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Perform differential expression analysis on fibrotic and non-fibrotic patients under 4 different treatments on HPC

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Background

Inflammatory bowel diseases(IBD)

Inflammatory bowel diseases (IBD) are a group of chronic conditions that cause inflammation and damage to the digestive tract. The two main types of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC). Both Crohn's disease and ulcerative colitis are chronic conditions, meaning they can last for a lifetime and require ongoing treatment to manage symptoms and prevent complications.

UC affects only the colon and rectum and causes symptoms such as bloody diarrhea, abdominal pain, and a frequent need to pass stools. It can also lead to complications such as inflammation of the skin, eyes, and joints. On the other hand, CD can affect any part of the digestive tract, like small or large intestine, and can cause symptoms such as abdominal pain, diarrhea, weight loss, and fatigue. It can also cause complications such as fistulas (abnormal connections between different parts of the intestine)

One of the most prevalent complication of CD is the onset of fibrotic complications and strictures (narrowing of the intestine)[1]. The molecular mechanisms involved in these phenotypes remain largely unknown, and as a result, there are currently no effective drugs to prevent or treat stricturing CD.

Induced Pluripotent Stem Cells(iPSCs)

Induced pluripotent stem cells (iPSCs) are a type of stem cell that are generated in the laboratory by reprogramming adult cells, such as skin or blood cells, to a pluripotent state. A pluripotent state means that the cells have the potential to develop into any type of cell in the body, just like embryonic stem cells.

iPSCs offer several advantages as they can be generated from the patient's own cells, avoiding issues with immune rejection, ethical concerns and the need for embryos.

iPSCs can be differentiated into multiple cell and tissue types, and can therefore be used to study the underlying causes of diseases, test new drugs and therapies, and potentially generate replacement tissues or organs for transplantation. As iPSCs possess the same genetic background as the patient they are derived from, they are considered an instrumental tool in the field of personalized and precision medicine[2].

Aim

In this project, we aim to take advantage of iPSC lines to unveil specific signaling pathways specifically affected in patients with fibrotic CD[3]. We will be analyzing RNA-seq data from 19 iPSC lines that were differentiated into gut mesenchymal organoids. This panel comprises 10 iPSC lines derived from Crohn's disease patients that suffered fibrotic complications, and 9 lines from patients with non-fibrotic disease. Each iPSC line was differentiated into mesenchymal organoids in two independent replicates, and each was subjected to 4 different treatments:

- untreated
- TGF β (a pro-fibrotic cytokine)[4]
- TNF α (a pro-inflammatory cytokine)

- and the combination of TGF-b+TNF-a

The final RNA-Seq dataset comprised a total of 151 samples(1 library failed). The objective is to

- investigate if the effect of the four different treatments in iPSC-derived organoids recapitulate the expected responses observed in-vivo[5].
- perform differential expression analysis to identify genes that show differential responses between fibrotic and non-fibrotic patients[6].

Pipelines

In HPC

Convert 151 Fastq to Fasta files

First, put all fasq.gz files into one folder and list all fastq files' name in fastqfiles.txt.

```
ls *q.gz > fastqfiles.txt
```

Cut redundant suffix "_R1_trimmed" and list all fastq files' name in libraryname.txt and preffix.txt.

```
ls *q.gz | cut -f 1 -d '.' | sed 's/_R1_trimmed//g' >libraryname.txt
ls *q.gz | cut -f 1 -d '.' | sed 's/_R1_trimmed//g' > preffix.txt
```

Form a table with 3 columns: fastqfiles.txt libraryname.txt preffix.txt.

```
paste fastqfiles.txt libraryname.txt preffix.txt > tofastatable.txt
```

Create small-sized fasta-formatted files. To submit this job to the cluster on HPC, you need to read the file, library, and prefix. Once you have done that, run the script "generatefastaFromFastaqz" which combine the script "DCfastaqTofastaLibraryId.pl". This results in small-sized fasta-formatted files contain only one header and one sequence per read. You can find all scripts in the "scripts" folder.

```
cat tofastatable.txt | awk '{print}' | while read file library preffix ; do qsub -
 cwd -o $PWD -e $PWD -l h_data=2048M,h_rt=8:00:00
 $HOME/scripts/generatefastaFromFastaqz $file $library $preffix
```

done

Figure 1 shows what each fasta-formatted file look like.

```
>527iP29TNFaM_S124_1
GNAGCAAAGTGGTACCCAAACCTAAGAGCTATTATCCTAAATTCAAAATCTAAAAAAAAACCTTAGAACCTC
>527iP29TNFaM_S124_2
GNGCTCATGCTGTTTCCAGGAGAAAAGTAAGATCCTCAGCCGTATTCGCTTAATATTCATTTCTAAA
>527iP29TNFaM_S124_3
CNTACCTTGAGTTCATTTTTCTATTCTATGCCATAACTAAATTCTGATTAGTTCTCCAATACAATGC
>527iP29TNFaM_S124_4
TNAGTATTGATTGTTAGCGGTGGTCGGGTGTATTCTGAATTGGGGAGGTTATGGGTTAATAG
>527iP29TNFaM_S124_5
GNCTAACTAAGAAGGAAATGTAGAATTAAAGGCAGAAATCTCATGAAAATCCTCCATGCAATAAAGGAGGCTT
>527iP29TNFaM_S124_6
CNCTTAATTGACTAAAGGGATGGTAGGTGGTCACATGCAGTCATGTGGGATTCTAACATGACATTAGTGAGT
>527iP29TNFaM_S124_7
ANTCTCCTTTGTTTGCATTAATTGAATAAAGTAATTCAAGGCTGTGCAGCTTCATTGCCGGTTGGTT
>527iP29TNFaM_S124_8
GCCATCCTTCGATTCTCAGGTGTCAGGCAGGCTGGTATTGGTGATTGTGATCTTATTGGTCCCTGTAC
>527iP29TNFaM_S124_9
GCCGGAAAACATCAGAGATGGAGGGCCCCAGCAGCAGGAAGACGTCAATGATGCCAGTCTGACATCCAGCGAAC
>527iP29TNFaM_S124_10
GCCGTGAATGCAGGACCATCCAGGTCTCAAAGTCTGTGAGGTTGTTCATATCCAAACAAGGGCCCTGCTGGC
```

Generate auxiliary files and directories for each sample

Put a list of names of all fasta files in the directory and save them in a text file named "fastafiles.txt".

```
ls *fasta.gz > fastafiles.txt
```

Cut the redundant suffix ".fasta.gz" from the names of all fasta files and generate a new list of file names with the suffix removed in a text file named "targetdirectories.GTF.txt".

```
cat fastafiles.txt |sed 's/.fasta.gz//g' > targetdirectories.GTF.txt
```

Create a separate directory for each sample listed in "fastafiles.txt".

```
cat fastafiles.txt |while read line ; do mkdir ${line/.fasta\.gz/GTFpass1/} ; done
```

Form the submission script called "sendmyof"

Add a shebang line at the beginning of your script file named "sendmyof" to indicate the interpreter that should be used to execute the script.

```
echo '#!/bin/bash/' > sendmyof
```

The command below runs the "generatesendscriptSingleGTFParam" script with several input parameters to map the RNA-seq data with STAR. The input parameters include the list of target directories containing the input data ("targetdirectories.GTF.txt"), the directory prefix for pass-1 alignments ("GTFpass1"), a parameter file containing settings for STAR alignment ("Parameters.txt"), a prefix for individual submission scripts to HPC ("myof"), the path to the STAR index directory ("~/home/luc/RNASEQ_MASTER/Hsapiens/GRC38/INDEXES/GRCh38.primary.33.basicselected.STAR2.7.3a/"), the path to the input data directory ("~/home/luc/iPSC/MYOFIBROBLAST/"), the amount of free memory to use ("mem_free=32G"), and the number of threads to use ("8"). In the end, it will generate a sample-specific sumission script called "processLaneSingleGTFParam" in each sample's folder:

```
./generatesendscriptSingleGTFParam targetdirectories.GTF.txt GTFpass1
Parameters.txt myof
~/home/luc/RNASEQ_MASTER/Hsapiens/GRC38/INDEXES/GRCh38.primary.33.basicselected.STAR2.7
~/home/luc/iPSC/MYOFIBROBLAST/ mem_free=32G 8 >> sendmyof
```

Change sendmyof into executable mode and run sendmyof.

```
chmod a+x sendmyof
. sendmyof
```

It will take less than one day to run through 151 samples and generate each sample a folder which contain every output from STAR.

Create a table summarizing the mapping statistics for each sample

Change directory into one sample file which ends with "GTFpass1". Extract the first column from the mapping statistics file and store it in "temp2.txt".

```
grep " |" 008iP22TGFbM_S71GTFpass1/008iP22TGFbM_S71GTFpass1Log.final.out | cut -f 1
```

```
-d "|" | sed 's/^ *//g' | awk 'NR>3 {print}' > temp2.txt
```

The first column from the mapping statistics file in Figure 2.

```
Mapping speed, Million of reads per hour
Number of input reads
Average input read length
Uniquely mapped reads number
Uniquely mapped reads %
Average mapped length
Number of splices: Total
Number of splices: Annotated (sjdb)
Number of splices: GT/AG
Number of splices: GC/AG
Number of splices: AT/AC
Number of splices: Non-canonical
Mismatch rate per base, %
Deletion rate per base
Deletion average length
Insertion rate per base
Insertion average length
Number of reads mapped to multiple loci
% of reads mapped to multiple loci
Number of reads mapped to too many loci
% of reads mapped to too many loci
Number of reads unmapped: too many mismatches
% of reads unmapped: too many mismatches
Number of reads unmapped: too short
% of reads unmapped: too short
Number of reads unmapped: other
% of reads unmapped: other
Number of chimeric reads
% of chimeric reads
```

Create an empty temporary file for storing intermediate results.

```
rm tempprev.txt
touch tempprev.txt
```

Extract the total mapped reads from each subsequent mapping statistics file and combine with previous results.

```
ls *pass1/*final.out | while read line ; do
grep "|\\" $line | cut -f 2 > temp.txt
paste tempprev.txt temp.txt > tempnew.txt
mv tempnew.txt tempprev.txt
```

done

Remove the first column and write the final results to a file called "mappingstatsFirstpass.txt".

```
cut -f 2- tempprev.txt | awk 'NR>3 {print}' > tempnew.txt
mv tempnew.txt tempprev.txt
paste temp2.txt tempprev.txt > mappingstatsFirstpass.txt
```

Figure 3 shows what mappingstatsFirstpass.txt look like.

Mapping speed, Million of reads per hour	452.92	344.45	460.17	487.96	331.34	441.99	560.78	487.93	532.33	351.69
Number of input reads	46046803	40855908	43588067	47982561	39392754	33886137	72122988			
Average input read length	75	75	75	75	75	75	75	75	75	75
Uniquely mapped reads number	42647348	36668193	39982295	43570276	36214207	31289999				
Uniquely mapped reads %	92.62%	89.75%	91.73%	90.80%	91.93%	92.34%	91.60%	90.47%	93.77%	90.29%
Average mapped length	75.21	75.21	75.20	75.20	75.19	75.21	75.20	75.20	75.20	75.24
Number of splices: Total	14107761	11743321	12025357	12627415	11851597	10196588				
Number of splices: Annotated (sjdb)	14029840	11673663	11945482	12536747	11787471	10140891				
Number of splices: GT/AG	14015583	11657575	11928403	12523568	11777228	10127320				
Number of splices: GC/AG	78050	74078	84368	87757	62434	59661	145139	96183	139381	88755
Number of splices: AT/AC	7048	6261	6785	8291	6179	5252	12249	9152	15682	8499
Number of splices: Non-canonical	7080	5407	5801	7799	5756	4355	9871	8901	10933	5153
Mismatch rate per base, %	0.27%	0.28%	0.29%	0.28%	0.28%	0.31%	0.28%	0.29%	0.29%	0.28%
Deletion rate per base	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%
Deletion average length	1.47	1.47	1.47	1.48	1.48	1.47	1.46	1.47	1.48	1.48
Insertion rate per base	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
Insertion average length	1.61	1.52	1.43	1.43	1.63	1.60	1.47	1.43	1.62	1.42
Number of reads mapped to multiple loci	2398878	3287402	2498197	3093230	2029231	1702575	4120922	3568843	3990452	2580456
% of reads mapped to multiple loci	5.21%	8.05%	5.73%	6.45%	5.15%	5.02%	5.71%	6.70%	4.80%	5.64%
Number of reads mapped to too many loci	222693	207795	314715	343251	343822	264218	498200	397171	262921	547542
% of reads mapped to too many loci	0.48%	0.51%	0.72%	0.72%	0.87%	0.78%	0.69%	0.75%	0.32%	1.20%
Number of reads unmapped: too many mismatches	1269	1089	1322	1446	1105	1001	2452	1565	1799	1824
% of reads unmapped: too many mismatches	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Number of reads unmapped: too short	759644	674719	771365	950053	788663	614921	1410179	1081555	902701	1286763
% of reads unmapped: too short	1.65%	1.65%	1.77%	1.98%	2.00%	1.81%	1.96%	2.03%	1.09%	2.81%
Number of reads unmapped: other	16971	16710	20173	24305	15726	13423	29546	25946	17237	24268
% of reads unmapped: other	0.04%	0.04%	0.05%	0.05%	0.04%	0.04%	0.05%	0.05%	0.05%	0.05%
Number of chimeric reads	0	0	0	0	0	0	0	0	0	0
% of chimeric reads	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

The summary statistics show good rates of unique alignments for all samples.

Counts

Generate a directory called "COUNTS" and copy all gene count files to this folder and then clean all file names.

```
mkdir COUNTS
cp *pass1/*PerGene* COUNTS/
```

```
ls *tab|while read line ; do mv $line ${line/GTfpass1ReadsPerGene.out/} ; done
```

For each count file, extract and create the five count tables.

```
ls *.tab | while read line ; do
echo $line
cat $line | awk 'NR==3{print}' | cut -f 2- > ${line/tab/nofeature}.tab
cat $line | awk 'NR==4{print}' | cut -f 2- > ${line/tab/ambiguous}.tab
cat $line | awk 'NR>4{print}' | cut -f 2 > ${line/tab/nostrand}.tab
cat $line | awk 'NR>4{print}' | cut -f 3 > ${line/tab/sense}.tab
cat $line | awk 'NR>4{print}' | cut -f 4 > ${line/tab/antisense}.tab
done
```

Make a Geneid list from one of the count tables as "countsannot_GRCh38.primary.Selected.Geneid.txt".

```
ls 008iP22TGFbM_S71.tab | head -1 | while read line; do
cut -f 1 $line | awk 'NR>4{print}' > countsannot_GRCh38.primary.Selected.Geneid.txt
done
```

Create a file listing the names of all samples as "RBarretTNFATGFBsamples.txt".

```
ls *.sense.tab | sed 's/.sense.tab//g' | tr -s " " "\n" | sed 's/_1//g' >
RBarretTNFATGFBsamples.txt
```

Make count tables for sense, anti-sense, nostrand, ambiguous, and nofeature reads.

```
# combine all sense counts into RBarretTNFATGFB_sense.ALL.cnt
paste *.sense.tab > RBarretTNFATGFB_sense.ALL.cnt

# combine all antisense counts into RBarretTNFATGFB_antisense.ALL.cnt
paste *.antisense.tab > RBarretTNFATGFB_antisense.ALL.cnt

# combine all nostrand counts into RBarretTNFATGFB_nostrand.ALL.cnt
paste *.nostrand.tab > RBarretTNFATGFB_nostrand.ALL.cnt

# combine all ambiguous counts into RBarretTNFATGFB_ambiguous.cnt
cat *ambiguous.tab > RBarretTNFATGFB_ambiguous.cnt

# combine all nofeature counts into RBarretTNFATGFB_nofeature.cnt
cat *nofeature.tab > RBarretTNFATGFB_nofeature.cnt
```

Next, I am going to use "RBarretTNFATGFB_antisense.ALL.cnt" file for the further analysis, as this matrix contains the counts matching the strand-specificity of the RNA-Seq libraries generated in this study.

In MATLAB

Transfer data and import annotation

Transfer the counts, annotation, and mappability data to your local laptop.

```
RBarretTNFATGFCnt = textread('RBarretTNFATGFB_antisense.ALL.cnt','');
RBarretsamplesTNFATGFB = textread('RBarretTNFATGFBsamples.txt','%s');
RBarretsampleskeysTNFATGFB = textread('samplekeys_Sam.txt','%s');

% calculate the sum of the counts in RBarretTNFATGFCnt, divides the result by
1000000, and rounds the result to the nearest integer.
RBarretTNFATGFBmeta_seqdepth=round(sum(RBarretTNFATGFCnt)/1000000);
```

The following files contain the annotation and gene effective lengths (mappabilities) for the human gene annotation used for alignment, and can be found in the "mappability and R code" folder.

```
Gencode_33_Selected_MappSS=textread('mappability and R
code/gencode.v33.Selected.ReadsPerGene.out.MappSS.txt','');
Gencode_33_Selected_MappUS=textread('mappability and R
code/gencode.v33.Selected.ReadsPerGene.out.MappUS.txt','');
Gencode_33_Selected_Geneid=textread('mappability and R
code/gencode.v33.annotation.Selected.geneid.txt','%s\n');
Gencode_33_Selected_Biotype=textread('mappability and R
code/gencode.v33.annotation.Selected.biotype.txt','%s\n');
Gencode_33_Selected_Genename=textread('mappability and R
code/gencode.v33.annotation.Selected.genename.txt','%s\n');
```

Compile counts

First, initialize a new variable called RBarretTNFATGFBTPM with the same count data as RBarretTNFATGFCnt. Then, iterates over each gene in the count data matrix. For each gene, the corresponding row in RBarretTNFATGFBTPM is updated by dividing the count data by the gene effective length from the "Gencode_33_Selected_MappSS", multiplying by 1000, and storing the result in RBarretTNFATGFBTPM.

Finally, iterates over each sample in the TPM data matrix. For each sample, the corresponding column in RBarretTNFATGFBTPM is updated by dividing the values in the column by the sum of the values in the column, multiplying by 1,000,000, and storing the result in RBarretTNFATGFBTPM. This step **normalizes the TPM values** across samples and scales the resulting values to TPM.

```
RBarretTNFATGFBTPM = RBarretTNFATGFCnt;
```

```

for i=1:size(RBarretTNFATGFBCnt,1)
% divid the gene count matrix RBarretTNFATGFBCnt by Gencode_33_Selected_MappSS
matrix, which is the sum of the transcript length of each gene
RBarretTNFATGFBTPM(i,:) =
RBarretTNFATGFBCnt(i,:)/Gencode_33_Selected_MappSS(i)*1000;
end
% set any NaN or Inf values resulting from the normalization process to 0
RBarretTNFATGFBTPM(isnan(RBarretTNFATGFBTPM)) = 0;
RBarretTNFATGFBTPM(isinf(RBarretTNFATGFBTPM)) = 0;
for i=1:size(RBarretTNFATGFBTPM,2)
% scale the TPM values so that the sum of expression values across each sample of
the matrix is equal to 1,000,000. This ensures that the expression values are
comparable across different samples and allows meaningful comparisons of gene
expression levels between different samples.
RBarretTNFATGFBTPM(:,i) =
RBarretTNFATGFBTPM(:,i)/sum(RBarretTNFATGFBTPM(:,i))*1000000;
end

```

Make the first dendrogram

Make a dendrogram to visualize the relationships among samples in the RBarretTNFATGFB dataset based on their gene expression profiles. To downgrade the effect of potential expression outliers in the dendrogram and compute a more robust sample clustering:

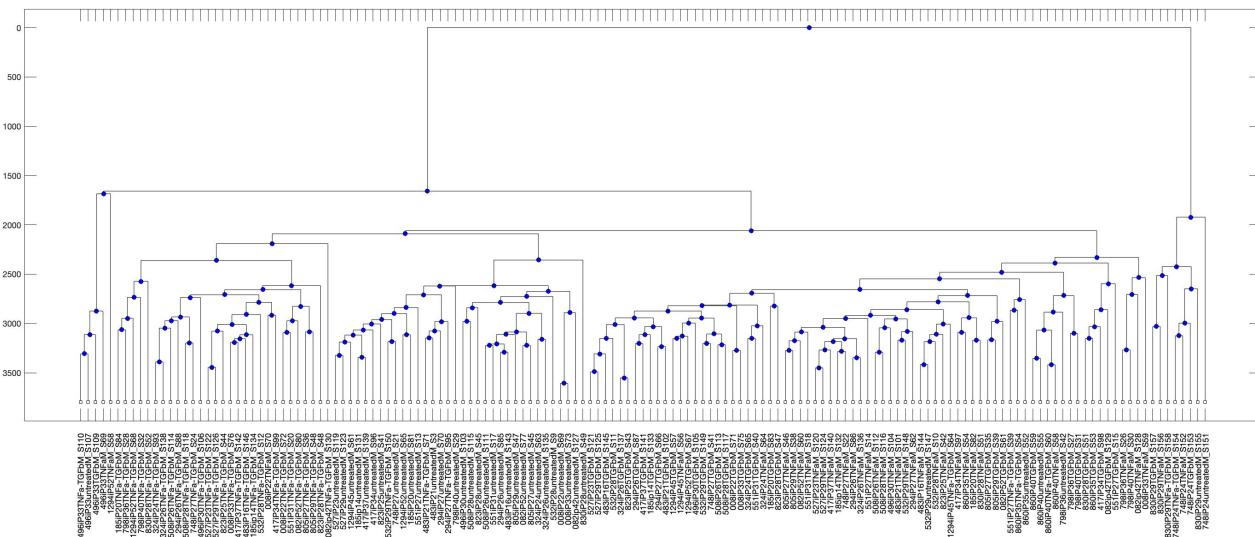
1. I generates a random selection of 1,000 genes from the TPM data matrix.
2. Calculates the pairwise distances between the selected genes.
3. Creates a for loop that iterates 9,999 times. For each iteration, a new random selection of 1,000 genes is generated, and the pairwise distances between these genes are added to the previous 'thisdist' calculation.
4. Converts the one-dimensional distance vector 'thisdist' into a distance matrix 'thisdistmat' using the 'squareform' function.
5. Generates a hierarchical clustering tree based on the distance matrix 'thisdistmat'.

Overall, I perform a clustering analysis on a subset of genes in the RBarretTNFATGFB dataset to visualize the relationships among samples based on their gene expression profiles in Figure 4.

```

thisrand = unique(randi([1 size(RBarretTNFATGFBTPM,1)],1,1000));
thisdist = pdist(RBarretTNFATGFBTPM(thisrand,:));
for i=1:9999
thisrand = unique(randi([1 size(RBarretTNFATGFBTPM,1)],1,1000));
thisdist = thisdist+pdist(RBarretTNFATGFBTPM(thisrand,:));
end
thisdistmat = squareform(thisdist/10000);
thistree = seqlinkage(thisdistmat,'average', RBarretsamplesTNFATGFB)
plot(thistree, 'ORIENTATION', 'top')

```



A first observation is that the major clusters are formed by samples from the same treatment, with exceptions. Therefore, the Treatment factor seems to be the dominant source of gene expression variation in this experiment.

All biotypes counts percents

As part of the preliminary quality control, the following estimates the relative contribution of each gene biotype to the expression matrix:

```

% use the unique function and stored the allbiotypes variable.
allbiotypes = unique(Gencode_33_Selected_Biotype);

% create A cell array allbiotypeslength to store the lengths of each biotype name.
allbiotypeslength = cell(length(allbiotypes),1);

% two new matrices, allbiotypescounts and allbiotypescountspercents, are
% initialized with zeros. These matrices have dimensions (number of unique biotypes)
% x (number of samples in the TPM data). They will be used to store the number of
% reads (counts) and the percentage of total reads (%TPM) for each biotype in each
% sample.
allbiotypescounts = zeros(length(allbiotypes),size(RBarretTNFATGFBCnt,2));
allbiotypescountspercents = zeros(length(allbiotypes),size(RBarretTNFATGFBCnt,2));

for i=1:length(allbiotypes)
    % finds all the indices of Gencode_33_Selected_Biotype that match the current
    % biotype. Then, returned a vector of **indices** where the biotype occurs in
    % Gencode_33_Selected_Biotype.
    temp = strmatch(allbiotypes{i}, Gencode_33_Selected_Biotype);
    % Stored the length of the temp vector represents the number of genes with the
    % current biotype in the Gencode_33_Selected_Biotype.
    allbiotypeslength{i} = length(temp);
    if length(temp)>1
        % Sum the expression values for all genes with the current biotype across all
        % samples. The resulting sums are stored in the corresponding row of the
        % allbiotypescounts matrix.

```

```
allbiotypescounts(i,:) = sum(RBarretTNFATGFBCnt(strmatch(allbiotypes{i},  
Gencode_33_Selected_Biotype),:));  
allbiotypescountspercents(i,:) =  
allbiotypescounts(i,:)./sum(RBarretTNFATGFBCnt)*100;  
end  
end  
  
dlmwrite('allbiotypescountspercents.txt',  
allbiotypescountspercents,'delimiter','\t')  
writetable(cell2table(allbiotypes),'allbiotypes.txt','WriteVariableNames',0)
```

Using Excel, create a spreadsheet using "allbiotypes.txt" and "allbiotypescountspercents.txt", and calculate the minimum, maximum, and average values for each biotype in Figure 5. You can access my completed spreadsheet [here](#). Notably, protein_coding genes exhibit an average of 98.65% among the various biotypes, consistent with my expectations. Moreover, I found no samples with excessive contributions from other biotypes (e.g. mitochondrial and non-coding RNAs), and therefore no library quality issues were found in this step.

Biotypes	min	max	average
IG_C_gene	0.000000000	0.000446580	0.000013340
IG_D_gene	0.000000000	0.000000000	0.000000000
IG_J_gene	0.000000000	0.000013813	0.000000235
IG_V_gene	0.000000000	0.000073058	0.000010604
Mt_rRNA	0.228350000	1.165600000	0.487934371
Mt_tRNA	0.000141280	0.002049800	0.000492376
TEC	0.011838000	0.029033000	0.018976556
TR_C_gene	0.000000000	0.002667100	0.000287637
TR_D_gene	0.000000000	0.000011542	0.000000580
TR_J_gene	0.000000000	0.000076445	0.000009219
TR_V_gene	0.000000000	0.000061956	0.000011904
lncRNA	0.537580000	1.612500000	0.825408079
miRNA	0.002352100	0.014811000	0.004202784
misc_RNA	0.000641580	0.002443400	0.001048973
protein_coding	97.675000000	99.144000000	98.658132450
rRNA	0.000000000	0.000028828	0.000005066
ribozyme	0.000000000	0.000007357	0.000000293
sRNA	0.000000000	0.000004680	0.000000135
scRNA	0.000000000	0.000000000	0.000000000
scaRNA	0.000002616	0.000324330	0.000036518
snRNA	0.000262070	0.001464200	0.000543351
snoRNA	0.001727500	0.004335800	0.002883131
vaultRNA	0.000000000	0.000000000	0.000000000

Protein coding genes

The "allbiotypes.txt" file contains multiple biotypes. For the next step, I will only retain the "protein_coding" biotype. I will also remove some gene classes that typically show very noisy or variable gene expression across different samples (e.g histone and ribosomal genes, among others).

```
allbiotypes=unique(Gencode_33_Selected_Biotype);
% finds the 15th unique value, which is protein_coding, of
Gencode_33_Selected_Biotype in the array proteincodingindx.
proteincodingindx = strmatch(allbiotypes{15}, Gencode_33_Selected_Biotype);
biotypeindx = proteincodingindx;
```

```
% creates an array additionalgenes contains the indices of genes that have certain
prefixes such as 'MT-', 'H1', 'H2', 'H3', 'H4', 'RPL', or 'RPS' in their names.
additionalgenes = [strmatch('MT-',Gencode_33_Selected_Genename) ;
strmatch('H1',Gencode_33_Selected_Genename);
strmatch('H2',Gencode_33_Selected_Genename);
strmatch('H3',Gencode_33_Selected_Genename);
strmatch('H4',Gencode_33_Selected_Genename) ;
strmatch('RPL',Gencode_33_Selected_Genename) ;
strmatch('RPS',Gencode_33_Selected_Genename)];
```

% creates an array nonadditionalgenes with the same length as the Gencode_33_Selected_Genename array.

```
nonadditionalgenes = 1:length(Gencode_33_Selected_Genename);
% remove the indices of genes in additionalgenes from the nonadditionalgenes array.
nonadditionalgenes(additionalgenes) = [];
```

% mappableindx contains the indices of elements in the Gencode_33_Selected_MappSS array that are greater than 50.

```
mappableindx = find(Gencode_33_Selected_MappSS>50);
```

% a new variable finalIndexGeneric which is the intersection of three other variables: biotypeindx, nonadditionalgenes, and mappableindx.

```
finalIndexGeneric =
intersect(biotypeindx,intersect(nonadditionalgenes,mappableindx));
% find the indices of rows in RBarretTNFATGFBCnt that have a sum greater than 150
(an average of >1 per sample).
countindx = find(sum(RBarretTNFATGFBCnt')>150);
```

% update finalIndexGeneric to be the intersection of finalIndexGeneric and countindx.

```
finalIndexGeneric=intersect(finalIndexGeneric,countindx);
```

% create a new variable RBarretTNFATGFBCnt_GMask which is a subset of RBarretTNFATGFBCnt corresponding to the rows indexed by finalIndexGeneric.

```
RBarretTNFATGFBCnt_GMask = RBarretTNFATGFBCnt(finalIndexGeneric,:);
```

Gencode_33_Selected_Geneid_GMask = Gencode_33_Selected_Geneid(finalIndexGeneric);
Gencode_33_Selected_Genename_GMask =
Gencode_33_Selected_Genename(finalIndexGeneric);
Gencode_33_Selected_MappSS_GMask = Gencode_33_Selected_MappSS(finalIndexGeneric);
Gencode_33_Selected_MappUS_GMask = Gencode_33_Selected_MappUS(finalIndexGeneric);

% normalize the expression data like we did previously, keeping only the filtered set of genes above:

```
RBarretTNFATGFBExpression_GMask = RBarretTNFATGFBCnt_GMask;
for i=1:size(RBarretTNFATGFBExpression_GMask,2)
```

```
RBarretTNFATGFBExpression_GMask(:,i) =
RBarretTNFATGFBCnt_GMask(:,i)/sum(RBarretTNFATGFBCnt_GMask(:,i))*1000000;
end
for i=1:size(RBarretTNFATGFBExpression_GMask)
RBarretTNFATGFBExpression_GMask(i,:) =
RBarretTNFATGFBExpression_GMask(i,:)/Gencode_33_Selected_MappSS_GMask(i)*1000;
end
RBarretTNFATGFBExpression_GMask(isnan(RBarretTNFATGFBExpression_GMask)) = 0;
RBarretTNFATGFBExpression_GMask(isinf(RBarretTNFATGFBExpression_GMask)) = 0;

% RBarretTNFATGFBCPM_GMask contains the expression data normalized only by CPM,
using the same normalization method as the code above.
RBarretTNFATGFBCPM_GMask = zeros(size(RBarretTNFATGFBCnt_GMask));
for i=1:size(RBarretTNFATGFBCnt_GMask,2)
RBarretTNFATGFBCPM_GMask(:,i) =
RBarretTNFATGFBCnt_GMask(:,i)/sum(RBarretTNFATGFBCnt_GMask(:,i))*1000000;
end

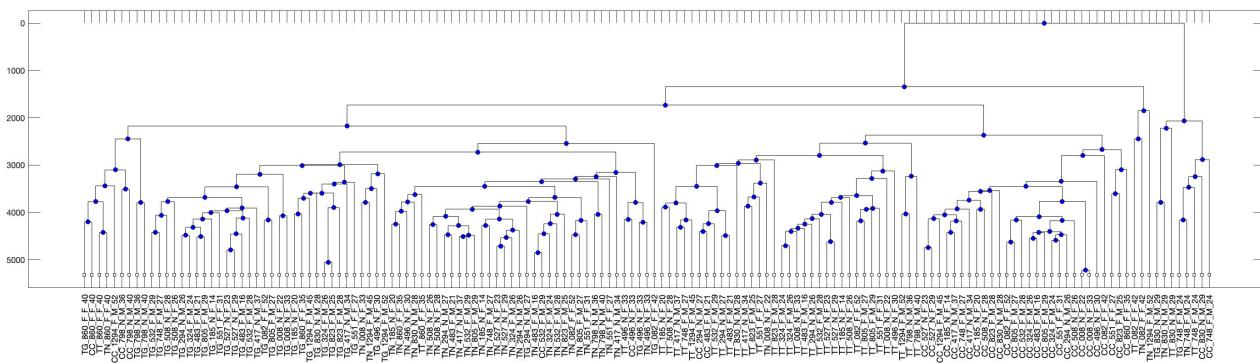
% RBarretTNFATGFBTPM_GMask contains the expression data normalized only by TPM.
RBarretTNFATGFBTPM_GMask = RBarretTNFATGFBCnt_GMask;
for i=1:size(RBarretTNFATGFBCnt_GMask,1)
RBarretTNFATGFBTPM_GMask(i,:) =
RBarretTNFATGFBCnt_GMask(i,:)/Gencode_33_Selected_MappSS_GMask(i)*1000;
end
RBarretTNFATGFBTPM_GMask(isnan(RBarretTNFATGFBTPM_GMask)) = 0;
RBarretTNFATGFBTPM_GMask(isinf(RBarretTNFATGFBTPM_GMask)) = 0;
for i=1:size(RBarretTNFATGFBTPM_GMask,2)
RBarretTNFATGFBTPM_GMask(:,i) =
RBarretTNFATGFBTPM_GMask(:,i)/sum(RBarretTNFATGFBTPM_GMask(:,i))*1000000;
end
```

Dendrogram with only protein coding genes

Perform hierarchical clustering on the filtered gene expression data stored in the variable RBarretTNFATGFBTPM_GMask:

```
thisrand = unique(randi([1 size(RBarretTNFATGFBTPM_GMask,1)],1,1000));
thisdist = pdist(RBarretTNFATGFBTPM_GMask(thisrand,:)');
for i=1:9999
thisrand = unique(randi([1 size(RBarretTNFATGFBTPM_GMask,1)],1,1000));
thisdist = thisdist+pdist(RBarretTNFATGFBTPM_GMask(thisrand,:)');
end
thisdistmat = squareform(thisdist/10000);
thistree = seqlinkage(thisdistmat,'average', RBarretsampleskeysTNFATGFB)
plot(thistree,'ORIENTATION','top')
```

Again, the samples are clustered largely by their treatment status but in a more consistent fashion as compared to the unfiltered dataset in Figure 6.



The percent of the top 100 genes

Another item for quality control is achieved by calculating the percent of signal attributed to the top 100 expressed genes in each sample based on their transcript per million (TPM) values in the RBarretTNFATGFBTPM_GMask matrix.

```
% iterates over 151 samples, it first sorts the TPM values of all genes in
descending order and stores the indices of the sorted genes in y. The top 100
expressed genes in the sample are obtained by selecting the first 100 indices in y,
and these indices are appended to a running list of all top 100 indices yall.
yall=[];
for i=1:151
[x y]=sort(RBarretTNFATGFBTPM_GMask(:,i),'descend');
yall=unique([y(1:100); yall]);
top100percent(i)=sum(RBarretTNFATGFBTPM_GMask(y(1:100),i))/1000000;
end
```

I find that, for some samples, the top 100 most-expressed genes accumulate ~40% of the total TPMs for the sample, while the average is ~25%. I will keep track of these number in case those samples show outlier behaviour in downstream analyses.

In the end, we store the Gencode_33_Selected_Geneid_GMask.txt, Gencode_33_Selected_Genename_GMask.txt, Gencode_33_Selected_MappSS_GMask.txt, RBarretTNFATGFBTPM_GMask.txt, and RBarretTNFATGFBCnt_GMask.txt for ours further analysis in R.

```
writetable(cell2table(Gencode_33_Selected_Geneid_GMask), 'Gencode_33_Selected_Geneid_GM'
writetable(cell2table(Gencode_33_Selected_Genename_GMask), 'Gencode_33_Selected_Genename_GM'
dlmwrite('Gencode_33_Selected_MappSS_GMask.txt',
Gencode_33_Selected_MappSS_GMask, 'delimiter', '\t')
dlmwrite('RBarretTNFATGFBTPM_GMask.txt', RBarretTNFATGFBTPM_GMask, 'delimiter', '\t')
dlmwrite('RBarretTNFATGFBCnt_GMask.txt', RBarretTNFATGFBCnt_GMask, 'delimiter', '\t')
```

Differential expression analysis in R

Install packages

First, install packages BiocManager, BiocLite, IHW, DESeq2[7], and ggplot2. Then, read in RBarretTNFATGFCnt_GMask.txt, RBarretTNFATGFBsamples.txt, samplekeys_Sam.txt, and Gencode_33_Selected_Genename_GMask.txt.

```
setwd("/Users/LuC/Desktop/Cedars-Sinai/PROJECTS/IBD_RNASeq/RBARRETTNFATGFB/")
#setwd("/Users/samuellu/Desktop/Cedars-Sinai/PROJECTS/IBD_RNASeq/RBARRETTNFATGFB/")

if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

#BiocManager::install("BiocLite")
#BiocManager::install("IHW")
#BiocManager::install("DESeq2")
#install.packages("ggplot2")

library(DESeq2)
library(IHW)
library(ggplot2)
library(ggrepel)

RBarretTNFATGFCntGMask = as.matrix(read.table("RBarretTNFATGFCnt_GMask.txt"))
sampleNameTNFATGFB = as.matrix(read.table("RBarretTNFATGFBsamples.txt"))
sampleKeyTNFATGFB = as.matrix(read.table("samplekeys_Sam.txt"))
genenames = as.matrix(read.table("Gencode_33_Selected_Genename_GMask.txt"))
```

Form samplekeys_Sam.tab

Separate samplekeys_Sam.txt by "_" to get samplekeys_Sam.tab before next step. Here are my code in terminal.

```
#In terminal
#Create an empty file to store the output
touch samplekeys_Sam.tab

#Loop over the sample names and split them by "_"
for sample in $(cat samplekeys_Sam.txt); do
  IFS=_ read -r col1 col2 col3 col4 col5 <<< "$sample"
  echo -e "$col1\t$col2\t$col3\t$col4\t$col5" >> samplekeys_Sam.tab
done
```

Generate a sampleTableTNFATGFB

The sampleTableTNFATGFB contains the experimental factors: Treatment, iPSC line (Line), fibrotic phenotype (Pheno), Sex, number of iPSC passages (Pass), the combination of phenotype and treatment (Factor), and the combination of line and passages (Batch) in Figure 7.

```
sampleTableTNFATGFB = read.table("samplekeys_Sam.tab")
rownames(sampleTableTNFATGFB)<-sampleKeyTNFATGFB
colnames(sampleTableTNFATGFB)<- c("Treatment","Line","Pheno","Sex","Pass")
sampleTableTNFATGFB$Factor <-
paste(sampleTableTNFATGFB$Treatment,sampleTableTNFATGFB$Pheno,sep="_")
#concatenating the "Line" and "Pass" columns with an underscore separator
sampleTableTNFATGFB$Batch <-
paste(sampleTableTNFATGFB$Line,sampleTableTNFATGFB$Pass,sep="_")
colnames(RBarretTNFATGFBCntGMask) <- sampleKeyTNFATGFB
write.table(sampleTableTNFATGFB,file="sampleTableTNFATGFB.txt", sep = "\t",
col.names = FALSE)
```

	Treatment	Line	Pheno	Sex	Pass	Factor	Batch
TG_008_N_F_22	TG	8	N	F	22	TG_N	8_22
TT_008_N_F_22	TT	8	N	F	22	TT_N	8_22
TN_008_N_F_22	TN	8	N	F	22	TN_N	8_22
CC_008_N_F_22	CC	8	N	F	22	CC_N	8_22
TG_008_N_F_33	TG	8	N	F	33	TG_N	8_33
TT_008_N_F_33	TT	8	N	F	33	TT_N	8_33
TN_008_N_F_33	TN	8	N	F	33	TN_N	8_33
CC_008_N_F_33	CC	8	N	F	33	CC_N	8_33
TG_082_F_F_52	TG	82	F	F	52	TG_F	82_52
TT_082_F_F_52	TT	82	F	F	52	TT_F	82_52
TN_082_F_F_52	TN	82	F	F	52	TN_F	82_52
CC_082_F_F_52	CC	82	F	F	52	CC_F	82_52

DESeq2 package

Different experimental factors are tested while creating the DESeq object for differential expression, to check if there are significant differences. The RBarretTNFATGFBCntGMaskBatch is created with the **Batch** information specified in the design formula, while the RBarretTNFATGFBCntGMaskFactor is created with the **treatment and phenotype** information specified in the design formula. I also tested if fitting the data to the first principal component (PC1, see below) makes a difference in the first steps.

The DESeq function is used to estimate size factors and dispersion values for the DESeqDataSet objects. Using this object, we first use the varianceStabilizingTransformation function to perform variance stabilizing transformation. This transformation is important for reducing the effect of noise and impose heteroscedasticity in the data, making it more suitable for downstream analyses such as linear modeling and clustering.

```
RBarretTNFATGFCntGMaskBatch <- DESeqDataSetFromMatrix(RBarretTNFATGFCntGMask,
colData= sampleTableTNFATGFB,design= ~Batch)
RBarretTNFATGFCntGMaskBatch <- DESeq(RBarretTNFATGFCntGMaskBatch)

RBarretTNFATGFCntGMaskFactor <- DESeqDataSetFromMatrix(RBarretTNFATGFCntGMask,
colData= sampleTableTNFATGFB,design= ~Factor)
RBarretTNFATGFCntGMaskFactor <- DESeq(RBarretTNFATGFCntGMaskFactor)

RBarretTNFATGFCntGMaskPC1 <- DESeqDataSetFromMatrix(RBarretTNFATGFCntGMask,
colData= pcabatchR,design= ~PC1)
RBarretTNFATGFCntGMaskPC1 <- DESeq(RBarretTNFATGFCntGMaskPC1)

RBarretTNFATGFCntGMaskBatch_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFCntGMaskBatch,blind=FALSE)
RBarretTNFATGFCntGMaskFactor_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFCntGMaskFactor,blind=FALSE)
RBarretTNFATGFCntGMaskPC1_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFCntGMaskPC1,blind=FALSE)
```

Principal Component Analysis (PCA)

```
#perform principal component analysis (PCA) on the variance-stabilized counts data
pcabatch <- prcomp(t(assay(RBarretTNFATGFCntGMaskBatch_vsd)))

#give the percentage of variance explained by each principal component
percentVarbatch <- round(100*pcabatch$sdev^2/sum(pcabatch$sdev^2))

#pcabatch$rotation is a matrix containing the loadings of the principal components.
aloadbatch <- abs(pcabatch$rotation)

#normalize the loadings in aloadbatch so that each column (i.e., PC) sums to 1.
aloadrelativebatch <- sweep(aloadbatch, 2, colSums(aloadbatch), "/")

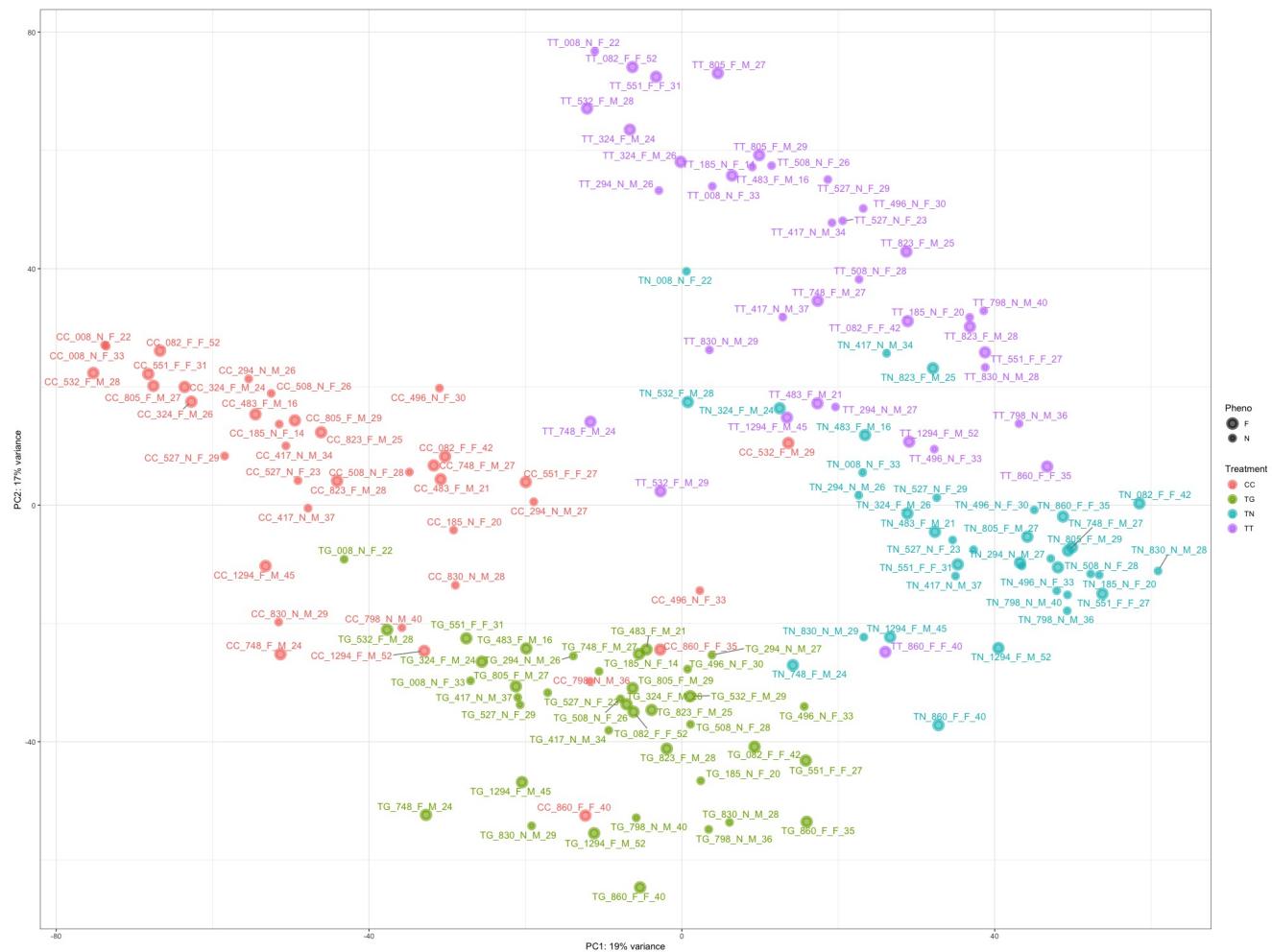
#pcabatch$x is a matrix containing each sample's coordinate on each principal
component
pcabatchALL <- pcabatch$x
pcabatchR<- cbind(pcabatchALL,sampleTableTNFATGFB)
```

PCA plots

```
ggplot(pcabatchR, aes(PC1, PC2, color= Treatment)) +
  geom_point(aes(size= Pheno), alpha=0.6, stroke = 3) +
  xlab(paste0("PC1: ",percentVarbatch[1],"% variance")) +
  ylab(paste0("PC2: ",percentVarbatch[2],"% variance")) +
  geom_text_repel(aes(label = sampleKeyTNFATGFB),size=4,box.padding = 0.35,
  point.padding = 0.5,segment.color = 'grey50')+ theme_bw()
```

The plot shows the clustering of all samples using the first two principal components, **PC1** and **PC2**, colored by **Pheno** variable, with the point size indicating the **Treatment** variable.

In Figure 8, four distinct groups were formed based on their treatment: the CC group (untreated) is located in the right corner, the TG group (treated with TGF-b) is located at the bottom, the TN group (treated with TNF-a) is located in the right corner, and the TT group (treated with both TGF-b and TNF-a) is located at the top. These 4 groups were differentiated based on the combination of both PC1 and PC2, which accounted for 19% and 17% of the variance, respectively.



A series of bar plots (one for each principal component)

Each bar plot represents the coordinates of all samples on a given principal component. These plots provide a quick visual evaluation of the potential association of each component with specific experimental factors.

```
#the resulting vector coul will contain 12 colors from the "Set3" palette.
library(RColorBrewer)
coul <- brewer.pal(12, "Set3")
#generates colors for a plot based on the batch variable
colors=pcabatchR$Batch
allbatches<-unique(pcabatchR$Batch)
for (i in 1:38){
  colors[pcabatchR$Batch==allbatches[i]]<-coul[i%%12+1]
}

thinlines=c(seq(4,72,8),75,seq(83,151,8))
thicklines=c(seq(8,72,8),79,seq(87,151,8))

#first half of the barplot would be the non-fibrotic group and the second part
would be the fibrotic group
#the order would be CC, TG, TN, TT
samplesorder=c(4,1,3,2,8,5,7,6,28,25,27,26,32,29,31,30,36,33,35,34,40,37,39,38,52,49,5

#create 38 barplots and saving each of them as a PNG file
for (i in 1:38) {
  filename = paste("PC_",i,".png", sep = "")
  png(filename)

  barplot(pcabatchALL[samplesorder,i],col=colors[samplesorder],las=2,xaxt='n',space=0)
  for (i in 1:length(thinlines)) {
    abline(v = thinlines[i], col = "black",lty = 3)
  }
  for (i in 1:length(thicklines)) {
    abline(v = thicklines[i], col = "black",lty = 1)
  }
  abline(v = 72, col = "red",lty = 1)
  dev.off()
}

write.csv(aloadrelativebatch,file="aloadrelativeMask_batchmodel_filtered.csv")
write.csv(pcabatch$x,file="pca_batchmodel_x.csv")
```

To facilitate visualization, a red line is drawn at position 72 in order to separate the non-fibrotic group from the fibrotic group. Within each patient, the treatment order would be CC, TG, TN, TT. The color of each bar represents the batch of the sample, with a unique color assigned to each batch. The vertical lines on the plot separate the data for individual iPSC lines and their two batches.

The Figure 9 below represents PC1 for all samples. From this plot, one can see that PC1 corresponds to extreme expression after treatment with TNF-a in all cases, even more than after its combination with TGF-b. Therefore, it seems to indicate that PC1 is associated with an interaction between TNF-a and TGF-b in iPSC mesenchymal organoids, an unexpected finding that warrants further analysis.

PCA rank matrix

For easier visualization, I next clean the PCA results for exporting into spreadsheets. Take csv files and converts it to the txt files with the second column onwards. It does this by first removing the first row using awk, replacing multiple commas with tabs using tr, and removing the first column using cut.

```
#In terminal
cat aloadrelativeMask_batchmodel_filtered.csv | awk 'NR>1{print}' | tr -s "," "\t"
| cut -f 2- > aloadrelativeMask_batchmodel_filtered.clean.txt
cat pca_batchmodel_x.csv | awk 'NR>1{print}' | tr -s "," "\t" | cut -f 2- >
pca_batchmodel_x.clean.txt
```

Read in the preprocessed data files created in the previous steps and store them in variables pcabatch_samples and pca_loadings, respectively.

```
#In Matlab
pcabatch_samples = textread('pca_batchmodel_x.clean.txt','');
pca_loadings = textread('aloadrelativeMask_batchmodel_filtered.clean.txt','');
```

Sort the three columns of pca_loadings in descending order and store the sorted values in variables x1, x2, and x3, and the corresponding indices in y1, y2, and y3.

```
%x = pca_loading number, y = its index

[x1 y1]=sort(pca_loadings(:,1),'descend');
[x2 y2]=sort(pca_loadings(:,2),'descend');
[x3 y3]=sort(pca_loadings(:,3),'descend');
```

Determine the rank of each row in the original order for the first three principal components and store the ranks in a matrix pcarankmatrix.

```
[x y z]=intersect(1:length(y1),y1);
pcarankmatrix(:,1)=z;
[x y z]=intersect(1:length(y2),y2);
pcarankmatrix(:,2)=z;
[x y z]=intersect(1:length(y3),y3);
pcarankmatrix(:,3)=z;

%contains the rank of each feature in the original order for the first three
```

principal components

```
dlmwrite('pcarankmatrix.txt', pcarankmatrix,'delimiter','\t')
```

To efficiently manage our data with a single glance, I have organized it into an Excel spreadsheet using a combination of command line, Excel, and R.

Spreadsheet

The Figure 10 (patients) built on excel contains patients order, patients id, phenotypes, and sex. You can visit the sheet by clicking [here](#).

ID	Phenotype	Gender		1	008IP2TGTGFM_571	N	F	TG_008_N_F_22			TG_008_N_F_22	TG	8	N	F	22_TG_N	8_22
16	082	Fibrotic	F	2	008IP2TNTFa-TGFbM_572	N	F	TT_008_N_F_22			TT_008_N_F_22	TT	8	N	F	22_TT_N	8_22
12	324	Fibrotic	M	3	008IP2TNTFa-M_570	N	F	TN_008_N_F_22			TN_008_N_F_22	TN	8	N	F	22_TN_N	8_22
13	483	Fibrotic	M	4	008IP2UntreatedM_569	N	F	CC_008_N_F_22			CC_008_N_F_22	CC	8	N	F	22_CC_N	8_22
17	532	Fibrotic	M	5	008IP3TGTGFM_575	N	F	TG_008_N_F_33			TG_008_N_F_33	TG	8	N	F	33_TG_N	8_33
11	551	Fibrotic	F	6	008IP3TNTFa-TGFbM_576	N	F	TT_008_N_F_33			TT_008_N_F_33	TT	8	N	F	33_TT_N	8_33
19	748	Fibrotic	M	7	008IP3TNTFa-M_559	N	F	TN_008_N_F_33			TN_008_N_F_33	TN	8	N	F	33_TN_N	8_33
10	805	Fibrotic	M	8	008IP3UntreatedM_573	N	F	CC_008_N_F_33			CC_008_N_F_33	CC	8	N	F	33_CC_N	8_33
14	823	Fibrotic	M	9	008IP5TGTGFM_561	N	F	TG_082_F_F_52			TG_082_F_F_52	TG	82	F	F	52_TG_F	82_52
15	860	Fibrotic	F	10	008IP5TNTFa-TGFbM_580	N	F	TT_082_F_F_52			TT_082_F_F_52	TT	82	F	F	52_TT_F	82_52
18	1294	Fibrotic	M	11	008IP5TNTFa-M_560	F	F	TN_082_F_F_52			TN_082_F_F_52	TN	82	F	F	52_TN_F	82_52
6	008	Non-fibrotic	F	12	008IP5UntreatedM_577	F	F	CC_082_F_F_52			CC_082_F_F_52	CC	82	F	F	52_CC_F	82_52
3	185	Non-fibrotic	F	13	008IP4TNTFa-M_5128	F	F	TG_082_F_F_42			TG_082_F_F_42	TG	82	F	F	42_TG_F	82_42
4	254	Non-fibrotic	M	14	008IP4TNTFa-M_5130	F	F	TT_082_F_F_42			TT_082_F_F_42	TT	82	F	F	42_TT_F	82_42
2	417	Non-fibrotic	M	15	008IP4TNTFa-M_5125	F	F	TN_082_F_F_42			TN_082_F_F_42	TN	82	F	F	42_TN_F	82_42
7	496	Non-fibrotic	F	16	008IP4TNTFa-M_5127	F	F	CC_082_F_F_42			CC_082_F_F_42	CC	82	F	F	42_CC_F	82_42
5	508	Non-fibrotic	F	17	008IP4MTGFM_557	F	F	TG_1294_F_M_45			TG_1294_F_M_45	TG	1294	F	M	45_TG_F	1294_45
8	527	Non-fibrotic	F	18	008IP4MTGFM_564	F	F	TT_1294_F_M_45			TT_1294_F_M_45	TT	1294	F	M	45_TT_F	1294_45
1	798	Non-fibrotic	M	19	008IP4MTGFM_556	F	F	TN_1294_F_M_45			TN_1294_F_M_45	TN	1294	F	M	45_TN_F	1294_45
0	830	Non-fibrotic	M	20	008IP4UntreatedM_561	F	F	CC_1294_F_M_45			CC_1294_F_M_45	CC	1294	F	M	45_CC_F	1294_45
			21	008IP5TGTGFM_567	F	F	TG_1294_F_M_52			TG_1294_F_M_52	TG	1294	F	M	52_TG_F	1294_52	
			22	008IP5TNTFa-TGFbM_568	F	F	TT_1294_F_M_52			TT_1294_F_M_52	TT	1294	F	M	52_TT_F	1294_52	
			23	008IP5TNTFa-M_558	F	F	TN_1294_F_M_52			TN_1294_F_M_52	TN	1294	F	M	52_TN_F	1294_52	
			24	008IP5UntreatedM_565	F	F	CC_1294_F_M_52			CC_1294_F_M_52	CC	1294	F	M	52_CC_F	1294_52	
			25	008IP2TGTGFM_583	N	F	TG_185_N_F_20			TG_185_N_F_20	TG	185	N	F	20_TG_N	185_20	
			26	008IP2TNTFa-TGFbM_584	N	F	TT_185_N_F_20			TT_185_N_F_20	TT	185	N	F	20_TT_N	185_20	
			27	008IP2TNTFa-M_582	N	F	TN_185_N_F_20			TN_185_N_F_20	TN	185	N	F	20_TN_N	185_20	
			28	008IP2UntreatedM_581	N	F	CC_185_N_F_20			CC_185_N_F_20	CC	185	N	F	20_CC_N	185_20	
			29	008IP4TGTGFM_5133	F	F	TG_185_N_F_14			TG_185_N_F_14	TG	185	N	F	14_TG_N	185_14	
			30	008IP4TNTFa-TGFbM_5134	F	F	TT_185_N_F_14			TT_185_N_F_14	TT	185	N	F	14_TT_N	185_14	
			31	008IP4TNTFa-M_5132	F	F	TN_185_N_F_14			TN_185_N_F_14	TN	185	N	F	14_TN_N	185_14	
			32	008IP4UntreatedM_5131	N	F	CC_185_N_F_14			CC_185_N_F_14	CC	185	N	F	14_CC_N	185_14	
			33	008IP2TGTGFM_587	N	M	TG_294_N_M_26			TG_294_N_M_26	TG	294	N	M	26_TG_N	294_26	
			34	008IP2TNTFa-TGFbM_588	N	M	TT_294_N_M_26			TT_294_N_M_26	TT	294	N	M	26_TT_N	294_26	
			35	008IP2TNTFa-M_586	N	M	TN_294_N_M_26			TN_294_N_M_26	TN	294	N	M	26_TN_N	294_26	
			36	008IP2UntreatedM_585	N	M	CC_294_N_M_26			CC_294_N_M_26	CC	294	N	M	26_CC_N	294_26	
			37	008IP2TGTGFM_566	N	M	TG_294_N_M_27			TG_294_N_M_27	TG	294	N	M	27_TG_N	294_27	
			38	008IP2TNTFa-TGFbM_595	N	M	TT_294_N_M_27			TT_294_N_M_27	TT	294	N	M	27_TT_N	294_27	
			39	008IP2TNTFa-M_562	N	M	TN_294_N_M_27			TN_294_N_M_27	TN	294	N	M	27_TN_N	294_27	
			40	008IP2UntreatedM_570	N	M	CC_294_N_M_27			CC_294_N_M_27	CC	294	N	M	27_CC_N	294_27	
			41	008IP2ATGFM_565	F	M	TG_324_F_M_24			TG_324_F_M_24	TG	324	F	M	24_TG_F	324_24	
			42	008IP2ATNFa-TGFbM_593	F	M	TT_324_F_M_24			TT_324_F_M_24	TT	324	F	M	24_TT_F	324_24	
			43	008IP2ATNFa-M_564	F	M	TN_324_F_M_24			TN_324_F_M_24	TN	324	F	M	24_TN_F	324_24	
			44	008IP2UntreatedM_563	F	M	CC_324_F_M_24			CC_324_F_M_24	CC	324	F	M	24_CC_F	324_24	
			45	008IP2TGTGFM_5137	F	M	TG_324_F_M_26			TG_324_F_M_26	TG	324	F	M	26_TG_F	324_26	
			46	008IP2ATNFa-TGFbM_5138	F	M	TT_324_F_M_26			TT_324_F_M_26	TT	324	F	M	26_TT_F	324_26	
			47	008IP2ATNFa-M_5136	F	M	TN_324_F_M_26			TN_324_F_M_26	TN	324	F	M	26_TN_F	324_26	
			48	008IP2UntreatedM_5135	F	M	CC_324_F_M_26			CC_324_F_M_26	CC	324	F	M	26_CC_F	324_26	

The Figure 11 (allbiotypes_percents) includes the names and percentages of all biotypes, along with their respective minimum, maximum, and average values, providing us with a comprehensive overview. You can visit the sheet by clicking [here](#).

```
#In terminal  
paste allbiotypes allbiotypescountspercents > combine_allbiotypes_percents.txt
```

```
#In R
#allbiotypes_percents
sheet2_1 <- list("Biotypes")
sheet2_2 <- sampleKeyTNFATGFB
combined_sheet2 <- c(sheet2_1, sheet2_2)
combined_spreadsheet2 <- as.matrix(read.table("combine_allbiotypes_percents.txt"))
colnames(combined_spreadsheet2) <- combined_sheet2
write.table(combined_spreadsheet2,file="combined_spreadsheet2.txt", sep = "\t",
row.names = FALSE)
#add their respective minimum, maximum, and average values on Excel
```

Biotypes	min	max	average	TG_008_N_F_22	TT_008_N_F_22	TN_008_N_F_22	CC_008_N_F_22	TG_008_N_F_33	TT_008_N_F_33	TN_008_N_F_33	CC_008_N_F_33
IG_C_gene	0.000000000	0.000446580	0.000013340	0.000000000	0.000000000	0.000000000	0.000000000	0.000003012	0.000011577	0.000000000	0.000000000
IG_D_gene	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000
IG_I_gene	0.000000000	0.000013813	0.000000235	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000
IG_V_gene	0.000000000	0.000073058	0.000010604	0.000012755	0.000010996	0.000008912	0.000002556	0.000015061	0.000008269	0.000010462	0.000011520
Mt_rRNA	0.228350000	1.165600000	0.487934371	0.495080000	0.584550000	0.439920000	0.826140000	0.508050000	0.550660000	0.508120000	0.859590000
Mt_tRNA	0.000141280	0.002049800	0.000492376	0.000214290	0.000274910	0.000199040	0.000345110	0.000240970	0.000219970	0.000237130	0.000336390
TEC	0.011838000	0.029033000	0.018976556	0.017090000	0.016613000	0.016773000	0.021052000	0.017133000	0.019370000	0.017489000	0.021066000
TR_C_gene	0.000000000	0.002667100	0.000287637	0.00002551	0.000000000	0.000005942	0.000000000	0.000018073	0.000011577	0.0000087182	0.000009216
TR_D_gene	0.000000000	0.000011542	0.000000580	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000
TR_I_gene	0.000000000	0.000076445	0.000009219	0.000000000	0.000000000	0.000000000	0.000010225	0.000009036	0.000000000	0.000006975	0.000002304
TR_V_gene	0.000000000	0.000061956	0.000011904	0.000000000	0.0000008247	0.000005942	0.000010225	0.000003012	0.000013231	0.000006975	0.000016129
lncRNA	0.537580000	1.612500000	0.825408079	0.832110000	0.782160000	0.759180000	1.060300000	0.858370000	0.828130000	0.792760000	1.102900000
miRNA	0.002352100	0.014811000	0.004202784	0.004000100	0.004865900	0.004536300	0.003474100	0.003707900	0.004582900	0.005147200	0.003327100
misc_RNA	0.000641580	0.002443400	0.001048973	0.001140300	0.001300300	0.001265500	0.001250100	0.001015100	0.001283400	0.001227500	0.001101300
protein_codi	97.675000000	99.144000000	98.658132450	98.647000000	98.607000000	98.775000000	98.084000000	98.608000000	98.592000000	98.671000000	98.008000000
rRNA	0.000000000	0.0000028828	0.000005066	0.000000000	0.000008247	0.000008912	0.000000000	0.000003012	0.000000000	0.0000010462	0.000002304
ribozyme	0.000000000	0.000007357	0.000000293	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000
sRNA	0.000000000	0.000004680	0.000000135	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000001654	0.000000000
scRNA	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000
scaRNA	0.000002616	0.000324330	0.000036518	0.000012755	0.000024742	0.000062386	0.000040902	0.000024097	0.000031424	0.000027898	0.000052994
snRNA	0.000262070	0.001464200	0.000543351	0.000413270	0.000519580	0.000534740	0.000682550	0.000590380	0.000583820	0.000606780	0.000580630
snoRNA	0.001727500	0.004335800	0.002883131	0.002719400	0.002650100	0.002857900	0.003037000	0.002970000	0.003486400	0.002978100	0.003205000
vaultRNA	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000

The Figure 12 is the main sheet that includes Genename, Geneid, Mapp, PC1, PC2, PC3, and patient's TPM values.

#In terminal

```
paste Gencode_33_Selected_Genename_GMask.txt Gencode_33_Selected_Genename_GMask.txt
Gencode_33_Selected_Geneid_GMask.txt Gencode_33_Selected_MappSS_GMask.txt
pcarankmatrix.txt > combine_test.txt
```

#In R

#spreadsheet

```
sheet3_1 <- list("Genename", "Genename", "Geneid", "Mapp", "PC1", "PC2", "PC3")
sheet3_2<- sampleKeyTNFATGFB
combined_headers <- c(sheet3_1, sheet3_2)
combined_spreadsheet <- as.matrix(read.table("combine_test.txt"))
colnames(combined_spreadsheet) <- combined_headers
combined_spreadsheet <- combined_spreadsheet[order(combined_spreadsheet[,1]),]
#sort by the first column
write.table(combined_spreadsheet,file="combined_spreadsheet.txt", sep = "\t",
row.names = FALSE)
```

You can sort this sheet with PC1, PC2, and so on to see the correlation between the experimental factors and the expression level of each gene, which allows a quick identification of genes and gene classes more associated with the dominant sources of gene expression variability in this experiment.

Genename	Genename	Geneid	Mapp	PC1	PC2	PC3	TG_008_N_F_22_TT_008_N_F_22_TN_008_N_F_22_CC_008_N_F_22_TG_008_N_F_33_TT_008_N_F_33_TN_008_N_F_33_CC_008_N_F_33	TG_082_F_TT_082_F_F_TN_082_F_F_CC_082_F_F_TG_082_F_F_TT_082_F_F_TN_082_F_F_CC_082_F_F
CXKL6	CXKL6	ENSG00000124875.10	1596.00	68.00	1.00	887.00	32.65 1058.00 5693.30 31.87 32.82 4913.60 870.40 33.79	13.20 4292.20 149.95 124.15 1.46 4126.20 943.95
CXKL8	CXKL8	ENSG00000169429.11	1868.00	1.00	2.00	389.00	0.24 4087.30 1728.20 0.40 0.29 1642.70 747.49 0.36	3.21 2633.80 804.62 7.38 0.67 8435.60 3672.60 0.48
CXKL1	CXKL1	ENSG00000163739.17	1075.00	111.00	3.00	1782.00	15.88 5467.70 2412.60 18.21 12.37 1673.10 351.36 19.33	17.31 2740.30 143.20 76.39 3.44 11957.00 1262.50 32.73
C3	C3	ENSG00000125739.17	989.00	114.00	1.00	1229.00	0.50 944.99 326.21 0.64 0.09 310.99 23.94 0.71	0.10 316.76 2.27 0.09 1.99 117.31 11.12 18.46
ABP1BP	ABP1BP	ENSG00000105664.11	4779.00	147.00	4.00	2037.00	49.71 1058.00 470.70 48.11 0.23 102.56 0.06 33.59	7.74 621.97 40.02 45.88 0.69 1.25 4.66 0.39
COMP	COMP	ENSG00000105664.11	5524.00	5644.00	7.00	1132.00	33.42 1.41 15.19 16.08 34.42 0.19 102.56 0.06 33.59	49.43 7.74 621.97 40.02 45.88 0.69 1.25 4.66 0.39
ELN	E LN	ENSG00000104947.11	5283.00	591.00	15.00	1695.00	8.00 1973.70 799.40 1.76 3.72 585.33 93.03 0.89	0.50 514.44 11.66 1.84 1.16 3769.20 322.23 4.70
CXKL3	CXKL3	ENSG00000163734.4	975.00	118.00	8.00	795.00	3.01 1973.70 799.40 1.76 3.72 585.33 93.03 0.89	0.50 514.44 11.66 1.84 1.16 3769.20 322.23 4.70
SOD2	SOD2	ENSG00000112096.18	13791.00	56.00	9.00	1172.00	12.85 272.70 798.31 23.34 8.93 913.94 202.64 19.33	9.63 780.30 230.05 43.23
CXKL5	CXKL5	ENSG00000163735.7	2337.00	81.00	10.00	550.00	0.34 869.40 225.78 0.64 0.17 151.01 0.00 0.05	0.31 1967.60 87.29 0.81
CCL5	CCL5	ENSG000001271503.1	1372.00	3.00	3.00	321.00	0.16 295.41 223.72 0.00 0.29 549.51 161.46 0.07	0.14 456.50 171.04 0.00 0.04 94.00 333.98 0.00
PTX3	PTX3	ENSG00000104947.11	1870.00	3158.00	12.00	4555.00	1.00 3158.00 3158.00 3158.00 3158.00 3158.00 3158.00 3158.00	3271.60 35.76 595.60 156.41 180.89 156.41 180.89 156.41 180.89
CXL2	CXL2	ENSG00000105661.9	4.00	1.00	1.00	1.00	1.72 1041.30 844.13 3.27 1.09 1050.00 805.00 5.13	0.30 542.91 292.03 3.37
CXL2	CXL2	ENSG000001081041.9	1016.00	333.00	14.00	1285.00	1.45 904.33 356.30 1.45 1.09 304.28 32.38 1.68	1.69 368.55 3.36 2.07 1.00 1356.60 140.94 9.89
VCAM1	VCAM1	ENSG00000163692.12	3283.00	591.00	15.00	1695.00	82.51 1391.60 462.26 24.01 2666.10 433.79 294.77 18.17 282.90 343.05	286.87 24.01 2666.10 433.79 294.77 18.17 282.90 343.05
MX1	MX1	ENSG00000157601.14	4555.00	413.00	16.00	495.00	1.94 278.74 62.79 2.31 2.09 116.67 6.18 2.21 0.68 148.83 9.95	1.84 378.71 22.66 15.53 1.48 378.71 22.66 15.53
IFI6	IFI6	ENSG00000126709.15	921.00	135.00	17.00	636.00	197.01 11438.00 392.40 263.68 106.44 4058.10 387.41 265.10 102.71 1193.00 705.40	290.36 68.81 1217.60 87.29 0.81
TP63	TP63	ENSG00000107282.13	6942.00	1074.00	18.00	434.00	0.13 74.20 3.17 0.37 0.54 76.34 8.95 0.22 0.10 139.07 0.62 3.43	0.09 0.18 0.08 0.02
ICAM1	ICAM1	ENSG00000137339.9	3063.00	20.00	19.00	1288.00	5.06 1045.60 430.16 27.88 2.54 7.49 2.45 7.25 8.45 493.27 60.84	7.25 493.27 60.84 2.27
BTG1	BTG1	ENSG00000105661.9	900.00	145.00	20.00	390.00	10.00 1170.00 201.45 14.94 9.55 538.00 71.06 12.89 201.08 160.41 150.41 150.41	150.41 150.41 150.41 150.41 150.41 150.41 150.41
Q4Z3	Q4Z3	ENSG00000113331.13	790.00	12.00	4.00	41.00	1.56 125.87 30.54 30.54 2.71 0.54 55.65 1.59 1.59 0.12 199.31 11.12 0.38 0.38 33.49 32.70 0.71	0.71 199.31 11.12 0.38 0.38 33.49 32.70 0.71
TRPA1	TRPA1	ENSG00000104321.11	5092.00	88.00	22.00	1008.00	0.03 57.40 30.22 0.04 0.03 98.37 10.63 0.00 0.10 151.80 9.66 1.15 156.78 3.19 38.96	30.22 0.04 0.03 0.03 359.15 55.77 3.51
DAS2	DAS2	ENSG00000113355.12	260.00	26.00	3.00	5.00	0.05 45.00 0.07 0.10 0.05 213.00 0.00 0.05 0.05 105.16 2.07 0.06 2582.70 252.83 0.54	0.05 213.00 0.00 0.05 0.05 105.16 2.07 0.06 2582.70 252.83 0.54
TRPA1P3	TRPA1P3	ENSG00000108501.5	5175.00	16.00	29.00	1485.00	11.01 508.75 471.41 12.45 12.45 527.70 334.22 12.31 5.71 745.53 304.41 1.11 147.71 15.05 14.70	471.41 12.45 527.70 334.22 12.31 5.71 745.53 304.41 1.11 147.71 15.05 14.70
COL10A1	COL10A1	ENSG00000123500.10	3719.00	426.00	30.00	258.00	14.14 0.84 4.44 0.23 22.50 0.71 19.39 0.29 0.45 0.00 0.28 0.00 0.22 2.40 4.16 0.00	0.84 4.44 0.23 22.50 0.71 19.39 0.29 0.45 0.00 0.28 0.00 0.22 2.40 4.16 0.00
C7	C7	ENSG00000112396.19	5583.00	100.00	31.00	8610.00	35.94 325.09 80.53 57.83 26.44 16.41 57.58 77.74 1392.20 45.82 500.07 1.89 0.91 0.22 76.14	99.34 26.44 16.41 57.58 77.74 1392.20 45.82 500.07 1.89 0.91 0.22 76.14
EPST1	EPST1	ENSG00000133106.14	3387.00	1787.00	32.00	1636.00	16.06 351.20 178.25 30.64 6.82 211.47 49.20 20.61 15.71 477.40 9.66 25.17 3.97 396.10 38.94	38.94 211.47 49.20 20.61 15.71 477.40 9.66 25.17 3.97 396.10 38.94
ANOS1	ANOS1	ENSG00000101201.12	5932.00	978.00	33.00	1324.00	85.09 2.89 35.76 0.63 241.05 9.27 50.30 0.80 219.43 1.02 98.02 1.15 156.78 3.19 5.04	11.22 241.05 9.27 50.30 0.80 219.43 1.02 98.02 1.15 156.78 3.19 5.04
SLC7A2	SLC7A2	ENSG00000105989.17	8019.00	162.00	34.00	1464.00	10.18 203.92 79.47 60.58 1.72 103.64 82.66 1.31 94.47 279.42 50.21 5.15 405.05 397.25 37.41	103.92 1.72 103.64 82.66 1.31 94.47 279.42 50.21 5.15 405.05 397.25 37.41
LRP1	LRP1	ENSG00000105671.11	179.00	17.00	1.00	1.00	10.67 1.51 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63	1.51 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63 233.89 0.63
SLC9A8	SLC9A8	ENSG00000138821.13	4772.00	380.00	36.00	634.00	4.03 520.69 96.33 2.46 3.16 143.62 19.82 2.39 0.84 238.80 9.36 31.61 31.61 11.27 2.46	520.69 96.33 2.46 3.16 143.62 19.82 2.39 0.84 238.80 9.36 31.61 31.61 11.27 2.46
BIRC3	BIRC3	ENSG00000232445.15	7397.00	8.00	37.00	468.00	0.20 43.90 23.12 0.22 0.05 47.92 19.87 0.16 0.12 49.16 19.24 0.23 0.08 30.37 29.72 0.44	43.90 23.12 0.22 0.05 47.92 19.87 0.16 0.12 49.16 19.24 0.23 0.08 30.37 29.72 0.44
CLDN11	CLDN11	ENSG00000103297.11	1477.00	44.00	38.00	3523.00	318.25 852.33 339.26 2145.20 54.92 748.84 39.03 2166.10 29.52 300.67 14.63 1357.10 18.26 2774.88 6.36 1463.40	3523.00 339.26 2145.20 54.92 748.84 39.03 2166.10 29.52 300.67 14.63 1357.10 18.26 2774.88 6.36 1463.40
CXKL10	CXKL10	ENSG00000169524.15	1076.00	336.00	39.00	406.00	0.00 69.21 10.80 0.00 0.00 107.42 16.86 0.00 0.00 365.14 1.00 0.20 0.00 0.00 0.00	0.00 69.21 10.80 0.00 0.00 107.42 16.86 0.00 0.00 365.14 1.00 0.20 0.00 0.00 0.00
IFT13	IFT13	ENSG00000115917.14	2424.00	4342.00	40.00	1342.00	24.02 301.95 73.64 59.05 8.70 180.05 25.11 53.31 5.28 700.96 21.53 96.16 2.61 47.62 45.89 38.15	24.02 301.95 73.64 59.05 8.70 180.05 25.11 53.31 5.28 700.96 21.53 96.16 2.61 47.62 45.89 38.15
IFI44L	IFI44L	ENSG00000137959.16	6048.00	539.00	41.00	1020.00	4.36 198.48 48.30 4.59 2.29 96.52 5.01 5.24 2.67 304.69 7.35 3.97 0.24 111.6 2.05	198.48 48.30 4.59 2.29 96.52 5.01 5.24 2.67 304.69 7.35 3.97 0.24 111.6 2.05
L32	L32	ENSG00000105675.11	2972.00	40.00	42.00	100.00	0.20 194.89 142.18 0.37 5.68 45.94 32.95 0.27 3.46 492.91 463.17 4.51 1.31 305.95 3.75	194.89 142.18 0.37 5.68 45.94 32.95 0.27 3.46 492.91 463.17 4.51 1.31 305.95 3.75
LBDAA44	LBDAA44	ENSG00000105674.11	4772.00	480.00	481.00	0.65	0.65 48.9 0.07 4.03 4.03 7.21 0.01 19.59 0.12 16.14 3.37 24.01 0.46 14.29 0.39	48.9 0.07 4.03 4.03 7.21 0.01 19.59 0.12 16.14 3.37 24.01 0.46 14.29 0.39
SPX	SPX	ENSG00000134548.11	2368.00	44.00	4466.00	41.82 662.12 155.00 146.01 12.20 111.61 32.33 143.49 0.30 89.01 2.96 4.23 0.42 18.83 0.46 1.18	4466.00 41.82 662.12 155.00 146.01 12.20 111.61 32.33 143.49 0.30 89.01 2.96 4.23 0.42 18.83 0.46 1.18	
CTSS	CTSS	ENSG00000131311.11	3937.00	94.00	45.00	604.00	0.37 86.80 24.61 0.20 1.25 16.63 1.25 0.93 129.91 15.59 2.78 0.59 79.83 36.88 1.16	86.80 24.61 0.20 1.25 16.63 1.25 0.93 129.91 15.59 2.78 0.59 79.83 36.88 1.16
CH3L2	CH3L2	ENSG00000064886.14	3501.00	224.00	46.00	1133.00	0.19 31.15 33.51 0.16 0.15 28.93 7.60 0.58 0.06 59.92 0.81 0.42 0.17 7.14 1.01 0.73	31.15 33.51 0.16 0.15 28.93 7.60 0.58 0.06 59.92 0.81 0.42 0.17 7.14 1.01 0.73
IL6	IL6	ENSG00000169524.14	1764.00	42.00	47.00	1049.00	0.26 432.09 40.78 0.00 0.23 63.80 1.31 0.22 0.67 117.20 13.02 0.12 0.27 330.18 47.31 0.51	432.09 40.78 0.00 0.23 63.80 1.31 0.22 0.67 117.20 13.02 0.12 0.27 330.18 47.31 0.51
TNFRSF1B	TNFRSF1B	ENSG000000208137.19	3534.00	4819.00	48.00	608.00	3.00 88.89 28.39 6.97 1.03 89.14 5.92 7.44 0.39 88.43 0.62 3.78 1.00 27.89 2.10 9.13	88.43 0.62 3.78 1.00 27.89 2.10 9.13
IFT1	IFT1	ENSG00000105745.10	4607.00	3926.00	49.00	1883.00	35.58 148.21 65.03 30.02 10.67 70.47 10.24 28.47 1.79 223.42 3.21 34.19 1.32 12.06 9.07 40.66	148.21 65.03 30.02 10.67 70.47 10.24 28.47 1.79 223.42 3.21 34.19 1.32 12.06 9.07 40.66

DESeq analysis for pairwise comparisons

Reorder data by phenotypes and patients (lines)

```
samplesorder=c(4,1,3,2,8,5,7,6,28,25,27,26,32,29,31,30,36,33,35,34,40,37,39,38,52,49,5
```

Add the PC1 coordinate of each sample into the experimental design, to be used as factor in the model

```
Genenames =
as.list(read.table("Gencode_33_Selected_Genename_GMask.txt",header=FALSE,as.is=TRUE))
```

```
#Find duplicate gene names
duplicated_genes <- Genenames$V1[duplicated(Genenames$V1)]
```

```

if(length(duplicated_genes) > 0){
  cat("Duplicate gene names found:", paste(duplicated_genes, collapse = ", "))
} else {
  cat("No duplicate gene names found.")
}

#Duplicate gene names found: TBCE, ATXN7, AHRR, MATR3, HSPA14, TMSB15B

#Find duplicate gene names at indices
duplicated_indices <- which(duplicated(Generenames$V1))
if(length(duplicated_indices) > 0){
  cat("Duplicate gene names found at indices:", paste(duplicated_indices, collapse
= ", "))
} else {
  cat("No duplicate gene names found.")
}
#Duplicate gene names found at indices: 1530, 3024, 4133, 4576, 7638, 15459

```

```

#Correct the duplicated gene names
#by appending "_1" to the end of each duplicate gene name
Generenames$V1[1530] <- "TBCE_1"
Generenames$V1[3024] <- "ATXN7_1"
Generenames$V1[4133] <- "AHRR_1"
Generenames$V1[4576] <- "MATR3_1"
Generenames$V1[7638] <- "HSPA14_1"
Generenames$V1[15459] <- "TMSB15B_1"

```

First round of pairwise comparisons:

All cell lines UNTREATED vs All cell lines TGF β

All cell lines UNTREATED vs All cell lines TNF α

All cell lines UNTREATED vs All cell lines TNF α /TGF β

We model the data correcting for PC1 and Line (patient-specific expression) and then test for the treatment effect

```

#The factor "Batch" is included as a covariate to account for potential batch
effects,
#and the factor "Treatment" is included as the variable of interest for
differential expression analysis.

```

```

RBarretMY0FCntGMaskTreatment <- DESeqDataSetFromMatrix(BarretMyofCnt, colData=
sampleTableMyof, design= ~ Batch + Treatment)
RBarretMY0FCntGMaskTreatment <- DESeq(RBarretMY0FCntGMaskTreatment)

```



README.md

case the independent hypothesis weighting (IHW) method.

```
#Calculate differential expression results for the pairwise comparisons of
#the TG vs CC, TN vs CC, and TT vs CC treatment groups, respectively.
```

```
#The resulting output for each comparison will contain a table of genes
#with their corresponding LFCs, p-values, and adjusted p-values based on the
#specified multiple testing correction method.
```

```
RBarretMYOFCntGMaskTreatment_TG <-
results(RBarretMYOFCntGMaskTreatment, contrast=c("Treatment", "TG",
"CC"), filterFun=ihw)
RBarretMYOFCntGMaskTreatment_TN <-
results(RBarretMYOFCntGMaskTreatment, contrast=c("Treatment", "TN",
"CC"), filterFun=ihw)
RBarretMYOFCntGMaskTreatment_TT <-
results(RBarretMYOFCntGMaskTreatment, contrast=c("Treatment", "TT",
"CC"), filterFun=ihw)
```

Visually check the names of the most significant genes (very low adjusted p-value)

```
Genenames$V1[which(RBarretMYOFCntGMaskTreatment_TG$padj<0.00000000000001)]
Genenames$V1[which(RBarretMYOFCntGMaskTreatment_TN$padj<0.00000000000001)]
Genenames$V1[which(RBarretMYOFCntGMaskTreatment_TT$padj<0.00000000000001)]
```

Second round of pairwise comparisons:

Untreated NON-FIBROTIC cell lines vs Untreated FIBROTIC cell lines

TNF α /TGF β NON-FIBROTIC cell lines vs TNF α /TGF β FIBROTIC cell lines

TGF β NON-FIBROTIC cell lines vs TGF β FIBROTIC cell lines

TNF α NON-FIBROTIC cell lines vs TNF α FIBROTIC cell lines

We model the data correcting for Line (patient-specific expression) and then test for the Factor (treatment+phenotype combination) effect.

```
RBarretMYOFCntGMaskFactor <- DESeqDataSetFromMatrix(BarretMyofCnt, colData=
sampleTableMyof, design= ~ Line + Factor)
RBarretMYOFCntGMaskFactor <- DESeq(RBarretMYOFCntGMaskFactor)
```

```
#Compute differential expression analysis results for the four contrasts of
interest "CC_F" vs "CC_N", "TG_F" vs "TG_N", "TN_F" vs "TN_N", and "TT_F" vs "TT_N"
in the dataset.
```

```
RBarretMYOFCntGMaskFactor_CC_Pheno <-
results(RBarretMYOFCntGMaskFactor, contrast=c("Factor", "CC_F",
"CC_N"), filterFun=ihw)
RBarretMYOFCntGMaskFactor_TG_Pheno <-
results(RBarretMYOFCntGMaskFactor, contrast=c("Factor", "TG_F",
"TG_N"), filterFun=ihw)
RBarretMYOFCntGMaskFactor_TN_Pheno <-
results(RBarretMYOFCntGMaskFactor, contrast=c("Factor", "TN_F",
"TN_N"), filterFun=ihw)
RBarretMYOFCntGMaskFactor_TT_Pheno <-
results(RBarretMYOFCntGMaskFactor, contrast=c("Factor", "TT_F",
"TT_N"), filterFun=ihw)
```

```
Genenames$V1[which(RBarretMYOFCntGMaskFactor_CC_Pheno$padj<0.01)]
Genenames$V1[which(RBarretMYOFCntGMaskFactor_TG_Pheno$padj<0.01)]
Genenames$V1[which(RBarretMYOFCntGMaskFactor_TN_Pheno$padj<0.01)]
Genenames$V1[which(RBarretMYOFCntGMaskFactor_TT_Pheno$padj<0.01)]
```

Export and process results

```
write.csv(as.data.frame(RBarretMYOFCntGMaskTreatment_TG), file="RBarretMYOFCntGMaskTreatment_TG.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskTreatment_TN), file="RBarretMYOFCntGMaskTreatment_TN.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskTreatment_TT), file="RBarretMYOFCntGMaskTreatment_TT.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_CC_Pheno), file="RBarretMYOFCntGMaskFactor_CC_Pheno.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TG_Pheno), file="RBarretMYOFCntGMaskFactor_TG_Pheno.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TN_Pheno), file="RBarretMYOFCntGMaskFactor_TN_Pheno.csv")
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TT_Pheno), file="RBarretMYOFCntGMaskFactor_TT_Pheno.csv")
```

Pairwise results shreadsheets

Paste Gencode_33_Selected_Geneid_GMask.txt, Gencode_33_Selected_Genename_GMask.txt, Gencode_33_Selected_MappSS_GMask.txt, RBarretMYOFCntGMaskFactor_CC_Pheno_test.txt, RBarretMYOFCntGMaskFactor_TG_Pheno_test.txt, RBarretMYOFCntGMaskFactor_TN_Pheno_test.txt, RBarretMYOFCntGMaskFactor_TT_Pheno_test.txt, RBarretMYOFCntGMaskTreatment_TG_test.txt, RBarretMYOFCntGMaskTreatment_TN_test.txt, and RBarretMYOFCntGMaskTreatment_TT_test.txt to formulate a shreadsheets.

```
#In terminal
```

```
tail -n +2 RBarretMYOFCntGMaskFactor_CC_Pheno.txt | cut -d"," -f3,7 >
```

RBarretMY0FCntGMaskFactor_CC_Pheno_test.txt

tail -n +2 RBarretMY0FCntGMaskFactor_TG_Pheno.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskFactor_TG_Pheno_test.txt

tail -n +2 RBarretMY0FCntGMaskFactor_TN_Pheno.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskFactor_TN_Pheno_test.txt

tail -n +2 RBarretMY0FCntGMaskFactor_TT_Pheno.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskFactor_TT_Pheno_test.txt

tail -n +2 RBarretMY0FCntGMaskTreatment_TG.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskTreatment_TG_test.txt

tail -n +2 RBarretMY0FCntGMaskTreatment_TN.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskTreatment_TN_test.txt

tail -n +2 RBarretMY0FCntGMaskTreatment_TT.txt | cut -d"," -f3,7 >

RBarretMY0FCntGMaskTreatment_TT_test.txt

paste Gencode_33_Selected_Geneid_GMask.txt Gencode_33_Selected_Genename_GMask.txt
 Gencode_33_Selected_MappSS_GMask.txt RBarretMY0FCntGMaskFactor_CC_Pheno_test.txt
 RBarretMY0FCntGMaskFactor_TG_Pheno_test.txt
 RBarretMY0FCntGMaskFactor_TN_Pheno_test.txt
 RBarretMY0FCntGMaskFactor_TT_Pheno_test.txt
 RBarretMY0FCntGMaskTreatment_TG_test.txt RBarretMY0FCntGMaskTreatment_TN_test.txt
 RBarretMY0FCntGMaskTreatment_TT_test.txt >
 Barret_Myofibroblast_TGFTNF_PAIRWISEResults.txt

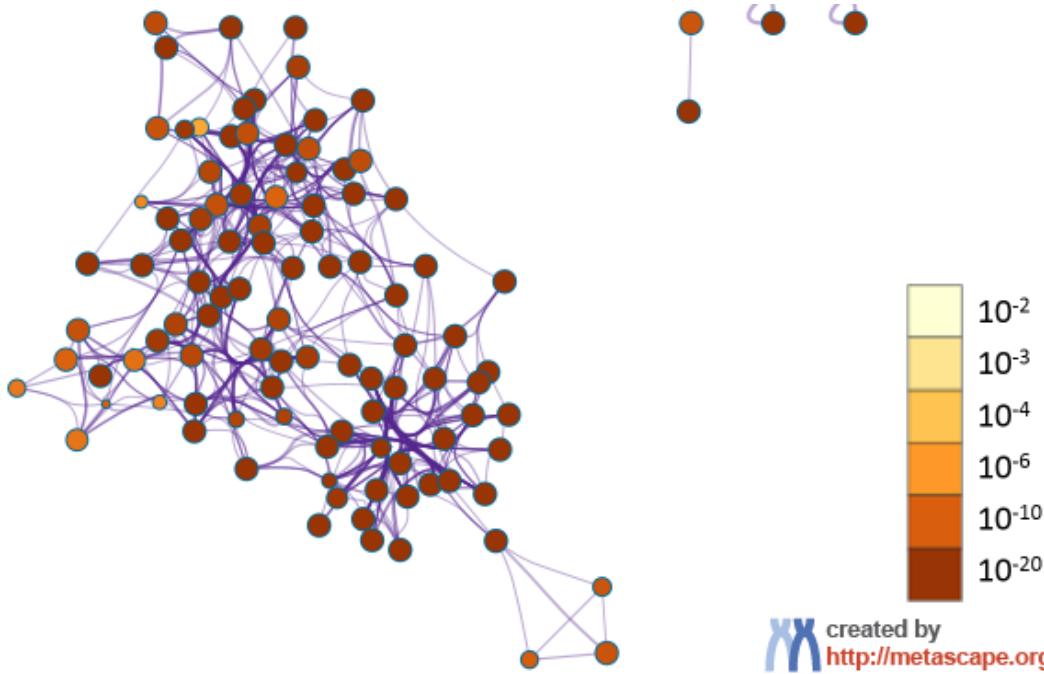
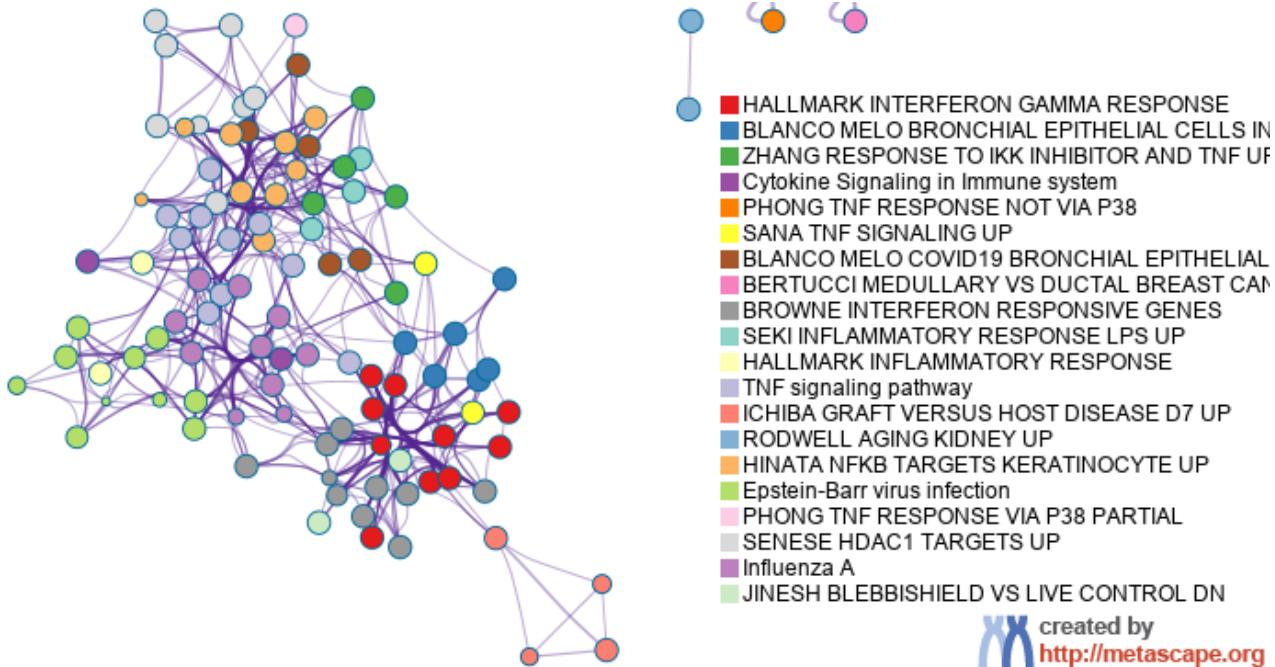
sed 's/,/\t/g' Barret_Myofibroblast_TGFTNF_PAIRWISEResults.txt >
 Barret_Myofibroblast_TGFTNF_PAIRWISEResults.txt

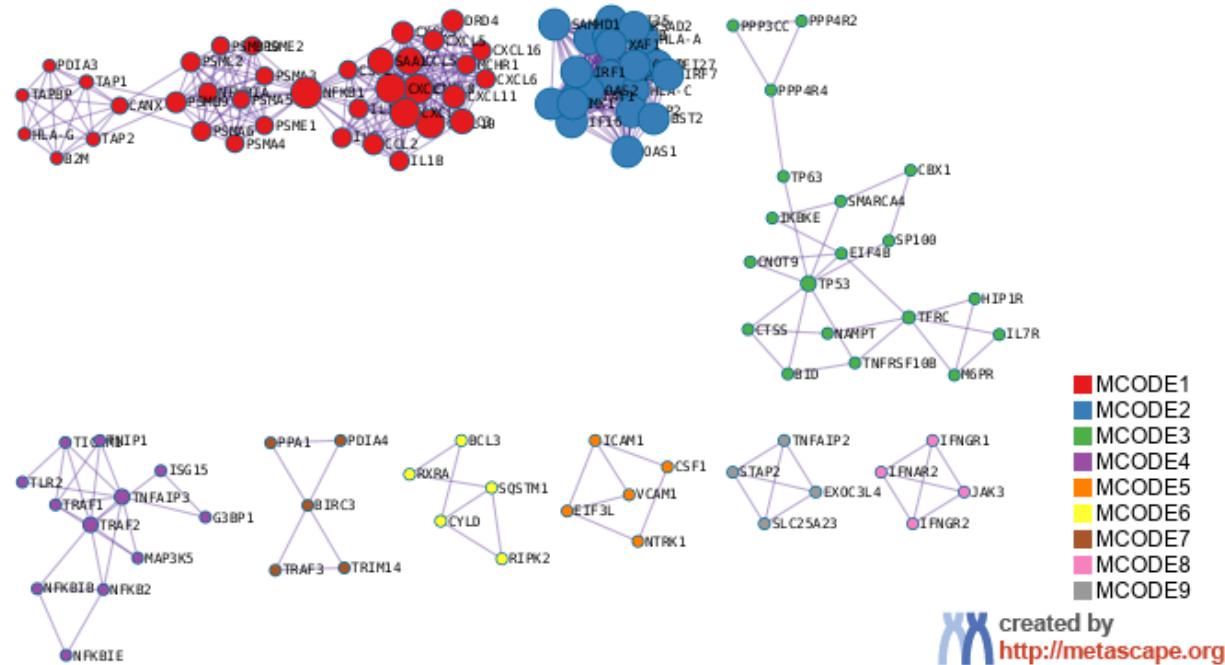
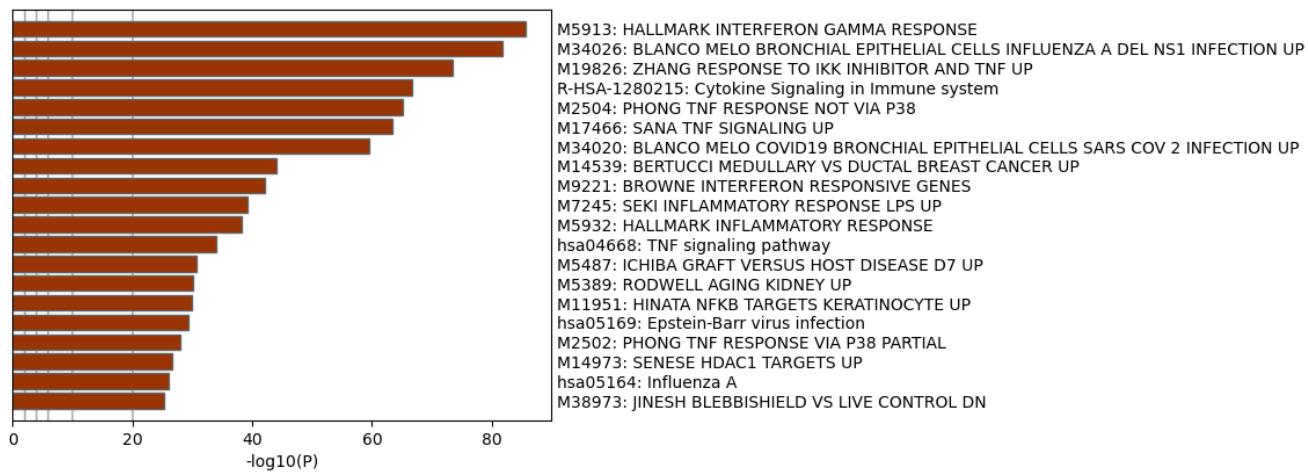
In Figure 13, it shows a clear formatted sheet containing Gene ID, Gene name, Mappability, fold change, and adjusted p-value. Then, you can sort each adjusted p-value column to focus on genes you are interested in. You can find the pairwise results spreadsheet [here](#).

Gene ID	Gene name	Mappability	Untreated NON-FIBROTIC cell lines vs Untreated FIBROTIC cell lines		TGFB NON-FIBROTIC cell lines vs TGFB FIBROTIC cell lines		TNFa NON-FIBROTIC cell lines vs TNFa FIBROTIC cell lines		TNFa/TGFB NON-FIBROTIC cell lines vs TNFa/TGFB FIBROTIC cell lines		All cell lines UNTREATED vs All cell lines TGFB		All cell lines UNTREATED vs All cell lines TNFa		All cell lines UNTREATED vs All cell lines TNFa/TGFB	
			fold change	adjusted p-value	fold change	adjusted p-value	fold change	adjusted p-value	fold change	adjusted p-value	fold change	adjusted p-value	fold change	adjusted p-value	fold change	adjusted p-value
ENSG00000187634.12	SAMD11	4521	0.596611691	1	0.226814098	1	0.378338171	1	0.919392421	0.336037463	2.706732474	5.206E-43	0.081053744	0.817710494	-0.73358626	2.77E-18
ENSG00000188976.11	NOC1L	2644	0.068821384	1	-0.000431352	1	0.040269268	1	0.019211781	2.36E-07	0.205588933	5.95E-14	0.10246128	0.000280382		
ENSG00000187961.14	KHL17	2896	0.172917015	0.832705293	0.136320927	0.989972294	0.08539404	0.120805040	0.61896504	0.327769911	2.15E-17	0.127323327	0.001907649	-0.138919428	0.00050619	
ENSG00000187583.11	PLEKH1N1	3613	-0.090864126	0.827806587	-0.028993874	1	-0.160520142	0.807752678	-0.134336653	0.824087967	1.788086587	3.48E-39	2.162682368	4.62E-57	1.071001939	7.40E-15
ENSG00000187642.9	PERM1	3296	-2.18570595	0.404394778	0.216761266	1	0.058135914	1	0.368492077	0.97846392	-0.598395081	0.081221314	-1.505134176	0.000323565	-0.870103019	0.01789074
ENSG00000188290.10	HES4	1133	0.153300669	1	0.122062697	1	0.218157143	0.887299992	-0.134392474	0.958923313	0.206496424	0.07818297	0.937081074	8.76E-19	0.37106490	0.000713975
ENSG00000187608.17	IG515	861	-0.102148425	1	0.024596762	1	0.021694639	1	0.279257826	1	0.263795161	0.186993258	8.39E-25	3.07608200	7.91E-89	
ENSG00000188157.15	AGRN	7570	-0.160468497	1	-0.075395693	1	-0.193898673	0.995982556	-0.163057511	1	0.58144503	1.72E-18	1.150947439	2.61E-68	1.20353386	3.41E-75
ENSG00000131591.17	Clof159	4519	-0.03309248	1	0.024669475	1	-0.027725627	1	-0.088624678	0.768141664	1.08E-17	0.084923253	0.012031563	0.278258925	1.32E-18	
ENSG00000186891.14	TGRSF18	1359	0.886403284	0.823129353	-0.446386643	0.96514689	0.117902521	1	0.338684822	1	-1.177307994	4.28E-06	5.451458563	4.38E-123	5.677301524	2.13E-15
ENSG00000186827.11	TGRSF4	976	-0.514160059	0.6644970317	-0.612637224	0.816114951	-0.860106585	0.46831063	-0.253356711	0.760286957	-0.334418897	0.330752548	4.49956402	3.17E-56	5.52886735	1.09E-85
ENSG00000187880.17	SDF4	2308	0.049625524	1	0.057274721	1	0.016151239	1	0.035605533	1	0.142337521	0.000149013	0.571263308	2.52E-58	0.932141946	2.09E-15
ENSG00000176022.7	B3GALT6	2706	0.085310973	1	0.056894942	1	0.097685668	0.722016938	0.028759473	1	0.249355441	5.52E-30	0.330182287	1.35E-51	0.31198219	1.12E-46
ENSG00000184163.3	C1QTNF12	935	0.040339553	0.950931075	0.01697861	1	0.123619172	0.971573052	0.096960593	0.795960145	0.027763009	0.684222846	0.27031544	0.000399295	0.252401655	0.001009205
ENSG00000160087.20	UBE2J2	4052	-0.018781721	1	0.0616056432	1	0.06902966	1	0.041120288	1	0.118911155	1.34E-05	0.359863102	6.77E-43	0.4020109	6.95E-54
ENSG00000162572.21	SCNN1D	4334	-0.072212678	1	-0.244865516	0.815898111	-0.181899086	0.825135208	-0.206986633	0.693142693	0.172373458	0.028499787	-0.37164395	6.50E-07	-0.34198392	5.80E-06
ENSG00000131584.19	ACAP3	3785	0.127844752	1	0.12791254	1	0.016340476	1	0.169561477	0.52509675	-0.195760833	1.71E-07	-0.43331158	1.06E-32	0.523908776	3.23E-47
ENSG00000169972.12	PUS11	1230	0.073770166	1	0.056988265	1	0.092046079	1	0.062409185	0.877004056	0.184795617	3.34E-07	0.094698149	0.008115119	-0.0585175	0.124565207
ENSG00000127054.20	INTS11	4763	-0.088556989	1	0.000764034	1	-0.148889871	1	-0.010827238	5.18E-07	-0.103127142	1.37E-16	-0.137617185	2.15E-15	-0.142479203	1.37E-16
ENSG00000224051.7	CPTP	2406	0.015653893	1	0.053973742	1	0.047698086	1	0.024181163	1	0.027714475	0.191445222	-0.004764667	1	-0.167596923	5.95E-15
ENSG00000169962.5	TAS1R3	3376	0.013513716	0.941888124	0.05216788	0.992719658	0.107758312	1	-0.176170461	0.853627636	0.341631389	0.003406764	0.17222661	0.1300722	-0.061516795	0.653158608
ENSG00000107404.20	DVL1	3522	-0.044768884	1	-0.03015666	1	-0.050538025	1	0.051134906	1	0.103787432	7.55E-05	0.02170496	0.797610809	-0.220666329	2.76E-17
ENSG00000162576.16	MXRAB8	3257	0.191313096	1	0.219490333	1	0.118577359	1	0.100133923	1	0.453824966	2.56E-17	-0.05839373	0.385826016	0.429853477	1.31E-15
ENSG00000175756.13	AURKA1P1	1520	-0.028795706	1	-0.037998442	1	0.087237629	1	-0.052937789	1	0.141171957	3.74E-05	0.245885007	1.31E-13	0.150746674	7.28E-06
ENSG00000123978.12	CNL2	4211	0.028521234	1	-0.005812613	1	-0.015013085	1	0.035759196	1	-0.118972028	6.80E-06	-0.326796888	5.25E-38	-0.323340944	1.81E-37
ENSG00000242485.6	MRPL20	1324	-0.07393538	1	-0.042639929	1	0.091903192	1	-0.117280624	0.754724517	0.157619322	8.45E-05	0.228739454	1.32E-08	0.155903354	0.000148877
ENSG00000235098.8	ANKRD65	2831	0.375439502	0.560677801	0.437439502	0.633271303	0.422432696	0.842296769	0.280112535	0.837926079	0.534111805	0.000250266	0.056395942	0.574281575	0.025426855	0.749726248
ENSG00000205116.3	TMEM88B	390	1.478490154	0.384576027	-0.032535513	1	-0.185697779	1	-0.112360105	1	1.037645284	0.021564088	0.298207391	0.342404805	0.29319792	0.394244566
ENSG00000179403.12	VWA1	5140	0.112048592	1	-0.12164659	1	-0.160268467	1	0.016458299	1	0.970974507	1.69E-22	-0.021517027	0.93781096	-0.217782531	0.038846874

Pathway analysis

Go to [Metascape](#) and enter the top 500 sorted gene list. After customizing your analysis, you can get their pathway analysis result.





Future works

To further our understanding of complex signaling pathways, we propose integrating data from both the myofibroblast experiment and the epithelial experiment, both of which involve treatments. By combining these datasets, we aim to identify novel ways to distinguish between cellular responses and gain insight into potential mechanisms underlying these responses. Specifically, I plan to leverage this combined dataset to model responses from induced pluripotent stem cell (iPSC) lines, which could provide a powerful tool for studying complex signaling pathways. By doing so, we hope to enhance our understanding of the intricate interplay between various signaling pathways and pave the way for future research in this field.

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