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Humanized medium (h7H) allows long-term primary follicular thyroid cultures from human normal thyroid, benign neoplasm and cancer

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Context: Mechanisms of thyroid physiology and cancer are principally studied in follicular cell lines. However, human thyroid-cancer lines were found to be heavily contaminated by other sources and only one supposedly normal-thyroid cell line, immortalized with SV40 antigen, is available. In primary culture, human follicular cultures lose their phenotype after passage. We hypothesized that loss of thyroid phenotype could be related to culture conditions in which human cells are grown in medium optimized for rodent culture (5H), including hormones with marked differences in affinity for the relevant rodent/human receptor.

Objective: To define conditions which allows proliferation of primary human follicular thyrocytes for many passages without losing phenotype.

Methods: Concentrations of hormones, transferrin, iodine, oligoelements, antioxidants, metabolites and ethanol were adjusted within normal homeostatic human serum ranges. Single cultures were identified by STRs. Human-rodent inter-species contamination was assessed.

Results: We defined an 'h7H medium' enabling growth of human thyroid cultures for more than twenty passages maintaining thyrocyte phenotype. Thyrocytes proliferated and grouped as follicle-like structures (FLS); expressed Na+/I- symporter, pendrin, cytokeratins, thyroglobulin and thyroperoxidase, showed iodine-uptake and secreted thyroglobulin and FT3. Using these conditions, we generated a Bank of Thyroid Tumors in Culture (BANTTIC) from normal thyroids, Grave's hyperplasias, benign neoplasms (goiter, adenomas) and carcinomas.

Conclusions: Using appropriate culture conditions is essential for phenotype maintenance in human thyrocytes. The BANTTIC generated under humanized h7H culture conditions will provide a much needed tool to compare similarly growing cells from normal versus pathological origins, and thus to elucidate molecular basis of thyroid disease.

In vitro study of the mechanisms of thyroid disease is very relevant since is a most frequently diagnosed disease in medical general practice. Congenital hypothyroidism appears in around 1:2000 newborns with only a handful of genes implicated. Thyroid autoimmune disease presents a high worldwide incidence. Moreover, thyroid cancer is the only cancer whose incidence has increased steadily in the

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Abbreviations: FLS: follicle-like structures; TSH: Thyroid Stimulating Hormone; DTC: Differentiated Thyroid Carcinoma; bINS: bovine insulin; r-hINS: recombinant human insulin; STRs: Short tandem repeats; FT3: free T3; FT4: free T4.; BANTTIC: Bank of thyroid tumors

last ten years, and currently has the sixth highest incidence of all cancers in women (1–5).

Thyroid hormones synthesis and secretion is a complex process requiring a series of minute steps under the control of the pituitary hormone Thyroid-Stimulating Hormone (TSH). To study the cell biology and physiological regulation of this process in humans, thyroid primary cultures and cell lines are employed. Thyroid cell lines have been obtained either from culturing dispersed cancers in a minimal medium (DMEM plus 10% FBS) (6). Also by introducing the SV40 oncogene in a primary culture of normal follicular thyrocytes in medium plus 10% FBS and 100 mIU/L TSH, a stable line -Nthy-Ori- was obtained but partially lost the thyroid phenotype (7). In 2008 a key report demonstrated cross-contamination of many human thyroid-cancer cell lines with other nonthyroid cancer lines (8). Moreover, for the remaining cancer lines of demonstrated thyroid origin there was a patent lack of thyroid phenotype both in lines coming from undifferentiated/anaplastic thyroid carcinomas (UTC), but also in those lines coming from differentiated thyroid carcinomas (DTC). This is not coincident with the patients' tumor biology since, contrary to UTC that lose differentiation, both types of DTC follicular (FTC) and papillary (PTC) maintain a substantial degree of differentiation.

Some years ago our group established a procedure to generate human primary thyroid cultures in a standardized fashion (9–13). We used a defined cell culture medium, 5H, successfully used in the 80s to generate a rat thyroid cell line that partially maintained the thyroid phenotype (FRTL-5) (14). 5H contains bovine TSH (bTSH), bovine Insulin (bINS), Cortisol, human Transferrin, and Somatostatin. In these conditions we were able to pass many of our primary cultures through a number of passages. But although expression of TTF1 and PAX8 was maintained overtime it was low and expression of thyroid proteins was minimal (NIS, PDS, TG) or undetectable (TPO).

We realized that hormone concentrations present in the 5H medium were empirically designed to culture rodent cells including hormones of heterologous animal origins used in high concentrations. For example, bTSH in 5H was used as 10 IU/L since the affinity of bTSH for rat TSH Receptor (TSHR) is low. However bTSH has a similar or even higher potency to human TSH (hTSH) on stimulating the hTSHR (15), and the normal serum range for hTSH in adults is 0.3–5 mIU/L, 2000 times lower than what was present in 5H. The same happened with bINS at $>$ 12 IU/L in the 5H medium. While bINS have a low affinity for rodent INS receptor (rINSR), it has a similar affinity for human INSR (hINSR) (16, 17), and normal insulin values in human serum are 8–11miliIU/L, 1000 times lower than those in 5H medium. The opposite happened with Cortisol at 10 nM in 5H. Physiologically human adrenal glands produce Cortisol (Hydroxy-corticosterone) while rodent adrenals produce Corticosterone. Although both are glucocorticoids, Corticosterone has 10 times less potency (18).

Importantly hormone receptors are easily downregulated in a negative feedback by an excess of hormone. Thus, results obtained in human cells cultured in these conditions could well be providing erroneous information for human biology.

Materials and Methods

A detailed version is added as Supplementary material.

Humanized seven homeostatic additives (h7h) medium

Concentrations of the components of the medium were based in the reference values for human serum of the Mayo Clinic (http://www.mayomedicallaboratories.com/test-catalog/) (Table S1).

Human thyrocyte primary culture

BANTTIC follows strictly legislated procedures of personal and biological data protection (LO 15/1999) and has been approved by the State Ethical Committee of Galicia, Spain (CEIC). We followed approved datasheets for informed consent and donation. Legislation applies to the experimental use of primary cells within our approved institution or in scientific collaborations. In parallel, this project was approved by the Bioethics Committee of USC.

A flow chart of the procedure to obtain the human thyrocyte primary cultures is summarized in Table S2. We follow our standardized protocol for thyroid primary culture from excedent fragments of thyroid surgical pieces (9–11, 13). During the following weeks cells were allowed to grow until dishes were fully covered and numerous FLSs were observed. We consider that this type of cultures never reach confluence since they grow in these threedimensional structures. Once the dish was full enough, cells were passaged.

Passage and transfection of the cultures

Cultures were very sensitive to trypsinization. We use a more gentle enzymatic treatment TrypLE (Gibco) and added glucose (1g/l) in every solution.

Transfection of pmaxGFP (Amaxa) and pTurboFp635/Katushka (Evrogen) was performed by Nucleofection (Amaxa) as described (13).

To demonstrate theedimensional growth 10,000 cells were resuspended in a drop of 25 mcl matrigel (ECM Gel, Sigma), seeded in a coverslip and allowed to solidify for 15 min. h7H

in culture. Cultures belonging to the bank are labeled with a T- followed by the pathology at diagnosis (example: T-MNG: culture from a multinodular goiter).

medium was added and cells allowed growing for a week. Fixation and immunofluorescence was performed similarly to the 2D cultures.

Nucleic acids extraction, Human vs. Rodent identification, STRs analysis, and Mutational profile

BRAF intron 14/exon 15 was amplified by PCR and sequenced while RET/PTC1 and RET/PTC3 was amplified by RT-PCR and run in a gel. (Table S5). For STRTs analysis the AmpFiSTR NGM Select kit (Applied Biosystems), using for the reading out Identifiler Plus Panel V1 (Applied Biosystems). The combined panel of 16 STRs has a probability of misidentification of $3.26 \times 10-21$.

Phenotypic mRNA expression was detected by TaqMan RT-PCR assays (Table S6). As described in various works, TBP is the gene presenting a more stable expression among different human tissues and cell types.

(http://www3.appliedbiosystems.com/cms/groups/mcb marketing/documents/generaldocuments/cms 042279.pdf)

Phenotypic immunodetection

The antibodies used were the same used for routine hospital diagnostic procedures (see Table S7). Immunohistochemical studies were performed using a peroxidase-conjugated labeled-dextran polymer (Dako EnVision Peroxidase/DAB; Dako, Glostrup, Denmark). Diluted H-E was used as counterstain. For immunofluorescence, appropriate secondary antibodies were applied at the end (Table S7). Nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma; 1:100). Photographs were obtained in a T-SP5 confocal microscope equipped with White Laser (Leica).

Iodide uptake

Iodide uptake was measured as described previously with slight modifications (Palos et al. 2010). Replicate wells were used to count cells. I- uptake was expressed as picomoles per 10^5 cells.

Detection of secreted Thyroglobulin and FT3

Cells were seeded in h7H medium. Four days later medium was changed to one of the three: complete h7H (10% serum), deprived h7H (the same but with only 0.5% NCS) and only TSH (TSH 40 miliIU/l plus 0.5%BSA in Coon's medium). After four days medium was collected (W1), and changed to fresh medium. After the four following days medium was again collected (W2). For hormone analysis, routine hospital assays were used in all cases (TG: Immulite 2000; FT3 and FT4: CLIA, ADVIA Centaur; all from Siemens Healthcare Diagnostics, UK). The detection limit for FT3 was 0.2 pg/ml and for FT4 was 0.1 ng/dl. Normal range for normal euthyroid subjects is 2.3–4.2 pg/ml for FT3 and 0.89–1.76 ng/dl for FT4. H7H medium with normal serum (FBS, NCS) presented detectable levels of FT3 and FT4 from the bovine components. Charcoal-dextran stripped FBS (Invitrogen) presented ten times less FT3 and three times less FT4.

Statistical Analysis

Results are expressed as media ± SEM. The experiments were repeated at least three times with three or more replicates when possible. A nonparametric T test was used for statistical analysis.

Results

We aim to improve the maintenance of the thyroid phenotype in long-term human cultures by designing new conditions based on human serum reference values. We considered thyroid phenotype as the expression of thyroid and epithelial markers and maintenance of thyroid cell physiology. As an initial experiment we compared two commercial human cell lines, 8305C, derived from ATC and thus not expected to conserve a strong thyroid phenotype, to FTC-238 derived from a lung metastasis of a follicular-type DTC and thus expected to express detectable NIS levels. These cell lines had been thawed and cultured in 5H for 10 passages (Figure 1A, Week 0). During the following weeks medium was changed towards humanized values (Weeks 1–3) by reducing successively TSH and Insulin while increasing Cortisol (see below and Table S1). mRNA expression of some thyroid phenotype genes was studied with TaqMan assays. We could detect an important increase in NIS, TSHR and TTF1 in FTC-238 but not in 8305C (Figure 1B).

Next, we refined humanized conditions generating a bank of primary cultures (Bank of Thyroid Tumors in Culture, BANTTIC). BANTTIC included normal thyroid (NT) and proliferative diseases either benign lesions (Graves' disease [GD], multinodular goiter [MNG], follicular adenomas [FA]), as well as carcinomas (PTC, FTC). It included many parallel samples to compare normal cells and neoplasia from the same patient. The culture medium was called h7H Medium in relation to the seven groups of additives which we have controlled: hormones, transferrin, iodine, oligoelements, antioxidants, and metabolites. In relation to hormones, we included, as in 5H, TSH, Insulin (INS), Cortisol and Somatostatin although within ranges found in human serum (Table S1). To allow some consumption by the cells in order to promote cell growth values of TSH were maintained in generous "hypothyroid" ranges (40 miliIU/l, normal upper range 6miliIU/l), although similar values are physiological in neonates. Since INS promotes growth but also induces differentiation, we tested a range of concentrations both with bINS and with recombinant hINS (r-hINS), finally using 25 miliIU/l, again allowing some consumption by the cells (Figure S1A). We maintained Somatostatin in the higher serum range (50 ng/L) since the half life of peptide hormones is low. A range of Cortisol concentrations was tested. We assumed that most Cortisol in the medium would be free Cortisol although bovine serum albumin can sequester a small fraction. High concentrations inhibited growth, and the best concentration was the middle range of early morning adult values (25 nM).

Human follicular thyroid cells express Growth Hor-

A

Week	bTSH	bINS	CORTISOL	SOMATOSTATIN	hTRANSFERRIN	IODIDE
0 (5H)	10 IU/L	>12 IU/L	10 nM (3.6µg/L)	10µg/L	5mg/L	-
1	40mIU/L	100mIU/L	300nM (120µg/L)	50ng/L	5mg/L	-
2	40mIU/L	100mIU/L	25nM (9µg/L)	50ng/L	5mg/L	-
3	40mIU/L	50mIU/L	25nM (9µg/L)	50ng/L	5mg/L	10µg/L

B

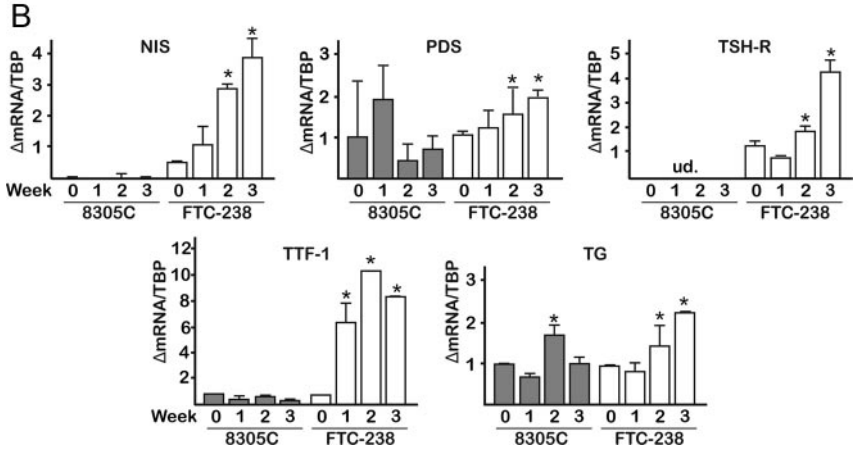

Figure 1. Adapting hormone concentrations from a rodent defined medium (5H) to humanized conditions by comparing a human follicular carcinoma cell-line (FTC-238) with an anaplastic thyroid carcinoma cell-line (8305C). A) Starting at week 0 with the well known 5H medium, 5% NCS plus shown hormones, every week a component was adjusted towards normal human serum values. At the end of the week RNA replicates were collected and the rest of the cells were passaged. The following day after seeding another hormone was changed. B) TaqMan RNA expression showing how NIS, TSHR or TTF1 were progressively increased in the follicular cells when hormone concentrations reach normal range values. (*$P < .05$).

mone (GH) receptors (19). Serum concentrations of GH are difficult to normalize since is secreted by the pituitary in peaks every few hours with a different range in males and females. We added a small but physiological dose of recombinant hGH that potentiated growth when r-hINS was used although not in the presence of bINS (Figure S1B). However we decided to maintain GH in the h7H mix assuming other actions on survival or maintenance of thyroid phenotype.

Iodine and some oligoelements are essential for thyroid function. Zinc and iron were already present in the liquid medium at serum reference concentrations. We added iodine and selenium. Iodine concentration was further adjusted through NIS mRNA expression (see Figure S3). Although hTransferrin was below the range found in serum it was virtually iron free in comparison with human serum, which presents around 40% iron saturation.

Antioxidants are essential to grow stem cells and iPS reducing radicals generated by the high oxygen percentage in the air (20). We tested the similar combination of antioxidants (vitamin C, tocopherols, glutathione and pyruvate) initially at similar concentrations. However, the combined antioxidants in those concentrations stop irreversibly the growth of the cultures in around two weeks. When diluted ten times the cultures grew well, and thus were maintained. We kept glucose levels at postfeeding physiological values (1.8 g/L) allowing again consumption during the following four days.

Finally, we improved the washing and trypsinization of the cells by including glucose (1 g/L) in every solution.

The new conditions were applied to all new cultures that arrived to the BANTTIC during the last two years and to some of the previous cultures (see Table S2 and S3). To distinguish the original tissue from the cultures we have added a T- to the name of each culture followed by the above abbreviation for each group, and a number to identify successive cultures within the same group (Table S3). Since cross-contamination is always a risk in this type of banks every culture was identified by STRs and rodent contamination excluded using a designed system based in a combination of two specific TaqMan assays (Table S6).

Cultures growing in h7H medium were able slowly but steadily to grow throughout passages. Moreover, above the basal layer of cells appeared cell clusters, appearing slightly threedimensional, reminding of follicles (Figure 2A and S2). We studied the expression of the thyroid -TTF1, NIS, PDS, TG and TPO- and epithelial phenotype (Cytokeratin 7, CK7, characteristic of thyroid follicular cells) in protein extracts of the cultures in comparison with normal thyroid tissue (Figure 2B-D). NIS was more easily detected in the cultures than in the normal thyroid tissue since the tissue extract was full of colloidal extracellular thyroglobulin. NIS and PDS presented a nonglycosilated lower band and a mature fully-glycosilated upper band in correlation with the bands detected in tissue. Cultures from papillary carcinomas maintained the expression of all these markers except TPO that was reduced. Metastatic cultures presented a marked reduction of NIS, PDS and TPO.

In the original description of the 5H medium for the rat FRTL-5 cell line, a low percentage of serum (0.5% NCS) was used in combination with hormones to maintain the thyroid phenotype while allowing growth (14). In the other hand, human primary cultures with only TSH as additive has been described although did not maintain the thyroid phenotype at long term (7). We also explored the importance of the mix of hormones in relation to the percentage of serum. In replicates of the same culture T-NT2

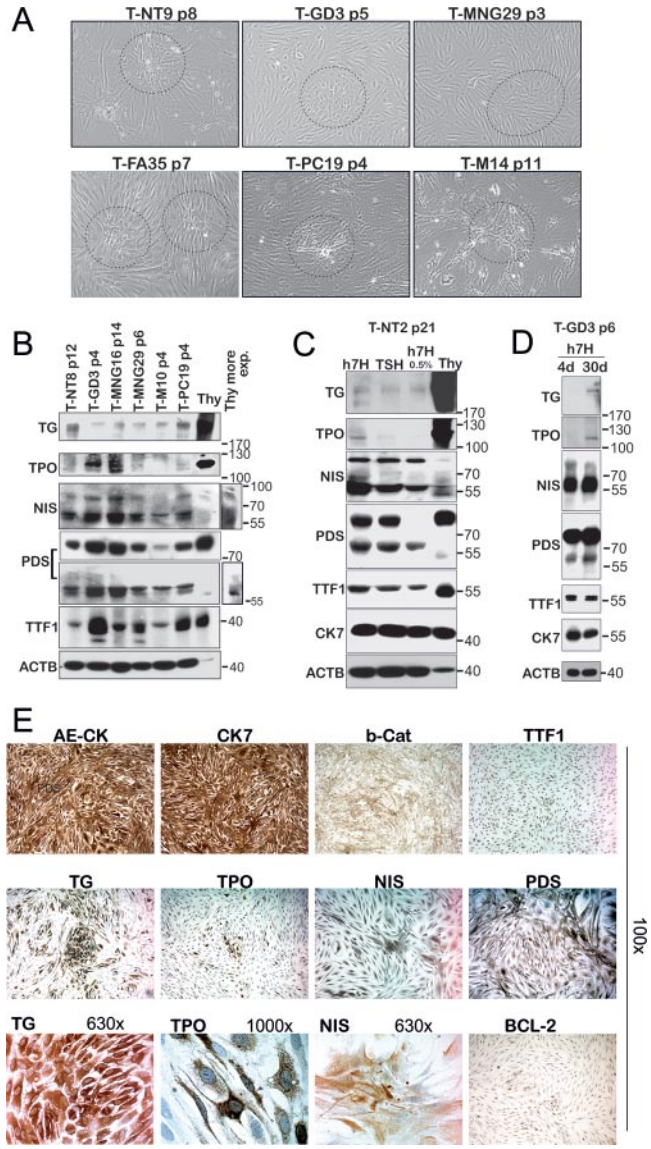

Figure 2. Medium h7H allows expansion of primary thyroid cultures, which cells cluster in follicle-like structures (FLS) and maintain thyroid phenotype. A) Pictures representing one of each of the six groups of thyroid cultures (normal thyroid T-NT, Grave's hyperplasia T-GD, multinodular goiter T-MNG, follicular adenomas T-FA, carcinomas T-PC, and metastasis T-M). Circles label groups of cells clustered and arranged in 3D (FLS). **B)** Western blot analysis of thyroid proteins from various cultures. As a reference an extract from normal thyroid tissue was included (Thy). NIS and PDS presents a double lower band (65 and 68 kDa respectively) corresponding to nonand partially- glycosilated intracellular protein and an upper band (90 kDa and 95 kDa respectively) corresponding to the fully-glycosilated plasma membrane transporters. **C)** Intensity of thyroid phenotype requires the presence of the full h7H medium. Cells were dispersed and seeded. The next day medium was kept as complete h7H (10% serum), or changed to serodeprived h7H medium (0.5% serum) or only TSH (0.5% BSA) for one week. Expression of TG, TPO, mature NIS and PDS, and TTF1 is more intense in complete h7H medium. However, thyroid phenotype is much related to the number of days in the dish after seeding in h7H as can be seen when compared extracts after 4 d with extracts after 30 d (FLS enrichment) **(D)**. **E)** Histochemical detection of pan-cytokeratins (AECK), cytokeratin 7, b-Catenin (b-Cat), TTF1, TG, TPO, NIS, PDS, and BCL2. Some proteins (cytokeratins, bCat, TTF1, NIS and PDS) were widely distributed while TG or TPO accumulate in the FLS. Magnification of an FLS shows cytoplasmic accumulation of TG and membrane location for TPO and NIS.

grown during ten days, we compared the full h7H medium including hormones and serum (5% FBS and 5% NCS), the h7H including hormones but only 0.5%NCS and a medium with only TSH instead of the whole hormone combination (Figure 2C). Reduction of serum in the medium with h7H resulted in reduction or loss of the mature iodine transporters and no TPO detection. Moreover, although TSH by itself was enough to maintain the expression of most the thyroid phenotype also failed to maintain TPO expression. This result indicated that complete h7H medium was required to maintain the thyroid phenotype in parallel to growth. However, there was another important factor that influenced the full expression of the thyroid phenotype, the number of days during the passage. In figure 2D it is shown how T-GD3 cells cultured for four days abundantly expressed NIS, PDS, TTF1 or CK7. However, only after many days in culture appeared the expression of TPO and TG. We related this result with the formation of the cell clusters since they increased with the number of days in culture.

To further assess the epithelial and thyroid phenotype we performed IH (Figure 2E and Table S4). Some proteins such as cytokeratins, β-catenin, TTF1, NIS and PDS were localized in the cells all over the dish. However, TG and TPO were easier to localize in the cell clusters, that we called Follicle-like Structures (FLS) (compare Figure 2A and E). FLS were maintained throughout passages. They were not present at the beginning immediately after dispersion, where few cells were present in the dish, appearing after the cells covered the dish (Figure S2A-B). Groups of cells that resembled FLS appear in established DTC cell lines cultured in h7H medium, such as BCPAP (papillary DTC) and FTC238 (follicular DTC), but not in cell lines from anaplastic carcinomas (shown Cal62, Figure S2C).

Colocalization of thyroid proteins in FLS was studied by immunofluorescence and confocal microscopy in cultures of different origins. Intense nuclear TTF1 was detected in more than 95% of the cells. CK7 was cytoplasmic and similar in intensity to pan-cytokeratin detection (AECK) (Figure 3A). β-catenin was mainly localized at the submembrane border and its pattern was suggestive of the presence of cell junctions. As we had seen in the immunohistochemistry (Figure 2E), both TG and TPO concentrated in FLSs. TG, more broadly distributed, colocalized with TPO at the center of the FLS (Figure 3B). As seen by immunohistochemistry, NIS was localized at plasma membranes all over the dish (Figure 1C and data not shown). At the FLSs NIS colocalized with TG but reached the plasma membrane (Figure 3C). Some cells were full of TG while others were less intense as if TG was being actively secreted. PDS was also broadly distributed. In cells outside FLS, PDS presented intracellular localization.

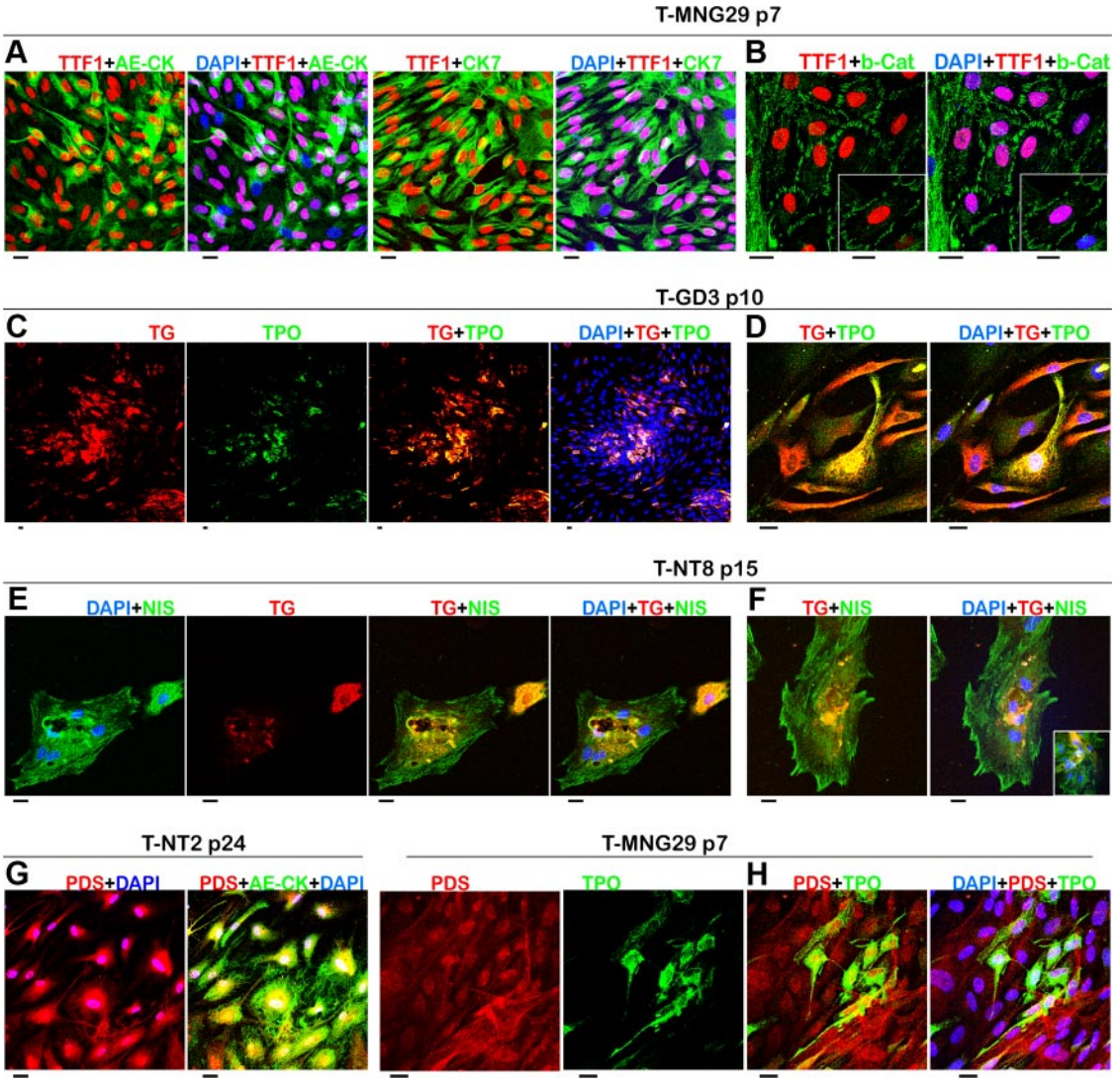

Figure 3. Colocalization of thyroid markers. A) TTF1 is present in all cytokeratin positive cells, either stained with the pan-cytokeratin antibody (AECK) or with the specific for Cytokeratin 7. **B)** While TTF1 is nuclear, b-Catenin is present at submembrane localization with a spiky pattern characteristic of cell-to-cell junctions. **C)** Shown are two FLS clusters where TG is intensively detected in many cells at small magnification. FLS coexpressed TPO at the center of the cluster. **D)** Colocalization of TG and TPO. **E)** TG and NIS were colocalized in the same cells but NIS presents an halo at the plasma membrane. In cell groups (**F**), some cells are full of TG (cartoon and arrow) while others are less intense. **G)** PDS colocalizes with cytokeratins, but only few cells in the FLS center coexpress TPO (**H**). (Black bar adjusted at 10 microns. Nuclei counterstained with DAPI.)

However, in cells within FLS PDS was concentrated with TPO at the plasma membrane (Figure 3D).

To summarize this part, primary cultures from normal and benign proliferative diseases maintained in h7H medium grow and preserve expression of the thyroid phenotype throughout passages both at the RNA and protein level (Figure S3 and Table S4). Functional regulation of thyroid genes was maintained. We tested the physiological regulation of phenotype by addition of increasing concentrations of iodine (10 or 100 microg/l) in a culture in passage 10 (T-GD3). As expected, excess iodine reduced NIS and PDS mRNA expression. TG and TPO expression were dramatically related to the length of the culture after plating the cells, and thus with the density of FLS (Table S4). Although still epithelial, the thyroid phenotype was less intense in cultures from papillary carcinomas or its lymph node metastasis. This correlated with the expression found in the original tissues (Figure S3A bottom).

Commercial cell lines from differentiated thyroid carcinomas thaw and cultured in h7H for more than 10 passages presented low but detectable levels of some of these proteins. Interestingly, FTC238 (follicular DTC) constantly expressed five-times more NIS than BCPAP (papillary DTC) or anaplastic cell lines (8305C, MB1, Cal62, BHT101) (Figure S4B).

We compared the h7H medium with two of the described mediums for thyroid cells: 5H and the minimal medium DMEM+10% FBS. We selected two primary cul-

tures, T-NT2 and T-FA31 and the cell line FTC-238. h7H significantly increased the growth of the primary cultures in comparison with the other media (Figure 4A). However, FTC-238 did not present differences in cell number with any of the media. But, again, when NIS expression was studied cells grown in h7H presented a significant difference in expression (Figure 4B).

FLS appeared threedimensional at the phase contrast microscope and we asked if they might be partially hollow reproducing the colloidal space of the real follicles in the thyroid. To assess if threedimensional FLS were generated de novo cells were independently transfected with GFP or Katushka (Fp635), and mixed after transfection, allowing them to grow for ten days until FLS were formed (Figure 5A and S4). Clones of green and red cells intermingled with nonfluorescent cells could be seen. Sometimes both fluorescences coincided in a yellow blended color indicating two layers of cells. Alternatively, fluorescent cell extensions could be detected mixed with other nonfluorescent cells, and elongating far away from the original cell. This again suggested a kind of dimensional organization very short in the z axis due to lack of support like the extracellular matrix. To allow this we grew the cells in matrigel and waited until FLS were formed. Using confocal reconstruction we observed threedimensional FLS that were hollow inside (Figure 5B-D, Videos 1, 2 and 3). Some of these FLS, contained TG in the inside recalling the colloid in the thyroid follicles.

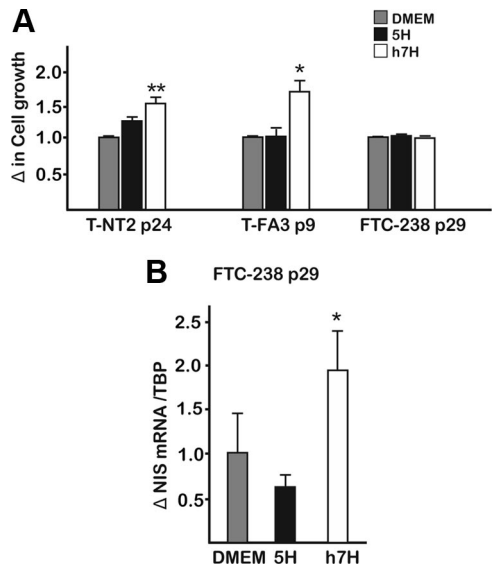

Figure 4. h7H medium improves growth and expression of thyroid phenotype. A) Two primary cultures (T-NT1 and T-FA31) and the cell line FTC-238 were seeded in the usual medium for cell lines DMEM+10%FBS (DMEM), the well known medium 5H with hormones and 5%NCS, or the h7H medium (with all additives and 5% NCS and 5% FBS). Cell numbers are expressed in relation to DMEM. Although in FTC-238 there was no difference in growth, replicate wells were analyzed for NIS expression that were significantly increased in h7H after one week (**B**) (*$P < .05$; ** $P < .01$).

When observed under the phase-contrast microscope a variety of extracellular materials could be seen over the FLS (Figure 5E): brown precipitates, a dense globular material or even fiber-like structures. These last were sometimes very long (see Video 4). We could stain many of these materials with H&E. We do not believe these materials are contaminants because although they appear at random, they have three different textures and present at different sizes but always on top of FLSs, and they were found in all culture types. However, the true nature(s) of these materials has not been identified.

Next we checked the functionally of our cultures. We measured iodine uptake since NIS expression was detected in the membrane of every cell and maintained throughout mitosis (Figure S4A). Cultures showed iodide uptake in the presence of h7H. Uptake was much increased with overnight incubation with only TSH and iodide deprivation. Uptake was blocked by competition with perchlorate (Figure 6A).

In the thyroid gland TG is secreted and iodized inside the colloid. TG is a heavily glycosilated long protein that we had detected inside the cells and in protein extracts (see Figures 2B-E, 3C-E, 5B-D). We measured secretion of TG into the medium (Figure 6B). TG was markedly secreted in cells cultured in h7H, in a process dependent on the number of FLS since increased with the number of days in culture. As for iodide uptake, TG secretion was highly stimulated by overnight incubation with only TSH.

Intracellular uptake and processing of iodized TG gives rise to thyroid hormones (T3, T4). Iodinated compounds in solution have brown color and we had seen a brown precipitate outside the FLSs (Figure 5E). Detection of thyroid hormones was technically problematic due to its presence in the culture medium per se, either in the bovine serum or the BSA (added when the TSH was the only hormone). In spite of this, Free T3 (FT3) was secreted actively by the cultures and, again, secretion was higher in the second week (Figure 6C). The fact that FT3 concentrations increased in the cultures in consecutive weeks suggested that the increase come from the cells in the FLSs processing their iodized TG. Interestingly, cells cultured with only TSH, a medium that had presented the higher iodide uptake and TG secretion (Figure 6 A-B), did not secrete FT3. This indicates that high values for iodine uptake and TG secretion in the presence of TSH-only are not reflecting the whole physiology, and that an important portion of the iodine/TG in cells cultured in h7H is converted into at least FT3.

Free Thyroxine (FT4) detection was markedly interfered by the medium alone and we could only detect some secretion when the medium had reduced serum (h7H with 0.5% NCS). One possible explanation was that the FT4

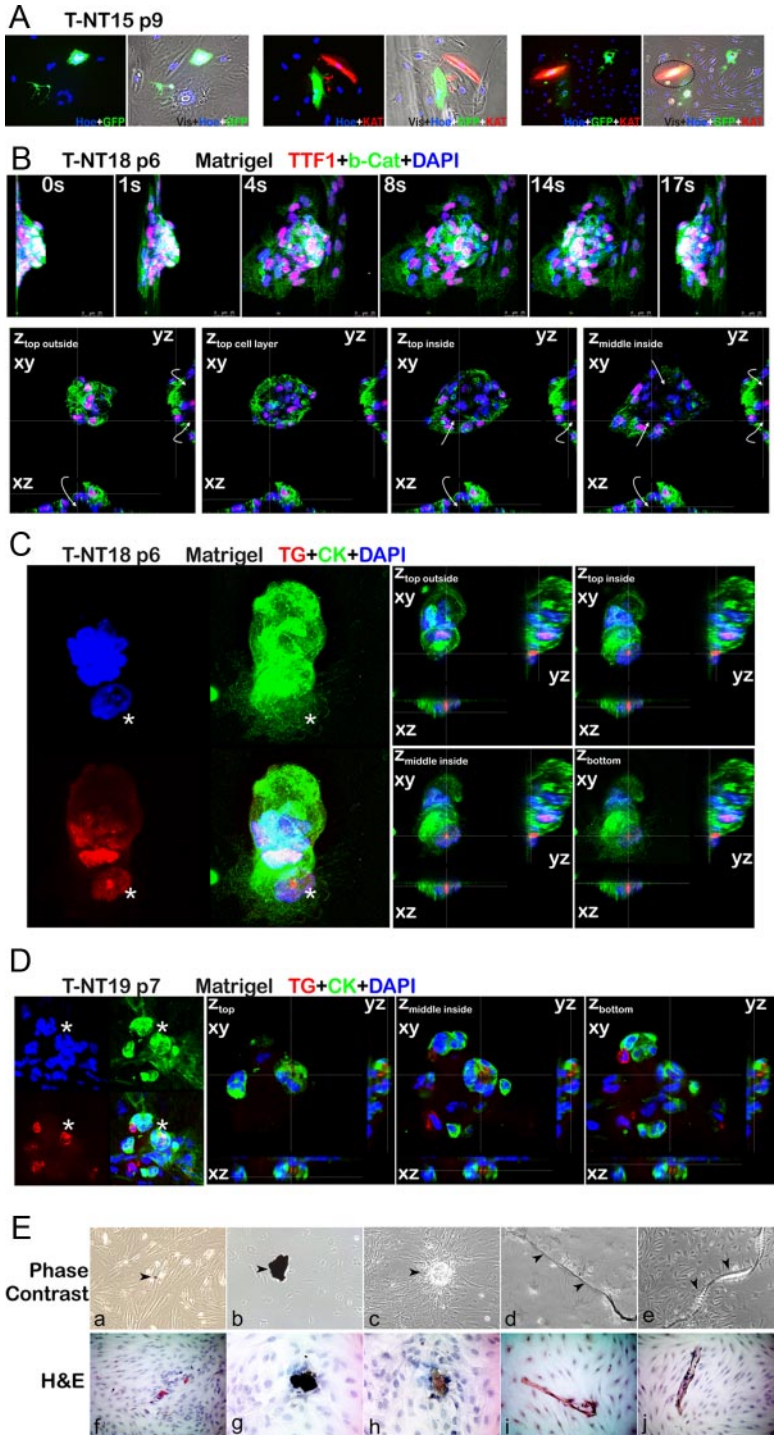

Figure 5. Thyroid cultures in h7H medium are threedimensional. A) Live microscopy of cells independently transfected with GFP or Fp635 and later combined and cultured for ten days. Nuclei are stained with Hoescht. Fluorescent cells are partially covered by untransfected cells and some FLS present combined fluorescence (yellow), indicating cell overlapping. **B-D)** T-NT18 p5 cells grown in matrigel. **B)** Threedimensinal confocal reconstruction of a FLS and stained with TTF1, b-Catenin and DAPI shown in video 1. Up: Shown are images at different time-course through the video. Down: Selected XY planes from top to bottom showing that the FLS is hollow inside (white arrows). Images include at the sides the XZ and YZ planes at the signaled crossing (hatched lines). **C)** FLS stained with TG, total CK and DAPI. Shown are the maximal projection for each channel and all together (left); and four different XY planes from top to bottom. Images include at the sides the XZ and YZ planes at the signaled crossing (hatched lines). In the FLS labeled with an asterisk TG is accumulated in the center reminding of the colloid in thyroid follicles. **D)** A similar staining can be seen in the FLS labeled with an asterisk in this picture. The progressive XY planes in Z show how inside the FLS there is TG staining inside the cells (delimited by cytokeratins in green) and TG staining in the inside hollow space. **E)** Phase-contrast pictures showing the variety of materials (arrows) of unknown nature found on the tip of FLS in the culture dish: brown precipitates (a,b), globular (c) and fibrous materials (d, e). Those materials can be stained with H&E.

assay (in ng/dl) was ten times less sensitive than the FT3 (in pg/ml). Another explanation could be that assays were standardized for normal human serum where the normal iodide concentration could reach 100 mcg/L, while in h7H medium there was only 10 mcg/L. This also could affect the ratio of T3/T4 secretion since when iodine is low there are modifications of thyroid activity leading to preferential synthesis and secretion of T3 (21). To solve these questions we repeat measurement of thyroid hormones in three different cultures (T-NT18, T-NT16, and T-NT9) using charcoal-dextran hormone stripped FBS and compared 10 and 100 mcg/L of iodide. As expected, this serum presented a very low interference with the FT3 assay so that all the cultures and conditions presented significant secretion independent of serum or iodide concentrations. FT4 in the stripped medium was also reduced but we could not detect secretion from any culture when the iodide was low. However, in the presence of 100 mcg/L of sodium iodide the three cultures presented significant secretion of FT4 indicating again that the cultures in h7H medium maintained the expected physiological regulation.

Discussion

Detailed mechanisms in cell physiology and pathophysiology are studied in cell culture. However, generalization of cell culture and simplified access to cell lines have led to some neglect in the culture techniques with the result of cell lines cross-contamination (22). Thyroid cancer cell lines have been specially affected by this contamination (8). And, unfortunately, much of the cell-culture research on thyroid cancer could be affected by this problem.

In this work we have used a care-

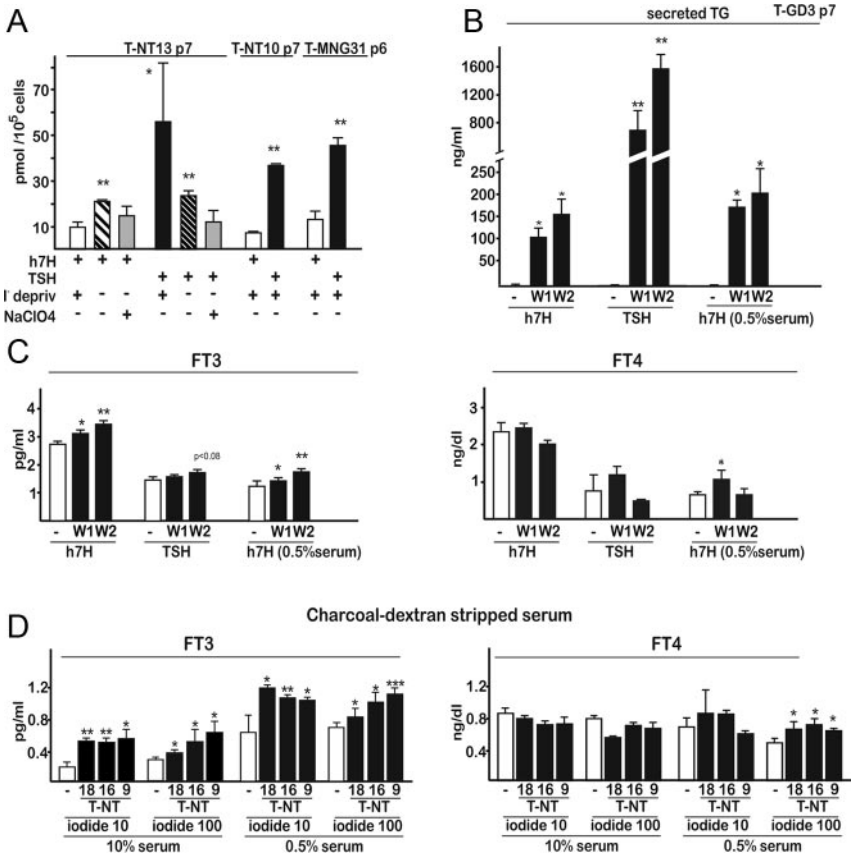

Figure 6. Thyroid cultures in h7H medium are functional. A) Cultures in h7H uptake iodine that is competed by perchlorate. Iodine uptake is enhanced by overnight iodide deprivation in the exclusive presence of TSH in comparison with the full h7H hormone mix. **B)** Secretion of human TG into the medium is high and again potentiated when only TSH is present. T-GD3 culture (passage 7) was allowed to cover the dish in h7H. After this, medium was maintained (h7H) or changed to TSH alone (0,5%BSA) or serodeprived h7H (0.5% NCS) during the following two weeks. Medium was replaced every four days and collected. Shown are values for the first 4 d in each week (W1, W2). TSH secretion increases with time. **C)** On the contrary, although Free T3 (FT3) secreted into the medium increases with time, is only significantly enhanced in the presence of h7H but not TSH alone. The assay for Free T4 (FT4) is ten times less sensitive and the hormones present in the serum interfere in excess. **D)** Assays were repeated in h7H medium with stripped serum and comparing two iodide concentrations (10 mcg NaI/L the usual in h7H with 100 mcg/L) in three independent cultures T-NT9, T-NT16 and T-NT18. Although FT3 was detected in all the conditions, FT4 was only detectable in deprived medium with 100 mcg/L. (*$P < .05$; ** $P < .01$; *** $P < .001$).

ful culture system to prevent cross-contaminations and identification of the cultures through a panel of sixteen STRs following the current recommendations of the ANSI (ANSI/ATCC ASN-0002–2011). However, STRs analysis "per se" does not detect interspecies (usually rodent) contamination (22). Thus we have added a panel of species-specific TaqMan mRNA assays to also exclude this type of contamination.

Unfortunately having perfectly identified human cells in culture does not preclude that the results obtained are going to be relevant for human biomedical research. In fact, it is worrying how many new drugs investigated in vitro with promising effects are not effective in vivo when tested in clinical trials. Recently, a call has been made to raise standards for preclinical cancer research to reduce the high drug attrition rates (23).

In relation to thyroid, our group has been using primary human thyroid cultures for some years (9–11, 13), using a well known defined medium developed to obtain normal rat thyroid cell lines (FRTL-5, (14)). However, with increasing passage number the cells lose the thyroid phenotype. By studying the possible reasons we realized that the concentrations of the medium components were designed for rodents and not humans. We have identified seven groups of additives and adjusted every one of them as much as possible to human serum concentrations. In the final concentrations it weighted the fact that many of the components where consumed by the cells during the subsequent days (up to four) until the medium was again changed. Other factor was the oxygen concentration, which was 18.65% in the incubator (95% humidity, 5%CO2) against 12–10%-5% in capillary blood. This excess oxygen needs a constant source of antioxidants and pyruvate. We had also improved the technique to passage the cells using a less damaging trypsin and maintaining constant the presence of glucose. Our gold standard was to obtain conditions to grow thyroid human cells for a relevant number of passages maintaining as much thyroid function as possible.

We have obtained a significant number of cultures able to expand for more than 15 passages, and maintaining thyroid functionality. The cultures are composed of epithelial cells expressing cytokeratins, TTF1 and PAX8. In discrete clusters, they reproduce threedimensional thin structures reminding of follicles (FLS) where cells expressing TPO, NIS, PDS and TG are concentrated. Other groups have demonstrated the feasibility to obtain rodent or porcine threedimensional thyroid cultures using matrixes (24, 25, 25, 26). However, for the sake of simplicity and focus in the conditions we had not routinely used materials to help threedimensional expansion. In spite of that, and after increasing the volume of medium per plate,

FLS are progressively formed as the cells grow. In a different set of experiments, we have used matrigel to expand the FLSs. We have demonstrated that they are hollow and can contain TG. Different materials of unknown nature seemed secreted from these FLS. Among them, TG, T3 and T4 are secreted into the medium. Importantly, either hormone and TG secretion, iodine uptake or expression of phenotypic proteins is regulated as it is in vivo. For example, overnight iodine deprivation increases iodine uptake while increasing iodine concentrations progressively decreases expression of NIS, PDS, TG, TPO and TSHR. Incubation with TSH alone, instead of complete h7H, increases TG secretion but reduces FT3 secretion. Low iodide promotes preferential synthesis of T3 while high iodide induces thyroxine secretion.

There was another possible danger of our culture system, the possibility of "overdifferentiation" of cancer cells when cultured in h7H, understood as the reacquisition of thyroid phenotypic markers that had disappeared in the cancer patient's sample. We had not the opportunity to include poorly or undifferentiated carcinomas in this study. Our data in papillary thyroid carcinoma and metastatic cultures indicates the absence of overdifferentiation as shown by the data regarding the marked reduced mRNA expression of NIS, TG, TPO genes in the carcinoma and the metastasis (Figure S3A) and the reduced TPO and NIS protein expression shown by the cultured metastasis (Figure 2B). Contrary to other carcinomas, well differentiated thyroid carcinomas maintain expression, although reduced, of the thyroid phenotype as it was shown in the immunohistochemistry performed in the surgery pieces of some tumors that we had in culture (Figure S3). Another study was made in human thyroid cancer cell lines cultured for many passages in h7H (Figure S3B). No anaplastic line presented remarkable NIS, TPO, TG, PDS or TTF1 mRNA expression. However, FTC-238 showed an intense NIS mRNA expression in correlation with what is expected in this pathology. And BCPAP presented marked PAX8 and some TTF1 and TSHR mRNA expression but barely NIS mRNA expression.

We can expect that results obtained in h7H conditions will be relevant for humans. Thus, we could compare relevant numbers of growing normal and hyperplastic thyrocytes to nodular goiter, adenoma or carcinoma thyrocytes to obtain the essential mechanisms altered in tumors. Moreover, these humanized conditions may help the search for new effective drugs for thyroid cancers and to clarify mechanisms of action of known drugs aiding in the best combinations of chemotherapies.

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