DRIVING PROLIFERATION OF FETAL PARATHYROID CELLS STABILIZES THE PARATHYROID PROGRAM

by

CHYNNA LEE POLLITT

(Under the Direction of Nancy Manley)

ABSTRACT

The parathyroid glands regulate blood calcium homeostasis through the secretion of parathyroid hormone. In mice, the parathyroid develops together with thymus in the third pharyngeal pouch. When these organs separate, cervical thymi and ectopic parathyroids are deposited in the neck. Parathyroid-derived cervical thymi arise when parathyroid cells spontaneously turn off the parathyroid program and transdifferentiate to a thymus fate. Occurring at a low frequency and increasing steadily until birth, this fate switch reveals the unstable differentiation program of fetal parathyroid cells. The molecular mechanism governing parathyroid cell fate instability is unclear. We hypothesized that the low proliferation rate of parathyroid cells may be a source of lineage instability. Our findings indicate that driving parathyroid cell proliferation stabilizes the parathyroid program. In our investigation of the order by which the parathyroid-to-thymus cell fate switch occurs, our data suggests that parathyroid cells downregulate the parathyroid program and subsequently upregulate the thymus program.

INDEX WORDS: parathyroid, cervical thymi, third pharyngeal pouch, cell fate instability

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The parathyroid glands are responsible for calcium regulation. Calcium is one of the most important minerals in the human body and is essential for muscle contraction and transmission of nerve impulses. Because intracellular cell signaling and other cell processes rely heavily on the maintenance of a narrow calcium concentration range, blood calcium levels must be tightly regulated. The parathyroid glands are located on the posterior surface of the thyroid gland and serve as the sole source of PTH production and secretion. Calcium-sensing receptors (CaSR) located on the surface of parathyroid chief cells, of which the gland is mostly comprised, detect aberrant calcium levels (R. A. Chen & Goodman, 2004). Able to detect even the slightest change in circulating calcium concentration, CaSR maintains calcium homeostasis through the modulation of PTH secretion. PTH plays a central role in the regulation of serum calcium and phosphate. When blood calcium is low, parathyroid glands begin releasing PTH. PTH then binds to cell receptors on the bone to trigger the release of calcium into the bloodstream.

Molecular mechanisms governing early thymus and parathyroid development

In mice, the thymus and parathyroid glands arise from the third pharyngeal pouch endoderm with contribution from surrounding neural crest cells (NCCs). From E9.5-E10.5, early third pouch patterning and formation of the thymus/parathyroid common primordium are mediated by a transcriptional network involving Hoxa3 (Chojnowski et al., 2014;

Manley and Capecchi, 1995), Pax1/9 (Peters et al., 1998; Wallin et al., 1996), Eya1 (Laclef et al., 2003; Xu et al., 2002; Zou et al., 2006) and Six1/4 (Zou et al., 2006). Mice heterozygous or homozygous for targeted mutations of these genes generally have normal initial pouch formation but exhibit thymic and parathyroid aplasia or severe hypoplasia. Additionally, *Hoxa3*, *Six*, and *Eya1* mutants also have severely reduced or absent *FGF8*, *BMP4*, and *Wnt5B* expression in the developing 3PP (Chojnowski et al., 2014; Zou et al., 2006). Transcription factors as well as components of the FGF, Wnt, and BMP signaling pathways present in the common primordium may, therefore, belong to a complex genetic network crucial for thymus and parathyroid organ specification.

Several secreted signaling molecules, including SHH (Sonic hedgehog), FGF8 (fibroblast growth factor 8), BMP4 (Bone Morphogenetic Protein 4), and Wnt5B (wingless/integrated family member 5B) are required to pattern the third pharyngeal pouch during early thymus and parathyroid organogenesis. During pouch formation, SHH signaling emanates from the pharynx and the NCC-derived mesenchyme. The opposing gradients of *Shh* expression and *Bmp4* expression promote dorsal-ventral polarity within the 3PP. SHH promotes *Gcm2* expression in the dorsal pouch, while Bmp4 induces *Foxn1* expression ventrally. In *Shh*-null mutant mice, the 3PP endoderm fails to activate *Gcm2* expression required for entry into the parathyroid differentiation program (Moore-Scott & Manley, 2005). Loss of *Shh* results in the downregulation of *Tbx1* expression and expansion of *Bmp4* and *Foxn1* expression domains. (Moore-Scott & Manley, 2005). Thus, the pouch progenitors give rise to thymus cells at the expense of parathyroid cell differentiation. Ectopic expression of *Shh* within the endoderm results in ventrally expanded Tbx1 expression and suppression of Foxn1 in the central domain (Bain, 2016).

However, expression of SHH-receptor Patched1 reveals that ectopic activation of the Shh pathway is insufficient to induce *Tbx1* expression and repress *Foxn1* expression in the most ventral primordium (Bain, 2016). The Shh pathway appears to repress thymus-specific gene expression, *Fgf8* and *Bmp4*, to promote parathyroid fate, although it is yet unknown exactly how SHH functions in parathyroid cell fate specification.

Transcription factors controlling organ patterning and initial organogenesis

Tbx1, a T-Box transcription factor, and Fgf8 in the Shh signaling pathway have been shown to regulate thymus and/or parathyroid fate specification and differentiation during organogenesis. *Foxn1* repression induced by ectopic *Tbx1* in the presumptive thymus domain suggests that Tbx1 acts downstream of Shh to inhibit thymus fate in the dorsal pouch (Reeh et al., 2014). Although early evidence supports a role for Tbx1 as a potential regulator of Gcm2, further studies are needed to clarify the role of Tbx1 in this context.

Hoxa3 is expressed in both the 3PP endodermal epithelium and NCC-derived mesenchyme and is required for thymus and parathyroid initial organogenesis. After initial pouch formation, *Hoxa3*-null mutants fail to form the shared organ primordia. Deletion of Hoxa3 from either the endoderm or NCCs results in normal initial stages of pouch patterning and organ formation. Additionally, *Gcm2* expression at E10.5 in the endoderm was reduced after global (*Hoxa3-/-*) or endoderm-specific deletion of *Hoxa3* but appeared unaffected by NCC-specific deletion of *Hoxa3* (L. Chen et al., 2010). Endoderm deletion embryos were able to upregulate and maintain control-like levels of *Gcm2* expression by E12.0 (Chojnowski et al., 2014; Chojnowski, Trau, Masuda, & Manley, 2016). Because only *Hoxa3*-expressing cells can adopt a parathyroid fate, this data suggests that Hoxa3 is

required for *Gcm2* upregulation in the 3PP but is not required for its expression. HOXA3 upregulates *Gcm2* but is not required for its expression or parathyroid lineage maintenance and differentiation.

Two closely related paired-box transcription factors Pax1 and Pax9 have a potential for upregulating *Gcm2* expression in the parathyroid anlage. *Pax1* mutants have thymic and parathyroid hypoplasia, an inactivation of *Pax9* results in thymus and parathyroid agenesis (Wallin et al., 1996; Peters et al., 1998; Su et al., 2001). *Hoxa3-/-* mutants exhibit reduced *Pax1/9* expression at E10.5, raising the possibility that these genes may function downstream of *Hoxa3* in the 3PP (Manley and Capecchi, 1995; Koushik and Manley, unpublished observations). Furthermore, $Hoxa3^{+/-}Pax1^{-/-}$ compound mutants display more severe developmental defects of the thymus and parathyroid than $Pax1^{-/-}$ single mutants (Su et al., 2001). *Pax1* single heterozygous mutants are able to initiate *Gcm2* expression, whereas Gcm2 expression is severely reduced or absent in $Hoxa3^{+/-}Pax1^{-/-}$ compound mutants (Su et al., 2001). These results suggest that Hoxa3 and Pax1/9 are required for the maintenance of *Gcm2* expression.

Mice heterozygous for the *Gata3* mutation have hypoparathyroidism and smaller parathyroid glands than controls (Grigorieva et al., 2010). Because *Gcm2* is never activated in *Gata3* knockout embryos, the parathyroid primordia are lost (Grigorieva et al., 2010). These results indicate that Gata3 is required for normal parathyroid organogenesis and cell proliferation.

Gcm2 is required for parathyroid cell differentiation and survival

After pouch formation, two molecularly distinct progenitor domains are established by E10.5 (Gordon et al., 2001). Regionally restricted expression of two organ-specific

transcription factors, *Foxn1* and *Gcm2*, is required for the differentiation of thymus- and parathyroid-fated cells in the 3PP, respectively.

The earliest known parathyroid-specific marker Gcm2 is critical for parathyroid gland organogenesis (Gunther et al., 2000). First identified in the embryonic central nervous system of Drosophila melanogaster, the glial cells missing (Gcm, also called glial cell deficient (glide)) transcription factor mediates the neuron-glial cell fate switch and functions as a master regulator of gliogenesis (Hosoya et al., 1995). Although expected to exhibit similar functionality to Drosophila Gcm, two mammalian homologs, *Gcm1/GCMa* and *Gcm2/GCMb*, are expressed predominantly in non-neural tissues. Mouse Gcm2 is expressed in the developing third pharyngeal pouch (3PP) and is required for parathyroid precursor cell differentiation and survival (Kim et al., 1998; Liu et al., 2007). After *Gcm2* is upregulated, markers of committed parathyroid precursors such as *CCL21*, *CaSR*, and *PTH* are expressed. GCM2 acts in cooperation with transcription factors GATA3 and MafB to upregulate *PTH* expression (Kamitani-Kawamoto et al., 2011; Han, Tsunekage, & Kataoka, 2015).

Around mouse embryonic day (E) 9.5, *Gcm2* expression initially encompasses the 2pp and 3pp but is upregulated and restricted to a discrete domain of the anterior-dorsal 3pp endoderm by E10.5 (Gordon et al., 2001). Homozygous Gcm2-null mice lack parathyroid glands because precursor cells fail to proliferate and undergo programmed cell death by E12.5 (Gordon et al., 2001; Gunther et al., 2000; Liu et al., 2007). While *Gcm2* expression is not required for progenitor domain specification during development, Gcm2 is necessary for parathyroid cell differentiation and survival. Thus, a complex network of signaling pathways and other transcription factors must govern parathyroid cell fate.

Third pharyngeal pouch derivatives

Starting around E12.5, the common primordia physically detach from the pharynx and being separating into one thymic lobe and a single parathyroid gland. As the thymus migrates to the superior mediastinum, the parathyroid glands remain adjacent to the thyroid in the cervical region (Manley & Capecchi, 1998). This organ separation is frequently incomplete as small clusters of parathyroid cells often are deposited along the pathway of thymic descent or remain attached to the thymus. Ectopic thymic tissue, called cervical thymi, can also persist along the migratory route.

Cervical thymi and stability of parathyroid cell fate

Cervical thymi localize along the path of thymic migration in about 50% of developing human and mouse embryos (Dooley et al., 2006; Terszowski et al., 2006; Corbeaux et al., 2010; Li et al., 2013). Accessory to the main thoracic thymus, cervical thymi can support T-cell development and have been found to arise from two distinct developmental lineages. Cervical thymi turn on *Foxn1* sometime after E12.5 and before about E15.5, which later than the onset of *Foxn1* expression in the thoracic thymus at about E11.25 (Corbeaux et al., 2010). Epithelial cells of cervical thymi express *Foxn1*, but at a lower level than TECs of orthotopic thymuses (Terszowski et al., 2006). Ablation of FOXN1+ cells between E10.5 and E12.5 resulted in the loss of the thoracic thymic anlage but did not prevent the formation of cervical thymi (Corbeaux et al., 2010). This suggests that FOXN1- TEC progenitor cells fated to become cervical thymi escaped detection and subsequent destruction by upregulating the thymus differentiation program later in development (Corbeaux et al., 2010).

More recent data has revealed the unstable differentiation program of fetal parathyroid cells. Though most cervical thymi result from delayed differentiation of endodermal primordia, around 25% of cervical thymi were determined to be of parathyroid lineage (Li et al., 2013). Parathyroid-derived cervical thymi (pCT) arise when PTH-expressing parathyroid cells spontaneously turn off the parathyroid program and transdifferentiate to a thymus fate. This fate switch occurs at a low frequency, late in development, and increases steadily until birth (Li et al., 2013). Following birth, the frequency of pCT remains unchanged, indicating the stabilization of parathyroid cell fate. Interestingly, inactivation of *Gcm2 in vivo* precludes the formation of cervical thymi suggesting that normal parathyroid organogenesis is a prerequisite for their development.

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CHAPTER 2

FETAL PARATHYROID FATE INSTABILITY

Introduction

In mice, the parathyroid glands and thymus are derived from the dorsal and ventral endoderm of the third pharyngeal pouch (3PP), respectively (Gordon, Bennett, Blackburn, & Manley, 2001). During organ separation, accessory cervical thymi and ectopic parathyroids develop along the course of thymic migration in the neck. Present in both humans and mice, cervical thymi can support T cell development in addition to the main thoracic thymus (Dooley et al., 2006; Terszowski et al., 2006). Beginning at embryonic day (E) 15, the incidence of cervical thymi in mice increases gradually and reaches a maximum of 50% at birth. Although cervical thymi predominantly arise from delayed differentiation of endodermal progenitors, a subset (25%) is derived from parathyroid cells. The presence of parathyroid-origin cervical thymi (pCT) suggests that parathyroid cell fate is unstable during late embryonic development (Li, Liu, Xiao, & Manley, 2013). There is currently no evidence to indicate that the same is true of the thymus program. Many aspects of parathyroid organogenesis well as the mechanisms underlying the parathyroid-to-thymus cell fate switch remain poorly understood. The main goal of this research is to investigate the source of fetal parathyroid cell fate instability and to develop a mechanistic understanding of how parathyroid cells transdifferentiate to a thymus fate.

During embryonic development, the differentiation potential of parathyroid progenitors is progressively restricted. However, the presence of parathyroid cells that

spontaneously transdifferentiated into thymus cells during the late stages of embryogenesis led us to examine the factors that regulate parathyroid cell fate stability. We hypothesized that the instability of the parathyroid cell fate may be due to the low and/or slow proliferation rate of parathyroid cells compared to that of thymus cells. The different proliferation rates may, therefore, contribute to the difference in program stability. If parathyroid cell proliferation is low during embryonic development, the parathyroid cell fate could potentially be unstable and susceptible to fate switch until birth. Thus, low-proliferating parathyroid cells may be slow to shut off the thymus program through DNA methylation, thereby permitting fate switch until late embryonic development. This led us to investigate whether the proliferation rate of parathyroid cells is a source of fate instability.

Previous work from our lab developed and characterized a novel mouse model to increase the proliferation rate of developing parathyroid cells by inducing a positive cell cycle regulator (Pollitt, unpublished). We used *PTH-Cre* to activate constitutive *Myc* (*iMyc* transgene) expression in parathyroid cells and their descendants. *129;FVB-Tg(PTH-cre)4167Slib/J* (hereafter referred to as *PTHCre*) mice were crossed with *R26iMyc* (strain name: C57BL/6N-Gt(ROSA)26Sor$^{tm13(CAG-MYC,-CD2*)Rsky}$/J) mice to generate newborn *PTH*$^{Cre/+}$*;R26*$^{iMyc/+}$ mutants and *PTH*$^{+/+}$*;R26*$^{iMyc/+}$ littermate controls. Mutant newborn parathyroids were approximately three times larger than those of the controls, suggesting parathyroid cell proliferation is increased in *PTH*$^{Cre/+}$*;R26*$^{iMyc/+}$ mice (Figure 1). Utilizing these transgenic mice, this study will examine the effects of increased fetal parathyroid cell proliferation on parathyroid cell fate stability.

Recently, it has been shown that forced expression of *Foxn1* in *PTH*-expressing cells is sufficient to downregulate *Gcm2* expression in the parathyroid (Peissig, unpublished data). Data from this experiment suggest that parathyroid cells first spontaneously activate a thymus differentiation program then downregulate and turn off the parathyroid program to switch to a thymus fate. The second part of this research will further investigate the order by which the parathyroid-to-thymus cell transdifferentiation occurs.

Results

Driving fetal parathyroid cell proliferation stabilizes the parathyroid program

For the investigation of parathyroid cell fate instability, *PTHCre*, *B6.Cg-Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$*/J* (henceforth *CAG-tdTomato*), *R26iMyc* and *Foxn1::EGFP* mice were obtained. To determine if increased proliferation rates in fetal parathyroid cells contribute to cell fate stability, I quantified the frequency of parathyroid-derived cervical thymi in *PTH*$^{Cre/+}$*;R26*$^{CAG-tdTomato/iMyc}$*;Foxn1::EGFP*$^{tg/0}$ mice. I used a *PTH-Cre* transgene to activate a *CAG-tdTomato* fluorescent reporter to permanently label parathyroid fate lineage (red) and induce human *Myc* (*iMyc*) expression to promote proliferation in fetal parathyroid cells and its descendants. TdTomato and iMyc will be activated in parathyroid cells at E11.5 when the expression of *PTH* is initiated. The *Foxn1::EGFP* reporter transgene was used to track endogenous *Foxn1* expression (green). Cervical thymi localize in the neck region between the thyroid and thymus and, if present, occur at a quantity of 1-4 ectopic cluster(s) per mouse (Li, Liu, Xiao, & Manley, 2013). Through live fluorescence imaging, I detected cervical thymi by expression of the

Foxn1::EGFP transgene and pCT by co-expression of *EGFP* and *tdTomato*. In 4-week-old PCMF mice, I found no occurrence of pCT (n=29 mice). Surprisingly, 100% of PCMF mutant mice had at least one ectopic cervical thymus (Table 1). In 4-week-old $PTH^{Cre/+};R26^{CAG\text{-}tdTomato/+};Foxn1::EGFP^{tg/0}$ controls, we detected cervical thymi in 3 out of 6 analyzed mice (50%) but no pCT (Table 1). These data support the hypothesis that parathyroid cell proliferation contributes to parathyroid cell fate instability.

Parathyroid cells switch fate by spontaneously downregulating the parathyroid program and then upregulating the thymus program

To further examine the order by which the parathyroid-to-thymus fate switch occurs, we used *PTH-Cre* to activate *Foxn1* (*iFoxn1* transgene) expression in parathyroid cells and their descendants. We crossed *PTHCre* mice, engineered to expresses Cre recombinase under the control of the human PTH promoter, to mice harboring a targeted allele of the *Rosa26* locus that permits conditionally activated *Foxn1* expression ($Rosa26^{CAG\text{-}STOP\text{-}Foxn1\text{-}IRES\text{-}GFP}$, henceforth designated *R26iFoxn1*). Induced exogenous expression of *Foxn1* in the Cre-positive progeny will begin at E11.5 when *PTH* is initiated.

The parathyroid glands and thymus were analyzed by detecting FOXN1 and PTH proteins via immunohistochemistry on alternating sagittal sections of whole neonates (Figure 2). Interestingly, newborn $PTH^{Cre/+};R26^{iFoxn1/+}$ mice exhibited a complete absence of *Foxn1* expression in the parathyroids (n=6). Consistent with the lack of *Foxn1* expression, PTH appeared to be normal. All six parathyroid glands appeared normal in size, though a volumetric analysis is needed to confirm this observation. Antibody staining

of TECs served as an internal positive control. Normal *Foxn1* expression was observed in the thymus, confirming the specificity of the immunostaining.

To verify whether *PTH*-Cre-mediated recombination was occurring in all *PTH*-expressing cells, we evaluated *Foxn1* expression in E16.5 $PTH^{Cre/+};R26^{iFoxn1/+}$ parathyroids (Figure 3). Immunostaining for FOXN1 protein showed that approximately 10% of parathyroid cells expressing *PTH* were also FOXN1 positive. These observations suggest that the absence of $FOXN1^{+}PTH^{-}$ cells from $PTH^{Cre/+};R26^{iFoxn1/+}$ newborns is not due to faulty Cre recombinase activity, but rather to poor survival of $FOXN1^{+}$ parathyroid cells.

Discussion

Recent work established that the failure to maintain the parathyroid cell fate during development leads to the formation of parathyroid-derived cervical thymi. Based on preliminary unpublished data from our laboratory, we hypothesized that the low and/or slow proliferation of parathyroid cells, compared to that of thymus cells, may confer fate instability. If true, then low-proliferating parathyroid cells may have the potential to switch fate because they are slow to shut down the thymus program. Thus, we may be able to stabilize the parathyroid program by driving parathyroid cells to proliferate, possibly enabling parathyroid cells to rapidly shut down the thymus program and disable the opportunity for fate switch. This led us to test whether forcing parathyroid cells to proliferate, using a positive cell cycle regulator, will result in decreased pCT formation. There were no instances of parathyroid-origin cervical thymi in 4-week-old PCMF mutant or control mice. It appears that constitutive *Myc* expression in parathyroid cells reduces or

prevents pCT formation, supporting the hypothesis that the low proliferation of parathyroid cells contributes to parathyroid cell fate instability. We also did not find any pCT in the control mice, likely due to the lack of sufficient *n* values. Although we expect 25% of cervical thymi to be parathyroid-derived, without the pCT frequency in the controls, we cannot exclude the possibility that the predicted Cre recombinase activity results in the underreporting of pCT.

The low levels of *Foxn1* expression exhibited by pCT compared to that of the thoracic thymus indicate that pCT do not fully upregulate the thymus program. Because Foxn1 is required for the maintenance of postnatal thymic epithelial cells (TECs), parathyroid cells that fail to maintain *Foxn1* expression upon transdifferentiation may subsequently undergo programmed cell death (Chen et al., 2009). Thus, this may be another reason why we did not observe pCT in PCMF adult mice. Before a conclusion can be drawn, data concerning the pCT frequency in 4-week-old wild type mice will be needed.

Cervical thymus frequency in mice increases with development from E15 until the newborn stage when a maximum of 50% incidence is reached. As this maximum frequency was observed at 0 to 10 days postnatal, we expected to observe a similar frequency of cervical thymi in both PCMF mutant and control mice (Li, Liu, Xiao, & Manley, 2013). Consistent with this prediction, we observed a 50% frequency of cervical thymi in 4-week-old $PTH^{Cre/+};R26^{CAG\text{-}tdTomato/+};Foxn1::EGFP^{tg/0}$ controls. However, in 4-week-old PCMF mice, *iMyc* surprisingly increased the overall frequency of cervical thymi to 100%. The reason why and mechanism by which inducing *Myc* in PTH-expressing cells resulted in a change of cervical thymi incidence is unclear. One possibility is that *iMyc* amplifies cervical thymi formation by allowing the survival and growth of any 3PP endodermal cells

that convert to a thymus fate after E12.5. It is also possible that constitutive expression of *Myc* somehow promotes the formation of cervical thymi. Additional experiments are necessary to substantiate these hypotheses.

At present, there is a limited understanding of the factors determining parathyroid cell fate switch to a thymus lineage and the order by which this transdifferentiation occurs. Therefore, we sought to understand the order of program switch by driving *Foxn1* expression in parathyroid cells. Based on previous data from our lab, it was demonstrated that inducing *Foxn1* in parathyroid cells is sufficient to downregulate *Gcm2* expression and that this downregulation occurs progressively. Since this study was conducted, the original antibody used to identify parathyroid cells (anti-GCM2) was discontinued; we instead utilized an anti-PTH antibody to circumvent this issue. It was expected that the newborn parathyroids in $PTH^{Cre/+;}R26^{iFoxn1/+}$ mice would present a higher percentage of $FOXN1^{+}$ cells with low or no *PTH* expression than the 15.5% and 23.7% of $FOXN1^{+}$ cells with low or no *Gcm2* expression observed in E13.5 and E16.5 parathyroids, respectively (Peissig, unpublished data). However, we observed a 0% frequency of $FOXN1^{+}PTH^{-}$ cells in Cre-positive neonatal parathyroid glands (n=8). As no $FOXN1^{+}PTH^{+}$ cells were observed, our data support the conclusion that *iFoxn1* expression is sufficient to downregulate *Gcm2*. Testing whether *Foxn1* misexpression is sufficient to downregulate the whole parathyroid program may address the phenotype we observe in $PTH^{Cre/+};R26^{iFoxn1/+}$ newborns. Additionally, repetition of these experiments at other time points will be necessary.

Analysis of $PTH^{Cre/+};R26^{iFoxn1/+}$ newborn mice revealed that the mutant parathyroid cells lack detectable *Foxn1* expression, which could be attributed to an absence of Cre activity in parathyroid cells. Based on unpublished data, Cre recombinase appeared to be

activated in only about 50% of parathyroid cells at E16.5 (Peissig, 2017). When we looked at E16.5 parathyroids, expression of *Foxn1* was induced in approximately 10% of cells. In the future, Cre recombinase activity can be visualized by crossing $PTH^{Cre/+};R26^{iFoxn1/+}$ mice with a reporter strain, such as the $R26^{CAG\text{-}tdTomato}$ mouse. When crossed, *tdTomato* expression is driven irreversible in the cells in which *Cre* is expressed. As all parathyroid cells express PTH, immunostaining of $PTH^{Cre/+};R26^{iFoxn1/CAG\text{-}tdTomato}$ parathyroids should reveal colocalization of tdTomato and Foxn1 in every PTH^+ cell (Liu, Yu, & Manley, 2007). Based on our preliminary data of E16.5 $PTH^{Cre/+};R26^{iFoxn1/+}$ parathyroids, it is likely that not every PTH^+ parathyroid cell will be both $FOXN1^+$ and $tdTomato^+$ in $PTH^{Cre/+};R26^{iFoxn1/CAG\text{-}tdTomato}$ mice. We show that this *PTH-Cre* mouse can induce parathyroid-specific *Foxn1* expression, though silencing of *PTH-Cre* transgene expression may account for the low proportion of $FOXN1^+PTH^+$ cells.

For the cells in which *Foxn1* is initiated, it is possible that we do not detect any $FOXN1^+$ parathyroid cells at the newborn stage because cells with no or low *Gcm2* expression cannot survive and subsequently undergo programmed cell death before the thymus program can be fully adopted. Because parathyroid-fated cells undergo apoptosis in the absence of Gcm2, Gcm2 is required for parathyroid cell survival (Liu, Yu, & Manley, 2007). If the remaining parathyroid cells, in which Cre recombination did not occur, are able to undergo sufficient compensatory proliferation, this might explain why the size of neonatal $PTH^{Cre/+};R26^{iFoxn1/+}$ parathyroids appears to be normal. These data thus indicate that $FOXN1^+$ parathyroid cells undergo programmed cell death rather than transdifferentiating into TECs *in vivo*, however, a definitive conclusion about this

possibility cannot be made without the presence of an apoptosis marker such as Caspase-3.

During thymus development, Foxn1 may act as a 'pioneer' transcription factor (TF). Pioneer TFs operate at the level of chromatin organization by binding directly to and opening condensed chromatin, triggering the recruitment of additional factors to target genes (Zaret and Carroll, 2011). While other Forkhead TFs have been implicated as having pioneer activity, it is unknown whether Foxn1 functions as a pioneer factor in initiating thymus-specific gene expression (Lee et al., 2005). Although *Foxn1* expression has not been demonstrated to establish thymus fate *in vivo*, Foxn1 is sufficient to drive thymus lineage in some cell types (Bredenkamp et al., 2014). Thus, we hypothesized that ectopic expression of *Foxn1* could activate the thymus program in parathyroid cells. As a potential pioneer factor, Foxn1 may first turn on the TEC program in parathyroid cells, followed by other transcription factors, to initiate TEC differentiation. If true, then *Foxn1* may only be capable of regulating key genes required for TEC proliferation and function with the help of a cofactor(s), *in vivo*. This requirement would explain why the misexpression of *Foxn1* alone in *PTH*-expression cells does not lead to the formation of parathyroid-derived cervical thymi in $PTH^{Cre/+;}R26^{iFoxn1/+}$ mice. It is possible that the $FOXN1^{+}$ parathyroid cells that are Gcm2 low or $Gcm2^{-}$ do not persist into postnatal life because they are unable to upregulate and/or maintain the level of *Foxn1* expression necessary for complete commitment to the thymus lineage. Because Foxn1 is required at an increasing dosage to progress through multiple stages of TEC development, cells that fail to fully upregulate the thymus program may subsequently undergo programmed cell death (Chen et al., 2009, Nowell et al., 2011). This might also explain why $FOXN1^{+}$ parathyroid cells are absent in

newborn *PTH*$^{Cre/+}$*;R26*$^{iFoxn1/+}$ mice. Additionally, the upregulation and maintenance of *Foxn1* expression might be necessary to retain accessible chromatin configurations of thymus-specific genes and recruit additional factors required for TEC differentiation. Altogether, if thymus-specific TFs do not cooperatively remodel chromatin to suppress the expression genes specific to the parathyroid cell fate and activate thymus lineage-specific genes, transdifferentiation will not occur. Though forced expression of *Foxn1* can turn off *Gcm2* at a low efficiency, Foxn1 is unable to induce a complete lineage switch in parathyroid cells, possibly due to incomplete extinguishment of the parathyroid program. To form a pCT, the initial parathyroid program must be fully shut off before Foxn1 and other factors can subsequently activate TEC-specific TFs to drive differentiation of the thymus lineage.

Experimentally, transdifferentiation of a pancreatic exocrine cell to an insulin-producing β-cell has been achieved *in vivo* through the expression of selected transcription factors (Zhou et al., 2008). This study demonstrated that expression of *Pdx1*, a TF required to specify pancreatic fate, and *Ngn3* and *Mafa,* genes associated with the induction and terminal differentiation of the β-cell lineage, induced exocrine-to-β-cell transdifferentiation. Other similar studies might improve our current understanding of how lineage switch occurs in pCT.

Data from these experiments indicate that parathyroid cells may, instead, spontaneously downregulate and shutdown the parathyroid program before upregulating the thymus program to switch from parathyroid to thymus fate. Based on our findings, the low proliferation of parathyroid cells is a source of parathyroid cell fate instability. Although misexpression of *Foxn1* is sufficient to downregulate *Gcm2,* our data suggest

that expression of the master regulator of the thymus program alone is not sufficient to generate parathyroid-derived cervical thymi. Further examination of the factors dictating the epigenetic status of thymus and parathyroid programs throughout embryonic development would help elucidate the mechanisms underlying parathyroid cell transdifferentiation to thymus cells.

In the future, as there is currently no evidence to suggest the thymus program is unstable, we could carry out an experiment to evaluate whether cells that express *Foxn1* during the early stages of development subsequently downregulate their thymus program to switch to a parathyroid fate. It might also be interesting to test whether ectopic *Tbx1* expression inhibits the formation of pCT. Tbx1 is a negative regulator of the thymus program by directly inhibiting *Foxn1* expression (Reeh et al., 2014). Preliminary data from our lab indicates that *Tbx1* expression diminishes around E15.5. If *Tbx1* is not on to inhibit *Foxn1*, this may explain why cervical thymi never occur before E15.5. Previous data from our lab also identifies TBX1 as a promoter of *Gcm2* expression, and ectopic expression of *Tbx1* could resultingly lead to a stabilization of parathyroid cell fate. Considering this, we expect that the prolonged expression of *Tbx1* will inhibit parathyroid cells from switching fate, therefore, preventing the formation of parathyroid-derived cervical thymi.

Methods

Mice

PTHCre (129;FVB-Tg(PTH-cre)4167Slib/J, stock number: 005989), *R26CAG-tdTomato* (B6;129S6-Gt(ROSA)26Sor tm9(CAG-tdTomato)Hze/J, stock number: 007905), and *R26iMyc* (C57BL/6N-Gt(ROSA)26Sortm13(CAG-MYC,-CD2*)Rsky/J, stock number: 020458) mice were sourced from The Jackson Labs. Individuals of a non-commercially available inbred mouse strain *R26iFoxn1* (Rosa26CAG-STOP-Foxn1-IRES-GFP) were also obtained for this study (Bredenkamp et al., 2014). *Foxn1:;EGFP* mice were obtained from Dr. Thomas Boehm (Max-Planck Institute of Immunobiology, Freiburg, Germany).

PTHCre;R26iFoxn1 Experiments

$PTH^{Cre/+}$ mice were crossed with $R26^{iFoxn1/iFoxn1}$, and the progeny were analyzed. Neonates were fixed in 4% PFA. After serial ethanol dehydration and paraffin wax infiltration, the newborns were sectioned and immunostained with anti-PTH (Quidel Corporation, 1:400) and anti-FOXN1 (Santa Cruz Biotechnologies, 1:400) antibodies. Nuclei were stained with DAPI (Invitrogen).

PTHCre;R26CAG-tdTomato;R26iMyc;Foxn1::EGFP Experiments

$PTH^{Cre/+;}R26^{CAG\text{-}tdTomato/CAG\text{-}tdTomato};Foxn1::EGFP^{tg/0}$ mice were crossed with $R26^{iMyc/iMyc}$ mice, and the progeny were analyzed. Tissues were examined by fluorescent microscopy.

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Figures

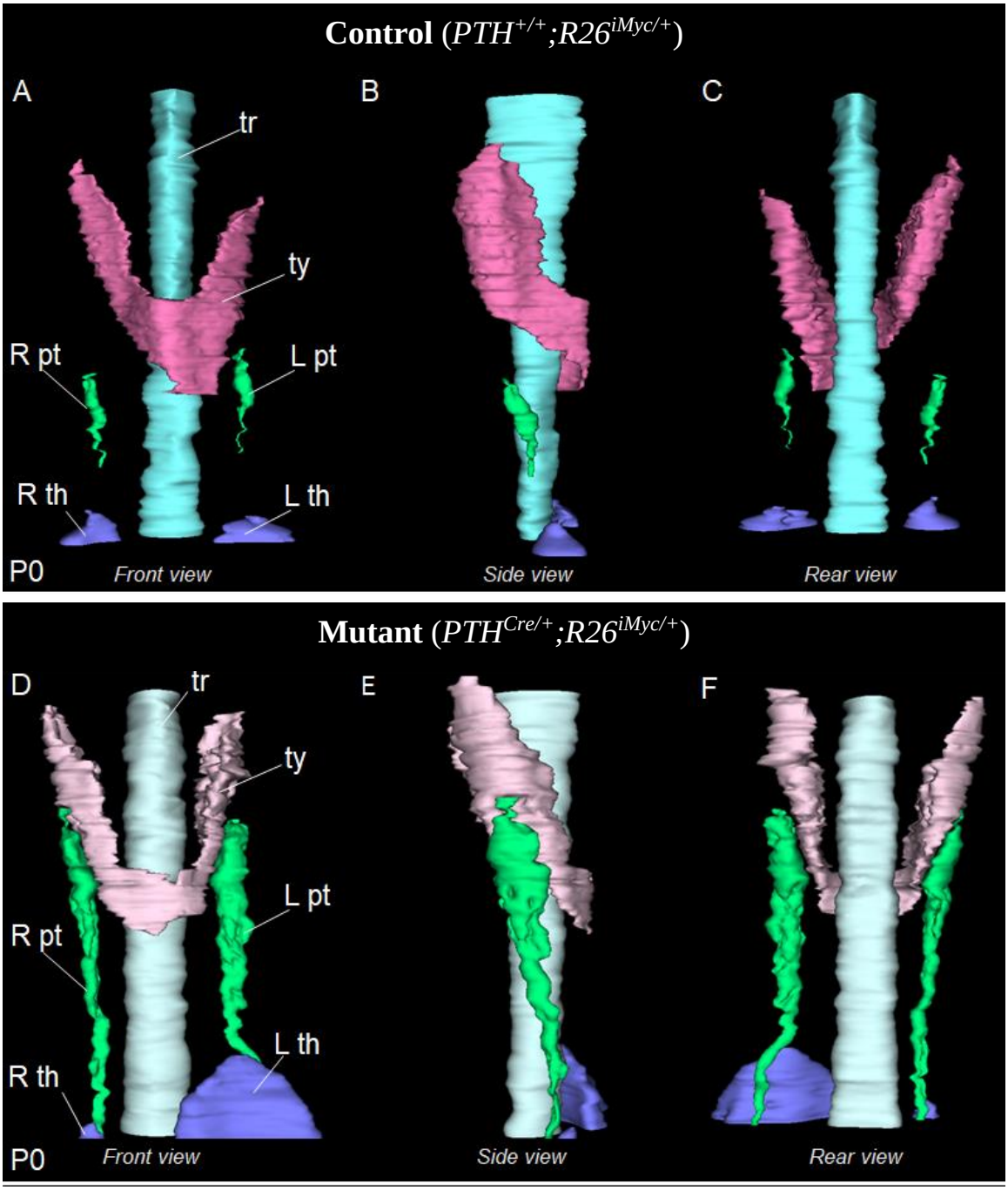

Figure 2-1. 3D reconstructions of newborn parathyroids. **A-C.** 3D reconstructions of control (*PTH*$^{+/+}$;*R26*$^{iMyc/+}$) newborn (postnatal day 0; P0) parathyroids. **D-F.** 3D reconstruction mutant (*PTH*$^{Cre/+}$;*R26*$^{iMyc/+}$) P0 parathyroids. parathyroid (pt) thyroid (ty) thymus (th) trachea (tr).

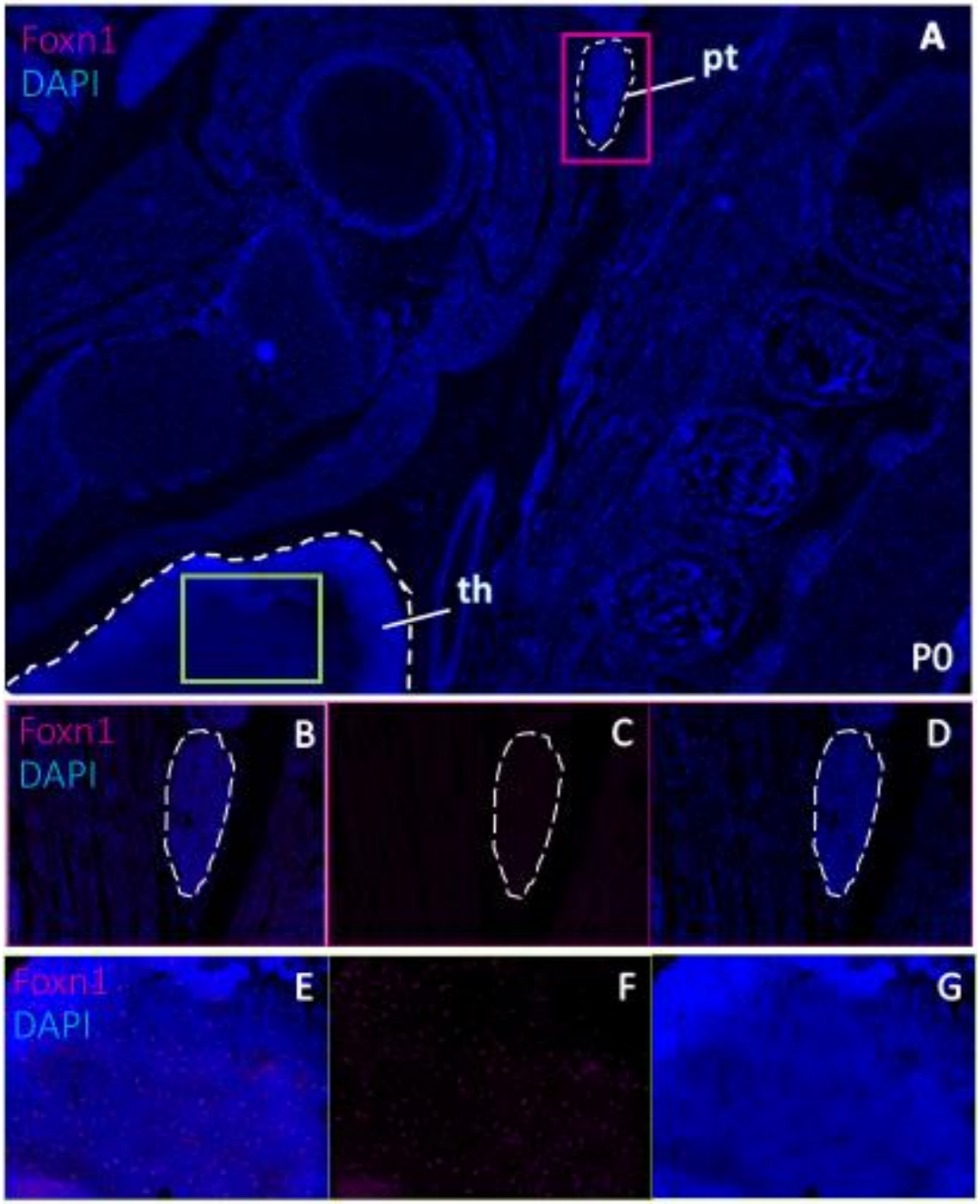

Figure 2-2. *PTH*$^{Cre/+}$;*R26*$^{iFoxn1/+}$ newborn mutant parathyroids lack Foxn1 expression. **A.** Parathyroid gland and thymus of newborn mutant. **B-D.** 20x magnification of the parathyroid (pink box in A). **E-G.** 20x magnification of the thymus (green box in A). parathyroid (pt). thymus (th).

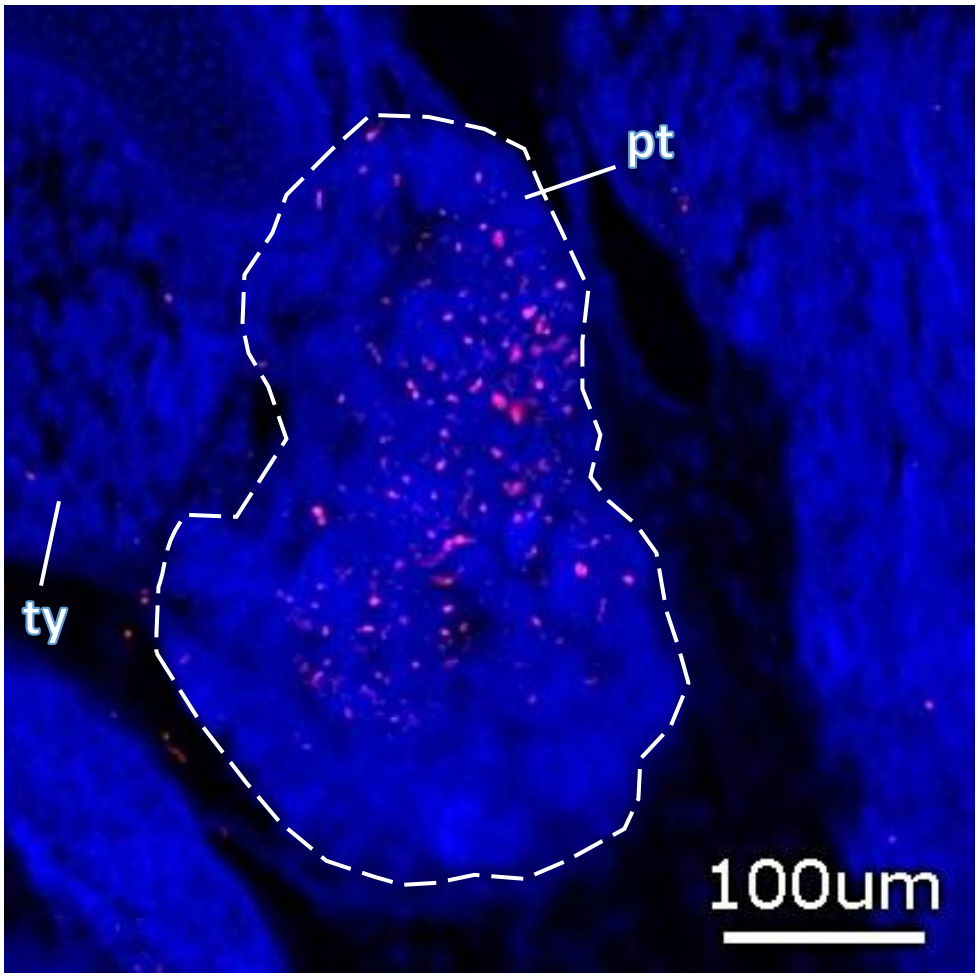

Figure 2-3. *Foxn1* expression in E16.5 $PTH^{Cre/+};R26^{iFoxn1/+}$ mutant parathyroid. parathyroid (pt) thyroid (ty).

Tables

Table 2-1. Different types of cervical thymi in 4-week-old *PTHCre;R26CAG-tdTomato;R26iMyc;Foxn1::EGFP* mice.

$PTH^{Cre/+};R26^{CAG\text{-}tdTomato/iMyc};Foxn1::EGFP^{tg/0}$ (mutants)							
	No CT	**1 CT/ mouse**	**2 CT/ mouse**	**3 CT/ mouse**	**4 CT/ mouse**	**5 CT/ mouse**	**Total**
Number of mice	0	6	9	5	8	1	29
Total number of CT/pCT	0	6	18	15	32	5	76
Frequency of CT (%)	0	100	100	100	100	100	-
Frequency of pCT (%)	0	0	0	0	0	0	-
$PTH^{Cre/+};R26^{CAG\text{-}tdTomato/+};Foxn1::EGFP^{tg/0}$ (controls)							
	No CT	**1 CT/ mouse**	**2 CT/ mouse**	**3 CT/ mouse**	**4 CT/ mouse**	**5 CT/ mouse**	**Total**
Number of mice	3	1	2	0	0	0	6
Total number of CT/pCT	0	1	4	0	0	0	5
Frequency of CT (%)	0	100	100	0	0	0	-
Frequency of pCT (%)	0	0	0	0	0	0	-