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Effect of iodine on early stage thyroid autonomy

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ABSTRACT

Thyroid autonomy is a frequent cause of thyrotoxicosis in regions with iodine deficiency. Epidemiological data suggest that iodide may influence the course of pre-existing thyroid autonomy.
Making use of FRTL-5 cells stably expressing a constitutively activating TSH receptor mutation as an *in vitro* model of thyroid autonomy, we investigated the impact of iodide on proliferation, function and changes in global gene expression.
We demonstrate that iodine inhibits growth in TSHR WT and L629F mutant FRTL-5 cells and downregulates e.g. protocadherin cluster (Pcdha1–13) and thyroid responsive element (Thrsp). In addition functional genes e.g. iodotyrosine deiodinase (*iyd*) and oncogen junB are upregulated, while sodium-iodide-symporter (Nis) and thyroid peroxidase (Tpo) are downregulated by iodide.
Iodide tunes down the biological activity of autonomous thyrocytes and may thus be of therapeutic benefit not only to prevent the occurrence of somatic TSHR mutations, causing thyroid autonomy, but also to slow down the development of clinically relevant disease.

1. Introduction

Thyroid autonomy is defined as the TSH-independent function and proliferation of thyroid follicles [4,24]. The most frequent cause of thyroid autonomy are TSH receptor mutations, that cause a constitutive activation of the cAMP cascade resulting in stimulation of thyroid function and growth [11,20]. Thyroid autonomy is highly prevalent in geographic regions with iodide deficiency and rarely occurs in iodide-sufficient regions [15].

The link between thyroid autonomy and iodine deficiency is not ultimately clarified on the molecular level, however thyroid hormone synthesis requires H_2O_2 as a co-substrate, which very likely represents an important source of reactive oxygen species (ROS). *In vitro*, an increased mRNA expression of oxidative defense genes during iodine deficiency has been shown in thyroids of rat and mice [17]. Increased oxidative stress may lead to DNA damage and may promote mutagenesis e.g. the occurrence of gain-of-function TSHR mutations.

The impact of iodine on the further evolution of clonal thyroid autonomy to clinically relevant thyroid disease has not been studied on the molecular level so far. Iodine plays an important role in the regulation of thyroid growth and function. Supraphysiological doses of iodide have long been known to cause a temporary inhibition of thyroid hormone synthesis, a process termed the Wolff–Chaikoff effect, which is still used pre-operatively as “plummering” in some thyrotoxic patients. In addition, iodine excess has been shown to inhibit cell growth, induce apoptosis and affect cell morphology [25]. Hypothetically, iodine exposure might also suppress proliferation of autonomous thyrocytes thereby slowing the development of autonomous growth and preventing hyperthyroidism. Early support for such hypothesis comes from the Pescopagano survey of patients with known thyroid autonomy. In this study, hyperthyroidism occurred twice as frequent in the iodide deficient vs. the iodide-sufficient patient group, suggesting that iodide deficiency may indeed be a selection factor for the clinically relevant development of thyroid autonomy [2].

In this paper we expand on previous studies by the group of Ludgate et al. using an *in vitro* model of thyroid autonomy [3,11] and investigate iodine-induced changes in the transcriptome as well as its effects on regulation of cell growth and function in early stage thyroid autonomy compared to normal thyroid cells.

2. Material and methods

2.1. Cell culture conditions

We have studied the SB5 sub-clone of FRTL-5 cells stably expressing wild-type (WT) TSH receptor or 2 different gain-of-function TSHR mutants, L629F and A623I, introduced by retroviral infection as previously described [11]. FRTL-5 cells were maintained in a 2:1:1 mixture of DMEM (PAA Laboratories, Cölbe, Germany): Ham's F12 (PAA Laboratories, Cölbe, Germany): MCDB104 (GIBCO Life Technologies,

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Karlsruhe, Germany) supplemented with 5% newborn calf serum (GIBCO Life Technologies, Karlsruhe, Germany), 10 μg/ml insulin, 0.4 μg/ml hydrocortisone (Calbiochem, San Diego, CA, USA), 45 μg/ml ascorbic acid (Sigma, Seelze, Germany), 5 μg/ml transferrin (Calbiochem, San Diego, CA, USA) and 5 mU/ml bovine TSH (Sigma, Seelze, Germany). Depending on the parameters analyzed, the cells were cultured in serum-containing medium, supplemented with TSH and varying concentrations of NaI (or NaCl) as indicated.

2.2. Direct cell counting

Cells were plated in complete (serum-containing) medium with or without TSH (1 mU/ml) in various iodide concentrations (1–50 mM NaI) in a density of 5×10^4 cells/well in 12-well plates. Cells were trypsinized on days 3, 6, 8 and 10, resuspended in phosphate buffered saline solution (PBS) and counted using a Neubauer counting chamber. Results are expressed as total counts per well. To monitor unspecific osmolaric effects of the salt solutions, sodium chloride was used in all experiments equivalent to the highest NaI concentration.

2.3. Apoptosis

Apoptosis was determined by staining cells with Annexin V-Cy5 (*Ex*/*Em* = 649 nm/670 nm). Annexin V is used to identify the externalization of phosphatidylserine during the progression of apoptosis and, therefore, is a marker for early phases of apoptosis. In brief, cells were incubated with 0–10 mM doses of sodium iodide for 72 h. Cells were washed twice with cold PBS, trypsinized and resuspended in 250 μl binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). 0.5 μl of Annexin V-Cy5 (PharMingen, San Diego, CA) and 10 μg propidium iodide was added to these cells prior to detection with a LSR II cytometer (Becton Dickinson UK Ltd, Oxford, Oxon, UK). Results were analyzed with FlowJo Software (Flow Cytometry Analysis Software; Tree Star, Inc., Leland Stanford, Jr. University).

2.4. Cell cycle analysis

FRTL-5 mTSHR cells cultured in complete medium with or without TSH and in the presence of varying NaI (0, 1 and 10 mM) concentrations, were plated in duplicates at a density of 3×10^5 cells/well in 6-well plates. After 72 h, trypsinized cells were washed in PBS and fixed in ice-cold 70% ethanol at 4 °C for 1 h, washed, then incubated in 250 μl PBS containing 50 μg/ml RNase for 30 min at 37 °C. Immediately prior to flow cytometry 10 μl of 1 mg/ml propidium iodide (Sigma) was added. In each case a minimum of 10,000 events were scanned on a LSR II flow cytometer (Becton Dickinson UK Ltd., Oxford, Oxon, UK). Results were analyzed with FlowJo Software (Flow Cytometry Analysis Software; Tree Star, Inc., Leland Stanford, Jr. University).

2.5. RNA extraction and quantitative real-time PCR

RNA extraction and cDNA synthesis were carried out as described and expression of the housekeeping gene *S6* was measured as a control in all samples by RT-PCR [9,10]. Real-time PCR (LightCycler system, LightCycler-DNA MasterSYBRGreen I kit; Roche, Mannheim, Germany) was performed using intron-spanning primers for slc5a5 (Nis), Tpo, Thrsp, Pcdha, Junb, Iyd and the housekeeping gene Rps6 (rat). For each PCR, annealing temperatures and $MgCl_2$ concentrations were optimized to create a one peak melting curve (primer sequences and PCR condition are available on request).

2.6. Microarray analysis

Before microarray analysis, RNA integrity and concentration was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6.000 LabChip Kit (Agilent Technologies) according to the manufacturer's instructions. Microarray analysis was conducted at the microarray core facility of the Interdisziplinäres Zentrum für klinische Forschung (IZKF) Leipzig (Faculty of Medicine, University of Leipzig). 3 μg of total RNA were used to prepare double-stranded cDNA (Superscript II, Life Technologies, Gaithersburg, MD USA) primed with oligo-dT containing an T7 RNA polymerase promoter site (Genset SA, Paris, France). cDNA was purified by phenol-chloroform extraction before *in vitro* transcription using the IVT labeling kit (Affymetrix, Santa Clara, CA, USA) to synthesize cRNA. The cRNA was fragmented and hybridized to Affymetrix Rat Genome, 230 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Washing and staining of the probe array were performed according to the manufacturer's instructions. The array was scanned with a third generation affymetrix GeneChipScanner 3000.

Affymetrix GeneChip data representing approximately 30,000 transcripts with complete Rat Genome coverage were extracted from fluorescence intensities and were scaled in order to normalize data for inter-array comparison using MAS 5.0 software according to the instruction of the manufacturer (Affymetrix). Our GeneChip data are submitted to the Gene expression omnibus and to track under the accession number GSE22118 (http://www.ncbi.nlm.nih.gov/geo/).

Microarray experiments were conducted in duplicate. Data was filtered to remove genes with a lower than 2-fold change between experimental conditions. Genes were functionally categorized using NetAffx (Affymetrix; http://www.affymetrix.com), KEGG (http://david.abcc.ncifcrf.gov/), DAVID2008 (http://david.abcc.ncifcrf.gov/) and GenMapp (http://www.genmapp.org).

3. Results

3.1. Effect of iodide on proliferation in normal and autonomous FRTL-5 cells

The effect of 1 up to 50 mM iodide on proliferation of FRTL-5 cells stably expressing the WT TSHR, the mutant TSHR L629F or A623I were analyzed by cell counting in the presence and absence of TSH.

In Fig. 1A TSH-independent growth is shown for clones expressing the constitutively active TSHR mutations L629F and A623I. In absence of TSH, the highest cell numbers were found for A623I FRTL-5 cells. Similar to the previous study [11] the L629F FRTL-5 cells showed only a moderate increase in cell number. When cells were grown in the presence of TSH, similar increases in cell numbers were observed for all three FRTL-5 clones (WT TSHR, L629F and A623I mutant TSHR).

Addition of iodide inhibited proliferation in WT TSHR, L629F and A623I FRTL-5 cells irrespective of the presence or absence of TSH (Fig. 1B). Inhibition of proliferation was more pronounced in mutant TSHR cells compared to WT TSHR cells and increased with long-term iodide exposure (>72 h). Relative growth inhibition after treatment with 1 mM NaI reached 43%, 58%, and 88% in WT TSHR, L629F mTSHR and A623I mutant TSHR FRTL-5 cells, respectively (Fig. 1A).

3.2. Effect of iodide on apoptosis and necrosis in normal and autonomous FRTL-5 cells

To examine whether iodide mediated growth inhibition is due to apoptosis or necrosis flow cytofluorometric analysis of Annexin V-Cy5 and propidium iodide (PI) staining was performed after 72 h of iodide incubation in FRTL-5 cells over-expressing the L629F mTSHR or the WT TSHR (Fig. 2). Increased Annexin V labeling (= indicator of early apoptosis) was observed with increased iodide doses in WT and mutant TSHR FRTL-5 cells. Furthermore, increased iodide exposure was associated with increased Annexin V and PI (= indicator of late apoptosis) labeling indices, without differences for WT and mutant TSHR expressing FRTL-5 cells. In contrast, PI labeling indices (= indicator of necrosis) increased in

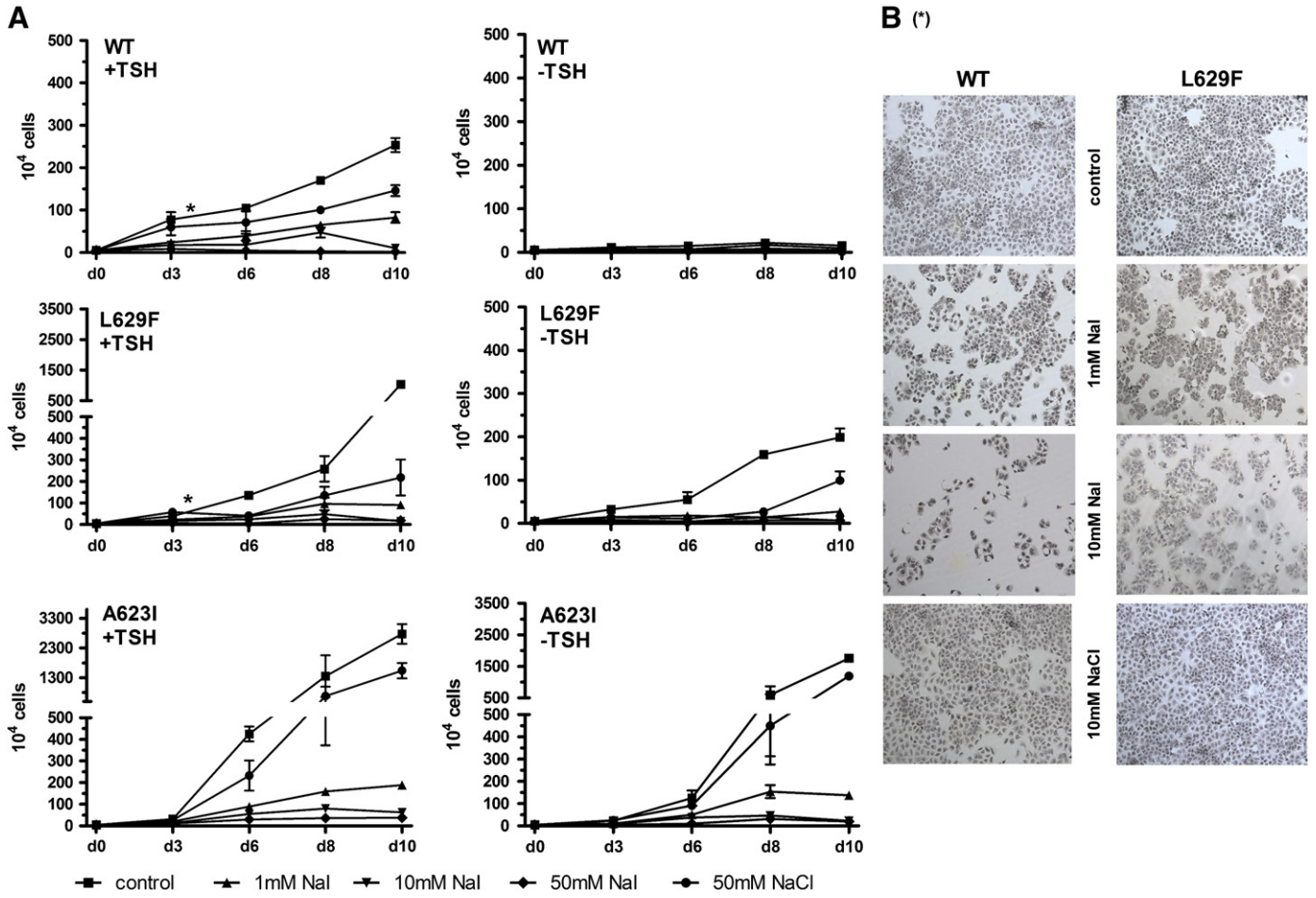

Fig. 1. Effects of iodide on cell proliferation in WT TSHR and mutant TSHR FRTL-5 cells (L629F and A623I mTSHR). Cell numbers were counted at day 3, 6, 8 and 10 after plating $5*10^4$ cells/well in 12-well plates in the presence or absence of TSH (control, black square). Iodide was added in 1 mM (black upward triangle), 10 mM (black downward triangle) and 50 mM (black rhombus) concentrations. To assess for tonicity effects sodium chloride was added in the highest iodide concentration (50 mM NaCl; black circle). (A) Dose dependent inhibition of iodide on cell growth. Three independent experiments were performed in duplicates. (B) Representative changes in cell numbers and morphology after 96 h stimulation with iodide in the presence of TSH in WT TSHR and L629F TSHR FRTL-5 cells (as assigned in (A) with *). NaCl in a concentration of 10 mM has no significant effect on proliferation unlike 50 mM NaCl (hematoxylin dye).

WT TSHR cells upon iodide exposure, while no increase in PI indices was found for L629F TSHR cells. This effect, however, was not significant.

These findings suggest that the lack of growth stimulation may be due to an increase of apoptosis in the presence of iodide.

3.3. Effect of iodide on cell cycle regulation in normal and autonomous FRTL-5 cells

To further assess, whether the lack of growth stimulation in cells stably expressing L629F or WT TSHR is associated with changes in cell cycle progression, cells were analyzed by flow cytometry after iodide exposure for 72 h. As shown in Fig. 3 iodide exposure results in a shift from G0/G1 to G2 phase in WT TSHR and L629F mTSHR FRTL-5 cells, which is less pronounced in L629F FRTL-5 cells and is accompanied by a S-phase reduction in these cells as previously shown by Al-Khafaji et al. [3]. This finding is in line with the known inhibitory effects of iodide on thyroid cell proliferation by causing G0/G1 and G2M arrest in high concentrations [22].

3.4. Effect of iodide on global gene expression in normal and autonomous FRTL-5 cells

To assess a broader spectrum of iodide effects on cell physiology, transcriptome profiles were obtained in the L629F and the WT TSHR FRTL-5 cell clones in the presence and absence of iodide, using microarray technology. Fig. 4 summarizes the number of genes and gene groups with at least 2-fold changes in the expression for iodide treated (1 mM NaI) vs. control (1 mM NaCl) WT TSHR and L629F mTSHR FRTL-5 cell clones.

To validate our GeneChip data we studied 6 iodine-regulated genes using quantitative real-time PCR (qPCR). With the main focus of genes involved in thyroid differentiation, we analyzed the thyroid peroxidase (Tpo), the thyroid hormone responsive element (Thrsp), the iodotyrosine deiodinase (Iyd) and the sodium-iodide symporter (Slc5a5/Nis). Differential regulation of the proto-oncogene jun B (Junb) was analyzed to validate a potential candidate with a focus on cell proliferation. In addition, Pcdha4 (protocadherin alpha 4) a member of the strongly regulated cadherin gene set was included in the qPCR validation. As shown in Fig. 5 quantitative real-time PCR analysis confirmed the reduction in mRNA expression in response to iodide in all investigated genes.

Using a cut-off of at least 2-fold expression difference our experiments showed iodide dependent upregulation of 6 genes (glucagon (Gcg), amphiregulin (Areg), ethanolamine kinase 1 (Etnk1), proline–serine–threonine phosphatase-interacting protein 1 (Pstpip1) and nuclear receptor interacting protein 3 (Nrip3) and downregulation of 68 genes (Table 3 — online supplement) in WT TSHR and L629F FRTL-5 cells.

Furthermore, comparison of gene expression pattern between L629F FRTL-5 cells and WT TSHR FRTL-5 cells in the presence of

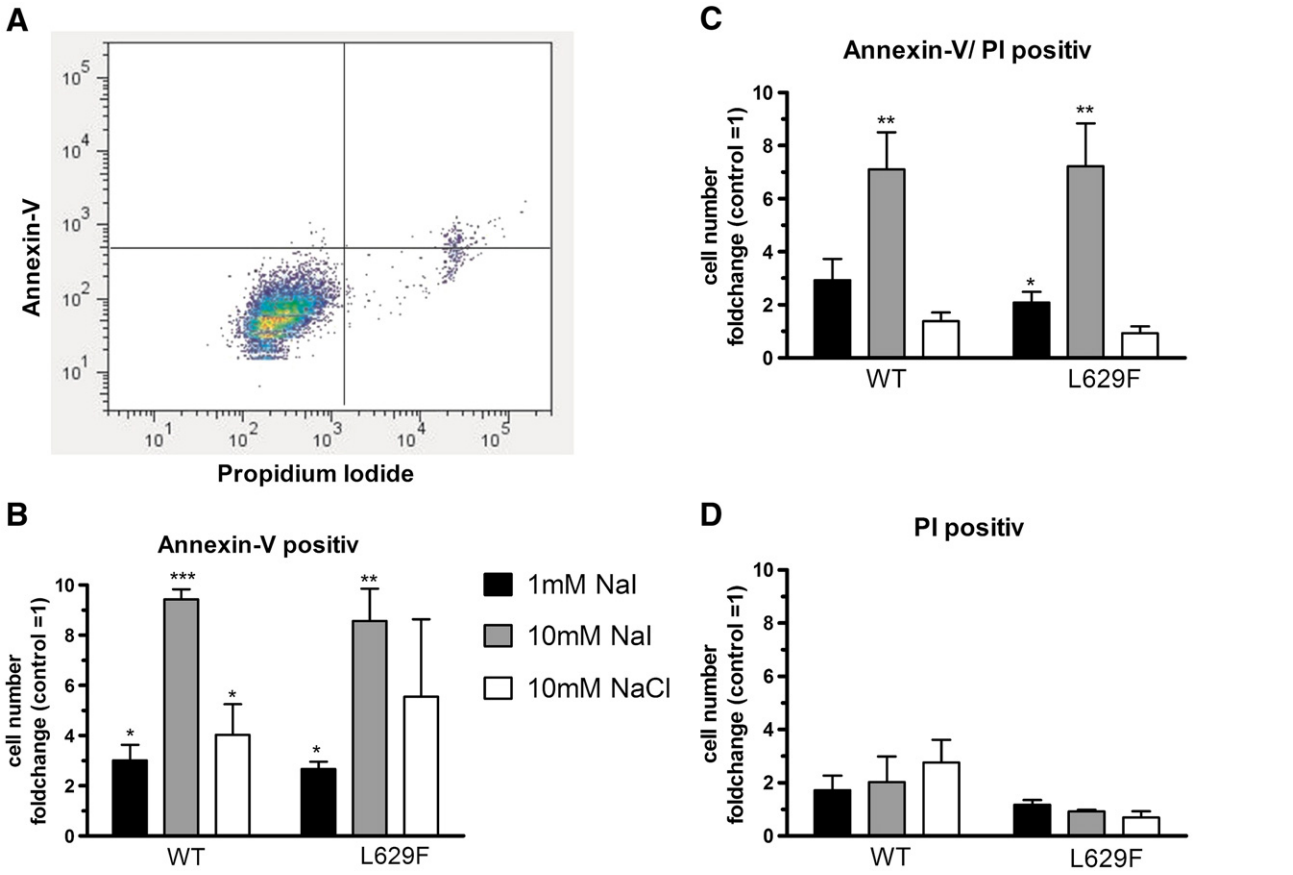

Fig. 2. Determination of apoptosis and necrosis using a flow cytometry based Annexin V assay. Cells treated with 1 mM, 10 mM NaI/ NaCl for 72 h, were stained with an anti-Annexin V antibody conjugated with Cy5 (ordinate). Propidium iodide (abscissa) was added before sorting. (A) Intact cells are located in the *lower left quadrant*, necrotic cells permeable to propidium iodide are shown in the *lower right quadrant*, apoptotic cells stained by Annexin V and propidium iodide are located in the *upper right and left quadrant*. (B) Iodine significantly upregulated apoptosis in WT TSHR and L629F mTSHR FRTL-5 cells. (C) Induction of iodine late apoptosis as demonstrated by Annexin V and propidium iodide labeling. (D) No significant induction of necrosis was seen by iodide exposure. Two different experiments were performed in triplicates.

1 mM NaI, showed 136 and 37 genes, which were up- and downregulated, respectively, between autonomous and normal thyroid cells (Table 3 — online supplement). Prominently upregulated genes in L629F cell clones were e.g. Rho GTPase activating protein 21 (Arhgap21), phosphatidylinositol-4-phosphate 5-kinase type 1 alpha (Pip5k1a), collagen type XII alpha 1 (Col12a1), ring finger protein 165 (Mf165) and neurotrophic tyrosine kinase, receptor, type 3 (Ntrk3) (Fig. 4A). Differentially downregulated genes in L629F cell clones were e.g. iodotyrosine deiodinase (Iyd), proto-oncogene jun B (JunB), tropomodulin 1 (Tmod1) and parvin alpha (Parva) (Table 3 — online supplement).

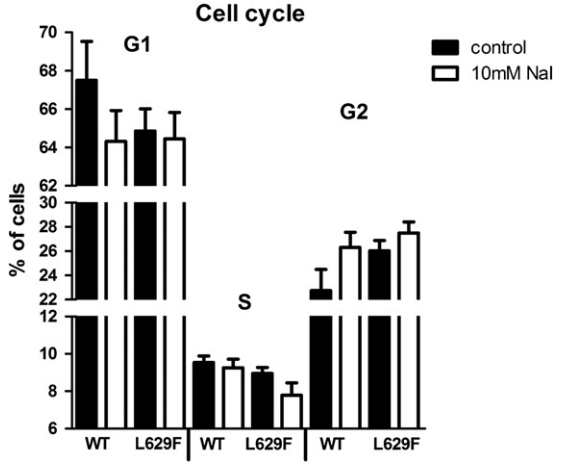

Fig. 3. Impact of iodide on cell cycle. L629F mTSHR and WT TSHR FRTL-5 cells were stimulated with 1 mM, 10 mM NaI or 10 mM NaCl in the presence of TSH for 72 h (data of 1 mM NaI and 10 mM NaCl are not shown). Subsequently, cells were stained with propidium iodide and were analyzed by flow cytometry. In both normal and autonomous thyroid cells iodine exposure induced a shift from G0/G1 to G2 phase without significant differences for 1 or 10 mM NaI. Values are the mean ± SD of two different experiments performed in triplicates.

When filtering genes with at least 1.5-fold expression difference using the DAVID2008 platform, the highest enrichment scores were obtained for categories of genes involved in cadherin signaling, cell adhesion, and ion binding (Table 1). Furthermore, analysis of microarray data by principal component analysis (PCA) showed a clear separation of the identified gene groups in WT vs. L629F TSHR FRTL-5 cells. Fig. 3B illustrates the PCA results comprising 74.6% of the identified genes with differential gene expression. The x-axis shows 36.8% difference in gene expression comparing WT and L629F FRTL-5 cells, while the y-axis shows 22.3% difference in iodide-induced gene expression. This illustrates, that the endogenous gene expression differences between autonomous thyrocytes (harboring the L629F TSHR mutation) and normal thyrocytes are more pronounced than effects of iodine on gene regulation. In other words, normal thyrocytes and autonomous thyroid cells are distinct molecular entities, with autonomy showing more impact on gene expression than iodine treatment. Iodine exposure affects both entities similarly but not identically. This finding is also illustrated by the total number of individually regulated genes (698 in WT clone versus 438 in L629F clone) and a total of only 74 genes, which are consistently regulated in normal and autonomous thyrocytes in response to iodide.

3.5. Effects of iodide on cell function in normal and autonomous FRTL-5 cells

Since iodide is known to inhibit thyroid function, besides proliferation, we were interested to investigate, whether this effect

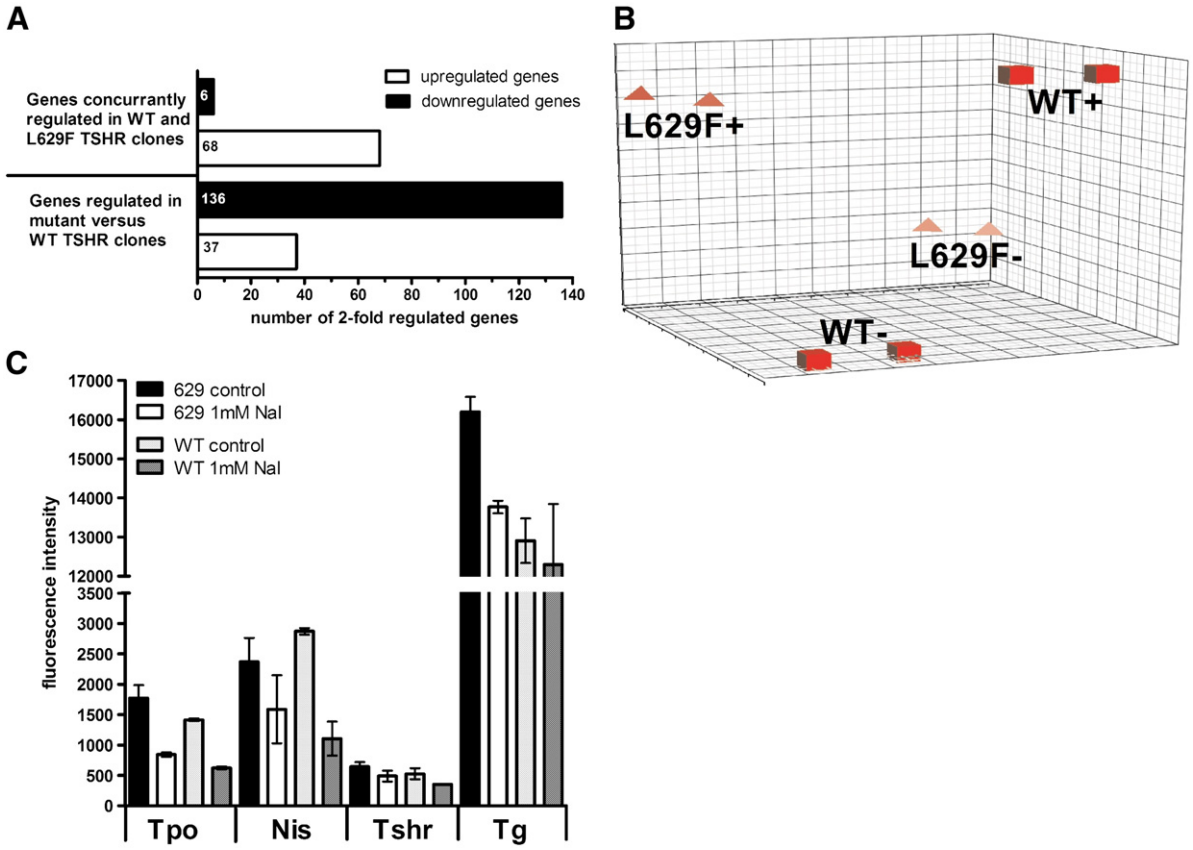

Fig. 4. Analysis of GeneChip data of L629F mTSHR and WT TSHR FRTL-5 cells in the presence and absence of 1 mM NaI. (A) Summary of upregulated genes (black bars) and downregulated genes (white bars) with at least 2-fold expression difference. Some genes are concurrently regulated in WT and L629F FRTL-5 cells. (B) 3-D visualization of a principal component analysis of the microarray data using Partek Genomics Suite software (Partek, St. Louis, MO, USA). (C) GeneChip data of thyroid differentiation genes. Fluorescence intensity of scan data of Tpo, Nis, Tshr and Tg in FRTL-5 hTSHR WT TSHR and L629F mTSHR FRTL-5 cells (control, black and bright shaded bars) and after 72 h 1 mM NaI treatment (white and dark shaded bars). Columns in the graph represent mean and SD values of two GeneChips per condition (WT/ L629F mutant). RNA extraction and array hybridization were performed in two different experiments.

also occurs in autonomously functioning thyrocytes and whether they react differently compared to normal thyrocytes.

Analysis of the sodium-iodide symporter and the thyroid peroxidase mRNA expression in WT TSHR FRTL-5 cells showed 1.5-fold downregulation (1.6-fold in 10 mM) and 3-fold downregulation (3.4-fold in 10 mM) in the presence of 1 mM iodide, respectively. In L629F TSHR FRTL-5 cells, incubation with 1 mM iodide resulted in 1.8-fold downregulation of Nis mRNA (2-fold in 10 mM) and in 3-fold downregulation of Tpo mRNA (3.5-fold in 10 mM) (Fig. 5B). Moreover, GeneChip analysis showed mRNA downregulation of other marker genes of thyroid function such as Tg and Tshr in WT and mutant TSHR FRTL-5 cells upon iodide exposure (Fig. 5C). Thus, iodide causes downregulation of thyroid function genes without significant difference between normal and autonomously functioning thyroid cells.

4. Discussion

Iodide is a pivotal factor in thyroid physiology and disturbed iodine homeostasis is an important trigger of thyroid pathophysiology. In this study we investigate an *in vitro* model of thyroid autonomy to determine the molecular effects of iodide on function, cell proliferation and global gene expression pattern in early stage autonomy.

We found reduced expression of genes important for thyroid function (Tpo, Nis, Thrsp, Iyd) in response to iodide exposure in normal and autonomously functioning FRTL-5 cell clones. Downregulation of functional genes has been reported in a recent SAGE analysis of the rat PCCl-3 cell line using similar iodide concentrations [16] and is in line with the Wolff–Chaikoff effect i.e. the inhibition of thyroid hormone synthesis and metabolism by iodide exposure [5,8].

Furthermore, in our experiments we observed an inhibition of cell proliferation in mutant and WT TSHR FRTL-5 cells clones, which was more pronounced in clones with constitutive TSHR activation and in the absence of TSH (i.e. the clinical equivalent of subclinical or overt hyperthyroidism). This effect has been reported in a previous study by Al-Kafahji et al. [3], but has now been demonstrated at much lower (1 mM) iodide concentrations. Further investigations showed that the reduction in growth is most likely due to increased apoptosis and G0/G1 and G2M arrest [22,23] with only marginal differences for WT TSHR and mutant TSHR cells. This could be due to the presence of TSH, which is a strong proliferation signal for thyrocytes. However, since the aim of our study was to assess the effect of iodide under physiological conditions we refrained from TSH deprivation of the investigated FRTL-5 cell clones.

Thus our *in vitro* data, which typify an artificial model however, suggest that iodide slows down growth and function in normal thyrocytes and early stage thyroid autonomy alike and may thus provide one explanation why thyroid autonomy is very rare in countries with iodide excess, e.g. Japan, despite the common molecular cause of activating TSHR mutations [13,19].

To get an insight into signaling events, which are altered by iodide, we performed global gene expression analysis using microarray technology.

Upon iodide exposure we found that a number of genes were simultaneously upregulated in WT TSHR and L629F mutant TSHR FRTL-5 cells. For example, upregulated genes included genes involved in cell cycle control (amphiregulin, Areg), cell metabolism (glucagon,

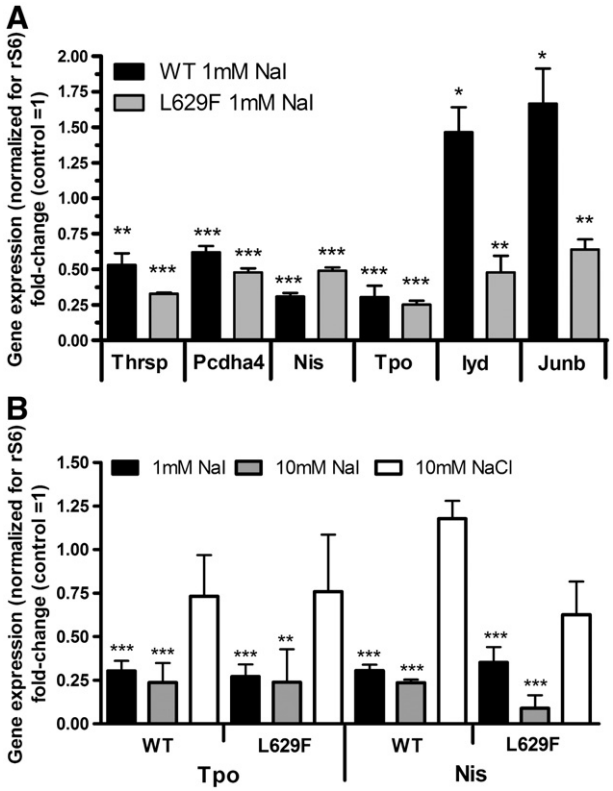

Fig. 5. Downregulation of thyroid function after iodide exposure and validation of expression difference by microarray analysis. (A) Quantitative Real-time PCR confirmation of GeneChip data. Iodide induced downregulation of *thrsp*, *pcdha4*, *nis*, *tpo*, *iyd* and *junb* was confirmed in WT TSHR and L629F TSHR FRTL-5 cells after 72 h treatment with 1 mM NaI. Data are presented as ratio of target mRNA normalized to the housekeeping gene ribosomal protein S6 and fold-change calculations to untreated control cells. (B) qPCR shows dose-dependent iodide-induced inhibition of *nis* and *tpo* mRNA expression in WT TSHR and L629F mutant TSHR FRTL-5 cells. Data are presented as fold-change of *nis* or *tpo* mRNA normalized to the housekeeping gene ribosomal protein S6 and untreated control cells. For all experiments cells were grown in the presence of TSH. The experiments were performed three times in duplicates.*$P<0.05$; **$P<0.01$; ***$P<0.001$ (ANOVA followed by Bonferroni's *post hoc* test and the Students t-test).

Gcg) and regulation of inflammatory processes (proline–serine–threonine phosphatase-interacting protein 1, Pstpip1).

In contrast, the complete gene cluster of protocadherin alpha was downregulated by iodide. We detected at least 13 highly similar coding sequences (Pcdha1-13) and two more distantly related coding sequences designated Pcdha c1 and Pcdha c2 in the Pcdha gene cluster, encoding N-terminal cadherin-like extracellular and transmembrane domains [26]. Those surface proteins are important in cell–cell adhesion [21]. A downregulation of this gene cluster could inhibit the cell–cell- and cell–surface adhesion and could reduce proliferation rate during iodide treatment.

In addition, downregulation of other genes involved in cell adhesion (laminin beta 3, Lamb3), cell metabolism (thyroid hormone responsive protein, Thrsp) and oxidative stress response (peroxidasin, Pxdn) underscore that iodide hampers cell viability in FRTL-5 cells.

Interestingly, more genes were found to be differential regulated by iodide in WT TSHR FRTL-5 cells compared to L629F mTSHR FRTL-5 cells. This could be due to the endogenous constitutive activity of the autonomous thyroid cells driving function and cell growth. However, of the distinctly regulated mRNAs between L629F TSHR and WT TSHR FRTL-5 cells three genes are worth highlighting due to their known relevance to thyroid physiology: i) iodotyrosine deiodinase (Iyd), ii) jun B proto-oncogene (Junb), and iii) the inhibitor of growth family, member 3 (Ing3). [14].

i) The iodotyrosine deiodinase (Iyd) also known as DEHAL1, facilitates iodide salvage in the thyroid cell thyroid by catalyzing the NADPH-dependent deiodination of mono (L-MIT) and diiodotyrosine (L-DIT) [12]. Inactivating mutations in the *iyd* gene have been identified in rare cases of thyroid dyshormonogenesis [1,18]. Downregulation of Iyd suggests reduced iodide salvage at the level of thyroid hormone synthesis (in line with iodide-induced downregulation of thyroid hormone synthesis) but hypothetically could also present an advantageous escape mechanism for the autonomous cells from "inhibitory" iodide excess.

Junb mRNA is increased by e.g. TSH and insulin and is besides c-fos a necessary mediator and coordinator of growth response in thyroid epithelial cells [6]. The tumor suppressor ING3 acts as a cofactor of p53 and regulates cell cycle progression, apoptosis, and DNA repair [7]. Thus downregulation of Junb and Ing3 is compatible with a more pronounced inhibition of cell growth in autonomous L629F FRTL-5 cells compared to WT TSHR cells.

In summary, we show that distinct gene regulation occurs between normal and autonomous thyroid cells in response to iodide and affects genes involved in e.g. cell cycle, proliferation and metabolic processes. Importantly, despite a constitutive TSHR activation, iodide still causes downregulation of proliferation and function in early stage autonomy. Future studies need to address this issue in a scenario closer to the *in vivo* situation. Either by making use of primary cultures from toxic thyroid nodules or an animal model of thyroid autonomy. This may open novel perspective for the prevention of clinically relevant thyroid autonomy through iodide treatment.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygeno.2010.10.007.

Table 1
GeneChip data analysis using the DAVID2008 platform. Shown are the gene counts of the first annotation cluster with the highest enrichment score. Detailed information on differential gene expression in WT TSHR and L629F mTSHR FRTL-5 cells and influence of iodide on gene expression are available as a supplemental info (online Acc number GSE22118).

Gene group	Gene counts	P value
Cadherin, N-terminal	13	2,60E-23
Cadherin	13	4,20E-17
Cadherin repeats	13	3,50E-16
Homophillic cell adhesion	14	2,90E-15
Cell adhesion	15	2,90E-13
Cell–cell adhesion	15	9,70E-13
Biological adhesion	18	6,80E-11
Cell adhesion	18	6,80E-11
Calcium ion binding	17	2,20E-08
Calcium	15	4,70E-08
Cation binding	24	8,50E-05
Metal ion binding	24	1,90E-04
Ion binding	24	2,80E-04
Receptor	14	6,10E-03
Transmembrane	20	3,60E-02
Membrane	23	4,50E-02

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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References

[1] G. Afink, W. Kulik, H. Overmars, J. de Randamie, T. Veenboer, A. van Cruchten, M. Craen, C. Ris-Stalpers, Molecular characterization of iodotyrosine dehalogenase

deficiency in patients with hypothyroidism, J. Clin. Endocrinol. Metab. 93 (2008) 4894–4901.
[2] F. Aghini-Lombardi, L. Antonangeli, E. Martino, P. Vitti, D. Maccherini, F. Leoli, T. Rago, L. Grasso, R. Valeriano, A. Balestrieri, A. Pinchera, The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano survey, J. Clin. Endocrinol. Metab. 84 (1999) 561–566.
[3] F. Al Khafaji, M. Wiltshire, D. Fuhrer, G. Mazziotti, M.D. Lewis, P.J. Smith, M. Ludgate, Biological activity of activating thyrotrophin receptor mutants: modulation by iodide, J. Mol. Endocrinol. 34 (2005) 209–220.
[4] K. Bauch, Epidemiology of functional autonomy, Exp. Clin. Endocrinol. Diabetes 106 (Suppl 4) (1998) S16–S22.
[5] P.C. Clemens, R.S. Neumann, The Wolff–Chaikoff effect: hypothyroidism due to iodine application, Arch. Dermatol. 125 (1989) 705.
[6] S. Deleu, I. Pirson, F. Clermont, T. Nakamura, J.E. Dumont, C. Maenhaut, Immediate early gene expression in dog thyrocytes in response to growth, proliferation, and differentiation stimuli, J. Cell. Physiol. 181 (1999) 342–354.
[7] Y. Doyon, C. Cayrou, M. Ullah, A.J. Landry, V. Cote, W. Selleck, W.S. Lane, S. Tan, X.J. Yang, J. Cote, ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation, Mol. Cell 21 (2006) 51–64.
[8] P.H. Eng, G.R. Cardona, S.L. Fang, M. Previti, S. Alex, N. Carrasco, W.W. Chin, L.E. Braverman, Escape from the acute Wolff–Chaikoff effect is associated with a decrease in thyroid sodium/iodide symporter messenger ribonucleic acid and protein, Endocrinology 140 (1999) 3404–3410.
[9] M. Eszlinger, K. Krohn, K. Berger, J. Lauter, S. Kropf, M. Beck, D. Fuhrer, R. Paschke, Gene expression analysis reveals evidence for increased expression of cell cycle-associated genes and Gq-protein–protein kinase C signaling in cold thyroid nodules, J. Clin. Endocrinol. Metab. 90 (2005) 1163–1170.
[10] D. Fuhrer, M. Eszlinger, S. Karger, K. Krause, C. Engelhardt, D. Hasenclever, H. Dralle, R. Paschke, Evaluation of insulin-like growth factor II, cyclooxygenase-2, ets-1 and thyroid-specific thyroglobulin mRNA expression in benign and malignant thyroid tumours, Eur. J. Endocrinol. 152 (2005) 785–790.
[11] D. Fuhrer, M.D. Lewis, F. Alkhafaji, K. Starkey, R. Paschke, D. Wynford-Thomas, M. Eggo, M. Ludgate, Biological activity of activating thyroid-stimulating hormone receptor mutants depends on the cellular context, Endocrinology 144 (2003) 4018–4030.
[12] S. Gnidehou, B. Caillou, M. Talbot, R. Ohayon, J. Kaniewski, M.S. Noel-Hudson, S. Morand, D. Agnangji, A. Sezan, F. Courtin, A. Virion, C. Dupuy, Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site, FASEB J. 18 (2004) 1574–1576.
[13] H.I. Gozu, R. Bircan, K. Krohn, S. Muller, S. Vural, C. Gezen, H. Sargin, D. Yavuzer, M. Sargin, B. Cirakoglu, R. Paschke, Similar prevalence of somatic TSH receptor and Gsalpha mutations in toxic thyroid nodules in geographical regions with different iodine supply in Turkey, Eur. J. Endocrinol. 155 (2006) 535–545.
[14] M. Gunduz, M. Ouchida, K. Fukushima, S. Ito, Y. Jitsumori, T. Nakashima, N. Nagai, K. Nishizaki, K. Shimizu, Allelic loss and reduced expression of the ING3, a candidate tumor suppressor gene at 7q31, in human head and neck cancers, Oncogene 21 (2002) 4462–4470.
[15] P. Laurberg, K.M. Pedersen, H. Vestergaard, G. Sigurdsson, High incidence of multinodular toxic goitre in the elderly population in a low iodine intake area vs. high incidence of Graves' disease in the young in a high iodine intake area: comparative surveys of thyrotoxicosis epidemiology in East-Jutland Denmark and Iceland, J. Intern. Med. 229 (1991) 415–420.
[16] S.G. Leoni, P.A. Galante, J.C. Ricarte-Filho, E.T. Kimura, Differential gene expression analysis of iodide-treated rat thyroid follicular cell line PCCl3, Genomics 91 (2008) 356–366.
[17] J. Maier, H. van Steeg, C. van Oostrom, R. Paschke, R.E. Weiss, K. Krohn, Iodine deficiency activates antioxidant genes and causes DNA damage in the thyroid gland of rats and mice, Biochim. Biophys. Acta 1773 (2007) 990–999.
[18] J.C. Moreno, W. Klootwijk, H. van Toor, G. Pinto, M. D'Alessandro, A. Leger, D. Goudie, M. Polak, A. Gruters, T.J. Visser, Mutations in the iodotyrosine deiodinase gene and hypothyroidism, N Engl J. Med. 358 (2008) 1811–1818.
[19] E. Nishihara, N. Amino, K. Maekawa, H. Yoshida, M. Ito, S. Kubota, S. Fukata, A. Miyauchi, Prevalence of TSH receptor and Gsalpha mutations in 45 autonomously functioning thyroid nodules in Japan, Endocr. J. 56 (2009) 791–798.
[20] R. Paschke, M. Ludgate, The thyrotropin receptor in thyroid diseases, N Engl J. Med. 337 (1997) 1675–1681.
[21] L. Shapiro, A.M. Fannon, P.D. Kwong, A. Thompson, M.S. Lehmann, G. Grubel, J.F. Legrand, J. Als-Nielsen, D.R. Colman, W.A. Hendrickson, Structural basis of cell–cell adhesion by cadherins, Nature 374 (1995) 327–337.
[22] P. Smerdely, V. Pitsiavas, S.C. Boyages, Evidence that the inhibitory effects of iodide on thyroid cell proliferation are due to arrest of the cell cycle at G0G1 and G2M phases, Endocrinology 133 (1993) 2881–2888.
[23] P. Smerdely, V. Pitsiavas, S.C. Boyages, The G2M arrest caused by iodide is unrelated to the effects of iodide at adenylate cyclase, Thyroid 5 (1995) 325–330.
[24] G. Vassart, J.E. Dumont, The thyrotropin receptor and the regulation of thyrocyte function and growth, Endocr. Rev. 13 (1992) 596–611.
[25] M. Vitale, T. Di Matola, F. D'Ascoli, S. Salzano, F. Bogazzi, G. Fenzi, E. Martino, G. Rossi, Iodide excess induces apoptosis in thyroid cells through a p53-independent mechanism involving oxidative stress, Endocrinology 141 (2000) 598–605.
[26] Q. Wu, T. Maniatis, A striking organization of a large family of human neural cadherin-like cell adhesion genes, Cell 97 (1999) 779–790.