 Localization of Gli1+ Expressing Cells in Adult Mouse Molars

Divya Puri
dpuri@uchc.edu

Follow this and additional works at: https://opencommons.uconn.edu/gs_theses

Recommended Citation
https://opencommons.uconn.edu/gs_theses/1542

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact opencommons@uconn.edu.
Localization of Gli1+ Expressing Cells in Adult Mouse Molars

Divya Puri
Doctor of Dental Medicine
Nova Southeastern University College of Dental Medicine, 2017

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Masters of Dental Science
At the
University of Connecticut
2020
Approval Page

Masters of Dental Science Thesis
Localization of Gli1+ Expressing Cells in Adult Mouse Molars

Presented by:
Divya Puri, DMD

Major Advisor
___________________________________________________________
Mina Mina, D.M.D., M.Dent.Sc., Ph.D.

Associate Advisor
___________________________________________________________
Bahar Houshmand, D.M.D

Associate Advisor
___________________________________________________________
Bina Katechia, B.D.S., M.Sc.

University of Connecticut
2020
Acknowledgements

My time at University of Connecticut has been a memorable experience that I will forever cherish. I am grateful to have received unconditional support and guidance from my advisors and teachers. I would like to express my gratitude to my advisor and primary investigator, Dr. Mina Mina, Chair Division of Pediatric Dentistry. I admire Dr. Mina’s passion for science and the quality of the work she and her laboratory produces. I am fortunate to have been accepted into an environment which has allowed me to blossom while pursuing my Masters project. Dr. Mina has taught me so much about dentistry, science, and how to approach the world as a critical thinker. Dr. Mina has always been a pillar of support to which to lean on and I could not be more grateful.

I am very fortunate to have matched at UConn and to have met Dr. Bina Katechia, Program Director, Pediatric Dentistry. Dr. Katechia is an amazing leader with a kind and gentle heart. Dr. Katechia has helped me grow into a more confident pediatric dentist and has passed on her wisdom concerning the real world of practice. She has calmed and guided me in times of uncertainty, and for this, I am thankful.

I would also like acknowledge my advisor Dr. Bahar Houshmand, Assistant Professor. Dr. Houshmand passed along her knowledge of scientific techniques and guided me through the gritty details of how to conduct my experiments. She motivates me to work harder, listens to my troubles, and helps in any way she can. Relationships such as the one I have with Dr. Houshmand made this project possible.

I would like to thank the members of Dr. Mina’s lab, who accepted me with welcoming arms. Anushree Vijaykumar helped me learn the scientific methods needed, and took the time to make me feel welcome through her patience and kindness. She helped me navigate as I first entered
the lab world. Sierra Root is a wonderful instructor who is very thoughtful and caring. She was always there to guide, edit, and adjust my thesis so that I could produce the best work possible. I am deeply appreciative of the time these individuals took out of their lives to assist me.

The faculty at UConn undoubtedly go above and beyond to help their residents flourish. I would like to thank all of the faculty of UConn Pediatric Dental department: Drs. Jorge Rabat, Gary Schulman, Yu-Hsiung Wang, Karen Prosterman Kemp, Moises Salas and Deborah Redford-Badwal. I would like to extend my appreciation towards all the administration, staff and assistants of the Pediatric Dental department. Special mentions: Ms. Josefina Ruiz, Carmen Vazquez, Paulette Chung, Johana Salgado, Daisey Alicea, and Pamela Heberle. I also want to thank my amazing co-residents for always having my back and keeping me sane through all my ups and downs. I could not have asked for a better group of people to experience this journey with.

I want to thank all the individuals who helped with various aspects of this research including technical support, providing the mice and providing reagents. I would like to thank Dr. Ivo Kalajzic and his lab for providing the necessary reagent for completion of my project. I would like to thank Ms. Chen Li (Dr. David Rowe’s lab) for always being kind and helpful especially when I was first learning to section. I would also like to thank NIDCR who provided the generous grant which made this project possible (NICDR(R21DE027807)).

Lastly, I would like to thank my family without whom I would not be here today. I want to thank my parents who have always supported me in all my endeavors. I want to thank my husband Brian for always being my rock.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>vi</td>
</tr>
<tr>
<td>Introduction and Background</td>
<td>1</td>
</tr>
<tr>
<td>A. Label Retaining and Lineage Tracing</td>
<td>2</td>
</tr>
<tr>
<td>B. Mouse Model</td>
<td>3</td>
</tr>
<tr>
<td>C. Gli1 and Sonic Hedgehog Pathway</td>
<td>4</td>
</tr>
<tr>
<td>D. MSCs and Neurovascular Niche</td>
<td>7</td>
</tr>
<tr>
<td>Hypothesis and Specific Aims</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>12</td>
</tr>
<tr>
<td>A. Animal Model</td>
<td>12</td>
</tr>
<tr>
<td>B. Histological Analysis</td>
<td>14</td>
</tr>
<tr>
<td>C. Immunohistochemical Analysis</td>
<td>14</td>
</tr>
<tr>
<td>D. Immunofluorescence Analysis</td>
<td>15</td>
</tr>
<tr>
<td>E. Image and Statistical Analysis</td>
<td>15</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>A. Specificity of Cre Activation</td>
<td>16</td>
</tr>
<tr>
<td>B. Location of Gli1 Expression in Mature Mouse Molars</td>
<td>17</td>
</tr>
<tr>
<td>C. Correlation of Gli1+ Expressing Cells with CD31+ Vascular Cells and CGRP+ Neuronal Cells in Three Week Mice</td>
<td>20</td>
</tr>
<tr>
<td>D. Correlation of Gli1+ Expressing Cells with CD31+ Vascular Cells and CGRP+ Neuronal Cells in Four Week Mice</td>
<td>25</td>
</tr>
<tr>
<td>E. Correlation of Odontoblasts with Lineage Traced Gli1-tdTomato+ Reporter Expression and CD31+ Blood Vessels in Four Week Maxillary Molars</td>
<td>30</td>
</tr>
<tr>
<td>Discussion and Conclusion</td>
<td>31</td>
</tr>
<tr>
<td>Future Direction</td>
<td>35</td>
</tr>
<tr>
<td>References</td>
<td>36</td>
</tr>
</tbody>
</table>
Abstract

Identification of the mesenchymal stem cell (MSC) population capable of giving rise to odontoblasts secreting dentin during growth (primary dentinogenesis) and repair (reparative dentinogenesis) is critical for the development of improved pulp therapy, regeneration of dentin and ultimately, the bioengineering of functional teeth. Recent lineage tracing studies have led to the identification of several new MSCs and their associated niche referred to as the neurovascular bundle, located at the apical end of the incisor. This newly identified niche contains several populations of MSCs including Gli1+ (glioma-associated oncogene homolog 1), cells that are activated by Shh secreted by sensory nerves (3, 6). Furthermore, these studies showed contribution of Gli1+ cells to odontoblasts during growth and repair, in incisors. Despite significant progress in the identification of MSCs within the mouse incisors in vivo, we know much less about MSCs in mouse molars that, unlike incisors, are not continuously growing and are more similar to human dentition. Therefore, the long-term goal of our studies is to gain insight into the roles of neurovascular niche and Gli1 expressing cells in primary and reparative dentinogenesis in mice molars.

As the first step in the present study we evaluated the neurovascular niche and Gli1 expressing in adult mice molars using Gli1-CreERT2/Ai9 tomato reporter mice. The expression of transgenes was correlated with the expression of vascular marker CD31 and neuronal marker CGRP. The results show that Gli1+ expressing cells were identified in close proximity to CD31+ blood vessels and CGRP+ nerve fibers in the apical portion of mature mouse molars, thereby indicating a close association of Gli1+ reporter cells to the neurovascular bundle. Understanding the supporting niche of Gli1+ cells within the dental pulp is an important step in identifying these
MSCs and their further lineage commitment to odontoblast-like cells, which could serve as a valuable cell source for the generation of reparative dentin after tooth injury.
Introduction

Mesenchymal stem cells (MSCs) play an important role in tissue repair and regeneration. Initially discovered in bone marrow, MSCs have since been localized in other tissues, including skeletal muscle, adipose tissue, placenta, teeth and bone (Zhao et al., 2014). MSCs are best characterized by their multipotent differentiation potential and immunomodulation abilities (Bernardo & Fibbe, 2013). To define MSCs is challenging and has, at times, been controversial. The current definition of MSCs is based on a set of criteria which includes their in vitro trilineage differentiation ability (osteogenic, chondrogenic and adipogenic differentiation potential), as well as their expression of various MSC surface markers (Bianco et al., 2013; Dominici et al., 2006; Keating, 2012). However, the in vivo markers of MSCs have yet to be fully defined and understood.

MSCs have been found in both the dental pulp and periodontal structures, collectively known as dental mesenchymal stem cells. Not all dental MSCs are equal in their phenotypic and functional properties (Sharpe, 2016). During tooth injury, the dental pulp stem cells (DPSCs) play a significant role in repair. Mild damage to the dentin allows the existing odontoblasts to generate reactionary dentin. However, when the damage is severe and penetrates the pulp, injury to the odontoblasts becomes lethal. It is this more extensive damage that triggers activation of the dental pulp stem cells to differentiate into odontoblast-like cells (Mitsiadis, Catón, Pagella, Orsini, & Jimenez-Rojo, 2017). These odontoblast-like cells can then generate reparative dentin, creating a bridge to protect the underlying pulp (Sharpe, 2016). DPSCs are housed in niches, or microenvironments, that allow cells to survive, repair and regenerate (Mitsiadis et al., 2017). To maintain homeostasis in the tooth, DPSCs remain active throughout life and differentiate into odontoblast-like cells when needed (Sharpe, 2016).
A. **Label Retaining and Lineage Tracing**

Recent *in vivo* studies sought to identify and localize MSCs in their supporting niches using label-retaining and lineage tracing techniques (Vidovic et al., 2017). Label-retaining is useful in the identification of adult stem cells. In this technique, cells are pulsed with a label (pulse phase), such as the thymidine analog bromodeoxyuridine (BrdU), at a time when the tissue is highly proliferative, so that the label may be incorporated into the newly formed DNA of these cells (Ishikawa et al., 2010). During the chase phase, an unlabeled compound replaces the labeled compound where the transit-amplifying cells dilute the label and the slow-cycling cells retain the label and thus, are marked as stem cells (Ceder, Aalders, & Schalken, 2017).

Lineage tracing techniques are used to identify and localize stem cells and their progenies over time *in vivo* (Y. C. Hsu, 2015). This method permanently marks the cell of interest *in vivo* and their progenies so that they can be examined at specific points in time. One of the widely used techniques to accomplish lineage tracing is through the Cre-lox system (Y.-C. Hsu, 2015). In this method, one mouse line expresses Cre recombinase under the control of a specific tissue or cell promotor. This Cre mouse line is crossed with a second mouse line, which houses a stop sequence, which is “floxed” or “flanked” between 2 specific loxP sites. Upon crossing of the two mouse lines, a new animal is produced which contains Cre and the reporter. When a cell contains the tissue or cell specific promotor, Cre recombinase excises the 2 loxP sites, thereby removing the stop cassette, and thus allowing for expression of the reporter thereby labeling the cells of interest for the life of the cell regardless of further tissue or cell specific promotor expression (Kretzschmar & Watt, 2012). This type of Cre cross is more of a historical marker of the cell as it is from the germline. To perform a more accurate genetic lineage tracing, the Cre-lox system was enhanced through addition of tamoxifen (TM) (Kim, Kim, Im, & Fang, 2018). Through this system, one is
able to control the precise timing of Cre activation in specific tissues (Kim et al., 2018). In the tamoxifen-inducible Cre system, the Cre enzyme is attached to a mutated estrogen receptor (ER) which binds only to tamoxifen; collectively this unit is called CreERT (Zhong et al., 2015). Upon binding of TM, the CreERT translocates from the cytoplasm into the nucleus and activates the reporter through Cre recombinase and loxP sites (Kim et al., 2018). Therefore, the cells of interest will express the reporter only when TM is administered. Additionally, the reporter will also be seen in the daughter cells from the cells of interest (Hosoya, Shalehin, Takebe, Shimo, & Irie, 2020). This method is very useful to study cells of interest and also their cell lineages at different points in time. Therefore, using the tamoxifen inducible Cre system, TM administration at specific time points in development allows for the cells of interest to be identified and their lineage traced with respect to time.

Three requirements need to be met to successfully perform a lineage tracing. These requirements include: a) the cells of interest at the initial time point are defined b) the marker selected must be exclusive to the original cell and its progenies as to not interfere with other non-related cells c) the markers should be stable and not toxic to the cells during the entire period of tracing (Y.-C. Hsu, 2015).

B. Mouse Model

Many studies have used the mouse incisor model to understand MSCs due to its continuous growth through the lifetime of the mouse. The mouse incisors undergo wear and attrition during feeding. To compensate for the loss of tooth structure at the incisal end, the incisors continue to grow to occlusion (Sharpe, 2016). The incisors are able to grow due to the continuous formation of enamel and dentin, which is made possible by the presence of active adult epithelial and
mesenchymal stem cells, respectively (Kuang-Hsien Hu, Mushegyan, & Klein, 2014). For the incisor, these quiescent and cycling adult epithelial stem cells can be found in the apical end of the incisor at the cervical loop region (Juuri et al., 2012). Unlike mouse incisors, after tooth eruption, mature mouse molars do not continuously grow and thus, can be compared to human teeth. Therefore, both murine incisors and molars are important models to use for the study of MSC populations and their regenerative potential.

C. **Gli1 and Sonic Hedgehog Pathway**

Sonic hedgehog pathway is responsible for cell proliferation, differentiation, patterning, growth and development (Bai, Auerbach, Lee, Stephen, & Joyner, 2002). Sonic hedgehog (Shh) signaling has been seen in the development of several craniofacial tissues such as the palate, salivary gland and teeth (Hosoya et al., 2020). This pathway is mediated through transmembrane proteins called Patched (Ptc1) and Smoothened (Smo), which form a receptor complex (Figure 1) (Cobourne, Miletich, & Sharpe, 2004). In the resting state without Shh ligand, Ptc1 inhibits the activity of Smo. Proteasome cleavage of Gli results in the formation of carboxyl-terminus-truncated repressor Gli, which move to the nucleus and repress the Gli-dependent transcription of target genes (Ruiz i Altaba, Palma, & Dahmane, 2002). However, when Shh binds to Ptc1, Smo inhibition is relieved and the active receptor complex with Smo is released thereby activating a macromolecular complex. Proteins within this macromolecular complex includes Su(fu) (Suppressor of fused), Fu (Fused), PKA, and Gli proteins. Activated Gli proteins are imported into the nucleus. Gli proteins have DNA-binding zinc finger domains that bind to specific sequences on their target genes to either initiate or inhibit transcription (Hosoya et al., 2020).
Figure 1: Sonic Hedgehog Pathway: Sonic hedgehog pathway represented with the a) absence of Shh ligand and b) presence of Shh ligand.

Gli1 is one of the downstream target genes of the sonic hedgehog pathway (Hosoya et al., 2020). It was initially discovered as an amplified gene and potential oncogene in a human glioblastoma (Kinzler et al., 1987). The sonic hedgehog pathway is mediated by the Gli gene family which includes Gli1, Gli2 and Gli3. These genes are expressed in many organs during mouse development (Hui, Slusarski, Platt, Holmgren, & Joyner, 1994; Ruppert et al., 1988). In the sonic hedgehog pathway, Gli1 acts as a transcriptional activator, whereas Gli3 acts as a transcriptional inhibitor (Merchant, Joseph, Wang, Brennan, & Matsui, 2010). This pathway has been found to be important for several developmental and stem cell processes including in the mouse incisor (Zhao et al., 2014).

Several studies have attempted to understand the role of Shh in stem cell populations by using the growing mouse incisor model. Seidel et al. showed that Hh is the principal ligand in the mouse incisor and is located in three regions including the labial cervical loop (laCL), lingual
cervical loop (liCL) and in the mesenchyme between the two loops. The cervical loops, in particular laCL, contain epithelial stem cells which house ameloblasts progenitors capable of differentiation into enamel producing cells, whereas the mesenchyme located between the cervical loops is believed to contain stem cells for dentin producing odontoblasts (Seidel et al., 2010). The three above regions contain Hh-responsive cells, which were identified through the expression of the Gli1 and Ptch1 reporters. Through genetic lineage tracing, it was found that Hh signaling regulates the generation of ameloblasts from adult stem cells in the mouse incisor (Seidel et al., 2010). Additionally, they found that there was evidence for a feedback loop where the differentiated cells were able to produce a signal that regulates their formation from the adult stem cells (Seidel et al., 2010).

Populations of MSCs in the dental pulp of incisors that have been discovered include slow cycling Thy1-expressing mesenchymal cells, peripheral nerve-associated glial cells, NG2-expressing pericytes, and Gli1-expressing (Gli1+) cells (Feng, Mantesso, De Bari, Nishiyama, & Sharp, 2011; Zhao et al., 2014). Glioma-associated oncogene homolog 1, or Gli1, is part of the sonic hedgehog (Shh) signaling pathway and has been suggested to be an important in vivo dental stem cell marker and has already been identified in mouse incisors (Seidel et al., 2010; Zhao et al., 2014). Therefore, Gli1+ cells could serve as potential MSCs of interest to study in vivo within other dental tissues, including the dental pulp of mouse molars. As the expression of Gli1 and supporting niche has not been studied in mature mouse molars, the objective of this study was to identify Gli1+ expressing cells near the neurovascular bundle in mature mouse molars. In this study, we refer to mature mouse molars as mice that are three to four weeks old.
D. MSCs and Neurovascular Niche

Defining MSCs has always been difficult. While multiple in vitro markers of MSCs are well known, defining in vivo markers and supporting niches of MSCs remains elusive in many tissues. Recent studies have investigated mouse incisor MSCs and not only identified, but also provided a regulatory and homeostatic role, for their neurovascular niches. One of the studies has shown the first evidence of a supporting neurovascular microenvironment for MSCs in mouse incisors (Zhao et al., 2014). These researchers identified the neurovascular bundle as an adequate in vivo marker of MSCs. In fact, MSCs in incisors were found to originate from periarterial cells in vivo and were supported throughout their lifespan in neurovascular bundles. We hypothesized that a similar niche may exist within mouse molars. The role of neuronal and vascular signals in homeostasis and differentiation of MSCs has been an area of focus in incisors and molars.

a. Neurons and MSCs in mouse incisor and molars

Several studies have utilized the calcitonin gene-related peptide (CGRP) as a nerve marker within murine incisors and molars. This marker has been used to identify locations of sensory nerves, as well as study their developmental patterns. One study looked at the different locations and developmental timing of peripherin (PER) and CGRP-immunoreactive nerve fibers in rat molars during tooth maturation by studying the molars of rats from 10 days to 1 year in age and looking at nerve patterns in comparison with odontoblast maturation (Veerayuthwilai, Luis, Crumpton, MacDonald, & Byers, 2006). The results showed that prior to tooth eruption, PER nerve fibers are present and innervate odontoblasts but not dentin. Two weeks after eruption of the first molars, CGRP beaded nerve fibers entered the odontoblast layer and then proceed to the dentin.
As the rat continued to age, they found that the CGRP nerve fibers continued to increase until root growth was completed (Veerayuthwilai et al., 2006).

CGRP marker was also utilized by Zhao et al. in their study to identify the neurovascular bundle as a MSC niche. Specifically, they demonstrated a link between CGRP sensory nerve fibers and Shh signaling in mouse incisors. They found that Shh produced by the sensory neurons of the trigeminal ganglia is transported through the inferior alveolar nerve which activates Gli1 expression in the adjacent periarterial mesenchymal cells (Zhao et al., 2014).

b. Perivascular Niche and MSCs in mouse incisors and molars

Studies of MSC populations in various tissues have shown that these cells are located within a perivascular niche. MSC populations have been found inside the dental pulp as this is a highly vascularized tissue with regenerative properties (Saghiri, Asatourian, Sorenson, & Sheibani, 2018). Additionally, these perivascular MSCs can be identified using markers which include CD146, chondroitin sulfate proteoglycan 4 (NG2), platelet-derived growth factor receptor-beta (PDGF-Rß), and alpha- smooth muscle actin (a-SMA) (Crisan et al., 2009; Crisan, Corselli, Chen, & Péault, 2011; Crisan et al., 2008; Vidovic et al., 2017). A study by Vidovic et al used lineage tracing to examine a-SMA expressing perivascular cells and their role in primary/reparative dentinogenesis in mouse incisors and molars in vivo. They looked at a-SMA expression during tooth growth and development as well as the effects after dental pulp injury. In their study, CD31 was used as the endothelial marker. The results showed that a-SMA expressing perivascular cells contributed to a small population of progenitor MSCs, which gave rise to second generation odontoblasts during reparative dentinogenesis (Vidovic et al., 2017). Furthermore, they
found that this progenitor MSC population makes a small contribution during primary dentinogenesis (Vidovic et al., 2017).

Additionally, NG2$^+$ pericytes have also been thought to represent a population of MSCs in the dental mesenchyme (Feng et al., 2011). Zhao et al. showed that NG2$^+$ pericytes surround vascular cells marked by CD31 also known as PECAM-1. While it is proposed that the perivascular space acts as a niche for the MSCs, the function of the vascular cells in regulating MSCs still remains unknown as well as whether arteries, veins and capillaries comprise different MSC niches (Zhao et al., 2014).

c. **Neurovascular Bundle (NVB)**

Neurovascular bundles contain both nerves and blood vessels that run together and provide an intricate environment for stem cells. The neurons and blood vessels are surrounded by loose connective tissue which forms the entire neurovascular bundle (Zhao et al., 2014). Several studies that have examined the murine incisor and molars have found that the nerves run with blood vessel, forming neurovascular bundles (Mahdee, Eastham, Whitworth, & Gillespie, 2019; Zhao et al., 2014). To understand the function of MSCs, we must also understand their supporting environment. NVB areas are rich with MSCs and control multiple molecular and cellular components (Zhao et al., 2014). Zhao et al. showed the importance of the NVB as an MSC niche. They found that the mouse incisor MSCs are localized near the arterioles and only the arterioles that were accompanied by nerves. Furthermore, they found that it is the NVB sensory neurons which secrete Shh that activates the Gli1 expression in the periarterial cells. Therefore, localizing the MSCs and their NVB niches is an important step in identifying MSCs and their function *in vivo*. 
Rationale:

In our preliminary studies, using Gli1-CreERT2;Ai9 reporter mice, we detected Gli1-tdTomato positive cells in the dental pulp of molars 4 days post TM injection from 4 weeks old mice. We hypothesized that the location of these Gli1+ expressing reporter cells would be in proximity to CD31+ blood vessels and CGRP+ nerve cells, providing us with information about Gli+ MSCs within the neurovascular bundle of mouse molars. This is an area that has not been studied before. Specifically, we wanted to study three and four week mice as this is the time where root formation and elongation takes place (Lungová et al., 2011).

Studying MSC populations within the dental pulp of teeth will provide a better understanding of their purpose, function and regenerative potential. Although several MSC populations have been discovered in the dental pulp of the continuously growing mouse incisor, it is imperative to study mouse molars as they are more comparable to human teeth. Identifying the stem cell niches in dental pulp expands our understanding of the mechanisms and environmental cues that supports these progenitor cells and their further lineage commitment and regenerative potential. Therefore, understanding the supporting niche of Gli1+ reporter cells within the dental pulp is an important step in identifying them as a potential cell source for the generation of reparative dentin after tooth injury.
**Hypothesis:**

Recent lineage tracing studies have led to the identification of several new MSCs and their associated niche referred to as the neurovascular bundle, located at the apical end of the incisors. This newly identified niche contains several populations of MSCs including Gli1\(^+\) (glioma-associated oncogene homolog 1) with contribution to odontoblasts during growth and repair, in incisors.

Despite significant progress in the identification of MSCs within the mouse incisors *in vivo*, we know much less about MSCs in mouse molars that, unlike incisors, are not continuously growing and are more similar to human dentition. Therefore, the long-term goal of our studies is to gain insight into the roles of neurovascular niche and Gli1 expressing cells in primary and reparative dentinogenesis in mice molars.

As the first step in the present study we evaluated the neurovascular niche and Gli1 expressing in adult mice molars.

**Specific aim:**

To evaluate the presence of neurovascular niche in adult mice molars by using Gli1-CreERT2;Ai9 reporter mice and immunohistochemistry for known vascular (CD31) and neuronal (CGRP) markers.
Materials and Methods

A. Animal Model

Gli1-CreERT2/Ai9:

All procedures were approved by the UConn Health Institutional Animal Care and Use Committee and performed in an AAALAC accredited facility. Mice were grouped and housed in ventilated cages with a 12 h light cycle. Water and irradiated rodent chow was provided ad libitum. Gli1tm3(cre/ERT2)Alj/J (The Jackson Laboratory, Stock 007913) mouse model was developed by Alexandra L Joyner, Memorial Sloan-Kettering Cancer Center, New York, NY. Gli1-CreERT2 mice (mixed background) were crossed with Ai9 reporter mice (The Jackson Laboratory, B6.Cg-Gt(Rosa)26Sor(tm9(CAG-tdTomato)Hze/J). For Cre positive animals, tamoxifen (TM) leads to Cre-mediated recombination, which further leads to deletion of the stop sequences resulting in permanent tomato expression in Gli1 expressing cells and their progenies (Figure 2).

Three and four-week-old Gli-Cre+/Ai9 transgenic mice were injected twice, 24 hours apart, with tamoxifen (75g/kg of body weight; Sigma) dissolved in corn oil. At day 4 post final TM injection, mice were injected with a lethal dose of xylazine/ketamine sedative and then sacrificed by CO₂ narcosis (Figure 3).

Vehicle (VH)-injected Gli1-CreERT2;Ai9 4 week old mice were used as controls to evaluate for spontaneous Cre activation using corn oil as the VH. Evaluation for spontaneous Cre activation was completed previously in our lab by Puranik et al, and the results will be used for this study as a control. In this experiment, 4 week Gli1-CreERT2;Ai9 mice were injected with VH twice within a 24 hour interval and sacrificed at different time points using CO₂ administration.
Figure 2: Animal Model showing the crossing of Gli1-CreERT2 mice with Ai9 reporter mice. The recombination leads to the creation of Gli1-CreERT2/Ai9 transgenic mice.

Figure 3: Timeline of experimental methods. Three and four-week old Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24hr apart. Maxillary arches were harvested and processed for various analyses at indicated time points.
B. Histological Analysis

Maxillae and mandibles from at least two mice per group were harvested and stored in 4% PFA for 24 hours and decalcified in 14% EDTA for 7 days with daily changes of solution under agitation at 4°C. Decalcified tissues were stored in sucrose solution (30%) overnight. Maxillae and mandibles were embedded in Cryomatrix (Thermo Fischer Scientific) and sectioned using a Leica cryostat with sections cut at 14 µm onto Japanese Cryotape (Cryofilm 2C, Section Lab) (Dyment et al., 2016). Sections were mounted and cross linked to glass slides using Norland Optical Adhesive 61 (Norland Optical) and a UV stratlinker 1800 (Stratagene).

C. Immunohistochemical Analysis

Prior to performing immunostaining for CD31 and CGRP on maxillary molar, cryosections were first rehydrated with PBS for 30 minutes. For CD31 staining, the sections were incubated in Powerblock (BioGenex) for 20 minutes at room temperature (RT). After washing with PBS, the sections were incubated with 0.3% TritonX in PBS for 30 minutes at RT. Sections were washed with PBS and incubated overnight with goat anti-mouse CD31 primary antibody at 4 °C (1:150, R&Dsystems). Sections were washed in PBST and then incubated with secondary antibody for 1 hour at RT (1:250, donkey anti-goat Alexa 647; Jackson Immuno Research). Hoescht dye (DAPI) was used as a nuclear counterstain.

For CGRP staining, after rehydration, frozen sections were washed in PBST (PBS with 0.1% Tween-20). Sections were permeabilized in 0.3% TritonX for 20 minutes and then blocked with 10% Normal Donkey Serum (NDS) for 1 hour at RT. Sections were incubated overnight with goat anti-rat CGRP primary antibody at 4 °C (Biorad CGRP 1:500). Sections were washed in PBST and then incubated with secondary antibody (1:250, Donkey anti-goat Alexa 488, Jackson Immuno
Research) for 1 hour at 20 °C. Sections were washed three times with PBST. Hoescht dye was used to stain for nuclei. Sections were coverslipped in 50% glycerol/PBS.

D. Immunofluorescence Analysis

Sections were examined and the maxillary first molar were imaged using the 5x, 10x and 20x objectives on a AxioImager.Z1 microscope (Zeiss) equipped with Zen Blue software and appropriate filter sets for brightfield, tdTomato (Cy3), GFP (FITC), Alexa647 (Cy5), and DAPI (UV). After threshold adjustments for each color were made, the images were exported as tiff files into Adobe Photoshop CS6 for further analysis. Adjacent sections were stained with hematoxylin and eosin Y solution (aqueous) and imaged with a color camera.

E. Image and Statistical Analysis

Images from two serial sections for Gli-tdTomato reporter expression were analyzed in ImageJ (Schindelin et al., 2012) to calculate average area of tdTomato+ signal for each molar analyzed within the specified region of interest in the root (from the cementoenamel junction to the periapical root portion or root tip). Both 3 and 4 week old TM treated animals were analyzed, 1-2 per group, and then graphed in Prism 6 (GraphPad software). Data is expressed as means ± standard error of the mean (SEM) with differences between the groups assessed by unpaired student’s t-test.
Results:

A. Specificity of Cre Activation

To ensure that there is no spontaneous Cre activation, it is important to have vehicle (VH) injected control mice alongside tamoxifen (TM) injected mice. In our study, we used the tamoxifen-inducible Cre system where Gli1-CreERT2 mice were crossed with Ai9 reporter mice with red fluorescent protein variant (tdTomato). This created the inducible mouse line-Gli-CreERT2/Ai9- whereby after TM injection, Gli-tdTomato\(^+\) cells and their progenies would be permanently labeled. CreERT activation is dependent on TM, however, it is important to ensure that we did not see any leaky transgene expression without Cre activation by TM. Previously in our lab, Puranik et al injected 4 week Gli/Ai9 mice with VH (twice at 24h interval) and assessed maxillary first molars for tdtomato reporter expression. The mice were sacrificed at different time points and serial sections were performed to evaluate molars for Gli1-tdTomato\(^+\) expression. They found very few Gli1-tdTomato\(^+\) cells in or around the first molars in the VH injected mice (Figure 4). This experiment showed that the activation of the transgene was dependent upon TM administration. As we were utilizing the same Gli1-CreERT2;Ai9 mice, we could proceed with our experiment as there is no spontaneous Cre activation that would interfere with our study.
Figure 4: Lack of spontaneous Cre activation in VH-injected mice 0d, 2d and 4d post Tamoxifen injection

A) Timeline of VH injection and tissue harvest

B) Darkfield (top row) and epifluorescent (bottom row) images of maxillary first molars in VH-injected mice. Very few if any Gli1-tdTomato+ cells were detected at any time points. Dotted lines represent the outline of the pulp. Scale bar =100µm. Figure provided by Puranik et al.

B. Location of Gli1 expression in mature mouse molars

Previous studies have demonstrated the presence of Gli1+ expressing cells in mouse incisors in the apical region (Seidel et al., 2010; Zhao et al., 2014). These Gli1+ cells reside near the neurovascular bundle niche and are activated by Shh secreted by CGRP+ sensory nerves (Zhao et al., 2014). The objective of our study was to localize Gli1+ expressing cells in mature mouse molars. We specifically wanted to examine the location of Gli1+ cells in three and four week molars to evaluate the expression of Gli1+ cells during a period of high root activity. Some studies
have found that the development of the first molar root in mice is initiated at 4 days postnatal (P4) with the emergence of Hertwig’s epithelial root sheath (HERs) (Lungová et al., 2011). Between P10 and P16 the root continues to grow and advanced tooth mineralization takes place. Additionally, during this time, the alveolar bone that surrounds the dental crown is removed creating a clear path for the eruption of the tooth (Lungová et al., 2011). After the first molar crown is fully developed, at age P16, the tooth emerges into oral cavity. Specifically during P16 to P27, root elongation takes place and the apical foramen remains open (Kawakami, Nakamura, & Karibe, 2015). Therefore, we specifically wanted to look at the time period between P16 and P27 to see the location of Gli1+ cells after eruption of the maxillary molar and during root development.

Our first aim was to, define the location of Gli1-tdTomato+ cells in the molars of TM treated three and four-week mice (Figure 5). In both three and four weeks TM treated animals, we identified Gli1-tdTomato+ cells at day 4 post TM injection localized to the apical region of the roots, as well as in the periodontal ligament space and alveolar bone (Figure 5). While in both three and four-week mouse molars we see the expression of Gli1+ cells at the apical region, there is a difference in the amount of expression between the two time points. Specifically, in three week molars, we are able to see Gli1-tdTomato+ cells localized to the apical third of the root with only a few cells dispersed in the coronal pulp chamber (Figure 5A). In four week molars, we also see Gli1-tdTomato+ cells however the expression is less compared to three weeks and is localized to the apical quarter of the root (Figure 5B). In addition, there are few Gli1-tdTomato+ cells present in the odontoblast layer of the molar root of the four week mouse (Figure 5B).
Figure 5: Expression of Gli1-tdTomato⁺ cells decreases during root elongation. Three and four week old Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24hr apart and were euthanized 4 days post TM injection. A-B) Gli-tdTomato expression (red) and H&E (insets) in TM treated A) three week maxillary molars and B) four week maxillary molars, Scale bars – 100µm. C) Percent area of td-Tomato expression in the pulp root from the cementoenamel junction (CEJ) to the periapical area and D) to the end of the root. Results represent mean and ±SEM from serial sections, n=2; *P<0.05 unpaired student t-test. Yellow areas indicate periapical border, white arrows indicate root tip.

Because there was a visual difference in Gli1-tdTomato⁺ expression in the developing root of TM treated three and four weeks old animals, a preliminary quantification into the average area of tdTomato⁺ signal was calculated on serial sections from a small cohort. Two regions of interest were analyzed in the root-from the cementoenamel junction (CEJ) to the periapical root portion or to the root tip. Our results showed that the average area of Gli1-tdTomato⁺ cells was 19.7% ± 1.9 for three week mice and 5.2% ± 2.6 for four week mice when the region of interest is from CEJ to the periapical area (Figure 5C). When we extend the region of interest from CEJ to the root tip, our results showed a similar significant decrease with the average area of Gli1-tdTomato⁺ cells being 13.3% ± 1.4 for three week mice and 2.7% ± 2.1 for four week mice (Figure 5D). Both
Figure 5C and 5D show that the expression of Gli1-tdTomato+ cells is significantly greater in three week mice compared to four week mice.

C. Correlation of Gli1+ expressing cells with CD31+ vascular cells and CGRP+ neuronal cells in three week mice

To further understand the niche of Gli1-tdTomato+ cells in mature molars, we utilized the molars from three-week-old mice. We wanted to look at the mouse molars during a time point where they had recently erupted into the oral cavity and were in the process of root development. The three-week mice were injected twice with tamoxifen (TM) 24 hours apart. Four days after the last TM injection, animals were euthanized and maxillae were removed for histology. Serial frozen sections of maxillary molars were stained with the endothelial marker (CD31) and neuronal marker (CGRP) to identify a possible neurovascular bundle near Gli1-tdTomato+ cells. In addition to CD31 and CGRP, the sections were counterstained with DAPI to identify nuclei.

First, the correlation between Gli1-tdTomato+ cells and CGRP cells was examined (Figure 6). The results in Figure 6 show that the majority of the Gli1-tdTomato+ cells were present in and near the apical area of the first molar, with some cells present in the periodontal ligament space and the alveolar bone (Figure 6D). CGRP staining was present mostly as long strands in the root of the molar going into the apical area. There was also some CGRP+ cells present in the pulp chamber (Figure 6E). Our results showed the presence of Gli1+ expressing cells in close proximity to the CGRP+ cells in the apical region of TM treated three-week molars (Figure 6G).

Next was to identify whether Gli1-tdTomato+ cells reside near CD31+ vascular cells in molars from three-week old animals treated with TM (Figure 7). Similarly, Gli1-tdTomato+ expression was found concentrated to the apical region of the molar (Figure 7D) however, CD31
expression (blood vessels) was shown throughout the molar including the apical region of the molar (**Figure 7E**). The results showed the presence of Gli1-tdTomato⁺ cells within the perivascular space due to their close proximity to blood vessels only at the apical area of the first molar (**Figure 7G**).
Figure 6: Correlation in expression of lineage traced Gli1-tdTomato+ cells with CGRP+ nerve fibers in three week maxillary molars. Three week old Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24hr apart and were euthanized 4 days post TM injection. Immunohistochemistry for CGRP of frozen sections were imaged at 5x for A) H&E, B) Brightfield, C) DAPI staining for nuclei (blue), D) Gli expression (red), E) CGRP (green) and merged images of F) CGRP and Gli, G) CGRP, Gli and DAPI, H) secondary only control (No CGRP antibody) which demonstrates Gli expression visible in apical area of maxillary molar and alveolar bone and CGRP staining visible as long strands in apical area of molar. Dotted white line outlines the pulpal region of the maxillary molar. Scale bars – 100µm.
Figure 7: Correlation in expression of lineage traced Gli1-tdTomato+ cells with CD31+ blood vessels in three week maxillary molars. Three week Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24 hrs apart and were euthanized 4 days post TM injection. Immunohistochemistry for CD31 on frozen sections were imaged at 5x for A) H&E, B) Brightfield, C) DAPI staining for nuclei (blue) D) Gli expression (red) E) CD31 (yellow) and merged images of F) CD31 and Gli, G) CD31, Gli and DAPI and H) secondary only control (No CD31 antibody) which demonstrates Gli expression visible in apical area of maxillary molar and alveolar bone and CD31 staining visible throughout the molar and alveolar bone. Dotted white line outlines the pulpal region of the maxillary molar. Scale bars – 100µm.

Correlation of Gli1-tdTomato+ expression with both CD31+ blood vessels and CGRP+ nerve fibers within the apical region of the maxillary three-week molars suggests Gli+ cells do reside in close proximity to the neurovascular bundle (Figure 8). Higher magnification of 10x and 20x shows the close interaction of the Gli1+ cells with both CD31+ and CGRP+ cells in the molar apical region (Figure 8). The results indicate that Gli1-tdTomato+ cells are present near both vascular cells and neuronal cells in the apical area of three-week molars.
Figure 8: Correlation in expression of lineage traced Gli1-tdTomato<sup>+</sup> cells with CD31<sup>+</sup> blood vessels and CGRP+ nerve fibers in three week maxillary molar apical region. A-D) Serial frozen sections were stained with either CD31 or CGRP to correlate neurovasculature with Gli1-tdTomato<sup>+</sup> reporter expression within the apical region 4 days post tamoxifen. Merged images of CD31 (yellow), Gli (red) and DAPI (blue) of the maxillary molar apical region at A) 10x magnification and B) 20x magnification. White arrows indicate perivascular Gli expression. C-D) Merged images of CGRP (green), Gli (red) and DAPI (blue) of the maxillary molar apical region at C) 10x magnification and D) 20x magnification. White arrows indicate region where nerves are localizing near Gli expression. White boxes indicate apical region. Scale bars – 50 µm.
D. Correlation of Gli1+ expressing cells with CD31+ vascular cells and CGRP+ neuronal cells in four week mice

To further examine Gli1+ expressing cells in mature molars, we examined the correlation of Gli1-tdTomato+ cells with CD31+ and CGRP+ cells in TM treated four week old mice. We wanted to look at four week time period to evaluate the location and expression of Gli1+ cells after significant root development had occurred but while the apical foramen was still open. Similar protocol was carried out to study the location of Gli1+ expression in correlation with CD31+ and CGRP+ cells in four week mice. First, we looked at the correlation of Gli1-tdTomato+ cells and CGRP+ cells (Figure 9). Maxillary molars from TM treated 4 week old mice showed the presence of Gli1+ cells at the apical region of the maxillary molar (Figure 9D) with CGRP+ cells present in the pulp chamber including the root as long strands of nerve fibers (Figure 9E). Specifically, in Figure 9G, we are able to see the close proximity of Gli1+ expressing cells near CGRP+ cells in the apical region.

Next, we examined the Gli1-tdTomato+ cells near CD31+ cells in TM treated four-week maxillary molars (Figure 10). Gli1-tdTomato+ expression was found localized near the apical region of the molar (Figure 10C). CD31 expression (blood vessels) was shown throughout the molar including the apical region of the molar (Figure 10D). The results showed the presence of Gli1-tdTomato+ cells in close proximity to the blood vessels found at the apical area of the four-week molars (Figure 10F).

To further investigate Gli1+ expressing cells and their correlation with CD31+ and CGRP+ cells in the molars of TM treated 4 week old mice, different areas in the apical region were analyzed including at higher magnification- 10x and 20x (Figure 11). Gli1+ reporter cells appeared in close proximity to blood vessels (Figure 11A-C). Specifically, at the distal apical area, there is close
interaction of Gli1+ reporter cells and CD31+ vascular cells (Figure 11C) and Gli1+ reporter cells near CGRP+ nerve fibers (Figure 11D-F). At the proximal apical area, CGRP+ cells present as bead-like structures going towards the distal apical area (Figure 11E). The close proximity of Gli1+ expressing cells with CGRP+ cells is more clearly seen at the distal apical area. Higher magnification shows the close interaction of the Gli1+ cells with both CD31 and CGRP in the molar apical region. Since Gli1-tdTomato+ cells reside near both vascular and neuronal cells in the apical area of four-week mouse molar, these results suggest that Gli+ cells reside near the neurovascular bundle of mature maxillary molars.
Figure 9: Correlation in expression of lineage traced Gli1-tdTomato$^+$ cells with CGRP$^+$ nerve fibers in four week maxillary molars. Four week old Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24hr apart and were euthanized 4 days post TM injection. Immunohistochemistry for CGRP on frozen sections were imaged at 5x for A) H&E, B) Brightfield, C) DAPI staining for nuclei (blue) D) Gli expression (red) E) CGRP (green) and merged images of F) CGRP and Gli, G) CGRP, Gli and DAPI and H) secondary only control (No CGRP antibody) which demonstrates Gli expression visible in the apical area and CGRP staining in the root and apical area of maxillary molar. Dotted white line outlines the pulpal region of the maxillary molar. Scale bars – 100µm.
Figure 10: Correlation in expression of lineage traced Gli1-tdTomato+ cells with CD31+ blood vessels in four week maxillary molars. Four week Gli1-CreERT2/Ai9 mice were injected twice with Tamoxifen (TM) 24 hrs apart and were euthanized 4 days post TM injection. Immunohistochemistry for CD31 on frozen sections were imaged at 5x for A) Brightfield, B) DAPI staining for nuclei (blue), C) Gli expression (red), D) CD31 (yellow) and merged images of E) CD31 and Gli, F) CD31, Gli and DAPI, G) secondary only control (No CD31 antibody) which demonstrates Gli expression visible in apical area of maxillary molar and odontoblast layer and CD31 staining visible throughout the molar. Dotted white line outlines the pulpal region of the maxillary molar. Scale bars – 100µm.
Figure 11: Correlation in expression of lineage traced Gli1-tdTomato+ cells with CD31+ blood vessels and CGRP+ nerves in the apical region of four week maxillary molar. A-F) Frozen sections were stained with either CD31 or CGRP to correlate neurovasculature with Gli1-tdTomato+ reporter expression within the apical region 4 days post tamoxifen. Merged images of CD31 (yellow), DAPI (blue) and Gli expression at the apical end of maxillary molar at A) 10x magnification, box indicates apical region, B) 20x magnification of proximal apical area and C) 20x magnification distal apical view of maxillary molar root. White arrows indicate CD31 and Gli correlation in the apex. Merged images of CGRP (green), DAPI (blue) and Gli (red) expression at the apical end of maxillary molar at D) 10x magnification, box indicates apical region where CGRP expression is present as bead-like structures throughout the root and apical area, E) 20x magnification of proximal apical area and F) 20x magnification distal apical area of maxillary molar root. Scale bars – 100µm.
E. Correlation of odontoblasts with lineage traced Gli1-tdTomato+ reporter expression and CD31+ blood vessels in four week maxillary molars

During the analysis of maxillary molars from four-week-old TM treated Gli+/Ai9 mice, we came across an interesting finding- the expression of Gli1+ cells in the odontoblast layer (Figure 12). As shown in Figure 12C, odontoblasts and odontoblast processes show strong Gli1-tdTomato+ reporter expression (Figure 12C). These results indicate one of two things; either Gli1 is expressed in a small number of odontoblasts under steady state conditions or after 5 days of TM treatment, Gli+ MSCs lineage traced to more mature cells- odontoblasts. The latter would suggest that the Gli1-tdTomato+ cells serve as a source of MSCs even under homeostatic conditions by differentiating into mature odontoblasts.

Figure 12: Correlation of odontoblasts with lineage traced Gli1-tdTomato+ reporter expression and CD31+ blood vessels in four week maxillary molars. A-C) Frozen sections stained with CD31 from 4 days post tamoxifen treated Gli1-tdTomato+ reporter mice show expression within more differentiated cell types. Merged images of CD31 (yellow), DAPI (blue) and Gli (red) expression present in four week maxillary molar at A) 5x magnification, B) 10x magnification of odontoblast layer with Gli+ cells and C) 20x magnification with Gli+ cells in odontoblast layer with odontoblast processes (white arrows). Orange dashes indicates the dentin region. Scale bars – 100µm.
Discussion:

For many researchers, mesenchymal stem cells have been an area of great interest due to their multipotent differentiation ability and consequently, their potential in regenerative medicine. The current *in vitro* definition that we have of MSCs pertains to their trilineage differentiation ability (osteoblasts, chondrocytes and adipocytes), colony forming ability, and the presence of specific MSC surface markers (Bianco et al., 2013; Dominici et al., 2006; Keating, 2012). The definition of MSCs has always been controversial, especially after research into the *in vivo* characteristics of MSCs, which have shown that they can have different tissue origins, properties and functions in different tissues that do not resemble the *in vitro* characteristics (Sharpe, 2016). With the use of label retaining and lineage tracing techniques, we have recently begun to understand the *in vivo* characteristics and properties of MSCs.

MSCs can be found in various tissues such as bone, skeletal muscle, adipose tissue and teeth (Zhao et al., 2014). The MSCs found in the dental pulp are called dental mesenchymal stem cells (DPSCs) which play a very significant role in tooth homeostasis and repair (Sharpe, 2016). Gli1, part of the Sonic Hedgehog pathway, was identified as an *in vivo* dental stem cell marker by Seidel et al (Seidel et al., 2010). Several studies have utilized this *in vivo* stem cell marker to try an identify MSC populations and niches in mouse incisors. **The objective of our study was to localize the Gli1+ expressing cells near a neurovascular bundle in three and four week mouse molars in order to identify a MSC niche.** We chose to analyze three and four-week mouse molars because it is the time-period after tooth eruption where we see high levels of root activity and development prior to apical closure.
In our results, we found Gli1+ expression near the apical portion of the maxillary molar root in both three week and four week maxillary molars. We found that the Gli1+ expression was significantly greater in the developing molar root of three week mice compared to four week mice. Lungová et al. showed that mouse molar tooth eruption is at 16 days (P16), at which point the root continues to develop and elongate. This process continues until around age day 27 (P27) when the molar root has reached its length while the apical foramen is open (Kawakami et al., 2015). A possible explanation for less Gli1+ expression seen in four week mice compared to three week mice could be that as the mouse molar root continues to develop and mature, there are less Gli1+ cells present in the apical region because less MSC activity is needed as the tooth matures.

Root development is dependent upon the guidance from Hertwig’s epithelial root sheath (HERS). It controls the induction, shape, size and number roots to be formed (Ten Cate, 1996). HERS is formed from the inner and outer enamel epithelium and is located between the dental papilla and dental follicle (Huang & Chai, 2013). As HERS migrates apically, the dental papilla cells and the epithelial basement membrane are induced to become odontoblasts to produce root dentin (Huang & Chai, 2013). Several studies have examined tooth root development and found that HERS is controlled by Shh signaling and Gli (Bae et al., 2016; Huang & Chai, 2013; Nakatomi, Morita, Eto, & Ota, 2006). Specifically, Gli1+ cells have been found in the dental mesenchyme around HERS and continue to proliferate as the tooth continues to grow (Feng et al., 2017; Li et al., 2015; Liu et al., 2015). The Gli1+ cells have been found to differentiate into root forming cells such as odontoblast, cementoblasts and fibroblasts in the dental pulp and PDL. Therefore, it may be a plausible explanation for why we encountered less Gli1+ expression at the apex for more mature, four-week-old mice.
Once we established the location of Gli1+ expressing cells, we wanted to examine the microenvironment that possibly support these cells. It is important to study the MSC niche, as it is a valuable step in understanding their function, origin, and regulation. In a previous study, Gli1 was used as an in vivo marker to identify MSCs and their niche in mouse incisor (Zhao et al., 2014). They identified the neurovascular bundle as a MSC niche. Therefore, the next step in this study was to identify a potential neurovascular bundle in the dental mesenchyme of mouse molars to examine the relationship of Gli1+ cells and the NVB. Similar to previous studies, to identify a neurovascular niche, we used neuronal (CGRP) and endothelial markers (CD31). We examined the location of CD31+ cells and CGRP+ cells in relation to Gli1+ expressing cells.

In our study, CD31+ endothelial cells were found throughout the maxillary molar dental pulp, periodontal ligament space and the alveolar bone in both three and four week mice. This finding is consistent with results from other studies which show the vasculature of mouse molars at different ages (Puranik, Mina, & Kalajzic, 2017; Vidovic et al., 2017). Vidovic et al. examined aSMA-expressing perivascular cells and their contribution to odontoblasts during primary dentinogenesis as well as during injury repair. They utilized the CD31+ endothelial marker to show that aSMA-expressing cells were near vasculature in dental pulp in mouse molar in mice ages P7 and P14. They identified a MSC population in mouse molars that was able to differentiate into odontoblasts allowing for secondary dentinogenesis during injury repair. However, they found that this population makes a small contribution during primary dentinogenesis. Puranik et al. also used the CD31+ endothelial marker to evaluate the vasculature in 4-week maxillary mouse molars. Their results showed the blood vessels were found throughout the dental pulp and the alveolar bone that was similar to the findings of this study.
CGRP+ cells, a marker for sensory nerves, was found in the coronal pulp as well as in the root leading towards the apex and in long strands parallel to the root. In addition, several CGRP+ cells were located at the apical end of the maxillary molar root. *Veerayuthwilai et al.* examined peripherin and CGRP immunoreactive nerve fibers in rat molars by looking at the timing and location of their development during tooth maturation. Their results showed that two weeks after eruption, many CGRP beaded fibers were seen in the dentin and that as the tooth continued to mature, CGRP nerve fibers continued to increase. In our study, we see similar findings of beaded and long stranded CGRP fibers located in both three and four week mouse molars.

Having established the location of both CD31 and CGRP in three and four week mouse molars, we examined the location of Gli1+ expressing cells and their relationship to CD31 and CGRP cells. In our results, Gli1+ expressing cells were identified in close proximity to CD31+ blood vessels and CGRP+ nerve fibers in the apical portion of the mature maxillary molars. These findings were consistent in both the three and four week mouse molars. The close interaction of both CD31+ and CGRP+ cells with Gli1+ expressing cell indicates a close association of Gli1+ reporter cells to the neurovascular bundle in three and four week mouse molars. It is possible that there is a neurovascular bundle present at the mouse molar apical root which may act as an MSC niche for Gli1+ expressing cells.

*Zhao et al.* found Gli1+ expressing cells in the incisor dental mesenchyme surrounded by a neurovascular bundle; specifically, they found Gli1+ cells near arterioles that were accompanied by nerves only. The neurovascular bundle was located at the proximal end of the mouse incisor. This was a critical finding in establishing the NVB as a MSC niche that supports the turnover of incisor mesenchyme. *Zhao et al.* showed that Shh is secreted by CGRP+ sensory neurons from the trigeminal ganglion and is transported to the inferior alveolar nerve into the incisor mesenchyme.
Upon arrival in the mesenchyme through the NVB, Shh activates Gli1 expression in the adjacent periarterial mesenchymal cells to regulate odontogenic differentiation such as giving rise to odontoblasts (Zhao et al., 2014).

Similar to Zhao et al, it is possible that the neurovascular bundle in the mouse molar is located at the apical region of the root near the MSC population. We know that HERS plays a very significant role in root development and is controlled by Shh signaling and Gli1 (Bae et al., 2016; Huang & Chai, 2013; Nakatomi et al., 2006). Furthermore, we know that Gli1+ cells are able to differentiate into different cells such as odontoblasts, cementoblasts, and fibroblasts (Feng et al., 2017; Liu et al., 2015). Thus, the neurovascular bundle at the apical region of the mouse molar can provide a possible niche for Gli1+ expressing cells as molar continues to develop. Therefore, a greater understanding of tooth formation may accelerate the development of novel regenerative and restorative therapies (Hosoya et al., 2020). Understanding the supporting niche of Gli1+ cells within the dental pulp is an important step in identifying these MSCs and their further lineage commitment to odontoblast-like cells which could serve as a valuable cell source for the generation of reparative dentin after tooth injury.

**Future Directions:**

The objective of this study was to localize in vivo Gli1+ cells near the neurovascular bundle in mature mouse molars. Identifying this area can allow us to study MSCs in the NVB as a possible niche for a supply of undifferentiated cells that can further differentiate to odontoblasts. This study will serve as an initial step towards future studies to uncover the populations of dental pulp stem cells that may be used in primary or reparative dentin formation.
References:


Juuri, E., Saito, K., Ahtiainen, L., Seidel, K., Tummers, M., Hochedlinger, K., … Michon, F.


Veerayuthwilai, O., Luis, N. A., Crumpton, R. M., MacDonald, G. H., & Byers, M. R. (2006). Peripherin- and CGRP-immunoreactive nerve fibers in rat molars have different locations and
developmental timing. *Archives of Oral Biology, 51*(9), 748–760. 
https://doi.org/10.1016/j.archoralbio.2006.03.011

