Impact of connexin32 deletion on E7 or RET/PTC3 oncogene-driven growth and neoplastic transformation of the thyroid gland

Gaelle Prost1,2, Françoise Bernier-Valentin1,2, Martine Croset1,2 and Bernard Rousset1,2

1Institut National de la Santé et de la Recherche Médicale, UMR 664, Lyon F-69372, France
2Faculté de Médecine Laennec, Université de Lyon, Lyon F-69372, France

(Correspondence should be addressed to B Rousset who is now at Faculté de Médecine Lyon, RTH Laennec, Inserm UMR 664, 7 Rue Guillaume Paradin, 69372 Lyon Cedex 08, France; Email: rousset@sante.univ-lyon1.fr)

Abstract

Connexins (Cx) form gap junctions and allow direct cell-to-cell communication. Cx through gap junctions or by themselves play regulatory roles on cell growth and differentiation. Using genetically modified mice, we previously found that Cx32 acts as a down-regulator of growth in normal thyroid gland. In this study, we examined the impact of Cx32 ablation on oncogene-driven thyroid growth and neoplastic transformation. Cx32 knockout (Cx32-KO) mice were crossed with transgenic mice expressing, selectively in the thyroid gland, either the E7 or RET/PTC3 (RP3) oncogene. As already described, Cx32-KO mice had no detectable thyroid alteration in physiological conditions and mice expressing E7 or RP3 exhibited time-dependent thyroid hypertrophy and variable changes in expression of differentiation. The thyroid of E7 mice evolved towards a large colloid goitre whereas RP3 mice developed a hyperplastic thyroid of variable size, and the largest glands (about 40% of total) represented a profound tissue remodeling with proliferative papillary formations. E7-induced thyroid hypertrophy was reduced by about 40% in Cx32-KO mice as compared with wild-type (WT) littermates. On the contrary, thyroid hypertrophy induced by thyrotropin stimulation (in response to goitrogen treatment) was enhanced by about 40% in Cx32-KO mice as compared with WT mice. Thyroid hypertrophy of RP3 mice and the proportion of glands showing extensive tissue remodeling were drastically reduced in mice devoid of Cx32. Our data show that Cx32, which negatively controls thyroid growth activated by thyrotropin via the cAMP pathway, would act as a positive effector of thyroid growth triggered by oncogenes acting through other signaling cascades.

Endocrine-Related Cancer (2009) 16 873–884

Introduction

Gap junctions forming intercellular channels are composed of two adjoining connexons, each made of six membrane proteins named connexins (Cx). So far, the Cx gene family is composed of 20 members in mouse and 21 members in human (Söhl & Willecke 2003). Cx are expressed in all organs and tissues except the skeletal muscle. Gap junctions, allowing direct cell-to-cell communication via small molecular mass hydrophilic molecules, are known to play key roles in many different biological processes such as the propagation of action potentials in the heart (Gros & Jongsma 1996, Bernstein & Morley 2006) or the contractility of endometrial muscle cells during parturition (Risck et al. 1990, Orsino et al. 1996, Döring et al. 2006). More generally, Cx through gap junctions (Loewenstein 1979, Kardami et al. 2007) or by themselves (Stout et al. 2004) are involved in the control of growth and expression of differentiation of numerous cell types. In a series of in vitro studies, we found that the reexpression of Cx32 in ‘gap junction-deficient’ thyroid cell lines causes: Firstly, a reduction
of the cell proliferation rate and secondly, an activation of expression of cell differentiation, i.e. expression of thyroid-specific genes (Statuto et al. 1997) and three-dimensional cell organization in follicle structures (Tonoli et al. 2000). Restoration of intercellular communication by the reexpression of Cx43, the other major Cx physiologically expressed in the thyroid gland (Meda et al. 1993, Munari-Silem et al. 1994), did not lead to any detectable change of thyroid cell growth or differentiation (Flachon et al. 2002).

Knowledge about the functional and/or regulatory roles of Cx obtained from in vitro studies on transfected cells has been more recently enriched by data deriving from genetically modified mice (Gros et al. 2004, Kretz et al. 2004, Söhl et al. 2004). We recently reported that the selective overexpression of Cx32 in the thyroid of mice (by targeted transgenesis) limits thyroid growth under physiological conditions, whereas the ablation of Cx32 (by gene inactivation) confers a growth advantage to thyroid epithelial cells subjected to TSH stimulation (Prost et al. 2008). The convergence between in vitro and in vivo data led us to conclude that Cx32 acts as a down-regulator of growth in normal thyroid gland.

Besides their role in the control of growth and differentiation of normal cells, gap junctions and Cx are involved in the processes of tumorigenesis and carcinogenesis. In their pioneering studies, Loewenstein & Kanno (1966) and Jamakosmanovicë & Loewenstein (1968a,b) described the lack of intercellular coupling in different types of tumors including thyroid carcinomas. Over the last 40 years, it has become clear that gap junction deficiency due to alterations of expression or localization of Cx is a common feature of cancer cells (Yamasaki et al. 1995, Trosko & Ruch 1998, Mesnil et al. 2005). Cx have been proposed as tumor suppressors (Rose et al. 1993, Mesnil 2002); however, very little is known about the molecular mechanisms by which Cx could influence tumorigenesis and carcinogenesis. The tumor suppressor function of Cx would be Cx- and cell type-specific (Mesnil et al. 1995, Mesnil 2002); a Cx would only exert its tumor suppressive effect in the cell type in which this Cx is physiologically expressed.

In the present study, we analyzed whether alterations of Cx32 expression level in the thyroid gland could influence oncogene-induced thyroid growth and neoplastic transformation in mice. Cx32 knockout (Cx32-KO) mice were crossed with transgenic mice expressing (selectively in the thyroid) either the E7 oncogene (from human papillomavirus type 16) or the RET/PTC3 (RP3) oncogene (found in human papillary thyroid carcinomas). We choose these oncogenes because firstly, they exert their cell transforming activity through different mechanisms and secondly, they differ in the time course and nature of thyroid tissue alterations, which they induce in mice (Ledent et al. 1995, Powell et al. 1998). Our data do not substantiate the tumor suppressor function of Cx32 but rather suggest that, in the thyroid gland, Cx32 would be involved in the growth-promoting activities of these two oncogenes.

Materials and methods
Origin and treatment of mice
Mice with a generalized inactivation of the Cx32 gene (Cx32-KO mice) were obtained from Prof. Willecke (Institute für Genetik Universität Bonn, Bonn, Germany). The main characteristics of these C57BL/6 strain mice have been described by Nelles et al. (1996); they are viable and fertile and exhibit a small reduction in body weight compared with wild-type (WT) littersmates.

Transgenic mice with a thyroid-targeted expression of the E7 oncogene (E7 mice) were kindly provided by J E Dumont, IRIBHM, Free University of Brussels, Brussels, Belgium. These mice (C57BL/6 genetic background) had been generated in 1995 by Ledent et al. (1995) using a construct composed of the human papillomavirus type 16 E7 oncogene under the control of the thyroglobulin gene regulatory region to drive the expression of E7 selectively in thyroid epithelial cells. The E7 oncoprotein has a transforming activity linked to its ability to interfere with the binding of Rb protein to the E2F transcription factor (Chellapappan et al. 1992). E7 mice first develop thyroid hyperplasia with large colloid-filled follicles and there is a progressive appearance (after 1 year) of nodular neoplasia with characteristics of follicular and papillary thyroid carcinomas.

Transgenic mice with a thyroid-targeted expression of the RP3 oncogene (RP3 mice) have been obtained from M Santoro & A Fusco (Università di Napoli ‘Federico II’, Naples, Italy) through the IRIBHM, Brussels, Belgium. The genetic background of these mice is C57BL/6. The RP3 oncogene, resulting from a rearrangement between RFG/ELE1 and RET genes, encodes a fusion protein with a constitutively active tyrosine kinase activity (Santoro et al. 1994). RP3 mice have been generated by Powell et al. (1998) using a construct composed of the RP3 fusion gene downstream the bovine thyroglobulin gene regulatory region; they develop thyroid hyperplasia and, later, solid tumor variants of papillary carcinomas.
All mice were housed in a conventional animal facility with a 12h light:12h darkness cycle; they were fed with standard laboratory chow (Safe Animal Food and Engineering, Augy, France) and water ad libitum. Groups of mice (at least 8 weeks old) received sodium perchlorate (NaClO₄) (Fluka, St Louis, MO, USA), 1% in drinking water, for up to 8 weeks. Mice were anesthetized by i.p. injection of 10 µl/g body weight of a solution composed of one volume of 2% xylazine (Rompun from Centravet, Dinan, France), five volumes of 5% ketamine (Imalgene from Centravet), and 15 volumes of saline. Animals were bled by cardiac puncture, and the thyroid gland was collected post mortem by careful dissection using a binocular magnifying lens. Thyroid tissue was weighed and subjected to either fixation or freezing in liquid nitrogen and storage at −80°C until use. All surgical and experimental procedures were conducted in accordance with French policies in animal use and care.

Because Cx32 gene is located on the X chromosome, experiments dealing with Cx32 ablation have been performed on male mice: WT (Cx32+/0) and Cx32-KO (Cx32−/−). In a concern of homogeneity, the other experiments were also performed on male mice. Thus, data presented in this paper is fully derived from thyroid phenotype analyses of male mice.

**Mouse genotyping**

WT and mutant Cx32 alleles were simultaneously detected by PCR using a set of three primers. A 500 bp fragment was amplified from the WT allele with the following primers: 5′-CCA TAA GTC AGG TGT AAA GGA GC-3′ and 5′-AGA TAA GCT GCA GGG ACC ATA GG-3′; the third primer (5′-ATC ATG CGA AAC GAT CCT CAT CC-3′) was complementary to a sequence in the neomycin resistance cassette of the disrupted allele. The PCR generated a 414 bp fragment from the mutant Cx32 allele. PCR was performed in a total volume of 30 µl containing 2 mM MgCl₂, 0.25 mM dGTP, dATP, dCTP, and dTTP, 0.3 µM of each primer, 1.5 U Taq DNA polymerase, and 300 ng genomic DNA. The reaction conditions were 5 min at 95°C followed by 40 cycles (30 s at 95°C, 45 s at 67°C, and 90 s at 72°C) and a final extension at 72°C for 10 min.

The presence of the oncogenes in the mouse genome was detected by PCR using the following primers: 5′-CAT GCA TGG AGA TAC ACC T-3′ and 5′-GAT TAT GGT TTC TGA GAA CA-3′ for E7 and 5′-GGC CAG AGC CCT AAG GTG GGC-3′ and 5′-AAG GGA TTC AAT TGC CAT CCA-3′ for RP3. PCR conditions for both E7 and RP3 identification included an initial step of denaturation at 94°C for 10 min followed by 40 cycles of amplification (60 s at 94°C, 60 s at 60°C, and 60 s at 72°C) and 10 min at 72°C for final extension. PCR products were fractionated on 2.0% agarose gels and visualized by ethidium bromide staining and u.v. transillumination.

**Histological analyses**

For conventional histological examinations by light microscopy, thyroid tissue was fixed in 10% formaldehyde and embedded in paraffin using the standard procedures. Five-micrometer sections were prepared and stained with hematoxylin at the ‘AniPath’ platform for histology (Faculté de Médecine Laennec, Lyon, France).

**Real-time PCR**

Total RNA was isolated on silica columns (RNeasy minikit from Qiagen) according to the manufacturer’s protocol. Potentially contaminating genomic DNA was eliminated by DNase I treatment (RNase-free DNase from Qiagen). Total RNA (500 ng) was reverse transcribed in a 20 µl reaction volume containing 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), 10 nmol of each dNTP, 24 U ribonuclease inhibitor (Promega), and 100 pmol random hexamers (Amersham Pharmacia) for 1 h at 37°C. PCR was performed on a LightCycler from Roche (Roche Diagnostics). After 5 min at 95°C, cDNAs were amplified in duplicate in a final volume of 10 µl using a FastStart DNA Master SYBR Green I kit from Roche. The reaction mixture contained 5 µM of the forward and reverse primers and 5 µl diluted cDNA template solution corresponding to 2.5 ng retrotranscribed RNA for Cx32, Nis, Tpo, Pax8, and RET TK domain and to 25 pg for 18S rRNA. Primers used for Cx32, Nis, Tpo, and Pax8 transcript assays were those previously described (Prost et al. 2008). Transcripts corresponding to the RET TK domain were assayed using the following primers: 5′-GAT CTC ACA GGG GAT GCA GA-3′ (forward) and 5′-CTG GCT CCT CTT CAC GTA GG-3′ (reverse). PCR conditions included an initial denaturation step of 10 min at 95°C followed by 35–40 cycles of 15 s at 95°C, 4 s at 58, 60, 60, or 58°C for Cx32, Nis, Tpo, Pax8, and RET TK domain respectively and 8 s at 72°C for the final extension step. Fluorescence intensity measurements were used to determine the crossing point value, i.e. the cycle number at which fluorescence was significantly greater than the background. The specificity of PCR amplification was assessed by determination of the Tm of amplicons, using a fusion program consisting of
increase in temperature of 0.1°C/s from 60 to 97°C. Standard curves were generated using dilutions of a calibrator (cDNA generated from a pool of normal mouse thyroid RNA). Results were expressed in fold change in comparison with the calibrator, after standardization using 18S rRNA or in mRNA copies per microgram total RNA; in that case, a cloned cDNA was used to generate calibration curves. Comparison of sample transcript contents was done by Student’s t-test.

**Hormone measurements**

Mouse serum TSH concentration was assayed using a double antibody precipitation RIA according to Pohlenz et al. (1999) with reagents provided by National Hormone and Pituitary Program (Harbor-UCLA-Medical Center, Torrance, CA, USA). Briefly, the assay mixture contained anti-rat TSH antibodies prepared in guinea pig, 125I-labeled rat TSH, and either mouse serum or a crude mouse TSH/LH reference preparation used as standard. Rat TSH was labeled using 125I Na and tubes coated with Iodogen (Pierce Chemical, Rockford, IL, USA); its specific radioactivity was ~30 μCi/μg TSH. TSH concentration was measured in 2–50 μl of mouse serum. Goat anti-guinea pig Ig precipitating antibodies (Antibodies, Davis, CA, USA) were used to precipitate immune complexes. Serum total thyroxine (T4) concentration was measured by RIA using anti-T4 antibodies (Valbiotech, Paris, France), 125I–T4 (specific radioactivity: 1300 mCi/mg) (PerkinElmer, Wellesley, MA, USA), increasing amounts of T4 in 10 μl or 10 μl of mouse serum and sheep anti-rabbit Ig antibodies immobilized on beads (Biogenesis, Brentwood, NH, USA). The sensitivity of the assay was ~60 ng/100 ml.

**Statistical analysis**

Comparisons between groups were made using the Student’s t-test or the nonparametric test of Mann–Whitney when samples did not follow a Gaussian distribution.

**Figure 1** The inactivation of Cx32 gene alters growth and functioning of the thyroid gland of mice expressing the E7 oncogene. Thyroid weight and serum T4 concentration were measured in wild-type mice (WT), mice without Cx32 (Cx32-KO), mice expressing the E7 oncogene (E7), and mice lacking Cx32 and expressing the E7 oncogene (Cx32-KO/E7) at 8 (A and C) and 20 (B and D) weeks of age. Columns and vertical bars represent the mean and S.E.M. of values from eight to 10 mice per group. Statistically significant difference at *P < 0.05 or **P < 0.01.

**Figure 2** Alterations of tissue-specific gene expression levels in the thyroid gland of mice expressing the E7 oncogene and expressing or not Cx32. Transcript levels of Nis (Na+/iodide symporter) and Pax8 (thyroid-specific transcription factor) genes were assayed by quantitative PCR in the thyroid of wild-type mice (WT), mice without Cx32 (Cx32-KO), mice expressing the E7 oncogene (E7), and mice lacking Cx32 and expressing the E7 oncogene (Cx32-KO/E7) at 8 and 20 weeks of age. Transcript levels are expressed in relative values, i.e. fold change in comparison with a calibrator. Columns and vertical bars represent the mean and S.E.M. of values from five to seven mice per group. Statistically significant difference at *P < 0.05 or **P < 0.01.
Results

Impact of Cx32 gene inactivation on the size and functioning of the thyroid gland of mice expressing the E7 oncogene

Mice with a generalized Cx32 gene inactivation (Cx32-KO mice) did not present any alteration in the size and function of the thyroid gland in basal conditions (Prost et al. 2008). These mice have been crossed with mice expressing the E7 oncogene. It is worth noting that the two strains of mice, Cx32-KO and E7 mice, have been generated in the same genetic background, C57Bl/6. At the age of 8 weeks, mice devoid of Cx32 and expressing the E7 oncogene (Cx32-KO/E7 mice) exhibited the same thyroid enlargement as E7 mice (Fig. 1A); their thyroid weight was five- to sixfold higher than that of WT mice (7.2 vs 1.3 mg). Serum T4 concentration of Cx32-KO/E7 and E7 mice remained at the control (WT mice) level (Fig. 1C). Between 8 and 20 weeks of age, the development of thyroid hyperplasia differed in Cx32-KO/E7 and E7 mice. At the age of 20 weeks (Fig. 1B), the average thyroid weight of Cx32-KO/E7

![Morphological analyses of the thyroid gland of mice expressing the E7 oncogene and expressing or not Cx32. Representative microscope images (at two different magnifications) of hematoxylin-stained thyroid tissue sections from wild-type mice (WT) at 8 weeks of age (A and B), E7 mice at 8 (C and D) or 20 (E and F) weeks of age and Cx32-KO/E7 mice at 20 weeks of age (G and H). Thyroid morphology of Cx32-KO mice (not illustrated on the figure) was indistinguishable from that of wild-type mice, both at 8 and 20 weeks of age. Bars represent 200 μm in A, C, E, and G and 100 μm in B, D, F, and H.](image-url)
Transgenic mice expressing the RP3 oncogene (RP3 mice) developed a thyroid hypertrophy (Fig. 4). At the age of 8 weeks, thyroid weight of RP3 mice was seven times higher than that of WT littermates (about 11.5 vs 1.56 mg), but individual values varied from 6–30 mg. The inter-animal heterogeneity was also 1.56 mg), but individual values varied from about 6–30 mg. The inter-animal heterogeneity was also

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Mice devoid of Cx32 and expressing the RP3 oncogene (Cx32-KO/RP3 mice) also exhibited an increase in thyroid weight (7.22 vs 1.56 mg in control mice), but the average gain in thyroid weight was 40% lower than that observed in RP3 mice. Interestingly, the inter-animal variation in thyroid weight was low in the group of Cx32-KO/RP3 mice as compared with that observed in the group of RP3 mice. Cx32-KO/RP3 mice did not show any change in body weight and exhibited a slight (not statistically significant) elevation of serum T4 concentration.

To further analyze differences in thyroid phenotype between RP3 and Cx32-KO/RP3 mice, we used two parameters: firstly, thyroid weight to body weight ratio, to take in account the contribution of mouse size variations in the changes of thyroid weight (Fig. 4B) and secondly, serum T4 concentration to thyroid weight ratio (Fig. 4D), to have an estimate of the specific (related to the mass) functional activity of thyroid tissue. As it can be seen in Fig. 4D, the latter parameter led to the distribution of RP3 mice into two subgroups, with average values of serum T4 to thyroid weight ratio of 0.67 ± 0.09 (n = 8) and 0.02 ± 0.01 (n = 6), corresponding to mice with an enlarged thyroid or subgroup.
L (open symbols) and mice with a very large thyroid or subgroup VL (closed symbols) respectively. Serum T4 to thyroid weight ratio of Cx32-KO/RP3 mice was equal to 0.49 ± 0.05, a value not statistically different from that obtained for RP3 mice of subgroup L. Values of thyroid weight to body weight ratio (Fig. 4B) also discriminated RP3 mice belonging to the subgroups L and VL defined above. Thus, whatever the parameter, RP3 mice of the subgroup L and Cx32-KO/RP3 mice appeared very similar.

The level of expression of thyroid-specific genes, *Nis* and *Pax8*, was only moderately altered in mice expressing the RP3 oncogene. *Nis* transcript level was similar in control mice (WT and/or Cx32-KO) and RP3 mice (subgroup VL) and significantly decreased (*P < 0.05), to about the same extent, in RP3 (subgroup L) and Cx32-KO/RP3 mice (Fig. 5); this decrease is probably secondary to a lowering of serum TSH concentration resulting from the slight elevation of serum T4 observed in RP3 (subgroup L) and Cx32-KO/RP3 mice (Fig. 4C). There was no statistically significant difference between Pax8 expression levels of the two subgroups of RP3 mice and Cx32-KO/RP3 mice.

To determine whether the expression of RP3 oncogene could influence Cx32 expression or vice versa, we compared 1) the thyroid Cx32 transcript content of WT and RP3 mice and 2) the thyroid RET/PTC transcript content of RP3 and Cx32-KO/RP3 mice. Data are reported in Fig. 5. Cx32 was expressed at comparable levels in the thyroid of RP3 mice (subgroup L), RP3 mice (subgroup VL), and WT mice. Similarly, transcripts corresponding to the RET TK domain were present at a comparable level in the thyroid of RP3 (subgroup L and VL) mice and Cx32-KO/RP3 mice; the transcript level of these mice was about 50-fold higher than that of WT mice (transcripts assayed in WT mice correspond to RET transcripts naturally present in thyroid C cells).

Thyroid tissue of RP3 and Cx32-KO/RP3 mice has been subjected to extended histological examinations (Fig. 6). Two distinct thyroid morphological subtypes were observed in RP3 mice. The first subtype corresponded to a rather normal thyroid architecture with large to very large follicle lumena (Fig. 6E and F), as compared with normal mouse thyroid tissue (Fig. 6A and B); cellular invaginations within follicles were occasionally observed. The second subtype was characterized by a profound tissue remodeling; the main structural features were heterogeneous zones composed of vast irregular lacunae replacing normal follicles and zones with multiple cell layers forming papillae-like structures projecting inside follicle

![Figure 5](https://example.com/figure5.png)
remnants (Fig. 6G and H). Interestingly, the first morphological subtype was found in RP3 mice of the L subgroup and the second subtype in mice of VL subgroup, without any exception. Glands of very large size (VL subgroup) exhibited signs of neoplastic transformation whereas other glands (L subgroup) corresponded to hyperplastic thyroid tissue. There was no histological difference between the thyroid of RP3 mice of the L subgroup (Fig. 6E and F) and the thyroid of Cx32-KO/RP3 mice (Fig. 6I and J) with one exception. Indeed, in one Cx32-KO/RP3 mouse (identified by an arrow in Fig. 4B) out of 10, thyroid tissue showed the remodeling with proliferative papillary formations (Fig. 6K and L) observed in the VL subgroup of RP3 mice.

**Cx32 gene inactivation promotes thyrotropin-stimulated thyroid growth**

A sodium perchlorate treatment (1% in drinking water) has been administered to WT and Cx32-KO mice (C57Bl/6 strain), for 8 weeks. Mice developed a thyroid hyperplasia in response to the elevation of serum thyrotropin concentration due to the inhibition of thyroid hormone synthesis caused by the suppression of iodide supply. At the end of the perchlorate treatment (Fig. 7), thyroid weight of WT mice was increased about three times as compared with that of untreated animals. Perchlorate treatment induced the formation of a larger goiter in Cx32-KO than in WT mice (8.60 ± 0.82 vs 6.25 ± 0.22 mg, *P* < 0.01). The increase in serum TSH concentration (about 100-fold...
over the basal level) was similar in WT and Cx32-KO mice. In both WT and Cx32-KO mice, perchlorate treatment induced the same reorganization of thyroid tissue characterized by an emptying of follicle lumena and an increase in thyroid epithelial cell volume (Fig. 6C and D).

Discussion

In this study, we demonstrate that Cx32 ablation influences the response of thyroid cells to two oncogenes, E7 and RP3.

Up to the age of 20 weeks, mice expressing the E7 oncogene developed a thyroid hypertrophy with no sign of neoplastic transformation. The effect of the E7 oncogene on thyroid activity appeared biphasic; after an activation in young mice, E7 caused a decrease in expression of thyroid differentiation leading to a colloid goitre; this has been documented in depth in a recent paper based on morphological analyses (Jin et al. 2008). We report that the E7 oncogene loses part of its ability to induce thyroid hypertrophy in Cx32-deficient mice.

Mice expressing the RP3 oncogene exhibited a thyroid hypertrophy comparable with that observed in E7 mice; but, in addition, signs of neoplastic transformation appeared with a rather high frequency (in six mice out of 14) after 8 weeks of life. From both morphological (Jin et al. 2008) and large-scale gene expression analyses (Burniat et al. 2008), RP3 mice at 2 months of age have been proposed as a valid but transient model of human papillary thyroid carcinomas. We show that the transforming activity of the RP3 oncogene is markedly reduced in Cx32-deficient mice.

These data indicate that Cx32, contrary to what was expected, could have a deleterious role in thyroid tumorogenesis in potentiating the activity of oncogenes. The apparent discrepancy between our data and the expected tumor suppressor function of Cx32 cannot be related to uncontrolled variations of the genetic background of mice; indeed, all experiments were conducted in a uniform genetic background. Parental strains: Cx32-KO, E7, and RP3 mice as well as WT mice used to obtain the progenies had the C57Bl/6 genetic background. The possibility of masked interferences between genetic alterations has been studied. We found that the level of expression of the E7 oncogene (data not shown) and the level of expression of RP3 oncogene (Fig. 5) were comparable in Cx32+/0 (WT) and Cx32−/0 (Cx32-KO) mice and, conversely, that the level of expression of Cx32 was comparable in WT, E7, and RP3 mice.

Additional experiments with transgenic mice overexpressing Cx32 selectively in thyroid have been performed in order to generate complementary and confirmatory information. Mice expressing a functional Cx32/EGFP fusion protein (Flachon et al. 2001, 2002, Prost et al. 2008) were crossed with E7 or RP3 mice. However, we did not get any informative data because the Cx32/EGFP transgene was only expressed at low levels in the thyroid of E7 and RP3 mice (unpublished data). This was probably due to the fact that Cx32/EGFP, E7, and RP3 transgenes are all placed under the control of the same bovine thyroglobulin gene regulatory region (which confers a strict thyroid expression specificity). A competition between transgene promoters for binding of transactivating factor probably took place in double transgenic mice.

Although most of the abundant literature on Cx and gap junctions is in favor of a tumor suppressor function...
of Cx, one can find several reports that do not support this proposal. One example is given by mice with a double deficiency in p27^Kip1 and Cx32. Mice lacking the tumor suppressor/cell cycle regulator p27^Kip1 exhibit increased tumorigenesis in a variety of tissues following chemical and radiation induction. Loss of p27^Kip1 in a Cx32-KO background results in an attenuation of liver and lung tumorigenesis (King & Lampe 2005). A second example also relates to liver tumorigenesis. Cx32 was found to be involved in tumor promotion by phenobarbital; Cx32-KO mice were almost entirely resistant to the promotional effects of the barbiturate (Schwarz et al. 2003). Another report deals with Cx32 in tumor progression in vivo; it was reported that intracellular accumulation of Cx32 enhances the motility and the metastatic ability of hepatocellular carcinoma cells (Li et al. 2007).

The regulatory functions of Cx32 in the thyroid gland appear very complex. Indeed, Cx32 behaves as a positive effector of thyroid growth triggered by E7 oncogene, which acts through Rb protein (Chellappan et al. 1992), has a promoting activity on the neoplastic transformation induced by RP3 oncogene, which primarily acts through the MAPK cascade (Santoro et al. 2004), and exerts a negative control on thyroid growth stimulated by thyrotropin, which activates the cAMP cascade (Dumont et al. 2002). This dual thyroid growth control exerted by Cx32 could be related to the coexistence of separate modes of mitogenic activation of thyroid epithelial cells (Kimura et al. 2001).

As already proposed, to explain the tumor suppressor function of different Cx in various organs or tissues, one can postulate that the tumor promoter role of Cx32 could bring into play either Cx32-gap junction-mediated cell-to-cell communication or the ability of Cx32 to interact with proteins belonging to signaling cascades and/or acting on the control of cell cycle machinery. Molecules that could play the role of messenger in Cx32-dependent cell-to-cell communication, on the one hand, and molecules interacting with Cx32 and endowed with a control activity of cell proliferation, on the other hand, remain to be discovered.

A quite common observation, now almost a dogma in the Cx field, is that cancer cells are deficient in gap junction and/or Cx (Yamasaki et al. 1995, Trosko & Ruch 1998, Mesnil et al. 2005). In the light of our finding, it is tempting to postulate that the reduction or loss of Cx expression and/or alterations of Cx localization in tumors could represent a cell response to either the action of oncogenes or the loss of tumor suppressor genes, to act against tumor formation and minimize its development.

In conclusion, Cx32 and/or Cx32-gap junctions, which are probably dispensable in the thyroid gland in physiological or steady-state conditions, are likely to be important in limiting thyroid hyperplasia caused by high and sustained serum TSH concentrations occurring in different pathological situations but could exert adverse effects by cooperating with certain oncogenes to induce thyroid neoplastic transformation; a down-regulation of expression of Cx32 in tumors would contribute to limiting tumor development as demonstrated here in Cx32-depleted mice.

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

**Funding**

This work was supported by a grant from Association pour la Recherche sur le Cancer and from la Ligue contre le Cancer (Comité du Rhône).

**Acknowledgements**

We are grateful to Klaus Willecke (Institute für Genetik, Universität Bonn, Bonn, Germany) for providing us with Cx32-KO mice. We address our most profound thanks to Catherine Ledent & Jacques Dumont (IRIBHM, Hôpital Erasme, Université libre de Bruxelles, Bruxelles, Belgique) and to Massimo Santoro and Alfredo Fusco (Università di Napoli ‘Federico II’, Naples, Italy) for providing us with E7 mice and RET/PTC3 mice respectively. We thank R Rabilloud for her technical assistance.

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