REVIEW

Current concepts and challenges to unravel the role of iodothyronine deiodinases in human neoplasias

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Abstract

Thyroid hormones (THs) are essential for the regulation of several metabolic processes and the energy consumption of the organism. Their action is exerted primarily through interaction with nuclear receptors controlling the transcription of thyroid hormone-responsive genes. Proper regulation of TH levels in different tissues is extremely important for the equilibrium between normal cellular proliferation and differentiation. The iodothyronine deiodinases types 1, 2 and 3 are key enzymes that perform activation and inactivation of THs, thus controlling TH homeostasis in a cell-specific manner. As THs seem to exert their effects in all hallmarks of the neoplastic process, dysregulation of deiodinases in the tumoral context can be critical to the neoplastic development. Here, we aim at reviewing the deiodinases expression in different neoplasias and exploit the mechanisms by which they play an essential role in human carcinogenesis. TH modulation by deiodinases and other classical pathways may represent important targets with the potential to oppose the neoplastic process.

Key Words

- ▶ iodothyronine deiodinases
- ▶ neoplasia
- ▶ carcinogenesis
- ▶ thyroid hormones

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Introduction

Thyroid hormones (THs) are essential modulators of several physiological processes, including organ development, cell differentiation and tissue growth. Since the description of 3,3',5-triiodothyronine (T₃) in human plasma by Gross and Pitt-Rivers (1952), numerous studies have demonstrated that it is mainly derived from the peripheral deiodination of 3,3',5,5'-tetraiodothyronine or thyroxine (T₄) (Braverman *et al.* 1970, Sterling *et al.* 1970). Monodeiodination of T₄ yields T₃ by enzymatic outer ring deiodination (ORD) of T₄ in the peripheral tissues such as the liver and the kidney, whereas the inactive form 3,3',5'-triiodothyronine (reverse triiodothyronine, rT₃)

is formed by inner ring deiodination (IRD) (Fig. 1). Both triiodothyronines are further degraded by a cascade of deiodination steps (Braverman *et al.* 1970, Sakurada *et al.* 1978, Visser 1979).

Despite an initial hypothesis that sequential deiodination was performed by two distinct enzymes acting either in the phenolic or the tyrosyl ring, evidence soon demonstrated that a single enzyme, type 1 deiodinase (*DIO1*, DIO1), was responsible for both ORD and IRD (Fekkes *et al.* 1982, St Germain & Morganelli 1989, Mandel *et al.* 1992). This process was classically studied in the liver, kidney and the thyroid and was subjected to

Figure 1

Schematic representation of the localization of deiodinases within the cell as well as the main steps of deiodination by which iodothyronines are generated. DIO1 and DIO3 are located in the plasma membrane, while DIO2 is located in the endoplasmic reticulum. DIO1 catalyzes both ORD and IRD, promoting both TH activation (generating T_3 from T_4) and inactivation (generating rT_3 from T_4). DIO2 is responsible for exclusive ORD, yielding T_3 from T_4 . DIO3 is an exclusive TH inactivating enzyme, generating T_2 from T_3 and rT_3 from T_4 . Cell graphic representation adapted from the Human Protein Atlas (https://www.proteinatlas.org/images_static/cell.svg).

6-propyl-2-thiouracil (PTU) inhibition (Visser *et al.* 1975, Leonard & Rosenberg 1978, Ishii *et al.* 1981). However, PTU did not inhibit the local deiodination of T_4 to T_3 in brain and pituitary tissues, suggesting the existence of two separate pathways of enzymatic ORD in these tissues. Investigation of the distinct biochemical properties of a possible second enzyme led to the identification of type 2 deiodinase (DIO2, DIO2) (Visser *et al.* 1981). DIO2 has a K_m for T_4 that is approximately three orders of magnitude lower than that of DIO1 in *in vitro* conditions. The observations that higher rates of IRD occur in neonatal tissues and that high levels of rT_3 are present in fetal serum, led to the identification of a specific enzyme responsible for IRD, generating rT_3 from T_4 and 3,3'-diiodothyronine (3,3'- T_2) from T_3 . This enzyme was subsequently demonstrated to be type 3 deiodinase (DIO3, DIO3) (Fig. 1) (Kaplan *et al.* 1983, Tanaka *et al.* 1992). DIO3 has a much lower K_m for T_4 than DIO1 and is the main enzyme involved in TH inactivation. This enzyme controls TH homeostasis locally, protecting the tissues, such as the brain and fetal tissues, from an excess of THs (Ciavardelli *et al.* 2014).

Deiodinases are selenoproteins, meaning they contain a single selenocysteine residue (SeC) in the catalytic center, which is highly conserved between the three enzymes. To incorporate the SeC into the amino acid chain, the cell must recognize the UGA as a Sec codon rather than a

STOP translation signal. This is performed by a stem-loop structure in the 3' untranslated region (UTR) called the Sec insertion sequence (SECIS) element. The SECIS element is the signal that recodes the UGA from a STOP to a Sec codon (Berry *et al.* 1991a,b). The three enzymes depend on a yet unidentified physiologic thiol cofactor that is substituted during *in vitro* reactions by reduced dithiols such as dithiothreitol (DTT). The group of selenoproteins still intrigues us due to their peculiar characteristics and mechanisms of action (Schweizer & Steegborn 2015). The mechanism of reductive deiodination of iodothyronines is not yet fully understood. Recently, the crystal structure of the type 3 deiodinase catalytic domain was identified, and it was shown to resemble the family of peroxiredoxin(s) (Prx). These findings can explain some previously enigmatic features of deiodinase biochemistry and confirms its thioredoxin (Trx) scaffold, suggesting that dimerization is mediated by the catalytic domain and primarily by the N-terminal region of the protein. Moreover, dimerization activates the enzyme by relaxing an autoinhibitory loop, providing access to the binding site. Analysis of Dio3 structure further reveals deiodinase-specific features classifying them as evolutionarily related to atypical 2-Cys Prx. Structure and biochemical data suggest that oxidized enzyme can be directly reduced by exogenous thiols *in vitro*. These data suggest an evolutionary pathway with Prx as an ancestor of iodothyronine deiodinase (Schweizer *et al.* 2014).

Deiodinases are Trx fold-containing dimeric enzymes with a molecular weight that varies between 29 and 33 kDa (each monomer) that are located in the plasma membrane (DIO1 and DIO3) and in the endoplasmic reticulum (ER) (DIO2) (Gereben *et al.* 2008). All three deiodinase enzymes are integral membrane proteins and are subject to dimerization (Curcio-Morelli *et al.* 2003a). While DIO1 and DIO3 expression are known to be controlled mainly through pretranscriptional mechanisms, DIO2 is uniquely known for its post-transcriptional activity-induced inactivation. The inactivation process involves ubiquitination of the active enzyme by WD repeat and SOCS box-containing protein 1 (WSB-1), which leads to an inactive DIO2 conformation, followed by proteasomal degradation (Steinsapir *et al.* 2000, Dentice *et al.* 2005, Sagar *et al.* 2007). However, DIO2 can also be reactivated through deubiquitination by ubiquitin-specific peptidase 33 (USP33) (Curcio-Morelli *et al.* 2003b). DIO1 activity is also regulated by rT_3 in a post-translational level through a mechanism that possibly involves post-catalytic structural changes in the DIO1 homodimer inactivating the enzyme (Zhu *et al.* 2012). The mechanism of substrate-

induced inactivation of DIO2 and DIO1 suggests that this regulation might be applicable to all three deiodinases (Schweizer & Steegborn 2015). There is also evidence of post-transcriptional regulation of DIO3. Drug-induced hepatotoxicity decreased DIO3 protein levels in rat liver, although *DIO3* mRNA levels were not changed (Dudek *et al.* 2013). Moreover, whole-cell deiodination assays with peroxiredoxin 3 (Prx3) knockdown strongly indicate that this DIO3-associated protein plays a specific role in DIO3 regeneration, contributing to the post-translational regulation of the enzyme (Aerts *et al.* 2009).

In humans, DIO1 is mainly expressed in the liver, the kidney and the thyroid gland (Maia *et al.* 2011). DIO2 expression, however, is more widely distributed. *DIO2* mRNA and/or DIO2 activity are found in the human thyroid, esophagus, heart, brain, pituitary, skeletal muscle, skin, brown adipose tissue and reproductive organs (Fig. 2) (Kaplan *et al.* 1988, Croteau *et al.* 1996, Salvatore *et al.* 1996a,b, de Jesus *et al.* 2001, Gouveia *et al.* 2005).

Figure 2
Graphic representation of mean mRNA levels of *DIO1*, *DIO2* and *DIO3* in human tissues. RNA-Seq data are reported as the median reads per kilobase per million mapped reads (RPKM) generated by the Genotype-Tissue Expression (GTEx) project (Consortium GT 2013). Data were downloaded from the Human Protein Atlas available at v18.proteinatlas.org (Uhlen *et al.* 2015).

The administration of PTU (which inhibits DIO1 activity) to hypothyroid individuals receiving levothyroxine supplementation reduces T_3 production by only approximately 25% (Saber *et al.* 1975), supporting *in vitro* studies that show that PTU-insensitive deiodination by DIO2 is a major source of T_3 in humans (Maia *et al.* 2005). DIO2 plays an essential role in different organs and systems regulating local T_3 production. In a system that transiently coexpresses DIO1 and DIO2, analysis of deiodination at physiologic free T_4 levels demonstrates that DIO2 has a much higher catalytic efficiency than DIO1 and is the primary source of extrathyroid-produced T_3 in the euthyroid state (Maia *et al.* 2005). DIO3, which is translated from a paternally imprinted gene and is located in the *DLK-DIO3* genomic region, is significantly increased in several tissues during embryogenesis, such as the embryonic liver, cerebral cortex, gonads, intestine and skin. It is critical for TH homeostasis in this context, as exposure of the embryo to high TH levels can be detrimental to proper development (Visser & Schoenmakers 1992, Ciavardelli *et al.* 2014). It is also expressed in the placenta, where it broadly protects the fetus from excessive TH exposure (Salvatore *et al.* 1995, Koopdonk-Kool *et al.* 1996, Huang *et al.* 2003). Importantly, DIO3 is reexpressed in normal and pathological hyperproliferative conditions. DIO3 reactivation has been demonstrated in the pathological context of cardiac hypertrophy, myocardial infarction, critical illness and several types of cancer (Huang 2005, Wajner *et al.* 2011, Lehnen *et al.* 2017).

Several signaling pathways and hormonal stimuli regulate deiodinase expression and activity in normal tissue. The human *DIO1* gene is under the control of GC-rich SP1 promoters and contains two thyroid hormone response elements that contribute to the T_3 responsiveness of the *DIO1* promoter (Toyoda *et al.* 1995, Jakobs *et al.* 1997, Zhang *et al.* 1998). The most potent modulator of DIO1 activity is T_3 . T_3 promotes transcriptional activation of the *dio1* gene in the rat and the mouse in a process that does not require protein synthesis (Berry *et al.* 1990, Maia *et al.* 1995a,b). Although studies of developmental changes with embryonic chickens showed a causal relationship between the increase in plasma growth hormone (GH) and T_3 levels, no changes in *DIO1* mRNA were observed (Darras *et al.* 1992, Van der Geyten *et al.* 1999). However, GH (and also dexamethasone) decreased DIO3 activity by acting at the pre-translational level, which could explain the increased levels of T_3 in this model (Van der Geyten *et al.* 1999). DIO1 is also positively regulated by the adenylyl cyclase (cAMP) cascade, hepatocyte nuclear factor 4 alpha (HNF4A),

liver X receptor alpha, thyroid-stimulating hormone (TSH), prolactin and beta-adrenergic stimulation (Ishii *et al.* 1983, Aceves *et al.* 1995, Beech *et al.* 1995, Ohguchi *et al.* 2008). Forkhead box (FOX) transcription factors play a key role in the regulation of crucial biological processes, including cell proliferation and metabolism (Lam & Gomes 2014). Interestingly, forkhead box A1 (FOXA1) and forkhead box A2 (FOXA2) regulate *DIO1* expression in liver. *DIO1* is positively regulated by FOXA1 and negatively regulated by FOXA2 (Kanamoto *et al.* 2012). *DIO1* has been shown to be negatively regulated by cytokines, such as interleukin-1 beta and tumor necrosis factor alpha. However, stimulatory or inhibitory effects of these molecules depend on the type of cytokine, the species and the organ studied (Ongphiphadhanakul *et al.* 1994, Davies *et al.* 1997, Baur *et al.* 2000, Jakobs *et al.* 2002, Boelen *et al.* 2004).

DIO2 mRNA and *DIO2* activity levels are upregulated by epidermal growth factor (EGF), cAMP, nuclear factor kappa B, forkhead box O3, peroxisome proliferator-activated receptor gamma, forskolin, bile acids and beta-adrenergic agonists (Song & Oka 2003, Fekete *et al.* 2004, Grozovsky *et al.* 2009, Dentice *et al.* 2010, Bianco 2011). In contrast, *DIO2* is negatively regulated at both the pre-translational and post-translational level by THs and at the pre-translational level by tumor necrosis factor alpha, dexamethasone, forkhead box protein O1 and liver X receptor/retinoid X-receptor pathway (Bianco *et al.* 1992, Burmeister *et al.* 1997, Tu *et al.* 1997, Hosoi *et al.* 1999, Song & Oka 2003, Christoffolete *et al.* 2010, Lartey *et al.* 2015). Platelet-derived growth factor and basic fibroblast growth factor (bFGF), which are essential mitogens for vascular smooth muscle cells, cause the induction of *DIO2*, which is mediated at least partially by the extracellular signal-regulated kinases (ERK)1/2 pathway (Maeda *et al.* 2003). GATA2, a transcription factor that determines thyrotroph differentiation, also stimulates *DIO2* promoter, as do GATA4 and Homeobox protein Nkx-2.5, central regulators of tissue-specific transcription in cardiomyocytes (Dentice *et al.* 2003, Matsunaga *et al.* 2015).

The hedgehog signaling pathway transmits the required information to embryonic cells for appropriate cell differentiation and is considered to be one of the critical regulators of vertebrate development (Ingham & McMahon 2001). Among the hedgehog homolog proteins, Sonic hedgehog (Shh) is the most studied and is involved in the development of the brain, skeleton, musculature, gastrointestinal tract and lungs (Villavicencio *et al.* 2000). The Shh pathway has also

been implied in neoplastic processes (Taipale & Beachy 2001) and importantly modulates both *DIO2* and *DIO3* expression. *DIO2* is downregulated post-transcriptionally primarily by ubiquitination (Dentice *et al.* 2005) while *DIO3* is subjected to Shh upregulation through the transcription regulator zinc finger protein GLI2 (GLI2) in normal keratinocytes (Dentice *et al.* 2007). T_3 , retinoic acid, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and bFGF induce *DIO3* expression and *DIO3* activity in rat astrocytes. The effects of TPA and bFGF seem to be mediated by the mitogen-activated protein kinase (MAPK) signaling pathway (Pallud *et al.* 1999). The *DIO3* gene is also transcriptionally induced by transforming growth factor beta (TGFB) via a Smad and MAPK-dependent pathway, by hypoxia-inducible factor 1 alpha (HIF1A) and by Wnt/beta-catenin pathway (Huang *et al.* 2005, Simonides *et al.* 2008, Dentice *et al.* 2012, Romitti *et al.* 2016).

Thyroid hormone levels and the neoplastic process

Depletion of THs or their excess promotes modifications in tumoral growth and development. These changes correspond to the ability of THs to promote or inhibit cell proliferation in a cell type-dependent manner, as well as to induce differentiation, in a process linked to growth arrest and exit from the cell cycle. Indeed, THs seem to exert their effects in all hallmarks of the neoplastic process, which include sustained proliferation signaling, resistance to growth suppressors, evasion of programmed cell death, replicative immortality, sustained angiogenesis and promotion of invasion and metastasis (Goemann *et al.* 2017).

In plasma, the amount of total T_4 exceeds the amount of T_3 by two orders of magnitude (Bianco & Kim 2006). Both T_4 and T_3 enter the cell via transporters, including the monocarboxylate transporter 8 (MCT8) and the organic anion transporting polypeptide C1 (Jansen *et al.* 2005, Friesema *et al.* 2006). T_4 can be deiodinated to T_3 in the intracellular environment by *DIO2*. In contrast, *DIO3* acts locally to decrease cellular T_3 concentrations. Thereby, the deiodinases are critical for the regulation of intracellular T_3 levels and therefore contribute to hormone nuclear concentration and saturation of thyroid hormone receptors (TRs) (Wu & Koenig 2000).

It is widely accepted that T_4 , which comprises the main secretory product of the thyroid gland is a prohormone and must be converted to the active form T_3 by *DIO1* or *DIO2* to promote TH metabolic

effects. However, increasing evidence suggests that T_4 can promote nongenomic effects through direct interactions with several pathways, particularly in the context of neoplasia. This broad issue has been recently reviewed by our group and others and will not be discussed here (Davis *et al.* 2016, Goemann *et al.* 2017). It should also be noted that rT_3 , which is generally regarded as an inactive metabolite, seems to be relevant to the structure of both normal cells and tumor cells, by supporting the integrity of the actin cytoskeleton (Farwell *et al.* 2005). Critical intracellular signaling pathways, such as MAPK, Wnt and Shh are dysregulated in tumoral cells, which may lead to upregulation or downregulation of the deiodinase enzymes depending on the context. Moreover, the theory of the stem cell origin of neoplastic cells, and the relevant role of DIO3 in stemness, suggests an essential role of DIO3 in tumor development (Ciavardelli *et al.* 2014). The yet unidentified reducing cofactor of deiodinases might be subject to alterations in redox and oxidative stress, which are well-known characteristics of the neoplastic microenvironment (Koppenol *et al.* 2011). Thus, the catalytic efficiency of deiodinases can be impaired by neoplastic conditions (Wajner *et al.* 2011).

Changes in deiodinase expression have been reported in several neoplasias (Table 1) (Meyer *et al.* 2007, Piekliko-Witkowska & Nauman 2011, Casula & Bianco 2012, Dentice *et al.* 2013). One of the best examples of how changes in deiodinases might alter TH concentrations is the clinical condition of consumptive hypothyroidism, a severe form of hypothyroidism due to high levels of DIO3 activity in the neoplastic tissues. It was first described in infantile liver hemangiomas (Huang *et al.* 2000). Subsequently, pediatric and adult liver vascular tumors were also associated with increased expression and activity of DIO3 (Huang *et al.* 2002, Weber Pasa *et al.* 2017). Indeed, large vascular tumors can express enough DIO3 sufficient to inactivate a significant amount of plasma T_3 and cause overt hypothyroidism. DIO3 upregulation occurs through the Shh pathway and the MAPK signaling cascade in these types of tumors (Aw *et al.* 2014). However, infantile hemangiomas origin is not fully elucidated. Current evidence suggests that hemangiomas are clonal proliferation of fetal endothelial cells, not hepatocytes. Another hypothesis speculates that these cells are derived from the placenta (Boye *et al.* 2001, Chen *et al.* 2013). Interestingly, either type of cells express a significant amount of DIO3 protein (Huang *et al.* 2003), what corroborates the suggestion that these tumors arise from other cells than hepatocytes.

As deiodinases control TH levels, they also contribute to the balance between proliferation and differentiation within the cell. Few studies have actually evaluated both deiodinase expression and intracellular TH concentrations at the same time (see the data below regarding basal cell carcinoma, glioblastoma and clear cell renal cell carcinoma) (Table 1) (Nauman *et al.* 2004, Dentice *et al.* 2007, Master *et al.* 2010). Nevertheless, it is important to keep in mind that disturbed deiodinase expression can go beyond the regulation of intracellular levels of T_4 and T_3 in the tumoral context. The upregulation or downregulation of deiodinases can reflect the overactivation or suppression of critical signaling pathways involved in carcinogenesis. Moreover, the expression of deiodinases may be a marker of hypermethylation or hypomethylation of the DNA regions where they are located, indicating that they may just represent a small portion of a bigger picture of aberrant cell function. Indeed, several tumor-related aberrations in the chromosomal regions of *DIO1*, *DIO2* and *DIO3* have been described (Piekliko-Witkowska & Nauman 2011). Thus, since the neoplastic process has distinct tissue-related features, it is reasonable to speculate that examining the role of deiodinases in specific tumoral contexts can render a better understanding whether they are a cause or a consequence of neoplastic cellular imbalance.

The thyroid: a classical model

Significant amounts of ORD are found in normal thyroid tissue due to the high expression of DIO1 and DIO2 (Ishii *et al.* 1981, Ishii *et al.* 1982, Sugawara *et al.* 1984, Salvatore *et al.* 1996b). DIO1 is the main enzyme responsible for T_3 production within the gland. DIO3 activity is regarded as being absent in the thyroid, but traces of *DIO3* mRNA transcripts have been found in human thyroid tissue samples (Romitti *et al.* 2012, Uhlen *et al.* 2015) (Fig. 2). When evaluating thyroid nodules, an increased 5' deiodination was observed in toxic and also follicular adenomas, while decreased activity was found in cold nodules when compared to healthy tissue (Sugawara *et al.* 1984, Brtko *et al.* 2002).

Differentiated thyroid cancer from follicular cells is the most common malignant neoplasia of the endocrine system. Papillary thyroid carcinoma (PTC) is the most prevalent histologic type accounting for more than 90% of cases, while follicular thyroid carcinoma (FTC) is responsible for the remaining cases (<https://seer.cancer.gov/statfacts/html/thyro.html>, Accessed on 02/09/2018) (Haugen *et al.* 2016). Genetic activation of the MAPK

Table 1 Expression of deiodinases in different types of cancer in humans.

Tumor	DIO1	DIO2	DIO3	Overall effect on intracellular T ₃ levels	Potential effect of T ₃ on hallmarks of cancer	References
Basal cell carcinoma	N/A	Increased <i>DIO2</i> mRNA levels, presence of <i>DIO2</i> activity levels	Increased <i>DIO3</i> mRNA, protein and enzymatic activity levels	Decreased*	Promotes proliferation and decreases apoptosis	Dentice et al. (2007) , Miro et al. (2017)
Breast cancer	Increased mRNA and enzyme activity levels, particularly in the most differentiated subtypes	<i>DIO2</i> mRNA upregulated in MDA-MB-231 cell line but not in MCF-7 cell line	<i>DIO3</i> mRNA upregulation is found in MCF-7 cells, and <i>DIO3</i> protein is present in breast cancer samples	Changes in deiodinases status involve T ₃ activation and inactivation and its 'consequences' on intracellular TH concentrations are unclear	Influences cell proliferation	Garcia-Solis and Aceves (2003) , Debski et al. (2007) , Rusolo et al. (2017) , I M Goemann, V Marczk, A L Maia (unpublished observations)
Clear cell renal carcinoma	Decreased mRNA and enzyme activity levels	N/A	N/A	Decreased (measured)	Stimulates proliferation and invasion, contributes to oxidative stress response	Pachucki et al. (2001) , Poplawski & Nauman (2008) , Poplawski et al. (2017a,b)
Colorectal cancer	N/A	N/A	Upregulation of <i>DIO3</i> mRNA, higher protein expression in cancer samples when compared to normal tissue	Decreased*	Induces proliferation	Dentice et al. (2012)
Glioma	N/A	Increased <i>DIO2</i> mRNA and <i>DIO2</i> activity levels	Variable levels of <i>DIO3</i> mRNA and <i>DIO3</i> activity	Decreased (measured)	Induces proliferation	Mori et al. (1993) , Murakami et al. (2000) , Nauman et al. (2004)
Hemangioma	Decreased <i>DIO1</i> activity levels	N/A	Increased <i>DIO3</i> mRNA and <i>DIO3</i> activity	Decreased (measured)	?	Huang et al. (2000) , Kornasiewicz et al. (2010) , Kornasiewicz et al. (2014)
Lung cancer	Decreased <i>DIO1</i> activity levels	<i>DIO2</i> activity similar to normal lung tissue	Decreased <i>DIO3</i> mRNA levels	Changes in deiodinases status involve T ₃ activation and inactivation and its 'consequences' on intracellular TH concentrations are unclear	?	Wawrzynska et al. (2003) , Molina-Pinelo et al. (2018)

(Continued)

Table 1 Continued.

Tumor	DIO1	DIO2	DIO3	Overall effect on intracellular T ₃ levels	Potential effect of T ₃ on hallmarks of cancer	References
Papillary thyroid cancer	Decreased <i>DIO1</i> mRNA and DIO1 activity levels	Decreased <i>DIO2</i> mRNA and DIO2 activity levels	Increased <i>DIO3</i> mRNA and DIO3 activity levels	Decreased*	Induces proliferation and invasion	Toyoda <i>et al.</i> (1992) , Köhrle <i>et al.</i> (1993) , Murakami <i>et al.</i> (2001) , Ambroziak <i>et al.</i> (2005) , de Souza Meyer <i>et al.</i> (2005) , Romitti <i>et al.</i> (2012)
Pituitary tumors	Both DIO1 and DIO2 activities are detected in tumoral and normal tissues in variable levels		Increased <i>DIO3</i> mRNA, variable DIO3 activity levels	Changes in deiodinases status involve T ₃ activation and inactivation and its 'consequences' on intracellular TH concentrations are unclear	?	Baur <i>et al.</i> (2002) , Tannahill <i>et al.</i> (2002)

*Intracellular T₃ levels were evaluated indirectly by intracellular T₃-responsive reporters or inferred according to changes in deiodinases expression. DIO1, deiodinase type 1; DIO2, deiodinase type 2; DIO3, deiodinase type 3; N/A: not available.

signaling pathway is a hallmark of PTC ([Cancer Genome Atlas Research Network 2014](#)). *DIO1* and *DIO2* seem to be underexpressed in PTC ([Toyoda *et al.* 1992](#), [Huang *et al.* 2001](#), [Murakami *et al.* 2001](#), [Ambroziak *et al.* 2005](#), [Romitti *et al.* 2012](#)). Earlier studies performed in human PTC samples have shown that *DIO1* mRNA levels were reduced in all the samples that were analyzed ($n=14$) when compared to the normal surrounding tissue. This was paralleled by a decrease in DIO1 activity with only one exception of a follicular variant of PTC ([de Souza Meyer *et al.* 2005](#)). [Arnaldi *et al.*](#) also reported significant *DIO1* and *DIO2* underexpression in most but not all PTC samples that were matched to normal tissue ([Arnaldi *et al.* 2005](#)). On the other hand, increased levels of *DIO3* mRNA and DIO3 activity have been demonstrated in human PTC samples. Of interest, PTC tumors carrying the BRAF^{V600E} mutation had the highest levels of DIO3 activity. Moreover, a positive correlation between tumor size and DIO3 activity, as well as an increased DIO3 activity was demonstrated in thyroid tumor samples from patients advanced disease at diagnosis ([Romitti *et al.* 2012](#)).

Taken together, one could speculate that changes in deiodinase expression in PTC could lead to decreased intracellular hormone levels and favor tumor proliferation. The increase in DIO3 and the decrease in DIO1 and

possibly DIO2 that lead to diminishing T₃ concentrations in the microenvironment could provide an advantage for tumor cell proliferation since THs can block the oncogenic Ras-mediated proliferation that interferes specifically with the activity of the MAPK pathway ([Garcia-Silva & Aranda 2004](#)). Recently, crosstalk between the MAPK and SHH pathways leading to *DIO3* upregulation has been demonstrated in human PTC cell lines ([Romitti *et al.* 2012, 2016](#)). In support of this line of reasoning, the inhibition of *DIO3* mRNA expression through siRNA decreases cyclin D1 expression and induces a partial G1 phase cell cycle arrest, thereby downregulating cell proliferation ([Romitti *et al.* 2016](#)). These observations indicate that SHH/Gli2 pathway contributes to DIO3 overexpression, suggesting that the consequent decrease in intracellular T₃ levels may be a critical factor for tumor proliferation in PTC. MAPK canonical signaling pathway is activated by the BRAF^{V600E}, the most commonly detected BRAF mutation in human PTC. Interestingly, mice expressing the BRAF^{V600E} mutation in thyroid follicular cells developed rapid clinical hypothyroidism (within 48h) ([Chakravarty *et al.* 2011](#)). This might indicate that the reactivation of DIO3 in PTCs that harbor the BRAF^{V600E} mutation through MAPK pathway. However, DIO3 expression was not evaluated in this model. Of note, increased immunostained DIO3

protein has been observed in FTC but not in medullary or anaplastic thyroid carcinoma samples (Romitti *et al.* 2012).

In FTC samples, we observed a significant increase in *DIO1* mRNA levels compared with nontumoral tissue, while others found comparable levels between the tumoral and the normal tissues or even decreased *DIO1* activity (Köhrle *et al.* 1993, Arnaldi *et al.* 2005, de Souza Meyer *et al.* 2005, Takano *et al.* 2006). *DIO1* activity was significantly higher in samples of metastases from follicular carcinoma (de Souza Meyer *et al.* 2005). Higher *DIO2* activity was found in samples of larger metastasis of FTC. However, no significant changes in *DIO2* mRNA levels were observed, which suggests that *DIO2* upregulation occurs mainly by post-transcriptional regulatory mechanisms (Kim *et al.* 2003, de Souza Meyer *et al.* 2005, Takano *et al.* 2006).

Although *DIO2* expression has not been evaluated in normal C cells, we described detectable *DIO2* activity in medullary thyroid cancer (MTC) samples, which was comparable to the amounts in the surrounding normal follicular tissue. *DIO2* mRNA and *DIO2* activity levels have also been demonstrated in the TT cell line (derived from MTC), which might suggest a potential role of intracellular T_3 in this neoplastic tissue (Meyer *et al.* 2008).

We performed an analysis of The Cancer Genome Atlas (TCGA) database (<http://cancergenome.nih.gov/>) through Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn>) (Tang *et al.* 2017), which is a bioinformatics research platform for the profiling and interactive analysis of cancerous gene expression based on TCGA and Genotype-Tissue Expression (GTEx) public databases (Consortium GT 2013). Intriguingly, analysis of TCGA database showed that *DIO1* and *DIO3* genes are downregulated in thyroid carcinoma (77% PTC, 21% FTC) ($n=512$, $P<0.01$ for *DIO1* and *DIO3*) compared to matched TCGA and GTEx data of normal tissue. *DIO2* expression in tumors is comparable to that in normal tissue (Fig. 3).

Basal cell carcinoma: insights on the imbalance of the proliferation/differentiation equilibrium

DIO2 and *DIO3* mRNA transcripts, as well as *DIO2* and *DIO3* activities, are present in normal human skin (Kaplan *et al.* 1988, Santini *et al.* 2003, Slominski *et al.* 2002). Basal cell carcinoma (BCC), which is the most common cutaneous malignancy, is a non-melanocytic skin cancer that arises from basal cells (the lower layer of the epidermis) (Sellheyer 2011). Dysregulated hedgehog (Hh) signaling is a hallmark of this neoplasia, due to inactivation of Protein

Figure 3

Expression of (A) *DIO1*, (B) *DIO2*, and (C) *DIO3* in different neoplasias (red) compared to matched TCGA and GTEx data from normal tissue (gray). Expression values are presented in log-scale ($\log_2(\text{transcripts per million (TPM)} + 1)$). Data were obtained from TCGA and GTEx databases and processed and analyzed with GEPIA. * $P<0.01$.

Patched Homolog 1 (PTCH1), which is an inhibitor of Hh signaling (Epstein 2008). Most of the research regarding deiodinases and BCC tumorigenesis has been performed by Dentice *et al.* (Dentice *et al.* 2007, Luongo *et al.* 2014, Di Girolamo *et al.* 2016, Miro *et al.* 2017). They demonstrated that sonic hedgehog (Shh), through Gli2, directly induces *DIO3* in human BCCs, reducing intracellular T_3 and thus increasing cyclin D1 and proliferation. Shh also mediates *DIO2* reduction through post-transcriptional mechanisms. Thus, *DIO3* knockdown blocks proliferation and reduces the oncogenic potential of BCC tumor cells. Indeed, the growth of BCC cells implanted in *DIO3*-knockdown mice was dramatically reduced, suggesting that T_3 reduces BCC proliferation and tumorigenic potential (Dentice *et al.* 2007). Moreover, in G2N2c keratinocyte cells, *DIO3* depletion led to the arrest of the cell cycle in G_1 and to decreases in cyclin D1 levels, further demonstrating that

cell proliferation is drastically reduced by DIO3 inhibition. Inversely, T₃ treatment decreases Gli2 protein levels through upregulation of cAMP/PKA signaling. Whether or not T₃ directly affects DIO3 levels in this context is unknown (Luongo *et al.* 2014). However, T₃ modulates DIO3 expression indirectly through another pathway. T₃ has a suppressive effect on the oncogenic microRNA (miRNA) miR21, which in turn induces DIO3 expression through downregulation of the Grainyhead-like protein 3 homolog (GRHL3). GRHL3 is tumor suppressor factor that is expressed in the skin and is essential for epidermal differentiation. Therefore, the existence of a miR21/GRHL3/DIO3 axis critically contributes to the intracellular TH imbalance in the context of BCC (Di Girolamo *et al.* 2016); thus, BCC is an excellent model to study the TH role in the delicate balance between cell proliferation and differentiation. *DIO3* mRNA and DIO3 activity have been reported in BCC cells, while only *DIO2* mRNA has been demonstrated in these cells. The authors infer the presence of DIO2 activity in BCC cells by the fact that genetically induced depletion of *DIO2* gene using CRISPR/Cas9 technology leads to decreased levels of T₃-responsive targets. Therefore artificial modulation of these enzymes can alter the local TH levels, and their effects on tumor growth can be evaluated (Miro *et al.* 2017). Interestingly, TH activity, as evaluated by a T₃-dependent artificial promoter that drives the luciferase gene, is reduced in BCC DIO2KO cells and enhanced in BCC DIO3KO cells. DIO2KO-BCC (low intracellular T₃) cells are characterized by a high proliferation rate, a high proportion of S-phase cells and decreased apoptosis. On the other hand, DIO3KO-BCC cells have decreased proliferation and low levels of cyclin expression. This interesting model could be expanded to other neoplasias aiming for a better understanding of the effects of THs on cancer (Miro *et al.* 2017).

Colorectal cancer: deiodinases mediate TH changes as differentiation agents

Colorectal cancer (CRC) is characterized by a complex array of genetic alterations, among which the mutation in the adenomatous polyposis coli (APC) gene is the most frequent (85% of cases). This mutation leads to a constitutively active Wnt pathway due to inadequate degradation of beta-catenin by the APC protein (Markowitz & Bertagnolli 2009). Dentice *et al.* provided the first evidence suggesting an interplay between the Wnt/beta-catenin pathway and the TH signaling pathway in the balance between proliferation and differentiation

in colorectal cancer (Dentice *et al.* 2012). The authors elegantly showed that exogenous T₃ treatment reduced proliferation and increased differentiation *in vitro* in CRC-derived cell lines. Moreover, they demonstrated that the activation of Wnt/beta-catenin induced the expression of *DIO3* mRNA while decreasing *DIO2* mRNA. This dual mechanism could result in intracellular hypothyroidism, favoring proliferation over differentiation. Additionally, they showed that DIO3 depletion had T₃-like effects and that xenografts of DIO3-depleted cells exhibited reduced tumor growth.

Dentice and colleagues have also shown DIO3 expression was associated with neoplastic transformation in CRC. Using immunohistochemistry in 105 human paraffin-fixed samples, they observed that DIO3 positivity was found in 10% of the normal tissues, whereas 80% of carcinomas and 90% of adenomas were DIO3 positive, suggesting that DIO3 is a Wnt/beta-catenin target in the context of colon carcinoma proliferation (Dentice *et al.* 2012). Moreover, T₃ treatment or DIO3 inhibition could be used to promote CRC-stem cell (CRC-SC) differentiation via Wnt and BMP4 signaling. Interestingly, TH-induced differentiation increased CRC-SC sensitivity to traditional chemotherapy (oxaliplatin and 5-FU), raising the possibility of combination therapy in the future (Catalano *et al.* 2016).

The analysis of the public TCGA database through GEPIA demonstrated low expression of *DIO1* in CRC and detectable levels of *DIO3* mRNA that were similar in tumor tissue and normal tissue (Fig. 3). These findings are in contrast with the findings of Dentice *et al.* (2012) who found that DIO3 protein levels were increased as evaluated through immunohistochemistry in 22 out of 24 tumors tissue samples compared with normal surrounding tissue. These differences could be attributed to post-translational mechanisms that regulate protein expression as well as to differences in the controls (normal predisposed surrounding tissue vs tissue from healthy individuals). *DIO2* was overexpressed in CRC tissue when compared to non-paired tissue ($n=275$, $P<0.01$) and was found to be a marker of good prognosis in this database (5-year survival: 66% in the high expression group vs 46% in the low expression group; $P<0.01$). This is a finding that should be further confirmed.

The glioma model: the adverse effects of T₃

Brain cells are uniquely sensitive to the effects of THs; therefore, they require even tighter control of TH homeostasis when compared to other organs.

THs stimulate the processes of myelination, the proliferation of glial cells as well as axon growth and formation. The necessity for strict control of TH levels might explain the detectable activity of DIO2 and DIO3 in glial cells (Visser *et al.* 1981, Crantz *et al.* 1982, Silva & Leonard 1985, Riskind *et al.* 1987, Ciavardelli *et al.* 2014). Indeed, studies performed in rat cerebral cortex demonstrate that approximately 80% of the T₃ bound to nuclear receptors is produced locally by monodeiodination of T₄ (Crantz & Larsen 1980, Crantz *et al.* 1982). DIO3 activity is present in adult human brain tissue, and high DIO3 levels in placental tissue play an essential role in protecting the developing brain of the fetus from excessive T₃ concentrations (Campos-Barros *et al.* 1996, Kester *et al.* 2004).

Gliomas are tumors arising from the brain parenchyma, with a broad range of aggressiveness. Grades I and II gliomas are referred to as low-grade gliomas, while the more rapidly progressive tumors are referred to as high-grade gliomas (grades III and IV) (Louis *et al.* 2016). The modulation of intracellular T₃ and T₄ levels by deiodinases in glioblastoma is of great importance, since the PI3K, Src kinase and ERK1/2 signaling cascades are parallel pathways that are stimulated by T₃ in U-87MG cells, a commonly studied human glioblastoma cell line (Liappas *et al.* 2011). These are among the most commonly dysregulated pathways in this type of cancer. DIO2 expression and DIO2 activity were found in glioblastoma and were the highest in a tissue sample from an anaplastic oligodendroglioma (Murakami *et al.* 2000). Nauman *et al.* made an effort to correlate deiodinase activity and the T₄/T₃ concentrations of gliomas and normal surrounding tissue. They found that T₄ and T₃ concentrations in the tumor tissues were lower than those in the non-tumor tissues in the majority of the patients, whereas DIO2 activity was higher in tumor tissues than in normal tissues (Nauman *et al.* 2004). In one study, DIO3 activity was detected in samples of gliomas, although no comparison to healthy tissues was performed (Mori *et al.* 1993). In another study, DIO3 was detected in a heterogeneous manner. In two samples, the DIO3 activity was lower than in the tumor tissue, but in all the high-grade gliomas (IV), the DIO3 activity was considerably higher in the tumoral tissue. These findings suggest that the expression of deiodinases and the metabolism of THs are altered in human brain tumors, and these changes might be related essential factors that contribute to tumorigenesis or tumor growth (Nauman *et al.* 2004). Finally, it is worth mentioning that limited clinical data suggest that medically induced hypothyroidism may increase patient

survival in high-grade glioblastoma (Hercbergs *et al.* 2003, Ashur-Fabian *et al.* 2013).

TCGA database analysis through GEPIA demonstrated that DIO2 expression was significantly downregulated in glioblastoma samples ($n=163$) when compared with non-paired normal tissue. Similarly, DIO3 tended to be underexpressed in glioblastoma tumors when compared to healthy tissue (Fig. 3B and C). However, post-transcriptional factors, as well as substrates and cofactor availability, could explain the discrepancies between these data and the results of previous studies that showed more heterogeneous patterns of enzyme activity. Moreover, TCGA data that were analyzed included only glioblastoma multiforme samples and does not comprise more differentiated tumor subtypes. It is of great importance to evaluate if altered deiodinase expression is modulated by signaling pathways that are often dysregulated in this type of cancer, such as the PI3K, Src kinase and ERK1/2 signaling pathways and to what extent this contributes to tumor aggressiveness.

Clear cell renal carcinoma: TRB1 and DIO1 as tumorigenesis protagonists

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer, accounting for approximately 75% of all cases. One of the first reports of the dysregulation of THs in cancer involved ccRCC, and there have been multiple and consistent reports supporting decreased expression of DIO1 mRNA and DIO1 protein in ccRCC, as well as decreased expression of thyroid hormone receptor beta (THRB)1 (Puzianowska-Kuznicka *et al.* 2000, Pachucki *et al.* 2001). Studies indicate that T₃ acts through THRB1 as the primary controller of DIO1 expression in the kidney, unlike in other tissues where T₃ acts through both thyroid hormone receptor alpha and THRB (Ammal *et al.* 2001).

Dysregulation of the splicing mechanisms in ccRCC and the existence of multiple splicing variants of DIO1 mRNA and THRB1 have been consistently reported, suggesting a cause for DIO1 disturbances (Piekliko-Witkowska *et al.* 2009, Master *et al.* 2010, Piekliko-Witkowska *et al.* 2010). Additionally, microRNAs targeting the 3'UTR region of DIO1 mRNA (miR-224 and miRNA-383) were reported to be upregulated in ccRCC. The transfection of pre-miRNA-224 or pre-miRNA-383 reduced DIO1 mRNA expression *in vitro*, confirming the suppressive effect of these miRs in DIO1 expression. Consistent with the downregulation of DIO1, intratumoral T₃ levels were 58% lower than in control tissue (Boguslawska *et al.* 2011).

More recently, Poplawski *et al.* provided clear evidence of DIO1 involvement in tumorigenesis by demonstrating that induction of *DIO1* inhibits proliferation and migration and improves adhesion to laminin in ccRCC-derived cell lines (KIJ265T and KIJ308T) (Poplawski *et al.* 2017a). Moreover, they noted decreased mRNA expression of the genes involved in the G1-to-S transition (cyclin D1, cyclin E2, Cdk2, E2F2) in the KIJ 265T cell line. These findings are in accordance with the previously reported *DIO1* downregulation in ccRCC, showing that a lower DIO1 activity could increase proliferation, promote migration and allow cells to detach from ECM proteins more easily. Indeed, *DIO1* expression is significantly downregulated in ccRCC when compared to normal samples based on TCGA data analysis through GEPIA ($n=523$, $P<0.01$) (Fig. 3A). However, no significant changes in *DIO2* and *DIO3* expression were found (Fig. 3B and C). Whether these effects are the result of intracellular hypothyroidism due to the insufficient conversion of T_4 to T_3 remains unclear. Supplementation of cells with T_3 did not reverse the effects of the diminished DIO1 activity. However, this finding might also be due to the inefficient transport of T_3 into the cell (Poplawski *et al.* 2017a). Restoration of *DIO1* expression in ccRCC 'downregulates' oncoproteins that promote proliferation, migration, and invasion while triggers proteins involved in regulation of anti-oxidative processes. Together, these results suggest that loss of *DIO1* expression could be an adaptive mechanism, protecting the cells against overstimulation of cancer metabolism and induction of apoptosis (Poplawski *et al.* 2017b).

Liver neoplasias: complex effects of THs, but few data on deiodinase expression

Classically, significant T_4 ORD is found in human liver homogenates (Hardy *et al.* 1986, Kornasiewicz *et al.* 2010). When evaluating liver microsomes, high DIO1 and DIO3 activities are detected in fetal liver but only DIO1 and mostly none DIO3 activities are found in adult liver. DIO2 activity is virtually absent in both fetal and adult tissues (Richard *et al.* 1998). Both human hepatocytes as well as human hepatoblastoma-derived cells HepG2 show an approximately ten-fold lower rate of iodothyronine metabolism when compared to rat hepatocytes (de Jong *et al.* 1993, van Stralen *et al.* 1993, 1996). However, deiodinase data in human liver tumors is scarce, and the influence of TH in hepatocellular carcinoma (HCC) is controversial. While THs seem to reduce growth, they also promote cell migration in hepatoma cell lines (Chen *et al.* 2008, 2014, Wu *et al.* 2013) THs can also induce cell

self-renewal and promote drug resistance of HCC CSCs (Wang *et al.* 2016).

In an analysis of 13 benign lesions and 7 samples of hepatocellular carcinoma that were compared to normal tissue, there was no difference in DIO1 activity (Kornasiewicz *et al.* 2014); however, DIO1 activity was decreased in hepatic hemangiomas (Kornasiewicz *et al.* 2010). HepG2 express functional DIO1 (van Stralen *et al.* 1996, Chen *et al.* 2016) but neither DIO2 or DIO3 activity has been observed in these cells (Kester *et al.* 2006).

Indirect clinical and basic data also point to a possibility that *DIO3* upregulation might occur in HCC. In a case-control trial, the high prevalence of hypothyroidism among patients with HCC (11.7%) suggested that long-term hypothyroidism was associated with HCC (Hassan *et al.* 2009). Studies in mouse HCC models have identified a cluster of microRNAs (miRNA) that are involved in the upregulation of the *DLK1-DIO3* genomic imprinted region, where the *DIO3* gene is located. More interestingly, overexpression of the *DLK1-DIO3* miRNA cluster was positively correlated with HCC stem cell markers and associated with a high level of serum α -fetoprotein, which is a conventional biomarker for liver cancer and for reduced survival rates in HCC patients (Luk *et al.* 2011). No differences in deiodinase expression between tumor tissue and normal tissue were observed in TCGA data (Fig. 3).

Breast cancer: deiodinases as markers or effectors?

THs are essential for mammary gland growth and development (Vonderhaar & Greco 1979). Studies in rats demonstrate that the mammary glands express significant amounts of functional DIO1 only during the functional stages (lactation) or during differentiation (puberty) (Aceves & Valverde 1989, Aceves *et al.* 1995, Navarro *et al.* 1997). In contrast, DIO2 activity is found in non-stimulated mouse mammary glands, and its expression decreases substantially in the lactating stage (Song *et al.* 2000). In human non-lactating tissue, low or undetectable DIO1 activity has been demonstrated (Debski *et al.* 2007) while no difference in *DIO1* mRNA expression was observed between normal and lactating tissue (Alyusuf *et al.* 2014). We were unable to find data on DIO2 and DIO3 expression in human normal breast tissue.

The first studies of TH metabolism in breast cancer were performed in rat mammary adenocarcinoma. The R3230AC mammary adenocarcinoma is an

estrogen-responsive autonomous tumor that has been maintained by serial transplantation in female Fischer rats since 1963 (Hilf *et al.* 1967). ORD has been demonstrated in this tumor and is insensitive to PTU, which would be compatible with DIO2 activity, as well as IRD, generating rT_3 from T_4 , suggesting that the deiodination pathways were preserved in this tumor model (Ong *et al.* 1986). More recently, abundant activity of DIO1 and high *DIO1* mRNA levels have been shown in human breast cancer, particularly in the most differentiated subtypes (Debski *et al.* 2007, Alyusuf *et al.* 2014). DIO1 activity was also found in the breast cancer cell line MCF-7 (differentiated epithelial carcinoma). However, the more dedifferentiated MDA-MB-231 (estrogen receptor negative) cell line did not express any deiodinase activity (Garcia-Solis & Aceves 2003). Despite these negative findings, upregulation of *DIO1*, *DIO2* and *DIO3* mRNAs has been shown in MCF-7 cells. On the other hand, only *DIO2* is upregulated in MDA-MB-231 when these cell lines were compared to non-tumoral human breast cells MCF-10A (Rusolo *et al.* 2017). The differences between deiodinase mRNA expression and protein activity could be explained by different subtypes of breast tumor as well as post-transcriptional regulatory mechanisms. Taken together, these results may suggest a role for DIO1 as a marker of differentiation in breast neoplasias. It is interesting to correlate these data with the effects of T_3 treatment on cell proliferation. T_3 leads to increased proliferation in MCF-7 cells but does not interfere with the growth of MDA-MB-231 cells (Nogueira & Brentani 1996, Hall *et al.* 2008, Cestari *et al.* 2009).

Analysis of TCGA public data demonstrates that *DIO2* expression is upregulated in breast cancer when compared to normal tissue ($n=1085$, $P<0.01$) (Fig. 3B), and the other deiodinases expression levels in breast cancer are comparable to normal tissue (Fig. 3A and C). As THs influence the proliferation of breast cancer cells, it is worthwhile to consider that DIO3 expression in mammary neoplasias may play a role in modulating intracellular T_3 levels and thus contribute to tumor progression (De Sibio *et al.* 2014). We observed moderate immunostaining of DIO3 in normal human mammary gland tissue and a significant expression of DIO3 staining in samples of invasive ductal carcinoma (I M Goemann, V Marczk, A L Maia, unpublished observations). *DIO3* has also been shown to be expressed in the MCF-7 cell line (Kester *et al.* 2006, Maynard *et al.* 2014). To what extent these alterations contribute to tumor growth due to the modulation of intracellular T_3 or represent markers of altered signaling pathways is yet to be demonstrated.

Deiodinase expression in other neoplasias

DIO2 expression is induced in most brain tumors derived from glial cells (Mori *et al.* 1993, Murakami *et al.* 2000, Nauman *et al.* 2004). Studies performed in 105 pituitary tumors demonstrated that *DIO2* and *DIO3* mRNA were significantly augmented in pituitary tumors when compared with normal pituitary tissue. However, in the rare TSH-secreting pituitary tumor subtype, the *DIO3* mRNA was strongly induced, while reduced *DIO2* mRNA levels were detected. Interestingly, in the case of TSH-secreting pituitary adenomas, the observed pattern of deiodinase mRNA expression may explain the 'resistance' of these tumors to TH feedback (Tannahill *et al.* 2002). When evaluating enzyme function in pituitary adenomas, Baur *et al.* found both DIO1 and DIO2 activity in normal and tumoral tissue. Of note, highest activities of both enzymes were found in TSH- and PRL-producing adenomas (Baur *et al.* 2002). These sets of deiodinase abnormalities may have functional consequences on pituitary tumor growth.

The clinical picture of consumptive hypothyroidism is not restricted to liver hemangiomas. A recent systematic review by our group revealed that among children, 97% had vascular tumors, with hepatic vascular tumors representing 88% of the cases, parotid hemangiomas 5%, cutaneous hemangiomatosis 2% and fibrosarcomas 2%. Because there is a high risk of bleeding associated with vascular tumor biopsy, only three patients underwent tissue sampling. High DIO3 activity was confirmed in all tumor specimens. Tumor histology in the adult population differs from that in pediatric patients. Hepatic vascular tumors represented only 33% of the cases. Gastrointestinal stromal tumors (GISTs) and fibrous tumors each accounted for 33% of the cases. Functional assays confirmed high DIO3 activity levels in all the adult patients (Maynard *et al.* 2014, Weber Pasa *et al.* 2017).

The analysis of deiodination in lung cancer tissue demonstrated that the activity of DIO1 is significantly lower in tumor tissue when compared to the peripheral normal lung tissue, while DIO2 activity is similar in peripheral lung and lung cancer tissue (Wawrzynska *et al.* 2003). Interestingly, *DIO3* was found to be hypermethylated in a subset of hematological malignancies, as assessed by microarray-based methylation analysis, suggesting that aberrant epigenetic modifications may confer *DIO3* tumor-associated properties (Martin-Subero *et al.* 2009). This is also supported by data that demonstrates *DIO3* hypermethylation in lung cancer, which was associated with lower levels of *DIO3* mRNA expression as compared

to normal tissue (Molina-Pinelo *et al.* 2018). Therefore, silencing of DIO3 gene by hypermethylation might be an epigenetic pattern common to different types of human cancer.

Future directions and conclusion

The set of data summarized here clearly indicates a potential role of alterations in deiodinase-related TH levels on the promotion of human carcinogenesis. In addition to the importance of this evidence, it is critical to keep in mind that these studies mostly show an association between changes in the levels of deiodinases and cancer, demonstrating that there is still a lack of knowledge regarding the direct effect of these enzymes on oncogenic processes. Indeed, the majority of data available so far have been obtained by studies performed in normal and tumor tissues from adult patients, human cancer cell lines and *in vivo* models of carcinogenesis. By using these models, the most relevant data that specifically analyzed the effect disruption of deiodinases on carcinogenesis were obtained by chemical inhibition or gene knockout/knockdown. Such approaches may imply the effects of enzyme reactivation on already established tumors and highlight the advantages of such inhibition on tumor behaviors. However, they add limited information to the knowledge of cell transformation and cancer development. Comparative analysis of TCGA and GTEx database can now provide further insight into deiodinases mRNA expression in different types of tumors. Likewise, this approach also has intrinsic limitations. TCGA and GTEx data were not collected in a single experiment. This may especially affect measurements of expression and correlation across different samples. Moreover, GTEx (normal tissue database) RNA are extracted from all tissues of postmortem donors with variable ischemic time (what could compromise RNA quality). On the other hand, TCGA comprises one of the largest and most comprehensive cancer genomics datasets in the world, providing analyses of high-throughput RNA sequencing data of 33 types of cancer, describing tumor tissue and matched normal tissues from more than 11,000 patients. These data are well validated and contributed to more than a thousand studies of cancer by different research groups (<https://cancergenome.nih.gov/>). Using the Human Proteome Map project and RNA-Seq measurements from the GTEx project, a comprehensive tissue- and gene-specific analysis of 16,561 genes and the corresponding proteins revealed that across the 14 tissues, the correlation between mRNA and protein expression was positive and ranged from 0.36

to 0.5 (Spearman correlation value) (Kosti *et al.* 2016). As deiodinases are subject to post-translational regulation as previously described, analysis confined to mRNA expression through public databases should be interpreted carefully, since it may not represent final protein levels and consequent TH changes within the tumor. TCGA also provides additional proteomics data, though only of a limited number of proteins, among which deiodinases are not included (Akbari *et al.* 2014).

Thus, *in vivo* and *in vitro* functional models are needed to fully understand how specific drivers, such as deiodinases, impact tumor initiation and maintenance. Genetically engineered mouse models have been recognized as powerful tools to investigate the impact of gene function on tumorigenesis (Frese & Tuveson 2007, Dow & Lowe 2012). Over the last several years, genome engineering and stem cell technologies have allowed the production of strains in which specific genes can be expressed in a tissue-specific manner. With the advent of Cre-lox and CRISPR/Cas9 technologies, conditional knockout and/or knockin alleles can precisely model events associated with human carcinomas. Furthermore, conditional gene expression systems based on the tetracycline (tet) response system or estrogen–receptor (ERT2) fusions allow temporal analysis of gene function. In addition, the insertion of fluorescent reporters enables lineage tracing and examination of activity (Huijbers *et al.* 2011, Saborowski *et al.* 2014). These new approaches in combination allowed the generation of genetically and histopathologically accurate *in vitro* and *in vivo* models of various human cancers that in turn can be applied to explore the role of the disruption of deiodinases (reactivation and/or downregulation) on cancer initiation and behavior.

In the context of the present review, most of the tumors mentioned above have already been modeled using stem cell technology, which has emerged as a great tool for this kind of research. With regard to the role of genes on the initiation and progression of thyroid cancer, the generation of 3D functional thyroid models derived from mouse embryonic stem cells (mESC) (Antonica *et al.* 2012) constitutes a major breakthrough in the field of thyroid research and raises opportunities for addressing questions related to thyroid organogenesis and diseases. By the induction of thyroid transcriptional factors (Antonica *et al.* 2012) or specific pathways with chemical timing in mESCs (Kurmann *et al.* 2015), these protocols allow us to obtain thyroid cells at different stages of differentiation. In addition, they provide the advantage of generating 3D functional follicles, which is a more sophisticated model

to address gene effects of thyroid cell transformation and can be easily manipulated using estrogen–receptor fusions, Cre-lox and/or CRISPR/Cas9 technologies (Antonica *et al.* 2012, 2017).

Human pluripotent stem cells (hPSCs) have been used as a valuable model for studying the development and progression of gliomas. In addition, neural differentiation protocols allow the derivation of relevant early neural stem cells that are often inaccessible. Thus, early tumorigenesis can be studied in the proper cellular context (Chambers *et al.* 2009, Funato *et al.* 2014). Gene function tests can be evaluated by mutational models, such as lentiviruses encoding constitutively active forms of mutated genes; and knockdown studies can be performed by using shRNA (Funato *et al.* 2014). Similarly, human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have been used to model human diseases of the large intestine. Through modulation of signaling pathways that are known to regulate normal mouse embryonic development, a stepwise strategy was designed for the progressive generation of definitive endoderm, hindgut endoderm (HE) and subsequently the formation of colonic organoids (COs). These 3D structures constitute the best models that resemble *in vivo* organ function and are one of the best tools for disease modeling and drug discovery for some types of cancer (Crespo *et al.* 2017). Therefore, we can make use of this technology to evaluate deiodinase dysregulation and consequent TH imbalance in the fine equilibrium of cellular proliferation and differentiation.

The expression of deiodinases in the neoplastic context is cancer specific and dependent on several clinical and tumoral characteristics. Despite the challenges in studying selenoenzymes *in vitro* and *in vivo*, the role of DIO1, DIO2 and DIO3 in each tumoral context is beginning to unravel (Table 1). Deiodinases can function as markers of disease and cell differentiation or play essential roles as intracellular TH regulators, though we still lack data on the potential concomitant function of all enzymes in each neoplastic context. Moreover, deiodinases can participate in signaling pathways through ‘TH-independent’ mechanisms that need to be further explored. In summary, the understanding of the myriad of mechanisms underlying the balance between tumor cell proliferation and differentiation promoted by THs through deiodinase regulation is critical for the development of new treatment strategies for cancer, inducing tissue-specific or even intracellular changes in TH status that could block excessive proliferation and/or induce tumor redifferentiation.

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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