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Thyroid Hormone in the Regulation of Hepatocellular Carcinoma and its Microenvironment

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Abbreviations: ALT, alanine amino transferase; BBC, basal cell carcinoma; CCL4, carbontetrachloride; CD, choline-deficient; CDK2, cyclin-dependent kinase; CSC, cancer stem cell DKK, dickkopf Wnt signaling inhibitor 4; DEN, diethylnitrosamine; DIO1-3, iodothyronine deiodinases; ECM, extracellular matrix; GSTP: glutathione S-transferase-positive ; HCC, hepatocellular carcinoma; HFD, high-fat diet; HSC, hepatic stellate cells; LPR5/6: low-density lipoprotein receptor-related protein; MF, myofibroblasts; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NCoR, nuclear receptor corepressor; PKA, protein kinase A; SMRT: silencing mediator for retinoid or thyroid-hormone receptors; RXR, retinoid X receptor; SBE, smad binding element, SRC: steroid receptor coactivator; Shh, sonic hedgehog; SMAD, mothers against decapentaplegic; STMN1, stathmin; rT3, reverse T3; T3, triiodothyronine; T4, thyroxine; TGF-β, transforming growth factor beta; TH, thyroid hormone; TR, thyroid hormone receptor TRE, thyroid hormone response element.

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Introduction

Liver cancer is the sixth most common cancer worldwide, with more than 782,500 new cases diagnosed in 2012 [1]. Although the incidence of hepatocellular carcinoma (HCC), the primary form of liver cancer, varies according to gender, etiology, age, and geographic region, it typically develops in a microenvironment that is characterized by pro-inflammatory, pro-angiogenic, and pro-fibrotic milieus. Liver fibrosis is a repair response to chronic injury that is recognized as the underlying pathogenic driver of carcinogenesis. Therefore, factors stimulating liver fibrosis may be potential therapeutic targets to limit tumor progression.

Several reports suggest that extrahepatic factors are key regulators of liver repair [2–5]. Dysregulation of thyroid hormone (TH) homeostasis and downstream signaling pathways have been shown to influence liver fibrogenesis, and accumulating data suggest that aberrant expression or mutations of the thyroid hormone receptor (TR) are associated with the development of human neoplasia. However, the association between TH and cancer remains controversial, with some investigators reporting that hyperthyroidism promotes either cancer development or progression [6–8], whereas others have reported a tumor suppressive role of TH [9].

The mitogenic effects of triiodothyronine (T3) have been extensively studied *in vivo* [10–13]. However, the effectiveness on normal hepatocytes *in vitro* has not been definitively established. As this criterion has not been met, it remains controversial whether T3 should be considered as a direct mitogen in the liver [10,14]. Nonetheless, T3 is well known for ameliorating liver regeneration after partial hepatectomy in rodent models [15–19]. In accordance with these findings, TH can be an important determinant of the regeneration process.

In contrast, T3 seems to have different effects on liver cancer cell growth as it inhibits liver cancer cell growth *in vitro* [20,21]. Moreover, clinical findings support the hypothesis of a procarcinogenic effect of hypothyroidism, as case-control studies demonstrated an independent positive association between hypothyroidism and HCC development [22,23].

Recent studies show that the tumor microenvironment plays an important role in regulating tumor growth and shaping tumor response to therapy **(reviewed in** [24]**)**. The liver tumor microenvironment consists of multiple cell types and the extracellular matrix (ECM). Activated hepatic stellate cells (HSC) or myofibroblasts (MF) are the major cell types responsible for the secretion of collagen, laminin, and elastin that constitute the ECM. Other stromal cell types include bone marrow-derived fibrocytes, resident portal fibroblasts, liver progenitor cells, as

well as resident and recruited immune cells which secrete cytokines and chemokines that modulate inflammatory and fibrogenic responses [25,26].

In this review, we will discuss the potential impact of TH on liver cancer biology and its effects on the tumor microenvironment. We will attempt to reconcile the apparent discrepant reports of TH-induced effects on cancer cells and will discuss how TH and related pathways modulate cancer cell proliferation, invasion, and metastasis.

Molecular basis of TH action

T3 and L-thyroxine, T4 are the two major thyroid hormones being critical for tissue and organ development, cellular growth, differentiation and (lipid-)metabolism [27]. The primary circulating thyroid hormone, T4 (the prohormone), is deiodinated within cells by iodothyronine deiodinases type I and type II (Dio1, Dio2) to become biologically active T3. In contrast, deiodinase type III (Dio3) reduces intracellular thyroid activity by degrading T4 and T2 into the "inactive" metabolites reverse T3 (rT3) and T2, respectively [28].

On entering the nucleus, the gene-regulating activity of T3 is mediated by binding to specific DNA sequences, known as thyroid hormone response elements (TREs), located on the promoter regions of thyroid hormone target genes **(Figure 1).** The two major thyroid receptor isoforms, thyroid hormone receptor α and β (TRα and TRβ), have tissue-specific distribution. While TRβ mediates the metabolic actions of T3 and is the known major receptor isoform expressed in the liver (hepatocytes), TRα is expressed predominantly in the heart, skeletal muscle, and adipose tissues, and specifically mediates adaptive thermogenesis. Transporter molecules such as MCT8 or OATP1 transport T4 and T3 into the cell. Unbound TR may heterodimerize with retinoid X receptor (RXR), which then binds to a TRE and to a corepressor complex. These corepressors include nuclear receptor co-receptor 1 (NCoR1) and silencing mediator for retinoid or thyroid-hormone receptors (SMRT), which may act to repress positively regulated genes and activate negatively regulated genes [27] **(Figure 1)**. T3-binding to the ligand-binding domain results in the movement of the carboxy-terminal helix 12, disruption of corepressor binding, and promotion of coactivator binding (among others, these include: steroid receptor coactivator 1 (SRC1), SRC2, and p300/CBP) which then leads to recruitment of polymerase III and initiation of positively regulated gene transcription [28].

Linking thyroid hormone and its receptors to chronic liver disease

TH is a major regulator of lipid metabolism [29–32]. By binding the cognate TRs, TH regulates cholesterol and carbohydrate metabolism through direct actions on gene expression. TH also

modulates hepatic insulin sensitivity, which is important for the suppression of hepatic gluconeogenesis **(reviewed in** [27]**)**.

Among individuals with non-alcoholic fatty liver disease (NAFLD), a condition characterized by perturbations in lipid metabolism and cellular injury, the prevalence of hypothyroidism is reported to range between 15.2% and 36.3% [33]. A population-based study reported that the prevalence of NAFLD and elevated alanine aminotransferase (ALT) – a proxy for liver inflammation, is higher among patients with hypothyroidism [34]. Moreover, hypothyroidism was also detected in patients with biopsy-proven nonalcoholic steatohepatitis (NASH) compared with simple steatosis [35]. Further evidence supporting the association between the severity of chronic liver disease and hypothyroidism is provided by a larger population-based, prospective cohort study from the Netherlands [36]. In this study, the investigators showed that elevated T4 levels were associated with a lower risk of NAFLD, while higher TSH levels were associated with an increased risk of liver fibrosis. Intriguingly, NAFLD risk decreased when TH levels increased (i.e. from hypothyroid state to hyperthyroid state) [36]. Apart from NAFLD, differences in TH levels have also been described for other chronic liver diseases. For example, hypothyroidism is more common among those with chronic HCV compared to healthy individuals, and higher TSH levels are also more common among those with more advanced liver fibrosis (compared with early fibrosis) [37,38]. A summary of these clinical studies is described in **table 1.**

In support of the above clinical observations are the studies performed in transgenic animal models. Mice with a thyroid receptor alpha (TRα) mutation (i.e., TRα-P398H mutant) exhibit hepatic steatosis and glycogen depletion in the liver [39]. The administration of a TRβ-selective agonist (GC-1, KB2115) reduces liver steatosis in genetic and dietary-induced models of obesity and NAFLD in mice and rats [40,41]. In a mouse model of advanced NASH-cirrhosis and cancer, the administration of T3 reduced liver triglycerides, repressed liver inflammation, and attenuated injury. Similar benefits were observed with TRβ-agonist GC-1 without significant effects on the heart, muscle, or the overall catabolic state [41]. Comparable outcomes were also seen when MB07811 (a liver-targeted TRβ agonist) was tested in rodent models of NAFLD. MB07811 treatment reduced liver steatosis and lowered plasma free fatty acid, triglyceride, and serum AST while upregulating lipid metabolism genes [42]. Finally, similar phenotypes were also noted in rabbits, where hypothyroidism induced moderate NASH [43].

Taken together, the findings described above illustrate the importance of TH in regulating chronic liver disease and the potential of TH/TR interaction to be a target for treatment of NASH/NAFLD.

TH and liver cancer

Other than NASH/NAFLD, hypothyroidism has also been reported to be associated with obesity and metabolic syndrome, all considered risk factors for the development of HCC, the primary form of liver cancer [44,45]. The association between hypothyroidism, NAFLD, and HCC is exemplified in a study of 160 HCC patients [23]. Hypothyroidism was more prevalent among those with unknown liver etiology than those with HCV or alcoholic liver disease related HCC, after adjustment for confounding factors (hypothyroidism was defined as TSH>5.0, history of hypothyroidism before HCC diagnosis, or a history of being on thyroid replacement at the time of HCC diagnosis) [23]. In a separate case-control study, hypothyroidism has been shown as an independent risk factor for HCC. Specifically, a history of hypothyroidism was associated with a 2-to-3-fold increased risk of cancer development in women. No such relationship, however, was found in men (see **Table 1**) [22,23,34–36,46–48]. The role of TH and TRs in HCC is further supported by studies describing the association between somatic TR mutation and human neoplasia **(reviewed in** [49]**)**. In an earlier study, it was shown that naturally occurring TRα mutations (V390A) (E350K/P398S) from HCCs of two patients abrogate the functions of TRs via a dominant negative effect. Indeed, TRE binding of those TRα mutants was reduced up to 90% compared to wild-type TRα1. Although differences in binding are dependent on the type of TR mutation, both mutants lost transcriptional activity and expressed dominant-negative functions [50]. In a later study, 9 out of 17 (53%) human HCC specimens presented different forms of somatic mutation including truncated cDNAs and point mutations. Unsurprisingly, all these TR mutants exhibited impaired TRE binding and loss of transcriptional activity [51]. Although no mechanistic information was provided in this study, findings were comparable to earlier studies of liver cancer cell lines (J7, HepG2, SK-Hep), where mutated TRs were unable to exchange coactivators for corepressors in response to physiological concentrations of T3, thereby resulting in a continued (dominant negative) inhibition of target genes (in contrast to wild-type TR) [52].

In a more recent study, HCC-derived TR cDNA mutants were individually transfected into a hepatoma cell line to functionally characterize their transcriptional and DNA recognition properties [53]. Confirming early studies, the majority of these 'HCC occurring' mutations were associated with a loss of transcriptional activation in response to T3. Moreover, TRα

mutants in HCC predominantly acted as dominant negative inhibitors at all levels of T3 concentration, while TRβ mutants exerted a dominant negative effect only at low and intermediate T3 levels. Interestingly, HCC-derived TR mutants repressed only a subset of the genes normally repressed by wild-type TRs in the absence of T3, and some mutants distinctively acquired an ability to trigger the transcription of a novel set of target genes, not regulated by the wild-type TRs [54]. These findings suggest that mutant TRs have a distinct and specific role in oncogenesis.

This hypothesis, however, has recently been challenged by other studies, which have failed to identify any TR mutations in deep sequencing analysis of HCC tumors [29,55–58]. A subsequent study reported that publicly available RNAseq data from 442 human HCC specimens [59] did not show any mutation for TRα and only two for TRβ. This is supported by rodent data where, in chemically induced rat HCC, no TR mutations have been found [60,61].

To summarize, patients with HCC tend to present with hypothyroidism. The pathophysiological role of TR mutations in human HCC remains unclear due to divergent reports.

TH homeostasis and action as part of physiological and pathological responses

During acute injury, the remaining healthy adult liver cells (hepatocytes) enter the cell cycle and replicate to replace lost or dying hepatocytes [62]. If the regenerative capacity of this process is exceeded by massive parenchymal injury or ongoing chronic injury, resident liver progenitor cells also participate in the regenerative response. This “alternative" restoration of liver mass and function in response to hepatocyte loss involves activation of progenitor cells within the liver (i.e., progenitor-associated repair response or ductular reaction) [63–65], which proliferate and differentiate into new hepatocytes and cholangiocytes [66,67].

During this injury-induced regenerative process, many genes that are normally quiescent become re-activated, and this resembles processes that occur during fetal development. Some of these re-expressed ‘fetal’ genes include several deiodinases which are involved in the regulation of T3 levels. Levels of Dio3, for example, are upregulated during liver injury, resulting in a reduced tissue concentration of T3 and an increased hepatocyte proliferation [68]. Similarly, elevated levels of Dio3 are also detected in the developing fetal and cancer tissues [69–71]. On the other hand, Dio1 is downregulated during liver injury, and the combination of a high Dio3 and low Dio1 results in low T3 and high reverse T3 **(rT3; an inactive form of**

T3) which are conditions observed during critical illness (also known as sick euthyroid or low T3 syndrome). These observations suggest that biochemical hypothyroidism may be a normal physiological response to liver injury. As tumor (or HCC) growth evokes similar responses to development and injury, it is plausible that a hypothyroid state could favor cancer cell survival, proliferation, and differentiation [72–74].

Impairment of TH homeostasis alone, however, is insufficient for HCC development and/or progression [75,76]. HCC generally arises from an underlying background of chronic liver injury and cirrhosis (i.e., a pro-fibrogenic, pro-inflammatory microenvironment) and from the premalignant lesions which range from dysplastic foci to hepatocyte nodules. Perturbations in TH homeostasis may act synergistically with pro-inflammatory and pro-fibrogenic factors to promote a pro-carcinogenic microenvironment and stromal milieu. This hypothesis is supported by a recent study in a rat model of HCC which showed that down-regulation of TRα1 and TRβ1 is an early event in the tumorigenic process, suggesting that a hypothyroid status of preneoplastic hepatocytes favors their progression to HCC [60]. In agreement with these studies, Ledda-Columbano and colleagues demonstrated that the switch from hypothyroid to hyperthyroid conditions resulted in regression of preneoplastic lesions seven days after initiation of T3 supplementation [75].

These results clearly suggest that hypothyroidism affects tumor progression and that TR in HCC act as tumor suppressors. However, it remains to be seen if the effects of hypothyroidism are related to TH's action on the tumor cell, the surrounding stroma or both.

Impact of TH signaling in HCC development, cell proliferation, and survival

Despite compelling evidence showing that T3 stimulates normal hepatocyte proliferation in animal models of liver injury and healthy liver [10–13,15–19] **(Figure 2)**, T3 and agonists appear to exert opposite effect on local tumor progression (i.e., inhibitory effect on HCC development in *vivo* [75–77] or on proliferation in *vitro* [20,78]) **(Figure 3)**.

HCC development

In male Fisher rats with diethylnitrosamine (DEN)-induced HCC, treatment with T3 led to a reduction in the number of hyperplastic lesions. Specifically, rats that were switched to a one-week diet containing T3, nine weeks after DEN administration exhibited a 70% reduction in the number of placental glutathione *S*-transferase (GSTP)-positive (an early marker of preneoplastic lesions) nodules in the liver compared to controls which did not receive T3. In an extended study, continued exposure to T3 for 16 weeks resulted in 50% reduced incidence

of HCC and a complete prevention of lung metastasis in the "rat resistant hepatocyte" (R-H) liver carcinogenesis model [75,79]. Notably, the reduction in GSTP-positive nodules negatively correlated with an increase in hepatocyte proliferative activity, both within the residual GSTP-positive nodules (64% *versus* 42% of controls) as well as in the surrounding liver (31% *versus* 7% of controls) [75]. Comparable results were observed in another rat HCC model, whereby DEN administration was coupled with a choline-deficient (CD) diet for ten weeks, followed by administration of either T3 or TRβ agonist GC-1 for one additional week. Short-term treatment with T3 or GC-1 reduced the number of preneoplastic foci [76]. Interestingly, the same group also reported that TRα1 and TRβ1 expressions were downregulated in early preneoplastic lesions in the R-H model, implicating the importance of TH signaling in HCC progression [60].

HCC proliferation and growth

In cell culture experiments, the addition of T3 to hepatoma HepG2 cells overexpressing wild-type TRs inhibited cell proliferation. Those results indicate that T3 only significantly suppresses the growth of HepG2-TR overexpressing cells, while the control cell line (HepG2-Neo, no ectopic TR expression) does not exhibit any T3 repressive effect on proliferation. It was also shown that T3 represses hepatoma cell growth by lengthening the G1 phase of the cell cycle. This was associated with a decreased expression of the major cell cycle mediators cyclin-dependent kinase 2 (cdk2) and cyclin E, as well as enhanced transforming growth factor (TGF)-β gene expression [20]. Another study confirmed the antiproliferative effect of T3 on HepG2 cells, achieved via a suppressive transcriptional regulation of stathmin (STMN1), a recognized oncoprotein in various cancers [78].

These studies, demonstrating an antiproliferative effect of TRs on hepatoma growth and proliferation, are in striking contrast with early studies [80,81]. As mentioned before, hepatoma SK-Hep1 cells ectopically expressing TRβ show less proliferation after inoculation into *nude* mice compared to control SK-Hep1 cells. However, tumor growth is even more impaired when hepatoma cells (SK-Hep1-TRβ and SK-Hep1) are inoculated into hypothyroid hosts. These findings indicate that TRβ has anti-proliferative characteristics, and non-bound TRβ seems to enhance those antiproliferative effects. However, questions remain about the particular role of T3 in this context [80,81].

Metastasis and Invasiveness

Interestingly, administration of T3 also promotes the invasive and metastatic potential of

hepatoma cells. Treatment of hepatoma cell lines which express endogenous TRα and TRβ (Huh7, J7, Mahlavu) with T3 results in higher metastasis rates. Moreover, SCID mice which were inoculated with TRα-expressing HepG2 cells show higher metastasis rates in the liver and lung when treated with T3 [82].

By contrast, there are conflicting results from other studies which show different effects on invasion and metastasis. Firstly, TRβ1-expressing HCC (SK-Hep-TRβ1) xenografts displayed reduced tumor growth (number of cells expressing the proliferation marker Ki-67), less vascularisation, and a less mesenchymal phenotype compared with parental controls, when injected in nude mice. Importantly, most hepatoma cells which had lost TRβ spontaneously had metastasized, compared with only 20% of transduced TRβ1-expressing cells. Additionally, tumors in a hypothyroid host are of a more mesenchymal phenotype, are more invasive, and show a higher metastatic potential. When cells were inoculated into hypothyroid mice, tumors from both parental and TRβ1 expressing SK-cell had a more mesenchymal phenotype with reduction of keratin 8/18 and beta-catenin and an increase in vimentin expression. However, in those hypothyroid hosts, the percentage of cells with a mesenchymal phenotype was higher in the parental cells in comparison to the TRβ1 bearing cells. These results led to the conclusion that T3 may oppose metastasis [80], which is in line with the notion that hypothyroidism leads to a more mesenchymal phenotype of the tumors [81]. However, despite the contradictory findings, the role of unbound TRβ still remains to be elucidated. In particular, it remains unclear if unbound TRβ has a ligand-independent impact on the metastatic characteristic of hepatoma cells [80,81].

Thyroid status of the tumor and liver microenvironment

Apparently divergent effects on oncogenesis (e.g., proliferation, migration, invasion) and different findings between groups may be due to cell-specific reasons, but they also highlight that the overall effects of T3 in cancer should be regarded as the sum of individual effects on multiple cell types within the tumor stromal microenvironment. *In vivo* studies appear to demonstrate that microenvironmental changes in hormone signaling have a specific role. As discussed above, TRβ-expressing SK-Hep1 cells show less proliferation after inoculation into *nude* mice compared to control SK-Hep1 cells. The reduced growth is more pronounced when hepatoma cells are xenografted into hypothyroid hosts. These findings suggest that ligand-bound TRβ has an anti-proliferative function, and non-bound TRβ seems to enhance those anti-proliferative effects. Additionally, tumors in a hypothyroid host have a more mesenchymal phenotype, are more invasive, and metastatic growth is enhanced. However, as increased

malignancy was also observed in cells which barely express TRs, these results show that changes in the stromal cells secondary to host hypothyroidism can modulate tumor progression and metastatic growth independently of the presence of TRs on the tumor cells [81].

Xenografted tumors formed by TRβ-overexpressing hepatoma cells develop a collagen pseudocapsule which prevents invasion. Intriguingly, tumors formed in hypothyroid hosts showed changes in the ECM with signs of increased ECM degradation [81]. The authors of this study concluded that a hypothyroid condition in the microenvironment promotes the release of collagen fibers which facilitates the invasion of the surrounding tissue by the tumor. Notably, it is surprising how the hormone status of the microenvironment impacts the metastatic potential of the hepatoma cells irrespective of the TR status of the cancer cell itself. This underscores the microenvironment's impact on cancer progression [81].

Additional conclusions in regard to TH's impact on the liver microenvironment comes from animal models of liver injury. Hyperthyroid mice developed less liver fibrosis than control mice following chronic exposure to carbon tetrachloride (CCl4) [2]. By contrast, TRα1/TRβ double knockout mice developed spontaneous liver fibrosis as compared to littermate controls [2]. Furthermore, the administration of glucagon-T3 (which selectively delivers T3 to the liver) prevented liver fibrosis in mice fed with a choline-deficient, high-fat diet (CD-HFD) [83].

TGF-β-related liver fibrogenesis

Liver fibrosis is defined as a wound healing, repair response to chronic injury and is the key predictor of HCC development and progression [84]. Chronic hepatocyte damage triggers a cascade of molecular and cellular reactions aimed at removing or repairing damaged/dying cells and stimulating regeneration. Multiple cell types are involved in this wound-repair process, including immune cells, liver progenitors, and stromal cells [85,86]. TGF-β is one of the most important pro-fibrogenic cytokines that is upregulated in diseased livers [87]. Recent data provide evidence for a direct relationship between TH and TGF-β signaling in a fibrotic context [2]. In detail, Luciferase reporter assay experiments in rat pituitary GH4C1 cells (highly responsive to T3) provided evidence for a TGF-β antagonistic effect of T3 on the SMAD binding element (SBE). The antagonistic effect of T3 was also observed in other cell types (e.g., hepatoma TRβ-expressing HepG2 cells) [2]. Incubation with TGF-β or transfection of SMAD3 and SMAD4 induced transcriptional activation on known SBEs, whereas T3 administration attenuated this activation [2]. Furthermore, ectopic expression of either TRα or TRβ in lung epithelial cells caused some ligand-independent SBE activation, and T3 administration repressed both basal activity and transactivation by TGF-β or SMADs [2].

These studies suggest that both TRα and TRβ can mediate an antagonistic effect of T3 on TGF-β/SMAD signaling. The disruption of TGF-β/SMAD activity provides a possible mechanism for previously mentioned *in vivo* findings of higher fibrosis rates in hypothyroid and TR deficient mice. It gives proof that T3/TR influence hepatic stromal cell activity and that this is related to interaction with TGF-β signaling. However, the impact on specific cells in the hepatic stroma and the impact of this interaction on the development of HCC remains unclear. **Figure 4** provides an overview of the above-mentioned findings.

β-catenin/Wnt pathway:

Wnt/β-catenin has been implicated in abnormal wound repair and fibrogenesis. Moreover, it is decisive in the mechanism of proliferation and has been indicated to be important in HCC development. The hallmark of this pathway is the activation of the multifunctional protein beta-catenin. Canonical Wnt-signaling deactivates glycogen synthase kinase (GSK)-3β which prevents β-catenin phosphorylation. This leads to an accumulation of non-phosphorylated cytoplasmatic β-catenin, which then translocates to the nucleus to regulate target gene expression [88,89].

As already mentioned, T3 cannot be ultimately considered a direct mitogen as the *in vitro* criterion has not been definitively met. However, *in vivo* findings undoubtedly suggest a proliferative potential on hepatocytes. In part, this mitogenic response is mediated via protein kinase A (PKA)β-catenin activation [13]. Intriguingly, F344 rats and C57BL/6 mice fed with T3 did not only show enhanced hepatocyte proliferation, but also had increased cytoplasmic stabilization and nuclear translocation of β-catenin with a resulting increase in cyclin D1 expression (proliferation mediator) in a T3-dependent manner. Additionally, no mitogenic response was detected in mice with a hepatocyte-specific conditional knockout of β-catenin [13].

In addition, using a conditional liver-specific mouse model knocked out for β -catenin and Wnt receptor LPR5/6 (downstream effectors of canonical Wnt signaling), it was demonstrated that thyreomimetics like T3 and GC-1 promote hepatocyte proliferation and that this is dependent on β-catenin activation. In line with those findings, disruption of canonical Wnt signaling abolishes T3 and GC-1 dependent β-catenin activation [90]. This suggests that thyreomimetics (T3, GC-1) induce hepatocyte proliferation through β-catenin activation via both Wnt-dependent and PKA mechanisms and contribute to a regenerative advantage following surgical resection of mice. However, given that the proliferative response was higher after T3

administration compared to GC-1 exposure, this leaves a possibility for the involvement of alternative pathways and or receptors [90].

Recent studies have also demonstrated that T3/TR interaction leads to a suppression of the Wnt/β-catenin pathway via dickkopf Wnt signaling inhibitor 4 (DKK4) (an antagonizer of canonical Wnt signaling), resulting in inhibition of hepatoma cell proliferation [91]. To discuss this in more detail, DKK4 is down-regulated in 67.5% of human HCC tissues, and DKK4 levels are decreased concomitantly with TRα1/TRβ1 levels in 29.3% of matched tissue samples. Additionally, ectopic expression of DKK4 in hepatoma cells increases β-catenin degradation, with a concomitant reduction of CD44, cyclin D1, and c-Jun expression, which results in reduced cell growth and migration [91]. Accordingly, mice inoculated with either DKK4-expressing J7 hepatoma cells or TRα-expressing J7 cells displayed a smaller tumor size and lower metastatic potential than control mice, supporting, therefore, the inhibitory role of a TR-DKK4 axis in HCC formation. However, the fact that xenografted mice with DKK4-expressing J7 hepatoma cells exhibited more lung metastasis than those xenografted with TRα-expressing J7 cells implies that additional pathways are regulated by TRα to accomplish these anti-migratory effects [91]. These findings were also confirmed *in vitro* by showing that T3 upregulated DKK4 transcription in a TR-dependent manner. Interestingly, the study also identified an atypical T3 response element (TRE) between nucleotides -1645 and -1629 in the DKK4 promoter in HepG2 cells [92]. Altogether, these studies collectively suggest that DKK4 upregulated by T3/TR antagonizes Wnt signaling to suppress tumor cell growth, thus providing new insights into the molecular mechanism underlying TH activity in HCC [91,92].

Considering the risk factor of liver fibrosis for HCC development, stromal cell activation represents a key modifying factor of the tumor microenvironment [93]. It is 15 years since the first involvement of Wnt in fibrogenesis was found [94]. Since then, many studies have emphasized a key role for canonical WNT signaling in fibrogenesis of different organ systems, including liver [95]. However, colleagues have just recently begun to investigate the role of Wnt signaling in liver fibrogenesis [96].

On the one hand, canonical Wnt signaling seems to promote liver fibrosis and HSC activation. *In vitro* experiments showed that treatment of human HSC (HSC line LX-2 and primary cells) with Wnt3a conditioned media (canonical Wnt pathway ligand) increased collagen 1α1 and α-SMA expression and attenuated HSC apoptosis [97]. Accordingly, the messenger RNAs for canonical Wnt genes, non-canonical Wnt gene, and related receptors were upregulated in culture-activated primary rat HSC. Moreover, blockade of this signaling by using the coreceptor antagonist DKK1 restored HSC quiescent state and reduced HSC apoptosis. In

addition, these results could be confirmed *in vivo* where Wnt antagonism by Dkk-1 inhibits cholestatic liver fibrosis (through bile duct ligation) in mice [98]. These findings are supported by further cell culture experiments where siRNA-mediated β-catenin knockdown reduces collagen I and III expression, inhibits cell proliferation, and induces apoptosis of HSC in vitro as well by human tissue samples from the cirrhotic liver which show an enhanced expression of canonical Wnt proteins and decreased expression of Dkk-1 [95,99].

The work of other groups shows quite contrary results. Although it could be confirmed that canonical Wnt is active in freshly isolated HSC from rats, cell-culture induced activation induced a striking change in expression from canonical Wnt proteins to non-canonical Wnt proteins which was accompanied by an increased expression of inhibitor of canonical Wnt signaling like DKK1/2. Moreover, mimicking canonical pathway activation of primary rat HSC in cell culture via treatment with TWS119 (an inhibitor of glycogen synthase kinase 3β which induces nuclear β-catenin translocation) reduced expression of pro-fibrogenic markers like α-SMA [100].

The theory of a specific role of non-canonical Wnts gains further support as proteomic analysis of LX-2 showed Wnt5a to be a part of the fibrotic ECM, and microarray experiments with KEGG pathway analysis showed the participation of non-canonical Wnt pathways in the activation of primary rat HSC [101,102]. In the same study, lentiviral-mediated suppression of Wnt5a in LX-2 showed a downregulation of profibrogenic markers like TGFβ-1 and collagen, as well as decreased proliferation. Upregulation of Wnt5a could be confirmed in an *in vivo* CCL4 rat model [101]. Further cell culture experiments using primary activated rat HSC cells demonstrated active secretion of Wnt5a, which leads not only to an autocrine suppression of HSC apoptosis, but also to a paracrine stimulation of fibrogenic factors including TGF-β1 by Kupffer cells [103].

These findings suggest an involvement of Wnt pathway in HSC activation; however, the system is highly complex, and if T3 and downstream signals interfere with this signaling pathway, it requires elucidation by future experiments.

Hedgehog signaling:

Hedgehog (Hh) is a developmental morphogen which is critical for liver regeneration[104]. Inhibiting the Hh pathway blocks hepatocyte proliferation and liver regeneration after partial hepatectomy, and the level of Hh pathway activity is associated with the severity of MF accumulation and liver fibrosis[105–107]. Recent studies link changes in intrahepatic TH homeostasis with liver MF activation and canonical Hh-signaling. By examining rat, mouse

and human liver tissue with fibrosing liver injury, it has been found that hepatocytes decrease their expression of Dio1 whereas stromal cells, such as HSC, upregulate Dio3 during ongoing liver injury. These changes seem to be regulated by Hh ligands [108]. Treating cultured MFs with Hh ligands, for instance, led to an increase of Dio3 mRNA. Conversely, targeted disruption of Hh signaling in liver MFs suppressed their myofibroblastic phenotype and prevented injury-related induction of Dio3. As Dio3 is transforming T4 into its "inactive" form rT3, this should counteract intracellular hypothyroidism [109]. In addition, disruption of Hh signaling also abrogated the loss of Dio1 expression in neighboring hepatocytes. This ultimately leads to the conclusion that impaired Hh signaling during liver injury prevents intrahepatic hypothyroidism [108].

This switch from 'active' to 'non-active' T3 during liver fibrosis may have important implications for liver repair because Dio3 predominance has been noted in relatively undifferentiated tissues, including developing embryos and various cancers [71,110,111]. Interestingly, stromal cells such as HSC undergo a dedifferentiation during chronic injury from an epithelial to a more mesenchymal-activated phenotype. In conclusion, Hh-regulated hepatic stromal cell responses that occur during adult liver repair shift the balance of local deiodinase expression to favor the accumulation of biologically inert TH at the expense of biologically active TH [108]. Thus, during chronic liver injury and fibrosis, Dio1 and Dio3 are reciprocally regulated. As Dio3 is promoting the availability of the inactive TH form rT3 while Dio1 is promoting deiodination of T4 to the active ligand from T3, chronic liver injury results in a functional intrahepatic hypothyroidism. To summarize, there is a switch from TH-activating to TH-deactivating enzyme predominance during liver fibrosis.

In accordance with the aforementioned findings, there are recent insights into Hh-TH-Dio3 crosstalk from murine skin cancer models. In the absence of TH in the serum, cultured keratinocytes grow faster. Additionally, topical treatment with T3 reduces basal cell carcinoma (BCC) tumor growth *in vivo*. Further experiments have shown that T3 inactivation by Dio3 plays a central role in the progression of BCC and that Dio3 expression is regulated by Hh ligands including sonic hedgehog (Shh) [112]. The mechanism in mouse and human BCC is a direct induction of Dio3 by Shh/Gli (Gli transcription factors are the key effectors of hedgehog signaling) in proliferating keratinocytes. Dio3 is under the control of Shh, which increases its expression by acting via a conserved Gli2 binding site on the human Dio3 promoter. This leads to reduced intracellular active TH levels (low T3, high rT3) and results in increased cyclin D1 and keratinocyte proliferation[70]. In addition, Dio3 depletion or T3 treatment induces

apoptosis of BCC cancer cells and attenuates Shh signaling via a direct impairment of Gli2 protein stability by T3 through PKA induction [112].

Conclusion

Recent studies have demonstrated the impact of hypothyroidism in patients with NAFLD of all types. This seems unsurprising considering the prominent role of TH in lipid metabolism in the liver. Notably, there is an accumulation of data to suggest that alterations in TH metabolism are also associated with the progression of NAFLD beyond simple steatosis. The association between NASH-related cirrhosis and HCC represents a growing area of concern, and even more alarming is the fact that HCC does also occur in the setting of noncirrhotic NASH. This highlights the importance of investigating factors which play a regulatory role in several aspects of liver carcinogenesis: (a) in the regulation of liver pathogenesis leading to HCC (i.e., NASH/-Fibrosis); (b) in regulating the development and maintenance of the cancer cell itself; and (c) in regulation of the specific tumor microenvironment once the tumor has developed.

TH is a factor which may make a functional contribution to those characteristics. First, TH has an impact on steatosis. Second, studies have demonstrated the importance of T3/TR interaction in the regulation of different patterns of liver cancer progression, including development and proliferation, as well as metastasis and invasiveness, which requires the participation of the tumor microenvironment.

Here, we have provided a more comprehensive view of the impact of TH on the chronic liver disease-HCC axis. Intriguingly, several of the important pathways involved in liver carcinogenesis, as well as liver fibrosis (i.e., TGF-β, Wnt, Hedgehog), feature regulation by TH. However, TH's actions are complex, tissue- and time-specific and even cell-specific within the liver, and dysregulation of TH-homeostasis appears to have different effects on different patterns of carcinogenesis (i.e., metastasis or proliferation).

It is interesting, though, that no study has examined the impact of TH action in HCC in a fibrotic context, even though cirrhosis is one of the major risk factors for developing HCC. In particular, recent findings of the possible influence of TH on TGF-β signaling in liver fibrogenesis and the theory of local hypothyroidism may inspire deeper investigations into TH signaling crosstalk between HCC and tumor microenvironment. Taken together, these findings suggest that TH and related pathways have several mechanisms to activate either the tumor cell or cells of the microenvironment. The challenge of future investigations will be to dissect actions of TH in the diverse system of cell types and pathways involved in the tumor microenvironment.

Conflicts of Interest Statement

Manuscript title:

The Role of Thyroid Hormone Signaling in the Regulation of the Liver Tumor Microenvironment

All participating authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Figure legends:

Figure 1: Nuclear action of Thyroid Hormone. Thyroid hormone (TH) and TH-signaling are critical for tissue and organ development, growth, differentiation, and metabolism (including lipid and cholesterol handling). The main circulating thyroid hormone T4 (the prohormone) is deiodinated within cells by deiodinases (DIO1, 2) to become the biologically-active T3. Deiodination can also lead to biologically inactive forms like T2 or rT3. On entering the nucleus, T3 binds to nuclear thyroid hormone receptors (TRs), which are transcription factors and usually form a heterodimer with the retinoid X Receptor (RXR). Those are bound to positive or negative thyroid hormone response elements (TREs) located in the regulatory region of target genes. In the unliganded state, TRs interact with one of the several corepressor proteins, while during the liganded state, a coactivator complex is present.

Figure 2: Partial hepatectomy in rodents. The proof of effectiveness of T3 on proliferation of normal hepatocytes in *vitro* has not been ultimately established yet. This means it remains controversial whether T3 can be considered a direct mitogen, as the in vitro criterion has not been definitively met. **(a)** However, T3 is well-known for ameliorating liver regeneration after partial hepatectomy (PHx) or subtotal hepatectomy in rodents. **(b)** Moreover, there are also models of hypothyroidism proving a delay in regeneration after PHx *in vivo*.

Figure 3: Effects of T3 (hyper-/ hypothyroidism) on different patterns of hepatocellular carcinoma (HCC). A hypothyroid status of HCC has been described in human HCC. However, still conflicting results are reported on development, proliferation, and migration. **(a)** Animal studies show that local hypothyroidism is an early event in the development of HCC and precedes neoplastic formation. Results from rodent studies suggest that a hypothyroid status of preneoplastic lesions may contribute to their progression to HCC and that the reversion of this condition may represent a possible therapeutic goal to interfere with the development of this tumor. **(b/c)** The impact of T3 on HCC cancer progression remains very controversial. Specifically, in benign tumors or early-stage cancer, T3/TR may inhibit cancer cell proliferation, but promote cancer cell migration and invasion in malignant tumors or late-stage cancer. However, while a consensus exists regarding the oncosuppressive role of TRβ1 in HCC, it is worth mentioning that some studies indicate its oncosuppressive role to be more severe in an unliganded status (hypothyroid state) pointing out the role of the tumor microenvironment.

Figure 4: Role of T3/TGF-β crosstalk in liver fibrogenesis and HCC. (a) Hepatic stellate cells (HSC) are the key liver cells responsible for the deposition of collagen and other components of the ECM. Activation of HSC leads to a myofibroblastic phenotype (motile, secretory). Putative factors involved in HSC wound healing – fibrogenic process. Particularly, TGF-β signaling is involved in HSC activation. Recently, it could be shown that T3 interacts with TGF-β downstream signaling proteins (SMADs) to reduce fibrogenic response. **(b)** T_3 signaling mediated by TGF-β inhibits the proliferation of hepatoma cells expressing high levels of TR-proteins. HepG2 cells with ectopic stable overexpression of TRα (HepG2-TRα) or TRβ (HepG2-TRβ) were compared with wild-type HepG2. T3 upregulates TGF-β mRNA

which leads to inhibition of HepG2-TRα cell proliferation compared to control cells. **(c)**Treatment of different hepatoma cell lines which express endogenous TRα and TRβ with T3 enhances expression of furin, leading to activation of matrix metalloproteinases (MMPs), which consequently results in higher metastasis rates. Also, the TGF-β pathway, particularly SMAD3 and SMAD4, is involved in furin induction by T3. The induction of furin by T3 was also demonstrated *in vivo*. SCID mice which were inoculated with HepG2-TRα cells had much higher metastasis rates in liver and lung when treated simultaneously with T3 and TGF-β and showed higher furin protein expression. Also, treatment with TGF-β and T3 led to a higher activity of MMP-2 and MMP-9, providing an explanation for the increased metastatic potential. Therefore, regulation of furin is partially dependent on the crosstalk between T3 and TGF-β pathway, and T3 and TGF-β seem to work synergistically to promote invasiveness and metastasis.

Figure 1

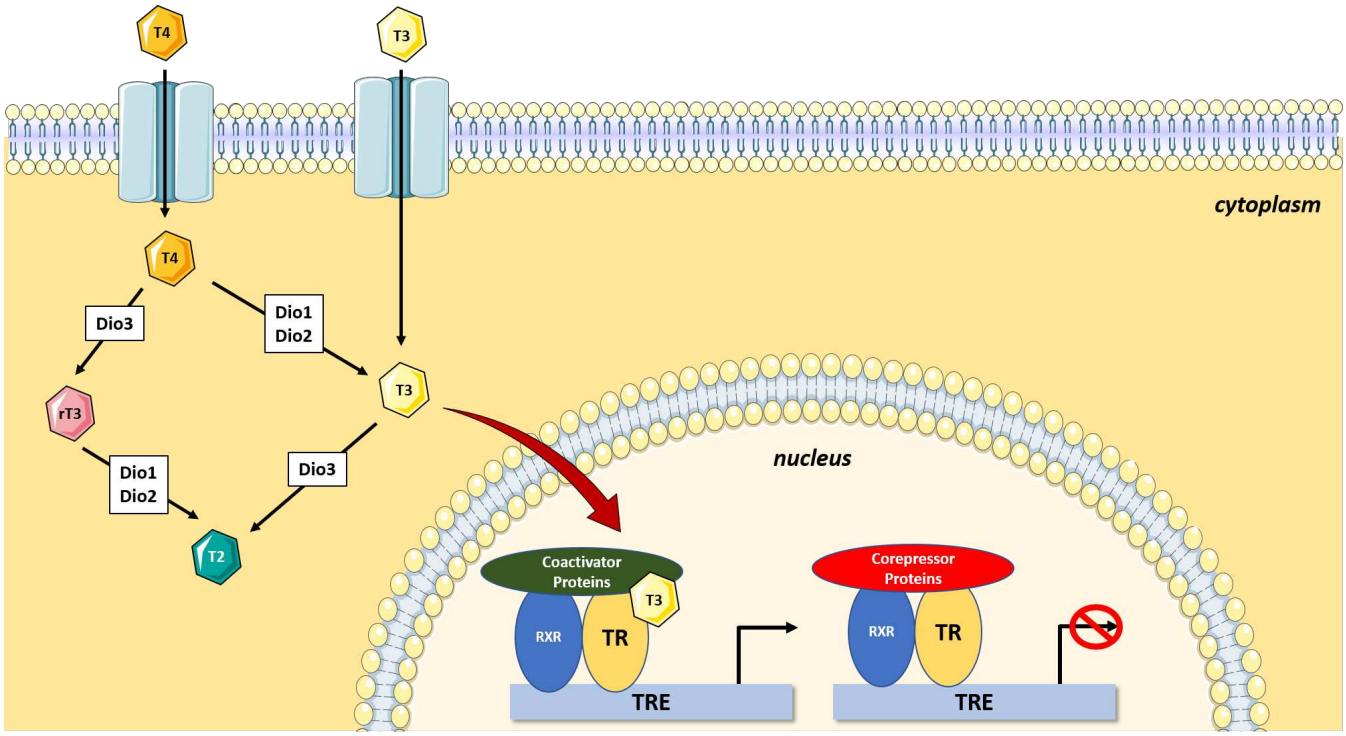
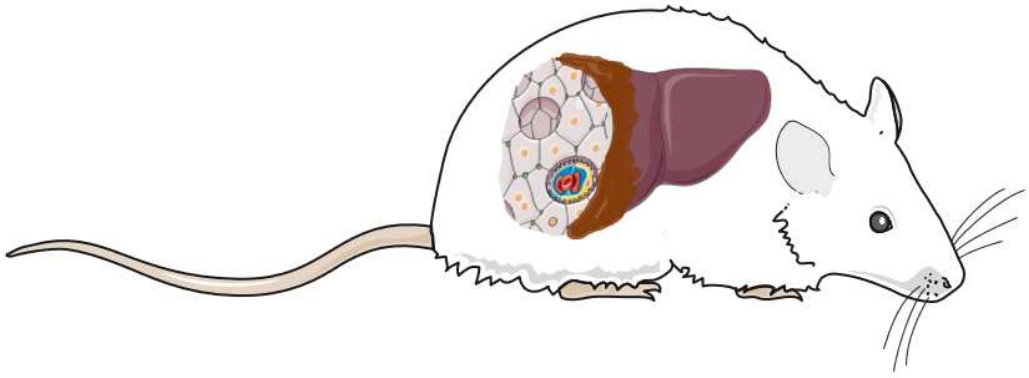

T4
T3
cytoplasm
T4
Dio3
Dio1
Dio2
T3
rT3
Dio1
Dio2
Dio3
T2
nucleus
Coactivator
Proteins
T3
RXR
TR
TRE
Corepressor
Proteins
RXR
TR
TRE

Figure 2

partial hepatectomy in rodents



Hyperthyroidism (induced by T3 treatment) *Rat models with 70%-90% hepatectomy* ***[15,16,18]***	**Hypothyroidism** (induced by thyroidectomy, pharmacological [PTU,MMI] or TR-knockout model) *Rat and mice models with 70% hepatectomy* ***[15,17,19]***
T3 treatment leads to increased hepatic regeneration • Hepatic proliferation (Ki67, BrdU, PCNA) ↑ • Liver-Body-Weight ratios ↑ • Expression of cell cycle progression regulatory proteins (e.g. Cyclin D1) ↑	**Hypothyroidism leads to a delay in hepatic regeneration** • Hepatic proliferation (PCNA, Ki67) ↓ • Liver-body-Weight ratios ↓ • Expression of cell cycleprogression regulatory proteins (e.g. Cyclin D1) ↓

Figure 3

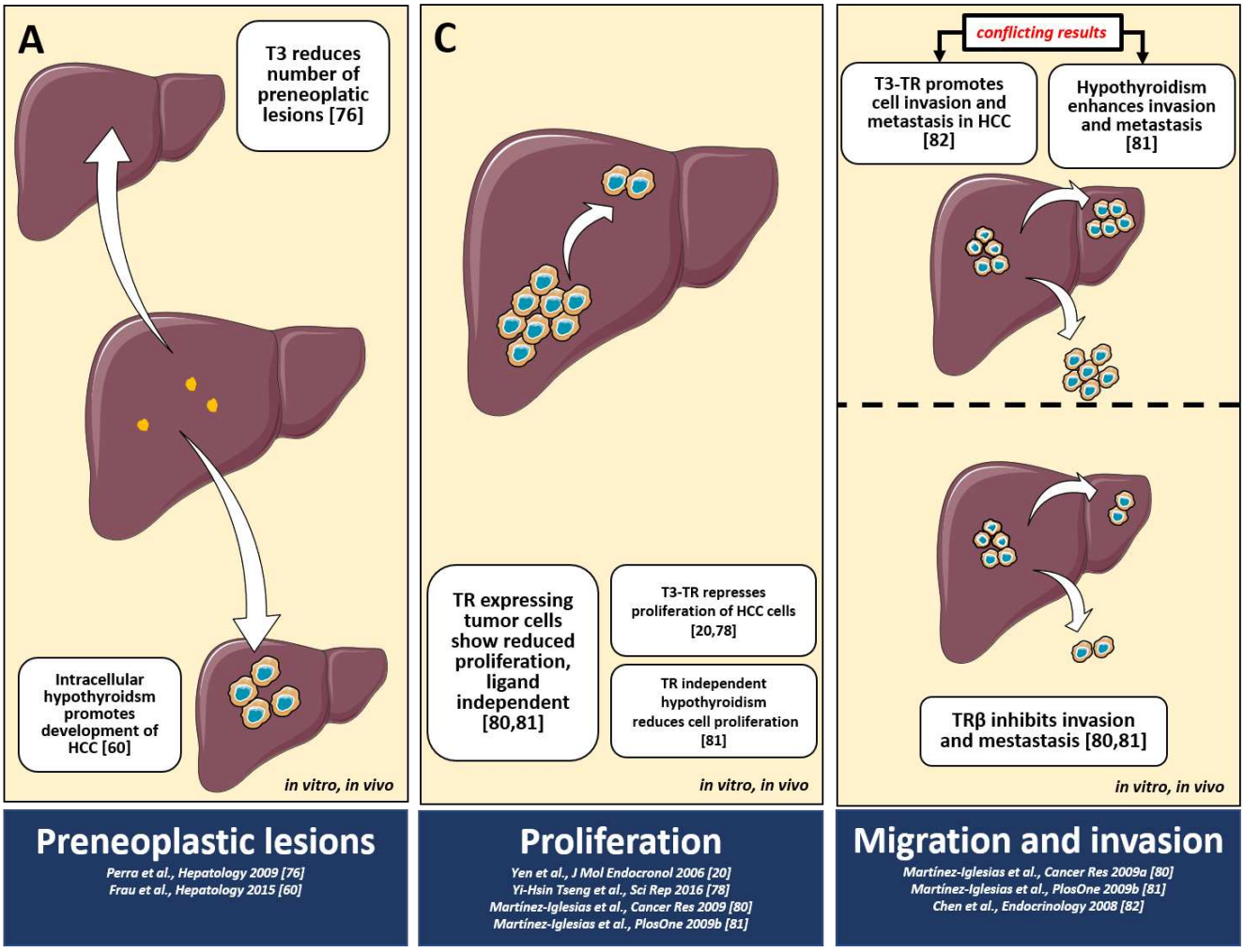
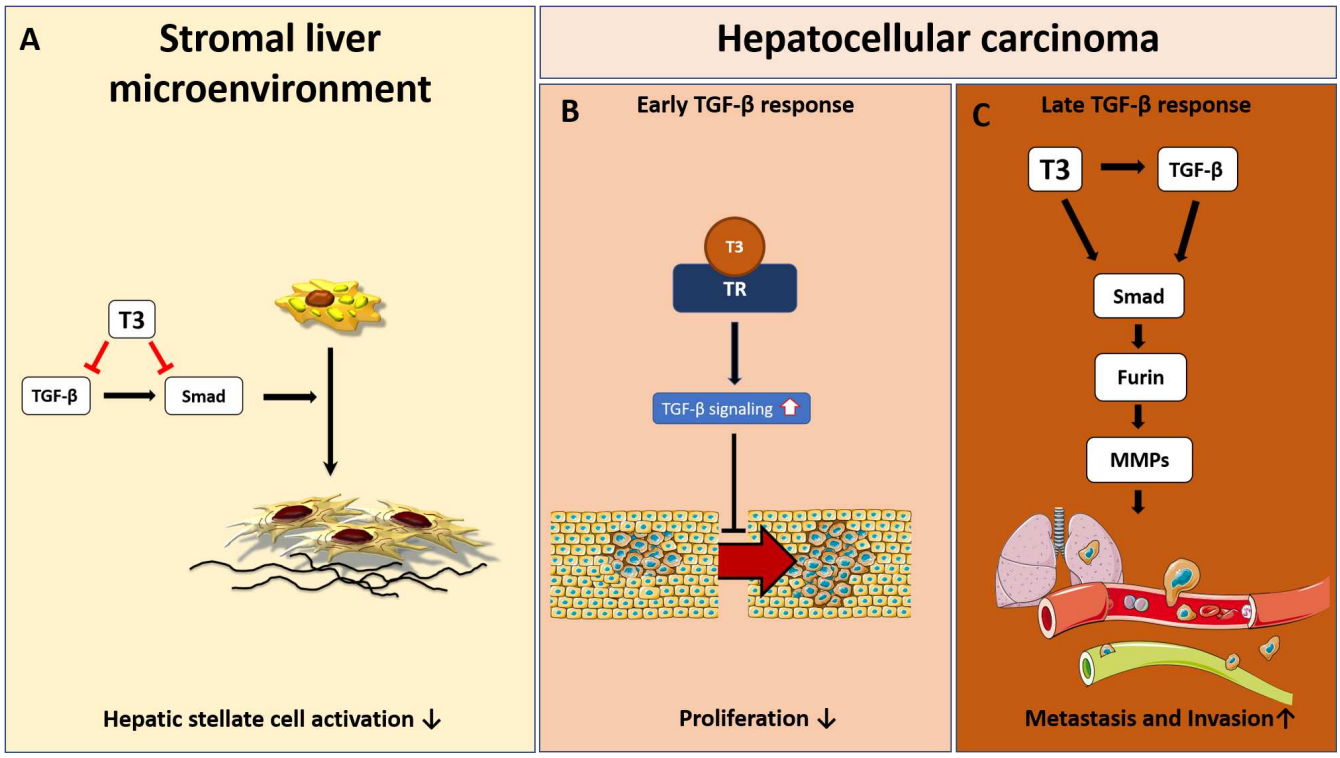

A
T3 reduces number of preneoplatic lesions [76]
Intracellular hypothyroidsm promotes development of HCC [60]
in vitro, in vivo
Preneoplastic lesions
Perra et al., Hepatology 2009 [76]
Frau et al., Hepatology 2015 [60]
C
TR expressing tumor cells show reduced proliferation, ligand independent [80,81]
T3-TR represses proliferation of HCC cells [20,78]
TR independent hypothyroidism reduces cell proliferation [81]
in vitro, in vivo
Proliferation
Yen et al., J Mol Endocronol 2006 [20]
Yi-Hsin Tseng et al., Sci Rep 2016 [78]
Martínez-Iglesias et al., Cancer Res 2009 [80]
Martínez-Iglesias et al., PlosOne 2009b [81]
conflicting results
T3-TR promotes cell invasion and metastasis in HCC [82]
Hypothyroidism enhances invasion and metastasis [81]
TRβ inhibits invasion and mestastasis [80,81]
in vitro, in vivo
Migration and invasion
Martínez-Iglesias et al., Cancer Res 2009a [80]
Martínez-Iglesias et al., PlosOne 2009b [81]
Chen et al., Endocrinology 2008 [82]

Figure 4


A
Stromal liver microenvironment
T3
TGF-β
Smad
Hepatic stellate cell activation ↓
Hepatocellular carcinoma
B
Early TGF-β response
T3
TR
TGF-β signaling
Proliferation ↓
C
Late TGF-β response
T3
TGF-β
Smad
Furin
MMPs
Metastasis and Invasion↑