

Microarray Gene Expression Analysis

Acute myeloid leukemia (AML)

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1 Microarray

A microarray is a laboratory tool used to detect the expression of genes from a sample at the same time.

Microarrays have thousands of tiny spots in defined positions, with each spot containing a known DNA sequence or gene. The DNA molecules attached to each slide act as probes to detect gene expression.

To perform a microarray, mRNA molecules are typically collected from two different groups. For example, one group is considered as reference, which includes healthy samples and another group is experimental samples collected from a disease individual such as cancer.

The two mRNA samples are then converted into complementary DNA (cDNA) and each sample is labeled with a fluorescent probe of a different color. For instance, the experimental cDNA may be labeled with a red fluorescent dye, whereas the reference cDNA may be labeled with a green fluorescent dye. The two samples are mixed together and allowed to bind to the microarray slide. cDNA molecules bind to the DNA probes on the slides is called hybridization. After hybridization, the microarray is scanned to measure the expression of each gene printed into the slide. The red spots show that the number of experimental samples is higher than the reference sample. If the expression of a particular gene is lower in the experimental sample than in the reference sample, then the corresponding spot on the microarray appears green. Finally, if there is an equal expression in the two samples, then the spot appears yellow.

The microarray output data is in the form of a matrix table, which is finally in three formats SOFT, MINiML and TXT saved. Raw data is provided as a supplementary file.

^{*}401208226

[†]400211513

[‡]401208204

2 Loading libraries

```
[ ]: library(GEOquery)
library(limma)
library(umap)
library(pheatmap)
library(gplots)
library(ggplot2)
library(reshape2)
library(plyr)
library(repr)
library(gridExtra)
library(ggpubr)
library(Rtsne)
library(MASS)
library(FactoMineR)
library(factoextra)
```

3 Loading the *GSE48558* series

The `getGEO()` method from the `GEOquery` package is used in order to download the GEO SOFT format of the *GSE48558* and then parse it to the R structure obtaining the GSE Matrix and the GPL annotations:

```
[2]: gset <- getGEO("GSE48558", GSEMatrix = TRUE, getGPL=T, destdir='../Data/' ,
  ↪AnnotGPL = TRUE)
gset <- gset[[1]]
```

Found 1 file(s)

GSE48558_series_matrix.txt.gz

Using locally cached version: ../Data//GSE48558_series_matrix.txt.gz

Using locally cached version of GPL6244 found here:
../Data//GPL6244.annot.gz

```
[3]: dim(gset)
```

Features	32321 Samples	170
----------	---------------	-----

4 Selecting proper samples

The *source name* column in the *GSE48558*, stands for the source of the biological material of the sample¹. There are 11 different sample sources in this data series, including:

- AML cell line
- AML patient
- B ALL cell line
- B ALL patient
- B normal
- T ALL cell line
- T ALL patient
- T normal
- Granulocytes normal
- Monocytes normal
- CD34+ normal

We will only select the **Leukemia AML** samples and those with the **normal** phenotype value. The selection and the grouping were done first in the **GE02R analysis tool** provided by *ncbi* website itself. A string will be generated, which can be used to select and group the samples in the R:

```
[4]: gsms <- paste0("000000000000XXXXXXXXXXXXXXXXXXXXXXXXX1XXX1XXXXX",
                  "XXXXXXXXXXXXXXXXXX2X3XXX1X1442X3XX33XX33X2X3X2X3X5",
                  "XXX5XXX5XXXXXXXXXXXXXXXXXXXXXXXXX111111003000",
                  "222222344413333333")
sml <- strsplit(gsms, split="")[[1]]
sel <- which(sml != "X")
sml <- sml[sel]
gset <- gset[,sel]
```

Samples are grouped according to the following rules:

- All the AML samples are grouped together.
- Normal samples are grouped based on their **source_name** (B, T, granulocytes, monocytes, and CD34+).

Each number in the provided string stands for a group, and the X character means the corresponding sample won't be selected.

Next we will create a **factor** from these class numbers and add the **group** column to the **gset**.

```
[5]: gs <- factor(sml)
groups <- make.names(c("AML", "Granulocytes", "B Cells", "T_
  ↳Cells", "Monocytes", "CD34"))
levels(gs) <- groups
gset$group <- gs
```

¹<https://www.ncbi.nlm.nih.gov/geo/info/qqtutorial.html>

5 Data Quality Control

5.1 Normalization

5.1.1 Log Transformation

A technique that we can perform to scale the data properly is the *log transformation*. Our data may contain huge feature values that are unevenly distributed. The easiest solution is to scale them down using the `log` operation.

But before that, we'll check if we need to perform the *log transformation* or not. For this purpose we will simply find the range of feature values to find out if they are already log-scaled or not:

```
[6]: ex <- exprs(gset) # Biobase.exprs() returns a matrix of expression values
      print(c(min(ex), max(ex)))
```

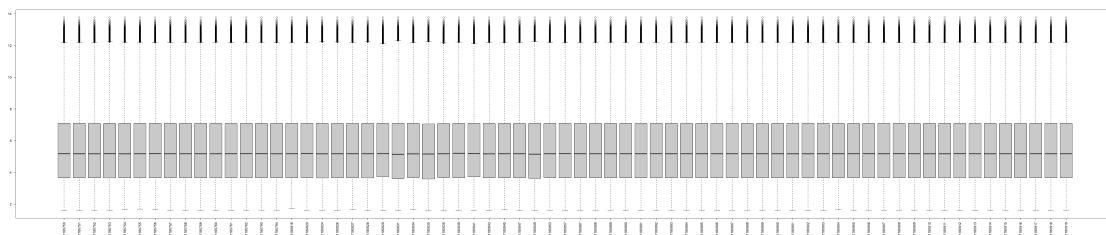
```
[1] 1.611473 13.761536
```

According to this, min value is 1.6 and max value is 13.76, so they are logarithmic already and data is normalized.

5.1.2 Plotting the *expression* matrix

We can also plot the data to check if any normalization process is needed. If samples vary in range and scale, it's imperative to perform the normalization:

```
[7]: options(repr.plot.width=55, repr.plot.height=12)
      boxplot(ex, las = 2)
```



As the plot represents, samples are well-scaled with approximately identical *5 number summary*² values.

²minimum, lower-hinge, median, upper-hinge, maximum

5.2 Dimensionality Reduction

First, let's check our data dimensions again:

```
[8]: dim(gset)
```

Features	32321 Samples	67
----------	---------------	----

As you see, we've got a 32321-dimensional data, which is enormous! What we're about to do, is reduce the dimensions of our data while keeping as much variation in the original data as possible.

but **How exactly is this *dimensionality reduction* going to help us?**

1. Lower dimensionality means less computational resources, less training time, and more performance (because in a high-dimensional space, most data points are likely far from each other, and it's hard for the model to train effectively on such data - ***curse of dimensionality***).
2. When there are many features, the model tends to become more complex, resulting in overfitting. So the *dimensionality reduction* avoids the problem of **overfitting**.
3. Imagine you want to **visualize** this 323321-dimensional data! It's not that practical, is it? But when you reduce the dimensionality of higher dimension data to 2 or 3 components, it can easily be plotted on a 2D or 3D plot.

4. multicollinearity

It occurs when features are highly correlated with one or more of the other features in the dataset and affect the performance of regression and classification models. *Dimensionality reduction* takes advantage of multicollinearity and combines the highly correlated variables into a set of uncorrelated variables.

5. When you keep the essential features and remove the others, the data **noise** is more likely to be removed, leading to higher model accuracy.

You can see different methods of *dimensionality reduction* in the diagram below:

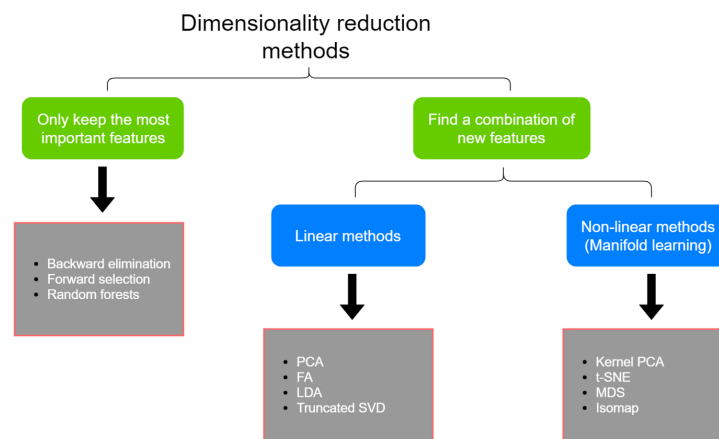


Image copyright: Rukshan Pramoditha

Figure 1: Dimensionality Reduction methods

5.2.1 Principal Component Analysis (PCA)

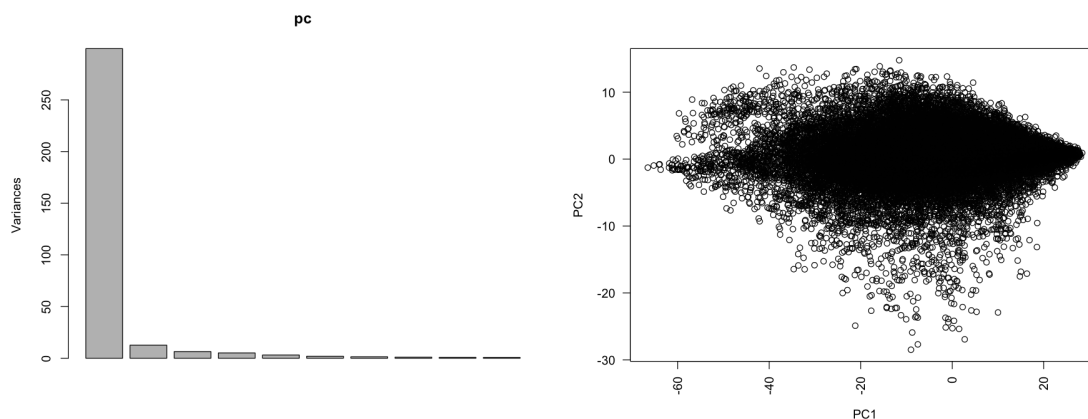
The first dimension reduction method that we're going to use is the *Principal Component Analysis (PCA)*. Principal component analysis is used to extract the important information from a multi-variate data table and to express this information as a set of few new variables called principal components. PCA assumes that the directions with the largest variances are the most **important** while the amount of variance retained by each principal component is measured by the **eigenvalue**. We will use the `prcomp()` method to obtain the *PCA* and then plot it:

```
[9]: pc <- prcomp(ex)
      get_eigenvalue(pc)[1:5, ]
```

		eigenvalue <dbl>	variance.percent <dbl>	cumulative.variance.percent <dbl>
A data.frame: 5 × 3	Dim.1	299.598423	87.2509394	87.25094
	Dim.2	12.643203	3.6820333	90.93297
	Dim.3	6.430822	1.8728244	92.80580
	Dim.4	5.144998	1.4983587	94.30416
	Dim.5	3.140644	0.9146382	95.21879

As you can see, about 90.93 of the variance is retained by the first two *principal components*. Before going further, let's first plot the data according to *PC1* and *PC2*:

```
[10]: options(repr.plot.width=15, repr.plot.height=6)
      par(mfrow = c(1, 2))
      p1 <- plot(pc)
      p2 <- plot(pc$x[, 1:2], las = 2)
```



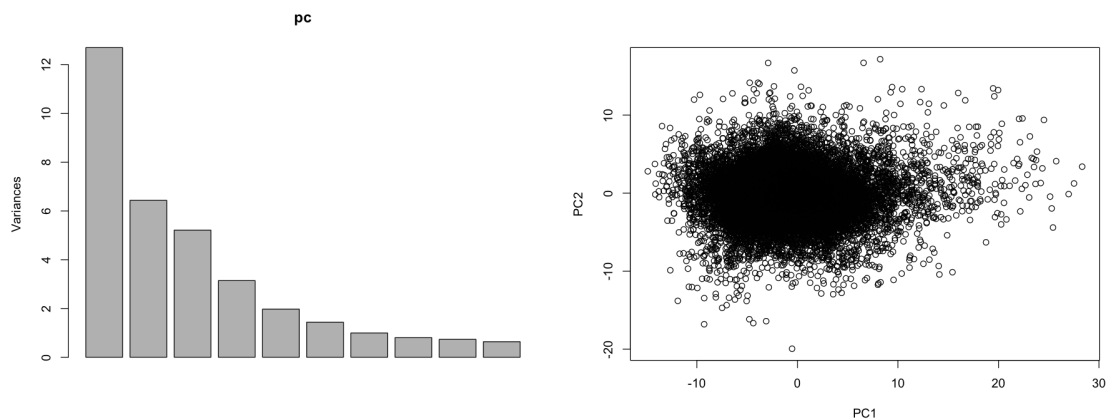
The problem is, *PC1* seems to present a misleading variance in the data points, acquired probably by the uninteresting expression differences between genes which often expressed (e.g. housekeeping genes) and those expressed rarely.

To overcome this problem, we are going to center³ our data columns using the `scale()` method:

```
[11]: ex.scale <- t(scale(t(ex), scale = F))
      pc <- prcomp(ex.scale)
      get_eigenvalue(pc)[1:5, ]
```

		eigenvalue <dbl>	variance.percent <dbl>	cumulative.variance.percent <dbl>
A data.frame: 5 × 3	Dim.1	12.698222	28.876999	28.87700
	Dim.2	6.434864	14.633510	43.51051
	Dim.3	5.211775	11.852086	55.36259
	Dim.4	3.142359	7.146032	62.50863
	Dim.5	1.972313	4.485232	66.99386

```
[12]: options(repr.plot.width=15, repr.plot.height=6)
      par(mfrow = c(1, 2))
      plot(pc)
      plot(pc$x[, 1:2])
```



As you can see, now other components are contributing as well.

³Subtracting columns mean from their corresponding columns.

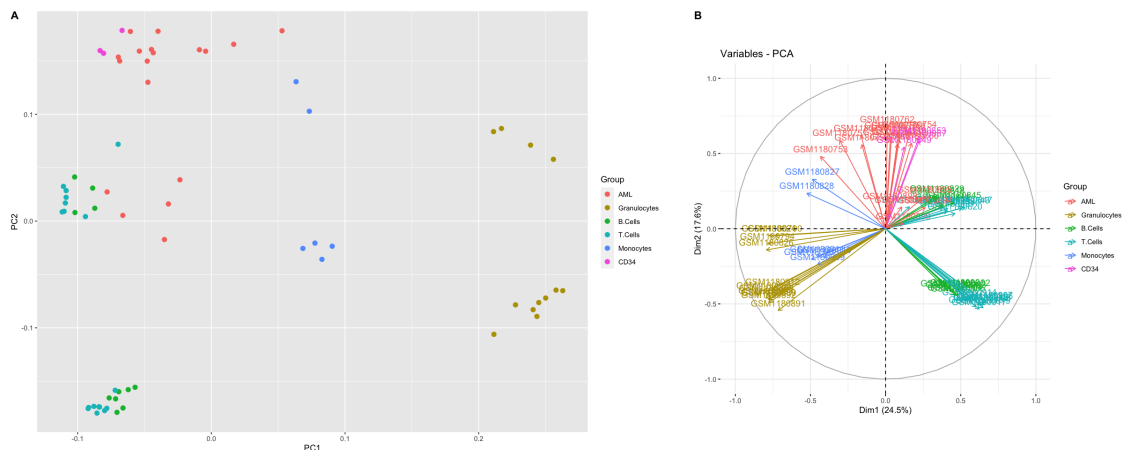
We can also plot the *PCA* results according to the *variables*(which are the samples in this case) instead of the *individuals*(which are the genes in this case) to demonstrate different sample groups and their correlation:

```
[13]: options(repr.plot.width=20, repr.plot.height=8)

pcr <- data.frame(pcr$rotation[, 1:3], Group=gs)
pca.plot1 <- ggplot(pcr,
                    aes(x = PC1,
                        y = PC2,
                        color = Group)) + geom_point(size=2.5) + theme_gray()

pca.plot2 <- fviz_pca_var(PCA(ex.scale, graph = FALSE),
                          col.var = gs,
                          legend.title = "Group")

ggarrange(pca.plot1, NULL, pca.plot2,
          widths = c(3, 0.1, 2),
          labels = c('A', '', 'B'),
          ncol = 3,
          nrow = 1)
```



About the figure B :

- Positively correlated variables are grouped together.
- Negatively correlated variables are positioned on opposite sides of the plot origin (opposed quadrants).

5.2.2 t-distributed stochastic neighbor embedding (tSNE)

The next *dimension reduction* algorithm that we're going to try is the *tSNE* which as mentioned in [figure 1](#), is a **non-linear algorithm**. Non-linearity means that it's capable of separating data which can't be separated by a straight line, unlike the linear algorithms(e.g. *PCA*).

We're going to use the `Rtsne()` method from the `Rtsne` package, to calculate the *tSNE* of our expression matrix. One parameter that we need to pass to the `Rtsne()` method is the **perplexity**. A perplexity is more or less a target number of neighbors for our central point. Basically, the higher the perplexity is, the higher the variance value.⁴

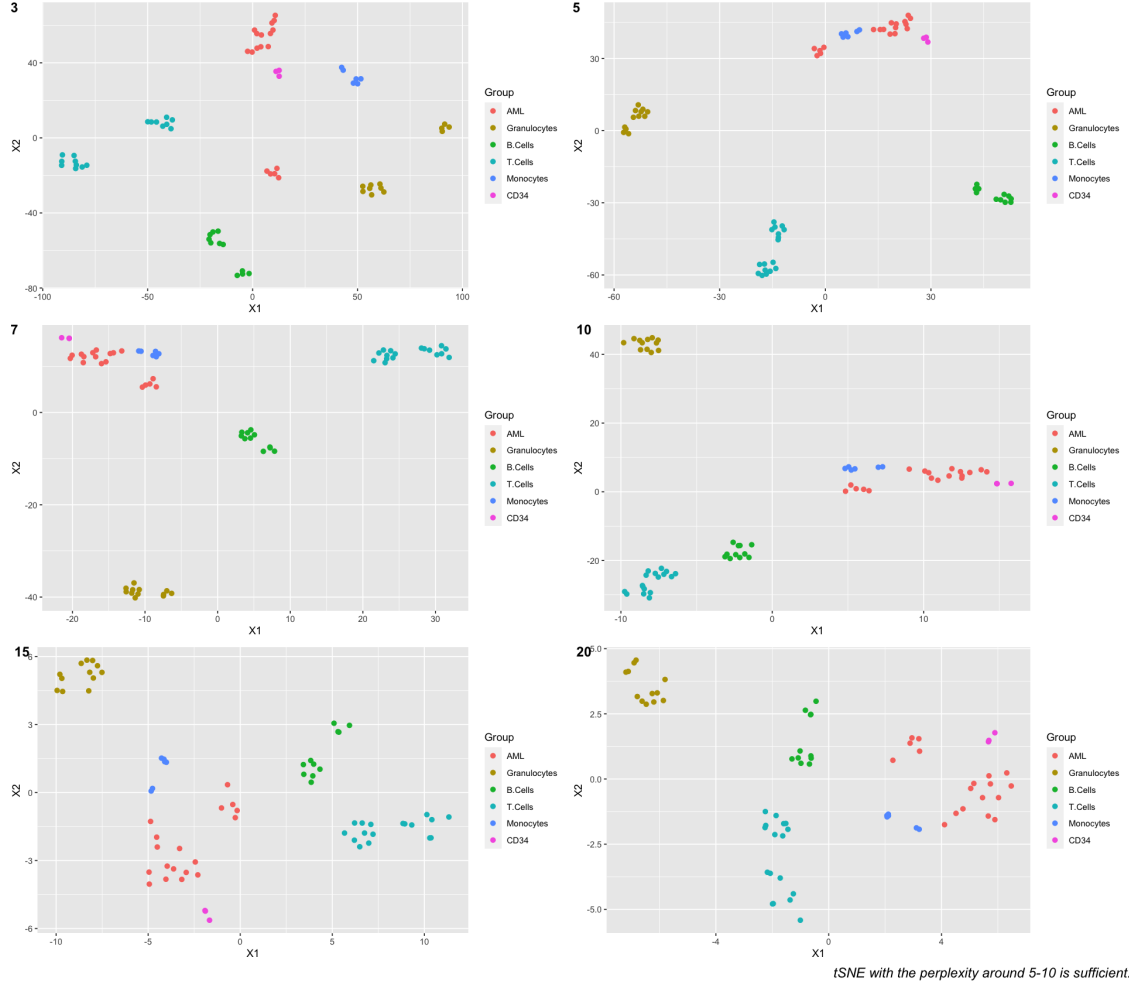
So we will calculate the *tSNE* for 3 perplexity values of 3, 5, 7, 10, 15 and 20. After plotting the result, we can choose the best value based on how well it separated the group clusters:

```
[14]: tsne_results <- list(Rtsne(t(ex), perplexity=3, check_duplicates = FALSE),
                          Rtsne(t(ex), perplexity=5, check_duplicates = FALSE),
                          Rtsne(t(ex), perplexity=7, check_duplicates = FALSE),
                          Rtsne(t(ex), perplexity=10, check_duplicates = FALSE),
                          Rtsne(t(ex), perplexity=15, check_duplicates = FALSE),
                          Rtsne(t(ex), perplexity=20, check_duplicates = FALSE))

options(repr.plot.width=16, repr.plot.height=14)
tsne.plots.list <- list()

for(i in seq_along(tsne_results)) {
  tsne <- data.frame(tsne_results[[i]]$Y[, 1:2], Group=gs)
  tsne.plots.list[[i]] <- ggplot(tsne, aes(X1, X2, color = Group)) +
    geom_point(size=2) + theme_gray()
}
ggarr <- ggarrange(plotlist = tsne.plots.list,
                   ncol = 2,
                   nrow = 3,
                   labels = c(3, 5, 7, 10, 15, 20))
annotate_figure(ggarr,
               bottom = text_grob("tSNE with the perplexity around 5-10 is
    ↪sufficient.",
                                hjust = 1,
                                x = 1,
                                face = "italic",
                                size = 14)
               )
```

⁴<https://towardsdatascience.com/t-sne-clearly-explained-d84c537f53a>



tSNE with the perplexity around 5-10 is sufficient.

According to the plots, *tSNE* with the perplexity in a range [5,10] was almost successful to separate different groups of samples **except for the AML, CD34+, and Monocytes** which brings this idea to mind that expressions in these groups were highly similar/correlated.

5.2.3 Multidimensional scaling (MDS)

MDS returns an optimal solution to represent the data in a lower-dimensional space, where the number of dimensions k is pre-specified by the analyst. For example, choosing $k = 2$ optimizes the object locations for a two-dimensional scatter plot.

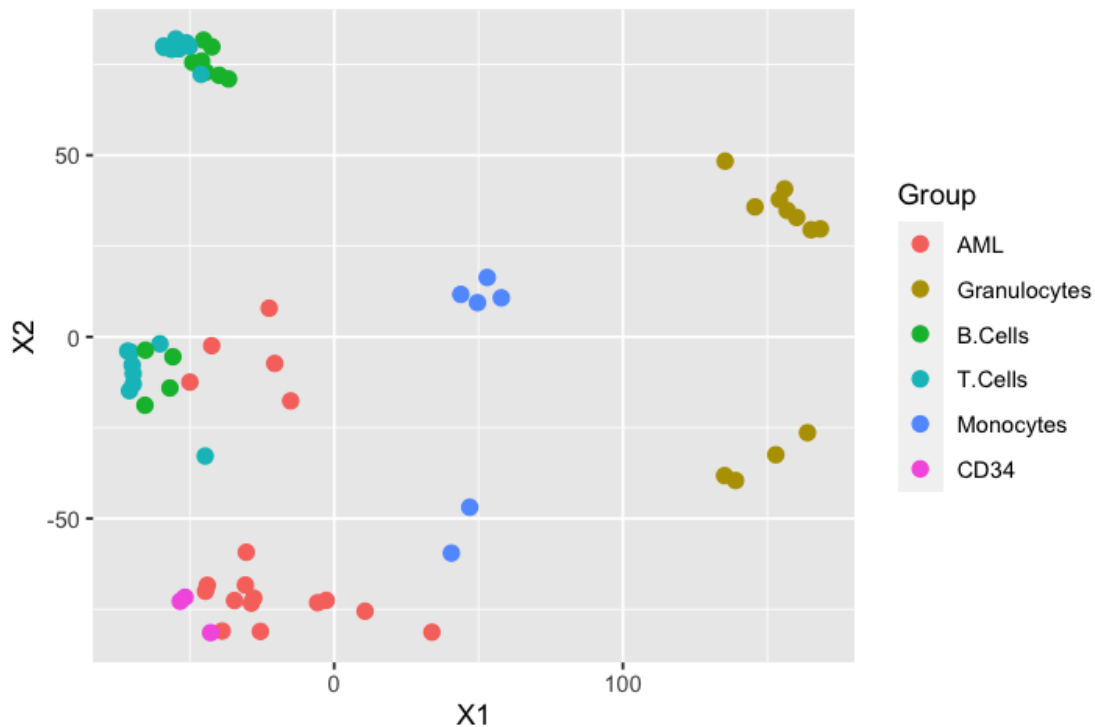
The input data for MDS is a dissimilarity matrix representing the distances between pairs of objects. So first we will compute the distance matrix using the `dist()` method:

```
[15]: ex_dist <- dist(t(ex.scale))
```

Now we can pass the `ex_dist` to the `cmdscale` from the `stats` package to compute the *mds* result:

```
[16]: options(repr.plot.width=6, repr.plot.height=4)

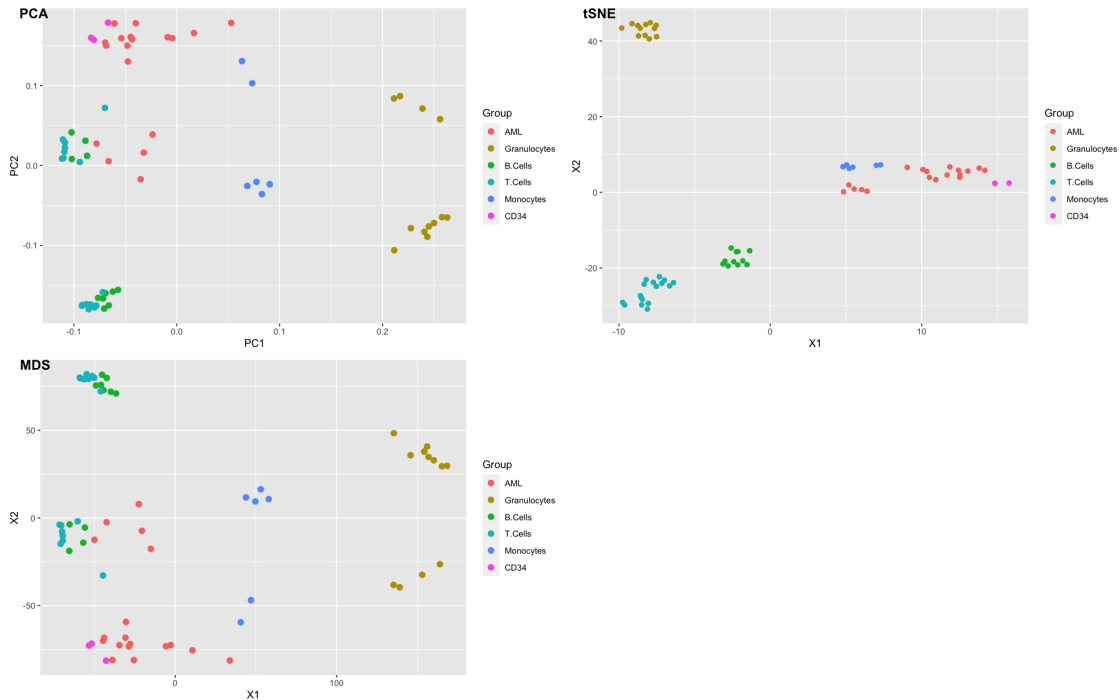
mds_result <- cmdscale(ex_dist, eig=TRUE, k=2)
mds_df <- data.frame(mds_result$points[,1:2], Group=gs)
mds.plot <- ggplot(mds_df, aes(X1,X2, color = Group)) + geom_point(size=2.5) +
  theme_gray()
mds.plot
```



5.2.4 PCA vs. tSNE vs. MDS

To compare the results of these 3 methods, we will plot their results one more time to make it easier for comparing:

```
[17]: options(repr.plot.width=16, repr.plot.height=10)
ggarrange(pca.plot1, tsne.plots.list[[4]], mds.plot,
          ncol = 2,
          nrow = 2,
          labels = c('PCA', 'tSNE', 'MDS'))
```



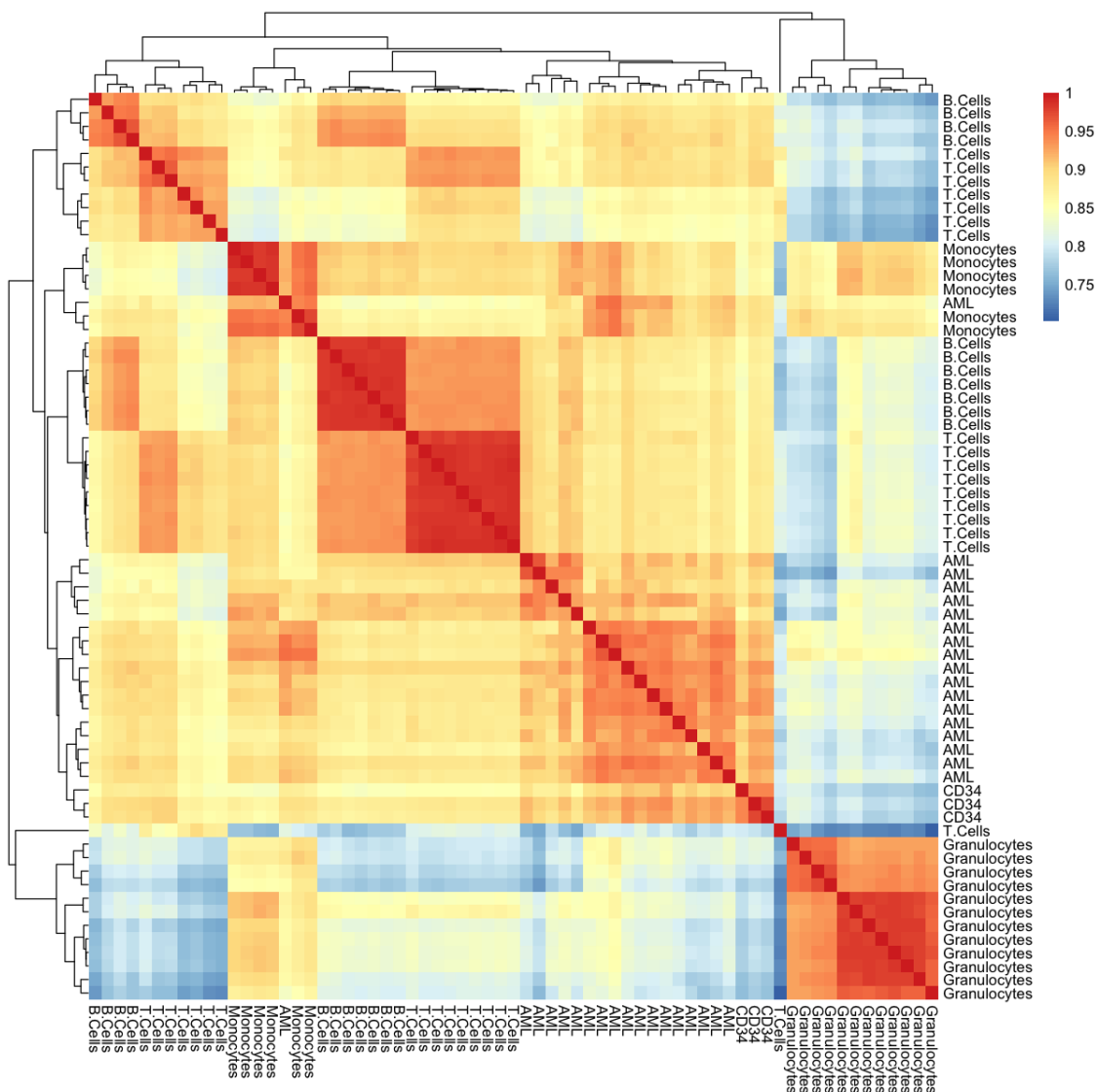
According to plots, we believe that the *tSNE* was able to separate groups of samples more precisely.

5.3 Correlation between samples

Heat map shows correlations between different samples. For example each group has high corelation with itself that is determined by red color or *Granulocytes* have a low correlation with *B-cells* and *T-cells* which is determined by blue color.

According to the heatmap, **AML** has a high correlation with **CD34+** and **Monocytes** which makes these 2 groups, proper candidates to check whether there are any significant expression differences between their genes and the *AML* ones.

```
[18]: options(repr.plot.width=10, repr.plot.height=10)
pheatmap(cor(ex),
  labels_row = gs,
  labels_col = gs,
  border_color = NA,)
```



6 Differential expression analysis

6.1 Fitting a linear model to the data

creating a *design matrix* which simply demonstrates which group, each sample belongs to:

```
[19]: design <- model.matrix(~ group + 0, gset)
      colnames(design) <- levels(gs)
```

```
[20]: head(design)
```

		AML	Granulocytes	B.Cells	T.Cells	Monocytes	CD34
A matrix: 6 × 6 of type dbl	GSM1180750	1	0	0	0	0	0
	GSM1180751	1	0	0	0	0	0
	GSM1180752	1	0	0	0	0	0
	GSM1180753	1	0	0	0	0	0
	GSM1180754	1	0	0	0	0	0
	GSM1180755	1	0	0	0	0	0

Now we try to fit a simple linear model to the gene expressions:

```
[21]: fit <- lmFit(gset, design)
```

Comparisons between groups(log fold-changes) are obtained as contrasts of these fitted linear models. Here we choose the **AML** and the **CD34** groups to compare:

```
[22]: cont.matrix <- makeContrasts(AML-CD34, levels=design)
```

now we use the `contrasts.fit()` to compute estimated coefficients and standard errors for the **AML-CD34** contrasts. It actually estimates contrast for each gene:

```
[23]: fit2 <- contrasts.fit(fit, cont.matrix)
```

Empirical Bayes smoothing of standard errors(shrinks standard errors that are much larger or smaller than those from other genes towards the average standard error):

```
[24]: fit2 <- eBayes(fit2, 0.01)
```

6.2 Top-ranked genes

Now we will extract a table of the top-ranked genes from our linear model `fit2`:

```
[25]: tT <- topTable(fit2, adjust="fdr", sort.by="B", number=Inf)
      tT <- subset(tT, select=c("Gene.symbol", "Gene.ID", "adj.P.Val", "logFC"))
```

As you can see below, `tT` consists of 32321 genes in the rows and the columns are *Gene.symbol*, *Gene.ID*, *adj.P.Val* and *logFC*. Genes are sorted according to the *B* values by passing the `sort.by="B"` argument to the `topTable()` method:

```
[26]: print(dim(tT))
      head(tT)
```

```
[1] 32321      4
```

		Gene.symbol	Gene.ID	adj.P.Val	logFC
		<chr>	<chr>	<dbl>	<dbl>
A data.frame: 6 × 4	8160088	MPDZ	8777	2.565924e-36	-3.670064
	8101284	PRKG2	5593	5.887234e-30	-5.874765
	7910915	CHRM3	1131	3.200429e-28	-4.378570
	8160168	FREM1	158326	1.119567e-23	-3.409825
	8121814	NKAIN2	154215	5.166675e-23	-4.673490
	8008588	HLF	3131	2.786807e-20	-4.140364

We can save the resulting differential expression matrix in a file:

```
[27]: write.table(tT,
                  file=" ../Results/AML_CD34.txt",
                  row.names=F,
                  sep="\t",
                  quote=F)
```

6.2.1 AML up genes

Now it's the time to achieve a list of *AML-CD34* genes which highly expressed in the case of AML patients:

```
[28]: aml.up <- subset(tT, logFC > 1 & adj.P.Val < 0.05)
aml.up.genes <- unique(as.character(strsplit2(aml.up$Gene.symbol, '///')))

write.table(aml.up.genes,
            file=" ../Results/AML_CD34_Up.txt",
            quote=F,
            col.names=F,
            row.names=F)
```

```
[29]: head(unique(aml.up))
```

	Gene.symbol <chr>	Gene.ID <chr>	adj.P.Val <dbl>	logFC <dbl>
A data.frame: 6 × 4	8111739	FYB	2533	7.381406e-13
	8080487	PRKCD	5580	1.411050e-12
	8034420	MAN2B1	4125	2.608491e-09
	7973108	ECRP///ECRP	643332///643332	1.590050e-08
	7907830	QSOX1	5768	1.749850e-08
	7970162	ATP11A	23250	2.212408e-08
				2.026483

6.2.2 AML down genes

Then also a list of *AML-CD34* genes which had a low-expression:

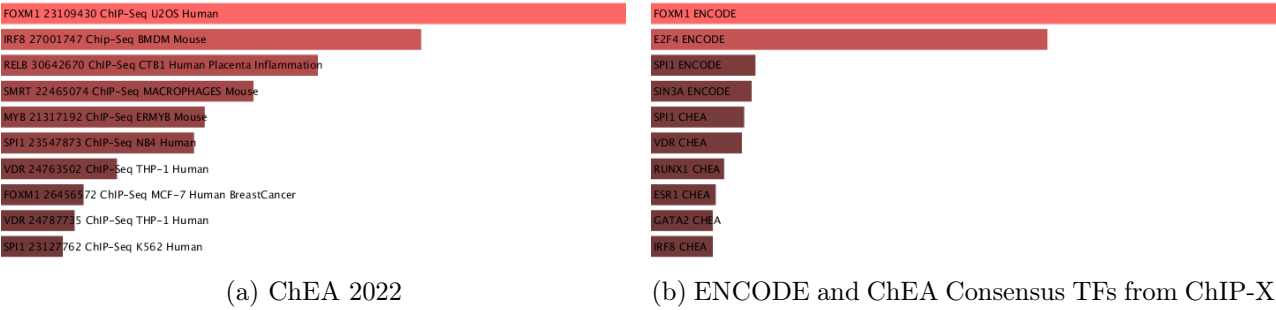
```
[30]: aml.down <- subset(tT, logFC < -1 & adj.P.Val < 0.05)
aml.down.genes <- unique(as.character(strsplit2(aml.down$Gene.symbol, '///')))

write.table(aml.down.genes,
            file=" ../Results/AML_CD34_Down.txt",
            quote=F,
            col.names=F,
            row.names=F)
```

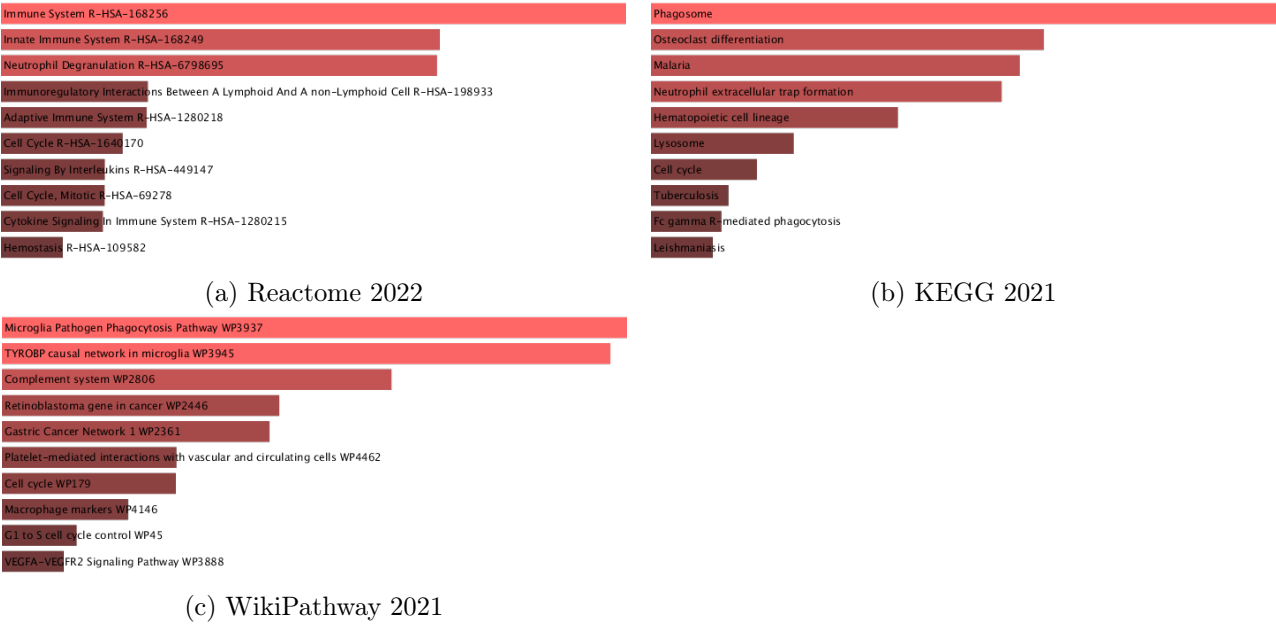
```
[31]: head(unique(aml.down))
```

	Gene.symbol <chr>	Gene.ID <chr>	adj.P.Val <dbl>	logFC <dbl>
A data.frame: 6 × 4	8160088	MPDZ	8777	2.565924e-36
	8101284	PRKG2	5593	5.887234e-30
	7910915	CHRM3	1131	3.200429e-28
	8160168	FREM1	158326	1.119567e-23
	8121814	NKAIN2	154215	5.166675e-23
	8008588	HLF	3131	2.786807e-20
				-4.140364

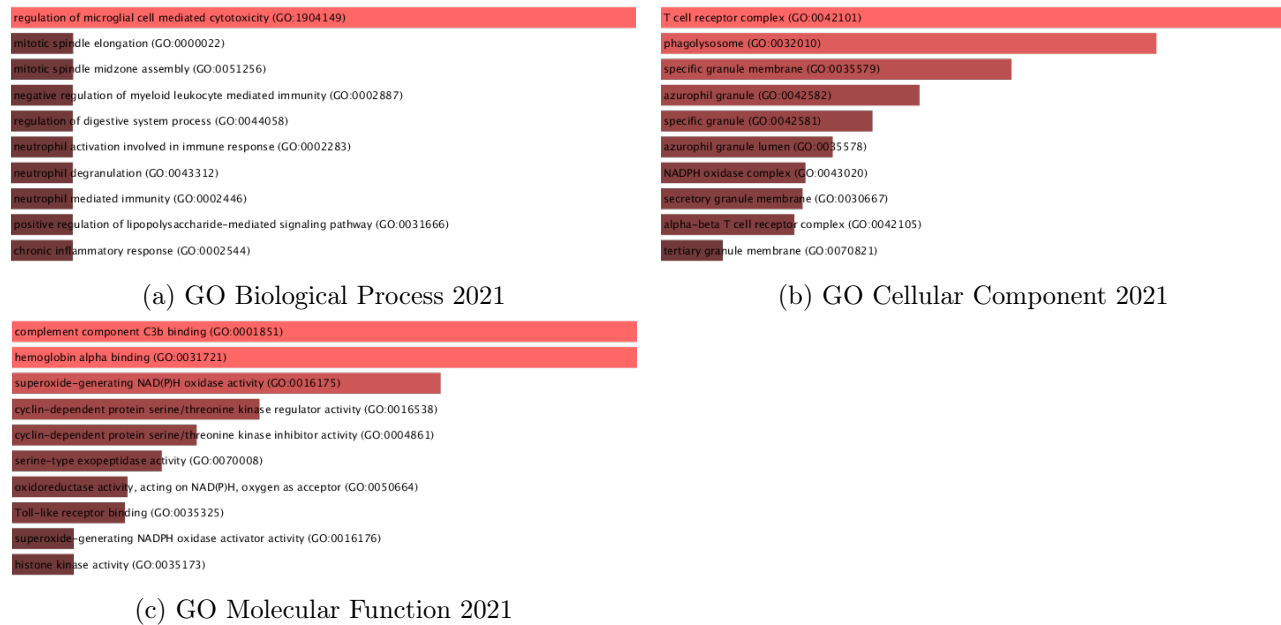
6.3 Transcription Analysis



6.4 Pathway Analysis



6.5 Gene Ontology Analysis



6.6 Validation

To validate our results, we will demonstrate some prior researches which can confirm our results.

6.6.1 FOXM1

FOXM1, a known transcription factor, promotes cell proliferation in a variety of cancer cells. our study shows a critical role and mechanisms of Foxm1 in MA9-LSCs, and indicates that FOXM1 is a potential therapeutic target for selectively eliminating LSCs in MLL-rearranged AML.⁵

6.6.2 E2F4

the experimental results showed that E2F4 was aberrantly overexpressed in human AML patients and cell lines. Depletion of E2F4 inhibited the proliferation, induced the differentiation and suppressed the growth of AML cells in a nude mouse model. By contrast, overexpression of E2F4 promoted the proliferation and inhibited the differentiation of AML cells in vitro.⁶

6.6.3 T Cell Receptor

In vitro studies show that coculture with AML cells and incubations with AML plasma inhibit the proliferation of T cells. One proven mechanism of T cell growth inhibition is the expression of AML surface receptors that interact with T cells and alter their function. This includes leukocyte immunoglobulin-like receptor B4 (LILRB4), an inhibitory immune checkpoint receptor restrictively expressed on monocytic leukemic cells (M4 and M5 subtypes).⁷

⁵FOXMI regulates leukemia stem cell quiescence and survival in MLL-rearranged AML

⁶E2F4 functions as a tumour suppressor in acute myeloid leukaemia via inhibition of the MAPK signalling pathway by binding to EZH2

⁷The Role of T Cell Immunotherapy in Acute Myeloid Leukemia

6.6.4 Phagosome

It is reported that phagosome pathway is the most optimal and common in both Kasumi-1 and MV4-11 AML cell lines induced by differentiation inducers, Trichostatin A and 5-Azacytidine (Asmaa et al., 2020)⁸

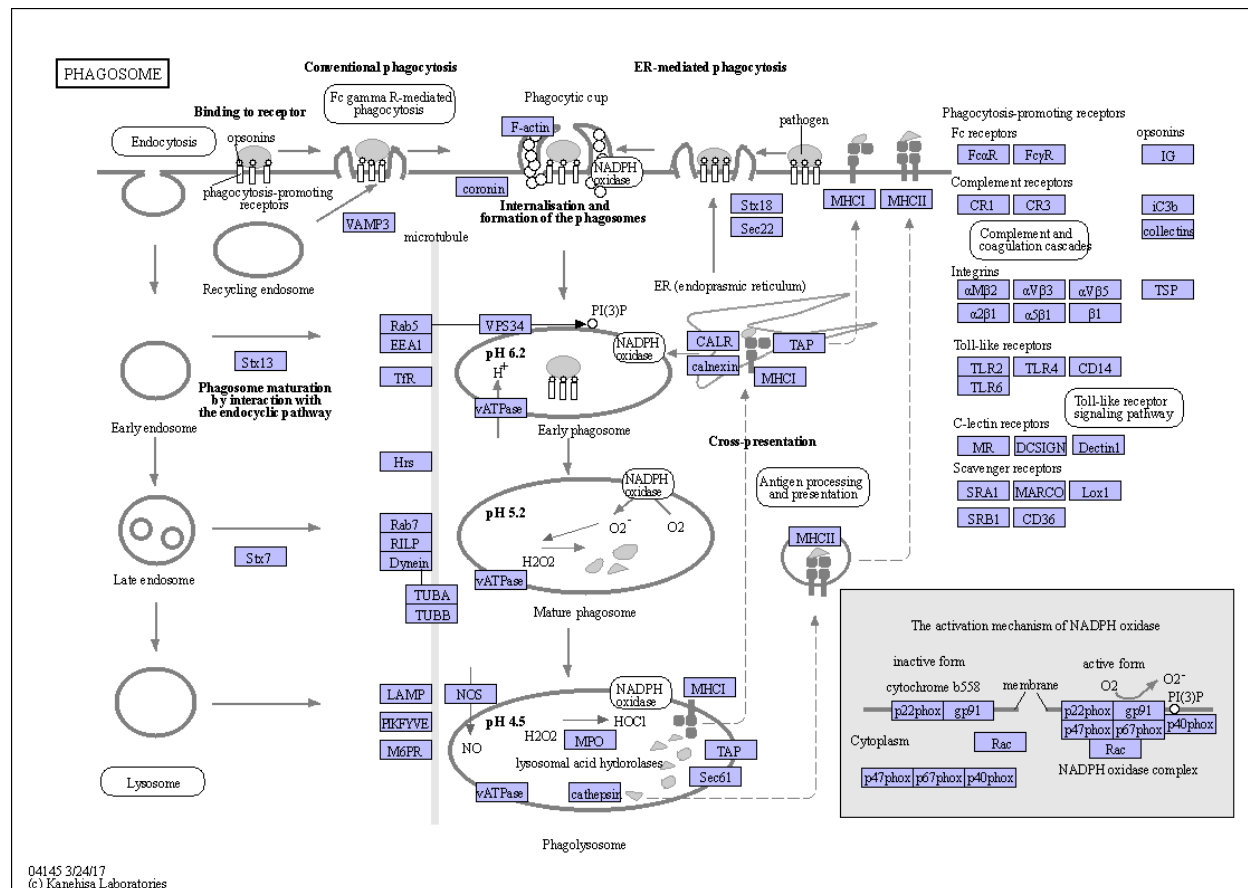


Figure 5: KEGG 2022 Phagosome*

* https://www.genome.jp/kegg-bin/show_pathway?ko04145

6.7 Related Drugs

6.7.1 Quizartinib and Crenolanib: FLT3 Inhibitors

Mutations in the FLT3 gene occur in approximately one third of all patients with newly diagnosed AML; 20% to 25% of these mutations are ITD and 5% to 10% are point mutations of the tyrosine kinase domain. Both types of mutations lead to constitutive activation of the FLT3 receptor tyrosine kinase, promoting cellular proliferation and survival and inhibiting differentiation.⁹

6.7.2 Venetoclax: BCL-2 inhibitors

Antiapoptotic proteins, including BCL2, BCL-XL, and MCL1, are frequently overexpressed in AML and are associated with resistance to chemotherapy (82). This observation led to the development of

⁸OGP46 Induces Differentiation of Acute Myeloid Leukemia Cells via Different Optimal Signaling Pathways

⁹Advances in the Treatment of Acute Myeloid Leukemia: New Drugs and New Challenges

BH3 mimetics that structurally mimic BH3-only proteins and are capable of binding to antiapoptotic proteins, effectively inhibiting their functional activity and inducing apoptosis.¹⁰

¹⁰Advances in the Treatment of Acute Myeloid Leukemia: New Drugs and New Challenges

7 References

- <https://maktabkhooneh.org> - Advanced Bioinformatics course
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