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Gcm2 is required for the differentiation and survival of parathyroid precursor cells in the parathyroid/thymus primordia

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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 and 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.

Keywords: Parathyroid; Thymus; Pharyngeal pouch; Organogenesis; *Gcm2*; Parathyroid hormone (PTH)

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,b; 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 are 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

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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, 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 begins 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 suggest 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