“Visualizing Axolotl Gene Expression During Limb Regeneration After Forelimb Amputation, Using R”

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Video Link:
https://youtu.be/zz2Sz-AvtF4

RScript:
https://github.com/cmorin3/Axolotl-Gene-Expression/blob/d0e45e94752fd029b3f1986e87342da325706647/Axolotl_Expression.R

Dataset:
https://www.axolomics.org/sites/default/files/Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt

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Purpose

This manual demonstrates the use of R to visualize gene expression variation during axolotl forelimb regeneration following amputation. The supplied data contains samples collected over 28 days of gene expression during blastema formation in seven juvenile axolotls. (Stewart, et. al. 2013). Each time, tissue samples were extracted and subject to Illumina sequencing. Genes that are important in axolotl regeneration have been identified in previous work (Demircan, et. al. 2016) and will be used in this analysis. With R, we will create plots to visually observe when gene expression occurs over the course of regeneration.

The six key genes we will extract from the data have prominent roles in blastema formation, which is a mass of undifferentiated, highly proliferative cells that are necessary for regenerating the limb. Bone morphogenic proteins (BMPs) support blastema formation and regulate patterning. Fibroblast growth factors (FGFs) are key signaling factors for blastema formation. Matrix metalloprotease (MMP) promotes blastema gene expression and reconstruction. Sonic hedgehog (Shh) is a determinant of the anterior-posterior patterning of regenerating limbs. Transforming growth factor-β (TGFB) promotes wound healing. Wingless and Int-1 (WNT) genes are key in developmental processes, specifically cell fate, proliferation, and migration. (Del Moral-Morales, et. al. 2024).

Background

Axolotls are amphibians that can fully regenerate body parts and organs after injury or amputation using regenerative-associated processes. (Demircan, et. al. 2016). Previous work has identified genes and pathways that are involved in axolotl tissue regeneration and development. (Stewart, et. al. 2013). They are model organisms used in developmental biology studies that warrant further examination of the regenerative mechanisms used during the process of wound healing. (Adamson, et. al. 2022).

After amputation, wound healing takes place before regeneration occurs. The blastema, a specialized structure, is one of the first steps of the regeneration process. Followed by a nerve supply (epithelium) and the development of functional cells to create new tissue. The blastema cells continue to differentiate into limb tissues throughout the process of regeneration. (Adamson, et. al. 2022).

The expression of genes can provide insights into the pathways involved in the process of regeneration by identifying when activation and deactivation occur. The genes specified for this tutorial will be visually examined during the measured course of limb regeneration among the sampled axolotls.

Tools/Dataset

"Comparative RNA-seq Analysis in the Unsequenced Axolotl: The Oncogene Burst Highlights Early Gene Expression in the Blastema" Stewart, et al. 2013. Data uploaded from: https://www.axolomics.org→ Axolomics → Data Downloads → Gene Expression Across Time: https://www.axolomics.org/sites/default/files/Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt

This dataset contains axolotl genes with their description, type, and ID alongside documented levels of expression over 28 days. The first day is broken up by intervals up to 12 hours, then continuing with day 1, intervals then progress from three, four, and seven until reaching day 28. Each time interval represents a collection of tissue samples from the amputation site of the seven axolotls used. Then, RNA was harvested and processed with Illumina sequencing. In the data, expression values were calculated by TPM (Transcripts Per Million), which are the normalized transcript abundance values from RNA-seq (adjusted for gene length and sequencing depth). TPM compares the expression levels of gene(s) across time or the relative expression within a sample. With the use of R and RStudio, we will use the measured expression of regenerative genes to create visual diagrams. This will allow us to further explore the correlation of these genes and evaluate expression levels/patterns during the development of the blastema within the samples provided.

RScript Guide: Step-by-Step Process

A. Preparing the Environment

```
#Installing and loading R packages
install.packages("tidyverse")
library(tidyverse)
```

Installing R Packages:
It is important to install R packages from Comprehensive R Archive Network (CRAN), to use while working on the script. These packages and not pre-installed in RStudio but can be easily uploaded. tidyverse is a collection of packages specific for data wrangling, visualization, and exploration. Some of which include:

- 'dplyr': data manipulation
- 'ggplot2': tool for creating and visualizing graphics
- 'tidyr': provides functions to reshape data into neater formats
- 'readr': reading rectangular data files

library() function loads the installed package into the current R session.

Uploading the Data:
The data that is used for this script can be uploaded directly into R using 'download.file' to download the information from the URL given. 'file.show' allows us to see the text file within the R environment.

```
#Setting Up The Environment:
#Download File With Gene Expression Across Time Course
download.file("https://www.axolomics.org/sites/default/files/Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt",
              destfile = "Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt",
              mode = "wb")

file.show("Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt", head = TRUE)
```

Refining the Data:

The uploaded data is still in a text file format, and we need to convert it to a readable file, using 'read.delim' to make the text file into a data file. After that, the data file now has extra information that we want to filter out, using 'group_by' to select the desired data. "symbol" was the labeled column that had the genes, and the numbers as columns have the gene expression values. I used 'rename_with' to remove the "X" in front of the time points.

```
#Convert Data to Readable Table
gene_expression <- tryCatch(
  read.delim("Stewart_Gene_Expression_Across_TimecourseTPMs_0.txt", header = TRUE, sep = "\t",
             stringsAsFactors = FALSE),error = function(e) {
               message("Read.delim failed, try read.csv with sep=\",\" or other");NULL})


#Aggregate Genes Expression Levels At Each Time, Removing "X" From Time
gene_count <- gene_expression %>%
  group_by(symbol, X0hr, X3hr, X6hr, X12hr, X1d, X3d, X5d, X7d, X10d, X14d, X21d, X28d) %>%
  summarise(frequency = n(), .groups = "drop") %>%
  rename_with(~ sub("^X", "", .x), starts_with("X"))
```

gene_count:

Filter

	symbol	0hr	3hr	6hr	12hr	1d	3d	5d	7d	10d
1	A2BP1	0.6548329	3.4216830	1.5526887	2.7156824	0.5133042	0.2668960	0.3152029	0.7218104	0.000000
2	A2LD1	0.0000000	1.8802456	0.0000000	0.0000000	0.0000000	1.9066027	4.5033777	6.4454210	3.545153
3	A2M	9.0297919	12.9567951	17.0868968	12.8759746	33.4729144	28.9674024	22.8993071	13.5638335	18.961760
4	A2ML1	6.3547423	6.5996538	10.9925354	11.1598964	31.8001490	26.3472508	17.8162427	16.4958729	21.564770
5	A4GNT	16.6379526	11.8106884	21.5713246	25.5863814	28.4762804	18.2898708	15.4523548	11.8984622	5.454425
6	AAAS	3.1163734	2.0041739	2.9557206	2.5848061	1.5878415	4.5726034	5.8502419	5.1526824	8.951071
7	AACS	2.8369051	2.2805559	2.6906592	3.7648118	4.8922854	1.8500196	3.2772968	2.5016550	1.719971
8	AADAC	6.7218387	3.1521060	7.5264192	4.2000432	10.9771893	15.5248462	18.0813197	24.0804212	15.282544
9	AADACL4	4.3860707	3.5796068	3.5194276	2.7699960	6.9809366	4.5372317	5.3584494	3.6812328	0.000000
10	AADAT	0.0000000	0.0000000	0.2425286	0.0000000	0.9669425	0.0000000	0.0000000	0.3382384	0.000000
11	AAGAB	4.5351166	3.8674636	9.1309380	8.2067782	8.5479558	11.2342982	16.9975410	10.8181525	13.611732
12	AAK1	0.0000000	0.0000000	0.0000000	0.3955707	0.0000000	0.3887650	0.4591296	0.0000000	1.445746
13	AAMP	7.2272365	7.1645164	8.4180081	5.0574392	28.0875509	28.9078570	33.3380845	42.1299477	28.931146
14	AARS	68.4507852	56.8906496	79.3559177	75.8183990	118.3439136	153.9819092	162.9640288	173.5013153	273.027068
15	AARS2	0.0000000	0.0000000	1.3341400	0.7000310	0.0000000	0.0000000	0.8125096	0.0000000	2.558498
16	AARSD1	2.3683319	2.5385003	1.8718679	1.9643598	3.7129308	5.1481695	9.1807459	10.4422763	14.358824
17	AASDH	1.0621263	2.1345783	3.3579080	2.6428706	2.4977083	3.4632010	3.0675182	5.8538123	1.609876
18	AASDHPPT	21.9283081	25.9779864	33.7053319	29.6750667	18.9983167	28.7505675	21.1103218	24.1711745	24.490400

B. Extracting Specific Genes

Create New Data Frame:

Instead of sorting through the refined *gene_count* data, which contains all genes in the axolotls' sampled tissues, we can create a new data frame with the genes we are interested in. Using 'filter()' to filter the genes in *gene_count* and 'grepl()' to search for specific matches, allows us to capture each unique gene using "paste0("^")'. So that genes from all listed families will be uploaded into new data frame. 'pivot_longer" reshapes the data from a "wide" to "long" format because it promotes consistency for analysis. As well as making time a factor, because the times listed are in hours and days, setting this parameter with 'factor()' to categorize them chronologically will ensure that they stay in the correct order moving forward.

```
#Create New Data Frame With Only Genes Of Interest In Regeneration
ReGen <- c("BMP", "FGF", "MMP", "Shh", "TGFB", "WNT")
ReGen <- gene_count %>%
  filter (grepl(paste0("^", ReGen, collapse = "|"), symbol, ignore.case = TRUE)) %>%
  pivot_longer(
    cols = matches("^(\\d+hr|\\d+d)$"),
    names_to = "time",
    values_to = "expression") %>%
  select(-frequency)

#Making Time A Factor
ReGen$time <- factor(ReGen$time,
                           levels =c("0hr","3hr","6hr","12hr",
                                     "1d","3d","5d","7d",
                                     "10d","14d","21d","28d"))
```

ReGen:

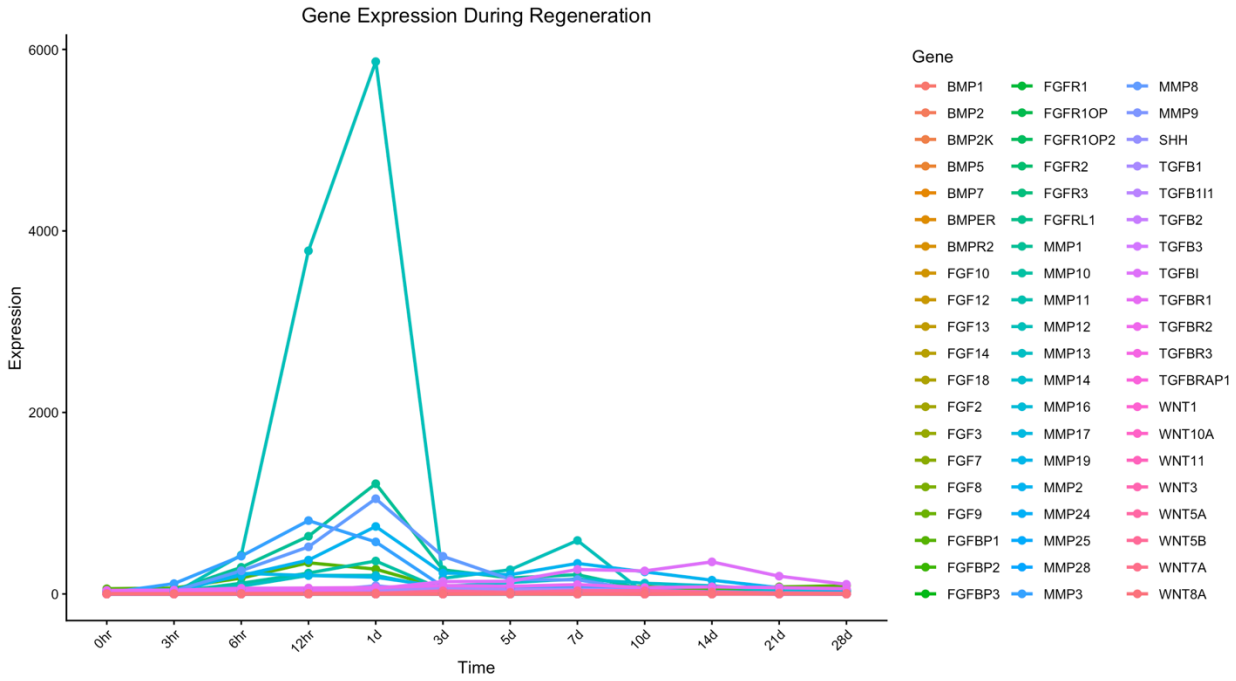
Filter

	symbol	time	expression
1	BMP1	0hr	3.2926860
2	BMP1	3hr	2.4965223
3	BMP1	6hr	4.2288579
4	BMP1	12hr	3.7856065
5	BMP1	1d	5.1840558
6	BMP1	3d	9.6471947
7	BMP1	5d	6.6159053
8	BMP1	7d	7.6190075
9	BMP1	10d	7.4851508
10	BMP1	14d	4.7682909
11	BMP1	21d	4.6154715
12	BMP1	28d	3.2528322
13	BMP2	0hr	5.9255091
14	BMP2	3hr	5.2535497
15	BMP2	6hr	6.5569782
16	BMP2	12hr	10.1891797
17	BMP2	1d	9.8951711
18	BMP2	3d	8.5947372

C. Gene Expression

Plotting Gene Expression:
'ggplot' creates data visualizations, using it allows us to better observe the data from our data frame. Here we are plotting all regenerative genes from our new dataset, *ReGen*. This will output a line graph of all gene expression levels over the course of time they were measured.

```
#Plotting Genes Expression Levels During Regeneration
ggplot(ReGen, aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "Gene Expression During Regeneration",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```

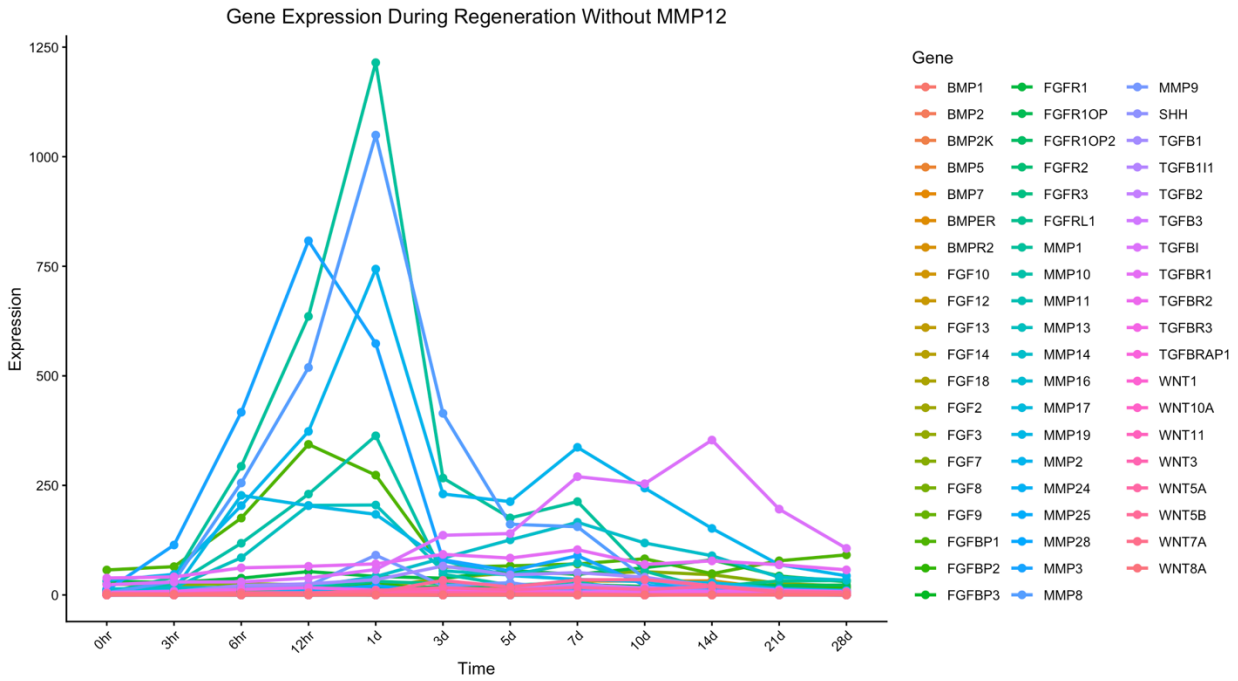


This represents the measured expression levels from all genes within the BMP, FGF, MMP, SHH, TGFB, and WNT families. The highest levels of expression are between 6hr to 1d, spiking around 12hr with an outlier from MMP12 showing the highest expression around 6000TPM. This decreases the magnification of the rest of the genes, distorting our visual observation. MMP12 is highly expressed after injury, and this is represented through this graph. (González-Orozco, et. al. 2023). It is the highest expressed gene throughout all the extracted regenerative genes in our data, indicating its significance in wound healing and blastema formation after amputation.

Re-Plotting Gene Expression and Excluding the Outlier:

With the same 'ggplot' from above, we will replot the genes while excluding MMP12, because its substantial values blocked the ability to observe the other genes expression levels. The 'dyplr::filter()' feature is from the 'dyplr' package and calls the filter function to avoid the statical filter ('stats::filter()'), then filters the data in *ReGen* to avoid the specific gene with 'symbol ! ='. This filters the data to include only the rows where the 'symbol' column does not match, "MMP12", then is plotted with 'ggplot'.

```
#Plotting Genes Expression Levels, Excluding MMP12
ggplot(dplyr::filter(ReGen, symbol != "MMP12"),
       aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "Gene Expression During Regeneration, Excluding MMP12",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```

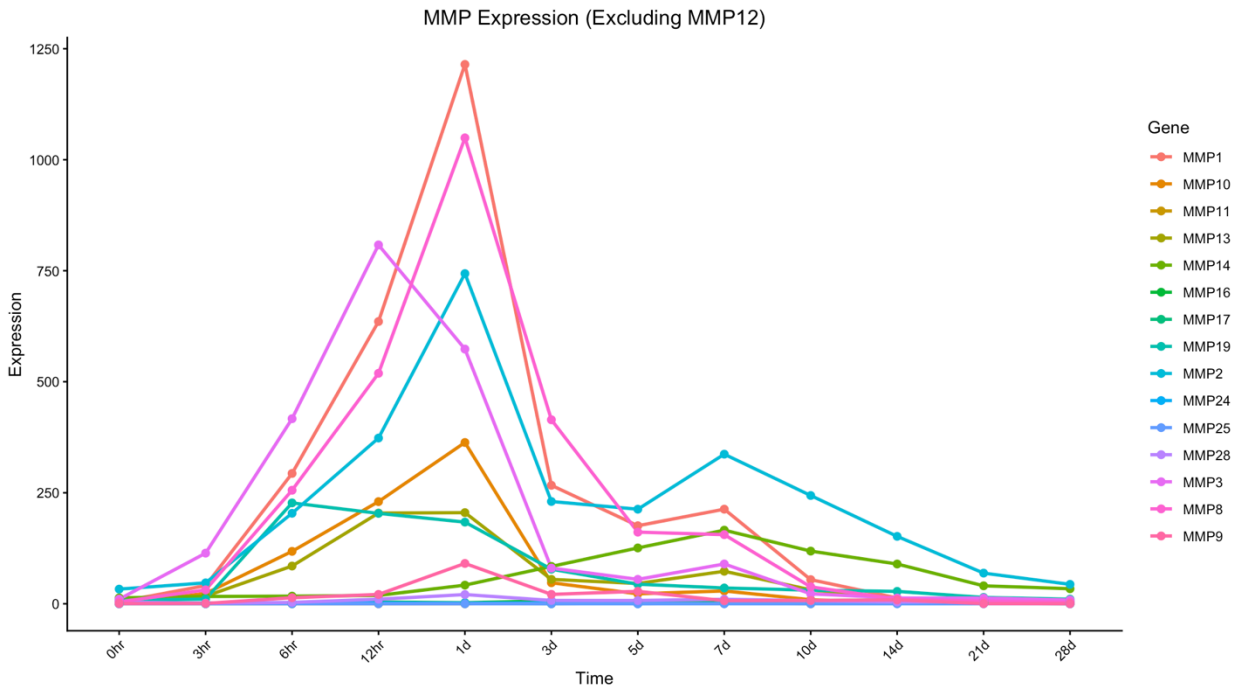


The plot shows a much clearer image of the expression levels of the included genes. We can now identify that the highest levels occur around 6hr-12hr, as well as some increases from 7d-14d. With this information and visual representation, we can now more closely examine these genes through further filtering.

D. Matrix Metalloprotease (MMP)

Plotting MMPs Expression Levels:
This will plot just the MMP genes, still excluding MMP12. The 'dyplr::filter()' filters the data in *ReGen* to search, using 'grepl()' to search for genes with "MMP" in their name within the column, while also using 'symbol ! =' to exclude MMP12. This filters the data to include only MMP genes and not include "MMP12", then is plotted with 'ggplot'.

```
#Plotting MMP Gene Family Expression Levels, Excluding MMP12
ggplot(dplyr::filter(ReGen,grepl("MMP", symbol, ignore.case = TRUE) &
                     symbol != "MMP12"),
  aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "MMP Expression (Excluding MMP12)",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```

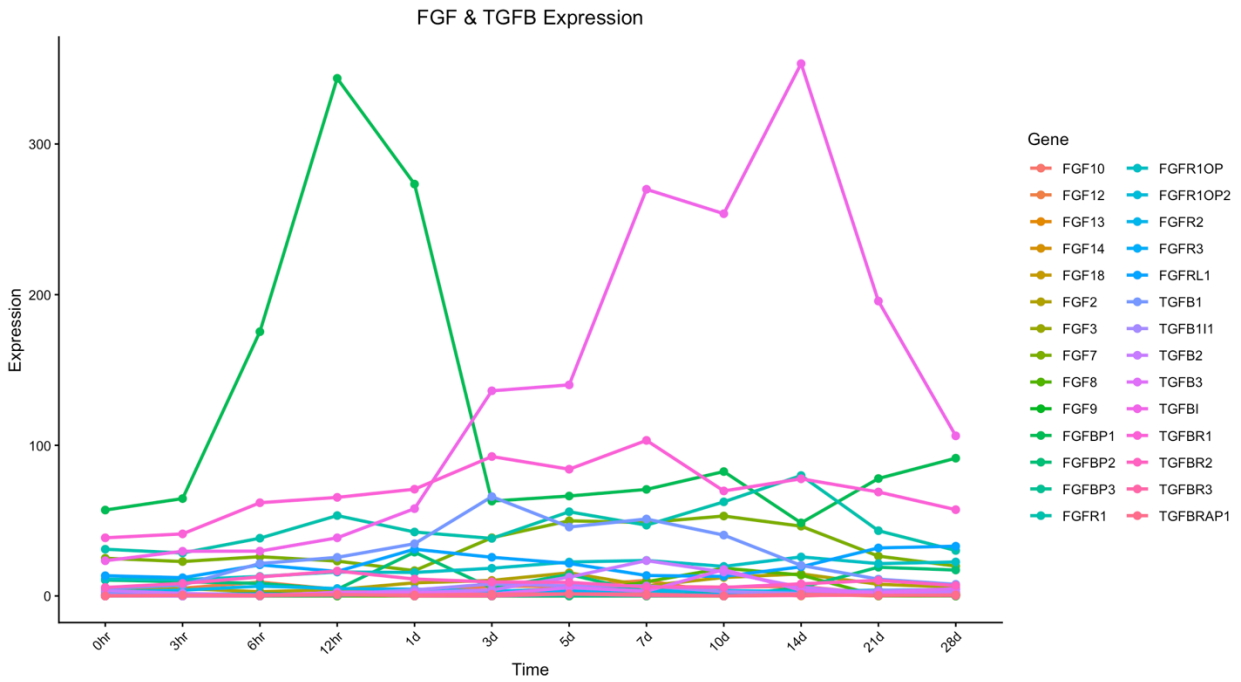


While still excluding MMP12, we can more clearly examine the other MMP levels. Expression is highest at 1d for all of them, starting with an incline at 6hr and a decline at 3d. MMPs break down the extracellular matrix (ECM) and are important in tissue remodeling during regeneration. Their expression analysis within axolotls has been well documented due to the ability to regulate ECM, which allows them to avoid scar formation and successfully regenerate tissues. (Cabral-Pacheco, et. al. 2020). This correlates to the spike early on after amputation; they are prepping the wound for blastema formation to begin regeneration.

E. Fibroblast Growth Factors (FGF) & Transforming Growth Factor-β (TGFB)

Expression Levels Between FGF & TGFB:
Now we will be plotting two gene families together. Using the same format with 'dyplr::filter()' to filter the *ReGen* data frame. To search for genes with 'grepl()' to find genes with "FGF" and "TGFB" in their name. This will filter the data and only include these genes when plotted with 'ggplot'.

```
#Plotting FGF and TGFB Gene Families Expression Levels Together
ggplot(dplyr::filter(ReGen, grepl("FGF|TGFB", symbol, ignore.case = TRUE)),
  aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "FGF & TGFB Expression",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
    plot.title = element_text(hjust = 0.5))
```

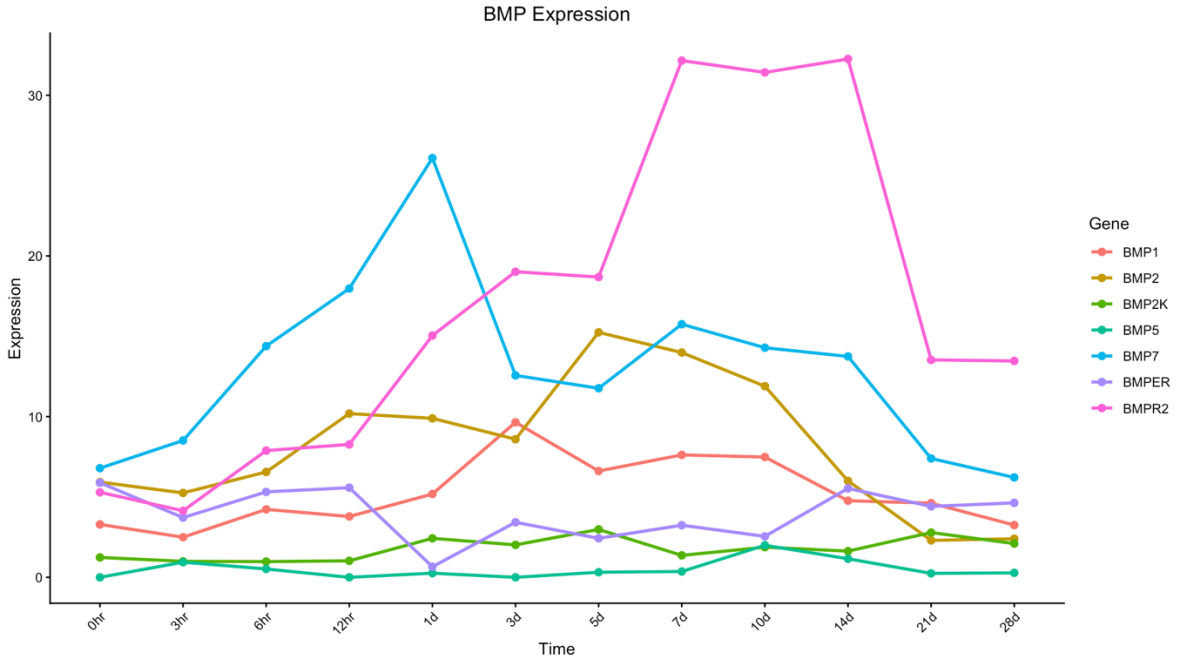


Typically, TGFBs are important during the initial wound healing phase and blastema formation, and FGFs are involved in later development of the limbs and patterning, after blastema formation. (Lévesque, et. al. 2007). However, this graph indicates two highly expressed genes, FGFBP1 (green/left) and TGFBPI (pink/right), occurring at different times, opposite to their "typical" expression. (Christensen, et. al. 2002). The FGFBP1 gene encodes the FGF-binding protein 1, which primarily releases FGFs from the extracellular matrix, enhancing signaling and promoting cell proliferation, differentiation, and migration. (Boutboul, et. al. 2006). FGFBP1 works with the FGF signaling pathway to aid in regeneration, acting as a substitute for nerve signals. The TGFBI gene provides instructions to make TGFB-1 protein and is important for the preparation of limb development. It is highly expressed during this initial preparation phase and decreases as the limb starts to rebuild. (Christensen, et. al. 2002). TGFBI is also involved in the formation of the apical epidermal cap, which maintains the proliferation and survival of cells during regeneration. Both specific genes and their familial pathways work in tandem, working to initiate healing and formation during the regenerative process. (Lévesque, et. al. 2007). In this graph, these genes are reaching similar expression levels as they mirror each other; when one increases, the others decrease.

F. Bone Morphogenic Proteins (BMPs)

Plotting BMPs Expression Levels:
We will examine the BMP genes using the 'dyplr::filter()' to filter in *ReGen* data and search with 'grepl()' to find all BMP genes, then plot with 'ggplot'.

```
#Plotting BMP Gene Family Expression Levels
ggplot(dplyr::filter(ReGen, grepl("BMP", symbol, ignore.case = TRUE)),
       aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "BMP Expression",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```

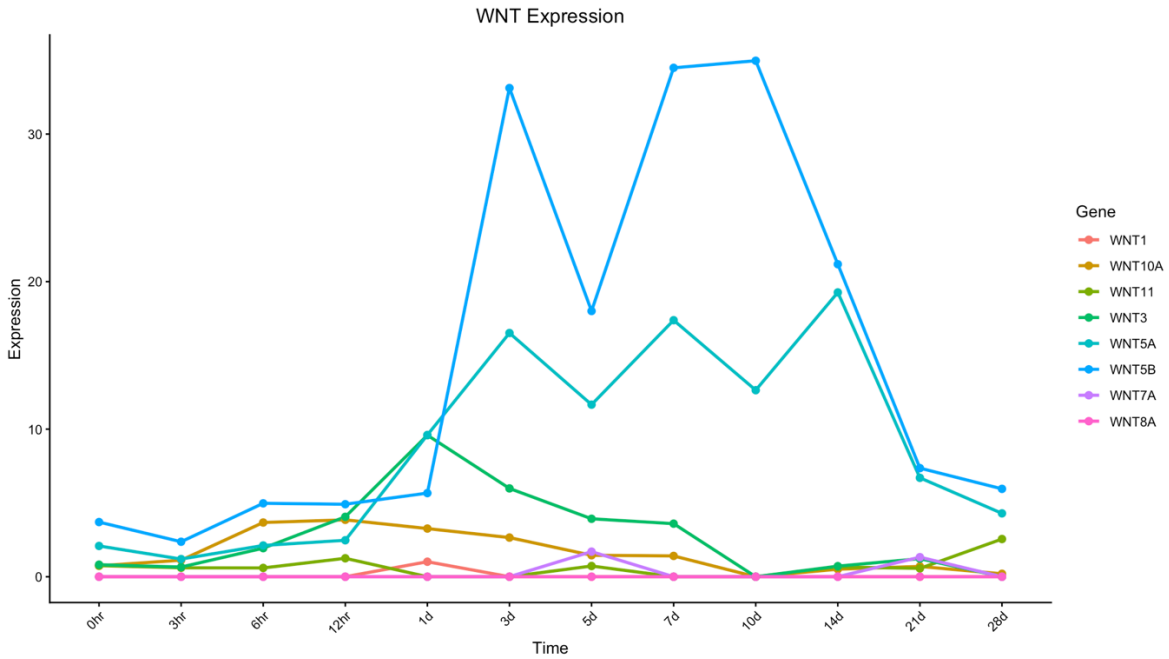


BMPs are involved in the regeneration of bone, cartilage, and other tissues, as well as the regulation of cell proliferation. (Machado, et. al. 2015). These expression levels vary throughout, some maintaining their levels, while others fluctuate. BMP5 stays consistently low; although it is important in the BMP signaling pathway, it is not highly expressed. However, BMP7 shows a variety of expression levels throughout; it has been identified through its role in blastema formation. (Saito, et. al. 2019). BMPR2 (pink) is a protein receptor 2 that is essential for the formation of new bone and cartilage. (Machado, et. al. 2015). Its expression can be detected in the blastema with involvement in maintaining proximo-distal progression of the regenerating limb.

G. Wingless and Int-1 (WNT)

Plotting WNTs Expression Levels:
We will examine the WNT genes using the 'dyplr::filter()' to filter in *ReGen* data and search with 'grepl()' to find all WNT genes, then plot with 'ggplot'.

```
#Plotting WNT Gene Family Expression Levels
ggplot(dplyr::filter(ReGen, grepl("WNT", symbol, ignore.case = TRUE)),
       aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "WNT Expression",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```

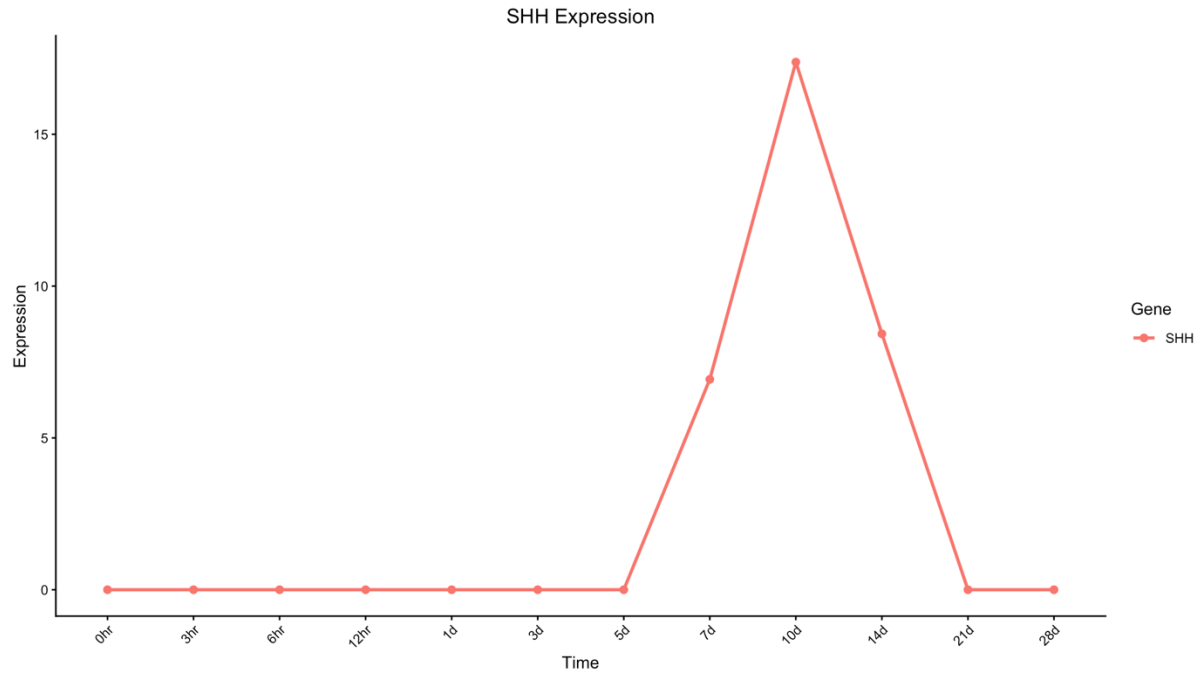


The Wnt signaling pathway has a significant influence throughout axolotl regeneration, promoting blastema formation, initiating limb regrowth, and cell proliferation. (Qingchao, Q., 2019). At 1d, there is an increase in many genes, while Wnt5A and Wnt5B have high levels from 1d to 21d. This could correspond with the pathway's initiation in blastema formation and signaling of limb regrowth. (Qingchao, Q., 2019). A complete limb regeneration takes about 40-50 days in juveniles, so we can see through this that the motion towards cell differentiation and limb growth is beginning to occur around this time when these genes have increased in expression.

H. Sonic Hedgehog (Shh)

Plotting SHH Expression Levels:
We will examine the SHH gene using the 'dyplr::filter()' to filter in *ReGen* data and searching with 'grepl()' to find all SHH gene, then plot with 'ggplot'.

```
#Plotting SHH Gene Expression Levels
ggplot(dplyr::filter(ReGen, grepl("SHH", symbol, ignore.case = TRUE)),
       aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "SHH Expression",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```



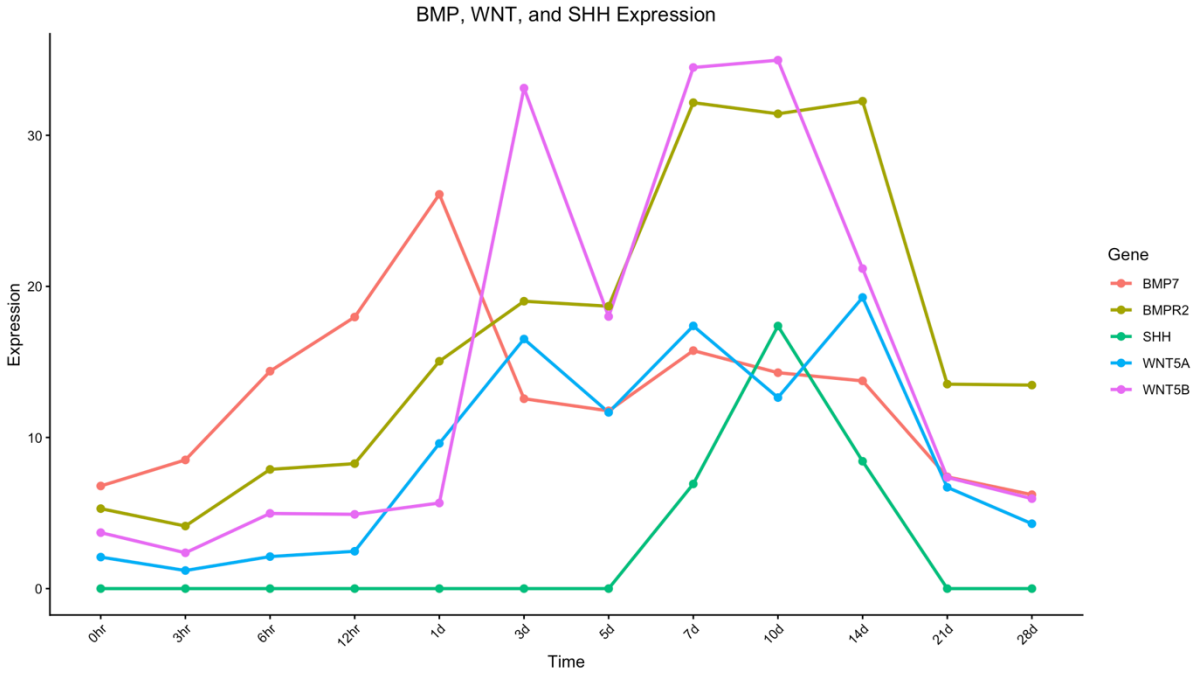
Shh has a crucial role in limb patterning during axolotl regeneration, as we can see this gene is not expressed until day 5 and peaks at day 10, then falls back to zero on day 21. While it is being expressed, it is telling the cells within the blastema where to position to form posterior structures (fingers). (Yamamoto, et. al. 2025). It is expressed only when it is required to give this information, which we can see in this graph.

I. Comparing Highly Expressed Genes in BMP, Shh, WNT

Comparing Expression Levels Between Highly Expressed Genes:

To examine the expression levels between highly expressed genes, we will filter them out of *ReGen* to plot. Using the 'dyplr::filter()' and 'symbol %in% c()' to identify the specific genes "BMPR2", "BMP7", "WNT5A", "WNT5B", "SHH" and then plotting them with 'ggplot' to compare.

```
#Plotting The Highest Expressed Genes From BMP, WNT, and SHH
ggplot(dplyr::filter(ReGen, symbol %in% c("BMPR2", "BMP7", "WNT5A", "WNT5B", "SHH")),
       aes(x = time, y = expression, color = symbol, group = symbol)) +
  geom_line(linewidth = 1) +
  geom_point(size = 2) +
  theme_classic() +
  labs(title = "BMP, WNT, and SHH Expression",
       x = "Time",
       y = "Expression",
       color = "Gene") +
  theme(axis.text.x = element_text(angle = 45, hjust = 1),
        plot.title = element_text(hjust = 0.5))
```



This graph allows us to visually combine the highest expressed genes in BMPs and WNTs, as well as Shh, because these signaling pathways are essential and contribute to the formation and patterning of the new limb. (McCusker, et. al. 2015). The influence these pathways have on each other is still being discovered, but evidence suggests coordinated interactions are occurring. At 1d, BMP7 peaks expression, while the others are increasing, following that BMP7 gradually declines. WNT5B increases from 1d to 3d where its stays relatively high until 14d where it begins to decline. As well as WNT5A and BMPR2 where they reach their peaks from 7d to 14d, notably Shh also peaking around 10d. The end of expression of these genes is 21d where they all decrease from 14d. They each play a specific role, which is expressed when needed to initiate that signaling. It is recorded that around day 10 post amputation, the blastema is continuing to develop and grow, which aligns with the presence of these expressed genes. (McCusker, et. al. 2015).

Analysis

The plots created in R help visualize the expression levels of the six gene families during limb regeneration. Over the course of 28 days, we can connect each gene's expression to the axolotl's regeneration. MMP12 and Shh were both expressed at different times, tied to distinct events in the blastema. MMP12 is important in promoting wound healing and breaking down the extracellular matrix, which prevents the wound from scarring. If inhibition of MMP12 occurs, scar tissue can form, leading to an inability to regenerate the limb. Its expression is crucial in the first steps of regeneration and is notable in visual diagrams. Both MMP12 and Shh were expressed for a set of time, then stopped once their roles were complete. In all plots, we see spikes in the first half (around 12 hours to one day) and the second half (10 days to 14 days). The first hours after amputation are vital for initial healing and signaling migrating cells. After initial healing, cell differentiation and positioning begin. These steps signal where the missing bone and tissue should be positioned. Examining when and how much these genes are expressed helps us identify their crucial roles in regeneration. This manual brings more precise visual insight and explanation to the genes playing key roles in axolotl limb regeneration. Further research into these genes will improve our understanding of regeneration in these animals.

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Appendices

Package Link:
https://cran.r-project.org/web/packages/tidyverse/index.html

Functions Used in Manual:
https://cran.r-project.org/doc/manuals/r-release/fullrefman.pdf