PARATHYROID CELL FATE INSTABILITY: AN INVESTIGATION OF PARATHYROID CELL CYCLE AND ORDER OF FATE SWITCHING

by

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(Under the Direction of Nancy Manley)

ABSTRACT

The parathyroid glands are essential for regulating calcium homeostasis in the body. During mouse development, the parathyroid develops in the 3rd pharyngeal pouch along with the thymus. The messy separation of these two organs leaves behind ectopic parathyroid glands and cervical thymi. When few cervical thymi were found to have a history of expressing parathyroid hormone, parathyroid cell fate stability was shown to be unstable. It is unclear why parathyroid cell fate is unstable or how the fate switch occurs. One possible reason for parathyroid cell fate instability is low proliferation and/or long cell cycle as proliferation has been linked to cell stability. My research investigates parathyroid cell proliferation and cell cycle length throughout embryonic development. To address how the fate switch occurs, my data suggests that activation of the thymus program in parathyroid cells is sufficient to downregulate the parathyroid program.

INDEX WORDS: Parathyroid, 3rd pharyngeal pouch, cervical thymi, cell fate instability

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DEDICATION

I would like to dedicate this work to my twin sister, Jenna Peissig. You are the sole reason why I chose to do this work. I hope that my research will contribute to a cure for hypoparathyroidism.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW: EMBRYOLOGY OF THE PARATHYROID GLANDS 1

Abstract 2

Introduction 2

Parathyroid Development in Mice 4

Gcm2: The Master Regulator of Parathyroid Development 5

Patterning of the 3rd Pharyngeal Pouch and Initiation of Gcm2 5

Gcm2 is Upregulated by HoxA3 and Interacting Transcription Factors 7

Terminal Differentiation of Parathyroid Cells 9

Parathyroid Development in Humans 9

The Future of Parathyroid Development Research 10

References 11

Figures 16

2 CELL FATE INSTABILITY OF DEVELOPING PARATHYROID CELLS 17

Abstract 18

Introduction 18

Results.....20

Discussion.....23

Methods.....27

References.....29

Figures.....31

APPENDICES

A Investigation of Bmp4 and Fgf8 Interaction in 3rd Pharyngeal Pouch.....36

Introduction.....36

Results.....37

Discussion.....38

Methods.....38

References.....40

Figures.....41

LIST OF FIGURES

Page

Figure 1-1: 3rd Pharyngeal Pouch at E11.5 16

Figure 1-2: Early Patterning of the 3rd Pharyngeal Pouch 16

Figure 2-1: Proliferation of parathyroid cells throughout embryonic development 31

Figure 2-2: Parathyroid cell cycle measurements at E11.5....32

Figure 2-3: Parathyroid cell cycle measurements at E13.5 and E16.5 33

Figure 2-4: Foxn1 and Gcm2 in E13.5 and E15.5 PTHcre;iFoxn1 parathyroid glands 34

Figure 2-5: CAGtdTomato and Foxn1 at E16.5 in PTHcre;iFoxn1 mouse 34

Figure 2-6: Downregulation of the parathyroid program 35

Figure 2-7: Abnormal Foxn1 positive, Gcm2 negative structure in 4 week old parathyroid gland....35

Figure A-1: Antibody staining of E11.5 3rd pharyngeal pouch primordia after 24-hr whole embryo culture 41

Figure A-2: Antibody staining of E11.5 3rd pharyngeal pouch primordia after 24-hr embryo culture 42

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW: EMBRYOLOGY OF THE PARATHYROID GLANDS[1]

[1] Peissig, K.B. and N.R. Manley. To be submitted to *Endocrinology & Metabolism Clinics*.

Abstract

Parathyroid glands are important for maintaining calcium homeostasis. When parathyroid glands do not function properly, this homeostasis is disrupted. There are two parathyroid disorders: hyper- and hypoparathyroidism, which result in excess and insufficient production of parathyroid hormone, respectively. Studying the development of parathyroid glands is essential for developing a strategy to cure and prevent parathyroid disorders. During mouse development, the parathyroid glands develop from the 3rd pharyngeal pouches in tandem with the thymus. Gcm2 is the master regulator of parathyroid development and its regulation is essential for proper parathyroid function. Initial patterning of the 3rd pharyngeal pouch allows for the initiation of Gcm2 expression. Upregulation of Gcm2 after initiation of its expression is necessary for parathyroid cell development and survival. This review covers the development of parathyroid glands in mice, and data from these studies can also be applied to human development.

Introduction

Maintaining a stable level of calcium in the body is important for proper muscle function, neurotransmission, and enzyme and hormone secretion, among many other functions (Ramasamy, 2006). This regulation of calcium is controlled by the parathyroid, which is a small gland of the endocrine system located in the neck next to the thyroid gland. Parathyroid glands contain receptors that monitor calcium levels called Calcium-Sensing Receptors (CaSR) (R. A. Chen & Goodman, 2004). When these receptors detect low levels of calcium, the parathyroid secretes parathyroid hormone (PTH), which interacts with G-protein-coupled receptors on bone and kidney cells. This allows for more calcium to be released from bones into the bloodstream. In the kidney, PTH increases resorption of calcium in the ascending limb of the loop of Henle and in the distal tube and additionally increases excretion of inorganic phosphate into the urine (Houillier, Nicolet-

Barousse, Maruani, & Paillard, 2003). When the parathyroid does not function properly, calcium/phosphorus homeostasis is disrupted. This culminates into one of two parathyroid disorders: hyper- and hypoparathyroidism. Hyperparathyroidism is usually caused by parathyroid adenomas, which are benign parathyroid tumors that cause an excess of PTH to be produced and secreted. For hyperparathyroidism, the current treatment option is to surgically remove the adenomas, and the patient is cured following this procedure. Hypoparathyroidism is observed much less frequently in the clinic and has a number of causes, including mutations in genes required for proper parathyroid development (Grigorieva & Thakker, 2011). There is currently no cure for hypoparathyroidism and individuals are prescribed calcium and vitamin D supplements in order to help regulate their calcium levels.

PTH has been detected in both the parathyroid and thymus, but the sole producer of PTH with endocrine function is the parathyroid. PTH was first detected in the thymus through work by Gunther et al., where PTH was detected in mice that lacked parathyroid glands. Upon further investigation of wild-type mice, the auxiliary source of PTH was said to be the thymus (Gunther et al., 2000). Subsequent investigation of PTH in the thymus revealed that thymus PTH originated from two sources: (1) parathyroid cells that were encapsulated by the thymic capsule during the messy separation of the parathyroid and thymus during embryonic development, and (2) medullary thymic epithelial cells (mTECs). It has been shown that mTECs express PTH as a self-antigen and do not have any endocrine function (Liu et al., 2010). Therefore, the sole source of endocrine-active PTH is the parathyroid.

Understanding the normal development process of the parathyroid is important so that we can learn how and why parathyroid disorders manifest. Embryonic development of the parathyroid is a topic that has been studied since the 1930s, and the findings up to the present will be described in this

review. Much of the research done in this area has been done in mice. One goal that these data will be used for is to develop a cure for hypoparathyroidism and prevent parathyroid disorders.

Parathyroid Development in Mice

During mouse development, endodermal outpocketings called pharyngeal pouches form in the pharyngeal region. These pouches are epithelial structures and are surrounded by neural-crest-derived mesenchymal cells, which play an important role in initial patterning. The parathyroid glands develop in the 3rd pharyngeal pouches, yielding one set of bilateral parathyroid glands (Cordier & Haumont, 1980). The dorsal region of each 3rd pharyngeal pouch develops into a parathyroid gland whereas the ventral region develops into one lobe of the thymus (Gordon, Bennett, Blackburn, & Manley, 2001). At about E12.5, the parathyroid and thymus begin to separate from each other. The thymus migrates to the top of the heart, leaving the parathyroid glands in the neck region next to the thyroid (Manley & Capecchi, 1998). This separation is a messy process, leaving small clusters of cells from both organs along the path to their final positions. These small clusters of cells become small parathyroid glands and cervical thymi, depending on the organ from which the cells originated. In some cases, these small parathyroid glands lose control of their cell cycle and turn into parathyroid adenomas that lead to hyperparathyroidism. Cervical thymi primarily arise from thymus cells that are left behind during the separation of the parathyroid and thymus. However, in 2013, it was revealed that some cervical thymi have a parathyroid lineage, having at one time expressed *PTH* but instead have a thymus fate (Li, Liu, Xiao, & Manley, 2013). This suggests that the parathyroid cell fate is unstable, and the mechanism behind the fate switch is unclear.

Gcm2: The Master Regulator of Parathyroid Development

Glial cells missing (Gcm) was first discovered in *Drosophila* as a transcription factor that acts as a binary switch for neural cells to become either neurons or glial cells (Hosoya, Takizawa, Nitta, & Hotta, 1995; Jones, Fetter, Tear, & Goodman, 1995). Characterization of the mammalian homologs of drosophila Gcm called Gcm1 and Gcm2 revealed high expression of Gcm2 in parathyroid glands in developing mice, suggesting a non-neuronal function of the transcription factor (Kim et al., 1998).

Gcm2 is required for parathyroid development and is also considered the master regulator of this process. Gcm2 expression can be detected at the dorsal end of the 2nd and 3rdpharyngeal pouches at E9.5, and by E10.5, Gcm2 is restricted to a small portion of the dorsal 3rd pouch endoderm (Gordon et al., 2001). In studies involving Gcm2 null mice, the failure to develop parathyroid glands leads to hypoparathyroidism (Gunther et al., 2000). Gcm2 is required for parathyroid cell differentiation. Expression of parathyroid genes CaSR and CCL21 is detected in parathyroid-fated cells, but their expression cannot be maintained without Gcm2. Additionally, PTH is never turned on in parathyroid-fated cells in Gcm2 null mice. Furthermore Gcm2 is required for parathyroid survival. Without Gcm2, developing cells of the parathyroid-fate undergo programmed cell death (Liu, Yu, & Manley, 2007).

Patterning in the 3rd Pharyngeal Pouch and Initiation of Gcm2

The 3rd pharyngeal pouch begins to form at E9.5. Initial patterning of the pouch by several genes in a spatial-temporal-dependent manner determines the cell fate of the endodermal cells within it, where parathyroid development occurs at the dorsal end marked by Gcm2 and thymus development at the ventral end marked by Foxn1 (Figure 1).

One of the earliest genes that can be detected in 3rd pharyngeal pouch patterning is the signaling molecule Sonic Hedgehog (Shh), which is expressed in neural crest cells and in the adjacent pharynx. Shh expression is necessary for parathyroid organogenesis, where in Shh null mutants, parathyroid glands are absent and the parathyroid master regulator gene, Gcm2, is never activated (Moore-Scott & Manley, 2005). This was also reported in a quail-chick study (Figueiredo et al., 2016). Recently, it was shown that Shh in either the neural crest cells surrounding the 3rd pharyngeal pouch or the pharyngeal endoderm itself is sufficient to activate Gcm2 expression (Bain et al., 2016). Another gene that is required for Gcm2 activation is the transcription factor Gata3. In Gata3 null mice, Gcm2 never is never activated. Additionally, Gata3 heterozygotes have fewer Gcm2-expressing cells during embryonic development and have hypoparathyroidism (Grigorieva et al., 2010). In addition to its role in initiating parathyroid differentiation, Shh has been shown to prevent parathyroid-specific genes such as Gcm2 from being activated in other pharyngeal pouches (Grevellec, Graham, & Tucker, 2011). At E11.5, Shh activates the T-box transcription factor, Tbx1, in the parathyroid domain, which is co-expressed with Gcm2. When Tbx1 is ectopically expressed in developing thymus cells, Foxn1 is suppressed, suggesting that Tbx1 blocks thymus differentiation at the dorsal end of the pouch. However, although Tbx1 suppresses Foxn1, Gcm2 expression remains restricted to the dorsal end of the 3rd pharyngeal pouch and does not expand into the ventral region (Bain et al., 2016; Reeh et al., 2014). Although there is no conclusive evidence to support the claim, a potential role for Tbx1 in the regulation of Gcm2, either directly or indirectly, has been proposed.

From E10.5 to E12.5, the signaling molecule Bmp4 is expressed at the ventral end of the 3rd pharyngeal pouch where the thymus will develop (Moore-Scott & Manley, 2005; Patel, Gordon, Mahbub, Blackburn, & Manley, 2006). At the dorsal parathyroid end of the pouch, the Bmp

antagonist Noggin is expressed, blocking the Bmp signal (Patel et al., 2006). The transcription factor Pax3 is expressed in the neural crest cells surrounding the 3rd pharyngeal pouch. At the dorsal end of the 3rd pharyngeal pouch, Pax3 acts to keep the thymus fate from expanding dorsally into the parathyroid domain. In Pax3(Sp/Sp) mutants, mixing of fates occurs in the middle region between the two organ primordia, indicating that Pax3 plays an important role in the refinement of the border between thymus and parathyroid primordia (Griffith et al., 2009). 3rd pharyngeal pouch patterning and initiation of Gcm2 is summarized in Figure 2.

Gcm2 is Upregulated by HoxA3 and Interacting Transcription Factors

At E9.5, Gcm2 is expressed at a low level at the dorsal end of the 3rd pharyngeal pouch. By E10.5, Gcm2 is upregulated in the parathyroid domain and parathyroid cells begin to proliferate. Failure to upregulate Gcm2 after initial expression leads to coordinated apoptosis in the parathyroid-fated cells. HoxA3 was the first Hox gene to be knocked out in mice via homologous recombination, and has been shown to play a role in the development of pharyngeal pouch derived organs, including the parathyroid and thymus (Chisaka & Capecchi, 1991). HoxA3 null mutants do not develop parathyroid glands or a thymus. Although there is overlapping function between the group 3 Hox paralogs, only HoxA3 has a specific role in the development of the parathyroid and thymus (Manley & Capecchi, 1998). Histologically, at E10.5, the formation of the 3rd pharyngeal pouch is normal in HoxA3 null mice. However, by E12, no parathyroid rudiment could be found, and the thymus rudiment was severely hypoplastic. At E12.5, all of the 3rd pharyngeal pouch derivatives had disappeared due to coordinated apoptosis (Chojnowski et al., 2014; Kameda, Arai, Nishimaki, & Chisaka, 2004; Manley & Capecchi, 1995).

HoxA3 is not required at the genetic level for the initiation of parathyroid development. In HoxA3 null mice, Gcm2 expression is initiated at a reduced level (L. Chen et al., 2010), however, by

E11.5, Gcm2 expression is undetectable. Neural crest cell- and/or endoderm-specific deletion of HoxA3 revealed that expression of HoxA3 in either tissue type is sufficient for the organogenesis of both the parathyroid and thymus. Additionally, HoxA3 expression in the 3rd pharyngeal pouch endoderm is required for the upregulation of Gcm2. In an endoderm-specific deletion of HoxA3, Gcm2 expression is low, like that seen in the HoxA3 null mice at E10.5. Only parathyroid-fated cells that express HoxA3 can upregulate Gcm2 by E12 (Chojnowski et al., 2014; Chojnowski, Trau, Masuda, & Manley, 2016). Without the upregulation of Gcm2, parathyroid-fated cells are unable to survive and undergo coordinated apoptosis.

The transcription factor Pax1 has also been shown to play a role in Gcm2 upregulation. In HoxA3 null mice, Pax1 expression is reduced in the 3rd pharyngeal pouch, suggesting that Pax1 may be downstream of HoxA3 (L. Chen et al., 2010). Pax1 null mutants show decreased Gcm2 expression at E11.5 and parathyroid hypoplasia. Pax1 is dependent on the expression of Eya1 and Six1 in the 3rd pharyngeal pouch. In Six1 null mutants, Gcm2 is initiated but cannot be maintained. These parathyroid-fated cells undergo coordinated apoptosis (Zou et al., 2006), similar to the parathyroid phenotype seen in HoxA3 null mutants. In HoxA3$^{+/-}$Pax1$^{-/-}$ compound mutants, Gcm2 expression is further reduced or absent by E11.5 (Su, Ellis, Napier, Lee, & Manley, 2001). These results suggests that HoxA3 and Pax1 interact to upregulate Gcm2 expression.

Additionally, Pbx1 null mutants show a similar parathyroid-thymus phenotype as the HoxA3 null mutant. Pbx1 null mice have small parathyroid and thymic primordia, leading the hypoplasia in both organs. Reduction in Gcm2 suggests that the HoxA3-Pax1 upregulation of Gcm2 is at least in part dependent on Pbx1 (Manley, Selleri, Brendolan, Gordon, & Cleary, 2004).

Terminal Differentiation of Parathyroid Cells

After the upregulation of Gcm2, downstream parathyroid genes are activated including PTH, CaSR, and CCL21. The initiation of CaSR and CCL21 in the parathyroid is Gcm2-independent, but their maintenance is Gcm2-dependent whereas PTH is dependent on Gcm2 for both initiation and maintenance (Liu et al., 2007).

The transcriptional activator MafB was found to be involved in the activation of PTH in 2011. MafB is expressed in the parathyroid after E11.5 and its expression is Gcm2-dependent. In MafB null mutants, PTH expression and secretion are greatly reduced. MafB was shown to physically associate with Gcm2 in order to interact with the PTH promoter via a Maf-recognition element and a Gcm2 binding sequence and turn on its expression (Kamitani-Kawamoto et al., 2011). In 2015, it was revealed that Gata3 also physically interacts with MafB and Gcm2 and with an additional ubiquitous transcription factor, Sp1, in order to activate PTH expression (Han, Tsunekage, & Kataoka, 2015).

Parathyroid Development in Humans

Parathyroid development in humans occurs in a similar manner as in mice. In humans, the parathyroid glands develop in tandem with the thymus in the 3rd pharyngeal pouch and with the ultimobranchial bodies in the 4th pharyngeal pouch, yielding two sets of bilateral parathyroid glands (Gilmour, 1937; Norris, 1938; Phitayakorn & McHenry, 2006; Weller, 1933). This was further strengthened when expression of the parathyroid marker, Gcm2, was found in both the 3rd and 4th pharyngeal pouches in human embryos (Liu et al., 2010). Genes that have been found to be important in human parathyroid development include: *GCMB*, *Gata3*, and *Tbx1* (Grigorieva & Thakker, 2011). Loss of function mutations in these three genes have all been shown to cause hypoparathyroidism in humans, which is consistent with data from mouse studies.

The Future of Parathyroid Development Research

There is still much to study in the field of parathyroid development. It will be important to identify all of the transcription factors and proteins that play a role in parathyroid cell differentiation during embryonic development. With the advent of –omics techniques, researchers will be able identify all of the genes that are being expressed, which genes are in areas of open chromatin, and what proteins are interacting with each other during parathyroid development.

For the genes that have already been identified as playing a role in parathyroid development, the precise pathways and mechanisms of action need to be elucidated. For example, Tbx1 is thought to play a role in the regulation of Gcm2, but further evidence is required to confirm this hypothesis. The interaction between HoxA3, Pax1, and Pbx1 also needs to be clarified in terms of their role in Gcm2 upregulation.

After the genes important for parathyroid development have been identified, an inducible parathyroid cell culture system could be made possible. An *in vitro* system with parathyroid cells will make protein interaction studies much easier and bring the cost of using live animals down. Having stable parathyroid cell lines may also lead to a cure for hypoparathyroidism as these cells could be implanted into patients to restore normal parathyroid function and calcium regulation. The question of parathyroid stability still needs to be addressed as parathyroid cell fate has been shown to be unstable with some parathyroid cells switching to a thymus cell fate (Li et al., 2013). The mechanism of this fate switch will need to be elucidated in order for cell therapy to become a viable treatment option.

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Figures

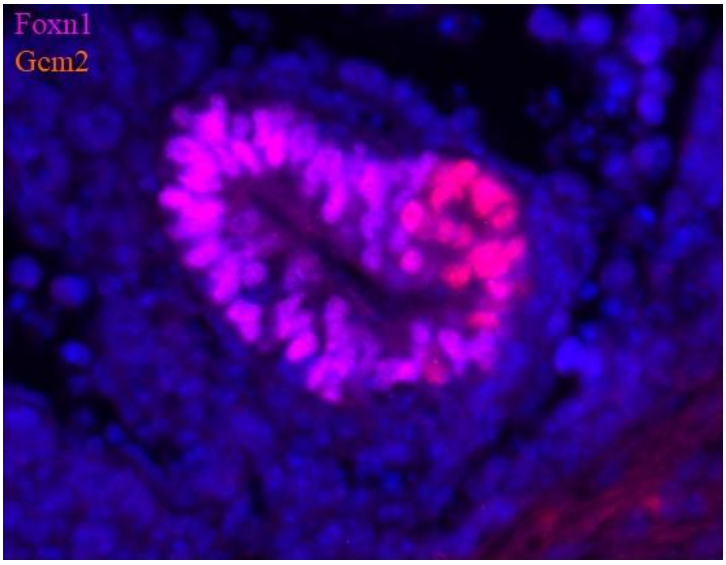

Figure 1-1: 3rd Pharyngeal Pouch at E11.5.

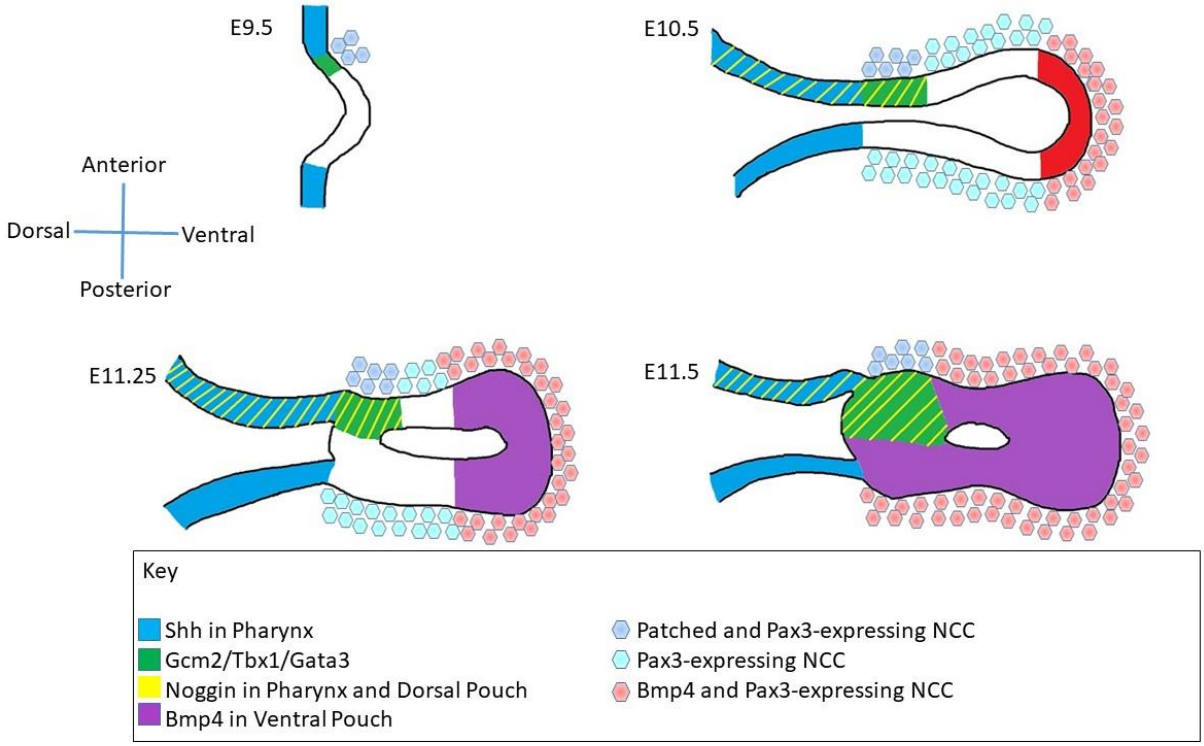

Figure 1-2: Early Patterning of the 3rd Pharyngeal Pouch.

CHAPTER 2

CELL FATE INSTABILITY OF DEVELOPING PARATHYROID CELLS[2]

[2] Peissig, K.B. To be submitted to *Development*.

Abstract

The parathyroid glands are essential for regulating calcium homeostasis in the body. During mouse development, the parathyroid develops in the 3rd pharyngeal pouch along with the thymus. The messy separation of these two organs leaves behind ectopic parathyroid glands and cervical thymi. When few cervical thymi were found to have a history of expressing parathyroid hormone, parathyroid cell fate stability was shown to be unstable. It is unclear why parathyroid cell fate is unstable or how the fate switch occurs. One possible reason for parathyroid cell fate instability is low proliferation and/or long cell cycle as proliferation has been linked to cell stability. My research investigates parathyroid cell proliferation and cell cycle length throughout embryonic development. To address how the fate switch occurs, my data suggests that activation of the thymus program in parathyroid cells is sufficient to downregulate the parathyroid program.

Introduction

The parathyroid is the organ responsible for maintaining calcium homeostasis in the body. During mouse development, the parathyroid develops in tandem with the thymus in the 3rd pharyngeal pouch, where the dorsal cells differentiate into parathyroid and the ventral cells differentiate into thymus (Gordon, Bennett, Blackburn, & Manley, 2001). When these two organs separate from each other, small clusters of cells are left in the migration path forming ectopic parathyroid glands and cervical thymi. Cervical thymi are ectopic clusters of thymus cells that are found in between the parathyroid and thoracic thymus. Although there is no evidence to suggest that thymus fate is unstable, parathyroid cell fate has been shown to be unstable with parathyroid cells transdifferentiating to thymus cells at low frequencies during late fetal stages when few cervical thymi were found to have a history of expressing parathyroid hormone (PTH) ((Li, Liu, Xiao, & Manley, 2013). It is unclear how the fate switch from the parathyroid program to the thymus

program occurs. The main focus of this research is to begin to elucidate the reason for the cell fate instability of developing parathyroid cells and the mechanisms that underlie the fate switch from parathyroid to thymus.

DNA methylation has been shown to be involved during cell fate commitment and cell differentiation by shutting down other fate pathways (Suelves, Carrio, Nunez-Alvarez, & Peinado, 2016). DNA is most accessible during DNA replication, so it is thought that *de novo* DNA methylation occurs most frequently during S-phase of the cell cycle. One possible reason that parathyroid cells have an unstable cell fate could therefore be that they do not proliferate rapidly and/or have a long cell cycle length. When we looked at proliferation in the developing 3rd pharyngeal pouch at embryonic day 10.5 (E10.5), it appeared to be uniform throughout the pouch. By E11.5, however, the parathyroid cells seem to be proliferating much more slowly than the developing thymus cells (Gordon, unpublished). There is no evidence to suggest that developing thymus cells also have an unstable cell fate, therefore the differing proliferation rates between the two cell types may play a role in the cell fate instability of developing parathyroid cells. It is possible that parathyroid cell proliferation remains low until the newborn stage, causing their fate to be unstable throughout embryonic development. Thus, DNA methylation may be slow to shut down the thymus fate pathway in developing parathyroid cells until late embryonic development, allowing the thymus program to be available for fate switch. The first part of this research will investigate proliferation and measure cell cycle length in developing parathyroid cells.

The transition from the parathyroid program to the thymus program during the formation of parathyroid lineage cervical thymi is unclear. Does the parathyroid program shut down first and then the thymus program turns on? Or is the thymus program initiated first in developing parathyroid cells, subsequently shutting down the parathyroid program? The second part of this

research aims to elucidate the order of initiation and downregulation of these two programs during the switch from the parathyroid program to the thymus program.

Results

Proliferation of parathyroid cells during embryonic development

To test the hypothesis that proliferation is low at E11.5 and increases near the newborn stage, I measured the proliferation of parathyroid cells throughout embryonic development. Proliferation can be measured in cells using nucleoside analogs that are incorporated into DNA during S-phase of the cell cycle when DNA is being replicated. These nucleoside analogs can be detected using antibody staining or, with more recent technology, using a Click-it Reaction. Cells that have incorporated the nucleoside analog must therefore have replicated their DNA at least once during the exposure to the analog and will be tagged. Proliferation of parathyroid cells was measured by dividing the number of $BrdU^{+}Gcm2^{+}$ cells by the total number of $Gcm2^{+}$ cells.

For all embryonic time points (E11.5, 13.5, 16.5, 17.5) a BrdU pulse time of 2 hours was used (Figure 1). The widest range in proliferation was seen at E11.5 at 16.87%, with the low proliferation rate at 9.19% and the high proliferation rate at 26.06% (n=8). This wide range in proliferation rate at E11.5 suggests that there is high variability in parathyroid proliferation at this time point. The greatest percentage of proliferating cells was seen at E13.5 at 27.25% (n=2, standard error=2.25%), and the lowest percentage was seen at E17.5 at 12.32% (n=1). This suggests that parathyroid proliferation is highest at E13.5 and decreases by E17.5, which disagrees with the hypothesis that proliferation remains low until near the newborn stage.

Cell cycle of parathyroid cells during embryonic development

To test the hypothesis that the cell cycle is long at E11.5 and decreases in length near the newborn stage, I measured cell cycle length in parathyroid cells throughout embryonic development. The

cell cycle can be measured in a similar fashion as cell proliferation using two different nucleoside analogs with staggered pulse times (See Methods). The nucleoside analogs BrdU and EdU were used to label proliferating cells at three stages of embryonic development (E11.5, E13.5, and E16.5) to measure cell cycle length in parathyroid cells.

At E11.5, I initially used three different pulse times to measure the cell cycle of parathyroid cells: 1 hour BrdU/ 30 minutes EdU, 2 hours BrdU/ 1 hour EdU, and 4 hours BrdU/ 2 hours EdU (Figure 2: Ai, Bi, C). The distribution of the cell cycle measurements was so wide, however, that no definitive measurement could be made (Range=18.35 hr). To try to generate more consistent data, I used a 1 hour BrdU/30 minutes EdU pulse time on three more litters of E11.5 embryos and measured the cell cycle length in one embryo from each litter (Figure 2: A ii, iii, iv). The distribution was not as wide in this dataset (Range=7.43 hr), but a definitive cell cycle measurement could still not be made. One reason for the inconsistent data could be that somite counts were not used to stage the embryos precisely. To address this concern, I used a 2 hour BrdU/ 1 hour EdU pulse time on one more litter of E11.5 embryos and counted somites in these embryos. I analyzed two embryos with somite counts of 45 and 54 (Figure 2: B ii, iii, respectively). The cell cycle measurement for the 45 somite embryo was nearly twice as long as the measurement for the 54 somite embryo (43.03 hr v 24.41 hr).

A summary of all of the data for the E11.5 time point culminated in an average cell cycle length of developing parathyroid cells of 18.25 hours with a standard error of 2.27 hours (n=20). Because the 45 somite cell cycle measurement was an outlier in the data set, a new average cell cycle measurement was calculated without those data. This gave an average cell cycle measurement of 15.5 hours, with a standard error of 1.36 hours (n=18) (Figure 2D).

At E13.5, three different pulse times were used to measure the cell cycle in parathyroid cells: 1 hour BrdU/ 30 minutes EdU, 2 hours BrdU/ 1 hour EdU, and 4 hours BrdU/ 2 hours EdU (Figure 3). The cell cycle measurement was consistent regardless of the pulse time used. The cell cycle length of parathyroid cells at E13.5 was calculated to be 13.08 hours, with a standard error of 0.36 hour (n=5).

At E16.5, a 2 hour BrdU/ 1 hour EdU pulse time was used to measure the cell cycle length in parathyroid cells (Figure 3). This was calculated to be 19.45 hours, with a standard error of 0.42 hour (n=4).

Taken together, these data suggest that cell cycle regulation in parathyroid cells at E11.5 is highly variable, but that it stabilizes by E13.5. These data also indicate that parathyroid cell cycle length is the shortest at E13.5 and the longest at E16.5, which is in disagreement with our hypothesis that the parathyroid cell cycle remains long from E11.5 to the newborn stage.

Activation of Foxn1 is sufficient to downregulate the parathyroid program

To investigate the order in which the parathyroid and thymus program switch, I activated a Foxn1 transgene (iFoxn1) in parathyroid cells using PTHcre. This addressed the question: Is the activation of Foxn1, the master regulator of thymus development, sufficient to downregulate or shut off the parathyroid program? For this experiment, PTHcre mice were crossed with iFoxn1 mice. In the resulting progeny, Foxn1 will be activated in parathyroid cells beginning at E12 when PTH expression is initiated.

In PTHcre;iFoxn1 mice, FOXN1 protein was detected by antibody staining in parathyroid cells. Interestingly, FOXN1 was detected in only 5% to 83% of the parathyroid cells in a gland, rather than the expected 100% (Figure 4). To determine the cause of this discrepancy, a tomato reporter was used to determie whether cre recombinase was activated in every PTH-expressing cell.

Surprisingly, not every $Gcm2^+$ parathyroid cell was labeled with the tomato protein, but FOXN1 protein was detected in all cells that were labeled with tomato protein (Figure 5). As PTH is known to be expressed in all parathyroid cells ((Liu, Yu, & Manley, 2007), it is possible that the PTHcre transgene is being silenced.

In cells where Foxn1 protein was detected, I looked to see whether or not Gcm2 was downregulated or turned off. An example of Gcm2 downregulation can be found in Figure 6. At E13.5, 1% to 15.7% of $Foxn1^+$ cells appeared to be Gcm2 low or $Gcm2^-$ (n=6). By E16.5, however, 13.5% to 23.7% of $Foxn1^+$ cells were Gcm2 low or $Gcm2^-$ (n=2). This suggests that Foxn1 activation is sufficient to downregulate the parathyroid program, but not in every cell that expresses it, and downregulation of the parathyroid program is progressive over time.

In 4-week old PTHcre;iFoxn1 mice, the parathyroid glands were still present, suggesting that the entire glands had not been converted to a thymus fate. This is consistent with data from earlier stages, where the parathyroid program does not appear to shut down in all cells. However, a Foxn1+ Gcm2- structure could be seen inside the parathyroid region at 4 weeks (Figure 7). This structure did not have the typical histology of a parathyroid gland. It is possible that this represents a small ectopic thymus that has formed inside the parathyroid gland itself as a result of the activation of the iFoxn1 transgene in the parathyroid. However, without staining for additional thymus markers, it is not possible to conclude whether or not downstream thymus genes were activated in this structure.

Discussion

Parathyroid cell fate instability is a phenomenon with unknown cause and mechanism. Based on unpublished data from our lab, it was hypothesized that cell fate instability in parathyroid cells may be related to cell cycle length and/or proliferation. These data claim that cell proliferation in

parathyroid cells at E11.5 is extremely low compared to thymus cells; however, the embryos used to produce this data were not precisely staged. Based on the data from my cell cycle experiments, proliferation appears to be quite variable during the period from E11-12, as shown by the wide range of measurements obtained for this time point. This is also reflected in the variable cell cycle lengths calculated for E11.5. Parathyroid cells at this developmental time point may be heterogeneous in their cell cycle regulation due to differences in Gcm2 levels. It is possible that a subset of parathyroid cells have recently initiated Gcm2, and will therefore only express at low levels. The cell cycle therefore may be quite long in these cells as increased proliferation in parathyroid cells occurs after Gcm2 upregulation (Su, Ellis, Napier, Lee, & Manley, 2001). The remaining population of parathyroid cells at E11.5 may already have upregulated Gcm2 and will therefore have a much shorter cell cycle compared to the subset of parathyroid cells that have recently initiated Gcm2. Such differences in cell cycle regulation could therefore explain the difficulty in obtaining consistent cell cycle length for parathyroid cells at this developmental time point.

The proliferation rate and cell cycle length of parathyroid cells became much more consistent after E13.5, suggesting that cell cycle regulation in parathyroid cells is stable by this time point. The highest proliferation rate was seen at E13.5, which is consistent with the cell cycle length data. The lowest proliferation rate was seen at E17.5 followed closely by E16.5. This is also consistent with cell cycle data showing that parathyroid cells had the longest cell cycle at E16.5.

Based on my findings from the proliferation and cell cycle studies of parathyroid cells throughout embryonic development, it is unclear whether cell proliferation and/or cell cycle length is related to parathyroid cell fate instability. It was hypothesized that parathyroid cell proliferation would remain low and parathyroid cell cycle length would remain high until near the newborn stage.

Compared to non-progenitor Plet1^{-} developing thymic epithelial cells (TECs), the percentage of proliferating parathyroid cells at time points between E13.5 and E17.5 during mouse embryonic development is lower than that of proliferating TECs. At E13.5, 27.25% of developing parathyroid cells had been in S-phase of the cell cycle within a two-hour time period compared to 43.7%, of developing TECs that were labeled in S/G2/M phase in one instant using DNA content analysis. At E16.5 and E17.5, respectively, 15.9% and 12.3% of developing parathyroid cells were labelled with BrdU in two hours whereas 17.4%, and 15.9% of developing TECs were in the proliferative phase of the cell cycle (Alistair Martin Cook dissertation 2010, University of Edinburgh). Because I used BrdU incorporation to measure cell proliferation, the number of proliferating parathyroid cells counted may be an overestimate, compared to the TEC proliferation data, of the actual number of proliferating parathyroid cells at one instant during each developmental time point since BrdU was allowed to be incorporated for a full two hours before analysis. However, the fact that this possible overestimate of parathyroid proliferation is still lower than that of thymus proliferation during embryonic development suggests that cell proliferation may play a role in the discrepancy in fate stabilization between developing thymus and parathyroid cells. Future studies will be required to determine whether parathyroid cell proliferation contributes to parathyroid cell fate instability, and will likely include an investigation of DNA methylation status of the thymus program in developing parathyroid cells throughout embryonic development. If DNA methylation of the thymus program is low in developing parathyroid cells compared to DNA methylation of the parathyroid program in TECs at E15.5 and increases to the newborn stage, then differences in proliferation and fate stability between the two cells types can be correlated with methylation status. Additionally, experiments where the proliferation rate of developing parathyroid cells is sped up by inducing a positive cell cycle regulator such as c-Myc or CyclinD1 may reveal that

higher proliferation leads to quicker stabilization of the parathyroid fate, yielding fewer parathyroid-derived cervical thymi.

The mechanism behind parathyroid fate switching to the thymus fate is unclear, and the order of the initiation of the thymus program and shutdown of the parathyroid program is unknown. My experiments in which Foxn1 was activated in parathyroid cells sought to clarify the order of the program switch.

Data from these experiments suggest that the thymus program is spontaneously activated in parathyroid cells, and then the parathyroid program is subsequently downregulated and shutdown during the fate switch from parathyroid to thymus. Activation of an iFoxn1 transgene in parathyroid cells was sufficient to downregulate the parathyroid program, where up to 23.7% of $Foxn1^{+}$ parathyroid cells showed low to no Gcm2 expression by E15.5. Interestingly, at 4 weeks, an abnormal $Foxn1^{+}Gcm2^{-}$ structure was seen within the parathyroid gland. The histology of this structure was not consistent of that of typical glandular tissue. It is possible that a small ectopic thymus lobe had developed in the parathyroid gland, but a definitive conclusion cannot be made without determining whether or not downstream genes from Foxn1 in the thymus program have been activated in the abnormal structure. If true, these data would provide some clues as to the mechanism of parathyroid fate switch to thymus in the formation of parathyroid-derived cervical thymi.

Methods

Mice

For all cell cycle experiments, C57BL6/J6 mice were sourced from Jackson Labs (stock number 000664) (Bl6). For PTHcre;iFoxn1 experiments, 129;FVB-Tg(PTH-cre)4167Slib/J (stock number 005989) (PTHcre) and B6;129S6-Gt(ROSA)26Sor $^{tm9(CAG\text{-}tdTomato)Hze}$/J (stock number 007905) (CAGtdTomato) were sourced from Jackson Labs. A non-commercially available mouse strain Rosa26CAG-STOP-Foxn1-IRES-GFP (iFoxn1) was also used for this experiment (Bredenkamp et al., 2014).

Proliferation and Cell Cycle Experiments

Male and female Bl6 mice were crossed. Pregnant female given a peritoneal injection with (10 x body weight (g)) ul of 5 mg/ml BrdU (Sigma-Aldrich). A set amount of time X (30 min, 1hr, 2hr) elapsed after the BrdU injection before injecting the mouse again with (body weight (g)) ul of 1 mg/ml EdU (Invitrogen). The same amount of time X elapsed before collecting embryos. Embryos were fixed and embedded in paraffin. Sections were immunostained using an anti-BrdU antibody (AbD Serotech, 1:10), anti-Gcm2 antibody (Abcam, 1:400), and the Click-iT EdU Alexa Fluor 555 Imaging Kit (invitrogen).

The length of S phase and the cell cycle were calculated using these formulas:

S phase= (Time between injections)/ (BrdU+EdU-/BrdU+EdU+)

Cell cycle= S phase/ (BrdU+EdU+/Total Gcm2+ cells)

Proliferation was calculated using this formula:

$Gcm2^{+}BrdU^{+}$/Total $Gcm2^{+}$ cells

$BrdU^{+}$ and EdU^{+} cells were counted manually.

PTHcre;iFoxn1 Experiments

PTHcre heterozygous mice were crossed with iFoxn1 mice. Embryos were collected at E13.5 and E15.5, with the plug date at noon being E0.5. Embryos were fixed in 4% PFA before dehydrating with increasing amounts of ethanol and embedding in paraffin wax. Embryos were sectioned and immunostained with anti-Gcm2 (Abcam, 1:400) and anti-Foxn1 (Santa Cruz Biotechnologies, 1:200) antibodies. Nuclei were stained with Dapi (Invitrogen).

PTHcre+/-;iFoxn1+/- mice were crossed with CAGtdTomato homozygous mice. E16.5 embryos and 4 week old dissected neck regions were collected. Samples were fixed in 4% PFA before undergoing a sucrose gradient and were embedded in OCT (Fisher Scientific) and frozen and stored at -80C. Samples were sectioned and immunostained with anti-Gcm2 (Abcam, 1:400) and anti-Foxn1 (Santa Cruz Biotechnologies, 1:200) antibodies. Nuclei were stained with Dapi (Invitrogen).

$Foxn1^+$ and $Gcm2^+$ cells were counted manually, and Gcm2 downregulation was counted based on fluorescent intensity by eye.

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Figures

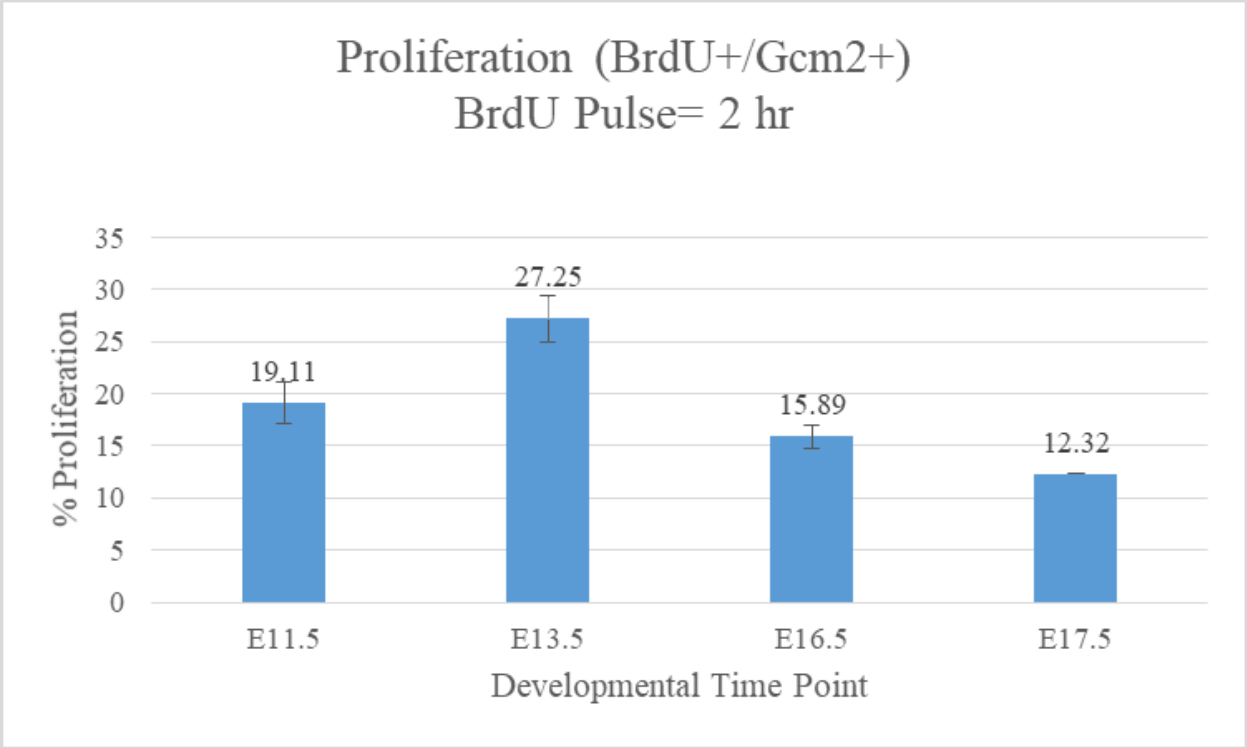

Figure 2-1: Proliferation of parathyroid cells throughout embryonic development.

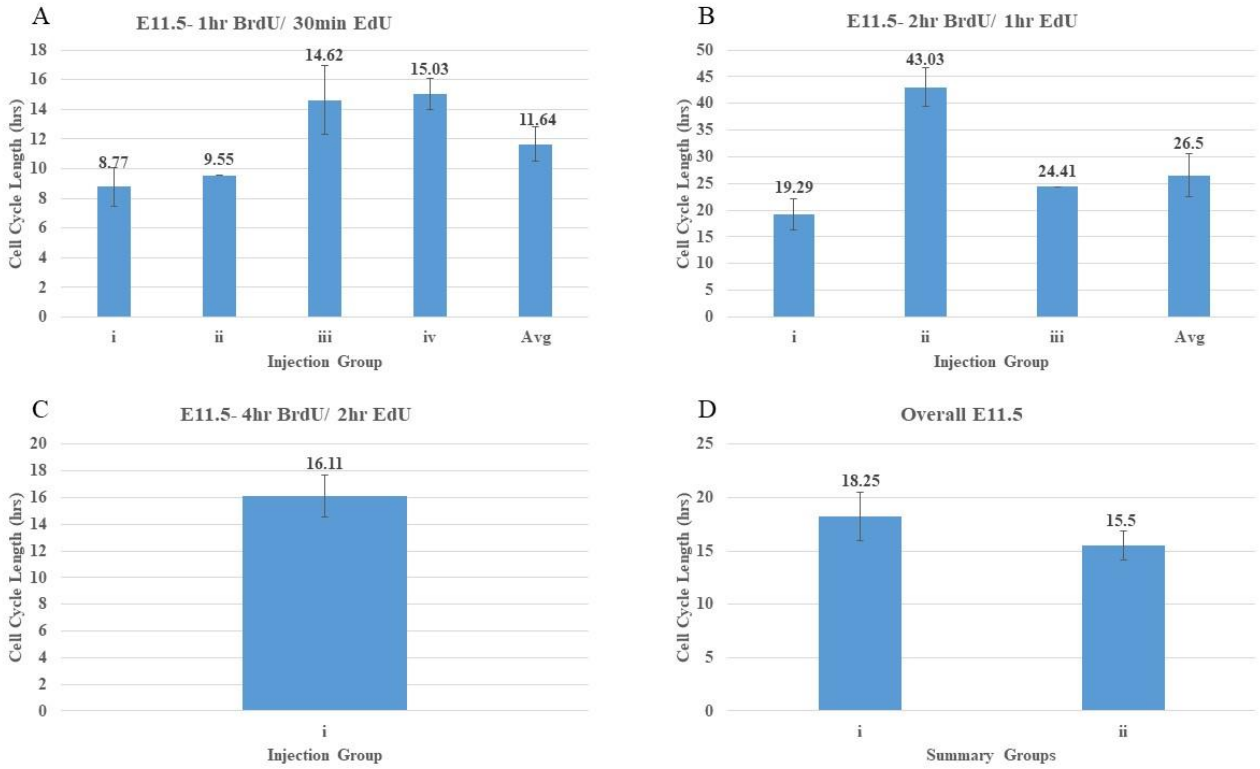

Figure 2-2: Parathyroid cell cycle measurements at E11.5.

A) 1 hr BrdU/ 30min EdU pulse time. i: initial injection group (n=3). ii: embryo 1/3 from second injection group (n=2). iii: embryo 2/3 from second injection group (n=2). iv: embryo 3/3 from second injection group (n=2). Avg: cumulative summary of pulse time data (n=9). B) 2hr BrdU/ 1hr EdU pulse time. i: initial injection group (n=4). ii: 45ss embryo (n=2). iii: 54ss embryo (n=2). Avg: cumulative summary of pulse time data. C) 4hr BrdU/ 2hr EdU pulse time. i: initial injection group (n=3). D) Cumulative summary of E11.5 data. i: includes all E11.5 data (n=20). ii: excludes 45ss data (n=18).

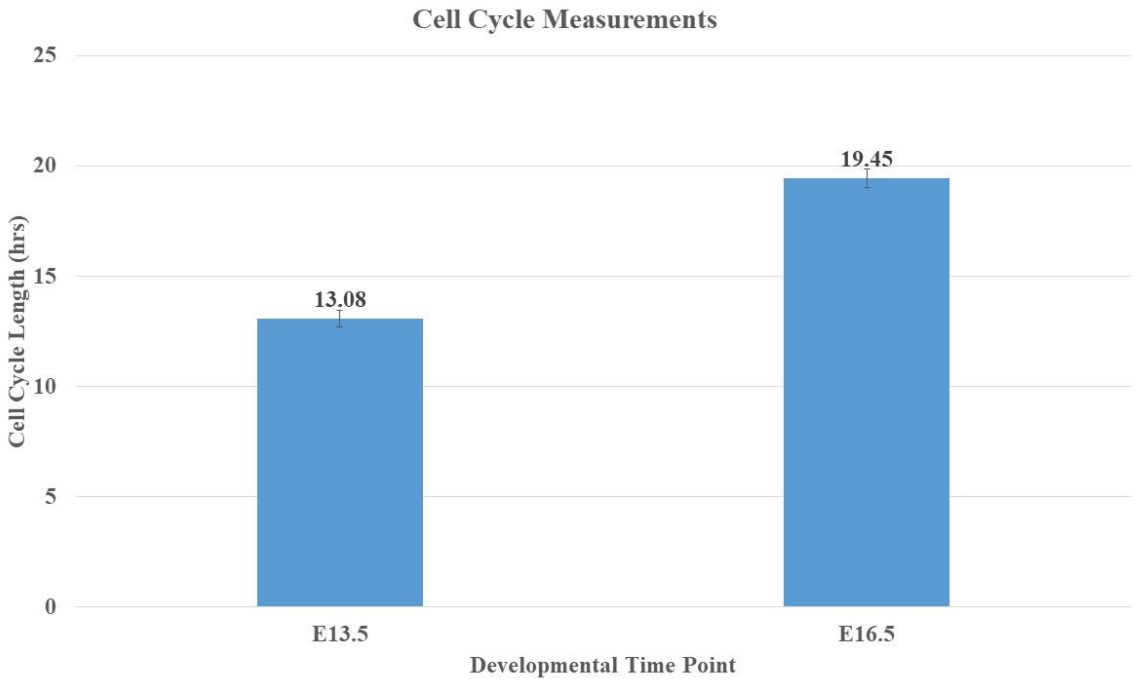

Figure 2-3: Parathyroid cell cycle measurements at E13.5 and E16.5.

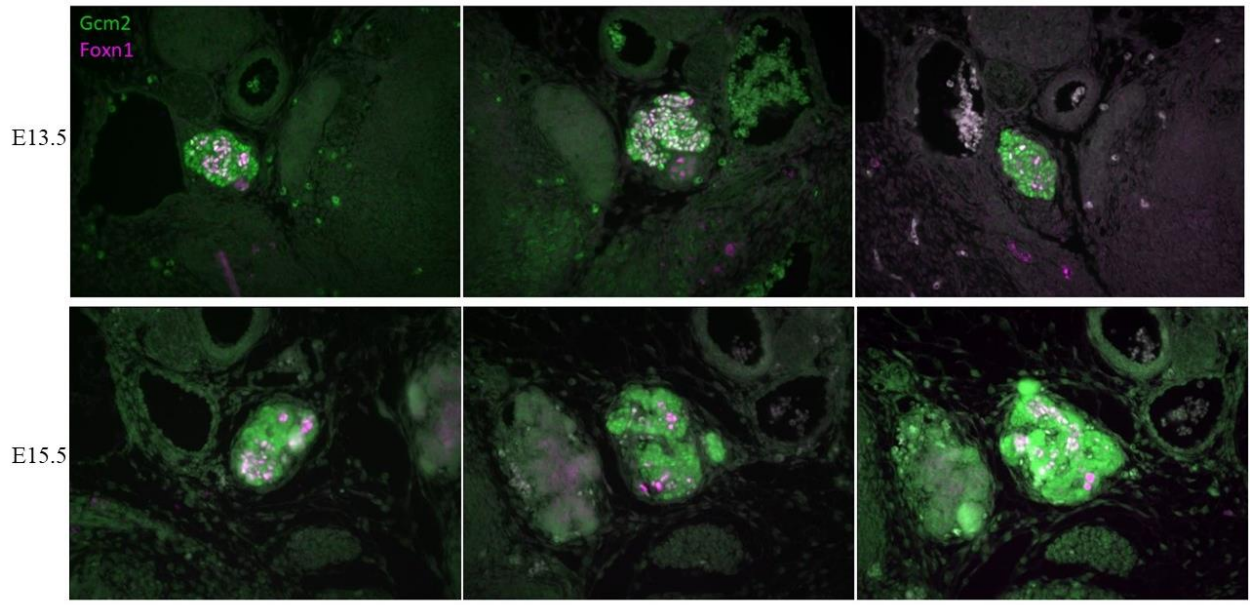

Figure 2-4: Foxn1 and Gcm2 in E13.5 and E15.5 PTHcre;iFoxn1 parathyroid glands. Three representative images for each developmental time point is shown.

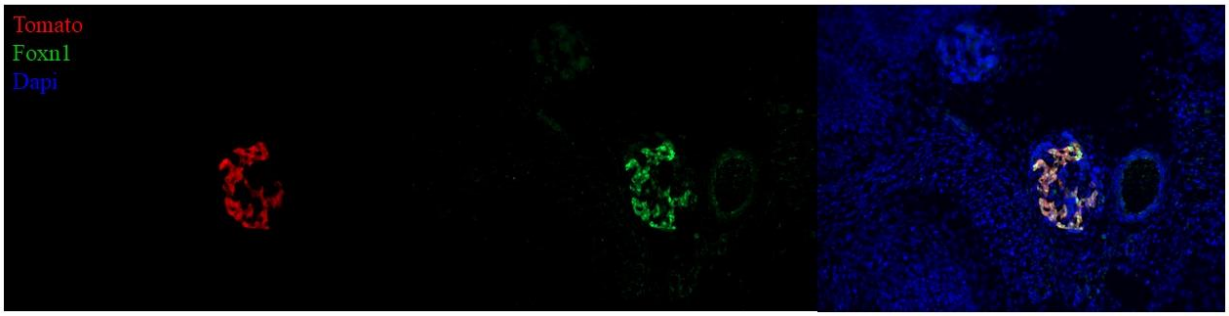

Figure 2-5: CAGtdTomato and Foxn1 at E16.5 in PTHcre;iFoxn1 mouse.

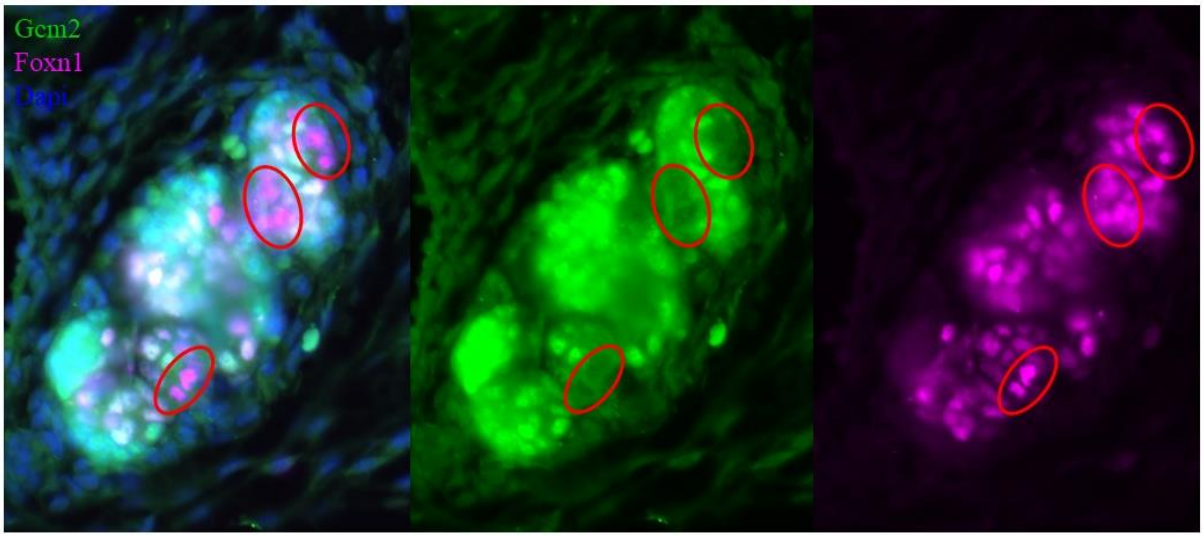

Figure 2-6: Downregulation of the parathyroid program. In red ovals, portions of E15.5 parathyroid gland with Gcm2 low or $Gcm2^{-}$ cells that are $Foxn1^{+}$.

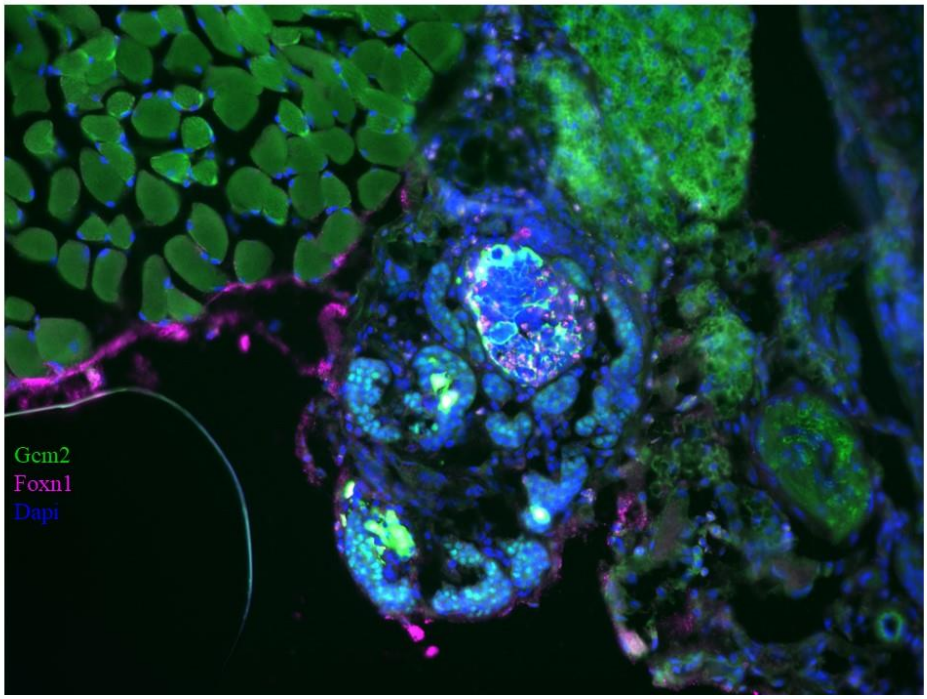

Figure 2-7: Abnormal Foxn1 positive, Gcm2 negative structure in 4 week old parathyroid gland.

APPENDIX A

INVESTIGATION OF BMP4 AND FGF8 INTERACTION IN 3RD PHARYNGEAL POUCH

Introduction

Summary of John O'Neil's doctoral research

One main aim of John O'Neil's doctoral research was to examine the role of Bmp4 and Fgf8 in the developing 3rd pharyngeal pouch in mouse development. This was done by culturing E10.5 embryos with beads soaked in recombinant protein (Bmp4, Noggin, or Fgf8) in media containing Bmp or Fgf small molecule inhibitors in various combination. The effect on Gcm2 and Foxn1 was recorded for each scenario.

When an Fgf8 bead was implanted in the embryos, Gcm2 decreased, and Foxn1 was at normal levels. When Fgf was inhibited, Gcm2 was normal, and the number of Foxn1-expressing cells was decreased. The overall size of the 3rd pharyngeal pouch was also smaller. It was concluded that Fgf8 promotes thymus fate but suppresses parathyroid fate and drives proliferation in the ventral region of the 3rd pharyngeal pouch.

Similar to the Fgf8 bead, when embryos were implanted with a Bmp4 bead, Gcm2 decreased, but Foxn1 was normal. When Bmp4 was inhibited, both Foxn1 and Gcm2 were reduced dramatically. It was concluded that Bmp4 promotes thymus fate and is required for both the thymus and parathyroid fates.

The inhibition of both Bmp4 and Fgf resulted in dramatically reduced Foxn1 and Gcm2, similar to inhibition of Bmp4 on its own.

Investigation of Bmp4 and Fgf8 interaction during 3rd pharyngeal pouch development

To continue O'Neil's research, I sought to investigate how Bmp4 and Fgf8 interact during the development of the 3rd pharyngeal pouch. I used the same whole embryo culture system used for O'Neil's experiments using a knock-in-knockout Bmp4 reporter mouse (Bmp4-CFP) to track changes in Bmp4 protein in response to inhibiting Fgf signaling with SU5402.

Results

Increased Bmp4 and Absent Gcm2 as a result of Fgf inhibition

To address how Bmp4 levels change in response to Fgf inhibition, Bmp4-CFP embryos were cultured in SU5402, a chemical inhibitor of Fgf signaling. Upon inhibition of Fgf, Bmp4 protein was increased by 21.9% (n=15) in the 3rd pharyngeal pouch (Figure 1B) compared to the DMSO treatment (Figure1A). Additionally, Gcm2 was absent in the 3rd pharyngeal pouch while Foxn1 levels remained normal. The effect on Gcm2 was not seen in a 37ss CFP^- embryo under the SU5402 treatment; however, as stated in O'Neil's dissertation, effects on Gcm2 and Bmp4 were only seen at or before 35ss when changes to Fgf or Bmp4 were made. These data suggest that when Fgf is inhibited, levels of Bmp4 increase.

Phenotype specific to Bmp4-CFP mouse

Because Bmp4-CFP mouse has half the amount of Bmp4 for signaling since it is a knock-in-knockout reporter mouse, it was hypothesized that after the 35ss Fgf signaling is required for maintenance of Gcm2 under the condition that Bmp4 is also inhibited. To test this hypothesis, wild-type Bl6 embryos were cultured using SU5402 to inhibit Fgf and beads soaked in Noggin recombinant protein to inhibit Bmp4 signaling. This experiment yielded similar results as those seen in O'Neil's dissertation. Before 35ss, the combination of SU5402 and Noggin beads resulted in dramatic decrease of both Foxn1 and Gcm2. After 35ss, normal expression was seen in both

Foxn1 and Gcm2 (Figure 2). Therefore, because the loss-of-expression phenotype in Gcm2 was not recapitulated, this phenotype is specific to Bmp4-CFP mice.

Discussion

To answer the question of how Bmp4 is affected by changes in Fgf, my results indicate that when Fgf is inhibited, Bmp4 levels increase. This could be because Fgf and Bmp4 play a redundant role in the 3rd pharyngeal pouch of promoting thymus fate.

To address the loss-of-expression phenotype in Gcm2 seen when Fgf was inhibited in Bmp4-CFP embryos, it is possible that this is a strain-specific phenotype. All wild-type embryo cultures done by John O'Neil and myself were performed with C57BL6/J6 mice while the Bmp4-CFP mouse line is maintained under a Swiss Webster background. To determine if this is the case, embryo culture using SU5402 and Noggin beads would need to be performed with Swiss Webster mice.

Methods

Mice

C57BL6/J6 mice were sourced from Jackson Labs (stock number 000664) (Bl6). A non-commercially available mouse strain Bmp4^{CFP} was also used in these experiments (Jang, Gao, Dickinson, & Behringer, 2010) (Bmp4-CFP).

Whole Embryo Culture

Whole embryo culture was performed according to previously published methods (Moore-Scott, Gordon, Blackburn, Condie, & Manley, 2003). Bl6 and Bmp4-CFP embryos were dissected between E10 and E10.5 and cultured at 37°C in KnockOut DMEM media (Life Technologies) containing 10% KnockOut serum replacement in a roller bottle culture apparatus under 95% O_2 with the Fgf small molecule inhibitor SU5402 (Selleck Chemicals) for 24 hours. Embryos were

also implanted with beads soaked in Noggin recombinant protein at 5μg/μL (R&D Systems). Control embryos were cultured with DMSO in the media and implanted with bead soaked in PBS.

Tissue Processing and Antibody Staining

Samples were collected after culture and fixed in 4% PFA. Samples were dehydrated in ethanol before embedding in paraffin wax. Samples were sectioned and immunostained with antibodies against Foxn1 (Santa Cruz Biotechnologies, 1:200), Gcm2 (Abcam, 1:400), and GFP (Abcam, 1:200). Nuclei were stained with Dapi (Invitrogen).

Fluorescent Intensity Measurements

Fluorescent intensity of GFP was measured using ImageJ.

References

Jang, C. W., Gao, L., Dickinson, M. E., & Behringer, R. R. (2010). Bmp4-directed nuclear cyan fluorescent protein provides a tool for live imaging and reveals cellular resolution of Bmp4 expression patterns during embryogenesis. *Int J Dev Biol, 54*(5), 931-938. doi:10.1387/ijdb.092911cj

Moore-Scott, B. A., Gordon, J., Blackburn, C. C., Condie, B. G., & Manley, N. R. (2003). New serum-free in vitro culture technique for midgestation mouse embryos. *Genesis, 35*(3), 164-168. doi:10.1002/gene.10179

Figures

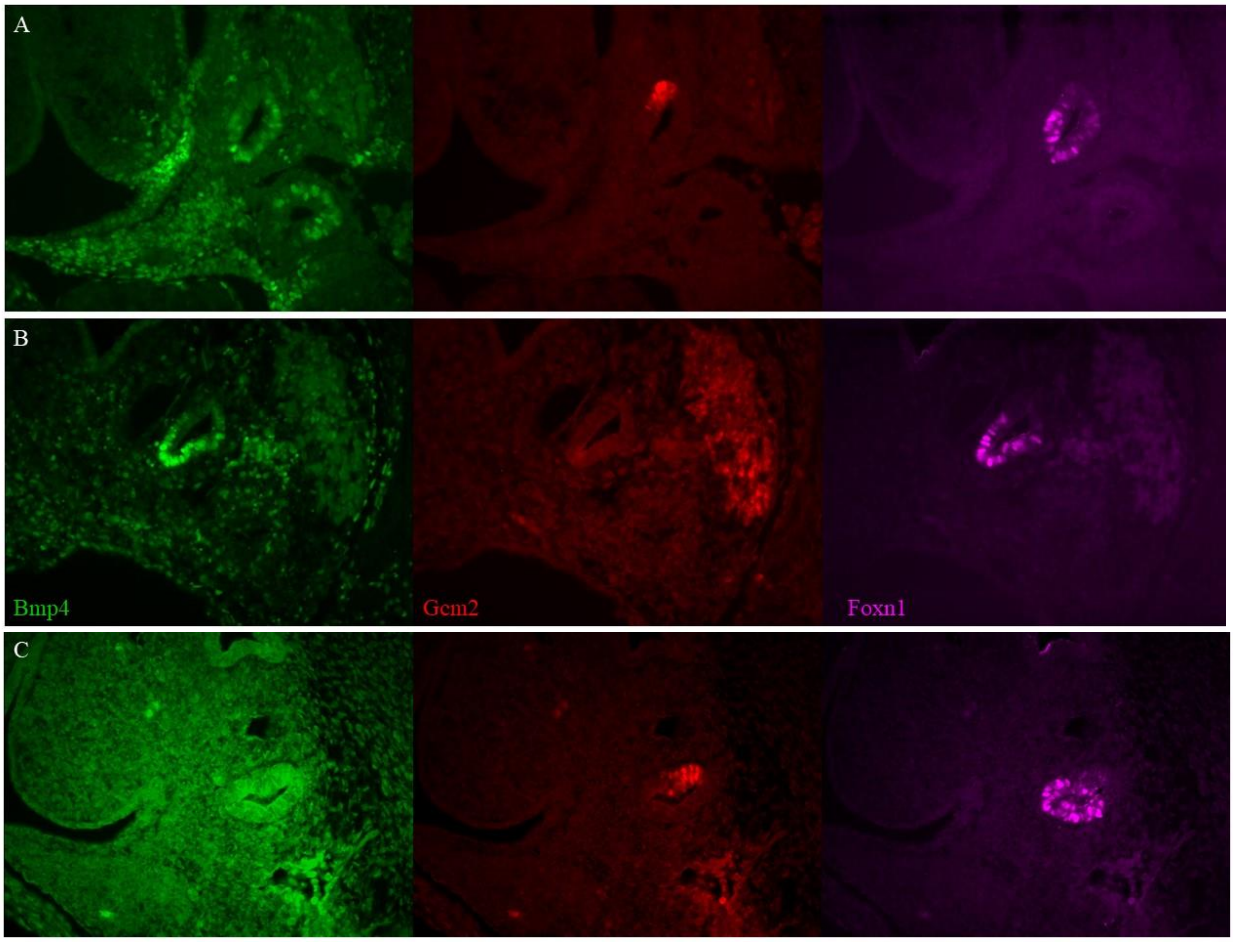

Figure A-1: Antibody staining of E11.5 3rd pharyngeal pouch primordia after 24-hr whole embryo culture. A) 36ss Bmp4-CFP^{+} with DMSO media. B) 37ss Bmp4-CFP^{+} with SU5402 media. C) 37ss Bmp4-CFP^{-} with SU5402 media.

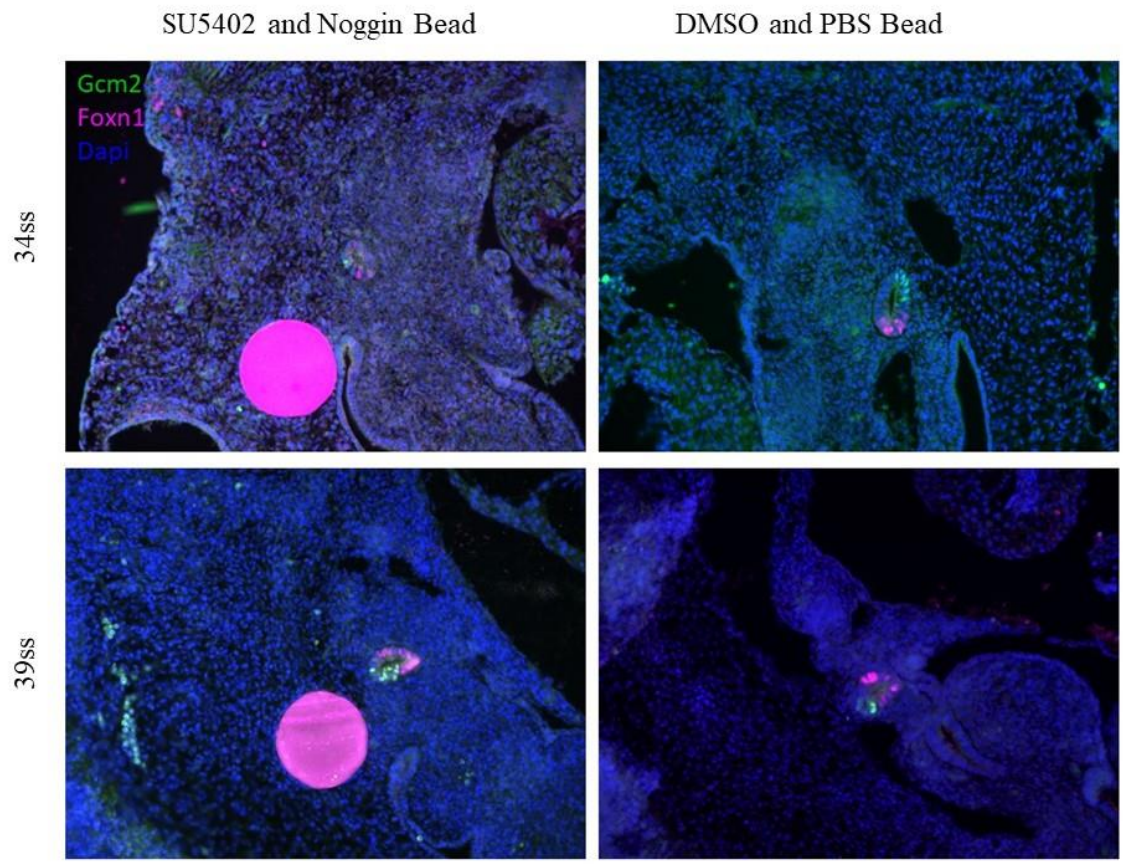

Figure A-2: Antibody staining of E11.5 3rd pharyngeal pouch primordia after 24-hr embryo culture. Top Panel) 34ss embryos with experimental and control treatments. Bottom Panel) 39ss embryos with experimental and control treatments.