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# Impacts of hypophosphatemia on gene expression needed for bone fracture repair

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**IMPACTS OF HYPOPHOSPHATEMIA ON GENE EXPRESSION NEEDED FOR  
BONE FRACTURE REPAIR**

by

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B.A., College of the Holy Cross, 2012

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

2021

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## **DEDICATION**

I would like to dedicate this work to my wife Jacqueline, my advisor Louis Gerstenfeld  
PhD, and my dogs Teddy and Sadie.

## **ACKNOWLEDGMENTS**

I would like to acknowledge and thank my advisor Louis Gersetenfeld Ph.D., for his help and patience throughout the thesis writing process. In the middle of a pandemic, he has been an incredible mentor.

# **IMPACTS OF HYPOPHOSPHATEMIA ON GENE EXPRESSION NEEDED FOR BONE FRACTURE REPAIR**

**CASEY NORTON**

## **ABSTRACT**

Bone fractures are one of the most common injuries in the United States, encompassing 6.2 million incidences, and costing the healthcare system roughly \$20 billion annually. The majority of this cost falls upon a unique type of fracture known as a non-union fracture, defined by incomplete healing after 9 months. The economic burden in combination with the frequency by which these incidences occur offer a unique opportunity for research and improvement in the healthcare field.

Previous research on the fracture repair process showed that dietary deficiency led to delayed healing producing a rachitic-like effect on the endochondral bone formation process that occurs during fracture healing. This research will build off the understanding of hypophosphatemia on bone fracture repair utilizing a unique temporal clustering approach to assess changes in the transcriptomic expression within callus tissues of control fed and dietary phosphate restricted animals.

Using a temporal cluster modeling technique developed by our group (Lu et al. 2019), twelve clusters were generated for the gene expression data extracted from the callus tissues of B6 strain mice at time points 3, 5, 7, 10, 14, 18-, 21-, 28-, and 35-days post fracture, in, control and phosphate restricted dietary groups. Groupings of clusters

were used to establish the temporal expression patterns over the time course of healing and identify the movement of genes that changed their temporal expression patterns between the control and phosphate restricted diets. Biological process categories were established for each cluster, grouping using both Ingenuity Pathway Analysis (IPA) and the NIH DAVID Bioinformatics Database ontology assessment programs. Genes based on their association with four key tissue developmental processes in fracture repair, skeletogenesis, myogenesis, vasculogenesis, and neurogenesis were analyzed.

The analysis showed shifts to later peak expression times for all four categories. Further analysis illuminated three specific regulatory pathways that were significantly impacted by hypophosphatemia, Hippo and WNT signaling pathways and the circadian rhythm pathway while oxidative phosphorylation was both shifted and showed reduced expression. The shifts in expression time and level of these pathways demonstrate their importance to bone fracture repair and their impacts on mesenchymal stem cell differentiation.

From the data analysis it is clear that limiting dietary phosphate results in impaired mesenchymal stem cell differentiation caused by delayed Hippo and WNT signaling. Further it is evident that the processes of skeletogenesis, myogenesis, vasculogenesis, and neurogenesis are heavily interconnected, often showing overlapping genes through all four processes. Based on these shifts and impairments in specific signaling we identified novel mechanisms by which hypophosphatemia can impair fracture callus growth and development and delay healing.

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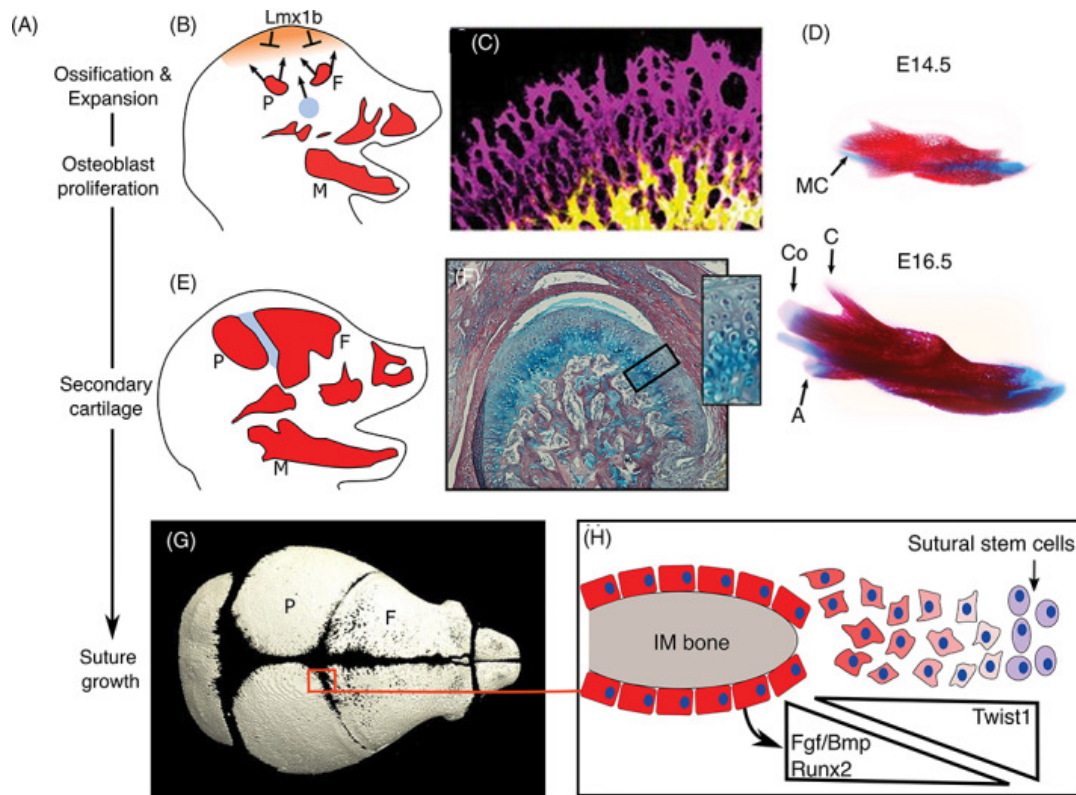
## LIST OF ABBREVIATIONS

BMP	Bone Morphogenic Protein
DAVID	Database for Annotation, Visualization and Integrated Discovery
FGF	Fibroblast Growth Factor
IPA	Ingenuity Pathway Analysis
TGF Beta	Transforming Growth Factor Beta
WNT	Wingless-related integration site

## INTRODUCTION

Fracture healing is a unique and complex process that can be broken down into two main systems, endochondral bone formation, and intramembranous bone formation over a specific time period (Einhorn 2005). A serious complication is something considered to be a non-union fracture, defined as an incomplete healing 9 months post injury with no signs of healing determined by repeated radiological scans 3 months after (Panteli 2015). Bone fractures and osteoporotic injury are a \$20 billion cost to the U.S. Healthcare system (Solomon 2014) and account for 6.2 million incidences in the United States alone each year (Dahabreh 2009). The economic consequences and the frequency by which fractures occur make it a topic in the medical field that is primed for analysis and improvement. Furthering the understanding of the process of fracture repair can ease economic constraints on lower income hospitals by reducing the incidence of non-union fractures and improving healing times will reduce loss in productive work productivity and disability after fracture injuries.

There are two methods of bone formation that are capable of operating independently and cooperatively during fracture repair. Intramembranous ossification begins with the condensation of mesenchymal stem cells and is demonstrated in figure 1.

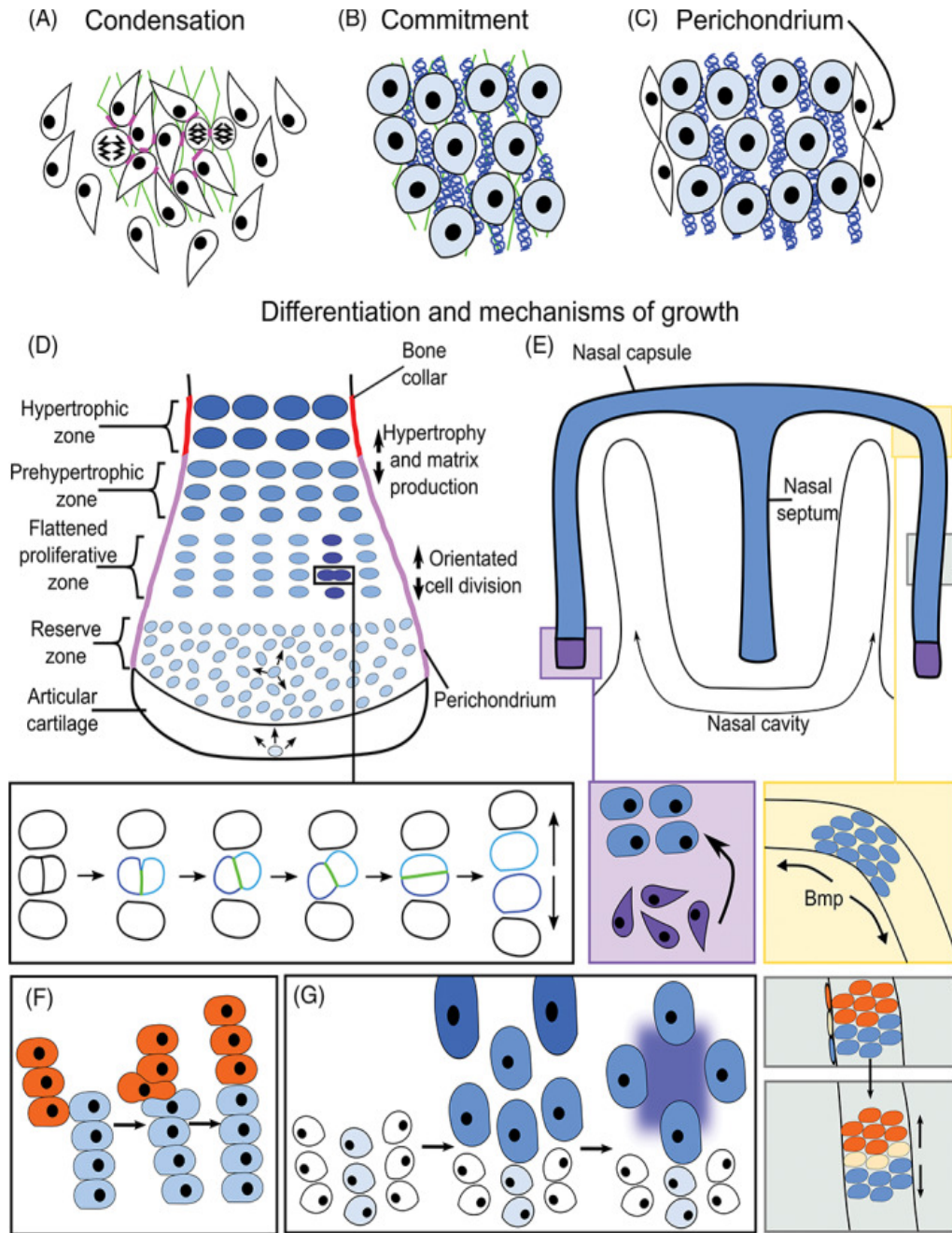


**Figure 1:** Intramembranous Bone Formation. This figure demonstrates the general steps of intramembranous bone formation. (Taken from Galea 2021)

These stem cells differentiate becoming osteoprogenitor cells, and then finally becoming osteoblast. These osteoblasts focus on what is known as the center of ossification, secrete collagen and produce unmineralized osteoid matrix. As the process progresses this area is infiltrated by calcium, mineralizing the area, trapping the osteoblasts, causing them to differentiate in osteocytes generating lacunar bone. A key to this process is the presence of high vascularization. The vasculature not only provides the nutrients, particularly calcium, but also introduces monocytes to the area which differentiate into osteoclasts, which break down the lacunar bone forming woven bone

(Dimitriou 2005) allowing later remodeling of the woven bone into its final lamellar structure of the original tissue.

Endochondral bone formation begins similarly, with the condensation of mesenchymal stem cells, and is demonstrated in figure 2. However, instead of differentiating into osteoprogenitor cells, in endochondral formation they differentiate into chondrocytes. These chondrocytes generate a cartilage template around the fracture site, an avascular frame for which the repair process can begin. Once the template is formed, the remaining mesenchymal stem cells differentiate to form a vascular periosteum. This introduction of a vascular network allows for chondrocyte proliferation, expanding cartilage in a longitudinal direction. As the cartilage continues to expand the chondrocytes in the middle of the template undergo programmed cell death, while the periosteum forms into a bone collar, stabilizing the cartilage and allowing for the vascular network to penetrate the cartilage introducing blood vessels, osteoblasts, and osteoclasts to the area to begin the ossification process as seen in intramembranous bone formation (Colnot 2005).



**Figure 2:** Endochondral bone formation. This figure demonstrates the general process of endochondral bone formation. (Taken from Galea 2021)

However, instead of differentiating into osteoprogenitor cells, in endochondral formation they differentiate into chondrocytes. These chondrocytes generate a cartilage

template around the fracture site, an avascular frame for which the repair process can begin. Once the template is formed, the remaining mesenchymal stem cells differentiate to form a vascular periosteum. This introduction of a vascular network allows for chondrocyte proliferation, expanding cartilage in a longitudinal direction. As the cartilage continues to expand the chondrocytes in the middle of the template undergo programmed cell death, while the periosteum forms into a bone collar, stabilizing the cartilage and allowing for the vascular network to penetrate the cartilage introducing blood vessels, osteoblasts, and osteoclasts to the area to begin the ossification process as seen in intramembranous bone formation (Colnot 2005).

### **Timeline of Fracture Repair**

To understand the intermingling of these two processes mouse models are used with fractures generally being induced in the femur.

As noted, bone formation occurs over a specific period of time. In this case, periods of time will be discussed based on the mouse model.

In the first 7 to 10 days of fracture healing, the process of chondrogenesis begins, next to the fracture while osteoprogenitor cells begin under the periosteum (Einhorn 2005). As with most injury, inflammatory response begins during the early phases, which triggers the invasion of macrophages, polymorphonuclear leukocytes, and lymphocytes.

At day 14 more bone forms under the periosteum and the cartilage begins to calcify. This calcification is conducted by the chondrocyte membrane delivering calcium

from the mitochondria of hypertrophic chondrocytes into the extracellular matrix, resulting in the formation of what is known as a hard callus, a cartilaginous mass that bridges the fracture (Einhorn 2005). Once critical mass is reached the callus will begin to calcify, as chondrocytes within the callus begin secreting phosphatases (Einhorn 2005). The secreted phosphate will precipitate with the delivered calcium forming cartilage, and secreted proteases, will degrade proteoglycans inhibiting mineralization, allowing chondrocytes to control the mineralization process (Einhorn 2005).

By day 21, the majority of the callus has calcified. The calcified callus becomes a major target for chondroclasts, which degrade the calcified cartilage, signaling the penetration of the area by blood vessels (Einhorn 2005). These blood vessels bring mesenchymal stem cells to the area, which differentiate into osteoprogenitor cells and ultimately osteoblasts. These osteoblasts begin the process of replacing the calcified cartilage. As this replacement occurs, the mineralized matrix is resorbed, and the chondrocytes removed (Einhorn 2005).

During days 28 to 35, the calcified cartilage combines with woven bone from osteoblasts. The remodeling of the callus begins with the osteoclasts converting the woven bone and calcified cartilage into lamellar bone capable of supporting weight (Einhorn 2005).

The transition from cartilage to bone is key in the healing process when considering intervention, specifically the precipitation caused by the calcium, phosphate, interaction.

Each of these periods throughout the repair process show differing peak genetic expressions. In the case of this research, the first will be considered overall decreasing expression (due to the immediate peak and then decreasing throughout the rest of the process), early peak time expression, middle peak time expression, and late peak time expression respectively.

### **Impairments of the Repair Process**

While the repair process seems direct, it is easily impaired by outside factors. For example, diabetes mellitus can cause impaired fracture healing due to premature resorption of the cartilaginous callus (Kayal 2007). The introduction of non-steroidal anti-inflammatory medications limit prostaglandins, which are key to the fracture repair process, thus suggesting avoidance of the medication (Zhang 2002). Even disruptions in circadian rhythm have been linked to mutation of the histone methyltransferase EZH2, which can control overall skeletal growth (Noguchi 2018).

Phosphate specifically has been shown to be a key to the fracture repair process. Hypophosphatemia is associated with rachitic disorders (Linde 2001, Robinson 2004), such as rachitic expansion of the hypertrophic chondrocyte layer in the growth plate as a consequence of impaired apoptosis of cells (Sabbagh 2005). Further molecular analyses have shown increasing extracellular phosphate results in an activation of the mitochondrial apoptotic pathway in hypertrophic chondrocytes (Wigner 2010). This is a key process in endochondral bone formation, suggesting that while phosphate is needed

for formation of the mineralized matrix, it is a key regulatory mechanism for hypertrophic chondrocyte differentiation and subsequent initiation of mineralized cartilage tissue resorption (Wigner 2010).

However, while it is evident that that phosphate is impacting repair, further exploration into how it is shifting genetic expression is required.

## **SPECIFIC AIMS AND OBJECTIVES**

While previous research has demonstrated the impacts of hypophosphatemia on fracture healing, this research will look to expand on the understanding of its impact by examining the global transcriptomic landscape within fracture callus tissues. A unique temporal clustering method (Lu 2019) will be used to assess changes in the transcriptomic expression within callus tissue mRNAs isolated from control and dietary phosphate restricted fed mice. This methodology will also allow for a broad understanding of how dietary phosphate levels effect fracture healing and how phosphate levels molecularly mediate shifts to the entire post-natal skeletal repair transcriptome.

## METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee at Boston University. Total RNA had been prepared from bone fracture callus tissues from N=6 mice per each time point. N=3 samples of RNA were then made for each time point by a randomized pooling of 2 samples per group of the six animal samples per time point. All microarray laboratory procedures were performed at the Boston University Microarray and Sequencing Resource. Methods for sample processing, normalization of microarray data, and quality control measures for examining for batch effects and comparing individual samples by principal components are as previously described ([Noguchi, et al 2018](#)). Raw CEL files and RMA-normalized, ComBat-adjusted values were archived in the National Center for Biotechnology Institute (NCBI) Gene Expression Omnibus Repository (Series [GSE99580](#)).

The analysis completed in this research is based on the genetic clustering method reported in Lu et al. 2019. This method is based on assessing N temporal gene-expression profiles at T time points. Each of these time points then generates R replicates resulting in each temporal gene expression having T x R measurements (Lu 2019). Using Gaussian finite mixture modeling generates the clustering of the N gene-expression profiles into K groups that predicts the total number of temporal profiles that best fits the gene expression data.

Lu et al. 2019 assumes that the number of replicates, R, for each gene is 1, for each time point, T. Lu continues that Gaussian mixture models assume temporal expression profiles for each cluster are generated using one component of a mixture of

Multivariate Normal distributions (Lu 2019). From here the  $k$ th cluster is the group has a probability of occurring equal to  $\alpha_k = P(z_i = k)$ . In this formula,  $z$  is missing cluster label for the temporal profile of gene  $I$  and  $\sum_{k=1} \alpha_k = 1$  (Lu 2019). Based on the parameters for each cluster, a probability density function can be determined, suggesting that when considering all parameters the unconditional probability density function for  $y_i$  can be represented as a weighted sum (Lu 2019). Using the expectation maximization algorithm (Dempster 1977), the maximum likelihood estimates for each parameter can be determined (Lu 2019). By then extending this formulation to account for a relationship with T-time points using polynomial regression models, the polynomial regression models can be solved using the expectation maximization algorithm (Lu 2019).

However, in most biological cases there are numerous replicates for each time point measure for gene-expression. Considering this concept of R replicates, for each time point T, T x R measurements are generated (Lu 2019). Using a mixed effects linear regression model accounts for the variability between the replicates for each time point by giving each gene for the time point its own intercept and/or slope (Celeux 2005, Ng 2006). Incorporating this mixed effect linear regression model with a random effects regression model maximizes the opportunity to detect unobserved missing data when used with the expectation maximization algorithm (Lu 2019). Further, a penalty term is added to the growing expectation maximization algorithm model, in order to account for increased data size and complexity, thus simplifying analysis (Lu 2019).

This model is then initialized by setting the number of clusters equal to the number of temporal gene expression profiles in the given data (Lu 2019). Cluster parameters are set using a fixed effect polynomial regression model, determining the median residual error for the gene and replicate models analyzed (Lu 2019). Finally the fixed and mixed effect polynomial order is determined prior to running the algorithm and thus applied to all clusters (Lu 2019).

The modeling technique described by Lu, while complex, was validated through numerous simulations of increasing sample size, and increasing complexity. The results were then compared to previously established multi-step clustering methodologies. Laboratory temporal gene expression data was conducted and a likelihood ratio test performed to determine if the Lu modeling technique was a better fit than the previously established modeling techniques using an alpha-level of 0.01 (Lu 2019).

This methodology was then applied to the given dataset for this study, establishing the 12 clusters for both the control and experimental diet groups for the B6 mouse strain.

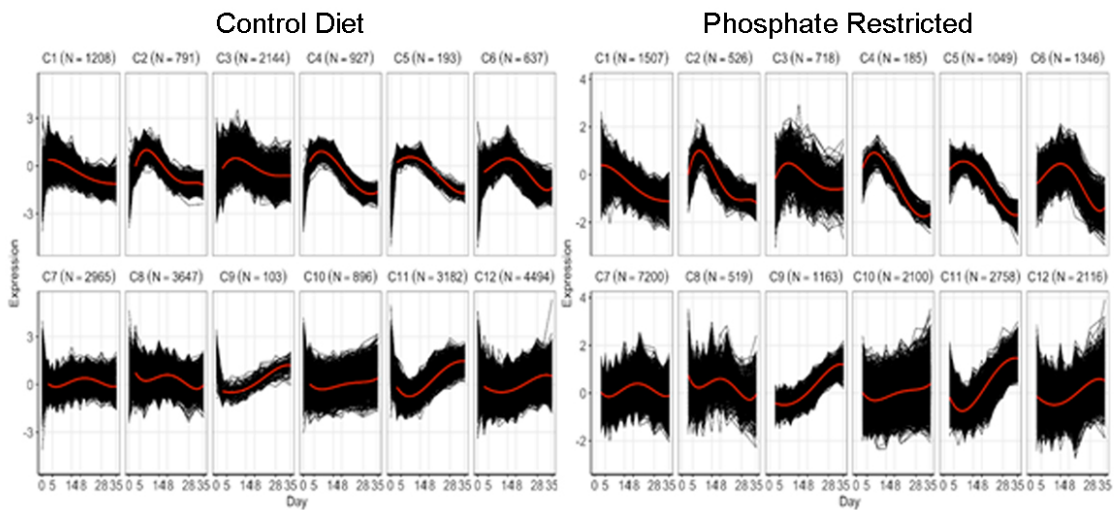
After the temporal clustering of various gene groupings based on the temporal analysis were completed, two different software tools were used to assess biological ontologies and biological pathways associated with the various gene groups. In the first, Ingenuity Pathway Analysis (IPA) was used for the analysis of the baseline clusters and groupings generated to assess significant movement based on the dietary changes. A core analysis was run on each cluster. This identified the various biological functions and canonical pathways that were associated with a given clusters and the genes associated

with these functions and pathways. For the core analysis only specific pathways and functions showing significance at ( $p < 0.05$ ) were considered.

The second method that was used was the DAVID Bioinformatics resource (<https://david.ncifcrf.gov/home.jsp>) (Hosack 2003, Huang 2007) This tool was used to identify pathways specifically associated with the development of four specific tissue types (muscle, skeletal tissues, neural tissues and vascular tissues. Only pathways with a  $p\text{-value} < 0.05$  were considered.

## RESULTS

Applying the temporal modeling to the control and phosphate restricted diet transcriptomic data across the 35-day time course produced twelve clusters. Two sets of identical clusters for both the control and phosphate restricted diet are seen in Figure 1. The mean expression of all genes in the cluster is depicted by the red line. While the clustering method sorts the total data sets from both control and phosphate restricted genes into the same twelve clusters a specific gene was assigned to a given cluster dependent on if it was from the control or phosphate restricted data set. Thus some genes for both control and diet restricted groups will be in the same cluster while others will be shifted into a different cluster.



**Figure 3.** Temporal clustering of control and phosphate restricted bone callus bone transcriptomes. Cluster numbers are denoted C1, 2, etc., while the number of genes contained in the cluster is indicated in parenthesis. Individual panels show the expression data for all genes (black) in the cluster as well as the mean (red).

By comparing the list of genes expressed in clusters for the control and phosphate restricted groups a cross tabulation table can be generated. This table can be used to determine the total number of genes that shifted from cluster to cluster based on the diet. For example, there were 648 genes that were in cluster 1 for both the control and phosphate restricted diet however 46 genes that had shifted from cluster 1 of the control diet, that were now in cluster 2 for the phosphate restricted diet and 66 in cluster 3 etc.

**Table 1.** Cross-Tabulation Table. This table is the cross tabulation of all the genes analyzed and shows how genes changed based on diet.

	Phosphate Res					Diet							
		1	2	3	4	5	6	7	8	9	10	11	12
control	1	648	46	66	10	94	124	81	22	0	91	0	26
Diet	2	160	258	52	44	150	99	21	5	0	1	0	1
	3	359	127	302	7	76	324	615	79	1	131	1	122
	4	40	78	8	112	574	113	2	0	0	0	0	0
	5	26	5	3	4	91	58	4	2	0	0	0	0
	6	18	3	8	6	56	437	82	14	0	10	0	3
	7	19	1	64	1	0	52	2207	117	24	229	41	210
	8	110	3	98	0	7	104	2544	157	4	346	34	240
	9	0	0	1	0	0	0	13	0	12	20	40	17
	10	24	1	21	1	0	13	193	21	24	363	108	127
	11	0	0	1	0	0	0	78	6	770	130	1947	250
	12	103	4	94	0	1	22	1360	96	328	779	587	1120

The graphical appearance of each of the twelve clusters was next used to identify cluster groups within specific temporal expression periods which show correspondence to the major biological process associated with fracture healing (Table 2).

**Table 2:** Clusters groups showing similar temporal expression profiles. This table demonstrates the established grouping and the appropriate clusters for those groupings.

Expression Peak	Clusters			
Overall Decreasing Expression	Cluster 1			
Early Peak Expression (Endochondral Period) Between days 5 -12	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Middle Peak Expression (Cartilage Resorption First Bone Formation) Between days 14-21	Cluster 6	Cluster 7	Cluster 8	
Late Peak Expression (Extended bone Remodeling Cortical and Marrow Bone Formation) Between days 21-35	Cluster 9	Cluster 10	Cluster 11	Cluster 12

This overview of the data shows the cluster groups within a temporal expression periods that correspond to specific periods of fracture healing over the 35 days period. This simplification then enables the significant pathways in the bone fracture repair process to be dynamically examined and identified within specific temporal period during fracture repair.

By grouping the genes in this manner, it offers the opportunity for significant gene groups in the bone fracture repair process to be dynamically examined and identified within specific temporal period during fracture repair. Using the cross tabulation table the total number of genes that shifted from cluster to cluster based on the diet within specific temporal period could be identified. For example, there were 2144 genes that were in cluster 3 for the control diet. Genes in this cluster have a peak expression of 9 days and contain the large majority of genes associated with chondrocytes and cartilage development. In contrast when placed on the phosphate

restricted diet only 302 genes show a common temporal expression with the control grouping while 1355 genes show a shifted expression to clusters with later peaks of expression while the remaining genes shifted to an early period of expression. Thus by analyzing what gene ontologies and signaling pathways these genes belong to, we can deduce how phosphate restriction has effected the callus tissues' developmental progression and identify the primary regulatory mechanisms that are mediating these temporal changes. Specific groupings of genes that are shifted from the major expression periods are colored coded in the cross-tabulation table and summarized in Table 3.

**Table 3.** Group Comparisons. This table shows the color coding for table 1. Color coding presents genes grouped together that showed similar types of temporal shifts.

Group 1: Same Cluster
Group 2: Decreasing vs. Early peak
Group 2.1: Early peak vs. Decreasing
Group 3: Decreasing vs. Middle peak
Group 3.1: Decreasing vs Middle peak
Group 4: Early peak vs Middle peak and Late (1579 genes)
Group 4.1: Middle peak vs. Early peak
Group 5 Decreasing: vs. Late peak
Group 5.1: Late Peak vs. Decreasing (91607 genes)
Group 6.1: Early peak vs Late Peak
Group 7: Middle vs Late peak (1141 genes)
Group 7.1: Late Peak vs Middle Peak (1607 gen)

These expression peaks were then compared to each other for the groups of genes that showed specific shifts in peak expression time based on diet, thus indicating particular genes that would be influenced by a change in dietary phosphate levels. The

aggregate gene groups were then placed into categories based on biological process using the DAVID ontology tool that associated with the development of four major tissue: myogenesis, skeletogenesis, neurogenesis, and vasculogenesis. It should be noted that genes that play a role in multiple categories were divided equally between the categories. For example, a gene that is expressed in both myogenesis and skeletogenesis counts as half a gene for each category. Using these four groups allows for the vast number of shifted and expressed genes to be viewed in a manner that relates their expression to a specific tissue. This will demonstrate the impact of phosphate restriction on these key tissues that contribute to callus tissue formation and bone regeneration after fracture.

### **Baseline Clusters**

Using the DAVID program to identify specific ontologies associated with the various tissues the number of genes expressed at the given peak expression time point for those tissues could be identified. Our initial focus was on biological processes of myogenesis, skeletogenesis, neurogenesis, and vasculogenesis, as the primary tissues that would have been injured by the blunt trauma of fracture and involved in tissue regeneration. Specific genes were given fractional values if they overlapped between biological processes. Myogenesis has 104.83 genes with an overall decreasing expression, 72 genes had an early peak expression time point, 13 genes with a middle peak expression time point, and 0 genes with a late peak expression time point. Skeletogenesis has 24.67 genes with an overall decreasing expression, 107.34 genes with an early peak expression time point, 43.67 genes with a middle peak expression time

point, and 0 genes with a late peak expression time point. Neurogenesis has 98.33 genes with an overall decreasing expression, 400.67 genes with an early peak expression time point, 353.17 genes with a middle peak expression time point, and 0 genes with a late peak expression time. Vasculogenesis has 41.17 genes with an overall decreasing expression, 110 genes with an early peak expression time, 42.17 genes with a middle peak expression time, and 0 genes with a late peak expression time.

### **Effect of Phosphate Restriction on Specific Tissue Development**

A comparison of how specific tissue development was impacted when placed on a phosphate restricted diet was then assessed by examining how they shifted. When placed on the phosphate restricted diet myogenesis had 27 genes shift to an overall decreasing expression, 15.58 genes shift to an later peak expression time, 4 genes shift to a middle peak expression time, and 0 genes shift to an early peak expression time. Skeletogenesis had 0 genes shift to an overall decreasing expression, 31.08 genes shift to a later peak expression time, 0 genes shift to a middle peak expression time, and 0 genes shift to an early peak expression time. Neurogenesis had 67 genes shift to an overall decreasing expression, 118.42 genes shift to a later peak expression time point, 0 genes shift to a middle peak expression time point, and 0 genes shift to a late peak expression time. Vasculogenesis had 0 genes shift to an overall decreasing expression, 27.92 genes shift to a later peak expression time point, 0 genes shift to a middle peak expression time point, and 0 genes shift to an early peak expression time point.

**Table 4.** Baseline peaks and number of genes shifted. This table demonstrates the total number of genes for a given biological process with the peak time expression in the baseline expression peak columns. The number of genes that shifted to a given expression peak consists of the number of genes that moved to the given expression based on the biological process.

Expression	Baseline				Shifted			
	Decreasing	Early	Middle	Late	Decreasing	Early	Middle	Late
<b>Myogenesis</b>	104.83 (8.68%) <sup>1</sup>	72.0 (1.78%) 1	13.0 (0.15%) 1	0.0 (0.00%) 1	27.0 (14.2%) <sup>2</sup>	0.0 (0.00%) 2	4.0 (2.1%) 2	15.58 (8.21%) 2
<b>Skeletogenesis</b>	24.67 (2.04%) <sup>1</sup>	107.34 (2.65%) 1	43.67 (0.50%) 1	0.0 (0.00%) 1	0.0 (0.00%) <sup>2</sup>	0.0 (0.00%) 2	0.0 (0.0%) 2	31.08 (17.7%) 2
<b>Neurogenesis</b>	98.33 (8.14%) <sup>1</sup>	400.67 (9.88%) 1	353.17 (4.07%) 1	0.0 (0.00%) 1	67.0 (7.86%) <sup>2</sup>	0.0 (0.00%) 2	0.0 (0.00%) ) <sup>2</sup>	118.42 (13.9%) 2
<b>Vasculogenesis</b>	41.17 (3.41%) <sup>1</sup>	110.0 (2.71%) 1	42.17 (0.49%) 1	0.0 (0.00%) 1	0.0 (0.00%) <sup>2</sup>	0.0 (0.00%) 2	0.0 (0.00%) ) <sup>2</sup>	27.92 (14.4%) 2

<sup>1</sup>indicates the number of genes as a percentage of the total number of genes with that expression peak for the biological process

<sup>2</sup>indicates the number of genes as a percentage of the total number of genes in the biological process

## **Canonical Pathways**

While the breakdown of genetic expression based on the biological process is useful, further analysis using IPA and DAVID was run on the groups and baseline clusters to understand various canonical pathways and genes that may not have been captured directly in the observed processes. The vast majority of the pathways produced by IPA and DAVID had no impact on bone repair or bone growth and were removed from the analysis.

Based on the IPA analysis of the baseline clusters there is only one canonical pathway that had an impact on fracture repair. IPA showed that cluster 8 was significant for the oxidative phosphorylation pathway.

Based on the DAVID analysis of the baseline clusters, cluster 2 was significant for cartilage and skeletal development, this is captured in the data above as cluster 2 has an early peak expression time. Clusters 5 and 6 were significant for genes involved in tissue development, again captured in the data above as cluster 5 has an early peak expression time and cluster 6 has middle peak expression time. Cluster 8 was significant for the oxidative phosphorylation pathway, the same as the IPA analysis had.

After establishing a baseline, the group that detected shift were entered into both systems. Again, numerous pathways that the systems produced were not significant to the bone fracture repair process and were removed from the analysis. Based on the IPA analysis of the groups, oxidative phosphorylation shifts in group 3, from a middle peak expression time to an overall decreasing expression. Further it shows that in group 6

circadian rhythm shifts from an early peak expression time to a later peak expression times.

Based on the DAVID analyses it is seen that WNT signaling shifts in group 2, from an early peak expression time to an overall decreasing expression. Group 4 showed a shift in tissue development from an early peak expression time to a late peak expression time. Group 6 continued to show a shift in circadian rhythm from an early peak expression time to a late peak expression time.

Finally due to its integration with almost every pathway a DAVID analyses was conducted to determine the shift of the metabolite pathway. Similar to most other pathways, when phosphate was restricted the metabolite pathway showed a shift from an early peak expression time point to a late peak expression time point.

Based on the DAVID pathway analysis the metabolite pathway was significantly involved in the control of the TCA cycle and nucleotide formation. It was evident that a large number of portions of the pathway were impacted by phosphate restriction however those of interest in the case of this analysis were the TCA cycle and nucleotide formation shift to a later peak expression time.

## DISCUSSION

Hypophosphatemia has a detrimental impact on the bone fracture repair process. Simply based on the data gathered here, shifts in processes clearly significant to the fracture repair cycle demonstrate changes in their peak expression time points.

In examining skeletogenesis a shift from earlier to a middle peak period of expression time was observed for a number of skeletal genes. While this biological process processes were observed for both chondrogenic and osteogenic cells consistent with our previous published studies (Noguchi et al 2018). However, the interconnectedness of these pathways, myogenesis, vasculogenesis, and neurogenesis, would further suggest this shift in skeletogenesis to an early peak expression time.

The decrease in mesenchymal stem cell differentiation caused by the phosphate restricted diet would trigger a response to increase the factors that can drive cellular differentiation. Hence, a shift to later expression of myogenic, vasculogenic, and neurogenic cells would be expected as all of these pathways interact to play key roles in cellular differentiation. Myogenesis promotes the vascularization of the area which promotes oxygen and nutrient flow to increase cellular differentiation and the earlier shift in neurogenic signaling continues to promote cellular differentiation. The interplay of these three biological processes would suggest that genes responsible for skeletogenesis would also shift to a later peak expression, as cellular differentiation would begin later and hence the expression of bone regeneration specific genes would also need to be expressed later.

This interconnectedness is further confirmed by the presence of numerous overlapping genes between the processes. Thus, not only suggesting that they work together to promote the fracture repair process, but also suggesting that any shifts we are seeing in one process, are most likely seen in the other processes as key genes overlap.

While it is clear that these shifts impair the fracture repair process, what is unclear is how these shifts relate to decreased size in callus formation (Wigner 2010). As mentioned, previous research points to the impaired signaling of BMP, WNT, and FGF and the detrimental impact this has on fracture repair. Direct impairment of these process would cause a decrease in the callus size. However, the shift of the other processes would be hypothesized to counteract these impaired signaling pathways. When considering the results of this research with previous evidence, it is clear that while these processes are interconnected, phosphate plays a directly role in the impairment of the signaling pathways of BMP, WNT, and FGF. Regardless of the compensation from other biological processes, impairment of these signals will limit callus growth.

The shifts observed and theories on phosphates impact on signaling in the biological process data are further supported by the canonical pathway shifts seen in the IPA and DAVID analyses. As shown by IPA oxidative phosphorylation begins in cluster 8, which demonstrates a middle peak expression time. It is then seen to shift as part of group 3, which indicates a shift from a middle peak expression time to an overall decreasing expression. Previous research suggests that when stem cells are differentiating there is a shift from glycolysis to the oxidative phosphorylation pathway, indicating that the oxidative phosphorylation pathway and mitochondrial dysfunction can impair

mesenchymal stem cell differentiation. The shift demonstrated in this data confirms the important of oxidative phosphorylation for stem cell differentiation, and the overall decreasing nature of cluster 1 in the phosphate restrict diet suggests that genes involved in oxidative phosphorylation are not being expressed at nearly the same level as they were previously in cluster 8 at baseline. Hence, a shift to an early peak expression with an overall decrease in total expression would conclude that the phosphate restriction in impairing oxidative phosphorylation, limiting mesenchymal stem cell differentiation, decreasing the possible callus size.

These concepts are again confirmed by the DAVID analyses. DAVID showed the same WNT shift in group 2, from an early peak expression time to an overall decreasing expression, a shift in tissue development in group 4, from middle peak expression to an early peak expression time, and a shift in circadian rhythm in group 6, from a late peak expression to an early peak expression time.

This shift seen in WNT signaling is confirmed by the shift seen in the biological process data, where neurogenesis has genes shift to overall decreasing expression time points. As mentioned WNT signaling promotes mesenchymal stem cell differentiation, which is impaired by phosphate restriction, and therefore would see a shift to an overall decreasing expression time point. Again, when considering the given cluster data for cluster 1 in the phosphate restricted diet (figure 3) it can be seen that the overall expression levels of the data in this cluster is lower than the expression levels for all other clusters, indicating that WNT signaling is impaired when phosphate is restricted. This is further confirmed by the DAVID pathway analysis of WNT signaling (figure 4).

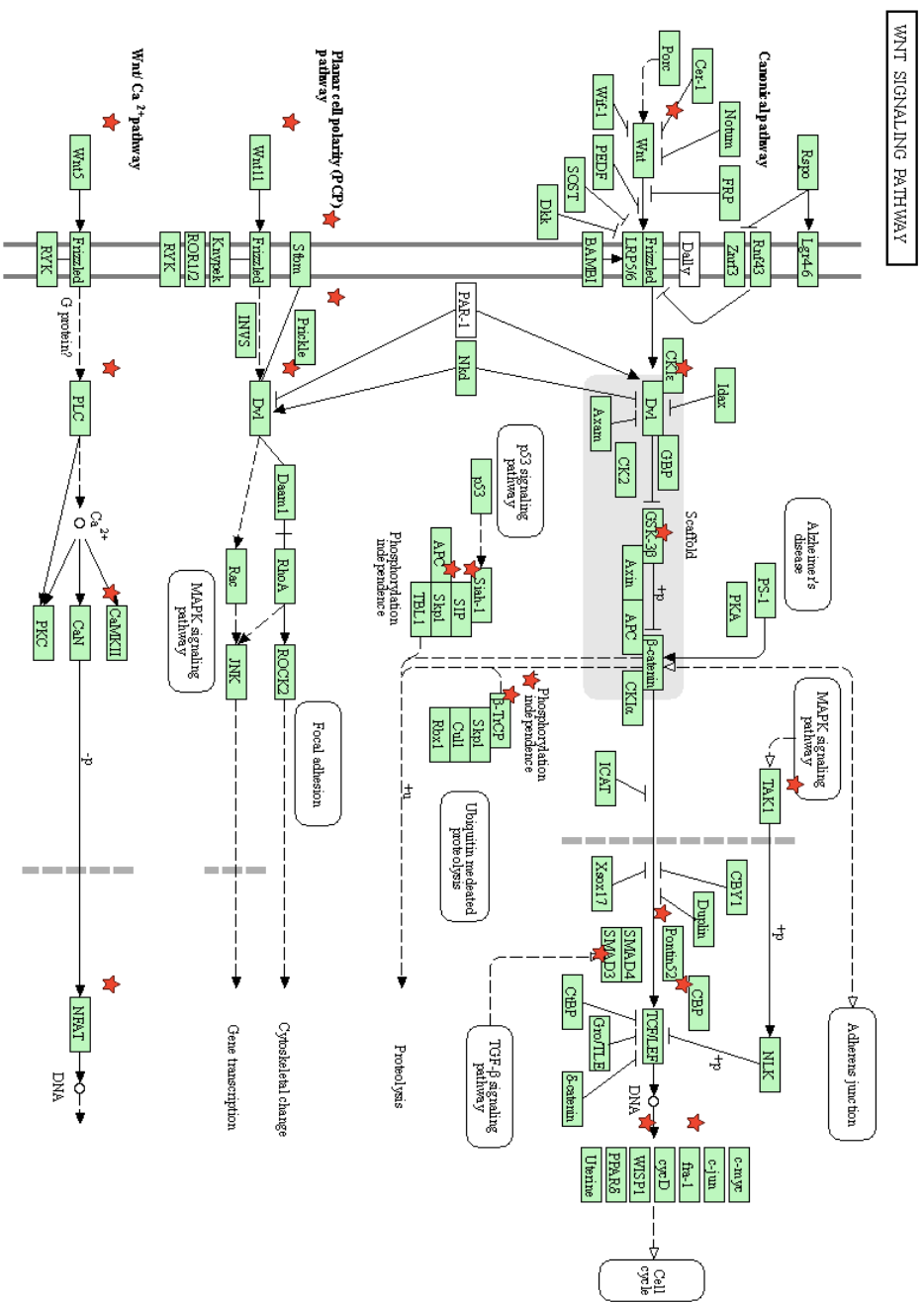


Figure 4. WNT Signaling Pathway. This diagram gathered from the DAVID Bioinformatics Database demonstrates the entire WNT signaling pathway. Those genes marked with red stars indicate genes impacted by phosphate restriction.

While not every gene in the WNT signaling pathway is directly impacted, it is clear that phosphate restriction significantly impacts the pathway. Decreased overall expression of the WNT signaling pathway would impair fracture repair.

Further it is seen that this phosphate restriction impacts other key pathways such as that TGF Beta signaling (figure 5) and HIPPO signaling (figure 6).

TGF Beta signaling is key to osteoblast differentiation and bone formation (Chen 2012). Previous research has already shown that impairment of this pathway impacts bone fracture repair (Wigner 2010). Further, there is close interaction between the pathways of WNT signaling as well as TGF Beta/BMP signaling (Wigner 2010). Thus impairment of one pathway would suggest impairment of the other pathway. When considering figure 5, it is clear that phosphate restriction impairs TGF Beta/BMP signaling, offering further explanation for the impaired fracture repair mechanisms, that coincide with the knowledge that phosphate restriction delays the fracture repair process (Wigner 2010).

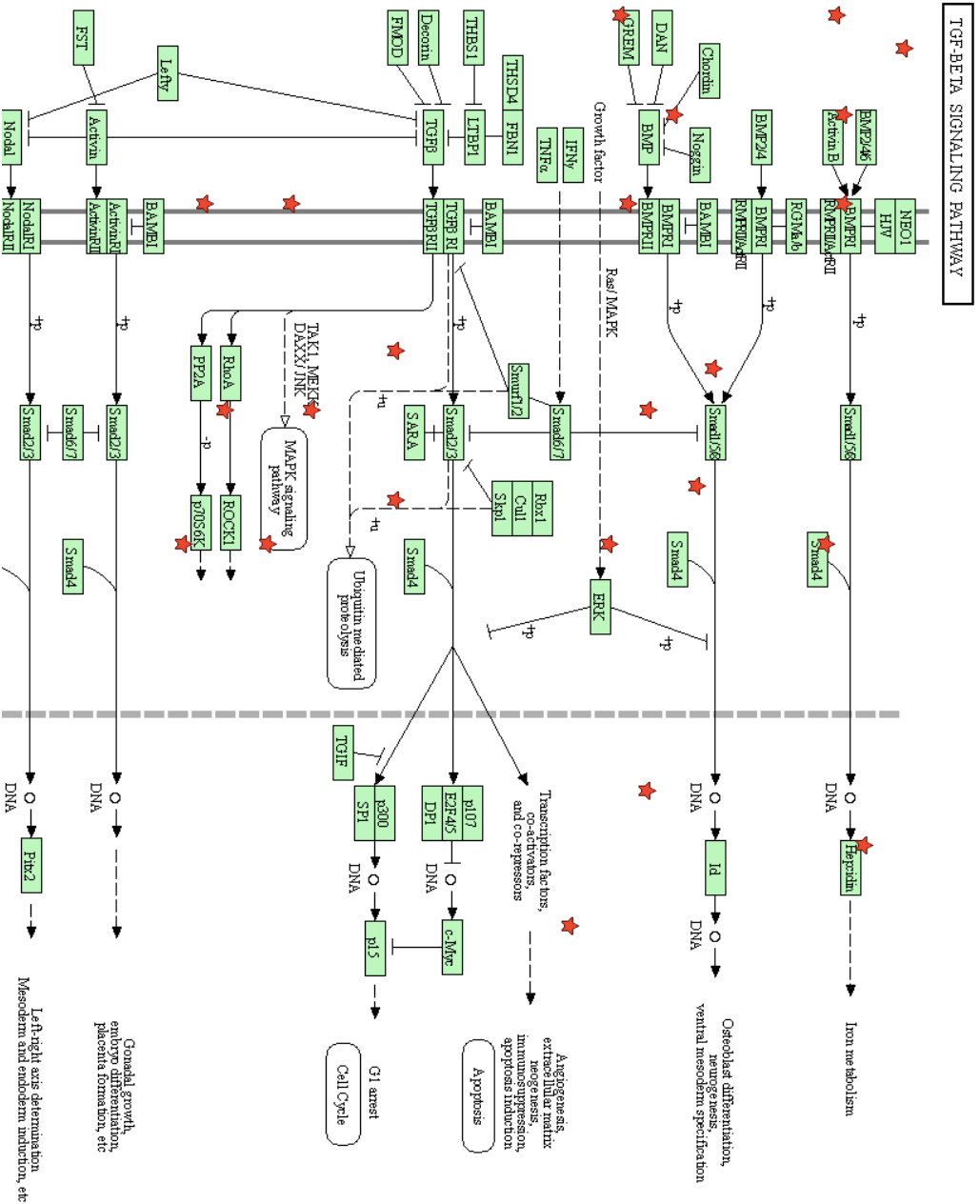


Figure 5: TGF-Beta Signaling Pathway. This diagram demonstrates the pathway for TGF-Beta Signaling. All genes indicated with a red star are impacted by phosphate restriction.

Finally, the HIPPO signaling pathway plays a key role in osteoblastic bone formation and osteoclastic bone resorption, as well as the mechanisms of bone metabolism and disease (Yang 2018). Again it would be assumed that impairment of this process would impact bone formation and fracture repair. This is confirmed when considering figure 6 as entire portions of the pathway are shifted based on changes in dietary phosphate.

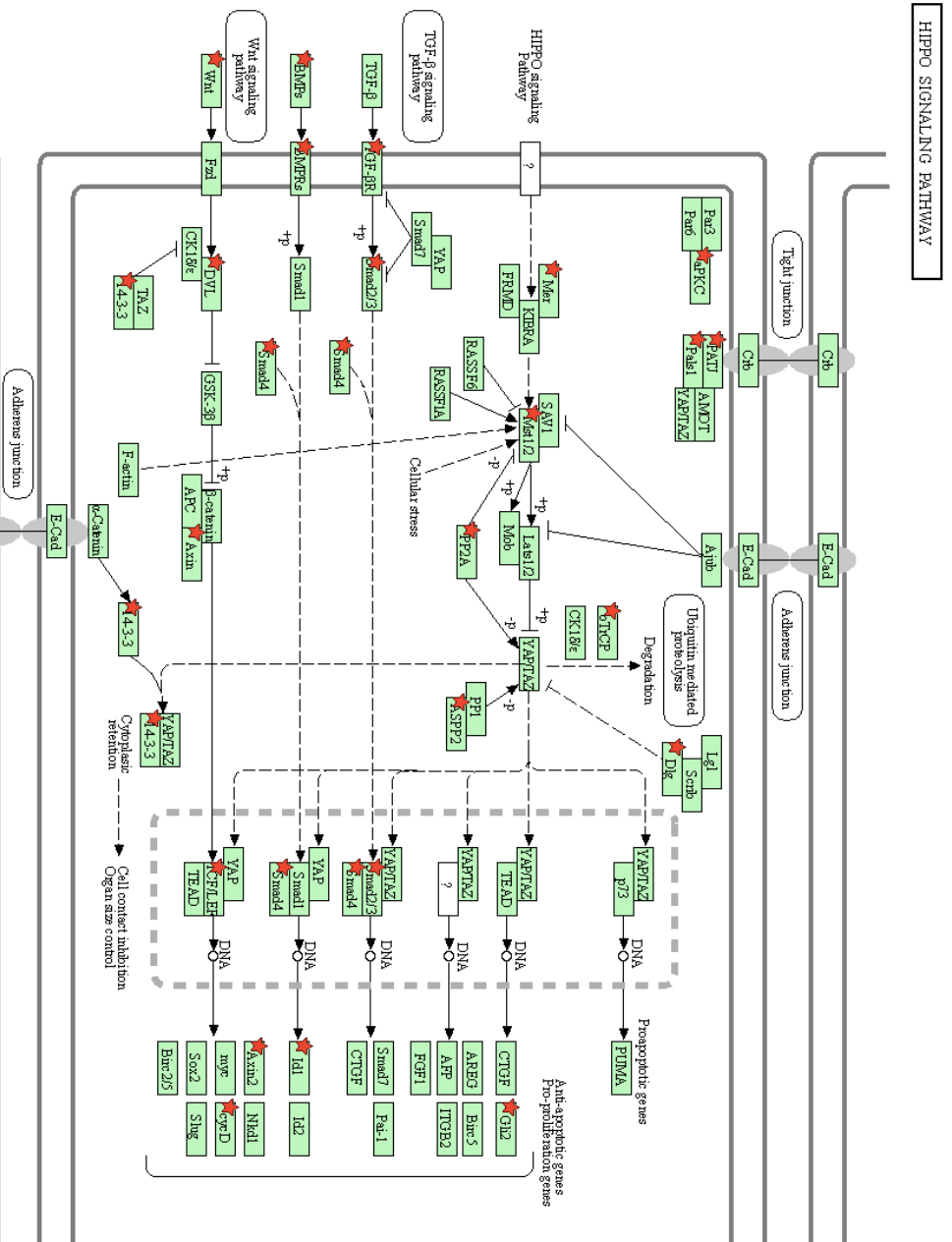


Figure 6: Hippo Signaling Pathway. This figure demonstrates the genes in the Hippo signaling pathway. All genes indicated with a red star are impacted by changes in dietary phosphate.

The shift in tissue development seen in group 4 from a middle peak expression to a later peak expression time is less specific but does coincide with the biological process shift data. Tissue development could encompass myogenesis, skeletogenesis, or vasculogenesis, all of which showed a shift from middle peak expression to an early peak expression time, thus confirming the ideas in the shift data.

Finally, the shift seen in circadian rhythm connects with the shift seen in oxidative phosphorylation. Circadian rhythm has been shown to have significantly altered expression in the fracture callus of mice and is linked to oxidative metabolism and skeletal growth in animals (Noguchi 2018). Based on the previous research it is seen that circadian rhythm takes its expression signaling from the expression levels of oxidative phosphorylation (Noguchi 2018). This would suggest that there should be a slight difference in the peak expression times between oxidative phosphorylation and circadian rhythm. This is confirmed by the fact that oxidative phosphorylation shifts to an overall decreasing expression and circadian rhythm shifts to an early peak expression time point. The impacts on circadian rhythm are further confirmed by the DAVID pathway analysis of circadian entrainment seen in figure 7.



The data gathered in this research confirms previously established research and offers direct changes in time point of peak expression and genetic expression levels based on phosphate restriction. These shifts in time point and overall expression levels offer opportunities for therapeutic targets during the fracture repair process. Later research can further differentiate the biological process groups and offer more insight in shifts of particular genes during the fracture repair process. In combination with further differentiation of the biological processes' groupings, comparison to varying mouse strains can confirm these shifts. By comparing to various mouse strains using both the biological process categories and further differentiated categories it can clarify which particular genes are being impacted by phosphate restriction thus impairing the fracture repair process.

First consider skeletogenesis. It is evident from previous research that under ricketic conditions there is significant retardation in the bone fracture repair process (Noguchi 2018). This is supported through the data gathered and analyzed in this trial. As demonstrated, phosphate is key to numerous pathways that have significant impact on signaling for cellular differentiation and the bone formation process. WNT, HIPPO, and Circadian rhythm have all been shown to have major implications during osteogenesis and have shown that during phosphate restriction are drastically impacted. This would offer explanation as to the shift to later expression of the genes responsible for skeletogenesis.

As discussed, WNT signaling promotes mesenchymal stem cell differentiation. If the expression of genes responsible for WNT signaling is decreased this would offer

minimal differentiation. This lack of stem cell differentiation would delay the beginning of chondrogenesis and osteogenesis, the main pathways of skeletogenesis, leading to the retardation in bone formation implicated by phosphate restriction.

This concept is supported again by the shift seen in the HIPPO pathway. One of the key steps in bone formation is interaction between the osteoblasts and osteoclasts, which generate and resorb bone respectively. This homeostatic process is one of the final steps in bone formation and controlled directly by the HIPPO pathway. As seen in the figure, phosphate drastically shifts the HIPPO signaling pathway, which would in turn justify the shift seen in the skeletogenesis pathway.

The shift noted in skeletogenesis is support by the shift seen in the circadian rhythm pathway. Previous research has shown that circadian rhythm is directly linked to oxidative phosphorylation and skeletal growth (Noguchi 2018), therefore a shift in circadian rhythm could be matched by a shift in oxidative phosphorylation. Thus with a decrease in the amount of available energy, the bone formation process and eventual callus size would be heavily impacted.

Next consider myogenesis and vasculogenesis, previous research has shown that myogenic gene expression in the area of the fracture is key for vascular penetration later in the cycle (Davis 2015), and that vasculogenesis is a highly regulated portion of the bone fracture repair cycle (Hankenson 2012). Simply between these two pathways, it would be expected that a shift in myogenesis would be paired with a shift in vasculogenesis, which is confirmed through the data of this analysis.

On a cellular level, vasculogenesis is controlled by growth factors and stromal cells (Hankenson 2012). The shift to a later expression by vasculogenesis is further confirmed when considering the shifts seen in TGF Beta signaling, a key growth factor signaling pathway. Again, the shift in vasculogenesis to a later time point, which would be responsible for bringing the chemical signaling necessary for bone formation and calcification to the fracture area, would then cause a shift in skeletogenesis to a later time point.

Lastly when considering neurogenesis it is again noted that the majority of the genes expressed in the pathway shift to a later peak expression time. BMP signaling is directly related to the differentiation of stem cells into osteopathic derivatives (Wigner 2010). WNT signaling acts, similarly, promoting the osteogenic differentiation of mesenchymal stem cells (Houschyar 2019). FGF plays a key role in the development and regeneration of various tissues, particularly bone regeneration (Charoenlarp 2017). These previous studies clearly demonstrate the impact of neurogenic pathways on cellular differentiation, a key component of the bone fracture repair process. Which confirms that a shift of neurogenesis to a later time point, would further confirm the shift seen in skeletogenesis.

When considering the pathways and shifts seen in the biological processes, it is evident that bone fracture repair is a heavily complex process that intertwines with numerous pathways and processes. Shifts in cellular differentiation, chemical signaling, and thus available material to conduct fracture, confirm the retardation noted in the bone fracture repair process.

Finally while there are potential explanations for almost every shift seen throughout this analysis, the most practical may be the shift seen in the metabolite pathway. The metabolite pathway is crucial in the formation of nucleotide and the TCA cycle. Without the building blocks of genetic material and the energy to conduct pathways it is comprehensible why bone fracture repair and bone formation may be hindered if the metabolite pathway is hindered for any reason.

In this case it is noted that the metabolite pathway shifts from an early peak expression time to a later peak expression time. The main window for chondrogenesis and osteogenesis occurs in the first fourteen days as mentioned previously. A lack of the crucial building blocks and energy needed for the cell during this time would impair the fracture repair process.

However, while it would impair the process, it further offers an explanation for why there is a decrease in the size of the callus formed while on a phosphate restricted diet. Again it all hinges on the lack of available nucleotides and energy in the cell to promote the necessary cell differentiation and tissue formation during the process.

The interconnection with the metabolite pathway and numerous other cellular pathways offers an interesting and simplistic explanation for the impairment of fracture repair and decreased callus size formation. Without changing any other pathways, by simply impacting the pathway that helps build the most basic units for protein formation and genetic expression you would significantly decrease the opportunity to quickly and effectively repair fractures or form bone.

In total, the bioinformatic analysis with this genetic expression data confirms previous research, showing that when phosphate is restricted, bone fracture repair and bone formation shows significant retardation.

### **Further Research**

While the data analyzed here confirms previous research on the impacts of phosphate on bone fracture repair and bone formation, it may not be capturing the full picture. In the case of this analysis, the methodology for establishing the groupings and running the comparisons may in fact be limiting the amount of gene shifts that are being captured and further being statistically significant.

In order to capture the full understanding picture of the genetic shifts based on phosphate restriction, the shift grouping analysis need to capture the largest number of shifted genes possible. Thus future research should potentially follow a new methodology.

Based on the modeling of Lu et. Al 2019, clusters are created based on genes that follow similar expression patterns. In this analysis those genes were then grouped based on general peak expression time, early, middle, and late peak genetic expression time. These peak groupings were used to establish baselines, and then were compared to the same groupings to assess shift. However, while this offered a simple analysis, it didn't necessarily capture all shifting genes and pathways.

As mentioned the bone formation and fracture repair process has peak chondrogenesis and osteogenesis for the first 14 days. When considering the clusters that

were placed into the early genetic peak expression time group, cluster 2, 3, 4, and 5, it is noted that they have varying peak expression days. Cluster 2 has a peak expression at day 7, cluster 3 at day 9, cluster 4 at day 10, and cluster 5 at day 11. While the max difference between these clusters of 4 days doesn't seem like a lot, when considering that the majority of chondrogenesis and osteogenesis occurs during the first 14 days, a shift of even a half a day would have a drastic impact bone fracture and bone formation.

Further analysis should seek to make the grouping more time specific. Instead of creating a large group of simply early peak expression, the groupings should be based on a specific peak expression time point. In this case seeing as each cluster has its own peak expression time point, they should each be considered as their own baseline group. Shifting from there should be considered any later time point.

By having the shift group be any later time point it allows for the maximum amount of shift to be detected and maximizes the number of genes of bioinformatic analyses. Furthermore, it allows for the entire necessary pathway and genetic expression profile to be captured in the shift group.

When again considering the early peak expression time group, it is seen that this spans clusters 2, 3, 4, and 5 and therefore days 7, 9, 10, and 11 respectively. It is understood that the switch between chondrogenesis and osteogenesis occurs between days 9 and 10. Therefore, based on the groupings established in this analysis the osteogenesis pathway is broken between the early peak expression time group and the middle peak expression time group. While this may be captured in the baseline or shift data, by breaking the pathway and genetic expression over two groups it decreases the

number of genes available for analysis. This decreased number of genes may allow for the shift to be detected in the analysis, but it may not be registered as significant, and would therefore be disregarded.

For example, to allow a more robust analysis, baseline genetic expression and pathways should be established using each individual cluster, in the case of this example consider cluster 2. From there shift analysis should be run by considering if any of the genes from cluster 2 shifted to anything later. This creates the largest number of genes available for analysis and will capture any significant shift. This process should be continued for each cluster, continuing to exam only shifts for later expression.

Once all clusters shifting to a later time point is assessed, the same process should be considered for shifts to an earlier time point. While this in contrary to the understanding of how phosphate impacts the bone formation process, it may suggest disease pathways that are shifting to early expression as the repair process slows and shifts later.

Ultimately after analyzing the data in this manner, the results should be verified against other genetic variations of mice. Comparison to other strains of mice offers two new points to consider. First, increase in genetic variation offers and increase in the number of clusters developed from the Lu et al. 2019 methodology. This is overcome by grouping the clusters based on matching peak expression time points. For example, anything that peaks on day 2 should be considered together. Second, it offers the opportunity for other genes to be shifted. If this is the case, it can further the understanding of how phosphate is impacting the fracture repair process.

By utilizing the Lu methodology with groupings that maximize the number of genes available for shift analysis, it offers the opportunity to get a complete picture of how the dietary phosphate is impacting genetic expression. Continuing analysis to assess both shifts to later expression and earlier expression not only offer insight into the fracture repair process but can suggest possible disease pathways that may arise from reduced phosphate intake following fracture.

## LIST OF JOURNAL ABBREVIATIONS

Ann Pharmacother	Annals of Pharmacotherapy
Dev Dyn	Developmental Dynamics
Front Cell Dev Biol	Frontiers in Cell and Developmental Biology
Inflamm Regen	Inflammation and Regeneration
Int J Biochem Cell Biol	International Journal of Biochemistry & Cell Biology
Int J Biol Sci	International Journal of Biological Sciences
J Bone Miner Res	Journal of Bone and Mineral Research
J Clin Invest	Journal of Clinical Investigation
J Foot Ankle Surg	Journal of Foot and Ankle Surgery
J Musculoskelet Neuronal Interact	Journal of Musculoskeletal & Neuronal Interactions
J Orthop Trauma	Journal of Orthopaedic Trauma
Nucleic Acids Res	Nucleic Acids Research
Proc Natl Acad Sci U S A	Proceedings of the National Academy of Sciences of the United States of America

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## CURRICULUM VITAE

