

Gene Expression in RET/PTC3 and E7 Transgenic Mouse Thyroids: RET/PTC3 But Not E7 Tumors Are Partial and Transient Models of Human Papillary Thyroid Cancers

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We studied gene expression profiles in two mouse models of human thyroid carcinoma: the Tg-RET/PTC3 (RP3) and Tg-E7 mice. RP3 fusion gene is the most frequent mutation found in the first wave post-Chernobyl papillary thyroid cancers (PTCs). E7 is an oncoprotein derived from the human papillomavirus 16 responsible for most cervical carcinoma in women. Both transgenic mice develop thyroid hyperplasia followed by solid differentiated carcinoma in older animals. To understand the different steps leading to carcinoma, we analyzed thyroid gene expression in both strains at different ages by microarray technology. Important biological processes were differentially regulated in the two tumor types. In E7 thyroids, cell cycle was the most up-regulated process, an observation consistent with the

huge size of these tumors. In RP3 thyroids, contrary to E7 tumors, several human PTC characteristics were observed: overexpression of many immune-related genes, regulation of human PTC markers, up-regulation of EGF-like growth factors and significant regulation of angiogenesis and extracellular matrix remodeling-related genes. However, similarities were incomplete; they did not concern the overall gene expression and were not conserved in old animals. Therefore, RP3 tumors are partial and transient models of human PTC. They constitute a good model, especially in young animals, to study the respective role of the biological processes shared with human PTC and will allow testing drugs targeting these validated variables. (*Endocrinology* 149: 5107–5117, 2008)

PAPILLARY THYROID CANCERS (PTC) are the most frequent malignant thyroid tumors. They represent 80% of all thyroid cancers. The mutations responsible for these tumors are known and lead to an activation of the MAPK pathway. This activation can be direct through BRAF or less frequently RAS mutations or indirect through tyrosine kinase receptor rearrangements (TRK, RET/PTC) (1). RET/PTC rearrangements are found in 20–40% of sporadic papillary cancers and in more than 70% of childhood radio-induced PTCs (2–6). More than 10 different RET/PTC rearrangements have been found until now. RET/PTC1 and RET/PTC3 are the most frequent. RET/PTC3 (RP3) is found in more than 80% of first-wave post-Chernobyl PTC and is often associated with a solid variant morphology (3, 4). This rearrangement results from the translocation on the long arm of chromosome 10 between two genes: RET and ELE1. This translocation leads to a fusion gene between the 3' tyrosine kinase domain of RET and the 5' domain of ELE1. RET is a

tyrosine kinase receptor expressed in neural crest-derived tissues and thyroid parafollicular C cells but not in normal thyrocytes. ELE1 is a ubiquitous androgen coreceptor. This rearrangement results in the abnormal expression and constitutive activation of RET tyrosine kinase in thyroid cells. RET activates several metabolic cascades, the RAS/RAF/MAPK and the phosphatidylinositol-3-kinase/Akt pathways, being the most important (7, 8). E7 is a viral oncoprotein derived from the human papillomavirus 16, responsible for most uterine cervix cancers (9). E7 binds several nuclear proteins, including the retinoblastoma susceptibility (Rb) protein (10, 11). E7 inactivates Rb and releases the E2F transcription factors, allowing cell cycle initiation.

In this study, we worked on two transgenic mouse populations: the Tg-RET/PTC3 (Tg-RP3) mice and the Tg-E7 mice (12–14). Both strains express the transgene (RP3 or E7) exclusively in the thyroid under the control of thyroglobulin (Tg) promoter. RP3 and E7 mice develop first a thyroid hyperplasia (a huge colloid goitre for E7) followed by differentiated carcinoma with papillary-looking structures in older animals (between 6 and 10 months) (Jin, L., A. Burniat, J. E. Dumont, F. Miot, B. Corvilain, and B. Franc, submitted for publication). Capsular invasion can be observed in E7 tumors, whereas RP3 tumors sometimes develop distant metastasis. Therefore, these two transgenic mice with different initiating mutations both develop thyroid carcinoma but

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Abbreviations: ECM, Extracellular matrix; EGF, epithelial growth factor; FDR, false discovery rate; GO, Gene Ontology; MDS, multidimensional scaling; PTC, papillary thyroid cancer; Rb, retinoblastoma; RP3, RET/PTC3; Tg, thyroglobulin; Tg-RP3, Tg-RET/PTC3.

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with different potential of growth and invasiveness. These models are of interest for three main reasons (1). Because these mice carry mutations known to promote cancer in humans, they allow the study of the different steps leading to carcinoma by analysis of gene expression kinetic (2). They also provide *in vivo* experimental models to define potential therapeutic targets and to test treatments (3). Finally, the analysis of similarities and differences in their gene expression may help to correlate physiopathological mechanisms and phenotype. We therefore analyzed gene expression in thyroids from Tg-RP3 and Tg-E7 mice at different ages by microarray technology. The results showed that only Tg-RP3 thyroids are partial and transient models of human papillary thyroid carcinoma.

Materials and Methods

Transgenic mice

Tg-RP3 transgenic mice were provided by Dr. M. Santoro (Napoli, Italy). The mice generated on a C57bL/6 background were crossed with wild-type C57bL/6 mice. Tg-E7 mice also generated on a C57bL/6 background were kindly provided by Dr. C. Ledent from our institute and crossed with wild-type C57bL/6. Both strain progenies were genotyped by PCR. Just after the mice were killed for thyroid removal, blood samples were taken by intracardiac puncture for T_4 and TSH activity measurements. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the university.

Genotyping PCR

Genomic DNA was extracted from tail biopsies. PCR was performed by adding 200 ng genomic DNA to a 25- μ l reaction medium containing 1 \times PCR buffer (QIAGEN, Valencia, CA), 0.4 mM deoxynucleotide triphosphate (QIAGEN), 0.2 μ M forward and reverse primers for RP3 or E7 gene, 1 μ l of homemade *Taq* polymerase for RET, or 0.75 U of QIAGEN *Taq* polymerase for E7. Then 0.1 mM of $MgCl_2$ (QIAGEN) and 0.25 μ l formamide were added for E7 PCR only. Specimens were placed into a thermocycler and subjected to 40 cycles for RET and 30 cycles for E7: denaturation at 94 C for 60 sec, annealing at 60 C for 60 sec, and elongation at 72 C for 60 sec. Primer sequences used for the detection of RP3 were the following: forward (Tg), 5'-GGCCAGAGCCCTAAGGTGGC-3', reverse (ELE1), 5'-AAGGGATTCAATTGCCATCCA-3', and for the detection of E7: forward, 5'-CATGCATGGAGATACACCT-3', reverse, 5'-GATTATGGTTTCTGAGAACA-3'.

The PCR products (~1300 bp for RP3 and 301 bp for E7) were detected by migration on a 2% agarose gel in the presence of ethidium bromide.

T_4 and TSH measurements

Total T_4 measurement was performed by solid-phase ^{125}I RIA (Coat-A-Count Canine T_4 ; Diagnostic Products Corp., Los Angeles, CA). TSH serum activity was evaluated by bioassay through the measurement of cAMP produced in TSH receptor expressing CHO cells (JP₂₆₋₂₆ clone) in response to mice sera (15).

RNA preparation for microarrays

RP3, E7, and wild-type mice were killed at 2, 6, or 10 months. Thyroid glands were removed, weighed, frozen in liquid nitrogen, and stored at -80 C. Tissue was homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA extracted using chloroform/ethanol procedure. RNA samples were further purified on RNeasy spin columns (QIAGEN). RNA concentration was assessed by NanoDrop ND-1000 spectrophotometer dosage (NanoDrop Technologies, Wilmington, DE) and quality checked using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) after pooling several RNA samples from thyroids of the same age and strain to get enough material. Only RNA samples showing a ratio 260:280 around 2 and 28S:18S around 1 were kept for the experiments. Two micrograms of total RNA were used to prepare cRNA probes.

RNA preparation and cDNA synthesis for real-time PCR

Four micrograms of RNA samples used for microarrays were treated by DNA-free (Ambion, Austin, TX). Two supplementary RNA samples were prepared from each thyroid type and age (wt, E7, and RP3 at 2, 6, or 10 months) following the same protocol as described before. The quality of RNA samples was assessed by analysis of the 28S:18S ratio on Experion RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA). Four micrograms of these RNAs were similarly treated by DNA-free (Ambion) to remove any contaminating DNA. One microgram of each RNA DNA free sample was then reverse transcribed (Superscript II; Invitrogen).

Microarray experiments

We used commercially available oligonucleotide DNA microarray chips (GeneChips Mouse Genome 430 2.0 array; Affymetrix, Santa Clara, CA). They contain 45,000 probe sets representing over 34,000 well-substantiated mouse genes. The probe sets correspond to 11 pairs of oligonucleotides synthesized *in situ* on the array. The array includes a set of mouse maintenance genes to facilitate the normalization and scaling of array experiments. Nine chips were used to test the three different strains (wt, RP3, E7) at three different ages (2, 6, and 10 months). Preparation of cRNA, hybridization, scanning, and image analysis of the arrays were performed according to the manufacturer's protocols.

Quantitative RT-PCR

Confirmation of expression modulations of 11 selected genes was performed by real-time PCR using a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA; 7500 Fast Software; 9600 emulsion run mode) with SYBR Green fluorescence. The oligonucleotide primers were designed to cross introns with the Primer Express software (Applied Biosystems). They are listed in supplemental Fig. 7, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>. Amplifications were performed in 25 μ l of reaction mixture containing 5 μ l of cDNA sample (4 ng cDNA), 12.5 μ l of Power SYBR Green PCR master mix (Applied Biosystems), and specific primer sets at the final concentration of 100 nM. PCR was carried out starting a 10-min hot start at 95 C followed by a denaturation step at 95 C for 15 sec and an annealing step at 60 C for 60 sec for 40 cycles. A standard curve was performed for each primer pair from six serial dilutions (ranging from 20 ng to 20 pg) of mouse thyroid cDNA to calculate individual amplification efficiencies. All reactions were done in triplicate. Dissociation curve analysis (95 C for 15 sec, 60 C for 60 sec, and 95 C for 15 sec) was performed at the end of the 40 cycles to verify PCR product identity and purity. Six housekeeping genes, selected according to microarray data, were tested and analyzed by GeNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>) software: JTB (jumping translocation breakpoint), RER1 (retention in endoplasmic reticulum 1 homolog), HPRT (hypoxanthine guanine phosphoribosyl transferase), Tbp (TATA box binding protein), Bub1 (budding uninhibited by benzimidazoles 1 homolog), and GA17 (Eif3m, eukaryotic translation initiation factor 3, subunit M). Only the two most stable genes, HPRT and RER1, were kept and used for normalization.

Data were analyzed and normalized with Q-base software version 1.3.5. For statistical analysis we used a two-way ANOVA test. Computations were performed with GraphPad Prism (GraphPad Software Inc., San Diego CA).

Data analysis

All calculations, but DAVID analysis, were programmed with the R statistical language (16) version 2.5 and Bioconductor (17) version 2.0. scans (*i.e.* CEL files) and were normalized with gcrma (17, 18). Normalized and raw data are available in the GEO database (www.ncbi.nlm.nih.gov/geo/, accession no. GSE10743). Multidimensional scaling was computed with R's isoMDS function. The immune gene signature was derived from the Novartis Foundation murine normal tissue gene expression atlas [(19); symatlas.gnf.org] as follows. The gcrma version of the data set was downloaded. The top 20% of genes of the most regulated between lymphocyte samples and non immune-related samples were selected. The collective regulation of these

genes in our mouse expression data were estimated with the standard Wilcoxon rank test.

Results and Discussion

Characterization of tumor phenotype

At all ages (from 2 to 10 months), E7 thyroids appeared much bigger than wild-type thyroids with huge colloid goiters early after birth. RP3 thyroids were also larger than wild-type thyroids but to a lesser extent, with early gland hyperplasia (Fig. 1). At all ages mean T_4 and TSH values were in the normal range in both transgenic mice (supplemental Fig. 1). However, some RP3 and few E7 mice were hyperthyroid with high T_4 and low TSH. Mean total T_4 was thus higher in transgenic compared with wild-type mice, at all ages in RP3 mice, and at 2 and 10 months in E7 mice. Similarly, mean TSH activity was lower in RP3 and E7 mice than wild-type mice. Few other RP3 mice (not E7) were hypothyroid with low T_4 level and high TSH. They displayed small size and hypomotricity. These hypothyroid mice did not survive after the age of 1 month and were therefore not included in the microarray study and the mean of T_4 and TSH measurements.

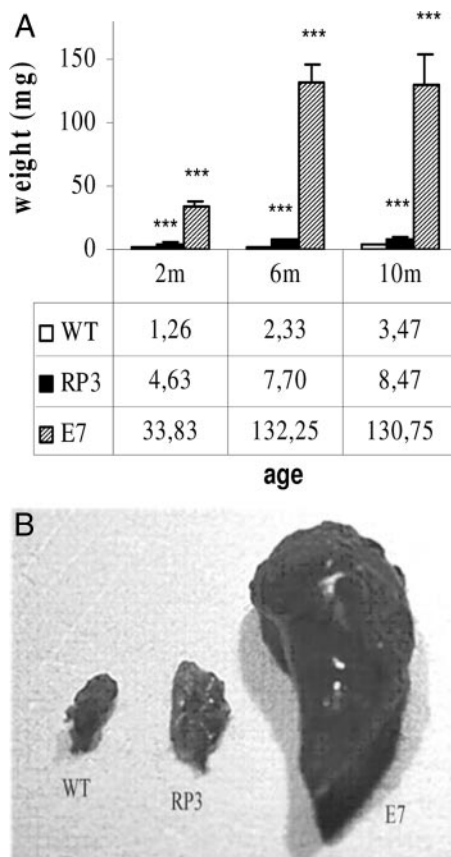


FIG. 1. A, Mean weights (milligrams) of wild-type, RP3, and E7 thyroids at 2, 6, and 10 months. After the mouse was killed, thyroids were dissected and freshly weighted. Unpaired Student *t* test was used for the statistical analysis. ***, $P < 0.0005$. The number of weighted thyroids was 11, 3, and 6 for wild types; 10, 12, and 6 for RP3; and 6, 6, and 4 for E7 at 2, 6, and 10 months, respectively. B, Thyroid lobe of wild-type, RP3, and E7 mice at 6 months.

TABLE 1. Microarray data analysis: number of modulated genes in E7 and RP3 tumors at 2, 6, and 10 months

20701 genes	E7 (months)			RP3 (months)		
	2	6	10	2	6	10
Up	913 <i>322</i>	823 <i>221</i>	655 <i>125</i>	550 <i>258</i>	425 <i>132</i>	391 <i>118</i>
Down	847 <i>274</i>	805 <i>230</i>	675 <i>141</i>	334 <i>142</i>	350 <i>122</i>	318 <i>147</i>

Italic numbers represent the number of genes modulated exclusively at the considered age. Up, fold change of 2 or greater; down, fold change of 0.5 or lower.

Global gene expression profiles in wild-type, E7, and RP3 thyroids

Genes were considered to have altered expression levels in tumor thyroids, compared with wild-type thyroids, when their expression (tumoral *vs.* wild-type expression ratio) was increased or decreased by a factor equal or superior to 2. E7 thyroid microarray data showed that 6.5% of genes were up-regulated and 6.3% of genes were down-regulated all ages taken together. In RP3 thyroids 4.1% of genes were up- and 3.2% were down-regulated at all merged ages (see details in Table 1). Interestingly, the number of up-regulated genes was maximal at 2 months in RP3 and E7 thyroids and then decreased with age, more quickly in RP3 thyroids. Thus, the ratio between up-regulated and down-regulated genes was relatively stable with age in E7: 1.18, 0.96, and 0.89 but decreased sharply in RP3 mice: 1.82, 1.08, and 0.80 at 2, 6, and 10 months, respectively. We compared global expression profiles between the different strains and ages by multidimensional scaling (MDS). MDS collapses the high dimensional gene space into two dimensions and preserves the mean distance relationships between all pairs of samples. This analysis showed similar expression profiles within one strain whatever the age, gene expression being thus more consistent within a given genotype than within a given age (Fig. 2A). Interestingly, when we performed the same analysis with the relative expression data (tumoral *vs.* wild-type expression ratios), expression profiles of 2-, 6-, and 10-month tumors remained together for E7 but split for RP3 strain (Fig. 2B). Thus, ageing changes in gene expression occurred in parallel in wild-type and E7 thyroids, contrary to RP3 tumors in which gene expression evolved in a different way with age. This explanation is compatible with the observation that wild-type and E7 thyroids demonstrated more homogenous histology with time, compared with RP3 thyroids. Wild-type thyroids were essentially composed of follicles at all ages, and even if E7 thyroids became slightly more heterogeneous with age, the appearing carcinoma lesions did not, however, represent a significant part of this huge follicular tumor. On the contrary, RP3 thyroids that were smaller than E7 demonstrated early remodeling, tumor areas representing a more significant part of the RP3 gland, compared with follicles of normal appearance (Jin *et al.*, submitted for publication).

Thyroid function gene expression

Thyroid function genes showed different regulations between tumor types (Table 2). In none of the two models, the determination genes TTF1 and PAX8 were down-regulated.

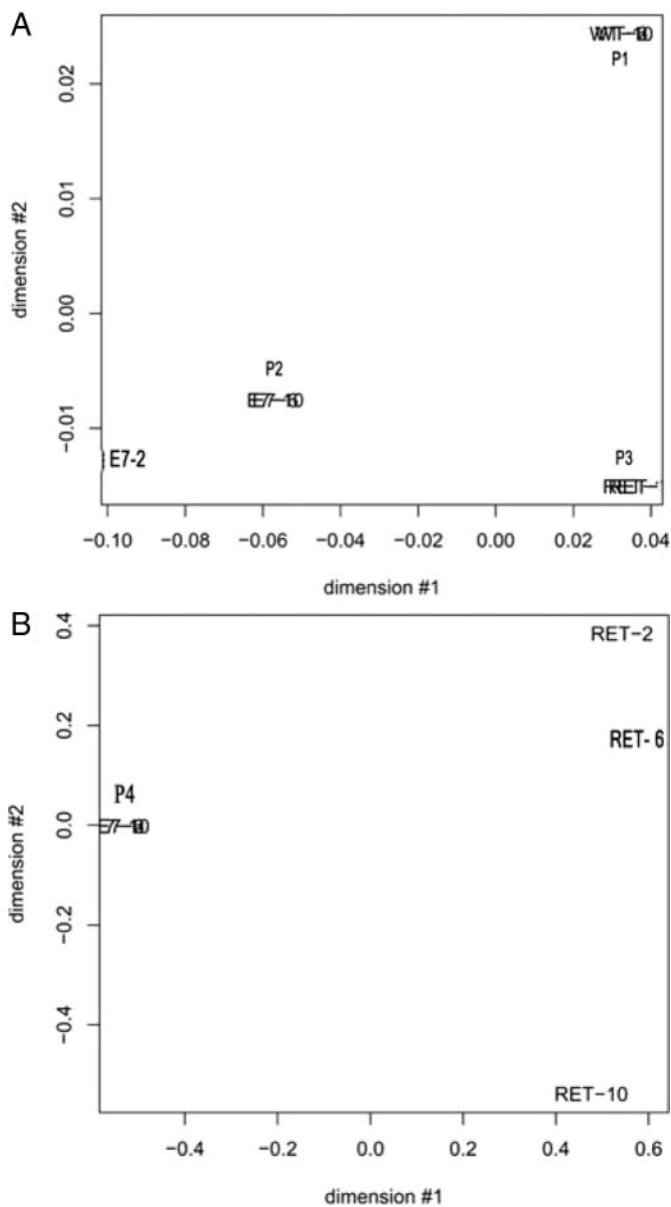


FIG. 2. Expression profiles of wild-type, E7, and RP3 thyroids represented by MDS. P1, Wild type at 2, 6, and 10 months; P2, E7 at 6 and 10 months; P3, RP3 at 2, 6, and 10 months; P4, E7 at 2, 6, and 10 months. The stress in panels A and B, *i.e.* the distortion between the two-dimensional distances and the actual gene space distances is less than 1%. A, MDS representing the absolute expression profiles of wild-type, E7, and RP3 thyroids; pool formation for one given genotype at different ages (P). B, MDS representing the relative expression profiles (tumoral *vs.* wild type) of E7 and RP3 thyroids.

TTF1 and PAX8 were even transiently up-regulated in E7 thyroids. TSH receptor was not regulated in the two models. All the thyroid function genes, NIS (sodium/iodide symporter), TPO (thyroperoxidase), Tg, Dio1, and Dio2 (deiodinase iodothyronine, types I and II) were progressively down-regulated in E7 mice, NIS being the most strongly repressed. These genes were moderately, transiently, or not regulated in RP3 thyroids except NIS, Dio1, and Dio2 that were significantly repressed. TPO and NIS microarray regulations in E7 and RP3 tumors were confirmed by real-time PCR (sup-

plemental Fig. 6). These results were consistent with a globally sustained T_4 production, an increase in the gland volume compensating a lower efficiency due to NIS (and TPO for E7) down-regulation. Thus, RP3 and E7 tumors retained some degree of differentiation and kept their identity (no significant down-regulation of the thyroid-specific transcription factors) as generally observed in differentiated human thyroid cancers (20–24).

Human PTC marker expression in our mouse models

When we looked at the up-regulated genes in the RP3 thyroids, we found many genes already described as overexpressed genes or coding for proteins overexpressed in human PTC (Table 3): mucin 1, TIMP1 (tissue inhibitor of metalloproteinase 1), SFTPB (surfactant associated protein B), Dusp6, cystatin 6 (E/M), CHI3L (chitinase 3-like 1), and to a lesser extent KRT19 (keratin 19) [microarrays (25)]; periostin and Dusp5 [microarrays (26)]; syndecan 1 [immunohistochemistry (27)]; CD10 (membrane metalloendopeptidase) [immunohistochemistry (28)]; lactotransferrin [immunohistochemistry (29)]; HMGA1 [high mobility group AT-hook1] (RT-PCR and immunohistochemistry (30)); HMGA2 (high mobility group AT-hook 2) [RT-PCR (31)]; uPAR (plasminogen activator, urokinase receptor) [RT-PCR, Western blotting (32)]; and S100A6 (member of the S100 protein family) [DIGE gels (33)]. Most of these genes were up-regulated more specifically in RP3 tumors at 2 months but not in E7 thyroids. Lactotransferrin and Dusp5 microarray regulations in RP3 and E7 tumors were confirmed by real-time PCR (supplemental Fig. 6). Other well-known PTC markers as FN1 (fibronectin 1), DPP4 (dipeptidyl peptidase 4), MET, CD44, and Bax were not significantly regulated in both mouse tumors. Galectin-3 and Gdf-15 (growth differentiation factor 15) were down-regulated in the later stages of RP3 tumor formation but not at 2 months. Finally, CITED1 was down-regulated at all ages in E7 and RP3 tumors. This last opposite regulation between RP3 tumors and human thyroid cancers could thus simply reflect interspecies differences.

Biological processes wherein E7 and RP3 up-regulated genes were involved

We analyzed our microarray data using the DAVID software (Database for Annotation, Visualization and Integrated Discovery; <http://david.abcc.ncifcrf.gov>). DAVID provides a comprehensive set of functional annotation tools to order large lists of genes according to biological functions. This software for example classifies submitted gene lists according to the most represented biological processes, cellular components, and molecular functions according to Gene Ontology (GO) terms. It also visualizes these genes on BioCarta and KEGG pathway maps.

We used Benjamini-Hochberg correction among the multiple testing *P* value correction procedures [false discovery rate (FDR)] (34) available in DAVID. We considered a process significantly represented when its FDR was inferior or equal to 0.05.

When we analyzed our data with DAVID, different biological processes could clearly distinguish between the two

TABLE 2. Fold change in expression of thyroid function and determination genes in E7 and RP3 tumors at 2, 6, and 10 months

Genes	E7 (months)			RP3 (months)			Human PTC
	2	6	10	2	6	10	
NIS	0.88	0.36	0.25	0.44	0.21	0.75	Down (24)
TPO	0.72	0.37	0.4	0.8	0.6	1.1	NR or down (21)
TSHr	0.8	0.8	0.85	1.1	1	1.3	NR (23)
TG	0.86	0.93	0.6	1.1	0.9	0.7	NR or down (22)
Dio1	0.63	0.34	0.46	0.18	0.1	0.27	Down (20)
Dio2	1	0.5	0.7	0.8	0.47	0.84	Down (20)
PAX8	1.8	2.1	2.3	1.2	1.2	1.3	NR or down (22)
TTF1	0.99	1.3	2.7	1	1.1	1.5	NR or down (22)

Last column is described regulations in human PTC in cited references. NR, Not regulated.

tumor types. The most significantly represented processes involving E7 up-regulated genes were cell cycle and related processes (Table 4 and Fig. 3). In RP3 the up-regulated genes were mainly representative of immune and inflammatory processes (Table 5). The major gene expression characteristics differentiating E7 and RP3 tumors are developed in the next paragraphs.

Cell cycle and proliferation in E7 and RP3 thyroids

Analysis of E7 up-regulated genes with the DAVID software showed a majority of genes involved in cell cycle and proliferation processes whatever the age (Table 4 and Fig. 3). Many cyclins and cyclin-dependent kinases were indeed up-regulated in E7 thyroids as were proteins implicated in replication checkpoints or DNA repair (supplemental Fig. 2). Cyclins D already described as down-regulated in Tg-E7 mice were an exception (35). Most of these cell cycle-related genes showed a decreasing up-regulation with age, which is consistent with the fact that thyroid growth rate is decreasing with time. Cell cycle-related genes up-regulated in E7 thy-

roids were also generally up-regulated in RP3 tumors but to a lesser extent and, as already pointed out for other genes, especially in RP3 2 month tumors. Moreover, some genes implicated in the inhibition of proliferation pathways were up-regulated in RP3 but not E7 thyroids (Table 6): several Dusp proteins (Dusp2, -5, -6) that inhibit the MAPK pathway; Mig6 or Errfi1 (ERBB receptor feedback inhibitor 1), a negative regulator of the epithelial growth factor (EGF) signaling pathway; and Reprimo, a candidate of p53-mediated cell cycle arrest at the G₂ phase. The up-regulation of Dusp proteins was previously described in human PTC (36). Several cyclin-CDK inhibitors were also slightly up-regulated in RP3 as in E7 tumors. Cyclin D1 and Dusp5 regulations were confirmed by real-time PCR (supplemental Fig. 6). Reprimo up-regulation was also confirmed in RP3 tumors. In E7 thyroids, real-time PCR demonstrated a significant down-regulation of Reprimo mRNA at all ages (no significant regulation in microarray data). Thus, we observed a much more important overexpression of cell cycle-related proteins in E7, compared with RP3 thyroids, an activation that was

TABLE 3. Fold change in expression of several genes known to be up-regulated in human PTC (*last column*) in E7 and RP3 tumors

PTC genes	E7 (months)			RP3 (months)			Human PTC
	2	6	10	2	6	10	
Mucin 1	4	5.8	9.4	15.4	9	14.3	Up
Timp1	0.78	7.3	1.22	23	21	5.6	Up
Dusp6	0.45	0.4	0.5	2.3	1.4	1.6	Up
SFTPB	0.93	0.94	0.97	169	286	242	Up
CHI3 liter	0.98	0.98	0.96	2.9	0.99	0.98	Up
Cystatin 6	0.44	0.58	0.24	5.1	4.1	2.2	Up
Dusp5	0.74	0.93	1.3	1.7	3.1	4.7	Up
KRT 19	0.97	1.4	1.3	1.6	1.9	1.5	Up
Periostin	1.1	1.2	1.1	2.2	1.2	1.1	Up (aggressive)
Lactotransferrin	0.85	0.18	0.17	33	0.54	0.36	Up
Periostin	1.1	1.2	1.1	2.2	1.2	1.1	Up (aggressive)
Syndecan 1	2	2.3	1.4	5.2	5.5	3	Up
CD10	0.3	0.2	0.2	17	17	10	Up; follicular variant
Hmga1	0.31	0.69	0.61	5.8	4.8	4.2	Up
Hmga2	0.24	0.22	0.19	5.1	2.6	2.7	Rat thyroid cell transformation <i>in vitro</i> ; thyroid carcinoma
uPAR	1.4	1.8	1	2.8	1.4	1	Up (more if metastasis)
S100a6	1.4	3.6	1.3	2.2	2.2	0.96	Up
MET	1.2	1.8	1.9	1.1	1.1	1	Up
DPP4	1.3	0.5	0.6	0.7	0.6	0.6	Up
CD44	0.83	1.1	1.2	1.6	1.6	1.3	Up
FN1	1.4	1.7	1	1.5	1.89	0.55	Up
Bax	0.85	0.82	0.82	1	0.93	1	Up
CITED1	0.2	0.13	0.35	0.25	0.16	0.4	Up
Gal-3	0.95	4.1	0.95	1.2	0.68	0.17	Up
Gdf-15	2.1	1.3	1.4	0.84	0.17	0.37	Up

TABLE 4. Statistical significance of the five most represented biological processes (GO level 4) among the E7 up-regulated genes after analysis with DAVID software

Processes	GO name	<i>P</i> value	FDR (Benjamini)
E7 2 months			
GO:0000279	M phase	2.3×10^{-24}	1.4×10^{-21}
GO:0000278	Mitotic cell cycle	6.7×10^{-23}	2.1×10^{-20}
GO:0043283	Bipolymer metabolism	1.3×10^{-15}	2.8×10^{-13}
GO:0006281	DNA repair	1.8×10^{-14}	2.7×10^{-12}
GO:0051726	Regulation of cell cycle	2.4×10^{-12}	3.1×10^{-10}
E7 6 months			
GO:0000278	Mitotic cell cycle	2.9×10^{-13}	9.4×10^{-11}
GO:0051726	Regulation of cell cycle	3×10^{-13}	1.8×10^{-10}
GO:0000279	M phase	5.9×10^{-12}	1.2×10^{-9}
GO:0006281	DNA repair	4.4×10^{-9}	6.9×10^{-7}
GO:0043283	Bipolymer metabolism	1.8×10^{-8}	2.3×10^{-6}
E7 10 months			
GO:0000279	M phase	6×10^{-13}	3.7×10^{-10}
GO:0000278	Mitotic cell cycle	1.1×10^{-12}	3.6×10^{-10}
GO:0051726	Regulation of cell cycle	1×10^{-9}	2.2×10^{-70}
GO:0043283	Bipolymer metabolism	2.3×10^{-9}	3.5×10^{-7}
GO:0006281	DNA repair	1.7×10^{-5}	2.2×10^{-3}

Benjamini-Hochberg FDR 0.05 or less.

only weakly counterbalanced by the up-regulation of cyclin-CDK inhibitor proteins. In RP3 tumors we observed an additive negative feedback, acting more upstream in cell signaling by inhibiting the MAPK pathway. This difference in cell proliferation could explain size differences observed between E7 and RP3 tumors and by extension could provide a good model for understanding the moderate growth of human PTC.

Angiogenesis, epidermidis development, and extracellular matrix (ECM) remodeling in E7 and RP3 thyroids

In addition to immune and inflammatory responses, blood vessel and epidermidis developments were also significantly represented among RP3 combined up-regulated genes (GO level 4; Table 5). Angiogenesis-related processes were particularly significant at 2 and 6 months, whereas epidermidis development was significant at 6 and 10 months (data not shown). None of these processes appeared significantly represented in E7 tumor up-regulated genes, whatever the age.

Angiogenesis is essential for tumor growth and is controlled by the balance between angiogenic and antiangiogenic factors. In human PTCs, this balance has been correlated with the level of neovascularization and invasiveness of the tumor (37). In our data we also observed an up-regulation of several proangiogenic factors like heparin-binding-EGF and other EGF-like factors (see paragraph on EGF pathway in E7 and RP3 thyroids), FGF2 or endothelin receptor type a, but also the up-regulation of thrombospondin-1 an anti-angiogenic protein and the down-regulation of endothelin 1 a pro-angiogenic factor. However in the RP3 data the balance was globally in favor of the pro-angiogenic factors (data not shown).

Epidermidis development is defined as followed in GO: "Process whose specific outcome is the progression of the epidermis over time, from its formation to the mature structure." More precisely we found among the up-regulated genes included in epidermidis development proteins like

stratifin, involucrin, transglutaminase, and small proline-rich protein 1A (Sprr1a; cornifinA or α). Involucrin, for example, up-regulated at all ages in RP3 thyroids, is a substrate for transglutaminase, which cross-links it to form the cornified envelope. Previous immunohistochemistry studies demonstrated a stronger staining of involucrin in human PTC, compared with human follicular thyroid carcinoma (38). Involucrin expression regulation was confirmed in RP3 (up-regulation) and E7 (no regulation) thyroids by real-time PCR (supplemental Fig. 6). In addition, epidermidis development was also one of the most significantly represented biological processes among human PTC up-regulated genes in microarray data of Delys *et al.* (36) (www.ncbi.nlm.nih.gov/geo; accession no. GSE3950) after analysis with the DAVID software (supplemental Fig. 3). Thus, it appeared that in RP3 tumors, as in human PTC, several up-regulated genes play a role in tissue formation and development. In this process, angiogenesis but also interactions with cell environment and extracellular space are crucial. Consistent with this, cell communication and ECM receptor interaction were the most significant pathways represented in RP3 up-regulated genes (Table 5) again as in human PTC (supplemental Fig. 4). Matrix degradation appears to be important for cancer invasion and metastasis. Changes in matrix components are related to an imbalance between their synthesis and their degradation. Several studies previously showed overexpression of genes implicated in ECM remodeling (36). Similarly, we found in RP3 tumors a significant regulation of ECM structural constituents (procollagen, laminin, fibrillin molecules), peptidase, and endopeptidase molecules (MMP3, MMP14, membrane metalloendopeptidase, cathepsin K, Sipi, lactotransferrin, tissue plasminogen activator), and peptidase and endopeptidase inhibitor molecules (cystatine E/M, cystatine 12, fetuin- β , serine peptidases inhibitor, Timp1, α 2-macroglobulin). Most of these ECM-related genes were up-regulated rather than down-regulated, and most of the regulations were found in RP3 2 month thyroids. All these proteins are involved in tissue remodeling, which goes parallel with the spatial progression of the tumor. We did not find such significant regulation of ECM-related genes in E7 thyroids, observation consistent with the more homogeneous growth of these tumors. Thus, it appeared that ECM remodeling could play an important role in RP3 thyroid tumorigenesis as in human PTC but not in E7 tumors.

Immunity in E7 and RP3 thyroids

Immunity plays an important role in many cancers. Previous studies reported links between RP3 expression and immune system. Russell *et al.* (39) showed macrophage infiltrates in RP3 mice thyroids (CD11b and F4/80 positive immunostaining) and up-regulation of several chemokines and cytokines by semiquantitative PCR (Cox2, IL-1 β , IL1 α , IL-6, Mcp1, TNF- α , Gmcsf) and/or Western blotting (Cox2, IL-6). In addition, Powell *et al.* (40) demonstrated that immunization with mouse RP3 fusion protein could induce strong immunogenic response. Therefore, we analyzed expression of immune genes in our microarray data. We found indeed an up-regulation of many cytokines, chemotactic genes, macrophage, mast cell, leukocyte, and lymphocyte

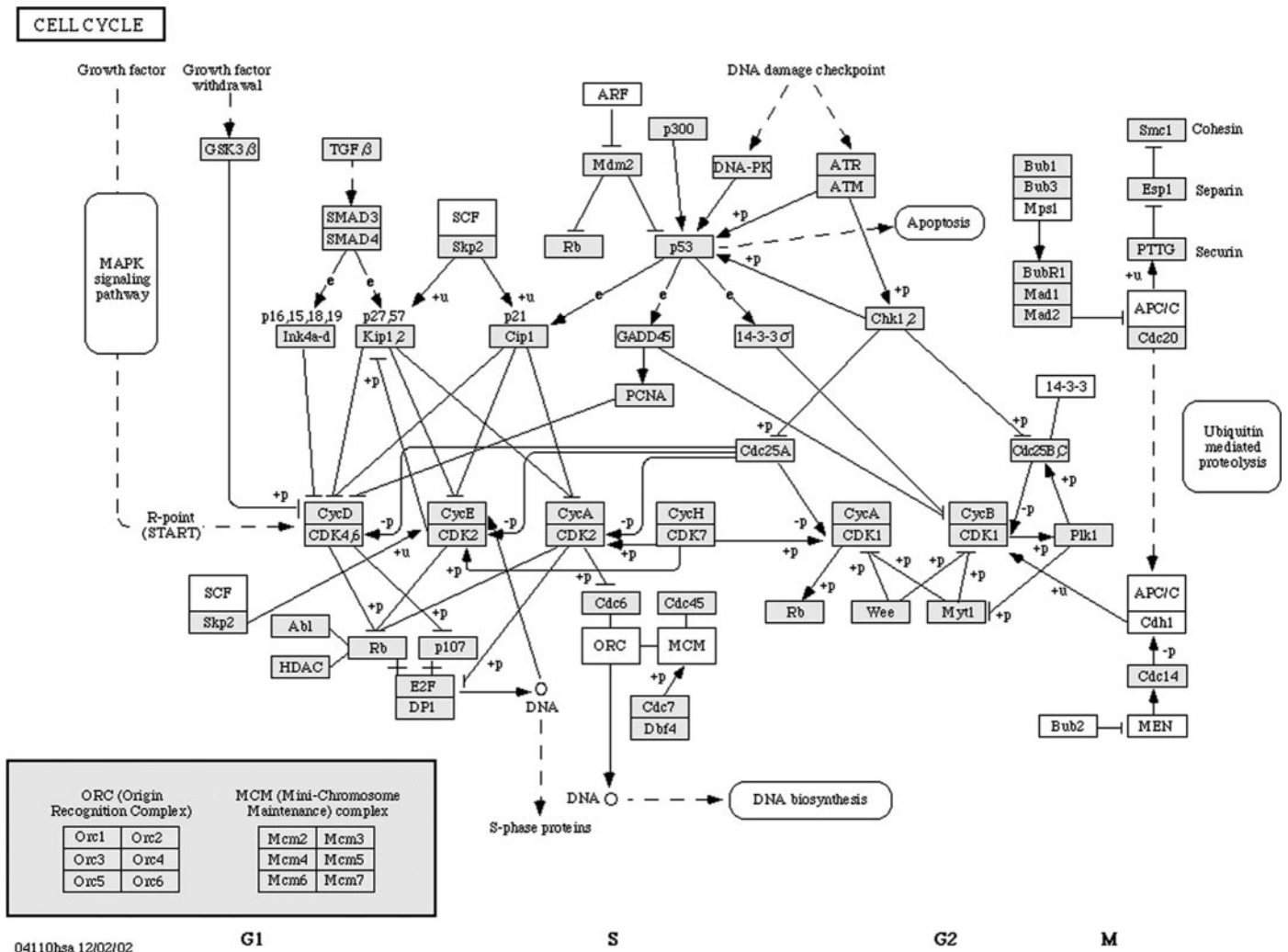


FIG. 3. Scheme of cell cycle including E7 up-regulated genes. Cell cycle (KEGG pathway) is the most significantly represented pathway among E7 up-regulated genes after analysis with DAVID software; up-regulated genes (fold change ≥ 2) are represented in gray squares.

markers, especially in RP3 tumors. In addition to this direct observation, we selected a list of lymphocyte-specific genes from published mouse normal tissue microarray data (see *Materials and Methods*) and compared their expression levels

TABLE 5. Statistical significance of the six most represented biological processes (GO level 4) and the significant pathways (KEGG) among RP3 up-regulated genes after analysis with DAVID software

RP3 (all ages)	GO name	P value	FDR (Benjamini)
GO:0006955	Immune response	7.9×10^{-9}	4.5×10^{-6}
GO:0006954	Inflammatory response	5.1×10^{-8}	1.6×10^{-5}
GO:0001568	Blood vessel development	3.6×10^{-6}	5.6×10^{-4}
GO:0048514	Blood vessel morphogenesis	7.5×10^{-6}	9.4×10^{-4}
GO:0051726	Regulation of cell cycle	2.6×10^{-5}	2.7×10^{-3}
GO:0008544	Epidermidis development	1.7×10^{-4}	1.5×10^{-2}
KEGG pathway	Cell communication	3.6×10^{-5}	7×10^{-3}
KEGG pathway	ECM receptor interaction	8.4×10^{-5}	8.2×10^{-3}

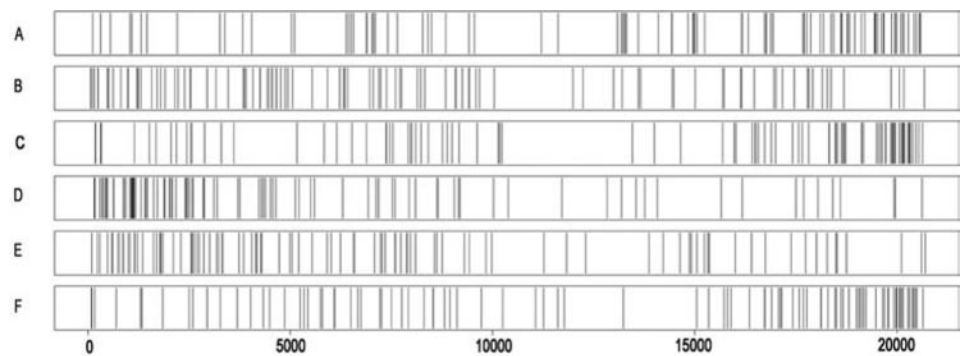
Benjamini-Hochberg FDR 0.05 or less.

in the different strains and ages. We represented the distribution of their expression levels in the form of graph in which each bar represents a lymphocyte gene and each row a thyroid type (Fig. 4). A lymphocyte signature appeared in RP3 2-month tumors and, to a lesser extent, in RP3 and E7 6-month tumors. On the contrary most of lymphocyte genes were down-regulated in RP3 and E7 10 month tumors. Similarly, among RP3 up-regulated genes, DAVID placed im-

TABLE 6. Fold change in expression of genes involved in the inhibition of cell proliferation in E7 and RP3 tumors

Genes	E7 (months)			RP3 (months)		
	2	6	10	2	6	10
p21	2.4	2.9	2.4	2.3	2.7	3
p15	1.9	2.6	2.6	1.96	2.2	1.96
p19	2	0.96	1.2	0.99	0.76	0.84
p27	2.3	1.5	1.4	1	0.94	1
Reprimo	0.76	0.76	0.66	13	20	22
Dusp5	0.74	0.93	1.3	1.7	3.1	4.7
Dusp6	0.45	0.4	0.5	2.3	1.4	1.6
Dusp2	1.2	1.9	1.36	3.6	4.5	3.9
Mig6	0.97	1.6	1.3	1.9	2.7	2.2

FIG. 4. Lymphocyte-specific gene repartition in E7 and RP3 strains at different ages. Each vertical bar represents a lymphocyte gene; each row represents a tumor type at one given age: A, E7 at 2 months. B, E7 at 6 months. C, E7 at 10 months. D, RP3 at 2 months. E, RP3 at 6 months. F, RP3 at 10 months. Not regulated genes are located in the middle of the scheme (point 10000), up-regulated genes on the left and down-regulated genes on the right. The significance of the collective regulation of the lymphocyte genes was assessed with the Wilcoxon rank test. Significance is less than 0.001 in all distributions.



immune and inflammatory responses respectively in first and second position (GO level 4; all ages combined) (Table 5). These two biological processes were also the two most representative of human PTC up-regulated genes from microarray data by Delys *et al.* (35) after DAVID analysis (supplemental Fig. 3). Immune response was particularly significant at 2 months in RP3 up-regulated genes and at 10 months in RP3 down-regulated genes, observation reinforcing the distribution of lymphocyte genes at these two ages. DAVID did not point out inflammatory and immune responses in E7 tumors (all ages combined). But when we considered the different ages separately, these biological processes appeared significant in E7 6-month up-regulated genes (supplemental Fig. 5). This result is consistent with the observed trend of a lymphocyte signature only at this age in E7 tumors. In conclusion, many immune response-related genes were up-regulated in our microarray data, especially at 2 months in RP3 tumors and at 6 months in E7 tumors. Globally immune processes appeared more significantly represented in RP3 than in E7 tumors. Thus, immune response could play a role in thyroid tumorigenesis, being a precocious event (RP3 tumors) or a later event (E7 tumors), depending on the initiating mutation. These gene expression profiles reinforce previous published observations in RP3 thyroid tumors, suggesting that inflammation could have an important role in these tumors like in human papillary thyroid cancers.

EGF pathway in E7 and RP3 thyroids

The EGF pathway has been implicated in the growth of several human cancers. In thyroid cancer EGF has been associated with dedifferentiation, tumor cell proliferation, and angiogenesis (40–42). We observed an important up-regulation of EGF-like peptides (amphiregulin, epiregulin, betacellulin, δ -like 1 homolog, and heparin-binding EGF) in mouse microarray data but exclusively in RP3 thyroids (Table 7). Amphiregulin and epiregulin strong up-regulation was confirmed by real-time PCR (Table 7 and supplemental Fig. 6). The slight up-regulation of amphiregulin in E7 6 months was not confirmed by real-time PCR. EGF receptor was not up-regulated in any mouse models but Neu (ErbB2) was slightly up-regulated in 10-month RP3 thyroids in our microarray data and slightly up-regulated at all ages by real-time PCR. As for amphiregulin, the slight up-regulation of Neu observed by microarray in 10-month old E7 mice was not confirmed by real-time PCR. On the contrary Neu

appeared significantly down-regulated at all ages in E7 (Table 7).

EGF-like proteins could promote cell proliferation in an auto- and/or paracrine way, reinforcing the MAPK cascade activation due to the RP3 mutation. The EGF pathway could also favor the neovascularization of the tumor. These results are consistent with previous literature data on human PTC (35) and support the potential role of EGF signaling in thyroid tumorigenesis.

General conclusion

Tg-RP3 and Tg-E7 are two transgenic mice expressing their mutation (RP3 or E7) exclusively in the thyroid. Both strains develop thyroid hyperplasia followed by thyroid carcinoma lesions at around 6 months from two different initial events. To better understand the mechanisms leading from simple hyperplasia to differentiated carcinoma, we compared gene expression in thyroids from Tg-E7, Tg-RP3, and wild-type (C57bL/6) mice at 2, 6, and 10 months by microarray technology. The microarray results on these two transgenic mouse models are probably valid and of physiological interest to understand thyroid tumorigenesis as: 1) we confirmed modulated expression of 11 genes by quantitative RT-PCR; 2) we confirmed previously observed regulations in RP3 and E7 mouse thyroids (thyroid specific markers, cyclins, CDK, and cyclin/CDK inhibitors for E7; thyroid specific markers and some cytokines for RP3); 3) we observed gene expression similarities between human papillary carcinomas

TABLE 7. Fold change in expression of EGF-like growth factors and Neu (ErbB2) genes in E7 and RP3 tumors

Genes	E7 (months)			RP3 (months)		
	2	6	10	2	6	10
Amphiregulin	0.8	2.2	0.95	53	35	23
<i>Amphir RT-PCR</i>	<i>0.99</i>	<i>0.9</i>	<i>0.55</i>	<i>22</i>	<i>7.1</i>	<i>7</i>
Epiregulin	0.99	0.99	0.96	232	120	138
<i>Epir RT-PCR</i>	<i>0.52</i>	<i>0.64</i>	<i>0.6</i>	<i>166</i>	<i>72</i>	<i>58</i>
HB-EGF	1.2	1.1	1.2	4.7	3.6	4.6
betacellulin	1.3	0.8	1.3	2.4	1.6	3.3
Dlk1	1	1	0.97	9.1	7.8	8.3
Neu (ErbB2)	1.3	1.2	2.5	1.6	1.9	2.9
<i>Neu RT-PCR</i>	<i>0.33</i>	<i>0.23</i>	<i>0.04</i>	<i>1.97</i>	<i>1.63</i>	<i>1.76</i>
EGFr	1	0.8	0.7	0.8	0.7	0.8
<i>EGFr</i>	<i>0.7</i>	<i>0.4</i>	<i>0.06</i>	<i>0.9</i>	<i>0.8</i>	<i>0.9</i>

Expression ratios obtained by real-time PCR (SYBR Green) for amphiregulin, epiregulin, and Neu are represented in *italics*.

and RP3 tumors (our model of human PTC) but not E7 thyroids.

The study of gene expression in a tissue is presently the most comprehensive way to define its molecular phenotype. This first work on gene expression in two mouse models of human differentiated tumors allows several interesting conclusions. The main question to be answered about a human disease model is to what extent does this model reproduce the characteristics of the human disease, *i.e.* how valid is the model? The answer to this question establishes, for instance, which variables can be used in the considered model to evaluate drug testing. Previous works had already demonstrated that the postulated human oncogene RET can induce thyroid tumors in mice with several morphological (papillae) and pathological (inflammation) characteristics of human papillary carcinoma (12). In the present microarray data, we did not find an overall similarity of gene expression with human thyroid papillary carcinoma. However, particularly in 2-month tumors, we found among the up-regulated genes several genes known either to be also overexpressed in human PTC or coding for overexpressed proteins. Expression similarities with human PTC decreased in 6- and 10-month RP3 tumors.

Several characteristics were shared by RP3 thyroids and human PTC: 1) the moderate decrease of differentiation gene expression, *i.e.* genes coding for proteins with thyrocyte-specific functions, with the maintenance of normal T₄ production; 2) the conserved expression of specific thyroid transcription factors that specify the identity of the cells, *i.e.* their determination; 3) the overexpression of genes involved in cell cycle and DNA repair (especially at 2 months); 4) the overexpression of genes coding for proteins involved in the EGF MAPK pathway, which could thus reinforce the oncogenic activation of this pathway by RET/PTC rearrangement and favor the neovascularization of the tumor; 5) the overexpression of some negative feedback proteins acting on the MAPK pathway, and therefore, the balance between positive and negative regulations on this pathway could explain the slow growth of RP3 tumors and by extension of human PTC; 6) the overexpression of genes involved in tissue remodeling that could explain the cohort type of progression and invasiveness of human PTC; and 7) the overexpression of immune response-related genes, which confirms previous pathological findings observed in RP3 mice and human PTC.

Thus, RP3 mouse thyroids, especially at 2 months, could constitute a valid model for several major properties of human PTC. Interestingly, most of these gene expression similarities were observed in 2-month tumors, whereas papillary-like structures appear later. Thus, most of the gene expression characteristics that sign malignancy appear soon in RP3 tumorigenesis, *i.e.* before the apparition of most differentiated carcinoma lesions (around 6–10 months). This observation is not linked to a loss of RP3 oncoprotein expression because RP3, like Tg, expression stays constant throughout the tumor progression (no down-regulation linked to the Tg promoter). The most plausible explanation is that microarray results at 2 months reflect the direct effect of RET activation. The lower degree of convergence at 6 and 10 months can be explained by secondary events that may be

different in two different cellular environments (mouse and human).

The validity of the RP3 model is reinforced by the absence of such gene expression similarities between E7, another cancer model, and human PTC. If E7 contrary to RET is not a recognized oncogene in human thyroid tumors, it was nevertheless interesting to compare tumor gene expression in these two transgenic mice models for three main reasons. First, both mutations lead to thyroid tumors in mice. Second, E7 and RP3 oncoproteins act at completely different positions of cell signaling pathway. RP3 is really upstream and is thus able to activate several metabolic pathways, some stimulating but also some inhibiting cell proliferation. E7 acts more downstream in cell signaling, acting directly in the nucleus. It was thus interesting to analyze the implication of these two different levels of action on gene expression and on the biological mechanisms leading to tumor formation. And third, we still do not know all the processes implicated in thyroid tumors. Some unknown mutations could also act more downstream in cell signaling, inactivating for example the Rb protein (as does E7) or affecting other cell cycle-related proteins as does the p53 mutation in many anaplastic cancers.

Thus, RP3 characteristics are not general properties of thyroid tumors and seem relatively specific of PTC or, at least, the activation of the MAPK pathway. In the absence of comparable data on human follicular carcinomas, we were not able to make a similar comparison between E7 thyroids and follicular carcinomas. E7 tumors (especially at 6 months) could indeed represent a better model to study human follicular thyroid carcinoma than human PTC, dysplastic follicular structures being clearly predominant, compared with papillary looking areas in these huge tumors. Results of our microarray analysis answer important questions.

First, as previously noted, the demonstration that RP3 expression induces thyroid tumors with several characteristics of human PTC reinforces the concept that this rearranged gene can cause a cancer similar to this human PTC. It is an oncogene *in vivo*. This is worth reemphasizing as the concept seems at variance with the reported findings that some normal thyrocytes would exhibit RET/PTC rearrangement and that in RET/PTC-positive cancers, the rearrangement would be found only in a fraction of cells (43, 44). The first finding could be explained by the hypothesis that RET/PTC rearrangement alone is not sufficient for tumorigenesis, postulating the appearance of secondary events, allowing tumor progression. This is reinforced by the observed heterogeneity in RP3 thyroids histology (Jin, L., A. Burniat, J. E. Dumont, F. Miot, B. Corvilain, and B. Franc, submitted for publication): all the cells express the RET/PTC mutation but only some lobes, and some areas will develop papillary-looking structures. We have no explanation for the second finding.

Second, the overexpression of genes involved in negative feedbacks on the activated MAPK pathway explains the relatively slow growth of a tumor related to the stimulation of this pathway and emphasizes the importance of the balance between activating and inhibiting signaling in tumor progression (45).

Third, the overexpression of many immune-related genes

in RP3 tumors by microarray analysis confirms previous data, demonstrating up-regulation of selected cytokines in RP3 thyroid tumors and reinforces findings, suggesting the induction, by RET/PTC, of some inflammation proteins in RP3-transfected PCCL3 cells (39, 46, 47). These observations tend to prove that, like in other tumors, it is the transformed thyrocyte that causes inflammation and not the reverse. Whether this inflammation promotes or impairs the growth of the tumor remains to be tested.

Fourth, as previously noted, the main papillary gene expression characteristics of the RP3 thyroids are exhibited at 2 months and seem to recede later. This suggests that the 2-month situation represents the best model to study some properties of human PTC. In particular, specific drugs could be tested on RP3 mice at this age to prevent the development of carcinoma lesions appearing in older animals.

Fifth, E7 thyroids display several characteristics of biological interest: 1) the absence of induction of negative feedbacks on the MAPK pathway; 2) the marked up-regulation of cell cycle-regulating proteins except cyclins D; 3) the up-regulation of immune and inflammatory responses, especially at 6 months. The first two findings could result from the downstream position of E7 in cell signaling, E7 playing the role of cyclins D/CDK complexes by releasing E2F transcription factors. The last finding could reflect either secondary events, mutations, appearing later in E7 than RP3 tumors, and/or the fact that E7 mutation by itself is less able than RP3 to activate pathways implicated in immune response activation, as, for example, the nuclear factor- κ B pathway.

In conclusion, expression of E7 and RP3 tumors lead to thyroid hyperplasia followed by differentiated carcinoma lesions. Despite this apparent similar evolution both mutations engage completely different tumorigenesis mechanisms. This work shows that both RP3 and E7 mice can be useful models to study human tumors, according to the validated variables reported here. They raise important general questions on cell cycle and tumorigenesis biology.

The results on E7 mice suggest that it would be interesting to look for mutations inactivating Rb in goiters and thyroid tumors. If our study demonstrates that Tg-RP3 mice are not the perfect model of human PTC described in literature, they constitute, however, especially at 2 months, a good model to study some properties of human PTC. This transgenic model will permit to study the role of the biological processes shared with human PTC and to test several drugs targeting these validated variables.

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