PRIMER

Investigating the role of nerves in regeneration in a primer for "Hyperinnervation improves Xenopus laevis limb regeneration"

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ABSTRACT

Xenopus laevis froglets have the ability to regenerate their limbs but not to a full extent. A regenerated limb consists of a limb "spike" with no digits. Along with this, many developmental genes are not expressed as much during regeneration as they are in development. The goal of this primer is to review a study done by Mitowaga et al. that examined whether increasing nerve count improves limb regeneration in *Xenopus laevis* froglets. Hyperinnervated limbs resulted in more complex bone structures called multiple cartilaginous and branched. Analysis of genes involved in limb development (*hoxa13, lmx1b,* and *shh)* indicated expression was upregulated post hyperinnervation. These results indicate that increasing nerve counts in limbs improves overall limb regeneration. Along with this, amplified gene expression in the regenerating blastemas occurred.

Keywords: *Xenopus laevis,* hyperinnervation, blastema, regeneration, gene expression

INTRODUCTION

Loss of human limbs has become a common occurrence due to accidents, infections, cancer, and many other diseases. In 2005 an estimated 1.6 million people were living with at least one missing limb, and the number is projected to grow to 3.6 million in 2050 (Ziegler-Graham et al., 2008). Missing limbs have an enormous impact on people's lives. An individual missing a limb can struggle to complete everyday tasks. Prosthetic advancement makes these tasks slightly easier but, in the end, the prosthetic cannot replace all of the functions of the missing limb.

Regeneration: The process of regrowing tissue that has been lost

Referred to as the African Clawed Frog, *Xenopus laevis* have been used to study the nervous system as well as general developmental patterns (Borodinsky, 2017; Lee-Liu et al., 2017). In recent years, scientists have come to utilize this frog to study **regeneration**. Like many amphibians, *Xenopus laevis* have the ability to regenerate limbs. Current research looks to use the frog as a model for regeneration in hopes that one day, scientists may be able to help humans regenerate lost limbs.

Previous research has shown that although *Xenopus laevis* have the ability to regenerate their limbs, the quality of the regeneration seems to decrease as they go from a tadpole to an adult frog (Dent, 1962; Mitogawa et al., 2018). Tadpoles can completely regenerate their limbs. In contrast, the regenerated limb of an adult *Xenopus laevis* is typically missing fingers and joints. When an adult frog regenerates, the limb can be seen coming to a point (Figure 1) (Suzuki et al., 2006). Scientists are now trying to understand this regeneration process in hopes to improve the quality of regenerated limbs.

There are a number of different stages in the limb regeneration process. The wound healing stage is the first stage that occurs immediately after amputation. Here, the apical epidermal cap (AEC) is formed over the wound site. The AEC is responsible for secreting growth factors which are known to have a role in limb development (Zielins et al., 2016).

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Blastema: A mass of undifferentiated cells that have the potential to differentiate

Next a **blastema** forms underneath the AEC. A blastema contains undifferentiated cells that then differentiate as the regeneration process occurs. For example, the undifferentiated cells can differentiate into muscle, connective tissue, bone and nerves until regeneration is complete (Zielins et al., 2016).

In most vertebrate blastemas, the genes *shh, lmx1b, and hoxa13* are expressed but in *Xenopus laevis* these genes are not expressed to that same extent (Matsuda et al., 2001; Mitogawa et al., 2018; Ohgo et al., 2010; Yakushiji et al., 2007). Sonic Hedgehog (*shh*) is extremely important for development as it contributes to processes such as angiogenesis, the development of blood vessels (Zavala et al., 2017) as well as the development of the anterior-posterior axis (Tickle and Towers, 2017). LIM homeobox transcription factor 1-beta (*lmx1b*) is a gene that has the vital role of determining the dorsal-ventral arrangement (Cross et al., 2014). *Hoxa13* is a gene responsible for the expression of *Fgf8* and *Bmp7* which target and activate other genes responsible for development. (Monsivais et al., 2017; Zhao and Potter, 2001). All these genes are crucial components for full regeneration. Therefore, since these genes are not being expressed fully in adult *Xenopus laevis*, it could explain why the limb does not fully develop after amputation.

Hyperinnervation: Artificially adding nerves into tissue

This primer looks at how **hyperinnervation**, the surgical increase of nerves in tissue, can enhance the quality of limb regeneration. Hyperinnervation generates improvement in regenerated limb quality. Also, increasing nerve count upregulates the gene expression of *shh, hoxa13, and lmx1b.* Therefore, it is possible that the upregulation of these genes could have caused the improved regeneration quality. The results of this experiment not only show how nerves play a role in regeneration, but also how specific genes affect regeneration. Understanding limb developmental gene expression could set up future experiments in understanding the role of those same genes in humans.

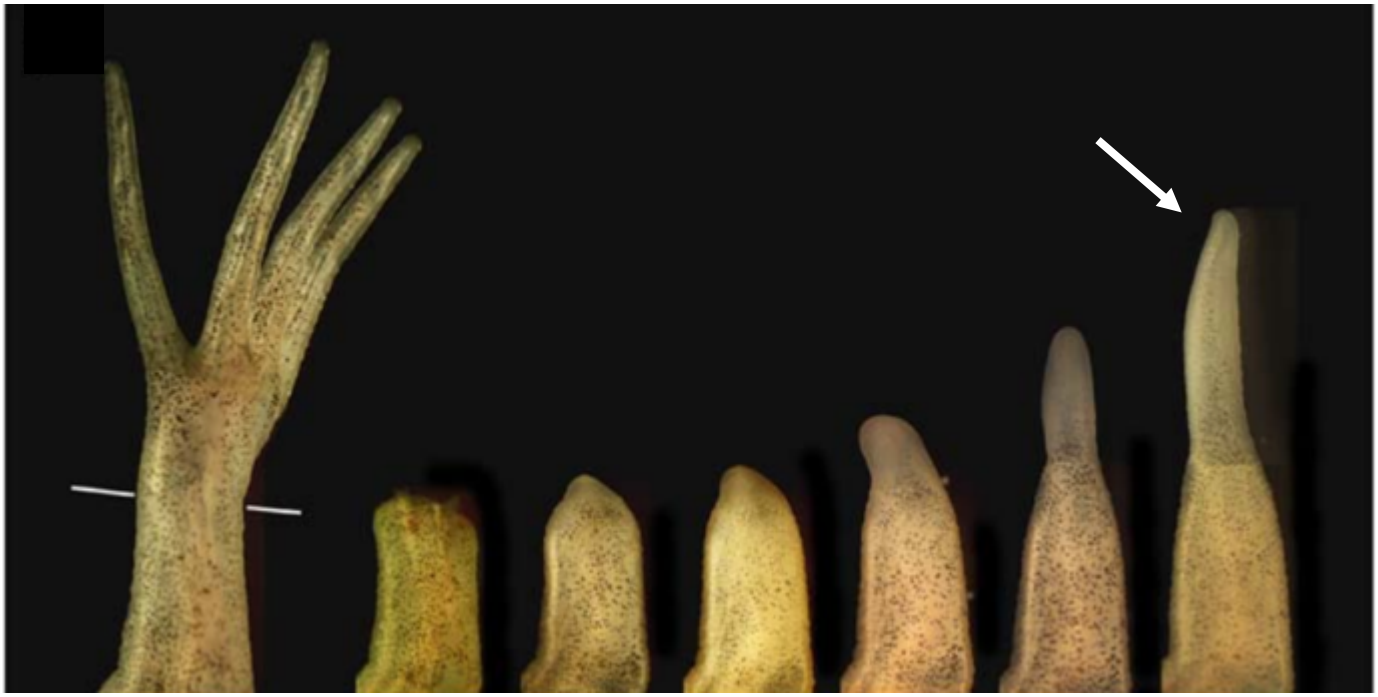


Figure 1: Progression of a regenerating adult *Xenopus laevis* limb. White line indicates amputation site. White arrow points to the extent and which the frog can regenerate naturally. Adapted from Suzuki et al. 2006 under a Creative Commons Atrribution License.

METHODS

These methods are paraphrased from Mitogawa et al., 2018.

Xenopus laevis and surgery

Xenopus laevis were acquired from a private breeder. The froglets had an average body length of 2-3cm and were kept in water at 22C. Before all surgeries were performed, the frogs were placed under anesthesia using MS222. Hyperinnervation surgery was executed by taking nerve bundles from a hind limb and placing them in a forelimb. Amputation surgery was completed 2 weeks after hyperinnervation surgery. Scissors and forceps were used to amputate a hyperinnerated forelimb as the experimental cut and a non-hyperinnervated hindlimb as a control cut.

Dyes for histology

Dehydrated tissue for analysis was stained with Alcian blue (used to dye bones) for 3 minutes. The tissue was then rinsed and exposed to a hematoxylin stain order to stain the nuclei of cells. The tissue was then rinsed and stained with eosin to dye the cytoplasm of the cells. Finally, the tissue was rinsed with 70% ethanol. Parts were then dehydrated and mounted with Softmount. The dye Alizarian red was also used to stain cartilage but the procedure was not specified in the methods.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and statistical analysis

In qRT-PCR, a reverse transcription is used to make complimentary DNA (cDNA) from mRNA. Once reverse transcription occurs, the PCR can move forward as normal. The RNA was prepared by using RNeasy Mini kit with DNase-treatment. The DNase treatment removes any DNA contamination in the RNA sample. The reverse transcription was executed with PrimeScript II, following the protocol set by the manufacturer. The PCR portion was completed by using KAPA SYBR FAST qPCR Master Mix kit. This kit contains a SYBR green I dye that stains nucleic acids. Statistical analysis was then carried out by using ANOVA and ABI StepOne software.

In Situ Hybridization

In situ hybridization was used to visualize gene expression.

RESULTS

This primer primarily focuses on the experiments regarding limb structure development and expression of limb development genes *hoxa13, lmx1b,* and *shh.* The original paper discussed that expression of *bmp, fgf,* and *shh* development genes played a role in nerve regulation. This topic is not discussed in depth because it focuses on nerves whereas the main focus on this primer is limbs.

Hyperinnervation created a more complex limb structure

The main goal of this study was to determine if hyperinnervation causes any changes in the limb regeneration process. Hyperinnervation surgery was performed on *Xenopus laevis* adult frog limbs in order to increase the nerve count. Hyperinnervation surgery entailed transferring nerve bundles from one limb to another limb on the same frog. Limb amputation was then followed on both hyperinnervated and non-hyperinnervated

limbs. The non-hyperinnervated limb was used as a control. The regenerated limbs were then examined through light microscopy 12 weeks after amputation surgery in order to assay the outer physical properties of the limb. Bone and cartilage formation were also assessed using Alcian blue and Alizarin dyes respectively.

The non-hyperinnervated control limb regenerated to form a spike shape with a simple bone structure and no digits forming (Fig. 2A, D). Hyperinnervated limbs had three different types of structure. One was a spike shape with also a single spike of cartilage (not shown). The second was a multiple cartilaginous limb. In this phenotype, the limb still came to a spike with no digits, but the bones had a more complex structure than the control limb (Fig. 2B and E). The third structure that arose was a branched limb. In this case, the regenerated limb showed two bones growing in opposite directions: one towards the posterior axis and the other towards the anterior axis (Fig. 2C and F). The branching structure is an abnormal outcome due to the fact that it is significantly different than the other two spiked limbs. These branching bones could have been the start of digit growth. This study was successful in determining that hyperinnervation causes change in limb regeneration in *Xenopus laevis* froglets.

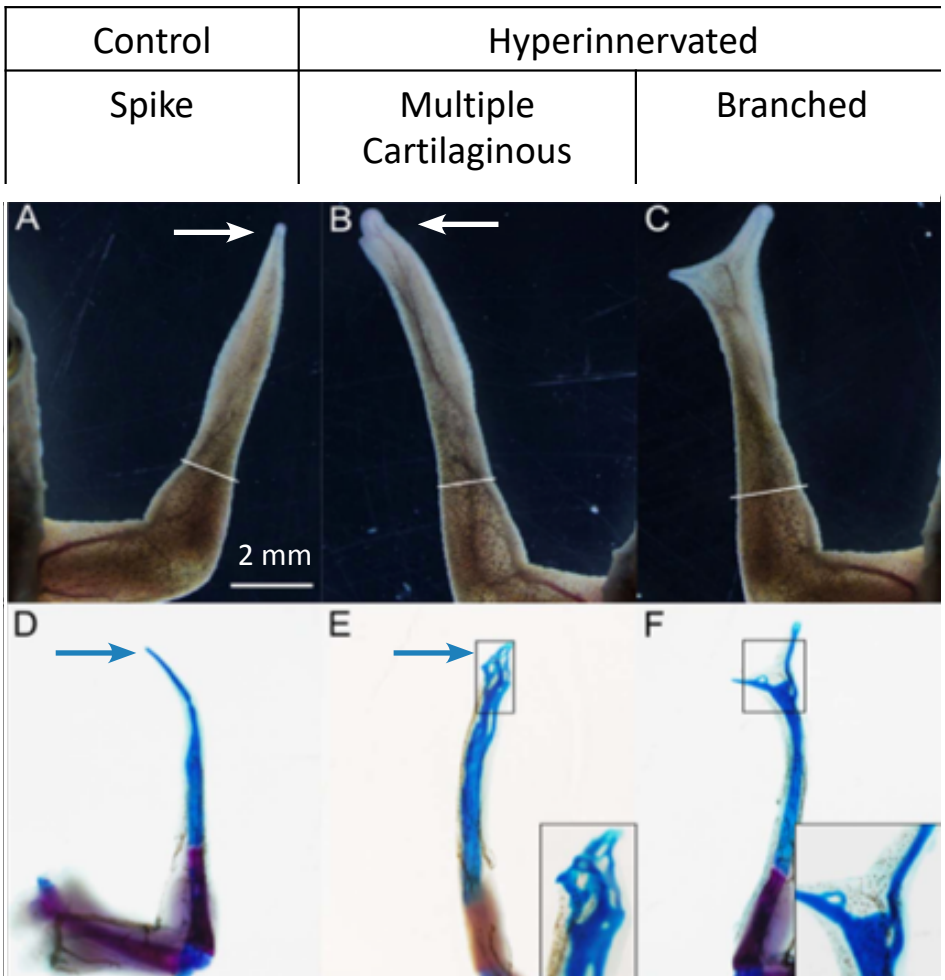


Figure 2: Hyperinnervated limbs have a more complex bone structure at the completion of regeneration. Limb regeneration 12 weeks after amputation. White lines indicate amputation sight. The total number of frogs that had surgery was not stated **(A)** A "spike" is the result of a normal regeneration (white arrow in control). **(B)** One type of abnormal limb generated with hyperinnervation has a spike that is thicker than spike control (compare are indicated by white arrows in A and B). **(C)** Second type of abnormal limb structure generated by hyperinnervation has two branching "fingers." **(D)** Distal bone of control limb has a spike (blue arrow). **(E)** Spike hyperinnervated is found to have a more complex bone structure than that of the control (compare blue arrows in D and E). **(F)** Branched limb also contains branching bones. **(A-C)** Light microscopy used to view limbs. **(D-F)** Alcian blue and Alizarin red dyes used to view bone and cartilage structures, respectively. Reprinted from Mitogawa et al., 2018 with permission from Elsevier.

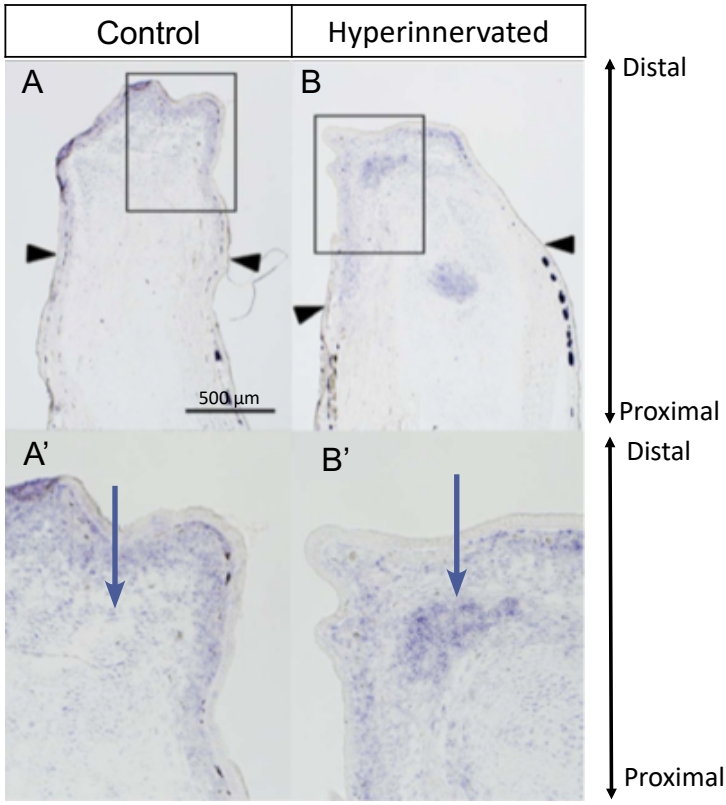


Figure 3: *hoxa13* gene expression was upregulated in hyperinnerated blastemas compared to control blastemas. Two weeks after limb amputation. *Hoxa13* gene expression levels are represented by purple stain. **(A)** *hoxa13 e*xpression level in control blastema. **(A')** Magnified image of the most distal portion of control blastema. **(B)** *hoxa13* expression level in hyperinnerated blastema is indicated by increased levels of purple stain. **(B')** Magnified image of most distal portion of hyperinnervated blastema. Note the difference between gene expression of *hoxa13* B' and A' (purple arrows). Black arrow heads in A and B indicate amputation site. **(C)** Gene expression levels of *hoxa13* in the proximal and distal regions of the limb blastema. Bars indicate standard deviation. Reprinted from Mitogawa et al., 2018 with permission from Elsevier.

Hoax13 and lmx1b and shh were upregulated in hyperinnervated blastemas

The main goal of the second experiment was to determine if expressions of genes involved in limb development (*hoxa13, lmx1b,* and *shh)* were upregulated in the hyperinnerated blastemas of the *Xenopus laevis* limbs. Analyzing these limb development genes may provide an explanation for why more complex bone structures occurred in hyperinnervated limbs. The limb development gene expressions were analyzed using in situ hybridization and qRT-PCR.

Acting as a transcription factor, hoxa13 can regulate many homeobox genes responsible for limb development (Genetics Home Reference). Expression of *hoxa13* was found all throughout the blastema in both regenerated hyperinnervated and control limbs (Fig. 3). What was different, though, was that the *hoxa13* expression tended to be more prominent in the distal regions of the hyperinnervatad blastema than the proximal regions (Fig. 3B and B'). qRT-PCR also confirmed this finding (Fig. 3E). The control blastema had more *hoxa13* expression in the distal region, but there was significantly more of a difference between expression in the distal and proximal regions in the hyperinnervated blastema (Fig. 3E). qRT-PCR also confirmed the significant expression difference between the limb regions (Fig. 3E).

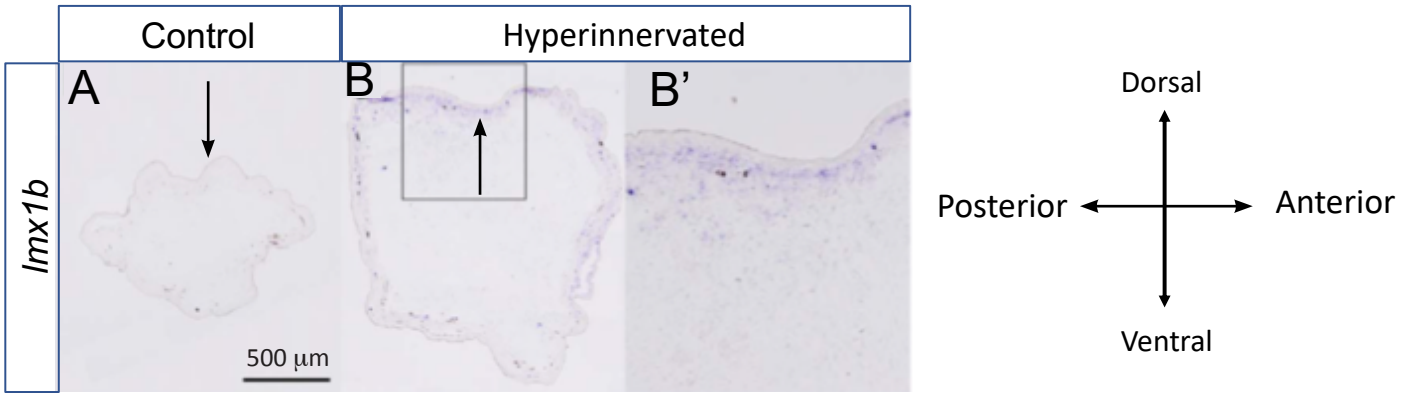


Figure 4: *lmx1b* expression increased in the hyperinnervated blastema compared to the control blastema. Two weeks after amputation. **(A)** Normal *lmx1b* expression in control blastema. **(B)** Upregulated *lmx1b* expression in a hyperinnervated blastema. **(B')** Magnified image of distal portion of B. Note the difference between expression between A and B (black arrows). Reprinted from Mitogawa et al., 2018 with permission from Elsevier.

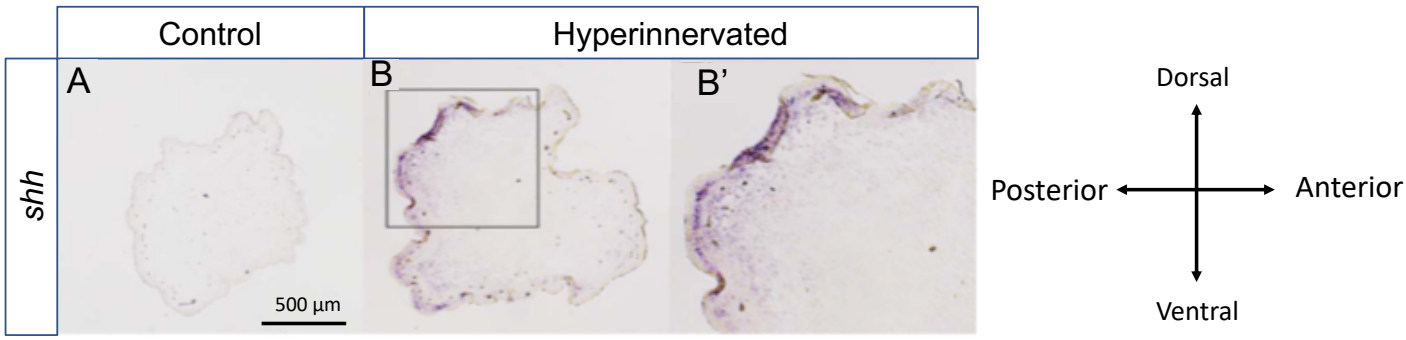


Figure 5: *shh* expression increased in the hyperinnervated blastema compared to the control blastema. (A) *shh* expression in control blastema. **(B).** *shh* expression upregulated in hyperinnerated blastema. **(B')** Magnified image of upregulated *shh* in B. Reprinted from Mitogawa et al., 2018 with permission from Elsevier.

lmx1b is responsible for the dorsal-ventral limb pattering . In *Xenopus laevis* development, it is known that *lmx1b* is expressed in a tadpole limb blastema but is not present in an adult frog blastema (Matsuda et al., 2001). Consistant with this, when expression of this gene was being analyzed in the control blastema, expression was not detected by the *in situ* hybridization (Fig. 4A). *lmx1b* expression in the hyperinnervated blastema, on the other hand, was detected (Fig. 4B). The location of the expression was only found underneath the epithelium of the blastema and surrounds the cartilage tissue found in the center (Fig. 4B and B').

shh is also important in development because it helps to specify the anterior-posterior axis of limbs (Tickle and Towers, 2017). *shh* mRNA is found in a tadpole limb blastema but not in an adult frog blastema (Yakushiji et al., 2007). The control blastema demonstrated low expression of *shh*. The hyperinnervated blastema, on the other hand, showed more expression (Fig 5). It was also presented that *shh* expression was found primarily on the posterior side of the blastema. Overall, experimental results were successful in indicating that hyperinnervation caused an upregulation in gene expression in the regenerating froglet limbs.

Bmp, fgf, and shh play a role in nerve regulation

Mitogawa et al. investigated the expression of *bmp, fgf,* and *shh* in froglet dorsal root

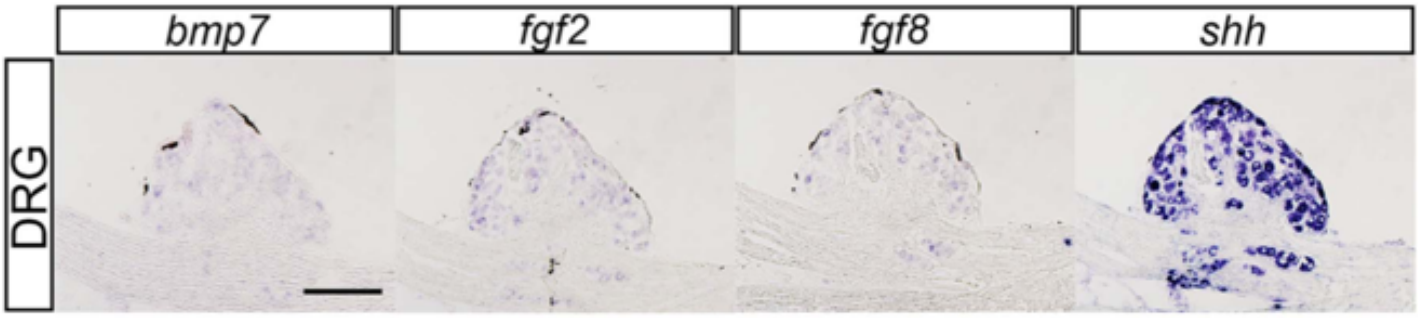


Figure 6: Expression of *bmp7, fgf2, fgf8,* and *shh* in a DRG neuron. Gene expression was visualized using *in situ* hybridization. Reprinted from Mitogawa et al., 2018 with permission from Elsevier.

ganglion (DRG) neurons. Previous research has shown that *bmp7, fgf2,* and *fgf8* have a role in nerves that induce the blastema (Makanae et al., 2016; Satoh et al., 2015). They found that *bmp* and *fgf* showed expression but *shh* revealed to have the most expression (Figure 6). This is an important finding because these genes also take part in limb development. By being expressed in the DRG, these developmental genes can induce the blastema and therefore, causing regeneration to occur.

DISCUSSION

Improvement in bone structure complexity

Results from Mitowaga et al have demonstrated that nerves can affect bone structure. Regenerated hyperinnevated limbs created a bone structure that was more complex than the regenerated control limb (Mitogawa et al., 2018). However, the limbs did not regenerate to its full potential. Hyperinnervation caused three different structures to form, but the cause of variability was not explained.

Reactivation of limb development genes

When *Xenopus laevis* are tadpoles, limb developmental genes are being expressed at very high levels. The control blastema in the amputation experiment had no expression of *lmx1b,* and some expression of *hoxa13* and *shh,* but at low levels. Hyperinnervation blastemas had higher expression of these limb development genes. This suggested that the limb development genes were "reactivated" when nerve bundles were added to the regenerating limb. The reactivated developmental genes could in turn, be the cause of the more complex bone structures. This indicates a promising connection between gene expression and improved bone regeneration.

Future directions

The authors did not discuss the importance of where the nerve bundles are coming from. The experimental methods states that the nerves are taken from the froglets own self and surgically placed in its own limb. Another future experiment that could be performed is seeing if using nerves from a froglets own body or if using nerves from a donor froglet would have a higher quality of limb regeneration.

The authors demonstrated that nerves have an important role in regeneration. Because nerves have a direct role in sensory, sensory systems and motor function would improve as well.

A hypothesis to test the sensory system is that the hyperinnervated limb would have greater sensitivity than a control limb. To test this, a frog limb (both control and

hyperinnervated) could be poked with a needle and reaction time measured. If the hypothesis is correct, the hyperinnervated limb would have a faster reaction time than the control limb.

In order to test the motor function ability of the limb, an obstacle course could be set up for the froglets. Food could be placed a distance away from the frog as an incentive. As the frog hops towards the food, visual observations could be made about the movement, such as distance hopped and speed, as well as the time it takes the frog to get to the food. A frog that has farther hops and a faster time to get to the food would be considered the one with better bone structure. In order to best test this, instead of having a frog with both a hyperinnervated limb and a control, the frogs should be separated into groups. Either they have a control limb, or they have a hyperinnervated limb, not both. This allows for the function of the limb to be better analyzed. This experiment could have a second part to it as well. In order to understand the impact of the amputated limb, the same experiment as before could be performed but no amputation of limbs would occur. There would still be a hyperinnervated limb and a non-hyperinnervated control limb. This allows for the nerves alone to be analyzed. Both of these experiments could help determine if the hyperinnervated gene had any impact on the function of the limb.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jennifer Liang for all the guidance she has given me throughout my college career. I would also like to thank Hannah Campbell, Madison Suess, and Maddie Chalmers for their excellent input and critics throughout this writing process. My primer would not be where it is today without them. Finally, the University of Minnesota Provost's Office and Center for Educational Innovation so graciously provided us with the finances needed for publication.

REFERENCES CITED

Borodinsky, L.N. (2017). Xenopus laevis as a Model Organism for the Study of Spinal Cord Formation, Development, Function and Regeneration. Front. Neural Circuits *11*.

Cross, S.H., Macalinao, D.G., McKie, L., Rose, L., Kearney, A.L., Rainger, J., Thaung, C., Keighren, M., Jadeja, S., West, K., et al. (2014). A Dominant-Negative Mutation of Mouse Lmx1b Causes Glaucoma and Is Semi-lethal via LBD1-Mediated Dimerisation. PLoS Genet. *10*.

Dent, J.N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. J. Morphol. *110*, 61–77.

Lee-Liu, D., Méndez-Olivos, E.E., Muñoz, R., and Larraín, J. (2017). The African clawed frog Xenopus laevis: A model organism to study regeneration of the central nervous system. Neurosci. Lett. *652*, 82–93.

Makanae, A., Mitogawa, K., and Satoh, A. (2016). Cooperative inputs of Bmp and Fgf signaling induce tail regeneration in urodele amphibians. Dev. Biol. *410*, 45–55.

Matsuda, H., Yokoyama, H., Endo, T., Tamura, K., and Ide, H. (2001). An Epidermal Signal Regulates Lmx-1 Expression and Dorsal–Ventral Pattern during Xenopus Limb Regeneration. Dev. Biol. *229*, 351–362.

Mitogawa, K., Makanae, A., and Satoh, A. (2018). Hyperinnervation improves Xenopus laevis limb regeneration. Dev. Biol. *433*, 276–286.

Monsivais, D., Clementi, C., Peng, J., Fullerton, P.T., Prunskaite-Hyyryläinen, R., Vainio, S.J., and Matzuk, M.M. (2017). BMP7 Induces Uterine Receptivity and Blastocyst Attachment. Endocrinology *158*, 979–992.

Ohgo, S., Itoh, A., Suzuki, M., Satoh, A., Yokoyama, H., and Tamura, K. (2010). Analysis of hoxa11 and hoxa13 expression during patternless limb regeneration in Xenopus. Dev. Biol. *338*, 148–157.

Reference, G.H. HOXA13 gene.

Satoh, A., Mitogawa, K., and Makanae, A. (2015). Regeneration inducers in limb regeneration. Dev. Growth Differ. *57*, 421–429.

Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H., and Tamura, K. (2006). Limb Regeneration in Xenopus laevis Froglet.

Tickle, C., and Towers, M. (2017). Sonic Hedgehog Signaling in Limb Development. Front. Cell Dev. Biol. *5*.

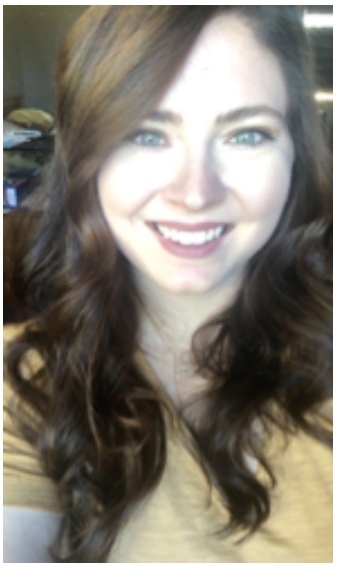
Yakushiji, N., Suzuki, M., Satoh, A., Sagai, T., Shiroishi, T., Kobayashi, H., Sasaki, H., Ide, H., and Tamura, K. (2007). Correlation between Shh expression and DNA methylation status of the limb-specific Shh enhancer region during limb regeneration in amphibians. Dev. Biol. *312*, 171–182.

Zavala, G., Prieto, C.P., Villanueva, A.A., and Palma, V. (2017). Sonic hedgehog (SHH) signaling improves the angiogenic potential of Wharton's jelly-derived mesenchymal stem cells (WJ-MSC). Stem Cell Res. Ther. *8*.

Zhao, Y., and Potter, S.S. (2001). Functional specificity of the Hoxa13 homeobox. Development *128*, 3197–3207.

Ziegler-Graham, K., MacKenzie, E.J., Ephraim, P.L., Travison, T.G., and Brookmeyer, R. (2008). Estimating the prevalence of limb loss in the United States: 2005 to 2050. Arch. Phys. Med. Rehabil. *89*, 422–429.

Zielins, E.R., Ransom, R.C., Leavitt, T.E., Longaker, M.T., and Wan, D.C. (2016). The role of stem cells in limb regeneration. Organogenesis *12*, 16–27.



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