CELL SIGNALING IN THE THIRD PHARYNGEAL POUCH: AN INVESTIGATION OF HOW BMP, FGF, AND SHH SIGNALS REGULATE CELL FATE SPECIFICATION

by

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(Under the Direction of NANCY MANLEY)

ABSTRACT

The third pharyngeal pouch is a columnar epithelium which is derived from the pharyngeal endoderm. In the mouse, it develops at roughly E9.0 and differentiates into the thymus and the parathyroids. At E11.5, the thymus and parathyroid domains of the pouch express the transcription factors Foxn1 and Gcm2 respectively. While thymus and parathyroid-specific domain markers, the mechanisms by which each domain is established are largely unknown. Here, we expand upon the current studies on the effects of sonic hedgehog (Shh), bone morphogenic protein (Bmp), and fibroblast growth factor (Fgf) signaling. We leverage several different approaches, including genetic manipulation by Cre-loxp technology and an *ex vivo* culture system to better understand the role Bmp, Fgf, and Shh signaling in in the patterning of the third pharyngeal pouch epithelium.

Our study shows that Bmp4 and Fgf8 act opposite Shh in the pouch in order to inhibit parathyroid fate and activate thymus fate within the third pharyngeal pouch while Shh activates Tbx1, a known inhibitor of Foxn1 expression in the dorsal pouch. We also

show that Bmp signaling is required for parathyroid fate, a result that parallels data from an earlier study in chick.

INDEX WORDS: Thymus, parathyroids, third pharyngeal pouch, endoderm, fate specification, Fgf, Bmp, Shh, genetics, ex vivo culture, Foxn1, Gcm2, Tbx1

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DEDICATION

I would like to dedicate this work to my wife, Estefania. Without her, I would not be writing this today. If you are reading this, thank you for everything that you have done for me. Words truly cannot express my gratitude.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Structure, function, and origins of the thymus and parathyroids

The thymus is an epithelial organ that is responsible for the selection and development of T-lymphocytes. It is made up of a stromal compartment consisting of two different cell layers, the medulla and cortex. Naïve T-cells, known as thymocytes, enter through the vasculature at the junction between cortex and medulla, and are presented with a series of antigens by the thymic stroma. The stroma selects these T cells in order to remove ineffective, or autoimmune cells before they are sent to the peripheral lymphatic system. All thymic epithelial cells express *Foxn1*, a forkhead box transcription factor, beginning at approximately E11.5 and mark a group of cells that is specified for thymus fate (Gordon et al., 2001).

The parathyroids are essential endocrine organs that are responsible for the regulation of blood calcium via the expression of parathyroid hormone (PTH). (Munson, 1955). Two health conditions, hyperparathyroidism and hypoparathyroidism, can both arise from improper parathyroid function. Parathyroids express the transcription factor known as the mammalian homolog of glial cells missing 2 (*Gcm2*) after E9.5, and begin expressing PTH at approximately E13.5 during development (Gordon et al., 2001).

In the mouse, thymic epithelial cells as well as parathyroid cells are derived entirely from the third pharyngeal pouch endoderm (Dossel, 1958; Schrier and Hamilton,

1952). This epithelium is generated as an evagination of the endodermal cell layer that forms at approximately E9.0. Cells of the third pharyngeal pouch are surrounded by pharyngeal mesenchyme, a combination of neural crest cells (NCCs) and mesoderm-derived mesenchymal cells. During development thymus and parathyroid domains are marked by *Foxn1* and *Gcm2* expression by E11.5, and the organs separate from one another at approximately E13.5. After migration, the lobes of the thymus sit on top of the heart, while the parathyroids embed within or outside of the thyroid gland in the neck.

Thymus and parathyroid glands are highly conserved over evolutionary time

Thymic epithelial cells (TECs) are responsible for the positive and negative selection of T-cells during thymopoiesis in all jawed vertebrates (Boehm, 2011). The thymus is found in all jawed vertebrates, and there evidence from *in situ hybridization* screens suggesting primitive thymi dubbed thymoids, are present in lampreys as well (Bajoghli et al., 2011). These thymoids have been shown to be similar to mammalian thymuses in gene expression analyses. They express *Foxn1* and have also been shown to attract lymphocytes to their locations (Bajoghli et al., 2011).

Parathyroids are also present in all tetrapods, and express *Gcm2* as an early domain marker (Gordon et al., 2001). While *Gcm2* demarcates parathyroid cells in tetrapods and their ancestors, *Gcm2* expression has been confirmed in the pharyngeal pouches of teleosts and chondrichthyans (Okabe and Graham, 2004) and has been shown to be required for gill budding in zebrafish in loss-of-function experiments using morpholinos to knock down *Gcm2* (Hogan et al., 2004). Interestingly, while all jawed vertebrates develop thymuses, and all ancestors of tetrapods develop parathyroids, the

pouches from which they originate are variable (Grevellec and Tucker, 2010). While the pouches that give rise to the thymus and parathyroids may differ between closely-related species, research suggests that the gene regulatory networks responsible for giving rise to thymus and parathyroid domains appear similar, as models for fate specification of thymus and parathyroid cells are relatively unchanged in mice and chick, despite roughly 300 million years of years of divergence between the two species (Gordon and Manley, 2011; Neves et al., 2012).

Foxn1 and Gcm2 may be required for the terminal differentiation and maintenance of third pharyngeal pouch derivatives as opposed to fate specification

While *Foxn1* and *Gcm2* are sometimes considered master regulators of thymus (Romano et al., 2013) and parathyroid (Kebebew et al., 2004) fates, it is important to make the distinction between domain markers which recognize fated cells, and master regulators of cell fate. While forced expression of *Foxn1* via the $R26^{iFoxn1}$ allele is sufficient to drive thymus fate in mouse embryonic fibroblasts (Bredenkamp et al., 2014). When primary embryonic fibroblasts are forced to express Foxn1 and then cultured and inserted into the kidney capsule of an adult $Foxn1^{nude/nude}$ mouse they are able to differentiate properly into a functional thymus with a cortex, a medulla, and the ability to attract and select thymocytes effectively (Bredenkamp et al., 2014). This data makes a compelling argument that in at least some cell types, *Foxn1* is sufficient to drive thymus fate. However, the phenotype of the null allele of *Foxn1* suggests that there must be another mechanism responsible for the initial fate specification of the thymus rudiment. In Foxn1 null mutants, a thymic rudiment is formed and expresses the *Foxn1*-

independent marker of thymus fate, *IL-7*, within the thymic rudiment at E12.5 (Zamisch et al., 2005). Then, the thymic rudiment separates from the parathyroids, but does not undergo proper differentiation and proliferation, which causes the thymus to be disorganized and nonfunctional (Pantelouris and Hair, 1970; Su et al., 2003). The thymic rudiment in adult Foxn1$^{nude/nude}$ mice has been reported to resemble lung tissue in both cellularity and gene expression (Dooley et al., 2005). This indicates that *Foxn1* is not specifying thymus fate. Likewise, *Gcm2*$^{-/-}$ mutants are capable of forming a parathyroid domain at E11.5, but the cells of the parathyroid undergo apoptosis shortly afterward (Liu et al., 2007). This confirms that *Gcm2* is required for the maintenance of parathyroid cells, but does not suggest a requirement for *Gcm2* in specification events. It is important that we make the distinction between the role of *Foxn1* and *Gcm2* as master fate regulators and their requirement for differentiation and maintenance. While *Foxn1* and *Gcm2* expression are routinely used to demarcate the respective thymus and parathyroid domains (Gordon et al., 2001), they cannot specify fate *in vivo*. Furthermore, some *Gcm2*-expressing cells of the parathyroids are able to switch fates during migration and form small structures between the parathryroids and the thymus. These structures, as well as small groups of *Foxn1*-expressing cells that may separate from the thymic rudiment during migration, are known as cervical thymi. While smaller than a normal thymus, these cervical thymi express *Foxn1* and are able to attract thymocytes from the periphery (Li et al., 2013). This suggests that *Gcm2*-expressing cells, while fated to become parathyroid, may remain plastic for a period of time after specification events and that changes in signaling, such as a change that might occur when they separate during migration, may be sufficient to force a fate change in them.

While we have a fairly thorough understanding of the phenotypes of *Foxn1* and *Gcm2* mutants, the gene regulatory network responsible for the specification of thymus and parathyroid fate is not as well understood. To date, no study has identified the transcription factors that act upstream of *Foxn1* and *Gcm2* to specify thymus and parathyroid fates. It is for this reason that genomic approaches should be used to identify candidate genes that are actively being expressed during this period in order to identify the transcription factors that are responsible for thymus and parathyroid specification events.

Transcription factors act upstream of Foxn1 and Gcm2 in pouch development

There are several transcription factors which act independently of *Foxn1* and *Gcm2* expression in the third pouch affect pharyngeal pouch development. These genes include *Hoxa3* (Chojnowski et al., 2014; Manley and Capecchi, 1995), *Pax1* (Peters et al., 1998; Wallin et al., 1996), *Six1* (Zou et al., 2006), and *Eya1* (Laclef et al., 2003; Xu et al., 2002; Zou et al., 2006). When genes within the *Hoxa3-Pax1-Six1-Eya1* pathway are knocked out, phenotypes ranging from patterning defects to a loss of pouch-derived structures can occur. This may be due to the inhibition of signaling molecules, such as FGF8, Wnt5B, and BMP4, that are normally present in the developing third pharyngeal pouch are absent or greatly reduced in *Hox*, *Six*, and *Eya* mutants (Chojnowski et al., 2014; Zou et al., 2006). While these transcription factors are critical to the development of the pharyngeal apparatus, these data do not prove a direct link between their expression and thymus fate (Gordon and Manley, 2011). However, endodermal deletions of *Hoxa3* have been shown to initiate *Foxn1* at a later stage than wild-type controls,

suggesting that *Hoxa3* may be required for the timing of early fate specification, but is not required to specify thymus fate (Chojnowski et al., 2014)

Foxg1 has also been shown to be expressed in the thymus domain of the third pharyngeal prior to the expression of *Foxn1*(Wei and Condie, 2011). *Foxg1* null mutants express *Foxn1* normally, but have thymic hypoplasia and fail to undergo proper TEC differentiation starting at E13.5-E14.5 (Jarvis et al., 2014). Foxg1 mutants also have delayed expression of Interleukin 7 (IL-7), within the thymus. *Foxg1* appears to be independent and upstream of *Foxn1*, and it appears to act in parallel in regulating terminal cell differentiation, as mutants of each appear to specify as thymus, but fail to properly differentiate into a functional and organized epithelial organ.

Another transcription factor, *Tbx1*, has been shown to be essential in the patterning of the third pharyngeal pouch. *Tbx1* mutations have been implicated in DiGeorge Syndrome, a disease that causes a number of pharyngeal and cardiovascular malformations (Chapman et al., 1996; Garg et al., 2001; Merscher et al., 2001). While *Tbx1*$^{-/-}$ mutations fail to generate a third pharyngeal pouch, ectopic expression of *Tbx1* from the R26^{iTbx1} allele driven by Foxn1Cre significantly reduces *Foxn1* expression in the thymus rudiment (Reeh et al., 2014). It also appears that Tbx1 is positively regulated by SHH within the developing third pouch. Ectopic SHH signaling in the ventral domain of the pouch via the SmoM2 allele causes expansions of *Tbx1* expression and reductions in *Foxn1* expression, but not *Gcm2*. This suggests that *Tbx1* is capable of regulating thymus fate within the third pharyngeal pouch, but is not sufficient to activate *Gcm2* expression outside of the parathyroid domain (Bain et al. submitted). The mechanism by which ectopic *Tbx1* is sufficient to inhibit *Foxn1* expression in the developing third

pharyngeal pouch is not known. However, *Tbx1* has been shown to be able to bind SMAD4, a downstream target of BMP signaling, *in vitro* (Fulcoli et al., 2009). This activity creates competition for the BMP receptor-phosphorylated SMADs, pSMAD1/5/8, causing a reduction in BMP signaling, which is a regulator of *Foxn1* in post-specification thymus (Bleul and Boehm, 2005; Dudakov et al., 2015). While SHH is a positive regulator of *Tbx1* expression, *Tbx1* appears to be negatively regulated by the microRNA cluster miR17-92 (Reeh et al., 2015), the transcriptional repressor Ripply3 (Okubo et al., 2011), and Chordin (Bachiller et al., 2003). All of these potential *Tbx1* inhibitors are present within the pouch, and mutations in them cause phenotypes similar to DiGeorge Syndrome, including lack of a thymus/parathyroid and craniofacial abnormalities. Therefore, tight regulation of *Tbx1* is not only required for proper thymus and parathyroid patterning within the third pharyngeal pouch, but for the proper development of pharyngeal arch and pouch derivatives as well.

Other cell autonomous factors may reinforce thymus and parathyroid fates within the third pharyngeal pouch

In addition to transcription factors and inhibitory microRNAs, the cell cycle may be an important cell autonomous factor in the establishment and reinforcement of thymus fate. Cells of the ventral pouch divide at a higher rate than dorsal cells as early as E10.5 (data not published). This fast cycling may affect receptiveness to extracellular signals due to the cells of the ventral pouch quickly transitioning between S phase and M phase, without much time to establish cell surface receptors. Furthermore, cell cycle machinery has been shown to be closely tied with the establishment of epigenetic DNA and histone

modifications (Probst et al., 2009). Therefore, it is possible that the rapid cycling of cells in the ventral domain of the pouch can quickly reinforce thymus fate by silencing genes associated with other fate choices through chromatin modifications. The comparatively low rate of cell divisions in the parathyroid domain may also account for increased plasticity and the formation of cervical thymi *in vivo*, as cells of the parathyroid may require more time to establish epigenetic modifications that inhibit thymus fate.

Cell non-autonomous signals act in permissive and instructive manners to pattern the third pharyngeal pouch

Non-autonomous cell signals are expressed from one cell and received by another. Because these signals work in *trans*, they can originate from other adjacent endodermal cells, surrounding mesenchymal cells, or even ectodermal cells, provided those cells are located close enough to the third pharyngeal pouch to expose them to a signal. While most of the signaling pathways that have been emphasized are expressed in the pharyngeal endoderm, the pharyngeal mesenchyme is important for the patterning and proper migration of the thymus and parathyroids. This is evidenced by the hypomorphic parathyroids and ectopic, but large thymuses in mutants for *Splotch,* a gene that causes neural crest cells to not populate the pharyngeal arches(Griffith et al., 2009).

Here, I focus on four of the main signaling pathways that have been identified as having being essential in proper pouch patterning and outgrowth: SHH (Grevellec et al., 2011; Moore-Scott and Manley, 2005), FGF (Gardiner et al., 2012; Revest et al., 2001; Zou et al., 2006), BMP (Bleul and Boehm, 2005; Gordon et al., 2010; Patel et al., 2006), and Wnt (Balciunaite et al., 2002; Zuklys et al., 2009). *Wnt5b* has been shown to be

present in the developing third pharyngeal pouch ahead of *Foxn1* expression at E10.0 (Zou et al., 2006). It has also been shown to regulate *Foxn1* in adult thymus in both positive (Balciunaite et al., 2002) and negative (Zuklys et al., 2009) manners. Expression of *Wnt5b* is ablated in the third pharyngeal pouch in *Eya1*$^{-/-}$ mutants, suggesting that it may act downstream of *Eya1* in early pouch patterning(Zou et al., 2006). Furthermore, over-activation of β-Catenin, a downstream regulator of Wnt signaling that participates in the APC complex, using a mesenchymal Cre causes mice heterozygous for *Fgf8* or *Tbx1* to exhibit DiGeorge symptoms resulting from a loss of *Tbx1* expression (Huh and Ornitz, 2010). These data suggest that Wnt is acting early to establish proper *Tbx1* expression patterns through localized inhibition of the transcription factor as opposed to later in the specification of thymus and parathyroid cell fates (Huh and Ornitz, 2010).

SHH ligand expression is restricted to the pharynx in wild-type mice, and the only cells within the third pharyngeal pouch that are normally responsive to this signal are in the dorsal region of the pouch, as shown by *in situ* hybridization for *Ptc1*, a downstream gene and receptor of SHH signals (Grevellec et al., 2011; Moore-Scott and Manley, 2005). In *Shh*$^{-/-}$ mice *Foxn1* and BMP4 expression expand into the dorsal pouch at E11.5 causing a complete loss of *Gcm2* expression (Moore-Scott and Manley, 2005). SHH has also been shown to positively regulate *Tbx1* in the developing pouch in both the chick (Garg et al., 2001) using culture techniques and the mouse (Bain et al. submitted) using Cre-lox technology to activate ectopic SHH within the Foxn1 domain of the third pharyngeal pouch. In the mouse, ectopic SHH signaling via the R26^{SmoM2} allele is sufficient to expand *Tbx1* into the thymus domain, causing reductions in the total number of *Foxn1*-expressing/thymus fated cells, but does not expand *Gcm2* expression outside of

the normal parathyroid domain (Bain et al. submitted). These data suggest that SHH is necessary and sufficient for the localized inhibition of *Foxn1* in the dorsal pouch via activation of *Tbx1*, but is not sufficient to activate *Gcm2* expression.

Several FGF signals are present in the pouch endoderm near the ventral pouch, as well as in the surrounding mesenchyme (Gardiner et al., 2012). Previous studies have shown that FGF8 is required for pouch outgrowth, as *Fgf8* null mutants fail to develop a pouch (Abu-Issa et al., 2002; Crump et al., 2004). Additionally mutants that display DiGeorge-like symptoms, including Wnt (Huh and Ornitz, 2010), *Tbx1*(Choe and Crump, 2014), and *Eya1*(Zou et al., 2006) mutants all show defects in *Fgf8* expression, suggesting that FGF8 may be responsible for the initial proliferation of the third pharyngeal pouch primordium from the rest of the endodermal cell layer. Mice deficient for the FGF receptor, *FgfR2-IIIβ,* as well as *Fgf10*$^{-/-}$ mice have decreased proliferation in *Foxn1*-positive cells, as well as a block in mature TEC differentiation (Revest et al., 2001). Additionally, deregulation of FGF signaling via mutations in the endogenous FGF inhibitors, *Sprouty1/2*, cause a delay in parathyroid fate specification (Gardiner et al., 2012). This delay may only affect the parathyroid domain for a limited period of time because FGF signals are not expressed beyond E11.5 in the third pharyngeal pouch during development (Gardiner et al., 2012). These data suggest that FGF signaling is necessary for initial pouch outgrowth, proper timing of Gcm2 expression and/or parathyroid fate specification, as well as the proliferation of *Foxn1*-expressing TECs.

BMP4 is expressed throughout the ventral pouch, and its inhibitor, Noggin, is localized to the parathyroid domain before fate specification at E10.5 (Patel et al., 2006). Studies using chick embryo culture have suggested a dual role for BMP4 in the activation

of both thymus and parathyroid fates (Neves et al., 2012). Inhibitions of Bmp in post-specified TECs using a Foxn1^{Noggin} transgene has shown that Bmp4 is required for the post-specification maintenance of *Foxn1* in the thymic rudiment, causing proliferative and migration defects in thymic cells (Bleul and Boehm, 2005). However, these small, ectopic thymi that do not express *Foxn1* are capable of attracting T-cells (Bleul and Boehm, 2005). This research also showed that Bmp signaling has a degree of redundancy in the developing pouch, as the inhibition of BMP signaling in these transgenic mice also activated BMP2, a ligand which is not normally expressed in wild-type third pouch (Bleul and Boehm, 2005). Bmp is expressed in adult thymic endothelial cells following injury in order to activate Foxn1 expression and proliferation in stem cells (Dudakov et al., 2015). Lastly, BMP has been shown to activate the *Tbx1* inhibitor, miR17-92, in facial clefting (Wang et al., 2013) and has been shown to be inhibited in turn by *Tbx1* expression by Tbx1 binding SMAD4 in the nucleus (Fulcoli et al., 2009). Despite these data suggesting that Bmp may be necessary in establishing the thymus domain of the third pouch, single gene knockouts of *Bmp4* and *Alk3* only result in mild thymic hypoplasia (Gordon et al., 2010). This relatively mild phenotype may have been due in part to functional redundancy between different Bmp ligands and receptor proteins in the developing pouch. While the localization and timing of BMP signals, as well as the role of BMP in the maintenance and proliferation of *Foxn1* cells suggests that BMP signals are important for fate specification, the mechanism by which BMP may act in third pharyngeal pouch patterning remains unclear.

Currently, no genetic studies have been able to effectively inhibit BMP and FGF signaling in the murine third pouch primordium prior to the activation of *Foxn1* without

preventing pouch formation itself. Because of the redundancy of BMP ligands and receptors, as well as the requirement for FGF8 to generate a pouch, it is technically difficult to perform loss-of-function genetic studies on these two pathways. Therefore, other approaches may be more appropriate to determine the roles of BMP and FGF in the patterning of the third pharyngeal pouch. These experiments are are necessary to determine how BMP and FGF establish the patterning of the thymus and parathyroid domains opposite SHH signaling in the dorsal pouch endoderm.

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CHAPTER 2

FGF AND BMP SIGNALS ARE REQUIRED FOR PATTERNING OF BOTH THE THYMUS AND PARATHYROID DOMAINS IN THE THIRD PHARYNGEAL POUCH.

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Abstract

In the mouse, the thymus and parathyroids are derivatives of third pharyngeal pouch endoderm. Previous studies have identified the transcription factors *Foxn1* and *Gcm2* as early domain markers of thymus and parathyroid fate, respectively. Shh from the pharynx has also been shown to positively regulate *Tbx1*, a transcription factor that is a repressor of thymus fate in the third pharyngeal pouch. While the relationships between *Tbx1*, *Foxn1*, and SHH have been linked using genetics to generate gain of function and loss of function alles, other pathways, such as BMP and FGF signaling, have a less clear role in the process of fate specification in the third pouch due to technical limitations preventing the use of knockout alleles. Here, we leverage an *ex vivo* culture system to test the hypothesis that BMP and FGF pathways are required for thymus fate and the inhibition of parathyroid fate within the ventral domain of the third pharyngeal pouch. Our evidence suggests an overlapping function for BMP4 and FGF8 in the inhibition of parathyroid fate, as well as a requirement for BMP signaling in activating both thymus and parathyroid fates in the third pharyngeal pouch.

Introduction

The thymus and parathyroids are two organs that are responsible for the selection of T cells and regulation of blood calcium, respectively. In the mouse, both of these organs are derived from the third pharyngeal pouch, an endoderm-derived epithelium (Blackburn and Manley, 2004). Ventral cells of the pouch cells express *Foxn1*, a forkhead box transcription factor which marks the thymus domain, at approximately E11.5 (Gordon and Manley, 2011). The dorsal cells of the third pouch express the

transcription factor mammalian homolog of *glial cells missing*, *Gcm2*, which is required for the survival of parathyroid cells and is the earliest marker of parathyroid fate (Gordon et al., 2001). These *Gcm2* expressing cells also express parathyroid hormone at a later stage. Research has shown that forced *Foxn1* expression driven using an inducible allele is sufficient to activate thymus fate in cultured mouse embryonic fibroblasts (Bredenkamp et al., 2014). However, null alleles of *Foxn1*, such as the *nude* mutants still generate a thymus (Gordon and Manley, 2011). Therefore, there must be another gene regulatory network separate from *Foxn1* that is required for the specification of thymus fate *in vivo*.

The relationship between *Gcm2* and the T-box transcription factor *Tbx1* has also been shown to be important in the process of pouch patterning. *Tbx1* has shown to be activated by SHH signals originating from the pharynx and is expressed along the dorsal end of the pouch (Garg et al., 2001; Moore-Scott and Manley, 2005; Gardiner, et al., 2012; Bain et al., submitted). $Shh^{-/-}$ embryos fail to form parathyroids, and thymus fate expands in these embryos such that the entire third pharyngeal pouch expresses *Foxn1* at E11.5 (Moore-Scott and Manley, 2005). When expressed ectopically using the $R26^{iTbx1}$ allele, Tbx1 is sufficient to cause a large reduction in the total number of *Foxn1* positive cells in the thymus domain in the pouch (Reeh et al., 2014). Ectopic SHH signaling using the $Foxa2^{CreERT2}$ driver expands *Tbx1* expression, and shifts *Foxn1* to only the most ventral cells of the pouch, but fails to expand the *Gcm2* domain of the pouch (data not published). This suggests that SHH signaling to the third pouch endoderm from the pharynx is responsible for activating *Tbx1* and inhibiting *Foxn1* in the developing pouch. While the interactions between SHH, *Foxn1*, *Tbx1*, and *Gcm2* have been studied, less is

currently understood about the gene regulatory networks that inhibit *Gcm2* expression and promote *Foxn1* expression in the ventral pouch. While *Shh*$^{-/-}$ embryos expand the *Foxn1* domain into the dorsal pouch, there are no equivalent mutations that expand *Gcm2* expression into the thymus domain. Therefore, there must be a mechanism in place by which the thymus domain is able to inhibit *Gcm2* expression during development.

Gene expression analyses have shown that the signaling ligand BMP4 is primarily expressed in the thymus domain prior to the onset of *Foxn1* expression, while Noggin, an endogenous BMP inhibitor is expressed throughout the dorsal pouch (Abu-Issa et al., 2002; Patel et al., 2006). BMP4 has also been shown to be important for the maintenance of *Foxn1* expression after initiation at E11.5 (Bleul and Boehm, 2005), as well as in response to injury after radiation (Dudakov et al., 2015). In addition, BMP4 has been shown in other tissues to function as a transcriptional activator of miR17-92, a micro RNA that negatively regulates *Tbx1 in vivo* (Reeh et al., 2015; Wang et al., 2013). Furthermore, BMP4 seems to be negatively regulated by SHH signals within the third pouch, as BMP4 expands into the dorsal domain of the pouch in *Shh*$^{-/-}$ mutants. In chick, sequential BMP4 and FGF8 signaling promote thymus fate, and in BMP knockdowns, neither *Foxn1* nor *Gcm2* is expressed in the thymus and parathyroid primordia (Neves et al., 2012). While these data all suggest that BMP4 may be regulating *Foxn1*, previous studies that have used conditional knockouts of *Bmp4* and its receptor, *Alk3*, to determine the role of BMP4 in third pharyngeal pouch fate specification have only shown delays in the activation of *Foxn1* throughout the thymus domain with hypomorphic thymuses (Gardiner et al., 2012; Gordon et al., 2010). This mild phenotype may be caused by redundancy, as previous studies knocking down BMP signaling in the pouch using a

Foxn1^{Noggin} transgene have shown that a second BMP ligand, BMP2, is expressed when BMP4 is inhibited (Bleul and Boehm, 2005).

Previous studies have also implicated FGF8 in third pharyngeal pouch patterning. Multiple FGF ligands are localized to subdomains of the ventral pouch prior to the activation of *Foxn1*, while the endogenous FGF inhibitors, *Sprouty1/2* are expressed in the posterior and dorsal portion of the pouch (Gardiner et al., 2012). When *Sprouty1/2* are knocked out, initiation of *Gcm2* expression is delayed relative to the wild-type (Gardiner et al., 2012) Furthermore, *Fgf8* expression has been greatly reduced in *Six1*$^{-/-}$ and *Eya1*$^{-/-}$ mutants. These mutants fail to maintain *Foxn1* and *Gcm2* expression in the pouch and undergo cell death (Zou et al., 2006). While these data suggest that FGF8 may be sufficient to inhibit *Gcm2* when added ectopically to the ventral domain, there are no current studies implicating *Fgf8* in the regulation of *Foxn1*. This is because there are multiple expressed FGF ligands and one of the most highly expressed in the pouch, FGF8, is required for initial third pharyngeal pouch outgrowth (Abu-Issa et al., 2002; Crump et al., 2004) making loss-of-function experiments using conditional or null alleles of *Fgf8* technically impractical for experiments to determine the role of *Fgf8* in fate specification. In addition, several other FGF ligands including FGF3, FGF10, and FGF15 are also expressed in and around the ventral pouch (Gardiner et al., 2012), so conditional knockouts of FGF8 may not be able to determine the function of FGF8 in specification events due to redundancy.

Current data suggests that SHH signaling activates the expression of *Tbx1* in the third pharyngeal pouch, which is then responsible for inhibiting *Foxn1* within the dorsal domain of the pouch (Garg et al., 2001; Bain et al., submitted). Meanwhile, mutations

causing gain of function in FGF signaling appear cause a delay in *Gcm2* expression, suggesting a role for FGF in the inhibition of parathyroid fate (Gardiner et al. 2012). Loss of function studies have shown no relationship between BMP signaling and thymus fate, and no gain of function studies for BMP in thymus fate have been performed in the mouse. However, BMP-mediated activation of microRNAs that inhibit *Tbx1* (Wang et al., 2004), as well as studies showing that BMP regulates *Foxn1* expression after initial fate specification (Bleul and Boehm, 2005) and is required for thymus fate in chick (Neves et al., 2012) suggest that BMP signaling may still be required for fate patterning within the third pharyngeal pouch. We therefore suggest a model in which BMP and FGF signaling each activate *Foxn1* and inhibit *Gcm2,* while SHH activates *Tbx1* which in turn inhibits *Foxn1* expression (Fig 1.).

In this study, we leverage an *ex vivo* culture system to manipulate BMP4 and FGF8 signals. Our system has several advantages over genetic manipulation in that we are able to tightly control the location, timing, and amount of signal that is added to the system. This approach also allows us to affect the pouch endoderm as well as the pharyngeal mesenchyme. This aspect of our experimental design is relevant because previous studies have shown that mesenchymal cell signaling is required for proper thymus and parathyroid development, and BMP and FGF ligands, as well as the BMP inhibitor Chordin are known to be present in the pharyngeal mesenchyme (Griffith et al., 2009). We also used this system to manipulate multiple signaling pathways simultaneously to determine the interactions between BMP, FGF, and SHH signaling during thymus and parathyroid development. Our data suggests that FGF8 and BMP4 are

each sufficient to inhibit *Gcm2* expression in third pharyngeal pouch endoderm, and that BMP signaling is required for the specification of both thymus and parathyroid cells.

Methods

Mice:

C57B6L/6J mice for all experiments were sourced from Jackson labs (Jackson Labs stock number 000664). Embryonic age was estimated as noon of the day of a vaginal plug as E0.5; stages were confirmed by somite number and morphology.

Embryo Culture: Embryo culture was performed according to our previously published methods. C57BL6 crosses were set up for timed pregnancies. Embryos were dissected at E10.0 and cultured as tissue explants with small molecule inhibitors (dorsomorphin, SU5402). Beads soaked in recombinant protein (BMP4, FGF8, Noggin) at 5µg/µL were implanted in whole embryos and cultured for 24hr at 37°C in KnockOut DMEM media (Thermo Fisher 10829018) containing 10% KnockOut serum replacement (Thermo Fisher 10828028) in a roller bottle culture under 95%O_2. For each treatment group, control embryos were cultured with suitable PBS or DMSO in media without inhibitors or proteins.

Tissue Processing: After culture, tissue was immediately fixed in 4% PFA for 1 hour at 4C. Embryos and tissue explants were then dehydrated in an EtOH gradient, washed twice in Xylenes and then transferred to three separate paraffin wax washes before embedding in paraffin. Sections were cut on a microtome to 8µM thickness and allowed to dry on a slide warmer at 30. All sections were de-waxed in xylenes and rehydrated in

an ethanol gradient. Antigen retrieval was performed by boiling the slides in a water bath in a 10mM sodium citrate solution for 30 minutes. After antigen retrieval, slides were washed once for five minutes in PBS.

Antibody Staining: Slides were stained with antibodies against Foxn1 (Santa Cruz sc-23566, 1:200) and Gcm2 (Abcam 64723, 1:200) in a 10% donkey serum/PBST solution and incubated at 4°C overnight. Slides were then washed in three times for five minutes in PBS. Donkey anti-rabbit 488 secondary (Thermo Fisher A-21206) and Donkey anti-goat 555 secondary (Abcam ab150130) were applied for one hour at room temperature. Slides were washed with PBS, incubated 5 minutes in DAPI diluted 1:10,000 in PBS, then washed for five minutes in PBS and coverslipped using Fluoro-gel mounting media (EMS 17985-10) before imaging. Cells expressing *Gcm2* and *Foxn1* were counted by hand and calculated as a percentage of all cells in the third pharyngeal pouch using DAPI. Samples with no more than 20% of sections missing were included in the dataset.

In Situ Hybridization: Paraffin section *in situ* hybridization was performed as previously described (Moore-Scott and Manley, 2005). Control and experimental embryos were hybridized and stained together. All probes were generated by PCR amplification. Probes for *Tbx1* (Chapman et al., 1996) and *Dusp6* (Gardiner et al., 2012) mRNA were previously described.

Results

Ectopic FGF8 is sufficient to inhibit Gcm2 expression in the dorsal pouch, but not to expand Foxn1:

We tested the requirement of ectopic FGF8 to promote thymus fate and suppress parathyroid fate by implanting beads soaked in recombinant FGF8 ligand (5µg/µL) into whole 35 somite stage embryos and culturing over a 24hr period. While the size of the pouch and the percentage of *Foxn1*-expressing cells was not significantly different in the treatment versus control groups, *Gcm2*-expressing cells were significantly reduced in the third pouch (n=3, p<0.05) (Fig 2). All effects were greatest when cultures began at the 35 somite stage or earlier. This mechanism of this reduction appears to be independent of Tbx1 expression, as ectopic FGF8 does not appear to change *Tbx1* expression patterns within the third pharyngeal pouch (Fig 2). This suggests that ectopic FGF8 signaling is sufficient for the local inhibition of *Gcm2*, but is not sufficient to drive *Foxn1* expression in the dorsal domain of the third pharyngeal pouch.

Ectopic BMP4 is sufficient to inhibit Gcm2 in the dorsal pouch, but not to expand Foxn1

We tested the hypothesis that ectopic BMP4 is sufficient to promote thymus fate and suppress parathyroid fate by implanting beads soaked in recombinant BMP4 ligand (5µg/µL) to whole embryos and culturing over a 24hr period. As with the FGF8 treatment, pouches were not significantly different in size between treatment and control groups, nor were the number of *Foxn1* expressing cells significantly different. The total number of *Gcm2* expressing cells was significantly lower when treated with BMP4 than in control groups. (n=3, p<0.05) (Fig 3). This suggests that high levels of ectopic BMP4,

like FGF8, is not sufficient to expand thymus fate, but is sufficient to inhibit *Gcm2* expression in the parathyroid domain of the third pharyngeal pouch (Fig 3).

FGF signaling drives Foxn1 expression and proliferation in the third pharyngeal pouch:

We tested the hypothesis that FGF8 is necessary for thymus fate in the third pharyngeal pouch development by inhibiting FGF signaling in 35 somite stage embryos using SU5402 at 10μM for 24hr in culture. FGF8 inhibition by SU5402 resulted in a significant decrease ($n=3, p<0.05$) in the percentage of *Foxn1*-expressing cells within the third pharyngeal pouch as well as a drop in overall third pouch size after 24hr in culture (Fig 4). However, the percentage of *Gcm2*-positive cells is not significantly different compared to the control group. While the boundaries of the *Foxn1* and *Gcm2* domains appear similar between the control and treatment groups, the total number of *Foxn1*-positive cells was only slightly reduced (Fig 4). This suggests that FGF8 does function to activate *Foxn1* expression in at least some third pharyngeal pouch cells, and is required for the proliferation of cells within the thymus domain of the third pharyngeal pouch.

BMP signaling is required for Foxn1 and Gcm2 expression:

We tested the hypothesis that BMP signals are required for the activation of thymus fate and inhibition of parathyroid fate using two separate knockdown models. The first treatment consisted of tissue explants soaked in dorsomorphin, a small molecule drug inhibitor. These treatments resulted in third pharyngeal pouches that expressed minimal, if any *Foxn1* and *Gcm2*, and were significantly smaller than control groups (Fig 5). These data suggest that BMP signaling is required for both thymus and parathyroid

fates, and that loss of BMP signals at E10.5 is sufficient to cause a loss of both domains of the third pharyngeal pouch.

Because of known off-target effects in TGF-β signaling in treatments with dorsomorphin, as well as technical difficulties verifying dorsomorphin knockdowns due to already low levels of endogenous BMP signaling in the third pharyngeal pouch, we used a second independent inhibitor of BMP signaling to verify our results. BMP signals were inhibited by implanting beads soaked in recombinant 5µg/5µL Noggin protein in 35 somite stage embryos. In this case, pouch size was similar to the controls, but *Foxn1* and *Gcm2* expressing cells were both significantly reduced in the treatment groups compared to PBS controls (n=3, p<0.05) (Fig 5E). This independent result shows that the loss of *Foxn1* and *Gcm2* expression in dorsomorphin treatments is due to the inhibition of BMP signals within the pouch, as opposed to off-target TGF-β signals or secondary effects due to reduced size in the primordium.

To test the hypothesis that BMP may act as a morphogen within the pouch and that low-level BMP signaling drives parathyroid fate, we implanted beads containing recombinant noggin were titrated from 5µg/µL 0.5µg/µL to determine if there was a threshold below which only thymus or only parathyroid domains were affected, but the thymus and parathyroid domains were affected equally in the titrated groups (Fig 6). This suggests that the requirement for BMP4 in the specification of parathyroid and thymus fates may not be dependent on expression levels, or that levels of BMP signaling outside of our titration range are required for parathyroid fating.

In experiments using BMP inhibitors, we noticed that the severity of fate changes sometimes varied between experiments. Therefore, we further analyzed the specific

timing of BMP receptiveness by staging embryos at one somite intervals and culturing over 24 hours in dorsomorphin. We show that receptiveness to changes in BMP signaling on *Foxn1* and *Gcm2* expression is extremely time-dependent, as embryos treated with dorsomorphin at or before the 35 somite stage result in severe patterning changes whereas embryos treated at the 36 somite stage and beyond are relatively normal (Fig 7). This suggests that the activity of BMP signaling in thymus and parathyroid fate specification is taking place within a very short time window, probably between 33 and 35 somites.

Addition of SHH signals to Bmp knockdown treatments is not sufficient to expand parathyroid fate

We hypothesized that BMP may act opposite SHH signals in a mutually antagonistic relationship. To test our hypothesis, we added a smoothened agonist (SAG) to cultures containing 10μM dorsomorphin to activate SHH signaling in BMP-deficient cells. However, in these experiments, we saw no change in *Gcm2* expression in the developing third pharyngeal pouch (Fig 8). This data suggests that the requirement for BMP signals in thymus and parathyroid fate specification may be independent of the requirement for SHH in *Tbx1/Gcm2* expression.

Inhibition of BMP and FGF together results in a loss of Foxn1 and Gcm2 expression:

In order to determine if BMP and FGF ligands are redundant for the inhibition of parathyroid fate in the ventral pouch, we cultured 35 somite embryos in media containing SU5402 and dorsomorphin inhibitors over a 24hr period. In this experiment, the pouches closely resembled those from BMP knockdown experiments with minimal *Foxn1* or

Gcm2 expression after culturing (Fig 9). This data suggests that, even in knockdowns of both BMP and FGF, endogenous SHH from the pharynx is not sufficient to activate or expand parathyroid fate within the third pharyngeal pouch.

Discussion

Previous models of third pharyngeal pouch specification suggest that a mutually antagonistic relationship between FGF/BMP and SHH signals are responsible for the patterning and specification of the thymus and parathyroid domains. Our data indicates that high levels of either ectopic FGF8 or BMP4 are sufficient to inhibit *Gcm2* expression in the dorsal pouch, and that BMP signaling, unlike FGF, is also required for the specification of parathyroid fate. We also show that BMP and FGF signals each promote thymus fate within the ventral pouch, but are not sufficient to expand thymus fate when added to the dorsal domain of the pouch. Lastly, our data supports that only a limited number of cells in the third pharyngeal pouch are capable of adopting parathyroid fate, even when signals that normally inhibit *Gcm2* when ectopically expressed, such as FGF and BMP, are inhibited.

No genetic manipulations found in current literature other than those that affect pouch outgrowth result in a total loss of *Foxn1* in the third pharyngeal pouch. Genetic studies deleting *Bmp4* or *BmpR1a* (Gordon et al., 2010) or adding an ectopic SHH signal throughout the pouch endoderm (Bain, et al., submitted) result in a hypomorphic thymus and shifts in fate boundaries with reductions in *Foxn1* respectively, but *Foxn1* is still activated in these mutants. Other mutant models, such as the $Foxn1^{Noggin}$ transgenic mouse, show that BMP signaling is required for the maintenance of *Foxn1* expression

after the initiation activation of *Foxn1*, but do not address whether BMP4 has any effect on initial Foxn1 expression patterns (Boehm, 2011). However, no loss-of-function studies have shown a requirement for a specific signaling molecule in establishing Foxn1 fate. This is likely due to two separate factors. The first factor is a series of technical problems limiting the genetic tools that are available to researchers. Several mutations of key genes related to third pouch fate specification including *Fgf8, Chordin, Tbx1, and Ripply3* cause pouch outgrowth phenotypes in which there is no pharyngeal pouch present (Bachiller et al., 2003; Crump et al., 2004; Liao et al., 2004; Okubo et al., 2011). Furthermore, conditional mutations may not be appropriate to determine the function of these genes, as outgrowth and specification may be overlapping events. These issues prevent the use of genetic tools in loss-of-function studies in the third pharyngeal pouch endoderm. Secondly, redundancy built into the system may mute any phenotypes that single gene knockouts would otherwise show. BMP4 loss-of-function experiments have shown that BMP signals may be redundant (Bleul and Boehm, 2005; Gordon et al., 2010). This redundancy may also be present in FGF signaling, as four separate FGF ligands are expressed in and around the third pharyngeal pouch at E10.5 (Gardiner et al., 2012). Because our experimental system allows us to precisely alter the timing, location, and dosage of drugs and proteins and affect BMP and FGF in a ubiquitous and manner, our data may show more severe defects in pouch domain specification than more targeted *in vivo* methods that have been previously published.

One technical challenge of our method is that the endogenous signals, particularly the endogenous BMP signal, are expressed at such low levels that our attempts to detect changes in signaling have been unsuccessful using several traditional reporters (Fig 10).

While we were unable to quantify the efficacy of our knockdowns in the culture system, other researchers have successfully used SU5402 (Jackman et al., 2004; Li et al., 2016) and dorsomorphin (Sherwood et al., 2011) in embryonic endodermal cells. Unlike dorsomorphin, which inhibits BMPR activity, Noggin acts at the level of the ligand, binding available BMP molecules in the extracellular space. Our results from cultures in which recombinant Noggin is added to the system show similar results to dorsomorphin treatments as well. Furthermore, recent studies in chick using similar knockdown methods with recombinant Noggin protein have also shown that BMP signals are required for the activation of thymus fate via the activation of FGF8 within the thymus domain and parathyroid fate (Neves et al., 2012). While the order of BMP and FGF signals are reversed in mouse (Neves et al., 2012), it is possible that the mechanism is similar between the two species.

The cells of the parathyroid domain appear to exhibit more plasticity than those of the thymus domain. In *Shh*$^{-/-}$ mutants, all cells within the parathyroid domain are able to convert to thymus fate (Moore-Scott and Manley, 2005). Additionally, we have shown that parathyroid cells can spontaneously convert from parathyroid to thymus fate in wild-type mice *in vivo* (Li et al., 2013). A role for FGF signaling in Gcm2 expression was also implicated by loss of the FGF inhibitor, *Sprouty,* which showed delayed parathyroid fate specification (Gardiner et al., 2012). This is a similar result to our study, as high levels of ectopic FGF signals were sufficient to inhibit *Gcm2* expression. It is possible that FGF signals may also be responsible for proliferation of thymus cells within the thymus domain. While our data shows that the percentage of *Foxn1* positive cells are reduced in treatments using SU5402, another study, knocking out the *FgfR2-IIIB* receptor shows that

loss of FGF signals cause a proliferative block in the thymus rudiment after E12.5 (Revest et al., 2012).

Based on our results and the current published data, we suggest a revised model of third pharyngeal pouch specification (Fig 11). In this model, BMP signals are required for both thymus and parathyroid fates and are sufficient to inhibit *Gcm2* expression at high levels. FGF signals are required for thymus but not parathyroid fate and are sufficient to inhibit *Gcm2* expression at high levels. Based on our results, it may be informative to revisit genetic experiments ablating BMP signaling in the third pharyngeal pouch using conditional *Alk3/6* double mutants to inhibit BMP signals at the level of the receptor.

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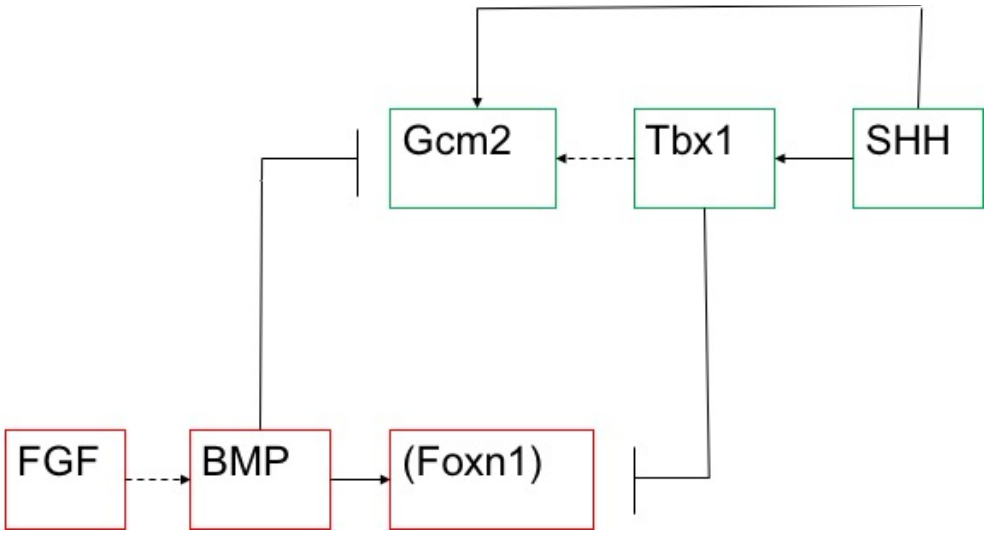

Figure 2-1: Current model of thymus and parathyroid fate specification suggests a mutually antagonistic role for FGF and BMP and SHH signaling.

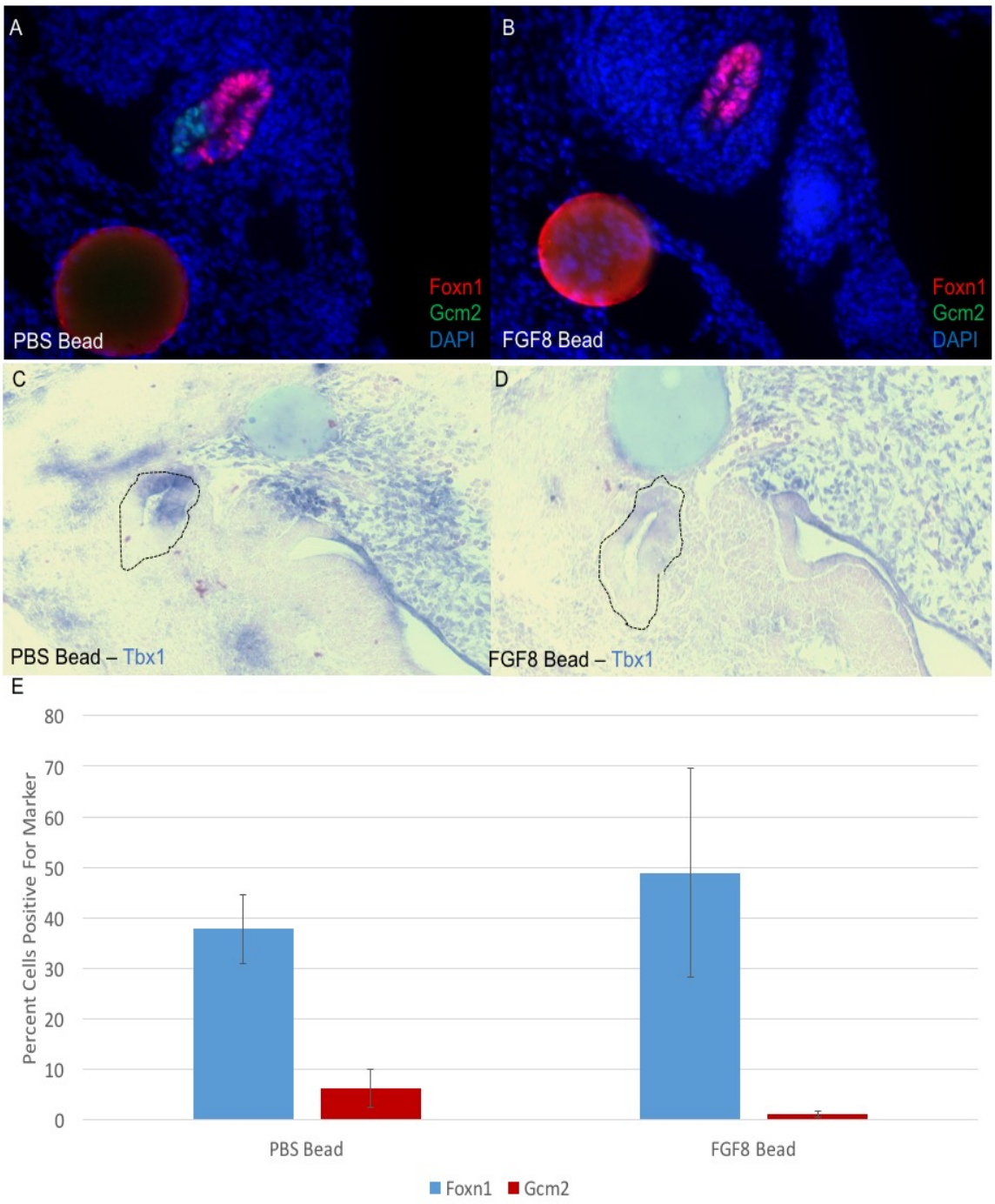

Figure 2-2: High levels of ectopic FGF8 from a bead soaked in recombinant protein and implanted at E10.5 is sufficient to inhibit Gcm2 expression via a Tbx1-independent mechanism. Beads soaked in PBS (A/C) or recombinant FGF8 (B/D) were implanted in 35 somite embryos and cultured for 24hrs. Control embryos were cultured with beads

containing PBS and cultured for 24hrs (B/D). Embryos were stained with anti-Foxn1 (red) and anti-Gcm2 (green). (E) The percentage of Gcm2 positive cells is significantly reduced in FGF8 treated embryos (n=3, $p<0.05$), but Foxn1 is not significantly expanded into the parathyroid domain.

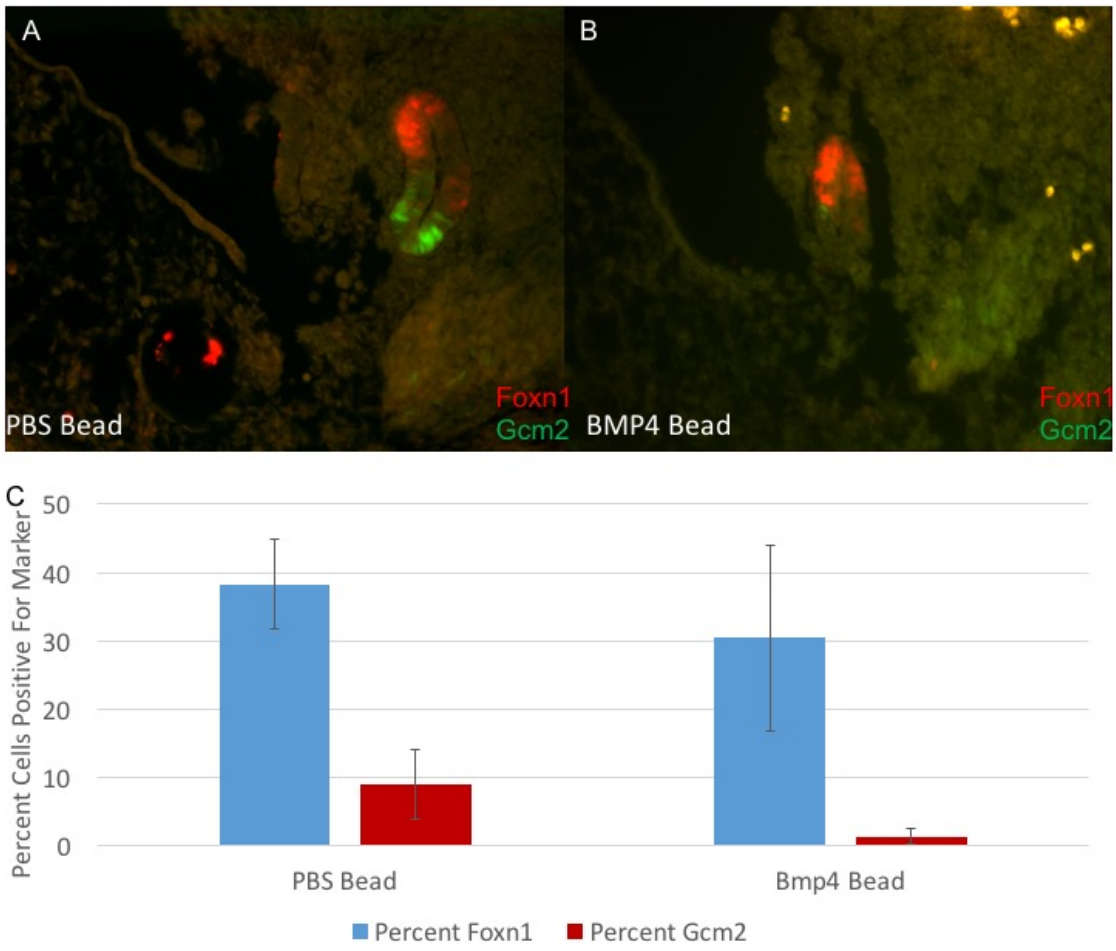

Figure 2-3: High levels of ectopic BMP4 signaling via beads soaked in recombinant protein and implanted in at E10.5 is sufficient to inhibit Gcm2 expression in the parathyroid domain. Beads soaked in PBS (A) or BMP4 (B) were implanted in 35 embryo and then cultured for 24hrs. Control beads (B) were soaked in PBS and cultured over 24hrs. Pouches were stained for Foxn1 (red) and Gcm2 (green). Cell counts (C) show that the percentage of cells expressing Foxn1 (blue) was not significantly changed with BMP4 treatment. However, the percentage of cells expressing Gcm2 (red) was significantly lower than in control embryos (n=3, p>0.05).

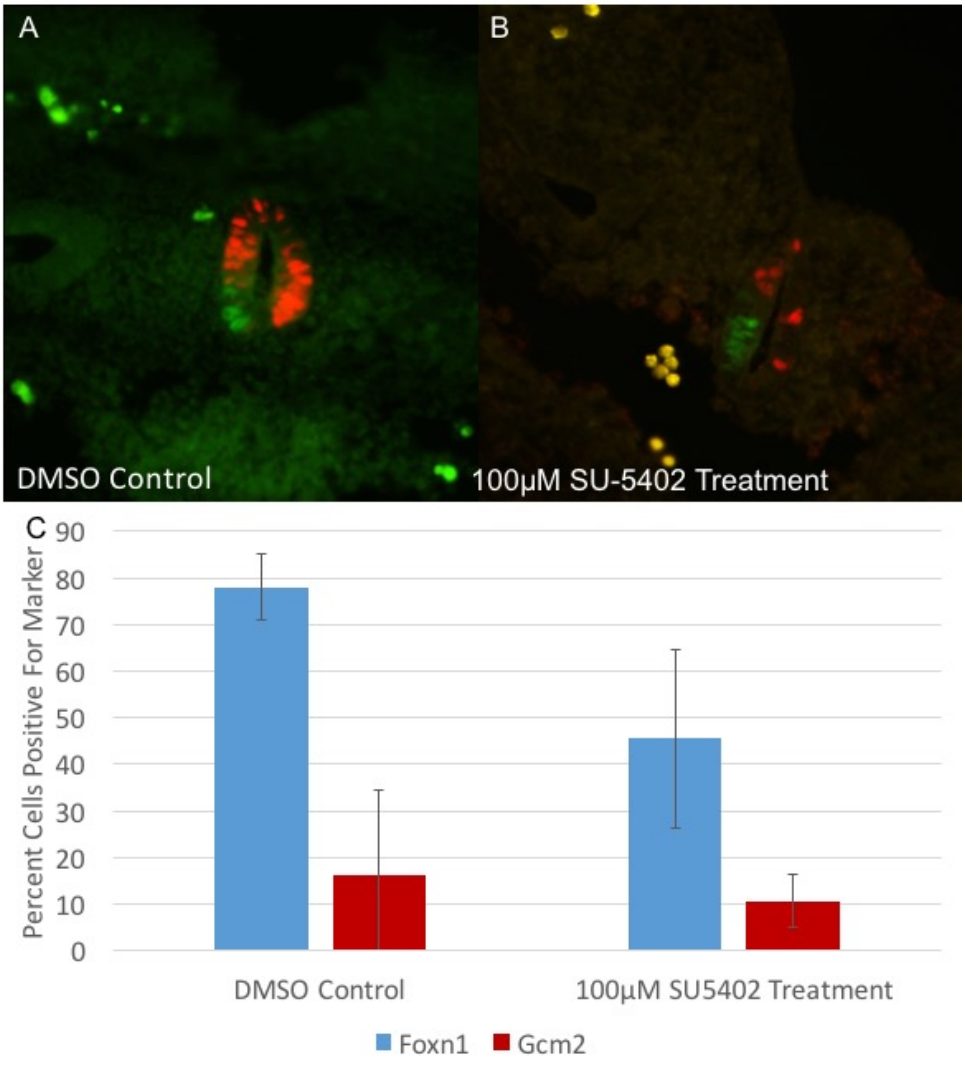

Figure 2-4: Loss of FGF signaling by SU5402 treatment significantly reduces Foxn1 expression in third pharyngeal pouch primordium. Embryos were cultured over 24hr period starting at 35 somites in media containing either DMSO (A) or 100µM SU5402 (B) and stained for Foxn1 (red) and Gcm2 (green). (C) Cell counts for Foxn1 (blue) and Gcm2 (red) show that Foxn1 expression is significantly reduced in the treatment group compared to the DMSO controls ($p<0.05$, $n=4$). However, Gcm2 was not significantly reduced between SU5402 treatments and DMSO controls.

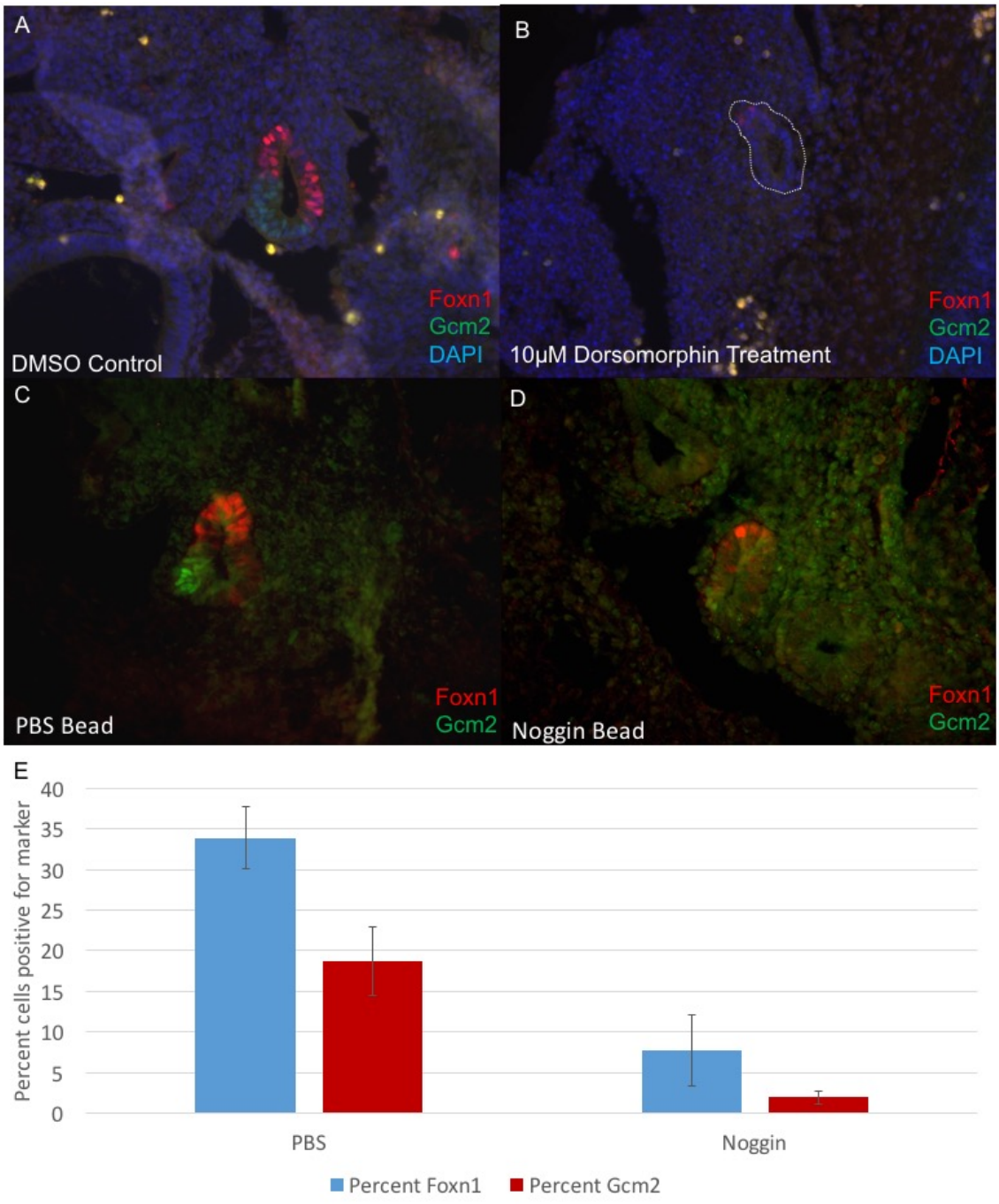

Figure 2-5: BMP signaling is required for thymus and parathyroid fates. 35 somite stage embryos cultured for 24hrs with media containing 10μg/μL dorsomorphin (B) were stained for Foxn1 (red), Gcm2 (green), and DAPI (blue). Dorsomorphin treatment results in a near-complete loss of both Foxn1 and Gcm2 (n=4). BMP knockdowns were

also achieved by implanting beads soaked in recombinant noggin protein (C) and culturing embryos over a 24hr period with PBS bead controls (D). While not as severe an inhibition as dorsomorphin treatments cultures with noggin showed a significant ($n=3$, $p<0.05$) reduction in both Foxn1 (blue) and Gcm2 (red) (E).

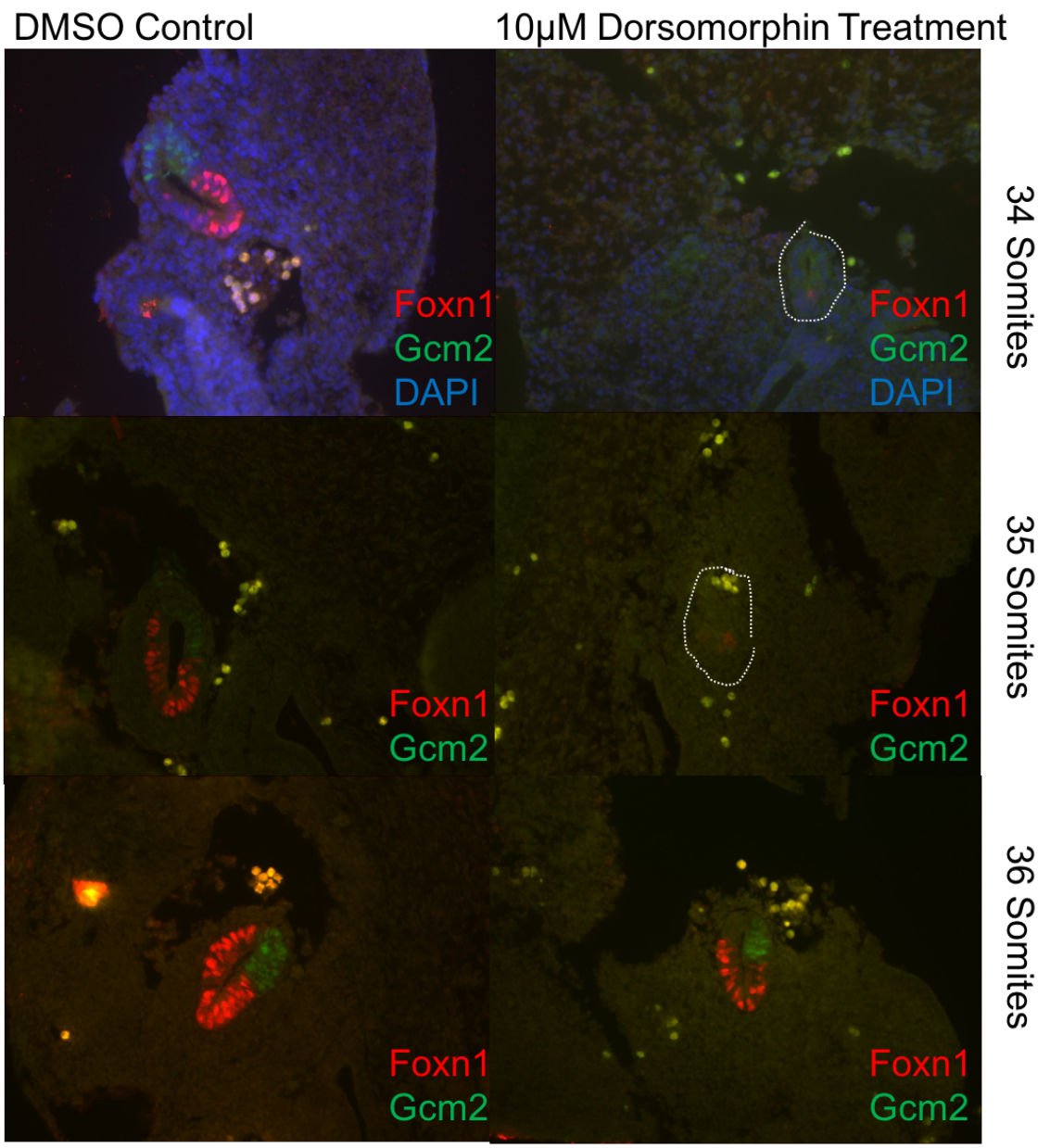

Figure 2-6: Responsiveness to changes in BMP signals are time-dependent. Embryos cultured with either 10μM dorsomorphin or DMSO were staged at the start of culture. All embryos were cultured over a 24hr period and stained for Foxn1 (red) Gcm2 (green) and DAPI (blue). When cultures were started at or beyond the 36 somite stage, changes in Foxn1 and Gcm2 domains were muted compared to cultures started at 35 somites and younger.

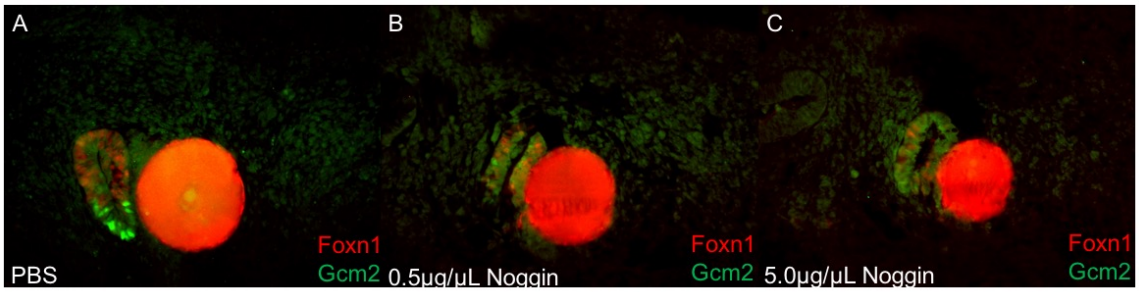

Figure 2-7: Noggin titration affects Foxn1 and Gcm2 domains proportionately. Embryos were implanted with beads containing either PBS (A) 0.5µg/µL (B) or 5.0µg/µL (C) recombinant noggin protein and stained for Foxn1 and Gcm2. Titrated noggin protein appears to affect Foxn1 and Gcm2 domains equally.

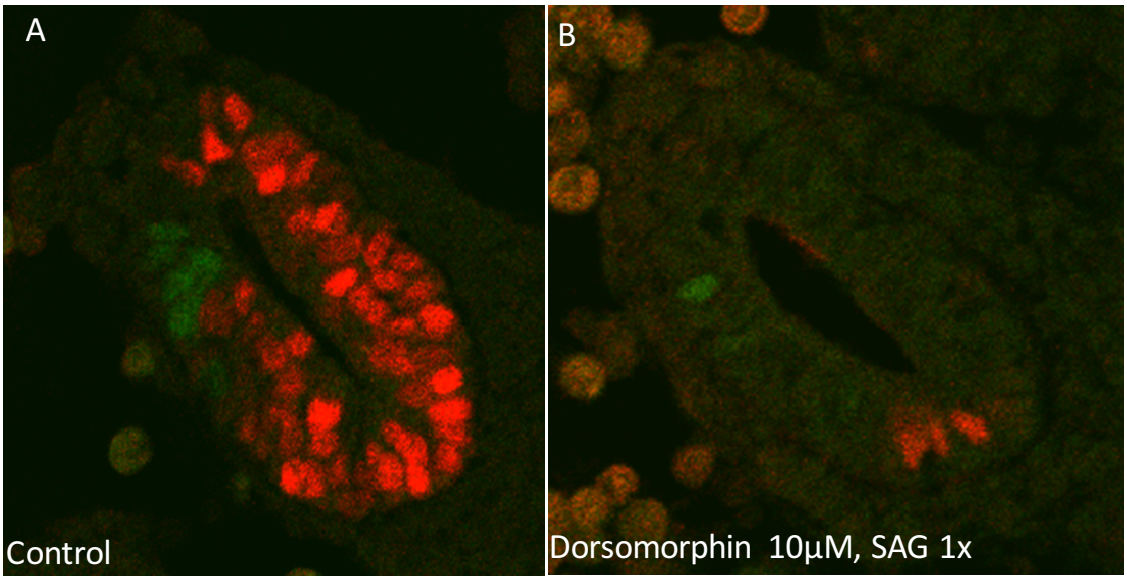

Figure 2-8: Activation of Sonic signaling is not sufficient to expand Gcm2 domain in BMP knockdown embryos. Embryos co-cultured with the BMP inhibitor dorsomorphin and the smoothened agonist (SAG) showed reduced Foxn1 (red) and Gcm2 (green) domains compared to the control (A) but no expansion of Gcm2 compared to embryos treated with dorsomorphin only.

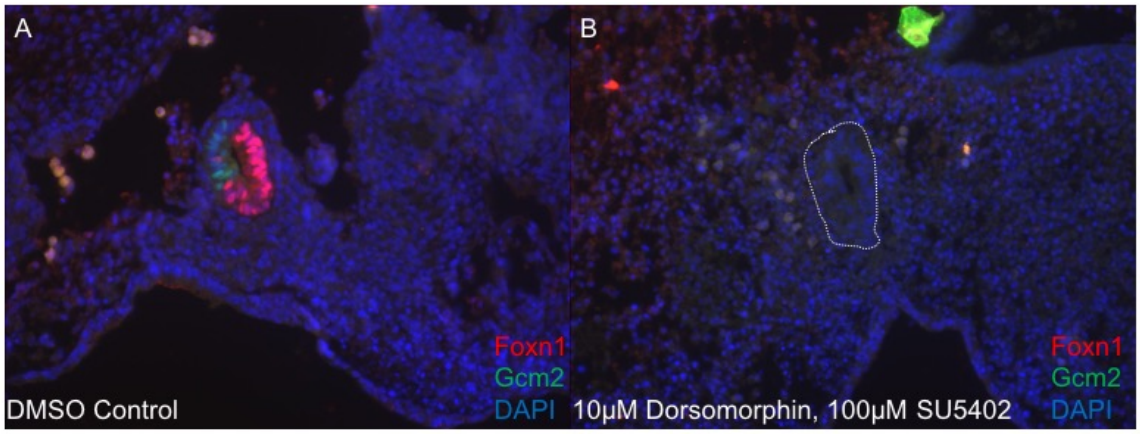

Figure 2-9: Combined BMP/FGF knockdowns express no Foxn1, Gcm2. Embryos were cultured with media containing DMSO (A) or 10μM dorsomorphin and 100μM SU5402 (B). Tissue was stained for Gcm2 (green), Foxn1 (red) and DAPI (blue). Double knockdown treatments have no Gcm2 or Foxn1 expressing cells within the third pharyngeal pouch.

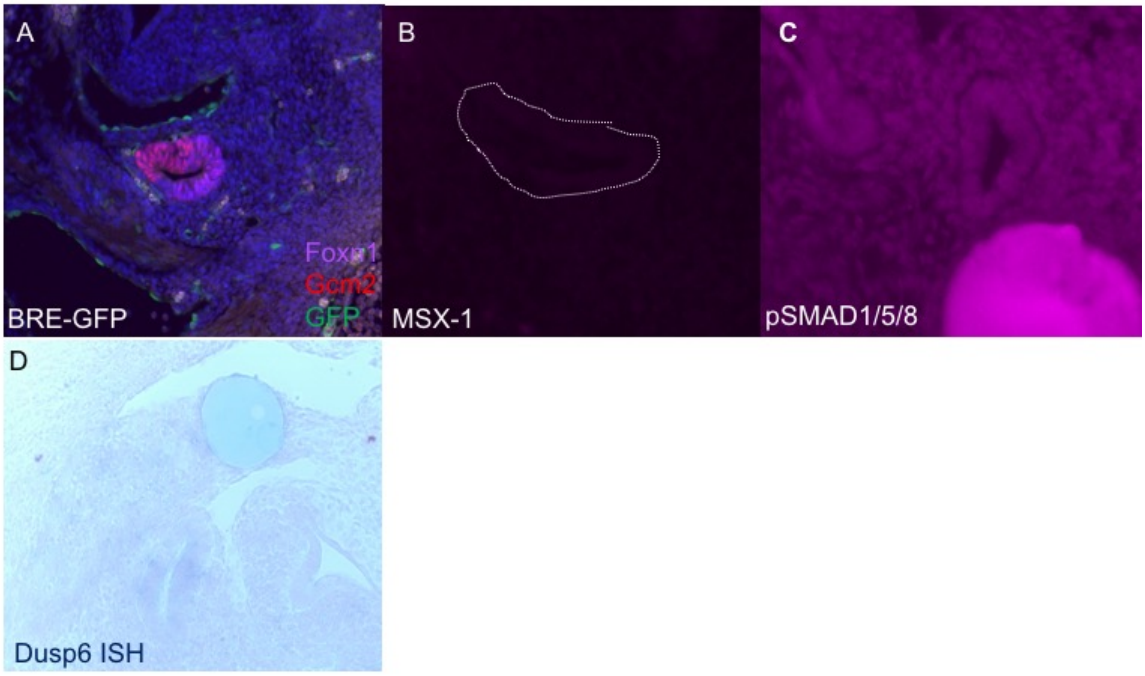

Figure 2-10: Reporters of BMP and FGF activity in the third pharyngeal pouch fail to confirm activity in control samples. (A) BMP response element (BRE) transgenic mouse driving GFP does not report in the pouch (green - GFP, red - Gcm2, magenta - Foxn1). (B) A downstream gene of BMP, Msx1, antibody fails to report in third pharyngeal pouch. (C) pSMAD1/5/8 fails to report in third pharyngal pouch. (D) In situ hybridization for Dusp6, a downstream gene of FGF signaling, only appears very lightly in 3pp at E11.5.

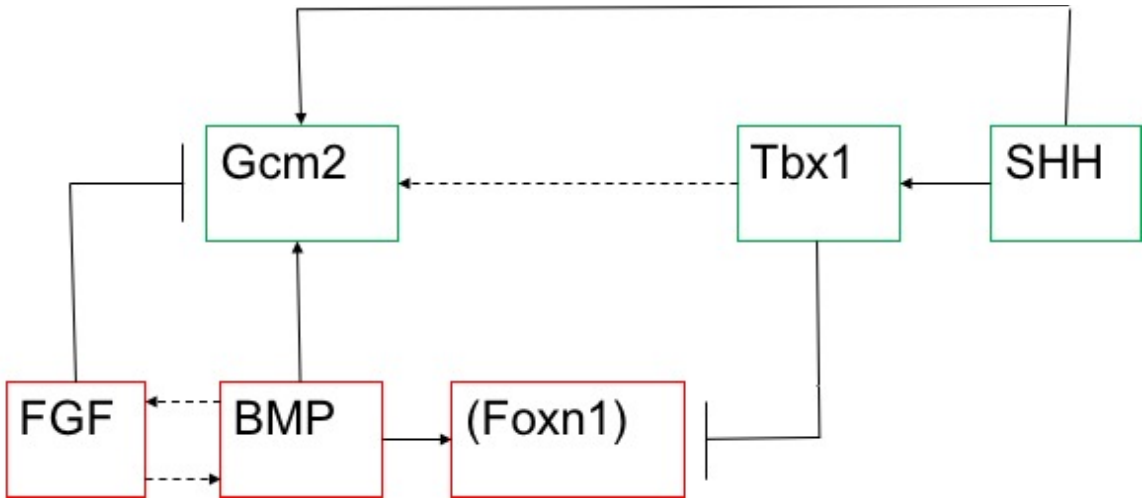

Figure 2-11: Our current model of thymus and parathyroid fate specification suggests that BMP is required in the specification of thymus and parathyroid domains while FGF8 acts to inhibit parathyroid while activating thymus fate.

CHAPTER 3

ECTOPIC SMOM2 EXPANDS TBX1 AND REDUCES FOXN1, BUT FAILS TO EXPAND GCM2 IN THE DEVELOPING THIRD PHARYNGEAL POUCH

This work was done as part of a larger project with Virginia Bain, a past graduate student of the Manley lab and a current post-doc in the Richie lab at UT-Austin. It is part of a publication elucidating the importance of SHH signals in third pharyngeal pouch development.

Bain, Virginia E., Gordon, Julie, O'Neil, John D., Ramos, Isaias, Richie, Ellen, Manley, Nancy R. Tissue-specific roles for Sonic hedgehog signaling in establishing thymus and parathyroid organ fate. 2016. Submitted to *Development*.

Introduction

Sonic Hedgehog (SHH) signaling has been shown to be required for parathyroid fate in the third pharyngeal pouch. *Shh*$^{-/-}$ mutants expand both *Foxn1* and *Bmp4* domains into the dorsal pouch, and completely lack expression of the parathyroid marker, *Gcm2*. These mutants ultimately lack parathyroids, and all cells of the pouch become thymus-fated. Because the loss-of-function model suggests that SHH is required for parathyroid fate, we hypothesized that a gain-of-function model may show that SHH is also sufficient to prevent thymus fate and expand parathyroid fate when expressed ectopically in the pharyngeal endoderm at early stages.

My role in these experiments was related to the ectopic expression of the R26^{SmoM2EGFP} allele within the pharyngeal endoderm using an endoderm-specific, tamoxifen-inducible Cre driver, *Foxa2*^{MCMCreERT2}. These experiments test whether SHH signals are sufficient to expand *Tbx1* and ablate *Foxn1* expression within the ventral pouch, as well as whether SHH signals are sufficient to expand *Gcm2* expression within the ventral third pouch primordium.

Methods

Mice

The R26^{SmoM2EGFP} mice were obtained from Jackson Labs (stock number 005130). Homozygotic R26$^{SmoM2/SmoM2}$ mice were bred to the Foxa2^{MCMCreERT2} line (Jackson Labs stock number 008464), a tamoxifen-inducible endodermal Cre. Mice were observed until a vaginal plug was present – this marked E0.5. At E6.0, pregnant females were injected with 0.75mg/g body weight of tamoxifen (Sigma) diluted in corn oil at a concentration of 1:10 to induce expression of the Cre within the endoderm. Mice were dissected at E11.5, somite staged, and genotyped according to Jackson Labs protocols.

Tissue Processing

Tissue was fixed in 4% PFA for 1 hour at 4C for antibody staining, and overnight for *in situ* hybridization. Embryos were dehydrated using an EtOH gradient, washed two times in xylenes, and embedded in paraffin wax. Sections were cut to 8µM and 12µM for antibody and *in situ* hybridization, respectively. After sectioned were dried on a slide warmer, excess wax was removed by washing two times for five minutes each in xylenes. Tissue was then rehydrated by passing through an ethanol gradient before antibody staining or *in situ* hybridization.

Antibody Staining

For antibody staining, antigen retrieval was carried out by boiling slides for 30 minutes in a 10mM sodium citrate buffer containing 0.05% Tween. Primary antibodies were diluted as follows Foxn1 – 1:200 (Santa Cruz Biotechnology), Gcm2 1:200 (Abcam) and Tbx1 1:200 (Abcam) and added to a 10% donkey serum/PBS-T solutions. Primary antibodies were incubated at 4C overnight and then washed two times in PBS. Anti-rabbit 488 (Alexafluor) and anti-goat 555 (Alexafluor) antibodies were bound to the Gcm2 and Foxn1 antibodies respectively by incubating for one hour at 1:400 concentrations in PBS containing 0.05% Tween. Excess antibody was washed from slides with two PBS washes and slides were washed in DAPI for 10 minutes before cover slipping cells with mounting media containing tris (Fluorogel).

In situ hybridization for Ptch1, Bmp4, and IL-7.

All *in situ* hybridization probe templates were generated using PCR from cDNA molecules. I would like to acknowledge Rodney Jarvis for providing the *IL-7* probe for these experiments. *In*

situ hybridization was performed using previously described methods (Manley and Capecchi, 1995). All probes were developed using BM Purple reagent (Rosche) and counterstained with nuclear fast red (Sigma).

Results

$R26^{SmoM2}$ is sufficient to activate ectopic SHH signaling in the third pharyngeal pouch

We assessed the effectiveness of the *Smoothened* allele in activating SHH signaling throughout the third pharyngeal pouch by performing *in situ* hybridization for *Ptch1*, a SHH receptor that is upregulated by the presence of SHH signals (Fig 1). In the Cre-positive embryos, *Ptch* was notably expanded relative to littermate Cre-negative controls, though *Ptc* expression does not expand entirely throughout the pouch in the mutant. While *Ptc*-negative cells generally appeared in the ventral pouch, their location was somewhat variable and did not appear to overlap entirely with Foxn1 expression patterns. This lack of expression may have been due to incomplete activation by $Foxa2^{CreER}$, which has been previously documented, or could be due to an endogenous inhibitor of SHH signaling in the ventral pouch.

Ectopic SHH signals in $R26^{SmoM2}$ mutants cause a loss of Foxn1 expression and an expansion of Tbx1 in the thymus domain, but no expansion of Gcm2

Ectopic SHH was sufficient to expand *Tbx1* into the ventral domain of the pouch at 48 somites (E11.5). It was also sufficient to reduce the overall number of *Foxn1*-expressing cells within the third pouch (Fig 2). However, ectopic SHH signals were not sufficient to expand *Gcm2* expression in the $R26^{SmoM2}$ mutants (Fig 3). This suggests that only a subset of pharyngeal pouch cells are able to become parathyroid cells despite an increase in SHH

signaling. These results indicate that SHH signaling is sufficient to drive *Tbx1* ectopically in the third pharyngeal pouch, but is not sufficient to expand *Gcm2*. As a result, a number of pharyngeal pouch epithelial cells express neither *Foxn1* nor *Gcm2*. We cannot definitively say that these double negative cells do not become parathyroid cells due to the lack of an early *Gcm2*-indpendent marker of parathyroid fate that is expressed at E11.5. However, the use of a new Cre-recombinase that does not require tamoxifen may allow us to determine if these double negative cells survive long enough to express parathyroid hormone.

Bmp4 signals are comparable in mutant and control pouch

I also performed *in situ* hybridization for *Bmp4* in the $R26^{SmoM2}$ mutants versus Cre-negative controls in order to determine if SHH signaling has an inhibitory effect on *Bmp4* expression within the third pharyngeal pouch. Our results showed that *Bmp4* expression patterns were not affected by ectopic SHH signals (Fig 4). This suggests that SHH-mediated *Tbx1* expression acts separately from *Bmp4* in suppressing thymus fate and does not regulate *Bmp4* at the level of expression. However, Tbx1 may still bind the downstream coactivator of BMP-mediated SMAD1/5/8, SMAD4, inhibiting BMP signals downstream of the ligand.

Foxn1, Gcm2 double negative cells do not specify as thymus in $R26^{SmoM2}$;$Foxa2^{CreER}$ mutants.

I performed *in situ* hybridization for a Foxn1-independent marker of thymus fate, *IL-7*, to determine whether Foxn1-negative, Gcm2-negative cells were not being specified as thymus. My data shows that these cells do not express *IL-7* (Fig 5). While it is not known whether these cells become fated as parathyroid, as parathyroids of *Gcm2* mutants die shortly after E11.5, so it is possible that any double-negative cells will undergo apoptosis shortly after the expression of

Foxn1. However, this is difficult to test using the *Foxa2*^{CreER} driver, as pregnant females have a high mortality rate more than 6 days post-injection with tamoxifen.

References

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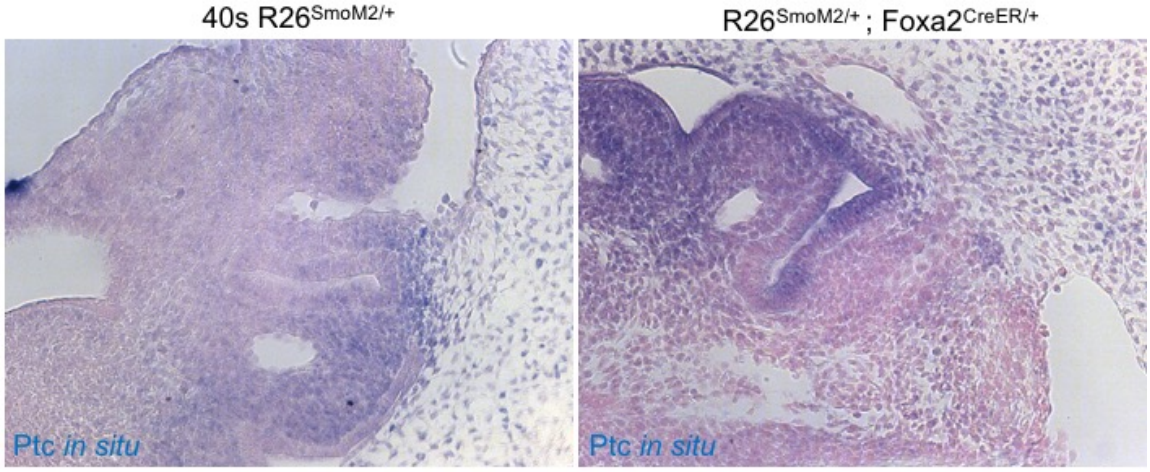

Figure 3-1: R26;SmoM2;Foxa2CreER efficiently drives ptc expression in the third pharyngeal pouch at E11.5. In situ hybridization for the downstream target of SHH signaling, Ptc shows an expansion of patched into the ventral domain of the third pharyngeal pouch.

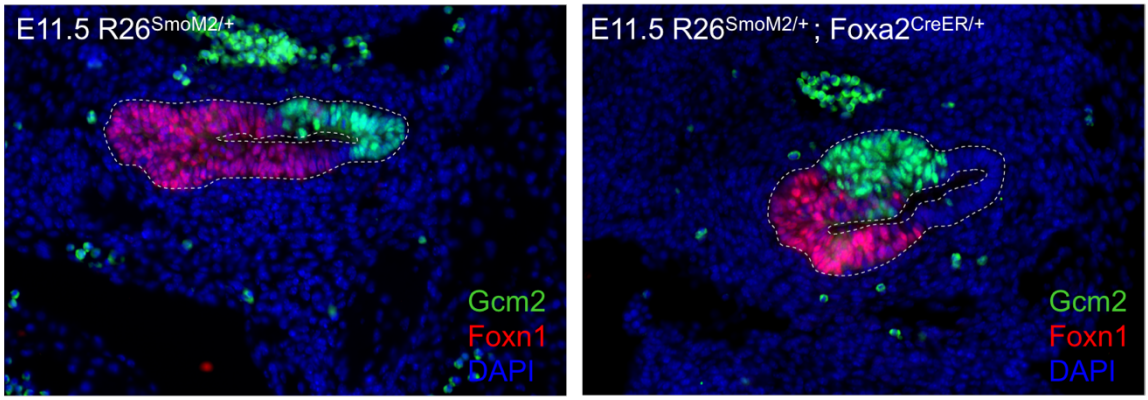

Figure 3-2: R26SmoM2:Foxa2CreER embryos show a reduction in total Foxn1 cells, but no expansion of Gcm2 expression. Sections stained for Foxn1 (red), Gcm2 (green) and DAPI (blue) show that cells which turn off Foxn1 in the mutant fail to express either Foxn1 or Gcm2 at E11.5.

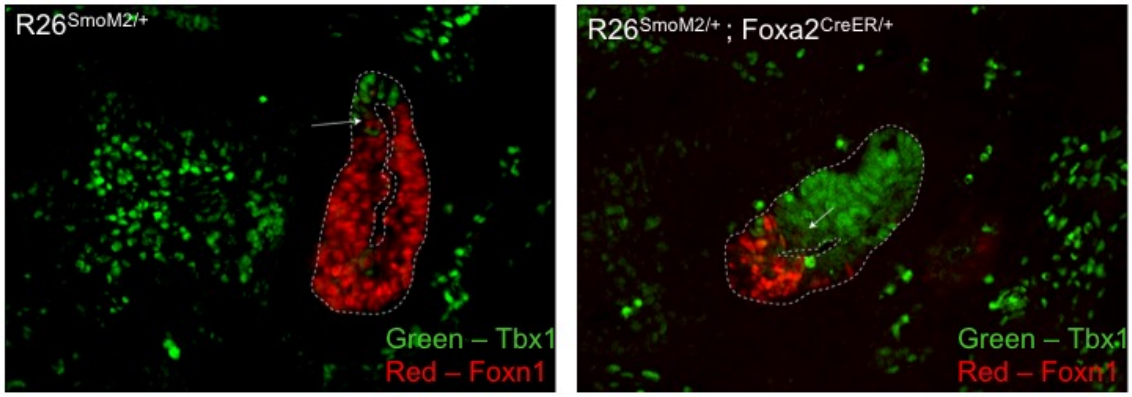

Figure 3-3: Ectopic SHH signaling expands Tbx1 into the ventral pouch. Tbx1 expression (green) appears throughout the dorsal pouch and expands into the ventral pouch in ectopic SHH mutants at E11.5. Arrows represent cells simultaneously expressing Tbx1 and Foxn1.

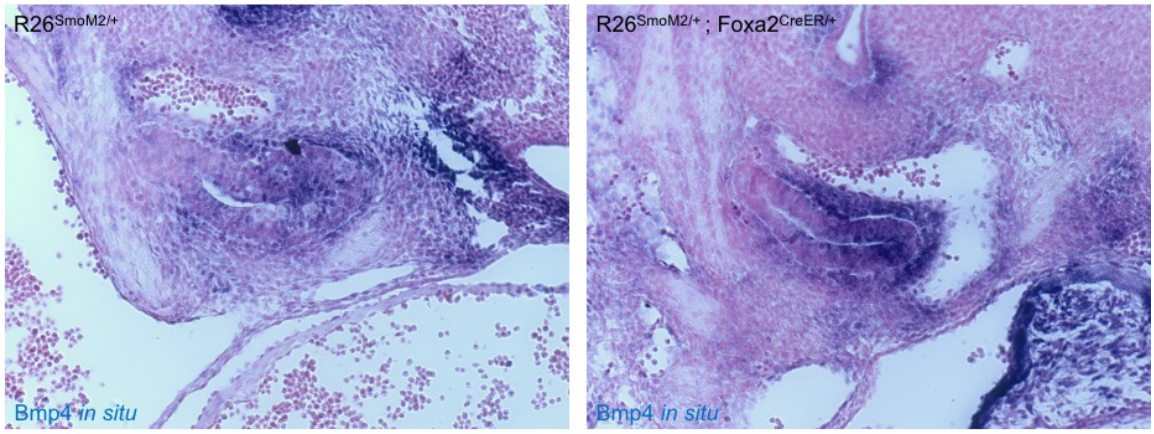

Figure 3-4: Bmp4 expression is similar in SmoM2 mutants and controls. In situ hybridization for Bmp4 shows similar expression patterns for Bmp4 between mutant and control embryos at E11.5

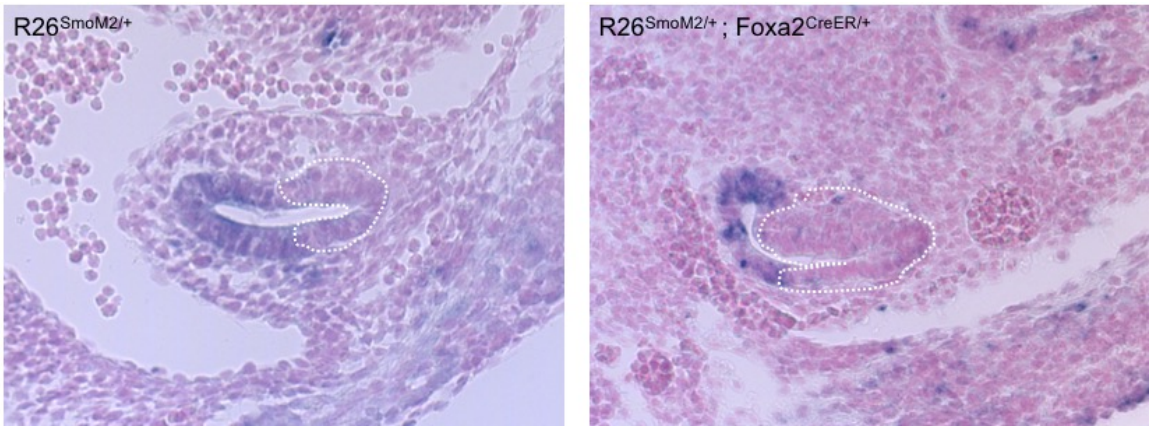

Figure 3-5: Reduction in IL-7 expression suggests that Foxn1/Gcm2 double negative cells are not becoming thymus fated. In situ hybridization for IL-7, a Foxn1-independent marker of thymus fate was performed on E11.5 mutant and control embryos. Results indicate that IL-7 is likely not expressed in most cells that do not express Foxn1 in SmoM2 mutants.

CHAPTER 4

CONCLUSIONS

These data give interesting new insights into murine thymus and parathyroid organogenesis that are a departure from current models of third pharyngeal pouch development. From the data that I have presented, it is clear that BMP signals are not just in a mutually antagonistic relationship with SHH signals, but are also required for *Gcm2* expression in the parathyroid domain. It also appears that parathyroid fate is very plastic. A number of alterations to paracrine signals are sufficient to ablate parathyroid fate, including ectopic BMP and FGF, as well as loss of BMP or SHH signaling. Parathyroid cell plasticity has also been shown in cervical thymi, small *Foxn1*-expressing organs which are derived from parathyroid cells that previously expressed *Gcm2*. While parathyroid cells can be shifted toward thymus fate, thymus fate has always been more robust, and only loss of BMP signaling via recombinant Noggin or dorsomorphin seems to fully ablate *Foxn1* expression in the developing third pharyngeal pouch. Furthermore, no study to date has identified cells that express *Foxn1* and then turn off *Foxn1* and inhibit *Gcm2*. Thymus fate is also capable of expanding throughout the pouch, signifying that all cells of the pouch are bipotent and become thymus or parathyroid, but no current research has succeeded in expanding parathyroid fate beyond its normal boundaries. Furthermore, *Gcm2*-expressing parathyroid cells have been shown to spontaneously express *Foxn1* and stop expressing *Gcm2*, suggesting that parathyroids remain plastic

beyond the initial events of third pharyngeal pouch fate specification occurring between E10.5 and E11.5.

The reason for this trend remains unclear, but we believe that it may be due to underlying epigenetic mechanisms that activate thymus-specific promoters and enhancers and inhibiting parathyroid promoters and enhancers in the thymus domain. While the cells within the thymus domain may contain more epigenetic modifications which reinforce thymus fate, the parathyroid domain is likely lacking in these marks. This may be due to an inherently higher rate of replication in the thymus versus parathyroid domain from stages as early as E10.5. Because the parathyroids are nearly quiescent at E10.5 and the cells of the thymus domain replicate much more quickly, it is possible that the DNA replication machinery of cells within the thymus domain is able to add more modifications to reinforce thymus fate than the histone and DNA modifiers in the parathyroid domain. Therefore, it is possible that by slowing the cell cycle of the thymus domain at a sufficiently early stage, some cells may become more receptive to signals that would cause fate shifts from thymus to parathyroid.

We had aimed to test the hypothesis that an abundance of epigenetic modifications within the thymus domain of the third pharyngeal pouch reinforces thymus fate and prevents fate changes using chromatin immuno precipitation followed by sequencing (ChIP-seq) technology. This would allow us to compare epigenomes of early stage thymus and parathyroid cells and would have given interesting insights into the genome-wide chromatin modifications between two characteristically different organs that develop within the same primordium. It may have also identified candidate transcription factors that may be involved in the specification of thymus and parathyroid

fate. However, the technology to build libraries from such small sample sizes has been on the edge of what is currently possible, making such experiments technically challenging during our several attempts at library building. Now, with new optimized methods and epigenome sequencing that is designed for use with small sample sizes, such as ATAC-seq and bisulfite-seq, we hope to be able to identify the mechanism by which cells of the thymus and parathyroid domains are specified.

APPENDIX A - A new method for the isolation of early stage third pharyngeal pouch cells

This work was done with assistance from Julie Gordon, a senior scientist in the Manley lab. We also had support from David Katz at Emory University for the development of our ChIP technique, validation, and library prep for ChIP-seq.

Introduction

One major hurdle in determining the mechanisms of fate specification in the developing third pharyngeal pouch is the lack of tools for the isolation of third pharyngeal pouch cells. Currently, no reporter is available that allows researchers to quickly and cleanly isolate cells. This prevents researchers from identifying novel candidates using gene expression analyses such as RNA-seq. Here, we develop a method using the Gcm2-EGFP transgenic mouse line to sort cells of the thymus and parathyroid domain of the pouch separately for analysis. The Gcm2-EGFP transgene is unique in its ability to separate out thymus and parathyroid cells at E10.5 because transgenic EGFP is originally expressed throughout all or nearly all of the developing pouch, but is only actively expressed in the parathyroid domain at E10.5. This allows us to separate pouch tissue based on high (actively expressed) GFP, low (residual) GFP, and negative GFP domains (Fig1). These domains roughly correlate to the parathyroid domain, thymus domain, and a middle domain, which appears to be the last to make fate decisions during

development and is more likely to exhibit plasticity after cells at the poles of the pouch become specified as thymus or parathyroid. The gene expression patterns of the Gcm2-EGFP transgene were confirmed by comparing Gcm2 protein expression against EGFP and *Gcm2* RNA (Fig 1). Once third pharyngeal pouch cells are separated from the surrounding mesenchyme and separated by domain, we are able to make comparisons based on gene expression or chromatin state which should help us better understand how these cells become specified as thymus and parathyroid during organogenesis.

Methods:

Mice

Gcm2-EGFP mice were received from the GENSAT program at Rockefeller University and maintained as heterozygotes on a C57BL6 background. Gcm2-EGFP mice were crossed to C57BL6 to generate embryos. Females were observed for the presence of a vaginal plug. The date of plug observation was considered E0.5. Females were sacrificed by cervical dislocation at E10.5.

Tissue Preparation

E10.5 embryos were dissected into sterile PBS containing 10% FBS. Third pharyngeal pouches were dissected out from the pharyngeal block, being careful to remove ectodermal or fourth pouch epithelia from the tissue. Third pharyngeal pouches were then spun at 4000rpm, removed from the PBS/FBS solution and incubated for 7-10 minutes at 37C in 0.7mg/mL collagenase (Sigma), 1.3mg/mL hyaluronidase (Sigma) enzyme solution in PBS, pipetting to mix every 60 seconds. Once pharyngeal pouches

were dissociated, cells were spun at 4C to pellet and the enzyme solution was decanted from the pellet. Dissociated cells were stained for EpCAM-APC (Biolegend) for 20 minutes on ice to isolate GFP negative epithelial cells from mesenchymal cells during sorting. Cells were then spun at 4000rpm at 4C to pellet and the supernatant was decanted off. Cells were resuspended in 150µL of PBS containing 10% FBS prior to sorting.

Cell Sorting

Cells were sorted on a MoFlo-XDP sorter and collected into three EpCAM+ groups by EGFP intensity. These groups included EGFP-negative cells, EGFP-low cells, and EGFP-high cells. EGFP sorting gates were designed to collect similar numbers of EGFP low and high cells from each sorting experiment and kept constant between experiments. EpCAM-negative cells were also collected, and used for validation controls during ChIP library preparation. Cells for RNA experiments were sorted into Ambion Cells-to-CT direct lysis buffer and lysed according to manufacturers' guidelines.

For the analysis of histone modifications, cells were sorted into PBS for subsequent ChIP-seq and frozen at -80C. Sorted cells were pooled for ChIP-seq until we reached cell populations of at least 20,000 for each population of third pouch cells. For RNA, cells were sorted into 50µL of Ambion cells-to-CT direct lysis reagent, spun at high speed for 60 seconds, mixed by pipette and then incubated at room temperature for 5 minutes before adding the Ambion stop solution to the lysis reagent.

ChIP-seq, and RNA-seq

ChIP-seq was performed with help from the Katz lab at Emory University. Sorted cells were processed for ChIP using previously published methods. Chromatin was broken into 500bp fragments using a Bioruptor (Diagenode) and then incubated at 4C overnight with antibodies to H3K4Me3, a histone marker. H3K4Me3 fragments were bound to beads and pulled down using centrifugation. Lastly, protein was separated from DNA and removed from samples by washing with SDS solutions and then extracting DNA using a phenol chloroform extraction. Input and H3K4Me3 DNA fragments were later used by the Katz and Corces labs to prepare sequencing libraries and validate known thymus and parathyroid-specific genes in separate cell populations.

Preparation of an RNA-sequencing library was performed by The Hudson Alpha Genomic Research Laboratory using the NuGen low input library kit. However, library preparation failed in our samples. We validated expression thymus and parathyroid specific genes using Taqman qPCR probes.

Results:

ChIP-seq

While genomic H3K4Me3 ChIP libraries failed to amplify properly, targeted ChIP using qPCR was successful in validating the presence of H3K4Me3 on known expressed genes (Fig 2). While ChIP libraries failed to amplify, targeted qPCR using known thymus and parathyroid specific transcripts was successful using as few as 1000 sorted cells (Fig 2) to validate that the sorting methods can separate third pharyngeal

pouch cells into discreet populations. We believe that this technique is promising, however the current state of the art in library preparation is not sufficient to amplify such small amounts of DNA for squencing. Other methods, such as ATAC-seq or bisulfite seq have been optimized for small samples of and may fare better with such small numbers of cells, despite measuring different aspects of gene regulation across the genome.

RNA-seq

RNA-seq was not possible due to issues with library building. However, we were able to validate the presence of thymus and parathyroid specific genes in separate cell populations using qPCR for known thymus and parathyroid domain-specific genes small samples of roughly 100 cells total (Fig 3). We aim to attempt RNA-seq again and perform the library preparation at the University of Georgia.

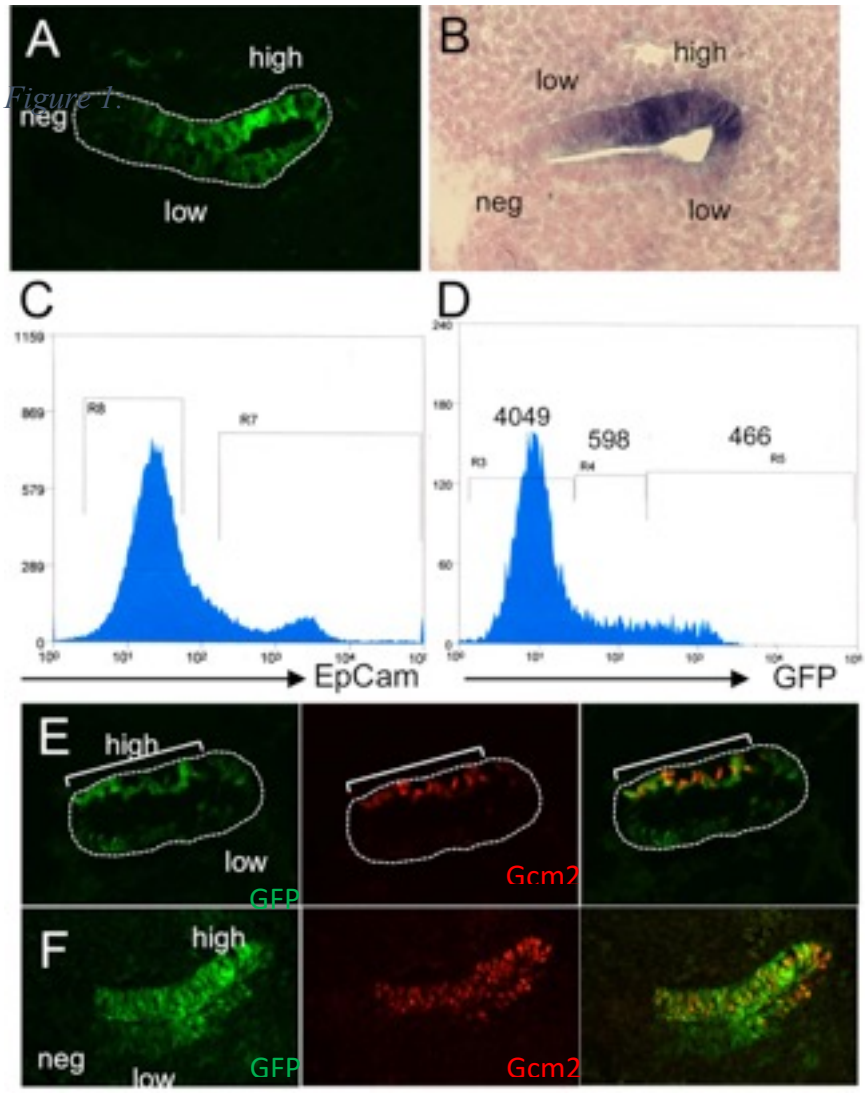

Figure A-1: Gcm2-EGFP transgenic mouse allows for the sorting of third pharyngeal pouch cells into discrete populations at E10.5. (A) GFP fluorescence of Gcm2-EGFP in an E10.5 pharyngeal pouch under a dissecting microscope shows three separate "bins" of fluorescence – high, low, and negative (B) Gcm2 RNA expression in an E10.5 pharyngal pouch shows zones of expression similar to GFP. (C/D) using EpCam and GFP, we are able to sort epithalial cells into discrete populations, with GFP low cells exhibiting 1/10 the fluorescence as GFP high cells. (E/F) Gcm2 protein is detectable only where GFP protein appears brightest, suggesting that bright GFP cells actively express Gcm2.

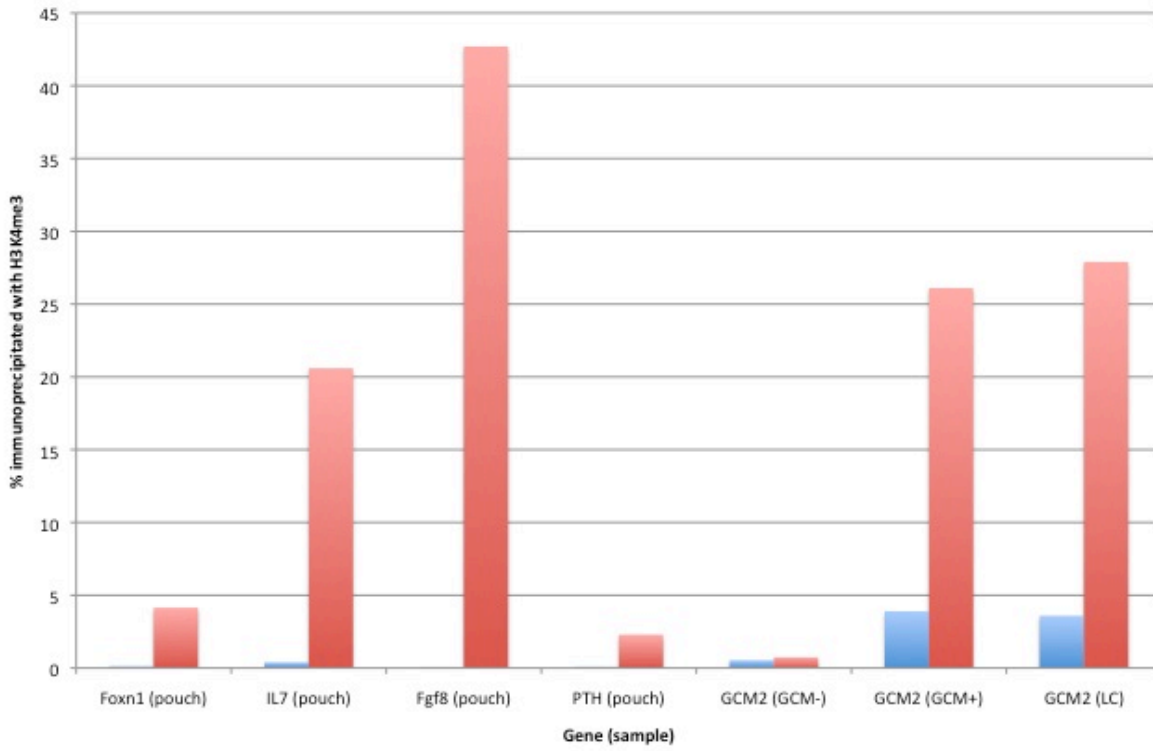

Figure A-2: qPCR validation for H3K4Me3 in pouch-specific genes amplifies genes in an expected manner. Red bars represent ChIP sample – blue bars represent input controls. From left to right: E10.5 whole dissected pharyngeal pouches were used to validate ChIP. Pouches were enriched for the known expressed pouch genes Foxn1, IL-7, Fgf8, and PTH. As expected, GFP negative cells were not enriched for Gcm2 over input controls, but 1000 GFP positive sorted cells were enriched over the input control. Lastly, laser capture microdissection of frozen third pharyngeal pouch showed enrichment of H3K4Me3 reads over input controls.

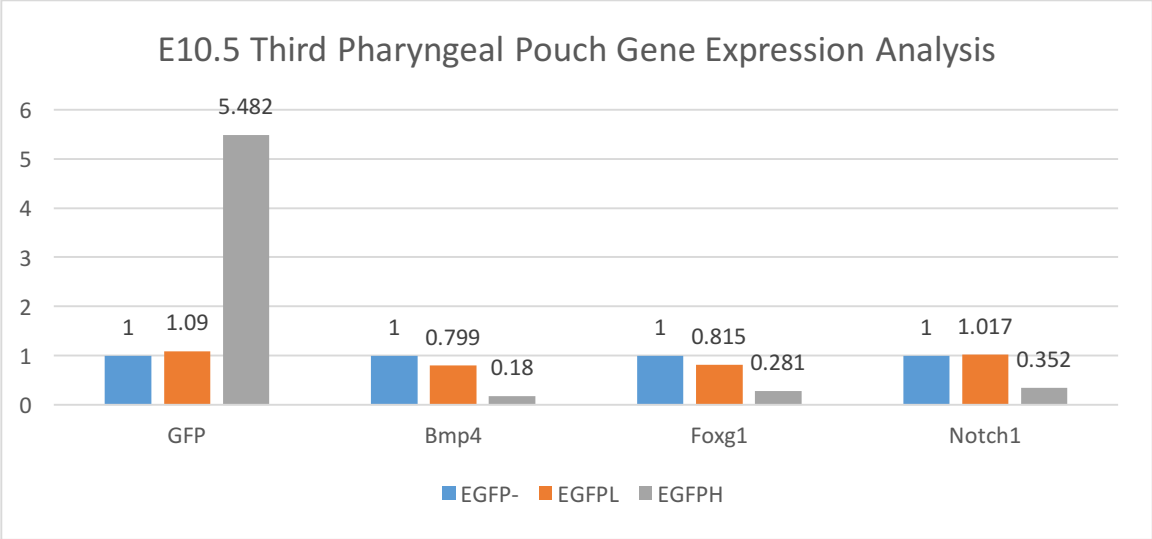

Figure A-3: qPCR validation of sorting methods confirms that EGFP low, medium, and high cells are transcriptionally different at E10.5. Known genes associated with the thymus domain at E10.5 (Bmp4, Foxg1, Notch) are most highly expressed in EGFP negative cells, but are not expressed in the EGFP high cells. EGFP low cells also appear to express significantly less Gcm2 transcript than EGFP high cells, confirming that most GFP fluorescence in this population is residual protein.

APPENDIX B - Foxg1;R26^{iGremlin} mutants have normal Foxn1/Gcm2 pouch patterning

This work was done with assistance from Trent Frisbee, a post-baccalaureate student participating in UGA's PREP program. A special acknowledgement to Steven Vokes at the University of Texas at Austin for generating and providing the R26^{iGremlinEGFP} allele.

Introduction

In light of our results suggesting that Bmp4 may be required for both thymus and parathyroid fates in the pre-specification third pharyngeal pouch, we attempted to use a new mouse model, the R26^{iGremlin} mouse to determine the *in vivo* effect of inhibiting Bmp4 within the developing third pharyngeal pouch. This mouse expresses Gremlin, an endogenous Bmp inhibitor that is similar to Noggin, under the R26 promoter, allowing us to manipulate Bmp signals in the third pharyngeal pouch during development. We crossed the R26iGremlin mouse line to Foxg1Cre, an endodermal/mesenchymal Cre that has confirmed expression patterns in the third pouch and surrounding pharyngeal arch mesenchyme. We chose this Cre driver because we believed that it was the most likely to generate a phenotype due to expression in multiple tissue types surrounding the third pouch primordium.

Here, we show that *Foxn1* and *Gcm2* expression patterns are similar between R26^{IGremlin} mutants and Cre-negative littermate controls. While fate patterning appears to

be unaffected, we cannot say if this is due to an inhibition of Bmp signaling in mutants, as attempts to show the knockdown efficiency at E10.5 and E11.5 have been unsuccessful.

Methods

Mice

Foxg1Cre mice were acquired from Jackson Labs (Stock number 00437) and crossed to the $R26^{iGremlin}$ allele, which was provided to us by the Vokes lab at University of Texas Austin. Females were observed for the presence of a vaginal plug. The day on which a plug was observed was considered E0.5. Females were sacrificed at E11.5 using cervical dislocation and embryos were dissected.

Tissue preparation

Embryos were dissected into PBS, and yolk sacks were kept for genotyping purposes. After dissection, embryos were fixed in 4% PFA for one hour at 4C. Embryos were dehydrated in an Ethanol gradient, washed two times for five minutes each in xylenes and embedded in paraffin wax. Tissue was sectioned to 8μM thickness using a microtome. Sections were then dried on a slide warmer and dewaxed by washing two times for five minutes in xylenes. Sections were rehydrated by passing through an EtOH gradient. Antigen retrieval was carried out before incubating slides with antibodies by boiling slides for 30 minutes in 10mM sodium citrate buffer containing 0.05% tween.

Antibody staining

Slides were incubated overnight at 4C with primary antibodies at concentrations of 1:200 for Foxn1 (Santa Cruz Biotechnology) and 1:200 for Gcm2 (Abcam) in a 10% donkey serum solution in PBS-T. Primaries were washed off with three five minute washes in PBS, followed by incubation anti-rabbit 488 (Alexafluor) and anti-goat 555 (Alexafluor) antibodies at 1:400 dilutions in PBS-T for one hour at room temperature. Excess secondary antibody was rinsed off with PBS before adding DAPI to stain nuclei. Slides were cover slipped using Fluorogel mounting media with tris before imaging.

Results

At the 48 somite stage (app E11.5), embryos appear to have normal fate boundaries between Foxn1 and Gcm2. This is confirmed by volumetric analysis of the pouch epithelium (Fig 1). Here Foxn1 and Gcm2 domain volumes are very similar between mutant and Cre-negative control embryos. While this is the opposite of the expected result, one major challenge in dealing with $R26^{IGremlin}$ is a lack of tools by which to determine the efficacy of the knockdown.

It is possible that Gremlin, driven from the R26 promoter is not sufficient to adequately knock down Bmp signaling within the pouch, whereas flooding the system with noggin or dorsomorphin is sufficient to knock down Bmp sufficiently to cause fate changes in the developing third pharyngeal pouch.

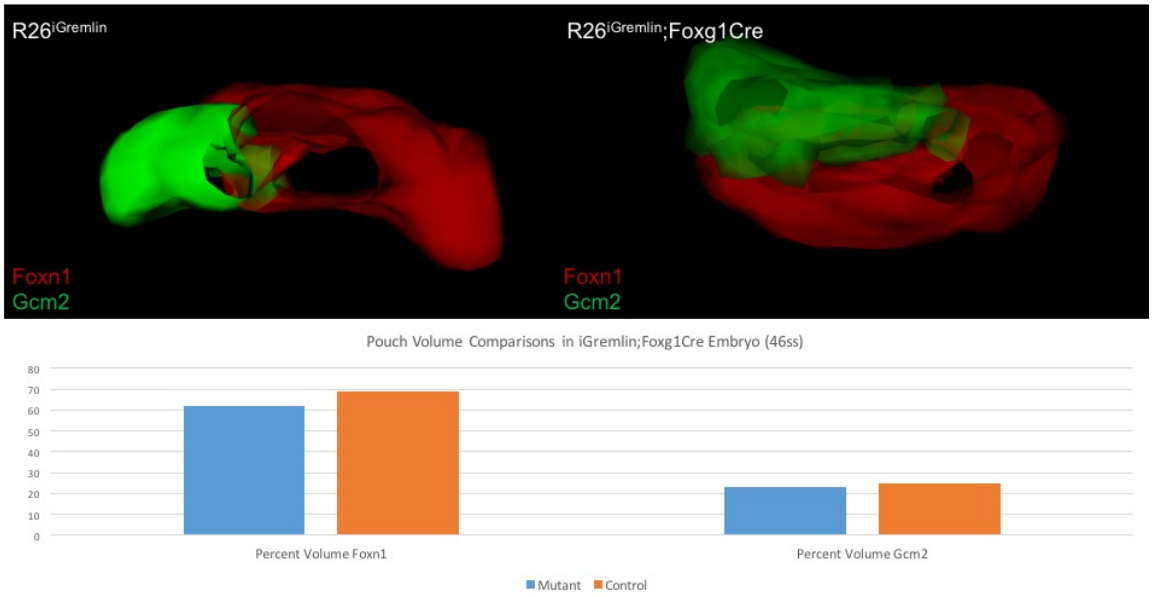

Figure B-1: Volumetric analyses of R26iGremlin;Foxg1Cre mutants confirms that patterning is similar between mutant and control embryos at E11.5.

Appendix C - PthCre; R26$^{imiR17-92}$ mutants express Gcm2 in parathyroids

This work was done with assistance from Barbara Del Castello, a post-baccalaureate student studying in the Manley lab who is now a PhD graduate student in the Integrated Life Sciences program at the University of Georgia. And in collaboration with the Richie lab (MD Anderson Cancer Center, Science Park, TX).

Introduction

Recent results indicate that miR17-92 may be sufficient to inhibit *Tbx1* and *Gcm2* when expressed ectopically in the parathyroid domain of the third pharyngeal pouch using an endodermal Cre and the inducible R26$^{imiR17-92}$ allele. Because we know how plastic parathyroid fate is, we wanted to determine if this microRNA was sufficient to inhibit *Gcm2* expression and possibly promote Foxn1 in differentiated parathyroid cells that express parathyroid hormone (PTH). We hypothesized that cells of the parathyroid may be able to change fates via expression using this microRNA, a known *Tbx1*/*Gcm2* inhibitor. Here, we use a PTH-Cre to activate the micro RNA in differentiated parathyroid cells.

Methods

Mice

$R26^{imiR17\text{-}92}$ mice were acquired from Jackson Labs (Stock number 008517) and crossed to PTH^{Cre} (Stock number 005989). Females were observed for the presence of a vaginal plug. The day on which a plug was observed was considered E0.5. Females were sacrificed at E11.5 using cervical dislocation and embryos were dissected.

Tissue preparation

Embryos were dissected into PBS, and yolk sacks were kept for genotyping purposes. After dissection, embryos were fixed in 4% PFA for one hour at 4C. Embryos were dehydrated in an Ethanol gradient, washed two times for five minutes each in xylenes and embedded in paraffin wax. Tissue was sectioned to 8μM thickness using a microtome. Sections were then dried on a slide warmer and dewaxed by washing two times for five minutes in xylenes. Sections were rehydrated by passing through an EtOH gradient. Antigen retrieval was carried out before incubating slides with antibodies by boiling slides for 30 minutes in 10mM sodium citrate buffer containing 0.05% tween.

Antibody staining

Slides were incubated overnight at 4C with primary antibodies at concentrations of 1:200 for Foxn1 (Santa Cruz Biotechnology) and 1:200 for Gcm2 (Abcam) in a 10% donkey serum solution in PBS-T. Primaries were washed off with three five minute washes in PBS, followed by incubation anti-rabbit 488 (Alexafluor) and anti-goat 555 (Alexafluor) antibodies at 1:400 dilutions in PBS-T for one hour at room temperature.

Excess secondary antibody was rinsed off with PBS before adding DAPI to stain nuclei. Slides were cover slipped using Fluorogel mounting media with tris before imaging.

Results

Preliminary results indicate that Gcm2 is still expressed in parathyroids of E13.5-newborn mice in mutants (Fig 1). MiR17-92 appears to not be sufficient to activate Foxn1 or inhibit Gcm2 expression. This indicates that PTH-expressing parathyroid cells may be terminally differentiated and are receptive to cues such as miR17-92 which may change cell fate at earlier stages.

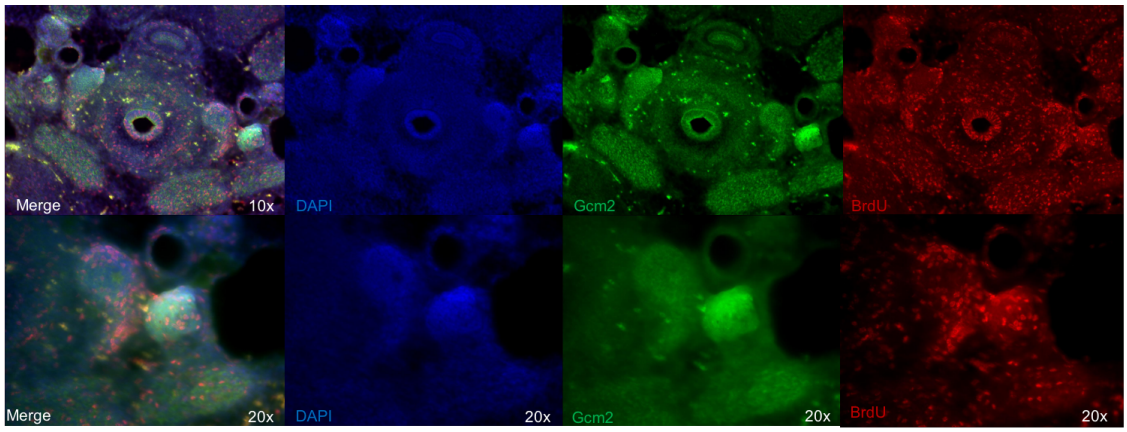

Figure C-1: Gcm2 expression is present in R26miR17-92 parathyroids at E13.5. Embryos were pulsed with BrdU for one hour before dissection and stained for DAPI, Gcm2, and BrdU.

Appendix D - Foxa2^{MCMCreERT2}; VegF$^{fx/fx}$ mutants have normal vascularization in postnatal thymus

This data was generated as part of a larger project assisting Jerrod Bryson, a former graduate student in the Manley Lab.

Introduction

VegF has been implicated in vascular endothelial growth in a number of vascularized tissues. However, knockouts of VegF in thymus show no phenotype. We knock out VegF in all thymic epithelial cells (TECs) using Foxa2^{MCMCreERT2}, an endodermal tamoxifen-inducible Cre crossed to the VegF$^{fx/fx}$ allele. After injection at E5.5, embryos were taken at E13.5 and stained for the vascular marker CD41.

Methods

Mice

VegF$^{fx/fx}$ mice were crossed to Foxa2^{CreER} (Jackson labs stock number 008464). Females were observed for the presence of a vaginal plug. The day on which a plug was observed was considered E0.5. Females were injected with tamoxifen at a dose of 0.75mg/g body weight. Females were sacrificed at E13.5 using cervical dislocation and embryos were dissected into PBS. Tail tissue was used for genotyping.

Tissue Processing

E13.5 embryos were flash frozen in liquid nitrogen and stored at -80C before sectioning. Sections were cut on a cryostat at 10μM thickness. Sectioned tissue was fixed in acetone and rinsed in PBS before staining.

Antibody Staining

Embryos were stained for the vasculature marker CD44 at a 1:200 dilution by incubating with antibody overnight at 4C in a 10% donkey serum/PBS solution. Excess antibodies were washed off with three five minute PBS washes before staining with secondary antibodies at 1:200 dilution. Slides were coverslipped using Fluorogel mounting media before imaging.

Results

Our results indicate that VegF in TECs is not required for thymus vascularization, as mutants express CD44 and appear to have normal vascular endothelial tissue (Fig 1). However, we are not able to confirm this deletion using q-PCR or PCR based methods.

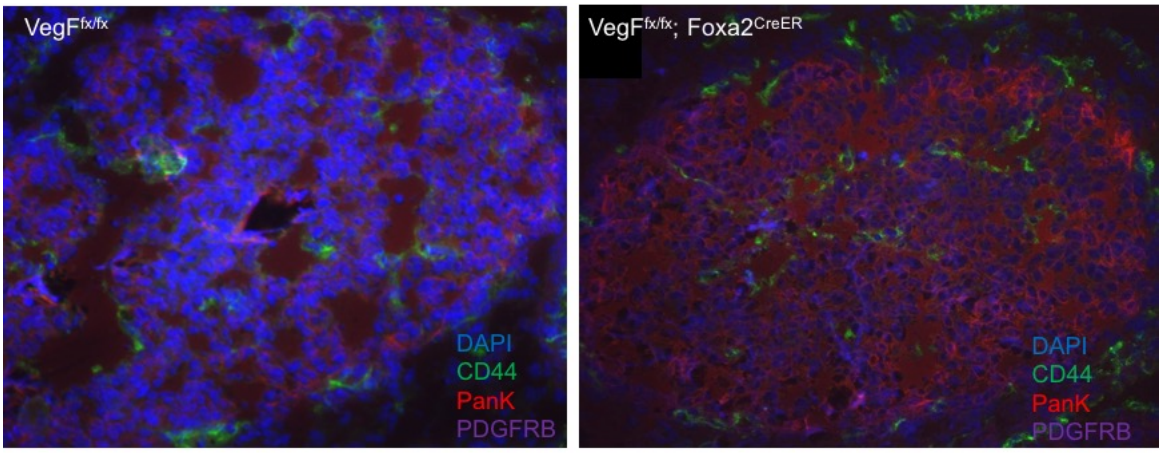

Figure D-1: VegF$^{fx/fx}$;Foxa2^{CreER} mutants have normal vasculature compared to Cre-negative controls. CD44, a vasculature marker is similar between mutant and control embryos at E13.5.