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Final Report

Abstract:

Mammals have evolved a variety of skin adaptations that allow them to thrive in many different environments. A fundamental challenge in biology is to understand how such adaptations originate at the molecular level. In this research project, we use a new model species, the sugar glider, to understand how gliding membranes form. We use cryosectioning, H&E staining, and image analysis to characterize the growth pattern of the sugar glider's gliding membrane, known as the patagium. We also seek to decipher the interactions of a gene regulatory network underlying patagium development.

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Project Context and Rationale:

Scholars have long been working to understand the developmental basis of adaptive evolution. In cases of trait loss, such as the morphological disappearance of limbs in snakes, evidence points to large-scale changes being driven by small numbers of critical enhancer regions (Kvon et al., 2016). In snakes, nucleotide changes causing loss of function in the highly conserved ZRS enhancer were shown to be responsible for the disappearance of limbs, and resurrection of snake enhancer function was even demonstrated in vivo on a mouse. This demonstrates the critical importance of cis-regulatory elements (promoters and enhancers) in adaptive evolution; rather than accruing mutations to important proteins which could be involved in multiple pathways, evolution takes the path of altering cis-regulatory elements, which produces a more modularized, controlled effect. While this has been demonstrated to be true for trait loss, it is less clear how organisms go about building totally novel structures. This process is likely to be more complicated, and subject to evolutionary constraint. The way that one lineage builds a trait is likely to be different to the way another lineage might, in large part due to restrictions posed by different mechanisms of development. The decreased morphological disparity of the marsupial shoulder girdle as compared to the placental shoulder girdle serves as a clear, observable example of this phenomenon. (Sears, 2004). Marsupials have short gestation times, with joeys being born very early and having to crawl from the mother's birth canal to the pouch. This reproductive strategy imposes a restriction on marsupial evolution which is reflected in their morphology; any significant divergence of the shoulder girdle poses a high risk of preventing joeys from getting to the pouch.

While the idea of evolutionary constraint is readily observable at the morphological level, this process of development must be reflected at molecular level. The project seeks to increase our understanding of how the structure of and changes in developmental genetic programs also changes a species' potential evolutionary path. The organisms of interest in this case are gliding mammals. As one of the most striking, often-cited examples of the phenomenon of convergent evolution, gliding marsupials (sugar gliders) and placentals (flying squirrels) have independently evolved a gliding membrane, known as the patagium. There are likely to be accrued molecular differences that are related to the ways these lineages have diverged adaptively from each other in the past (e.g. placental reproduction versus marsupial reproduction). These already "fixed" differences may have a significant impact on their future evolution. The patagium serves as an ideal model for studying how distant species may or may not be constrained by existing developmental genetic programs when independently deriving a novel structure.

The first step of the project, which I am working on, is characterizing the fundamental mechanisms of patagium development in sugar gliders. Sugar gliders were chosen as the organism in which to do this initial characterization because of a few key experimental advantages: they breed year-round, and the entirety of patagium development occurs after birth, while the developing joey is accessible in the mother's pouch. This can be broken down into two main branches: characterizing the growth pattern of the patagium, and deciphering the interactions of a gene regulatory network that regulates patagium development.

For the gene regulatory network branch, my mentor and I are running cell culture experiments to validate and add directionality to a preliminary gene regulatory network identified with RNA-Seq experiments. This gene network consists of several promising

candidate genes, which are up-regulated in cultured primary cell lines via transfection. The transcription levels of other genes in the preliminary regulatory network are then monitored by qPCR analysis, thereby allowing us to analyze the effects of each gene on the network at large. One of these candidates is Tbx3, a transcription factor that has been shown to play an important role in stratified epithelium development (Ichijo et al., 2017) and shows increased expression in the bat plagiopatagium, a similar structure to the patagium. Another promising candidate is Wnt5a, a gene involved in cell proliferation during limb outgrowth (Yamguchi et al., 1999). In the future, this network model can be compared to other species, like the flying squirrel. This will allow us to assess whether the species have significantly different developmental programs for arriving at the same structure, and to determine if these differences are due to happenstance, or evolutionary constraint, giving us further insight into the molecular mechanisms of evolution.

Methodology:

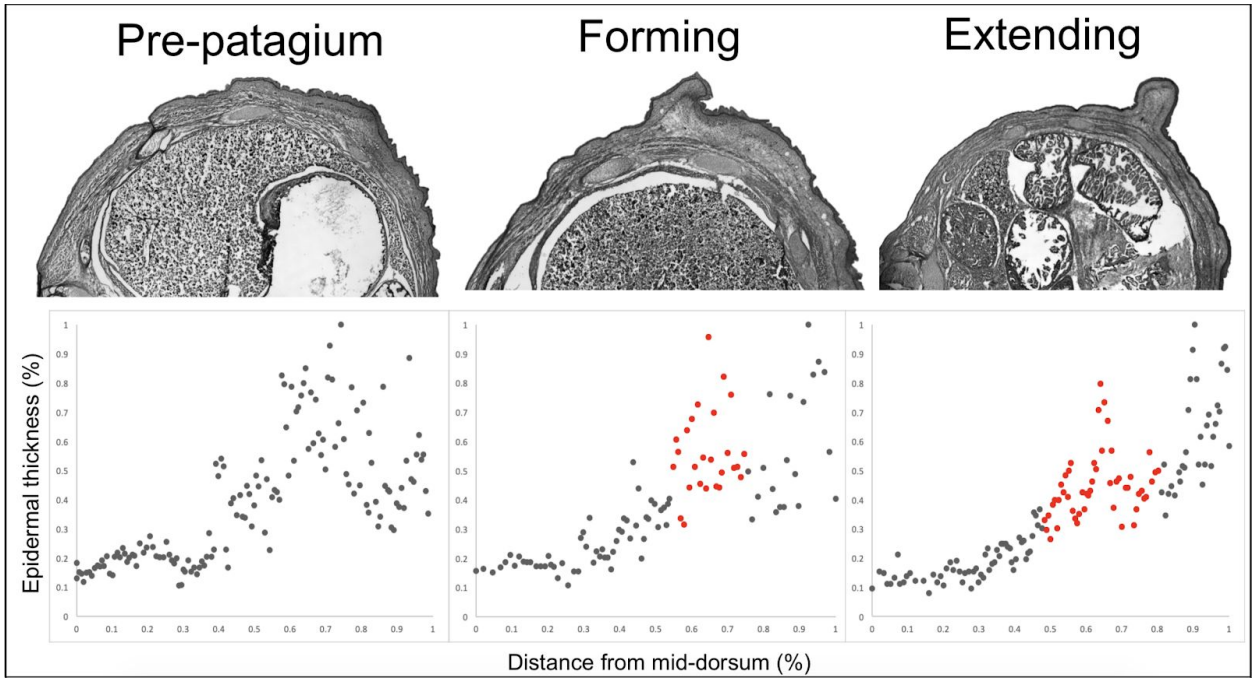
To characterize the growth pattern of the patagium, I performed cryosectioning of glider joeys at the pre-patagium, patagium forming, and patagium extending stages. I then used H&E staining to visualize and image these samples. Finally, I used a computational image analysis approach to analyze the samples, taking epidermal thickness measurements at equal increments along the epidermis to epidermal thickness to distance from the mid-dorsum in order to get an idea of the relative thickness of dorsal, lateral, and ventral skin at each stage.

To use the identified candidate genes to decipher the interactions of the gene regulatory network underlying patagium development, I have tried to optimize a transfection protocol on NIH/3T3 immortalized mouse fibroblasts as well as a primary mouse fibroblast cell line. The idea behind using mouse cells rather than primary sugar glider cells is that they have been shown to grow far more easily than primary sugar glider cells, and would provide a useful preliminary result. The process of transfection used involves the Lipofectamine 3000 reagent, which coats a concentrated plasmid(exogenous DNA) in a lipid layer that allows it to pass through the cell membrane and be transcribed by the host cell. The first two weeks of the project involved my mentor training me to grow up and passage the NIH/3T3 cells in order to prepare for transfection. To optimize this transfection efficiency, a GFP plasmid called LVGFP was used. Unfortunately, transfection efficiency was found to be extremely low with this plasmid, with very few cells demonstrating successful uptake of the GFP plasmid by glowing green. Another GFP plasmid, PLKO, was transformed into *E. coli* and isolated via miniprep to use in another round of transfection. However, at a variety of cell densities the transfection efficiency was still very low. To adjust for this setback, my mentor helped me grow up primary mouse cells rather

than immortalized ones in the hopes that they would uptake the plasmid with higher efficiency than the immortalized cells. Another round of optimization took place in which we transfected primary mouse cells with LVGFP and PLKO, both of which worked at higher efficiencies than the immortalized cells had. However, these efficiencies were still too low to produce qPCR results.

Preliminary Results:

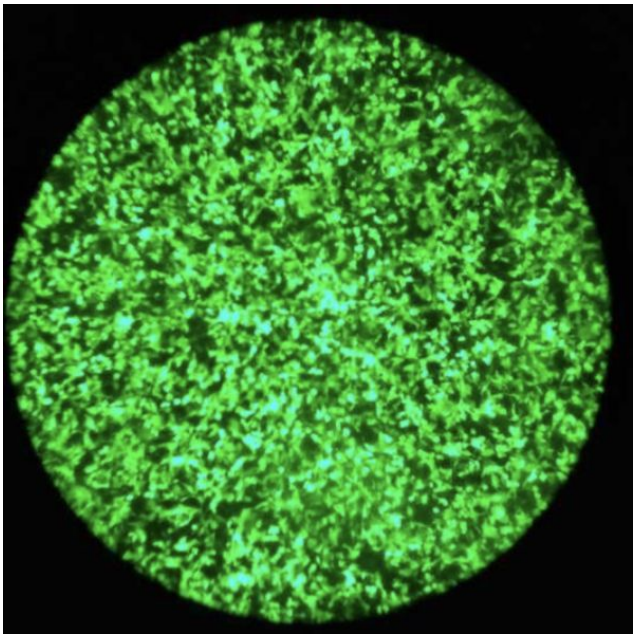
The growth pattern of the patagium was characterized using image analysis (as described in the methodology section) resulted in fairly conclusive results for joeys in the pre-patagium, forming, and extending stages.



Prior to patagium outgrowth, the epidermis increases in thickness along the dorso-ventral axis. Later, lateral skin undergoes a marked increase in epidermal thickness, as the patagium forms. These results suggest that lateral/ventral epidermis proliferates at faster rates than dorsal skin.

For the research question, "What are the interactions between the genes that regulate patagium development?", results were less conclusive. In order to decipher the interactions of the preliminary gene regulatory network underlying patagium development, I was tasked with transfecting immortalized mouse fibroblasts (NIH-3T3 cells) to work out an optimized protocol for having these cells express candidate genes to be monitored by RT-qPCR. However,

extremely poor transfection efficiency across the board with this cell line has prevented these data from being obtainable.



However, just this past week we were able to successfully transfect primary sugar glider fibroblasts with GFP (see picture above). We had initially tried to do the transfections with an established cell line like the NIH-3T3's, but it now seems that the primary sugar glider fibroblasts are working quite well. In future experiments, I will be moving to using these primary fibroblasts to validate and add directionality to the preliminary gene regulatory network.

Conclusion and future directions:

The growth pattern of the patagium was successfully characterized, suggesting a faster rate of proliferation of the lateral and ventral skin as compared to the dorsal skin. While this does not change the ongoing scholarly conversation, it does allow us to contextualize our future findings when deciphering the interactions of the gene regulatory network controlling patagium development. In future experiments, I will transfect primary sugar glider fibroblasts with candidate genes. From the characterization of the patagium's growth pattern, it seems that it could be informative to design the experiment such that the fibroblasts being transfected are taken from dorsal, lateral, and ventral skin separately. In addition to validating and adding directionality to the gene regulatory network, this would allow us to compare across different skin regions to see how the network is modified in different locations to produce the glider's dramatic phenotype.

Works Cited

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