Parathyroid Development and the Role of Tubulin Chaperone E

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Key Words

Parathyroid glands development · Hypoparathyroidism · HRD syndrome · Tubulin chaperone E

Abstract

The development of the parathyroid glands involves complex embryonic processes of cell-specific differentiation and migration of the glands from their sites of origin in the pharynx and pharyngeal pouches to their final positions along the ventral midline of the pharyngeal and upper thoracic region. The recognition of several distinct genetic forms of isolated and syndromic hypoparathyroidism led us to review the recent findings on the molecular mechanisms of the development of the parathyroid glands. Although far from being understood, a special emphasis was given to the possible role of tubulin chaperone E (TBCE), which was implicated in the pathogenesis of the hypoparathyroidism, retardation and dysmorphism (HRD) syndrome. The novel finding that TBCE plays a critical role in the formation of the parathyroid opens a novel domain of research, not anticipated previously, into the complex process of parathyroid development.

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Introduction

The principal function of the parathyroid glands is to regulate calcium ion concentration by release of parathyroid hormone (PTH).

The parathyroid glands develop in humans between 5 and 12 weeks of gestation from the 3rd and 4th pharyngeal pouches. The epithelium of the dorsal part of the 3rd and 4th pouches proliferates during the 5th week and forms small nodules on the dorsal aspect of each pouch. Vascular mesenchyme soon grows into these nodules, forming a capillary network. The principal cells differentiate during the embryonic period and are believed to become functionally active in regulating fetal calcium metabolism. The epithelium of the dorsal wing of the third pouch differentiates into the inferior parathyroid gland while the ventral wing forms the thymus, then the gland primordia disconnect from the pharyngeal wall and migrate in a caudal and medial direction and become closely associated with the inferior region of the thyroid gland. The parathyroids can become embedded within the thyroid, but always remain a separate organ. The superior parathyroid glands are derived by the 6th week from the dorsal part of the 4th pharyngeal pouches and lie on the dorsal surface of the thyroid gland. The superior glands are more constant in position than the inferior ones. Occasionally an inferior gland fails to descend and remains near the bifurcation of the common carotid artery. In other cases it may accompany the thymus into the thorax. Uncommonly there are more than four parathyroid glands. Supernumerary parathyroid glands probably result from division of the primordia of the original glands. Absence of a parathyroid gland results from failure of one of the primordia to differentiate or from atrophy of a gland early in development [1].

Table 1. Genetic disorders associated with congenital hypoparathyroidism

Gene	Chromosomal location	Disease	Inheritance	Main clinical features	OMIM (No.)
PTH	11p15	hypoPTH	AR/AD	isolated hypoPTH	146200 168450
GCMB	6p23-p24	hypoPTH	AR	isolated hypoPTH	146200 603716
CaSR	3q13	hypocalcemia with hypercalciuria	AD	low or inappropriate 'normal' PTH levels	146200 601199
Regulatory elements of SOX3	Xq26-27	hypoPTH	X-linked recessive	isolated hypoPTH	307700
TBX1, CrkL and others	22q11.2 10p13	DiGeorge syndrome	AD or sporadic	hypoPTH, thymic hypoplasia outflow tract defects of the heart facial dysmorphism	188400 601362
GATA 3	10p15	HDR syndrome	AD	hypoPTH, deafness, renal failure	146255
TBCE	1q42-43	HRD syndrome	AR	hypoPTH, growth and developmental retardation dysmorphism	241410
Unknown	Unknown	hypoparathyroidism- lymph edema syndrome	AR or X-linked recessive	renal failure	247410

AR = Autosomal recessive, AD = autosomal dominant.

Molecular Mechanisms Determining Parathyroid Glands Development

Extensive research, mainly in murine models, has identified secreted molecules and transcription factors important in the formation of the parathyroid glands. Mice have only two parathyroid glands that are generated exclusively from the third pharyngeal pouch and are morphologically distinct by embryonic (E) day 8, fully developed by E12, and in their final position by E15.5 [2 and references therein]. The molecular mechanisms defined in mice have proven to be relevant to human parathyroid development as well and defects in these pathways were found to play a role in various forms of congenital hypoparathyroidism (table 1). A summary of the molecules predicted to have a role in parathyroid development and their presumed regulators is presented in table 2.

Signals from pharyngeal endoderm, that gives rise to the thyroid, parathyroid and thymus glands, pattern the neural crest arch and mesoderm [3–7]. Ablation of the cranial neural crest in the chick results in aplasia or dysplasia of the thymus, parathyroid and thyroid and the heart [8]. The neural crest mesenchyme contributes connective tissue elements to the thymus and parathyroid

[9–11]. However, the precise role of neural crest cells in the development of organs derived from the pharyngeal region remains unclear.

Secreted Signaling Regulators

Retinoid Signaling. Retinoid signaling has been shown to be essential for the formation of the 3rd and 4th pharyngeal arches [12, 13]. Mice deficient in retinoic acid (RA) due to targeted inactivation of retinaldehyde dehydrogenase 2, the enzyme responsible for early embryonic RA synthesis, die in utero because of cardiac malformations. Transient maternal RA supplementation from E7.5 rescues these embryos, but they exhibit impaired development of the 3rd–6th branchial arch region [14]. RA is known to induce the expression of the family of Hox genes transcription factor (see below) in sequential order corresponding to their chromosome location according to the time and level of RA [15]. Expression of Hoxb1 and Hoxa1 were indeed demonstrated to be markedly reduced, but the expression of Hoxa3 was not verified. The secreted factor Fgf8 is expressed at abnormally low levels in the RA mutants [14] suggesting this growth factor as a downstream

Table 2. Genes suggested being involved in the development of the murine parathyroid glands

Gene	Function	Downstream of	Upstream of
Retinoic acid	secreted signaling protein		Hox genes, Fgf8
Shh	secreted signaling protein		Fgf8, Bmp4
Fgf8	secreted signaling protein	retinoic acid, Shh, Tbx1, Crkl	Crkl
Bmp4	secreted signaling protein	Shh	
TGF β	secreted signaling protein		Tbx1, Crkl
Vegf	secreted signaling protein	Tbx1, Crkl	Tbx1, Crkl
Crkl	adaptor protein, tyrosine kinase signaling	TGF β , Fgf8	
Hoxa 3	transcription factor	RETINOIC acid (?)	Pax1, Gcm2, Eya1
Pax9	transcription factor	Tbx1	Eya1
Pax1	transcription factor	Hoxa 3	Gcm2, Eya1
GCM2	transcription factor	Hoxa 3, Pax1, Pax9, Eya1, Six1, Tbx1	
Eya1	transcription factor	Hoxa 3, Pax1, Pax9	Six1, Gcm2
Six1	transcription factor	Eya1	
Sox3	transcription factor		
Tbx1	transcription factor		Pax9, Gcm2
GATA3	transcription factor	TGF β , Vegf	

target of RA signaling. The transcription factor Tbx1 expression is not affected in the mutant, thus it is not considered to be a critical determinant of the phenotype [14].

Shh, Fgf8 and Bmp4. These secreted signaling molecules produced by the pharyngeal endoderm and ectoderm have been demonstrated to have a role in the development of the parathyroid glands [16 and references therein]. Multiple cell populations of the pharyngeal arches require Shh activity for survival, but not for initial formation and patterning. Shh appears to act predominantly as a repressor of the key pharyngeal pouch identity genes Fgf8 and Bmp4 since the parathyroid glands are missing in Shh $^{-/-}$ mice while the thymus domain and the population of Bmp4 expressing cells are expanded [16]. Shh and the transcription factor Hoxa3 (see below) appear to be in different pathways since their expression patterns are not affected in the reciprocal null mice [16].

TGF β . Specific inactivation of TGF β signaling in neural crest stem cells by conditional inactivation of the TGF β receptor type II gene (T β RII) in mice causes the characteristic cardiovascular defects and the thymic, parathyroid and craniofacial anomalies of the DiGeorge syndrome (DGS) [17]. Tbx1 and Crkl mutants, two genes encoded in the 3-Mb common deletion of 22q11 causing the DGS, are also implicated in neural crest cell migration. Knockout of each of these gene phenocopies DGS and interferes with neural crest migration into the pharyngeal arch complex [18, 19] and the actions of Fgf8 and Vegf [18]. Crkl is an adaptor protein that is highly ex-

pressed in neural crest during mouse development and which may mediate TGF β and Fgf8 signaling. 40% of Crkl $^{-/-}$ embryos had abnormalities in the development of the thymus, thyroid and parathyroid glands [20]. Phosphorylation of Crkl is detectable at E10 and restricted to a narrow time window as it is no longer observed from E11.5 onward. This phosphorylation is impaired in T β RII-deficient neural crest cells [17]. Fgf8 induces tyrosine phosphorylation of FgfRs 1 and 2 and their binding to Crkl [20]. Moreover, Crkl is required for normal cellular responses to Fgf8, including survival and migration, Erk activation, and target gene expression [20]. Crkl functions in additional signaling pathways activated by VEGF, EGF, PDGF and BMP/TGF β [20 and references therein]. A potential association between TGF β , Src and Crkl signaling was suggested since TGF β signaling has been reported to induce phosphorylation and activation of Src tyrosine kinase regulating TGF β -dependent apoptosis, cell migration or tumor cell invasiveness [17].

Mice that are compound heterozygotes for null alleles of CRKL and the transcription factor TBX1 (see below), display a striking increase in the penetrance and expressivity of a DGS-like phenotype compared to heterozygosity at either locus alone. Furthermore, these two genes have critical dose-dependent functions in pharyngeal segmentation, patterning of the pharyngeal apparatus along the anteroposterior axis, and local regulation of RA metabolism and signaling [21]. However the mechanisms mediating this synergy are not known.

The Role of Transcription Factors

Several transcription factor genes have been implicated in the development of the pharyngeal organs.

Hoxa3. This member of the Hox gene family, which specifies positional identity in the developing embryo [15], is strongly expressed in the 3rd pharyngeal arch and pouch from which the two parathyroid glands of mice develop, and in the 4th pharyngeal pouch endoderm and neural crest mesenchyme [22]. Inactivation of the *Hoxa3* gene in mice results in parathyroid and thymic aplasia and persistent ultimobranchial bodies [22–24].

Pax1 and Pax9. These are closely related members of the paired-box gene family, which play critical roles in the development of multiple organs [25–28]. Inactivation of *Pax9* results in early failure of thymus, parathyroid and ultimobranchial body formation [29], while *Pax1* mutants have hypoplastic parathyroid and thymus and disturbed thymocyte maturation [30, 31]. The initiation of *pax-1* expression in the 3rd pouch is not disturbed in *Hoxa3* null mice. However, by E10.5, *Hoxa3* homozygotes show a marked reduction in the level of *pax-1* expression in the 3rd pouch and by E11, *pax-1* expression is not detectable in the 3rd pouch of the mutant. This observation suggests that *pax-1* is downstream of *Hoxa3* [22].

GCMB. The human and murine (*Gcm2*) homologs of the *Drosophila* *glial cells missing* gene play a key role in the organogenesis of the parathyroid gland [32–34]. GCMB is exclusively expressed in the PTH-secreting cells of the developing and mature parathyroid gland [33]. During parathyroid organogenesis, *Hoxa3* is required for the initiation of *Gcm2* expression in the 3rd pouch endoderm, and both *Hoxa3* and *Pax1* are required for the maintenance of *Gcm2* expression [31]. *Gcm2* null mice lacked parathyroid glands and exhibited biological hypoparathyroidism [35]. However, these mice were viable and fertile and had only a mildly abnormal bone phenotype. Despite their lack of parathyroid glands, *Gcm2*-deficient mice had PTH serum levels identical to those of wild-type mice, as did parathyroidectomized wild-type animals. Thus, mice appear to have an auxiliary mechanism for the regulation of calcium homeostasis, suggested to be a downregulatable production of PTH from the thymus [35].

In contrast to *Gcm2* null mice, a homozygous deletion of the 5'-end of the GCMB gene was described in a 9-month-old girl with parathyroid agenesis accompanied by hypoparathyroidism characterized by hypocalcemia, hyperphosphatemia, and absent or very low circulating PTH levels [36]. This discrepancy might indicate that the

thymus plays a less important role as an auxiliary source of PTH in humans than in mice. Loss of the GCMB gene appears to cause a highly specific embryological defect that is limited to parathyroid cells, thus confirming its restricted role in the control of differentiation of cells into parathyroid glands. The correlation between GCMB expression and differentiation into parathyroid cells is exemplified by the upregulation of GCMB expression in abnormal parathyroid glands of hyperparathyroidism and the decreases in expression in response to hypocalcemia [37].

Eyes Absent 1 (*Eya1*). *Eya* genes regulate organogenesis in both vertebrates and invertebrates. Targeted inactivation of murine *Eya1* impairs early developmental processes in multiple organs including ear, kidney, thymus parathyroid and thyroid [38]. *Eya1* is expressed in all three cell types of the 3rd pharyngeal clefts: neural crest-derived arch mesenchyme, pouch endoderm and possibly the surface ectoderm, during thymic and parathyroid development from E9.5. The organ primordia for both of these structures failed to form in *Eya*^{-/-} embryos [39]. Expression of the transcription factor *Six1* is markedly reduced in all 3rd pharyngeal cleft derivatives in *Eya*^{-/-} embryos, indicating that its transcription is *Eya1*-dependent. In addition, *Gcm2* expression is undetectable at E10.5, but the expression of *Hox* and *Pax9* and *Pax 1* genes is preserved at E9.5–10.5 [39]. This expression pattern fits with the finding that *Eya1* functions in an evolutionarily conserved cassette downstream of *Pax* genes and upstream of *Six* genes during the development of pharyngeal pouch endoderm. There are no significant changes of *Eya1* expression in the pharyngeal pouch endoderm at E9.5 of *Pax9* null embryos [39]. Similarly, *Gcm2* expression is initiated normally at E9.5–10.5 in *Pax1* single mutants [31]. As this finding contradicts the theory of *Eya1* functioning downstream of the *Pax* genes, the possibility has been suggested that *Pax1* and *Pax9* function redundantly in the pharyngeal pouches and that a critical threshold of *Pax1*/*Pax9* protein expression in pharyngeal endoderm regulates *Eya1* expression. Such compensatory upregulation has been noted for *Pax1* and *Pax9* in the formation of other organs [40]. However, *Pax1* apparently does not compensate for *Pax9* deficiency during embryonic parathyroid and thymus formation. Thus, it was suggested that *Gcm2* expression is regulated by the *Hoxa3*-*Pax1/9*-*Eya1* pathway during parathyroid organogenesis [31].

Sox3. *Sox3* is a transcription factor belonging to a family of high mobility group box proteins that includes SRY, the Y-linked sex determining gene and other master reg-

ulator genes [reviewed in 41]. It was recently implicated to have a role in the development of the parathyroid glands by the finding that the X-linked recessive form of hypoparathyroidism is caused by an interstitial deletion-insertion involving chromosomes 2p25.3 and Xq27.1 near the Sox3 gene [42]. The deletion of 23–25 kb telomeric to Sox3 was postulated to ablate transcriptional regulatory regions. The demonstration that Sox3 is transcribed between 10.5 and 15.5 dpc in the pharyngeal pouches and developing parathyroids of the mouse embryo supports this hypothesis. Mutations causing altered transcription levels of SOX3 do not result in parathyroid abnormalities but in hypopituitarism and a mutation in the SOX3 coding region causes hypopituitarism, mental retardation and growth hormone deficiency. Sox3-null mice have abnormal pituitary development, craniofacial abnormalities and midline central nervous system (CNS) defects, but no parathyroid abnormality has been reported [42 and references therein]. These observations support the hypothesis that the X-linked form of hypoparathyroidism is caused by disruption of regulatory elements of Sox3.

Tbx1. The role of the genes present in the deletion causing the DGS has been extensively studied [18]. The deletion mutants Df1 or Lgdel, which reduce the 22 genes of the deletion to hemizyosity [43], recapitulate the DGS phenotype. The embryological phenotype of the transcription factor Tbx1+/- mice is indistinguishable from the Df1 mutants [44–46], and thus haploinsufficiency of Tbx1 is proposed to be the major contributor to the syndrome. Microarray analysis of mice lacking Tbx1 [47] identified additional transcription factors (Pax9, Gcm2 and Gbx2) that are quantitatively reduced in expression, thus suggesting that they are Tbx1 target genes.

GATA3. The syndrome of hypoparathyroidism, deafness and renal anomalies (HDR) was found to be caused by mutations causing loss of function in the GATA3 transcription factor, consistent with the expression pattern of GATA3 during human and mouse embryogenesis [48]. GATA3 is also expressed in the developing CNS and the hematopoietic organs in human and mice, and accordingly, homozygous Gata3 knockout mice have defects of the CNS and T-cell development, but not hypoparathyroidism [49]. The manner in which GATA3 mutations cause the syndrome remains to be elucidated since direct correlation between the phenotypic presentation and the mutations could not be established [50].

Tubulin Chaperone E (TBCE) and Parathyroid Development

The syndrome of hypoparathyroidism, growth and mental retardation, and characteristic dysmorphism (HRD syndrome) known alternatively as autosomal recessive Kenny-Caffey syndrome [51] (MIM244460), HRD [52] (MIM241410), or Sanjad-Sakati syndrome [53] is caused by mutations in the tubulin-specific chaperone E gene, *TBCE* (fig. 1) [54]. The cardinal manifestations of this disease include parathyroid aplasia, growth failure, ocular malformations and microcephalic mental retardation (fig. 2). Secondary features include characteristic facial dysmorphism, cryptorchidism/micropenis, susceptibility to bacterial infection and variable osteosclerosis [55]. The hypoparathyroidism is due to the absence of the parathyroid glands [Hershkovitz, unpubl. data, and 56].

Coincident with this discovery, a missense mutation in murine *Tbce* (W527G) was described in a mouse model of peripheral motor neuropathy, *pmn* [57, 58]. The W527G mutation destabilized the chaperone, resulting in diminished protein levels [56]. The original *pmn* mice had low birth weight, decreased brain size, and hypogonadism, reminiscent of the human trait, but no hypoparathyroidism was noted [59]. As HRD patients have survived to late adolescence, some have developed a hypoventilation syndrome and lower motor neuropathy [N. Sakati, pers. commun.], presumably secondary to a neurodegenerative process similar to that described in *pmn* mice. Since mice that lack the parathyroid glands have PTH serum levels identical to those of wild-type mice, as do parathyroidectomized wild-type animals, are viable and fertile and have only a mildly abnormal bone phenotype [35], it is possible that the parathyroid defect in *pmn* mice had been overlooked. Thus, the concordance between the human and murine phenotypes is greater than previously appreciated.

Presently, the only known function for TBCE is a chaperone of α -tubulin.

Tubulin Folding and Assembly

Microtubules are polymerized from α/β -tubulin heterodimers [60]. Newly synthesized α - and β -tubulin polypeptides undergo a sequence of folding steps catalyzed by chaperones. Initially, the tubulins are associated with the hexameric prefoldin complex that passes them on to the cytosolic chaperonin complex [61], and they are then further processed by tubulin-folding cofactors [62,

Fig. 1. TBCE mutation analysis. **a** Sequence traces from affected subjects. Middle Eastern subjects were homozygous for 155–166del, a Belgian subject was compound heterozygous for 66–67delAG and 1113T→A. Deleted sequences underlined above electropherograms, point mutant shown. **b** Two truncating mutants (Val23fsTer48, Cys371Ter) are indicated by arrows, the in-frame del52–55 is indicated by an arrowhead. CAP-Gly and leucine-rich repeat domain locations are indicated. Approximate amino acid positions are below cartoon. [Taken from 54].

Fig. 2. A 14-year-old HRD patient with severe growth retardation and lack of pubertal signs.

Fig. 3. Tubulin-specific chaperone folding in mammalian cells. After folding in cytoplasmic chaperonin (CCT), tubulin subunits are folded in parallel pathways that converge to produce a supercomplex that assembles native GTP-tubulin, stimulates GTP hydrolysis and then releases native GDP-tubulin. Cofactor complex-bound heterodimers can also undergo subunit exchange or sequestration of subunits.

63]. In vitro folding assays suggest that in mammals α -tubulin binds to cofactors B and E, whereas β -tubulin binds to cofactors A and D. α -Tubulin/TBCE and β -tubulin/TBCD are bound by TBCC, forming a supercomplex from which α/β -tubulin heterodimers are released by GTP hydrolysis of β -tubulin. The small G-protein Arl2 appears to play a regulatory role, binding to and sequestering cofactor D [64] (fig. 3). Budding yeast mutants lacking tubulin cofactor homologs have only conditional effects and are normally not lethal [65 and references therein]. In contrast, null mutations in fission yeast TFC genes cause abnormal cell shapes and mostly result in lethality [66–70]. Genetic analysis of tubulin cofactor function in fission yeast has led to a different model of tubulin folding: an essential pathway of α -tubulin folding involves, successively, cofactors B, E, and D, with the Arl2 homolog acting upstream of D, whereas a non-essential pathway of β -tubulin folding involves cofactor A passing β -tubulin on to cofactor D to associate with α -tubulin [67–69]. Results in the plant *Arabidopsis* suggest that cofactors C–E and Arl2 are stringently required for microtubule formation, similar to the requirements for in vitro assays using purified mammalian cofactors [71]. *PFI*, the ortholog of the vertebrate *Tbce* in *Arabidopsis*, is necessary for continuous microtubule organization, mitotic division and cytokinesis but do not mediate cell cycle progression [72, 73]. Vesicle trafficking to the division plane during cytokinesis but not to the cell surface during interphase was impaired [72].

Our studies on the effect of a homozygous four amino acid deletion of TBCE (del52–55) in patients' cells demonstrated that lymphoblastoid cells showed aberrant microtubule polarity and the microtubules arrays are not centered on centrosomes in disease cells. This effect was more pronounced in fibroblasts than in keratinocytes.

Thus, the cellular phenotype may be tissue-specific despite ubiquitous transcriptional *TBCE* expression. Organization of the Golgi complex in patients' fibroblasts was diffuse and surrounded the nucleus, in contrast to its compact and localization near one side of the nucleus in control cells. The distribution of late endosomes which, like the Golgi complex, is microtubule-dependent, revealed an abnormally diffuse pattern in the whole cell in contrast to the predominantly perinuclear pattern observed in control cells.

Tubulin is needed for many essential life processes, including cell division and proper organelle positioning in the cell. Because chaperones are required to produce tubulin, most mutations in the genes encoding these proteins are probably lethal at a very early stage of embryogenesis. Therefore, we assume that the genetic lesions in *TBCE* which cause HRD syndrome in humans and progressive motor neuropathy in mouse could not totally abrogate the function of TBCE. Indeed, verification of a number of patients with phenotypes similar to HRD has not identified additional mutations in this gene [R.P. and G.D., unpubl. data].

Conclusion and Future Directions in the Study of TBCE and Parathyroid Development

The specific absence of parathyroid glands, with accompanying normal development of the thyroid and other branchial pouch derivatives, is an intriguing and unexpected aspect of a derangement in tubulin physiology. Microarray transcription analysis of *TBX1* null mice did not show effect on the transcription of *TBCE*, or any other tubulin chaperone. However, there was a reduction to half in the transcription of the tubulin α 8 isotype. *TBCE*

transcription in the parathyroid is amongst the lowest compared to other organs [Electronic Database: GEO <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=geo>]. This may render the parathyroid particularly sensitive to compromised action caused by mutation of the TBCE. Alternatively, or in addition, the development of the parathyroid glands may require a function that is particularly sensitive to the function of the microtubule cytoskeleton, such as migration, or particular cell morphology. As an example, migration of neurons and specifically 'neurokinesis' depend upon the microtubule network [74]. It was recently demonstrated that the tubulin cytoskeleton and two microtubule-associated proteins, LIS1 and doublecortin, function with dy-

nein to mediate coupling of the nucleus to the centrosome in neuronal migration [75], raising the possibility of a similar requirement for the microtubuli for the migration of the parathyroid glands. The lethality observed in TBCE mutants of fission yeasts [70] in contrast to the non-lethality of the budding yeast may demonstrate the differential requirements for the tubulin microtubule cytoskeleton. Still another possibility is that TBCE may have additional roles to its tubulin chaperone function for which the parathyroid is specifically sensitive. The interplay between the known factors involved in the parathyroid development and the TBCE and/or microtubule cytoskeleton remains to be elucidated by future studies.

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