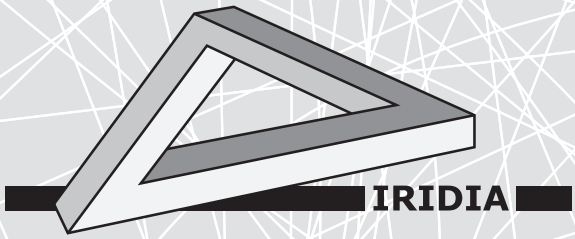

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Gene expression in human thyrocytes and autonomous adenomas reveals suppression of negative feedbacks in tumorigenesis

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The cAMP signaling pathway regulates growth of many cell types, including somatotrophs, thyrocytes, melanocytes, ovarian follicular granulosa cells, adrenocortical cells, and keratinocytes. Mutations of partners from the cAMP signaling cascade are involved in tumor formation. Thyroid-stimulating hormone (TSH) receptor and Gsα activating mutations have been detected in thyroid autonomous adenomas, Gsα mutations in growth hormone-secreting pituitary adenomas, and PKAR1A mutations in Carney complex, a multiple neoplasia syndrome. To gain more insight into the role of cAMP signaling in tumor formation, human primary cultures of thyrocytes were treated for different times (1.5, 3, 16, 24, and 48 h) with TSH to characterize modulations in gene expression using cDNA microarrays. This kinetic study showed a clear difference in expression, early (1.5 and 3 h) and late (16–48 h) after the onset of TSH stimulation. This result suggests a progressive sequential process leading to a change of cell program. The gene expression profile of the long-term stimulated cultures resembled the autonomous adenomas, but not papillary carcinomas. The molecular phenotype of the adenomas thus confirms the role of long-term stimulation of the TSH–cAMP cascade in the pathology. TSH induced a striking up-regulation of different negative feedback modulators of the cAMP cascade, presumably insuring the one-shot effect of the stimulus. Some were down- or nonregulated in adenomas, suggesting a loss of negative feedback control in the tumors. These results suggest that in tumorigenesis, activation of proliferation pathways may be complemented by suppression of multiple corresponding negative feedbacks, i.e., specific tumor suppressors.

cyclic AMP | microarrays | papillary tumors | thyrotropin

Tight regulation of the second messenger cAMP is of crucial importance for cells because it regulates function, differentiation, and proliferation (1). In cells in which cAMP stimulates growth, activating mutations in partners of this pathway induce uncontrolled growth. In most benign thyroid autonomous adenomas, activating mutations have been found in the thyroid-stimulating hormone (TSH) receptor (TSHR) (2) and, to a lesser extent, in the Gsα protein, an activator of the cAMP-producing adenylyl cyclase (1, 3). These mutations result in a TSH-independent growth and lead to hyperfunction (1). In addition, activating mutations of the Gsα protein have been detected in growth hormone-secreting pituitary adenomas (4) and inactivating mutations in the type I-α regulatory subunit inhibiting protein kinase A (PKAR1A) in Carney complex, a multiple neoplasia syndrome (5).

Our knowledge of the genes regulated by the cAMP–protein kinase A cascade and its uncontrolled activation is still sketchy (6). To gain more insight into the cAMP-activated signal transduction cascade in tumors, human primary cultures of thyrocytes treated for different times with the TSH growth and differentiation stimulus were used as a model. Thyrocytes in primary culture are expected to be better models than immortalized cell lines that are already well on the way to transformation. Cells under prolonged stimulation by TSH should be an informative model of the thyrocytes chronically stimulated by the same cascade in autonomous adenomas (7). First, these cultures contain only thyrocytes, and therefore thyrocyte-specific gene expression is studied without interference of other cell types (8). Second, cell-culture conditions are strictly controlled. The cells are submitted to a single stimulus at a time, and the exposure to the stimulus can be exactly timed. Third, TSH-treated and untreated cells are handled identically at all time points; hence, artifacts related to specimen handling are greatly reduced.

In this study, primary thyrocytes were stimulated by TSH for various times, and their gene expressions were analyzed by using microarrays. The results demonstrate a sequential program of gene expression after TSH stimulation and a similarity of gene expression between cells submitted to prolonged TSH stimulation and autonomous adenomas, but not with papillary tumors. Interestingly, a number of genes that were up-regulated in the cultures treated with TSH are involved in negative feedback mechanisms demonstrating the importance of the natural brakes of the activating pathway, some of which disappear in adenomas.

Materials and Methods

Primary Culture of Human Thyrocytes. Thyroid tissue was obtained from seven human subjects undergoing surgery for Graves' disease, solitary or multiple nodules (either hyperfunctioning or hypofunctioning). Nodules were always removed for pathology, and only separate, healthy tissues were used for the preparation of the cell cultures. Protocols were performed according to the rules of the ethic committees of the institutions. Follicles were prepared as described in ref. 9. Cultures then were stimulated with 0.3 milliunits/ml bovine TSH (Sigma) during 1.5, 3, 16, 24, or 48 h or were treated for 24 h with 10^{-5} M forskolin. At each time point, controls were included.

Tissue Samples. Diagnosis of autonomous adenomas was based on the low TSH serum levels, the demonstration by scintigraphy of a highly radioactive nodule with poorly radioactive surrounding and contralateral tissue, and on postsurgical histological analysis demonstrating encapsulation. Validation of the diagnosed identity of the analyzed tumors was made by measuring the increase in sodium/iodide symporter mRNA expression (10).

For the papillary thyroid carcinomas, paired samples of nontu-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MDS, multidimensional scaling; qRT-PCR, quantitative real-time PCR; SAM, Significance Analysis of Microarray; TSH, thyroid-stimulating hormone; TSHR, TSH receptor.

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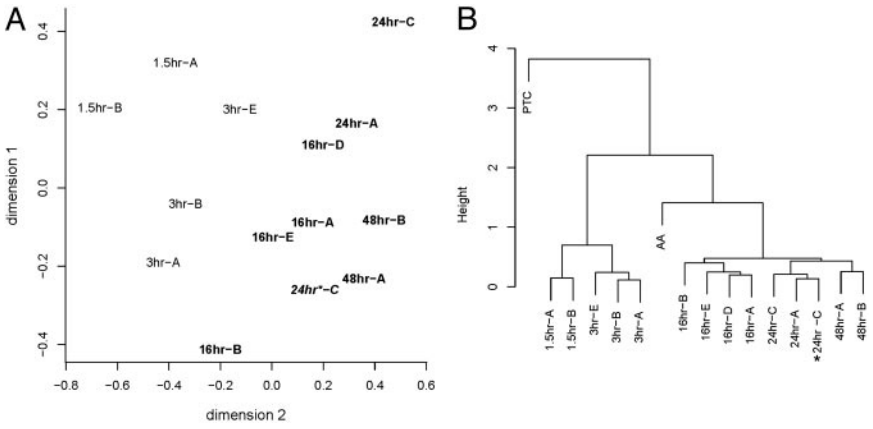

Fig. 1. Visualization of gene expression data. (*A*) MDS based on five independent human primary thyroid cell cultures, labeled A–E, treated with 0.3 milliunits/ml TSH for 1.5, 3, 16, 24, and 48 h or with 10^{-5} M forskolin for 24 h (24 h*). The MDS is based on all of the genes present on the array. The distortion of distances (stress) between the MDS 2D space and the actual gene space is 13.4%. (*B*) Hierarchical clustering of the microarray data from five independent human primary thyroid cultures. Labeling is identical to that in *A*. In addition, shown is the expression profile of a pool of autonomous adenomas (AA) and from a group of papillary tumors (PTC). Clustering was made based on considering only differentially expressed genes in the primary thyroid cell cultures selected by SAM ($q < 0.05$). 24h* still clusters at 24 h if excluded from SAM analysis (data not shown).

mor and tumor thyroid tissues were obtained from patients undergoing surgery for thyroid disease at the Ambroise Pare Hospital (Boulogne, France) ($n = 10$) or at the Institute of Oncology and Metabolism (Kiev, Ukraine) ($n = 6$). The protocol was approved by the ethics committees of the institutions. All tissues were immediately dissected, placed on ice, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

RNA Purification. Total RNA was extracted from thyroid samples by using a TRIzol Reagent kit (Invitrogen) followed by a purification on RNeasy columns (Qiagen, Hilden, Germany). The RNA concentration was spectrophotometrically quantified, and its integrity was verified by visualization of intact 18S and 28S ribosomal RNA bands after gel migration. For the adenoma samples, equal quantities of total RNA were pooled from the tumors of five different patients and from their normal adjacent tissues. Papillary thyroid tumors were individually analyzed by comparing each tumor with its corresponding adjacent tissue.

cDNA Synthesis, Labeling, and Microarray Hybridization. From 3 μg of total RNA, double-stranded cDNA was synthesized followed by production of antisense RNA using an Ampliscribe T7 high-yield transcription kit (11). Next, samples were incubated with 5-(3-aminoallyl)-dUTP (Sigma–Aldrich), followed by a labeling with Cy3 and Cy5 (Amersham Pharmacia Biosciences). Samples were hybridized onto in-house-manufactured slides containing 23,232 spots with 7,541 different identified cDNAs (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site).

Data Analysis. ***Data acquisition and preprocessing.*** Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA). Expression levels were quantified with GENEPIX PRO 5.0 (Axon Instruments). Each array was scanned at three different photomultiplier gains to achieve a broader range of measurement (12). After merging the multiple scans (12), background fluorescence intensities were subtracted, and negative intensity probes were removed. Spatial- and intensity-based LOESS normalizations (13) were carried out by using functions of the MARRAY 1.6.3 package (14) for the R 2.1.0 language (15). All hybridizations were replicated with dyes swapped. Log_2 ratios averaged over replicates were considered in subsequent analysis.

Search for regulated genes. Regulated genes were selected by a threshold method (see Tables 2 and 3, which are published as supporting information on the PNAS web site) and the Significance Analysis of Microarray (SAM) (15) method as implemented in the SIGGENES package (Version 1.2.17) (16) for R (see Table 4, which is published as supporting information on the PNAS web site). In Tables 2 and 3 (threshold method), features above a tumor/control $\log_2$-ratio of 1 in at least two biological replicates at one time point at least were selected as up-regulated (below −1 defined as down-regulated). For Table 4, SAM was run with 10^4 permutations and a moderated five-classes F-statistic, one class per time point. Genes with a q value of <0.05 were considered regulated.

Nonsupervised analysis. Nonsupervised analysis was performed on the basis of between-sample correlation distances. In Fig. 1*A*, multidimensional scaling (MDS) (as implemented by the isoMDS function in R) was performed on all of the features. In Fig. 1*B*, average linkage was used for hierarchical clustering (as implemented by the hclust function in R) on the features selected by SAM (Table 4).

Validation of Gene Expression by Quantitative Real-Time PCR (qRT-PCR). Bradykinin receptor B2 (*BDKRB2*), dual-specificity phosphatase 2 (*DUSP2*), growth arrest- and DNA damage-inducible gene GADD45-β (*GADD45B*), homogentisate 1,2-dioxygenase (*HGD*), heat shock 70-KD protein 5 (*HSPA5*), regulator of G protein signaling 2 (*RGS2*), *RGS16*, and SNF1/AMPK-related protein kinase (*SNARK*) mRNA expressions were investigated in four independent cultures per time point and in autonomous adenomas by using qRT-PCR (SYBR Green) (Eurogentec, Liege, Belgium) (see *Supporting Materials and Methods* and also Table 5, which is published as supporting information on the PNAS web site).

Results

Human Thyrocytes Treated with TSH Show a Progression in Overall Gene Expression Through Time with a Separation into an Early and Late Program on a Subset of Genes. Gene expression in thyrocytes was investigated in human primary thyrocyte cultures treated with 0.3 milliunits/ml TSH for 1.5, 3, 16, 24, and 48 h, compared with their nontreated controls for the corresponding time points. Treatment of human thyrocytes with 0.3 milliunits/ml TSH stimulates essentially the cAMP pathway and has a strong effect on differentiation and proliferation (9). Primary thyrocytes were also treated with the adenylate cyclase activator forskolin (10 μM) for 24 h. Differentially expressed genes were searched for by using two approaches. First, a gene was considered differentially expressed at a given time point if it was at least 2-fold up or down in two cultures or more (Tables 2 and 3). This threshold method detected 468 regulated spots, representing 141 genes. Second, the F test-based SAM (17) method was used. SAM detects genes with highly consistent regulation among cultures within one time point but shows widely contrasted expression between different time points [$q < 0.05$; i.e., the q value is a measure of statistical confidence taking into account multiple testing (18)] (Table 4). SAM detected 316 differentially expressed spots, representing 109 genes. SAM takes into account multiple testing, the fact that tens of thousands of genes are being tested at once. Another advantage is that even genes that did not reach the conventional 2-fold modulation could be detected. Genes such as *RGS16* or *BDKRB2*, up-regulated in all

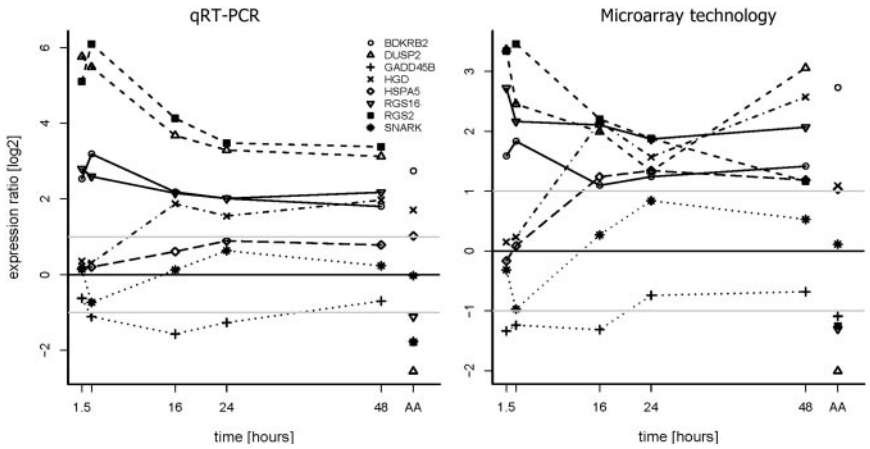

Fig. 2. Comparison of differential gene-expression data obtained by microarray and qRT-PCR (SYBR Green) from human thyroid cell primary cultures treated with 0.3 milliunits/ml TSH for different times (1.5, 3, 16, 24, and 48 h) and of a pool of autonomous adenomas (AA) for *BDKRB2*, *DUSP2*, *GADD45B*, *HGD*, *HSPA5*, *RGS2*, *RGS16*, and *SNARK*. For each gene, averages over two to four cultures (microarray) or four cultures (qRT-PCR) per time point are depicted. Correlation factors between microarray and qRT-PCR data for each gene were 0.61 (*BDKRB2*), 0.93 (*DUSP2*), 0.16 (*GADD45B*), 0.92 (*HGD*), 0.88 (*HSPA5*), 0.98 (*RGS2*), 0.99 (*RGS16*), and 0.92 (*SNARK*). The low correlation factor of *GADD45B* is due to the outstanding stability of this gene. It is fluctuating around -1 $\log_2$-ratio for both qRT-PCR and microarray measurements.

cultures but with strong variations per culture, were not retained in the SAM list because their q-values are high ($q \approx 0.50$).

Overall gene expression was investigated from the profiles of two to four primary cultures and analyzed by using MDS. The MDS algorithm reduces the high-dimension genes space into two dimensions while preserving distances between the profiles and thereby visualizes the similarity relationships between samples. MDS based on all of the genes present on our microarrays reflected a time evolution (Fig. 1*A*). Time points were ordered sequentially along the top-left to bottom-right axis, with late time points at the bottom-right. The 24-h forskolin-treated cells showed a gene-expression profile very similar to the long-term TSH-treated thyrocytes (Fig. 1*A*) (19, 20). Hierarchical clustering, based on the subset of genes detected by SAM (Table 4), demonstrated a clear separation into the short-time (1.5 and 3 h) and long-time TSH treatments groups (16, 24, and 48 h, including forskolin 24 h), further indicating a change of cell program (Fig. 1*B*).

Autonomous Adenomas Are Most Similar to Long-Term Stimulated Primary Cultures. The gene expression profiles of the TSH-treated primary cultures were compared with those of a pool of benign hyperfunctioning autonomous adenomas and a group of malignant papillary tumors. We pooled the adenoma samples because autonomously functioning adenomas are homogenous tumors with consistent gene-expression modulations among the different tumors (10). Clustering based on the differential expression detected by SAM showed that autonomous adenomas were the closest to long-term stimulated cultures (Fig. 1*B*). In contrast, our negative control, papillary tumors did not cluster preferentially with cultures at any time point and were far apart from both the TSH-treated cultures and the autonomous adenomas (Fig. 1*B*).

Validation of Microarray Data. A more detailed analysis of the lists of regulated genes (Tables 2–4) confirms previous work on thyroid cells or other cells treated with TSH- or cAMP-enhancing agents for the genes *RGS2* (21, 22), *RGS16* (23), *PDE4* (24, 25), *DIO2* (26–28), *JUNB* (19, 29), *NR4A1* (30), cAMP response element modulator (*CREM*) (31), *TGFβ* (32), *IL8* (33), *RRAD* (34), and *EGR1* (19, 29).

Eight modulated genes, selected by the threshold method, SAM, or both, in four TSH-stimulated primary cultures and in autonomous adenomas were investigated by qRT-PCR (Fig. 2). Some of them had been hybridized previously on the microarray slides. The investigated genes were *BDKRB2*, *DUSP2*, *GADD45B*, *HGD*, *HSPA5*, *RGS2*, *RGS16*, and *SNARK*. Similar modulation patterns were found for the expression of the eight genes comparing microarray analysis with qRT-PCR. The average correlation of the eight genes between the two methods was 0.8. The results of both techniques were also comparable for *EGR1* and *KLF6* (data not shown).

Kinetics of mRNA Expressions. ***Early up-regulated genes.*** Examples of different patterns of kinetics derived from the microarray data are shown in Fig. 3. Among the 22 genes up-regulated >2-fold after 1.5 h of TSH treatment, 11 did not reach higher levels later on (pattern A; Fig. 3*A* and Table 1), and 5 of these 11 were up-regulated only at early times: the immediate early genes. Of 5, 3 are known transcription factors (*JUNB*, *KLF10*, *ZFP36*). Among the 38 genes up-regulated at 3 h, 17 had their peak expression at that time (pattern B; Fig. 3*B* and Table 1): the early genes. Among these 38 genes, 29 of them were still up-regulated at later times. They code for proteins involved in different types of cellular processes, such as signal transduction (G-protein coupled receptors, *EBI2*, *EDG2*), membrane traffic (*VAPA*), metabolism (*DIO2*), cell adhesion (*LOC90139*), and some long-lived transcription factors (*NR4A1*, *NR4A3*, *CREM*, *GATA3*).

Late up-regulated genes. After 16 and 24 h of TSH treatment, 54 and 48 genes, respectively, were up-regulated. Among all of the genes up-regulated at 16 or 24 h, 40 had not been up-regulated at earlier times (pattern C; Fig. 3*C*). At 48 h, from the 44 up-regulated genes, only 6 of them had not been regulated earlier (pattern D; Fig. 3*D*). The latter code for proteins involved in transport (*SLC39A14*), lipid metabolism (*LPL*), phosphorylation (*PTPN4*), chaperoning (*HSPA9B*), and immunity (*CD74*, *HLA-DRA*). All these genes can be considered as late genes.

Down-regulated genes. Of the 53 genes down-regulated at least at one time point, 8 were down-regulated after 1.5 h of TSH treatment. These 8 genes were still down-regulated at 3 h, and, in addition, 18 other genes were down-regulated at this time point, resulting in a total number of 26 down-regulated genes at 3 h. These genes are early depressed genes (pattern E; Fig. 3*E*). They code for transcription factors (*EGR1*, *KLF6*) and signal transduction proteins such as protein kinases (*JUN*) or a kinase modulator (*PIK3R1*). Twenty-six genes were only down-regulated at later times (pattern F; Fig. 3*F*): the late down-regulated genes. They code for functional, signal transduction, and structural proteins.

Comparison of Gene Expression in TSH-Treated Thyrocytes and in Autonomous Adenomas: Down-Regulation of Negative Feedbacks in Adenomas. Differentially expressed genes from the TSH-treated primary cultures were compared with their expression in autonomous adenomas. In the cultured thyrocytes, 88 genes were up-regulated and 53 were down-regulated at least at one time point. In contrast, 84 genes were up-regulated and 196 genes were down-regulated in the adenomas. The greater number of down-regulated compared with up-regulated genes in adenomas has been reported

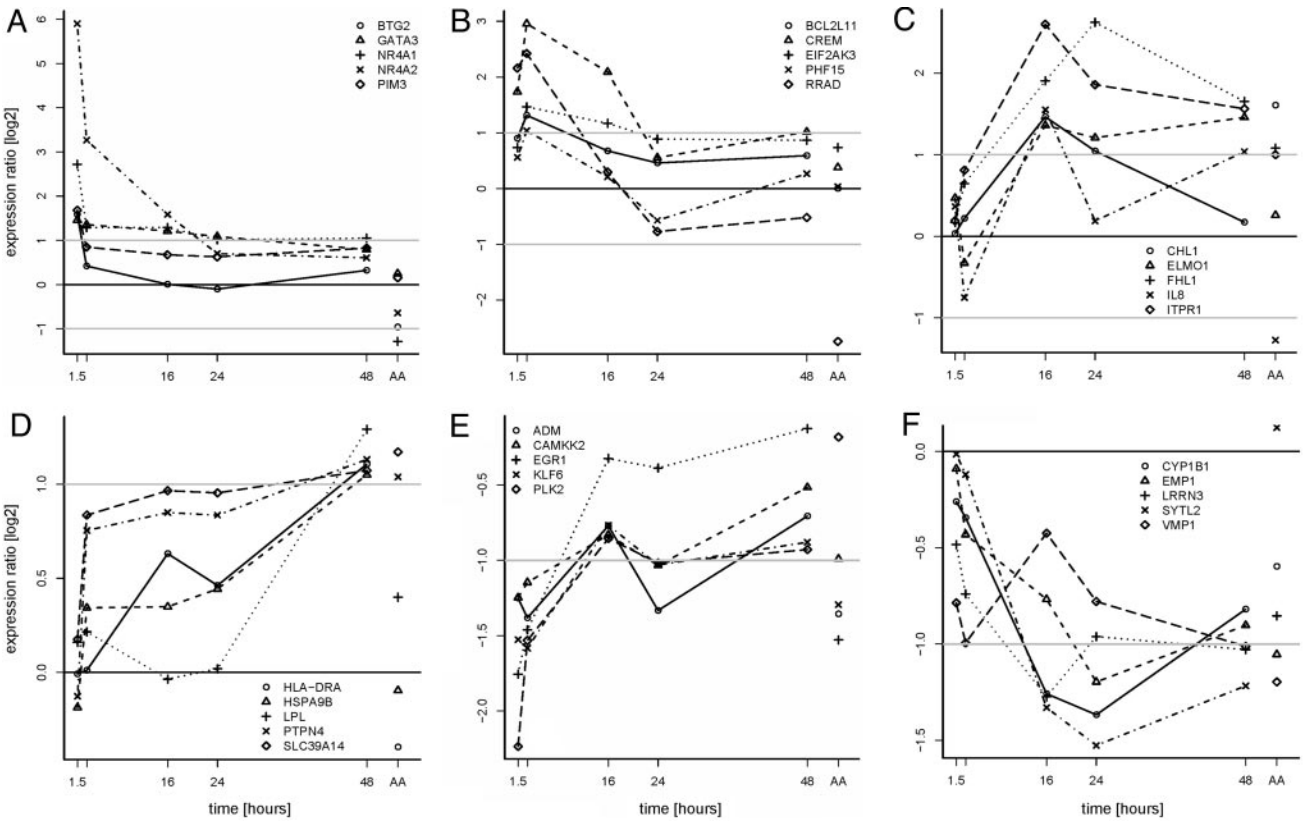

Fig. 3. Kinetics of gene-expression profiles in primary cultures treated for different times with TSH compared with the gene expression profile of autonomous adenomas (AA). For each time point, the results represent the averages of the $\log_2$ intensity ratios (TSH-treated/control or tumor/control). Plots *A–F* represent different expression patterns as described in the text for immediate early up-regulated (*A*), early up-regulated (*B*), late up-regulated (*C*), very late up-regulated (*D*), early down-regulated (*E*), and late down-regulated (*F*) genes.

(10). It probably reflects a change in cell population (decreased lymphocytic infiltration, red cell content).

From the total of 22 genes up-regulated at 1.5 h, one gene (*BDKRB2*) also was up-regulated in the adenomas (1/22; 4.5%) (Table 1). The proportions of genes modulated in both cell culture and adenomas were 21.0% (8/38) at 3 h, 29.6% (16/54) at 16 h, 33.3% (16/48) at 24 h, and 40.9% (18/44) at 48 h (Table 1). This finding parallels our clustering result: the longer the stimulation, the closer the relation between *in vitro* thyrocytes and *in vivo* adenomas. This progression does not apply to the down-regulated genes (Table 1). The largest overlap between down-regulated genes and adenomas was found at short-term TSH treatment. Genes down-regulated only at early time points and down-regulated in the adenomas were *EGR1*, *CLC*, *JUN*, *PMAIP1*, *PPP1R15A*, *IER2*, and *CDC42EP2*. From the genes only regulated at late time points, *EMP1* and *VMP1* also were down-regulated in the adenomas.

From the 8 genes (*NR4A1*, *DUSP2, RGS2*, *RGS16*, *EBI2*, *CREM*, *CXCL2*, *BDKRB2*) that were up-regulated after 1.5 h of TSH treatment and were still up-regulated at late time points, 6 were down-regulated in the autonomous adenomas, 1 was up-regulated (*BDKRB2*), and 1 was not modulated (*CREM*). The modulation of *DUSP2*, *RGS2*, *RGS16*, and *BDKRB2* in the cultures and adenomas also was confirmed by qRT-PCR (Fig. 2). These four genes were still up-regulated in the cultures after 72 h of TSH treatment (data not shown). *RGS2* (an inhibitor of adenylyl cyclases), *RGS16* (inhibitor of Gq), and *DUSP2* (MAPK phosphatase) all operate as negative regulators of signal transduction.

Discussion

Hormonal stimulation of the cAMP-signaling pathway regulates growth of several cell types. Activating mutations of genes of this pathway lead to pituitary (4) and thyroid (2, 3) tumors. In this study we tried to elucidate the genes that are modulated by the cAMP-signaling pathway in thyrocytes *in vitro* treated with their physiological stimulus TSH. These gene expressions then were compared with chronically stimulated autonomous adenomas, benign thyroid tumors.

Table 1. Microarray data obtained from human primary thyroid cell cultures treated with TSH for 1.5, 3, 16, 24, or 48 h

TSH treatment, h	Total no. of up-regulated genes	No. of up-regulated genes with peak value	Total no. of regulated genes in AA	Total no. of down-regulated genes	No. of down-regulated genes with peak value	Total no. of regulated genes in AA
1.5	22	11	1 up, 10 down, 2 NA	8	4	5 down, 0 up, 1 NA
3	38	17	8 up, 8 down, 4 NA	26	20	12 down, 0 up, 1 NA
16	54	27	16 up, 9 down, 4 NA	18	10	2 down, 0 up, 1 NA
24	48	19	16 up, 5 down, 5 NA	11	6	3 down, 0 up, 1 NA
48	44	15	18 up, 6 down, 1 NA	17	12	2 down, 0 up, 2 NA

The expression of each regulated gene in the culture at each time point was compared with the expression in autonomous adenomas (AA). NA, missing value.

Our analysis of gene expression in primary culture of TSH-stimulated human thyrocytes shows (*i*) a remarkable congruence of microarray results derived from independent cultures confirmed by qRT-PCR and also with results from reported studies on thyroid cells; (*ii*) a clear distinction between an early and a late program of gene expression after TSH stimulation; (*iii*) genes modulated by the TSH–cAMP pathway in thyrocytes with their kinetics and in particular negative feedback modulators of the cAMP pathway; and (*iv*) a number of genes modulated in both the TSH-treated cultures and the adenomas in a similar or an inverse way.

Besides the confirmation by the literature and qRT-PCR, validation of microarray data and the model was done in several ways. (*i*) Clustering showed that the time course of TSH treatment could be completely recovered even though SAM gene selection and hierarchical clustering operate in a time-independent order. (*ii*) Clustering of the results of forskolin-treated cells, a known activator of the adenylyl cyclase, further confirmed that the TSH-induced expression profile of the primary cultures reflects cAMP action. In addition, the fact that it grouped with the 24-h TSH-treated cells further shows a similar activation kinetics by TSH and forskolin. (*iii*) Genes characteristic of TSH stimulation detected by SAM produce a clustering in which TSH-treated cultures are grouped with autonomous adenomas, but far apart from papillary thyroid tumors. Indeed, the latter tumors are malignant and characterized by an activation of growth factor pathway, not the cAMP cascade (35).

A large number of genes that are modulated by cAMP in the primary cultures also were modulated in the autonomous adenomas. Many early up-regulated genes were down-regulated in adenomas, whereas the longer the TSH stimulus, the more genes that were up-regulated in the adenomas. However, this relation was not found for the down-regulated genes. Nevertheless, hierarchical clustering shows that on the whole, autonomous adenomas are the closest to long-term stimulated cultures.

Although the cAMP pathway is activated in TSH-treated cultures and in adenomas, there are discrepancies when comparing both expression profiles. Many discrepancies can be explained by the existence of nonthyrocyte cell populations in the *in vivo* tumor (36). On one hand, the purity of the thyrocyte cultures reveals regulations operating in the thyrocytes themselves independently of the possible variations in gene expression in the different cell populations present in the tissue specimens. On the other hand, a drawback of the model is that the tissue organization and the effect of other cell types on thyrocytes was not studied. In addition, the presence of different cell types in tissues explains the clustering of adenomas with papillary tumors, but not with the TSH-treated cultures, when clustering is based on the overall gene expression (data not shown).

Recently, we showed that a change in cell population occurs in the autonomous adenomas with greatly decreased lymphocytes, macrophage infiltration, and increased endothelial cell content when compared with normal tissue (10). Such changes cannot be studied in our model, but our data show that thyrocytes in culture treated with TSH produce lymphocyte attractants/stimulators such as *IL8* (33), *CXCL2*, or *IRF4*, which are down-regulated in the autonomous adenomas, in line with our previous findings (10). In addition, thyrocytes could be modified by the culturing itself, and the model does not fully mimic the years of stimulation of the cAMP pathway in adenomas. Nevertheless, genes regulated in both models may reflect more the primary physiopathological mechanisms involved.

The gene-expression data reported here relate the molecular and biological phenotypes of stimulated thyroid cells. The profiles show a sharp distinction between early and late effects of TSH. Successive waves of very early, early, and late genes suggest a sequential process induced by the stimulus. Accordingly, the predominance of transcription factors among the very early up-regulated genes likely induce genes responsible for a sustained activation state. Some early down-regulated genes such as *SNARK*, which inhibits biosyntheses, fall in the same category. Conversely, some of the early up-regulated genes remain up-regulated. These genes, as well as the late up-regulated genes, code for signal-transduction and functional proteins reflecting the passage from one phenotype to another and an on/off regulation between two different states of the system. Similarly, early depressed genes code for transcription factors and signal-transduction proteins, whereas late depressed genes code for signal-transduction, functional, and structural proteins.

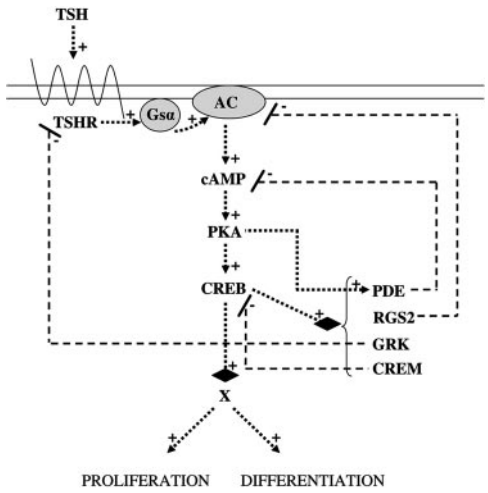

Fig. 4. TSH–cAMP signaling pathway in thyrocytes and its negative feedback regulators. AC, adenylyl cyclase; CREB, cAMP response element binding protein; PKA, cAMP-dependent protein kinase. Data on GRK are from ref. 46. ⋯▶, activation; ——, inhibition; ⋯◆, gene induction.

The main consequence of TSH action is cAMP accumulation, which induces cell activation, proliferation, and differentiation. Unsurprisingly, genes of proteins involved in specialized (*DIO2*, *ELMO1*, *VAPA*) or general metabolism (mitochondrial, *CYCS*, *ATP5B*; other, *TSPYL4*), transport (*SLC7A7*, *SLC39A14*), protein export (*VAPA*, *SNX1*), and chaperoning (*HSPA5*, *TRA1*, *CANX*, *HSPA9B*) were up-regulated in accordance with the known positive effect of TSH and cAMP on the function, growth, and differentiation of these cells (1). The protein export and chaperoning mediators would certainly help the increased thyroglobulin secretion and turnover by the stimulated cells. The decreased expression of three proapoptotic genes (*DAXX*, *PMAIP2*, *GADD45B*) and the increased expression of two antiapoptotic genes (*HSPA9B*, *BCL2L11*) give some indication about the mechanisms of the antiapoptotic effects of TSH on such cultured thyroid cells (1). There is no clear trend in the regulation of positive and negative regulators of cell mitogenesis.

Comparing gene expression in TSH-treated cultures, autonomous adenomas, and papillary tumors sheds light on the biological function of the genes. Genes regulated in a similar direction in cultured thyrocytes and autonomous adenomas, but that are regulated in the inverse way in papillary carcinomas, might reflect the differentiating action of the TSH–cAMP pathway [*DIO2* (26, 37), *HGD* (38), *FHL1* (39), *ITPR1* (39, 40), *CRABP1* (40, 41), and *ADM* (40)], whereas common expression in the TSH-stimulated thyrocytes, autonomous adenomas, and papillary carcinomas concerns possibly proteins involved in the control or support of cell growth [*EFHD2*, *IER2*, *KLF6* (10), *EGR1* (10), *GADD45B*, and JUN]. One puzzling common property of autonomous adenomas and papillary carcinomas is the down-regulation of a number of immediate early genes [*NR4A1*, *JUNB* (10), *KLF10*, and *ZFP36*]. Absence of up-regulation may reflect the relatively low rate of cell proliferation in both tumors (42, 43) and thus the low fraction of cells in early G_1, but down-regulation is more difficult to explain.

One striking result is the importance of the negative feedbacks induced by the stimulating cascade itself (Fig. 4), leading to the

self-limitation of the effects of this cascade. The enhancement of cAMP accumulation is itself later counteracted by multiple negative feedbacks resulting from the increased transcription of genes such as phosphodiesterases (*PDE1A*, *PDE4B*) (24) and *RGS2*, which inhibits some cyclases [e.g., the III and VI, which are well expressed in human thyroid cells (44, 45)]. These genes are already up-regulated at 1.5 h and are still up-regulated after 48 h of TSH treatment; however, some are down-regulated in the adenomas. In autonomous adenomas, increased transcription of G protein-coupled receptor kinase 3 (*GRK3*) (46) and decreased transcription of some adenylate cyclases have similarly been reported (47). Together with the well described direct activation of some phosphodiesterases by cAMP-dependent kinases (48, 49), these negative feedbacks, already described separately in various thyroid and other cell models (25, 50), account for the well known biphasic character of the cAMP accumulation (1). The induction of a repressor of cAMP-induced genes (*CREM*) (31) operates in the same direction.

The remarkable induction or activation of negative feedbacks on cAMP accumulation and action explains not only the decrease of cAMP levels after the first day (9, 51) but also the brief duration of early immediate gene expression and the one-shot nature of the mitogenic effect in thyrocytes in primary culture (9). It explains why chronic stimulation by TSH in pituitary TSH hypersecretion or by stimulating thyroid receptor antibodies leads only to mild hyperplasia and goiter (52). Conversely, it suggests that the important mitogenesis necessary to generate an autonomous adenoma of 1 g, i.e., $\approx 5 \times 10^8$ cells from one mutated cell (>30 divisions), requires additional events besides the initial constitutive activation of the TSHR. The most obvious supplementary events would be the relief of some of the negative feedbacks, i.e., the silencing of the genes involved, thus qualifying as tumor suppressor genes. It is therefore interesting that the up-regulations of *RGS2* and *CREM* observed in TSH-treated thyrocytes were not found in autonomous adenomas. Also, other negative regulators of cell growth induced by TSH *in vitro* were not modulated or were down-regulated in autonomous adenomas: *IGFBP4*, *RGS16*, *DUSP2*, *PTP4A1*, and *JUNB*. When reviewing the consequences of TSHR-constitutive activation in the genesis of autonomous adenomas, Derwahl *et al.* (53) suggested that other complementary mechanisms must be involved. The suppressed negative feedbacks would be good candidates.

The induction of negative feedbacks by signal-transduction pathways, in particular those of cell growth, is a general, well recognized phenomenon. cAMP induces specific phosphodiesterases; ERK induces DUSP, which then inactivates it; NFKB induces its inhibitor IKB; and STATs induce SOCS. The main result of this study is that one specific activated signal transduction pathway (cAMP) induces a multiplicity of negative feedbacks, some of them being lost or not regulated in the tumor. It would be interesting to compare mitogenic pathways in primary cultures of other cell types treated with their specific growth-stimulating factor and to investigate whether multiple negative feedbacks also exist and whether they are affected in corresponding tumors. Inactivation of multiple negative feedbacks induced by one specific pathway might be part of the relief of tumor suppressor action in cancer cells (54).

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