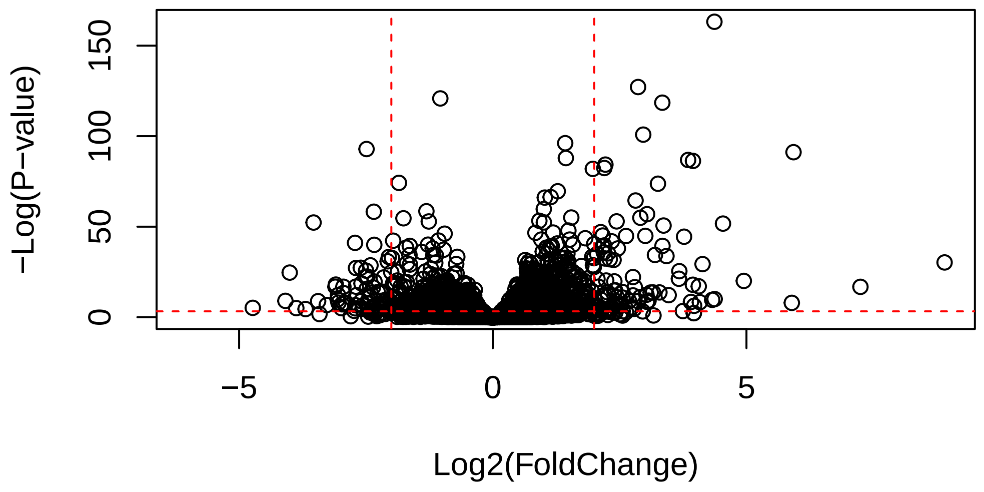
padj values are stricter to help establish which genes we should consider.

Volcano Plot

This is a common summary result figure from these types of experiments and plot the log2 fold-change vs the adjusted p-value.

```r
plot(res$log2FoldChange, -log(res$padj),xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")

abline(v=c(-2,2), col="red", lty=2)
abline(h=-log(0.04), col="red", lty=2)
```

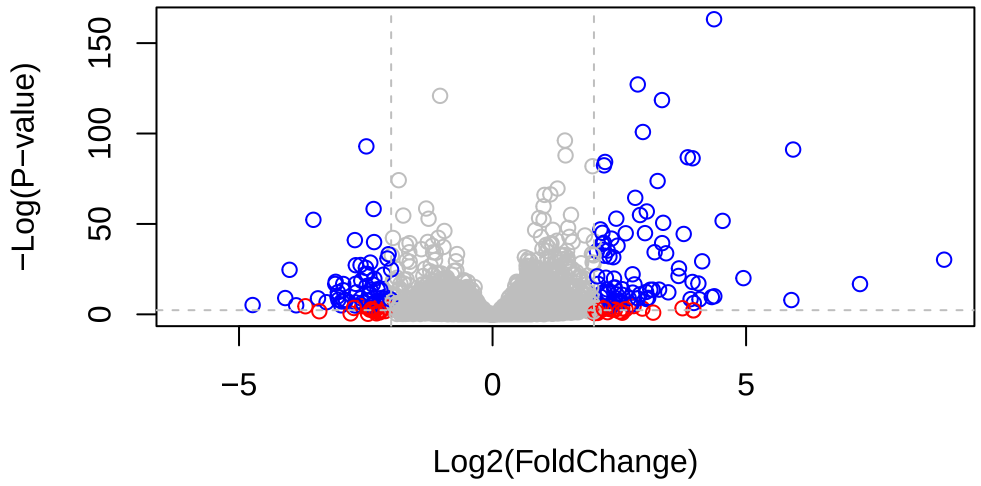


```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

inds <- (res$padj < 0.01) & (abs(res$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"

# Volcano plot with custom colors
plot( res$log2FoldChange,  -log(res$padj),
 col=mycols, ylab="-Log(P-value)", xlab="Log2(FoldChange)" )

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.1), col="gray", lty=2)
```



Save our results

```
write.csv(res, file="my_results.csv")
```

Add gene annotation

To help make sense of the results and communicate them to other people we need to add some more annotations to our main `res` object.

We will use two bioconductor packages to first map IDs to different formats, including the classic gene "symbol" gene name.

Install the following in the console. `BiocManager::install("AnnotationDbi")` `BiocManager::install("org.`

```
library(AnnotationDbi)
library(org.Hs.eg.db)
```

Let's see what is in `org.Hs.eg.db` with the `columns()` function:

```r
columns(org.Hs.eg.db)
```

```
 [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
 [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
[11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
[16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
[21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
[26] "UNIPROT"
```

We can translate or "map" IDs between any of these 26 databases using the `mapIds()` function.

```r
res$symbol <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "SYMBOL"      # the format/DB to map to
      )
```

```
'select()' returned 1:many mapping between keys and columns
```

```r
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 7 columns
                 baseMean log2FoldChange     lfcSE      stat    pvalue
                <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195      -0.350703  0.168242 -2.084514 0.0371134
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160       0.206107  0.101042  2.039828 0.0413675
ENSG00000000457 322.664844       0.024527  0.145134  0.168996 0.8658000
ENSG00000000460  87.682625      -0.147143  0.256995 -0.572550 0.5669497
ENSG00000000938   0.319167      -1.732289  3.493601 -0.495846 0.6200029
                     padj      symbol
                <numeric> <character>
ENSG00000000003  0.163017      TSPAN6
ENSG00000000005        NA        TNMD
ENSG00000000419  0.175937        DPM1
```

```
ENSG00000000457  0.961682       SCYL3
ENSG00000000460  0.815805       FIRRM
ENSG00000000938        NA         FGR
```

Add mappings for "GENENAME" and "ENTREZID" and store as `res$genename` and `res$entrez`

```
res$genename <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "GENENAME"    # the format/DB to map to
      )
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$entrez <- mapIds(keys = row.names(res), # our current Ids
       keytype = "ENSEMBL" ,  # the format of our Ids
       x = org.Hs.eg.db,      # where to get the mappings from
       column = "ENTREZID"    # the format/DB to map to
      )
```

```
'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 9 columns
                 baseMean log2FoldChange     lfcSE      stat    pvalue
                <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195      -0.350703  0.168242 -2.084514 0.0371134
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160       0.206107  0.101042  2.039828 0.0413675
ENSG00000000457 322.664844       0.024527  0.145134  0.168996 0.8658000
ENSG00000000460  87.682625      -0.147143  0.256995 -0.572550 0.5669497
ENSG00000000938   0.319167      -1.732289  3.493601 -0.495846 0.6200029
                     padj      symbol               genename      entrez
                <numeric> <character>            <character> <character>
ENSG00000000003  0.163017      TSPAN6          tetraspanin 6        7105
ENSG00000000005        NA        TNMD            tenomodulin       64102
```

```
ENSG00000000419  0.175937        DPM1 dolichyl-phosphate m..        8813
ENSG00000000457  0.961682       SCYL3 SCY1 like pseudokina..       57147
ENSG00000000460  0.815805       FIRRM FIGNL1 interacting r..       55732
ENSG00000000938        NA         FGR FGR proto-oncogene, ..        2268
```

Pathway analysis

There are lots of bioconductor packages to do this type analysis. For now, let's try one called **gage** again we need to install this if we don't have it already.

```
library(gage)
library(gageData)
library(pathview)
```

To use **gage** I need two things:

- a named vector of fold-change values for our DEGs (our geneset of interest)
- a set of pathways or genesets to use for annotation.

```
foldchanges <- res$log2FoldChange
names(foldchanges) <- res$entrez
head(foldchanges)
```

```
       7105       64102        8813       57147       55732        2268
-0.35070296          NA  0.20610728  0.02452701 -0.14714263 -1.73228897
```

```
data(kegg.sets.hs)

keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

In our results object we have:

```
attributes(keggres)
```

```
$names
[1] "greater" "less"    "stats"
```

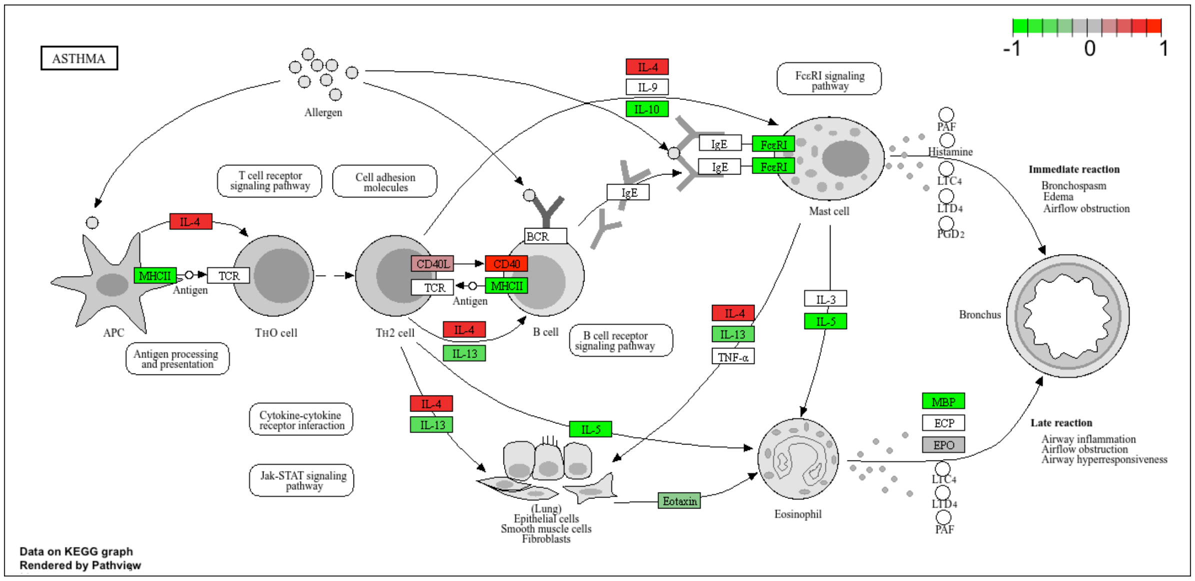
```
head(keggres$less, 5)
```

```
                                                      p.geomean stat.mean
hsa05332 Graft-versus-host disease                 0.0004250607 -3.473335
hsa04940 Type I diabetes mellitus                  0.0017820379 -3.002350
hsa05310 Asthma                                    0.0020046180 -3.009045
hsa04672 Intestinal immune network for IgA production 0.0060434609 -2.560546
hsa05330 Allograft rejection                       0.0073679547 -2.501416
                                                          p.val      q.val
hsa05332 Graft-versus-host disease                 0.0004250607 0.09053792
hsa04940 Type I diabetes mellitus                  0.0017820379 0.14232788
hsa05310 Asthma                                    0.0020046180 0.14232788
hsa04672 Intestinal immune network for IgA production 0.0060434609 0.31387487
hsa05330 Allograft rejection                       0.0073679547 0.31387487
                                                   set.size         exp1
hsa05332 Graft-versus-host disease                       40 0.0004250607
hsa04940 Type I diabetes mellitus                        42 0.0017820379
hsa05310 Asthma                                          29 0.0020046180
hsa04672 Intestinal immune network for IgA production       47 0.0060434609
hsa05330 Allograft rejection                             36 0.0073679547
```

Let's look at one of these pathways with our genes colored up so we can see the overlap

```r
pathview(pathway.id = "hsa05310", gene.data = foldchanges)
pathview(gene.data=foldchanges, pathway.id="hsa05310")
```

Add this pathway figure to our lap report



Save our main results

```
write.csv(res, file="myresults_annotated.csv")
```