Class 12: Transcriptomics and the analysis of RNA-Seq data

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Background

Today we will analyze some RNAseq data from Himes et al. on the effects of a common steroid (dexmethasone also called "dex") on airway smoot muscle cells (ASMs).

For this analysis we need to main inputs

- `countData`: a table of **counts** per gene(in rows) accross experiments (in columns) (amount of genes in rows and experiments in columns)
- `colData`: **metadata** about the design of the experiments. The rows match the columns in `countData`

Data Import

```
counts <- read.csv("airway_scaledcounts.csv", row.names = 1)
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
```

and the `metadata`

```
metadata <- read.csv("airway_metadata.csv")
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?

```
nrow(counts)
```

```
[1] 38694
```

Q2. How many experiments (i.e columns are in `counts` or rows in `metadata`) are there?

```
ncol(counts)
```

```
[1] 8
```

Q3. How many "control" experiments are there in the dataset?

```
sum(metadata$dex == "control")
```

```
[1] 4
```

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

Toy analysis example

1. Extract the "control" columns from `counts`
2. Calculate the mean value for each gene in these "control" columns

3-4. Do the same for the "treated" columns 5. Compare these mean values for each gene

Step 1.

```
control.inds <- metadata$dex == "control"
control.counts <- counts[ , control.inds]
```

Step 2.

```
control.mean <- rowMeans(control.counts)
```

Step 3.

```
treated.inds <- metadata$dex == "treated"
treated.counts <- counts[ , treated.inds]
```

Step 4.

```
treated.mean <- rowMeans(treated.counts)
```

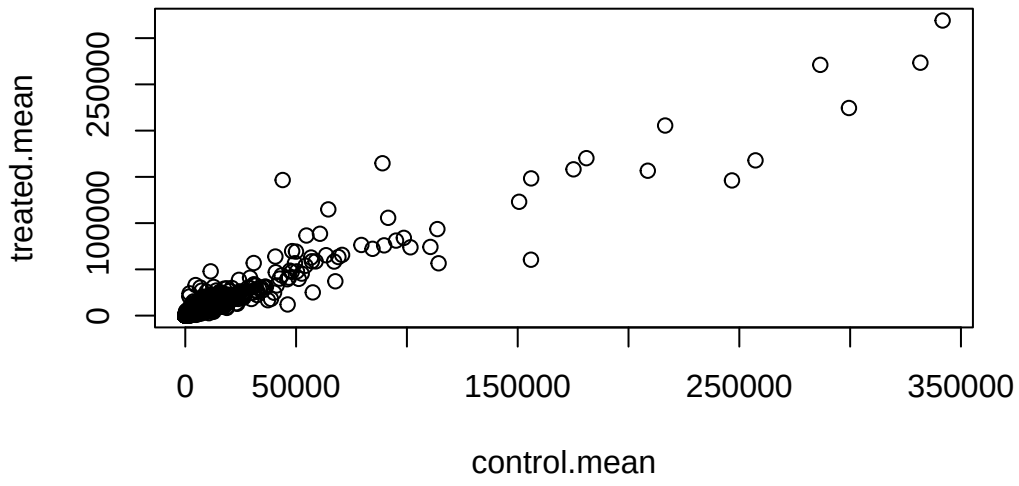
For ease of book-keeping we can store these together in one data frame called `meancounts`

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

```
                control.mean treated.mean
ENSG00000000003       900.75       658.00
ENSG00000000005         0.00         0.00
ENSG00000000419       520.50       546.00
ENSG00000000457       339.75       316.50
ENSG00000000460        97.25        78.75
ENSG00000000938         0.75         0.00
```

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?

geo_point

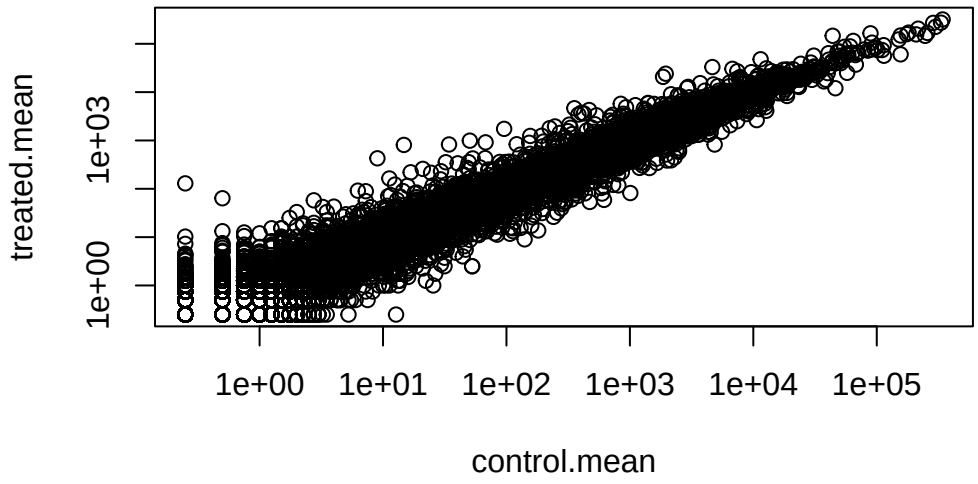
This is screaming at me to log transform this data!

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

```
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 use log2 "fold-change" as a way to compare

```
#treated/control
log2(10/10) #no change
```

```
[1] 0
```

```
log2(20/10) #up regulated
```

```
[1] 1
```

```
log2(10/20) #down regulated
```

```
[1] -1
```

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

```
[1] 2
```

```
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
```

Filter out zeero count genes

```
nonzero.inds <- rowSums(counts) != 0
mycounts <- meancounts [nonzero.inds,]
```

```
zero.inds <- which(meancounts[,1:2] == 0, arr.ind = T)[,1]
mygenes <- meancounts[-zero.inds,]
```

```
y <- data.frame(a=c(1,5,0,5), b=c(1,0,5,5))
y
```

```
  a b
1 1 1
2 5 0
3 0 5
4 5 5
```

```
which(y==0, arr.ind=TRUE)
```

```
     row col
[1,]   3   1
[2,]   2   2
```

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 arr.ind = TRUE option makes which() return a matrix of (row, column) positions of zeros instead of a single index vector. We take the first column to get the row numbers of those zeros, and use unique() so each row is only listed once before removing it.

A common "rule-of-thumb" threshold for calling something "up" regulated is a log2-fold-change of +2 or greater. For "down" regulated -2 or less.

Q8. How many genes are "up" regulated at the +2 log2FC threshold?

```
sum(mygenes$log2fc >= 2)
```

```
[1] 314
```

Q9. How many genes are "down" regulated at the -2 log2FC threshold?

```
sum(mygenes$log2fc <= -2)
```

```
[1] 485
```

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

Can't trust it yet until we make sure the differences are statistically significant with further analysis.

DESeq analysis

Let's do this woth DESeq2 and put some stats behind these numbers.

```
library(DESeq2)
```

```
Warning: package 'IRanges' was built under R version 4.4.2
```

```
Warning: package 'GenomeInfoDb' was built under R version 4.4.2
```

```
Warning: package 'MatrixGenerics' was built under R version 4.4.2
```

DESeq wants 3 things for analysis, countData, colData and design.

```
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 the DESeq package to rn analysis is called `DESeq()`.

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

Q11. Run the mapIds() function two more times to add the Entrez ID and UniProt accession and GENENAME as new columns called res$entrez$, res$uniprot and res$genename.

Get the results of this DESeq object with the function `results()`

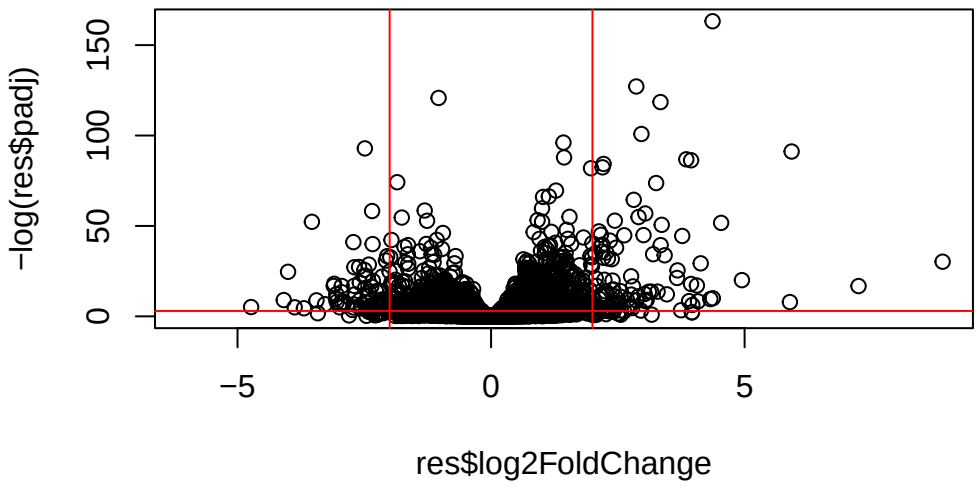
```
res <- results(dds)
head(res)
```

```
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     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163035
ENSG00000000005        NA
ENSG00000000419  0.176032
ENSG00000000457  0.961694
ENSG00000000460  0.815849
ENSG00000000938        NA
```

Volcano Plot

This is a plot of log2FC vs adjusted p-value

```
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(-2,2), col="red")
abline(h=-log(0.05), col = "red")
```



Save our results

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

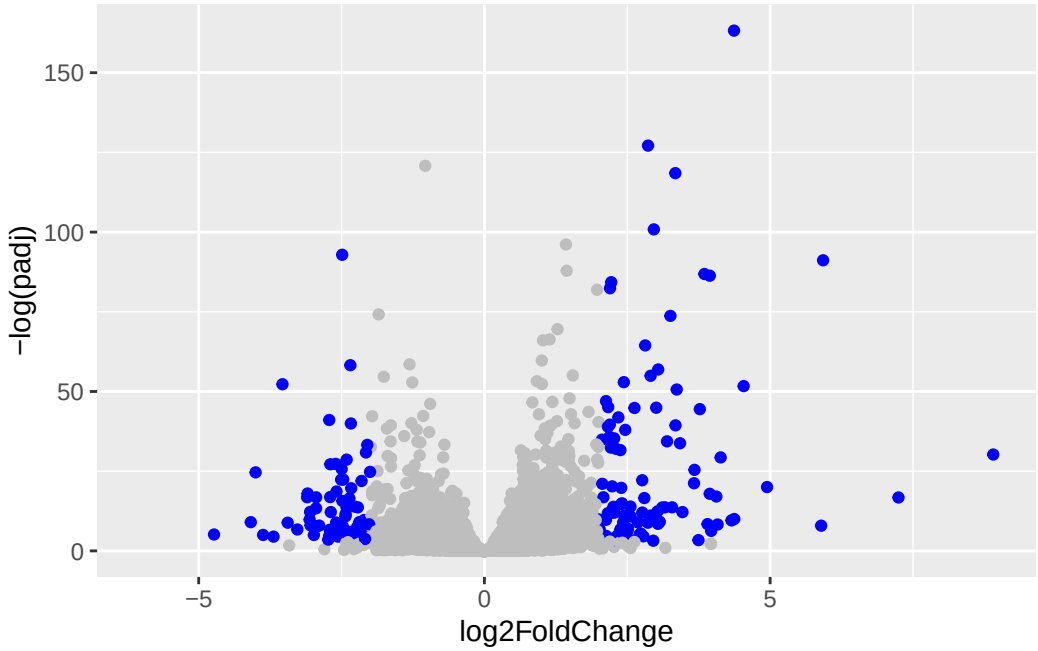
A nicer ggplot volcano plot

```
library(ggplot2)

mycols <- rep ("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ] <- "blue"
mycols[ res$padj >= 0.05 ] <- "gray"

ggplot(res) +
  aes(log2FoldChange, -log(padj)) +
  geom_point(col = mycols)
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



Add annotation data

We need to add gene symbols, gene names and other database ids to make my results useful for further analysis.

```
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     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163035
ENSG00000000005        NA
```

```
ENSG00000000419  0.176032
ENSG00000000457  0.961694
ENSG00000000460  0.815849
ENSG00000000938        NA
```

```
head(rownames(res))
```

```
[1] "ENSG00000000003" "ENSG00000000005" "ENSG00000000419" "ENSG00000000457"
[5] "ENSG00000000460" "ENSG00000000938"
```

We can use the `mapIds()` function from bioconductor to help us.

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

Let's see what database id formats we can translate between

```
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"
```

```
res$symbol <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="SYMBOL")          # The new format we want to add
```

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

```
head(res$symbol)
```

```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
       "TSPAN6"          "TNMD"          "DPM1"         "SCYL3"         "FIRRM"
ENSG00000000938
          "FGR"
```

Add `GENENAME` then `ENTREZID`

```
res$genename <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="GENENAME")          # The new format we want to add
```

```
'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 8 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj      symbol               genename
                <numeric> <character>            <character>
ENSG00000000003  0.163035      TSPAN6          tetraspanin 6
ENSG00000000005        NA        TNMD            tenomodulin
ENSG00000000419  0.176032        DPM1 dolichyl-phosphate m..
ENSG00000000457  0.961694       SCYL3 SCY1 like pseudokina..
ENSG00000000460  0.815849       FIRRM FIGNL1 interacting r..
ENSG00000000938        NA         FGR FGR proto-oncogene, ..
```

```
res$entrezid <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="ENTREZID")          # The new format we want to add
```

```
'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     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj      symbol               genename    entrezid
                <numeric> <character>            <character> <character>
ENSG00000000003  0.163035      TSPAN6          tetraspanin 6        7105
ENSG00000000005        NA        TNMD            tenomodulin       64102
ENSG00000000419  0.176032        DPM1 dolichyl-phosphate m..        8813
ENSG00000000457  0.961694       SCYL3 SCY1 like pseudokina..       57147
ENSG00000000460  0.815849       FIRRM FIGNL1 interacting r..       55732
ENSG00000000938        NA         FGR FGR proto-oncogene, ..        2268
```

Save my annotated results

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

Pathway analysis

We will use the **gage** function from bioconductor.

```
library(gage)
```

```
library(gageData)
```

```
library(pathview)
```

```
Warning: package 'pathview' was built under R version 4.4.2
```

```
##############################################################################
Pathview is an open source software package distributed under GNU General
Public License version 3 (GPLv3). Details of GPLv3 is available at
http://www.gnu.org/licenses/gpl-3.0.html. Particullary, users are required to
formally cite the original Pathview paper (not just mention it) in publications
or products. For details, do citation("pathview") within R.

The pathview downloads and uses KEGG data. Non-academic uses may require a KEGG
license agreement (details at http://www.kegg.jp/kegg/legal.html).
##############################################################################
```

```
data(kegg.sets.hs)

# Examine the first 2 pathways in this kegg set for humans
head(kegg.sets.hs, 2)
```

```
$`hsa00232 Caffeine metabolism`
[1] "10"   "1544" "1548" "1549" "1553" "7498" "9"

$`hsa00983 Drug metabolism - other enzymes`
 [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
 [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
[17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
[25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
[33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
[41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
[49] "8824"   "8833"   "9"      "978"
```

What **gage** wants as input is a named vector of importance i.e. a vector with labeled fold-changes.

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

```
[1] -0.35070302          NA  0.20610777  0.02452695 -0.14714205 -1.73228897
```

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

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

```
                                      p.geomean stat.mean p.val q.val
hsa00232 Caffeine metabolism                 NA       NaN    NA    NA
hsa00983 Drug metabolism - other enzymes     NA       NaN    NA    NA
hsa01100 Metabolic pathways                  NA       NaN    NA    NA
hsa00230 Purine metabolism                   NA       NaN    NA    NA
hsa05340 Primary immunodeficiency            NA       NaN    NA    NA
                                      set.size exp1
hsa00232 Caffeine metabolism                 0   NA
hsa00983 Drug metabolism - other enzymes     0   NA
hsa01100 Metabolic pathways                  0   NA
hsa00230 Purine metabolism                   0   NA
hsa05340 Primary immunodeficiency            0   NA
```

Let's look at just one of these hsa05310

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

```
Warning: None of the genes or compounds mapped to the pathway!
Argument gene.idtype or cpd.idtype may be wrong.
```

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

```
Info: Working in directory /Users/mariatavares/Desktop/BGGN213/class12
```

```
Info: Writing image file hsa05310.pathview.png
```

Insert figure for this pathway

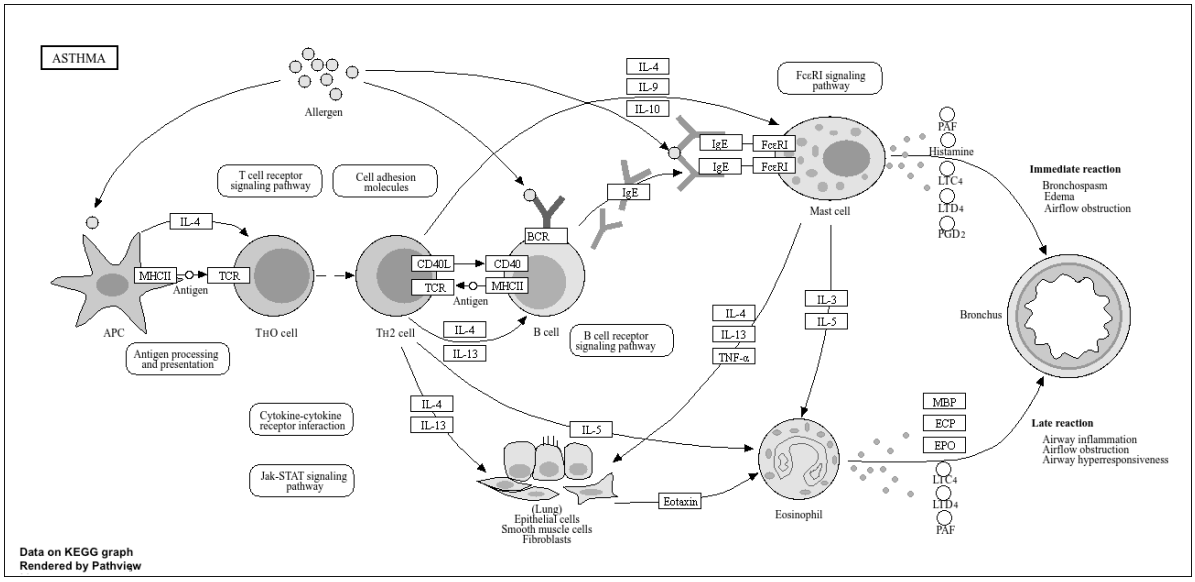


Figure 1: Asthma pathway from KeGG with my differentially expressed genes highlighted