GENETIC ANALYSIS OF THE ROLE OF GCM2 GENE IN MOUSE EMBRYONIC DEVELOPMENT

by

ZHIJIE LIU

(Under the Direction of Nancy R. Manley)

ABSTRACT

The molecular mechanism of parathyroid organogenesis is poorly understood. The mouse *Gcm2* gene was found to be expressed predominantly at the anterior/dorsal side of the 3rd pouch endoderm and subsequently in the parathyroid domain of the parathyroid/thymus common primordium. The *Gcm2* null mutation causes loss of parathyroids in mouse, indicating that *Gcm2* is an important regulator gene for parathyroid organogenesis. Based on this observation, *Gcm2* has been thought to be a cell fate determinant, like its orthologous *Drosophila Gcm* genes, to specify the parathyroid domain in the 3rd pouch. Besides its function in parathyroid organogenesis, low level expression of *Gcm2* has been detected in the mouse brain and otic region, although its function here is unknown. In my doctoral study, I genetically dissected how *Gcm2* functions in parathyroid organogenesis and whether *Gcm2* has a role in the development of other organs.

My loss-of-function studies in the *Gcm2*$^{-/-}$ mutants showed that the specification of the parathyroid/thymus was normal, but that the parathyroid domain did not differentiate properly and subsequently underwent apoptosis. This suggests that *Gcm2* is required for the

differentiation and subsequent survival of parathyroid precursor cells, but not for the specification of the parathyroid domain.

It has been proposed that parathyroid hormone (PTH) expression in the thymus is a backup mechanism for the parathyroids in *Gcm2*$^{-/-}$ mutants. Interestingly, we identified two cellular sources for thymic PTH: misplaced parathyroid cells and medullary thymic epithelial cells (mTECs). PTH expression in the former source is regulated by *Gcm2*, whereas mTECs express PTH in a *Gcm2*-independent way. Our further studies showed that mTEC-derived PTH did not contribute to serum PTH, suggesting that the lethality of *Gcm2*$^{-/-}$ mutants may not be related to the reduction of serum PTH levels but is caused by non-parathyroid defects.

To further study Gcm2 function, we ectopically expressed *Gcm2* in other tissues. Mice with ubiquitous *Gcm2* expression displayed multiple abnormal phenotypes, including defective eyelid and inner ear structures, and neonatal lethality. *Gcm2* has been shown to be expressed in the brain and otic region. Thus our data suggest that *Gcm2* may have a role in the development of other organs.

INDEX WORDS: parathyroid organogenesis, *Gcm2*, 3rd pouch endoderm, parathyroid/thymus common primordium, cell fate determinant, loss-of-function, specification, differentiation, apoptosis, parathyroid hormone (PTH), thymic PTH, misplaced parathyroid cells, ectopically express.

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DEDICATION

I would like to dedicate my dissertation to my wife Lizhen Chen, who is also a doctoral student studying in the same lab. Without her endless love and support, I can not believe I can make the achievement in my doctoral study. She is not only a good wife taking care of my living, but also an invaluable scientific partner to work with. She gave me numberless helpful advice, technique support and even the inspiration for new scientific ideas.

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CHAPTER 1

GENERAL INTRODUCTION

I. Mouse *Gcm2* gene and Gcm transcription factor family

1. Gcm transcription factor family

Glial Cells Missing (Gcm) family is a novel transcription factor family. All *Gcm* genes in this family contain a DNA-binding domain called the Gcm domain. The Gcm domain, which consists of about 150 amino acids at the N-terminal, is highly conserved from fly to mouse and human (Akiyama et al., 1996; Cohen et al., 2003). Additionally, this Zn-containing DNA binding domain can specifically bind the conserved sequence 5'-ATGCGGGT-3'. However, the C-terminal domain of Gcm proteins, which functions as transcription-activator domain, is poorly conserved (Wegner and Riethmacher, 2001).

The first two *Gcm* genes were identified in *Drosophila*, and their expression has been demonstrated in the central nervous system, visual nervous system, and the blood cells. In the central nervous system, these two *Gcm* genes function redundantly as the binary switch genes between glial cells and neurons (Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001; Vincent et al., 1996). The loss of function of *Drosophila Gcm* genes can cause all presumptive glial cells to differentiate into neurons. Conversely, ectopic expression of *Drosophila Gcm* genes promotes the transformation of presumptive neurons to a glial cell fate. Moreover, *Drosophila Gcm* genes also function as cell fate determinants between macrophages/plasmatocytes and crystal cells in blood cell development (Alfonso and Jones,

2002; Lebestky et al., 2000). The mutation of either *Drosophila Gcm* gene results in a decrease in macrophage/plasmatocyte number; however, misexpression of *Drosophila Gcm* genes can cause crystal cells to be transformed into macrophages/plasmatocytes. Recently, *Drosophila Gcm1* and *Gcm2* genes were found to be expressed in lamina precursor cells in the larval visual system. Besides their function in the generation of a subset of glial cells, in their absence, lamina neurons are not produced, indicating that these two genes are also required to induce a neuronal cell fate from the lamina precursor cells (Chotard et al., 2005; Yoshida et al., 2005). Thus, *Drosophila Gcm* genes have diverse functions during *Drosophila* embryonic development and function as cell fate determinants of both glial cells and macrophages/plasmatocytes.

Gcm genes also have been identified in a number of vertebrate species with diverged expression and function. In zebrafish, *Gcm2* is expressed in the ectodermal portion of pharyngeal region and regulates the formation of gills and cartilage (Hanaoka et al., 2004; Hogan et al., 2004). Zebrafish *Gcm2* is also expressed in the macrophage cell lineage, but its function there is unknown (Hanaoka et al., 2004). Interestingly, the zebrafish gill has been proposed as the evolutionarily related structure of the parathyroid glands in chickens and mammals, where *Gcm2* is also expressed in the pharyngeal region (Okabe and Graham, 2004).

In chicken, there are two *Gcm* genes, and their expression has been detected in the central nervous system and several other tissues. Chicken *Gcm1* is expressed in the developing spinal cord and regulates the differentiation of neurons (Soustelle et al., 2007). In addition to its expression in the central nervous system, chicken *Gcm1* is also expressed in extraembryonic tissues (Hashemolhosseini et al., 2004). Chicken *Gcm2* was found to be expressed in the hindbrain, but its function in this region has not been reported (Soustelle et al., 2007). Chicken *Gcm2* expression has been detected in the 3rd and 4th pharyngeal pouch endoderm at early

embryonic stages. As development proceeds, *Gcm2* is restricted to the parathyroid glands overlapping with parathyroid hormone (PTH) and CasR gene expressions (Okabe and Graham, 2004).

Two mammalian *Gcm* genes (*Gcm1* and *Gcm2*) have been identified in mouse, rat and human, both of which were found to be expressed predominantly in non-neural tissues with low expression levels in the central neural system (Akiyama et al., 1996; Altshuller et al., 1996; Kammerer et al., 1999; Kanemura et al., 1999; Kim et al., 1998). In mouse, *Gcm1* was found to be strongly expressed in the labyrinth layer and to function as a critical regulator for placental development (Schreiber et al., 2000). In mouse, *Gcm1* null mutation results in the failure of placenta development due to loss of the labyrinth layer, which is necessary for nutrient and gas exchange (Schreiber et al., 2000). This suggests a conserved extraembryonic function of the *Gcm1* gene in both bird and mammals.

2. The expression and function of mouse *Gcm2* gene

In mouse, *Gcm2* is predominantly expressed in the 3rd pharyngeal pouch endoderm and subsequently in the parathyroid domains or parathyroid glands that develop from the 3rd pharyngeal pouches (Gordon et al., 2001; Kim et al., 1998). *Gcm2* expression is initiated at E9.5 in the caudal pharyngeal pouch endoderm that encompasses the 3rd and 4th pouches (Gordon et al., 2001). By E10.5, it is restricted to the anterior/dorsal side of the 3rd pharyngeal pouch and is subsequently maintained in the parathyroid domain at the anterior/dorsal side of the parathyroid/thymus common primordium (Gordon et al., 2001). After the parathyroid glands form, *Gcm2* expression is still maintained (Kim et al., 1998). The expression pattern of *Gcm2* in the 3rd pharyngeal pouch and subsequently in the parathyroids suggests that *Gcm2* plays an important role in parathyroid organogenesis in mammals. Studies on *Gcm2* null mutant mice

support this conclusion (Gunther et al., 2000). *Gcm2* null mutant mice have failed parathyroid glands and exhibit hypoparathyroidism (Gunther et al., 2000). Based on these observations, mouse *Gcm2* was proposed to be a master regulator gene in parathyroid organogenesis (Gunther et al., 2000).

Similar to mouse *Gcm1*, mouse *Gcm2* is also expressed in the brain (Iwasaki et al., 2003; Kim et al., 1998), and was shown to induce glial cell characteristics in fibroblast cell and embryonic brain cells (Iwasaki et al., 2003). However, a function for these two genes in mouse brain development has not been reported. In addition to the brain, low level *Gcm2* expression can also be detected in the otic region by *in situ* hybridization in E10.5 mouse embryos (http://genex.hgu.mrc.ac.uk/das/jsp/browse.jsp#table) (Gray et al., 2004). Furthermore, *Gcm2* expression was also detected in placental tissue (Kim et al., 1998). Despite these reports of *Gcm2* expression in these non-parathyroid organs, the function of *Gcm2* outside parathyroid is still unknown.

II. Parathyroid organ function

1. Parathyroid-a key endocrine organ for the regulation of ionized calcium and inorganic phosphorus homeostasis

Mammals have evolved an efficient system to regulate ionized calcium and inorganic phosphorus homeostasis in their extracellular environment to facilitate their successful adaptation to land life. This system comprises the parathyroid glands, bones, kidneys and intestines. The primary role of parathyroids is to function as an endocrine regulator, to modulate the physiologic activities of bones, kidneys and intestines (Ramasamy, 2006).

As an endocrine organ, parathyroids perform their functions by secreting an 84-amino acid hormone called parathyroid hormone (PTH). PTH is secreted into the circulation and finally targets its receptors located at the membrane of osteoblast cells in the bone or in the distal renal cells of the kidney (Houillier et al., 2003). In bone, the binding of endogenous PTH to its receptors stimulates osteoclastic bone digestion, which can release ionic calcium and inorganic phosphorus into the circulation (Chambers, 1980; Fuller and Chambers, 1998; Fuller et al., 1998). In the kidney, PTH acts on the distal tubule epithelial cells to enhance calcium reabsorption and to inhibit phosphate reabsorption from renal tubules. Simultaneously, PTH stimulates renal production of calcitriol (1,25-$(OH)_2D_3$), which can enhance intestinal absorption of calcium and phosphate from food (Deluca, 1981; Houillier et al., 2003; Ramasamy, 2006; Stumpf et al., 1980). Collectively, PTH is an endocrine hormone that can directly regulate physiologic activities of bone and kidney and indirectly regulate intestine action. The net effect of PTH on calcium and phosphate metabolism is an increase in the plasma calcium concentration with no change or a decrease in the plasma phosphate concentration.

In order to tightly control the concentration of the extracellular ionized calcium and inorganic phosphorus in a narrow range that is suitable for physiologic activities, the production of PTH in the parathyroids is regulated by feedback from changes in serum calcium concentration (Ramasamy, 2006). To achieve this aim, parathyroid cells use a membrane protein called calcium-sensing receptor (CasR) to sense changes in the extracellular ionized calcium concentration (Chen and Goodman, 2004). When the calcium concentration is too low, CasR regulates parathyroid cells to produce and secrete more PTH, whereas a high level of Ca^{2+} inhibits PTH synthesis and secretion. In addition, the high concentration of plasma phosphate and calcitriol also can decrease PTH production and secretion (Ramasamy, 2006).

2. The importance of calcium homeostasis

In mammals, the circulating ionized Ca^{2+} is involved in a wide range of physiological activities including neuromuscular excitability, muscle contraction, bone mineralization, blood coagulation and cardiovascular functions (Bootman et al., 2001; Clapham, 1995). Disorders of calcium homeostasis can result from failure of parathyroid function. In humans, there are two different kinds of the disorders of parathyroid function: hyperparathyroidism (HPT) and hypoparathyroidism, both of which can affect the concentration of calcium in the extracellular fluid (Marx, 2000). An overactive parathyroid gland or parathyroid tumors can secrete excess PTH, which causes the condition known as hyperparathyroidism (HPT). HPT is a consequence of excessive calcium in the blood (Krebs and Arnold, 2002). The symptoms of HPT include skeletal problems (bone pains, osteoporosis, and fractures), mental disturbances, myopathy (muscular disease), kidney stones and abdominal discomfort. On the other hand, too low PTH production can cause hypoparathyroidism, which can result from defects in parathyroid development (Garfield and Karaplis, 2001). Hypoparathyroidism leads to decreased blood calcium concentration (hypocalcemia) and increased blood phosphorus concentration (hyperphosphatemia). Severly reduced blood calcium levels may cause symptoms such as tingling of the lips, fingers, and toes, and muscle cramps or spasms.

3. Parathyroid organogenesis disorders

Despite its important physiologic function in extracellular calcium homeostasis, there are few reports on parathyroid organogenesis. In humans, both hyperparathyroidism and hypoparathyroidism disorders can be caused by mutations in a number of genes that function in parathyroid organogenesis (Krebs and Arnold, 2002; Thakker, 2001; Thakker, 2004). Among these parathyroid diseases, one type of parathyroid tumor was demonstrated to be associated with

lower *Gcm2* expression, which suggests that maintaining proper *Gcm2* expression levels is required to maintain a fully differentiated parathyroid cell state (Correa et al., 2002). In humans, parathyroid adenomas can be found in the thymus, and can cause hyperparathyroidism (Chandran et al., 2003; Maret et al., 2004). Surprisingly, this PTH-secreting adenoma also expresses *Gcm2*, suggesting that these tumors might derive from parathyroid cells that migrated aberrantly during parathyroid/thymus organogenesis (Kronenberg, 2004; Maret et al., 2004). *Gcm2* mutations with a deletion at exons 1-4 or with a point mutation at the DNA-binding domain, which can affect Gcm2 function, result in hypoparathyroidism (Ding et al., 2001; Thomee et al., 2005). Analogous to mouse *Gcm2*, human *Gcm2* mutations were found to underlie these heritable parathyroid diseases, suggesting that human *Gcm2* gene also functions as a key regulator gene in parathyroid gland development (Berg, 2002).

III. Parathyroid organogenesis

1. Mouse organogenesis

During mouse embryonic development, the early postimplantation stages are preoccupied with the formation of the extraembryonic structures including the placenta, yolk sac, allantois, and the amnion, which function to sustain the viability of the embryos by supporting fetal-maternal exchanges (Rossant and Tam, 2002). Subsequently, the vital organs will start to be fully installed in the embryo to prepare for an independent existence after it is delivered (Rossant and Tam, 2002). The definitive endoderm is a population of multipotent stem cells that will give rise to many organs including the thyroid, lung, pancreas, liver, thymus and the parathyroid glands (Grapin-Botton and Melton, 2000; Wells and Melton, 1999). The organogenesis of these organs

involves several discrete phases (Rossant and Tam, 2002): Positioning of the competent endodermal cells at the correct location in the embryo; specification of these competent endodermal cells as a specific cell fate and formation of a bud rudiment for the respective organ; growth and differentiation of the organ bud rudiment and integration with other components to become a functional organ.

The adoption of similar morphogenetic strategies in the formation of different endodermal organs is paralleled by the application of similar molecular mechanisms. It has been proposed that there is a conserved 'morphogenetic code'-a set of different combinations of common rules that are repeatedly used to make different organs (Hogan, 1999). For example, the same intercellular signaling pathways, like Sonic hedgehog (Shh), Fibroblast growth factors (Fgfs), and Bone morphogenetic proteins (BMPs), have been shown to be repeatedly involved in the mesenchyme-endoderm interactions responsible for patterning of these endodermal organs (Duncan, 2003; Grapin-Botton and Melton, 2000; Hogan, 1999; Moore-Scott and Manley, 2005; Patel et al., 2006; Rossant and Tam, 2002). Endodermal organs use very similar strategies to develop through specification and differentiation. In response to the different inductive signals through mesenchyme-endoderm interactions, these endodermal organs express lineage-specific transcription factors to acquire organ-specific phenotypes during the specification and differentiation steps (Duncan, 2003; Grapin-Botton and Melton, 2000; Hogan and Yingling, 1998; Manley and Blackburn, 2004). For example, the thyroid expresses *Nkx2.1* and *Ttf2* (Grapin-Botton and Melton, 2000). The pancreas expresses *Pdx1* and *Hlxb9* (Grapin-Botton and Melton, 2000). The liver expresses *Hex* and *Prox1* genes (Duncan, 2003). The thymus expresses *Foxn1,* and parathyroid expresses *Gcm2* transcription factor (Gordon et al., 2001). Loss of function of these genes results in failure or dysgenesis of organ formation, suggesting that these

organs use these lineage-specific transcription factors to regulate the specification or differentiation steps. Due to the similarity in the development of these endodermal organs, knowledge we gained from studies of other endodermal organs will provide the clues to understand the molecular mechanism of parathyroid organogenesis.

The molecular mechanisms responsible for determining a specific organ cell fate from endodermal precursor cells during organogenesis is poorly understood. Several studies on the cell fate specification have shown that the specification step can be controlled by 'master regulator' genes. A 'master regulator' gene is defined by two characteristics: an indispensable role in cell fate specification and the sufficient ability to induce a cell fate itself. Several genes have been identified as 'master regulators' of cell fate specification. *Pax6* is one among these genes. Targeted ectopic expression of *Drosophila Eyeless* or its ortholog mouse *Pax6* gene in various imaginal disc primordia in the fly is sufficient to induce the formation of eye (Halder et al., 1995). In Xenopus embryos, ectopic expression of *Pax6* also induces lens tissue (Altmann et al., 1997; Chow et al., 1999). These observations lead to the proposal of *Pax6* being a 'master regulator' gene for eye development (Halder et al., 1995; Pichaud and Desplan, 2002). *Foxp3* has also been shown to be a master regulator gene for CD4+CD25+ regulatory T cell fate. Besides the loss of CD4+CD25+ regulatory T cells in *Foxp3* null mutants, *Foxp3* has also been shown to be sufficient to program CD4+CD25+ regulatory T cell development (Fontenot et al., 2003; Hori et al., 2003). Another master regulator gene is *Pax5*, which functions in B cell lineage fate commitment (Cotta et al., 2003). In chicken, gain-function experiments demonstrated that a novel homeobox gene MNR2 was sufficient to direct somatic motor neuron fate (Tanabe et al., 1998). These examples show that it is a common phenomenon for cells to use master regulator gene to control the programming of a cell fate during the specification development step. *Gcm2*

has been proposed as ‘master regulator’ gene in parathyroid organogenesis as a result of its expression pattern, and loss-of-function phenotype (Balling and Erben, 2000; Berg, 2002; Gunther et al., 2000; Manley and Blackburn, 2004). Based on the fact that the structure of all *Gcm* genes is evolutionarily conserved, it will be interesting to determine whether mouse *Gcm2* also plays a role as a parathyroid cell fate determinant during parathyroid organogenesis, like its orthologous *Drosophila Gcm* genes.

2. Parathyroids develop with the thymus from two common primordia in the 3rd pharyngeal pouch

In mouse, the parathyroid glands are bilateral organs that develop together with the thymus from two common parathyroid/thymus primordia derived from the 3rd pharyngeal pouch endoderm (Manley and Blackburn, 2004). Thus, the early stages of parathyroid organogenesis are closely linked with thymus organogenesis. The development of parathyroid and thymus can be divided into several distinct stages: The formation of the 3rd pharyngeal pouch from the foregut endoderm; the formation of two parathyroid/thymus primordia at the lateral sides of 3rd pharyngeal pouch endoderm; the specification of the parathyroid- and thymus-specific domains in the 3rd pharyngeal pouches or parathyroid/thymus common primordia; the differentiation of parathyroid- and thymus-specific domains into functional organs; the separation of parathyroid glands from the thymus lobes (Manley and Blackburn, 2004). This developmental process is very similar to the organogenesis of the other endoderm-derived organs as previously mentioned, which includes position, specification, differentiation, and maturation.

As shown in Figure 1.1, beginning at E8.0, the pharyngeal endoderm folds into four pairs of transient outpockets called the pharyngeal pouches, which will give rise to several different organs including the parathyroids and thymus (Graham, 2003; Graham and Smith, 2001). The 3rd

pharyngeal pouches are formed at E9.5-10. They then proliferate to form two bilateral parathyroid/thymus common organ primordia at E11-11.5. During these stages, the anterior/dorsal side of the 3rd pharyngeal pouch or of the subsequent parathyroid/thymus common primordium is specified as parathyroid domain and the posterior/ventral side is specified as thymus domain (Gordon et al., 2001; Moore-Scott and Manley, 2005; Patel et al., 2006). These two domains begin to separate from each other at E12.5. By E13.5, each parathyroid/thymus primordium divides into one parathyroid gland and one thymus lobe. In the adult mouse, these two parathyroid glands are localized to bilateral sites near the thyroid, and the thymus organ, which consists of two lobes, is situated in the anterior chest cavity (Manley, 2000; Manley and Blackburn, 2003; Manley and Blackburn, 2004).

Although the thymus has a close relationship with the parathyroid glands during their organogenesis stages, the thymus functions as a primary lymphoid organ, which provides a specialized microenvironment where T lymphocyte maturation occurs (Miller, 1994; Miller, 2002; Miller and Osoba, 1967). The thymus is an epithelial organ composed in part of thymic epithelial cells (TECs), which develop from endodermal precursor cells in the thymus domain in the 3rd pharyngeal pouch (Gordon et al., 2004). To accomplish its full function, the thymus also contains other non-lymphocytic components, like macrophages, fibroblasts, dendritic cells and vascular components (Anderson et al., 1996). All these non-lymphocytic cells including TECs are referred as thymic stroma. Thymic stroma consisting of different stromal cells collectively provide the signals for various stages of T cell maturation such as cytokines, cell surface molecules and extracellular matrix elements (Anderson et al., 1996; Moore and Zlotnik, 1995; Pongracz et al., 2003; Wiles et al., 1992).

3. Molecular mechanism of the parathyroid/thymus organogenesis

Our understanding of the molecular mechanisms that control parathyroid and thymus development was gained mostly by studying mice with null mutations in genes that function in the different stages of parathyroid/thymus development. *Chordin*, *Tbx1* and *Fgf8* were found to function in the formation of the 3rd pharyngeal pouch. Null mutations in any one of these three genes result in the loss of the 2nd, 3rd and 4th pharyngeal pouches and subsequently athymic and aparathyroid phenotypes (Abu-Issa et al., 2002; Bachiller et al., 2003; Frank et al., 2002; Jerome and Papaioannou, 2001). The failure of parathyroid and thymus organogenesis as the secondary consequence of the loss of 3rd pharyngeal pouch suggests that 3rd pouch formation is required for the formation of parathyroid/thymus common primordium.

A *Hoxa3-Pax1/9-Eya1-Six1/4* regulatory network also has been identified to function in the formation of the parathyroid/thymus primordia from the 3rd pharyngeal pouch endoderm. Disruption of this pathway specifically affects the formation of parathyroid/thymus common primordia without affecting pharyngeal pouch formation (Chisaka and Capecchi, 1991; Hetzer-Egger et al., 2002; Peters et al., 1998; Su et al., 2001; Su and Manley, 2000; Wallin et al., 1996; Xu et al., 2002; Zou et al., 2006). *Hoxa3* performs its role in parathyroid/thymus organogenesis through the regulation of *Pax1* and *Pax9* genes, since *Pax1* and *Pax9* are specifically down-regulated in *Hoxa3*$^{-/-}$ embryos at E10.5 (Manley and Capecchi, 1995) (Koushik and Manley, unpublished observations). *Hoxa3*$^{+/-}$*Pax1*$^{-/-}$ compound mutants have more severe defects in parathyroid and thymus development than *Pax1*$^{-/-}$ single mutants (Su et al., 2001; Su and Manley, 2000). Furthermore, *Hoxa3-Pax1/9-Eya1-Six1/4* was proposed as an endodermal regulatory network that functions to the 3rd pouch endoderm into organ-specific domains, based on the expression pattern and mutant phenotypes of *Hoxa3* and *Eya1* (Xu et al., 2002). In *Eya1* null

mutant embryos, *Hoxa3-Pax1/9* expression is normal, but *Six1* expression is reduced (Xu et al., 2002).

The mechanism by which the parathyroid- and thymus-specific domains in the 3rd pharyngeal pouches and subsequent common primordia are specified is just beginning to be understood. Regulation of cell fate specification involves two important aspects: the specific extracellular signaling that will provide the special microenvironment cues to instruct precursor cell specification and an intracellular transcriptional cascade pathway that receives the instructional signal from the extracellular microenvironment and completes the programming of cell fate specification (Korswagen, 2002; Medina and Singh, 2005; Mondal et al., 2004; Schonhoff et al., 2004). Data from previous studies in our lab on the expression pattern of Shh and Bmp4 signaling pathways in the 3rd pharyngeal pouches indicate that *Shh* and *Bmp4* may be involved in the specification of parathyroid- and thymus-specific domains in the 3rd pouch (Moore-Scott and Manley, 2005; Patel et al., 2006). *Shh* is expressed at a higher level at the entrance of the dorsal side of the 3rd pouches (Moore-Scott and Manley, 2005). *Bmp4* was found to be expressed at higher levels at the ventral side, and its antagonist, *Noggin* gene, is expressed at the dorsal side, overlapping with *Gcm2* expression (Patel et al., 2006). Based on these data, a working model for Shh and Bmp4 signaling function in the specification of parathyroid/thymus domains has been proposed (Moore-Scott and Manley, 2005; Patel et al., 2006). According to this model, these two signaling molecules are expressed in an opposing gradient pattern in the 3rd pouches. Shh induces a parathyroid organ-specific differentiation program at the dorsal pharynx. Conversely, the presence of Bmp4 and absence of Shh in the ventral pharynx induces a thymus organ-specific differentiation program and thymus organogenesis. Consistent with this model, Shh was found to be required for parathyroid domain formation (Moore-Scott and Manley, 2005).

In *Shh* null mutant mice, *Gcm2* was never expressed and no parathyroid domain was formed, but an expanded thymus domain marked with *Bmp4* and *Foxn1* was formed (Moore-Scott and Manley, 2005). Based on these functional evidences, we propose that the transcription factor, Gcm2, functions as a binary cell fate switch to relay the signals from Shh/Bmp4 signaling and regulate the intracellular regulation events responsible for parathyroid cell specification. At the same time, Gcm2 may also prevent thymus cell fate specification in the anterior/dorsal side by repressing *Foxn1* expression. We still have a big gap to fully understand the molecular mechanisms controlling the differentiation of parathyroid-specific domains into functional parathyroid organ. Besides its early expression in the 3rd pharyngeal pouches and its possible role in the specification of parathyroid domain from the common primordium, *Gcm2* expression is also maintained in the parathyroid domain or parathyroid glands at the late stages (Kim et al., 1998), suggesting that *Gcm2* may also function at the differentiation stage. However further evidence is required to confirm this hypothesis.

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Figure 1.1. The organogenesis model of parathyroid glands and thymus in the 3rd pharyngeal pouch endoderm. Panel A is the foregut endoderm at the pharynx at E8 stage when the pharyngeal pouches are not formed. Panel B shows the pharyngeal endoderm structure at E9.5 stage when pouches (P1-P4) are formed. Panel C is one pharyngeal pouch that is squared in panel B. The yellow color marks the endodermal cells that will give rise to the parathyroid and thymus cell fates at late stages. Panel D shows a parathyroid/thymus common primordium formed in one 3rd pharyngeal pouch. At this stage, the common primordium has not been separated from the 3rd pharyngeal pouch. Panel E is the parathyroid/thymus common primordium at E12-12.5 stages. In panel D and E, parathyroid and thymus domains are under differentiation development step. From E12.5, the parathyroid domain starts to separate from the thymus domain. By E13.5 (F), the parathyroid gland already has been separated from the thymus lobe. p3, third pouch.

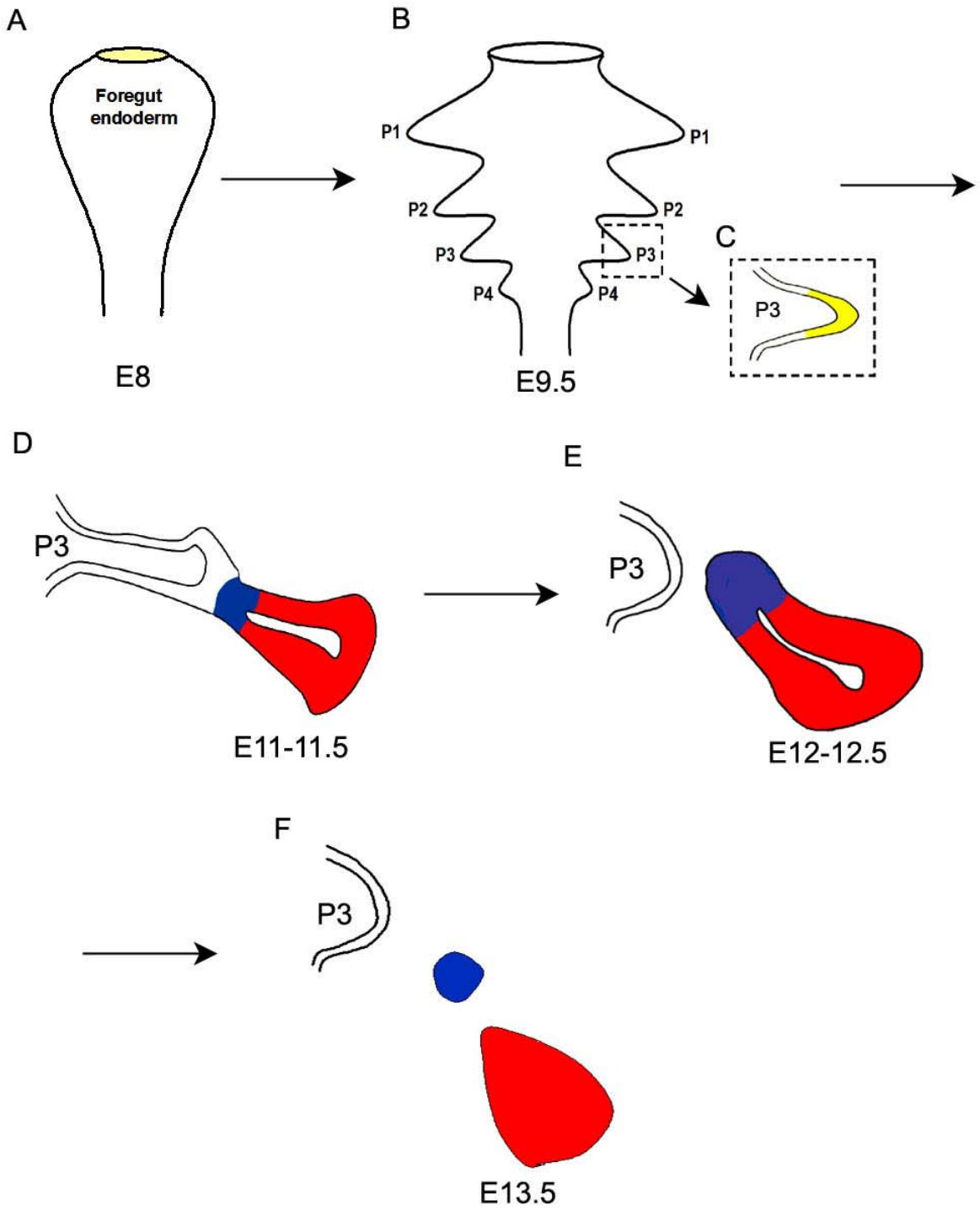
A
Foregut endoderm
E8
B
P1
P1
P2
P2
P3
P3
P4
P4
E9.5
C
P3
D
P3
E11-11.5
E
P3
E12-12.5
F
P3
E13.5
Parathyroid/thymus common precursors
Parathyroid domain
Thymus domain

Figure 1.1

CHAPTER 2

GCM2 IS REQUIRED FOR THE DIFFERENTIATION AND SURVIVAL OF PARATHYROID PRECURSOR CELLS IN THE PARATHYROID/THYMUS PRIMORDIA[1]

[1]Zhijie Liu, Shannon Yu, Nancy R. Manley 2007. Dev. Bio. 305:333-346.
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ABSTRACT

The parathyroid glands develop with the thymus from bilateral common primordia that develop from the 3rd pharyngeal pouch endoderm in mouse embryos at about E11, each of which separates into one parathyroid gland and one thymus lobe by E13.5. *Gcm2*, a mouse ortholog of the *Drosophila Glial Cells Missing* gene, is expressed in the parathyroid-specific domains in the 3rd pouches from E9.5. The null mutation of *Gcm2* causes aparathyroidism in the fetal and adult mouse, and has been proposed to be a master regulator for parathyroid development. In order to study how *Gcm2* functions in parathyroid development, we investigated the mechanism that causes the loss of parathyroids in *Gcm2* null mutants. Analysis of the 3rd pouch-derived primordium in *Gcm2*$^{-/-}$ mutants showed the parathyroid-specific domain was present before E12.5, but underwent programmed cell death between E12-12.5. RNA and protein localization studies for parathyroid hormone (*Pth*) in wild-type embryos showed that the presumptive parathyroid domain in the parathyroid/thymus primordia started to transcribe *Pth* mRNA and produce PTH protein from E11.5, before the separation of parathyroid and thymus domains. However in *Gcm2*$^{-/-}$ mutants, the parathyroid-specific domain in the common primordium did not express *Pth* and could not maintain the expression of two other parathyroid marker genes, *CasR* and *CCL21*, although expression of these two genes was initiated. Marker gene analysis placed *Gcm2* downstream of the known transcription and signaling pathways for parathyroid/thymus organogenesis. These results suggest that *Gcm2* is not required for pouch patterning or to establish the parathyroid domain, but is required for differentiation and subsequent survival of parathyroid cells.

INTRODUCTION

Mammals are equipped with an efficient system to regulate ionized calcium and phosphorus homeostasis in the extracellular environment that is composed of parathyroid glands, bone, kidney, and intestine. In this system, the parathyroid glands are the most important endocrine regulator to maintain the calcium homeostasis in the circulation (Ramasamy, 2006). The primary function of the parathyroids is to produce and release an 84-amino acid hormone called parathyroid hormone (PTH), which directly targets receptors on osteoblasts to regulate bone resorption and on distal tubule epithelial cells in the kidney to increase renal calcium reabsorption (Houillier et al., 2003). PTH also indirectly stimulates intestinal calcium absorption by increasing $1,25(OH)_2D_3$ production in the kidney (Ramasamy, 2006). The requirement of PTH in the regulation of calcium homeostasis was found not only postnatally, but also at fetal stages (Kovacs et al., 2001a; Kovacs et al., 2001b; Miao et al., 2002). PTH is also essential for fetal bone formation (Miao et al., 2002). The production and secretion of PTH in the parathyroid glands is controlled by the membrane-bound calcium-sensing receptor (CasR), which regulates PTH secretion by sensing the changes of extracellular ionized calcium concentration (Chang and Shoback, 2004; Chen and Goodman, 2004).

Serum calcium plays many physiological functions including neuromuscular excitability, muscle contraction, blood coagulation and bone mineralization (Ramasamy, 2006). Due to the importance of PTH in the calcium homeostasis, PTH deficiency (hypoparathyroidism) caused by the failure of or disorders in parathyroid development causes disease in humans (Thakker, 2001). Hypoparathyroidism can be caused by the mutation of the genes that are required for normal parathyroid physiological functions, including *Pth* (Ahn et al., 1986; Goswami et al., 2004) and *CasR* (Suzuki et al., 2005; Thakker, 2004). It also can result from the mutation of genes that

function in parathyroid development, like *Gata3*(Van Esch et al., 2000), *Sox3* (Bowl et al., 2005), and *Gcm2* (Ding et al., 2001; Thomee et al., 2005). The study of parathyroid organogenesis can therefore help us to understand the mechanisms of human hypoparathyroidism.

In mouse, the parathyroids are bilateral organs that develop with the thymus from two common parathyroid/thymus primordia originating from the 3rd pharyngeal pouch endoderm. Beginning at E8.0, the pharyngeal endoderm develops four bilateral pouches that give rise to several organs, including the thymus and parathyroids (Graham, 2003; Graham and Smith, 2001). The 3rd pharyngeal pouches are formed at E9.5-10 days, and are patterned into dorsal/anterior parathyroid and ventral/posterior thymus domains (Gordon et al., 2001; Moore-Scott and Manley, 2005; Patel et al., 2006). The 3rd pouch endoderm proliferates to form bilateral parathyroid/thymus common primordia at E11-11.5. Each primordium separates into one parathyroid gland and one thymus lobe at E12.5-13.5, which then migrate to their eventual adult locations by about E14.5 (Blackburn and Manley, 2004; Manley, 2000; Manley and Blackburn, 2003). In the adult mouse, the parathyroids are located near or embedded within the thyroid gland, and the thymus is situated in the anterior chest cavity. Thus, the early stages of parathyroid organogenesis are closely linked with thymus organogenesis.

The molecular mechanisms that regulate pouch patterning and early parathyroid/thymus organogenesis are beginning to be identified. The Hoxa3, Pax1/9, Eya1, and Six1/4 transcriptional regulators have been implicated as a pathway/network regulating early organogenesis of both organs, since mice that lack these genes have normal initial pouch formation, but then fail to form or have hypoplastic parathyroids and thymus. The *Hoxa3* null mutation causes the most severe defects in parathyroid/thymus organogenesis, as the *Hoxa3*$^{-/-}$ mutants fail to initiate the formation of the parathyroid/thymus primordia (Chisaka and Capecchi,

1991; Kameda et al., 2004; Manley and Capecchi, 1995; Manley and Capecchi, 1998; Su and Manley, 2002). A Pax1/9-Eya1-Six1/4 network has been identified to act downstream of Hoxa3 during patterning and early organogenesis of both the thymus and parathyroids (Dietrich and Gruss, 1995; Manley and Capecchi, 1995; Neubuser et al., 1995; Peters et al., 1998; Su et al., 2001; Su and Manley, 2000; Wallin et al., 1996; Xu et al., 2002; Zou et al., 2006).

The mechanism by which the parathyroid- and thymus-specific domains in the 3rd pouch and subsequent primordia are specified is beginning to be understood. Gcm2 and Foxn1 are organ-specific transcription factors that are localized to the parathyroid- or thymus-specific domains of the common primordia before their separation (Gordon et al., 2001). *Foxn1* expression beings at E11.25 in a domain that is complementary to *Gcm2* expression in the parathyroid/thymus primordia (Gordon et al., 2001). The *Foxn1* null mutation, nude, causes failure of thymic epithelial cell differentiation, but does not affect the initiation of thymus organogenesis (Blackburn et al., 1996; Nehls et al., 1996). The *Gcm2* null mutation has been reported to cause complete and specific failure of parathyroid development (Gunther et al., 2000). *Gcm2* expression begins at E9.5 in the dorsal-anterior pharyngeal endoderm of the 3rd pouch and is maintained in the presumptive parathyroid domain at later stages (Gordon et al., 2001). The early expression pattern and apparent failure of parathyroid organogenesis suggests that *Gcm2* may specify the parathyroid domain in the 3rd pharyngeal pouch prior to primordium formation, and be required for initial organogenesis.

Gcm2 is member of the Glial Cells Missing (Gcm) transcription factor family, which have a conserved Gcm DNA binding domain (Cohen et al., 2003). The first *Gcm* gene was found in *Drosophila*, which was shown to function to as a binary switch between neuronal and glial cells determination in *Drosophila* central nervous system (Hosoya et al., 1995; Jones et al., 1995). In

mammals, there are two *Gcm* orthologs: *Gcm1* and *Gcm2* (Kim et al., 1998). However, neither gene is required in the nervous system in mice. *Gcm1* is expressed at the placenta and is required for labyrinth formation (Schreiber et al., 2000), while *Gcm2* expression is restricted to the parathyroid gland (Gordon et al., 2001; Gunther et al., 2000; Kim et al., 1998). The role of *Gcm* as a binary switch specifying glial cell fate in *Drosophila* nervous system development and the complementary expression domains of *Foxn1* and *Gcm2* in the common primordium suggest that parathyroid organogenesis may fail in *Gcm2*$^{-/-}$ mutants because the parathyroid domain is transformed to a thymus fate. This possibility is supported by previous studies in our lab of the *Sonic hedgehog (Shh)* mutant phenotype. In the *Shh* null mutant, *Gcm2* is never expressed, and no parathyroid domain forms. In contrast, there is an expanded thymus domain in the 3rd pouch, marked by expanded *Bmp4* and subsequently *Foxn1* positive domains (Moore-Scott and Manley, 2005; Patel et al., 2006). These results are consistent with a model in which in the absence of Shh, and therefore of *Gcm2*, the parathyroid domain may be transformed to a thymus fate.

In the current study, we determined the role of *Gcm2* in parathyroid organogenesis by studying the mechanism of aparathyroidism in *Gcm2*$^{-/-}$ mutants. In contrast to previous reports, we showed that the parathyroid-specific domain was present and morphologically normal until E12 in *Gcm2*$^{-/-}$ embryos. However, parathyroid-specific markers were either not expressed or not maintained at E11.5, and the parathyroid domain underwent coordinated programmed cell death at E12 and was totally lost by E12.5. Consistent with these and previous results, marker gene analysis showed normal expression of the Hoxa3-Pax1/9-Eya1 transcription factor and the Shh-Bmp4 signaling networks in *Gcm2*$^{-/-}$ mutants, indicating that these pathways act upstream of *Gcm2*. We further found that *Tbx1* expression, which is also restricted to the parathyroid-specific domain in the 3rd pouch and/or the parathyroid/thymus common primordia, was not affected by

the *Gcm2* null mutation. This raises the possibility that *Tbx1* may function to specify the parathyroid-specific domain downstream of Shh and upstream of *Gcm2*. Our data indicate that in spite of its early expression at E9.5, *Gcm2* is not required for early patterning of the dorso-anterior parathyroid domain or initiation of parathyroid organogenesis. Also, *Gcm2* does not act as a binary cell fate switch between parathyroid and thymus fates in the 3rd pouch, but is instead required for the differentiation and survival of parathyroid precursor cells after initial organ domain formation.

MATERIALS AND METHODS

Mice

The generation of the *Gcm2* null mutant and genotyping have been described (Gunther et al., 2000). *Gcm2* mutant mice used for experiments were originated on 129/SvEv-C57BL/6J and had been backcrossed to C57BL/6J mice for more than 4 generations.

Pax9^{lacZ} (Peters et al., 1998), *Bmp4*^{lacZ} (Lawson et al., 1999), and *Noggin*^{lacZ} (McMahon et al., 1998) alleles were each crossed with *Gcm2*$^{+/-}$ to obtain double heterozygous F1 mice, which were then crossed with *Gcm2*$^{+/-}$ to produce *Gcm2* homozygous mutants carrying the marker alleles for analysis.

Embryos were collected with the day of the vaginal plug designated as E0.5. We also used somite number, eye pigment, and the morphology of parathyroid/thymus primordium to stage the embryos. All experiments were carried out with the approval of the UGA institutional animal care committee.

TUNEL Assay

The TUNEL assay was performed as described (Su et al., 2001). Staged embryos were fixed in 4% paraformaldehyde for 2 hours and processed for paraffin embedding. Sections were cut at 8μm. The TUNEL assay was performed on the paraffin-embedded tissue sections following the manufacturer's guidelines (Roche Diagnostics).

Immunohistochemistry

PTH immunohistochemistry was performed using an anti-PTH antibody as described (Wurdak et al., 2005). Staged embryos were fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. Sections were cut at 8-10μm and stained with a goat anti-PTH antibody using the Vectastain method (VectorLab).

Section and whole mount *in situ* hybridization

Paraffin section *in situ* hybridization was performed as described (Moore-Scott and Manley, 2005). Staged embryos were fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. 8-10μm sections were hybridized with digoxigenin-labeled RNA probes at 0.5 µg/ml. Alkaline phosphatase-conjugated antidigoxigenin Fab fragments were used at 1:5000. BM-purple (Roche) was used as a chromagen to localize hybridized probe. Probes for *Gcm2* and *Foxn1* (Gordon et al., 2001), *Tbx1* (Chapman et al., 1996), *CasR* (Bowl et al., 2005), *Shh* (Echelard et al., 1993), and *Ptc1* (Goodrich et al., 1996) have been described. *CCL21* probe was a gift from Yousuke Takahama. *Pth* probe was cloned using the primers: 5-CTGCAGTCCAGTTCATCAGC-3 and 5-AAGCTTGAAAAGGTAGCAGCA-3.

For the *Foxn1* section *in situ* hybridization on the alternative sections with the sections used for TUNEL experiment, the paraffin sections prepared for TUNEL experiment were refix in 4%

paraformaldehyde for 20 minutes after rehydrate, then preformed section *in situ* hybridization like normal procedure.

Whole-mount *in situ* hybridization was performed as described (Carpenter et al., 1993; Manley and Capecchi, 1995). Probes for *Hoxa3* (Manley and Capecchi, 1995), *Pax1*(Manley and Capecchi, 1995), and *Eya1*(Xu et al., 1997) have been described. *Gcm2*, *Tbx1*, *CasR* and *CCL21* probes were the same as the above used for section *in situ* hybridization.

X-gal staining

Whole-mount X-gal staining was performed to the staged embryos as described (Patel et al., 2006). After lacZ staining, embryos were embedded in paraffin and 8 μm sections cut and counterstained with nuclear fast red.

RESULTS

Initial parathyroid domain formation is normal in *Gcm2* null mutants

Previous studies had concluded that the parathyroids were absent in *Gcm2*$^{-/-}$ embryos as early as E11.5 (Gunther et al., 2000). To investigate how parathyroid organogenesis fails in *Gcm2*$^{-/-}$ mutants, we performed a detailed morphological and cell fate analysis of the 3rd pouch-derived primordium in control and *Gcm2* mutant embryos from E10.5-E13.5. Gcm2 expression is normally restricted to a small dorso-anterior domain in the third pouch and in the subsequent shared organ primordium, with *Foxn1* expressed in the remainder of the primordium after E11.5 (Gordon et al., 2001) (Fig. 2.1A-C). As *Gcm2* mRNA is not present in the mutant embryos, we used *Foxn1* gene expression as a marker for thymus cell fate to assess the formation of the

parathyroid domain in *Gcm2* mutants. The *Foxn1*-expressing thymus domain was normal at all time points assayed, and the presumptive parathyroid domain was clearly identifiable as a *Foxn1*-negative domain at the dorsal and anterior aspect of the common parathyroid/thymus primordium at E11.25, E11.5 and E12 in both wild-type and mutant embryos (Figs. 2.1C-F, and data not shown). At E12.5-13, we saw presumptive parathyroids consisting of *Foxn1*-negative cells separating from the thymus domain in the wild-type embryos (Fig. 2.1G, I). However, at these stages the parathyroid domain was absent in the *Gcm2*$^{-/-}$embryos (Figs. 2.1H, J). Thus, the parathyroid domain did form in *Gcm2*$^{-/-}$embryos and initially appeared morphologically normal, but was lost by E12.5. Furthermore, the parathyroid domain does not appear to be transformed to a thymus-specific fate, since in *Gcm2*$^{-/-}$embryos the *Foxn1*-positive thymus domain did not expand to the whole primordium at E11.25-E12 (Figs. 2.1D, F).

Loss of the parathyroid domain in *Gcm2*$^{-/-}$ mutants by programmed cell death

To determine whether the presumptive parathyroid domain underwent programmed cell death in *Gcm2*$^{-/-}$ mutants, we used the TUNEL assay at E11.5-12.5. At E11.5, there was no difference between *Gcm2* null mutant and WT control embryos (Figs. 2.2A and B). As we have previously reported, at this stage apoptosis is normally present at in the region of endoderm where the primordium is separating from the pharyngeal pouch (Gordon et al., 2004). This apoptosis is very transient, and at E12 few or no TUNEL-positive cells were seen in the wild-type primordium (Fig. 2.2E). In contrast, there was a concentration of apoptotic cells in the presumptive parathyroid-specific domain at E12 in the *Gcm2* null mutants (Fig. 2.2F). We confirmed that the apoptotic domain at E12 corresponded to the presumptive parathyroid domain by *in situ* hybridization for *Foxn1* on alternate sections (Fig. 2.2C, D). At E12.5, apoptosis could not be detected in the *Gcm2*$^{-/-}$ mutants (data not shown), consistent with the absence of the

parathyroid domain at this time point (Fig. 2.1H). These results indicated that loss of the presumptive parathyroid domain was via coordinated apoptosis between E12 and E12.5.

Parathyroid differentiation initiated, but was subsequently blocked in *Gcm2*$^{-/-}$ mutants

To determine the earliest time point when parathyroid differentiation markers initiate and whether they were ever expressed in the *Gcm2*$^{-/-}$ mutants prior to loss of the parathyroid domain at E12.5, we assayed the expression of calcium sensor receptor (*CasR*), the chemokine *CCL21*, and parathyroid hormone (*Pth*), in wild-type and *Gcm2*$^{-/-}$ embryos. This analysis was performed to determine whether parathyroid specification and differentiation was initiated in *Foxn1* negative domain in the *Gcm2* mutants at E10.5-11.5.

The two earliest differentiation markers for the parathyroid domain other than Gcm2 are the calcium sensing receptor (CaSR) and the chemokine CCL21. CasR is functionally required for parathyroid cells to respond to modulating calcium concentrations. Our previous study showed that CCL21 protein is produced by the parathyroid-specific domain at E11.5, and is absent from the parathyroid domain in *Gcm2*$^{-/-}$ mutants at E11.5 (Liu et al., 2006). This result indicated that Gcm2 regulates parathyroid-specific expression of *CCL21*, and providing functional evidence that the parathyroids play a role in the attraction of hematopoietic-derived T-lymphoid progenitor cells to the developing parathyroid/thymus primordium. Both *CCL21* and *CaSR* mRNA are expressed in the dorso-anterior *Gcm2* positive parathyroid domain of the 3rd pouch at E10.5 in wild type embryos (Fig. 2.3 A, C, E) (Bowl et al., 2005). *CasR* is also expressed in a similar domain of the other three pharyngeal pouches at E10.5 (Fig. 2.3E), but by E11, *CasR* expression was specifically maintained in the dorsal parathyroid domain of the 3rd pouch but down regulated in the other three pouches (Fig. 2.3G). In *Gcm2*$^{-/-}$ mutants, both *CCL21* and *CasR* expression in the pharyngeal region was initiated normally, although CCL21 was reduced compared to

controls (Fig. 2.3D, F). At E11, CaSR was not maintained in pouch 3 (Fig. 2.3H). Thus, expression of both of these early parathyroid markers was initiated in the *Gcm2* mutants, indicating that the initial patterning of the parathyroid domain in the 3rd pouch is normal in the *Gcm2*$^{-/-}$ mutants

At E11.5 in control embryos, both *CasR* and *CCL21* were present specifically in the parathyroid domain of the common primordium (Fig. 2.4A, B, D). In contrast, both of these markers were absent from the presumptive parathyroid domain in the common primordium in E11.5 *Gcm2*$^{-/-}$ mutants (Fig. 2.4C, E). These results show that while the initial expression of parathyroid-specific markers does not absolutely require *Gcm2*, *Gcm2* is required for wild type initial levels of *CCL21* and for maintenance of both *CCL21* and *CasR* expression in the parathyroid domain of the shared primordium.

Perhaps the most characteristic parathyroid marker is parathyroid hormone. The major function for the mature parathyroid glands is to produce and secrete PTH in the circulation to regulate extracellular calcium concentration. *Pth* mRNA has been detected in the presumptive parathyroid domain as early as E11.5 (Gunther et al., 2000). To determine whether this represented the earliest time point of *Pth* expression, we checked *Pth* mRNA expression and protein localization in the wild-type embryos beginning at E9.5, when *Gcm2* is first expressed in the 3rd pouch. At E9.5 and E10.5, *Pth* mRNA was not detected in the 3rd pouch (Fig. 2.5A and data not shown). The initial expression of *Pth* mRNA was seen at E11.5 in the parathyroid-specific domain (the anterior and dorsal part) of the parathyroid/thymus primordium (Fig. 2.5B), consistent with previous data (Gunther et al., 2000). *Pth* mRNA was then maintained in the parathyroid at all subsequent stages examined (Figs. 2.5C-F). The timing of initial *Pth* expression characterizes *Pth* as a late differentiation marker for parathyroid cells, compared to

CCL21 and *CasR*. Immunohistochemistry using a PTH antibody also showed that PTH protein was produced in the parathyroid-specific domain as early as E11.5, as soon as *Pth* mRNA was detected, and was maintained in the parathyroid at all subsequent stages examined (Figs. 2.5G-I). These *Pth* mRNA and protein studies suggest that the parathyroid precursor cells in the parathyroid-specific domain can produce PTH at E11.5, prior to separation from the thymus in wild-type embryos. However, *Pth* mRNA and protein were never present in the parathyroid domain in *Gcm2*$^{-/-}$ mutant embryos (Figs. 2.5J-N), even though the parathyroid domain is morphologically normal before E12.5 in *Gcm2*$^{-/-}$ mutant embryos (Figs. 2.1D, F). This result confirms that *Pth* expression requires *Gcm2* as in the previous report (Gunther et al., 2000).

Taken together, these marker studies present a time course of parathyroid differentiation, initiating with *Gcm2* at E9.5, followed by *CCL21* and *CasR* at E10.5 and *Pth* at E11.5. The earliest markers of the parathyroid domain, *CaSR* and *CCL21*, are both initiated at the right time and place in *Gcm2* mutants, and are entirely or partially independent of Gcm2 at this stage. However, subsequent differentiation, as indicated by initiating *Pth* expression and maintenance of the early markers, failed in the *Gcm2* mutants.

Tbx1 expression in the dorsal pouch is normal in *Gcm2* mutants

Our previous studies and others have shown that the transcription factor Tbx1 is also expressed in the dorsal and anterior 3rd pouch endoderm in wild-type embryos at E10.5, strikingly similar to the *Gcm2* expression domain (Manley et al., 2004; Vitelli et al., 2002; Zhang et al., 2005) (Fig. 2.6A, C). *Tbx1* has also been identified as a downstream target of Shh signaling in the pharyngeal endoderm (Garg et al., 2001; Yamagishi et al., 2003), as has *Gcm2* (Moore-Scott and Manley, 2005). *Gcm2* expression at E10.5 and parathyroid organogenesis are also absent in *Tbx1*$^{-/-}$ mutants, although in this case the loss is secondary to the absence of the 3rd

and 4th pouches (Ivins et al., 2005; Jerome and Papaioannou, 2001; Vitelli et al., 2002). Consistent with this early similarity between *Tbx1* and *Gcm2* expression, at E11.5 *Tbx1* expression was restricted to the parathyroid-specific domain (Fig. 2.6E). There was no change in *Tbx1* expression in *Gcm2*$^{-/-}$ mutant embryos at E10.5 or E11. 5 (Fig. 2.6B, D, F). The restricted expression pattern of *Tbx1* in the 3rd pouch endoderm and in the parathyroid-specific domain of the common primordium in the *Gcm2* mutants places *Tbx1* upstream of *Gcm2* in the parathyroid domain.

Gcm2 is downstream of known transcription factor and signaling networks in parathyroid/thymus organogenesis

A number of transcription factor and signaling pathways have been identified as playing a role in the patterning of the third pouch into parathyroid and thymus domains and/or in the initiation of primordia formation. Mutants for *Hoxa3, Pax1, Pax9, Eya1*, and *Shh* all have absent or reduced *Gcm2* expression at E10.5-11.5 (Moore-Scott and Manley, 2005; Su et al., 2001; Xu et al., 2002). These transcription factor and signaling pathways should function upstream to *Gcm2*, based on their expression patterns and mutant phenotypes.

To confirm that *Gcm2* is downstream of the *Hoxa3-Pax1/9-Eya1* pathway, we studied these genes' expression in *Gcm2*$^{-/-}$ mutants using whole mount *in situ* hybridization or LacZ reporter transgenic mice. *Hoxa3* (Figs. 2.7A and B), *Pax1* and *Pax9* (Figs. 2.7C-F), and *Eya1* expression (Figs. 2.7G and H) were all the same in both wild-type and *Gcm2*$^{-/-}$ mutant embryos. Combined with previous data, these results show that *Gcm2* expression is down stream of this transcriptional network.

Our previous data have implicated the Shh and Bmp4 signaling pathways in the patterning of the organ-specific domains in the 3rd pouch, with Shh required for parathyroid domain formation and *Gcm2* expression, and opposing Bmp4 in the presumptive thymus domain (Moore-Scott and Manley, 2005; Patel et al., 2006). The Bmp antagonist Noggin is also expressed in the dorsal anterior 3rd pouch overlapping with *Gcm2* at E10.5-11.5 (Patel et al., 2006). *In situ* hybridization for *Shh* and *Ptc* in wild-type and *Gcm2*$^{-/-}$ mutant embryos did not show any difference (Figs. 2.8A-D), consistent with this signaling pathway functioning upstream *Gcm2*. We used *Bmp4*^{lacZ} and *Noggin*^{lacZ} transgenic mouse strains to perform a detailed study of *Bmp4* and *noggin* expression from E10.5-11.5 in the wild-type and *Gcm2*$^{-/-}$ mutant embryos. Both *Bmp4* and *noggin* expression were unchanged in wild-type and *Gcm2*$^{-/-}$ mutant embryos at all stages tested (Fig. 2.9A-L), suggesting that this pathway is also not affected by *Gcm2* mutation, and confirming co-localization of *Bmp4* with presumptive thymus cells and normal patterning of the pouch and primordium in the *Gcm2*$^{-/-}$ mutants.

DISCUSSION

Our data suggest that *Gcm2* is required for the differentiation of parathyroid precursor cells in the parathyroid-specific domain, but is not required for initial patterning or initial expression of differentiation markers of the parathyroid domain in the 3rd pharyngeal pouches and common parathyroid/thymus primordia. Our data also show that *Gcm2* acts downstream of the known transcription and signaling pathways that function in parathyroid/thymus organogenesis. In this gene network, *Gcm2* acts as a parathyroid-specific regulator gene for parathyroid differentiation, such that in the absence of *Gcm2*, the parathyroid precursor cells form and express initial differentiation markers, but cannot complete differentiation, and subsequently undergo apoptosis.

Based on its expression pattern, the initial description of the *Gcm2* mutant phenotype in mouse, the *Shh* mutant phenotype, and the role of the *Drosophila Gcm* gene in cell fate specification, *Gcm2* has been proposed by us and others to act as the master regulator gene that establishes the initial specification of the parathyroid domain (Balling and Erben, 2000; Berg, 2002; Manley and Blackburn, 2004). This role for *Gcm2* predicted that aparathyroidsim in the *Gcm2* mutants would be due to failure to specify the parathyroid domain in the third pouch. This failure might then result in either early apoptosis of the cells that would normally form the parathyroid domain in the third pouch, or transformation of the parathyroid domain to a thymus fate.

Our results did not support either of these predicted roles for *Gcm2*. The cell fate analysis of the 3rd pouch-derived parathyroid/thymus primordium showed that the parathyroid domain formed and was morphologically normal before E12.5 in *Gcm2*$^{-/-}$ mutants. *Gcm2* also did not act as a binary switch between parathyroid and thymus fates, since the presumptive parathyroid domain did not express the thymus-specific marker *Foxn1* in *Gcm2*$^{-/-}$ mutants, and did express the early parathyroid differentiation markers *CaSR* and *CCL21*. The *Foxn1* expressing domains were also a similar size in *Gcm2*$^{-/-}$ mutants and wild-type controls, and *Bmp4* expression in the presumptive thymus domain was also normal in *Gcm2*$^{-/-}$ embryos. These results further showed that Gcm2 does not normally act to suppress thymus fate or thymus-specific gene expression in the parathyroid domain in the third pouch.

Our results show *Gcm2* is required for the differentiation of the parathyroid domain after it is formed. Among three different parathyroid cell marker genes we test in *Gcm2*$^{-/-}$ mutants, we found three different regulation models. *Gcm2* is required for initial *Pth* expression at E11.5. On the other hand, the initial expression of *CasR* and *CCL21* genes is at least somewhat independent

of *Gcm2*, but do require *Gcm2* to maintain their expression. For *CasR*, this may not be surprising, as it is initially expressed in all four pouches at E10.5 – in this case, *Gcm2* seems to take the role of maintaining expression only in the parathyroid domain. However, *CCL21* expression is initially restricted to the 3rd pouch, but only partially depends on *Gcm2* for full initial expression levels. The expression analysis of these three parathyroid differentiation markers reveals a surprising complexity in the regulation of parathyroid differentiation, and indicates that at least one other transcription factor is required to establish correct initial expression of these factors. The maintenance of all of these genes at later stages may also require *Gcm2*, since *Gcm2* expression is maintained in adult parathyroid cells, although that remains to be experimentally determined.

Gcm2 not only regulates the differentiation procedure of parathyroid precursor cells, it is also required for their survival. This phenotype may reflect a direct role for *Gcm2* in promoting cell survival. Our previous analysis of $Hoxa3^{+/-}Pax1^{-/-}$ compound mutants showed that loss of *Gcm2* expression after E11.5 resulted in progressive loss of parathyroids (Su et al., 2001). Interestingly, there was a delay of up to two days before this phenotype was evident, similar to the time delay between initial expression of *Gcm2* at E9.5 and cell death at E12 in the *Gcm2* mutants. Alternatively, in the absence of normal differentiation parathyroid cells either fail to acquire responsiveness to survival signals in the environment, or activate a default apoptotic fate.

The restricted expression of several genes in the presumptive parathyroid domain even in the absence of *Gcm2* strongly suggests that this domain is specified and initiates parathyroid differentiation in these mutants. If *Gcm2* does not specify the parathyroid domain, what does? From its expression pattern and its position upstream of Gcm2, Tbx1 is a likely candidate to specify the parathyroid domain in the 3rd pouch. At E10.5, *Tbx1* expression is restricted to the

dorsal and anterior part in the 3rd pouch endoderm that will become the parathyroid domain (Manley et al., 2004; Vitelli et al., 2002; Zhang et al., 2005), and remains restricted to the parathyroid domain in the parathyroid/thymus primordia (Fig. 2.6E). This expression pattern is consistent with *Tbx1* functioning in early parathyroid organogenesis. The Shh pathway is also required to establish the parathyroid domain, as *Gcm2* expression is lost and the thymus domain extended into the pharynx in *Shh* mutants (Moore-Scott and Manley, 2005). *Tbx1* is also thought to be a down stream target of the Shh signaling pathway (Garg et al., 2001; Yamagishi et al., 2003). Taken together, these data support a model in which specification of the parathyroid domain is regulated through a Shh-Tbx1-Gcm2 pathway.

In addition to the Shh pathway, a Hoxa3-Pax1/9-Eya1 pathway is also required for 3rd pouch patterning and initiation of parathyroid/thymus primordium formation, and may directly regulate *Gcm2* expression. *Gcm2* expression is absent or down regulated in *Hoxa3*$^{-/-}$ and *Eya1*$^{-/-}$ embryos at E10.5 (our unpublished data)(Xu et al., 2002). Consistent with this data, the expression of the *Hoxa3-Pax1/9-Eya1* pathway in *Gcm2*$^{-/-}$ embryos is normal at E10.5. How the Shh-Tbx1 and Hoxa3-Pax1/9-Eya1 pathways converge to regulate *Gcm2* and parathyroid cell fate is a key remaining question in understanding parathyroid organogenesis.

The current results indicate that in spite of their different timing for initial expression, both *Gcm2* and *Foxn1* play analogous roles in the development of the parathyroid and thymus domains in the common primordium – both genes are not required for initial specification of the organ domains or initial primordium formation, but are required for subsequent tissue-specific differentiation events. However, while *Foxn1* initial expression is at E11.25, similar to the appearance of the first identifiable phenotype, *Gcm2* expression in the 3rd pouch endoderm is initiated from E9.5, well before the parathyroid/thymus primordium is formed. The decreased

initial expression of *CCL21* does indicate some role for *Gcm2* at least at E10.5, even though it is not required for domain specification. The earlier expression could also be due to differences in the timing of expression of upstream regulation of the two genes. The location of parathyroids within the endoderm of the pharyngeal pouches is evolutionarily conserved, and in teleost fishes is required for gill bud formation, which precedes thymus organogenesis in these animals (Graham et al., 2005; Okabe and Graham, 2004; Schorpp et al., 2002; Willett et al., 1997). The molecular pathways that specify the parathyroid domain may turn on earlier than those of the thymus due to this evolutionary legacy. A consequence of this earlier expression may also be to carve out the parathyroid domain within the third pouch and protect it from a thymus fate.

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Figure 2.1. Cell fate analysis of the 3rd pouch-derived parathyroid/thymus common primordia in wild-type and *Gcm2*$^{-/-}$ mutant embryos. Section *in situ* hybridization of *Gcm2* on sections of wild-type embryos at E11.5 (A) and E12.5 (B) is shown for comparison. *Foxn1* in situ hybridization was performed on the sections from wild-type (C, E, G and I) and *Gcm2*$^{-/-}$ (D, F, H and J) embryos at E11.5 (C, D), E12 (E, F), E12.5 (G, H), and E13 (I, J) stages. Sections were cut in the sagittal plane. In all figures, anterior is up, and dorsal is to the right. Ages of embryos are indicated in the upper right corner of each panel. The thymus domains in panels A and B, and the parathyroid domains in panels C-J are outlined. pt, parathyroid; th, thymus.

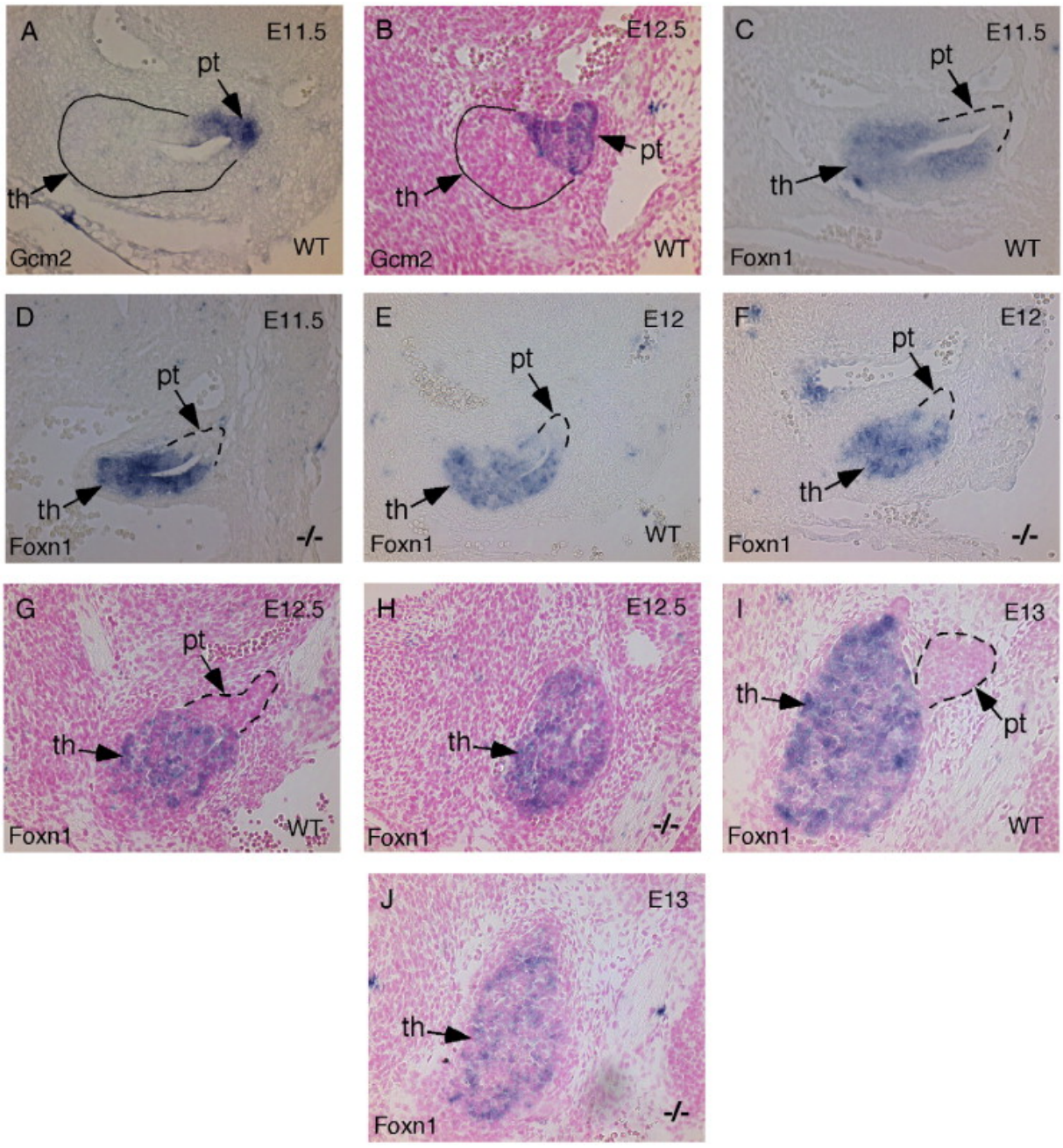
A
E11.5
pt
th
Gcm2
WT
B
E12.5
pt
th
Gcm2
WT
C
E11.5
pt
th
Foxn1
WT
D
E11.5
pt
th
Foxn1
-/-
E
E12
pt
th
Foxn1
WT
F
E12
pt
th
Foxn1
-/-
G
E12.5
pt
th
Foxn1
WT
H
E12.5
th
Foxn1
-/-
I
E13
th
pt
Foxn1
WT
J
E13
th
Foxn1
-/-

Figure 2.1

Figure 2.2. TUNEL analysis of cell death in the parathyroid/thymus primordia in wild-type and *Gcm2*$^{-/-}$ mutant embryos. TUNEL was performed on a complete sagittal section series prepared from wild-type controls (A, E) and *Gcm2*$^{-/-}$ embryos (B, F) at E11.5 (A and B) and E12 (E and F) stages. Anterior is up, and dorsal is to the right. In panels A-B, the common parathyroid/thymus primordium is outlined in white, and the 3rd pouch from which the primordium is undergoing separation by apoptosis is outlined in yellow (PIII). At E12, *in situ* hybridization for *Foxn1* to indicate the thymus domain (C, D) was performed on alternate sections with the sections used for TUNEL (E, F) to confirm that the location of the apoptotic cells are in the parathyroid domain in *Gcm2* null mutant. The dorso-anterior parathyroid domain at E12 is indicated by a dashed line in C-F. The apoptotic cells in the parathyroid domain are indicated with a white arrow in panel F.

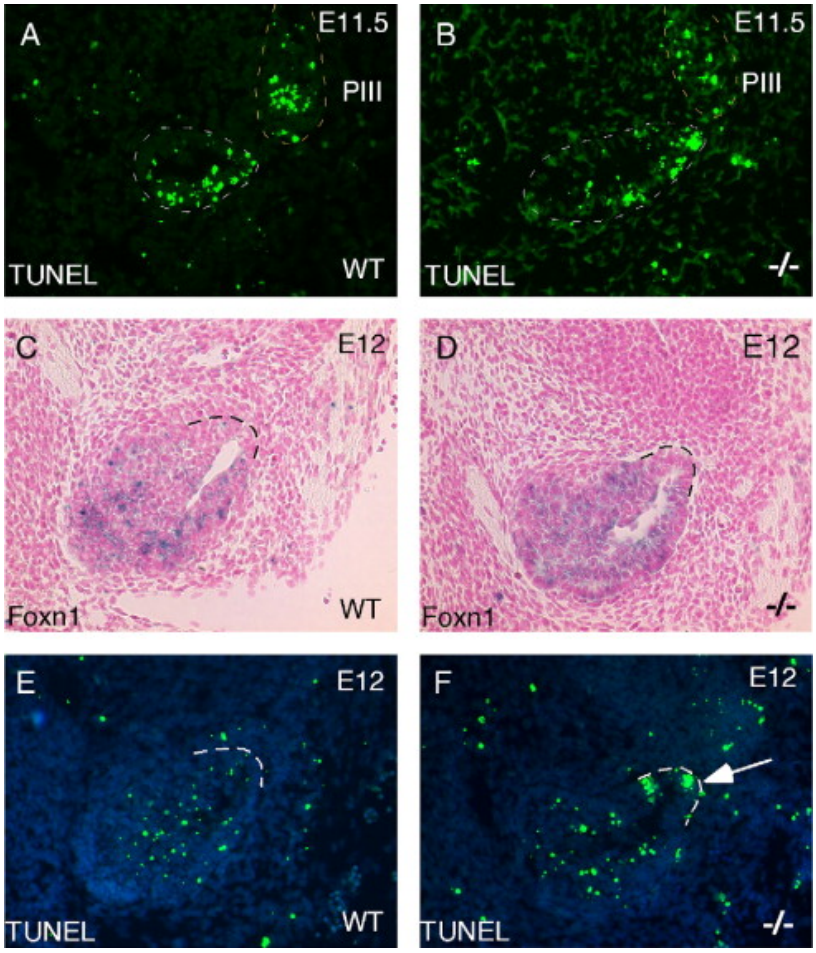
A
E11.5
PIII
TUNEL
WT
B
E11.5
PIII
TUNEL
-/-
C
E12
Foxn1
WT
D
E12
Foxn1
-/-
E
E12
TUNEL
WT
F
E12
TUNEL
-/-

Figure 2.2

Figure 2.3. The expression of *CasR* and *CCL21* in wild-type and *Gcm2*$^{-/-}$ mutant embryos. Whole mount *in situ* hybridization for *Gcm2* at E10.5 (A) and E11 (B) is shown for comparison. *CCL21* (C, D) and *CasR* (E-H) expression is shown in wild-type (C, E, G) and *Gcm2*$^{-/-}$ (D, F, H) embryos at E10.5 (C-F). *CasR* expression is also shown at E11 (G, H). In all panels, dorsal is to the right, anterior is up. *CasR* expression was initiated at the dorsal sides of all four pouches (p1-p4) in the wild-type E10.5 embryo (E), and this expression was not affected by *Gcm2* null mutation (F). The initiation of CCL21 expression at the dorsal side of 3rd pouch (C) partially required *Gcm2* function (D). *CasR* expression was maintained only in the parathyroid domain at E11 in the wild-type (G), and this expression required *Gcm2* function (H). In A-D, arrows indicate dorsal/anterior parathyroid domains. a1, first arch; a2, second arch.

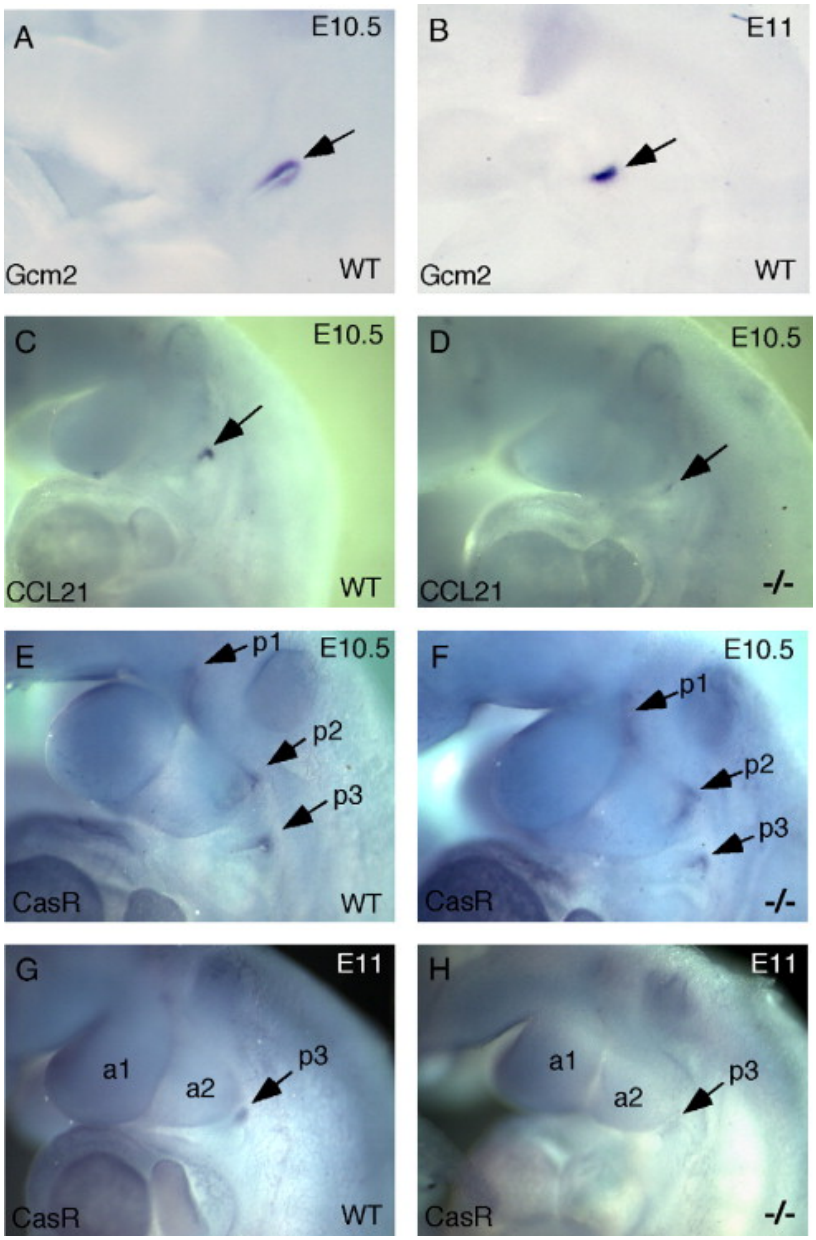
A
E10.5
Gcm2
WT
B
E11
Gcm2
WT
C
E10.5
CCL21
WT
D
E10.5
CCL21
-/-
E
p1
E10.5
p2
p3
CasR
WT
F
E10.5
p1
p2
p3
CasR
-/-
G
E11
a1
a2
p3
CasR
WT
H
E11
a1
a2
p3
CasR
-/-

Figure 2.3.

Figure 2.4. Maintenance of *CCL21* and *CasR* expression in the parathyroid domain at E11.5 requires *Gcm2*. Sections were cut at sagittal plane. In all figures, anterior is up, and dorsal is to the right. The common parathyroid/thymus primordium is outlined and the parathyroid domain is indicated with an arrow in A, B, D. *In situ* hybridization for *Gcm2* at E11.5 (A) is shown for comparison. *CCL21* (B) and *CasR* (D) are restricted to the parathyroid domain at E11.5 in wild-type embryos. Neither is expressed in *Gcm2* mutants (C, E).

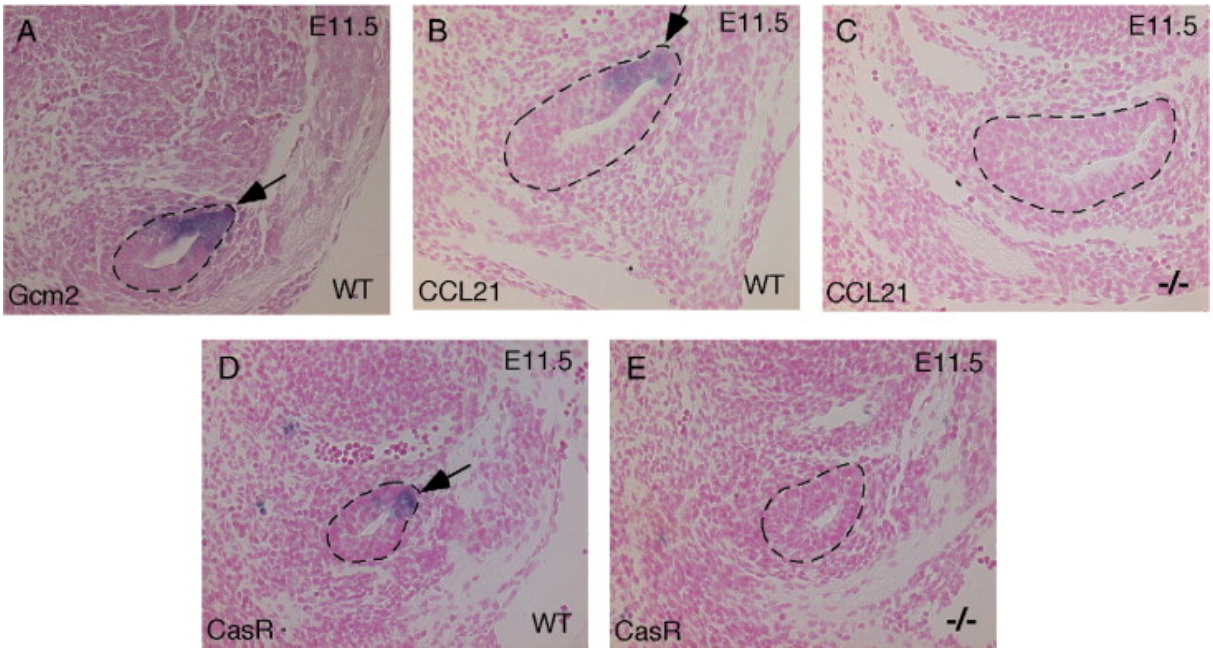
A
E11.5
Gcm2
WT
B
E11.5
CCL21
WT
C
E11.5
CCL21
-/-
D
E11.5
CasR
WT
E
E11.5
CasR
-/-

Figure 2.4

Figure 2.5. The expression of *Pth* in wild-type and *Gcm2*$^{-/-}$ mutant embryos. Section *in situ* hybridization of *Pth* was performed on the sections prepared from the wild-type embryos at E10.5 (A), E11.5 (B), E12 (C), E12.5 (D), E13.5 (E), and E18.5 (F). Immunohistochemistry of PTH was performed on the sections prepared from the wild-type embryos at E11.5 (G), E12.5 (H), and E18.5 (I). The initiation of *Pth* mRNA and protein expression was not present in the *Gcm2*$^{-/-}$ mutants at E11.5 (J and M), and *Pth* mRNA and protein expression was also not present in late stages at E12 (K and N), and E12.5 (L). Sections were cut at sagittal plane. In all figures, anterior is up, and dorsal is to the right. Arrows in panels B-I indicate the parathyroid domains or parathyroid glands. The common parathyroid/thymus primordium is outlined in panels B, C, G, H, J-N. p3, third pouch; a1, first arch; a2, second arch; h, heart; th, thymus; tr, thyroid; pt, parathyroid.

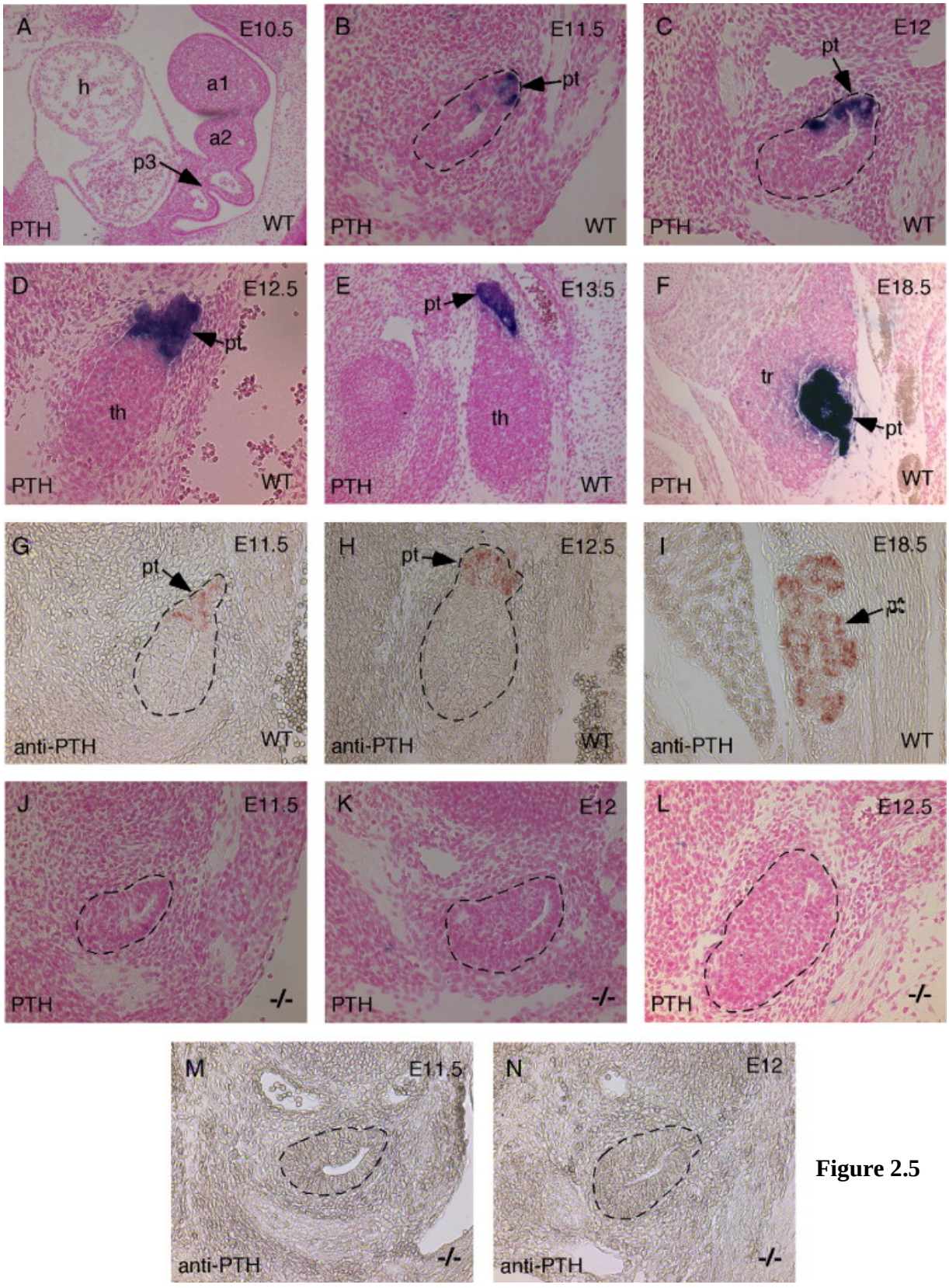
A
E10.5
h
a1
a2
p3
PTH
WT
B
E11.5
pt
PTH
WT
C
E12
pt
PTH
WT
D
E12.5
pt
th
PTH
WT
E
E13.5
pt
th
PTH
WT
F
E18.5
tr
pt
PTH
WT
G
E11.5
pt
anti-PTH
WT
H
E12.5
pt
anti-PTH
WT
I
E18.5
pt
anti-PTH
WT
J
E11.5
PTH
-/-
K
E12
PTH
-/-
L
E12.5
PTH
-/-
M
E11.5
anti-PTH
-/-
N
E12
anti-PTH
-/-

Figure 2.5

Figure 2.6. Expression of *Tbx1* in wild-type and *Gcm2*$^{-/-}$ mutant embryos. Whole mount (A, B) or paraffin section (C-F) *in situ* hybridization for *Tbx1* at E10.5 (A-D) and E11.5 (E, F). In the wild-type, *Tbx1* expression was present at the dorsal side of 3rd pouch at E10.5 (A, C) and at the parathyroid-specific domain in the parathyroid/thymus primordium at E11.5 (E). This expression is not affected by *Gcm2* null mutation (B, D, F). In A and B, anterior is up, and dorsal is to the left. Sections were cut in the saggital plane. In C-F, anterior is up, and dorsal is to the right. Arrows in E and F indicate the parathyroid domain. p3, third pouch; a1, first arch; a2, second arch.

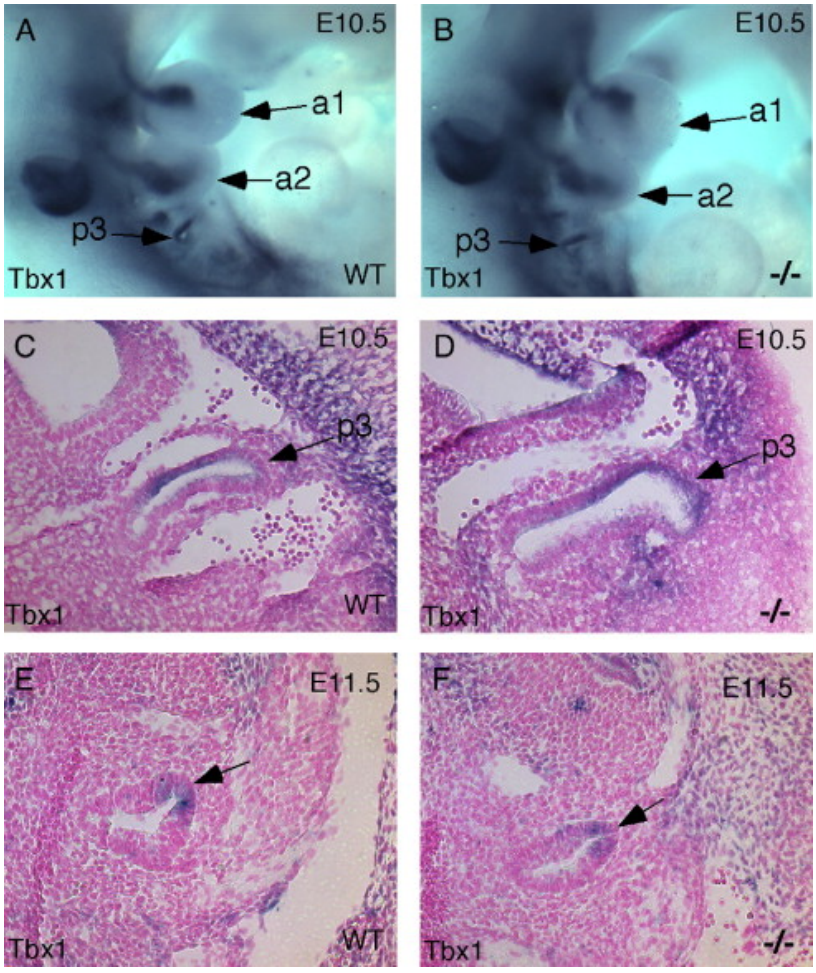
A
E10.5
a1
a2
p3
Tbx1
WT
B
E10.5
a1
a2
p3
Tbx1
-/-
C
E10.5
p3
Tbx1
WT
D
E10.5
p3
Tbx1
-/-
E
E11.5
Tbx1
WT
F
E11.5
Tbx1
-/-

Figure 2.6

Figure 2.7. Expression of the *Hoxa3-Pax1/9-Eya1* pathway in wild-type and *Gcm2*$^{-/-}$ mutant embryos. Whole mount *in situ* hybridization (A-D, G and H) or LacZ staining (E and F) was performed on wild-type (A, C, E and G) and *Gcm2*$^{-/-}$ (B, D, F, and H) embryos at E10.5 for Hoxa3 (A and B), Pax1 (C and D), Pax9 (E and F), and Eya1 (G and H). The expression of these genes in the 3rd pouch was normal in *Gcm2* mutants. The 3rd pouch is indicated in each panel (p3).

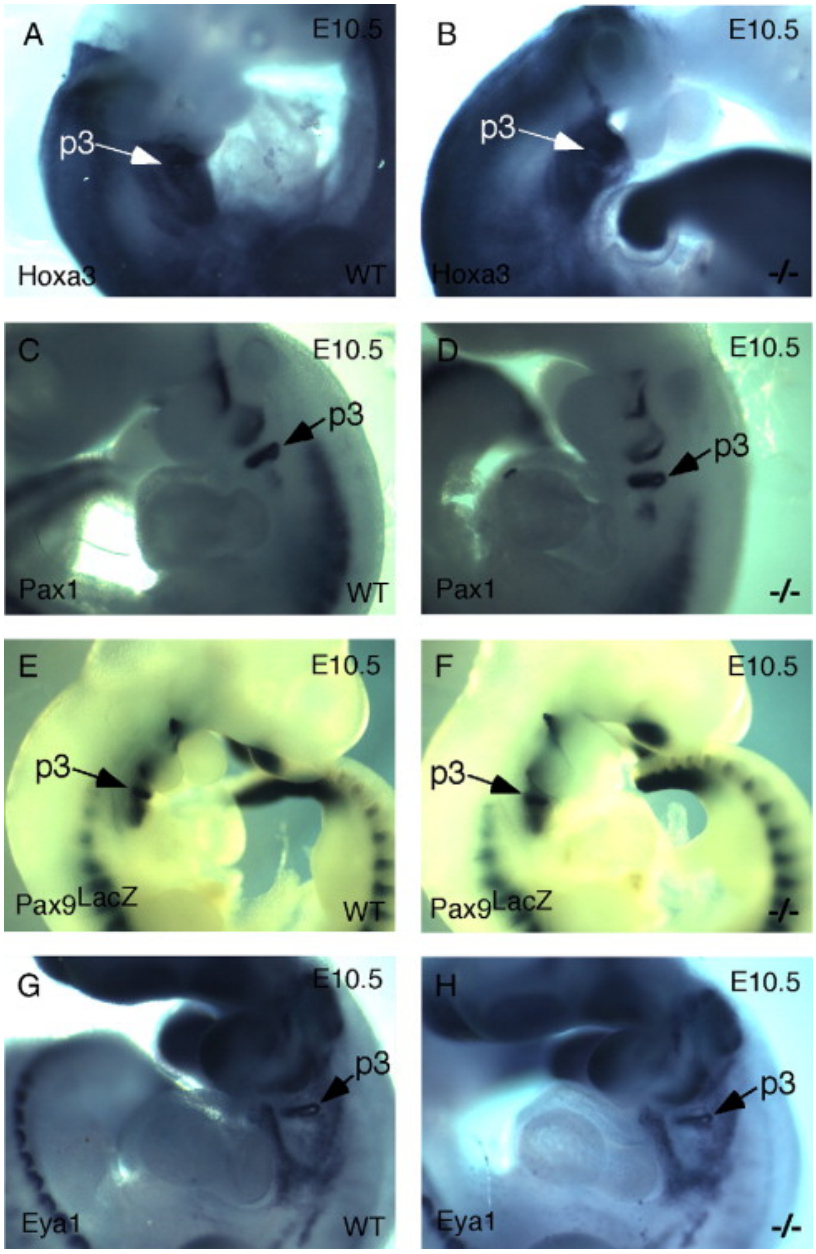
A
E10.5
p3
Hoxa3
WT
B
E10.5
p3
Hoxa3
-/-
C
E10.5
p3
Pax1
WT
D
E10.5
p3
Pax1
-/-
E
E10.5
p3
Pax9LacZ
WT
F
E10.5
p3
Pax9LacZ
-/-
G
E10.5
p3
Eya1
WT
H
E10.5
p3
Eya1
-/-

Figure 2.7

Figure 2.8. Expression of *Shh* and *Ptc1* in the 3rd pouch of wild-type and *Gcm2*$^{-/-}$ mutant embryos. Section *in situ* hybridization for *Shh* (A and B) and *Patched1* (C and D) was performed on transverse paraffin sections from wild-type (A and C) and *Gcm2*$^{-/-}$ (B and D) embryos at E10.5. The expression of these genes in the 3rd pouch was normal in *Gcm2* mutants. Dorsal is up. p3, third pouch.

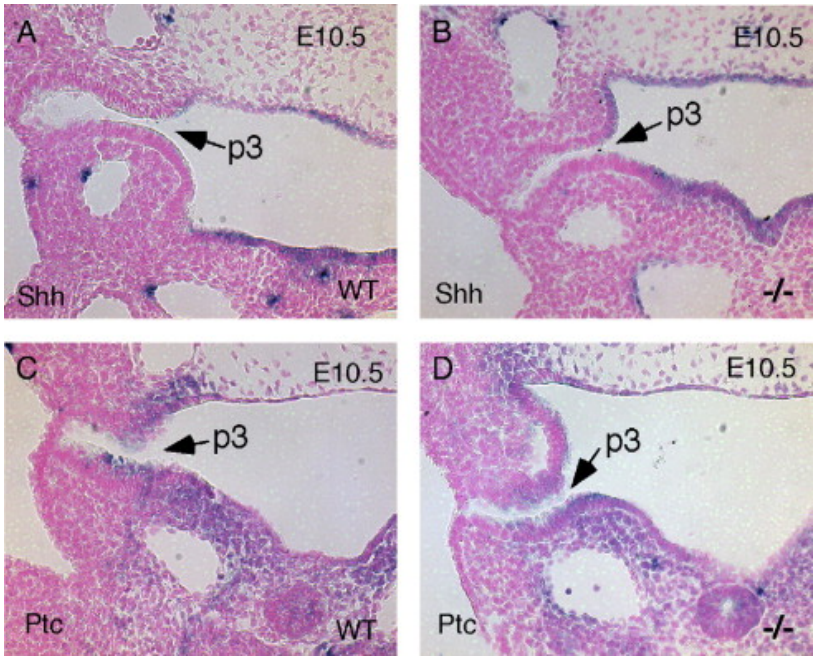

Figure 2.8

Figure 2.9. Expression of *Bmp4* and *Noggin* in the 3rd pouch of wild-type and *Gcm2*$^{-/-}$ mutant embryos. Whole mount LacZ staining for *Bmp4*^{LacZ} (A- F) and *Noggin*^{LacZ} (G-L) was performed on wild-type (A, C, E, G, I and K) and *Gcm2*$^{-/-}$ (B, D, F, H, J and L) at E10.5 (A, B, G and H), E11 (C, D, I and J), and E11.5 (E, F, K and L). In panels A-F, the black arrows indicate *Bmp4* expression restricted to the ventral/posterior thymus domain in the 3rd pouch (p3) and common primordium. In panels E and F, the Bmp4-negative dorsal domain is indicated with a white arrow. In panels G-L, arrows indicate dorsally restricted *noggin* domain. Arrowheads in panels G-L indicate the ventral/posterior thymus domain.

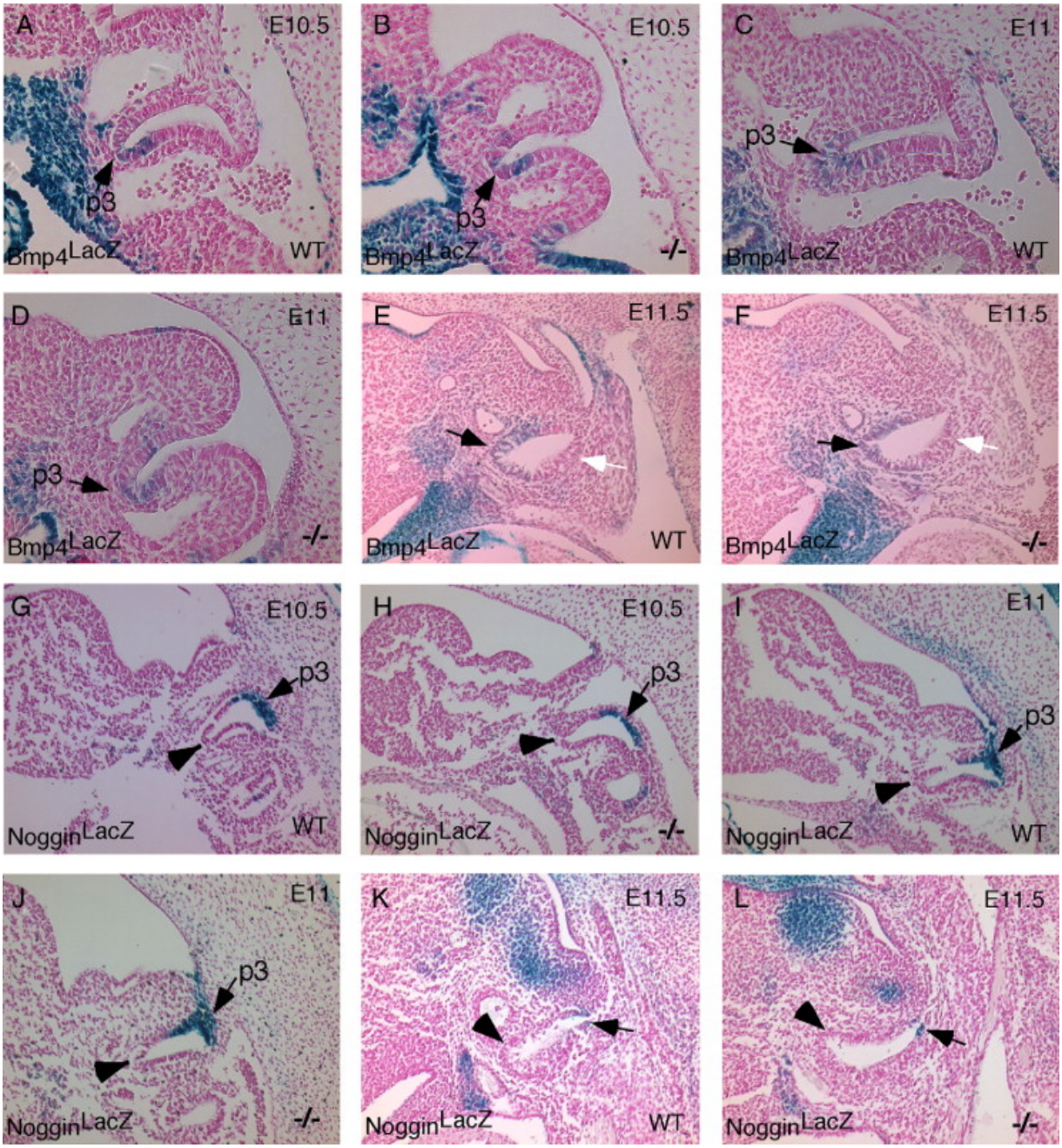
A
E10.5
p3
Bmp4LacZ
WT
B
E10.5
p3
Bmp4LacZ
-/-
C
E11
p3
Bmp4LacZ
WT
D
E11
p3
Bmp4LacZ
-/-
E
E11.5
Bmp4LacZ
WT
F
E11.5
Bmp4LacZ
-/-
G
E10.5
p3
NogginLacZ
WT
H
E10.5
p3
NogginLacZ
-/-
I
E11
p3
NogginLacZ
WT
J
E11
p3
NogginLacZ
-/-
K
E11.5
NogginLacZ
WT
L
E11.5
NogginLacZ
-/-

Figure 2.9

CHAPTER 3

PARATHYROID HORMONE EXPRESSION IN THE THYMUS IS NOT A BACKUP MECHANISM FOR THE PARATHYROID GLANDS[1]

[1]Zhijie Liu, Lizhen Chen, Nancy R. Manley. To be submitted to PNAS.

ABSTRACT

In the classic view, parathyroid hormone (PTH), a key regulator hormone for calcium and inorganic phosphorus homeostasis in the extracellular environment in mammals, was thought only to be produced by parathyroid glands. The initial report of the aparathyroid phenotype on *Gcm2* mutants has changed this view. Normal PTH levels and a mild lethality phenotype in *Gcm2*$^{-/-}$ mutants were reported and proposed to result from a backup mechanism of PTH expression from the thymus, an organ that shares a common origin with parathyroids at their early organogenesis stages.

In this paper, we explored the mechanism of the regulation of thymic PTH expression in mouse. We found that the major thymic PTH expression in the wild-type mice was from clusters of misplaced parathyroid cells that result from the incomplete separation of the parathyroid and thymus organs. In *Gcm2* null mutants, thymic PTH expression was greatly reduced due to the ablation of these misplaced parathyroid cells. We further identified a second source of PTH in medullary thymic epithelial cells (mTECs), which is *Gcm2*-independent but requires the differentiation of mTECs. We also found that serum PTH levels were similarly reduced in *Gcm2*$^{-/-}$ mutants and *Gcm2*$^{-/-}$; *Foxn1*$^{nu/nu}$ double mutants with no PTH from either parathyroids or thymus, suggesting that mTEC-derived PTH in *Gcm2*$^{-/-}$ mutants can not backup the endocrine function of parathyroids. Furthermore, we demonstrated that the lethality phenotype of *Gcm2*$^{-/-}$ mutants was exacerbated on C57BL/6J genetic background with no change in serum PTH levels, which further confirmed that the lethality phenotype of *Gcm2*$^{-/-}$ mutants was not correlated with thymic PTH. Collectively, our data show that the lethality of *Gcm2*$^{-/-}$ mutants is not related to PTH reduction, suggesting extra-parathyroid functions for *Gcm2*.

INTRODUCTION

Mammals have evolved a very fine system, consisting of the parathyroid glands, bone, kidney and the intestine, to regulate ionized calcium and inorganic phosphorus homeostasis in the extracellular environment (Ramasamy, 2006). In this system, an 84 amino acid peptide called parathyroid hormone (PTH) produced by parathyroids plays as the key endocrine regulator to modulate the physiological actions in the bone, kidney and the intestine to maintain the homeostasis of ionized calcium and inorganic phosphorus concentrations in the extracellular environment (Houillier et al., 2003). Circulating ionized Ca^{2+} is involved in a wide range of physiological activities, including neuromuscular excitability, muscle contraction, bone mineralization, blood coagulation and cardiovascular functions (Bootman et al., 2001; Clapham, 1995). Failure of calcium homeostasis, which can result from PTH production disorders, causes diseases in human (Thakker, 2001).

For a long time, parathyroid glands were thought to be the sole source of PTH production and secretion. However, a recent study on the aparathyroid mutation phenotype of the *Gcm2* gene, an important regulator gene required for parathyroid organogenesis (Gordon et al., 2001; Kim et al., 1998; Liu et al., 2007), identified the thymus, a primary lymphoid organ, as an auxiliary source of circulating PTH to backup the parathyroids in mice (Gunther et al., 2000). Thymic PTH was found to come from small clusters of unidentified cells under the thymic capsule. However, the ontogeny of these PTH-expressing cells and the regulation of PTH expression in these cells are not clear.

Intriguingly, despite their distinct physiological functions and final locations, parathyroids and thymus have a close relationship during their organogenesis. At early organogenesis stages, these two organs develop together from the 3rd pharyngeal pouch endoderm and share two

parathyroid/thymus common primordia originating from the 3rd pouches (Manley and Blackburn, 2004). These two organs are also controlled by the same regulatory molecules, including Hoxa3, Pax1, Pax9 and Eya1 (Chisaka and Capecchi, 1991; Peters et al., 1998; Su et al., 2001; Xu et al., 2002), although their differentiation is regulated by two different transcription factors, Gcm2 and Foxn1 (Blackburn et al., 1996; Liu et al., 2007).

The mature thymus provides a microenvironment for T lymphocyte maturation, which consists of a phenotypically diverse group of cells, including thymic epithelial cells (TECs), macrophages, dendritic cells, fibroblasts, and endothelial cells (Gray et al., 2002). Among these different cells, TECs play an important role in the production of a self-restricted and self-tolerant T-cell repertoire through positive selection and negative selection (Anderson et al., 1996). Negative selection occurs in the medullary region, where mTECs promiscuously express many tissue-restricted self-antigens (TRAs) that are required for negative selection to establish central tolerance and prevent autoimmunity (Derbinski et al., 2001; Farr et al., 2002). For example, insulin, a hormone produced by the pancreas, was found to be expressed in the thymus and be required to prevent autoimmunity disease of the pancreas (French et al., 1997; Nakayama et al., 2005; Pugliese et al., 2001). Similarly, although the thymus has been proposed to function as a backup mechanism of the parathyroids by providing circulating PTH (Gunther et al., 2000), which is required for the extracellular calcium homeostasis, it is possible that PTH may also function as a self-antigen produced in the medullary thymic epithelial cells (mTECs) for negative selection.

To clarify the physiological role of thymic PTH, we investigated the origin of the PTH-expressing cells in the thymus and the regulation mechanism of PTH expression in these cells by studying parathyroid and thymus organogenesis. We observed that some parathyroid cells are

misplaced between the parathyroid and thymus and within the thymus, resulting from incomplete separation of these two organs during normal organogenesis. The ablation of these misplaced parathyroid cells in the thymus in *Gcm2*$^{-/-}$ mice caused a significant decrease of thymic PTH expression but still left a low level of thymic PTH expression, which we identified as originating from mTECs. Compared with the misplaced parathyroid cells, which express PTH by a *Gcm2*-dependent mechanism, mTECs express PTH in a *Gcm2*-independent but *Foxn1*-dependent pathway. Furthermore, our research indicates that mTEC-derived PTH, the only source of thymic PTH in *Gcm2*$^{-/-}$ mice, is not secreted into the general circulation to function as the backup mechanism of parathyroid glands, but may function as a self-antigen for negative selection.

MATERIALS AND METHODS

Mice

The generation and genotyping of *Gcm2* null mutant has been described (Gunther et al., 2000). *Gcm2* mutant mice were originated on 129/SvEv-C57BL/6J genetic background, but were backcrossed to C57BL/6J mice for more than 5 generations. For the lethality phenotype study, we crossed some *Gcm2* mice with 129S6 mice (Taconic) to obtain 129S6/C57BL/6 F1 hybrid mice.

Foxn1 nude mice were obtained from Jackson lab and maintained on a C57BL/6J and 129SvJ hybrid background. Rag2 null mutant mice were a generous gift from Dr. Ellen V. Rothenberg and were maintained on a C57BL/6J background. R26YFP reporter mice have been previously described (Srinivas et al., 2001; Zhang et al., 2005). This colony was maintained on C57BL/6J and 129SvJ hybrid background. Foxn1Cre is a knockin allele of *Foxn1* constructed by inserting IRES-Cre cassette into the 3' UTR of the *Foxn1* locus (Gordon et al., 2007).

For staging of embryos, noon on the day of the vaginal plug was designated as E0.5. All experiments were carried out with the approval of the University of Georgia institutional animal care committee.

RT-PCR and semi-quantitive RT-PCR

Isolation of RNA and RT-PCR were performed as described (Su et al., 2001). Thymi or other tissues were dissected from different stages of embryos or mice and total RNA was isolated with Trizol. Genomic DNA was removed using DNase I. DNase I enzyme was inactivated using 25 mM EDTA and being heated for 15 min at 65°C. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen). RNA was removed using RNase H and RNase A. cDNA was subjected to PCR using Qiagen PCR Taq polymerase. The following primers were used: β-actin forward 5'-TGGAATCCTGTGGCATCCATGAAAC-3', β-actin reverse 5'-TAAAACGCAGCTCAGTAACAGTCCG-3', *Pth* forward 5'-CTGCAGTCCAGTTCATCAGC-3', *Pth* reverse 5'-AAGCTTGAAAAGGTAGCAGCA-3', *Gcm2* forward 5'-CATCAATGACCCACAGATGC-3', *Gcm2* reverse 5'-GGCACTTCTTCTGCCTTCTG-3', *Foxn1* forward 5'-TGACGGAGCACTTCCCTTAC-3', *Foxn1* reverse 5'-GGGAAAGGTGTGGGTAGGTC-3', *Gcm1* forward 5'-TGAAAAACAAGCCCTTCAGC-3' and *Gcm1* reverse 5'-TCTGGCTTTGTCACAGATGG-3'.

Section in situ hybridization

Paraffin section *in situ* hybridization was performed as described (Liu et al., 2007). Staged embryos were fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. 8-10μm sections were hybridized with digoxigenin-labeled RNA probes at 0.5 µg/ml. Alkaline phosphatase-conjugated antidigoxigenin Fab fragments were used at 1:5000. BM-purple (Roche) was used as a chromagen to localize hybridized probe. Nuclear fast red was used as counterstain

before mounting. Probes for *Gcm2* (Gordon et al., 2001) and *Pth* (Liu et al., 2007) have been described.

Immunostaining

Immunostaining was performed as described (Gray et al., 2002). To detect YFP positive cells more easily, we used rabbit anti-GFP labeled by Alexa Fluor 488 (1:200, Molecular Probes).The following primary antibodies were used for immunolabeling of mTECs or cTECs: rabbit anti-K5 (1:200, Covance), rabbit anti-K14 (1:200, Covance), UEA-1-biotin (1:200, Vector) and rat anti-K8 (1:200, Troma-1 supernatant). Secondary antibodies were supplied from Jackson ImmunoResearch Laboratories: Donkey anti-rat IgG-Cy3 (1:100), Donkey anti-rabbit IgG-Texas Red (1:100) and Streptavidin-Cy3 (1:200).

Thymic epithelial cells purification

Thymic stromal cell isolation was modified from a previously described method (Gray et al., 2002). Thymi were dissected, minced into small pieces and agitated in RPMI1640 with 2% FBS to remove most of thymocytes in the supernatant. The remaining tissue pieces were collected and resuspended in RPMI1640 containing 0.2mg/ml collagenase for 20 minutes at 37^{o}C with gentle stirring. The tissue pieces were settled with gravity sedimentation for 5 minutes, the supernatant was discarded, and the tissue was resuspended in dispase media (0.2mg/ml of dispase, 0.2mg/ml of collagenase and 25ug/ml of DNaseI in RPMI 1640) for 20 minutes at 37 ^{o}C with gentle stirring. The supernatant was discarded and the tissue chunks were allowed to settled and resuspended in fresh dispase media for 30-45 minutes at 37 ^{o}C. The whole digested products were then passed through a 25G needle to break down the remaining pieces. The cells were centrifuged at 800 x g for 3minutes and resuspended in PBS containing 2% FBS and 5mM EDTA, and then filtered through a 70um cell strainer.

The filtered cells were stained with anti-mouse CD45-PE (BD pharmingen) antibody before being subjected to MoFlo cell sorter (Dako) for sorting CD45-PE^{-}, YFP^{+} TECs. The yield of TECs from each adult thymus using this method was about 20,000 cells with about 93% purity.

RNA preparation and quantitative RT-PCR

Total RNA for sorted thymic epithelial cells were extracted with RNeasy Micro kit (QIAGEN). For total RNA preparation from thymi from *Gcm2* and *Foxn1* double mutants, we also used RNeasy Micro kit (QIAGEN). For *Gcm2* and *Rag2* double mutants, total thymus was homogenized in trizol (Invitrogen) and total RNA was extracted according to manufacturer's instructions.

First-strand cDNA was reverse transcribed using superscript III (Invitrogen). Quantitative PCR was performed on an ABI 7500 real time PCR system with Taqman universal PCR mix (Applied Biosystems). 18S rRNA VIC/TAMRA primer-probe (Applied Biosystems) was used as endogenous control. *Pth* FAM primer-probe (Assay ID: Mm00451600_g1) was purchased from Applied Biosystems. The PCR program was as follows: 50 ^{o}C, 2min; 95 ^{o}C, 10min; 40 cycles of 95 ^{o}C for 15sec and 60 ^{o}C for 1min. The relative quantity of the gene expression was determined using 7500 SDS software (Applied Biosystems).

Serum Biochemistry

Serum sample collection from newborn mice or adult mice has been described (Kovacs et al., 2001; Woodrow et al., 2006). For newborn mice, blood samples were collected after neck incision. For adult mice, blood samples were collected into capillary tubes from tail vein right after mice were sacrificed by cervical dislocation, whereas a cardiac puncture was done to obtain larger samples. Serum samples were prepared by centrifugation to remove blood cells, then

stored in -20 ^{o}C until assayed .Serum PTH concentration was determined using a rat PTH 1–34 ELISA kit (Immutopics).

RESULTS

Misplaced parathyroid cells contribute the major part of thymic PTH expression

Since the thymus and parathyroid organs originate from the same primordia at their early developmental stages, we first investigated the possibility that thymic PTH expression was produced from some parathyroid cells that incorrectly migrate with the thymus following the separation of these two organs.

RT-PCR using cDNA made from total thymus showed that *Gcm2* and *Pth* were both expressed in the adult thymus (Fig. 3.1A) and the expression of these two genes was found only in the thymus and not in other organs we tested (Fig. 3.1A). Both *Gcm2* and *Pth* RT-PCR products were confirmed by sequencing. We also found that the expression of *Gcm2* and *Pth* in the thymus could be detected as early as E13.5, when the thymus had just separated from the parathyroids, and at all other later stages we tested (Fig. 3.1B). The expression of *Gcm2*, a parathyroid cell differentiation regulator gene, with *Pth* in the thymus led us to further analyze the entire separation procedure in mice using *in situ* hybridization for *Gcm2* and *Pth*. At E12, in the parathyroid/thymus common primordium, *Gcm2*/*Pth* expression specifically marked the anterior/dorsal parathyroid domain with a clear boundary with the posterior/ventral thymus-specific domain (Fig. 3.1C-I). At E13, the *Gcm2*/*Pth* positive parathyroid domain had started to separate from the thymus domain and some parathyroid cells were found to locate outside the major parathyroid domain (Fig. 3.1C-II). At E18.5, when the separation of the

parathyroid/thymus was completed, we observed some misplaced clusters of parathyroid cells located between the parathyroids and thymus, and some clusters of parathyroid cells associated with the thymus and even embedded beneath the thymic capsule (Fig. 3.1C-III and D). This phenotype was detected in all 11 E16.5-18.5 stages wild-type embryos on multiple genetic backgrounds (C57BL/6J, 129/C57BL/6J F1 hybrid, or 129S6) (Fig. 3.1E and data not shown), which indicates that this incomplete separation pattern is a common phenomenon in the mouse.

In *Gcm2* null mutant mice, all parathyroid cells undergo apoptosis before E12.5 (Liu et al., 2007). If these misplaced parathyroid cells are authentic parathyroid cells, *Gcm2* should also regulate their differentiation and survival, and we should not expect to see any misplaced parathyroid cells in *Gcm2*$^{-/-}$ mutants. As predicted, we found all the misplaced parathyroid cells were ablated together with the parathyroids in *Gcm2*$^{-/-}$ mutants (Fig. 3.2A). The ablation of misplaced parathyroid cells in the thymus was also confirmed by the semi-quantitive RT-PCR analysis of *Pth* expression in whole thymi from wild-type and *Gcm2*$^{-/-}$ mice. In *Gcm2*$^{-/-}$ mutants, the thymic *Pth* expression level was greatly reduced. However, the expression of the TEC marker *Foxn1* was not affected by *Gcm2* null mutation (Fig. 3.2B). These data suggest that misplaced parathyroid cells in the thymus are the primary source of thymic PTH.

RT-PCR using total thymus cDNA from *Gcm2*$^{-/-}$ mice still could amplify *Pth* at high cycle numbers (Fig. 3.2B), which suggested that the misplaced parathyroid cells were not the only source of thymic PTH. Real time RT-PCR using total thymus cDNA from wild-type and *Gcm2*$^{-/-}$ mice on a C57BL/6 genetic background showed that the second source of thymic PTH in the *Gcm2*$^{-/-}$ mice is about 1/250-1/300 of the level in the wild-type mice (Fig. 3.2C). This ratio was affected by genetic background, as 129/C57BL/6J F1 mice had a ratio of about 1/20 due to lower

levels of thymic *Pth* expression in wild-type mice, as the level in *Gcm2*$^{-/-}$ mutants was similar to the C57BL/6J background (Fig. 3.2C and D).

Thymic epithelial cells express thymic PTH in a *Gcm2*-independent pathway

According to the data described above, the ablation of misplaced parathyroid cells in the thymus in *Gcm2*$^{-/-}$ mutants revealed another source of thymic Pth expression, which is not regulated by *Gcm2*. The thymus is a complex immune organ composed of hematopoietic cell-derived thymocytes and many different types of stromal cell (Anderson et al., 1996). Medullary thymic epithelial cells (mTECs) have been reported to express many different tissue-restricted antigens (TRAs) for negative selection during T cell maturation (Farr et al., 2002). To test whether thymic PTH expression is a TRA from mTECs, we sorted TECs using a Foxn1Cre mouse, which has been shown to express Cre recombinase in all TECs (Gordon et al., 2007). We genetically marked all TECs with YFP fluorescence by mating Foxn1Cre with R26YFP, an inducible reporter mouse (Fig. 3.3A). To confirm that YFP positive cells marked by this genetic method are TECs, we performed immunostaining for YFP and several different TEC makers. The results showed that the cTEC marker K8 and mTEC markers K5, K14 and UEA-1 overlapped with YFP, suggesting that YFP marks both cTECs and mTECs (Fig. 3.3B and C). After immunostaining confirmation, we used FACS to sort CD45- and YFP+ TECs. The purity of sorted TECs could reach to 93% (Fig. 3.3D).

RT-PCR using cDNA made from the sorted TECs showed they expressed *Foxn1*, which confirmed that the sorted cells are TECs (Fig. 3.4A). *Pth* was expressed in sorted TECs, and the expression level was similar in the TECs samples that were sorted from wild-type controls and *Gcm2*$^{-/-}$ mutants (Fig. 3.4A and B), confirming that *Pth* expression in these cells is not controlled by *Gcm2*.

Pth expression was not found in other cell types, including T cells, macrophages, and dendritic cells in the thymus (Fig. 3.4B and personal communication with Bruno Kyewski). We have crossed *Gcm2* with *Foxn1-nude* to obtain double mutant mice that have no parathyroid and in which TEC differentiation is blocked (Blackburn et al., 1996; Gunther et al., 2000; Liu et al., 2007). We failed to detect any thymic PTH expression in *Gcm2* and *Foxn1* double mutants (Fig. 3.4C), which confirmed that thymic PTH has only two sources: misplaced authentic parathyroid cells that express *Pth* in a *Gcm2*-dependent way; and TECs that express *Pth* independent of *Gcm2*. Our data also show that PTH expression in TECs depends on their differentiation, which is regulated by *Foxn1*.

We genetically confirmed the expression of PTH in TECs was from medulla region by using $Rag2^{-/-}$ mutant mice, which has a normal cortical structure but lacks an organized medulla (Hollander et al., 1995; Klug et al., 1998; Penit et al., 1996). Although thymic PTH expression was not totally ablated (due to only partial blocking on mTEC differentiation by Rag2 null mutation), it was greatly reduced in $Gcm2^{-/-}$-$Rag2^{-/-}$ double mutants (Fig. 3.5). Microarray data using RNA samples from mTECs or cTECs also showed that PTH transcripts existed only in mTECs (personal communication with Bruno Kyewski).

In a previous report, *Gcm1* was proposed to regulate thymic PTH in $Gcm2^{-/-}$ mutants (Gunther et al., 2000). However, our data did not show detectable *Gcm1* or *Gcm2* expression in the purified TECs (Fig. 3.4A), which rules out the possibility that *Gcm1* might regulate thymic PTH expression in TECs by contributing to the differentiation of PTH-expressing cells in thymus.

Thymic PTH is not the backup mechanism in *Gcm2* null mutant mice

To check if thymic PTH from mTECs also participates in endocrine function, we measured the serum PTH concentration in wild-type, $Gcm2^{-/-}$ (no parathyroids or misplaced parathyroid

cells), *Foxn1*$^{nu/nu}$ (no mTEC-derived PTH), and *Gcm2* and *Foxn1* double mutants (no parathyroids or misplaced parathyroid cells or mTEC-derived PTH) (Fig. 3.4C). The PTH concentration in newborn mice was not significantly different between wild-type and *Foxn1*$^{nu/nu}$ mutant mice, or between *Gcm2*$^{-/-}$ mutants and *Gcm2*;*Foxn1* double mutants (Fig. 3.6A), suggesting that Foxn1-dependent PTH expression in mTECs does not contribute to serum PTH. Surprisingly, in contrast to the original report that has showed normal PTH level in *Gcm2*$^{-/-}$ mutants (Gunther et al., 2000), our results showed a dramatic reduction of PTH levels in both *Gcm2*$^{-/-}$ mutants and *Gcm2-Foxn1* double mutants (Fig. 3.6A). This reduction in serum PTH levels in *Gcm2*$^{-/-}$ mutants has also been previously reported (Tu et al., 2003).

To determine if the difference on the serum PTH levels in *Gcm2*$^{-/-}$ mutants between our data and the previous report (Gunther et al., 2000) was caused by different genetic backgrounds, we checked serum PTH concentrations in the *Gcm2*$^{-/-}$ mutants on the C57BL/6J background or129S6/C57BL/6J F1 hybrid background. Compared to wild-type controls, *Gcm2*$^{-/-}$ mutants on both genetic backgrounds have similar serum PTH levels (Fig. 3.6B), which indicates that the reduction of serum PTH is not affected by genetic background.

Gcm2$^{-/-}$ mutants with 129/C57BL/6J hybrid background had been reported to have a mild lethality phenotype (Gunther et al., 2000). However, we found that *Gcm2*$^{-/-}$ mutants on C57BL/6J genetic background had nearly 100% lethality rate (Fig. 3.7A). After crossing the mice back to 129S6, the lethality rate of the *Gcm2*$^{-/-}$ mutants decreased to 56% on the 129/C57BL/6J F1genetic background (Fig. 3.76B), confirming the mild phenotype in the previous report (Gunther et al., 2000). These data suggest that the lethality phenotype of *Gcm2*$^{-/-}$ mutants is exacerbated on a C57BL/6J genetic background. To check if the surviving *Gcm2*$^{-/-}$ mutants had a higher serum PTH concentration than the *Gcm2*$^{-/-}$ mutants that failed to survive,

we measured serum PTH levels in surviving adult *Gcm2*$^{-/-}$ mutants. Surprisingly, these mice had the same reduction in serum PTH levels including some mice from both groups with no detectable serum PTH (Fig. 3.6C). This strongly suggests that the lethality phenotype observed in *Gcm2*$^{-/-}$ mutants is not related to PTH.

DISCUSSION

The results from our study of the regulation of thymic PTH expression reveal two cellular sources of thymic PTH: the first source is from the misplaced authentic parathyroid cells, which express *Pth* in the same way as the parathyroids and are ablated in the *Gcm2* null mutants; the second source is mTECs, which express *Pth* in a way different from the parathyroids and is ablated in *Foxn1* null mutants. In contrast to the previous report (Gunther et al., 2000), we show that thymic PTH expression is not a backup mechanism of the parathyroid glands but a TRA for negative selection.

The thymus is an epithelial organ composed of a diverse group of cells that form a complex microenvironmental network required for T cell maturation and repertoire selection (Gray et al., 2002). Based on the literature, the expression of PTH in the thymus has two possible functions: a backup mechanism for the parathyroids specially when they fail to secrete PTH (Gunther et al., 2000); or one of the TRAs expressed in mTECs required for negative selection (Farr et al., 2002). Our current data showed that *Gcm2*-independent PTH expression from mTECs, the only source of thymic PTH in *Gcm2*$^{-/-}$ mutants, is much lower than the expression level in parathyroid cells (Fig. 3.2B, C and D). Furthermore, our data showed that thymic PTH from mTECs was not responsible for serum PTH or endocrine function, because the same reduction in serum PTH was observed in both *Gcm2*$^{-/-}$ mutants and *Gcm2*;*Foxn1* double mutants (Fig. 3.6A). Based on these

data, we conclude that thymic PTH in *Gcm2*$^{-/-}$ mutants is not a backup mechanism for the parathyroids, but functions as a TRA for negative selection.

Using our data, we propose a new model for the PTH production and function from parathyroid glands and thymus (Fig. 3.8). In this model, parathyroid cells, including parathyroid glands and the misplaced parathyroid cells in thymus (or scattering between parathyroid and thymus), is the major source of serum PTH. Furthermore, PTH production in these cells is *Gcm2*-dependent since the *Gcm2* null mutation can totally ablate all of these cells. mTECs also express PTH as a self-antigen for negative selection in a *Gcm2*-independent way, but does not contribute to serum PTH. This result is consistent with the lack of the secretory machines, which are used in the parathyroid cells to secrete PTH into the circulation (Habener and Kronenberg, 1978; Hendy et al., 1995; Setoguti et al., 1995; Wild et al., 1985), and the likelihood that the PTH translated in the mTECs is degraded into short peptides of about 10 amino acids to be used as self-antigens.

Humans usually have two pairs of parathyroid glands that develop from the 3rd and 4th pharyngeal pouches. However, in addition to their variable location, ectopic and supernumerary parathyroids have been found (Wang, 1976). Analogous to our observations in the mouse, the extra parathyroids may result from the loose architecture of parathyroid organ and the incomplete separation of the parathyroids from the thymus. Moreover, parathyroid adenomas have been found in the human thymus and *Gcm2* was showed to co-express with these intrathymic parathyroid adenomas in the thymus, indicating that these adenomas could be the result of uncontrolled growth of the misplaced parathyroid cells in the thymus (Kronenberg, 2004; Maret et al., 2004).

The promiscuous expression of tissue-restricted self-antigens in medullary thymic epithelial cells is required for the negative selection to establish central tolerance. However, the mechanism that regulates the expression of TRAs in mTECs is still not clear and is controversial (Derbinski et al., 2005; Derbinski et al., 2001; Dooley et al., 2005; Farr et al., 2002; Kyewski et al., 2002). Two models have been proposed: The mosaic model proposes the presence of a mosaic of differentiated tissue cells within the medulla expressing tissue-specific TRAs following the same tissue-specific regulation rules (Dooley et al., 2005; Farr et al., 2002; Gillard and Farr, 2006). The permissive model proposes that some mTECs can obtain an autonomous property to express TRAs in a different way compared to the tissue-specific regulation rules (Derbinski et al., 2005; Gotter and Kyewski, 2004; Kyewski et al., 2002). Our data showed that *Pth* expression in mTECs was regulated by a *Gcm2*-independent pathway that is different to the *Gcm2*-dependent pathway used by parathyroid cells. This finding strongly supports the permissive model. It remains unclear which pathway directly regulates PTH expression in mTECs. It would also be interesting to know if the regulation mechanism for thymic PTH in mTECs is common to other TRAs, particularly those of other endoderm-derived organs.

Mild lethality has been reported in *Gcm2*$^{-/-}$ mutants on 129/C57BL/6J hybrid background (Gunther et al., 2000), and our data showed that this lethality rate was increased when they were backcrossed to C57BL/6J. It had been proposed that this lethality was due to loss of parathyroids, which can result hypocalcemia and hyperphosphatemia, and that thymic PTH could in part rescue this and produce only a mild lethality phenotype (Gunther et al., 2000). Our data did not support this proposal, and we suggest that the lethality phenotype in *Gcm2*$^{-/-}$ mutants is not related to the reduction of serum PTH levels. Furthermore, recent data showed that *Pth*$^{-/-}$ mice survive normally due to the compensation from another hypercalcaemic hormone vitamin D3

(Miao et al., 2002; Miao et al., 2004; Xue et al., 2005). The difference of the lethality phenotype in *Gcm2*$^{-/-}$ and *Pth*$^{-/-}$ suggest that there may be an additional requirement for *Gcm2* outside parathyroid cells. The expression of *Gcm2* gene in non-parathyroid tissues, including brain and placenta, has been reported (Iwasaki et al., 2003; Kim et al., 1998). It will be worth to further study whether *Gcm2* plays a role in the development of these organs.

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Figure 3.1 Misplaced parathyroid cells resulted from messy separation of parathyroid and thymus contributes to thymic PTH expression. (A) RT-PCR using cDNA made from different tissues to show the co-expression of *Gcm2* with *Pth* only in thymus, but not in other organs tested. (B) RT-PCR using thymus cDNA from different stages. The expression of *Gcm2* and *Pth* was detected in all stages tested. (C) Section *in situ* hybridization of *Gcm2* and *Pth* was performed on the sections of C57BL/6J wild-type embryos at E12 (I), E13 (II) and E18.5 (III) to show the separation procedure of parathyroid/thymus organs from common primordium. (D) Section *in situ* hybridization of *Pth* on the sections of C57BL/6J wild-type embryos at E18.5 to show the location of misplaced parathyroid cells. (E) Section *in situ* hybridization of *Pth* on the sections of 129S6 and C57BL/6J hybrid embryos at E18.5 to show the messy separation of parathyroid and thymus is a common phenomenon in mouse. In C-E, sections were cut in sagittal plane. In all figures, anterior is up, and dorsal is to the right. Ages of embryos were indicated at the upper left corner of each panel. The parathyroid/thymus common primordia in panels C-I were outlined. Black arrows point to parathyroid domain or parathyroid glands. White arrows point to thymus domain. Arrow heads point to misplaced parathyroid cells. pt, parathyroid; th, thymus; tr, thyroid.

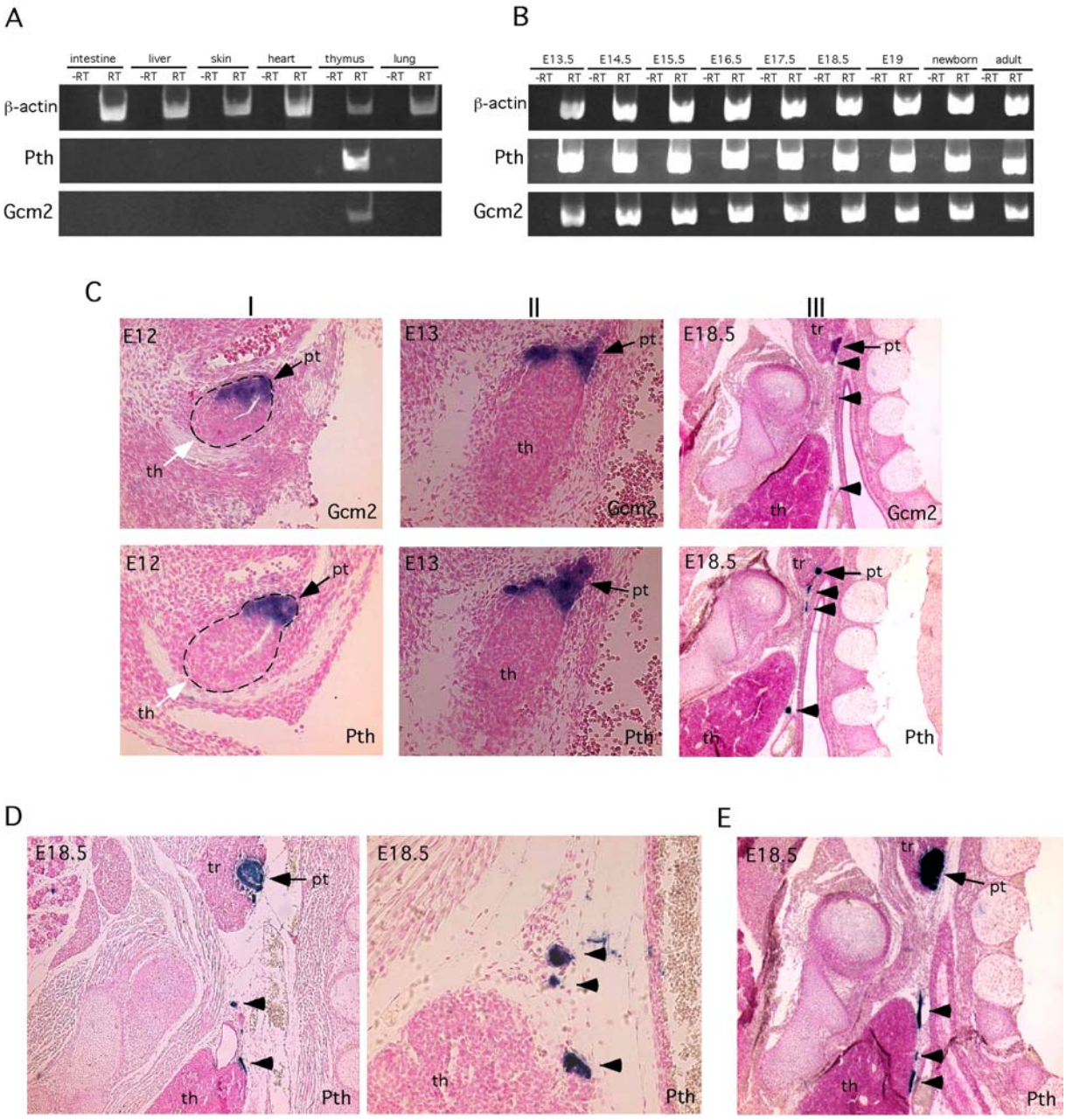
A
intestine
liver
skin
heart
thymus
lung
-RT RT
β-actin
Pth
Gcm2
B
E13.5
E14.5
E15.5
E16.5
E17.5
E18.5
E19
newborn
adult
C
I
II
III
E12
E13
E18.5
pt
th
tr
Gcm2
Pth
D
E
E18.5

Figure 3.1

Figure 3.2 Ablation of misplaced parathyroid cells by *Gcm2* null mutation. (A) Section *in situ* hybridization of *Pth* was performed on the sections of wild-type and *Gcm2*$^{-/-}$ mutant embryos at E13.5, E14.5 and E18.5 to show the loss of parathyroid gland and misplaced parathyroid cells in *Gcm2*$^{-/-}$ mutants. Sections were cut in sagittal plane. In all figures, anterior is up, and dorsal is to the right. Ages of embryos were indicated at the upper left corner of each panel. Arrows point to parathyroid glands. Arrow heads point to misplaced parathyroid cells. pt, parathyroid; th, thymus; tr, thyroid. The mice used are on C57BL/6J background. (B) Semi-quantitive RT-PCR was performed to thymus cDNA from newborn wild-types and *Gcm2*$^{-/-}$ mutants. β-actin was used as standard PCR to show the same amount cDNA was used from *Gcm2*$^{-/-}$ mutant and wild-type. *Foxn1* was a positive control. *Gcm2* transcript was confirmed not present in *Gcm2*$^{-/-}$ mutant. (C) Realtime PCR of *Pth* was performed for total thymus cDNA samples from wild-types and *Gcm2*$^{-/-}$ mutants with C57BL/6J background. (D) Realtime PCR of *Pth* was performed for total thymus cDNA samples from wild-types and *Gcm2*$^{-/-}$ mutants with 129S6 (129) and C57BL/6J (B6) hybrid background. In panel C and D, n is the number of mice analyzed. RQ is the relative quantitive expression level of Pth.

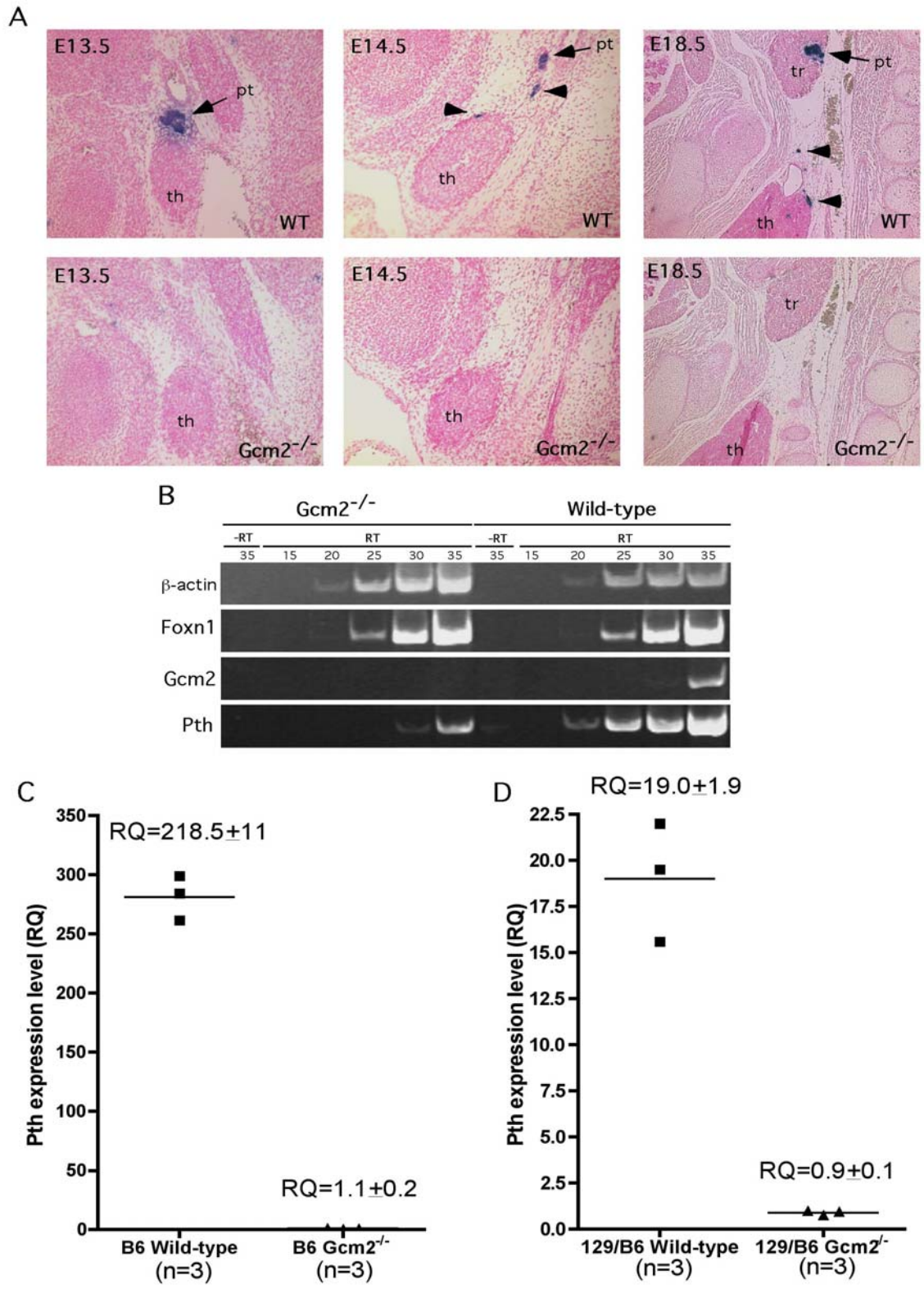
A
E13.5
pt
th
WT
E14.5
pt
th
WT
E18.5
tr
pt
th
WT
E13.5
th
Gcm2-/-
E14.5
th
Gcm2-/-
E18.5
tr
th
Gcm2-/-
B
Gcm2-/-
Wild-type
-RT
RT
35
15
20
25
30
35
β-actin
Foxn1
Gcm2
Pth
C
Pth expression level (RQ)
RQ=218.5±11
RQ=1.1±0.2
B6 Wild-type
(n=3)
B6 Gcm2-/-
(n=3)
D
RQ=19.0±1.9
RQ=0.9±0.1
129/B6 Wild-type
(n=3)
129/B6 Gcm2-/-
(n=3)

Figure 3.2

Figure 3.3 Purification of genetically marked thymic epithelial cells by FACS sorting. (A) The mating strategy used to genetically mark all TECs with YFP fluorescence. (B) Co-localization of cTEC marker keratin 8 and YFP in the 1 month old adult thymus. The section of thymus was stained with anti-K8 (red) and anti-GFP (green). Anti-GFP antibody can recognize YFP protein. m, medulla region. c, cortex region. (C) Co-localization of mTEC makers (keratin 5, keratin 14 and UEA-1) and YFP in the 1 month old adult thymus. The section of thymus was stained with anti-GFP (green) and anti-K5, or K14, or UEA-1(red). m, medulla region. c, cortex region. (D) TECs sorting from Foxn1Cre$^{+/-}$; R26YFP$^{+/-}$ wild-type or *Gcm2*$^{-/-}$ adult thymus. Before sorting the cells were also stained with anti CD45-PE, a marker of hematopoietic cells, to help to get rid of CD45^{+} thymocytes. FACS analyses of the samples before sorting and after sorting showed here were used to check the purity after cell sorting. Two single color controls were also analyzed.

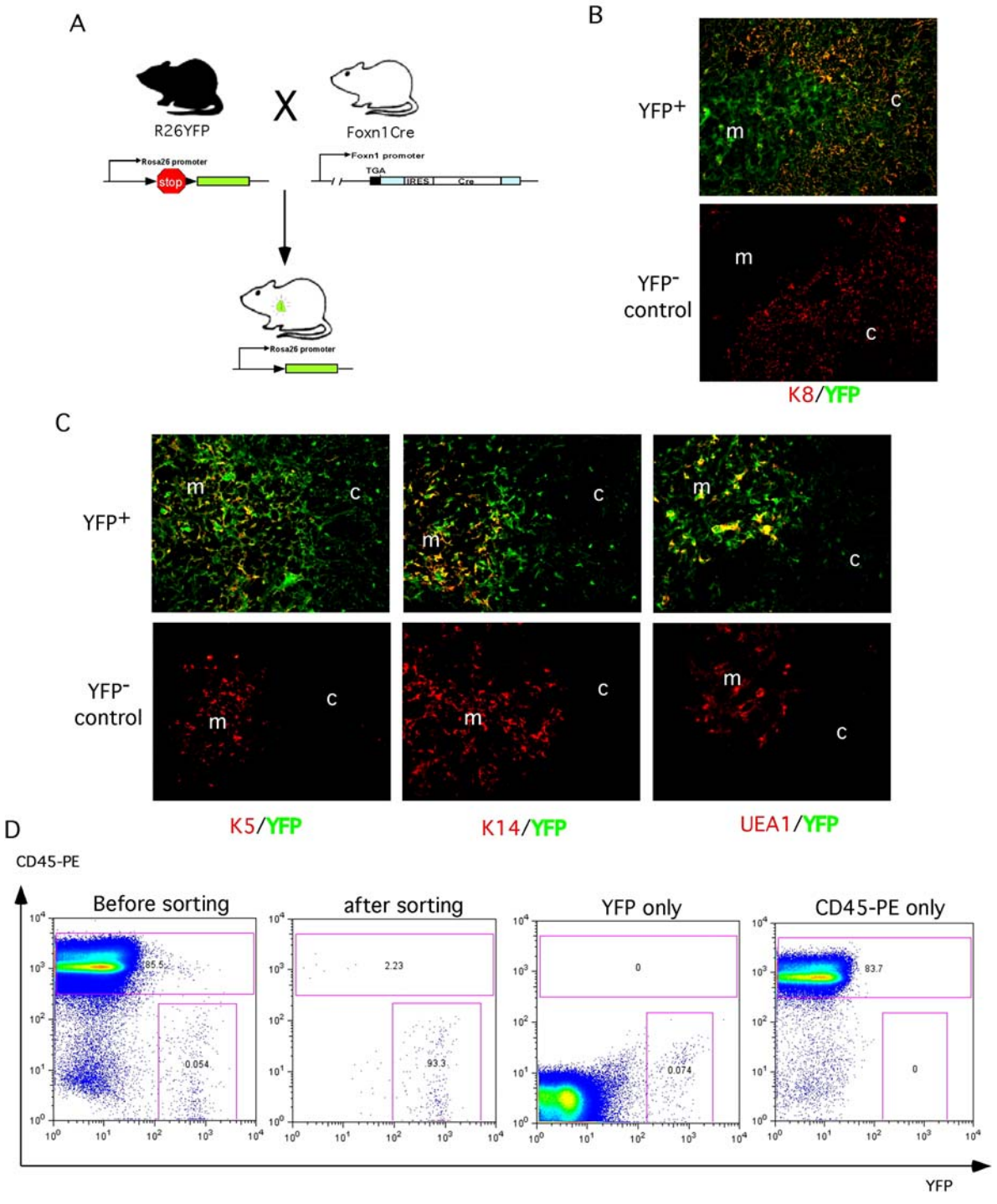
A
R26YFP
X
Foxn1Cre
Rosa26 promoter
stop
Foxn1 promoter
TGA
IRES
Cre
Rosa26 promoter
B
YFP+
YFP-
control
m
c
K8/YFP
C
YFP+
YFP-
control
K5/YFP
K14/YFP
UEA1/YFP
D
CD45-PE
Before sorting
after sorting
YFP only
CD45-PE only
85.5
0.054
2.23
93.3
0
0.074
83.7
0
YFP

Figure 3.3

Figure 3.4 *Gcm2*-independent and *Foxn1*-dependent thymic PTH expression in TECs. (A) RT-PCR using cDNA synthesized from sorted TECs from wild-type (WT) *Gcm2*$^{-/-}$ adult thymi. (B) Realtime PCR of *Pth* was performed for cDNA synthesized from sorted TECs from wild-type and *Gcm2*$^{-/-}$ adult thymi. (C) Realtime PCR of *Pth* was performed for total thymus cDNA samples from newborn wild-types, *Foxn1*$^{nu/nu}$ mutants, *Gcm2*$^{-/-}$ mutants and *Gcm2*$^{-/-}$ and *Foxn1*$^{nu/nu}$ double mutants. These mice were on 129S6 and C57BL/6J hybrid background. In panels B and C, n is the number of mice analyzed.

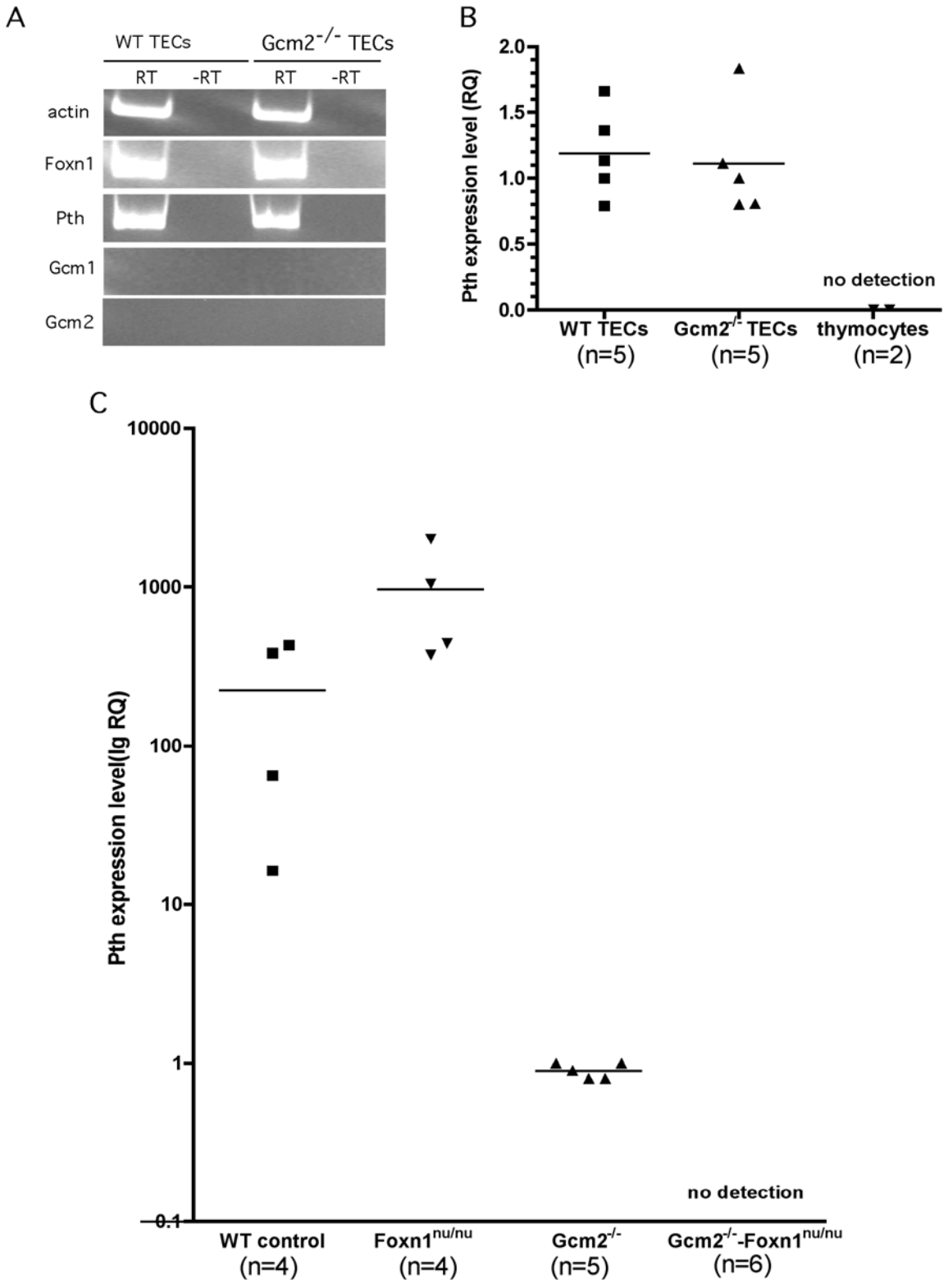
A
WT TECs
Gcm2-/- TECs
RT
-RT
RT
-RT
actin
Foxn1
Pth
Gcm1
Gcm2
B
Pth expression level (RQ)
2.0
1.5
1.0
0.5
0.0
no detection
WT TECs
(n=5)
Gcm2-/- TECs
(n=5)
thymocytes
(n=2)
C
Pth expression level(lg RQ)
10000
1000
100
10
1
0.1
no detection
WT control
(n=4)
Foxn1nu/nu
(n=4)
Gcm2-/-
(n=5)
Gcm2-/--Foxn1nu/nu
(n=6)

Figure 3.4

Figure 3.5 PTH expression in TECs was affected by mTECs defects. (A) Realtime PCR of *Pth* was performed for total thymus cDNA samples from newborn wild-types (WT), Rag2$^{-/-}$ mutants, *Gcm2*$^{-/-}$ mutants and *Gcm2*$^{-/-}$ and Rag2$^{-/-}$double mutants. These mice were maintained under 129S6 and C57BL/6J hybrid background. (B) *Pth* expression levels of *Gcm2*$^{-/-}$ mutants and *Gcm2*$^{-/-}$ and Rag2$^{-/-}$double mutants shown in (A) were compared especially here. In panels A and B, n is the number of mice analyzed. RQ is the relative quantitive expression level of Pth.

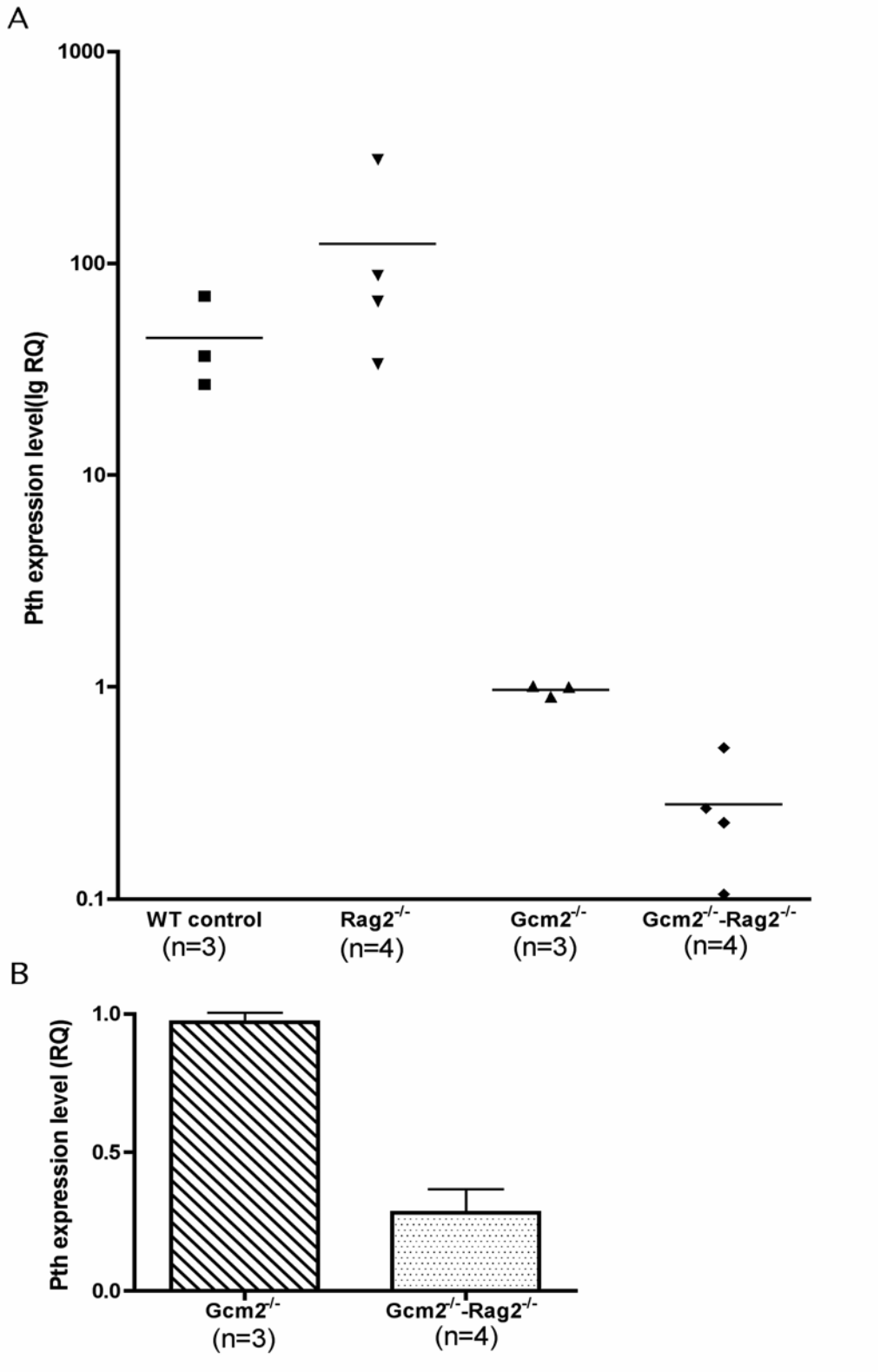
A
1000
100
10
1
0.1
Pth expression level(lg RQ)
WT control
(n=3)
Rag2-/-
(n=4)
Gcm2-/-
(n=3)
Gcm2-/--Rag2-/-
(n=4)
B
1.0
0.5
0.0
Pth expression level (RQ)
Gcm2-/-
(n=3)
Gcm2-/--Rag2-/-
(n=4)

Figure 3.5

Figure 3.6 Thymic PTH from mTECs does not backup the parathyroid glands for Serum PTH homeostasis. (A) PTH concentrations of wild-types (WT), *Foxn1*$^{nu/nu}$ mutants, *Gcm2*$^{-/-}$ mutants and *Gcm2*$^{-/-}$ and *Foxn1*$^{nu/nu}$ double mutants. The serum samples were collected from newborn stage mice. These mice were 129S6 and C57BL/6J hybrid genetic background. (B) PTH concentrations of wild-type, *Gcm2*$^{-/-}$ mutants with C57BL/6J (B6) genetic background or 129S6 (129) and C57BL/6J hybrid genetic background. All serum samples were collected from newborn stage mice. (C) PTH concentrations of wild-type and *Gcm2*$^{-/-}$ mutant survivor mice with 129S6 and C57BL/6J hybrid genetic background. The serum samples were collected from adult mice. In panels A-C, n is the number of samples analyzed.

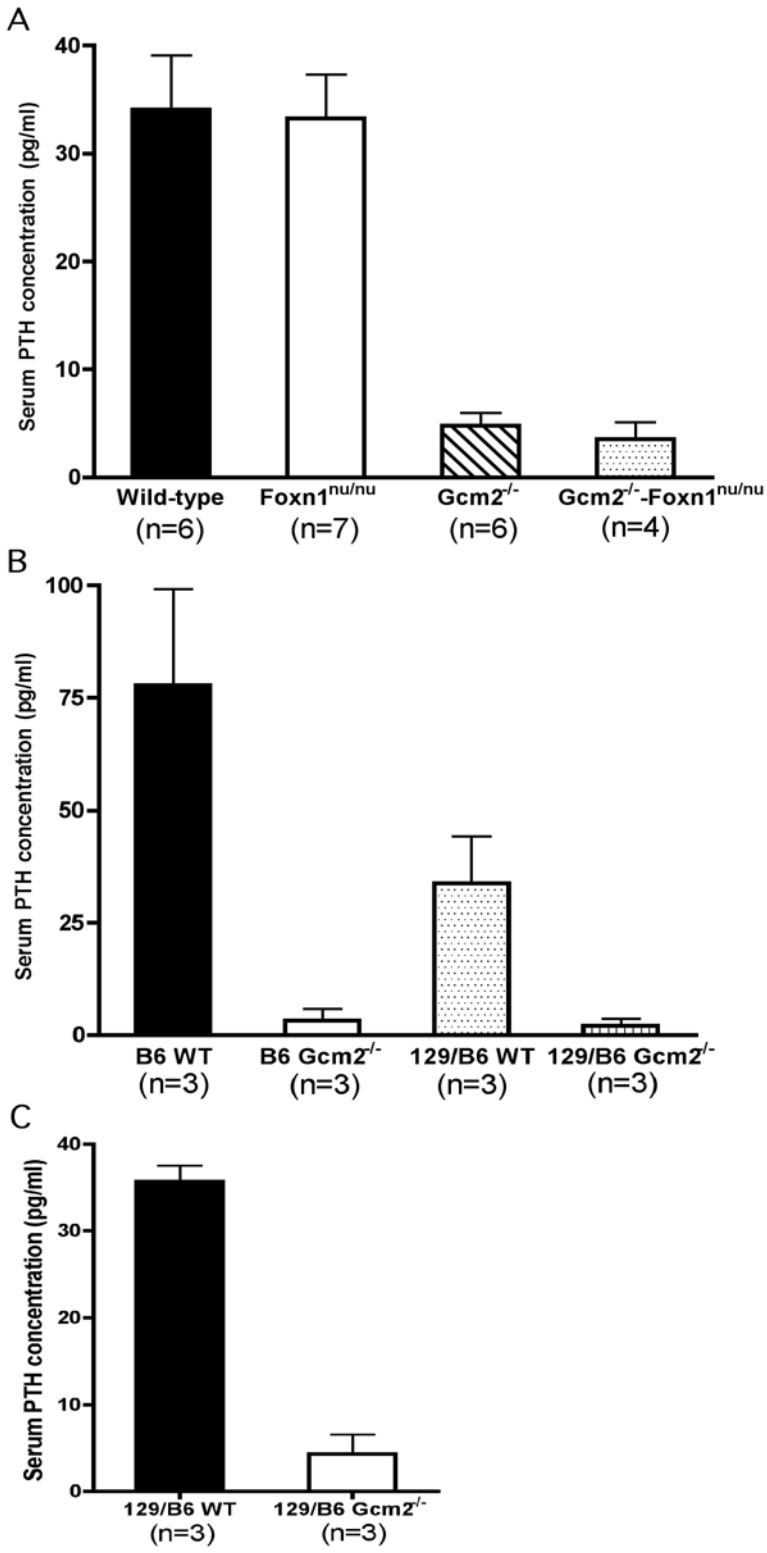
A
Serum PTH concentration (pg/ml)
40
30
20
10
0
Wild-type
(n=6)
Foxn1nu/nu
(n=7)
Gcm2-/-
(n=6)
Gcm2-/--Foxn1nu/nu
(n=4)
B
Serum PTH concentration (pg/ml)
100
75
50
25
0
B6 WT
(n=3)
B6 Gcm2-/-
(n=3)
129/B6 WT
(n=3)
129/B6 Gcm2-/-
(n=3)
C
Serum PTH concentration (pg/ml)
40
30
20
10
0
129/B6 WT
(n=3)
129/B6 Gcm2-/-
(n=3)

Figure 3.6

Figure 3.7 Lethality rate of the *Gcm2*$^{-/-}$ mutants was affected by genetic background. (A) The survival rate of mice with different genotypes born by the mating of *Gcm2*$^{+/-}$ mice with C57BL/6J genetic background. The lethality of *Gcm2*$^{-/-}$ mice happened mostly at newborn stage. The mice that survived more than 1 month were counted as survivors. (B) The survival rate of mice with different genotypes born by the mating of *Gcm2*$^{+/-}$ mice with 129S6 and C57BL/6J hybrid genetic background. In panels A and B, n is the survival adult mice number.

A

$Gcm2^{+/-}$ X $Gcm2^{+/-}$ (C57BL/6J)

genotype	Survival (%)	n
+/+	95	69
+/-	100	145
-/-	4	3

B

$Gcm2^{+/-}$ X $Gcm2^{+/-}$ (50% 129S6 and 50% C57BL/6J)

genotype	Survival (%)	n
+/+	100	44
+/-	92	81
-/-	44	19

Figure 3.7

Figure 3.8 Model of PTH production and function in the parathyroids and thymus. In this model, the serum PTH mostly comes from parathyroid glands and misplaced parathyroid cells. Thymic PTH has two sources: one is from misplaced parathyroid cells that locate in thymus; the other source is mTECs. Thymic PTH from mTECs is not responsible to maintain serum PTH concentration. Instead this source PTH functions as a self-antigen for negative selection. *Gcm2* is required for the differentiation of parathyroid cells in both parathyroid glands and misplaced parathyroid cell clusters. In *Gcm2*$^{-/-}$ mutants, misplaced parathyroid cells also are lost together with parathyroid glands, which cause the reduction of serum PTH concentration. *Foxn1* is required for TECs differentiation. In *Foxn1*$^{nu/nu}$ mutants, TECs cannot differentiate normally and lose the ability to expression PTH for negative selection.

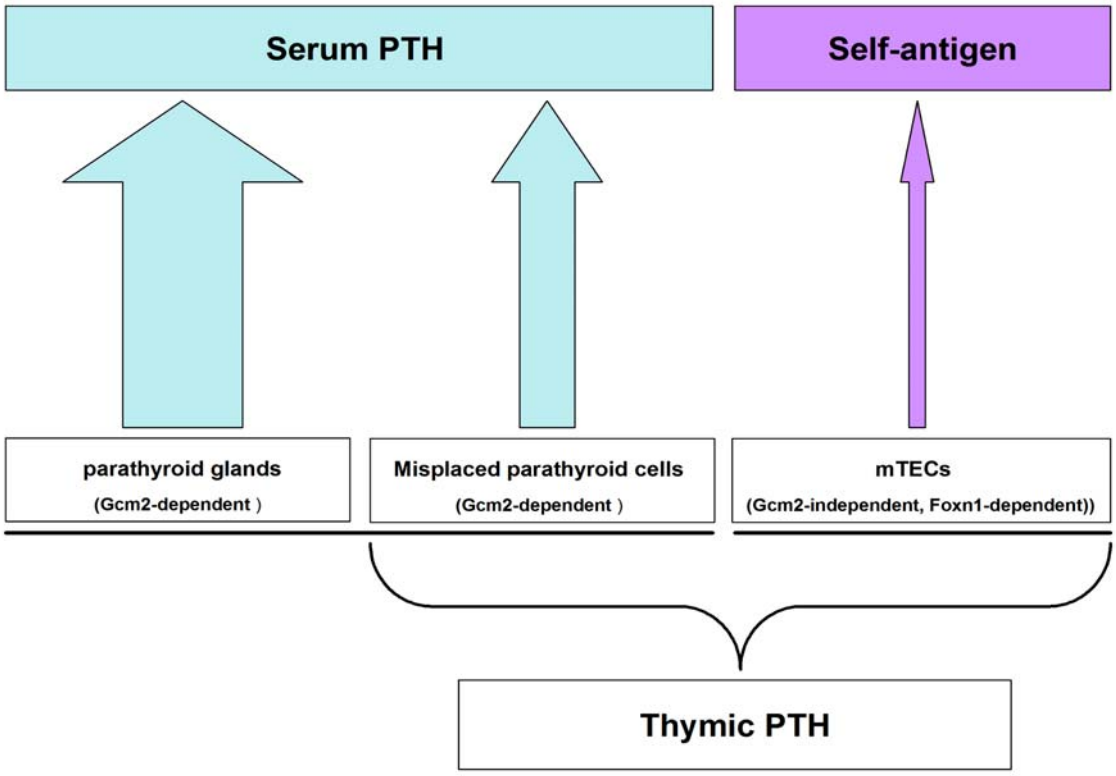
Serum PTH
Self-antigen
parathyroid glands
(Gcm2-dependent)
Misplaced parathyroid cells
(Gcm2-dependent)
mTECs
(Gcm2-independent, Foxn1-dependent))
Thymic PTH

Figure 3.8

CHAPTER 4

GENETIC ANALYSIS OF *GCM2* FUNCTION IN MOUSE EMBRYONIC DEVELOPMENT BY AN INDUCIBLE GAIN-OF-FUNCTION APPROACH[1]

[1]Zhijie Liu, Albert C. Noyes, Suzanne Mansour, Brian Condie, Nancy R. Manley. Unpublished data.

ABSTRACT

To test the ability of *Gcm2* to induce ectopic parathyroid fates in mouse embryos, we made a Gcm2-inducible transgenic mouse, which can express Gcm2 in specific tissues using the loxP-Cre recombination system. The expression level of ectopic Gcm2 expression induced from Rosa26 promoter was found to be much lower than endogenous Gcm2 expression and could not rescue the *Gcm2* null mutation phenotype. However, we found that mice with ubiquitous Gcm2 expression displayed eyelid defects and had a high lethality rate at neonatal stages. These mice also had circling and tossing behaviors that were found to be associated with vestibular defects in inner ear development. Since mouse *Gcm* genes have been reported to be expressed in the brain and otic region during embryonic stages, we postulate that *Gcm2* may have extra roles in mouse embryonic development besides its role in parathyroid organogenesis.

INTRODUCTION

Although our previous studies indicated that *Gcm2* is not necessary to specify parathyroid fate in the third pouch, we also wanted to test the ability of *Gcm2* to induce ectopic cell fates. In this study, we generated a Gcm2 inducible mouse by targeting the Rosa26 locus with a Gcm2 inducible cassette (Soriano, 1999; Zambrowicz et al., 1997). Using different Cre transgenic mouse strains, we induced Gcm2 expression in the presumptive thymus domain to determine if *Gcm2* is sufficient to specify parathyroid cells fate. Due to the low expression level from the Rosa26 promoter, forced Gcm2 expression could not rescue the *Gcm2* null mutation phenotype and did not change thymus cell fate specification in the third pouch. However, gain-of-function mutants with ubiquitous Gcm2 expression showed some unexpected phenotypes. These mice had a lethality phenotype at neonatal stages and eyelid defects. We also found that they had tossing and circling behaviors, which were found to be associated with vestibular defects in the inner ear. Together with the expression of *Gcm2* in the brain and otic region, the inner ear defects in the gain-of-function mutant mice indicate that *Gcm2* may have a role in the development of the neural tissue or ear. Further studies are still ongoing to determine if endogenous *Gcm2* has normal function in the development of these tissues.

MATERIALS AND METHODS

Mice

To generate the *Gcm2* inducible mouse-R26Gcm2, a targeting vector was constructed by inserting a SA-loxP-βgeo-pA-STOP-loxP-Gcm2-pA *Gcm2* inducible cassette into the XbaI site of the vector pROSA26-1, which contains a 5-kb genomic fragment of Rosa26 locus and a diphtheria toxin (DTA) expression cassette for negative selection (Soriano, 1999). The construct

of *Gcm2* inducible cassette was generated from plasmid pSAβgeo (Friedrich and Soriano, 1991) by inserting a loxP site into HindIII site and being ligated with STOP (Lakso et al., 1992) and a loxP-Gcm2-pA fragment. The loxP-Gcm2-pA fragment was constructed from pGEM-T Easy vector (Promega) by TA ligation of *Gcm2* RT-PCR product, which was amplified using cDNA made from neck tissue of E15.5 mouse embryos using primers: 5'-AGGGCCCTGACTAGAGAGAAC-3' and 5'-TTCCTTGTCACCGTGTGTGTATCC-3'. A LoxP site was inserted into SacII site and a pA sequence from pGL3-promoter vector (Promega) was inserted into SpeI site.

The targeting vector was linearized and introduced into a C57BL/6J mouse ES cell line by electroporation at the MCG Transgenic and Knockout Mouse Core Facility. Correctly targeted cell lines were screened with PCR and Southern Blot with a 5'flanking probe, both of which have been described previously (Soriano, 1999). The internal Southern Blot using Neo probe and karyotyping were performed for the targeted ES cell line that was used for microinjection into blastocysts to generate mice chimera (Fig. 4.1C, D and E). The R26Gcm2 colony was maintained by PCR genotyping, which can distinguish between the wild-type Rosa26 allele and the R26Gcm2 allele (Fig. 4.1F). The Cre-excised R26Gcm2 allele was confirmed by Southern Blot and PCR. For R26Gcm2 genotyping, we used the following primers: Rosa26 forward 5'-TTGCAATACCTTTCTGGGAGTT-3', Rosa26 reverse 5'-AACCCCAGATGACTCCTATCCT-3' and β-galactosidase reverse 5'-GACAGTATCGGCCTCAGGAAG-3'. This genotyping PCR will amplify a 568bp band from the R26Gcm2 allele and a 298bp band from the wild-type Rosa26 allele. R26Gcm2 colony was maintained on C57BL/6J genetic background.

Foxn1Cre is a knockin allele of *Foxn1* constructed by inserting IRES-Cre cassette into the 3' UTR of the *Foxn1* locus (Gordon et al., 2007). B6Cre, a transgenic mouse, has Cre recombinase activity in the female germline (Koni et al., 2001). Cre positive females from this colony were mated with R26Gcm2 males to activate *Gcm2* expression from the fertilization stage and have a ubiquitous expression pattern. The generation and genotyping of *Gcm2* null mutant has been described (Gunther et al., 2000). *Gcm2* mutant mice used for experiments were originated on 129/SvEv-C57BL/6J background and were backcrossed to C57BL/6J mice for more than 4 generations. Nestin-Cre (Graus-Porta et al., 2001), Syn1-Cre (Zhu et al., 2001), and PTH-Cre (Libutti et al., 2003) transgenic mice have been described previously and were purchased from the Jackson Laboratory. The Wnt1Cre transgenic mouse has been described (Danielian et al., 1998), and was a gift from Henry Sukov (USC).

For timed embryos, the day of the vaginal plug was designated as E0.5. All experiments were carried out with the approval of the UGA institutional animal care committee.

RT-PCR

Isolation of RNA and RT-PCR were performed as described (Su et al., 2001). Thymi or other tissues were dissected from different stage embryos or mice and total RNA was isolated with Trizol. Genomic DNA was removed using DNase I. DNase I enzyme was inactivated by using 25 mM EDTA and being heated for 15 min at 65°C. Reverse transcription was performed by using SuperScript III Reverse Transcriptase (Invitrogen). RNA was then removed using RNase H and RNase A. The cDNA was subjected to PCR using Qiagen PCR Taq polymerase. The following primers have been used: β-actin forward 5'-TGGAATCCTGTGGCATCCATGAAAC-3', β-actin reverse 5'-TAAAACGCAGCTCAGTAACAGTCCG-3', *Gcm2* forward 5'-

CATCAATGACCCACAGATGC-3', and *Gcm2* reverse 5'-GGCACTTCTTCTGCCTTCTG-3'. For RT-PCR to check the Cre-mediated recombination excision event, we used the following primers: splice acceptor forward 5'-ACAAACTCTTCGCGGTCTTTC-3' and *Gcm2* reverse 5'-TCTTCTCCTGGCTGCTGTAGAT-3'.

H&E histology staining

H&E staining was performed as previously described (Moore-Scott and Manley, 2005). Staged embryos were fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. Sections were cut at 10μm and stained with hematoxylin and eosin using standard methods.

Section *in situ* hybridization

Paraffin section *in situ* hybridization was performed as described (Moore-Scott and Manley, 2005). Staged embryos were fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. 8-10μm sections were hybridized with digoxigenin-labeled RNA probes at 0.5 μg/ml. Alkaline phosphatase-conjugated antidigoxigenin Fab fragments were used at 1:5000. BM-purple (Roche) was used as a chromagen to localize the hybridized probe. Probes for *Gcm2*, *Foxn1* and *Pth* have been described (Gordon et al., 2001; Liu et al., 2007).

Paint injection of inner ear

The technique of paint injection of inner ear has been described (Kiernan, 2006). E15.5 stage embryos were harvested and fixed overnight in Bodian's fixative. Specimens were then dehydrated in ethanol and cleared in methyl salicylate. The inner ears were visualized by injecting 0.1% white latex paint in methyl salicylate into the membranous labyrinth as previously described (Kiernan, 2006). At a minimum, three embryos for each different genotype were injected.

RESULTS

Construct *Gcm2* inducible mouse by targeting Rosa26 locus

We made an Gcm2-inducible transgenic mouse based on the R26R β-galactosidase reporter mouse (Soriano, 1999). The Rosa26 promoter has been characterized to be a ubiquitous promoter. We targeted the Rosa26 locus by introducing a Gcm2-inducible DNA fragment into the first intron of this locus (Fig. 4.1A). Before Cre-mediated recombination, only a fusion protein of the neomycin resistance gene and β-galactosidase will be expressed from the Rosa26 locus (Fig. 4.1B) (Lakso et al., 1992). After deletion of the β-Geo-STOP sequence, *Gcm2* will be transcribed from the Rosa26 locus (Fig. 4.1B). We screened 38 $G418^r$ cell lines and obtained 30 targeted cell lines, indicating that nearly 80% of $G418^r$ clones had homologous recombination at the Rosa26 locus. We named this targeted Rosa26 allele as R26Gcm2. We did not find any obvious defects in $R26Gcm2^{+/-}$ and $R26Gcm2^{-/-}$ mice. Due to the lethality caused by Rosa26 locus when both alleles are targeted (Zambrowicz et al., 1997), the recovery of $R26Gcm2^{-/-}$ was low.

Low level of *Gcm2* expression from Rosa26 promoter did not affect thymus development or rescue the $Gcm2^{-/-}$ phenotype

To induce ectopic Gcm2 expression, we first used Foxn1Cre to activate Gcm2 expression in the thymus domain. The mice with induced Gcm2 expression were totally normal, and we did not find any defects in the development of the parathyroid and thymus organs (data not shown).

To induce earlier Gcm2 expression in the 3rd pouch, we chose to use B6Cre transgenic mice, which have been shown to have Cre recombinase activity in female germline cells (Koni et al., 2001) (Fig. 4.2A). RT-PCR using cDNA from different tissues showed that Cre excision and *Gcm2* transcript were present in all tissues from $R26Gcm2^{+/-}$ newborn pups but not in the wild-

type littermates (Fig. 4.2B). We did not find defects in the parathyroid and thymus organs in the mice with ubiquitous Gcm2 expression (Fig. 4.2C and data not shown). However, we found the expression level from Rosa26 promoter was very low. *Gcm2 in situ* hybridization could detect only the endogenous *Gcm2* transcript in the parathyroid gland, but not the forced *Gcm2* transcripts from Rosa26 promoter (data not shown). This means the Rosa26 promoter is much weaker than the endogenous *Gcm2* promoter. We also tested if *Gcm2* expression from Rosa26 promoter could rescue the aparathyroid phenotype caused by the null mutation of endogenous *Gcm2* (Gunther et al., 2000) (Fig. 4.3A). Compared with the controls (Fig. 4.3B and 4.3C), the *Gcm2*$^{-/-}$ mutant with ubiquitous Gcm2 expression did not have parathyroids (Fig. 4.3D and 4.3E), indicating that forced Gcm2 expression from Rosa26 promoter could not substitute the function of the endogenous Gcm2 expression in the parathyroid development. Thus, the induced ectopic Gcm2 expression from Rosa26 promoter cannot be used to test whether ectopic *Gcm2* can induce parathyroid cell fate determination.

Phenotypes in mice with ubiquitous *Gcm2* expression-extra roles of *Gcm2*?

Although the forced *Gcm2* expression from Rosa26 promoter is not sufficient to specify a parathyroid cell fate, we found that nearly 100% of mice with ubiquitous *Gcm2* expression died at the newborn stage (Fig. 4.4A). We recovered only one male mouse that had complete Cre-excision from several hundred mice born by B6Cre (Fig. 4.4C). This surviving mouse with ubiquitous *Gcm2* expression could mate with C57BL/6J wild-type females and we recovered another two mice with ubiquitous *Gcm2* expression. We also found some R26Gcm2$^{+/-}$ mice born by B6Cre female have partial Cre-excision. These chimeric Gcm2 expressing mice had low frequency neonatal lethality. We recovered 8 mice with chimeric Gcm2 expression in the mating of R26Gcm2 with B6Cre. Some of the mice with ectopic Gcm2 expression died right after the

birth, and others died in several hours to 2 days. The dying pups had purple skin color, breathed with difficulty, and moved weakly. Milk spots were found in the pups that survived more than several hours. The histological analysis did not show any obvious defects.

Interestingly, we found that the B6Cre induced mutants had eyelid problems during development. At the newborn stage, when the eyelids are supposed to be closed, these mutants had open eyelids although they had normal eyeball structure (Fig. 4.4B). Surviving adult mice with complete Cre-excision could not open eyelids (Fig. 4.4C). These results show that ectopic *Gcm2* expression affects eyelid development.

We also found tossing and circling behaviors in all surviving mice with ubiquitous *Gcm2* expression and in 6 of the 8 chimeric Gcm2 expressing mice. The tossing and circling behaviors indicated possible neural or inner ear defects. The vestibular part of the inner ear in mammals functions to detect and send information to the brain about the attitude and rotation of the head using fluids and detection cells (hair cells). We checked the inner ear structure in the B6Cre forced embryos that had complete or partial Cre-excision. Compared to wild-type littermates (Fig. 4.5A), all embryos that had the compete Cre-excision were missing the posterior and lateral canals and cristae (Fig. 4.5B). In the partial Cre-excised mosaic embryos, the defects were not as serious as the complete Cre-excised embryos. Some lacked the lateral canal and part of the posterior canal (Fig. 4.5C). The loss of semicircular canals in the vestibular system of inner ear is likely to contribute to the tossing and circling behaviors.

Using different Cre transgenic mice, we induced Gcm2 expression in different tissues to determine the tissue that caused the defects we saw in the ubiquitous expressing mice. First, we used two neural specific Cre transgenic mice: Nestin-Cre with Cre recombinase activity in neuronal and glial cell precursors starting around E10.5 (Graus-Porta et al., 2001), and Syn1-Cre

with Cre activity in neuronal cells from E12.5 (Zhu et al., 2001). The ectopic expression of Gcm2 in neural tissues using these two mice did not cause any detectable phenotypes. We also used PTH-Cre mice to induce Gcm2 expression in the parathyroids from E11.5 (Libutti et al., 2003) to test whether Gcm2 over-expression in parathyroid cells could cause hyperparathyroidism and lethality. However, these mice survived at a normal rate, suggesting that the over-expression of Gcm2 in the parathyroids does not cause lethality. We also did not see any defects by activating the R26Gcm2 allele with Wnt1Cre to force Gcm2 expression in neural crest cells (Danielian et al., 1998; Jiang et al., 2000).

DISCUSSION

Here we used gain-of-function experiments to examine the function of *Gcm2* in mouse embryonic development. We hypothesized that the *Gcm2* gene functioned to specify parathyroid domain in the 3rd pharyngeal pouches and that the presumptive thymus precursor cells would be specified as parathyroid cells if we forced ectopic *Gcm2* expression in the entire 3rd pouch or the whole parathyroid/thymus primordium. However, our data showed that the induced Gcm2 expression from the Rosa26 promoter was too low to rescue *Gcm2* null mutation phenotype. Due to this problem, we cannot conclude whether *Gcm2* can function to specify parathyroid cell fate. We have constructed two new Gcm2-inducible transgenic mouse strains using a chimeric promoter consisting of the CMV immeadiate early enhancer and the chicken β-actin promoter, which has been proved to be a strong promoter (Garg et al., 2004). In one construct, the Gcm2 cDNA had the inhibitory domain deleted to extend the half-life of the Gcm2 protein (Tuerk et al., 2000). We are currently testing these two new strains to see whether high level ectopic Gcm2 expression can induce parathyroid cell fate.

Although our experiments could not conclusively test our hypothesis about the role of *Gcm2* in parathyroid cell fate determination, the gain-of-function mutants with ubiquitously forced *Gcm2* expression caused neonatal lethality and revealed several defects in the development of non-parathyroid organs. Our histology studies did not find any obvious defects in the organs that could result in the lethality. However, the dying newborn pups had purple skin color and had difficulty breathing. Dissection showed that there was no or little air in their lungs. This suggests that the respiratory problem may be the direct cause of lethality. Further study is necessary to find the cause of the breathing failure in these mutants. For the eyelid problem, since we do not have any evidence to show the expression of endogenous *Gcm2* in that region, the possible explanation is that the ectopic Gcm2 expression in the eyelid cells can turn on or repress some genes to change normal cell development procedure. These defects are not likely due to an effect in neural nest cells, as induction with the Wnt1-Cre did not result in this same phenotype.

Although ectopic *Gcm2* expression appears to cause defects in tissues that have no normal *Gcm2* expression, the defects in tissues that have endogenous *Gcm2* expression may reveal a normal function of *Gcm2* in these tissues. The inner ear defects can be caused directly by the mutation of the genes that function in inner ear development (Depew et al., 1999; Merlo et al., 2002), or be caused secondarily from disturbances in the hindbrain, which can function as the signaling center to turn on Fgf3 and regulate the early development of inner ear (Pasqualetti et al., 2001; Phillips et al., 2004; Torres and Giraldez, 1998). There is some *in situ* hybridization data showing a low level of endogenous *Gcm2* expression in otic region in E10.5 embryos (Emage data base: http://genex.hgu.mrc.ac.uk/das/jsp/browse.jsp#table) (Gray et al., 2004). This suggests that endogenous *Gcm2* may have a role in ear development. In this case, forced *Gcm2* expression

from the Rosa26 locus could cause an overdose or misexpression of *Gcm2* in the inner ear, which then results in development defects. However, further studies are required.

Another possible cause of the inner ear defects in the *Gcm2* gain-of-function mutants is that endogenous *Gcm2* has a role in hindbrain development and forced Gcm2 expression affects its normal function in hindbrain development. Defects in the hindbrain influence it to send inductive signals for inner ear development. There is evidence supporting the notion that *Gcm* genes may conserve the function in central nervous system from *Drosophila* to vertebrates. In a recent report, chick *Gcm* genes were shown to be expressed in central nervous system including hindbrain (Soustelle et al., 2007). Moreover, RT-PCR experiments also revealed the presence of mouse Gcm genes transcripts in mouse brain (Iwasaki et al., 2003). It is possible our mice with ubiquitously ectopic *Gcm2* expression have hindbrain defects, which then secondarily affect inner ear development. However, our experiments using Nestin-Cre and Syn1-Cre to induce ectopic Gcm2 expression in the neural tissues did not result in any tossing and circling behaviors. Therefore, we need to further confirm if these two Cre transgenic mice can efficiently induce Gcm2 expression through Cre-excision. We also need to check if inner ear development is normal in the mice with Gcm2 expression in the neural tissues.

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Figure 4.1 Construct of *Gcm2* inducible R26Gcm2 mouse. (A) Targeting strategy for making *Gcm2* inducible R26Gcm2 mouse. The genomic organization of Rosa26 locus is on the top. The middle is the structure of targeting vector. (B) Cre-mediated excision of targeted R26Gcm2 allele. The top is the targeted Rosa26 allele, which we named it as R26Gcm2. This allele expresses β-galactosidase and Neo^r fusion gene mRNA. The bottom is the Cre-excised R26Gcm2 allele, which starts to transcribe *Gcm2* mRNA. In panels A-B, H3, HindIII. RV, EcoRV, βgeo, a fusion gene of β-galactosidase and Neomycin resistant gene. pA, polyA signal. STOP, a transcription stopper sequence. (C) PCR screening for the targeting event. One pair of primers shown in A were used to amplify a 1.2 kB size fragment from the R26Gcm2 allele, but not wild-type Rosa26 allele. (D) Southern Blot analysis of genomic DNA prepared from Neo resistant ES cell lines after electroporation after HindIII digestion. This 5' probe can hybridize with a 3.7kb fragment from R26Gcm2 allele, and a 4.4kb fragment from Rosa26 allele. (E) Southern Blot analysis of genomic DNA prepared from R26Gcm2 heterozygous and wild-type mice after XhoI digestion. Neo^r probe can hybridize with a 7.1 kb fragment from R26Gcm2 allele. We did not get extra bands, which indicated no random insertion event happened. (F) A PCR genotyping used to distinguish different genotype mice of R26Gcm2 colony. This PCR can amplify a 568 bp band from mutant allele and a 298 bp band from wild-type allele.

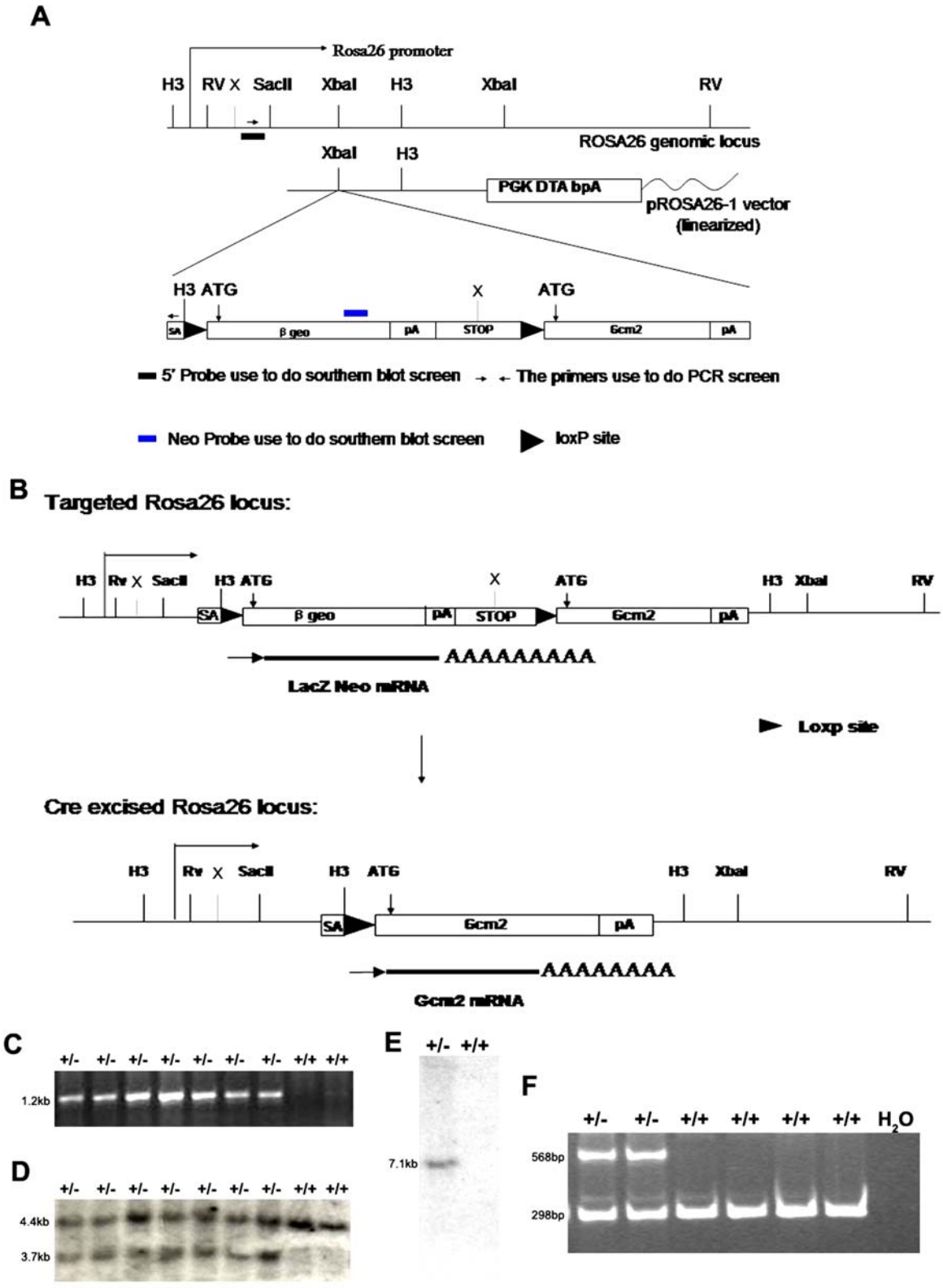
A
Rosa26 promoter
H3
RV
SacII
XbaI
H3
XbaI
RV
ROSA26 genomic locus
XbaI
H3
PGK DTA bpA
pROSA26-1 vector
(linearized)
H3 ATG
X
ATG
SA
β geo
pA
STOP
Gcm2
pA
5' Probe use to do southern blot screen
The primers use to do PCR screen
Neo Probe use to do southern blot screen
loxP site
B
Targeted Rosa26 locus:
H3
Rv
SacII
H3 ATG
X
ATG
H3
XbaI
RV
SA
β geo
pA
STOP
Gcm2
pA
AAAAAAAAA
LacZ Neo mRNA
Loxp site
Cre excised Rosa26 locus:
H3
Rv
SacII
H3
ATG
H3
XbaI
RV
SA
Gcm2
pA
AAAAAAAA
Gcm2 mRNA
C
+/- +/- +/- +/- +/- +/- +/- +/+ +/+
1.2kb
D
+/- +/- +/- +/- +/- +/- +/- +/+ +/+
4.4kb
3.7kb
E
+/- +/+
7.1kb
F
+/- +/- +/+ +/+ +/+ +/+ H2O
568bp
298bp

Figure 4.1

Figure 4.2 Ubiquitously forced Gcm2 expression does not affect thymus development. (A) The mating strategy used to produce mice with forced Gcm2 expression in all tissues. B6Cre (Cre^{+}) females can provide female germline cells with Cre recombinase activity. Fertilization of R26Gcm2 allele with female germline cell makes the Cre excision event happen at a single cell stage, and all somatic cells have Gcm2 expression. (B) RT-PCR using cDNA made from different tissue from newborn $R26Gcm2^{+/-}$ and wild-type littermate delivered by B6Cre (Cre^{+}) female to check for Cre excision and Gcm2 expression in all tissue in $R26Gcm2^{+/-}$ mice but not in wild-type. (C) H&E staining to check thymus formation in newborn $R26Gcm2^{+/-}$ and wild-type littermate delivered by B6Cre (Cre^{+}) female. Sections were cut in the transverse plane. In all the figures, dorsal is up. th, thymus.

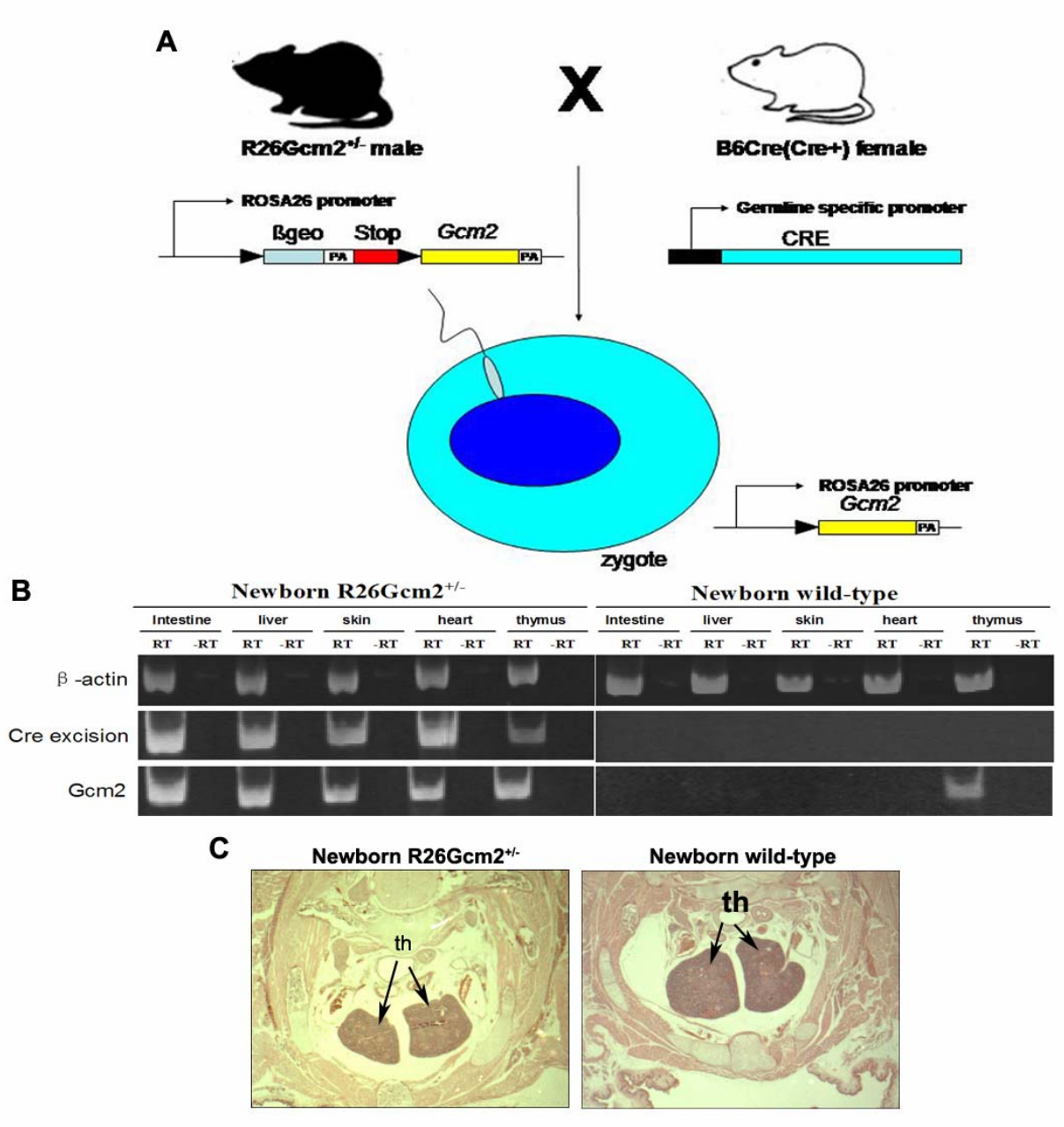
A
X
R26Gcm2+/- male
B6Cre(Cre+) female
ROSA26 promoter
ßgeo
Stop
Gcm2
PA
PA
Germline specific promoter
CRE
ROSA26 promoter
Gcm2
PA
zygote
B
Newborn R26Gcm2+/-
Newborn wild-type
Intestine
liver
skin
heart
thymus
RT
-RT
β -actin
Cre excision
Gcm2
C
Newborn R26Gcm2+/-
Newborn wild-type
th
th

Figure 4.2

Figure 4.3 Ubiquitously forced Gcm2 expression does not rescue aparathyroid phenotype in *Gcm2* null mutants. (A) The mating strategy used to get *Gcm2*$^{-/-}$; R26Gcm2$^{+/-}$ genotype embryos delivered by B6Cre (Cre^{+}) female that have ubiquitously forced Gcm2 expression and other genotype embryos for control. (B) Section *in situ* hybridization of *Pth* on the sections of E13.5 embryos delivered by B6Cre (Cre^{+}) female to check if ubiquitously forced Gcm2 expression can rescue parathyroid organogenesis in *Gcm2*$^{-/-}$ mutants. In panel B-E, anterior is up and dorsal is to the right. Ages of embryos are indicated at the upper right corner of each panel. Arrows point to parathyroid glands. Arrow heads point to misplaced parathyroid cells. pt, parathyroid; th, thymus; tr, thyroid.

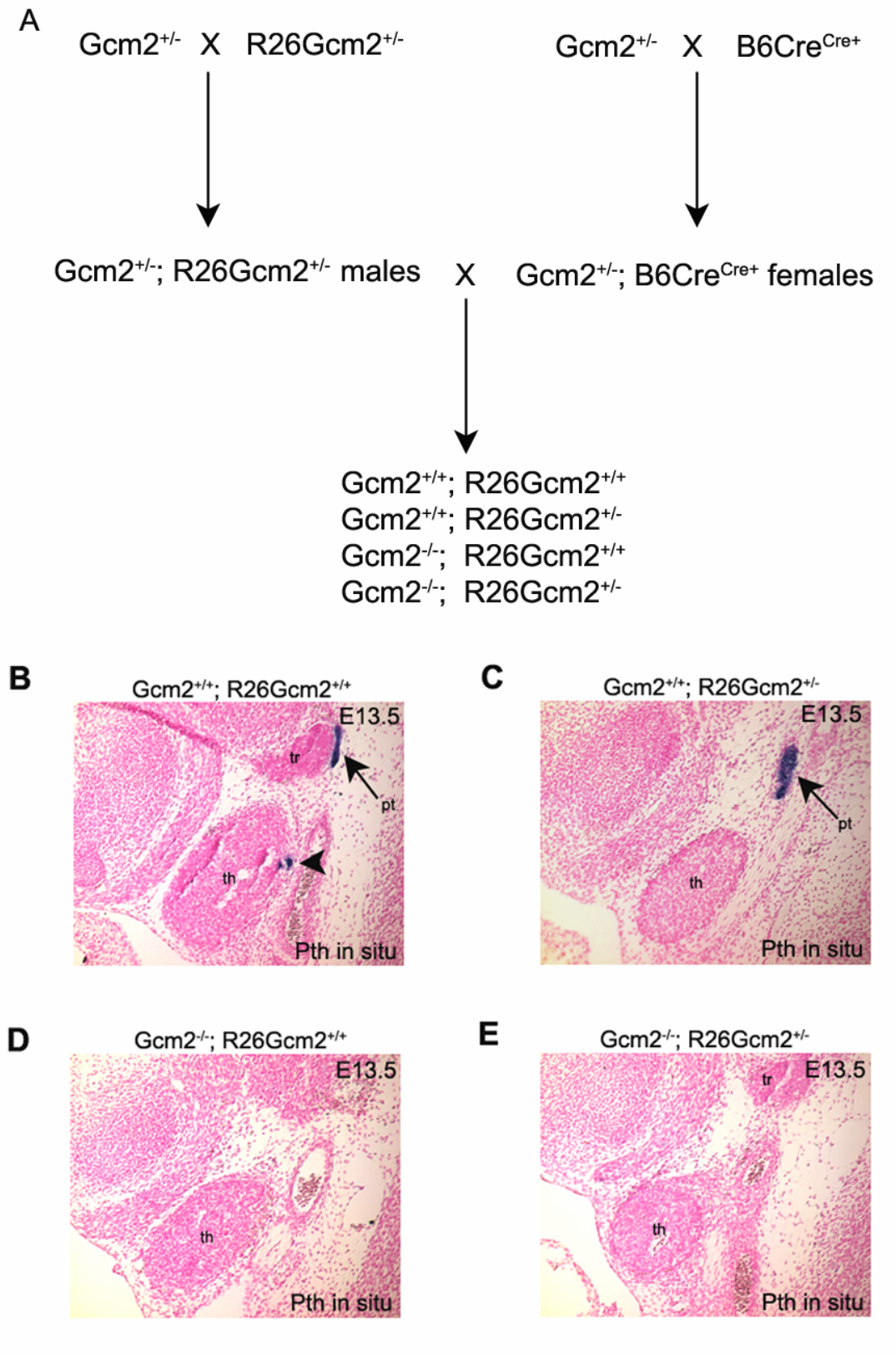
A
Gcm2+/- X R26Gcm2+/-
Gcm2+/- X B6Cre Cre+
Gcm2+/-; R26Gcm2+/- males X Gcm2+/-; B6Cre Cre+ females
Gcm2+/+; R26Gcm2+/+
Gcm2+/+; R26Gcm2+/-
Gcm2-/-; R26Gcm2+/+
Gcm2-/-; R26Gcm2+/-
B
Gcm2+/+; R26Gcm2+/+
E13.5
tr
pt
th
Pth in situ
C
Gcm2+/+; R26Gcm2+/-
E13.5
pt
th
Pth in situ
D
Gcm2-/-; R26Gcm2+/+
E13.5
th
Pth in situ
E
Gcm2-/-; R26Gcm2+/-
tr
E13.5
th
Pth in situ

Figure 4.3

Figure 4.4 Lethality and eyelid phenotypes in gain-of-function mutant mice with ubiquitously forced Gcm2 expression. (A) Lethality phenotype of newborn R26Gcm2$^{+/-}$ mice delivered by B6Cre (Cre^{+}) female. Arrows point to dead newborn R26Gcm2$^{+/-}$ mice delivered by B6Cre (Cre^{+}) female. The others are wild-type littermates. (B) Eyelid phenotype of newborn R26Gcm2$^{+/-}$ mice delivered by B6Cre (Cre^{+}) female. Wild-type littermate was used as control. White arrows point to eyelids in the top two panels. The bottom two panels are H&E staining of transverse sections of newborn mice shown at the top. Arrow heads point to the eyelids. (C) The eyelid phenotype of adult R26Gcm2$^{+/-}$ mice delivered by B6Cre (Cre^{+}) female. Wild-type littermate was used as control. Arrow heads point to the eyelids.

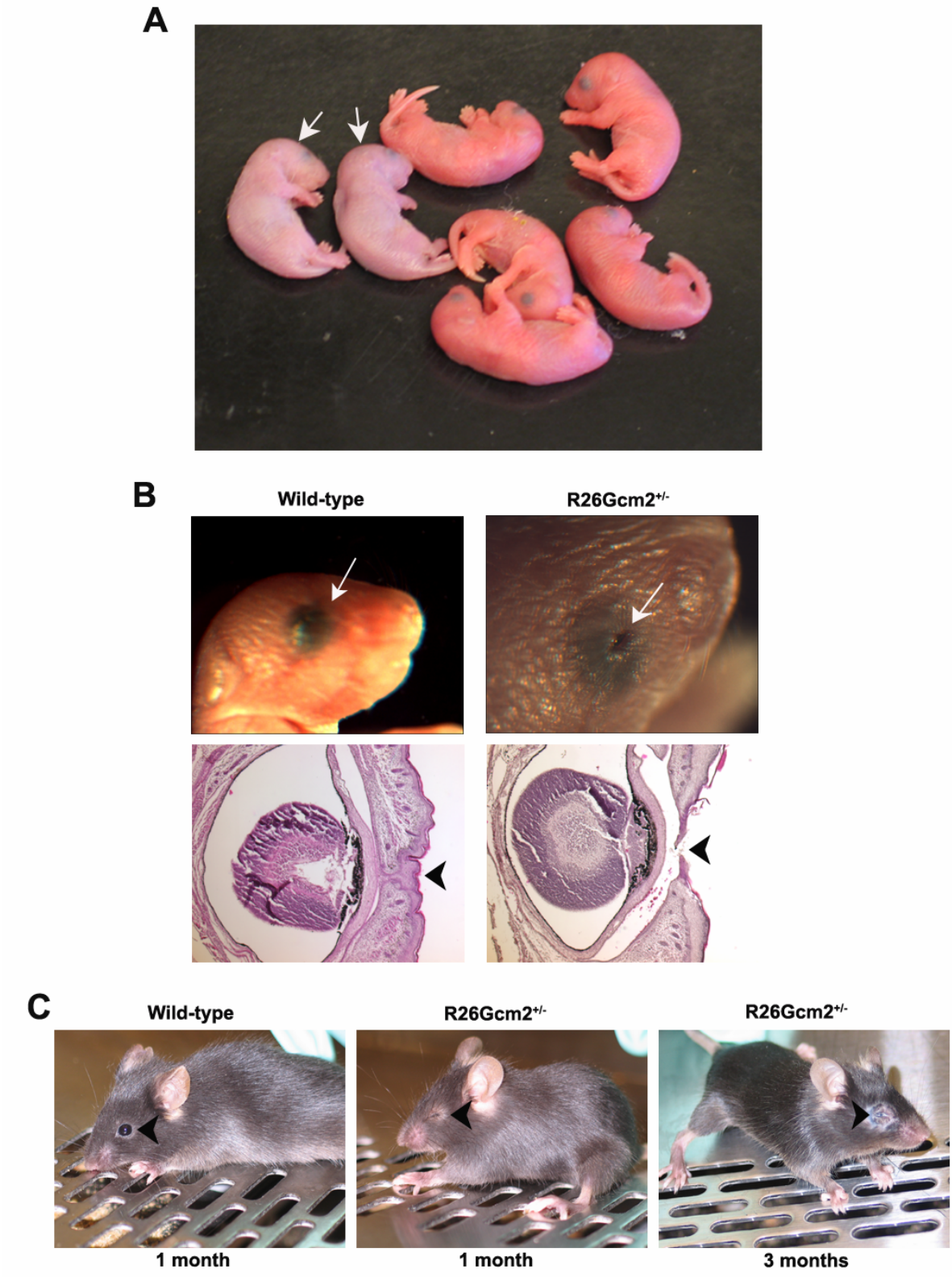
A
B
Wild-type
R26Gcm2+/-
C
Wild-type
R26Gcm2+/-
R26Gcm2+/-
1 month
1 month
3 months

Figure 4.4

Figure 4.5 Vestibular defects in the inner ears of mice with ubiquitously forced Gcm2 expression. (A) The normal inner ear structure of wild-type E15.5 embryos. Names of the detailed structure parts are shown in panel D. (B) The inner ear structure of an E15.5 R26Gcm2$^{+/-}$ embryo from B6Cre (Cre^{+}) female that have complete Cre-excision in all somatic cells. This embryo is missing the posterior and lateral semicircular canals. It is also missing all cristae, including anterior crista, posterior crista and lateral crista. (C) The inner ear structure of an E15.5 R26Gcm2$^{+/-}$ embryo from B6Cre (Cre^{+}) female that have Cre-excision only in part of somatic cells. This embryo has mosaic forced Gcm2 expression. This embryo is missing lateral semicircular canal and part of posterior semicircular canal. The posterior semicircular canal defect is evident on the right than the left. (D) This diagram for the normal structure of mouse inner ear (from Mouse Development, Rossant, J. et al., Chapter 22 Development of Mouse Inner Ear, pp548, Academic Press, San Diego, 2002. Reprinted here with the permission of publisher.). The abbreviations of all parts are marked. ac, anterior crista; asc, anterior semicircular canal; cc, common crus; csd, cochlear saccular duct; ed, endolymphatic duct; es, endolymphatic sac; lc, lateral crista; lsc, lateral semicircular canal; oC, organ of Corti; pc, posterior crista; psc, posterior semicircular canal; s, saccular macula; u, utricular macula; usd, utricular saccular duct.

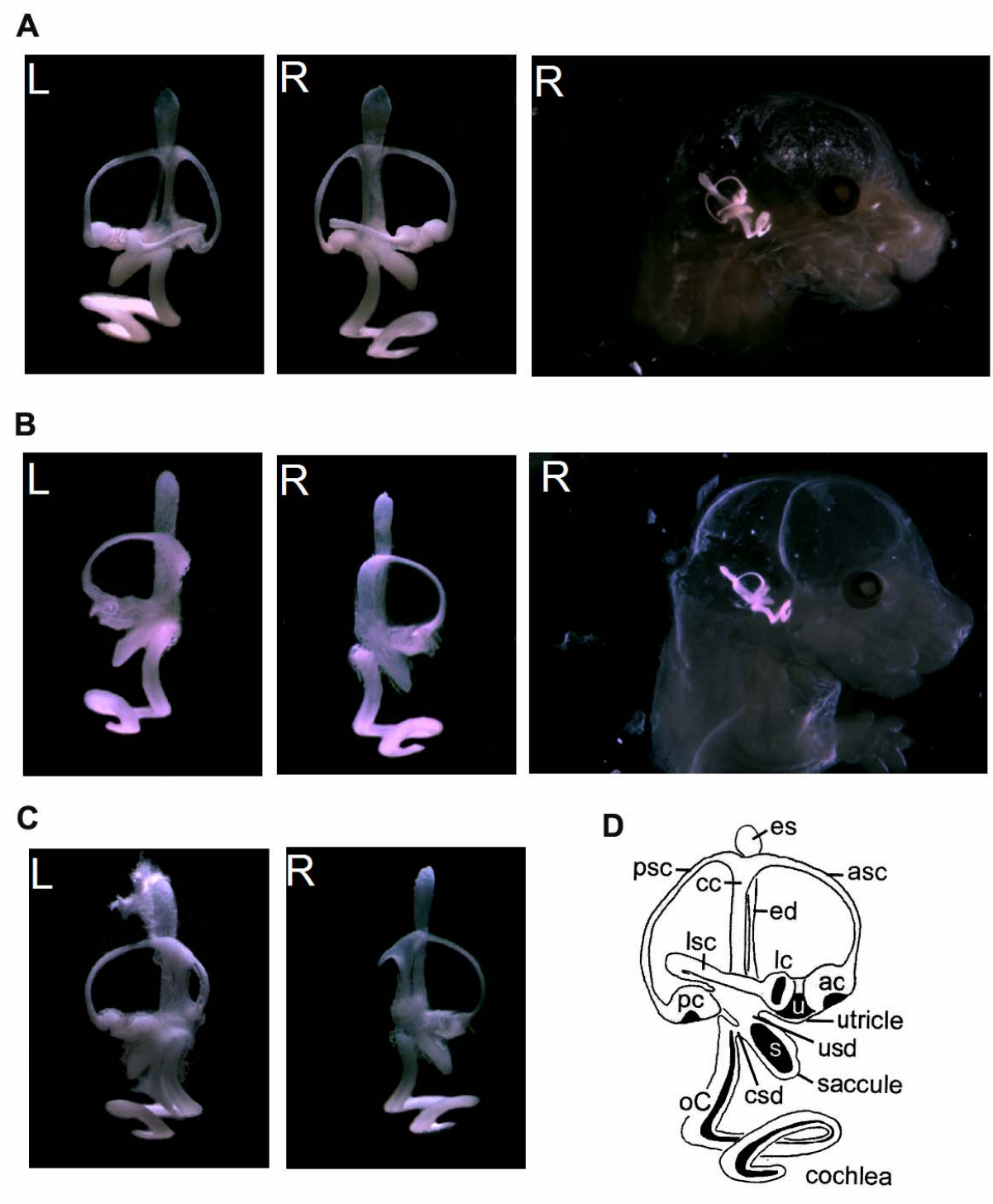
A
L
R
R
B
L
R
R
C
L
R
D
es
psc
cc
asc
ed
lsc
lc
ac
pc
u
utricle
s
usd
saccule
oC
csd
cochlea

Figure 4.5

CHAPTER 5

CONCLUSION AND PERSPECTIVE

The development of most organs shares some common mechanisms, such as using different combinations of the same molecular pathways and important regulator genes. To acquire organ-specific phenotypes many organs express lineage-specific transcription factor genes to regulate the specification and differentiation stages. For example, the thyroid expresses *Nkx2.1* and *Ttf2* (Grapin-Botton and Melton, 2000), the pancreas has *Pdx1* and *Hlxb9* (Grapin-Botton and Melton, 2000), and the parathyroids use the *Gcm2* gene. Thus, the study of *Gcm2* as an important regulator gene in parathyroid organogenesis will provide us with insights into the mechanisms of how these lineage-specific transcription factor genes function in organogenesis. In addition to this significance on organogenesis, our experiments will also help us to understand the causes of some parathyroid diseases in humans that are caused by *Gcm2* gene mutations (Correa et al., 2002; Maret et al., 2004; Sticht and Hashemolhosseini, 2006; Thakker, 2001). In this dissertation, I investigated two aspects of *Gcm2* function in mouse embryonic development: 1. whether *Gcm2* functions as a master regulator gene to specify parathyroid cell fate in parathyroid organogenesis as previously proposed; 2. whether *Gcm2* has a role in the development of other organs.

Gcm2 and parathyroid organogenesis

Based on its expression pattern, the role played by its orthologous genes in fly embryonic development, and the aparathyroid phenotype in its null mutants, *Gcm2* was proposed to be the master regulator gene in parathyroid organogenesis by defining the anterior/dorsal side of the 3rd pharyngeal pouch endoderm or of the parathyroid/thymus common primordium as the

parathyroid-specific domain (Balling and Erben, 2000; Berg, 2002; Manley and Blackburn, 2004). However, in contrast with this hypothesis, our data show that the mouse *Gcm2* gene functions only in the differentiation step but not the specification step of the parathyroid organogenesis. Without *Gcm2* function, early differentiation can be initiated, as shown by CasR and CCL21 expression at the anterior/dorsal side of the 3rd pouch. However the later differentiation step is blocked, and cells lose the expression of all parathyroid marker genes in the parathyroid domain. From E12.5, the parathyroid domain undergoes apoptosis.

According to these results, there is another key gene(s) acting upstream of *Gcm2* that specifies parathyroid cell fate. In Chapter2, we hypothesized that *Tbx1* might act as a parathyroid determinant via a Shh-Tbx1-Gcm2 regulatory pathway. To further explore this possibility, we can perform *Tbx1* conditional gain-of-function experiment to see if the ectopic expression of *Tbx1* in the 3rd pouch can change the parathyroid/thymus specification event.

Another remaining question is how *Gcm2* regulates the differentiation and survival of parathyroid precursor cells. A key experiment is to identify target genes regulated by the Gcm2 transcription factor. Since the target sequence for Gcm2 is known, we can search the whole mouse genome for Gcm2 binding sites and identify the candidate genes for further genetic analyses or biochemical studies. Another approach would be to use microarray technology to determine how gene expression profile changes in the parathyroid domain in the *Gcm2* null mutant (this domain is present before E12, and can be picked out by laser capture technique) by comparing with the wild-type parathyroid domain. This experiment will identify all the genes that are affected by *Gcm2* null mutation. Among these genes, we can further identify the direct or indirect targets of *Gcm2*.

In my dissertation, I performed gain-of-function studies for the *Gcm2* gene to test whether the entire parathyroid/thymus common primordium could acquire a parathyroid cell fate following forced ectopic Gcm2 expression in the presumptive thymus cells. Unfortunately, due to the low level of forced Gcm2 expression from Rosa26 promoter, which was much lower than the endogenous Gcm2 expression level and was unable to rescue *Gcm2* null mutation, we were unable to conclude whether or not the mouse *Gcm2* gene is sufficient to specify parathyroid cell fate. We have an ongoing experiment using a stronger promoter to generate the new Gcm2 inducible mice that will test our hypothesis.

Does Gcm2 have a role in the development of other organs?

In *Drosophila*, *Gcm* genes were originally found to function as glial determinant genes during central nervous system development. Further studies revealed additional roles for *Drosophila Gcm* genes in the development of blood cells and the visual nervous system (Chotard et al., 2005; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001; Lebestky et al., 2000; Vincent et al., 1996). This indicates that *Gcm* genes have diverse functions in embryonic development. The major expression site of *Gcm2* in mouse was demonstrated to be the parathyroid domain and later the parathyroid gland (Gordon et al., 2001; Kim et al., 1998), but it was also found to be expressed in other tissues (Gray et al., 2004; Iwasaki et al., 2003; Kim et al., 1998). Although there is no doubt about the important role of *Gcm2* in parathyroid development, its role in the development of other organs remains unclear.

We have data to suggest that *Gcm2* may have function outside parathyroids. In our study of the origin and regulation of thymic PTH expression, we showed that the lethality in *Gcm2*$^{-/-}$ mutants was not related to the reduction of serum PTH levels, suggesting that the *Gcm2* null mutation may cause defects in non-parathyroid organ(s). In another study using gain-of-function

experiments, we showed that ectopic *Gcm2* expression in all tissues caused defects in several non-parathyroid organs and a lethality phenotype. Although we still do not know the reason of the lethality phenotype and the eyelid defects, the expression of the endogenous *Gcm2* gene in the brain and otic region suggests that the inner ear defects may result from the overdose of Gcm2 in the hindbrain or otic region. However we need further studies to investigate whether the endogenous *Gcm2* gene functions during hindbrain and ear development, and how Gcm2 overexpression in these tissues affects normal *Gcm2* function.

To further define the role of the *Gcm2* gene in mouse embryonic development, we will first confirm the expression pattern of *Gcm2*. High-sensitive *Gcm2 in situ* hybridization should be performed to cover earlier stages like E7.5 to E9.5, which might have been ignored before. Especially we need to carefully check *Gcm2* expression in the hindbrain and otic region. We will also further examine *Gcm2* null mutants to determine the cause of their neonatal lethality and check if they have defects in the central nervous system and the inner ear. Since *Gcm1* may function redundantly with *Gcm2* in these organs, as has been shown in *Drosophila* (Alfonso and Jones, 2002; Chotard et al., 2005; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001; Lebestky et al., 2000; Vincent et al., 1996), when we study *Gcm2* expression it is important to examine whether *Gcm1* is expressed in the same tissues. If we find *Gcm1* and *Gcm2* are expressed together in these organs and we cannot detect the defects in the *Gcm2* null mutants, we would have to analyze *Gcm1*;*Gcm2* double mutants to address whether these two genes function redundantly in the development of these organs.

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