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REVIEW

Follicular cell lineage in persistent ultimobranchial remnants of mammals

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Abstract

It has been a subject of much debate whether thyroid follicular cells originate from the ultimobranchial body, in addition to median thyroid primordium. Ultimobranchial remnants are detected in normal dogs, rats, mice, cattle, bison and humans and also in mutant mice such as Eya1 homozygotes, Hox3 paralogs homozygotes, Nkx2.1 heterozygotes and FRS2$\alpha^{2F/2F}$. Besides C cells, follicular cell lineages immunoreactive for thyroglobulin are located within these ultimobranchial remnants. In dogs, the C cell complexes, i.e., large cell clusters consisting of C cells and undifferentiated cells, are present together with parathyroid IV and thymus IV in or close to the thyroid lobe. In addition, follicular cells in various stages of differentiation, including follicular cell groups and primitive and minute follicles storing colloid, are intermingled with C cells in some complexes. This review elaborates the transcription factors and signaling molecules involved in folliculogenesis and it is supposed why the follicular cells in the ultimobranchial remnants are sustained in immature stages. Pax8, a transcription factor crucial for the development of follicular cells, is expressed in the fourth pharyngeal pouch and the ultimobranchial body in human embryos. Pax8 expression is also detected in the ultimobranchial remnants of Eya1 and Hes1 null mutant mice. To determine whether the C cells and follicular cells in the ultimobranchial remnants consist of dual lineage cells or are derived from the common precursor, the changes of undifferentiated cells in dog C cell complexes are examined after chronically induced hypercalcemia or antithyroid drug treatment.

Keywords Ultimobranchial body remnants · C cell complexes · Immature follicular cells · Thyroglobulin · Pax8 · Folliculogenesis

Introduction

The thyroid gland of mammalian species is made up from two types of cells, i.e., thyroid hormone-producing follicular cells and calcitonin-producing C cells. The thyroid primordium originates from the ventral floor of the anterior pharyngeal endoderm, moves caudally down along the midline and expands laterally to form two lobes eventually giving rise to follicular cells. The ultimobranchial body develops from the fourth pharyngeal pouch, comes into contact with the thyroid primordium and fuses with it. Subsequently, the ultimobranchial cells differentiate into C cells while spreading along the follicular cell cords; the C cells disperse throughout the thyroid parenchyma (see Kameda 2016 for references).

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The thymus and parathyroid primordia develop from the third and fourth pharyngeal pouches, although in murine species, the organs develop from only the third pharyngeal pouch. The thymus III arisen from the third pharyngeal pouch reaches its final destination, i.e., anterior mediastinum, close to pericardium. Parathyroid III forms in the third pharyngeal pouch together with thymus III. Parathyroid IV develops from the fourth pharyngeal pouch, together with thymus IV and the ultimobranchial body. During downward migration, the parathyroid gland is located on the top of the thymus and at the lateral side of the thyroid lobe, where the gland detaches from the thymus and comes into contact with the thyroid.

It has been a subject of much debate whether thyroid follicular cells originate from the ultimobranchial body, in addition to median thyroid primordium. In dog thyroid glands, C cells are usually gathered in groups. In addition to the ordinary C cell groups, large C cell clusters termed "C cell complexes" are present near the parathyroid IV (Fig. 1a, b; Kameda 1971). The organs are regarded as persistent remnants of ultimobranchial bodies. Some of the complexes contain the

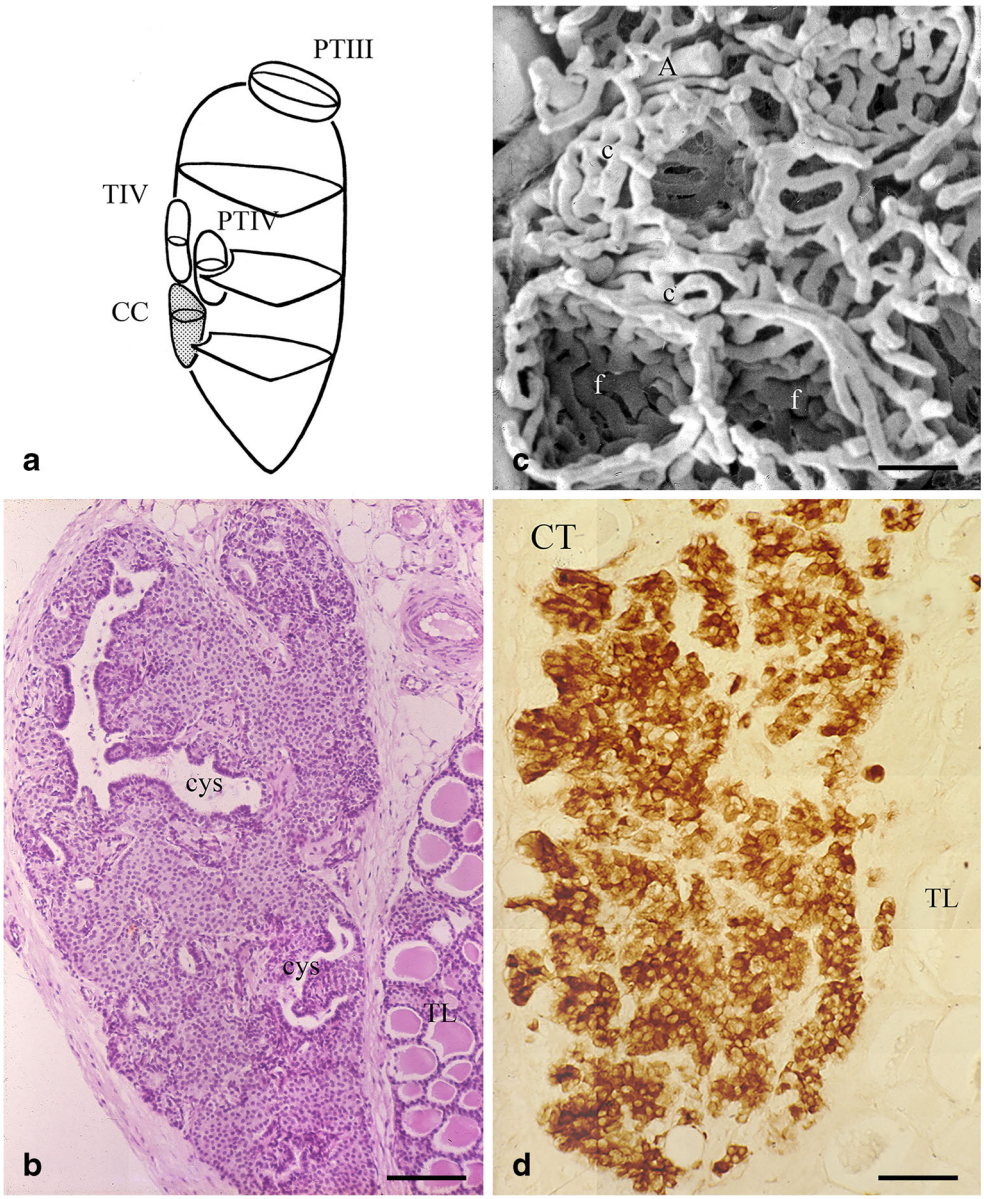


Fig. 1 **a** Schematic illustration of the C cell complex (CC), parathyroid III (PTIII), parathyroid IV (PTIV) and thymus IV (TIV) located in or adjacent to the thyroid lobe in dog. **b** A canine C cell complex consisting of C cells and cysts (cys) is observed close to the thyroid lobe (TL). Hematoxylin–eosin staining. Modified from Kameda (1971). **c** Vascular cast of the thyroid gland in dog, observed by scanning electron microscopy. Blood capillaries encapsulating a follicle (f) constitute a round or oval basket-like structure, whereas those of C cell groups (c) show a check-like network. The bloods of both follicles and C cell groups are intimately connected. A an interfollicular artery. Modified from Kameda (1977). **d** The C cell complex after chronically induced hypercalcemia. A dog was treated with $CaCl_2$ and vitamin D_3 for 20 days. Immunostaining with calcitonin (CT) antiserum. The complex is occupied by C cells showing hyperplastic and hypertrophic features and also degranulation. TL thyroid lobe. Modified from Kameda and Ikeda (1980b). Bars 100 μm (**b**), 65 μm (**c**), 80 μm (**d**)

follicular cell lineages intermingled with C cells. The follicular cells in the complexes exhibit immunoreactivity for thyroglobulin and the colloid-containing follicles show uptake of radioactive iodine, as well as those in thyroid parenchyma (Kameda and Ikeda 1980a; Kameda et al. 1981). In rat thyroid glands, the possibility that the ultimobranchial body gives rise to both C cells and follicular cells has been suggested by an electron microscopic study (Calvert 1972). In thyroid glands of a variety of animals including normal rats, normal mice, cows, bull, bison and humans, the occurrence of ultimobranchial remnants has been reported; the remnants are referred to as the ultimobranchial follicles, tubules, or cysts in rats and mice and the solid cell nests (SCNs) in humans, cows, bull and bison (see below).

In humans, the ectopic lingual thyroid gland that failed to descend downward is rarely encountered. In that case, the ultimobranchial body cannot fuse with the thyroid primordium and remains as a separate organ for life. The isolated ultimobranchial remnant contains follicular cell elements immunoreactive for thyroglobulin in addition to C cells (Williams et al. 1989). Furthermore, in human thyroid glands, "SCNs," which are composed of main cells and C cells, have been considered by many authors as the ultimobranchial remnants. The SCNs exhibit the follicular cell elements immunoreactive for thyroglobulin (Harach 1985; Cameselle-Teijeiro et al. 1994). In the early 1980s, some authors started describing the "mixed medullary and follicular thyroid carcinoma" constituted by a predominantly medullary pattern admixed with areas of follicular differentiation (Hales et al. 1982; Pfaltz et al. 1983). The intimate relationship between the C cells and follicular cells has been suggested on the cell origin of the tumors.

Recently, interest in persistent ultimobranchial remnants has been revived by their existence in some mutant mice such as FRS2$\alpha^{2F/2F}$, Nkx2.1 heterozygote, Eya1 homozygote and Hox3 paralogs homozygote (Kameda et al. 2009; Kusakabe et al. 2006a; Xu et al. 2002; Manley and Capecchi 1998). In these mutant mice, the ultimobranchial remnants do not associate or only partially associate with the thyroid tissue. The small primitive follicles containing colloid immunoreactive for thyroglobulin are located in the ultimobranchial remnants of these mutant mice.

Thus, the follicular cell lineages, i.e., colloid-containing primitive and small follicles and cell clusters immunoreactive for thyroglobulin, are detectable in the ultimobranchial remnants of different normal mammals and mutant mice. These observations indicate that the follicular cells are derived not only from the median thyroid primordium but also from the ultimobranchial body. The ultimobranchial body may contribute both C cells and follicular cells to the thyroid gland. This review provides the recent view concerning the ultimobranchial remnants and discusses the origin of thyroid follicular cells, with a focus on the follicular cell lineages located in the ultimobranchial remnants. In addition, transcription factors and signaling molecules involved in folliculogenesis are highlighted. The consideration of these genes may solve why the follicular cells in the C cell complexes remain in immature phenotypes and many undifferentiated cells are present even after birth. The changes of undifferentiated cells after chronically induced hypercalcemia or antithyroid drug treatment have been examined in dog C cell complexes to clarity whether the undifferentiated cells consist of dual lineage cells or are common precursors for both C cells and follicular cells.

Mammalian C cells are derived from pharyngeal endoderm but not from neural crest cells

Ultimobranchial primordium of non-mammalian vertebrates does not merge with thyroid primordium, remaining as an independent organ throughout life. Except for the ultimobranchial gland of birds and some reptiles, in which C cells are dispersed among cysts, the organ of other lower vertebrates usually consists of follicular structures (see Kameda 2017 for references). The cranial neural crest cells contribute to the mesenchyme of the pharyngeal arches. Classic experiments using the quail–chick chimeric technique have demonstrated that connective tissues of pharyngeal organs, including ultimobranchial gland, thymus, parathyroid and thyroid glands, are derived from the mesenchymal neural crest cells (Le Lièvre and Le Douarin 1975). In particular, the neural crest cells give rise to C cells (Le Douarin et al. 1974). The avian ultimobranchial primordium forms close to the nodose ganglion and enteric ganglia and neuronal progenitors originating from the ganglia invade the primordium, resulting in C cells (Kameda 1995a, 2017). The ultimobranchial primordium of the other vertebrates including mammals does not have topological relations with any ganglia. Without formal proof, the concept of neural crest origin has often been accepted as valid for mammalian thyroid C cells. It has been evidenced by using both Connexin (Cxn)43-LacZ and Wnt1-Cre/R26R transgenic mice, which allow us to perform neural crest lineage tracing, that neither the fourth pharyngeal pouch nor ultimobranchial body is colonized by neural crest-derived cells at any stages of the development (Kameda et al. 2007a; Kameda 2016). Furthermore, calcitonin and E-cadherin, an epithelial cell marker, are colocalized in the mouse C cells. More recently, lineage tracing of the endoderm using Sox17-Cre-mediate labeling demonstrates that the foregut endoderm gives rise to C cells in mouse embryos (Johansson et al. 2015). Thus, murine thyroid C cells are derived from the endodermal epithelial cells of the fourth pharyngeal pouch and not from the neural crest cells. The C cells of non-mammalian vertebrates also appear to be of endodermal origin (Kameda 2017). Exceptionally, the avian C cells have dual origins: neuronal progenitors and endodermal epithelium.

Development of thyroid primordium from the ventral pharyngeal floor

Simultaneous expression of Nkx2.1, Pax8, Hhex and Foxel is required for the organogenesis of the thyroid gland (De Felice and Di Lauro 2004). The regulatory interactions among Nkx2.1, Pax8 and Hhex have been confirmed and Foxel is located downstream in the thyroid regulatory network. In the absence of Nkx2.1, Pax8, Hhex, or Foxe1, the thyroid primordium is correctly formed but the subsequent thyroid morphogenesis is severely impaired. In particular, both Nkx2.1 and Pax8 play significant roles for controlling the survival and/or proliferation of thyroid cell precursors (Parlato et al. 2004; De Felice and Di Lauro 2011). In Nkx2.1 or Pax8 null mutant mice at E15.5, the thyroid tissue is undetectable. Concerning development of the thyroid gland, see the recent comprehensive review by Nilsson and Fagman (2017).

Folliculogenesis and thyroid differentiation

The thyroid follicles of mature animals are delineated by a monolayer of follicular cells resting on a continuous basement membrane. The cells face lumen at the apical pole and are attached to the basement membrane at their basal pole (Colin et al. 2013). In addition, a dense network of blood vessels surrounds each follicle (Fig. 1c). They form angiofollicular units responsible for thyroid hormone synthesis and storage.

The thyroid folliculogenesis in mouse embryos has been examined in detail immunohistochemically using several kinds of antibody (Fagman et al. 2006; Kameda et al. 2009; Andersson et al. 2011; Hick et al. 2013; Villacorte et al. 2016). After the downward migration and bilateral expansion of the midline thyroid primordium, the parenchyma begins to be organized into cell cords at E13.5. At this stage, the ultimobranchial anlage is engulfed in the thyroid primordium and localized in the central portion of the lobe. The endothelial cells progressively invade the expanding thyroid cell mass and separate into the cell cords by a network of a microvascular loop. At E14.5, the cell cords are oriented radially from the center to the periphery. Simultaneously, the differentiating C cells begin to disperse along the follicular cell cords. Prior to folliculogenesis, branching-like morphogenesis is stimulated by fibroblast growth factor (Fgf)10 derived from the adjacent mesenchyme, controlling the growth of prospective thyroid parenchyma (Liang et al. 2018). The peripheral or distal parts of the thyroid parenchyma are occupied by rapidly multiplying progenitors. Sox9, a key factor in branching organ development and Fgf2b are co-expressed distally in the branching follicular epithelium. Fgf10 is a major and specific growth stimulus of the orthotopic thyroid gland before birth (Ohuchi et al. 2000; Teshima et al. 2016). Absence of Fgf10 leads to defective branching and disorganized angiofollicular units.

At E15.5, the primitive follicles storing colloid droplets immunoreactive for thyroglobulin are first detected and the punctate labeling for ezrin, the subapical marker, becomes apparent in the thyroid parenchyma; the cell masses initiate apical pole formation (Kameda et al. 2007b; Hick et al. 2013). Subsequently, the cord-like structures begin to be broken up into small follicles. The vasculature differentiates into an elaborated capillary network that encircles the individual follicles. In addition, the basement membrane surrounds the individual follicles; the basement membrane assembly seems to be necessary for folliculogenesis (Villacorte et al. 2016). At E16.5, a majority of follicular cells are organized in distinctive follicle structures made of polarized monolayer, i.e., acquisition of apical–basal polarity and luminal structure. The expression of tight junction marker, ZO-1 and storage of colloid showing immunoreactivity for iodinated thyroglobulin are recognized in almost all follicles (Hick et al. 2013).

Factors involved in thyroid folliculogenesis

▪Pax8

The paired box transcription factor Pax8 is expressed in the thyroid primordium and is involved in the development of the thyroid gland (Kameda et al. 2009). The gene is also expressed in the pharyngeal pouch IV and ultimobranchial anlage in human embryos (Trueba et al. 2005). In Eya1 and Hes1 null mutant mouse embryos, the Pax8 expression is detected in the ultimobranchial remnants (Xu et al. 2002; Carre et al. 2011). It raises the possibility that the expression of Pax8 in the ultimobranchial body is normally suppressed by a transcriptional machinery; Shh seems to function as the suppressor. Shh signaling represses inappropriate thyroid differentiation in non-thyroid embryonic tissues (Fagman et al. 2004). In mouse embryos at E9.5, Shh is expressed in the entire foregut endoderm except for the thyroid primordium (Parlato et al. 2004; Fagman et al. 2004). In Shh null mutant mice, ectopic thyrocytes expressing thyroglobulin aberrantly develop from the respiratory epithelium. Concerning the development of ultimobranchial primordium, fate mapping of Shh expressing cells has been examined with β-galanin (β-gal) immunostaining in Shh-Cre/R26R mice (Westerlund et al. 2013). At E11.5, only the ventral portion of the endoderm of the fourth pharyngeal pouch in which the ultimobranchial primordium forms is positive for β-gal. At E12.5, β-gal-positive Shh cells appear in the ultimobranchial body. At this stage, the trachea also expresses intense β-gal staining for Shh. Thus, the expression of Pax8 in the ultimobranchial body seems to be normally repressed by Shh signaling. The development of follicular cells in the ultimobranchial remnants might reflect dosage differences and/or variations in the efficiency of these genes in the ultimobranchial body.

It has been demonstrated using the PCPy cell line that the introduction of Pax8 expression vectors activates transcription of thyroid-specific genes encoding thyroglobulin, thyroid peroxidase (TPO), sodium/iodide symporter (NIS) and TSH receptor; Pax8 plays a role for the maintenance of functional differentiation in thyroid cells (Pasca di Magliano et al. 2000). In Pax8 null mutant mice, the thyroid diverticulum is able to evaginate from the pharyngeal endoderm but subsequently disappears at E12.5; the thyroid gland is absent (Mansouri et al. 1998). On the other hand, the ultimobranchial body is normally developed and is observed as a cell mass in the newborn null mutant mice.

A follicular structure is required for thyroid hormone synthesis, storage and secretion. It has been demonstrated using a three-dimensional epithelial culture model system that Pax8 and its target cadherin-16 function for the generation of polarized follicle-like structures (Koumarianou et al. 2017). Silencing Pax8 expression inhibits the acquisition of apical–basal membrane polarity and impairs lumen formation.

▪Nkx2.1

The homeodomain transcription factor Nkx2.1 is expressed in the developing thyroid gland and is crucial for morphogenesis of the organ (Lazzaro et al. 1991). The gene functions for the production of thyroid hormones, including thyroglobulin, TPO and thyrotropin receptor. In addition, Nkx2.1 is expressed in the ultimobranchial body at E11.5–E13.0 in

mouse embryos (Fagman et al. 2006; Kameda et al. 2009). Nkx2.1 expression in the thyroid follicular cells is maintained throughout adulthood. In Nkx2.1 null mutant mice, the primordia of the thyroid and ultimobranchial body are initially formed but are eliminated at E12–E13 through apoptosis; neither thyroid follicular cells nor C cells are present (Kimura et al. 1996). Thus, Nkx2.1 is required for proliferation and survival of the progenitors for both follicular cells and C cells.

Nkx2.1 contains two redundant domains and is post-translationally modified by phosphorylation. A Nkx2.1 mutant, encoding a phosphorylation defective protein, brings about critical defects in the organization of thyroid follicles (Silberschmidt et al. 2011). In PM/PM embryos in which phosphorylable serine residues of Nkx2.1 have been mutagenized, the thyroid size is reduced, although the thyroid cells express Nkx2.1, Pax8, thyroglobulin and NIS. Furthermore, the organization of follicular structures with lumina is missing in the mutants; the lumen defined by the presence of ZO-1 and storing thyroglobulin-positive substances is absent. In thyroid follicular cell–specific conditional knockout mice, Nkx2.1(fl/fl);TPO-Cre, altered thyroid structures with extraordinary dilated follicles are observed (Kusakabe et al. 2006b). Thus, Nkx2.1 seems to maintain the normal thyroid histoarchitecture postnatally.

Overexpression of two transcription factors Pax8 and Nkx2.1 promotes differentiation of mouse embryonic stem cells into thyroid follicular cells when treated with thyrotropin (Antonica et al. 2012). Thus, Pax8 and Nkx2.1 cooperate in the functional differentiation of thyroid cells.

▪Dicer

Micro-RNAs (miRNAs) are small non-coding RNAs that regulate gene expression mainly at the mRNA post-transcriptional level. Functional maturation of most miRNAs requires processing of the primary transcript Dicer, an RNaseIII-type enzyme. In thyrocyte-specific Dicer conditional knockout mice (Pax8(Cre/+);Dicer$^{flox/flox}$), hypothyroidism gradually develops after birth (Frezzetti et al. 2011). A severely disorganized follicular architecture is detected in 1-month-old mutants. Expression of thyroid markers is also affected. On the other hand, Pax8(Cre/+);Dicer$^{flox/flox}$ mice used by Rodriguez et al. (2012) are severely affected and die soon after weaning due to conspicuous hypothyroidism. With T4 supplement, however, some animals survive to adulthood. A profoundly damaged architecture of the thyroid tissue is observed in Dicer mutant mice at 4-weeks old. Most follicular cells do not form follicles, aggregating in cell clusters. In thyroid-specific Dicer deletion in later stages during thyroid development (thyroglobulin (Cre/+);Dicer$^{flox/flox}$ mutant mice), the thyroid gland indicates hypothyroidism associated with low T4 plasma levels (Rodriguez et al. 2012). The disruption of thyroid architecture is conspicuous in 4-week-old mutant mice. Thus, loss of miRNA maturation due to Dicer inactivation severely disturbs functional thyroid differentiation.

▪VEGF-A

VEGF-A (vascular endothelial growth factor-A) is a major regulator of blood vessel development and the VEGF-AmRNA expression is increased in thyroid with age (Hick et al. 2013). In mouse thyroid at E14.5, the gene is exclusively detected in E-cadherin-positive thyroid cells, whereas a cell mass of ultimobranchial body is devoid of VEGF-A. VEGF receptor2, the main VEGF-A receptor involved in blood vessel angiogenesis, is expressed in the endothelial cells of the thyroid lobe except for ultimobranchial body cell mass. In thyroid-specific conditional VEGF-A knockout mice, the reduction of microvasculature is induced in the thyroid lobe. The folliculogenesis of thyroid cells is impaired and a multilayered cell mass with defective polarization and apical lumen formation appears in VEGF-A mutant mice. Thus, the affected follicular cells are sustained in clusters with few interspersed endothelial cells.

▪Smad

Smad transcription factors, the intracellular signal transducers, play central roles for the signal transduction pathways that mediate the numerous effects of the TGF-β superfamily (Macias et al. 2015). Bone morphogenetic proteins (Bmps) are members of the TGF-β family that control many developmental processes such as epithelial differentiation and tissue morphogenesis. Bmp signaling is transduced through a canonical pathway involving phosphorylation of Smad1/5/8 (R-Smad) from the ligand–receptor complex.

Bmp–Smad signaling is required for thyroid follicle formation. Lumen enlargement of the follicles depends on correct apicobasal polarization, i.e., defined basal pole apposition on basement membrane and coordinated assembly of the apical pole (Colin et al. 2013). In thyroid-specific Smad1 and Smad5 double-knockout (Smad1/5^{dKO}) mice, defective follicular architectures are induced (Villacorte et al. 2016). The cells remain associated in large clusters and form small follicles. Reduced expressions of laminin α1 and collagen type IV are detected in the extracellular matrix of thyroid lobes in Smad1/5^{dKO}. In contrast to VEGF-A knockout mice in which decreased blood capillaries are induced in addition to defective follicle formation, Smad1/5^{dKO} exhibits normal endothelial cell density. Both VEGF-A knockout and Smad1/5^{dKO} mice display impaired basement membrane assembly. Thus, the epithelial Smad signaling and endothelial cell invasion seem to promote folliculogenesis via assembly of the basement membrane.

Mammalian ultimobranchial body contributes to both C cells and follicular cells

The opinion that the thyroid follicular cells originate from ultimobranchial body, in addition to median thyroid primordium, has been supported by increasing evidence as follows.

Canine C cell complex

In mammalian species, C cells are usually concentrated in the central portion of the thyroid lobe. However, in some species including monkeys, humans and rabbits, the C cell distribution is restricted to the place close to the parathyroid IV derived from the pharyngeal pouch IV. Especially, in monkeys, the cells are detected only in a narrow space of the thyroid parenchyma right below parathyroid IV and large portions of the thyroid lobe lack C cells (Kameda 1983). In dog thyroid glands, C cells are also densely distributed in the proximity of the parathyroid IV. In addition, the C cell complex, a special C cell group composed of C cells in various stages of differentiation and other types of epithelial cells, is located around the parathyroid IV in or close to the thyroid parenchyma (Fig. 1a, b) (Kameda 1971). The C cell complex is the remnant of the ultimobranchial body derived from the pharyngeal pouch IV together with parathyroid IV and thymus IV. In mammals, the ultimobranchial anlage fuses with the thyroid primordium and then differentiates into C cells during dispersion along the follicular cell cords within the thyroid parenchyma (Andersson et al. 2011). Since dog C cells usually disperse gathering in large cell groups, markedly large cell masses may be unable to scatter, remaining as C cell complexes in the places not far away from the invasion spot. When one lobe per dog, from newborns to young adults, was examined, one or more C cell complexes were detected in 24 (70.6%) out of 34 lobes. They are more frequently encountered in younger animals than older ones.

The C cell complexes are classified into two types: one type is made up of C cells and cysts, whereas the other type contains follicular cells immunoreactive for thyroglobulin in addition to C cells. Undifferentiated cells and high-columnar epithelial cells, which exhibit no immunoreactivity for antisera examined, such as calcitonin, somatostatin, calcitonin gene-related peptide (CGRP), chromogranin A and thyroglobulin, are also present in the both types of the complex; the younger the animals are, the more numerous are undifferentiated cells and high-columnar epithelial cells. The C cell complex is usually oval in shape. The complexes consisting of C cells and cysts are surrounded by connective tissue and separated from thyroid parenchyma (Fig. 1b). Cysts are lined with single columnar or cuboidal epithelial cells and they are empty or contain foamy, flocculent and colloid-like substances, which are intensely immunoreactive for thyroglobulin but cannot accumulate radioiodine (Kameda et al. 1981; Kameda 1982a).

The complexes containing the follicular cell lineage frequently fuse with the neighboring thyroid parenchyma. Immature follicular cells sustained in cell groups, primitive follicles accumulating colloid droplets and minute follicles storing colloid are observed in this type of complex (Figs. 2 and 3). Furthermore, follicles storing pretty amounts of colloid are rarely encountered in the peripheral portion of the complex. Both the follicular cells in various stages of development and colloid are immunoreactive for thyroglobulin. The follicles in the C cell complexes, as well as those in the thyroid lobe, can store radioiodine (^{125}I) injected on colloid in the follicular lumen (Fig. 3d) (Kameda et al. 1981). Thus, the functional follicles are present in the C cell complexes. The follicular cells in the complexes are not engulfed from neighboring thyroid parenchyma because almost all follicular cells in the thyroid lobe are mature and form typical colloid-containing follicles, whereas the cells in the complexes are maintained in immature conditions.

▪Ultrastructure of C cell complex

The C cell complex retains the features of the fetal ultimobranchial body; undifferentiated epithelial cells and immature C cells are detected. Undifferentiated cells are characterized by their small dark cytoplasm, poorly developed cell organelles and chromatin dense irregular nucleus (Fig. 4a) (Kameda 1973, 1977). Immature C cells are distinguished from the undifferentiated cells by the presence of membrane-bound secretory granules. Every transitional form between immature C cells and the mature cells filled with many secretory granules is seen in the complexes. In addition, primitive and minute follicles, which consist of fewer cells but exhibit apical differentiation (tight junction), are detectable in some C cell complexes (Fig. 4c). A few microvilli or cytoplasmic processes protrude at the apical surface of follicular cells, which is sealed by the junctional complexes. Furthermore, there are some small follicles displaying similar structures yet entirely intracellular (no visible junctions) (Fig. 4b).

▪Development of C cell complex

In the thyroid gland of fetal dogs at around 39 days of gestation, the follicular cells begin to exhibit immunoreactivity for 19S-thyroglobulin and C cells exhibit immunoreactivity for calcitonin and at 40 to 41 days, the primitive follicles storing colloid droplets appear (Kameda et al. 1980). At these developmental stages, the C cell complexes are identified near the parathyroid IV as large cell masses containing numerous undifferentiated cells that exhibit a small cytoplasm and chromatin-dense nucleus and no immunoreactivity for any of the antisera to calcitonin, CGRP, somatostatin and thyroglobulin. During fetal periods, the undifferentiated cells occupy the greatest part of the complex. The C cells

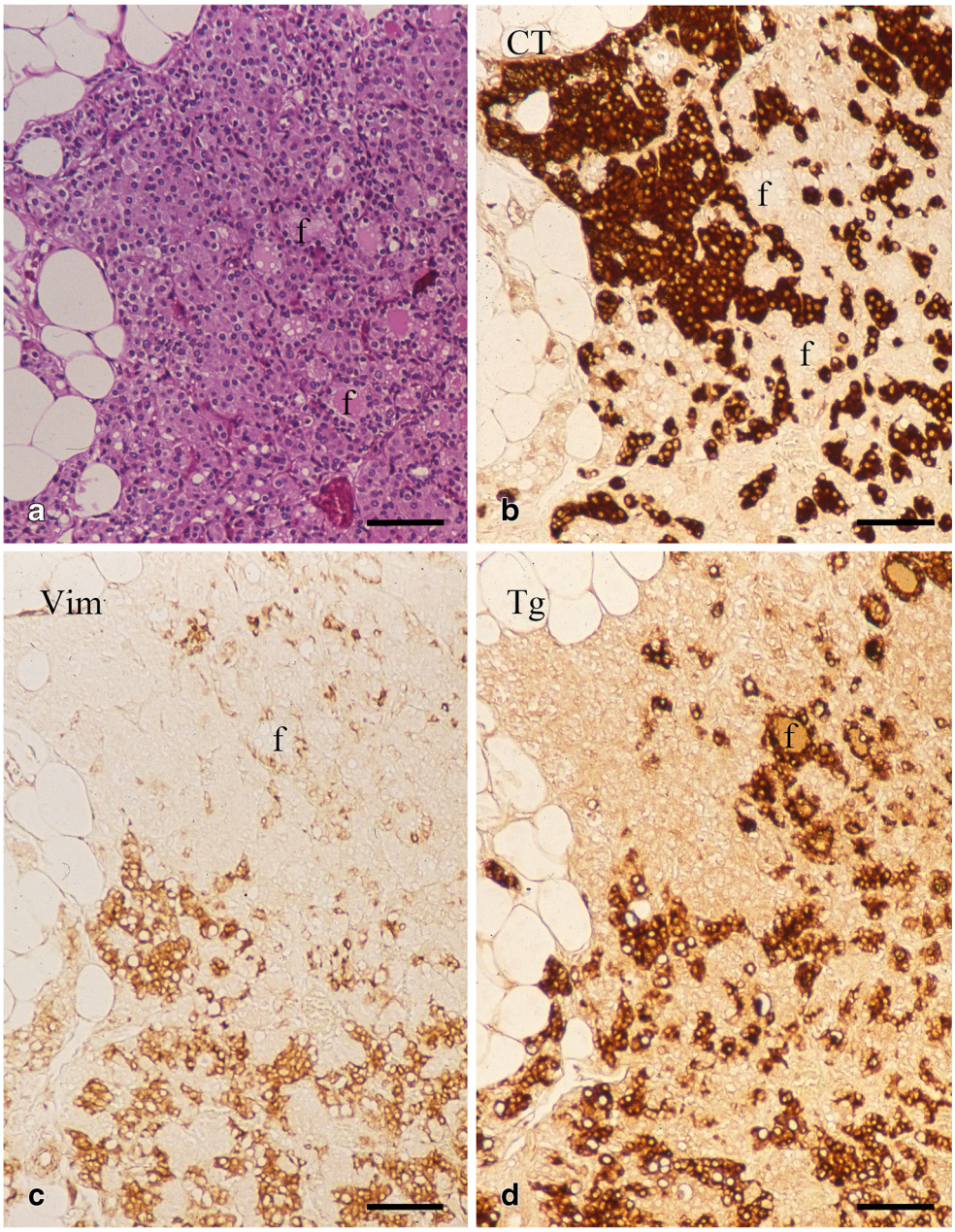


Fig. 2 **a**–**d** Serial sections of the C cell complex in a dog, in which C cells are mingled with follicular cells in various stages of differentiation. The sections were stained by different methods: hematoxylin–eosin staining (**a**), immunostaining with calcitonin (CT) (**b**), vimentin (Vim) (**c**) and 19S-thyroglobulin (Tg) (**d**) antibodies. **a** Colloid-containing small follicles (f) are observed in the complex. **b** The greater portion of the complex is occupied by C cells immunoreactive for calcitonin. **c** Vimentin immunoreactivity is expressed in immature follicular cells gathered in cell clusters and primitive follicles not yet storing colloid. **d** The cells immunoreactive for vimentin show immunoreactivity for 19S-thyroglobulin. Modified from Kameda (1995b). Bars 80 μm

immunoreactive for calcitonin gradually increase in number with age. Furthermore, in some complexes, the immature follicular cells, which are immunoreactive for 19S-thyroglobulin but not yet organized into follicles, are detected. The follicular cells in the complexes are maintained in cell groups and do not form follicles during fetal development.

▪Regulation of C cell complex (hypercalcemia, hypothyroidism)

Since both the C cells and follicular cells in various stages of development are located in the C cell complexes, it is necessary to clarify whether the undifferentiated cells are common precursors for both types of cells or two cell lines are present in the complex. Their responses to induced hypercalcemia or antithyroid drug treatment have been examined; a rapid differentiation of the undifferentiated cells is caused after these treatments (Kameda 1973, 1974a; Kameda and Ikeda 1980b).

Hypercalcemia

C cells react sensitively to changes of serum calcium levels (Kameda 2016). After hypercalcemia induced by chronic administrations of $CaCl_2$ and vitamin D3, dog C cells exhibit hypertrophic features and secretory granules immunoreactive

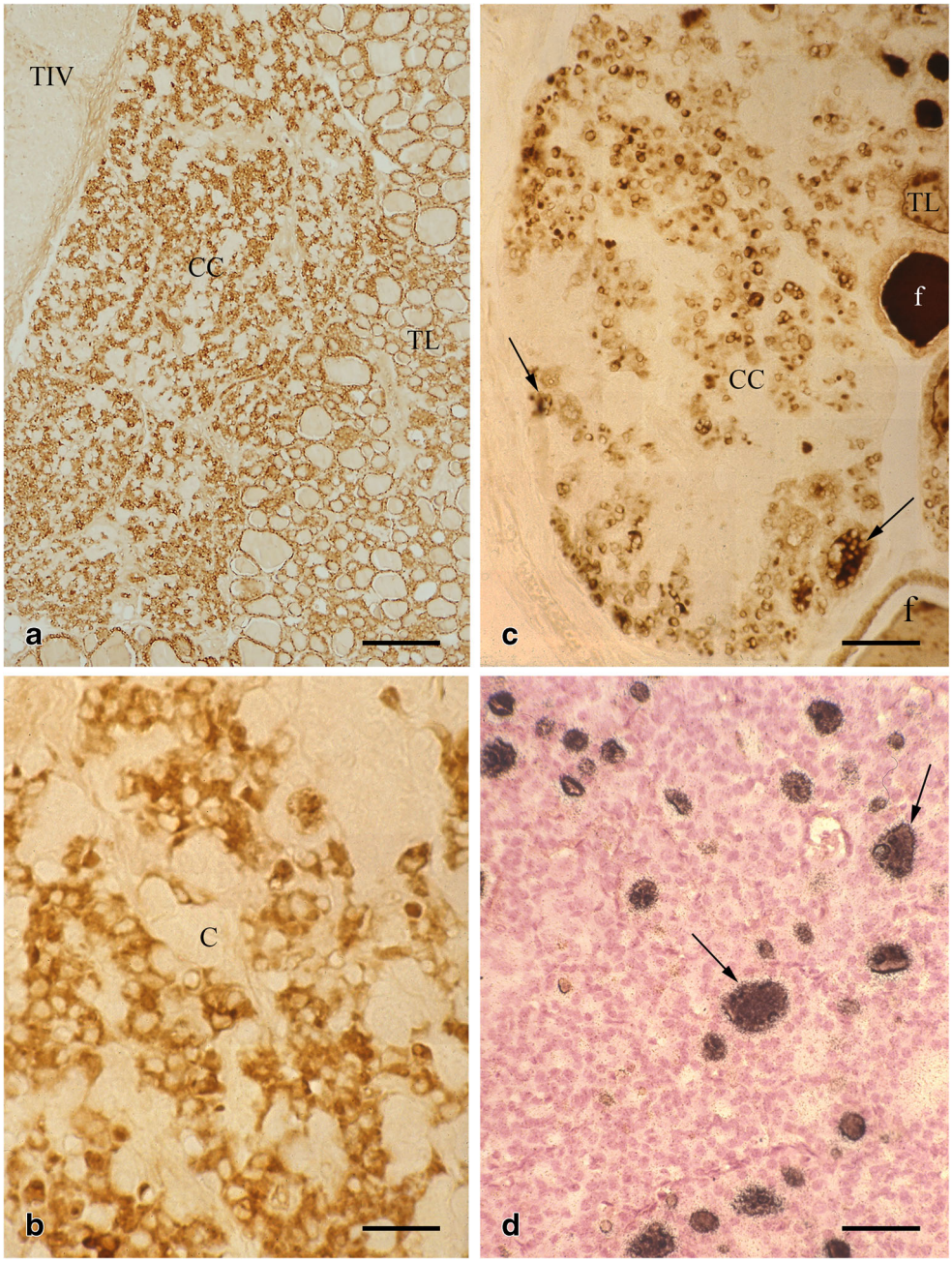
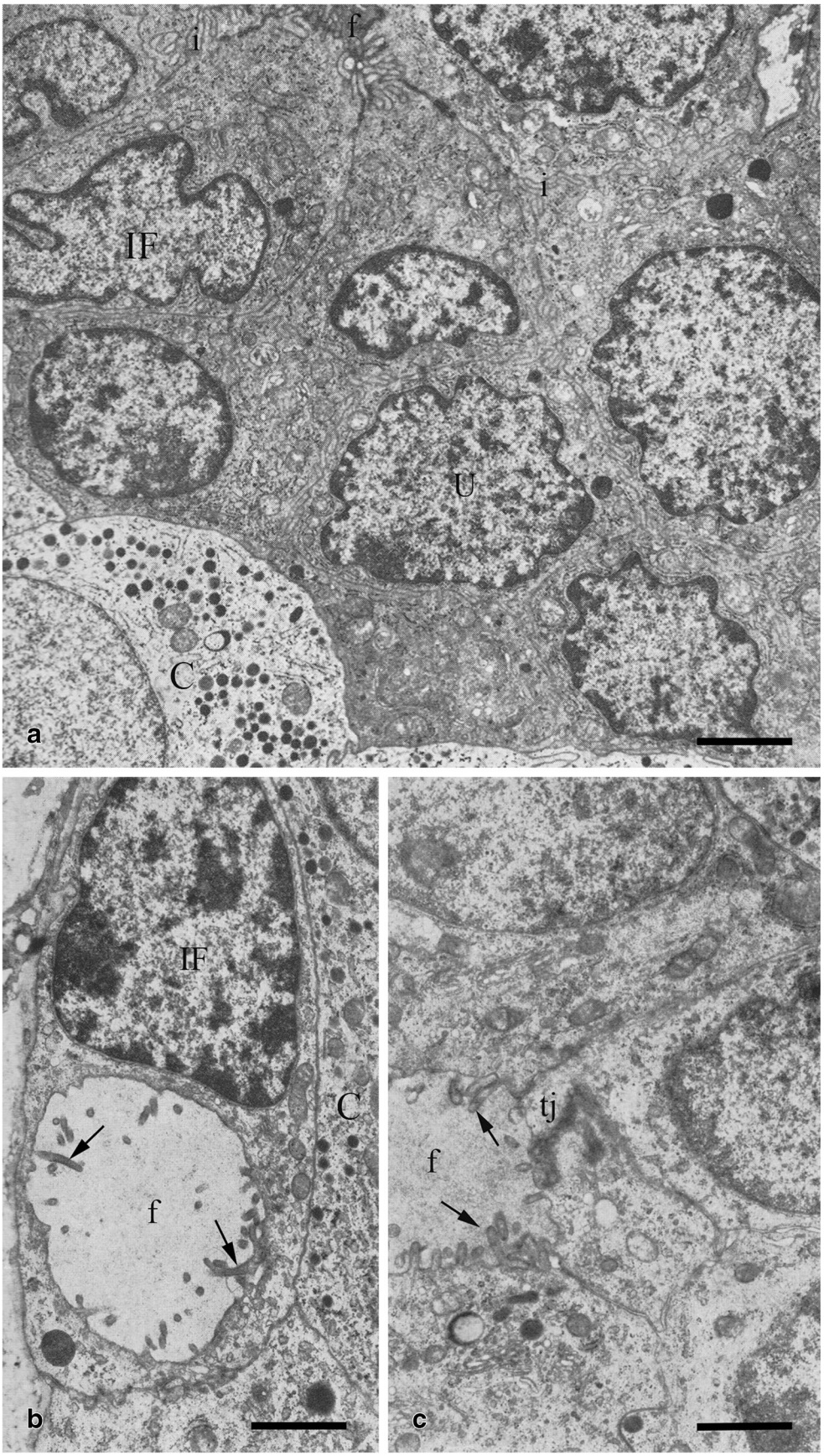


Fig. 3 **a**–**d** The canine C cell complexes (CC) contain follicular cell lineages in addition to C cells. Immunostaining with 19S-thyroglobulin antiserum (**a**–**c**). **a** The complex displays immature follicular cells sustained in clusters. The organ is embedded in the thyroid lobe (TL). TIV thymus IV in contact with the complex. **b** Higher magnification of figure **a**. The follicular cell clusters immunoreactive for 19S-thyroglobulin are intermingled with C cells (C). **c** The complex exhibits primitive and minute follicles (arrows) storing colloid immunoreactive for 19S-thyroglobulin. (f) typical follicles in thyroid lobe. **a**–**c** modified from Kameda and Ikeda (1980a). **d** Autoradiography of the complex from a dog injected with $Na^{125}I$. Hematoxylin–eosin staining. Accumulation of silver grains indicating radioactive iodine is detected on colloid in the follicles (arrows). Modified from Kameda et al. (1981). Bars 165 μm (**a**), 23 μm (**b**), 63 μm (**c**), and 50 μm (**d**)

for calcitonin are decreased in proportion to the duration of hypercalcemia. In addition, a conspicuous increase in number of C cells occurs in association with increased mitotic activity (Kameda 1970).

The C cells in the complexes, as well as the cells in thyroid parenchyma, display hypertrophic and hyperplastic features and conspicuous degranulation after chronically induced hypercalcemia (Fig. 1d) (Kameda and Ikeda 1980b). Neither immature C cells nor undifferentiated cells are observed in the complexes after the chronical hypercalcemic, in contrast to the normal controls. On the other hand, in the complexes containing follicular cell lineages, the immature follicular cells are detectable and remain unchanged (Kameda 1973; Kameda and Ikeda 1980b).

Fig. 4 **a**–**c** Electron micrographs of the C cell complexes in dogs. **a** ▶ Undifferentiated cells (U) and immature follicular cells (IF) show an irregular and chromatin dense nucleus and small cytoplasm. Extensive cellular interdigitations (i) are formed among cells. The C cell (C) contains characteristic secretory granules. (f) primitive follicle. **b** An intracytoplasmic follicle (f) furnished with microvilli (arrows) is seen in the immature follicular cell. **c** Minute follicle (f) storing colloid is lined with follicular cells. The cells protrude cytoplasmic processes (arrows) into the follicular lumen and exhibit tight junction (tj) at the apical surface. Modified from Kameda (1977). Bars 1.9 μm (**a**), 1.2 μm (**b**), 1 μm (**c**)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Antithyroid drug treatment

Antithyroid drugs such as thiourea derivatives inhibit the biosynthesis of thyroid hormones, through interruption of TPO, which catalyzes the iodination of tyrosyl residues of thyroglobulin, leading an excess secretion of TSH. TSH, via its receptor (TSHr), stimulates the thyroid hormone secretion by upregulating the expression of NIS, TPO and thyroglobulin (Colin et al. 2013). Dog thyroid glands are markedly enlarged after chronic administrations (1–4 months) of antithyroid drugs, thiourea, or ethylenethiourea (Kameda and Ikeda 1980b). Follicular cells become conspicuously hyperplastic and hypertrophic and follicular lumina become irregular and vary in size. Typical colloid completely disappears from the lumina and, in turn, scanty foamy or reticular substances appear in them. Some follicles exhibit a papillary pattern and are covered by stratified epithelium. Furthermore, the marked increase of blood capillaries surrounding the follicles is induced.

Thyroglobulin is composed of a heterogenous group of proteins. In addition to the main component with a sedimentation coefficient of 19S, several components with slower (3-8S, 12S) and faster (27S, 32S, 37S) sedimentation coefficients are present. In dog thyroid glands after chronic administrations of antithyroid drug, large molecular weight components including 27S are completely lost and instead small molecular weight components including 12S are markedly increased (Kameda 1984). 19S-thyroglobulin is conspicuously decreased, although the follicular cells exhibit more intense immunoreactivity for thyroglobulin than the controls. Albumin is increased in amount in affected thyroid glands and many follicular cells display intense immunoreactivity for albumin.

Not only follicular cells but also C cells show hypertrophic and hyperplastic changes after treatment of antithyroid drugs (Kameda 1974a, 1982b; Martin-Lacave et al. 2009). In rats fed a low-iodine diet for more than 1 year, goiters are induced and abundant C cells are distributed in them (Peng et al. 1975). Although the thyroid lobes are markedly increased in size in dogs treated with ethylenethiourea for 6 months, the proportion of C cells to follicular cells did not particularly differ from that in the normal control dogs and mitoses of the C cells are sometimes observed (Kameda 1982b). In addition, the C cells exhibit a marked decrease of secretory granules immunoreactive for calcitonin. In some C cells, almost complete loss of the secretory granules is caused. Furthermore, vesicular inclusions of various sizes and dilated nuclear envelopes, which are immunoreactive for calcitonin, are encountered (Kameda 1982b). These are regarded as dilated cisternae of rough endoplasmic reticulum accumulating the biosynthetic precursors of calcitonin. Calcitonin is derived from glycosylated precursors with larger molecular weights (Jacobs et al. 1981). Taken together, it is supposed that the enzymes that convert the biosynthetic precursors to calcitonin are inhibited by the antithyroid drugs, resulting in the accumulation of precursors in cisternae of rough endoplasmic reticulum. In fact, dilated cisternae of rough endoplasmic reticulum and decreased secretory granules have been observed at the electron microscopic level in the C cells of dog thyroid glands after thiourea administrations (Kameda 1974a). The follicular cells exert a paracrine effect on the C cells (Andersson et al. 2011). Basolateral expression of EphA4, tyrosine kinase receptor, is observed in the follicular cells. The C cells, however, are devoid of EphA4. In EphA4$^{EGFP/EGFP}$ mutant adult mice in which functional impairment of EphA4 is induced, the follicle architecture becomes very flat and enlarged and the follicular cells exhibit an elongated nucleus (Andersson et al. 2011). The C cell number decreases to 42% of wild-type mice. Thus, both follicular cells and C cells become inactive in the mutants; the intimate functional relationship between both cell types is considerable. After chronic administration of antithyroid drugs, C cells markedly changed in appearance. In addition to direct effects of antithyroid drugs on C cells, the affected follicular cells may induce inhibitory effects on calcitonin synthesis of C cells.

After antithyroid drug treatment, almost all complexes observed are of follicle-containing type. Successive stages of folliculogenesis are detected in the complexes in proportion to the duration of treatment (Kameda and Ikeda 1980b). After administration for 2 months, primitive follicles containing colloid droplets are vigorously formed among the follicular cell clusters throughout the complexes. Typical undifferentiated cells are not observed. The rapid differentiation of undifferentiated cells into follicular cells is possibly caused by the treatment. After 4 months of treatment, the complexes are occupied by the minute follicles covered with columnar follicular cells. The follicles contain no colloid and often display papilliform protrusions of the wall into the lumen. The complexes become large and some are difficult to distinguish from the affected thyroid parenchyma. Remarkably, enlarged blood capillaries are rich in the complexes after the antithyroid drug treatment.

In rats that received propylthiouracil or thyroxine for 2 or 5 weeks, alterations of both C cells and follicular cells have been reported (Martin-Lacave et al. 2009). The administration of the goitrogen provokes a hypertrophy in both the cells, whereas exogenous thyroxine treatment induces the inactive follicular cells and a decrease of C cell number. The numerical relation between follicular cells and C cells is maintained independent of the thyroid status. Thyrotrophin receptors are expressed in the rat and human C cell lines and in thyroid C cells (Morillo-Bernal et al. 2009). After hypophysectomy, however, the C cells of dog thyroid glands show no light and electron microscopically recognizable changes, in contrast to the follicular cells that become flat and inactive (Kameda 1974b). Thus, the relationship between the pituitary gland and C cells does not represent the typical hypophysis–target gland feedback system. On the other hand, the hypophysectomy prior to the induction of chronical hypercalcemica

results in a marked suppression of C cell response to hypercalcemia (Kameda 1974b).

Follicular cells in the ultimobranchial remnants of other mammals

▪Rats

In rat thyroid glands, the second kind of follicle, distinguished from the usual thyroid follicles, is designated as a so-called "ultimobranchial follicle" (Wollman and Nève 1971a, b). The ultimobranchial outpocketings, before fusion with the thyroid primordium, in rat embryos exhibit the second kind of follicle, when they are implanted into kidney capsules of adult rats (Wollman and Hilfer 1977). The follicular cells immunoreactive for thyroglobulin and PAS-positive colloid-containing microfollicles are observed in the wall of ultimobranchial follicles of rats (Conde et al. 1992). Recently, immunohistochemical reactions of the ultimobranchial follicles have been demonstrated in rat thyroid glands (Vázquez-Román et al. 2017). The rat ultimobranchial follicles are immunoreactive for both P63 and high-molecular weight cytokeratin. Some thyroglobulin-positive cells are present in the mixed follicles emerging from the wall of ultimobranchial follicles. The so-called mixed follicles have been defined as the follicle structures that are lined on one side by follicular epithelium and on the other side by multilayered squamous epithelium. It is not clear whether all "ultimobranchial follicles," "ultimobranchial tubules" and "ultimobranchial cysts" in the rat thyroid lobes reported in the past are actually derived from the ultimobranchial body, especially in the cases in which the distribution of C cells is scarce around the structures. In dog thyroid glands, various sizes of cysts and follicular structures immunoreactive for epidermal keratins are distributed throughout the thyroid lobe (Kameda 1987). Some of these structures have no topological relation with the C cell complexes and only a few C cells are dispersed around them.

▪Mice

Similar to those of the rat embryos, the ultimobranchial outpocketings of mice embryos, implanted into kidney capsules of adult mice, form small follicles (Wollman and Hilfer 1978).

▪Humans

In humans in which the thyroid lobes do not fully descend, ectopic lingual thyroid is detectable in the upper neck and the ultimobranchial body cannot fuse with the thyroid, sustaining as a glandular nodule (Williams et al. 1989). In this nodule, solid cell areas consisting of C cells immunoreactive for calcitonin and CGRP intermingle with follicular structures immunoreactive for thyroglobulin. Thus, it has been suggested that the follicular cells in addition to C cells are formed in the ultimobranchial gland of humans.

SCNs

Humans

In normal and pathologically altered human thyroid glands, SCNs consisting of small cell clusters are present. They are usually located in the middle third of the thyroid lobe and their frequency occurrence is 7% (Janzer et al. 1979). The SCNs have been thought to be remnants of the ultimobranchial body by many authors (Janzer et al. 1979; Harach 1985, 1988; Autelitano et al. 1987; Mizukami et al. 1994), because the anatomical position of SCN is comparable to that of the ultimobranchial anlage in human fetuses and numerous C cells are distributed in the proximity of the SCNs. Furthermore, the parathyroid and thymus tissues are rarely observed around them. On the other hand, there are opinions that the SCN may be difficult to distinguish from squamous metaplasia, papillary thyroid carcinoma, medullary thyroid carcinoma and C cell hyperplasia (Reis-Filho et al. 2003). The SCNs are composed of numerous main cells and a few scattered C cells. The main cells are intensely immunoreactive for P63, Bcl-2, cytokeratins and carcinoembryonic antigen (Cameselle-Teijeiro et al. 1994; Reis-Filho et al. 2003; Preto et al. 2004; Burstein et al. 2004; Rios Moreno et al. 2011). P63, a member of the p53 tumor-suppressor gene family, is a reliable immunohistochemical marker for the main cells of SCNs. P63 is expressed in the basal or progenitor layers of many epithelial tissues and functions to maintain proliferative potential (Senoo et al. 2007; Koster and Roop 2004). In P63 null mutant mice, a striking decrease of all squamous epithelia and their derivatives are induced; the gene is critical for maintaining the progenitor cell populations that are necessary to sustain epithelial development and morphogenesis (Yang et al. 1999; Senoo et al. 2007). Bcl-2 is associated with an increased differentiation potential and blocks the apoptotic cell death. Thus, the main cells have been suggested to be multipotential stem cells contributing to the histogenesis of both C cells and follicular cells (Rios Moreno et al. 2011). Cystic structures and follicles of various sizes, which are immunoreactive for thyroglobulin, intermingle with SCNs. In addition, the so-called mixed follicles are observed in continuity with the SCNs (Harach 1985, 1988; Cameselle-Teijeiro et al. 1994; Preto et al. 2004; Rios Moreno et al. 2011). Both follicular cells and microfollicles immunoreactive for thyroglobulin are detectable in the SCNs (Harach 1985; Cameselle-Teijeiro et al. 1994), whereas the mixed follicles in the human SCNs

are thyroglobulin-negative (Cameselle-Teijeiro et al. 1994; Rios Moreno et al. 2011).

▪P63 is not expressed in ultimobranchial body, C cells and follicular cells

In mouse embryos, P63 is intensely exhibited in the nuclei in the exodermal epithelium, endodermal epithelium lining the pharyngeal cavity, pharyngeal pouch III and thymus, whereas ultimobranchial body cells are not immunoreactive for P63 (Kameda et al. 2009; Kameda et al. 2013). In E13.5 mouse embryos, only a few cells of the ultimobranchial body are immunoreactive for P63 (Kusakabe et al. 2006a). Furthermore, the C cells and follicular cells in thyroid glands are negative for P63 (Vázquez-Román et al. 2017; Reis-Filho et al. 2003; Rios Moreno et al. 2011). In P63 null mutant mice, normal thyroid glands in which calcitonin-positive C cells are normally distributed develop (Ozaki et al. 2011).

Cattle and bison

In cattle, as well as humans, SCNs mingled with colloid containing follicles of various sizes are located in thyroid glands (Ljungberg and Nilsson 1985; Harmon and Kelley 2001). The SCNs are composed of undifferentiated cells, C cells immunoreactive for calcitonin, cystic structures and follicles immunoreactive for thyroglobulin. In addition to SCNs, diffuse or multifocal hyperplasia of C cells showing features of both a follicular and C cell tumor are frequently observed in bull thyroid glands (Ljungberg and Nilsson 1985). The follicular cell groups and small follicles immunoreactive for thyroglobulin are dispersed in the tumors (Ljungberg and Nilsson 1985).

In the thyroid gland of European bison, persistent ultimobranchial remnants are observed (Sawicki and Zabel 1997). Aggregations of C cells immunoreactive for calcitonin, CGRP and neuron-specific enolase mingle with follicular structures that vary in size, shape and luminal contents.

Conclusion The ultimobranchial remnants are different from species to species in appearances, positions and constituents. In dogs, the remnants (C cell complexes) consisting of cell clusters and cysts frequently form large separate organs in and around thyroids close to thymus IV and parathyroid IV. Some are buried in the thyroid parenchyma. In addition, there are remnants containing follicular cell groups and small primitive follicles immunoreactive for thyroglobulin. Dense distributions of C cells are observed in the vicinity of the ultimobranchial remnants in thyroid parenchyma. In rats and mice, the ultimobranchial remnants usually show follicular structures of various sizes and features. C cells occur to an extent comparable to those in the usual thyroid follicles. In cattle and bison, cell aggregates (SCNs) are dispersed among cysts and follicular structures. Mixed follicles immunoreactive for thyroglobulin are located in the cell aggregates. In humans, SCNs consisting of small cell clusters mingle with cystic structures and follicles of various sizes. The human SCNs are composed of numerous main cells immunoreactive for P63 and a few scattered C cells immunoreactive for calcitonin. C cells are densely distributed near the SCNs.

Mixed medullary and follicular thyroid carcinoma

Humans

The medullary thyroid carcinoma (MTC) is a malignant tumor derived from C cells. The tumor cells are immunoreactive for calcitonin and carcinoembryonic antigen (Schmid 2015). The MTC sometimes combines with follicular and papillary thyroid carcinoma and its lymphonodi metastases also show mixed phenotypes of tumors (see Matias-Guiu 1999 for review). The tumor has been designated as "mixed medullary and follicular thyroid carcinoma." It exhibits a great variability in the morphological appearance of the follicular component. Thyroglobulin immunoreactivity and mRNA are expressed in the neoplastic follicles and/or solid foci (Hales et al. 1982; Noel et al. 1991; Papotti et al. 1997; Nilsson and Williams 2016). The follicles of lymphonodi metastases also display thyroglobulin immunoreactivity and concentrate injected radioactive iodine ^{131}I (Hales et al. 1982). Thus, the mixed medullary and follicular carcinoma display morphological, immunohistochemical and genetic phenotypes of both medullary and follicular neoplasm.

Concerning the origin of the mixed medullary and follicular thyroid carcinoma, one has hypothesized that the tumor results from a coincidental malignant change in both follicular and C cells, i.e., dual origin of tumor components; RET mutations are present in the MTC and BRAF mutations in the papillary thyroid carcinoma (Volante et al. 1999). The other says that tumor cells are derived from the common stem cells with the potentiality of differentiating into both follicular and C cells (Ljungberg et al. 1983). It has been demonstrated by both immunohistochemistry and in situ hybridization that rare cells in the neoplasmic elements display the dual expression of calcitonin and thyroglobulin (Papotti et al. 1997). The recent review has also supported that the mixed medullary and follicular thyroid carcinoma may be of a single cell origin from an ultimobranchial stem cell (Nilsson and Williams 2016).

Mouse tumor models

In addition to the tumors showing typical features of medullary thyroid carcinoma, the mixed medullary and follicular thyroid carcinoma have been produced in a subset of

transgenic mice by expressing v-Ha-ras under control of calcitonin/CGRP promoter (Johnston et al. 1998). Rare cells with concurrent calcitonin and thyroglobulin expression are detectable in the carcinoma.

The RET proto-oncogene encodes a receptor tyrosine kinase. Transgenic mice expressing multiple endocrine neoplasia type2A (MEN2A) RET51 mutation, Cys-634-Arg, under the control of the human calcitonin promotor, develop both C cell tumors resembling human medullary thyroid carcinoma and follicular tumors resembling human papillary thyroid carcinoma depending on the founder line examined (Reynolds et al. 2001). The tumors are occupied by both C cell masses and large irregular cystic follicles. Tumor cells are positive for RET and Nkx2.1. The colloid-like substances in the cystic lumen are intensely immunoreactive for thyroglobulin.

Follicular cells in the ultimobranchial remnants of mutant mice

Eya1 homozygous mutant

The eyes absent (Eya)1, which encodes a transcription coactivator, is ubiquitously expressed in the pharyngeal mesenchyme, pouch endoderm and surface ectoderm in the pharyngeal region of mouse embryos (Xu et al. 2002). Particularly, the Eya1 expression is seen in the third and fourth pharyngeal pouches and also in the primordia for thymus and parathyroid. In Eya1 null mutant mice, the thymus and parathyroid primordia fail to form, whereas the ultimobranchial body primordium normally forms in the fourth pharyngeal pouch and expresses Pax1 and Pax9 at E11.5–E12.5 (Xu et al. 2002). At E15.5, a persistent ultimobranchial body, which is not fused with the thyroid lobe, is detected in the null mutants. Calcitonin-positive C cells and follicle-like structures containing colloid are present in the persistent ultimobranchial body (Xu et al. 2002). The persistent ultimobranchial body shows Pax8 expression at E15.5, when thyroglobulin begins to be expressed. Pax8 may be required for the expression of thyroglobulin in the follicular cells of the persistent ultimobranchial body, as well as thyroid lobe.

Hox3 paralogs homozygous mutants

The Hox family encodes a class of transcription factors that mediate formation of the animal body plan along the anteroposterior axis. Hoxa3 is expressed in the third and fourth pharyngeal pouch endoderm and pharyngeal arch mesenchyme (Manley and Capecchi 1995). Targeted disruption of Hoxa3 results in the absence of the thymus and parathyroid glands, because the third pharyngeal pouch fails to differentiate (Chisaka and Kameda 2005). The mutant mice lacking for three Hox3 paralogs, i.e., Hoxa3, Hoxb3 and Hoxd3, exhibit a failure of migration of the ultimobranchial body to the normal position, i.e., within thyroid parenchyma (Manley and Capecchi 1998). Thus, various degrees of ultimobranchial body defects, including persistent, ectopic and a partially fused ultimobranchial body and also absence of the organ, are induced in the mutant mice. The colloid-containing follicles immunoreactive for thyroglobulin, in addition to calcitonin-positive C cells, are detected in the ultimobranchial remnants.

Nkx2.1 heterozygous mutant

Homeobox transcription factor Nkx2.1 is required for proliferation and survival of the progenitors for both thyroid follicular cells and C cells. In Nkx2.1 null mutant mice, thyroid primordium forms and migrates but is lost by E12–E13 through apoptosis. Similarly, the ultimobranchial body anlage is detected at E10 but disappears at E12–E13 (Kimura et al. 1996). In wild-type mice, Nkx2.1 is expressed in the ultimobranchial body at E11.5–E12.5 (Fagman et al. 2006; Kameda et al. 2013). After the organ is embedded in the thyroid lobe, immunoreactivity for Nkx2.1 disappears from the ultimobranchial body, although thyroid follicular cells exhibit intense immunoreactivity for Nkx2.1. In Nkx2.1 heterozygote mice at E19.0, the vesicular structure regarded as the ultimobranchial body remnant is located in the dorsal part of the thyroid lobe in all cases examined (Kusakabe et al. 2006a). Both a cluster of C cells immunoreactive for calcitonin and colloid-containing small follicles immunoreactive for Nkx2.1 exist around the vesicle, that is, the ultimobranchial anlage fails to integrate with the thyroid lobe and C cells are unable to disperse throughout the thyroid lobe in Nkx2.1 heterozygote mice. The transcription factor P63 is a marker for epidermal stem cells and is required for proliferation of stem cells in stratified epithelia. P63 is also expressed in epithelial cells with high-proliferative capacity. The P63-positive cells always surround the ultimobranchial-derived vesicular structure in which the inner wall is lined by P63-negative cells. Thus, it has been supposed that both C cells and follicular cells originate from the ultimobranchial-derived vesicular structures immunoreactive for P63 (Kusakabe et al. 2006a).

FRS2α$^{2F/2F}$ mutant

The docking protein FRS2α mediates FGF signaling by providing a link between FGF receptors and a variety of intracellular signaling pathways. The FRS2α protein contains four binding sites for an adaptor protein Grb2 and two binding sites for tyrosine phosphatase Shp2 (Hadari et al. 2001). Disruption of the FRS2α results in embryonic lethality at E7.0–7.5, due to multiple defects in FGF-mediated signaling. However, mice carrying a targeted disruption of the Shp2-binding site of FRS2α (FRS2α$^{2F/2F}$) are viable until E18.5. FRS2 is widely

expressed in the pharyngeal region, especially in the pharyngeal endoderm and arch mesenchyme (Kameda et al. 2009).

In the FRS2$\alpha^{2F/2F}$ mouse embryos, the formation of pharyngeal pouches and thyroid primordium is normally initiated. However, the epithelial buds of pharyngeal pouches, including the primordia for parathyroid, thymus and ultimobranchial body, remain affiliated with the pharyngeal epithelium and the thyroid primordium becomes hypoplastic at E11.5–12.5 (Kameda et al. 2009). Thus, the developments of pharyngeal organs are affected in all mutant mice (the numbers of FRS2$\alpha^{2F/2F}$ mutants examined at E15.5–E18.5 are 17, i.e., 34 cases). Thyroid lobes are absent in 14 out of 34 cases (41.2%) and are rudimental or hypoplastic in 15 cases (44.1%). C cells are absent in 19 out of 34 cases (55.9%). In seven cases, the C cell clusters, composed of a compact mass of C cells and capsulated by the connective tissue, were detected in the connective tissue around the pharyngeal cavity (Fig 5a–c) or attached to the pharyngeal epithelium. In the C cell clusters, primitive follicles containing colloid droplets immunoreactive for thyroglobulin are localized (Fig. 5c) (Kameda et al. 2009). Parathyroid glands are absent in 9 out of 34 cases (26.5%). Thymus and parathyroid are most often identified near the pharyngeal cavity, failing in descent. Thus, the migration and development of all pharyngeal organs are affected in FRS2$\alpha^{2F/2F}$ mutants.

Conclusion In mutant mice, such as Eya1−/−, Hox3 paralogs −/−, Nkx2.1+/− and FRS2$\alpha^{2F/2F}$, the migration of the ultimobranchial body into the thyroid primordium is prevented. Therefore, the ultimobranchial body remains as a separate organ. The colloid-containing small follicles immunoreactive for thyroglobulin are detected in the ultimobranchial remnants of these mutant mice.

Vimentin expression in the immature follicular cells derived from the ultimobranchial body

In post-natal dogs, follicular cells in various stages of differentiation are detectable in the C cell complexes and large C cell groups. The immature follicular cells not yet organized in follicles and primitive follicles starting to store colloid droplets are immunoreactive for vimentin in addition to thyroglobulin (Fig. 2a–d) (Kameda 1995b). When the follicles accumulate colloid in the lumen, vimentin immunoreactivity disappears from the follicular cells. Typical thyroid follicles in the thyroid gland exhibit no immunoreactivity for vimentin. In canine fetuses, neither follicular cells arranged in cell clusters nor the cells forming primitive follicles exhibit immunoreactivity for vimentin. Thus, only the ultimobranchial body–derived immature follicular cells after birth are immunoreactive for vimentin.

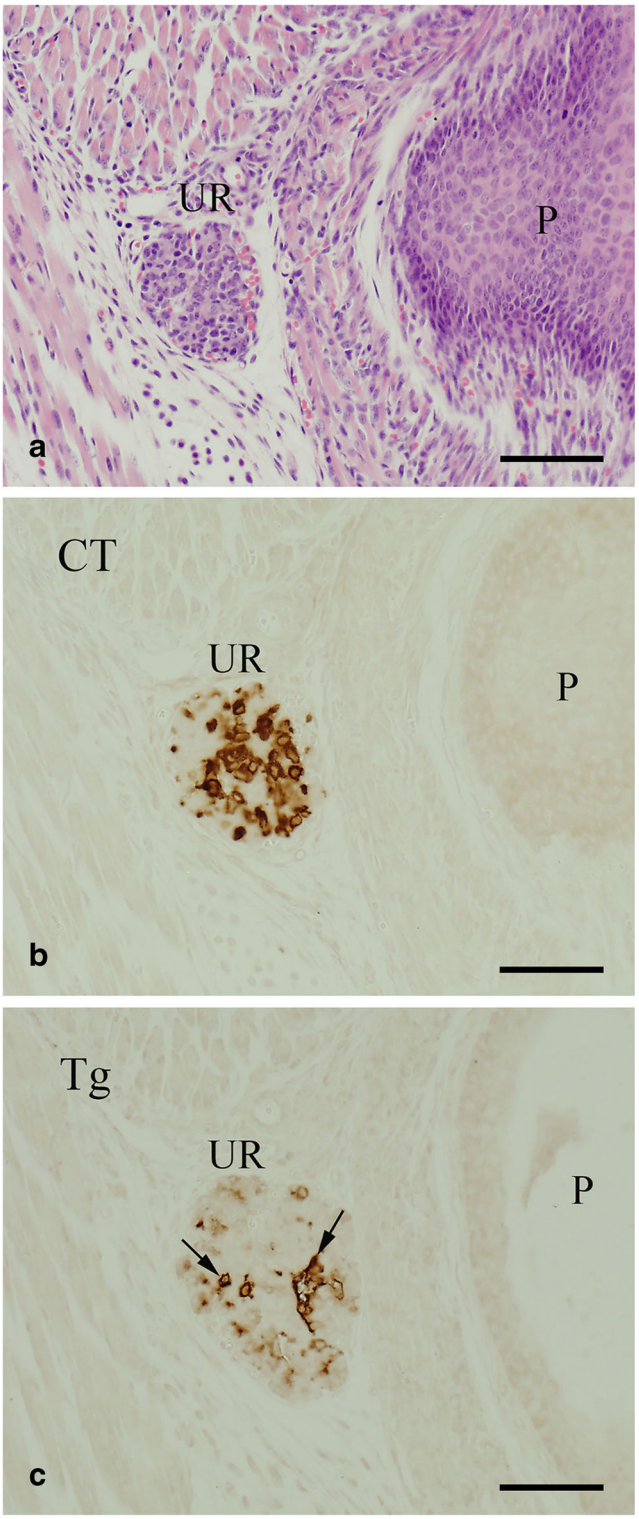

Fig. 5 **a**–**c** Serial frontal sections of an ultimobranchial remnant (UR) located close to the pharynx (P) in FRS2$\alpha^{2F/2F}$ mouse were stained by different methods. **a** Hematoxylin–eosin staining. **b** Immunostaining with calcitonin (CT) antiserum. A majority of the remnant is occupied by C cells immunoreactive for calcitonin. **c** Immunostaining with thyroglobulin (Tg) antiserum. Minute follicles immunoreactive for thyroglobulin (arrows) are seen. Modified from Kameda et al. (2009). Bars 80 μm

Vimentin is an intermediate-sized filament expressed in mesenchymal cells. Furthermore, vimentin is expressed in non-mesenchymal cell types such as epithelial cells and neurons during embryonic development (Lane et al. 1983; Bignami et al. 1982). Some epithelial cells in mature animals express vimentin in addition to cytokeratin (Kameda 1996).

A completely differentiated thyroid follicular epithelium can transform into mesenchymal-like cells when cultured in type I collagen gels (Greenburg and Hay 1988). The cells bordering the follicle lumen retain epithelial polarity, whereas at the basal surface, the cell processes extend into the collagenous matrix; the basal cells acquire the mesenchymal phenotype and express vimentin. Thus, in cultured follicular cells, cytokeratin-positive intermediate filaments change to a vimentin cytoskeleton and thyroglobulin immunoreactivity is lost during epithelial–mesenchymal transformation. In cultured keratinocytes, vimentin co-exists with keratin in the same cell at the leading edge of the migrating epidermal cells and knockdown of vimentin impairs colony growth (VelezdelValle et al. 2016). Furthermore, in cultured neo-natal hepatocytes (Pagan et al. 1996) and corneal epithelial cells (Castro-Munozledo et al. 2017), vimentin and keratin filaments are co-expressed in the cells localized at the proliferative/migratory rim of the growing colonies. Thus, vimentin expression reflects a phenotypic characteristic that contributes to the migration and appears to be related to changes in cell–cell contact and cell shape. Taken together, it is supposed that vimentin is essential for a highly motile phenotype. The vimentin-positive cells in thyroid lobes might retain the ability for expansion throughout the parenchyma and also associate with increased cellular activities, i.e., folliculogenesis.

Summary and future directions

Follicular cells in various stages of differentiation, including follicular cell groups and small primitive follicles containing colloid, are located in the ultimobranchial remnants of different mammals. The remnants are observed in normal dogs, humans, rats, cattle and bison and also in mutant mice such as Eya1−/−, Hox3 paralogs−/−, Nkx2.1+/− and FRS2$\alpha^{2F/2F}$. The follicles in the remnants are immunoreactive for thyroglobulin and can accumulate radioiodine (NaI^{125}) injected on colloid in the lumina, as well as those in thyroid lobes. Both median thyroid primordium and the ultimobranchial body derived from the pharyngeal pouch IV are derivatives of the pharyngeal endoderm (see Fig. 6).

Pax8 and Nkx2.1 play crucial roles for the development of thyroid follicular cells. These genes are also expressed in the ultimobranchial body and its remnants. The slow development of follicular cell lineages in the ultimobranchial remnants might reflect dosage differences and/or variations in the efficiency of the genes in the ultimobranchial body, compared with thyroid

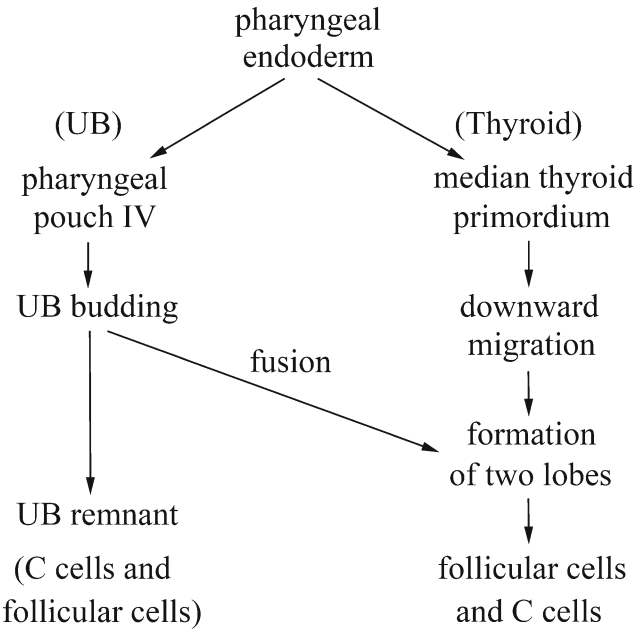

Fig. 6 Scheme for the formations of ultimobranchial body (UB) and thyroid gland primordia. Both organs are derived from the pharyngeal endoderm. A majority of the ultimobranchial body invades the thyroid lobe. A part of the organ, however, remains separated as the ultimobranchial remnants in normal dogs, humans, rats, mice, cattle and bison and also in mutant mice such as Eya1−/−, Hox3 paralogs−/−, Nkx2.1+/− and FRS2$\alpha^{2F/2F}$

lobes. Studies on folliculogenesis in the thyroid gland indicate that a sufficient blood supply is necessary for architectures of ripened follicles that exhibit basal–apical orientation, lumen formation, storage of colloid and accumulation of basement membrane. The development of blood capillaries in the ultimobranchial remnants may be less than that in thyroid lobes, because VEGF-A and its receptor involved in the angiogenesis are not expressed in the ultimobranchial body, in contrast to the thyroid lobe. Dicer and Bmp–Smad signaling are also required for the formation of follicle structures. However, no information is available concerning their functions on the follicular cells in ultimobranchial body remnants. In addition, it remains to be elucidated why vimentin is specifically expressed in the ultimobranchial-derived immature follicular cells.

Not only follicular cells but also C cells in the canine C cell complexes are sustained in immature stages for a long time. In Pax8 null mutant mice at E18.5 in which the thyroid gland is missing, almost all cells in the ultimobranchial remnant express Nkx2.1, in addition to calcitonin (Mansouri et al. 1998). Neither the ultimobranchial body fused with the thyroid primordium nor C cells in the thyroid express Nkx2.1 in the mouse (Kusakabe et al. 2006a; Liang et al. 2018). The Nkx2.1-positive ultimobranchial remnant in Pax8 mutants at later stages of development may consist of immature C cells. Interactions with follicular cells appear to be necessary for the differentiation and development of C cells in mammalian species.

Cyst structures showing various sizes and irregular shapes are localized in ultimobranchial remnants in different mammals. Colloid-like, foamy and flocculent substances in the cyst lumina are intensely immunoreactive for thyroglobulin, although they cannot accumulate radioiodine (Kameda 1982a). In non-mammalian vertebrates including birds, the ultimobranchial body remains as an independent organ throughout life (Kameda 2017). The ultimobranchial glands of non-mammalian species never contain typical follicles storing colloid immunoreactive for thyroglobulin. Furthermore, the contents of cysts in the gland are not immunoreactive for thyroglobulin. From the phylogenetic aspect, only the ultimobranchial body of mammals obtains the ability to form follicular cells, as well as the median thyroid primordium. Consequently, the organ of mammalian species might fuse with the thyroid primordium to establish an efficient functional relationship between C cells and follicular cells.

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