

Relationships between cell division, expression of growth factors and microcirculation in the thyroids of Tg-A2aR transgenic mice and patients with Graves' disease

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Abstract

Tissue heterogeneity and nodule formation are hallmarks of thyroid growth. This is accounted for by the clonality theory that acknowledges different individual cellular abilities to respond to trophic stimuli. In order to test the hypothesis that functional and mitotic properties of thyrocytes could be influenced by paracrine interactions with neighbour endothelial cells, studies were conducted in both mouse and human goitre models. In the first part of the study, homogenous goitres in C57 black mice were compared with heterogeneous goitres in transgenic hyperthyroid mice expressing the A2 adenosine receptor (Tg-A2aR). The second part of the study concentrated on comparing human thyroid tissue of control individuals and of patients with Graves' disease. The rate of cell division was evaluated by immunohistochemical detection of cells positive for proliferating cell nuclear antigen (PCNA). Their spatial distribution was then correlated with immunohistochemical cellular expression of growth- and vasoactive-related factors (fibroblast growth factor-2, transforming growth factor- β , endothelin-1, vascular endothelial growth factor, nitric oxide synthase III), and with microcirculation expansion. Observations were made on digitalised images of histological serial sections. The

nearest-neighbour method was used to distinguish between random or clustered distribution. PCNA-positive cells were both randomly and uniformly distributed in homogenous goitres from C57 black mice, and were clustered in tissue areas identified as papillary and hyperplastic zones in heterogeneous goitres from Tg-A2aR mice. However, they were absent in the so-called compact cellular zones featuring resting cells. Moreover, whereas papillary and hyperplastic zones were highly vascularised, compact zones were nearly free of microvessels. Spatial distribution of dividing cells was positively correlated with the expression of growth-related factors. A similar pattern was observed in the thyroids of patients with Graves' disease. In accordance with the recent demonstration of the presence of angiofollicular units in the thyroid, these data strongly support the hypothesis that functional and mitotic properties of each single thyrocyte, likely to be responsible for growth heterogeneity of hyperplastic glands, may be adjusted at tissue level by specific interactions with neighbour endothelial cells that, in turn, could alter the mitotic rate of thyrocytes through paracrine signals.

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Introduction

Changes in the microcirculation have an important role during tumour growth. Goitre development can be considered as a model of regulated growth, as opposed to cancer, which is characterised by uncontrolled growth and loss of organic functions. We previously demonstrated, both in mouse and in human thyroids, the existence of angiofollicular units with complex paracrine interactions between endothelial and epithelial cells, the spreading of

the vascular bed being correlated with thyrocyte function – that is to say, with iodination performance (Gérard *et al.* 2000, 2002).

The identification of angiofollicular units could explain the great heterogeneity in growth and functional properties of thyrocytes. The thyroid gland is indeed characterised by the presence of either active or hypofunctioning follicles in variable proportions. *In vitro* experiments showed that dividing cells organise themselves into patchy clusters in response to growth stimuli (Derwahl *et al.* 1990,

Roger *et al.* 1992), indicating that all follicular cells do not have the same growing potential (Peter *et al.* 1985, Smeds *et al.* 1987). Spatial and temporal modulations in the expression of paracrine/autocrine factors that alter either the growth or the function of epithelial cells could therefore be associated with changes in the vascular network.

In order to establish whether relationships exist between vascular growth, cell proliferation and expression of regulatory factors, we investigated the tissue expression of nitric oxide synthase III (NOSIII), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor β (TGF β) and fibroblast growth factor-2 (FGF-2) in parallel with the proliferation of both microvessels and thyrocytes as analysed by the detection of proliferating cell nuclear antigen (PCNA). We addressed this question in a two-step approach. We first used a model of spontaneous severe hyperthyroidism in mice transgenic for the A2 adenosine receptor (Tg-A2aR). In this model, thyroid-specific expression of the A2 receptor transgene that constitutively activates the cAMP cascade promotes glandular hyperplasia. These transgenic glands are characterised by a considerable tissue enlargement. Histological sections demonstrate that they are highly heterogeneous, with papillary-like structures secondary to increased proliferation of thyrocytes (Ledent *et al.* 1992, Coppée *et al.* 1996). They were compared with both control and iodine-deficient C57 black mice. We then carried out a similar study in the normal human thyroid gland and in hyperstimulated thyroids of patients with Graves' disease.

Materials and Methods

Animals and treatment

Goitre was induced in 3-month-old C57 black mice by giving a low iodine diet (LID; 0.1 $\mu\text{g/day}$; Animalabo, Brussels, Belgium) supplemented with perchlorate (ClO_4^-) for 10 days, followed by LID alone for 2 further days. This model of mildly stimulated thyrocytes was compared with spontaneous highly stimulated goitres obtained in 3-month-old Tg-A2aR mice (Ledent *et al.* 1992). In order to test the effect of the goitrogen treatment (LID/ ClO_4^-) on epithelial and endothelial proliferation, another group of Tg-A2aR mice also received the LID/ ClO_4^- diet. In another set of experiments, 1-month-old C57 black mice received LID/ ClO_4^- for 22, 46 and 96 days in order to achieve the same period of stimulation as the 3-month-old Tg-A2aR mice. Mice were maintained in accordance with the principles of laboratory animal welfare.

Preparation of tissues samples for microscopy

Animals were anaesthetised with pentothal and perfused as described previously (Gérard *et al.* 2000). One thyroid lobe

was fixed in glutaraldehyde for 1.5 h, postfixed in 1% osmium tetroxide for 1 h, and embedded in LX112 resin (Ladd Research Industries, Burlington, VT, USA). Each thyroid lobe was weighed immediately after dissection. Thin sections (0.5 μm) were cut and stained with toluidine blue and used for the morphometric analysis. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and examined with an EM301 electron microscope (Philips, Eindhoven, The Netherlands). The second lobe of these C57 black mice was immersed in Bouin Allen. For both groups of Tg-A2aR mice, the second lobe was divided into two parts: the first was fixed in Bouin Allen and the second in paraformaldehyde (4%), for 36 h each. They were then embedded in paraffin. Thick serial sections (5 μm) were cut, mounted on superfrost glasses and used for immunohistochemistry. In a separate group of Tg-A2aR untreated mice, thyroid lobes were dissected out and rapidly frozen. Cryostat sections were cut and used for CD31 immunohistochemistry in order to identify endothelial cells (see Table 1).

Five human thyroid glands were obtained postmortem and considered as normal tissue. Five thyroid glands from patients with Graves' disease were selected from the tissue bank in the pathology department. All samples were fixed in Bouin's solution and embedded in paraffin. Thick sections were cut, mounted on superfrost slides and used for immunohistochemistry.

Morphometric analysis

The morphometric measurements were performed by the point-counting method of Weibel *et al.* (1966) on one section crossing the centre of the lobe, considered to be representative of the entire gland (Denef *et al.* 1979). For each thyroid, 1000 points were then counted and the relative volume (Vv) of capillaries was measured. The number of capillary sections per field was also counted.

Immunohistochemistry

Mouse and human paraffin-embedded tissue sections were dewaxed and dehydrated. Cryostat sections were plunged into acetone. All sections were then washed with phosphate-buffered saline (pH 7.4) supplemented with 1% bovine serum albumin (PBS-BSA). The sections were incubated in a solution containing normal rabbit or goat serum (Vector Labs, Burlingame, CA, USA), at a dilution of 1/50 for 30 min at room temperature, in order to inhibit non-specific binding. The first antibody was then applied (see Table 1 for experimental conditions) at room temperature. After two washes with PBS-BSA, the antibody binding was detected using the ABC Perox kit (Vector Labs) for mouse sections, or the ABC alkaline phosphatase kit (Vector Labs) for human sections and for detection of CD31 in Tg-A2aR mice. The peroxidase activity was revealed using the diaminobenzidine tetrahydrochloride

Table 1 Experimental conditions for immunohistochemistry

	Analysed tissue	First antibody	Concentration	Incubation time
Section				
Paraffin	Mouse/human	PCNA (polyclonal affinity-purified antibody; Dako, Glostrup, Denmark)	1/75	1 h
Paraffin	Mouse/human	NOSIII (polyclonal affinity-purified antibody; Transduction Laboratories, Lexington, KT, USA)	1/75	1 h
Paraffin	Mouse	Endothelin (monoclonal antibody; Biogenesis, Poole, UK)	1/100	Overnight
Paraffin	Mouse/human	VEGF (polyclonal affinity-purified antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA)	1/100	Overnight
Paraffin	Mouse	TGFβ (polyclonal affinity-purified antibody; Santa Cruz Biotechnology)	1/50	1 h
Paraffin	Mouse	bFGF (polyclonal affinity-purified antibody; Santa Cruz Biotechnology)	1/400	1 h
Paraffin	Human	CD31 (monoclonal antibody; Dako)	1/20	48 h
Cryostat	Mouse	CD31	1/50	1 h

(Aldrich, Bornem, Belgium)–H₂O₂ reaction. The alkaline phosphatase activity was revealed using the fast red reaction (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's haematoxylin.

For NOSIII, VEGF, TGFβ and FGF-2 detection, sections were pretreated in a microwave oven in citrate buffer (0.01 M, pH 6) for one cycle of 3 min at 750 W, followed by four cycles of 3.5 min each at 350 W.

Several negative controls were performed: absence of the first antibody and peptide immunoneutralisation for ET-1, VEGF, TGFβ and FGF-2. A control staining was also performed before and after inhibition of endogenous peroxidase, when the ABC Perox kit was used.

Morphological aspects and mapping analysis of immunostainings

All immunostained sections from C57 black and Tg-A2aR mice were digitalised using a Fujix HC-2000 camera (Fuji Photo Film Co. Ltd, Tokyo, Japan). The entire section of each gland was then rebuilt by computer (Imagemagick (version 5.1.1.) <http://www.imagemagick.org>). The same procedure was applied after PCNA immunostaining for human thyroid sections, as defined in serial sections.

Three different histological zones (hyperplastic, papillary and compact cellular zones) were identified on the basis of the morphological setting of epithelial cells and mapped in Tg-A2aR mice. They were then superimposed on PCNA mapping and corresponding areas immunostained for regulatory factors.

Distribution of PCNA-labelled nuclei

PCNA-labelled nuclei were ticked off to define their spatial distribution in all tissue sections. In order to

determine whether proliferating cells were distributed in a random or clustered manner, we used the nearest-neighbour method (Clark & Evans 1954). Using digitalised maps of PCNA-labelled nuclei, the X–Y centre coordinates and the section areas were calculated using Scion Image Software (Scion Corporation, Frederick, MD, USA). The nearest-neighbour distance was then calculated. The analysis was based on the assumption that, in a random distribution, the probability that an area of specified size will contain exactly x points is given by Poisson's exponential function. This allows calculation of the mean distance to nearest neighbour expected in an infinitely large random distribution of density, ρ . The measured mean distance to nearest neighbour can be compared with the calculated distance and the significance of the departure can be tested by the normal curve. If N =the number of points for which nearest neighbour was measured, ρ =the density of the observed distribution expressed as the number of points per area unit, and r =the nearest-neighbour measured distance, then $R=rA/rE$, where rA (mean of the series of distances measured)= $\Sigma r/N$ and rE (mean of the series of distances expected)= $1/2\sqrt{\rho}$. The ratio R can be used as a measure of the degree to which the observed distribution approaches or departs from random expectation. $R=1$ if distribution is random, $R<1$ if distribution is clustered, and $R>1$ if distribution is dispersed. The formula used in the test of significance of the departure of rA from rE is: $c=(rA-rE)/\sigma_{rE}$ (standard variate of the normal curve), where $\sigma_{rE}=0.26136/\sqrt{N\rho}$ (standard error of the mean distance to nearest neighbour in a randomly distributed population of the same density as that of the observed population). Two populations were compared by comparison of the two R values by analysis of variance.

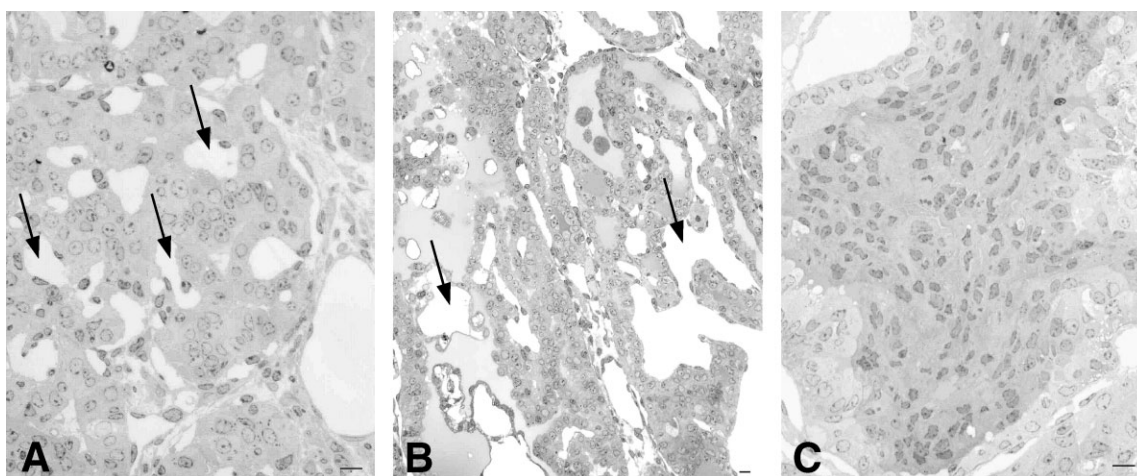


Figure 1 Morphological aspect of Tg-A2aR thyroids: semi-thin sections stained with toluidine blue (0.5 µm). Scale bars represent 10 µm. Three morphological zones coexist: (A) the hyperplastic zone with numerous enlarged microvessels (arrows), (B) the papillary zone with vascular lakes (arrows), and (C) the poorly vascularised cellular compact zone.

Results

Mouse thyroids

Morphological analysis and weight of the thyroid gland in C57 black and Tg-A2aR mice Thyroids from iodine-deficient C57 black mice were highly vascularised and contained many hypertrophic thyrocytes, with little colloid compared with controls (data not shown). The thyroids of both untreated and iodine-deficient Tg-A2aR mice were highly heterogeneous, as treatment of Tg-A2aR mice with $\text{LID}/\text{ClO}_4^-$ did not induce significant added changes in glandular morphology. Three different histological zones were considered: the hyperplastic zone with hypertrophic thyrocytes and enlarged capillaries (Fig. 1A), the papillary zone with papillary-like structures (Fig. 1B), and the compact cellular zone with clustered cells and no follicular-like structure (Fig. 1C). The electron microscopy study of cells in the compact zone showed ultrastructural characteristics of normal resting functional cells without necrotic or apoptotic features. Nevertheless, they exhibited morphological signs of thyroid differentiation, such as microvilli and little accumulation of colloid in the extracellular spaces (not shown).

The glandular weight in Tg-A2aR mice was more than 10 times greater than that in control C57 black mice. It was still significantly increased when compared with 96-day treated goitrous C57 black mice. There was, however, no observed difference in weight compared with that in $\text{LID}/\text{ClO}_4^-$ -treated Tg-A2aR mice (Fig. 2).

Number and spatial distribution of PCNA-labelled cells

In C57 black goitrous mice, the number of PCNA-positive cells was significantly increased after 12 days of treatment, but diminished thereafter to reach control values at 96 days of treatment. The number of

PCNA-positive cells in untreated Tg-A2aR mice was increased compared with that in control C57 black mice. In $\text{LID}/\text{ClO}_4^-$ -treated Tg-A2aR mice, the number of PCNA-labelled nuclei reached the same values as the 12-day treated goitrous C57 black mice (Fig. 3). We can therefore conclude that goitrogen treatment in Tg-A2aR mice increased the number of dividing cells without inducing qualitative morphological changes in the gland.

The R analysis indicated that the spatial distribution of PCNA-positive cells was random in control C57 black mice ($R=1$), whereas it tended to be dispersed in goitrous C57 black mice ($R>1$). By contrast, untreated and goitrous Tg-A2aR mice had R values always less than 1, suggesting a significant clustered spatial distribution of dividing cells (Table 2).

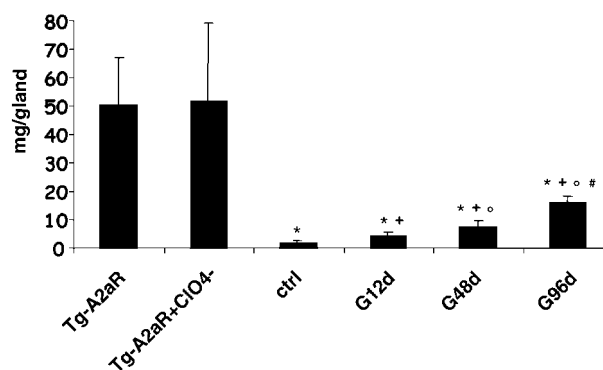


Figure 2 Weight of thyroid glands in control C57 black mice (ctrl), in untreated Tg-A2aR mice (Tg-A2aR), in goitrous Tg-A2aR mice (Tg-A2aR+ ClO_4^-), and in goitrous C57 black mice analysed after 12 days (G12d), 48 days (G48d) or 96 days (G96d) of goitrogen treatment. Results are expressed as means $\pm$ s.d. ($n=5$). $P<0.05$: °compared with G12d; $P<0.01$ compared with: *untreated Tg-A2aR mice; +control C57 black mice; #G48d.

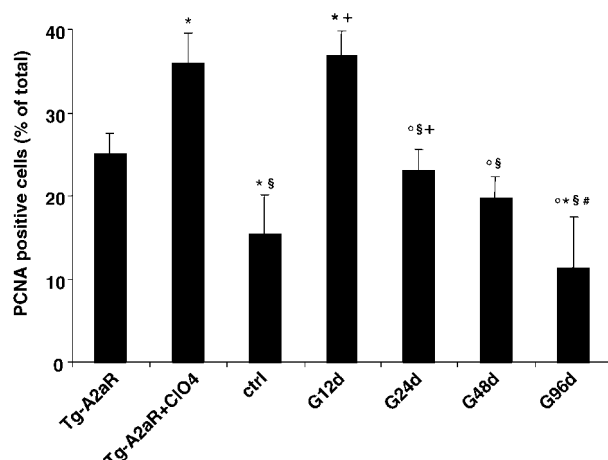


Figure 3 Percentage of PCNA-positive cells (expressed from all cells) in control C57 black mice (ctrl), in untreated Tg-A2aR mice (Tg-A2aR), in goitrous Tg-A2aR mice (Tg-A2aR+ClO₄⁻), and in goitrous C57 black mice analysed after 12 days (G12d), 24 days (G24d), 48 days (G48d) or 96 days (G96d) of goitrogen treatment. Results are expressed as means $\pm$ S.D. ($n=5$). $P<0.05$ compared with: °G12d; #G24d; $P<0.01$ compared with: *untreated Tg-A2aR mice; §goitrous Tg-A2aR mice; +control C57 black mice.

Table 2 Dispersion of PCNA-labelled cells: *R* values

	Group	<i>R</i> values
Mouse	C57bl ctrl	1.11 $\pm$ 0.0624
	C57bl+G12d	0.921 $\pm$ 0.0259*
	C57bl+G96d	1.26 $\pm$ 0.0854**+
	Tg-A2aR	0.699 $\pm$ 0.0089*+
	Tg-A2aR+LID+ClO ₄ ⁻ 12d	0.642 $\pm$ 0.1109*+
Human	Normal	1.103 $\pm$ 0.0766**+
	Graves' disease	0.727 $\pm$ 0.0717*+°

Values are means $\pm$ S.D. bl, black; ctrl, control; G, goitrogen diet; d, days; LID, low-iodine diet. $P<0.001$ compared with: *control C57 black mice (ctrl); °G12d; #control Tg-A2aR mice; °normal human thyroid $R=rA/rE$ where rA (mean of the series of distances measured) = $\Sigma r/N$ and rE (mean of the series of distances expected) = $1/2\sqrt{p}$.

When PCNA and morphological maps were superimposed, goitrous C57 black mice showed a homogenous distribution of PCNA-labelled cells over the entire gland, except in strands of connective tissue (Fig. 4IA). By contrast, wide cellular zones lacking PCNA-positive cells were observed in Tg-A2aR mice which, when superimposed on morphology maps, corresponded to the compact cellular zones. However, PCNA-positive cells were numerous and clustered in the hyperplastic and papillary zones (Fig. 4IB).

Immunohistochemical analysis of growth and vasoactive factors In goitrous C57 black mice, the different factors studied were uniformly distributed throughout the entire gland, irrespective of the duration of

treatment. NOSIII expression increased in thyrocytes up to 12 days of treatment and decreased afterwards. ET-1, VEGF and FGF-2 immunostainings increased after 12 days of treatment and remained unchanged up to 96 days. The TGF β signal increased slightly after 12 days of treatment, but continued increasing with treatment duration (data not shown).

In control and goitrous Tg-A2aR mice, all factors analysed were strongly expressed. However, their expression was patchy across the gland. Superimposition of immunohistochemical and morphological maps demonstrated that there was no immunostaining in areas defined as compact cellular zones, except in peripheral cells in contact with microvessels. By contrast, hyperplastic and papillary zones were strongly labelled (Fig. 4IC–F).

Spatial organisation of the microcirculation The relative volume of capillaries of Tg-A2aR mice was significantly greater than that of control C57 black mice, but smaller than that of iodine-deficient C57 black mice (Fig. 5A). Nevertheless, the number of capillaries per microscopic field in Tg-A2aR mice was lower than that in iodine-deficient C57 black mice (Fig. 5B). No difference was observed between Tg-A2aR-treated and -untreated mice. Electron microscopic analysis of the different zones from the Tg-A2aR mice showed that compact cellular zones contained only scarce endothelial cells, whereas microvessels were mostly located in hyperplastic and papillary zones. This was confirmed by the CD31 labelling of endothelial cells (Fig. 4IG).

Human thyroids

Spatial repartition of PCNA-labelled cells In normal human thyroids, the *R* value for the spatial distribution of PCNA-positive cells indicated that dividing cells tended to be either uniformly or randomly distributed (Table 2). The *R* value was not statistically different from that of control C57 black mice, but the difference compared with that of Tg-A2aR mice was statistically significant.

Thyroids from patients with Graves' disease had a different pattern. PCNA-positive cells were clustered and the *R* value was therefore statistically different from that of normal human thyroids. It was similar to that of Tg-A2aR mice (Table 2). As in mice, mapping of the PCNA-positive cells made it possible to demarcate three different cellular zones (Fig. 4 IIA): inflammatory compartments in which these cells were numerous (1), papillary structures in which they were scattered (2), and compact cellular zones in which they were absent (3).

Immunohistochemical analysis of growth and vasoactive factors In the normal thyroids, NOSIII was uniformly distributed. VEGF was detected in only a few follicles (data not shown).

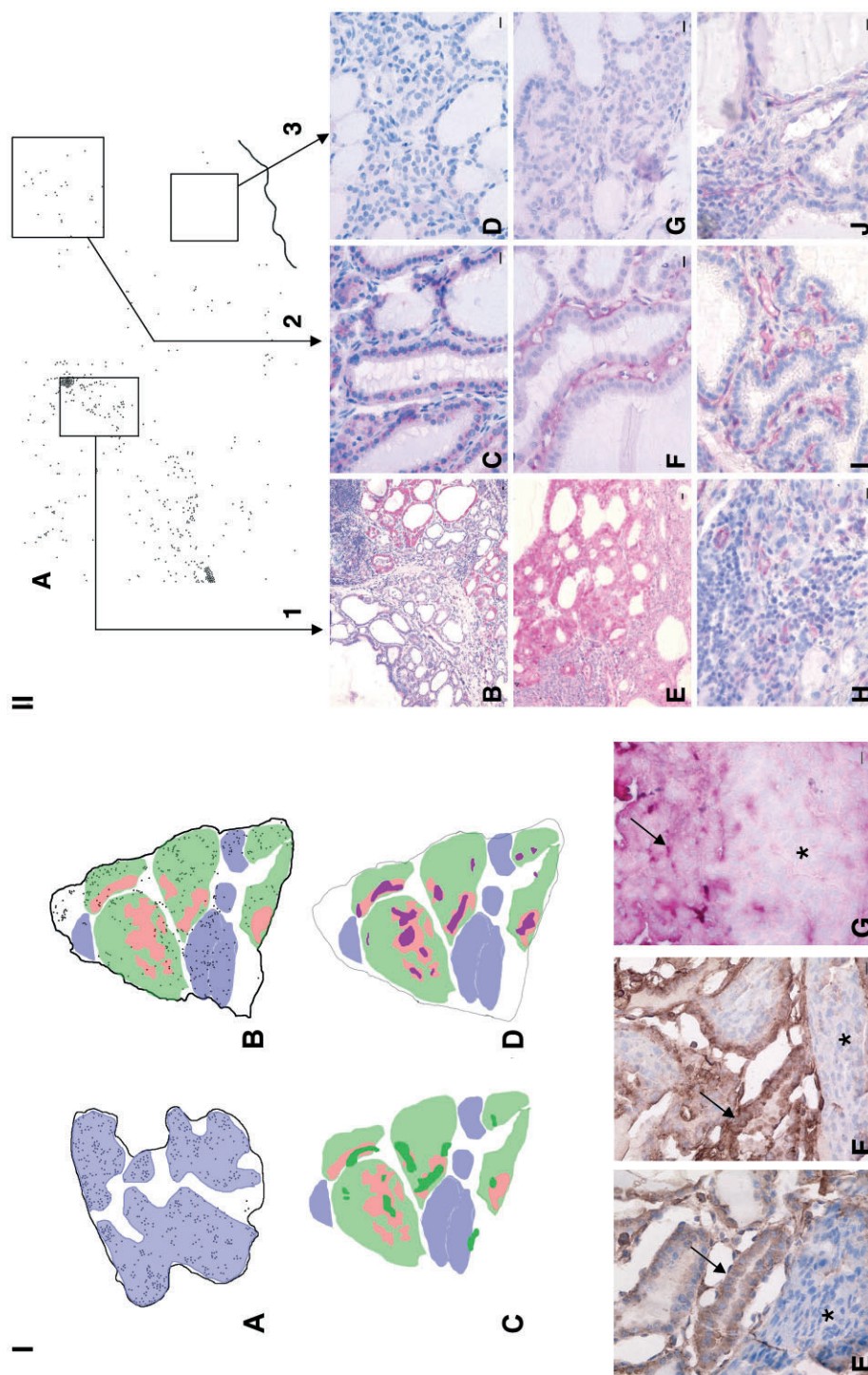


Figure 4 (I) Example of spatial distribution analysis of cells in sections from (A) goitrous C57 black mice and (B–D) Tg-A2aR mice, and (E–G) immunohistochemical detection of expression of growth and vasoactive factors in Tg-A2aR mice. Scale bars represent 10 µm. (A) Each dot corresponds to a PCNA-positive nucleus. The blue zones represent goitrous thyrocytes. White zones correspond to strands of connective tissue. (B) Each dot corresponds to a PCNA-positive nucleus. Blue = hyperplastic zones; green = papillary zones; red = cellular compact zones. (C) The dark green colour corresponds to ET-1-negative cellular areas superimposed on (B). (D) The purple colour corresponds to FGF-2-negative cellular compact areas. In contrast, they were all present in most hyperplastic and papillary zones analysed growth-related factors was immunodetected in cellular compact areas. (E) Detection of VEGF. (F) Detection of FGF-2. (G) Detection of FGF-2. None of the depicted in blue and green. (H) Endothelial cells are detected in hyperplastic and papillary zones but not in compact cellular. *Cellular compact zones. Arrows indicate hyperplastic/papillary zones. (II) Thyroid tissue from a patient with Graves' disease. Scale bars represent 10 µm. (A) Mapping of PCNA immunostaining. Negative areas correspond to cellular compact zones (3), whereas positive areas correspond to either hyperplastic zones (2) or inflammatory zones (1). (B–D) Immunodetection of NOSIII in (B) inflammatory, (C) hyperplastic and (D) cellular compact zones. (E–G) Immunodetection of VEGF in (E) inflammatory, (F) hyperplastic and (G) cellular compact zones. (H–J) Immunodetection of CD31 in (H) inflammatory, (I) hyperplastic and (J) cellular compact zones.

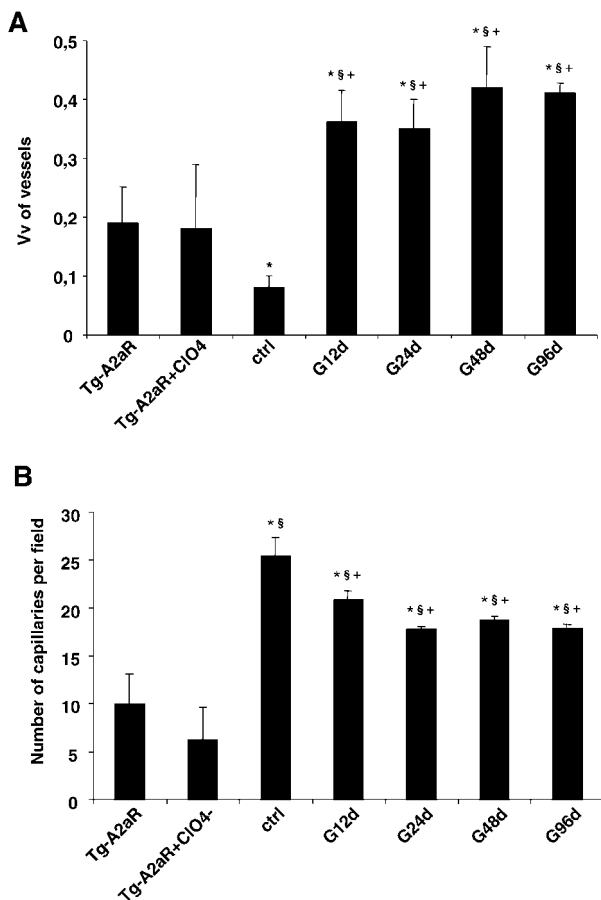


Figure 5 (A) Relative volume (Vv) of vessels and (B) number of capillaries per field, in control C57 black mice (ctrl), in untreated Tg-A2aR mice (Tg-A2aR), in goitrous Tg-A2aR mice (Tg-A2aR+ClO₄⁻), and in goitrous C57 black mice analysed after 12 days (G12d), 24 days (G24d), 48 days (G48d) or 96 days (G96d) of goitrogen treatment. Results are expressed as means $\pm$ s.d. ($n=5$). $P<0.05$: § compared with goitrous Tg-A2aR mice; $P<0.01$ compared with: *untreated Tg-A2aR mice, +control C57 black mice.

In the thyroid glands of patients with Graves' disease, the distribution of these factors was not uniform. Inflammatory zones showed many follicles intensively stained for NOSIII and VEGF (Fig. 4 IIB and E). In papillary structures, NOSIII was present in follicular cells (Fig. 4 IIC). VEGF labelling was strong around capillaries surrounding follicles (Fig. 4 IIF). In the compact cellular zones, there was immunolabelling neither for NOSIII nor for VEGF (Fig. 4 IID, G).

Spatial organisation of the microcirculation Vessels, detected by CD31 antibody positivity, were more abundant in papillary structures (Fig. 4 III) than in compact cellular zones (Fig. 4 IJJ). Inflammatory compartments also displayed a well developed vascularisation (Fig. 4 IIH).

Discussion

Vascular supply is an obvious requirement for cellular inflow of nutrients and removal of waste or secretory products. In endocrine organs, the microangioarchitecture is also adapted to functional specificity and is suspected to have a role in the homeostatic control of hormone release. This is particularly true in the thyroid gland, where the microcirculation is extensively reformed during goitre formation and involution. Thus, in iodine-deficient goitres, the expansion of the vascular tree acts to prevent iodine shortage by enhancing clearance of iodine from thyrocytes (Wollman *et al.* 1978, Denef *et al.* 1989, Michalkiewicz *et al.* 1989). Conversely, the vascular supply in iodine-induced goitre involution is promptly restricted in order to limit access of iodide to cells and the subsequent formation of toxic free radicals (Mahmoud *et al.* 1986, Toussaint-Demyelle *et al.* 1990, Many *et al.* 1992). The link between epithelial function and microcirculation has recently been clarified by the identification of angiofollicular units both in mouse and in human thyroids (Gérard *et al.* 2000, 2002). Hence, each follicle is surrounded by its own capillary network that reacts in response to nearby epithelial activity (Imada *et al.* 1986). In cold hypofunctioning follicles, the development of the microcirculation is usually marginal. By contrast, in hot active follicles, microvessels are enlarged and numerous. In the human, it is even possible to establish the existence of a positive correlation between the expression of differentiation proteins (pendrin, Na⁺/I⁻ symporter, thyroid oxidases) and the development of the microcirculation (Gérard *et al.* 2002). The present study demonstrates that positive correlations also exist between epithelial cell proliferation, expression of growth and vasoactive factors and the extent of vascularisation, indicating that the thyroid microcirculation is intimately involved in the control of the thyroid economy.

The study was first carried out using two animal experimental models of goitres, and was then extended to the human. In the first model, in C57 black mice, increased plasma concentrations of thyroid-stimulating hormone (TSH) in response to iodine deficiency represented a somewhat mild and physiological stimulus. As a consequence, epithelial and endothelial compartments enlarged homogeneously and synchronously, as commonly described in the early steps of endocrine hyperplasia (Derwahl & Studer 2002). Iodine deficiency induced an increase in the number of dividing cells for the first 12 days and then a gradual return to control values at 3 months, indicating that autocrine and paracrine regulatory mechanisms were activated within the gland to limit goitre size (Bidey *et al.* 1999). In the mouse Tg-A2aR transgenic model, the level of stimulation was markedly stronger. Indeed, as the adenosine A2 receptor transgene constitutively activates the cAMP cascade, a heterogeneous glandular hyperplasia evolves, associated with severe

hyperthyroidism and causing premature death of the animal. The structural heterogeneity appears as early as 3 months of age, and growing cells are still present in the adult (Ledent *et al.* 1992, Coppée *et al.* 1996). In our study, growing tissue compartments with numerous PCNA-positive cells were also the most vascularised, in contrast to compact cellular areas, which were poorly irrigated and characterised by the presence of resting, non-dividing cells. Likewise, cell proliferation was correlated with the expression of growth-related factors. In C57 black mice with $\text{LiD}/\text{ClO}_4^-$ -induced goitre, growth and vasoactive factors were uniformly detected in the whole gland, as were PCNA-positive cells and microvessels. In Tg-A2aR mice, compact cellular zones were negative for all growth and vasoactive factors analysed, while proliferative and vascularised areas were positively immunostained for these factors. Similar results were obtained in the human. In control tissue, PCNA-positive cells were randomly distributed, in addition to the microcirculation and the expression of growth-related factors. In the thyroids of patients with Graves' disease, the distribution of PCNA-positive cells was patchy. They were particularly concentrated in highly vascularised inflammatory areas, where the number of epithelial and endothelial cells immunostained for NOSIII and VEGF was also increased. These results strongly favour the existence of interactive paracrine regulatory loops between endothelial and epithelial thyroid cells. Hence, thyrocytes, uniformly distributed in C57 black mice or clustered in hyperplastic areas in Tg-A2aR mice, may express vasoactive and angiogenic factors that induce vasodilatation and proliferation of endothelial cells. In turn, endothelial cells could stimulate the proliferation of thyrocytes via paracrine factors and modulate their differentiating properties, as previously suggested (Gérard *et al.* 2002).

Nodule formation and structural heterogeneity resulting from differences in growth and functional properties of each individual follicle – or even each individual cell – are actually the rule during endocrine hyperplasia, especially in the thyroid. As suggested recently, some cells scattered throughout the tissue may retain an intrinsic tendency to proliferate autonomously, independently of trophic stimuli (Kopp *et al.* 1994, Studer & Derwahl 1995). At some point, goitre growth could result from the uncoordinated proliferation of cell foci spread throughout the gland (Derwahl & Studer 2002). However, on the basis of the Folkman's hypothesis, one can suppose that a minimal vascular supply, for instance to provide at least oxygen and nutrients, might be required to maintain the ability of these cells to proliferate (Folkman 1972, Folkman & Shing 1992). In our study, we are obviously confronted by the 'chicken and egg' conundrum, because our results do not permit discrimination between the cause and the consequence, and do not explain, for example, the absence of growth factors in poorly vascularised areas of non-dividing cells. We can only refer to previous experiments that

demonstrated that the earliest tissue modification occurring during goitre formation involves the proliferation of endothelial cells that takes place just before the proliferation of epithelial cells (Denef *et al.* 1981, Many *et al.* 1984). It is therefore conceivable that endothelial cells are the first to be stimulated in the presence of trophic factors. They are known to carry TSH receptors on their surface (Milgrom *et al.* 1997) and thus stimulation of TSH receptors could promote endothelial cell proliferation, leading to enlarged capillary networks and increased production of growth factors that could then act on thyrocytes. In our study, endothelial cells were positive for FGF-2, ET-1, NOSIII, VEGF and TGF β . All these factors have already been shown to regulate the growth of thyrocytes (Eggo & Sheppard 1994, Bidey *et al.* 1999). Alternatively, thyrocytes themselves could be the main cellular targets and could release, in response to increased TSH concentrations, vasoactive and angiogenic factors that in turn will stimulate endothelial cells (Emoto *et al.* 1991, Colin *et al.* 1992, 1995, 1997, Bechtner *et al.* 1993, Pekari *et al.* 1995, Sato *et al.* 1995, Patel *et al.* 1996, Viglietto *et al.* 1997, Toda *et al.* 1999). To date, the sole objective fact that can be firmly established is the spatial correlation between thyrocyte proliferation, expression of growth factors and development of the vascular bed. The same conclusion can be drawn for Graves' disease. In this case, though, the distinction between avascularised resting zones and highly active ones is more difficult, because of the presence of inflammatory cells that could also release factors competent to activate endothelial cell proliferation (Toda *et al.* 1999, Carmeliet & Jain 2000).

In conclusion, we show in the present study that the spatial distribution of the microcirculation in benign thyroid tumours is correlated with the ability of thyrocytes to proliferate and to express growth-related factors. This reinforces the recently proposed hypothesis of the existence of angiofollicular units in the thyroid that could represent, via the setting of interactive loops between endothelial and epithelial cells, the tissue expression of the clonality theory that accounts for the heterogeneous growth of hyperplastic endocrine tissues.

References

- Bechtner G, Rieder B, Linsenmaier U, Kellerman J, Greil W & Gärtner R 1993 Isolation and partial molecular characterisation of basic fibroblast growth factor from isolated porcine thyroid follicles and entire porcine thyroid glands. *Acta Endocrinologica* **129** 458–466.
- Bidey SP, Hill DJ & Eggo MC 1999 Growth factors and goitrogenesis. *Journal of Endocrinology* **160** 321–332.
- Carmeliet P & Jain RK 2000 Angiogenesis in cancer and other diseases. *Nature* **147** 249–257.
- Clark PJ & Evans FC 1954 Distance to nearest neighbor as a measure of spatial relationships in populations. *Ecology* **35** 445–453.
- Colin IM, Berbinschi A, Denef J-F & Ketelslegers J-M 1992 Detection and identification of endothelin-1 immunoreactivity in rat and porcine thyroid follicular cells. *Endocrinology* **130** 544–546.
- Colin IM, Nava E, Toussaint-Demyle D, Maiter DM, vandenHove M-F, Lüscher TF, Ketelslegers J-M, Denef J-F & Jameson JL 1995

- Expression of nitric oxide synthase isoforms in the thyroid gland: evidence for a role of nitric oxide in vascular control during goiter formation. *Endocrinology* **136** 5283–5290.
- Colin IM, Kopp P, Zbären J, Grizzle W & Jameson JL 1997 Expression of nitric oxide synthase III in human thyroid follicular cells: evidence for increased expression in hyperthyroidism. *European Journal of Endocrinology* **136** 649–655.
- Coppée F, Gérard A-C, Denef J-F, Ledent C, Vassart G, Dumont JE & Parmentier M 1996 Early occurrence of metastatic differentiated thyroid carcinomas in transgenic mice expressing the A2a adenosine receptor gene and the human papillomavirus type 16E7 oncogene. *Oncogene* **13** 1471–1482.
- Denef J-F, Cordier AC, Mesquita M & Haumont S 1979 The influence of fixation procedure, embedding medium and section thickness on morphometric data in thyroid gland. *Histochemistry* **63** 163–171.
- Denef J-F, Haumont S, Cornette C & Beckers C 1981 Correlated functional and morphometric study of thyroid hyperplasia induced by iodine deficiency. *Endocrinology* **108** 2352–2358.
- Denef J-F, Ovaert C & Many M-C 1989 Experimental goitrogenesis. *Annals of Endocrinology* **50** 1–15.
- Derwahl M & Studer H 2002 Hyperplasia versus adenoma in endocrine tissues: are they different? *Trends in Endocrinology and Metabolism* **13** 23–28.
- Derwahl M, Studer H, Huber G, Gerber H & Peter HJ 1990 Intercellular propagation of individually programmed growth burst in FRTL-5 cells. Implications for interpreting growth factor actions. *Endocrinology* **127** 2104–2110.
- Eggo MC & Sheppard MC 1994 Autocrine growth factors produced in the thyroid. *Molecular Cell Endocrinology* **100** 97–102.
- Emoto N, Iozaki O, Arai M, Murakami H, Shizume K, Baird A, Tsushima T & Demura H 1991 Identification and characterization of basic fibroblast growth factor in porcine thyroids. *Endocrinology* **128** 58–64.
- Folkman J 1972 Anti-angiogenesis: new concept for therapy of solid tumor. *Annals of Surgery* **175** 409–416.
- Folkman J & Shing Y 1992 Angiogenesis. *Journal of Biological Chemistry* **267** 10931–10934.
- Gérard A-C, Xhenseval V, Colin IM, Many M-C & Denef J-F 2000 Evidence for co-ordinated changes between vascular endothelial growth factor and nitric oxide synthase III immunoreactivity, the functional status of the thyroid follicles, and the microvascular bed during chronic stimulation by low iodine and propyl-thiouracil in old mice. *European Journal of Endocrinology* **142** 651–660.
- Gérard A-C, Many M-C, Daumerie C, Costagliola S, Miot F, DeVijlder JJM, Colin IM & Denef J-F 2002 Structural changes in the angiofollicular units between active and hypofunctioning follicles align with differences in the epithelial expression of newly discovered proteins involved in iodine transport and organification. *Journal of Clinical and Endocrinological Metabolism* **87** 1291–1299.
- Imada M, Kurosuni M & Fugita H 1986 Three-dimensional aspects of blood vessels in thyroids from normal, low iodine diet-treated, TSH-treated, and PTU-treated rats. *Cell and Tissue Research* **245** 291–296.
- Kopp P, Aeschmann S, Asmis L, Tobler A, Fey MF & Studer H 1994 Polyclonal and clonal nodules may coexist within multinodular goiters. *Journal of Clinical Endocrinology and Metabolism* **79** 134–139.
- Ledent C, Dumont JE, Vassart G & Parmentier M 1992 Thyroid expression of an A2 adenosine receptor transgene induces thyroid hyperplasia and hyperthyroidism. *EMBO Journal* **11** 537–542.
- Mahmoud I, Colin IM, Many M-C & Denef J-F 1986 Direct toxic effect of iodide in excess on iodine-deficient thyroid glands: epithelial necrosis and inflammation associated with lipofuscin accumulation. *Experimental Molecular Pathology* **44** 259–271.
- Many M-C, Denef J-F & Haumont S 1984 Precocity of endothelial proliferation during a course of rapid goitrogenesis. *Acta Endocrinologica* **105** 487–491.
- Many M-C, Mestdagh C, vandenHove M-F & Denef J-F 1992 In vitro study of acute toxic effects of high iodide doses in human thyroid follicles. *Endocrinology* **131** 621–630.
- Michalkiewicz ML, Huffman J, Connors JM & Hedge GA 1989 Alterations in thyroid flow induced by varying levels of iodine intake in the rat. *Endocrinology* **125** 54–60.
- Milgrom E, de Roux N, Ghinea N, Beau I, Loosfelt H, Vannier B, Savourek JF & Misrahi M 1997 Gonadotrophin and thyrotrophin receptors. *Hormone Research* **48** (Suppl 4) 33–37.
- Patel VA, Hill DJ, Eggo MC, Sheppard MC, Becks GP & Logan A 1996 Changes in the immunohistochemical localization of fibroblast growth factor-2, transforming growth factor- β 1 and thrombospondin-1 are associated with early angiogenic events in the hyperplastic rat thyroid. *Journal of Endocrinology* **148** 485–499.
- Pekari AE, Berg L, Wang J, Lee P, Dubinett SM & Hershan JM 1995 TNF- α , TSH and aging regulate TGF- β synthesis and secretion in FRTL-5 rat thyroid cells. *American Journal of Physiology* **268** R808–R815.
- Peter HJ, Gerber H, Studer H & Smeds S 1985 Pathogenesis of heterogeneity in human multinodular goiter. A study on growth and function of thyroid tissue transplanted onto nude mice. *Journal of Clinical Investigation* **76** 1992–2002.
- Roger PP, Baptist M & Dumont JE 1992 A mechanism generating heterogeneity in thyroid epithelial cells: suppression of the thyrotropin/cAMP-dependent mitogenic pathway after cell division induced by cAMP-independent factors. *Journal of Cell Biology* **117** 383–393.
- Sato K, Yamazaki K, Shizume K, Kanaji Y, Obara T, Ohsumi K, Demura H, Yamaguchi S & Shibuya M 1995 Stimulation by thyroid-stimulating hormone and Graves' immunoglobulins of vascular endothelial growth factor mRNA expression in human thyroid follicles in vitro and flt mRNA expression in the rat thyroid in vivo. *Journal of Clinical Investigation* **96** 1295–1302.
- Smeds S, Peter HJ, Jortso E, Gerber H & Studer H 1987 Naturally occurring clones of cells with high intrinsic proliferation potential within the follicular epithelium of mouse thyroids. *Cancer Research* **47** 1646–1651.
- Studer H & Derwahl M 1995 Mechanisms of nonneoplastic endocrine hyperplasia – a changing concept: a review focused on the thyroid gland. *Endocrine Reviews* **16** 411–426.
- Toda S, Nishimura T, Yamada S, Koike N, Yonemitsu N, Watanabe K, Matsumura S, Gärtner R & Sugihara H 1999 Immunohistochemical expression of growth factors in subacute thyroiditis and their effects on thyroid folliculogenesis and angiogenesis in collagen gel matrix culture. *Journal of Pathology* **188** 415–422.
- Toussaint-Demyle D, Many M-C, Theisen H, Kraal G & Denef J-F 1990 Effects of iodide on class II-MHC antigen expression in iodine deficient hyperplastic thyroid glands. *Autoimmunity* **7** 51–62.
- Viglietto G, Romano A, Manzo G, Chiappetta G, Paoletti I, Califano D, Galati MG, Mauriello V, Bruni P, Lago CT, Fusco A & Persico MG 1997 Upregulation of the angiogenic factors PIGF, VEGF and their receptors (Flt-1, Flk-1/KDR) by TSH in cultured thyrocytes and in the thyroid gland of thiouracil-fed rats suggests a TSH-dependent paracrine mechanism for goiter hypervascularization. *Oncogene* **27** 2687–2698.
- Weibel ER, Kistler GS & Scherle WF 1966 Practical stereological methods for morphometric cytology. *Journal of Cell Biology* **30** 23–28.
- Wollman SH, Herve J-P, Zeligs JD & Ericson LE 1978 Blood capillary enlargement during the development of thyroid hyperplasia in the rat. *Endocrinology* **103** 2306–2314.

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