

Tissue-restricted antigens (TRAs) in embryonic development

Alina Aksianova, Nina Bank, Letizia Holube and Lydia Steiner

2022-07-18

Final Report - Datascience MoBi 2022

Topic 04 - Team 02

Supervisor: Dr. Maria Dinkelacker

Tutor: Ian Fichtner

Institute of Pharmacy and Molecular Biotechnology

Heidelberg University

Abstract

Embryogenesis is difficult to study and therefore still not fully understood. Yet, a novel approach borrowed from immune biology may provide the necessary tools to embryo research in the scope of an interdisciplinary application. Tissue restricted antigens are utilised by the immune system for the production of t-cells to prevent autoreactive events. Therefore, they are expressed simultaneously with other genes in the corresponding tissue. Tissue specific antigens seem to be a promising biological indicator to depict the intensity of gene expression to a given time during tissue development. Such markers would be a useful method to not only further analyse and understand embryogenesis and organogenesis but inspect general tissue development with high accuracy.

This project aims to explore embryogenesis in mice by analysing the expression levels of tissue restricted antigens over time and summarising these findings in an applicable dataframe.

1 Introduction

The immune system is responsible for recognising and eliminating threatening pathogens, such as bacteria, viruses or fungi, however, distinguishing these from endogenous cells and not harming them. CD8+ T cells (CD8s), also referred to as cytotoxic T cells precisely recognise antigens via specific T cell receptors (TCRs). Thus, playing a vital role in this process. In order to prevent an autoreactive self-antigen-CD8 complex that would be harmful to the organism, T cells undergo negative selection in addition to positive selection, in the thymus. This negative selection process is mediated by medullary thymic epithelial cells that are presented to developing T cells, leading to the elimination of binding T cells (mTECs) (Kyewski and Derbinski, 2004).

Auto-antigens fall in two categories: housekeeping antigens (HAs) and tissue restricted antigens (TRAs). While HAs are expressed in a multitude of tissues, TRAs are found to be expressed rather uniquely. This results in HAs experiencing only insignificant amounts of epigenetic inactivation. TRAs in contrast, display silencing extensively in a majority of tissues. To classify as a TRA, an antigen is required to exceed five times the median gene expression within one to four tissue expression profiles (Dinkelacker, 2021). An unpublished dataset by Dr. Maria Dinkelacker will be utilised to analyse embryonic development.

Antigen expression levels constantly vary during embryogenesis, since different stem cells undergo differentiation. This includes TRA expression levels that display a temporal connection to the development of specific organs. Due to this connection they offer an interesting approach to study organ development. Despite spatiotemporally mapping of organogenesis in mice recently, specific timing and expression levels of TRAs remain underexplored. Hence, this project aims to investigate TRA expression profiles within mice over a course of mid- to late embryogenesis. Data by Irie and Kuratani (2011) supported this undertaking.

In addition to the TRA analysis, the embryonic data will be examined regarding chemokine expression levels. Chemokines, chemotactic cytokines, are signalling molecules that do not only direct immune cells to pathogenic locations, but also orchestrate cell migration during developmental processes. Upon secretion, they promote cell survival, proliferation, strengthening of immune responses and direction of migrating cells. More than 40 chemokines are expected to operate within vertebrates, each one with a grand influence over the immune system and embryogenesis Alanko and Sixt (2018)

This project aims to utilise the datasets on time-dependent embryonic transcriptomes and TRAs to catalogue established TRAs in respect to specific stages of embryonic development to offer a base of data that may support further studies in tissue development.

2 Methods

2.1 Datasets

Irie and Kuratani (2011) provided comparative data to study the expression-levels of tissue-restricted antigens (TRAs) during embryonic development. TRA data was isolated from the Microarray data with the help of a TRA dataset (Dinkelacker, 2021). The statistical open source programming language R (R Core Team, 2022), bioconductor packages, and an annotation file (Cunningham et al., 2022) served to prepare data for the following analysis.

Embryonic data set GSE28389 There were two main criteria for choosing a dataset. Firstly, it had to use Affymetrix microarray analysis. Secondly it was required to display expression levels of the whole mouse during different stages of embryogenesis. For the purpose of this work the whole RNA of multiple wild type C57BL/6 mice embryos was collected at eight different stages (Microarray chips: 3x E7.5, 3x E8.5, 3x E9.5, 3x E10.5, 2x E12.5, 2x E14.5, 2x E16.5, 2x E18.5). Triplicates or Duplicates were homogenised before application on the Affymetrix Mouse Genome 430 2.0 Array (Irie and Kuratani, 2011).

TRA dataset Our TRA data stems from unpublished data by Dr. Maria Dinkelacker. The data included in the “tra.2014.mouse.4301.5x.table.tsv” table includes a larger quantity of TRAs and was thus chosen to work with.

Table 1: Overview of datasets used for TRA analysis

dataset	datatype	source
embryonic dataset	Affymetrix Mouse Genome 430 2.0 microarray	Irie and Kuratani, 2011
TRA dataset	specific dataframe for tissue specific genes and their main tissue	unpublished data from Dr. Maria Dinkelacker

2.2 Packages

The essential Bioconductor packages for the analyses were “affy” (version 1.74.0), “limma” (version 3.52.1) and “vsn” (version 3.64.0). The package “affy” was applied for exploratory oligonucleotide array analysis (Gautier et al., 2004). Linear Models for Microarray and RNA-Seq Data (limma) was used for differential expression analysis of microarray data. The package uses voom method, linear modelling and empirical Bayes moderation to assess differential expression and give stable results even with a small number of arrays (Ritchie et al., 2015). “Limma” can be utilised for all gene expression technologies including microarray data. Variance stabilising normalisation via package “vsn” was employed for data normalisation. VSN executes data calibration, quantification of differential expression, and the quantification of measurement error (Huber et al., 2002).

2.3 Quality control (QC) of the embryonic microarray data

Four major quality complications are commonly encountered when working with microarray data. Low quality chips, imprints such as fingerprints or marks from pipette tips, irregularities in dye distribution, and light intensity extremes. To detect possible damages, we performed single-chip control for all twenty chips. Conspicuous chips should be removed at this point, however the number of biological replicates contained in the data was quite scarce. Some development stages only included data on two chips. Excluding an entire chip could thereby affect the significance of further statistical work.

2.3.1 Single Chip Control

There are no visual damages such as fingerprints present on the chips. Nevertheless the Affy chips of E12.5_1 and E14.5_1 are considerably lighter in intensity. This is also observable in the boxplot before normalisation.

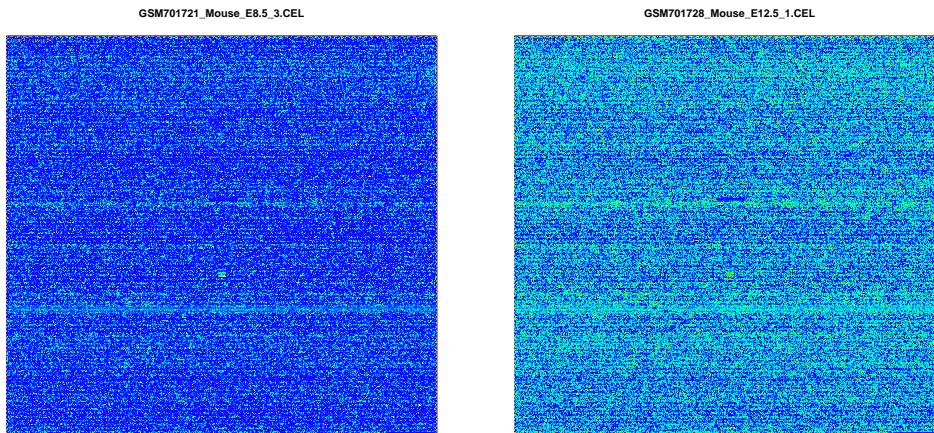


Figure 2.3.1: **Single chip control** Probe three of day E8.5 shows no salience, whereas the first probe of day E12.5 is noticeably lighter in intensity compared to other chips.

2.3.2 RNA Degradation

RNA Degradation plots serve as another possibility to detect physical artefacts on the chips. It stands out that probe intensities are lower towards the 5' end of a transcript than towards the 3' end which is expected due to the nature of RNA degradation. Irregularities would appear as individual lines that have slopes differing from the group pattern. The RNA degradation plot overall shows a regular group pattern without slopes that deviate significantly.

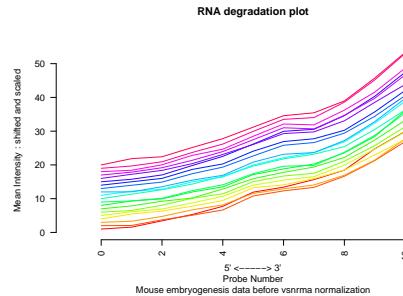


Figure 1: RNADeg

Figure 1.2: **RNA degradation plot** This plot is used to verify the quality of RNA. Slopes with a higher steepness compared to the overall pattern indicate low quality RNA.

2.3.3 Normalisation

The data had to be normalised before conducting further calculations necessary to obtain differentially expressed genes (limma). Variance stabilising normalisation (VSN) is specific for Affymetrix GeneChip probe level data and thus a reasonable choice for this step. VSN does probe-wise background correction and between-array normalisation. The function returns an ExpressionSet that can be used for further analysis. In the boxplot with VSN-normalised data it stands out that the mean values of the different chips are similar and the intensity differences equalised. Yet, it should be noted that even after normalisation, there is still a slight increase visible in the means of chip E12.5 and E14.5.

2.3.4 Scatter Plot

Furthermore, scatter plots can be used to review the quality of data. We plotted the normalised chips against each other according to the following pattern: E7.5_1 against E7.5_2,..., E8.5_3 against E 9.5_1,... Plots comparing the same measurement points do not show scattering. Some plots comparing different measurement points however display skewed scattering.

2.3.5 MeanSdPlot

To verify the previous normalisation in regards to variance-mean dependence a MeanSDPlot was used on the normalised data. A horizontal line would indicate no dependence. In the plot below the line slightly ascends with increasing mean value. However this is still in range, so no data was excluded.

3 Results

3.1 Analysis of TRA gene expression

In order to determine expression levels of TRAs, the aforementioned data was run through statistical devices and then visualised with different methods. Within the framework of this project, the data was first explored

with k-means clustering, applying elbow method and silhouette method to determine the optimal number of clusters. This was followed by limma analysis with p values 0.001 and 0.005 on TRA and Chemokine data and yielded respective tables. TRA and Chemokine expression were plotted in different ways to visualise expression development in different tissues. Some significant plots will be shown here, whereas consecutive data can be found in supplementary material.

3.1.1 Dimensionality Reduction and Clustering (PCA, UMAP, t-SNE)

Dimensionality reduction, including Principal Component Analysis (PCA) served as additional quality control and was followed by t-distributed stochastic neighbour embedding (t-SNE) and uniform manifold approximation and projection (UMAP) to further reduce dimensionality.

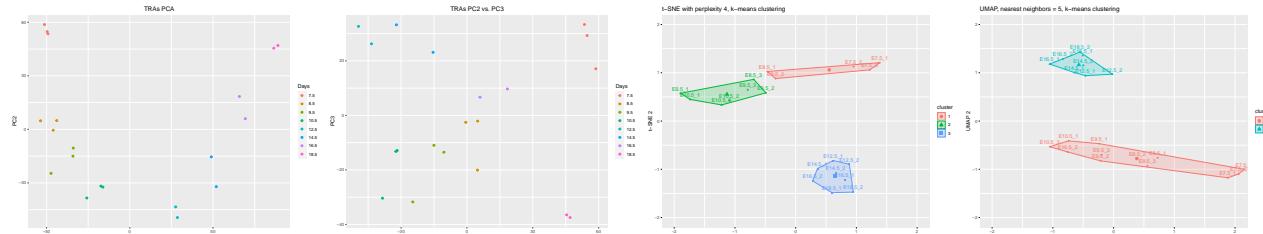


Figure 3.1.1: Reduction of dimensions and indirect Quality Control. The 20 chips are shown as data points that contain gene expression as features, respectively as loadings for the principle components (PCs). The first 14 PCs add up to 95% of cumulative proportion. Hence the remaining 6 were removed before further dimensionality reduction. t-SNE and UMAP further reduce the 14 dimensions given by PCA to 2 dimensions with maximal maintenance of information and clusters.

The PCA plots give clusters pairing data points of the same day along the axis of individual PCs. The orientation of coherent data points allows to foreclose batch errors rendering the chosen dataset as reliable. UMAP, with nearest neighbours 5 results in data with an ideal number of two clusters. One of which contains probes from days E7.5 to E10.5 and the other from E12.5 to E18.5. t-SNE, with perplexity 4, shows three clusters (optimal number of clusters was determined via Silhouette method). Again, separating chips with early and late embryonic data.

3.1.2 Differentially Expressed Gene Analysis (DEGA)

In an effort to determine the expression profile of TRAs over the course of mouse embryogenesis, every cons was plotted against the next (E7.5 against E8.5, E8.5 against E 9.5) during Differential Expressed Gene Analysis (DEGA).

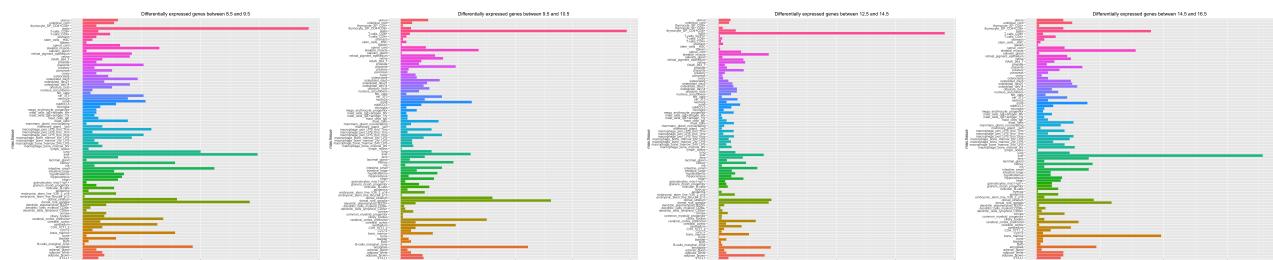


Figure 3.1.2: Difference in TRA expression over the course of mid- to late embryogenesis. Here shown are the quantities of differentially expressed genes in different organs from one time point to the next. Tissues are enlisted on the y-axis, while the x-axis provides scaling for an absolute count of differentially expressed TRA genes.

These bar plots show prominent TRA expression in testicles, liver, dorsal root ganglia and amygdala. Testicles account for the highest amount of differentially expressed TRA genes in a majority of phases, highlighted by the abundance of differentially expressed genes (DEGs) at phase E12.5 to E14.5, E14.5 and E16.5 being an exception within this profile. Liver-specific TRA expression especially stands out during development of E14.5

to E16.5, while dorsal root ganglia associated genes peak during developmental phases of E8.5 to E9.5 to E10.5 to E12.5; mid-embryogenesis and late embryogenesis. Comparatively, amygdala-related TRAs display significant levels during E9.5 to E10.5 to E12.5, but do not stand out as intensely as testicles, liver and dorsal root ganglia. It is noticeable that in the interval of E7.5 to E8.5 and E12.5 to E14.5 gene expression seems more differentiated, with testicle-associated genes skyrocketing.

3.1.3 Volcano plots

In an effort to determine TRAs with significant gene regulation, DEG analysis was visualised in volcano plots.

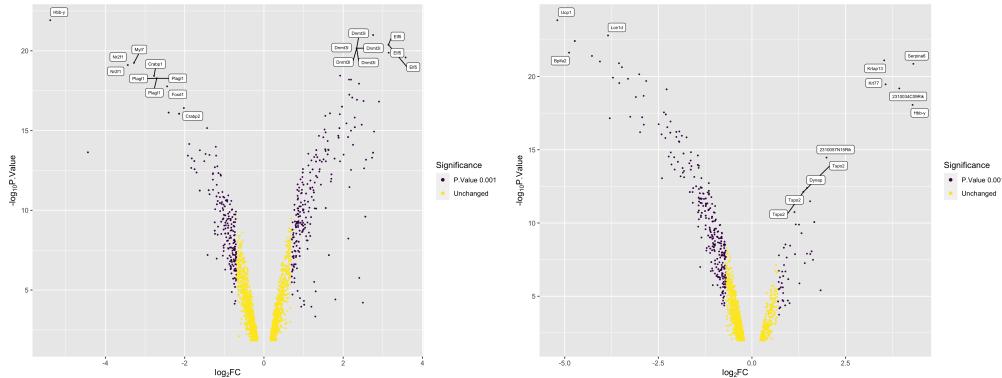


Figure 3.1.3: Differentially expressed TRAs labelled at maximum divergence and significance. Differentially expressed genes are plotted according to the intensity (x-axis) and significance (y-axis) of their up or down regulation. On the left from E7.5 to E8.5 and on the right from E16.5 to E18.5.

Results of the volcano plot provide a collection of significantly up or down regulated genes being upregulated in tissues of liver, umbilical cord and testis during E7.5 to E8.5 and in tissues of the umbilical cord, epidermis and liver as well during E16.5 to E18.5. It is noticeable that the plots from days E12.5 to E14.5, E14.5 to 16.5, and E16.5 to E18.5 have a left skewed distribution indicating more significantly down regulated genes. The plot from day E8.5 to E9.5 generally shows lower p-values and thus greater significance.

3.1.5 Venn diagram

Venn diagrams quantify the overlap of TRA expression levels at different embryonic stages.

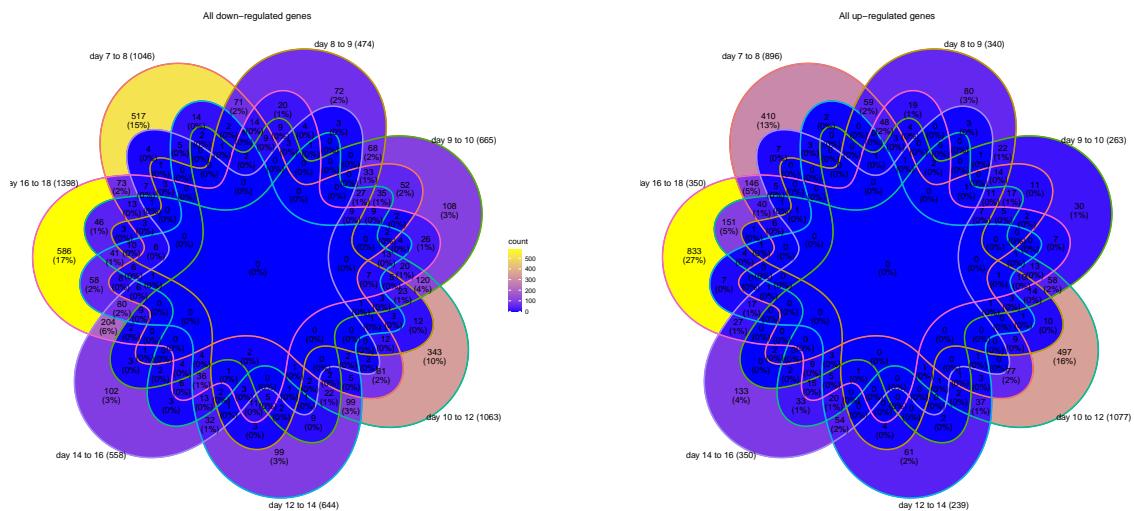


Figure 3.1.6: Venn Diagram of upregulated TRAs and downregulated TRAs. Upregulated (right side) and downregulated (left side) genes from the different stages are plotted against each other. The key on

the right indicates an absolute TRA count. Intersections visualise an overlap of TRA expression profiles at the given time.

Mid-embryonic phase E7.5 to E8.5 (517) as well the late embryonic phase E16.5 to E18.5 (586) stand out in independent downregulation. Phases E16.5 to 18.5 also show a pronounced count of upregulated differentially expressed genes (833). However, it can also be noted that between E10.5 and E12.5 DEGs are both significantly up and down regulated independently. Phase E16.5 to E18.5 exhibits the highest count of upregulated as well as downregulated TRAs. There are no differentially expressed genes that are up- or downregulated during all development stages.

3.1.6 Gene Set Enrichment Analysis (GSEA)

In order to retrieve a functional profile of every gene set, gene set enrichment analysis (GSEA) was performed for each day of measurement and visualised.

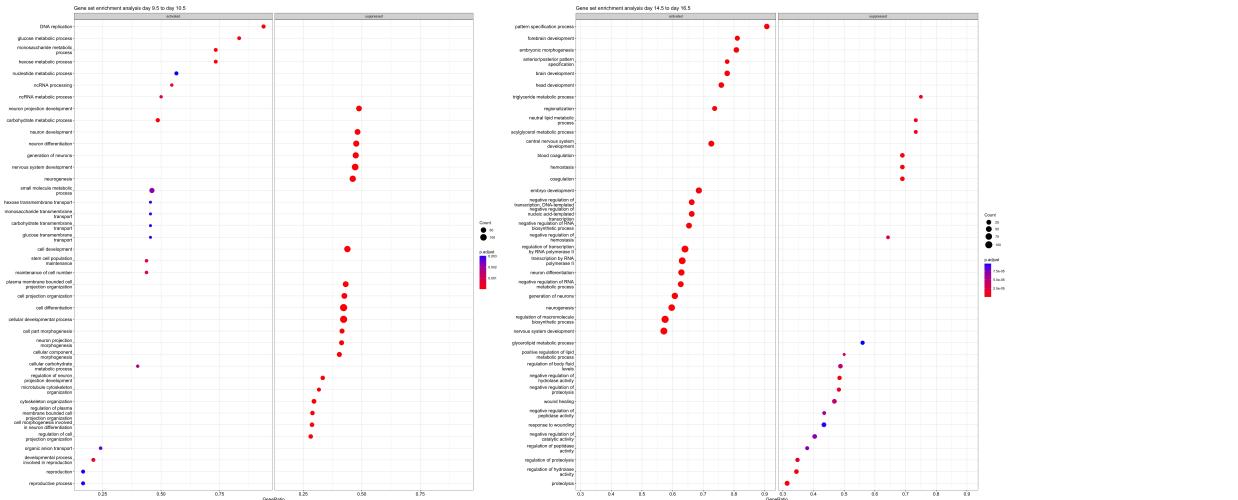


Figure 3.1.5: **Gene functions in the course of mouse embryogenesis.** Activated and suppressed DEGs and their functions are shown including absolute count and p-value.

GSEA shows an inversion of gene function profiles between mid-embryogenesis and late embryogenesis. Especially a shift in metabolism-related genes from activation to suppression. Organ development-related genes, however, are activated with time. Especially neuron and nervous system related processes stand out.

3.1.7 Overview of TRA expression throughout embryogenesis

In order to research time-dependent expression levels of some tissue-specific genes, we picked 5 genes that displayed the biggest absolute difference between following days and had a p-value less than 0.01. The following dot plot aims to present distinct TRA genes, with regard to days of embryogenesis. Gene *Lin28a* was amongst top 5 differentially expressed genes from day 9 to 10 as well as from 10 to 12, *Serpina6* - from 10 to 12 and from 16 to 18, *Hbb-y* - from 7 to 8 and from 16 to 18.

Figure 3.1.7: Most differentially expressed TRAs through embryogenesis. Each subplot illustrates change in the expression level of one gene from day 7 to day 18. Points are colored by the tissue of maximal expression.

Each subplot illustrates one or more peaks in expression of a TRA gene, within which it is noticeable that late embryogenesis accumulates a higher expression of a certain gene in contrast to mid-embryogenesis and vice versa. A considerable amount of highly expressed TRA genes are associated with umbilical cord, especially with a tendency of peaks towards late embryogenesis and a pronounced peak of Hba-x and Hbb-y. Further tissues that are frequently represented are liver and dorsal root ganglia.

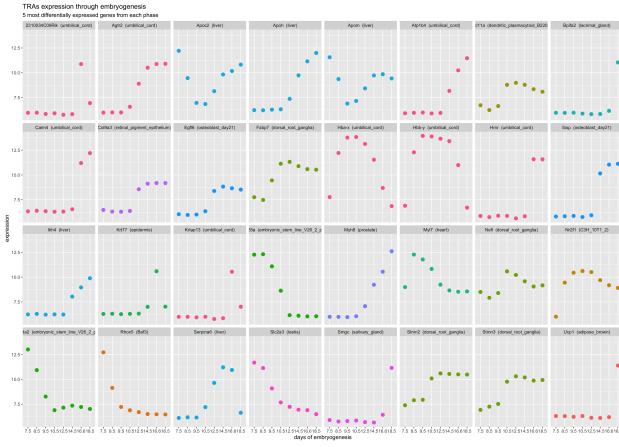


Figure 2: TRA_dotplot

This plot underlines the main goal of the project. It was possible to generate an applicable dataframe and observe TRA expression characteristics through several different plots.

3.2 Analysis of chemokine expression

Following the analysis of TRA expression levels, PCA and differential expression analysis were also performed on chemokine and chemokine receptor genes that were isolated from the studied gene expression set. Annotated tables of differentially expressed genes with p-value less than 0.05 and 0.01 for each consecutive daily comparison are provided in supplementary data. In order to analyse change of chemokine expression through day 7 to 18 we picked 5 genes with the greatest absolute difference between following days and had a p-value less than 0.01.

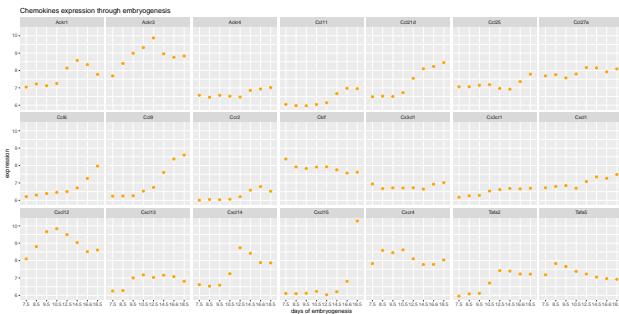


Figure 3: chem_dotplot

Figure 3.2.1: Most differentially expressed chemokine and chemokine receptor genes through embryogenesis. Each subplot illustrates change in the expression level of one gene from day 7 to day 18.

In order to analyse the amount of up- and downregulated chemokines, two venn diagrams were generated. In this diagram days of embryonic development are compared with each other, resulting in intersections that visualise an overlap of chemokine expression profiles. The major upregulation occurred between E16.5 and E18.5. The major downregulation of chemokine-related genes was from E7.5 to E8.5 as well as from E16.5 to E18.5.

4 Discussion

This project aimed to generate a data frame containing TRA expression levels connected to organogenesis in the time of mid- to late embryonic mice development. In Chen et al. (2022)'s similar work, the location of expression was additionally taken into account. This resulted in a spatiotemporal map of transcriptomes at different stages of embryogenesis. The research of Chen et al. (2022) thereby helps to validate certain results within this project.

4.1 Differential Expressed Gene Analysis (DEGA)

We analysed TRA expression levels to achieve a better understanding of embryogenesis and organogenesis. An applicable dataframe was built and multiple ways of analysing the data were applied. By plotting differentially expressed genes over time it was possible to receive an estimate on the amount of different tissue types developing during different stages of embryogenesis. The expression activity, translated into bar length, can be assumed to be proportional to organ development. This enables further understanding of organogenesis in mice. Reflecting on the observed results, three tissue types especially stand out during mid-to late embryogenesis: testicles, the liver, and brain-associated tissues. Based on this observation we can assume that brain-related tissue is actively generated in a slightly fluctuating, yet constant manner during the whole course of embryogenesis, especially in areas of the dorsal root ganglia. Other studies observed similar developmental patterns in brain related gene expression (Hartl et al., 2008). They hypothesised on the basis of their results that this expression rate is rather constant due to limited cellular resources like energy, space and water. This would match the assumption that brain development is shown as dominant in the plots. Due to its importance over the whole course of embryonic development this organ is constantly in need of the full capacity of cellular resources, with the exception of the phylotypic period.

In a similar fashion, liver-associated TRA genes experience expression levels comparable to those of dorsal root ganglia. And only exceed brain gene activities during late embryogenesis. This may result from liver development which starts with the liver bud formation at E9.5 (Keng et al., 2000). Lastly, testicle-related TRAs stand out due to their extreme amount of differentially expressed genes in contrast to remaining tissues. According to (Dinkelacker, 2021) a potential reason for such high expression levels could be the fact that they are an immune deprived site as in the case of spermatogenesis. Another attempt to explain the strong expression of testicular TRAs could be based on their very nature. They inhabit a germline expression character, mainly to perform spermatogenesis (Law et al., 2019). Similar developmental patterns can be observed in the research work of Chen et al. (2022), in which brain and liver development stand out during analysis. However, other important tissue developments during DEGA might have been underestimated due to the sheer intensity of testis and liver TRAs present. This may be a major limitation of this attempt. Furthermore a constant scaling of the axis for all plots would have been more beneficial for analysis in retrospect.

4.2 Volcano Plots

Data visualisation with volcano plots aided the isolation of prominent TRA genes that were up- or down-regulation with a relevant significance. Genes associated with reproductive functions, such as the placenta (*Sfmbt2*, *Fmr1nb*) and testicles (*Tex19.1*) stand out. Researching potential pathways corresponding to the intense regulation of these genes should be considered. Generally, visualising genes of high and low regulation can be later used to extract TRAs that might have some greater significance in embryonic development. Interestingly, plots from days 12 to 14, 14 to 16, and 16 to 18 indicated down regulation of genes with a left skewed distribution. At the same time, they displayed higher significance than the upregulated genes. It could be promising to further validate this trend of downregulated genes towards the end of embryo development and explore the main genes that contribute to this trend.

4.3 Dot Plots

Although some genes that are specific to the same tissue demonstrate similar trends regarding expression levels (e.g. *Hbb-y* and *Hba-x*, both specific to liver), others show different trends (e.g. *Agtr2*). *Hbb-y* plays an important role in the primitive erythroid lineage, it is expressed in primitive erythroid cells (Capellera-Garcia

et al., 2016), this may be the reason why it is distinctively regulated over the course of embryogenesis. However *Agtr2* which is coding for the Angiotensin II type 2 receptor is specifically expressed in the vascular muscle cell during late gestation (Akishita et al., 1999). This might be the reason why expression of this gene increases on days 12 to 18, probably exceeding the time frame of the study that provided us with this gene expression set. The plot of most differentially expressed chemokines over the course of embryogenesis provided valuable insights into tissue development. For example *Cxcl15*, also known as lungkine, that is strongly up-regulated on day 18, is expressed through embryogenesis specifically by lung bronchoepithelial cells (Rossi et al., 1999), suggesting that this tissue is already formed on the 18th day of embryogenesis.

4.4 Venn Diagram

Another method to monitor time-related TRA activity was the venn diagram, highlighting up- and downregulation of an absolute count of TRA genes. The plot empathises with the original hypothesis of the paper we chose our embryonic dataset from: the hourglass theory. The hourglass theory supports the idea of a high and divergent gene expression during early embryogenesis and late embryogenesis and a low and more conversed expression during mid-embryogenesis (Irie and Kuratani, 2011). As mentioned before, the venn diagram generated in our project presents an hourglass theory-supporting character with noticeable increase in up- and downregulation of genes during the earliest and latest embryonic stage. Nevertheless analysis by venn diagram did not provide any information on specific TRAs that experienced regulation, nor on general tissue development. Another issue with this plot arose from the exclusive structure of intersections, which complicated the information within the plot.

4.5 Gene Set Enrichment Analysis (GSEA)

GSEA enabled us to analyse molecular mechanisms, such as the intensity of metabolic activities, molecular transport mechanisms, biosynthesis and cell cycle activities. However, in contrast to Chen et al. (2022), as well as DEGA, brain development received suppression of genes, which is contradictory to previous research. Hence accuracy of GSEA should be handled with caution regarding this project. Additionally, data that stood out was presented in absolute numbers, instead of specific TRA gene names, leaving the same issue as the venn diagram that was not able to provide neither information on specific TRA genes nor on organ development as a whole.

4.6 Problems and Limitation

During this project we encountered the general problem of plot incomparability. Most plots differ in what they observe: singular, specific genes or holistic tissue development, time-dependent TRA expression or general regulatory profiles with no regard to time. For example GSEA plots do not display gene names, whereas the Volcano plot does. Another example is that the Venn and Volcano plots generally analyse at the gene regulation independent of time, while GSEA and DEG show at the expression levels over time. But in the end, this project was able to successfully create an applicable dataframe with an overview of organogenesis in mice.

4.7 Methods

At each point during the statistical assessment of our data a multitude of options were available, such as the following: To determine the optimal number of clusters, elbow method, silhouette method, and gap statistics were performed, serving reliable information for consecutive steps. However, when clustering t-SNE results, the optimal number of clusters was predicted to be three. After visualisation, assigning the number k=2 would have been more reasonable. Generally, clustering the t-SNE and UMAP plots according to the PCs would have yielded more insightful information. Although performance of t-SNE is said to be (Van der Maaten and Hinton, 2008) robust to changes in the perplexity, different values (2, 3, 4) were tested. They gave similar biologica results. However, according to the original paper, typical values should range between 5 and 50. In this case that was not possible, due to a low sample count. The highest possible perplexity value equaled 4. For UMAP a count of nearest neighbours had to be given to indicate the local connectivity of the data. The maximum number possible in this case equaled 20, since this is the amount of samples, displayed

as datapoints(McInnes et al., 2018). Thus UMAP was conducted with perplexities 5, 15, and 20 to cover the possible cases of having close clusters in the high dimensional dataset as well as more global ones. Clusters within the UMAP plots were determined with k-means clustering, using the elbow method as an indication for the optimal count. In contrast to the t-SNE plots, there were more differences between respective plots and the clustering was biologically less intuitive.

4.8 Perspectives

The combination of immunology and embryonic research may be a breakthrough. Similar work on the basis of immune biology and tissue related antigens may shape the future of embryonic research. Additionally further exploration of TRAs can not only contribute to research in embryonic development, but also play a considerable role in cancer research and therapy.

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