Research Article
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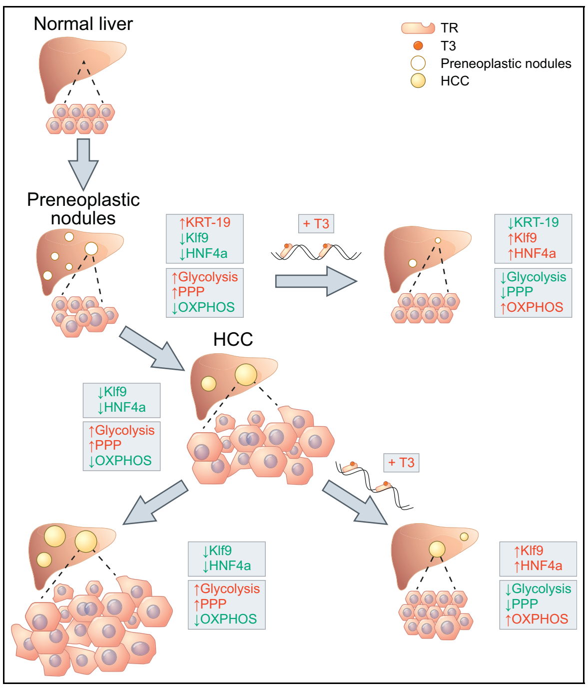
Thyroid hormone inhibits hepatocellular carcinoma progression via induction of differentiation and metabolic reprogramming

Graphical abstract



Highlights

- The T3/TRβ axis is altered in human HCC.
- T3 induces a rapid differentiation program in hepatic preneoplastic lesions.
- Repeated cycles of T3 impair HCC progression.
- The antitumorigenic effect of T3 is long-lasting and is maintained after withdrawal.
- T3 reverts the metabolic profile of HCC to that of a normal hepatocyte.

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Lay summary

Hepatocellular carcinoma (HCC) represents an important challenge for global health. Recent findings showed that systemic or local hypothyroidism is associated with HCC development. In rat models, we showed that administration of the thyroid hormone T3 impaired HCC progression, even when given at late stages. This is relevant from a translational point of view as HCC is often diagnosed at an advanced stage when it is no longer amenable to curative treatments. Thyroid hormones and/or thyromimetics could be useful for the treatment of patients with HCC.

https://doi.org/10.1016/j.jhep.2019.12.018
 J. Hepatol. 2020, 72, 1159–1169

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Thyroid hormone inhibits hepatocellular carcinoma progression via induction of differentiation and metabolic reprogramming

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Background & Aims: Only limited therapeutic options are currently available for hepatocellular carcinoma (HCC), making the development of effective alternatives essential. Based on the recent finding that systemic or local hypothyroidism is associated with HCC development in humans and rodents, we investigated whether the thyroid hormone triiodothyronine (T3) could inhibit the progression of HCCs.

Methods: Different rat and mouse models of hepatocarcinogenesis were investigated. The effect of T3 on tumorigenesis and metabolism/differentiation was evaluated by transcriptomic analysis, quantitative reverse transcription PCR, immunohistochemistry, and enzymatic assay.

Results: A short treatment with T3 caused a shift in the global expression profile of the most aggressive preneoplastic nodules towards that of normal liver. This genomic reprogramming preceded the disappearance of nodules and involved reprogramming of metabolic genes, as well as pro-differentiating transcription factors, including Kruppel-like factor 9, a target of the thyroid hormone receptor β (TRβ). Treatment of HCC-bearing rats with T3 strongly reduced the number and burden of HCCs. Reactivation of a local T3/TRβ axis, a switch from Warburg to oxidative metabolism and loss of markers of poorly differentiated hepatocytes accompanied the reduced burden of HCC. This effect persisted 1 month after T3 withdrawal, suggesting a long-lasting effect of the hormone. The antitumorigenic effect of T3 was further supported by its inhibitory activity on cell growth and the tumorigenic ability of human HCC cell lines.

Conclusions: Collectively, these findings suggest that reactivation of the T3/TRβ axis induces differentiation of neoplastic cells towards a more benign phenotype and that T3 or its analogs, particularly agonists of TRβ, could be useful tools in HCC therapy.

Lay summary: Hepatocellular carcinoma (HCC) represents an important challenge for global health. Recent findings showed that systemic or local hypothyroidism is associated with HCC development. In rat models, we showed that administration of the thyroid hormone T3 impaired HCC progression, even when given at late stages. This is relevant from a translational point of view as HCC is often diagnosed at an advanced stage when it is no longer amenable to curative treatments. Thyroid hormones and/or thyromimetics could be useful for the treatment of patients with HCC.

Introduction

Hepatocellular carcinoma (HCC), the third most common cause of cancer mortality worldwide,[1] represents an important challenge for global health. Despite an important increase in incidence, current therapeutic options for HCC remain unsatisfactory.[2]

Thyroid hormones, namely 3,5,3'-triiodo-L-thyronine (T3) and 3,5,3',5'-tetraiodo-L-thyronine (thyroxine or T4), influence a variety of physiological processes, including development, metabolism, cell growth and proliferation.[3,4] Although it has been proposed that rapid non-genomic mechanisms initiated at the cell membrane could mediate thyroid hormone activity,[5] most of the effects of thyroid hormones on cellular proliferation and differentiation are driven by the thyroid hormone receptors (TRs), TRα and TRβ.[3,4] The liver is an important target organ of thyroid hormones where TRβ represents the most abundant isoform.[6]

In the last years, growing evidence has demonstrated that thyroid hormones and TRs are implicated in HCC development. Indeed, 2 case-control studies suggested that hypothyroidism represents a risk factor for HCC.[7,8] In the first study, Hassan *et al.* observed an increased association between hypothyroidism and

Keywords: T3; Thyroid hormone receptors; HCC; Rats; Differentiation; Metabolic reprogramming; KLF9; PPP; OXPHOS.

Received 14 May 2019; received in revised form 18 December 2019; accepted 20 December 2019; available online 15 January 2020

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https://doi.org/10.1016/j.jhep.2019.12.018

HCC in women, independent of established HCC risk factors; in the second study, hypothyroidism was significantly more prevalent in patients with HCC of unknown etiology. In addition, a recent work showed that TRβ expression correlated with more progressed stages of non-alcoholic steatohepatitis (NASH), a protumorigenic condition.[9] These results suggest that hypothyroidism may represent a permissive factor for HCC development.

In addition, a state of severe local hypothyroidism, revealed by downregulation of *TRβ* and Deiodinase1 (*Dio1)* mRNA levels has been identified in the most aggressive rat hepatic preneoplastic lesions, characterized by their positivity for the stem/progenitor cell marker cytokeratin-19 (KRT-19),[10] and in rat and human HCCs.[11,12] Finally, the finding that increased expression of these genes upon T3 treatment was associated with preneoplastic nodule regression[10] suggests that local reactivation of the T3/TR axis in cancer cells may impact on HCC development.

Based on this premise, we investigated whether reactivation of the T3/TR axis in fully developed HCCs could impact on their progression, even when the hormone is administered at late phases. This is relevant, as HCC is often diagnosed at an advanced stage when it is not amenable to curative treatments. Collectively, our findings show that T3 acts as a potent antitumoral agent, most likely by prompting a differentiation program and a metabolic switch.

Materials and methods

Animals and treatments

Male Fischer and Wistar rats (75-100 g) and CD1 nude mice were obtained from Charles River (Milano, Italy). Guidelines for Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health.

Six experimental protocols were used for the study of rat hepatocarcinogenesis. CD-1 experiments were performed as described.[13] For details see supplementary materials and methods and Fig. S1-3.

Histology, immunohistochemistry, enzyme histochemistry and immunofluorescence

Liver sections were fixed in 10% formalin or snap-frozen in liquid nitrogen. For details see supplementary materials and methods.

Laser-capture microdissection

Nodules positive for both the placental form of glutathione S-transferase (GSTP) and KRT-19 or for GSTP alone were identified by immunohistochemistry (IHC) of 6 μm-thick frozen liver sections. Nodule microdissection was performed on 16 μm serial sections with a Leica LMD6000 (Leica Microsystems), as previously described.[14] Equivalent areas of normal livers were similarly microdissected.

mRNA expression profiling

Total RNA was extracted from 8/10 preneoplastic nodules (obtained from 3/4 animals) with the MirVana kit, and from HCCs with TRIzol (Thermo Fisher Scientific). For the gene expression profile, 150 ng of RNA were amplified (Illumina TotalPrep RNA Amplification Kit), labeled and hybridized on Illumina microarray (RatRef-12 V1 BeadChips, Illumina Inc.), including 21,791 genes. Gene array data are available at GEO, accession number GSE131034 mRNAs validation was performed using specific TaqMan assays (Life Technologies). For further details and data analysis, see supplementary materials and methods.

Chromatin immunoprecipitation assays.

Chromatin immunoprecipitation (ChIP) assays were performed as previously described[15] with some modifications. For details see supplementary materials and methods.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was performed as described in the supplementary materials and methods.

Cell cultures and *in vitro* experiments

Human HepG2 and Mahlavu cell lines were cultured as described in the supplementary materials and methods. Cell transduction, growth and soft-agar assays were performed as described in the supplementary materials and methods.

Enzymatic activity measurement of the respiratory chain complex I and II and G6PD, and radioactive assay

Liver tissues from HCC-bearing rats with or without T3 treatment were frozen in liquid nitrogen-cooled isopentane and cut in 16 μm-thick sections. For each sample, 50 mg of liver tissue were homogenized in different buffers depending on the assay. For details see supplementary materials and methods.

Patients

The cohort consisted of HCC and cirrhotic tissues obtained from 45 randomly selected patients (33 males and 12 females, median age ± SD: 69.3 ± 4.9) undergoing resection for HCC at the Department of Surgery of the University of Bologna. The characteristics of patients are described in Table S1. For further details, see supplementary materials and methods.

Statistical analyses

Data are expressed as mean ± either SD or SE. Analysis of significance was done by either Student's *t* test or Mann-Whitney and by one-way ANOVA using the GraphPad software (La Jolla, California).

Results

T3 induces a global shift of the expression profile of preneoplastic lesions towards that of fully differentiated hepatocytes

Using the resistant hepatocyte (R-H) model of hepatocarcinogenesis (see supplementary materials and methods) we previously showed that a 7-day treatment with T3 to rats bearing hepatic nodules induced a rapid regression of these preneoplastic lesions, in the absence of clear signs of cell death.[15] To investigate the molecular mechanisms underlying the observed regression, we performed a transcriptomic analysis of laser-microdissected preneoplastic nodules at 2 and 4 days after T3 treatment, times that precede the disappearance of the nodules. A total of 869 out of 21,791 genes included in the array were selected as described in the supplementary materials and methods. Unsupervised hierarchical cluster analysis stratified the rat lesions of T3-untreated animals into 2 major clusters: i) normal liver and preneoplastic lesions negative for KRT-19; ii) the most aggressive preneoplastic KRT-19+ lesions.[14] T3 administration for 4 days induced a shift in the gene expression profile of KRT-19+ lesions towards that of normal liver and indolent preneoplastic KRT-19- lesions (as evidenced by unsupervised co-clustering). Conversely, no major gene expression profile changes were observed after 2 days of treatment (Fig. 1A). These results suggest that the T3-induced reprogramming of poorly differentiated hepatocytes to a more mature phenotype precedes the disappearance of preneoplastic lesions. Indeed, the ratio between the most aggressive and poorly differentiated KRT-19+ nodules and those KRT-19- before and after T3 treatment was 0.33 and 0.14, respectively. According to our previous findings,[16] nodule regression was not associated with increased apoptosis (compared to untreated rats), as revealed by histology and immunohistochemical detection of activated caspase-3 (data not shown).

T3 treatment modulates pathways and transcription factors related to the acquisition/maintenance of a differentiated phenotype

Ingenuity Pathway Analysis (IPA) of the genes differentially expressed in KRT-19+ preneoplastic lesions after 4 days of T3 treatment *vs.* untreated lesions, revealed their involvement in metabolic pathways, such as those related to lipopolysaccharide (LPS)/interleukin (IL)-1-mediated inhibition of retinoic-X-receptor (RXR) function, NRF2-mediated oxidative response and glutathione-mediated detoxification (Fig. 1B). Remarkably, T3 treatment caused activation of hepatocyte nuclear factor 1-alpha (*Hnf1a*), hepatocyte nuclear factor 4-alpha (*Hnf4a*) and CCAAT-enhancer binding protein alpha (*Cebpa*) involved in the

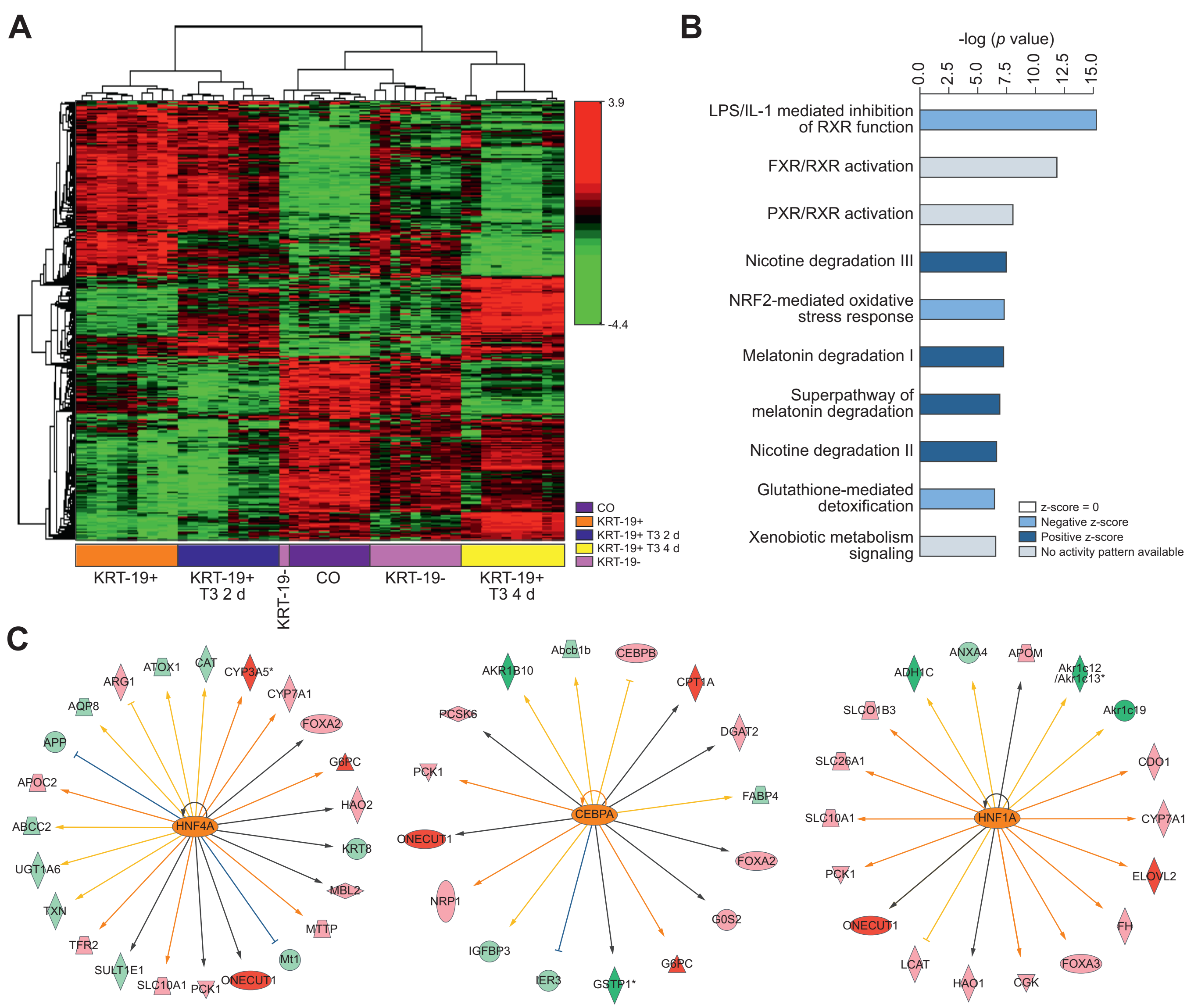

Fig. 1. T3 modifies the global gene expression profile of preneoplastic lesions. (A) Hierarchical clustering of 869 genes in normal liver (CO), KRT-19-, KRT-19+ preneoplastic lesions, and KRT-19+ lesions treated with T3 for 2 (T3 2d) or 4 days (T3 4d). Each row represents the expression of a gene and each column a sample. Red and green colors represent higher or lower mRNA expression levels (median-centered), respectively. (B) Enriched pathways in 4 days T3-treated *vs.* untreated KRT-19+ preneoplastic lesions. *P* values were determined using the Ingenuity scoring system. Threshold $p < 0.05$. Color bars indicate predicted pathway activation (light blue) , or inhibition (dark blue). (C) Network visualization of the transcription factors HNF4a, CEBPa and HNF1a. TF → target gene. Red: upregulated genes; green: downregulated genes.

acquisition/maintenance of the differentiated hepatocyte phenotype[17] (Fig. 1C). Among the T3-activated transcription factors, it was of particular interest to find *Klf9*, a Kruppel-like factor that contains a thyroid hormone response element and is implicated in the regulation of the balance between pluripotency, self-renewal, differentiation, and metabolism[18–20] (Fig. S4). Of note, some of the most deregulated genes were direct targets of TRβ or Klf9, as shown by ChIP analysis (Fig. S5).

To further investigate the pro-differentiating effect of T3, we determined the expression of genes involved in hepatocyte differentiation. As previously shown,[10] hepatic preneoplastic nodules are characterized by a local hypothyroid state. Quantitative reverse transcription PCR (qRT-PCR) analysis showed that T3 treatment reverted this status, as revealed by the strong induction of the TRβ-target gene deiodinase 1 *(Dio1)* (Fig. 2A). *Dio1* upregulation was accompanied by a profound downregulation of the stem/progenitor cell marker *Krt-19* (Fig. 2B,E). Concomitantly, the expression of the pro-differentiating transcription factors *Klf9* and *Hnf4a* – strongly decreased in preneoplastic nodules – was upregulated following T3 treatment (Fig. 2C and D). The T3-induced shift towards a differentiated phenotype was further supported by histochemical analysis showing reacquisition of glucose-6-phosphatase (G6Pase) activity, an established marker of differentiated hepatocytes that occurred concomitantly with loss of activity of the preneoplastic marker gamma-glutamyltransferase (GGT), which is not expressed by differentiated hepatocytes (Fig. 2F).

HCCs are still responsive to T3

Since human HCC is often diagnosed at late stages and treatment options are limited and generally poorly effective, to attribute a clinical relevance to T3 it was important to determine whether not only preneoplastic lesions but also HCCs were responsive to the hormone. Therefore, animals exposed to the R-H model were sacrificed 10 months after diethylnitrosamine (DEN) treatment. At necropsy, tumors were macroscopically evident in all animals and displayed the typical histological features of HCC, such as irregular edges, with an infiltrating pattern of growth, a marked cellular pleomorphism with mild nuclear atypia, and the normal lobular architecture being replaced by thick trabeculae of neoplastic hepatocytes (Fig. 3A). Remaining rats were divided into 2 groups and fed for 1 week with either basal diet or T3-supplemented diet (Fig. S1B). qRT-PCR analysis showed that HCCs from untreated rats, similarly to preneoplastic nodules, exhibited diminished *Dio1* mRNA levels that returned to basal values following 1 week of treatment with T3 (Fig. 3B). Restoration of the T3/TR axis in HCCs of T3-treated rats was associated with a strong reduction of *Gstp* and *Krt-19* expression (Fig. 3C,E). Notably, while a significant increase of *Klf9* was observed after T3 (Fig. 3D), no such change was found for Cebpα, Cebpβ, Foxa2 and Foxa3 (Fig. S6).

T3 reduces HCC number and burden

Having established that HCCs are still responsive to T3 administration, we investigated whether T3 treatment of HCC-bearing animals could interfere with tumor progression. To this aim, 10 months after DEN treatment rats were exposed to 5 cycles of T3-supplemented diet (1 week/every 3 weeks) and sacrificed 3.5 months afterward (Fig. S2A). Livers of T3-exposed rats were characterized by very few macroscopic HCCs, while animals fed normal diet displayed large and multiple HCCs (Fig. 4A,B). A

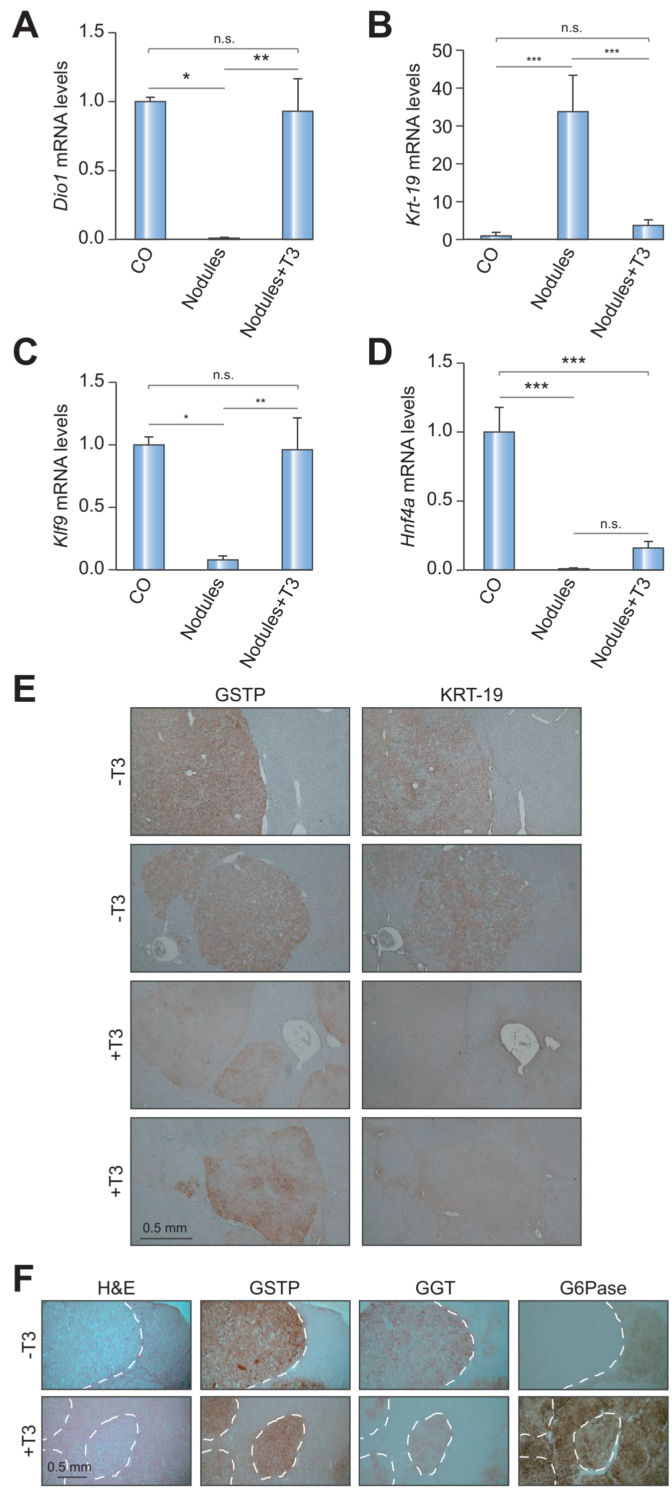

Fig. 2. T3 induces the expression of genes involved in hepatocyte differentiation. (A-D) Quantitative reverse transcription PCR analysis of *Dio1*, *Krt-19*, *Klf9* and *Hnf4a* mRNA in control livers, preneoplastic GSTP/KRT-19+ nodules, with/without 4 days T3 treatment. Gene expression is reported as fold-change relative to livers from untreated rats. The histogram represents mean values ± SD of 6/8 nodules/group (3 to 4 rats per group) (ANOVA test). (E) GSTP and KRT-19 immunohistochemical staining of preneoplastic nodules of untreated (−T3) or 4 days T3-treated (+T3) animals. (F) H&E, GSTP, GGT and G6Pase staining of representative preneoplastic lesions of untreated (−T3) or 4 days T3-treated (+T3) animals (X5). n.s., not significant. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001.

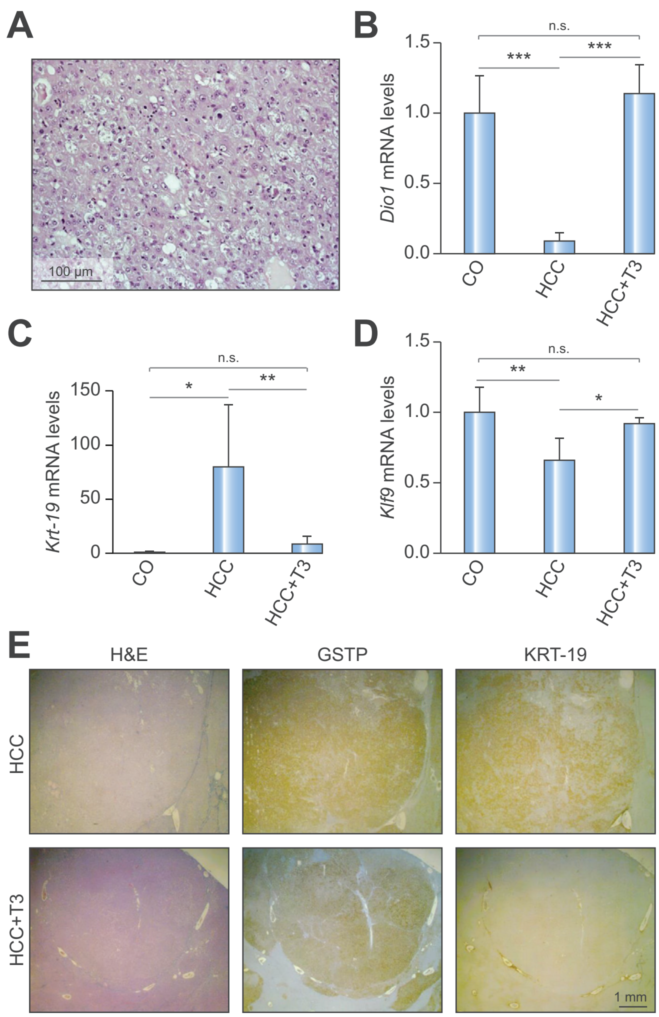

Fig. 3. HCCs maintain the ability to respond to T3. (A) HCC displaying a marked cellular pleomorphism in a rat exposed to the RH protocol, killed 10 months after DEN (H&E ×20). (B-D) qRT-PCR analysis of *Dio1, Krt-19, Klf9* mRNA in control livers (CO), HCC from rats 10 months after DEN administration, fed (HCC+T3) or not (HCC) a T3-supplemented diet for 1 week. For each liver, qRT-PCR analysis was performed on 25 randomly cut sections obtained from different segments (treated or untreated with T3). Gene expression is reported as fold-change relative to livers from controls. The histogram represents mean values + SD of 4-6 rats/group. (ANOVA test) (E) HCC serial sections in rats treated as before, stained for H&E, GSTP and KRT-19 (X1.25). n.s., not significant. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; qRT-PCR, quantitative reverse transcription PCR; RH, resistant hepatocyte.

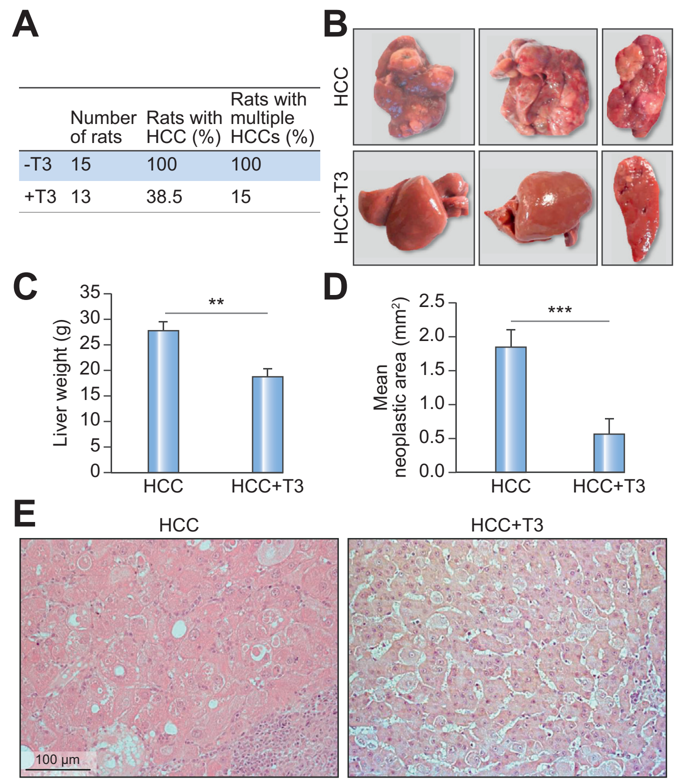

Fig. 4. T3-induced reduction of HCC number and burden. (A) Table showing the number of animals carrying HCCs and of HCCs/rat. (B) Livers of rats exposed to the RH protocol and sacrificed 13.5 months after DEN, and treated (HCC+T3) or not (HCC) with 5 cycles of T3. (C) Liver weight of the same rats. Results are expressed as means ± SD of 15 rats. (*t* test) (D) Average of liver area occupied by neoplastic hepatocytes. Tumor burden was evaluated in H&E stained histological sections from at least 5 to 10 liver sections/rat. (*t* test) (E) Poorly differentiated HCC in a rat not given T3 (HCC) and a more differentiated HCC with features of adenomas in the liver of a T3-treated animal (HCC+T3) (H&E ×20). $^{**}p$ <0.01; $^{***}p$ <0.001. DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; RH, resistant hepatocyte.

reduction in tumor burden was associated with a significant decrease in liver weight (Fig. 4C). Light microscopy examination confirmed a significant reduction of the neoplastic area in T3-treated rats (Fig. 4D). Moreover, microscopic observation revealed that lesions with features of adenomas were predominant in the livers of T3-treated animals – with only rare tumors displaying HCC features – while well- or poorly differentiated HCCs predominated in rats fed a basal diet (Fig. 4E). This result reinforces the hypothesis that thyroid hormone induces differentiation of neoplastic cells towards a more benign phenotype.

As observed in preneoplastic nodules, restoration of the T3/TR axis, confirmed by the increase of TRβ-target genes *Dio1* and *Spot14* expression, was paralleled by *Gstp* and *Krt-19* downregulation (Fig. 5A-E). Concomitantly to the loss of the fetal markers GSTP, KRT-19 and GGT, reacquisition of G6Pase activity was observed in the livers of T3-treated rats (Fig. S7A and Table S2). T3 treatment also induced several HNF4a-positive hepatocyte nuclei (Fig. 5F), together with increased *Klf9* mRNA levels and nuclear localization (Fig. 5G,H). Interestingly, the expression of *Notch3* and *Hey2* genes, belonging to the Notch signaling pathway involved in the maintenance of stemness and known to display a T3-response,[20] was downregulated upon T3 treatment (Fig. 5I,J). Altogether, these results demonstrate that T3 massively impairs HCC progression, likely promoting differentiation of neoplastic hepatocytes.

Notably, as observed in early preneoplastic lesions, no sign of increased apoptosis was observed in the livers of HCC-bearing animals treated with T3, as determined by caspase-3 immunostaining (Fig. S7B).

An additional rat model was used to test the antitumorigenic ability of T3, wherein the clonal expansion of DEN-initiated cells is promoted by a steato-necrogenic environment generated by 4 month-feeding with a choline-devoid, methionine-deficient (CMD) diet (Fig. S3A). Ten months after DEN treatment at least 1 small HCC was observed in all animals. Treatment with 5 cycles of T3-supplemented diet (1 week/every 3 weeks) caused a

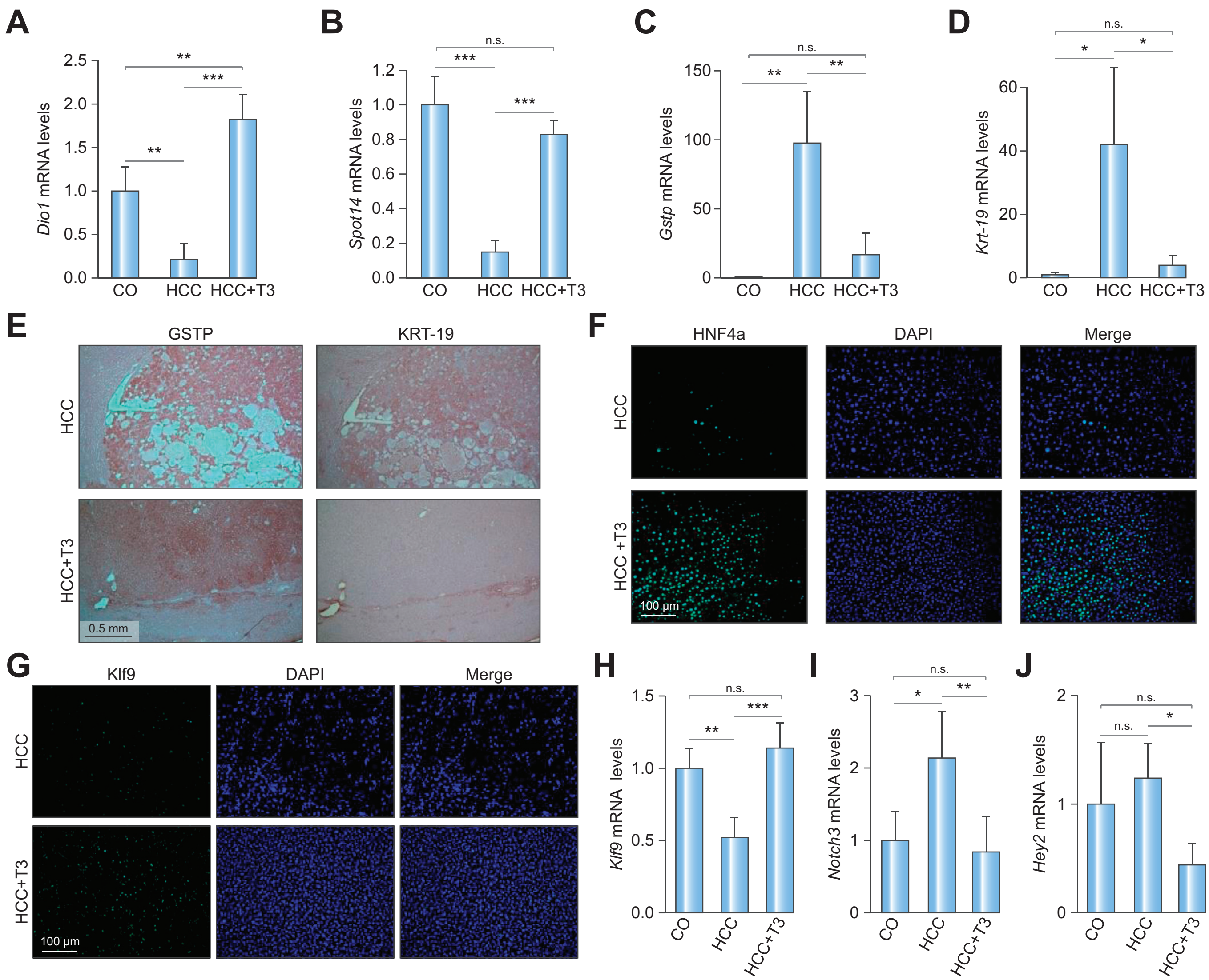

Fig. 5. T3 induces hepatocyte differentiation. (A-D) qRT-PCR analysis of *Dio1, Spot-14, Gstp* and *Krt-19* mRNA in control liver and in the livers of rats killed 13 months after DEN, with/without 5 cycles of T3. For each liver, qRT-PCR analysis was performed on 25 randomly cut sections obtained from different segments (treated or untreated with T3). Gene expression is reported as fold-change relative to livers from untreated rats. The histogram represents mean values ± SD of 6/8 livers/group. (ANOVA test) (E) GSTP and KRT-19 immunohistochemistry of livers from animals treated as described in A (X5). (F,G) HNF4a and KLF9 immunofluorescence of livers of rats treated as in A (X20). (H-J) qRT-PCR analysis of *Klf9, Notch3* and *Hey2* mRNA levels in control livers and in the livers of rats treated as in A. The histogram represents mean values ± SD of 6/8 livers/group. (ANOVA test) n.s., not significant; **p* <0.05; ***p* <0.01; ****p* <0.001. DEN, diethylnitrosamine; qRT-PCR, quantitative reverse transcription PCR.

striking reduction in the number of rats displaying macroscopic tumors (2/8; 25%), when compared to that of animals not exposed to the hormone (4/5; 80%) and in the number of tumors/rat (average: 0.25 *vs.* 2.4) (Fig. S7C,D,E).

T3 inhibits the growth of human HCC cells and their *in vivo* tumorigenic ability

To directly establish the antitumorigenic properties of T3, we evaluated its effect on cell growth and colony forming ability of human HCC cells. While TRβ transduction did not significantly modify the basal growth ability of either the cell lines (data not shown), treatment with T3 severely impaired the capacity of Mahlavu and HepG2 cells to grow both in adhesion (Fig. 6A and Fig. S8A) and in the absence of anchorage (Fig. 6B and Fig. S8B). This effect was more evident in cells transduced with TRβ. Notably, the mRNA levels of *TRβ* were similar in both Mahlavu and HepG2 cells (Fig. S8C). As *in vitro* treatment with T3 significantly induced *KLF9* expression in both Mahlavu and HepG2 cells (Fig. 6C and Fig. S8D), we investigated whether the antitumorigenic activity of T3 was linked to KLF9-mediated neoplastic cell differentiation. As shown, *KLF9* silencing significantly impaired the T3 inhibitory effect on anchorage-independent growth (Fig. 6D and Fig. S8E,F). Moreover, T3 treatment greatly reduced tumor growth of both *TRβ*-transduced and not transduced Mahlavu cells when injected into the posterior flank of nude mice (Fig. 6E).

TRβ/KLF9 axis is affected in a chronic model of rat hepatocarcinogenesis and in human HCC

Evidence supporting the role of TRβ/KLF9 axis in HCC development was further investigated in an additional rat model consisting of chronic treatment with DEN (Fig. S3B).

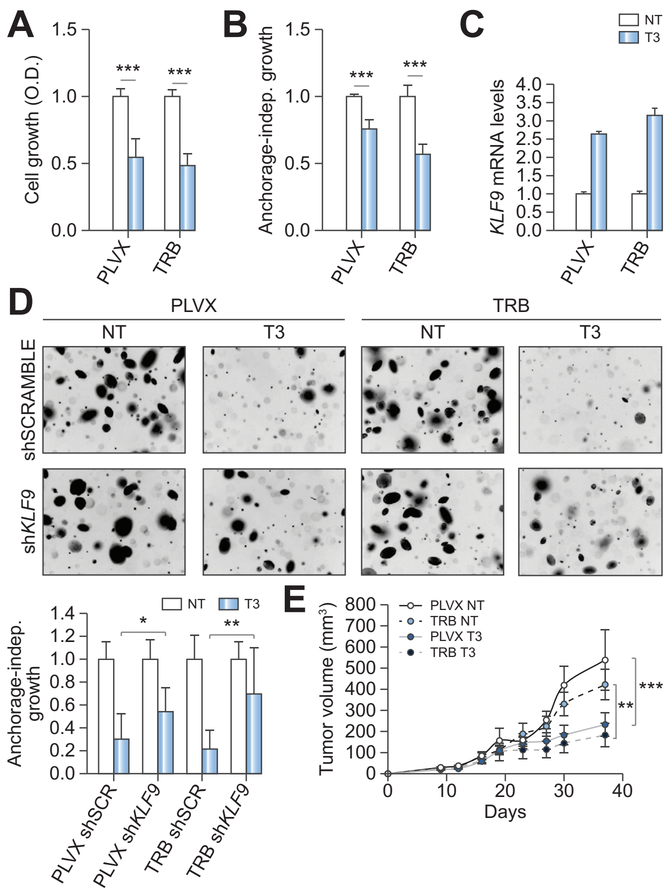

Fig. 6. T3 treatment impairs growth and tumorigenic ability of human HCC cells. (A) Growth curve of Mahlavu cells, transduced with TRβ or an empty vector (PLVX) and treated (T3) or not (NT) for 6 days with 100 nM T3. O.D.= optical density at 590 nm. (multiple *t* test) (B) Anchorage-independent growth of cells described in A. Visible colonies were counted after 15 days. (multiple *t* test) (C) Quantitative reverse transcription PCR analysis of *KLF9* mRNA levels from cells described in A. (D) Mahlavu cells described in A were stably transduced with a lentiviral KLF9 short hairpin mix (shKLF9) or a control (shSCR) and grown in anchorage-independent conditions. (multiple *t* test) (E) Tumor growth curves of Mahlavu cells subcutaneously injected in nude mice fed either T3 diet or basal diet until sacrifice (n = 6/group). (two-way ANOVA) Histograms show mean + SD of 3 independent experiments. (*t* test) $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. HCC, hepatocellular carcinoma.

qRT-PCR analysis indeed showed a highly significant downregulation of *Klf9* and *TRβ* in HCCs *vs.* surrounding livers and a strong positive Pearson correlation between the 2 genes (Fig. S9A,B).

To investigate whether a similar correlation also exists in human HCC – and thus if this finding has translational value – *KLF9* and *TRβ* mRNA levels were determined in a cohort of 45 patients subjected to HCC resection (study population characteristics are described in Table S1). As observed in rats, *TRβ* and *KLF9* were downregulated in 66% and 60% of human HCCs, respectively, when compared to matched non-cancerous cirrhotic tissues (Fig. S9C,D, left panels). Importantly, a significantly positive correlation between *KLF9* and *TRβ* levels was observed (Fig. S9C, right panels). Notably, in both rat and human HCCs we also found a highly significant positive correlation between *TRβ* and *KLF9*, and between *HNF4a* and *KLF9* (Fig. S9A-D, right panels).

The antitumorigenic effect of T3 is long-lasting and is maintained upon hormone removal

To investigate whether the inhibition of HCC progression observed in T3-fed rats is a reversible process that depends on the presence of exogenous T3 or it is long-standing, one further group of animals was switched back to basal diet following the 5th cycle with T3-supplemented diet and sacrificed 1 month afterward (See Fig. S2B). The results showed that the livers of rats exposed to the hormone were smaller than those of untreated animals and displayed very few macroscopic tumors even after T3 withdrawal (Fig. 7A). Light microscopy analysis showed that the average neoplastic area remained significantly reduced in T3-treated animals (Fig. 7B). Intriguingly, the T3/TR axis was still partially restored compared to untreated animals, as evidenced by the significantly higher expression of *Dio1* and *Spot14*, even 1 month after T3 withdrawal (Fig. 7C,D). Of note, the levels of the neoplastic marker *Krt-19* did not significantly increase following T3 removal but stayed at levels similar to those in rats killed immediately after the 5th cycle, remaining downregulated compared to T3-untreated rats (Fig. 7E).

T3-induced metabolic rewiring precedes preneoplastic nodule regression

Recent evidence supports a link between metabolism and cell differentiation.[21–23] Based on our previous findings that aggressive preneoplastic lesions and HCCs are characterized by a rewiring of cellular metabolism towards an enhanced glycolytic phenotype (*i.e.* Warburg),[24] we investigated whether the T3-prodifferentiative effect was associated with a rerouting of metabolites from a Warburg metabolism to oxidative phosphorylation (OXPHOS).

We first analyzed the expression profile of the 16 metabolic genes involved in glycolysis, significantly altered in preneoplastic lesions in the array described in Fig. 1A. Unsupervised hierarchical cluster analysis stratified rat lesions into 2 major clusters: i) preneoplastic KRT-19+ lesions, and ii) normal liver and preneoplastic KRT-19+ lesions exposed to T3 for 2 or 4 days (Fig. 8A). Interestingly, T3 treatment severely affected the expression of metabolic genes as early as 2 days after treatment, a time when no major effect in the global expression profile was observed (Fig. 1A). The most marked T3-induced changes were observed in genes belonging to the pentose phosphate pathway (PPP). Indeed, the expression levels of glucose-6-phosphate dehydrogenase (*G6pd*, the rate-limiting enzyme of the oxidative branch of the PPP), phosphogluconate dehydrogenase (*Pgd*) and Transaldolase1 (*Taldo1*) were significantly downregulated by T3 (Fig. 8A).

Along with diversion of glycolytic metabolites into the PPP, mitochondrial respiration is impaired in preneoplastic lesions.[24] Crucially, T3 administration rapidly restored the activity of succinate dehydrogenase (SDH), an indicator of mitochondrial functionality, hence impacting the metabolic phenotype of preneoplastic lesions (Fig. 8B).

T3 administration reverts HCC metabolic profile

Next, we assessed if the inhibitory effect of T3 on HCC progression is accompanied by a reversion of the tumor metabolic profiles. One-week T3 treatment of HCC bearing rats (10 months after DEN) rescued normal glycolytic activity, as revealed by decreased expression of hexokinase 2 (*Hk2)* and glucose

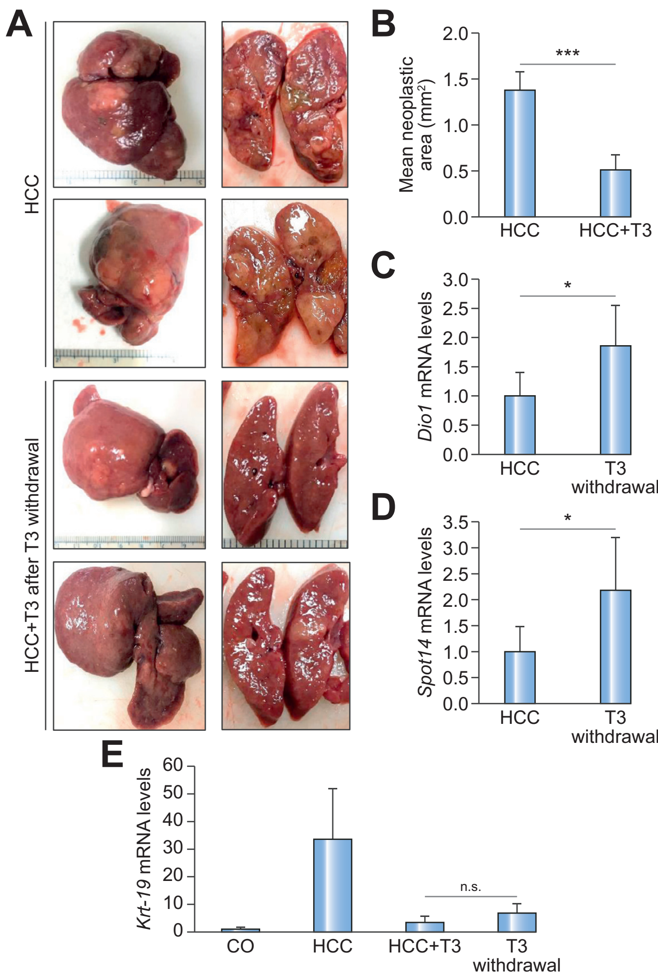

Fig. 7. T3 antitumorigenic effect persists after hormone withdrawal. (A) Livers of rats treated with 5 cycles of T3-supplemented diet and killed 1 month after the last T3 cycle. (B) Average liver area occupied by neoplastic hepatocytes. (*t* test) (C-E) qRT-PCR analysis of *Dio1, Spot14, Krt-19* mRNA levels in the livers of the rats described in A. For each liver, qRT-PCR analysis was performed on 25 randomly cut sections obtained from different segments (treated or untreated with T3). Gene expression is reported as fold-change relative to livers from untreated rats. The histogram represents mean values + SD of 6 rats/group. (*t* test and ANOVA) n.s., not significant. *p <0.05; ***p <0.001. qRT-PCR, quantitative reverse transcription PCR.

transporter-1 (*Glut1*) (Fig. S10A). As observed in preneoplastic nodules, T3 also strongly downregulated the expression and activity of *G6pd* (Fig. S10B), *Taldo1* and Transketolase (*Tkt*) levels in these tumors (Fig. S10C).

Based on the finding that G6PD and Taldo1 are targets of the transcription factor Nrf2, PPP activation has been proposed as one of the mechanisms by which deregulation of Nrf2/Keap1 signaling promotes tumorigenesis.[25] Since T3 strongly decreased *G6pd, Taldo1 and Tkt* expression (Fig. 8D), we investigated whether thyroid hormone could impact the Nrf2-Keap1 pathway. As shown in Fig. S11, while the Nrf2/Keap1 pathway was strongly activated in HCCs, after 5 cycles of the T3 regimen we observed a strong decrease of the mRNA (Fig. S11A) and protein levels (Fig. S11B) of *Nqo1*, the best known Nrf2 target gene, proving Nrf2 pathway inhibition.

Enzymatic activity assays displayed increased activity of complex I and II of the respiratory chain in liver tissues belonging to the hormone-treated group (Fig. S10D). These results are in line with a previous study reporting that the reduction of glycolysis caused by *HK2* knockdown in hepatoma cell lines led to a compensatory upregulation of OXPHOS.[26]

T3-induced changes in metabolic genes were even more pronounced following 5 cycles of the T3-supplemented diet (Fig. 8C,D). Indeed, we observed a clear switch from high *Hk2* expression to that of glucokinase (*Gck*), the enzyme that, in normal hepatocytes, catalyzes the first committed step in glucose metabolism.[27] This switch was accompanied by *Glut1* and *Mct4* downregulation (Fig.8C). A similar effect on metabolic genes was observed in HepG2 cells treated with T3 (Fig. S12A). Five cycles of T3 diet also reversed the enhanced expression of *Taldo1*, *Tkt* and *G6pd* usually associated with HCC (Fig. 8D). G6PD activity was also significantly decreased by T3 (Fig. 8D). T3's ability to interfere with PPP activation was also demonstrated in HepG2 and Mahlavu cells, as shown by decreased CO_2 production from glucose radiolabeled in position 1 (CO_2 derived from both OXPHOS and PPP) when subtracting glucose radiolabeled in position 6 (CO_2 exclusively derived from OXPHOS) (Fig. 8E and S12B). These findings are further reinforced by mass spectrometry analysis showing a significant reduction of ribose-5-phosphate (*i.e.* the final product of the oxidative branch of the PPP) in tumor tissue derived from HCC-bearing rats undergoing T3 treatment (Fig. S12C). In agreement with the results shown in Fig. S12C, enzymatic activity assays performed on the livers of rats exposed to 5 cycles of T3 exhibited increased activity of complex I and II of the respiratory chain (Fig. 8F). These data were further confirmed by histochemical analysis of serial frozen sections showing reactivation of SDH activity after T3 treatment (Fig. S13). Interestingly, the reduced levels of R5P in the T3-treated samples are accompanied by a significant increase in phosphoenol pyruvate (PEP) and pyruvate, metabolites that can fuel the tricarboxylic acid cycle, assayed using GC-MS. Indeed, citrate and malate levels are increased and can be considered as a readout of an enhanced tricarboxylic acid cycle (Fig. S12C).

Discussion

The most important findings achieved in this study are: i) fully developed HCCs are responsive to T3, ii) giving repeated cycles of T3 to HCC-bearing rats strongly impairs HCC progression, iii) the antitumorigenic effect of T3 is durable, lasting after T3 removal, iv) re-differentiation and the metabolic switch from glycolysis to OXPHOS precede the regression of early lesions and impair HCC progression, v) the T3/TR axis is altered in human HCCs.

Thyroid hormones and their receptors are involved in differentiation and metamorphosis[28] and T3 accelerates differentiation of oval/liver progenitor cells to hepatocytes, leading to loss of oval cell-specific markers and acquisition of a mature hepatocyte phenotype.[29]

In the RH model used in the present study, the biochemical phenotype of preneoplastic nodules resembles that of fetal hepatocytes, as these lesions lack the expression of enzymes normally present in differentiated hepatocytes, while exhibiting high levels of proteins poorly expressed/absent in fully differentiated hepatocytes.[30] Here, we demonstrate that T3 exerts its effects on preneoplastic nodules and HCCs by inducing genes responsible for acquisition/maintenance of hepatocyte differentiation, such as *Klf9* and *Hnf4a*, and by inhibiting genes expressed in less-differentiated hepatocytes, such as *Krt-19, Gstp, Ggt.* Our

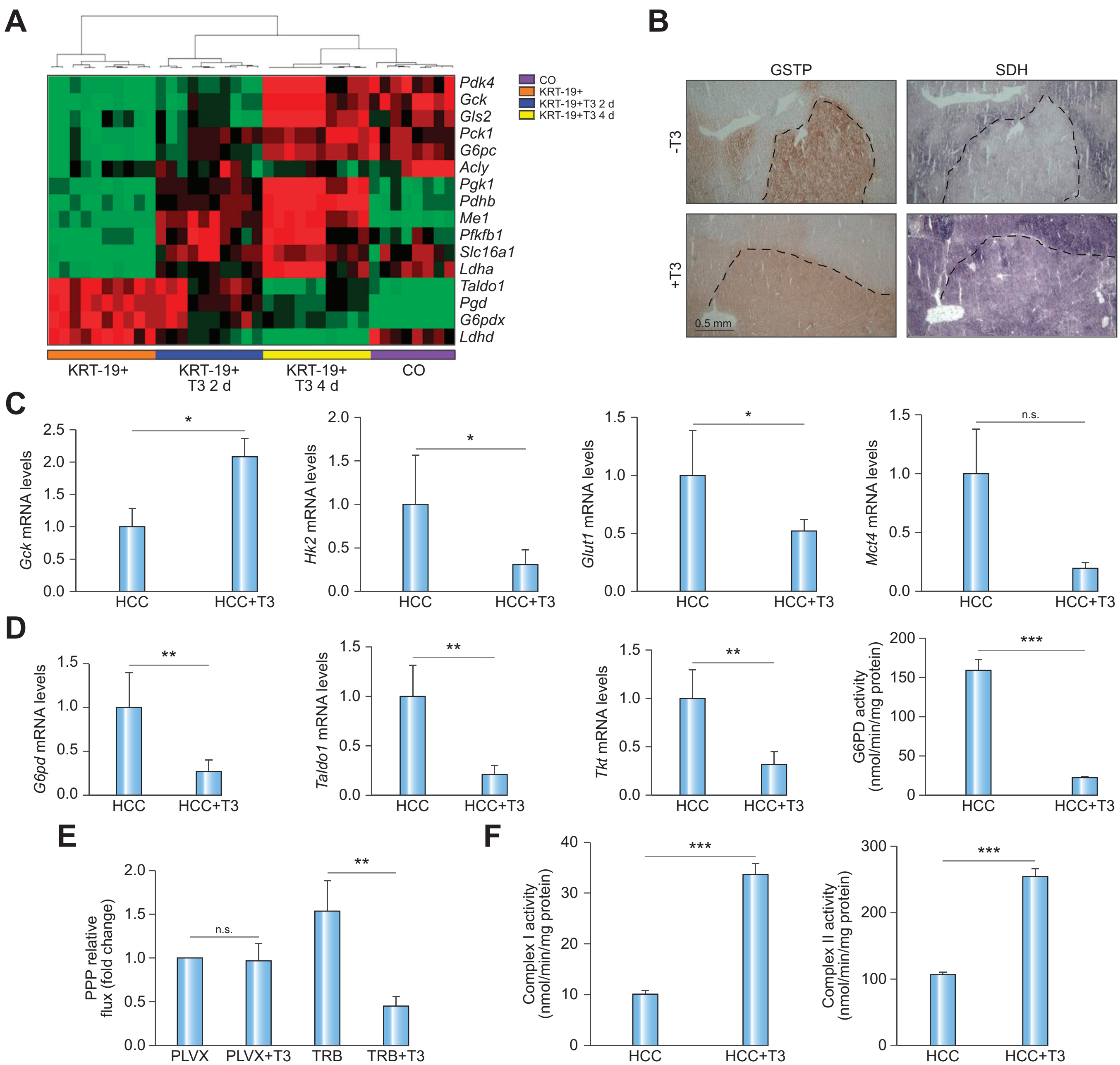

Fig. 8. T3 induces metabolic changes which precede preneoplastic lesion regression and impairment of HCC growth. (A) Hierarchical clustering of 16 genes involved in metabolic reprogramming in normal liver (CO), KRT-19+ preneoplastic lesions, and KRT-19+ lesions, treated with T3 for 2 (T3 2d) or 4 days (T3 4d). Each row represents the expression of a gene and each column a sample. Red and green colors represent higher or lower mRNA expression levels (median-centered), respectively. (B) Histochemical reaction evaluating SDH activity in preneoplastic lesions untreated (−T3) or T3-treated (+T3) for 4 days (X5); (C) qRT-PCR analysis of the glycolytic genes *GCK, HK2, Glut1* and *Mct4* mRNA levels in the livers of rats killed 13.5 months after DEN, with (HCC+T3) or without (HCC) 5 cycles of T3. Gene expression is reported as fold-change relative to livers from untreated rats. The histogram represents mean values + SD of 6/8 livers/group. (*t* test) (D) qRT-PCR analysis of *G6pd, Taldo1, Tkt* performed and reported as in C, and G6PD activity in livers of rats treated as in C. (*t* test) (E) Radioactive assay using [14C]-glucose labeled in position C1 or in position C6. Radiolabeled glucose was added for the last hour to HepG2 cells that were cultured either in the presence or absence of T3 for 72 h, before subjecting to CO_2 radioactive analysis (see Methods). PPP CO_2 production results from subtracting the radioactive signal derived from [1-^{14}C]-glucose to that of [6-^{14}C]-glucose. (*t* test) (F) Complex I and complex II activity in the livers of rats treated as in C. (*t* test) n.s., not significant; **p* <0.05; ***p* <0.01; ****p* <0.001. PPP, pentose phosphate pathway; qRT-PCR, quantitative reverse transcription PCR.

data also imply that the TR/KLF9 network is involved in the reactivation of the differentiation program. The Sp1/KLF transcription factor family plays different roles in cellular growth, development, differentiation and inflammation.[18,20,31] In particular, *Klf9* expression greatly influences cell differentiation processes. Recently, Cvoro *et al.*[20] showed that TRs cooperate with KLF9 to regulate hepatocyte differentiation and TR activation leads to KLF9 induction in transformed and non-transformed liver cells, and in stem cells. *KLF9* was down-regulated in human HCCs while KLF9 transduction in HCC cell lines inhibited proliferation and tumorigenesis.[32] Remarkably, we observed a strong decrease of *Klf9* mRNA levels in preneoplastic lesions and HCCs, paralleled by reduced TRβ expression, and its significant upregulation following T3 treatment. These data were confirmed in an additional model of rat hepatocarcinogenesis and in human HCCs. The finding that T3 impaired growth of human hepatocarcinoma cell lines and that this effect was partially reverted upon *KLF9* silencing supports a role of the TR/KLF9 axis in the antitumoral effect of T3.

Interestingly, in the model of HCC+T3 administration and T3 withdrawal we observed that the antitumoral effects were maintained 1 month after T3 removal. We hypothesize that restoration of the T3/TR axis, persisting even at 1 month after T3 withdrawal – as evaluated by the increased expression of Dio1 and Spot14 – might in part explain the long-lasting activity of thyroid hormone.

Another important observation stemming from this study is that a metabolic switch from glycolysis to oxidative-dependent metabolism is an early event in the antitumorigenic effect exerted by T3. This is in line with the notion that differentiation requires a fundamental shift in the metabolic landscape of the cell.[33] Unlike normal cells, most neoplastic cells undergo an important metabolic shift, known as the Warburg effect, in which glucose utilization is favored and OXPHOS is downregulated, even when oxygen availability is plentiful.[34] Nwosu *et al.* found more than 600 consistently altered metabolic genes in human HCCs.[35] Moreover, in the R-H model, metabolic reprogramming is a feature observed from very early stages that persists up to fully developed HCC, suggesting that it has critical role in HCC onset and progression. Concerning T3, several reports have described its effect on the metabolic behavior of cancer cells. Indeed, T3 sensitized mitochondrial metabolism in triple-negative breast cancer cells displaying a marked Warburg effect.[36] In addition, T3 was shown to increase mitochondrial function and respiration in HCC cells.[37,38] In our study, the most marked T3-induced changes affected PPP, in particular G6PD, the limiting enzyme of the PPP oxidative branch.[39,40] G6PD expression was completely abolished after T3 treatment, both in nodules and in HCCs, and remained low after T3 removal. Notably, in human HCC, G6PD expression is upregulated and associated with high tumor grade, metastasis and poor overall survival.[24]

Our findings demonstrate that T3 acts as a potent antitumoral agent and are in line with other reports suggesting that T3 administration interferes with HCC development.[38,41] In fact, T3 suppressed HCC onset in DEN-treated mice via activation of mitophagy[37] and in HBV-encoded X protein-induced hepatocarcinogenesis.[38]

Collectively, these findings shed light on the possibility of targeting the T3/TR axis in HCC therapy. Unfortunately, T3-based therapies often result in undesired side effects, particularly cardiac dysfunctions,[42] which hamper their clinical use. Nonetheless, new thyroid hormone analogs devoid of the cardiac toxic effects of thyroid hormone are now available and worth testing in clinical trials.

Abbreviations

2-AAF, 2-acetylaminofluorene; ATPase, adenosine triphosphatase; Cas-3, caspase 3; ChIP, chromatin immunoprecipitation; CMD, choline-devoid methionine-deficient; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; DEN, diethylnitrosamine; DIO1, deiodinase 1; G6Pase, glucose-6-phosphatase; G6PD, glucose-6-phosphate dehydrogenase; GCK, glucokinase; GGT, gamma glutamyl transpeptidase; GLUT1, glucose transporter 1; GST-P, placental form of glutathione-S-transferase; HCC, hepatocellular carcinoma; HK2, hexokinase 2; HNF, hepatocyte nuclear factor; IPA, Ingenuity Pathway Analysis; KLF9, Kruppel-like factor 9; KRT-19, cytokeratin-19; MCT4, monocarboxylate transporter 4; OXPHOS, oxidative phosphorylation; NQO1, NAD(P)H quinone dehydrogenase 1; PCK1, phosphoenolpyruvate carboxykinase 1; PDK1, pyruvate dehydrogenase kinase 1; PEP, phosphoenolpyruvate; PGC-1a, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PGK1, phosphoglycerate kinase 1; PPP, pentose phosphate pathway; qRT-PCR, quantitative reverse transcription PCR; R5P, ribose 5-phosphate; R-H, resistant hepatocyte; SDH, succinate dehydrogenase; T3, 3,5,3'-triiodo-L-thyronine; T4, 3,5,3',5'-tetraiodo-L-thyronine; TALDO1, transaldolase 1; TKT, transketolase; TR, thyroid hormone receptor.

Financial support

This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC, Grants IG-20176 to AC; IG 20210 to SG; IG-20749 to AR, AIRC and Fondazione Cassa di Risparmio di Firenze 19515 to AM); Fondazione Banco di Sardegna to AC and AP. MAK is a recipient of Fondazione Umberto Veronesi fellowship. The work of AM was funded by Associazione Italiana Ricerca sul Cancro and Fondazione Cassa di Risparmio di Firenze (Multiuser program - grant 19515).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest concerning this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

MAK, LC, EP, CO; performed the in vivo experiments and analyzed data. APet, EP; performed in vitro experiments. SM, AF; performed the mouse experiment with inoculated hepatoma cells; LG, FF; provided human HCC and analyzed data. PS; performed bioinformatics and data analysis. APer, GMLC; performed in vivo experiments, histopathologic classification and contributed to the study design. AR, CSM; performed studies on the activity of G6PD and mitochondrial Complex I and Complex II. PS; performed bioinformatics analyses. AM; performed the radioactive assay. MP; performed the metabolomics assay; CS, SEB; performed ChIP assay; AC, SG; conceived and supervised the study, provided funding, wrote the manuscript.

Acknowledgements

We thank Dr. VP Leoni and Dr. M. Serra for their valuable technical support.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.12.018.

References

Author names in bold designate shared co-first authorship

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.

[2] Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–390.

[3] Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev 2010;31:139–170.

[4] Brent GA. The molecular basis of thyroid hormone action. N Engl J Med 1994;331:847–853.

[5] **Davis PJ**, **Goglia F**, **Leonard JL**. Nongenomic actions of thyroid hormone. Nat Rev Endocrinol 2016;12:111–121.

[6] Lazar MA. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev 1993;14:184–189.

[7] Hassan MM, Kaseb A, Li D, Patt YZ, Vauthey JN, Thomas MB, et al. Association between hypothyroidism and hepatocellular carcinoma: a case-control study in the United States. Hepatology 2009;49:1563–1570.

[8] Reddy A, Dash C, Leerapun A, Mettler TA, Stadheim LM, Lazaridis KN, et al. Hypothyroidism: a possible risk factor for liver cancer in patients with no known underlying cause of liver disease. Clin Gastroenterol Hepatol 2007;5:118–123.

[9] Cable EE, Finn PD, Stebbins JW, Hou J, Ito BR, van Poelje PD, et al. Reduction of hepatic steatosis in rats and mice after treatment with a liver-targeted thyroid hormone receptor agonist. Hepatology 2009;49:407–417.

[10] **Frau C**, **Loi R**, Petrelli A, Perra A, Menegon S, Kowalik MA, et al. Local hypothyroidism favors the progression of preneoplastic lesions to hepatocellular carcinoma in rats. Hepatology 2015;61:249–259.

[11] Aranda A, Martínez-Iglesias O, Ruiz-Llorente L, García-Carpizo V, Zambrano A. Thyroid receptor: roles in cancer. Trends Endocrinol Metab 2009;20:318–324.

[12] **Liao CH**, **Yeh CT**, Huang YH, Wu SM, Chi HC, Tsai MM, et al. Dickkopf 4 positively regulated by the thyroid hormone receptor suppresses cell invasion in human hepatoma cells. Hepatology 2012;55:910–920.

[13] **Angioni MM**, **Bellofatto K**, Merlin S, Menegon S, Perra A, Petrelli A, et al. A long term, non tumorigenic rat hepatocyte cell line and its malignant counterpart, as tools to study hepatocarcinogenesis. Oncotarget 2017;8:15716–15731.

[14] **Petrelli A**, **Perra A**, **Cora D**, Sulas P, Menegon S, Manca C, et al. MicroRNA/gene profiling unveils early molecular changes and nuclear factor erythroid related factor 2 (NRF2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). Hepatology 2014;59:228–241.

[15] Sebastián C, Serra M, Yeramian A, Serrat N, Lloberas J, Celada A. Deacetylase activity is required for STAT5-dependent GM-CSF functional activity in macrophages and differentiation to dendritic cells. J Immunol 2008;180:5898–5906.

[16] Ledda-Columbano GM, Perra A, Loi R, Shinozuka H, Columbano A. Cell proliferation induced by triiodothyronine in rat liver is associated with nodule regression and reduction of hepatocellular carcinomas. Cancer Res 2000;60:603–609.

[17] Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. Hepatology 2003;38:1331–1347.

[18] Denver RJ, Williamson KE. Identification of a thyroid hormone response element in the mouse Kruppel-like factor 9 gene to explain its postnatal expression in the brain. Endocrinology 2009;150:3935–3943.

[19] Cui A, Fan H, Zhang Y, Zhang Y, Niu D, Liu S, et al. Dexamethasone-induced Krüppel-like factor 9 expression promotes hepatic gluconeogenesis and hyperglycemia. J Clin Invest 2019;129:2266–2278.

[20] Cvoro A, Devito L, Milton FA, Noli L, Zhang A, Filippi C, et al. A thyroid hormone receptor/KLF9 axis in human hepatocytes and pluripotent stem cells. Stem Cells 2015;33:416–428.

[21] **Bracha AL**, **Ramanathan A**, Huang S, Ingber DE, Schreiber SL. Carbon metabolism-mediated myogenic differentiation. Nat Chem Biol 2010;6:202–204.

[22] White KP, Hurban P, Watanabe T, Hogness DS. Coordination of Drosophila metamorphosis by two ecdysone-induced nuclear receptors. Science 1997;276:114–117.

[23] McGraw TE, Mittal V. Stem cells: metabolism regulates differentiation. Nat Chem Biol 2010;6:176–177.

[24] **Kowalik MA**, **Guzzo G**, **Morandi A**, Perra A, Menegon S, Masgras I, et al. Metabolic reprogramming identifies the most aggressive lesions at early phases of hepatic carcinogenesis. Oncotarget 2016;7:32375–32393.

[25] **Mitsuishi Y**, **Taguchi K**, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell 2012;22:66–79.

[26] DeWaal D, Nogueira V, Terry AR, Patra KC, Jeon SM, Guzman G, et al. Hexokinase-2 depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin. Nat Commun 2018;9:446.

[27] Wilson JE. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J Exp Biol 2003;206:2049–2057.

[28] Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev 2001;81:1097–1142.

[29] László V, Dezso K, Baghy K, Papp V, Kovalszky I, Sáfrány G, et al. Triiodothyronine accelerates differentiation of rat liver progenitor cells into hepatocytes. Histochem Cell Biol 2008;130:1005–1014.

[30] Roomi MW, Ho RK, Sarma DS, Farber E. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. Cancer Res 1985;45:564–571.

[31] Cao Z, **Sun X**, **Icli B**, **Wara AK**, Feinberg MW. Role of Kruppel-like factors in leukocyte development, function, and disease. Blood 2010;116:4404–4414.

[32] Sun J, Wang B, Liu Y, Zhang L, Ma A, Yang Z, et al. Transcription factor KLF9 suppresses the growth of hepatocellular carcinoma cells in vivo and positively regulates p53 expression. Cancer Lett 2014;355:25–33.

[33] Agathocleous M, Harris WA. Metabolism in physiological cell proliferation and differentiation. Trends Cell Biol 2013;23:484–492.

[34] Warburg O. On the origin of cancer cells. Science 1956;123:309–314.

[35] Nwosu ZC, Megger DA, Hammad S, Sitek B, Roessler S, Ebert MP, et al. Identification of the consistently altered metabolic targets in human hepatocellular carcinoma. Cell Mol Gastroenterol Hepatol 2017;4:303–323.e1.

[36] Suhane S, Ramanujan VK. Thyroid hormone differentially modulates Warburg phenotype in breast cancer cells. Biochem Biophys Res Commun 2011;414:73–78.

[37] Sinha RA, Singh BK, Zhou J, Wu Y, Farah BL, Ohba K, et al. Thyroid hormone induction of mitochondrial activity is coupled to mitophagy via ROS-AMPK-ULK1 signaling. Autophagy 2015;11:1341–1357.

[38] Chi H-C, Chen S-L, Lin S-L, Tsai C-Y, Chuang W-Y, Lin Y-H, et al. Thyroid hormone protects hepatocytes from HBx-induced carcinogenesis by enhancing mitochondrial turnover. Oncogene 2017;36:5274–5284.

[39] Patra KC, Hay N. The pentose phosphate pathway and cancer. Trends Biochem Sci 2014;39:347–354.

[40] Kowalik MA, Columbano A, Perra A. Emerging role of the pentose phosphate. Pathway in hepatocellular carcinoma. Front Oncol 2017;7:87.

[41] Chi H-C, Chen S-L, Tsai C-Y, Chuang W-Y, Huang Y-H, Tsai M-M, et al. Thyroid hormone suppresses hepatocarcinogenesis via DAPK2 and SQSTM1-dependent selective autophagy. Autophagy 2016;12:2271–2285.

[42] Klein I, Ojamaa K. Thyroid hormone and the cardiovascular system. N Engl J Med 2001;344:501–509.