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Development of thyroid gland and ultimobranchial body cyst is independent of p63

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The ultimobranchial body (UBB) and thyroid primordium are the origins of the thyroid gland that fuse around embryonic day 14.5 of mouse gestation, ultimately giving rise to calcitonin-producing C cells and thyroglobulin-producing follicular cells, respectively. A homeodomain transcription factor NKX2-1 is expressed both in the UBB and the thyroid primordium, and is critical for development of the thyroid gland. In this study, the role of p63 in development of UBB and the thyroid gland was analyzed by histological, immunohistochemical, and electron microscopic analyses using mice with various combinations of *Nkx2-1* and *p63* wild-type, heterozygous, and null alleles. In the absence of p63, a normal thyroid gland develops, as revealed by expression of thyroglobulin and calcitonin, thus showing that p63 is not required for thyroid development. However, in mice carrying the *Nkx2-1*-null allele, the UBB remains as a cystic vesicular structure and/or in nested patterns consisting of p63-positive cells surrounding the vesicle and undifferentiated immature cells with occasional cilia lying inside. The cystic UBB was present even in the *Nkx2-1;p63* double-null mice. The structure and p63 expression pattern of the UBB cyst strikingly resemble the solid cell nest. These results show that in the absence of NKX2-1, UBB becomes cystic independent of p63, which is likely the origin of SCN.
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The thyroid gland of mammals has two distinct cell types: follicular cells and C cells.[1–4] The follicular cells are derived from the thyroid primordium, outpocketing of the foregut endoderm that loses its connection to the foregut tube and subsequently descends in front of the pharyngeal gut as a bilobed diverticulum. The follicular cells eventually synthesize and secrete thyroid hormones. In contrast, C cells, or parafollicular cells are derived from the ultimobranchial body (UBB) that migrates from the fourth pharyngeal pouch to which neural crest cells have invaded. The UBB fuses with the thyroid primordium around mouse embryonic day (E) 14.5, and the cellular components of the UBB disseminate within the thyroid, ultimately giving rise to the calcitonin-producing C cells.[1–5] From the analogy to chick ultimobranchial C cells in the ultimobranchial gland, which express neuronal markers[6] and extend long neurite-like processes when cultured,[7] it is generally believed that the C cells in mammals also originate from the neural crest cells. However, it was recently shown that murine thyroid C cells are derived from the endodermal epithelial cells of the fourth pharyngeal pouch that expresses E-cadherin and do not originate from the neural crest cells.[8]

NKX2-1 (TTF1, TITF1, T/EBP, NKX2.1)[9,10] is a homeodomain transcription factor that is critical for the genesis of the thyroid, lung and ventral forebrain.[11] It is also essential for the regulation of lung and thyroid-specific expression of genes, the latter of which includes those encoding thyroglobulin and thyroid peroxidase.[2,12] NKX2-1 is expressed in the thyroid primodium and it is required for the maintenance of ordered architecture and function of the differentiated thyroid.[13] NKX2-1 is also expressed in the UBB rudiment,[5,11,14,15] and is responsible for the survival of the UBB cells and their dissemination into the thyroid diverticulum.[5] In *Nkx2-1*-null mice, the thyroid primordium starts degenerating around E10.5 before the commencement of its caudal migration[16] while the UBB remains as a cystic vesicular structure after NKX2-1-positive cells disintegrate.[5] This vesicular structure is lined by a monolayer of p63-negative cells, surrounded by a cluster and/or single layer of p63-positive cells.[5] The vesicular structure resembles the solid

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cell nests (SCNs) described in humans that contain both solid cell proliferation and follicular-like structures, and has been considered to be the embryonic remnants of the UBB.[17–20] SCNs are reported to be found in normal human fetal thyroid with approximately 32.5% in multi-step sections and 87.5% in serial sections.[21]

P63 is a member of the p53 tumor-suppressor family, which consists of several isotypes having full-length (TAp63) and N-terminal truncated forms (ΔNp63) as well as having three alternative splicing at the C-terminus (α, β, and γ).[22] TAp63α is the predominant isoform expressed in human thyroid cancer specimens and cell lines, while normal human thyroids do not express p63.[23–25] P63 is used as a marker for the main cells of the SCNs in humans.[18–20,24] P63 is also expressed in a subset of papillary thyroid carcinomas and/or Hashimoto's thyroiditis, often associated with SCN, suggesting a possible link between p63 expression, papillary thyroid cancer, Hashimoto's thyroiditis, and SCN.[18,25–27]

In this study, the role of p63 in thyroid development and the nature of UBB cysts were examined using embryos with various combinations of *p63* and *Nkx2-1* alleles (wild-type, heterozygous, and null) by histological and immunohistochemical methods, and electron microscopy (EM). The results showed that p63 is not required for thyroid development and the UBB rudiment remains as a cystic and/or vesicular structure even without *Nkx2-1* nor *p63* alleles, which consists of one layer of cells that seem to be very poorly differentiated.

MATERIALS AND METHODS

Animals

p63(+/−);*Nkx2-1*(+/−) mice (C57BL/6J background) were established by crossing *Nkx2-1*(+/−) mice[11] with *p63*(+/−) mice, both of which had been backcrossed six times to C57BL/6J mice. The *p63*(+/−) mice were produced by mating *p63*(*fl/fl*) mice (kindly provided by Dr Alea Mills[28]) with EIIa-Cre transgenic mice.[29] Animals were maintained under conditions outlined in the 'Guide for the Care and Use of Laboratory Animals' by the National Institutes of Health. Different genotypes of embryos in nine combinations were obtained at embryonic day (E) 12.5, 14.5, 17.5, and 18.5 by intercrossing *Nkx2-1*(+/−);*p63*(+/−) mice. A noon of the day when a vaginal plug was observed was considered as E 0.5. The genotypes of embryos used in this study were as follows (tentative name shown in the parenthesis); *p63*(+/+);*Nkx2-1*(+/+) (wild-type), *p63*(+/+);*Nkx2-1*(+/−) (*Nkx2-1*-Ht), *p63*(+/+);*Nkx2-1*(−/−) (*Nkx2-1*-null), *p63*(+/−);*Nkx2-1*(+/+) (*p63*-Ht), *p63*(+/−);*Nkx2-1*(+/−) (*p63*;*Nkx2-1*-dHt), *p63*(+/−);*Nkx2-1*(−/−) (*p63*-Ht;*Nkx2-1*-null), *p63*(−/−);*Nkx2-1*(+/+) (*p63*-null), *p63*(−/−);*Nkx2-1*(+/−) (*p63*-null;*Nkx2-1*-Ht), *and p63*(−/−);*Nkx2-1*(−/−) (*p63*;*Nkx2-1*-d null).

Genotyping was performed by PCR using DNAs isolated from yolk sacs or tails with the following conditions: 1 cycle for 3 min at 94 °C, 30 cycles for 30 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C, and 1 cycle for 5 min at 72 °C. The primers used were 5′-GGCGAGCGGCATGAATATGA-3′ (forward) and 5′-TCTTGTAGCGGTGGTTCTGGA-3′ (reverse) for *Nkx2-1* wild-type allele, and 5′-TCGCCTTCTATCGCCTTCTTGA-3′ was paired with the reverse primer for the detection of the *Nkx2-1* targeted allele. The primers for detection of *p63* (+/−) allele were 5′-CAGAGGAGGCAACACAGGATAGA-3′ and 5′-CCGGGGGATCCGAATTCATCGA-3′.[28] The loxP sites of *p63*(fl/fl) allele flank exons in the DNA-binding domain of p63, thus rendering all isotypes of p63 inactive in the presence of active Cre recombinase.[28]

Histopathological Examination

Histological analysis was carried out using at least three embryos or adult thyroids from each genotype and/or developmental stage. A whole embryo or a cervical region of an adult mouse containing the thyroid, larynx, and trachea was dissected, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4 °C overnight, dehydrated, and embedded in paraffin. Serial sections of 3 μm thickness were prepared, which were treated with xylene and graded ethanol, and were stained with hematoxyline and eosin (H&E). Disruption of the *p63* gene was confirmed by the presence of aplastic skin in *p63*(−/−) mice from E14.5 to postpartum as described.[30]

Immunohistochemistry

Sections were treated with 1% hydrogen peroxide in methanol for 30–45 min to block endogenous peroxidase activity, followed by rinsing three times for 10 min each with PBS. Epitope retrieval was carried out by heating sections at 100 °C for 3 min, five times using a microwave oven in 10 mM citrate buffer, pH 6.0, followed by cooling for 30 min at room temperature, and washing in PBS. Sections were then treated with 5% skim milk in PBS for 15 min to block nonspecific protein binding. Incubation with primary antibody was carried out overnight at 4 °C in a humidified chamber using the following antibodies: anti-p63 antibody (mouse monoclonal, 1:1000 dilution; BD Biosciences, San Diego, CA, USA), anti-TTF-1 antibody (mouse monoclonal, 1:1000 dilution; DAKO, Carpinteria, CA, USA), anti-SOX10 antibody (rabbit polyclonal, 1:1000 dilution; Abcam, Cambridge, MA, USA), anti-Nestin antibody (rabbit polyclonal, 1:1000 dilution; Abcam), anti-NSE antibody (goat polyclonal, 1:200 dilution, DAKO), anti-S-100 antibody (mouse monoclonal, 1:3000 dilution, Abcam), and anti-calcitonin antibody (rabbit polyclonal, 1:1000 dilution, ICN, Irvine, CA, USA). After washing in 0.01 M PBS, the sections were treated using labeled streptavidin-biotin method (LSAB2 System-HRP, ready-to-use, DAKO) or the ABC method with commercially available kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Immunocomplexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA).

Electron Microscopy

The tissue sample preparation for the EM analysis has been described in a great detail.[31] Briefly, mouse tissue was fixed in formaldehyde (4%) and glutaraldehyde (2%) in cacodylate buffer (0.1 M, pH 7.4) (Tousimis, Rockville, MD, USA) followed by a post-fixation in 1% osmium in same buffer. The tissue was *en bloc* stained in 0.5% uranyl acetate in acetate buffer (0.1 M pH 4.5) and dehydrated in a graded ethanol (35, 50, 70, 95, and 100%) and propylene oxide. The infiltration was carried out in an equal mixture of epoxy resin (Embed 812, Electron Microscope Sciences, Fort Washington, PA, USA) and propylene oxide overnight. The tissue was embedded in a pure epoxy resin and cured in 55 °C oven. Thin sections were mounted on copper grids and stained in uranyl acetate and lead citrate. The grids were examined in the EM (Hitachi H7600, Tokyo, Japan) operated at 80 kV and digital images were taken by CCD camera (AMT, Danvers, MA, USA).

RESULTS

We previously showed using *Nkx2-1*-null mice that the thyroid primordium disintegrates in the absence of NKX2-1.[11,16] As p63 is used as a marker for the SCN in humans that is considered to be embryonic remnant of UBB,[19,20,23] the role of p63 in development of UBB and/or thyroid was examined using mice with various combinations of *p63* and *Nkx2-1* alleles (wild-type, Ht, and null). The presence of UBB was confirmed in all genotyped mice at E12.5 (Figure 1). The UBB was composed of columnar cells having high nucleus/cytoplasm ratio, which configured the tubular or cystic patterns. At this gestational stage, the size of UBB was not significantly different among wild-type (Figure 1a), *Nkx2-1*-Ht, (Figure 1d) and *Nkx2-1*-null mice (Figure 1g) as previously described.[5] Similarly, p63 did not seem to have a significant effect on the size of UBB (Figures 1a–f). In contrast, the UBB appeared to look slightly smaller in *p63* and *Nkx2-1*-double-null mutants (Figure 1i). All the UBBs were histologically

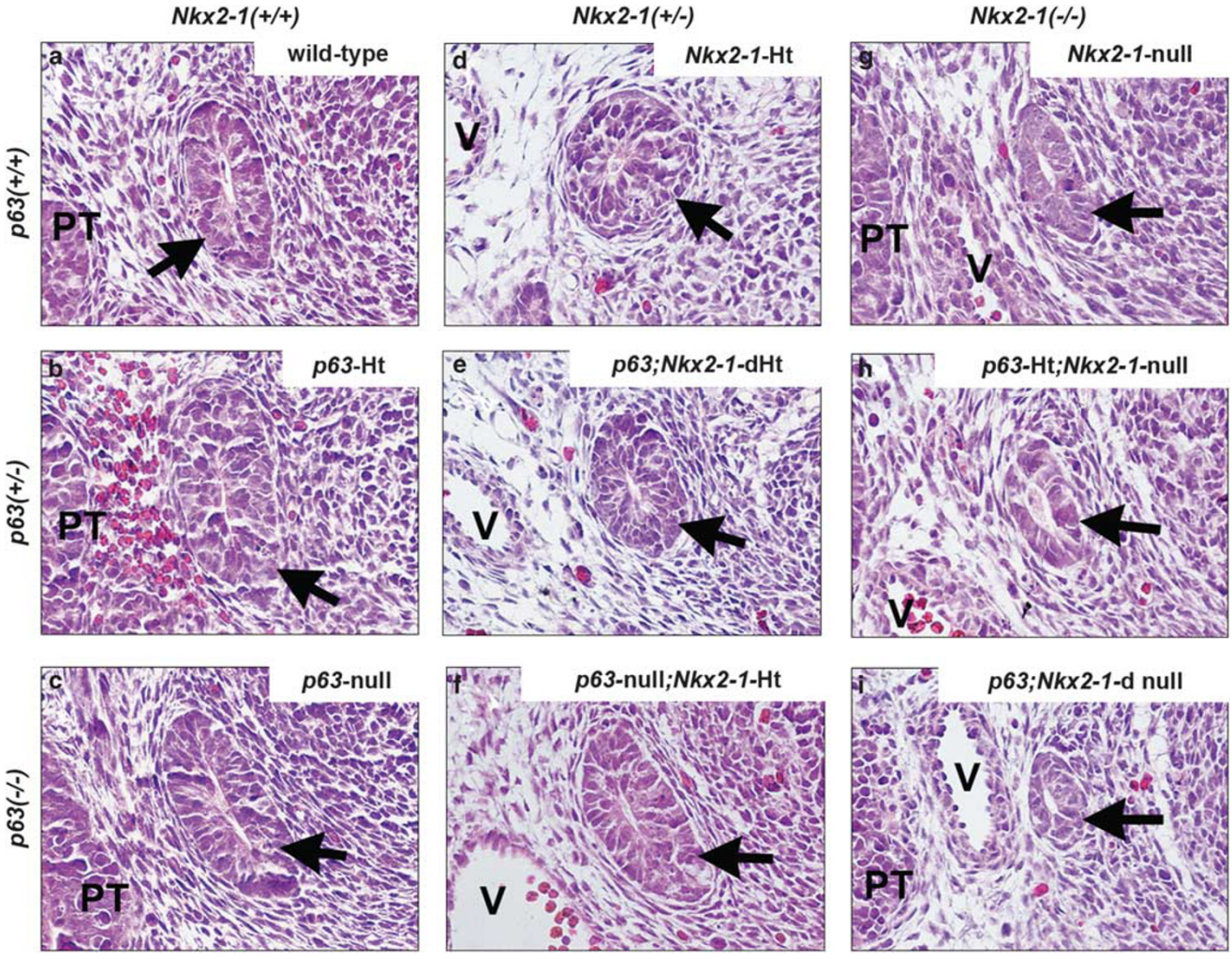

Figure 1 Ultimobranchial body (UBB) of E12.5 mouse embryos. Transverse sections are shown. (**a–i**) Each panel shows representative UBB (indicated by an arrow) from mice with various combinations of *p63* and *Nkx2-1* alleles as indicated. PT: parathyroid, V: vessel. Magnification: × 400.

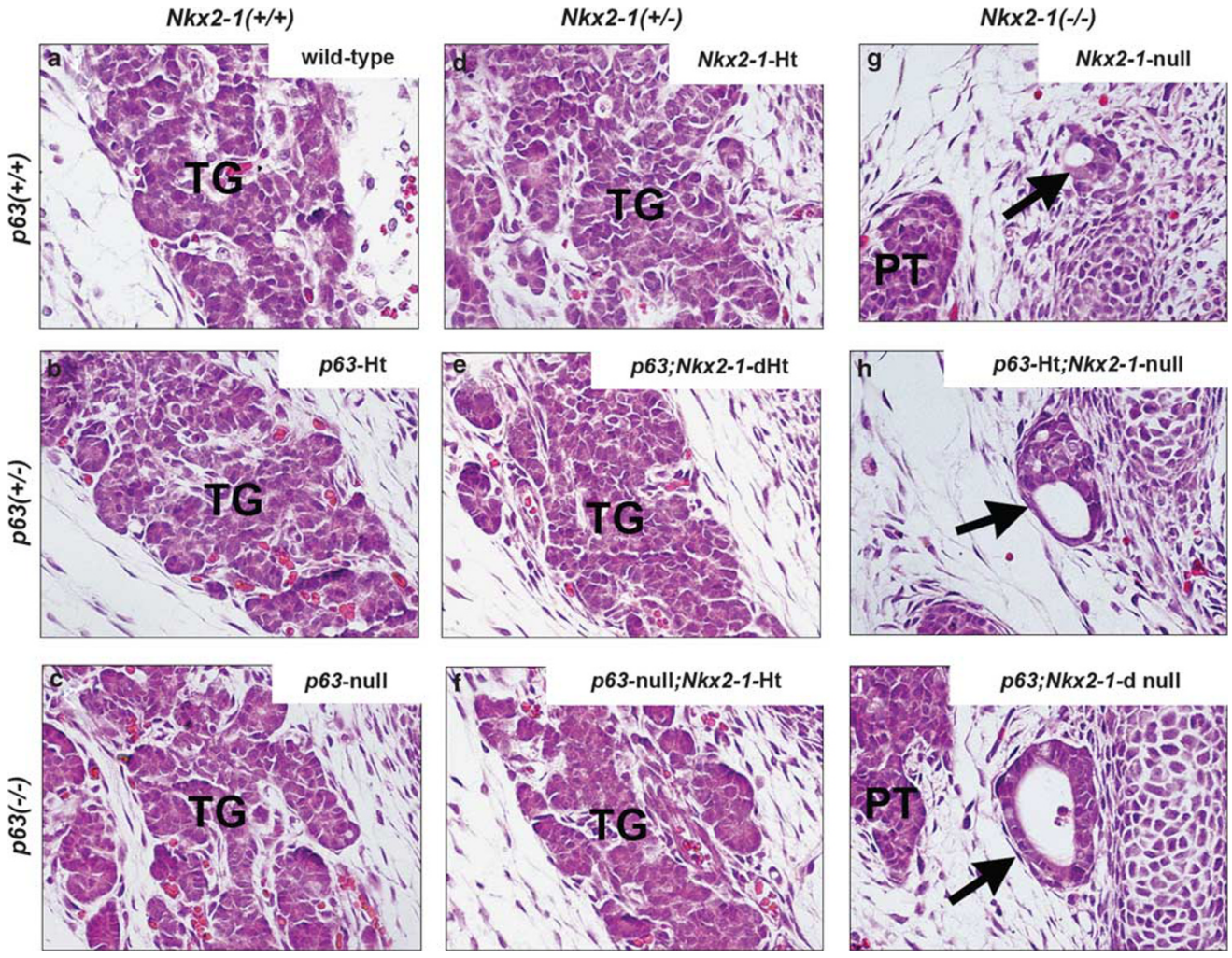

Figure 2 Thyroid gland and ultimobranchial body (UBB) of E14.5 mouse embryos. Frontal sections are shown. (**a**–**f**) Each panel shows representative thyroid (TG) from mice with various combinations of *p63* and *Nkx2-1* wild-type or Ht alleles as shown. (**g**–**i**) Each panel shows representative UBB cyst (indicated by an arrow) from mice with three different combinations of *p63* and *Nkx2-1*-null alleles as shown. PT: parathyroid. It is to be noted that each size of UBB cyst seems to be different because of section's orientation. Magnification: × 400.

indistinguishable. These results indicate that the development of UBB does not require either NKX2-1 or p63.

By E14.5 of mouse gestation, thyroid primordium cells meet with UBB cells, forming the thyroid gland.[1–5] Indeed, both wild-type and *Nkx2-1*-Ht mice formed a thyroid gland composed of cells in trabecular or microfollicular patterns, regardless of the presence of the *p63* allele (Figures 2a–f). As gestation proceeded, the thyroids from six different combinations of *Nkx2-1* (wild-type and Ht) and *p63* alleles (wild-type, Ht, and null) further increased in size, and by E18.5, many immature follicles of small and/or various sizes were clearly visible, in which colloid was present (Figures 3a–f). No significant histological differences in terms of size, follicle formation, and the presence of C-cells were found among these six different genotype thyroids. As the thyroid primordium of *Nkx2-1*-null mice starts degenerating around E10.5,[16] no thyroid with follicular forming cells and C-cells was found in mice carrying *Nkx2-1*-null allele. Instead, the UBB remained in tubular or cystic patterns, which was found even in the *p63*;*Nkx2-1*-double-null mutants (Figures 2g–i and 3g–l). By E18.5, the UBB consisted of increased number of cells (Figures 3g–i *vs* j-l), which appeared dilated with monolayer of cells in tubular structure in the *p63*;*Nkx2-1*-double-null embryos (Figure 3i). Occasionally ciliated cells were found in the UBB cysts (Figures 3g–l). These results show that p63 is not required for the development of thyroid gland and further suggest that cells in the *p63*;*Nkx2-1*-double-null UBB cyst resemble to those found in the SCN having occasional cilia.[18–20]

To examine whether p63 has any role in the function of thyroid, E17.5 embryonic thyroids from wild-type, *p63*-Ht, and *p63*-null mice were subjected to immunohistochemitry for thyroglobulin and calcitonin (Figure 4). Thyroid starts producing thyroid hormone around E15.[2,12] Thyroglobulin

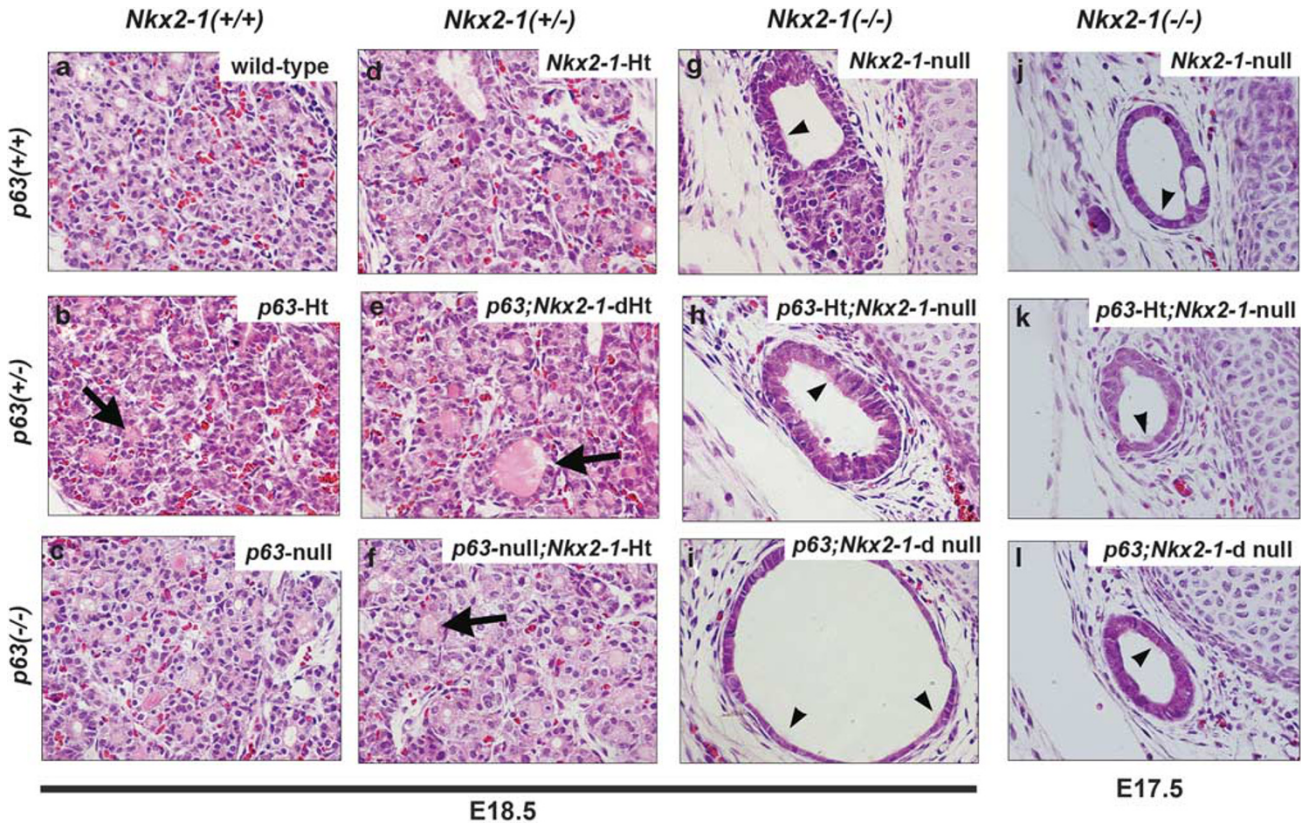

Figure 3 Thyroid and/or ultimobranchial body (UBB) cyst of mouse embryos at E17.5 and E18.5. Frontal sections are shown. (**a**–**f**) Each panel shows a representative thyroid from E18.5 mice with various combinations of *p63* and *Nkx2-1* wild-type or Ht alleles as shown. Many small follicles are present, some of which contain colloid (seen in pink color, indicated by an arrow). (**g**–**l**) Each panel shows representative UBB cyst from E18.5 (**g**–**i**) and E17.5 (**j**–**l**) mice with three different combinations of *p63* and *Nkx2-1*-null alleles as shown. Inside cysts, occasional ciliated cells are found (indicated by an arrowhead). Magnification: × 400.

was highly expressed in the thyroids of wild-type, and *p63*-Ht and null mice at similar immunohistochemical intensity (Figures 4a–c). Further, calcitonin was clearly detected in parafollicular cells of all three thyroids at similar extent (Figures 4d–f). These results show that p63 may not be essential for the function of thyroid.

To further characterize the UBB cysts, expression of p63 was next examined. It was reported that NKX2-1 is strongly expressed in the UBB of normal mouse embryos at E11.5–13.5,[5,11,14,15] while p63-positive cells are scarce.[5] In *Nkx2-1*-null mice, p63 expression was detected in a very small number of cells in E12.5 UBB (Figure 5a), while the expression was found in more cells as the gestational age increased (Figures 5b, c, e, and f). At later gestational ages, the p63-expressing cells were located either at the outer layer surrounding the vesicular structure (Figure 5b, c, e and f) or uniformly situated in the cystic structure (Figure 5e). This compartmentalized p63 staining is strikingly similar to those found in the SCN.[18–20] No expression of p63 was observed in the UBB cysts in mice carrying *p63*-null allele as expected (Figures 5d and g). It is to be noted that low levels of p63 expression were found in normal mouse thyroids (Figure 5h). This was in contrast to humans, in which no expression of p63 was reported.[23–25]

To further characterize the *p63*;*Nkx2-1*-double-null UBB cysts, immunohistochemistry was next carried out using antibodies for NSE (neuron-specific enolase) and S100 as neuron-specific markers, Oct 4 as a stem cell marker, nestin and SOX10 as a neural crest marker, and vimentin as a mesenchymal marker. Positive staining was not found with any of the antibodies examined in E12.5, 14.5, and 17.5 embryos (data not shown). When E14.5 *p63*;*Nkx2-1*-double-null UBB was subjected to electron microscopic examination, irregular shaped cells with scarce cytoplasm and fairly modestly electron dense nucleus were found (Figure 6b). Further, no clear intracellular organelles or adhesion structures were found. This was in sharp contrast to wild-type thyroid follicular cells, in which mitochondria, rough endoplasmic reticulum, tight junction, and desmosome were clearly observed. (Figure 6a). These results show that cells in the *p63*;*Nkx2-1*-double-null UBB are very poorly developed.

DISCUSSION

In this study, we showed using mice with various combinations of *p63* and *Nkx2-1* alleles, that p63 is not required for thyroid development and function. Further, UBB remains as a tubular structure even without *Nkx2-1* and *p63* alleles,

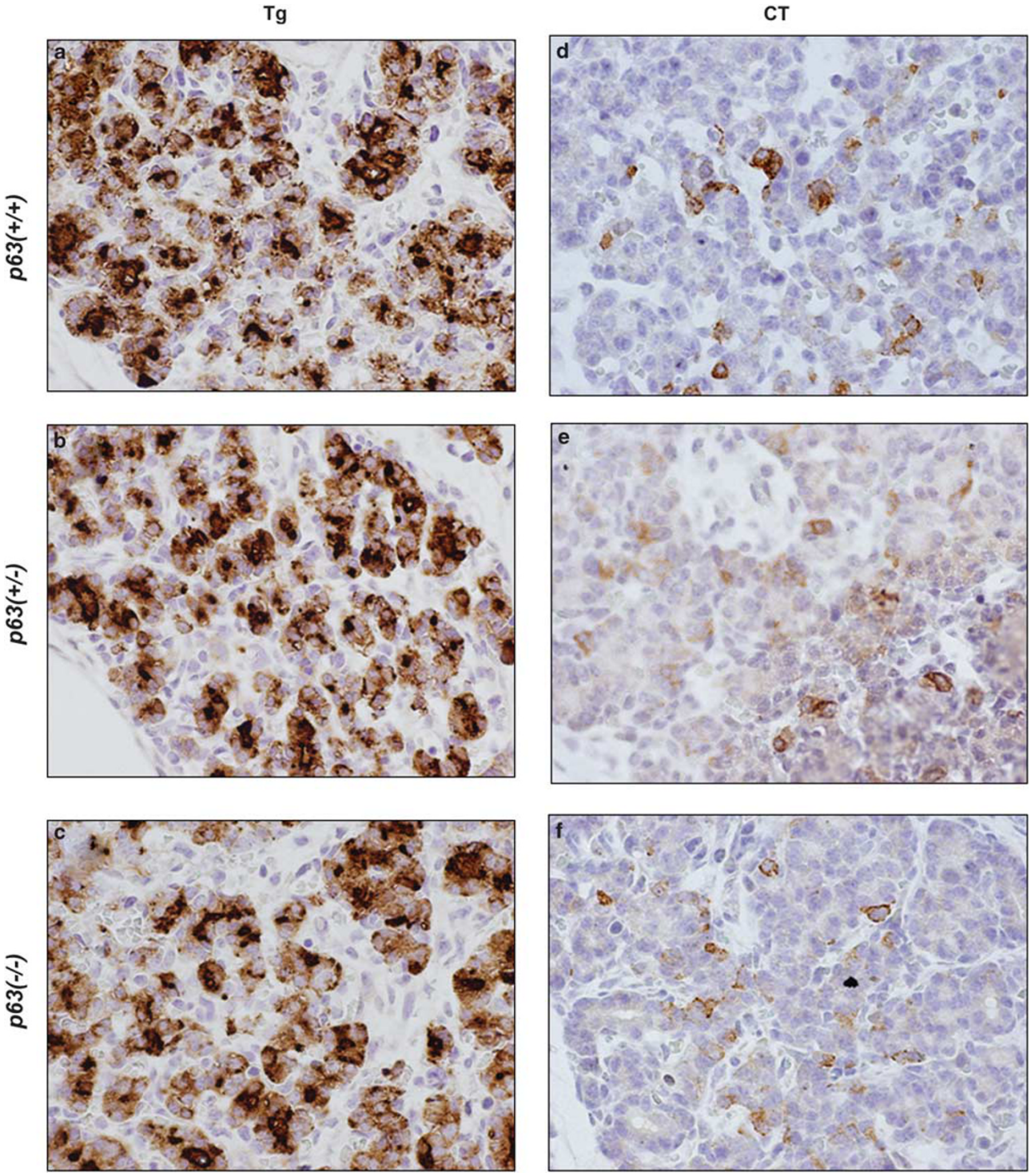

Figure 4 Thyroglobulin and calcitonin expression in E17.5 thyroid from mice with three different *p63* alleles. *Nkx2-1* allele is wild-type. Both thyroglobulin (Tg, **a**–**c**) and calcitonin (CT, **d**–**f**) expression are found equally in distribution and intensity in three different genotype mouse thyroids by immunohistochemistry. Positives are shown in brown color. Magnification: ×400.

whose cells are poorly developed as shown by electron microscope. This indicates that UBB consists of three types of cells; those expressing NKX2-1 and p63, and immature cells not expressing either gene. As calcitonin is expressed in *p63*-null mouse thyroids, NKX2-1-positive UBB cells may be the origin of C cells. In fact, the expression of NKX2-1 was previously reported in C cells.[32] Sometimes, ciliated cells are found in the UBB cells. In the presence of p63 as found in the

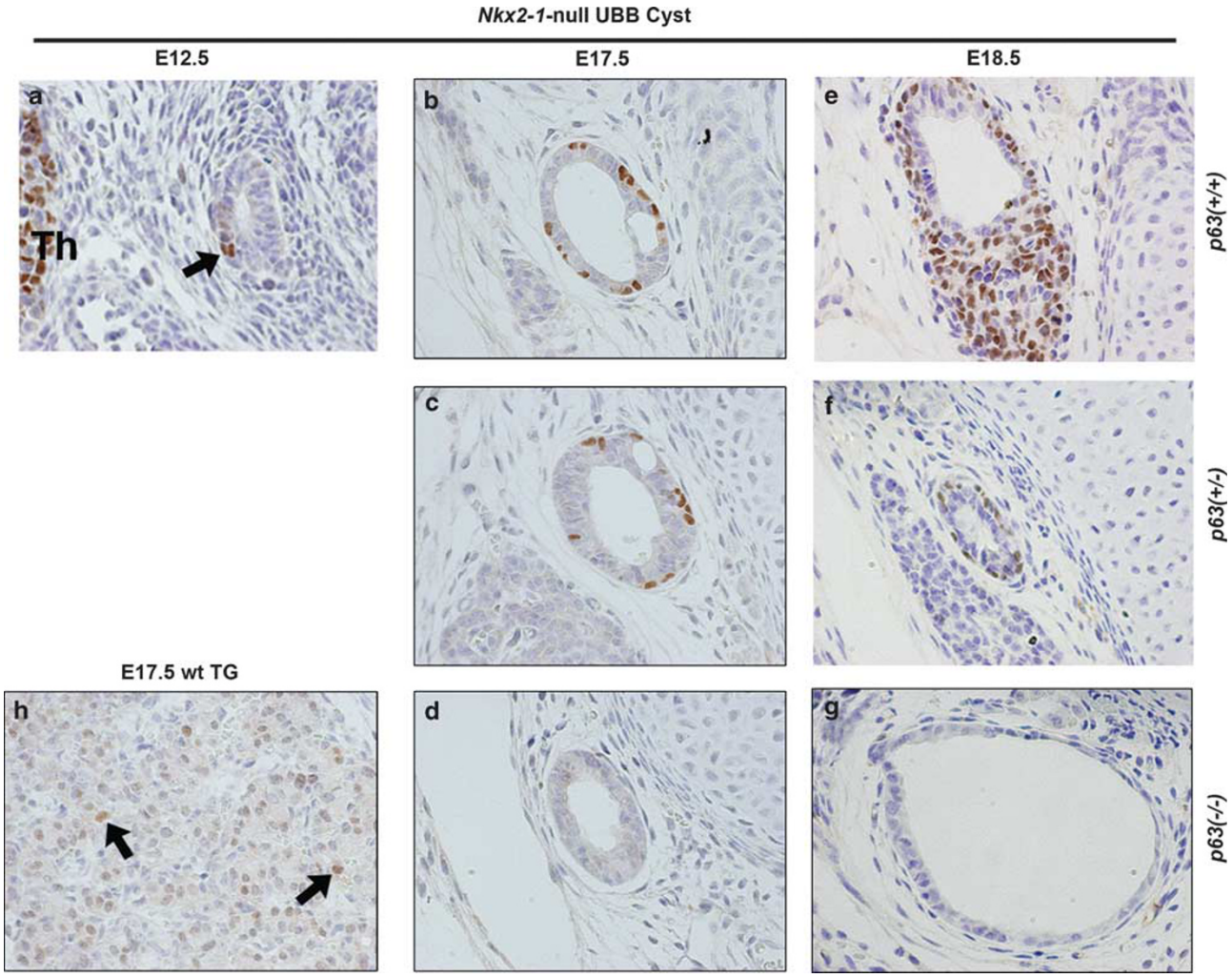

Figure 5 p63 expression in the *Nkx2-1*-null mouse ultimobranchial body (UBB) cyst. p63-positive cells in UBB of E12.5 *Nkx2-1*-null mouse (**a**) and E17.5 normal mouse thyroid (**h**, TG) are shown by an arrow. Thm: thymus. p63-positive cells in E17.5 (**b**–**d**) and E18.5 (**e**–**g**) *Nkx2-1*-null mouse UBBs with various p63 genotypes are observed outside of vesicular structure (**b**, **c**, **e**, **f**) or solid cystic pattern of cells (**e**). It is to be noted that the size of UBB appears to look smaller in (**f**) because of orientation of the section. Magnification: $\times 400$.

Nkx-2-1-null and *p63*-Ht;*Nkx2-1*-null mice, the cystic structure of UBB is surrounded by p63-positive outer layer of cells, which sometimes continued to p63-positive cells in nested pattern. This compartmentalized p63-positive structure strikingly resembles the SCN in humans. The SCN is present in most human thyroids.[21] It contains both solid cell proliferation and cyst-like structures, having strong p63-positive cells at the periphery with centrally located p63-negative cells of unknown phenotype.[18–20] The occasional presence of ciliated cells is noted.[18–20] Furthermore, the presence of second kind of follicle in so-called mixed follicles has been known for decades in normal rats and mice.[33] This second kind of follicle is characterized by a nonhomogeneous or foamy colloid and the presence of occasional ciliated cells,[33] and is thought to originate from the UBB.[34] Taken together, the *p63*;*Nkx2-1*-double-null UBB cyst may indeed be the origin of SCN in humans and the second kind of follicles in rats and mice.

The *p63*;*Nkx2-1*-double-null UBB cells appeared to be undifferentiated at least when examined at E14.5 by electron microscope. In particular, the presence of scarce narrow cytoplasm (high ratio of nucleus/cytoplasm) and moderately electron dense nucleus with clumps of nuclear heterochromatin is reported for immature cells and undifferentiated ES cells.[35–37] This could be the reason that no positive immunoreactivity was obtained with the antibodies examined (NSE, S100, Oct 4, nestin, SOX10, and vimentin). Thus, the *p63*;*Nkx2-1*-double-null UBB cells may represent immature stem-like cells. If they are stem-like cells, one may expect the expression of Oct 4. However, Oct 4 expression was not detected in the *p63*;*Nkx2-1*-double-null UBB cells by immunohistochemitry. Oct 4 protein is expressed in

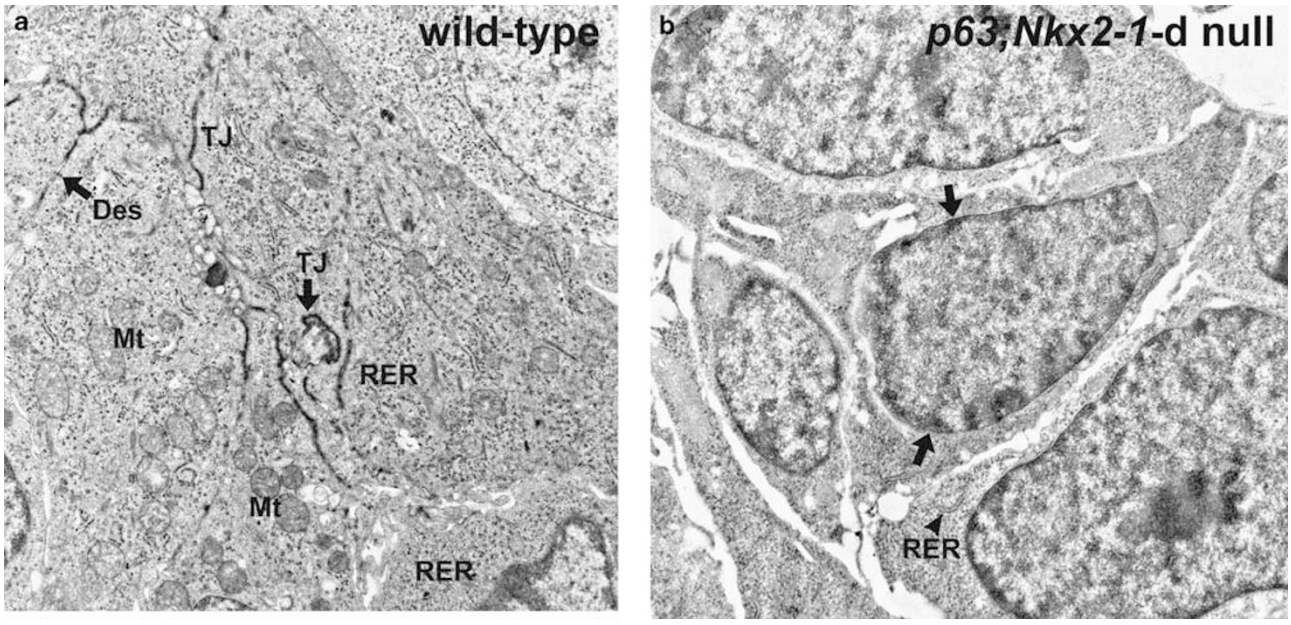

Figure 6 Electron microscopic analysis. Representative electron microscopic images of E14.5 wild-type thyroid (**a**) and *p63;Nkx2-1*-d null mouse ultimobranchial body (UBB) cyst (**b**) are shown. (**a**) Many intracellular organelles and cell attachments are clearly observed as dense membranes. Numerous cytoplasmic organella are well developed in the immature follicular cells at this embryonic stage. No colloid formation is shown but surrounded by well developed tight junctions. A few microvilli are observed. Des: desmosome, Mt: mitochondria, RER: rough endoplasmic reticulum, TJ: tight junction. (**b**) Very scant intracellular organella is observed. RER are minimally found (shown by an arrowhead). Clumps of nuclear heterochromatin are indicated by arrows. Magnification: $\times$ 3000.

embryonic/primordial germ and embryonic stem cells. Although some reports have described Oct 4 expression in adult normal stem cells and tumors, they are mainly determined by RT-PCR, but not by detection of protein such as immunohistochemistry or western blotting.[38,39]

The undifferentiated UBB cells may correspond to cells found in SCN that were described by Burstein *et al*[18] as centrally located p63-negative cells of unknown phenotype. Previously, electron microscopic study showed that many desmosomes and hemidesmosomes are present in SCN, however, these cell adhesion structures and their cytoplasmic organella are poorly developed.[40] As p63 is used as a marker for the SCNs,[18–20,24] it seems that the undifferentiated UBB cells are always associated with the p63-positive cells. In this regard, it is interesting to note that p63 is expressed in the basal/stem cells of several types of epithelia such as skin, esophagus, urethra, and secretory epithelial tissues including lacrimal, mammary, and prostate glands.[41–46] On the basis of these studies together with those of p63-null mice,[41,42] it was suggested that p63 has a role in commitment, maintenance, and differentiation of epithelial cells. It was reported that a subset of papillary thyroid carcinoma and Hashimoto's thyroiditis are often associated with the SCN and express high levels of p63.[18,22,25–27] Further, ciliated cells are occasionally identified in some histological types of follicular cell neoplasms.[47] Whether the basal/stem cell characteristics of p63-expressing cells together with the associated undifferentiated cells have any role in the pathogenesis of SCN-associated lesions such as papillary thyroid carcinoma and Hashimoto's thyroiditis requires further experimentation.

On the basis of the fact that almost all normal human fetal thyroids have SCN,[21] at least two questions arise. Why and for what purpose do the SCN originating from cystic UBB cells remain in the thyroid, and whether they stay dormant throughout adult life or disintegrate at some point, most notably after birth. What is the nature of the undifferentiated UBB cells? Additional studies are required to address these questions and firmly establish correlations with development of thyroid lesions if there are any.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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