# $T_3$/TRs axis in hepatocellular carcinoma: new concepts for an old pair

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## Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide, and its burden is expected to further increase in the next years. Chronic inflammation, induced by multiple viruses or metabolic alterations, and epigenetic and genetic modifications, cooperate in cancer development via a combination of common and distinct aetiology-specific pathways. In spite of the advances of classical therapies, the prognosis of this neoplasm has not considerably improved over the past few years. The advent of targeted therapies and the approval of the systemic treatment of advanced HCC with the kinase inhibitor sorafenib have provided some hope for the future. However, the benefits obtained from this treatment are still disappointing, as it extends the median life expectancy of patients by only few months. It is thus mandatory to find alternative effective treatments. Although the role played by thyroid hormones (THs) and their nuclear receptors (TRs) in human cancer is still unclear, mounting evidence indicates that they behave as oncosuppressors in HCC. However, the molecular mechanisms by which they exert this effect and the consequence of their activation following ligand binding on HCC progression remain elusive. In this review, we re-evaluate the existing evidence of the role of TH/TRs in HCC development; we will also discuss how TR alterations could affect fundamental biological processes, such as hepatocyte proliferation and differentiation, and consequently HCC progression. Finally, we will discuss if and how TRs can be foreseen as therapeutic targets in HCC and whether selective TR modulation by TH analogues may hold promise for HCC treatment.

**Key Words**
- thyroid hormone receptor
- triiodothyronine
- hepatocellular carcinoma
- microRNA
- TRβ agonists
- local hypothyroidism
- cell differentiation

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## TH/TR axis

Thyroid hormones, namely 3,5,3′-triiodo-L-thyronine ($T_3$) and 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine or $T_4$), influence a variety of physiological processes, including development, metabolism, cell growth and proliferation. THs are produced in response to signals deriving from the hypothalamus, which synthesises the thyrotrophin-releasing hormone (TRH). TRH induces the expression of the thyroid-stimulating hormone (TSH) which, in turn, stimulates the follicular cells of the thyroid gland to secrete mainly $T_4$. $T_4$ is then transported across the cell membrane of responsive cells by specific transporters, including the monocarboxylate anion transporters 8 and 10 (MCT8 and MCT10) (Heuer & Visser 2009, van der Deure *et al* 2010), and is converted

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into the active $T_3$ hormone in peripheral tissues, such as liver and kidney. This conversion, carried out by type I 5′-deiodinase (DIO1) and, to a lesser extent, by DIO2 (Gereben *et al.* 2008), leads to increased levels of circulating $T_3$. On the opposite, type III deiodinase (DIO3) is responsible for thyroid hormone inactivation as it converts $T_4$ and $T_3$ to the inactive metabolites rT3 and 3,3′-diiodothyronine ($T_2$), respectively.

Although it has been proposed that rapid non-genomic mechanisms initiated at the cell membrane could be involved in mediating the actions of thyroid hormones (for a review, see Davis *et al.* 2016), most of the effects of THs on cellular proliferation and differentiation are mediated through the thyroid hormone nuclear receptors (Brent 1994, Cheng *et al.* 2010). TRs are members of the steroid/thyroid superfamily of nuclear hormone receptors, which are ligand-modulated transcription factors (Mangelsdorf *et al.* 1984, Lazar 1993, Forrest & Vennstrom 2000). The human TRs are encoded by two genes, *THRA* (*NR1A1*) and *THRB* (*NR1A2*), localised in human chromosomes 17 and 3, respectively. Both genes encode for several mRNA isoforms, generated by use of different promoters and alternative splicing (Mitsuhashi *et al.* 1988, Chassande *et al.* 1997, Harvey *et al.* 2007). Although the TR isoforms are widely distributed, differences exist concerning their expression in various tissues and/or during developmental stages. For example, TRα1 is the dominant receptor in the brain and skeletal system and mediates most of the synergism between $T_3$ and the sympathetic signalling pathway in the heart; TRβ, abundant in liver, is responsible for most of $T_3$ effects on lipid metabolism and on metabolic regulation (Hsu & Brent 1998, Weiss & Murata 1998, Wikstrom *et al.* 1998, Brent 2000, Kaneshige *et al.* 2000). All TRs have a similar domain organisation that is shared by all nuclear hormone receptors: an amino-terminal A/B domain which recruits co-regulatory proteins; a central DNA-binding domain (DBD), or C region, consisting of two zinc-finger motifs intercalating with the major and minor grooves of DNA; a linker D region, necessary for nuclear translocation of the receptor; and a carboxy-terminal ligand-binding domain (LBD), forming a pocket that binds $T_3$ (Yen 2001). Acting as transcription factors, TRs bind to specific DNA sequences known as thyroid hormone response elements (TREs), located in the regulatory regions of $T_3$-target genes, activating or repressing transcription in response to the hormone. *In vivo,* TRs bind TREs predominantly as heterodimers with the retinoid X receptor (RXR), another member of the nuclear hormone receptor superfamily (Lazar 2003).

## TH/TRs and cancer

In the last years, emerging evidence has shown that TRs and THs are implicated in cancer. The first evidence came from the demonstration that the *v-ErbA* oncogene, isolated from an avian retrovirus, is an altered form of the *TRα* gene (Thormeyer & Baniahmad 1999) that antagonises TRs activity by competing for TREs or co-activator binding (Yen 1994). Later on, several studies reported *TR* lower and/or aberrant expression and/or somatic mutations in human cancers (Table 1), supporting the hypothesis that they might play a role in tumour development. Further evidence that TRβ1 acts as a tumour suppressor came from the observation that transgenic mice harbouring a dominant negative mutant *TRβ (TRβPV)*, originally identified in a patient with thyroid hormone resistance (RTH), spontaneously develop thyroid cancer (Suzuki *et al.* 2002). Moreover, Zhu and coworkers reported that mice devoid of functional TRs ($TR\alpha1^{-/-}/TR\beta1^{-/-}$) spontaneously develop follicular thyroid cancer and lung metastases (Zhu *et al.* 2010). On the contrary, thyroid hormones and TRα1 receptor promote intestinal tumourigenesis, as they control intestinal epithelial cell proliferation and expression of components of the Wnt pathway (Kress *et al.* 2009, 2010). Thus, while the body of evidence indicates that partial or complete loss of TRs function stimulates the proliferative, invasive and metastatic capacity of tumour cells, other findings show that TRs activation may facilitate tumour progression. Until now, these often contradictory data did not allow to clearly establish whether TRs play an oncogenic or a tumour suppressor role. It is, however, worth underlying that TRs play tissue-specific functions (Kress *et al.* 2009), probably accounting for these contradictory results.

## TRs alterations and HCC

Hepatocellular carcinoma (HCC) is the second cause of cancer-related death worldwide, and its burden is expected to increase further in the next future (Jemal *et al.* 2011, Njei *et al.* 2015). While HCC has historically been more common in the developing world, its incidence in developed countries has almost doubled in the last two decades, largely as a result of liver cirrhosis. Since the efficacy of traditional chemotherapeutic agents and of new developed drugs and their ability to produce a significant survival benefit is questionable, there is, thus, an urgent need to develop novel molecular targeted therapies for HCC.



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**Table 1** Different cancer types displaying *TRβ* downregulation/mutation.

| Type of cancer | Species | Regulation | Reference |
|---|---|---|---|
| Breast cancer | Human | Loss of heterozygosity | Ali *et al*. (1989), Chen *et al*. (1994) |
| Breast cancer | Human | A variable degree of *TRβ1* promoter hypermethylation | Li *et al*. (2002) |
| Breast cancer | Human | Lack of *TRβ1* nuclear staining | Li *et al*. (2002) |
| Breast cancer | Human | ↓ *TRβ1* transcripts in breast cancer cell lines | Li *et al*. (2002) |
| Breast cancer | Human | ↓ *TRβ1* RNA (semi-quantitative RT-PCR) | Silva *et al*. (2002) |
| Breast cancer | Human | ↓ protein levels (Western blot) | Silva *et al*. (2002) |
| Breast cancer | Human | Tumour-specific truncated *TRβ1* RNA | Silva *et al*. (2002) |
| Breast cancer | Human | ↓ *TRβ* mRNA (RT-PCR) | Ling *et al*. (2010) |
| Breast cancer | Human | Hypermethylation in cancer tissues and plasma samples | Ling *et al*. (2010) |
| Breast cancer | Human | Low *TRβ* mRNA levels associated with poor outcome | Gu *et al*. (2015) |
| Colon cancer | Human | ↓ *TRβ* mRNA levels (Northern blot) | Markowitz *et al*. (1989) |
| Colon cancer | Human | ↓ nuclear TRβ1 (IHC) | Hörkkö *et al*. (2006) |
| Colon cancer | Human | ↓ TRβ1 protein levels (WB) | Hörkkö *et al*. (2006) |
| Hepatocellular carcinoma | Human | Point mutations in *TRβ1* | Lin *et al*. (1999) |
| Hepatocellular carcinoma | Human | ↓ *TRβ1* mRNA levels (qRT-PCR) | Frau *et al*. (2015), Martínez-Iglesias *et al*. (2016) |
| Hepatocellular carcinoma | Human | ↓ TRβ protein levels (WB) | Liao *et al*. (2012) |
| Lung carcinoma | Human | Loss of heterozygosity | Leduc *et al*. (1989) |
| Lung carcinoma | Human | Absent TRβ1 expression (RT-PCR) | Iwasaki *et al*. (2010) |
| Lung carcinoma | Human | TRβ1 promoter methylation | Iwasaki *et al*. (2010) |
| Renal carcinoma | Human | ↓ mRNA levels (Northern blot) | Puzianowska-Kuznicka *et al*. (2000) |
| Renal carcinoma | Human | ↓ protein levels (WB) | Puzianowska-Kuznicka *et al*. (2000) |
| Renal carcinoma | Human | *TRβ1* mutations | Kamiya *et al*. (2002) |
| Renal carcinoma | Human | ↓ *TRβ1* mRNA levels (qRT-PCR) | Master *et al*. (2010) |
| Renal carcinoma | Human | ↓ TRβ1 protein levels (WB) | Master *et al*. (2010) |
| Renal carcinoma | Human | ↓ *TRβ* mRNA levels (qRT-PCR) | Wojcicka *et al*. (2014) |
| Pituitary tumour | Human | Somatic mutation in the ligand-binding domain of *TRβ* | Ando *et al*. (2001*a*) |
| Pituitary tumour | Human | Alternative splicing of *TRβ2* mRNA | Ando *et al*. (2001*b*) |
| Thyroid cancer | Human | ↓ mRNA levels (Northern blot) | Wallin *et al*. (1992), Brönnegård *et al*. (1994) |
| Thyroid cancer | Human | ↓ mRNA levels (Northern blot) | Puzianowska-Kuznicka *et al*. (2002) |
| Thyroid cancer | Human | *TRβ* mutations | Puzianowska-Kuznicka *et al*. (2002) |
| Thyroid cancer | Human | Hypermethylation of the *TRβ gene* in tumour samples and in thyroid tumour cell lines | Joseph *et al*. (2007) |
| Thyroid cancer | Human | Loss of heterozygosity | Joseph *et al*. (2007) |
| Thyroid cancer | Human | ↓ mRNA levels (qRT-PCR) | Takano *et al*. (2003), Rosignolo *et al*. (2015) |
| Uveal melanoma | Human | Loss of heterozygosity (LOH) | Sisley *et al*. (1993) |
| Human hepatocarcinoma and breast cancer cells inoculated into nude mice | Mouse | TRβ1 re-expression reduces tumour growth and has an inhibitory effect on invasiveness, extravasation and metastasis formation | Martínez-Iglesias *et al*. (2009) |
| Skin tumours (chemical skin carcinogenesis in mice) | Mouse | ↓ TRβ expression (IHC) | Martínez-Iglesias *et al*. (2009) |
| Mammary tumour | Mouse | *TRβ* mutations increase the risks of mammary hyperplasia and tumour | Guigon *et al*. (2010) |
| Mammary tumour | Mouse | Mutation of a single copy of *TRβ* doubles the percentage of $Pten^{+/-}$ females to develop mammary tumours | Guigon *et al*. (2010) |
| Thyroid carcinoma | Mouse | Mice deficient in total functional TRs or with a targeted homozygous mutation of the *TRβ* gene spontaneously develop metastatic thyroid carcinoma | Suzuki *et al*. (2002), Kato *et al*. (2004), Zhu *et al*. (2010) |
| Hepatocellular carcinoma | Rat | ↓ mRNA levels (qRT-PCR) | Frau *et al*. (2015) |
| Hepatocellular carcinoma | Rat | ↓ protein levels (WB) | Frau *et al*. (2015) |



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### *TR* mutation

Search of activating mutations in *TR* genes in HCC gave rise to contrasting findings. Lin and coworkers (1996) showed that 65% of the tumours analysed display mutations in *TRα* and 76% in *TRβ* genes, with a subgroup exhibiting mutations in both loci (Lin *et al.* 1999). The majority of the mutated TRs act as dominant negative molecules, hampering the activity of the wild-type receptor, leading to impaired transcriptional activation; moreover, many of them display defects in $T_3$-dependent co-repressor release and/or co-activator binding (Chan & Privalsky 2006). Notably, these TR mutants activate several pro-proliferative genes, such as colony-stimulating factor 1 (*CSF1*), neuronal cell adhesion molecule (*NRCAM*) and repress tumour suppressor genes, such as dickkopf-related protein 1 (*DKK1*) and tissue inhibitor of metalloproteinase 3 (*TIMP3*) (Yang & Privalsky 2001). Although these findings suggest that *TR* mutations may play an oncogenic role in HCC development, evidence for such a high frequency of *TR* mutations in human HCC has not been confirmed in more recent studies. Indeed, several works based on the powerful deep sequencing analysis did not detect any mutation in TRs, although they were able to detect mutations in other genes, at frequencies as low as 1% (Guichard *et al.* 2012, Cleary *et al.* 2013, Ahn *et al.* 2014, Totoki *et al.* 2014, Schulze *et al.* 2015). In addition, publicly available RNAseq data for TRs (442 human HCC) (http://www.cbioportal.org/index.do) reported only two mutations for *TRβ* and none for *TRα*, further confirming that *Tr* mutations are virtually absent in human HCC. Moreover, no TR mutations have been described in experimentally induced HCCs, including a recent study aimed at detecting *Trs* mutations in chemically induced rat HCC (Frau *et al.* 2015).

### Methylation

Alternative to mutations, dysregulation of TR expression could be a mechanism by which these receptors play a suppressive role in the carcinogenic process. Hypermethylation of the *TRβ* gene promoter region is frequent in several human cancers and leads to *TRβ* silencing (Joseph *et al* 2007, Dunwell *et al* 2009, Iwasaki & Sunaga 2010, Ling *et al.* 2010), while reactivation of the silenced murine *Trβ* gene delays thyroid tumour progression (Kim *et al.* 2013). Unfortunately, data on the possible role of *TR* methylation in human HCC are only available from the Liver Hepatocellular Carcinoma (TGCA, Provisional), which contains methylation data for 379 of 442 samples. This topic is particularly relevant since *TRβ1* mRNA levels were found significantly downregulated in the vast majority of HCCs compared with matched cirrhotic tissues, in two independent studies on human patients (Frau *et al.* 2015, Martínez-Iglesias *et al.* 2016). Decreased levels of *Trβ1* induced target genes, such as *DIO1,* and glucose-6-phosphatase (*G6PC*) were detected in the same HCCs samples. However, in neither of these studies, the methylation status of the TR promoters was investigated. In the only extensive study of DNA methylation in human HCC (Villanueva *et al.* 2015), no aberrant methylation of *TRs* has been reported. Thus, whether hypermethylation of *TR* promoters might contribute to the decreased expression of the receptors and affect HCC onset and progression is still an unsolved question.

Animal studies confirmed a highly significant downregulation of *Trβ1* expression in HCCs, both at mRNA and protein levels (Frau *et al.* 2015). As expected, decreased levels of target genes positively regulated by TRβ1, such as *Dio1, G6pc* and *Spot14* (Feng *et al.* 2000), and upregulation of *App*, a gene negatively regulated by TRβ1 (O'Barr *et al.* 2006), were associated with *Trβ1* downregulation. When methylation of *Tr* promoter was analysed in these rodent HCCs, no difference was found compared with normal liver. Thus, whether hypermethylation occurs in human HCC and plays a role in the downregulation of TRβ1 observed in these tumours still remains an unsolved question.

### microRNA

microRNAs (miRNAs) are small non-coding RNAs, which negatively control gene expression by binding to complementary sequences present in untranslated regions of the target transcripts. The involvement of microRNAs in cancer pathogenesis is well established as they behave as oncogenes or tumour suppressor genes depending on the cellular function of their mRNA targets (Calin & Croce 2006, Lujambio & Lowe 2012). The importance of microRNAs in cancer progression is also underlined by the observation that they can influence both the response to chemotherapy and the development of drug resistance (Tomokuni *et al.* 2011, Zhou *et al.* 2011, Giordano & Columbano 2013).

Recently, a number of studies provided evidence that *TR*β expression could be repressed through microRNA regulatory mechanisms (Master *et al.* 2010, Jazdzewski *et al.* 2011, Nishi *et al.* 2011, Ruiz-Llorente *et al.* 2014). In a study on papillary thyroid cancer (PTC) patients, lower



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levels of TR$\beta$ transcripts were observed in most tumour samples (Jazdzewski *et al*. 2011), and *TRβ* downregulation was associated with high levels of miR-21, -146a, -181a and -221, all predicted to target TRβ. Moreover, while downregulation of *TRβ* expression in human clear cell renal carcinomas (ccRCC) is not associated with changes in DNA methylation of *TRβ* promoter region, it is inversely correlated with the levels of miR-204 (for which a putative interaction site was identified in the TRβ1 3′UTR) (Master *et al*. 2010).

Unfortunately, not many studies have investigated the possible role of miRNAs in the regulation of *TR* expression in HCC. We have recently analysed in rat HCCs the expression of miRNAs (miR-21, miR-27a, miR-181a, miR-221, miR-146a and miR-204) known to target *Trβ1* (Master *et al*. 2010, Jazdzewski *et al*. 2011, Nishi *et al*. 2011, Tomokuni *et al*. 2011). Interestingly, miR-27a, miR-146a, miR-181a and miR-204 were upregulated in rat HCCs displaying *Trβ1* downregulation (Frau *et al*. 2015). Among these miRNAs, miR-27a showed an inverse relationship with TRβ expression in human HCCs and in the five HCC cell lines examined, suggesting that this miRNA might negatively regulate *TRβ1* expression in human HCC. Accordingly, transfection of HuH7, HepG2 and Mahlavu cells with an miR-27a mimic, led to a significant decrease in *TRβ1* expression. MiRNAs may modulate TH-mediated effects also indirectly, i.e. by acting on enzymes involved in the $T_4$–$T_3$ conversion or on their degradation. Interestingly, tumour-specific changes in intracellular $T_3$ concentration correlate with changes in the *DIO1*-targeting miR-224 (Boguslawska *et al*. 2011). Notably, this miRNA is one of the most upregulated in human and rat HCC (Imbeaud *et al*. 2010, Giordano & Columbano 2013), and the inverse relationship between miR-224 levels and *Trβ1* expression is present since the very early stages of experimental models of hepatocarcinogenesis (Petrelli *et al* 2014, Frau *et al*. 2015).

Not only miRNAs regulate the expression of TRs, but $T_3$/TRs receptor signalling is, in turn, able to regulate miRNA expression (Diniz *et al*. 2013, 2015, Huang *et al*. 2013, Lin *et al*. 2013, Lu *et al*. 2013, Yap *et al*. 2013, Ruiz-Llorente *et al*. 2014). In fact, $T_3$ treatment of HepG2-TR-expressing cells stimulates miR-21 expression with subsequent T-cell lymphoma invasion and metastasis 1 (TIAM1) suppression and promotion of hepatoma cell migration and invasion (Huang *et al*. 2013). Work by the same group also showed that $T_3$ downregulates miR-17 expression in HepG2-TR-expressing cells and that the $T_3$/TR axis promotes cell migration through miR-17 downregulation (Lin *et al*. 2013). Based on these data, the authors proposed a novel metastatic pathway involving the activities of $T_3$/TR, miRNA-17, p-AKT and metallo-proteinase3 (MMP3) in hepatoma cells. In the same context, but with opposite conclusions, studies from Ruiz-Llorente and coworkers (Ruiz-Llorente *et al*. 2014) showed that $T_3$ increases the levels of miR-424 and miR-503 in SK-TR HCC cells. These authors postulated that this induction plays an important role in the anti-tumourigenic and anti-invasive actions mediated by $T_3$. $T_3$-induced expression of these miRNAs was also found in non-transformed hepatocytes and in MDA-TRβ overexpressing breast cancer cells, indicating that the phenomenon is not specific for the HCC cell line. Furthermore, miR-424 or miR-503 depletion enhanced extravasation to the lungs of hepatocarcinoma cells injected in the tail vein of mice (Ruiz-Llorente *et al*. 2014).

Although we have a yet limited knowledge about the relevance of the cross-regulation between miRNAs and TRs, nevertheless, the impact of this mutual regulation on HCC development and metastatic capacity represents a very promising topic that it is worth to be actively pursued (Table 2).

## TRs, hypo- and hyperthyroidism and HCC

An aberrant activity of TRs is associated with several human cancers; however, it is still unclear whether changes in thyroid hormone levels affect cancer development and progression. Indeed, conflicting results are reported. Population-based case–control studies of risk factors associated to development of ovarian and pancreatic cancers found that hyperthyroidism is associated with a two-fold increase in cancer risk (Ness *et al*. 2000, Ko *et al*. 2007). Moreover, while hyperthyroidism is associated with more advanced clinical stage and higher risk of recurrence in prostate cancer (Lehrer *et al*. 2002), hypothyroidism is associated with a lower risk of carcinoma and a reduced progression to more invasive stages for mammary cancer (Cristofanilli *et al*. 2005). Consistent with hypothyroidism being beneficial, pharmacologically induced hypothyroidism, together with tamoxifen treatment, resulted in better survival of glioblastoma patients, and a significantly longer survival was observed in patients with recurrent high-grade gliomas treated with tamoxifen and the anti-thyroid drug propylthiouracil (Hercbergs *et al*. 2003).

An opposite conclusion stems from two case–control studies suggesting that hypothyroidism represents a risk factor for HCC development. In one of these studies, women with a history of hypothyroidism had a 2.8-fold higher risk of HCC (Hassan *et al*. 2009); in the second



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**Table 2** miRNAs and TRs/$T_3$ axis.

| | Tissue | Species | miRNA | Regulation | Reference |
|---|---|---|---|---|---|
| TRα | Breast | Human | miR-10a | Positive correlation between miR-10a and THRα gene expression | Khan *et al*. (2015) |
| TRα | Liver | Human | miR-17 | miR-17 expression negatively associated with TRα1 | Lin *et al*. (2013) |
| TRα | Liver | Human Rat Mouse | miR-21 | In patients with increased TRα1 expression in tumour tissues, miR-21 is concomitantly increased | Huang *et al*. (2013) |
| TRβ | Heart | Rat Human | miR-27a | TRβ1 is a target of miR-27a | Nishi *et al*. (2011) |
| TRβ | Heart | Rat Human | miR-27a | miR-27a regulates β-MHC gene expression by targeting TRβ1 in cardiomyocytes | Nishi *et al*. (2011) |
| TRβ | Heart | Mouse | miR-208a miR-199a | miR-208a and miR-199a contribute to THRβ-mediated cardiac hypertrophy | do Império *et al*. (2015) |
| TRβ | Liver | Rat | miR-27a miR-146a miR-181a miR-204 | Upregulation in HCC displaying TRβ1 downregulation | Frau *et al*. (2015) |
| TRβ | Liver | Human | miR-27a | Inverse correlation between TRβ1 and miR-27a in HCC cell lines | Frau *et al*. (2015) |
| TRβ | Liver | Human | miR-181a | Inverse relationship between TRβ1 and miR-181a in human cirrhotic peritumoural tissue | Frau *et al*. (2015) |
| TRβ | Kidney | Human | miR-204 | miR-204 reported as a candidate regulator of TRβ1 expression | Master *et al*. (2010) |
| TRβ | Kidney | Human | miR-155 miR-425 | THRβ is targeted by miR-155 and miR-425 | Wojcicka *et al*. (2014) |
| TRβ | Kidney | Human | miR-155 miR-425 | Inverse correlation between TRβ and miRs in clear cell renal cell carcinoma | Wojcicka *et al*. (2014) |
| TRβ | Kidney | Human | miR-452 | miR-452 directly regulates the expression of TRβ1 in renal cancer cells | Boguslawska *et al*. (2014) |
| TRβ | Thyroid | Human | miR-21 miR146a miR181a miR-221 | An inverse correlation between TRβ and miRs in papillary thyroid carcinoma tumours | Jazdzewski *et al*. (2011) |
| TRβ | Thyroid | Human | miR-21 miR-146a miR-181a miR-221 | An inverse correlation between TRβ and miRs in papillary thyroid carcinomas | Rosignolo *et al*. (2015) |
| TH | Breast cancer cells, hepatocarcinoma cells expressing TRβ | Human | miR-424 miR-503 | $T_3$ treatment induces miR-424 and miR-503 | Ruiz-Llorente *et al*. (2014) |
| TH | Breast cancer cells, hepatocarcinoma cells expressing TRβ | Human | miR-424 miR-503 | Reduced expression of miR-424 and miR-503 in tumours developed in hypothyroid mice | Ruiz-Llorente *et al*. (2014) |
| TH | Heart | Rat | miR-208a miR-208b | miR-208a is upregulated in response to $T_4$ treatment | Diniz *et al*. (2013) |
| TH | Heart | Rat | miR-208a miR-208b | miR-208b is downregulated in response to $T_4$ treatment | Diniz *et al*. (2013) |
| TH | Heart | Rat | miR-133 | miR-133 expression is reduced in TH-induced cardiac hypertrophy | Diniz *et al*. (2015) |
| TH | Heart | Rat | miR-133 | miR-133 mimic prevents the cardiomyocyte hypertrophy in response to $T_3$ *in vitro* | Diniz *et al*. (2015) |
| TH | Hepatic cell line | Human | miR-181d | $T_3$ treatment increases miR-181d expression | Yap *et al*. (2013) |
| TH | Liver | Mouse | miR-1 miR-206 miR-133a miR-133b | miRs upregulation in hypothyroid mice | Dong *et al*. (2010) |
| TH | Proximal tubular epithelial cell line | Human | miR-34a | $T_3$ treatment induces miR-34a expression | Lu *et al*. (2013) |



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study, hypothyroidism was significantly more prevalent in patients with HCC of unknown aetiology than in HCC patients with alcoholic liver disease or HCV. Thus, these results suggest that hypothyroidism may be a permissive factor for the development of HCC (Reddy *et al.* 2007).

A hypothyroid status of HCC has been described in human HCC (Liao *et al.* 2012, Frau *et al.* 2015, Martinez-Iglesias *et al.* 2016). Moreover, animal studies showed that downregulation of TRs, especially *Trβ1*, associated to severely reduced *Dio1* expression, is a very early event in the multistage process of hepatocarcinogenesis and precedes neoplastic transformation (Frau *et al.* 2015). The latter results suggest that alterations of *Trβ1* expression may play a critical role not only in the progression but also in the onset of HCC. Based on these observations, a regulatory loop can be hypothesised, wherein decreased expression of *Trβ1* leads to diminished transcription of its classical target gene *Dio1*; in turn, *Dio1* inhibition leads to reduced $T_4$ to $T_3$ conversion causing local hypothyroidism (Fig. 1). The data obtained from the rat model suggest that this loop is an early event and may be critical in conferring a growth advantage to preneoplastic hepatocytes. Notably, downregulation of *Trβ1* is particularly evident in the most aggressive lesions, endowed with a higher proliferative capacity, further supporting the relevance of the hypothyroid status in cancer development.

Collectively, these studies demonstrate that TRβ1 downregulation is associated to HCC onset and progression and that local hypothyroidism takes place in a species- and aetiology-independent fashion.

In this context, a very interesting study unveiled a possible mechanism by which astrocyte-elevated gene-1 (AEG-1), an oncogene overexpressed and an independent prognostic factor in HCC (Jung *et al.* 2015), links non-thyroidal illness syndrome (NTIS) to cancer development. NTIS, a condition often associated with malignancy, is characterised by low serum $T_3$, but normal $T_4$ levels due to decreased *DIO1* activity in the liver (Srivastava *et al.* 2015). In a study, aimed at investigating the role of AEG-1 in NTIS in the context



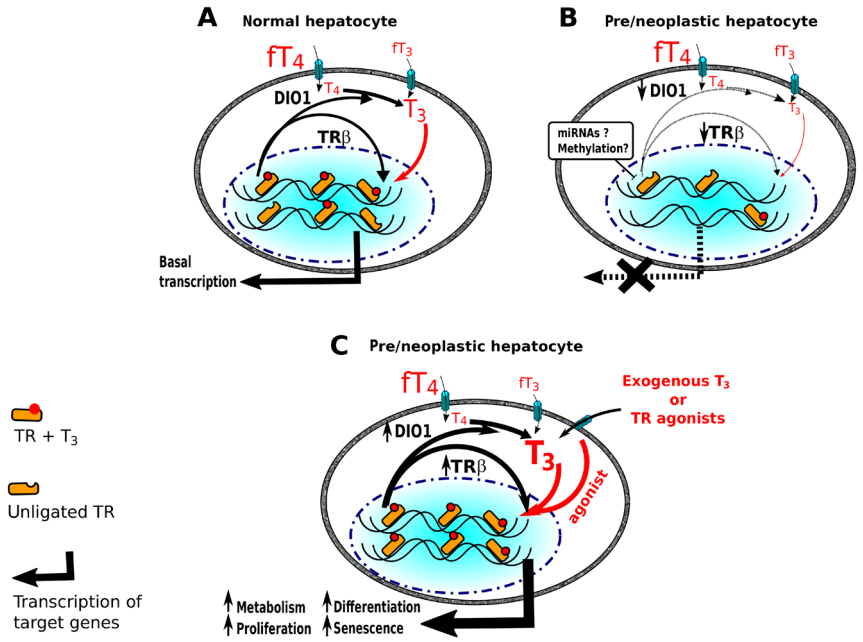


**Figure 1**
Hypothyroid status of preneoplastic/neoplastic hepatocytes and effect of $T_3$ and/or TRβ agonists. (A) In normal hepatocytes, $T_4$ is converted in $T_3$ by deiodinase 1 (DIO1). Binding of $T_3$ to TR activates transcription of TR-target genes, including DIO1, and stimulates transcription of *TRβ*. (B) In preneoplastic and neoplastic hepatocytes, downregulation of *TRβ* due to different mechanisms (methylation of promoter gene, upregulation of TR-targeting miRNAs) results in decreased expression of *DIO1* and consequently, in an impaired conversion of $T_4$ into $T_3$. This generates a hypothyroid status in preneoplastic/neoplastic hepatocytes that favors cancer progression. (C) Exogenous $T_3$ or TRβ agonists bind and activate TRβ. TRβ activation leads to its increased expression as well as to enhanced expression of its target genes, including *DIO1* which, unlike in hypothyroid pre/neoplastic hepatocytes (B), is now able to convert $T_4$ into $T_3$. The hyperthyroid status induced by $T_3$ or TRβ1 agonists and the consequent robust activation of TRβ triggers several biological processes, such as proliferation, senescence and differentiation, which negatively interfere with cancer progression. Arrow width is representative of the modifications of the activity of the TH/TRβ axis.

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of HCC, the authors found that AEG-1 inhibits ligand-dependent TR/RXR activity and downstream *DIO1* gene expression in human HCC cells and AEG-1 transgenic hepatocytes. Moreover, an inverse correlation was observed between AEG-1 and *DIO1* levels in human HCC patients. AEG-1 thus links NTIS with cancer development, including HCC.

In agreement with the possible critical role of local hypothyroidism in HCC development, the switch from hypothyroid to hyperthyroid status of preneoplastic lesions caused by $T_3$ administration was associated with the disappearance of most preneoplastic lesions and with a significant inhibition of HCC development and lung metastases (Ledda-Columbano *et al.* 2000).

## $T_3$ and HCC

While a general consensus exists regarding the oncosuppressor role of TRβ1 on HCC, controversial data have been reported about its role upon $T_3$ activation. *In vivo* studies showed that 1-week treatment with $T_3$ accelerates the regression of chemically induced hepatic preneoplastic lesions in rats subjected to the resistant hepatocyte (RH) model of hepatocarcinogenesis (Ledda-Columbano *et al.* 2000). Moreover, repeated cycles of $T_3$ reduced the frequency of HCC by 50% and completely inhibited lung metastases. Similarly, a short $T_3$ treatment leads to the disappearance of preneoplastic hepatic foci in rats exposed to another well-established model of rat hepatocarcinogenesis, namely the choline-deficient diet model (Perra *et al.* 2009). These results, together with the finding that *TRβ* and *DIO1* are profoundly downregulated in preneoplastic lesions and in rat and human HCC, and that an increase in their levels upon $T_3$ treatment is associated with preneoplastic nodule regression, suggest that reactivation of the $T_3$–TR axis may impact on the fate of preneoplastic and neoplastic lesions (Fig. 1). It follows that increasing the intracellular levels of $T_3$ might represent a novel therapeutic approach.

An anti-tumourigenic action of $T_3$ was also described by Liao and coworkers (2012); in this work, the authors proposed that $T_3$ stimulates the expression of suppressor genes, such as Dickkopf 4 (DKK4), a secreted protein that antagonises the Wnt signal pathway (Liao *et al.* 2012). Intriguingly, however, in two other studies, the same authors suggested that $T_3$ promotes cancer progression inducing either lipocalin-2 (Chung *et al.* 2015) or furin (Huang *et al.* 2012). To explain these contrasting results, the authors hypothesise that $T_3$ may inhibit cancer cell proliferation at early stages of HCC development, while it promotes cancer cell migration and invasion in malignant tumours or late-stage cancer. Although conceptually valid, this hypothesis is not yet supported by experimental data suggesting a dual role of $T_3$ in different steps of the hepatocarcinogenic process; moreover, in the latter studies, the effect of $T_3$ was investigated only at the final step of the tumourigenic process, namely in fully transformed HCC cell lines.

In addition, it is unclear why in the study showing an anti-tumourigenic activity of $T_3$ (Liao *et al.* 2012) TR levels were downregulated in HCC compared with non-tumoural liver, while in the one where $T_3$ was shown to exert a pro-tumourigenic effect, TR levels were higher in the tumours and correlated with a poor survival (Chung *et al.* 2015). Thus, whether $T_3$ inhibits or promotes HCC development has still to be clarified.

## $T_3$/TRs axis and hepatocyte proliferation

Increased cell proliferation has long been associated to cancer development. Therefore, it is not surprising that many studies aimed to investigate the effect of the TH/TR axis on liver cell proliferation. TR downregulation occurs concomitantly with increased DNA synthesis in rat liver regeneration after 2/3 partial hepatectomy (PH) and returns towards control values once proliferation ceases (Frau *et al.* 2015). These *in vivo* data are in agreement with those, *in vitro,* showing that *Trβ*, when not bound to $T_3$, acts as a cell cycle inhibitor by inhibiting Cyclin D1 expression (González-Sancho *et al.* 2002) or repressing its induction by the oncogene Ha-Ras$^{val12}$ (García-Silva *et al.* 2004). However, they are in contrast with the finding that a significant delay in the restoration of liver mass post-PH occurs in mice lacking *Trα1*, *Trβ1* or both receptors (López-Fontal *et al.* 2010).

Activation of TR by $T_3$ has been long recognised as a potent hepatomitogenic event (Short *et al.* 1972, Francavilla *et al.* 1994, Pibiri *et al.* 2001). Although the molecular mechanisms through which $T_3$ induces hepatocyte proliferation are unclear, it is of interest to note that its mitogenic effect occurs in the absence of activation of transcription factors such as AP-1, NF-κB or $STAT_3$, and it is not associated with an increased expression of *c-fos*, *c-jun* or *c-myc* proto-oncogenes (Pibiri *et al.* 2001). On the other hand, Cyclin D1 mRNA and protein levels increase very rapidly after $T_3$ treatment, suggesting that Cyclin D1 could be implicated in the $T_3$-triggered rapid entry into S phase of hepatocytes (Pibiri *et al.* 2001). An additional mechanism responsible



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for $T_3$-induced mitogenesis has recently implicated β-catenin, an important nuclear effector of the Wnt signalling pathway (Nejak-Bowen & Monga 2011). Indeed, while in wild-type mice administration of $T_3$ induces a robust wave of hepatocyte proliferation, no mitogenic response is seen in the hepatocyte-specific β-catenin knockout mice (Fanti *et al.* 2014).

Importantly, $T_3$ is not only mitogenic for intact liver, but it also improves the regenerative response of rodent livers after 70 or 90% hepatectomy and stimulates the regenerative response of the liver of old rats when given before 70% PH (Bockhorn *et al.* 2007, Columbano *et al.* 2008, Malik *et al.* 2008, Taki-Eldin *et al.* 2011).

Liver expresses both TRα and TRβ receptors; however, although TRα is predominant in the hepatocyte precursors and in the stellate cells, and could play a critical role in hepatocyte maturation during the perinatal period (Rodd *et al.* 1992), TRβ1 is the predominant $T_3$ receptor in adult liver (Schwartz *et al.* 1992). Gene profiling of livers from *Trα* or *Trβ* KO mice identified a large number of differentially regulated genes, revealing a clear predominance of *Trβ* over *Trα* in adult liver function (Flores-Morales *et al.* 2002, Yen *et al.* 2003). Accordingly, $T_3$ is able to induce proliferation in KO mice devoid of *Trα* (Kowalik *et al.* 2010), and the TRβ-specific agonist GC-1 mimics the effect of $T_3$ on hepatocyte proliferation (Columbano *et al.* 2006), thus indicating that the mitogenic activity of $T_3$ on fully differentiated hepatocytes mainly depends on TRβ.

Collectively, these studies provide substantial evidence that $T_3$ induces hepatocyte proliferation both in intact and injured liver, leading to the hypothesis that while in the absence of its ligand TRβ exerts an antiproliferative effect on hepatocytes, its activation by $T_3$ rapidly stimulates their proliferation. They also suggest that the possible use of $T_3$ or its analogues could represent a useful tool in pathological conditions characterised by an impaired regenerative ability (i.e. aged livers) or when a rapid growth stimulation of the liver is required (i.e. size transplantation).

While the mitogenic effect of $T_3$ on normal hepatocytes seems unquestionable, several *in vitro* works have reported its inhibitory role on cancer cells. Indeed, TRβ stably expressing hepatocarcinoma cell line SK-hep1 is poorly able to grow in soft agar when treated with $T_3$ (Martinez-Iglesias *et al.* 2009), and thyroid hormone inhibits proliferation of a HepG2-TRα cell line, possibly by stimulating the expression of transforming growth factor-β (TGF-β) (Yen *et al.* 2006). However, it should be noted that, *in vivo*, $T_3$ maintains its powerful mitogenic activity for preneoplastic hepatic nodules (Ledda-Columbano *et al.* 2000).

In conclusion, even though it is unclear whether $T_3$ exerts an opposite effect on normal and transformed hepatocytes, it is clear that $T_3$ is a strong inducer of hepatocyte proliferation. It follows that while in the absence of ligand TRβ acts an oncosuppressor, it delivers mitogenic signals once activated by binding to $T_3$.

## $T_3$/TRs axis and cell differentiation

$T_3$-induced regression of hepatic preneoplastic lesions occurs concomitantly with an increased proliferative activity (Ledda-Columbano *et al.* 2000). Since no signs of increased apoptosis are present, a possible explanation for such a paradoxical result is that $T_3$ exerts both pro-proliferative and pro-differentiating effects on preneoplastic hepatocytes. In several models of rat hepatocarcinogenesis, the biochemical phenotype of preneoplastic hepatocytes closely resembles that of foetal or neonatal hepatocytes; indeed, they lack the expression of enzymes normally present in differentiated hepatocytes (P-450, ATPase, glucose-6-phosphatase) (Roomi *et al.* 1985), while exhibiting high levels of enzymes expressed at a low level or absent in fully differentiated hepatocytes (γ-glutamyl transpeptidase, glutathione S-transferase P, glucose-6-phosphate dehydrogenase, α-fetoprotein). During the carcinogenic process, most preneoplastic lesions show a slow regression over time and their immature phenotype is replaced by the acquisition of adult differentiated features (Enomoto & Farber 1982). These findings suggest the existence of an active remodelling, which involves the modulation of specific genes through a genetically programmed process. Notably, a rapid loss of several markers associated with preneoplasia occurs after treatment with $T_3$ or TRβ agonists (Perra *et al.* 2009), suggesting that $T_3$-induced mitogenesis is associated with – or followed by – a process of differentiation.

Interestingly, $T_3$ induces differentiation of oval cells (considered a progenitor type) to hepatocytes, as shown by the loss of oval cell markers and the acquisition of mature hepatocyte markers (László *et al.* 2008). Even more intriguingly, a recent work (Catalano *et al.* 2016) showed that $T_3$ treatment induces differentiation of colorectal cancer stem cells (CR-CSCs), by increasing the levels of the bone morphogenetic protein (BMP4, a known promoter of differentiation in normal colonic epithelium) and of its downstream targets. The same work also showed that increasing intracellular levels of $T_3$ results in reduced



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clonogenic and tumourigenic potential and establishes a higher sensitivity of CR-CSCs to chemotherapy, supporting the hypothesis that $T_3$ may inhibit tumour development by activating a differentiation program. Since high doses of exogenous BMP4 promote CD133-positive HCC-CSC differentiation and inhibit the self-renewal and tumourigenic capacity of these cells (Zhang *et al.* 2012), one can speculate that treatment with $T_3$ may also induce differentiation of highly aggressive HCC-CSC and reduce their tumourigenic capacity by inducing BMP4 levels.

The potential relevance of $T_3$-induced differentiation in modulating tumour development is also highlighted by a work implicating Krüppel-like factors (KLFs) in TR-induced differentiation (Cvoro *et al.* 2015). KLFs are classified as a part of Sp1/KLF family of zinc-finger-containing transcription factors and play an important role in proliferation, differentiation, apoptosis, inflammation and development (Cao *et al.* 2010). They act as transcriptional activators or repressors, depending on the type and developmental stage of the cell. TR actions on neuronal differentiation in mammalian and amphibian are mediated by KLF9 induction (Denver *et al.* 1999, Dugas *et al.* 2012). KLF9 and KLF4 control NOTCH1 gene expression and exert opposite effects on its transcription, thereby influencing the Notch signalling pathway (Ying *et al.* 2011). Notch signalling, in turn, cross-reacts with other signalling pathways including Wnt, FGF, TGFβ/BMP and Hedgehog (Katoh *et al.* 2007) and converges on a transcriptional network that involves OCT4, NANOG and SOX2 to regulate stem cell maintenance and differentiation (Schnerch *et al.* 2010). Thus, alterations in KLF9 levels could greatly influence cell differentiation processes. In line with these findings, TRs induce KLF9 in several normal and transformed liver cell lines and/or progenitors, including HepG2 cells, non-transformed liver cells, human induced pluripotent stem cells (hiPSC) and human embryonic stem cells (hESC).

It is worth to underline that complex cross-regulations between $T_3$/TRα and key signalling pathways including Wnt, Notch and BMP have been largely described in the intestinal epithelium, where $T_3$/TRα controls the balance between cell proliferation and cell differentiation of the crypt precursor cells (Plateroti *et al.* 2006, Kress *et al.* 2009, 2010, Sirakov *et al.* 2015). Interestingly, studies in human HCC showed that KLF9 expression is downregulated compared with the normal liver counterpart, and that exogenous KLF9 expression inhibits proliferation of HCC cell lines and their tumourigenic capacity when xenografted in immunodepressed mice (Sun *et al.* 2014). However, the relationship between $T_3$/TRs and KLF9 in this same setting was not investigated. Given the importance of KLF9 and Notch signalling in regulating the balance between cell proliferation and cell differentiation, further studies aimed at investigating how the $T_3$/TR axis in conjunction with KLF9 regulates HCC development are of paramount importance. Towards this direction, preliminary data provide evidence for a decrease in *Klf9* mRNA levels in rat preneoplastic lesions exhibiting reduced *Trβ* expression, and its strong upregulation following $T_3$ treatment (A Columbano and A Perra, personal communications). These findings indicate an important avenue for future investigations on the $T_3$/TR/KLF9 axis in preneoplastic and neoplastic hepatocytes to obtain more insights into the ways by which TRs modulate HCC development by reactivating a differentiation programme.

## TRs and $T_3$ analogues

Hyperthyroidism is associated with a wide range of harmful effects, in particular cardiac dysfunction, i.e. tachycardia, arrhythmias and precipitation of ischaemic episodes or heart failure. These and other adverse effects have strongly limited the possible use of $T_3$ as a therapeutic agent. Therefore, several efforts have been made to develop $T_3$ analogues that could induce some beneficial effects (triglyceride, cholesterol, obesity and body mass lowering), without most of the adverse $T_3$/TR-dependent side effects. This purpose was achieved by the synthesis of TRβ agonists. Among the several analogues so far generated, GC-1, KB2115 and the Hep-Direct prodrug MBO07811 have reproduced most of the beneficial effects of $T_3$, in the absence of deleterious effects (Chiellini *et al.* 1998, Erion *et al.* 2007, Berkenstam *et al.* 2008). Experimental studies showed that treatment with GC-1 causes a reduction in triglyceride levels greater than that produced by equimolar doses of $T_3$ (Grover *et al.* 2004). These effects were achieved at doses devoid of relevant side effects on heart rate and that did not cause muscle loss or an increase in the overall catabolic state (Trost *et al.* 2000). GC-1 also prevents the development and progression of rat hepatic steatosis by increased mitochondrial and peroxisomal fatty acid β-oxidation and reduced levels of inflammatory markers (Perra *et al.* 2008). Similarly, MB07811 exhibits anti-steatotic activity in different animal models, through increased fatty oxidation in rats and mice (Cable *et al.* 2009). Interestingly, analogues such as GC-1, KB2115 and MB07344 display liver selectivity (Martínez *et al.* 2009). Due to their beneficial effects, two of the aforementioned designed analogues, GC-1



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and KB2115, commercially known as Sobetirome and Eprotirome, respectively, entered human clinical trials for dyslipidaemia, displaying encouraging results in the absence of harmful effects typically associated with TH high levels (for reviews, see Tancevski *et al*. 2011, Meruvu *et al*. 2013). Unfortunately, no phase II trials for GC-1 are planned. As to KB2115, a phase III trial was terminated due to unexpected effects on animal studies; in addition, reduction in $T_3$, as well as some degree of liver toxicity, occurred in homozygous patients affected by familiar hypercholesterolaemia, treated with 50 or 100 µg of eprotirome for only 6 weeks (Sjouke *et al*. 2014).

Although caution about the use of these analogues should be maintained, as they could have some deleterious effects, still the possibility of their therapeutic use in a disease that currently does not offer any satisfactory alternative, such as HCC, might be considered. Indeed, animal studies support the possible use of $T_3$ analogues to interfere with HCC development and progression. A short-term treatment with GC-1 of rats carrying chemically induced preneoplastic nodules induces a rapid disappearance of the vast majority of these lesions in two distinct experimental protocols (Perra *et al*. 2009), one of which characterised by extensive fatty liver, a condition often preceding HCC development in humans. Similar to what observed with $T_3$, regression of preneoplastic hepatocytes occurred concomitantly with GC-1-induced proliferation and is associated to downregulation of preneoplastic markers; these results again suggest that, when activated by ligand binding, TRβ exerts both pro-proliferative and pro-differentiating effects on preneoplastic hepatocytes. In a quite similar way, treatment with KAT-68, a liver-selective thyromimetic with hypocholesterolaemic properties and devoid of the cardiotoxicity elicited by $T_3$, shows inhibitory effects in the early and late phases of hepatocarcinogenesis (Hayashi *et al*. 2005). Indeed, in this study, KAT-68 treatment was shown to induce a reduction in the number and mean size of preneoplastic lesions when given early in the tumourigenic process. Notably, similar to the GC-1 study (Perra *et al*. 2009), the reduction in preneoplastic lesions induced by KAT-68 was associated with enhanced cell proliferation.

Based on these results, it is conceivable that treatment with $T_3$ analogues may prove to be beneficial in HCC therapy. Assuming that a condition of local hypothyroid status exists in both rodent and human HCCs, it will be critical to investigate whether these agents can induce a differentiation programme not only in preneoplastic lesions, but also in fully transformed HCCs. Our preliminary results (A C and A P) indicate that $T_3$ is indeed able to elicit, in early HCC, a modification of genes/pathways similar to that observed in preneoplastic nodules (i.e. a switch from a local hypothyroid to hyperthyroid status, downregulation of the NRF2-Keap1 pathway, loss of the putative progenitor cell marker cytokeratin-19). If $T_3$ analogues prove to be able to induce the same effects, one could seriously consider the possibility of interfering with HCC progression by inducing a differentiation programme to which even fully transformed cells may not be resistant (Fig. 1).

**Table 3** Open questions.

| |
|---|
| Is methylation of the TR promoter responsible for the downregulation of thyroid hormone receptors commonly observed in HCC? |
| Do microRNAs play a role in downregulating TR expression? |
| Is the differentiating effect of $T_3$ intimately associated with its mitogenic activity? |
| Can $T_3$ agonists represent possible therapeutic drugs in HCC? |

## Conclusions

Emerging evidence highlights the relevance of the $T_3$/TRs axis in the regulation of HCC development. Activation of TRs is required for normal growth and proliferation of liver cells, but the exact role played in the tumourigenic process by their unliganded vs liganded form is unclear. Absence or low expression of TRs seems to be a common event in many human cancers, including HCC, and in experimental studies; downregulation of TRs is observed at very early stages of the tumourigenic process, suggesting its critical role in HCC development. However, the identification of the regulatory mechanisms responsible for their altered expression (gene promoter methylation? microRNAs?) remains a still unsolved topic that needs to be carefully addressed (Table 3). Although several targets and mechanisms of anti-tumourigenic actions of TRs have been hypothesised to date, several issues remain elusive. In particular, the effect of TR activation following $T_3$-binding on proliferation and differentiation of normal and genetically altered hepatocytes, the mechanisms by which activated TRs trigger these two fundamental processes and the impact of these effects on HCC progression remain to be clarified. Undoubtedly, elucidation of the consequences of $T_3$/TRs signalling crosstalk with other pathways and specific co-regulators may help in clarifying the anti-tumourigenic effects exhibited by $T_3$ in experimental studies and in determining the crucial therapeutic implications.



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Finally, the availability of a number of recently developed TRβ1 agonists, devoid of $T_3$-induced adverse side effects, offers the fascinating perspective to consider their usefulness as therapeutic cancer drugs.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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