

Regulation of Thyroid Cell Proliferation by TSH and Other Factors: A Critical Evaluation of *in Vitro* Models

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TSH via cAMP, and various growth factors, in cooperation with insulin or IGF-I stimulate cell cycle progression and proliferation in various thyrocyte culture systems, including rat thyroid cell lines (FRTL-5, WRT, PC Cl3) and primary cultures of rat, dog, sheep and human thyroid. The available data on cell signaling cascades, cell cycle kinetics, and cell cycle-regulatory proteins are thoroughly and critically reviewed in these experimental systems. In most FRTL-5 cells, TSH (cAMP) merely acts as a priming/competence factor amplifying PI3K and MAPK pathway activation and DNA synthesis elicited by insulin/IGF-I. In WRT cells, TSH and insulin/IGF-I can independently activate Ras and PI3K pathways and DNA synthesis. In dog thyroid primary cultures, TSH (cAMP) does not activate Ras and PI3K, and

cAMP must be continuously elevated by TSH to directly control the progression through G₁ phase. This effect is exerted, at least in part, via the cAMP-dependent activation of the required cyclin D3, itself synthesized in response to insulin/IGF-I. This and other discrepancies show that the mechanistic logics of cell cycle stimulation by cAMP profoundly diverge in these different *in vitro* models of the same cell. Therefore, although these different thyrocyte systems constitute interesting models of the wide diversity of possible mechanisms of cAMP-dependent proliferation in various cell types, extrapolation of *in vitro* mechanistic data to TSH-dependent goitrogenesis in man can only be accepted in the cases where independent validation is provided. (*Endocrine Reviews* 22: 631–656, 2001)

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Abbreviations: bFGF, Basic fibroblast growth factor; cdk, cyclin-dependent kinase; CREB, cAMP response element binding protein; EGF, epidermal growth factor; 6H, six hormone mixture containing TSH, high concentrations of insulin that activate IGF-I receptors (insulin/IGF-I), transferrin, somatostatin, gly-his-lys acetate, and hydrocortisone; HGF, hepatocyte growth factor; IRS, insulin receptor substrate; MEK, MAPK kinase; PKB, protein kinase B; TPA, 12-O-tetradecanoylphosphorol 13-acetate.

I. Introduction

AN ABNORMAL THYROID CELL proliferation has a very important role in human disease. Its dysregulation causes goiter, thyroid adenomas, and carcinomas or primary hypothyroidism resulting from hypoplasia. To understand the pathogenic processes, endocrinologists are therefore trying to elucidate the mechanisms of the control of normal and pathological human thyroid cells *in vivo*. With regard to proliferation and differentiation, this is difficult or impossible for ethical and experimental reasons. We thus resort to the use of *in vitro* models or *in vivo* transgenic mice models. The aim of this review is to critically summarize our knowledge and the relevance *in vivo* of the various available *in vitro* models of thyroid cells.

Our analysis is mainly focused on the regulation of cell proliferation by TSH acting through cAMP and IGF-I, which are the main controls considered *in vivo*. Understanding the respective roles of TSH and IGF-I in their synergistic regulation of cell proliferation is indeed considered a question of major interest in the field of thyroid regulation. A central question addressed to the different models is whether TSH and IGF-I, through distinct signaling cascades, exert similar or complementary functions required for cell proliferation. In the latter hypothesis, a second question is whether one of these factors merely amplifies the effect of the other, which is thus qualified as the only genuine mitogen. Although specifically considered here in various thyroid cell systems, the complex problem of the identification of the integration of cAMP and IGF-I signaling cascades has a broader relevance to other endocrine cells targeted by both IGF-I and pituitary trophic hormones, including ACTH, LH, and FSH.

Unexpectedly, the mechanisms demonstrated in the various *in vitro* thyroid models are different.

II. The *in Vitro* Models

The *in vitro* models used for the study of thyroid cell proliferation and differentiation belong to two main classes, the cell lines and the primary cultures. Tissue slices from dog, calf, sheep, and human thyroid have been extensively used for functional studies. As they have no time to reprogram themselves, they are very good replicas of the tissue *in vivo*. However, they survive 24 h at most, and the cells do not enter DNA synthesis.

Cell lines are derived from normal and cancer tissues. Human cancer cell lines can obviously not be used to study the normal process of thyrocyte growth and division. Differentiated sheep thyroid cell lines (OVNIS) have been little used for proliferation investigations (1). The most studied models are immortal rat thyroid cell lines [FRTL-5 (2), WRT (3), and PC Cl3 cells (4)], which present a very appealing set of properties that resemble those ascribed to normal differentiated thyrocytes (5–8), such as TSH dependence for growth and differentiated functions, iodide uptake, and thyroglobulin and thyroperoxidase gene transcriptions. Because of their simplicity and accessibility, because they allow permanent transfections and genetic experiments, and also because of the increasing difficulty in obtaining animals for experimentation, these rat thyroid cell lines are now the preferred and often only used systems in the majority of *in vitro* studies of thyroid biology (>800 entries in MEDLINE concern FRTL-5 cells). Due in part to the availability of these untransformed rat cell lines, the proliferation of thyroid epithelium has been studied *in vitro* to a greater extent than that of any other endocrine gland.

The immortality of the cell lines is sufficient evidence that they have lost some of the basic mechanisms of cell cycle control, PC Cl3 cells to a lesser extent than the apparently similar FRTL-5 cells. The transformation of PC Cl3 cells requires the combination of two retroviral oncogenes, while only one is sufficient for the full transformation of FRTL-5 cells, which suggests their precancerous nature (4). In fact, in nude mice FRTL-5 cells present a thymidine labeling index 4-fold higher than endogenous thyrocytes (9) and develop TSH-dependent tumors (10). It is also important to keep in mind that most cell lines result from selections, fortuitous or not, and that they are often maintained only when they present desired characteristics, *i.e.*, when they correspond to *a priori* expectations. Depending on the culture medium (presence or not of serum), primary cultures of rat thyrocytes develop cell lines that stably display the phenotypes of FRTL cells (unpolarized but expressing thyroid differentiation) or FRT cells (morphologically polarized but lacking the expression of differentiation proteins) (2). FRTL-5 have been “adapted” to the presence of 5% serum and survive in the absence of TSH (2). However, they derive from FRTL cells for which TSH and insulin not only support proliferation, but also are necessary for survival (11). Some vital functions might thus have come to depend on these hormones, which were present during the establishment of the cell strain.

Recently, a survival function of TSH has been unmasked in FRTL-5 cells (12). By contrast, the WRT cell line displays the interesting characteristics of slow proliferation in response to insulin alone, and survival in the absence of hormones while retaining the capacity to respond to TSH as a full mitogen (3). Nevertheless, the inventors of this cell line were correct to point out that the WRT clone was the only one (of 27 clones obtained by limited dilution plating) presenting these characteristics (3). Two other clones with characteristics more similar to FRTL-5 cells were discarded (3).

Although often left unsaid, it is widely acknowledged that cell lines may evolve and deviate from their parental counterparts. Perhaps due in part to their very broad dissemination, this is especially well documented in the case of FRTL-5 cells. This cell line was indeed reported to suffer from increasing instability (13, 14) and clonal variability (15–17), which explains the opposite results sometimes obtained in different laboratories. A monthly monitoring of thymidine incorporation characteristics has been recommended (5, 18). Several studies have now compared the characteristics of “young” and “aged” FRTL-5 cells (18–23). Repeatedly passaged FRTL-5 cells often display a larger size (16), lose the TSH responsiveness of growth, which then is only enhanced by insulin (24), lose thyroglobulin production (25) or the okadaic acid-induced apoptosis (24), or acquire the TSH-dependent capacity to grow in semisolid medium (26). In contrast to their initial characterization, FRTL-5 cells supplied by the ATCC (Manassas, VA) have been recently reported to be tetraploid (27). Aged WRT cells also lose the TSH responsiveness of growth (our unpublished observations). Until now, no such changes have been reported for the PC Cl3 cells.

Cells in short-term primary cultures are not selected by their propagation *in vitro* and are thus expected to be less remote from physiology. Although their environment and their organization may differ markedly from the *in situ* situation, they are probably not modified genetically or epigenetically. The contamination of primary cultures by nonepithelial cells should be evaluated, especially after long-term culture in the presence of serum. In the dog thyroid primary culture model, this problem has been solved by the initial enrichment of thyrocytes by the seeding of thyroid follicles rather than isolated cells, and plating and culture maintenance in serum-free conditions that do not support the attachment and proliferation of fibroblasts. The existence of rapidly proliferating phenotypic variants of thyrocytes reported in cultures of human thyroid might be more problematic (28).

Primary cultures can be distinguished by the species studied and by their architecture. Four species have been mainly used: man (29–32), dog (33, 34), pig (35–39), and sheep (40–42). Pig and calf (43) thyroid cultures, while useful for the investigation of function and gene expression, respond poorly to TSH as a growth stimulus, for still unknown reasons (44). In sheep thyroid primary cultures, TSH potentiates the increase of cell numbers induced by insulin and IGF-I (45). However, this effect might require very precise conditions, because it was not found in earlier reports by this group using the same system (41, 46), and it has not been further investigated. Although they were very useful for the

demonstration of growth and differentiation effects of growth factors (47), pig, sheep, and calf thyroid cultures have generally been little used for the study of proliferative effects of TSH, and thus are no longer considered in the present review. Human cells are the obvious choice, but it is very difficult to obtain normal tissue in sufficient amounts; these cells, obtained mostly from rather elderly patients undergoing surgery for single nodules, grow poorly. Mostly dog thyroid cells have been studied by our group (34). As discussed in this review article, their properties are similar to those of human cells in several respects, but they grow much better and allow biochemical studies of the mechanisms. However, this material is also difficult to obtain in most centers.

Cells in primary cultures can be studied as monolayers (29, 34, 41), as reorganized follicles in suspension (36, 48, 49) or in collagen gels (32, 50), or as monolayers on filters set between two chambers (37, 51). The latter system is used mostly for transport studies. Reorganized follicles can produce high amounts of thyroid hormones and thus are used mostly for secretion and functional studies. Because they generally exhibit higher cell multiplication responses and are easily handled, monolayers are considered the material of choice for the investigation of cell proliferation mechanisms. However, as observed very early, thyroglobulin iodination and the synthesis and secretion of thyroid hormones are lost in monolayers in the absence of the spatial constraints of follicular architecture, even though the key enzymatic processes are preserved. Indeed, appropriately stimulated (TSH) dog thyrocyte monolayers have been demonstrated to perform all the thyroid-specific functions required for the synthesis and secretion of thyroid hormones, including thyroglobulin and thyroperoxidase gene expression, iodide uptake, H₂O₂ generation, iodide efflux, and macropinocytosis, and to remarkably retain the regulation of these functions (34). Nevertheless, the extent to which some of the proliferation characteristics of monolayer cell primary cultures might have been affected by their profoundly modified *in vitro* organization and environment has not been systematically investigated.

The confrontation of the different thyroid primary culture systems has pointed out the importance of possible species differences (52), but also the influence of culture conditions, and the fact that cells have a “memory,” which means that their characteristics are not fully stabilized and may evolve depending on their previous *in vivo* and *in vitro* history. For instance, dog thyrocytes specifically lose their mitogenic response to TSH and cAMP (but not their response to growth factors or to the differentiation effects of TSH) after having proliferated in the presence of serum or growth factors (53). This might also apply to human thyrocytes, which have never been reported to maintain their responsiveness to TSH as a mitogen after exposure to high serum concentrations. The necessity to obtain fresh tissue for each culture constitutes a difficulty, especially when available quantities are low and scarce. Moreover, modern approaches based on gene transfection cannot be easily applied to primary cultures, although retroviral vectors allow efficient transfections of a fraction of human thyrocytes in primary cultures (54). The very limited proliferation capacity of thyrocytes in primary culture, of course, prevents permanent transfections.

On the other hand, although there is some quantitative variability from one primary culture to another, such material exhibits a remarkable qualitative reproducibility over many years.

With all these caveats in mind, we shall analyze results obtained in the most studied models, the FRTL-5, WRT, and PC Cl3 cell lines, and the primary cultures of dog and human thyroid, and compare them with our real subject of interest, the elusive human thyroid cell *in vivo*.

III. Methods of Measuring Cell Proliferation

The best index of cell proliferation is the number of cells in a growing population. However, this measures the balance between cell proliferation and cell death, and, if used for evaluating cell proliferation, an important rate of cell death must be ruled out. Moreover, cells in primary cultures do not detach easily from the substratum or from each other. Substitutes of cell counting are therefore used, such as measurements of total DNA in a culture, or indexes of cell mass (protein content) or metabolic activities assayed using various colorimetric measurements. DNA measurements count multiploidy as cell division. Protein contents and metabolic activities measure cell volume independently of cell proliferation.

Autoradiographs of cells incubated with labeled thymidine or immunocytochemistry of bromodeoxyuridine-labeled cells reveal the number of cells having entered into DNA synthesis. A 24- to 48-h incubation with the tracer labels all the cells having entered into DNA synthesis during this period, thus providing a cumulative measure that reflects the mathematical integration of the asynchronous cell cycle progression within the cell population. The continuous availability of the tracer during the incubation period must be checked. A half hour labeling provides a precise estimate of the number of cells synthesizing DNA at this time (this measurement thus represents a mathematical derivative rate of the overall cell proliferation process).

Measurement by fluorescence-activated cell sorter of DNA content allows a rough estimate of the number of cells in between the diploid and tetraploid state to be obtained at the time of measurement, *i.e.*, cells in DNA synthesis. This is also a derivative rate measure. Derivative measurements are especially useful for kinetic studies, but their use for an overall evaluation of the proliferation process requires many time points.

Incorporation of labeled thymidine into the whole DNA of the culture (or often into the trichloroacetic acid-precipitable material) is also often used as an index of proliferation. However, such measurements are sensitive to all the effects on cell pools of thymidine phosphates. For instance, they are often increased in the presence of hormones that enhance thymidine uptake. Several studies have shown qualitative dissociations of cell number increases and ³H-thymidine uptake into acid-insoluble material (3, 18, 55–58). Unless specifically validated for each condition, the latter assay is unreliable.

A detailed analysis of these methods and their pitfalls has been presented previously (59). Because they are the most reliable, we shall take into account mainly studies based on

DNA measurements (including by fluorescence-activated cell sorter), cell counting, and the frequency of labeled nuclei.

IV. Proliferation Characteristics of the Various Systems

Results obtained in different laboratories using the most reliable methodologies lead to the following conclusions:

A. *FRTL-5* rat thyroid cell line

FRTL-5 cells (Table 1) proliferate rapidly (doubling time ≈

36 h) in the presence of 5% FCS and the six hormone mixture (6H) containing TSH, high concentrations of insulin that activate IGF-I receptors (insulin/IGF-I), transferrin, somatostatin, gly-his-lys acetate, and hydrocortisone. According to the initial characterization, this proliferation was absolutely dependent on TSH, the cells remaining quiescent in the same medium as above without TSH (5H medium) (2, 5). Very soon, however, many reports of growth stimulation in the absence of TSH by insulin/IGF-I or serum, and additive effects of insulin and serum, have appeared, which correspond to the present characteristics of the cell line in most

TABLE 1. Variations of proliferation responses assayed by different methods in *FRTL-5* rat thyroid cells in different laboratories

Mitogen	Methods		
	No. cells or DNA content	S-phase nuclei (FACS) ³ H-thymidine- or BrdU-labeled nuclei	³ H-thymidine incorporation into acid-insoluble material
TSH			
TSH alone	Increase (60, 61)	Induce S phase (62) (very weak), (24, 63)	Increase (64) (very weak), (65) (weak), (18, 56, 62, 63, 66–70)
TSH + insulin/IGF-I	No effect (18, 57) Increase/additive (60) Increase/synergistic (57, 68, 72)	No effect (64) Induce S phase/additive (24) Induce S phase/synergistic (62–64, 73)	No effect (57, 71) Increase/synergistic (56–58, 62–64, 66, 67, 71, 72, 74)
TSH + serum	Increase (56, 57, 60, 67, 70, 75–77)	Induce S phase/additive (73) (very weak)	Increase/additive (65, 76)
TSH + serum + insulin/IGF-I	No effect (78) Increase/additive (56, 57, 60) Increase synergistic (11, 18, 78–80)	Induce S phase/synergistic (69, 73)	Increase/synergistic (65, 76) Increase/additive (57, 75)
Insulin/IGF-I			
Insulin/IGF-I alone	Increase (57, 60)	Induce S phase (24, 63, 64, 73)	Increase (56, 57, 62–65, 67–72, 74, 81) No effect (82)
Insulin/IGF-I + serum	Increase/additive (56, 57, 60, 67, 68) No effect (11, 18, 78–80)	Induce S phase/additive (73)	Increase additive (57, 69) Same as serum (65, 80)
Calf serum			
Serum alone	Increase (57) (weak)	Induce S phase (73)	Increase (57, 65)
EGF			
EGF alone		Induce S phase (73) (very weak)	No effect (63, 83)
EGF + insulin/IGF-I		Induce S phase/synergistic (73)	No effect (63) Increase (58)
EGF + insulin + serum	Increase (84) (weak) No effect (58)		
EGF + TSH + serum	Increase (84)		
Phorbol esters (TPA)			
TPA alone		Induce S phase (73)	Increase (67) (weak) (69)
TPA + insulin/IGF-I		Induce S phase/additive (73)	Increase/additive (67, 82)
TPA + insulin/IGF-I + serum	Increase (67)	Induce S phase/additive (69, 73)	
bFGF			
bFGF alone			Increase (63)
bFGF + IGF-I			Increase/synergistic (63)
bFGF + insulin + serum			Increase (85, 86)
HGF			
HGF alone		Induce S phase (73) (very weak)	
HGF + insulin		Induce S phase/synergistic (73)	No effect (87)

FACS, Fluorescence-activated cell sorter; BrdU, bromodeoxyuridine.

laboratories (56, 57, 63, 64, 73). As reported in 1990 by the laboratory of Kohn and associates (18), the basal incorporation of ^3H -thymidine into DNA supported by insulin and 5% serum strikingly increases during repeated passages of FRTL-5 cells, which is accompanied by a relative attenuation of the TSH response. The ability of insulin/IGF-I to increase ^3H -thymidine incorporation in "aged" cells even exceeded levels induced by TSH plus insulin/IGF-I in "young" cells. Although no such changes were observed by this group when growth was measured as cell number (implicitly casting grave doubts on the reliability of the thymidine incorporation assay) (18), many other investigators, including Tramontano and colleagues (60) and Takahashi *et al.* (63), report significant increases of cell number or DNA content by insulin/IGF-I in the absence of TSH.

Despite this controversy, the view that in FRTL-5 cells proliferation and DNA synthesis are synergistically activated by TSH and insulin/IGF-I is almost unanimously accepted. In some reports, TSH and insulin/IGF-I are only additive on cell number, while a marked synergy is shown by the incorporation of thymidine (56). Some confusion about the respective roles of TSH and insulin/IGF-I in this synergy also results from an additional controversy about the capacity of TSH to elicit a limited proliferation in the absence of insulin or IGF-I. While many investigators reported variable stimulations of thymidine incorporation by TSH alone (56, 61, 63, 66, 68), others did not find such an effect (57, 64, 71) or failed to reproduce it when measuring proliferation by cell number (18). According to Zakarija and McKenzie (57), TSH alone is devoid of stimulatory effects on thymidine incorporation and cell number, but as little as 0.2% FCS suffices to support these TSH effects in the absence of insulin. By contrast, Isozaki and Kohn (78) reported that 5% serum does not support TSH-dependent proliferation in the absence of insulin. The demonstration of an important mitogenic effect of TSH *per se* (but in the presence of 0.2% FCS) is facilitated by the removal of the cytostatic somatostatin from the usual 6H culture medium in the FRTL-5 cells used by Santisteban and collaborators (61). When observed, the effect of TSH alone on thymidine incorporation was found to depend, at least in part, on an autocrine IGF-II production (66). Collectively, results are therefore generally compatible with TSH merely amplifying the mitogenic response to insulin or IGF, either endogenous or exogenous.

Despite a first contradictory report (80), the mitogenic effects of TSH, either alone or in the presence of insulin/IGF-I, are reproduced totally (58, 62, 88), or only partially (76, 89), by various cAMP enhancers in FRTL-5 cells, as first demonstrated using dog thyroid primary cultures (90, 91).

Proliferation of FRTL-5 cells can be stimulated independently of TSH and cAMP. Phorbol ester and insulin effects are additive (67, 73). Basic fibroblast growth factor (bFGF) in synergy with insulin strongly induces DNA synthesis (63, 85, 86). We observed a similar stimulation using hepatocyte growth factor (HGF) (73), but others did not get this effect in FRTL-5 cells that, nevertheless, express functional HGF receptors (87). The recent findings of a slight to moderate growth stimulation by epidermal growth factor (EGF) in the presence of TSH or insulin (73, 84) contrast with earlier

negative reports (63, 83), and might be a characteristic developed by "aged" FRTL-5 cells (92).

In conclusion, the marked regulatory differences between young and old cultures, as well as the discrepancies between effects of the same agents in different laboratories (Table 1), suggest that there are no "FRTL-5 cells," but different batches or subclones of such cells, each with its peculiar properties, and therefore that findings from one laboratory cannot necessarily be extrapolated to others. According to a majority of recent reports, FRTL-5 cells proliferate in response to insulin/IGF-I alone and the TSH/cAMP cascade amplifies this effect. By contrast, the group of Santisteban and colleagues (93) investigates the mechanisms involved in the stimulation of DNA synthesis in FRTL-5 cells that can proliferate in response to TSH alone (Fig. 1).

B. WRT rat thyroid cell line

WRT cells proliferate rapidly in the presence of TSH, insulin, and/or serum (doubling times 42 h with 5% serum and 80 h with 0.5% serum) (3). These cells proliferate more

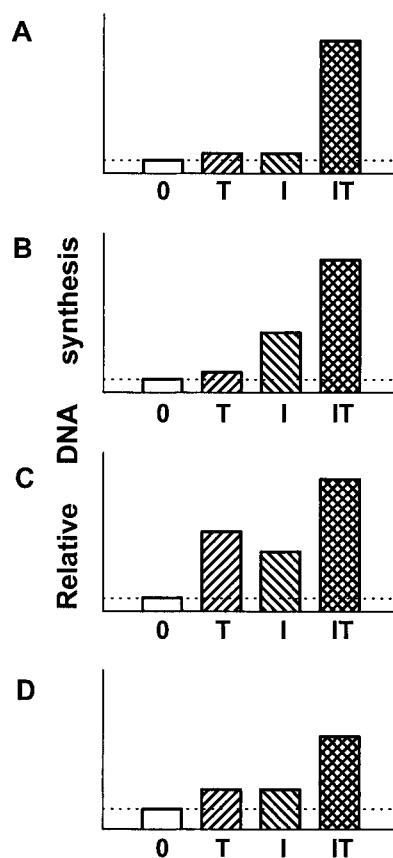


FIG. 1. Summary of relative DNA synthesis rates reported in the various *in vitro* thyroid culture systems, in response to TSH alone (T), IGF-I or high insulin concentrations alone (I), or the combination of both stimulations (IT) (0, no treatment). A, The situation corresponding to dog thyroid primary cultures and initial reports on FRTL-5 cells. B, The situation observed in rat thyroid follicles in suspension cultures, in PC Cl3 cells, and in FRTL-5 cells presently used in most laboratories. C, The situation studied in WRT cells, and in FRTL-5 cells used by Santisteban and collaborators. D, The situation generally observed by the present authors in primary cultures of normal human thyroid epithelium. See text for references.

slowly, but can be propagated in the absence of TSH, using only insulin and a low serum concentration. TSH also can support a sustained proliferation in the presence of 0.5% serum but in the absence of insulin, and TSH alone can trigger DNA synthesis in WRT cells deprived of serum and insulin. Insulin/IGF-I and TSH synergistically increase thymidine incorporation but produce less than additive effects when proliferation curves are analyzed (3). At variance with the initial characterization of FRTL-5 cells, TSH may thus appear as a full mitogen in WRT cells (3, 94). Nevertheless, its mitogenic activity has been most frequently investigated in the presence of high insulin concentrations (95). Moreover, it is not totally clear whether the induction of DNA synthesis by TSH alone after insulin deprivation might depend on endogenous IGF production, as shown in FRTL-5 cells, or even on some persistence of a permissive effect of the high insulin concentration present in the 6H medium used for routine cell maintenance. cAMP enhancers fully mimic the mitogenic effects of TSH in WRT cells (94). In our hands, DNA synthesis in WRT cells is not stimulated by phorbol esters and HGF (our unpublished data). When maintained in low-serum conditions, they are also unresponsive to EGF. Nevertheless, two months after shifting to a 5% serum medium, WRT cells reversibly develop a moderate responsiveness to EGF proliferative effects (55).

In summary, in WRT cells, insulin/IGF-I and the TSH/cAMP cascade independently induce proliferation (Fig. 1).

C. PC Cl3 rat thyroid cell line

Like FRTL-5 and WRT cells, PC Cl3 cells are routinely maintained in the 6H medium containing TSH, insulin, and 5% FCS (4). Insulin at high concentrations or IGF-I alone can stimulate DNA synthesis in the absence of serum (96). At variance with WRT cells, TSH or cAMP enhancers alone are almost inactive, but they markedly potentiate the effect of insulin/IGF-I (96), and they can stimulate DNA synthesis in the presence of a low, inactive *per se*, concentration of insulin (73). FGF stimulates the proliferation of PC Cl3 cells (97). In our hands, DNA synthesis is also induced by phorbol esters in the presence or not of insulin, but not by EGF or HGF (73). Thus, as in most FRTL-5 cells, insulin/IGF-I stimulates the proliferation of PC Cl3 cells, and this effect is revealed or amplified by the TSH/cAMP cascade (Fig. 1).

D. Rat thyroid follicles in primary culture

Rat thyroid follicles in suspension culture have been little used for cell multiplication studies, because of their poor capacity for proliferation and the insufficient amount of cell material they provide for biochemical studies. Nevertheless, this model has given us the first unambiguous *in vitro* demonstration of the mitogenic effect of TSH, observed in the presence of 0.5% serum (48). In serum-free medium, the stimulation of DNA synthesis by TSH absolutely requires the presence of insulin/IGF-I, which alone weakly increases the proportion of cells in S phase (98) (Fig. 1). The effect of TSH is mimicked by forskolin (99). EGF is devoid of mitogenic effect, either alone or in the presence of insulin (98).

E. Dog thyrocytes in primary culture

Dog thyrocytes in monolayer culture can proliferate rapidly (doubling time \approx 36 h) in response to a combination of TSH, insulin, EGF, and serum (1–10%), and then abruptly stop growing after four to six generations (53, 100). After initial plating, cells remain quiescent and healthy in a serum-free medium supplemented or not with insulin. They have thus proliferated only slightly *in vitro* when stimulation is applied. Insulin, IGF-I, or IGF-II alone have in general only marginal effects on DNA synthesis, but they support the induction of DNA replication and cell cycle progression by TSH, EGF, bFGF, or phorbol esters (100–105). TSH and EGF triggering effects in the presence of insulin are additive (100). Insulin/IGF-I are generally required for the mitogenic stimulation by these various factors, but in one-third of the experiments a significant stimulation of DNA synthesis can be achieved in response to TSH alone (100, 105). When observed, this effect is inhibited in part by neutralizing antibodies blocking IGFs or IGF-I receptors (104). It thus depends, at least in part, on an autocrine IGF production, according to a paradigm first introduced by Eggo and collaborators (106) in the thyroid field. In general, the permissive effect of insulin is obtained at high concentrations and is mediated by IGF-I receptors, which are constitutively expressed in dog thyrocytes (104, 105). However, TSH in the absence of insulin induces the synthesis and accumulation of insulin receptors, which then allows low physiological insulin concentrations, instead of IGF-I, to act as a comitogenic permissive factor for the cell cycle progression induced by TSH (100). HGF is the only growth factor so far that can induce DNA synthesis and proliferation in dog thyrocytes cultured without insulin/IGF-I, thus acting as a full mitogenic factor (105, 107). In several experiments, its action is nevertheless potentiated by insulin (104). The mitogenic effects of TSH in dog thyrocytes are perfectly mimicked by forskolin, cholera toxin, and various cAMP analogs (90, 91, 102, 108), which has provided the first direct evidence that cAMP fully accounts for TSH-stimulated mitogenesis (109, 110).

Thus, in dog thyrocytes the cascade activated by IGF-I or insulin is necessary for the TSH/cAMP mitogenic effect, but, by itself, it is inactive on cell proliferation (Fig. 1).

F. Human thyrocytes in primary culture

We have restricted our analysis to studies performed using culture conditions that allow the *in vitro* demonstration of the mitogenic effect of TSH. Unlike growth factor effects, the stimulation of DNA synthesis and proliferation by TSH may be weak or absent if cells originate from goiter, from tissue from old people, and/or from previously frozen cells, subcultivated cells, or cells exposed to high serum concentrations (32, 50, 111–113). In serum-free primary cultures of normal human thyrocytes organized as cell monolayers or cell aggregates, the mitogenic effect of TSH is best demonstrated by the induction of DNA synthesis (29, 30, 114, 115). Nevertheless, marked stimulations of proliferation by TSH, as reflected by increases of cell numbers, were obtained using human fetal thyrocytes (114, 116). The mitogenic effect of

TSH absolutely depends on the presence of IGF-I or insulin [including, at very low physiological concentrations, acting exclusively through insulin receptors (117)], which alone weakly stimulate DNA synthesis (29, 30, 105). In the absence of exogenous insulin or IGF-I, the weak stimulation by TSH of thymidine incorporation in human thyrocytes cultured with 1% serum was inhibited by an IGF-neutralizing monoclonal antibody, suggesting that it depended on autocrine IGF production (118, 119). In primary cultures of human thyroid follicular adenomas, this autocrine IGF production is exacerbated, leading to an escape from exogenous IGF dependence for proliferation, without reducing the requirement for TSH (30, 120). In monolayer cultures, the effect of TSH is mimicked in large part, but not totally, by cAMP enhancers [forskolin, cholera toxin, (Bu)₂cAMP (29)]. cAMP-independent stimulations of proliferation and DNA synthesis can be achieved using serum, EGF in the presence of insulin and/or serum, or phorbol esters in the presence of insulin (29, 112, 121–123). HGF, even in the presence of insulin, fails to induce DNA synthesis, as observed in our group (S. Dremier, unpublished data). However, the Cardiff group has demonstrated a strong stimulation of DNA synthesis by HGF in the presence of 10% serum (124). Possibly, a serum factor might increase the abundance of HGF receptors (c-met), which are poorly expressed in normal human thyroid tissue, at variance with their high abundance in many papillary carcinomas (125).

Thus, as in dog thyroid cells, the signaling cascade of IGF-I or insulin is necessary for the TSH/cAMP-induced DNA synthesis, but it is weakly mitogenic by itself (Fig. 1).

G. Comparison of cell systems (Table 2)

In all these systems, at variance with thyroid cell cultures from porcine or bovine origins, TSH exerts a major stimulatory effect on cell proliferation, and cAMP is a sufficient mediator of at least a large part of this effect. In all the systems, the TSH/cAMP stimulatory effects on DNA synthesis and cell proliferation are best demonstrated in the presence of insulin or IGF-I, which reflects an important synergy between both kinds of factors. When studied, the effects of insulin/IGF-I have been related to an effect on the IGF-I receptor in rat thyroid cell lines and primary cultures (56, 98, 126). By contrast, in canine thyroid primary cultures and human thyrocytes, insulin receptors expressed in response to TSH can also mediate the insulin comitogenic effects (104, 117). Low concentrations of insulin were also reported to be mitogenic in sheep thyroid primary cultures (45).

Whether TSH or insulin/IGF-I alone can elicit a significant DNA synthesis response is particularly relevant to the question of the respective roles of these factors in their synergistic regulation of cell proliferation (Fig. 1). In the different rat thyroid cell lines and in rat thyroid primary cultures, insulin/IGF-I alone significantly stimulates proliferation. By contrast, it generally produces marginal effects on DNA synthesis in canine primary cultures. In PC Cl3 cells as in rat thyroid primary cultures, TSH alone is devoid of proliferation-inducing effect, but it merely potentiates the action of insulin/IGF-I. In FRTL-5 cells, the situation is more controversial, depending on the proliferation assay (³H-thymidine incorporation *vs.* more reliable methods), the culture me-

TABLE 2. Proliferation index of various mitogens in rat, dog, and human thyroid cells

	DNA synthesis or proportion of S-phase cells					
	FRTL-5	WRT	PC Cl3	Rat	Dog	Human
TSH						
TSH alone	0/+	+	0	0	0/+	±
TSH + insulin	+++	++	+++	+++	+++	+++
Insulin/IGF-I						
Insulin/IGF-I alone	+/0	+	+	+	0/±	+
EGF						
EGF alone	0/±	0/+	0	0	0	nd
EGF + insulin	0/+++	nd	0 same as insulin alone	0	+++	+
Phorbol esters (TPA)						
TPA alone	+	0	+	nd	0	nd
TPA + insulin	++	0 same as insulin alone	++	nd	+++	+
HGF						
HGF alone	0/±	0	0	nd	+	nd
HGF + insulin	0/+	0 same as insulin alone	0 same as insulin alone	nd	++	0
HGF + serum	nd	nd	nd	nd	nd	+++
cAMP						
cAMP alone	0/+	+	0	0	0/+	0
cAMP + insulin	++(+)/+++	++	+++	+++	+++	++(+)
Calf serum						
Serum alone	+	+	+	nd	+	+

0, No effect; ±, very weak or marginal effect; +, increase; ++, additive increasing effects; +++, synergistic increasing effects; nd, not determined.

dium (presence of 0.2% serum, absence of somatostatin, possible incomplete removal of the very high concentration of insulin used for routine cell maintenance), and possibly the subclone used and the number of repeated passages. TSH alone may induce, or not, a significant mitogenic effect, which has been suggested to depend on an autocrine effect of endogenous IGF-II (66). By contrast, in WRT cells, TSH alone generates an important stimulation of proliferation, which is additive to the one exerted by high insulin concentrations. In the different rat thyroid cell systems, the respective roles of TSH and insulin/IGF-I might thus be different. In canine and normal human thyroid primary cultures, the triggering of DNA replication by TSH absolutely depends on insulin, IGF-I, or IGF-II. In some primary cultures of dog and human thyrocytes, as in FRTL-5 cells, endogenous IGF production may partially fulfill this requirement.

Thyroid cell proliferation is also stimulated independently of cAMP by various growth factors and phorbol esters. EGF in the presence of insulin/IGF-I stimulates DNA synthesis and proliferation in human and canine thyroid primary cultures, and in most other species (sheep, pig, calf), but not in rat thyrocytes in primary culture and in PC Cl3 cells. Moderate EGF responses in FRTL-5 cells might be restricted to “aged” cells, and in WRT cells *de novo* EGF responsiveness only appears after weeks of culture in the presence of high serum concentrations. The mitogenic responsiveness to bFGF in the presence of insulin first shown in dog thyrocytes is also observed in pig and calf (43) thyrocytes and in FRTL-5 and PC Cl3 cells, but it has not been reported so far in WRT cells and in human thyrocytes. The very potent mitogenic stimulation by HGF demonstrated in dog thyrocytes has been confirmed in human thyrocytes, but only in the presence of serum, and in porcine thyrocytes (87). Among the rat cell lines, we have found only FRTL-5 cells to be responsive to HGF. In our hands, phorbol esters stimulate DNA synthesis in all the cell systems including human thyrocytes, with the exception of WRT cells. There are thus again some important differences between the three rat cell lines, PC Cl3 cells being more similar to rat thyroid primary cultures. Dog thyrocytes are the only system responding to the full range of factors that stimulate DNA synthesis in human cells. This has prompted an investigation of the mitogenesis induced by TSH via cAMP by comparison with the more general mechanisms of growth factors and phorbol esters, which have been delineated in many other cell types.

V. Kinetics of TSH-Insulin/IGF-I Synergy and Cell Cycle Progression

In the following sections, we have only considered the stimulation of quiescent G_0 cells to enter the cell cycle and the DNA synthesis phase, not the control of the G_1 to S phase transit in continuously stimulated cycling cells, which has been little studied in thyroid culture models.

In the stimulation of quiescent BALB/c 3T3 fibroblasts, synergizing comitogens have been sorted in two categories. The first one increases the capacity (competence) of cells to respond to the second category, which supports cell cycle progression (127). For instance, platelet-derived growth fac-

tor stimulates proliferation, at least in part, by inducing IGF-I receptors, thus increasing cell competence to progress into the cell cycle in response to IGF-I (128). Similarly in FRTL-5 cells, a 12- to 24-h preincubation with TSH or a cAMP enhancer suffices to shorten G_1 phase and to strongly amplify the DNA synthesis response induced by insulin/IGF-I added afterward (62–64, 72, 129). The continuous presence of TSH is dispensable during cell cycle progression triggered and supported by insulin/IGF-I (63, 64, 72). TSH is thus identified as a competence factor that exerts a priming effect facilitating the action of the progression factor insulin/IGF-I. The mechanism is not fully understood. As reported by Takahashi and colleagues (130, 131), the TSH pretreatment does not increase the number and activity of IGF-I receptors, but it potentiates the IGF-I-dependent tyrosine phosphorylation of insulin receptor substrate (IRS)-2 and activation of PI3K, and the phosphorylation and up-regulation of Shc leading to increased binding of Grb2 to Shc and activation of p42/p44 MAPKs.

When TSH and insulin/IGF-I are administered simultaneously in FRTL-5 cells, the elevation of cellular cAMP levels is biphasic, as cAMP activates a type IV phosphodiesterase (132). While cAMP unambiguously mediates the mitogenic effect of TSH, preventing the further decline of cAMP levels by the administration of phosphodiesterase inhibitors or a phosphodiesterase-resistant cAMP analog impairs the initiation of DNA synthesis in both FRTL-5 (133, 134) and PC Cl3 cells (135). In our hands, when PC Cl3 cells are stimulated by the combination of insulin and the adenylyl cyclase activator forskolin, the washing out of forskolin 16 or 20 h afterward accelerates, rather than prevents, the entry of cells into DNA synthesis phase (S. Demartin and P. P. Roger, unpublished data). In FRTL-5 and PC Cl3 cells, cAMP effects on cell cycle are therefore biphasic. After its initial priming/competence effects, cAMP is no longer required for G_1 phase progression supported by insulin/IGF-I, and it even inhibits it when maintained at too high a level. No such data are available from WRT cells.

The observation that the presence of TSH or cAMP enhancers is not continuously required for G_1 phase progression of FRTL-5 cells is compatible with the hypothesis that cAMP could also indirectly stimulate cell proliferation by inducing the production of autocrine growth factors (63). Part of the synergism between TSH and IGF-I is eliminated when the culture media are renewed every 4 h with fresh media (63). The conditioned medium from TSH-treated FRTL-5 cells potentiates the mitogenic effect of IGF-I on human fibroblasts (63). The priming action of TSH and its potentiation of IGF-I-dependent DNA synthesis were therefore suggested to be mediated, in part, by an autocrine amplification factor (63). bFGF was proposed as a likely candidate (63, 136). Not only does it strongly induce DNA synthesis in synergy with insulin/IGF-I, but also it is produced by FRTL-5 cells (136). During TSH-dependent thyroid hyperplasia in rats, mRNA expression of both bFGF and FGF receptor 1 are increased (137). Very recent findings confirm the induction by TSH and cAMP of the expression of both FGF and FGF receptor 1 in FRTL-5 cells (138). Immunoneutralization of bFGF slightly decreases the basal rate of DNA synthesis observed in the absence of TSH (136). Additional FGF immunoneutralization experiments are crucial to dem-

onstrate to which extent this autocrine mechanism indeed contributes to the mitogenic action of TSH in this cell line. However, other possible explanations for the discontinuous requirement for TSH during the G₀-to-S phase progression have not been considered in FRTL-5 cells. For instance, one can imagine that TSH could be required for the assembly of a stable structure, such as a prereplication complex subsequently required for S phase initiation.

In canine thyroid primary cultures, quite at variance with FRTL-5 cells, the stimulation of DNA synthesis requires the *simultaneous* presence of TSH and insulin/IGF-I. When TSH is added 24 h after insulin/IGF-I or when insulin/IGF-I is administered 24 h after TSH, DNA synthesis follows with a similar 16- to 20-h lag phase the first time that TSH and insulin/IGF-I are present together, regardless of which factor is added first (100). Furthermore, in canine thyrocytes cultured with insulin, the induction of DNA synthesis by forskolin requires its continuous presence for at least 16 h until a very late G₁ phase restriction point situated approximately 2 h before DNA synthesis initiation. In response to forskolin, dog thyrocytes progress toward S phase, but if this adenylyl cyclase activator is withdrawn for as little as 2 h before cells reach the commitment point, they regress to an earlier part of G₁, from which they can be rescued by forskolin readdition (139). Moreover, elimination of forskolin at later time points arrest without detectable delay the entry of cells into DNA synthesis phase (140). cAMP thus directly supports G₁ phase progression in dog thyrocytes. This implies a very late rate-limiting event, which must be labile to explain the rapid consequence of forskolin deprivation on DNA synthesis initiation (139, 140). Similar observations were made in dog thyrocytes stimulated by the phosphodiesterase-resistant cAMP analog (Bu)₂cAMP in the presence of carbamylcholine, which can substitute for insulin as a supportive comitogenic factor (141). In this case, the immediate inhibition of carbamylcholine signaling by atropine, unlike the removal of (Bu)₂cAMP, still permitted the entry of G₁ cells into DNA synthesis phase for 6–8 h. This suggests that cAMP can exert alone the last crucial control that determines the cell commitment toward DNA replication (141).

Nevertheless, when TSH is administered to dog thyrocytes 24 h before insulin/IGF-I, a higher rate of DNA synthesis is often observed (100), and various responses to insulin, IGF-I, and IGF-II are enhanced, including autophosphorylation of insulin/IGF-I receptors, tyrosine phosphorylation of IRS-like proteins, MAPK activation, and c-Fos expression (104). In dog and human thyrocytes, TSH clearly increases insulin responsiveness by inducing the expression of insulin receptors, which allows low physiological insulin concentrations to exert a sufficient comitogenic activity (104). However, in dog thyrocytes as in FRTL-5 cells, the expression of IGF-I receptors is unaffected. The mechanism of the potentiation of IGF-I receptor activity by TSH remains to be defined in dog thyrocytes (104). It might be similar to the above detailed mechanism recently suggested in FRTL-5 cells by Takahashi and co-workers (131).

In dog thyrocytes, an additional synergy between TSH (cAMP) and EGF is observed in the presence of insulin, as evidenced by a shortening of G₁ phase and an increase of the fraction of cells that enter S phase (100, 142, 143).

In summary, in dog thyrocytes TSH through cAMP acts mainly as a progression factor. Secondly, it also acts as a competence factor increasing responsiveness to insulin and IGF-I, which then cooperate with cAMP as progression factors (104). In most FRTL-5 cells, TSH through cAMP exerts a sufficient competence/priming action augmenting the responsiveness to IGF-I, but cAMP is not required for G₁ phase progression supported by insulin/IGF-I, which is inhibited even by the maintenance of high cAMP levels. Therefore, cAMP exerts opposite effects on G₁/S transition and DNA synthesis initiation in FRTL-5 cells and dog thyrocyte primary cultures (Fig. 2).

VI. Hypertrophy vs. Mitogenesis

For a cell to divide, or at least to sustain several divisions, it must double its mass as well as its DNA content before each division. Strikingly, while potently stimulating DNA synthesis in the presence of insulin/IGF-I, TSH does not increase the overall protein synthesis and protein accumulation in primary cultures of canine and human thyrocytes (105). Insulin and IGF-I, on the contrary, while not being sufficient mitogens, stimulate protein accumulation leading to the hypertrophy of these cells. This increase of cell mass is probably necessary, but clearly not sufficient, for the permissive action of these factors on the TSH/cAMP mitogenic response (105, 141).

The three rat thyroid cell lines are again different in this regard. Not only insulin/IGF-I, but also TSH, activate protein synthesis and induce cell growth (73). In our hands, these effects are less than additive (73). By contrast, Koide *et al.* (71) reported a marked synergy on FRTL-5 cells and a priming effect of IGF-I potentiating the stimulation of protein synthesis by TSH. Interestingly, the converse situation is observed for the induction of DNA synthesis (71).

Therefore, the regulations of protein synthesis and DNA synthesis are dissociated in all the systems. Nevertheless, as the final effects of hormones in the primary cultures and in

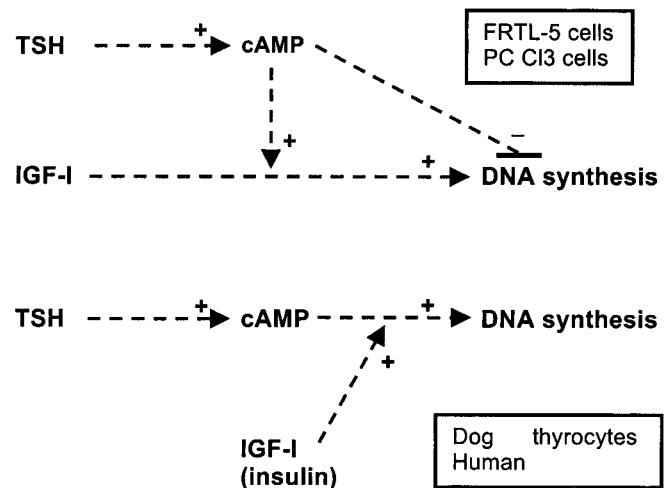


FIG. 2. Respective effects of TSH (cAMP) and IGF-I (insulin) on DNA synthesis in FRTL-5 and PC Cl3 cell lines *vs.* dog and human thyroid primary cultures. Arrows pointing to other arrows indicate permissive effects of the second factor, as recently proposed (144).

the cell lines are different, their effects on the intracellular cascades are thus probably also different. Even in the different rat cell lines, different mechanisms might be involved. For instance, the stimulation of protein synthesis by insulin is blocked by the HMG-CoA reductase inhibitor lovastatin in FRTL-5 and WRT cells, but not in PC Cl3 cells (73).

Thus, in rat thyroid cell lines insulin/IGF-I and TSH independently stimulate protein synthesis, whereas only IGF-I or insulin do so in dog and human thyrocytes.

VII. Signaling Cascades

The problem of the delineation of the various signaling cascades involved in the mitogenic stimulation of thyrocytes is especially complex. It includes the identification of the respective intermediaries of the different comitogenic pathways (*i.e.*, TSH *vs.* insulin/IGF-I or growth factor *vs.* insulin/IGF-I) and of the possible cross-signalings by which one comitogenic factor influences the activity of another one. This may help to define the respective roles of such factors. Various pharmacological probes and genetic tools are available to “specifically” activate or inhibit most known signaling cascades. Nevertheless, it should be reemphasized that a very strong expression by transfection of a constitutively activated (mutated) intermediary of a cascade may produce effects that do not mimic a physiological activation. On the other hand, the simple fact that the action of a given stimulus is blocked by an inhibitor of a signaling cascade does not demonstrate that this stimulus transmits a signal through this cascade, as even its basal activity might exert a required permissive influence [*e.g.*, the simple observation of an inhibition of TSH-stimulated proliferation of FRTL-5 cells by wortmannin does not warrant the claim that TSH activates PI3K (93)]. Absence of oxygen blocks most cascades even though cell respiration is not part of these cascades. Moreover, the specificity of most inhibitors is only relative and should be checked for each model at the concentration used.

A. Expression of membrane receptors

The regulation of the expression of key membrane receptors markedly differs in the different systems. In FRTL-5 cells, insulin and IGF-I receptors are constitutively expressed as suggested by binding experiments (126, 145), and the expression of TSH receptor mRNA depends on insulin/IGF-I but is attenuated by TSH (146, 147). Such a down-regulation has not been observed in murine thyroid gland *in vivo* (148). Conversely, in dog and human thyrocytes the expression of insulin receptor protein is induced by TSH and inhibited by insulin (104, 117), while the constitutive expression of TSH receptor mRNA is independent of insulin but transiently and moderately enhanced by TSH (149, 150).

B. Coupling of TSH receptor

Studies using human and dog thyroid membrane preparations have shown that the TSH receptor can be coupled to G proteins of each of the four main classes, Gs, Gq, Gi, and G0 (151, 152). Nevertheless, in intact cells a more restricted selectivity of G protein coupling has been demonstrated

(152). In all the thyroid cell systems, TSH activates the Gs α /adenylyl cyclase/cAMP cascade. In dog and human thyrocytes, TSH also activates Gi, as demonstrated by an inhibition of adenylyl cyclase, which partially opposes the stimulation through Gs and can be relieved by pertussis toxin (152). In human thyrocytes (153, 154), but not in dog thyrocytes (155, 156), TSH also stimulates the Gq/PLC/Ca⁺⁺ cascade. This activation requires 10 times higher concentrations of TSH than adenylyl cyclase activation. A similar effect has been reported in FRTL-5 (Refs. 157 and 158 but see Ref. 159 for a contradictory report) and PC Cl3 cells (160), but with TSH concentrations 100–1000 times higher than those required for cAMP accumulation, which raises questions about the role of this effect in the cell lines and the possible effect of TSH contaminants. However, Sho *et al.* (161) have shown in FRTL-5 cells that adenosine through A1 receptors potentiates the stimulation by TSH of the PLC/Ca⁺⁺ cascade and inhibits the activation of the adenylyl cyclase/cAMP cascade in a pertussis toxin-sensitive manner. A similar phenomenon has been shown in human thyrocytes (154). In FRTL-5 cells, the TSH receptors have also been claimed to be coupled to PLA₂ through a pertussis toxin-sensitive pathway leading to arachidonic acid release and PG synthesis (162), but no evidence of a direct coupling through G proteins was provided (163). On the contrary, in dog thyroid slices TSH and cAMP inhibit arachidonate release (164). Finally, the common $\beta\gamma$ -subunits of the various G proteins are potentially coupled to different effector pathways including the Ras/Raf/MAPK pathway, raising the possibility of a Ras activation in response to TSH.

C. Involvement of cAMP and PKA (Fig. 3)

As stated above, cAMP enhancers (forskolin, cholera toxin, cAMP analogs . . .) mimic totally or in great part the effects of TSH on DNA synthesis and cell proliferation in the different experimental systems. In FRTL-5 cells, whether cAMP may totally account for the effects of TSH and thyroid-stimulating Igs remains a matter of controversy. The additional involvement of PLC and PLA₂ cascades has been suggested (163), but the high concentrations of TSH required make this very doubtful. The cyclooxygenase inhibitor indomethacin is claimed to partially inhibit the stimulation of thymidine incorporation by TSH, suggesting a role of PG synthesis (165). Pertussis toxin was also reported to inhibit DNA synthesis in FRTL-5, but this inhibition was related to the G₁ phase progression supported by IGF-I, not to the priming effect of TSH (72). In sharp contrast, in dog primary thyrocytes, the mitogenic effects of TSH are perfectly mimicked by the cAMP enhancers (34). TSH does not activate PLC (155), nor does it enhance PG production (166). Proliferation effects of TSH in the presence of insulin are insensitive to indomethacin (166) and pertussis toxin (152) in dog thyrocytes. As exemplified by this system and WRT cells, cAMP is thus a fully sufficient mediator of the comitogenic effects of TSH.

In WRT cells, dog thyrocytes, FRTL-5 cells, and human thyrocytes, the activity of PKA is required for the mitogenic stimulation by TSH (Refs. 93, 108, 167, and 168 and A. Van Keymeulen, S. Dremier, and P. P. Roger, unpublished data), possibly through downstream targets such as CREB/CREM

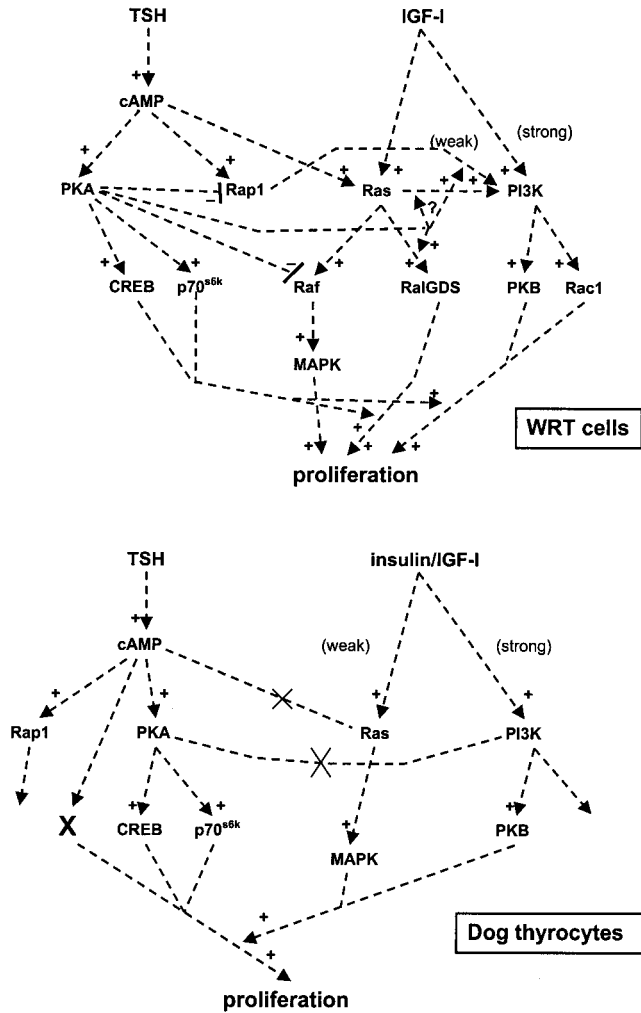


FIG. 3. Mitogenic signaling cascades of TSH (cAMP) and insulin/IGF-I suggested in WRT cells *vs.* dog thyrocytes in primary culture. The “X” event(s) in dog thyrocytes represents a unknown cAMP-dependent, but PKA-independent, effect required for cAMP-dependent mitogenesis. Arrows pointing to other arrows indicate potentiating permissive effects, and crossed out lines represent demonstrated absence of effects (144). In our tentative representation of the complex situation resulting from the intricate signaling cascades reported in WRT cells, we have been unable to depict the apparent paradox recently raised by the observations that the cAMP-dependent activation of Rap1 appears to be necessary and sufficient for the activation of the PI3K pathway by cAMP (174), but is neither sufficient nor even necessary for the PI3K-dependent mitogenesis elicited by cAMP (174). See text for references, details, and explanations.

(169, 170) or p70^{S6k} kinase (95, 171). However, as first shown in dog thyrocytes, PKA activation is not sufficient for cAMP-dependent induction of DNA synthesis (167, 172), and the role of other cAMP-dependent but PKA-independent pathways remains to be defined.

In WRT cells, TSH and cAMP weakly stimulate the activating phosphorylation of protein kinase B (PKB)/Akt (173), as a likely consequence of PI3K activation. This effect is probably superfluous in the presence of insulin, which strongly activates the PI3K/PKB pathway. Nevertheless, it might help to explain the relative insulin independence, which is a peculiarity of the cAMP-elicited mitogenesis in

WRT cells. Initially, the cAMP stimulation of PKB/Akt phosphorylation was reported to be independent of PKA because it was not inhibited by the PKA inhibitor H89 used at 25 μ M (172, 173). However, in a more recent report by the same authors, 10 μ M H89 abolished the cAMP-dependent phosphorylation of Akt in WRT cells, which is now interpreted as indicating a requirement for PKA activity in this process (174). This illustrates the weakness of evidence based solely on the use of such inhibitors. By contrast, in dog thyrocytes, TSH and cAMP do not activate PI3K and PKB/Akt (171). In this system, the inhibition of cAMP-dependent mitogenesis by PI3K inhibitors such as wortmannin thus bears on the permissive activity of PI3K strongly stimulated by insulin/IGF-I (171).

cAMP activates the small G protein Rap1 independently of PKA in dog thyrocytes (175) and WRT cells (172). However, Rap1 activation is a common step of various signaling cascades, which have different effects on dog thyrocytes, and it is thus neither characteristic nor sufficient for mitogenic stimulation (175). Moreover in WRT cells, expression of activated Rap1A (A63E) or a putative dominant negative Rap1A (A17N) did not affect the TSH stimulation of DNA synthesis in the presence of insulin (174). Because Rap1A (A17N) abolishes the PI3K-dependent phosphorylation of Akt by TSH (174), this also raises questions with regard to the involvement of the latter event in TSH-stimulated DNA synthesis, at least in the presence of insulin.

D. Involvement of Ras and its effector pathways

Ras has long been thought to play an important role in the regulation of proliferation and differentiation of thyrocytes. Activating mutations of Ras genes are a frequent early event in thyroid follicular adenomas and carcinomas (176). Activated (val12 mutation) H-Ras induces a sustained proliferation compatible with differentiation expression in primary cultures of normal human thyrocytes (177, 178), while it provokes both a TSH/insulin-independent growth and a suppression of differentiation expression in rat thyroid cell lines (4, 179–181). In WRT cells, each of various potential effectors of Ras has been reported by Meinkoth and collaborators (182) to be sufficient to elicit a TSH-independent proliferation, including Raf/MEK kinase (MEK)/MAPK, PI3K (183) and Ral GDS (184). Nevertheless, this group did not consider the paradox between the potent induction of proliferation by Ras Val12 mutants defective for binding of Raf (183, 184) and the inhibition by strategies that block the Raf/MEK pathway of the DNA synthesis caused by overexpression of wild-type Ras (185). This might indicate that high concentrations of microinjected Ras mutant proteins could signal through effectors not normally activated by wild-type Ras. In FRTL-5 cells, constitutively activated MEK only weakly affects proliferation (186), and in human thyrocytes Raf/MEK is a necessary, but not sufficient, intermediary in the stimulation of proliferation by oncogenic Ras (187). Effects of oncogenic Ras are thus partly different in human thyrocytes *vs.* rat cell lines, and, even among the latter, downstream mechanisms may vary. In dog thyroid primary cultures, normal Ras activation by extracellular stimuli, including EGF and the very potent phorbol ester,

12-O-tetradecanoylphosphol 13-acetate (TPA), is not sufficient to trigger mitogenesis (188).

As demonstrated in dog thyrocytes, unlike growth factors and TPA, TSH and cAMP do not stimulate the phosphorylation and activity of p42/p44 MAPKs (189), which was a first indication of a lack of Ras activation in this pathway. However, in WRT cells Ras is suggested by Meinkoth and collaborators (168) as an intermediary in the cAMP-dependent mitogenesis, because microinjected neutralizing antibodies and dominant interfering mutants of Ras partly inhibit TSH/cAMP-stimulated DNA synthesis. In this cell line, the lack of MAPK activation by cAMP is ascribed to PKA inhibition of c-Raf and redirection by cAMP of Ras signaling toward other effectors such as Ral GDS and PI3K (182). Very recently, Meinkoth and collaborators (172) have demonstrated the activation by TSH and cAMP of human H-Ras ectopically overexpressed in stably transfected WRT cells. As in the case of Rap1 activation, this effect is resistant to PKA inhibitors (172). However, the endogenous Ras proteins and thus their activation were undetectable (172), and a possible alteration of signaling pathways by cellular Ras overexpression has yet to be excluded. In sharp contrast in dog thyrocytes, while the activity of endogenous Ras, as reflected by its GTP-loading, is strongly stimulated by EGF, HGF, and TPA and weakly by insulin, TSH and cAMP reduce the basal levels of GTP-Ras (188). This lack of Ras activation explains and confirms the lack of MAPK activation in the cAMP-dependent pathway, which therefore does not result from the uncoupling by cAMP of c-Raf from Ras. Such a uncoupling is also made unlikely by the fact that MAPK phosphorylation and nuclear translocation induced by EGF are not affected by TSH and forskolin in dog thyrocytes (143). In these cells, we have not excluded a requirement for Ras activity in the TSH-stimulated DNA synthesis. We have indeed consistently observed a low basal level of Ras-GTP in dog thyrocytes (188). In these cells [but not in FRTL-5 cells (93)], PD098059, which specifically inhibits a MAPK kinase and thus p42/p44 MAPKs, inhibits DNA synthesis triggered by TSH in the presence of insulin, even though TSH does not activate MAPKs. This suggests a requirement for a basal activity of MAPKs, and thus perhaps of Ras, as one condition permitting the cAMP-dependent mitogenesis. However, the activation of Ras and MAPKs does not contribute as a *signal* in the still enigmatic mechanism by which cAMP can trigger mitogenesis in dog thyrocytes.

In FRTL-5 cells, active Ras was very recently shown to be required for cAMP-dependent mitogenesis (190), as in WRT cells, but Ras was mentioned not to be directly activated by cAMP (190). TSH and cAMP, through a PKA-dependent mechanism, rapidly stimulate the formation of Ras-PI3K complexes, indicating that cAMP can redirect Ras signaling toward PI3K, but PI3K activity was not investigated (190). Again, the significance of this observation is unclear for the cAMP-dependent proliferation in the presence of insulin/IGF-I, which strongly activates PI3K through its association with tyrosine-phosphorylated IRS proteins, an effect enhanced by cAMP (131) as detailed in *Section V*.

Commercial preparations of bovine pituitary or human recombinant TSH were recently found to activate p42/p44 MAPKs in FRTL-5 cells, but this cAMP-independent effect

was also observed in cell lines that do not express TSH receptors, and thus was ascribed to contaminants of TSH preparations (191). A cAMP-independent activation of p42/p44 MAPKs by TSH was also reported in human thyrocytes (192). In our laboratory this effect is not inhibited by antibodies neutralizing TSH or blocking TSH receptors (F. Vandepuit, unpublished data). It may thus also reflect the contamination of TSH with a growth factor.

E. Summary

In all the systems, the mitogenic effects of TSH are mainly or totally mediated by cAMP and require PKA activity. However, as shown in dog thyrocytes and WRT cells, the activation of PKA is not sufficient to reproduce the cAMP-dependent mitogenic activity, and cAMP activates Rap1 independently of PKA. In all the systems, the cAMP-dependent signaling cascade does not activate the p42/p44 MAPKs, unlike insulin/IGF-I, growth factors, and phorbol esters. In other respects the various models are very different. In WRT cells, overactivation of Ras and each of its potential effectors (Raf/MEK/MAPKs, PI3K, RalGDS) is reported to be sufficient to elicit mitogenesis. In dog thyrocytes, normal activation of Ras, MAPKs, or PI3K by extracellular stimuli is possibly necessary but not sufficient for mitogenesis. In WRT cells, cAMP and insulin/IGF-I are reported to independently activate Ras and PI3K (PKB), and the lack of MAPK activation by cAMP is ascribed to inhibition of c-Raf by PKA. In dog thyrocytes, cAMP does not activate Ras and PI3K. In FRTL-5 cells, the effects of cAMP alone on the various signaling pathways have been poorly explored, but cAMP exerts delayed potentiating effects on the PI3K and MAPK pathways activated by insulin/IGF-I. In the different systems, the different relative effects of cAMP and insulin/IGF-I on the intracellular signaling cascades are thus consistent with their relative effects on proliferation.

VIII. Immediate/Early Genes

Expression of immediate/early genes encoding potentially oncogenic transcription factors is generally considered to function as a third phase in the transduction of mitogenic stimuli. For instance, c-Myc is a strong inducer of proliferation, and its role in cell cycle control has been intensively investigated as it is believed to be critical for its oncogenic properties (193, 194). How c-Myc affects the cell cycle is still unclear. A plethora of mechanisms have been described, mostly derived from overexpression studies, whose physiological relevance is not always supported by genetic information. A sustained increase of c-myc expression is generally considered to be required for induction of important cell cycle-regulatory proteins (such as cyclin D2, E2F2, cdc25A, cyclin E . . .), G₁ phase progression, and DNA synthesis.

The expression of *c-myc* is stimulated by TSH and cAMP enhancers in FRTL-5 cells (77, 78, 195) and in dog (196, 197) and human thyrocytes (198). It is also induced by insulin/IGF-I and TPA in dog thyrocytes (196, 199, 200) and FRTL-5 cells (78), and by EGF and HGF in dog thyrocytes (199, 200). In FRTL-5 cells, TSH/cAMP effects on *c-myc* expression are sustained (77). By contrast, in dog thyrocytes, they are bi-

phasic and the cAMP-dependent increase of *c-myc* expression is very transient, at variance with the sustained effects of growth factors and phorbol esters (197, 199). After a first phase of 1 h of higher levels of *c-myc* mRNA and protein, *c-myc* expression is even decreased below basal levels in TSH or forskolin-treated cells. At 1 h the effects of EGF and cAMP are additive, but at 3 h and thereafter cAMP markedly inhibits the stimulation of *c-myc* expression by EGF (197, 199). *c-Myc* expression is clearly not sufficient for mitogenesis in dog thyrocytes (155, 200), nor in PC Cl3 cells as shown by *c-myc* transfection (4). The involvement of *c-myc* in the cAMP-dependent mitogenic pathway of dog thyrocytes is unclear. *c-Myc* expression is too transient to explain the continuous requirement for high cAMP during the progression into G₁ phase (139). Unlike the relatively durable *c-myc* expression in TSH-stimulated FRTL-5 cells, in dog thyrocytes *c-Myc* is repressed by cAMP just when it is expected to transactivate genes encoding important cell cycle-regulatory proteins. Moreover, the activity of *c-Myc* as a transcription factor has been reported to require its stabilization by its phosphorylation by MAPKs (201), which are not activated by cAMP.

Dimers of proteins of Fos and Jun families compose the AP-1 transcription factors, which are also involved in the synthesis of cell cycle-regulatory proteins such as cyclin D1. *c-Fos* is induced by all studied (co)mitogenic stimuli, including TSH (cAMP), insulin/IGF-I, phorbol esters, serum and growth factors in FRTL-5 (77, 78, 202) and WRT cells (203), and in dog (143, 199, 200) and human thyrocytes (116, 204). *c-Fos* expression has been claimed to be required for TSH-dependent proliferation of FRTL-5 cells (205). However, in dog (143) and human thyrocytes (204) and in WRT cells (203), the effects of TSH and cAMP on *c-fos* expression are very weak compared with the effects of growth factors and phorbol esters, and to the effects of TSH in FRTL-5 cells. In FRTL-5 cells (78) and human fetal thyrocytes (116), TSH and insulin/IGF-I effects are additive, but in WRT cells cAMP represses the induction of *c-fos* mRNA by IGF-I (203). In dog thyrocytes, TSH and forskolin potentiate the induction of *c-fos* mRNA and protein by EGF (143, 199). By contrast, in human thyrocytes (in conditions which do not allow the demonstration of TSH-dependent mitogenesis), TSH inhibits the induction of *c-fos* by TPA and EGF (204). In contrast to *c-fos*, *fos B* is markedly induced by cAMP but weakly or not at all by other factors in dog thyrocytes. Interestingly, this effect is potentiated by insulin (200).

The expression of *c-jun* is stimulated by TPA, growth factors, and insulin/IGF-I in dog (200, 206) and human thyrocytes (204), and in WRT cells (203). By contrast, in these different systems basal and/or stimulated accumulations of *c-jun* mRNA are repressed by TSH and cAMP (200, 203, 204, 206). This difference between cAMP-dependent and -independent factors might be particularly significant, as *c-jun* is frequently considered the rate-limiting factor in the formation of AP-1 transcriptional complexes, downstream to the activation of MAPKs (207). Indeed, AP-1 transcriptional activity stimulated by serum or TPA is repressed by TSH in WRT cells (203). On the contrary, TSH induces *c-jun*-like *c-fos* in FRTL-5 cells (208, 209). Unlike *c-jun*, *jun B* expression is stimulated by cAMP-dependent and cAMP-independent

factors in dog thyrocytes (210), WRT (203), and FRTL-5 cells (209). However, *Jun B* has been found as a repressor of AP1-activity by competition with *c-Jun* in *Jun-Fos* heterodimeric complexes, including on the cyclin D1 promoter (211, 212).

Egr-1 has also been reported as an important transactivator of the cyclin D1 promoter (213). Like *c-jun*, the expression of *egr-1* is induced by insulin/IGF-I, growth factors, and TPA but repressed by TSH (cAMP) in WRT cells (203) and dog thyrocytes (200). By contrast, TSH has been found to stimulate *egr-2* expression in FRTL-5 cells (208).

In no system, a single immediate/early gene expression could thus account for mitogenesis. Collectively, the investigations of the pattern of immediate/early gene expression in dog thyrocytes, WRT cells, and possibly human thyrocytes have shown some overlap, but also major differences, between the signaling pathways of TSH through cAMP, on the one hand, and of insulin/IGF-I, growth factors, and phorbol esters on the other hand. Remarkably, in these cells the expression pattern generated by cAMP is similar to the one observed in other cell types in which the proliferation is stimulated by growth factors but inhibited by cAMP (110, 214). At variance, in FRTL-5 cells available data indicate that TSH and cAMP induce an early gene expression pattern that resembles the one generally described in the response of fibroblasts to growth factors.

IX. Cell Cycle-Regulatory Proteins

The key events in the complex signaling cascades of TSH and insulin/IGF-I are still largely hypothetical. However, these cascades and those of growth factors are expected to finally modulate the level and activity of proteins that are the primary regulators of the cell cycle machinery. As generally considered, mitogenic signals regulate mammalian cell cycle by stimulating the accumulation of D-type cyclins and their assembly through a ill-defined mechanism with their partners the cyclin-dependent kinases (cdk) 4 and 6 (215). These complexes operate in mid-to-late G₁ phase to promote progression through the restriction point, and thus commit cells to replicate their genome. In the current model, this key decision depends on the initiation by cyclin D-cdk complexes of the phosphorylation of the growth/tumor suppressor protein pRb, which triggers the activation of transcription factors, including those of the E2F family, the synthesis of cyclin E and then cyclin A, and cdk2 activation by these cyclins. Activated cdk2, in turn, further phosphorylates pRb and other substrates and initiates and organizes the progression through the DNA synthesis phase (216, 217). The down-regulation of cdk inhibitors of the CIP/KIP family, including p27^{kip1}, by mitogenic factors and/or their sequestration by cyclin D-cdk complexes participate in cdk2 activation, but their proposed role of adaptor and/or nuclear anchor for cyclin D-cdk complexes suggests positive influences on cell cycle progression as well (218).

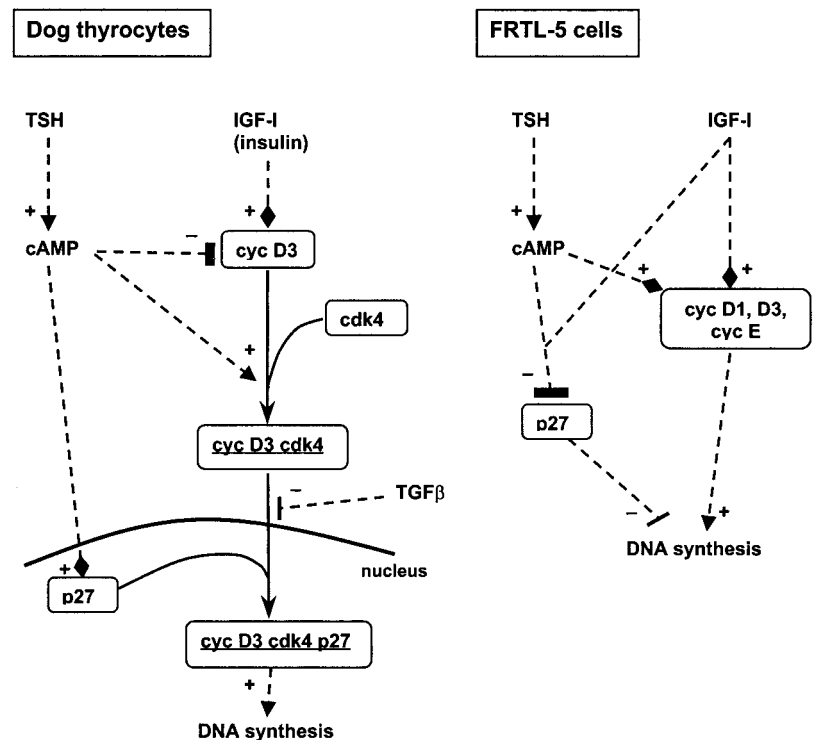
In dog thyrocytes, the different mitogenic stimulations (TSH, cAMP, growth factors) require the activity of cdk4 (219) and converge on the inactivating phosphorylation of pRb and related proteins p107 and p130 (220), on the phos-

phorylation and nuclear translocation of cdk2 (221), and on the induction of cyclin A and cdc2 (221). These effects are dependent on insulin (220, 222). Cyclin D3 is the predominant D-type cyclin expressed in dog thyrocytes (223) and in mouse thyroid *in vivo* (224). TSH, unlike all the other known mitogenic factors, does not induce the accumulation of D-type cyclins (223), but it stimulates the expression of p27^{kip1} (225). Nevertheless, cyclin D3 is required for the proliferation stimulated by TSH, but not in the proliferation of dog thyrocytes stimulated by EGF or HGF, which induce cyclins D1 and D2 in addition to increasing cyclin D3 levels (223). As depicted in Fig. 4, the formation and the nuclear translocation of essential cyclin D3-cdk4 complexes depend on the synergistic interaction of TSH and insulin in dog thyrocytes (222, 223). These complexes are absent from cells stimulated by TSH or insulin alone. Paradoxically, in the absence of insulin, TSH strongly inhibits the basal accumulation of cyclin D3 (222). In contrast, insulin alone stimulates the required cyclin D3 accumulation, and it overcomes in large part the inhibition by TSH (222), but it is unable to assemble cyclin D3-cdk4 complexes in the absence of TSH. In the presence of insulin, TSH (cAMP) unmasks some epitopes of cyclin D3 and induces the assembly of cyclin D3-cdk4 complexes and their import into nuclei (222, 223), where these complexes are presumably anchored by their association with p27^{kip1} (Fig. 4), which is then sequestered away from cdk2 complexes (226), thus contributing to cdk2 activation. TGF β selectively inhibits the cAMP-dependent proliferation of dog thyrocytes by preventing the association of cyclin D3 and cdk4 with nuclear p27^{kip1}, but it does not affect the assembly of cyclin D3-cdk4 complexes probably formed in the cytoplasm (226). Moreover, cAMP exerts an additional crucial function in very late G₁ phase, because pRb phos-

phorylation and DNA synthesis initiation still depend on sustained cAMP elevation, even after cAMP induction of stable nuclear cyclin D3-cdk4-p27 complexes (140). The investigation of cell cycle-regulatory proteins has thus clearly established that both cdk4 activation and pRb phosphorylation result from distinct but complementary actions of TSH and insulin, rather than from their interaction at an earlier step of the signaling cascades (141, 222) (Figs. 4 and 5).

In very sharp contrast, in FRTL-5 cells, TSH (cAMP) and insulin/IGF-I are both able to induce cyclin D1 and cyclin E (64, 93) (Fig. 4). In addition, TSH accelerates the IGF-I-stimulatory effects on cyclin D1 accumulation (64). These effects are probably related to the induction of *c-jun* and *egr-2*, which are known to transactivate the cyclin D1 promoter (212, 213). Cyclin D3 levels are also increased (227). In FRTL-5 cells, the activation of cdk2 is ascribed to the down-regulation of p27^{kip1} induced by TSH alone (61, 93) and probably even more by the combination of TSH and IGF-I (228, 229). The delay of the onset of S phase provoked by the up-regulation of the PKA pathway by exogenous cAMP during G₁ phase was associated with an inhibition of p27 decay (134). In FRTL-5 cells, the synergy of TSH and insulin on cell cycle progression is thus mediated by an increase of cyclin D levels and a decrease of p27 levels likely resulting from an earlier integration of TSH and insulin cascades (Figs. 4 and 5). The mechanism of the TSH mitogenic action, therefore, much resembles the action of IGF-I in these cells and of growth factors in other cell types. The possible contribution of the autocrine loop involving FGF and FGF receptors should be examined. In PC Cl3 cells, as in FRTL-5 cells, TSH and insulin additively induce the expression of cyclin D1, cyclin D2, and cdk4 and enhance cyclin D3 levels (S. Demartin and P. P. Roger, unpublished data). However, in

FIG. 4. Respective effects of TSH (cAMP) and insulin/IGF-I on cell cycle-regulatory proteins in dog thyrocytes in primary culture *vs.* FRTL-5 cells. *Straight lines* indicate transport or chemical associations or reactions. *Diamond/rectangle arrowheads* represent inductions/repressions; the other *dashed arrows* are activations (+) and inhibitions (-) (144). cAMP exerts opposite effects on the accumulation of p27^{kip1} and D-type cyclins in dog thyrocytes *vs.* FRTL-5 cells.



our hands, the high levels of p27^{kip1} are relatively unaffected by TSH and insulin alone or in combination. Cell cycle-regulatory proteins have not been analyzed in WRT cells.

In summary, the expression of G₁ phase regulatory proteins, which constitute the end points at which mitogenic signaling pathways are expected to be integrated, still markedly differ in FRTL-5 and PC-C13 cells *vs.* dog thyroid primary cultures. In FRTL-5 cells, D-type cyclins are induced, and p27 down-regulated, in response to both TSH and insulin/IGF-I, as generally observed in fibroblasts stimulated by growth factors. In dog thyrocytes, TSH does not induce D-type cyclins, but it increases p27 expression and activates cyclin D3 synthesized in response to insulin, which results in the assembly of required nuclear cyclin D3-cdk4-p27 complexes (Fig. 4).

According to our preliminary results in primary cultures of human thyrocytes, cdk4 is constitutively expressed, and cyclin D1 accumulation is stimulated by EGF and serum, but not affected or even markedly repressed by TSH and forskolin, as in dog cells. Cyclin D2 is undetectable, and the high basal accumulation of cyclin D3 is slightly enhanced by TSH or insulin in different experiments. p27 Levels are not down-regulated by TSH in the presence of insulin. Thus, these important cell cycle-regulatory proteins are not subjected to significant modulations of expression during TSH-stimulated G₁ phase progression of human thyrocytes. This is in sharp contrast to rat cell lines and also to the increased expression of cyclin D1 and down-regulation of p27 associated with the sustained proliferation induced by oncogenic Ras in human thyrocytes (178). The molecular target(s) of the mitogenic action of TSH through cAMP, therefore, remains to be identified in human thyrocytes.

X. *In Vivo* Models

The thyroid cell that obviously most interests us is the human thyrocyte *in vivo*. Clinical investigation has allowed us to validate in this context several concepts developed on the basis of *in vitro* models, especially human cells in primary culture.

To develop to adult stage, the human thyroid requires the number of divisions necessary to generate about 2.10⁹ cells of the adult from 1 or a few cells in the embryo: about 30 divisions if there are no cell deaths. At the adult stage, the human thyrocytes divide once every 8 yr, *i.e.*, about six times (230). This by itself does not imply a limit of the lifespan. However in rats, chronic stimulation by TSH causes thyroid growth up to a plateau (231), and in dog thyrocyte primary cultures stimulated proliferation abruptly stops after four to six divisions (53), which might suggest such a limit. Auto-crine mechanisms, such as the TSH-stimulated production by thyrocytes of growth inhibitors including TGFβ, could also be involved in the specific desensitization of the proliferative response to TSH, as first suggested from the FRTL-5 model (232), and validated in rats *in vivo* (233, 234).

The mitogenic role of the TSH receptor and of the cAMP cascade that it activates is supported, in man, by the growth of the thyroid in patients with TSH-secreting pituitary adenomas and in patients with Graves' disease (235). The serum

TSAb found in the latter disease, *i.e.*, antibodies against the TSH receptor, stimulate predominantly the cAMP cascade (236). TSH and TSAAb massively stimulate DNA synthesis in nonneoplastic human thyroid tissues xenotransplanted in nude mice (237). In contrast, TSH deficiency or TSH receptor-inactivating mutations are accompanied by thyroid hypothyrophy (238, 239). The predicted role of the TSH receptor and its cAMP cascade has also been validated by the discovery of somatic and germline mutations of the TSH receptor (240, 241) and Gsα (242–245), causing constitutive activation of these proteins and their subsequent cascades in autonomous adenomas and congenital hyperthyroidism.

On the other hand, goiter is a frequent clinical finding in acromegalic patients, an effect mediated by chronically elevated IGF-I levels (246, 247). Nevertheless, the presence of basal TSH levels might be a prerequisite for the growth-promoting action of IGF-I, because a GH replacement therapy did not increase thyroid size in patients deficient for both GH and TSH (248). The anomalously low endemic goiter prevalence among pygmies living in iodine-deficient areas (249), who are genetically resistant to IGF-I, is also compatible with an *in vivo* permissive effect of IGF-I and IGF-I receptor on TSH mitogenic action. The *in vitro* demonstrated role of PI3K in the supportive proliferation effects of IGF-I and insulin is also consonant with the high incidence of thyroid tumors in patients with Cowden disease (250). These patients are congenitally deficient in PTEN (250), the 3'-phosphatase that catabolizes intracellular PIP3 and PIP2 signals generated by PI3K. Somatic hemizygous deletions of PTEN are also frequently found in follicular adenomas and a few thyroid carcinomas (251).

Transgenic and natural mutant mouse models have also validated *in vivo* many conclusions of *in vitro* studies. The role of the TSH receptor in thyroid growth is demonstrated by the hypothyrophy of the thyroid in mice with natural TSH receptor-inactivating mutations (hyt/hyt) (252). The *in vivo* mitogenic role of the cAMP cascade is supported by the phenotype of TgA2R mice in which the constitutive activation of adenylyl cyclase by the adenosine A2 receptor expressed in thyroid leads to goiter and hyperthyroidism (253). Similar, albeit weaker, phenotypes are obtained in mice expressing constitutive Gs (the G protein activating adenylyl cyclase) (254), cholera toxin (255), or constitutive adrenergic α2 receptor (256) (which activates Gs). By contrast, transgenic mice overexpressing both human IGF-I and IGF-I receptor in their thyroid (TgIGF-I-TgIGF-IR) develop only a mild thyroid hyperplasia and respond to a goitrogenic effect of antithyroid drugs while maintaining a comparatively low serum TSH level. This indicates some autonomy of these thyroids, as in acromegalic patients, and a much greater sensitivity to endogenous TSH (S. Clément, S. Refetoff, B. Robaye, J. E. Dumont, and S. Schurmans, unpublished results). To distinguish the relative importance of the two mechanisms will require the cross of TgIGF-I-TgIGF-IR and hyt/hyt mice. Thus, the phenotype of TgIGF-I-TgIGF-IR mice supports the concept of the permissive role of the IGF-I system on the cAMP mitogenic cascade, and also of some independent effects of the overactivation of this system on growth and function. The functional and goitrogenic effects of endogenous TSH elevations by antithyroid drugs are im-

paired in rats and mice made diabetic by streptozotocin. These defects are corrected by insulin (257, 258), but whether they primarily involve the thyroid gland, pituitary, or peripheral tissues remains unclear (259).

In rats, mice, and humans *in vivo*, chronic stimulation by TSH secondary to treatment with antithyroid drugs induces thyroid hyperplasia but also a marked hypertrophy of follicular cells. This apparent discrepancy with the lack of *in vitro* effect of TSH and cAMP on cell size in dog and human thyroid primary cultures (105) might be explained by the stimulation by TSH of IGF-I secretion by thyrocytes, which could activate the cells *in vivo* through an autocrine mechanism, but be diluted out *in vitro*. Stimulating effects of TSH on the accumulation by thyrocytes of IGF-I mRNA and peptide have indeed been demonstrated in mice (260).

Growth factor-signaling cascades demonstrated *in vitro* can exert similar effects *in vivo*. In nude mice, the injection of EGF promotes DNA synthesis in thyroid and inhibits iodide uptake in xenotransplanted rat (261) and human thyroid tissues (262). By contrast, the injection of FGF induces a colloid goiter in mice with no inhibition of iodide metabolism or thyroglobulin and thyroperoxidase mRNA accumulation (263). These effects are the exact replica of initial observations from the dog thyroid primary culture system (101, 102) and other thyroid primary culture systems (36, 41, 122, 123). EGF and FGF have since been found to be locally synthesized in the thyroid gland, as a possible response to T₄ (264) and TSH (137), respectively. Their exact role as autocrine and/or paracrine agents in the development, function, and pathology of the thyroid gland of different species has yet to be clarified (47, 265). The overexpression of both FGF and FGF receptor 1 in thyrocytes from human multinodular goiter might explain its relative TSH independence (266). On the other hand, the subversion of tyrosine kinase pathways similar to those normally operated by local growth factors [*i.e.*, the activation of Ret/PTC (267) and TRK (268), the overexpression of Met/HGF receptor sometimes in association with HGF (269), or erbB/EGF receptor in association with its ligand TGF α (270)] can be causally associated with TSH-independent thyroid papillary carcinomas, as demonstrated in transgenic mice in the case of different forms of Ret (271, 272).

The role of some *in vitro* studied downstream elements of the thyroid mitogenic cascades is also supported by studies of transgenic mice. The expression in thyroid of a dominant negative CREB provokes a marked thyroid hypotrophy, suggesting the crucial role of CREB and its activating phosphorylation by PKA (273). TgE7 mice, which express the HPV16E7 gene in their thyroid, develop an euthyroid goiter (148). The E7 protein sequesters the Rb protein, thus releasing its negative control of E2F transcription factors. Rb protein inactivation by phosphorylation has been shown *in vitro* to be a common control point of all the thyroid mitogenic cascades. The massive cAMP-dependent thyroid hyperplasia of TgA2R mice is not associated with a down-regulation of nuclear p27^{kip1} (224), as in TSH-stimulated dog thyroid primary cultures (225), but at variance with the stimulation of FRTL-5 cells by TSH (61, 228).

Thus, experimental evidence on mice *in vivo* and clinical evidence in human disease, when they exist, validate the

mitogenic regulatory scheme derived from *in vitro* studies on dog and human thyroid cells in primary culture.

XI. Discussion

In this review article, we have examined the hypothesis that the hyperplasia of thyroid gland, as generated by the activation of TSH receptors, can be validly investigated by means of the available *in vitro* experimental models. Rat thyroid cell lines and primary cultures of canine and human origins maintain *in vitro* an excellent expression of TSH-dependent thyroid-differentiated functions. In all the systems, TSH, in large part or totally through cAMP, and insulin or IGF-I synergize and induce cell proliferation. Both in FRTL-5 cells (274) and in dog thyroid primary cultures (53), the TSH/cAMP-dependent mitogenesis has been described as a differentiated trait, which is adjunctive to the more general mechanisms of growth control by growth factors. Its unique characteristics might explain how it can be compatible with differentiation expression (275, 276), which is repressed by growth factors (277), phorbol esters (67, 103), or Ras activation (278). Quite unexpectedly, however, beyond this framework of common features, the confrontation of the experimental systems strikingly illustrates that similar phenotypes may rely on quite divergent mechanisms. In fact, it is now clear that the respective roles of TSH and insulin/IGF-I are different in the different systems.

The main divergences include 1) the insulin/IGF-I dependence of TSH receptor expression in FRTL-5 cells but not in dog and human thyrocytes and, conversely, the TSH dependence of insulin receptor expression and of insulin receptor-mediated mitogenesis in dog and human thyrocytes but not in FRTL-5 cells; 2) the increase of cell mass caused by both TSH and insulin/IGF-I in rat cell lines, but only by insulin/IGF-I in dog and human thyrocytes; 3) the independent activation of PI3K and Ras by both insulin/IGF-I and TSH (cAMP) in WRT cells, but not by TSH and cAMP in dog thyrocytes; 4) the profoundly different effects of TSH and insulin/IGF-I on cell cycle kinetics and cell cycle-regulatory proteins in FRTL-5 and PC Cl3 cells *vs.* dog and possibly human thyrocytes.

In FRTL-5 cells, TSH induces early genes such as *c-jun* and *c-myc*, stimulates the expression of D-type cyclins, and down-regulates p27. Possibly because this reinforces similar actions of insulin/IGF-I, and also because cAMP augments the signaling pathways of IGF-I leading to the activations of MAPKs and PI3K (131) (Fig. 5), TSH exerts a priming effect, making the cell more competent to progress into G₁ phase in response to insulin/IGF-I alone, which can thus be qualified as the only genuine mitogen. Further TSH presence is dispensable during G₁ phase progression, and maintenance of high cAMP levels even delays DNA synthesis initiation. In fact, in FRTL-5 cells, TSH and insulin/IGF-I induce a similar pattern of responses, and TSH mostly potentiates IGF-I action (Fig. 5). Whether part of these TSH/cAMP comitogenic signaling events are indirectly mediated by autocrine growth factors such as FGF has yet to be definitively demonstrated.

In dog thyrocytes, TSH has a similar delayed potentiating action on IGF-I transduction (104). However, its main actions

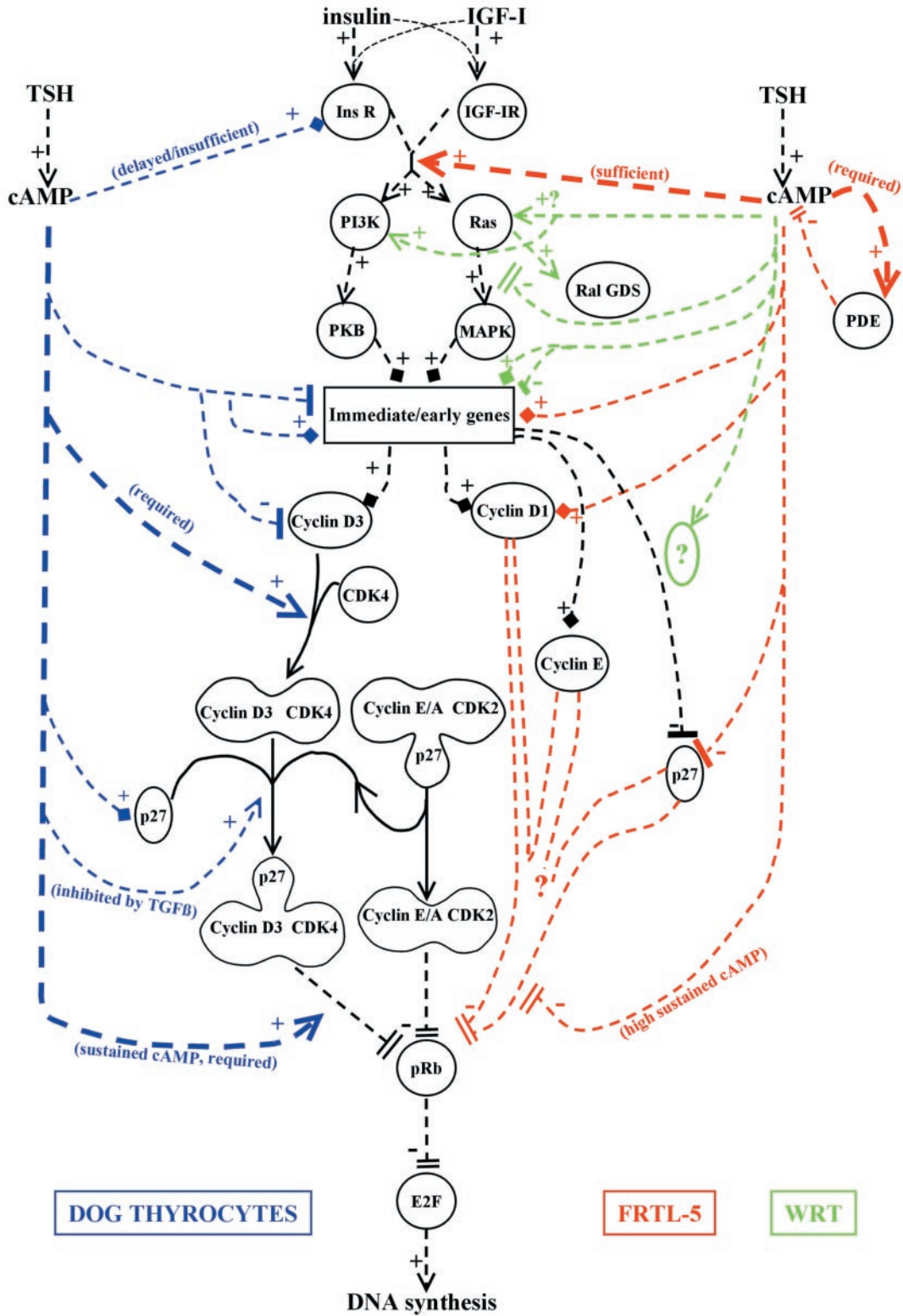


FIG. 5. Overall summary of synergistic interactions of comitogenic pathways of TSH and insulin/IGF-I mapped in dog thyroid primary cultures *vs.* FRTL-5 and WRT rat thyroid cell lines. Only the main regulations are included. TSH/cAMP actions demonstrated in dog thyrocytes, FRTL-5, and WRT cells are displayed in blue, red, and green, respectively. The symbolic representation of signal transduction controls is as proposed recently (144). Straight lines indicate transport or chemical associations or reactions. Diamond/rectangle arrowheads represent inductions/repressions; the other dashed arrows are activations and inhibitions. See text for references, details, and explanations.

are essentially different in these cells and involve more direct effects of cAMP on G₁ phase progression (Fig. 5). Unlike growth factors and/or insulin, TSH does not activate Ras, PI3K, PKB, or MAPKs. It down-regulates the expression of *c-myc* (after a short initial induction), *c-jun*, and *egr-1*. As a likely consequence, TSH rather inhibits the accumulation of D-type cyclins but stimulates the expression of p27^{kip1}. In dog thyrocytes, TSH must continuously elevate cAMP levels to directly control the passage through the restriction point. This requires, at least in part, critical actions on the assembly and nuclear translocation of cyclin D3-cdk4 complexes, which depend on the cAMP-dependent activation of the necessary cyclin D3, itself synthesized in response to insulin/IGF-I. Thus, at least in a first stage, the formation of cyclin D3-cdk4 complexes and the phosphorylation of pRb result from distinct but complementary actions of TSH and insulin/IGF-I, rather than their interaction at an earlier step of the signaling cascades. Together with the fact that the necessary increase of cell mass before division depends on insulin/IGF-I but not TSH, these observations provide a molecular basis for the well established physiological concept that in the regulation of normal thyroid cell proliferation, TSH is the “decisional” mitotic trigger, while locally produced IGF-I and/or circulating insulin are supporting “permissive” factors (52).

The reasons for these major differences between different experimental models of the same cell are unclear. They may obviously reflect species differences (52). Nevertheless, even among the apparently similar rat thyroid cell lines, major differences have been observed. The induction of *c-jun* by TSH/cAMP in FRTL-5 cells and its repression by cAMP in WRT cells, as in dog and human thyrocytes, likely reflect major differences in upstream signaling cascades and should result in divergent expression of downstream target genes, such as cyclin D1. Some signaling features, when they lead to selective proliferative advantages, might have been acquired during the establishment and continuous cultures of the cell lines and stabilized by subcloning. In the FRTL-5 cell line, this is facilitated by its instability exemplified by the spontaneous generation of variants with altered TSH dependence for growth or escaping from TGF β inhibition (14, 17). Single mutations may completely change the complex interplay of signaling cascades. A model for this has been evidenced in WRT cells, where introduction of a mutated Ras^{V12 G37}, but not other Ras^{V12} mutants, leads to a strong MAPK activation by cAMP (182), which is expected to profoundly influence the mechanism of cAMP-dependent mitogenesis. Among rat cell lines, PC Cl3 cells might more closely correspond to rat thyroid primary cultures. Unlike FRTL-5 cells, their transformation requires a two-hit mechanism, and their characteristics appear more stable until now. They are thus the most suitable for the investigation of mechanisms of multistep oncogenic transformation.

In the present analysis, we have considered the interest of model systems on the point of view of the closeness of their properties with those of the normal human cell *in vivo*. We were not concerned about the basic cell biology interest they have by themselves. Rat cell lines are valuable models for the investigation of mechanisms that underlie the thyroid-specific expression of differentiation genes (7, 279) [never-

theless oncogenic Ras was recently shown not to inhibit differentiation expression in human thyrocytes (177), at variance with rat cell lines]. Moreover, the different thyrocyte systems constitute interesting models of the wide diversity of possible mechanisms of the cAMP-dependent proliferation observed in other cell types including several endocrine epithelia (110, 280). However, clues gathered in the present review article are sufficient to suggest caution to the investigator contemplating the examination of human thyroid cell proliferation with rat thyroid cell lines as model systems. Although up-regulation of cyclin D1 or cyclin D3 and down-regulation or cytoplasmic retention of p27^{kip1} may play an essential role in human thyroid tumorigenesis (281–283), they are not observed during the normal stimulation of human thyrocyte proliferation by TSH.

Dog thyrocyte primary cultures are the only system responding to the full range of (co)mitogens demonstrated in human thyrocytes. However, this system does not allow the analysis of the cAMP-independent component(s) of TSH action. It is marred by its restricted accessibility and its limited proliferation capacity. Most mechanisms demonstrated in this system so far apply to normal human thyrocytes, but much remains to be defined. This constitutes part of our current efforts, but will prove an especially difficult and frustrating task, because of the difficulty of obtaining normal tissue in sufficient amount, and the inherent variability between individuals (which nevertheless reflects rather than distorts reality). Moreover, comparison of data obtained for human thyrocytes in different centers is prevented by the absence of a consensus on culture protocols to be applied. However, the predictions based on the extensive *in vitro* investigation of dog thyroid cells in primary culture and the more scarce data on human cells have been supported by the available *in vivo* experimental data on transgenic mice and the clinical data in man. In both these systems, chronic activation of the TSH/cAMP cascade leads to hyperfunction and growth, and, conversely, in both systems IGF-I does not induce marked growth but sensitizes the thyroid to the action of TSH. One conclusion is also established: there is no such thing as “the thyroid cell” and many (but hopefully not all) extrapolations from results of *in vitro* model systems to the normal human thyroid cell are presently unwarranted, unless validated.

Acknowledgments

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References

1. Aouani A, Hovsepian S, Fayet G 1987 cAMP dependent and independent regulation of thyroglobulin synthesis by two clones of the OVNIS 6H thyroid cell line. *Mol Cell Endocrinol* 52:151–160

2. **Ambesi-Impiombato FS, Picone R, Tramontano D** 1982 Influence of hormones and serum on growth and differentiation of the thyroid cell strain FRTL. In: Sato GH, Pardee AB, Sirbaku DA, eds. Growth of cells in hormonally defined media, vol 9. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 483–492
3. **Brandi ML, Rotella CM, Mavilia C, Franceschelli F, Tanini A, Toccafondi R** 1987 Insulin stimulates cell growth of a new strain of differentiated rat thyroid cells. *Mol Cell Endocrinol* 54:91–103
4. **Fusco A, Berlingieri MT, Di-Fiore PP, Portella G, Grieco M, Vecchio G** 1987 One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes. *Mol Cell Biol* 7:3365–3370
5. **Ambesi-Impiombato FS, Villone G** 1987 The FRTL-5 thyroid cell strain as a model for studies on thyroid cell growth. *Acta Endocrinol Suppl (Copenh)* 281:242–245
6. **Bidey SP, Lambert A, Robertson WR** 1988 Thyroid cell growth, differentiation and function in the FRTL-5 cell line: a survey. *J Endocrinol* 119:365–376
7. **Damante G, Di Lauro R** 1994 Thyroid-specific gene expression. *Biochim Biophys Acta* 1218:255–266
8. **Medina DL, Santisteban P** 2000 Thyrotropin-dependent proliferation of *in vitro* rat thyroid cell systems. *Eur J Endocrinol* 143:161–178
9. **Peter HJ, Gerber H, Studer H, Groscurth P, Zakarija M** 1991 Comparison of FRTL-5 cell growth *in vitro* with that of xenotransplanted cells and the thyroid of the recipient mouse. *Endocrinology* 128:211–219
10. **Ossendorp FA, Bruning PF, Schuurin EM, Van Den Brink JA, van der Heide D, De Vijlder JJ, De Bruin TW** 1990 Thyrotropin dependent and independent thyroid cell lines selected from FRTL-5 derived tumors grown in nude mice. *Endocrinology* 127:419–430
11. **Ambesi-Impiombato FS, Parks LA, Coon HG** 1980 Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc Natl Acad Sci USA* 77:3455–3459
12. **Li X, Lu S, Miyagi E, Katoh R, Kawaoi A** 1999 Thyrotropin prevents apoptosis by promoting cell adhesion and cell cycle progression in FRTL-5 cells. *Endocrinology* 140:5962–5970
13. **Huber G, Derwahl M, Kaempf J, Peter HJ, Gerber H, Studer H** 1990 Generation of intercellular heterogeneity of growth and function in cloned rat thyroid cells (FRTL-5). *Endocrinology* 126:1639–1645
14. **Asmis LM, Kaempf J, Von Gruenigen C, Kimura ET, Wagner HE, Studer H** 1996 Acquired and naturally occurring resistance of thyroid follicular cells to the growth inhibitory action of transforming growth factor- β 1 (TGF- β 1). *J Endocrinol* 149:485–496
15. **Davies TF, Yang C, Platzer M** 1987 Cloning the Fisher rat thyroid cell line (FRTL-5): variability in clonal growth and 3',5'-cyclic adenosine monophosphate response to thyrotropin. *Endocrinology* 121:78–83
16. **Endo T, Shimura H, Saito T, Onaya T** 1990 Cloning of malignantly transformed rat thyroid (FRTL) cells with thyrotropin receptors and their growth inhibition by 3',5'-cyclic adenosine monophosphate. *Endocrinology* 126:1492–1497
17. **Coppa A, Mincione G, Mammarella S, Ranieri A, Colletta G** 1995 Epithelial rat thyroid cell clones, escaping from transforming growth factor β negative growth control, are still inhibited by this factor in the ability to trap iodide. *Cell Growth Differ* 6:281–290
18. **Bellur S, Tahara K, Saji M, Grollman EF, Kohn LD** 1990 Repeatedly passed FRTL-5 rat thyroid cells can develop insulin and insulin-like growth factor-I-sensitive cyclooxygenase and prostaglandin E_2 isomerase-like activities together with altered basal and thyrotropin-responsive thymidine incorporation into DNA. *Endocrinology* 127:1526–1540
19. **Chen G, Pekary AE, Hershman JM** 1992 Aging of FRTL-5 rat thyroid cells causes sensitivity to cytotoxicity induced by tumor necrosis factor- α . *Endocrinology* 131:863–870
20. **Pekary AE, Berg L, Wang J, Lee P, Dubinett SM, Hershman JM** 1995 TNF- α , TSH, and aging regulate TGF- β synthesis and secretion in FRTL-5 rat thyroid cells. *Am J Physiol* 268:R808–R815
21. **Pekary AE, Hershman JM** 1998 Tumor necrosis factor, ceramide, transforming growth factor- β 1, and aging reduce Na⁺/I⁻ symporter messenger ribonucleic acid levels in FRTL-5 cells. *Endocrinology* 139:703–712
22. **Pekary AE, Levin SR, Johnson DG, Berg L, Hershman JM** 1997 Tumor necrosis factor- α (TNF- α) and transforming growth factor- β 1 (TGF- β 1) inhibit the expression and activity of Na⁺/K⁽⁺⁾-ATPase in FRTL-5 rat thyroid cells. *J Interferon Cytokine Res* 17:185–195
23. **Taniguchi SI, Shong M, Giuliani C, Napolitano G, Saji M, Montani V, Suzuki K, Singer DS, Kohn LD** 1998 Iodide suppression of major histocompatibility class I gene expression in thyroid cells involves enhancer A and the transcription factor NF- κ B. *Mol Endocrinol* 12:19–33
24. **Golstein J, Kimura T, Miot F, Dumont JE** 1999 Loss of several cell functions including okadaic acid-induced apoptosis after multiple passages in FRTL-5 cells. *Mol Cell Endocrinol* 150:141–149
25. **Zimmermann-Belsing T, Rasmussen AK, Feldt-Rasmussen U** 1998 Lack of thyroglobulin synthesis as an indicator of early random dedifferentiation of the Fischer rat thyroid cell line FRTL-5. *Scand J Clin Lab Invest* 58:529–535
26. **Pajer Z, Cor A, Stiblar-Martincic D** 2000 Morphology of FRTL-5 cell colonies in a semi-solid medium. *J Endocrinol Invest* 23:218–222
27. **Tasevski V, Benn D, Peters G, Luttrell B, Simpson A** 1998 The Fischer rat thyroid cell line FRTL-5 exhibits a nondiploid karyotype. *Thyroid* 8:623–626
28. **Bond JA, Wyllie FS, Ivan M, Dawson T, Wynford-Thomas D** 1993 A variant epithelial sub-population in normal thyroid with high proliferative capacity *in vitro*. *Mol Cell Endocrinol* 93:175–183
29. **Roger P, Taton M, Van Sande J, Dumont JE** 1988 Mitogenic effects of thyrotropin and adenosine 3',5'-monophosphate in differentiated normal human thyroid cells *in vitro*. *J Clin Endocrinol Metab* 66:1158–1165
30. **Williams DW, Williams ED, Wynford-Thomas D** 1988 Loss of dependence on IGF-1 for proliferation of human thyroid adenoma cells. *Br J Cancer* 57:535–539
31. **Eggo MC, King WJ, Black EG, Sheppard MC** 1996 Functional human thyroid cells and their insulin-like growth factor-binding proteins: regulation by thyrotropin, cyclic 3',5' adenosine monophosphate, and growth factors. *J Clin Endocrinol Metab* 81:3056–3062
32. **Kraiem Z, Sadeh O, Yosef M** 1991 Iodide uptake and organification, tri-iodothyronine secretion, cyclic AMP accumulation and cell proliferation in an optimized system of human thyroid follicles cultured in collagen gel suspended in serum-free medium. *J Endocrinol* 131:499–506
33. **Rapoport B** 1976 Dog thyroid cells in monolayer tissue culture: adenosine 3',5'-cyclic monophosphate response to thyrotropic hormone. *Endocrinology* 98:1189–1197
34. **Roger PP, Christophe D, Dumont JE, Pirson I** 1997 The dog thyroid primary culture system: a model of the regulation of function, growth and differentiation expression by cAMP and other well-defined signaling cascades. *Eur J Endocrinol* 137:579–598
35. **Fayet G, Michel-Bichet M, Lissitzky S** 1971 Thyrotrophin-induced aggregation and reorganization into follicles of isolated porcine thyroid cells in culture. 2. Ultrastructural studies. *Eur J Biochem* 24:100–111
36. **Westermarck K, Karlsson FA, Westermarck B** 1983 Epidermal growth factor modulates thyroid growth and function in culture. *Endocrinology* 112:1680–1686
37. **Mauchamp J, Mirrione A, Alquier C, Andre F** 1998 Follicle-like structure and polarized monolayer: role of the extracellular matrix on thyroid cell organization in primary culture. *Biol Cell* 90:369–380
38. **Ericson LE, Nilsson M** 1996 Effects of insulin-like growth factor I on growth, epithelial barrier and iodide transport in polarized pig thyrocyte monolayers. *Eur J Endocrinol* 135:118–127
39. **Gartner R, Greil W, Demharter R, Horn K** 1985 Involvement of cyclic AMP, iodide and metabolites of arachidonic acid in the regulation of cell proliferation of isolated porcine thyroid follicles. *Mol Cell Endocrinol* 42:145–155
40. **Westermarck K, Westermarck B** 1982 Mitogenic effect of epidermal growth factor on sheep thyroid cells in culture. *Exp Cell Res* 138:47–55
41. **Eggo MC, Bachrach LK, Fayet G, Errick J, Kudlow JE, Cohen MF, Burrow GN** 1984 The effects of growth factors and serum on DNA

- synthesis and differentiation in thyroid cells in culture. *Mol Cell Endocrinol* 38:141–150
42. Hill DJ, Phillips ID, Wang JF, Becks GP 1994 Basic fibroblast growth factor (basic FGF) in isolated ovine thyroid follicles: thyrotropin stimulation and effects of basic FGF on DNA synthesis, iodine uptake and organification, and the release of insulin-like growth factors (IGFs) and IGF-binding proteins. *Thyroid* 4:77–85
 43. Gerard CM, Roger PP, Dumont JE 1989 Thyroglobulin gene expression as a differentiation marker in primary cultures of calf thyroid cells. *Mol Cell Endocrinol* 61:23–35
 44. Dumont JE, Maenhaut C, Pirson I, Baptist M, Roger PP 1991 Growth factors controlling the thyroid gland. *Baillieres Clin Endocrinol Metab* 5:727–754
 45. Eggo MC, Bachrach LK, Burrow GN 1990 Interaction of TSH, insulin and insulin-like growth factors in regulating thyroid growth and function. *Growth Factors* 2:99–109
 46. Bachrach LK, Eggo MC, Hintz RL, Burrow GN 1988 Insulin-like growth factors in sheep thyroid cells: action, receptors and production. *Biochem Biophys Res Commun* 154:861–867
 47. Bidey SP, Hill DJ, Eggo MC 1999 Growth factors and goitrogenesis. *J Endocrinol* 160:321–332
 48. Nitsch L, Wollman SH 1980 Thyrotropin preparations are mitogenic for thyroid epithelial cells in follicles in suspension culture. *Proc Natl Acad Sci USA* 77:2743–2747
 49. Munari-Silem Y, Mesnil M, Selmi S, Bernier-Valentin F, Rabiloud R, Rousset B 1990 Cell-cell interactions in the process of differentiation of thyroid epithelial cells into follicles: a study by microinjection and fluorescence microscopy on *in vitro* reconstituted thyroid follicles. *J Cell Physiol* 145:414–427
 50. Kraiem Z, Sadeh O, Heinrich R 2000 Preparation and culture of a serum-free human thyroid follicle system and its application for measuring thyroid hormone secretion, iodide uptake and organification, cyclic adenosine monophosphate formation, gene expression, and cell growth. *Thyroid* 10:53–57
 51. Nilsson M, Husmark J, Nilsson B, Tisell LE, Ericson LE 1996 Primary culture of human thyrocytes in Transwell bicameral chamber: thyrotropin promotes polarization and epithelial barrier function. *Eur J Endocrinol* 135:469–480
 52. Dumont JE, Lamy F, Roger P, Maenhaut C 1992 Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. *Physiol Rev* 72:667–697
 53. 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. *J Cell Biol* 117:383–393
 54. Lemoine NR, Wynford-Thomas D 1997 Transfection and transformation of human thyroid epithelial cells. *Methods Mol Biol* 75:441–447
 55. Rotella CM, Mavilia C, Frediani U, Toccafondi R 1989 Calf serum modifies the mitogenic activity of epidermal growth factor in WRT thyroid cells. *Mol Cell Endocrinol* 65:63–74
 56. Tramontano D, Cushing GW, Moses AC, Ingbar SH 1986 Insulin-like growth factor-I stimulates the growth of rat thyroid cells in culture and synergizes the stimulation of DNA synthesis induced by TSH and Graves'-IgG. *Endocrinology* 119:940–942
 57. Zakarija M, McKenzie JM 1989 Variations in the culture medium for FRTL5 cells: effects on growth and iodide uptake. *Endocrinology* 125:1253–1259
 58. Jin S, Hornicek FJ, Neylan D, Zakarija M, McKenzie JM 1986 Evidence that adenosine 3',5'-monophosphate mediates stimulation of thyroid growth in FRTL5 cells. *Endocrinology* 119:802–810
 59. Dumont JE, Roger PP, Ludgate M 1987 Assays for thyroid growth immunoglobulins and their clinical implications: methods, concepts, and misconceptions. *Endocr Rev* 8:448–452
 60. Veneziani BM, Di Marino C, Salvatore P, Villone G, Perrotti N, Frunzio R, Tramontano D 1992 Transfected insulin-like growth factor II modulates the mitogenic response of rat thyrocytes in culture. *Mol Cell Endocrinol* 86:11–20
 61. Medina DL, Velasco JA, Santisteban P 1999 Somatostatin is expressed in FRTL-5 thyroid cells and prevents thyrotropin-mediated down-regulation of the cyclin-dependent kinase inhibitor p27 kip1. *Endocrinology* 140:87–95
 62. Tramontano D, Moses AC, Veneziani BM, Ingbar SH 1988 Adenosine 3',5'-monophosphate mediates both the mitogenic effect of thyrotropin and its ability to amplify the response to insulin-like growth factor I in FRTL5 cells. *Endocrinology* 122:127–132
 63. Takahashi S, Conti M, Van Wyk JJ 1990 Thyrotropin potentiation of insulin-like growth factor-I dependent deoxyribonucleic acid synthesis in FRTL-5 cells: mediation by an autocrine amplification factor(s). *Endocrinology* 126:736–745
 64. Yamamoto K, Hirai A, Ban T, Saito J, Tahara K, Terano T, Tamura Y, Saito Y, Kitagawa M 1996 Thyrotropin induces G₁ cyclin expression and accelerates G₁ phase after insulin-like growth factor I stimulation in FRTL-5 cells. *Endocrinology* 137:2036–2042
 65. Damante G, Cox F, Rapoport B 1988 IGF-I increases *c-fos* expression in FRTL5 rat thyroid cells by activating the *c-fos* promoter. *Biochem Biophys Res Commun* 151:1194–1199
 66. Maciel RM, Moses AC, Villone G, Tramontano D, Ingbar SH 1988 Demonstration of the production and physiological role of insulin-like growth factor II in rat thyroid follicular cells in culture. *J Clin Invest* 82:1546–1553
 67. Lombardi A, Veneziani BM, Tramontano D, Ingbar SH 1988 Independent and interactive effects of tetradecanoyl phorbol acetate on growth and differentiated functions of FRTL5 cells. *Endocrinology* 123:1544–1552
 68. Tsuzaki S, Moses AC 1990 Somatostatin inhibits deoxyribonucleic acid synthesis induced by both thyrotropin and insulin-like growth factor-I in FRTL5 cells. *Endocrinology* 126:3131–3138
 69. Vainio M, Saarinen P, Tornquist K 1997 Adenosine inhibits DNA synthesis stimulated with TSH, insulin, and phorbol 12-myristate 13-acetate in rat thyroid FRTL-5 cells. *J Cell Physiol* 171:336–342
 70. Villone G, Veneziani BM, Picone R, De Amicis F, Perrotti N, Tramontano D 1993 In the thyroid cells proliferation, differentiated and metabolic functions are under the control of different steps of the cyclic AMP cascade. *Mol Cell Endocrinol* 95:85–93
 71. Koide T, Ono Y, Ito Y, Akahori M, Nedachi T, Hakuno F, Takenaka A, Takahashi SI, Noguchi T 1998 Insulin-like growth factor-I potentiates protein synthesis induced by thyrotropin in FRTL-5 cells: comparison of induction of protein synthesis and DNA synthesis. *Endocr J* 45:151–163
 72. Takada K, Amino N, Tada H, Miyai K 1990 Relationship between proliferation and cell cycle-dependent Ca²⁺ influx induced by a combination of thyrotropin and insulin-like growth factor-I in rat thyroid cells. *J Clin Invest* 86:1548–1555
 73. Kimura T, Dumont JE, Fusco A, Golstein J 1999 Insulin and TSH promote growth in size of PC Cl3 rat thyroid cells, possibly via a pathway different from DNA synthesis: comparison with FRTL-5 cells. *Eur J Endocrinol* 140:94–103
 74. Dai Z, Takahashi SI, Van Wyk JJ, D'Ercole AJ 1992 Creation of an autocrine model of insulin-like growth factor-I action in transfected FRTL-5 cells. *Endocrinology* 130:3175–3183
 75. Taniguchi S, Yoshida A, Mashiba H 1989 Direct effect of methimazole on rat thyroidal cell growth induced by thyrotropin and insulin-like growth factor I. *Endocrinology* 124:2046–2051
 76. Damante G, Russo D, Foti D, Grasso G, Filetti S 1990 Effect of thyrotropin and cAMP on FRTL5 cell growth in a serum free medium. *J Endocrinol Invest* 13:397–402
 77. Tramontano D, Chin WW, Moses AC, Ingbar SH 1986 Thyrotropin and dibutyryl cyclic AMP increase levels of *c-myc* and *c-fos* mRNAs in cultured rat thyroid cells. *J Biol Chem* 261:3919–3922
 78. Isozaki O, Kohn LD 1987 Control of *c-fos* and *c-myc* proto-oncogene induction in rat thyroid cells in culture. *Mol Endocrinol* 1:839–848
 79. Tramontano D, Rotella CM, Toccafondi R, Ambesi-Impiombato FS 1986 Thyrotropin-independent mutant clones from FRTL5 rat thyroid cells: hormonal control mechanisms in differentiated cells. *Endocrinology* 118:862–868
 80. Valente WA, Vitti P, Kohn LD, Brandi ML, Rotella CM, Toccafondi R, Tramontano D, Aloj SM, Ambesi-Impiombato FS 1983 The relationship of growth and adenylate cyclase activity in cultured thyroid cells: separate bioeffects of thyrotropin. *Endocrinology* 112:71–79
 81. Brenner-Gati L, Berg KA, Gershengorn MC 1988 Thyroid-stimulating hormone and insulin-like growth factor-1 synergize to elevate 1,2-diacylglycerol in rat thyroid cells. *Stimulation of DNA*

- synthesis via interaction between lipid and adenylyl cyclase signal transduction systems. *J Clin Invest* 82:1144–1148
82. **Takada K, Amino N, Tetsumoto T, Miyai K** 1988 Phorbol esters have a dual action through protein kinase C in regulation of proliferation of FRTL-5 cells. *FEBS Lett* 234:13–16
 83. **Eggo MC, Burrow GN** 1985 Cultured thyroid: is immortality the answer? In: Eggo M, Burrow GN, eds. *Thyroglobulin: The prothyroid hormone*. New York: Raven Press; 201–210
 84. **Asmis LM, Gerber H, Kaempf J, Studer H** 1995 Epidermal growth factor stimulates cell proliferation and inhibits iodide uptake of FRTL-5 cells *in vitro*. *J Endocrinol* 145:513–520
 85. **Black EG, Logan A, Davis JR, Sheppard MC** 1990 Basic fibroblast growth factor affects DNA synthesis and cell function and activates multiple signalling pathways in rat thyroid FRTL-5 and pituitary GH3 cells. *J Endocrinol* 127:39–46
 86. **Isozaki O, Emoto N, Tsushima T, Sato Y, Shizume K, Demura H, Akamizu T, Kohn LD** 1992 Opposite regulation of deoxyribonucleic acid synthesis and iodide uptake in rat thyroid cells by basic fibroblast growth factor: correlation with opposite regulation of *c-fos* and thyrotropin receptor gene expression. *Endocrinology* 131:2723–2732
 87. **Bergstrom JD, Nilsson M, Heldin NE** 2000 Impaired response to hepatocyte growth factor in FRTL-5 rat thyroid cells expressing a functional hepatocyte growth factor receptor. *Thyroid* 10:631–640
 88. **Dere WH, Rapoport B** 1986 Control of growth in cultured rat thyroid cells. *Mol Cell Endocrinol* 44:195–199
 89. **Yun K, Yamashita S, Izumi K, Yonemitsu N, Sugihara H** 1986 Effects of forskolin on the morphology and function of the rat thyroid cell strain, FRTL-5: comparison with the effects of thyrotrophin. *J Endocrinol* 111:397–405
 90. **Roger PP, Hotimsky A, Moreau C, Dumont JE** 1982 Stimulation by thyrotropin, cholera toxin and dibutyryl cyclic AMP of the multiplication of differentiated thyroid cells *in vitro*. *Mol Cell Endocrinol* 26:165–176
 91. **Roger PP, Servais P, Dumont JE** 1983 Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin. *FEBS Lett* 157:323–329
 92. **Freiberger R, Richter C, Weber K, Wiss F, Schatz H** 1989 Influence of IGF-I, EGF and anti-EGF-receptor antibody on basal and TSH-stimulated (³H)thymidine incorporation into fresh and aged FRTL-5 cell strains. In: Ambesi-Impimbato FS, Perrild H, eds. *FRTL-5 today*. Amsterdam: Elsevier Science Publishers; 177–178
 93. **Medina DL, Toro MJ, Santisteban P** 2000 Somatostatin interferes with thyrotropin-induced G₁-S transition mediated by cAMP-dependent protein kinase and phosphatidylinositol 3-kinase. Involvement of RhoA and cyclin E × cyclin-dependent kinase 2 complexes. *J Biol Chem* 275:15549–15556
 94. **Meinkoth JL, Goldsmith PK, Spiegel AM, Feramisco JR, Burrow GN** 1992 Inhibition of thyrotropin-induced DNA synthesis in thyroid follicular cells by microinjection of an antibody to the stimulatory G protein of adenylate cyclase, G_s. *J Biol Chem* 267:13239–13245
 95. **Cass LA, Meinkoth JL** 1998 Differential effects of cyclic adenosine 3',5'-monophosphate on p70 ribosomal S6 kinase. *Endocrinology* 139:1991–1998
 96. **Florio T, Scorizello A, Fattore M, D'Alto V, Salzano S, Rossi G, Berlingieri MT, Fusco A, Schettini G** 1996 Somatostatin inhibits PC Cl3 thyroid cell proliferation through the modulation of phosphotyrosine activity. Impairment of the somatostatinergic effects by stable expression of E1A viral oncogene. *J Biol Chem* 271:6129–6136
 97. **Battaglia C, Berlingieri MT, Martelli ML, Trapasso F, Delli BP, Fusco A** 1993 Mitogenic and dedifferentiating effect of the K-fgf/hst oncogene on rat thyroid PC clone 3 epithelial cells. *Cell Growth Differ* 4:185–192
 98. **Smith P, Wynford TD, Stringer BM, Williams ED** 1986 Growth factor control of rat thyroid follicular cell proliferation. *Endocrinology* 119:1439–1445
 99. **Wynford-Thomas D, Smith P, Williams ED** 1987 Proliferative response to cyclic AMP elevation of thyroid epithelium in suspension culture. *Mol Cell Endocrinol* 51:163–166
 100. **Roger PP, Servais P, Dumont JE** 1987 Induction of DNA synthesis in dog thyrocytes in primary culture: synergistic effects of thyrotropin and cyclic AMP with epidermal growth factor and insulin. *J Cell Physiol* 130:58–67
 101. **Roger PP, Dumont JE** 1982 Epidermal growth factor controls the proliferation and the expression of differentiation in canine thyroid cells in primary culture. *FEBS Lett* 144:209–212
 102. **Roger PP, Dumont JE** 1984 Factors controlling proliferation and differentiation of canine thyroid cells cultured in reduced serum conditions: effects of thyrotropin, cyclic AMP and growth factors. *Mol Cell Endocrinol* 36:79–93
 103. **Roger PP, Reuse S, Servais P, Van Heuverswyn B, Dumont JE** 1986 Stimulation of cell proliferation and inhibition of differentiation expression by tumor-promoting phorbol esters in dog thyroid cells in primary culture. *Cancer Res* 46:898–906
 104. **Burikhanov R, Coulonval K, Pirson I, Lamy F, Dumont JE, Roger PP** 1996 Thyrotropin via cyclic AMP induces insulin receptor expression and insulin co-stimulation of growth and amplifies insulin and insulin-like growth factor signaling pathways in dog thyroid epithelial cells. *J Biol Chem* 271:29400–29406
 105. **Deleu S, Pirson I, Coulonval K, Drouin A, Taton M, Clermont F, Roger P, Nakamura T, Dumont JE, Maenhaut C** 1999 IGF-1 or insulin, and the TSH cyclic AMP cascade separately control dog and human thyroid cell growth and DNA synthesis and complement each other in inducing mitogenesis. *Mol Cell Endocrinol* 149:41–51
 106. **Eggo MC, Bachrach LK, Burrow GN** 1987 Role of non-TSH factors in thyroid cell growth. *Acta Endocrinol Suppl (Copenh)* 281:231–237
 107. **Dremier S, Taton M, Coulonval K, Nakamura T, Matsumoto K, Dumont JE** 1994 Mitogenic, dedifferentiating, and scattering effects of hepatocyte growth factor on dog thyroid cells. *Endocrinology* 135:135–140
 108. **Van Sande J, Lefort A, Beebe S, Roger P, Perret J, Corbin J, Dumont JE** 1989 Pairs of cyclic AMP analogs, that are specifically synergistic for type I and type II cAMP-dependent protein kinases, mimic thyrotropin effects on the function, differentiation expression and mitogenesis of dog thyroid cells. *Eur J Biochem* 183:699–708
 109. **Dumont JE, Jauniaux JC, Roger PP** 1989 The cyclic AMP-mediated stimulation of cell proliferation. *Trends Biochem Sci* 14:67–71
 110. **Roger PP, Reuse S, Maenhaut C, Dumont JE** 1995 Multiple facets of the modulation of growth by cAMP. *Vitam Horm* 51:59–191
 111. **Westermarck B, Karlsson FA, Walinder O** 1979 Thyrotropin is not a growth factor for human thyroid cells in culture. *Proc Natl Acad Sci USA* 76:2022–2026
 112. **Errick JE, Ing KW, Eggo MC, Burrow GN** 1986 Growth and differentiation in cultured human thyroid cells: effects of epidermal growth factor and thyrotropin. *In Vitro Cell Dev Biol* 22:28–36
 113. **Curcio F, Ambesi-Impimbato FS, Perrella G, Coon HG** 1994 Long-term culture and functional characterization of follicular cells from adult normal human thyroids. *Proc Natl Acad Sci USA* 91:9004–9008
 114. **Huber GK, Davies TF** 1990 Human fetal thyroid cell growth *in vitro*: system characterization and cytokine inhibition. *Endocrinology* 126:869–875
 115. **Huber GK, Fong P, Concepcion ES, Davies TF** 1991 Recombinant human thyroid-stimulating hormone: initial bioactivity assessment using human fetal thyroid cells. *J Clin Endocrinol Metab* 72:1328–1331
 116. **Huber GK, Safirstein R, Neufeld D, Davies TF** 1991 Thyrotropin receptor autoantibodies induce human thyroid cell growth and *c-fos* activation. *J Clin Endocrinol Metab* 72:1142–1147
 117. **Van Keymeulen A, Dumont JE, Roger PP** 2000 TSH induces insulin receptors that mediate insulin co-stimulation of growth in normal human thyroid cells. *Biochem Biophys Res Commun* 279:202–207
 118. **Ollis CA, Hill DJ, Munro DS** 1989 A role for insulin-like growth factor-I in the regulation of human thyroid cell growth by thyrotrophin. *J Endocrinol* 123:495–500
 119. **Tode B, Serio M, Rotella CM, Galli G, Franceschelli F, Tanini A, Toccafondi R** 1989 Insulin-like growth factor-I: autocrine secretion by human thyroid follicular cells in primary culture. *J Clin Endocrinol Metab* 69:639–647

120. Williams DW, Williams ED, Wynford-Thomas D 1989 Evidence for autocrine production of IGF-1 in human thyroid adenomas. *Mol Cell Endocrinol* 61:139–143
121. Kasai K, Hiraiwa M, Suzuki Y, Emoto T, Banba N, Nakamura T, Shimoda S 1987 Presence of epidermal growth factor receptors on human thyroid membranes. *Acta Endocrinol (Copenh)* 114:396–401
122. Lamy F, Taton M, Dumont JE, Roger PP 1990 Control of protein synthesis by thyrotropin and epidermal growth factor in human thyrocytes: role of morphological changes. *Mol Cell Endocrinol* 73:195–209
123. Kraiem Z, Sadeh O, Yosef M, Aharon A 1995 Mutual antagonistic interactions between the thyrotropin (adenosine 3',5'-monophosphate) and protein kinase C/epidermal growth factor (tyrosine kinase) pathways in cell proliferation and differentiation of cultured human thyroid follicles. *Endocrinology* 136:585–590
124. Eccles N, Ivan M, Wynford-Thomas D 1996 Mitogenic stimulation of normal and oncogene-transformed human thyroid epithelial cells by hepatocyte growth factor. *Mol Cell Endocrinol* 117:247–251
125. Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, Belfiore A, Costantino A, Vigneri R, Pierotti MA 1992 Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. *Oncogene* 7:2549–2553
126. Tramontano D, Moses AC, Ingbar SH 1988 The role of adenosine 3',5'-monophosphate in the regulation of receptors for thyrotropin and insulin-like growth factor I in the FRTL5 rat thyroid follicular cell. *Endocrinology* 122:133–136
127. O'Keefe EJ, Pledger WJ 1983 A model of cell cycle control: sequential events regulated by growth factors. *Mol Cell Endocrinol* 31:167–186
128. Baserga R, Sell C, Porcu P, Rubini M 1994 The role of the IGF-1 receptor in the growth and transformation of mammalian cells. *Cell Prolif* 27:63–71
129. Nedachi T, Akahori M, Ariga M, Sakamoto H, Suzuki N, Umesaki K, Hakuno F, Takahashi SI 2000 Tyrosine kinase and phosphatidylinositol 3-kinase activation are required for cyclic adenosine 3',5'-monophosphate-dependent potentiation of deoxyribonucleic acid synthesis induced by insulin-like growth factor-I in FRTL-5 cells. *Endocrinology* 141:2429–2438
130. Takahashi S, Conti M, Prokop C, Van Wyk JJ, Earp HS 1991 Thyrotropin and insulin-like growth factor I regulation of tyrosine phosphorylation in FRTL-5 cells. Interaction between cAMP-dependent and growth factor-dependent signal transduction. *J Biol Chem* 266:7834–7841
131. Ariga M, Nedachi T, Akahori M, Sakamoto H, Ito Y, Hakuno F, Takahashi S 2000 Signalling pathways of insulin-like growth factor-I that are augmented by cAMP in FRTL-5 cells. *Biochem J* 348:409–416
132. Oki N, Takahashi SI, Hidaka H, Conti M 2000 Short term feedback regulation of cAMP in FRTL-5 thyroid cells. Role of PDE4D3 phosphodiesterase activation. *J Biol Chem* 275:10831–10837
133. Villone G, De Amicis F, Veneziani BM, Salzano S, Di Carlo A, Tramontano D 1997 Sustained *vs.* transient cyclic AMP intracellular levels: effect on thyrotropin-dependent growth of thyroid cells. *Cell Growth Differ* 8:1181–1188
134. Feliciello A, Gallo A, Mele E, Porcellini A, Troncone G, Garbi C, Gottesman ME, Avvedimento EV 2000 The localization and activity of cAMP-dependent protein kinase affect cell cycle progression in thyroid cells. *J Biol Chem* 2000 275:303–311
135. Villone G, De Vita G, Chieffi P, Picascia A, Stanzone R, Santoro M, Fusco A, Tramontano D 2000 Association between the expression of E1A oncogene and increased sensitivity to growth inhibition induced by sustained levels of cAMP in rat thyroid cells. *Eur J Endocrinol* 142:286–293
136. Logan A, Black EG, Gonzalez AM, Buscaglia M, Sheppard MC 1992 Basic fibroblast growth factor: an autocrine mitogen of rat thyroid follicular cells? *Endocrinology* 130:2363–2372
137. Becks GP, Logan A, Phillips ID, Wang JF, Smith C, DeSousa D, Hill DJ 1994 Increase of basic fibroblast growth factor (FGF) and FGF receptor messenger RNA during rat thyroid hyperplasia: temporal changes and cellular distribution. *J Endocrinol* 142:325–338
138. Cocks HC, Ramsden JD, Davies EL, Watkinson JC, Eggo MC 2000 TSH increases angiogenic growth factor expression in FRTL-5 cells. *Endocr J* 47(Suppl):142 (Abstract)
139. Roger PP, Servais P, Dumont JE 1987 Regulation of dog thyroid epithelial cell cycle by forskolin, an adenylate cyclase activator. *Exp Cell Res* 172:282–292
140. Roger PP, Demartin S, Dumont JE 1999 Nature of the critical labile event that controls RB phosphorylation in the cyclic AMP-dependent cell cycle of thyrocytes in primary culture. *Exp Cell Res* 252:492–498
141. Van Keymeulen A, Deleu S, Bartek J, Dumont JE, Roger PP 2001 Respective roles of carbamylcholine and cyclic AMP in their synergistic regulation of cell cycle in thyroid primary cultures. *Endocrinology* 142:1251–1259
142. Baptist M, Dumont JE, Roger PP 1993 Demonstration of cell cycle kinetics in thyroid primary culture by immunostaining of proliferating cell nuclear antigen: differences in cyclic AMP-dependent and -independent mitogenic stimulations. *J Cell Sci* 105:69–80
143. Baptist M, Dumont JE, Roger PP 1995 Intercellular heterogeneity of early mitogenic events: cAMP generalizes the EGF effect on c-Fos protein appearance but not on MAP kinase phosphorylation and nuclear translocation in dog thyroid epithelial cells. *Exp Cell Res* 221:160–171
144. Pirson I, Fortemaion N, Jacobs C, Dremier S, Dumont JE, Maenhaut C 2000 The visual display of regulatory information and networks. *Trends Cell Biol* 10:404–408
145. Trischitta V, Damante G, Foti D, Filetti S 1987 Insulin binding and biological activities in the FRTL-5 rat thyroid cell line. *Metabolism* 36:379–383
146. Saji M, Akamizu T, Sanchez M, Obici S, Avvedimento E, Gottesman ME, Kohn LD 1992 Regulation of thyrotropin receptor gene expression in rat FRTL-5 thyroid cells. *Endocrinology* 130:520–533
147. Lalli E, Sassone-Corsi P 1995 Thyroid-stimulating hormone (TSH)-directed induction of the CREM gene in the thyroid gland participates in the long-term desensitization of the TSH receptor. *Proc Natl Acad Sci USA* 92:9633–9637
148. Coppee F, Gerard AC, Deneff JF, 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 16 E7 oncogene. *Oncogene* 13:1471–1482
149. Maenhaut C, Brabant G, Vassart G, Dumont JE 1992 *In vitro* and *in vivo* regulation of thyrotropin receptor mRNA levels in dog and human thyroid cells. *J Biol Chem* 267:3000–3007
150. Huber GK, Concepcion ES, Graves PN, Davies TF 1991 Positive regulation of human thyrotropin receptor mRNA by thyrotropin. *J Clin Endocrinol Metab* 72:1394–1396
151. Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, Schultz G 1996 The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci USA* 93:116–120
152. Allgeier A, Laugwitz KL, Van Sande J, Schultz G, Dumont JE 1997 Multiple G-protein coupling of the dog thyrotropin receptor. *Mol Cell Endocrinol* 127:81–90
153. Laurent E, Mockel J, Van Sande J, Graff I, Dumont JE 1987 Dual activation by thyrotropin of the phospholipase C and cyclic AMP cascades in human thyroid. *Mol Cell Endocrinol* 52:273–278
154. Yanagita Y, Okajima F, Sho K, Nagamachi Y, Kondo Y 1996 An adenosine derivative cooperates with TSH and Graves' IgG to induce Ca²⁺ mobilization in single human thyroid cells. *Mol Cell Endocrinol* 118:47–56
155. Raspe E, Reuse S, Roger PP, Dumont JE 1992 Lack of correlation between the activation of the Ca(2+)-phosphatidylinositol cascade and the regulation of DNA synthesis in the dog thyrocyte. *Exp Cell Res* 198:17–26
156. Mockel J, Laurent E, Lejeune C, Dumont JE 1991 Thyrotropin does not activate the phosphatidylinositol bisphosphate hydrolyzing phospholipase C in the dog thyroid. *Mol Cell Endocrinol* 82:221–227
157. Bone EA, Alling DW, Grollman EF 1986 Norepinephrine and thyroid-stimulating hormone induce inositol phosphate accumulation in FRTL-5 cells. *Endocrinology* 119:2193–2200
158. Field JB, Ealey PA, Marshall NJ, Cockcroft S 1987 Thyroid-stimulating hormone stimulates increases in inositol phosphates as well

- as cyclic AMP in the FRTL-5 rat thyroid cell line. *Biochem J* 247: 519–524
159. Singh J, Hunt P, Eggo MC, Sheppard MC, Kirk CJ, Michell RH 1996 Thyroid-stimulating hormone rapidly stimulates inositol polyphosphate formation in FRTL-5 thyrocytes without activating phosphoinositidase C. *Biochem J* 316:175–182
 160. Meucci O, Berlingieri MT, Fusco A, Scorziello A, Santoro M, Grieco M, Grimaldi M, Schettini G 1993 Norepinephrine and thyrotropin stimulation of $[Ca^{++}]_i$ in PC C13 a rat thyroid epithelial cell line: effect of transformation by E1A gene adenovirus and polyomavirus middle-T antigen gene. *Life Sci* 52:891–899
 161. Sho KM, Okajima F, Abdul MM, Kondo Y 1991 Reciprocal modulation of thyrotropin actions by P1-purine agonists in FRTL-5 thyroid cells. Inhibition of cAMP pathway and stimulation of phospholipase C- Ca^{2+} pathway. *J Biol Chem* 266:12180–12184
 162. Tahara K, Grollman EF, Saji M, Kohn LD 1991 Regulation of prostaglandin synthesis by thyrotropin, insulin or insulin-like growth factor-I, and serum in FRTL-5 rat thyroid cells. *J Biol Chem* 266:440–448
 163. Di Cerbo A, Corda D 1999 Signaling pathways involved in thyroid hyperfunction and growth in Graves' disease. *Biochimie* 81: 415–424
 164. Boeynaems JM, Waelbroeck M, Dumont JE 1979 Cholinergic and α -adrenergic stimulation of prostaglandin release by dog thyroid *in vitro*. *Endocrinology* 105:988–995
 165. Di Paola R, Menzaghi C, De Filippis, V, Corda D, Di Cerbo A 1997 Cyclooxygenase-dependent thyroid cell proliferation induced by immunoglobulins from patients with Graves' disease. *J Clin Endocrinol Metab* 82:670–673
 166. Roger PP, Dumont JE, Boeynaems JM 1984 Lack of prostaglandin involvement in the mitogenic effect of TSH on canine thyroid cells in primary culture. *FEBS Lett* 166:136–140
 167. Dremier S, Pohl V, Poteet-Smith C, Roger PP, Corbin J, Doskeland SO, Dumont JE, Maenhaut C 1997 Activation of cyclic AMP-dependent kinase is required but may not be sufficient to mimic cyclic AMP-dependent DNA synthesis and thyroglobulin expression in dog thyroid cells. *Mol Cell Biol* 17:6717–6726
 168. Kupperman E, Wen W, Meinkoth JL 1993 Inhibition of thyrotropin-stimulated DNA synthesis by microinjection of inhibitors of cellular Ras and cyclic AMP-dependent protein kinase. *Mol Cell Biol* 13:4477–4484
 169. Woloshin PI, Walton KM, Rehfuss RP, Goodman RH, Cone RD 1992 3',5'-Cyclic adenosine monophosphate-regulated enhancer binding (CREB) activity is required for normal growth and differentiated phenotype in the FRTL5 thyroid follicular cell line. *Mol Endocrinol* 6:1725–1733
 170. Uyttersprot N, Costagliola S, Dumont JE, Miot F 1999 Requirement for cAMP-response element (CRE) binding protein/CRE modulator transcription factors in thyrotropin-induced proliferation of dog thyroid cells in primary culture. *Eur J Biochem* 259: 370–378
 171. Coulouval K, Vandeput F, Stein R, Kozma S, Lamy F, Dumont JE 2000 Phosphatidylinositol 3-kinase, protein kinase B and ribosomal S6 kinases in the stimulation of thyroid epithelial cell proliferation by cAMP and growth factors in the presence of insulin. *Biochem J* 348:351–358
 172. Tsygankova OM, Kupperman E, Wen W, Meinkoth JL 2000 Cyclic AMP activates Ras. *Oncogene* 19:3609–3615
 173. Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL 1999 Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. *Mol Cell Biol* 19:5882–5891
 174. Tsygankova OM, Saavedra A, Rebhun JF, Quilliam LA, Meinkoth JL 2001 Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. *Mol Cell Biol* 21:1921–1929
 175. Dremier S, Vandeput F, Zwartkruis FJ, Bos JL, Dumont JE, Maenhaut C 2000 Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. *Biochem Biophys Res Commun* 267:7–11
 176. Lemoine NR, Mayall ES, Wyllie FS, Williams ED, Goyns M, Stringer B, Wynford-Thomas D 1989 High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene* 4:159–164
 177. Gire V, Wynford-Thomas D 2000 RAS oncogene activation induces proliferation in normal human thyroid epithelial cells without loss of differentiation. *Oncogene* 19:737–744
 178. Jones CJ, Kipling D, Morris M, Hepburn P, Skinner J, Bounacer A, Wyllie FS, Ivan M, Bartek J, Wynford-Thomas D, Bond JA 2000 Evidence for a telomere-independent "clock" limiting RAS oncogene-driven proliferation of human thyroid epithelial cells. *Mol Cell Biol* 20:5690–5699
 179. Fusco A, Pinto A, Tramontano D, Tajana G, Vecchio G, Tsuchida N 1982 Block in the expression of differentiation markers of rat thyroid epithelial cells by transformation with Kirsten murine sarcoma virus. *Cancer Res* 42:618–626
 180. Avvedimento VE, Musti AM, Ueffing M, Obici S, Gallo A, Sanchez M, DeBrasi D, Gottesman ME 1991 Reversible inhibition of a thyroid-specific trans-acting factor by Ras. *Genes Dev* 5:22–28
 181. Kupperman E, Wofford D, Wen W, Meinkoth JL 1996 Ras inhibits thyroglobulin expression but not cyclic adenosine monophosphate-mediated signaling in Wistar rat thyrocytes. *Endocrinology* 137:96–104
 182. Miller MJ, Rioux L, Prendergast GV, Cannon S, White MA, Meinkoth JL 1998 Differential effects of protein kinase A on Ras effector pathways. *Mol Cell Biol* 18:3718–3726
 183. Cass LA, Meinkoth JL 2000 Ras signaling through PI3K confers hormone-independent proliferation that is compatible with differentiation. *Oncogene* 19:924–932
 184. Miller MJ, Prigent S, Kupperman E, Rioux L, Park SH, Feramisco JR, White MA, Rutkowski JL, Meinkoth JL 1997 RalGDS functions in Ras- and cAMP-mediated growth stimulation. *J Biol Chem* 272: 5600–5605
 185. al-Alawi N, Rose DW, Buckmaster C, Ahn N, Rapp U, Meinkoth J, Feramisco JR 1995 Thyrotropin-induced mitogenesis is Ras dependent but appears to bypass the Raf-dependent cytoplasmic kinase cascade. *Mol Cell Biol* 15:1162–1168
 186. Cobellis G, Missero C, Di Lauro R 1998 Concomitant activation of MEK-1 and Rac-1 increases the proliferative potential of thyroid epithelial cells, without affecting their differentiation. *Oncogene* 17:2047–2057
 187. Gire V, Marshall CJ, Wynford-Thomas D 1999 Activation of mitogen-activated protein kinase is necessary but not sufficient for proliferation of human thyroid epithelial cells induced by mutant Ras. *Oncogene* 18:4819–4832
 188. Van Keymeulen A, Roger PP, Dumont JE, Dremier S 2000 TSH and cAMP do not signal mitogenesis through Ras activation. *Biochem Biophys Res Commun* 273:154–158
 189. Lamy F, Wilkin F, Baptist M, Posada J, Roger PP, Dumont JE 1993 Phosphorylation of mitogen-activated protein kinases is involved in the epidermal growth factor and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway. *J Biol Chem* 268: 8398–8401
 190. Ciullo I, Diez-Roux G, Di Domenico M, Migliaccio A, Avvedimento EV 2001 cAMP signaling selectively influences Ras effector pathways. *Oncogene* 20:1186–1192
 191. Correze C, Blondeau JP, Pomerance M 2000 The thyrotropin receptor is not involved in the activation of p42/p44 mitogen-activated protein kinases by thyrotropin preparations in Chinese hamster ovary cells expressing the human thyrotropin receptor. *Thyroid* 10:747–752
 192. Saunier B, Tournier C, Jacquemin C, Pierre M 1995 Stimulation of mitogen-activated protein kinase by thyrotropin in primary cultured human thyroid follicles. *J Biol Chem* 270:3693–3697
 193. Nasi S, Ciarapica R, Jucker R, Rosati J, Soucek L 2001 Making decisions through Myc. *FEBS Lett* 490:153–162
 194. Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K 1999 Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* 253:63–77
 195. Dere WH, Hirayu H, Rapoport B 1985 TSH and cAMP enhance expression of the myc proto-oncogene in cultured thyroid cells. *Endocrinology* 117:2249–2251
 196. Reuse S, Roger PP, Vassart G, Dumont JE 1986 Enhancement of cmyc mRNA concentration in dog thyrocytes initiating DNA synthesis in response to thyrotropin, forskolin, epidermal growth fac-

- tor and phorbol myristate ester. *Biochem Biophys Res Commun* 141:1066–1076
197. **Pirson I, Coulonval K, Lamy F, Dumont JE** 1996 *c-Myc* expression is controlled by the mitogenic cAMP-cascade in thyrocytes. *J Cell Physiol* 168:59–70
 198. **Yamashita S, Ong J, Fagin JA, Melmed S** 1986 Expression of the *myc* cellular proto-oncogene in human thyroid tissue. *J Clin Endocrinol Metab* 63:1170–1173
 199. **Reuse S, Maenhaut C, Dumont JE** 1990 Regulation of protooncogenes *c-fos* and *c-myc* expressions by protein tyrosine kinase, protein kinase C, and cyclic AMP mitogenic pathways in dog primary thyrocytes: a positive and negative control by cyclic AMP on *c-myc* expression. *Exp Cell Res* 189:33–40
 200. **Deleu S, Pirson I, Clermont F, Nakamura T, Dumont JE, Maenhaut C** 1999 Immediate early gene expression in dog thyrocytes in response to growth, proliferation and differentiation stimuli. *J Cell Physiol* 181:342–354
 201. **Sears R, Leone G, DeGregori J, Nevins JR** 1999 Ras enhances *Myc* protein stability. *Mol Cell* 3:169–179
 202. **Colletta G, Cirafici AM, Vecchio G** 1986 Induction of the *c-fos* oncogene by thyrotropic hormone in rat thyroid cells in culture. *Science* 233:458–460
 203. **Tominaga T, Dela Cruz J, Burrow GN, Meinkoth JL** 1994 Divergent patterns of immediate early gene expression in response to thyroid-stimulating hormone and insulin-like growth factor I in Wistar rat thyrocytes. *Endocrinology* 135:1212–1219
 204. **Heinrich R, Kraiem Z** 1997 The protein kinase A pathway inhibits *c-jun* and *c-fos* protooncogene expression induced by the protein kinase C and tyrosine kinase pathways in cultured human thyroid follicles. *J Clin Endocrinol Metab* 82:1839–1844
 205. **Foti D, Damante G, Rapoport B** 1990 Studies on the role of *c-fos* in TSH-stimulated thyroid cell proliferation. *Cell Mol Biol* 36:363–373
 206. **Reuse S, Pirson I, Dumont JE** 1991 Differential regulation of protooncogenes *c-jun* and *jun D* expressions by protein tyrosine kinase, protein kinase C, and cyclic-AMP mitogenic pathways in dog primary thyrocytes: TSH and cyclic-AMP induce proliferation but downregulate *C-jun* expression. *Exp Cell Res* 196:210–215
 207. **Karin M** 1995 The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 270:16483–16486
 208. **Colletta G, Cirafici AM** 1992 TSH is able to induce cell cycle-related gene expression in rat thyroid cell. *Biochem Biophys Res Commun* 183:265–272
 209. **Kambe F, Miyazaki T, Seo H** 1996 Differential induction of *fos* and *jun* family genes by thyrotropin in rat thyroid FRTL-5 cells. *Thyroid* 6:123–128
 210. **Pirson I, Dumont JE** 1994 *Jun B* expression is regulated differently by three mitogenic pathways in thyrocytes. *Exp Cell Res* 214:561–569
 211. **Deng T, Karin M** 1993 *JunB* differs from *c-Jun* in its DNA-binding and dimerization domains, and represses *c-Jun* by formation of inactive heterodimers. *Genes Dev* 7:479–490
 212. **Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M** 2000 Cell cycle-dependent variations in *c-Jun* and *JunB* phosphorylation: a role in the control of cyclin D1 expression. *EMBO J* 19:2056–2068
 213. **Yan YX, Nakagawa H, Lee MH, Rustgi AK** 1997 Transforming growth factor- α enhances cyclin D1 transcription through the binding of early growth response protein to a *cis*-regulatory element in the cyclin D1 promoter. *J Biol Chem* 272:33181–33190
 214. **Mechta F, Piette J, Hirai SI, Yaniv M** 1989 Stimulation of protein kinase C or protein kinase A mediated signal transduction pathways shows three modes of response among serum inducible genes. *New Biol* 1:297–304
 215. **Sherr CJ** 1995 D-type cyclins. *Trends Biochem Sci* 20:187–190
 216. **Weinberg RA** 1995 The retinoblastoma protein and cell cycle control. *Cell* 81:323–330
 217. **Bartek J, Bartkova J, Lukas J** 1996 The retinoblastoma protein pathway and the restriction point. *Curr Opin Cell Biol* 8:805–814
 218. **Sherr CJ, Roberts JM** 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13:1501–1512
 219. **Lukas J, Bartkova J, Bartek J** 1996 Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G₁ checkpoint. *Mol Cell Biol* 16:6917–6925
 220. **Coulonval K, Maenhaut C, Dumont JE, Lamy F** 1997 Phosphorylation of the three Rb protein family members is a common step of the cAMP-, the growth factor, and the phorbol ester-mitogenic cascades but is not necessary for the hypertrophy induced by insulin. *Exp Cell Res* 233:395–398
 221. **Baptist M, Lamy F, Gannon J, Hunt T, Dumont JE, Roger PP** 1996 Expression and subcellular localization of CDK2 and *cdc2* kinases and their common partner cyclin A in thyroid epithelial cells: comparison of cyclic AMP-dependent and -independent cell cycles. *J Cell Physiol* 166:256–273
 222. **Van Keymeulen A, Bartek J, Dumont JE, Roger PP** 1999 Cyclin D3 accumulation and activity integrate and rank the comitogenic pathways of thyrotropin and insulin in thyrocytes in primary culture. *Oncogene* 18:7351–7359
 223. **Depoortere F, Van Keymeulen A, Lukas J, Costagliola S, Bartkova J, Dumont JE, Bartek J, Roger PP, Dremier S** 1998 A requirement for cyclin D3-cyclin-dependent kinase (cdk)-4 assembly in the cyclic adenosine monophosphate-dependent proliferation of thyrocytes. *J Cell Biol* 140:1427–1439
 224. **Coppee F, Depoortere F, Bartek J, Ledent C, Parmentier M, Dumont JE** 1998 Differential patterns of cell cycle regulatory proteins expression in transgenic models of thyroid tumours. *Oncogene* 17:631–641
 225. **Depoortere F, Dumont JE, Roger PP** 1996 Paradoxical accumulation of the cyclin-dependent kinase inhibitor p27 kip1 during the cAMP-dependent mitogenic stimulation of thyroid epithelial cells. *J Cell Sci* 109:1759–1764
 226. **Depoortere F, Pirson I, Bartek J, Dumont JE, Roger PP** 2000 Transforming growth factor β (1) selectively inhibits the cyclic AMP-dependent proliferation of primary thyroid epithelial cells by preventing the association of cyclin D3-cdk4 with nuclear p27(kip1). *Mol Biol Cell* 11:1061–1076
 227. **Carneiro C, Alvarez CV, Zalvide J, Vidal A, Dominguez F** 1998 TGF- β 1 actions on FRTL-5 cells provide a model for the physiological regulation of thyroid growth. *Oncogene* 16:1455–1465
 228. **Hirai A, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Tatsuno I, Oeda T, Tahara K, Terano T, Narumiya S, Kohn LD, Saito Y** 1997 Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27Kip1 and facilitate the progression from G₁ to S phase in growth-stimulated rat FRTL-5 cells. *J Biol Chem* 272:13–16
 229. **Noguchi Y, Nakamura S, Yasuda T, Kitagawa M, Kohn LD, Saito Y, Hirai A** 1998 Newly synthesized Rho A, not Ras, is isoprenylated and translocated to membranes coincident with progression of the G1 to S phase of growth-stimulated rat FRTL-5 cells. *J Biol Chem* 273:3649–3653
 230. **Coclet J, Foureau F, Ketelbant P, Galand P, Dumont JE** 1989 Cell population kinetics in dog and human adult thyroid. *Clin Endocrinol (Oxf)* 31:655–665
 231. **Wynford-Thomas D, Stringer BM, Harach HR, Williams ED** 1983 Control of growth in the rat thyroid—an example of specific desensitization to trophic hormone stimulation. *Experientia* 39:421–423
 232. **Morris JC3, Ranganathan G, Hay ID, Nelson RE, Jiang NS** 1988 The effects of transforming growth factor- β on growth and differentiation of the continuous rat thyroid follicular cell line, FRTL-5. *Endocrinology* 123:1385–1394
 233. **Logan A, Smith C, Becks GP, Gonzalez AM, Phillips ID, Hill DJ** 1994 Enhanced expression of transforming growth factor- β 1 during thyroid hyperplasia in rats. *J Endocrinol* 141:45–57
 234. **Contempre B, Le Moine O, Dumont JE, Deneff JF, Many MC** 1996 Selenium deficiency and thyroid fibrosis. A key role for macrophages and transforming growth factor β (TGF- β). *Mol Cell Endocrinol* 124:7–15
 235. **Zakarija M, Jin S, McKenzie JM** 1988 Evidence supporting the identity in Graves' disease of thyroid-stimulating antibody and thyroid growth-promoting immunoglobulin G as assayed in FRTL5 cells. *J Clin Invest* 81:879–884
 236. **Laurent E, Van Sande J, Ludgate M, Corvilain B, Rocmans P, Dumont JE, Mockel J** 1991 Unlike thyrotropin, thyroid-stimulating antibodies do not activate phospholipase C in human thyroid slices. *J Clin Invest* 87:1634–1642
 237. **Smeds S, Boeryd B, Jörtsö E, Lennquist S** 1989 Normal and stim-

- ulated growth of different human thyroid tissues in nude mice. In: Goretzki PE, Röher HD, eds. Growth regulation of thyroid gland and thyroid tumors. Basel, Switzerland: Karger; 98–108
238. **Abramowicz MJ, Duprez L, Parma J, Vassart G, Heinrichs C** 1997 Familial congenital hypothyroidism due to inactivating mutation of the thyrotropin receptor causing profound hypoplasia of the thyroid gland. *J Clin Invest* 99:3018–3024
 239. **Biebermann H, Schoneberg T, Krude H, Schultz G, Gudermann T, Gruters A** 1997 Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. *J Clin Endocrinol Metab* 82:3471–3480
 240. **Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G** 1993 Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* 365:649–651
 241. **Duprez L, Parma J, Van Sande J, Allgeier A, Leclere J, Schwartz C, Delisle MJ, Decoux M, Orgiazzi J, Dumont J** 1994 Germline mutations in the thyrotropin receptor gene cause non-autoimmune autosomal dominant hyperthyroidism. *Nat Genet* 7:396–401
 242. **Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh QY, Clark OH, Kawasaki E, Bourne HR** 1990 Two G protein oncogenes in human endocrine tumors. *Science* 249: 655–659
 243. **O'Sullivan C, Barton CM, Staddon SL, Brown CL, Lemoine NR** 1991 Activating point mutations of the *gsp* oncogene in human thyroid adenomas. *Mol Carcinog* 4:345–349
 244. **Suarez HG, du Villard JA, Caillou B, Schlumberger M, Parmentier C, Monier R** 1991 *gsp* Mutations in human thyroid tumours. *Oncogene* 6:677–679
 245. **Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM** 1991 Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. *N Engl J Med* 325: 1688–1695
 246. **Miyakawa M, Saji M, Tsushima T, Wakai K, Shizume K** 1988 Thyroid volume and serum thyroglobulin levels in patients with acromegaly: correlation with plasma insulin-like growth factor I levels. *J Clin Endocrinol Metab* 67:973–978
 247. **Cheung NW, Boyages SC** 1997 The thyroid gland in acromegaly: an ultrasonographic study. *Clin Endocrinol (Oxf)* 46:545–549
 248. **Cheung NW, Lou JC, Boyages SC** 1996 Growth hormone does not increase thyroid size in the absence of thyrotropin: a study in adults with hypopituitarism. *J Clin Endocrinol Metab* 81:1179–1183
 249. **Dormitzer PR, Ellison PT, Bode HH** 1989 Anomalously low endemic goiter prevalence among Efe pygmies. *Am J Phys Anthropol* 78:527–531
 250. **Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R** 1997 Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 16:64–67
 251. **Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C** 1997 Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. *Cancer Res* 57:4710–4713
 252. **Stein SA, Oates EL, Hall CR, Grumbles RM, Fernandez LM, Taylor NA, Puett D, Jin S** 1994 Identification of a point mutation in the thyrotropin receptor of the *hyt/hyt* hypothyroid mouse. *Mol Endocrinol* 8:129–138
 253. **Ledent C, Dumont JE, Vassart G, Parmentier M** 1992 Thyroid expression of an A2 adenosine receptor transgene induces thyroid hyperplasia and hyperthyroidism. *EMBO J* 11:537–542
 254. **Michiels FM, Caillou B, Talbot M, Dessarps-Freichy F, Maunoury MT, Schlumberger M, Mercken L, Monier R, Feunteun J** 1994 Oncogenic potential of guanine nucleotide stimulatory factor α subunit in thyroid glands of transgenic mice. *Proc Natl Acad Sci USA* 91:10488–10492
 255. **Zeiger MA, Saji M, Gusev Y, Westra WH, Takiyama Y, Dooley WC, Kohn LD, Levine MA** 1997 Thyroid-specific expression of cholera toxin A1 subunit causes thyroid hyperplasia and hyperthyroidism in transgenic mice. *Endocrinology* 138:3133–3140
 256. **Ledent C, Deneff JF, Cottecchia S, Lefkowitz R, Dumont J, Vassart G, Parmentier M** 1997 Costimulation of adenylyl cyclase and phospholipase C by a mutant α 1B-adrenergic receptor transgene promotes malignant transformation of thyroid follicular cells. *Endocrinology* 138:369–378
 257. **Pericas I, Jolin T** 1977 The effect of streptozotocin-induced diabetes on the pituitary-thyroid axis in goitrogen-treated rats. *Acta Endocrinol (Copenh)* 86:128–139
 258. **Bagchi N, Brown TR, Shivers B, Lucas S, Mack RE** 1981 Decreased thyroidal response to thyrotropin in diabetic mice. *Endocrinology* 109:1428–1432
 259. **Steger RW, Rabe MB** 1997 The effect of diabetes mellitus on endocrine and reproductive function. *Proc Soc Exp Biol Med* 214:1–11
 260. **Thomas GA, Davies HG, Williams ED** 1994 Site of production of IGF1 in the normal and stimulated mouse thyroid. *J Pathol* 173: 355–360
 261. **Ozawa S, Spaulding SW** 1990 Epidermal growth factor inhibits radioiodine uptake but stimulates deoxyribonucleic acid synthesis in newborn rat thyroids grown in nude mice. *Endocrinology* 127: 604–612
 262. **Paschke R, Eck T, Herfurth J, Usadel KH** 1995 Stimulation of proliferation and inhibition of function of xenotransplanted human thyroid tissue by epidermal growth factor. *J Endocrinol Invest* 18:359–363
 263. **De Vito WJ, Chanoine JP, Alex S, Fang SL, Stone S, Huber CA, Shalhoub V, Lian JB, Stein GS, Braverman LE** 1992 Effect of *in vivo* administration of recombinant acidic fibroblast growth factor on thyroid function in the rat: induction of colloid goiter. *Endocrinology* 131:729–735
 264. **Ozawa S, Sheflin LG, Spaulding SW** 1991 Thyroxine increases epidermal growth factor levels in the mouse thyroid *in vivo*. *Endocrinology* 128:1396–1403
 265. **Derwahl M, Broecker M, Kraiem Z** 1999 Thyrotropin may not be the dominant growth factor in benign and malignant thyroid tumors. *J Clin Endocrinol Metab* 84:829–834
 266. **Thompson SD, Franklyn JA, Watkinson JC, Verhaeg JM, Sheppard MC, Eggo MC** 1998 Fibroblast growth factors 1 and 2 and fibroblast growth factor receptor 1 are elevated in thyroid hyperplasia. *J Clin Endocrinol Metab* 83:1336–1341
 267. **Fusco A, Santoro M, Grieco M, Carlomagno F, Dathan N, Fabien N, Berlingieri MT, Li Z, De Franciscis V, Salvatore D** 1995 RET/PTC activation in human thyroid carcinomas. *J Endocrinol Invest* 18:127–129
 268. **Pierotti MA, Bongarzone I, Borrello MG, Mariani C, Miranda C, Sozzi G, Greco A** 1995 Rearrangements of TRK proto-oncogene in papillary thyroid carcinomas. *J Endocrinol Invest* 18:130–133
 269. **Trovato M, Villari D, Bartolone L, Spinella S, Simone A, Violi MA, Trimarchi F, Batolo D, Benvenia S** 1998 Expression of the hepatocyte growth factor and c-met in normal thyroid, non-neoplastic, and neoplastic nodules. *Thyroid* 8:125–131
 270. **Aasland R, Akslen LA, Varhaug JE, Lillehaug JR** 1990 Co-expression of the genes encoding transforming growth factor- α and its receptor in papillary carcinomas of the thyroid. *Int J Cancer* 46: 382–387
 271. **Jhiang SM, Sagartz JE, Tong Q, Parker-Thornburg J, Capen CC, Cho JY, Xing S, Ledent C** 1996 Targeted expression of the ret/PTC1 oncogene induces papillary thyroid carcinomas. *Endocrinology* 137:375–378
 272. **Powell DJ, Russell J, Nibu K, Li G, Rhee E, Liao M, Goldstein M, Keane WM, Santoro M, Fusco A, Rothstein JL** 1998 The RET/PTC3 oncogene: metastatic solid-type papillary carcinomas in murine thyroids. *Cancer Res* 58:5523–5528
 273. **Nguyen LQ, Kopp P, Martinson F, Stanfield K, Roth SI, Jameson JL** 2000 A dominant negative CREB (cAMP response element-binding protein) isoform inhibits thyrocyte growth, thyroid-specific gene expression, differentiation, and function. *Mol Endocrinol* 14:1448–1461
 274. **Veneziani BM, Villone G, Romano R, Di Carlo A, Garbi C, Tramontano D** 1990 The tissue-specific pathways regulating cell proliferation are inherited independently in somatic hybrid between thyroid and liver cells. *J Cell Biol* 111:2703–2711
 275. **Pohl V, Roger PP, Christophe D, Pattyn G, Vassart G, Dumont JE** 1990 Differentiation expression during proliferative activity induced through different pathways: *in situ* hybridization study of

- thyroglobulin gene expression in thyroid epithelial cells. *J Cell Biol* 111:663–672
276. Pohl V, Abramowicz M, Vassart G, Dumont JE, Roger PP 1993 Thyroperoxidase mRNA in quiescent and proliferating thyroid epithelial cells: expression and subcellular localization studied by *in situ* hybridization. *Eur J Cell Biol* 62:94–104
277. Roger PP, Van Heuverswyn B, Lambert C, Reuse S, Vassart G, Dumont JE 1985 Antagonistic effects of thyrotropin and epidermal growth factor on thyroglobulin mRNA level in cultured thyroid cells. *Eur J Biochem* 152:239–245
278. Missero C, Pirro MT, Di Lauro R 2000 Multiple ras downstream pathways mediate functional repression of the homeobox gene product TTF-1. *Mol Cell Biol* 20:2783–2793
279. Missero C, Cobellis G, De Felice M, Di Lauro R 1998 Molecular events involved in differentiation of thyroid follicular cells. *Mol Cell Endocrinol* 140:37–43
280. Richards JS 2001 New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Mol Endocrinol* 15:209–218
281. Lazzereschi D, Sambuco L, Carnovale SC, Ranieri A, Mincione G, Nardi F, Colletta G 1998 Cyclin D1 and cyclin E expression in malignant thyroid cells and in human thyroid carcinomas. *Int J Cancer* 76:806–811
282. Wang S, Wu J, Savas L, Patwardhan N, Khan A 1998 The role of cell cycle regulatory proteins, cyclin D1, cyclin E, and p27 in thyroid carcinogenesis. *Hum Pathol* 29:1304–1309
283. Baldassarre G, Belletti B, Bruni P, Boccia A, Trapasso F, Pentimalli F, Barone MV, Chiappetta G, Vento MT, Spiezia S, Fusco A, Viglietto G 1999 Overexpressed cyclin D3 contributes to retaining the growth inhibitor p27 in the cytoplasm of thyroid tumor cells. *J Clin Invest* 104:865–874

**15th International Symposium of the
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“RECENT ADVANCES IN STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY”

May 17–20, 2002—Munich (München), Germany

The 15th International Symposium of the *Journal of Steroid Biochemistry & Molecular Biology*. “Recent Advances in Steroid Biochemistry & Molecular Biology” will be held in Munich (München), Germany, on May 17–20, 2002. The following topics will be considered: 1. Steroid receptors, structure, gene expression, and mechanism of action; 2. Nongenomic effect of steroid hormones; 3. Steroid membrane receptors; 4. Steroids and cancer; 5. Steroids in the central and peripheral nervous systems; 6. Steroids and menopause; 7. Enzyme modulators; 8. Steroid hormones, phyto-, xeno-estrogens, and the environment; 9. Steroids and sport.

Lectures (approximately 30–35) will be by invitation of the Scientific Organizing Committee and, in addition, there will be poster sections. All posters presentations will be subject to selection by the Scientific Organizing Committee.

Abstracts (maximum 200 words) must be sent to Prof. J. R. Pasqualini by Monday, February 4, 2002 (postmark) (*original* + 12 copies).

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Erratum

In the June 2001 issue of *Endocrine Reviews*, an error appears in Table 1 in the article by Palter *et al.* (Palter SF *et al.* 2001 Are estrogens of import to primate/human ovarian folliculogenesis? *Endocr Rev* 22:389–424). Under the heading “ER α status,” the entry for Rosenfeld *et al.* (1999) should read N/A instead of TC.