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The Effect of Fibrodysplasia Ossificans Progressiva on the Tongue

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Abstract:

FOP is a rare genetic disorder in which skeletal muscle and associated connective tissue progressively turn to bone through a process called heterotopic ossification (HO). The extra skeletal bone growth is cumulative, eventually trapping patients in a second skeleton that eventually leads to death by asphyxiation. The FOP mutation is autosomal dominant that can be inherited or acquired sporadically. Unfortunately, FOP is currently incurable with no therapeutic options to inhibit bone growth or reduce existing bone nodules. My project intends to further our understanding of the cellular mechanisms of the disease within the tongue muscle. A population of cells known to be an origin of HO resides in the skeletal muscle of the tongue, but patients have clinically never been afflicted with HO in the tongue. The goal of this research is to consider the cellular environment of the tongue and the population of cells that reside there as possible inhibiting factors for bone growth. Using an FOP accurate mouse model, experiments priming the tissue for HO found that chemical injury in the presence of a specific cell population induced HO in the tongue. Cross transplantation experiments confirmed this finding. Further analysis into the heterogenous population of causal cells in the tongue is necessary.

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Introduction

Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disorder in which skeletal muscle and associated connective tissue progressively turn to bone through a process called heterotopic ossification (HO)[5,7,10,13,14]. HO can be provoked by soft tissue injury or spontaneously, with no known cause. The extraskeletal bone growth causes chronic pain for patients and progresses throughout their life. HO fuses with endogenous bone, locking afflicted tissue into place, eventually trapping patients into positions in which they are wheelchair bound and reliant on a feeding tube[10,13]. Progressive HO often leads to death due to asphyxiation. Since the disease is so rare, affecting one in every million individuals, physicians often misdiagnosed FOP due to lack of awareness and education. The only sign of FOP at birth is malformation of the big toes, which is found in all afflicted individuals, but not unique to FOP. This, paired with early swelling of soft tissue and HO presentation can lead to a proper diagnosis[10,13]. Once diagnosed, medical intervention is limited. There are currently no approved therapeutics that block bone growth or diminish pre-existing lesions. Surgical removal of bone lesions is not an option, because it acts as a form of injury, eliciting a painful, larger HO to form in its place. Rather, medicines are often prescribed to alleviate symptoms as they present[5,10,13,14].

The FOP mutation is an autosomal dominant mutation that can be inherited or acquired sporadically[5,7,10,13]. In most patients, there is a point mutation in the gene encoding the bone morphogenetic protein (BMP) type 1 signaling receptor, Activin A Receptor Type 1 (ACVR1). This mutation leads to an amino acid switch of an arginine to histidine at position 206 in the ACVR1 receptor ($ACVR1^{R206H}$)[5,7,10,13]. Only one mutated allele is required to induce a malfunctioning ACVR1(R206H) receptor. This amino acid switch alters the receptor's ligand binding activity, creating a hyperactive BMP receptor that has a newfound responsiveness to the

ligand activin A, a member of the transforming growth factor-ß family[5,7,14,17]. ACVR1 without the R206H mutation activates SMAD 2/3 phosphorylation and inhibits BMP activity when activin A is bound. In the presence of the mutation, signaling is altered, so upon activin A binding, SMAD 1/5/8 phosphorylation is activated, leading to an osteogenic response in competent cells. Activin A is secreted in response to injury or illness, which explains why HO is often found following soft tissue injury[5,7,14].

An FOP mouse model which accurately mimics clinical FOP was created to further research the disease. This is a conditional knockin mouse model of FOP in which the expression of the FOP mutation at the *Acvr1* loci is present in exon 5 (**Figure 1a)**[7]. Expression of this mutation is controlled by activity of a site-specific recombinase, Cre, which establishes temporal and cell specific activation[7,18]. Cre is controlled by *Tie*2 or *Pdgfra* promoters[7]. Upstream of the *Acvr1*^{tnR206H} mutation, in intron 4, is a stop cassette with a tdTomato reporter and a CAG promoter, flanked by two LoxP sites[7,18]. The CAG promoter is a constitutively active promoter that allows the expression of the tdTomato, which provides a red fluorescent label to indicate that the cell contains the *Acvr1*^{R206H} mutation and Cre is not active. The

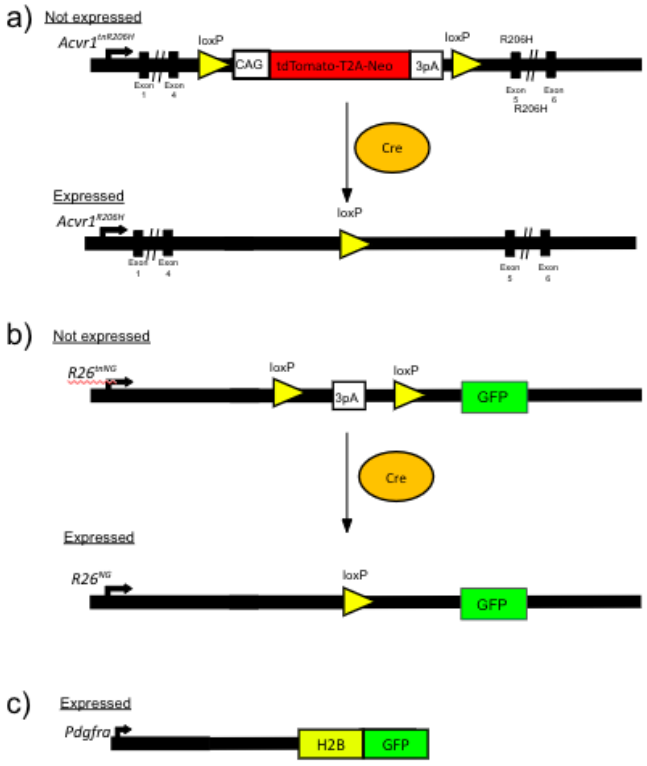

Figure 1: FOP accurate Mouse model. a) Knockin of Cre-dependent FOP mutation at the ACVR1 loci with a red fluorescently labelled stop cassette. When Cre is not active, red fluorescence is present and *Acvr1*^{R206H} is not expressed. When Cre is active, the tdTomato and Stop cassette (3pA) are cut at the loxP sites, and the *Acvr1*^{R206H} mutation is expressed. b) Knockin of Cre-dependent eGFP at the Rosa26 loci. When Cre is not active, no fluorescence is present. When Cre is active, the Stop cassette is cut at the loxP sites, and the GFP fluoresence is expressed. c) A knock-in fusion gene targeted to the *Pdgfra* gene. GFP expression reflects current *Pdgfra* expression. The GFP will be nuclear localized in the cell due to the H2B tag.

stop cassette inhibits the read out of the downstream *Acvr1*^{tnR206H}. When Cre is active, the tdTomato reporter stop cassette will be cleaved at the LoxP sites, the cell will lose its red fluorescence, and the mutated *Acvr1* gene will be transcribed (**Figure 1a)**[7]. This cleavage only occurs once, and then the gene will be indefinitely turned on. Additionally, a Cre dependent enhanced green fluorescent protein (eGFP) reporter is knocked in at the *Rosa26* locus, providing a lineage tracer to confirm Cre activity[7] (**Figure 1b**). In other words, when a cell loses its red fluorescence and gains green fluorescence, it is understood that is Cre is active and the cell is recombined. The *Rosa26* locus is transcriptionally active throughout development and adulthood, as well as uniformly and ubiquitously expressed. Additionally, mice with knockin mutations at the *Rosa26* loci are still viable and develop normally, making it an efficient, safe loci to use for lineage labeling. In a different mouse model, green fluorescence is controlled by current *Pdgfra* expression. An H2B-eGFP fusion gene is knocked in downstream of the the *Pdgfra* promoter, so GFP fluorescence indicates current *Pdgfra* expression (*Pdgfra*$^{H2B\text{-}GFP/+}$)(**Figure 1c**).

Using this accurate mouse model of FOP, the Goldhamer lab identified fibro/adipogenic progenitors (FAPs) as a major causal cell of injury induced and spontaneous HO[7]. To decipher which cell type was responsible for HO, many different Cre drivers were tested for all cell types implicated in HO. *MyoD*^{iCre} and VE-Cadherin-Cre were used to recombine muscle stem cells and endothelial cells, respectively. When these cells expressed the *Acvr1*^{R206H} mutation they did not form HO[7]. Tie2-Cre was used to recombine FAP cells. The *Tie2* gene, also known as *Tek*, codes for a tyrosine kinase receptor found on endothelial, hematopoietic and FAP cells[8]. When these cells expressed the *Acvr1*^{R206H} mutation, HO occurred. To confirm FAP origin rather than endothelial or hematopoietic, Pdgfra-Cre was used. PDGFRa (platelet-derived growth factor

receptor alpha) is a membrane receptor specific to FAP cells found in adult muscle tissue[1,7,9]. In a separate experiment, FAPs expressing the *Acvr1*^{R206H} mutation and the TIE2+ receptor were isolated and transplanted into SCID mice. Results from both experiments proved that recombined FAPs are sufficient to produce HO[7,9,].

Specific cell surface receptors and lack of myogenic capacity distinguish FAPs from other cell types. They are found within the skeletal muscle interstitium but are distinct from endothelial and hematopoietic cells due to their cell surface receptors[1,7,8,9]. FAPs display the membrane markers platelet-derived growth factor receptor alpha (PDGFRa) and stem cell antigen 1 (SCA1). FAPs also exhibit multipotency, capable of forming fibrotic or adipogenic tissue in a non-diseased state[1,7,8,9]. When FAPs actively express the *Acvr1*^{R206H} mutation, they respond to activin A binding by initiating osteogenic signaling[5,7,9]. FAPs are a heterogenous population with multiple subtypes[7,8,9]. In uninjured muscle, these subtypes upregulate specific genes for different biological processes. The TIE2 receptor labels a subtype of FAPs implicated in BMP signaling, while the PDGFRa receptor is found on all FAPs[7,8,9].

In a healthy individual FAPs are imperative for skeletal muscle homeostasis and regeneration[1,8]. Muscle has an impressive regenerative capacity because of the muscle stem cells present. Muscle stem cells efficiently enter the cell cycle to increase the myogenic cells present for repair of tissue[12,13]. To do this, muscle stem cells rely on signaling from non-myogenic cells in the environment, and FAPs are a major contributor in this process[1,8,13]. Similar to muscle stem cells, FAPs enter the cell cycle following injury or illness, but FAPs have a quicker response than the muscle stem cells[1,9,13]. FAP cell count peaks around three days post-injury to promote neutrophil and monocyte recruitment. FAPs work to regulate the activity of these immune cells from their pro-inflammatory to anti-inflammatory phenotypes[1,9]. Simutaneously, these immune

cells work to regulate FAP density and activity in injured tissue. FAPs also interact with myogenic cells, stimulating differentiation and aiding with muscle maturation[1,8,9]. In non-diseased cells, FAPs return to a similar state to non-injured tissue around seven to ten days after injury[9]. In the presence of the *Acvr1*^{R206H} mutation, this precise balance of cell communication is disturbed. By day six post-injury, there is a difference in FAP behavior in diseased tissue compared to non-disease tissue[8,9]. At this time point, non-diseased tissue exhibits regenerated fibers, while diseased tissue shows a high number of chondrocytes. By day 14 post-injury, non-diseased tissue has completely regenerated, while cartilage in diseased tissue has turned to bone through endochondral ossification[7,9].

Flow cytometry is a technology that uses fluorescent markers that are analyzed and standardized to identify cell populations[10]. Using flow cytometry, a population of FAP cells was identified in the tongue, a skeletal muscle with dense myofibers surrounded by connective tissue[3,12,16]. In the presence of the active *Acvr1*^{R206H} mutation, the tongue presumably has optimal conditions for HO. For unknown reasons there have been no clinical or experimental HO findings in the tongue. The absence of HO in the tongue is an understudied topic. My project was intended to increase our understanding of inhibiting factors for HO in an FOP diseased tongue.

Many skeletal muscles contain densely packed myofibers which are aligned parallel, but the tongue is organized in a complex way[3,16]. There are separate bundles of myofibers in the tongue that run in different directions to accommodate the versatile movement required of the tongue. The tongue is unique in that there is no tendentious attachment of the muscle to a joint and it is not connected to bone at both ends[3,16]. The mid-belly of the tongue is most densly packed with myofibers and little connective tissue[16]. Connective tissue, adispose tissue, and interstitial space are found in greater quantity anterior, near the tip of the tongue. Most

connective tissue resides between the bundles of myofibers that run on different planes, in different directions[16]. Unfortunately, information on structural organization, regeneration, and cell types within the tongue is limited[3,12]. Interestingly, there have been five clinical cases of osteolipomas in the oral cavity unrelated to FOP, and at least one reported case specifically within the tongue[15]. Lipomas with osseous metaplasia are scarce, and rarely found in the oral cavity. These tumors often come from deep soft or subcutaneous tissues. This is significant because it indicates that the tongue has the capacity to ossify in the presence of other mutations[15].

To further elucidate why an FOP diseased tongue is not known to ossify, we considered the subpopulation of FAPs that reside in the tongue, and the extracellular environment of the tongue as possible inhibitory factors. FAPs are a heterogenous population, and the presence and behavior of the subpopulations of FAPs present in the tongue are unknown. The specific subpopulation of FAPs within the tongue may dictate the tissue's ability to ossify. Additionally, skeletal muscle regeneration and growth involves recruitment of surrounding cells to stimulate specific signals for muscle stem cells to correctly differentiate into myofibers; therefore, the extracellular environment can also influence a tissue's ability to ossify. We utilized injury assays to determine if the tongue forms bone following injury. It was found that Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice did not form bone following pinch injury, and cardiotoxin injury results were ambiguous. Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice did not form bone following pinch injury, but did form bone from cardiotoxin injury. This led us to consider a difference in osteogenic capacity of Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ and Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ FAPs. We performed histological analysis on injured and uninjured tissue to evaluate the structure, organization, and regenerative process in nondiseased tongues and diseased tongues. Histology

proved that HO, cartilage and/or lack of structural integrity were found following a sufficient injury to induce regeneration in an FOP diseased tongue. More research into the regenerative processes in the tongue could prove beneficial in understanding the range in results. We conducted cross transplantation assays to separately evaluate the osteogenic capacity of tongue FAPs and the extracellular environment of the tongue. We evaluated tongue FAP osteogenic competence by transplanting tongue FAPs in an environment known to be competent for HO. Similarly, we transplanted HO competent FAPs into the tongue to test osteogenic capacity of the tongue environment. We found that Tie2-Cre; *Acvr1*^{R206H} hindlimb FAPs transplanted into the tongue reproducibly formed HO, Tie2-Cre; *Acvr1*^{R206H} tongue FAPs did not form HO in the tongue or hindlimb, and Pdgfra-Cre, *Acvr1*^{R206H} tongue FAPs formed HO in the tongue. This further supported the results from the injury assays that there could be an osteogenic deficit in the TIE2+ FAP subpopulation. Further research on intrinsic differences in the subpopulation of FAPs within the tongue is necessary.

My intention was to help the FOP community further understand the cellular mechanisms of the disorder. The data stimulates the need for further research into the mechanisms responsible for the varying phenotypes in the different mouse models. This could be helpful in understanding HO origins, leading to a treatments that inhibit this process.

Methods

Genotyping

To conduct experiments on FOP accurate mice, new litters were genotyped to ensure that the experimental mice had the phenotypes necessary to see intended results. This was done by ear punching the mice in a specific location that also acts to number them within their litter. This tissue was visualized under a Leica MZ FL III stereoscope for preliminary information on the

gene expression. Then the tissue was transferred to a 100 µL of alkaline lysis buffer with 25 mM NaOH and 0.2 mM of disodium ethylenediamine tetra acetic acid (EDTA) in water (Fisher Scientific, BP1201). This was placed in a heat block and heated to ~96℃ and remained there for an hour. After an hour, 100 µL of neutralizing buffer with 40 mM of Tris hydrochloride salt in water, was added to the DNA, and this solution was spun down. The DNA was amplified using traditional PCR methods and then run on a gel to identify which mice have the genes of interest. For this project, I was interested in the presence of expressed Tie2-Cre; Pdgfra-Cr;, *Acvr1*^{tnR206H}*;* *R26*$^{NG/+}$*;* and *Pdgfra*$^{H2B\text{-}GFP/+}$.

Pinch Injury

To initiate a regenerative response, and create an environment primed for HO, a pinch injury was used as a physical form of injury. Following IACUC protocols, the experimental mice were placed in a chamber to be anesthetized using a steady flow of O2 and isoflurane, from Animal Care services. Following this, the mice were placed on their back, and forceps were used to gently guide the tongue out of the mouth to expose the tissue. Once exposed, the serrated forceps were used to pinch down on the mid-belly of the tongue, a few micrometers from the tip of the tongue. As much force as possible was used for every tongue injury, in an effort to maintain consistent pressure between injuries, and limit human error. The mice were then placed back in the chamber to anesthetize in the same way previously mentioned. Once anesthetized, the mice were placed on their back to expose their right hindlimb. Holding the foot of the hindlimb to be pinched, the serrated forceps were used to pinch-injure the gastrocnemius muscle of the right hindlimb in the same manner as the tongue. At the spot of injury, observations were made to confirm that marks were made suitable to signify injury occurred. The injured mice were housed separately from non-injured mice and given wet food. The mice were weighed every

other day to verify they were not losing too much weight, following IACUC protocol. 14 days post-injury the mice were placed in a chamber with kimtech paper that had four drops of isoflurane to anesthetize the mice. While the mice were being anesthetized, a dissection station was made. The area was cleansed with ethanol and covered with a layer of aluminum foil and multiple kimtech wipes. The area was sprayed once more with ethanol. Once the mouse's breaths were spaced out a few seconds, they were sacrificed by cervical dislocation. A small incision with scissors was made in the skin to expose the musculoskeletal structures. The entire hindlimb was collected by cutting the limb with dissection scissors at the hip joint. The feet were also cut off. Harvested hindlimbs were placed directly into 4% Paraformaldehyde (PFA) for three days and then transferred to PBS. 4% PFA was made by diluting a stock solution of 16% Paraformaldehyde (Electron microscopy science, 15710) with PBS. To harvest the tongue the jaw bones were cut using dissection scissors. The lingual frenulum was cut at the base of the tongue, and the larynx was cut at the back of the tongue far enough to collect the tongue tissue and hyoid bone. Harvested tongues were placed directly into 4% PFA for 24 hours and then transferred to PBS. Once in PBS, the tissue was scanned in the In Vivo Imaging System (IVIS), a μCT machine, which scans for bone growth. The IVIS was set on CT, photograph, and visual field C. The tongue tissue was scanned with the hindlimb to maintain a consistent threshold for endogenous bone. Any exogenous bone growth found was quantified using 3D Slicer. This is an online software created for image processing and three-dimensional visualization.

Cardiotoxin Injury

To initiate a regenerative response, and create an environment primed for HO, cardiotoxin injury was used as a chemical form of injury. Following IACUC protocols, the mice were cardiotoxin injured using 10 μM cardiotoxin (Latoxan, L8102-1MG). The experimental

mice were placed in a chamber to anesthetize the mice for the injury using the same steps as pinch injury. During this time, 20 µL of cardiotoxin was measured using a lo-dose insulin syringe 50 cc, (MedPlus services, EXEL 26028). From previous optimization experiments, we found that 20 µL of liquid was the threshold the mouse tongue could maintain without bursting. Following this, the mice were placed on their back and forceps were used to gently guide the tongue out of the mouth to expose it. Once exposed, the needle was inserted parallel to the tongue to minimize the chance of it poking out and ensure the cardiotoxin was injected into the mid-belly of the tongue (**Figure 2a)**. As the cardiotoxin was injected, the needle was slowly pulled out of the tongue, dispersing the liquid, to decrease the chance of bursting. The mice were then placed back in the chamber to anesthetize once more. Once anesthetized again, the mice were placed on their back to expose their hindlimb. Holding the foot of the hindlimb to be injured a needle with 50 µL of cardiotoxin was injected into the right hindlimb into the gastrocnemius muscle (**Figure 2b)**. The injured mice were housed separately from non-injured mice and given wet food. The mice were weighed every other day to verify they were not losing too much weight, following IACUC protocol. 14 days post-injury the mice were sacrificed and harvested using the same methods as pinch injury. Once in PBS, the tissue was ready to be scanned in the IVIS, to visualize for HO and then quantified.

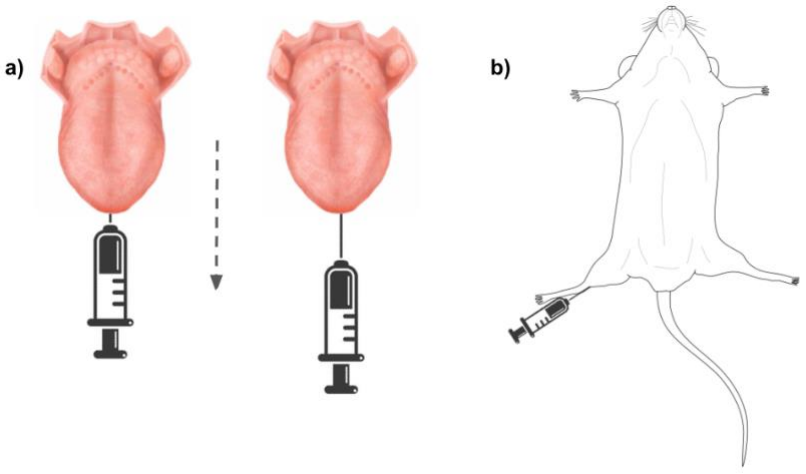

Figure 2: Cardiotoxin method for the tongue and hindlimb. a) The needle is inserted parallel to the tongue, far enough to get the tip in the center. Depth of the needle was estimated based on how much needle was showing. The needle was slowly pulled out, maintaining a parallel position, as the cardiotoxin was expelled. b) The needle was injected along the achilles tendon into the gastrocnemius muscle. The cardiotoxin was expelled when most of the needle was in the tissue, verifying it was deep in the mid-belly of the muscle.

Histology

To prepare harvested tissue for histological analysis, there is a month-long process to properly embed and section it. From dissection, a tongue went into 4% PFA for 24 hours, and a hindlimb for three days, to fix the tissue. Both tissues were then washed with PBS for three cycles and then placed in 12% EDTA in water. A harvested tongue requires two weeks for full decalcification, while a hindlimb takes 4 weeks to decalcify. The EDTA solution is changed every other day. Following their respective timelines, both tissues were then transferred to a 1% sucrose solution (Sigma-Aldrich, S5016-1KG) for at least 24 hours. Once this process was complete, the tissue was flash frozen in OTC. Embedded tissue was kept in a -80℃ freezer until ready to be sectioned.

A cryostat, which maintains low cryogenic temperatures of samples, was used to slice the tissue into 10 µm sections. The cryostat was set to -25℃. The tissue was captured on histology slides. The slides were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, 10236276001) or Hematoxylin and Eosin (H&E). A conventional DAPI stain procedure was used to fluorescently label nuclei as blue. For the H&E procedure, slides were washed in water twice, for three minutes and then sat in Mayer's hematoxylin (Sigma-Aldrich, MHS16-500ML) for ~8 minutes. The slides then went through three more water washes for 30 seconds each, followed by a minute bluing step, which is a 0.2% ammonia water solution (Sigma-Aldrich, 221228-500ML-A). The slides were transferred to two 70% ethanol bathes, a minute each, followed by three washes in 95% ethanol for 30 seconds each. Slides were dunked in an Eosin Y working solution (0.25%), (Sigma-Aldrich, E4382-25G) for 5-10 seconds. The slides went through two more 95% ethanol washes for a minute each, followed immediately by two washes in 100% ethanol for three minutes each. The staining process ends with two washes in xylene

(Fisher Scientific, X3P-1GAL) for five minutes each, and then cover slipped using a thin line of paramount down the middle of the slide. This had to set overnight in the histology hood. An inverted confocal microscope with three excitation wavelengths and bright field was used to visualize histology. The microscope contained fluorescent channels to visualize green, red, and blue, as well as a bright field feature. Magnitudes of 100x, 200x and 400x were used to focus on certian areas of the sections.

Cross Transplantations

Cross transplantations were conducted to test the osteogenic capacity of the tongue tissue and the FAPs within the tongue. Mice were genotyped for expression of Tie2-Cre or Pdgfra-Cre; $Acvr1^{tnR206H}$; $R26^{NG/+}$ or $Pdgfra^{H2B\text{-}GFP/+}$. Tongue tissue and hindlimb tissue from mice expressing these genes of interest were dissected in the same manner as the pinch-injured tissue. The harvested tissue was minced in digestion media with 2 mg/mL of collagenase type 2, (Worthington Biochemical, LS004177) and 0.3 mg/mL of dispase (Invitrogen, 17105041) in DMEM for 8-10 minutes, until no large clumps were visible. Minced tissue was placed in 10 mL of the same digestion media and sat in a hot bath for an hour and vortexed every 10 minutes. The cells were plated at a seating density to promote growth with growth media containing 10% fetal bovine serum (FBS) (R&D systems, S11510) and 1% Penicillin-Streptomyocin, (Sigma-Aldrich, P4333-100ML) in DMEM. Growth media was changed every other day and cells were split if they were over 80% confluent. To split cells, growth media is aspirated up and the cells were washed with DPBS. After the wash, the DPBS was aspirated and replaced with trypsin (Thermo-Fisher Scientific, 15090046) and placed back in the incubator for 3-5 minutes. After confirming the cells had detached by visualizing under a Nikon Exlipse E600 compound microscope, the

trypsin was neutralized with an equal volume of quench buffer made with 10% FBS in DMEM and transferred to a 15 mL conical. The cells were spun down at 4°C for five minutes at 1500 rpm. The supernatant was aspirated up and replaced with growth media. The cells were then split 1:2 or 1:3 depending on their confluency prior to splitting. 5-7 days after the cells were plated, they were prepared for the FACS (fluorescence activated cell sorting) machine to isolate the FAPs. Cells were stained with fluorescently conjugated antibodies that bind surface receptors. APC, BV7/11, BV421, and 7AAD, stain for PDGFRa, CD31/45, SCA1, and live/dead marker, respectively. When an *Pdgfra*$^{H2B\text{-}GFP/+}$ mouse was used for transplantations, the APC was omitted, because PDGFRa was denoted by endogenous green fluorescence. Some samples contained only one antibody stain in each, while some samples had all stains but one, to calibrate the FACs machine. Experimental samples to be isolated were stained with all fluorescent markers. These stains and endogenous fluorescence allowed the FACS machine to calibrate which cells to keep and which to discard. The result of the FACS was an isolated population of recombined FAP cells from the tongue and the hindlimb. After isolation, the FAPs were plated in growth media for another 5-7 days to increase population size. Growth media was changed every other day and cells were split according to their confluency. Previous research has shown that transplanting 50 µL of 1 million FAP cells is sufficient to initiate HO in a pre-injured HL. 20 µL of 400k cells were transplanted into the tongue to maintain an equal density. The density of recombined FAPs transplanted into no color SCID mice remained constant for both mouse models, Tie2-Cre and Pdgfra-Cre. To have enough cells for the transplantation, the goal is to collect around 1.5 million cells per SCID mouse. When a sufficient population of cells was reached, they were collected from culture. Growth media was aspirated out and replaced with DPBS for a wash. DPBS was aspirated and replaced with trypsin, and the cells were moved back

into the incubator for 3-5 minutes. To fully detach the cells from the plate, the trypsin was pipetted up and down a few times around the plate to add physical force. Trypsin was neutralized with an equal volume of quench buffer. The cells were spun down and counted using a hemocytometer. This number was used to calculate the volume of DPBS to resuspend the cells in to have 1 million cells in every 50 µL of volume. The SCID mice to be used were anesthetized in the same way previously mentioned. Cells isolated from the tongue were transplanted into an injured hindlimb and tongue, and those from the hindlimb into an injured hindlimb as a control. FAPs isolated from the hindlimb were transplanted into an injured tongue and an injured hindlimb as a control. These mice were housed separately and given wet food. 14 days post-transplantation the mice were collected. Tongue and hindlimb tissue were dissected using the same methods previously mentioned. Prior to a 4% PFA wash, whole mounts images were observed of the tissue under a Leica MZ FL III stereoscope. Tongues and hindlimbs then went through the same process as injured tissue for IVIS scans, embedding and histological analysis.

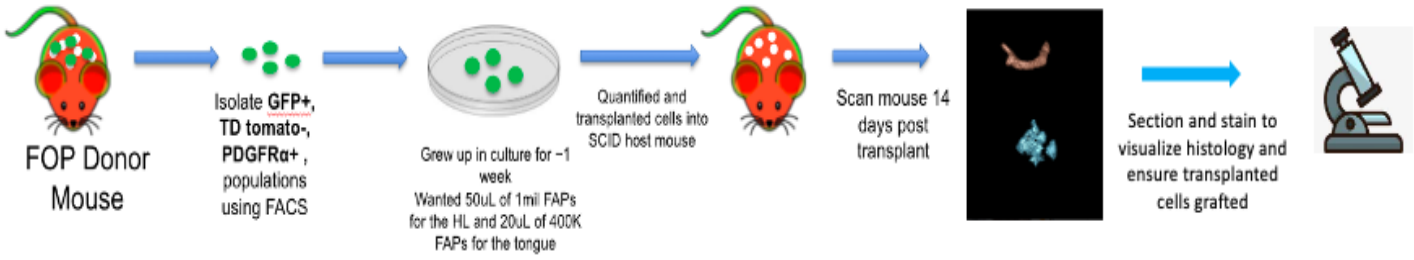

Figure 3: FAP isolation and Cross-transplantation procedure. Genotype mice for Tie2-Cre or Pdgfra-Cre, *Acvr1*^{R206H}, *R26*$^{NG/+}$ or *Pdgfra*$^{H2B\text{-}GFP/+}$. Collect tongue and hindlimb tissue to culture cells from host mice with the genes of interest. Isolate the FAP cells using FACS, gating for CD31/45-, SCA1+, PDGFRa+, GFP+ and tdTomato- cells. Grow up FAPs in culture for a week. Resuspend cells in DPBS to a calculated volume resulting in 1 million cells/50 µL and transplant cells into SCID mice. Isolated tongue FAPs were transplanted into the tongue and left hindlimb, using the same methods as cardiotoxin injection. Hindlimb FAPs were transplanted into the right hindlimb of these SCID mice as a control. Hindlimb FAPs were also transplanted into the tongue and right hindlimb of other experimental SCID mice. 14 days post-injury tissue was collected and scanned for HO. Harvested tissue was then prepared for histological analysis.

Results

Testing HO Capacity of the Tongue through Injury Assays

Pinch injury does not induce HO in the tongue of a mouse with FOP. Pinch injury assays were conducted on the tongue to test whether the tongue is competent for HO. The right hindlimb was also injured as a positive control. Initially, five Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice were pinch-injured and one Tie2-Cre; $R26^{NG/+}$ mouse was pinch-injured as a control. 14 days post-injury, the mice were sacrificed and imaged. All six mice did not from HO (**Figure 4)**. Bone growth was seen in the hindlimbs of the experimental mice, confirming the mice were competent to form HO (data not shown). The tissue was then fixed and decalcified for two weeks to prepare it for the embedding process. Embedded tissue was sectioned assess regeneration with histological analysis. Two experimental tongues were sectioned and stained with DAPI to visualize centralized nuclei. Centralized nuclei were difficult to find in both tongues, because they were dispersed and in low concentration (**Figures 5a)**. The section stained is from the mid-belly of the tongue in a medial location that was pinch-injured. A few myofibers show regeneration, but they are not in a centralized location. From histology, we speculate that not enough myofibers were injured by the force of the pinch to provoke injury-induced HO. The injured tongue maintains structural and organizational integrity compared to the uninjured tissue (**Figure5a, c**). Following these results, we pinch-injured Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice. From previous data we know that Pdgfra-Cre has a higher recombination efficiency and reproducibly results in a more robust HO response to injury[7]. Four Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice were pinch-injured at the midbelly of the tongue in a medial location. All four of the mice were negative for HO (**Figure 4)**. HO was found in all four injured hindlimbs, confirming that the mice were comptent to form HO (not shown). Two of the tongues were

sectioned and stained using DAPI. Histology showed that few myofibers contained centralized nuclei (**Figure 5b**). This section represents the midbelly of the tissue that was pinch-injured. Myofibers presenting centralized nuclei were found in closer proximity to each other compared to the Tie2-Cre model. Injured tissue lacked structural integrity and organization found in uninjured tissue. (**Figure 5b, d**).

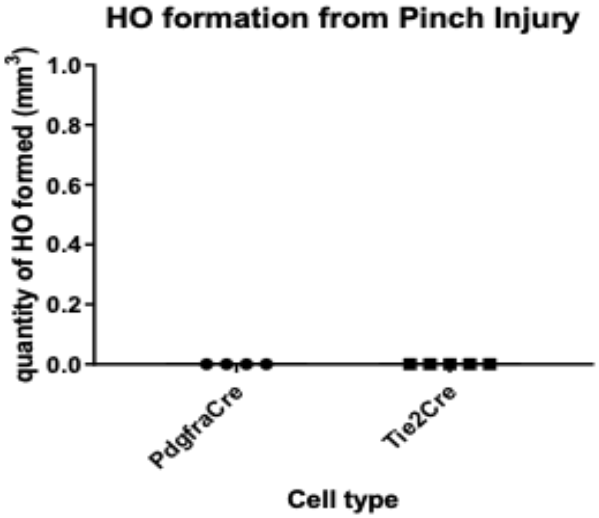

Figure 4: HO data from pinch-injured mice. Four Pdgfra-Cre; *Acvr1*^{tnR206H}; *R26*$^{NG/+}$ (PdgfraCre) mice were pinch-injured on their tongue. Two weeks post-injury the tongues were harvested and scanned for bone growth. None of these mice formed HO in their tongue. Five Tie2-Cre; *Acvr1*^{tnR206H}; *R26*$^{NG/+}$ (Tie2Cre) mice were pinch-injured on their tongue. Two weeks post-injury the tongues were harvested and scanned for bone growth. None of these mice formed HO in their tongue. The right HL of each experimental mouse was also pinch-injured as a positive control; these limbs did form HO (data not pictured).

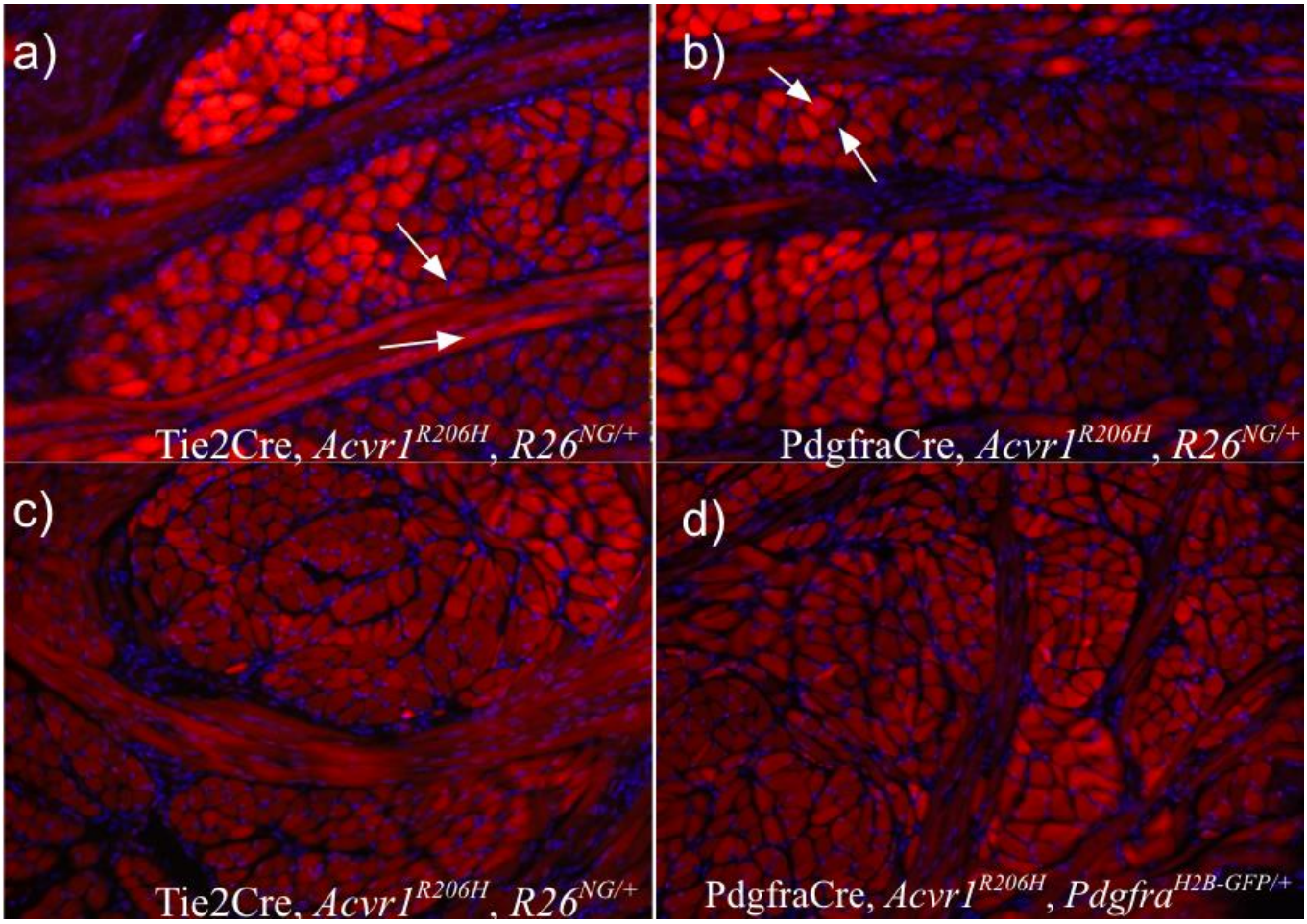

Figure 5: Pinch-injured tongues in comparison to uninjured tongues of mice with the *Acvr1^{tnR206H}* mutation. a) A DAPI stained image of a Tie2-Cre; *Acvr1^{tnR206H}*; *R26$^{NG/+}$* pinch-injured tongue at 200x magnification. This image shows the myofibers in the midbelly of the tongue at the location the injury was performed. White arrows point to DAPI stained nuclei that can be seen in a centralized location within the cell. Few centralized nuclei were seen. Organzation and structure of myofibers remain intact **b)** A DAPI stained image of a Pdgfra-Cre; *Acvr1^{tnR206H}*; *R26$^{NG/+}$* pinch-injured tongue at 200x magnification. This image shows the myofibers in the midbelly of the tongue, where the injury was administered. White arrows point to DAPI stained nuclei that can be seen in a centralized location within the cell. This indicates regeneration. Additionally, the DAPI stain shows a lot of cellularization, also commonly seen in regenerating tissue. Organization and structure of myofibers remain intact **c)** A DAPI stained image of a Tie2-Cre; *Acvr1^{tnR206H}*; *R26$^{NG/+}$* uninjured tongue at 200x magnification. No centralized nuclei are seen here. Organization and structure of myofibers are highly complex **d)** A DAPI stained image of a Pdgfra-Cre; *Acvr1^{tnR206H}*; *Pdgfra$^{H2B-GFP/+}$* uninjured tongue at 200x magnification. No centralized nuclei are seen here. Organization and structure of myofibers are highly complex.

Cardiotoxin injury is sufficient to produce HO in a Pdgfra-Cre; *R26*$^{NG/+}$; *Acvr1*^{tnR206H} tongue, but not in a Tie2-Cre; *R26*$^{NG/+}$; *Acvr1*^{tnR206H} tongue. Cardiotoxin injury was used as a chemical form of injury to test if the tongue is competent to form HO. A consistent volume of toxin is injected, limiting human error, and injection can be directed to the center of the dense myofiber and connective tissue. After multiple trials, 3 of 15 Tie2-Cre mice presented HO following cardiotoxin injury on the IVIS machine. The volume of these bone nodules was determined using Slicer; quantification revealed nodules of 0.040 mm^3, 0.203 mm^3 and 0.126 mm^3 (**Figure 6**). All the hindlimbs injured did form HO (data not shown). Two tongues that formed HO and two tongues that did not form HO were sectioned. Bone morphology was not seen following DAPI or H&E staining of sections of the tongues that showed HO on the IVIS. Disorganization and degeneration of myofibers was evident in these sections (**Figure 7a, c**). Further analysis using stains for bone markers is necessary to confirm an absence of bone tissue. This section is from the mid-belly of the tongue where the cardiotoxin injection initiated. There are still intact myofibers present which have maintained some organization (**Figure 7a, c**). The injured tissue is highly cellularized and centralized nuclei can be seen near the site of disorganization (**Figure 7c**). We see centralized nuclei in the mid-belly of the tissue in more abundance from the cardiotoxin injury than the pinch injury (**Figure 5a, 7c**). Multiple trials were done on Pdgfra-Cre mice, and 6 of 7 injured mice formed HO (**Figure 6**). Quantification of the HO was 10.045 mm^3, 0.578 mm^3, 5.198 mm^3, 4.141 mm^3, 0.298 mm^3, and 0.443 mm^3 (**Figure 6**). All the hindlimbs injured did form HO (data not shown). Three tongues that formed HO were sectioned and analyzed. Consistently, there was a complete loss of structural integrity and organization of the myofibers in the midbelly of the tongue (**Figure 7b, d, f)**. There was a large GFP presence, and minimal tdTomato presence. As expected, the bone nodule was surrounded

by GFP+ cells. The loss of myofibers expands the width of the tongue, far greater than the size of the bone nodule (**Figure 7b**). H&E stain of the tissue surrounding the bone nodule shows a cartilage morphology (**Figure 7d**). If the injured tissue was collected at a later time point, this area of cartilage may have ossified, forming a larger bone nodule. The HO volume was consistently greater in the Pdgfra-Cre mice (>0.250 mm^3) than the Tie2-Cre mice (<0.250 mm^3) (**Figure 6**). Similarly, there was consistently greater structural damage to myofibers n the Pdgfra-Cre model (**Figure 7a, b**). This pattern suggests there could be a difference in osteogenic competence of both FAP populations or recombination efficiency can have a large effect on phenotype. There was no apparent sex difference in HO formed in the Pdgfra-Cre model.

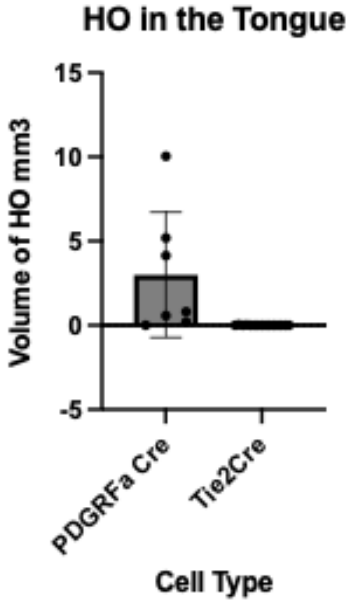

Figure 6: Cardiotoxin injury Assay results in Tie2-Cre mice and Pdgfra-Cre mice. Cardiotoxin was sufficient to constitently produce HO in the Pdgfra-Cre; *Acvr1*^{tnR206H}; *R26*$^{NG/+}$ (Pdgfra-Cre) model, but not consistently in the Tie2-Cre; *Acvr1*^{tnR206H}; *R26*$^{NG/+}$ (Tie2-Cre) model. Tongues were collected and scanned two weeks post-cardiotoxin injection. There was bone growth in 6 of 7 Pdgfra-Cre models, with a variation in quantity. In contrast, only 3 of 14 Tie2-Cre tongues exhibited osteogenic capability, to a much lesser extent than the Pdgfra-Cre model. HO volume in Tie2-Cre model was consistently <0.250 mm^3, while Pdgfra-Cre consistently formed HO >0.250 mm^3. There was no discernible pattern in quantity of HO in Pdgfra-Cre mice.

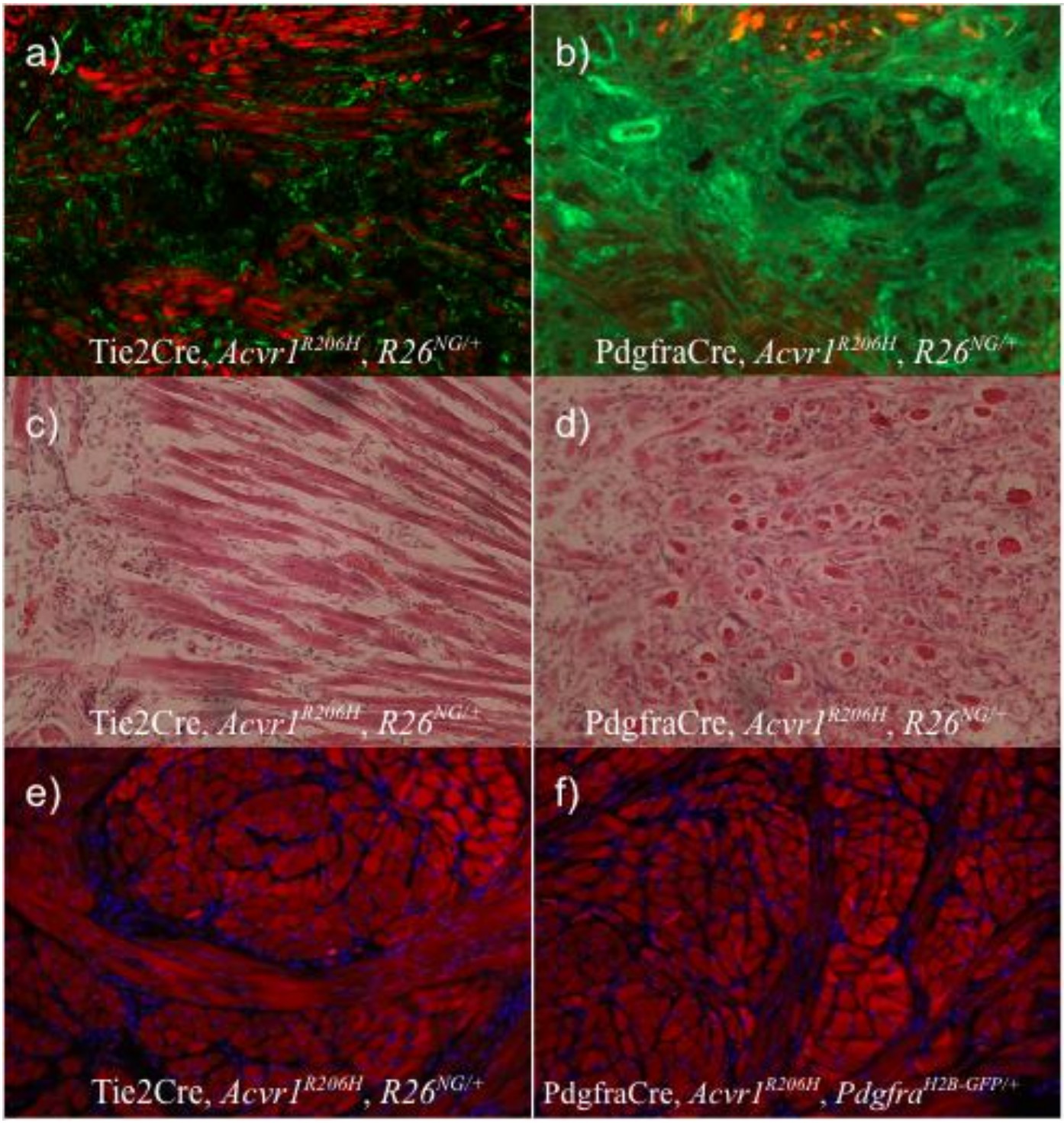
a)
Tie2Cre, Acvr1R206H, R26NG/+
b)
PdgfraCre, Acvr1R206H, R26NG/+
c)
Tie2Cre, Acvr1R206H, R26NG/+
d)
PdgfraCre, Acvr1R206H, R26NG/+
e)
Tie2Cre, Acvr1R206H, R26NG/+
f)
PdgfraCre, Acvr1R206H, PdgfraH2B-GFP/+

Figure 7: DAPI and H&E stained cardiotoxin injured *Acvr1^{tnR206H}* tongue in comparison to uninjured tongue. a)A 100x image of a cardiotoxin injured Tie2-Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* tongue. This image is of the tissue in the midbelly of the tongue, where the cardiotoxin was injected and the small bone nodule was seen in the CT scan. Green fluorescence is indicating recombined TIE2 labelled FAPs; red labels unrecombined cells with the *Acvr1^{R206H}* mutation. Disorganization and lack of myofibers is evident. No bone nodule morphology is observed. **b)** A 100x image of a cardiotoxin injured Pdgfra-Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* tongue. This image is of the tissue in the midbelly of the tongue, where the cardiotoxin was injected and the bone nodule was seen in the CT scan. Green fluorescence is indicating all recombined FAPs; red labels unrecombined cells with the *Acvr1^{R206H}* mutation. Disorganization and lack of myofibers is evident. A white arrow points to a bone nodule in the upper right corner of the image. There is no retention of myofibers surrounding the bone nodule, just complete disorganization. **c)** A 200x image of an H&E stain of a cardiotoxin injured Tie2Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* tongue. This image is shows part of the disorganization seen in **a**, and a view of the intact myofibers. There is a great amount of cellularization (marked by blue dye) normally seen in injured tissue at the sight of disorganization. Disorganization of myofibers is visible on the left side of the image, and myofibers can be seen on the right. **d)** A 200x image of an H&E stain of a cardiotoxin injured Pdgfra-Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* tongue. This image was taken of the area surrounding the bone nodule in **b**, reflecting the morphology of cartilage, indicating the disorganization seen in **b** is at least partially cartilage. There is no retention of myofibers surrounding the bone nodule. **e)** A DAPI stained image of a Tie2-Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* uninjured tongue at 200x magnification. Complexity and structure of myofibers is apparent, in contrast to the section in **a,** where myofibers are seen, but not in structured bundles. **f)** A DAPI stained image of a Pdgfra-Cre; *Acvr1^{tnR206H}; R26$^{NG/+}$* uninjured tongue at 200x magnification. Complexity and structure of myofibers is apparent, in contrast to the section in **b** and **d**, where no myofiber morphology is present.

Cross Transplantations

Cross transplantation of Tie2-Cre, *R26$^{NG/+}$*, *Acvr1^{R206H}* Hindlimb FAPs into a SCID tongue forms HO. In the first cross transplantation experiment, tongue and hindlimb cells from Tie2-Cre; *R26$^{NG/+}$; Acvr1^{tnR206H}* mice were collected and grown in culture for a week. During this week, the tongue cells were contaminated, making them inviable for the experiment. The hindlimb FAPs were FACS isolated and then spent another week in culture. After the week in culture, there were just under 6 million hindlimb FAPs, providing enough cells to transplant into four SCID mice (n=4). The tissue was pinch-injured prior to transplantation to offer an environment primed for HO. 400k cells/20 μL was transplanted into each tongue and 1 million cells/50 μL was transplanted into the right hindlimb of each SCID mouse. The right hindlimb was a control in each mouse. 14 days after the injury and transplantation, the mice were collected and scanned for HO using the IVIS machine. We found that all four SCID mice formed

HO in the tongue and the hindlimb. Quantification of HO for each mouse was 0.494 mm^3, 0.0961 mm^3, 0.958 mm^3, and 0.734 mm^3. These tongues were prepared for histological analysis, but histology was not completed for this tissue. These results support findings that the tongue is competent to form HO.

Tie2-Cre; *R26$^{NG/+}$; Acvr1^{R206H}* tongue and hindlimb FAPs were transplanted into the hindlimb and tongue of SCID mice, respectively. From the prior experiment, we found that we needed to collecte more tongue cells to have a sufficient number for transplantations. In the second experiment, tongues from three Tie2-Cre; *R26$^{NG/+}$; Acvr1^{tnR206H}* mice were pooled together to increase the number of starting cells. The cells were grown in culture for a week, and then FAPs were FACS isolated, and grown in culture for one more week. Just under 3 million tongue FAPs were collected at the end of two weeks in culture, providing enough cells to transplant into an injured tongue and left hindlimb of two SCID mice. Hindlimb FAPs were transplanted into the right hindlimb of these SCID mice as a control. 14 days after injury and transplantation, the tissue was harvested and scanned using the IVIS. Tongue FAPs transplanted into the left hindlimb and tongue did not form HO in both experimental mice. The hindlimb FAPs did form HO in the right hindlimb of both mice. The tissue was fixed and decalcified following their respective protocols, and embedded for histological analysis. The two tongues were sectioned and stained. GFP+ cells were present in the tongue tissue that received tongue FAPs indicating that the transplanted cells engrafted, but did not form HO(**Figure 8**). Wholemount observations of the hindlimb's that received tongue FAPs showed GFP+ cells (data not shown). Histology was not completed on the hindlimbs that received tongue FAPs. Due to the lack of histology, we cannot confidently claim that transplanted tongue FAPs engrafted into

the hindlimbs of the SCID mice. Additionally, cells from two hindlimbs of Tie2-Cre; *R26*$^{NG/+}$*; Acvr1*^{tnR206H} mice were pooled together. After a week in culture, FAPs were FACS isolated from this population, and grown for another week. These hindlimb FAPs were used as controls for the two SCID mice that received tongue FAPs. Just under 3 million hindlimb FAPs were still available to also transplant hindlimb FAPs into the tongue and right hindlimb of two SCID mice. The tongues and hindlimbs that received hindlimb FAPs formed HO in both SCID mice after 14 days. Using Slicer, quantifications of these bone nodules in SCID tongues that received hindlimb cells were 1.077 mm^3 and 1.499 mm^3. This data further confirms injury assay data and the first cross transplantation experiment, showing a deficit in the Tie2-Cre tongue FAPs and osteogenic capacity of tongue tissue.

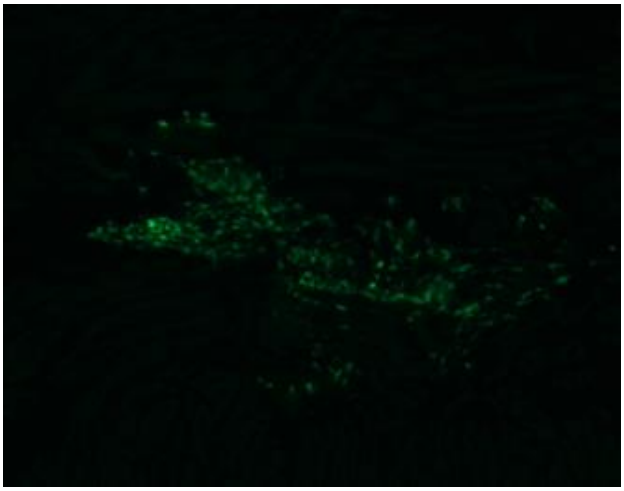

Figure 8: GFP Fluorescence of transplanted tongue FAP cells indicate successful engraftment. This image shows a section from the mid-belly of the tongue, where the transplanted FAPs were injected. Presence of GFP fluorescence indicates that FAPs from the host, Tie2-Cre; *Acvr1*^{tnR206H}*; R26*$^{NG/+}$, engrafted into the SCID tongue. The GFP+ cells are surrounded by no color WT myofibers of the SCID mouse. The results confirm that the transplantation was successful.

Tongue FAPs from a Tie2-Cre, *Pdfra*$^{H2B\text{-}GFP/+}$, *Acvr1*^{tnR206H} mice and Pdgfra-Cre, *Pdgfra*$^{H2B\text{-}GFP/+}$, *Acvr1*^{tnR206H} mice were transplanted into the tongues of SCID mice. In the third transplantation experiment, only two tongues of each mouse model were pooled together due to available mice. These cells were grown in culture for a week, then FAPs were FACS

isolated, and grown in culture for another week. An equivalent amount of recombined FAPs were collected from FACS from each mouse type, which eliminates the difference in recombination efficiency between the two mouse models. Pdgfra-Cre FAP cells grew more quickly in culture than Tie2-Cre FAPs and had to be split two extra times. To keep an equal density of cells being transplanted and the same amount of experimental SCID mice for each model, Tie2-Cre FAPs were a limiting factor. Hindlimb cells were not transplanted in this experiment, as adequate data proved the hindlimb FAPs induce HO in the tongue. *Pdgfra*$^{H2B\text{-}GFP/+}$ mice were used in this experiment, because endogenous fluoresence offers a more accurate read out for PDGFRa expression than the antibody, providing a more precise isolation method. At the end of the two weeks in culture, there were 1 million Tie2-Cre FAPs, and more Pdgfra-Cre FAPs, so 400k cells/20 μL were transplanted into two SCID mice tongues each. Not enough cells were collected to transplant the isolated tongue FAPs into the hindlimb of the SCID mice. 14 days after injury and transplantation, the tongues were harvested and scanned on the IVIS to verify HO formation. The two SCID mice that received Tie2-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} FAPs in the tongue did not form HO. These tongues were fixed, decalcified, embedded and sectioned for histological analysis. GFP+ cells were present in these tongues, suggesting engraftment of transplanted cells (**Figure 9a**). H&E stain does not show bone morphologies within the area of engrafted cells. Degradation of myofibers, a common phenotype found in a tongue with the *Acvr1*^{tnR206H} mutation, is observed in the area of engrafted cells (**Figure 9a, b**). The two SCID mice that received Pdgfra-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} FAPs in the tongue did form HO. Bone nodules were quantified on Slicer and found to be 0.290 mm^3 and 0.537 mm^3. These are comparable volumes to bone nodules formed upon cardiotoxin injury in Pdgfra-Cre; *R26*$^{NG/+}$*; Acvr1*^{tnR206H} mice. GFP+ presence suggests that transplanted FAPs engrafted into

the SCID host tissue (**Figure 9c**). H&E stain of this tissue shows a bone nodule and high cellularization to the area of engrafted cells (**Figure 9c,** d). Tissue surrounding the bone nodule looks like it maintained structural integrity, which is a different phenotype than cardiotoxin injury of Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice. This data supports and enhances the findings from the previous cross transplantation experiments, and the injury assays.

A summarization of all the transplantation results from the three experiments can be found in **Table 1.**

Table 1: A summary of the cross-transplantation data from all three experiments.

Experiment	HO from Tie2Cre Tongue Cells into Tongue	HO from Tie2Cre HL cells into Tongue	HO from Tie2Cre Tongue cells into the HL	HO from PdfraCre Tongue cells into Tongue
1	N/A	4/4	N/A	N/A
2	0/2	2/2	0/2	N/A
3	0/2	N/A	N/A	2/2

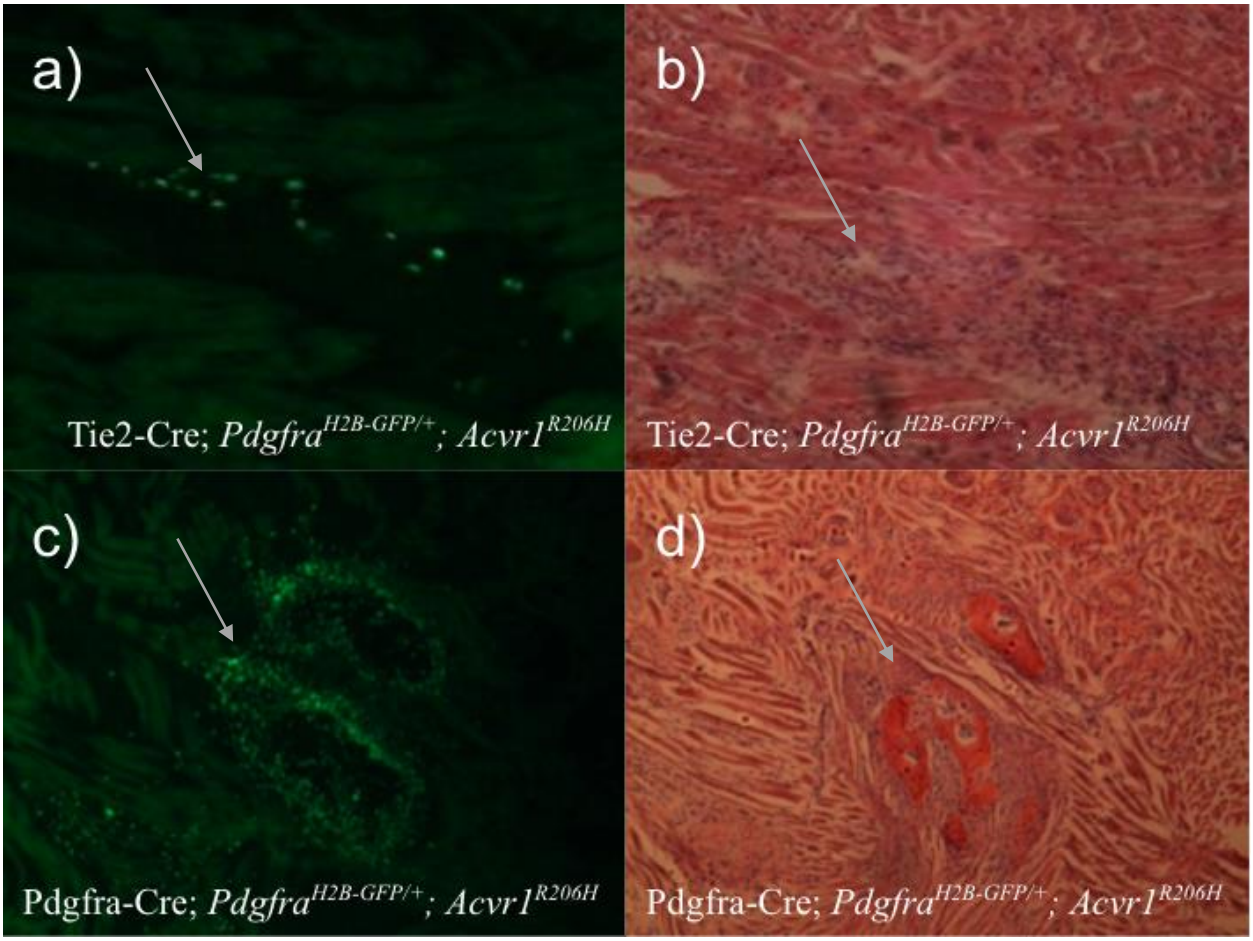

Figure 9: Histology of tissue from cross transplantations using Tie2-Cre; *Acvr1*^{tnR206H}; *Pdgfra*$^{H2B\text{-}GFP/+}$ and Pdgfra-Cre; *Acvr1*^{tnR206H}; *Pdgfra*$^{H2B\text{-}GFP/+}$ mice. Transplanted cells have endogenous GFP fluorescence when upregulating PDGFRa. H&E stains show exogenous bone growth and muscle tissue morphology. **a)**200x image of a section of a SCID tongue that received Tie2-Cre; *Acvr1*^{R206H}; *Pdgfra*$^{H2B\text{-}GFP/+}$ tongue FAP cells. This image shows the midbelly of the tongue where the host cells were transplanted. GFP presence supports engrafting of the transplanted cells. This is surrounded by a larger area where no myofibers are present. This could indicate that the cells engrafted into surrounding connective tissue, or degeneration of surrounding muscle tissue. **b)**200x image of an H&E stain of the same area, on the same section of the tongue tissue as **a**. Arrows point to very cellularized area and an absence of myofibers. GFP+ cells seen in **a** are at the same location of the disorganization and degeneration of myofibers seen in **b**. **c)**100x image of a section of a SCID tongue that received Pdgfra-Cre; *Acvr1*^{R206H}; *Pdgfra*$^{H2B\text{-}GFP/+}$ tongue FAP cells. GFP presence supports engrafting of the transplanted cells. The pattern of GFP+ cells is comparable to what is normally seen in FOP induced HO, in which it surrounds the bone nodules. **d)**100x image of an H&E stain of the same area, on the same section of tongue tissue as **c**. Arrows point to bone morphologies on this section. GFP+ cells seen in **c** are at the same location of the HO seen in **d**.

Discussion

Fibrodysplasia ossificans progressive is characterized by heterotopic ossification, the debilitating growth of exogenous bone in soft tissue[5,7,11,14]. Since the work of the Goldhamer lab identified a cell of origin for this bone growth, our understanding of the disease has changed substantially. FAPs are now a large focus for FOP research, as our knowledge of the behavior of mutated FAPs could lead to therapeutics. FAPs are a heterogenous population that play a role in growth and regeneration in response to environmental signals[1,7,8,9]. In diseased tissue, FAPs inappropriately signal for osteogenesis during muscle regeneration[5,7,10,14]. The tongue muscle contains a population of FAPs, but does not from HO clinically in an FOP diseased tongue. In this report we conducted research to evaluate the osteogenic capacity of the population of FAPs present within the tongue muscle.

We used an FOP accurate mouse model to test the effect of the disease on the tissue in the tongue[7,19]. Pinch injury did not form HO in Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ and Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ mice. Histology showed that there were few centralized nuclei throughout the mid-belly of the tongue. Previous literature states that the anterior of the tongue has the lowest percent of muscle tissue, but the highest percent of connective tissue. In the medial tissue of the tongue there is more muscle tissue and less connective tissue[17]. The pinch injuries were more medial, where the myofibers are more densely packed and complex. Due to the nature of the myofiber organization and the minimal centralized nuclei, we speculate that the pinch injury did not have enough force to provoke injury and regeneration in the tongue. Clinically, the major forms of injury of the tongue tissue are burns, which would only affect the outer mucosal layers, and bites. As the pinch injury did not provide enough force to cause regeneration within the

tongue to induce HO, we could speculate that biting the tongue would not either. These could serve as reasons we do not clinically see HO in the tongue, but we do in a lab setting.

The limitations of pinch injury assay were resolved by cardiotoxin injury. Cardiotoxin is a myonecrotic agent that spreads throughout the soft tissue[2,7]. We injected the cardiotoxin into the medial tongue area to affect the myofibers in the mid-belly of the tongue. This form of injury reproducibly induced HO in Pdgfra-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ tongues. These findings indicate that the extracellular environment of the tongue is competent for HO in the presence of the $Acvr1^{tnR206H}$ mutation. Interestingly, histology of this tissue showed complete disorganization of the myofibers in the mid-belly of the tongue that was far more expansive than the size of the bone nodule. This is an atypical phenotype of regenerative tissue with the $Acvr1^{tnR206H}$ mutation [15]. Research on tongue muscle satellite cell biology is limited, hindering our understanding of the regenerative process in the tongue[12]. Further research into the regenerative process and cellular composition of the tongue may explain this injury induced phenotype.

In contrast, HO formation in Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ tongues following cardiotoxin injury had ambiguous results. This mouse model formed HO less consistently, and in smaller quantities. These tongues were fixed in 4% PFA before they were scanned using the IVIS CT machine; in recent conversations with members of my lab, it has been brought to my attention that fixing tissue prior to CT scans can have an effect on the readings from the IVIS machine. Previous scans with wild-type mice, which should not have any HO, showed positive results for HO only if they were fixed beforehand. Since the bone nodules seen in the Tie2-Cre model were notably small, this is worthy of consideration for the three Tie2-Cre mice that had HO. Additionally, histological analysis showed cellularization and disorganization of injured tissue, but no bone nodules were observed. While bone morphologies were not seen with an

H&E stain, we cannot confidently state that osteocytes were not present until this tissue is stained for bone markers.

Our next goal was to understand why there were different findings in osteogenic ability, quantity of HO and myofiber retention between the two mouse models. We speculated that these results could be explained by recombination efficiency or an intrinsic difference in the two FAP populations. Previous research using flow cytometry, shows that Pdgfra-Cre has almost a three-fold greater recombination efficiency than that of Tie2-Cre[7]. Therefore, more FAPs are recombined to have the *Acvr1*^{R206H} mutation when controlled by the *Pdgfra* promoter, than the *Tie2* promoter. This could explain the deficit of HO observed in the Tie2-Cre; *R26*$^{NG/;}$ *Acvr1*^{tnR206H} model in the injury assay. Additionally, this difference could be due to the specific FAP population that is recombined in each model. Subpopulations of FAPs are present at different times throughout regeneration of muscle tissue[8,9]. A distribution profile of subpopulations of FAPs showed that TIE2+ FAPs peaked a day after acute injury, and slowly returned to baseline levels[8,9]. Interestingly, ossification occurs at later timepoints following injury, when different subpopulations of FAPs peak [8,9]. Therefore, there could be a population of osteo-competent FAPs that are recombined in the Pdgfra-Cre; *R26*$^{NG/+}$*; Acvr1*^{tnR206H} mouse model that are not recombined in the Tie2-Cre; *R26*$^{NG/;}$*, Acvr1*^{tnR206H} mouse model.

The cross-transplantation experiments revealed that there may be an intrinsic cell difference between the Tie2-Cre FAPs and the Pdgfra-Cre FAPs. We collected an equal number of recombined tongue FAPs from both mouse models through FACS isolation and transplanted an equal density of isolated FAPs. This eliminated the recombination efficiency difference seen *in vivo* between Tie2-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} and Pdgfra-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} FAPs. With the same population of recombined FAPs, cells with a Pdgfra-Cre driver

still showed a greater capacity for HO. Pdgfra-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} tongue FAPs formed HO when transplanted into the tongue, supporting findings from the injury assays. In contrast Tie2-Cre; *Pdgfra*$^{H2B\text{-}GFP/+}$; *Acvr1*^{R206H} and Tie2-Cre; *R26*$^{NG/+}$; *Acvr1*^{R206H} tongue FAPs did not form HO in the tongue or the hindlimb.

These results suggest that Tie2-Cre; *R26*$^{NG/+}$*; Acvr1*^{R206H} tongue FAPs have an intrinsic inability to produce HO. More cross transplantation and cardiotoxin injury experiments must be done to increase the *n* to make a confident claim. Further, cardiotoxin injury of tongue tissue of Tie2-Cre; *R26*$^{NG/+}$*; Acvr1*^{tnR206H} mice must be redone without fixing the tissue prior to IVIS scans. This will eliminate the possibility that there are artifacts from fixing the tissue that register as ossified tissue. From the present data, we speculate that there is a subpopulation of FAPs that are imperative for tongue ossification that are not recombined when the *Tie2* promoter is the driver of Cre expression. This can be analyzed through FACS isolation and transplantation of PDGFRa+, TIE2- tongue FAPs. Testing osteogenic ability of this population will confirm if the osteogenic origin resides in a different subpopulation of FAPs.

Notably, Tie2-Cre; *R26*$^{NG/+}$*; Acvr1*^{R206H} tongue FAPs did not form HO when transplanted into the hindlimb. In contrast, Tie2-Cre; *R26*$^{NG/+}$*; Acvr1*^{R206H} hindlimb FAPs can form bone in the hindlimb. This would indicate that there is an intrinsic difference between Tie2-Cre tongues FAPs and hindlimb FAPs. FAPs are dynamic throughout the regenerative process, upregulating subpopulations at different time points depending on extracellular signals[8,9]. However, there is no literature on different subpopulations within the Tie2+ subpopulation of FAPs. One hypothesis is that environmental signals in the tongue shutoff certain genes in Tie2+ cells during development that are active in the hindlimb Tie2+ cells, or vice versa. Single cell mRNA sequencing is a technology that can identify the expression of genes and distinguish cells at the

molecular level[6]. This would be an advantageous next step to detect differences in cell surface markers or internal cellular components at play.

Conclusion

The findings in these experiments indicate that the extracellular environment of the tongue is competent for HO. While there have been no reported cases of clinical HO in an FOP diseased tongue, upon thorough injury HO can be provoked.

It is also important to acknowledge a possible intrinsic deficit in the Tie2+ FAP cells within the tongue. However, injury of Tie2-Cre; $R26^{NG/+}$; $Acvr1^{tnR206H}$ without fixing the tissue prior to IVIS scans and repeated cross transplantation experiments need to be conducted before we can confidently draw this conclusion. These results will dictate the need for further exploration of this population.

Ultimately, the tongue is a supportive environment for HO. More information on the unique behaviors of the subpopulations of FAPs that reside within the tongue can be very beneficial for our understanding of FOP.

Literature Cited:

1. Biferali, B., Proietti, D., Mozzetta, C., & Madaro, L. (2019). Fibro–Adipogenic Progenitors Cross-Talk in Skeletal Muscle: The Social Network. *Fronteirs in Physiology*, *10:1074*(10.3389). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6713247/
2. Dalle, S., Hiroux, C., Poffe, C., Ramaekers, M., Deldicque, L., & Koppo, K. (2020). Cardiotoxin-induced skeletal muscle injury elicits profound changes in anabolic and stress signaling, and muscle fiber type composition. *SpringerLink*, *41*(10.1007), 375–387. https://link.springer.com/article/10.1007/s10974-020-09584-5
3. Dotiwala, A. K., & Samra, N. S. (2021). Anatomy, Head and Neck, Tongue. *StatPearls*, *NBK507782*. https://www.ncbi.nlm.nih.gov/books/NBK507782/

4. Giuliani, G., Rosina, M., & Reggio, A. (2021). Signaling pathways regulating the fate of fibro/adipogenic progenitors (FAPs) in skeletal muscle regeneration and disease. *Federation of European Biochemical Societies*, *10.1111*. https://febs.onlinelibrary.wiley.com/doi/pdf/10.1111/febs.16080
5. Hatsell, S. J., Idone, V., Alessi Wolken, D. M., Huang, L., Kim, H. J., Wang, L., Wen, X., Nannuru, K. C., Jimenez, J., Kie, L., Das, N., Makhoul, G., Chernomorsky, R., D'Ambrosio, D., Corpina, R. A., Schoenherr, C., Feeley, K., Yu, P. B., Yancopoulos, G. D., … Economides, A. N. (2015). ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. *Science Translational Medicine*, *7*, 303–303. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6164166/
6. Hwang, B., Hyun Lee, J., & Bang, D. (2018). Single-cell RNA sequencing technologies and bioinformatics pipelines. *Experimnetal and Molecular Medicine*, *50*, 1–14. https://www.nature.com/articles/s12276-018-0071-8
7. Less-Shepard, J. B., Yamamoto, M., Biswas, A. A., Stoessel, S. J., Nicholas, S. E., Cogswell, C. A., Devarakonda, P. M., Schneider, M. J., Cummins, S. M., Legendre, N. P., Yamamoto, S., Kaartinen, V., Hunter, J. W., & Goldhamer, D. J. (2018). Activin-dependent signaling in fibro/adipogenic progenitors causes fibrodysplasia ossificans progressiva. *Nature Communications*, *9*(471). https://www.nature.com/articles/s41467-018-02872-2
8. Malecova, B., Gatto, S., Etxaniz, U., Passafaro, M., Cortez, A., Nicoletti, C., Giordani, L., Torcinaro, A., de Bardi, M., Bicciato, S., de Santa, F., Madaro, L., & Lorenzo Puri, P. (2018). Dynamics of cellular states of fibro-adipogenic progenitors during myogenesis and muscular dystrophy. *Nature Communications*, *9*(3670). https://www.researchgate.net/publication/327559642_Dynamics_of_cellular_states_of_fibro-adipogenic_progenitors_during_myogenesis_and_muscular_dystrophy/fulltext/5b96a46e4585153a531d8bdf/Dynamics-of-cellular-states-of-fibro-adipogenic-progenitors-during-myogenesis-and-muscular-dystrophy.pdf
9. McKinnon, K. M. (2018). Flow Cytometry: An Overview. *U.S. National Library of Medicine*, *120*, 1–11. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5939936/
10. Molina, T., Fabre, P., & Dumont, N. A. (2021). Fibro-adipogenic progenitors in skeletal muscle homeostasis, regeneration and diseases. *The Royal Society*, *12*(210110). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8651418/
11. Pignolo, R. J., Shore, E. M., & Kaplan, F. S. (2011). Fibrodysplasia Ossificans Progressiva: Clinical and Genetic Aspects. *U.S. National Library of Medicine*, *1*(6), 80.
12. Randolph, M. E., & Pavlath, G. K. (2015). A muscle stem cell for every muscle: variability of satellite cell biology among different muscle groups. *Frontiers in Aging Neuroscience*, *7*(190). https://www.frontiersin.org/articles/10.3389/fnagi.2015.00190/full
13. Relaix, F., & Zammit, P. S. (2012). Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *The Company of Biologists*, *139*(16). https://journals.biologists.com/dev/article/139/16/2845/45218/Satellite-cells-are-essential-for-skeletal-muscle
14. Shore, E. M. (2012). Fibrodysplasia ossificans progressiva (FOP): A human genetic disorder of extra-skeletal bone formation, or - How does one tissue become another? *U.S.*

National Library of Medicine, *1*(1), 153–165. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3297114/

15. Tasic, D., Pavlovic, M., Stankovic, D., Dimov, I., Stanojevic, G., & Dimov, D. (2012). Ossifying chondrolipoma of the tongue. *U.S. National Library of Medicina*, *69*(11), 1009–1012. https://scindeks.ceon.rs/
16. Miller, J. L., Watkin, K. L., & Chen, M. F. (2002). Muscle, Adipose, and Connective Tissue Variations in Intrinsic Musculature of the Adult Human Tongue. *EBSCO Host*, *45*, 51–65. https://web-s-ebscohost-com.ezproxy.lib.uconn.edu/ehost/pdfviewer/pdfviewer?vid=0&sid=3acc0b44-1451-410c-a848-be214c322769%40redis
17. Wosczyna, M. N., Biswas, A. A., Cogswell, C. A., & Goldhamer, D. J. (2012). Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. *Journal for Bone and Mineral Research*, *27*(5), 1004–1017. https://asbmr.onlinelibrary.wiley.com/doi/10.1002/jbmr.1562
18. Yamamoto, M., Shook, N. A., Kanisicak, O., Yamamoto, S., Wosczyna, M. N., Camp, J. R., & Goldhamer, D. J. (2009). A Multifunctional Reporter Mouse Line for Cre- and FLP-Dependent Lineage Analysis. *U.S. National Library of Medicina*, *47*(2), 107–114. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8207679/