Results of pathway analysis of Kimball ATVG dataset

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Abstract

Here we found that only two genes, PGAM1 and CHCHD2, were significantly differentially expressed in DIO mice vs. ND mice when comparing the differential expression between the two mice of the difference in expression between Ly6C$^{\mathrm{Hi}}$ and Ly6C$^{\mathrm{Lo}}$ cells. Specifically, PGAM1 was upregulated and CHCHD2 was downregulated. It is known that upregulation of PGAM1 induces the upregulation of IL-1β expression and apoptotic cell death (Song et al., 2018). Similarly, it is known that when mitochondrial CHCHD2 is downregulated, apoptosis increases (Liu Y, 2015). It then makes sense that these two genes are most differentially expressed when comparing DIO and ND mice, as both of these gene regulation differences increase inflammation and apoptosis, which are associated with the initial tissue destruction phase of wound healing.

1 Objective

We went ahead and went through the pathway analysis pipeline based on your pipeline as well as the workflow given by https://dockflow.org/workflow/rnaseq-gene-edgerql and have gotten results, however we're not entirely sure where to go from here. Through this writeup, we would like to show you all the results we obtained and how we obtained them to verify that we obtained them correctly, as well as ask what direction we might take from here.

2 Converting RNASeq data to read counts

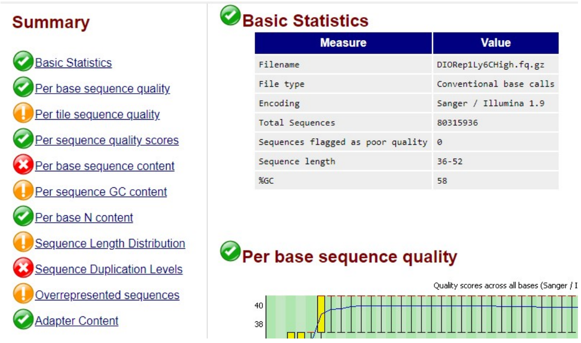
Here we'd just like to list all the commands we used to verify that we're indeed using them correctly. This is the first time we've done any sort of trimming and alignment and so the commands we used mainly were copy-pasted from random forums online, trying to find the most concise versions closest to default settings.

2.1 FastQC and Trimming

We first ran through the RNASeq with fastqc to see what the quality was. We found that some adapter sequences still existed so we trimmed them off with Trimmomatic with the following command:

```
 java -jar trimmomatic-0.39.
↪ jar SE -phred33 in.fastq.gz
↪  trimmed.fq.gz ILLUMINACLIP:
↪ TruSeq3-SE:2:30:10 LEADING:3
↪ TRAILING:3 SLIDINGWINDOW:4:15
↪ MINLEN:36
```

After that, we ran it through fastqc again and generated the following report:



Summary

- ✓ Basic Statistics
- ✓ Per base sequence quality
- ! Per tile sequence quality
- ✓ Per sequence quality scores
- ✗ Per base sequence content
- ! Per sequence GC content
- ✓ Per base N content
- ! Sequence Length Distribution
- ✗ Sequence Duplication Levels
- ! Overrepresented sequences
- ✓ Adapter Content

✓ Basic Statistics

Measure	Value
Filename	DIORep1Ly6CHigh.fq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	80315936
Sequences flagged as poor quality	0
Sequence length	36-52
%GC	58

✓ Per base sequence quality

Figure 1: FastQC report from trimmed data

What was concerning was that Per base sequence content and Sequence Duplication Levels were marked as X. It seems that this is experiment dependent, so we went ahead with the analysis with the assumption that this is expected. However we're curious whether this is ok.

2.2 Alignment read counting

We then aligned the data using hisat, using the **built in** mm10 genome.

```
  ./hisat2-2.0.4/hisat2 -x mm10
↪ /genome -U trimmed.fq.gz -S
↪ aligned.sam
```

Then we converted it to bam format:

```
  samtools view -bS aligned.sam >
↪  compressed.bam
```

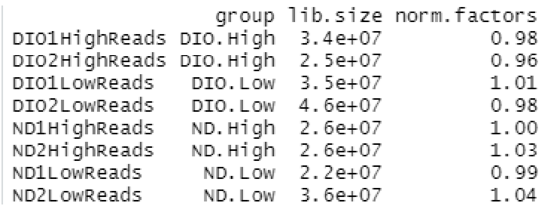
Lastly, we counted it with the following featureCounts command with the in-built mm10 RefSeq exon annotation:

```
  featureCounts -a annotation/
↪ mm10_RefSeq_exon.txt -o reads.
↪ txt ./*.bam -F SAF
```

This gave us our read count file.

3 Data Cleaning

Once we read in the read count data into a DGEList object, several other processes were performed in order to refine the data further. After mapping our Entrez Gene Ids to Gene Symbols using the NCBI database, we removed low count genes that didn't have a CPM above 0.5 for at least two libraries, and then normalized our data for composition bias.



```
                   group lib.size norm.factors
DIO1HighReads DIO.High  3.4e+07         0.98
DIO2HighReads DIO.High  2.5e+07         0.96
DIO1LowReads   DIO.Low  3.5e+07         1.01
DIO2LowReads   DIO.Low  4.6e+07         0.98
ND1HighReads   ND.High  2.6e+07         1.00
ND2HighReads   ND.High  2.6e+07         1.03
ND1LowReads     ND.Low  2.2e+07         0.99
ND2LowReads     ND.Low  3.6e+07         1.04
```

Figure 2: Normalization Factors of Samples

4 Data Exploration

We then plotted our data to see if there were interesting patterns. Plotting the data with an MDS plot we found that one of the DIO Ly6C High replicates had a significant amount of upregulated genes. (Figure 3).

5 Dispersion Estimations

We found that our data had high dispersion (Figure 4), or at least higher than the workflow we referenced. Since the workflow we referenced mentioned that quasi-likelihood F-tests were better than the more popular likelihood-ratio tests for data with high dispersion, we decided to follow suit and use QLF-tests as well.

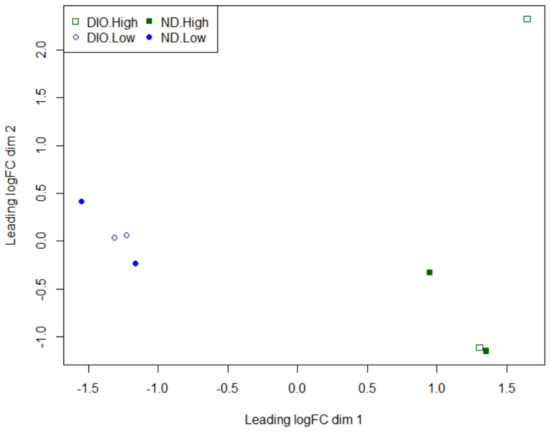


Figure 3: MDS Plot of Samples

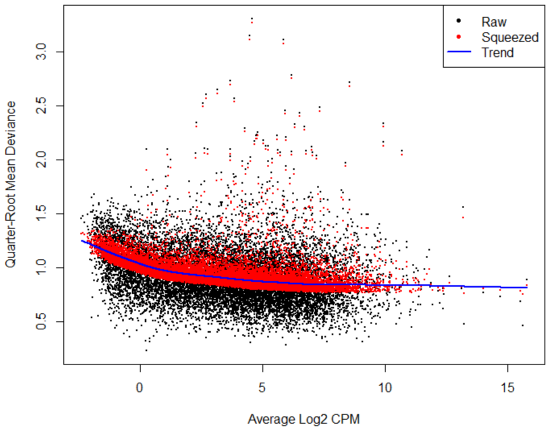


Figure 4: Dispersion Plot

6 Differentially expressed data

We looked into the differential expression between (DIO Ly6C^{Hi} - DIO Ly6C^{Lo}) and (ND Ly6C^{Hi} - ND Ly6C^{Lo}) in order to remove the variability from just DIO vs. ND genes or Ly6C^{Hi} vs. Ly6C^{Lo}, and instead see how the change in expression was different between DIO and ND groups.

Running our data through glmQLF tests taking into account QL dispersion, we ended up finding that only two genes were significantly differentially expressed under default conditions, shown in the MD plot (Figure 5). The next most significantly differentially expressed genes had FDR cutoffs above 0.05 (Figure 6).

Specifically, of the only two significantly differentially expressed genes, PGAM1 was upregulated

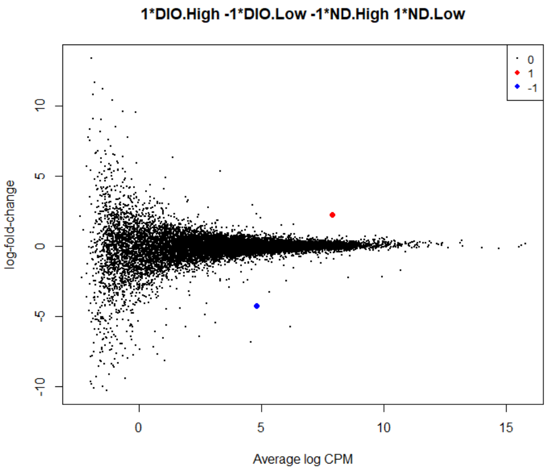
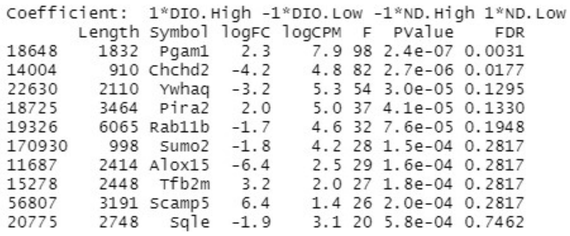


Figure 5: Differential expression MD plot



```
Coefficient:  1*DIO.High -1*DIO.Low -1*ND.High 1*ND.Low
       Length Symbol logFC logCPM  F  PValue    FDR
18648    1832  Pgam1   2.3    7.9 98 2.4e-07 0.0031
14004     910 Chchd2  -4.2    4.8 82 2.7e-06 0.0177
22630    2110  Ywhaq  -3.2    5.3 54 3.0e-05 0.1295
18725    3464  Pira2   2.0    5.0 37 4.1e-05 0.1330
19326    6065 Rab11b  -1.7    4.6 32 7.6e-05 0.1948
170930    998  Sumo2  -1.8    4.2 28 1.5e-04 0.2817
11687    2414 Alox15  -6.4    2.5 29 1.6e-04 0.2817
15278    2448  Tfb2m   3.2    2.0 27 1.8e-04 0.2817
56807    3191 Scamp5   6.4    1.4 26 2.0e-04 0.2817
20775    2748   Sqle  -1.9    3.1 20 5.8e-04 0.7462
```

Figure 6: Top differentially expressed genes

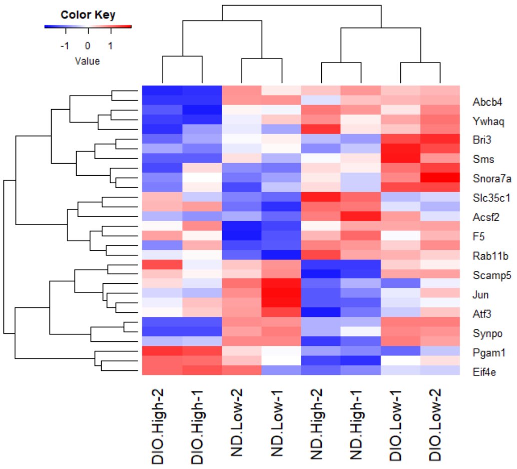


Figure 7: Heatmap

and CHCHD2 was downregulated. It appears that upregulation of PGAM1 induces the upregulation of IL-1β expression and apoptotic cell death (Song et al., 2018). Similarly, it appears that when mitochondrial CHCHD2 is downregulated, apoptosis increases (Liu Y, 2015). It then makes sense that these two genes are most differentially expressed when comparing DIO and ND mice, as both of these gene regulation differences increase inflammation and apoptosis, which are associated with the initial tissue destruction phase of wound healing.

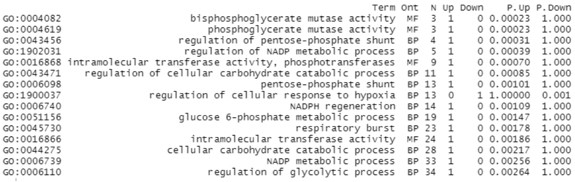
7 Heatmap clustering

Figure 7 is the heatmap showing the differential expression across all groups for the top differentially expressed genes between DIO and ND (Ly6C^{Hi}-Ly6C^{Low}).

8 Pathway analysis

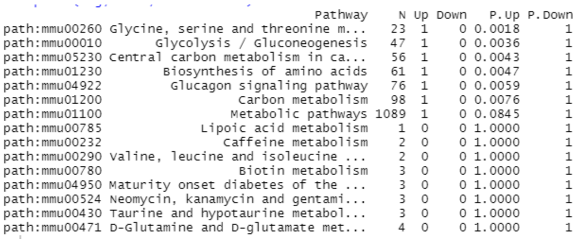
As there were only two differentially expressed genes, GO and KEGG pathway analysis yielded limited results as they were based on only two genes. The next most significantly differentially expressed gene had a FDR of 0.13, making it and all other genes with higher FDRs unviable for GO and KEGG analysis.

From the GO and KEGG pathway analysis (Figure 8 9) for these two genes, we see a variety of pathways that are affected by the up and down regulation of the PGAM1 and CHCHD2 genes.



```
                                                          Term Ont  N Up Down    P.Up P.Down
GO:0004082                    bisphosphoglycerate mutase activity  MF  3  1    0 0.00023  1.000
GO:0004619                       phosphoglycerate mutase activity  MF  3  1    0 0.00023  1.000
GO:0043456                    regulation of pentose-phosphate shunt  BP  4  1    0 0.00031  1.000
GO:1902031                   regulation of NADP metabolic process  BP  5  1    0 0.00039  1.000
GO:0016868 intramolecular transferase activity, phosphotransferases  MF  9  1    0 0.00070  1.000
GO:0043471    regulation of cellular carbohydrate catabolic process  BP 11  1    0 0.00085  1.000
GO:0006098                                pentose-phosphate shunt  BP 13  1    0 0.00101  1.000
GO:1900037             regulation of cellular response to hypoxia  BP 13  0    1 1.00000  0.001
GO:0006740                                     NADPH regeneration  BP 14  1    0 0.00109  1.000
GO:0051156                   glucose 6-phosphate metabolic process  BP 19  1    0 0.00147  1.000
GO:0045730                                      respiratory burst  BP 23  1    0 0.00178  1.000
GO:0016866                        intramolecular transferase activity  MF 24  1    0 0.00186  1.000
GO:0044275                 cellular carbohydrate catabolic process  BP 28  1    0 0.00217  1.000
GO:0006739                                 NADP metabolic process  BP 33  1    0 0.00256  1.000
GO:0006110                         regulation of glycolytic process  BP 34  1    0 0.00264  1.000
```

Figure 8: Top GO results



```
                                              Pathway    N Up Down   P.Up P.Down
path:mmu00260 Glycine, serine and threonine m...   23  1    0 0.0018      1
path:mmu00010       Glycolysis / Gluconeogenesis   47  1    0 0.0036      1
path:mmu05230 Central carbon metabolism in ca...   56  1    0 0.0043      1
path:mmu01230        Biosynthesis of amino acids   61  1    0 0.0047      1
path:mmu04922         Glucagon signaling pathway   76  1    0 0.0059      1
path:mmu01200                  Carbon metabolism   98  1    0 0.0076      1
path:mmu01100                 Metabolic pathways 1089  1    0 0.0845      1
path:mmu00785            Lipoic acid metabolism    1  0    0 1.0000      1
path:mmu00232                Caffeine metabolism    2  0    0 1.0000      1
path:mmu00290 Valine, leucine and isoleucine ...    2  0    0 1.0000      1
path:mmu00780                  Biotin metabolism    3  0    0 1.0000      1
path:mmu04950 Maturity onset diabetes of the ...    3  0    0 1.0000      1
path:mmu00524 Neomycin, kanamycin and gentami...    3  0    0 1.0000      1
path:mmu00430 Taurine and hypotaurine metabol...    3  0    0 1.0000      1
path:mmu00471 D-Glutamine and D-glutamate met...    4  0    0 1.0000      1
```

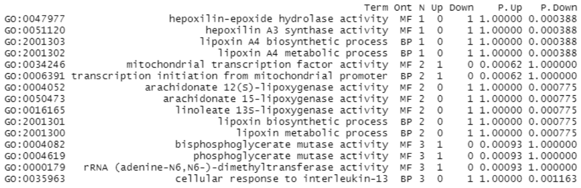
Figure 9: Top KEGG results

In particular, from the GO results we see that the biophosphoglycerate mutase activity pathway is strongly upregulated. This main function of this pathway is the synthesis of 2,3-BPG from 1,3-BPG (an intermediate in glycolysis) which is found only in red-blood cells and placental cells. Specifically, 2,3-BPG binds with high affinity to Hemoglobin causing a release of oxygen. From the fact that this pathway is highly upregulated, and that the cells are taken from epithelial cells, we see that there is significantly more oxygen being released at the site of the wound.

Similarly, from the KEGG results we see that the Glycine, Serine and Threonine Metabolism pathway and Glycolysis pathway are upregulated. Both of these pathways are involved in glycolysis.

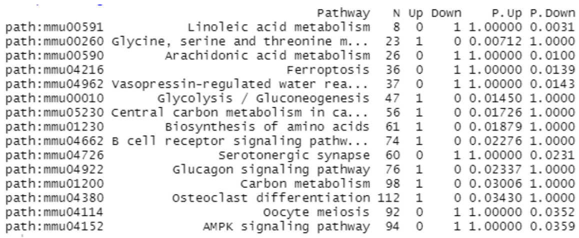
As both oxygen release and glycolysis are associated with increased ATP production, this suggests that there is increased cell activity. This likely has to do with the fact DIO mice have an extended tissue destruction phase and chronic inflammation, which causes increased blood flow to deliver nutrients and white blood cells to the wound area.

If we however increase the FDR cutoff of genes included in the GO and KEGG analysis to 0.3, we now include 9 rather than 2 genes and receive the following results (Figure 10 11)



```
                                                           Term Ont N Up Down    P.Up   P.Down
GO:0047977                 hepoxilin-epoxide hydrolase activity  MF 1  0    1 1.00000 0.000388
GO:0051120                       hepoxilin A3 synthase activity  MF 1  0    1 1.00000 0.000388
GO:2001303                      lipoxin A4 biosynthetic process  BP 1  0    1 1.00000 0.000388
GO:2001302                         lipoxin A4 metabolic process  BP 1  0    1 1.00000 0.000388
GO:0034246          mitochondrial transcription factor activity  MF 2  1    0 0.00062 1.000000
GO:0006391 transcription initiation from mitochondrial promoter  BP 2  1    0 0.00062 1.000000
GO:0004052             arachidonate 12(S)-lipoxygenase activity  MF 2  0    1 1.00000 0.000775
GO:0050473                arachidonate 15-lipoxygenase activity  MF 2  0    1 1.00000 0.000775
GO:0016165                  linoleate 13S-lipoxygenase activity  MF 2  0    1 1.00000 0.000775
GO:2001301                         lipoxin biosynthetic process  BP 2  0    1 1.00000 0.000775
GO:2001300                            lipoxin metabolic process  BP 2  0    1 1.00000 0.000775
GO:0004082                  bisphosphoglycerate mutase activity  MF 3  1    0 0.00093 1.000000
GO:0004619                     phosphoglycerate mutase activity  MF 3  1    0 0.00093 1.000000
GO:0000179   rRNA (adenine-N6,N6-)-dimethyltransferase activity  MF 3  1    0 0.00093 1.000000
GO:0035963                  cellular response to interleukin-13  BP 3  0    1 1.00000 0.001163
```

Figure 10: Top GO results



```
                                        Pathway   N Up Down    P.Up P.Down
path:mmu00591          Linoleic acid metabolism   8  0    1 1.00000 0.0031
path:mmu00260 Glycine, serine and threonine m...  23  1    0 0.00712 1.0000
path:mmu00590       Arachidonic acid metabolism  26  0    1 1.00000 0.0100
path:mmu04216                       Ferroptosis  36  0    1 1.00000 0.0139
path:mmu04962 Vasopressin-regulated water rea...  37  0    1 1.00000 0.0143
path:mmu00010      Glycolysis / Gluconeogenesis  47  1    0 0.01450 1.0000
path:mmu05230 Central carbon metabolism in ca...  56  1    0 0.01726 1.0000
path:mmu01230       Biosynthesis of amino acids  61  1    0 0.01879 1.0000
path:mmu04662 B cell receptor signaling pathw...  74  1    0 0.02276 1.0000
path:mmu04726              Serotonergic synapse  60  0    1 1.00000 0.0231
path:mmu04922        Glucagon signaling pathway  76  1    0 0.02337 1.0000
path:mmu01200                 Carbon metabolism  98  1    0 0.03006 1.0000
path:mmu04380        Osteoclast differentiation 112  1    0 0.03430 1.0000
path:mmu04114                    Oocyte meiosis  92  0    1 1.00000 0.0352
path:mmu04152            AMPK signaling pathway  94  0    1 1.00000 0.0359
```

Figure 11: Top KEGG results

9 Closing

This are the results we've received. Two significantly differentially expressed genes, both with a role in inflammation. The most significantly affected pathways seem to involve the increased release of oxygen and upregulation of glycolysis. We are now left with a few questions:

- Did we process the RNA-seq data to read counts correctly?
- Is it ok that Per base sequence content and Sequence Duplication Levels were marked as X for this experiment?
- Did we find differential expression correctly?
- Was it ok to use quasi-likelihood F-tests rather than likelihood ratio tests given the amount of dispersion?
- With only two genes significantly differentially expressed, would it be fine to increase the FDR cutoff to 0.13 (3 more genes) or 0.3 (7 more genes) for pathway analysis?
- Are we really able to do pathway analysis with just two significantly expressed genes?
- **What direction do we go from here?**

References

Leslie PL Di J Tollini LA He Y Kim TH Jin A Graves LM Zheng J Zhang Y. Liu Y, Clegg HV. 2015. Chchd2 inhibits apoptosis by interacting with bcl-x l to regulate bax activation. *Cell Death Differ*.

Jinsoo Song, In-Jeoung Baek, Churl-Hong Chun, and Eun-Jung Jin. 2018. Dysregulation of the nudt7-pgam1 axis is responsible for chondrocyte death during osteoarthritis pathogenesis. *Nature Communications*, 9.