Activated thyroid hormone promotes differentiation and chemotherapeutic sensitization of colorectal cancer stem cells by regulating Wnt and BMP4 signaling

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Abstract

Thyroid hormone (TH) is a pleiotropic factor that controls many cellular processes in multiple cell types such as cancer stem cells (CSCs). TH concentrations in the blood are stable, but the action of the deiodinases (D2-D3) provides cell-specific regulation of TH activity. Deregulation of deiodinase function and TH status has been implicated in tumorigenesis. Therefore, we investigated the role of TH metabolism and signaling in colorectal CSCs (CR-CSCs), where deiodinases control cell division and chemosensitivity. We found that increased intracellular TH concentration through D3-depletion induced cell differentiation and sharply mitigated tumor formation. Upregulated BMP4 expression and concomitantly attenuated Wnt signaling accompanied these effects. Furthermore, we demonstrate that BMP4 is a direct TH target, and is involved in a positive autoregulatory feedback loop that modulates TH signaling. Collectively, our findings highlight a cell autonomous metabolic mechanism by which CR-CSCs exploit TH signaling to facilitate their self-renewal potential, and suggest that drug-induced cell differentiation may represent a promising therapy for preventing CSC expansion and tumor progression.
Introduction

T3 is a pleiotropic hormone, which controls several cellular processes, including growth, development and homeostasis (1). The main thyroid product thyroxine (T4) is inactive until converted into the active hormone T3 via type 1 or type 2 deiodinase (D1 and D2), while type 3 deiodinase (D3) converts T4 and T3 into inactive metabolites (2, 3).

T3 primarily acts as a transcription factor upon binding to its nuclear thyroid hormone receptors (TR). TRs heterodimerize with many other nuclear receptors and bind the chromatin to TR response elements (TREs) for the positive or negative regulation of target genes transcription (4).

Many in vitro and in vivo studies have indicated that the thyroid status affects tumorigenesis. Type 3 deiodinase (D3) is an oncofetal protein frequently expressed in proliferating and neoplastic cells, where it controls aspects of diseases, injury responsiveness and tumorigenesis (5). Congruently, the actions of the deiodinases provide tissue-specific regulation of TH action at an intracellular level (2). In different tumoral contexts, deiodinases are under the control of relevant pathways in cancer, such as Wnt and Shh (5).

Many in vitro and in vivo studies have indicated that thyroid status affects tumor formation, growth and metastasis in experimental laboratory animals and humans (5). However, the relationship between the cell-specific mechanisms, which control thyroid hormone ligand availability and properties of cancer cells, is still unknown.

Colorectal cancer stem cells (CR-CSCs) represent a small subset of cells within the tumor mass with self-renewing potential and the ability to engraft and generate tumors in immunodeficient mice (6, 7). According to the CSC model, these cells are difficult to kill and their relative insensitivity to chemotherapeutic drugs may explain the frequent failure of conventional treatments used against advanced tumors (8).

The intestine is a highly dynamic tissue, characterized by rapid and continuous regeneration and supported by crypt intestinal stem cells (ISCs) (9). The Wnt signal, which rigorously controls the sequential events that lead up to the transition from normal colon mucosa to adenocarcinoma, is one of the major forces that maintain the stem cells’ fate and capacity to self-renew as well as their ability to escape conventional chemotherapy-
induced apoptosis (10, 11). We recently demonstrated that the Wnt-β-catenin pathway drives an inverse, coordinated regulation of D2 and D3 in colon cancer cells (12).

Here, we demonstrate that CR-CSCs are highly sensitive to intracellular T3. Following T3-treatment or D3-depletion CR-CSCs undergo differentiation, a process that under normal serum conditions requires intracellular T4 to T3 conversion. This is achieved through increases in BMP-4 levels and its downstream targets and significant attenuation of the Wnt pathway. Strikingly, increasing intracellular T3 results in reduced clonogenic and tumorigenic potential and establishes a higher sensitivity of CR-CSCs to conventional chemotherapeutics.

Materials and Methods

Cell culture
Sphere purification and propagation from CRC patients were assessed as previously described (8, 13, 14). Cells were monthly tested for mycoplasma contamination as previously described (14). To evaluate the asymmetric division, single CR-CSCs were also labeled with PKH26 dye (2×10⁻⁶ M, Sigma), cultured for up to 14 days and subjected to flow cytometry analysis in order to yield the PKH positivity. BMP4, at a concentration of 100 ng/ml, in combination with rT3, was added to CR-CSCs and cultured up to 48 hours. Human colorectal adenocarcinoma cells (CaCo-2, obtained in 2005 from ATCC, and authenticated by RT-PCR analyses), cultured in adherent conditions in presence of DMEM medium and supplemented with 2mM L-glutamine and 10% FBS (American Type Culture Collection (ATCC); Manassas, VA, USA).

Reagents and Plasmids
The BMP4 reporter plasmid was generated by PCR on genomic DNA with two sets of oligonucleotides (5mBMP4pU and 5mBMP4pL, and 3mBMP4pU and 3mBMP4pL). Two regions were amplified: 859-bp (containing the first exon) and 552-bp (containing the 3' UTR of the mouse bmp4 gene). The PCR products were digested with SacI/Xhol and cloned in pGL3basic (5'-UTR BMP4) or TKpGL3 (3'-UTR BMP4).
**Lentiviral and luciferase reporter assays**

Cells were transiently transfected (FuGENE 6; Roche) with a mixture of inducible TCF/LEF-responsive firefly luciferase and constitutively expressed *Renilla* luciferase (40:1), or with a negative control containing a mixture of non-inducible firefly luciferase and constitutively expressed *Renilla* luciferase (40:1). The relative quantification of gene expression was calculated on triplicate reactions using the comparative Ct method (ΔΔCt).

**Invasion Assay**

Cell migration was measured using growth factor-depleted Matrigel-coated (BD Biosciences) transwell inserts. Dissociated CR-CSCs (5x10³), transduced with a D3 specific MiRNA (iD3) or control scramble MiRNA (iCTR), were placed on 8-μm pore size Matrigel-coated transwell (Corning). NIH-3T3-stem cell conditioned medium was used as a chemo-attractant and plated in the lower compartment of transwell. After plating, migrated cells were counted up to 72 hours. Cell viability was evaluated on spheres transfected with a D3 specific RNAi pool.

**Real-time PCR**

The mRNAs were extracted with Trizol reagent (LifeTechnologies, Ltd). The cDNAs were prepared with Superscript III (Life Technologies, Ltd) as indicated by the manufacturer. The cDNAs were amplified by PCR in an iQ5 Multicolor Real Time Detector System (BioRad, Hercules, CA, USA) with the fluorescent double-stranded DNA-binding dye SYBR Green (BioRad). The relative amounts of gene expression were calculated with CyclophillinA expression as an internal standard (calibrator). The results, expressed as N-fold differences in target gene expression, were determined as follows: N *target=2*(Ct sample Ct calibrator).

**Western Blot**

Cells were re-suspended in ice-cold NP40 lysis buffer, fractioned on SDS-polyacrylamide gels and blotted on nitrocellulose membranes. Membranes were exposed to specific antibodies for Notch Full length (sc-6014-R, Santa Cruz), Cleaved Notch-ICD (Cell signaling, 2421), E-cadherin (rabbit polyclonal; CST), pAkt (9271, Ser 473, rabbit IgG; CST), Akt (9272, rabbit IgG; CST), pGSK3 (Ser 9, rabbit polyclonal; CST), GSK3 (rabbit polyclonal; CST), PTEN (138G6, rabbit IgG; CST), or tubulin (T9026, Sigma), and

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detected using HRP-conjugated anti-mouse or anti-rabbit Abs (Amersham). p21 (sc-397) and p27 (sc-1641) antibodies were purchased by Santa Cruz. Densitometry analyses were performed by Scion Image (Frederick, MD). Results were expressed as protein/tubulin optical density ratio.

**Immunofluorescence/Immunohistochemistry**

For the immunofluorescence, CR-CSC cytospins, untreated and treated with T3 and rT3, were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and washed in PBS. Thereafter, slides were exposed to antibodies against cytokeratin 20 (Ks20.8, mouse IgG2a; DAKO Cytomation) and BMP4 (3H2, mouse IgG1; Novocastra), both diluted in PBS plus 3% BSA and 0.05% Tween 20 (PBS-T). Cells were treated with fluorochrome-conjugated anti-mouse antibodies (Invitrogen) plus RNaseA (200 ng/mL, Sigma) and counterstained with Toto-3 iodide (Invitrogen). Samples were analyzed on a Nikon C1-Si confocal microscope equipped with EZ-C1 software.

Paraffin-embedded sections of xenografts were subjected to specific antibodies for CK20 (Ks20.8, mouse IgG2a; DAKO Cytomation), Ki67 (MIB-1, mouse IgG1; Dako Cytomation), CD133 (AC133, mouse IgG2b; Miltenyi), or isotype-matched controls at appropriate dilutions. Apoptotic events were determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling using the *In Situ* Cell Death Detection, AP Kit (Boehringer Mannheim, Ingelheim am Rhein, Germany). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Dako Cytomation) substrate.

Cell viability was assessed by using a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Milano, Italy) on sphere cells, treated with T3 or rT3, up to 6 days, and then exposed to oxaliplatin (100nM) and 5-fluorouracil (50 μg/mL), alone or in combination. Cell death was evaluated by orange acridine (50 μg/mL)/ethidium bromide (1 μg/mL) staining. To evaluate the proportion of differentiated and undifferentiated cells, CR-CSCs were cultured in stem cell medium in adherent conditions with 100 ng/ml BMP4 for up to 72 hours.

**CR-CSCs transduction**
CR-CSCs were transduced with lentiviral-TOP-dGFP-reporter as previously reported (14). TOP-dGFP High and Low (Wnt^{high} and Wnt^{Low}) populations were enriched by FACSaria (BD Biosciences). The relative quantification of gene expression was calculated on triplicate reactions using the comparative Ct method (ΔΔCt). Block-IT Lentiviral technology (Life Technologies, Ltd, Paisley, Scotland) was used to the expression of microRNA silencing D3 (iD3) or negative control miR (iCTRL) into dissociated sphere cells, as previously reported (12).

**Clonogenic Assay**

Dissociated sphere cells were cultured in presence or absence of FBS and exposed to 30nM rT3 or T3, on ultra low-adhesion 96-well plates at a single cell concentration. rT3 and T3 were added every 48 hours to the end of experiments. Wells containing either none or more than one cell were excluded for analysis. Sphere formation in culture was monitored and counted under light microscope for up to 21 days. The CSC frequency was calculated using the ELDA algorithm (http://bioinf.wehi.edu.au/software/elda/).

**RT² Profiler PCR array**

The RT² Profiler PCR array was performed to simultaneously evaluate the mRNA levels of 89 genes responsive to and related to WNT signal transduction, following the manufacturer’s protocol (PAHS-243Z, PAHS-043Z; SuperArray Bioscience). Cycle threshold values were normalized using the average of 5 housekeeping genes in 96-well plates (B2M, HPRT1, RPL13A, GAPDH, and ACTB). The comparative cycle threshold method was used to calculate the relative quantification of gene expression (cells treated with T3 and rT3 in presence of 10% FBS medium vs. untreated or cultured with 10% FBS). RT² Profiler PCR Array Data Analysis was represented by clustergrams based on Pearson’s Correlation of 2^ (ΔCt).

**Flow Cytometry**

Cell-cycle analysis was evaluated on dissociated CR-CSCs as previously described (13).

**Tumor xenografts**

Dissociated CR-CSCs (5x10^5) and those transfected with a D3 specific RNAi pool were injected subcutaneously with Matrigel GF reduced (BD Biosciences, Erembodegem,
Belgium) at a 1:2 ratio in a total volume of 100 µL. Tumor size was calculated once a week up to 10 weeks according to the following formula: \( \frac{n}{6} \times \text{larger diameter} \times \text{(smaller diameter)}^2 \).

**Human and Animal studies and Approval**

CRC specimens were obtained from 14 patients (age range 50–57 years) undergoing cancer resection (Table 1), in accordance with the ethical standards of the Institutional Committee on human experimentation (Convention on Human Rights and Biomedicine, Oviedo, 4.IV.1997). None of the patients was under thyroid hormone treatment before surgery. Informed consent for all patients was obtained prior to their participation in this study. All research was conducted within the Ethical principles for Medical Research involving Human Subjects expressed by the Declaration of Helsinki (revised version, Seul 2008, nn.14-15).

Male NOD.CB17-Prkdcscid/J (NOD/SCID) mice, 7 weeks old, were acquired from Charles River Laboratories (Milan, Italy). Mice were maintained, in accordance to institutional guidelines of the University of Palermo Animal Care committee, in an animal house authorized by the Italian Ministry of Health (DGSAF #0020301-P-03.10.2014).

**Statistics**

Differences between samples were assessed by a Student’s 2-tailed t test for independent samples. \( P \) values less than 0.05 were considered significant. Relative mRNA levels (in which the first sample was arbitrarily set as 1) are reported as results of real-time PCR in which expression of cyclophilin A was used as a housekeeping gene. All experiments were repeated and analyzed three to five times. Extreme limiting dilution assay analysis was performed to determine the statistical differences in stem cell-like frequency among the treatment groups.
Results

*Intracellular thyroid hormone concentration is adapted by CR-CSCs to control multiple cell properties.*

To assess the sensitivity of CSCs to T3 signaling, we initially demonstrated the expression of thyroid hormone receptors TRα and TRβ and transporters in freshly purified CD133+ cells from two different CRC samples (Figs. S1-3). Thereafter, we analyzed deiodinases expression in CR-CSCs during FBS-induced differentiation. Both D2 and D3 were expressed and oppositely regulated during spontaneous differentiation (Fig. 1A). D3 mRNA was at its highest level in proliferating CR-CSCs and diminished during differentiation. Contrarily, D2 expression, barely detectable in proliferating CRCs, drastically increased in differentiated cells. We have previously demonstrated that sub-populations of CR-CSCs with high-expression of Wnt (Wnt\textsuperscript{High}) differ from those with a low-expression of Wnt (Wnt\textsuperscript{Low}), with respect to their stemness and ability to differentiate (15). In FACS sorted cells, we observed that Wnt\textsuperscript{High} cells are characterized by a strong expression of type 3 deiodinase, and a corresponded reduction in putative TH-target genes (Fig. S2), thus confirming that D3 expression marks the highly proliferating subset of CR-CSCs and is regulated by the Wnt pathway (Fig. 1B). In order to assess the effect of exogenous T3-treatment, we cultured CR-CSCs under different T3 conditions and evaluated their morphology after 3 and 6 days. T3 treatment alone is sufficient to induce morphologic cell changes similar to what observed when using FBS treatment, i.e. large, flatted, polygonal–shape cells. Contrastingly, rT3 treatment (by abolishing D2-mediated T4 to T3 conversion and the consequent reduction in intracellular T3) impairs FBS-induced differentiation markedly reducing the expression of CK20 (Fig. 1C). Overall, these data indicated that, while exogenous T3 treatment is sufficient to promote cell differentiation, FBS-induced differentiation occurs via endogenous T3, which derives from T4-to-T3 conversion.

*T3 signal is essential for the BMP4-induced CSCs’ differentiation.*

It has been proven that BMP4 is a major promoter of differentiation in normal colonic stem cells (13). To get insights into the mechanisms by which T3 induces cellular differentiation, we investigated the existence of a putative interplay between T3 and BMP4 signals.
Notably, we observed that T3 treatment sustains BMP4 synthesis while rT3 inhibits FBS-mediated BMP4 induction (Fig. 2A). We hypothesized that BMP4 could be a novel T3-target gene as suggested by a ChIP-seq and in silico analyses (unpublished data and Fig. 2B, upper). To prove this, we cloned a 859-bp (part of 5'-UTR) and a 552-bp DNA region (part of 3'-UTR) containing two putative TR-binding sites upstream of the luciferase reporter gene (5'-UTR BMP4 and 3'-UTR BMP4, Fig. 2B, upper). Functional assays demonstrated that T3 strongly induces the activation of both the 5’ and 3’ regions of the BMP4 gene (Fig. S4). Finally, ChIP assay confirmed that the 5’-UTR and 3’-UTR region of the BMP4 gene are physically associated with TRα in CRC cells and this interaction is potentiated by T3 (Fig. 2B, bottom). Altogether, these results indicate that BMP4 is a novel T3 responsive gene in CRC cells.

Considering that BMP4 induces a non-canonical pathway that involves phospho-inositol 3-kinase (PI3K)/AKT activation and modulates the Wnt signaling, we tested the effects of T3-depletion on PI3K/AKT. Western blot analysis revealed that T3 attenuation by rT3 potently induces phosphorylation of AKT and GSK-3β in FBS- and BMP4-treated cells. Accordingly, E-Cadherin was induced by T3 and down modulated by T3 signal reduction (Fig. 2C).

Interestingly, BMP4 was able to induce the expression of the T3 producing enzyme D2, and reduce the T3 inactivating enzyme, D3 (Fig. 2D). Such a double regulation generates an auto-sustaining loop driven by BMP4, which is a T3 target and simultaneously aims to increase intracellular T3 by modulating deiodinases (Fig. 2E). Overall, these data indicate that T3 induces differentiation of CRC cells by reducing BMP/PI3K/AKT signaling, a process that is auto-sustained by BMP.

**T3 treatment alters the clonogenic capacity and cell cycle kinetics of CRC cells by affecting active Notch.**

We evaluated the clonogenic potential of CRC spheres, which were characterized for the expression of the putative and functional stem cell markers CD133 and CD44v6 (Fig. S3) cultured at different T3 concentrations. As shown in Fig. 3A, the clonogenic capacity of CRC cells was significantly reduced in the presence of serum (by 54%) or T3 (by 58%), compared to those cultured in stem medium. In contrast, rT3 treatment significantly preserved the clonogenic capacity, even in the presence of serum. CSC frequency
confirmed the ability of T3 to induce differentiation (1/13.43 vs. 1/6.75) and thereby decrease the clonogenic activity of CR-CSCs (Fig. 3A, lower panel).

Of note, we observed that the exogenous exposure to FBS or T3 significantly decreased the quiescent cell compartment in the G0/G1 phase of CR-CSCs (Fig. S5). This is in accordance with the notion that T3 awakens CR-CSCs from their dormant state by enhancing the p21 and p27 expression levels (Fig. S6A and B).

Subsequently, we monitored cell division by analyzing the distribution of the membrane dye PKH26 expression via FACS (16). While 81% of cells in stem medium underwent asymmetric division, the vast majority of them, grown in stem medium plus T3 as well as in FBS, arrested cell division (Fig. 3B), the latter completely reversed via rT3 treatment. Altogether, these results suggest that T3 alters the percentage of asymmetric cell division in CSCs, thus affecting their ability to maintain the stem-like properties.

Since Notch is a critical regulator of asymmetric cell division and cell fate in CRC cells (17, 18), we sought to assess whether T3 might induce variations in Notch activity. Hence, we measured the expression levels of the active Notch (Notch-ICD). As shown in Fig. 3C and Fig. S7, T3 or D3-depletion greatly induced the intracellular cleavage and activation of Notch. FBS-induced Notch activation was completely revoked by rT3, thus indicating a key role of intracellular T3 in the regulation of Notch pathway.

T3 down-modulates the Wnt pathway, inhibits the tumorigenic potential and sensitizes CR-CSCs to chemotherapy-induced death.

Given the relevance of the Wnt pathway with regard to the functional state of CR-CSCs (15, 19) and its cross talk with the BMP-4 pathway, we aimed to investigate the possible influence of T3 on the Wnt pathway. For this purpose, we compared the transcriptional profile of Wnt targets and Wnt-pathway-relevant genes in T3-treated sphere cells. Clustergrams showed Wnt activation in CSCs when untreated and treated with FBS plus rT3. Vice versa, T3-treatment resulted in a considerable reduction of relevant Wnt targets, including β-catenin, associated with the up-regulation of negative regulators of Wnt-receptor signaling (Fig. S8). This analysis significantly points to T3 as a potent repressor of Wnt signaling.

To assess the capacity of T3 to inhibit the tumorigenic potential, we measured the effects of T3 treatment on the CR-CSCs engrafting capacity. We injected CR-CSCs, pre-treated
with 30nM T3 for 6 days, into immunodeficient NOD/SCID mice. Untreated CR-CSCs generated palpable tumors within 3-4 weeks, which were strikingly reduced in T3-treated cells (Fig. 3D). Tumors derived from T3-treated cells show elevated CK20 expression levels and abundant cell death. Furthermore, the CD133 and Ki67 expression was drastically reduced in CR-CSCs exposed to T3 (Fig. S9), indicating a substantial effect on proliferating cells endowed with self-renewal capacity.

D3 is the T3-inactivating enzyme often over-expressed in human cancer (5). To assess the role of D3 in the modulation of intracellular T3 and its effect on tumorigenic potential, we infected CR-CSCs with a lentivirus able to efficiently knockdown D3 (12). D3 knockdown in CR-CSCs cells dramatically reduced the in vitro clonogenicity and their invasive capability as well as tumor growth in immunocompromised mice (data not shown). D3-depleted CR-CSCs failed to give rise to detectable tumor outgrowth. In line with the observed T3 effects, D3-depletion potently reduced the xenograft’s growth, which is consistent with an increase of the intracellular T3 availability in absence of D3 (Fig. 3E). These data underline that T3 signaling is a strong determinant in the CSCs’ ability to promote tumorigenesis in vivo.

We have previously reported that CSCs are widely resistant to chemotherapeutic drugs (8). To assess whether such a complex regulation of T3 on the BMP4/Wnt pathways in CR-CSCs might influence their ability to respond to chemotherapy, we investigated whether T3 is able to alter the resistance to oxaliplatin (OX), 5-fluorouracil (5-FU) and a combination of them (FOX), at clinically relevant doses. Time course treatment showed that, while untreated spheres were largely inert to chemotherapeutic drug-induced apoptosis, T3 treatment caused an increased percentage of cell death (up to 75%), when combined with FOX. RT3 treatment did not alter the cell resistance to drugs (Fig. 3F).

Discussion

Despite improvements in therapeutic strategies, CRCs remain the third leading cause of cancer related-deaths in western countries, due to the failure in curing the metastatic disease. Although canonical cancer treatments have been designed to reduce the rapidly growing tumor cells, CSCs are spared and are still able to mediate cancer relapse after chemotherapy and radiation (20). This suggests that curative therapies can be effective
only by targeting the sub-populations of those tumor cells with self-renewing potential.

Drug-induced differentiation represents a promising approach to hamper CSCs’ self-renewal ability. Although differentiation therapies do not selectively kill CSCs, they make them more sensitive to the conventional therapies and ultimately eradicate the tumor-driving cell population. Contrary to the haematological malignancies, the clinical use of differentiation–inducing agents to treat solid tumors is very limited (21, 22).

We demonstrate that CR-CSCs with β-catenin activation display high levels of D3, which correlate to an enhanced self-renewal capacity. Drug-induced differentiation could represent a promising approach to hamper CSCs’ self-renewal ability. Our data provide evidence that, while D3 contributes to maintain the undifferentiated status, T3 induces differentiation, affects the Wnt targets-related genes, and sensitizes CR-CSCs to the standard chemotherapeutic drugs by down-modulating the AKT/PI3K pathway. Furthermore, D3-induced inactivation of TH stabilizes the quiescence status of CR-CSCs, altered by T3 exposure. Targeting D3 abrogated the tumorigenic activity in vivo of CR-CSCs.

It was reported that the Notch1 hinders the β-catenin activation by reducing the levels of the available unphosphorylated (active) form (23). Triggering the Notch signal pathway induces the cytoplasmic cleavage of Notch-ICD, which activates nuclear Notch target genes who mainly promote differentiation (24). It was also demonstrated that the overexpression of Notch has a negative effect on CRCs progression, thus suggesting Notch as a favourable prognostic marker (25). In line with these data, we show that T3 controls the self-renewal pathway of CR-CSCs through the activation of canonical Notch pathway. While T3 induces the cleavage of Notch-ICD, D3 restores the levels of activated β-catenin drastically decreasing the expression levels of Notch that are sustained by T3.

In CR-CSCs, Notch signalling increases asymmetric division, which generates one daughter cell that retains stem cell properties (17, 18). Meanwhile, the second daughter cell undergoes a differentiation process via multiple division rounds. In this scenario, the stem cell pool can be expanded by a series of symmetric cell divisions that is essentially controlled by the Notch inhibitors. Importantly, D3 action, by reducing intracellular T3, increases the frequency of symmetric self-renewing divisions of CR-CSCs, which could explain the enhanced tumor growth.
We have previously demonstrated that BMP4 displays high anti-tumor activity in CRC by inducing CSC differentiation, targeting survival, proliferation and chemoresistance (13). Interestingly T3, a potent BMP4 inducer, impairs clonogenic activity and xenograft tumor outgrowth, suggesting this molecule as a key effector in the activation of the CRC differentiation program. Being that T3 reduces nuclear β-catenin accumulation, the PI3K/AKT pathway may represent a connection point between BMP4 and Wnt pathways, implying an active cross talk that balances stemness and differentiation in CR-CSCs. The ability of this hormone to regulate the BMP4 gene activation is confirmed by the ChIP-seq and silico analysis that provide a feedback control of the differentiation pathway. Likewise, a D3-induced increase of BMP4 inhibitors restores their self-renewal capacity, tumorigenic capacity and refractoriness to conventional anti-cancer therapies.

In summary, our findings suggest that intracellular T3 exerts a pro-differentiative effect that prevents CSC expansion and triggers a differentiation program. The latter may result in CSCs depletion, thereby hampering tumor development. While D3 enhances tumor growth, T3 signalling appears particularly effective in inducing differentiation, growth reduction and chemosensitization of CR-CSCs. The therapeutic effects observed by the combined action on intracellular T3 and chemotherapy, further substantiate the necessity to target the stem-like population of cancer cells in order to improve CRC treatment and open new avenues for the use of locally manipulated deiodinases for treating proliferative disorders or hormone-sensitive tumors such as CRCs.

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REFERENCES

Table 1. Patient and tumor descriptions

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FIGURE LEGENDS

Figure 1. Thyroid hormone signal induces in vitro differentiation of colorectal cancer stem cells (CR-CRCs). (A) Real time PCR (RT-PCR) analysis of D2 and D3 expression in primary CR-CRCs cultured in stem medium (0) or 10% FBS-supplemented medium for the indicated time points. Cyclophilin A was used as internal control. Values are mean ± SEM for at least 3 independent experiments. (B) FACS-sorted TOP-dGFP high and low (Wnt$^{\text{high}}$ and Wnt$^{\text{low}}$) CR-CSCs were analyzed by RT-PCR. Data are mean ± Standard Deviation (SD) from independent experiments performed with CR-CSCs from 6 different patients. (C left) Phase contrast and CK20 staining (green) of CR-CRCs cultured as sphere-like aggregates in different media as indicated for 3 and 6 days. Nuclei were stained with Toto-3 (blue color). One representative experiment of 5, performed with tissues derived from different patients, is shown. Scale bars represent 20 μm. (C right) Percentage of differentiated CR-CRC cells (upper panel) and percentage of CK20$^+$ cells cultured as before mentioned at the indicated time (lower panel).

Figure 2. Thyroid hormone signal promotes BMP pathway activation. (A) Representative confocal analysis for BMP4 (green color) on CR-CSCs treated as indicated for 48 hours. Scale bars represent 20 μm. (B, upper panels) Schematic representation of BMP4 gene. In the boxes the ChIP-Seq positive region (yellow box) and cloned region are indicated (dashed box); TSS: transcription start site; TES: transcription end site (top). Physical binding of TR to the 5’-UTR and 3’-UTR of BMP4 gene was evaluated by ChIP assay. (B, lower panels) CR-CSCs cells were cultured for 6 days in the presence or absence of T3, and harvested for the ChIP assay with a TR$^\alpha$ antibody. Data are expressed as mean ± SD of 5 independent experiments performed with cells from different patients. (C) Representative Western blot analysis of BMP downstream targets in CR-CSCs cultured in stem medium (UT), plus and minus rT3 or supplemented with BMP4 and rT3 for 48 hours. Tubulin levels were measured as loading control. One representative of 7 immunoblots for BMP downstream targets is shown. (D) D2 and D3 mRNA were measured by RT-PCR in CR-CSCs treated with BMP at indicated times (* : p<0.05). (E) Schematic diagram illustrating the proposed interplay between thyroid hormone and BMP4 pathway modulating the differentiation and stemness of CSC.
Figure 3. Thyroid hormone significantly reduces the tumorigenic potential of CR-CRCs and enhances the sensibility to chemotherapeutic treatments. (A, upper panel) Clonogenic assay was performed in CR-CSCs treated as indicated. Data are expressed as mean ± SD of 6 independent experiments performed with cells from different patients (**: p<0.01). (Lower panel) Frequency of CSCs in CR-CSCs cultured as before mentioned. (B) PKH-26 labeling of CR-CSCs cultured as in (A) was measured by FACS analysis. Bars represent the mean ± SD of results obtained from 6 independent experiments derived by using cells from different patients. (C) Activated and full-length Notch protein levels were measured in CR-CSCs cultured as indicated. One representative of 5 immunoblots for the Notch pathway is shown. (D) Size of subcutaneous tumor growth after injection of dissociated sphere cells pre-cultured in vitro for 6 days as indicated. Representative set of xenografts derived from the injection of dissociated sphere cells as in left panel. (E) Representative set of xenografts and tumor sizes derived from the injection of sphere cells knocked down for D3 (iD3) or transduced with control RNAi lentivirus (iCTR). Each tumor set was obtained using cells from 4 different donors. (F) Cell death percentage of CR-CSCs cells cultured as in (A) and treated with oxaliplatin (OX), 5-fluorouracil (5-FU) and a combination of them (FOX), up to 96 hours. Values are expressed as the mean ± SD of 6 independent experiments performed with cells from different patients.
Fig. 1
Fig. 2
Activated thyroid hormone promotes differentiation and chemotherapeutic sensitization of colorectal cancer stem cells by regulating Wnt and BMP4 signaling

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