Validating Hedgehog-Responsive Chondrogenic Lizard Blastema Cells

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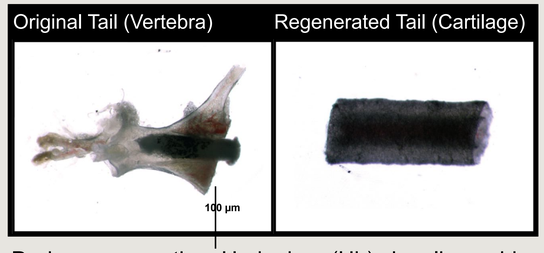
BACKGROUND

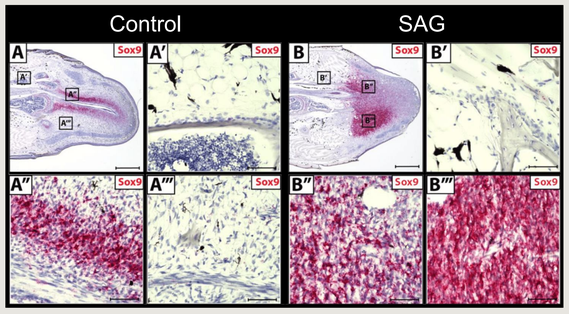
Lizards are the closest relatives to mammals that retain remarkable regenerative capacities as adults. Some, such as the green anole (*Anolis carolinensis*) have the ability to naturally regenerate tails through epimorphic or blastema-based regeneration. Interestingly, regenerated lizard tails consist of an unsegmented, cartilaginous tube instead of a patterned, ossified vertebra (Fig. 1).

Fig. 1



During regeneration, Hedgehog (Hh) signaling guides cellular differentiation and patterning. Sulfatase 1 (*sulf1*) is known to modulate Hh signaling, and is presumed to increase Hh responsiveness in the area surrounding the regenerating spinal cord that ultimately becomes cartilage. During chondrogenesis, Hh activates SRY-box transcription factor 9 (*sox9*), leading to the differentiation of precursor cells into chondrocytes. Chondrocytes produce collagen type II alpha 1 chain (encoded by *col2a1*), which is essential for cartilage structure and function. In the blastemas of green anoles, fibroblastic connective tissue cells respond to Hedgehog signaling to create cartilage.

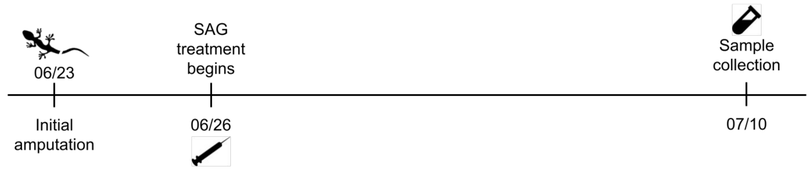
Fig. 2



Smoothened agonist (SAG) mimics the effects of Hh ligands; thus, the presence of SAG initiates Hh signaling without the presence of Hh ligands. During tail regeneration, undifferentiated blastema cells begin to express *sox9* and differentiate into chondrocytes, while others differentiate into muscle, fat, blood vessel, dermis, and other key tissue types in regenerated tails. We hypothesize that actively regenerating fibroblasts found in the blastema will respond to Hh signaling via SAG injections, causing most, if not, all undifferentiated blastema cells to take on a cartilage program.

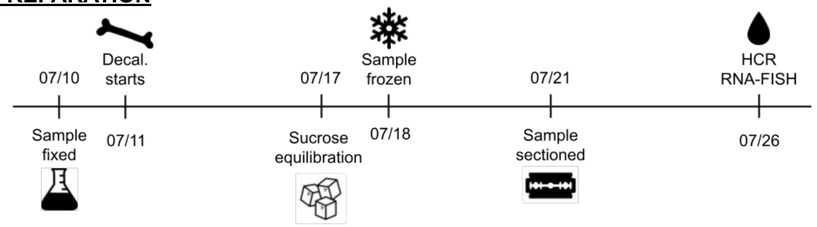
MATERIALS & METHODS

SAG TREATMENT



Lizards were injected with 300 µl of 800 µg/mL concentration SAG solution every other day, beginning three days post amputation (DPA) and continuing until tail collection. The same protocol was performed using PBS instead of SAG for the control group. All samples were collected ~14 DPA.

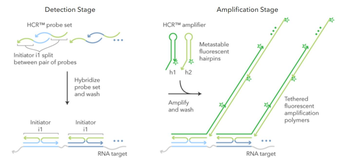
HCR PREPARATION



After being triple washed in PBS, samples were fixed in 10% NBF overnight at 4°C for 16 hours before being moved to 20% EDTA to decalcify for approximately one week. Samples were then equilibrated in sucrose through sucrose gradient washes, then positioned and frozen in OCT compound. Next, samples were sectioned in 16 µm-thick increments and placed onto slides, in preparation for hybridized chain reaction RNA fluorescence *in situ* hybridization.

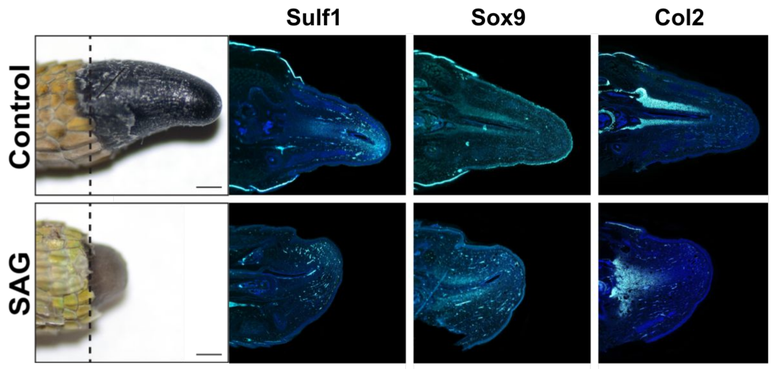
HCR RNA FISH

The fluorescence *in situ* hybridization process was adapted from the HCR RNA-FISH protocol for fresh frozen or fixed tissue sections. *Sulf1* and *col2a1* were targeted using B1-647, as part of the HCR RNA-FISH bundle for target mRNA1, and *sox9* was targeted with B2-647.



RESULTS

Fig. 3



Comparing the PBS and SAG-treated tails at roughly 14 DPA, it is clear that the regeneration of the SAG tail is severely inhibited. Externally, this is indicated by the SAG tail's notably shorter length, as well as its relatively lighter color, which is similar to that of cartilaginous tissues. On a molecular level, chondrogenesis is also shown by the SAG tail's extensive expression of *sulf1*, *sox9*, and *col2a1*. Both of these attributes indicate that the SAG treatment successfully induced cartilage throughout the entire blastema of the regenerating tail.

SUMMARY

In vivo treatment with exogenous SAG-induced ectopic cartilage formation in green anole lizard blastemas, in accordance with our hypothesis. Two groups of lizards had their tails amputated, then regenerated under the influence of either PBS (control group) and SAG (experimental group). After about 14 DPA, their tails were amputated once more and compared – both externally, and via hybridized chain reaction RNA fluorescence *in situ* hybridization. Our results showed stunted growth in the regenerating SAG tail, as well as expression of cartilage/chondrogenesis markers *sulf1*, *sox9*, and *col2a1* throughout the blastema. This indicated a lack of blastema cell differentiation into tissues such as fat, dermis, muscle, and blood vessels, thereby confirming that the introduction of SAG causes the majority of the blastema to differentiate into cartilage due to enhanced Hh signaling.

FUTURE DIRECTIONS

Future directions will include repeating the SAG/PBS treatment with a new batch of green anoles to be analyzed via flow cytometry, which is now possible because HCR RNA-FISH can mark single-cell populations. Steps to be taken after this will include transcriptomic comparisons between homeostatic fibroblasts, chondrocytes, and SAG-stimulated chondrocytes for insights into differential gene expression. Data from this study will provide target genes that will help to elucidate the mechanism of cartilage formation in the regenerating lizard tail. In understanding the processes and patterns exhibited, we hope to use this knowledge to stimulate the regeneration of appendages in mammals in future experiments, and ultimately create new regenerative therapies for humans.

ACKNOWLEDGEMENTS

Rest in peace to the green anoles who nobly gave and lost their lives to science. Their sacrifices were not in vain, and their legacies will forever be remembered through the advancement of regenerative medicine. Additionally, thank you to Dr. Thomas Lozito and Darian Gamble for generously providing me the chance to work in the Lozito Lab, and for being wonderful mentors!

REFERENCES

1. Vonk, et al. Lizard Blastema Organoid Model Recapitulates Regenerated Tail Chondrogenesis. *J. Dev. Biol*. 2022, 10, 12.

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