README_loc.md

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)

On 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
- TGFb (a pro-fibrotic cytokine)[4]
- TNFa (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
GNAGCAAAGTGGTACCCAAACCTAAGAGCTATTATCCCTAAATTTCAAAATCTAAAAAAAAAAAACCTTAGAACCTC
>527iP29TNFaM_S124_2
GNGCTCATGCTGTTTTTCCAGGAGAAAAGTAAGATCCTTCAGCCGGTATTTCTGCTTTAATATTTCCATTTCTAAA
>527iP29TNFaM_S124_3
CNTACCTTGAGTTCATTTTTTTCTATTTCTATAGCCATAACTTAAATTTCTGATTTAAGTTCTTCCAATACAATGC
>527iP29TNFaM_S124_4
TNAGTATTGATTGTTAGCGGTGTGGTCGGGTGTGTTATTATTCTGAATTTTGGGGGAGGTTATATGGGTTTAATAG
>527iP29TNFaM_S124_5
GNCTAACTAAAGAAGGAAAATGTAGAATTAAGGCAGAAATCTCATGAAAATCCTTCCCATGCAATAAAGGAGGCTT
>527iP29TNFaM_S124_6
CNCTTAATTTGTACTAAAGGGATGGTAGGTGGTCACATGCAGTCCATGTGGGATTCTAACATGACATTTAGTGAGT
>527iP29TNFaM_S124_7
ANTCTCCTTTTGTTTTTGCATTAATTGAATAAAGGTAATTTCAGGCTGTGCAGCTTTCAATTGCCGGTTGGTGGTT
>527iP29TNFaM_S124_8
GCCATCCTTTCGATTTCTTCAGGTGTCAGGCGATTCTGGTCATTGGTGATTGTGATCTTATTTTTGTTCCCTGTAC
>527iP29TNFaM_S124_9
GCCGGAAAACATCAGAGATGGAGGGCCCCAGCAGCAGGAAGACGTCAATGATGCCAGTCTCTGACATCCAGCGAAC
>527iP29TNFaM_S124_10
GCCGTGAATGCAGGACCATCCAGGTCCTCAAAGTCTGTGAGGTTTGTTCATAATCCCAAACAAGGGCCCTGCTGGC
```

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
/home/luc/iPSC/MYOFIBROBLAST/ mem_free=32G 8 >> sendmyof
```

Change sendmyof into executable mode and run sendmyof.

```
chmod a+x sendmyof
```

```
. sendmyof
```

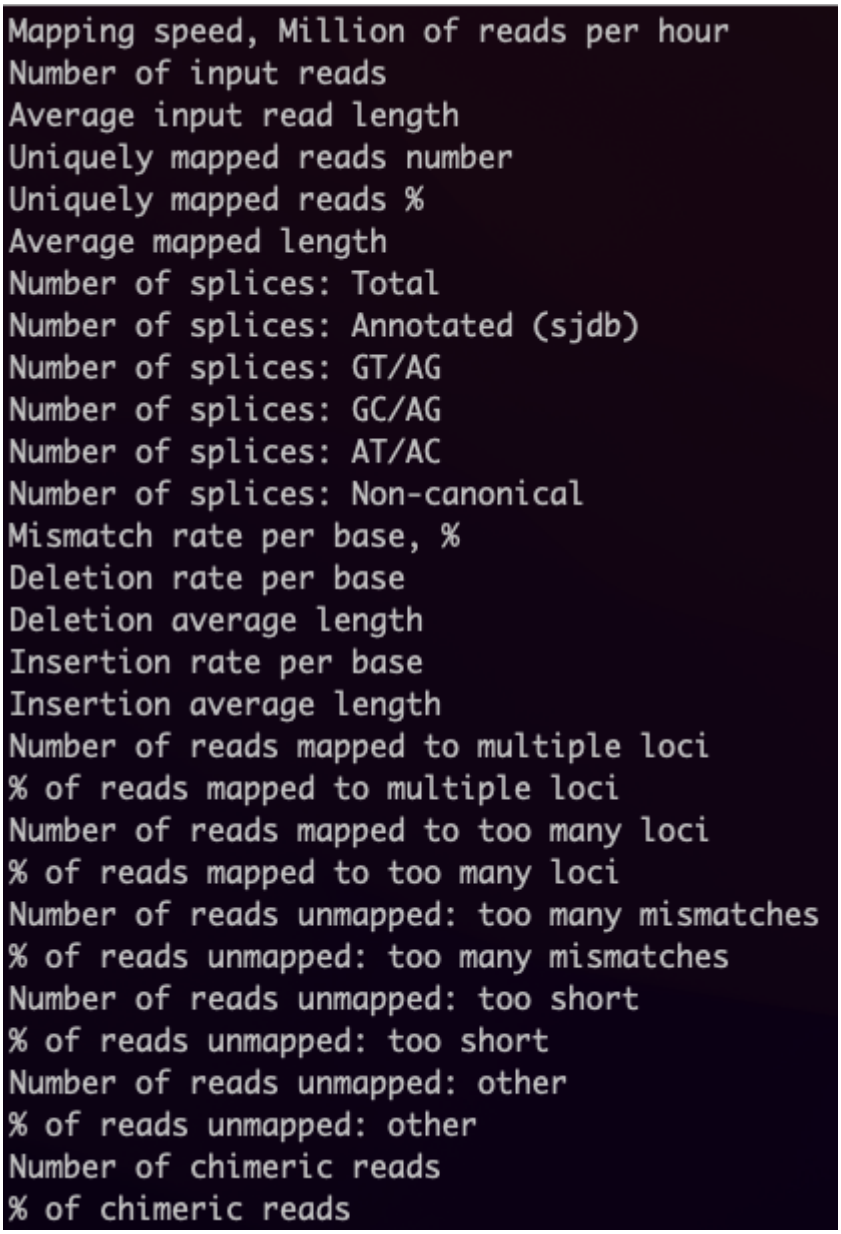
It will take less than one day to run through 151 samples and generate each sample a folder which contain every output from STAR.

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.



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
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%	90.29%	90.70%
Average mapped length	75.21	75.21	75.21	75.20	75.20	75.20	75.19	75.21	75.20	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	60185	79428
Number of splices: AT/AC		7048	6261	6785	8291	6179	5252	12249	9152	15682	8499	6008	7801
Number of splices: Non-canonical		7080	5407	5801	7799	5756	4355	9871	8901	10933	5153	3764	
Mismatch rate per base, %		0.27%	0.28%	0.29%	0.28%	0.28%	0.28%	0.31%	0.28%	0.29%	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%	0.02%	0.02%	0.02%
Deletion average length		1.47	1.47	1.47	1.47	1.48	1.48	1.47	1.46	1.47	1.48	1.50	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%	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	1.38	1.44
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%	6.11%
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%	0.84%
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	815091
% 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%	2.69%	2.07%
Number of reads unmapped: other		16971		16710	20173	24305	15726	13423	29546	25946	17237	24268	22820
% of reads unmapped: other		0.04%		0.04%	0.05%	0.05%	0.04%	0.04%	0.04%	0.05%	0.02%	0.05%	0.08%	0.05%
Number of chimeric reads		0		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%	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.

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

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

The following files contain the annotation and gene effective lenghts (mappabilities) for the human gene annoation 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 RBarretTNFATGFBCnt. 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 = RBarretTNFATGFBCnt;
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

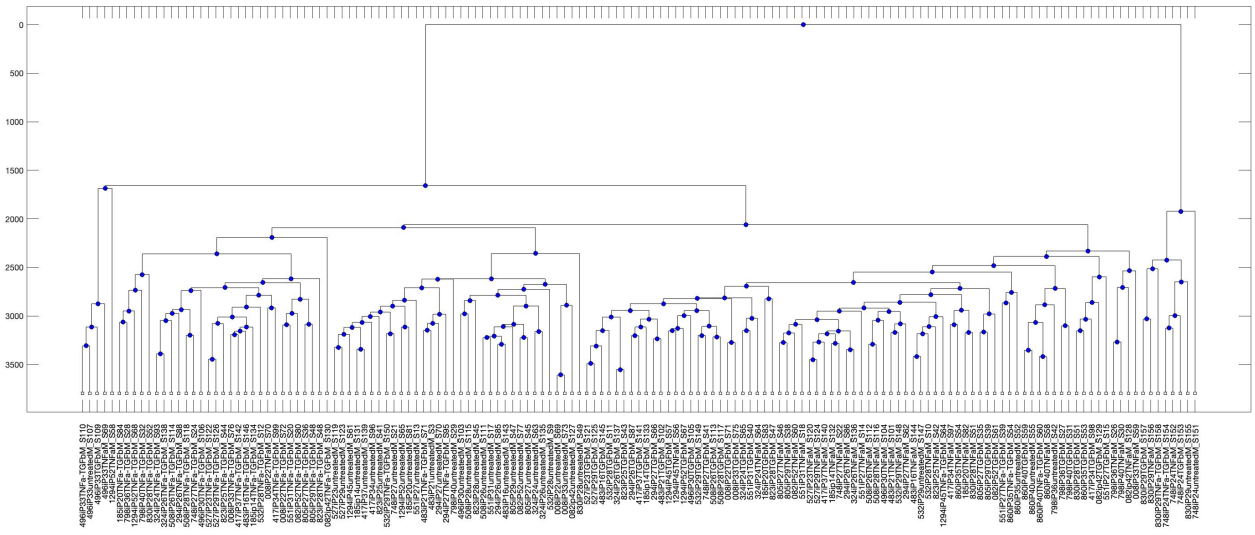
Make a dendrogram to visualize the relationships among samples in the RBarretTNFATGFB dataset based on their gene expression profiles. To downgrade the effect of potential expression outliers in the dendrogram and compute a more robust sample clustering:

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 tipically 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)
```

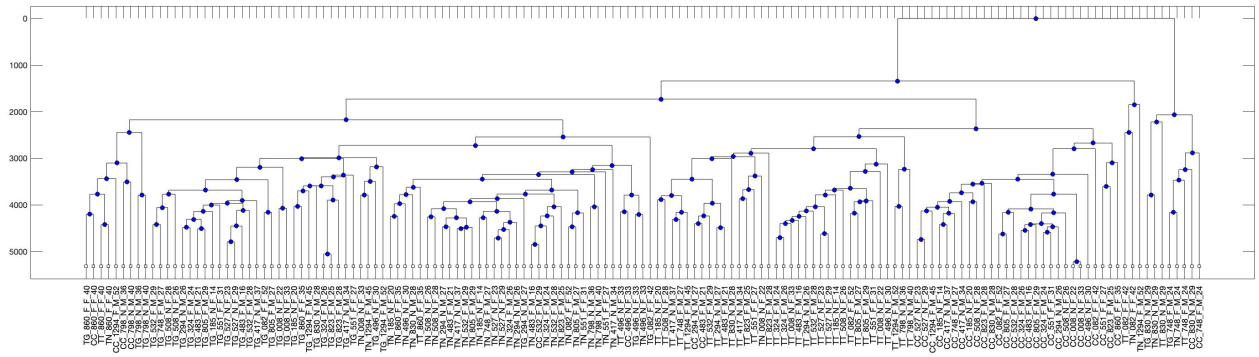
```
RBarretTNFATGFBTPM_GMask(:,i) =
RBarretTNFATGFBTPM_GMask(:,i)/sum(RBarretTNFATGFBTPM_GMask(:,i))*1000000;
end
```

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')
```

Agains, 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_G
writetable(cell2table(Gencode_33_Selected_Genename_GMask),'Gencode_33_Selected
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 RBarretTNFATGFBCnt_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)

RBarretTNFATGFBCntGMask =
as.matrix(read.table("RBarretTNFATGFBCnt_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.

```
RBarretTNFATGFBCntGMaskBatch <-
DESeqDataSetFromMatrix(RBarretTNFATGFBCntGMask, colData=
sampleTableTNFATGFB,design= ~Batch)
RBarretTNFATGFBCntGMaskBatch <- DESeq(RBarretTNFATGFBCntGMaskBatch)

RBarretTNFATGFBCntGMaskFactor <-
DESeqDataSetFromMatrix(RBarretTNFATGFBCntGMask, colData=
sampleTableTNFATGFB,design= ~Factor)
RBarretTNFATGFBCntGMaskFactor <- DESeq(RBarretTNFATGFBCntGMaskFactor)

RBarretTNFATGFBCntGMaskPC1 <-
DESeqDataSetFromMatrix(RBarretTNFATGFBCntGMask, colData= pcabatchR,design=
~PC1)
RBarretTNFATGFBCntGMaskPC1 <- DESeq(RBarretTNFATGFBCntGMaskPC1)

RBarretTNFATGFBCntGMaskBatch_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFBCntGMaskBatch,blind=FALSE)
RBarretTNFATGFBCntGMaskFactor_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFBCntGMaskFactor,blind=FALSE)
RBarretTNFATGFBCntGMaskPC1_vsd <-
varianceStabilizingTransformation(RBarretTNFATGFBCntGMaskPC1,blind=FALSE)
```

Principal Component Analysis (PCA)

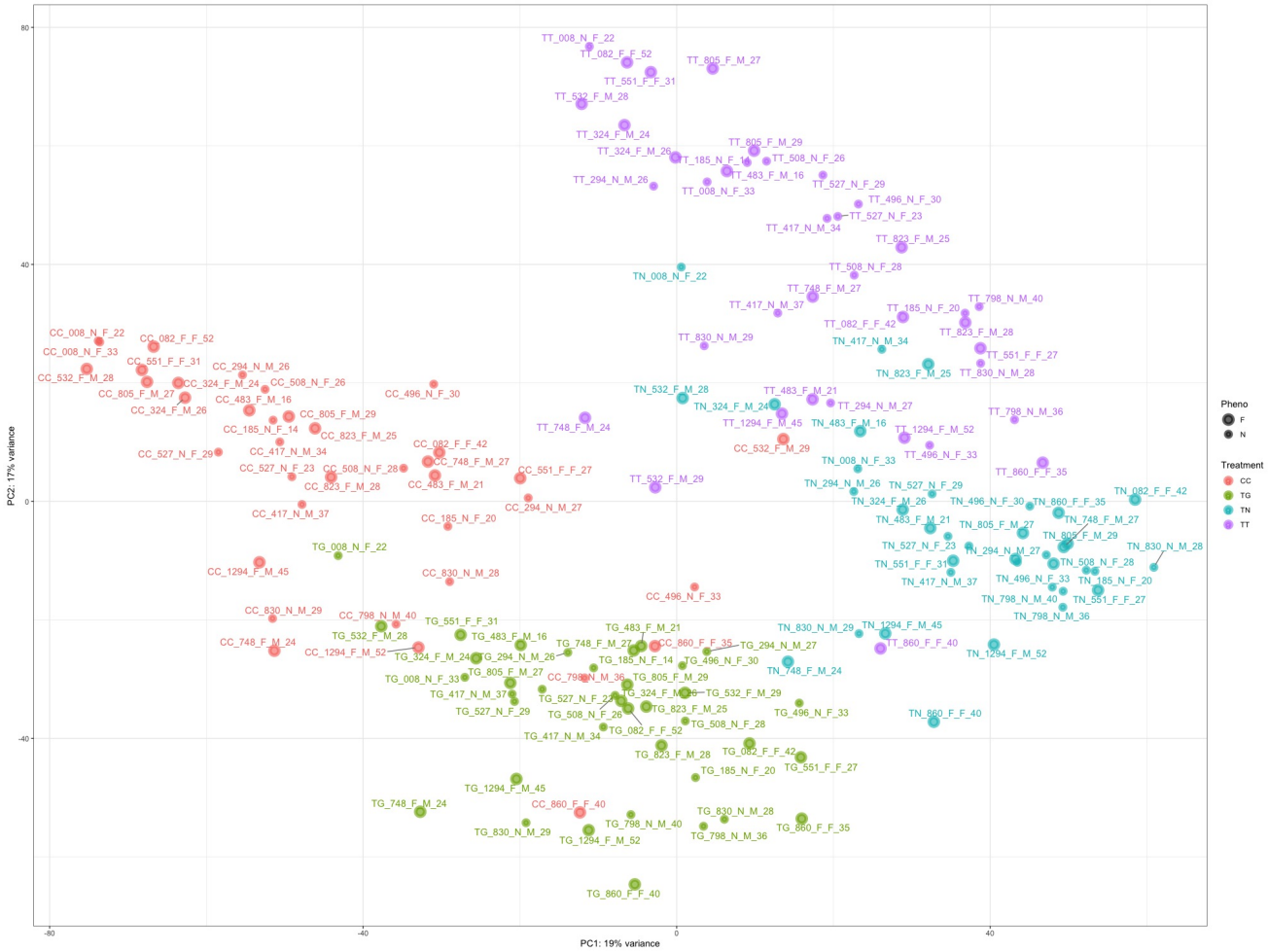
```
#perform principal component analysis (PCA) on the variance-stabilized
counts data
pcabatch <- prcomp(t(assay(RBarretTNFATGFBCntGMaskBatch_vsd)))

#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

#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',sp
  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 that 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');
```

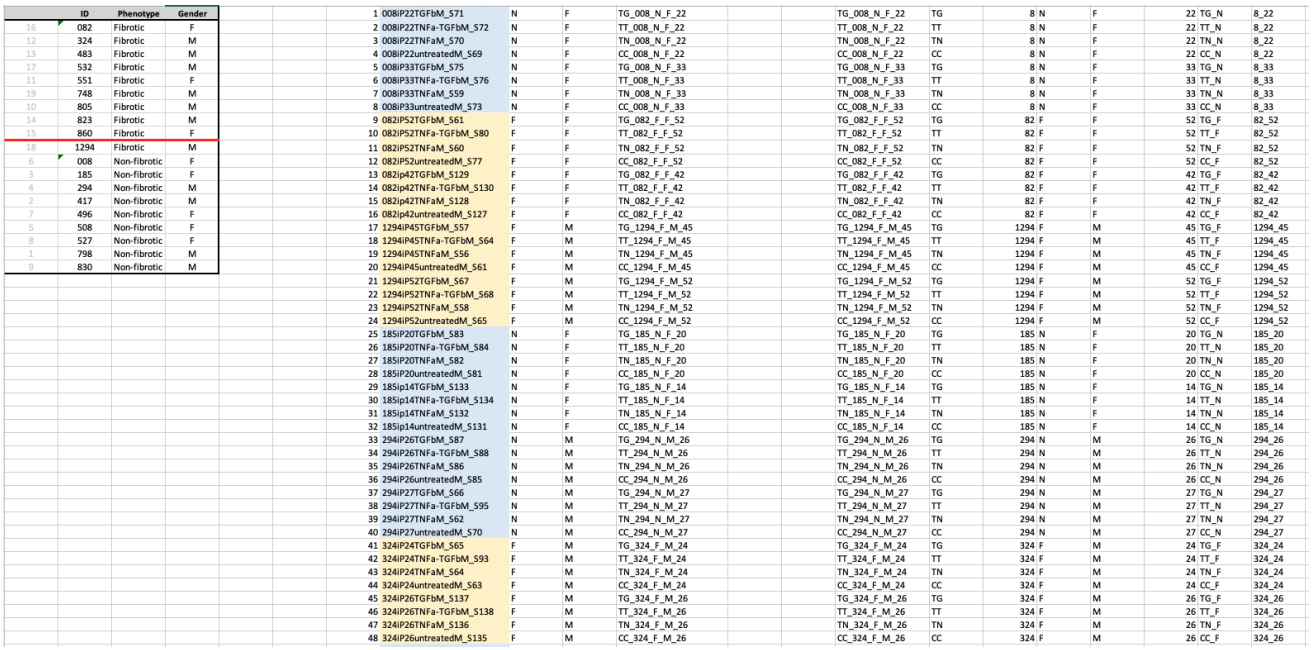
Determine the rank of each row in the original order for the first three principal components and store the ranks in a matrix pcarankmatrix.

```
[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
16	082	Fibrotic	F
12	324	Fibrotic	M
13	483	Fibrotic	M
17	532	Fibrotic	M
11	551	Fibrotic	F
19	748	Fibrotic	M
10	805	Fibrotic	M
14	823	Fibrotic	M
15	860	Fibrotic	F
18	1294	Fibrotic	M
6	008	Non-fibrotic	F
3	185	Non-fibrotic	F
4	294	Non-fibrotic	M
2	417	Non-fibrotic	M
7	496	Non-fibrotic	F
5	508	Non-fibrotic	F
8	527	Non-fibrotic	F
1	798	Non-fibrotic	M
9	830	Non-fibrotic	M

1	008iP22TGFbM_S71	N	F	TG_008_N_F_22	TG_008_N_F_22	TG	8	N	F	22	TG_N	8_22
2	008iP22TNFa-TGFbM_S72	N	F	TT_008_N_F_22	TT_008_N_F_22	TT	8	N	F	22	TT_N	8_22
3	008iP22TNFaM_S70	N	F	TN_008_N_F_22	TN_008_N_F_22	TN	8	N	F	22	TN_N	8_22
4	008iP22untreatedM_S69	N	F	CC_008_N_F_22	CC_008_N_F_22	CC	8	N	F	22	CC_N	8_22
5	008iP33TGFbM_S75	N	F	TG_008_N_F_33	TG_008_N_F_33	TG	8	N	F	33	TG_N	8_33
6	008iP33TNFa-TGFbM_S76	N	F	TT_008_N_F_33	TT_008_N_F_33	TT	8	N	F	33	TT_N	8_33
7	008iP33TNFaM_S59	N	F	TN_008_N_F_33	TN_008_N_F_33	TN	8	N	F	33	TN_N	8_33
8	008iP33untreatedM_S73	N	F	CC_008_N_F_33	CC_008_N_F_33	CC	8	N	F	33	CC_N	8_33
9	082iP52TGFbM_S61	F	F	TG_082_F_F_52	TG_082_F_F_52	TG	82	F	F	52	TG_F	82_52
10	082iP52TNFa-TGFbM_S80	F	F	TT_082_F_F_52	TT_082_F_F_52	TT	82	F	F	52	TT_F	82_52
11	082iP52TNFaM_S60	F	F	TN_082_F_F_52	TN_082_F_F_52	TN	82	F	F	52	TN_F	82_52
12	082iP52untreatedM_S77	F	F	CC_082_F_F_52	CC_082_F_F_52	CC	82	F	F	52	CC_F	82_52
13	082ip42TGFbM_S129	F	F	TG_082_F_F_42	TG_082_F_F_42	TG	82	F	F	42	TG_F	82_42
14	082ip42TNFa-TGFbM_S130	F	F	TT_082_F_F_42	TT_082_F_F_42	TT	82	F	F	42	TT_F	82_42
15	082ip42TNFaM_S128	F	F	TN_082_F_F_42	TN_082_F_F_42	TN	82	F	F	42	TN_F	82_42
16	082ip42untreatedM_S127	F	F	CC_082_F_F_42	CC_082_F_F_42	CC	82	F	F	42	CC_F	82_42
17	1294iP45TGFbM_S57	F	M	TG_1294_F_M_45	TG_1294_F_M_45	TG	1294	F	M	45	TG_F	1294_45
18	1294iP45TNFa-TGFbM_S64	F	M	TT_1294_F_M_45	TT_1294_F_M_45	TT	1294	F	M	45	TT_F	1294_45
19	1294iP45TNFaM_S56	F	M	TN_1294_F_M_45	TN_1294_F_M_45	TN	1294	F	M	45	TN_F	1294_45
20	1294iP45untreatedM_S61	F	M	CC_1294_F_M_45	CC_1294_F_M_45	CC	1294	F	M	45	CC_F	1294_45
21	1294iP52TGFbM_S67	F	M	TG_1294_F_M_52	TG_1294_F_M_52	TG	1294	F	M	52	TG_F	1294_52
22	1294iP52TNFa-TGFbM_S68	F	M	TT_1294_F_M_52	TT_1294_F_M_52	TT	1294	F	M	52	TT_F	1294_52
23	1294iP52TNFaM_S58	F	M	TN_1294_F_M_52	TN_1294_F_M_52	TN	1294	F	M	52	TN_F	1294_52
24	1294iP52untreatedM_S65	F	M	CC_1294_F_M_52	CC_1294_F_M_52	CC	1294	F	M	52	CC_F	1294_52
25	185iP20TGFbM_S83	N	F	TG_185_N_F_20	TG_185_N_F_20	TG	185	N	F	20	TG_N	185_20
26	185iP20TNFa-TGFbM_S84	N	F	TT_185_N_F_20	TT_185_N_F_20	TT	185	N	F	20	TT_N	185_20
27	185iP20TNFaM_S82	N	F	TN_185_N_F_20	TN_185_N_F_20	TN	185	N	F	20	TN_N	185_20
28	185iP20untreatedM_S81	N	F	CC_185_N_F_20	CC_185_N_F_20	CC	185	N	F	20	CC_N	185_20
29	185ip14TGFbM_S133	N	F	TG_185_N_F_14	TG_185_N_F_14	TG	185	N	F	14	TG_N	185_14
30	185ip14TNFa-TGFbM_S134	N	F	TT_185_N_F_14	TT_185_N_F_14	TT	185	N	F	14	TT_N	185_14
31	185ip14TNFaM_S132	N	F	TN_185_N_F_14	TN_185_N_F_14	TN	185	N	F	14	TN_N	185_14
32	185ip14untreatedM_S131	N	F	CC_185_N_F_14	CC_185_N_F_14	CC	185	N	F	14	CC_N	185_14
33	294iP26TGFbM_S87	N	M	TG_294_N_M_26	TG_294_N_M_26	TG	294	N	M	26	TG_N	294_26
34	294iP26TNFa-TGFbM_S88	N	M	TT_294_N_M_26	TT_294_N_M_26	TT	294	N	M	26	TT_N	294_26
35	294iP26TNFaM_S86	N	M	TN_294_N_M_26	TN_294_N_M_26	TN	294	N	M	26	TN_N	294_26
36	294iP26untreatedM_S85	N	M	CC_294_N_M_26	CC_294_N_M_26	CC	294	N	M	26	CC_N	294_26
37	294iP27TGFbM_S66	N	M	TG_294_N_M_27	TG_294_N_M_27	TG	294	N	M	27	TG_N	294_27
38	294iP27TNFa-TGFbM_S95	N	M	TT_294_N_M_27	TT_294_N_M_27	TT	294	N	M	27	TT_N	294_27
39	294iP27TNFaM_S62	N	M	TN_294_N_M_27	TN_294_N_M_27	TN	294	N	M	27	TN_N	294_27
40	294iP27untreatedM_S70	N	M	CC_294_N_M_27	CC_294_N_M_27	CC	294	N	M	27	CC_N	294_27
41	324iP24TGFbM_S65	F	M	TG_324_F_M_24	TG_324_F_M_24	TG	324	F	M	24	TG_F	324_24
42	324iP24TNFa-TGFbM_S93	F	M	TT_324_F_M_24	TT_324_F_M_24	TT	324	F	M	24	TT_F	324_24
43	324iP24TNFaM_S64	F	M	TN_324_F_M_24	TN_324_F_M_24	TN	324	F	M	24	TN_F	324_24
44	324iP24untreatedM_S63	F	M	CC_324_F_M_24	CC_324_F_M_24	CC	324	F	M	24	CC_F	324_24
45	324iP26TGFbM_S137	F	M	TG_324_F_M_26	TG_324_F_M_26	TG	324	F	M	26	TG_F	324_26
46	324iP26TNFa-TGFbM_S138	F	M	TT_324_F_M_26	TT_324_F_M_26	TT	324	F	M	26	TT_F	324_26
47	324iP26TNFaM_S136	F	M	TN_324_F_M_26	TN_324_F_M_26	TN	324	F	M	26	TN_F	324_26
48	324iP26untreatedM_S135	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_J_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	0.000002551	0.000000000	0.000005942	0.000000000	0.000018073	0.000011577	0.000087182	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_J_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	0.000008247	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	0.000028828	0.000005066	0.000000000	0.000008247	0.000008912	0.000000000	0.000003012	0.000000000	0.000010462	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	0.000001654	0.000000000	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_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
CXCL6	CXCL6	ENSG00000124875.10	1596.00	68.00	1.00	887.00	32.65	10587.00	5693.30	31.87	32.82	4913.60	870.45	33.79	13.20	4292.20	149.95	124.15	1.46	4126.20	943.95	7.07
CXCL8	CXCL8	ENSG00000169429.11	1868.00	1.00	2.00	389.00	0.24	4089.30	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
CXCL1	CXCL1	ENSG00000163739.5	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	ENSG00000125730.17	5851.00	21.00	4.00	153.00	0.50	944.99	236.21	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
ABI3BP	ABI3BP	ENSG00000154175.17	9390.00	1164.00	5.00	1275.00	10.58	412.98	49.70	48.11	10.23	659.79	59.99	49.43	7.72	621.97	40.02	45.88	0.69	15.85	4.66	3.53
COMP	COMP	ENSG00000105664.11	2779.00	147.00	6.00	2037.00	49.71	0.04	67.18	0.08	34.12	0.19	102.56	0.00	54.69	0.00	33.59	0.00	134.30	0.11	12.07	0.12
ELN	ELN	ENSG00000049540.17	5524.00	5644.00	7.00	1132.00	33.42	3.41	15.19	0.64	13.08	0.78	5.12	0.59	64.26	0.31	1.65	0.48	1012.30	12.42	20.23	11.67
CXCL3	CXCL3	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	1272.70	798.31	23.34	8.93	913.94	202.64	22.62	8.31	1386.80	84.31	19.33	9.63	780.30	230.05	43.23
CXCL5	CXCL5	ENSG00000163735.7	2337.00	81.00	10.00	550.00	0.34	869.40	225.78	0.64	0.17	151.01	14.67	0.17	0.00	95.85	2.82	0.05	0.31	1967.60	87.29	0.81
CCL5	CCL5	ENSG00000271503.6	1372.00	3.00	11.00	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	833.98	0.00
PTX3	PTX3	ENSG00000163661.4	1781.00	3156.00	12.00	4555.00	1386.10	9236.90	3935.60	2258.20	184.73	6763.80	768.90	2271.60	55.27	3225.10	140.89	150.15	41.85	468.01	43.16	1158.50
CCL2	CCL2	ENSG00000108691.9	1970.00	4.00	13.00	172.00	1.72	1043.10	834.13	3.22	1.09	1280.90	806.48	3.09	4.20	1968.40	1513.90	5.13	0.30	542.81	292.03	3.57
CXCL2	CXCL2	ENSG00000081041.9	1016.00	333.00	14.00	1285.00	1.45	904.33	356.30	4.76	1.59	304.28	32.38	3.23	0.68	368.55	3.36	2.07	1.00	1236.60	140.94	9.89
VCAM1	VCAM1	ENSG00000162692.12	3283.00	591.00	15.00	1695.00	82.51	1391.60	462.26	286.87	24.01	2666.10	433.79	294.77	18.17	2822.90	343.05	256.84	7.87	238.71	61.08	99.14
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	0.61	487.83	9.96	15.53	1.48	37.89	22.66	19.59
IFI6	IFI6	ENSG00000126709.15	921.00	135.00	17.00	636.00	197.01	11438.00	3923.40	263.68	106.44	4058.10	387.41	265.10	102.71	11933.00	705.00	290.36	68.81	1217.60	878.34	214.17
TP63	TP63	ENSG00000073282.13	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	ENSG00000090339.9	3063.00	20.00	19.00	1288.00	5.06	1045.60	430.16	27.88	2.54	1143.40	413.10	29.38	27.86	1830.80	1044.70	129.72	7.40	482.19	926.08	50.29
BST2	BST2	ENSG00000130303.13	900.00	145.00	20.00	390.00	12.06	1170.20	201.45	14.94	9.55	531.86	71.06	12.89	0.55	1587.70	7.71	5.38	2.72	54.49	50.72	27.43
OAS3	OAS3	ENSG00000111331.13	7694.00	127.00	21.00	471.00	1.50	125.87	39.64	2.71	0.54	65.66	9.59	1.97	0.12	190.81	11.51	2.29	0.73	23.47	22.70	8.72
TRPA1	TRPA1	ENSG00000104321.11	5092.00	88.00	22.00	1008.00	0.04	57.40	30.22	0.04	0.03	98.37	10.63	0.00	0.10	35.51	0.33	0.02	0.93	359.15	55.77	3.51
MMP1	MMP1	ENSG00000196611.5	1864.00	17.00	23.00	236.00	3.15	377.17	978.50	0.52	3.24	141.31	83.54	1.24	0.05	105.16	2.07	0.00	0.26	2582.70	252.83	0.54
OAS1	OAS1	ENSG00000089127.13	4894.00	316.00	24.00	494.00	0.25	83.85	21.32	1.32	0.33	38.14	2.10	1.84	0.10	149.43	3.93	2.78	1.11	14.71	15.05	14.70
BDKRB2	BDKRB2	ENSG00000168398.6	6478.00	5115.00	25.00	2145.00	3.46	299.40	167.19	35.96	3.17	169.33	146.70	35.20	1.84	550.72	12.32	119.07	9.39	297.21	165.28	64.21
TNFAIP2	TNFAIP2	ENSG00000185215.9	4879.00	47.00	26.00	768.00	2.90	480.29	114.66	7.49	2.45	324.42	59.14	7.25	8.45	493.27	60.84	6.93	0.72	108.12	66.09	2.27
PTGES	PTGES	ENSG00000148344.11	1648.00	13160.00	27.00	927.00	22.78	937.77	605.45	120.14	6.60	201.08	56.81	130.62	1.55	477.40	9.66	25.17	3.77	398.15	38.91	38.84
OAS2	OAS2	ENSG00000111335.12	6417.00	205.00	28.00	304.00	0.05	56.65	6.50	0.07	0.10	27.13	0.60	0.08	0.05	109.23	0.55	0.15	0.10	1.54	1.64	0.82
TNFAIP3	TNFAIP3	ENSG00000118503.15	5175.00	16.00	29.00	1485.00	11.01	508.75	471.41	12.45	12.07	527.70	324.42	12.31	5.71	743.53	304.41	9.60	7.48	586.30	359.82	18.54
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
C7	C7	ENSG00000112936.19	5583.00	100.00	31.00	8610.00	35.94	325.09	80.53	57.83	26.44	99.34	16.41	57.58	77.74	1392.20	45.82	500.07	1.89	0.91	0.22	76.14
EPSTI1	EPSTI1	ENSG00000133106.14	3387.00	1787.00	32.00	1636.00	16.06	351.20	178.25	30.64	6.82	211.47	49.20	26.46	4.30	277.19	18.16	38.45	1.66	25.39	13.08	10.65
ANOS1	ANOS1	ENSG00000011201.12	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
SLC7A2	SLC7A2	ENSG00000003989.17	8019.00	162.00	34.00	1464.00	10.18	203.92	79.47	60.58	1.72	103.64	82.66	56.99	1.31	94.47	279.42	50.21	5.15	405.05	797.25	37.41
CRLF1	CRLF1	ENSG00000006016.11	1713.00	139.00	35.00	1053.00	104.67	0.13	228.89	0.63	52.76	0.46	230.10	0.62	103.95	0.19	138.65	0.74	776.71	8.46	777.11	1.70
SLC39A8	SLC39A8	ENSG00000138821.13	5772.00	338.00	36.00	634.00	4.03	522.69	96.33	2.46	3.16	143.62	19.82	2.39	0.84	233.80	5.71	3.36	1.61	31.61	11.27	2.46
BIRC3	BIRC3	ENSG00000023445.15	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
CLDN11	CLDN11	ENSG00000013297.11	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	277.48	6.36	1463.40
CXCL10	CXCL10	ENSG00000169245.6	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	1.06	0.20	0.00	12.87	6.02	0.00
IFIT3	IFIT3	ENSG00000119917.14	2424.00	3432.00	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
IFI44L	IFI44L	ENSG00000137959.16	6048.00	539.00	41.00	423.00	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	11.16	7.05	2.59
IL32	IL32	ENSG00000008517.16	2856.00	2.00	42.00	1031.00	0.20	194.89	142.13	0.87	5.08	451.84	329.95	0.27	3.48	492.91	463.17	4.06	4.91	114.73	305.85	3.75
LDLRAD4	LDLRAD4	ENSG00000168675.18	11732.00	486.00	43.00	981.00	0.65	0.02	0.59	0.02	4.03	0.36	7.21	0.01	19.99	0.12	16.14	3.37	24.01	0.46	14.29	0.25
SPX	SPX	ENSG00000134548.11	2268.00	1773.00	44.00	4460.00	41.82	662.12	115.00	146.01	12.20	111.61	32.33	143.45	0.30	89.01	2.96	4.22	0.42	1.83	0.46	1.18
CTSS	CTSS	ENSG00000163131.11	3937.00	94.00	45.00	604.00	0.37	86.80	24.61	0.90	0.20	84.66	16.63	1.25	0.93	129.91	15.59	2.78	0.59	79.83	36.88	1.16
CHI3L2	CHI3L2	ENSG00000064886.14	3501.00	224.00	46.00	1133.00	0.19	31.15	33.51	0.46	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	ENSG00000136244.12	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
TNFRSF1B	TNFRSF1B	ENSG00000028137.19	3534.00	4819.00	48.00	608.00	3.01	88.89	28.39	6.97	1.03	89.14	5.92	7.44	0.39	88.43	0.64	3.78	1.00	27.89	2.10	9.13
IFIT1	IFIT1	ENSG00000185745.10	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

DESed 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
```

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

```
BarretMyofCnt=RBarretTNFATGFBCntGMask[,samplesorder]
sampleTableMyof=cbind(sampleTableTNFATGFB[samplesorder,],pcabatchALL[samplesor
sampleTableMyof$PC1 <- scale(sampleTableMyof$PC1 , center = TRUE)

#normalized and batch-corrected expression matrix
#using the variance stabilizing transformation
BarretMYO_Batch_vsd=RBarretTNFATGFBCntGMaskBatch_vsd[,samplesorder]

write.csv(assay(BarretMYO_Batch_vsd),file="BarretMYO_Batch_vsd.csv")
```

Check gene name duplicates

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

```
#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(Genenames$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
Genenames$V1[1530] <- "TBCE_1"
Genenames$V1[3024] <- "ATXN7_1"
Genenames$V1[4133] <- "AHRR_1"
Genenames$V1[4576] <- "MATR3_1"
Genenames$V1[7638] <- "HSPA14_1"
Genenames$V1[15459] <- "TMSB15B_1"
```

First round of pairwise comparisons:

All cell lines UNTREATED vs All cell lines TGFβ

All cell lines UNTREATED vs All cell lines TNFα

All cell lines UNTREATED vs All cell lines TNFα/TGFβ

We model the data correcting for 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.

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

The filterFun argument specifies a multiple testing correction method to apply to the results, in this 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<0.000000000000001)]
Genenames$V1[which(RBarretMYOFCntGMaskTreatment_TN$padj<0.000000000000001)]
Genenames$V1[which(RBarretMYOFCntGMaskTreatment_TT$padj<0.000000000000001)]
```

Second round of pairwise comparisons:

Untreated NON-FIBROTIC cell lines vs Untreated FIBROTIC cell lines

TNFα/TGFβ NON-FIBROTIC cell lines vs TNFα/TGFβ FIBROTIC cell lines

TGFβ NON-FIBROTIC cell lines vs TGFβ FIBROTIC cell lines

TNFα NON-FIBROTIC cell lines vs TNFα 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="RBarretMYOFCntG
write.csv(as.data.frame(RBarretMYOFCntGMaskTreatment_TN),file="RBarretMYOFCntG
write.csv(as.data.frame(RBarretMYOFCntGMaskTreatment_TT),file="RBarretMYOFCntG
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_CC_Pheno),file="RBarretMYOFC
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TG_Pheno),file="RBarretMYOFC
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TN_Pheno),file="RBarretMYOFC
write.csv(as.data.frame(RBarretMYOFCntGMaskFactor_TT_Pheno),file="RBarretMYOFC
```

Pairwise results shreadsheet

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 shreadsheet.

```
#In terminal

tail -n +2 RBarretMYOFCntGMaskFactor_CC_Pheno.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskFactor_CC_Pheno_test.txt

tail -n +2 RBarretMYOFCntGMaskFactor_TG_Pheno.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskFactor_TG_Pheno_test.txt

tail -n +2 RBarretMYOFCntGMaskFactor_TN_Pheno.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskFactor_TN_Pheno_test.txt

tail -n +2 RBarretMYOFCntGMaskFactor_TT_Pheno.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskFactor_TT_Pheno_test.txt

tail -n +2 RBarretMYOFCntGMaskTreatment_TG.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskTreatment_TG_test.txt

tail -n +2 RBarretMYOFCntGMaskTreatment_TN.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskTreatment_TN_test.txt

tail -n +2 RBarretMYOFCntGMaskTreatment_TT.txt | sed 's/,/\t/g' | cut -
d$'\t' -f3,7 > RBarretMYOFCntGMaskTreatment_TT_test.txt

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
RBarretMYOFCntGMaskTreatment_TT_test.txt >
```

`Barret_Myofibroblast_TGFTNF_PAIRWISEResults.txt`

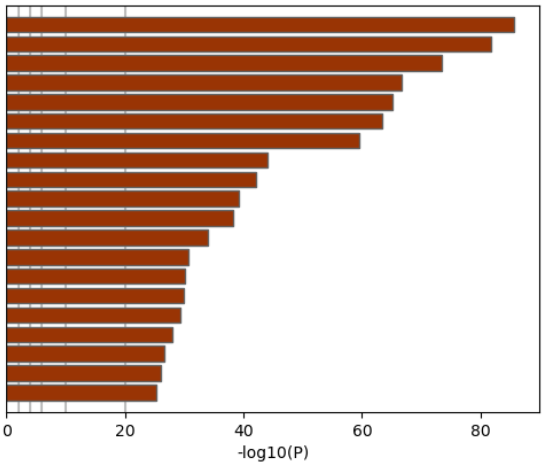
In Figure 13, it shows a clear formatted sheet containing Gene ID, Gene name, Mappability, fold change, and adjusted p-value. Then, you can sort each adjusted p-value column to focus on genes you are interested in. You can find the pairwise results spreadsheet here.

			Untreated NON-FIBROTIC cell lines vs Untreated FIBROTIC cell lines		TGFβ NON-FIBROTIC cell lines vs TGFβ FIBROTIC cell lines		TNFα NON-FIBROTIC cell lines vs TNFα FIBROTIC cell lines		TNFα/TGFβ NON-FIBROTIC cell lines vs TNFα/TGFβ FIBROTIC cell lines		All cell lines UNTREATED vs All cell lines TGFβ		All cell lines UNTREATED vs All cell lines TNFα		All cell lines UNTREATED vs All cell lines TNFα/TGFβ	
Gene ID	Gene name	Mappability	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	5.20E-43	0.081053744	0.817710494	-1.73358626	2.77E-18
ENSG00000188976.11	NOC2L	2644	0.068821384	1	-0.000431352	1	0.040269268	1	0.019211781	1	0.145174491	2.36E-07	0.205588933	5.95E-14	0.10246128	0.000280382
ENSG00000187961.14	KLHL17	2896	0.172917015	0.832705293	0.136320927	0.989972294	0.085394042	1	0.208050404	0.618960068	0.327769911	2.15E-17	0.127323327	0.001907649	-0.138919428	0.00050619
ENSG00000187583.11	PLEKHN1	3613	-0.090864126	0.827806587	-0.028993874	1	-0.160520142	0.807752678	-0.134336653	0.824087967	1.788086873	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	0.975846392	-0.598395081	0.081221314	-1.505134176	0.000323565	-0.870103019	0.017890754
ENSG00000188290.10	HES4	1133	0.153300669	1	0.122066297	1	0.218157143	0.887299992	-0.134392474	0.958923313	0.20649624	0.07818297	0.937081074	8.76E-19	0.371064905	0.000713975
ENSG00000187608.10	ISG15	861	-0.102148425	1	0.024596762	1	0.021694639	1	-0.279257826	1	0.263795161	0.186993258	1.601800299	8.39E-25	3.076082001	7.91E-89
ENSG00000188157.15	AGRN	7570	-0.160468497	1	-0.075395693	1	-0.193898673	0.995982556	-0.163057511	1	0.584144503	1.72E-18	1.150947439	2.61E-68	1.20353386	3.41E-75
ENSG00000131591.17	C1orf159	4519	-0.03309248	1	0.024669475	1	-0.027725627	1	-0.088624678	0.768141664	-0.282644916	1.08E-17	0.084923253	0.012031563	0.278258925	1.32E-18
ENSG00000186891.14	TNFRSF18	1359	0.886403284	0.823129353	-0.446386643	0.96514689	0.117902521	1	0.338684822	1	-1.177370894	4.28E-06	5.451458563	4.38E-123	5.677301524	1.23E-135
ENSG00000186827.11	TNFRSF4	976	-0.514160059	0.664970317	-0.612637224	0.816114951	-0.860106585	0.46831063	-0.253356711	0.760286957	-0.333418897	0.330752548	4.49956402	3.17E-56	5.52886735	1.09E-85
ENSG00000078808.17	SDF4	2308	0.049625524	1	0.057274721	1	0.016151239	1	0.003560533	1	0.142337521	0.000149013	0.571263308	2.52E-58	0.932141946	2.09E-155
ENSG00000176022.7	B3GALT6	2706	0.085310973	1	0.056899424	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	0.004339553	0.950931075	0.01697861	1	0.123619172	0.971573052	0.096906593	0.795960145	0.027763009	0.684222846	0.270031544	0.000399295	0.252401656	0.001009205
ENSG00000160087.20	UBE2J2	4052	-0.018781721	1	-0.005616432	1	0.06902968	1	-0.041120288	1	0.118911155	1.34E-05	0.359863102	6.77E-43	0.40201098	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	-0.433311158	1.06E-32	-0.523908776	3.23E-47
ENSG00000169972.12	PUSL1	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	-0.008556989	1	0.000764034	1	-0.014889871	1	-0.010872238	1	-0.103127142	5.18E-09	-0.137611785	2.15E-15	-0.142479203	1.37E-16
ENSG00000224051.7	CPTP	2406	0.015653893	1	0.053973742	1	0.047689086	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	0.172222661	0.1300722	-0.061516795	0.653158608
ENSG00000107404.20	DVL1	3522	-0.044768884	1	-0.03015666	1	-0.050538025	1	0.051143906	1	0.107387432	7.55E-05	0.012170496	0.797610809	-0.220666329	2.76E-17
ENSG00000162576.16	MXRA8	3257	0.191313096	1	0.219490333	1	0.118577359	1	0.100133923	1	0.453824966	2.56E-17	-0.055839373	0.385826016	-0.429853477	1.31E-15
ENSG00000175756.13	AURKAIP1	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
ENSG00000221978.12	CCNL2	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	0.000148887
ENSG00000235098.8	ANKRD65	2831	0.375416773	0.560677801	0.437439502	0.633271303	0.422432696	0.842296769	0.280112535	0.837926079	0.534111805	0.00025026	0.056395942	0.574281575	0.025426855	0.749726248
ENSG00000205116.3	TMEM88B	390	1.478490154	0.384576027	-0.032535513	1	-0.185967779	1	-0.11236015	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
ENSG00000215915.10	ATAD3C	3451	0.783851656	0.318345814	1.012888717	0.216598782	0.432984931	0.835849093	0.25993902	0.782237837	1.139276693	1.41E-08	0.427454615	0.036459678	0.129881993	0.591273577

Pathway analysis

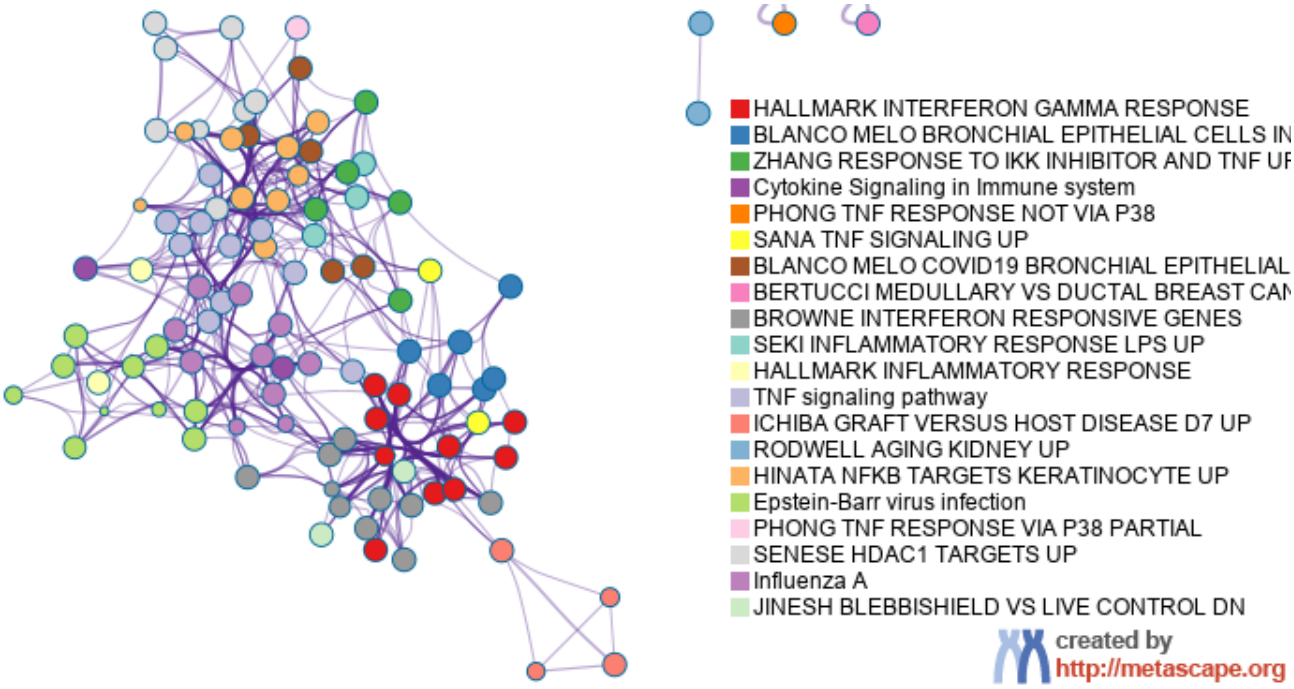
To run a first test on the reliability of the differential expression results above, we retrieve the top most significant genes (by adjusted p-value) and run functional enrichment analysis using Metascape. An example of the enrichment analyses that can be used for validation are below, using the top 500 most significant genes by their overall response to the TNFa-TGFb combination.

The following Figure 14 shows the top functional terms ranked by significance (hypergeomtric p-value):

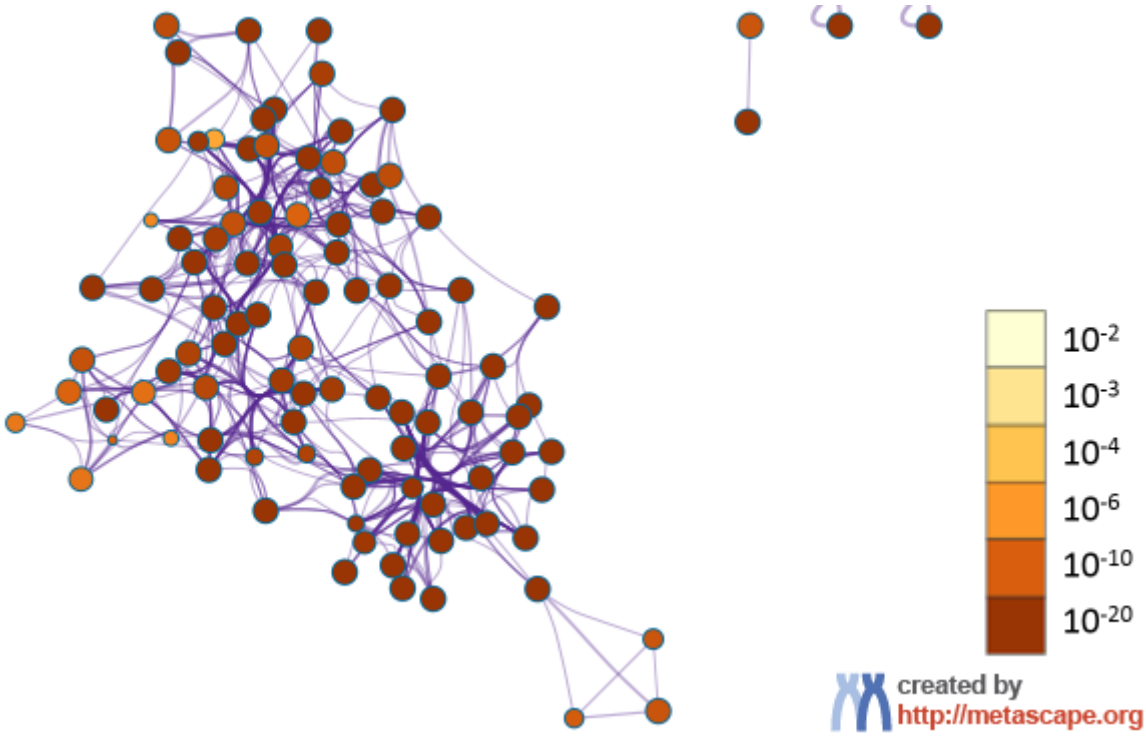


M5913: HALLMARK INTERFERON GAMMA RESPONSE
M34026: BLANCO MELO BRONCHIAL EPITHELIAL CELLS INFLUENZA A DEL NS1 INFECTION UP
M19826: ZHANG RESPONSE TO IKK INHIBITOR AND TNF UP
R-HSA-1280215: Cytokine Signaling in Immune system
M2504: PHONG TNF RESPONSE NOT VIA P38
M17466: SANA TNF SIGNALING UP
M34020: BLANCO MELO COVID19 BRONCHIAL EPITHELIAL CELLS SARS COV 2 INFECTION UP
M14539: BERTUCCI MEDULLARY VS DUCTAL BREAST CANCER UP
M9221: BROWNE INTERFERON RESPONSIVE GENES
M7245: SEKI INFLAMMATORY RESPONSE LPS UP
M5932: HALLMARK INFLAMMATORY RESPONSE
hsa04668: TNF signaling pathway
M5487: ICHIBA GRAFT VERSUS HOST DISEASE D7 UP
M5389: RODWELL AGING KIDNEY UP
M11951: HINATA NFKB TARGETS KERATINOCYTE UP
hsa05169: Epstein-Barr virus infection
M2502: PHONG TNF RESPONSE VIA P38 PARTIAL
M14973: SENESE HDAC1 TARGETS UP
hsa05164: Influenza A
M38973: JINESH BLEBBISHIELD VS LIVE CONTROL DN

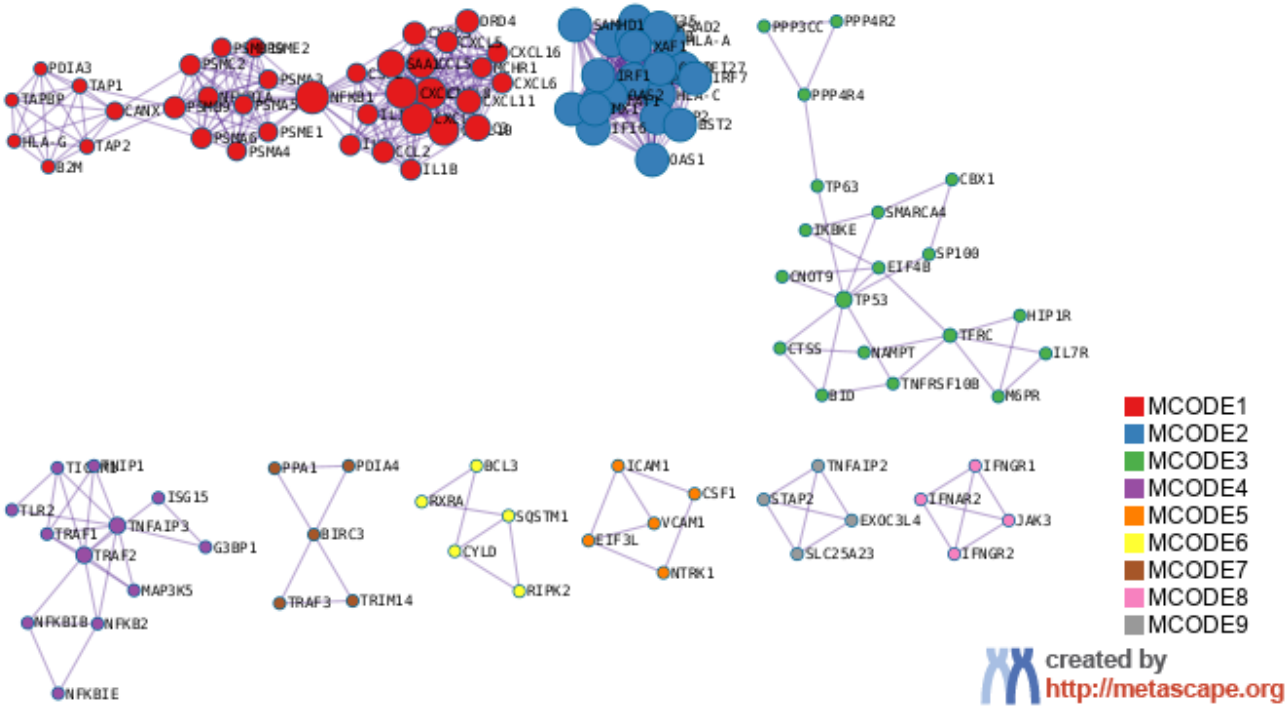
Two observations are noted: these results recapitulate the expected response to TNFa, but on the other hand too many categories are related with rather synonymous functional terms. The following Figure 15 shows an alternative representation, an enrichment network of the same significant pathways and functional terms. The network is arranged by the similarity between the genes contributing to each term, and colored according to functional groups with high overlap. A highly connected network indicates that most of the top significant pathways are highly redundant, and only a few of them are needed for further consideration.



The following Figure 16 shows the same enrichment network colored by statistical significance (hypergeomtric p-value):



Finally, we can screen how our differential results capture previously known protein-protein interactions. The figures below aggregate treatment-responsive genes in interaction modules. Many of the interactions are expected (e.g. groups of inflammation-related genes, modules of genes known to interact in the control of cell-cyle or NFKB-mediated regulation), and facilitate the validation of the experimental results and the identification of the most regulated genes in specific pathways.



Future works

To further our understanding of these complex signaling pathways, my future activities in this project will include:

- The identification of patient-specific responses.
- The identification of phenotype-specific responses, to better understand if our iPSC models can shed light into the predisposition of some patients to develop fibrotic complications.
- The integration of this experiment with a matched dataset (same iPSC lines, same treatments) obtained from iPSC lines differentiated into epithelial organoids (currently being analyzed). My goal is to integrate data from both the myofibroblast experiment and the epithelial experiment. By combining these datasets, I aim to identify patient-specific and phenotype-specific epithelial-mesenchymal interactions under inflammatory (TNFa) and pro-fibrotic (TGFb) conditions. These epithelial-mesenchymal interactions are known to be involved in the recurrence of fibrotic complication in some IBD patients. One possible way to do this is to merge both datasets to analyze differential changes in the levels of ligand-receptor interactions

between both cell types. If successful, leveraging this combined dataset to model responses from iPSCs lines could provide a powerful tool for studying personalized interventions for these diseases.

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