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Circadian clock characteristics are altered in human thyroid malignant nodules

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Context: Circadian clock represents the body's molecular time-keeping system. Recent findings revealed strong changes of clock gene expression in various types of human cancers.

Objective: Due to emerging evidence on the connection between circadian oscillator, cell cycle and oncogenic transformation, we aimed to characterize the circadian clockwork in human benign and malignant thyroid nodules.

Design: Clock transcript levels were assessed by quantitative RT-PCR in thyroid tissues. To provide molecular characteristics of human thyroid clockwork, primary thyrocytes established from normal or nodular thyroid tissue biopsies were subjected to *in vitro* synchronization with subsequent clock gene expression analysis by circadian bioluminescence reporter assay, and by quantitative RT-PCR.

Results: The expression levels of *Bmal1* were up-regulated in tissue samples of Follicular Thyroid Carcinoma (FTC), and in Papillary Thyroid Carcinoma (PTC), as compared to normal thyroid and benign nodules, while *Cry2* was down-regulated in FTC and PTC. Human thyrocytes derived from normal thyroid tissue exhibited high-amplitude circadian oscillations of *Bmal1-luciferase* reporter expression, and of endogenous clock transcripts. Thyrocytes established from FTC and PTC exhibited clock transcript oscillations similar to those of normal thyroid tissue and benign nodules (except for *Per2* altered in PTC), while cells derived from Poorly Differentiated Thyroid Carcinoma (PDTC) exhibited altered circadian oscillations.

Conclusions: This is the first study demonstrating a molecular makeup of human thyroid circadian clock. Characterization of the thyroid clock machinery alterations upon thyroid nodule malignant transformation contributes to understanding the connections between circadian clocks and oncogenic transformation. Moreover, it might help improving the thyroid nodule pre-operative diagnostics.

Thyroid nodules are frequent but only 5% of them are malignant (1). Thyroid malignancies of follicular cell origin include well-differentiated papillary and follicular thyroid carcinomas (PTC, FTC), which are the most common. Poorly differentiated (PDTC) and undifferentiated (anaplastic) thyroid carcinomas (ATC) (2) are less fre-

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Abbreviations: FTC Follicular Thyroid Carcinoma, PTC Papillary Thyroid Carcinoma, PDTC Poorly Differentiated Thyroid Carcinoma, PDTC-PTC Poorly Differentiated Thyroid Carcinoma developed on Papillary Thyroid Carcinoma, ATC Anaplastic (Undifferentiated) Thyroid Carcinoma, FNA Fine Needle Aspiration, TRH Thyrotropin Releasing Hormone, TSH Thyroid Stimulating Hormone, BMAL1 Brain and muscle ARNT-like protein 1, CRY Cryptochrome, PER Period, DBP D-site albumin promoter binding protein, TIMP1 Tissue Inhibitor of Metalloproteinase 1, GADD153 Growth arrest and DNA damage induced gene 153, MOI Multiplicity of infection, qPCR Quantitative PCR.

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quent. Fine-needle aspiration (FNA) biopsy is recommended for the clinical evaluation of thyroid nonsecreting nodules ≥ 1cm. FNA is currently the most accurate and safe tool in the management and classification of patients with thyroid nodules. Thyroid FNA represents a test of choice for preoperative diagnostic in PTC cases, allowing reliable recognition of this malignancy type. In cases of FTC, however, FNA does not allow clear discrimination between benign follicular lesions (nodular hyperplasia, adenomatous goiter and follicular adenoma) and a malignant follicular lesion (FTC), and thus represents only screening test. Therefore, surgery is required for all lesions diagnosed as suspicious for a follicular neoplasm, and for some of the lesions of undetermined significance (3). Postoperatively, 70%–90% of these cases are found to be benign, revealing a significant rate of unnecessary surgery, complications, and morbidity (4). Multiple ultrasonography, immunohistochemical or genetic features have been associated with malignancy, but none of them is by itself sufficiently accurate to distinguish follicular adenoma from carcinoma with high probability (5). The search for preoperative markers for thyroid malignancies stays therefore of utmost clinical importance.

Circadian oscillation of biological processes has been described in virtually all light-sensitive organisms. It reflects the existence of intrinsic clocks with near 24 h oscillation periods. The mammalian clock has a hierarchical structure, in which a master pacemaker residing in the brain's suprachiasmatic nuclei must establish phase coherence in the body by synchronizing billions of individual cellular clocks every day (6). Central and peripheral clocks have a similar molecular makeup. Moreover, this rhythm generating circuitry is functional in most cell types (7). In addition to intrinsic circadian oscillator, another fundamental attribute of a cell is its ability to divide and multiply. While the circadian clock is the body's molecular time-keeping system, the cell division clock executes a precise temporal control mechanism with multiple checkpoints for proper cell division. Recent findings revealed that circadian and cell cycle clocks might be linked (7–9). Furthermore, clock genes have been linked to the cell cycle, DNA damage, apoptosis control, and carcinogenesis (10, 12). Perturbation of circadian rhythms both in humans (shift workers) and animals has been associated with malignant transformations (13). Taken together, these data suggest a strong link between the circadian clock and the cell cycle.

Turning to the thyroid gland regulating hormones, both Thyrotropin Releasing Hormone (TRH) and Thyroid Stimulating Hormone (TSH) exhibit pronounced circadian oscillations in the blood with a peak between 2AM and 4AM in healthy subjects. Moreover, low amplitude circadian variations were reported for the thyroid hormones (total thyroxine (T3) and triiodothyronine (T4) (14)), suggesting circadian function for the thyroid gland. We therefore aimed at characterizing the clock machinery in human healthy thyroid tissue, and in benign and malignant thyroid nodules.

Materials and Methods

Study participants and thyroid tissue sampling

Fresh thyroid tissue samples ($1cm^3$) were obtained from patients undergoing thyroidectomy for thyroid cancer or suspicious nodule, with the written informed consent. Donor characteristics are summarized in Table 1. The subjects were not kept on constant routine prior to the surgery. The biopsy material was collected in a daytime dependent manner, with all the surgeries performed in the time-window between 8AM and 2PM. The study protocol was approved by the local Ethics Committee (CER 11–014). Malignant tumors were classified by histopathological analysis according to the World Health Organization Histological Classification of Thyroid Tumors (15) and staged according to the AJCC Cancer Staging Manual seventh ed. One part of the obtained thyroid tissue was deep-frozen and kept for tissue transcript analysis; the other part was immediately processed to establish primary culture.

Human primary thyroid cell culture

Fresh tissue biopsies were subjected to Type II collagenase (Life Technologies) digestion for one hour. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Primary thyrocytes were used for the experiments after reaching confluence, typically after 7 d of culture.

In vitro cells synchronization

To synchronize thyrocytes, dexamethasone was added to the culture medium at a final concentration of 100 nM. Following 30 min incubation at 37°C in a cell culture incubator, dexamethasone was washed away and replaced with normal medium, as described (16). Cells were harvested every 6 h during 36 h, deep-frozen and kept at −80°C.

RNA extraction and quantitative RT-PCR (qPCR) analysis

Total RNA from frozen thyroid biopsies or from thyrocytes was prepared using RNA spin II kit (Macherey-Nagel). Tissue biopsies were first homogenized using a Polytron homogenizer. Half a μg of RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) and random hexamers, PCR amplified and quantified as previously described (17). Mean values for each experiment were calculated from technical triplicates of PCR assays for each sample, and normalized to the mean of those obtained for *GAPDH* and *9S* transcripts served as internal controls. Primers used for this study are listed in Supplementary Table 1.

Table 1. Patient characteristics and diagnosis

	DONOR	SEX	AGE (YEARS)	TIME OF SURGERY	CYTOLOGICAL DIAGNOSTICS
BENIGN THYROID SAMPLES					
	H11014994*,$	F	48	10h30	Adenoma
	H11011188*,$	F	80	9 h	Hyperplasia
	H11001549	F	70	9h30	Adenoma
	H12000211↑,$	F	39	10h30	Hyperplasia
	H12011256*,↑,$	F	58	9h30	Hyperplasia
	H12012922↑,$	F	40	9h00	Multinodular goiter
	H12013740	F	57	10h30	Multinodular goiter
	H12013289	M	49	10h30	Adenoma
	H12013281	F	52	8h20	Adenoma
	H12010459	F	46	8h25	Adenoma
	H12014865	F	53	9 h	Multinodular goiter
	H12012567	F	39	10 h	Multinodular goiter
Total benign	**12 patients**	**M = 1; F = 11**	**52.5 ± 12.4**	**9h40 ± 0h40**	
MALIGNANT THYROID SAMPLES					
FTC	H12009322↑	F	38	9h30	FTC pT3
	H13000118↑	F	48	13 h	FTC pT2
	H13001153	F	70	11h05	FTC pT3N1b
	H13000118	F	49	12h25	FTC pT2
	H09012394	M	32	10 h	FTC pT3Nx
	H08014112	F	36	12h30	FTC pT2Nx
	H07008276	F	36	ND	FTC pT2Nx
	H12014817	F	48	10 h	FTC pT2
Total FTC	**8 patients**	**M = 1; F = 7**	**44.6 ± 12.2**	**11h15 ± 1h30**	
PTC	H12007145↑	M	29	9h30	PTC pT3pN0
	H12014994↑	F	38	11 h	FV PTC
	H13001254	M	64	13 h	PTC pT3 pN0
	H12013965	F	51	12h50	PTC pT3 pN1a
	H12012290	M	26	8h30	PTC pT2 pN1a (6/11)
	H12012070	M	56	13h30	PTC pT1b pN0
	H12011540	F	73	14h00	PTC pT3 pN1a
	H12010429	F	57	13h10	PTC pT3 Nx
	H12003781	F	55	12h30	PTC pT3 pN0
	H11009795	F	29	11 h	PTC pT2 pN0
	H10003462	F	65	8h30	PTC pT3Nx
	H10001114	F	50	8h30	PTC pT3 pN1a
	H12006309	F	38	12h30	PTC pT3 pN1a
	H12007402↑	F	40	13h00	PTC pT3 pN1b
Total PTC	**14 patients**	**M = 4; F = 10**	**50.3 ± 14.5**	**11h45 ± 2 h**	
PDTC	H12010398↑	M	47	9 h	PTC / PDTC pT3
PDTC-PTC	H13000386*↑	F	20	13 h	PTC/ PDTC pT2
Total malignant	**24 patients**	**M = 6; F = 18**	**45.6 ± 14.2**	**11h15 ± 1h40**	
Total	**36 patients**	**M = 7; F = 29**	**49 ± 14.5**	**11 h ± 1h40**	

↑ Tissue samples used for primary thyrocyte culture

$ Healthy thyroid tissue samples adjacent to the nodule were used as healthy control

*Tissue samples used for circadian bioluminescence assay

ND Surgery timing was not defined

TN(M) classification:

Primary Tumor (T)

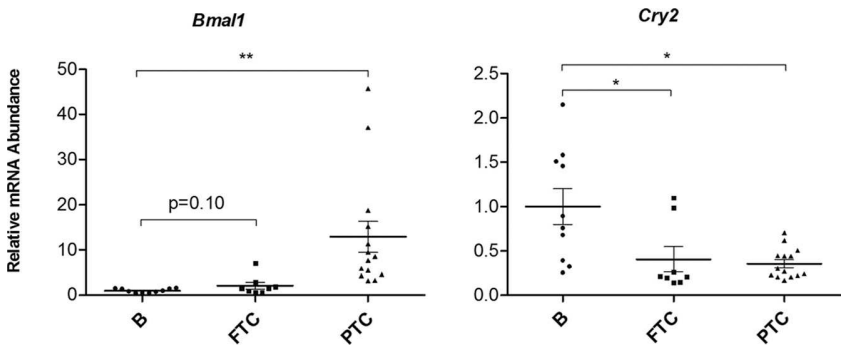

Figure 1. Expression of core-clock genes in tissue biopsies of benign thyroid nodules, FTC and PTC qPCR for *Bmal1* and *Cry2* was performed on cDNAs obtained from tissue samples from nodules with benign (N = 10), FTC (N = 8) and PTC (N = 14) postoperatory diagnosis. The relative mRNA expression of each transcript was normalized to average of *Gapdh* and *9S,* and then reported to the mean value of the respective transcript levels in nodular benign tissues. Results were expressed as mean ± SEM. Mann-Whitney test was applied to assess the significance in the transcript expression level differences between FTC or PTC as compared to benign nodules (*: $P < .05$, **: $P < .01$ and ***: $P < .001$).

Lentivectors and lentiviral production

Bmal1-luciferase (Bmal1-luc) (18) lentiviral particles were produced as previously described (17), 100-fold concentrated, and used for the transduction of thyrocytes at MOI = 5.

Bioluminescence monitoring

Bioluminescence patterns were monitored from dexamethasone synchronized human thyrocytes four days following *Bmal1-luc* lentiviral transduction, as previously described (16, 17). Briefly, synchronized cells were transferred to Actimetrics LumiCycle placed in a 37°C light-tight incubator and bioluminescence from each dish was continuously monitored using a Hamamatsu photomultiplier tube detector assembly. Actimetrics LumiCycle Analysis program was used to assess the rhythm parameters.

Cosinor Analysis

To quantify circadian oscillatory gene expression profiles, we have developed new software CosinorJ, based on a cycling function model representing the extension of the standard Cosinor method (19):

where T is the period, A+Bt is the range of oscillations, C is the mesor and ω is the acrophase (see Supplementary Methods for details). It is freely available at *http://bigwww.epfl.ch/algorithms.html*

Statistics

The results are expressed as the means ± SEM unless stated otherwise. Mann-Whitney test was applied to compare transcript expression levels between the different tissue types.

Results

Bmal1 transcript is up-regulated, while *Cry2* is down-regulated in FTC and PTC nodule tissues

In an attempt to assess core-clock transcript levels in benign and malignant thyroid tissues, biopsies from human normal thyroid tissue, benign follicular nodules, FTC or PTC were obtained after thyroidectomy (see Table 1 for patient characteristics). qPCR analysis of core-clock transcripts was performed in healthy thyroid tissue (N = 4 subjects), benign thyroid nodules (N = 10 subjects), FTC (N = 8 subjects) and PTC nodules (N = 14 subjects). No difference was observed between healthy tissue and benign nodules for all of the analyzed transcript expression levels (data not shown). By contrast, *Bmal1* expression was 13 fold up-regulated, while *Cry2* expression was about two fold down-regulated in PTC compared to benign nodules (Figure 1, Supplementary Table 2). In FTC, *Bmal1* exhibited two fold up-regulated levels, while *Cry2* was two-fold down regulated (Figure 1, Supplementary Table 2). Transcript levels of *Cry1*, *Per1*, *Per2*, *Per3*, *Reverbα* and *Dbp* in PTC and FTC were either indistinguishable or not significantly changed, as compared to benign counterparts (Supplementary Table 2). In addition to the core-clock transcript changes, we found up-regulated levels of *Timp1* and down-regulated levels of *Gadd153* transcripts in PTC (Supplementary Figure 1A, left panel, and 1B). This finding is in a good agreement with previous publications (20, 21). Of note, significant correlation was observed between *Timp1* and *Bmal1* transcript levels obtained in PTC (Supplementary Figure 1A, right panel), further validating our conclusion regarding *Bmal1* up-regulation.

Self-sustained circadian oscillators are operative in human primary thyrocytes

Prompted by marked differences in core-clock transcript levels between benign and malignant thyroid tissues, we next aimed at characterizing the human thyroid clock molecular makeup. Given obvious obstacles for studying peripheral clocks in human beings, we employed cultured human primary thyrocytes synchronized in vitro. Cells were established from fresh normal thyroid tissue obtained after thyroidectomy (Table 1, samples labeled with #). To confirm thyrocyte cell identity, thyroglobulin expression was assessed by indirect immunofluorescence. Thyroglobulin was detected in 75.3% of cells (1232 positive out of total 1634 cells) after 7 d in culture (Supplementary Figure 2).

To assess the endogenous core-clock transcript expression profiles around-the-clock, confluent primary thyrocytes were synchronized by a dexamethasone pulse, since

Legend to Figure 1 Continued. . .
T2 Tumor size > 2 cm but ≤ 4 cm, limited to the thyroid
T3 Tumor size >4 cm, limited to the thyroid or any tumor with minimal extrathyroidal extension (*e.g.* extension to sternothyroid muscle or perithyroid soft tissues)
Regional lymph nodes (N)
NX Regional nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Regional lymph node metastasis
N1a Metastases to level VI (pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes)
N1b Metastases to unilateral, bilateral, or contralateral cervical (levels I, II, III, IV, or V) or retropharyngeal or superior mediastinal lymph nodes (level VII).

dexamethasone has been previously demonstrated to synchronize efficiently circadian oscillators in various cellular systems (17, 22). mRNA accumulation patterns from synchronized thyrocytes were monitored every 6 h during 36 h by quantitative RT-PCR, using amplicons for *Bmal1*, *Cry1, Cry2, Per1, Per2, Per3, Reverbα,* and *Dbp*. The values were normalized to the average obtained for *Gapdh* and *9S* transcripts, which accumulated to similar levels throughout the day. Endogenous *Bmal1* transcript abundance exhibited high-amplitude circadian oscillations, in phase with *Cry1*, and antiphasic to those of *Reverbα, Per1–3* and *Dbp* transcripts (Figure 2), in good agreement with previous studies on human skin dermal fibroblasts (23) and human pancreatic islets (17). *Cry2* mRNA exhibited weak oscillatory profile, in phase with *Reverbα*, as expected from previous studies (17). For the quantification of these datasets, we used CosinorJ software, representing the optimization of the existing Cosinor method (19) to the cases with few circadian time points over short time (see Materials and Methods). CosinorJ analysis revealed that *Bmal1, Per1–3* and *Reverbα* exhibited clear circadian oscillations with period length of 28.06 ± 1.44 h (mean ± SEM), mesor comprised in 0.63 - 1.00 interval, and acrophase of 17.31 h for *Bmal1*, and 31.01–34.78 h for *Reverbα* and *Per1–3* (Table 2). On the other hand, *Cry1, Cry2* and *Dbp* were considered as nonoscillating according to the fit (Supplementary Figure 3). Taken together, our experiments reveal the presence of cell-autonomous self-sustained circadian oscillators in in vitro synchronized human primary thyrocytes, with the characteristics comparable to other human peripheral tissues.

Table 2. CosinorJ analysis

			Bmal1		
	H	**B**	**PTC**	**PDTC-PTC**	**PDTC**
Amplitude	**0.59**	**0.41**	**0.37**	**0.02**	**0.38**
Mesor	**1.00**	**0.99**	**0.98**	**0.92**	**0.88**
Acrophase, h	**17.312**	**17.67**	**18.50**	**14.28**	**9.98**
	Per1				
	H	**B**	**PTC**	**PDTC-PTC**	**PDTC**
Amplitude	**0.47**	**0.49**	**0.35**	**Non circadian**	**Non circadian**
Mesor	**0.69**	**0.66**	**0.77**		
Acrophase, h	**31.01**	**31.91**	**27.93**		
	Per2				
	H	**B**	**PTC**	**PDTC-PTC**	**PDTC**
Amplitude	**0.34**	**0.39**	**Non circadian**	**0.40**	**0.43**
Mesor	**0.85**	**0.93**		**0.77**	**0.71**
Acrophase, h	**33.31**	**34.34**		**39.79**	**25.549**
	Per3				
	H	**B**	**PTC**	**PDTC-PTC**	**PDTC**
Amplitude	**0.46**	**0.39**	**0.35**	**0.16**	**0.49**
Mesor	**0.72**	**0.71**	**0.84**	**0.25**	**0.64**
Acrophase, h	**34.78**	**33.1**	**35.96**	**31.67**	**27.85**
	Reverbα				
	H	**B**	**PTC**	**PDTC-PTC**	**PDTC**
Amplitude	**0.42**	**0.46**	**0.36**	**0.10**	**0.69**
Mesor	**0.63**	**0.77**	**0.78**	**0.54**	**0.84**
Acrophase, h	**34.04**	**30.54**	**29.55**	**31**	**20.7**

Data obtained with qPCR analysis and presented at Figures 2 and 3 were fit by CosinorJ, and the oscillation parameters (amplitude, mesor and acrophase) were calculated by this software. The values significantly altered in comparison to normal and benign nodular thyrocytes are labeled in red color. The profiles with the periods out of circadian range (20–35 h) or ablated circadian amplitude were defined as non-circadian by CosinorJ.

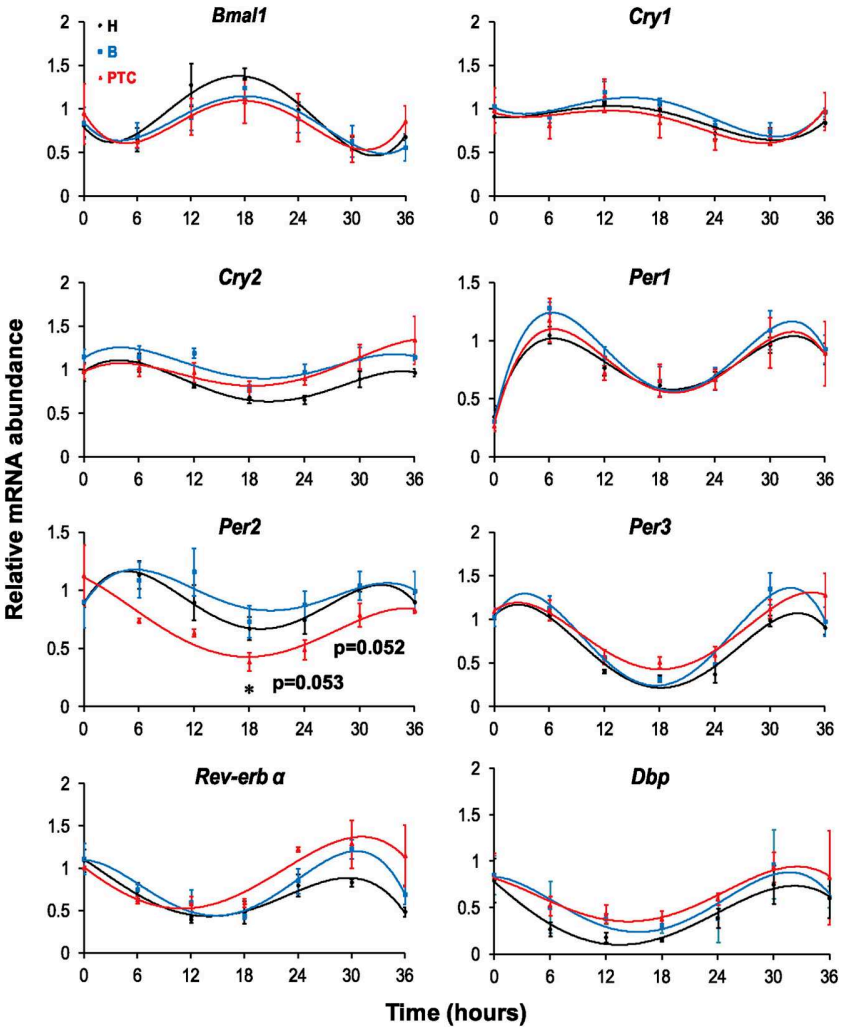

Figure 2. Cell-autonomous high-amplitude circadian oscillations of clock genes in primary human thyrocytes derived from healthy tissue, benign nodule and PTC Oscillation of endogenous clock transcripts was monitored in human primary thyrocytes synchronized with dexamethasone pulse (100 nM, 30 min pulse). qPCR was performed on cDNAs obtained from thyrocyte samples for core-clock (*Bmal1, Reverbα*, *Per1–3* and *Cry1–2*) and clock-controlled (*Dbp*) transcripts, and normalized to the average of *Gapdh* and *9S* housekeeping gene expression for each transcript. Profiles are representative of N = 3 experiments (mean ± SEM), each using cells from one donor for every cell type. H stands for healthy thyrocytes (black lines); B for benign nodule derived thyrocytes (blue lines); PTC for PTC derived thyrocytes (red lines).

Circadian characteristics of the human nodular primary thyrocytes established from benign and malignant thyroid nodules

To study possible clock alterations in thyroid malignancies, we assessed oscillatory profiles of human primary thyrocytes derived from nodules with benign or malignant histopathological diagnosis such as FTC (Supplementary Figure 4), PTC (Figure 2), and PDTC (Figure 3; see Table 1 for diagnosis details). Primary thyrocytes derived from nodular tissues were established and grown as described above for normal thyrocytes. Thyroglobulin staining was performed in all cell types after 7 d in culture, and was positive in 77.3% of cells for benign nodule derived thyrocytes (729 positive out of 942 cells), 72% for FTC derived thyrocytes (532 out of 738 cells), 71.9% for PTC thyrocytes (295 out of 410 cells), and 93% for PDTC thyrocytes (243 out of 261 cells; Supplementary Figure 2). Therefore, our analyses report circadian properties of 71.9% to 93% of human primary thyrocytes with the residual amount of contaminant cells, mostly fibroblasts.

Human primary thyrocytes established from benign nodules exhibited circadian oscillations with similar characteristics to those of normal thyrocytes (Figure 2, compare blue and black lines). CosinorJ analysis (performed in all cases as depicted in Supplementary Figure 3) revealed that similarly to normal primary thyrocytes, primary cells established from benign nodules exhibited oscillation period of 27.99 ± 1.93 h. *Bmal1*, *Per1–3* and *Reverbα* transcripts exhibited pronounced circadian oscillatory patterns with mesor, amplitude and acrophase close to those of normal thyrocytes (Table 2), while *Cry1–2* and *Dbp* profiles did not fit CosinorJ criteria for circadian pattern.

Analysis of endogenous clock transcripts in primary thyrocytes established from FTC revealed no significant differences for all of the analyzed clock transcripts when compared to the thyrocytes derived from benign nodules (Supplementary Figure 4), or to normal thyrocytes (not shown). Period length, amplitude and acrophase were similar between FTC and thyrocytes established from benign nodules for all of the transcripts, with *Bmal1*, *Per1–3* and *Reverbα* showing pronounced circadian oscillations. Oscillatory profiles of *Bmal1*, *Cry1*, *Cry2*, *Per1*, and *Per3* transcripts assessed in synchronized PTC derived thyrocytes were similar to those of benign nodular and normal thyrocytes (Figure 2). CosinorJ analysis revealed however reduced circadian amplitude for *Per2* in PTC, and no fit to the circadian pattern for this transcript (Table 2). Thus, clock transcripts in PTC thyrocytes keep their oscillatory properties, except for

Per2 exhibiting altered circadian profile (Figure 2, Table 2). Remarkably, analysis of the primary thyrocytes established from a single case of PDTC revealed altered oscillatory profiles for all of the analyzed transcripts (Figure 3). Namely, *Per1* transcripts exhibited ablated circadian amplitude, while *Bmal1*, *Per2–3,* and *Reverba* transcripts were strongly phase-shifted in comparison to the thyrocytes derived from benign nodules (marked differences in acrophase values, see Table 2). Furthermore, an additional single case analysis of primary thyrocytes established from a mixed PDTC-PTC showed flatter amplitude and phase-shifts for all of the transcripts (Figure 3, Table 2). Taken together these experiments reveal that while thyrocytes from benign nodules perfectly keep their circadian properties, alterations in the clock gene profiles are observed in in vitro synchronized thyrocytes established from PTC and PDTC.

The rhythm alterations we observed in PTC and PDTC derived thyrocytes might be attributed to the circadian phase alteration solely, or to the changes in the circadian period length. To assess the human thyrocyte circadian properties beyond the first cycle kinetics, we turned to more sensitive methodology based on long-term continuous circadian bioluminescence oscillation recording with high temporal resolution. Indeed, human fibroblasts and human islet cells expressing firefly luciferase from circadian promoters were previously shown to exhibit robust circadian bioluminescence rhythms following in vitro synchronization (17, 23). We thus monitored circadian bioluminescence of human primary thyrocytes transduced with *Bmal1-luciferase* lentivectors, synchronized by dexamethasone pulse. Oscillation patterns were assessed in healthy, nodular benign and PDTC-PTC derived thyrocytes (Figure 4). High-amplitude circadian bioluminescence cycles with the period length of 27.45 ± 0.81 h and 27.90 ± 0.76 h (N = 3 subjects, labeled with * in Table 1) were detected in primary thyrocytes derived from normal tissues and benign nodules for at least three consecutive days, in a good agreement with the endogenous transcript analysis. Of note, PDTC-PTC thyrocytes exhibited remarkably shifted or even antiphasic profiles of *Bmal1-luc* reporter oscillatory expression over the first cycle only, while getting in phase with healthy and benign nodular counterparts over the following second and third circadian cycles. This experimental approach outcome provides a clear-cut confirmation of the presence of functional circadian oscillators in human primary thyrocytes originated from healthy and benign nodular tissues. Moreover, it suggests that the phase shifts observed in PDTC-PTC derived cells as compared to the benign counterparts are attributed to the first cycle kinetics change and do not imply the circadian period length alterations.

Discussion

Molecular makeup of human thyroid clock

Our study provides for the first time an evidence for cell-autonomous high-amplitude circadian oscillators functional in cultured human primary thyrocytes established from healthy thyroid tissue. Thyrocyte oscillations were assessed by two approaches. Continuous recording of circadian bioluminescence produced by a stably integrated *Bmal1-luciferase* reporter gene represents a powerful and rather unique tool for circadian oscillator studies in living human primary explants or cells for several consecutive days with high temporal resolution. Dexamethasone synchronized human thyrocytes transduced with *Bmal1-luciferase* lentivectors exhibited pronounced circadian oscillations for at least 90 h following synchronization (Figure 4). In line with the outcome of reporter experiments, endogenous clock gene expression measurements by qPCR suggested that the core- clock transcripts exhibit circadian oscillatory patterns in synchronized thyrocytes (Figure 2). Both approaches suggested circadian period of about 27 h for this type of cells. Previously circadian timekeepers were characterized in human primary skin fibroblasts and in human pancreatic islets synchronized in culture (17, 23). In terms of the phase of core-clock transcripts our work is in a good agreement with previous studies. Human thyrocytes exhibit longer oscillation period if compared to skin fibroblasts (24.5 h) or

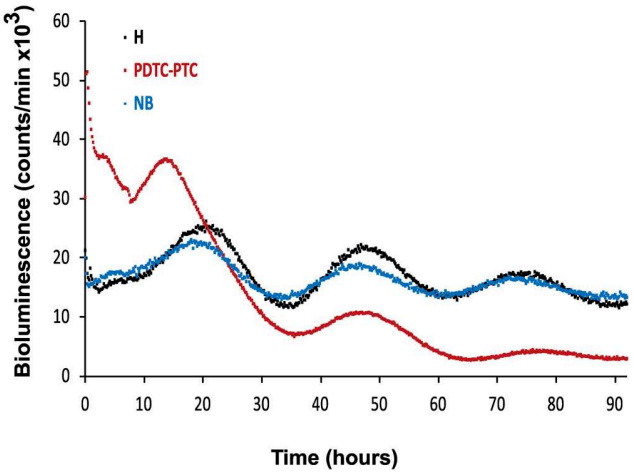

Figure 4. Circadian oscillations of *Bmal1-luciferase* reporter in human primary thyrocytes *Bmal1-luciferase* oscillations were recorded in in vitro synchronized human primary thyrocytes derived from healthy tissue, benign thyroid nodule, or PDTC-PTC (H, NB, and PDTC-PTC: black, blue and red lines respectively). Cells were transduced with *Bmal1-luciferase* lentivectors, synchronized 4 d later with dexamethasone pulse, and *Bmal1-luciferase* bioluminescence profiles were recorded for two parallel dishes for each donor; N = 3 donors for thyrocytes derived from healthy tissue and benign nodules; N = 1 donor for PDTC-PTC. The curves represent an average of all the performed recordings for each cellular type.

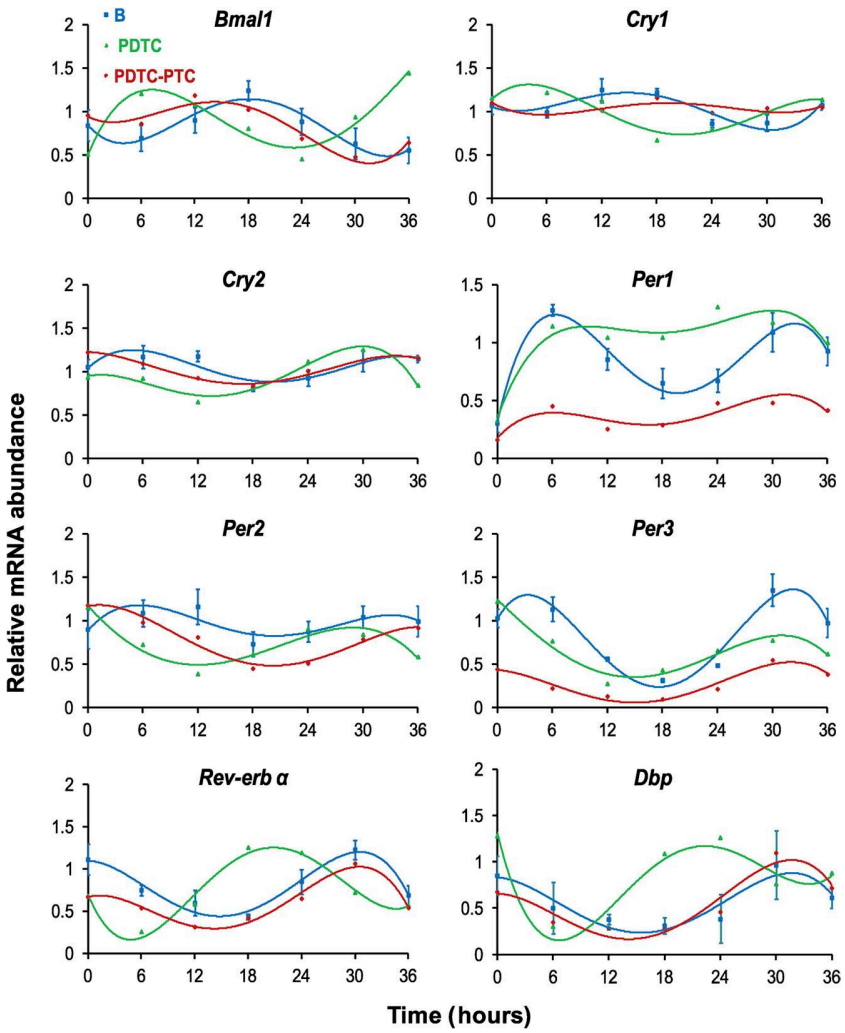

Figure 3. Oscillation profiles of clock genes are altered in primary thyrocytes derived from PDTC and PDTC-PTC (single case studies) qPCR analysis was performed in dexamethasone synchronized thyrocytes in the samples collected around-the-clock for *Bmal1*, *Reverbα*, *Per1–3, Cry1–2* and *Dbp* transcripts, and normalized to the average of *Gapdh* and *9S* house-keeping gene expression for each transcript. Profiles for benign thyroid nodule (blue lines) correspond to those presented in Figure 2 as mean ± SEM of N = 3 experiments. Profiles for PDTC (green lines) and PDTC-PTC derived thyrocytes (red lines) represent single donor each.

human pancreatic islets (23.6 h; (17, 23)). Of note, circadian oscillation period length varies significantly among different mouse organ explants cultured in vitro (24), reflecting probably the differences in the general transcriptional rates and cell size among these organs, if comparing liver and lung for example (16, 25).

Thyrocyte oscillator properties change upon thyroid nodule malignant transformation

Central question addressed in this study was whether circadian clockwork is altered upon thyroid malignancies. We found that transition of normal thyroid tissue to the benign nodule does not alter circadian oscillator function. This held true for both circadian bioluminescence analysis of the cells expressing circadian reporter, and for the endogenous transcript analysis (Figures 4 and 2). However around-the-clock analysis of cells established from PTC and PDTC suggested that malignant transformation of human primary thyrocytes might change their circadian oscillator properties (Figures 2–4, Table 2). While in general the oscillators are functional in FTC and PTC derived thyrocytes, *Per2* transcript oscillatory profile was altered in PTC (Supplementary Figure 4, Figure 2, Table 2). This tendency becomes more obvious when looking at *Per1–3* transcript profiles in PDTC samples (Figure 3, Table 2), where the amplitude is flattened with complete loss of the oscillatory pattern in *Per1*. Most importantly we observed dramatic changes in all circadian transcript phase in PDTC, with some of the transcripts being antiphasic to benign counterparts (Figure 3, Table 2). Observing circadian profile in PDTC-PTC derived thyrocytes over several consecutive days with high temporal resolution (Figure 4), we conclude that these changes are limited to the phase shift of the first circadian peak, in an agreement with our qPCR measurements (Figure 3), while later on PDTC cell profile aligns with healthy and benign counterparts (Figure 4). This change attributed to the kinetics of the first circadian cycle might suggest the alteration of clock immediate resetting response in PDTC derived cells. To get further insights to the nature of these alterations, single cell analysis by bioluminescence time-lapse microscopy of benign versus PDTC thyrocytes will be required. It is not excluded that glucocorticoid hormones, commonly used by us and others for in vitro cell synchronization and playing an important role for peripheral clock synchronization in vivo (6, 26), might also play a role in thyroid clock regulation in vivo. Another plausible candidate for the thyroid clock synchronization stimulus would be TSH, exhibiting strongly oscillating profile in the blood (14, 27). Of note, the level of TSH receptor in thyrocytes was found to be decreased in PTC (28). It will be interesting to address in the future if indeed TSH might play a role in thyroid cell synchronization, and whether

down-regulation of TSH receptor in PTC might impact on oscillator synchronization properties in these cells.

Two major factors limit the interpretation of our experiments: the low number of available fresh postoperative material of the malignant nodules, and the low number of the primary cells we could establish from each biopsy. Therefore, although the reproducibility among the "around-the-clock" experiments was high, these experiments must be taken with caution due to low number of the analyzed cell lines. The number of cells derived per biopsy allowed performing around-the-clock analysis with 6 h resolution over 36 h, 7 circadian time points in total. To make these dataset's analysis more robust, we developed an adaption of the existing software packages already available for this type of datasets analysis (29–31). Our new software, CosinorJ, represents an efficient, fast, user-friendly tool for oscillating dataset analysis, freely available for the research community.

Bmal1 and *Cry2*: potential candidates for FTC and PTC preoperative diagnostic markers

Importantly, our study reveals strong alterations of core-clock transcripts *Bmal1* and *Cry2* in FTC and PTC, when compared to benign nodules and healthy tissue samples taken at a similar time window of the day (Figure 1). We demonstrate for the first time that core-clock gene expression levels are altered in thyroid malignancies, namely the up-regulation of *Bmal1* and down-regulation of *Cry2* in FTC and PTC. Moreover, *Bmal1* transcript levels were positively correlated with those of *Timp1*, also up-regulated in the same PTC samples (Supplementary Figure 1A; (20)). This correlation further underscores a potential link between *Bmal1* expression level and possibly function, and thyroid nodule tumor progression. Of note, we observe more pronounced differences for core-clock transcript levels in biopsy tissue analysis when compared to our experiments done in in vitro synchronized PTC derived thyrocytes. This discrepancy might be associated with the drastic changes in the cellular environment in in vitro culture situation, associated with the absence of neural and hormonal regulation, disruption of three dimensional tissue structure and alterations in cell-cell communication, changes in epigenetic regulation, and influence of additional factors. Those limitations must be taken into account when drawing conclusions from clock in vitro studies in synchronized cultured cells, although this approach stays rather unique when applied to human circadian clock studies.

There is accumulating evidence on the important role of core-clock components for cell cycle progression and timing of cell division. The circadian oscillator gates cytokinesis to defined time windows in in vitro cultured fibroblasts (7), and regulates key components of the cell cycle *wee1*, *cyclinB1* and *cdc2* in mouse liver cells in vivo (9). Of note, *Per2* plays a key role as a tumor suppressor by regulating DNA damage responsive pathways (10). Moreover, altered expression levels of several clock genes have been described in various types of malignancies: *Per1–3* were down-regulated in breast cancer (32); *Per1–2*, *Clock* and *Cry1* were decreased in skin melanoma (33); most of the core-clock transcripts were down-regulated in head and neck squamous cell carcinoma (34), in pancreatic ductal adenocarcinoma (35), and in chronic myeloid leukemia (36). In line with these studies, our experiments demonstrate down-regulation *Cry2* levels upon thyroid malignancies. *Bmal1* levels were strongly up-regulated in PTC and moderately up-regulated in FTC, suggesting that up-regulation of this core-clock transcript might be associated with the pathological conditions as well as its down-regulation, demonstrated in human studies cited above for its association with malignancies, or in *Bmal1* knockout study revealing an association with reduced life span and premature aging in mice (37). Taken together these findings further underscore the link between circadian oscillatory function and cellular malignant transformation. Given that preoperative diagnostics of malignant thyroid nodules is still far from providing reliable responses in numerous cases of suspicious or indeterminate nodules (5, 38), it would be important to launch a prospective study for *Bmal1* and *Cry2* transcripts in thyroid nodules to explore the potential employment of these transcript changes for improving FTC and PTC preoperative diagnostics.

In conclusion, we present the first detailed characterization of the human thyroid circadian clockwork and its changes upon malignant transformation. These new insights into the core-clock changes upon thyroid malignancies should contribute to the unresolved issue of the malignant nodule preoperative diagnosis. Exploring human thyroid clock function and its potential role in thyroid nodule malignant transformation represents an important step forward in our understanding of the molecular link between clock function, thyroid tissue physiology and pathophysiology of malignant thyroid nodules.

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