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C-cell differentiation in the wall of an aberrant ultimobranchial sinus in the thyroid gland of an old rat

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Abstract

Background: In mammals, the thyroid gland possesses two types of endocrine cells, follicular cells and C cells, which have different functions but share a similar endodermal origin (although from different regions of the primitive pharynx). Specifically, follicular cells derive from the ventral pharyngeal floor, while C cells derive from the fourth pair of pharyngeal pouches through the ultimobranchial bodies (UBBs). Disruptions to human midline thyroid morphogenesis are relatively frequent and known as thyroid dysgenesis, which is the leading cause of congenital hypothyroidism. In contrast, fourth branchial apparatus anomalies are very rare clinical entities.

Objectives: The aim of this study was to analyze the morphological features and the immunohistochemical pattern of an aberrant ultimobranchial remnant, align with its persistent contribution to the formation of new C cells.

Methods: The thyroid gland of an old rat was serially sectioned and immunostained for the following markers: calcitonin, thyroglobulin, cytokeratins, PCNA, P63, E-cadherin, beta-tubulin and CD3.

Results: We detected a spontaneous congenital defect in the organogenesis of the UBB in an old rat, giving rise to an 'ultimobranchial sinus', which was accompanied by thymic tissue and an abscess. The epithelium contained basal/stem cells and contributed to the formation of abundant C cells and scarce follicular cells.

Conclusions: The ultimobranchial sinus is an exceptional finding for representing the first spontaneous abnormality in the development of UBB reported in rats, and the opportunity to observe sustained C-cell differentiation from stem cells in an old rat. These findings are consistent with a common origin of both C cells and follicular cells from UBB.

KEYWORDS
ultimobranchial, sinus, thyroid, C cells, follicular cells

1 | INTRODUCTION

In mammals, the thyroid gland consists of two endocrine cell populations, follicular cells and C cells, with similar endodermal origins, although from different regions of the primitive pharynx. Specifically, follicular cells, the most abundant cells, are responsible for the biosynthesis of thyroid hormones and are derived from the ventral pharyngeal floor. C cells, however, are rather scarce and synthetize calcitonin and other regulatory peptides (Utrilla et al., 2013), originating from the ultimobranchial bodies (UBBs) that are separated from the fourth pair of pharyngeal pouches without being infiltrated by neural crest cells, as was recently confirmed (Kameda, 2016). Neural crests give rise only to the ectomesenchyme, which forms the stromal compartment of the thyroid gland, but definitively are not the source of C cells (Kameda et al., 2007; Johansson et al., 2015).

Thyroid organogenesis from the midline thyroid anlage is a very complex process that is regulated by a number of key thyroid transcription factors, although four of them, namely, Hhex, Nkx2-1, Pax8 and Foxe1, stand out as crucial and may thus be considered a thyroid signature within the anterior foregut endoderm (Fernandez et al., 2015; Nilsson & Fagman, 2017). Disruptions to human thyroid morphogenesis result in different phenotypes that are known as thyroid dysgenesis, which is the leading cause (85% of cases) of congenital hypothyroidism (De Felice & Di Lauro, 2004). The most characteristic alterations are thyroid agenesis or athyreosis, ectopia, hypoplasia and hemiagenesis (Fernandez et al., 2015; Nilsson & Fagman, 2017). In contrast, fourth branchial apparatus anomalies related to the development of UBB are rare clinical entities (representing only 1% of branchial anomalies), whose diagnosis in humans can be difficult due to the absence of evident clinical signs, needless to say in other mammals (Li et al., 2020).

UBBs, apart from forming C cells during prenatal life, remain in the adult thyroid gland as rather complex structures that are considered embryonic remnants with unknown significance. These structures show differences among species and have been described in the literature under different names; specifically, in rats, they are mainly known as 'ultimobranchial follicles' (UBFs) (Martin-Lacave et al., 1992; Vazquez-Roman et al., 2013, 2017), while in humans, they are termed 'solid cell nests' (SCNs) (Harach, 1985).

The developmental progression of UBFs throughout rat postnatal life has been extensively studied by our research group (Vazquez-Roman et al., 2013). We have found UBFs at all ages in every lobe, and they are clearly differentiable from normal thyroid follicles, although they showed differences in both structure and size according to age and sex. At any age, cystic UBFs were composed of epithelial-type cells surrounding a lumen, together with undifferentiated cells located at the periphery that were p63(+) (Vazquez-Roman et al., 2017). Nevertheless, despite the existence of undifferentiated cells, the so-called 'U-cells' (Wollman & Hilfer, 1977), no calcitonin-positive cells were observed in the wall of any UBF, except in sporadic newborn rats. Moreover, no increased C-cell number was seen around UBFs; consequently, C-cell formation from UBBs must be restricted to rat prenatal life (Martin-Lacave et al., 1992; Vazquez-Roman et al., 2013). This finding is in clear contrast to that observed in human SCNs, where numerous C cells are intermingled with undifferentiated cells or main cells (Cameselle-Teijeiro et al., 1994, 2005; Rios Moreno et al., 2011).

Therefore, the case reported in the present paper is exceptional and interesting since it consists of an old rat with aberrant UBB development, giving rise to a conspicuous UBF partially included in the left thyroid lobe and surrounded by ectopic thymic tissue, which still maintains sustained C-cell differentiation, accompanied by follicular-cell formation, although to a much lesser extent.

2 | MATERIALS AND METHODS

2.1 | Selection of samples

We analyzed the thyroid gland of a 15-month-old female Wistar rat that presented aberrant spontaneous development of UBB. The thyroid gland together with the trachea were fixed in formaldehyde, embedded in paraffin and serially sectioned at a 5 μm thickness.

2.2 | Immunohistochemical analysis

Consecutive sections mounted on silane-coated slides were dewaxed in xylene and hydrated through a series of graded alcohols. Next, an antigen retrieval step using a high- or low-pH retrieval solution was performed in a heating instrument (PTLink, Dako) at 96°C for 20 min, following the manufacturer's instructions. After treatment with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, the slides were incubated with a panel of primary antibodies (see Table 1) at 4°C overnight in a humidified chamber. EnVision Flex/HRP (Dako, Glostrup, Denmark) or a Vectastain ABC-HRP Kit (Vector, USA) was used as the labeling system, following the manufacturers' instructions, and 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma–Aldrich, Darmstadt, Germany) was used as the chromogen. The slides were counterstained with Harris hematoxylin, dehydrated and coverslipped. Photomicrographs were taken using an Olympus photomicroscope and a Nikon DS-Fi3 camera.

2.3 | Immunofluorescence analysis

Thyroid sections were dewaxed, hydrated and subjected to antigen retrieval using Target Retrieval High pH buffer, as described above. Non-specific binding was blocked with 10% normal donkey serum for 15 min (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Then, the polyclonal primary antibody anti-calcitonin or anti-E-cadherin was added and incubated at 4°C overnight in a humidified chamber. Subsequently, the slides were washed and incubated with Cy3-labelled donkey anti-rabbit IgG secondary antibody (1:100, Jackson ImmunoResearch Laboratories) for 30 min at room temperature.

TABLE 1 Antibodies and experimental conditions for immunohistochemical (IHC) and immunofluorescence (IF) analysis

Antigen	Primary antibody	Dilution	Antigen retrieval and labelling system
Calcitonin	Rabbit, polyclonal (A0576, DAKO, Denmark)	1:2000	-ABC/HRP IgG Cy2 labelled
CD3	Rabbit, polyclonal (DAKO, Denmark)	Ready to use	Heating, pH6 ABC/HRP
Pan-cytokeratins (Low and high molecular weight –HMW- CKs)	Mouse, monoclonal (clones AE1/AE3, DAKO, Denmark)	1:50	Heating, pH 9 ABC/HRP
HMW-CKs	Mouse, monoclonal (clone 34βE12, DAKO, Denmark)	Ready to use	Heating, pH 9 Flex/HRP
E-cadherin	Rabbit, polyclonal (Santa Cruz Biotechnology, USA)	1:100	Heating, pH9 IgG Cy3 labelled
p63	Mouse, monoclonal (clone 4A4, Santa Cruz Biotechnology, USA)	1:50	Heating, pH 9 Flex/HRP IgG Cy3 labelled
PCNA	Mouse, monoclonal (clone PC10, DAKO, Denmark)	1:100	Heating, pH 6 ABC/HRP kit
Thyroglobulin	Rabbit, polyclonal (A 0251, DAKO, Denmark)	1:400	-ABC/HRP
Beta-tubulin	Mouse, monoclonal (clone Z023, Zymed laboratories INC., USA)	1:200	Heating, pH 9 ABC/HRP

After washing in PBS, DAPI (Sigma–Aldrich) was added for nuclear counterstaining, and the slides were coverslipped with antifading mounting medium (Mowiol 4-88, Sigma–Aldrich).

Additionally, double immunofluorescence (IF) was performed to identify different cell populations at the UBF wall. We followed the same procedure described above for the first antigen. Specifically, we incubated the cells with a monoclonal anti-p63 antibody and then with a Cy3-labelled donkey anti-mouse IgG antibody (1:100, Jackson ImmunoResearch Laboratories). Then, the slides were incubated with a second specific polyclonal anti-calcitonin antibody and, subsequently, with Cy2-labelled donkey anti-rabbit IgG antibody (1:100, Jackson ImmunoResearch Laboratories) under the same conditions as before. DAPI was used for nuclear counterstaining, and slides were mounted using Mowiol. Finally, samples were observed under a fluorescence microscope (Olympus BX50) equipped with a scientific digital camera (Hamamatsu ORCA-03G).

Controls for determining immunostaining specificity consisted of replacing the primary antibody with an appropriate dilution of mouse or rabbit IgG serum (Sigma–Aldrich), followed by the immunohistochemical (IHC) or IF protocol as outlined above.

3 | RESULTS

3.1 | Histological features of an aberrant UBF located in the rat thyroid gland

An apparent UBF of considerable size (500 × 100 μm maximal diameter, 40,000 μm^2 surface area) was accidentally found in the left thyroid lobe of a 15-month-old female rat (Figure 1). Its proximal portion was in continuity with a duct, which was located next to the cricoid cartilage, and ascended as a tube, apparently toward the neck; its central portion entered into the thyroid lobe, closely surrounded by thymic tissue (mostly CD3-positive), where it acquired a cystic appearance. Eventually, the cyst became ramified into several branches at its distal portion. Most of the UBF wall was composed of a squamous stratified epithelium of variable thickness (Figure 1B).

Along the UBF wall, scattered mucous and ciliated cells were seen facing the lumen (Figures 1B1,B3). The lumen contained an inflammatory exudate in which neutrophils predominated, although both lymphocytes and tissue debris were also observed, which was compatible with the existence of a chronic abscess (Figure 1B). Taken together, these traits suggested that this structure corresponded to an ultimobranchial sinus (UB sinus), whose duct had likely not detached from the hypopharynx wall during fetal development, hence keeping it in contact with the content of the digestive cavity, which could explain the origin of the abscess.

To assess whether the left parathyroid gland was also affected by aberrant development, we completely seriated the whole thyroid gland and found a normal parathyroid gland in each lobe. We also located a UBF at the centre of the right thyroid lobe, which instead showed a normal morphology.

3.2 | Immunofluorescence and immunohistochemical analyses of the wall of the UB sinus

Most epithelial cells of the UB sinus wall were immunopositive for pan-CKs (clones AE1/AE3; Figures 1C and 2a) and E-cadherin (Figure 2g);

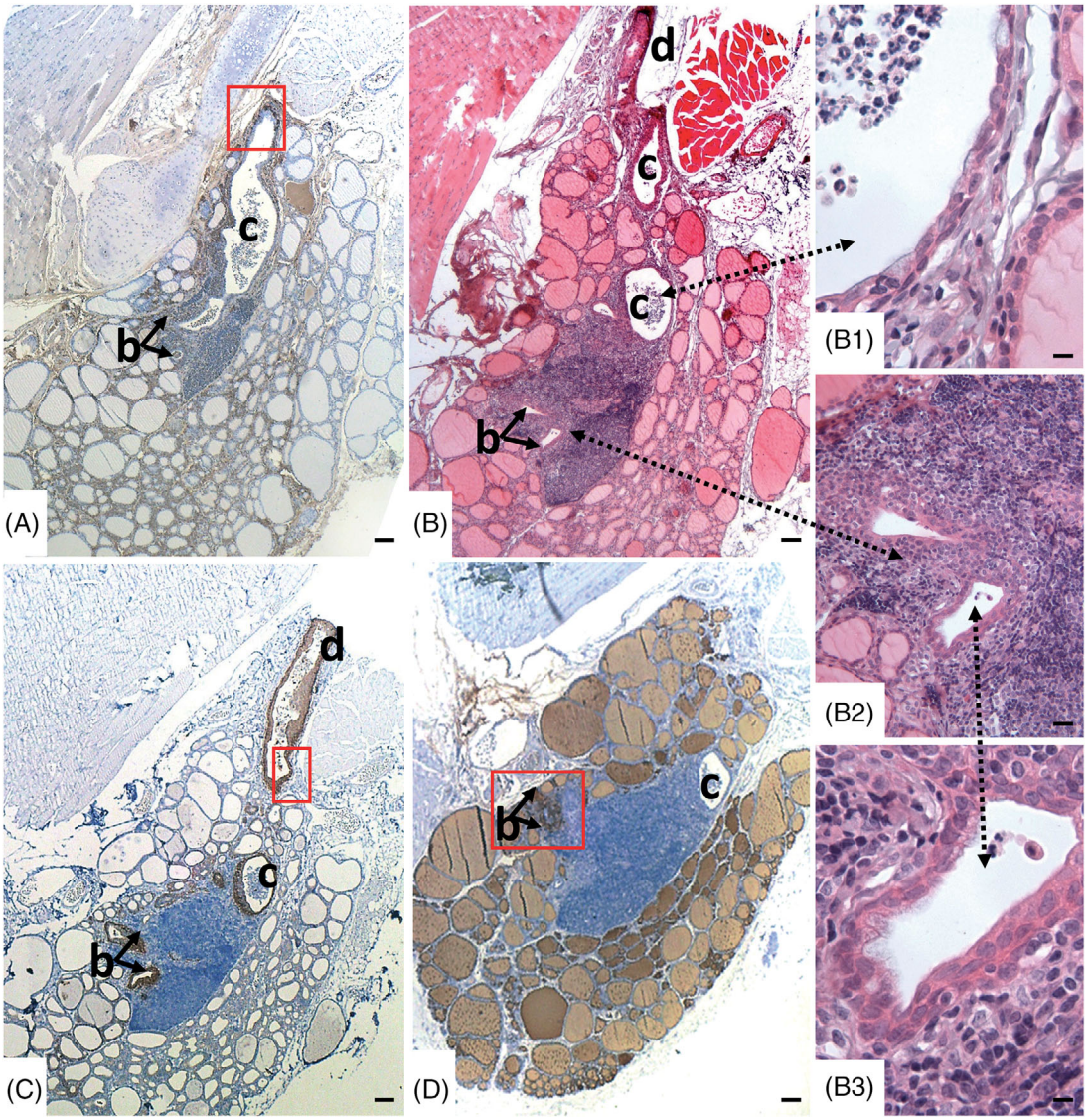

FIGURE 1 Histological structure and trajectory of the ultimobranchial sinus observed by Hematoxylin–Eosin and immunohistochemistry in serial sections. (A) calcitonin, (B) Hematoxylin–Eosin, (C) pan-CKs, and (D) thyroglobulin. The proximal end of the ultimobranchial sinus (UB) sinus is continuous with an ascendent duct (d) situated under the cricoid cartilage, which becomes dilated at the thyroid level, forming a wide cyst (c), that eventually bifurcates into two branches (b) at the most distal end. The lumen contains inflammatory cells and is surrounded by a squamous stratified epithelium immunopositive for CKs. The cyst and branches are completely surrounded by lymphoid tissue. The red squares overlapped to the immunohistochemical images mark the zones that are amplified in the correspondent photographs of Figure 2. Bars A–D: 150 μm. Bar B2: 50 μm. Bar B1, B3: 20 μm

however, only those cells that formed the basal stratum exhibited immunostaining for p63 (Figure 2c,f) and, less specifically, for HMW-CKs (clone 34βE12; Figure 2b), the two characteristic markers of undifferentiated cells or U cells. Moreover, at the duct and the proximal portion of the UB sinus, PCNA expression was restricted to basal cells (Figure 2d), in contrast to the rest of the wall where numerous immunopositive cells for PCNA were observed at different heights of the epithelium.

Nevertheless, the most interesting finding was the existence of abundant calcitonin-positive cells located along the wall of the UB sinus (Figures 1A and 2e,f). These cells were also immunopositive for E-cadherin (Figure 2g) and beta-tubulin (Figure 2h), but not for p63 (Figure 2f) or HMW-CKs. C cells were seen at the basal stratum and emigrated to the connective tissue that surrounded the UB sinus, where they even formed C-cell clusters (Figure 2i). Numerous C cells were also observed in the rest of the thyroid tissue (Figure 1A), according to a pattern that was similar to that observed in the right thyroid lobe.

The pattern of expression for beta-tubulin, a neural-phenotype marker, changed depending on C-cell maturity; thus, C cells recently formed in the UBF wall or emigrated through the connective tissue were strongly immunostained (Figure 2h,j). In contrast, C cells that

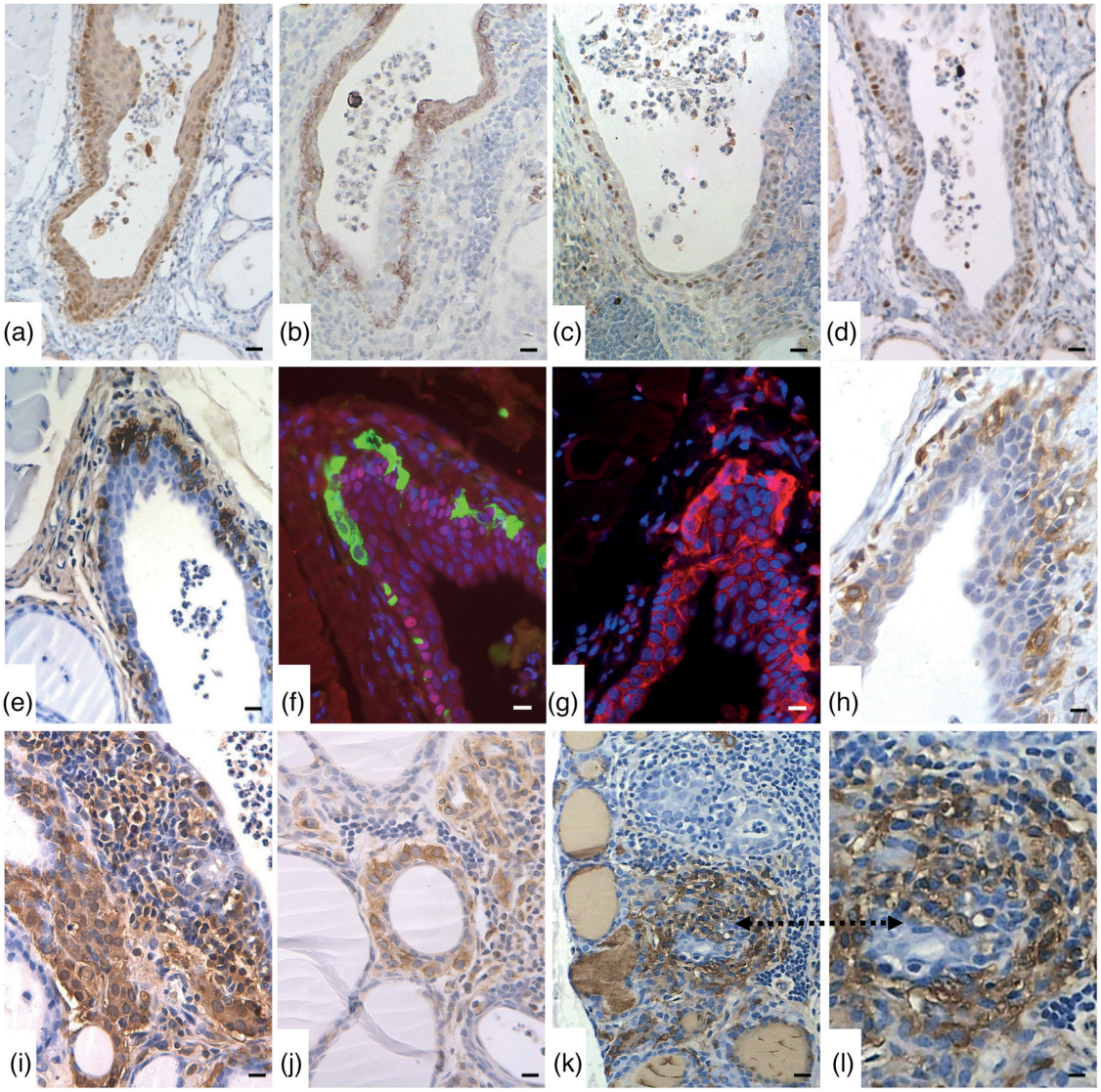

FIGURE 2 Immunohistochemical staining of the epithelial wall of the ultimobranchial sinus, where undifferentiated cells (a–d) and thyroid endocrine cells (e–l) are identified. (a) Pan-cytokeratins (pan-CKs), (b) high molecular weight cytokeratins (HMW-CKs), (c) p63, (d) PCNA, (e and i) calcitonin, (f) double immunofluorescence (IF) for calcitonin (green) and p63 (purple), (g) immunofluorescence for E-cadherin, (h and j) beta-tubulin, (k and l) thyroglobulin. Undifferentiated cells are specifically marked by p63 (c) and PCNA (d) antibodies, and less specifically for HMW-CKs (b), whereas all epithelial cells are immunostained by a cocktail of pan-CKs (a). In contrast, C cells are marked for calcitonin (e and i), E-cadherin (g), and beta-tubulin (h and j), but not for p63 (f). Finally, follicular cells are recognized using thyroglobulin immunostaining (k and l). Bars a–k: 20 μm. Bar l: 10 μm

reached their definitive position at the centre of the thyroid lobe exhibited only faint positivity for beta-tubulin. Nerve fibres of the connective tissue were also immunopositive for beta-tubulin, as well as smooth muscle cells of the blood vessels (Figure 2h,j).

Finally, at the most distal end of the UB sinus, in close proximity to the rest of the thyroid tissue, differentiation to thyroglobulin-producing cells was also observed, but to a much lesser extent than that for C cells. These cells stayed around the UB sinus branches and eventually originated small follicles containing colloid that was also immunopositive (Figures 2k,l).

4 | DISCUSSION

In the present study, we describe, for the first time to our knowledge, a spontaneous congenital defect in the organogenesis of UBB in a rat that had likely not detached from the hypopharynx wall during fetal development, giving rise to an 'ultimobranchial sinus', which was accompanied by thymic tissue and an abscess. Moreover, after entering the thyroid lobe, the UB sinus wall persisted in contributing to the formation of abundant C cells and very scarce follicular cells, as shown in the diagram represented in Figure 3.

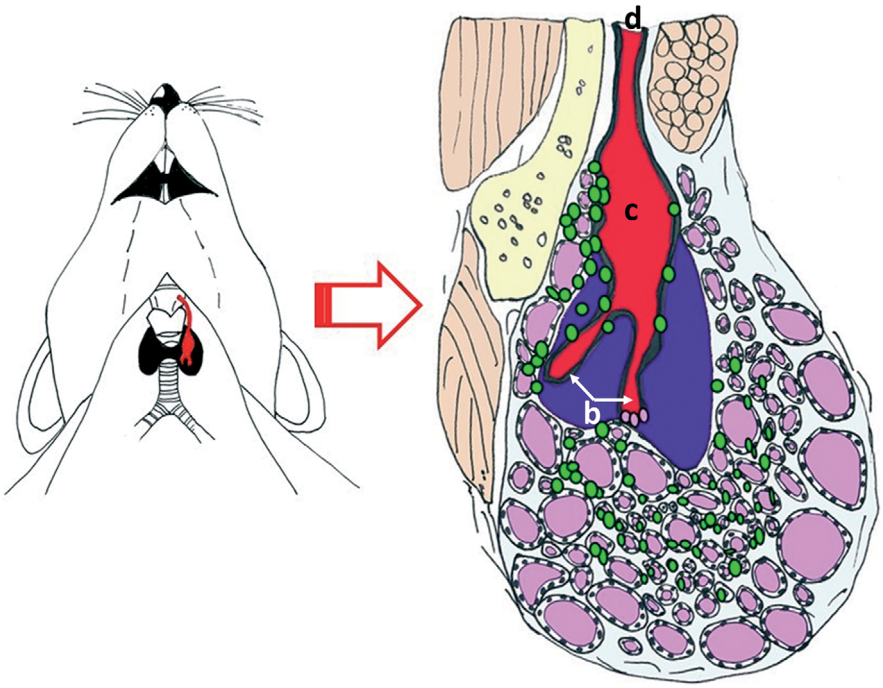

FIGURE 3 Drawing of the ultimobranchial sinus. The representation of the morphology of the ultimobranchial sinus, as well as its way from the hypopharynx to enter into the left thyroid lobe, it has been deduced from the microscopic observation along consecutive thyroid sections up to its complete disappearance. (d) Duct, (c) cyst, and (b) branches

This rat UB sinus is an exceptional case that corresponds to fourth branchial apparatus anomalies described in humans, which are very scarce (1% congenital branchial anomalies) and often manifest quite late in life as a recurrent neck abscess and suppurative thyroiditis (Contreras et al., 2006; Li et al., 2020; Pal et al., 2018). Fourth branchial apparatus anomalies include three possibilities: cyst, sinus and fistula. A cyst is an epithelial-lined structure without an external opening; a sinus is a blind tract that opens either externally through the skin or internally into the foregut (specifically, the apex of the piriform fossa). A fistula is a tract that communicates with the skin externally and the foregut internally (Contreras et al., 2006; Li et al., 2020; Pal et al., 2018). Consequently, our case must correspond to a UB sinus that, similar to what happens in humans, occurred in a female rat on the left neck side, with a proximal opening likely in the hypopharynx and accompanied by ectopic thymic tissue into the thyroid lobe.

During the complex process of thyroid organogenesis, the development of midline thyroid anlage is mainly regulated by four thyroid transcription factors (Hhex, Nkx2-1, Pax8 and Foxe1), whose mutations can give rise to several anomalies termed thyroid dysgenesis, which may be accompanied by congenital hypothyroidism (De Felice & Di Lauro 2004; Fernandez et al., 2015; Trueba et al., 2005). Nkx2-1, but not Pax8, is also involved in the development of fourth pharyngeal pouch derivatives (Mansouri et al., 1998; Kusakabe et al., 2006; Ozaki et al., 2011). Nevertheless, there are additional genes, such as Hox3 paralogs and Hes1, that are specifically committed to the development of UBBs (Manley et al., 2001; Kameda et al., 2013). Mutations in some of those genes cause various degrees of UBB defects, including persistent, ectopic, partially fused UBB, and even the absence of the organ, as reported in different heterozygous or homozygous mutant mouse models (Manley et al., 2001; Kameda, 2016; Kusakabe et al., 2006; Ozaki et al., 2011).

Specifically, in Hoxa3-mutant homozygotes, ectopic thymus and unilateral persistent UBB appeared at the anterior end of one thyroid lobe at the level of the cricoid cartilage (Manley et al., 2001), similar to what occurred in our old rat, which likely carries some unknown mutations. In Nkx2.1 heterozygous mice at E19.0, the UB anlage also failed to integrate with the thyroid and persisted as a vesicular structure located in the dorsal part of the thyroid lobe in which both calcitonin-immunoreactive C cells and colloid-containing small follicles Nkx2.1(+) existed (Kusakabe et al., 2006; Ozaki et al., 2011). This cystic structure reminds us the characteristics of our abnormal UB remnant and is likewise composed of undifferentiated p63(+) cells at the periphery. The presence of numerous cells with a basal/stem phenotype is not exclusive to these anomalies in the development of UBBs since we have also observed them at the UBF wall throughout the postnatal life of the rat (Vazquez-Roman et al., 2017). What is outstanding in the present case is the uninterrupted proliferation and migration of C cells from the wall of this abnormal UB remnant after 15 months of rat life. In fact, C cells originating from rat UBFs are normally restricted to fetal life (Wollman & Hilfer, 1977) or, exceptionally, to just a few days after birth, as also occurs with thyroglobulin-positive cells, as we have previously published (Martin-Lacave et al., 1992; Vazquez-Roman et al., 2013, 2017). In normal circumstances, both C cells and follicular cells renovate themselves exclusively by mitosis along postnatal life (Conde, Martin-Lacave et al., 1992), staying UBFs as closed structures in the centre of the thyroid lobes, without forming additional

calcitonin-positive cells. In our understanding, the sustained formation of C cells could likely be a consequence of a special microenvironment at the UB sinus wall due to the maintenance of the connection to the primitive hypopharynx.

A similar case, but in humans, may be that reported by Williams et al. (1989) about the fate of UBB in congenital abnormalities of the thyroid gland, specifically, in several cases of lingual thyroid, where both anlages remained separated as different entities. The UBB remnants persisted as nodules composed of multilobulated cystic structures at the neck, which showed numerous calcitonin-positive cells intermingled with follicular structures that stained for thyroglobulin; hence, the authors concluded that UBB may contribute to both C cells and follicular cells in humans (Williams et al., 1989).

The presence of both types of endocrine cells in UB remnants has been documented in different species, such as human SCNs (Harach, 1985), dog C-cell complexes (Kameda, 2019) and rat UBF (Conde, Moreno et al., 1992; Martin-Lacave et al., 1992; Vazquez-Roman et al., 2017), as we have stated previously. This finding is more understandable in light of the recent evidence brought forward by Kameda et al. (Kameda et al., 2007; Kameda, 2016) and Johansson et al. (Johansson et al., 2015), demonstrating that C cells do not derive from neural crests, as it was generally accepted, but they share a similar endodermal origin with follicular cells, although from different pharyngeal anlages. Furthermore, according to Nilsson and Fagman (2017), a unifying origin of thyroid follicular cells and C cells, albeit from different endoderm domains, could help to answer questions regarding the histogenesis of mixed thyroid tumours that were previously difficult to explain (Nilsson & Fagman, 2017).

5 | CONCLUSIONS

In summary, the case reported in the present study is an exceptional one, which was fortuitously discovered after observing hundreds of rats over the years, because it represents (1) the first described spontaneous abnormality in the development of the fourth branchial apparatus giving rise to a rat UB sinus still connected to the pharynx and (2) the opportunity to observe sustained C-cell differentiation from the basal/stem cells located at the periphery of the UBF wall in an adult rat. These findings are consistent with a common origin of both C cells and follicular cells from the UBB.

AUTHOR CONTRIBUTION

Investigation, methodology, visualization, writing-original draft, and writing-review and editing: Victoria Vázquez-Román. *Methodology, microphotographs, Funding, and writing-review and editing*: José M. Fernández-Santos. *Conceptualization, resources, supervision, and writing-review and editing*: Inés Martín-Lacave.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Experiments were conducted in accordance with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving the use of laboratory animals. Tissue samples were collected with approval from the Research Ethics Committee of the Virgen Macarena University Hospital (C.P.-C.I. 1921).

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