THE ROLE OF PRICKLE1 IN THE DEVELOPMENT OF THE PITUITARY GLAND IN THE *BEETLEJUICE* MOUSE

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Prickle1 is a protein that is a core component of the Wnt/Planar Cell Polarity pathway and is a key player in establishing cell polarity. To study the role of Prickle1, we are using the *Beetlejuice* mouse line. *Beetlejuice* mice have a missense mutation in Prickle1, and we used them to study the effects of *Prickle1* in the development of the pituitary gland. We studied *Beetlejuice* mice at embryonic days 9.5 and 10.5 by utilizing hematoxylin and eosin (H&E) and immunofluorescence staining in order to visualize gland morphology and protein localization. Experiments utilizing dual immunofluorescence staining were performed using primary antibodies to Prickle1 and β-catenin and to Pitx1 and ROR2. We found that Prickle1 is expressed in the cell membrane and in the nucleus of the wild type animals. However, in the *Beetlejuice* mutants, Prickle1 is scattered throughout the cytoplasm, and we did not see obvious cell membrane localization. We observed fewer Pitx1-positive cells in the *Beetlejuice* mutant, and the cells which are positive for Pitx1 are located in the edge of Rathke's pouch. In the wild type, Pitx1 is localized to the nucleus. *Beetlejuice* mice have ROR2 dispersed throughout the cytoplasm, while in the wild type animals it is localized to the cell membrane. Based on our findings, we conclude that Prickle1 is required for cell polarization in normal pituitary gland development.

TABLE OF CONTENTS

LIST OF FIGURES

1.0 BACKGROUND

1.1 PITUITARY GLAND ANATOMY & PHYSIOLOGY

The pituitary gland, also known as the hypophysis, is an endocrine gland that functions in many physiologic processes throughout life. Feedback mechanisms aid the gland in integrating signals from the central and peripheral nervous system to bring about these physiologic processes. As a result, gene transcription, hormone synthesis and hormone cell proliferation are affected in order to bring about growth and development, maintain homeostasis, manage stress responses, influence metabolism, as well as many other functions. The location of the gland comes about from its dual origin, the roof of the oral cavity and the floor of the brain. As a result, the gland sits in the sella turcica, a shallow depression in the sphenoid bone, with the fully formed pituitary consisting of three main lobes: anterior, intermediate, and posterior (Skowronska-Krawczyk et al.).

The posterior lobe of the pituitary gland, also referred to as the *neurohypophysis*, consists mainly of axons from the paraventricular nucleus of the hypothalamus. It is derived from neural ectoderm and is connected to the ventral diencephalon of the brain via the infundibulum. It consists of a large portion, the *pars nervosa*, and the smaller infundibulum, or neural stalk. Blood supply is derived from the internal carotid artery via the superior and inferior hypophyseal arteries. The primary function of the posterior lobe is to release the hormones vasopressin and

oxytocin. These hormones are released into the blood due to impulses in the nerve fibers from the hypothalamus and have effects on the permeability of collecting tubules of the kidney and the contraction of myoepithelial cells in the mammary glands, respectively (Junqueira et al.).

The term *adenohypophysis* refers to the anterior lobe (*pars distalis*) and the intermediate lobe (*pars intermedia*) of the pituitary, which are derived from oral ectoderm. The anterior and intermediate lobes function as the primary regulatory site for the endocrine glands of the body (Rossant et al). The hormones released from the anterior pituitary influence both the development and function of their target organs and are derived from five primary cell types: corticotropes, thyrotropes, somatotropes, lactotropes, and gonadotropes. Adrenocorticotropin (ACTH), produced by corticotropes, influences glucocorticoid production in the adrenal cortex. Thryotropes produce thyroid-stimulating hormone (TSH), which is necessary for organization of thyroid follicles, growth of the thyroid gland, and normal development of the brain. Growth hormone (GH) is produced by somatoropes and is responsible for regulating body growth through stimulation of insulin-like growth factor production in the liver. Prolactin (PRL) is produced by lactotropes and influences milk production in the mammary gland. Gonadotropes release both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and are responsible for spermatogenesis and ovulation (Zhu, Gleiberman and Rosenfeld).

1.2 DEVELOPMENT OF THE ADENOHYPOPHYSIS

The pituitary gland originates from the neural plate, with the anterior neural ridge giving rise to Rathke's pouch, the precursor to the adenohypophysis. During development, the anterior neural ridge moves ventrally to form the oral epithelium which will eventually contribute to the

development of the adenohypophysis. Pituitary development is highly conserved throughout the animal kingdom, though it forms at different developmental stages across species (Skowronska-Krawczyk et al.). In the mouse, development of the adenohypophysis and Rathke's pouch occurs from approximately embryonic day 8.5 to embryonic day 17.5 (Khonsari et al.).

Development of the adenohypophysis begins when bone morphogenic protein 4 (BMP4) in the ventral diencephalon stimulate cells of the pituitary placode in the oral ectoderm to thicken and invaginate (Davis et al.). This invagination is referred to as Rathke's pouch and first appears at E9.0 rostral to the oropharyngeal membrane. As development progresses, the pouch thickens and grows upward from the roof of the mouth toward the floor of the diencephalon. By E9.5, cell differentiation is evident in the anterior wall, but the peripheral boundary is still wide (Zhu, Gleiberman and Rosenfeld). Eventually, the anterior wall comes to be in direct apposition with the floor of the diencephalon and all intervening mesenchyme is obliterated, so that the two tissues are in direct contact (Kauffman). At this period of development, the interaction between Rathke's pouch and the ventral diencephalon is essential for further pituitary development (Zhu, Gleiberman and Rosenfeld).

At E11 to E11.5, the rostral portion of Rathke's pouch expands upwards on either side of the pituitary stalk, while the lateral part is in close association with the internal carotid arteries. The wall of the pouch becomes well differentiated, and by E12 to E12.5 the connection between the pouch and the roof of the oral cavity is lost (Kauffman). At this time, cell division increases in the pituitary primordium, most notably in the anterior portion of Rathke's pouch. As cell division continues, progenitors of the five primary cell types of the anterior pituitary begin to arise, and eventually the ventral portion of Rathke's pouch will give rise to the anterior lobe. Alternatively, the dorsal portion will form the intermediate lobe as it comes into contact with the

infundibular processes and the hypophyseal cleft. Rathke's pouch, being the precursor to the anterior pituitary, is the source of all endocrine pituitary cells. The timing of commitment of the pituitary precursor to producing endocrine cells coincides with the formation Rathke's pouch (Skowronska-Krawczyk et al.).

1.2.1 *The Role of* Pit1 *and* Pitx1 *in Development*

Pit1 and Pitx1 are transcription factors that control cell commitment and differentiation of the pituitary gland (Skowronska-Krawczyk et al.). Signals originating from the diencephalon and the mesenchymal cells interact to bring about specific domains of transcription factor expression (Rossant et al.). Normal expression of these factors would imply that normal cell differentiation is occurring in the developing pituitary.

Pit1 is expressed exclusively in the pituitary gland, and its structure is evolutionarily conserved throughout the animal kingdom, although it may play different roles in individual species. In mice, the initial expression of Pit1 can be detected by E13.5 and are confined to the anterior ventral pituitary. The initial activation of the *Pit1* gene requires the concerted actions of Prop1 and the Wnt/B-catentin signaling pathway . In adulthood, Pit1 co-localizes with expression of GH, PRL, and TSHb genes (Skowronska-Krawczyk et al.).

Due to the fact the *Pit1* gene is required for their differentiation and expansion, somatotropes, lactotropes, and thyrotropes are referred to as the Pit1 lineage of hormones (Zhu, Gleiberman and Rosenfeld). In addition, Pit1 is a direct transcriptional activator of *Gh, Prl*, and *Tshb*. In the absence of Pit1, gonadotrope differentiation is enhanced, likely because Pit1 acts synergistically with GATA2 to promote differentiation of thyrotropes (Rossant et al.).

Pitx1 and *Pitx2* are expressed throughout the oral ectoderm during the earliest stages of pituitary development. *Pitx2* is required for expansion of the pouch, specification of gonadotropes, and expansion of the *Pit1* cell lineage. *Pitx1*, conversely, has a minor role in expansion of the individual specialized cells, affecting only the relative proportions of each cell type.

1.3 WNT SIGNALING PATHWAY

The Wnt family of proteins are major signaling molecules required for normal growth and homeostasis (Logan and Nusse). Their expression is highly regulated during development, and alteration in their expression can result in a host of diseases and cancers. By binding to specific receptors, Wnts trigger activation of signaling pathways within the cell. Due to their pleiotropic nature, Wnt signals control mitogenic stimulation, cell fate specification, and cell differentiation (Kikuchi, Yamamoto and Sato).

Wnt genes are defined by sequence homology to the original discovered members, *Wnt*-1 in the mouse and *wingless (wg)* in *Drosophila*. They encode secreted glycoproteins, usually 350-400 amino acids in length (Cadigan and Nusse). The Wnt proteins are defined by sequence rather than by functional properties and contain a signal sequence followed by a highly conserved distribution of cysteines (Logan and Nusse). Wnt signaling molecules are important to this discussion because they are emerging as contributors to pituitary development (Rossant et al.).

1.3.1 *CANONICAL WNT SIGNALING PATHWAY*

The canonical Wnt signaling pathway is also known as the β-catenin-dependent signaling pathway and is highly conserved among various species (Logan and Nusse). It is well known that the alterations of the β-catenin-dependent pathway lead to various diseases (Kikuchi, Yamamoto and Sato). Wnt proteins bind to Frizzled (Fz) receptors on the cell surface in order to act on target cells. These receptors then transduce a signal to several intracellular proteins, including β-catenin. Cytoplasmic β-catenin levels are normally kept low, but when Wnt proteins bind to cell-surface receptor complexes, cytoplasmic β-catenin and its entry into the nucleus are stabilized. When cells receive Wnt signals, β-catenin accumulates in the cytoplasm and nucleus due to inhibition of the degradation pathway. Nuclear β-catenin then interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to affect transcription (Logan and Nusse).

1.3.2 *NON-CANONICAL WNT SIGNALING PATHWAY*

The Wnt non-canonical pathway is also known as the Wnt non-canonical planar cell polarity pathway (Wnt/PCP pathway) and it regulates cell polarity. The core components of the Wnt/PCP pathway are three integral membrane proteins (Frizzled, Flamingo/Stan/Celsr, and Vangl/Strabismus) and three membrane-associated proteins (Dishevelled, Diego/Inversin, and Prickle).

Prickle-like 1 (Prickle1) is a core component in the Wnt non-canonical planar cell polarity (PCP) pathway. It consists of a PET (Prickle, Espinas, and Testin) domain in its N-terminus and three LIM (Lin11, Isl-1, and Mec3) domains in its C terminus. It belongs to the group of LIM proteins that are associated with actin and the transcriptional machinery, thus playing a dual role in gene regulation and cargo transportation (Gubb et al.). Prickle joins with Vangl at the cell membrane and becomes asymmetrically localized to one side of the cell, thus establishing the foundation of cell polarity. However, the function of Prickle1 remains poorly understood. Currently, there is limited data on the role of Prickle1 in the Wnt/PCP pathway, with most of our knowledge of Wnt/PCP function in mammals coming from *Wnt5a* and *Vangl2* mouse mutants (Yang et al.).

1.4 BEETLEJUICE MOUSE LINE

The *Beetlejuice* mouse line stems from a novel *Prickle1* missense allele, termed *Beetlejuice (Bj)*. The missense mutation (p:C161F) occurs in the first Lim domain of the Prickle1 protein and replaces the cysteine at amino acid location 161 with a phenylalanine (Figure 2). The *Beetlejuice* mutant is different from other *Prickle1* mutants in that the mice survive to term. However, these mice have developmental anomalies including cochlea defects, congenital heart defects, and skeletal and craniofacial anomalies. In Figure 3, one can appreciate the microcephaly and midfacial clefting that occurs in the mutant mice (Gibbs et al.) (Wan et al.).

As previously mentioned, Prickle1 is part of the Wnt non-canonical planar cell polarity pathway. It is important because it is a key player in establishing cell polarity. Humans with recessive missense mutations in the PET domain of the *Prickle1* gene have shown an association with progressive myoclonus epilepsy (PME) (Bassuk et al.) (Tao et al.). Mice with a reduced dosage of the *Prickle1* gene are prone to drug-induced seizures. Still, specific expression and function of Prickle genes as components of the PCP pathway have not been fully elucidated (Liu et al.).

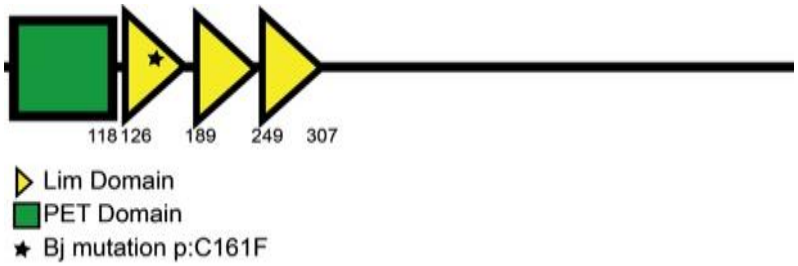

Figure 1. Schematic of Prickle1 protein. Prickle1 protein contains 1 PET domain and 3 Lim domains- *Beetlejuice* mutation noted (Gibbs et al. 2016)

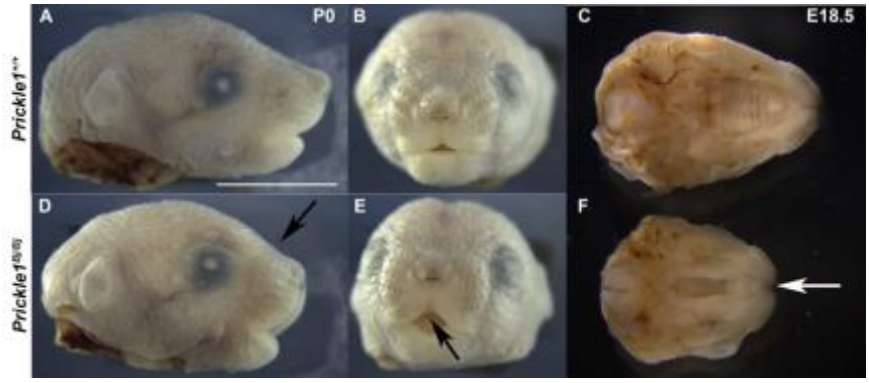

Figure 2. External phenotype of *Beetlejuice* Mouse Line. A-C) Wild type D-F) *Beetlejuice* mutants. (A,D) Lateral view, (B,E) Frontal view, (C,F) View of palate (Wan et al. unpub)

2.0 PURPOSE OF THE PRESENT STUDY

The overall goal of this investigation is to determine the role of the *Prickle1* gene mutation on the development of the craniofacial structures in the mouse model. Specifically, this project will examine the effects of the *Prickle1* mutation on the development of the anterior pituitary gland. We hypothesize that, in *Beetlejuice* mutants, the development of the pituitary gland at E10.5 is disrupted because Prickle1 is required for cell polarization in the pituitary gland.

3.0 MATERIALS & METHODS

Embryos were collected at E9.5 and E10.5 via Cesarean section. The day of the plug is designated E0.5. The harvested embryos were fixed overnight in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin wax. Specimens were sectioned to 10μm, placed on TESPA-coated Fisherbrand® Superfrost®/Plus slides (Fisher Scientific), and placed on a slide warmer at 25°C to dry overnight.

3.1 HEMATOXYLIN & EOSIN STAINING

Hematoxylin and eosin staining was performed on a subset of the slides using a standard protocol. Specimens were deparaffinized in xylene, rehydrated using ethanol and tap water, and placed in hematoxylin (ThermoScientific #7221) for one minute. Then, specimens were washed with tap water followed by acid alcohol (Richard-Allan Scientific #7401) to eliminate background staining. The specimens were then returned to tap water in order to stop the process of differentiation initiated with the acid alcohol. Bluing agent (Richard-Allan Scientific #7301) was applied for one minute, and specimens were again washed with tap water followed by ethanol. Eosin stain (ThermoScientific #7111) was applied for one minute, and specimens were

washed with ethanol and xylene. Mounting medium (Richard-Allan Scientific) was placed on the slides, and a coverslip was added.

3.2 IMMUNOFLUORESCENCE STAINING

Two dual immunofluorescence staining experiments were performed on a subset of the slides. The first experiment was a dual staining for Prickle1 (Catalog Number GR46168-1) and β-catenin (Catalog Number 610153, BD Biosciences) primary antibodies. Corresponding secondary antibodies AlexaFluor594 (Catalog Number 1739294, Life Technologies) and AlexaFluor488 (Catalog Number 1484573, Invitrogen) were used, respectively. The second experiment was a dual staining for Pitx1 (Catalog Number PA5-14219, Thermo Scientific) and ROR2 (Catalog Number Q2633066) primary antibodies. Corresponding secondary antibodies AlexaFluor594 and AlexaFluor488 were used, respectively. The specimens were deparaffinized with xylene then rehydrated with ethanol followed by protein buffer solution (PBS). Antigen unmasking was performed by placing the slides in sodium citrate buffer solution, microwaving for 10 minutes, refilling with distilled water, and microwaving for an additional 10 minutes. The slides were left in the citrate buffer solution to cool to room temperature for 2 hours up to 6 hours. Blocking was performed using 5% goat serum at a dilution of 1:100 in PBS for 30 minutes up to 3 hours. Then, the primary antibodies diluted at 1:200 were added and left to sit overnight at 4°C. The next day the primary antibodies were washed off with PBS at 3 minutes per cycle for a total of 3 cycles. Secondary antibody diluted 1:200 was then added and allowed to sit in the dark for 30 minutes. The slides were kept in the dark and washed in PBS at 5

minutes per cycle for a total of 3 cycles. Mounting was performed using both Prolong gold and DAPI methods, and a coverslip was added. Slides were stored in the dark at 4°C.

3.3 PHOTOGRAPHING THE SLIDES

Photographs of the specimens were taken using a Zeiss AXIO microscope and AxioCam MRc 35 camera. The slides stained with H&E were photographed at 5x and 40x magnification. The slides stained via immunofluorescence were photographed at 40x magnification.

4.0 RESULTS

4.1 PRICKLE1 EXPRESSION IN NORMAL TISSUE

In the wild type animals at E9.5, Prickle1 protein is expressed in the epithelium of Rathke's pouch as well as in the mesenchymal cells surrounding the pouch. In the mesenchymal tissue, Prickle1 is expressed more profoundly in the cells located anterior to Rathke's pouch than in the cells located posterior to the pouch.

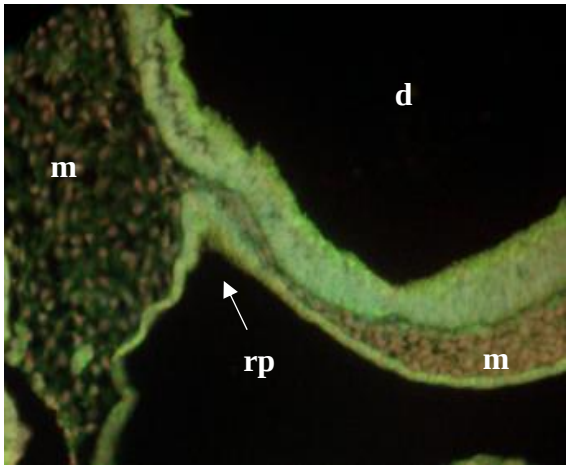

Figure 3. Prickle1 protein is expressed in the epithelium and in the surrounding mesenchyme. Mid-sagittal section through Rathke's pouch at E9.5. Immunoflourescence of *Prickle1* (red) and DAPI (blue) in wild type E9.5 mouse. Prickle1 protein is expressed in Rathke's pouch (rp) epithelium, the mesenchyme (m) of the cranial base and the diencephalon (d) at E9.5 40X

4.2 MORPHOLOGY

Rathke's pouch in *Beetlejuice* mice is underdeveloped at E10.5 when compared to wild type mice. The epithelium of the anterior wall lacks the pronounced developmental curvature seen in the wild type. Also, there is little to no mesenchymal tissue separating the anterior epithelium of the pouch and the floor of the brain in the *Beetlejuice* mouse. In addition, the thickness of the epithelium is approximately 4 cell layers thick in the wild type animals but is approximately 2 cell layers thick in the *Beetlejuice* mice.

Prickle1$^{+/+}$ *Prickle1*$^{Bj/Bj}$

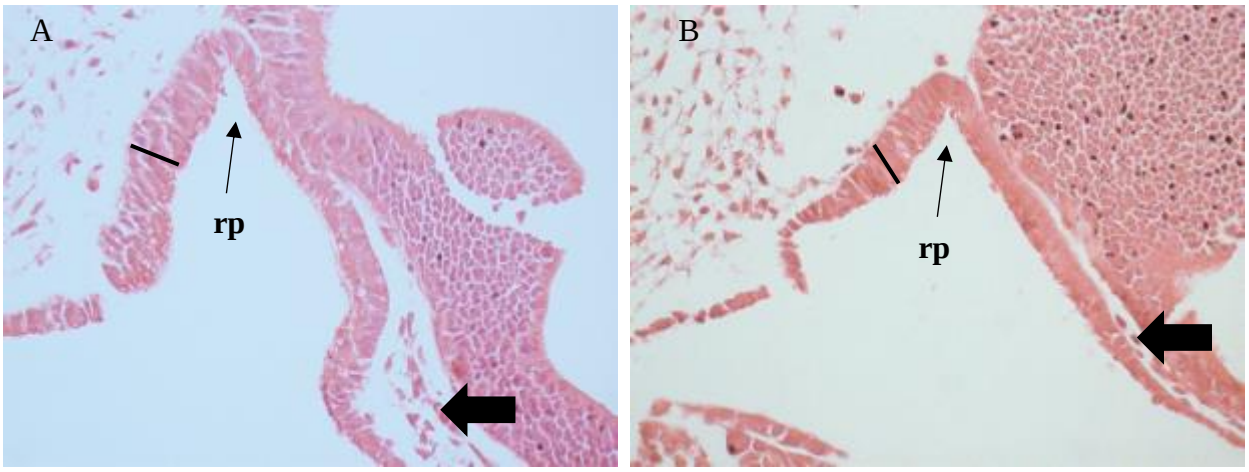

Figure 4. Prickle1$^{Bj/Bj}$ Rathke's pouch is misshapen (A) *Prickle1*$^{+/+}$ (B) *Prickle1*$^{Bj/Bj}$ Mid-sagittal section through Rathke's pouch at E10.5. Hematoxylin and eosin staining of *Prickle1*$^{+/+}$ (A) and *Prickle1*$^{Bj/Bj}$ (B). Epithelium of Rathke's pouch (rp) is approximately four cell layers thick in *Prickle1*$^{+/+}$ and approximately two cell layers thick in *Prickle1*$^{Bj/Bj}$. There is very little mesenchyme (bold arrows) in *Prickle1*$^{Bj/Bj}$ when compared to *Prickle1*$^{+/+}$ at E10.5 40X

4.3 PRICKLE1 AND β-CATENIN STAINING AT E10.5 IN PRICKLE1$^{+/+}$ *AND PRICKLE1*$^{Bj/Bj}$

The *Prickle1*^{Bj} is a missense mutation, and as such we expect to see *Prickle1* protein produced in the mutant animals. Because the mutation is located in the Lim domain, and Lim domains act as protein interacting domains we hypothesize that *Prickle1* protein in the mutants will be mislocalized. In addition, to visualize the cell membranes, we used an antiboy to β-catenin. We first looked at the expression of *Prickle1* in wild type animals. We found that

Prickle1 is expressed in the cell membrane and in the nucleus of the wild type animals. However, in the *Beetlejuice* mutants, Prickle1 is scattered throughout the cytoplasm and we did not see obvious cell membrane localization (Fig 5).

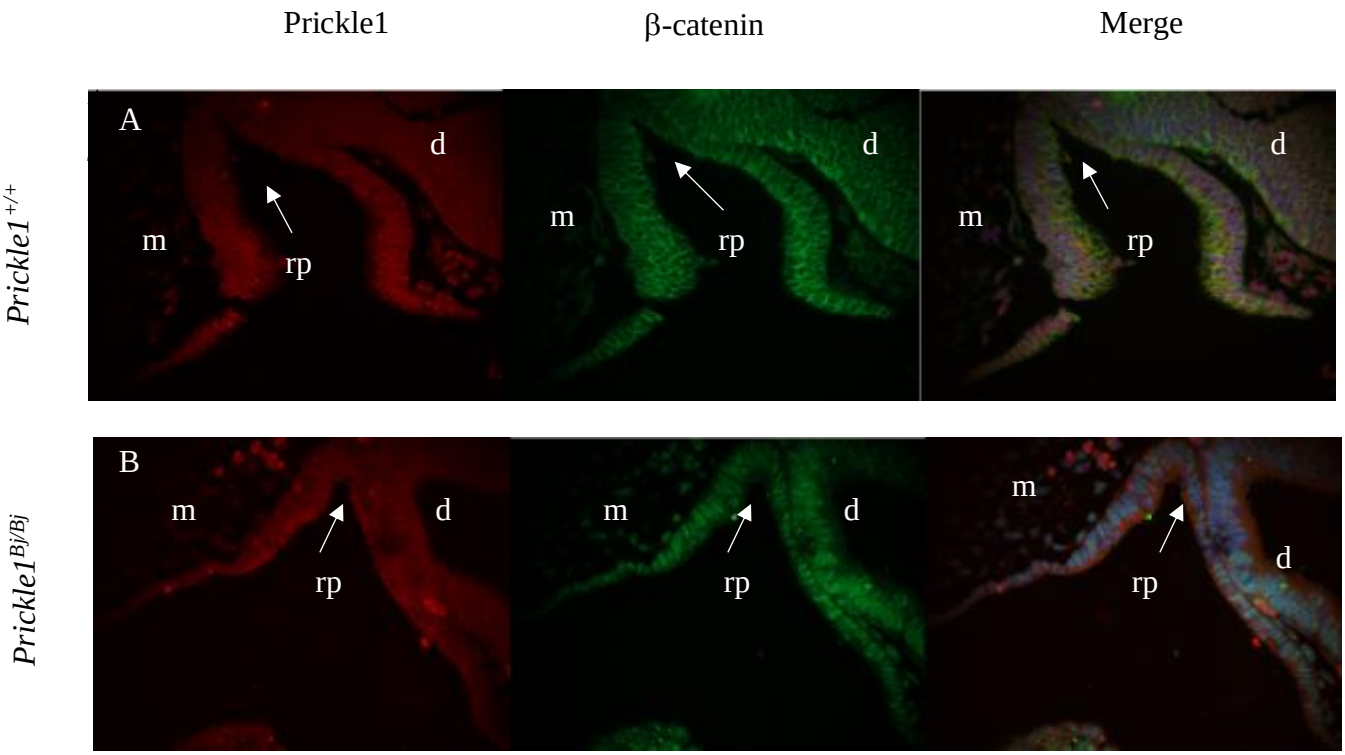

Figure 5. Prickle1$^{+/+}$ (A) and Prickle1$^{Bj/Bj}$ (B) stained for Prickle1 Primary Antibody at E10.5 40X

Mid-sagittal section through Rathke's pouch (rp) at E10.5 in Prickle1$^{+/+}$ (A) and Prickle1$^{Bj/Bj}$ (B). Immunoflourescence of *Prickle1* (red), DAPI (blue) and β-catenin (green). Prickle1 protein is expressed in the cell membrane and nucleus in Prickle1$^{+/+}$ but is dispersed throughout the cytoplasm in Prickle1$^{Bj/Bj}$. β-catenin is expressed in the cell membrane in Prickle1$^{+/+}$ but is in the cytoplasm in Prickle1$^{Bj/Bj}$. Mesenchyme (m), diencephalon (d).

4.4 PITX1 AND ROR2 STAINING

We looked at the role of Pitx1 because it is a transcription factor for pituitary development and indicates cell differentiation. We looked at the role of ROR2 because it is a kinase that is downstream of Prickle1 in the Wnt/PCP signaling pathway, and a mutation in Prickle1 could potentially affect ROR2 since it is located downstream. We observed fewer Pitx1-positive cells in the *Beetlejuice* mutant, and the cells which are positive for Pitx1 are located in the edge of Rathke's pouch. In the wild type, Pitx1 is localized to the nucleus. *Beetlejuice* mice have ROR2 dispered throughout the cytoplasm, while in the wild type animals it is localized to the cell membrane.

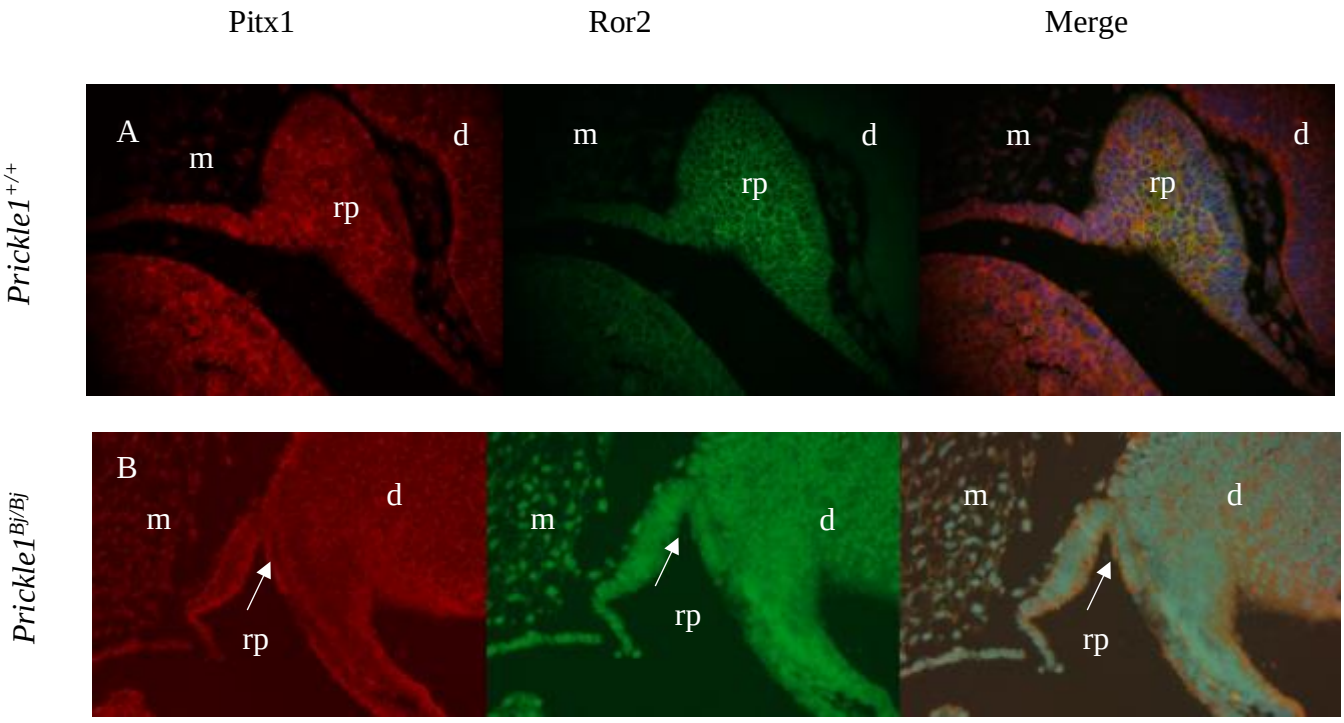

Figure 6. Prickle1$^{+/+}$ (A) and Prickle1$^{Bj/Bj}$ (B) stained for Pitx1 and ROR2 Primary Antibodies at E10.5 40X

Mid-sagittal section through Rathke's pouch (rp) at E10.5 in Prickle1$^{+/+}$ (A) and Prickle1$^{Bj/Bj}$ (B). Immunoflourescence of *Pitx1* (red), DAPI (blue) and ROR2 (green). Pitx1 is expressed in the cell membrane and nucleus in Prickle1$^{+/+}$ but is dispersed throughout the cytoplasm in Prickle1$^{Bj/Bj}$. ROR2 is expressed in the cell membrane in Prickle1$^{+/+}$ but is in the cytoplasm in Prickle1$^{Bj/Bj}$. Mesenchyme (m), diencephalon (d).

5.0 DISCUSSION

The aim of this project was to examine the effects of a missense mutation in Prickle1 on the developing craniofacial structures in the *Beetlejuice* mouse. Specifically, we studied effects of the mutation on the developing anterior pituitary gland at E9.5 and E10.5. Data from previous experiments in our lab showed abnormal pituitary gland development and defects in the cranial base at E12.5 when compared to wild type animals. These findings inspired us to examine the *Beetlejuice* mouse at earlier time points in development in order to better understand the effects of the mutation in Prickle1.

At E9.5, immunofluorescence staining with Prickle1 primary antibody in the wild-type animals revealed that the Prickle1 protein is expressed in both the epithelium and the mesenchyme surrounding Rathke's pouch (Figure 4). Using these findings as a reference for normal Prickle1 expression in Rathke's pouch, we expanded our experiment by examining wild-type and mutant animals at E10.5.

When *Beetlejuice* mice were examined at E10.5, Rathke's pouch was underdeveloped and no mesenchyme was present between the anterior portion of the epithelium of the pouch and the diencephalon, suggesting that the development of the anterior pituitary is abnormal much earlier than E12.5 in the mutant animals. In addition, there were fewer epithelial cells present in Rathke's pouch in the *Beetlejuice* mice. The fact that Pitx1 expression is altered in *Beetlejuice* could partially account for the fact that there are fewer epithelial cells present in these mice, as

Pitx1 is a stem cell precursor in pituitary development. β-catenin and ROR2 expression were also altered in the *Beetlejuice* mutants, suggesting that mutations in Prickle1 somehow influence the expression and/or function of these proteins.

In future studies, it would be important to further explore the effect of the Prickle1 mutation in the *Beetlejuice* mesenchyme since there appears to be a difference in expression in regions of the wild type as well as in the mutant. It is also interesting to note that studies by Bassuk et al. (2008) and Tao et al. (2011) found that humans who have a recessive missense mutation in the PET domain of the human *Prickle1* gene develop progressive myoclonus epilepsy (PME). It could prove beneficial to examine the pituitary gland in these individuals in order to test for interactions between pituitary development and function as a result of the mutation.

6.0 CONCLUSIONS

The Prickle1 protein is expressed in the normal pituitary gland at E9.5 and in the mesenchyme surrounding it. By histological analysis, morphogenesis of the pituitary gland is altered in the *Beetlejuice* animals. Prickle1 is still expressed in the mutants but is located in a different domain than in the wild-type animals. β-catenin, ROR2 and Pitx1 domains are also altered in *Beetlejuice* mutants. Based on our findings, we conclude that Prickle1 is required for cell polarization in normal pituitary gland development.

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